



Faculty of Resource Science and Technology

**Evaluation of Strategies for Lysing Spent Baker's Yeast Generated Upon
Production of Sago Bioethanol**

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Evaluation of Strategies for Lysing Spent Baker's Yeast Generated Upon Production of Sago Bioethanol

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DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of Universiti Malaysia Sarawak. Except where due acknowledgements have been made, the work is that of the author alone. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.



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ABSTRACT

Spent Baker's yeast (*Saccharomyces cerevisiae*), a by-product of the ethanol production industry, is mainly discarded as a waste. The spent yeast can be converted to yeast extract by the lysis process, in which the yeast cells are disintegrated under specific conditions to release significant bioactive components. To date, studies on the lysis strategies of spent *S. cerevisiae* produced after sago bioethanol synthesis is still limited. The present work was aimed to evaluate the feasibility of autolysis and enzymatic hydrolysis of spent *S. cerevisiae* produced following sago bioethanol fermentation. The autolysis of spent *S. cerevisiae* was studied according to the effect of initial pH (3, 5, and 7) and incubation time (24, 48, 72, and 96 hours). Secondly, the enzymatic hydrolysis was investigated using two different enzyme types (alcalase and cellulase) with varying enzyme doses (0.1, 0.2, 0.3, 0.4, and 0.5 %, v/v). The resulting lysates were analysed for protein and carbohydrate concentrations using Lowry assay and phenol-sulphuric acid assay, respectively. The lysed yeast cells were observed for their morphological characteristics using a scanning electron microscope. The results indicated that after 72 hours of incubation, the autolysis of spent Baker's yeast at an initial pH of 3 produced the maximum concentration of protein and carbohydrate in the autolysate. These were 2.5 and 2-fold higher than the concentrations of protein and carbohydrates in the control samples, respectively. Meanwhile, in enzymatic hydrolysis, the highest protein and carbohydrate concentration of lysate were achieved from the hydrolysis of spent Baker's yeast using cellulase (0.5%, v/v) and alcalase (0.4%, v/v), respectively. The enhancement of protein and carbohydrate concentrations were 8.6-fold and 11.4-fold, respectively as compared to that achieved by the control experiment. The surface morphology analysis of lysed yeast cells resulting from autolysis and enzymatic hydrolysis of spent *S. cerevisiae* under the aforementioned conditions were parallel with the lytic events occurred during both

processes. The best strategies for lysing spent Baker's yeast generated upon production of sago bioethanol was enzymatic hydrolysis, considering to the high release of protein and carbohydrate concentration as compared to autolysis. The current work provides early insights into the potential strategies of valorising spent *S. cerevisiae*, generated from sago bioethanol production, where the resulting yeast extract can be used for various industrial applications.

Keywords: Autolysis, enzymatic hydrolysis, *Saccharomyces cerevisiae*, spent Baker's yeast, yeast extract

Penilaian terhadap Strategi untuk Memecahkan Sisa Yis Roti yang Terhasil daripada Produksi Bioetanol Sagu

ABSTRAK

Sisa yis roti (*Saccharomyces cerevisiae*), iaitu produk sampingan daripada industri fermentasi etanol kebanyakannya dibuang sebagai hasil buangan. Sisa yis boleh ditukar menjadi yis ekstrak melalui proses lisis, di mana sel-sel yis dipecahkan dalam keadaan yang spesifik untuk merembeskan kandungan-kandungan bioaktif yang penting. Sehingga kini, kajian mengenai strategi lisis terhadap sisa *S. cerevisiae* yang dihasilkan berikutan produksi bioetanol sagu masih terhad. Penyelidikan ini bertujuan untuk menyiasat kebolehlaksanaan autolisis dan hidrolisis enzim terhadap sisa *S. cerevisiae* daripada fermentasi bioetanol sagu. Autolisis yis roti dikaji berdasarkan kesan pH awal (3,5, dan 7) dan tempoh inkubasi (24, 48, 72, dan 96 jam). Kedua, hidrolisis enzim telah dikaji menggunakan dua jenis enzim yang berbeza (alcalase dan cellulase) dan kepekatan enzim yang berbeza (0.1, 0.2, 0.3, 0.4, dan 0.5%, v/v). Produk lisis yang terhasil dianalisa untuk kepekatan protein dan karbohidrat masing-masing menggunakan ujian Lowry dan ujian asid fenol-sulfurik. Ciri-ciri morfologi sel yis yang dipecahkan, dicerap menggunakan mikroskop elektron pengimbas. Hasil kajian menunjukkan selepas 72 jam inkubasi, autolisis sisa yis roti pada pH permulaan 3 menghasilkan kepekatan protein dan karbohidrat tertinggi dalam produk autolisis. Ini adalah 2.5 dan 2 kali ganda lebih tinggi daripada kepekatan protein dan karbohidrat, masing-masing dalam sampel kawalan. Sementara itu, dalam hidrolisis enzim, kepekatan protein dan karbohidrat tertinggi dalam produk hidrolis enzim telah tercapai daripada hidrolisis sisa yis roti masing-masing menggunakan cellulase (0.5%, v/v) dan alcalase (0.4%, v/v). Peningkatan untuk kepekatan protein dan karbohidrat masing-masing adalah 8.6 kali ganda dan 11.4 kali ganda berbanding yang telah dicapai daripada eksperimen

kawalan. Analisa morfologi permukaan sel yis yang telah dipecahkan hasil daripada autolisis dan hidrolisis enzim terhadap sisa S. cerevisiae menggunakan keadaan yang telah dinyatakan adalah selari dengan keadaan lisis yang berlaku daripada kedua-dua proses. Strategi terbaik untuk memecahkan sis yis Baker yang dihasilkan selepas penghasilan bioetanol sagu ialah hidrolisis enzim, berikutan penghasilan kepekatan protein dan karbohidrat yang tinggi berbanding dengan autolisis. Kajian ini memberikan pemahaman awal tentang strategi yang berpotensi dalam meningkatkan penggunaan sisa S. cerevisiae yang dihasilkan daripada produksi bioetanol sagu, di mana ekstrak yis yang terhasil boleh digunakan untuk pelbagai aplikasi perindustrian.

Kata kunci: Autolisis, hydrolisis enzim, Saccharomyces cerevisiae, sisa yis roti, ekstrak yis

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LIST OF ABBREVIATIONS

β	Beta
μm	Micrometer
$\mu\text{g/mL}$	Microgram per millilitre
AN	Amino nitrogen
ANOVA	Analysis of variance
AU	Avson unit
BSA	Bovine serum albumin
CAZyme	Carbohydrate active enzyme
CBD	Cellulose binding domain
CBM	Carbohydrate binding module
CD	Catalytic domain
CG	Commercial glucose
DNA	Deoxyribonucleic acid
EC	Enzyme commission
ER	Endoplasmic reticulum
FAO	Food and agricultural organisation
g/L	Gram per litre
GHs	Glycoside hydrolases
GPI	Glycosylphosphatidylinositol
GRAS	Generally recognised as safe
GTs	Glycosyltransferases
HPLC	High performance liquid chromatography
hr	Hour

Leu	Leucine
M	Molarity
Met	Methionine
MOS	Mannanoligosaccharides
mg/g	Miligram per gram
mg/mL	Miligram per mililitre
mg/L	Miligram per litre
min	Minute
mm	Milimetre
mM	Milimolar
MSG	Monosodium glutamate
nm	Nanometre
N	Normality
OD	Optical density
OH	Hydroxyl group
PDA	Potato dextrose agar
Phe	Phenylalanine
PLs	Polysaccharide lyases
RNA	Ribonucleic acid
rpm	Revolutions per minute
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
SEM	Scanning electron microscopy
SFH	Sago fibre hydrolysate
SFP	Sago fibre powder
TN	Total nitrogen

Tyr	Tyrosine
U/g	Unit per gram
v/v	Volume per volume
w/v	Weight per volume
w/w	Weight per weight
YCW	Yeast cell wall
YDE	Yeast distillery effluent
YE	Yeast extract
YMB	Yeast malt broth

CHAPTER 1

INTRODUCTION

1.1 Study Background

The exploitation of Baker's yeast (*Saccharomyces cerevisiae*) in the fermentation sector has been widely utilised. This in turn, generates large amount of waste products, including spent yeast (Jacob et al., 2019a). The spent yeast in general, is typically used either as a low-cost protein source in animal feed formulations or discarded to the environment causing severe ecological impacts (Podpora et al., 2015; Jaeger et al., 2020). However, the waste constitutes of many valuable compositions such as proteins, essential amino acids, RNA, vitamins B, and minerals which essentially serves as a raw material for the production of yeast extract (Vieira et al., 2016; Jacob et al., 2019a).

Yeast extract refers to soluble portions of yeast cells after the separation of the insoluble components after the lysis of yeast cells (Vukašinović-Milić, 2007). Spent yeast extracts show promise as a source of valuable nutrients such as bio-active components and amino acids for use in functional food, nutritional supplements, and as a component for microbial growth medium. In addition, yeast extract has also been widely used as a flavouring agent and flavour enhancer in food industry (Boonraeng et al., 2000). It is possible to recover the extracts by disrupting the yeast cells by the processes of autolysis or enzymatic hydrolysis, which involve the utilisation of either endogenous or exogenous enzymes (Li et al., 2015; Takaloo et al., 2020).

1.2 Problem Statement

Previously, studies on the autolysis and enzymatic hydrolysis of spent Baker's yeast have commonly utilised either fresh yeast or yeast generated from fermentations, supplemented with synthetic feedstocks. Currently, the information regarding the exploitation of *S. cerevisiae* obtained from the fermentation of bioethanol employing agricultural waste, such as sago fibre is limited. Bioethanol generation from sago fibre has been documented in the literature (Awg-Adeni et al., 2010). While sago bioethanol has the potential to meet expanding industrial demand, its growth will almost certainly be accompanied by an increase in waste output. The possible fate of the waste produced after sago bioethanol production is necessary to be investigated to ensure the sustainability of the entire process in sago bioethanol biorefinery.

One of the sources of spent Baker's yeast is bioethanol fermentation. The spent Baker's yeast is discarded from the fermentation as a solid waste. Following bioethanol production, the wastes generated were discarded to landfill or as wastewater. Previously, utilisation of sago bioethanol liquid waste for production of an industrial biocatalyst has been reported by Mamat et al. (2021). However, there is no work reported for the autolysis and enzymatic hydrolysis of spent *S. cerevisiae* generated from sago bioethanol fermentation. Bioethanol solid waste is currently used as a component of animal feeds in ruminants and aquaculture for its cheap sources of protein, B vitamins, and minerals (Oliveira et al., 2016; San Martin et al., 2020). Additionally, it was reported that spent *S. cerevisiae* before the primary fermentation in a brewing process was recycled in small amounts to start the following batch of fermentation before being discarded (Vieira et al., 2012; Marson et al., 2019). Therefore, appropriate techniques to manage and exploit the wastes created following the bioethanol production should be proposed in order to ensure that the whole process is

both cost-effective and sustainable. A fundamental inquiry of the characteristics and uses of the sago bioethanol waste stream, as investigated in this research, will help to illuminate the potential application of the waste stream.

1.3 Aim and Objectives

The aim of this work is to establish effective strategies for lysing spent Baker's yeast generated after the production of sago bioethanol. The objectives of the work are as follow:

- i. To evaluate the effectiveness of different lysis strategies (autolysis and enzymatic hydrolysis) for lysing spent Baker's yeast generated after the production of bioethanol from sago fibre.
- ii. To determine the properties of the resulting yeast lysates obtained from autolysis and enzymatic hydrolysis with particular emphases on its protein and carbohydrate contents, and surface morphology of the lysed yeast cells.

CHAPTER 2

LITERATURE REVIEW

2.1 Baker's Yeast (*Saccharomyces cerevisiae*)

In biological terms, *Saccharomyces cerevisiae* is classified as a unicellular eukaryote under kingdom fungi. It was the first eukaryotic organism that was sequenced in 1996 (Goffeau et al., 1996), and to date has proven to be the most ideal organism for study in biology. It is ellipsoid in shape and the size of an individual cell has a diameter ranging from 5–10 μm and 1-7 μm for large and smaller diameter, respectively (Montes de Oca et al., 2016). *S. cerevisiae* is ubiquitous and may be isolated from variety of sources, including soil, water, plants, animals, and even insects (Starmer & Lachance, 2011). The structural components of *S. cerevisiae* include a rigid cell wall, nucleus, mitochondria, endoplasmic reticulum (ER), Golgi apparatus, vacuoles, microbodies, and secretory vesicles with a complex network of membranes within and outside of the cell (Montes de Oca et al., 2016). Figure 2.1 shows the surface morphology of *S. cerevisiae*.

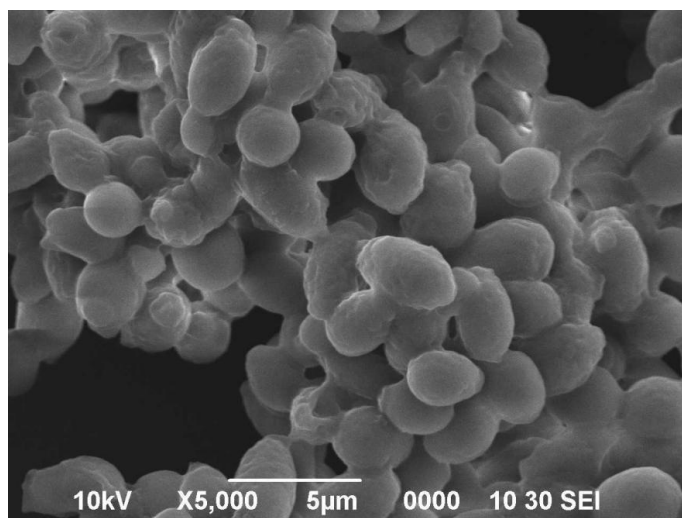


Figure 2.1: Scanning electron micrograph of *S. cerevisiae* ($\times 5000$).
Adapted from Mahmud et al. (2021)

The cell reproduces both vegetatively and sexually through the process of ascospore production and multilateral budding, respectively (Stewart, 2014). *S. cerevisiae* thrives in environments with a pH between 4.5 and 6.5 and a temperature range of about 28 to 33°C for optimal growth due to its acidophilic nature (Jach et al., 2022). In addition to water and oxygen, *S. cerevisiae* requires sufficient amounts of macronutrients including carbon, free amino nitrogen, sulphur, phosphorus potassium, and magnesium to meet its nutritional needs. Apart from that, calcium, copper, iron, manganese, and zinc are required serving as micronutrients, known as trace elements for the yeast cells (Walker & Stewart, 2016). According to Ljungdahl and Daignan-Fornier (2012), yeast cells prefer nitrogen sources derived from amino acids alanine, arginine, asparagine, aspartate, glutamate, glutamine, and serine.

Among all yeast strains, *S. cerevisiae*, referred to as Baker's yeast, is most recognised for its role in fermentation processes for the food, beverage, and biofuel production industries (Parapouli et al., 2020). In biotechnological application, *S. cerevisiae* is the world's most exploited microorganism, responsible for the development of ethanol production, one of the most valuable biotechnological products, by converting carbohydrates (glucose or maltose) into ethanol and carbon dioxide (Chang et al., 2018). Moreover, the ethanol fermentation by *S. cerevisiae* involves in anaerobic respiration through glycolysis pathway. In the absence of oxygen, glucose provides the cell with essential molecules such as carbon, hydrogen and oxygen. During fermentation of ethanol ($C_3H_4O_3$), two molecules of pyruvic acid are formed from a single molecule of glucose ($C_6H_{12}O_6$). Two molecules of acetaldehyde (CH_3CHO) are produced by further decarboxylating pyruvic acid, and these molecules are then reduced to ethanol (C_2H_5OH). Finally, two molecules of ATP are gained for every molecule of glucose converted into two molecules of ethanol and two molecules of carbon dioxide (CO_2)

during the process (Akhtar et al., 2017). Figure 2.2 shows the glycolysis pathway during ethanol fermentation by *S. cerevisiae*.

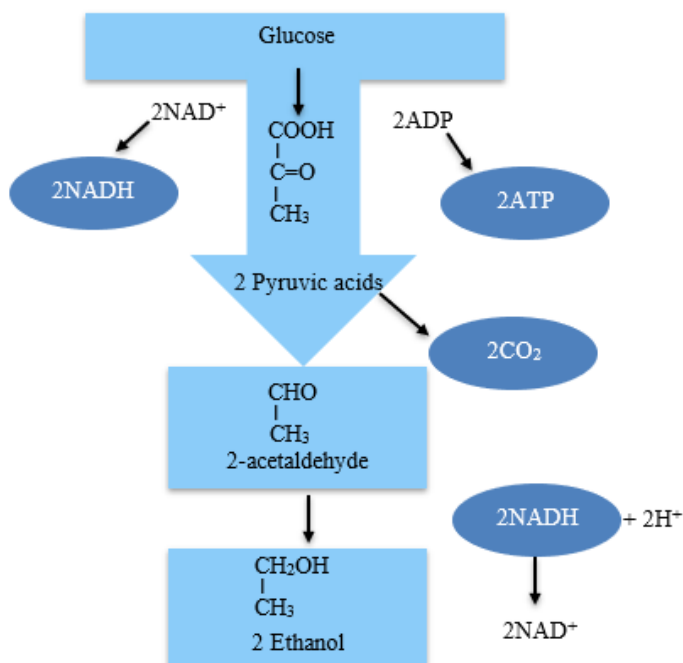


Figure 2.2: Ethanol fermentation pathway by *S. cerevisiae*. Adapted from Akhtar et al. (2017)

This well-studied organism dominates in fermentation process in ethanol production due to its high fermentation ability, fast growth rates, high ethanol yield as well as keeping low cost of distillation. *S. cerevisiae* is an ideal microorganism for industrial-scale fermentation due to its ability to survive high temperatures, acidic environment, and a high concentration of substrates and ethanol (Kasavi et al., 2012; Lin et al., 2012; Morales et al., 2015). There are many strains that can thrive in an environment with 8–12% ethanol (v/v), including Variant M1 and Yeast distillery effluent (YDE) strain (Tikka et al., 2013; Qiu & Jiang, 2017). Moreover, it was reported that a UV-C mutagenesis mutant strain, UVNR56 and genetically modified industrial fuel ethanol CAT-1 strain, exhibited considerably higher viability during ethanol fermentation in the presence of 15% and 16% (v/v), respectively

(Thammasittirong et al., 2013; Varize et al., 2022). Table 2.1 shows the composition of baker's yeast as dry matter.

Table 2.1: Composition (as % dry matter) of *S. cerevisiae* (Klis et al., 2006)

Component	Percentage
Protein	37-40
Carbohydrates	35-40
Lipids	4-5
Ribonucleic acid	5-9
Ashes	4.5-7.5

2.2 Components of Yeast Cell Wall

S. cerevisiae has a cell wall with a thickness of 100 to 150 nanometers and is critical for maintaining cell morphology and structural integrity as well as protecting cells from osmotic shock and mechanical stresses (Levin, 2011; Wang et al., 2018). The wall of yeast cells makes up an estimated 15-30% of their total dry mass, with 85% of it composed of polysaccharides, 15% proteins, and 5% lipids. The innermost layer of the bilayer cell wall comprises of 30-45% of β -1,3-glucan, 5-10% of β -1,6-glucan, and 1.5-6% of chitin. On the other hand, the outermost layer is made up of the highly glycosylated mannoproteins which comprised of 30-50% of the cell wall (Klis et al., 2002; Klis et al., 2006; Lesage & Bussey, 2006). The inner layers of yeast cell walls offer structural support and serve as sites for protein attachment to the cell wall's outermost layer. According to Figure 2.3, these components are located in the cell wall from outside to inside in the following order: mannoproteins $\rightarrow$ β -1,6-glucan $\rightarrow$ β -1,3-glucan $\rightarrow$ chitin (Anwar et al., 2017).

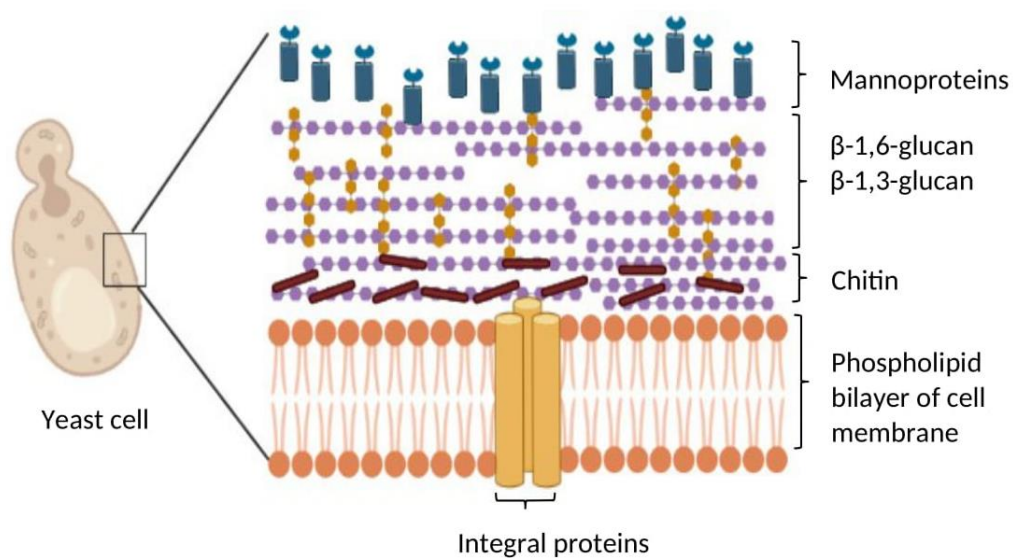


Figure 2.3: Schematic representation of *S. cerevisiae* cell wall. Adapted from Anwar et al. (2017)

2.2.1 Mannoproteins

These mannose-rich mannoproteins act as structural components at the yeast cells outermost layer, extending from the cell surface (Quiros et al., 2012). The carbohydrate content of these mannose polysaccharides is approximately 95% (weight/weight), and they are referred as mannanoligosaccharides (MOS). These mannose polysaccharides consist of several single residues of α -D-mannose that are linked to protein either as N- or O-glycosidic bond. Mannoproteins in the cell wall are linked indirectly by β -1,6-glucan via a glycosylphosphatidylinositol (GPI) anchor and directly by β -1,3-glucan via alkali bond (Sanz et al., 2017). The majority of cell wall proteins are glycosylphosphatidylinositol cell-wall proteins, and indirectly connected to the β -(1,3)-glucan chains.

2.2.2 β -glucan

The main structural component of the yeast cell wall is known as β -glucan. As shown in Figure 2.4, yeast β -glucans are composed of a main chain of β -(1,3)-D-glucose residues and are highly branched glucose oligosaccharide, with randomly dispersed side chains of β -(1,3)-D-glucose units that are attached by β -(1,6) linkages. The structure of these molecules can be altered by a random coil or more ordered conformations, such as a network of single helix chains linked together to form a triple helix and stabilised by inter- and intra-hydrogen bonds (Zekovi et al., 2005; Zhu et al., 2016).

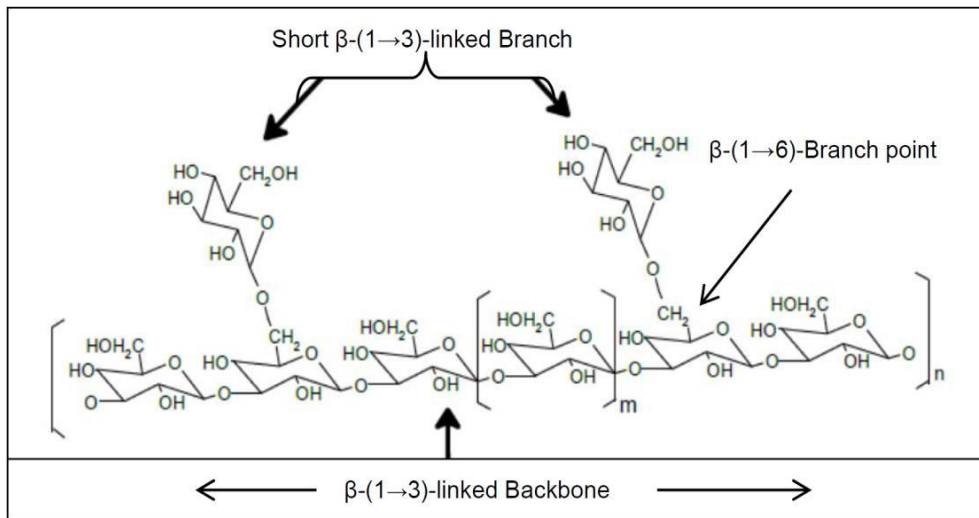


Figure 2.4: Molecular structure of soluble yeast β -glucan. Adapted from Waszkiewicz-Robak et al., 2013

According to Klis et al. (2006), the flexible and helical structure of each individual β -1,3-glucan molecule, which functions like wire springs that can extend in various states of extension, is responsible for the cell wall's elasticity. Yeast glucans are formed from glucose monomers with the attachment of β -1,3-glucan to the highly branched β -1,6-glucan, whilst chitin attaching to these monomers through its reducing ends (Klis et al., 2006). The degree of glucose polymerisation for both β -1,3-glucan and β -1,6-glucan are 1500 glucose units and 140 glucose units, respectively (Varelas et al., 2014; Fesel et al., 2016).

2.3 Spent *S. cerevisiae*

Spent *S. cerevisiae* is an abundant, low-cost by-product of the bioethanol fermentation process. Figure 2.5 depicts the spent *S. cerevisiae* obtained from this study, which was after the fermentation of sago bioethanol. It has various useful applications. In 2019, approximately 900 000 tons of spent yeast were generated, making it the second largest by-product in the brewing sector (San Martin et al., 2021). It was determined that the average weight of spent yeast recovered upon production of sago bioethanol was $15.81 \text{ g/L} \pm 0.77$ (refer to Appendix C). Despite of high nutritional density in the spent yeast, it is generally disposed off and posing a serious effect to the environment. To date, the valorisation of spent yeast is still restricted to animal feed formulations in ruminants, poultry, and aquaculture as a source of proteins, minerals and B vitamins (Oliveira et al., 2016; Chollom et al., 2017; San Martin et al., 2020).



Figure 2.5: Spent *S. cerevisiae* slurry, after bioethanol fermentation obtained from this study

In addition, it was reported that spent *S. cerevisiae* prior to the primary fermentation in brewing process was employed in small quantities to inoculate the subsequent fermentation batch before being discarded (Vieira et al., 2012; Marson et al., 2019). Several attempts had been taken in converting this yeast surplus into value-added ingredients which may be the best option for a low environmental footprint. The yeast harvested at the end of fermentation has been reported to be reused in subsequent fermentations, for up to four to six times before being discarded in the brewing process, while it can be used up to fifteen times. This practice is termed as serial repitching (Vieira et al., 2016). Cells can divide two to three times throughout a fermentation cycle, resulting in 13-25 replications (Powell et al., 2007). As a result, it causes genetic drift, which brings stability to the environment in which it flourishes. The effect of serial repitching on the enzymatic hydrolysis of spent yeast was previously studied (Marson et al., 2019). It has been documented that the yeast cell wall thickens due to the stability of growth media during serial repitching. This in turn, makes hydrolysis of the repitched spent yeast more difficult up to 3.5 times as opposed to unrepitched yeast.

The nutrients and functional components found within the spent Baker's yeast can be extracted and processed into yeast extract, by disintegrating and removing the yeast cell membrane (Vieira et al., 2016). The soluble components of yeast cells, specifically amino acids, peptides, nucleotides, and other water-soluble substances, are referred to as yeast extract. The biodiversity of the generated spent yeast can substantively influence the future composition in YE that contains physiologically important elements. There are significant differences in terms of protein, fat and ash contents when using various kinds of spent yeasts (Jacob et al., 2019a). Over the years, much effort has been given to the development of

alternative applications for yeast extracts from the spent Baker's yeast. Figure 2.6 shows the flow of yeast extract production from spent yeast upon production of alcoholic fermentation.

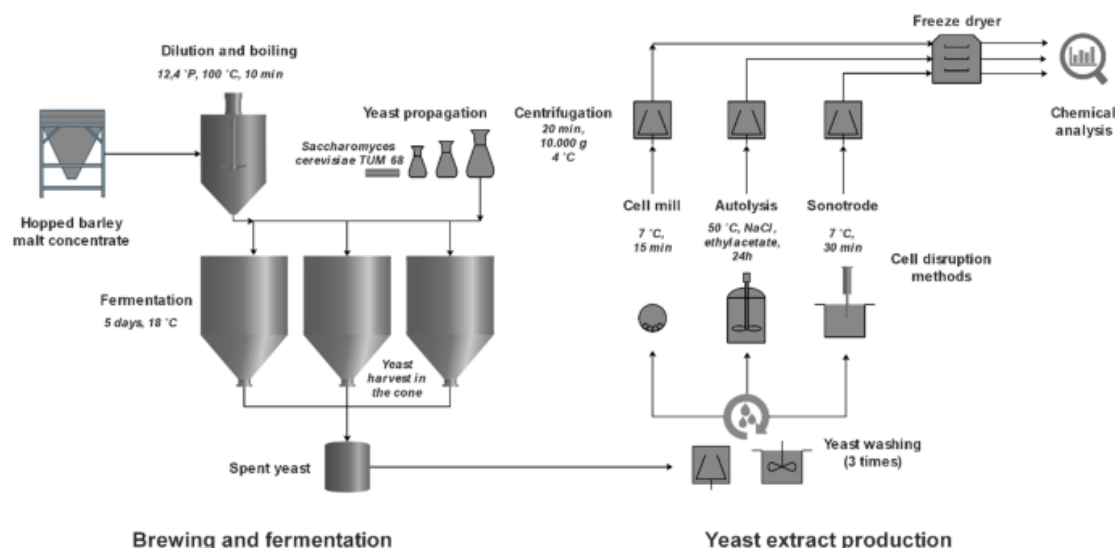


Figure 2.6: Pilot alcoholic fermentation process and yeast extract production. Adapted from Jacob et al., 2019b.

2.4 Potential Valorisation of Spent Baker's Yeast

2.4.1 Food Additives

The utilisation of brewer's yeast cells from the brewing industry to produce food-grade yeast extract seems promising since it has been categorised as generally recognised as safe (GRAS) by the US Food and Drug Administration (Shalaby et al., 2014). Thammakiti and colleagues (2004) demonstrated that spent yeast's β -glucans can be employed in food products as a thickening, water-holding, or oil-binding agent and emulsifying stabiliser. Several studies have shown that β -glucan extracted from yeast biomass can be beneficial in a variety of dietary applications. In a study by Worrasinchai et al. (2006), β -glucans are used as fat-replacing ingredient which functions as emulsifier in mayonnaise, which is an attractive alternative in low-fat food production. In another study, β -glucans from spent yeast were included into the manufacturing of β -glucan-rich flour. It was reported that the β -

glucans stabilised the dough's gas cells, producing higher volume and the porosity of the bread becomes more consistent (Martins et al., 2018).

2.4.2 Health and Nutritional Applications/ Functional Food Ingredient

Functional foods are being developed using some of the bioactive compounds found in spent brewer's yeast. The proteins present in spent yeast extracts made up of 64%, and they contained all of the essential amino acids, as well as RNA, vitamins (B3, B6, and B9), and minerals, which can be used as protein supplements and energy boosters (Podpora et al., 2015; Vieira et al., 2016). Several studies have also indicated that spent yeast contains higher quantities of functional elements such as polyphenols, flavonoids, carotenoids, and antioxidants (Vieira et al., 2016; Leon-Gonzalez et al., 2018; Marson et al., 2019). As a result of their immune-stimulating properties, these chemicals have a significant effect in preventing diseases such as cancer, cardiovascular disease, and neurological disorders (Pandey & Rizvi, 2009). A sufficient amount of B vitamins is required for the human body's optimal neurological and physiological processes (Moyad, 2008). Therefore, consumption of supplements derived from the spent yeast would be beneficial.

2.4.3 Flavour Enhancing Ingredients

There is a strong demand in the food industry for yeast extract as a flavour enhancer for foodstuffs, particularly for meaty, “umami” flavours (Alim et al., 2019). According to the authors, subjecting yeast cells to a heat treatment involving Maillard reaction can accelerate the process of cell death, which in turn enhances the production of new aroma compounds. These components including glutamic acid, 5'-guanosine monophosphate and 5'-inosine monophosphate nucleotides along with different amino acids and peptides function synergistically, contributing to the enhancement of the flavour in the foodstuffs (Rakowska et al., 2017). Vieira and co-workers (2013) reported that 5'-adenosine

monophosphate is a precursor to 5'-inosine monophosphate, a flavour enhancer 100 times more powerful than monosodium glutamate. This explains that yeast extract has the capability in replacing MSG as flavour enhancing ingredients.

The high nucleic acid concentration of yeast biomass, particularly ribonucleic acid (RNA), restricts its use for human consumption. An excess of purines in the human diet is known to elevate uric acid levels in the blood, potentially leading to uric acid deposition in joint tissues, especially in patients with purine metabolic failure (Nasseri et al., 2011; Saeed et al., 2016). Thus, yeast biomass must be further processed to reduce the nucleic acid content to 1%, in order to be considered as safe for human consumption (Nasseri et al., 2011).

2.4.4 Fermentation Substrate

Several studies have been carried out to assess the feasibility of using spent yeast extract as growth promoters in microbiological media. The use of yeast extracts as a nitrogen source for microbial growth has been demonstrated, particularly for the cultivation of fastidious bacteria. The low carbon-to-nitrogen ratio of spent yeast makes it useful as a component of fermentation media (Mathias et al., 2015). This is consistent with the findings by Mathias et al. (2017), who discovered that the addition of spent yeast's extract to lactic acid bacteria fermentation increased the acidity. In another study, the addition of yeast extract to lactic acid bacteria fermentation improved the nutritional and protective characteristics of vegetable juices (Rakin, et al., 2005).

As suggested by Sawisit et al. (2012), the extract from spent Baker's yeast could potentially serve as a nitrogen source in the fermentation of *Actinobacillus succinogenes* in order to produce succinate. Rojas and colleagues (2021) described the use of yeast protein hydrolysate as nitrogen supplementation in grape must fermentation. The yeast-based enzymatic protein hydrolysate was recovered from a prior wine fermentation. The results

showed significant positive influence of yeast extract as nitrogen supplementation on the increase of production of volatile compounds.

2.4.5 Ethanol Fermentation Supplementation

Studies on the effect of supplementing spent yeast on ethanol fermentation is still limited. York and Ingram (1996) reported that the high content of nitrogen and essential minerals in the autolysate derived from brewer's yeast was found to aid in the ethanol fermentation by recombinant *Escherichia coli*. Several studies suggested that yeast extracts can be used to supplement high-gravity fermentation to increase ethanol concentration and accelerate fermentation, possibly as a substitute for commercial yeast extracts (Kawa-Rygielska & Pietrzak, 2014; Palasak et al., 2019).

2.5 Composition of Baker's Yeast Extract

The composition of the growth substrates as well as the media determines the components that make up the yeast extract. According to a study conducted by Haddish (2015), the inclusion of glucose as a carbon source to the yeast cells, resulted in an increase in the yeast biomass.

Spent Baker's yeast extracts presented a rich source of proteins (64%) containing essential amino acids, RNA, vitamins (B3, B6, and B9) and minerals (Vieira et al., 2016). It was found that the yeast extract derived from fermentation serves a valuable source of amino acids such as tryptophan (11.9 mg/g), valine (69.7 mg/g), threonine (61.3 mg/g) and phenylalanine+tyrosine (90.6 mg/g). In addition, yeast protein biomass is abundant in all essential amino acids as recommended by FAO, making it an appealing option for food supplements (Podpora et al., 2015). The remaining quantity of nitrogen in the cell is mostly derived from nucleic acids, specifically RNA. Amino acids are the primary source of nitrogen, which accounts for 70–80% of the total nitrogen content of the cell. Compared to

more conventional protein sources, yeast protein biomass contains a comparatively high percentage of nucleic acids. This is a feature shared by all microbes capable of rapid growth (Ritala & Hakkinen, 2017).

Apart from that, polysaccharides and carbohydrates derived from the yeast cell wall constitute between 31% and 51% of the dry biomass (Haddish, 2015). Yeasts can produce saccharides with a broad range of molecular weights. The disaccharide trehalose and the oligosaccharides glucans and mannans are two examples. The highly stable molecule trehalose is composed of two glucose units (Otero et al., 2011).

2.6 Lysis Strategies of Spent Baker's Yeast

The techniques in disrupting the yeast cell are categorised as mechanical such as bead mill, homogenisation or ultrasonic treatment (Dimopoulos et al., 2020; Avramia & Amariei, 2022; Eom et al., 2022) and non-mechanical methods using electrical, physical, chemical and enzymatic treatment (Bzducha-Wrobel et al., 2014; Ganeva et al., 2015; San Martin, 2021). The number of compounds, such as amino acids, proteins, and sugars released from yeast cells is highly dependent on the lysing method used (Jacob et al., 2019a). The amount of energy expended in the process and the efficiency to recover the products, determines the cost-effectiveness of disruption method, which needed to take into consideration (Liu et al., 2016). The energy required to disrupt cells and the amount of recovered product from various approaches are compared in Table 2.2.

Table 2.2: Comparison of energy required for various techniques of cell disruption (Liu et al., 2016)

Method of disruption	Selective product recovery	Consumption of energy
Bead mill	Low	High/medium
High pressure homogenisation	Low	High/medium
Ultrasound	Low	High/medium
Physical treatment	Modest	Medium/low
Electrical treatment	Modest	Medium/low
Chemical treatment	High	Medium/low
Enzymatic lysis	High	Low

Non-mechanical procedures, such as autolysis and enzymatic hydrolysis, were employed in this study because due to practical and financial considerations. They are frequently used at laboratory scale, and are also gentler, resulting in high selective product recovery (Liu et al., 2016).

2.6.1 Autolysis

One of the conventional methods for lysing spent Baker's yeast is autolysis. The term "yeast autolysis" refers to a process that cannot be reversed, in which the yeast cell is disintegrated by its own enzymes. The process occurs spontaneously when a yeast cell's stationary phase of growth ends and the death phase begins (Cebollero et al., 2005). This process occurs after fermentation, when the nutrients have been used up and maximum ethanol content has been reached (Xu et al., 2014). The key event of autolysis is the degradation of cellular proteins which is the principal constituent of yeast extract. As a result,

proteins, peptides, and amino acids are released in the autolysate (Lukondeh et al., 2003). However, there are a few drawbacks associated with yeast autolysis, some of which include a lower extract yield, difficult phase separation, reduced quality in terms of taste and aroma as well as increased likelihood of microbial contamination due to autolysate residues (Bayarjargal et al., 2011).

A study on the kinetic of Baker's yeast autolysis and the role of related enzymes in hydrolysing the intracellular compounds (proteins, DNAs and RNAs) were previously studied by Verduyn et al. (1999) and Boonraeng et al. (2000). At an early phase of autolysis, proteinase and glucanase would start working first, by breaking down the yeast cell wall and glucans, respectively. When the yeast cell wall is disrupted, glucanase will be readily start functioning due to its location near the cell wall and mainly at periplasmic space (Reichelt & Fleet, 1981; Fleet, 1991). The components that are embedded in the cell wall or covalently bound to β -glucans particularly glucose, oligosaccharides, and mannoproteins are released as a result of β -glucan chains with β -O-glycosidic linkages hydrolysed by β -glucanases. Later, carboxypeptidase and proteolytic activity acts on the protein portion of the mannoproteins and digest the protein from different parts of cell into smaller components and producing amino acids (Charpentier & Feuillat, 1993). Proteolytic enzymes present in the yeast cells such as trypsin and chymotrypsin hydrolyse the proteins. Lysyl and argininy residues of protein, which supply the carbonyl portion of the peptide bond, are hydrolysed by trypsin, whereas tyrosine, phenylalanine, and tryptophan residues, which supply the carbonyl group of the peptide bond, are hydrolysed by chymotrypsin (Mirzaeia et al., 2015).

The morphological changes that take place in *S. cerevisiae* during autolysis are depicted schematically in Figure 2.7. There are four steps describing yeast autolysis. Firstly, simple proteins in the vacuole are degraded at the beginning of yeast autolysis due to the

depletion of nutrients. Following this, many hydrolytic enzymes are released into the cytoplasm and degradation of membrane-bound proteins begins. This is due to the simultaneous breakdown of the vacuolar membrane. These activated enzymes reacted with their specific substrate which then resulting in internal cell structure hydrolysis including the cell wall composition. The hydrolysis action on the cell wall weakens the cell membrane's selective permeability (cell wall becomes porous and crumpled) and the hydrolytic products are released across the cell wall via pores into the extracellular environment leading to the increase of autolysate contents. The process comes to the end when the yeast cell consumes the hydrolytic enzymes (Li et al., 2015).

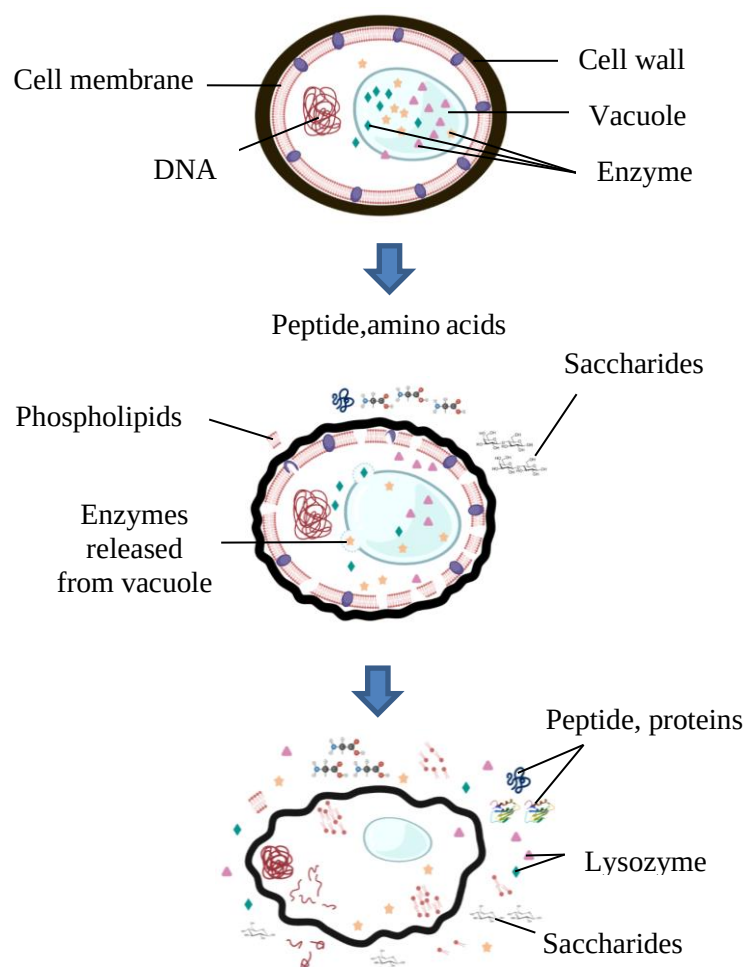


Figure 2.7: A schematic representation of morphological changes during autolysis in *S. cerevisiae*. Adapted from Wang et al., (2018)

In intracellular vacuoles of living cells, enzymes are found as inactive precursors. As energy is consumed within the cell, the structure of the cell begins to deteriorate. This gradual process is frequently accelerated by cell disintegration, rising temperatures, and the release of digestive enzymes. Autolysis is the most frequently manufacturing practice and the production of YE is required on a large scale within a shorter time for industrial purposes. This process can be initiated by physical means, for instance, thermolysis, alternate freezing, and melting, and osmotic pressure. Chemical inducers are also used including pH, detergents and antibiotics. It can be induced biologically by aeration and starvation (Alexandre, 2011).

2.6.1.1 Effect of pH on Autolysis

The optimal approach for producing yeast extracts involves modifying environmental variables, such as pH, to allow yeast cells to fully utilise the hydrolytic enzyme system in living cells.

The effect of pH on autolysis was previously studied by Li et al. (2015) using freshly grown baker's yeast cells. It is reported that the degradation by acid hydrolysis is preferable in the autolysis environment with pH value lower than 4.0 while pH value greater than 4.0 involves in enzymatic hydrolysis (Li et al, 2015). It indicates that pH 3.0 resulted in higher yields of total nitrogen (TN) and amino nitrogen (AN) in yeast autolysates compared to pH 4.0 until 7.0. However, pH 5.5 was chosen as the optimal pH in yeast extract powder production considering variety of factors including the flavour, TN and AN since pH 3.0 gave poor and bitter taste of YE powder.

The pH of the autolysis environment was reported to have a remarkable effect on the hydrolytic enzymes' activity in yeast cells particularly proteases (Slaughter & Nomura, 1992). Proteases A, B, and C are the three significant proteases that may be found in *S. cerevisiae*. The other two proteases are activated by protease A, which is an acidic protease

that functions most effectively in the pH range of 2 to 3. Protease B is most active at a pH of 9, whereas protease C, known as carboxypeptidase, performs digestion most effectively at a pH range of 5–6. In order for protease A to become active, there must first be some quantity of protease B present (Behalova et al., 1979; Boonraeng et al., 2000). The activity of yeast endogenous proteases is beneficial in slightly acidic or near-neutral conditions. The nucleases' activity is significantly affected when pH 3.0 or 4.0 is subjected and it is reported that nucleases work the best at pH 5.0-6.0, proposing as the optimal pH (Li et al., 2015).

The yield of final product was significantly influenced by the reaction time and pH value (Huang, 2010). The result shows that the optimum condition for the protein extraction from the yeast cell wall is 3 hr and pH value 6-7, where the maximum protein content was maximal up to 66.52% as compared to other pH values; pH 1-2 and 11-12. This can be explained that the proteins are easy to be hydrolysed into peptides with low polymerization degree. The cell membrane and vacuole membrane of yeast cells become more permeable due to acid hydrolysis under highly acidic conditions. It is due to the vacuolar degradation, protease inactivation and hydrolysis of macromolecular components released into the yeast autolysate. As a result, there are differences in the osmotic pressure across the membrane leading to a high amount of intracellular water loss and plasmolysis (Li et al., 2015).

The endogenous enzymes possess different optimum temperature, Protease A (35-40 °C), Protease B (45-55 °C) and carboxypeptidase (60 °C). This justifies the high level of extracted yeast components as temperature rises (Alves, 2021). Despite their effectiveness above 40 °C, these enzymes are inhibited at 50 °C, except for carboxypeptidase, which works effectively at 50 °C and is resistant to higher temperatures. At 40 °C, the activity of protease B is primarily responsible for proteolysis. Carboxypeptidase produces free amino acids during the final phase of autolysis (Behalova et al., 1979; Boonraeng et al., 2000).

2.6.2 Enzymatic Hydrolysis

Researchers are currently focusing primarily on enzymatic hydrolysis which is an efficient way to facilitate the degradation of the yeast cell and to release intracellular compounds. In contrast to autolysis, this mild hydrolysis technique provides greater process specificity, which is accomplished with the addition of enzymes derived from microbes or other exogenous enzymes responsible in the breakdown of proteins in the cell wall (Chae et al., 2001; Martinez-Rodriguez et al., 2001). Furthermore, the activity of endogenous enzymes is increased by these enzymes, which leads to the release of intracellular compounds and the production of hydrolysates that have a superior sensory quality, improved functional and biological functions, and low salt content (Torresi et al., 2014; Takaloo et al., 2020).

The exogenous proteases efficiently hydrolysed the cell wall proteins by cleaving bonds within the polysaccharide matrix. This action gradually collapses the intact cell wall structure, followed by cytoplasmic membrane collapse and leakage of intracellular contents such as hydrolysed proteins, which are rich in non-glycosylated soluble proteins (Comuzzo et al., 2012; Martinez-Maqueda et al., 2013; Takaloo et al., 2020). Protein recovery is a key factor in yeast enzymatic hydrolysis (Chae et al., 2001). Proteolysis is termed as solubilisation or hydrolysis of yeast insoluble protein and protein-bound intracellular molecules into smaller peptides and into the respective constituent amino acid residues due to the action of protease enzymes. The amount of carbohydrate that is liberated in the yeast hydrolysate is another important indicator of enzymatic hydrolysis. This carbohydrate is primarily obtained from the yeast cell wall polysaccharides (Vukašinović-Milić et al., 2007).

2.6.2.1 Previous Works on Enzymatic Hydrolysis

Enzymatic hydrolysis is influenced by a variety of parameters, including the selected enzyme preparations and the reaction conditions, which are influenced by the type and dosage of enzyme used, affecting the total yeast extract content recovery (Vukašinović-Milić et al., 2007; Marson et al., 2020). The usage of commercial lytic enzymes has been documented in literatures, such as glucanase, papain, pancreatin, flavourzyme, brauzyn, lyticase and alcalase (Conway et al., 2001; Vukašinović-Milić et al., 2007; Bayarjargal et al., 2011; Marson et al., 2019). According to past studies, proteolytic activity (proteases or peptidases), RNA degrading activity (nucleases), or deaminase activity (deaminases) are among the enzyme activities employed in yeast enzymatic hydrolysis (Chae et al., 2001; Vukašinović-Milić et al., 2007; Cao et al., 2017). Several enzymes such as β -glucanase, lyticase and β -mannanase have been utilised in catalysing the hydrolysis of yeast cell wall (Conway et al., 2001; Vukašinović-Milić et al., 2007; Cao et al., 2017).

In a recent study, a combination of proteases (alcalase, brauzyn, and protamex) was found to have an effect on the solids and crude protein contents recovered from spent yeast. These contents accounted for 57% (w/w) and 70% (w/w), respectively, after protein hydrolysis. These proteolytic enzymes synergistically improved the physicochemical and antioxidant characteristics of the spent yeast protein hydrolysate (Marson et al., 2020). A combined hydrolysis treatment using proteases (protamex and flavourzyme) followed by nucleases (5'-phosphodiesterase and AMP-deaminase) were developed in hydrolysing spent yeast (Chae et al., 2001). A high solid yield of 55.1% and 5'-nucleotides content of 3.67% were obtained after the treatments. The protein recovery, degree of hydrolysis and sensory properties have been greatly influenced by the exoprotease dosage, indicating that

exoprotease (flavourzyme) is the main factor in hydrolysing yeast protein rather than endoprotease (protamex).

A strategy on transforming spent *S. cerevisiae* into biomolecules of added value by sequential enzymatic digestion using brauzyn and alcalase has been documented (Marson et al., 2019). Brauzyn is a plant-derived protease that possesses both endo and exoactivities, whereas alcalase only displays endoprotease activity. The enzymes possess distinct enzymatic functions, which contributes to the higher degree of hydrolysis. The authors reported that the hydrolysate has the potential to be used in the food sector as a peptide-rich product with antioxidant properties since it constitutes maximum solid recovery and antioxidant properties. Furthermore, proteolytic hydrolysis on spent yeast was optimised by using alcalase and flavourzyme via continuous overflow system, resulted in the yield of hydrolysis as $59.62\% \pm 0.027$ is (Ngoc et al., 2016).

The specificity of the enzymes and the type of enzymes utilised both play a role in determining the peptides profile produced during the enzymatic treatment. The influence of exogenous enzymes (flavourzyme and pancreatin) on enzymatic hydrolysis was determined by Bayarjargal et al. (2011). Pancreatin's enzymatic treatment is more extensive than flavourzyme, resulted in the release of 14% and 49.5 mg/ml of soluble solids content and soluble protein concentration in hydrolysates, respectively. In this case, yeast endogenous enzymes and pancreatic enzymes complex including proteases with different specificities are involved in a synergistic activity. The enzymatic hydrolysis of spent yeast yielded 222.27 mg/g of low molecular weight peptides by employing Neutrase under optimum conditions (yeast concentration of 22.72 % (w/w), protease concentration of 4.07 % for 16.73 hours (Hien., 2016).

The enzyme dosage has a significant impact on the recovery of yeast cell constituents. In a study by Vukašinović-Milić and colleagues (2007), they found that 2.5% and 0.025% of papain and lyticase, respectively were the optimal dosages for the recovery of solids, proteins, and carbohydrates. Lyticase was found to be a great enzyme in poly-(β -1,3-glucose) hydrolysis, releasing cell wall carbohydrates such as yeast cell wall glucan, resulted in maximum degradation. Under optimum conditions, yeast extract treated with papain had a protein concentration of 56.38 mg/ml and a solid recovery of 61.95%. It was discovered that a considerable number of larger molecular polysaccharides are degraded into smaller oligosaccharides via the hydrolysis of spent yeast utilizing β -mannanase (Cao et al., 2017). As a consequence, it improves the solubility of spent yeast which facilitates their functional use. After the enzymatic treatment, more compounds were released accounting for the improvement of the antioxidant properties and solid content.

2.6.2.2 Alcalase

Protein recovery is important in yeast enzymatic hydrolysis (Chae et al., 2001). Yeast peptides and their associated amino acid residues are released from insoluble yeast proteins and protein-bound intracellular components due to the proteolytic activity of proteases. The proteins derived from cell wall are hydrolysed by the proteolytic activity, releasing mannoproteins (Chae et al., 2001; Marson et al., 2020). Proteases falls under class 3, and subclasses 3,4.-, of the hydrolase class of enzymes, as defined by the international enzyme classification and nomenclature system, enzyme commission (EC number) (McDonald, 1985). Proteases have different specificities and selectivity based on the enzymes used to hydrolyse the final product or protein extract. The configuration (tertiary structure) of the enzyme active site in which catalytic amino acid residue is involved influences the interaction of the catalytic sites with the protein substrate chain (Polgár, 2005).

Subtilisin is a protease initially obtained from *Bacillus subtilis* and it belongs to subtilases, a group of serine proteases that attack on peptide bond through a serine residue at the active site (Siezen & Leunissen, 1997). Subtilisins are categorised into subfamilies: true subtilisins, highly alkaline proteases, intracellular proteases, intermediate subtilisins, and high-molecular-weight subtilisins (Rozanov et al., 2021). Initially, the first widely used alkaline serine protease was subtilisin A (EC 3.4.21.62). It was isolated from *Bacillus subtilis* and formerly known as “Subtilisin Carlsberg” and subtilopeptidase A (Smith et al., 1996). The industrial subtilases were obtained from various *Bacillus* species, and it is commercially known as Alcalase®, Esperase®, Savinase®, subtilisin DY and bacillopeptidase (Eschenburg et al., 1998; Ellaiah et al., 2002; Omura et al., 2005; Vasconcelos et al., 2006; Garcia-Mora et al., 2014).

Alcalase is an endoprotease of serine type, produced from *Bacillus licheniformis* (Ellaiah et al., 2002). This enzyme, which Novozymes Corporation marketed under the brand name "Alcalase," has the activity of ≥ 2.4 U/g. It is made up of glycerol (50%, w/w), water (41%, w/w), and protease extract (9%, w/w). Despite being nonspecific, alcalase is an alkaline enzyme that has great ability for protein hydrolysis, with an optimal temperature range between 50 and 70 °C and an optimal pH range between 8 and 10, which may mitigate the risk of microbial contamination (Chabeaud et al., 2009). Alcalase has been applied in cleaning products, textiles, and food production (Kim et al., 2016; Singh & Bajaj, 2017).

A number of studies have been published on the utilisation of alcalase in enzymatic hydrolysis of *S. cerevisiae*, to accelerate the release of intracellular contents into the extracellular media (Ngoc et al., 2016; Xie et al., 2017; Marson et al., 2019; Takaloo et al., 2020). It has been reported to be one of the highly efficient bacterial proteases used to prepare yeast protein hydrolysates. Yeast cells treated with alcalase hydrolysis were more efficiently

hydrolysed than those treated with papain, as reported by Xie et al. (2017). Furthermore, alcalase has been documented to be a better candidate for hydrolysing yeast cells as compared to autolysis and plasmolysis. Takaloo et al. (2020) demonstrated that the treatment of yeast cells with alcalase (0.2%, w/w) releases 52.1% of soluble solids and 27.9% proteins in the hydrolysate at its optimum conditions (pH 7.0, 55 °C). After 48 h, the protein recovery and degree of hydrolysis for alcalase reached up to 67.74% and 47.50%, respectively. Both of these proteases are endoproteases but however, they are different since alcalase is a subtilisin protease while papain is a cysteine protease. Moreover, at high solid concentration (28%, w/w), their research demonstrated that hydrolysis strengthened the interaction between enzyme and substrate, leading to a significant degree of hydrolysis. *B. licheniformis* serine endoprotease is a protein hydrolase that cleaves mostly at the amino acids such as tyrosine, phenylalanine, leucine, and methionine, leading to shorter peptides and proteins.

Alcalase is a group of serine protease and the reaction mechanism of a serine protease is shown in Figure 2.8. The catalysis begins when the carbonyl carbon of the scissile peptide bond of the substrate is attacked by the nucleophilic group of serine (-OH) (1). The serine hydroxyl group donated proton (hydrogen) to the nitrogen of histidine. The catalytic triad facilitates the proton transfer, with aspartate stabilising the increasing positive charge. As a result, a tetrahedral intermediate bearing an oxyanion is generated. The peptide bond joining the carbon and the nitrogen cleaved as a result of nitrogen binding to hydrogen from histidine, releasing the first half of polypeptides (N-terminus of the peptide) from the enzyme (2). The other half of polypeptide is covalently bounded to serine. The acylation step (1. and 2.) of the reaction is completed when the amino-terminal component of the substrate

esterified to the enzyme serine in a covalent acyl intermediate, releasing the carboxy-terminal portion

The deacylation (3. and 4.) is an acylation reversal in which a water molecule replaces the amine component of the substrate. At the end of the reaction, water is introduced into the catalytic site of the enzyme, and the nitrogen (histidine) attacks the proton (hydrogen) from the water molecule, activating oxygen for nucleophilic attack. The hydroxide from the water molecule attacks carbonyl carbon on serine, forming another tetrahedral intermediate. Oxygen attacks hydrogen on histidine, resulted in the cleavage of bond with serine, releasing second half of polypeptide chain (C-terminus of the peptide). The cycle is completed when the enzyme returned to its original state (Schaller, 2004; Polgár, 2005).

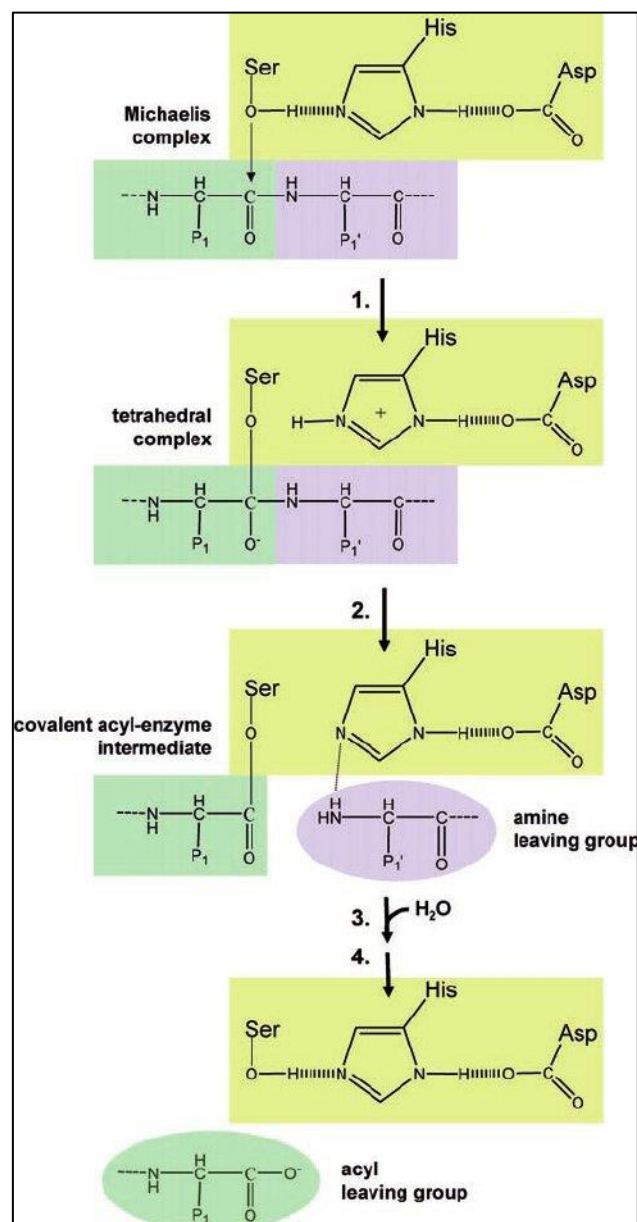


Figure 2.8: A schematic representation of reaction mechanism of a serine protease.

Adapted from Schaller (2004). The catalytic triad of the protease is shown in yellow, and the portions of the substrate that are amino-terminal and carboxy-terminal to the scissile bond are marked in green and purple, respectively. The presence of hydrogen bonds and the continuing of the polypeptide chain in the substrate are represented by the broken lines

2.6.2.3 Cellulase

The carbohydrate-active enzyme (CAZyme), carbohydrases are a group of enzymes that can degrade or synthesize glycosidic bonds found in carbohydrate-based structures. CAZymes are divided into three classes based on their activities: glycoside hydrolases (GHs), glycosyltransferases (GTs), and polysaccharide lyases (PLs) (Lombard et al., 2014). The usage of cellulase in the enzymatic hydrolysis of *S. cerevisiae* is scarcely reported. However, in a recent study several CAZymes has been utilised in hydrolysing the yeast cell wall, being cellulase one of them (Gameiro, 2017; Garcia, 2017).

Cellulase is categorised as glycoside hydrolases and can be synthesised by microorganisms such as fungi and bacteria during their growth on cellulosic material (Ejaz et al., 2021). Out of all the organisms, *Trichoderma reesei* is the most studied organism in the production of cellulase. This fungal cellulase optimum conditions was determined at pH 5.0 and exhibit maximum activity at 55 °C (Herlet et al., 2017). The hydrolysis of cellulose into fermentable sugars and ethanol requires the usage of cellulase, which has seen a significant growth in its application, particularly in the biofuel sector (Srivastava et al., 2015). It has also been employed in pulp and paper industry, to improve paper properties (Garcia et al., 2002) and improving the digestibility of animal feeds (Murad & Azzaz, 2010). Figure 2.9 shows mode of action of various components of cellulase.

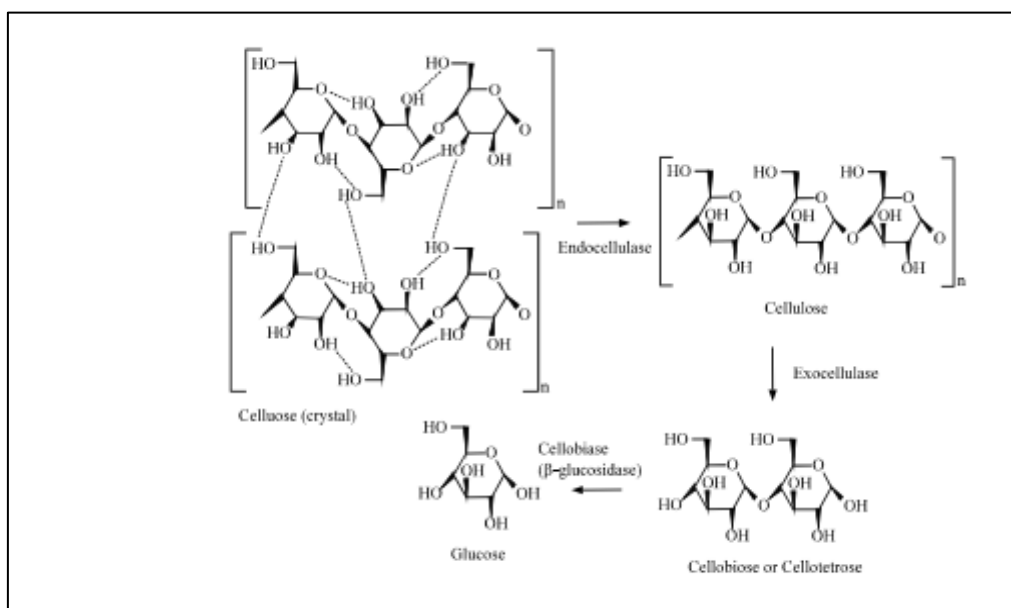


Figure 2.9: The mechanism of action of cellulase and its constituent parts.
Adapted from Karmakar & Ray, (2011)

T. reesei can simultaneously produce multiple of enzymes. It was reported that *T. reesei* produces seven β -glucosidases, eight endo- β -1,4-glucanase and two cellobiohydrolase components (Sakka et al., 2000). In cellulase system, β -glucosidase, endo-1,4- β -D-glucanase (endoglucanase) and exo-1,4- β -D-glucanase (exoglucanase) act synergistically. The cellulose binding domain (CBD) or the carbohydrate binding module (CBM) which are connected through a short poly-linker region and the catalytic domain (CD) are the two components that make up cellulases. The catalysis of the enzyme occurs in CD, whereas the cellulose binding occurs in the CBDs (Ohmiya et al., 1997; Sakka et al., 2000).

A complete hydrolysis of the β -1,4-glycosidic linkages in cellulose present in plant cell wall requires synergistic actions of these three enzymes to release glucose (Patel et al., 2019). The enzymes function as follows; endoglucanases (β -glucanases) catalyse the scission of cellulose chains on the inner sites of oligosaccharides found in carboxymethyl cellulose, opening the reducing and non-reducing ends and producing cello-oligosaccharides. In addition, the cleavage of cellulose chains at their non-reducing ends by

exoglucanases yields cellobiose or glucose. The hydrolysis stage is completed when β -glucosidases facilitate the release of glucose from cellobiose (Murad & Azzaz, 2010).

Cellulase from *T. reesei* present activities specifically to digest plant cell walls not yeast cell wall since it does not exhibit β -(1,4) bonds (Klis et al., 2006). However, this enzyme has been reported to release reducing and free sugars in hydrolysates from yeast cells. This is supported by the activity of this enzyme, which releases glucose chains from yeast β -(1,3)/(1,6)-glucans (Gameiro, 2017; Garcia, 2017). Furthermore, enzymes from *T. reesei* with β -glucanase activity attacked β -(1,3)-glucans which are the major polysaccharide component of the yeast cell wall, releasing oligosaccharides from glucan or mannan (Vukašinović-Milić et al., 2007). According to Gameiro (2017) and Garcia (2017), these enzymes present other unknown side activities that have not been described since *T. reesei* can produce many enzymes simultaneously.

2.7 Bioethanol Production from Sago Fibre Fermentation

A growing demand for fuel as an energy source, which relies mostly on fossil fuels, have prompted concern throughout the globe due to the scarcity of petroleum reserves and the ever-increasing price of fuel (Schubert, 2006; Aditya, 2016). In addition, this non-renewable fuel caused problems worldwide, due to the accumulation of greenhouse gases that contribute to environmental pollution and climate change. All of which have encouraged an alternative environmentally friendly, renewable and sustainable energy sources to be promoted by government, industry, and energy companies (Demirbas, 2009; Shafiee & Topal, 2009). Therefore, at present, biofuels are the most feasible and attractive way to substitute fossil fuels as the main source of energy, accounting for 40% of global energy consumption (Tan et al., 2008).

Bioethanol (C_2H_5OH) or ethyl alcohol is referred to as a widely used biofuel, serves as a promising, renewable energy alternative to fossil fuel which is used widely in automobile sector (Azhar et al., 2017). As of now, ethanol is typically used purely or blended with gasoline to produce gasohol, which is a cleaner alternative for gasoline and helps keep the price of fuel more economical (Staniszewski, 2007; Balat et al., 2008). Biofuel has a higher-octane number (108), a wider flammability range, a faster flame, and more heat of vaporisation than gasoline (Balat & Balat, 2009). Growing the biomass feedstocks used in biofuel production may balance the carbon dioxide released when biofuels are consumed which in turn, reduces the greenhouse gas emissions (Schubert, 2006). Commercial bioethanol production has traditionally relied heavily on sugarcane, corn, and potatoes (referred to as "first generation feedstock") due to the accessibility of their starch sources during the fermentation process (Sadia et al., 2021). However, the practice is undesirable and controversial considering food sustainability, high production costs, and high market prices (Aditya et al., 2016; Daylan et al., 2016). Therefore, second generation bioethanol has been explored by utilising non-food substrates such as lignocellulosic agricultural residues (corn stove, rice straw, and sago fibre) (Awg-Adeni et al., 2013; Anil et al., 2020; Zhao et al., 2020). Tye et al. (2016) suggested that these resources can be an excellent raw material for bioethanol production since they are not in competition with agricultural crops.

After the extraction of starch from the pith of the sago palm, a starchy lignocellulosic by-product known as sago fibre is produced (*Metroxylon sagu*, Rottb.). In Malaysia, the plantation of sago palm is mostly concentrated in Sarawak, also as the biggest exporter of sago starch worldwide. In 2013, it was estimated that Sarawak had a total planted area of 54,087 hectares of sago, and approximately 47,946 tonnes of sago starch were exported, primarily to Peninsular Malaysia, Japan, Singapore, and Thailand (Bukhari et al., 2017).

Awg-Adeni et al. (2010) found that after the extraction of starch, organic wastes (organic-rich effluent and solid residues) were discarded near the factory compound or in the nearby streams which lead to a reduction in water quality and pollution of the environment (Cecil, 2002). According to sago manufacturers, one tonne of sago pith residue (SPR) is generated for every tonne of sago starch produced (dry weight basis) (Bukhari et al. 2017). Figure 2.91 shows sago fibre powder after the grinding process.



Figure 2.91: Ground dried sago fibre

The feasibility of sago residues as a feedstock in bioethanol production by *S. cerevisiae* was previously studied (Awg-Adeni et al., 2013; Thangavelu et al., 2014, Bukhari et al., 2017). In addition to its free and abundant nature, sago fibre contains a mixture of 58% starch, 23% cellulose, 9.2% hemicellulose, and 4% lignin (Linggang et al., 2012). The residue contains a significant amount of starch and cellulose, which is a crucial carbon source for the synthesis of bioethanol. The process of creating bioethanol from sago waste should go through the following stages: pre-treatment, hydrolysis, and fermentation process. The main purpose of pre-treatment is to minimise particle size, which increases surface area. As

a result, the decrystallisation of cellulose is improved, making subsequent operations more effective and simpler (Rajendran et al., 2018).

In hydrolysis process, there are two stages involved (liquefaction and saccharification) which facilitate the conversion of complex carbohydrates into simpler sugar such as glucose which makes them suitable to be used as a substrate for fermentation (Galbe & Zacchi, 2014). During the hydrolysis of sago fibre, the enzymes serve as catalysts to break down the glycosidic bonds of the starch, which results in the production of glucose. A gelatinisation process is an essential step before saccharification in order to get a high hydrolysis yield. It involves the degradation of the intermolecular bonds that are present in starch molecules, reducing the crystallinity of the starch granules and making them more susceptible to the action of enzymes (Kumoro et al., 2008). The next step is liquefaction by α -amylase, which partially hydrolyse starch to oligosaccharides. During saccharification of the partially-hydrolysed sago fibre, glucoamylase is employed to hydrolyse glucosidic bonds in starch, releasing glucose units (Bukhari et al., 2017). The hydrolysate obtained after the sago fibre is hydrolysed serves as a feedstock for *S. cerevisiae* to ferment into bioethanol. (Mohammad et al., 2020).

According to Awg-Adeni et al. (2013), substrate loading of 7% (w/v) was suitable for the hydrolysis process, both for the gelatinization reaction and for the saccharification reaction. As substrate loading increases to 9% (w/v) or more, enzymatic hydrolysis becomes more challenging, and yields are reduced. It was demonstrated that enzymatic reactions carried out with high soluble solid consistency caused the mixture to thicken, requiring more energy for mixing, to shear inactivate the enzymes, and to cause poor heat transfer in dense fibrous suspensions (Gupta et al., 2012). Figure 2.92 shows A flow diagram for bioethanol production from sago fibre residues by using enzymatic hydrolysis.

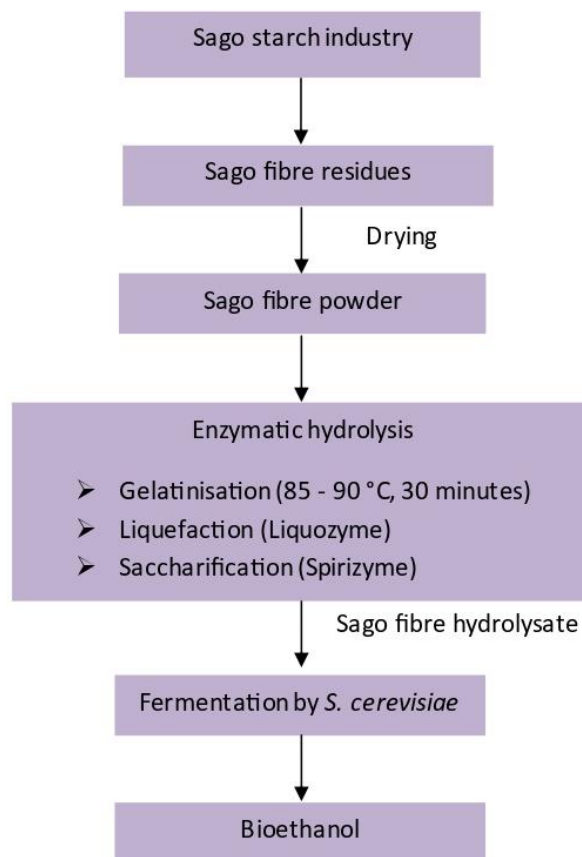


Figure 2.92: A flow diagram for bioethanol production from sago fibre residues.
Adapted from Mohammad et al., 2020

Following the sago bioethanol fermentation, spent *S. cerevisiae* constitutes as a by-product apart from the spent broth. It was recently reported that the liquid waste could be used as a feedstock for the production of recombinant laccase (Mamat et al., 2021). However, the potential and fate of the solid waste produced after sago bioethanol fermentation, particularly the spent *S. cerevisiae*, are not well understood. Therefore, further study is required for the exploitation of the solid waste generated from sago bioethanol production.

CHAPTER 3

MATERIALS AND METHODS

3.1 Pre-treatment and Hydrolysis of Sago Fibre

The pre-treatment procedure was based on a protocol developed by Awg-Adeni et al. (2013). The sago fibre was supplied by Herdsen Sago Mill, Pusa, Saratok, Sarawak and dried in an oven (Memmert UNB 400, Germany) at 80 °C for 24 hr. The dried sample was ground by using a grinder machine (Khind BL1012, Malaysia) and sieved to pass through a 1 mm (20 mesh) screen sieve prior to produce sago fibre powder (SFP). The resulting SFP was used as the raw material for bioethanol fermentation. Approximately, 7% (w/v) of sago fibre suspension was gelatinised at 90-100 °C for 30 min. Subsequently, the resultant slurry was then subjected to a 30-minute liquefaction stage during which 2 µL/g of Liqueozyme® (Novozymes, Denmark) was added and continuously agitated for 30 min to ensure enzyme and substrate homogeneity was achieved. The suspension was allowed to cool down and 1 µL/g of Spirizyme® (Novozymes, Denmark) was added for the saccharification process. After that, the mixture was left to incubate at 50 °C for overnight. Sago fibre hydrolysate (SFH) was recovered by separating the remaining lignocellulosic fibre from the solution using a 100-mesh sieve filter. The glucose concentration of the recovered SFH was analysed by using High Performance Liquid Chromatography (HPLC). Figure 3.1 shows the hydrolysate obtained after the sago fibre hydrolysis.

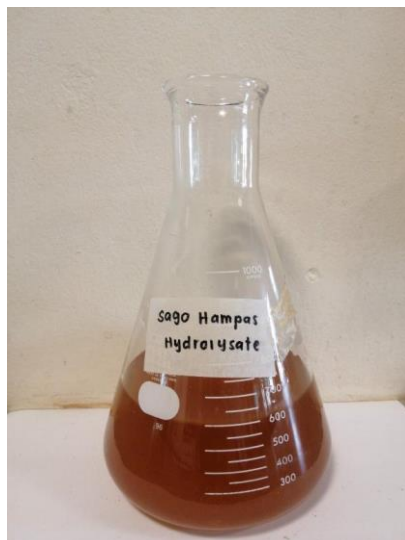


Figure 3.1: Hydrolysate collected after the hydrolysis process of sago fibre

3.2 Bioethanol Fermentation using Sago Fibre

3.2.1 Preparation of Inoculum

An instant dry baker's yeast, *S. cerevisiae* (Mauripan Baking Industry, Malaysia) was bought from a local market. The growth medium including yeast malt broth (YMB) and potato dextrose agar (PDA) were prepared aseptically in a laminar air flow. In a 50 mL sterile flask containing 30 g/L of yeast malt broth (YMB), 1 g of yeast grain was inoculated and incubated on a rotary shaker (Digital Orbital Shaker, TS-560D, Yihder Co., Ltd, Taiwan) for 24 hr at room temperature with shaking speed of 100 rpm (Awg-Adeni et al., 2013). Then, the yeast culture in YMB was streaked onto PDA and incubated at 30 °C for overnight (Figure 3.2).

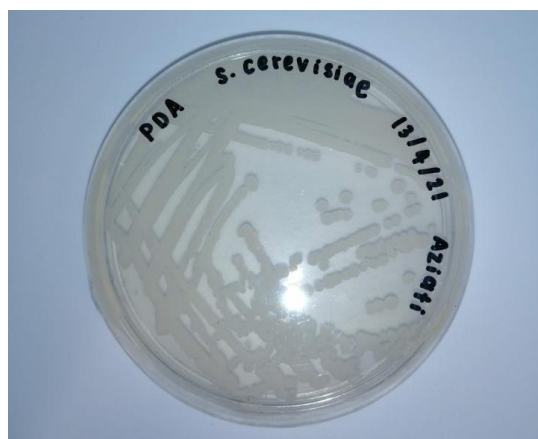


Figure 3.2: *S. cerevisiae* cultivated on plate

Single yeast colony from the PDA was inoculated into a sterilised inoculum media that had been supplemented with 20 g/L commercial glucose (Sigma-Aldrich Company, USA) and 5 g/L yeast extract (Oxoid, UK). Then, it was incubated at 30 °C for 7-9 hr or until the reading of optical density (OD) reached 0.5–0.6 using a spectrophotometer (UVmini-1240, Shimadzu, Japan) at 600 nm. The inoculum media was centrifuged (LC-8 Series microcentrifuge, Benchmark Scientific, USA) for 5 minutes at 8000 rpm to recover the yeast pellets for the fermentation process (Awg-Adeni et al., 2013).

3.2.2 Bioethanol Fermentation

Bioethanol fermentation was carried out by *S. cerevisiae* using the SFH generated (Figure 3.3) from the previous section (Section 3.1) as the fermentation media (Awg-Adeni et al., 2013). A working volume of 250 mL of fermentation media was prepared in triplicates and supplemented with 5 g/L of yeast extract (Oxoid, UK). The fermentation process was conducted via batch system and the initial concentration of glucose in fermentation media was obtained to approximately 50.0 g/L. The initial pH of the medium was set to 5.5 by using a pH meter (Benchtop pH Meter, PHS-3BW, Bante Instruments, China) and adjusted by using 2M NaOH or 10% (v/v) H₂SO₄ before sterilisation. The cultures were incubated at room temperature on an orbital shaker (Digital Orbital Shaker, TS-560D, Yihder Co., Ltd,

Taiwan) at 150 rpm for 48 hr. For control experiment, the fermentation was conducted using commercial glucose (Sigma-Aldrich Company, USA) as a feedstock with an initial glucose content of 50 g/L and supplemented with YE (5 g/L). The initial pH was set at pH 5.5.

Aliquots of the fermentation broth was sampled at every 8 hours to analyse for the glucose and ethanol profiles using HPLC. Yeast cells were separated from the culture broth via centrifugation after fermentation was completed and then subjected to autolysis and enzymatic hydrolysis. Figure 3.3 shows the spent broth after 48 hr of fermentation derived from fermentation of (a) sago bioethanol and (b) control experiment.



Figure 3.3: (a) Sago bioethanol; (b) Control experiment

3.3 Recovery of Spent Baker's Yeast

The spent yeast was recovered following sago bioethanol fermentation (Figure 3.4) by centrifuging the fermentation broth for 10 minutes at 3000 rpm and at 4 °C (Eppendorf, 5810 R). The resulting yeast sediment was subjected to three washing processes to remove any fermentation residues by diluting it with sterile distilled water using a volume ratio of 1:3 (yeast sediment: distilled water) (Boonyeun et al., 2011).

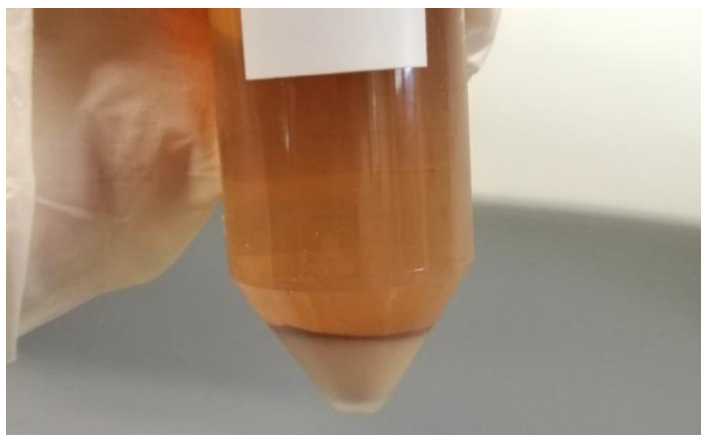


Figure 3.4: Spent yeast recovered at the bottom of a 50 mL falcon tube

3.4 Autolysis of Spent Baker's Yeast

Modified methods proposed by Boonyeun et al. (2011) and Li et al. (2015) were used to study the effect of different initial pH (3,5, and 7) in autolysing the spent *S. cerevisiae*. The yeast suspensions with final concentration of 14% (w/v) were prepared via batch system in triplicates by mixing the spent yeast slurry in 100 mL of 0.05 M sodium phosphate buffer. The buffers were prepared according to the pre-lab calculations as shown in Appendix A. Based on Figure 3.5, the flasks containing yeast suspensions were incubated in a shaking water bath (Digital Precise Shaking Water Bath, Daihan, Korea) at a controlled temperature of 55 °C, at 100 rpm for 96 hr. The control sample was prepared without adding any inducers of autolysis to the yeast suspension as a benchmark to compare against the effect of inducers

in test results in lysing the spent *S. cerevisiae*. This was done by diluting the suspension in sterile distilled water to achieve a final concentration of 14% (w/v) and left at room temperature without stirring. The initial pH in the control flask was determined at pH 5.6 by using a pH meter.



Figure 3.5: Incubation of yeast suspension in a waterbath shaker

During the autolysis, aliquots of the batch suspension were withdrawn at every 24 hours to analyse protein, carbohydrate concentrations and surface morphology. The harvested aliquots were transferred into two tubes. Figure 3.6 shows the two portions of the aliquots withdrawn from the suspension. The first tube (a), 2 mL of the aliquots was immediately centrifuged to recover the yeast pellet for the observation of the cell morphology. For the second tube (b), another 2 mL of the aliquots was heated at 100 °C for 5 minutes to inhibit the activity of the enzyme before it was centrifuged at 3000 rpm for 10 minutes to recover the supernatant (yeast extract) (Takaloo et al., 2020). The resulting supernatant was analysed for protein and carbohydrate contents.

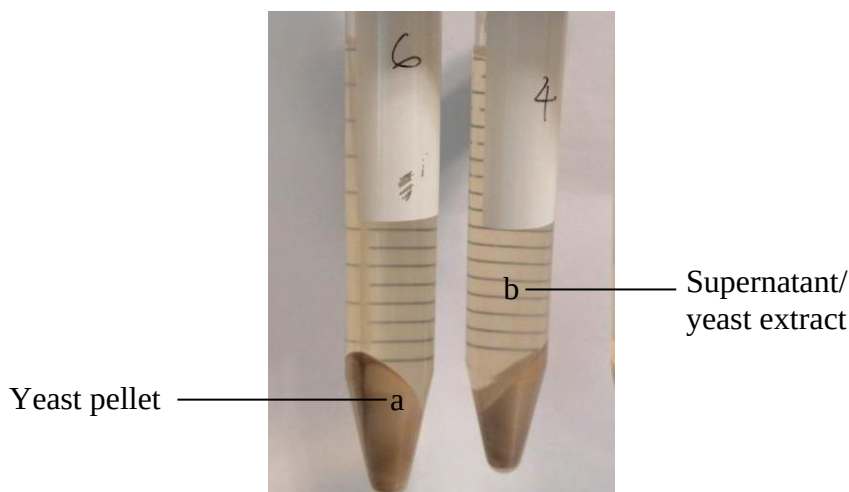


Figure 3.6: Two portions of the sampled suspensions, after centrifugation to recover (a): yeast pellet and (b): supernatant or yeast extract

3.5 Enzymatic Hydrolysis of Spent Baker's Yeast

In this section, the effect of different types of enzymes and different concentrations on yeast hydrolysis were studied. Two types of enzymes were used in enzymatic hydrolysis of spent *S. cerevisiae* namely, alcalase (EC 3.4.21.14) (Millipore, USA) from *B. licheniformis* and cellulase (EC 3.2.1.4) (Sigma-Aldrich, Denmark) from *T. reesei*. The enzymatic hydrolysis was carried out following the methods described by Xie et al. (2017) and Takaloo et al. (2020). The spent yeast slurry was mixed with sterile distilled water to a final concentration of 14% (w/v). The enzymes were added at different concentrations (0.1-0.5%, v/v) into the batch suspension culture, separately. The initial pH was adjusted accordingly to the type of enzymes. The alcalase hydrolysis was carried out at an initial pH of 7.0 and 55 °C (Takaloo et al., 2020). On the other hand, the cellulase hydrolysis was carried out at the same temperature but with an initial pH of 5.5 (Nataraja et al., 2010). Aliquots of the batch suspensions were withdrawn at every 24 hr during the enzymatic hydrolysis to analyse protein, carbohydrate and surface morphology.

3.6 Analyses

3.6.1 High Performance Liquid Chromatography (HPLC)

HPLC was used for the quantitative analysis of bioethanol and glucose (Shimadzu, Kyoto, Japan). The system used a refractive index detector to monitor the temperature within the column oven, which was kept at 65 °C and equipped with an Aminex HPX-87H column (300 mm x 7.8 mm, Bio-Rad Co., USA). The supernatant from the fermentation broth was retrieved from section 3.6. The samples were filtered through 0.45 µm nylon membrane filters and injected into the HPLC with a volume of 20 µL. At a temperature of 60 °C, the column was eluted with a mobile phase consisting of 0.005 M sulphuric acid at a flow rate of 0.8 mL/min (Vincent et al., 2015). Glucose and bioethanol had retention periods of 4.1 and 8.9 minutes, respectively (refer to Appendix F).

3.6.2 Protein Analysis

The protein content of the hydrolysate was assayed based on Lowry method by using bovine serum albumin, (BSA) as a standard at 2 mg/mL (Lowry et al., 1951). In a 500 mL volumetric flask, BSA stock solution was prepared at 100 mg/L and diluted (refer to Appendix A). In each test tube, 0.1 mL of solution from each dilution was transferred, and 2N NaOH was added. The mixtures were mixed using a vortex mixer (Vortexer, Heathrow Scientific, USA) and hydrolysed at 100 °C for 10 min. Then, 1 mL of freshly mixed Lowry solution (refer to Appendix A) was added into each tube and incubated for 10 min. The mixture was then added with 0.1 mL of diluted Folin reagent and incubated for another 30 min at room temperature in a dark environment. In order to determine the absorbance value, a spectrophotometer (Shimadzu UV Mini 1240 UV-vis) was used at a wavelength of 750 nm and a protein standard curve was constructed. The obtained lysates were first diluted with

distilled water, and the absorbance values were then converted to equivalent protein concentrations based on the protein standard curve (refer to Appendix B).

3.6.3 Carbohydrate Analysis

The carbohydrate concentration of the hydrolysate was determined according to a modified phenol-sulphuric acid assay, using glucose (20 g/L) (Sigma-Aldrich, USA) as a standard (Agrawal et al., 2015). The working standard of glucose solution was prepared (refer to Appendix A). The mixtures in the tubes were mixed using a vortex mixer (Vortexer, Heathrow Scientific, USA) and incubated for 15 minutes at 25-30 °C in a water bath (Digital Precise Water Bath WB-11, Daihan, Korea). At a wavelength of 490 nm, the absorbance value of each tube was measured with a spectrophotometer (Shimadzu UV Mini 1240 Uv-vis), and a standard curve was generated as a result of these measurements. The lysates were diluted with distilled water and mixed with the reagents. The absorbance values were then converted to equivalent carbohydrate concentrations based on the glucose standard curve constructed (refer to Appendix B).

3.6.4 Surface Morphology Analysis

The morphological characteristics of the lysed yeast cells were visualised using scanning electron microscope (SEM) (JSM-IT500HR, JEOL, Japan), according to a modified protocol developed by Li and co-workers (2015). Firstly, the lysed cells retrieved upon sampling were treated with by 2.5% glutaraldehyde solution (Merck Millipore Corp., USA) for 30 min and mixed using a vortex machine (Heathrow Scientific, USA) until homogenous before being centrifuged at 10 000 rpm for 3 min. The resulting yeast pellet was rinsed with sterile distilled water and gradually dehydrated with ethanol solution (HmbG Chemicals, Malaysia) at different concentrations (25%, 50%, 70%, 95%, 100%, v/v) for 15 min at each concentration. After mixing with ethanol, the mixtures were centrifuged at 8 000

rpm for 2 min and left to air dry. After being allowed to dry, the samples were placed on carbon tape and mounted on aluminium sample stubs. The cells were viewed under scanning electron microscope with a magnification of 5000 x with accelerating voltage of 5.0 kV.

3.6.5 Statistical Analysis

All the experiments in bioethanol fermentation and lysis of spent *S. cerevisiae* were carried out in triplicate and the data was analysed using Minitab®21 by performing a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Level of statistical significance was set at 5% ($p < 0.05$).

The overall project overview is shown in Figure 3.7.

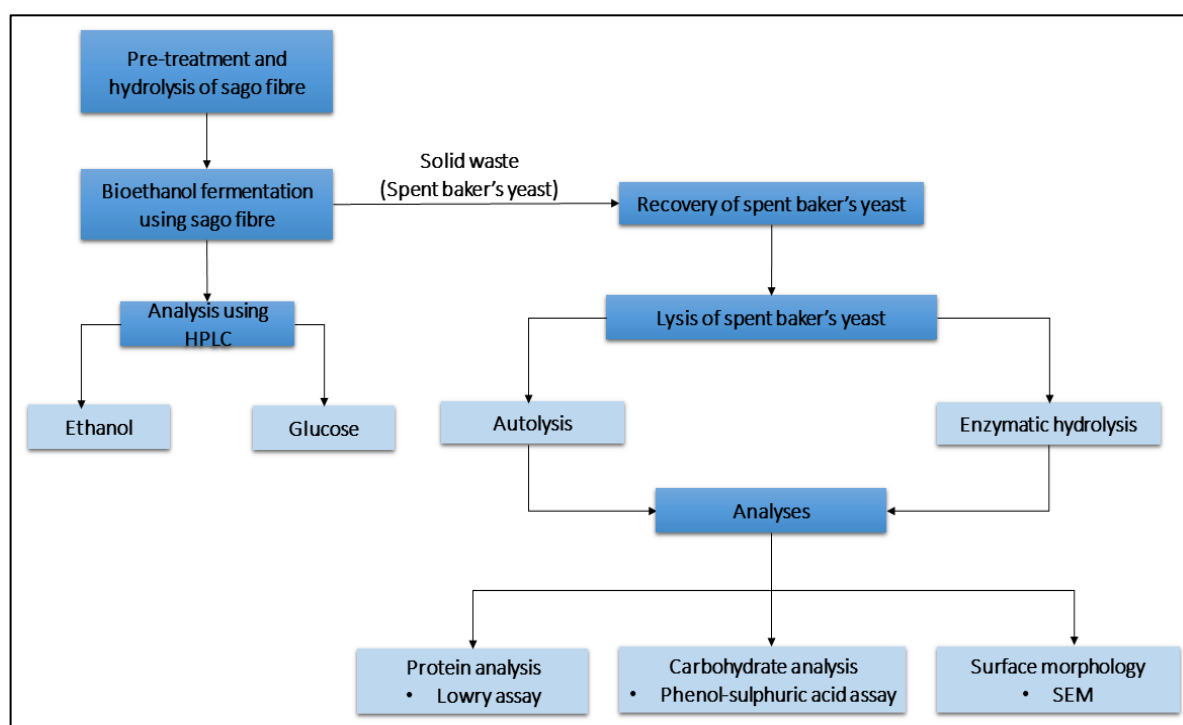


Figure 3.7: General overview of the research project

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Bioethanol Fermentation from Sago Fibre

In this study, SFH media supplemented with nitrogen source, 5 g/L of YE was used as the fermentation media with an initial glucose of 50 g/L. A control experiment, using CG (50 g/L) with addition of YE (5 g/L) was used as comparison to the glucose consumption efficiency with the ethanol production from fermentation using SFH. *S. cerevisiae* was chosen for bioethanol fermentation due to its fermentation ability to create a high ethanol output and can grow optimally in a pH varied from 4 to 6 (Kasavi et al., 2012; Lin et al., 2012; Liu et al., 2015). Figure 4.1 represents the fermentation profiles obtained from this study, which indicates the glucose consumption and bioethanol production in SFH and CG media throughout 48 hours of fermentation.

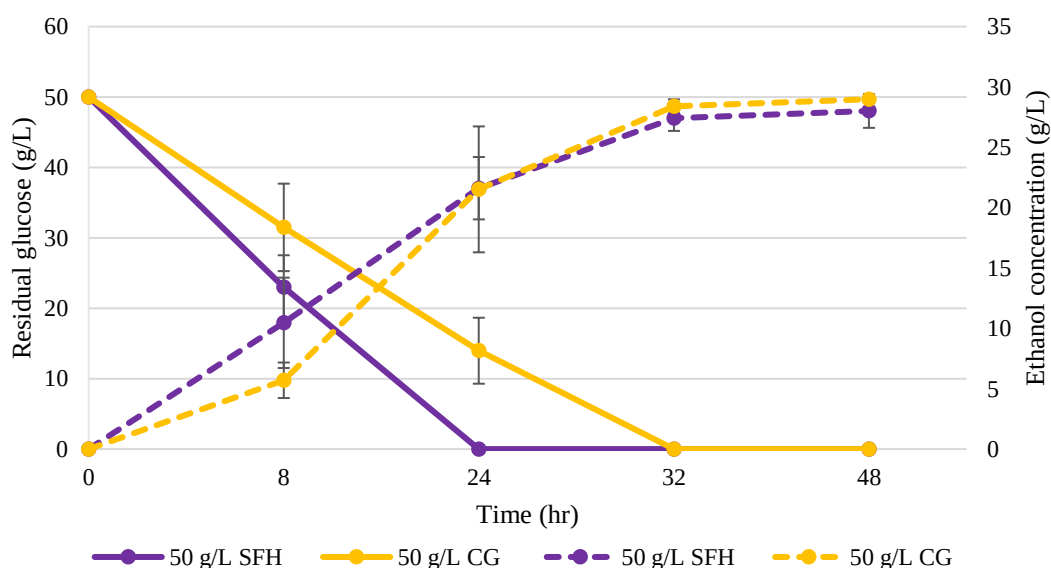


Figure 4.1: Glucose consumption and bioethanol production by *S. cerevisiae* during bioethanol fermentation using sago fibre hydrolysate as a feedstock. Data represent mean $\pm$ S.D. (n=3)

Based on the fermentation profiles, it was observed that, glucose in SFH media was fully consumed by *S. cerevisiae* during the first 24 hours of fermentation. Meanwhile, in CG media, the remaining glucose was still at 13.97 g/L at this hour and replenished at 32 hours of fermentation. This indicates that fermentation using 50 g/L glucose in SFH media demonstrated better performance on glucose consumption with 100% glucose consumption efficiency at 24 hours of fermentation as compared to 50 g/L glucose in CG media. The result in this study is in line with the results obtained by Mohammad et al. (2020), in which the glucose consumption efficiency by SFH (97.78%) was higher than that achieved by CG media (83.81%) at 24 hours of fermentation.

The results demonstrated that the concentration of glucose dropped concurrently with the increase of ethanol production. This indicates that *S. cerevisiae* utilises glucose as carbon source to obtain energy and produces ethanol as the main product in the absence of oxygen. This is consistent with earlier researches (Ahmad et al., 2011; Chang et al., 2018; Phukoetphim et al., 2019). Ethanol production from yeast fermentation does not associate with the growth since it is excreted extracellularly by the growing yeast cells and could not be utilised as carbon source under anaerobic condition (Ahmad et al., 2011; Lin et al., 2012). The ethanol production entered stationary phase after 24 hours of fermentation which may be due to the accumulation of ethanol in the fermentation broth which can become a significant stress factor during fermentation. The comparatively high ethanol concentrations may reduce cell growth and viability, which in turn reduces fermentation efficiency and ethanol yield (Galeote et al., 2001; Aguilera et al., 2006).

It was observed that the maximum concentration of ethanol at 28.01 g/L was generated using 50 g/L glucose in SFH media. Similar ethanol concentration was obtained in 50 g/L glucose in CG media, which was at 28.89 g/L. The maximum peak of bioethanol

production attained by fermentation using 50 g/L glucose SFH was not significantly different than that obtained by control experiment based on the statistical analysis shown in Appendix D. This study showed that the conversion of starch entrapped in sago fibre into glucose through enzymatic hydrolysis, could be used as a carbon source by *S. cerevisiae* due the significant amount of glucose that was used up in the production of ethanol. This is in accordance with the findings from past studies that demonstrated the viability of sago residues as a feedstock for the manufacture of bioethanol (Awg-Adeni et al., 2013; Thangavelu et al., 2014; Bukhari et al., 2017). Following the bioethanol fermentation, the fermentation broth was centrifuged and the resulting solid referred to as spent *S. cerevisiae* henceforth, was characterised.

4.2 Autolysis of Spent Baker's Yeast

4.2.1 Effect of Different Initial pH Values and Incubation Period on Protein Concentration of Autolysate

The changes in concentration of protein and carbohydrate in the autolysate was studied throughout the autolysis of spent Baker's yeast. The study emphasised on the influence of different initial pH values on the autolysis process at different interval times. The degradation of proteins, peptides, and amino acids is important in yeast lysis process, in which it determines the amount of protein released in the autolysate (Lukondeh et al., 2003). The protein concentration profile of the autolysate during the course of 96 hours of autolysis is shown in Figure 4.2.

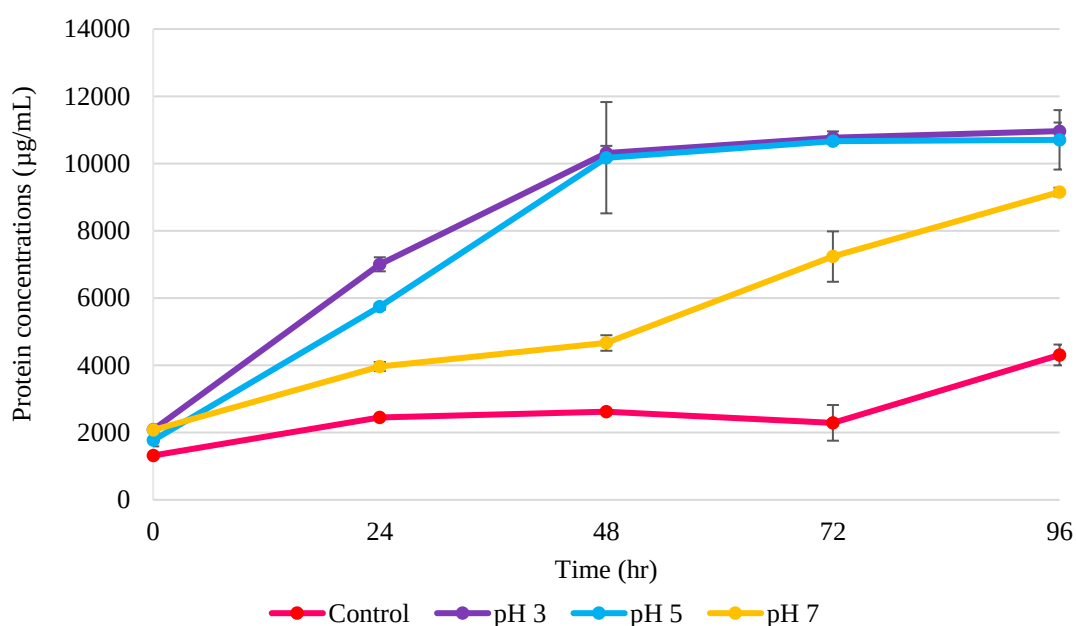


Figure 4.2: Changes of protein concentration of autolysate over time. Data represent mean $\pm$ S.D. (n=3)

The autolysis of spent *S. cerevisiae* for 96 hours resulted in a significant increase in protein concentration when it was conducted at pH values of 3, 5, and 7, indicating that yeast lysis occurred successfully. On the other hand, the control samples, showed a minimal change in protein concentration over the same time period. This is because, the control samples were incubated in an environment unfavourable to autolysis such as no agitation provided with unaltered initial pH of the media and incubated at room temperature. Autolysis conducted at initial pH 3 and 5 exhibited remarkably comparable trends, with a significant rise in protein content during the first 48 hours and a subsequent plateau stage following the prolonged incubation. The statistical analysis in Appendix D shows that there was no significant difference between the protein concentrations obtained by autolysis conducted at initial pH 3 and 5 from 48 hours until 96 hours of incubation.

The autolysis studies on yeast cells were tested at pH 3, 5, and 7 in accordance with past researches (Tanguler & Erten, 2008; Huang, 2010; Li et al., 2015; Alves et al., 2021). The yeast intracellular components released when autolysis was conducted at an initial pH 7 resulted in lower concentrations as compared to the lower pHs. Due to this, increasing the pH is not advisable since autolysis favours acidic conditions for it to take place (Li et al., 2015). At both aforementioned pH settings, the highest concentration of protein in the autolysate attained during autolysis exceeded the control experiment by 2.5-fold. These findings are consistent with earlier studies (Oliveira & Oliva Neto, 2011; Li et al., 2015; Alves et al., 2021), that have reported that a pH range between 3.8 and 5 is optimal for autolysis.

It is possible to link the rise in the concentrations of proteins released into the autolysate with the exposure of yeast cells to an acidic environment that stimulates yeast autolysis. The cell membrane and vacuole membrane of *S. cerevisiae* becomes more permeable due to the action of yeast autolysis. According to Li et al. (2015), the degraded proteins, peptides, and amino acids are released into the autolysate as a result of the action of the endogenous enzymes. The degradation of vacuole, protease inactivation and hydrolysis secrete macromolecular components into the yeast autolysate. As a result, there are differences in the osmotic pressure across the membrane leading to a high amount of intracellular water loss and plasmolysis (Li et al., 2015).

In contrast, yeast autolysis is primarily mediated by the action of yeast's own endogenous enzymes when the lysis process is carried out at a milder condition such as pH 7. This causes the intracellular components of yeast cells to be released at a much slower rate (Li et al., 2015). Yeast proteases and other hydrolytic enzymes are profoundly affected by the pH of autolysis environment (Slaughter & Nomura, 1992). Protease A, a crucial

enzyme in autolysis, has been shown to function best at a pH in the acidic range (2–6) (Maddox & Hough, 1970). Recently, Alves and Oliva Neto (2021) developed an acid shock method during autolysis, and this strategy was found to be effective in shortening the autolysis time.

The autolysis of spent *S. cerevisiae* reached its maximum protein concentration at 96 hours of incubation, but there was no significant change in the protein concentration between 48 and 96 hours (refer to Appendix D). This suggests that autolysis for 48 hours may be preferable and more cost-effective over incubation for 96 hours. This is consistent with the findings of several earlier investigations that reported optimal duration of autolysis between 24 and 70 hours (Li et al., 2015; Takaloo et al., 2020; Alves & Oliva Neto, 2021). The results of this study suggest that the production of protein in autolysate and the time needed to attain its maximal concentration are significantly affected by adjusting the pH before autolysis.

4.2.2 Effect of Different Initial pH Values and Incubation Period on Carbohydrate Concentration of Autolysate

The carbohydrate content of the autolysate was also analysed, in addition to the protein content. The carbohydrate concentration of the autolysate produced over the course of 96 hours of autolysis at different values of pH is shown in Figure 4.3.

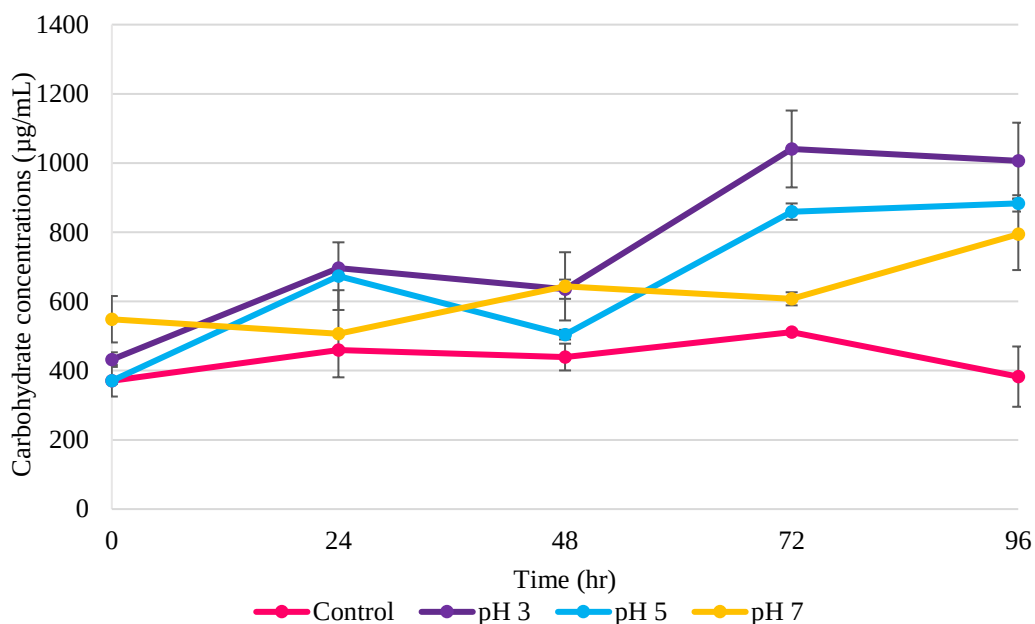


Figure 4.3: Changes of carbohydrate concentration of autolysate over time. Data represent mean $\pm$ S.D. (n=3)

The lysates produced by the autolysis carried out at initial pH 3 and 5 showed a significant increase in carbohydrate concentration after 48 hours of incubation. However, extending the incubation to 96 hours had no significant influence on the carbohydrate concentration based on the statistical analysis in Appendix D. Meanwhile, during the 96-hour incubation period, the carbohydrate concentration in the autolysate released during the autolysis performed at pH 7 was found to be the lowest as compared to the other two pH levels. The autolysis process at an initial pH of 3 resulted in a 2-fold increase in carbohydrate concentration over the control experiment. According to the amount of protein and carbohydrates released from the yeast cells, it is evident that the optimal incubation period

for autolysis of spent Baker's yeast is 72 hours. It is undesirable to extend the incubation period since it increases costs and contamination risks owing to pathogen development (Alves et al., 2021).

The cell walls of *S. cerevisiae* are composed of polysaccharides, range from 80% to 90%, with β -glucans and mannoproteins serving as the primary components. These polysaccharides account for 15% to 30% of the dry mass of the cell (Wang et al., 2018). As reported in this study, a high content of carbohydrates was attained in the autolysate when autolysis was conducted at an initial pH of 3. This is due to the fact the yeast cell polysaccharides are gradually hydrolysed, resulting in the deformation of the yeast cell wall when autolysis is carried out in an acidic environment such as pH 3 (Wang et al., 2018). During the autolysis process, yeast cells were found to release a greater quantity of proteins than carbohydrates, according to this study. This is in agreement by several studies which that yeast extract is primarily consisted of protein (Takalloo et al., 2020). According to Klis et al. (2002), the yeast cell wall degrades in two stages. Firstly, glucanase hydrolyses the glucans in the cell wall, releasing mannoproteins. Then, the remaining glucanase activities releases β -glucans from the amorphous matrix of cell wall (Alexandre & Guilloux-Benatier, 2006; Jane Lombardi et al., 2015).

The polysaccharides derived from yeast cell walls are a promising raw material in the creation of functional foods and nutraceuticals due to their positive health effects (Rakowska et al., 2015). It was reported that β -glucans from yeast cell wall possess the ability to inhibit oxidation, hence preventing cancer, whilst absorbing potentially pathogens and promote cellular immunity (Tang et al., 2017). Thus, this study provides a preliminary understanding on the feasibility of lysing spent Baker's yeast generated after the production of sago bioethanol. In the future, this insight will serve as a starting point for developing

potential side processes that could be developed alongside with the sago bioethanol industry, for example, those related to yeast cell wall products.

4.2.3 Morphological Analysis of Autolysed Cells

The microscopic observation was conducted to analyse the morphological alteration of the autolysed yeast cells that occurred during the autolysis. Figure 4.4 depicts the changes in surface morphology of yeast cells resulting from autolysis at an initial pH of 3 (Figure 4.4(b)), 5 (Figure 4.4(c)), 7 (Figure 4.4(d)), and untreated yeast cells (Figure 4.4(a)).

At 0-hour, yeast cells appeared generally plump with smooth surfaces in all treatments. The majority of the untreated yeast cells (Fig. 4.4 (a)) remained to have plump appearance after 96 hours of incubation. The reason is that in control samples, the yeast cells were not induced for autolysis since the cells were incubated at room temperature, without pH alteration under static condition. However, a significant change with a decreased number of plump cells were observed throughout incubation from the majority of yeast cells produced from autolysis conducted at pH 3 (Figure 4.4 (b)), pH 5 (Figure 4.4 (c)) and pH 7 (Figure 4.4 (d)). In addition, it was discovered that some of the yeast cells in Figure 4.4 (b) and (c) at 96 hours had smaller diameters, more uneven structures, and irregular fragments than those at 0 hr. The autolysis-induced disintegration of yeast cells is responsible for the aforementioned appearance. According to Wang et al. (2018), the yeast cell wall is characterised with 100-150 nm and primarily influence the overall shape of the cells (Wang et al., 2018). During yeast autolysis, hydrolytic enzymes are produced after the rupture of vacuoles to hydrolyse the organelles and cell membrane components found in yeast cells (Wang et al., 2018). Consequently, the cell wall becomes shrivelled and reduced in cell diameter as autolysis progressed, which is consistent with several previously reported studies (Li et al., 2015; Wang et al., 2018). Furthermore, the findings of this study indicate the

success of yeast cells lysis due to autolysis, which occurs concurrently with protein and total carbohydrate released in the autolysate, as previously documented.

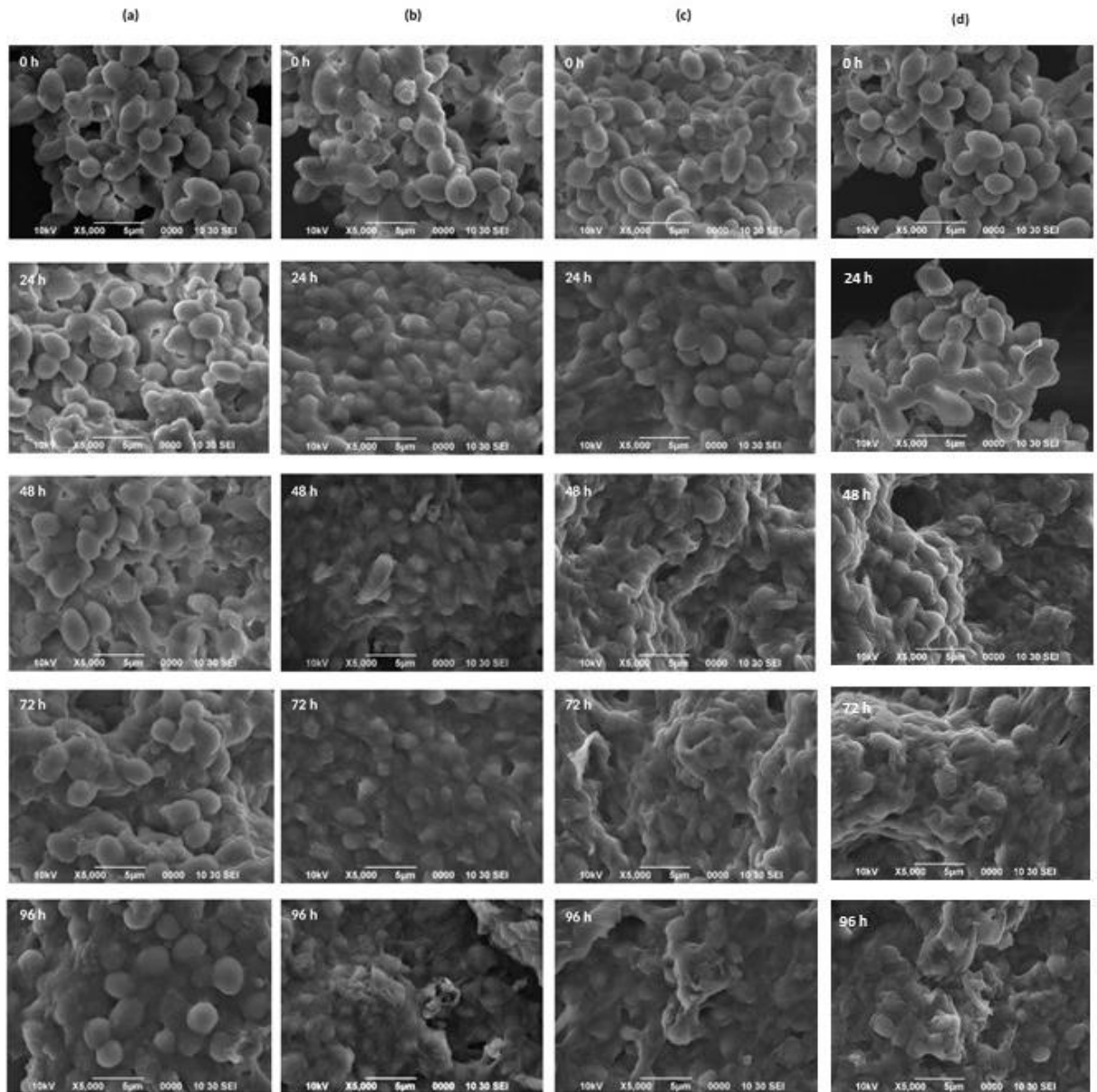


Figure 4.4: Changes of surface morphology of (a) control samples; (b) yeast cells derived from autolysis conducted at pH 3 (c) autolysis conducted at pH 5 and (d) autolysis conducted at pH 7

4.3 Enzymatic Hydrolysis of Spent Baker's Yeast

4.3.1 Effect of Different Enzymes and Their Concentrations on Protein Concentration of Yeast Hydrolysate

The hydrolysis of spent *S. cerevisiae* produced from sago bioethanol fermentation was studied in terms of the protein liberated in the hydrolysate using different enzymes, (alcalase and cellulase), and their concentrations (0.1%-0.5%, v/v). Figure 4.5 and 4.6 show the profiles of protein concentration of the yeast hydrolysates obtained from the hydrolysis using alcalase and cellulase at different time intervals, respectively. The concentration of both enzymes during the hydrolysis was adjusted separately from 0.1 to 0.5% (v/v).

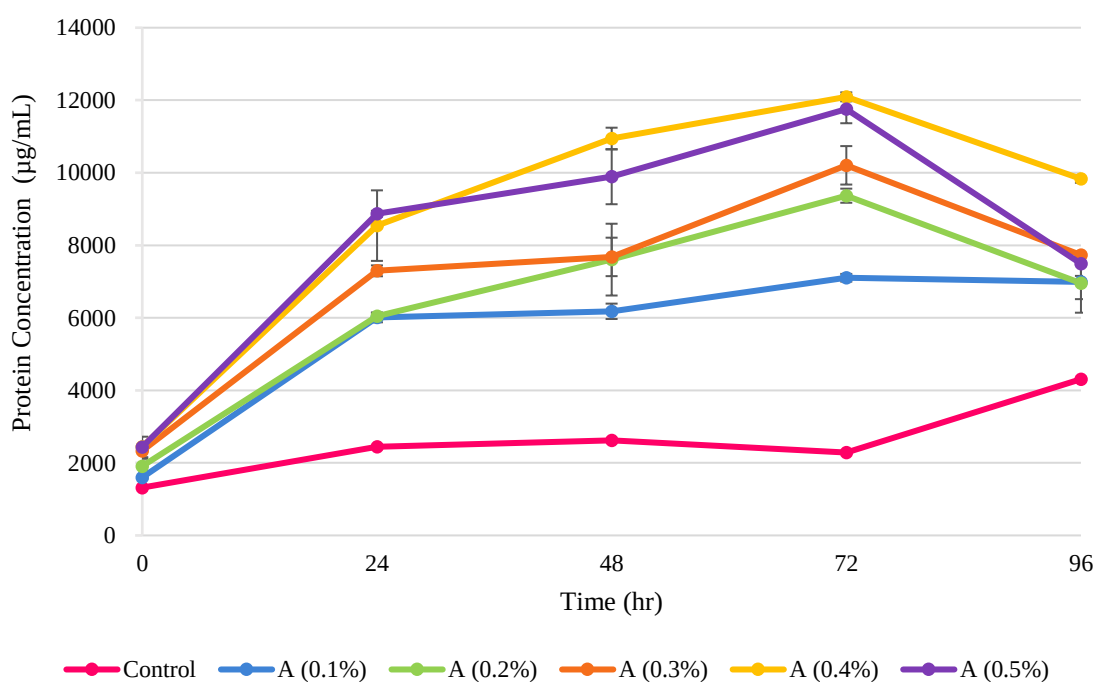


Figure 4.5: Changes of protein concentration of hydrolysates over time. The hydrolysates were derived from hydrolysis using alcalase at different concentrations. A: alcalase. The concentration of alcalase is indicated in the parenthesis. Error bars indicate one standard deviation about the mean (n=3).

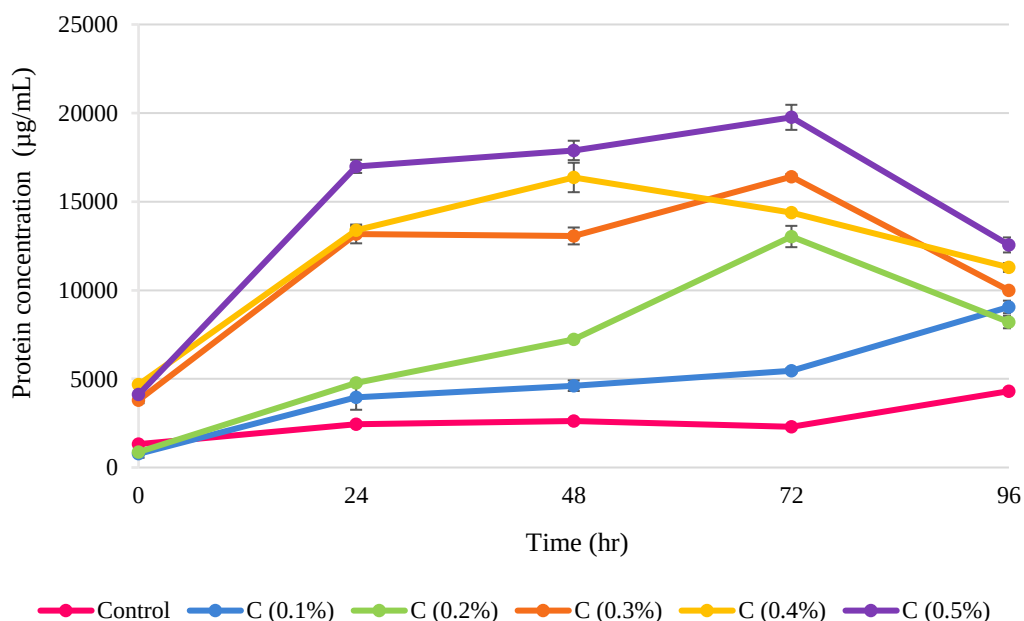


Figure 4.6: Changes of protein concentration of hydrolysates over time using cellulase. The hydrolysates were derived from hydrolysis using cellulase at different concentrations. C: cellulase. The concentration of cellulase is indicated in the parenthesis. Error bars indicate one standard deviation about the mean (n=3).

The results showed that in general, the hydrolysis using both enzymes have shown positive results where the protein concentrations in the hydrolysates were found to increase over time. This suggests that the hydrolysis of spent *S. cerevisiae* due to the action of alcalase and cellulase which resulted in the release of protein in the hydrolysate. The highest protein profile for alcalase was shown by the hydrolysates derived from the hydrolysis using 0.4% (v/v) alcalase with the maximum peak of 12092.5 µg/mL occurred after 72 hr of hydrolysis, representing an increment of 5-fold than that of the control samples. Based on the statistical analysis as shown in Appendix D, increasing the alcalase concentration to 0.5% (v/v), however, was found to result in a negligible difference of the maximum peak of protein concentration.

The protein profiles gradually increase when the enzyme concentration is increased from 0.1 to 0.5% (v/v) in the hydrolysis of spent yeast by cellulase, as illustrated in Figure 4.6. The maximum profile was shown by the hydrolysis using 0.5% (v/v) cellulase with the maximum peak of 19760 $\mu\text{g/mL}$ representing an increment of 1.6-fold over the maximum peak achieved in the hydrolysis using alcalase. This suggests the effectiveness of cellulase over alcalase in releasing the protein content from the lysed yeast cells.

In general, yeast endoenzymes and exoenzymes act synergistically which resulted in the degradation of the yeast cell (Vukašinović-Milić et al., 2007). Consequently, the recovery of the intracellular components from the lysed yeast cells strongly depends on this action. The choice of enzymes and their concentrations play important roles in the enzymatic hydrolysis. Alcalase is a serine protease produced by *Bacillus licheniformis* (Razzaq et al., 2019). The enzyme acts as an endopeptidase, in which it selectively cleaves peptide bond between amino acids within proteins in a particular position causing the cytoplasmic membrane to rupture, allowing intracellular materials to flow out (Vukašinović-Milić et al., 2007; Tacias-Pascacio et al., 2020). As a result, the protein is released into the hydrolysates as the enzymatic hydrolysis progresses. The hydrolysed proteins are rich in non-glycosylated soluble proteins and free amino acids originated from cytoplasm (Comuzzo et al., 2012). Meanwhile, cellulase is a carbohydrate enzyme derived from *Trichoderma reesei*, which contains (1 $\rightarrow$ 3)- β -glucanase activity. The primary components of the yeast cell wall, β -1,3-glucans, are broken down by β -1,3-glucanases via glycosidic bonds in order to hydrolyse the yeast cell wall (Souza et al., 2016; Garcia, 2017).

The concentration of the enzyme influences the degree of the hydrolysis as well as the solubilisation of protein (Demirhan et al., 2011). This is demonstrated by the present work, which shows that increasing the enzyme concentration from 0.1 to 0.5% (v/v) resulted

in a gradual increase in the protein profiles. As the enzyme concentration increases, more substrates bind to its active sites, producing an enzyme-substrate complex that further speed up the reaction rate until reaching maximum point, in which all of the enzyme's active sites are bound to substrates at the highest concentration (Blanco & Blanco, 2017). Further increase in the enzyme concentration beyond the saturation point will have no significant influence on the enzymatic hydrolysis.

The effectiveness of alcalase in hydrolysing spent yeast cells as reported in this work is in parallel with the several findings reported in the literature. In a work by Xie et al. (2017), alcalase was found to be more effective than papain in hydrolysing spent Baker's yeast. Meanwhile, Takaloo et al. (2020) reported the efficiency of alcalase in producing yeast hydrolysates with an antioxidative properties. In the same work, alcalase is also revealed as one of the most effective proteases in recovering the intracellular content from the yeast cells. Meanwhile, the use of cellulase for hydrolysing spent yeast cells have also been reported in the literature. It was demonstrated that β -(1,3/1,6)-glucans of the yeast cell wall were hydrolysed by cellulase, leading to a significant release of free glucose. Comparison between the performance of alcalase and cellulase in hydrolysing spent *S. cerevisiae* particularly those derived from sago bioethanol fermentation, however, has yet to be reported in any studies before. The results from this work have confirmed the feasibility of alcalase and cellulase for hydrolysing spent *S. cerevisiae* generated from sago bioethanol fermentation. The maximum recovery of protein from spent *S. cerevisiae* via enzymatic hydrolysis using alcalase and cellulase as reported in the current work was found to be 1.1 to 1.8-fold higher than that obtained via autolysis. The statistical analysis in Appendix D showed that the maximum protein contents liberated by enzymatic hydrolysis using cellulase was significantly different than that of hydrolysis using alcalase and autolysis.

4.3.2 Effect of Different Enzymes and Their Concentrations on Carbohydrate Concentration of Yeast Hydrolysate

Similar to protein, the amount of carbohydrate released in the yeast hydrolysate can be associated with the degree of the enzymatic hydrolysis and hence, it is referred to as one of the important indicators. Figure 4.7 and 4.8 illustrate the change of total carbohydrate of hydrolysate derived from the hydrolysis using alcalase and cellulase, respectively conducted at different enzyme concentrations throughout the 96 hr of hydrolysis.

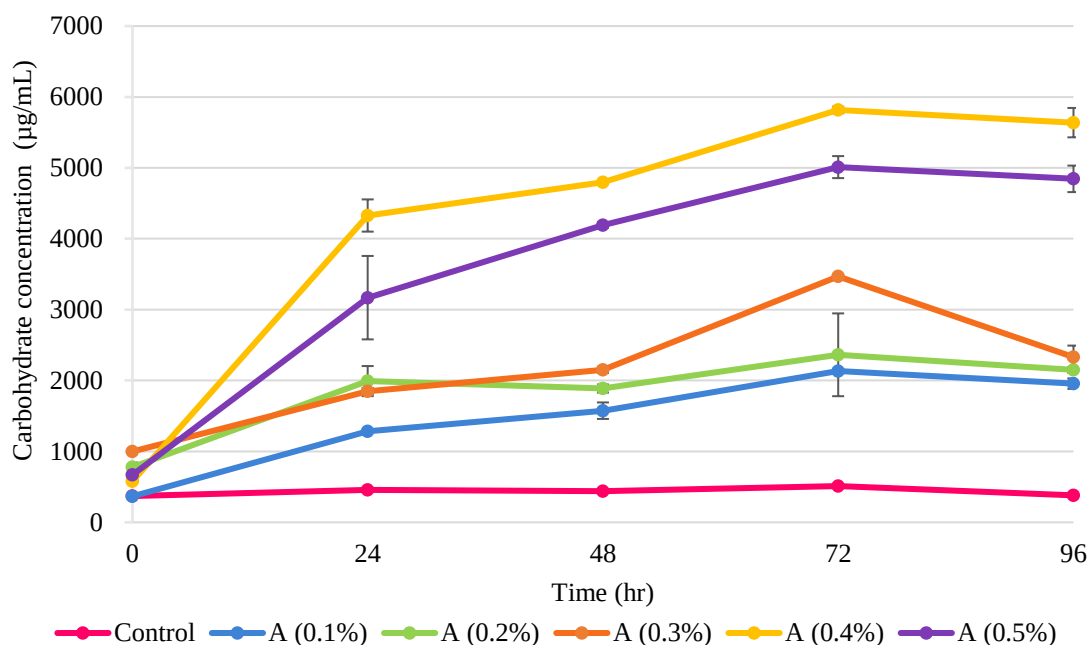


Figure 4.7: Changes of total carbohydrate concentration of hydrolysate over time. The hydrolysates were derived from hydrolysis using alcalase at different concentrations. A: alcalase. The concentration of alcalase is indicated in the parenthesis. Error bars indicate one standard deviation about the mean (n=3).

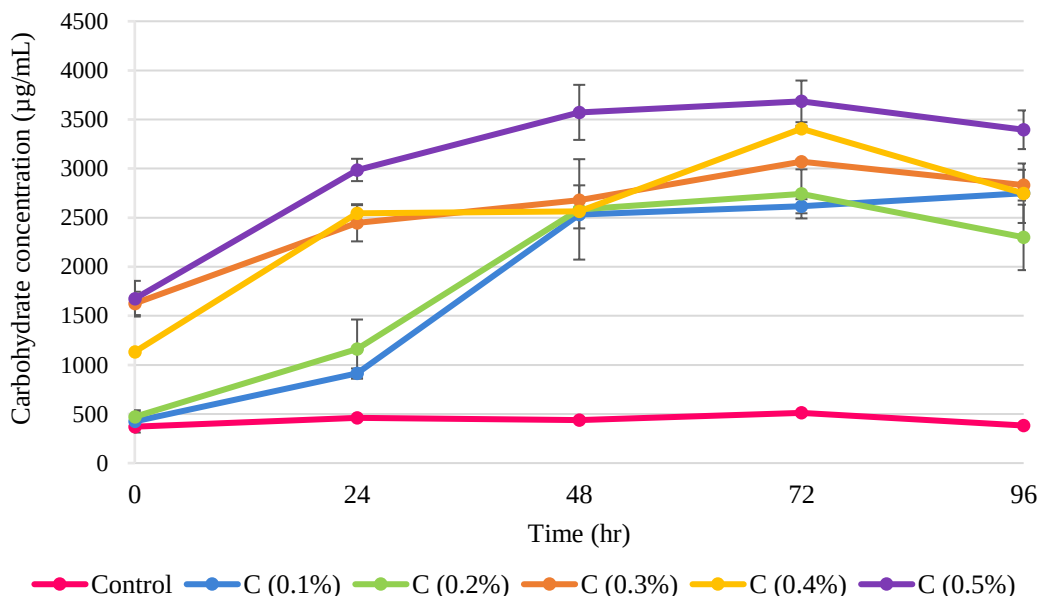


Figure 4.8: Changes of total carbohydrate concentration of hydrolysate over time. The hydrolysates were derived from hydrolysis using cellulase at different concentrations. C: cellulase. The concentration of cellulase is indicated in the parenthesis. Error bars indicate one standard deviation about the mean (n=3).

It was demonstrated that after 48 hours of hydrolysis with both enzymes, the carbohydrate concentration had increased substantially. However, there was no significant difference in carbohydrate concentration was observed when the hydrolysis period was extended to 96 hours (refer to Appendix D). Additionally, as the enzyme concentration is increased from 0.1 to 0.5% (v/v), more carbohydrates are released into the hydrolysate. The variation of the performance of the two enzymes in releasing protein and carbohydrate can be associated with their different efficiency in degrading the yeast cells and consequently, in recovering intracellular components. Furthermore, the protein and carbohydrate contents of the hydrolysate are affected by the yeast endoenzymes and exogenous enzymes that act synergistically (Vukašinović-Milić et al., 2007). The maximum profiles of carbohydrate concentration were achieved in the hydrolysis conducted using 0.4% (v/v) alcalase and 0.5% (v/v) cellulase where the highest peaks were at 5816.8 µg/mL and 3684.3 µg/mL,

respectively. The values were found to be 5.6 and 3.5-fold over that achieved via autolysis of spent yeast as reported in previous section. The maximum peak of carbohydrate contents attained by enzymatic hydrolysis using alcalase was significantly different than that of enzymatic hydrolysis using cellulase and autolysis, based on the statistical analysis in Appendix D.

The enzymatic degradation of cell wall components causes cell wall rupture, membrane lysis, and intracellular content leakage, all of which contribute to an increase in the concentration of carbohydrates in the hydrolysates (Vukašinović-Milić et al., 2007). Therefore, an increased carbohydrate content in the yeast hydrolysate is to be expected. Unlike autolysis where the release of carbohydrates occurs in two phases, degradation of structural carbohydrates during the enzymatic hydrolysis starts from the beginning of the process (Vukašinović-Milić et al., 2007). The results from the present work are in agreement with some of previous works that reported the release of carbohydrate in the hydrolysate upon the hydrolysis of spent yeast using proteases (Vukašinović-Milić et al., 2007; Cao et al., 2017; Garcia, 2017).

It can be concluded here that alcalase and cellulase are suitable to be employed for hydrolysing spent *S. cerevisiae* derived from sago bioethanol fermentation with their suggested concentration of 0.4% (v/v) and 0.5% (v/v), respectively. Further increase of the enzyme concentration may not just increase the cost of the process but also results in minimal benefits of the recovery of protein and carbohydrate. The results also showed that 72 hours is the optimal duration of the hydrolysis of spent *S. cerevisiae* by both alcalase and cellulase with regards to the release of protein and carbohydrate in the hydrolysate. It is undesirable to extend the hydrolysis period since it increases costs and contamination risks due to pathogen development such as *Clostridium* sp., which prefers protein-rich and low oxygen

condition found during yeast hydrolysis (Bataeva et al., 2020). Similar to autolysis, it was also shown that upon the enzymatic hydrolysis of spent *S. cerevisiae*, the concentration of protein in the hydrolysate was found to be higher than carbohydrate. This is in line with the findings by Takaloo et al. (2020), that the main constituent of yeast extract is protein.

4.3.3 Morphological Analysis of Hydrolysed Yeast Cells

Figures 4.9 and 4.10 depict the morphological changes that occurred in the yeast cells during the enzymatic hydrolysis utilising alcalase and cellulase. Similar as in autolysis treatment, the yeast cells had an ellipsoidal shape with a plump and smooth surface at 0 hr in all treatments. The yeast cells that were subjected to alcalase and cellulase hydrolysis, on the other hand, changed drastically in their appearance and cell volume over the course of the 96-hr incubation period. The cells became flattened, shrivelled up and became severely deformed, losing their plump appearance. In Figure 4.9 (a) and (b), some of the yeast cells when treated with alcalase at 0.1% and 0.2% (v/v) still appeared in spherical shape during the 24 hr of hydrolysis. However, the yeast cells from the hydrolysis using other concentrations of alcalase began to flatten and distort at this hour. In contrary to the yeast cells from the hydrolysis using cellulase (Figure 4.10), the yeast cells started to flatten after 24 hr of hydrolysis in all treatments. These findings imply that the enzymatic hydrolysis aided by alcalase and cellulase results in the destruction of yeast cell wall, hence altering the structure of the yeast cell surface. It is observed that in general, the yeast cells treated with cellulase showed more prominent damage as compared to those treated by alcalase. This is in line with the high release of protein content than that catalysed by alcalase as discussed earlier.

The loss of cell wall components predominantly comprised of β -glucans and mannoproteins will alter the wall porosity, hence, allowing intracellular macromolecules to

be released (Hernawan & Fleet, 1995). During the enzymatic hydrolysis, the cell wall comes into direct contact with the external culture media, triggering a sequence of biological activities in the cell to adapt to the changing environment. The external hydrolysing enzymes breakdown the cell membrane components, causing holes to appear in the cell membrane. Consequently, the increase in the cell porosity leads to the leakage of large amount of the intracellular components into the hydrolysate (Wang et al., 2018). The cell wall shrivelled and the cell diameter was reduced throughout the lysis, which are in agreement with several other studies (Cao et al., 2017; Takaloo et al., 2020). In summary, the morphological analysis data obtained supports the lysis of spent *S. cerevisiae* via the enzymatic hydrolysis using alcalase and cellulase.

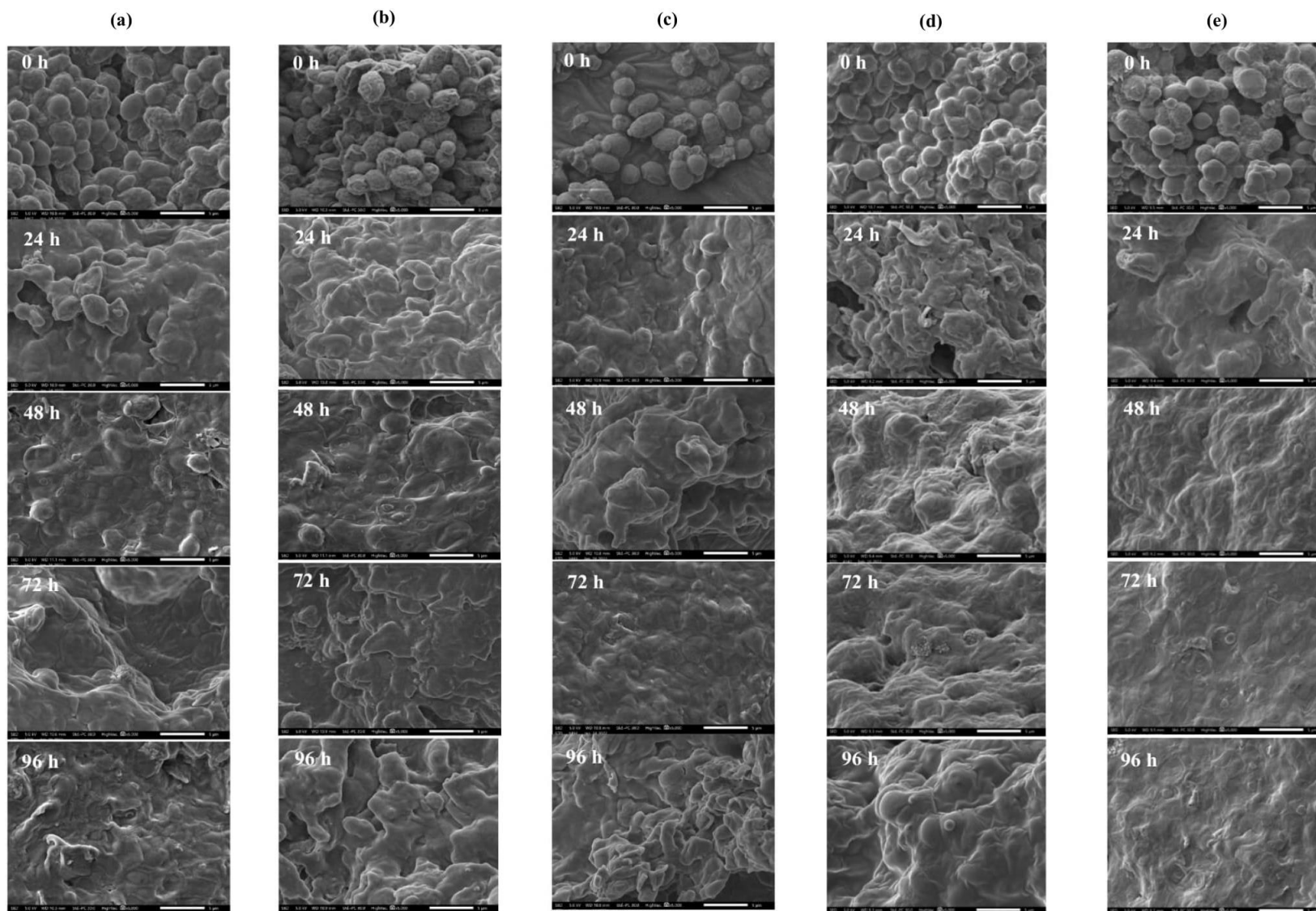


Figure 4.9: Changes of surface morphology of yeast cells treated with alcalase at (a) 0.1% (v/v); (b) 0.2% (v/v); (c) 0.3% (v/v); (d) 0.4% (v/v) and (e) 0.5% (v/v). Magnification is 5000 ×

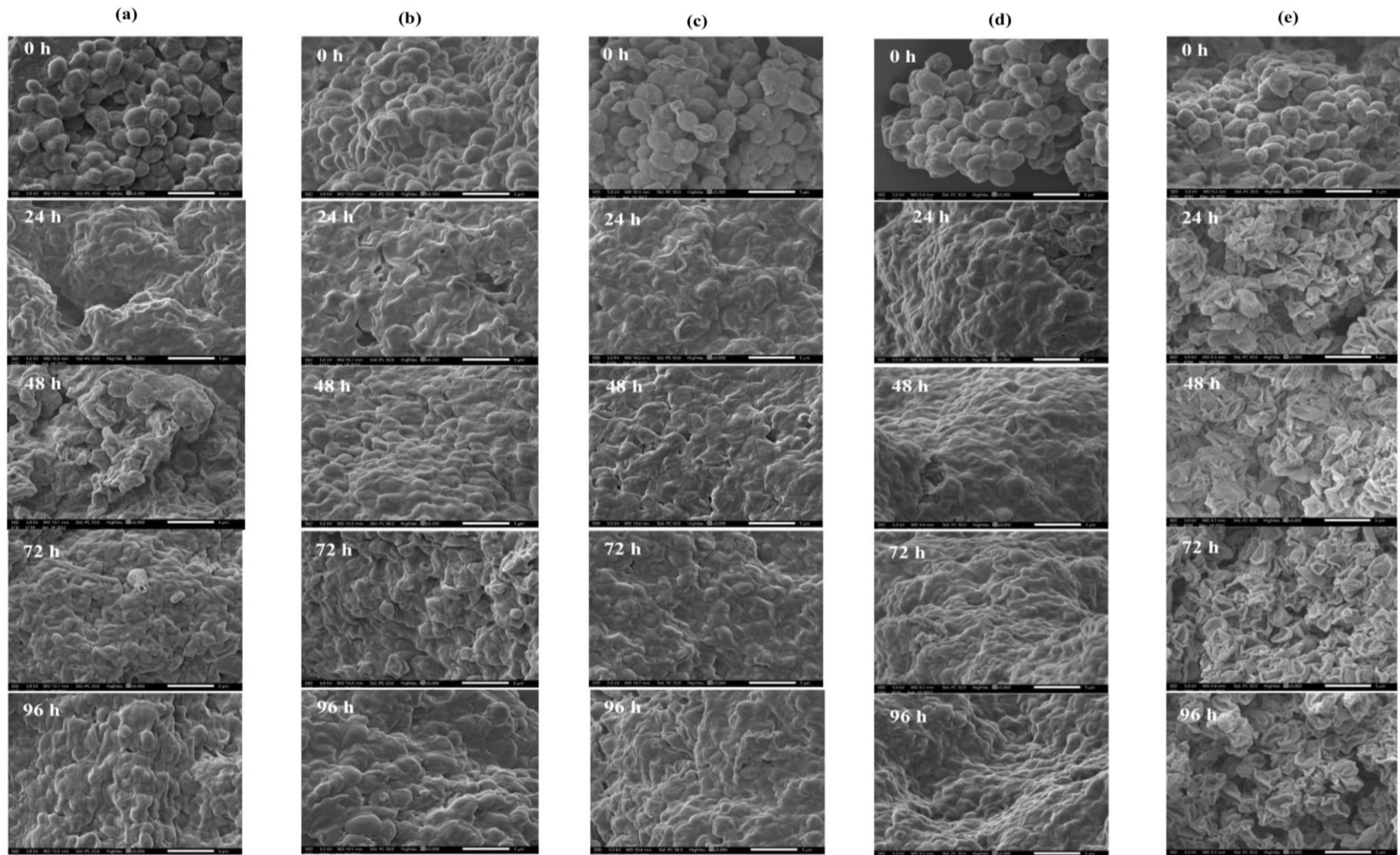


Figure 4.10: Changes of surface morphology of yeast cells treated with cellulase at (a) 0.1% (v/v); (b) 0.2% (v/v); (c) 0.3% (v/v); (d) 0.4% (v/v) and (e) 0.5% (v/v). Magnification is 5000 ×

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

The growing demand for cost effective and sustainable productions of bioethanol has prompted the use of renewable resources to optimise their manufacture whilst minimising environmental impact. In order to ensure the sustainability of the entire sago bioethanol production, it is essential to look into the prospective fate of the waste generated during the process. The present study offers helpful insight into the potential strategies of reusing sago bioethanol solid waste, facilitating the development of sago biorefinery in the future. Additionally, this work may help to minimise sago bioethanol waste while generating side revenue to the sector. Several key findings and their significance are presented as following.

The feasibility of autolysis and enzymatic hydrolysis in lysing spent *S. cerevisiae* derived from the production of sago bioethanol were studied. It was found that pH 3 achieved the highest protein concentration and carbohydrate concentration in autolysis of spent *S. cerevisiae*, corresponding to 2.5-fold and 2-fold increments over the control experiment, respectively. This study suggests that 48-hour autolysis may be sufficient and more cost-effective than 96-hour incubation. Moreover, the morphological changes of the lysed yeast cells were analysed and supported the findings that proteins and carbohydrates were released into the autolysate.

For enzymatic hydrolysis, the treatment using alcalase and cellulase in hydrolysing spent *S. cerevisiae* were evaluated. The optimal concentration of alcalase and cellulase in hydrolysing spent *S. cerevisiae* were found to be 0.4% and 0.5% (v/v), respectively. The maximum recovery for protein was obtained at 19760 µg/mL from 0.5% (v/v) cellulase treatment, representing an increment of 1.6-fold in compared to the maximum peak achieved

in the hydrolysis using alcalase. This suggests the effectiveness of cellulase over alcalase in releasing the protein content from the lysed yeast cells. The maximum carbohydrate recovery was obtained at 5816.8 µg/mL by 0.4% (v/v) alcalase treatment, representing 5.6-fold over that achieved via autolysis of spent yeast.

In both cases of autolysis and enzymatic hydrolysis, the increase of protein and carbohydrate concentration along the incubation period suggests that the lysis of spent *S. cerevisiae* took place. The optimal duration of the hydrolysis of spent *S. cerevisiae* by both alcalase and cellulase with regards to the release of protein and carbohydrate in the hydrolysate was 72 hours. It is undesirable to extend the incubation period since it increases costs and contamination risks owing to pathogen development.

The concentration of protein in the lysates was found to be greater than that of carbohydrates, as protein is the major constituent of yeast extract. The highest protein and carbohydrate concentration of lysate achieved from cellulase treatment and alcalase treatment with the titre represented about 8.6-fold and 11.4-fold increments, respectively over the concentrations obtained by the control experiment. The release of yeast extracts into the lysates corresponds to the morphological analysis in which the cells were severely ruptured, suggesting successful occurrence of yeast lysis. As compared to control samples, the cells showed plump structure with minimal increase for both protein and carbohydrate concentrations. It can be concluded that the best strategy for lysing spent *S. cerevisiae* generated upon production of sago bioethanol was enzymatic hydrolysis, due to the high release of protein and carbohydrate as compared to autolysis. The statistical analysis also shows that both protein and carbohydrate release from enzymatic hydrolysis was found to be significantly different than that obtained from autolysis. However, there is a drawback to consider which is the cost of the enzyme.

Future work should consider several aspects. Application of yeast lysates as components in microbiological media can be used for growing microorganism in fermentations. This will provide insightful information on the utilisation of spent yeast from the fermentation of sago bioethanol. Other than that, the enzymatic hydrolysis of spent Baker's yeast should also utilise other types of enzymes, such as lyticase, papain, and pancreatin. Last but not least, more analyses such as α -amino nitrogen, nucleic acid, antioxidant properties and total solid contents may be conducted for evaluating the chemical and biological properties of the yeast lysates. These will give more detailed pictures of the lysis process.

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APPENDICES

Appendix A: Media / Reagents and Solution

i. Potato Dextrose Agar (PDA)

Approximately, 19.5 g of Difco™ PDA powder was dissolved in 500 mL of distilled water. The solution was stirred until fully dissolved and autoclaved at 121 °C for 20 minutes. Then, the agar was poured into petri dishes and left to solidified.

ii. Yeast Malt Broth (YMB)

About 1.05 g of yeast malt broth powder (Sigma-Aldrich Company, USA) was added into 50 mL of distilled water.

iii. 0.05 M Sodium Phosphate Buffer (500 mL)

The buffer solutions were prepared according to the pre-lab calculations as in Table 1. Then, 300 mL of distilled water was added and the pH of the solutions was adjusted by using 10% (v/v) phosphoric acid (H_3PO_4) or 0.5 M sodium chloride (NaCl). Finally, the final volume (500 mL) of the solutions was adjusted by adding distilled water.

Table 1: Preparation of 0.05 M sodium phosphate buffer		
Buffer (pH)	NaH_2PO_4 (g)	Na_2HPO_4 (g)
3	0.35994	3.123
5	1.4878	0.014196
7	1.7997	1.4196

Lowry Solution (15 mL)**Table 2: Preparation of complex forming reagent mix stock solution, 100:1:1**

Solution	Chemical
A	2% (w/v) Na ₂ CO ₃ in distilled water
B	1% (w/v) CuSO ₄ ·5(H ₂ O) in distilled water
C	1% (w/v) C ₄ H ₄ KNaO ₆ ·4(H ₂ O) in distilled water

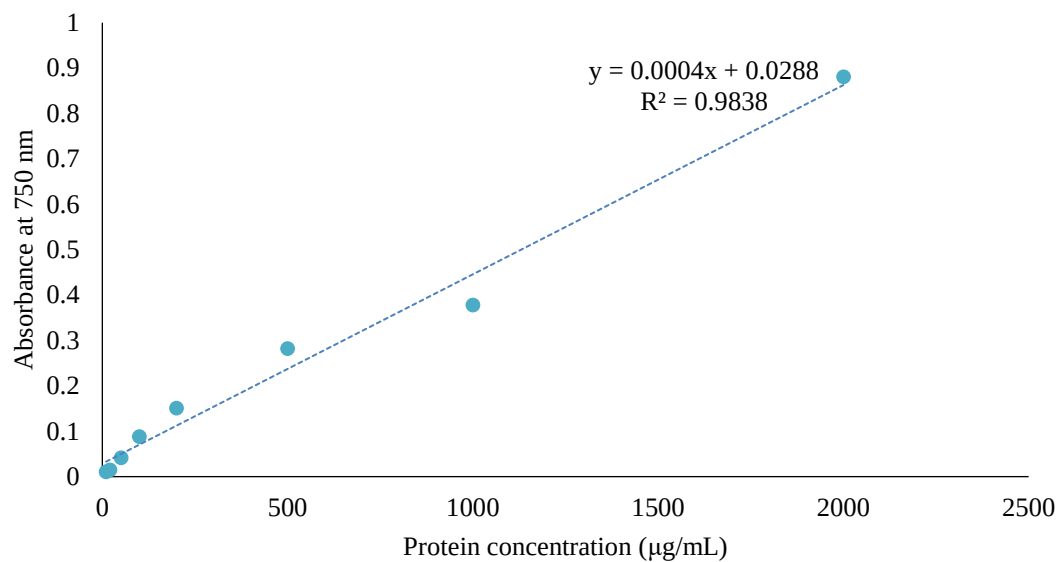
Table 3: Dilution from BSA stock solution

BSA stock solution (μL)	Distilled water (μL)	Protein concentration (μg/L)
0	500.0	0
2.5	498.0	10
5.0	495.0	20
12.5	488.0	50
25.0	475.0	100
50.0	450.0	200
125.0	375.0	500
250.0	250.0	1000
500.0	0	2000

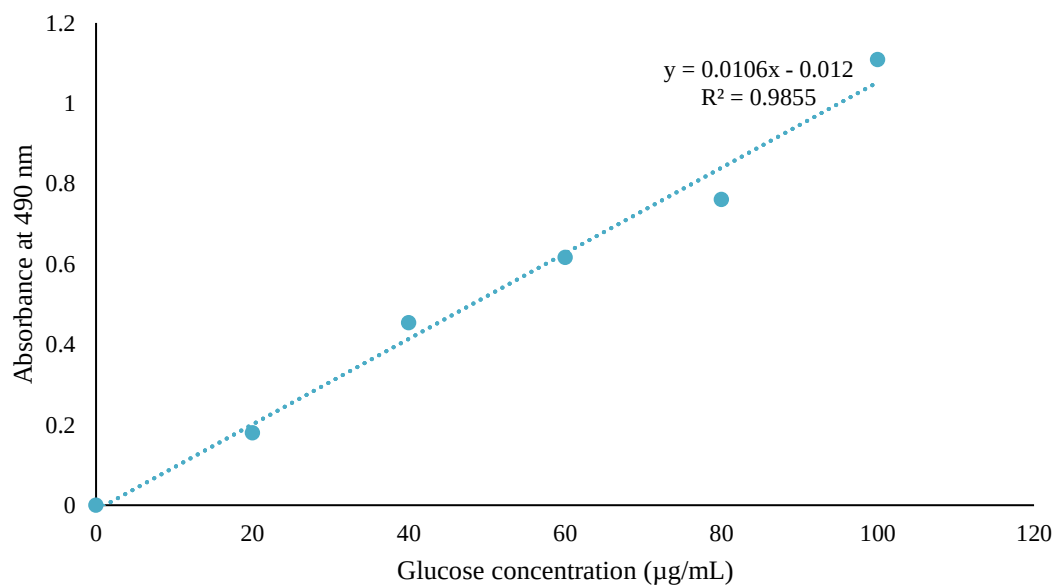
Table 0.1: Working standard of glucose solution				
Tube	Glucose solution (μL)	Distilled water (μL)	5% phenol solution (mL)	96% sulphuric acid solution (mL)
Blank	0	1000	1	5
1	20	980	1	5
2	40	960	1	5
3	60	940	1	5
4	80	920	1	5
5	100	900	1	5

Appendix B: Standard Calibration Curve

4.2.3.1.1.1 Standard Curve of Protein (Bovine Serum Albumin as a standard)



4.2.3.1.1.2 Standard Curve of Glucose



Appendix C: Tabulated Data

i. Fermentation using sago fibre hydrolysate

Hour	Residual Glucose (g/L)	Ethanol Concentration (g/L)
0	50.00 $\pm$ 0	0
8	22.97 $\pm$ 4.56	10.46 $\pm$ 3.74
24	0	21.61 $\pm$ 2.58
36	0	27.40 $\pm$ 1.05
48	0	28.01 $\pm$ 1.40

ii. Fermentation Control (Glucose + YE)

Hour	Residual Glucose (g/L)	Ethanol Concentration (g/L)
0	50.00 $\pm$ 0	0
8	31.48 $\pm$ 6.21	5.70 $\pm$ 1.47
24	13.97 $\pm$ 4.69	21.52 $\pm$ 5.22
36	0	28.39 $\pm$ 0.58
48	0	28.98 $\pm$ 0

Table 1: Average weight of spent yeast collected after sago bioethanol fermentation

Replicate	Spent yeast (g/L)
R1	16.61
R2	15.07
R3	15.75
Mean $\pm$ sd.	15.81 $\pm$ 0.77

Table 2: Average protein and carbohydrate concentrations ($\mu\text{g/mL}$) of autolysate obtained from control sample

Time (hr)	Protein concentration ($\mu\text{g/mL}$)	Carbohydrate concentration ($\mu\text{g/mL}$)
0	1319.0 $\pm$ 0.02	370.8 $\pm$ 45.49
24	2446.5 $\pm$ 0.04	459.6 $\pm$ 6.31
48	2619.0 $\pm$ 0.02	439.4 $\pm$ 38.71
72	2290.0 $\pm$ 530.33	511.4 $\pm$ 2.52
96	4308.8 $\pm$ 309.40	382.8 $\pm$ 87.13

Autolysis of Spent *S. cerevisiae*

Table 3: Average protein and carbohydrate concentrations ($\mu\text{g/mL}$) of autolysate obtained from autolysis of spent *S. cerevisiae* conducted at initial pH 3

Time (hr)	Protein concentration ($\mu\text{g/mL}$)	Carbohydrate concentration ($\mu\text{g/mL}$)
0	2086.5 $\pm$ 106.07	432.0 $\pm$ 21.11
24	7004.0 $\pm$ 208.81	696.8 $\pm$ 0.95
48	10314.0 $\pm$ 212.13	635.4 $\pm$ 27.78
72	10770.7 $\pm$ 185.83	1040.7 $\pm$ 111.12
96	10964.0 $\pm$ 255.34	1006.3 $\pm$ 110.33

Table 4: Average protein and carbohydrate concentrations ($\mu\text{g/mL}$) of autolysate obtained from autolysis of spent *S. cerevisiae* conducted at initial pH 5

Time (hr)	Protein concentration ($\mu\text{g/mL}$)	Carbohydrate concentration ($\mu\text{g/mL}$)
0	1771.5 $\pm$ 180.31	370.8 $\pm$ 9.47
24	5742.8 $\pm$ 90.16	673.2 $\pm$ 97.73
48	10174.0 $\pm$ 1654.63	504.1 $\pm$ 13.89
72	10664.0 $\pm$ 28.28	859.7 $\pm$ 23.66
96	10705.0 $\pm$ 883.88	883.5 $\pm$ 23.62

Table 5: Average protein and carbohydrate concentrations ($\mu\text{g/mL}$) of autolysate obtained from autolysis of spent *S. cerevisiae* conducted at initial pH 7

Time (hr)	Protein concentration ($\mu\text{g/mL}$)	Carbohydrate concentration ($\mu\text{g/mL}$)
0	2076.5 $\pm$ 93.17	548.7 $\pm$ 67.08
24	3966.5 $\pm$ 133.39	506.7 $\pm$ 125.95
48	4664.0 $\pm$ 230.65	643.7 $\pm$ 98.55
72	7234.0 $\pm$ 749.53	607.7 $\pm$ 18.94
96	9154.0 $\pm$ 127.28	794.3 $\pm$ 103.54

Enzymatic Hydrolysis of Spent *S. cerevisiae* using Alcalase

Table 6: Average protein and carbohydrate concentrations ($\mu\text{g/mL}$) of hydrolysate obtained from enzymatic hydrolysis of spent *S. cerevisiae* using Alcalase (0.1%, v/v)

Time (hr)	Protein concentration ($\mu\text{g/mL}$)	Carbohydrate concentration ($\mu\text{g/mL}$)
0	1599.0 $\pm$ 53.03	366.34 $\pm$ 0.63
24	6008.75 $\pm$ 132.58	1283.57 $\pm$ 10.10
48	6180.0 $\pm$ 212.13	1574.64 $\pm$ 116.17
72	7105.0 $\pm$ 106.07	2134.29 $\pm$ 30.31
96	6992.5 $\pm$ 477.30	1956.16 $\pm$ 73.87

Table 7: Average protein and carbohydrate concentrations ($\mu\text{g/mL}$) of hydrolysate obtained from enzymatic hydrolysis of spent *S. cerevisiae* using Alcalase (0.2%, v/v)

Time (hr)	Protein concentration ($\mu\text{g/mL}$)	Carbohydrate concentration ($\mu\text{g/mL}$)
0	1910.3 $\pm$ 121.98	779.3 $\pm$ 63.14
24	6046.3 $\pm$ 97.23	1992.1 $\pm$ 212.13
48	7605.0 $\pm$ 989.95	1887.5 $\pm$ 56.82
72	9367.5 $\pm$ 194.45	2362.9 $\pm$ 583.99
96	6955.0 $\pm$ 813.17	2148.7 $\pm$ 41.04

Table 8: Average protein and carbohydrate concentrations ($\mu\text{g/mL}$) of hydrolysate obtained from enzymatic hydrolysis of spent *S. cerevisiae* using Alcalase (0.3%, v/v)

Time (hr)	Protein concentration ($\mu\text{g/mL}$)	Carbohydrate concentration ($\mu\text{g/mL}$)
0	2333.8 $\pm$ 220.97	999.4 $\pm$ 1.89
24	7296.3 $\pm$ 150.26	1849.3 $\pm$ 45.46
48	7680.0 $\pm$ 530.33	2150.9 $\pm$ 44.19
72	10205.0 $\pm$ 530.33	3467.9 $\pm$ 18.94
96	7730.0 $\pm$ 35.36	2331.7 $\pm$ 160.99

Table 9: Average protein and carbohydrate concentrations ($\mu\text{g/mL}$) of hydrolysate obtained from enzymatic hydrolysis of spent *S. cerevisiae* using Alcalase (0.4%, v/v)

Time (hr)	Protein concentration ($\mu\text{g/mL}$)	Carbohydrate concentration ($\mu\text{g/mL}$)
0	2446.3 $\pm$ 114.91	578.2 $\pm$ 10.10
24	8542.5 $\pm$ 972.27	4327.9 $\pm$ 227.28
48	10942.5 $\pm$ 300.52	4796.3 $\pm$ 11.36
72	12092.5 $\pm$ 123.74	5816.8 $\pm$ 45.46
96	9830.0 $\pm$ 106.07	5637.9 $\pm$ 207.08

Table 10: Average protein and carbohydrate concentrations ($\mu\text{g/mL}$) of hydrolysate obtained from enzymatic hydrolysis of spent *S. cerevisiae* using Alcalase (0.5%, v/v)

Time (hr)	Protein concentration ($\mu\text{g/mL}$)	Carbohydrate concentration ($\mu\text{g/mL}$)
0	2440.0 $\pm$ 282.84	668.4 $\pm$ 61.87
24	8871.3 $\pm$ 79.55	3168.9 $\pm$ 588.41
48	9892.5 $\pm$ 760.14	4193.6 $\pm$ 57
72	11755.0 $\pm$ 388.91	5010.5 $\pm$ 155.31
96	7492.5 $\pm$ 335.88	4845.0 $\pm$ 186.88

Enzymatic Hydrolysis of Spent *S. cerevisiae* using Cellulase

Table 11: Average protein and carbohydrate concentrations ($\mu\text{g/mL}$) of hydrolysate obtained from enzymatic hydrolysis of spent *S. cerevisiae* using Cellulase (0.1%, v/v)

Time (hr)	Protein concentration ($\mu\text{g/mL}$)	Carbohydrate concentration ($\mu\text{g/mL}$)
0	771.3 ± 220.97	424.6 ± 113.64
24	3965.0 ± 707.11	913.6 ± 50.51
48	4617.5 ± 300.52	2532.6 ± 142.05
72	5467.5 ± 123.74	2615.9 ± 71.97
96	9060.0 ± 353.55	2748.6 ± 303.05

Table 12: Average protein and carbohydrate concentrations ($\mu\text{g/mL}$) of hydrolysate obtained from enzymatic hydrolysis of spent *S. cerevisiae* using Cellulase (0.2%, v/v)

Time (hr)	Protein concentration ($\mu\text{g/mL}$)	Carbohydrate concentration ($\mu\text{g/mL}$)
0	871.3 ± 114.91	472.0 ± 46.72
24	4771.3 ± 8.84	1161.8 ± 300.52
48	7230.0 ± 70.71	2583.9 ± 511.39
72	13035.0 ± 601.04	2741.8 ± 250.01
96	8210.0 ± 353.55	2298.6 ± 333.35

Table 13: Average protein and carbohydrate concentrations ($\mu\text{g/mL}$) of hydrolysate obtained from enzymatic hydrolysis of spent *S. cerevisiae* using Cellulase (0.3%, v/v)

Time (hr)	Protein concentration ($\mu\text{g/mL}$)	Carbohydrate concentration ($\mu\text{g/mL}$)
0	3796.3 $\pm$ 167.93	1626.4 $\pm$ 118.69
24	13180.0 $\pm$ 530.33	2447.5 $\pm$ 189.40
48	13067.5 $\pm$ 477.30	2677.5 $\pm$ 151.52
72	16410.0 $\pm$ 141.42	3070.0 $\pm$ 20.20
96	9985.0 $\pm$ 106.07	2830.7 $\pm$ 156.57

Table 14: Average protein and carbohydrate concentrations ($\mu\text{g/mL}$) of hydrolysate obtained from enzymatic hydrolysis of spent *S. cerevisiae* using Cellulase (0.4%, v/v)

Time (hr)	Protein concentration ($\mu\text{g/mL}$)	Carbohydrate concentration ($\mu\text{g/mL}$)
0	4677.5 $\pm$ 0.00	1133.6 $\pm$ 15.15
24	13392.5 $\pm$ 229.81	2543.9 $\pm$ 83.34
48	16367.5 $\pm$ 830.85	2565.0 $\pm$ 37.88
72	14385.0 $\pm$ 35.36	3405.7 $\pm$ 30.31
96	11285.0 $\pm$ 247.49	2745.0 $\pm$ 25.25

Table 15: Average protein and carbohydrate concentrations ($\mu\text{g/mL}$) of hydrolysate obtained from enzymatic hydrolysis of spent *S. cerevisiae* using Cellulase (0.5%, v/v)

Time (hr)	Protein concentration ($\mu\text{g/mL}$)	Carbohydrate concentration ($\mu\text{g/mL}$)
0	4115.0 $\pm$ 194.45	1674.3 $\pm$ 181.83
24	16992.5 $\pm$ 371.23	2985.7 $\pm$ 113.64
48	17892.5 $\pm$ 548.01	3572.1 $\pm$ 280.32
72	19760.0 $\pm$ 707.11	3684.3 $\pm$ 212.13
96	12560.0 $\pm$ 424.26	3395.0 $\pm$ 196.98

Appendix D: Statistical Analysis Data

1. Bioethanol production at 48 hours of fermentation using SFH and CG media

BIOETHANOL

One-way ANOVA: Bioethanol (48 hr) versus Fermentation media

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	$\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels Values
Fermentation media	2 SFH, CG

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Fermentation media	1	1.411	1.4114	1.45	0.295
Error	4	3.898	0.9745		
Total	5	5.309			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.987142	26.58%	8.23%	0.00%

Means

Fermentation media	N	Mean	StDev	95% CI
SFH	3	28.010	1.396	(26.428, 29.592)
CG	3	28.98	0.00	(27.40, 30.56)

Pooled StDev = 0.987142

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Fermentation media	N	Mean	Grouping
CG	3	28.98	A
SFH	3	28.010	A

Means that do not share a letter are significantly different.

2. Effect of different initial pH on protein concentrations

WORKSHEET 2
PH LEVELS

One-way ANOVA: Protein versus pH level

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	$\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
pH level	3	3, 5, 7

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
pH level	2	16502533	8251267	40.74	0.007
Error	3	607600	202533		
Total	5	17110133			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
450.037	96.45%	94.08%	85.80%

Means

pH level	N	Mean	StDev	95% CI
3	3	10834	212	(9821, 11847)
5	3	10664.0	28.3	(9651.3, 11676.7)
7	3	7234	750	(6221, 8247)

Pooled StDev = 450.037

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

pH level	N	Mean	Grouping
3	3	10834	A
5	3	10664.0	A
7	3	7234	B

Means that do not share a letter are significantly different.

3. Effect of incubation period on protein concentrations at initial pH 3

INCUBATION PERIOD

One-way ANOVA: Protein (pH 3) versus Incubation Period

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	$\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Hour	3	48, 72, 96

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Hour	2	443200	221600	3.09	0.187
Error	3	215000	71667		
Total	5	658200			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
267.706	67.34%	45.56%	0.00%

Means

Hour	N	Mean	StDev	95% CI
48	3	10314	212	(9712, 10916)
72	3	10834	212	(10232, 11436)
96	3	10934	354	(10332, 11536)

Pooled StDev = 267.706

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Hour	N	Mean	Grouping
96	3	10934	A
72	3	10834	A
48	3	10314	A

Means that do not share a letter are significantly different.

4. Effect of incubation period on protein concentration at initial pH 5

INCUBATION PERIOD

One-way ANOVA: Protein (pH 5) versus Hour

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	$\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Hour	3	48, 72, 96

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Hour	2	349161	174581	0.15	0.868
Error	3	3519850	1173283		
Total	5	3869011			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
1083.18	9.02%	0.00%	0.00%

Means

Hour	N	Mean	StDev	95% CI
48	3	10174	1655	(7736, 12612)
72	3	10664.0	28.3	(8226.5, 13101.5)
96	3	10705	884	(8267, 13143)

Pooled StDev = 1083.18

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Hour	N	Mean	Grouping
96	3	10705	A
72	3	10664.0	A
48	3	10174	A

Means that do not share a letter are significantly different.

5. Effect of incubation period on carbohydrate concentrations at initial pH 3

INCUBATION PERIOD

One-way ANOVA: Carbohydrate (pH 3) versus Incubation period

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	$\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Hour	3	48, 72, 96

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Hour	2	178904	89452	20.35	0.018
Error	3	13190	4397		
Total	5	192094			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
66.3068	93.13%	88.56%	72.53%

Means

Hour	N	Mean	StDev	95% CI
48	3	635.4	27.8	(486.1, 784.6)
72	3	1040.7	111.2	(891.5, 1189.9)
96	3	942.65	7.85	(793.44, 1091.86)

Pooled StDev = 66.3068

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Hour	N	Mean	Grouping
72	3	1040.7	A
96	3	942.65	A
48	3	635.4	B

Means that do not share a letter are significantly different.

6. Effect of incubation period on carbohydrate concentration at initial pH 5

INCUBATION PERIOD

One-way ANOVA: Carbohydrate (pH 5) versus Incubation Period

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	$\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Hour	3	48, 72, 96

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Hour	2	245710	122855	171.87	0.000
Error	6	4289	715		
Total	8	249999			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
26.7359	98.28%	97.71%	96.14%

Means

Hour	N	Mean	StDev	95% CI
48	3	521.7	32.0	(483.9, 559.5)
72	3	859.7	23.7	(821.9, 897.5)
96	3	883.5	23.6	(845.7, 921.3)

Pooled StDev = 26.7359

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Hour	1	N	Mean	Grouping
96		3	883.5	A
72		3	859.7	A
48		3	521.7	B

Means that do not share a letter are significantly different.

7. Effect of Alcalase concentrations on protein concentrations

ALCALASE

One-way ANOVA: Protein versus Alcalase concentrations

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	$\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Alcalase concentrations	5	0.1, 0.2, 0.3, 0.4, 0.5

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Alcalase concentrations	4	32453125	8113281	81.64	0.000
Error	5	496875	99375		
Total	9	32950000			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
315.238	98.49%	97.29%	93.97%

Means

Alcalase concentrations	N	Mean	StDev	95% CI
0.1	3	7105.0	106.1	(6532.0, 7678.0)
0.2	3	9368	194	(8794, 9941)
0.3	3	10205	530	(9632, 10778)
0.4	3	12092.5	123.7	(11519.5, 12665.5)
0.5	3	11755	389	(11182, 12328)

Pooled StDev = 315.238

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Alcalase concentrations	N	Mean	Grouping
0.4	3	12092.5	A
0.5	3	11755	A
0.3	3	10205	B
0.2	3	9368	B
0.1	3	7105.0	C

Means that do not share a letter are significantly different.

8. Effect of Alcalase concentrations on carbohydrate concentrations

ALCALASE

One-way ANOVA: Carbohydrate versus Alcalase concentrations

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	$\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Alcalase concentrations	5	0.1, 0.2, 0.3, 0.4, 0.5

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Alcalase concentrations	4	20948112	5237028	71.05	0.000
Error	5	368532	73706		
Total	9	21316644			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
271.489	98.27%	96.89%	93.08%

Means

Alcalase concentrations	N	Mean	StDev	95% CI
0.1	3	2134.3	30.3	(1640.8, 2627.8)
0.2	3	2363	584	(1869, 2856)
0.3	3	3467.9	19.0	(2974.4, 3961.4)
0.4	3	5816.8	45.5	(5323.3, 6310.2)
0.5	3	5011	155	(4517, 5504)

Pooled StDev = 271.489

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Alcalase concentrations	N	Mean	Grouping
0.4	3	5816.8	A
0.5	3	5011	A
0.3	3	3467.9	B
0.2	3	2363	C
0.1	3	2134.3	C

Means that do not share a letter are significantly different.

9. Effect of incubation period on carbohydrate concentrations using alcalase

INCUBATION PERIODS

One-way ANOVA: Alcalase versus Hour

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	$\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Hour	3	48, 72, 96

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Hour	2	1187811	593905	39.51	0.007
Error	3	45092	15031		
Total	5	1232903			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
122.600	96.34%	93.90%	85.37%

Means

Hour	N	Mean	StDev	95% CI
48	3	4796.25	11.38	(4520.36, 5072.14)
72	3	5816.8	45.5	(5540.9, 6092.6)
96	3	5638	207	(5362, 5914)

Pooled StDev = 122.600

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Hour	N	Mean	Grouping
72	3	5816.8	A
96	3	5638	A
48	3	4796.25	B

Means that do not share a letter are significantly different.

10. Effect of incubation period on carbohydrate concentrations using cellulase

INCUBATION PERIODS

One-way ANOVA: Cellulase versus Hour

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	$\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Hour	3	48, 72, 96

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Hour	2	85103	42551	0.79	0.532
Error	3	162415	54138		
Total	5	247518			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
232.677	34.38%	0.00%	0.00%

Means

Hour	N	Mean	StDev	95% CI
48	3	3572	280	(3049, 4096)
72	3	3684	212	(3161, 4208)
96	3	3395	197	(2871, 3919)

Pooled StDev = 232.677

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Hour	N	Mean	Grouping
72	3	3684	A
48	3	3572	A
96	3	3395	A

Means that do not share a letter are significantly different.

11. Effect of different lysis treatments on protein concentrations

AUTOLYSIS VS ENZYMATIC HYDROLYSIS

One-way ANOVA: Protein versus Treatments

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	$\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Treatments	3	A (0.4%), C (0.5%), pH 3

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatments	2	92020636	46010318	215.57	0.001
Error	3	640312	213437		
Total	5	92660949			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
461.993	99.31%	98.85%	97.24%

Means

Treatments	N	Mean	StDev	95% CI
A (0.4%)	3	12092.5	123.7	(11052.9, 13132.1)
C (0.5%)	3	19760	707	(18720, 20800)
pH 3	3	10934	354	(9894, 11974)

Pooled StDev = 461.993

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Treatments	N	Mean	Grouping
C (0.5%)	3	19760	A
A (0.4%)	3	12092.5	B
pH 3	3	10934	B

Means that do not share a letter are significantly different.

12. Effect of different lysis treatments on carbohydrate concentrations

AUTOLYSIS VS ENZYMATIC HYDROLYSIS

One-way ANOVA: Carbohydrate versus Treatments

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	$\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Treatments	3	A (0.4%), C (0.5%), pH 3

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatments	2	22897745	11448873	578.00	0.000
Error	3	59423	19808		
Total	5	22957168			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
140.740	99.74%	99.57%	98.96%

Means

Treatments	N	Mean	StDev	95% CI
A (0.4%)	3	5816.8	45.5	(5500.0, 6133.5)
C (0.5%)	3	3684	212	(3368, 4001)
pH 3	3	1040.7	111.2	(724.0, 1357.4)

Pooled StDev = 140.740

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Treatments	N	Mean	Grouping
A (0.4%)	3	5816.8	A
C (0.5%)	3	3684	B
pH 3	3	1040.7	C

Means that do not share a letter are significantly different.

Appendix E: Lysates harvested after lysis of spent Baker's yeast

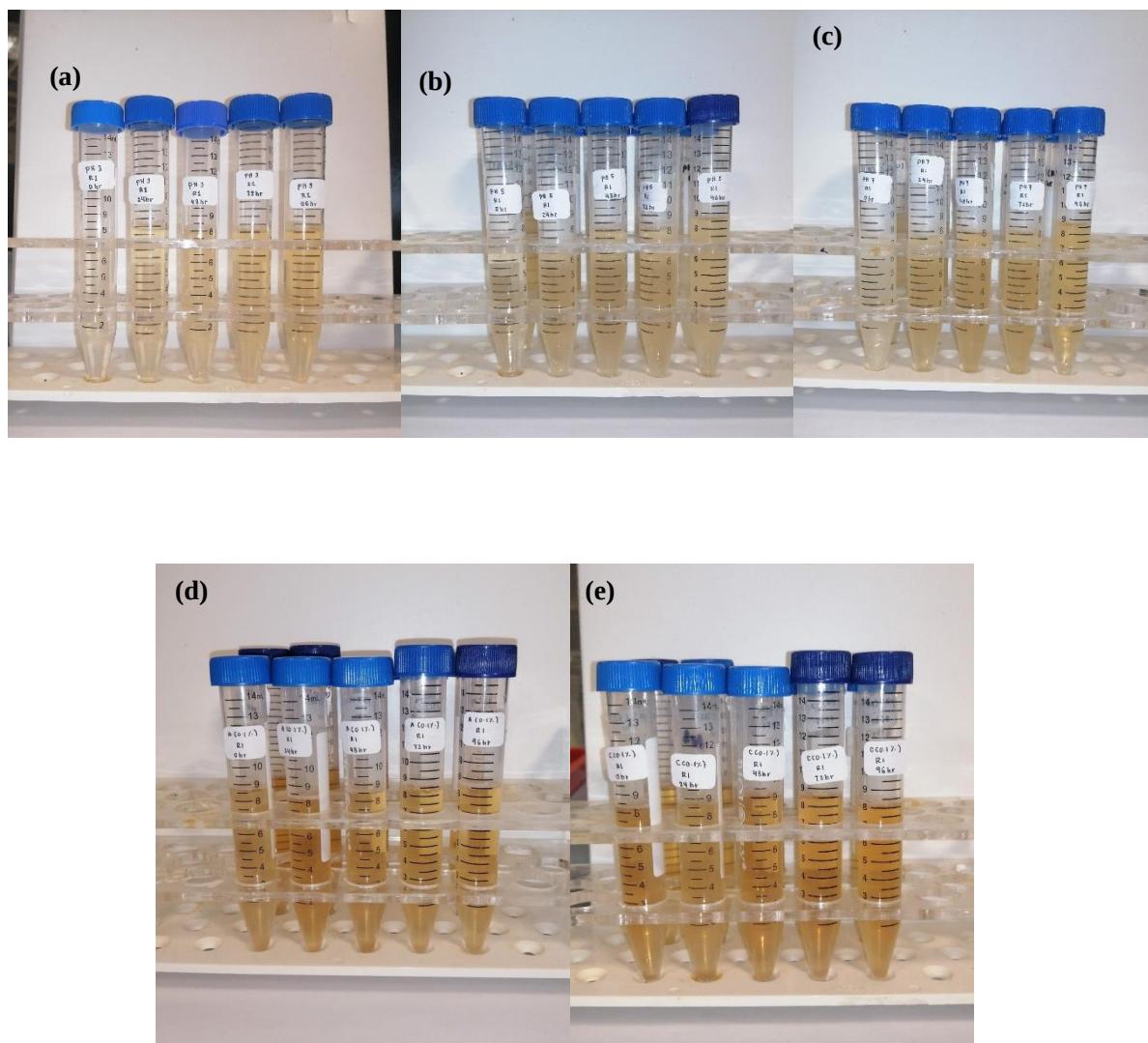


Figure 1: The supernatants (yeast extracts) collected from 0 hr (a) to 96 hr (e)

Appendix F: Chemical analyses and HPLC results

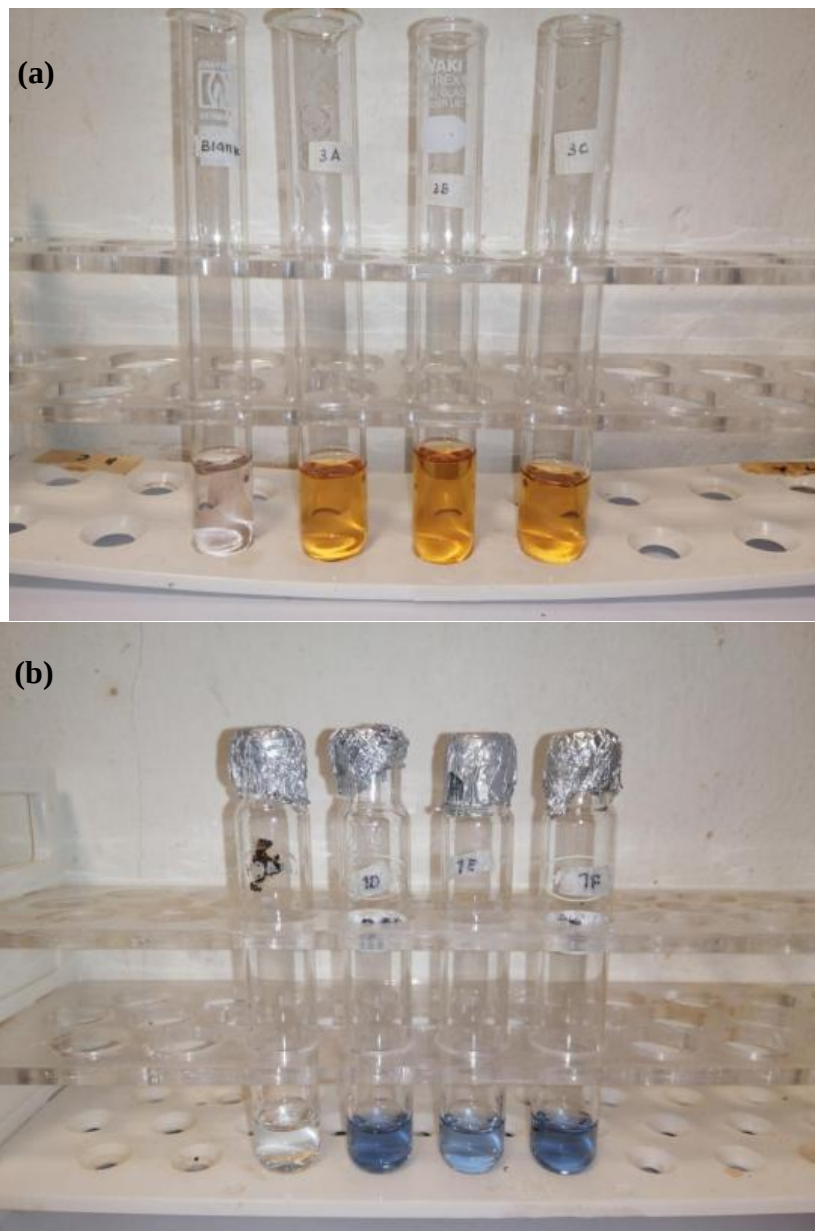


Figure 1: Chemical analysis conducted for quantifying (a) protein concentration using Lowry assay and (b) carbohydrate concentration using Phenol-sulphuric assay

HPLC results:

Fermentation using SFH media (50 g/L glucose)

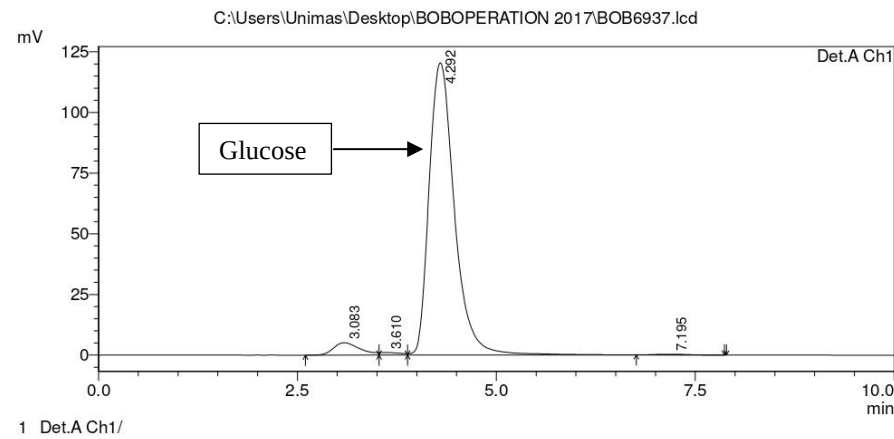
(a) Ethanol fermentation at 0 hr (Replicate 1)

2/22/2023 13:59:29 1 / 1

==== Shimadzu LCsolution Analysis Report ====

C:\Users\Unimas\Desktop\BOBOPERATION 2017\BOB6937.lcd
Acquired by : Admin
Sample Name : SFH R1 0h
Sample ID :
Vial # :
Injection Volume : 20 µL
Data File Name : BOB6937.lcd
Method File Name : Adilla-method.lcm
Batch File Name :
Report File Name : Default.lcr
Data Acquired : 2/22/2023 1:46:42 PM
Data Processed : 2/22/2023 1:56:45 PM

<Chromatogram>



PeakTable

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.083	118117	5133	4.180	4.044
2	3.610	19604	1079	0.694	0.850
3	4.292	2679063	120419	94.811	94.869
4	7.195	8899	301	0.315	0.237
Total		2825683	126933	100.000	100.000

C:\Users\Unimas\Desktop\BOBOPERATION 2017\BOB6937.lcd

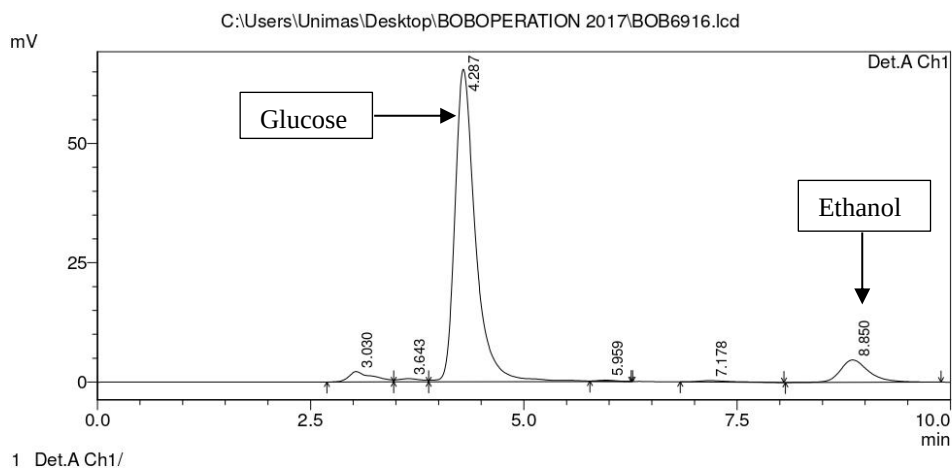
(b) Ethanol fermentation at 8hr (Replicate 1)

2/21/2023 14:51:31 1 / 1

==== Shimadzu LcSolution Analysis Report ====

Acquired by : Admin
Sample Name : SFH R1 8h
Sample ID :
Vial # :
Injection Volume : 20 uL
Data File Name : BOB6916.lcd
Method File Name : Adilla-method.lcm
Batch File Name :
Report File Name : Default.lcr
Data Acquired : 2/21/2023 2:17:18 PM
Data Processed : 2/21/2023 2:27:21 PM

<Chromatogram>



Detector A Ch1

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.030	42478	2168	3.334	2.947
2	3.643	12618	677	0.990	0.920
3	4.287	1097712	65508	86.154	89.037
4	5.959	2750	209	0.216	0.284
5	7.178	8209	308	0.644	0.419
6	8.850	110368	4704	8.662	6.394
Total		1274135	73574	100.000	100.000

C:\Users\Unimas\Desktop\BOBOPERATION 2017\BOB6916.lcd

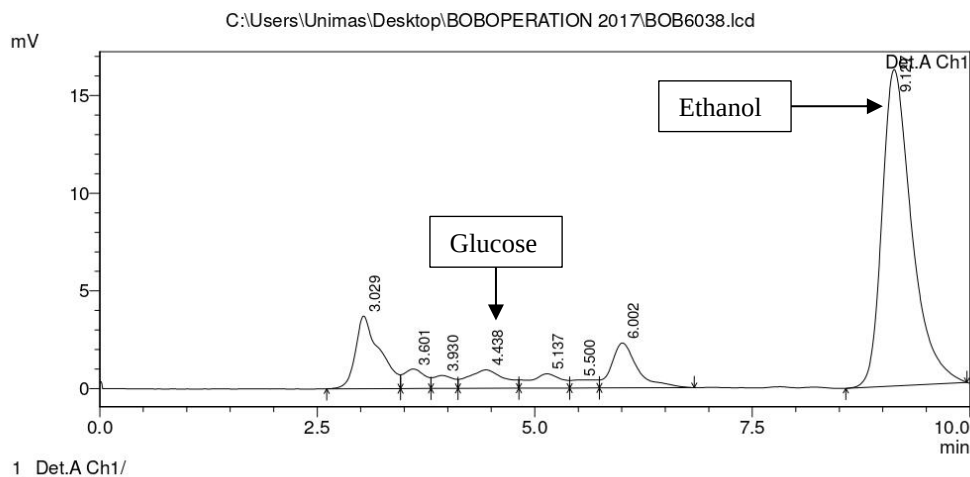
(c) Ethanol fermentation at 24hr (Replicate 1)

10/22/2021 10:45:40 1 / 1

==== Shimadzu LCsolution Analysis Report ====

Acquired by : Admin
Sample Name : R1-24hr
Sample ID :
Vial # :
Injection Volume : 20 uL
Data File Name : BOB6038.lcd
Method File Name : Adilla-method.lcm
Batch File Name :
Report File Name : Default.lcr
Data Acquired : 10/22/2021 10:34:32 AM
Data Processed : 10/22/2021 10:44:35 AM

<Chromatogram>



PeakTable

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.029	72481	3718	12.378	14.325
2	3.601	16820	999	2.872	3.849
3	3.930	10654	662	1.819	2.549
4	4.438	27198	942	4.645	3.630
5	5.137	18131	728	3.096	2.807
6	5.500	8337	412	1.424	1.588
7	6.002	47212	2297	8.062	8.848
8	9.127	384742	16197	65.703	62.404
Total		585575	25955	100.000	100.000

C:\Users\Unimas\Desktop\BOBOPERATION 2017\BOB6038.lcd

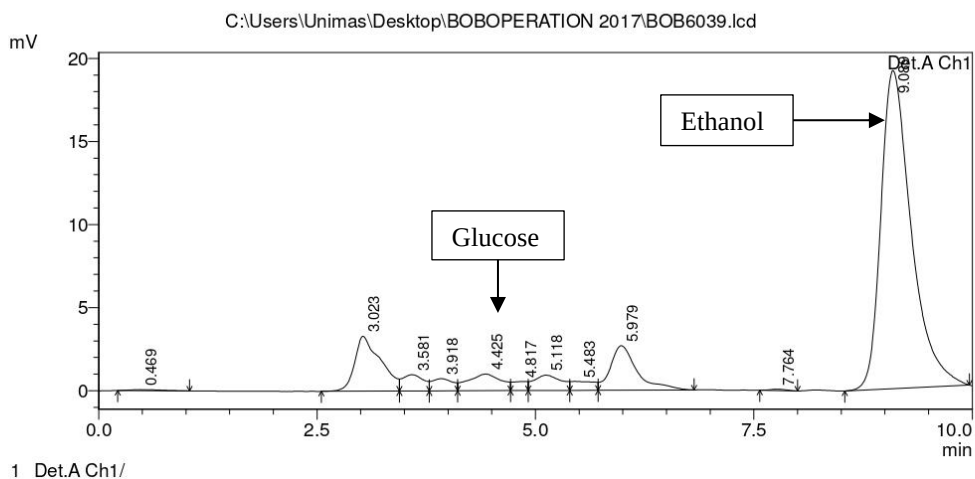
(d) Ethanol fermentation at 32 hr (Replicate 1)

10/22/2021 10:58:43 1 / 1

==== Shimadzu LCsolution Analysis Report ====

Acquired by : Admin
Sample Name : R1-32hr
Sample ID :
Vial # :
Injection Volume : 20 uL
Data File Name : BOB6039.lcd
Method File Name : Adilla-method.lcm
Batch File Name :
Report File Name : Default.lcr
Data Acquired : 10/22/2021 10:47:02 AM
Data Processed : 10/22/2021 10:57:04 AM

<Chromatogram>



Detector A Ch1

Peak#	Ret. Time	Area	Height	Area %	Height %
1	0.469	1533	61	0.224	0.204
2	3.023	68292	3306	10.000	11.020
3	3.581	16500	982	2.416	3.272
4	3.918	12214	737	1.789	2.456
5	4.425	26347	1001	3.858	3.338
6	4.817	6372	537	0.933	1.791
7	5.118	20147	921	2.950	3.071
8	5.483	10038	534	1.470	1.779
9	5.979	56611	2681	8.290	8.937
10	7.764	1083	85	0.159	0.283
11	9.089	463768	19154	67.911	63.851
Total		682904	29998	100.000	100.000

C:\Users\Unimas\Desktop\BOBOPERATION 2017\BOB6039.lcd

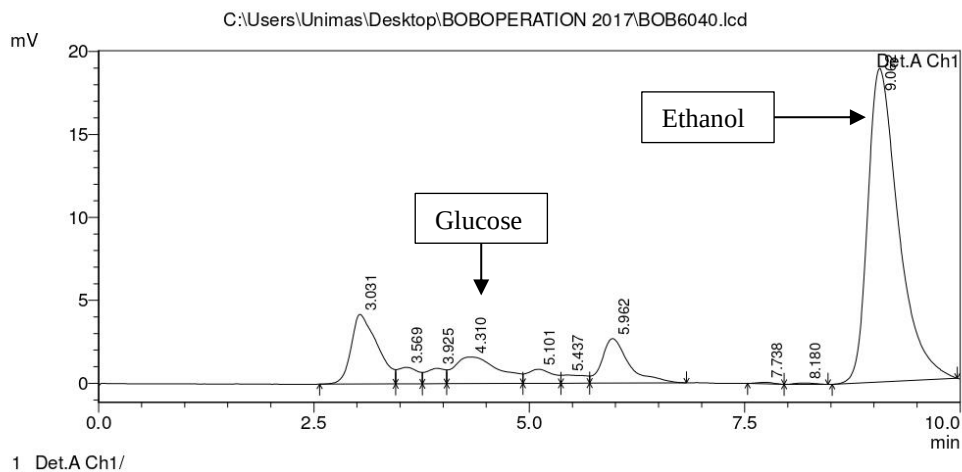
(e) Ethanol fermentation at 48 hr (Replicate 1)

10/22/2021 11:12:33 1 / 1

==== Shimadzu LCsolution Analysis Report ====

Acquired by : Admin
Sample Name : R1-48hr
Sample ID :
Vial # :
Injection Volume : 20 uL
Data File Name : BOB6040.lcd
Method File Name : Adilla-method.lcm
Batch File Name :
Report File Name : Default.lcr
Data Acquired : 10/22/2021 11:00:27 AM
Data Processed : 10/22/2021 11:10:29 AM

<Chromatogram>



PeakTable

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.031	89198	4198	12.126	13.614
2	3.569	16184	996	2.200	3.230
3	3.925	14225	925	1.934	2.999
4	4.310	57660	1603	7.838	5.198
5	5.101	17794	850	2.419	2.755
6	5.437	9390	493	1.277	1.600
7	5.962	58199	2686	7.912	8.712
8	7.738	1209	95	0.164	0.309
9	8.180	1363	84	0.185	0.272
10	9.062	470379	18906	63.945	61.312
Total		735600	30836	100.000	100.000

C:\Users\Unimas\Desktop\BOBOPERATION 2017\BOB6040.lcd

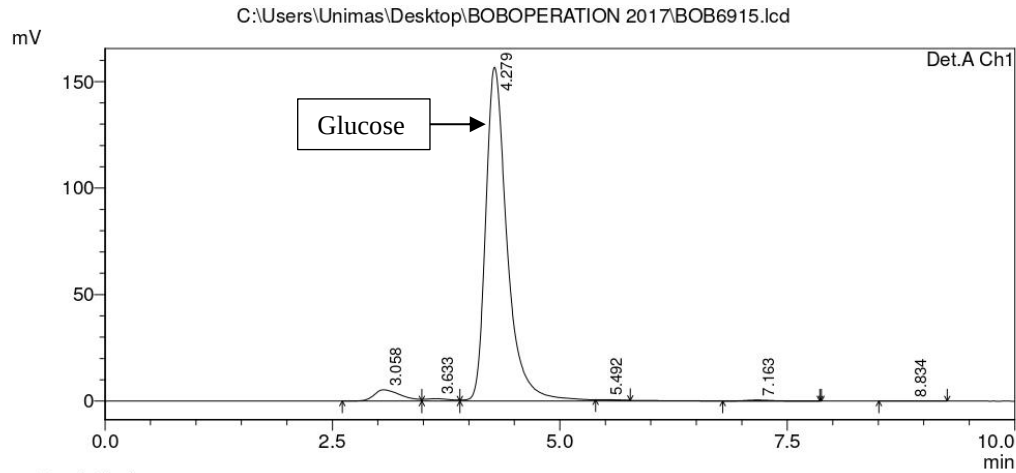
(f) Ethanol fermentation at 0 hr (Replicate 2)

2/21/2023 14:15:33 1 / 1

==== Shimadzu LCsolution Analysis Report ====

Acquired by : Admin
Sample Name : SFH R2 0h
Sample ID :
Vial # :
Injection Volume : 20 uL
Data File Name : BOB6915.lcd
Method File Name : Adilla-method.lcm
Batch File Name :
Report File Name : Default.lcr
Data Acquired : 2/21/2023 2:04:59 PM
Data Processed : 2/21/2023 2:15:02 PM

<Chromatogram>



PeakTable

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.058	108686	5321	3.969	3.246
2	3.633	21722	1153	0.793	0.703
3	4.279	2594717	156801	94.762	95.650
4	5.492	1390	98	0.051	0.060
5	7.163	9630	460	0.352	0.281
6	8.834	1991	99	0.073	0.060
Total		2738135	163932	100.000	100.000

C:\Users\Unimas\Desktop\BOBOPERATION 2017\BOB6915.lcd

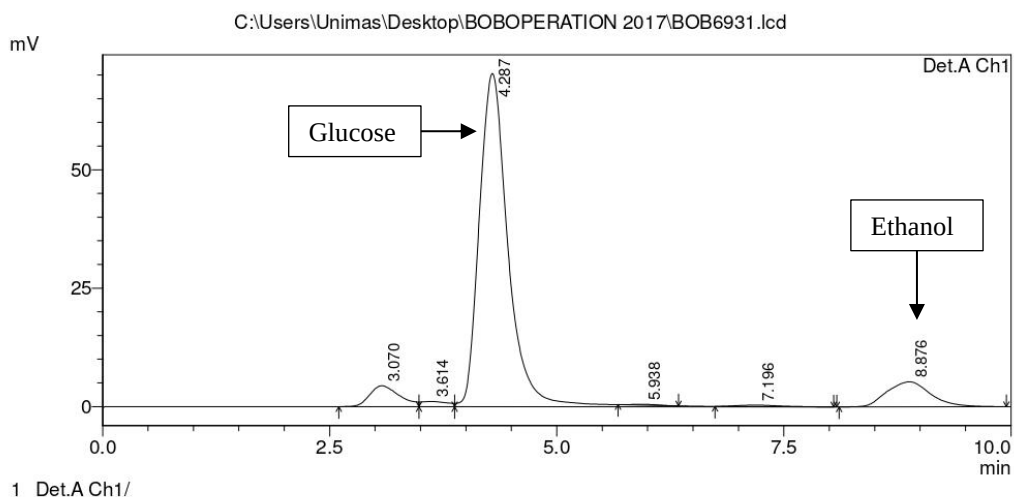
(g) Ethanol fermentation at 8 hr (Replicate 2)

2/22/2023 12:08:29 1 / 1

==== Shimadzu LCsolution Analysis Report ====

Acquired by : Admin
Sample Name : SFH R2 8h
Sample ID :
Vial # :
Injection Volume : 20 uL
Data File Name : BOB6931.lcd
Method File Name : Adilla-method.lcm
Batch File Name :
Report File Name : Default.lcr
Data Acquired : 2/22/2023 11:57:49 AM
Data Processed : 2/22/2023 12:07:52 PM

<Chromatogram>



PeakTable

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.070	100584	4411	5.358	5.404
2	3.614	22406	1097	1.194	1.344
3	4.287	1563526	70349	83.294	86.198
4	5.938	3512	173	0.187	0.212
5	7.196	10239	311	0.545	0.381
6	8.876	176851	5274	9.421	6.462
Total		1877117	81613	100.000	100.000

C:\Users\Unimas\Desktop\BOBOPERATION 2017\BOB6931.lcd

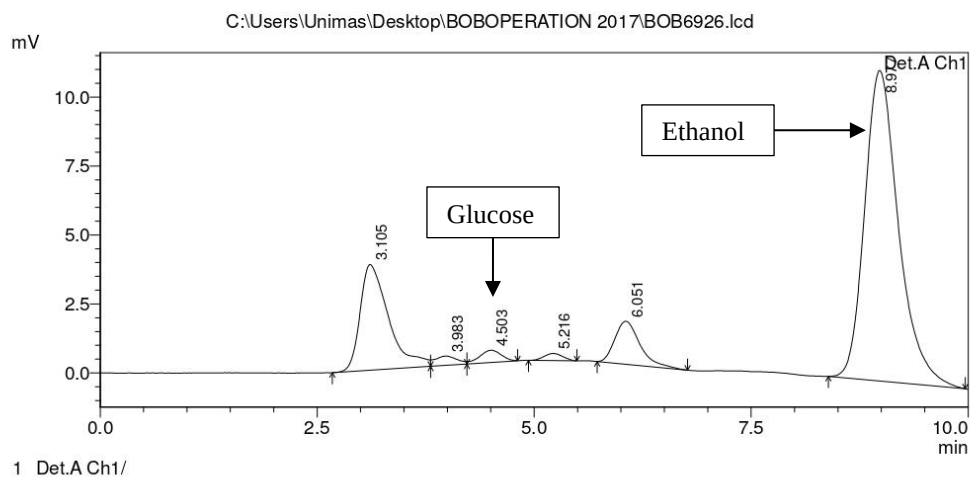
(h) Ethanol fermentation at 24 hr (Replicate 2)

2/22/2023 11:05:23 1 / 1

==== Shimadzu LCsolution Analysis Report ====

Acquired by : Admin
Sample Name : SFH R3 24h
Sample ID :
Vial # :
Injection Volume : 20 uL
Data File Name : BOB6926.lcd
Method File Name : Adilla-method.lcm
Batch File Name :
Report File Name : Default.lcr
Data Acquired : 2/22/2023 10:54:22 AM
Data Processed : 2/22/2023 11:04:23 AM

<Chromatogram>



PeakTable						
Detector A Ch1						
Peak#	Ret. Time	Area	Height	Area %	Height %	
1	3.105	87223	3836	19.759	21.692	
2	3.983	5926	336	1.342	1.899	
3	4.503	7715	441	1.748	2.496	
4	5.216	3875	259	0.878	1.462	
5	6.051	31455	1557	7.126	8.806	
6	8.977	305233	11254	69.147	63.645	
Total		441427	17682	100.000	100.000	

C:\Users\Unimas\Desktop\BOBOPERATION 2017\BOB6926.lcd

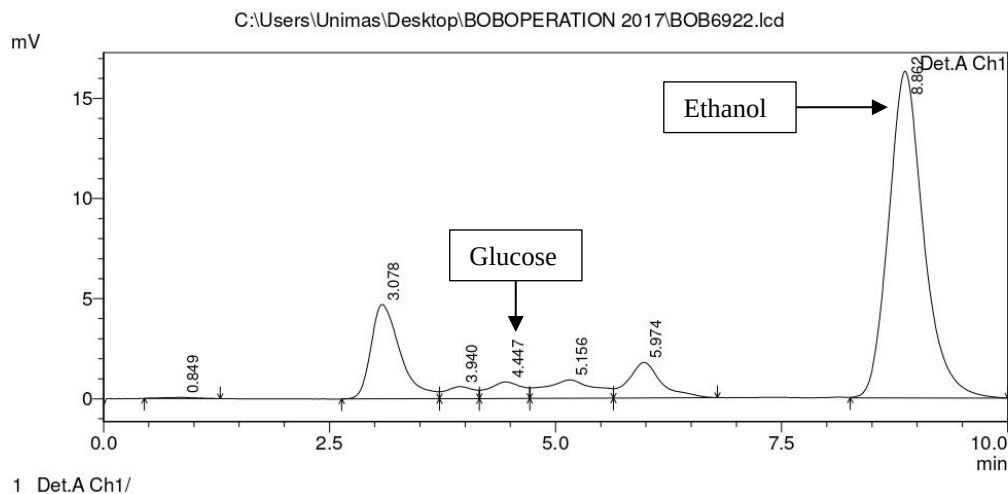
(i) Ethanol fermentation at 32 hr (Replicate 2)

2/21/2023 16:02:36 1 / 1

==== Shimadzu LCsolution Analysis Report ====

Acquired by : Admin
Sample Name : SFH R2 32h
Sample ID :
Vial # :
Injection Volume : 20 uL
Data File Name : BOB6922.lcd
Method File Name : Adilla-method.lcm
Batch File Name :
Report File Name : Default.lcr
Data Acquired : 2/21/2023 3:52:03 PM
Data Processed : 2/21/2023 4:02:06 PM

<Chromatogram>



PeakTable					
Detector A Ch1					
Peak#	Ret. Time	Area	Height	Area %	Height %
1	0.849	1477	57	0.223	0.225
2	3.078	106559	4717	16.085	18.709
3	3.940	12841	597	1.938	2.368
4	4.447	21174	824	3.196	3.269
5	5.156	35806	916	5.405	3.634
6	5.974	44575	1775	6.729	7.041
7	8.862	440030	16324	66.423	64.753
Total		662463	25210	100.000	100.000

C:\Users\Unimas\Desktop\BOBOPERATION 2017\BOB6922.lcd

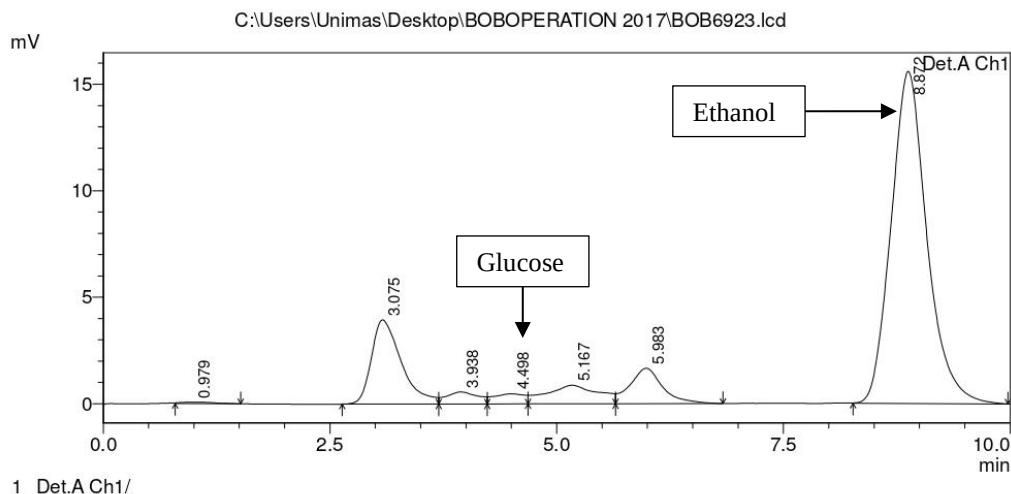
(j) Ethanol fermentation at 48 hr (Replicate 2)

2/21/2023 16:14:16 1 / 1

==== Shimadzu LCsolution Analysis Report ====

Acquired by : Admin
Sample Name : SFH R2 48h
Sample ID :
Vial # :
Injection Volume : 20 uL
Data File Name : BOB6923.lcd
Method File Name : Adilla-method.lcm
Batch File Name :
Report File Name : Default.lcr
Data Acquired : 2/21/2023 4:03:35 PM
Data Processed : 2/21/2023 4:13:39 PM

<Chromatogram>



PeakTable

Peak#	Ret. Time	Area	Height	Area %	Height %
1	0.979	1803	70	0.290	0.300
2	3.075	90745	3955	14.574	17.045
3	3.938	13724	563	2.204	2.425
4	4.498	11182	473	1.796	2.039
5	5.167	34702	873	5.573	3.763
6	5.983	41548	1668	6.673	7.189
7	8.872	428966	15601	68.891	67.240
Total		622669	23202	100.000	100.000

C:\Users\Unimas\Desktop\BOBOPERATION 2017\BOB6923.lcd

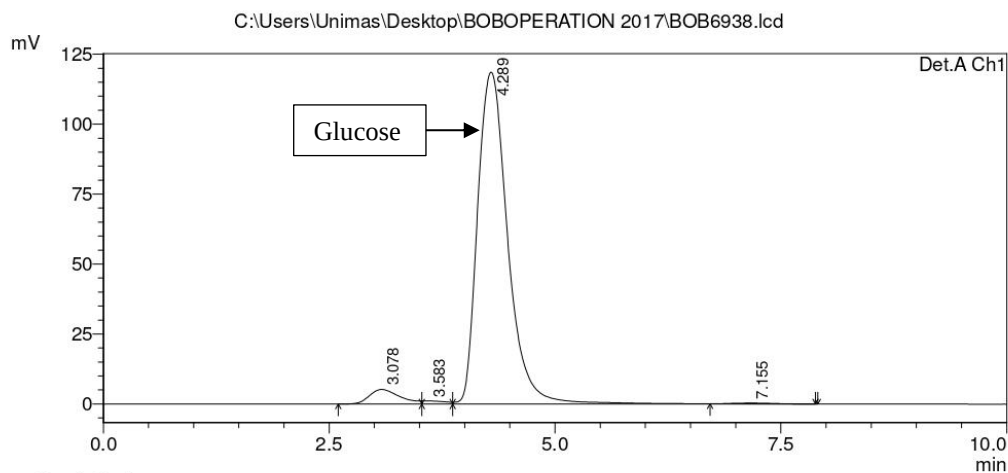
(k) Ethanol fermentation at 0 hr (Replicate 3)

2/22/2023 14:13:21 1 / 1

==== Shimadzu LCsolution Analysis Report ====

Acquired by : Admin
Sample Name : SFH R3 0h
Sample ID :
Vial # :
Injection Volume : 20 uL
Data File Name : BOB6938.lcd
Method File Name : Adilla-method.lcm
Batch File Name :
Report File Name : Default.lcr
Data Acquired : 2/22/2023 2:02:42 PM
Data Processed : 2/22/2023 2:12:45 PM

<Chromatogram>



Detector A Ch1

PeakTable					
Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.078	121778	5209	4.190	4.161
2	3.583	19146	1094	0.659	0.874
3	4.289	2755939	118598	94.827	94.730
4	7.155	9425	294	0.324	0.235
Total		2906288	125196	100.000	100.000

C:\Users\Unimas\Desktop\BOBOPERATION 2017\BOB6938.lcd

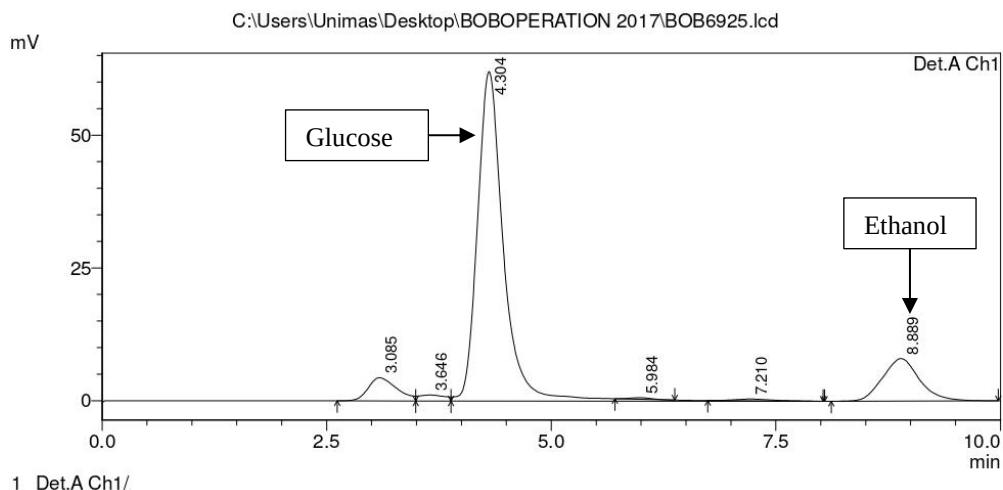
(I) Ethanol fermentation at 8 hr (Replicate 3)

2/21/2023 16:38:51 1 / 1

==== Shimadzu LCsolution Analysis Report ====

Acquired by : Admin
Sample Name : SFH R3 8h
Sample ID :
Vial # :
Injection Volume : 20 uL
Data File Name : BOB6925.lcd
Method File Name : Adilla-method.lcm
Batch File Name :
Report File Name : Default.lcr
Data Acquired : 2/21/2023 4:28:19 PM
Data Processed : 2/21/2023 4:38:23 PM

<Chromatogram>



PeakTable

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.085	96615	4364	5.932	5.732
2	3.646	22602	1119	1.388	1.469
3	4.304	1263560	62008	77.585	81.441
4	5.984	4976	294	0.306	0.387
5	7.210	10364	337	0.636	0.443
6	8.889	230505	8015	14.153	10.528
Total		1628622	76138	100.000	100.000

C:\Users\Unimas\Desktop\BOBOPERATION 2017\BOB6925.lcd

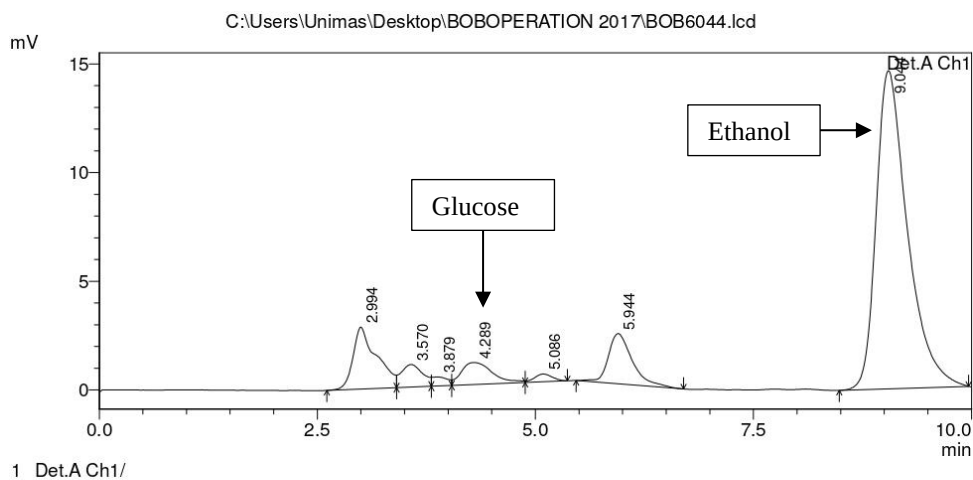
(m) Ethanol fermentation at 24 hr (Replicate 3)

11/1/2021 16:57:45 1 / 1

==== Shimadzu LCsolution Analysis Report ====

Acquired by : Admin
Sample Name : R3-24hr
Sample ID :
Vial # :
Injection Volume : 20 uL
Data File Name : BOB6044.lcd
Method File Name : Adilla-method.lcm
Batch File Name :
Report File Name : Default.lcr
Data Acquired : 11/1/2021 4:47:11 PM
Data Processed : 11/1/2021 4:57:13 PM

<Chromatogram>



PeakTable

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.994	53680	2852	10.395	12.613
2	3.570	17813	1040	3.449	4.599
3	3.879	5006	415	0.969	1.834
4	4.289	24989	1010	4.839	4.468
5	5.086	4958	351	0.960	1.554
6	5.944	44312	2309	8.581	10.211
7	9.044	365667	14636	70.807	64.721
Total		516424	22615	100.000	100.000

C:\Users\Unimas\Desktop\BOBOPERATION 2017\BOB6044.lcd

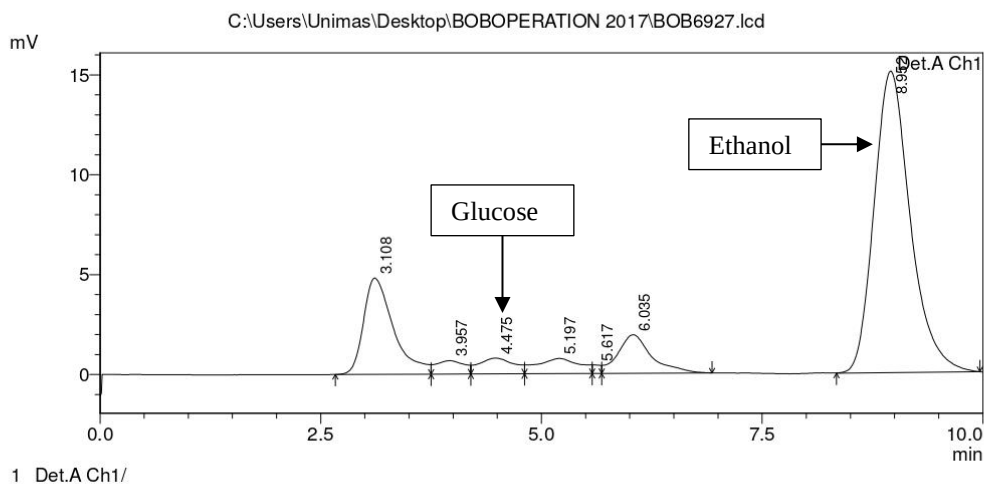
(n) Ethanol fermentation at 32 hr (Replicate 3)

2/22/2023 11:18:29 1 / 1

==== Shimadzu LCsolution Analysis Report ====

Acquired by : Admin
Sample Name : SFH R3 32h
Sample ID :
Vial # :
Injection Volume : 20 uL
Data File Name : BOB6927.lcd
Method File Name : Adilla-method.lcm
Batch File Name :
Report File Name : Default.lcr
Data Acquired : 2/22/2023 11:07:48 AM
Data Processed : 2/22/2023 11:17:51 AM

<Chromatogram>



Detector A Ch1

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.108	113755	4822	17.224	19.697
2	3.957	14985	667	2.269	2.725
3	4.475	21698	788	3.285	3.217
4	5.197	25282	756	3.828	3.089
5	5.617	2689	419	0.407	1.712
6	6.035	50843	1927	7.698	7.870
7	8.952	431202	15103	65.289	61.689
Total		660453	24483	100.000	100.000

C:\Users\Unimas\Desktop\BOBOPERATION 2017\BOB6927.lcd

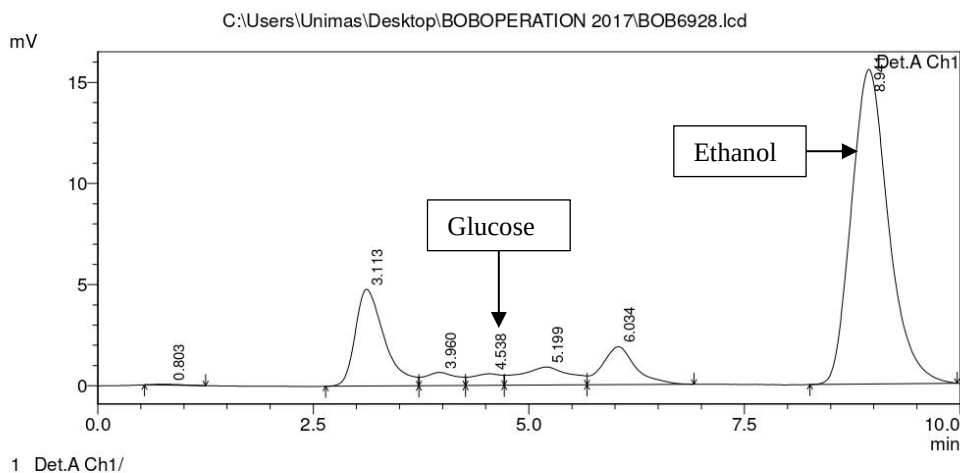
(o) Ethanol fermentation at 48 hr (Replicate 3)

2/22/2023 11:30:21 1 / 1

==== Shimadzu LCsolution Analysis Report ====

Acquired by : Admin
Sample Name : SFH R3 48h
Sample ID :
Vial # :
Injection Volume : 20 uL
Data File Name : BOB6928.lcd
Method File Name : Adilla-method.lcm
Batch File Name :
Report File Name : Default.lcr
Data Acquired : 2/22/2023 11:19:43 AM
Data Processed : 2/22/2023 11:29:45 AM

<Chromatogram>



PeakTable

Peak#	Ret. Time	Area	Height	Area %	Height %
1	0.803	1007	43	0.145	0.177
2	3.113	112515	4790	16.180	19.650
3	3.960	16963	654	2.439	2.685
4	4.538	13699	573	1.970	2.350
5	5.199	36454	894	5.242	3.666
6	6.034	49911	1872	7.177	7.679
7	8.941	464869	15551	66.847	63.791
Total		695419	24378	100.000	100.000

C:\Users\Unimas\Desktop\BOBOPERATION 2017\BOB6928.lcd

ii. Fermentation Control; Commercial glucose (50 g/L)

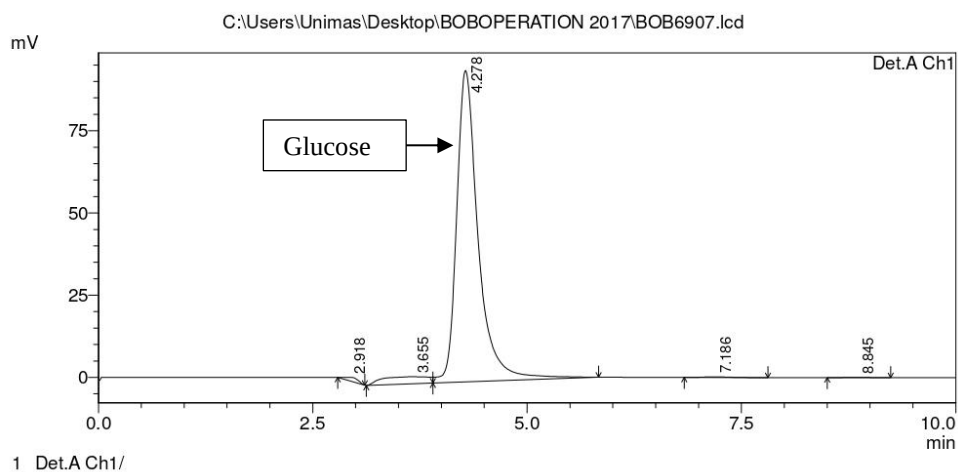
(a) Ethanol fermentation at 0 hr (Replicate 1)

2/20/2023 16:42:45 1 / 1

==== Shimadzu LcSolution Analysis Report ====

Acquired by : Admin
Sample Name : Ctrl R1 0h
Sample ID :
Vial # :
Injection Volume : 20 uL
Data File Name : BOB6907.lcd
Method File Name : Adilla-method.lcm
Batch File Name :
Report File Name : Default.lcr
Data Acquired : 2/20/2023 4:32:10 PM
Data Processed : 2/20/2023 4:42:13 PM

<Chromatogram>



PeakTable

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.918	12546	987	0.710	1.006
2	3.655	83911	2147	4.746	2.188
3	4.278	1664755	94695	94.164	96.506
4	7.186	5323	231	0.301	0.235
5	8.845	1396	64	0.079	0.066
Total		1767932	98123	100.000	100.000

C:\Users\Unimas\Desktop\BOBOPERATION 2017\BOB6907.lcd

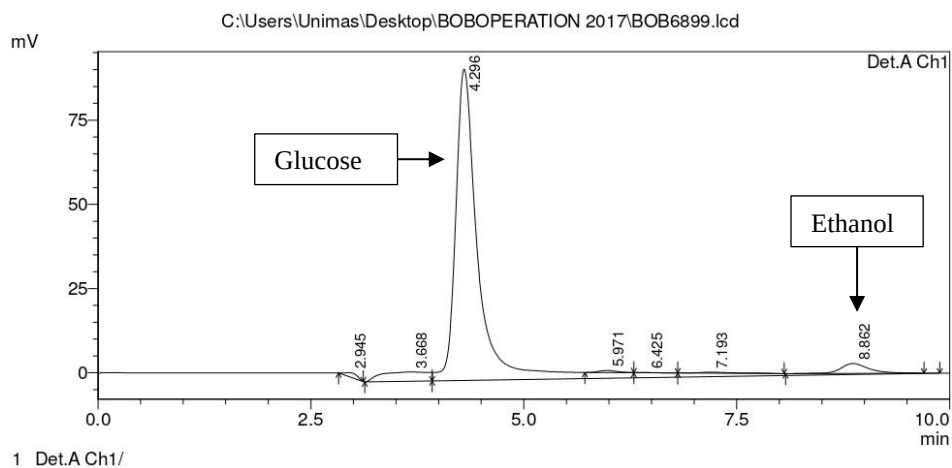
(b) Ethanol fermentation at 8 hr (Replicate 1)

2/20/2023 14:33:17 1 / 1

==== Shimadzu LCsolution Analysis Report ====

Acquired by : Admin
Sample Name : Ctrl R1 8h
Sample ID :
Vial # :
Injection Volume : 20 uL
Data File Name : BOB6899.lcd
Method File Name : Adilla-method.lcm
Batch File Name :
Report File Name : Default.lcr
Data Acquired : 2/20/2023 2:20:35 PM
Data Processed : 2/20/2023 2:30:37 PM

<Chromatogram>



PeakTable

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.945	12673	1116	0.607	1.114
2	3.668	106462	2729	5.103	2.723
3	4.296	1874778	92372	89.864	92.184
4	5.971	9991	620	0.479	0.619
5	6.425	1813	110	0.087	0.109
6	7.193	10754	301	0.515	0.300
7	8.862	69761	2956	3.344	2.950
Total		2086232	100204	100.000	100.000

C:\Users\Unimas\Desktop\BOBOPERATION 2017\BOB6899.lcd

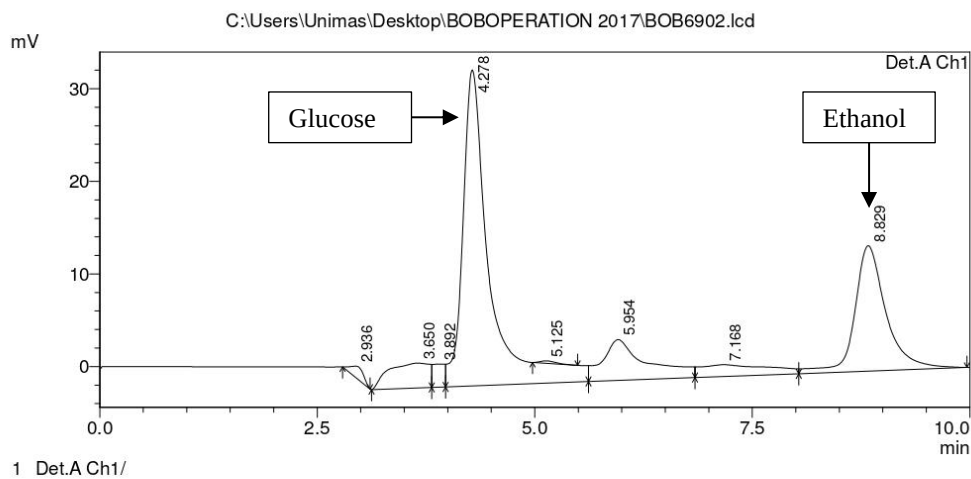
(c) Ethanol fermentation at 24 hr (Replicate 1)

2/20/2023 15:11:51 1 / 1

==== Shimadzu LCsolution Analysis Report ====

Acquired by : Admin
Sample Name : Ctrl R1 24h
Sample ID :
Vial # :
Injection Volume : 20 uL
Data File Name : BOB6902.lcd
Method File Name : Adilla-method.lcm
Batch File Name :
Report File Name : Default.lcr
Data Acquired : 2/20/2023 3:00:50 PM
Data Processed : 2/20/2023 3:10:53 PM

<Chromatogram>



PeakTable

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.936	13430	1178	0.941	1.965
2	3.650	87818	2673	6.153	4.460
3	3.892	23471	2475	1.645	4.129
4	4.278	732964	34114	51.357	56.920
5	5.125	3283	259	0.230	0.432
6	5.954	157583	4411	11.041	7.359
7	7.168	69222	1275	4.850	2.128
8	8.829	339417	13549	23.782	22.607
Total		1427189	59934	100.000	100.000

C:\Users\Unimas\Desktop\BOBOPERATION 2017\BOB6902.lcd

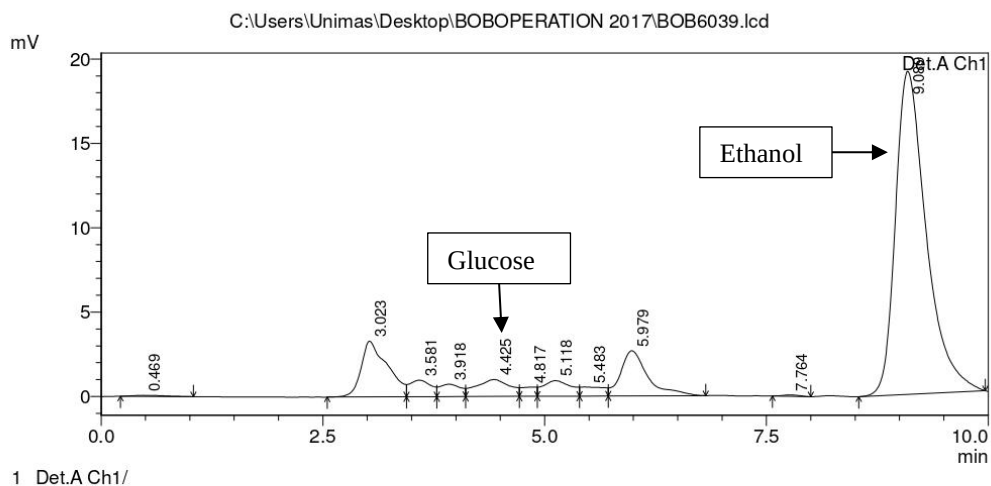
(d) Ethanol fermentation at 32 hr (Replicate 1)

10/22/2021 10:58:43 1 / 1

==== Shimadzu LCsolution Analysis Report ====

C:\Users\Unimas\Desktop\BOBOPERATION 2017\BOB6039.lcd
Acquired by : Admin
Sample Name : R1-32hr
Sample ID :
Vial # :
Injection Volume : 20 uL
Data File Name : BOB6039.lcd
Method File Name : Adilla-method.lcm
Batch File Name :
Report File Name : Default.lcr
Data Acquired : 10/22/2021 10:47:02 AM
Data Processed : 10/22/2021 10:57:04 AM

<Chromatogram>



PeakTable

Peak#	Ret. Time	Area	Height	Area %	Height %
1	0.469	1533	61	0.224	0.204
2	3.023	68292	3306	10.000	11.020
3	3.581	16500	982	2.416	3.272
4	3.918	12214	737	1.789	2.456
5	4.425	26347	1001	3.858	3.338
6	4.817	6372	537	0.933	1.791
7	5.118	20147	921	2.950	3.071
8	5.483	10038	534	1.470	1.779
9	5.979	56611	2681	8.290	8.937
10	7.764	1083	85	0.159	0.283
11	9.089	463768	19154	67.911	63.851
Total		682904	29998	100.000	100.000

C:\Users\Unimas\Desktop\BOBOPERATION 2017\BOB6039.lcd

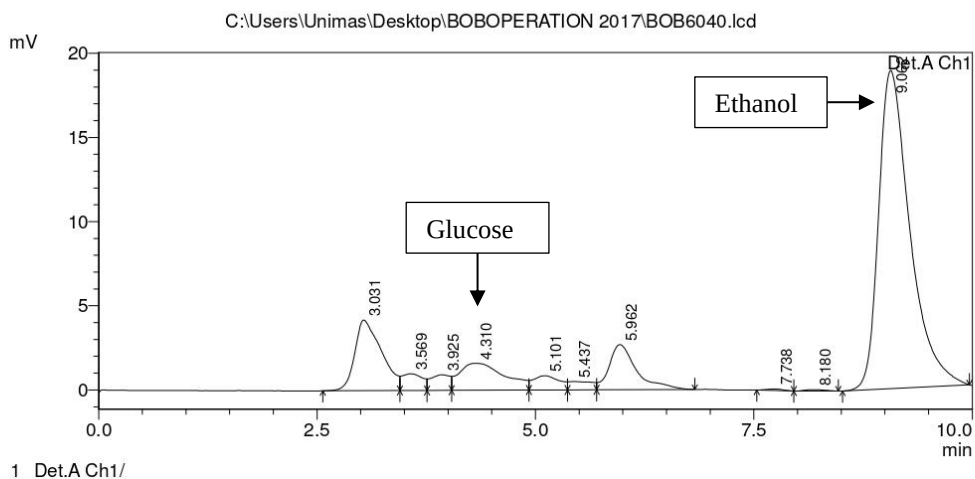
(e) Ethanol fermentation at 48 hr (Replicate 1)

10/22/2021 11:12:33 1 / 1

==== Shimadzu Lcsolution Analysis Report ====

Acquired by : Admin
Sample Name : R1-48hr
Sample ID :
Vial # :
Injection Volume : 20 uL
Data File Name : BOB6040.lcd
Method File Name : Adilla-method.lcm
Batch File Name :
Report File Name : Default.lcr
Data Acquired : 10/22/2021 11:00:27 AM
Data Processed : 10/22/2021 11:10:29 AM

<Chromatogram>



PeakTable

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.031	89198	4198	12.126	13.614
2	3.569	16184	996	2.200	3.230
3	3.925	14225	925	1.934	2.999
4	4.310	57660	1603	7.838	5.198
5	5.101	17794	850	2.419	2.755
6	5.437	9390	493	1.277	1.600
7	5.962	58199	2686	7.912	8.712
8	7.738	1209	95	0.164	0.309
9	8.180	1363	84	0.185	0.272
10	9.062	470379	18906	63.945	61.312
Total		735600	30836	100.000	100.000

C:\Users\Unimas\Desktop\BOBOPERATION 2017\BOB6040.lcd

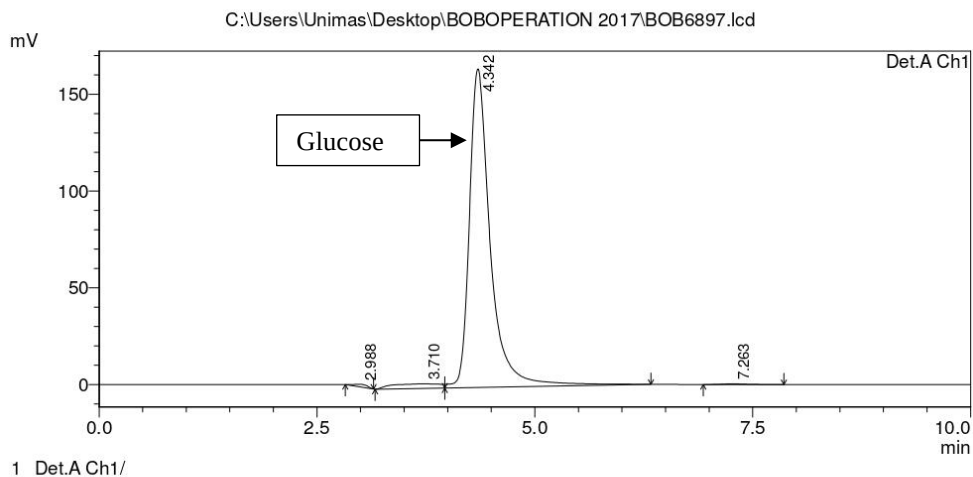
(f) Ethanol fermentation at 0 hr (Replicate 2)

2/20/2023 14:06:25 1 / 1

==== Shimadzu Lcsolution Analysis Report ====

Acquired by : Admin
Sample Name : Ctrl R2 0h
Sample ID :
Vial # :
Injection Volume : 20 uL
Data File Name : BOB6897.lcd
Method File Name : Adilla-method.lcm
Batch File Name :
Report File Name : Default.lcr
Data Acquired : 2/20/2023 1:55:22 PM
Data Processed : 2/20/2023 2:05:25 PM

<Chromatogram>



PeakTable

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.988	14798	1306	0.515	0.774
2	3.710	93251	2376	3.246	1.409
3	4.342	2757880	164557	95.985	97.599
4	7.263	7325	367	0.255	0.217
Total		2873254	168605	100.000	100.000

C:\Users\Unimas\Desktop\BOBOPERATION 2017\BOB6897.lcd

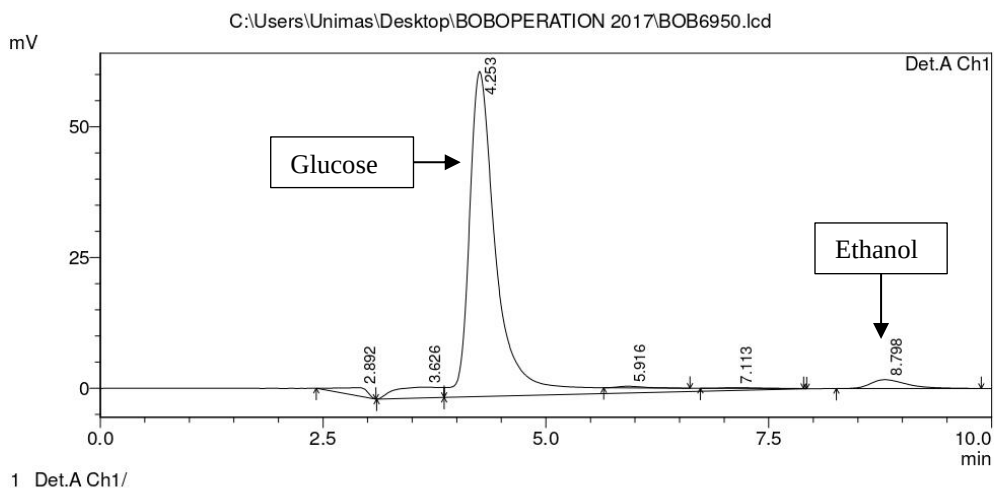
(g) Ethanol fermentation at 8 hr (Replicate 2)

2/23/2023 12:37:28 1 / 1

==== Shimadzu LcSolution Analysis Report ====

Acquired by : Admin
Sample Name : CTRL R2 8h
Sample ID :
Vail # :
Injection Volume : 20 uL
Data File Name : BOB6950.lcd
Method File Name : Adilla-method.lcm
Batch File Name :
Report File Name : Default.lcr
Data Acquired : 2/23/2023 12:23:43 PM
Data Processed : 2/23/2023 12:33:45 PM

<Chromatogram>



Detector A Ch1

PeakTable

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.892	32838	1558	2.115	2.293
2	3.626	75866	2074	4.886	3.052
3	4.253	1383873	62158	89.126	91.462
4	5.916	6978	316	0.449	0.465
5	7.113	4651	158	0.300	0.232
6	8.798	48516	1696	3.125	2.496
Total		1552722	67961	100.000	100.000

C:\Users\Unimas\Desktop\BOBOPERATION 2017\BOB6950.lcd

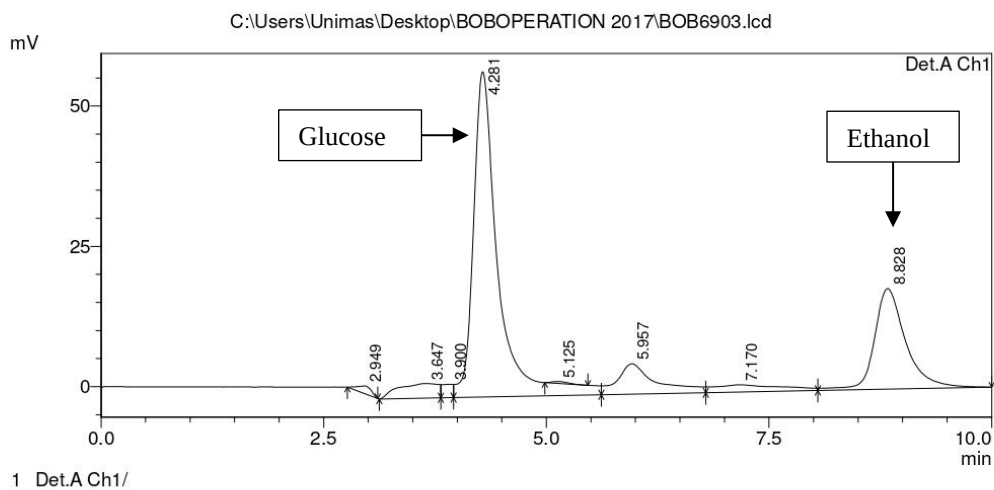
(h) Ethanol fermentation at 24 hr (Replicate 2)

2/20/2023 15:24:16 1 / 1

==== Shimadzu LCsolution Analysis Report ====

C:\Users\Unimas\Desktop\BOBOPERATION 2017\BOB6903.lcd
Acquired by : Admin
Sample Name : Ctrl R2 24h
Sample ID :
Vial # :
Injection Volume : 20 uL
Data File Name : BOB6903.lcd
Method File Name : Adilla-method.lcm
Batch File Name :
Report File Name : Default.lcr
Data Acquired : 2/20/2023 3:13:24 PM
Data Processed : 2/20/2023 3:23:25 PM

<Chromatogram>



PeakTable

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.949	14680	1290	0.767	1.446
2	3.647	82046	2607	4.287	2.923
3	3.900	20036	2362	1.047	2.648
4	4.281	1120322	57959	58.534	64.983
5	5.125	4221	338	0.221	0.379
6	5.957	165716	5415	8.658	6.071
7	7.170	67849	1291	3.545	1.447
8	8.828	439091	17930	22.942	20.103
Total		1913961	89192	100.000	100.000

C:\Users\Unimas\Desktop\BOBOPERATION 2017\BOB6903.lcd

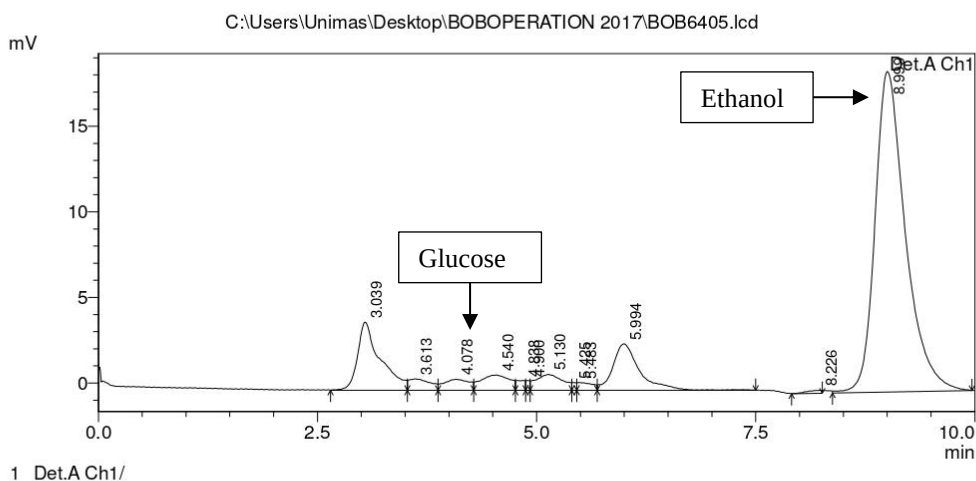
(i) Ethanol fermentation at 32 hr (Replicate 2)

6/24/2022 12:35:08 1 / 1

==== Shimadzu LCsolution Analysis Report ====

Acquired by : Admin
Sample Name : R2 32hr
Sample ID :
Vial # :
Injection Volume : 20 uL
Data File Name : BOB6405.lcd
Method File Name : Adilla-method.lcm
Batch File Name :
Report File Name : Default.lcr
Data Acquired : 6/24/2022 12:22:40 PM
Data Processed : 6/24/2022 12:34:45 PM

<Chromatogram>



PeakTable

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.039	71459	3982	10.874	12.956
2	3.613	11174	662	1.700	2.153
3	4.078	12733	640	1.938	2.084
4	4.540	20154	889	3.067	2.892
5	4.838	3910	570	0.595	1.854
6	4.900	1683	569	0.256	1.850
7	5.130	19464	925	2.962	3.009
8	5.425	1545	450	0.235	1.463
9	5.483	5453	454	0.830	1.477
10	5.994	56872	2712	8.654	8.822
11	8.226	2076	170	0.316	0.553
12	8.999	450610	18715	68.572	60.887
Total		657134	30737	100.000	100.000

C:\Users\Unimas\Desktop\BOBOPERATION 2017\BOB6405.lcd

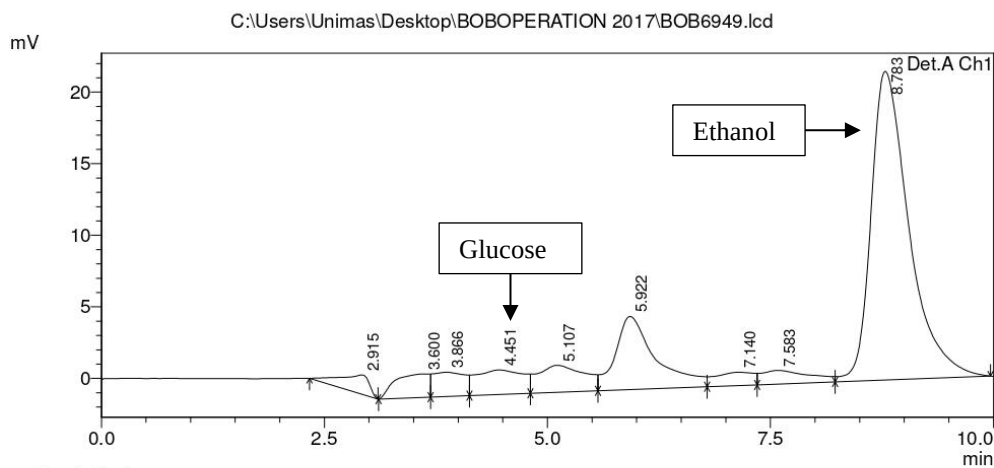
(j) Ethanol fermentation at 48 hr (Replicate 2)

2/23/2023 12:22:15 1 / 1

==== Shimadzu LCsolution Analysis Report ====

Acquired by : Admin
Sample Name : CTRL R2 48h
Sample ID :
Vial # :
Injection Volume : 20 uL
Data File Name : BOB6949.lcd
Method File Name : Adilla-method.lcm
Batch File Name :
Report File Name : Default.lcr
Data Acquired : 2/23/2023 12:09:53 PM
Data Processed : 2/23/2023 12:19:56 PM

<Chromatogram>



PeakTable

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.915	29579	1336	2.671	3.625
2	3.600	44351	1641	4.005	4.456
3	3.866	41686	1699	3.764	4.612
4	4.451	62817	1720	5.672	4.668
5	5.107	68352	1895	6.172	5.142
6	5.922	160553	5111	14.497	13.873
7	7.140	27821	931	2.512	2.527
8	7.583	35959	957	3.247	2.597
9	8.783	636400	21552	57.462	58.499
Total		1107517	36841	100.000	100.000

C:\Users\Unimas\Desktop\BOBOPERATION 2017\BOB6949.lcd

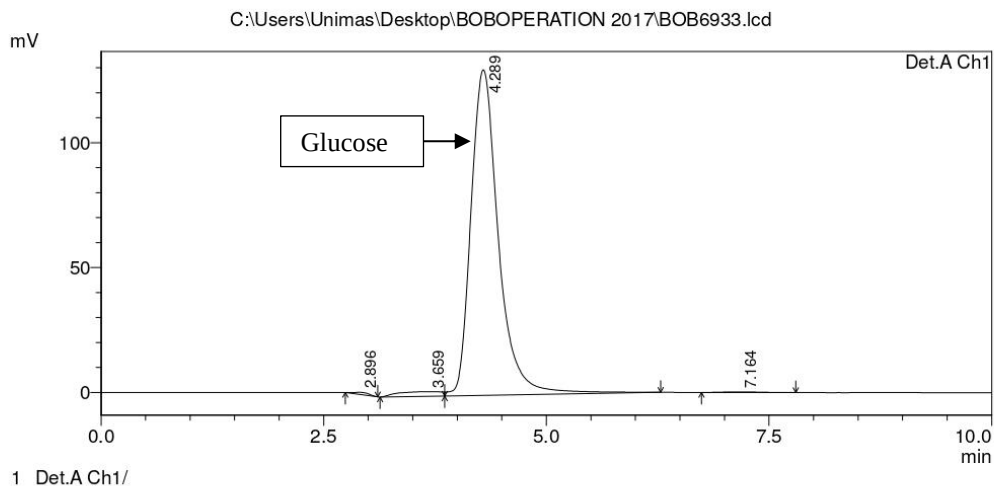
(k) Ethanol fermentation at 0 hr (Replicate 3)

2/22/2023 12:34:08 1 / 1

==== Shimadzu LCsolution Analysis Report ====

C:\Users\Unimas\Desktop\BOBOPERATION 2017\BOB6933.lcd
Acquired by : Admin
Sample Name : Ctrl R3 0h
Sample ID :
Vial # :
Injection Volume : 20 uL
Data File Name : BOB6933.lcd
Method File Name : Adilla-method.lcm
Batch File Name :
Report File Name : Default.lcr
Data Acquired : 2/22/2023 12:22:45 PM
Data Processed : 2/22/2023 12:32:48 PM

<Chromatogram>



PeakTable

Detector A Ch1					
Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.896	10674	812	0.370	0.609
2	3.659	63513	1846	2.200	1.386
3	4.289	2805449	130299	97.183	97.814
4	7.164	7129	255	0.247	0.191
Total		2886766	133211	100.000	100.000

C:\Users\Unimas\Desktop\BOBOPERATION 2017\BOB6933.lcd

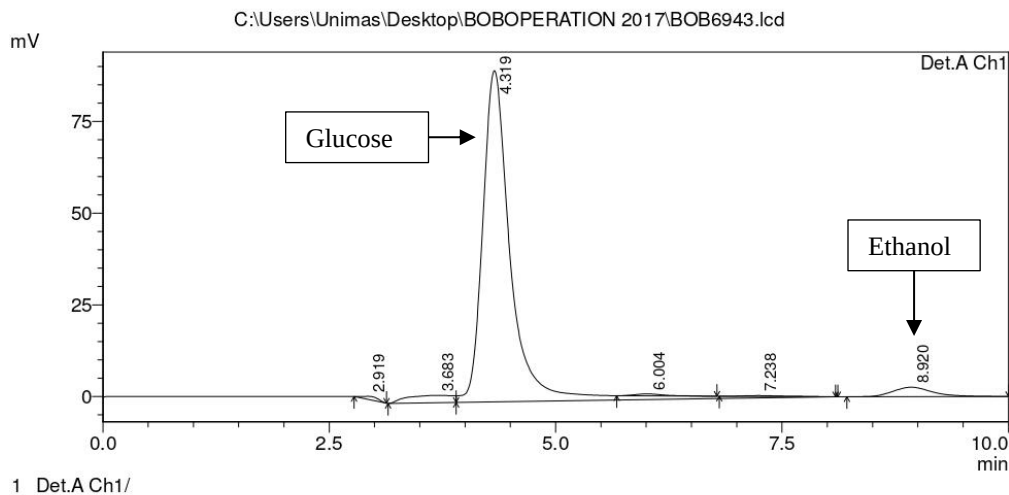
(l) Ethanol fermentation at 8 hr (Replicate 3)

2/22/2023 16:30:09 1 / 1

==== Shimadzu LCsolution Analysis Report ====

Acquired by : Admin
Sample Name : Cntrl R3 8h
Sample ID :
Vial # :
Injection Volume : 20 uL
Data File Name : BOB6943.lcd
Method File Name : Adilla-method.lcm
Batch File Name :
Report File Name : Default.lcr
Data Acquired : 2/22/2023 4:18:29 PM
Data Processed : 2/22/2023 4:28:33 PM

<Chromatogram>



PeakTable

Detector A Ch1

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.919	10779	790	0.496	0.819
2	3.683	70989	2002	3.268	2.076
3	4.319	1989575	90283	91.600	93.602
4	6.004	12900	544	0.594	0.564
5	7.238	8440	249	0.389	0.258
6	8.920	79337	2586	3.653	2.681
Total		2172021	96454	100.000	100.000

C:\Users\Unimas\Desktop\BOBOPERATION 2017\BOB6943.lcd

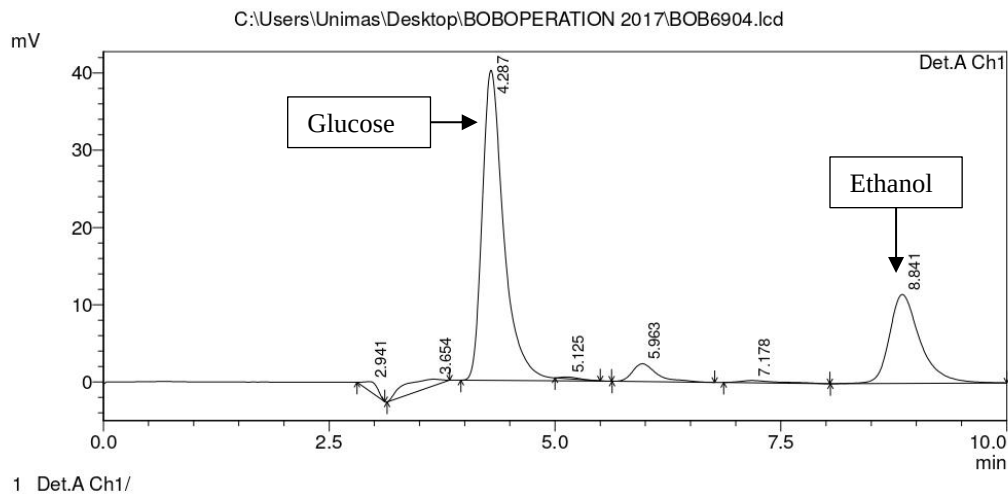
(m)Ethanol fermentation at 24 hr (Replicate 3)

2/20/2023 15:35:51 1 / 1

==== Shimadzu LCsolution Analysis Report ====

Acquired by : Admin
Sample Name : Ctrl R3 24h
Sample ID :
Vial # :
Injection Volume : 20 uL
Data File Name : BOB6904.lcd
Method File Name : Adilla-method.lcm
Batch File Name :
Report File Name : Default.lcr
Data Acquired : 2/20/2023 3:25:16 PM
Data Processed : 2/20/2023 3:35:19 PM

<Chromatogram>



PeakTable

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.941	13363	1168	1.268	2.067
2	3.654	38902	867	3.693	1.533
3	4.287	673382	40167	63.918	71.043
4	5.125	2584	204	0.245	0.361
5	5.963	43635	2307	4.142	4.080
6	7.178	9174	296	0.871	0.524
7	8.841	272469	11530	25.863	20.392
Total		1053509	56539	100.000	100.000

C:\Users\Unimas\Desktop\BOBOPERATION 2017\BOB6904.lcd

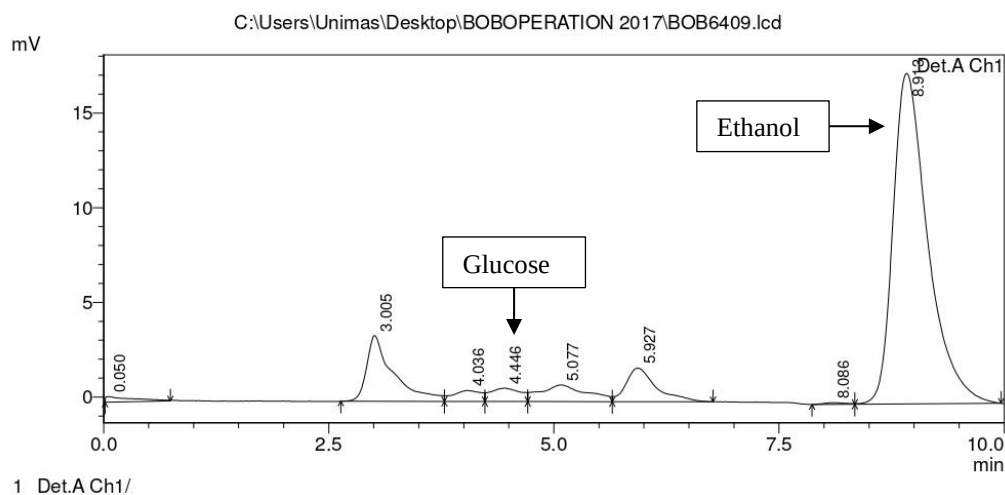
(n) Ethanol fermentation at 32 hr (Replicate 3)

6/24/2022 13:52:23 1 / 1

==== Shimadzu LCsolution Analysis Report ====

Acquired by : Admin
Sample Name : R3 24hr
Sample ID :
Vial # :
Injection Volume : 20 uL
Data File Name : BOB6409.lcd
Method File Name : Adilla-method.lcm
Batch File Name :
Report File Name : Default.lcr
Data Acquired : 6/24/2022 1:41:08 PM
Data Processed : 6/24/2022 1:51:10 PM

<Chromatogram>



PeakTable

Peak#	Ret. Time	Area	Height	Area %	Height %
1	0.050	6401	295	0.986	1.170
2	3.005	71755	3472	11.053	13.757
3	4.036	12378	579	1.907	2.293
4	4.446	16410	702	2.528	2.781
5	5.077	31269	879	4.817	3.483
6	5.927	41166	1769	6.341	7.007
7	8.086	1526	87	0.235	0.344
8	8.913	468294	17456	72.134	69.164
Total		649198	25239	100.000	100.000

C:\Users\Unimas\Desktop\BOBOPERATION 2017\BOB6409.lcd

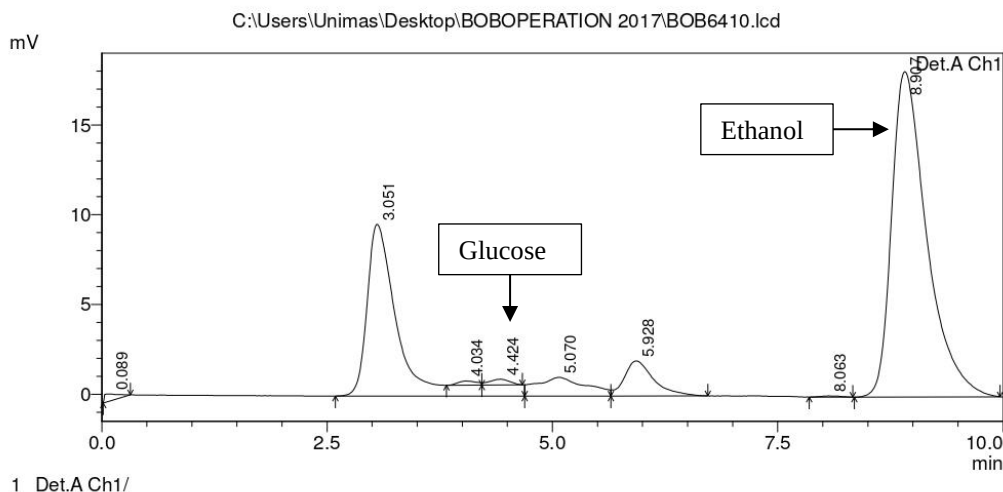
(o) Ethanol fermentation at 48 hr (Replicate 3)

6/24/2022 14:05:10 1 / 1

==== Shimadzu LCsolution Analysis Report ====

Acquired by : Admin
Sample Name : R3 48hr
Sample ID :
Vial # :
Injection Volume : 20 uL
Data File Name : BOB6410.lcd
Method File Name : Adilla-method.lcm
Batch File Name :
Report File Name : Default.lcr
Data Acquired : 6/24/2022 1:54:10 PM
Data Processed : 6/24/2022 2:04:12 PM

<Chromatogram>



PeakTable

Peak#	Ret. Time	Area	Height	Area %	Height %
1	0.089	4343	390	0.523	1.231
2	3.051	240734	9567	29.002	30.194
3	4.034	3404	230	0.410	0.726
4	4.424	4940	314	0.595	0.991
5	5.070	39818	1041	4.797	3.285
6	5.928	44221	1950	5.327	6.156
7	8.063	1019	66	0.123	0.208
8	8.907	491592	18127	59.223	57.209
Total		830070	31685	100.000	100.000

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Appendix G: Publication

Mahmod Nik Nur Aziati, Suhaili Nurashikin, Awang Husaini Awang Ahmad Sallehin and Awang Adeni Dayang Salwani. (2022). Autolysis of spent baker's yeast generated from sago bioethanol fermentation: a preliminary study. *Res. J. Biotech.*; Vol. 17(7); 91-97.

Autolysis of spent baker's yeast generated from sago bioethanol fermentation: a preliminary study

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Abstract

Spent Baker's yeast (*Saccharomyces cerevisiae*) is obtained as a by-product of ethanol fermentation industry and is mainly discarded as waste. The spent yeast can be potentially converted to yeast extract through autolysis process in which the yeast cells are lysed under certain conditions to release valuable and bioactive substances. To date, there are still limited studies on the autolysis of spent *S. cerevisiae* generated following the production of sago bioethanol. This research aims to investigate the feasibility of autolysis of spent Baker's yeast generated from sago bioethanol fermentation by focusing on two important parameters namely initial pH and incubation time. The spent Baker's yeast was autolysed at different initial pH values (3, 5 and 7) and at different incubation times (24, 48, 72 and 96 h). The protein and carbohydrate concentrations of the resulting lysates were analysed using Lowry assay and phenol-sulphuric acid assay respectively. Additionally, the surface morphology of the autolysed yeast cells was observed using Scanning electron microscope.

Our results showed that the autolysis conducted at the initial pH of 3 gave the highest concentration of protein and total carbohydrate released from the lysate which were 2.5-fold and 2-fold over that in the control samples respectively.

Meanwhile, the optimal incubation time for the autolysis of spent Baker's yeast for both maximum concentrations of protein and carbohydrate released was found to be 72 h whilst prolonging beyond that did not result in any significant change of both the protein and total carbohydrate concentration. The morphological analysis of the lysed yeast cells clearly showed a significance change of the surface morphology indicative of cell lysis throughout the incubation period. In conclusion, the present work gives significant insights into the feasibility of autolysis of spent Baker's yeast generated from sago bioethanol fermentation. This will in turn serves as useful fundamental information for developing potential side process streams within an integrated sago biorefinery in the future.

Keywords: Autolysis, Bioethanol fermentation, *Saccharomyces cerevisiae*, Spent Baker's yeast, Yeast extract.

Introduction

Baker's yeast (*Saccharomyces cerevisiae*) is commonly utilised in the fermentation industry and the production generates large quantities of spent yeast as their by-products^{9,24}. About 400 000 tons of spent Baker's yeast are produced as a by-product by the brewing industry annually. The waste, in general, is still underused and is normally discarded into the environment⁵. The spent yeast is a rich source of proteins, essential amino acids, RNA, vitamins B and minerals which essentially serve as a raw material in the production of yeast extract²³. The extracts from the spent yeast can serve as promising sources of valuable ingredients such as bio-active compounds and amino acids that can be used for the production of functional food and dietary supplements¹⁸.

The extracts can be recovered by disrupting the yeast cell walls with the use of endogenous enzymes via autolysis¹¹. Autolysis is termed as an irreversible process in which the yeast cell is degraded by the endogenous enzyme activity when the stationary phase of the cell growth ended⁷. The main reaction in this process is demonstrated by the hydrolysis of cellular proteins (primary component in yeast extract) releasing degraded proteins, peptides and amino acids into yeast extract¹⁴.

Previous reports on autolysis in the literature focused on the use of either fresh yeast or spent yeast from bioethanol fermentation using commercial feedstock. There is still limited information on the valorisation of spent Baker's yeast generated from bioethanol production using agricultural waste. One of the potential agricultural wastes for producing bioethanol is sago fibre. The feasibility of sago fibre as a feedstock for bioethanol fermentation has been reported in our previous report⁴.

Considering the potential of sago bioethanol in meeting the increasing industrial demand in the future, it is timely to uncover the potential direction of the waste generated from sago bioethanol production. In our prior report¹⁶, we reported the utility of liquid waste stream from sago bioethanol as a potential feedstock for recombinant laccase production. However, the information on the potential and fate of the solid waste generated from sago bioethanol production namely the spent *S. cerevisiae* still remains unexplored.

Therefore, the aim of the current work is to investigate the feasibility of autolysis of spent Baker's yeast generated from sago bioethanol fermentation with the specific focus on the effect of pH and incubation time on protein and carbohydrate concentration released in the yeast lysate. In general, the present work provides useful insight into the development of on-site process stream which may benefit the establishment of an integrated sago biorefinery in the future. Furthermore, the present work will help to promote sustainable development of sago bioethanol production with reduced waste whilst generating side revenue to the industry.

Material and Methods

Microorganism: *Saccharomyces cerevisiae* used in this work was obtained from Biochemistry Lab, Faculty of Resource Science and Technology, UNIMAS.

Pre-treatment and hydrolysis of sago fibre: Sago fibre used in this work was provided by Herdsen Sago Mill. The fibre was dried until a constant weight was achieved. Then, it was ground into powder form. The hydrolysis of sago fibre was based on an established protocol⁴. A suspension of sago fibre 7% (w/v) was prepared and boiled for gelatinisation at 90-100 °C for about 30 minutes.

Then, the suspension was added with Liquozyme® (Novozymes, Denmark) at 2 µL/g. The suspension was constantly stirred for 30 minutes and it was left to cool down to 50-60 °C before adding Spirizyme® (Novozymes, Denmark) at 1 µL/g for saccharification. The suspension was incubated overnight at 50 °C before it was filtered through a fabric mesh filter in order to harvest the sago fibre hydrolysate (SFH).

Bioethanol fermentation: The SFH produced from the previous section was used as a substrate for bioethanol fermentation by *S. cerevisiae*. The SFH was supplemented with 5 g/L yeast extract (Oxoid, UK). The initial pH of the medium was adjusted to 5.5 before the sterilisation. The fermentation was carried out in triplicate using 500 mL flasks with the working volume of 250 mL. The cultures were incubated on an orbital shaker with a shaking speed of 150 rpm at a room temperature for 48 h. An aliquot of culture broth was withdrawn every 8 h during the fermentation. The sampled broth was centrifuged at 4000 rpm for 3 minutes. The resulting pellet, which consisted of spent *S. cerevisiae*, was recovered and used for autolysis.

Autolysis: The autolysis of spent *S. cerevisiae* was based on modified methods^{6,11}. The spent yeast with the final concentration of 14% (w/v) was suspended in 100 mL of 0.05 M sodium phosphate buffer in 250 mL Erlenmeyer flasks. The control sample was prepared by suspending the spent yeast in sterile distilled water and left at room temperature without agitation. The flasks were then incubated in a shaking water bath (Digital Precise Shaking Water Bath, Daihan, Korea) at a temperature of 55 °C with a shaking speed of 100 rpm for 96 h. The effect of pH on

autolysis of spent *S. cerevisiae* was investigated at different pH values (3, 5 and 7). The temperature during the autolysis was set at 55 °C.

An aliquot of the suspension was withdrawn at different time interval during the autolysis for the analysis of protein, carbohydrate and surface morphology. The sampled suspension was divided into two portions. The first portion was directly centrifuged and the resulting pellet was recovered for the morphological analysis. The second portion was heated at 100 °C for 5 minutes upon sampling in order to terminate the enzymatic activity. The samples were then centrifuged at 3000 rpm for 10 minutes²¹. The resulting supernatant (autolysate) was kept for the protein and carbohydrate analysis.

Analyses

Protein: The protein analysis in this work was based on modified methods^{8,13}. The autolysate was diluted with distilled water and mixed with 0.1 mL of 2 N NaOH before being heated at 100 °C for 10 minutes. Then, 1 mL of freshly mixed Lowry solution was added and the suspension was left for 10 minutes. After the incubation, 0.1 mL of freshly prepared diluted Folin Reagent was added and the mixture was then incubated at room temperature in a dark environment for 30 minutes. The samples were transferred into microcuvettes and the absorbance of the samples was measured using a spectrophotometer (Shimadzu UV Mini 1240 UV-vis) at 750 nm. The absorbance reading was then translated into equivalent protein concentration based on an established protein standard curve.

Carbohydrate: The carbohydrate analysis in this work was based on a published method¹. The autolysate was diluted by adding distilled water to the autolysate. Then, 1 mL of 5% (v/v) phenol and 5 mL of 96% (v/v) concentrated sulphuric acid were added to the mixtures and mixed evenly. Then, the samples were placed in a water bath for 15 minutes at 25-30 °C. The absorbance of the suspension was measured at 490 nm using a spectrophotometer. The total carbohydrate concentration of the sample was determined based on an established standard curve of glucose.

Scanning electron microscopy: The microscopic analysis of the autolysed cells in this work was based on a modified protocol¹¹. First the autolysed cells was fixed by 2.5% glutaraldehyde solution for 30 minutes. Then the suspension was centrifuged at 10 000 rpm for 3 minutes and the resulting pellet was recovered. The pellet was then rinsed with sterile distilled water. The fixed samples were gradually dehydrated with a series of ethanol gradients (25%, 50%, 70%, 95%, 100% (v/v)) for 15 minutes. The mixtures were mixed and centrifuged at 8000 rpm for 2 minutes.

Then, the samples were air dried. After drying, the samples were attached on carbon tape on cover slips. The cover slips were mounted on aluminum sample stubs and the samples were then observed under a Scanning electron microscope

(SEM) (JSM-IT500HR, JEOL, Japan) with a magnification of 5000x.

Statistical analysis: The results were analysed using SPSS Statistics 23 Software (IBM, United States). Analysis of Variance was used to compare the means using Tukey's test (ANOVA). When the p-value was < 0.05 , the results were regarded highly dissimilar.

Results and Discussion

Effect of different initial pH values and incubation period on protein concentration of autolysate: The effect of different initial pH values on autolysis of spent Baker's yeast was investigated by examining the concentration of protein and carbohydrate present in the autolysate at different interval times throughout the autolysis process. The protein content in the autolysate is one of the important indicators of yeast lysis process since the process releases degraded proteins, peptides and amino acids¹⁴. Fig. 1 shows the protein concentration profile of the autolysate throughout the 96 h of autolysis.

In general, there was a notable rise of protein concentration in the autolysate when the spent Baker's yeast was autolysed for 96 h at different initial pH values ranging from 3 to 7 which clearly suggests the successful occurrence of yeast lysis. In contrast, the control samples, which were incubated in a non-conductive environment for autolysis (static state, unaltered initial pH and at room temperature), showed a minimal change of protein concentration during similar time frame. It was noticed that the trend shown by the autolysis conducted at an initial pH of 3 and 5 was very similar where

a sharp increase of protein concentration occurred during the first 48 h whilst incubating beyond that has led to a plateau stage.

The highest protein concentration of autolysate achieved in the autolysis conducted at both the aforementioned pH values represented about 2.5-fold increment over that achieved by the control experiment. The results of this study are in accordance with some previous studies^{3,11,17} which reported that the optimal result of autolysis conducted at pH ranged from 3.8 to 5.

The increasing amount of proteins released into the autolysate can be associated with the increasing permeability of cell membrane and vacuole membrane when subjected to a condition that induces the autolysis such as in an acidic condition¹¹. This, in turn, resulted in a significant breakdown of cellular proteins by the endogenous enzymes leading to the release of degraded proteins, peptides and amino acids into the autolysate. In contrast, in autolysis with the initial pH under mild conditions such as in pH 7, the yeast lysis is mainly due to the action of the endogenous enzymes¹¹ which may result in slower release of the intracellular components.

The activity of hydrolytic enzymes in yeast cells, especially proteases, is greatly influenced by the pH of the autolysis²⁰. Acidic pH is revealed to be beneficial for some key enzymes responsible for the autolysis such as protease A which has an optimum pH between 2 and 6¹⁵. In a recent work³ which introduced acid shock strategy during autolysis, the acidification was reported to be useful in reducing the autolysis period.

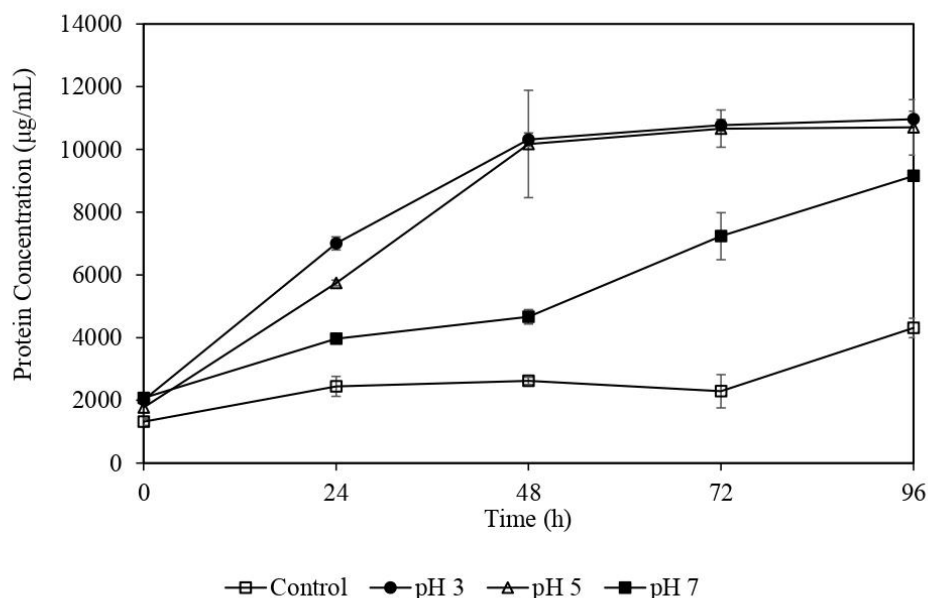


Fig. 1: Changes of protein concentration of autolysate over time

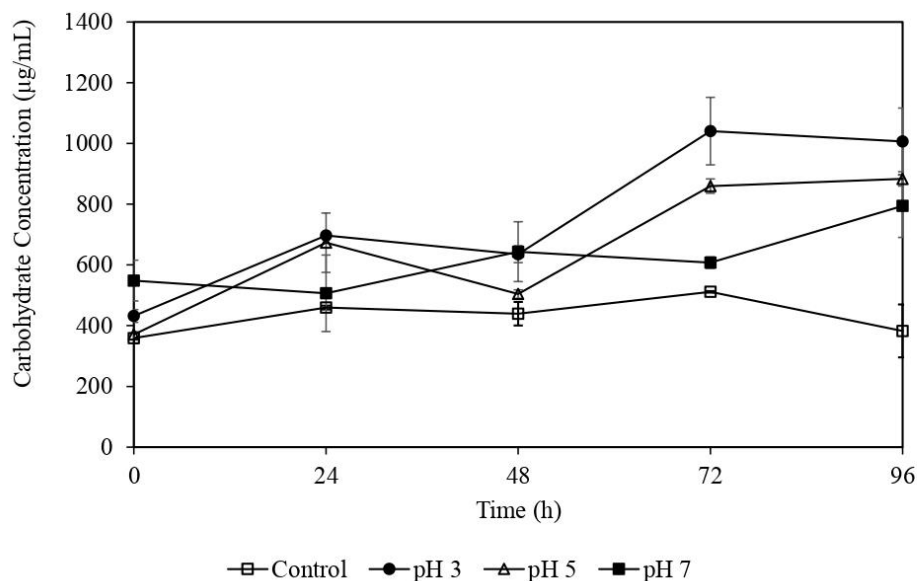


Fig. 2: Changes of total carbohydrate concentration of autolysate over time

In terms of the effect of incubation period on autolysis, although the highest protein concentration of autolysate was achieved after 96 h of autolysis, the increase of the protein titre between 48 and 96 h was insignificant. This suggests that conducting the autolysis up to 48 h may be sufficient and is more cost effective in comparison to the 96-hour incubation. This is in parallel with several previous studies that reported the range of an optimal autolysis which is from 24 to 70 h^{3,11,21}. The results from this study confirm that the alteration of pH before the autolysis has a significant effect on the amount of protein released in the autolysate as well as the time taken to reach the maximum protein concentration.

Effect of different initial pH values and incubation period on carbohydrate concentration of autolysate: Apart from the protein content, another important indicator of autolysis is the total carbohydrate released in the resulting autolysate.

Fig. 2 illustrates the changes of the concentration of total carbohydrate of autolysate derived from the autolysis conducted at different initial pH values throughout the 96 h of autolysis.

The results showed that there was a sharp increase of carbohydrate concentration after 48 h of incubation in the lysates derived from the autolysis conducted at pH 3 and 5 whilst prolonging the incubation to 96 h resulted in a negligible change of the carbohydrate concentration. Meanwhile, the change of carbohydrate concentration released in the autolysis conducted at pH 7 was very minimal throughout the 96-hour incubation. The highest

carbohydrate concentration released was in the autolysis conducted at the initial pH of 3 with the titre representing 2-fold enhancement over that in the control experiment.

Considering the release of both protein and carbohydrate from the lysed yeast cells, it can be concluded that 72 h of incubation is the best period for autolysis of spent Baker's yeast in this work. Unnecessary extension of incubation period during the autolysis may not be favourable since it may increase the cost and also the risk of contamination due to the growth of pathogens over a long period.

Saccharomyces cerevisiae has a cell wall made up of 80 to 90% polysaccharides consisting of β -glucans and mannoproteins as the main components, which comprise 15 to 30% of the dry mass of the cell²⁵. During the yeast autolysis, the cell wall becomes crumpled and the polysaccharides will be gradually hydrolysed²⁵. This will be triggered faster in an acidic condition like in pH 3. Thus, this may explain the higher concentration of total carbohydrates in the autolysate derived from the autolysis performed at the initial pH of 3 as obtained in this work. It was also observed in this study that in general, the yeast cells released higher concentration of proteins compared to the carbohydrates during the autolysis process. This is in line with the fact that protein is the main component of yeast extract²¹.

There are two phases in the yeast cell wall degradation during the autolysis. First, glucans within the cell wall inner layer are hydrolysed by glucanase and mannoproteins are released¹⁰. Secondly, the residual glucanase activities cause β -glucans to be released from the amorphous matrix of cell

wall^{2,12}. Recently, there is a growing interest in yeast cell wall products since the polysaccharides have been revealed to possess health benefits that can be used for production of functional food and nutraceuticals¹⁹. For example, β -glucans, which are the most prevalent compound of cell wall polysaccharides, have been reported to have antioxidant and anti-cancer properties besides able to absorb pathogenic bacteria and enhance cellular immunity²². Hence, early insight into the feasibility of lysing the cell wall of spent Baker's yeast generated from sago bioethanol production as

demonstrated in this work will benefit the development of potential side process such as those that are related to yeast cell wall products that can be developed along with the sago bioethanol industry in the future.

Morphological analysis of autolysed cells: In addition to the protein and total carbohydrate quantification, the microscopic analysis of the autolysed cells was also carried out in order to examine the change of the surface morphology of the yeast cells throughout the autolysis.

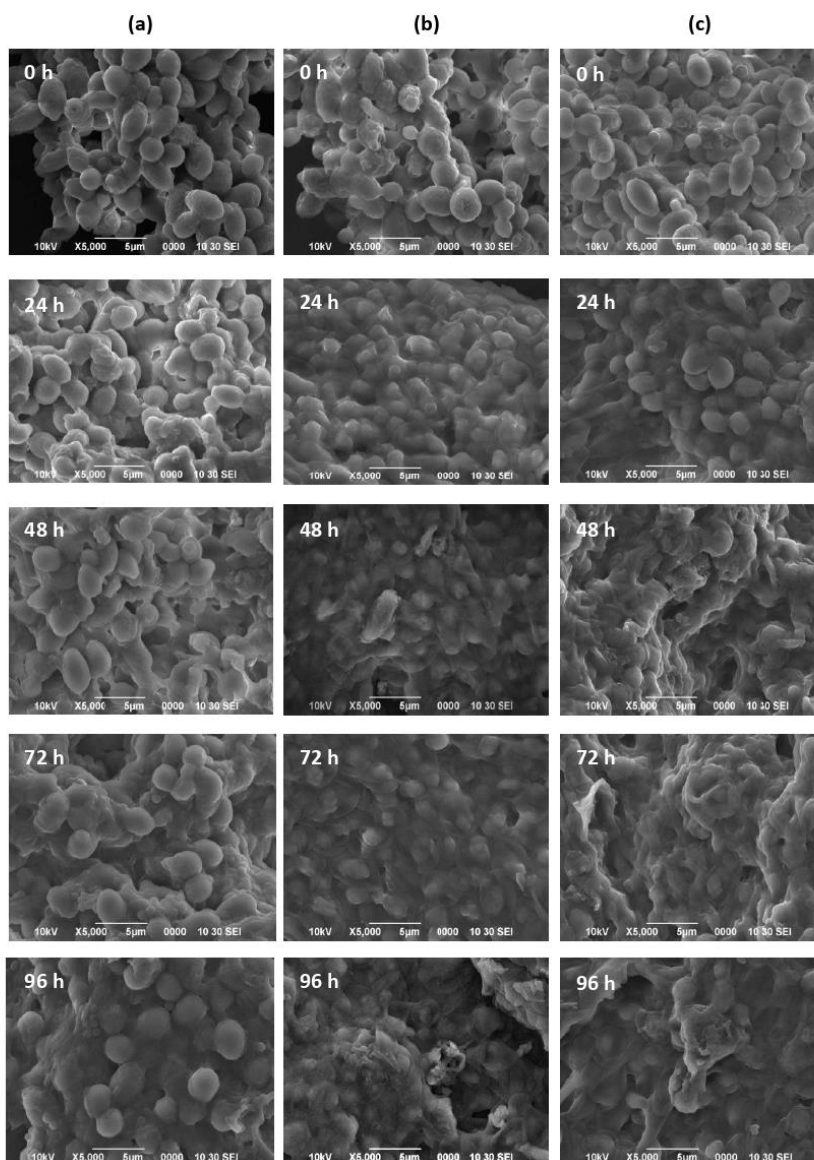


Fig. 3: Changes of surface morphology of (a) untreated yeast cells (control samples); (b) yeast cells derived from autolysis conducted at pH 3 and (c) yeast cells derived from autolysis conducted at pH 5

Fig. 3 shows the changes of surface morphology of the yeast cells derived from the autolysis conducted at the initial pH of 3 (Fig. 3b), 5 (Fig. 3c) and also the untreated yeast cells (Fig. 3a).

It is clearly shown that for all cases at 0 h, the yeast cells in general appeared plump, mellow and with smooth surfaces. Whilst most of the untreated yeast cells (Fig. 3a) still appeared plump and unaffected throughout the 96 h of incubation, the surface morphologies of most of the yeast cells derived from the autolysis conducted at pH 3 (Fig. 3b) and pH 5 (Fig. 3c) were found to show a notable change with reduced number of plump cells observed throughout the incubation.

Furthermore, more uneven structures and irregular fragments could be observed after 96 h of autolysis. Besides that, some of the yeast cells in fig. 3 (b) and (c) at 96 h were also found to have a reduced diameter than those at 0 h. The aforementioned appearance can be associated with the breakage of the yeast cells due to the autolysis.

The yeast cell wall, which has a thickness of 100-150 nm, is a key component that determines the overall shape of the yeast cells²⁵. During the lysis of the cells, the vacuole will be ruptured leading to the release of the hydrolytic enzymes that are responsible for the degradation of the organelles and cell membrane components²⁵. This in turn resulted in the wrinkling of the cell wall and subsequently the reduction of the diameter of the cells throughout the autolysis. The results obtained in this study are in parallel with some published reports in the literature^{11,25} which reported similar change of surface morphology of the lysed yeast cells upon the autolysis. In addition, the results of this work also confirm the lysis of the yeast cells as a result of the autolysis which is in parallel with the release of protein and total carbohydrates in the autolysate as described earlier.

Conclusion

In conclusion, we have demonstrated the feasibility of autolysis of spent Baker's yeast generated from sago bioethanol fermentation. Our results showed that the initial pH of 3 and incubation period of 72 h were proven as the best conditions for the autolysis on the basis of protein and total carbohydrate released in the lysate.

The highest concentrations of protein and carbohydrate in the lysate produced by the autolysis under the aforementioned conditions were 2.5-fold and 2-fold over that in the control samples respectively. Future work should consider the influence of other parameters such as organic solvents and mechanical intervention on the efficiency of autolysis.

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