Towards Upstream Process Development Using Mixed Induction Strategy and Continuous System in Cultivation of *Komagataella phaffii*

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Declaration

I, Almir Yamanie, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed:…….

Date:……..

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Abstract

In recent years, the biopharmaceutical market has grown a lot because of rising demand and the fast growth of biotechnology in terms of new ways to treat a wide range of diseases. Using yeast, like *Komagataella phaffii*, as a platform for making biopharmaceuticals is becoming increasingly popular because it is more cost-effective, easier to scale, and more consistent product quality. However, the standard fed-batch cultivation process using a pure methanol induction strategy that is currently commonly used in the cultivation of *K. phaffii* may not be able to keep up with the demands due to challenges such as a large carbon footprint due to scale-up of cultivation to increase production, a higher risk of hazard for using large volumes of pure methanol, and extended downtime for batch preparation, which causes high operating and utility costs. Thus, a new way of combining the continuous cultivation process with methanol and sorbitol mixed induction strategies is needed to reduce the production size and the amount of methanol used.

In this thesis, the impact of both a methanol/sorbitol mixed induction strategy and continuous cultivation process on *K. phaffii* fermentation in the production of KEX2 protease was studied and evaluated in terms of cell growth, product content and volumetric yield (ug.mL^{-1}), as compared with commonly used yeast-based cultivation method that uses pure methanol as an induction agent in fed-batch mode. Furthermore, a techno-economic feasibility study of the processes was compared in a pilot bioreactor and on simulated industrial scales.

The standard pure methanol induction method was compared to the 50% (C-mol/C-mol) methanol with sorbitol mixed induction method in the fed-batch cultivation process for the production of KEX2 protease, which improved the specific growth by almost 2-fold while also increasing the KEX2 protease yield (mg) in one batch by 2.3-fold when 2 L of broth was harvested 24 hours after the start of the induction process. In addition, the impurities inside the supernatant after separating the cells from the broth were also found to be lower when using the mixed induction method when compared to the pure methanol induction method, increasing the KEX2 protease content by 7% of the total protein bands found from the SDS PAGE result.

The continuous cultivation process of *K. phaffii* for the production of KEX2 protease was also done using pure methanol induction and a 50% (C-mol/C-mol) methanol with sorbitol mixed induction method using the data previously acquired from the fed-batch cultivation process. Using a mixed induction strategy with a continuous cultivation method created a synergistic effect due to the increase in specific growth rate, which resulted in an increased daily volume harvested. The biomass could be kept at 80 $g.L^{-1}$ and 100 $g.L^{-1}$ for pure methanol induction and mixed induction, respectively, and the process was kept for at least 240 hours and harvested every 24 hours. The final harvested volume in one batch when using the pure methanol and mixed induction methods was found to be 8 L and 13.5 L, respectively. It resulted in a yield (mg) of 8.6-fold increase in one “batch.” Also, the yield (mg) of continuous cultivation using a mixed induction strategy was compared to the yield of fed-batch cultivation processes in one batch, which showed an increase of 8.9 times for mixed induction and 21.3 times for pure methanol induction.

A simulation of a 400-litre fed-batch cultivation system with the methanol induction strategy was used as a benchmark. Then, a simulation of a combination of continuous cultivation with the mixed induction strategy at the same scale and on a smaller scale was compared with the benchmark to determine its benefits and cost savings. The combination method allowed for a 10x reduction in production scale while maintaining similar output and time. Other benefits also included lower capital costs, operating costs, and a smaller carbon footprint.

Impact Statement

Komagataella phaffii is often used to make heterologous proteins because it has clear advantages over mammalian cells and *E. coli*. However, more than the standard fed-batch fermentation process using pure methanol induction may be needed to produce biopharmaceutical products for *K. phaffii* cultivation as their demands increase, which requires multiple larger-scale productions. This thesis has established a novel combination technology of induction strategy using sorbitol as a co-substrate and continuous fermentation process. The strategy has been shown to produce synergy, improving the daily volume harvested, increasing product content and volumetric yield, and reducing production costs in the production of KEX2 protease.

In this research, the data acquired from the project will guide how to use a methanol/sorbitol mixed induction strategy, convert the fed-batch process to a continuous fermentation process, and use both simultaneously. Continuous fermentation and sorbitol/methanol mixed induction strategy used concurrently can be considered over standard fed-batch fermentation with pure methanol induction when:

1. There are limitations in terms of the availability of large-scale bioreactors and downstream processes
2. The high operating costs because of downtime and preparation for multiple batches of cultivation
3. The risks of using a large volume of pure methanol are too high
4. The host cell is genetically robust and stable
5. Many host cell proteins are co-released with the product, and proteases quickly degrade the products

Combining a mixed induction strategy with a continuous fermentation process will reduce the scale needed, increase volumetric yield, and lessen oxygen consumption to reduce capital and operating costs. In addition, reducing the usage and concentration of a highly flammable solution (methanol) will make the manufacturing process less hazardous and easier to manage. Other benefits of using a sorbitol/methanol induction strategy and continuous process include reducing batch-to-batch variation and excreting less host cell protein, which may

cause target product degradation and increase cell viability throughout the excretion process.

The key findings in this research can be used as a foundation for the biopharmaceutical manufacturer to improve their process to be more efficient and effective when using *K. phaffii* by lowering the scale and risks of production. In the future, a compact, robust, and self-sufficient manufacturing facility would be possible with the further development of continuous processing in the downstream process. In addition, two research manuscripts are being drafted to be published in academic journals to spread the knowledge of the findings.

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Nomenclature

Symbol	Description	Units
ATM	Standard atmospheric pressure	kPa
C	Correlation factor	-
%Content	Target content band percentage	%band
D	Dilution rate	h^{-1}
DCW	Dry cell weight	$g.L^{-1}$
F_{O2}	Volumetric fraction of oxygen	-
F_{N2}	Volumetric fraction of nitrogen	-
F_{CO2}	Volumetric fraction of carbon dioxide	-
k_La	Oxygen transfer coefficient	h^{-1}
$m.v^{-1}$	Mass by volume	$g.L^{-1}$
q_p	Specific productivity	$mg.gDCW^{-1}.h^{-1}$
q_v	Specific volumetric productivity	$mg.L^{-1}.h^{-1}$
Q	Liquid flow rate	$mL.h^{-1}$
Q_c	Current Harvest flow rate	$mL.h^{-1}$
r_{met}	Carbon fraction of methanol	%
R	Gas constant	$J.mol^{-1}.K^{-1}$
RT	Retention time	h
Temp	Temperature	^oC
TSP	Total Soluble Protein	$mg.L^{-1}$
μ	specific growth rate	h^{-1}
V	Volume	L

V_c	Current Volume	L
$v.v^{-1}$	Volume by volume	-
WCW	Wet cell weight	$g.L^{-1}$
ΔH	Enthalpy of combustion	$kJ.mol^{-1}$

Abbreviation and symbols

ABTS	2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)
AOX	Alcohol oxidase
ATP	Adenosine triphosphate
ATPS	Aqueous two-phase system
BAPNA	Nα-Benzoyl-DL-Arginine-p-Nitroanilide
BCA	Bicinchoninic acid
BMGY/BMMY	Buffered glycerol/methanol complex medium
BPTI	Bovine pancreatic trypsin inhibitor
BSA	Bovine serum albumin
BSM	Basal salts medium
CFD	Computational fluid dynamics
CHO	Chinese hamster ovary
CHAPS	3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate hydrate
CNBr	Cyanogen bromide
CQA	Critical quality attribute
DCW	Dry cell weight
DE-MF	Dead end microfiltration
DO	Dissolved oxygen
DTT	Dithiothreitol
EBA	Expanded bed absorption
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FDA	Food and Drug Administration
GC/MS	Gas Chromatography tandem-mass spectrometry
HCHO	Formaldehyde
HCl	Hydrogen chloride
HCPs	Host cell proteins
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
IPG	Immobilized pH gradient
LC-MS/MS	Liquid chromatography tandem-mass spectrometry

LDS	Lithium dodecyl sulfate
MLFB	Methanol limited fed-batch
MOPS	3-(N-morpholino) propanesulfonic acid)
$Mut^{(+,s,-)}$	Methanol utilisation phenotype positive, slow or negative
MW	Molecular weight
NaCl	Sodium chloride
NAD^+	Nicotinamide adenine dinucleotide
NADH	Reduced NAD^+
OD	Optical density
OLFB	Oxygen limited fed-batch
pAOX	Promoter of alcohol oxidase
pGAP	Promoter of glyceraldehyde-3-phosphate dehydrogenase gene
pI	Isoelectric point
PTMs	Post–translational modifications
PTM_1	*Pichia* trace metals
RCT	Research Corporation Technologies
rHuEPO	Recombinant human erythropoietin
rIFN-α	Recombinant Interferon α
scFv	Single chain antibody fragment
SCP	Single cell protein
TCA	Tricarboxylic acid
TE	Tris-EDTA
TFF-MF	Tangential flow microfiltration
USD	Ultra scale down
WCW	Wet cell density
YNB	Yeast nitrogen base
YPD	Yeast extract peptone dextrose

Chapter 1: Introduction

1.1 Biopharmaceutical manufacturing and its recognized hosts

Recombinant technology was founded in the early 1970s and succeeded in expressing a mammalian protein in *Escherichia coli*, a bacteria-based host called somatostatin in 1977 (Science History Institute, 2017). Soon after, production of insulin was successful in 1978 by Herbert W. Boyer and Stanley N. Cohen, which further proved previous successes. It was the birth of biotech industry and the technology progressed into using a more complex expression system other than bacteria, such as mammalian cells, yeast, insect cells, and plants, to produce even more complex products such as large antibodies and anti-cancer agents (Nielsen et al., 2014).

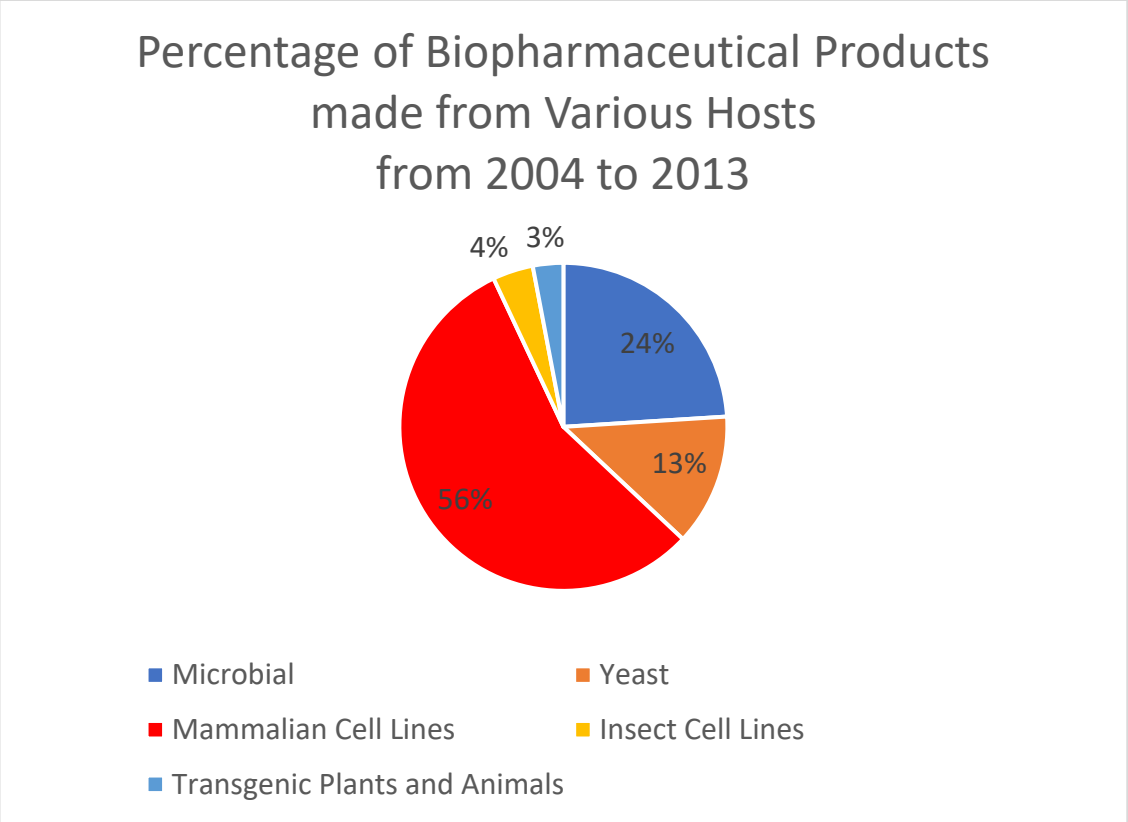


Figure 1-1 The worldwide percentage of various hosts being used in the production of biopharmaceutical products that made it into the market. Mammalian cell lines are the most common hosts used at 56%, the second most common host is microbial, which is mostly *E. coli* at 24% and the third is yeast at 13% (Nielsen et al., 2014).

Each available host has benefits and drawbacks that can be exploited according to how complex the product is and how much time the production and research need to be done before reaching the market. A simple comparison table of the three most commonly used hosts was made (table 1-1). *E. coli* is generally inexpensive to grow and the fastest to develop. However, it has the drawback of potentially producing its proteins as inclusion bodies, being inactive and insoluble, and requiring further refolding processes to make them functional (Jana & Deb, 2005). Mammalian cells are the most preferred expression system on an industrial scale, as they can produce complex and functional human-like proteins. Thus, as shown in Figure 1-1, mammalian cells produced the most biopharmaceutical products that entered the market from 2004 to 2013, at 56%. Nevertheless, they are generally more expensive, take longer to develop when compared to *E. coli*, and are more susceptible to viral and bacterial contamination (Wurm, 2004). On the other hand, yeast is a widely used expression system. Similar to *E. coli*, yeast-based expression systems can be made for a reasonable amount of money. They are also faster and less complicated than the mammalian cell expression system. Yeast-based expression system is also capable of excreting its product into the medium, producing a higher overall yield than *E. coli. It is* capable of producing post-translational modifications (PTMs), such as disulphide bonds and glycosylation, unlike *E. coli*-based expression system(Karbalaei et al., 2020). However, yeast-based expression systems also have their challenges, such as the use of methanol in cultivation and protein target expression processes, especially for methylotrophic yeast in the induction step. Furthermore, yeast has a lower secretion capability than mammalian cells (Hartner & Glieder, 2006; Wang, 2019)Changing its genes may help or hurt its growth rate, the rate and structure of the target protein it makes, and the way it triggers itself to make the target protein. To overcome these challenges, *Komagataella phaffii,* which is categorized as a yeast-based expression system, has been used to produce biopharmaceutical products such as vaccines for Hepatitis B and Human Papilloma Virus (HPV)(Cregg et al., 1987; Wetzel et al., 2018).

Table 1-1 Characteristics comparison of expression systems (Wang et al., 2019).

	Mammalian cells/ CHO	Yeast/ *Komagataella phaffii*	Microbial/ *Escherichia coli*
Nutrient Diversity Requirement	High	Low	Low
Time of Cell Line Development	6-12 months	1-2 weeks	2-3 days
Protein Folding and Disulphide Bond Formation	Yes	Yes	No
Secretion Capability Mg. (gDCW.h)$^{-1}$	2-3	0-0.01	None
Glycosylation Pattern	Human-like	High mannose	None
Volumetric Yield (g.L^{-1})	1-10	1-20	< 1
Cost of Goods	High	Low	Low
Viral Risk	High	Low	Low

1.1.1 Trends in biopharmaceutical manufacturing

The biopharmaceutical industry is constantly changing, with new technologies and methods to make biological drugs more efficient and cost-effective. Some of the most recently developed and used technological developments are using single-use bioreactors to replace traditional stainless-steel bioreactors and continuous bioprocessing, allowing operations from upstream to downstream to connect into a single continuous process. These developments were established to reduce production costs and time and increase productivity. Single-use bioreactors allow for more flexibility in production, require less cleaning, and eliminate the risk of cross-contamination (Klutz et al., 2015). While continuous bioprocessing offers consistent product quality, reduced costs, and a reduced industrial carbon footprint (Hoskisson & Hobbs, 2005). These technologies were made to produce varying biopharmaceutical products, which shows the technology's flexibility and benefits when used in industry.

Table 1-2 List of examples of biopharmaceutical products ranging from vaccines, protease, and biosimilars, with their description of possible uses.

Name	Host	Product Type	Description	References
Bromelain	Plants	Cysteine / endoprotease	Anti-inflammatory compound, immunomodulator, antimicrobial, anti-anticoagulant, anticancer, antiplaque, antiulcer, wound healing, dermatological disorders, post-surgery recovery, antibiotic absorption, treatment of osteoarthritis, sinusitis and diarrhea, antiviral agent against COVID-19	(Verma et al., 2017)
Trypsin	Mammalian	Serine protease	Anti-inflammatory enzyme, debriding agent for cleaning of necrotic wounds, ulcers, empyema, and fistulas	(Chanalia et al., 2011)
KEX2 protease	Mammalian/ Microbial / Yeast	Serine Endoprotease	Enhanced production of recombinant secretory protein, conversion of proinsulin to insulin.	(Rockwell et al., 2002)
Insulin (Aspart, glargine, etc)	Microbial / Yeast	Insulin Analogues	Hormone used in regulating sugar concentration inside the blood	(Polez et al., 2016)
Influenza Vaccine	Egg-based/ Mammalian/ Microbial	Vaccine	Vaccine for influenza usually given based on regions and seasonal, giving	(PT Bio Farma, 2022)
Hepatitis B Vaccine	Microbial / Yeast	Protein Recombinant Vaccine	Vaccine for hepatitis B	(PT Bio Farma, 2022)
Polio Vaccine	Mammalian	Inactivated Vaccine/ Attenuated Vaccine	Vaccine for Polio virus	(PT Bio Farma, 2022)

The products listed in Table 1-2 provide a small example of biopharmaceuticals for therapeutic indication and prophylactic use, such as protease, an anti-viral agent against COVID-19, insulin, and vaccines. The majority of biopharmaceutical products are based on recombinant protein technology. Their production requires host cells such as mammalian, plant, and microbial cells. Each host platform has its characteristics that drive the use of the platform in biopharmaceutical products.

1.1.2 *K. phaffii* as an emerging host for biopharmaceutical manufacturing

Multiple yeast-based expression systems have been used in the industry as a host to produce biopharmaceutical products, such as *Saccharomyces cerevisiae* (non-methylotrophic), *Yarrowia lipolyticaa* (non-methylotrophic), Hansenula polymorpha (methylotrophic), *Komagataella phaffii* (methylotrophic), and *Candida boidinii* (methylotrophic) (Baghban et al., 2019). However, each of these yeast-based expression systems has advantages and disadvantages. For example, some of them are easier to be genetically modified, whereas others are not that easy to modify (Ahmad et al., 2014). In addition, the intellectual property of most expression systems is protected by patents.

Komagataella phaffii, also known as Pichia pastoris, is a yeast-based expression system. It is one of the most commonly used expression system candidates, capable of producing non-animal-based food products, recombinant proteins, vaccines, and biosimilars (Ahmad et al., 2014; Richelle et al., 2014) . Besides, the reason for its utilization in the field is because it is capable of producing a high yield of functional proteins, even when they are considered toxic to the cells; it can excrete or retain the target protein internally; and it has readily available genetic modification protocols that will attract even more biopharmaceutical companies to use *K. phaffii* (Juturu & Wu, 2018). Furthermore, *K. phaffii* can grow up to 130 $g.L^{-1}$ dry cell weight (DCW) of biomass, which is considered high, is genetically stable, and has site-specific gene integration (Kim et al., 2015).

However, *K. phaffii* also has some strain and product-specific challenges. One of the main challenges of utilizing *K. phaffii* is that the most common substrate to be used as an induction solution is methanol, a highly flammable material that can generate a substantial amount of heat during the cultivation process, which will

be more apparent in a larger scale. Despite the drawbacks, *K. phaffii* is still an appealing expression system for both researchers and industries because of its potential to produce high cell density and product yield.

1.2 Introduction to *Komagataella phaffii* expression system

To analyse the advances that have been done in improving *K. phaffii* as an expression system, the history of the expression system, the strains that have been developed, and the genetic modifications that have been developed will be explored.

1.2.1 History of *Komagataella phaffii* as an expression system

Komagataella phaffii is a methylotrophic yeast that the Phillips Petroleum Company made in the late 1960s to make protein for animal feed (Ahmad et al., 2014). However, its use was not successfully spread during that period because of the oil crisis in the early 1970s, which increased the cost of methane gas, the precursor of methanol, which is the primary material utilized for the induction process of *K. phaffii,* and thus rendered this process unfeasible (Wegner, 1990). However, the use of yeast as an expression system or host cell for producing a variety of biological products other than animal feed, such as biopharmaceutical products that include vaccines and hormones, biofuels such as methane gas, and food products such as meatless burgers branded as “impossible foods” (Senior, 2019), which is a recently popular product stated to outsell beef.

1.2.2 *Komagataella phaffii* Strains

Komagataella phaffii has many strains, each with its own characteristics and specializations in terms of their applications. Some of the most common strains of *K. phaffii* are *GS115*, *KM71*, *SMD1168*, and *X-33*. Differences between each strain of *K. phaffii* concerning their characteristics and application are shown in table 1-3(Çelik & Çalik, 2012; Cereghino & Cregg, 2000). The expected differences between the strains are the addition or deletion of the methanol utilization genes and other genes, such as protease deficient strain.

Table 1-3 Examples of strains for *Komagataella phaffii* and their genotype and possible applications such as having possible certain antibiotic resistance for clone selection, have some of its genes disrupted to create different methanol utilisation phenotype, protease deficient strain and expression vectors containing histidine (Çelik & Çalik, 2012; Cereghino & Cregg, 2000).

Strain	Genotype	Application
GS115	his4	Expression vectors containing *HIS4*
X-33	Wild Type	Antibiotic (e.g. Zeocin) -resistant expression vectors
KM71	his4, AOX1;;ARG4, arg4	Expression vectors containing *HIS4* to generate strains with Mut^S phenotype
KM71H	AOX1;;ARG4, arg4	Antibiotic-resistant expression vectors to generate strains with Mut^S phenotype
SMD1168	his4, pep4	Expression vectors containing *HIS4* to generate strains without protease A activity
SMD1168H	pep4	Antibiotic-resistant expression vectors to generate strains without protease A activity

1.2.3 Phenotype of *Komagataella phaffii* strains

Alcohol oxidase (AOX) promoter is the most commonly used and preferred promoter for *K. phaffii,* as it is one of the simplest promoters for induction. The AOX promoter can be activated by adding methanol, and it is also a tightly regulated expression pathway that results in a naturally strong expression level (Kim et al., 2015). Furthermore, this promoter is also widely known to be repressed in the presence of glycerol, which enables the cells to grow until they have a high cell concentration before beginning the gene expression.

K. phaffii have three types of methanol utilization phenotypes that correspond to the presence of *AOX1* and *AOX2* genes, and they are called positive methanol utilization type (Mut^+), slow methanol utilization type (Mut^s) and negative methanol utilization type (Mut^-) (Hartner & Glieder, 2006). Depending on how the AOX genes are coded, a cell can have one of three different methanol utilization types.

Firstly, when both *AOX1* and *AOX2* genes are present, the phenotype of *K. phaffii* will be the positive methanol utilization type (Mut^+). This phenotype can metabolize methanol at a high rate, which will be used for both growth and expression of antigens (Vogl & Glieder, 2013). Therefore, this phenotype is preferred for research because it can proliferate and make antigens. However,

the Mut^{+} phenotype is not preferred for industrial purposes because it will require a high volume of methanol for the induction phase, which will incur higher production costs. Also, methanol is very flammable, making it much more dangerous when used in large amounts.

Secondly, when *the AOX1* gene is disrupted while *the AOX2* gene becomes the primary gene that utilizes methanol, the *K. phaffii* will have a slow methanol utilization type or Mut^{s} phenotype. The *AOX2* gene has significantly less activity, with only 5–10% of the expression level compared to *AOX1,* which is capable of producing 30% of the total soluble protein, making this phenotype have a lower growth rate and consume less methanol during the induction phase compared to the Mut^{+} phenotype (Vogl & Glieder, 2013). Even though the Mut^{s} phenotype seemed less effective, Cregg et al. (1987), Polez et al. (2016), and Tam et al. (2012) found that this trait can make recombinant proteins more efficiently for products like hepatitis B and insulin precursors.

Lastly, when both the *AOX1* and *AOX2* genes are disrupted, rendering the cells unable to use methanol as their primary carbon source. In that case, the *K. phaffii* will have a Mut^{-} phenotype, or negative methanol utilization type. This phenotype is only beneficial in producing recombinant products that require a low growth rate to ensure that the product expressed is in the correct structure and is active (Macauley-Patrick et al., 2005).

1.2.4 Promoters used in *Komagataella phaffii* system

Other than the AOX promoters explained in the previous section, other promoters can be used for *K. phaffii* (shown in Table 1-4). Glyceraldehyde 3-phosphate dehydrogenase (*GAP*) promoter and formaldehyde dehydrogenase (*FLD1*) promoter are two other preferred promoters that have successfully expressed soluble heterologous proteins and have a comparably strong expression level when compared to the AOX promoter. As was previously mentioned, there are other promoters. However, they tend to have lower expression levels than the *AOX1* promoter, and each has its own regulations that can be better in specific situations.

The GAP promoter is a constitutive promoter with a strong expression level similar to the *AOX1* promoter. A *constitutive promoter* is an unregulated promoter that will not require induction through methanol to be active and express an

antigen, eliminating the need to use and store a highly flammable and heat-generating material such as methanol on a large scale to express an antigen (Çalik et al., 2015). However, this promoter has the drawback of having the capability of expressing antigens correlated to its growth rate and the condition of its growth. Therefore, the condition of cultivation and the type of antigen expressed will significantly impact the culture's growth rate and ultimately affect the antigen's expression rate. For example, the expression rate of the system will lower if the system is working at a low temperature or if the antigen to be expressed is toxic to the cells(Çalik et al., 2015). In addition, different carbon sources will also affect the expression rate considerably. Thus, many optimizations must be done to find the optimum cultivation parameter. A comparison study was done between *the GAP* promoter and the AOX promoter, and it was found that it varies for different products as they require varying specific cultivation conditions. Some products that could be expressed using *a GAP* promoter are human chitinase and β-Lactamase (Çalik et al., 2015; Cos et al., 2006).

The FLD1 promoter is another famous promoter that uses formaldehyde dehydrogenase, a key enzyme in the methanol utilization pathway. This promoter can use methanol as an inducer just like the AOX promoter and uses methylamine as an alternative inducer because methanol and methylamine produce formaldehyde (HCHO) for either energy production or biomass production in the *K. phaffii* metabolic pathway. The expression strength of this promoter is also known to be comparable to that of AOX, which makes it as attractive as the AOX promoter. *FLD1* promoter can also be regulated using a nitrogen source such as ammonium sulphate when an alternative carbon source such as glycerol or sorbitol is used instead of methanol(Resina et al., 2009). A comparison study was done expressing varying products, such as β-Lactamase and ROL, between *the FLD1* promoter and the *AOX1* promoter. It shows a similar or higher product yield according to its product (Resina et al., 2005; Shen et al., 1998).

Table 1-4 Example of promoters that can be used for *K. phaffii.* The table also shows how the expression is regulated and expression level strength compared to *AOX1* (Vogl & Glieder, 2013)

No.	Promoter Name	Regulation	Strength
1.	AOX1	Induced by methanol	Strong (naturally ~5% of mRNA and ~30% of total protein
2.	AOX2	Induced by methanol	~5-10% of P_{AOX1}
3.	GAP	Constitutive	Strong (similar to P_{AOX1})
4.	FLD1	Induced by methanol and methylamine	Strong (similar to P_{AOX1})
5.	AOD	Expression on glucose but not on methanol or upon glucose depletion if integrated in natural locus	~40% P_{GAP}
6.	DAS	Induced by methanol	Strong (similar to P_{AOX1})
7.	ENO1	Constitutive	~20-70% P_{GAP}
8.	GPM1	Constitutive	~15-40% P_{GAP}
9.	ICL1	De-repression and ethanol induction	Not compared to P_{AOX1} or P_{GAP}
10.	ILV5	Constitutive	~15% P_{GAP}
11.	PET9	Constitutive	~10-1700% P_{GAP}
12.	PHO89 or NSP	Induced by phosphate limitation	Strong (similar to P_{GAP})
13.	TEF1	Constitutive and strong growth association	Strong (similar to P_{GAP})

1.2.5 Secretion signals

One of the benefits of using *K. phaffii* as an expression system is that it can release a high number of recombinant proteins that are correctly folded and active into the medium. Because *K. phaffii* does not release many proteins from the host cell (Digan et al., 1989; Paifer et al., 1994), releasing the recombinant protein into the medium outside of the cells makes it possible to get the target product without disrupting the cells and reducing the number of purification steps. However, *K. phaffii* must meet a few conditions before being able to successfully excrete the target protein into the medium. The prerequisites are that the target protein is also secreted by its native hosts, such as human catalase (Shi et al., 2007), and that the target protein is not too complex or large (Maccani et al., 2014). In addition, to get the cells to release the target protein into the medium, secretion signals must be encoded. Some of the most popular and well-known secretion

signals are those derived from *Saccharomyces cerevisiae: the* α-mating factor *(α-MF),* invertase (*SUC2*), and *K. phaffii* endogenous acid phosphatase (*PHO1*) (Ahmad et al., 2014). Other secretion signals have also been used to excrete proteins, such as the human serum albumin (*HSA*) secretion signal and the protein with internal repeat (*PIR1*) secretion signal in *Komagataella phaffii,* and some successes have been reported (Karaoglan et al., 2014).

α-mating factor (*α-MF*) is one of the most commonly used secretory signals for *K. phaffii* to direct the target protein into the secretory pathway because of its consistently high success rate when used for excreting various proteins. The *α-MF* comprises of two regions, pre- and pro-, and they must be appropriately aligned during the gene modification stage for the secretion signal to work. Nevertheless, *α-MF* has the drawback of having one of its secreted products to have inconsistent variation of its N-terminal amino acid number, in which case this problem could be resolved by using other secretion signals. Therefore, other secretion signals such as *SUC2*, *HSA*, *PIR1*, and *PHO1* have also been compared with *α-MF* resulting in comparable or even higher results for products such as enzymes and proteins. However, the results vary for the production of different products (Kallel, 2016; Karaoglan et al., 2014; Paifer et al., 1994)

1.2.6 Genomic Stability Capability

When it comes to expressing foreign proteins, *K. phaffii* is known to be more genetically stable than other bacterial host systems. This is because the AOX promoter is a tightly regulated expression system inducible by methanol, and it is integrated into its genome, which makes it less likely to change. Thus, it is possible to easily and stably insert the expression cassette into the host gene (Cregg et al., 1987). In comparison, *E. coli* is known to be more susceptible to mutation or plasmid instability, which may result in losing the plasmid (Waegeman & Soetaert, 2011). Therefore, many studies were done on *K. phaffii* to verify its capability to retain genes, and they were shown in terms of how many generations they could retain them. Also, its ability to stay genetically stable was tested in several ways, such as its environment, copy number, and the complexity of the target protein.

A previous study found a *K. phaffii* integrated with 11 copies of the human gene Cu/Zn superoxide dismutase on the chromosome stable for up to 28 generations

(Marx et al., 2009). Another study found that gene copies as high as 29 of the porcine insulin precursor gene were stable after 35 generations in a non-methanol environment (Zhu et al., 2009). This study proves it can be stable even for high copy numbers in a low-stress environment (without methanol). In a methanol environment, a study shows that a *K. phaffii* strain expressing human serum albumin (two copies) was found to be very stable for up to 83 generations in a methanol environment, proving its capability even in large-scale fermentation (Ohi et al., 1998). However, this may not apply if the broth's methanol concentration is higher than 0.4% $v.v^{-1}$, as it will significantly inhibit cell growth (Trinh et al., 2003; Zhang, Bevins, et al., 2000), at which point methanol will become toxic to the host. In a study by (Curvers et al., 2002), a *K. phaffii* strain encoded with a single copy of recombinant human chymotrypsinogen B lost its ability to make the protein when it was grown in methanol at a level above 4 $g.L^{-1}$. Thus, in other situations that may cause stress to the host, they may also reduce genetic stability, such as unsuitable pH, low oxygen, and low nutrients.

1.3 Progress of cultivation process of *Komagataella phaffii*

From the history of *K. phaffii*, the development of *K. phaffii* as a host started in the 1960s, when it was called *P. pastoris* for the production of animal feed protein, which then progressed to producing human insulin in the 1970s. Many studies were done to further optimize and develop *K. phaffii* as a host, from the medium used, parameters used to grow and express, and types of cultivation, which will be further explored in this section.

1.3.1 Cell culture media

Some of the most common media used for the cultivation of *K. phaffii* are buffered glycerol/methanol complex medium (BMGY/BMMY), yeast extract peptone dextrose medium (YPD), minimal glycerol medium (MG), and basal salts medium (BSM). Hanko and Rohrer (2004) found that both BMGY/BMMY medium and YPD medium have all the amino acids yeast needs to grow and provide nutrients that can be used well so cells can proliferate. However, complex components (such as yeast extract and peptone) were used in BMGY/ BMMY and YPD. Complex components will increase the cost of medium and cause batch-to-batch variations, which is not preferred by industries and regulatory agencies (van der Valk et al., 2010). Therefore, YPD and BMGY/BMMY media are usually used for

lab or small-scale cell culture. In contrast, chemically defined media such as BSM and MG medium are more prevalent in industrial-scale cultivation.

A chemically defined medium is a medium that has all the required nutrients for cells to grow and has its exact chemical composition known (Matthews et al., 2018). BSM is a chemically defined medium comprising carbon and nitrogen sources and trace elements (vitamins and metals) that act as growth catalysts, salts, and buffers. Some carbon sources that can be used are glycerol, methanol, and sorbitol. In contrast, the nitrogen source that can be used is ammonium hydroxide, which adjusts pH during cultivation, and amino acids. Trace elements such as Pichia trace metals (PTM_1) are generally added to BSM after sterilization and before inoculation to improve cells' growth and product synthesis (Hélène et al., 2001). Compared to more complex media like YPD and BMGY/BMMY medium, BSM medium is less expensive and gives more consistent results. However, it was also reported that cells cultured on a BSM medium had to synthesize all metabolic intermediates, slowing cell growth rate (Matthews et al., 2018).

1.3.2 Fed-batch cultivation of *Komagataella phaffii*

Fed-batch cultivation is the most common cultivation method when using *K. phaffii* for producing recombinant proteins. An example of a fed-batch cultivation flow process for *K. phaffii* that uses an AOX promoter and glycerol as its primary carbon source for growth is shown in figure 1-2. The flow process shown starts with thawed seed (which was frozen for storage purposes) and ends in the fermenter. There are other variations in the flow process before entering the fermenter, such as using an agar medium to create a single colony first from the seed or using smaller flasks or tubes to help the cells adapt and acquire a higher cell concentration before entering the fermenter.

To successfully grow *K. phaffii* in a fermenter, the temperature, pH, agitation, and dissolved oxygen (DO) must always be controlled. For *K. phaffii,* its typical growth temperature is 30 ^{o}C, stable in pH conditions of 3.0-7.0, the agitation can be as high as 1200 RPM in a 1 L fermenter because *K. phaffii* cells are relatively durable, and the DO concentration needs to be maintained above 20%. *K. phaffii* can be grown at low temperatures, outside the stable pH range, and in higher or lower DO concentrations. However, this may affect the cell's growth rate, product

yield, and quality of the expressed product as it changes the protease activity and productivity of the cells and the stability of the product (Huang et al., 2009). The total time needed for a complete fed-batch cultivation process of *K. phaffii* is usually around 5-8 days and changes according to the strain, media, and product (Invitrogen, 2013).

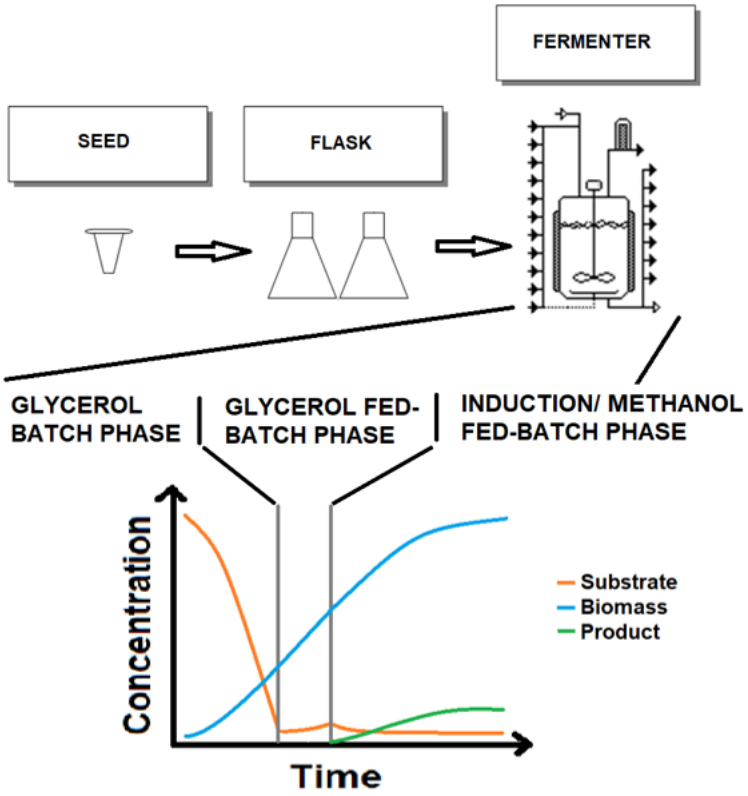


Figure 1-2 Flow chart of an example of upstream process of *Komagataella phaffii* starting from seed to flasks and then finally to fermenter, increasing in scale in terms of volume of medium inside each vessel. It also shows that there will be 3 phases inside the fermenter which starts from glycerol batch phase to glycerol fed-batch process and ending at methanol fed batch phase.

To explain the figure above, a typical *K. phaffii* cultivation process starts from a frozen seed of *K. phaffii* in glycerol stock, usually stored at -20 ^{o}C or -80 ^{o}C. A frozen seed could be thawed completely and inoculated into flasks containing buffered complex glycerol with yeast extract medium (BMGY). Next, the cells in the flasks could be incubated at 30 ^{o}C and 250 RPM for 18-24 hours. This first process prepares the cells before entering a fermenter by increasing the population of the cells and adaptability after being stored at -20 ^{o}C (Invitrogen, 2013).

In the fermenter, the cells could be grown in a controlled environment. BSM is a typical medium used inside a fermenter, and glycerol could be used as the initial carbon source. Both BMGY in the flasks and BSM inside the fermenter contain glycerol as the primary carbon source because glycerol will inhibit the AOX promoter and be used solely to increase biomass (Ahmad et al., 2014; Juturu & Wu, 2018). he initial phase for cultivating *K. phaffii* is called the "glycerol batch phase" and generally lasts around 18–30 hours until the glycerol inside the medium is depleted. The next phase, the "glycerol fed-batch phase," could increase biomass further (Huang et al., 2009; Invitrogen, 2013) and generally takes 6-12 hours. Before moving on to the last step of the "methanol fed-batch phase," glycerol must be entirely consumed by stopping the glycerol feed, called the "glycerol starvation phase." The presence of glycerol must be exhausted because glycerol inhibits the AOX promoter, which causes methanol to build up in the medium if methanol is added too early (Ahmad et al., 2014; Juturu & Wu, 2018). After the glycerol is depleted, the methanol adaptation phase, or "transition phase," could be started by injecting the methanol at a lower feed rate before increasing the feed rate to sustain a larger growth rate and expression rate. Starting with a lower feed rate is done to ensure that the methanol does not build up to a level that is toxic to the cells in the medium (Invitrogen, 2013). An accumulation of methanol inside the medium is toxic because it will result in toxic by-products from alcohol oxidation, such as hydrogen peroxide (H_2O_2) and formaldehyde (HCHO). Accumulating toxic by-products will naturally demolish cell structures and produce inhibitory effects on the AOX enzyme (Kupcsulik & Sevella, 2004). The methanol fed-batch phase starts the expression of the target protein and will sustain the growth rate. The methanol fed-batch phase will take 3-5 days, depending on the type of strain and product being produced. The range of days for the fed-batch process during the methanol induction mostly depends on when the product is expressed the most and may be linked to the specific growth rate of the cells. The growth rate of *K. phaffii* will vary even if the same cultivation process parameters, medium, and promoter (AOX) are used. Examples of them are shown in table 1-5.

Table 1-5 Maximum specific growth rates (μ_{max}) of various cultivation process using *K. phaffii* in different phenotypes and producing different target product.

Target Product	Phenotype	Maximum Specific Growth μ_{max} (h^{-1})	References
Recombinant human chymotripsynogen B	Mut^+	0.084±0.005	(Simon Curves, Jorg Linnemann, Thomas Klauser, Christian Wandrey, 2001)
Single chain variable fragment antibody	Mut^+	0.02-0.04	(Peebo & Neubauer, 2018)
Multiple protein product	Mut^+	0.08-0.12	(Peebo & Neubauer, 2018)
mAb4813	Mut^+	0.012	(Cos et al., 2006)
Hepatitis B surface antigen	Mut^+	0.009	(Rahimi et al., 2019)
Angiostatin	Mut^S	0.011-0.018	(Cos et al., 2006)
rFSH	Mut^S	0.012	(Cos et al., 2006)

1.3.3 Methanol limited fed-batch cultivation

Fed-batch cultivation of *K. phaffii* can be further divided into methanol-limited fed-batch (MLFB) cultivation and oxygen-limited fed-batch (OLFB) cultivation. The former is a control strategy in which the methanol feeding rate is controlled to maintain the dissolved oxygen (DO) inside the broth at a set point, usually around 20–30% saturation level (Invitrogen, 2013). There are multiple methods by which this can be done. One commonly used method for maintaining the DO is setting the feeding pump of the methanol to stop when the DO falls below the set point and start when it rises above the set point. A study was done using this method of maintaining DO by Lim et al. (2003), and it was found to have increased the growth rate and volumetric yield of rGuamerin. Cascading the DO saturation rate with the agitation rate and then the pure oxygen feed rate is another standard way to keep DO saturation at a certain level in a bioreactor (Invitrogen, 2013). Another way to keep the DO saturation level steady is to use feedforward control based on fed-batch substrate balance for a quasi-steady state. This method uses an equation to calculate the amount of methanol feed rate while keeping the DO above the set point (Barrigón et al., 2013). This method is closely related to the

other method of fed-batch, which is an oxygen-limited fed-batch. The methanol feed rate is kept in a pseudo-steady state, which keeps up with the methanol intake of the cultivation process.

The previous methods of maintaining DO were created because it was found that dissolved oxygen affected the expression of foreign proteins (Barrigón et al., 2013; Cregg, 2007; H. K. Lim et al., 2003). Thus, DO saturation is a critical parameter that must be maintained to achieve an optimized cultivation process. However, the MLFB cultivation process is not always the most optimal type of fed-batch cultivation because the presence of methanol is significant in ensuring the expression of the target product, and limiting methanol will reduce its expression rate (Niu et al., 2013).

1.3.4 Oxygen limited fed-batch cultivation

Oxygen-limited fed-batch (OLFB) cultivation is another type of fed-batch cultivation method of *K. phaffii* at which oxygen is not maintained. Instead, the methanol feed rate is maintained to ensure enough or excess for growth and expression. Therefore, OLFB may also be called methanol unlimited fed-batch cultivation (MULFB) or methanol non-limited fed-batch (MNLFB) (Charoenrat et al., 2005; H. K. Lim et al., 2003).

Many studies have been done on OLFB, which show that it can increase product yield and purity when compared with MLFB. For example, in one study by Charoenrat et al. (2005) to produce recombinant Thai rosewood B-glucosidase, the cell density was found to be 5% higher, the product concentration 16% higher, and the purity higher while still retaining the same level of cell viability of around 90-95% when compared to its MLFB counterpart. However, like MLFB, OLFB is not always the most optimal method of fed-batch production, as it may vary according to the strain of *K. phaffii* and the type of product being expressed. For instance, in a study by Khatri and Hoffman (2006), increasing the methanol concentration from 0.3% to 3% ($v.v^{-1}$) increased the volumetric yield of the target product (single chain antibody fragment) by almost 6-fold. However, another study by Barrigon et al. (2013) shows that the cell growth rate of *Rhizopus oryxae* and target product expression were inhibited when the methanol concentration reached 10 $g.L^{-1}$.

In the same way that DO saturation is a critical factor for MLFB, methanol concentration is a critical factor for OLFB. The methanol concentration inside the bioreactor can be controlled and maintained using an online methanol sensor, which can be cascaded with the methanol feed rate. Nevertheless, if a methanol sensor is not applicable to be added to the system, it can be checked outside of the system using HPLC, an enzymatic reaction, or GC/MS. On another note, maintaining methanol concentration during the process may also have an adverse effect on the target product and thus not be suitable for that particular strain.

1.4 Continuous cultivation of *K. phaffii*

Another type of cultivation process other than batch and fed-batch that can be used for culturing *K. phaffii* is called a "continuous cultivation process." Compared to batch and fed-batch cultivation processes, the continuous cultivation process is much more complex regarding setup and operations.

1.4.1 Continuous cultivation

The continuous cultivation process can also be described as an extension of the fed-batch cultivation process, where the fed-batch cultivation process ends in a single harvest. In contrast, the product for the continuous cultivation process is taken out continuously. The benefits of using a continuous cultivation process compared to a fed-batch or batch cultivation process are numerous, such as producing less carbon footprint to set up and producing a higher product yield (mg) within a single cultivation process. Another benefit of the continuous process is that it reduces the downtime from cleaning, sterilization, and preparation, and the product quality will be more consistent (Hazeu & Donker, 1983; Simon Curves, Jorg Linnemann, Thomas Klauser, Christian Wandrey, 2001). However, the drawbacks of the continuous cultivation process are also apparent. It is much more complicated in terms of mass balance calculations and setting up equipment and processes. If the continuity of the process cannot be maintained, the system will collapse. Continuous cultivation also requires a genetically stable strain because the process takes longer to grow and express and is more likely to get contaminated because there are more inlets and outlets. Thus, the benefits and drawbacks of each type of cultivation can be summarized in table 1-6.

Table 1-6 Comparison of benefits and drawbacks of batch, fed-batch and continuous cultivation methods in general in the cultivation of *K. phaffii.*

	Batch	Fed-batch	Continuous
Benefits	1. Simplest cultivation process 2. Have the least chance of contamination occurring	1. Simple and standard process for *K. phaffii* 2. High Cell Density (HCD) cultivation process 3. Greater product yield compared to batch	1. Smaller carbon footprint 2. Lower downtime 3. Increase overall product yield within one inoculation 4. More uniformed product
Drawbacks	1. High downtime for preparation and sterilisation 2. High labour cost 3. Low product yield 4. Not recommended for cultivation of *K. phaffii*	1. Moderate downtime 2. Have small chance of contamination	1. Complex process 2. Requires genetically stable strain 3. Highest chance towards contamination

1.4.2 Types of continuous cultivation

Like types of fed-batch cultivation processes that separate based on the system's limitations, the continuous cultivation process also varies in terms of the limitations set on the process. Therefore, the continuous cultivation process can be separated into the classical and alternative approaches (changestats) (Nieto-Taype et al., 2020).

The classical approach can be divided into chemostats and turbidostats, mainly separated by whether the system is set to be nutrient- or non-nutrient-limited. Most parameters are kept the same and stable throughout the cultivation process to reach a steady state for both classical approaches. On the other hand, in terms of differences, the chemostat is controlled by continuously adding a culture medium to which a single primary nutrient is kept as a limiting nutrient. By having a limiting nutrient, the specific growth rate of a chemostat process may not be close to the maximum specific growth rate (μ_{max}). On the other hand, turbidostats are not limited to nutrients. Instead, they use sensors to control growth and a feedback loop to keep the biomass constant. By doing this, the growth of the cells can be kept close to its μ_{max}.

The alternative approach of continuous cultivation or the changestats was proposed as a much more novel approach to a continuous process. As was implied from the name, the changestats continuous process continuously changes its parameters in a single cultivation to achieve a quasi-steady state. Like the classical approach, the changestats can be further separated into two types of continuous cultivation and are also based on whether the system is nutrient-limited or non-nutrient-limited, which have the impact of not being close to μmax or being close to μmax respectively. There are three variations of changestats for a nutrient-limited system: accelerostats and deceleration-stats (studying the increase and decrease of specific growth rates throughout the cultivation process), dilution-rate-stats (the environment condition changes while keeping the dilution rate constant), and retentostats (biomass is retained using internal and external filters). On the other hand, there are also three types of non-nutrient limited changestats: auxo-accelerostats (growth in nutrient excess condition), nutristats (setting the substrate concentration point and the dilution rate as a function), and adaptastats (substrate limiting but near μmax condition).

Although both the classical approach and changestats have their benefits and drawbacks, the classical approach of the chemostat continues to be the best choice regarding the characterization of the microorganism and ease of operation (Hoskisson & Hobbs, 2005; Monod, 1950; Ziv et al., 2013).

1.4.3 Continuous cultivation phases and strategies

A typical simple fermenter setup for a continuous cultivation process system has a similar setup to a fed-batch cultivation process while adding a new substrate that is fed in and cell broth that is taken out at the same rate as the total volume being fed into the fermenter (which includes acids and bases, fresh medium without glycerol, and induction solution) to keep the volume inside the fermenter constant as depicted in figure 1-3. For a fed-batch process, the carbon source will initially be consumed during the batch process before adding a new carbon source, which should be kept at a low concentration through the addition of glycerol and methanol during the glycerol fed-batch phase and the methanol fed-batch phase, respectively. The addition of the carbon source into the medium in the fed-batch process will result not only in an increase in biomass but also in an increase in volume. As for a continuous cultivation process, by adding new substrates and induction solution and harvesting at the same rate as all the

substrates being fed in, the volume in the fermenter is kept constant, and by creating a chemostat condition, the substrate, biomass, and product concentration will be able to be kept constant.

During the broth's harvesting in continuous cultivation, both the cells and the medium could be taken from the broth as it is. However, continuous cultivation can also allow the cells to be recycled into the bioreactor. In continuous cultivation, the cells are kept in constant growth rather than allowed to enter a stationary phase. As a result, the cells harvested from the bioreactor are still actively growing and dividing and can be quickly reintroduced into the culture without significant loss of viability or productivity. The usage of this method depends on where the target protein is retained (excreted into the medium or inside the cells) and how stable their genetic modifications are. Recycling the harvested cells back into the bioreactor would benefit the cultivation process if the target protein was not retained inside the cells. There are three main benefits of recycling the cells back into the bioreactor; they reduce the cost of production by eliminating the need to add new cells to the bioreactor constantly; they increase the efficiency of the process by ensuring that the bioreactor is continuously operating at optimal cell densities; and, lastly, they help maintain consistency of the final product by ensuring that the same strain of cells is used throughout the entire production process. However, the drawbacks of cell recycling are apparent. It required additional equipment such as filtration system which increased the complexity of the system, membrane filter fouling may occasionally occur and accumulation of inhibitory by-products because of incomplete removal of spent medium and waste products (Meier et al., 2014).

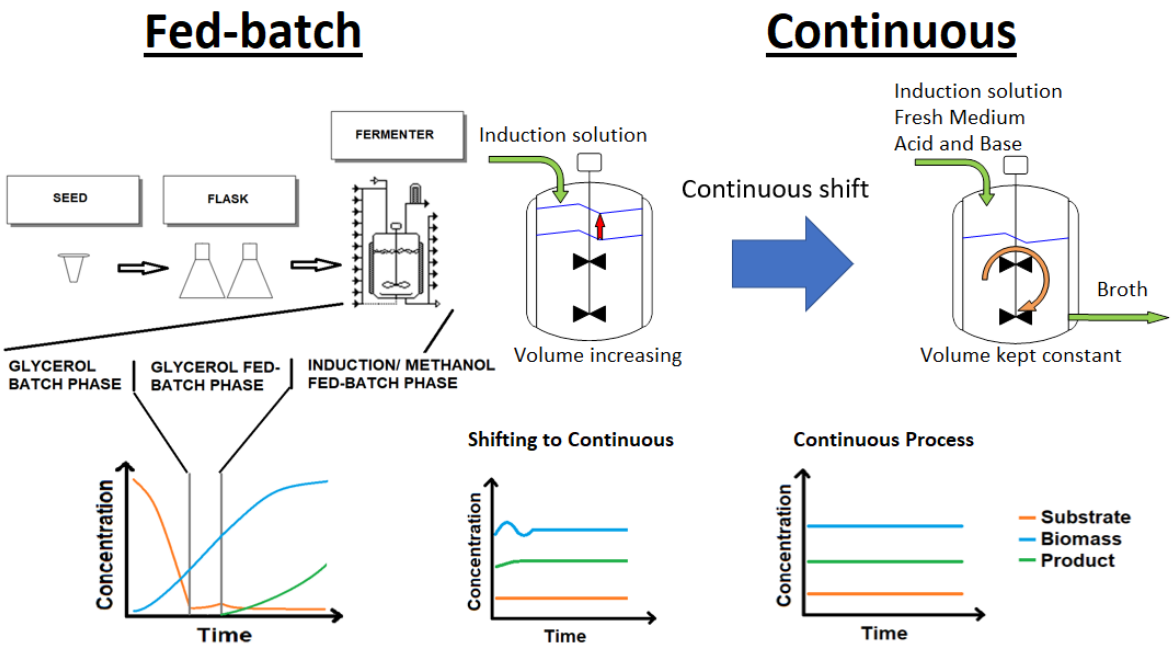


Figure 1-3 A theoretical graph of a fed-batch cultivation process (left) and a continuous cultivation process (right), showing changes in terms of substrates, biomass, product concentration, and volume inside the fermenter (Peebo & Neubauer, 2018).

Initially, a continuous cultivation process typically has the same three phases as the fed-batch cultivation process inside a fermenter. However, an additional phase after the methanol fed-batch phase will continuously feed substrates and harvest cell broth. The rate at which the substrate is fed is equal to the cell broth harvested out and is set as the dilution rate (D). The dilution rate of continuous cultivation is crucial as it may accumulate proteases, by-products that inhibit growth and harm protein production and consistency of quality. Thus, optimizing the cell density and dilution rate in continuous cultivation is necessary. The dilution rate is the most critical parameter in a chemostat continuous cultivation process. It is related to the current volume inside the fermenter and the biomass, which results in the cells' specific growth rate (μ). Researchers have tried to determine how fast different kinds of *K. phaffii* grow by comparing their phenotypes, carbon sources, and end products. The result shows varying maximum specific growth rates that can be used. For example, a typical maximum specific growth rate of *K. phaffii* ranged between 0.01 to 0.12 h^{-1} for the Mut^{+} phenotype (Cos et al., 2006; Peebo & Neubauer, 2018). Table 1-7 shows an example of varying dilution rates used when using *K. phaffii* as their host and basal salt medium (BSM) as their medium.

Table 1-7 A few examples of previous studies of the strain, target product, and dilution rate used of various continuous cultivation process using *K. phaffii* and their titre using basal salt medium (BSM).

Strain	Target Product	Dilution Rate used D (h^{-1})	Titre $mg.L^{-1}.h^{-1}$	References
X-33, Mut^+	IFN-tau	0.0333	2.73	(Zhang et al., 2004)
X-33, Mut^+	IFN-tau	0.0361	2.566	(Zhang et al., 2004)
X-33, GAP	Rh-chitinase	0.042	6	(Schilling et al., 2001)
GS115, Mut^+	Chymotrypsin	0.085	Growth based ~5.9	(Curves et al., 2001)
GS115, Mut^+	HbsAg	0.009	1.13	(Rahimi et al., 2019)

1.5 Mixed induction strategies

The most common induction strategy for *K. phaffii* with the phenotypes Mut^+ and Mut^s is pure methanol and Pichia trace minerals (PTM1). However, alternative carbon sources used with pure methanol for the induction phase may have beneficial effects. It is a simple strategy to increase the energy supply for the cells to increase growth rate and productivity by increasing the carbon source inside the medium (Looser et al., 2017; Zhang et al., 2003). Furthermore, mixing pure methanol with other carbon sources will also have the effect of lowering the methanol concentration, which will reduce the flammability of the induction solution, lower the toxicity by lowering the concentration of methanol, increase cell viability, and lower the amount of protease released from lysing cells (Ahmad et al., 2014). Glycerol and sorbitol are two carbon sources that can be mixed with methanol during the induction phase. Adding a co-substrate can also lower much of the heat made by digestion during the growing process compared to only digesting methanol. The energy released for methanol ($H_{S,\ methanol}$= 22.70 $kJ.g^{-1}$ of methanol) during combustion is higher than when using other carbon sources such as glycerol ($H_{S,\ glycerol}$= 17.98 $kJ.g^{-1}$ of glycerol), sorbitol ($H_{S,\ sorbitol}$= 16.52 $kJ.g^{-1}$ of sorbitol) and glucose ($H_{S,\ glucose}$= 15.58 $kJ.g^{-1}$ of glucose) (Çalik et al., 2015).

1.5.1 Metabolic pathway of methanol

When methanol is introduced into the *K. phaffii* cell, it is first metabolized and oxidized into formaldehyde (HCHO) by the AOX enzyme in the peroxisome. The metabolized methanol will be divided into two pathways; biomass synthesis and energy production (or formaldehyde dissimilatory) (Gao et al., 2012). When methanol is oxidized into HCHO and broken down in the biomass synthesis pathway, no ATP is made, and little to no oxygen is used. On the other hand, when the methanol is metabolized through the energy-producing pathway, it produces 2 moles of ATP from 1 mole of methanol and consumes 1.5 moles of oxygen. A simplified methanol metabolic pathway in *K. phaffii* is represented in Figure 1-4. The distribution of methanol into these two pathways varies and changes according to the condition of the cultivation process. It varies according to the product being made and the temperature of the cultivation process, which affects the specific growth rate of the culture (Jahic et al., 2002). For example, in one study by Gao et al. (2012), the methanol metabolic flux favoured the biomass synthesis pathway more when the cultivation temperature decreased from 30 ^{o}C to 20 ^{o}C, significantly increasing the maximum specific activity (IU.mL^{-1}) of IFN-α.

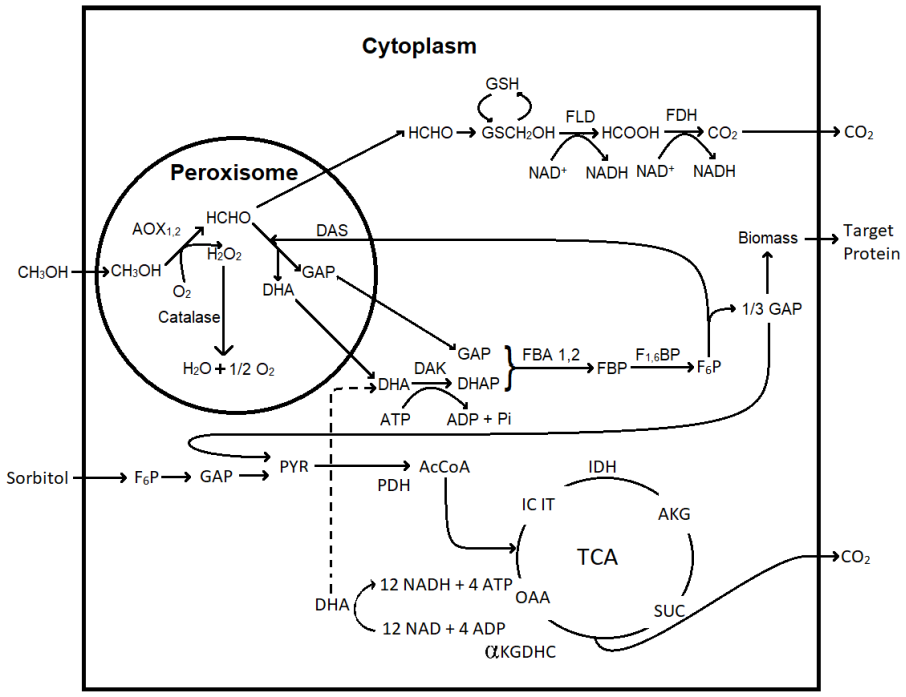


Figure 1-4 The utilisation of methanol (CH_3OH) and sorbitol through metabolism pathway of *K. phaffii*. Methanol is consumed for its growth (producing biomass) and energy, while sorbitol is consumed for energy which eventually used for growth. The enzymes involved as shown in the pathways includes AOX (alcohol oxidase), catalase, *FLD* (formaldehyde dehydrogenase), *FDH* (formate dehydrogenase), *DAS* (dehydrogenase synthase), *DAK* (dihydroxyacetone kinase), *FBA 1,2* (fructose 1,6-biphosphate aldolase), $F_{1,6}BP$ (fructose 1,6-biphosphatase), *PDH* (Pyruvate dehydrogenase), *IDH* (Isocitrate dehydrogenase), and *αKGDHC* (oxoglutarate dehydrogenase complex). One of the in-between products that needs to be mentioned is formaldehyde (HCHO) which is used by *FLD1* promoter to induce the start of antigen expression (Çalik et al., 2015; Wang, 2019)

1.5.2 Metabolism in mixed inductions

Alternative carbon sources such as glycerol, acetic acid, lactic acid, and sorbitol are metabolized and used through a different pathway from methanol. They are metabolized in the cytoplasm instead of the peroxisome, as shown in figure 1-5. When the alternative carbon source mentioned before is introduced into the cytoplasm, glycolysis converts it into glyceraldehyde 3-phosphate (GAP). It will then be utilized through a biomass synthesis pathway or energy-producing pathway. For the alternative carbon source, the energy-producing pathway will go through the tricarboxylic acid (TCA) cycle (Niu et al., 2013). By conversion, for every three moles of carbon consumed from an alternative carbon source and three moles of O_2, around 8 moles of ATP are produced in the TCA cycle.

1.5.3 Glycerol/methanol mixed induction

Glycerol is the primary carbon source for BSM that is mainly used for growth, and it can also be used as an alternative carbon source for induction when mixed with pure methanol. In the *K. phaffii* metabolic pathway, glycerol is utilized in a different pathway from methanol, and by glycolysis, it is converted to glyceraldehyde 3-phosphate (GAP). Then, GAP will be converted to biomass and energy (ATP) through the TCA cycle (Niu et al., 2013). Although glycerol is known to have an inhibitory effect on the AOX promoter, some studies have shown that it is possible to be used in the induction phase when mixed with pure methanol.

A study was done on the production of recombinant CD40 ligand comparing the induction solution between a mixture of glycerol and pure methanol at a ratio of 1:1 C-mol:C-mol and pure methanol, and it was found that the former produced twice the volumetric yield when compared to the latter (McGrew et al., 1997). However, another study using glycerol co-feed by Cos et al. (2006) showed varying results. On the production of monoclonal antibody 4813 with the Mut^+ phenotype, although the growth rate doubles when comparing pure methanol to glycerol co-feeding with a carbon content ratio of 1:1 C-mol:C-mol, the productivity fell to zero. Only when the carbon content ratio is set to at least 4:1 does monoclonal antibody production start, albeit with a lower final volumetric yield. The previous study proved the repressive reaction of the AOX promoter in the presence of glycerol and that during the induction phase, the glycerol concentration will need to be kept as low as possible to avoid inhibiting the *AOX1* promoter (Huang et al., 2009).

1.5.4 Sorbitol/methanol mixed induction

Sorbitol is a non-repressive alternative carbon source for the *AOX1* promoter*. It has* been widely used as a co-substrate mixed with pure methanol in *the K. phaffii* induction process. As shown in Figure 1-5, sorbitol is utilized in a different metabolic pathway from methanol and a similar pathway to glycerol by glycolysis. First, it is converted to GAP and then to biomass and energy (ATP) through the TCA cycle (Niu et al., 2013). So, most sorbitol will be broken down for biomass and energy, while methanol will still be the primary expression-inducing agent. Sorbitol is also found to be similarly priced, if not slightly more expensive when compared to methanol. So, it is feasible to use it with methanol to get the same or a higher yield (Bustos et al., 2022).

The use of sorbitol as a co-substrate in the fed-batch cultivation process can be separated into batch addition and constant addition. A study by Celik et al. (2009) shows that sorbitol does not repress the AOX promoter when the concentration is below 5% ($w.v^{-1}$), which shows that using sorbitol in fed-batch cultivation does not require a complex process. Furthermore, a study was done by Xie et al. (2005) comparing a fed-batch process using glycerol mixed feed and sorbitol mixed feed in the cultivation of a Mut^S phenotype, *K. phaffii,* expressing and excreting angiostatin into the medium. The result shows that glycerol mixed feed induction produces a lesser amount of recombinant protein of 108 $mg.L^{-1}$ than sorbitol mixed feed induction of 141 $mg.L^{-1}$. Also, the glycerol mixed feed induction yields a lower average specific growth rate of cells of 0.012 h^{-1} than the sorbitol mixed feed. However, other studies show that the target protein yield and cell growth rate still vary for different strains, showing that glycerol mixed feed can have a higher average specific growth rate while having a lower volumetric recombinant protein yield than sorbitol mixed feed (Thorpe et al., 1999).

Although using sorbitol as a co-substrate produced varying results in increasing the volumetric yield of the cultivation process, it could be used with a low temperature strategy to ensure the increase in volumetric yield produced during the cultivation process. For example, a study by Gao et al. (2012) used both sorbitol co-feeding and low temperature to increase the maximum specific activity in the cultivation of a strain of *K. phaffii* expressing recombinant porcine interferon-α. In exchange for the high operating cost of keeping the temperature low at 20 ^oC during induction in the large-scale cultivation process, sorbitol co-feeding was done at 30 ^oC and increased maximum specific activity of recombinant porcine interferon-α by 10- to 200-fold.

1.5.5 Other possible mixed induction combination

Acetic acid and lactic acid are other non-repressive carbons capable of being used as a co-substrate to be mixed with pure methanol in the *K. phaffii* induction process. Sorbitol is used in a different part of the metabolic process than methanol but in the same part as glycerol. Glycolysis converts it to GAP, which will then be converted to biomass and energy (ATP) through the TCA cycle (Niu et al., 2013).

Xie et al. (2005) compared a fed-batch process using multiple mixed feed strategies to cultivate a Mut^S phenotype, *K. phaffii,* expressing and excreting angiostatin into the medium. For the use of acetic acid and lactic acid as an alternative carbon source, the result shows that they produced half the amount of average angiostatin of 52 $mg.L^{-1}$ for the former and almost double that of 191 $mg.L^{-1}$ for the latter when compared to glycerol mixed feed induction of 108 $mg.L^{-1}$. However, even though lactic acid shows promise by producing almost double the volumetric yield of angiostatin, the growth rate was slightly lower, the final biomass was lower, and the induction time was longer than when using glycerol as an alternative carbon source. Only a few studies have been done on lactic acid as an alternative carbon source and, thus, may vary according to the target protein being expressed.

1.6 Recombinant Proteins in biopharmaceutical manufacturing

From previous sections, it has been mentioned that many types of products can be produced using *K. phaffii*, such as vaccines (e.g., hepatitis B surface antigen, Dengue VLP vaccine serotypes 1, 2, 3, and 4), biosimilars (e.g., angiostatin, human hormone irisin, insulin), and antibodies (fAb, mAb). The manufacturing of these products starts with cell culture, product purification, formulation, and fill and finish. The research scope would focus on the cell culture stage or the fermentation processes, and thus its importance would be emphasized. Insulin and KEX2 protease will be described further to see how they have developed as biopharmaceutical products.

1.6.1 Importance of cell culture of *K. phaffii* in overall process

Cell culture is the initial production step in biopharmaceutical products, as shown in figure 1-5, as it mainly focuses on the mass production of the target product by growing cells and inducing them to start production of the target protein. After the protein is made in bioreactors, the first step in processing is to harvest or clean up the cell culture. Then, in the clarification step, cells, cell debris, or solid contaminants are separated with particle-free liquid that will be used for subsequent downstream processing steps such as chromatography and diafiltration, depending on whether the protein is expressed intracellularly or extracellularly (Sampath et al., 2014).

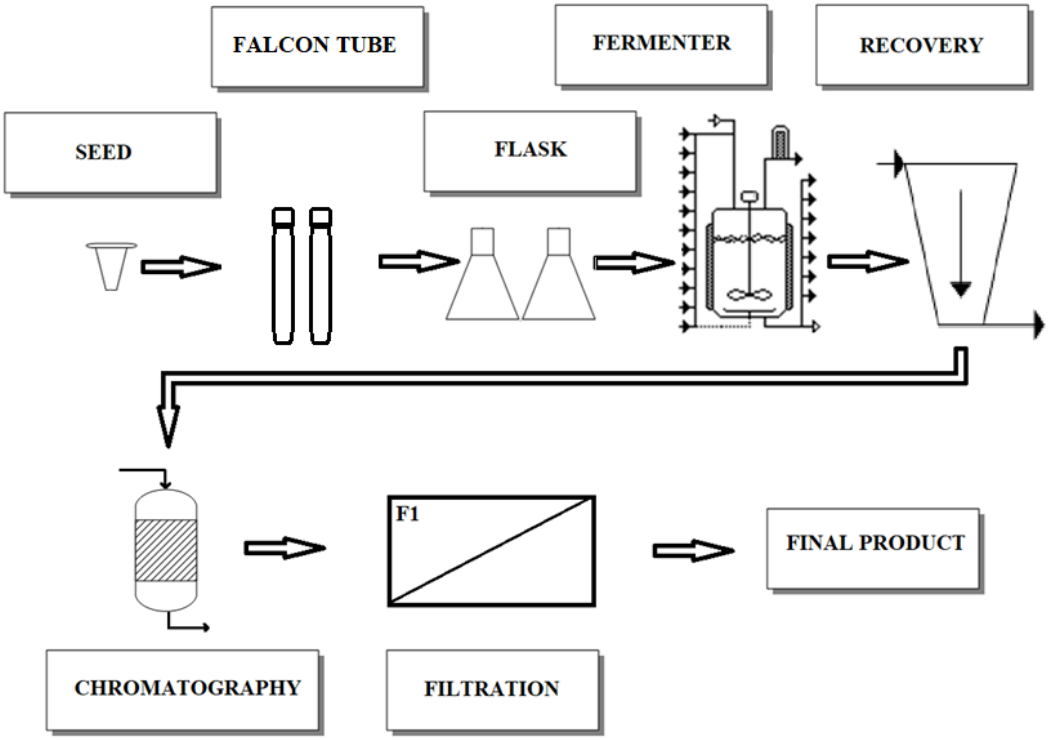


Figure 1-5 An example of an overall flow process of the production of recombinant protein in lab scale using *K. phaffii* as its host from seed to final product

For the host *K. phaffii*, it is pretty standard for the cell culture process to use a high cell density system, which can be a challenge for the proceeding clarification process and may require a dilution step for the harvesting process or the cell disruption process to increase the efficiency and effectivity of the process (Wang et al., 2019). Multiple studies have been done for the primary clarification process using centrifugation, which dilutes the sample to 80 to 100 gDCW.L^{-1} or even lower (Brady et al., 2001; Gurramkonda et al., 2013). Dilution of samples will affect the overall purification process by increasing the sample volume to be processed, which will increase the scale of the equipment, and the time required to purify the target product and increase the risk of product loss through the longer process time. These points will be further explored in this chapter.

1.6.2 Harvest/ Clarification processes

Numerous clarification processes can be used in harvesting and separating the cells and supernatant. Yavorsky et al., (2003) state that the steps of clarification are usually primary recovery, secondary clarification, and virus filtration, which are done in order if and when needed.

The most commonly used processes for primary recovery or clarification are centrifugation, filtration, and depth filtration (Sampath et al., 2014). Most of the whole cells and cell debris were removed in the primary recovery step. However, there may still be smaller particles that remain in the sample, such as smaller debris, particles, and viruses, which will affect the efficacy of further purification processes. The limitations of the primary clarification process depend on the type of process. For example, particle size is a limit for clarification processes that use filtration, while density is a limit for clarification processes that use gravity, like centrifugation. For secondary clarification and virus filtration, the results acquired from primary recovery may be further filtered at the smaller-sized filter to remove residual cells, cell debris, viruses, DNA, and any remaining particles not removed in primary recovery. The main purpose of secondary clarification is to protect the further downstream by removing any particles that may lower the efficiency of the chromatography process or break or dirty the resin.

Multiple options and combinations of primary and secondary clarification processes can be chosen based on whether the product stays in the cells or is released, the type of cell, cell density, and the bulk volume. The main parameters to be optimized for when selecting these clarification processes are product recovery, process time, capital and operating cost, consistency, and reproducibility (Wang et al., 2019). The process's availability and need may also be considered when selecting the clarification process. For example, a study by Polez S. et al. (2016) completed the clarification process by small-scale diafiltration using Prostak™ prototype Tangential Flow filtration system by Merck before entering the cation chromatography process, which does not use a secondary filtration process. Another study was done for the technical and economic considerations comparing disc stack centrifuge (DSF) and depth filter after the flocculation/acid precipitation process, which shows that DSF gives a similar overall cost for volumes above 5000 L (Dryden et al., 2021). The second study also compared the pros and cons of different processes to clarify capital, labour, and process time costs.

Changes to the processes that come before clarification, like genetic modification, cultivation, and pre-clarification, have been suggested and studied as ways to improve the efficiency of the clarification process. For example, a study by Wang et al. (2019) compared the clarification process of *K. phaffii* when

using different sorbitol and methanol mixed induction strategies during the induction stage. It showed that it reduced impurities and improved centrifugal dewatering. Another study also used flocculation to increase particle size distribution, which helps clarification when using filtration during the clarification process, and acid precipitation to reduce HCP and DNA impurities (Dryden et al., 2021). These studies were done to make clarification processes less limited and to make the chosen process easier to use.

1.7 KEX2 Protease

In this study, KEX2 protease was used as a model protein and was analysed in terms of its volumetric yield and condition. KEX2 protease is used to produce insulin (Sreenivas et al., 2014). KEX2 protease works by cutting the C-peptide domain and the two chains of proinsulin (chains Alpha and Beta) to create insulin, which will then be ready for the subsequent purification step (Polez et al., 2016). In the context of targeting KEX2 protease expression, the KEX2 expressed in *K. phaffii* using continuous cultivation and a mix of methanol and sorbitol for induction has never been studied individually. The strategy of using continuous cultivation and mixed induction during the expression of design not only gives more insight into design choice and how it is expressed, which is needed for rational design, but it also opens up exciting business opportunities. Then, the commercialization of *K. phaffii*, which makes insulin or other biopharmaceutical products, will be scaled up.

It was found that SCKEX2 (*Saccharomyces cerevisiae* KEX2) can perform precursor cleavage not only in *Saccharomyces cerevisiae* but also in *K. phaffii* and mammalian cells, which recognizes cleavage sites in precursor proteins and processes them to produce active proteins. Since KEX2 protease cuts in a more specific place than trypsin, it can help researchers study the properties of enzymes and their uses in industry and applications in Muslim countries, which is vital for the issue of halal properties. For example, in Indonesia, where the majority of the population is Muslim, halal certification is not only mandated in food industry but also in medicinal products.

1.8 Thesis objectives

Knowing that fed-batch cultivation of *K. phaffii* using an AOX promoter requires a large amount of methanol, which has high potential risks in handling it in large-

scale biological product production processes, the objective of this study is to investigate a novel cultivation methodology to reduce the need for methanol during the induction and improve the volumetric yield of the target molecule to be expressed. It is thought that mixing sorbitol and methanol as an alternative feeding strategy or carbon source during the induction step will reduce the need for methanol without affecting the condition of the target protein. Furthermore, it is hypothesized that the mixed-mode feeding strategy delivered in a continuous cultivation mode, as compared to a fed-batch mode in the cultivation of *K. phaffii*, will improve the productivity of the protein target.

1.8.1 Research Goals

This research aims to improve the cultivation process methodology in terms of productivity while still ensuring the quality (activity or identity of the target protein is confirmed) of a recombinant protein product using the KEX2 protease protein as a model expressed in *K. phaffii* as an expression system. The objectives of the research are:

1. To investigate a mixed feed induction strategy with sorbitol as an alternative carbon source as co-feed with methanol and assess the impact of the strategy on volumetric yield and content of KEX2 protease as a model.
2. To investigate fed-batch and continuous cultivation and assess the impact of the strategies on volumetric yield and content of KEX2 protease as a model.
3. To compare the effects of mixed feed strategy and continuous culturing method on manufacturing aspects, including cell properties, product content, productivity, volumetric yield, overall cost, and process time.

1.8.2 Methodology

Four modes of cultivation were used to achieve the goals of this study: (1) fed-batch mode of cultivation utilizing methanol only; (2) mix of methanol and sorbitol as carbon sources; and (3) continuous mode of cultivation utilizing methanol only or (4) mix of methanol and sorbitol as carbon sources. The processes were compared and studied in terms of the condition of the target protein, including its activity, identity, and productivity, as well as how they affected the manufacturing process.

Chapter 2: Materials and methods

2.1 Materials

Methanol and sorbitol were purchased from Merck (Darmstadt, Germany). All the other materials were purchased from Sigma-Aldrich Cooperation (Dorset, UK) at a material grade of at least molecular biology to analytical grade unless otherwise specified.

2.2 Preparation of cell culture medium

The types of media used in this research can be separated according to their use, with a minimum medium and complex medium used mainly for lab scale and a defined basal salt medium (BSM) for fermenters. The method of preparation of these media is shown below.

2.2.1 Minimum and buffered complex agar media

The minimum glycerol medium used in this research was prepared by dissolving 2.0 g of glycerol, 2.36 g of potassium dihydrogen phosphate (KH_2PO_4), 0.46 g of di-potassium hydrogen phosphate (K_2HPO_4), and 3.0 g of bacteriological agar in 200 mL of milli-Q water. The agar medium was then sterilized using an autoclave. After sterilization and cooling down slightly, a zeocin antibiotic was added to a working concentration of 0.1 $mg.mL^{-1}$ if required and then poured into a petri dish accordingly and further cooled down before use.

The buffered complex agar medium that was used in this research was prepared by dissolving 20 g of peptone, 10 g of yeast extract, 20 g of bacteriological agar, 11.813 g of potassium dihydrogen phosphate (KH_2PO_4), and 2.3 g of di-potassium hydrogen phosphate (K_2HPO_4) in 900 mL of milli-Q water. The medium was then sterilized using an autoclave. Then, the yeast nitrogen base (YNB) solution was prepared by dissolving 13.4 g of yeast nitrogen base in 100 mL of Milli-Q water and filtering it using a Pall 0.22 µm sterile syringe filter. The YNB solution was then added sterilely into the previous solution to produce the buffered complex medium. The buffered complex medium was then added with glycerol or methanol to obtain BMGY or BMMY agar medium. Zeocin antibiotic was added to a working concentration of 0.1 $mg.mL^{-1}$ if required before pouring them into the petri dish accordingly.

2.2.2 Buffered complex medium

The buffered complex medium used in this research was prepared by dissolving 20 g of peptone, 10 g of yeast extract, 11.813 g of potassium dihydrogen phosphate (KH_2PO_4), and 2.3 g of di-potassium hydrogen phosphate (K_2HPO_4) in 900 mL of milli-Q water. The medium was then sterilized using an autoclave. Then, the yeast nitrogen base (YNB) solution was prepared by dissolving 13.4 g of yeast nitrogen base in 100 mL of Milli-Q water and filtering it using a Pall 0.22 µm sterile syringe filter. Biotin (500x) solution was also prepared by dissolving 0.4 mg of biotin in 2 mL of Milli-Q water and filtering it using a Pall 0.22 um sterile syringe filter. The YNB and biotin solutions were added sterilely to the previous solution to produce the buffered complex medium and stored at 4 ^oC. Before use, glycerol or methanol was added to the buffered complex medium to make BMGY or BMMY medium.

2.2.3 Basal salts medium (BSM)

Invitrogen^{TM} Thermo Fisher Scientific developed the basal salt medium (BSM) recipe used in this research. The medium was prepared by dissolving 26.7 mL of 85% ($v.v^{-1}$) phosphoric acid (H_3PO_4), 0.93 g of calcium sulphate (Ca_2SO_4), 18.2 g of potassium sulphate (K_2SO_4), 14.9 g of magnesium sulphate heptahydrate ($MgSO_4.7H_2O$), 4.1 g of potassium hydroxide (KOH), and 40.0 g of glycerol per litre of BSM. The pH was then adjusted to 4.0 using 15% ($m.v^{-1}$) ammonium hydroxide and sterilized using an autoclave. 4.35 mL of Pichia trace metals (PTM_1) were added per litre of BSM before use.

PTM_1 solution was prepared by dissolving 6.0 g of cupric sulphate pentahydrate ($CuSO_4.5H_2O$), 0.08 g of sodium iodide (NaI), 3.0 g of manganese sulphate monohydrate ($M_nSO_4.H_2O$), 0.2 g of sodium molybdate dihydrate ($Na_2MoO_4.2H_2O$), 0.02 g of boric acid, 0.5 g of cobalt chloride ($CoCl_2$), 20.0 g of zinc chloride ($ZnCl_2$), 65.0 g of ferrous sulphate heptahydrate ($FeSO_4.7H_2O$), 0.2 g of biotin and 5.0 mL of sulfuric acid in one litre Milli-Q water. The PTM_1 solution was sterilized using Sartorius® Sartolab™ filtration system and stored at 4 ^oC for further use.

2.3 *K. phaffii* strain and working cell bank

A recombinant strain X-33 of the *K. phaffii* Mut^{+} strain, producing KEX2 protease, was provided and used in a cultivation process at PT Bio Farma, Bandung. This strain of *K. phaffii* will be used for further research in producing KEX2 protease to produce insulin by converting pro-insulin to insulin.

A plasmid with a sequence for 6x his-tagged KEX2 protease with α-MF has been stably transformed into *K. phaffii,* and the recombinant protein is predicted to be expressed extracellularly. After a recent investigation, the KEX2 protease was found to have been successfully expressed extracellularly. 6x his-tagged KEX2 protease without α-MF has a molecular weight of 90.8 kDa and a theoretical isoelectric point (pI) of 4.8.

```
        10         20         30         40         50         60
MKVRKYITLC FWWAFSTSAL VSSQQIPLKD HTSRQYFAVE SNETLSRLEE MHPNWKYEHD

        70         80         90        100        110        120
VRGLPNHYVF SKELLKLGKR SSLEELQGDN NDHILSVHDL FPRNDLFKRL PVPAPPMDSS

       130        140        150        160        170        180
LLPVKEAEDK LSINDPLFER QWHLVNPSFP GSDINVLDLW YNNITGAGVV AAIVDDGLDY

       190        200        210        220        230        240
ENEDLKDNFC AEGSWDFNDN TNLPKPRLSD DYHGTRCAGE IAAKKGNNFC GVGVGYNAKI

       250        260        270        280        290        300
SGIRILSGDI TTEDEAASLI YGLDVNDIYS CSWGPADDGR HLQGPSDLVK KALVKGVTEG

       310        320        330        340        350        360
RDSKGAIYVF ASGNGGTRGD NCNYDGYTNS IYSITIGAID HKDLHPPYSE GCSAVMAVTY

       370        380        390        400        410        420
SSGSGEYIHS SDINGRCSNS HGGTSAAAPL AAGVYTLLLE ANPNLTWRDV QYLSILSAVG

       430        440        450        460        470        480
LEKNADGDWR DSAMGKKYSH RYGFGKIDAH KLIEMSKTWE NVNAQTWFYL PTLYVSQSTN

       490        500        510        520        530        540
STEETLESVI TISEKSLQDA NFKRIEHVTV TVDIDTEIRG TTTVDLISPA GIISNLGVVR

       550        560        570        580        590        600
PRDVSSEGFK DWTFMSVAHW GENGVGDWKI KVKTTENGHR IDFHSWRLKL FGESIDSSKT

       610        620        630        640        650        660
ETFVFGNDKE EVEPAATEST VSQYSASSTS ISISATSTSS ISIGVETSAI PQTTTASTDP

       670        680        690        700        710        720
DSDPNTPKKL SSPRQAMHYF LTIFLIGATF LVLYFMFFMK SRRRIRRSRA ETYEFDIIDT

       730        740        750        760        770        780
DSEYDSTLDN GTSGITEPEE VEDFDFDLSD EDHLASLSSS ENGDAEHTID SVLTNENPFS
```

```
       790         800         810         820
DPIKQKFPND ANAESASNKL QELQPDVPPS FSGRSHHHHHH
```
Seq. 1 Amino acid sequence of KEX2 protease (Uniprot, N.A.)

Working cell banks were made using the recombinant strain X-33 of the *K. phaffii* Mut^{+} strain, producing KEX2 protease by thawing frozen cells and culturing them in one baffled shake flask with one litre of 150 mL BMGY. The flask was cultured in an incubator at 30 ^{o}C and agitated at 250 RPM. The cell culture's optical density at 600 nm (OD_{600}) was observed and recorded by a Genesys 10S UV-VIS spectrophotometer (Thermo Fisher Scientific, Cramlington, UK). The culture grew for over 20 hours until the OD_{600} reached above 15. Then, it was harvested into 50 mL centrifuge tubes (Thermo Fisher Scientific, Cramlington, UK) and centrifuged at 4000 RPM for 10 mins using an SL 40R benchtop centrifuge (Thermo Fisher Scientific, Cramlington, UK). After the supernatant was discarded, the cell pellet was re-suspended in sterilized BMGY with a glycerol concentration of 30% ($v.v^{-1}$) and aliquoted into 2 mL cryotubes for long-term storage at -80 ^{o}C.

2.4 Cell culture methods

The cell culture methods that were used in this research ranges from agar plates, 1 L baffled shake flasks and bioreactor at lab scale with total volume of 5 L.

2.4.1 Petri dish or agar plates

The *K. phaffii c*ells were grown on agar plates with glycerol, sucrose, or methanol as a carbon source. First, 20 mL of buffered complex medium was supplemented with 1% ($v.v^{-1}$) glycerol or methanol and poured into a 10 cm diameter petri dish. After the agar plates were inoculated using the four-quadrant streak method, they were cultured at 30 ^{o}C for 48 hours in an incubator.

2.4.2 Shake flask culture

The *K. phaffii c*ells were grown on glycerol for adaptation and growth or methanol for expression as a carbon source in baffled shake flasks. 100 mL of buffered complex medium was added to a 1000 mL baffled shake flask and supplemented with 1% ($v.v^{-1}$) glycerol or methanol. After flasks were inoculated to obtain a starting OD_{600} of 0.6 ±0.3, they were cultured at 30 ^{o}C and agitated at 250 RPM for 24, 48, and 72 hours in an incubator. The OD_{600} of cell culture was measured every 24 hours using a Genesys 10S UV-VIS spectrophotometer.

2.4.3 bioreactor culture (Fed-batch)

The fed-batch cultivation used Sartorius 5000 mL benchtop fermenter with Sartorius B plus tower (Germany, Göttingen). The system consists of fermenter vessels, acid, base, antifoam, and induction solution feed bottles with their respective pumps. The fermenter uses air as its main source of oxygen, and two 6-bladed Rushton impellers were used to meet oxygen demands.

The fed-batch cultivation process was based on the Invitrogen™ *K. phaffii* cultivation process (Invitrogen, 2013). The preparation of the fermenter started by calibrating the pH probes before they were installed. First, they were calibrated using pH buffer solutions at pH 4 and 7 in successive order. Then, the pH and oxygen probes were installed on the fermenter cap. Next, 2000 mL of BSM medium was added to the vessel, which was then closed and sterilized using an autoclave. The vessel was then put back onto the laminar airflow unit. The probes (pH probe, oxygen probe, temperature probe, and antifoam probe) attached to the vessel were connected to the Sartorius tower system for at least 8 hours before use. When the dissolved oxygen (DO) concentration reading stabilized, the oxygen probe was calibrated using the current oxygen reading of 100%, and the pH was adjusted to 5.0 using 12.5% ($v.v^{-1}$) ammonium hydroxide. Lastly, 2.5 mL of sterilized PTM_1 solution was added to the vessel before inoculation.

The working cell bank was grown in one baffled flask with 150 mL of sterile BMGY medium to make the cells used for inoculation. It was then incubated at 30 ^{o}C and 250 RPM for 18-24 hours. An initial OD_{600} target of 1.0 was used to ensure a similar starting point. Throughout the fed-batch cultivation process, the pH was maintained at 5.0 using 12.5% ($m.v^{-1}$) ammonium hydroxide, while the DO was kept above 30% by cascading with agitation speed and oxygen concentration. For instance, the agitation would go from 300 RPM to 1000 RPM to ensure that the amount of oxygen in the medium is above 30%.

At the start of cultivation, the *K. phaffii* cells were inoculated into the fermenter and grew in batch mode using the glycerol inside the BSM. There would be a DOC spike when the glycerol inside the medium was consumed. Therefore, at the start of the glycerol fed-batch phase, 50% ($w.v^{-1}$) glycerol + PTM1 was fed at 36 $mL.h^{-1}$ until the culture's OD_{600} reached ~100 and then stopped. After stopping

the glycerol, the cells were starved for at least 15-30 minutes to ensure that all the glycerol inside the fermenter had been consumed. Then, the induction solution was fed into the fermenter using either pure methanol or a mixture of methanol and sorbitol (Wang, 2019). To help the cells adapt to the new carbon source, the induction solution was initially fed at a rate of 7 $mL.h^{-1}$ for one hour, then increased and kept at 14 $mL.h^{-1}$ for two hours afterward, and finally increased and kept at 21 $mL.h^{-1}$ until the end of the cultivation process. The cell broth was harvested through a sampling port and centrifuged at 4000 RPM, 8 ^{o}C, and 30 minutes to separate the pellets and the medium. The samples were then stored at -20 ^{o}C for further use.

Throughout the cultivation process, samples were taken regularly and analysed by recording their optical density at 600 nm and using the analytical methods mentioned in chapter 2.6.

2.4.4 bioreactor culture (Continuous)

The continuous cultivation used a Sartorius 5000 mL benchtop fermenter with a Biostat B plus tower (Germany, Göttingen). The system consists of a fermenter vessel, acid, base, antifoam, an induction solution feed bottle, a fresh medium without glycerol feed bottle, and harvest bottles with their respective pumps. The fermenter uses air as its primary source of oxygen, and two 6-bladed Rushton impellers were used to meet oxygen demands.

The continuous cultivation process was based on the fed-batch cultivation used in the Invitrogen™ *K. phaffii* cultivation process (Invitrogen, 2013). The preparation of the fermenter started by calibrating the pH probes before they were installed. They were calibrated using pH buffer solutions at pH 4 and 7 in successive order. Then, the pH probe and the oxygen probe were installed on the fermenter cap. Next, 2000 mL of BSM were filled into the vessel, which were then closed and sterilized using an autoclave. The vessel was then put back onto the laminar airflow unit. All the probes (pH probe, oxygen probe, temperature probe, and antifoam probe) attached to the vessel were connected onto the sartorius tower system for at least 8 hours before use. When the dissolved oxygen (DO) concentration reading stabilized, the oxygen probe was calibrated using the current oxygen reading of 100%, and the pH was adjusted to 5.0 using

12.5% ($v.v^{-1}$) ammonium hydroxide. Lastly, 2.5 mL of sterilized PTM_1 solution was added to the vessel before inoculation.

The continuous cultivation process started similarly to the fed-batch process. However, the process started to diverge after the induction phase continued for 24 hours. After 24 hours of induction, the fresh BSM + Antifoam 0.5% $v.v^{-1}$ without glycerol and PTM_1 was fed into the fermenter, while the broth inside the fermenter was harvested simultaneously and continuously according to the dilution rate and current volume of broth. The dilution rate was crucial in ensuring that the chemostat condition was established and wash out did not occur. The cell broth was harvested daily during the continuous phase was kept below 8 ^oC. At the end of the cultivation process, the broth inside the fermenter would be harvested too. The harvested cell broth was centrifuged at 4000 RPM, 8 ^oC, and 30 minutes to separate the pellets and the medium/ supernatant. The supernatant samples were then stored at -20 ^oC for further use.

Throughout the cultivation process, samples were taken regularly and analysed by recording their optical density at 600 nm and using the analytical methods mentioned in chapter 2.6.

2.5 Oxygen transfer coefficient characterization

Oxygen transfer coefficient (k_La) of the sartorius 5 L vessels were measured using the static method (Garcia-Ochoa & Gomez, 2009). DO probe was calibrated between 0% and 100% using nitrogen and air flow. 2000 mL BSM medium was added into each vessel and temperature was set at 30 ^oC. Nitrogen flow was initially introduced into the vessels until dissolved oxygen dropped to 0%. Oxygen was then sparged into the vessels at the rate of 1vvm and DO reading was recorded every 5 seconds until it reached 100%. k_La values of bioreactor were calculated at agitation speeds of 100, 500, and 1000 RPM, respectively.

2.6 Analytical methods

The analytical methods were used to measure, verify, and quantify the results of the cultivation process. The samples used were mainly the supernatants of the broth harvested from cultivation at a specified time after induction, the cells themselves at a specified time after induction, or the purified target product through the 1-step purification process of affinity chromatography through the use of the his-tagged KEX2 protease.

2.6.1 Cell Density measurement

Throughout the cultivation process, OD_{600}, wet cell weight (WCW) and dry cell weight (DCW) were measured by the process of sampling.

OD_{600} of the samples were measured by diluting the samples using Milli-Q water when needed and loaded into 1 mL disposable cuvette and read for absorbance measurement at 600nm by Genesys 10S UV-VIS spectrophotometer. Three readings were done for each individually diluted sample to ensure the accuracy of the readings.

Dry cell weight (DCW) and wet cell weight (WCW) were measured by weighing empty 15 mL falcon tubes before sample loading. 5 mL of samples were pipetted into tubes and centrifuged at 4000 RPM for 10 minutes using an Thermoscientific SL 40R benchtop centrifuge. after the supernatant was removed, the tube with the wet cells were weighted again and recorded as the wet cells with tube to be further analysed for WCW. Then, the wet cells were dried at 120 ^{o}C for 20-45 minutes using the KERN moisture balance analyser which the result was then recorded as the dry cells to be further analysed for DCW.

The equation for wet cell weight (WCW) can be simplified to equation 2.1 (Wang et al., 2019).

$$\text{WCW}\ (\text{g}.L^{-1}) = \frac{\left(\text{wet cells with tube}\ (\text{g}) - empty\ tube\ (\text{g})\right)}{\text{Volume}\ of\ sample(L)} \qquad \text{Eq2.1}$$

where WCW is the concentration of cells after being separated with the medium (supernatant) before being dried. While the equation for dry cell weight (DCW) which was done using the KERN moisture analyser can be simplified to equation 2.2.

$$\text{DCW}\ (\text{g}.L^{-1}) = \frac{dry\ cells\ (g)}{\text{Volume}\ of\ sample(L)} \qquad \text{Eq2.2}$$

where the DCW is the concentration of cells after being dried.

2.6.2 Specific growth rate calculation

The specific growth rate (μ) of the *Komagataella phaffii* is the rate of increase of biomass of a cell population per unit of biomass concentration. In this thesis, it was calculated using equation 2.3 (Rahimi et al., 2019).

$$\mu\ (h^{-1}) = \frac{\ln\ (V_2.\text{x}_2) - \ln\ (V_1.\text{x}_1)}{\text{t}_2 - \text{t}_1} \qquad \text{Eq2.3}$$

where x_2 and x_1 refer to the biomass per dry cell weight (DCW) at sampling time of t_2 and t_1 at their respective specified volume V_1 and V_2. The cultivation process that was used in this chapter is a fed-batch cultivation process. The continuous feeding process of glycerol and methanol increases the volume of the broth inside the fermenter throughout the cultivation process. Thus, for the calculation of the specific growth rate, the change of volume was also taken into account with their respective biomass and time at which the sample was taken. The values of μ was set to be shown in three significant figures.

2.6.3 Dilution rate and retention time

Based on an equation for a continuous stirred tank reactor, dilution rate was calculated using equation 2.4.

$$\text{D}\ (h^{-1}) = \frac{Q_C\ (mL.h^{-1})}{V_C\ (mL)} \qquad \text{Eq2.4}$$

Where D (h^{-1}) is the dilution rate, Q_C ($mL.h^{-1}$) is the current flow rate of harvest or total volume being fed in (when the volume inside the bioreactor was kept constant), and V_C (mL) is the current volume inside the bioreactor which was being kept constant throughout the process after shifting to continuous phase.

While the retention time was calculated using equation 2.5.

$$\text{RT}\ (h) = \frac{V_C\ (mL)}{Q_C\ (mL.h^{-1})} \qquad \text{Eq2.5}$$

Where RT (h) is the retention time, which were the time it took to entirely exchange the volume of the bioreactor, Q_C (mL.h^{-1}) is the current harvest rate which is the same as the harvest rate or total feed rate into the bioreactor, and V_C (mL) is the current volume inside the bioreactor which was being kept constant throughout the process after shifting to continuous phase.

2.6.4 Harvest rate and feed rate of substrates

In a chemostat continuous cultivation process, dilution rate is equal to the steady state of specific growth rate, which can be depicted into equation 2.6 (Fernandes et al., 2015)

$$\mu\ (h^{-1}) = D\ (h^{-1}) \qquad \text{Eq2.6}$$

Where μ is the specific growth rate and D is the dilution rate.

For the initial calculations of the harvest rate, induction solution feed rate, fresh medium feed, and base feed rate for continuous cultivation, the equation can be simplified into the equation 2.7 to 2.9.

$$Harvest\ Rate\ or\ Q_C(mL.h^{-1}) = D\ (h^{-1}).V_C\ (mL) \qquad \text{Eq2.7}$$

$$Flow\ out\ (mL.h^{-1}) = Flow\ in\ (mL.h^{-1}) \qquad \text{Eq2.8}$$

$$\begin{aligned} Harvest\ Rate\ (mL.h^{-1}) \\ &= Induction\ feed\ rate\ (mL.h^{-1}) \\ &+ Fresh\ medium\ feed\ rate\ (mL.h^{-1}) \\ &+ Acid\ and\ Base\ feed\ rate\ (mL.h^{-1}) \end{aligned} \qquad \text{Eq2.9}$$

Where the harvest rate is the rate at which the broth is taken out of the fermenter (mL.h^{-1}), D is the dilution rate (h^{-1}), and V_c is the current volume of the fermenter

when the transition to continuous phase occurred. For a chemostat process, flow out (harvest rate) must be equal to the total of flow in, to ensure the volume of broth inside the bioreactor is constant (Zhang et al., 2004). The flow in consisted of induction feed rate, which was the amount of methanol or 50%C-mol methanol and sorbitol feed rate ($mL.h^{-1}$), the fresh medium feed rate, which was the rate of the fresh medium without carbon source and PTM_1, and the acid and base feed rate, which was the average hourly feed rate ($mL.h^{-1}$) of NH_4OH and H_3PO_4.

2.6.5 Total soluble protein quantification

The total soluble protein content of both the methanol induction and the mixed induction cultivation process for both fed batch and continuous process were found using Pierce™ bicinchoninic acid (BCA) protein assay kit (Thermoscientific). Pierce™ BCA Protein Assay Kit includes the bovine serum albumin (BSA) standard and BCA reagent A and reagent B. The BSA standard solution were prepared by diluting the BSA standard in phosphate buffer saline (PBS) to concentrations of 2, 1.5, 1, 0.75, 0.5, 0.25, 0.125, 0.025, and 0 $mg.mL^{-1}$, with pure PBS for the concentration of 0 $mg.mL^{-1}$. The samples that were used in the BCA assay were the supernatant of the medium after the cells were separated using centrifuge because the target protein was predicted to be excreted into the medium.

96 wells plate with flat bottom were used and each well was firstly pipetted with 25 µl BSA standard protein or sample and then added with 200 µl BCA solution which is a mix of reagent A and reagent B at a ratio of 50:1. After the plate was incubated at 37 ^oC for 30 minutes, absorbance was measured at 562 nm by a microplate reader.

2.6.6 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The samples that were used for SDS PAGE analysis were the supernatant after separating the cells (pellets) and the medium (supernatant). The supernatant was mixed with SDS Sample loading buffer 4x in 3: 1 ratio and heated at 95 ^oC for 10 minutes. Samples were then spun down at 8000 RPM for 8 seconds to cool down and ensure any heated-up liquid were collected at the bottom of the tube. Any kD™ Mini-PROTEAN® TGX™ Precast Protein Gels (Biorad, California, USA) was set up in a gel tank and was then immersed in running buffer solution at 1x

concentration. PageRuler Prestained ladder standard (Thermo Fisher Scientific, Cramlington, UK) was used as the standard protein ladder. 3 µl of the ladder or 10 µl samples mixed with loading buffer was loaded into each well of the gel. Electrophoresis process of the SDS gel was performed at a constant voltage of 120 V for 80 minutes using Bio-rad Powerpack Basic (Bio-rad, Watford, UK).

After the electrophoresis process, the gel was then taken out of the gel tank and cast, and then was immersed in 50 mL Coomassie blue staining solution (Thermoscientific, Poole, UK) and shaken gently overnight for staining. The staining solution was then discarded, and the gel was destained and washed using destaining solution for four times for 30 minutes each, which consists of Milli-Q water, methanol, and glacial acetic acid at a ratio of 8:1:1. Picture of the protein bands were then captured using biostep ViewPix 700 after destaining the gel.

2.6.7 Western Blot

This process was done on the SDS PAGE gels after the electrophoresis step. After the gel was taken out of the gel tank and cast, it was immersed in running before transferring the samples from the gel into the membrane using iBlot® and iBlot® gel transfer stacks nitrocellulose, Mini kit (Invitrogen, Israel, Kiryat Shmona). The transfer process was done in dry transfer process which lasts 7 minutes.

After the transfer process, the membrane was taken out and immersed in 25 mL 5% skim milk TBST 0.1% (tris buffer saline with tween20 0.1%) for 16 hours or an overnight at 4-8 ^{o}C and shaken at 40 RPM using Stuart roller mixer SRTD6. Then, the membrane was washed using TBS for 3 minutes at lab temperature once after the blocking solution was decanted. Then, the primary antibody in 2% skim milk TBST 0.1% was poured into the membrane for 2 hours at 4-8 ^{o}C and shaken at 40 RPM.

for a direct antibody method, after decanting the primary antibody, the membrane was washed using TBST 0.1% for 3 minutes x 3 at lab temperature. Then the substrate HRP was added to reveal the bands that was compatible with the antibody. Lastly, the membrane was washed using milli-Q water, dried and have the image captured using biostep ViewPix 700.

for an indirect antibody method, after decanting the primary antibody, the membrane was washed using TBST 0.1% for 3 minutes x 3 at lab temperature. Then, the secondary antibody in 2% skim milk TBST 0.1% was poured into the membrane and shaken for 1 hour at 4-8 ^{o}C and 40 RPM. Afterward, the secondary antibody was decanted, and the membrane was washed using TBST 0.1% for 3 minutes x 3 at lab temperature. Then the substrate BCIP/NBT was added to reveal the bands that was compatible with the antibody. Lastly, the membrane was washed using milli-Q water, dried and have the image captured using biostep ViewPix 700.

2.6.8 Determination of methanol, glycerol, and sorbitol

Methanol concentration of the supernatant samples was determined using Agilent systems GC/MS (Agilent, Santa Clara, USA) with a minimum reading of 7-10 ppm of methanol. A standard with known concentration was used to calibrate and verify the results based on the same condition of the process. A semi-polar column DB 624 was benchmarked with nitrogen gas at constant flow rate of 30 $cm^3.s^{-1}$ before use to detect methanol. After 1 µl samples were loaded manually and helium gas was used as the carrier at 30 $cm^3.s^{-1}$, with the temperature of the oven set at 40 ^{o}C for 5 minutes, then increased the temperature at 10 $^{o}C.min^{-1}$ until it reached 260 ^{o}C and held at that point for 3 minutes. The output was detected by FID Detector (Agilent, Santa Clara, USA) set at 300 ^{o}C.

Sorbitol and glycerol concentrations were determined using UltiMate 3000 HPLC (Thermo Fisher Scientific, Cramlington, UK) with Aminex HPX-87h column (Bio-rad Laboratories In., Watford, UK). The column was balanced with 0.5% ($v.v^{-1}$) trifluoroacetic acid at constant flow rate of 0.6 $mL.h^{-1}$ before use. After 20µl samples were loaded through the column for 30 minutes, the output was detected by Thermo Scientific™ RefractoMax 520 Refractive Index Detector (Thermo Fisher Scientific, Cramlington, UK) at 55 ^{o}C (Parpinello and Versari, 2000).

2.6.9 Cell size measurement

Cell size was measured by using microscope Olympus CX31 (Olympus corporation, Tokyo, Japan). After the fresh samples were prepared on glass slides and covered by using cover glass, immersion oil was dropped on top of the cover glass and an image of the sample was captured at 10,000x magnification.

The fresh samples were captured for all samples for each of the cultivation types, after 18, 24, 48, and 72 hours of inoculation for fed-batch cultivation, and after 18, 24, 48, 168, and 290 hours after inoculation for continuous cultivation. The size of the cells was measured using the calibrated measuring ruler in the capturing program.

2.6.10 KEX2 protease content, yield and productivity quantification

The content and volumetric yield of KEX2 protease was quantified by using image analysis software Totallab Quant version 13.2 (TotalLab, Newcastle, UK) on the results acquired from SDS PAGE and western blot following the instruction manual from the manufacturer. After the picture of the gel with the protein bands were captured using biostep ViewPix 700, the lanes were then assigned, the background were normalized using the rolling method at 800, and the bands were detected automatically at the recommended settings of the program. The size of the bands was quantified by comparing the target bands with the bands in the lane containing a standard protein with known size values (Thermo Scientific Pageruler) with a size between 180 kDa and 10 kDa. The content of the bands was quantified by using a known concentration of bovine serum albumin (BSA) in the same gel as the samples.

Volumetric yield calculation was done by using the total soluble protein (TSP) acquired from Bicinchoninic Acid (BCA) assay to acquire the total soluble protein in the supernatant of the broth after cell separation process, and from the densitometry assay analysis from the scanned SDS PAGE result. The densitometer reading was analysed to acquire the content of the target protein by comparing the target band in comparison with the total number of bands in the same lane where the target band is known to be purely target protein based on previous SDS PAGE and Western blot results. Then the volumetric yield and specific yield was simplified to equation 2.10 and equation 2.11.

$$Volumetric\ Yield\ (mg.L^{-1}) = \%\text{Content}\ (\%\text{band}).\text{TSP}\ (mg.L^{-1}) \quad \text{Eq2.10}$$

$$Specific\ Yield\ (mg.gDCW^{-1}) = \frac{\%Content\ (\%band).TSP\ (mg.L^{-1})}{DCW\ (g.L^{-1})} \quad \text{Eq2.11}$$

Where the volumetric yield (mg.L^{-1}) is the concentration of target protein in mg of target protein per mL of supernatant of the broth at specified time acquired after separating the cells and the specific yield (mg.gDCW^{-1}) is in mg of target protein per gram of biomass in DCW. The %Content is the percentage of band (%band) of the target product acquired inside the supernatant of the broth sample from densitometer analysis. The TSP is the total soluble protein (mg.L^{-1}) in the supernatant of the broth taken at specified time acquired through the total protein concentration quantification process. The DCW is the dry cell weight concentration (g.L^{-1}) acquired from cell density measurement.

The specific volumetric productivity of the continuous process can then be calculated by using the volumetric yield and dilution rate to produce equation 2.12.

$$Specific\ Volumetric\ Productivity\ (mg.L^{-1}.h^{-1}) = \text{Average Continuous Volumetric Yield}\ (mg.L^{-1}).\mathrm{D}\ (h^{-1}) \quad \text{Eq2.12}$$

Where the specific volumetric productivity (mg.L^{-1}.h^{-1}) is the concentration of the target product being harvested in one hour of continuous process, the average continuous volumetric yield is the acquired average volumetric yield (mg.L^{-1}) during the continuous process, and D (h^{-1}) is the dilution rate used during the continuous phase.

2.6.11 Analytical purification

KEX2 samples were analytically purified by using AKTApurifier (GE healthcare, Chicago, USA) and XK50 column containing Ni-NTA resin Ni Sepharose® High performance (Cytiva, Massachusetts, USA). The sample was prepared before injection into the column by having the supernatant of the centrifuged sample filtered using 0.45um sartolab to ensure no cells or cell debris were retained and then diluted to have a total soluble concentration of the sample at 500 ug.mL^{-1}.

The target sample were eluted using 80 mM of imidazole and the purified sample was stored at -20 ^{o}C.

2.6.12 Techno-Economic Analysis

The calculations for the techno-economic analysis were done based on the initial calculations on the lab scale of the process, which were then used in the simulation of the whole upstream process that was done using the SuperPro Designer® simulation tool on the production scale. SuperPro Designer® version 12, Build 3, Special Build 1100, is a tool for simulation, design, and scheduling for the process manufacturing industries by Intelligen, Inc. (New Jersey, US).

The costs to be calculated would be separated into capital and operating costs. The capital costs would be further separated into land, patent, equipment, and laboratory creation costs. In contrast, operating costs would be separated into labour, utility, and material. The material costs were calculated by multiplying the amount of medium/ solution used for the process from the subculture to the end of the cultivation process in litres (L) with the price of the solutions acquired in USD$ in July 2022. This is shown as equation 2.13.

$$Medium\ Cost\ (\$USD) = \text{Price of medium } (\$\text{USD. L}^{-1}) \text{ x Volume of medium used } (L) \quad \text{Eq2.13}$$

The overall costs prediction would be calculated in terms of ratio, with the base method chosen set as 100% of the costs and the modified/ developed method cost to make an increase or decrease from the 100% cost based on the changes made for comparison. For example, if the other method increased the equipment cost as it required additional pump, the equipment cost in the other method would increase to more than 100% of the base cost in accordance to the set cost of the pump in terms of the overall percentage cost. This is simplified to equation 2.14.

$$Modified\ method\ cost\ (\%) = \text{Base method cost } (100\%) + \text{Change cost } (\%) \quad \text{Eq2.14}$$

The change cost (%) would be based on weighted cost (%) and previous studies done on the modification to the base method.

The total price of KEX2 per batch would also be calculated using the equation 2.15.

$$Total\ Price\ of\ KEX2\ \ (USD\$) = \text{KEX2 Content (g) x KEX2 price } (USD\$.g^{-1}) \quad \text{Eq2.15}$$

Chapter 3: Production of KEX2 protease in fed-batch system using mixed induction strategy

3.1 Introduction

In this chapter, the aims were as follows:

1) To check the cell characteristics of the cell line producing KEX2 protease and small-scale expression of KEX2 protease as the target protein.
2) To study the impact of sorbitol and methanol mixed induction on cell growth and the condition of the cells at the bioreactor scale.
3) To compare volumetric yields in pure methanol and sorbitol and methanol mixed induction strategies and determine the harvest time of fermentation.
4) To acquire the fed-batch cultivation parameters and product yield in one batch to be compared in the next chapter.

Methanol is the primary induction solution for *K. phaffii* with an AOX promoter. It is known for its tightly regulated expression pathway, which results in a naturally strong expression level (Kim et al., 2015). However, the downsides of using methanol are apparent as it will require a high volume and concentration of methanol, which will incur high production costs and is a highly flammable material.

Sorbitol can be used as a carbon source and an alternative feeding strategy by co-feeding sorbitol or a mixed-mode strategy (Çelik et al., 2009). Sorbitol is a non-volatile and non-repressive carbon source. Combined with methanol, it can reduce the amount of methanol needed and handled during the fermentation process. Thus, this mixed-mode strategy is thought to be a method of choice for addressing the drawbacks of methanol utilization for *K. phaffii*. In addition, implementing the strategy can increase the scalability of the fermentation as it will reduce oxygen uptake and risks of methanol storage and handling, a hazardous material.

In a previous study by Wang et al. (2019) and Woodhouse S. (2015), the mixed induction strategy was further studied in terms of its effectiveness and efficiency in detail, with aprotinin as its target product. It was shown that by mixing sorbitol with methanol, the amount of cooling and oxygen needed throughout the process

and the amount of HCP produced lessened and improved the overall cultivation process. Furthermore, the ratio of sorbitol and methanol was also investigated, and the effect of the ratios was assessed, indicating that methanol to a co-substrate ratio of equal or lower than 4:6 in the induction solution may lower the volumetric yield significantly (Berrios et al., 2017; Wang, 2019).

Moreover, several studies have evaluated volumetric yield as the effect of using a mixed induction strategy using sorbitol and methanol. The results vary depending on the strain used and the target product being produced. For example, a study by Çelik et al. (2009) showed that using sorbitol as a co-substrate instead of just pure methanol increased the volumetric yield of recombinant human erythropoietin from 80 $mg.L^{-1}$ to 130 $mg.L^{-1}$ after 24 hours of induction. However, another study done by Wang et al. (2019) on using sorbitol as a co-substrate was also found to decrease the volumetric yield of aprotinin by almost 40% compared to its pure methanol induction counterpart. Thus, the volumetric yield results in using sorbitol as a co-substrate were found to have varying results according to the cell lines and target product.

In this study, to better understand the effect of sorbitol and methanol mixed induction strategy in fed-batch cultivation methodology, KEX2 protease was used as a target protein model. Thus, a method of quantifying KEX2 protease was developed and produced content could be quantified. Furthermore, the impact of the feeding induction strategy and mode of cultivation on cell growth, cell condition, and volumetric yield were studied.

3.2 Results and discussion

In this chapter, the strain of *K. phaffii* seed and the target product, KEX2 protease protein, were verified in a small-scale cultivation process. Then the process was upscaled to a 5 L bioreactor, and a mixed induction strategy of a 50% C-mol or 1:1 C-mol sorbitol to methanol ratio was implemented (calculation shown in the Appendices A). The results acquired from the bioreactor were then studied and compared.

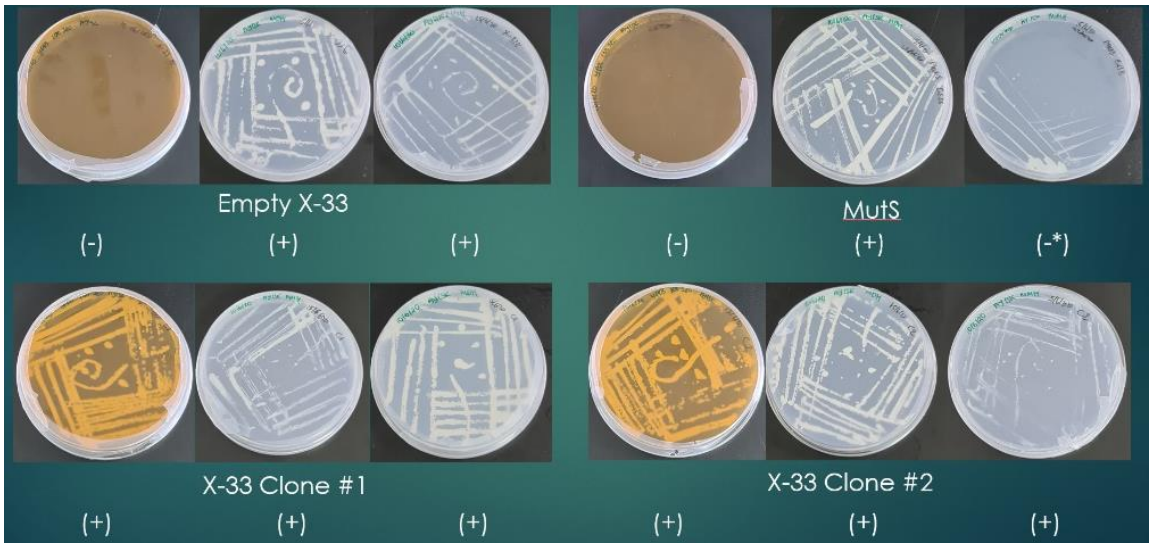
3.2.1 KEX2 protease gene insertion and strain check

An essential initial step in protein expression using *K. phaffii* is to find the characteristics of seed cells carrying the gene that encodes KEX2 protease. The characterization of the seed determined the type of medium and protocol used

during the scaling up of the cultivation process. The seed used in this study was presented to have the characteristics of a Mut^+ strain by the report from Protein Expression Facility (PEF) in Queensland and have a resistance towards zeocin as an indicator of whether the gene insertion expresses and excretes KEX2 protease (gene insertion proof shown in Appendices B). Initially, there are ten clones to be chosen. From the small-scale expression results of the clones acquired from the report from Queensland, Clone #1 and Clone #2 showed the most promising expression with the least number of impurities being expressed in the medium (data not shown) and thus will be chosen as the seed to be re-verified and compared in terms of colony characterisation and small-scale expression.

According to a protocol developed by Invitrogen (User Manual EasySelect TM Pichia Expression Kit for Expression of Recombinant Proteins Using PPICZ and PPICZα in *Pichia pastoris*, 2010), the *K. phaffii* seed can be characterized by growing the seed in an agar plate medium with minimum nutrients. An empty X-33 strain of *K. phaffii* (also known as the wild type or a strain before being genetically modified), which was the same as what was used in the creation of the strain producing KEX2 protease, and a *K. phaffii* strain with a known slow methanol utilization (Mut^S) type of methanol utilization (further mentioned as strain B) were used as a benchmark in conjunction with two clones of *K. phaffii* containing a plasmid with a sequence for 6x his-tagged KEX2 protease. The agar plate media used in this experiment were: YPD with Zeocin 100 $\mu g.mL^{-1}$, Minimum Dextrose Histidine, and Minimum Methanol Histidine. The seed was grown in the respective media on agar plates at 30 ^oC for 48 hours.

Figure 3-1 Agar plates with seeds of a wild type X-33 which were used for the gene insertion (top left panel), a seed containing a strain with MutS characteristic but without zeocin resistant gene (top right panel) and two clones of X-33: clone #1 (bottom left panel) and Clone #2 (bottom right panel) with KEX2 protease expression gene inserted. The three media agar used from left to right were YPD with Zeocin 100 ug.mL^{-1} agar medium, minimum dextrose with histidine agar medium, and lastly minimum methanol with histidine agar medium.



The results in Figure 3-1 show that the wild type and strain B did not grow in YPD with Zeocin 100 µg.mL^{-1} agar medium as they did not contain genetic material resistant to Zeocin. In contrast, the clones containing the KEX2 protease expression gene could grow because they were resistant to zeocin. Therefore, YPD with 100 µg.mL^{-1} Zeocin Agar medium also verified the success of the insertion of the gene expressing KEX2 protease. Thus, the gene insertion was successful.

The second agar medium was a minimal medium with dextrose as a source of carbon and histidine to help the bacteria grow as well as possible. Because there is no limitation, such as an antibiotic or a specific carbon source, all cultures could grow. The third agar medium contained methanol as a carbon source. This medium separated the methanol utilization type of the applied seed or the capability of the seed to use methanol as a carbon source. A wild-type X-33 strain was naturally capable of using methanol as a carbon source and still had both *AOX1* and *AOX2* promoters to metabolize the methanol. Similar growth can be seen for the two clones containing the KEX2 protease expression gene. However, although strain B could grow on methanol as a carbon source as well, it grew

much slower when compared to the others. Thus, it was shown that the clones containing the KEX2 protease expression gene were successfully inserted and were confirmed as a positive methanol utilization (Mut^+) strain.

3.2.2 Cultivation at small scale and verification of KEX2 protease expression

To confirm the expression of the target protein, small-scale cultivation of *K. phaffii* was done in 1 L baffled Erlenmeyer using a 100 mL complex medium with 1% glycerol (BMGY) for negative control or 1% methanol (BMMY) for expression and grown for 72 days at 30 ^oC at 250 RPM with two duplicates each. The process of the small-scale expression was based on the established protocol by Invitrogen (User Manual EasySelect TM Pichia Expression Kit for Expression of Recombinant Proteins Using PPICZ and PPICZα in Pichia pastoris, 2010). Since the design of the seed was made to excrete the KEX2 protease into the medium, the sample's supernatant was taken and analysed using SDS PAGE Coomassie blue and the western blot process. The ladders used in the SDS PAGE results were benchmark prestained protein ladder (10 to 190 kDa) by Invitrogen and Pageruler^{TM} prestained protein ladder (10 to 180 kDa) by Thermo Fisher.

Figure 3-2A shows that the wild-type strain, negative control medium, and KEX2 clone produced no protein target before induction. However, the target protein was expressed in the KEX2 clones that contain the protein expression genes after methanol induction, as indicated by species of protein bands with molecular weights between 82 – 115 kDa. According to the Saccharomyces database (KEX2 Protein | SGD, 2022), the size of the KEX2 protease protein is 90.8 kDA with a sequence length of 814. In this study, the sequence length of KEX2 protease is 813. This result shows that the size of KEX2 protease expressed by the induced seed is consistent with KEX2 protease in the databank.

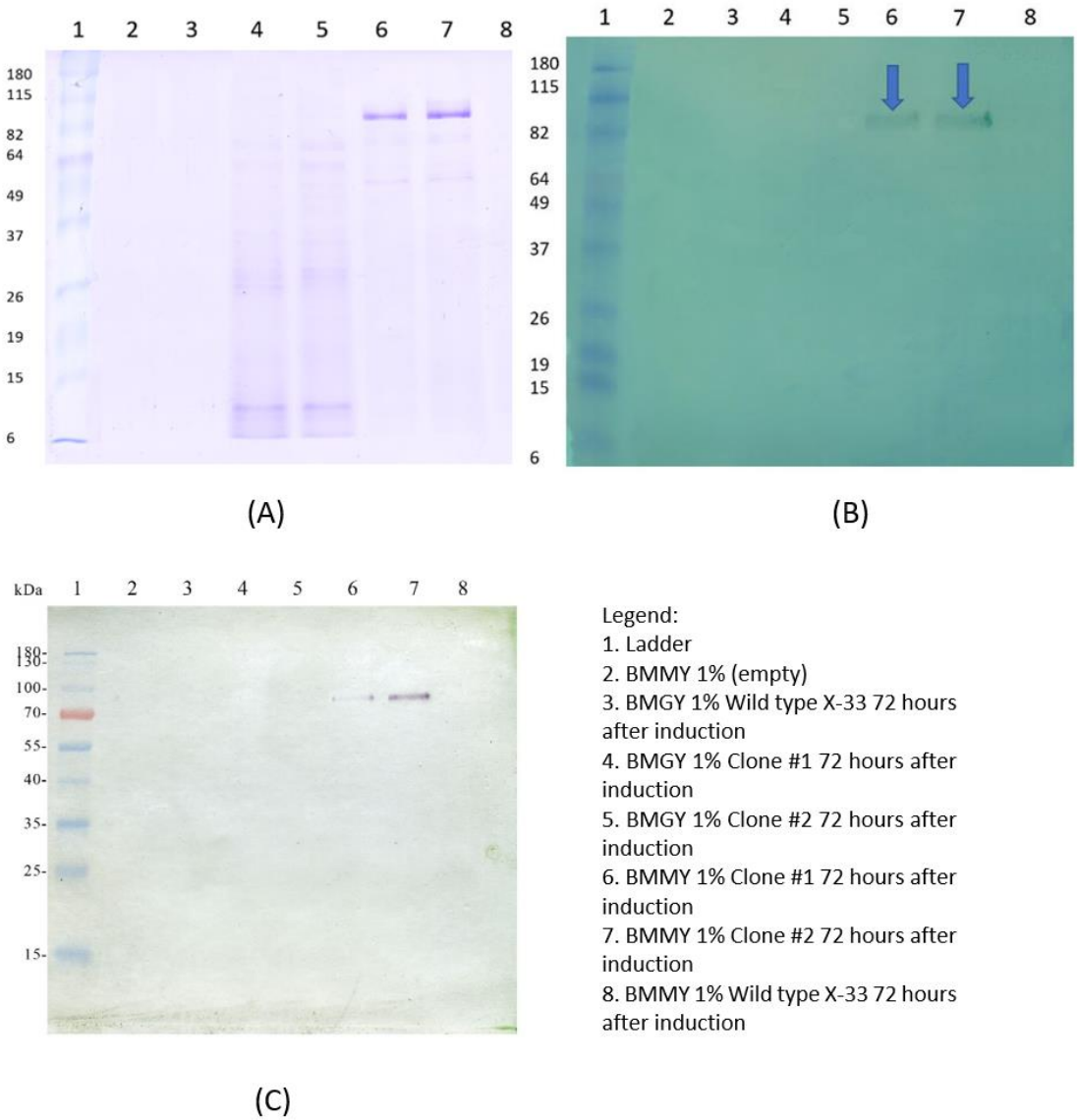


Figure 3-2 Characterisation of KEX2 protease expressed in small-scale expression process using either 1% glycerol complex buffer (BMGY) for negative control or 1% methanol complex buffer (BMMY). The protein was analysed by SDS PAGE Coomassie blue (A) and western blot with histag antibody (B), and KEX2 protease antibody (C).

To further verify the identity of KEX2 protease, western blot analysis was performed using the histidine tag antibody and the KEX2 antibody shown in Figures 3-2B and 3-2C. The results show that the target band with molecular weight between 82 – 115 kDa when a prestained benchmark protein ladder was used was recognized by the histidine tag antibody, and a target band with molecular weight between 70 and 100 kDa when the Pageruler^{TM} prestained protein ladder was used was recognized by the KEX2 antibody. Furthermore, based on the intensity of the target band, clone #2 exhibited a more intense band than clone #1, which depicted the capability of the cell to express the protein. Therefore, clone two was chosen for scaling up in a bioreactor.

3.2.3 Fed-batch cultivation at bioreactor scale

Pure methanol and sorbitol and methanol mixed induction strategies were implemented and compared at a bioreactor scale. The fed-batch cultivation was done using the procedure recommended by Invitrogen (Invitrogen, 2013). Cells were grown on 4% ($m.v^{-1}$) glycerol in the medium. After the glycerol in the medium was consumed, 50% ($m.v^{-1}$) glycerol was fed to enhance biomass further. Production was then induced by feeding a pure methanol or a sorbitol and methanol (1:1, C-mol ratio of methanol and sorbitol) mixture (calculation of the C-mol ratio shown in the Appendices A) at a constant rate of 10.8 $mL.L^{-1}.h^{-1}$ (270 $mmol\ Carbon.L^{-1}.h^{-1}$) for 72 hours while keeping the pH and temperature constant at 5.0 and 30 ^{o}C respectively. The induction phase was kept for 72 hours to find the optimum harvest time to acquire the highest yield of KEX2 protease in a single batch. Cultivation conditions and parameters, growth, and cell morphology were studied.

3.2.3.1 Growth curve and volume of fed-batch cultivation at bioreactor scale

For the cultivation process using pure methanol as its induction solution, the initial glycerol in the medium was consumed after 18-20 hours, and DCW reached around 30 $g.L^{-1}$, as shown in figure 3-3.

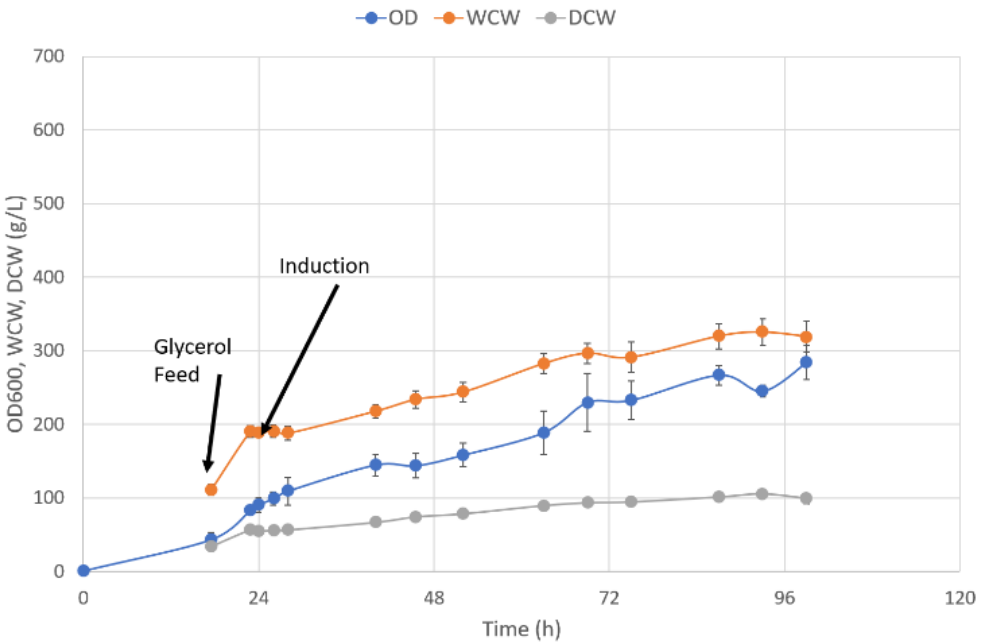
Then, additional glycerol was added by using 50% ($m.v^{-1}$) glycerol to increase the DCW to around 55-60 $g.L^{-1}$. Then, the cells were starved for 15-30 minutes after glycerol feeding was stopped to ensure all glycerol had been consumed and to prevent glycerol from inhibiting *pAOX* activity (Cregg et al., 1987). In the early stage of pure methanol induction, an adaptation phase was observed (see figure

3-3A), where biomass did not increase as the AOX enzyme consumed methanol in the peroxisome. It will take a couple of hours of adaptation before the AOX enzyme is produced and the cells can safely consume methanol (Cámara et al., 2017).

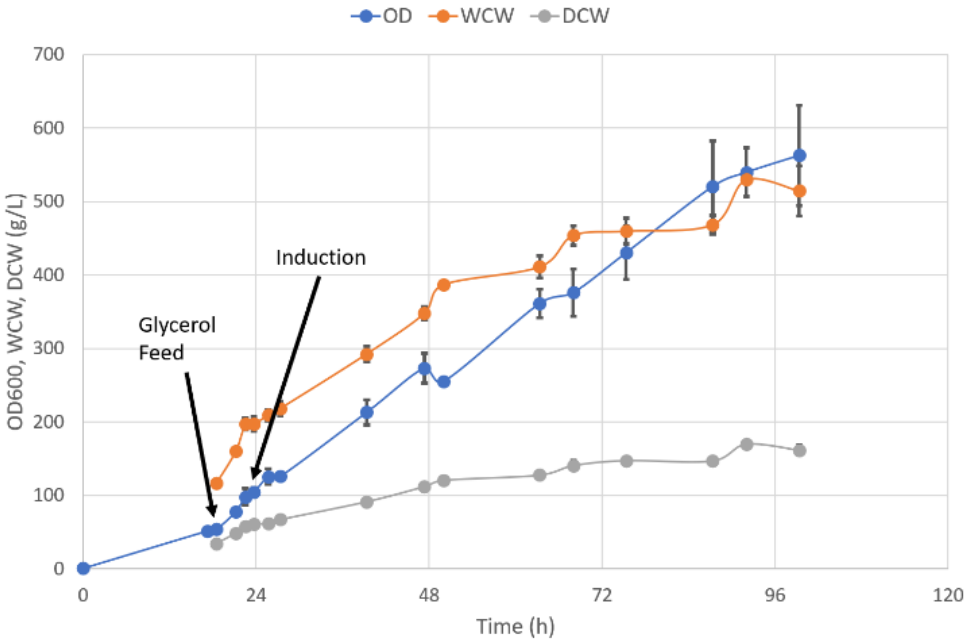
On the other hand, an adaptation phase was not observed in the early stage of mixed induction, as shown in figure 3-3B. Unlike methanol induction, an increase in biomass was observed in the adaptation phase because sorbitol is metabolized through the same pathway as glycerol. Furthermore, unlike glycerol, it does not have any inhibition effect on expression and does not need to wait for the AOX enzyme to be produced.

At the harvest time of 72 hours, the DCW reached 99.1 $g.L^{-1}$ for pure methanol induction, while for mixed induction, the DCW reached 161.7 $g.L^{-1}$. However, the maximum DCW was reached 66 hours after the start of induction at 105.3 $g.L^{-1}$ and 170.1 $g.L^{-1}$ for pure methanol and mixed induction, respectively. It was observed that the biomass peaked at 66 hours of induction and started to stagnate, indicating the start of the stationary phase. The biomass in mixed induction was much higher than in methanol induction, up to 1.6 times higher.

At the start of the cultivation process, the initial volume of basal salt medium was 2000 mL. The volume was predicted to increase because of the addition of acids and bases, glycerol, and induction solution, even after including the reduction from the samples taken throughout the process. After 72 hours, the final volume of the cultivation process using pure methanol as its induction solution was 2500 mL, while the final volume for mixed induction was slightly higher at 2820 mL. The main difference in volume was due to the higher number of cells inside the broth for the mixed induction than for the methanol induction. Another reason the volume for mixed induction was higher than pure methanol was that the volume increase from the amount of pure methanol is lesser because of the possible evaporation of pure methanol as it has a lower boiling point.



(A)



(B)

Figure 3-3 Growth curve of the fed-batch cultivation using pure methanol as induction solution (A) and 50%C-mol ratio of methanol and sorbitol as mixed induction solution (B). The growth was represented in terms biomass in three different readings; OD_{600} (optical density at 600nm) OD , wet cell weight (WCW) in $g.L^{-1}$ WCW , and dry cell weight (DCW) in $g.L^{-1}$ DCW from the average of three cultivation experiments with the same parameters with three samples each.

It was observed that there are some differences in the parameters recorded for both induction strategies used. The benefits of using mixed induction were reported in a previous study (Woodhouse, 2015). It was found that the specific heat production in the mixed induction method is lower when compared with methanol induction because the combustion rate of sorbitol is lower than that of methanol. As can be seen in figures 3-4 and 3-5, although the temperature was maintained at 30 ^{o}C, the temperature in the methanol induction method fluctuated more compared to that in the mixed mode method when using the same control process parameter. This result was consistent with previous studies when more heat was produced during induction. Although the difference in energy required to maintain the temperature may have a lesser impact in shorter cultivation periods, the impact will be significant in more extended cultivation periods and on larger scales (Niu et al., 2013). The amount of base used was also higher in the mixed induction strategy, as the growth of *K. phaffii* was much faster than in the methanol induction strategy. This result was consistent because the growth rate was higher when using a mixed induction rate, resulting in a higher base used to maintain the pH at 5.0.

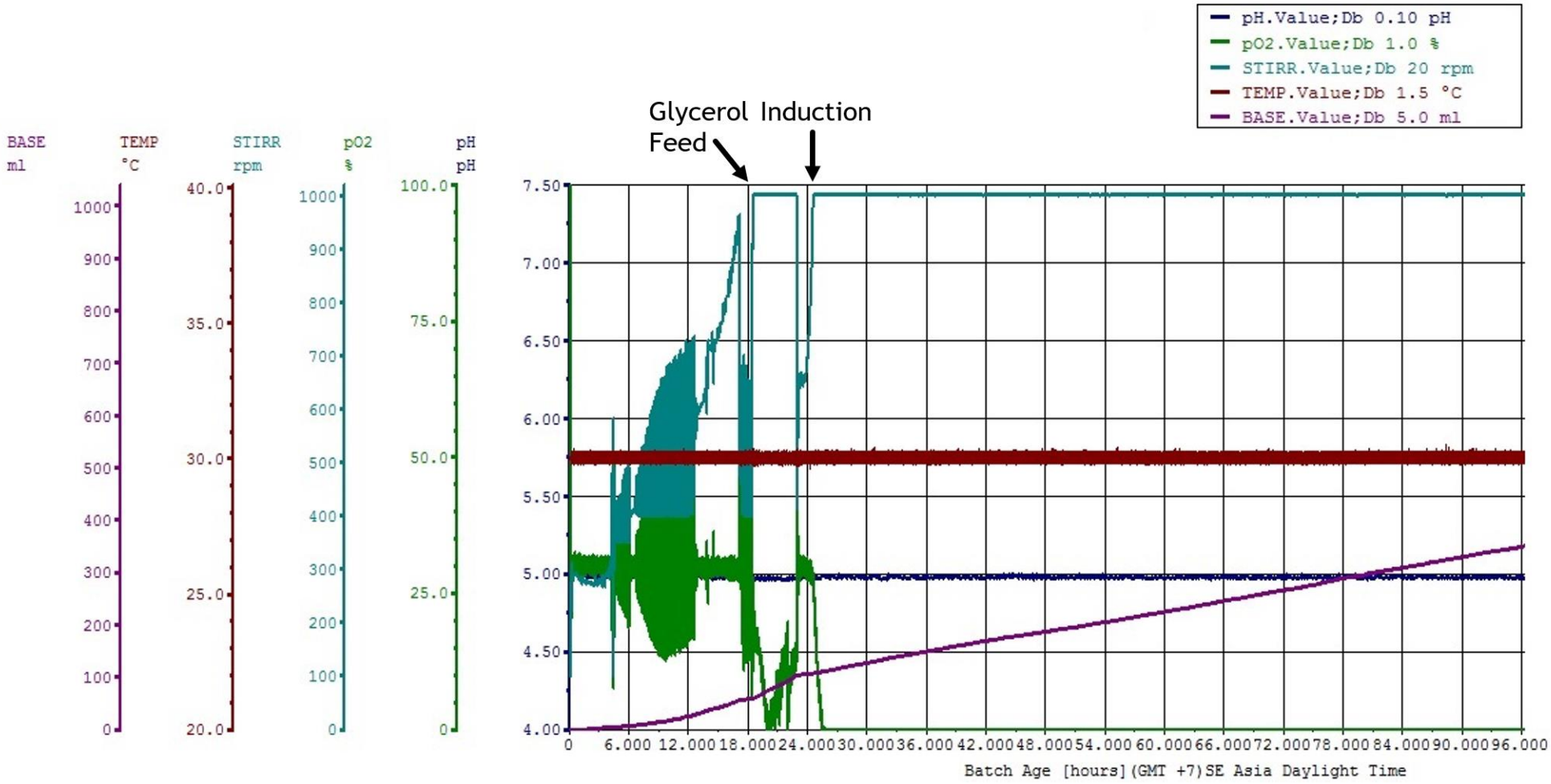


Figure 3-4 The recorded parameters of a fed-batch cultivation process using pure methanol as its induction solution. This cultivation chart is a representative of three cultivation experiments with the same parameters producing consistent results.

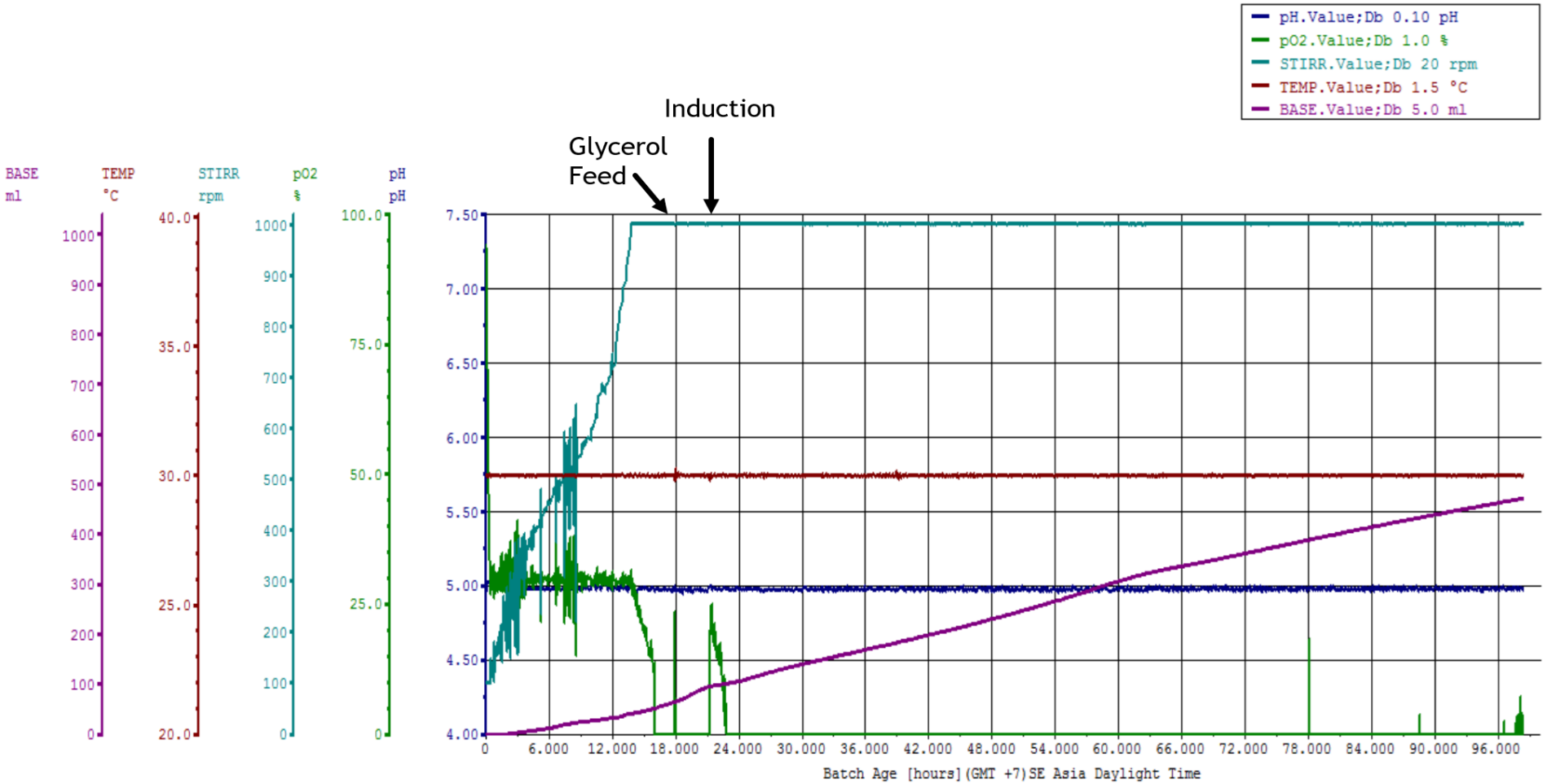


Figure 3-5 The recorded parameters of a fed-batch cultivation process using 50%C-mol methanol/ sorbitol as its induction solution. This cultivation chart is a representative of three cultivation experiments with the same parameters producing consistent results.

The residual methanol concentration inside the supernatant from the broth during the cultivation process was quantified using GC/MS every 24 hours from the start of the induction process (Standard used for the GC/MS results shown in Appendices C). As shown in Fig 3-6, residual methanol concentrations were below 0.13 %v.v^{-1} at the start of the induction as it was still transitioning the carbon source being used and increased to 0.67 %v.v^{-1} after 24 hours of the induction when using pure methanol induction strategy. Furthermore, the residual methanol concentration was steadily increasing and reached 3 %v.v^{-1} at 72 hours after induction and may increase further if no changes were made to the methanol flow rate. On the other hand, the residual methanol concentration for the mixed induction strategy was initially lower than 0.1% v.v^{-1} because of the transition phase and then dropped to an undetectable level until the end of the cultivation process. The cultivation procedure followed the Invitrogen protocol for the cultivation of *K. phaffii*. This procedure ensured that the cultivation processes could be directly compared; no changes were made to the flow rates of substrates when changing the induction strategy to a mixed induction strategy. Although the residual methanol concentration for the methanol induction strategy was steadily increasing, it did not significantly impact the growth unless it reached above 0.4 % v.v^{-1} (Khatri & Hoffmann, 2006; Surribas et al., 2007; Zhang, Bevins, et al., 2000). A mixed induction strategy was found to have a below-detectable range of residual methanol throughout the process. This result shows that both digestion processes may happen simultaneously because methanol and sorbitol have different digestion pathways. Thus, a lower methanol concentration in the mixed induction solution helps lower the chances of methanol build-up. Furthermore, a study by Çelik et al. (2009) shows that inhibiting production and growth from substrate accumulation does not apply to sorbitol, as it does not inhibit production even at a concentration above 50 g.L^{-1}.

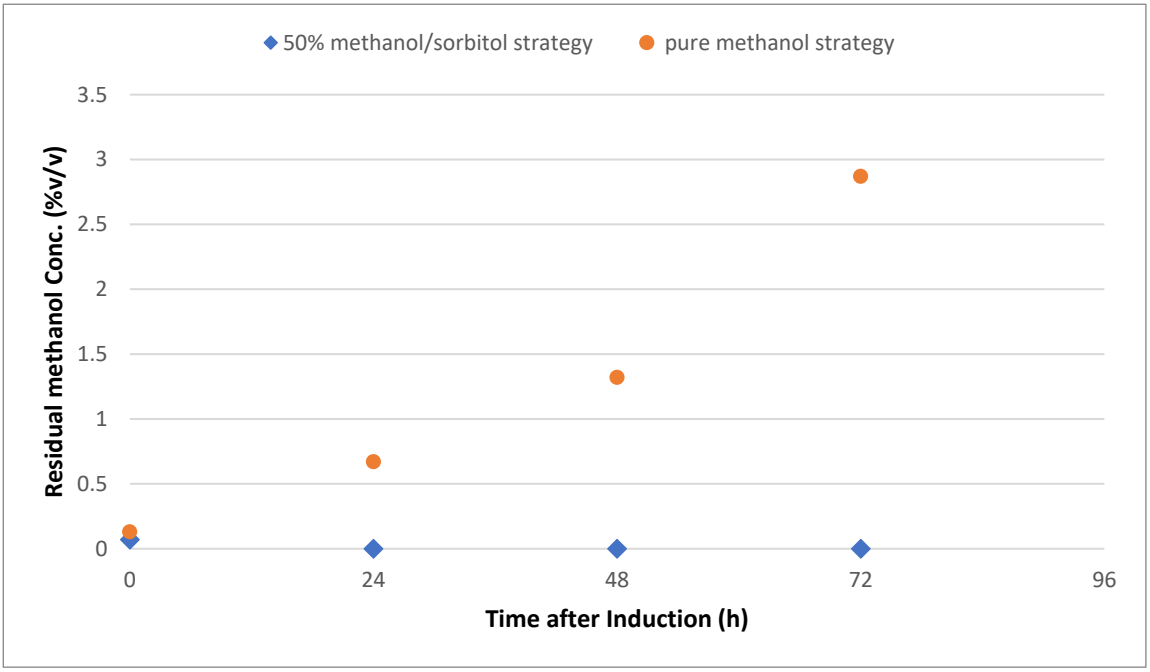


Figure 3-6 The residual methanol concentration (%v/v) after induction taken every 24 hours for the fed-batch cultivation of *K. phaffii* using both pure methanol induction strategy and 50% C-mol methanol/ sorbitol induction strategy from the average of three cultivation experiments with the same parameters.

A simple methanol, glycerol, and sorbitol digestion equation (Eq) was made to find out how the different induction strategies work. 3.1 for methanol digestion and Eq. 3.2 for glycerol and sorbitol digestion, each represented as a carbon molecule. The equation shows that for every methanol consumed, it will require 1.5 moles of oxygen and produce 2 ATP as energy. On the other hand, when using glycerol or sorbitol as the carbon source, for every 1 mole of carbon, 1 mole of O_2 was required and produced 2.67 moles of ATP as energy. These calculations show that a mixed induction strategy that used sorbitol to make more biomass produced more energy and used less oxygen. As a result, there was more oxygen available. Furthermore, the studies by Wang et al. (2019) showed that the trend of less HCP being produced was apparent, and thus, more energy could be used to produce target proteins.

Methanol digestion equation (Gao et al., 2012; Niu et al., 2013)

$$CH_3OH + 1.5O_2 + 2ADP + 2P_i = 2ATP + CO_2 + 3H_2O \qquad \text{Eq. 3.1}$$

Glycerol and sorbitol digestion equation (Gao et al., 2012; Niu et al., 2013)

$$C + O_2 + 2.67ADP + 2.67P_i = 2.67ATP + CO_2 + 2H_2O \qquad \text{Eq. 3.2}$$

3.2.3.2 Specific growth rate of fed-batch cultivation at bioreactor scale

The specific growth rate (μ) was calculated using Equation 2.3 (Rahimi et al., 2019) and shown in Figure 3-7 (raw data calculations shown in Appendices D). Initially, μ reached a maximum of around 0.14 -0.18 h^{-1} during the glycerol batch and fed-batch processes. According to past studies (Çalik et al., 2015), the specific growth rate of *K. phaffii* of the Mut^+ type when using glycerol as its carbon source was around 0.14-0.3 h^{-1} which is consistent with the results acquired. However, μ during the induction phase dropped to 0.0126 h^{-1} and 0.0234 h^{-1} for the first day of induction for pure methanol induction and mixed induction, respectively. The specific growth rate was lowered further to 0.0106 h^{-1} and 0.0164 h^{-1} after 48 hours and 0.0077 h^{-1} and 0.0123 h^{-1} after 72 hours of pure methanol and mixed induction, respectively. This result is consistent with previous studies (Wang, 2019), which found that the growth rate decreased as cultivation progressed because of the accumulation of biomass, which limited the amount of carbon, oxygen, and other nutrients available per unit of biomass. Another reason the growth rate decreased was the accumulation of toxic by-products; thus, cell growth almost stopped and may decay soon after (Zhang et al., 2003). The specific growth rate when using mixed induction was higher than when using pure methanol, at almost twice the growth rate of 0.0234 h^{-1} for mixed induction and 0.0134 h^{-1} for pure methanol after 24 hours of induction.

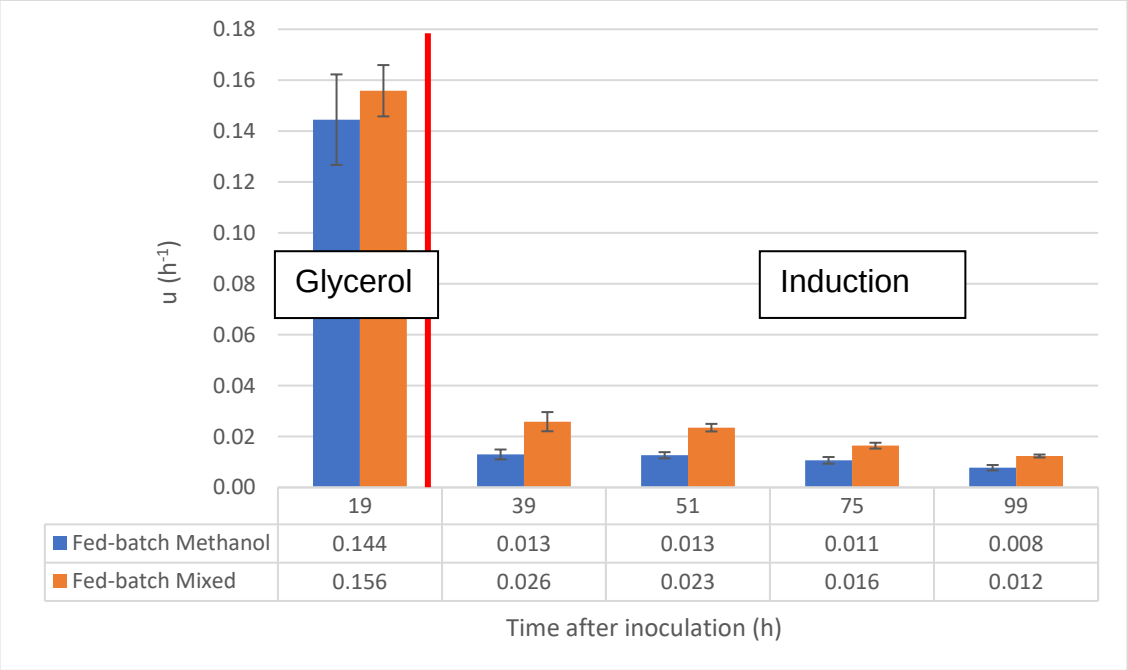


Figure 3-7 The average specific growth rate (μ) in h^{-1} from three repetitions each before induction (glycerol) at hour 20 after inoculation and 12, 24, 48, and 72

hours after induction for the cultivation of *K. phaffii* using both pure methanol induction strategy and 50% C-mol methanol/ sorbitol induction strategy in fed-batch (FB) cultivation process.

The condition of the cells after the process was photographed and analysed in terms of their size and condition (Figure 3-8). From the photograph, the cells between pure methanol and mixed induction have similar sizes of around 10-13 µm for both types of cultivation. In a study by Krainer et al., (2013), the standard size of a wild-type *P. pastoris* was 3-6 µm when it was no longer in the growth stage. However, the size of the cells can double for different cells according to the time at which it is almost splitting into two cells or when a target protein is being produced other than its wild type. Thus, the size of the cells was found to be average as it was taken when it was still growing.

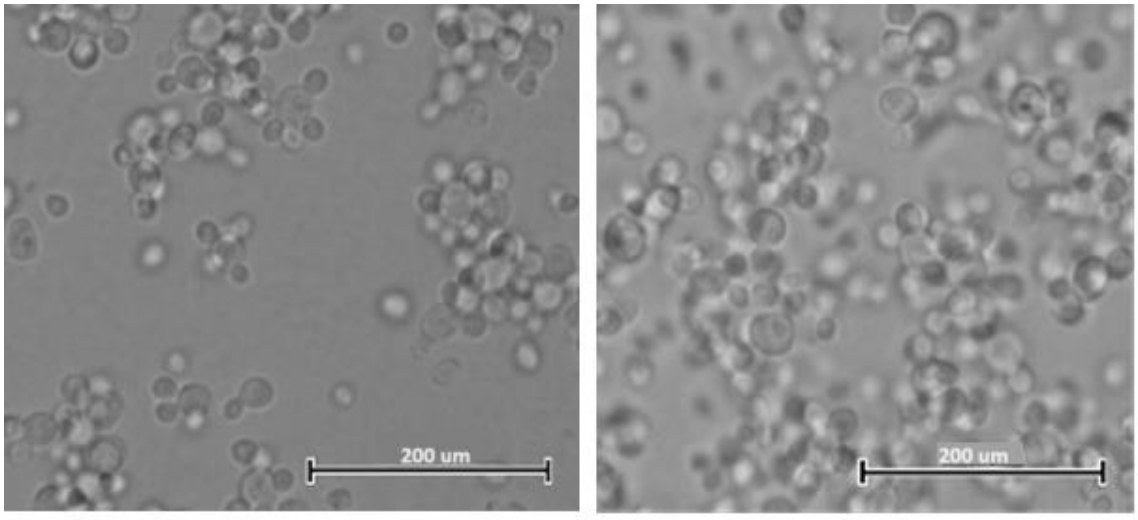


Figure 3-8 Photographs of the cells after 72 hours of induction in fed-batch cultivation process for pure methanol (A) and 50% C-mol methanol/sorbitol (B) induction.

3.2.4 Impact of mixed induction and comparison on product content

Both fed-batch cultivation processes using pure methanol and mixed methanol and sorbitol (50% C-mol) as induction solutions were done using the same cultivation procedure and time. The presence of KEX2 protease had been verified for both cultivation processes. The differences in the product content were studied and compared based on these results.

3.2.4.1 KEX2 protease content and calculations

Before calculating the KEX2 protease content in the cultivation of *K. phaffii* using both induction strategies, the results must again be verified to determine whether

KEX2 protease existed and their concentrations. Therefore, the SDS PAGE results and total soluble protein quantification results were acquired, and the quantification method mentioned in Chapter 2 was used. As shown in figures 3-9A and 3-9C, although the biomass kept increasing throughout the 72-hour induction process, it was observed from the SDS PAGE result that the target protein decreased after 12–24 hours of induction and was hard to discern at the end of the 72-hour induction. Therefore, the decrease in target protein after 24 hours of induction was probably due to the decreased amount of methanol and oxygen available per cell, which affects productivity. Besides, as the induction process continued, more cells were generated, and more impurities were accumulated, which could degrade KEX2 protease more than its production in the broth. Thus, to determine the optimum harvest time for the production of KEX2 protease, a similar method was used in a study by Wilson et al. (2019), which looked at the condition, content, impurities, and volumetric yield of the IgG1 system made by CHO cells and determined the optimum time to harvest.

The KEX2 profile in the first 24 hours was taken from the supernatant using both induction strategies and then checked with the Western blot method to see if the protein that was made was KEX2 protease. This procedure determined what happened to the target protein after 24 hours of induction. KEX2 protease antibodies were used in the Western blot process with a known internal positive control containing KEX2 protease and a negative control of bovine serum albumin (BSA). It was verified that KEX2 protease exists after 12 hours and 24 hours of induction with a protein size between 70-100 kDa (Figures 3-9B and 3-9D), which complies with a KEX2 protein size of 90.8 kDa. In addition, no band appeared between 70-100 kDa before induction for the SDS PAGE Coomassie blue and Western blot results.

Based on previous studies done by Wang (2019) and Woodhouse (2015), a mixed induction strategy was developed by replacing 50% (%C-mol) methanol with sorbitol, resulting in higher biomass but a reduced volumetric yield of aprotinin. However, in this case, the volumetric yield of the protein target (KEX2 protease) was found to increase instead of decrease.

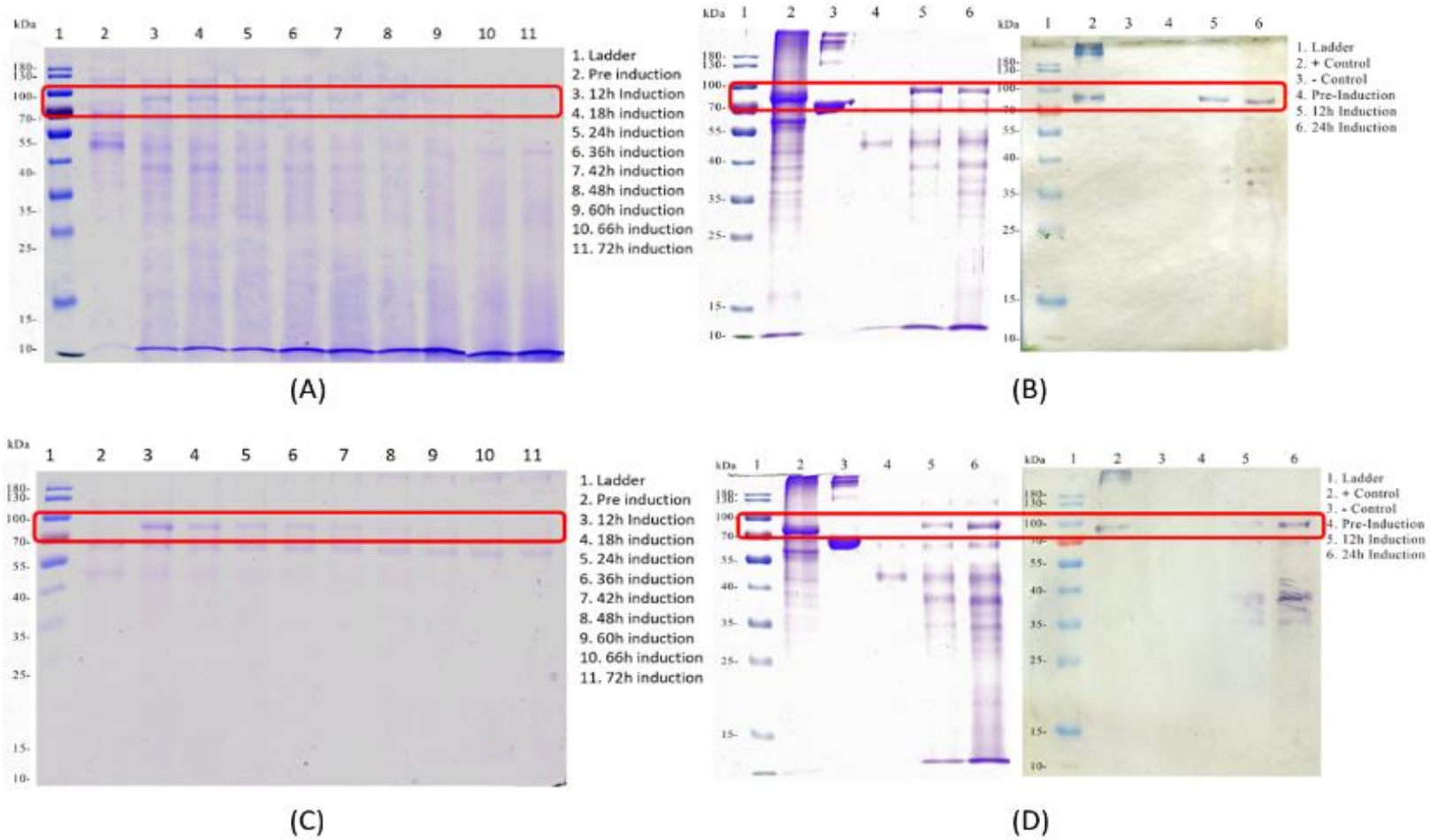
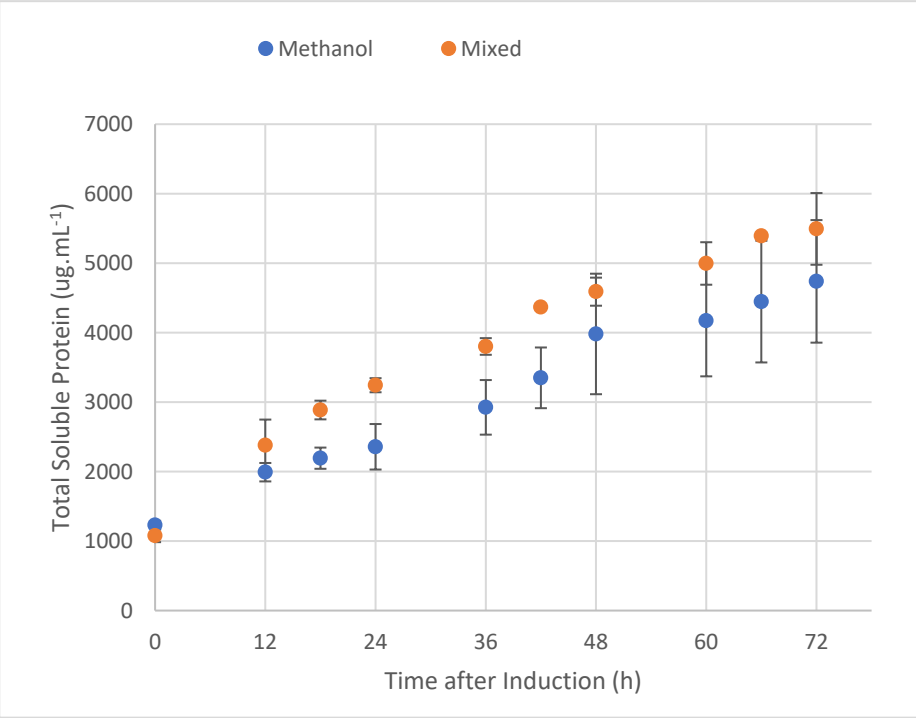
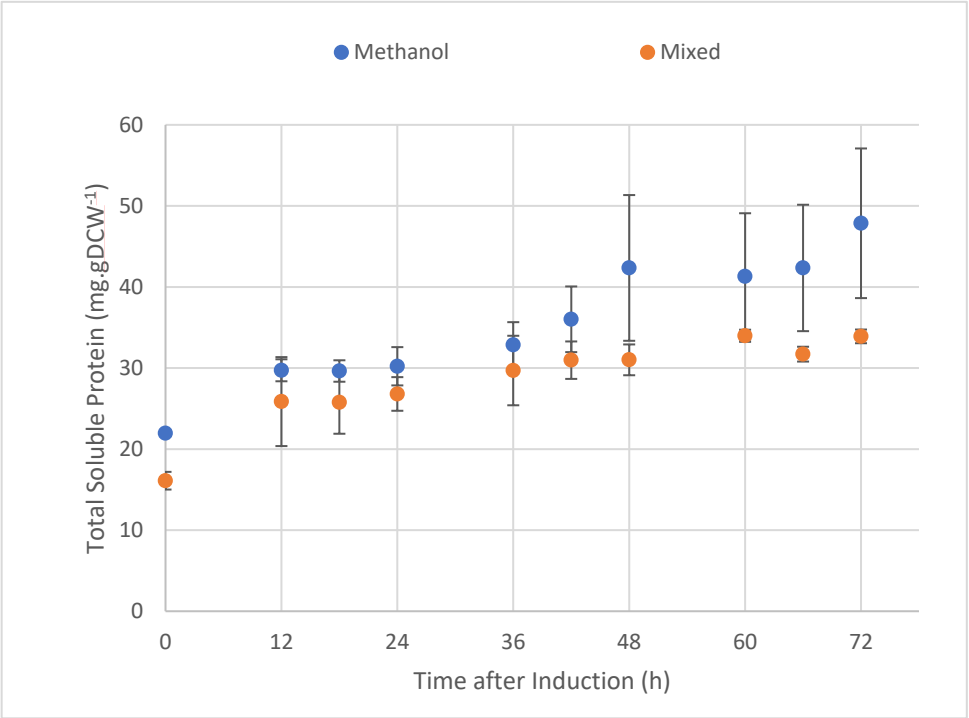


Figure 3-9 SDS PAGE and Western blot results using commercial KEX2 antibody of the cultivation process of *K. phaffii* using pure methanol induction strategy (A and B) and 50% C-mol methanol/sorbitol induction strategy (C and D), respectively. This SDS PAGE result is a representative of three cultivation experiments with the same parameters producing consistent results. The positive control used was from the one-step purified KEX2 protease through affinity chromatography acquired in small scale expression previously produced.



(A)



(B)

Figure 3-10 The average total soluble protein in terms of concentration (ug.mL^{-1}) (A) and in terms of mg.gDCW^{-1} (B) acquired through the quantification method BCA assay of the supernatant from the fed-batch cultivation process of *K. phaffii* using pure methanol induction and mixed (50% C-mol methanol and sorbitol) induction from three cultivation experiments and three samples each.

From the results of figures 3-10A and 3-10B, it was found that although the total soluble protein concentration (ug.mL^{-1}) of the mixed induction solution was found to be higher than that of the pure methanol induction, the amount of total soluble protein excreted per unit of biomass was found to be lower in a mixed induction than in a pure methanol induction. These results show that the amount of total soluble protein quantified when using the mixed induction strategy was higher than when using the pure methanol induction strategy because there was more biomass in the mixed induction strategy.

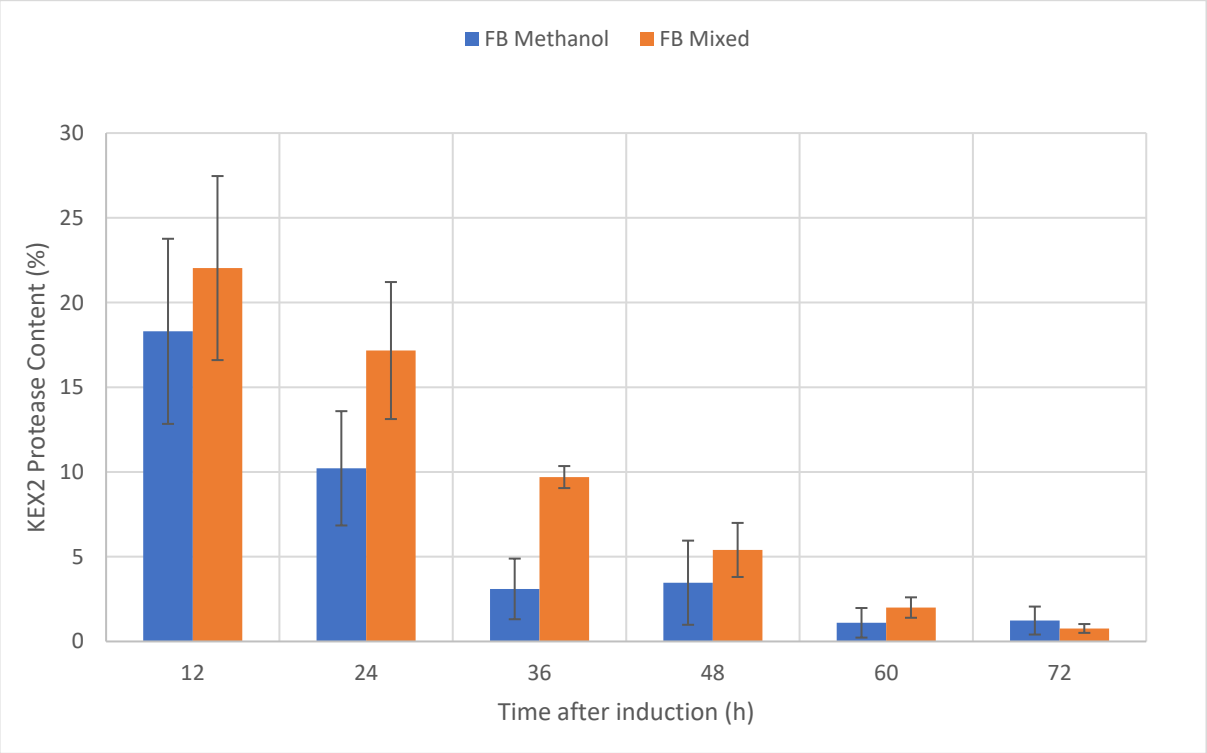


Figure 3-11 The average percentage of KEX2 protease content detected through Totallab Quant^{TM} densitometer analysis from three cultivation experiments each. Comparison between the fed-batch (FB) cultivation of *K. phaffii* using pure methanol induction and mixed (50% C-mol methanol and sorbitol) induction process.

The amount of KEX2 protease content was acquired through TotallabTM Quant densitometry by analysing the SDS PAGE results of both induction strategies (raw densitometry analysis shown in Appendices E). As shown in figure 3-11, the content peaked within the first 12 hours of induction for both types of inductions and decreased to around 1% at the end for both strategies after 72 hours of induction. From the results in Figures 3-10 and 3-11, the KEX2 protease content in the broth supernatant could be obtained. It was found that although the KEX2 protease content decreased over the induction period, the total soluble protein

concentration was increasing, which shows that more impurities may be produced in methanol induction than in mixed induction. This result is consistent with a previous study by Wang et al. (2019), which shows that a mixed induction strategy may produce lower excreted HCP when compared to a pure methanol induction strategy.

To determine whether a mixed induction strategy could affect volumetric yield of KEX2 protease, cultivation process was performed using pure methanol and mixed induction methodologies with the methods explained in chapter 2. Based on the harvest time of 24 hours after induction, samples were taken before induction, and 12 and 24 hours after induction. Total soluble protein (TSP), KEX2 protease content (%), KEX2 protease concentration ($mg.L^{-1}$), and total yield (mg) at the harvest (24 hrs after induction) were then analysed. Table 3-1 shows that the total soluble protein concentration is higher in cultivation with mixed induction than with pure methanol at 12 or 24 hours after induction. The profile is also consistent with the target-to-non-target protein ratio and KEX2 protein content.

Table 3-1 The average yields of KEX2 protease in one batch through the use of the quantified total soluble protein and KEX2 content acquired from Totallab^{TM} Quant from three cultivation experiments and three samples each.

1 batch	Total Soluble Protein (TSP) ($mg.L^{-1}$)	KEX2 Protease Content (Densitometer) (%band in lane)	KEX2 Protease Concentration (TSP x Content) ($mg.L^{-1}$)	KEX2 Protease Yield from 24 hours harvest (mg)
Methanol				~2 L Harvested
Before induction	1573.4 ±160	0	0	481.6 ±80
12 hours after induction	1991.6 ±130	18.3 ±5.5	364.5 ±90	
24 hours after induction	2357.1 ±330	9.9 ±3.6	240.8 ±40	
Mixed				~2 L Harvested
Before induction	1604.0 ±210	0	0	1113.5 ±240
12 hours after induction	2379.2 ±370	22.0 ±5.4	524.2 ±210	
24 hours after induction	3243.1 ±80	17.2 ±4.0	556.7 ±120	

Moreover, the volumetric yield of KEX2 protease resulting from the mixed induction methodology is higher than that from the pure methanol method, with a total amount of 556.7±120 $mg.L^{-1}$ and 240.8±40 $mg.L^{-1}$, respectively. A non-parametric Mann-Whitney test was also done to investigate whether there is a significant difference between the calculated harvested KEX2 protease yields of both induction strategies in the fed-batch process. The result was shown in Figure

3-12 as a one-tailed p-value, which shows that the result was significantly different with a p-value of 0.05. Again, this shows how much better mix-mode induction is than using pure methanol as an inducer when growing *K. phaffii* with an AOX promoter. Thus, the optimum harvest time with the highest KEX2 protease content was 12–24 hours after induction for both strategies.

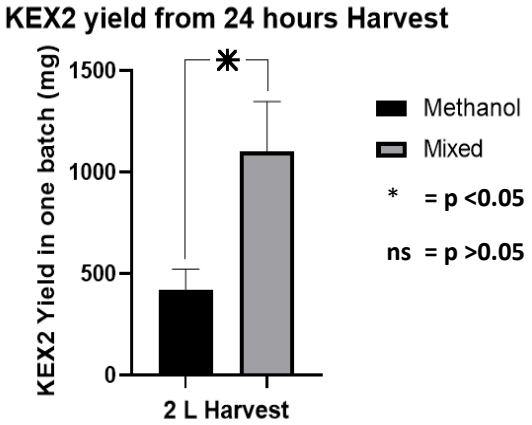


Figure 3-12 The average yields of KEX2 protease (mg) comparison between the fed-batch cultivation of *K. phaffii* using pure methanol induction and mixed (50% C-mol methanol and sorbitol) induction process from three repetitions.

3.3 Conclusion

When the AOX promoter makes recombinant proteins in *K. phaffii*, the induction step is crucial, and pure methanol is often used as the inducing agent. Even though this method works, it is hard to use much methanol in an industrial setting because it could be dangerous. In this study, we sought to find an alternative method to improve the strategy and performance of the induction step. Two induction strategies were compared: the mixed mode of induction method with methanol and sorbitol as alternative carbon sources and the methanol induction strategy. The mixed-mode induction method is promising and can address the challenges. The results showed the optimum harvest time was 12–24 hours for pure methanol and mixed induction. The specific growth rates for both cultivation processes at the optimum harvest time were 0.0119 h^{-1} and 0.0234 h^{-1} for pure methanol and mixed induction, respectively.

Furthermore, this study's results demonstrated that the fed-batch cultivation process of *K. phaffii* using the mixed mode induction strategy could improve the yield (mg) 2.4-fold compared to pure methanol as the inducing agent. Moreover,

the total consumption of methanol is reduced two-fold when the mixed mode induction method is used. Therefore, this method is safer for the manufacturing processes and environment because it uses less methanol and improves the volumetric yield of the cultivation process of *K. phaffii* for expressing recombinant proteins like KEX2 protease, which constitutes a method of choice and warrants further exploration and investigation. However, using a fed-batch cultivation process with a mixed induction strategy may not be feasible for producing KEX2 protease in an industrial setting because of the requirement of a large-scale cultivation process. Thus, another development strategy of the continuous cultivation process was studied.

Chapter 4: Evaluation of Continuous cultivation Method and Mixed Induction Strategy

4.1 Introduction

The main objectives of this chapter were:

1) To establish the parameters such as dilution rate, harvest rate, and time to shift to the continuous phase used in the continuous cultivation process and investigate the challenges of a continuous process for the expression of KEX2 protease.
2) To determine how the induction strategy affects cell growth and protein condition at the bioreactor scale in a continuous process and to specify the continuous phase change parameter in the production of KEX2 protease.
3) to compare KEX2 protease volumetric yields and the impact of methanol and sorbitol-methanol mixed induction strategies in continuous processes.

Fed-batch cultivation is the most commonly used method for *K. phaffii* in bioreactors because of its simplicity, convenience, reliability, and decent overall yield. The use of methanol as its induction solution is also the norm because pAOX1 is the most extensively used promoter (Potvin et al., 2010). However, as the demand for biopharmaceutical products increases and thus causes a rise in their price, several strategies were developed to improve process yield and reduce production costs and time. As described in Chapter 3 and other studies (Azadi et al., 2017; Jungo, Marison, et al., 2007; Jungo, Schenk, et al., 2007; Thorpe et al., 1999), one of the strategies was to use a mixed induction strategy in a fed-batch cultivation method. This method showed promising results in improving the efficiency of recombinant protein production in *K. phaffii*. Another method to further improve the productivity and efficiency of the process was then investigated, which was a continuous cultivation process.

Continuous cultivation has several advantages, including a smaller carbon footprint, lower downtime, and more consistent batch-to-batch production. However, it has several disadvantages: a more complex and tedious process, contamination risk, and a high cell stability requirement (Curvers et al., 2002; Hazeu & Donker, 1983). In addition, the feasibility of continuous process application was inconsistent, as it produced varying results for different strains.

Furthermore, it may not increase productivity and use more resources than the fed-batch process. Hence, the continuous cultivation process may cost more than the fed-batch process (H. Lim & Shin, 2011) and is not a popular method of choice for increasing the overall yield of the target recombinant protein.

Nevertheless, if the right balance could be found where the improvement of process productivity could surpass the impact of the use of the continuous cultivation method as compared to the fed batch, it would be beneficial for the biopharmaceutical industry to apply the method. Some of the drawbacks of the continuous process can be resolved as the technology progresses. One of the ways to resolve the limitation is by using a mixed induction strategy in combination with the continuous cultivation method. However, such a combination has yet to be thoroughly studied and has only been studied in detail individually without being combined.

In a previous study by Rahimi et al. (2019), a chemostat continuous cultivation strategy was performed to produce recombinant HBsAg as its target protein expressed in *K. phaffii* and methanol as its induction. It was shown that the average daily rHBsAg was found to be 187.71 $mg.L^{-1}$, which was similar to the volumetric yield harvested at the end of the fed-batch process. If this is true, implementing a continuous process may be feasible. It was also found that the specific growth rate is a significant attribute directly related to the dilution rate, which will raise the volume of broth harvested during a continuous process daily. This finding highlights the benefit of using the continuous cultivation method.

This study hypothesized that using sorbitol as a mixed induction solution, known to increase the growth rate of *K. phaffii*, could create a synergistic effect with the continuous cultivation method, increasing the overall yield without altering the condition of the target protein and giving rise to more benefits. Two methods were implemented to verify the hypothesis: expression of the KEX2 protein was done using fed-batch and continuous cultivation methods and a mixed induction strategy. The impact of the methods mentioned previously on cell growth condition, and volumetric yield of the expressed target protein were studied.

4.2 Results and discussion

To determine whether continuous cultivation process has more advantages with respect to improving volumetric yield and feasibility of cultivation process for a

recombinant protein production, continuous cultivation process of *K. phaffi* in the production of KEX2 protease was evaluated in 5 L bioreactor scale using pure methanol or mixed induction strategy using 50% C-mol sorbitol to methanol ratio as an inducing solution. The growth profile of the cells and volumetric yield of KEX2 protein expression were determined and compared. The growth rate of the fed-batch cultivation processes, when the KEX2 protease volumetric yield was at its highest, calculated in chapter 3 were used in defining the initial parameters of continuous process to acquire the highest amount of KEX2 protease harvested.

4.2.1 Defining the continuous cultivation parameters

The critical parameters in continuous cultivation for *K. phaffii* have been progressively studied and defined, which include the setting of the continuous cultivation process, dilution rate, harvest, and substrates feed rate (Digan et al., 1989; Monod, 1950; Nieto-Taype et al., 2020). An overall flow process of a continuous cultivation process is depicted in figure 4-1.

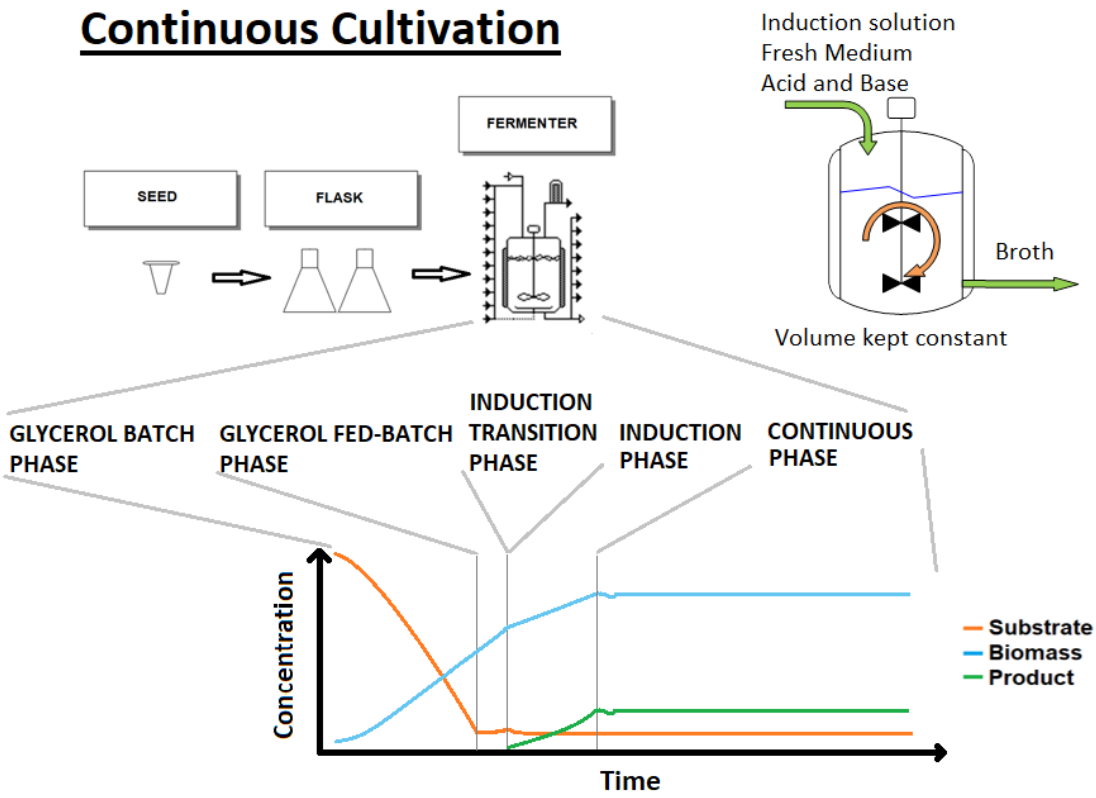


Figure 4-1 An example of continuous cultivation process steps inside the fermenter consists of five phases, which are: glycerol batch phase, glycerol fed-batch phase, transition phase, induction phase and continuous phase (Zhang, Inan, et al., 2000).

4.2.1.1 Setting the continuous cultivation process

Determining the type of continuous cultivation process to be used is crucial as it dictates the methodology of the continuous cultivation process. The two most common cultivation processes for *K. phaffii* are chemostat and turbidostat (Nieto-Taype et al., 2020). Both are similar, but the main difference is in the method for controlling the growth rate of the cultivation process. Chemostat controls the growth of the cells by limiting a single nutrient from the fresh medium that is added. In contrast, a turbidostat controls the growth of the cells not by limiting the nutrients but by adding fresh medium in a feedback loop that controls the biomass inside the broth. Although other continuous processes exist, such as changestats, the chemostat, and turbidostat are still considered the most practical methods. In this study, the chemostat continuous cultivation process was used as it has been considered the most effective type of continuous process and the simplest to implement in multiple studies (Hoskisson & Hobbs, 2005; Nieto-Taype et al., 2020; Peebo & Neubauer, 2018). For a chemostat cultivation process, the conditions of the bioreactors, such as their parameters and substrate concentration, were kept constant.

The continuous cultivation process initially follows the same phases as the fed-batch cultivation process (glycerol batch, glycerol fed-batch, and induction phase). The process parameters, such as pH and temperature, were kept the same as those in the fed-batch cultivation process, which were 5.0 and 30^{o}C, respectively. The induction solution feed rate during the continuous cultivation process was kept the same to ensure the condition was the same as when the growth and KEX2 protease volumetric yield was the highest. After 24 hours of the induction phase, a shift to the continuous phase was done. The broth inside the bioreactor was then continuously harvested at a rate according to the dilution rate (D) calculated using equation 2.4. Fresh BSM medium without any carbon source was also fed at the start of the continuous phase at a rate where the volume inside the fermenter was kept constant using equations 2.7 to 2.9. The harvested broth from the bioreactor was held in a bottle submerged in a water bath at 4-6 ^{o}C to keep the broth in good condition before being harvested daily. A schematic diagram of the fed-batch and continuous processes is depicted in figure 4-2, showing the differences in adding fresh medium feed and a harvest line with a bottle from which the broth can be taken daily.

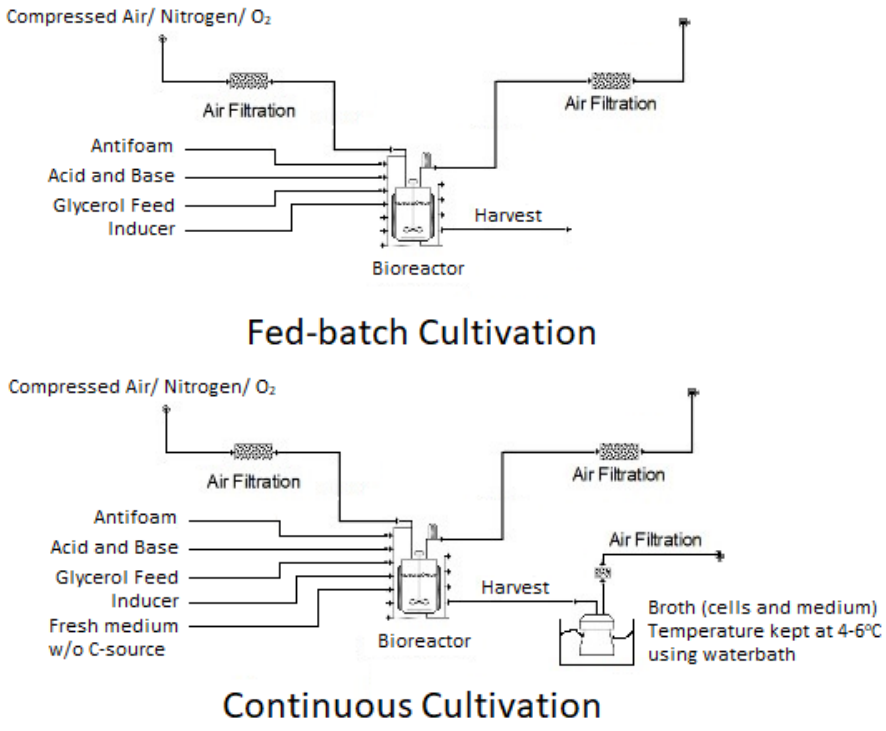


Figure 4-2 Schematic diagrams comparing the fed-batch and continuous cultivation set up used in this research.

In the continuous cultivation process, the timing at which the process shifted from the fed-batch phase to the continuous phase was critical as it affected the KEX2 protease volumetric yield harvested and the cells' condition. Based on the results found and reported in chapter 3, the optimum time to shift from fed-batch to continuous processing would be after 12–24 hours of induction, when the biomass was at 80 to 100 gDCW.L^{-1}. A study by Rahimi et al. (2019) reported shifting to a continuous process when the growth of *K. phaffii* reached the end of the exponential phase and when the biomass reached 80 gDCWL^{-1}, which was later kept at 100 gDCWL^{-1} which took 140 hours after inoculation to reach. Therefore, it was implied that the highest productivity of rHBsAg was at that point in fed-batch cultivation before shifting to a continuous process. Another study by Zhang et al. (2004) showed that the optimal time to shift was set using the predicted highest volumetric productivity or the predicted highest specific production rate. From the results acquired in fed-batch cultivation, the highest volumetric yield was 12–24 hours after induction with a DCW of 80 g.L^{-1} and 100 g.L^{-1} for pure methanol and mixed induction, respectively. Thus, the shift to continuous phase was set at 24 hours after induction started at biomass with 80 gDCW.L^{-1} when using the methanol induction strategy and 100 gDCW.L^{-1} when using mixed 50% C-mol methanol and sorbitol induction strategy.

4.2.1.2 Dilution rate, the harvest rate and total feed rate calculations

The dilution rate is the most significant parameter in the continuous cultivation process, as it impacts whether the biomass inside the bioreactor can be maintained (Monod, 1950; Nieto-Taype et al., 2020). The dilution rate could not be too high as it would cause a gradual loss of biomass because the cells taken out at the harvest rate would exceed the number of cells grown inside the bioreactor (washout). On the other hand, the dilution rate could not be too low as it would cause a build-up of biomass, which may further cause a build-up of impurities or a reduction of available nutrients per cell. Multiple studies have been done in the cultivation of *K. phaffii* using BSM as its medium, and the dilution rate was found to range as high as 0.085 h^{-1} and as low as 0.009 h^{-1} (Rahimi et al., 2019; Curves et al., 2001; Zhang et al., 2004). From equation 2.6, the dilution rate used was equal to the steady state of the specific growth rate in real-time. From the previous chapter, the specific growth rate (μ) of both fed-batch cultivation processes using pure methanol induction and mixed 50% C-mol methanol and sorbitol induction was found to be at 0.0126 and 0.0234 h^{-1} during the first 24 hours of induction for pure methanol induction and mixed induction, respectively. The specific growth rate was then lowered to 0.0106 h^{-1} and 0.0164 h^{-1} after 48 hours for pure methanol induction and mixed induction strategies, respectively. The specific growth rate of 24-hour induction was not used as it was the condition before the shift to the continuous phase happened. Thus, the real-time specific growth rate was set at 0.0106 h^{-1} and 0.0164 h^{-1} for pure methanol induction and mixed induction strategies, respectively.

After setting the dilution rate, the harvest and feed rates can be quantified using Equations 2.7–2.9. After 24 hours of induction, the volume for both induction strategies were verified to be 2200 mL for pure methanol and 2500 mL for the mixed induction strategy. The feed rate of the acid and base was set to be reactive in maintaining the pH at 5.0. The new medium feed rate will be set to balance the volume of the total feed rate with the harvest rate to keep the volume inside the bioreactor the same as when the continuous shift phase occurred.

The methanol feed rate was kept as set during the fed-batch. However, maintaining the methanol feed rate at the required amount is crucial, as feeding less than required means less growth and volumetric yield and is thus not an optimum cultivation process. While feeding too much will result in methanol

excess, which may cause build-up and toxicity and cause cell lysis if it reaches above 0.4% $v.v^{-1}$, resulting in an unintentional decrease in biomass and breaking the continuity. After initial tests, it was found that maintaining the methanol feed rate at the same level as the fed batch would cause methanol build-up. As shown in figure 4-3, after four days of maintaining the process, the biomass suddenly decreased and continued to decrease significantly, breaking the continuity. The excess of methanol was observed in the experiment by stopping the feeding momentarily, but even after 3 hours of not feeding methanol, no oxygen spike was observed. The excess of methanol was also observed when the broth was analysed, which reached higher than 0.4% $v.v^{-1}$.

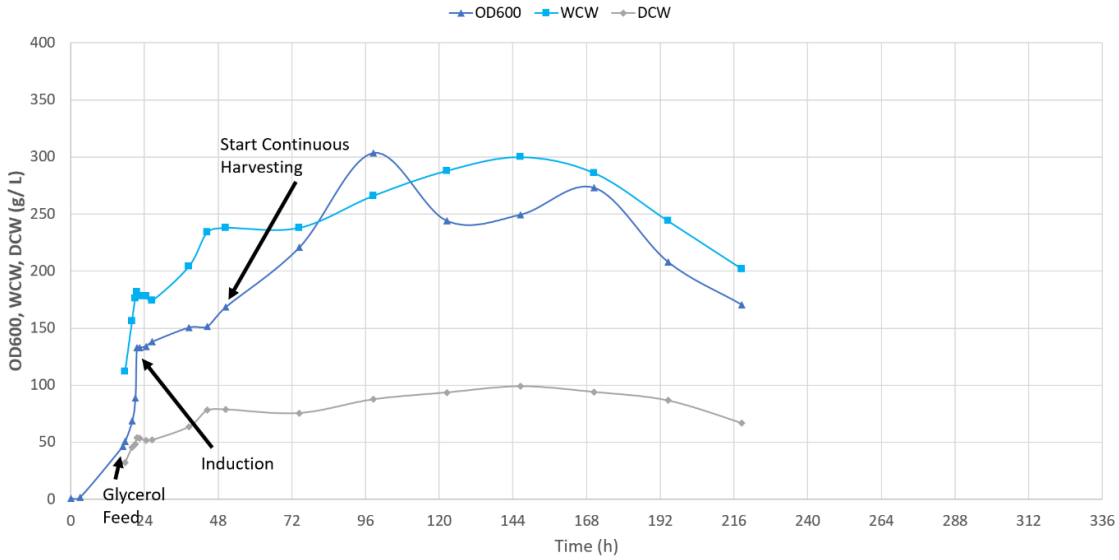


Figure 4-3 An example of growth curve presented in terms of biomass of optical density (OD_{600}), wet cell weight (WCW) in $g.L^{-1}$ and dry cell weight (DCW) in $g.L^{-1}$ of *K. phaffii* when an excess of methanol occurred on the 4th day after shifting to continuous process.

After multiple trials, with the methanol feed rates of 10.8, 9.6, 8.4, and 7.2 $mL.h^{-1}.L^{-1}$, the optimum pure methanol induction feed rate was 8.4 $mL.h^{-1}.L^{-1}$. It was able to maintain continuity throughout the process. This new feed rate lowered the methanol feeding rate from fed-batch to continuous processing by a ratio of 0.778. The methanol content was also observed daily throughout the cultivation process, and an oxygen spike was observed after stopping methanol feeding within 2 minutes on average. The changes to the methanol feeding rate are consistent with Rahimi et al. (Rahimi et al., 2019), as the calculated ratio of total produced rHBsAg to the total consumed methanol for chemostat, and fed-batch fermentation was 0.732, which is close to 0.778. This result suggests that less methanol was needed for the expression of target proteins and growth in

continuous fermentation. The main reason for this result might be related to the cellular age range (Madadi et al., 2017). In the fed-batch process, there were older cells inside the broth, which might not be as efficient as young cells in the growth and production of the target product.

Different from pure methanol as an induction solution, there was no need to lower the feeding rate of mixed induction solution between fed-batch and continuous process. The main reason is that sorbitol is metabolized using a different pathway than methanol. Therefore, sorbitol would not affect the expression or growth rate even if an excess existed. Furthermore, the methanol feed rate in the mixed induction strategy at 50% C-mol was already lower than the pure methanol induction strategy (Jungo, Marison, et al., 2007; Jungo, Schenk, et al., 2007; Zhu et al., 2011).

Thus, the initial parameters of the continuous cultivation process were set, as shown in Table 4-1.

Table 4-1 Initial parameters set for continuous cultivation process of both induction strategies based on previously mentioned challenges. *Reactive was used for the acid and base feed rate as it was set to keep the pH of cultivation process at 5.0 and thus was not controlled. **Balancing was used for fresh medium feed rate as the amount of fresh medium was adjusted to balance the amount of volume being harvested according to equation 2.9 to keep the volume inside the cultivation process constant

Initial parameters set for continuous process	Methanol Induction	Mixed Induction
Biomass during continuous phase ($gDCW.L^{-1}$)	80.0	100.0
Dilution rate (h^{-1})	0.0106	0.0164
Real time volume (V_c) after shifting to continuous phase (mL)	2200	2500
Harvest rate ($mL.h^{-1}$)	24	41
Acid and Base feed rate ($mL.h^{-1}$)	Reactive* to keep pH at 5	Reactive* to keep pH at 5
Fresh medium feed rate ($mL.h^{-1}$)	Balancing**	Balancing**
Induction feed rate ($mL.h^{-1}$)	8.4	10.8

4.2.2 Continuous cultivation at 5 L bioreactor scale

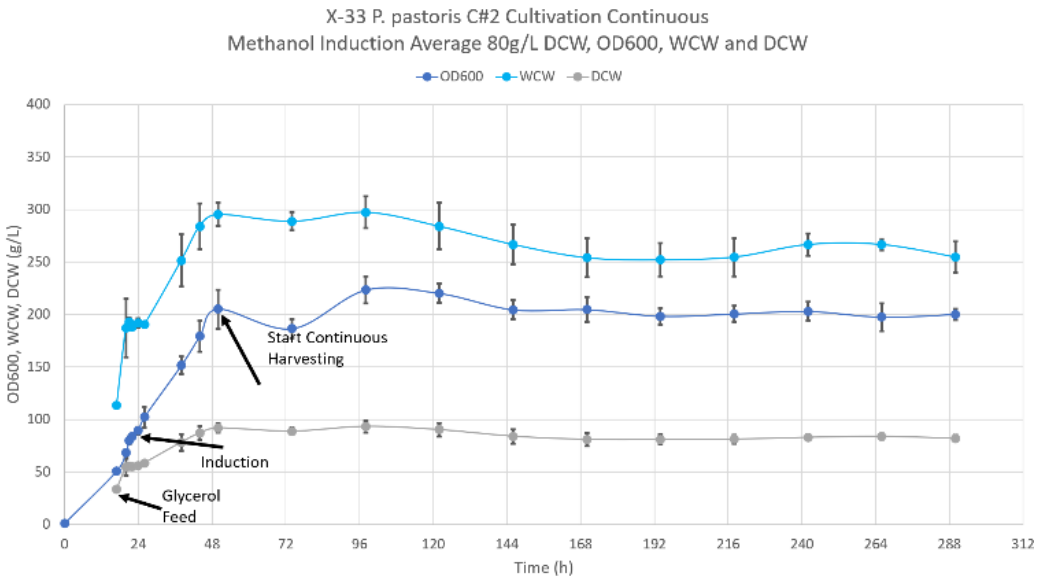
Continuous cultivation using pure methanol and mixed 50% C-mol methanol and sorbitol as its induction solution was done on a bioreactor scale to verify whether a volumetric yield increase was achievable for the expression of KEX2 protease when compared to a conventional fed-batch cultivation process. The start of the cultivation process before shifting to the continuous cultivation phase at a certain point was done using the procedure recommended by Invitrogen (Invitrogen, 2013). Cells were grown on a medium containing 0.4% ($v.v^{-1}$) glycerol. After the glycerol in the medium was consumed, 50% ($m.v^{-1}$) glycerol was fed to enhance biomass further. Production was then induced by feeding pure methanol at a constant rate of 8.4 $mL.L^{-1}.h^{-1}$ (210 mmol $Carbon.L^{-1}.h^{-1}$) and a sorbitol and methanol (1:1, C-mol:C-mol or 50% C-mol methanol and sorbitol) mixture at a constant rate of 10.8 $mL.L^{-1}.h^{-1}$ (270 mmol $Carbon.L^{-1}.h^{-1}$) for 72 hours. The pH and temperature were kept constant at 5.0 and 30 ^{o}C, respectively. The continuous phase was kept for 240 hours (10 days) to determine whether the continuity of the cultivation process could be kept and whether the volumetric yield of KEX2 protease was consistent throughout the continuous process. Cultivation conditions and parameters, growth, and cell morphology were studied.

4.2.2.1 Growth curve and parameters of continuous cultivation process

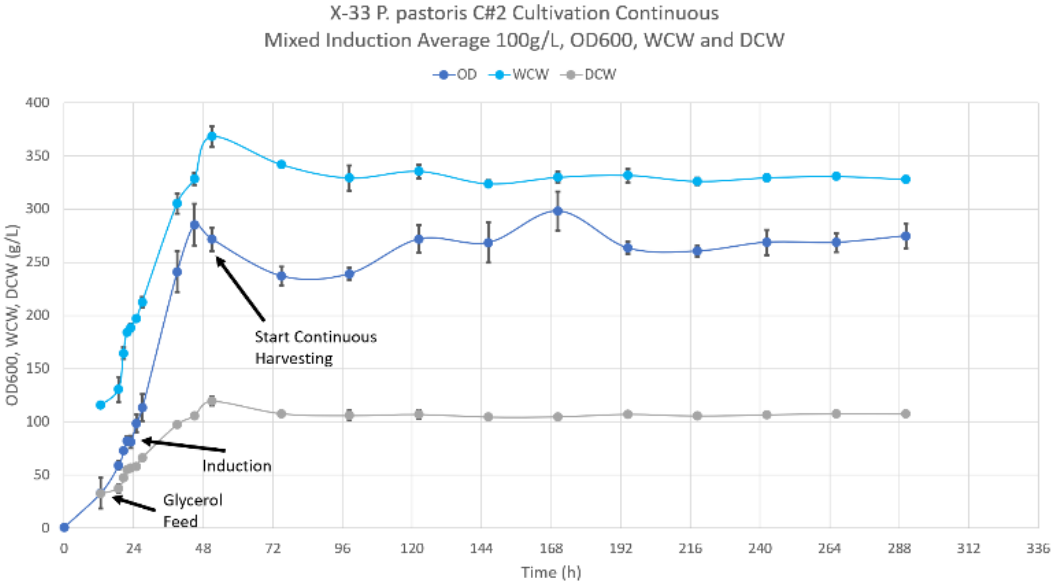
For the continuous cultivation process of *K. phaffii* in the expression of KEX2 protease, the starting process was similar to the fed-batch cultivation process until 24 hours after induction, which is the same as the time at which the volumetric yield was the highest. After 24 hours of induction, the biomass reached around 84±4 $gDCW.L^{-1}$ and 106±2 $gDCW.L^{-1}$ for pure methanol and mixed induction, respectively. The biomass of both induction strategies was slightly higher than what was acquired in the fed-batch process before shifting to the continuous phase. However, the biomass was found to drop until it gradually reached the set amount. Then, the biomass dropped and was kept at 80 $gDCW.L^{-1}$ for the pure methanol continuous cultivation process.

In contrast, the biomass was kept at 100 $gDCW.L^{-1}$ for the mixed induction continuous cultivation process. As a result, continuous pure methanol processes took around four days before the biomass stabilized at 80 $g.L^{-1}$. At the same time, the mixed induction continuous process took slightly less time, at three days, before stabilizing. The retention time, based on the volume of broth currently

inside the bioreactor and the amount harvested daily from the dilution rate, was the main factor in the longer time it took to stabilize. For a pure methanol induction strategy, the amount of broth harvested daily was 27.3% of the volume of broth inside the bioreactor, or a retention time of 3.67 days, while the amount of broth harvested daily for mixed induction was 40% of the volume of broth inside the bioreactor, or a retention time of 2.5 days. The biomass of both cultivation process using pure methanol induction and mixed induction were shown as figure 4-4A and 4-4B, respectively.



(A)



(B)

Figure 4-4 Growth curve of the continuous cultivation using (A) pure methanol at 80 gDCW.L^{-1} biomass kept during continuous phase and (B) mixed 50% C-mol methanol/ sorbitol induction at 100 gDCW.L^{-1} biomass kept during continuous phase. This cultivation chart was obtained from average of three cultivation experiments with the same parameters with three samples each.

At the start of the cultivation process, the initial volume of basal salt medium was set at 2000 mL. The volume increased because of the addition of acids and bases, glycerol, and induction solution, even after including the reduction from the samples taken throughout the process. After 24 hours of induction, the volume of the cultivation process using pure methanol as its induction solution was found to be 2063±179 mL, while the volume for mixed induction was found at 2467±44 mL. Similar to the previously discussed difference in final volume in the fed-batch cultivation process, the main difference in volume was because of the higher number of cells inside the broth for the mixed induction compared to methanol induction. The volume was then kept the same throughout the continuous process.

It was observed from Figures 4-5 and 4-6 that the trend of the parameters before shifting into the continuous phase was similar to their fed-batch counterpart. The temperature of the continuous cultivation process was kept at 30 ^{o}C for both induction strategies and had the same process control parameters. However, the recorded temperature fluctuated more when using the pure methanol induction strategy than the mixed induction strategy. This fluctuation was because the combustion energy used to digest methanol was higher than that used to digest sorbitol, causing the temperature to increase faster and requiring more energy to maintain it. In a study, lowering the set temperature was done to maintain the rise of temp caused by methanol induction (Woodhouse, 2015). A study was done by Zhu et al. (2011) on using sorbitol, lowering the temperature, or a combination of both that increases the production of β-mannanase. Although this effect of requiring more energy to cool down the system for the pure methanol strategy was almost negligible in a shorter fed-batch cultivation, it had a more significant impact on the longer duration of the cultivation process, such as the continuous cultivation process (Niu et al., 2013). This difference would affect the overall amount of energy required in the continuous cultivation process. The longer the continuous cultivation process, the more energy is required to maintain it. The same could be seen about the pH, as fluctuating temperature reflects the fluctuation in the recorded pH value. The volume of NH_4OH used to keep the pH at 5.0 was much higher for mixed induction, using, on average, 1500 mL, while pure methanol induction used an average of 980 mL. The difference in the

amount of NH_4OH used was because the biomass and growth for mixed induction were higher than for pure methanol.

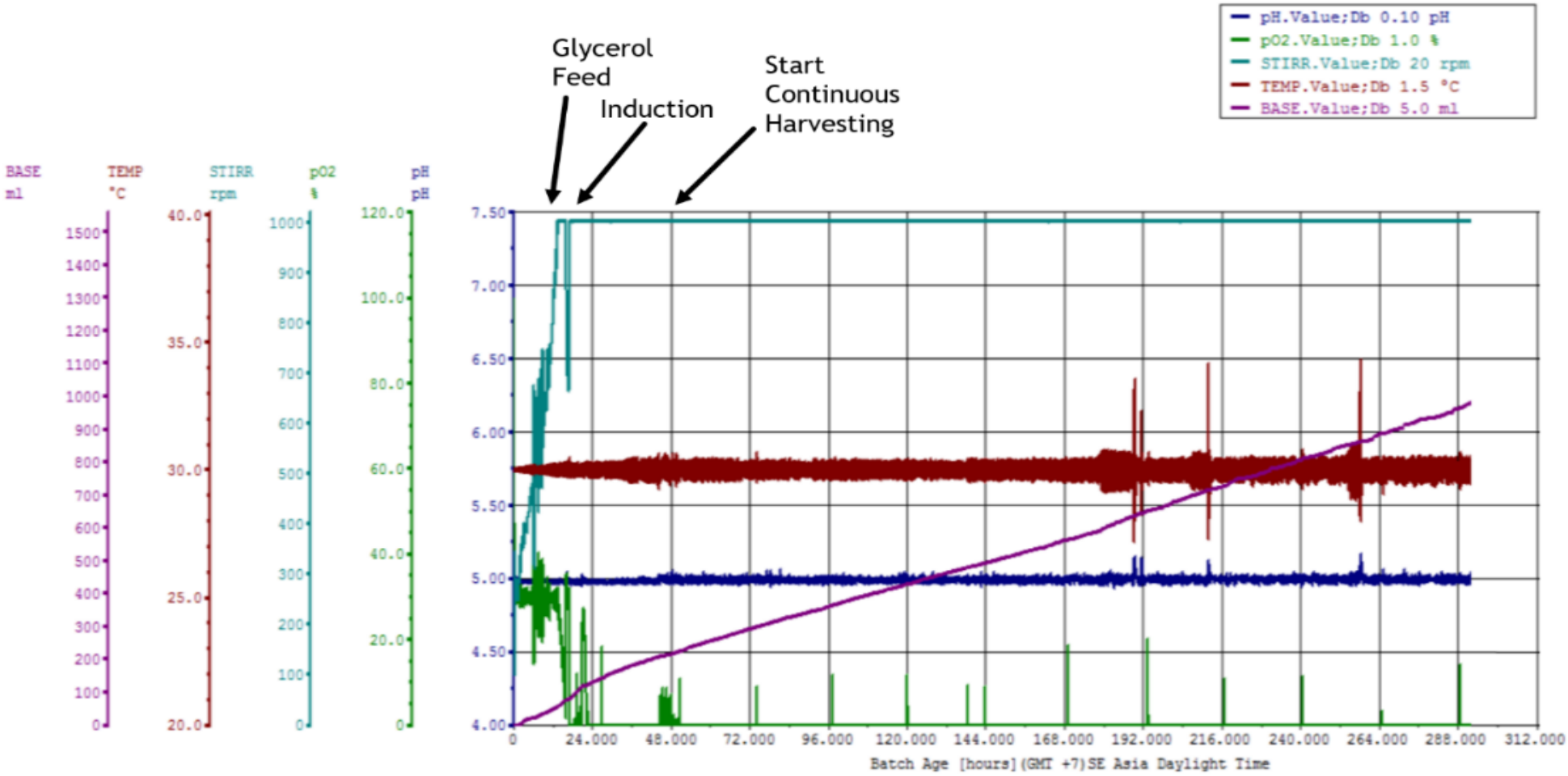


Figure 4-5 The recorded parameters of a continuous cultivation process using pure methanol as its induction solution. This cultivation chart is a representative of three cultivation experiments with the same parameters producing consistent results.

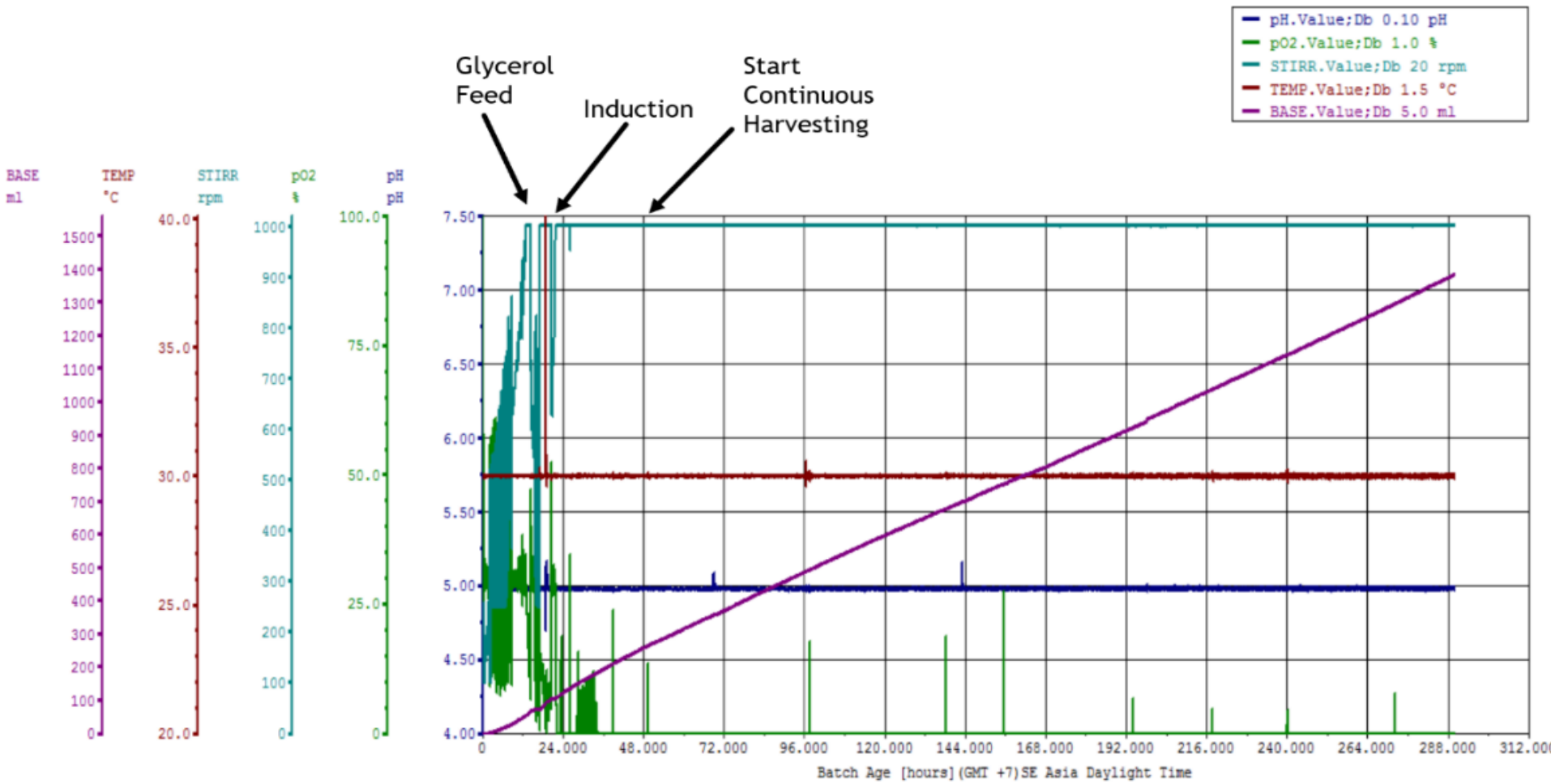


Figure 4-6 The recorded parameters of a continuous cultivation process using 50% C-mol methanol/ sorbitol as its induction solution. This cultivation chart is a representative of three cultivation experiments with the same parameters producing consistent results.

The residual methanol concentration inside the supernatant from the broth during the cultivation process was quantified using GCMS every 24 hours from the start of the induction process until the end of the continuous cultivation process. As shown in Fig 4-7, residual methanol concentrations were below 0.1 % $v.v^{-1}$ at the start of the induction for both induction strategies as it was still transitioning the carbon source being used. However, the residual methanol concentration dropped below the detectable level and continued to be below the detectable level until the end of the cultivation process. This result shows that a continuous state was able to be maintained, and lowering the feed-rate of the pure methanol feeding rate from the previously used fed-batch process ensures the residual methanol concentration level was below 0.4% $v.v^{-1}$ (Khatri & Hoffmann, 2006; Surribas et al., 2007; Zhang, Bevins, et al., 2000; Zhang, Inan, et al., 2000). Furthermore, in a study by Çelik et al. (2009), the inhibition of production and growth from sorbitol accumulation did not happen, as it does not inhibit production even at a concentration above 50 $g.L^{-1}$.

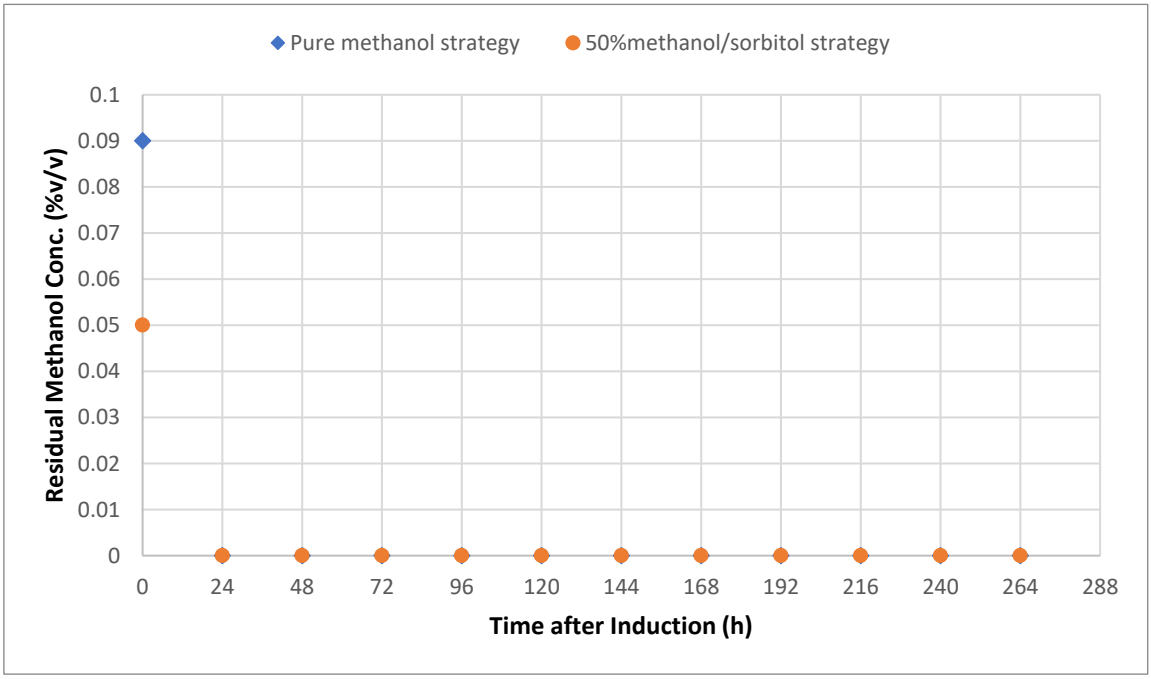


Figure 4-7 The average residual methanol concentration (%v/v) from three cultivations each after induction taken every 24 hours, including the daily harvested material during the continuous phase for the continuous cultivation of *K. phaffii* using both pure methanol induction strategy and 50% C-mol methanol/ sorbitol induction strategy.

4.2.2.2 Dilution rate and the harvest and feed rates of continuous cultivation at bioreactor scale

The dilution rate (D) was set to be the same as the specific growth rate (μ) in a steady-state chemostat process, which was calculated using equations 2.3 and 2.6 during the induction phase. Although the dilution rate was initially predicted for the continuous process using the results from the fed-batch cultivation process at 0.0106 h^{-1} and 0.0164 h^{-1} for pure methanol induction and mixed induction, the real dilution rate was found to be slightly higher for both induction strategies at 0.0119 h^{-1} and 0.0181 h^{-1} for pure methanol induction and mixed induction, respectively. The reason why it was slightly higher was that, as time progressed, the old cells and impurities were gradually taken out through the broth, which helped maintain not only the condition of the cells inside the fermenter but also maintained a healthy number of young cells inside the broth (Nieto-Taype et al., 2020; Rahimi et al., 2019). The applied condition of continuous cultivation is summarized in Table 4-2.

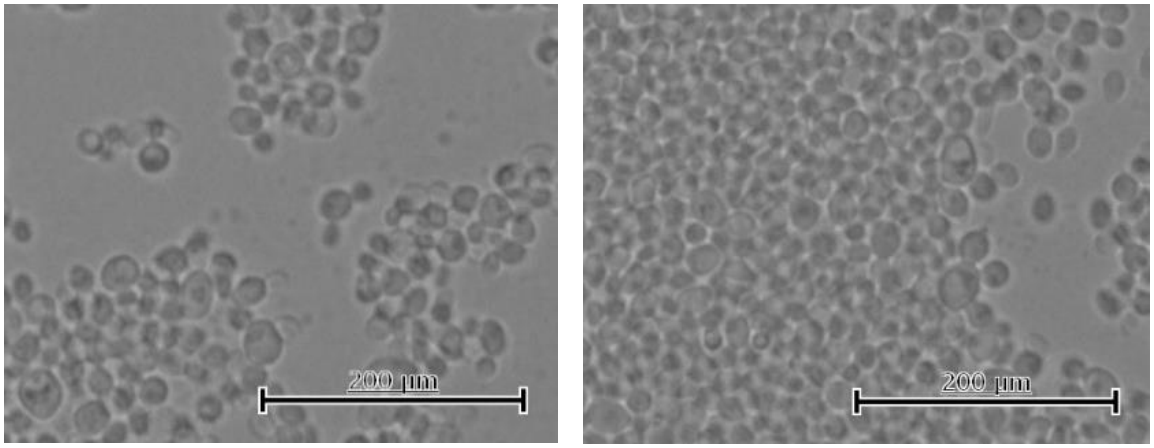
Table 4-2 The average cultivation parameters of continuous cultivation process for both pure methanol induction strategy and mixed (50% C-mol sorbitol/methanol) induction strategy. It was obtained from three cultivation repetitions using the same parameters. Data parameter is presented as mean ± SEM (standard error margin) of the experiment repeated three times.

Parameters	Methanol Continuous 80 $g.L^{-1}$ DCW Average	Mixed Continuous 100 $g.L^{-1}$ DCW Average
Initial volume (mL)	2000	2000
Volume kept during continuous phase (mL)	2063 ±179	2467 ±44
Fed-batch induction time	24	24
Continuous time (day)	10	10
Dilution rate predicted (h^{-1})	0.0106	0.0164
Dilution rate set (h^{-1})	0.0119 ±0.0019	0.0181 ±0.0005
Biomass kept constant at ($g.L^{-1}$)	84 ±4	106 ±2
Average daily harvested volume (mL)	631 ±36	1087 ±11
Methanol usage (L)	4.43 ±0.06	2.88 ±0.06
Average daily NH_4OH volume added (mL)	80 ±4	120 ±4
Average daily induction solution volume added (mL)	403 ±5	523 ±5
Average daily fresh medium volume added (mL)	148 ±27	444 ±2

From the dilution rate, the daily harvested volume and daily addition of fresh BSM medium without a carbon source and PTM_1 could be calculated using equations 2.7 and 2.9. The current volume was maintained during the continuous phase. The daily harvested volume of broth when using pure methanol as its induction solution was 631.3±36 mL, while for mixed induction, it was 1087±11 mL. The amount of broth harvested daily using mixed induction was almost double that of the method using pure methanol as its induction solution. This result correlates to the dilution rate used for the continuous cultivation process. However, the amount of fresh medium fed daily for mixed induction was almost triple the amount used in the method using pure methanol induction.

Although the continuous cultivation process was done daily, the harvest rate was kept consistent by gradually harvesting using a pump with a flow rate calculated using equation 2.7. The harvest rate of the continuous cultivation process was held at 26.18 $mL.h^{-1}$ and 45.25 $mL.h^{-1}$ for pure methanol induction and mixed induction, respectively. In addition, the broth was harvested into a bottle kept at 4-6 ^{o}C throughout the continuous harvesting process. This storage procedure aimed to ensure that the harvested broth was kept at a cool temperature to reduce the risk of the cells undergoing lysis after being harvested daily (Berrios et al., 2014). It was also found that although the broth's temperature before entering the bottle was not monitored, it had almost no impact on the condition of the broth.

The condition of the cells after the ten days of continuous cultivation was photographed and analysed in terms of their size and condition (Figure 4-8). The cells of both induction strategies during the continuous phase have similar sizes of around 10-13 µm for both types of cultivation. It was the same as the previous chapter's results from the fed-batch cultivation. In a study by Krainer et al. (2013), the standard size of a wild-type *P. pastoris* was 3-6 µm at the condition where it was no longer growing. However, the size of the cells can be larger for different cells according to the time at which some proportion of cells exists, where a cell is almost splitting into two cells, or when a target protein is being produced in cells other than its wild type.



(A) (B)

Figure 4-8 Photographs of the cells after 240 hours of induction in continuous cultivation process for pure methanol (A) and 50% C-mol methanol/sorbitol (B) induction.

4.2.3 Impact of induction strategy in continuous process on KEX2 protease content and volumetric yield

Both continuous cultivation processes using pure methanol and mixed methanol and sorbitol (50% C-mol) as its induction solution were done using the same cultivation procedure and continuous phase shift time, and the presence of KEX2 protease was verified for both cultivation processes. From these results, the differences in the product content and volumetric yield were studied and compared.

4.2.3.1 KEX2 protease content

To calculate the KEX2 protease content in the continuous cultivation of *K. phaffii*, the results must be verified to determine whether KEX2 protease exists and its concentration and quantity. Therefore, using the quantification method mentioned in Chapter 2, their profiles were examined by SDS PAGE assay, and total soluble protein quantification results were acquired. The continuous cultivation processes were run for 290 hours, or ten days of continuous harvesting.

Figures 4-3A and 4-3B show that the biomass increased until 24 hours after induction. The biomass was then consistently kept at 80 $gDCW.L^{-1}$ and 100 $gDCW.L^{-1}$ for pure methanol induction and mixed induction, respectively, throughout the 240 hours of the continuous process. It was observed from the SDS PAGE results (Figures 4-9A, 4-9C, 4-10A, and 4-10C) that the target protein

decreased after the first day of harvest for the continuous process but was then increased again at the end of the continuous process for both induction processes. The target protein decreased after the first day of harvest because the cells were still adapting to the changes made when the process was switched to continuous. Furthermore, most of the impurities generated during the shift to the continuous process were still present inside the broth. They had not been taken out through the process of dilution, which could degrade KEX2 protease more than its production in the broth. After seven days of continuous cultivation for pure methanol induction, the amount of KEX2 protease slightly went up after going down. It took five days of a continuous cultivation process using mixed induction. These changes could be a tipping point at which the impurities and the old cells were taken out through the daily harvesting process, which left only the new cells in a more optimum growing and expressing. A study showed a trend during the continuous process when there was an increase in productivity because of the regeneration of new cells inside the broth, which reduced the amount of methanol for its induction process (Nieto-Taype et al., 2020; Rahimi et al., 2019).

The KEX2 protease profile from the broth harvested daily was taken. Its supernatant was analysed by the Western blot method to verify whether the expressed protein was truly KEX2 protease for both induction strategies. KEX2 protease antibodies were used in the Western blot process with a known internal positive control containing KEX2 protease and a negative control of bovine serum albumin (BSA). It was verified that KEX2 protease existed in the induction and continuous phases with a protein size between 70-100 kDa (Figure 4-9B, 4-9D, 4-10B, and 4-10D), which complied with a KEX2 protein size of 90.8 kDa. No band appeared between 70-100 kDa before induction for the SDS PAGE Coomassie blue and Western blot results. However, the western blot results for continuous cultivation of KEX2 protease using pure methanol induction after five days of induction were found to be faint. This trend could be because of the low concentration of KEX2 protease, which was below the antibodies' limitations to detect it and shown in the scanned results.

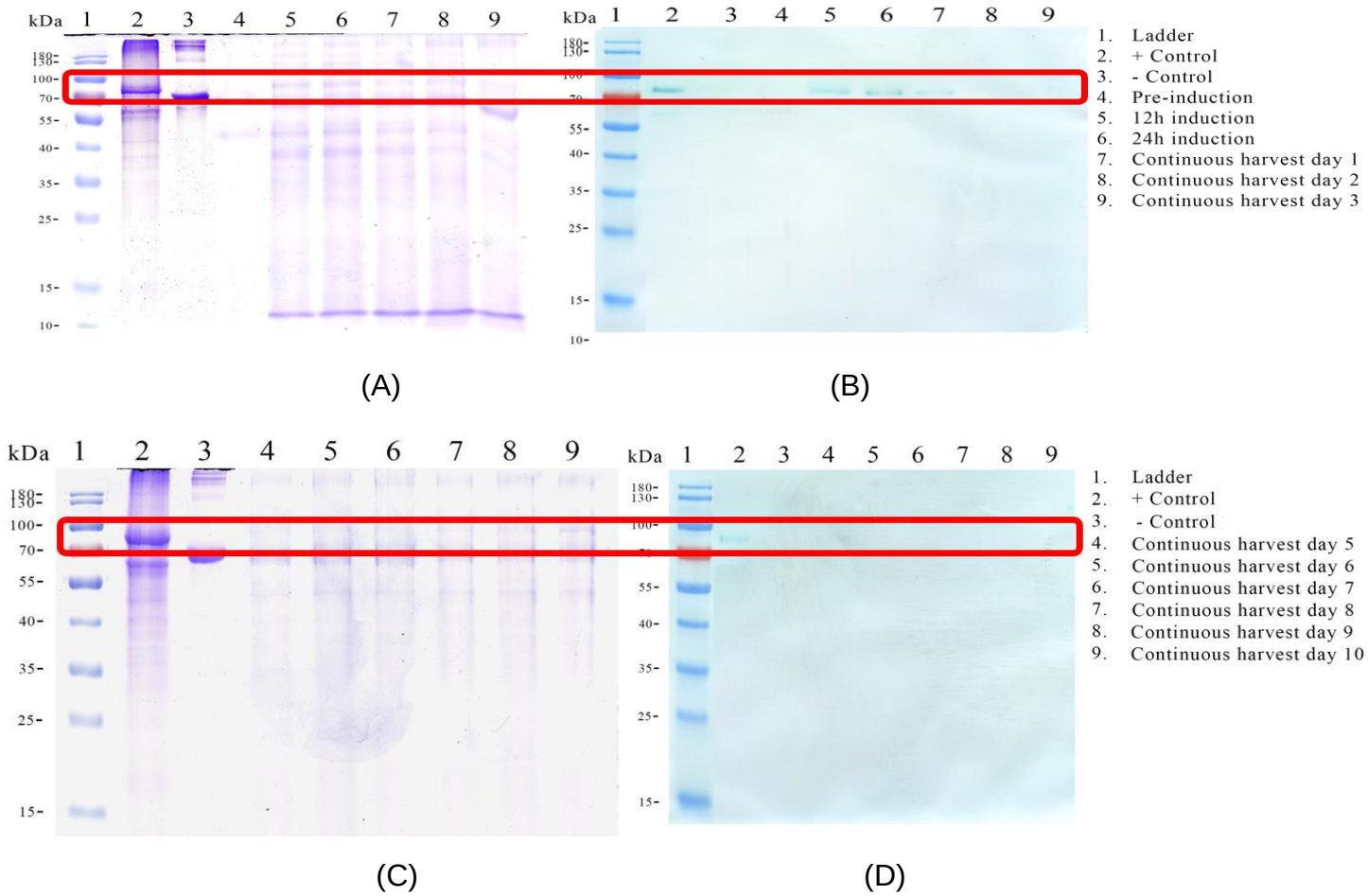


Figure 4-9 SDS PAGE results (A for part 1 and C for part 2) and western blot results using commercial KEX2 antibody (B for part 1 and D for part 2) of the cultivation process of *K. phaffii* using pure methanol induction strategy. This SDS PAGE result is a representative of three cultivation experiments with the same parameters producing consistent results. The positive control used was from the one-step purified KEX2 protease through affinity chromatography acquired in small scale expression previously produced.

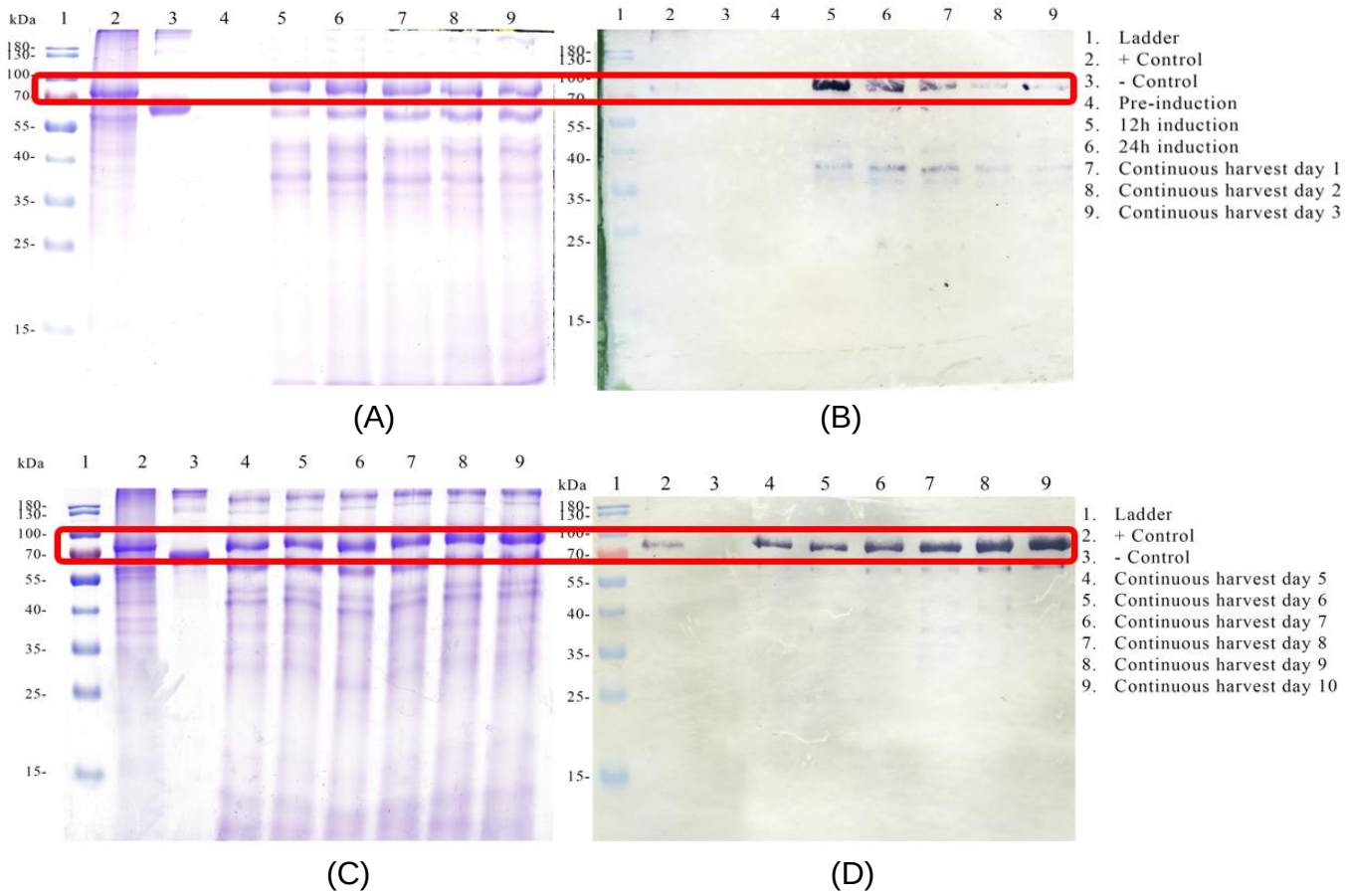
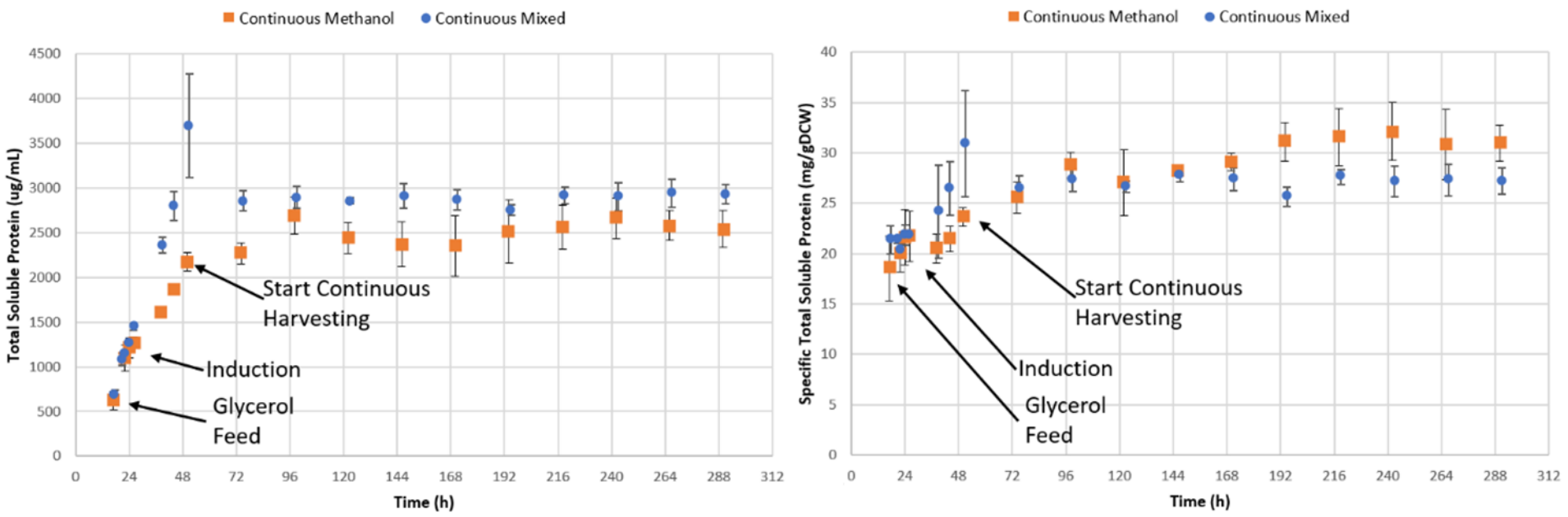


Figure 4-10 SDS PAGE results (A for part 1 and C for part 2) and western blot results using commercial KEX2 antibody (B for part 1 and D for part 2) of the cultivation process of *K. phaffii* using 50% C-mol methanol/sorbitol induction strategy. This SDS PAGE result is a representative of three cultivation experiments with the same parameters producing consistent results. The positive control used was from the one-step purified KEX2 protease through affinity chromatography acquired in small scale expression previously produced.

The results (Figures 4-9, 4-10) also showed that using a mixed induction strategy gave rise to a better expression level of KEX2 protein, as indicated by the intensities of both SDS-PAGE and western blot analysis data, where the intensity of the target band at 90.8 kDa is denser as compared to those in a pure methanol strategy. This finding of the results before the shift to the continuous phase was consistent with what was observed in the fed-batch cultivation method.

Based on previous studies done by Wang (2019) and Woodhouse, (2015), a mixed induction strategy was developed by replacing 50% C-mol of methanol with sorbitol, which resulted in higher biomass but reduced volumetric product yield.

From the results of the figure 4-11A, it was found that although the total soluble protein concentration (ug.mL^{-1}) of mixed induction solution was found to be higher than pure methanol induction because of its higher biomass, the amount of total soluble protein excreted per biomass was found to be lower in mixed induction than pure methanol induction. This trend indicates that the amount of total soluble protein quantified, which could be composed of target and non-target proteins when using a mixed induction strategy, is higher than when using a pure methanol induction strategy due to the higher biomass generated in the mixed induction strategy. Nevertheless, total soluble protein per biomass cell (Figure 4-11B) shows that more total soluble protein per cell was produced in the methanol induction strategy compared to the mixed induction strategy. Overall, the total soluble protein concentration for both induction processes was consistent throughout the continuous process, fluctuating around 2500 ug.mL^{-1} and 2900 ug.mL^{-1} for pure methanol and mixed induction, respectively.



(A) (B)

Figure 4-11 Average total soluble protein in terms of concentration (ug.mL^{-1}) (A) and in terms of mg.gDCW^{-1} (B) acquired through the quantification method BCA assay of the supernatant from the fed-batch cultivation process of *K. phaffii* using pure methanol induction and mixed (50%C-mol methanol and sorbitol) induction during the induction process from three cultivation experiments with three samples each.

The amount of KEX2 protease content in the harvested supernatant was quantified using Totallab^{TM} Quant densitometry by analysing the SDS PAGE results of both induction strategies (raw data calculation shown in Appendices F). As shown in figure 4-12, the content peaked within the first day of harvest for both inductions and decreased to around 1% for pure methanol induction before increasing again. As for the results for mixed induction, the highest KEX2 protease content was also on the first day of harvest before decreasing slightly and increasing again to near or above the first day of harvest on the tenth day of harvest. The KEX2 protease concentration could be obtained using the results in Figures 4-11 and 4-12. It was found that although the KEX2 protease content for pure methanol induction fluctuated and was lower than for mixed induction strategy over the continuous period, the specific total soluble protein concentration for the pure methanol induction strategy was higher, which shows that there may be more impurities being produced in methanol induction when compared with mixed induction. This result is consistent with a previous study by Wang et al. (2019), which shows that a mixed induction strategy may produce lower HCP than a pure methanol induction strategy.

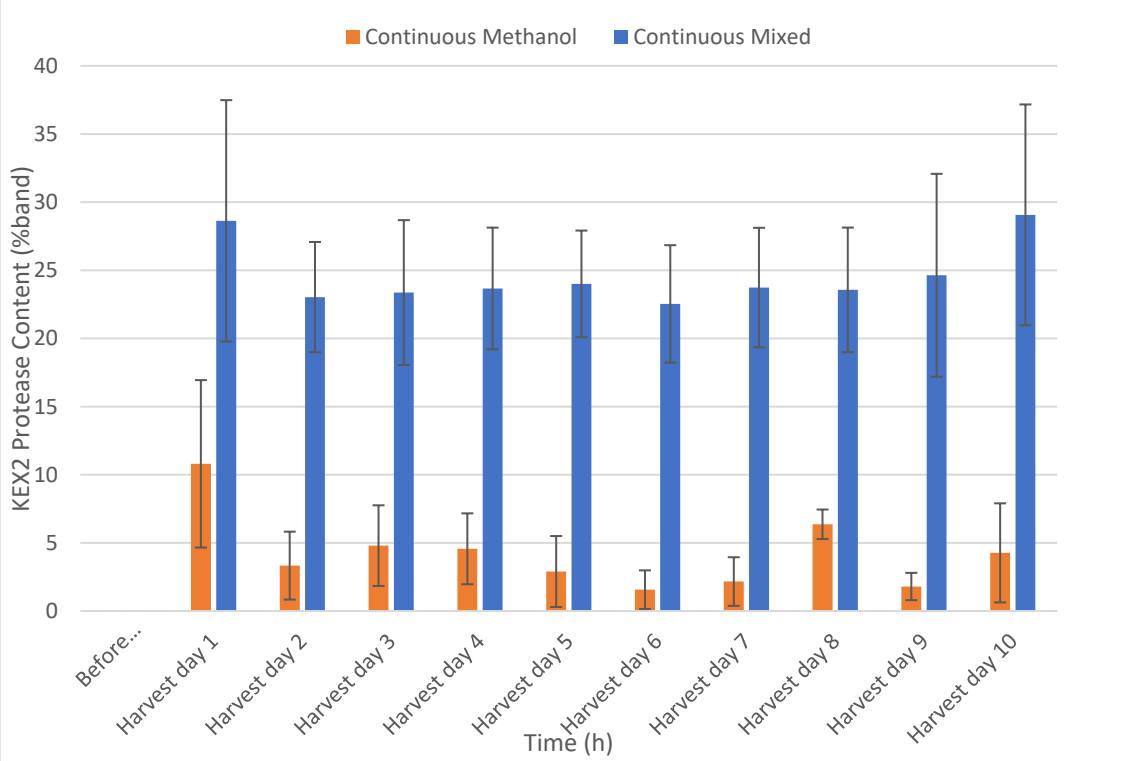


Figure 4-12 The average percentage of KEX2 protease content (%band) detected through Totallab Quant^{TM} densitometer analysis compared between the continuous cultivation of *K. phaffii* using pure methanol induction and mixed (50% C-mol methanol and sorbitol) induction during the continuous process from three cultivation experiments with three samples each.

After acquiring the total soluble protein (TSP) and KEX2 protease content (%band), the KEX2 protease concentration or volumetric yield of the continuous phase's start, middle, and end, and the final yield (mg) from a single 'batch' were acquired. Table 4-3 shows that the total soluble protein concentration is higher in cultivation with mixed induction than pure methanol induction because of the difference of biomass of 100 gDCW.L^{-1} and 80 gDCW.L^{-1}, respectively. The profile is also consistent with the target-to-non-target protein ratio and KEX2 protein content. Moreover, the yield (mg) of KEX2 protease from a 'batch' of the continuous process resulting from mixed induction methodology is higher than that from the pure methanol method, with total amounts of 1154.9 ±870 mg per batch and 9932.7 ±2980 mg per batch, respectively, when adding the average daily harvest volumetric yield and also the final harvest volumetric yield acquired from harvesting the broth inside the fermenter. A non-parametric Mann-Whitney test was also done to investigate whether there is a significant difference between the calculated harvested yields of both induction strategies in the continuous process. The result was shown in Figure 4-13 as a one-tailed p-value, which shows that the result was significantly different with a p-value smaller than 0.05. This KEX2 protease overall yield analysis result further highlights the benefit of implementing mix-mode induction instead of pure methanol as an inducing agent in continuous cultivation involving *K. phaffii*.

Table 4-3 The average yields of KEX2 protease in continuous cultivation process from three cultivation experiments and three samples each through the use of the quantified total soluble protein and KEX2 content acquired from Totallab™ Quant.

'1 batch'	Total Soluble Protein (TSP) (mg.L^{-1})	KEX2 Protease Content (Densitometer) (%band in lane)	KEX2 Protease Concentration (TSP x Content) (mg.L^{-1})	KEX2 Protease Yield from final harvest (10 days) (mg)
Methanol				~8.2 L Harvested
Before induction	620.6 ±105	0	0	1154.9 ±870
Harvest day 1	2268.8 ±120	10.8 ±6.1	245.0 ±130	
Harvest day 5	2354.1 ±340	2.9 ±2.6	68.3 ±80	
Harvest day 10	2540.4 ±200	4.3 ±3.6	109.2 ±110	
Mixed				~13.5 L Harvested
Before induction	697.2 ±40	0	0	9932.7 ±2980
Harvest day 1	2853.4 ±110	23.9 ±8.3	682.0 ±260	
Harvest day 5	2868.9 ±110	25.0 ±3.2	717.2 ±120	
Harvest day 10	2927.8 ±110	27.6 ±8.8	808.0 ±290	

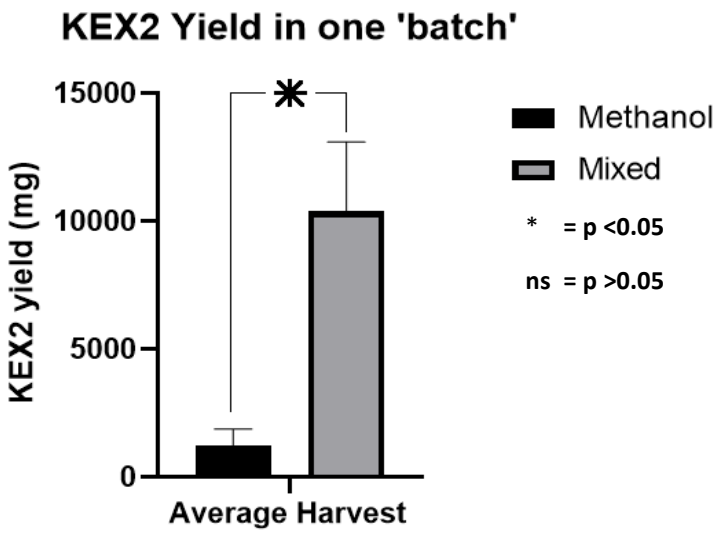


Figure 4-13 The average yields of KEX2 protease in a batch comparison between the continuous cultivation of *K. phaffii* using pure methanol induction and mixed (50%C-mol methanol and sorbitol) induction process from three cultivation experiments each.

The benefit of the synergy of combining mixed 50%C-mol methanol/ sorbitol inductions strategy with continuous cultivation process would also further increase if, hypothetically, the time of continuous phase were done were more than 10 days. Multiple studies (Marx et al., 2009; Ohi et al., 1998; Zhu et al., 2009) showed that *K. phaffii* could grow and express stably for at least 28 and up to 83 generations in a methanol environment, according to the design of the strain. It was calculated from the 12 days of the continuous process; the cells were grown for a maximum of 18 generations. Furthermore, when grown in a more flexible environment, such as the mixed induction method (lower methanol concentration), the number of generations of *K. phaffii* can be grown in a much longer process time.

4.3 Comparison of Fed-batch and Continuous results

The effect of fed-batch cultivation method in terms of KEX2 yield (mg) after 24 hours of induction using pure methanol and mixed induction strategies were described and compared in the previous chapter. In this chapter, the KEX2 yield (mg) produced using both induction strategies in continuous cultivation mode is discussed. The yield of KEX2 produced per batch using all four combinations of cultivation modes and induction strategies were then compared through a non-parametric one-way ANOVA Kruskall-Wallis multiple comparison test to determine whether there is any significant difference between the mean calculated harvested yields.

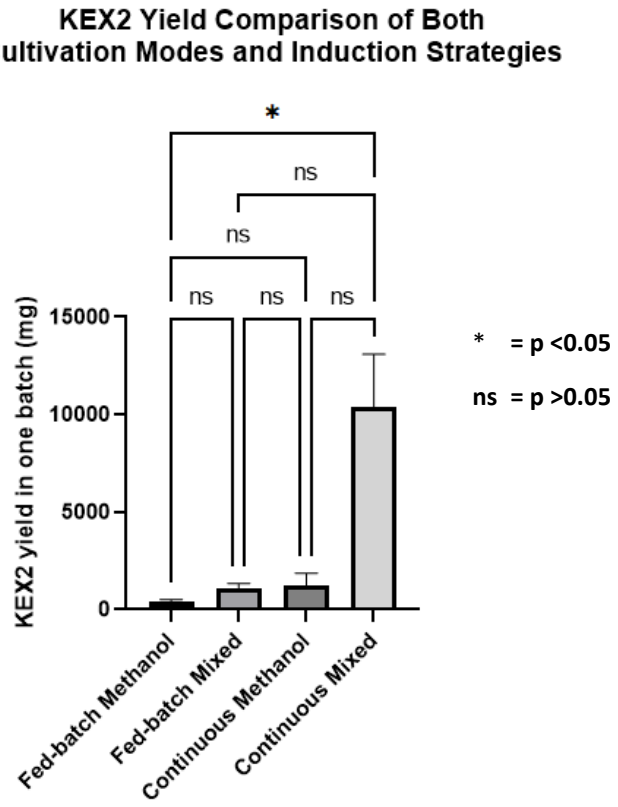


Figure 4-14 The average yields of KEX2 protease in a batch comparison between the two cultivation modes (fed-batch and continuous cultivation) of *K. phaffii* using either pure methanol induction or mixed (50%C-mol methanol and sorbitol) induction process from three cultivation experiments each.

The analysis result (Figure 4-14) showed that the continuous cultivation with mixed induction strategy produced the highest yield per batch when compared to the other methods. It was also found that the difference was statistically significant when compared to the fed-batch cultivation using pure methanol induction strategy, with a p-value lower than 0.05. Even though the rest of the methods are not significantly different when compared to continuous cultivation with mixed induction method, with a p-value higher than 0.05, the fold difference of the yield is pronounced: 8.6-fold, 8.9-fold, and 21.3-fold increase for continuous mixed compared to continuous methanol, fed-batch mixed, and fed-batch methanol method respectively. Given that each method was done only in triplicates, the statistical analysis could be impacted which needs further evaluation such as by increasing the number of replicates for each method that could improve the sensitivity of the analysis. However, the trend of the method's performance presented as KEX2 yield per batch that was shown in the data already demonstrated the superiority of the continuous mixed method compared to the others.

4.4 Conclusion

This chapter evaluated KEX2 protease production using continuous cultivation with pure methanol, and mixed induction solution strategy. It was found that using a continuous cultivation process with a mixed induction strategy had a synergistic effect and produced about 8.6-fold increase of the KEX2 protease yield (mg) when compared to continuous cultivation with a methanol induction strategy. The main reason for the increase in KEX2 protease yield in a single 'batch' is the increase in the growth rate of *K. phaffii*, which led to an increase in the volume of daily harvested broth. Thus, KEX2 protease content increased as old cells were washed out, leaving younger cells to produce. In addition, a benefit of the mixed induction method was also found that it used less oxygen and produced less heat.

A statistical comparison analysis was done on the yield of KEX2 for all four combinations of cultivation modes and induction strategies. Continuous cultivation using a mixed induction strategy produced the highest KEX2 yield per batch. It was significantly different (p-value below 0.05) when compared with the fed-batch method using pure methanol induction, when analysed using one way ANOVA Kruskall-Wallis multiple comparison test. Although other methods did not show a significant statistical difference compared to the continuous mixed

method, the yield differences were substantial. However, further research would be needed to enhance the statistical robustness, such as increasing the number of replicates in each method.

K. phaffii is known to have a stable genomic capability that can extend the continuous process by up to 3 months, which should improve the continuous process's efficiency. However, the potential drawbacks were also detected, as the method requires more medium to be added daily to keep the volume inside the fermenter constant. Thus, further investigation is needed to determine how much a mixed induction strategy benefits the continuous cultivation process in terms of economic parameters. Additionally, the condition of the KEX2 protease should be further investigated using other methods such as protease activity assay from the results of continuous fermentation after being purified to verify the activity of the product. This thesis is the first study to examine how the continuous cultivation process with mixed induction works, especially when making KEX2 protease.

Chapter 5: Simulating Fed-batch and Continuous Cultivation Process for Expression of Recombinant Protein using *K. phaffii*

5.1 Introduction

The main objectives of this chapter were:

1) To compare harvested KEX2 protease yield from a batch, process time, and costs, focusing on the difference in 5 L cultivation scale for fed-batch or continuous cultivation methods with pure methanol or a combination of sorbitol and methanol induction strategies.
2) To simulate and compare the impacts of using the conventional method (fed-batch process with pure methanol induction) and the combination method (50% C-mol sorbitol and methanol mixed induction and continuous cultivation) on an industrial scale.

Increasing demand for recombinant protein-based products has led to increased use of a promising host, *K. phaffii*. However, *K. phaffii* mainly uses fed-batch cultivation involving methanol as the primary inducing agent, which emphasizes a need for a more efficient and cost-effective methodology for its production process.

In addition, process variability is impacted by how robust and efficient the production process's methodology is. The variability often occurs due to changes in critical process parameters (CPPs), key parameters of interest (KPIs), or critical material attributes (CMAs), which can contribute to changes in critical quality attributes (CQAs). CPPs can change during cultivation and need appropriate control strategies to reduce process variability. Advances in process analytical technology (PAT) benefit biopharmaceutical product development. Various research projects towards developing new and more efficient technologies in the production of biopharmaceutical products using *K. phaffii* with goals to reduce the cost of producing recombinant proteins have been developed, such as strain engineering, process engineering (Dai et al., 2021; de Sá Magalhães & Keshavarz-Moore, 2021). According to Fernandes et al. (2015), continuous bioprocesses offer many advantages over the fed-batch

methodology, and it is essential to identify CPPs, CQAs, and PATs during the process transition.

This study investigated a novel processing technology to improve volumetric recombinant protein yield, with KEX2 protease used as a model target protein. The feasibility of producing the protein using the existing strategy, a fed-batch cultivation method with methanol as an inducing agent (conventional method), and a novel approach that combines two strategies that have been studied, a continuous cultivation method with a mixed induction agent of 50% C-mol sorbitol and methanol (combination method), was evaluated. Furthermore, an analysis of how this method will affect the industry regarding its techno-economic feasibility was also conducted. The evaluation was done by performing a modelling analysis that scaled the process to the production scale. Even if only a few cents per gram of recombinant protein is produced, a cost reduction is highly desirable for industries as it will impact their market price and profitability or return on product investments. Therefore, the ability to predict the costs of production prior to implementing new technology in production operations is of paramount importance.

The investigation was done by analysing the known effects of the continuous cultivation method with a mixed induction agent, 50%C-mol sorbitol and methanol (combination method), in comparison to the conventional method and its effects in the industry from a practical point of view, then designing the model to simulate both methodologies and their operation conditions, which allows manufacturers to estimate the results of alternative processes with confidence. Studies investigating the benefit of using the novel strategy compared to the conventional method were done and reported in previous chapters 3 and 4. An analysis was performed to simulate the processing technologies in a pilot plant operation by simulating and estimating the economic parameters impacted by both processes. The following section describes the modelling analysis of both methodologies and the analysis to determine the effect of utilizing conventional and combination methods in pilot plant operations on a 1000-litre scale bioreactor.

5.2 Theoretical considerations

5.2.1 Industrial simulation assumptions

Some assumptions were made to reduce the difficulty of the calculations so that a simulation of the traditional and combination methods could be done. These assumptions are:

- The focus of the simulations will be on the production of KEX2 protease, and the bioreactor at which the process is occurring
- All materials and utilities used will have the same defined value for all methodologies unless stated otherwise
- All equipment used was of the same quality, scale, and cost unless stated otherwise
- The simulated production scale fermenter used was equal to or smaller than the 1000 L total bioreactor volume
- The total working volume of the pilot scale simulations were done at 400 L for fed-batch and continuous cultivation process, and 40 L for continuous cultivation process which would be predicted to have the same volume being harvested at the end of the process
- Two workers as operational staff per day with three shifts of eight hours daily which comprised equipment preparation, process, and maintaining the condition of the process.
- All liquid materials entering the bioreactor were going through the same type of sterilization process unless mentioned otherwise
- Utilities such as compressed air, cooling water, and steam are generated outside of the system and are thus not included in the costs as they will be assumed to have the exact costs when comparing the strategies.
- Supporting equipment that produced compressed air, cooling water, and steam will not be included in the capital cost calculation as they were the same for both processes.

5.3 Results and discussion

The results from the previous chapters were analysed to determine whether the combination (continuous cultivation and mixed induction) process has more benefits in terms of its industrial impacts and techno-economic feasibility on a 5 L scale. Subsequently, a simulation of the conventional and combination processes was performed at an industrial scale, and the difference in impact from both methodologies was then compared.

5.3.1 Process Model Simulation

Using two induction strategies, a simulation of the industrial production of KEX2 protease was made for fed-batch cultivation and continuous cultivation processes using the SuperPro Designer® process simulator. Figure 5-1 shows simplified flow diagrams for the fed-batch and continuous cultivation methods. The actual industrial process on a production scale would involve more than 100 pieces of equipment and unit operations, such as equipment to ensure the sterility and condition of the lab and the process, which includes the production of steam and pure water to ensure the operations of the main production processes. This analysis is not intended to replicate whole production processes of recombinant protein production, but rather a generic plant design simulation mainly containing equipment and unit operations necessary to produce recombinant protein KEX2 protease, specifically the processes that represent the differences between the conventional and the combination method. Also, because the process and equipment will be the same, the main focus of the analysis was on the lab scale, production bioreactor, and how it affects the subsequent downstream processes.

In this simulation, the size of bioreactors would be differentiated into lab and production scales. In a lab-scale simulation, the bioreactor size that would be used would be a 5 L bioreactor, which was the same as the scale that was used in the previous chapters 3 and 4. On a lab scale, other equipment that will be used to support the activity of the bioreactor and preparation for the solutions and medium would also be of similar or smaller size. On the other hand, lesser than or equal to 1000-litre size bioreactors would be used in the simulation for production scale. Bioreactors with capacities of up to 10,000 L are standard in industrial settings. However, having a large bioreactor would also require a larger facility and produce a larger carbon footprint. Another drawback of a bigger

bioreactor is that the process would be harder to control and might not be as productive as a smaller-scale version. Furthermore, continuous cultivation requires a more advanced monitoring and control system, which will require higher capital cost. Setting the size of the simulation of the bioreactor to 1000 L or less would also open up the possibility of using the results for a single-use bioreactor, as the standard size of a single-use bioreactor was found to be between 15 mL and 2000 L (Collignon Marie-Laure & Williams Alex, 2020).

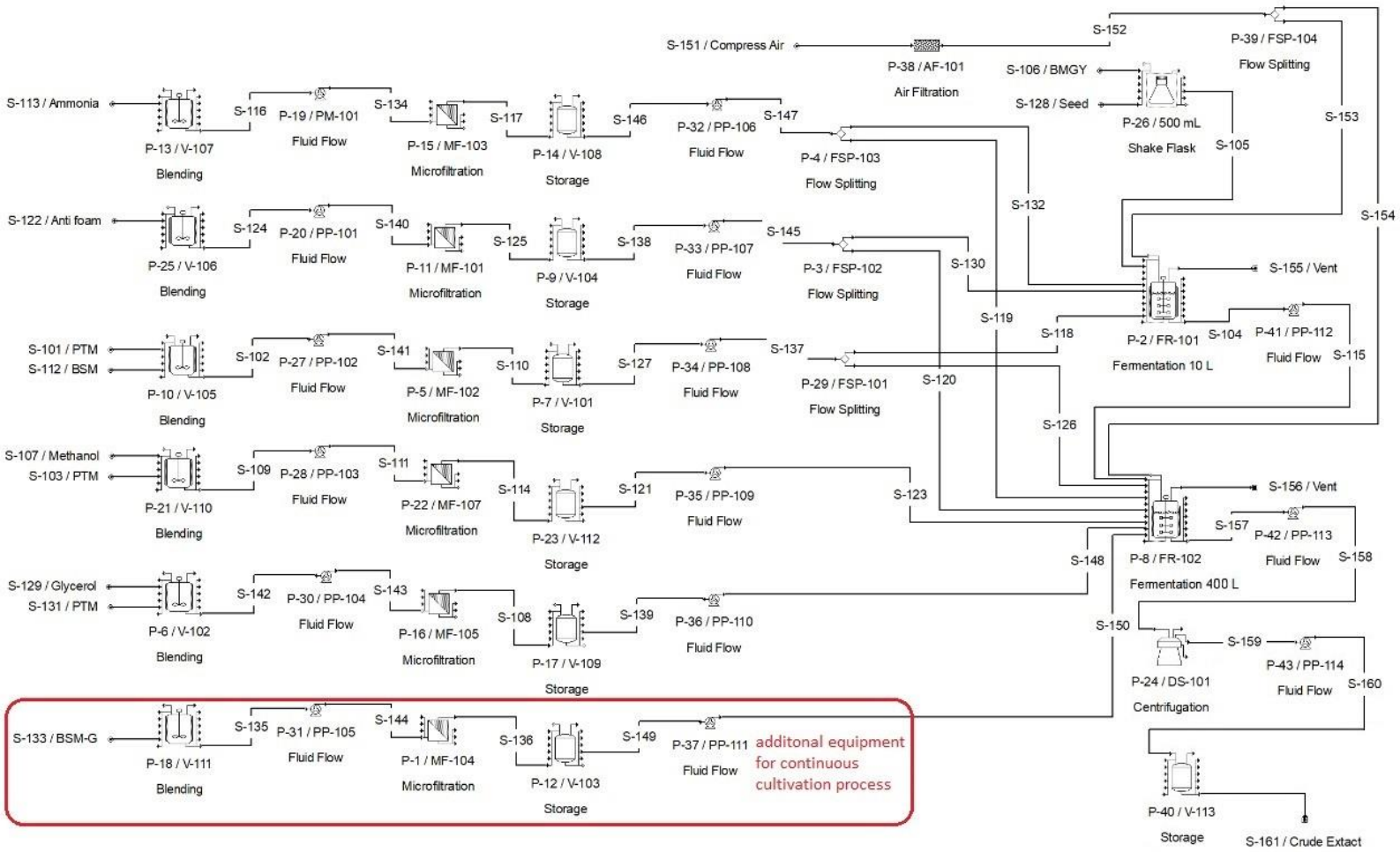


Figure 5-1 Simplified schematic diagram flow process of fed-batch cultivation processing and continuous cultivation processing (with additional equipment in the red square) for production of KEX2 protease at production scale of 400 L. The schematic diagram is also applicable for 40 L production scale for continuous cultivation; however, the scale of all other equipment must be made smaller accordingly.

The model simulation shows how the process works, how much energy it needs, and how the equipment works for the given operating scenario. To conduct the analysis, volumes, the composition of the medium and other physical characteristics of the input and output streams for each piece of equipment in the studied processes were identified. The information was used for utility consumption and purchased equipment costs for each item.

From figure 5-1, the main difference in the simplified schematic diagram of a simulated industrial production of KEX2 protease using a fed-batch cultivation process and a continuous cultivation process was mainly due to adding a continuous feed of BSM without glycerol and having the harvesting process done daily and on a smaller scale for the continuous cultivation process instead of at the end of the batch in the fed-batch cultivation process.

The medium composition used in fed-batch and continuous processes is the same. However, the total amount of material fed into the bioreactor differs in both methods. Nevertheless, based on the experimental data generated by all the methods, as reported in previous chapters, the KEX2 protease volumetric yield and the number of solutions and media could be estimated. Table 5-1 demonstrates an overview of critical unit operations and setting in the process model from small scale operations.

5.3.2 Techno-economic analysis of mixed induction and continuous cultivation process on lab scale results

The previous chapters highlighted the benefits of using mixed induction by comparing cell growth rate and volumetric yield in both fed-batch and continuous processes. However, comparing only the cell growth rate and volumetric yield will not mean the modification is more feasible than its base process. Therefore, an economic evaluation of the methods was done to assess the impact of the methodologies on the efficiency of the process. Table 5-1 presented the yield from a single batch (mg), materials, and process time of the fed-batch and continuous cultivation processes when using pure methanol as the induction solution or 50% C-mol methanol/sorbitol mixed induction strategy in the production of KEX2 protease. In this chapter, a comparison will be made on the lab scale (5 L bioreactor) of production for both cultivation strategies, and the

results will be further used and discussed on the production scale simulated using SuperPro Designer® simulation tool.

Table 5-1 Fed-batch and continuous cultivation KEX2 protease yield using pure methanol and mixed (50% C-mol methanol/sorbitol) in the production of KEX2 protease in 2 L working volume scale.

1 Batch	Fed-batch		Continuous	
	Methanol	Mixed	Methanol	Mixed
Yield (mg)	466.8 ±80	1115.6 ±240	1154.9 ±870	9932.7 ±2980
Initial Volume (L)	2	2	2	2
Total Process Time (day)	5	5	15	15
Total Harvested Volume (L)	~2	~2	~8.2	~13.5
Total Additional Medium (L)	-	-	1.5	4.5
Total Induction Volume (L)	0.6	0.6	4.6	5.9
Total NH_4OH Volume (L)	0.26 ±0.03	0.3 ±0.03	1 ±0.3	1.5 ±0.3
Total Antifoam Volume (mL)	5 ±3	5 ±3	5 ±3	5 ±3

5.3.2.1 Yield and time

In the production of KEX2 protease, Table 5-1 shows that, for a single batch of 2 L working volume, the conventional cultivation method using pure methanol induction resulted in the lowest yield (mg). On the other hand, using a mixed induction strategy increased the yield in the production of KEX2 protease by more than 130% in the fed-batch process and by more than 830% in the continuous cultivation process. However, from another point of view, when the total process time was made to be the same for the continuous cultivation process at 15 days of process time for the fed-batch processes (which multiplies the number of batches for fed-batch cultivation to 3 batches), the continuous process using pure methanol would have the lowest overall KEX2 protease yield. Thus, although using a mixed induction strategy was found to have varying results for different products, it had an overall increase in yield in the production of KEX2 protease. On the other hand, continuous cultivation on its own, which also had varying results for different products, had a negative impact on the production of KEX2 protease but had a significant positive Impact when combined with a mixed induction strategy.

As previously mentioned, mixed induction had varying impacts on the product volumetric yield according to the target product. Thus, it was recommended to be tested on a small scale first to determine whether it was a feasible method to increase volumetric yield. A study by Çelik et al. (2009) in producing rHuEPO found a higher volumetric yield of 130 $mg.L^{-1}$ with the presence of sorbitol during

induction compared to the sole presence of methanol at a volumetric yield of 80 $mg.L^{-1}$ of rHuEPO after 24 hours of induction. However, in the same study, the specific yield coefficient ($mgrHuEPO.gcell^{-1}$) was lower in the presence of sorbitol during the induction phase when compared to the sole presence of methanol. In another study done by Wang (2019) using a mixed induction strategy as its leading research, the volumetric yield ($g.L^{-1}$) of aprotinin was found to be lower when using sorbitol and methanol mixed induction when compared to pure methanol after 90 hours of induction, which were 1.05 $g.L^{-1}$ and 1.69 $g.L^{-1}$ respectively.

On the other hand, the continuous cultivation process, which was also found to have varying volumetric yield results, has a different impact on the production of KEX2 protease compared to the mixed induction strategy. In the production of KEX2 protease, the continuous cultivation process with pure methanol as its induction solution only slightly increased the yield acquired in a single ‘batch’, which shows that it is not a feasible process for increasing the productivity. However, in a study by Yamawaki et al. (2007), the production of single-chain fragment antibody (scFv) using a continuous cultivation process with pure methanol as its induction solution yielded volumetric productivity of 7-9 $mg.L^{-1}.h^{-1}$. In another study by Paulová et al. (2012), the production of extracellularly produced trypsinogen with a mixture of methanol and glucose as its induction solution yielded a volumetric productivity of up to 10.9 $mg.L^{-1}.h^{-1}$. As productivity varies for different products, using a continuous process is not attractive. Furthermore, there are other reasons that make the continuous cultivation process less appealing than other technologies for increasing yield, such as increasing the amount of material used, requiring a more consistent quality of materials to ensure the consistency of the process, a more complex process, a greater susceptibility to contamination, and regulatory hurdles. These are considered the main challenges in utilizing the continuous process.

Using mixed induction and continuous cultivation (combination) processes was hypothesized to eliminate or reduce the drawbacks of each method when done individually and increase the cultivation process's efficiency, promising a more definite and feasible process. This hypothesis was found to be true in the previous chapter. Combining a mixed induction strategy with continuous cultivation increased the KEX2 protease yield to 9932.7 mg in a batch that lasted fifteen

days (including preparation and cleaning). The KEX2 protease volumetric yield was also found to be harvested on average of 735.8 $mg.L^{-1}$ or specific volumetric productivity of 13.3 $mg.L^{-1}.h^{-1}$ (or 0.133 $mg.gDCW^{-1}.h^{-1}$). In comparison, the fed-batch cultivation process using pure methanol induction strategy (conventional) only produced 466.8 mg in a batch that lasted five days, which equals a volumetric yield of 233.4 $mg.L^{-1}$. Table 5-2 shows a comparison of the volumetric yield and specific volumetric productivity acquired in this thesis when compared to results from other studies and shows the significance of combining continuous cultivation mode with mixed induction strategy.

Table 5-2 Comparison of volumetric yield ($mg.L^{-1}$), and specific volumetric productivity ($mg.L^{-1}.h^{-1}$) of KEX2 when compared to other products when using *K. phaffii* in specified combination of cultivation modes and induction strategies.

Product FB= Fed-batch C= Continuous PM= Pure Methanol Mix= Methanol/Sorbitol	Volumetric Yield of Target Protein ($mg.L^{-1}$)	Specific Volumetric Productivity ($mg.L^{-1}.h^{-1}$)	Source
KEX2 (FB +PM)	233.4	-	Chapter 3
KEX2 (FB +Mix)	577.8	-	Chapter 3
rHuEPO (FB +PM)	130	-	(Çelik et al., 2009)
rHuEPO (FB +Mix)	80	-	(Çelik et al., 2009)
Aprotinin (FB +PM)	1690	-	(Wang, 2019)
Aprotinin (FB +Mix)	1050	-	(Wang, 2019)
HBsAg (FB +PM)	191.8	-	(Rahimi et al., 2019)
KEX2 (C +PM)	144.4	1.7	Chapter 4
KEX2 (C +Mix)	735.8	13.3	Chapter 4
single-chain fragment antibody (scFv) (C +PM)	810	7.6	(Yamawaki et al., 2007)
Trypsinogen (C +Glucose/Methanol)	157	11.0	(Paulová et al., 2012)
HBsAg (C +PM)	187.1	1.7	(Rahimi et al., 2019)

When comparing the amount of the target product (KEX2 protease) made by increasing the number of batches in a conventional fed-batch process to a 15-day continuous cultivation process, the fed-batch cultivation process only made about 4.6% of the amount of KEX2 protease made by a combination of mixed induction and continuous processes. Thus, in terms of KEX2 protease yield (mg), at the same period of production time of 15 days in a 5 L bioreactor scale, the continuous cultivation method using mixed induction resulted in a significantly higher KEX2 protease yield than that acquired from fed-batch cultivation with a pure methanol induction strategy.

5.3.2.2 Capital costs and operational costs

In defining whether it is more feasible to use a combination process compared to a conventional process, the main points of comparison were set to be capital expenditure cost and operating cost.

The capital costs can be separated into equipment cost, patent cost, laboratory construction cost, and land cost. However, on the lab scale, since the same lab and land were used in the cultivation process, it can be summarized as having the same lab and land cost. The patent cost will not be introduced in this comparison as the technologies will be assumed to not use any patent and would not incur any cost. Thus, the main difference in capital costs at the same scale between using fed-batch and continuous cultivation was the additional pump, bottle, and cooling apparatus for the fresh medium feed and harvest. The additional equipment for continuous cultivation can be an extra 30% of the fed-batch cultivation process.

The operating costs in a cultivation process can be separated into media, utility, disposable equipment and materials, and labour costs (Amasawa et al., 2021). The number of people working each day will be the same to simplify the labour cost. However, the time for one continuous batch process was equivalent to three times fed-batch cultivation. Therefore, regarding labour costs, the continuous cultivation process can be simplified to three times the rate at which fed-batch cultivation was set.

As for utility costs, Niu et al. (2013) predicted that the constant amount of cooling needed for a bioreactor with a mixed induction strategy would be lower because it would release less energy during digestion than pure methanol. This difference

would mean less energy would be needed to keep the temperature constant. However, the continuous process processing time was three times that of the fed-batch process, and thus the utility cost would still be at least three times that of the fed-batch process.

Lastly, the material cost will be defined by the medium amount and other used liquids such as medium, base, glycerol, and induction solution. The price of the materials was defined, converted into $USD, and shown in Table 5-3 in terms of per litre, and Figure 5-2 shows the price of materials used for the medium for each cultivation type. By using the price in table 5-3, the cost of materials was calculated and shown in figure 5-2, which shows the cost of materials used in a single batch for continuous cultivation process using mixed induction strategy was calculated to be USD$479.04, while fed-batch cultivation using pure methanol was calculated to be USD$131.17 from a single batch (raw data calculation for material costs shown in Appendices G).

Table 5-3 The price of the media used in cultivation process per 1L predicted as of 11th July 2022 in $USD.

Media	Price per Litre ($USD)
BMGY	63.99
BSM	28.30
PTM	21.90
Ammonia	7.73
Antifoam	86.61
Induction (pure)	43.64
Induction (mix)	52.85
Glycerol Feed	206.20
BSM-G	11.71

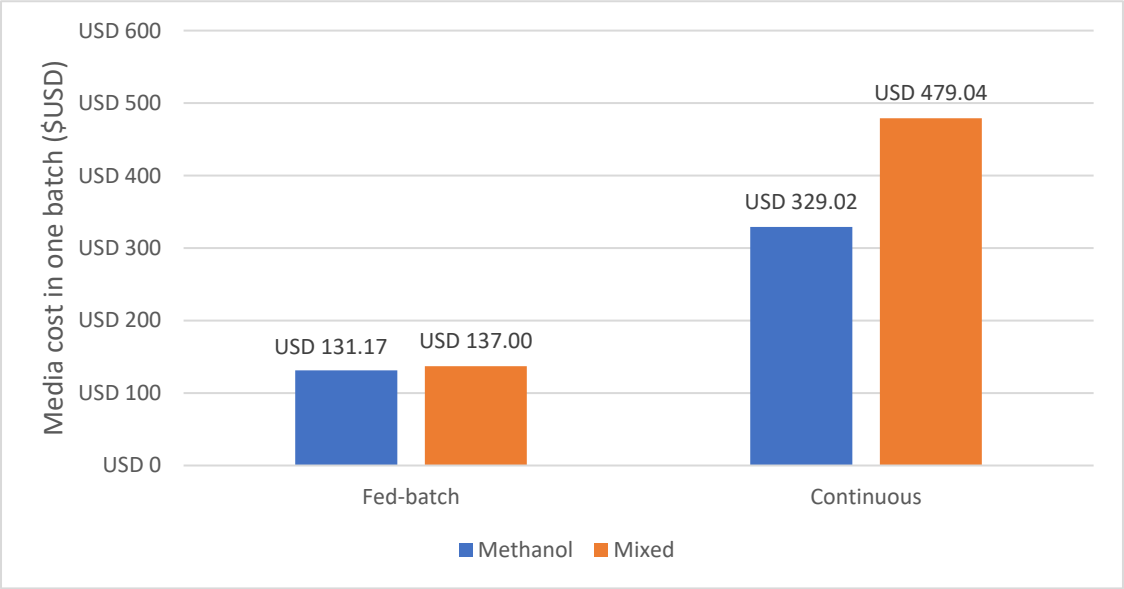


Figure 5-2 The cost of material comparison of each cultivation type (fed-batch with pure methanol, fed-batch with mixed induction, continuous with pure methanol, and continuous with mixed induction) from a single batch in 2 L working volume scale.

5.3.2.3 Overall techno-economic analysis on lab scale results

Although the combination method was found to have a much larger yield in one batch and productivity, it also required a more considerable amount of investment in terms of capital cost and operating cost in a single batch. However, the increase in costs can be covered by the much higher productivity that it produces. As shown in Figure 5-3, even though the increase in operating costs was at least tripled and there was a third increase in capital costs, the amount of yield increased in the combination method in a single 'batch' by at least 20 times compared to the conventional method. Increasing the processing time of the fed-batch to be the same as the combination method was also unable to meet the harvested yield acquired in the combination method in a single batch. Therefore, even though the method is feasible, it still needs to be investigated to see how much is being made from the price of KEX2 protease and how much the capital and operating costs of the conventional method are. The methodology with the highest probability of being feasible is the combination method.

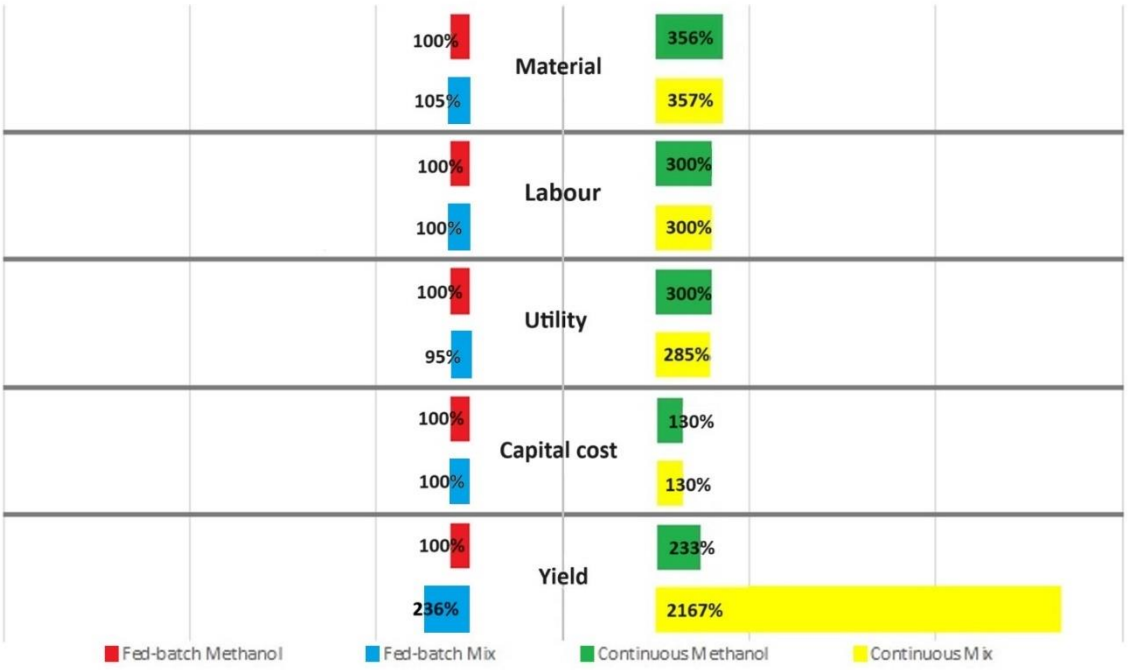


Figure 5-3 Comparison of the costs (material, labour, utility and capital expenditure) and yield harvested in one batch of fed-batch with pure methanol, fed-batch with mixed induction, continuous with pure methanol, and continuous with mixed induction in terms of percentage. The comparison was done by using fed-batch cultivation process using pure methanol as its induction solution as the baseline for cost and volumetric yield and was set as 100%.

The results showed that although the cost of materials of 2 L scale in the continuous cultivation method is 2.5 – 3.4-fold higher than that in the fed-batch cultivation method, the volumetric yield of the target protein is significantly higher, reaching as high as 233% for methanol alone and 2167% for the combination induction strategy used. This result indicates that the benefit of using the continuous cultivation method with a combination of induction agents is much more significant than the cost of the process.

5.3.3 Simulation of Cultivation Process for *K. phaffii* in production scale

Two methodologies were chosen from the four possible combinations to focus on the comparison point in the production scale simulation. The first methodology chosen was the fed-batch process using the data from the conventional method (fed-batch cultivation process using a methanol induction strategy) as a base process, also known as the most common cultivation process of *K. phaffii* in industry. The second methodology chosen was the continuous process, which

was simulated using the data from the combination method (continuous cultivation process using 50% C-mol methanol/sorbitol mixed induction strategy), as it had the highest probability of being feasible from the lab scale techno-economic analysis. Although there are other hurdles, such as regulations and real-time sensor requirements, when changing the fed-batch process to a continuous process in the industry, this could not be analysed through the simulation tool but can be resolved through documentation techniques such as Quality by Design (QbD) and Process Analytical Technology (PAT), which will smooth the validation processes.

For the simulation of the production scale, a 1000 L total bioreactor volume was set as the base production scale for the fed-batch cultivation process. Two production scales were simulated for the continuous process: another 1000-litre bioreactor for the same scale comparison and a 100-litre bioreactor for a similar yield (mg) produced and process time comparison with the 1000-litre bioreactor for the fed-batch cultivation process. The target protein in this simulation of the production scale bioreactor is the same as in the lab scale, which was KEX2 protease, and the lab scale results were used as a base for the production scale simulation. The scope of the simulation includes the facility and media preparation up until the first clarification step, which was the centrifugation process, which was the process that was studied and directly compared.

Unlike at the lab scale, scaling the cultivation process to a 1000-litre bioreactor with 400 L of medium required an additional step for increasing the biomass (sub-culture), using a 30 L total volume bioreactor containing 10 L of the medium. Furthermore, to simplify the creation and sterilization of all media and solutions, they were all sterilized using a sterile filtration process. The sterilization of large equipment would be done using the CIP or SIP method. The larger scale also means that the medium and other solutions would all be stored in their storage vessels before use. This can be a challenge in industry as the keeping the quality of the stored materials is crucial in ensuring the consistency of the cultivation process, especially in ensuring consistent cell growth, productivity and steady state. A pump would be used each time a solution or medium was transferred for filtration and use in the bioreactor. An additional media creation line was made for the fresh medium without a carbon source for the continuous cultivation

process. Multiple storage vessels would be used to contain the daily harvest after processing it through the centrifugation process.

The process of increasing biomass started with adding 12.5 mL of working seed of *K. phaffii* to 500 mL of BMGY medium sterilized through microfiltration, cultivated in a 2 L pre-sterilized Erlenmeyer flask, and then incubated at 30 ^{o}C at 250 RPM agitation rate for 24 hours. Then, 10 L of BSM medium and PTM1 sterilized through microfiltration were transferred to a 30 L pre-sterilized bioreactor at a rate of approximately 200 L.h^{-1}. The 500 mL of inoculum acquired from the incubation in the shake flask would then be transferred into a 30 L bioreactor containing 10 L of basal salt medium (BSM). The culture was then incubated at increasing agitation from 100-800 RPM with a temperature of 30 ^{o}C, pH of 5.0, and an airflow rate of 1 Vvm for 24 hours, or until the glycerol inside the medium was consumed.

Four hundred litres of BSM containing PTM1 sterilized through microfiltration were transferred to a 1000-litre pre-sterilized bioreactor at a rate of approximately 2000 L.h^{-1}. 10 litres of inoculum acquired from the incubation in a 30-litre bioreactor were then transferred into a 1000-litre bioreactor containing the sterilized 400-litre BSM. The culture was then incubated at increasing agitation from 100 to 500 RPM with a temperature of 30 ^{o}C, pH of 5.0, and an airflow rate of 1vvm for 24 hours or until the glycerol inside the medium was consumed. The process was then continued through the phases on a 5 L scale: the glycerol fed-batch phase for 4 hours, the transition phase, and the induction phase for 24 hours. For the conventional method or fed-batch cultivation process, at least 400 litres of broth were harvested after 24 hours of induction.

For the combination method or continuous cultivation process with a 50% methanol/sorbitol mixed induction strategy, fresh BSM without glycerol and PTM$_1$ would be fed into the fermenter after 24 hours of induction, and the broth inside the fermenter was harvested simultaneously and continuously according to the dilution rate after 24 hours of induction. The harvested cell broth would be centrifuged at 4000 RPM, 8 ^{o}C, and for 30 minutes to separate the pellets from the medium (crude extract). The crude extract harvested during the continuous phase was stored in a storage vessel to be kept below 8 ^{o}C and kept for 24 hours of harvesting before being further processed and purified.

The smaller production scale for the combination method (continuous cultivation using 50% C-mol methanol/sorbitol induction strategy) was simulated to produce a similar product yield and process time to the 1000-litre bioreactor production scale using the conventional method (fed-batch cultivation using pure methanol induction strategy), which would use a 100-litre bioreactor containing 40-litres of BSM as its initial medium volume. The subculture process would follow the same number of processes. However, the initial seed would be set at 2 mL. The initial subculture would be in a 1 L baffled Erlenmeyer flask containing 200 mL of complex medium, and the second would be in a 5 L bioreactor containing 2 L of BSM. From this, it was calculated to have slightly more product yield harvested in one batch.

The time taken for simulation at the production scales (400 L and 40 L) for fed-batch cultivation and continuous cultivation on an industrial scale was much longer than at the 5 L scale. The time taken for a fed-batch process in 1000 L was simulated (details of process schedule shown in Appendices H) and was found to take 7 days instead of 5 days, while it took 17 days instead of 15 days for the continuous process. This is one of the noticeable drawbacks of scaling up, which requires a longer start up time. The increase in large-scale processing time was due to the additional subculture step of the cultivation process. To have a similar processing time, two batches of the fed-batch cultivation using 400 L of BSM as its initial volume would be compared to one batch of the smaller production scale of continuous cultivation using 40 L of BSM as its initial medium which results in 14 days of cultivation time for fed-batch and 17 days of cultivation time for the continuous process as shown.

5.3.4 Downstream processing

For the fed-batch method, the culture was designed to be harvested only at the end of the cultivation process, and thus 400 litres of broth were predicted to be harvested at the end of the cultivation process. The target protein was designed to be excreted into the broth. Therefore, it must be processed immediately after harvest, separating the cells and the medium to produce a crude extract to ensure that fewer impurities were introduced in the medium because of cell lysis. Thus, the cell separation process should be done at 400 L scale in one lot or separated into three sub lots with 134 L being processed in each. This cell separation process will also affect the proceeding purification processes, such as diafiltration

and chromatography steps, which require a large scale capable of processing 400 litres of harvested supernatant within three batches to reduce the probability of degrading the product because of the long process time.

For the continuous method, the harvest process was designed to be done daily after the continuous phase started. The amount of broth harvested in the simulation was set to be 220 L per day or at a rate of 9.2 $L.h^{-1}$. The process was also designed to do the separation process directly to reduce the storage condition requirement to the optimum storage condition of the target protein and reduce the possibility of introducing more impurities because of cell lysis. An hourly harvest rate can reduce the scale of the cell separation process to as low as 10 $L.h^{-1}$. Furthermore, the combination method has the capability of having a flexible option to pool the medium into batches of either 12 or 24 hours after the separation between cells and medium, which can lower the scale of the purification process to 120 L or 240 L in exchange for increasing the number of workers shifts and a more intense process time. However, to prevent any build-up of products harvested, the subsequent processes have a time limit of 12 or 24 hours (the same as harvest time), which includes the diafiltration and chromatography in succession. Although the harvest time in the simulation is set to ten days of the continuous phase, the continuous phase can be increased further up to three months, which will impact the durability of each piece of equipment if used continuously for the same period.

In a smaller-scale production at a 100-litre bioreactor containing 40 litres of BSM as its initial medium, the impact on the downstream will be apparent as the scale of the equipment downstream will be adjusted to be smaller accordingly. These differences will significantly impact the capital cost, carbon footprint, process time, utility cost, material cost, and labour cost, reducing the risk of product deteriorating because of processing a large volume.

5.3.5 Profitability of the simulated process

The product's price must be set so that the total price of KEX2 produced can be calculated based on the price and how much was produced to calculate the profitability of the simulated processes. For example, from multiple sources, the price of KEX2 protease in the market ranged between USD$2740 and USD$600 for 1 mg of KEX2 protease, as shown in tables 5-4 with their respective sources.

Therefore, for the calculation of total price of KEX2 produced in one batch, the minimum price of USD$600 for 1 mg of KEX2 protease would be used for the rough calculation.

Table 5-4 Price of KEX2 protease from yeast system in terms of USD$ for 1 mg of KEX2 protease as of July 2022 in $USD.

1 mg of KEX2 price (USD$)	Source
1620	(Abcam, 2022)
600	(Peprotech, 2022)
600	(Signalchem, 2022)
2740	(Mybiosource, 2022)

5.3.5.1 Comparison of 400 L production scale for both cultivation method

The price of media used in the 400 L BSM medium scale could be predicted using the base scale of 2 L BSM. As a result, the price of the media in a 1000-litre scale was calculated to be USD$21,389.56 and USD$191,906.47 for the conventional (fed-batch with pure methanol induction strategy) process and combination process (continuous cultivation process with 50% methanol/sorbitol mixed induction strategy), respectively, in one batch (raw calculations are shown in the Appendices G). This difference shows that the price of media used during the continuous process is almost nine times higher than that used in fed-batch cultivation. However, the KEX2 protease yield produced would also be much higher, at around 0.09 kg and 1.98 kg of KEX2 protease, respectively, for fed-batch and continuous processes in one batch, which made the latter produce 21.3-fold more than the former. Based on the price of KEX2 protease set previously, the total price of KEX2 produced was shown in Table 5-7. The total price of KEX2 produced is significantly higher than the total media cost of their respective cultivation types. However, in terms of total price of KEX2 produced to total cost ratio, the combination method (continuous cultivation using 50% C-mol methanol and sorbitol induction strategies) had a much higher ratio when compared to the conventional method.

Other costs need to be clarified to determine whether the simulated process was profitable, such as capital, operating, labour, and utility costs. However, based on the information presented previously, if the scale was set to be the same, the

continuous cultivation process should require 30% more capital cost compared to fed-batch because of the extra equipment and storage vessel for preparing the

BSM without glycerol and for storing and moving the crude factor during the daily harvest. In addition, the utility, labour, and operating costs may be more than double when compared to the fed batch as the processing time for the continuous cultivation process was almost double at 17 days compared to the fed batch at seven days. Based on these results, the combination process (continuous cultivation using a mixed146 induction strategy) may appear slightly more attractive when compared to the conventional method (fed-batch cultivation with a pure methanol induction strategy).

5.3.5.2 Comparison of 400 L fed-batch production scale with 40 L continuous production scale

The setting was to compare the two batches of the 1000-litre scale bioreactor, each containing 400 litres of an initial medium, to a single batch of the 100-litre scale bioreactor containing 40 litres of an initial medium to set a similar KEX2 protease yield being produced and process time. The medium price for both simulated processes was calculated. It was found to be 37% higher for the 40-litre scale continuous cultivation when compared to the 400-litre scale fed-batch cultivation. The price of medium and total price of KEX2 produced with their process times (two batches of fed-batch and one batch of continuous process) was tabulated into Table 5-5 and showed that they were set to be similar.

Table 5-5 Rough media cost, yield and total price for the production in $USD of KEX2 protease in 400 L initial medium volume scale for both conventional process and combination process and 40 L for combination process.

$USD	Conventional 400L (1 batch)	Combination 400L (1 batch)	Conventional 400L (2 batches)	Combination 40L (1 batch)
Total Media Cost ($thousands)	21.39	191.91	43.42	59.62
Yield (g)	93.36	1986.54	186,72	198.65
YieldxPrice ($millions)	56.02	1,191.92	112.03	119.19
Process time (days)	7	17	14	17
Capital Cost	100%	~130%	100%	~40%
Utility Cost	100%	~285%	200%	~30%
Labour Cost	100%	~243%	200%	~243%

However, the smaller production scale had a significant impact on other costs, such as capital cost, labour cost, utility cost, and operating costs. Reducing the scale from 400 L to 40 L can reduce the capital cost by 70-80% and similarly reduce the other costs by more than 50% as it reduces the complexity of large-scale production and maintenance. A smaller production scale would have an impact not only by lowering the costs but also by lowering the land requirements for building the factory and having a smaller carbon footprint, making it more environmentally friendly. These benefits also further impact smaller equipment sizes in the subsequent processes (downstream processes).

5.4 Conclusion

This study showed that the cultivation of *K. phaffii* to express the KEX2 protease protein using the mix induction method utilizing sorbitol and methanol implemented in a continuous cultivation process gave rise to the highest productivity of KEX2 as compared to other methods. As for the cost analysis, even though the cost of materials was highest in the continuous process with the mix induction strategy, the benefits from the increase in product yield produced by the process exceeded the cost of material and process. Furthermore, the use of a continuous cultivation process in combination with a mixed induction strategy in lowering the scale of production to acquire a similar production rate on a larger scale using the fed-batch cultivation method was also shown to have a significant impact in terms of lowering the overall costs such as capital cost, utility cost, labour cost, and operating cost. Therefore, this promising methodology for developing efficient cultivation of *K. phaffii*-based processes warrants further investigation.

Chapter 6: Conclusions and future work

6.1 Conclusions

This research aims to improve the cultivation process methodology regarding productivity while still ensuring the condition of the recombinant protein product is maintained using the KEX2 protease protein as a model expressed in *K. phaffii* as an expression system. Objectives of the research were:

1) To investigate a mixed feed induction strategy with sorbitol as an alternative carbon source as co-feed with methanol and assess the impact of the strategy on volumetric yield and content of KEX2 protease as a model.
2) To investigate fed-batch and continuous cultivation and assess the impact of the strategies on overall yield and content of KEX2 protease as a model.
3) To compare the effects of mixed feed strategy and continuous culturing method on manufacturing aspects, including cell properties, productivity, overall cost, and process time.

In this thesis, these objectives will be answered step by step. First, the effects of the sorbitol/methanol mixed induction strategy and continuous cultivation on *K. phaffii* fermentation were studied and evaluated in terms of cell growth, product content, and KEX2 protease volumetric yield. These results were compared to the most common yeast-based cultivation method, which uses pure methanol as an induction solution in fed-batch mode. Furthermore, a techno-economic feasibility study of the processes was compared on the pilot bioreactor and simulated industrial scales.

Pure methanol induction and 50% C-mol sorbitol/methanol mixed induction strategies were compared using a 5-litre bioreactor in the fed-batch cultivation process. It was observed that mixed induction enhanced cell growth and increased KEX2 protease content significantly. In addition, the volumetric product yield in the mixed induction strategy was also higher than that in pure methanol induction, with a 2.4-fold increase.

An evaluation of a novel cultivation methodology, a mix induction method utilizing 50% C-mol sorbitol and methanol in a continuous process for *K. phaffii*, and its impact on improving productivity and other industrial aspects, was conducted. As a result, the volumetric yield of the target protein, KEX2 protease, in one batch expressed using the mixed induction method is higher than that expressed using the methanol induction strategy in the fed-batch cultivation method. Furthermore, a much higher protein yield was observed when cultivation was done using a mixed induction strategy with the continuous cultivation method. For comparison, the average yield (mg) of the continuous cultivation batch with mixed induction was 810% higher than that of the continuous cultivation batch with pure methanol induction. This increase could be due to the synergistic effect of increased cells growth and harvest rates, which significantly impacts the dilution rate as the crucial parameter in the use of this novel cultivation methodology.

Techno-economic impacts from using the induction and cultivation methods studied were also assessed. Although in one batch, the continuous method requires more materials and time to complete a batch of the production process, which implies a higher cost (e.g., methanol consumption used in the novel cultivation methodology is higher by 5-fold than that in the fed-batch cultivation method), the volumetric yield of target protein produced is 21.3-fold higher. Furthermore, other benefits of using the continuous method were assessed, including lowering the scale, reducing the time and sterilization number required for the production process, and potentially lowering the overall capital and operating costs. Thus, the benefits of utilizing the method outweigh the cost needed to run the process. These benefits highlight the advantages of a mixed induction strategy in a continuous cultivation method for *K. phaffii* that requires methanol as an induction agent.

Finally, this is the first study that evaluates the methodology using *K. phaffii* for producing KEX2 protease protein using a continuous process and mixed induction strategy. This finding is essential to be implemented in industries to reduce the handling of methanol and, at the same time, improve the productivity of target protein, hence the production process's efficiency.

6.2 Future work

A combination of 50% C-mol sorbitol/methanol mixed induction and continuous cultivation was evaluated as an alternative *K. phaffii* induction strategy to increase productivity and process feasibility. As a benchmark, a common pure methanol induction strategy in the fed-batch cultivation process was used to compare cell growth, product expression, and product harvest.

In Chapter 3, clone #2 was chosen as the cell line for this research. A protease activity assay could be used as a quality attribute in choosing between the ten clones in the future, which could further verify which clone to use for the production/ research. The sorbitol: methanol (1:1, C-mol ratio) mixed induction used was established previously by Wang (2019) and Woodhouse (2015) by screening ratios of sorbitol and methanol mixtures in shake flasks and parallel bioreactors. Although it resulted in a higher product yield for mixed induction using the 1:1 C-mol ratio compared to pure methanol (from 240.8 $mg.L^{-1}$ to 556.7 $mg.L^{-1}$), it was not confirmed if it was the optimum ratio to produce the highest volumetric yield. Therefore, screening other C-mol ratios for continuous cultivation is necessary to ensure that the optimum conditions used in the fed-batch process apply to the continuous process in future work. In addition, the impact of mixed induction on volumetric product yield was described as being influenced by cell strains. Therefore, the impact of the mixed induction method on other cell strains must be evaluated.

In Chapter 4, it was shown that using both mixed induction and continuous cultivation was a better strategy in terms of improving productivity in a bioreactor. The target protein content was predicted to be consistent throughout the continuous phase, but it was found to have fluctuated, initially decreasing and then increasing again until reaching optimum volumetric yield. It would be interesting to find out whether it is possible to maintain optimum volumetric yield, which will increase the overall productivity of the process, in a continuous process over a more extended time in future work. A further study should also be done in terms of the activity of the product to ensure that the KEX2 protease produced in continuous cultivation over an extended cultivation time is of similar condition to the KEX2 produced from the fed-batch. Furthermore, another study should be done using the combination of continuous cultivation mode and mixed induction

strategy in producing different target products to determine whether this method would increase their yield and how effective it would be.

The simplified techno-economic feasibility of sorbitol/methanol mixed induction and continuous cultivation was analysed to ensure the feasibility of the process in an industrial setting. However, in future work, it would be more beneficial to go into in-depth analysis and focus on how to combine continuous cultivation with the downstream process more efficiently and the impact on the downstream process of mixed induction with a continuous setting to acquire a more accurate prediction of the overall productivity of the continuous purification process.

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Chapter 8: Appendices

Appendices A: Sorbitol and Methanol %C-mol calculations

For determining how much methanol and sorbitol were needed for the 50% C-mol methanol/sorbitol (or 1:1 C-mol methanol/sorbitol) induction feed, the carbon was going to come from each substrate in the same amount:

Carbon, $Mr_C = 12.011\ g.mol^{-1}$

Methanol, $Mr_{CH4} = 32.042\ g.mol^{-1}$

Mass of carbon (Mr_C) in methanol as percentage

Mr = ((Mr_Cx1): Mr_{CH4}) x 100

= (12.011: 32.042) × 100

= 37.48 %

Sorbitol, $Mr_{C6H14O6} = 182.17\ g.mol^{-1}$

Mass of carbon (Mr_C) in sorbitol as percentage

Mr = ((Mr_Cx6): $Mr_{C6H14O6}$) x 100

= (72.064: 182.17) × 100

= 39.56 %

Thus, carbon mass ratio of methanol : sorbitol = 37.48 : 39.56

= 1 : 1.056

Methanol has a density of $0.792\ g.mL^{-1}$

Thus, for the same C-mol ratio (1:1)

For 1 L of methanol (pure 100%), 1 L of sorbitol will consist of (0.792x1000):1.056 = 750 g of sorbitol.

Appendices B: Queensland Report of Initial Gene Insertion

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RESULTS:

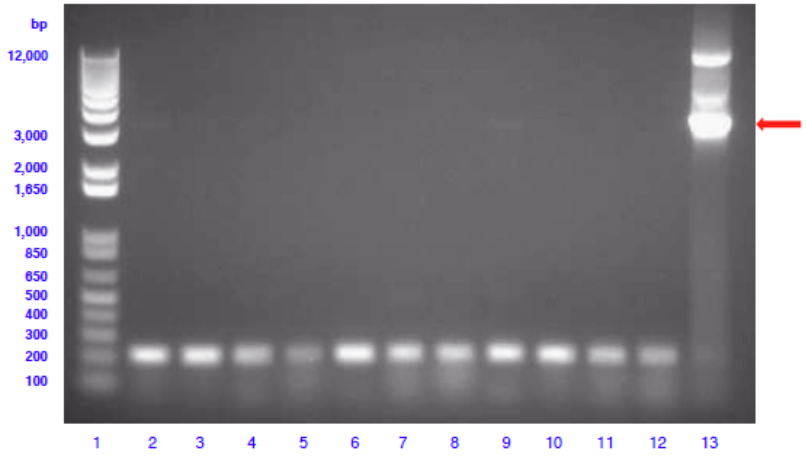


Figure 1 | 2018-1657 Transformation analysis into X-33. Agarose gel image showing results of PCR on individual yeast clones. Expected size of PCR product using vector-specific F- and gene-specific R-primers is 3447 bp.

Lane	Sample	Lane	Sample
1	1 Kb Plus DNA ladder	8	Clone 7
2	Clone 1	9	Clone 8
3	Clone 2	10	Clone 9
4	Clone 3	11	Clone 10
5	Clone 4	12	Negative Control (Untransformed X-33)
6	Clone 5	13	Positive Control (Plasmid DNA)
7	Clone 6		

COMMENTS:

- Faint PCR products at the expected size were amplified for all clones, confirming gene integration. Note that faint bands were visible on raw gel image files.
- There was a predominant DNA artefact observed above 200 bp. This may be due to non-specific amplification of the host genome by the primers.

Appendices C: GCMS Standard Result

Method C:\USERS\PUBLIC\DOCUMENTS\CHEMSTATION\1\METHODS\cooling.M

Calibration Table

General Calibration Setting

Calib. Data Modified : Thursday, January 6, 202211:59:10 AM
Signals calculated separately : No

Rel. Reference Window : 5.000 %
Abs. Reference Window : 0.000 min
Rel. Non-ref. Window : 5.000 %
Abs. Non-ref. Window : 0.000 min
Uncalibrated Peaks : not reported
Partial Calibration : Yes, identified peaks are recalibrated
Correct All Ret. Times: No, only for identified peaks

Curve Type : Linear
Origin : Included
Weight : Equal

Recalibration Settings:
Average Response : Average all calibrations
Average Retention Time: Floating Average New 75%

Calibration Report Options :
Printout of recalibrations within a sequence:
Calibration Table after Recalibration
Normal Report after Recalibration
If the sequence is done with bracketing:
Results of first cycle (ending previous bracket)

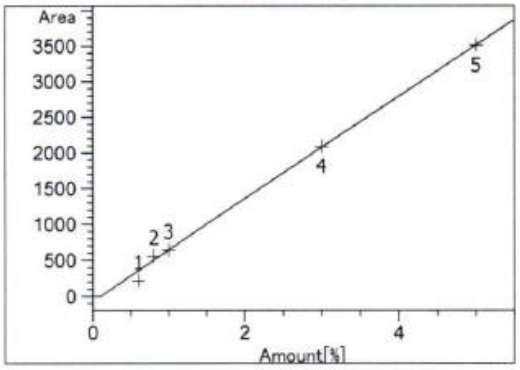
Signal Details

Signal 1: FID2 B, Back Signal

Overview Table

RT	Sig	Lvl	Amount [%]	Area	Rsp.Factor	Ref	ISTD	#	Compound
3.295	1	1	6.00000e-1	216.92755	2.76590e-3	No	No		
		2	8.00000e-1	557.13660	1.43591e-3				
		3	1.00000	642.36572	1.55675e-3				
		4	3.00000	2092.00415	1.43403e-3				
		5	5.00000	3512.90330	1.42332e-3				

Calibration Curves



at exp. RT: 3.295
FID2 B, Back Signal
Correlation: 0.99847
Residual Std. Dev.: 84.26171
Formula: y = mx + b
m: 717.08693
b: -72.72780
x: Amount[%]
y: Area

Appendices D: Specific growth rate (μ) Calculation for Fed-batch Cultivation

In determining the specific growth rate (μ) for fed-batch cultivation from the average of three cultivation experiments with three samples each, Equation 2.3 were used on the data acquired from experiments:

$$\mu\ (h^{-1}) = \frac{\ln\ (V_2.\mathrm{x}_2) - \ln\ (V_1.\mathrm{x}_1)}{\mathrm{t}_2 - \mathrm{t}_1} \qquad \text{Eq2.3}$$

The average of three repetitions of both induction method is shown in the table below:

Fed-batch Methanol				**Fed-batch Mixed**			
Time After Inoculation	Time After Induction	DCW $g.L^{-1}$	Volume L	Time After Inoculation	Time After Induction	DCW $g.L^{-1}$	Volume L
0			2	0			2
18		34.0	2.06	19		34.8	2.06
22	-5	56.6	2.06	22	-5	57.9	2.06
23	-4	55.2	2.06	23	-4	60.7	2.07
25	-2	55.8	2.07	25	-2	61.9	2.07
27	0	56.5	2.08	27	0	67.1	2.09
39	12	66.7	2.16	39	12	91.5	2.27
45	18	74.2	2.18	45	18	112.1	2.35
51	24	78.4	2.2	51	24	120.7	2.47
63	36	93.6	2.31	63	36	127.7	2.61
69	42	98.6	2.34	69	42	140.8	2.66
75	48	102.8	2.36	75	48	147.6	2.75
87	60	109.2	2.46	87	60	147.1	2.91
93	66	112.8	2.47	93	66	170.1	2.96
99	72	111.8	2.49	99	72	161.7	3.02

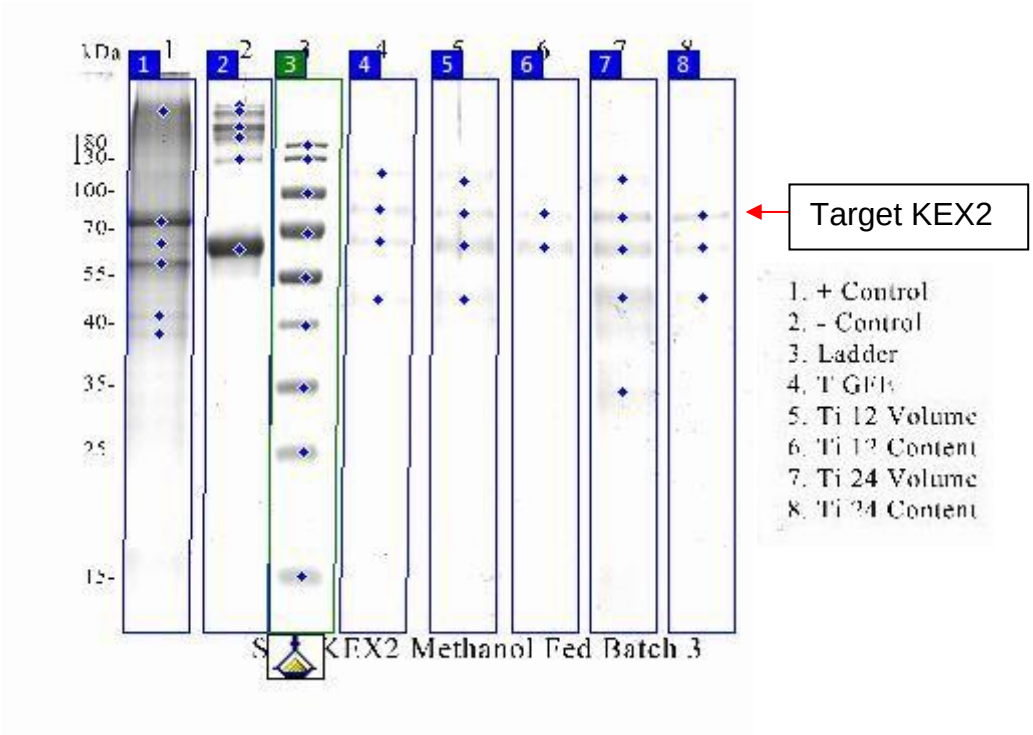
From these data, the results are as shown below with their error margin from the results acquired for the three cultivation experiments with three samples each:

			Methanol		Mixed	
AVE	Methanol	Mixed	Std	SEM	Std	SEM
Time	μ	μ				
h	h^{-1}	h^{-1}				
19	0.144	0.156	0.0308	0.0178	0.0175	0.0101
39	0.013	0.026	0.0034	0.0019	0.0065	0.0038
51	0.013	0.023	0.0021	0.0012	0.0026	0.0015
75	0.011	0.016	0.0023	0.0013	0.0020	0.0011
99	0.008	0.012	0.0018	0.0011	0.0010	0.0006

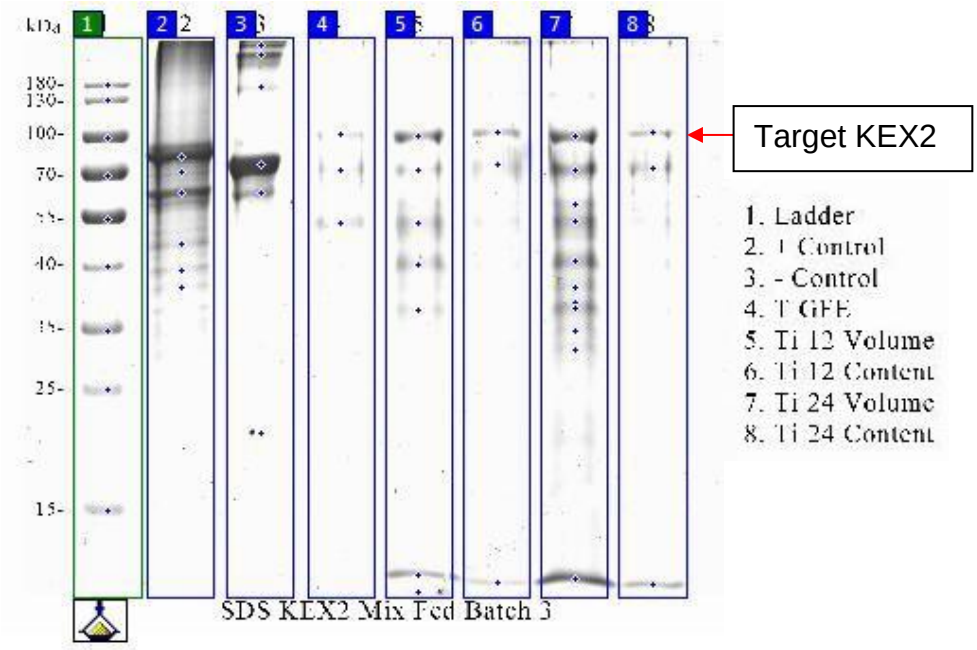
Appendices E: Data from densitometry analysis for fed-batch cultivation to acquire the KEX2 protease content

Reporting Tool version 13.2

Analysis Report: kex2 fb methanol



Analysis Report: kex2 fb mix



From the %band that was acquired from TotalLab analysis tool and total soluble protein acquired from BCA assay analysis process; the data can be summarized below to acquire the average of the three cultivation experiments with three samples each:

Methanol Induction strategy			
Fed-batch 1	Time after induction	Total Soluble Protein Concentration	KEX2 protease
Sample	hour	ug.mL^{-1}	%band
Ti0	0	1893.3	0
Ti12	12	2256.3	10
Ti24	24	2920.3	4
Fed-batch 2			
Ti0	0	1426.4	0
Ti12	12	1871.8	28.6
Ti24	24	1838.3	9.1
Fed-batch 3			
Ti0	0	1400.3	0
Ti12	12	1846.7	16.3
Ti24	24	1786.3	16.5
Average			
Ti0	0	1573.4	0.0
Ti12	12	1991.6	18.3
Ti24	24	2181.7	9.9

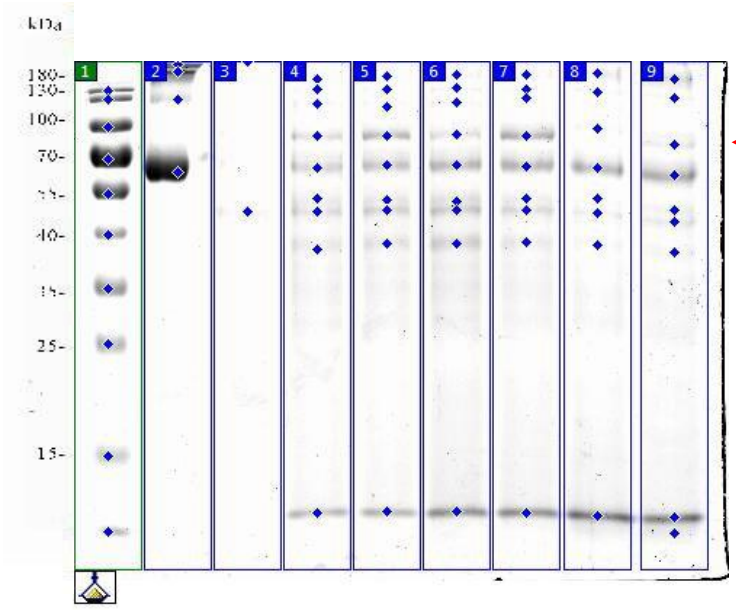
Mixed Induction strategy			
Fed-batch 1	Time after induction	Total Soluble Protein Concentration	KEX2 protease
Sample	hour	ug.mL^{-1}	%band
Ti0	0	1258.0	0
Ti12	12	2388.7	24.0
Ti24	24	3184.7	24.1
Fed-batch 2			
Ti0	0	1968.3	0
Ti12	12	1734.4	11.8
Ti24	24	3399.7	10.1
Fed-batch 3			
Ti0	0	1585.7	0
Ti12	12	3014.7	30.3
Ti24	24	3145.0	17.3
Average			
Ti0	0	1604.0	0.0
Ti12	12	2379.2	22.0
Ti24	24	3243.1	17.2

The %band was calculated through the analysis tool by comparing the number of pixels of the target band in comparison to the number of pixels of all the bands in the specified lane.

Appendices F: Data from densitometry analysis for continuous cultivation to acquire the KEX2 protease content

Reporting Tool version 13.2

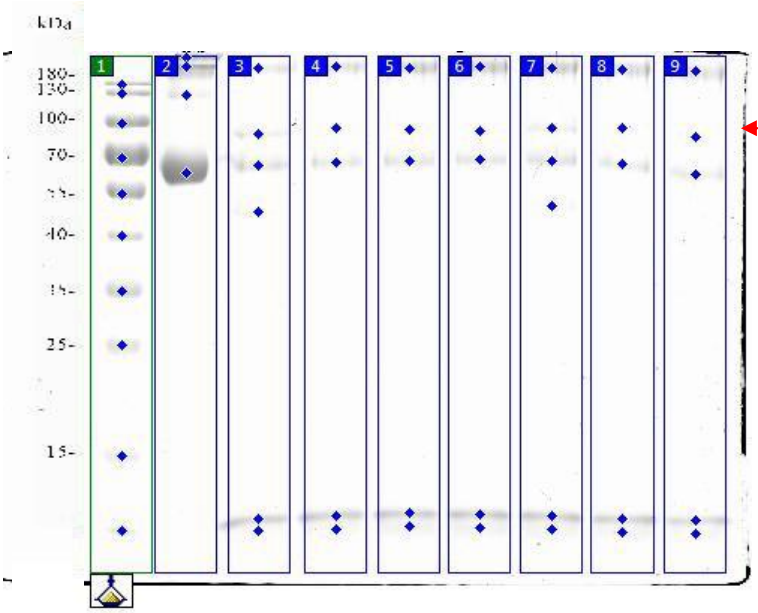
Analysis Report: sds kex2 c9 methanol cultivation part 1.tif



Target KEX2

1. Ladder
2. – Control
3. Pre-induction
4. 12h induction
5. 18h Induction
6. 24h induction
7. Continuous harvest day 1
8. Continuous harvest day 2
9. Continuous harvest day 3

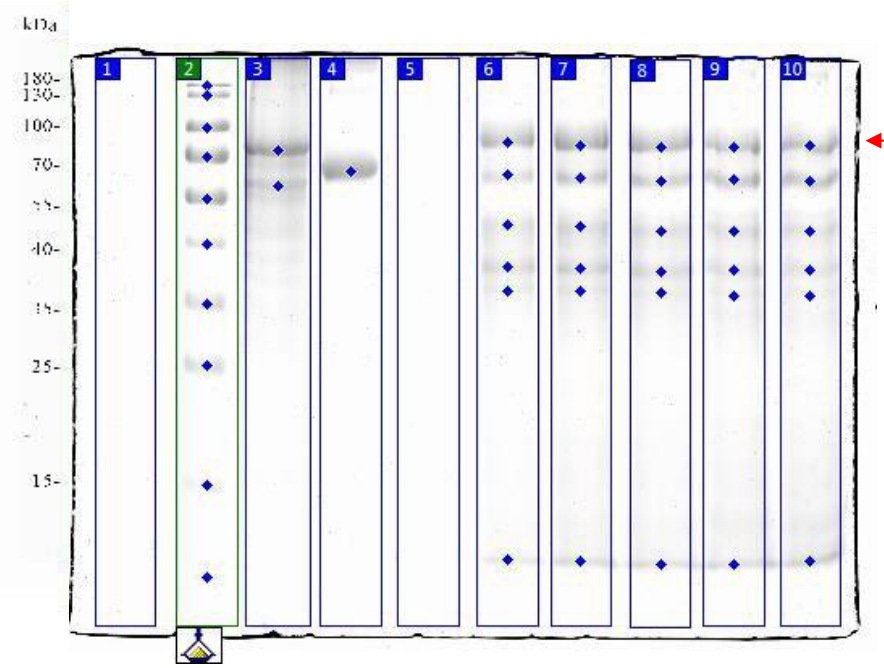
Analysis Report: SDS kex2 c9 methanol cultivation part 2.tif



Target KEX2

1. Ladder
2. – Control
3. Continuous harvest day 4
4. Continuous harvest day 5
5. Continuous harvest day 6
6. Continuous harvest day 7
7. Continuous harvest day 8
8. Continuous harvest day 9
9. Continuous harvest day 10

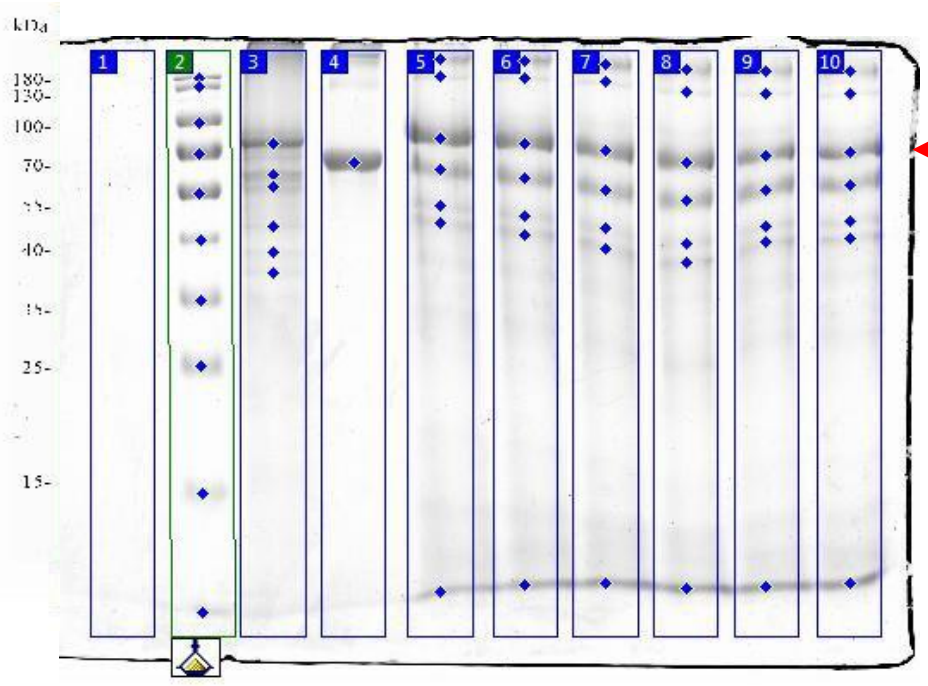
Analysis Report: SDS kex2 c1 mix volume wb.tif



Target KEX2

1. Buffer Sample
2. Ladder
3. + Control
4. – Control
5. Pre-induction
6. 12h induction
7. 24h induction
8. Continuous harvest day 1
9. Continuous harvest day 2
10. Continuous harvest day 3

Analysis Report: SDS kex2 c1 mix part 2.tif



Target KEX2

1. Buffer Sample
2. Ladder
3. + Control
4. – Control
5. Continuous harvest day 10
6. Continuous harvest day 9
7. Continuous harvest day 8
8. Continuous harvest day 7
9. Continuous harvest day 6
10. Continuous harvest day 5

From the %band that was acquired from TotalLab analysis tool and total soluble protein acquired from BCA assay analysis process; the data can be summarized below to acquire the average of the three cultivation experiments with three samples each:

Methanol Induction strategy			
Sample	Time after induction	Total Soluble Protein Concentration	KEX2 protease
Continuous 1	hour	ug.mL^{-1}	%band
Ti-5	0	436.9	0.0
Ti12	12	1636.8	24.1
Ti24	24	2161.3	8.2
day 1	48	2504.3	10.3
day 5	144	2992.7	8.1
day 10	264	2915.1	11.5
Continuous 2	hour	ug.mL^{-1}	%band
Ti-5	0	801.0	0.0
Ti12	12	1660.1	12.2
Ti24	24	2365.0	5.5
day 1	48	2180.9	21.7
day 5	144	2230.9	0.5
day 10	264	2490.9	0.0
Continuous 3	hour	ug.mL^{-1}	%band
Ti-5	0	624.0	0.0
Ti12	12	1518.0	26.0
Ti24	24	1998.4	17.9
day 1	48	2121.1	0.4
day 5	144	1838.7	0.1
day 10	264	2215.4	1.3
Average	hour	ug.mL^{-1}	%band
Ti-5	0	620.6	0.0
Ti12	12	1605.0	20.8
Ti24	24	2174.9	10.5
day 1	48	2268.8	10.8
day 5	144	2354.1	2.9
day 10	264	2540.4	4.3

Mixed Induction strategy			
Sample	Time after induction	Total Soluble Protein Concentration	KEX2 protease
Continuous 1	hour	$ug.mL^{-1}$	%band
Ti-5	0	772.3	0.0
Ti12	12	2290.3	41.0
Ti24	24	4821.3	43.4
day 1	48	2979.3	39.5
day 5	144	3049.3	31.3
day 10	264	3088.8	45.2
Continuous 2	hour	$ug.mL^{-1}$	%band
Ti-5	0	690.2	0.0
Ti12	12	2247.5	20.6
Ti24	24	2913.0	18.6
day 1	48	2628.7	11.1
day 5	144	2659.3	17.9
day 10	264	2717.3	22.3
Continuous 3	hour	$ug.mL^{-1}$	%band
Ti-5	0	628.9	0.0
Ti12	12	2535.7	39.4
Ti24	24	3347.0	36.2
day 1	48	2952.3	35.3
day 5	144	2898.0	22.8
day 10	264	2977.3	19.7
Average	hour	$ug.mL^{-1}$	%band
Ti-5	0	697.2	0.0
Ti12	12	2357.8	33.7
Ti24	24	3693.8	32.7
day 1	48	2853.4	28.6
day 5	144	2868.9	24.0
day 10	264	2927.8	29.1

The %band was calculated through the analysis tool by comparing the number of pixels of the target band in comparison to the number of pixels of all the bands in the specified lane.

Appendices G: Calculations for material costs in lab and industrial setting

Material cost Calculations for 2 L BSM:

Buffer	1 Batch			
	FB Methanol	FB mixed	Con Methanol	Con Mixed
Initial volume (L)	2	2	2	2
Total Process (day)	5	5	15	15
Total harvested Volume (L)	2	2	8.2	13.5
Total additional medium (L)	-	-	1,5±0,5	4,5±0,3
Total induction volume (L)	0.6	0.6	4.6	5.9
Total NH_4OH volume (L)	0.26	0.3	1	1.5
Total Antifoam Volume (L)	0.005	0.005	0.005	0.005
Total Glycerol feed (L)	0.1452	0.1452	0.1452	0.1452

no.	Media	Price per L as of July 2022
1	BMGY	USD$ 63.99
2	BSM	USD$ 28.30
3	PTM	USD$ 21.90
4	Ammonia	USD$ 7.73
5	Antifoam	USD$ 86.61
6	Induction (pure methanol)	USD$ 43.64
7	Induction (mix)	USD$ 52.85
8	Glycerol Feed	USD$ 206.20
9	BSM-G	USD$ 11.71

Buffer: 2 L initial medium as of July 2022	Costs in USD			
	FB Methanol	FB mixed	Con Methanol	Con Mixed
Initial volume (L)	$56.60	$56.60	$56.60	$56.60
Total induction volume (L)	$26.19	$31.71	$200.76	$311.79
Total NH_4OH volume (L)	$2.01	$2.32	$7.73	$11.59
Total Antifoam Volume (mL)	$0.43	$0.43	$0.43	$0.43
Total Glycerol feed	$29.94	$29.94	$29.94	$29.94
TOTAL	$115.17	$121.00	$295.47	$410.36

Appendices H: Industrial scale process schedule based on the established process flow simulation

Table Appendices H-1. KEX2 protease production in industrial scale process time for fed-batch (conventional) process

	Fed-batch (conventional) process	Personnel	Equipment	Time (h)	Material
Day 1	Equipment and materials preparation	3	Washing machine	3	
	Equipment and materials sterilisation		Autoclave		
	Bioreactor 30 L preparation	2	Bioreactor 30 L, pH meter	3	
	SIP bioreactor 30 L		Bioreactor 30 L		
	bioreactor 1000 L Preparation	2	Bioreactor 1000 L, pH meter	2	
Day 2	Production of subculture 1 medium	1		1	500 mL BMGY
	Sterilisation of Subculture 1 medium 500 mL BMGY		Sterile filtration, pumps		
	Production of medium subculture 2	2		5	10 L BSM +PTM
	Sterilisation and transfer subculture 2 medium into bioreactor 30 L		Sterile filtration, pumps		
	Glycerol feed and Antifoam (AF) production	2		3	30 L Glycerol 50% + PTM
	Sterilisation and transfer of Glycerol feed, Antifoam (AF) to storage tank		Sterile filtration, pumps		1 L AF
Day 3	Inoculation subculture 1	1		1	
	SIP bioreactor 1000 L	2	Bioreactor 1000 L	1	
	Cultivation medium production	2		4	400 L BSM + PTM
	Sterilisation and transfer of cultivation medium to bioreactor 1000 L		Sterile filtration, pumps, bioreactor 1000 L		
	Bioreactor 30 L calibration with BSM	2	bioreactor 30 L	2	

	Fed-batch (conventional) process	Personnel	Equipment	Time (h)	Material
Day 4	Inoculation for subculture 2	2	Bioreactor 30 L	2	
	Subculture 2 cultivation		Bioreactor 30 L		
	Bioreactor 1000 L calibration with BSM	2	Bioreactor 1000 L	2	
	Disinfection dan cleaning SC1	1		1	
Day 5	Inoculation for cultivation process 1000 L	2	Bioreactor 1000 L	2	
	Cultivation process day 1		Bioreactor 1000 L		
	Induction solution production	2		4	105 L methanol + PTM
	Sterilisation and transfer induction solution to storage tank		Sterile filtration, pumps		
	Preparation and sterilisation of equipment and materials	2	Washing machine, autoclave	2	
	SIP bioreactor 30 L	2	Bioreactor 30 L	4	
	CIP bioreactor 30 L		Bioreactor 30 L		
Day 6	Cultivation process day 2	2	Bioreactor 1000 L	5	
Day 7	Final Harvest	2	Pumps	4	
	SIP bioreactor 1000 L	2	Bioreactor 1000 L	4	
	CIP bioreactor 1000 L		Bioreactor 1000 L		

Table Appendices H-2. KEX2 protease production in industrial scale process time for continuous (combination) process

	Continuous (combination) process	Personnel	Equipment	Time (h)	Material
Day 1	Equipment and materials preparation	3	Washing machine	3	
	Equipment and materials sterilisation		Autoclave		
	Bioreactor 30 L preparation	2	Bioreactor 30 L, pH meter	3	
	SIP bioreactor 30 L		Bioreactor 30 L		
	bioreactor 1000 L Preparation	2	Bioreactor 1000 L, pH meter	2	
Day 2	Production of subculture 1 medium	1		1	500 mL BMGY
	Sterilisation of Subculture 1 medium 500 mL BMGY		Sterile filtration, pumps		
	Production of medium subculture 2	2		5	10 L BSM +PTM
	Sterilisation and transfer subculture 2 medium into bioreactor 30 L		Sterile filtration, pumps		
	Glycerol feed and Antifoam (AF) production	2		3	30 L Glycerol 50% + PTM
	Sterilisation and transfer of Glycerol feed, Antifoam (AF) to storage tank		Sterile filtration, pumps		1 L AF
Day 3	Inoculation subculture 1	1		1	
	SIP bioreactor 1000 L	2	Bioreactor 1000 L	1	
	Cultivation medium production	2		6	400 L BSM + PTM
	Sterilisation and transfer of cultivation medium to bioreactor 1000 L		Sterile filtration, pumps, bioreactor 1000 L		
	Bioreactor 30 L calibration with BSM	2	bioreactor 30 L	2	

	Continuous (combination) process	Personnel	Equipment	Time (h)	Material
Day 4	Inoculation for subculture 2	2	Bioreactor 30 L	2	
	Subculture 2 cultivation		Bioreactor 30 L		
	Continuous medium preparation	2		6	900 L BSM-G
	Sterilisation and transfer continuous medium to storage tank		Sterile filtration, pumps		
	Bioreactor 1000 L calibration with BSM	2	Bioreactor 1000 L	2	
	Disinfection dan cleaning SC1	1		1	
Day 5	Inoculation for cultivation process 1000 L	2	Bioreactor 1000 L	2	
	Cultivation process day 1		Bioreactor 1000 L		
	Induction solution production	8		4	1200 L mixed + PTM
	Sterilisation and transfer induction solution to storage tank		Sterile filtration, pumps		
	Preparation and sterilisation of equipment and materials	2	Washing machine, autoclave	2	
	SIP bioreactor 30 L	2	Bioreactor 30 L	4	
	CIP bioreactor 30 L		Bioreactor 30 L		
Day 6	Cultivation process day 2	2	Bioreactor 1000 L	5	
	Harvest tank for continuous harvest preparation	2	Storage tank 200 L	2	
Day 7	Continuous start	2	Pumps	4	
	Continuous Harvest	2	Pumps	4	
	Cleaning harvest tank	2	Storage tank 200 L	2	
	Harvest tank preparation	2	Storage tank 200 L	2	

	Continuous (combination) process	Personnel	Equipment	Time (h)	Material
Day 8	Continuous Harvest	2	Pumps	4	
	Cleaning harvest tank	2	Storage tank 200 L	2	
	Harvest tank preparation	2	Storage tank 200 L	2	
Day 9	Continuous Harvest	2	Pumps	4	
	Cleaning harvest tank	2	Storage tank 200 L	2	
	Harvest tank preparation	2	Storage tank 200 L	2	
Day 10	Continuous Harvest	2	Pumps	4	
	Preparation and sterilisation of equipment and materials	2	Washing machine, autoclave	2	
	Cleaning harvest tank	2	Storage tank 200 L	2	
	Harvest tank preparation	2	Storage tank 200 L	2	
Day 11	Continuous Harvest	2	Pumps	4	
	Cleaning harvest tank	2	Storage tank 200 L	2	
	Harvest tank preparation	2	Storage tank 200 L	2	
Day 12	Continuous Harvest	2	Pumps	4	
	Cleaning harvest tank	2	Storage tank 200 L	2	
	Harvest tank preparation	2	Storage tank 200 L	2	
Day 13	Continuous Harvest	2	Pumps	4	
	Cleaning harvest tank	2	Storage tank 200 L	2	
	Harvest tank preparation	2	Storage tank 200 L	2	
Day 14	Continuous Harvest	2	Pumps	4	
	Cleaning harvest tank	2	Storage tank 200 L	2	
	Harvest tank preparation	2	Storage tank 200 L	2	

	Continuous (combination) process	Personnel	Equipment	Time (h)	Material
Day 15	Continuous Harvest	2	Pumps	4	
	Preparation and sterilisation of equipment and materials	2	Washing machine, autoclave	2	
	Cleaning harvest tank	2	Storage tank 200 L	2	
	Harvest tank preparation	2	Storage tank 200 L	2	
Day 16	Continuous Harvest	2	Pumps	4	
	Cleaning harvest tank	2	Storage tank 200 L	2	
	Harvest tank preparation	2	Storage tank 200 L	2	
Day 17	Final Harvest	2	Pumps	4	
	SIP bioreactor 1000 L	2	Bioreactor 1000 L	4	
	CIP bioreactor 1000 L		Bioreactor 1000 L		