Adapted Substrate Delivery Strategies to Enhance Production in Ethanol Fermentation

by

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Abstract

Bioethanol, a renewable liquid biofuel, has been widely used to reduce carbon emissions from road transportation. Lignocellulosic biomass, such as forestry wood biomass or agricultural residues, is a sustainable resource for fuel ethanol production which does not compete with food supply. However, bioethanol production from lignocellulosic biomass still faces technological and economic challenges; hence the production steps – pretreatment, hydrolysis, fermentation, and purification – require further advancements. Despite fermentation being extensively applied to lignocellulosic ethanol production, studies on the management of substrate delivery to improve fermentation performance are limited. For instance, self-cycling fermentation (SCF), a semi-continuous fermentation approach, enables sustained yeast growth in the exponential phase and enhanced ethanol productivity through cycling operations. However, the final ethanol titer is restricted in this system by low substrate loading. The primary objective of this thesis is to advance efficient control of substrate delivery in the fermentation process to enhance ethanol production.

In the first study two adapted feeding strategies, in which feed medium addition was adjusted to increase the supply of fermentable sugar, were investigated in pulsing fed-batch fermentation with *Saccharomyces cerevisiae*. Specifically, a linear adapted feeding strategy was established based on changes in cell biomass, and an exponential adapted feeding strategy was developed based

on cell biomass accumulation. These two adapted feeding strategies led to increases in overall ethanol productivity of ~20% compared to fixed feeding operations. The results suggest that adjusting sugar feedings based on cell biomass in pulsing fed-batch fermentation leads to higher ethanol productivity than the fixed feeding modes. As the pulsed feeding operation spiked the feed medium into the 5-L bioreactor, glucose concentration increased dramatically followed by depletion until the next feeding.

To maintain lower glucose content during the feeding period, a second study explored adapted feeding strategies using a continuous feeding approach. Evolved gas production, which positively correlated with glucose consumption, was used to adjust the sugar feed rate in fed-batch fermentations. As the sugar feed rate gradually increased, the residual glucose content remained near zero until the yeast was metabolically impacted by the ethanol accumulated. The results showed that the adapted feeding strategy enhanced ethanol productivity by 21% compared to the fixed continuous feeding strategy, in which the sugar feed rate was stable. In addition, the adapted continuous feeding strategy maintained the same ethanol productivity even under low-nitrogen feeding conditions. This study suggests that evolved gas production, a metabolic monitoring parameter, can be used to guide the continuous feeding approach for effectively delivering sugar in yeast ethanol fermentation.

Finally, in the third study, the continuous adapted feeding strategy was integrated into SCF operation. Additionally, a single pulse feed second stage was implemented to further enhance ethanol titer and productivity. The two-stage integrated high-cell density SCF system was successfully applied to the fermentation of hydrolysate from steam-exploded poplar, achieving ~11% (v/v) ethanol, which is considered economically favorable for subsequent recovery in the bioethanol industry.

Overall, this thesis demonstrates that adapted feeding strategies, based on the yeast's metabolic responses, can efficiently manage sugar delivery through either pulsing or continuous feeding approaches in ethanol fermentation, thereby enhancing ethanol production. Furthermore, this thesis acts as a foundation to promote lignocellulosic ethanol production, especially when developing an integrated fermentation approach for large-scale production.

Preface

This thesis is an original work performed by Yueh-Hao Ronny Hung. A part of the thesis has been published, with other chapters submitted or in preparation for publication.

Chapter 2 will be submitted for publication. David C. Bressler, Dominic Sauvageau, and Yueh-Hao Ronny Hung have contributed to the published version of the manuscript with Yueh-Hao Ronny Hung serving as the primary author and major contributor. David C. Bressler and Dominic Sauvageau helped in editing the manuscript.

Chapter 3 was published as Hung, Y. H. R., Chae, M., Sauvageau, D., & Bressler, D. C. Adapted feeding strategies in fed-batch fermentation improve sugar delivery and ethanol productivity. Bioengineered. 2023; 14:2250950. David C. Bressler, Dominic Sauvageau, Michael Chae, and Yueh-Hao Ronny Hung conceived and designed experiments. Yueh-Hao Ronny Hung performed experiments and analyzed data. Yueh-Hao Ronny Hung and Dominic Sauvageau wrote the manuscript. David C. Bressler, Dominic Sauvageau and Michael Chae provided assistance in data analysis and troubleshooting and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

Chapter 4 will be submitted for publication. David C. Bressler, Dominic Sauvageau, and Yueh-Hao Ronny Hung conceived and designed experiments. Yueh-Hao Ronny Hung performed experiments and analyzed data. Yueh-Hao Ronny Hung and Dominic Sauvageau wrote the manuscript. David C. Bressler and Dominic Sauvageau provided assistance in data analysis, troubleshooting, and edited the manuscript.

Chapter 5 will be submitted for publication. As the primary author, I was responsible for designing, performing, and analyzing experiments, and writing and editing the manuscript. Under my guidance, Mr. Les Dean, an electronics technician in Faculty of Engineering (University of

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List of Abbreviations

5-HMF	5-hydroxymethylfurfural
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
CBP	Consolidated bioprocessing
ccm	Cubic centimeter per minute (the unit of gas flow rate)
CDW	Cell dry weight
CER	Carbon dioxide evolution rate
CNCs	Cellulose nanocrystals
FPU	Filter paper unit
g/L	Gram per liter
GC	Gas chromatography
h	Hours
HPLC	High performance liquid chromatography
IEA	International Energy Agency
mL	Milliliter
mM	Millimolar
N:C	Nitrogen-to-carbon
OD_{600}	Optical density at 600 nm
RID	Refractive index detector
rpm	Revolutions per minute
SCF	Self-cycling fermentation
SEP	Steam-exploded poplar
SHF	Separate hydrolysis and fermentation
SSCF	Simultaneous saccharification and co-fermentation
SSF	Simultaneous saccharification and fermentation
v/v	Volume by volume
w/v	Weight by volume
w/w	Weight by weight
YNB	Yeast nitrogen base
YPDA	Yeast extract-peptone-dextrose agar

Chapter 1 Introduction

1.1 Research background

Developing biofuels from renewable materials is essential to enhancing the sustainability of energy sources and reducing carbon emissions in transportation. Bioethanol, as a promising liquid biofuel, is widely used as an alternative fuel for road transportation in many countries, such as the United States of America, Brazil, and Canada, offsetting the usage of gasoline. As the bio-industrial economy continues to develop, the use of bioethanol and, thus, its related research have widely expanded [1]. According to the International Energy Agency (IEA), under the projected Net Zero Scenario, the global demand for bioethanol will increase by about 63% from 2022 to 2030 [2]. Lignocellulosic biomass, such as forestry-provided woody materials, straw and other agricultural biomass, are viewed as sustainable feedstocks for bioethanol production that is not in competition with food sources [3]. Furthermore, the integration of bioethanol production and co-production of value-added products from lignocellulosic biomasses is a potential strategy to further enhance economic competitiveness. However, it should be noted that compared to the sugar- or starch-based bioethanol pathways, the commercial availability of lignocellulosic ethanol is relatively limited and requires both technical and non-technical advancements for broader adoption [4]. Reduction of processing costs for lignocellulosic ethanol production is a subject of great interest for the industry moving forward. A generalized process for lignocellulosic ethanol includes pretreatment, hydrolysis/saccharification, fermentation, and ethanol recovery [1]. Among these steps, fermentation is a key processing step for converting substrate to ethanol using microorganisms, which is the primary focus of this thesis.

Self-cycling fermentation (SCF) is a cyclic, semi-continuous approach which comprises three parts: harvest, refill, and fermentation [5]. In each cycle, half of the volume of the fermentation

broth is harvested and replaced with an equal volume of fresh medium to continue the fermentation [6]. A major advantage of using SCF is to keep the microorganism growing in exponential phase, which means that lag phase, stationary phase, and death phase are eliminated, or at least greatly reduced, during the fermentation [7]. SCF has been widely applied for the production of secondary metabolites, such as biosurfactant [8], antibiotics [9], biodegradable plastics [10] under aerobic conditions. However, studies on the potential and capability of the SCF system for bioethanol production are limited. In previous work from our laboratory, an effective SCF system for lignocellulosic ethanol production was explored and established. Firstly, Wang *et al.* [7] showed that volumetric ethanol productivity in shake flasks improved by 43.1±11.6% compared to batch fermentation. This study demonstrated a proof-of-concept that SCF can be applied to ethanol production using a commercial distiller yeast strain. In a second study, Wang *et al.* [11] further scaled up the fermentation to 5-L bioreactors and developed an automated SCF system for precisely controlling the fermentation process. The results showed that ethanol productivity improved by 37.5–75.3% in SCF cycles compared to batch fermentation. In a third study, Wang *et al.* [12] successfully applied the SCF to a lignocellulosic hydrolysate made from wood pulp and indicated that ethanol productivity increased by 54–82% compared to batch operation. In addition, they designed a two-stage fermentation process, incorporating an additional stage after cycling operations to make the yeast further consume xylose for ethanol production; as a result, overall ethanol productivity was improved by 63–95% beyond than of a traditional batch operation. These three studies achieved a great milestone for potentially utilizing SCF for lignocellulosic ethanol production.

On the other hand, in this earlier work, the ethanol titer achieved in each SCF cycle was only 2.0–2.5% (w/v) when using a synthetic medium [11] and around 2.0% (w/v) when using the wood

pulp hydrolysate [12]. To make lignocellulosic ethanol production more economically viable, the ethanol titer must be increased to lower the energy demand and recovery costs from the subsequent downstream processes [13]. The minimal ethanol titer required for economically viable recovery in industrial settings is 4% (w/v) [14, 15]. As a comparison, fuel ethanol made from first-generation feedstocks (sugar- and starch-based biomass) generally reaches above 11% (v/v) [16]. In this context, fed-batch fermentation is a strategy with the potential to address the low final ethanol titer seen in previous SCF studies.

It is known that fed-batch fermentation helps to mitigate substrate inhibition through adding substrates gradually or stepwise [17]. In addition, fed-batch fermentation can improve ethanol yield [18] and productivity [19] by intermittently or continuously adding fresh nutrients in bioreactors to increase cell density beyond what is possible in batch fermentation. Hence, higher ethanol concentration can be achieved in fed-batch operation compared to batch mode. It is worth noting that feeding strategies or regimes are critical to impact the effectiveness of fed-batch fermentation. The addition of fermentable sugar especially influences overall ethanol yield and productivity in the fermentation process [20]. However, only a few studies have focused on the management of substrate delivery in fed-batch fermentation for lignocellulosic ethanol production.

Therefore, my research first aims to develop adapted feeding strategies in fed-batch fermentation, allowing the efficient management of substrate delivery. Secondly, these strategies will be integrated into the SCF system to further enhance final ethanol titer, as compared to a single fermentation system. Finally, the integrated fermentation system will be investigated for the utilization of lignocellulosic feedstock for ethanol production, providing critical insights on the implementation of multiple fermentation approaches for lignocellulosic ethanol production in the bioethanol industry.

1.2 Research objectives

The overall objective of this research is to investigate adapted fed-batch strategies, which enable efficient control of substrate delivery in ethanol fermentation, followed by their integration into a SCF system. This is expected to further improve ethanol titer and productivity. The research suggested opportunities for applying the integrated SCF system in the lignocellulosic ethanol industry, highlighting the prospects of commercial bioethanol production.

- The specific objectives investigated and achieved in this research included:
  (1) The development of adjustable and efficient feeding strategies for substrate delivery via either pulsing (Chapter 3) or continuous (Chapter 4) feeding in fed-batch fermentation for improving ethanol titer and productivity.
  (2) The integration of the adapted feeding strategies into SCF and subsequent evaluation of ethanol concentration and volumetric ethanol productivity gains made through the integrated fermentation system using synthetic media, as compared to a batch mode (Chapter 5).
  (3) The investigation of the potential for an integrated fermentation system to be applied to the fermentation of steam-exploded poplar hydrolysate for ethanol production and the assessment of the ability for this advanced fermentation system to further improve ethanol titer and volumetric productivity, as compared to a batch mode (Chapter 5).

- The promising outcomes from those specific objectives are:
  (1) The first study (Chapter 3) demonstrates that cell growth is a significant variable for adjusting sugar delivery in pulsing fed-batch fermentation of *Saccharomyces cerevisiae* for ethanol production. The following major hypothesis was tested: *if glucose is fed as*

pulses adjusted based on changes in cell biomass, then ethanol productivity would be improved compared to constant pulse feeding. This study provides an efficient method to adjust glucose addition in a pulsing fed-batch system to enhance ethanol production.

(2) The second study (Chapter 4) shows that evolved gas production from ethanol fermentation can be used as an effective monitoring parameter to estimate glucose consumption in the reactor and to guide the continuous, adapted sugar delivery in fed-batch fermentation of *S. cerevisiae*. The major hypothesis tested was: *if glucose addition is adjusted according to the evolved gas production when 10% (v/v) ethanol is achieved in the fermentation of S. cerevisiae, then ethanol productivity can be improved, as compared to a fixed continuous feeding mode*. This study contributes to a robust and adjustable feeding strategy for sugar delivery in continuous fed-batch fermentation.

(3) The third study (Chapter 5) integrated the continuous adapted feeding strategy into a SCF system. Ethanol was accumulated at higher levels than in previous SCF studies [11], and was further increased (to above 10% (v/v)) by adding a second stage in which an additional pulse feed was added to complete fermentation. *The integrated fermentation system was successfully applied to both synthetic medium and the steam-exploded poplar hydrolysate.* This study provides baseline information to efficiently control ethanol fermentation using the advanced integrated fermentation system, suggesting its potential applicability to the cellulosic ethanol industry for commercial production.

Chapter 2 Literature review

The focus of this review is to provide a better understanding of the impact of fermentation modes on lignocellulosic ethanol production and address pathways to shorten the technical limitations for commercial lignocellulosic ethanol production from a fermentation perspective. Specifically, in this review article, fundamental and advanced fermentation modes for producing lignocellulosic ethanol will be investigated. Moreover, we will highlight possible limitations in various approaches to the fermentation of lignocellulosic ethanol and suggest opportunities for fermentation modes to provide opportunities to overcome hindrances. Finally, the opportunity for the integration of fermentation modes is discussed in terms of their potential in industrial lignocellulosic ethanol production and advanced processes.

*A version of this chapter will be submitted for publication.

2.1 Ethanol fermentation

Although various microorganisms can produce ethanol, the yeast *S. cerevisiae* has been widely used by the bioethanol industry, due to its high ethanol yield and environmental stress tolerance [21]. For example, the wild type *S. cerevisiae* can tolerate ethanol stress up to an ethanol content of approximately 15% (v/v) [22]. However, some industrial strains can tolerate ethanol contents above 20% (v/v) to purse higher ethanol yield [23]. Ethanol production under anaerobic conditions is a key aspect of the primary metabolism of *S. cerevisiae* consuming fermentable sugars, such as sucrose, maltose, glucose, fructose, galactose, and mannose. During this process, when sugars are metabolized, it is converted into ethanol and carbon dioxide. This conversion occurs through glycolysis and subsequent fermentation pathways. Additionally, the process generates adenosine triphosphate (ATP), which is essential for the yeast's cellular functions, including growth,

maintenance, and reproduction. [3, 24]. Based on this metabolism, glucose utilization is coupled with cell biomass formation, ethanol and carbon dioxide production, and cell maintenance in ethanol fermentation of *S. cerevisiae* [20].

These metabolic functions can be modeled in a batch culture according to the following kinetic equations [25, 26]:

(i) Cell biomass (X) growth rate: $\frac{dX}{dt} = \mu X$ Eq. (2.1)

where μ is the specific growth rate (h^{-1})

(ii) Substrate (S) utilization rate: $\frac{\mathrm{dS}}{\mathrm{dt}} = -\frac{\mu \mathrm{X}}{\mathrm{Y_{x/s}}}$ Eq. (2.2)

where $Y_{X/S}$ is the yield coefficient of cell biomass on substrate (g-biomass/g-substrate)

(iii) Product (P) formation rate: $\frac{\mathrm{dP}}{\mathrm{dt}} = \mu X \cdot Y_{P/X}$ or $\frac{\mathrm{dP}}{\mathrm{dt}} = Y_{P/S} \cdot \left(\frac{dS}{dt}\right)$ Eq. (2.3)

where $Y_{P/X}$ is the yield coefficient of product on biomass (g-product/g-biomass) and $Y_{P/S}$ is the yield coefficient of product on substrate (g-product/g-substrate).

The information of the growth kinetic of the yeast is crucial for optimizing ethanol fermentation in fuel production [21]. Substrate concentration or the approach of substrate delivery becomes an important variable that influences the overall fermentation performance. In the following sections, fermentation approaches to substrate delivery for ethanol production are reviewed.

2.2 Fundamental fermentation modes for ethanol production

2.2.1 Batch fermentation

Batch fermentation is a closed operation mode. In batch operation, most of components, such as starter culture, base media, and substrates, are loaded into the bioreactor prior to fermentation,

and the desired product is harvested in the end of operation [27-29]. It should be noted that the end of batch operation is not exactly equal to the end of fermentation. For example, after ethanol fermentation in the brewing process, most of beer requires additional conditioning (or aging) to form aromatic compounds for the purpose of enhancing flavors [30]. In this case, the batch reactor can also be used as the intermediate storage vessel. In addition, during batch fermentation, traditionally the volume of fermentation medium does not change substantially, and the change usually comes from sampling, gas exchange, or other essential control agents (e.g. antifoam, acid/alkali). Thus, compared to other fermentation operations, batch fermentation is easily controlled which makes it widely applied for food, chemical, pharmaceutical, and agricultural products.

To optimize microbial growth, batch fermentation is appropriate for use as a benchmark approach. Cell growth is influenced by intrinsic factors (e.g. pH, nutrient), extrinsic factors (e.g. gaseous environment, processing conditions), and implicit factors (e.g. specific growth rate) [31]. Certain levels of metabolic inhibiting compounds in lignocellulosic hydrolysate, such as furans, organic acids, and phenolic compounds can limit yeast growth and its ethanol production [32, 33]. However, through batch fermentation, the growth-related variables can be optimized for cell growth and ethanol production. As an example, the concentration of inhibitors has been evaluated and regulated in batch fermentation for lignocellulosic ethanol production, allowing the yeast to grow with inhibitors and reducing the potential of growth of microbial contaminants under a non-sterile condition [34]. Although fermentation is a complex bioprocess, interactions between growth-related variables in ethanol fermentation can be explored via batch approaches for optimizing cell growth.

To maximize ethanol yield utilizing a batch approach, substrate concentration is a key design parameter. Chang *et al.* [35] pointed out that glucose concentration in the corncob hydrolysate influenced the alcoholic metabolism and cell accumulation of the yeast *Saccharomyces (S.) cerevisiae*. Either excessive or insufficient sugar loadings resulted in inefficient ethanol production, leading to high residual sugars or low ethanol content at the end of fermentation, respectively. It should be noted that the residual sugars in starchy ethanol production are typically kept lower than 2 g/L to prevent the growth of contaminants and to ensure high ethanol yield [22]. Studies have focused on optimizing substrate loadings in simultaneous saccharification and fermentation (SSF) to improve ethanol concentration and to reduce energy expenditure in subsequent distillation [36-38]. High amounts of solid substrate could limit mass transfer in the reactor and decrease the efficiency of enzymatic hydrolysis [37]. However, this limitation can be addressed by investigating fundamental batch operations, gaining a proper understanding of solid loading in SSF.

Compared to other fermentation approaches, applying batch fermentation alone can limit the efficiency of fermentation in lignocellulosic ethanol production. For instance, the initial substrate content is restricted in batch mode due to the potential substrate inhibition on microorganisms, thereby limiting the final ethanol concentration in the fermentation process [39]. Furthermore, intensive manpower has to be taken into consideration for batch operations since every batch requires the repetition of the same preliminary works for fermentation, such as medium preparation, cleaning, and harvesting [29, 40]. These time-consuming steps may reduce overall ethanol productivity and cause economic loss, as compared to a fermentation system working continuously [41]. As a result, to further enhance bioethanol production from different lignocellulosic feedstocks, various fermentation operations beyond batch mode are required, including fed-batch, continuous, self-cycling, and other advanced fermentation approaches.

2.2.2 Fed-batch fermentation

Fed-batch fermentation is a fermentation mode that combines batch and feeding operations. Through fed-batch approaches, substrates can be transferred intermittently or continuously into the bioreactor. Based on this characteristic, substrate inhibition on microorganisms can be minimized in the fed-batch pathway [42]. For example, Chang *et al.* [35] reported that fed-batch fermentation could gain higher sugar-to-ethanol ratio and biomass-to-ethanol ratio than the batch fermentation, due to low substrate inhibition in fermentation. When sugar concentration is greater than 25% (w/v), the environmental osmotic pressure can have significant impacts on reducing the yeast *S. cerevisiae* viability and its ethanol production [22]. As such, compared to batch fermentation that loads all the medium at once, fed-batch approach has an advantage in that it relieves the substrate inhibition by introducing fresh substrate at a controlled lower concentration during fermentation. Furthermore, with feeding controls, the log phase of the yeast is extended for ethanol production at the highest rate [20]. Due to this characteristic, high cell density can be also achieved by fed-batch fermentation [43]. Overall, fed-batch fermentation is a well-established fermentation approach in various industries [42], suggesting a promising pathway for large-scale commercial production of lignocellulosic ethanol.

2.2.2.1 Feeding strategies

In the fed-batch approach, feeding strategy is a crucial variable to control the fermentation process. Based on different feeding strategies, fermentation can be driven in a variety of corresponding directions. For instance, adding high amounts of sugars or salts in fermentation led to higher glycerol accumulation in yeast cells, due to changes in environmental osmotic pressure [20, 44]. Or supplying additional vitamins to yeasts could improve cell viability and the strain's overall ethanol tolerance [45]. In terms of limiting nutrients, fermentable sugar is a major carbon

source and thus impacts the yeasts' metabolism, ethanol yield, and ethanol productivity [20]. Controlling the sugar stream in the fed-batch fermentation is particularly critical for optimizing ethanol production. However, studies of adaptable feeding strategies for sugar delivery in lignocellulosic ethanol production are still very limited. A fixed rate substrate supply may not address limited fermentation performance and maximize ethanol production.

In terms of fed-batch strategies, substrate delivery can be achieved by one or multiple streams in either intermittent or continuous feeding operations until the desired end-point is reached [42]. Intermittent feeding strategy, also named pulsed feeding, features an approach in which specific quantities of limiting nutrients are added either at one time or at multiple times [18, 46]. On the other hand, continuous feeding strategy relies on pumping limiting nutrients into the bioreactor continuously [47, 48]. When it comes to feeding lignocellulosic hydrolysate for ethanol production, a continuous feeding strategy may favor ethanol fermentation more than pulsed feeding. This is supported by the realization that the exposure of microorganisms to metabolically inhibiting compounds, such as furfural derivatives, is reduced under continuous delivery. However, both pulsed and continuous feeding operations can reach the maximum concentration of the desired product [42]. The selection of feeding pathway should be aligned to the entire process design.

When developing feeding strategies, aligning to the chosen approach of fermentation system for the production process is also critical. In terms of lignocellulosic ethanol production, both separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) can fit with fed-batch operations. In a SHF system, fermentable sugars can be transported using either hydrolysates or synthetic media. Reducing substrate inhibition, increasing final ethanol concentration, and other advantages are achieved by fed-batch operations in SHF [35, 49, 50]. Alternatively, fed-batch operations act slightly differently in SSF system because hydrolysis and

fermentation are combined in the same step. As a result, feeding strategy can either focus on delivering substrates, enzymes, or both in combination, suggesting more opportunities to improve production efficiency. For example, high solid substrate loading in a batch SSF may have issues with mass transfer in the reactor [51]. However, by adjusting substrate or enzyme loadings with fed-batch operations, these limitations can be resolved for enhancing ethanol production [52, 53]. It should be noted that as compared to SHF system, the physical-chemical characteristics of substrates need more assessments when developing a feeding strategy in SSF. Variables like viscosity or contents of fermentable components may influence the overall efficiency of ethanol fermentation [54].

2.2.2.2 Limitations in fed-batch fermentation

Compared to batch fermentation, control variables (such as one stream or multiple streams feeding pathways) and maintenance in fed-batch fermentation is more complex, including an increase in capital costs for infrastructure and manpower [55]. Moreover, frequent feedings or adjustments to bioreactors may increase the risk of cross-contamination in the process. When substrates are fed into bioreactors, the working volume and composition of fermentation medium change. Additionally, the heterogeneous properties of the fermentation medium often affect the microbial growth in fermentation. For example, metabolic inhibitory compounds (e.g. furfural, 5-HMF) can be introduced and accumulated in the fermentation medium when feeding the non-detoxified lignocellulosic hydrolysate for ethanol fermentation. Once the inhibitor concentration exceeds the tolerance of the microorganism, cell growth cannot be controlled by the feeding strategy and the fermentation efficiency may reduce [49, 56]. In addition, ethanol concentration is another variable known to induce environmental stress on microorganisms which requires further investigation and optimization in fed-batch approaches [22, 57]. However, these metabolic

limitations can be addressed or alleviated with effective feeding designs, thereby achieving high cell density, high product titer, or maximal product formation rate in fermentation. To reduce unfavored accumulation issues in fed-batch fermentation, various modified approaches can be introduced to the fermentation operational system, such as combining it with cycling operations.

Overall, fed-batch fermentation is viewed as a valuable approach for bioethanol production that can benefit final ethanol concentration and ethanol productivity. An adjustable feeding strategy plays a critical role in controlling yeast growth and maximizing ethanol production in the fed-batch fermentation. Feeding objectives, fermentation systems, or substrate characteristics can be suitable candidates to investigate when developing effective fed-batch approaches for lignocellulosic ethanol production.

2.2.3 Continuous fermentation

Continuous fermentation is an open culture system, including both feeding and harvesting operations continuously which at steady state results in a constant culture volume during the fermentation process. Two forms of steady states are mainly recognized in continuous fermentation. The first is a chemostat and the second is the turbidostat, which either focus on controlling culture environment or cell biomass concentration, respectively [58]. Most ethanol production conducted in continuous fermentation is conducted under the chemostat approach, due to various production goals of the fermentation. For example, a chemostat provides a potential pathway to address the effect of a single limiting nutrient on both yeast growth and ethanol productivity at the same time [59]. In terms of reactor designs, plug-flow reactors and continuous stirred tank reactors (CSTR) are known classes applied in continuous fermentation for ethanol production [60].

Compared to batch fermentation, continuous fermentation provides several advantages in ethanol production. Primarily, it enhances the product throughput. With continuously feeding and

harvesting, the growth of yeast is kept in the exponential phase, leading to the highest ethanol productivity in the growth curve during ethanol fermentation [20, 60]. Ahn *et al.* [61] showed that ethanol productivity was enhanced 1.57 times by continuous fermentation over batch fermentation. When the dilution rate increases, ethanol productivity can boost as well [62, 63]. Due to the improvement in ethanol productivity, bioreactors are likely to be designed with smaller working volumes for continuous fermentation which reduces the capital cost of infrastructure [64]. Furthermore, operating a continuous fermentation approach can cut down the processing time required for non-fermentation stages, such as cleaning, sterilization, or other unproductive activities [65, 66]. Secondly, negative impacts generated from toxic metabolites can be reduced in continuous fermentation. In the case of *S. cerevisiae*, as ethanol accumulates to a certain level during fermentation, the yeast eventually become metabolically inactive due to the toxic nature of the ethanol [22]. However, continuous fermentation could dilute the level of ethanol by feeding fresh substrate and harvesting the fermentation medium, leading to a higher ethanol productivity, as compared to batch fermentation [67]. It should be noted that although increasing the dilution rate can enhance ethanol productivity, cell biomass may be washed out if the dilution rate is higher than the specific growth rate of the cell. To address this limitation, cell retention has been investigated to minimize the wash out effect and reach high cell density in continuous fermentation. Cell immobilization [68], cell recycling [67, 69], or using flocculating yeast [70] are known cell retention techniques for enhancing the efficiency of continuous fermentation in this system.

On the other hand, certain limitations in continuous fermentation provide obstacles to extensive application at an industrial scale for bioethanol production. Continuous fermentation systems have been recognized as having severe microbial contamination issues [30]. They require high hygiene standards across the entire operations, increasing extra costs for quality control.

Moreover, operational skills for controlling a continuous fermentation system are more demanding and complicated than for a batch mode operation. For example, if the dilution rate is not controlled properly, the wash-out effect can be significant in the bioreactor, where cell density may decrease and more substrates will accumulate in the outlet stream. Additionally, due to the dilution effect, final ethanol concentration is generally low in continuous fermentation system which is not favored for downstream recovery in the bioethanol industry. However, it is worth noting that continuous fermentation system can combined with very-high-gravity fermentation by applying multistage reactors to increase substrate feedings and further improve final ethanol concentration [71]. With this approach, there is still a requirement to avoid substrate inhibition in the first reactor, as extra osmotic stress may inhibit yeast viability and growth. In terms of strain stability, microbial selection can occur in a continuous fermentation system, which may increase the probability and risks of selecting for mutants. Although continuous fermentation can be a useful approach to select high ethanol-tolerant yeasts by adapting the environmental pressure [72], mutants may perform different metabolisms and characteristics, disturbing the original steady state. Unexpected new traits, such as foaming, flocculation, or flotation, may disrupt fermentation systems and cause economic losses [73].

A well-controlled continuous fermentation can demonstrate steady states in both the microorganism's metabolism and the culture environment, which provides opportunity to study the physiology of the microbe. Overall, the continuous fermentation approach offers the advantage of high productivity, low substrate/product inhibition, and less workload in terms of maintenance, as compared to traditional batch approach. On the other hand, the stability of the continuous fermentation still requires further investigation and improvement, especially in large scale ethanol production. [74]

2.3 Advanced fermentation modes for ethanol production

In addition to the traditional fundamental fermentation approaches (batch, fed-batch, and continuous), various derivative fermentation modes have been constructed for different purposes in lignocellulosic ethanol production.

2.3.1 Repeated batch fermentation

One central principle in repeated batch fermentation is to re-use the starter culture in the process, i.e., cell recycling. For example, after a batch completion, the yeast has been collected, centrifuged, and then transferred into a fresh fermentation medium to start a new batch fermentation [75]. Through recycling cells, time and cost can be saved on the preparation of inoculum for repeated processes [76, 77]. In addition, cell density have been increased by cell recycling in the reactor, resulting in further improvement of ethanol productivity [78]. Fan *et al.* [79] stated that there was no lag phase for ethanol production in repeated batch fermentation, and the average ethanol productivity was improved compared to batch fermentation mode. Slininger *et al.* [80] introduced a supportive perspective that a high pitching rate might reduce the metabolic demand for cell growth in a new batch, enabling more carbon to flow to ethanol production instead of biomass accumulation and thereby enhancing sugar-to-ethanol conversion and fermentation rate. Furthermore, cell recycling operations might lead to cell adaption in the fermentation process in which the principle of the genetic drift aligns with the continuous fermentation system. Interestingly, the operational process of repeated batch fermentation acts similarly to adaptive laboratory evolution, a potential technique for selecting highly inhibitor-tolerant yeast for lignocellulosic ethanol production. In this process, cells are harvested, washed, and re-inoculated into fresh medium with gradually increasing inhibitor concentrations [81]. Landaeta *et al.* [82]

reported that the adapted yeast strain showed improvements in ethanol productivity and cell growth rate when cultured in inhibitors-containing lignocellulosic hydrolysate, as compared to the non-adapted strain. However, it should be noted that the adaptive evolution occurring in cell recycling may or may not be directed to the desired fermentation process [73], requiring further investigations for the design of an efficient fermentation approach for lignocellulosic ethanol production.

Repeated batch fermentation is a semi-continuous fermentation approach for semi-continuous multiple products formation, expanding it applicability. Dasgupta *et al.* [83] reported that glucose and xylose were collected separately from the sugarcane bagasse, and *Kluyveromyces marxianus* IIPE453 produced ethanol and xylitol semi-continuously in different reactors by applying the cell recycling operations. This aligns with the concept of biorefinery for ethanol production and co-production of valued-added products from lignocellulosic feedstocks. In terms of cell recovery in the repeated batch mode, morphology of microorganisms is also worth investigating. For example, a flocculating yeast can benefit and support high cell recovery rates through the successive fermentation processes [78]. Furthermore, cell immobilization is a known technique enabling the recovery of cells in repeated batch mode. Mishra *et al.* [84] immobilized *S. cerevisiae* in calcium alginate beads and subjected them to rice straw enzymatic hydrolysate. The authors successfully demonstrated 17 batch cycles with a consistent ethanol titer and yield, along with low residual sugars at the end of fermentation. It is essential to note that as the number of repetitions increases, monitoring and evaluating changes in the integrity of the carrier is required when applying cell immobilization in repeated-batch fermentation. Khanpanuek *et al.* [85] observed that the morphology of the carrier changed as the number of repeated batches increased, and the lowest ethanol production occurred at the last batch. To achieve viable ethanol fermentation via repeated

batch fermentation, the repeatability of the strain's growth pattern is critical, especially for a long-term production.

2.3.2 Sequential batch fermentation

Sequential batch fermentation is a semi-continuous operation, and it initiates new fermentation by harvesting fermentation medium, cell recycling, and refilling fresh medium in reactors sequentially. Cells can be recycled in bioreactors by filtration, centrifugation, cell immobilization, cell flocculation, or cell sedimentation. The concept of re-using cells in sequential batch mode shares similarities with the repeated batch approach, both of which enable high throughput fermentation processes [86]. Differently, sequential batch fermentation can be categorized as a fermentation approach without re-using the same culture. Kumar *et al.* [87] reported a sequential fermentation system for 2-stages ethanol production, where ethanol was produced from glucose in the first stage by *Zymomonas (Z.) mobilis* and produced from xylose in the second stage by *Pichia (P.) stipitis*. This further extends the applicability of using sequential batch approach in a biorefinery process.

A major application of sequential batch that has been achieved is the utilization of multiple strains to improve sugar utilization from lignocellulosic hydrolysates. Pentose utilization is viewed as a fermentation challenge in bioethanol production, especially for lignocellulosic feedstocks. Known wild-type ethanol-producing strains, such as *S. cerevisiae* and *Z. mobilis*, are not able to utilize pentose for ethanol fermentation. On the other hand, pentose-consuming strains, such as *P. stipitis* and *Candida shehatae*, can be used for pentose utilization by sequential batch approaches to address this limitation. For example, Song *et al.* [88] inoculated *S. cerevisiae* in the hardwood hydrolysate for initial glucose consumption. When glucose was almost utilized, *P. stipitis* was introduced in the same reactor for fermenting xylose. The results indicated that ethanol content

improved around 12% with both glucose and xylose utilization, as compared to glucose conversion alone [88]. To enhance fermentation efficiency in a co-culture system, the compatibility of mixed strains required further investigation. Kumar *et al.* [87] demonstrated that if *Z. mobilis* wasn't removed before the addition of *P. stipitis* in the sequential operations, the growth of *P. stipitis* was inhibited, and the strain didn't utilize xylose as efficiently for ethanol production. In terms of avoiding the strain incompatibility, the initial strain can be inactivated before the second culture was introduced [88, 89]. In addition, the inhibition of xylose utilization of *P. stipitis* may result from accumulated ethanol or the catabolite repression by high glucose contents. Switching the order of sequential operations for different inoculums may address the limitation. For example, Singh *et al.* [90] inoculated *P. stipitis* first for xylose utilization and then introduced *Z. mobilis* for further glucose utilization. This sequential approach allowed *P. stipitis* to expose less ethanol when taking xylose and finally displayed 97.7% sugar consumption in overall fermentation [90]. Furthermore, sequential batch approach can involve multi-stage fermentation. By switching aerobic to anaerobic condition, *Candida tropicalis* W103 was able to produce xylitol first and then generate ethanol from the non-detoxified acid pretreated corncob [91]. This approach further suggests opportunities for lignocellulosic ethanol production and the co-production of value-added products from lignocellulosic feedstocks, expanding economic competitiveness in the bioethanol industry. While the challenges of co-culture in sequential fermentation for bioethanol production require addressing, the sequential approach can act as a practical configuration for high ethanol throughput and designing fermentation in a mixed sugars system.

2.3.3 Self-cycling fermentation

Self-cycling fermentation (SCF), a semi-continuous operation and consists of three stages in each cycle including: nutrient supplementation; fermentation; and the harvesting of the medium.

Between each cycle in SCF, half volume of fermented medium is taken out and half volume of fresh nutrient is refilled into a bioreactor [7]. SCF can not only provide a high throughput process but also further achieve cell synchronization during fermentation [6]. The cell division cycle for a budding yeast cell is composed of four phases, G1 (the first gap phase), S (DNA synthesis phase), G2 (the second gap phase), and M (mitosis phase). Cell synchrony is described as the simultaneous division of cells in the entire population [92]. Ideally, each single cell becomes coordinated at the same stage in cell division, in which case the cell population can be simply regarded as a single cell and show almost identical behaviors in cell growth. This characteristic can benefit the understanding of cell physiology. For example, the relationship between the life cycle and protein expression of *Escherichia (E.) coli* was studied via SCF system [5].

To set up a SCF system, selecting a reliable monitoring parameter for determining the length of each cycle is critical. The control variable monitored must indicate the growth pattern of the microorganism, enabling to trigger cycling operations in SCF system. Dissolved oxygen, carbon dioxide evolution rate (CER), and redox potential are known candidates to indicate the stage of fermentation in SCF systems [6]. For instance, Sauvageau *et al.* [93] utilized CER as the control parameter and demonstrated that the first derivative of CER can correspondingly indicate the growth transition from exponential phase to stationary phase of *E. coli*. Moreover, multiple control indicators can be implemented to support the SCF system. Tan *et al.* [94] manipulated the cycle based on four variables, including minimal running time, carbon dioxide concentration, and the first derivatives of carbon dioxide concentration and CER for shikimic acid production by the engineered yeast. Through real-time feedback controls, SCF can be designed as an automated fermentation system, reducing repeated manual operations in a long-term fermentation process.

Currently, studies using SCF in bioethanol production are limited, as compared to other fundamental fermentation approaches. However, it is worth noting that a relative comprehensive series has been reported by Wang *et al*. [7, 11, 12]. The authors first demonstrated the concept of SCF in ethanol fermentation in a flask scale [7]. Next, the authors further explored evolved gas flow rate, a real-time monitoring parameter during ethanol fermentation, to control SCF in a bioreactor scale [11]. Finally, Wang *et al.* [12] applied the SCF to wood pulp hydrolysate and developed a biorefinery system for ethanol and co-production of the value-added product. Ethanol productivity was improved by 63–95% in the wood pulp hydrolysate by SCF, as compared to the batch mode [12]. This was supported by the fact that *S. cerevisiae* was kept in the exponential phase by cycling operations, leading to a higher ethanol productivity than growing in the lag and stationary phase [11]. SCF can also be applied to very-high-gravity fermentation for high ethanol titer and productivity. Although the stability of the control parameter was affected by the medium composition, Feng *et al.* [95] successfully utilized the slope of redox potential to indicate the end of glucose consumption and to determine the cycle time for ethanol fermentation. Compared to batch and continuous fermentation modes, performing SCF could gain higher annual ethanol productivity by 2.4% and 13.2%, respectively [95]. However, risks of microbial selection require attention in SCF. It was pointed out that more flocculation of the yeast obtained in the later cycles of the SCF which might influence the stability of the control parameter [12]. Overall, as compared to batch fermentation, SCF is an advanced fermentation mode for bioethanol production, providing a high throughput process. It is also known that SCF has been applied to various fields for biofuel, bioplastic, and antibiotic productions [6]. In terms of cell synchrony techniques, SCF is a suitable approach to investigate cell physiology in various biomass hydrolysates to further optimize overall

fermentation efficiency. Addressing the stability of the SCF system for a long-term process and integrating SCF to other fermentation approaches are promising pathways to focus on.

2.3.4 Extractive fermentation

In addition to the above operational approaches mainly controlling substrate delivery, extractive fermentation focusses on removing components from fermentation medium to avoid metabolic inhibition during the process. For instance, as ethanol concentration increases in fermentation medium, it can gradually inhibit the metabolic pathway of the yeast, such as reducing ATP synthesis, cell growth, and further impacting ethanol productivity in ethanol fermentation [96]. Extractive techniques for removing ethanol include pervaporation, gas stripping, vacuum, solvent extraction, adsorption, and distillation, which are clearly reviewed by Zentou *et al.* [97]. Depending on operational designs, the separation approaches can be integrated into various fermentation modes. For example, gas stripping has been demonstrated in batch [98], fed-batch [50, 99], and continuous [100] fermentation modes for ethanol production, improving either final ethanol concentration, yield, or productivity by mitigating ethanol stress in the fermentation medium. The concentration of recovered ethanol is worth investigating for optimizing those extractive techniques. Kongkaew *et al.* [101] explained that ethanol concentration might affect downstream designs, such as whether the dehydration step is further necessary to produce the fuel-grade ethanol.

In terms of metabolic inhibitory compounds, ethanol is not the only stress in ethanol fermentation. For example, certain amount of organic acids either from biomass hydrolysate or fermentation itself can exhibit inhibitory effects on cell growth as well [22]. In the report of Kongkaew *et al.* [101], although ethanol was removed, ethanol productivity was still limited since organic acids accumulated without pH control in the fermentation system. Extractive techniques can also remove other inhibitory compounds to relieve the related stress. Furfural, an inhibitory

byproduct formed by hydrolysis in lignocellulosic hydrolysates, can be selectively removed by adsorption using activated carbon [102] or zeolites [103]. Both studies [102, 103] indicated that sugar consumption and ethanol production were improved with removal of inhibitors, as compared to untreated groups. It is worth noting that extractive approaches can operate either in-situ or ex-situ with ethanol fermentation [97]. Although ethanol and other inhibitory compounds may impact fermentation systems significantly in certain cases, energy requirement of extractive technique limits its application. Spending extra costs on developing extractive fermentation for ethanol production may not be attractive to the bioethanol industry [97].

Overall, extractive techniques are useful approaches to optimize bioethanol production by removing ethanol or other inhibitory compounds. Based on the improvement of overall ethanol productivity, Rodrigues *et al.* [100] indicate that smaller bioreactors may be considered to set up to potentially reduce the production cost. Furthermore, extracting platform chemicals, such as 5-HMF, furfural, and vanillin, from lignocellulosic hydrolysates may create extra values in bioethanol production from lignocellulosic feedstocks. To evaluate the trade-off between energy spending and potential benefits, further economic analysis of integrating extractive techniques into bioethanol production is necessary, especially in industrial scale production [97].

In summary, this section of review highlights the operational concepts and examples for fundamental and advanced fermentation approaches in bioethanol production. Applications and limitations of these fermentation modes are summarized in Table 2.1. It should be noted that these fermentation modes can be further integrated together in different scales and biomass systems. For instance, Farias and Maugeri-Filho [86] incorporated fed-batch, sequential, and vacuum operations for lignocellulosic ethanol production, enhancing ethanol productivity by 10-fold. The operational

approach plays a critical role in substrate delivery and even inhibitory compound removal for bioethanol production.

Table 2.1 Applications and limitations of fermentation modes on bioethanol production

Fermentation modes	Applications	Limitations
Fundamental approaches		
Batch	• Can explore/set up an unknown fermentation • Can be a benchmark for optimizations • Easily integrate to other fermentation modes • Intermediate storage (e.g. aging beer)	• Substrate inhibition • Long lag phase • Intensive manpower and long downtime between each batch
Fed-batch	• Can reduce substrate inhibition • Can reach high product content and productivity • Can reach high cell density • Control desired metabolic reactions by feeding strategies	• Complex control settings on feeding strategies • Product inhibition • Inhibitory compounds may be accumulated (e.g. furfural, 5-HMF, organic acids, phenols)
Continuous	• Can reduce substrate inhibition • Can reach high productivity (less working volume) • Can reach high cell density (with cell retention techniques) • Can be used to evaluate the cell physiology at a certain cell growth phase	• Complex control requirements for maintaining the steady state • Wash-out effect • Usual low product concentration • Microbial selection and contamination

Derivative approaches		
Repeated batch (semi-continuous)	• Can re-use the starter culture • Can save preparations of inoculum • Can experience cell adaptation • Can gain benefits listed in fed-batch and continuous (depending on operational designs)	• Microbial selection and contamination • Consistency of long-term processing
Sequential batch (semi-continuous)	• Multiple sugar utilizations and product formations (single or co-culture system) • Can deal with multiple stage of fermentation • Can gain benefits listed in fed-batch and continuous (depending on operational designs)	• Microbial selection and contamination • Compatibility of mixed cultures
Self-cycling (semi-continuous)	• Can achieve cell synchrony • Can gain benefits listed in sequential batch	• Microbial selection and contamination • A stable feedback control is required
Extractive fermentation -Pervaporation -Gas stripping -Vacuum -Adsorption -Solvent extraction etc.	• Can reduce metabolic inhibitory compounds (e.g. ethanol, organic acids, furfural etc.) • Can recover valuable compounds (biorefinery)	• Extra energy consumption • Cost-effective materials and techniques are required.

2.4 Control strategy in feeding regimes for ethanol fermentation

As fermentation modes control substrate delivery, the control strategy can impact the effectiveness of the feeding regimes. For example, Hemansi and Saini [104] evaluated six feeding strategies for both biomass and hydrolytic enzymes in the SSF of dilute acid-alkali pretreated sugarcane bagasse. As substrate concentration increases in the fermentation medium, the efficiency of ethanol fermentation is greater [105]. However, the authors also noted that overloading the substrate could cause the substrate inhibition on the ethanolic strain [105]. Hence, determining an effective control in substrate delivery is required.

2.4.1 Open-loop control

Open-loop control is an operational approach based on pre-known outputs from the process [106]. Under this control system, a generic feeding strategy can be developed without any online monitoring or adjustments. The amount of substrate can be determined by previous batch experiences or other research references that already offered a concept for feeding operations. For example, Hjersted and Henson [107] reported the model-based optimization of ethanol production with an open-loop control in fed-batch fermentation, where the feeding strategy was developed by a predetermined scheme. The open-loop control simplified the modeling process, enabling to evaluate the trade-off between ethanol productivity and yield for optimizations. However, open-loop control may not adapt to disturbances in fermentation because it relies on the predetermined model [108]. To minimize disturbances (e.g. structural errors and parametric errors), adding feedback measurements to the open-loop controller was feasible, improving ethanol titer by 8–15% in the modified system [109].

2.4.2 Feedback control

Feedback control is a closed loop system that adjusts feeding operations based on fermentation outputs. In terms of ethanol fermentation with *S. cerevisiae*, the carbon source primarily flows to product formation (ethanol and carbon dioxide), cell biomass production, and cell maintenance. Monitoring this metabolic information is critical for applying feedback control in ethanol fermentation. For example, viable cell sensor is used to estimate the cell number by measuring the capacitance of the fermentation medium, determining the time for supplying fresh medium in the fed-batch fermentation [110]. In addition, applying spectroscopy is a promising pathway for on-line monitoring of ethanol fermentation, such as FTIR [111], Near-IR, Mid-IR, or Raman spectroscopy [112]. Hirsch *et al.* [113] adjusted the feed rate to maintain glucose at 100 g/L based on Raman spectroscopy in ethanol fermentation, and this feeding approach improved ethanol yield from 75% to 86%, as compared to the batch mode. However, it should be noted that validation and calibration are required when applying spectroscopy. The measurement may be interfered by non-dissolved particles, such as bubbles or cell particles, in the fermentation medium [114-116]. Besides, gas composition in ethanol fermentation is a crucial variable for developing feedback control strategies. Release of carbon dioxide is coupled with ethanol production and glucose consumption in ethanol fermentation with *S. cerevisiae* ($C_6H_{12}O_6 \rightarrow 2C_2H_5OH+2CO_2$). Nilsson *et al.* [56] utilized carbon dioxide evolution rate (CER) and cell biomass to estimate residual sugar concentration, adjusting the feed rate to control sugar at a certain level with the lignocellulosic hydrolysate. The results demonstrated that increasing the sugar set-points from 0.5 g/L to 3 g/L improved the average ethanol productivity (g/g/h), as compared to the batch mode. With an effective monitoring approach, establishing an adjustable feeding strategy based on feedback control to enhance ethanol fermentation is feasible.

Modeling approaches, such as adaptive control, model predictive control (MPC), artificial neural networks (ANN), can be integrated into feedback control strategies for process optimization and automation in fed-batch fermentation [117]. Prior to a modeling simulation, variables and objectives are critical to define. For example, the target reactions (e.g. ethanol fermentation, glucose oxidation) are clearly defined to control continuous fermentation for ethanol production in the study of Ciesielski and Grzywacz [74]. In addition, the optimizing theory and its mathematical rational also need to be stated specifically in the modeling approach. Even though different kinetic models are built in the same control strategy, they can aim at various objectives that may not be comparable under a single benchmark. To address the pathway from model simulation to real processing, inputs of economic and energy are required in modeling controls. For instance, economic impact was used to assess the trade-off between ethanol productivity, yield, and concentration in different operating strategies [118]. Energy balance in fed-batch SSF for bioethanol production was considered in the control model [119]. Furthermore, it is also critical to validate the simulated model through database [119], experiments [120, 121], or real plant demonstrations for final applicability.

2.5 Potential limitations in ethanol fermentation for lignocellulosic ethanol production

Currently, bioethanol is primarily produced from the first-generation feedstocks (sugar- and starch-based biomass). The United States of America and Brazil, the first and the second largest bioethanol producers, take advantage of corn and sugarcane for ethanol production, respectively [122]. On the other hand, when it comes to the food-fuel competition in bioethanol production, exploring alternative feedstocks is still imperative. Lignocellulosic biomass is a promising

bioresource for long-term bioethanol production without competing with food crops for land utilization [123]. However, production of lignocellulosic ethanol faces challenges for industrialization due to its high production cost, as compared to the first-generation ethanol [124]. Optimizing the production process is one approach to address the limitation. Pretreatment and hydrolysis in lignocellulosic ethanol production have been widely reviewed [125-127]. Therefore, in this section, we focus on updating the fermentation process and its known limitations, and then highlight potential fermentation pathways to enhance lignocellulosic ethanol production.

2.5.1 Separated hydrolysis fermentation

In the principle of SHF, hydrolysis and fermentation are conducted separately with respective optimal conditions. Despite this characteristic, SHF may not be an attractive choice for lignocellulosic ethanol production, as compared to SSF [128-130]. For example, activity of cellulases (or other hydrolases) can be reduced by end-product accumulation during SHF [131]. However, due to different operational conditions, comparison of SHF and SSF may not be based on the same benchmark, such as the timeline of the fermentation process. In the report of Dahnum *et al.* [132], the experimental time for SHF (72 h) was considered longer than SSF (24 h); however, most glucose was converted to ethanol in the first 24 h in SHF. Additionally, xylose, a co-product released in SHF (1.0–1.5%) was likely higher than in SSF (0.10–0.85%) [132]. This may result from the higher hydrolysis temperature in SHF, accelerating the release of xylose. Depending on different design perspectives, the effectiveness of SHF and SSF in lignocellulosic ethanol production can vary. Furthermore, characteristics of feedstock also impact the effectiveness of SHF and SSF. Mithra *et al.* [54] indicated that with steam pretreated lignocellulosic feedstocks, SSF acquired higher ethanol recovery (mL/kg dry biomass) than SHF. However, SHF generally performed greater when using dilute acid pretreated lignocellulosic feedstocks. To further prompt

the development of lignocellulosic ethanol in the bioethanol industry, SHF can potentially serve as a short-term solution by integrating first-generation and the second-generation feedstocks. D3MAX, a technology company (https://www.d3maxllc.com/), built a fermentation process with both SHF and SSF, enabling bioethanol production from lignocellulosic sugars and starchy sugars with the same biomass.

In terms of the fermentation performance of lignocellulosic ethanol, final ethanol concentration, sugar-to-ethanol ratio (or biomass-to-ethanol ratio), and ethanol productivity are critical variables. Especially, final ethanol concentration is important for the subsequent distillation. The minimal concentration of ethanol for an economic recovery is 4% (w/v) [14, 15]. To compete with the first-generation bioethanol in distillation cost, achieving more than 10% (v/v) ethanol via fermentation for lignocellulosic ethanol is necessary [16]. High substrate loading possibly releases more fermentable sugar by hydrolysis, boosting the final ethanol concentration [51, 133]. López-Linares *et al.* [51] demonstrated that ethanol fermentation in SHF with 20% (w/v) solid substrate (pretreated rapeseed straw) performed a higher final ethanol concentration than in SSF, where the yeast might be stressed by high solid loading in SSF. It was also pointed out that high solid content might increase viscosity in the medium, limiting agitation in SSF and reducing the efficiency of ethanol fermentation [36, 134]. SHF enables various biomass to be utilized in lignocellulosic ethanol production, as reactors and operating conditions for hydrolysis and fermentation are designed individually. To further optimize the fermentation performance in SHF, integrating it with other fermentation approaches is potential for lignocellulosic ethanol production.

Advancing fermentation modes can address limitations in substrate delivery in SHF for ethanol fermentation. Wang *et al.* [12] investigated an automated self-cycling fermentation system using lignocellulosic hydrolysate, achieving improvements in ethanol volumetric productivity by

63–95%, as compared to batch fermentation. Additionally, sugar delivery by fed-batch approaches can be impactful for accelerating ethanol production in SHF system. For example, final ethanol concentration and ethanol yield were enhanced when pulsed fed-batch operations were applied in the corncob hydrolysate [35]. Furthermore, recycling techniques for recovering yeast, ethanol, or other valuable products in SHF system may be considered in biorefinery processes as well. Cubas-Cano *et al.* [135] applied sequential batch fermentation to lignocellulosic hydrolysate for ethanol and lactic acid co-productions, addressing xylose utilization in SHF. When lignocellulosic feedstock is hydrolyzed into fermentable sugars readily, efficient fermentation modes can be involved in SHF. Although SHF may not be as attractive as SSF in developing advanced fermentation systems for lignocellulosic ethanol production recently, its well-known principle makes it more flexible to be enhanced by integrating with fermentation modes. The fermentation integration in SHF is a promising pathway for lignocellulosic ethanol production by a short-term period in the industry.

2.5.2 Simultaneous saccharification and fermentation

SSF, a fermentation configuration, combines hydrolysis and fermentation in a single reactor, addressing end-product inhibition during hydrolysis and enhancing fermentation performance [52]. Currently, SSF is extensively utilized for starchy bioethanol production in the ethanol industry [122]. The use of SSF for lignocellulosic ethanol production has been studied in various lignocellulosic feedstocks [53, 136-144]. However, this technology still faces several challenges to implement lignocellulosic ethanol production at industrial scale. For example, further improvements in final ethanol concentration are required in SSF for lignocellulosic ethanol production. High ethanol concentration in the fermentation medium contributes to cost reductions in the subsequent downstream processes, such as ethanol recovery or waste water treatment [133].

Increasing feedstock loading in SSF brings more substrates for converting fermentable sugars to ethanol production [51, 145]. To achieve high substrate loadings and maintain evenly agitation in reactor, fed-batch operation is a suitable approach for delivering substrates in SSF, as noted in the literature [146-148].

In terms of the development of fed-batch strategies in SSF, feeding material, feed loading, and feeding time-point are critical variables to investigate in lignocellulosic biomass systems. For instance, feeding material is not restricted to solid (or semi-solid) substrates. In addition to the fermentable substrate, enzymes or supplemental nutrients can also be fed into bioreactors. In SSF, ethanol productivity is limited by enzymatic hydrolysis (or sugar releasing rate), instead of fermentation rate [133]. However, as cells grow during fermentation, the demand of fermentable sugar for the culture also increases. To further improve ethanol production in SSF, the degree of enzymatic hydrolysis needs to be gradually increased during the process. Hence, fed-batch approach in either substrate or enzyme is worth investigating in SSF for lignocellulosic ethanol production [52]. Zhang and Zhu [145] established substrate and enzyme feeding modes in the SSF of pretreated sugarcane bagasse. They demonstrated that the enzyme feeding mode could enhance ethanol productivity and reduce enzyme loadings in the process, as compared to the batch SSF and the substrate feeding mode [145]. It should be noted that different lignocellulosic feedstocks may vary in their adaptability to feeding strategies. Jin *et al.* [149] developed substrate and enzyme feeding modes for the SSF of pretreated rice straw. With 25% (w/v) solid loading, final ethanol concentration by applying substrate and enzyme feeding modes reached 116.8 g/L and 108.6 g/L, respectively. Depending on substrate composition, feeding time-point can impact overall fermentation performance as well. Li *et al.* [150] established a two-step fed-batch simultaneous saccharification and co-fermentation (SSCF) for ethanol production from pretreated corn stover.

The results indicated that final ethanol concentration was higher when the feed was added in the second stage of fermentation (noted as strategy C in their study), as compared to the addition in the first stage (noted as strategy B in their study). The metabolic inhibitors introduced from feeds might have more negative impacts in the first stage of fermentation, possibly leading to a lower xylose utilization and ethanol production. While increasing the lignocellulosic substrate loading in SSF, the composition of the fermentation medium changes, requiring further adjustments for maximizing the fermentation process.

High solid loading may impact the stirring effectiveness in bioreactors, leading to adverse effects on mass transfer in enzymatic hydrolysis as well as fermentation. Qiu *et al.* [151] reported that 15% (w/w) pretreated wheat straw was fully liquified within 48 h while the 20% (w/w) solid loading required 72 h, resulting from limited mass transfer. This indicates the trade-off between high solid loading of lignocellulosic feedstocks and the effectiveness of the agitation. To address the limitation, bioreactor design and settings are important for effective agitation and lignocellulosic substrate transfer in SSF. Zhang *et al.* [152] investigated two stirring systems in the SSF of pretreated corn stover (solid loading, 30%, w/w). The pretreated corn stover could be evenly agitated with the helical impeller in SSF, resulting in a higher glucose consumption rate, ethanol production rate, and lower stirring power consumption than using the Rushton impeller. At the end of SSF, ethanol concentration achieved 51.0 and 43.9 g/L by the helical and the Rushton impeller agitation, individually [152]. To reduce the viscosity of solid substrate, hydrolysis can be applied to lignocellulosic feedstocks before feeding. Paulová *et al.* [153] set up two bioreactors, one was for SSF; another was for pre-saccharifying the feed, for producing ethanol from pretreated wheat straw. The outcome indicated that the final ethanol concentration was improved in the fed-batch SSF, as compared to the batch SSF which was limited by the viscosity of the fermentation medium.

Beside fed-batch approaches, other fermentation modes, such as repeated batch fermentation, can be integrated into SSF system for enhancing ethanol production. Watanabe *et al.* [154] combined repeated batch fermentation with SSF of 20% (w/w) alkali-pretreated rice straw by using immobilized *S. cerevisiae*. The authors observed a gradual increase in final ethanol concentration and a reduction of lag growth over five consecutive batch fermentation. In addition to cell immobilization, free cells can be recycled in SSF as well. Choi *et al.* [78] utilized a flocculent yeast, enabling the recovery of cells from sediment flocs and achieving repeated batch cycles in SSF. However, it should be noted that lignocellulosic SSF contains undigested solid in the reactor while SHF does not. When applying repeated batch mode or other cell recycling techniques in the lignocellulosic SSF, the operational standards for cell recycling require further investigations. For example, evaluating the morphology of the carrier and its mechanical strength are important, which may impact the fermentation performance of the immobilized cells in consecutive cycling processes [85, 155].

Overall, further advancements need to be accomplished in SSF system for lignocellulosic ethanol production. One fermentation limitation in lignocellulosic SSF results from the physical and chemical characteristics of lignocellulosic feedstocks. Fermentation mode can be applied in substrate or enzyme deliveries to control fermentation process in SSF. Through our reviews, fed-batch approach is a promising and applicable pathway to enhance the efficiency of hydrolysis and fermentation in lignocellulosic SSF.

2.5.3 Pentose utilization from lignocellulosic feedstocks

In terms of fermentable sugars, hexose and pentose are the primary units in lignocellulosic feedstocks for ethanol fermentation [156]. However, utilizing both hexose and pentose may be limited to ethanolic microorganisms. For example, while *S. cerevisiae* is a known yeast used in the

ethanol industry for the first-generation ethanol production, it cannot ferment pentose to ethanol directly without genetic modifications [157, 158]. To improve ethanol yield from lignocellulosic biomass, pentose utilization in fermentation is worth investigating.

Co-fermentation is one fermentation approach to enhance both hexose and pentose utilizations in lignocellulosic ethanol production, either using single or multiple strains in fermentation process [159-161]. Demiray *et al.* [159] demonstrated that co-culture of *S. cerevisiae* and the isolated *Candida boidinii* improved ethanol concentration by approximate 70% in the tea waste hydrolysate, as compared to the average ethanol concentration in individual culture. In this study, the *S. cerevisiae* could utilize glucose, and the isolated *Candida boidinii* could utilize both glucose and xylose for ethanol production. When it comes to the co-culture of multiple strains, it is necessary to consider the fermentation kinetic for each strain. Fermentation temperature, aeration, and environmental stress could impact the cell growth and glucose/xylose utilization in co-culture [162]. It should be noted that the fermentation kinetic of the strain may perform differently when the strain is cultured solely or co-cultured with other strains. In the report of Zhu *et al.* [163], the xylose-utilizing strain had higher glucose/xylose consumption and ethanol concentration in low temperature (30 and 35ºC), as compared to high temperature cultivation (38 and 42ºC) which might result from decreased cell viability. Intriguingly, when the co-culture was applied, the cell viability of the xylose-utilizing strain was improved at 42ºC. In comparison of single sugar utilization, co-culture system can improve overall ethanol concentration by utilizing two sugars [159] and may provide additional benefits for cell growth. On the other hand, finding an optimized culture condition for co-culture of multiple strains is a challenge. For example, when co-culturing *S. cerevisiae* and *Scheffersomyces stipitis*, maintaining partial aerobic condition in fermentation may lead to an increase in cell growth for *Scheffersomyces stipitis*, instead of in ethanol production

[164]. In addition to co-culture, using a single strain to ferment both hexose and pentose in lignocellulosic hydrolysates for ethanol production has been studied, such as recombinant *E. coli* [160] and genetic engineering *S. cerevisiae* [165-167]. Comprehensive details in genetic engineering for pentose utilization and ethanol production were reviewed by Dien *et al.* [168], Jeffries and Jin [169], and Kuhad *et al.* [170]. Overall, co-fermentation is a promising approach to utilize both hexose and pentose for lignocellulosic ethanol production.

In addition to using pentoses for ethanol production, pentose can be derived into different valuable products utilizing biorefinery based approaches. To increase cost-competitiveness of lignocellulosic ethanol and biomass utilization, integrating biorefinery approaches into the process is viewed as a potentially advantageous [171]. Xylose and arabinose, major pentoses in the heteropolysaccharide of lignocellulose, have been derived into various products and applications. For example, xylitol, a widely used sugar substitute in food industry, can be produced from xylose via chemical or biological process [172, 173]. L-ribose, a rare sugar used as one of the precursors for developing antiviral drugs, can be converted from L-arabinose via microbial pathways [174]. Suhartini *et al.* [173] reported that co-production of bioethanol and xylitol from oil palm empty fruit bunches (lignocellulosic resource) is a promising strategy in Indonesia to increase the capacity of renewable energy production, although challenges in the scale-up technique, policy, and supply chain still need to be addressed.

2.5.4 Inhibitory compounds in lignocellulosic hydrolysate

When degrading lignocellulosic biomass to acquire fermentable sugars for ethanol production, smaller molecular weights of compounds are generated from the lignocellulosic matrix. Part of them are identified as metabolic inhibitory compounds, such as furan-derivatives, aromatic compounds, and aliphatic acids, negatively impacting ethanol fermentation [175]. In the example

of furfural (furan-derivative), as furfural concentration in the fermentation medium are increased, proportional increases in inhibition on yeast's hexose utilization and ethanol productivity have been observed [176].To reduce the negative impacts from inhibitors, three major approaches have been considered including: (1) optimizing 'pre'-fermentation processes (such as pretreatments or hydrolysis); (2) detoxification; and (3) enhancement of microbial tolerance. Firstly, optimizing the processes prior to fermentation is feasible for low inhibitor productions. Pretreating lignocellulosic biomass can also lead to different levels of degradation. For instance, pretreating the olive tree pruning with water produced less furfural and hydroxymethylfurfural (HMF) than with 2% sulfuric acid at the same temperature condition [177]. The severity of dilute sulfuric acid hydrolysis on spruce could impact the contents of inhibitors in the hydrolysate [178]. In addition, researchers are addressing the concern by investigating the structure of lignocellulosic biomass. Through reducing lignin content or recalcitrant interaction between lignin and cellulosic structure in lignocellulosic biomass, inhibitor formation from pretreatment or hydrolysis has been reduced [175, 179].

Secondly, detoxification approaches aim to remove inhibitors or decrease their contents from lignocellulosic hydrolysates. Approaches include applying chemical additives, extractive fermentation, or biological treatments, either ex-situ or in-situ [180, 181]. Zhang *et al.* [102] reduced furfural content from 4 g/L to 0.1 g/L using activated carbon sorption and improved the sugar utilization and ethanol production. In addition, the furfural-sorption group exhibited similar cell growth, sugar consumption, and ethanol production to the no furfural group [102]. Through detoxification, the performance of ethanol fermentation can be enhanced. Interestingly, detoxification requirement varies by strains and inhibitors. In the report of Hou *et al.* [182], *Z. mobilis* generally exhibited greater tolerance on lignocellulose-derived inhibitors than *E. coli* based on the assessment of the half-maximal inhibitory concentration (IC50). Based on this study, when

Z. mobilis is used as a fermentation strain for lignocellulosic ethanol production, the detoxification requirement in lignocellulosic hydrolysates can be relatively mild, as compared to fermenting with *E. coli*. This may benefit the production cost for lignocellulosic ethanol. However, the effectiveness and the necessity of detoxification in lignocellulosic ethanol production are dependent on various process scenarios [183]. It is also required to evaluate a risk of losing fermentable sugars from detoxifications [184]. However, inhibitory compounds may act both negatively and/or positively to microorganisms. For example, acetic acid, a by-product from degrading hemicellulose, has been shown to reduce ethanol volumetric productivity, but also enhance the sugar-to-ethanol conversion ratio during ethanol fermentation [185]. The choice of detoxification methods can vary based on individual fermentation design. To further mitigate negative impacts from metabolic inhibitory compounds, understanding the inhibitor-tolerance of the microorganisms is also critical.

In addition to reducing inhibitor content through detoxification approaches, improving the strain's metabolic tolerance to inhibitors is a known approach. Evolution engineering and tolerance engineering are potential pathways to enhance the inhibitor tolerance of microorganisms from genetic levels [186]. For instance, adaptive evolution was demonstrated to improve the inhibitor tolerance of the yeast by increasing the concentration of inhibitors gradually in the cultivations. Compared to the parent strain, the adapted strain enhanced ethanol fermentation efficiency by 80% in the inhibitor-containing medium [81]. It is worth noting that the concept of adaptive evolution on inhibitor tolerance improvements can be achieved by applying specific fermentation modes, such as cycling or sequential fermentation, allowing the strain to experience different inhibitor titers by repeating the fermentation operations. Palakawong Na Ayutthaya *et al.* [187] indicated that the yeast adapted from acclimatization in serial concentrations of lignocellulosic hydrolysate demonstrated higher ethanol yield and volumetric productivity, as compared to the non-adapted

yeast. Improving the inhibitor tolerance for microorganisms is potential to benefit the product yield and fermentation cost [186]. Understanding the morphological characteristic of microorganisms is also important for improving their inhibitor tolerance. As an example, flocculation among *S. cerevisiae* could lead to more efficient performance on ethanol fermentation in the lignocellulosic hydrolysate containing several inhibitors [188]. Interestingly, self-cycling fermentation, a semi continuous fermentation approach, can facilitate the flocculation of the yeast during ethanol fermentation and also improve ethanol productivity [11]. From our perspective, investigating the applications of fermentation modes to enhance microbial inhibitor tolerance suggests opportunities for addressing hindrances in industrial lignocellulosic ethanol production.

2.6 Integrations of fermentation modes for bioethanol production

Combining different fermentation approaches into an integrated fermentation system is a promising pathway to efficiently regulate material transfer and enhance lignocellulosic ethanol production. For example, fed-batch operational mode is a known approach to improve ethanol concentration and productivity in fermentation. Triwahyuni *et al.* [37] applied fed-batch operations for substrate loadings in the SSF of oil palm empty fruit bunches. The results demonstrated that the fed-batch mode (15+10 g) could improve higher ethanol concentration and reduced the enzyme usage as compared to the batch substrate loading (25 g) regime in SSF. The batch regime was suggested to show limitations in mass transfer of the lignocellulosic feedstock, leading to a low efficiency of enzymatic hydrolysis and ethanol fermentation. This observation is supported by Liu *et al.* [189] on the SSF of the corncob residues. Furthermore, Kang *et al.* [190] introduced a continuous feeding operation in the SSF of the lignocellulosic feedstock, improving ethanol concentration and productivity, as compared to the batch mode. The lignocellulosic feedstock was

liquified more efficiently with the continuous supplement than the batch mode in SSF. To further promote industrial lignocellulosic ethanol production, developing a continuous and repeatable fermentation system is also attractive, which relies on the integration of fermentation modes. For example, self-cycling fermentation or sequential batch fermentation can address multiple sugars utilization for ethanol production under the consecutive fermentation process [12, 87]. These fermentation approaches can manage the substrate utilization efficiently throughout the process.

Integrating different fermentation approaches can enhance the advantage of a single operational mode and address its limitation. For example, Farias and Maugeri-Filho [86] combined fed-batch, sequential, and vacuum operations for lignocellulosic ethanol production, improving ethanol productivity by 10-fold. Fed-batch approach led to high ethanol production, while cell recycling and vacuum operation reduced ethanol toxicity in the fermentation medium. Depending on the primary fermentation target, fermentation mode can be chosen accordingly. Jiang *et al.* [191] combined fed-batch and sequential modes for co-cultivation of cellulolytic and saccharolytic microorganism for ethanol production from Solka Floc (powdered cellulose), generating 474 mM bioethanol (~22 g/L). Further improvements in the integrated fermentation system are still required, particularly in addressing limitations such as a low final ethanol concentration from lignocellulosic feedstocks. However, only a few studies are investigating the integration of fermentation approaches for lignocellulosic ethanol production. The significant breakthrough on either SHF or SSF system with a single operational mode is limited to managing lignocellulosic substrate efficiently.

The use of biorefinery approaches for the conversion of lignocellulosic biomass to fuel ethanol and other value-added products has drawn attention as a pathway for the creation of a circular bioeconomy [192, 193], where integration of fermentation approaches plays a crucial role. For

example, not only ethanol is generated from lignocellulosic biomass but also xylitol, xylooligosaccharides, vanillin, or other value-added products can be produced [194]. These fermentation processes can be further optimized by integrating with appropriate fermentation modes. Unrean and Ketsub [195] reported that both ethanol and xylitol could be produced from sugarcane bagasse by two strains, *S. cerevisiae* and *Candida tropicalis*, individually. The authors maximized the product titer by applying the fed-batch fermentation approach. In the above study, the lignocellulosic biomass was pretreated and separated into solid and liquid fractions, and fractions were followed by ethanol and xylitol productions respectively. Another scenario for two products formations without using the separation process can be achieved by applying the specific fermentation mode. For example, sequential fermentation mode is used in the multiple stage of fermentation process for direct ethanol and value-added products formations [91, 196]. Moreover, to promote industrial lignocellulosic ethanol production, it is worth highlighting that configuration integration of lignocellulosic ethanol and the first-generation bioethanol is attractive [197]. Rather than constructing new infrastructure for lignocellulosic ethanol production, it is more feasible for the ethanol industry to integrate lignocellulosic biomass into existing first-generation bioethanol plants. For instance, ICM is a technology company (https://icminc.com/) and they develop a fermentation process (GEN 1.5) that generates both hexoses and pentoses from corn (its starch and fiber) and utilizes them separately for ethanol fermentation. As a result, arranging both fermentation systems and fermentation modes are required when designing an efficient fermentation process for lignocellulosic ethanol production. From a fermentation perspective, integrating effective fermentation modes can greatly manage substrate delivery and address potential fermentation limitations in lignocellulosic ethanol production, strengthening its feasibility in the bioethanol industry.

2.7 Summary

This literature review provides an updated overview of fermentation approaches for lignocellulosic ethanol production. Limitations in final ethanol concentration, ethanol yield, and volumetric ethanol productivity from a singular fermentation system (such as SHF or SSF) can be addressed via integrating different fermentation operational modes. Advancing fermentation operational modes can further guide material transfer, thereby improving the overall fermentation process. In this context, approaches to substrate delivery require deeper exploration and understanding in future research. In addition, as value-added lignocellulosic products suggest more opportunities for industrial lignocellulosic ethanol production, the practice of fermentation operational mode plays more critical roles in it. This can be facilitated by managing multiple substrate deliveries with the proper integration of fermentation operational modes. Further studies on advancing fermentation approaches to be implemented into the current industrial infrastructures are thus a promising pathway for broadening lignocellulosic ethanol production.

In the following chapters, I will focus on fed-batch fermentation and self-cycling fermentation to discover more efficient approaches to guide substrate delivery in ethanol fermentation. In addition, Table 2.1 and previous sections summarized the possible hindrances from these two fermentation systems, such as microbial contamination, product inhibition in fed-batch, or a reliable cycling control in self-cycling fermentation. These risks and/or difficulties will be further discussed in the following chapters as well.

Chapter 3 Adapted pulsed feeding strategies in fed-batch fermentation improve sugar delivery and ethanol productivity

3.1 Abstract

Bioethanol is a renewable fuel widely used in road transportation and is generally regarded as a clean energy source. Although fermentation is one of the major processes in bioethanol production, studies on improving its efficiency through operational design are limited, especially compared to other steps (pretreatment and hydrolysis/saccharification). In this study, two adapted feeding strategies, in which feed medium addition (sugar delivery) was adjusted to increase the supply of fermentable sugar, were developed to improve ethanol productivity in 5-L fed-batch fermentation by *Saccharomyces cerevisiae*. Specifically, a linear adapted feeding strategy was established based on changes in cell biomass, and an exponential adapted feeding strategy was developed based on cell biomass accumulation. By implementing these two feeding strategies, overall ethanol productivity reached 0.88±0.04 and 0.87±0.06 g/L/h, respectively. This to ~20% increases in ethanol productivity compared to fixed pulsed feeding operations. Additionally, there was no residual glucose at the end of fermentation, and final ethanol content reached 95±3 g/L under the linear adapted operation and 104±3 g/L under the exponential adapted feeding strategy. No statistical difference was observed in the overall ethanol efficiency (sugar-to-ethanol ratio)

between fixed and adapted feeding strategies (~91%). These results demonstrate that sugar delivery controlled by adapted feeding strategies was more efficient than fixed feeding operations, leading to higher ethanol productivity. Overall, this study provides novel adapted feeding strategies to improve sugar delivery and ethanol productivity. Integration into the current practices of the ethanol industry could improve productivity and reduce production costs of fermentation processes.

Keywords

Bioethanol, Fed-batch fermentation, Adapted feeding strategy, Sugar delivery, Ethanol productivity, Bench-scale, Bioreactor

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3.2 Background

To enhance the prospects of lignocellulosic ethanol production at an industrial scale, more efficient fermentation processes need to be developed. As ethanol productivity is greatest during exponential growth of *Saccharomyces cerevisiae* [20], most commonly used for bioethanol production, there are incentives to develop processing strategies that extend this phase of growth, such as fed-batch fermentation [41]. This approach consists of continuously or intermittently feeding essential nutrients to the fermenter in order to control and sustain cell growth and product formation [42]. Fed-batch operation also helps overcoming substrate inhibition during the fermentation process and can lead to greater ethanol yield [35] and ethanol productivity [48],

compared to batch fermentation. Interestingly, the above two studies have shown that feeding strategies play an important role in fermentation performance for ethanol production. This highlights the importance of developing effective feeding strategies for fed-batch fermentation. Once the efficient fermentation process is designed in the laboratory, bench-scale assessments can bring practical knowledge in transitioning the process from laboratory experiments to commercial production [198]. For example, Kang *et al.* [199] performed economic and sensitivity analyses based on a bench-scale plant for lignocellulosic ethanol production, providing feasible viewpoints to the development of a commercial production plant. Reproducibility of ethanol production by yeast between laboratory- and bench-scale is also crucial to understanding possible hindrances to industrial ethanol production [200]. Nonetheless, most studies on the improvement of lignocellulosic ethanol production have been carried out at laboratory-scale. It is thus worth further investigating the efficient bioethanol fermentation processes at bench-scale to enhance the prospect of commercialization.

Overall, fed-batch fermentation has the potential to greatly improve bioethanol production. The feeding strategy is a key factor in fed-batch fermentation; it can be designed based on nutrient contents, cell growth, or other factors impacting the final product [56, 76, 108]. However, there are few studies on the role of operational design for fed-batch fermentation in improving ethanol productivity. The development of adapted feeding strategies enables better management of dynamic fermentation processes and improves both substrate utilization of the yeast and ethanol productivity. In this context, the present chapter aims to develop adapted feeding strategies for sugar delivery to enhance ethanol production in cultures of *S. cerevisiae* at bench-scale. It compares the performance of a fixed and two adapted pulsing fed-batch strategies in terms of ethanol titer, efficiency, substrate utilization and ethanol productivity. All fermentations were performed in 5-L

stirred tank fermenters with monitoring of multiple parameters related to ethanol fermentation. This work highlights the potential of these feeding strategies for the improvement of the performance of industrial bioethanol production from first- and second-generation feedstocks.

3.2 Methods and Materials

3.2.1 Yeast, medium, and cultivation

Superstart™ active distillers dry yeast, *Saccharomyces cerevisiae*, was purchased from Lallemand Ethanol Technology (Milwaukee, WI, USA) and was used for ethanol fermentation in this study. Methods of yeast cultivation and preparation of glycerol stock followed Wang *et al.* [7]. Briefly, glycerol stock was thawed and streaked on a YPD agar (YPDA) plate. YPDA was made from 50 g/L yeast-peptone-dextrose medium (BD Difco™, Bergen Country, NJ, USA) and 15 g/L agar (Thermo Fisher Scientific, Waltham, MA, USA), and was sterilized by autoclaving (121°C/15 min). After culturing the yeast on YPDA at 30°C for 2–3 days, an isolated colony was selected, transferred to 20 mL of yeast nitrogen base (YNB) medium (50-mL shake flask), and cultured at 30°C with shaking at 230 rpm for 18–20 h to produce the first seed culture. YNB medium, prepared from 50 g/L glucose, 6.7 g/L YNB with amino acids (MilliporeSigma, Burlington, MA, USA) in 0.1 M sodium phosphate buffer ($NaH_2PO_4 \cdot 2H_2O/Na_2HPO_4 \cdot 2H_2O$, pH 6.0, Thermo Fisher Scientific), was sterilized by filtration (0.2 μm Sartolab™ P20 Pressure Filters, Sartorius, Göttingen, Germany). The first seed culture was transferred into 200 mL of fresh YNB medium (500-mL shake flask) to reach an initial optical density at 600 nm (OD_{600}) ~0.2, resulting in the second seed culture. It was then incubated at 30°C with shaking at 250 rpm until OD_{600} reached ~0.5. At this point, the active cell culture was ready to be inoculated into the fermenter.

3.2.2 Fermentation

3.2.2.1 Fermentation settings

All fermentation experiments were performed in 5-L stirred tank fermenters (Infors-HT, Bottmingen, Switzerland) based on the settings from Wang *et al.* [11]. Dissolved oxygen probe (VisiFerm DO ECS 325 mm H0, Hamilton Company, Reno, NV, USA) and pH probe (EasyFerm Plus PHI K8 325 mm, Hamilton Company) were attached to the fermenter. 2 N NaOH (Thermo Fisher Scientific) for pH control (set point at pH > 4.0). Pall AcroTM 50 vent filters (Pall Canada ULC., Mississauga, ON, Canada) were connected to the gas inlet and outlet of the fermenter. A water trap was installed between the gas outlet and the mass flow meter (MW-200SCCM-D/5M, Alicat Scientific Inc., Tucson, AZ, USA). The water trap was used to prevent environmental air from being introduced to the fermenter and maintain the anaerobic conditions for ethanol fermentation. The flow meter was calibrated with pure CO_2 by the supplier, and it was used to monitor the flow of gas released from the fermenter. Data from the gas flow meter was reported at standard atmospheric conditions (25°C, 1 atm) and the flow rate was reported as the average value over a 15-min timespan. An external condenser, set at 0°C, was used as the cooling system for the fermenter and vented gas. The bioreactor system was controlled by the software Iris 6.0 (Infors-HT). The initial working volume of all fed-batch operations was 2 L.

3.2.2.2 Fed-batch fermentation

The YNB fermentation medium was prepared, filter-sterilized (as mentioned above) and transferred to the fermenter aseptically. Before inoculation, the medium was flushed with N_2 (99.998% purity, Praxair Canada Inc., Mississauga, ON, Canada) for 30 min through the bioreactor gas inlet/sparger. Yeast grown to OD_{600} ~0.5 in the second seed culture was added to the fermenter as inoculum, comprising 8% (v/v) of the fermentation medium. Following inoculation, N_2 was

purged into the fermenter for 10 min to ensure anaerobic conditions. Fermentation was conducted at 30°C and 600 rpm using two Rushton impellers. The initial stage of fermentation acted as batch fermentation.

The outlet gas flow rate was used to monitor ethanol fermentation in real-time, as per Wang *et al.* [11], and served as an indicator to trigger pulse feeding. When the gas flow rate approached zero, a pulse of fresh feed medium was fed to the fermenter to increase the sugar concentration to a given value, depending on the experiment. The feed medium was a modified YNB medium: 500 g/L glucose and 6.7 g/L YNB with amino acids in 0.1 M sodium phosphate buffer (pH 6.0). The fermentation broth was sampled throughout experiments for biological and chemical analyses described in section 3.2.3.

3.2.2.3 Feeding strategies

Pulsed feeding was conducted in all fed-batch fermentation. Adapted feeding strategies were developed to improve sugar delivery and ethanol productivity in the fed-batch fermentation, while fixed feeding strategies were used for comparison. We hypothesized that the adjustment of glucose addition based on the change in cell biomass by pulsed feeding operations would enhance substrate utilization and ethanol productivity, compared to fixed glucose additions. Overall, three strategies were developed.

1) In a fixed feeding operation, the fresh feed medium was fed to increase sugar concentration to either 50 or 100 g/L (described as 5% pulsed feeding and 10% pulsed feeding, respectively).

2) In linear adapted feeding operation, the first pulsed feed increased glucose concentration to 5% (50 g/L), while the subsequent pulsed feeds were adjusted based on the change of OD_{600}, according to equation 3.1:

$$C_f = 5 \cdot (OD_t / OD_{t-1}) \qquad \text{Eq. (3.1)}$$

where C_f is glucose concentration (%, w/v) to reach in the fermenter, OD_t is the OD_{600} reading at a given sampling time t, and OD_{t-1} is the OD_{600} reading at the previous sampling time.

3) In exponential adapted feeding operation, the pulsed feeding was adapted based on cell biomass changes and cell accumulation. The glucose addition was based on equation 3.2:

$$C_f = 10 \cdot (1 - \exp(-OD_t / OD_{t-1})) \quad \text{Eq. (3.2)}$$

Equation 3.2 ensures the glucose concentration in the fermenter never exceeds 10 %, generally regarded as the maximum concentration to avoid inhibiting yeast growth and ethanol production [49, 66]. For all feeding strategies, the required fresh feed volume was calculated based on the volume in the fermenter and the glucose concentration in the fresh feed medium (500 g/L).

3.2.3 Analytical methods

OD_{600} was measured for 1-ml samples using a UV-Vis spectrophotometer (Ultrospec 4300 Pro, Amersham Biosciences, Mississauga, ON, Canada). Cell dry weight (CDW) analysis was performed as follows. A 9-ml sample was filtered using a pre-weighed 0.45-µm filter paper (Whatman™ type WCN cellulose nitrate membranes, Cytiva, Maidstone, Kent, UK). The retentate was washed by filtering through an equal volume of 10 mM sterile sodium phosphate buffer. The retentate and filter paper were then removed and dried at 60°C in an oven (Fisher Scientific Isotemp oven model 750F, Dubuque, IA, USA) until the mass was constant. The CDW was calculated and reported in g/L. During fed-batch fermentation, OD_{600} and CDW were used to evaluate yeast growth. Supplementary Figure 1 (Appendix A) shows the strong correlation between these two parameters ($R^2 = 0.995$).

Glucose and ethanol concentrations were determined by high-performance liquid chromatography (HPLC) and gas chromatography (GC), respectively [201]. Briefly, glucose analysis was carried out on the Agilent 1200 series HPLC system (Agilent, Santa Clara, CA, USA)

with an Aminex HPX-87H column (300×7.8 mm; Bio-Rad Laboratory, Hercules, CA, USA) held at 60ºC and a refractive index detector. 5 mM sulfuric acid was used as the mobile phase with a constant flow rate of 0.5 mL/min. Ethanol analysis was conducted in the Agilent 7890A series GC system (Agilent Technologies, Mississauga, ON, Canada) equipped with the Agilent 7693 series autosampler (injector temperature: 170ºC; pressure: 7.5 psi; septum purge flow: 3 mL/min; spilt ratio: 10 to 1), Restek™ Stabilwax-DA column (30 m×0.53 mm×0.5 μm; Restek, Bellefonte, PA, USA) and a flame ionization detector (temperature: 190ºC; airflow: 400 mL/min; hydrogen flow: 40 mL/min; make-up nitrogen flow: 25 mL/min). The following method was applied to the oven for ethanol analysis: temperature was kept at 35ºC for 3 min, then increase by 20ºC/min up to 190ºC, which was held for 1 min. Helium was used as the carrier gas (constant pressure: 51.710 kPa), and 1% (v/v) 1-butanol was added as the internal standard in the sample.

3.2.4 Statistical analysis

All fermentation experiments were run in independent triplicates. Data were expressed as means ± standard deviation. Statistical analysis was performed by one-way analysis of variance (ANOVA) and Tukey's honestly significant difference test, where $p < 0.05$ indicated the significant differences.

3.3 Results and Discussion

3.3.1 Impact of adapted pulsed feeding strategies

Pulsed feeding is a general mode of operation commonly used in fed-batch fermentation, as it is simple and easy to control [19, 46]. Optimizing feeding strategies can improve productivity which helps reduce production time and cost [42] and increase cell biomass and ethanol concentration during fermentation [105]. To improve sugar utilization and ethanol production in

fed-batch fermentation, linear adapted and exponential adapted pulsed feeding strategies were developed and compared to fixed pulsed feeding strategies.

Figures 3.1 (a–d) show cell dry weight (CDW) for each strategy tested. Although the settings of bioreactors and initial fermentation conditions (such as the amount of substrate, pH, and anaerobic status) were controlled consistently among the four strategies tested, CDW showed slight differences at the end of the batch stage. This is likely due to inter-batch variations, and/or experimental errors in sampling and the measurement of CDW. However, the accumulation of CDW was greater in the adapted feeding strategies than in the fixed feeding strategies during feeding period. For example, CDW remained relatively constant (~ 2 g/L) in the fixed pulsed feeding experiments but reached ~ 4 g/L by the end of fermentation undergoing linear adapted feeding. Similar results were observed when using the exponential adapted feeding strategy. This suggests that adapted feeding strategies benefit cell growth during ethanol fed-batch fermentation compared to fixed feeding strategies. It has been shown that cell biomass concentration can be enhanced by increasing glucose addition [35]; and this was observed in our two adapted feeding strategies in which glucose supplementation was gradually increased based on cell content and accumulation. On the other hand, Phukoetphim *et al.* [48] reported that viable cell count did not increase following pulsed feedings, which might be due to high glucose addition causing osmotic pressure on the yeast. In this study, we limited the glucose addition to 10% (w/v) to reduce the effect of substrate inhibition on the yeast; as a result, cell growth greatly improved in both adapted pulsed feeding strategies. In addition, it should be noted that flocculation was observed during all fermentations carried out in this study. Indeed, flocculation is a common characteristic of many *S. cerevisiae* cultures, and is affected by pH, temperature, nutrients composition and other fermentation parameters [202]. In our experiments, ethanol accumulation and pulsed feeding (rapid,

punctual addition of sugars) might have contributed to flocculation. Yeast flocculation can offer a cost-effective way to separate yeast from the liquid fraction at the end of fermentation; a widely applied strategy in the brewery industry [203]. It is worth exploring flocculation in fuel ethanol production as it may benefit the downstream process for ethanol recovery.

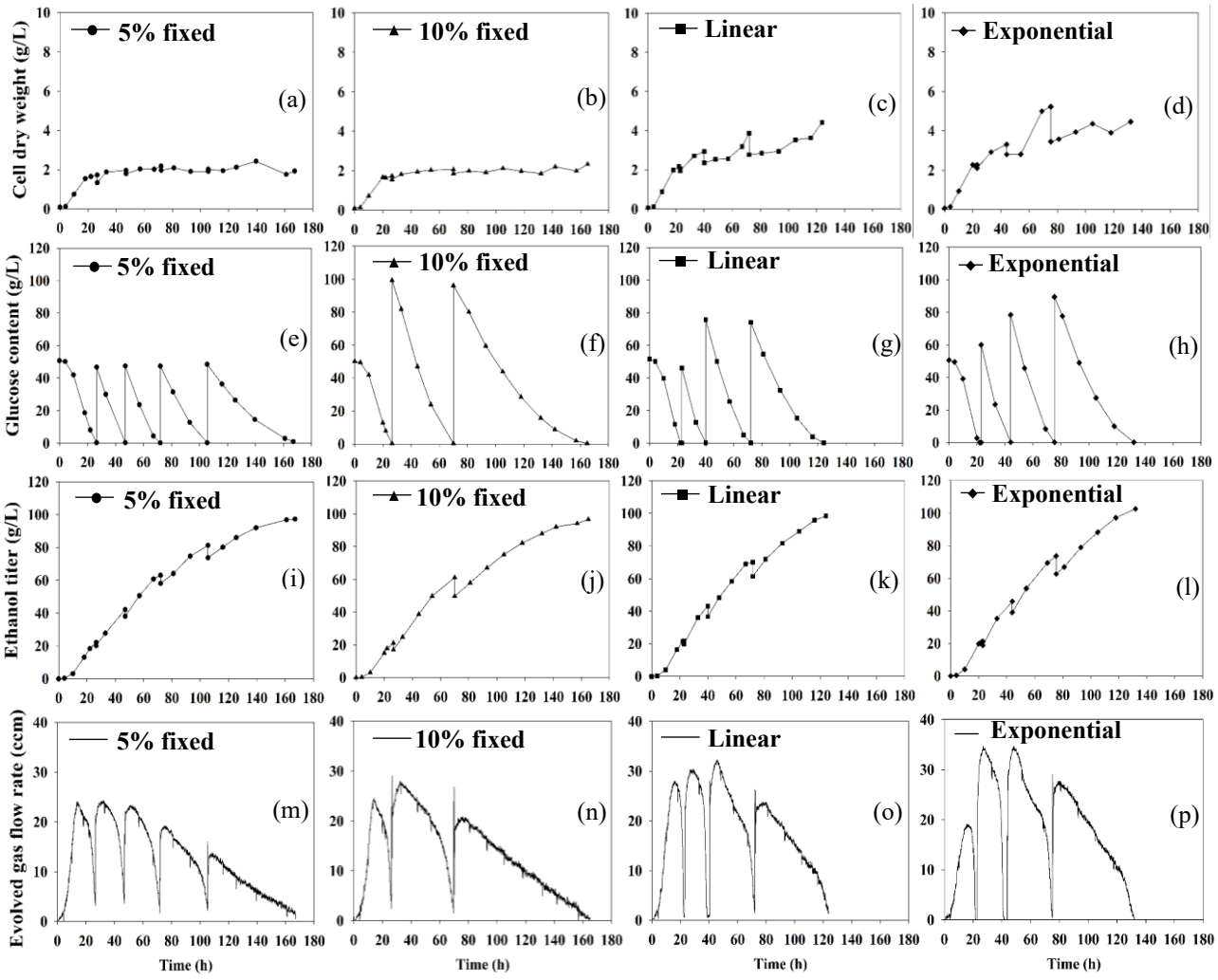

Figure 3.1 Changes in cell dry weight (a–d), glucose content (e–h), ethanol titer (i–l), and evolved gas flow rate (m–p) from 5% fixed (circle), 10% fixed (triangle), linear adapted (square), and exponential adapted (diamond) feeding strategies.

Changes in glucose (Figures 3.1e–h) and ethanol (Figures 3.1i–l) concentrations during 5% fixed, 10% fixed, linear adapted, and exponential adapted feeding strategies are shown in Figure 3.1. Firstly, it should be noted that was no residual glucose at the end of the fermentation regardless of the fed-batch strategy used, indicating complete utilization of the glucose supplied. Although fixed pulsed feeding operations were easy to implement, it had the poorest performance of the methods tested. A faster glucose consumption rate was observed with the linear adapted and exponential adapted feeding strategies (1.9 g/L/h), compared to the fixed feeding groups (1.5–1.6 g/L/h) (Table 3.1). In all cases, the glucose concentration was kept below 10% (w/v) to avoid metabolic inhibition of yeast growth [49, 118]. This implies that the differences in glucose consumption rates observed between the adapted and fixed pulses could be attributed to better substrate management by the yeast. Glucose concentration can play a critical role in the growth rate of *S. cerevisiae* [204]. When substrate concentration is higher in both adapted feeding strategies, the cell growth rate is expected to be faster compared to 5% fixed feeding, as observed in Figures 3.1 (a, c, and d). Similar phenomena were observed by Chang *et al.* [105], where the specific growth rate of *S. cerevisiae* increased when glucose input was increased from 10 to 100 g/L, but the cell growth rate started decreasing when glucose exceeded 100 g/L. On the other hand, although 10% fixed feeding strategy provided more substrate than the adapted feedings per addition, cell growth did not show an increased trend during fermentation. This demonstrates that a one-time addition of 10% (w/v) glucose may lead to more metabolic stress on the yeast. In comparison, the adapted feeding strategies have less risk of substrate inhibition, as they gradually increase the substrate additions. Our study also indicates that feeding strategy or sugar delivery can influence the performance of ethanol fermentation in the fed-batch mode. As the target substrate concentration increases in the fed-batch fermentation, there may be a trade-off between high final ethanol

concentration and high ethanol productivity [50]. Out of the feeding strategies tested, exponential adapted feeding strategy was able to enhance both final ethanol concentration and productivity, compared to the fixed feeding strategies.

As seen in Figures 3.1 (i–l), ethanol accumulated between each pulsed feeding for all modes of operations. The final ethanol titers reached 97, 97, 98 and 103 g/L for the 5% fixed, 10% fixed, linear adapted, and exponential adapted pulsed feeding strategies, respectively. In other words, between 9–10% (w/v) ethanol was achieved at the end of fermentation. In industrial operation, increasing final ethanol concentration can reduce the cost of ethanol recovery in the downstream process. The minimal concentration of ethanol for an economic recovery in industrial settings is 4% (w/v) [14, 15]. Our work demonstrates that 9–10% (w/v) ethanol production is achievable in 5-L fermenters, increasing the probability of applying adapted pulsed feeding strategies at larger scale. As an example, Fujii *et al.* [205] expanded the bioethanol production from a laboratory scale to a bench-scale and achieved higher sugar concentration from saccharification and higher ethanol concentration in fermentation with the bench-scale settings. Similar benefits from scaling up the bioprocess were found that ethanol yield was improved by 11.77% through the scale-up from working with 150-mL serum bottles to 5-L bioreactors [206].

On the other hand, high ethanol accumulation might induce inhibitory effects on the yeast during fermentation. After the last feeding, as ethanol concentration exceeded 9% (w/v), the ethanol production rate decreased compared to previous feeding intervals (Figures 3.1i–l). Ethanol can influence the structure of membranes in cells, such as nuclear and mitochondrial membranes, and other cell organelles, and further impact yeast metabolism, like fatty acid synthesis [22, 207]. Hence, when it comes to optimizing the fermentation process for bioethanol production, it is important to consider the ethanol tolerance of a strain [208, 209]. Overall, based on the trends

shown in Figures 3.1 (e–l), the two adapted feeding strategies improved glucose utilization and ethanol production, compared to the fixed feeding modes. Ethanol productivity was enhanced by 23–24% when applying the linear adapted and exponential adapted approaches, respectively, compared to the 10% fixed strategy.

Table 3.1 Overall performance in ethanol fermentation by four pulsed feeding strategies

Modes	Glucose consumption (g/L)	Glucose consumption rate (g/L/h)	Ethanol production (g/L)	Final ethanol concentration (g/L)	Ethanol efficiency (%)*	Ethanol productivity (g/L/h)
5% fixed	241 ± 5^{B}	1.6 ± 0.1^{B}	114 ± 2^{B}	95 ± 2^{B}	92 ± 3^{A}	0.74 ± 0.04^{B}
10% fixed	245 ± 4^{B}	1.51 ± 0.07^{B}	114 ± 3^{B}	98 ± 2^{AB}	92 ± 2^{A}	0.71 ± 0.04^{B}
Linear adapted	250 ± 10^{B}	1.9 ± 0.1^{A}	113 ± 3^{B}	95 ± 3^{B}	91 ± 4^{A}	0.88 ± 0.04^{A}
Exponential adapted	279 ± 7^{A}	1.9 ± 0.2^{A}	127 ± 5^{A}	104 ± 3^{A}	89 ± 2^{A}	0.87 ± 0.06^{A}

Means from independent triplicates (n=3) are reported with error bars representing standard deviation.

*: Ethanol efficiency (%) = Amount of ethanol produced/(Amount of glucose consumed·0.511)·100.

$^{A-B}$: Different superscript letters in the same column indicate significant differences ($p < 0.05$) based on statistical analyses performed through Tukey HSD test.

The overall performance of fixed and adapted feeding strategies in ethanol fermentation is shown in Table 3.1. Ethanol productivity improved by approximately 20% in both linear and exponential adapted feeding strategies compared to fixed feeding strategies. This demonstrates that adjusting substrate feeding based on cell biomass concentration can benefit both substrate utilization and ethanol production, and the improvements in ethanol productivity support our hypothesis. To deal with metabolic changes of the yeast and optimize the metabolite productivity in fed-batch fermentation, an adapted feeding strategy can be a more promising operation than a fixed feeding mode. This concept is consistent with the report of Alfenore *et al.* [45] who developed

an exponential feeding strategy for vitamin supplement in ethanol fermentation, which improved the viability of *S. cerevisiae* and ethanol productivity by 10–18%. Maximizing the product formation rate can benefit the entire process design by, for example, reducing the required bioreactor size which reduces the cost of equipment and its maintenance [42] or facilitating separation.

In addition to ethanol productivity, ethanol efficiency (based on the maximum theoretical sugar-to-ethanol ratio) and final ethanol concentration are crucial metrics of the performance of ethanol fermentation. Firstly, as shown in Table 3.1, ethanol efficiency reached between 89–92% for the four feeding strategies tested, with no statistical difference. In ethanol fermentation, the maximum theoretical yield of ethanol is 0.511 g per g glucose. In this study, fed-batch operations with 10% (w/v) glucose improved the ethanol efficiency by 12.5% compared to the batch stage. Using pulsed fed-batch to extend the active growth phase of cultures enabled the extended conversion of glucose to ethanol and carbon dioxide [20], leading to improvements in ethanol efficiency (Table 3.1) and increases in evolved gas flow rate (Figure 3.1). Similar results were observed by Laopaiboon *et al.* [18], who found that ethanol yield improved by 14% in fed-batch fermentation with pulsed feeding operations, compared to batch fermentation, due to reduced byproduct formation. Secondly, in terms of ethanol content, the highest ethanol production was observed under the exponential adapted feeding (Table 3.1). By considering cell accumulation during fermentation, the exponential adapted feeding can provide more substrate for ethanol fermentation and achieve higher ethanol concentration at the end of fermentation. Dilution effects from feeding operation translated into a final ethanol content slightly lower than the overall ethanol production. However, all four feeding strategies led to final ethanol contents of around 9–10% (w/v). Fed-batch operations can regulate various feeding policies to relieve the potential substrate

inhibition towards the microorganism and further enhance final ethanol concentration and yield, as compared to the batch [105]. Based on these advantages, it would be promising to combine fed-batch operation with other fermentation modes, such as extractive fermentation, as such integrated fermentation systems could enable the reduction of both substrate and product inhibition effects [210]. This is of importance as increasing ethanol concentration in the fermentation medium can play a significant role in the cost and energy spent on the downstream process [15].

Optimizing ethanol productivity, efficiency, and final concentration is a trade-off for the bioethanol industry. Different decision points can result in vastly different approaches to process design. The economical balance between operational strategy and ethanol production needs to be considered [211]. This can be better evaluated through scaling up the bioprocess; for example, scaling up to the bench-scale enables the provision of operational [205] and economical [199] information from advanced bioethanol productions, which may offer further insights into the potential commercial phase. In our current study, although exponential adapted feeding required more glucose, it showed higher final ethanol content than the linear adapted feeding strategy (Table 3.1). Importantly, both adapted feeding strategies could be implemented in the 5-L fermenters. Overall, we developed two adapted feeding strategies to improve ethanol production, which helps reduce the production costs associated with fermentation. The concept of adapted feeding strategies can be integrated into current ethanol production; for example, it can be applied to either sugar-based or lignocellulosic ethanol production. The bench-scale performance demonstrated in this work is promising for future pilot-scale processes. The work can thus benefit the economic viability of commercial bioethanol.

It is worth noting that although sugar-based and starch-based feedstocks are the main sources for current bioethanol production in the industry, many companies, such as GranBio, POET

Biorefining, and Raízen, are working on cellulosic ethanol as well. To produce cellulosic ethanol on an industrial scale, implementing a single SSF presents challenges, particularly due to limited fermentation performance, despite extensive research on the use of simultaneous saccharification and fermentation for lignocellulosic ethanol production [53, 136-144] (Table 3.2).

Table 3.2 Lignocellulosic ethanol production via simultaneous saccharification and fermentation using *Saccharomyces cerevisiae*

Feedstock	Mode	Ethanol concentration (g/L)	Ethanol productivity (g/L/h)	Reference
Agro-industrial wastes	Batch	30.0	3.0	[136]
Banana peels	Batch	28.2	2.3	[137]
Barley straw	Fed-batch	51.7	0.54	[138]
Cassava pulp	Batch	27.4	0.37	[139]
Green coconut fiber	Batch	7.9	-	[140]
Rice straw	Batch	25.2	1.05	[141]
Sugarcane bagasse	Fed-batch	75.6	0.63	[142]
Spruce	Fed-batch	40.0	0.42	[53]
Pomegranate peel	Batch	12.9	0.54	[143]
Potato peel wastes	Batch	22.5	1.51	[144]

-: not indicated

On the other hand, separate hydrolysis and fermentation (SHF) has been used to degrade the complex structure of cellulosic materials and produce ethanol separately, aiming to optimize the individual steps [208]. Further enhancement of the production efficiency of SHF requires overcoming some of the limitations of current fermentation practices. Based on the results shown in this study, two adapted feeding strategies have the potential to be applied to direct fermentation and SHF for boosting sugar-based ethanol and lignocellulosic ethanol production in the short term. Feeding or controlling actions in fed-batch fermentation may impact the heterogeneity of fermentation media when scaling up the bioprocess at an industrial scale [212]. To further explore

the feasibility of the adapted pulsed feeding strategies, studies should be conducted in larger-scale bioreactors. However, it is clear that continuously adapted feeding is a promising alternative that enables the mitigation of substrate inhibition and fermentation heterogeneity during feeding, potentially leading to greater ethanol production performance.

3.3.2 Monitoring evolved gas during fed-batch fermentation

Appropriate monitoring of ethanol fermentation is important to optimize production and regulate dynamic changes during fermentation and reduce production costs [213]. For example, spectroscopy has been studied for monitoring glucose and ethanol contents in the fermentation medium. However, specific concerns need to be taken into account for this technique, such as the applicability of the calibration model for the independent process [214]. Additionally, instrument fluctuations may contribute to response errors when building the calibration model [111]. Considering fed-batch fermentation introduces fresh substrate to bioreactors intermittently or continuously, spectroscopy may not be the most effective option to monitor real-time changes during fermentation. Alternative monitoring tools need to be explored for fed-batch fermentation.

Wang *et al.* [11] showed that evolved gas flow rate could be used as a real-time monitoring parameter for ethanol fermentation in a self-cycling fermentation system. It was pointed out that this parameter could be used to monitor metabolic activity and determine the divide between the exponential phase and stationary phase of the yeast [11]. In the presented study, we monitored the evolved gas flow rate and applied it to determine the appropriate feeding points for the different fed-batch operations. Trends in evolved gas flow rate during 5% fixed, 10% fixed, linear adapted, and exponential adapted feeding operations are shown in Figures 3.1 (m–p). When evolved gas flow rate decreased sharply and reached values close to zero – indicating a rapid decline in metabolic activity, fresh feed medium was added to the bioreactor to extend substrate availability.

After each feeding, the gas flow rate increased strongly, indicative of the re-establishment of rapid metabolic activity. For example, greater evolved gas flow rates were observed after 10% fixed pulses compared to 5% fixed pulses, consistent with greater specific growth rate expected at higher substrate concentration.

When investigating the effects of the adapted feeding strategies on the evolved gas flow rate, we could see that this parameter increased rapidly after the pulse and reached greater values than for the fixed feeding strategies (with the exponential adapted operation reaching as much as 34 ccm. This is interesting as it indicates that the rate of conversion of substrate to gas was greater in the adapted strategies even if less glucose was fed than in the 10% fixed pulsed strategy. This suggests a more rapid and efficient utilization of glucose, and in turn faster production of ethanol and carbon dioxide. In the 10% fixed pulsed strategy, the substrate addition may push the influence of substrate inhibition on the yeast metabolic activity. This surmise can echo the report from Chang *et al.* [105] which showed that the growth rate of the yeast starts decreasing when glucose concentration exceeds 100 g/L. For this reason, it is critical to consider the substrate inhibition in ethanol fermentation to prevent loss of product yield [215]. Our study shows that adapted feeding strategies can further improve ethanol productivity through tailored sugar delivery (Table 3.1).

For all feeding strategies tested, the magnitude of the evolved gas flow rate decreased as the number of pulse feedings increased, and the curves showed that it took a long time to utilize the substrate (Figures 3.1m–p). This is likely due to the accumulation of ethanol in the fermentation broth. After the last feeding, ethanol contents increased from 74 to 97 g/L, from 50 to 97 g/L, from 61 to 98 g/L, and from 63 to 103 g/L in the 5% fixed pulsed, 10% fixed pulsed, linear adapted, and exponential adapted operations, respectively (Figures 3.1i–l). Considering that ethanol concentration reached 90–100 g/L, it is reasonable to expect that, by this point, most cells were not

fully activated for ethanol production, especially compared to the earlier pulsed feedings. Zhang *et al.* [216] reported that when the initial exogenous ethanol concentration exceeded 70 g/L, the yeast ceased growing and producing ethanol even if sufficient substrate was present. We were able to push ethanol production toward the boundary of high ethanol accumulation through pulsed feeding operations. Under ethanol stress, the physiology and gene expression of yeast can change to balance cellular energy expenditure, resulting in cell growth inhibition [207]. This aligns with the reduced evolved gas flow rate following the last feedings (Figures 3.1m–p). In this context, the evolved gas flow rate proved to be a valuable real-time monitoring parameter for the determination of pulsed feeding and the evaluation of ethanol production and potency of feeding strategies.

3.4 Conclusions

In this study, two adapted feeding strategies developed for fed-batch fermentation led to significant enhancements in ethanol productivity (increases of approximately 20%) compared to fixed pulsed feeding strategies. Evolved gas flow rate, a result of sugar utilization, from ethanol fermentation could be viewed as a determining factor for pulsed feeding operations in ethanol production by *S. cerevisiae*. In addition, ethanol efficiency of 91% and titers of 13% (v/v) were achieved by using these adapted feeding strategies. The results demonstrated the advantages of adjusting feeding based on cell biomass during fed-batch fermentation. This approach, which was successfully established in 5-L fermenters, improved the efficiency of ethanol production. To sum up, linear adapted and exponential adapted pulsed feeding strategies show great potential for integration into sugar-based ethanol and lignocellulosic ethanol production to enhance productivity.

Chapter 4 Adapted continuous feeding strategies in fed-batch fermentation improve ethanol productivity

*A version of this chapter will be submitted for publication

4.1 Abstract

Advances in the ethanol fermentation process are essential to improving the performance of bioethanol production. In the present work, different modes of substrate delivery – fixed feeding, adapted feeding – were investigated in fed-batch cultures of *Saccharomyces cerevisiae* in 5-L bioreactor. Evolved gas production, which was positively correlated with glucose consumption, was used to adjust the sugar feed rate in fed-batch fermentations under an adapted feeding strategy. The adapted feeding strategy enhanced ethanol productivity by 21% compared to the fixed feeding strategy, in which the sugar feed rate was stable, and ethanol titer reached 91 g/L (~11.5%, v/v) at the end of fermentation. Moreover, cell biomass accumulation and cell growth rate were significantly improved when using the adapted feeding strategy. The effect of nitrogen availability on the performance of the adapted feeding strategy was explored using a low-nitrogen content medium. The results showed that, even under low nitrogen feeding conditions (N:C = 0.046:10), the adapted feeding strategy maintained the same ethanol productivity as nitrogen-rich medium feeding. Overall, these results suggest that sugar delivery with low-nitrogen content using the

adapted feeding strategy could help reduce medium and preparation costs, and improve productivity of current facilities in the ethanol industry.

4.2 Background

Ethanol fed-batch fermentation typically relies on intermittent or continuous feeding. In the former, also named pulsed feeding, specific amounts of a limiting nutrient are added intermittently, leading to spikes in substrate concentration followed by depletion until the next feeding [18, 46], which we investigated in Chapter 3 for adapted pulsing fed-batch approaches. On the other hand, continuous feeding consists of a continuous addition of a limiting nutrient (typically through pumping) to the bioreactor either at a fixed or changing (adapted) rate [47, 48]. When lignocellulosic hydrolysate is used as substrate, continuous feeding results in relatively less exposure of the microorganisms to inhibitors, such as furfural derivatives, compared to pulsed feeding, in which higher concentrations of substrate and inhibitor are observed immediately after the pulsed addition. Some ethanologenic microorganisms have the capability to convert inhibitors, which reduces the metabolic stress on cells [217, 218]. Moreover, continuous feeding can mitigate heterogeneity between the fermentation broth and the fresh feed medium, which may lead to greater performance in ethanol fermentation as compared to pulsed feeding. The heterogeneity of a fermentation broth becomes critical especially when the fermentation process is implemented at industrial scale [212]. It is worth noting that, although both pulsed and continuous feeding operations lead to elevated product formation [42], developing an efficient feeding strategy for substrate delivery in fed-batch fermentation is essential to optimize the metabolic performance of microorganisms.

Therefore, the present work focused on developing adapted continuous feeding strategies for sugar delivery to enhance ethanol productivity in cultures of *S. cerevisiae*, and comparing their performance with fixed continuous feeding in 5-L bioreactor. To this end, evolved gas production from the yeast during ethanol fermentation was assessed as a monitoring parameter to guide sugar feeding rate. Fermentation performance was determined based on sugar utilization, ethanol titer, efficiency (sugar-to-ethanol conversion), and productivity. In addition, the impact of nitrogen content in the feed medium on the performance of the fermentations was investigated. Overall, this work provides practical insights to develop and/or implement adapted feeding strategies for efficient substrate delivery and further improving the performance of industrial bioethanol production.

4.3 Methods and Materials

4.3.1 Yeast, media, and cultivation

The industrial yeast for fuel ethanol production, Superstart^{TM} active distillers dry yeast, *Saccharomyces cerevisiae*, was purchased from Lallemand Ethanol Technology and used in this study. Media for seed cultures and ethanol fermentation as well as the cultivation conditions were used as described in Chapter 3

4.3.2 Fed-batch fermentation

4.3.2.1 Settings and accessories for 5-L bioreactors

All fermentation experiments were performed in 5-L stirred tank bioreactors (Infors-HT), and the operation settings and accessories were used as described in Chapter 3.

4.3.2.2 Continuous feeding strategies

The feed medium added during fed-batch fermentation was a modified YNB medium: 500 g/L glucose (MilliporeSigma), 6.7 g/L yeast nitrogen base with amino acids (MilliporeSigma), and 47.7 g/L urea (MilliporeSigma) in 0.1 M sodium phosphate buffer (pH 6.0). The nitrogen-to-carbon (N:C) ratio in the feed medium was 0.95:10 (mol:mol).

Continuous feeding was performed using a peristaltic pump (MFLX78001-62, Masterflex Ismatec Reglo Independent Channel Control Peristaltic Pumps, Avantor, Mississauga, Ontario, Canada).

Evolved gas produced per reactor volume (E with units of mL/L) during fermentation positively correlated with glucose consumed per reactor volume (G with units of g/L), as expressed in equation 4.1 ($R^2 = 0.895$; Figure 4.1):

$$G = 0.005 \cdot E + 0.302 \qquad \text{Eq. (4.1)}$$

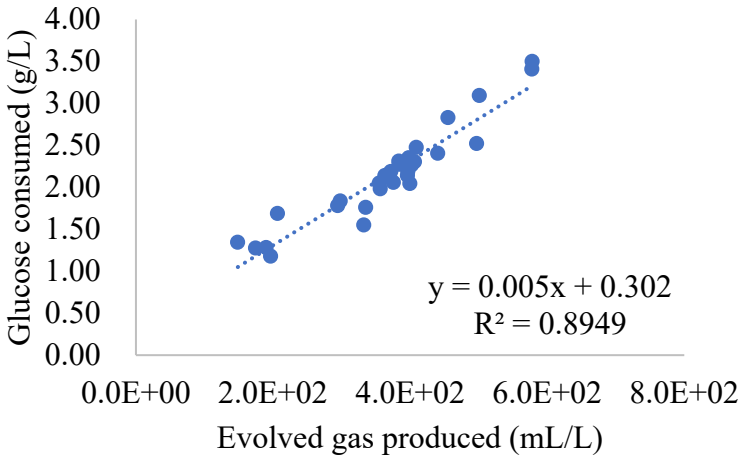

Figure 4.1 The correlation between evolved gas produced (mL/L) and glucose consumed (g/L) per reactor volume in ethanol fermentation of *S. cerevisiae*.

Evolved gas from the bioreactor was used to control the glucose addition in adapted continuous feeding, and real-time evolved gas flow rate was used as a monitoring parameter to indicate the end of the initial batch phase, at which point the fed-batch phase was initiated.

Details for fixed and adapted feeding are stated as follows.

1) In the fixed continuous feeding strategy, glucose was fed at a rate of 2.1 g/L/h. This glucose feed rate was set based on the average glucose consumption rate in batch fermentation of *S. cerevisiae* in previous works.

2) In the adapted continuous feeding strategy, glucose was fed at a rate of 2.1 g/L/h at the beginning of the fed-batch fermentation. Glucose consumed per reactor volume (g/L) was evaluated every 12 h based on the evolved gas production (using equation 4.1). The glucose feed rate (F) was adjusted according to equation 4.2:

$$F_t = F_{(t-1)} \cdot \frac{(E_{(t-1)} + \Delta E)}{E_{(t-1)}} \qquad \text{Eq. (4.2)}$$

where F_t is the adapted glucose feed rate at time t (g/L/h), $F_{(t-1)}$ is the glucose feed rate in the previous time interval (g/L/h), $E_{(t-1)}$ is the total evolved gas at the previous sampling time (mL), and ΔE is the increase in evolved gas over the time interval (mL/h). When the estimated glucose consumption rate was lower than the current feed rate, the glucose feed rate was maintained to ensure that ethanol fermentation was not limited due to insufficient substrate input. Overall, for both fixed and adapted feeding strategies, glucose was continuously fed until the total glucose added reached approximately 200 g/L, this ensured the ethanol contents could reach above 11% (v/v), a common target for the fuel industry for recovery in downstream process.

To further investigate the application of adapted feeding strategies on ethanol production, the composition of the feed medium was modified to reduce its nitrogen content. A lower nitrogen-to-

carbon ratio was obtained by removing urea from the medium. The modified feed medium thus contained 500 g/L glucose and 6.7 g/L yeast nitrogen base with amino acids in 0.1 M sodium phosphate buffer (N:C = 0.046:10, mol:mol). This feed medium composition was tested with both the adapted and fixed continuous feeding strategies, with all other fermentation conditions kept the same.

4.3.3 Analyses of fermentation samples

Biomass content of samples from the bioreactors was assessed by OD_{600} using a UV-Vis spectrophotometer (Ultrospec 4300 Pro) and converted to cell dry weight (CDW, g/L) using a calibration curve ($CDW = 0.4412 \cdot OD_{600} + 0.049$; $R^2 = 0.967$; Supplementary Figure 2, Appendix A). Fermentation samples were also centrifuged at 10,100·*g* for 10 min (Eppendorf® Centrifuge 5418), and the supernatants were analyzed by HPLC and GC for determining glucose and ethanol concentrations, respectively, which were stated in the methods and materials of Chapter 3.

All fermentation experiments were run in independent triplicates. Data were expressed as means ± standard deviation. Statistical analysis for comparing multiple groups was performed by ANOVA and Tukey's honestly significant difference test, where $p < 0.05$ indicated significant differences.

4.4 Results and Discussion

4.4.1 Impact of continuous feeding strategies on ethanol fermentation

Fed-batch fermentation has been shown to improve ethanol production through enabling higher cell density and avoiding substrate inhibition [41]. However, in that operational approach, the feeding regime must be properly controlled [76]. Ethanol production under anaerobic conditions is linked to the primary metabolism of *S. cerevisiae*: when glucose is consumed, ethanol and carbon

dioxide are produced, and ATP is generated for cell growth, maintenance, or reproduction [3, 24] .In this study, we developed a feeding strategy in which the sugar delivery was adapted to the glucose consumption rate to improve ethanol productivity, and compared its performance with a fixed feeding strategy.

Figure 4.2 shows the trends in yeast cell dry weight, glucose concentration, ethanol concentration, and evolved gas flow rate for the fixed (2a–2d), adapted (2e–2h) and low-nitrogen adapted (2i–2l) continuous feeding strategies. The comparison of the fixed and adapted feeding at a N:C ratio of 0.95:10 yields a direct assessment of the impact of substrate addition strategies on growth and ethanol production. Firstly, cell dry weight increased with both fixed and adapted feedings; however, the latter led to higher final biomass (~3.4 g/L) than the fixed feeding (~2.6 g/L) (Figures 4.2a and 4.2e). This suggests that the adapted feeding strategy provides more efficient sugar delivery for cell growth. Sugar concentration is an essential variable for the performance of ethanol fermentation. Chang *et al.* [105] reported that the specific growth rate and ethanol productivity increased in batch cultures of *S. cerevisiae* as the initial glucose concentration was increased from 10 g/L to 100 g/L. It is worth mentioning that, beyond 100 g/L, the sugar concentration needs to be controlled to avoid strong osmotic stress on the microorganism [118]. This is also supported by Chang *et al.* [105] who showed decreases in yeast specific growth rate and ethanol productivity when the initial glucose concentration increased from 100 g/L to 260 g/L. In the present work, glucose addition was tightly controlled to stay well below 100 g/L and mitigate the risk of substrate inhibition on the yeast.

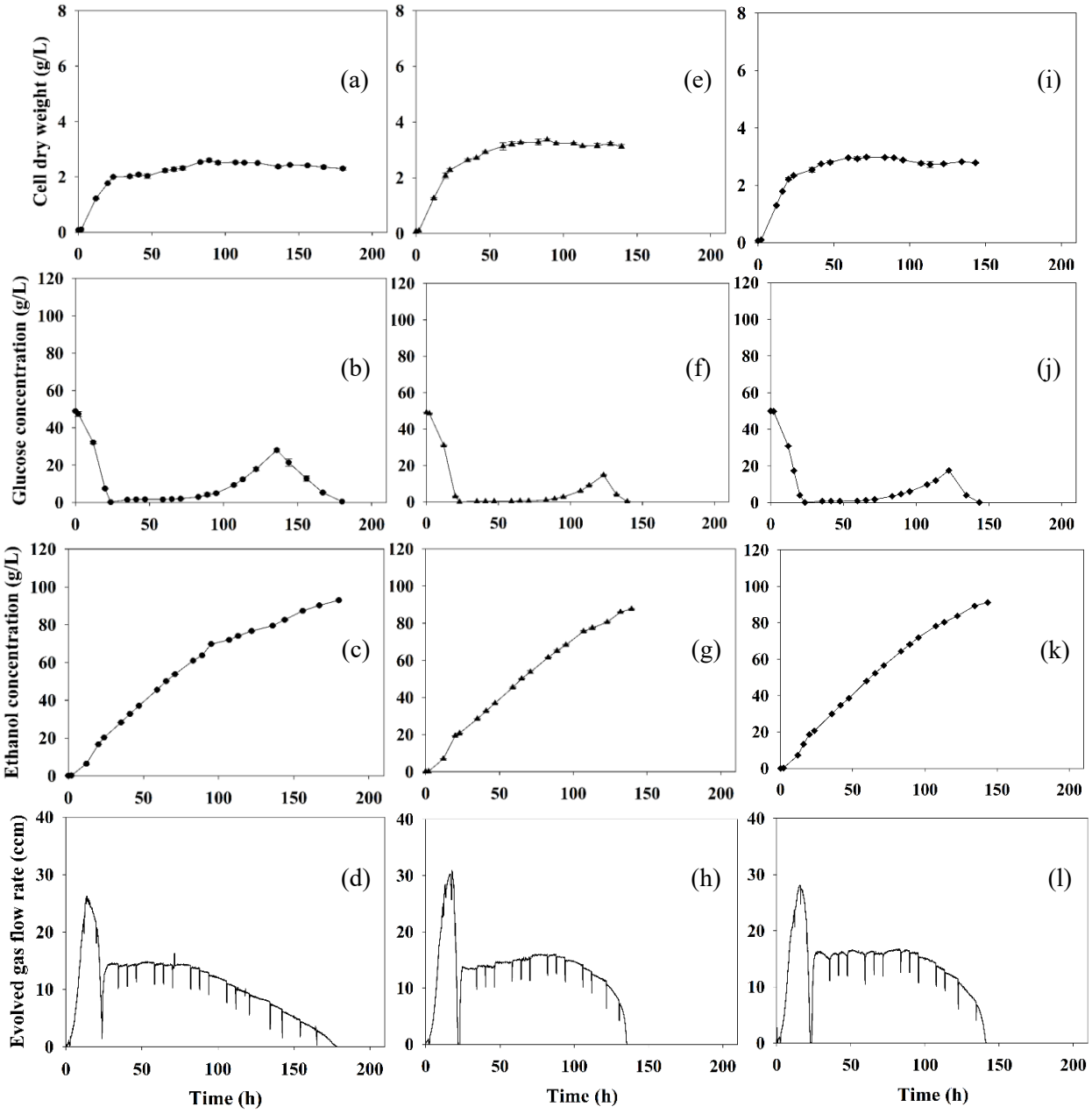

Figure 4.2 Cell dry weight (a, e, i), glucose concentration (b, f, j), ethanol concentration (c, g, k), and evolved gas flow rate (d, h, l) in fed-batch fermentation of *S. cerevisiae* cultures undergoing fixed (a–d, circle) and adapted (e–h, triangle) continuous feeding strategies, and adapted continuous feeding strategy with low nitrogen feed medium (i–l, diamond). Means are reported for analytical triplicates (n=3), with error bars representing standard deviation.

The impacts of the adapted feeding strategy were also observed in trends in glucose concentration, ethanol concentration and evolved gas flow rate. The residual glucose content at the end of each feeding period was at or near zero (Figures 4.2b and 4.2f) until approximately 83 h for fixed feeding and until 95 h for adapted feeding. This indicates that, over that time period, the glucose consumption rate was likely equivalent to or limited by the glucose addition rate; in fact, it should be noted that the glucose addition rate was increased gradually in the adapted feeding strategy. Considering that from approximately 24 h onward the cell dry weight did not change substantially, this suggests that more glucose was utilized by the yeast for producing ethanol or cell biomass when applying the adapted feeding strategy. This is supported by the glucose consumption rate (Figure 4.3) which was greater in cultures undergoing adapted feeding compared to those undergoing fixed feeding.

Moreover, the comparison of the areas under the curve of the glucose consumption rate – which is an indicator of performance based on the overall glucose consumed per liter of reactor – demonstrates that the adapted feeding strategy enhanced overall glucose consumption (115 g/L compared to 105 g/L for fixed feeding). The gradual increase in glucose addition in adapted feeding may benefit the glucose consumption rate. Unrean and Nguyen [19] reported that substrate feeding rate could impact the cell growth rate and the optimal ethanol productivity in fed-batch fermentations of *Scheffersomyces stipitis*. In the present study, we noticed a similar impact from fed-batch fermentation of *S. cerevisiae*, showing that the adapted feeding strategy can enhance glucose consumption rate and, consequently, ethanol productivity.

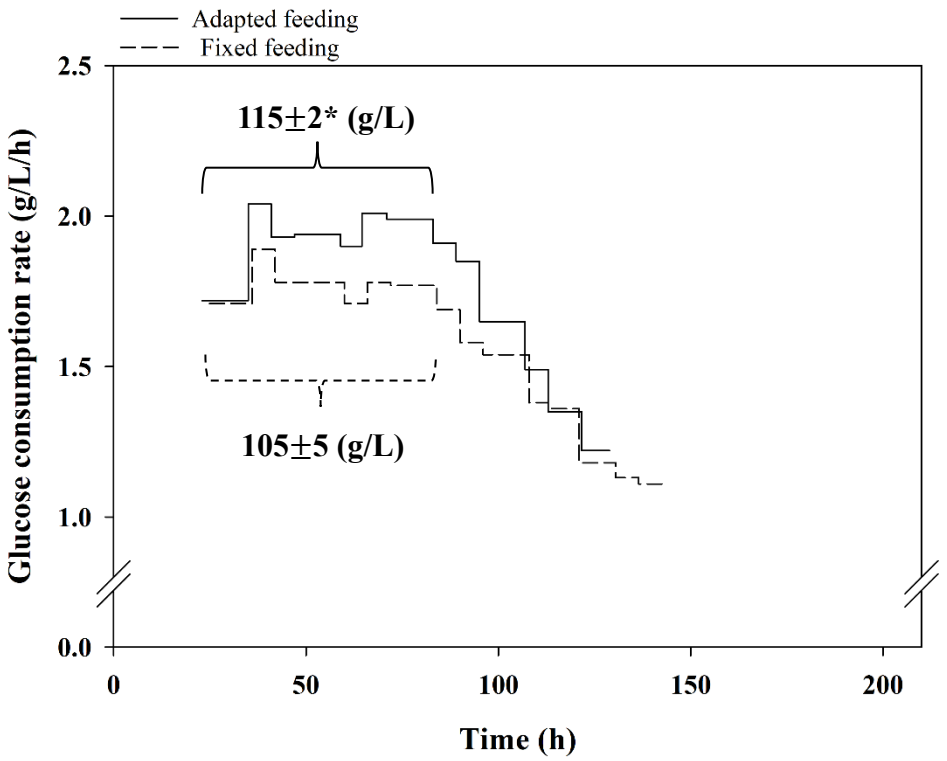

Figure 4.3 Glucose consumption rate in fed-batch fermentation of *S. cerevisiae* cultures undergoing fixed and adapted continuous feeding strategies.

The numbers shown indicate the areas under the curve of glucose consumption rate, i.e. the sum of glucose consumption (g/L) from the start of fed-batch operation to the mid-feeding period, beyond which the rate decreased. The two values were statistically different ($p < 0.05$) based on a two-tailed t-test, indicated by the asterisk sign.

The rise in residual glucose at ~83 h for fixed feeding and ~95 h for adapted feeding (Figures 4.2b and 4.2f) is indicative of a decrease in glucose conversion rate. This occurred later in adapted feeding, likely because this strategy improved cell biomass accumulation which correlates with glucose consumption. In a report, Cruz *et al.* [219] showed that at the same fermentation time, temperature and initial substrate concentration, inoculating at higher cell concentration led to low residual sugar and increased ethanol concentration. In addition, the total glucose fed was controlled in both fixed and adapted feeding strategies to ensure they could reach the same ethanol concentration at the end of fermentation for comparison. In the fixed feeding strategy, glucose

addition was stopped at ~136 h, while in the adapted feeding strategy it was stopped earlier, at ~123 h. Moreover, the peak in residual glucose was lower in the adapted feeding strategy (15 g/L) than in the fixed feeding strategy (28 g/L). This also suggests that the glucose consumption was more efficient when operating the adapted feeding strategy. Interestingly, for both feeding strategies, the onset in glucose accumulation aligned with the ethanol concentration reaching ~60 g/L (Figures 4.2b and 4.2c, and 4.2f and 4.2g). Since ethanol is a product that can stress yeast cells [20, 22], we surmise that 60 g/L ethanol was likely a threshold to trigger metabolic inhibition. This is supported by Cot *et al.* [220], who showed that, in fed-batch fermentation, cell viability was reduced when ethanol reached 60–80 g/L; and that cell viability decreased sharply when ethanol reached 100 g/L. As ethanol is accumulated during fermentation, its negative metabolic impact on the yeast can be viewed differently, such as the trigger for mild or completed inhibition. It is also important to note that after stopping the feeding, glucose stopped accumulating and decreased to complete depletion (Figures 4.2 b and f), which shows that the cells retained metabolic activity.

Evolved gas flow rate was monitored over the entire fermentation process to track metabolic activity and infer glucose consumption rate (Figures 4.2d and 4.2h). Based on the metabolic pathway of ethanol fermentation in *S. cerevisiae*, ethanol production is coupled with carbon dioxide formation. Wang *et al.* [11] showed that evolved gas flow rate could be used as a real-time monitoring parameter for operating ethanol fermentation, which is also supported by our study. As shown in Figures 1d and 1h, evolved gas flow rate from the fixed and the adapted feeding strategies were maintained at ~14 ccm in the beginning of the feeding period. However, as glucose addition was increased in the adapted feeding strategy, the evolved gas flow rate increased to ~16 ccm; inferring that glucose consumption rate might increase under this mode of operation. Indeed, as shown in Figure 4.3, glucose consumption rate in the adapted feeding strategy was generally greater

than in the fixed feeding strategy. It is worth noting that as evolved gas flow rate increased, the evolved gas production increased as well, indicating more glucose was required for ethanol fermentation. Through monitoring changes in evolved gas flow rate, the performance of ethanol fermentation can be tracked, which was also demonstrated in Wang *et al.* [11] and Chapter 3. This allowed us to identify events, such as the accumulation of glucose and its complete depletion (Figures 4.2d and 4.2h), corresponding to trends observed by HPLC analysis (Figures 4.2b and 4.2f). In fact, as fresh medium was continuously fed until ~83 h and ~95 h in the fixed and adapted feeding strategy respectively, evolved gas flow rate started to decrease, aligning with the increase in residual glucose (Figure 4.2).

The potential application of the adapted continuous feeding strategy was further investigated by testing the system with reduced nitrogen content (N:C = 0.046:10) in the feed medium (Figures 4.2i–l). Much like in nitrogen replete experiments (N:C = 0.95:10; Figures 4.2a and 4.2e), the adapted feeding strategy led to more efficient glucose feeding and higher cell growth rate compared to the fixed feeding strategy with low-nitrogen feed medium. Interestingly, although the cell dry weight in the nitrogen-replete experiment with adapted feeding was similar to that in the low-nitrogen operation, it showed a higher cell growth rate before the cell content reached a plateau during the feeding period (Figure 4.4) (Table 4.1).

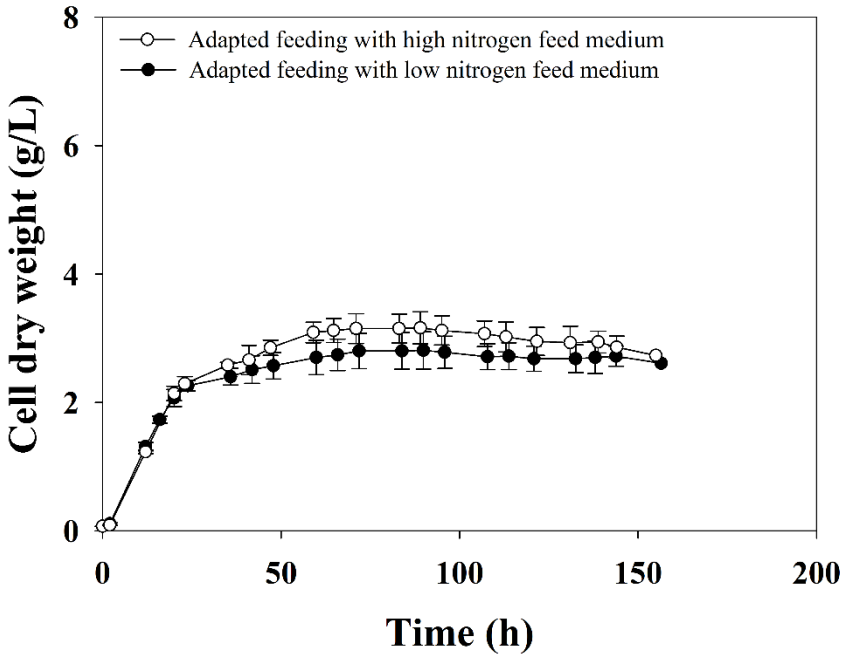

Figure 4.4 Cell growth in adapted continuous feeding strategies with high (white circle) or low nitrogen feed medium (black circle). Means are reported for independent triplicates (n=3), with error bars representing standard deviation.

Kiran and Liu [221], who found that lower *S. cerevisiae* cell dry weight was measured at 24 h with media at low nitrogen-to-carbon ratio (N:C = 0.29:10), as compared to high nitrogen-to-carbon ratio (N:C = 2:10). Indeed, nitrogen is essential to yeast cell formation (Russell, 2003). However, the impact of nitrogen content on the performance of ethanol production may not necessarily align with the above situation. For examples, high ethanol concentration was observed in a low N:C ratio conditions with *S. cerevisiae* [221] and *Escherichia coli* KO11 [222], respectively. Furthermore, Pinilla *et al.* [223] reported that ethanol yield and specific ethanol productivity were higher when N:C ratio was 0.22:10, as compared to either lower or higher N:C ratio at 0.11:10 and 1.1:10 in the fermentation of *Zymomonas mobilis*. The minimal nitrogen requirement of a yeast determines the

point below which sluggish or stuck fermentation may take place [224]. Mendes-Ferreira *et al.* [225] reported that 267 mg-Nitrogen/L was the minimal nitrogen content for growing *S. cerevisiae* PYCC 4072 (an industrial wine yeast) in synthetic grape juice medium, avoiding sluggish or stuck fermentation. In the present study, the feed lower nitrogen content (1.06 g-Nitrogen/L) had no impact on ethanol productivity when applying the adapted feeding strategy. This suggests that the low-nitrogen feed medium was applicable for growing the yeast strain in adaptive fed-batch fermentation, providing more flexibility to the bioethanol industry.

The trends in glucose and ethanol concentrations were similar with low or high nitrogen content for both adapted feeding strategies (Figures 4.2e–h and 4.2i–l); with ethanol reaching 89 g/L (~11.3%, v/v) at the end of fermentations with low nitrogen content. Here again, glucose accumulation was observed once the ethanol concentration reached 60 g/L (at ~83 h). Residual glucose concentration reached 18 g/L in the adapted feeding, at which point feeding was stopped and the residual was consumed until depletion (Figure 4.2j). The complete fermentation process was, again, faster using the adapted feeding strategy. For comparison, the results of fixed feeding strategy with low N:C feed medium can be found in Figures 4.5 and 4.6. These results suggest that the substrate delivery in the adapted feeding strategy could enhance the rates of glucose consumption and ethanol production compared to the fixed feeding operation, even when the low nitrogen feed medium was used.

Lignocellulosic hydrolysates used in bioethanol production can have a range of types and contents of nitrogenous compounds [226-228]. Although nitrogen is essential to the growth of microorganisms, quantifying and unifying the nitrogen content in every batch of lignocellulosic hydrolysate may not be practical. Thus, we further demonstrate the flexibility of conducting the adapted feeding strategy with a low nitrogen feed medium in ethanol fermentation. Even using low

nitrogen (N:C = 0.046:10) feed medium, the adapted feeding strategy still enhanced ethanol productivity compared to the fixed feeding strategy.

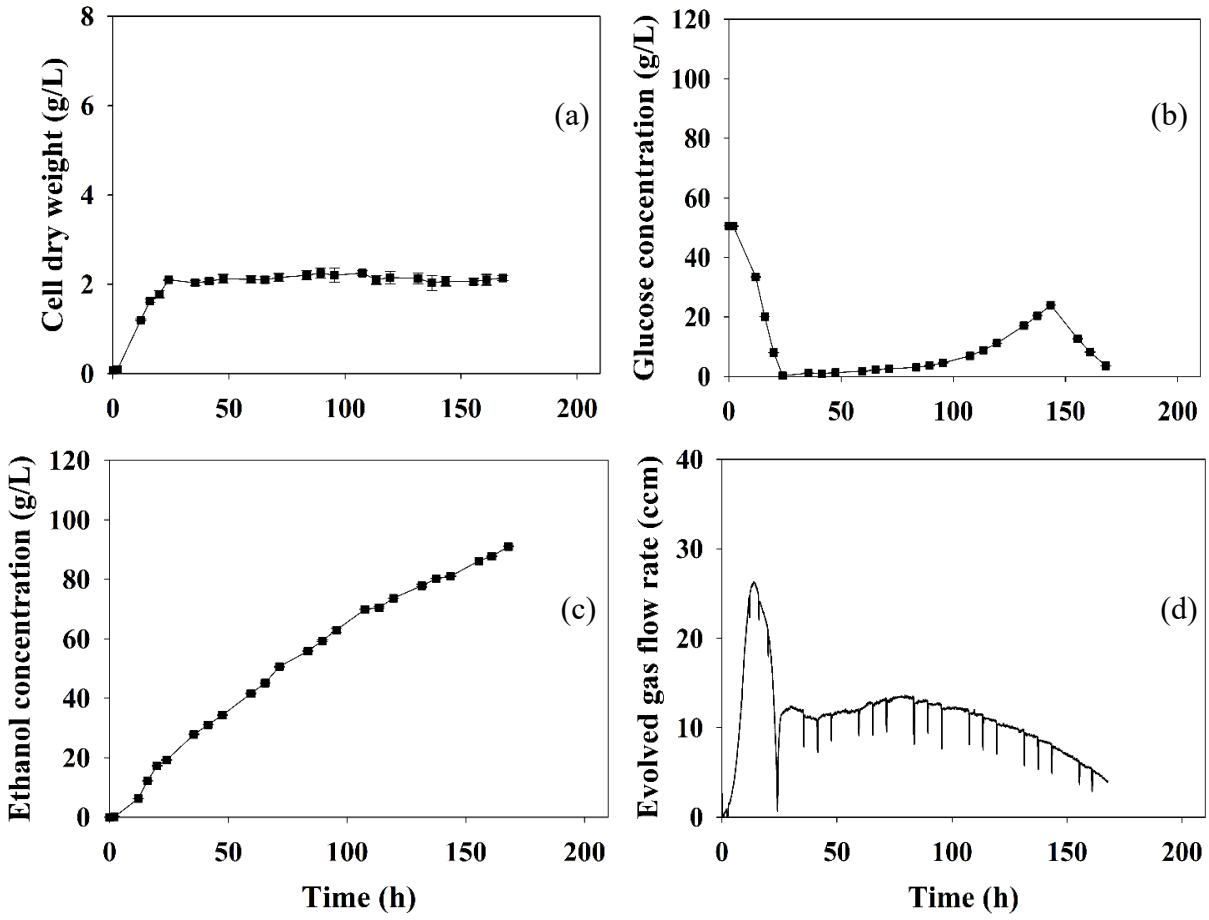

Figure 4.5 Cell dry weight (a), glucose concentration (b), ethanol concentration (c), and evolved gas flow rate (d) in fed-batch fermentation of *S. cerevisiae* cultures undergoing fixed continuous feeding strategies with low nitrogen medium. Means are reported for analytical triplicates (n=3), with error bars representing standard deviation.

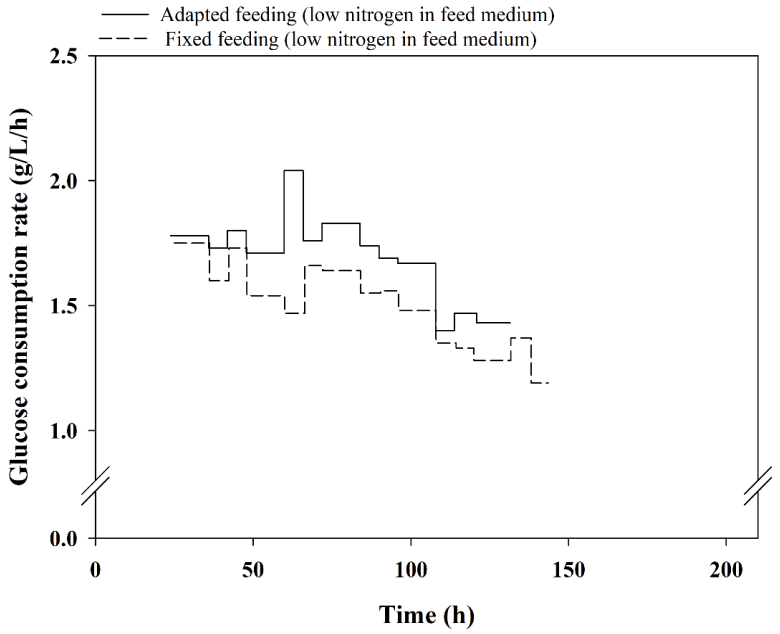

Figure 4.6 Glucose consumption rate in fed-batch fermentation of *S. cerevisiae* cultures undergoing fixed and adapted continuous feeding strategies with low nitrogen medium.

4.4.2 Overall advancements in ethanol fermentation by adapted feeding strategies

Table 4.1 highlights critical fermentation performance parameters from fixed and adapted feeding strategies with high- and low-nitrogen feed media. In all cases, the residual glucose contents at the end of the fermentation were near zero and represented less than 0.5% of the total glucose added. Since the glucose addition was controlled to reach a given ethanol content, all fermentations reached statistically equivalent ethanol concentrations (~90 g/L or ~11%, v/v) and efficiency (~70.5% of the theoretical maximum sugar-to-ethanol conversion). However, it is worth noting that ethanol productivity was improved by approximately 21% when the adapted feeding strategy was applied, regardless of nitrogen content in the feed medium. This supports our hypothesis that adjusting glucose addition based on the evolved gas production during ethanol fermentation can enhance sugar utilization and ethanol productivity. The adapted feeding strategy

provides a more efficient way for continuous sugar delivery in fed-batch fermentation, while the fixed feeding strategy may limit substrate addition and even ethanol production. Regulating sugar concentration in fermentation becomes important because it can affect yield and efficiency [48, 229].

Table 4.1 Overall fermentation performance from fixed and adapted continuous feeding strategies

Feed media	Mode	Residual glucose (g/L)	Cell biomass increase (g/L)*	Cell biomass increase rate (g/L/h)**	Ethanol titer (g/L)	Ethanol efficiency (%)***	Ethanol productivity (g/L/h)
High nitrogen	Fixed	3 ± 3^{a}	0.4 ± 0.2^{a}	0.007 ± 0.004^{ab}	91 ± 3^{a}	67.5 ± 0.6^{a}	0.51 ± 0.02^{a}
	Adapted	0.18 ± 0.05^{a}	0.9 ± 0.2^{b}	0.017 ± 0.001^{c}	91 ± 3^{a}	72.4 ± 0.6^{a}	0.62 ± 0.02^{b}
Low nitrogen	Fixed	1 ± 2^{a}	0.2 ± 0.1^{a}	0.003 ± 0.001^{a}	90 ± 3^{a}	70 ± 4^{a}	0.52 ± 0.02^{a}
	Adapted	0.7 ± 0.6^{a}	0.6 ± 0.2^{ab}	0.010 ± 0.003^{b}	89 ± 4^{a}	72 ± 3^{a}	0.60 ± 0.03^{b}

* Cell biomass increase indicates the difference in cell dry weight between the start of the feeding period to the time at which the highest cell dry weight is reached during that period.

**Cell growth rate over the feeding period.

***Ethanol efficiency (%) = (amount of ethanol produced/amount of glucose consumed/0.511)·100.

Means are reported for independent triplicates (n=3), with error bars representing standard deviation. Different letters (a, b) in the same column represent a statistical difference ($p < 0.05$), performed by ANOVA and Tukey HSD test.

In the bioethanol industry, high ethanol concentration is important to reduce the cost and improve the efficiency of ethanol recovery in the downstream process. For example, the minimal ethanol content for an economic recovery at industrial scale is 4% (w/v) [14, 15], but fermentation ethanol contents commonly reach up to 11% (v/v) in the industry [16]. In the present work, with both fixed and adapted feeding strategies, final ethanol contents reached 11% (v/v). The bench-scale performance demonstrated in this work is promising for large-scale processes. Operational and economic information from advanced bioethanol production can be imparted through scaling

up to the bench-scale [199], contributing further insights into potential commercial-scale production. Moreover, the adapted feeding strategy showed the highest ethanol productivity and reached above 11% (v/v) ethanol, regardless of the nitrogen contents (Table 4.1). Such advancements can be attractive to the bioethanol industry as they can be implemented in the current infrastructures without major changes in equipment or operation (e.g. merely changing the substrate feed rate).

4.5 Conclusions

In summary, this study successfully demonstrated how an adapted feeding strategy could improve the ethanol productivity by 21% in fed-batch fermentation based on evolved gas, as compared to a fixed feeding strategy. The performance of the adapted feeding strategy was shown to be maintained even when using a feed medium with low nitrogen content (N:C = 0.046:10). In all cases tested the final ethanol concentration was ~90 g/L (~11%, v/v) in 5-L bioreactors. These advancements can serve in the development of efficient practices for substrate delivery and improving ethanol production from starchy or lignocellulosic feedstocks. Efficient yeast ethanol fermentation could be identified using the evolved gas increased, as a correlation of glucose consumed per liter of reactor. Moreover, we showed that the point at which ethanol concentration can impede the glucose utilization of the yeast. Overall, this study presents a flexible and effective sugar feeding strategy for fed-batch fermentation which could be potentially integrated into existing fermentation systems to further optimize production in the ethanol industry.

Chapter 5 Ethanol production from steam-exploded poplar hydrolysate through a two-stage high-cell density self-cycling fermentation system

*A version of this chapter will be submitted for publication.

5.1 Abstract

Improving fermentation systems is an important path to improve the economics of lignocellulosic ethanol production by *Saccharomyces cerevisiae*. The present study combined the concepts of self-cycling fermentation (SCF) with those of continuous adapted feeding for ethanol production. Additionally, a second stage, in which an additional pulse feed was implemented to further enhance ethanol titer and productivity. As a result, the high cell density SCF system, with a second stage pulse fermentation, was successfully applied to lignocellulosic fermentation medium using steam-exploded poplar hydrolysate as feedstock. The patterns of glucose consumption, ethanol production, and evolved gas flow rate were all reproducible, suggesting that the integrated fermentation system could reliably be used for lignocellulosic ethanol production. This two-stage fermentation approach led to final ethanol titer of ~11% (v/v). Overall, this study presents a robust fermentation system for lignocellulosic ethanol production, and highlights the feasibility of implementing it in biorefinery processes for the bioethanol industry.

5.2 Background

The production steps for converting lignocellulosic biomass to bioethanol include pretreatment, hydrolysis (or saccharification), fermentation, and purification. Many studies have focused on pretreatment and hydrolysis to improve the yield and recovery efficiency of fermentable sugars for fermentation [125, 230]. On the other hand, fermentation itself still requires advancements to enhance microbial performance towards ethanol production. The different types of fermentation – e.g. batch, fed-batch, and continuous – rely on different modes of substrate delivery, which can enhance the performance of ethanol fermentation [48].

SCF is a semi-continuous fermentation approach in which cycling half the volume of the fermenter upon depletion of the substrate provides the microorganisms with fresh nutrients and enhances growth in the log phase [6]. SCF has found success in various applications, such as production of citric acid [231], antibiotics [9], shikimic acid [94] and bacteriophages [232]. In terms of achievements in ethanol production, Wang *et al.* [11] built up a precise cycling trigger point for the SCF in a 5-L bioreactor to efficiently manage ethanol fermentation process, resulting in a 37.5–75.3% increase in ethanol productivity compared to batch mode. However, final ethanol titer was reported 2.0–2.5% (w/v) at the end of each cycle, which is not sufficient for economic industrial production. In commercialized starch- and sugar-based bioethanol production, ethanol titer typically reaches above 11% (v/v) [16]. Thus, the potential implementation of SCF for ethanol production necessitates a significant increase in ethanol titer. Fed-batch fermentation, which involves the addition of substrates or nutrients during operation, has been shown to enhance the final product concentration in many bioprocesses [42]. We previously evaluated how adapted feeding strategies – through either pulsing (Chapter 3) or continuous feeding (Chapter 4) – in which the substrate delivery rate was adjusted based on the metabolic response from *S. cerevisiae*

improved ethanol titer and productivity. Hence, integrating fed-batch approaches into SCF is of interest in this chapter to further address the limitation in the fermentation process for lignocellulosic ethanol production.

To this end, the primary objective of the present study is to explore the performance of an integrated fermentation system for lignocellulosic ethanol production. Firstly, a continuous adapted feeding strategy was introduced to the SCF system for ethanol production. Then, a single pulse feed addition was applied to the harvested medium from the SCF system to further enhance ethanol titer to above 11% (v/v). Finally, the applicability of the integrated fermentation system to steam-exploded poplar hydrolysate was shown, providing a proof-of-concept in lignocellulosic ethanol production for the bioethanol industry.

5.3 Methods and Materials

5.3.1 Fermentation media

Synthetic fermentation media

A synthetic fermentation medium, as described in Chapter 3, was used to test the feasibility of the integrated fermentation system. Briefly, the synthetic fermentation medium was composed of 50 g/L glucose and 6.7 g/L yeast nitrogen base with amino acids in 0.1 M sodium phosphate buffer (pH 6.0). For both continuous and pulsed feeding operations, the same synthetic medium was used but with glucose concentration increased to 500 g/L.

Lignocellulosic fermentation medium

Steam-exploded poplar (SEP), a potential lignocellulosic feedstock for the production of biofuels and other high value-added products, was prepared based on Haddis *et al.* [233]. The steam-exploded poplar underwent enzymatic hydrolysis as described in Beyene *et al.* [234] with

some modifications. 10% (w/v) steam-exploded poplar was prepared in 0.05 M sodium citrate buffer (pH 4.8). 20 FPU/g of cellulase cocktail NS 51129, a non-commercial proprietary research formulation (Novozymes® A/S, Bagsvaerd, Denmark), was added to the suspension. The mixture was incubated at 50ºC and 150 rpm for 24 h. After enzymatic hydrolysis, the hydrolysate was filtered through Whatman® qualitative filter papers (Grade 3, diameter: 110 mm, pore size: 6 μm; MilliporeSigma) to remove solid residues. The liquid hydrolysate was then autoclaved at 121ºC/15 min to terminate the enzyme reaction. Hydrolyzed samples were taken for analyses of fermentable sugars and furfural-derived compounds (described in section 5.3.4). The liquid hydrolysate was supplemented with 6.7 g/L yeast nitrogen base with amino acids, 0.02 g/L ergosterol (MilliporeSigma), and 0.8 g/L Tween 80 (MilliporeSigma), forming the lignocellulosic fermentation medium for subsequent experiments. Ergosterol and Tween 80 were added to the medium for the SCF system to compensate for the reduced sterol and unsaturated fatty acid synthesis in *S. cerevisiae* grown under anaerobic conditions [11].

5.3.2 Yeast cultivation

Superstart™ active distillers dry yeast, *Saccharomyces cerevisiae*, was used in this chapter. The seed cultivation followed the procedure from Chapter 3. In the present study, the cultivation medium for the seed culture was the same as the fermentation medium used in the bioreactor (synthetic or lignocellulosic fermentation medium).

5.3.3 Fermentation configurations

5.3.3.1 Self-cycling fermentation with continuous adapted feeding strategy (stage 1)

Stage 1 fermentation consisted of an SCF system combined with continuous adapted feeding. It was operated in a 5-L stirred tank bioreactor (Infors-HT). The settings of the SCF system was reported in Wang *et al.* [11] with modification, and it was controlled by using a custom LabVIEW

program monitoring temperature (30ºC), agitation, pH, evolved gas flow rate, and cumulative evolved gas volume. Gas released from the bioreactor was monitored in real-time during the fermentation using a mass flow meter (MW-200SCCM-D/5 M). The flow rate was recorded at standard atmospheric conditions (25ºC, 1 atm) and reported as an average value over a 15-min timespan. A basic solution of 2 N NaOH was used to maintain pH at or above 3.5. Changes in pH during SCF are shown in Supplementary Figure 3 (Appendix A).

The first SCF cycle consisted of a batch fermentation carried out using either synthetic or lignocellulosic fermentation medium until the carbon source was depleted (identified by the evolved gas flow rate decreasing to less than 5 ccm), followed by a fed-batch period in which a feed pump initiated the continuous transfer of concentrated glucose synthetic medium to the bioreactor. The feed rate was adjusted according to the parameters described in Chapter 4. Thirdly, when ethanol titer reached approximately 60 g/L, the feed pump stopped, and the cycling sequence was triggered. Ethanol production during the feeding period was monitored and estimated by using evolved gas production (Supplementary Figure 4, Appendix A) and the setpoint of 60 g/L was set to avoid sugar accumulation in the system (as described in Chapter 4). The cycling sequence consisted of: 1) harvest of culture broth until 1 L remained in the reactor, 2) fresh synthetic or lignocellulosic fermentation medium was added to the bioreactor until the 2-L level was reached, and 3) a new cycle was started. Nitrogen gas was purged through the bioreactor to maintain anaerobic conditions and balance the pressure of the bioreactor. The harvested medium was further transferred to shake flasks for the stage 2 fermentation.

5.3.3.2 Pulsed feed second fermentation (stage 2)

200 mL of harvested fermentation medium was transferred to a sterile 500-mL shake flask, and the concentrated glucose synthetic medium was pulsed into the flask to extend ethanol fermentation

and increase ethanol titer to above 11% (v/v). A S-lock was installed on the shake flask and filled with distilled water to maintain anaerobic conditions.

5.3.4 Analytical methods

Culture samples (50 mL) were collected and centrifuged at 10,100×g for 10 min (Eppendorf centrifuge 5418). Supernatant was taken out for analysis of sugar and ethanol contents, and the residual pellet was used for cell dry weight analysis. Sugar composition (glucose, xylose, mannose, arabinose, and galactose) of the steam-exploded poplar hydrolysate was analyzed by HPLC based on Beyene *et al.* [234] with slight modifications. HPLC was equipped with Aminex HPX-87P column (Bio–Rad Laboratory) held at 85ºC, in which deionized water was used as the mobile phase with a constant flow of 0.3 mL/min and a RID for signal detection. Potential fermentation inhibitors, furfural and 5-hydroxymethyl furfural (5-HMF), and glucose content of culture samples were analyzed by HPLC equipped with Aminex HPX-87H column (Bio–Rad Laboratory) using RID [235]. Ethanol titer in the fermentation medium was analyzed by GC, as described in Chapter 3. Cell dry weight was measured by gravimetric analysis [11], in which experiments were performed and analyzed in at least triplicate ($n \geq 3$).

5.4 Results and Discussion

5.4.1 High-cell density SCF system with synthetic fermentation medium (stage 1)

In previous work, Wang *et al.* [11] developed a SCF system for enhancing ethanol productivity in 5-L bioreactor, yielding approximately 2% (w/v) ethanol at the end of cycles. To further increase ethanol titer and make the process more economically viable, we implemented combined the SCF with fed-batch operation, using a continuous adapted feeding strategy in each cycle. The fed-batch strategy was selected based on improvements in ethanol productivity observed in Chapter 4.

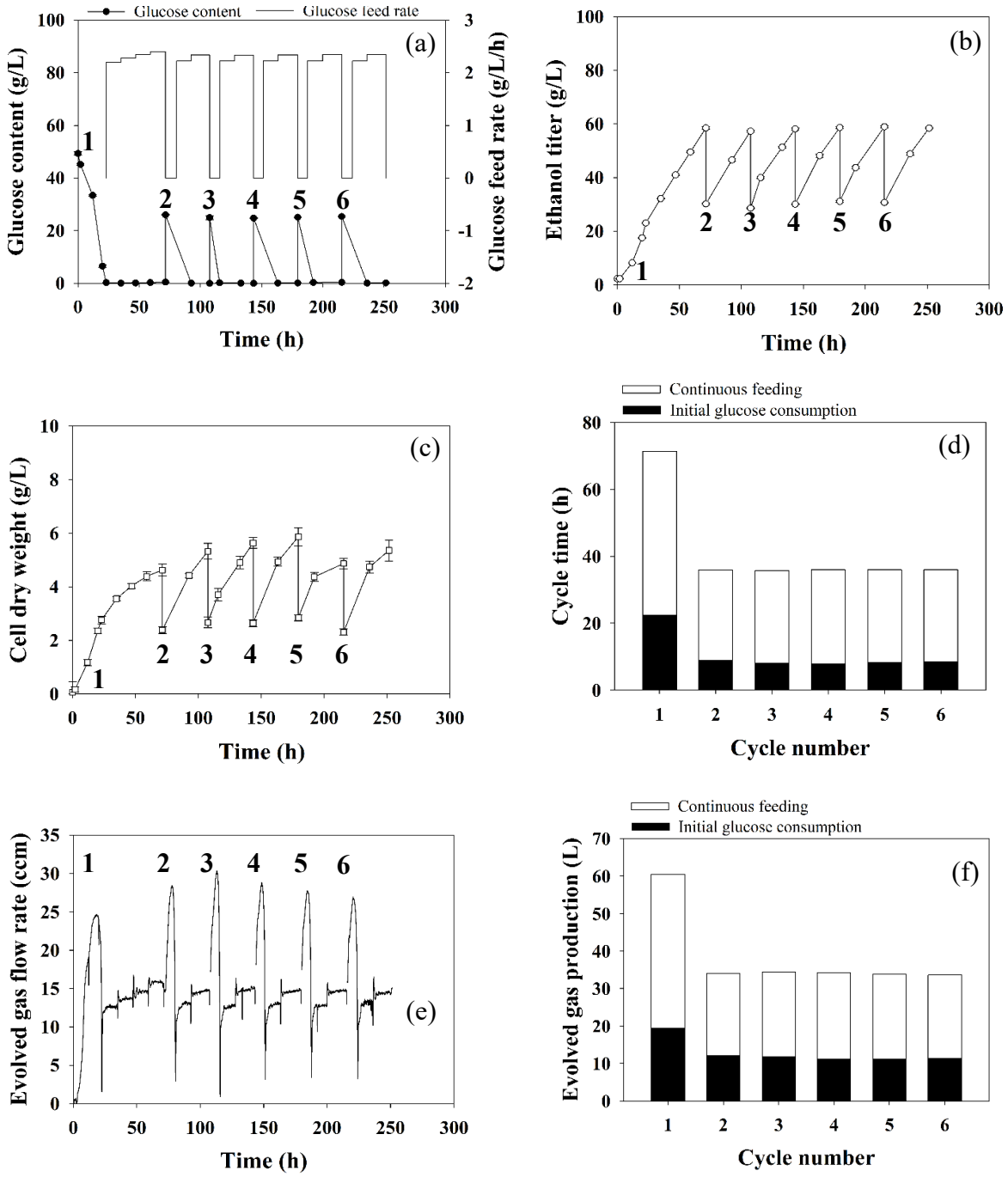

Figure 5.1 Glucose (a), ethanol (b), cell dry weight (c), cycle time (d), evolved gas flow rate (e), and evolved gas production (f) in synthetic fermentation medium undergoing SCF with continuous adapted feeding (stage 1). Cycle numbers are indicated at the beginning of the cycle. Means are reported for analytical triplicates (n=3), with error bars representing standard deviation.

Figure 5.1 shows trends in the fermentation parameters, including glucose concentration, ethanol titer, cell dry weight, cycle time, and evolved gas measured over six cycles of SCF operation with continuous adapted feeding. As seen in Figure 5.1 (a), the trend in glucose contents was regular for cycles 2 to 6 (these cycles are all conducted under the same feeding conditions, unlike cycle 1). These cycles were initiated at an initial glucose concentration of 25 g/L, and, upon depletion (indicated by evolved gas flow rate dropping below 5 ccm), adapted continuous feeding was initiated until the ethanol contents reached 60 g/L. At this point cycling was triggered. It is worth noting that the residual glucose content remained low (< 0.5 g/L) over the continuous feeding period, demonstrating that this feeding strategy did not result in substrate accumulation. Maintaining the low residual sugar in each cycle and maintaining high ethanol titer also reduced the risk of growth of contaminants, through low substrate availability in the former and ethanol metabolic inhibition in the later [236]. It is worth noting that no contamination was found through microscopy during fermentation, as aseptic techniques were also applied for media preparation and bioreactor setup.

During SCF operation, ethanol titer was monitored in real time using evolved gas production as a proxy (Figure 5.1f). The calculated ethanol contents were validated by GC analysis of samples (Figure 5.1b), which showed that 60 g/L ethanol was attained at the end of cycles 2 to 6. As with glucose contents, the ethanol titer pattern was stable between cycles. Change in cell biomass is shown in Figure 5.1 (c). In cycle 1, cell biomass reached 2.8 g/L at the end of initial batch fermentation and then increased to 4.6 g/L through the continuous feeding operation. In cycles 2 to 6, cell biomass increased from 2.6±0.2 g/L to 5.4±0.4 g/L, which was approximately a two-fold improvement within a cycle. It should be noted that the inclusion of continuous feeding to the SCF cycle led to greater biomass contents than SCF alone, as developed by Wang *et al.* [11]. Cycle time

was 71.5 h for cycle 1, and then shortened to 36 h for cycles 2 to 6 (Figure 5.1d), indicating that the same level of ethanol could be achieved in the subsequent cycles over a shorter time. When comparing between the initial batch period in SCF alone, cycle time reduced around 36% in cycle 2 to 6 as compared to cycle 1. This result is consistent with the findings of Wang *et al.* [11], where cycle time stabilized to 1/3 of that of cycle 1. Shortening the lag phase of the yeast and keeping its exponential growth during the subsequent cycles via cycling operation thus contributes to a reduction in cycle time for ethanol fermentation. This is supported by Feng *et al.* [95], who showed that their sequential batch system eliminated the lag phase of the yeast in very high gravity conditions and enhanced annual ethanol productivity compared to the batch fermentation. Tan *et al.* [94] further investigated the mechanism of increasing volumetric productivity by using transcriptomic analysis in the SCF of the engineered yeast, and the authors demonstrated that genes related to DNA replication and cell cycle were up-regulated in the early stage of SCF, leading to a higher product yield and productivity for shikimic acid production.

Under anaerobic conditions, when glucose is consumed by *S. cerevisiae*, the carbon metabolic flow is directed towards ethanol and carbon dioxide formation [22]. It is important to understand the metabolic balances during ethanol fermentation. In the present work, evolved gas was monitored in real-time (Figures 5.1e and 5.1f), allowing direct tracking of the performance of ethanol fermentation. The curve of evolved gas flow rate displayed a bell shape in the first part of SCF cycles (corresponding to the utilization of the initial glucose loaded) (Figure 5.1e). The narrower pattern in cycles 2–6 suggests less time was required to complete a cycle compared to the initial cycle (Figure 5.1d). It is worth noting that during the continuous feeding period, the evolved gas flow rate increased as glucose feed rate increased. This finding suggests a corresponding enhancement in ethanol production since it is coupled with carbon dioxide formation. This real-

time monitoring also enables the establishment of automation for the SCF system, which would be expected to reduce manpower requirements [6]. The stable patterns in glucose consumption, ethanol production and evolved gas production also suggest the high-cell density SCF is a robust and reproducible fermentation system for ethanol production (Figure 5.1f).

5.4.2 Single pulsed feed fermentation with synthetic fermentation medium (stage 2)

In the present study, a second stage was implemented in which harvested culture broth from stage 1 was transferred and supplemented with additional sugar through a single pulse addition of concentrated glucose synthetic medium. This prolonged the fermentation process to achieve higher ethanol titer. Figure 5.2 shows the glucose, ethanol, and cell biomass trends in stage 2 cycles 1 to 6. In each of these second stage cycles, 88±2 g/L of glucose was reduced to 7±2 g/L within 24 h, indicating that around 90% of the total glucose was consumed. In terms of ethanol titer, it should be noted that the ethanol contents reached approximately 60 g/L in stage 1; this was then diluted to ~49 g/L in stage 2 by adding concentrated glucose medium at 0 h (Figures 5.2a–f). Additional ethanol was then rapidly produced to reach 89±1 g/L (~11%, v/v) at the end of fermentation. High ethanol titer can reduce the costs of downstream processing, such as ethanol recovery or waste water treatment [133]. Elliston *et al.* [16] pointed out that first-generation ethanol fermentation typically reaches above 11% (v/v) to economically recover the ethanol via distillation. In our present study, ~11% (v/v) of ethanol was produced through the two-stage high-cell density SCF system. This finding suggests that a single pulse feed in the second stage is a feasible practice to reach a higher ethanol titer.

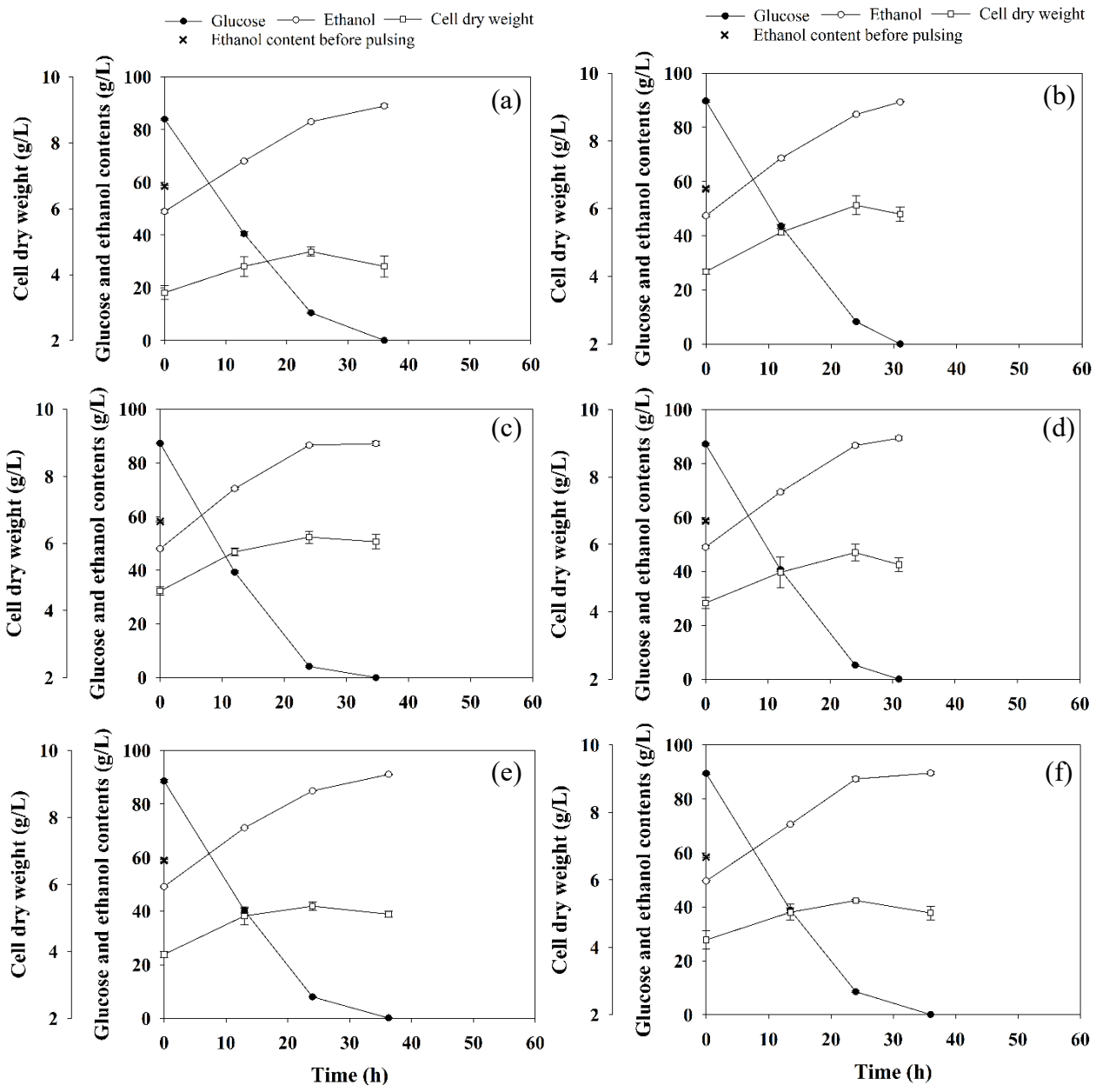

Figure 5.2 Glucose, ethanol, and cell dry weight in the harvested cycles 1–6 synthetic fermentation medium (a–f) undergoing stage 2. Symbol "x" shown at 0 h indicates the ethanol content in the initial harvested medium before the single pulse addition. Means are reported for analytical triplicates (n=3), with error bars representing standard deviation.

5.4.3 Integrated two-stage system

It is important to note that fed-batch operation has been indicated as an effective practice for final product accumulation in many fermentation processes [42]. As demonstrated in Figure 5.3, volumetric ethanol productivity in stage 2 was 60–64% higher than stage 1, showing that the single pulse approach not only elevated the final ethanol titer but also improved the ethanol productivity from the high-cell density SCF system.

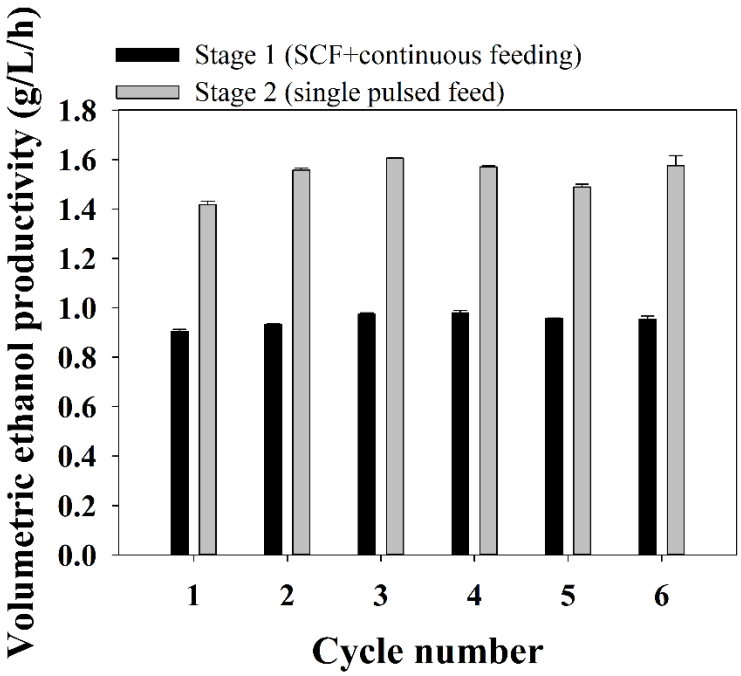

Figure 5.3 Volumetric ethanol productivity in the SCF with continuous adapted feeding (stage 1) and the single pulsed feed fermentation (stage 2) in synthetic fermentation medium. Means are reported for analytical triplicates (n=3), with error bars representing standard deviation.

As SCF can be designed for a long-term production, a reproducible feeding strategy is critical for applying fed-batch approach into the SCF. Intriguingly, pulsing fed-batch operation may not be

favorable in a long-term operation of SCF. In Chapter 3, we reported that, while adapted pulsed feeding strategies could improve ethanol productivity compared to fixed pulsing strategies, yeast flocculation may occur and disrupt cell biomass homogeneity in long-term fermentation processes. Wang *et al.* [11] observed yeast aggregation and flocculation in their SCF system. In our integrated SCF system, yeast flocculation was observed in cycles 5 and 6. Although flocculation occurred, the feed adjustment with the continuous adapted feeding strategy is not hindered by cell aggregation as I rely on evolved gas to monitored glucose consumption rate and ethanol production during fermentation (Chapter 4). As shown in Figures 5.1 and 5.2, with a reproducible measurement of evolved gas, the continuous adapted feeding strategy could effectively support SCF.

Overall, we successfully demonstrated the feasibility of a two-stage high-cell density SCF system, integrating a continuous fed-batch approach into a SCF system, for ethanol fermentation. Table 5.1 highlights the fermentation performance of the high cell density SCF system (stage 1) and single pulse fermentation (stage 2) using a synthetic fermentation medium. Among cycles 2 to 6, ethanol yield fluctuated from 0.427 to 0.437 (w/w). Considering the theoretical maximum ethanol production from glucose (0.511 g-ethanol/g-glucose) [22], the fermentation efficiency of our fermentation system reached approximately 84.5%. Interestingly, volumetric ethanol productivity shown in cycles 2 to 6 was greater than in cycle 1. This finding aligns with the previous report by Wang *et al.* [11], who found that the subsequent SCF cycles generally had higher ethanol productivity than the first cycle. While the fermentation system proposed in the present study is not optimized, it provides proof-of-concept of the integration of different fermentation approaches to improve the overall performance of ethanol fermentation. In the following section, we further investigated the feasibility of applying the two-stage high-cell density SCF system to a lignocellulosic hydrolysate medium.

Table 5.1 Fermentation performance of the high-cell density SCF system (stage 1) with single pulsed feed fermentation (stage 2) in synthetic fermentation medium

Cycle number	Phase	Final ethanol titer (g/L)	Ethanol yield (w/w)[#]	Volumetric ethanol productivity (g/L/h)
Cycle 1	First stage	58.5±0.4	0.431±0.005	0.906±0.007
	Second stage	89.0±0.6	0.476±0.002	1.42±0.01*
	Overall		0.454±0.002	0.968±0.006*
Cycle 2–6	First stage	58.3±0.6	0.404±0.005	0.960±0.002
	Second stage	89±1	0.459±0.009	1.56±0.04*
	Overall		0.432±0.005	1.14±0.02*

#: Ethanol yield (w/w) = (g-produced ethanol/g-consumed glucose).

*: volumetric ethanol productivity is calculated based on the ethanol production for 24 h from the second stage fermentation. Cycle 1: means are reported for analytical triplicates (n=3), with error bars representing standard deviation. Cycle 2–6: means are reported for cycle 2 to 6 (n=5), with error bars representing standard deviation.

5.4.4 Enzymatic hydrolysate from steam-exploded poplar

Table 5.2 shows the sugar composition of the steam-exploded poplar hydrolysate. Glucose was the primary fermentable sugar (31 g/L) in the hydrolysate, with a hydrolysis yield of 27.0%, while xylose was the second most abundant sugar (5.2 g/L), with a hydrolysis yield of 4.0%. Little arabinose, galactose, and mannose were detected in the hydrolysate with relatively low hydrolysis yield, which means they may not significantly contribute to ethanol formation compared to glucose. Furfural and 5-HMF, potential metabolic inhibitors to ethanol fermentation [180], were not detected in the hydrolysate (Table 5.2), making them unlikely to impact fermentation. In terms of fermentable sugars, it should be noted that although xylose was present in the hydrolysate, our fermentative strain, *S. cerevisiae*, is not able to utilize it for ethanol production. Xylose utilization can be further addressed by introducing a pentose-fermenting strain [170], or xylose can be converted to xylitol or other value-added products for the co-production of ethanol in a market-

attractive manner [173, 237]. In the present study, our research objective was to explore the applicability of the two-stage high-cell density SCF system using a lignocellulosic hydrolysate as feedstock. Composition of the lignocellulosic biomass, optimization of enzymatic hydrolysis, and xylose utilization are crucial for future research. In addition to carbohydrates, the minor components in the lignocellulosic biomass become important in the large-scale biorefinery process [227]. For example, protein or nitrogenous contents of the lignocellulosic hydrolysate can lead to cost-savings if they are sufficient as sources of nitrogen to support growth and ethanol production.

Table 5.2 Sugar composition and inhibitors in the steam-exploded poplar hydrolysate

Steam-exploded poplar hydrolysate	Concentration, g/L	Hydrolysis yield, % (g-sugar/g-biomass)
Sugars		
Glucose	31±1	27.0±0.6
Xylose	5.2±0.1	4.0±0.7
Arabinose	0.22±0.03	0.17±0.02
Galactose	0.08±0.05	0.06±0.04
Mannose	0.52±0.09	0.43±0.06
Inhibitors		
Furfural	ND	
5-hydroxylmethylfurfural (5-HMF)	ND	

ND: not detected, the concentration was less than 0.03 g/L.

Means are reported from independent replicates (n=6) with error bars representing standard deviations.

5.4.5 High-cell density SCF system with lignocellulosic fermentation medium (stage 1)

In these experiments, we operated the two-stage system using the steam-exploded wood poplar hydrolysate as fermentation medium to further evaluate the effectiveness of the integrated system on a lignocellulosic feedstock. Figure 5.4 shows the major parameters monitored in the first stage of the high-cell density SCF system using the lignocellulosic fermentation medium. As seen in

Figures 5.4 (a–c), the patterns of glucose concentration, ethanol contents, and cell biomass were reproducible for cycles 2 to 6. Following the same operational approach, in each cycle, when the initial glucose was depleted (monitored by evolved gas flow rate), continuous adapted feeding was started to supplement substrate. It is worth noting that there was little residual glucose (< 0.5 g/L) in the fermentation medium even with a gradual increase in glucose addition during the feeding period. These findings suggest that high-cell density SCF could be effectively applied to the lignocellulosic fermentation medium. Interestingly, in terms of cell biomass, approximately 0.3 g/L of cell biomass was produced from the initial glucose consumption in cycles 2, 3, 5, and 6 (~4.5 h after cycling), while cell dry weight reached ~2.1 g/L after implementation of the continuous adapted feeding strategy (Figure 5.4c). This result also aligned with the results obtained with the synthetic medium (Figure 5.1c). Here again, the reduction in SCF cycle times compared to the first cycle was substantial (64% reduction for cycles 2 to 6, Figure 5.4d).

As with the synthetic medium, evolved gas flow rate was used to monitor glucose consumption, manage the continuous adapted feeding, and trigger the cycling process (Figure 5.4e). Wang *et al.* [12] identified that the slope of evolved gas flow rate was an effective parameter to determine the onset of stationary phase of the yeast *S. cerevisiae* growing on wood pulp hydrolysate. As seen in Figure 5.4f, evolved gas production was reproducible in cycles 2 to 6, and the ethanol production in the subsequent cycles remained consistent. This observation suggests that evolved gas production is a practical monitoring parameter to estimate ethanol production from the lignocellulosic medium.

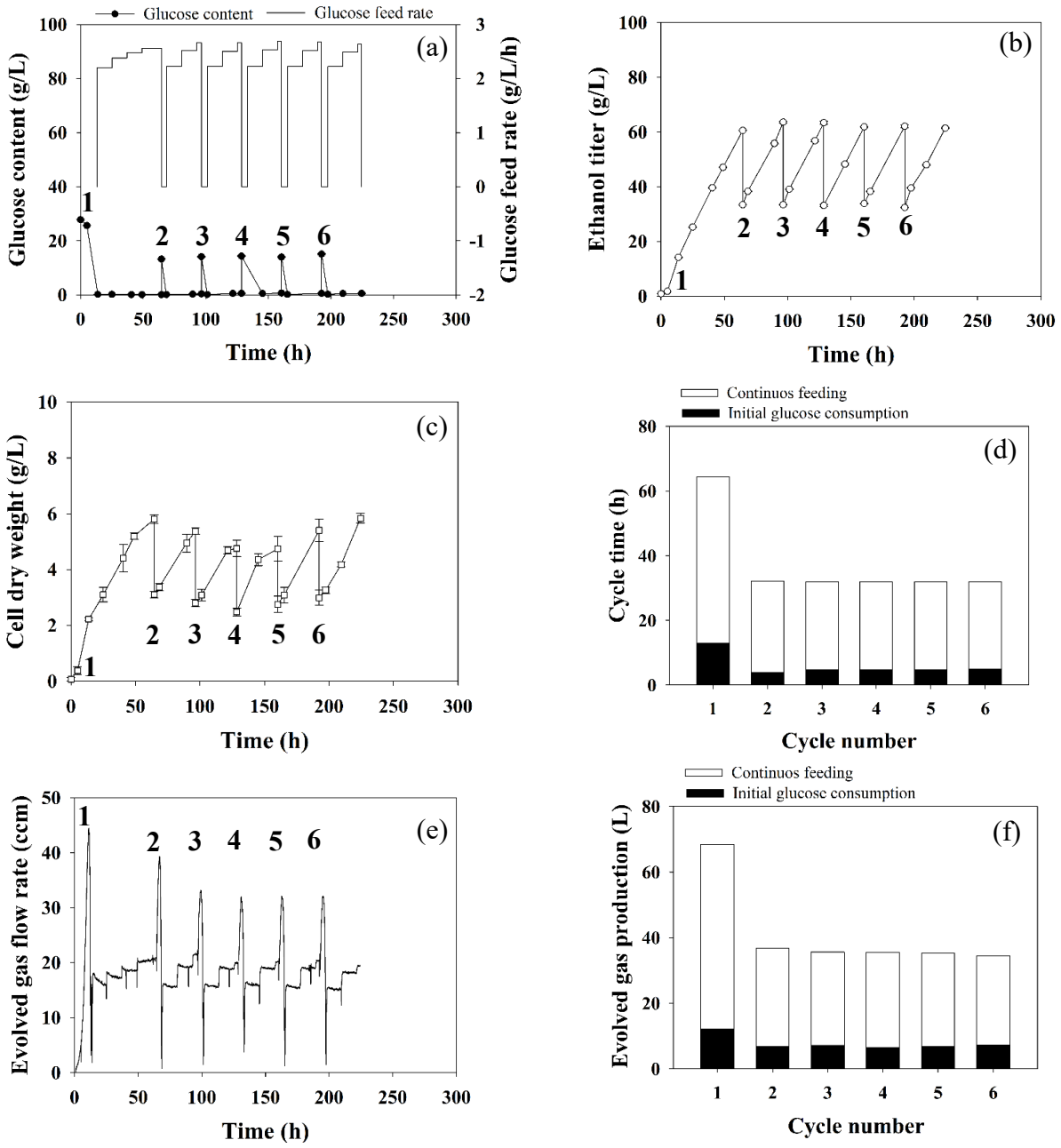

Figure 5.4 Glucose (a), ethanol (b), cell dry weight (c), cycle time (d), evolved gas flow rate (e), and evolved gas production (f) in lignocellulosic fermentation medium undergoing SCF with continuous adapted feeding (stage 1). Cycle numbers are indicated at the beginning of the cycle. Means are reported from analytical triplicates (n=3) with error bars representing standard deviations.

Evolved gas production from continuous feeding was greater than for the initial glucose utilization, indicating most ethanol was produced in the continuous feeding stage. However, it is worth noting that should the enzymatic hydrolysis of steam-exploded poplar be further optimized, more fermentable sugars could be generated in the hydrolysate, thus improving ethanol production in the system.

As with the synthetic medium, evolved gas flow rate was used to monitor glucose consumption, manage the continuous adapted feeding, and trigger the cycling process (Figure 5.4e). Wang *et al.* [12] identified that the slope of evolved gas flow rate was an effective parameter to determine the onset of stationary phase of the yeast *S. cerevisiae* growing on wood pulp hydrolysate. As seen in Figure 5.4f, evolved gas production was reproducible in cycles 2 to 6, and the ethanol production in the subsequent cycles remained consistent. This observation suggests that evolved gas production is a practical monitoring parameter to estimate ethanol production from the lignocellulosic medium. Evolved gas production from continuous feeding was greater than for the initial glucose utilization, indicating most ethanol was produced in the continuous feeding stage. However, it is worth noting that should the enzymatic hydrolysis of steam-exploded poplar be further optimized, more fermentable sugars could be generated in the hydrolysate, thus improving ethanol production in the system.

5.4.6 Single pulsed feed fermentation with lignocellulosic fermentation medium (stage 2)

Trends in glucose, ethanol, and cell biomass in the lignocellulosic fermentation medium undergoing the single pulse feed stage 2 are summarized in Figure 5.5, where Figures 5.5 (a–f) correspond to the harvested medium from cycles 1 to 6, respectively.

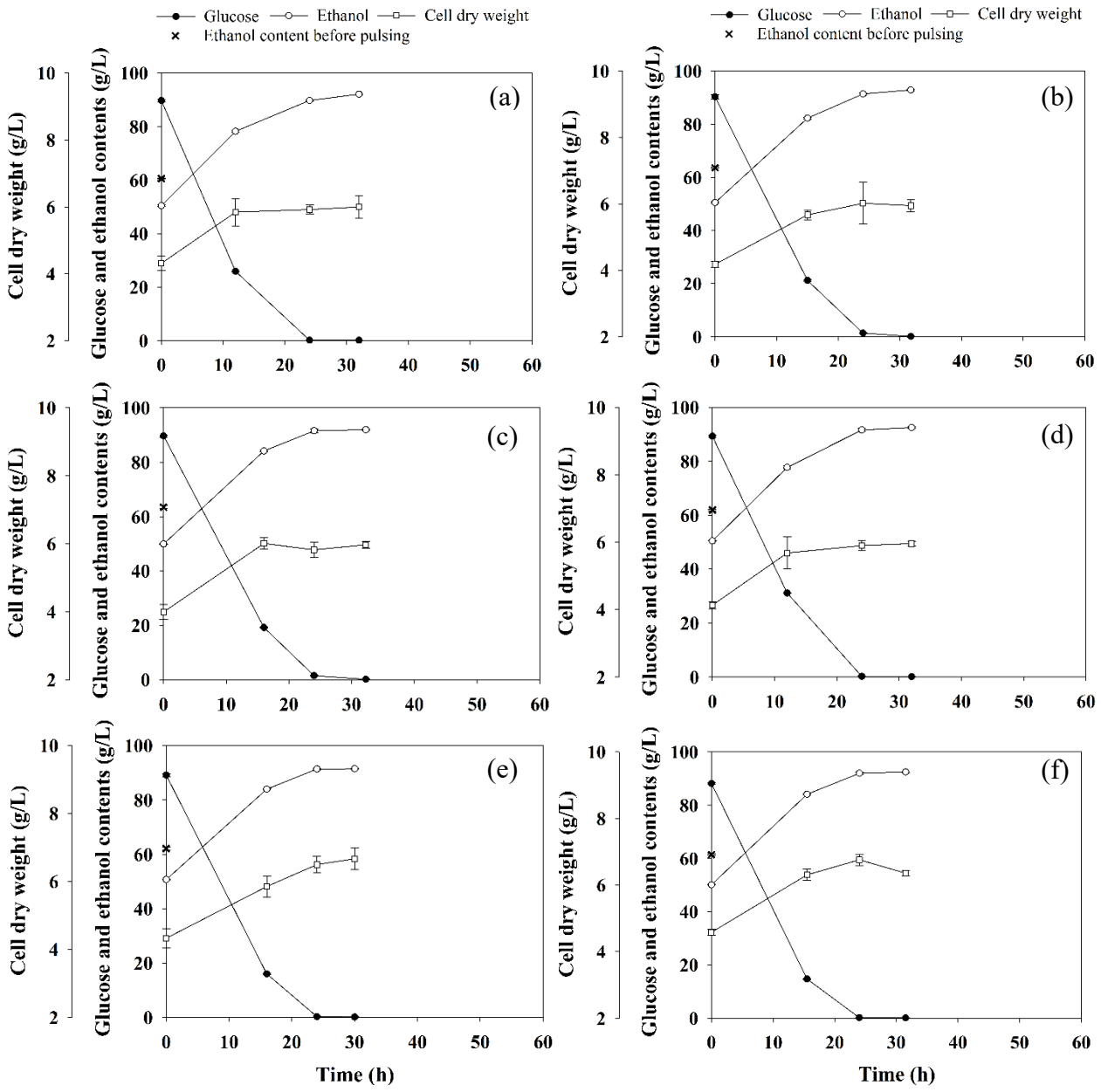

Figure 5.5 Glucose, ethanol, and cell dry weight in the harvested cycles 1–6 lignocellulosic fermentation medium (a–f) undergoing stage 2. Symbol "x" shown at 0 h indicates the ethanol content in the initial harvested medium before the single pulse addition. Means are reported from analytical triplicates (n=3) with error bars representing standard deviations.

Most glucose was consumed within 24 h in each case, and, importantly, the residual glucose was less than 0.3 g/L in Figures 5.5a, d, e, f (pulsed no. 1, 4, 5, 6) at 24 h, indicating the completion of ethanol fermentation. Glucose was utilized faster in the lignocellulosic fermentation medium than in the synthetic fermentation medium of the stage 2 fermentation (Figure 5.2). At 24 h, the ethanol titer reached 91.3±0.8 g/L (~11.6%, v/v) in all single pulse experiments (Figure 5.5). This finding suggests that the integrated two-stage system can successfully be applied to lignocellulosic-based medium for high ethanol titer, a crucial indicator for the commercial potential of cellulosic ethanol production [16]. In a report by Chang *et al.* [35], pulsing fed-batch fermentation was used to increase ethanol titer from corncob hydrolysate.

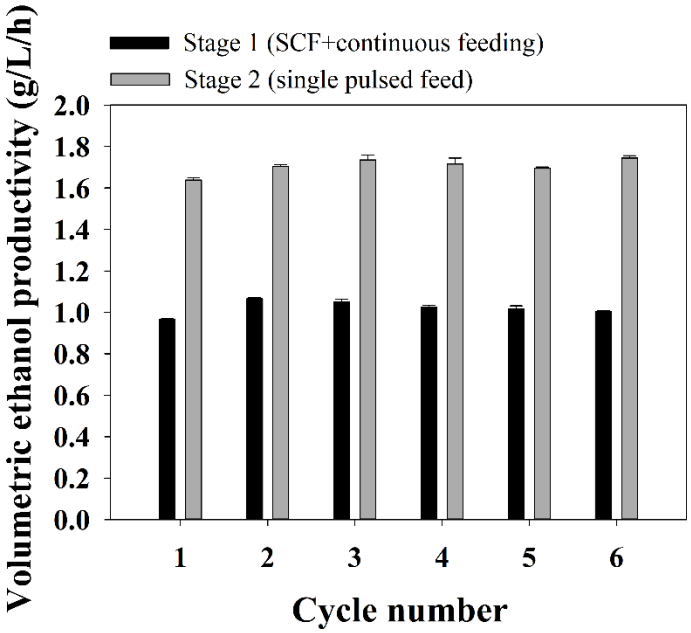

Figure 5.6 Volumetric ethanol productivity in the SCF with continuous adapted feeding (stage 1) and the single pulsed feed fermentation (stage 2) in lignocellulosic fermentation medium. Means are reported from analytical triplicates (n=3) with error bars representing standard deviations.

Figure 5.6 shows volumetric ethanol productivity from the high-cell density SCF system (stage 1) and the subsequent single pulse fermentation (stage 2). In addition to reaching high ethanol titer, the second stage also improved the volumetric ethanol productivity by 66±3% in cycles 1 to 6, as compared to stage 1.

This is also supported by Table 5.3, which highlights the final ethanol titer and volumetric ethanol productivity in the lignocellulosic fermentation medium. Volumetric ethanol productivity was 1.03±0.03 g/L/h in cycles 2 to 6 of the stage 1 and was 1.33±0.02 g/L/h in cycles 2 to 6 of the overall system. Table 3 also summarizes the ethanol yield in different phases of the fermentation system, with cycle 1 reaching 0.447 (w/w) and the subsequent cycles 2 to 6 achieved 0.453 (w/w). Considering the theoretical maximum conversion of glucose (0.511 g-ethanol/g-glucose) [22], the fermentation efficiency was approximately 88% in the integrated fermentation system. It should be noted that the concentrated glucose synthetic medium was fed to the stage 2 fermentation in the present study.

Table 5.3 Fermentation performance of the high-cell density SCF system (stage 1) with single pulsed feed fermentation (stage 2) in lignocellulosic fermentation medium

Cycle number	Phase	Final ethanol titer (g/L)	Ethanol yield (w/w)[#]	Volumetric ethanol productivity (g/L/h)
Cycle 1	First stage	60.6±0.1	0.430±0.001	0.966±0.002
	Second stage	92.1±0.4	0.465±0.005	1.64±0.01*
	Overall		0.447±0.003	1.14±0.01*
Cycle 2–6	First stage	63±1	0.43±0.01	1.03±0.03
	Second stage	92.3±0.6	0.470±0.008	1.72±0.02*
	Overall		0.453±0.006	1.33±0.02*

#: Ethanol yield (w/w) = (g-produced ethanol/g-consumed glucose).

*: volumetric ethanol productivity is calculated based on the ethanol production for 24 h from the second stage fermentation. Cycle 1: means are reported from analytical triplicates (n=3) with error bars representing standard deviations. Cycle 2–6: means are reported from cycle 2 to 6 (n=5) with error bars representing standard deviations.

To further approach the industrial practice for lignocellulosic ethanol production, the feed medium needs to be made economically, possibly utilizing bioresources. Sugar beet molasses, a concentrated sugar by-product from processing sugar beet, has been used for ethanol production [238, 239] and it may serve as a potential feedstock to replace the concentrated glucose synthetic medium in a short-term solution. On the other hand, using concentrated lignocellulosic hydrolysates as the fermentation substrate can be more sustainable for lignocellulosic ethanol production in the long-term perspective. Furthermore, xylose utilization could lead to further optimization and improvement of the economic viability of lignocellulosic ethanol production. Xylose can be metabolized to ethanol for higher titer production by pentose-utilizing microorganisms [170], or it can be converted to other valuable products from the lignocellulosic fermentation medium through a biorefinery platform, such as xylitol [237].

5.5 Conclusions

This study successfully demonstrated a two-stage high-cell density SCF system for lignocellulosic ethanol production, providing a proof-of-concept of the integration of adapted feeding strategies into the SCF system for ethanol fermentation. Moreover, implementing a second stage fermentation promoted the original operational fermentation pathway, ultimately achieving a high ethanol titer (~11%, v/v). The repeatable patterns of fermentation parameters (glucose, ethanol, and evolved gas) support the robustness and reproducibility of the integrated fermentation system. Hence, this study can act as a foundation for developing an advanced fermentation system for lignocellulosic ethanol production, thereby enhancing its feasibility for commercialization.

Chapter 6 Discussion, conclusions, and future perspectives

6.1 Discussion and conclusions

This thesis successfully demonstrates adapted feeding strategies in either pulsing or continuous operational configurations that can manage efficient sugar delivery in fed-batch fermentation of *S. cerevisiae*, thereby enhancing ethanol production. Specifically, pulsed adapted feeding strategies were structured to regulate sugar addition based on cell density, while continuous adapted feeding strategies utilized evolved gas production to control the sugar feed rate. Both adapted feeding pathways improved ethanol productivity, as compared to the fixed feeding modes. These adapted feeding strategies based on yeast metabolism address difficulties in flexible sugar adjustments in fed-batch fermentation, instead of random or fixed feeding delivery. Furthermore, this thesis is the first attempt to integrate adapted fed-batch fermentation into SCF, forming a high-cell density SCF system with robustness for improving ethanol production. The increased ethanol titer and low residual sugar in the integrated SCF also mitigate the risk of growth of contaminants in a long-term process since SCF is a semi-continuous fermentation system. The integrated SCF system was successfully utilized for the fermentation of lignocellulosic feedstock (steam-exploded poplar hydrolysate) and achieved approximately 11% (v/v) ethanol at the end of the process.

In a previous SCF system using low sugar media for bioethanol production, 2–3% (v/v) ethanol contents was achieved by the end of SCF cycles [11, 12]. In comparison, this work integrated fed-batch and SCF operations which further increased ethanol above 11% (v/v). This production level is promising for implementation in the bioethanol industry, where requirements are typically within the 10–14% (v/v) ethanol range. Beyond this concentration, ethanol can contribute significantly to the metabolic inhibition of the yeast growth. Figure 6.1 displays ethanol production during fermentation and highlights regions of the impact of ethanol contents on

metabolic inhibition to the yeast and the economic prospects for recovering ethanol. Ethanol contents up to 3% do not greatly affect metabolism; this was the regime demonstrate in previous studies on ethanol production in SCF [11, 12]. Within the range of 3–10%, ethanol starts to inhibit some metabolic processes of the yeast [220]. This aligns with our observation in Chapter 4 that glucose utilization rate was reduced as ethanol contents reached above 60 g/L during fermentation. At contents above 10%, ethanol stress on the yeast is significant and growth and production both significantly slow down [220]. However, higher ethanol titer is more attractive for the downstream process to purify ethanol in bioethanol industry. The trade-off between fermentation performance and economic recovery is critical when advancing the fermentation system for bioethanol production. The present study has shown that the fermentation strategies used led to high production of ethanol at 11% (v/v) with improved productivity.

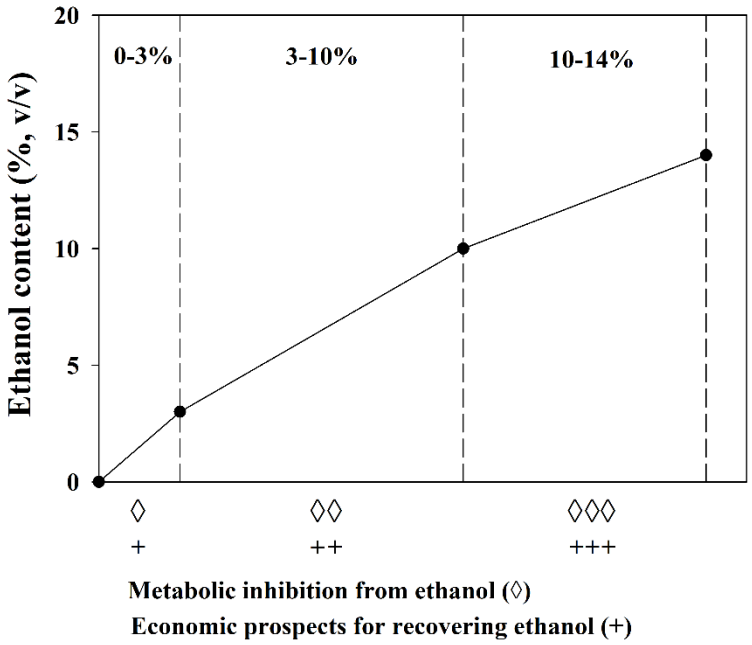

Figure 6.1 Effect of ethanol content on metabolic inhibition and economic prospects of ethanol recovery. More numbers of symbols indicate more impacts on either metabolic inhibition or economic prospects for recovering ethanol.

Overall, this thesis suggests opportunities for improving ethanol production through advanced substrate delivery in ethanol fermentation and demonstrates the potential of integrating different fermentation approaches to further strengthen the fermentation performance for lignocellulosic ethanol production. The critical contributions from each specific research study are summarized as follows.

The first research study (Chapter 3) began by investigating the effect of pulsed feeding approaches with sugar additions on ethanol fermentation in 5-L bioreactors. As cell biomass accumulated, the yeast sugar demand increased. Hence, the pulsed adapted feeding strategies were developed to address the increased sugar consumption in pulsing fed-batch fermentation of *S. cerevisiae*. A linear adapted feeding strategy was established based on changes in cell biomass, and an exponential adapted feeding strategy was developed based on cell biomass accumulation. These two pulsed adapted feeding strategies led to higher ethanol productivity (20% greater than for the fixed feeding strategy), in which the sugar addition was equivalent.

Compared to adding a substrate to a high concentration in every pulse, continuous feeding can minimize the residual sugar content during the feeding period, which helps in reducing the risk of substrate inhibition and growth of microbial contaminants in a long-term process. In the second research study (Chapter 4), the exploration of the continuous adapted feeding strategy was based on evolved gas production in ethanol fermentation, which was positively correlated with glucose consumption. This correlation was used to adjust the sugar feed rate in fed-batch fermentation undergoing adapted feeding strategy. The results demonstrated that the sugar delivery managed by the continuous adapted feeding strategy improved ethanol productivity by 21% compared to the fixed feeding mode. Furthermore, the continuous adapted feeding strategy could be applied using a low-nitrogen feed medium (N:C = 0.046:10) and maintained the same ethanol productivity as

when feeding with a nitrogen-rich medium (N:C = 0.95:10). As such, the second study demonstrated the feasibility of sugar adjustment in fed-batch fermentation with continuous feeding pathways for enhancing ethanol productivity, in which the advancement can be attractive to the bioethanol industry to improve their processes without major changes in current infrastructures.

Finally, the third research study (Chapter 5) integrated the continuous adapted feeding strategy into the SCF, forming a high-cell density SCF system in which cell biomass reaches higher concentrations than in a traditional SCF system. The integrated SCF system successfully demonstrates recurring patterns in glucose, ethanol, cell biomass, and evolved gas flow in each cycle, indicating the robust fermentation performance using a lignocellulosic fermentation medium derived from steam-exploded poplar. In addition, a single pulse feed second stage was implemented in the harvested fermentation broth from the integrated SCF, further leading to higher ethanol titer (11%, v/v) and productivity (by 66±3%) compared to the integrated SCF alone. This two-stage fermentation approach addressed the challenge of managing high concentrations of substrate delivery in the single SCF for high concentration ethanol production. In summary, the findings and implications of this thesis advance the approach of substrate delivery in ethanol fermentation and act as the foundation for integrating various fermentation approaches to promote lignocellulosic ethanol in large-scale production.

6.2 Future perspectives

This thesis points out that advancing fermentation approaches is a potential pathway to enhance ethanol production. However, further studies can be explored to strengthen the current work and to further make lignocellulosic ethanol more economically favorable in the bioethanol industry. Suggested studies are listed below.

(I) Xylose utilization

As reported in Chapter 5, the lignocellulosic hydrolysate, made from steam-exploded poplar via enzymatic hydrolysis, contained xylose. However, the yeast *S. cerevisiae* used in the present thesis could not ferment xylose for ethanol production. Two potential pathways can further address xylose utilization. The first pathway is to introduce pentose-fermenting microorganisms, either using co-cultures [159, 164] or a single strain with both hexose and pentose utilizing ability [160, 166], for converting xylose to ethanol, further enhancing ethanol yield from lignocellulosic feedstocks. The second pathway to utilize xylose involves converting it into other value-added chemicals, such as xylitol, levulinic ester, and furfural, increasing the commercial viability of lignocellulosic biorefinery [237]. In terms of the operational flexibility offered by semi-continuous SCF, these two pathways can be introduced either during or after the SCF, in a second stage approach. Improving xylose utilization can create more opportunities for implementing lignocellulosic ethanol production at an industrial scale.

(II) Bio-based feedstocks for feed medium

The present work used a synthetic feed medium (contained 500 g/L glucose and 6.7 g/L yeast nitrogen based with amino acids) for all fed-batch approaches (Chapters 3–5). Future studies can investigate bio-based feedstocks to prepare the feed medium, which will help to reduce the preparation cost. For example, lignocellulosic hydrolysates could be concentrated with consideration given to inhibitor management, or sugar beet molasses, a concentrated sugar by-product from processing sugar beet, that has been used for ethanol production [238, 239], may serve as a promising bio-based feedstock to replace the current synthetic feed medium.

(III) Removal of ethanol from fermentation broth

As reported in Chapter 4, when ethanol titer reached 60–70 g/L, glucose started to accumulate undergoing with the continuous adapted and/or fixed feeding strategies, in which the yeast might be negatively impacted on glucose utilization. Future work can perform extractive techniques to remove, either in batches or continuously, ethanol from the fermentation broth, thereby mitigating the possible metabolic inhibition caused by accumulated ethanol content. Extractive techniques for removing ethanol include pervaporation, gas stripping, vacuum, solvent extraction, adsorption, and distillation [97]. In the current fermentation configuration, adsorption, or even modern advanced continuous membrane separations, could be options to investigate as they are less likely to have impact on the evolved gas readings during ethanol fermentation when applying the continuous adapted feeding strategy.

(IV) Characteristics of SCF

In this thesis, we have integrated fed-batch approaches into SCF to enhance ethanol production (Chapter 5). To further improve its performance, the characteristics of SCF are worth investigating. For example, cell synchronization is a unique metabolic process that often occurs in SCF [6]. Exploring whether this characteristic benefits ethanol yield and productivity in the integrated SCF system will be interesting. On the other hand, strain drift is critical to evaluate whether the microbial selection is positive or negative in a long-term SCF process.

(V) Large-scale assessment and economic/environmental analysis

Although the present work demonstrates the bench-scale evaluations carried out by 5-L bioreactors for adapted substrate managements in ethanol fermentation (Chapters 3–5), further

scale-up campaigns are still required to better understand the future applicability of the advanced fermentation approaches for lignocellulosic ethanol production at an industrial scale. Assessing economic analysis from the developed bench-scale fermentation system will help infer the possibility of large-scale production. In addition, life cycle assessment (LCA) is another attractive methodology to evaluate the environmental impacts and energy balance of the developed process for lignocellulosic ethanol production, providing further information and insights into the bioethanol industry.

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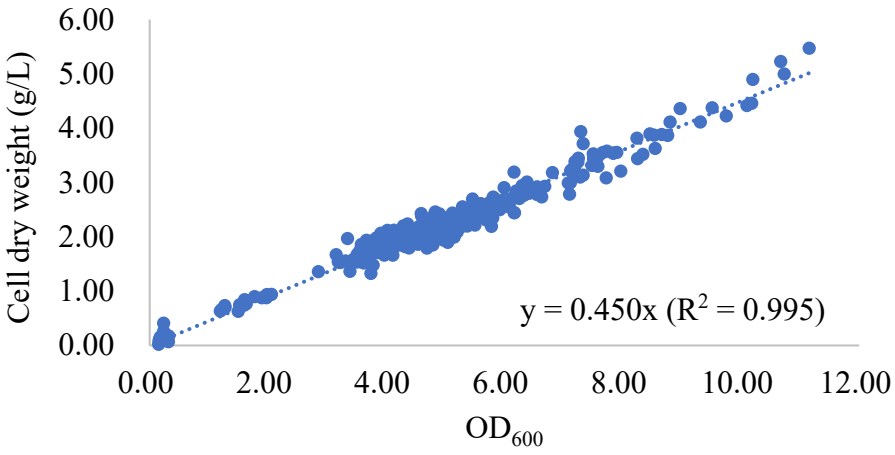
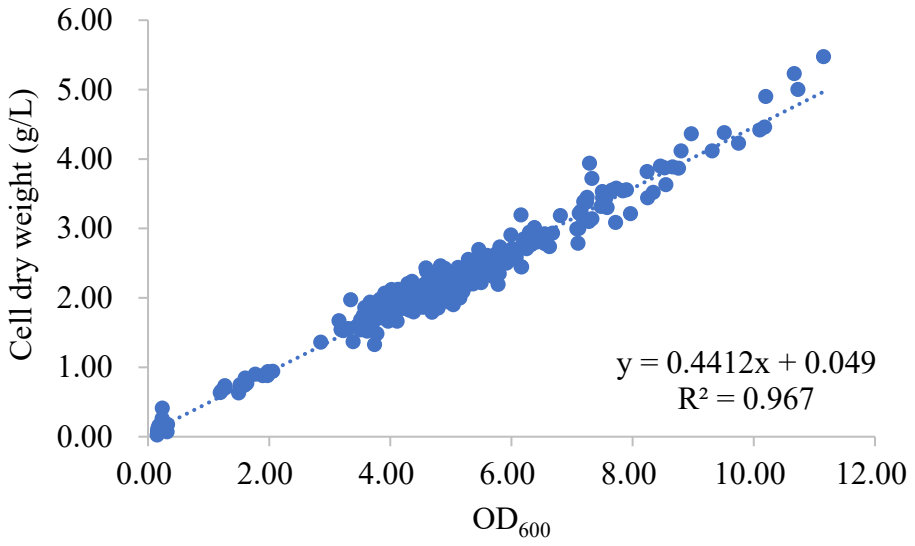
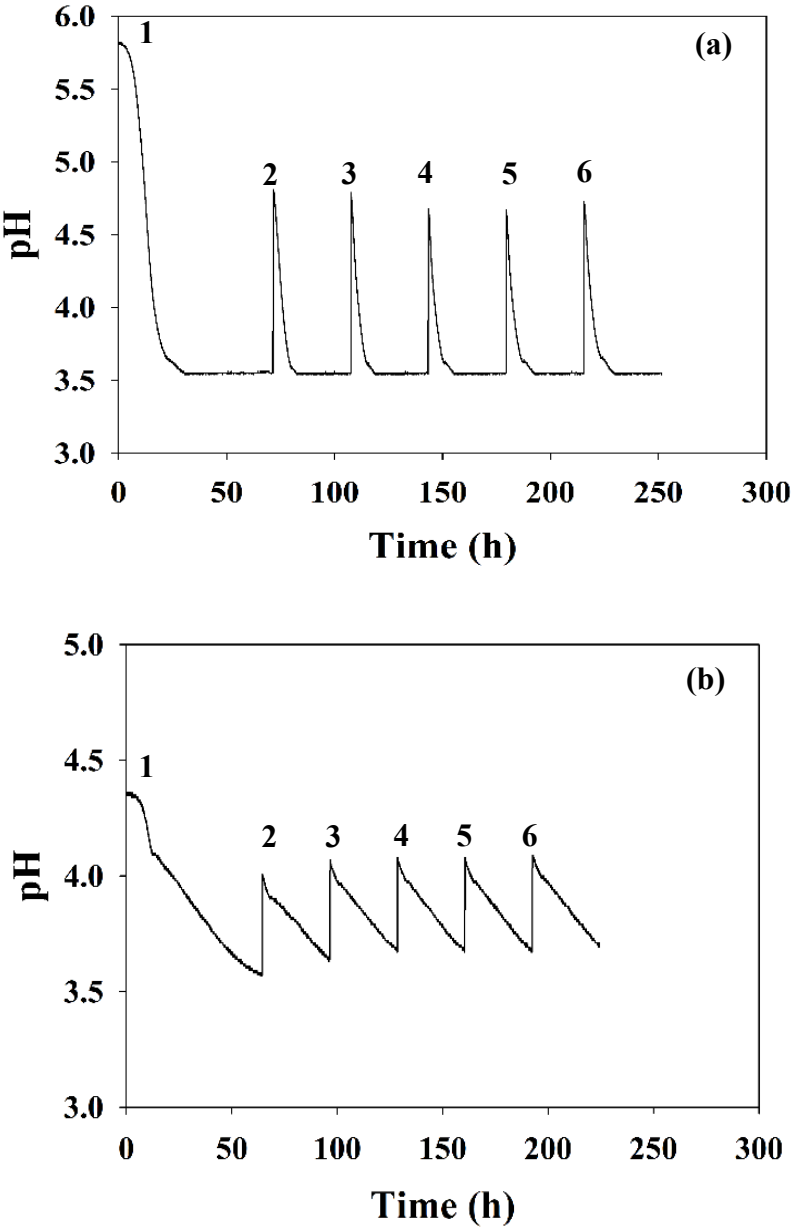
Appendix A: supplementary materials



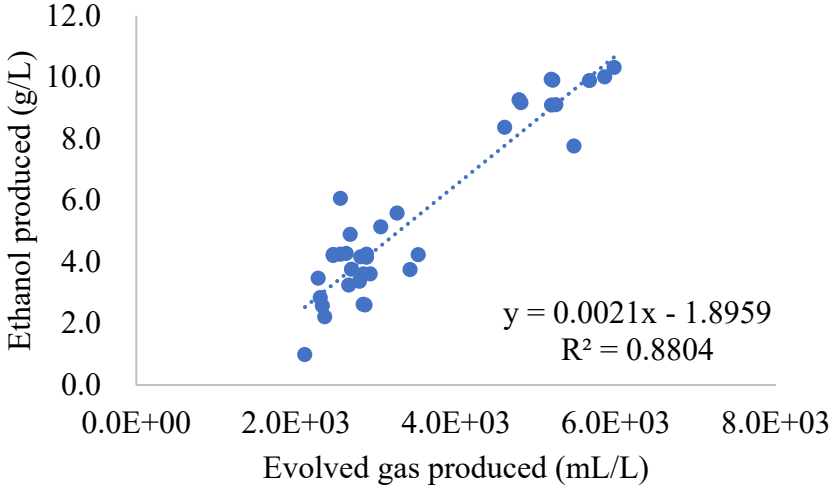
Supplementary Figure 1. The correlation between OD_{600} and cell dry weight (CDW, g/L) in Chapter 3.



Supplementary Figure 2. The correlation between OD_{600} and cell dry weight (CDW, g/L) used in Chapter 4.



Supplementary Figure 3. pH in the SCF with continuous adapted feeding (stage 1) from synthetic fermentation medium (a) and lignocellulosic fermentation medium (b). Cycle numbers are indicated at the beginning of the cycle.



Supplementary Figure 4. The correlation between evolved gas produced (mL/L) and ethanol produced (g/L) per reactor volume in ethanol fermentation of *S. cerevisiae*. The datapoints are acquired from adapted pulsing fed-batch fermentation in Chapter 3.