Enset Biomass: A promising Feedstock for Biorefinery Valorisation

Zur Erlangung des akademischen Grades einer

DOKTORIN DER INGENIEURWISSENSCHAFTEN (DR.-ING.)

von der KIT-Fakultät für Chemieingenieurwesen und Verfahrenstechnik des Karlsruher Instituts für Technologie (KIT)

genehmigte

DISSERTATION

von

M.Sc. Nebyat Abdu Seid

aus Äthiopien

Tag der mündlichen Prüfung: 17.06.2024

Erstgutachter: Prof. Dr. rer. nat. Christoph Syldatk

Zweitgutachter: Prof. Dr.-Ing. Alexander Grünberger

Zweitgutachterin: Dr. rer. nat. Anke Neumann

For our GOD እግዚአብሔር (igizī'ābiḥēri)(Father, Son, Holy Spirit);

For the Holy Virgin Mary, the Holy Angels, the Holy Martyrs and the Righteous Fathers.

``The Fear of God is the Beginning of Wisdom``

(Proverbs 1:7) EOTC Holly Bible

Acknowledgments

First and foremost, I praise the almighty God (እግዚአብሔር), the Creator of the Universe for giving me the strength and wisdom to understand His creation.

It was a great honor for me to join the Institute of Technical Biology, the research group of Prof. Dr. Christoph Syldatk, and I would like to express my sincere gratitude to him for giving me this opportunity.

I extend my heartfelt appreciation to my supervisor, Dr. Anke Neumann for her guidance and encouragement throughout this journey. Her constructive suggestions and insightful idea have always motivated me. Her kindness and support have been priceless to me, for which I am deeply grateful.

I would like to thank Dr.-Ing. Katrin Ochsenreither for believing in me and giving me the chance to join this amazing group. I am also very grateful to Dr. Habibu Aliyu for mentoring me and being a friend throughout this journey.

I am grateful to all the staff members at TeBi and EBT for their constant help and support. Especially, I would like to thank Michaela Kugel and Pascal Gorenflo for introducing and teaching me the laboratory equipments. I thank Dr. Ulrike, Dr. Jens, Daniela, Delphine, Laura, Marvin, and Sandra for their assistance and kindness whenever I needed. Furthermore, I want to thank Beate and Susanne for their help with all administrative matters. I had an amazing time at TeBi thanks to all of you!

I express my gratitude to all my past and present PhD colleagues at TeBi and EBT; Alba, Alberto, Aline, André, Christin, Fei, Flávio, Kevin, Magda, Marcus, Olga, Rebecca, Teresa, and Vanessa for the fruitful discussions and the friendly atmosphere. I also appreciate the opportunity to supervise Lea Wießner for her bachelor thesis, which was a rewarding experience.

A special thanks to my family and friends, whose unwavering support has been my backbone throughout this journey.

Last but not least, I express my love and gratitude to my wonderful husband, Kibrom Hailu Tafere. His unconditional love, support, and patience have been my greatest strength. አንተ እግዚአብሔር የሰጠኝ በረከቴ ነህ። አፈቅርሃለሁ።

Preamble

Parts of this thesis are based on peer reviewed research articles. All articles have been drafted during this work and describes the major findings of studying the potential of Enset biomass for biorefinery processes with a focus on the production of high-value biofuels and biochemicals. The texts of the chapters are therefore partly identical to these publications, but the layout, citation style, figures and formatting have been adapted to the style of this dissertation.

Chapter 1 presents an overview of the theoretical background regarding the biorefinery concept, the Enset plant, and the biorefinery conversion process.

Chapter 2 outlines the outcomes of analysing the composition of different parts of Enset biomass, evaluating different pretreatment methods for Enset biomass, and conducting a bottle experiment to investigate the potential of Enset biomass as a feedstock for butanol production using the separate hydrolysis fermentation (SHF) method. This chapter is based on the publication:

Investigating the Processing Potential of Ethiopian Agricultural Residue Enset/*Ensete ventricosum* for Biobutanol Production

Nebyat Seid, Pia Griesheimer and Anke Neumann (2022)

Bioengineering, 9(4), 133.

DOI: 10.3390/bioengineering9040133

Chapter 3 presents the process optimization results for the simultaneous saccharification and fermentation (SSF) process in bottles experiments aimed at maximizing hydrogen and butanol production. Additionally, it describes the finding of scaling up the process in a bioreactor, including the evaluation of the effects of pH control and pressure on hydrogen and butanol production. This chapter is based on the submitted manuscript:

Stirring the Hydrogen and Butanol production from Enset Fiber via Simultaneous Saccharification and Fermentation (SSF) process.

Nebyat Seid, Lea Wießner, Habibu Aliyu and Anke Neumann (2024)

Manuscript submitted for publication

DOI: 10.21203/rs.3.rs-3836946/v1

Chapter 4 describes the results of assessing the growth of *Neocallimastix cameroonii* on Enset fiber without pretreatment methods, as well as the development of a one-pot two-step fermentation process from untreated Enset fiber by merging anaerobic fungi fermentation and chain elongation process for caproate production. This chapter is based on the publication:

Caproate production from Enset fiber in one-pot two-step fermentation using anaerobic fungi (*Neocallimastix cameroonii* strain G341) and *Clostridium kluyveri* DSM 555 (2023).

Nebyat Seid, Katrin Ochsenreither and Anke Neumann

Microbial Cell Factories, 22(1), 216.

DOI: 10.1186/s12934-023-02224-w

List of publications

Peer reviewed original publications

Seid, N., Griesheimer, P., & Neumann, A. (2022). Investigating the Processing Potential of Ethiopian Agricultural Residue Enset/*Ensete ventricosum* for Biobutanol Production. *Bioengineering*, 9(4), 133. https://doi.org/10.3390/bioengineering9040133

Seid, N., Ochsenreither, K., & Neumann, A. (2023). Caproate production from Enset fiber in one-pot two-step fermentation using anaerobic fungi (*Neocallimastix cameroonii* strain G341) and *Clostridium kluyveri* DSM 555. Microbial Cell Factories, 22(1), 216. https://doi.org/10.1186/s12934-023-02224-w

Original manuscripts submitted to peer reviewed journals

Seid, N., Wießner, L., Aliyu, H., & Neumann, A. (2024). Stirring the Hydrogen and Butanol production from Enset Fiber via Simultaneous Saccharification and Fermentation (SSF) process. *Manuscript submitted for publication:* https://doi.org/10.21203/rs.3.rs-3836946/v1

Poster presentation

Optimization of fermentation parameters in ethanol production from cane molasses *(the case of Metahara Sugar Factory Ethanol Plant)*

Nebyat Seid, Gizachew Shiferaw and Anke Neumann

Annual Conference of the Association for General and Applied Microbiology – VAAM (2020), Leipzig, Germany

Investigating the Processing Potential of Ethiopian Agricultural Residue Enset/*Ensete ventricosum* for Biobutanol Production

Nebyat Seid, Pia Griesheimer and Anke Neumann

DECHEMA Himmelfahrtstagung on Bioprocess Engineering 2022 – Future Bioprocesses for a Sustainable Industry, Mainz, Germany.

Caproate production from Enset fiber in one-pot two-step fermentation using anaerobic fungi (*Neocallimastix cameroonii* strain G341) and *Clostridium kluyveri* DSM 555

Nebyat Seid, Katrin Ochsenreither and Anke Neumann

Annual Conference of the Association for General and Applied Microbiology –VAAM (2023), Göttingen, Germany.

Abstract

Global warming and environmental pollution are major global challenges resulting from the high consumption of petroleum fuels. Furthermore, factors such as population growth, ongoing depletion of fossil fuels, and escalating petroleum prices led to a global energy crisis. A key strategy to address these challenges is to replace fossil fuels with renewable energy sources. Lignocellulosic biomass is one of the renewable resources that plays a crucial role in creating a circular bioeconomy and minimizing environmental impact. However, the main obstacle to use lignocellulosic biomass as a feedstock for biorefinery process is the higher proportion of lignin, which tightly binds cellulose and hemicellulose, and it is recalcitrant in nature. This limits the effectiveness of microorganisms in breaking down for bioconversion processes. Enset plant is a potential food source for about 20 million Ethiopians. Despite its significance, a massive amount of residual byproduct is discarded from traditional Ethiopian Enset food processing. However, the potential of Enset biomass for biorefinery applications has not been extensively explored. To effectively utilize Enset biomass as a feedstock for biorefinery process, understanding its composition and selecting an appropriate bioconversion production method are crucial. Thus, the main objective of this thesis is to investigate the processing potential of Ethiopian agricultural residue Enset/*Ensete ventricosum* for biorefinery process, focusing on the production of high-value biofuels and biochemicals.

The first step in utilizing Enset biomass as a feedstock for biorefinery process was to characterize its compositions. The compositional analysis results showed that the majority of Enset biomass parts contained 36–67% cellulose, 16–20% hemicelluloses, and less than 6.8% lignin. The Enset fiber had a high cellulose content at 67.1%, followed by the midrib and leaf sheath peels, at 40% and 34.1%, respectively. All parts of the Enset biomass contained small amounts of furfural, ranging from 0.43% to 1.03% (w/w), and hydroxymethylfurfural (HMF) was not detected. After selecting the most fibrous parts, the effect of different pretreatment methods on enzymatic hydrolysis was evaluated to determine the most suitable pretreatment method for each Enset biomass part. The results indicated that in all alkali-pretreated Enset biomass samples, the enzyme converted 80–90% of the biomass to glucose within 24 hours, while it took 60 hours to convert 48–80% of the acid-pretreated Enset biomass. In addition, the alkali pretreatment method released more glucose than the acid pretreatment in all Enset biomass samples.

To convert Enset biomass into high-value products, acetone-butanol-ethanol (ABE) fermentation was carried out using *Clostridium saccharoperbutylacetonicum* DSM 14923 strain. These experiments were conducted in serum bottles using the separate hydrolysis fermentation (SHF) method. After 72 hours of fermentation, 2.8 g/L acetone, 9.9 g/L butanol, and 1.6 g/L ethanol were produced from mixed Enset waste biomass pretreated with alkali, achieving an ABE yield of 0.32 g/g and productivity of 0.2 g $L^{-1}h^{-1}$. However, the SHF method faced some challenges such as substrate inhibition, low butanol yield and productivity, and complicated operational steps. Hence, the simultaneous saccharification and fermentation (SSF) process was examined to develop a more efficient process from Enset biomass.

The SSF process was initially performed in serum bottles to optimize the process parameters for butanol production from Enset fiber. The SSF process in bottles achieved a higher butanol concentration of 11.36 g/L with a yield of 0.23 g/g and a productivity of 0.16 g $L^{-1}h^{-1}$ compared to the SHF process. These results were observed under optimal process parameters, including 5% (w/v) substrate loading, 16 FPU/g cellulase loading, and 100 rpm agitation speed. After determining the optimal process parameters for the SSF process, a scale up process was established using a 2.5 L bioreactor with a total volume of 1 liter. In addition, the effects of pH control and pressure on butanol production in the SSF process were examined. A comparable butanol yield to that of the bottle experiment was achieved in the bioreactor under the pH-uncontrolled and overpressure conditions; however, the butanol productivity decreased to 0.095 g $L^{-1}h^{-1}$. To maximize the butanol productivity and resolve mixing problem in the bioreactor, prehydrolysis simultaneous saccharification and fermentation (PSSF) process was implemented with the same process parameters except for a substrate loading of 7% (w/v). The PSSF process resulted in the highest butanol concentration and productivity of 12.84 g/L and 0.104 g $L^{-1}h^{-1}$ respectively, however, the butanol yield was the lowest at 0.18 (g/g) compared to the SSF process.

In addition, we observed significant potential in the *C. saccharoperbutylacetonicum* strain for producing hydrogen from Enset fiber in the SSF process. However, to achieve maximum yields for both hydrogen and butanol, it is crucial to optimize the fermentation conditions separately. By further optimizing the SSF process in serum bottles, we achieved 18.86 mmol of hydrogen with a yield of 168.99 mL/g-Enset fiber under the optimal parameters of temperature (37 °C), initial pH (8.0) and low hydrogen partial pressure. This study also examined the influence of hydrogen partial pressure on hydrogen production and concluded

that lower hydrogen partial pressure was beneficial for maximizing hydrogen production. The bottle experiments demonstrated a 21.5% increase in hydrogen production at lower hydrogen partial pressure compared to higher values. To validate these results, a scale up SSF process was implemented under atmospheric pressure and without a pH control system at the optimal process parameters. The results confirmed a 79.7% increase in hydrogen production compared to the SSF process (pH-uncontrolled) at 30 °C, initial pH of 6.8, and under overpressure, which favored butanol production. However, butanol production decreased by 21.4% under the optimal process conditions for hydrogen production.

Finally, to expand the biorefinery product range derived from Enset biomass and produce other valuable products, we developed a one-pot two-step fermentation using anaerobic fungi (*Neocallimastix cameroonii strain* G341) and *Clostridium kluyveri* DSM 555. In two step fermentation, the process started by growing *N. cameroonii* on Enset fiber as a carbon source in serum bottles for 7 days. Subsequently, the fungal culture was inoculated with active *C. kluyveri* preculture and further incubated. The results showed that *N. cameroonii* grew on 0.25 g untreated Enset fiber as the sole carbon source and produced 1.16 mmol acetate, 0.51 mmol hydrogen, and 1.34 mmol formate. In addition, lactate, succinate, and ethanol were detected in small amounts, 0.17 mmol, 0.08 mmol, and 0.7 mmol, respectively. After inoculating with *C. kluyveri*, 0.3 mmol of caproate and 0.48 mmol of butyrate were produced, and hydrogen production also increased to 0.95 mmol compared to sole *N. cameroonii* fermentation. Moreover, after the culture was supplemented with 2.18 mmol of ethanol during *C. kluyveri* inoculation, caproate, and hydrogen production was further increased to 1.2 and 1.36 mmol, respectively, and the consumption of acetate also increased.

In summary, this thesis demonstrates the potential of Enset biomass for biorefinery applications due to its high cellulose and low lignin content. Enset biomass holds great promise for efficient glucose production and can be used as a feedstock for hydrogen and butanol production without requiring additional detoxification steps or sugar supplementation, as it has very low levels of inhibitory compounds that hinder the process. Furthermore, the study introduces a novel microbial cell factory method to convert untreated Enset fiber into caproate and hydrogen, providing valuable insights for future research and industrial applications. Utilizing inexpensive raw materials for the biorefinery process, such as Enset biomass, can be an effective way to create a sustainable system and minimize environmental impacts. In addition, it opens a new value chain for Enset farmers as the process only requires locally available raw materials and low-price fermenters.

Zusammenfassung

Die globale Erwärmung und die Umweltverschmutzung sind große globale Herausforderungen, die sich aus dem hohen Verbrauch von Erdölkraftstoffen ergeben. Darüber hinaus haben Faktoren wie das Bevölkerungswachstum, die fortschreitende Erschöpfung der fossilen Brennstoffe und die eskalierenden Erdölpreise zu einer weltweiten Energiekrise geführt. Eine wichtige Strategie zur Bewältigung dieser Herausforderungen besteht darin, fossile Brennstoffe durch erneuerbare Energiequellen zu ersetzen. Lignozellulose-Biomasse ist eine der erneuerbaren Ressourcen, die eine entscheidende Rolle bei der Schaffung einer Kreislauf-Bioökonomie und der Minimierung der Umweltauswirkungen spielt. Das Haupthindernis für die Verwendung von Lignozellulose-Biomasse als Ausgangsmaterial für Bioraffinerieprozesse ist jedoch der hohe Anteil an Lignin, das Zellulose und Hemizellulose fest bindet und von Natur aus widerspenstig ist. Dies schränkt die Wirksamkeit der Mikroorganismen beim Abbau für Biokonversionsverfahren ein. Die Enset-Pflanze ist eine potenzielle Nahrungsquelle für rund 20 Millionen Äthiopier. Trotz ihrer Bedeutung fällt bei der traditionellen äthiopischen Enset-Verarbeitung eine große Menge an Nebenprodukten an. Das Potenzial von Enset-Biomasse für Bioraffinerie-Anwendungen wurde jedoch noch nicht umfassend erforscht. Um Enset-Biomasse effektiv als Rohstoff für Bioraffinerieprozesse nutzen zu können, ist es wichtig, ihre Zusammensetzung zu verstehen und eine geeignete Methode für die Biokonversion auszuwählen. Das Hauptziel dieser Arbeit ist es daher, das Verarbeitungspotenzial des äthiopischen landwirtschaftlichen Reststoffs Enset/*Ensete ventricosum* für Bioraffinerieprozesse zu untersuchen, wobei der Schwerpunkt auf der Herstellung hochwertiger Biokraftstoffe und Biochemikalien liegt.

Der erste Schritt bei der Nutzung von Enset-Biomasse als Ausgangsmaterial für Bioraffinerieprozesse war die Charakterisierung ihrer Zusammensetzung. Die Ergebnisse der Analyse der Zusammensetzung zeigten, dass die meisten Teile der Enset-Biomasse 36–67% Zellulose, 16–20% Hemizellulose und weniger als 6,8% Lignin enthielten. Die Enset-Faser wies mit 67,1% einen hohen Zellulosegehalt auf, gefolgt von den Schalen der Mittelrippe und der Blattscheide mit 40% bzw. 34,1%. Alle Teile der Enset-Biomasse enthielten geringe Mengen an Furfural, die zwischen 0,43% und 1,03% (w/w) lagen, und Hydroxymethylfurfural (HMF) wurde nicht nachgewiesen. Nach der Auswahl der faserigsten Teile wurden die Auswirkungen verschiedener Vorbehandlungsmethoden auf die enzymatische Hydrolyse bewertet, um die am besten geeignete Vorbehandlungsmethode für

jeden Teil der Enset-Biomasse zu ermitteln. Die Ergebnisse zeigten, dass das Enzym bei allen alkalivorbehandelten Enset-Biomasseproben 80–90% der Biomasse innerhalb von 24 Stunden in Glukose umwandelte, während es 60 Stunden dauerte, um 48–80% der säurebehandelten Enset-Biomasse umzuwandeln. Darüber hinaus setzte die Alkalivorbehandlungsmethode bei allen Enset-Biomasseproben mehr Glukose frei als die saure Vorbehandlungsmethode.

Um Enset-Biomasse in hochwertige Produkte umzuwandeln, wurde eine Aceton-Butanol-Ethanol-Fermentation (ABE) mit dem Stamm *Clostridium saccharoperbutylacetonicum* DSM 14923 durchgeführt. Diese Versuche wurden in Serumflaschen nach der Methode der separaten Hydrolysefermentation (SHF) durchgeführt. Nach 72 Stunden Fermentation wurden 2,8 g/L Aceton, 9,9 g/L Butanol und 1,6 g/L Ethanol aus gemischter, mit Alkali vorbehandelter Enset-Abfallbiomasse hergestellt, wobei eine ABE-Ausbeute von 0,32 g/g und eine Produktivität von 0,2 g $L^{-1}h^{-1}$ erreicht wurde. Die SHF-Methode hatte jedoch mit einigen Problemen zu kämpfen, wie z. B. Substrathemmung, geringe Butanolausbeute und -produktivität sowie komplizierte Arbeitsschritte. Daher wurde das Verfahren der gleichzeitigen Verzuckerung und Fermentation (SSF) untersucht, um ein effizienteres Verfahren aus Enset-Biomasse zu entwickeln.

Der SSF-Prozess wurde zunächst in Serumflaschen durchgeführt, um die Prozessparameter für die Butanolproduktion aus Enset-Fasern zu optimieren. Das SSF-Verfahren in Flaschen erzielte eine höhere Butanolkonzentration von 11,36 g/L mit einer Ausbeute von 0,23 g/g und einer Produktivität von 0,16 g $L^{-1}h^{-1}$ im Vergleich zum SHF-Verfahren. Diese Ergebnisse wurden unter optimalen Prozessparametern beobachtet, einschließlich 5% (w/v) Substratbeladung, 16 FPU/g Cellulasebeladung und 100 U/min Rührgeschwindigkeit. Nach der Bestimmung der optimalen Prozessparameter für den SSF-Prozess wurde ein Scale-up-Prozess unter Verwendung eines 2,5-Liter-Bioreaktors mit einem Gesamtvolumen von 1 Liter durchgeführt. Darüber hinaus wurden die Auswirkungen der pH-Kontrolle und des Drucks auf die Butanolproduktion im SSF-Prozess untersucht. Eine vergleichbare Butanolausbeute wie im Flaschenexperiment wurde im Bioreaktor unter den Bedingungen mit ungeregeltem pH-Wert und Überdruck erzielt; allerdings sank die Butanolproduktivität auf 0,095 g $L^{-1}h^{-1}$. Um die Butanolproduktivität zu maximieren und das Mischungsproblem im Bioreaktor zu lösen, wurde ein Vorhydrolyseprozess mit gleichzeitiger Verzuckerung und Fermentation (PSSF) mit denselben Prozessparametern implementiert, mit Ausnahme einer Substratbeladung von 7% (w/v). Das PSSF-Verfahren führte zu der höchsten

Butanolkonzentration und Produktivität von 12,84 g/L bzw. 0,104 g $L^{-1}h^{-1}$, jedoch war die Butanolausbeute mit 0,18 (g/g) im Vergleich zum SSF-Verfahren am geringsten.

Darüber hinaus haben wir festgestellt, dass der *C. saccharoperbutylacetonicum*-Stamm ein erhebliches Potenzial für die Produktion von Wasserstoff aus Enset-Fasern im SSF-Verfahren hat. Um jedoch maximale Ausbeuten sowohl für Wasserstoff als auch für Butanol zu erzielen, ist es entscheidend, die Fermentationsbedingungen separat zu optimieren. Durch weitere Optimierung des SSF-Prozesses in Serumflaschen erzielten wir 18,86 mmol Wasserstoff mit einer Ausbeute von 168,99 mL/g-Enset-Faser unter den optimalen Parametern Temperatur (37 °C), anfänglicher pH-Wert (8,0) und niedriger Wasserstoffpartialdruck. Diese Studie untersuchte auch den Einfluss des Wasserstoffpartialdrucks auf die Wasserstoffproduktion und kam zu dem Schluss, dass ein niedrigerer Wasserstoffpartialdruck für die Maximierung der Wasserstoffproduktion von Vorteil ist. Die Flaschenversuche zeigten, dass die Wasserstoffproduktion bei niedrigerem Wasserstoffpartialdruck um 21,5% höher ist als bei höheren Werten. Um diese Ergebnisse zu bestätigen, wurde ein SSF-Verfahren unter Atmosphärendruck und ohne pH-Kontrollsystem mit den optimalen Prozessparametern in größerem Maßstab durchgeführt. Die Ergebnisse bestätigten eine 79,7% ige Steigerung der Wasserstoffproduktion im Vergleich zum SSF-Prozess (ohne pH-Kontrolle) bei 30 °C, einem anfänglichen pH-Wert von 6,8 und unter Überdruck, was die Butanolproduktion begünstigte. Unter den optimalen Prozessbedingungen für die Wasserstoffproduktion ging die Butanolproduktion jedoch um 21,4% zurück.

Um die Produktpalette der Bioraffinerie aus Enset-Biomasse zu erweitern und weitere wertvolle Produkte herzustellen, entwickelten wir eine zweistufige Ein-Topf-Fermentation mit anaeroben Pilzen (*Neocallimastix cameroonii* Stamm G341) und *Clostridium kluyveri* DSM 555. Bei der zweistufigen Fermentation begann der Prozess mit dem Wachstum von *N. cameroonii* auf Enset-Fasern als Kohlenstoffquelle in Serumflaschen für 7 Tage. Anschließend wurde die Pilzkultur mit einer aktiven *C. kluyveri* Vorkultur beimpft und weiter bebrütet. Die Ergebnisse zeigten, dass *N. cameroonii* auf 0,25 g unbehandelter Enset-Faser als einzige Kohlenstoffquelle wuchs und 1,16 mmol Acetat, 0,51 mmol Wasserstoff und 1,34 mmol Formiat produzierte. Darüber hinaus wurden Laktat, Succinat und Ethanol in geringen Mengen (0,17 mmol, 0,08 mmol bzw. 0,7 mmol) nachgewiesen. Nach der Beimpfung mit C. kluyveri wurden 0,3 mmol Caproat und 0,48 mmol Butyrat produziert, und auch die Wasserstoffproduktion stieg auf 0,95 mmol im Vergleich zur alleinigen *N.*

cameroonii-Fermentation. Nachdem die Kultur während der Inokulation von *C. kluyveri* mit 2,18 mmol Ethanol ergänzt wurde, stieg die Caproat- und Wasserstoffproduktion weiter auf 1,2 bzw. 1,36 mmol, und auch der Acetatverbrauch nahm zu.

Zusammenfassend zeigt diese Arbeit das Potenzial von Enset-Biomasse für Bioraffinerieanwendungen aufgrund ihres hohen Zellulose- und geringen Ligningehalts. Enset-Biomasse ist vielversprechend für eine effiziente Glukoseproduktion und kann als Ausgangsmaterial für die Wasserstoff- und Butanolproduktion verwendet werden, ohne dass zusätzliche Entgiftungsschritte oder Zuckerzusätze erforderlich sind, da sie nur sehr geringe Mengen an hemmenden Verbindungen enthält, die den Prozess behindern. Darüber hinaus wird in der Studie eine neuartige mikrobielle Zellfabrik-Methode zur Umwandlung unbehandelter Enset-Fasern in Caproat und Wasserstoff vorgestellt, die wertvolle Erkenntnisse für künftige Forschung und industrielle Anwendungen liefert. Die Verwendung von kostengünstigen Rohstoffen für den Bioraffinerieprozess, wie z.B. Enset-Biomasse, kann ein effektiver Weg sein, ein nachhaltiges System zu schaffen und die Umweltauswirkungen zu minimieren. Darüber hinaus eröffnet es eine neue Wertschöpfungskette für Enset-Bauern, da für den Prozess nur lokal verfügbare Rohstoffe und preisgünstige Fermenter benötigt werden.

Table of contents

Acknowledgments I

Preamble II

List of publications IV

Abstract VI

Zusammenfassung IX

Table of contents XIII

1. Theoretical Background and Research Proposal 1

1.1 Biorefinery concept 2

1.1.1 Butanol 3

1.1.2 Hydrogen 4

1.1.3 Caproate 5

1.2 Lignocellulosic biomass 6

1.3 Enset biomass 8

1.3.1 Enset plant 8

1.3.2 Ethiopian traditional Enset food processing 10

1.3.3 Enset plant biorefinery 11

1.4 Pretreatment and enzymatic hydrolysis of lignocellulosic biomass 14

1.4.1 Pretreatment methods 15

1.4.2 Enzymatic hydrolysis 16

1.5 Bioconversion of lignocellulosic biomass 18

1.5.1 ABE fermentation 18

1.5.2 From anaerobic fungi fermentation to chain elongation 20

1.6 Scale-up in anaerobic fermentation 24

1.6.1 Mass transfer in simultaneous saccharification and fermentation (SSF) process 25

1.6.2 Gas-liquid mass transfer in anaerobic fermentation 27
1.7 Research proposal 30
2. The Potential of Enset Biomass as a Substrate for ABE Fermentation 32
2.1 Introduction 33
2.2 Materials and methods 34
2.2.1 Raw materials and sample preparation 34
2.2.2 Compositional analysis 36
2.2.3 Pretreatment and enzymatic hydrolysis 36
2.2.4 Microorganism and culture maintenance 37
2.2.5 ABE fermentation 37
2.2.6 Analytical methods 38
2.3 Results and discussion 39
2.3.1 Compositional analysis 39
2.3.2 Effect of dilute alkali and acid pretreatment method on enzymatic hydrolysis 42
2.3.3 ABE fermentation 45
2.4 Conclusions 48
3. Enhancing Hydrogen and Butanol Production from Enset Fiber 49
3.1 Introduction 50
3.2 Materials and methods 52
3.2.1 Enset fiber preparation 52
3.2.2 Bacterial strain and culture medium 52
3.2.3 Preparation of SSF medium from pretreated Enset fiber for the bottle experiment 52
3.2.4 Influence of SSF process parameters on butanol and hydrogen production in the bottle experiment 53
3.2.5 Bioreactor setup for the SSF process from pretreated Enset fiber 54

3.2.6 PSSF process from pretreated Enset fiber in the bioreactor 55
3.2.7 Analytical methods 55
3.3 Results 56
3.3.1 Effect of SSF process parameters on butanol production 56
3.3.2 The impact of controlled and uncontrolled pH values in the SSF process on butanol production 59
3.3.3 PSSF process at high substrate loading with Enset fiber 61
3.3.4 Effect of SSF process parameter on hydrogen production 62
3.3.5 Hydrogen production in the SSF process at atmospheric pressure 65
3.4 Discussion 67
3.4.1 Maximizing butanol production from Enset fiber using the SSF process 67
3.4.2 Scaling up butanol production from Enset fiber 72
3.4.3 Hydrogen production from Enset fiber using SSF process 74
3.5 Conclusions 76
4. Merging Anaerobic Fungi Fermentation and Chain Elongation Process 77
4.1 Introduction 78
4.2 Materials and methods 79
4.2.1 Enset fiber preparation 79
4.2.2 Anaerobic fungi cultivation and growth culture conditions 79
4.2.3 Effect of substrate loading on *N. cameroonii* growth 80
4.2.4 Bacterial strain cultivation 81
4.2.5 Two-step fermentation process 81
4.2.6 Carbon balance calculations 82
4.2.7 Analytical methods 82
4.3 Results 83
4.3.1 Determination of *N. cameroonii* growth on Enset fiber 83

4.3.2 Caproate production from Enset fiber in one-pot two-step fermentation using *N. cameroonii* and *C. kluyveri* 87
4.3.3 Carbon balance 89
4.4 Discussion 90
4.4.1 Anaerobic fungal growth on Enset fiber 90
4.4.2 One-pot two-step fermentation 93
4.5 Conclusions 96
5. Final Conclusions and Future Perspectives 97
List of References 100
List of Figures 129
List of Tables 131
List of Abbreviations 132
Appendix A 133
Appendix B 134
Appendix C 136

1. Theoretical Background and Research Proposal

1.1 Biorefinery concept

The biorefinery concept, as defined by the International Energy Agency (IEA) bioenergy task 42, involves the sustainable transformation of biomass into various commercially viable products including food, feed, materials, and chemicals, as well as the production of energy in the form of fuels, power, and heat [1]. The concept of biorefinery emerged in the late 1990s as a response to the energy crisis and the environmental concerns associated with the use of fossil fuels [2]. Over time, this concept has evolved, incorporating different types of feedstocks and technologies. Biorefineries are classified into first, second, third, and fourth generations based on the utilization of feedstock types [3,4]. First-generation biorefineries use edible crops such as corn, sugar cane, wheat, potatoes, and vegetable oil as feedstocks to produce biofuel and other bioproducts, however, this raises the issue of food versus fuel competition, especially during unpredictable weather, events like droughts and floods [5]. Lignocellulosic biomass, such as agricultural and forest residues, is the main source of feedstock for the second-generation biorefineries. This resource is very appealing because it is abundant, cheap, and does not compete with food production [6]. The primary feedstocks for producing third-generation biorefineries are algae and seaweeds, which are efficient and economical because they have high amounts of oil/lipid, carbohydrate, or protein and do not need fertile land or other farming resources. However, the biorefineries based on algae are not very advanced technologically, and only a few genetically modified algal types can produce a lot of oil [7]. Recently, researchers have become interested in a fourth generation biorefineries, which use genetically modified plants and microorganisms to capture more carbon and produce biofuels and bio-chemicals [4].

To convert the feedstocks into usable products in the biorefinery approach, various technologies can be applied individually or in combination, these may include mechanical, chemical, biochemical, or thermochemical processes. The choice of processing technologies are primarily influenced by factors such as the nature of the feedstocks, the platform utilized, and the desired end products [2]. The biorefineries have also became more integrated and diversified, aiming to produce a variety of products and co-products. The biorefineries products can be broadly categorized into two main groups: biofuels and biochemical products. Biofuels consist of gaseous (biogas, syngas, hydrogen, and biomethane), solid (pellets, lignin, and charcoal), and liquid fuels for transportation (biobutanol, bioethanol, biodiesel, and bio-oil). In addition, important biochemical products include fine chemicals, building blocks, and bulk chemicals. The biorefineries also produces polymers and resins,

as well as biomaterials such as wood panels, pulp, paper, cellulose, and products for food, feed, and fertilizers [8].

1.1.1 Butanol

n-Butanol, also known as 1-butanol or n-butyl alcohol, is the simplest form with a straight chain of four carbon atoms and a hydroxyl group (-OH) at the end. It is an organic compound with the chemical formula C_4H_9OH [9]. Butanol is a colourless, flammable liquid with a characteristic of alcoholic odour. It is commonly used as a solvent in various industries, including the production of cosmetics, drugs, antibiotics, hormones, vitamins, paints, coatings, and resins. In addition, butanol is used as a precursor in the manufacturing of chemicals, plastics, and textiles [10]. It can also be used as a fuel, either alone or blended with gasoline. Butanol has gained interest in recent years as an alternative to ethanol, which is widely used as a gasoline additive or as E85. **Table 1** compares the properties of butanol with other fuels. Butanol has a much higher energy density and a higher air-fuel ratio than ethanol, which means it can generate more power with richer mixtures. The octane number of butanol is close to that of gasoline, but ethanol has a higher one, and its lower vapor pressure enhances its safety during handling. Moreover, butanol is non-hygroscopic, meaning that unlike ethanol, it does not absorb and retain water, this gives it a longer shelf life. Additionally, due to its lower corrosiveness compared to ethanol, butanol can be blended with gasoline in higher proportions without modifying the gasoline engine, transported through existing pipelines, and stored in conventional tanks without the need for any modifications.

Table 1. Properties of fuels [11–13].

Fuel	Energy density (MJ/L)	Motor octane number	Air-fuel ratio	Vapour pressure at 20 °C (kPa)	Flammability limits (%vol)	Boiling point (°C)
Gasoline	32–35	81–89	14.6	0.7–207	0.6–0.8	27–225
Butanol	27–29.2	78	11.2	0.53	1.4–11.2	117–118
Ethanol	19.6–21.2	102	9.0	7.58	4.3–19	78–78.4
Methanol	16	97–104	6.5	12.8	6–36.5	64.5–65

Butanol can be produced by chemical or biological methods. Chemical methods use materials derived from fossil fuels such as ethylene, propylene, carbon monoxide, and hydrogen, to synthesize butanol through reactions such as Oxo-synthesis, Reppe synthesis or crotonaldehyde hydrogenation and aldol condensation. These methods are energy intensive and not environmentally friendly [14]. The biological methods use microorganisms to ferment sugar obtained from renewable feedstock into butanol in a process called acetone-butanol-ethanol (ABE) fermentation. It can be produced from various biomass sources, such as agricultural residues, forest biomass, municipal solid waste, or algae. This method is renewable and sustainable, but has some challenges, such as low product yield and productivity, high cost and energy consumption associated with pretreatment and hydrolysis, the inhibitory effects of degradation products, solvent toxicity, and the complexities of butanol recovery [15,16].

1.1.2 Hydrogen

Hydrogen is the lightest and simplest of all the elements, usually found as a colourless, odourless, and highly flammable gas. It is also the most abundant element on Earth, but it does not occur naturally as a gas on Earth, it is always combined with other elements, such as oxygen, carbon, and nitrogen [17]. Hydrogen has many important applications in various industries. In the petrochemical industry, it serves as a key component in the production of ammonia, refining petroleum, and the synthesis of methanol. In addition, hydrogen acts as a catalyst in various reactions including hydrocracking, hydrodesulfurization, and hydroformylation [18]. Moreover, it plays a crucial role in generating electricity, heat, and mechanical power through devices like fuel cells, internal combustion engines, or turbines [19,20]. Hydrogen has a lot of potential as a fuel for various purposes because it has a high amount of energy per unit mass (140 MJ/kg) compared to other fuels, and it only emits water as a by-product when it is combusted [21]. Hydrogen can be produced through various methods from renewable or non-renewable resources. These methods differ in terms of costs, efficiencies, environmental impacts, and resource availability. One of the main sources of hydrogen is fossil fuel, and it can be transformed into hydrogen by different technologies, such as hydrocarbon reforming, plasma reforming, aqueous reforming, and pyrolysis. This method is the cheapest and most widely used source of industrial hydrogen, however it emits a lot of greenhouse gases [22]. Hydrogen can also produce from water by splitting it into hydrogen and oxygen using electrolysis method or solar thermochemical method. These

methods have the potential to produce high purity of hydrogen without any emissions, however it requires a significant amount of energy and water [23,24]. Another method for hydrogen production is biological methods, which use bacteria and algae to produce hydrogen from organic matter. These methods can be divided into light-dependent and light-independent (dark fermentation) processes. Light-dependent methods such as photofermentation and biophotolysis use algae or cyanobacteria to produce hydrogen, while dark fermentation uses heterotrophic bacteria that can produce hydrogen from organic material such as biomass or wastewater without the need for light. Although biological methods have the potential to produce sustainable and low-carbon hydrogen, they face some challenges such as low efficiency, scalability, and stability [25].

1.1.3 Caproate

Caproate, also called hexanoic acid, is a carboxylic acid with the chemical formula $CH_3(CH_2)_4COOH$. It has a goat-like smell, is a colourless oil, and occurs naturally in some animal oils and fats [26]. Caproate has various applications, such as precursors for fuels, fragrances, lubricants, paint additives and pharmaceuticals. It is also used directly as plant growth promoters, antimicrobial agents, flavour additives, and feed additives. Caproate can be extracted from oils such as palm and coconut oils, however, this is expensive method because the oils have very low levels of caproate (less than 1%) [27]. Another way to produce caproate is to utilize fossil fuels using various methods such as thermochemical, electrochemical, or photochemical conversion. However, these methods also have some disadvantages and challenges, such as low efficiency, high cost, and environmental impact [28,29]. Moreover, researchers have developed a biological process to produce caproate from organic waste such as food waste and wastewater using fermentations. The biological production process of caproate involves two main steps: hydrolysis and chain elongation. In the hydrolysis step, organic waste is broken down by acidogenic bacteria or other methods into short-chain carboxylic acids, such as acetate, propionate, and butyrate, along with other intermediates such as lactate, ethanol, and hydrogen. Subsequently, chain-elongating bacteria, including *C. kluyveri*, *Eubacterium pyruvativorans*, *Clostridium sp. Bs-1*, *Ruminococcaceae bacterium* CPB6, and *Megasphaera elsdenii*, convert the short-chain acids and other compounds into caproate. Producing caproate from organic waste is a promising way to recover valuable chemicals from low-grade biomass and reduce greenhouse gas emissions. However, there are still some challenges and limitations that need

to be overcome, such as low production rates, product inhibition, reactor configuration, and downstream separation [29–31].

1.2 Lignocellulosic biomass

Lignocellulosic biomass is the fibrous part of plant material, and the most abundant and renewable resource on Earth. It is composed of cellulose, hemicellulose, and lignin, which are complex organic compounds that are the main components of plant cell walls and provide structural support to the plant. It also contains small amounts of extractives such as chlorophyll, gums, fatty acids, resins, terpenoids and ash, which are minerals rich in Ca, K, Mg, and Si. The polymers within lignocellulosic biomass are connected through specific bonds and form a complex structure [4,32]. **Figure 1** illustrates the structure of lignocellulosic biomass. Cellulose is the main component of lignocellulosic biomass, which is a linear polymer of glucose units linked by β-1,4-glycosidic bonds. Its chemical formula is $(C_6H_{10}O_5)_n$, where 'n' shows how many glucose units are in one molecule and it has 7000–15,000 glucose monomers. The polymer chains are arranged parallel to each other and held together by hydrogen bonds, glycosidic linkages and van der Waals forces, forming crystalline and amorphous regions. Most of the cellulose is crystalline cellulose, a well-organized structure that is resistant to enzyme breakdown, while amorphous cellulose is not [33,34]. The second main component of lignocellulosic biomass is hemicellulose, it is more complex structure than cellulose, composed of different kinds of sugars, such as xylose, arabinose, mannose, glucose, galactose, and glucuronic acid [32]. Hemicellulose has a shorter and more branched structure than cellulose, and it forms a network with cellulose, acting as a link between lignin and cellulose fiber. This integration enhances the overall strength and toughness of the cellulose–hemicellulose–lignin network. Hemicellulose can be classified into four groups based on its backbone structure and side groups: xylans, mannans, mixed linkage β-glucans, and xyloglucans. The polymers in hemicellulose are more susceptible to thermal-chemical pretreatment and readily undergo hydrolysis into monomer sugars, unlike crystalline cellulose [33,35]. Lignin is another component of lignocellulosic biomass; it is a complex organic polymer consisting of phenylpropanoid units that are randomly and nonlinearly linked together by ester bonds. Lignin consists of three main types of building blocks called monolignols, which are derived from phenylpropane: coniferyl alcohol, sinapyl alcohol, and p-coumaryl alcohol. These monolignols vary in their degree of methoxylation (0, 1, and 2, respectively). Lignin is synthesized by enzymatic dehydrogenation of these monolignols, which form both C–O and C–C bonds, leading to a

heterogeneous and three-dimensional structure. The relative amounts and types of monolignols vary according to the plant source, resulting in different lignin classes, such as G (guaiacyl), S (syringyl), and H (p-hydroxyphenyl) [34,36]. Lignin does not have a regular or uniform structure like cellulose, but is a physically and chemically diverse material, with additional components such as hydroxycinnamic acids and flavonoids that further complicate the structure and functionality of lignin. Lignin connects hemicelluloses and cellulose in the cell wall, making plant tissues strong and stiff, and defending them from microbial attacks. It is recalcitrant to degradation and acts as a physical barrier that prevents enzymes from reaching cellulose [32,33,37].

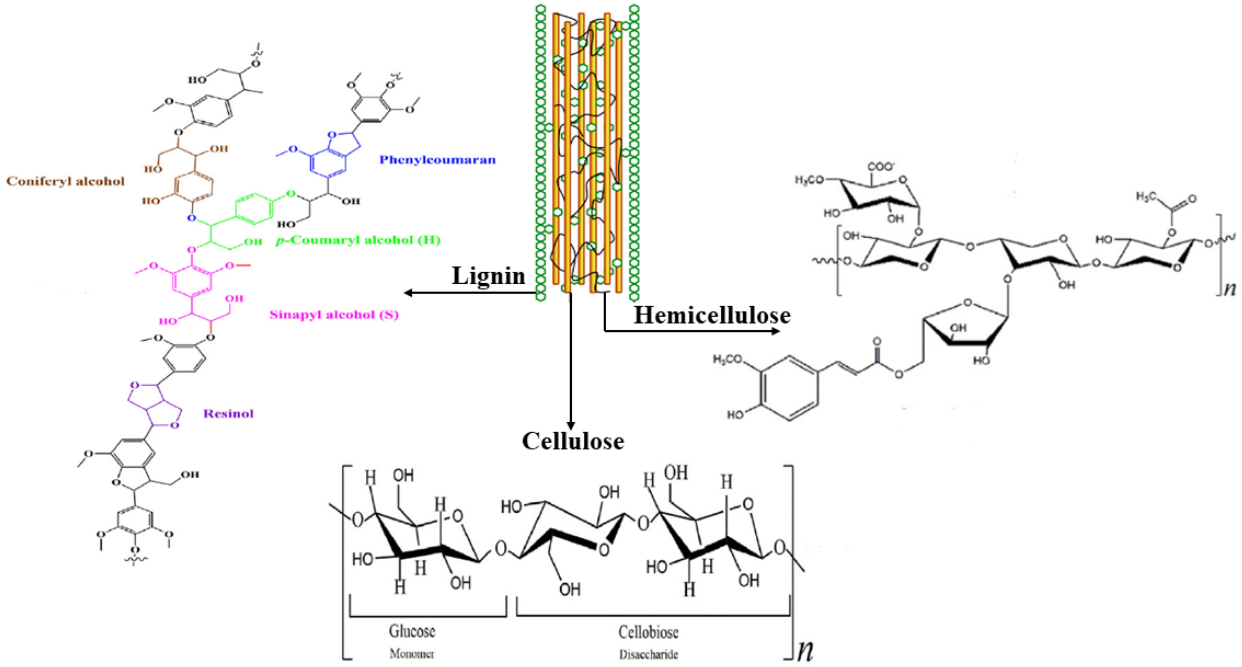

Figure 1. Lignocellulosic biomass structure. Modified from [4,38].

Lignocellulosic biomass is obtained from various sources such as forest residues (hardwood and softwood), agriculture residues (crop straw, rape seed, and sugarcane), herbaceous crops (grasses and weeds), and various wastes (industrial, municipal and food) [39]. **Table 2** shows the composition of different lignocellulosic biomass from a variety of sources. The type of plant species, growth of stage and area, harvest time and other conditions affect the chemical composition and structure of lignocellulosic [32,40]. The cellulose, hemicellulose, and lignin content in lignocellulosic biomass plays a crucial role in the processing and conversion of biomass into valuable products.

Table 2. Chemical compositions of lignocellulosic biomass from a variety of sources.

Lignocellulosic biomass	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Reference
Enset fiber	67.1	15.6	5.1	[41]
Enset midrib	40	19.7	3.1	[41]
Banana fiber	60–65	6–19	5–10	[42]
Cotton stalk	67	16	13	[43]
Sponge gourd fiber	66.6	17.4	15.5	[4]
Hemp stalk	52	25	17	[43]
Wheat straw	30.2	21.0	17	[44]
Barley straw	36–43	24–33	6.3–13.1	[32]
Rice straw	31.1	22.3	13.3	[44]
Corn cobs	45	35	15	[33]
Corn stalks	50	20	30	[40]
Sugarcane bagasse	43.1	31.1	11.4	[45]
Sorghum straw	32–35	24–27	15–21	[32]
Aspen	52.7	21.7	19.5	[4]

1.3 Enset biomass

1.3.1 Enset plant

The Enset plant (*Ensete ventricosum* [Welw.] Cheesman), commonly referred to as the false banana, is a plant species classified under the order Scitamineae, the family Musaceae, and the genus Ensete [46]. Enset and banana plants belong to the same family, however, the banana plant is classified under the genus Musa, and they share similar morphology. Despite these similarities, there are differences between the Enset plant and the banana plant; the banana plant produces edible fruits that are widely consumed around the world, while Enset

plant produces inedible fruits. Enset plant is taller than banana plant and can reach a height of up to 13 meters. Enset plant is very resilient and can tolerate droughts better than other staple crops, and is sometimes called the ‘tree against hunger’ [47,48]. The morphology of Enset plant is shown in **Figure 2**. The Enset plant has a corm, which is an adventitious underground stem structure. Above the ground, the plant has a pseudostem that is wide at the base and provides support to its large leaves, forming the overall structure of the plant. The Enset pseudostem consists of many leaf sheaths that wrap around each other. As the Enset plant matures, a stalk emerges from the inner part of the pseudostem and develop an inflorescence at the top, which hangs down with a flower [49]. Depending on the variety, an Enset plant can grow between 4 and 13 m tall and has a thick pseudostem that measures 1.5 to 3.0 m around and 2 to 5 m long. The leaves are long and wide, reaching 4 to 6 m in length and 0.6 to 0.9 m in width. The corms are 0.7 ± 1.8 m in length and 1.5 ± 2.5 m across when fully grown [49,50]. In addition, the ratio of Enset plant components is estimated as follows: the laminas 6-16%, the midribs 4-21%, the pseudostem 46-60%, the corm 10-30% and stalk 9-11% [51,52].

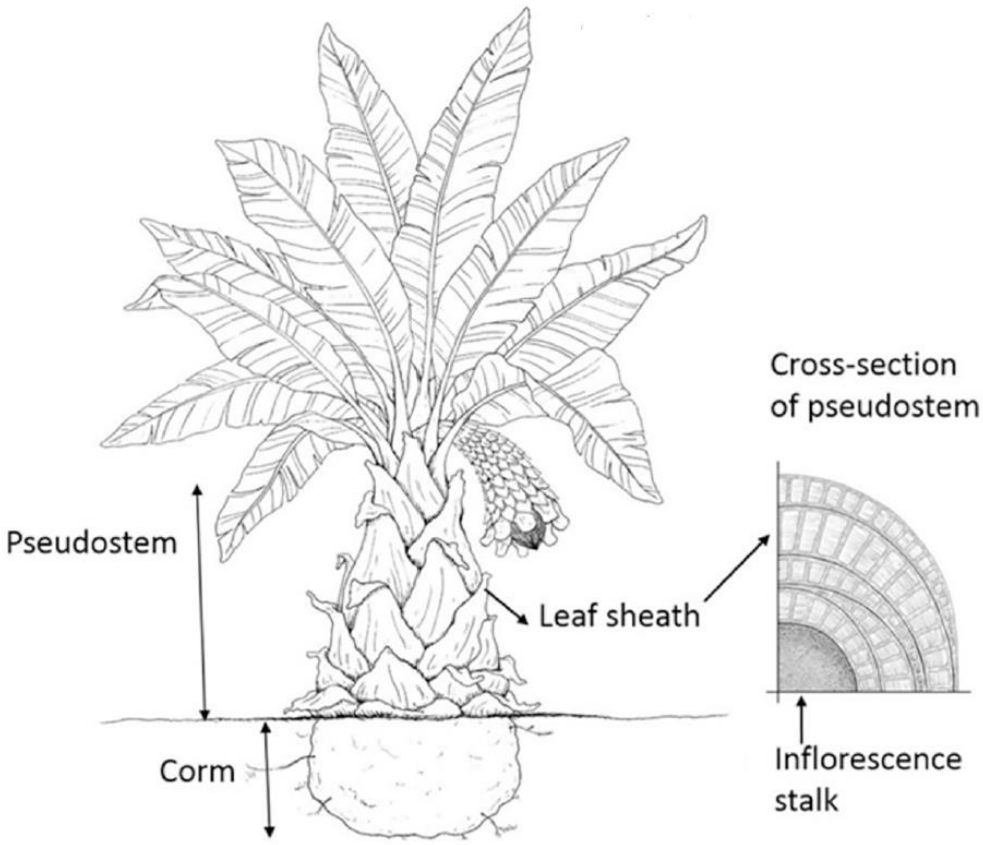

Figure 2. Morphology of Enset plant [49].

Enset plant has both wild and domesticated varieties, which differ in their genetic diversity, propagation, and morphology. The Enset plant, taxonomically classified as *Ensete ventricosum* has been domesticated only in the Ethiopian highlands at elevations ranging from 1,100 to 3,000 meters above sea level and cultivated as a staple food crop by southern Ethiopian farmers. Enset plant grows best in moist, high-altitude areas with fertile soils [47,53]. There are 106 varieties of Enset plant documented in Ethiopia from different farmers and Angacha Enset field gene bank (Angacha district, South Ethiopia) [54]. The central statistics agency of Ethiopia (CSA, 2020) reported that 206,659,076 Enset trees were harvested across the country in 2020/21 year, and the majority of Enset crop production is in the regional state of South Nations and Nationalities of People [55]. In addition, there are other species of wild Enset, such as *Ensete livingstonianum* and *Ensete glaucum*, found in central, eastern, and southern Africa and Asia [56].

1.3.2 Ethiopian traditional Enset food processing

The Enset plant is a potential source of food for around 20 million Ethiopians. In addition, a recent study suggests that cultivation of this plant can be expanded to provide food for an additional 87.2 to 111.5 million people in Ethiopia and potentially expand its domestication to other parts of sub-Saharan Africa [57]. Enset plays a crucial role in the diet and culture of many communities in Ethiopia, particularly in the southern and southwestern parts of the country. Enset farming offers sustainable food supply that can cope with both regular and occasional food shortages [58]. The traditional processing of Enset involves several steps to convert the pseudostem and corm of the Enset plant into a staple food known as Kocho, Bulla, and Amicho. All these three food items are rich in carbohydrate and minerals content, such as calcium, potassium, and zinc [59].

Enset processing is a labour intensive and time-consuming traditional practice that requires knowledge and skill, the process also varies from one community to the others. Enset is typically propagated through suckers from the appropriately prepared corm of a young plant. The plant is grown in a field until it reaches maturity, which can take 4 to 12 years depending on the variety, climatic conditions, and agricultural practices [60]. To harvest Enset, the leaf and midrib of the mature plant are cut off and discarded from the pseudostem. Then, the leaf sheath layers of the pseudostem are peeled off. The first layer is usually dry and starchless, so it is thrown away, the remaining leaf sheath layers are scraped with traditional wooden tools, and the resulting pulp is collected. The discarded leaf sheath peel and Enset fiber are not utilized. In some traditions, the pulp from the scraped leaf sheath layers is mixed with

the chopped corm. The pulp is then squeezed by hand to remove the excess water and the solid part undergoes fermentation. The extracted water is subsequently gathered and filtered to produce Bulla, a white and starchy powder that can be prepared into a porridge. Moreover, the corm can be boiled and consumed as a food called Amicho, which resembles potato. Amicho is made from young Enset plants, while Kocho and Bulla are made from older plants that are close to flowering. Kocho is a pancake-like bread and a fermented product obtained from the scraped pulp. The fermentation process varies depending on the climate conditions. At high altitudes, the scraped pulp is fermented in a pit with a traditional starter for about 2-5 months. At low altitudes, the fermentation is carried out in a two-step process; the scraped pulp is fermented on the surface for 2-4 weeks, and then in a pit for another 2-4 months [61]. The fermentation pit is prepared by lining it with leaves to facilitate the process, and the scraped pulp is carefully placed within. The pit is covered with leaves, other plant materials and stones to create an anaerobic fermentation environment. After fermentation, the Enset is removed from the pit and ground into a fine flour using a traditional grinder to eliminate any remaining fiber. The resulting Enset flour is kneaded into a dough-like consistency. This final product can be prepared in various ways, including baking, and can be stored for an extended period.

1.3.3 Enset plant biorefinery

A massive amount of residual byproduct is discarded from traditional Ethiopian Enset food processing. Traditionally, Enset biomass has multiple applications such as animal feeds and medicine. In addition, the Enset fiber obtained from Enset plant can be crafted into different things, such as sacks, ropes, mats, and sieves [47]. Enset processing is mainly produced by small-scale farmers using indigenous knowledge and practices. However, there are some challenges and limitations of the traditional Enset processing, such as long fermentation time, low nutritional quality, high moisture content, and microbial spoilage [62]. Furthermore, the valorisation of Enset plant has not been widely adopted or commercialized. Thus, more research and development are required to optimize the production and utilization of Enset plant. Up to date there is no established industrial process for the utilization of the Enset plant.

Enset plant parts can be converted into various value-added products, and the possibility of converting this plant into high-value industrial products would strengthen the bioeconomy. **Figure 3** shows the possible routes for the Enset plant biorefinery process and products. The proposed integrated biorefinery concepts are designed based on both traditional Ethiopian

Enset food processing methods and information gathered from existing literature. Seid et al. [41] reports the composition of Enset plant parts, such as ash content, moisture content, elemental analysis, calorific value, cellulose, hemicellulose, and lignin content. The study concluded that Enset biomass can be used as a viable feedstock for biological processes but may not be optimal for thermochemical processes due to its relatively low calorific value. For more details, additional information can be found in **chapter 2**. Butanol, hydrogen, caproate, and other biochemicals can be derived from Enset biomass using biological processes, such as ABE fermentation, anaerobic fungi fermentation, and chain elongation process [41,63,64]. Furthermore, Enset fiber has the potential to be converted into ethanol [65], pulp [66], textiles [67], nanocomposite [68], and construction materials [69]. The Enset plant is rich in starch content, and apart from its traditional use as food, the extracted starch can be used in the pharmaceutical industry as a tablet binder and disintegrant [70]. Research indicates that the starch obtained from the Enset plant is of better quality compared to corn and other crops [71]. In addition, the waste products from integrated Enset plant biorefineries are rich in organic nutrients, which can be utilized as a substrate for biogas production or recycled into the fermenter. Integrated Enset plant biorefineries can contribute to sustainable agriculture, economic development, and the creation of valuable products for both local and global markets. However, to industrialize it, the performance of the integrated Enset biorefinery needs to be evaluated through further research on feasibility, process modelling and simulation, and optimization.

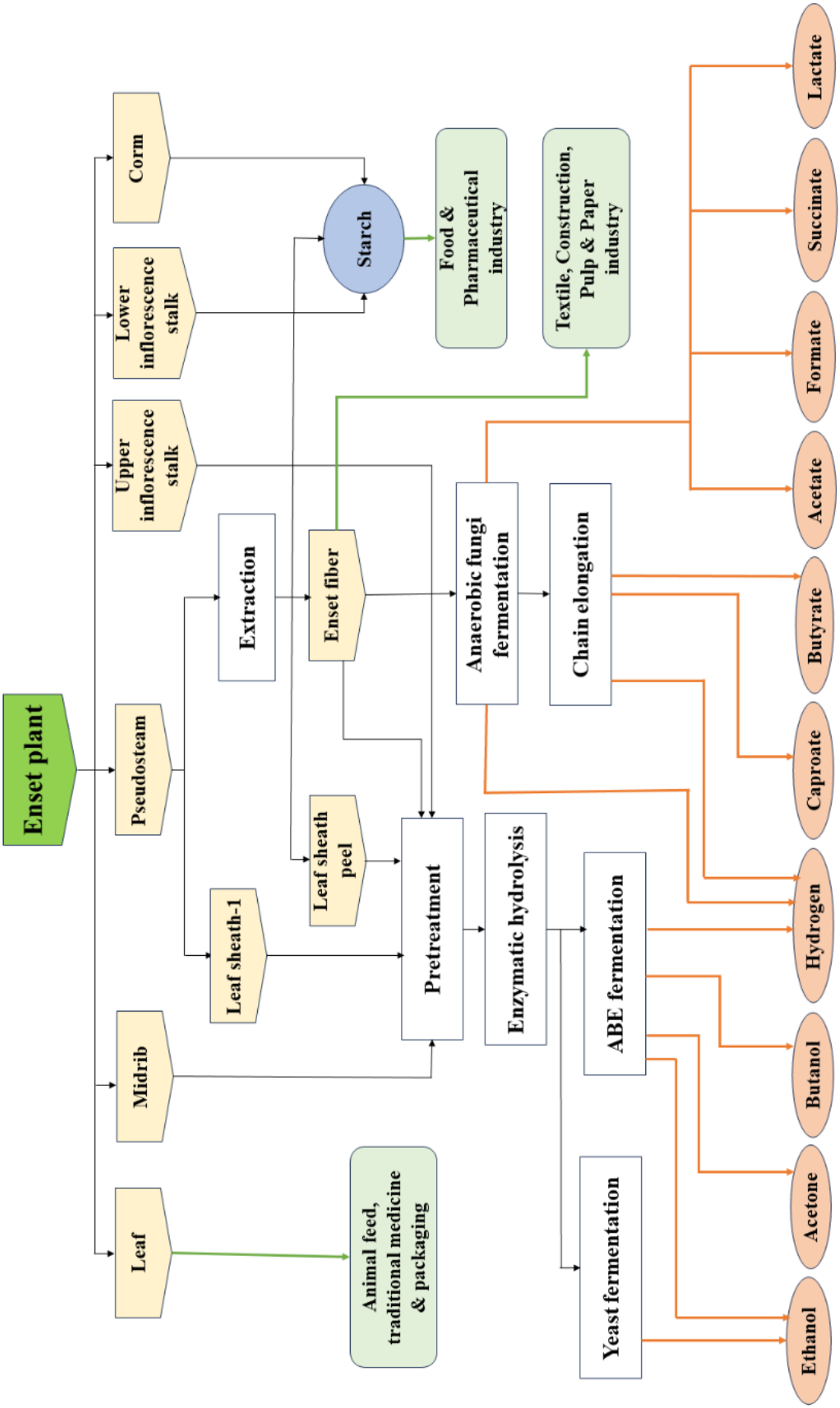

Figure 3. Potential of Enset plant for integrated biorefineries.

1.4 Pretreatment and enzymatic hydrolysis of lignocellulosic biomass

As previously mentioned, lignocellulosic biomass has a complicated structure in which cellulose, hemicelluloses and lignin are tightly bound together through the formation of lignin-carbohydrate complexes. This makes the biomass recalcitrant, which impedes the accessibility of cellulose to enzymes. Moreover, the recalcitrance of the biomass depends on the crystallinity of cellulose and the degree of lignification. To overcome this challenge, pretreatment of lignocellulosic biomass is essential to disrupt the ordered structure and improve the accessibility of cellulose and hemicellulose. During this process, the lignin barrier is broken, the hemicellulose is either completely or partially degraded, and crystalline cellulose is converted into an amorphous form, which enzymes can degrade more easily [72–74]. In addition, different degradation products can be formed from the lignocellulosic biomass, depending on the type of biomass, pretreatment method and pretreatment conditions. Some of the common degradation products of lignocellulosic biomass are shown in **figure 4**. Cellulose and hemicellulose can be degraded into glucose, xylose, arabinose, mannose, galactose, and other sugars by the pretreatment and enzymatic hydrolysis process. However, these components can also undergo degradation, resulting in the production of furans (furfural and 5-hydroxymethylfurfural (HMF)) and organic acids (acetic acid, formic acid, and levulinic acid). In addition, lignin may degrade into phenolic compounds and aromatic aldehydes. The degradation products of lignocellulosic biomass can affect the subsequent conversion processes positively or negatively. Some of the products, such as glucose, xylose, and other sugars, can be utilized as substrates or co-products, while others, such as furans, organic acids, phenolic compounds, and aromatic aldehydes, can inhibit the enzymes or microorganisms involved in the hydrolysis and fermentation steps [44,75,76]. Therefore, it is important to optimize the pretreatment conditions and select the appropriate pretreatment method to minimize the formation of undesirable degradation products and maximize the yield of the desired products.

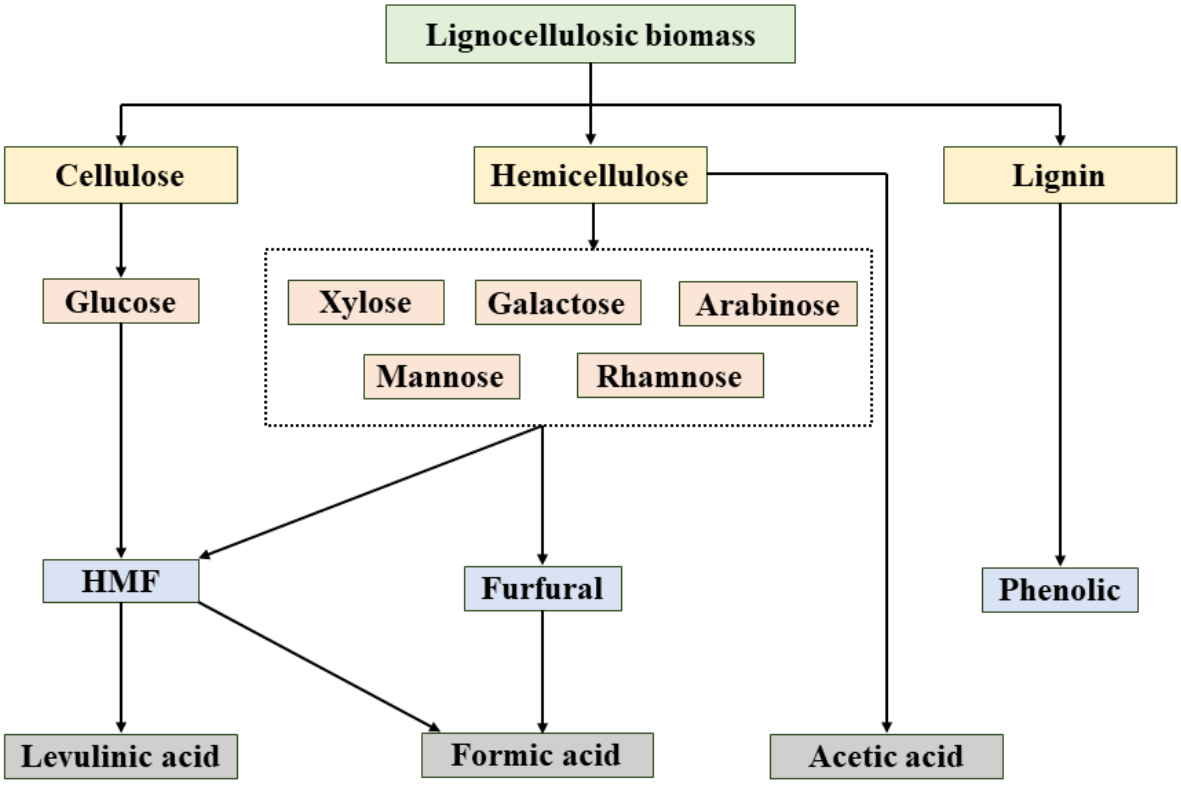

Figure 4. Degradation products from lignocellulosic biomass. Modified from [44].

1.4.1 Pretreatment methods

Pretreatment methods can be categorized into physical, chemical, physicochemical, and biological, depending on the type of agent or process used to disrupt the structure of lignocellulosic biomass. Each method has its own advantages and disadvantages. The efficiency of a pretreatment method relies on its ability to remove lignin from lignocellulosic biomass without changing its original structure, reduce the cellulose crystallinity index, decrease the particle size of lignocellulosic biomass to increase the surface area, prevent the formation of enzyme inhibitors, operate at a low cost, and use environmentally friendly chemicals [40,77].

Before applying any other pretreatment method, physical pretreatment is required. This includes milling, microwave irradiation, ultrasonication or extrusion techniques that increase the surface area and porosity of the biomass. This method can improve the accessibility of the biomass to further treatments and is an environmentally friendly method. However, it requires high energy costs and may not effectively remove lignin or hemicellulose [73].

Chemical pretreatment involves the use of acids, alkali, ionic liquids, organic solvents, or deep eutectic solvents to alter the structure of lignocellulosic biomass [73]. This method can

effectively remove or modify the lignin and hemicellulose components and make the cellulose more porous, larger in surface area, and more crystalline. Chemical pretreatment can be applied to various types and compositions of biomass, and optimized by adjusting the parameters such as temperature, pressure, time, and chemical concentration. However, it has some disadvantages such as high costs and the potential formation of inhibitory compounds [78,79]. In addition, chemical pretreatment can be combined with physical methods to improve overall performance and overcome the limitations of each method. Physicochemical methods make the lignocellulosic material more accessible to hydrolysis and reduce the formation of inhibitors. Some examples of physicochemical pretreatment methods are steam explosion, ammonia fiber explosion (AFEX), liquid hot water, and CO_2 explosion pretreatment [73,80].

Biological pretreatment is an attractive option due to its low energy consumption, mild operating conditions, and environmental friendliness. In addition, it produces fewer inhibitory compounds than chemical pretreatment. The microorganisms that are often used for biological pretreatment include white rot fungi, brown rot fungi, soft rot fungi, actinomycetes, and lignin-degrading bacteria. These microorganisms can produce various extracellular enzymes, such as lignin peroxidase, cellulase, hemicellulase, xylanase, pectinase, and laccase. Biological pretreatment has the potential to replace chemical pretreatment, but has some drawbacks, such as slow reaction rate, low sugar yield, and possible contamination, which make it less attractive for commercial use [73,81,82]. Therefore, it needs careful design and optimization to achieve the best performance and efficiency in biomass conversion.

1.4.2 Enzymatic hydrolysis

Enzymatic hydrolysis is a process that uses enzymes to break down the polysaccharides (cellulose and hemicellulose) in lignocellulosic biomass into simple sugars. This method is considered a promising technology for converting biomass to sugars due to its advantages, such as mild operating conditions, high specificity and affinity, and low environmental impact [83]. Generally, enzymes can be classified into three main types to hydrolyse lignocellulosic biomass: cellulase, hemicellulase, and accessory enzymes, including those responsible for removing hemicellulose branches, phenolic acid esterase, and lignin degrading enzymes [84].

Cellulase is an enzyme that degrades the cellulose component of lignocellulosic biomass and can be derived from diverse sources such as microbes and fungi. Filamentous fungi such as *Trichoderma reesei, Acremonium sp*., *Aspergillus niger*, *Penicillium oxalicum* and *Thermoascus aurantiacus* are some examples of fungal sources of cellulase [83]. One of the most common and effective enzyme blends to break down cellulose and hemicellulose is the commercial cellulase (Cellic® CTec2). It contains cellulase, β-glucosidase and hemicellulase, and is produced by Novozyme using various cellulolytic microbes. Cellulase can be classified as endoglucanase, exoglucanase, and β-glucosidase based on their structure and mode of action [84]. The mechanism of cellulase hydrolysis involves several steps, such as adsorption, complex formation, bond cleavage, and product release. First, the endoglucanase absorbs on the solid cellulose surface, penetrates the inner polymer chain, cleaves it, and creating two new chain ends. Then, the exoglucanase release cellobiose units from the nonreducing end of the cellulose chain. Lastly, in a liquid-phase reaction, β-glucosidase breaks down cellobiose units into glucose [85,86]. In addition, hemicellulase plays a crucial role in the degradation of hemicellulose, which is categorized into various groups according to the sugar type they target and the bonds they cleave. These groups include arabinofuranosidase, mannanase, galactomannase, esterase, xylanase, and xylosidase [83].

Enzyme hydrolysis is a complex process that depends on various factors related to the substrate, enzyme, inhibitors, and hydrolysis conditions. The substrate related factors are the characteristics of the biomass that affect the rate and yield of sugar production by enzymes. The presence of lignin in the biomass is the primary substrate related factor that hinders cellulolytic and hemicellulolytic processes. In addition, the structural properties of cellulose, including particle size, crystallinity, degree of polymerization, and accessible surface area, play a crucial role in determining the efficiency of enzymatic hydrolysis. Furthermore, the enzymatic hydrolysis of lignocellulosic biomass faces significant challenges, especially at high substrate loadings; these challenges includes obstacles such as poor mass transfer rates, high concentrations of inhibitory compounds, and inhibition caused by end-products [87,88].

Another crucial factor affecting the enzymatic hydrolysis of lignocellulosic biomass is enzyme related factors such as enzyme type, concentration, activity, stability, and synergy. The enzyme type plays a crucial role in determining substrate specificity and the overall reaction mechanism, and different enzymes have different active sites and only bind to specific substrates. In addition, the enzyme concentration influences the availability of

enzyme molecules for substrate binding; higher enzyme concentrations increase the probability of enzyme-substrate collisions, resulting in faster hydrolysis, however, it also increases the cost and risk of inhibition by products or co-products. Enzyme activity is also used as a measure of the enzyme's catalytic efficiency, which means that higher activity indicates a faster turnover rate of substrate to product, and it depends on factors such as enzyme structure, substrate affinity, and reaction conditions. Furthermore, higher enzyme stability contributes to a longer shelf life and operational life for the enzyme and is influenced by parameters such as temperature, pH, ionic strength, and the presence of additives or inhibitors. In addition, the synergy of enzymes involves the cooperative effect of multiple enzymes working together to improve hydrolysis efficiency; this synergy results in a higher sugar yield and lower enzyme dosage [89,90].

Besides, the hydrolysis of lignocellulosic biomass is influenced by inhibitory compounds formed during biomass pretreatment, as well as the enzymatic hydrolysis conditions, such as temperature, pH, and hydrolysis time and agitation speed. Therefore, various approaches should be developed to achieve high sugar yield and concentration with low enzyme loading and a short reaction time, which can make enzymatic hydrolysis more economical and feasible for industrial applications [83,87].

1.5 Bioconversion of lignocellulosic biomass

1.5.1 ABE fermentation

ABE fermentation is a process that uses bacterial fermentation to produce acetone, butanol, and ethanol in a final ratio of approximately 3:6:1 from carbohydrates such as starch and sugar [91]. The history of ABE fermentation can be traced back to 1862, when Louis Pasteur first reported butanol production through microbial fermentation. However, the first industrial-scale ABE fermentation started operating in 1916 due to the increased need for acetone in World War I. However, after the war ended in 1918, most of the plants were closed. Then, the oil crisis in the 1970s revived interest in ABE fermentation, and it has been growing ever since [92,93].

The ABE fermentation process is an anaerobic process that typically uses a strain of bacteria from the *Clostridia* genus. These bacteria are characterized by their rod-shaped cell morphology and are gram-positive and capable of forming endospores. Several *Clostridium* species have demonstrated the ability to produce butanol, including *Clostridium acetobutylicum*, *Clostridium beijerinckii*, *Clostridium saccharoperbutylacetonicum*,

Clostridium saccharobutylicum, Clostridium sporogenes, Clostridium pasteurianum, Clostridium carboxidivorus, Clostridium tetanomorphum, and *Clostridium aurantibutyricum* [94]. Strain selection is an important factor for industrial ABE fermentation that affects the product yield and the production cost. The choice of strains depends on their compatibility with the type of raw material, their production of desired products, and their resistance to solvent, low pH, and high temperature [95].

C. saccharoperbutylacetonicum is a mesophilic and strictly anaerobic bacteria, and originally isolated from soil [96]. It is known for its high capacity for n-butanol and hydrogen production and low tendency to form spores, which is desirable for industrial applications. It can also utilize a broad range of substrates, such as glucose, cellobiose, xylose, arabinose, mannose, galactose, pentose, and starch, and tolerates high solvent stress [97,98]. The metabolic pathway of *C. saccharoperbutylacetonicum* resembles that of *C. acetobutylicum*, as shown in **Figure 5.** These pathways involve several steps, enzymes, and intermediates to convert sugars into acetone, butanol, and ethanol. The process begins with the uptake of sugars through Emden-Meyerhof-Parnas (EMP) pathways, a series of enzymatic reactions that transform glucose into pyruvate. Pyruvate is then reduced to acetyl-CoA by pyruvate-ferredoxin oxidoreductase, this pathway also generates reduced ferredoxin, which can be used to produce hydrogen gas by hydrogenase. Acetyl-CoA can enter two different pathways: the acidogenesis pathway or solventogenesis pathway. In the acidogenesis pathway, acetyl-CoA is converted to acetate by phosphotransacetylase and acetate kinase. It can also be reduced to ethanol by alcohol dehydrogenase. In the solventogenesis pathway, acetyl-CoA is first converted to acetoacetyl-CoA by acetoacetyl-CoA transferase and then further to acetone by decarboxylation. Alternatively, acetoacetyl-CoA can be reduced to butyryl-CoA, and then to butyraldehyde using butyraldehyde dehydrogenase, or butyryl-P using phosphotransbutyrylase. Butyryl-P can be converted to butyrate using butyrate kinase. Butyraldehyde is also converted to butanol by NADH-dependent butanol dehydrogenase [95,97]. The balance between the production of these solvents is influenced by various factors such as nutrient availability, the pH of the medium, The NADH/NAD+ ratio, and fermentation conditions.

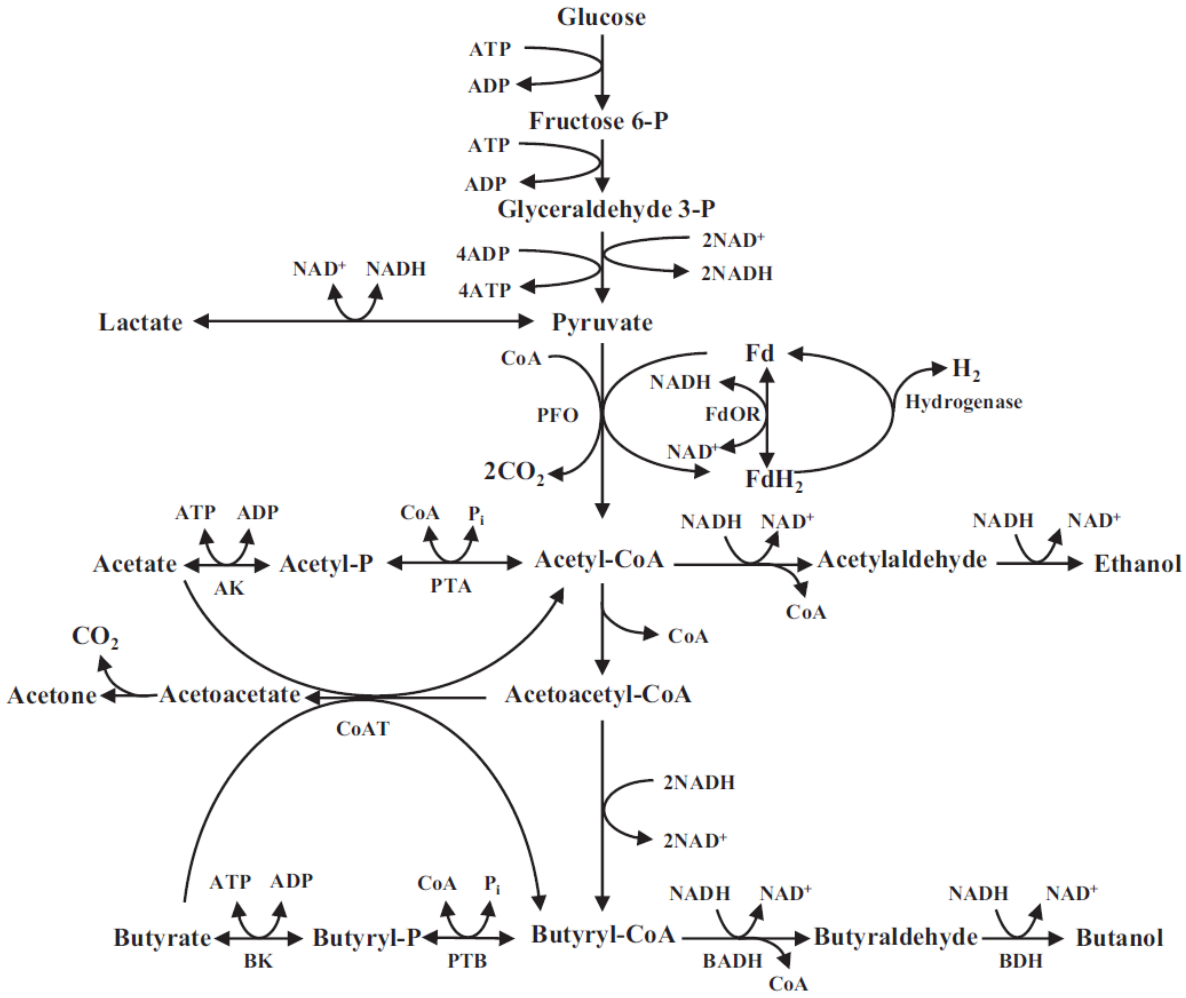

Figure 5. Metabolic pathway of *C. saccharoperbutylacetonicum* [99]. ***Note:*** PFO, pyruvate-ferredoxin oxidoreductase; FdOR, ferredoxin oxidoreductase; AK, acetate kinase; PTA, phosphotransacetylase; CoAT, CoA transferase; BK, butyrate kinase; PTB, phosphotransbutyrylase; BADH, butyraldehyde dehydrogenase; BDH, butanol dehydrogenase. Not all reactions are stoichiometrically correct.

1.5.2 From anaerobic fungi fermentation to chain elongation

Anaerobic fungi are a group of fungi that inhabit the gastrointestinal tract of herbivorous animals and play a crucial role in the digestion of fibrous plant material in the host animal. They belong to the phylum *Neocallimastigomycota*, which lacks mitochondria, the organelles that produce energy in most cells, but they have hydrogenosome, which produce hydrogen gas and ATP [100]. *Neocallimastigomycota* have been categorized in the literature based on morphological characteristics, including the number of flagella attached to their zoospores, the growth form of the rhizoid, and the number of reproductive centers. This classification has identified 20 distinct genera, namely: *Neocallimastix*, *Piromyces*, *Caecomyces*, *Cyllamyces*, *Anaeromyces*, *Orpinomyces*, *Oontomyces*, *Buwchfawromyces*, *Pecoramyces*, *Liebetanzomyces*, *Feramyces*, *Agrisomyces*, *Aklioshmyces*, *Capellomyces*,

Ghazallomyces, *Joblinomyces*, *Khoyollomyces*, *Tahromyces*, *Aestipascuomyces* and *Paucimyces* [101].

Anaerobic fungi can be isolated from the alimentary tract, saliva, and feces of a variety of herbivorous animals [102]. They have a complex life cycle that involves formation of motile zoospores, establishment of rhizoidal networks, and development of sporangia. These stages allow anaerobic fungi to adapt and thrive in the complex environments of the digestive systems of herbivorous animals [103,104]. Anaerobic fungi produce carbohydrate-active enzymes (CAZymes), which are large enzyme complexes playing a key role in breaking down lignocellulosic biomass such as Enset fiber, wheat straw, cellobiose, xylan, cellulose, starch, pectin, chitin, inulin, alginate, maltose, sucrose, and lactose without requiring any pretreatments. Furthermore, these fungi produce hydrogen, ethanol, and various organic acids such as acetate, succinate, formate and lactate as part of their growth cycle [63,101]. Anaerobic fungi offer promising applications for biorefinery conversion of lignocellulosic biomass, as they can utilize the biomass without the need for a treatment step. The metabolites of anaerobic fungi fermentation can be used as industrial chemicals, or further converted into valuable compounds called medium-chain carboxylates, such as caproate and butyrate through a chain elongation process [105].

In chain elongation process, medium-chain carboxylates can be produced from mixed organic waste, food waste, or wastewater using open culture. However, the process may be affected by an unknown group of microorganisms that could break down the substrates and generate undesired products in an unpredictable manner [106]. In other way, *Clostridium kluyveri* can produce caproate and other medium chain fatty acids using acetate, ethanol, succinate, and hydrogen in pure culture fermentation [26]. *C. kluyveri* is a type of bacterium that belongs to the genus *Clostridium*, which is anaerobic, gram-positive, and spore-forming. The first enrichment of *C. kluyveri* from the canal mud in Delft, Netherlands was done by H. A. Barker in 1937. *C. kluyveri* is found in various environments, such as the bovine rumen, silage, and oil reservoirs. It is unique among the *Clostridia* and can elongate volatile fatty acids using ethanol as an electron donor through a reaction known as the reverse β-oxidation pathway.

By merging anaerobic fungi fermentation and chain elongation, Seid et al. [63] developed a novel method to produce caproate from untreated Enset fiber. The method uses *Neocallimastix cameroonii* (G341) and *C. kluyveri* in a single bottle for a two-step fermentation process. This innovative approach allows for the direct conversion of Enset

fiber into caproate without the need for pretreatment or the introduction of external enzymes. **Figure 6** illustrates the two-step fermentation process metabolites involving anaerobic fungi fermentation and chain elongation process. In the two-step process, *N. cameroonii* strain isolated from the feces of zoo animals by Stabel et al. [101]. First, *N. cameroonii* degrades the untreated Enset fiber through the secretion of CAZymes such as cellulosome, which consists of cellulase and hemicellulase. This enzymatic activity leads to the breakdown of complex polysaccharides and produce valuable monosaccharides. These monosaccharides, such as glucose and xylose, are then metabolized through the Embden-Meyerhof pathway to generate energy carrier compounds. Following that, pyruvate metabolism occurs in the cytosol and hydrogenosome, resulting in various metabolic end products, such as acetate, succinate, ethanol, hydrogen, formate, and lactate [107]. After inoculation with *C. kluyveri,* the metabolic pathway enters to the reverse β-oxidation pathway. The cyclic process of the reverse β-oxidation pathway involves the conversion of all substrates into acetyl-CoA, which generates reducing equivalents (NADH) to maintain electron flow. A portion of the acetyl-CoA molecules (one-sixth) undergoes oxidation to form acetate, simultaneously generating ATP. The rest of acetyl-CoA combines with acetyl-CoA cycled within the reverse β-oxidation cycle, leading to the synthesis of butyryl-CoA [31]. Butyryl-CoA can be further transformed into butyrate or immediately cycled again, producing caproyl-CoA, which can be converted into caproate. At the same time, 6 protons are moved through the cell membrane per cycle, which helps to keep the balance of NADH/NAD+ pool for the whole metabolism. This process creates a proton motive force, which helps to use more of the energy made in the cycle (2.5 ATP in total per cycle) [108].

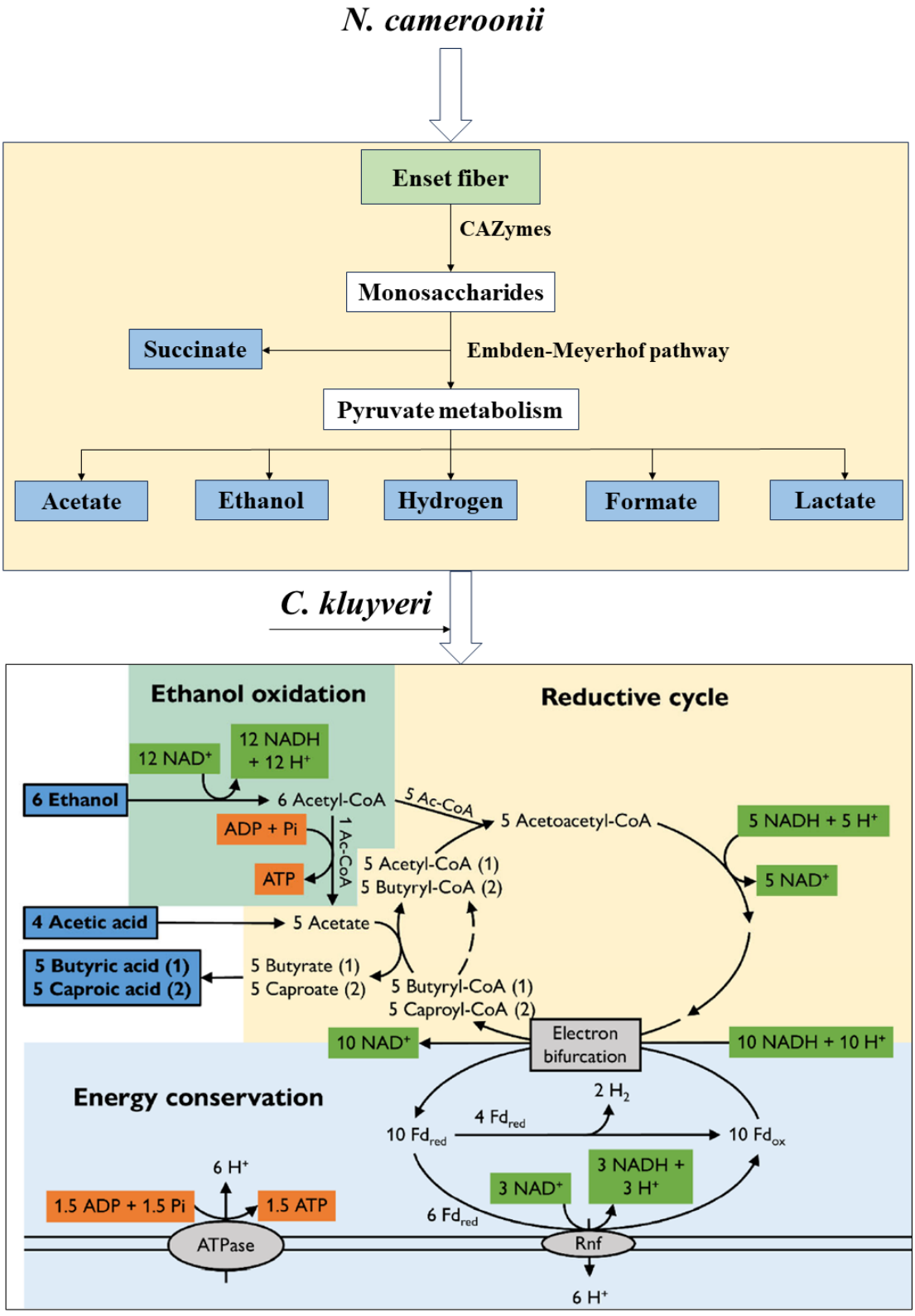

Figure 6. Main metabolites in one-pot two-step fermentation process using *N. cameroonii* and *C. Kluyveri*. Modified from [107,109]. ***Note:*** CoA, coenzyme A; Fdox, oxidized ferredoxin; Fd-red, reduced ferredoxin; Rnf, ferredoxin-NAD reductase complex.

1.6 Scale-up in anaerobic fermentation

Anaerobic fermentation is a metabolic process that occurs in the absence of oxygen and in which microorganisms convert organic compounds into energy and metabolic products. The gas composition of anaerobic fermentation can vary depending on the specific microorganisms involved and the environmental conditions. However, in typical anaerobic fermentation, the primary gases produced include hydrogen, methane, carbon dioxide, and small amounts of other gases such as water vapor, ammonia, and hydrogen sulfide. Scaling up anaerobic fermentation involves expanding the fermentation process from the laboratory scale to the pilot scale and then to a massive industrial scale. The goals of scaling up are to achieve the same level of fermentation performance, maintain similar product yields, and ensure consistent product quality as at the laboratory scale while reducing costs [110].

The fundamental requirement for a successful scale-up lies in establishing similarity between the model and the prototype. Various conditions, such as geometric similarity, dynamic similarity, kinematic similarity, thermal similarity, and chemical similarity, must be met to ensure similarity between the model and prototype [111]. In most cases, achieving geometric and dynamic similarity are the main requirements for scaling up. Geometric similarity means that the shape and the ratio of dimensions between the model and prototype are related by a constant factor, such as the height to diameter ratio, the impeller diameter to reactor diameter ratio, and the number of impellers. Dynamic similarity is another concept which means the flow velocities and forces of each fluid particle in the model and prototype are proportional. It ensures that the flow regimes and turbulence characteristics are similar in both scales. To achieve dynamic similarity, the dimensionless numbers of the model and the prototype should be equal. Several dimensionless numbers are commonly used to assess dynamic similarity and provide insights into the fluid dynamics, heat transfer, and mass transfer. Some relevant dimensionless numbers in stirred tank reactors includes, Reynolds number (Re), Power number (Np) and Froude number (Fr) [112].

The Reynolds number helps predict the type of flow pattern as laminar or turbulent. At low Reynolds numbers, the flow tends to be laminar and smooth, while at high Reynolds numbers, the flow is more likely to be turbulent. It is defined as:

$$Re = \frac{nd^2}{\nu} \quad (1.1)$$

where n is stirrer speed, d is stirrer diameter and ν is kinematic viscosity.

The Power number also known as Newton number is used to quantify the efficiency of an impeller in terms of power transfer to the fluid. Different impeller designs and configurations can result in different power numbers, impacting the overall performance of the mixing equipment. It is expressed as:

$$Ne = \frac{P}{\rho n^3 d^5} \dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots (1.2)$$

where P is impeller power and ρ is liquid density.

The Froude number (Fr) is used to characterize the ratio of inertial forces to gravitational forces in a flowing fluid, which can be relevant for predicting fluid flow patterns and the effectiveness of mixing in the bioreactor during the scale-up process. The Froude number is defined as:

$$Fr = \frac{nd^2}{\mathrm{g}} \dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots (1.3)$$

where g is gravitational acceleration [110,113].

Furthermore, achieving a successful scale-up in anaerobic fermentation requires careful consideration of various theoretical factors, such as mass transfer, heat transfer, thermodynamics, and solubility of gas.

1.6.1 Mass transfer in simultaneous saccharification and fermentation (SSF) process

Mass transfer in fermentation is the process of transferring substances, including substrates, products, or gases, between different phases, such as gas, liquid, or solid, in a fermenter. It is important for the growth and metabolism of microorganisms, as well as the quality and yield of the desired products. The mass transfer phenomenon takes place in the direction of a decreasing concentration gradient through various transfer modes such as diffusion and convection. Mass transfer by diffusion takes place when molecules move randomly in laminar flowing fluids and transport mass through their movement. This phenomenon is driven by, concentration gradient, temperature gradient, or hydrostatic pressure difference. Mass transfer by diffusion can be described by the Fick's law of diffusion, it is a fundamental equation of molecular diffusion to understand how particles is transferred through a medium. It can be written as:

$$J_A = -D_{AB}\frac{dC_A}{dz} \dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots (1.4)$$

where J_A is molar flux of a component A relative to the average molar velocity of all constituents, D_{AB} is diffusion coefficient of component A through B, and dC_A/dz is concentration gradient [112].

Convective mass transfer is a process in which the bulk movement of fluids, where mass transfer occurs between the surface boundary and a moving fluid. Unlike mass transfer by diffusion, convective mass transfer involves the movement of the fluid itself. It can be classified into two types: forced convection and natural convection. Forced convection occurs when the fluid motion is caused by an external force, such as a pump or a fan. Natural convection occurs when the fluid motion is caused by density differences due to temperature or concentration gradients [114,115].

As the scale of the fermentation system increases, mass transfer becomes more challenging and complex, especially in SSF process. SSF is a viable option for production of butanol and hydrogen from lignocellulosic biomass. In this process, enzymatic hydrolysis of cellulose and hemicellulose occurs simultaneously with the fermentation of the resulting sugars into butanol and hydrogen by *Clostridium.* In SSF processes, mass is transferred through the movement of various substances including lignocellulosic biomass, enzyme, nutrient, microorganism, products, and gases between different phases within the bioreactor. The mass transfer paths in the SSF process are shown in **Figure 7**. The SSF mass transfer typically occurs in three primary phases: solid (lignocellulosic biomass), liquid (aqueous phase containing enzymes, nutrient, and microorganisms), and gas (hydrogen and CO_2). The enzymatic hydrolysis of lignocellulosic biomass occurs at the liquid-solid interface. Initially, enzyme molecules migrate from the liquid medium into the lignocellulosic biomass particles through convective mass transfer. Subsequently, the enzymes are adsorbed at active sites on the solid surface and release fermentable sugars from the polysaccharides in the lignocellulosic biomass. This release occurs both on the external surface of the particles and within their pores. After this process, the enzyme molecules desorb from the particles and are ready to initiate the hydrolysis of additional polysaccharides [116]. Once the fermentable sugars are in the liquid phase, microorganisms consume these sugars and produce fermentation products (liquids and gases). In addition, in the SSF process, there is an additional path for gas-liquid mass transfer, which is explained in more detail below. Various factors can significantly affect the mass transfer in the SSF process. Key factors affecting mass transfer in SSF process includes, particle size and solid loading of lignocellulosic

biomass, enzyme loading, temperature, type and size of the bioreactor, agitation, nutrient availability, microorganism, produced gasses, inhibitors, and product formation.

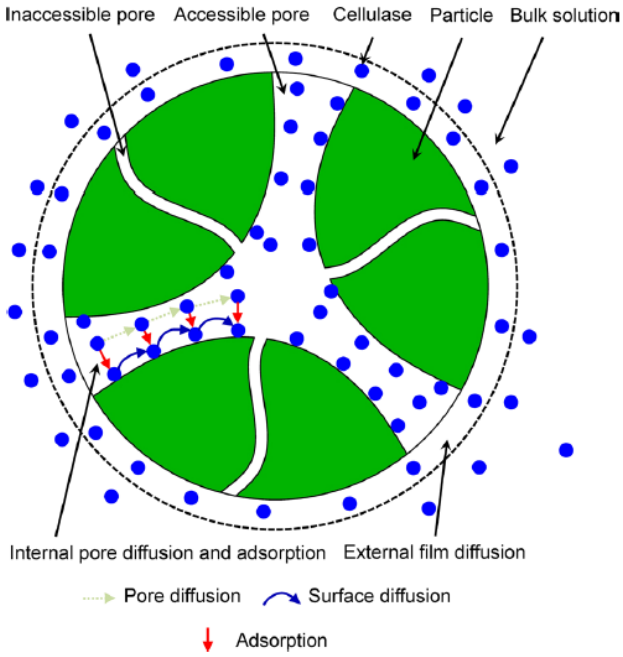

Figure 7. Mass transfer paths in the SSF process [117].

1.6.2 Gas-liquid mass transfer in anaerobic fermentation

In anaerobic fermentation, microorganism produce or consume different gases, such as hydrogen and carbon dioxide. Hydrogen gas plays a pivotal role in facilitating microbial growth and metabolite production, but its solubility in the liquid medium is low and can limit process efficiency. On the other hand, overaccumulation of hydrogen gas in the fermentation broth can have adverse effects on the overall process, and high levels of hydrogen gas affect the ratios of reducing equivalents within the microbial cells and metabolic pathways [118]. Therefore, understanding the gas-liquid mass transfer in anaerobic fermentation is important.

Various theories and models have been developed to explain mass transfer phenomena, including the two-film theory, penetration theory, and surface renewal theory. These theories are based on different assumptions and simplifications, and can provide insights into the mass transfer coefficients, the concentration gradients, the diffusion mechanisms, and the effects of hydrodynamic conditions on the mass transfer rate. Among these theories, the two-film theory is widely used to describe gas-liquid mass transfer in cellular systems due to its simple calculations [119]. The two-film theory assumes that there are thin stagnant films on both sides of the gas and liquid interface in which mass transfer occurs through molecular diffusion (**Figure 8**). The theory also assumes that mass transfer within these films occurs

under steady state conditions [120], and can be used to calculate the overall mass transfer coefficients and the interfacial concentrations.

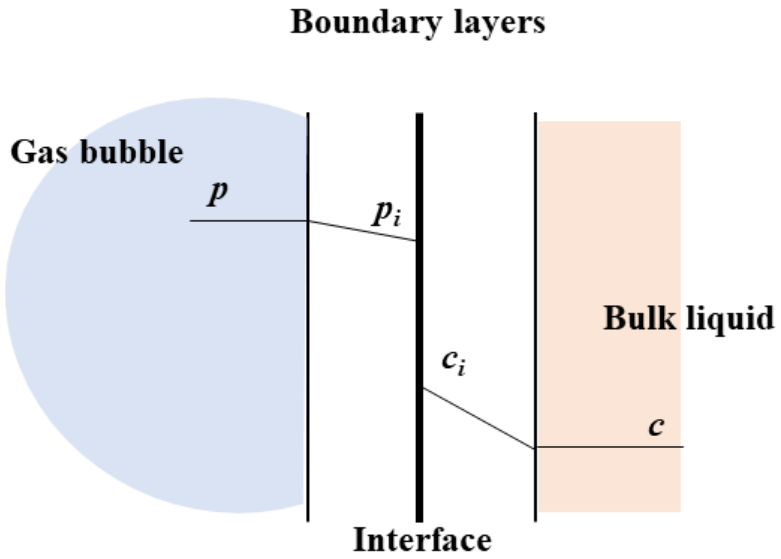

Figure 8. Two-film theory for gas-liquid mass transfer. Modified from [121].

According to the two-film theory, the solute flux from the gas phase to the interface and from the interface to the liquid phase must be equal, and this can be formulated as:

$$J = k_G(p - p_i) = k_L(C_i - C) \quad (1.5)$$

where k_G and k_L are the individual mass-transfer coefficient for the gas and liquid phase respectively, p and p_i are the partial pressure of the gas at the bulk and the interface, respectively, C_i and C are the liquid concentration at the interface and the bulk, respectively, as shown in **Figure 8.**

The concentration of gas and liquid phase at the interface are related by Henry´s law. The law states that the amount of a dissolved gas in a liquid is directly proportional to the partial pressure of the gas above the liquid at a constant temperature.

$$p_i = H^{cp} C_i \quad (1.6)$$

where H^{cp} is the Henry's solubility constant defined by concentration.

In practice, the interfacial values are not directly measurable. Therefore, they are excluded from the equations, and the mass flow is formulated as a function of the concentrations in both bulk phases.

$$J = k_G(p - p^*) = k_L(C^* - C) \quad (1.7)$$

where C^* is the saturation value in the liquid phase according to Henry`s law ($p^*=H^{cp}C^*$). By combining the above equation, the following equation obtained:

$$\frac{1}{K} = \frac{1}{H^{cp}k_G} + \frac{1}{k_L} \quad \text{(1.8)}$$

Since the gas phase film resistance is insignificant compared to the liquid film resistance, the general equation can be simplified as $1/(H^{cp}k_G) << 1/k_L$, and the volumetric mass transfer rate can be written as:

$$Ja = k_L a(C^* - C) \quad \text{(1.7)}$$

where a is the gas-liquid interfacial area per unit of liquid volume [121].

Mass transfer is commonly expressed per unit volume of the bioreactor, rather than per unit interfacial area. Since measuring `$k_L$´ and `a´ separately is challenging, the product k_La is commonly measured as a combined term, representing the mass transfer from gas to liquid. This combined term is referred to as the volumetric mass transfer coefficient. The volumetric mass transfer coefficient (k_La) depends on the physical and chemical properties of the gas and liquid phases, the geometry and operation of the bioreactor, and the intensity and type of mixing. The k_La values for different gases can vary significantly depending on their solubility and diffusivity in the liquid phase. Highly soluble gases, such as carbon dioxide, has high k_La values and thus reach equilibrium between the gas and liquid phases quickly. Poorly soluble gases, such as hydrogen, has low k_La values and thus tend to accumulate in the liquid phase, resulting in overconcentration. The overconcentration of dissolved hydrogen can have significant implications for the anaerobic process, as it affects the thermodynamics and kinetics of the microbial reactions, the gas production and composition, and the stability and performance of the bioreactor. The gas-liquid mass transfer can be enhanced by increasing the gas flow rate, the agitation speed, the gas sparger design, and the use of additives or surfactants to reduce the surface tension of the liquid phase [110,113,122].

1.7 Research proposal

Transitioning from conventional refinery processes to sustainable biorefinery practices holds great promise in addressing energy and environmental problems. However, the search for suitable and sustainable feedstocks for biorefinery processes remains a major challenge. Lignocellulosic biomass is a widely studied renewable source for biorefinery process. Despite this, there are still some barriers that need to be overcome, such as the recalcitrant nature of lignocellulosic biomass, low yield and productivity, and inhibition by degradation products, which make biofuels less competitive than fossil fuels. Moreover, the feedstock is the main cost driver in biorefinery process, which affects the economic viability of the process. Hence, there is a need to find suitable, cheap and non-food competitive feedstock for biorefinery process.

Enset biomass could be a promising feedstock for biorefinery process, however, its potential for biorefinery applications remains largely unexplored. Instead, most researchers have focused primarily on studying the mineral and nutritional content of the Enset plant parts, particularly its suitability as food for humans or animals. Thus, this thesis aims to investigate the processing potential of Enset biomass for biorefinery process, focusing on the production of high-value biofuels and biochemicals. This was addressed through the following chapters:

Chapter 2

- Compositional analysis of Enset biomass parts to identify the most suitable biomass for biorefinery processes,
- Evaluation of dilute alkali and acid pretreatment methods to enhance enzymatic hydrolysis efficiency of Enset biomass,
- Examination of the inhibitory compounds in the Enset biomass parts,
- Investigation of the processing potential of Enset biomass for ABE fermentation using SHF methods.

Chapter 3

- Process optimization of SSF process to maximize butanol and hydrogen production from Enset fiber,
- Investigate the scalability of the SSF process from Enset fiber for industrial scale production,
- Evaluation of the effects of pH control and hydrogen partial pressure on the SSF process from Enset fiber in the bioreactor,

- Development of PSSF process from Enset fiber to improve butanol productivity and mixing efficiency.

Chapter 4

- Evaluation of the growth of *N. cameroonii* on Enset fiber without pretreatment methods,
- Development of a one-pot two-step fermentation process from Enset fiber using *N. cameroonii* and *C. kluyveri* for caproate production

2. The Potential of Enset Biomass as a Substrate for ABE Fermentation

This chapter is based on the publication:

Investigating the Processing Potential of Ethiopian Agricultural Residue Enset/*Ensete ventricosum* for Biobutanol Production

Nebyat Seid, Pia Griesheimer and Anke Neumann (2022)

Bioengineering, 9(4), 133.

DOI: 10.3390/bioengineering9040133

Author Contributions:

Nebyat Seid: Conceptualization, methodology, formal analysis, investigation, resources, data curation, writing–original draft preparation, visualization, funding acquisition.

Pia Griesheimer: Investigation.

Anke Neumann: Conceptualization, methodology, formal analysis, resources, data curation, writing–review and editing, project administration, funding acquisition.

2.1 Introduction

Currently, most industrial and transport sectors rely on petroleum fuels as their main source of energy [123]. However, researchers predict that the global supply of petroleum fuels will be depleted by 2070–2080. In addition, the consumption of petroleum products contributes to global warming and environmental pollution [124]. In developing countries like Ethiopia, the scarcity of energy increases poverty and unemployment [125], and over 80% of energy needs are met by hydropower and biomass production [126]. Moreover, 75% of foreign earnings are spent on importing petroleum fuels [127]. In this context, the rising demand for energy on the planet and our pressing environmental problems can be addressed by better utilizing biofuels [128].

Biobutanol (C_4H_9OH) holds great promise as a biofuel for the next generation. Compared to bioethanol, it produces higher energy per gallon, with a greater heat of combustion and higher-octane numbers. Above all, it mixes better with gasoline without adapting the gasoline engine and is safe to use due to its lower vapor pressure [129–131]. Biobutanol can be produced from sugar, starch, or certain food crops [5], but the production process creates food and energy competition problems primarily due to sudden climate changes, including dry seasons and flooding [132]. Researcher have suggested that lignocellulosic biomass could represent a promising raw material for biobutanol production because of its abundance, high availability, renewability, and versatility [6]. Biobutanol has been produced from lignocellulosic biomass such as barley straw [133], corn stover [134], wheat straw [135], rice hull [136], and sugar cane bagasse [137]. However, the main obstacle to using lignocellulosic biomass as a raw material for biobutanol production is the higher proportion of lignin, which firmly binds cellulose and hemicelluloses, and cannot be used by microorganisms [138]. For the majority of agricultural biomass, the lignin mass fraction is around 10–25% [139], though the lignin mass fraction for Enset biomass is estimated to be lower. Berhanu et al. [49] estimated that the low lignin contents of the Enset fiber and the inflorescence stalk main are 10.53% and 5.72%, respectively. Further going in its favor, the Enset plant is well adapted to growing in many soil conditions, has favorable growth measurements (generally 4–8 m high, sometimes reaching up to 11 m), and high draught resistance [50,140]. The Enset plant could thus represent a promising source of lignocellulosic biomass for biobutanol production.

The Enset plant (*Ensete ventricosum* [Welw.] Cheesman) (false banana) is a herbaceous monocarpic plant in the Musaceae family [47]. It is similar to the banana plant in that it has an underground corm, a bundle of sheaths, and large leaves, but the seedy fruit from the Enset plant is not edible like a banana [48]. The Enset plant is composed of multiple components; the ratio of each component varies with the varieties of the plant. Nurfeta et al. [52]. estimated the ratios of its components to be in the range of 6–16% lamina, 4–21% midribs, 46–60% pseudostem, and 10–30% corm. The Enset plant is a potential food source for about 20 million Ethiopians [141]. In addition, in Uganda, Enset plant parts are used for therapeutic purposes and local beer brewing [142]. A recent study predicted that the crop can be grown more and provide food for an additional 87.2 to 111.5 million Ethiopians, with the potential to expand its farming into sub-Saharan Africa [57]. Kocho, bulla, and amicho are foods made from Enset plants by scraping and fermenting leaf sheaths and corm [143]. In traditional Ethiopian Enset food processing, a massive amount of residual byproducts is discarded; only the Enset fibers from this waste are used, to make bags, ropes, mats and sieves [49]. In a recent study, Erebo, [144] attempted to assess the processing potential of Enset wastes for ethanol production, and showed that ethanol can be produced. The main steps to produce biobutanol from lignocellulosic biomass are pretreatment, enzyme hydrolysis, and ABE fermentation [145]. To date, no comprehensive study has been carried out to evaluate the production process of biobutanol from Enset plant biomass. This study aimed to analyze the composition of the Enset biomass and examine the processing potential for biobutanol production.

2.2 Materials and methods

2.2.1 Raw materials and sample preparation

The samples of Enset biomass were collected from a privately owned Enset plantation in Wolkite, Ethiopia. The Enset plant was selected at random and separated into different parts based on traditional Enset food processing: leaf sheath layers (leaf sheath-1 (LS1), leaf sheath-2 (LS2), leaf sheath-3 (LS3), and leaf sheath-4 (LS4)), upper inflorescence stalk (UIS) and lower inflorescence stalk (LIS), upper corm (UC) and lower corm (LC), leaf sheath peel (LSP), Enset fiber (EF), midrib (M), and leaf (L) (**Figure 9**). Samples were manually chopped into smaller 3–6 cm pieces using a stainless-steel knife. The materials were dried separately in the sun for 4–5 days, pulverized with the knife mill, and sieved with various sieve sizes. The dry powder material was stored in a plastic bag at room temperature.

The sample powder was subjected to composition analysis and pretreatment. The chemicals used in this study were purchased from Sigma-Aldrich Chemie GmbH (Hamburg, Germany) or Carl Roth GmbH + Co. KG (Karlsruhe, Germany).

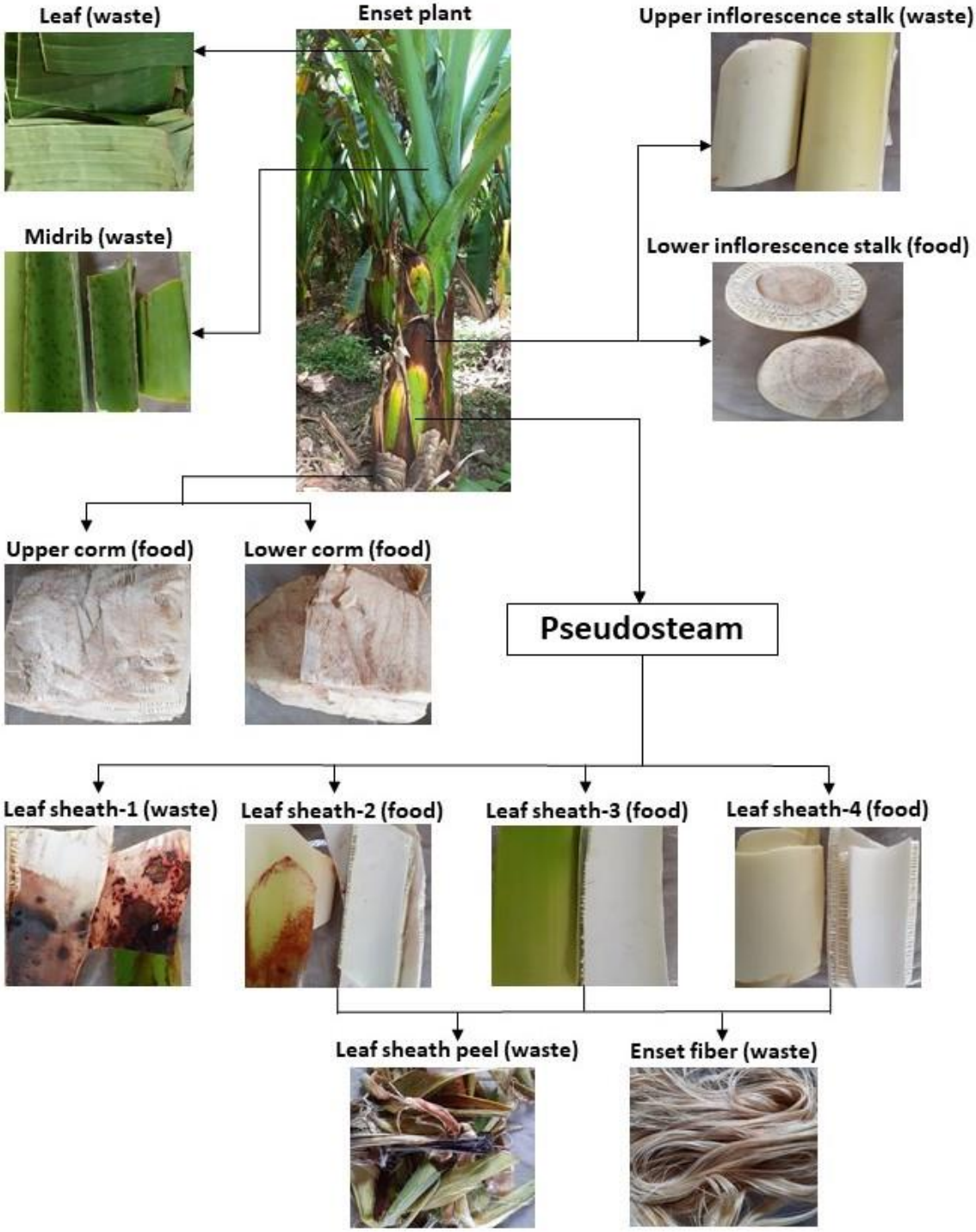

Figure 9. Morphology of Enset biomass based on traditional Enset food processing. (This photo was taken by one of the authors at a private Enset plantation in Wolkite, Ethiopia).

2.2.2 Compositional analysis

The compositions of the Enset biomass parts were analyzed. The acid detergent fiber (ADF), acid detergent lignin (ADL), and neutral detergent fiber (NDF) of the sample were determined by Gesellschaft für Analysentechnik HLS using the ANKOM technology method (ANKOM^{A2000} fiber analyzer) [146–148] at the University of Hohenheim, Stuttgart, Germany. Based on the van Soest method [149], the cellulose content of the sample was determined by subtracting ADL from ADF; the difference between NDF and ADF gives the hemicellulose content, and the lignin content was acid detergent lignin (ADL) [150]. The elemental analysis which is carbon, hydrogen and nitrogen content, moisture content, ash content and calorific value of the Enset biomass samples were determined at the Karlsruhe Institute of Technology (KIT), Institut für Katalyseforschung und -technologie (IKFT) according to the the ISO 16948: 2015, ISO 18134, ISO 18122 and ISO 181251 protocol, respectively. Conversion of the results to a dry reference condition was carried out with the respective analytical moisture contents of the samples. Thus, the parameters of the ash content, calorific value, carbon, and nitrogen were corrected upward. An exception was hydrogen, for which a downward correction was made since the H component from the water (moisture) must first be subtracted from the measured value of the total hydrogen. Waste samples with a high cellulose content were selected for analysis of the monomeric sugar and degradation products of the biomass; analyses were performed according to the National Renewable Energy Laboratory (NREL/TP-510-42623) standard using the two-step hydrolysis method [151]. The monomeric sugar results were corrected with the respective sugar recovery standards.

2.2.3 Pretreatment and enzymatic hydrolysis

The following samples were selected for pretreatment and enzymatic hydrolysis experiments: leaf sheath peel, Enset fiber, Midrib, and mixed Enset waste, which was a mixture of leaf sheath-1, upper inflorescence stalk, leaf sheath peel, Enset fiber, midrib, and leaf. Then, 20 g dried and milled (1 mm particle size) samples were placed in a 500 mL Erlenmeyer flask, mixed with 200 mL 2% (*w/v*) NaOH or 2% (*v/v*) H_2SO_4. The mixture was autoclaved at 121 °C for 20 min. At the end of the autoclave cycle, the samples were cooled, centrifuged at 4700 × *g* for 30 min, and filtered. The filtrate was analyzed for sugars and degradation products. The residue was washed repeatedly with 2 L deionized water, and the pH was adjusted to 5 before it was filtered again using a muslin cloth [152]. According to

the NREL (NREL/TP-510-42621) standard, residue samples were dried at 105 °C for 24 h using a convection oven to determine their moisture content [153], and subjected to enzymatic hydrolysis.

The enzymatic hydrolysis experiment was performed in a 500 mL Erlenmeyer flask, where 5 g (dry weight) alkali- or acid- pretreated biomass was mixed with 100 mL liquid containing 28 FPU/g cellulase enzyme (Cellic CTec2) (Sigma-Aldrich Chemie GmbH, Hamburg, Germany) and 9.6 g/L citrate buffer (pH 5.0) to keep the pH at 5. The experiment was conducted at 50 °C and 130 rpm (Infors Thermotron, Infors AG, Bottmingen, Switzerland) for 72 h [154]. A 1.5 mL aliquot was withdrawn every 12 h, chilled on ice, centrifuged at 10,000 rpm for 10 s, and the glucose concentration was measured. For the control experiment, a wheat straw sample and blank (enzyme without substrate) were used. All experiments were performed in triplicate. The glucose concentration was corrected by subtracting the respective blank controls.

2.2.4 Microorganism and culture maintenance

Clostridium saccharoperbutylacetonicum DSM 14923 was acquired from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The preculture medium was tryptone-glucose-yeast extract (TGY), which contained 30 g/L tryptone, 20 g/L glucose, 10 g/L yeast extract, and 1 g/L cysteine-HCl.H_2O. The strain was regularly maintained in 50% glycerol stocks at −80 °C [155]. The glycerol stocks were prepared according to the method described by Infantes et al. [156], whereby 5 mL culture grown for 13–14 h at 30 °C was placed in a Hungate-type (sterilized and anaerobized) tube, and centrifuged at 3000 × *g* and 4 °C for 5 min. The supernatant was removed from the tube, and 1 mL equal volumes of culture medium and a 50% (*v/v*) glycerol solution were added to the pellet and frozen at −80 °C. For cultivation, 1 mL glycerol stocks were anaerobically revived in a 118 mL serum bottle (Glasgerätebau Ochs, Bovenden, Germany) with 50 mL TGY medium, until the optical density (OD) at 600 nm reached 1.0–2.0.

2.2.5 ABE fermentation

ABE fermentation was performed with mixed Enset waste hydrolysate as a carbon substrate, prepared by enzymatic hydrolysis after alkali pretreatment, and supplemented with 1% (*v/v*) P2 stock medium. The latter contained buffer stock solution (50 g/L KH_2PO_4, 50 g/L K_2HPO_4, and 220 g/L CH_3COONH_4), mineral stock solution (20 g/L $MgSO_4.7H_2O$, 1 g/L $MnSO_4.H_2O$, 1 g/L $FeSO_4.7H_2O$, and 1 g/L NaCl), and vitamin stock solution (0.1 g/L para-

aminobenzoic acid, 0.1 g/L thiamin, and 0.001 g/L biotin) [157]. Next, 48.3 mL hydrolysate was mixed with 1 g/L yeast extract and 1 g/L resazurin, and the pH was adjusted to 6.8 with $NaOH/H_3PO_4$ [158]. The medium was then poured into 250 mL serum bottles, which were sealed with a rubber stopper and an aluminum cap, and anaerobized. The anaerobization process was carried out by flashing the bottles with a mixture of 20% CO_2 and 80% N_2 gas using needles connected to the gas lines. After anaerobization process, 0.2 mL Cys-HCl (100 g/L) was added to the bottles using syringes and needles, and then autoclaved. After autoclaving, 0.5 mL each of sterile-filtered and anaerobic P2 stock solutions was added. The bottles were inoculated with 5% (*v/v*) actively growing culture and incubated for 72 h at 30 °C (Infors Thermotron, Infors AG, Bottmingen, Switzerland) [158]. During the fermentation, 1 mL of a sample was taken for analysis within 8 h, and the pH value of the sample was measured without controlling it. For the control experiment, 40 g/L glucose solution was used as a substrate. All experiments were carried out in triplicate. The overall biobutanol production process from mixed Enset waste is illustrated in **Figure 10.**

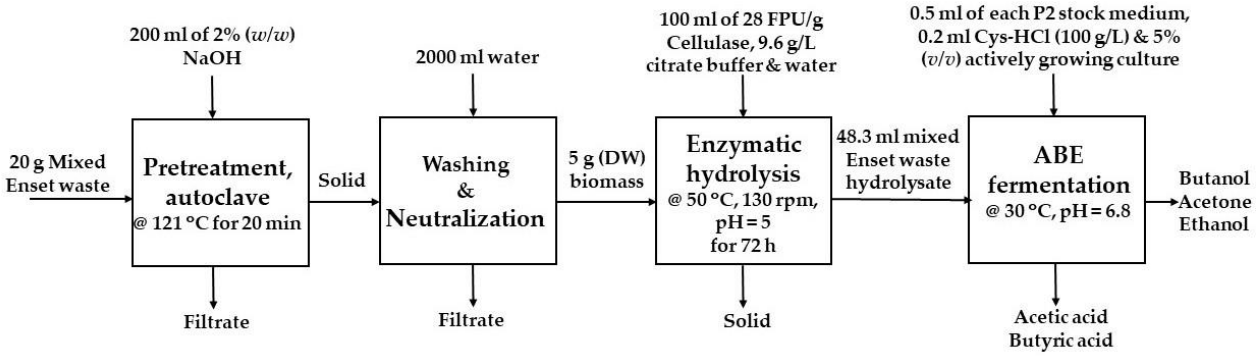

Figure 10. Biobutanol production process from mixed Enset waste.

2.2.6 Analytical methods

Cell growth was measured by taking absorbance measurements (OD_{600}) using an Ultrospec 1100 pro spectrophotometer (Amersham Biosciences, Uppsala, Sweden), and Profilab pH 597 (Xylem Analytics, Weilheim, Germany) was used for pH measurement. The monomeric sugars, degradation products, and fermentation metabolites in the samples were analysed by high-performance liquid chromatography (HPLC) in an 1100 Series System (Agilent Technologies, Waldbronn, Germany), with the column model a Rezex ROA-Organic acid H^+ (8%) and 5 mM sulfuric acid eluent, as described by Stabel et al. [101]. The method was modified with a column temperature of 55 °C and an eluent flow rate of 0.6 mL/min to detect furfural and hydroxymethylfurfural (HMF) [159]. Butyric acid was analyzed separately

using the reversed-phase column Synergi™ 4 μm Fusion-RP 80 Å (150 mm × 4.6 mm) (art. No. 00F-4424-E0, Phenomenex Inc., Aschaffenburg, Germany) at 30 °C column temperature, with eluent compound 20 mM KH_2PO_4 at pH 2.5, and a flow rate of 1 mL/min [160]. The acetone pick in HPLC was overlapped with butyric acid pick; hence, acetone was analyzed with headspace-gas chromatography (GC) using a 6890 N Network GC-System (Agilent Technologies Deutschland GmbH, Waldbronn, Germany) equipped with a flame ionization detector (FID). The chromatographic column was an Agilent FFAP, with capillary as the stationary phase (30.0 m × 320 μm × 0.50 μm nominal). The carrier gas was helium at 1 bar and 3.2 mL/min. The acetone pick was separated by a temperature gradient that was initially held at 40 °C for 2 min, raised at 20 °C/min to 180 °C, and held for 3 min. The headspace GC sample was prepared by adding 100 μL sample into 10 mL serum bottles sealed with butyl septa and screw caps; these contained 0.5 g NaCl, 100 μL 20% (*v/v*) H_3PO_4 and 100 μL 1-propanol as the internal standard. The bottles were heated to 95 °C for 1 h. A gas-tight syringe was used to withdraw 0.5 mL samples from the gas phase and place these into the GC [161].

2.3 Results and discussion

2.3.1 Compositional analysis

The ash content, moisture content, elemental analysis, and calorific value of the Enset biomass parts are shown in **Table 3.** The carbon content of the Enset biomass ranged from 37.1–42.8 %. The majority of the Enset biomass parts had a lower carbon content than the Enset fiber and the leaf, i.e., 41.2% and 42.8%, respectively. The Enset fiber had a relatively similar carbon content to barley straw, at 40.69% [162], but this was lower than that of wheat straw, at 45.58% [163]. The hydrogen content ranged from 5.2 to 6.2%, similar to what is found in most lignocellulose biomasses [162]. The nitrogen was relatively low (<2.5%) in most parts of the Enset biomass except for the leaf (3.3%), though this leaf content would have little impact on the environment during the thermochemical process [164]. Then, the calorific value of most of the Enset biomass parts varied from 14.3 MJ/kg to 17.4 MJ/kg, which was lower than that of banana leaves, at 19.8 MJ/kg [165]. Studies have shown that the biomass used in thermochemical processes must have a calorific value between 17.0 and 22.0 MJ/kg [166]. As it falls below this range, Enset biomass could not be recommended for thermochemical processes. We also noted that the ash content of the Enset biomass parts

varied from 4.7% to 19.1%, which was relatively high compared to other lignocellulosic biomasses [167].

Table 3. Proximate and elemental analysis results for Enset biomass parts.

Analysis	LS1	LS2	LS3	LS4	UIS	LIS	UC	LC	LSP	EF	M	L
Proximate analysis (% dry weight, *w/w*)												
Ash	12.9	7.4	10.8	8.8	19.1	8.5	7.1	4.8	14.4	4.7	15.4	13.7
Moisture	7.4	9.7	9.4	10.3	7.8	9	10.2	10.6	7.7	7.3	6.5	5.7
Elemental analysis (% dry weight, *w/w*)												
C	37.8	38.5	37.9	38.2	37.3	39.1	38.4	38.4	37.9	41.2	37.1	42.8
H	5.5	6.1	6	6.2	5.2	5.9	6.2	6.2	5.5	6.1	5.2	5.7
N	0.5	0.5	0.8	0.6	2.5	1.2	0.6	0.6	0.7	0.3	1.2	3.3
O[a]	43.3	47.5	44.5	46.2	35.9	45.3	47.7	50	41.5	47.7	41.1	34.5
Calorific value (CV) (MJ/kg)												
CV	14.4	15.02	14.7	14.8	14.7	15.3	14.9	14.98	14.62	16.14	14.28	17.4

Note: All experiments were done in triplicate, and the mean is reported here. [a] The percentage of O calculated from the difference between CHN and ash by assuming the sulfur content is small compared to oxygen [168].

Table 4 lists the lignocellulosic composition of Enset biomass parts. The results show that the Enset fiber had a high cellulose content of 67.1%, followed by the midrib and leaf sheath peels, at 40% and 34.1%, respectively. In contrast, the upper and lower corm had lower cellulose contents of 2.2% and 3.8%, respectively. The cellulose content of Enset fiber was higher than that of *pandanus amaryllifolius* fiber (48.8%) [169], wheat straw (34.6%) [164], barley straw (33.25%) [170], corn stover (31.32%) [171], and sugarcane bagasse (54.87%) [172], but quite similar to banana fibers (60–65%) [42] and pineapple leaf fiber (62.5%) [173]. The majority of the Enset biomass had less than 20.4% hemicellulose, except for the leaf, which contained 27%. Furthermore, the Enset biomass parts contained less than 6.8% lignin, which was significantly less than that of most lignocellulosic biomass, which typically have a lignin content of 14–25% [174]. Our findings lead us to propose that Enset biomass can be used to produce biofuels, especially from its fibrous parts, which are an excellent source of fermentable sugars due to their high cellulose content, i.e., the main source of glucose. In addition, the low lignin content of the Enset biomass makes it easier for the pretreatment process to release more fermentable sugars [175].

Table 4. Lignocellulosic composition of Enset biomass parts.

Analysis (% Dry Matter)	LS1	LS2	LS3	LS4	UIS	LIS	UC	LC	LSP	EF	M	L
Cellulose	26.4	5.6	8	6.1	32	6.7	2.2	3.8[a]	34.1	67.1	40	20
Hemicellulose	18.6	10.2	9.5	6.6	20	20	6.2	11.4	15.7	15.6	19.7	27
Lignin	6.8	0.3	0.6	0.5	6.5	1.7	0.4	0.7	6.3	5.1	3.1	3.8

Note: All experiments were done in duplicate and the mean is reported here. [a] The sample was very difficult to grind, which increased the variance in the NDF measurements.

Complete hydrolysis of the cellulose and hemicellulose contents of the biomass is necessary to determine the amount of monomeric sugar in the biomass. Monomeric is key as oligomeric sugars may further break down into other compounds on hydrolysis with concentrated sulfuric acid [151]. **Table 5** shows the composition of sugars and degradation products we found in Enset biomass parts hydrolysate. The cellobiose content of all samples was low 0.8–2.5% (*w/w*), indicating that the oligomeric sugars were completely converted to monomeric forms [176]. High percentages of glucose were found in the Enset fiber (65.4% *w/w*) and leaf sheath peel (56.4% *w/w*) compared to the Midrib (39.1% *w/w*) and mixed Enset waste (45.0% *w/w*). However, the amount of arabinose in the Enset fiber was significantly lower, at 0.93% (*w/w*), than other samples in the range of 2.35–3.28% (*w/w*). Other sugars, including xylose, mannose, and galactose, all had similar amounts of between 10.5% and 12.7% (*w/w*), which were found across all samples. The acetic acid content of Enset biomass samples ranged from 5.0 to 9.0% (*w/w*), while the formic acid was less than 2.24% (*w/w*). All samples contained low levels of furfural, between 0.43 and 1.03% (*w/w*), and HMF was not detected. In this study, further degradation of the hemicellulose to organic acids and a small amount of furfural was observed. The formation of high levels of acetic acid could be due to xylose degradation [177].

Table 5. Sugars and degradation products in liquid hydrolysate of Enset biomass parts after two-step acid hydrolysis. Values are given in % weight per weight dry Enset biomass part.

Compounds [% (w/w)]	Leaf Sheath Peel	Enset Fiber	Midrib	Mixed Enset Waste
Cellobiose	0.8 ± 0.3	2.5± 0.4	1.1± 0.2	1.3±0.4
Glucose	56.4 ± 0.56	65.5 ± 4.73	39.1 ± 2.22	45.1± 0.21
Arabinose	2.4 ± 0.42	0.9 ± 0.16	3.1± 0.65	3.3 ± 0.04
Other sugar (xylose, mannose, and galactose)	11.4 ± 0.61	12.8 ± 0.68	10.5 ± 1.27	10.5 ± 0.84
Formic acid	2.0 ± 0.08	2.2 ± 0.23	2.1± 0.13	1.9 ± 0.12
Acetic acid	5.0 ± 0.46	6.7± 0.57	7.4± 0.55	9.0± 0.7
Furfural	0.8 ± 0.02	1.0 ± 0.04	0.4 ± 0.03	0.7 ± 0.00

Note: All experiments were done in triplicate, and the mean is reported here

2.3.2 Effect of dilute alkali and acid pretreatment method on enzymatic hydrolysis

In this study, an enzymatic hydrolysis experiment was performed to evaluate the effect of the dilute alkali or acid pretreatment method on the glucose release from each Enset biomass part. **Figure 11** shows the glucose concentration results produced from alkali- or acid-pretreated biomass after enzyme hydrolysis with 5% (dry weight) solid loading. In samples pretreated with alkali (**Figure 11A**), after 36 h, the Enset fiber contained 45.8 g/L glucose, while in midrib, the same amount of glucose was found after 48 h. A similar amount of glucose (44 g/L) was found in leaf sheath peel and mixed Enset waste after 72 h. However, in samples pretreated with acid (**Figure 11B**), after 72 h, the Enset fiber glucose level was reduced to 41.5 g/L and the converted glucose from midrib and leaf sheath peel was slightly higher than that of the mixed Enset waste, which was showing as 35 g/L. Overall, 24 h was sufficient to convert 80 to 90% of all alkali-pretreated Enset biomass samples to glucose, while it took 60 h to convert 48 to 80% of the acid-pretreated Enset biomass. One possible explanation could be the influence of the pretreatment process on the structural properties of the Enset biomass. One study showed that despite the enzyme mechanism, various factors influence the enzymatic hydrolysis of lignocellulosic biomass, such as the physical, chemical, and morphological properties of the materials [178]. Zhang et al. [179] investigated the effect of structural features of biomass on enzymatic hydrolysis and found that, in addition to the lignin content, the crystallinity of the biomass was an important factor in reducing the enzyme hydrolysis rate. According to this study, for samples with a low

lignin biomass, those with high biomass crystallinity took longer to complete enzymatic hydrolysis than samples with low biomass crystallinity [179]. Even though the lignin content of Enset biomass was low, acid-pretreated samples did not necessarily lose their crystallinity. It is important to conduct several tests on the structural properties of Enset biomass before and after pretreatment to better understand this material. In our research, with both methods, the glucose concentration in all Enset biomass samples was higher than in wheat straw samples (control), except for the acid-pretreated mixed Enset waste hydrolysate, which had a similar concentration to the acid-pretreated wheat straw hydrolysate.

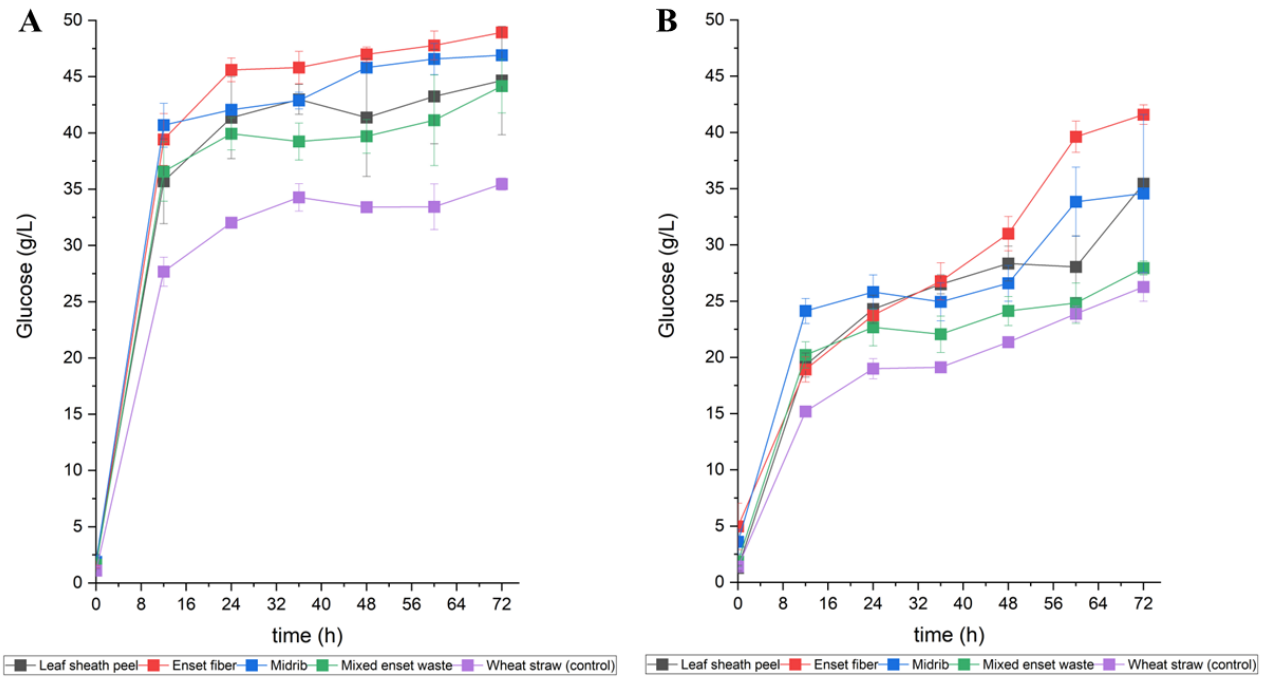

Figure 11. Glucose produced from enzymatic hydrolysis of Enset biomass samples with 5% (dry weight) solid loading, pretreated using different pretreatment methods: (A) alkali pretreatment method; (B) acid pretreatment method.

A comparison of the glucose yield for Enset biomass samples pretreated with dilute acid and alkali after 72 h of enzymatic hydrolysis is shown in **Figure 12.** The percentage of glucose yield was calculated from each pretreated Enset biomass samples. The difference between the acid- and alkali-pretreated Enset fiber and Leaf sheath peel yields was less than 20% (*w/w*). However, in the mixed Enset waste and midrib samples, the alkali-pretreated samples had higher glucose yields by 33% (*w/w*) and 35% (*w/w*), respectively, than the acid-pretreated samples. In this study, the alkali pretreatment method released more glucose than the acid method did for all Enset biomass samples; this was due to the compositional

differences between each Enset biomass sample. Research has shown that the most effective pretreatment methods vary significantly depending on the type of biomass [178]. The alkali pretreatment method enhances cellulose digestibility, which makes it easier to remove lignin from the biomass than with the acid pretreatment method [180]; several lignocellulosic materials, such as corn stover, switchgrass, and Bermuda grass, have been successfully pretreated in this way [152]. However, acid pretreatment method is primarily responsible for eliminating hemicellulosic materials from biomass and releasing sugars, such as xylose and arabinose, into the liquid stream [181]. In this study, we observed that between all of the samples, greater monomeric sugars were found in acid-pretreated than in alkali-pretreated liquids (**Appendix A, Table A1**), showing that the hemicellulose portion was more strongly solubilized than alkali-pretreated samples. Yet, following the washing process, the monomeric sugars were lost from the acid-pretreated samples. When comparing our findings with those for different biomasses from a previous study under similar alkaline conditions and enzymatic hydrolysis, 44.81 g/L glucose was found in switchgrass [152] and 48.68 g/L glucose in corn cobs [182] after 72 h, values that are comparable to the findings for most Enset biomass samples. This shows that Enset biomass could represent a potential raw material for biobutanol production. However, further investigations should be carried out to optimize the enzymatic hydrolysis process.

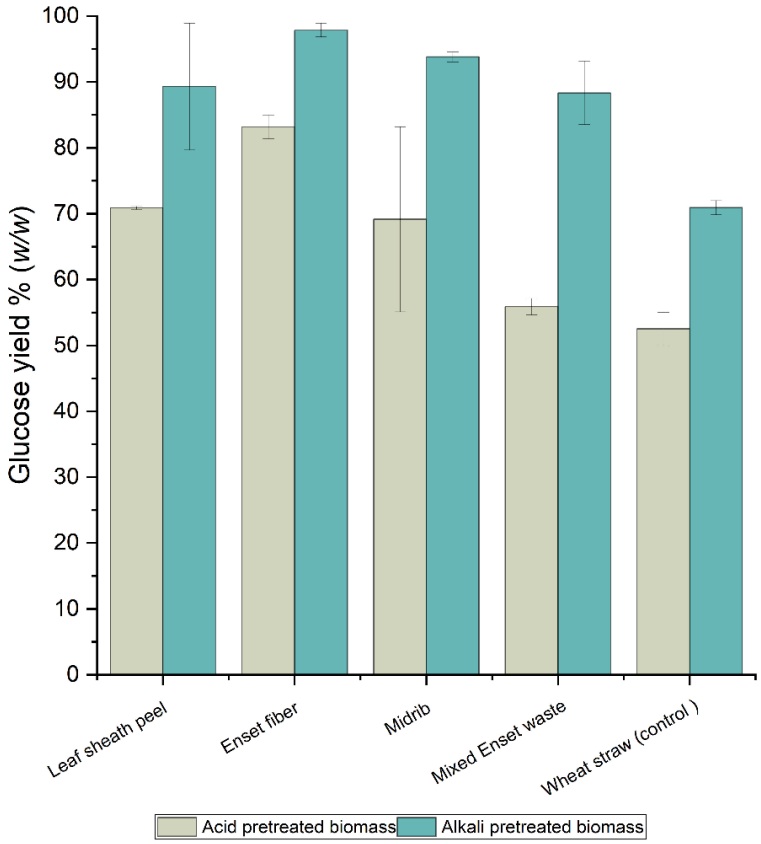

Figure 12. Comparison of the glucose yield after 72 h of enzymatic hydrolysis for acid- and alkali-pretreated Enset biomass samples.

2.3.3 ABE fermentation

ABE fermentation was carried out using alkali-pretreated mixed Enset waste hydrolysate with *Clostridium saccharoperbutylacetonicum DSM 14923*. The fermentation results for mixed Enset waste hydrolysate were compared to a control medium containing pure glucose (40 g/L) as the substrate. The growth profiles of the cells differed when the hydrolysate and pure glucose were used as different carbon sources, but they achieved the same maximum OD (**Figure 13**). Cell growth started 8 h faster in the control than in the hydrolysate medium, and the maximum OD_{600} of 9 was reached after 16 and 48 h, respectively. This could be due to the effect of the preculture medium since the preculture was grown on a glucose medium and it took a while for the cell to adapt to the mixed Enset waste hydrolysate medium. In addition, there might have been growth inhibition by citrate and other sugars in the mixed Enset waste hydrolysate medium (**Figure 14A**). However, after 32 h, the strain converted all of the citrate into acetic acid. Research has shown that during ABE fermentation, acetic acid helps increase the buffering capacity, prevent degeneration, and increase CoA transferase activity [183].

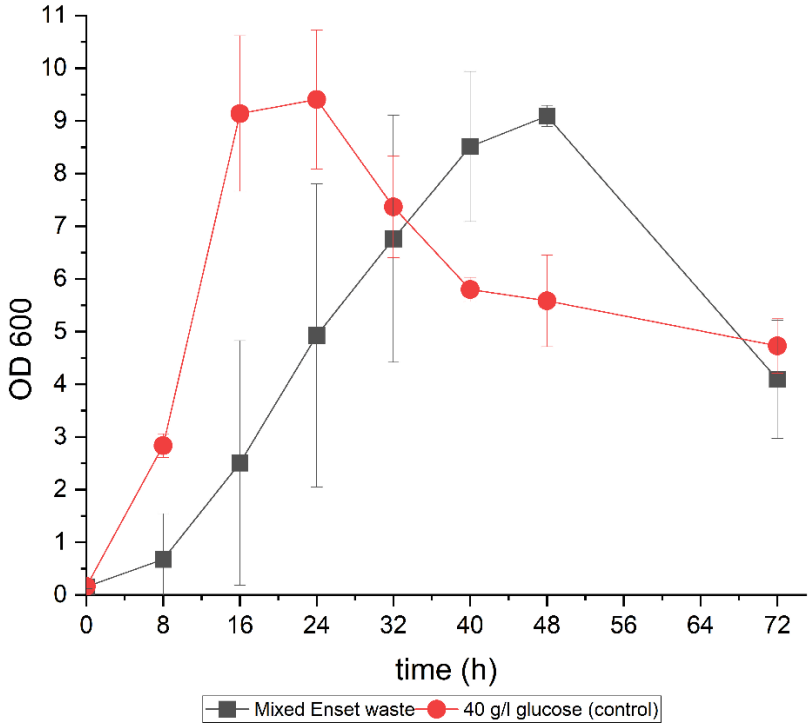

Figure 13. Growth profiles of *C. saccharoperbutylacetonicum* DSM 14923 during fermentation of mixed Enset waste hydrolysate and pure glucose (40 g/L) as a control.

Figure 14 shows the concentrations of ABE fermentation metabolites produced from alkali-pretreated mixed Enset waste hydrolysate and a control medium with 40 g/L glucose using *C. saccharoperbutylacetonicum DSM 14923*. The sugar consumption varied in fermentation depending on the initial sugar concentration; initially, the hydrolysate medium contained 37.8 g/L glucose, 7.5 g/L other sugars (xylose, mannose, and galactose), and 9.6 g/L citrate for the enzymatic hydrolysis process to maintain the pH. After 72 h of fermentation, only 1.9 g/L glucose and 1.5 g/L other sugar remained unused for the hydrolysate. Furthermore, it was observed in this study that all the citrate was consumed by the strain after 32 h. Similarly, after 72 h, 9.9 g/L butanol, 2.8 g/L acetone, and 1.6 g/L ethanol were obtained, achieving an ABE yield 0.32 g/g and productivity of 0.2 g/(L h). After 16 h, 0.6 g/L butyric acid was produced, and this reached 1.5 g/L after 32 h. Overall, 3.4 g/L acetic acid was initially present in the hydrolysate medium and this gradually increased to 9.6 g/L after 32 h (**Figure 14A**). Acetic acid is produced in relatively high amounts, presumably due to the presence of citrate in the medium. It should be noted that after 32 h, the amount of acetic acid produced was quite small. Studies have shown that *clostridial* fermentation of citrate produces acetate and ethanol as the main products, along with negligible amounts of butanol and acetone [184].

During 72 h of fermentation on a glucose control medium, 28.7 g/L glucose was depleted by the culture and 7.8 g/L butanol, 1.6 g/L acetone, and 0.6 g/L ethanol were produced, resulting in an ABE yield of 0.25 g/g and a productivity of 0.14 g/(L h). In addition, during the first 8 h, 0.8 g/L butyric acid was detected, and a maximum of 5.5 g/L acetic acid was found at 24 h (**Figure 14B**). The ABE yield was calculated as the ratio between the total solvents produced and the sugar consumed, showing that the hydrolysate´s yield and thus its productivity were higher than those of the control fermentation, which could be due to the presence of sugars other than pure glucose. Yao et al. [158] reported that *C. saccharoperbutylacetonicum* is capable of utilizing glucose, cellobiose, xylose, arabinose, mannose, and galactose, but the rate depends on the type of sugar used. Additionally, in our research, once cell growth started, the rates of glucose consumption were similar in both cultures, but in the control medium, the cell growth and glucose consumption stopped at around 40 h due to the low pH. In the mixed Enset waste medium, meanwhile, the pH was higher due to the consumption of citrate during the first growth phase, which enabled the complete consumption of glucose. The ABE yield in this study supported the findings of previous studies carried out on the same strain with different biomass, but the ABE productivity was lower than those found in previous studies [152,185]. Mixed Enset waste hydrolysate was utilized by *C. saccharoperbutylacetonicum* without extra detoxification or sugar supplementation. However, further research should be carried out to determine how we can maximize the yield and productivity.

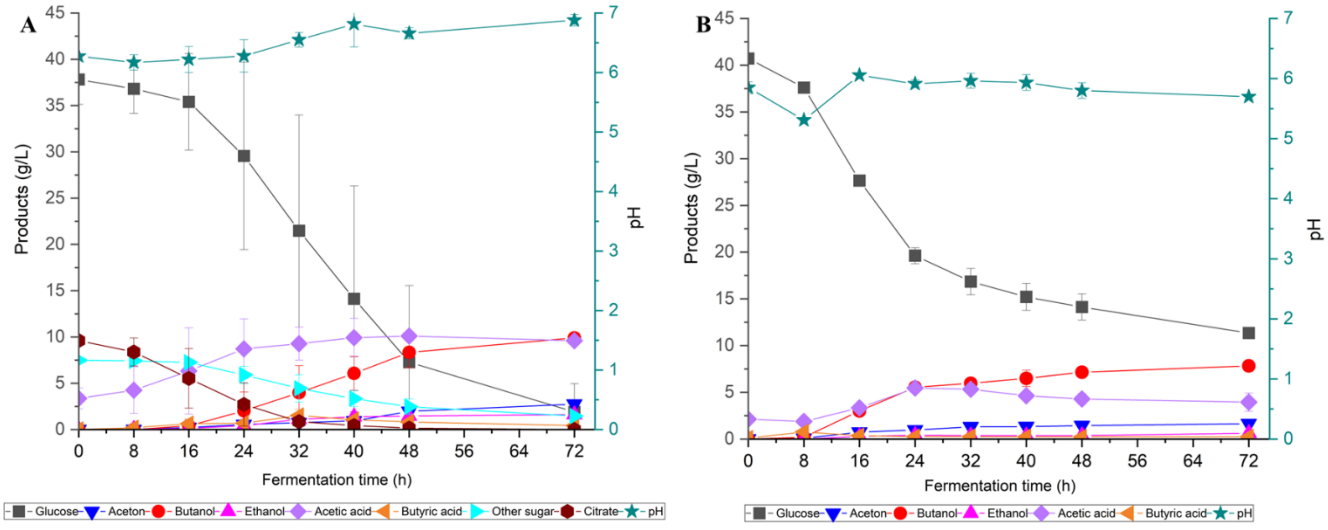

Figure 14. ABE fermentation using *C. saccharoperbutylacetonicum* DSM 14923 from different carbon source: (A) enzymatic hydrolysates of mixed Enset waste, pretreated with 2% (*w/w*) NaOH; (B) control medium with 40 g/L glucose.

2.4 Conclusions

This study found that Enset biomass parts contained high cellulose and low lignin, which contributed to producing a high level of glucose. In addition, low levels of inhibitory compounds were detected in all samples. The alkali-pretreatment method released more glucose from Enset biomass than the acid-pretreatment method. *C. saccharoperbutylacetonicum* utilized mixed Enset waste hydrolysate and produce 9.9 g/L butanol, 2.8 g/L acetone and 1.6 g/L ethanol, achieving an ABE yield 0.32 g/g and productivity of 0.2 $g \times L^{-1} \times h^{-1}$. Enset biomass could represent an ideal candidate for biobutanol production. As part of our ongoing research, we are investigating the possibility of using biological pretreatment methods to reduce biomass loss during acid or alkaline pretreatments while protecting the environment at the same time.

3. Enhancing Hydrogen and Butanol Production from Enset Fiber

This chapter is based on the submitted manuscript:

Stirring the Hydrogen and Butanol production from Enset Fiber via Simultaneous Saccharification and Fermentation (SSF) process.

Nebyat Seid, Lea Wießner, Habibu Aliyu and Anke Neumann (2024)

Manuscript submitted for publication

DOI: 10.21203/rs.3.rs-3836946/v1

Author Contributions:

Nebyat Seid: Conceptualization, methodology, formal analysis, investigation, resources, data curation, writing–original draft preparation, visualization, funding acquisition.

Lea Wießner: Investigation.

Habibu Aliyu: Methodology, writing–review and editing.

Anke Neumann: Methodology, resources, writing–review and editing, supervision, project administration, funding acquisition.

3.1 Introduction

The impacts of human-driven global warming, such as the melting of sea ice, rising sea levels, unpredictable heat waves, wildfires, and intense rainfall, are getting worse day by day. Although some countries have made progress in adapting their climate action plans to achieve net-zero emissions, the situation remains challenging [186,187]. According to the International Energy Agency (IEA) report (2022), CO_2 emissions increased by 6% from 2020 to 2021, the highest emission level recorded for a single year. This increase in emissions clearly indicates that the world is far from achieving the goal of limiting warming to 1.5 °C, as set by the United Nations [187,188]. The goal can be achieved by shifting global energy consumption from fossil fuels to biofuels such as hydrogen and butanol, which can potentially reduce carbon emissions and establish a more sustainable energy system [189]. In recent years, hydrogen has become increasingly popular as an energy carrier for fuelling vehicles, generating electricity, and various industrial applications [190,191]. The high energy content of hydrogen (140 MJ/kg) and the fact that its combustion produces only water as a byproduct make it an ideal clean fuel for various applications [21]. Similarly, butanol is a highly versatile fuel that can be blended with gasoline in high proportions, used in existing engines, and easily transported through current pipeline infrastructure. This versatility arises from its excellent properties, such as higher energy density (29 MJ/L), lower vapor pressure (0.53 kPa), lower hygroscopicity, and lower volatility than ethanol [192,193]. Several studies have shown that biological processes can successfully produce butanol and hydrogen. However, there are still numerous challenges that need to be addressed to make this process competitive with other processes. These includes substrate availability [194], low production yield [190,195], scalability, high processing cost [196,197], and maintaining optimal fermentation conditions [198,199].

Lignocellulosic biomass is readily available and economically viable worldwide and can be obtained from agricultural residues, industrial wastes, forest residues, and municipal wastes [194]. It is composed of cellulose, hemicellulose, and lignin, which are the three components of plant cell walls. These components can be broken down using enzymes and other chemicals, allowing subsequent utilisation by microorganism as a substrate for biofuel production, such as hydrogen and butanol [200]. Enset fiber is Ethiopian agricultural residue obtained from the Enset plant. A large amount of Enset fiber goes to waste after the traditional Enset food processing in Ethiopia, which feeds more than 20 million people [141]. Enset contains 67.1% cellulose, 15.6% hemicellulose, and 5.1% lignin, which makes Enset

fiber a promising substrate for biofuel production due to its high cellulose and very low lignin content [41]. A recent study conducted by Seid N. et al. [41] examined the potential of Enset fiber for butanol production using the separate hydrolysis and fermentation (SHF) method compared with various other Enset plant residues. The results revealed that Enset fiber holds significant promise as a viable resource for butanol production and has a higher hydrolysis conversion efficiency than other Enset plant residues. However, the main challenges for the SHF process were substrate inhibition, low butanol yield and productivity, the complexity of operational procedures requiring multiple distinct steps, and the economic viability of the process [16,201].

Several studies showed that butanol can be produced by combining pretreated biomass, enzymes, and *Clostridia* species in a single bioreactor. This process is called simultaneous saccharification and fermentation (SSF), enables the simultaneous occurrence of sugar release and fermentation [194,202,203]. Compared with the SHF process, SSF has many advantages, including minimising substrate inhibition, reducing operating costs, the overall risk of contamination, and maximising butanol productivity and yield [204]. However, the SSF process faces several challenges that limit its industrial application. These include the different optimal operating conditions for the saccharification process and acetone-butanol-ethanol (ABE) fermentation, which depend on the type of substrate and microorganism used. Therefore, finding a balance between the two processes is challenging and may affect the efficiency and productivity of butanol production [205,206]. ABE fermentation consists of two distinct phases: acidogenic and solventogenic phases. During the acetogenic phase, the strain undergoing exponential growth produces hydrogen, as well as acids such as acetic acid and butyric acid. Due to the accumulation of these acids, cell growth begins to slow down, and the metabolism of the strain shifts into the solventogenic phase. In this phase, the strain produces solvents such as acetone, butanol, and ethanol as a survival mechanism [190,207]. *Clostridium saccharoperbutylacetonicum* DSM 14923 is well known for producing butanol and hydrogen as a sugar fermentation product, making it ideal for biofuel production [208]. The strain is versatile, capable of using a variety of substrates, resistant to solvents, and can thrive in harsh environments [158]. Studies showed that several factors, including temperature, pH, electron flow and reducing equivalents, play a significant role in *C. saccharoperbutylacetonicum* fermentation, influencing the amount of hydrogen and butanol [208–211]. These factors contribute to a competitive relationship between the two products. Therefore, optimal fermentation conditions are crucial for maximising both hydrogen and

butanol production. This study aimed to optimize the process parameters for producing hydrogen and butanol from Enset fiber using the SSF process. We examined the impact of controlling pH on the SSF process from Enset fiber. Finally, we attempted to maximize butanol productivity through the prehydrolysis simultaneous saccharification and fermentation (PSSF) process at a high substrate loading with Enset fiber. It is worth noting that this report is the first study on the simultaneous production of hydrogen and butanol from Enset fiber using the SSF process.

3.2 Materials and methods

3.2.1 Enset fiber preparation

Enset fiber was obtained from a privately owned Enset plantation in Wolkite, Ethiopia. It was ground to a particle size of 2 mm and then pretreated with 2% (w/v) NaOH according to the methods previously described by Seid N. et al. [41].

3.2.2 Bacterial strain and culture medium

Clostridium saccharoperbutylacetonicum DSM 14923 was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). To prepare the inoculum, glycerol stock of the strain (1 mL) was added to 50 mL of anaerobic TGY medium, and the culture was incubated at 30 °C until the optical density (OD_{600}) reached 1.0–1.2. TGY medium contained 30 g/L tryptone, 20 g/L glucose, 10 g/L yeast extract, and 0.4 g/L cysteine-HCl·H_2O. The main fermentation medium comprises 1% (v/v) P2 stock solutions , which was prepared separately, buffer stock solution (50 g/L KH_2PO_4, 50 g/L K_2HPO_4, and 220 g/L CH_3COONH_4), mineral stock solution (20 g/L $MgSO_4 \cdot 7H_2O$, 1 g/L $MnSO_4 \cdot H_2O$, 1 g/L $FeSO_4 \cdot 7H_2O$, and 1 g/L NaCl), and vitamin stock solution (0.1 g/L para-aminobenzoic acid, 0.1 g/L thiamin, and 0.001 g/L biotin) [158]. All stock solutions were filter-sterilized and anaerobized using N_2 gas.

3.2.3 Preparation of SSF medium from pretreated Enset fiber for the bottle experiment

To prepare the SSF medium, different amounts of pretreated Enset fiber were added as the sole carbon source into 250 mL Duran pressure plus bottles, each containing a total volume of 50 mL of medium. Subsequently, 1 g/L of yeast extract, 1 mL of 1 g/L resazurin and 0.4 g/L of cysteine-HCl·H_2O were added, and the initial pH was adjusted to different pH values.

The bottles were sealed using a rubber stopper and an aluminum cap, anaerobized with N_2 gas, and autoclaved. Following autoclaving, 1% (v/v) of each P2 stock solutions and 5 mL cellulase-water mixed solution (Cellic CTec2) (Sigma-Aldrich Chemie GmbH, Hamburg, Germany) at varying cellulase loading were added. The bottles were inoculated with 5% (v/v) active *C. saccharoperbutylacetonicum* culture and incubated in Infors Thermotron incubators (Infors AG, Bottmingen, Switzerland) for 72 hours at different temperatures and agitation speeds. In each experiment, the pressure inside the bottles was measured with a GMH 3100 Series manometer (Greisinger, Mainz, Germany), and samples of 3 mL of gas and 1.5 mL of liquid were collected using needles and syringes. In addition, the saccharification experiment was conducted as a control, and the medium was prepared following the procedure outlined by Seid N. et al. [41].

3.2.4 Influence of SSF process parameters on butanol and hydrogen production in the bottle experiment

Preliminary experiments were carried out to find out which temperature ranges were suitable for the cultivation of *C. saccharoperbutylacetonicum* in the SSF process using Enset fiber at various temperatures, including 28, 30, 33, 35, 37, 40, 45, and 50 °C. It was performed with a substrate loading of 5% (w/v), which was 2.5 g pretreated Enset fiber (50 g/L), a cellulase loading of 24 FPU/g, an initial pH of 6.8 and an agitation speed of 130 rpm.

The influences of agitation speed, cellulase loading and substrate loading on butanol production in the SSF process were investigated at a constant temperature of 30 °C and an initial pH of 6.8. Initially, the effects of agitation were tested at different speeds (0, 100, 130 rpm) while maintaining a constant substrate loading of 5% (w/v) and a cellulase loading of 24 FPU/g. After determining an optimal agitation speed (100 rpm), experiments were conducted to examine the effect of different cellulase loadings (9, 16, 24, 30 FPU/g) while maintaining a constant substrate loading of 5% (w/v). Subsequently, the effects of substrate loading on butanol production were tested at different substrate loadings of Enset fiber (2, 3, 5, 7% (w/v)) while maintaining constant agitation (100 rpm) and cellulase loading (16 FPU/g). Finally, a validation experiment was performed under optimal conditions with an agitation speed of 100 rpm, a cellulase loading of 16 FPU/g and a substrate loading of 5% (w/v). As a control experiment, optimization of the saccharification process was performed in a similar manner to determine how changes in process parameters affect the efficiency of enzymatic hydrolysis.

In addition, the influence of temperature, initial pH, and hydrogen partial pressure on hydrogen production in the SSF process were examined under optimal process parameters, which included an agitation speed of 100 rpm, a cellulase loading of 16 FPU/g, and a substrate loading of 5% (w/v) Enset fiber. First, to study the effects of temperature on hydrogen production, bottles were placed in incubators set at various temperatures (30, 35, 37 °C) while maintaining a constant initial pH of 6.8 in closed bottles. Following the selection of the best temperature (37 °C) for hydrogen production, the effects of initial pH were examined by adjusting the SSF medium to the initial pH (5.0, 6.0, 7.0, 8.0, and 9.0) while maintaining a constant temperature. Afterwards, the effects of hydrogen partial pressure were determined at a constant temperature of 37 °C and an initial pH of 8.0. The following experimental setup was established to investigate the effect of hydrogen partial pressure on hydrogen production, the 250 mL bottles containing the fermentation medium were connected to empty bottles (anaerobized 1-L and 2-L bottles) by tube. This setup allowed the hydrogen-rich gas produced by fermentation to flow from the smaller bottles to the larger ones. All experiments were performed in triplicate and lasted 72 hours.

3.2.5 Bioreactor setup for the SSF process from pretreated Enset fiber

A 2.5 L bioreactor (Minifors, Infors HT, Bottmingen, Switzerland) with two six-bladed Rushton turbine impellers was used for the SSF process. The impellers had a diameter of 4.6 cm and a width of 1.1 cm and positioned 6.0 cm apart on the stirrer. For the SSF medium preparation in the bioreactor, 50 g of pretreated Enset fiber with a substrate loading of 5% (w/v) was added to the bioreactor as the sole carbon source, and the medium had a total volume of 1 liter. The bioreactor was filled with liquids containing 1 g/L yeast extract and 1 mL of 1 g/L resazurin and autoclaved. After autoclaving, all tubes and the bioreactor were sealed airtight and anaerobized overnight using filter-sterilized N_2 gas while being mixed at a stirring speed of 500 rpm. After anaerobization, the bioreactor was supplemented with 1% (v/v) of each P2 stock solution and 0.4 g/L of cysteine-HCl·H_2O using needles and syringes. For the main fermentation, the temperature was adjusted to the desired level, and the stirring speed was reduced to 150 rpm. Subsequently, the N_2 gas flow was stopped, 10 mL of a cellulase-water mixed solution (Cellic CTec2) with a cellulase loading of 16 FPU/g was added to the bioreactor and inoculated with 5% (v/v) active *C. saccharoperbutylacetonicum* culture. 5 mL gas and 2 mL liquid samples were collected and analyzed.

The bioreactor setup varied depending on the type of fermentation. For butanol production, fermentations were performed at a temperature of 30 °C, with an initial pH of 6.8 and under overpressure, with the gases partially kept in the bioreactor. The bioreactor outlet tube was connected to the pressure relief valve (V07, IMI Norgren, Lichfield, United Kingdom) and set to an absolute pressure of 1.55 bar. After the pressure reached 1.55 bar, the valve was opened, and the gases produced were passed through BlueVCount (BlueSens gas sensor GmbH, Herten, Germany) to measure the volume of gases accumulated in the gas bags. In addition, for the pH-controlled experiment, the pH of the medium was controlled at pH ≥ 5.0 by using the bioreactor's base pump containing 4 M NaOH. For hydrogen production, fermentation was performed at 37 °C, with an initial pH of 8.0 and under atmospheric pressure, releasing the gas from the bioreactor. In this fermentation, the bioreactor outlet tube was directly connected to the BlueVCount and accumulated in the gas bags. All experiments were incubated for 120 hours and performed in duplicate.

3.2.6 PSSF process from pretreated Enset fiber in the bioreactor

The PSSF medium was prepared similarly to the SSF medium except for the substrate loading, which was 7% (w/v) of pretreated Enset fiber (70 g/L), and operating conditions. Before inoculation with an active preculture of *C. saccharoperbutylacetonicum*, the media was hydrolyzed for 2 hours. The prehydrolysis experiment was started by adjusting the pH to 5.0 and the temperature to 40 °C. Then, the cellulase enzyme was added at 16 FPU/g cellulase loading and stirred at 500 rpm. Afterwards, the temperature and stirrer speed were minimized to 30 °C and 150 rpm, respectively, and the initial pH was adjusted to 6.8, then inoculated with 5% (v/v) active preculture of *C. saccharoperbutylacetonicum*. During the fermentation, the pH was controlled with 4M NaOH to pH ≥ 5.0 and the pressure to 1.5 bar. The experiment was incubated for 120 hours and performed in duplicate.

3.2.7 Analytical methods

The liquid samples were analysed following the method described by Seid N. et al. [41]. The gas pressure developed during fermentation was measured using a manometer, and the gas composition was determined using the Micro GC Fusion® gas analyzer (Inficon, Bad Ragaz, Switzerland) equipped with PLOT and WCOT columns and utilizing Argon and Helium as carrier gases [63]. The volume of gas generated from the bioreactor was measured using BlueVCount. The total moles of the gas were calculated by summing the moles of gas within the bioreactor and the moles of gas passing through the BlueVCount.

3.3 Results

3.3.1 Effect of SSF process parameters on butanol production

This work investigates process parameters to improve butanol production from Enset fiber using the SSF process. Important parameters, such as agitation speed, cellulase loading and substrate loading were selected and optimized. The preliminary results showed that *C. saccharoperbutylacetonicum* fermentation produced metabolites only between temperatures of 28 °C and 37 °C; however, no metabolites were detected above 37 °C. Unless otherwise stated, all subsequent experiments were carried out at a constant temperature of 30 °C and an initial pH of 6.8. **Table 6** shows the effect of agitation speed on butanol production in the SSF process and glucose production in the saccharification process (control experiment) from Enset fiber. After 72 hours of the SSF process, a maximum butanol concentration of 11.48 g/L was produced at an agitation speed of 100 rpm compared to other fermentations. At this speed, the strain consumed almost all glucose, resulting in a butanol yield of 0.24 g/g and productivity of 0.16 g/(L h). However, at 130 rpm, butanol production was lower (10.33 g/L), and 9.48 g/L glucose remained unconsumed. The saccharification process showed that the enzyme released more glucose (44.57 g/L) at 130 rpm than at other speeds after 72 hours.

Table 6. Effect of agitation speed on butanol production in the SSF process and glucose production in the saccharification process in bottles [a, b]

	SSF process				Saccharification process	
Agitation speed (rpm)	**Final glucose concentration (g/L)**	**Butanol concentration (g/L)**	**Butanol yield (g/g)**	**Butanol productivity (g/(L h))**	**Glucose released (g/L)**	**Maximum glucose production rate (g/(L h))**
0	7.93 ± 1.12	9.64 ± 0.97	0.19	0.13	37.13 ± 0.14	2.93
100	0.24 ± 0.17	11.48 ± 0.22	0.23	0.16	38.94 ± 1.99	3.29
130	9.48 ± 1.36	10.33 ± 0.08	0.21	0.14	44.57 ± 1.01	2.96

[a] All calculations accounted for 2.5 g Enset fiber in 50 mL medium at 72 hours fermentation period; [b] Values are means from triplicate bottles.

In addition, the effects of cellulase loading on butanol production from Enset fiber were investigated. **Table 7** presents the effects of different cellulase loadings on butanol production in the SSF process and glucose production in the saccharification process from Enset fiber. The result showed no significant difference in butanol production in the SSF process between 16 and 24 FPU/g, while 9 FPU/g cellulase loading resulted in a slightly lower butanol production of 10.73 g/L. The saccharification process released a higher glucose concentration of 47.47 g/L at 30 FPU/g cellulase loading, but the SSF process showed the lowest butanol concentration of 9.6 g/L and a yield of 0.19 g/g.

Table 7. Effect of cellulase loading on butanol production in the SSF process and glucose production in the saccharification process in bottles [a, b]

	SSF process				Saccharification process	
Cellulase loading (FPU/g)	**Final glucose concentration (g/L)**	**Butanol concentration (g/L)**	**Butanol yield (g/g)**	**Butanol productivity (g/(L h))**	**Glucose released (g/L)**	**Maximum glucose production rate (g/(L h))**
9	3.73 ± 0.34	10.73 ± 0.29	0.21	0.15	29.67 ± 0.33	1.11
16	4.99 ± 0.18	11.23 ± 1.28	0.22	0.16	39.47 ± 0.62	1.85
24	0.24 ± 0.19	11.48 ± 0.22	0.23	0.16	38.94 ± 1.99	3.29
30	6.82 ± 1.42	9.60 ± 0.59	0.19	0.13	47.47 ± 0.75	3.21

[a] All calculations accounted for 2.5 g Enset fiber in 50 mL medium at 72 hours fermentation period; [b] Values are means from triplicate bottles.

The effects of substrate loading with Enset fiber on butanol production in the SSF process were also examined. **Table 8** shows how substrate loading affects butanol production in the SSF process and glucose production in the saccharification process from Enset fiber. The results showed that the same butanol concentration of 11 g/L was observed at both 5 and 7% (w/v) substrate loading of Enset fiber. However, at 7% (w/v) substrate loading, the butanol yield was much lower (0.16 g/g), and 12.41 g/L of glucose remained unfermented compared to other substrate loadings. On the other hand, higher butanol yields were achieved at 2 and 3% (w/v) substrate loadings despite lower butanol concentration and productivity. In the saccharification process, the highest glucose concentration (44.74 g/L) was released at a

substrate loading of 7% (w/v), but the glucose production rate was slower than other substrate loadings.

Table 8. Effect of substrate loading on biobutanol production in the SSF process and glucose production in the saccharification process in bottles [a, b]

	SSF process				Saccharification process	
Substrate loading (% (w/v)	**Final glucose concentration (g/L)**	**Butanol concentration (g/L)**	**Butanol yield (g/g)**	**Butanol productivity (g/(L h))**	**Glucose released (g/L)**	**Maximum glucose production rate (g/(L h))**
2	0.01 ± 0.00	5.23 ± 0.14	0.26	0.07	16.02 ± 1.05	1.87
3	0.01 ± 0.00	7.96 ± 0.17	0.27	0.11	25.44 ± 1.54	1.83
5	4.99 ± 0.18	11.23 ± 1.28	0.22	0.16	39.47 ± 0.62	1.85
7	12.42 ± 0.43	11.06 ± 0.09	0.16	0.15	44.74 ± 1.78	1.64

[a] All calculations accounted for different substrate loading of Enset fiber in 50 mL medium at 72 hours fermentation period; [b] Values are means from triplicate bottles.

Overall, at the end of fermentation, the validation experiment confirmed that under the optimum process parameters, including 5% (w/v) substrate loading, 16 FPU/g cellulase loading, and 100 rpm agitation speed, the SSF process from Enset fiber resulted in a maximum butanol concentration of 11.36 g/L, with a corresponding yield of 0.23 g/g and a productivity of 0.16 g/(L h). In addition, 4.33 g/L acetone and 0.71 g/L ethanol were produced, while 1.77 g/L glucose, 0.41 g/L acetic acid, and 1.08 g/L butyric acid remained unconsumed (**Figure 15A**). The validation experiment in the saccharification process also showed an almost similar glucose concentration (37.4 g/L) after 72 hours. However, the maximum glucose production rate of this experiment was slightly faster than the previous one at 2.34 g/(L h) (**Figure 15B**).

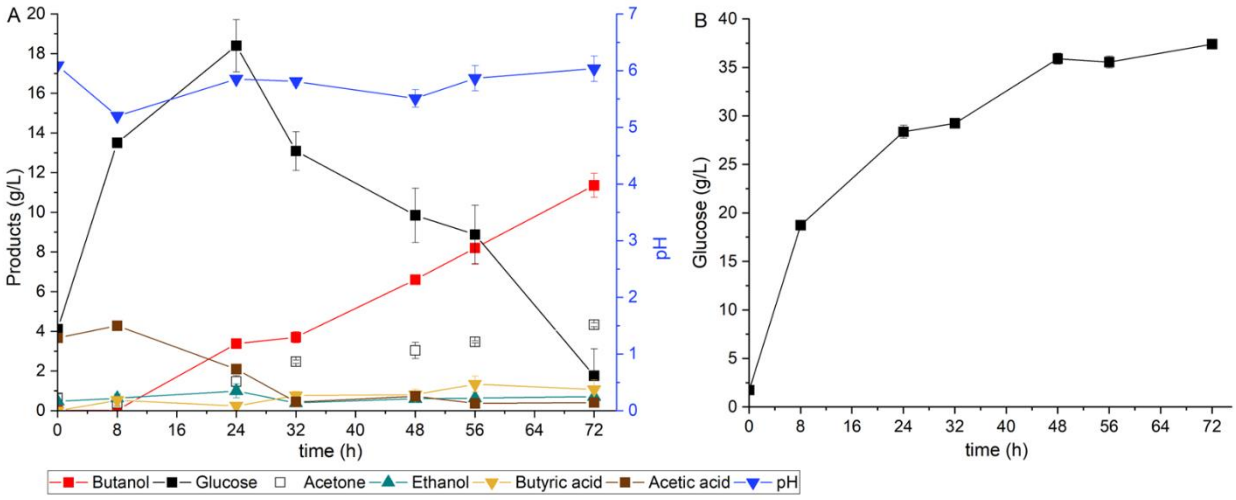

Figure 15. Validation experiment at 5% substrate loading, 16 FPU/g cellulase loading, and 100 rpm (A) SSF process, (B) saccharification process**.** All values are means from triplicate bottles.

3.3.2 The impact of controlled and uncontrolled pH values in the SSF process on butanol production

This study aimed to establish a small-scale SSF process for butanol production from Enset fiber in a 2.5 L bioreactor and to investigate the influence of pH control on the process. All fermentations were carried out under the optimal conditions of 5% (w/v) substrate loading, 16 FPU/g cellulase loading, a temperature of 30 °C, and an initial pH of 6.8, as determined from the previous bottle experiments, except for the stirrer speed, which was 150 rpm. The SSF process was scaled up in a 2.5 L stirred tank reactor with a pressure relief valve that maintained the hydrogen-rich gas in the medium. The bioreactor pressure reached an absolute pressure of 1.55 bar in about 5 hours; after that, the valve opened and regulated the pressure, while the excess gas was released through BlueVCount and collected in a gas bag. The pressure dropped to 1.52 bar after 24 hours of fermentation and remained constant until 54 hours. Then, the pressure fluctuated between 1.23 and 1.48 bar until the end of fermentation. The influence of pH control on butanol production in the SSF process was examined using the same bioreactor setup, and the results were compared with those of pH-uncontrolled fermentation. **Figure 16A &B** shows the metabolites and pH profiles in the SSF process from Enset fiber with the pH-controlled and pH-uncontrolled fermentations. The pH value in both fermentations decreased from 6.8 to 5.0 within 8 hours of inoculation. In the pH-controlled fermentation, the base pump was turned on after 8 hours to keep the pH above 5.0; however, after 19 hours, no addition of NaOH was observed. Within 19 hours,

the pH of this fermentation reached a peak pH of 5.96 and then decreased to 5.53 after 68 hours. Afterwards, it fluctuated between 5.51 and 5.60. In contrast, the pH-uncontrolled fermentation showed a slight increase in pH to 5.29 after 19 hours and a gradual decrease to 4.83 after 68 hours, where it remained constant until the end of the fermentation.

In addition, a comparison was made between the pH-controlled and pH-uncontrolled SSF processes regarding their effects on liquid metabolites. In both fermentations, as shown in **Figure 16A&B**, the enzyme released a maximum glucose concentration of about 8 g/L after 8 hours, which was similar to the time when the pH was lowered to 5.0. Following that, a higher glucose concentration was observed in the pH-uncontrolled fermentation until the end of the fermentation than in the pH-controlled fermentation. Similarly, other sugars such as xylose, mannose and galactose were detected in higher concentrations in the pH-uncontrolled fermentation than in pH-controlled. In both fermentations, the strain began producing butanol around 19 hours. However, the pH-uncontrolled fermentation had a faster production rate of 0.23 g/(L h) than the pH-controlled one. It also had a higher butanol concentration of 10.54 g/L after 72 hours compared to 8.67 g/L in the pH-controlled fermentation. At the end of fermentation, butanol concentration was increased to 11.35 g/L, yielding 0.23 g/g in the pH-uncontrolled fermentation. However the productivity decreased to 0.095 g/(L h) compared to a fermentation time of 72 hours. Likewise, in the pH-controlled fermentation, the butanol concentration increased to 9.86 g/L, yielding 0.20 g/g and productivity of 0.082 g/(L h). In addition, both fermentations produced other solvents, such as acetone and ethanol. However, the difference in acetone production between the pH-controlled and pH-uncontrolled fermentations was insignificant at 3.61 g/L and 3.00 g/L, respectively, and ethanol was detected in small amounts in both fermentation, which was lower than 0.5 g/L. The acid compositions, such as acetic acid and butyric acid, also differed in both fermentations. Acetic acid and butyric acid levels were higher in the pH-controlled fermentation than in the pH-uncontrolled fermentation. As with butanol production, hydrogen production showed the opposite trend when comparing pH-controlled and pH-uncontrolled fermentations. A higher amount of hydrogen was observed in the pH-controlled fermentation at 308.02 mmol than in the pH-uncontrolled fermentation at 246.47 mmol.

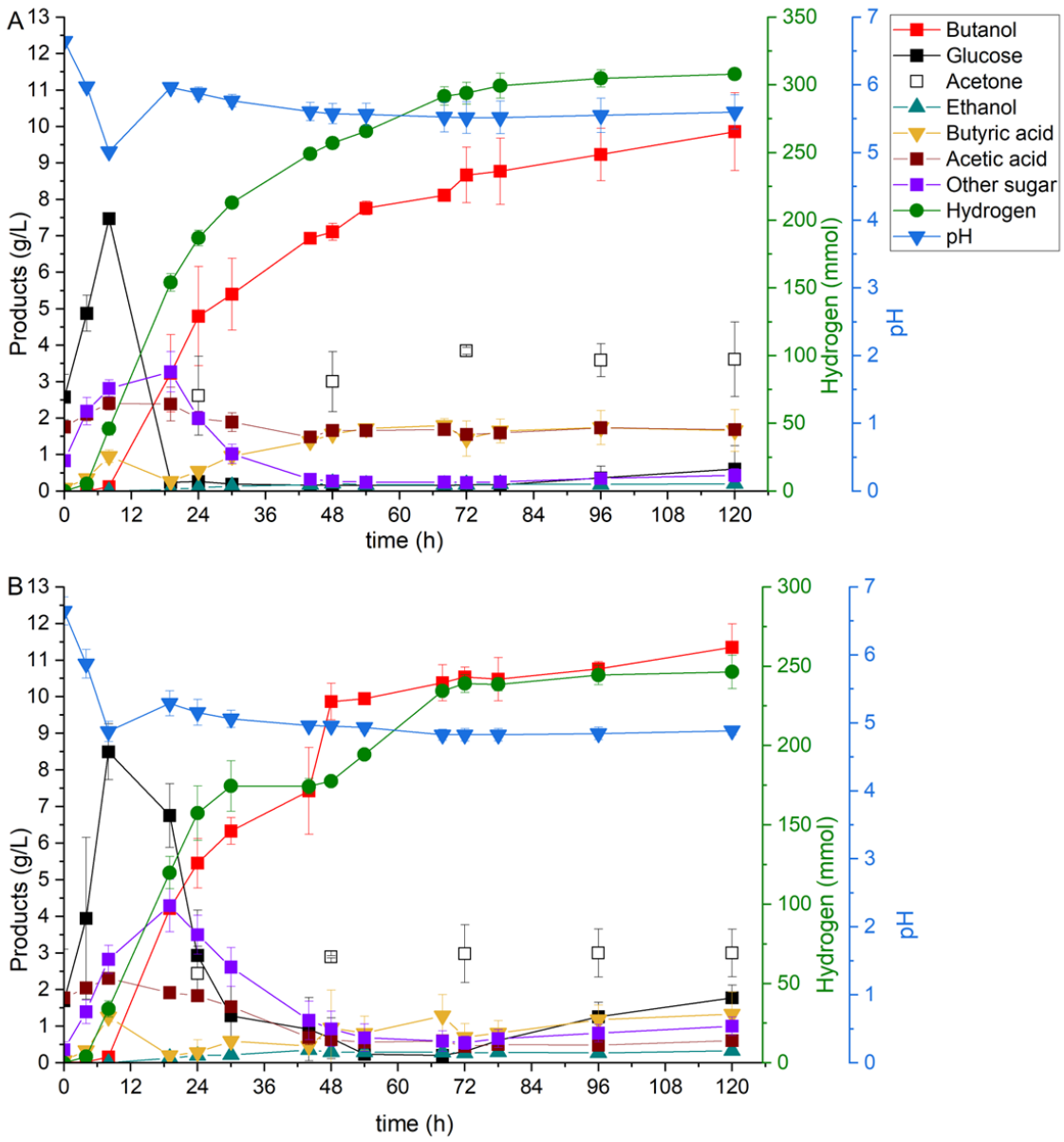

Figure 16. Metabolites in the SSF process from Enset fiber with (A) pH-controlled and (B) pH-uncontrolled**.** All values are means from duplicate fermentations in the bioreactor.

3.3.3 PSSF process at high substrate loading with Enset fiber

The purpose of this study was to maximize butanol productivity using the PSSF process at high substrate loading with Enset fiber. This experiment was done in a bioreactor with pH-controlled fermentation similar to the experiment described above. **Figure 17** shows the metabolites produced in the PSSF process at a substrate loading of 7% (w/v) using Enset fiber as a carbon source. The prehydrolysis step lasted for 2 hours, followed by a 1 hour transition period to adjust the temperature and initial pH value, and the enzyme hydrolysed the Enset fiber into 12.53 g/L of glucose and 2.66 g/L of other sugars, such as xylose,

mannose, and galactose. In addition, we observed that the Enset fiber was partially degraded, and the medium was homogenized at the end of this process. After inoculation with *C. saccharoperbutylacetonicum*, the PSSF process achieved a faster butanol production rate of 0.21 g/(L h) than the pH-controlled SSF process but a slightly similar rate to the pH-uncontrolled SSF process. However, the butanol concentration after 72 hours was higher at 11.04 g/L in the PSSF process than in the SSF process in both pH-controlled and uncontrolled fermentation. At the end of fermentation, the butanol concentration and productivity in the PSSF process were increased to 12.84 g/L and 0.104 g/(L h), respectively. Although the butanol concentration and productivity were higher in the PSSF process than in the SSF process, the butanol yield of 0.18 (g/g) was significantly lower than in the SSF process. Furthermore, the PSSF process produced a higher concentration of acetone (5.64 g/L) and a higher amount of hydrogen (378.69 mmol) than the SSF process. However, at the end of the fermentation, we found that 4.51 g/L glucose, 2.58 g/L acetic acid and 1.37 g/L butyric acid remained unused.

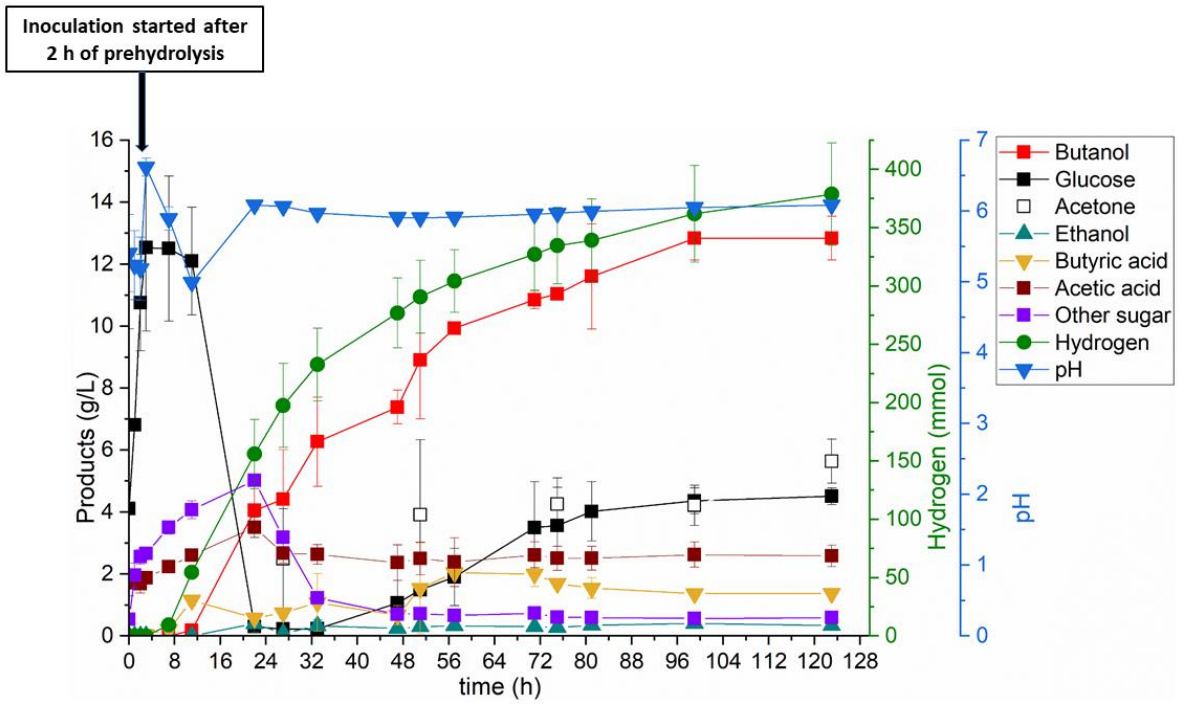

Figure 17. Metabolites in the PSSF process at high substrate loading with Enset fiber**.** All values are means from duplicate fermentations in the bioreactor.

3.3.4 Effect of SSF process parameter on hydrogen production

The objective of this study was to examine the influence of SSF process parameters on hydrogen production from Enset fiber. The key process parameters affecting hydrogen

production were selected and optimized, including temperature, initial pH, and hydrogen partial pressure. At the same time, other factors were kept constants for all experiments based on results from previous bottle experiments. **Figure 18** shows the effect of temperature on hydrogen production from Enset fiber in the SSF process. After 44 hours of incubation, at both 35 °C and 37 °C, hydrogen production reached a maximum of 15.75 mmol and then slightly declined at both temperatures. At the end of fermentation, hydrogen production was lower at 30 °C (12.03 mmol) than at other temperatures. However, the butanol yield and productivity were much higher at 30 °C, with 0.23 g/g and 0.17 g/(L h), respectively. In contrast, at 37 °C, the butanol yield was lower at 0.17 g/g, while the hydrogen yield was higher at 133.50 mL/g-Enset fiber compared to others (**Appendix B, Table B1**).

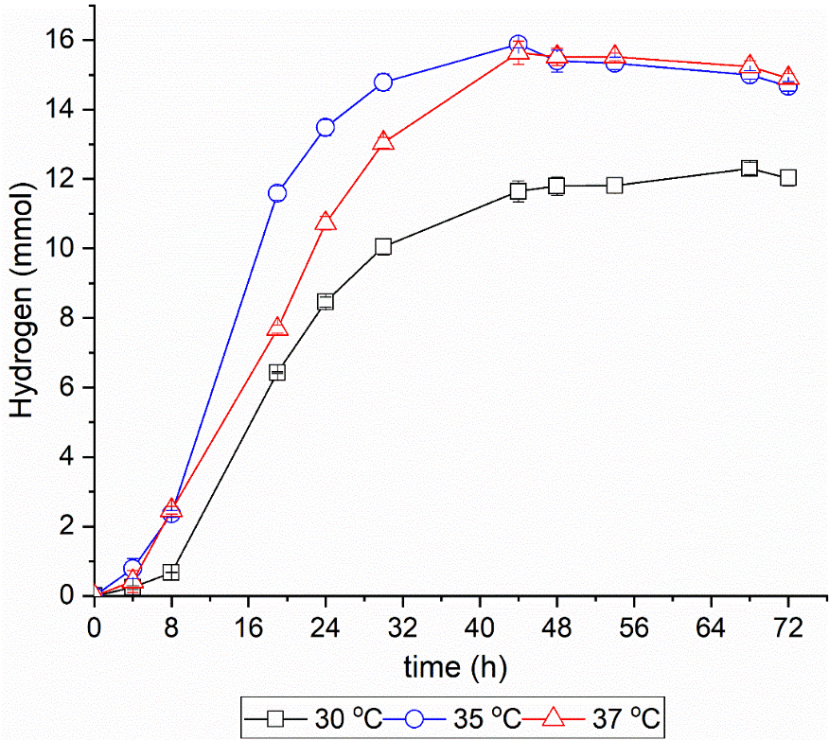

Figure 18. Effect of temperature on hydrogen production in the SSF process**.** All values are means from triplicate bottles.

In addition, the effects of initial pH value on hydrogen production from Enset fiber were investigated. **Figure 19A** shows the pH changes during the SSF process with Enset fiber at different initial pH values. The initial pH of 6.0, 7.0, and 8.0 showed similar pH trends, decreasing at 8 hours, and increasing to about 6.23 after 24 hours. At the end of fermentation, they had nearly the same pH of 6.01, except for the initial pH of 6.0, which was slightly lower at 5.76. In contrast, the initial pH of 5.0 and 9.0 showed similar pH trends, dropping below 5.0 after 8 hours and remaining low until the end of fermentation. Similarly, the initial

pH values affected the amount of hydrogen produced by the SSF process, as shown in **Figure 19B**. After 44 hours of fermentation, the highest hydrogen production was observed at 16.32 mmol at initial pH values of 7.0 and 8.0. However, the initial pH of 8.0 had a slightly faster rate of 0.53 mmol/h than the others. At the end of fermentation, a similar hydrogen yield (139 mL/g-Enset fiber) was observed at all initial pH values of 6.0, 7.0 and 8.0. The lowest amounts of hydrogen were found at both initial pH of 5.0 and 9.0, with 1.74 mmol and 5.33 mmol, respectively. Similar to the amount of hydrogen, there was no significant difference in butanol yield (0.18 g/g-Enset fiber) between the initial pH of 6.0, 7.0, and 8.0 at the end of fermentation. In contrast, at the end of fermentation, the lowest butanol yield was observed at initial pH of 5.0 and 9.0; however, a significantly higher glucose concentration of 38 g/L and 30 g/L were observed at an initial pH of 5.0 and 9.0, respectively (**Appendix B, Table B2**).

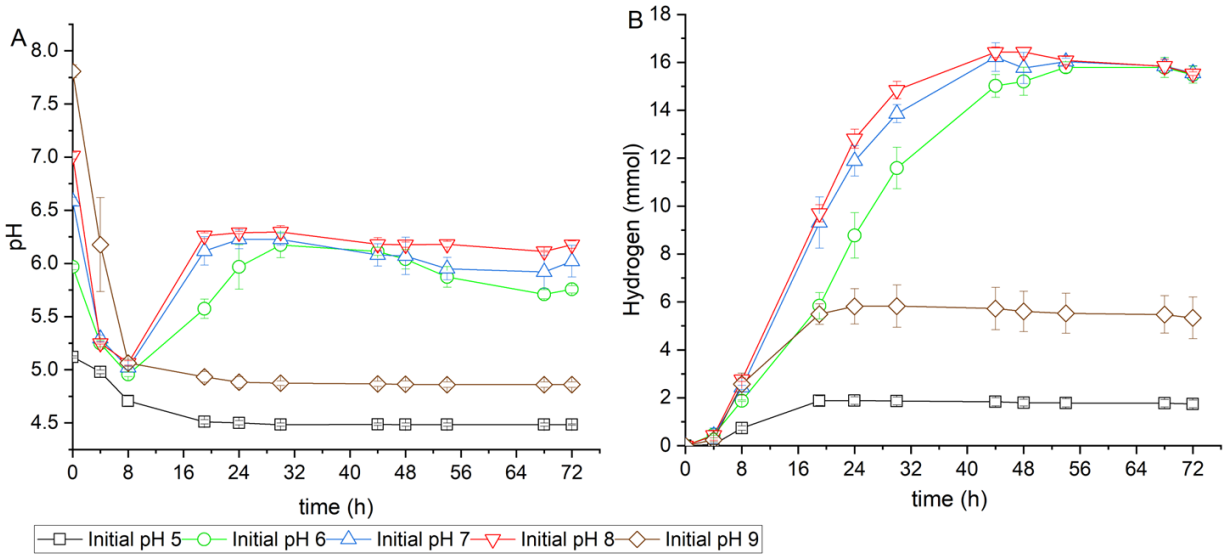

Figure 19. Effect of initial pH values on hydrogen production in the SSF process from Enset fiber (A) pH profile and (B) hydrogen production. All values are means from triplicate bottles.

The effects of hydrogen partial pressure on hydrogen production in the SSF process from Enset fiber were also investigated. **Figure 20A&B** shows the hydrogen partial pressure and hydrogen production in the SSF process from Enset fiber under different gas release strategies. In both experiments, where gas was released into 1-L and 2-L bottles, the hydrogen partial pressure was lower compared to the control experiment without gas released. However, the gas released into the 1-L bottle resulted in a slightly lower hydrogen partial pressure than that released into the 2-L bottle **(Figure 20A)**. Similarly, at the end of

fermentation, there was a slight difference in hydrogen production of 17.24 mmol and 18.86 mmol between the fermentation, where the gas was released into 1-L and 2-L bottles, respectively. However, a smaller amount of hydrogen (15.52 mmol) was observed in the control experiment **(Figure 20B).** Furthermore, it was found that there was little difference in butanol production between all experiments (**Appendix B, Table B3**). Overall, at the optimum process parameters, including a temperature of 37 °C, an initial pH of 8.0, and the lowest hydrogen partial pressure, a maximum hydrogen yield of 168.99 mL/g-Enset fiber was achieved in the SSF process from Enset fiber.

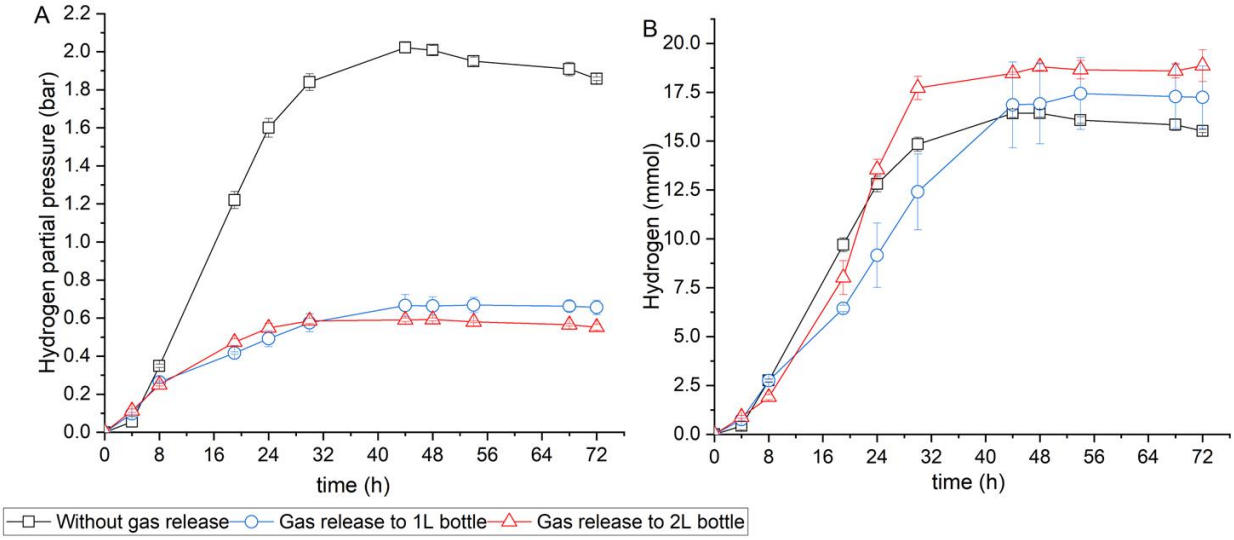

Figure 20. Effect of gas release strategy in the SSF process from Enset fiber (A) hydrogen partial pressure (B) hydrogen production. All values are means from triplicate bottles.

3.3.5 Hydrogen production in the SSF process at atmospheric pressure

This study aimed to develop a small-scale SSF process for hydrogen production from Enset fiber in a 2.5 L bioreactor. This was achieved by using optimal process parameters, including temperature (37 °C), initial pH (8.0), and atmospheric pressure as determined in bottle experiments. The SSF process was scaled up using a 2.5 L stirred tank reactor operated at atmospheric pressure and without a pH control method. Initially, the outlet tube was closed for 4 hours to maintain an anaerobic environment in the bioreactor. During this period, the strain produced its gas, and the pressure was increased to an absolute pressure of 1.3 bar. Following this, the bioreactor outlet was connected to the BlueVCount, which released gases into the gas bag, thereby reducing the pressure to the atmospheric level of 1 bar**. Figure 21** shows the metabolites and pH profiles in the SSF process from Enset fiber at atmospheric

pressure. The result of this study was compared to a similar SSF process, except that the SSF was performed at 30 °C, with an initial pH of 6.8, without pH control and under overpressure (see **Figure 16B**). Unless otherwise stated, this was the reference process for the comparison. After inoculation, hydrogen gas production was started in 4 hours in both fermentations, and the amount was similar at 3.64 mmol. However, after that, the hydrogen production rate in the SSF process at 37 °C was faster, with a maximum production rate of 8.38 mmol/h, than in the SSF process at 30 °C with 6.56 mmol/h. Further, after 72 hours, a higher amount of hydrogen was found at 428.53 mmol in the SSF process at 37 °C than in the SSF process at 30 °C with 239.14 mmol. However, hydrogen production did not change significantly when the fermentation time was extended to 120 hours. Both fermentations showed a slight increase in hydrogen production with 442.57 mmol and 246.47 mmol for the SSF processes at 37 °C and 30 °C, respectively, resulting in relatively low hydrogen productivity 1.65 $mLg^{-1}h^{-1}$ and 0.92 $mLg^{-1}h^{-1}$, respectively. In addition, a higher hydrogen yield of 198.27 mL/g-Enset fiber was achieved at the end of fermentation in the SSF process at 37 °C compared to the yield of 110.42 mL/g-Enset fiber at 30 °C.

Furthermore, the pH profiles and liquid metabolites of the SSF processes showed different results at different temperatures and initial pH values (**Figure 21** and **Figure 16B**). The pH of both fermentations decreased to 5.0 in the first 8 hours of fermentation and then increased slightly above 5.0 in the next 19 hours. After that, the pH value in the SSF process at 37 °C remained above 5.0 and fluctuated between 5.5 and 5.76, while the pH value in the SSF process at 30 °C dropped again for about 44 hours and varied between 4.82 and 4.96. At the end of fermentation, the SSF process at 37 °C had a higher pH of 5.6 than the SSF process at 30 °C, which had a lower pH of 4.89. Similarly, the glucose concentration released by the enzyme after 8 hours of fermentation was the same in both SSF processes (8.2 g/L). However, after 19 hours, the SSF process at 37 °C reached a maximum glucose concentration of 15.54 g/L, while the SSF process at 30 °C did not increase further. At the end of fermentation, the strain consumed almost all of the glucose in the SSF process at 37 °C, while 1.77 g/L glucose remained unused at 30 °C. Moreover, both SSF processes had a maximum of 4.25 g/L of other sugars, such as xylose, mannose and galactose, which were also consumed by the end of fermentation. At the end of fermentation, the liquid metabolites such as butanol and acetone also differed depending on the temperature and initial pH value. The SSF process at 30 °C produced more butanol (11.35 g/L) than the SSF process at 37 °C (8.92 g/L). However, the opposite was observed for acetone, which was higher in the SSF

process at 37 °C (6.04 g/L) than the SSF process at 30 °C (3.00 g/L). In addition, the SSF process at 37 °C contained more acetic acid and less butyric acid than the SSF process at 30 °C.

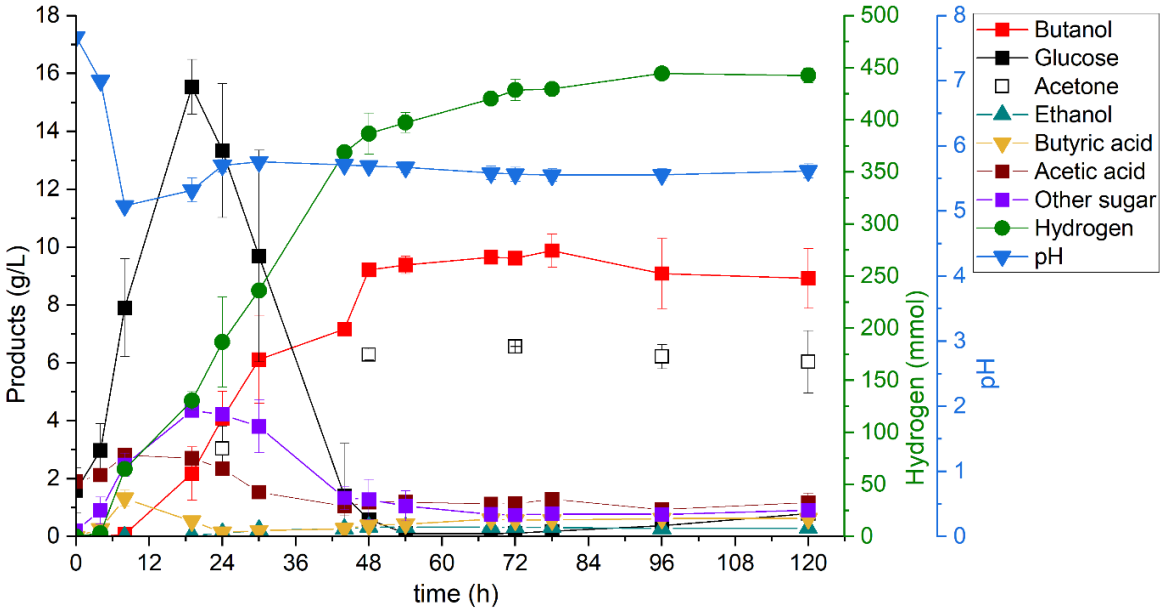

Figure 21. Metabolites in the SSF process from the Enset fiber SSF process at atmospheric pressure. All values are means from duplicate fermentations in the bioreactor.

3.4 Discussion

3.4.1 Maximizing butanol production from Enset fiber using the SSF process

The SSF process is a widely recognized method for enhancing product yield, minimizing equipment and energy costs, and offering the advantage of faster processing time compared to conventional methods [16]. However, one of the primary challenges in maximizing butanol production in the SSF process using *C. saccharoperbutylacetonicum* is the large temperature difference required for ABE fermentation and saccharification, typically between 30 °C and 50 °C [212]. The cellulase enzymes require higher temperatures of 40–50 °C to efficiently degrade lignocellulosic biomass [213], while the strain prefers lower temperatures of 30 °C for optimal butanol production [158]. This makes it challenging to choose an optimal temperature for the SSF process to maximize butanol production. In this study, we observed that *C. saccharoperbutylacetonicum* was able to grow and produce butanol from pretreated Enset fiber using the SSF process within a temperature range of 28 to 37 °C, and the maximum butanol production was observed at 30 °C. This could be due to

the structural properties of Enset fiber, which has a high cellulose content (67.1%) and a low lignin content (5.1%), making it a promising feedstock for the SSF process [41]. Lignin is a natural polymer that acts as a barrier to prevent the enzymatic hydrolysis of cellulose and hemicellulose. It can be removed by various pretreatment methods such as dilute acid pretreatment, dilute alkali pretreatment, steam explosion, and liquid hot water; however, depending on the biomass and the specific type of pretreatment, the pretreatment methods can lead to lignin degradation. The resulting lignin degradation products, such as aromatic alcohols, phenolic compounds, furan derivatives, and organic acids, act as soluble inhibitors that can affect enzyme activity and strain growth [214,215]. Previous studies have shown that the SSF process is affected by inhibitory compounds resulting from the degradation of lignin in alkali-pretreated switchgrass [202] and acid-pretreated corn stover [216], which inhibits the *clostridial* strain. However, alkali-pretreated Enset fiber did not generate any inhibitory compounds, according to a study [41].

In this study, the effects of other process parameters, such as agitation speed, cellulase loading and substrate loading on the SSF process were further optimized while maintaining a constant temperature (30 °C) and initial pH (6.8), which were found to be the optimal parameters for ABE fermentation using *C. saccharoperbutylacetonicum* [158]. Mixing the SSF medium improves the mass transfer between biomass, enzyme, microorganism, and nutrients in the SSF process. However, agitation speed affects the ABE fermentation and saccharification process differently. The bottle experiments in the current study revealed that the SSF process produced the highest butanol at an agitation speed of 100 rpm. However, higher rotation reduced the butanol concentration by 10% in the SSF process. On the other hand, the saccharification process benefited from high rotation; more glucose was produced at higher agitation speeds, possibly due to the enhanced interaction between the enzyme and the Enset fiber. The results of this study were similar to those of Al-Shorgani N. et al. [217], who investigated the effects of agitation on butanol production in ABE fermentation using the same strain as in this study. They found that 100 rpm was the optimal agitation speed for maximum butanol production and at higher speeds, butanol production decreased.

Maintaining an optimal cellulase loading during the SSF process is essential for maximizing the butanol production and ensuring the overall cost-effectiveness of the process. In this study, it was observed that there was only a 4% difference in butanol yield between the cellulase loadings of 16 and 24 FPU/g, despite a 33% increase in the cellulase loading. For this reason, a cellulase loading of 16 FPU/g was selected as an optimal point for the SSF

process from Enset fiber. Study shows that enzymes contribute to 40% of the total cost of biofuel production from lignocellulosic biomass [218] and reducing cellulase loading is economically beneficial when the yield loss is less than 6–7% [212]. Further increasing cellulase loading to 30 FPU/g in the SSF process resulted in a 16% decrease in butanol production. This could be due to the inhibitory effect of excessive cellulase on the strain [219]. On the other hand, in the saccharification process, higher cellulase loading was beneficial for releasing more glucose from Enset fiber. Compared to the previous study, this result was similar to the research conducted by Md R. et al. [206], which investigated the effect of cellulase loading in the SSF process using the *C. acetobutylicum* strain and reported a reduction in butanol production from pretreated oil palm empty fruit bunch as the cellulase loading increased from 20 to 30 FPU/g.

One of the challenges in butanol production from lignocellulosic biomass using the SSF process is balancing the required fermentable sugars and substrate loading, which can lead to mass transfer limitations. This study revealed that even at low substrate loading of 2% and 3% (w/v) Enset fiber, the SSF process achieved a maximum butanol yield of 0.26 (g/g) and 0.27 (g/g), respectively. In addition, 80–84% of Enset fiber was converted into glucose during the saccharification process. These proved that the SSF and saccharification processes using Enset fiber were efficient, despite the reduced enzyme activity due to the low process temperature. However, the butanol productivity was significantly lower at low substrate loading than in fermentations with high substrate loading. Butanol productivity was maximized by 45% when the substrate loading increased from 3% to 5% (w/v) of Enset fiber. However, further increases in substrate loading to 7% (w/v) showed no significant difference; instead, the butanol yield decreased by 27%, and 12.42 g/L glucose remained unconsumed. This might be due to poor mixing caused by high solid and low water content, leading to a mass transfer problem in the SSF process [200]. A similar challenge was identified in a study by Guan W. et al. [202], which focused on butanol production from kraft paper mill sludge using the SSF process; they found that the slurry became too viscous, limiting mass transfer at substrate loadings of 6.3% and higher. Several studies suggest that the PSSF process can solve the mixing problem during the initial stages of fermentation in the SSF process [198,200,220].

Table 9 compares the butanol production from different biomass and *Clostridial* strains using the SSF process in bottle experiments. Compared with other studies, Enset fiber had the highest butanol concentration (11.36 g/L), yield (0.23 g/g), and productivity (0.16 g/(L

h)) among rice straw, oil palm empty fruit bunch, and avicel at optimal process parameters. However, wheat straw had a higher butanol concentration (12.64 g/L) than Enset fiber, as reported by Qi G. et al. [203], while Enset fiber had a higher butanol yield and productivity. To our knowledge, no studies have yet reported butanol production in the SSF process using *C. saccharoperbutylacetonicum*. Compared to the SHF method described in our previous study [41], which used the same strain and biomass with separate fermentation and saccharification steps, the butanol concentration was 9.9 g/L, which was lower than the current study. Furthermore, the SSF process enhanced butanol yield by 20% and productivity by 21% compared to the SHF process. This could be because the SSF process can avoid substrate inhibition by releasing the sugar gradually rather than all at once, as in the SHF process.

Table 9. Comparison of butanol production from different biomass using the SSF process in bottle experiments

Biomass	Pre-treatment method	Strain	Process parameters	Butanol titer (g/L)	Butanol yield (g/g biomass)	Butanol productivity (g/(L h))	Ref.
Enset fiber	2% (w/v) NaOH	*C. saccharoperbutylacetonicum* DSM 14923	5% (w/v) substrate loading, Cellic CTec2 (16 FPU/g-substrate), initial pH=6.8, 30 °C, 100 rpm, 72 h	11.36	0.23	0.16	This study [a]
Rice straw	Microwave-assisted hydro-thermolysis	*C. beijerinckii* DSM 6422	9% (w/v) substrate loading, Cellic CTec2 (12 FPU/g-glucan, initial pH=6.4, 37 °C, 150 rpm, 48 h	5.5	0.06	0.11	[196]
Oil palm empty fruit bunch	2% (w/v) NaOH	*C. acetobutylicum* ATCC 824	5% (w/v) substrate loading, acremonium cellulase (15 FPU/g-substrate), initial pH=5.5, 35 °C, 150 rpm, 120 h	3.97	0.08	0.03	[208]
Avicel	None	*C. acetobutylicum* ATCC-824	5.8% (w/v) substrate loading, Cellic CTec2 (20 FPU/g-glucan), initial pH=6.7, 36 °C, 150 rpm, 120 h	9.5	0.16	0.08	[204]
Wheat straw	10% (w/v) Ammonium sulfite	*C. acetobutylicum* ATCC 824	9% (w/v) substrate loading, Cellulase (5 FPU/g-substrate) & xylanase (10 IU/g-substrate), initial pH=5.0, 37 °C, 150 rpm, 144 h	12.64	0.14	0.088	[205]

[a] All calculations accounted for different substrate loading of Enset fiber in 50 mL medium at 72 hours fermentation period.

3.4.2 Scaling up butanol production from Enset fiber

To develop butanol production from Enset fiber at an industrial scale, a scale-up process that matches the bottle-scale experiment performance is essential. This study successfully established a small-scale SSF process from pretreated Enset fiber using a 2.5 L stirred tank bioreactor by maintaining a slight overpressure inside the bioreactor. In this study, the bioreactor fermentation using the pH-uncontrolled method showed a comparable butanol production result as the bottle experiment under similar optimal process parameters, except for the stirrer speed in the bioreactor. In both experiments, there was only a small difference in butanol concentration of 7.8% after 72 hours. However, after 120 hours, a similar butanol concentration of 11.35 g/L was observed with a yield of 0.23 g/g. These comparable results were achieved due to the utilization of a partially similar configuration when setting up the bioreactor system, which included maintaining the hydrogen-rich gas in the bioreactor. Several studies showed that maintaining hydrogen gas during fermentation is beneficial for maximizing biobutanol production in *clostridial* strains. Brosseau J. et al. [221] observed that *C. saccharoperbutylacetonicum* requires hydrogen gas to produce butanol, and when the hydrogen gas reaches a certain level, the bacteria reduce their growth rate and start producing butanol. Similarly, in this study, the highest hydrogen production rate was observed during the initial cell growth phase of *C. saccharoperbutylacetonicum*, which remained faster until 24 hours. Afterwards, the rate of hydrogen gas production slowed down, and the strain started producing butanol and entered the solventogenic phase. Furthermore, a separate study conducted by Stein U. et al. [222], focusing on the effect of pressure on ABE fermentation using *C. acetobutylicum*, concluded that butanol production could be enhanced through overpressure fermentation compared to lower pressure.

In addition, this study investigated the influence of controlled and uncontrolled pH values on butanol production from Enset fiber in the SSF process. The results showed that controlling the pH above 5.0 was not essential to achieve optimal butanol production in the SSF process from Enset fiber. In this study, butanol concentration decreased by 18% in pH-controlled fermentation compared to pH-uncontrolled fermentation, this could be due to the influence of pH value on the efficiency of the cellulase enzyme. Previous research has shown that the optimal pH range for commercial cellulase is typically between 4.8 and 5.5 [155,213]. However, in this study, during the SSF process in the pH-controlled fermentation, there were fluctuations in the pH values after 8 hours, which ranged between 5.5 to 5.96. The primary cause of these fluctuations was the substantial addition of NaOH. When the

base pump started adding NaOH to the medium, challenges arose due to the high solid content of the medium and inadequate mixing, impacting the accurate operation of the bioreactor's pH sensor. As a result, the base pump introduced an excessive amount of NaOH, resulting in an elevated pH level. The observed deviation from the optimal pH range reduced enzyme activity, ultimately limiting the release of glucose during pH-controlled fermentation compared to pH-uncontrolled fermentation. In contrast, the pH values in the pH-uncontrolled fermentation after 8 hours were between 4.8 and 5.29, which was in the optimal pH range for cellulase enzyme activity. This pH range resulted from the two-phase fermentation of the *Clostridia* strain. Initially, the strain produced acids such as acetic acid and butyric acid, leading to a pH drop to 4.8 during the first phase. Subsequently, the strain transitioned to the solventogenic phase, producing butanol, acetone, and a small amount of ethanol, causing the pH to rose to 5.29 [223]. Numerous studies have used pH control methods for ABE fermentation to prevent the occurrence of an acid crash, which is often triggered by the presence of inhibitory compounds in the fermentation medium resulting from pretreatment of the lignocellulosic biomass [224]; however, in this study, there were no inhibitory compounds that could have led to an acid crash. The pH is crucial for SSF process, keeping the pH low after the start of the solventogenic phase might increase butanol production rate and yield but also comes with the costs for pH control.

In this study, the major challenges observed in the bioreactor SSF process were inadequate mixing and reduced butanol productivity. To address these issues, an experiment was conducted using the PSSF method with a 7% (w/v) substrate loading of Enset fiber. The PSSF method alleviated the mass transfer problem by prehydrolysing for 2-3 hours before inoculation. This was because the SSF process had poor mixing from the start of fermentation until the Enset fiber was saccharified by the enzyme [225]. Furthermore, the butanol concentration and productivity were increased by 30% and 27%, respectively, compared to the SSF process with pH control method. This might be due to the initial glucose concentration at the end of the prehydrolysis step, which could facilitate the initial growth of the *C. saccharoperbutylacetonicum* strain [200], and the high substrate loading might have contributed to maximizing the butanol concentration. Studies showed that high substrate loading is crucial for large-scale operations to minimize overall production costs and maximize productivity [226]; by increasing the substrate loading from 5% (w/v) to 8% (w/v) of biomass, the production cost can be reduced by 19%, as this can lower the volume of downstream processing equipment and the energy consumption that is related to the

dilution level [227]. Compared to previous PSSF studies with different biomass and *Clostridial* strains, the highest butanol concentration and yield were observed in Enset fiber than in sugarcane straw [228], corn stover [198] and napier grass [220]. However, the butanol productivity of this study was lower than that of Dong J. et al. [200], who performed the PSSF process using *C. saccharobutylicum* from corn stover at an SSF temperature of 37 °C. This could be due to the lower working temperature of the SSF process in this study, which slowed down enzyme activity. To enhance the butanol productivity of this study, utilizing a strain with high-temperature tolerance [229] or cold-active cellulase [230] may enable an effective PSSF process from Enset fiber.

3.4.3 Hydrogen production from Enset fiber using SSF process

Producing hydrogen and butanol from affordable materials such as Enset fiber is essential to ensure the sustainability of the biofuel industry. The choice between butanol or hydrogen as a biofuel depends on application, cost considerations, and environmental sustainability [231,232]. In this study, butanol and hydrogen were simultaneously produced by the SSF process from Enset fiber using *C. saccharoperbutylacetonicum*. However, the fermentation conditions must be adjusted separately to achieve maximum yields for both products. This is because fermentation conditions affect cell metabolism and can potentially shift the metabolic pathway in favor of hydrogen or butanol production [210]. In addition, it has been shown that the amount of hydrogen and butanol produced in the *Clostridia* strain is influenced by factors such as electron flow and the balance of reducing equivalents in the metabolic pathway, leading to a competitive relationship between the two products [209].

Temperature is a key factor in the SSF process, as it influences both the growth of the cells and the enzyme activity in their metabolism. The results indicated that higher temperatures (35 and 37 °C) improved hydrogen production compared to lower temperature (30 °C); however, butanol production decreased with increasing temperature in the SSF process using *C. saccharoperbutylacetonicum*. A possible explanation could be that the cells grew faster at the higher temperature in the initial growth phase [190], which coincided with the highest hydrogen production rate in the acidogenic phase observed in this study. The result of this study was similar to those of Alalayah W. et al. [208] and Yadav S. et al. [190], who investigated the influence of temperature on hydrogen production in ABE fermentation using *C. saccharoperbutylacetonicum* and found the highest hydrogen values at 37 °C in both studies.

Another factor that affected hydrogen production in the SSF process was the initial pH value of the medium, which was influenced by the buffers in the medium and the metabolites in the cell growth phase. As *C. saccharoperbutylacetonicum* initiated growth, it began by producing acetic acid, butyric acid, and hydrogen. The buildup of organic acids resulted in a decrease in the pH of the medium and created stressful conditions for cell growth. Subsequently, the culture converted these acids into solvents such as butanol, acetone, and ethanol as it entered the stationary phase, increasing in pH value. Under optimal conditions favorable for cell growth, these phase changes represent the natural pathway for ABE fermentation [223]. However, in the presence of inhibitors or unfavorable conditions, the cells struggle to survive, leading to increased acid production and eventual cell death [233]. In this study, the pH profile of the SSF process at initial pH of 6.0, 7.0, and 8.0 followed a similar phase change and showed no significant difference in hydrogen and butanol production. However, at initial pH of 5.0 and 9.0, the strain could not enter the solventogenic phase, indicating that lower and higher initial pH values inhibited the cell. A study using the same medium and strain for ABE fermentation as in this study found that hydrogen production was maximized in the initial pH range of 7.0-8.5 [234]. In contrast, other studies suggested an optimal initial pH of 6.5, and beyond this point, hydrogen production decreased, although they used a different medium [207,208].

In addition, this study examined the effect of hydrogen partial pressure on hydrogen production and found that lower hydrogen partial pressure was beneficial for maximizing hydrogen production. The results of bottle experiments showed that hydrogen production was increased by 21.5% at lower hydrogen partial pressure compared to higher values. To validate the bottle experiment results and maximize hydrogen production, a scale up SSF process without a pH control system was implemented at the optimal process parameters with a temperature of 37 °C, an initial pH of 8.0 and under atmospheric pressure. The results confirmed that hydrogen production increased by 79.7% compared to the SSF process (pH-uncontrolled) at 30 °C, with an initial pH of 6.8 and under overpressure. However, butanol production was reduced by 21.4% at optimum process conditions for hydrogen production. Several studies have shown that hydrogen partial pressure is an essential factor in maximizing hydrogen production; it could also potentially inhibit cell growth and limit the thermodynamics of the process, depending on the microorganisms involved [211,222]. Electrons play a crucial role in the *Clostridial* metabolic pathways, and the strain can release excess electrons as hydrogen gas to balance the nicotinamide adenine dinucleotide

(NADH/NAD^+) ratio. However, when the hydrogen gas levels are high, it becomes more difficult for the bacteria to continue producing more hydrogen gas; this means the bacteria have too many electrons and insufficient energy, which can stop their growth and activity. As a consequence of this electron backlog, the strain utilises alternative pathways in which electrons are transferred from NADH to ferredoxin, which can serve as an electron carrier to reduce $NADP^+$ to NADPH, which facilitates butanol production [209,235,236]. For this reason, removing hydrogen gas from the medium might help the strain to produce more hydrogen.

This study obtained a similar hydrogen yield of 170 mL/gCOD in the bottle experiment as the previous study by Singh V. et al. [21], which used starchy wastewater and acid-treated water hyacinth as substrates with a similar strain. However, a higher hydrogen yield of 198.27 mL/g-Enset fiber from alkali-pretreated Enset fiber was achieved in the bioreactor experiment at optimal process parameters. This was also higher than the hydrogen yield of 68 ml/g-biomass from steam-exploded straw using the SSF process with *C. butyricum* AS1.209 strain, reported by Li D. et al. [237].

3.5 Conclusions

This study showed the potential of Enset fiber for hydrogen and butanol production in the SSF process. By optimizing the SSF process with *C. saccharoperbutylacetonicum*, we achieved high yields and concentrations of both biofuels under different conditions. Furthermore, a scalable process for both products was developed to achieve similar results to bottle-scale experiments, indicating potential applicability on an industrial scale. This is the first study to use this strain and substrate combination for the SSF process, and it contributes to the development of sustainable energy sources. However, more research on the 5L, 10L bioreactors and pilot scale processes, along with a feasibility study, is required to make industrial-scale butanol production feasible using the SSF process from Enset fiber.

4. Merging Anaerobic Fungi Fermentation and Chain Elongation Process

This chapter is based on the publication:

Caproate production from Enset fiber in one-pot two-step fermentation using anaerobic fungi (*Neocallimastix cameroonii* strain G341) and *Clostridium kluyveri* DSM 555 (2023).

Nebyat Seid, Katrin Ochsenreither and Anke Neumann

Microbial Cell Factories, 22(1), 216.

DOI: 10.1186/s12934-023-02224-w

Author Contributions:

Nebyat Seid: Conceptualization, methodology, formal analysis, investigation, resources, data curation, writing–original draft preparation, visualization, funding acquisition.

Katrin Ochsenreither: Writing–review and editing.

Anke Neumann: Conceptualization, methodology, resources, writing–review and editing, supervision, project administration, funding acquisition.

4.1 Introduction

International Energy Agency (IEA) bioenergy task 42 defines a biorefinery concept as a process that converts biomass into a variety of valuable products such as chemicals, materials, energy, food, and feed. The goal is to expand the range of products made from biomass to create other valuable products [1,238]. The use of renewable resources is crucial for sustainable development and minimizing environmental impact [239]. Lignocellulosic biomass is the most attractive resource due to its plentiful availability, low cost, and lack of competition with food [6]. An enormous amount of byproduct known as Enset biomass is thrown away during traditional Ethiopian Enset food processing. It is estimated that more than twenty million Ethiopians are dependent on the Enset plant for their food supply [57,141]. A major source of cellulose from the Enset plant is the Enset fiber, midrib, and leaf sheath peel, which contain 67.1%, 40%, and 34.1% cellulose, respectively [41]. Enset fiber can be used in a number of traditional products such as ropes, sacks, mats and bags [49]; however, the possibility of turning this waste into high value industrial products would strengthen the bioeconomy. Recent studies have shown that Enset biomass is a very promising lignocellulosic biomass for biobutanol [41] and pulp [66] production. However, to produce biofuel from Enset biomass, the material must undergo a degradation process that includes chemical and physical pretreatments before being hydrolyzed by enzymes. As a result, there is a substantial loss of biomass, environmentally unfriendly chemicals are used, and the process is energy intensive and expensive [240].

Research showed that anaerobic fungi can utilize lignocellulosic biomass without any pretreatment process and produce valuable products. As they grow, anaerobic fungi penetrate lignocellulosic biomass with their hyphal tips and degrade it with the enzyme complexes they produce [241,242]. Anaerobic fungi can be extracted from the alimentary tract, saliva, and feces of a variety of herbivorous animals [102]. From the feces of zoo animals, Stabel M. et al. [101] isolated six different anaerobic fungal strains, including *Neocallimastix cameroonii*, *Pecoramyces ruminantium*, *Caecomyces spec.*, *Khoyollomyces ramosus*, *Orpinomyces joyonii*, and *Aestipascuomyces dubliciliberans*. According to the study, of all isolates *Neocallimastix cameroonii* strain G341 proved to be the most efficient organism for utilizing wheat straw and was capable of producing acetate, succinate, ethanol, hydrogen, formate and lactate [107]. These products by itself can be already used to produce other bulk industrial chemicals [243], however, industrializing the use of mixed fermentation products may be challenging due to expensive and energy-intensive separation methods

[105,244]. Moreover, the market value of these products is not high compared to other products available on the market. To make this process economically more attractive, the products can be transformed into higher value products through the chain elongation process.

In chain elongation process, volatile fatty acids are changed to more valuable medium chain fatty acids through anaerobic fermentation [105]. A medium chain fatty acid like caproate, with its low solubility and high energy content, is used for various applications, such as antibiotics, feed additives, and as a component of products such as fragrances, lubricants, paint additives, and pharmaceuticals [26,27]. Researchers have conducted several studies on the production of caproate from mixed organic waste [27], food waste [105] and wastewater [245] using an open culture. However, due to an undefined consortium of microorganisms potentially involved in the process, it is possible that substrates might be degraded and unwanted products might be produced in a hardly controllable way [106]. In pure culture fermentation, *Clostridium kluyveri* is capable of utilizing acetate, ethanol, succinate, and hydrogen to produce caproate and other medium chain fatty acids [246,247]. Therefore, fermentation metabolites of *N. cameroonii* strain G341 from lignocellulosic biomass could be a suitable substrate for *C. kluyveri* fermentation. The objectives of this study were to evaluate the growth of *N. cameroonii* on Enset fiber, and to develop and characterize a one-pot two-step fermentation process for caproate production using *N. cameroonii* strain G341 and *C. kluyveri* DSM 555 from Enset fiber. To date, no research has been conducted on the growth of *N. cameroonii* on Enset fiber as well as the production of caproate from Enset fiber using *N. cameroonii* and *C. kluyveri* in a single pot two-step fermentation.

4.2 Materials and methods

4.2.1 Enset fiber preparation

Enset fiber was collected from Wolkite, Ethiopia, and was prepared according to previous instructions [41]. It was dried for four days in the sun, cut into 6 cm pieces with scissors, and ground using a knife mill. The pulverized sample was sieved to 1 mm particle size and stored in a plastic bag.

4.2.2 Anaerobic fungi cultivation and growth culture conditions

In a previous study, Stabel M. et al. [101] isolated *Neocallimastix cameroonii* strain G341 from giraffe feces. The strain was grown anaerobically in 250 mL serum bottles containing 50 mL of sterilized basal minimal medium and 0.25 g Enset fiber (5 g/L) as a carbon source

corresponding to a substrate loading of 0.5% (w/v). 1 L minimal medium consisted of 150 mL of salt solution I (K_2HPO_4 (3.0 g/L)), 150 mL of salt solution II (KH_2PO_4 (3.0 g/L), $(NH_4)_2SO_4$ (6.0 g/L), NaCl (6.0 g/L), $MgSO_4.7H_2O$ (0.6 g/L), $CaCl_2.2H_2O$ (0.6 g/L)), 2 mL of 0.05% (w/v) hemin solution (a mixture of 0.05 g hemin, 50 mL ethanol, and 50 mL of 0.05 M NaOH), 2 mL of 0.1% (w/v) resazurin solution, $NaHCO_3$ (6 g/L), cysteine-HCl·H_2O (1 g/L) and 10 mL of trace elements solution (a mixture of 0.25 g $MnCl_2.4H_2O$, 0.25 g $NiCl_2.6H_2O$, 0.25 g $NaMoO_4.2H_2O$, 0.25 g H_3BO_3, 0.20 g $FeSO_4.7H_2O$, 0.05 g $CoCl_2.6H_2O$, 0.05 g $Na_2SeO_3.5H_2O$, 0.05 g $NaVO_3.4H_2O$, 0.025 g $ZnSO_4$, 0.025 g $CuSO_4.2H_2O$, and 1 L 0.2 M HCl). The media was anaerobized by flashing with 100% CO_2 until the color turned yellow, and the pH was corrected to 6.9 with 5 M NaOH. During anaerobization, a serum bottle containing 0.25 g of Enset fiber was filled with 49.5 mL of medium, enclosed with a rubber stopper and aluminum cap, and autoclaved. After autoclaving, 0.5 mL filter-sterilized vitamin solution (0.01 g/L thiamine, 0.2 g/L riboflavin, 0.6 g/L calcium pantothenate, 0.6 g/L nicotinic acid, 1.0 g/L nicotinamide, 0.05 g/L folic acid, 0.02 g/L vitamin B12, 0.2 g/L biotin, 0.1 g/L pyridoxamine, and 0.05 g/L p-aminobenzoic acid) was added [101,248]. The main fermentation culture was inoculated with 10% (v/v) actively growing *N. cameroonii* preculture propagated every seven days for two consecutive cultivations before being used as a preculture. The bottle was incubated at 39 °C for 7 days in the dark in an incubator (Infors Thermotron, Infors AG, Bottmingen, Switzerland) without shaking. The pressure of the bottle was measured with manometer GMH 3100 Series (Greisinger, Mainz, Germany) at 39 °C, and 5 mL gas and 1.5 mL liquid samples were taken with needles every day and centrifuged at 10,000 rpm for 10 minutes [101]. 0.25g wheat straw (5 g/L) was used as a substrate for the control experiment, and all experiments were performed in three replicates.

4.2.3 Effect of substrate loading on *N. cameroonii* growth

The same conditions as mentioned above were used to cultivate *N. cameroonii* to investigate the effects of substrate loading. A total of 50 mL of media was prepared with Enset fiber at different substrate loadings (0.5, 1, 3, 5, 7% (w/v)) as carbon source, inoculated with 5 mL of anaerobic fungal culture, and incubated at 39 °C for 7 days [101]. At the beginning and the end of fermentation, 5 mL gas and 1.5 mL liquid samples were taken. All experiments were conducted in triplicate.

4.2.4 Bacterial strain cultivation

Freeze-dried *Clostridium kluyveri* DSM 555 culture was acquired from the German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany (DSMZ), and revived according to DSMZ protocol. The DSM-52 medium contained buffer stock solution (K_2HPO_4 (31 g/L), KH_2PO_4 (23 g/L), NH_4Cl (25 g/L)), vitamin stock solution (B12 (0.1 g/L), p-aminobenzoic acid (0.08 g/L), biotin (0.02 g/L), nicotinic acid (0.2 g/L), D-Ca-pantothenate (0.1 g/L), pyridoxine-HCl (0.3 g/L), thiamine-HCl.$2H_2O$ (0.2 g/L)), yeast extract stock solution (100 g/L), Na_2CO_3 stock solution (100 g/L), $Na_2S.9H_2O$ stock solution (100 g/L), L-Cysteine-HCl.H_2O stock solution (100 g/L), and mineral stock solution ($MgSO_4$.7 H_2O (20 g/L), 100 mL of trace element solution, 100 mL of selenite-tungstate solution. The trace element solution SL-10 consisted of 10 mL of (25%) HCl in 1 L, $FeCl_2.4H_2O$ (1.5 g/L), $ZnCl_2$ (0.07 g/L), $MnCl_2.4H_2O$ (0.1 g/L), H_3BO_3 (0.006 g/L), $CoCl_2.6H_2O$ (0.19 g/L), $CuCl_2.2H_2O$ (0.002 g/L), $NiCl_2.6H_2O$ (0.024 g/L), $Na_2MoO_4.2H_2O$ (0.036 g/L). The selenite-tungstate solution was prepared by mixing NaOH (0.5 g/L), $Na_2SeO_3.5H_2O$ (0.003 g/L), and $Na_2WO_4.2H_2O$ (0.004 g/L). All the stock solutions were filter sterilized and anaerobized with 100% N_2 gas. For the cultivation experiment, 10 g/L CH_3CO_2K and 1 g/L resazurin were added into 47.75 mL distilled water. The pH was corrected to 6.9 with 1M NaOH/1M HCl and filled into 250 mL serum bottles, enclosed with an aluminum cap and rubber stopper. Each bottle was flashed with a mixture of 80% N_2 and 20% CO_2 gases using needles and autoclaved. Then, after autoclaving, 1 mL of (99.5%) ethanol was injected with a needle and 1% (v/v) of each stock solution was added. 10% (v/v) of active culture with an OD of 0.8-1.0 was inoculated into bottles and incubated at 37 °C and 150 rpm. The culture was transferred three times before being used as a preculture [26].

4.2.5 Two-step fermentation process

N. cameroonii was first grown on 0.25 g Enset fiber at 39 °C for 7 days in 250 mL serum bottles containing 50 mL of the basal medium described above. Subsequently, the fungal culture was supplemented with 1% (v/v) sterilized DSM-52 stock medium without acetate, ethanol, and reducing agents. The bottle containing the grown fungal culture was then inoculated with 10% (v/v) of the actively growing *C. kluyveri* culture with an OD of 0.8-1.0 and incubated at 37 °C and 130 rpm for 7 days. During the second fermentation, the pressure was measured, and the 5 mL gas and 1.5 mL liquid samples were taken using needles (**Figure 22).** In addition, to investigate the effect of ethanol in two-step fermentation, another

experiment was conducted separately by supplementing the bottle with 2.18 mmol ethanol during *C. kluyveri* inoculation. As a control, *N. cameroonii* and *C. kluyveri* were grown separately using the standard media. All experiments were conducted in triplicate.

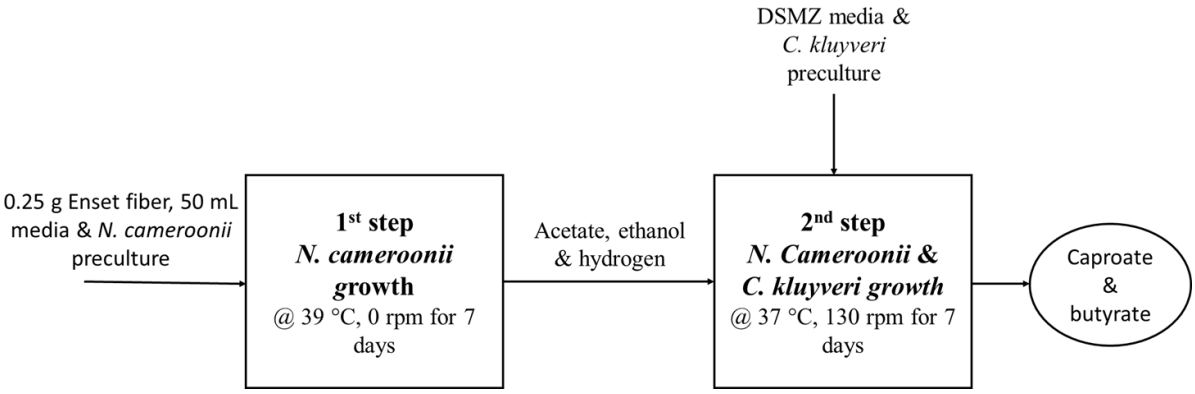

Figure 22. Caproate production from Enset fiber in two-step fermentation using *N. cameroonii* and *C. Kluyveri*

4.2.6 Carbon balance calculations

The carbon balance calculation was estimated for *N. cameroonii* growth on Enset fiber, and one-pot two-step fermentation using *N. cameroonii* and *C. kluyveri* from Enset fiber without additional carbon source. Carbon recovery was calculated by balancing the carbon moles contained in all substrates and products (**Appendix C, Table C1**). The calculation was done without considering the amount of biomass from fungal and bacterial cells. In addition, the Enset fiber carbon content was estimated from its elemental composition, which was estimated by Seid N. et al. [41] with 41.2% (w/w) dry weight and calculated as follows:

$$Amount\ of\ carbon\ in\ Enset\ fiber\ (mol) = \frac{mass\ of\ Enset\ fiber\ (g) \times 41.2\ \% \left(\frac{g}{g}\right)\ Carbon}{Molar\ mass\ of\ carbon\ \left(\frac{g}{mol}\right)} \ldots\ldots (4.1)$$

4.2.7 Analytical methods

After inoculation, the gas pressure inside the bottle was monitored using a manometer with a needle. As anaerobic fungi grew and produced gases, the manometer recorded changes in pressure over time. By correlating gas pressure measurements with fungal growth, this method provided insights into anaerobic fungal growth [101]. Ultraspec 1100 pro spectrophotometer (Amersham Biosciences, Uppsala, Sweden) was used to measure the optical density (OD_{600}) of bacterial cells. For liquid samples, the pH was measured by Profilab pH 597 (Xylem Analytics, Weilheim, Germany) and metabolites were determined by high-performance liquid chromatography (HPLC) in a 1100 Series System (Agilent

Technologies, Waldbronn, Germany). HPLC was performed with a Rezex ROA Organic Acid H^+ (8%) column at 55 °C, and 5 mM H_2SO_4 eluent with a flow rate of 0.6 mL/min.[41] The gas samples were analyzed by Micro GC Fusion® gas analyzer (Inficon, Bad Ragaz, Switzerland) with Porous Layer Open Tubular (PLOT) and Wall Coated Open Tubular (WCOT) capillary columns using Argon and Helium as carrier gases.

4.3 Results

4.3.1 Determination of *N. cameroonii* growth on Enset fiber

To evaluate the growth of *N. cameroonii* on Enset fiber, and to analyze the fermentation products, the fungi were grown initially on 0.25 g Enset fiber as sole carbon source. Before fermentation, the bottles were filled with 8.06 mmol CO_2 gas at atmospheric pressure (≈1.00 bar). As shown earlier by Theodorou M. et al. [249], pressure increase can be used as an indicator of growth and metabolite production, therefore, the bottle pressure was measured regularly. **Figure 23A** shows the amount of gas produced by the anaerobic fermentation of *N. cameroonii* along with the absolute pressure built up. Within 24 h, an absolute pressure of 1.2 bar was developed and 0.97 mmol of CO_2 were produced. As fermentation progressed, the gas composition in the bottle headspace changed to hydrogen and CO_2, and a maximum pressure of 1.39 bar was generated on the fourth day. After 5 days, 0.51 mmol of hydrogen was produced with no significant changes in hydrogen quantity in the following days. However, there was a slight decrease in the amount of CO_2 gas as well as the pressure. In the control experiments with 0.25 g wheat straw as substrate, the gas profiles were similar and the fungi developed a maximum pressure of 1.25 bar and 0.39 mmol hydrogen, which was lower than with Enset fiber (**Figure 23B**).

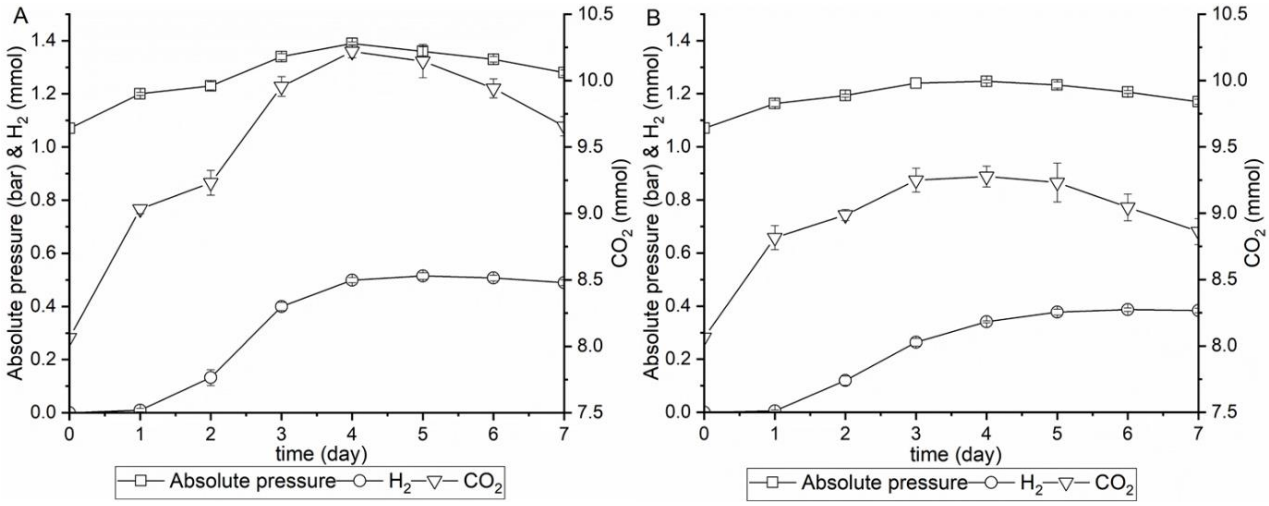

Figure 23. Gas production and pressure generated during *N. cameroonii* growth on 0.25 g (A) Enset fiber or (B) wheat straw. All values are means from triplicate bottles. Note the offset of the right (CO_2) axis.

The amount of soluble products released during growth of *N. cameroonii* on 0.25 g of Enset fiber is shown in **Figure 24A**. The cultures produced 0.37 mmol acetate and 0.41 mmol formate from Enset fiber within two days, however, lactate and succinate were not detected. After 5 days, the production was increased to 1.16 mmol acetate and 1.26 mmol formate. In addition, lactate and succinate were detected in small amounts, 0.16 mmol and 0.08 mmol, respectively. During the media preparation, the hemin solution containing ethanol was added to the media; but after 5 days of fermentation, the amount of ethanol increased to 0.8 mmol, indicating that ethanol was produced during the process. At 7 days, all amounts of metabolites remained constant except for formate, which increased to 1.34 mmol. For both substrates, the maximum total metabolite production rate was observed on the third day, but with Enset fiber a higher production rate than with wheat straw was calculated, 0.73 mmol/day compared to 0.49 mmol/day for wheat straw. At the end of the fermentation process, it was observed that the anaerobic fungi produced a total of 3.94 mmol of metabolites from Enset fiber, which was considerably higher than the 2.86 mmol of metabolites produced from wheat straw (**Figure 24A&B**). When comparing the percentage of each metabolite, acetate, hydrogen, and succinate were all produced from Enset fiber in the same proportions as from wheat straw, 29, 13, and 1.9% (mmol/mmol), respectively. However, there was a significant difference in the percentage of formate (34% (mmol/mmol)) as well as lactate (4% (mmol/mmol)), and it was higher with Enset fiber than with wheat straw (**Figure 24C**).

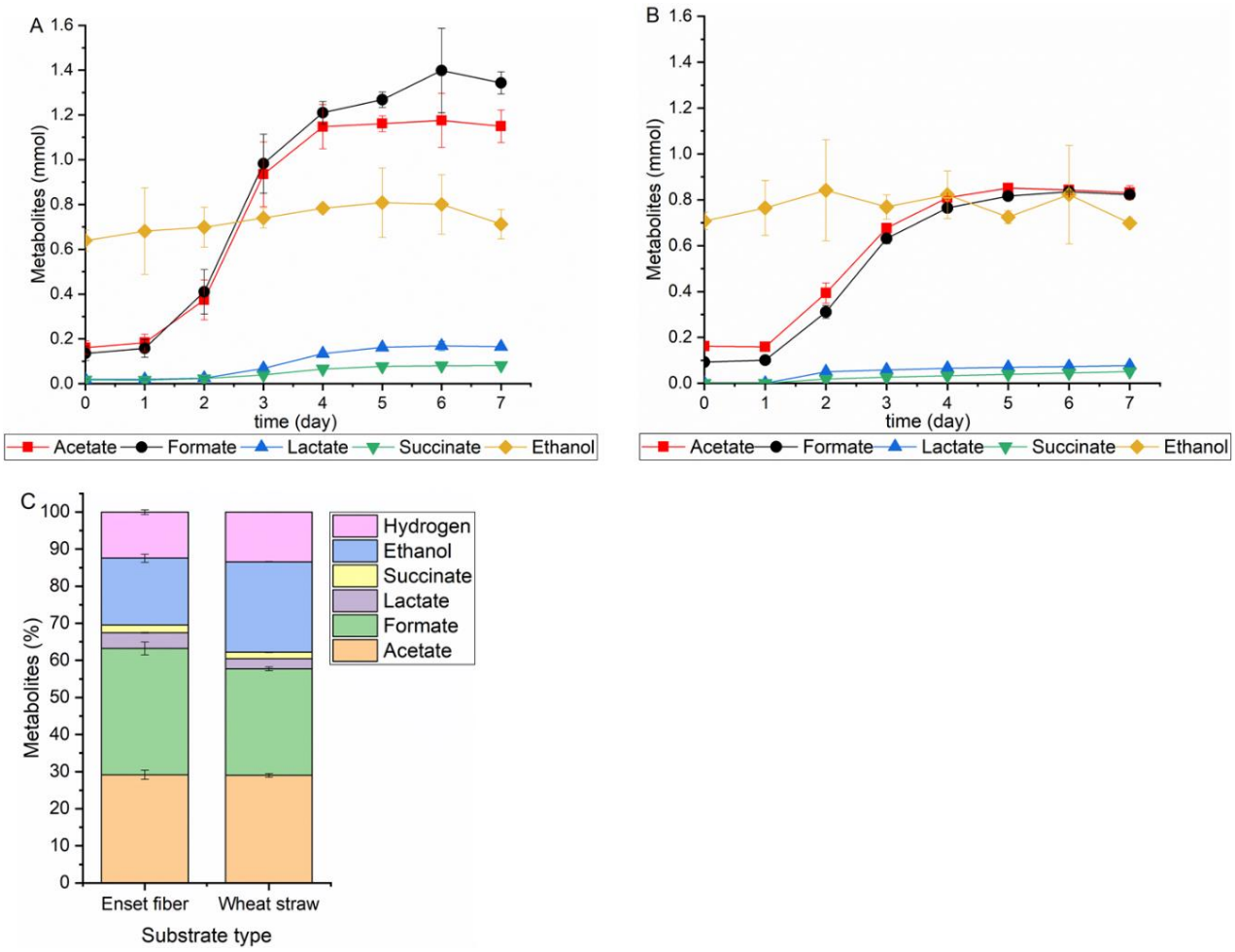

Figure 24. Soluble products of *N. cameroonii* growth on 0.25 g (A) Enset fiber, (B) wheat straw (C) mol percentage of all metabolites. All values are means from triplicate bottles.

In addition, to investigate the influence of substrate loading on the growth of *N. cameroonii*, 0.5, 1, 3, 5 and 7% (w/v) of Enset fiber were tested. **Figure 25** shows the pressure generated at the end of fermentation during *N. cameroonii* growth at different substrate loading of Enset fiber. The highest pressure of 1.72 bar was recorded with 1% (w/v) of Enset fiber, but the pressure decreased with increasing substrate loading. Statistical analysis with $p < 0.05$ showed a significant difference between different substrate loadings of Enset fiber on pressure development by *N. cameroonii* (**Appendix C, Table C2**). Moreover, the metabolite results shown in **Figure 26** indicate that all fermentation products increased when substrate loading was increased from 0.5 to 3% (w/v) except hydrogen. At 3% (w/v) substrate loading, acetate and formate increased to 2.44 mmol and 1.88 mmol. There was also an increase in lactate and succinate to 0.49 mmol and 0.24 mmol, respectively, but no significant difference in ethanol production was observed. On the other hand, a maximum hydrogen production of 0.88 mmol was observed at the substrate loading of 1% (w/v). All metabolites decreased at

both 5 and 7% (w/v) substrate loadings, except acetate which increased to 3.18 mmol. The percentage of each metabolite produced during *N. cameroonii* growth showed that there was a similarity between the acetate and formate percentages of 30% (mmol/mmol) when the substrate loading was low, however, the acetate percentages increased to 53% (mmol/mmol) when substrate loading was high. Additionally, as substrate loading increased, the hydrogen percentage tended to decrease from 14 to 8% (mmol/mmol) as well (**Appendix C, Figure C1**).

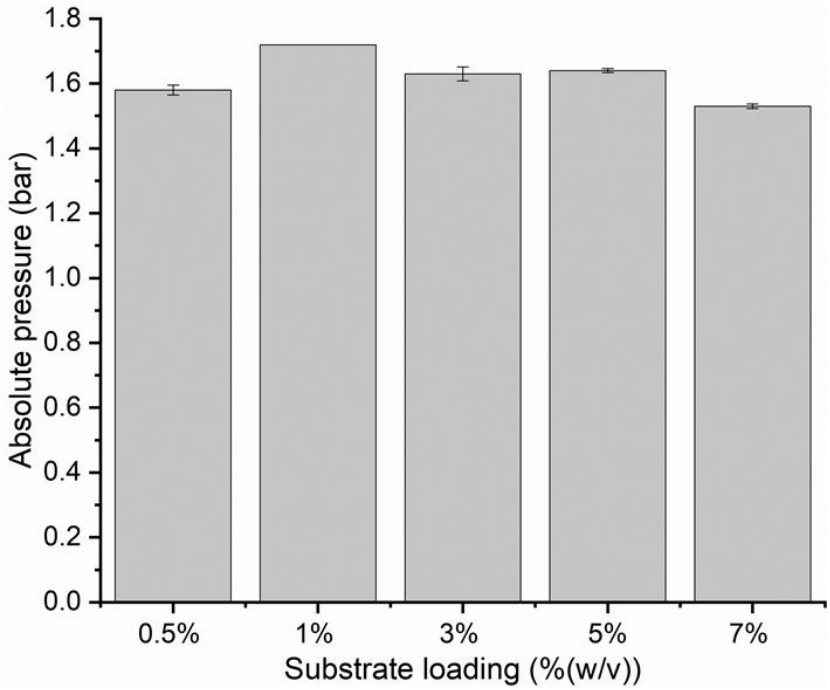

Figure 25. Pressure value measured at different substrate loading with Enset fiber during *N. cameroonii* growth at the end of fermentation. All values are means from triplicate bottles.

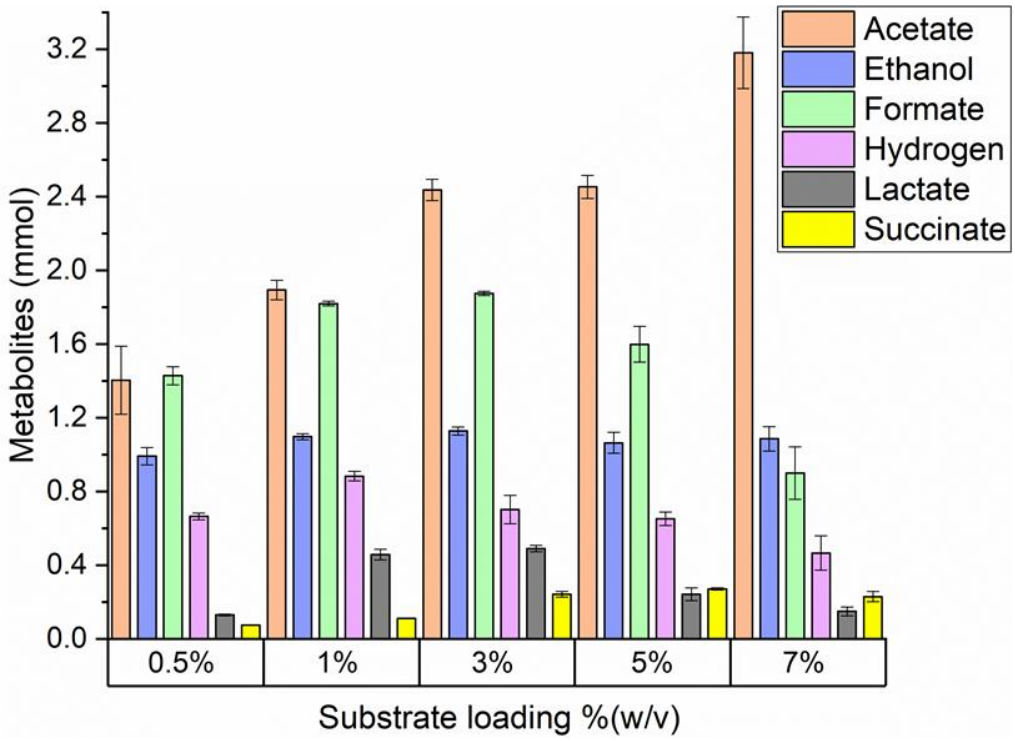

Figure 26. Fermentation products of *N. cameroonii* growth on different substrate loading of Enset fiber at the end of fermentation. All values are means from triplicate bottles.

4.3.2 Caproate production from Enset fiber in one-pot two-step fermentation using *N. cameroonii* and *C. kluyveri*

The purpose of this study was to produce caproate directly from Enset fiber without any pretreatment or addition of external enzymes. *N. cameroonii* was cultivated on Enset fiber and then fermented with *C. kluyveri* to produce caproate in an one-pot two-step fermentation. **Figure 27A** shows the metabolites produced in one-pot two-step fermentation using *N. cameroonii* and *C. kluyveri* from Enset fiber as sole carbon source. In the first step fermentation, *N. cameroonii* grew on 0.25 g Enset fiber for 7 days and produced 1.36 mmol acetate, 0.69 mmol hydrogen, and 1.11 mmol of ethanol. Within 18 h of starting the second fermentation, *C. kluyveri* produced 0.21 mmol caproate and 0.33 mmol butyrate. After 42 h, a maximum of 0.3 mmol caproate and 0.46 mmol butyrate were produced and thereafter there was no significant difference in production. In addition, the hydrogen production increased to 0.95 mmol and ethanol was completely consumed within 27 h. However, the remaining 0.76 mmol of acetate and other metabolites from anaerobic fungi were not consumed at the end of fermentation. As a control experiment, 2.63 mmol acetate and 1.1 mmol ethanol were used as a substrate for the *C. kluyveri* fermentation, and after 42 h 0.27 mmol caproate, 0.68 mmol butyrate and 0.34 mmol hydrogen were produced (**Figure 27B**).

Similarly to the two-step fermentation, all ethanol was consumed at the end of the fermentation, but 1.57 mmol of acetate remained unconsumed.

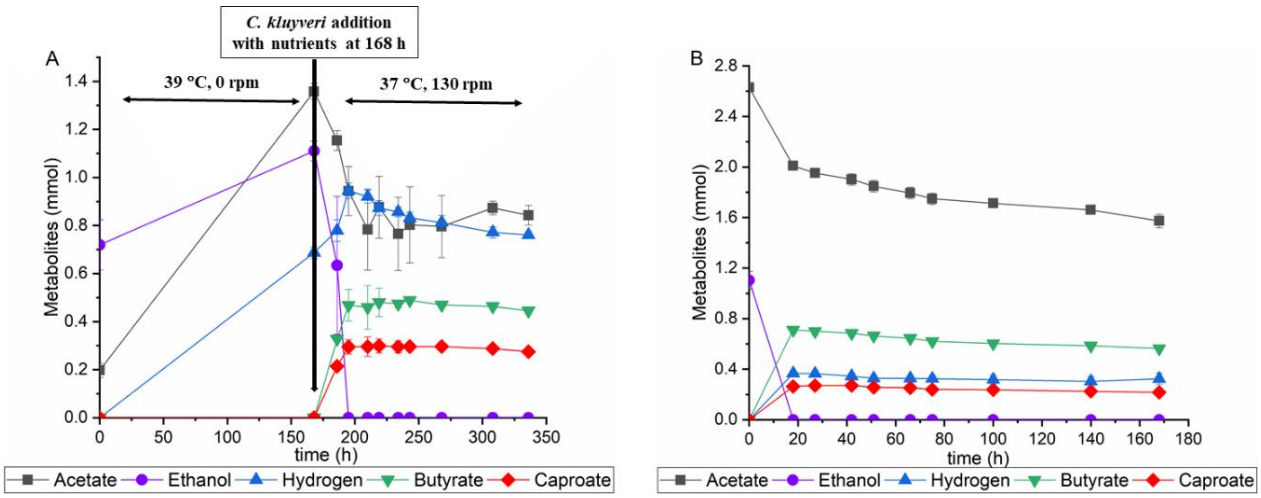

Figure 27. Fermentation products (A) one-pot two-step fermentation using Enset fiber without additional carbon source (B) *C. kluyveri* fermentation using acetate and ethanol as a carbon source. All values are means from triplicate bottles.

In addition, separate experiments were conducted in which ethanol was added during *C. kluyveri* inoculation in the two-step fermentation to maximize acetate consumption and study the effect of ethanol. **Figure 28** shows the results of two-step fermentation using Enset fiber and with the addition of 2.18 mmol ethanol. After second fermentation, there was no difference in the production of metabolites during the first 27 h of the experiment compared to previous experiments. However, the fermentation products after 42 h were significantly different from the previous experiment, more caproate was found than butyrate, 1.13 mmol and 0.5 mmol, respectively, and hydrogen production increased to 1.35 mmol. It was also found that all ethanol was consumed within 42 h and only 0.32 mmol acetate was not consumed.

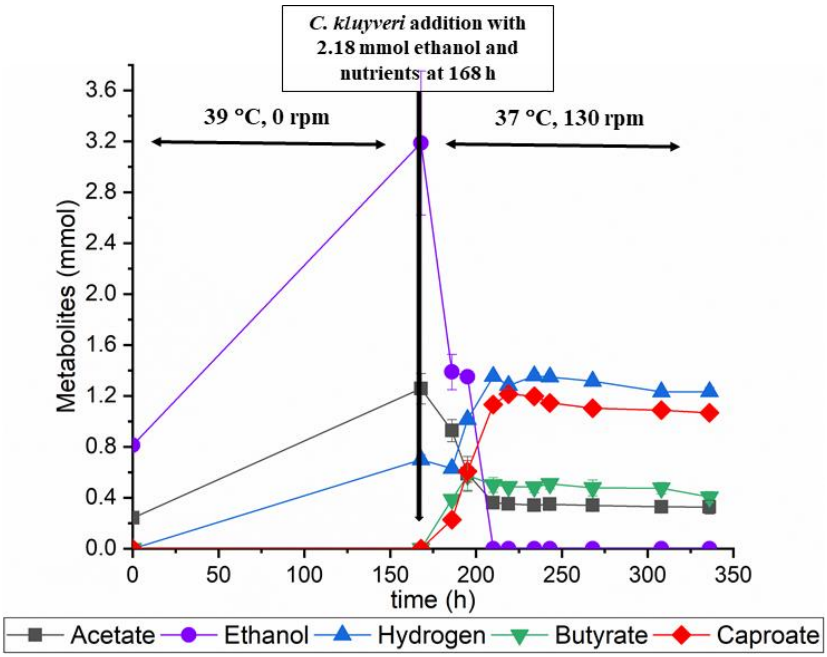

Figure 28. Metabolites from one-pot two-step fermentation using Enset fiber and with the addition of 2.18 mmol ethanol.

4.3.3 Carbon balance

Carbon balance estimation was performed for *N. cameroonii* growth on Enset fiber, and for one-pot two-step fermentation using *N. cameroonii* and *C. kluyveri* without additional carbon source (**Table 10**). In a study using Enset fiber as carbon source for growing *N. cameroonii*, 86.3% of the carbon was recovered as metabolites. The remaining carbon in the Enset fiber might not be degraded by anaerobic fungi. In the two-step fermentation, *C. kluyveri* achieved 94.1% carbon recovered as metabolites after the fungi were grown on Enset fiber, therefore it is likely that the missing carbon is bacterial cell biomass.

Table 10. Carbon balance for *N. cameroonii* growth on Enset fiber, and two-step fermentation using *N. cameroonii* and *C. kluyveri* on Enset fiber

Compound	Carbon per compound (mmol) for *N. cameroonii* growth on Enset fiber		Carbon per compound (mmol) for two-step fermentation [a]	
	Substrates	**Products**	**Substrates**	**Products**
Enset fiber	8.58	-	-	-
Acetate	-	2.22	2.72	1.69
Ethanol	1.28[b]	1.43	2.22	0
Succinate	-	0.32	0.44	0.24
Lactate	-	0.49	0.27	0.25
Formate	-	1.34	1.14	1.11
CO_2	8.06	9.66	9.19	8.32
Caproate	-	-	-	1.65
Butyrate	-	-	-	1.78
Sum of carbon	17.92	15.47	15.97	15.03
Carbon recovery (%)[c]	86.3		94.1	

[a] The two step fermentation was without addition of ethanol; [b] The amount of ethanol came from the hemin solution in the medium;[c] Carbon recovery (%) =(Sum of carbon in products/Sum of carbon in substrate)×100%

4.4 Discussion

4.4.1 Anaerobic fungal growth on Enset fiber

Anaerobic fungi release carbohydrate-active enzymes (CAZymes) such as cellulases and hemicellulases that can degrade lignocellulosic biomass, and produce several organic acids, and hydrogen as byproducts of its growth process [104]. In this study, we observed that *N. cameroonii* grew on Enset fiber and produced acetate, succinate, ethanol, hydrogen, formate and lactate. During the fermentation process, the pressure in the headspace of the culture bottles increased due to the build-up of fermentation gases within the headspace of the bottles and indicated that anaerobic fungal growth was taking place [249]. Growth and production of metabolites by *N. cameroonii* started within 24 h on Enset fiber and remained constant after 5 days, suggesting that the growth process was completed by this time. Previous studies showed that after inoculation, the fungi were able to colonize and grow on the biomass by developing rhizoids, which then became a highly branched rhizoid system

within 24 h [104,250]. Stabel M. et al. [101] characterized the growth of *N. cameroonii* on different substrates such as wheat straw, cellobiose, xylan, cellulose, starch, pectin, chitin, inulin, alginate, maltose, sucrose, lactose, glucuronic acid, and monosaccharides. They found that *N. cameroonii* was capable of growing on all substrates except arabinose, chitin, alginate, galactose, ribose, and glucuronic acid, and produced hydrogen, acetate, succinate, ethanol, formate and lactate, when grown on these substrates. The study concluded that on wheat straw more hydrogen (0.35 mmol) was produced compared to monosaccharide substrates, which was less than when Enset fibers were used as the substrate. Furthermore, in our study, the amount of CO_2 increased until the fourth day, similar to what Borneman W. et al. [251] observed in their study of *Neocallimastix sp*. Strain RI grown on Italian ryegrass hay. However, it was not sure whether it came from bicarbonate buffer or anaerobic fungal growth [249]. It is necessary to conduct further research to confirm the cause of the CO_2 sources.

One of the factors that affect the metabolite production and the growth of *N. cameroonii* is the type of substrate. *N. cameroonii* grew better on Enset fiber and produced more metabolites than with wheat straw, compared to the control experiment shown in **Figure 24A&B**. This could be due to the structural difference in the lignocellulosic biomass. It has been found that Enset fiber contains 67.1% cellulose, 15.6% hemicellulose, and 5.1% lignin while wheat straw contains 35–45% cellulose, 20–30% hemicellulose, and around 15.5% lignin [41,252]. A significant difference between Enset fiber and wheat straw can be seen in the lower percentage of lignin and the higher percentage of cellulose, which could make Enset fiber a more suitable substrate for the fungi growth. Geoffrey L. et al. [253] conducted experiments in order to determine whether lignin is degraded by ruminal fungi, *Neocallimastix sp.* (strain LM-1), *Sphaeromonas sp.* (strain NM-1) and *Piromonas sp.* (strain SM-1), and the results showed that the lignin component of straw was not degraded. However, other researchers observed a high percentage of dry weight and lignin loss in perennial ryegrass stem cell walls [254], Bermudagrass leaf, cordgrass fiber [255], wood [256], and Italian ryegrass hay [257], when treated with different species of anaerobic fungi. However, most of the losses occurred due to the solubilization of lignin-polysaccharide complexes such as phenolic compounds [258]. In addition, there is some evidence that phenolic compounds might inhibit the growth of anaerobic rumen fungi [256]. However, further investigations should be carried out on the cell wall degradation of lignocellulosic biomass and on the effects of phenolic compounds on *N. cameroonii* growth.

There is no doubt that anaerobic fungal growth is highly dependent on the proper substrate loading, and an excessive solid substrate in the system can reduce the anaerobic fungi growth. In the current study, *N. cameroonii* growth on Enset fiber was observed at higher substrate loading. Even though there was a mixing problem due to the high amount of solid content in the bottles, at 7% (w/v) substrate loading of the Enset fiber a pressure of 1.53 bar was developed. However, the carbon balance calculation showed that at maximum substrate loading, the fungi's carbon conversion efficiency was minimized from 98.8% to 16.5% (**Table 11**). 3% (w/v) was a suitable substrate loading to obtain a higher amount of soluble products, whereas 1% (w/v) substrate loading was more suitable for producing maximum hydrogen. In addition, it was observed that the maximum pressure exerted by *N cameroonii* on Enset fiber varied between 1.53 and 1.72 bar (**Figure 25**). Thereafter, metabolism shifted from gaseous to soluble products, possibly due to the thermodynamic limitations of the microorganism [107]. The results of this study were similar to those of Zhu et al, [259], who conducted batch fermentation ranging from 5 to 80 g dry matter/L of wheat straw to determine the growth of *Neocallimastix hurleyensis*, indicating that about 45% wheat straw were degraded by the fungi at 5 g dry matter/L of substrate loading, whereas degradation efficiency was lowered with 10–80 g dry matter/L. There could be a number of reasons for this, including mass transfer between nutrients and fungi being limited [260], which might affect the rate at which nutrients are transferred to fungi. In the case of higher substrate loading levels, we observed that homogeneity of the substrate was causing a problem and the fungus only grew on one side of the bottle. In addition, according to the study [107], the mass transfer problem in *N. cameroonii* fermentation could also occur between the liquid and the gas phases, where the produced hydrogen gas might accumulate on the fungus mat and thereby inhibit its growth. To consolidate the reason behind the conclusions, further research should be conducted on product inhibition of *N. cameroonii* growth.

Table 11. Carbon conversion efficiency for *N. cameroonii* growth on different substrate loading of Enset fiber

	Carbon per compound (mmol)				
	0.5% (w/v)	**1% (w/v)**	**3% (w/v)**	**5% (w/v)**	**7% (w/v)**
Compound	**Substrates**				
Enset fiber	8.58	17.2	51.5	85.8	120.1
Ethanol*	1.28	1.28	1.28	1.28	1.28
CO_2	8.06	8.06	8.06	8.06	8.06
	Products				
Acetate	2.81	3.79	4.88	4.91	6.36
Ethanol	1.98	2.20	2.26	2.13	2.17
Succinate	0.30	0.45	0.97	1.09	0.92
Lactate	0.39	1.37	1.47	0.73	0.45
Formate	1.43	1.82	1.88	1.60	0.90
CO_2	10.8	11.1	10.9	11.2	10.6
Carbon recovery (%)	98.8	78.3	36.7	22.7	16.5

*The amount of ethanol came from the hemin solution in the medium

4.4.2 One-pot two-step fermentation

In this study, a novel approach was developed to produce caproate from Enset fiber in a two-step fermentation process using *N. cameroonii* and *C. kluyveri* in a single bottle. Using this method, Enset fiber was directly converted into caproate without pretreatment or the addition of an external enzyme. Using Enset fiber as a raw material for caproate production contributes to a circular bioeconomy that creates sustainable systems. Furthermore, the two-step fermentation process could have several advantages for industrial applications, including reducing equipment costs because it used a single reactor, as well as chemical costs, as fewer reducing agents, antibiotics, and pH-adjusting chemicals are needed. Several studies have shown that caproate can be produced in one bioreactor by merging the chain elongation process with syngas fermentation [261,262]. However, one of the biggest challenges was the pH unsuitability of acetogens and chain-elongating microorganisms [263]. Researchers found that *C. kluyveri* growth was inhibited at an acidic pH, which is the ideal pH for acetogens strains to maximize solvent production [264,265]. During this study, a neutral pH was used in a one-pot two-step fermentation process that was suitable for the growth of both *N. cameroonii* and *C. kluyveri*, making it ideal for caproate production.

In this study, anaerobic fungi were able to directly convert Enset fiber into acetate, succinate, ethanol, hydrogen, formate, and lactate. The organic compounds collected from anaerobic fungal fermentation were successfully converted into high-value medium-chain carboxylates (MCC) such as caproate and butyrate. In addition, it was observed that hydrogen production was maximized in a two-step fermentation process compared to a single fermentation of anaerobic fungi (**Figure 27A**). In this study, *C. kluyveri* metabolites production stopped around 42 h and entered a stationary phase, showing a similar growth process as in the study by Yin Y. et al. [26]. Furthermore, there was faster consumption of acetate and ethanol, as well as a small amount of succinate consumption. *C. kluyveri* is known to be able to consume a wide range of substrates and studies on this strain have demonstrated that succinate can also be consumed [246]. In addition, organic compounds such as formate and lactate, produced by anaerobic fungi, accumulated in the bottles. It appears that none of the metabolites accumulated from anaerobic fungal fermentation inhibited *C. kluyveri* growth.

Numerous studies have been conducted to evaluate the potential of anaerobic fungi for co-cultivation with other strains. Li Y. et al. [266] studied a co-culture experiment between an anaerobic fungus (*Pecoramyces* species) and a methanogen (*Methanobrevibacter* species), and the results showed that methane can be produced from various parts of corn stover through coculturing. However, in another study, Dollhofer V. et al. [267] showed that the co-cultivation of anaerobic fungi with mixed cultures from biogas plants faced many challenges, including unfavorable conditions in the digester due to extreme temperatures and long retention time. Moreover, it is also possible that there might be competition between the strains for nutrients and substrates [268]. In this study, besides suitable fermentation conditions such as an anaerobic environment, optimal pH, and temperature for the two strains, apparently, there was no competition between the anaerobic fungi and *C. kluyveri* for the availability of nutrients and substrates, since the strains were added sequentially and the nature of metabolic pathways between them were different. In the two-step fermentation process, *N. cameroonii* degraded lignocellulosic biomass by carbohydrate-active enzymes (CAZymes) to monosaccharides such as glucose and xylose, which are then metabolized through the Embden-Meyerhof pathway to generate energy carrier compounds. After that, pyruvate metabolism takes place in the cytosol and hydrogenosomes, producing various metabolic end products like acetate, succinate, ethanol, hydrogen, formate, and lactate [107,269]. The end products of anaerobic fungi are important substrates in *C. kluyveri* metabolism and are involved in energy production and biosynthesis of cellular components

[270]. *C. kluyveri* can break down fatty acids into medium-chain carboxylates (MCC) through the reverse β-oxidation pathway [31]. Despite this, it has been found that anaerobic fungi are unable to produce high concentrations of ethanol, which can be used as a source of energy, carbon, and reducing equivalents for the production of caproate through a reverse β-oxidation pathway [247].

Some studies have shown that hydrogen can potentially act as an electron donor in the production of caproate from acetate [26,271]. However, the strain in this study did not use hydrogen or lactate as electron donors, likewise, previous results have shown that ethanol cannot be substituted by neither hydrogen nor lactate as electron donors in pure cultures of *C. kluyveri* [246,247]. Ding, H. et al. [272] studied the metabolic pathways of caproate formation in *C. kluyveri* and concluded that caproate is not formed through hydrogenotrophic processes but by hydrogenogenic processes. Moreover, we observed that metabolite production ceased after ethanol was consumed completely, and 0.76 mmol of acetate produced by anaerobic fungi remained unconsumed (**Figure 27A**). Similar results were also observed in the control experiment, where 2.63 mmol acetate and 1.1 mmol ethanol were used as a substrate for *C. kluyveri* fermentation, however, even though 1.57 mmol of acetate was present in the media, the strain could only produce 0.27 mmol of caproate (**Figure 27B**). This shows that as soon as the ethanol content was reduced to zero, the strain stopped producing caproate since a biochemical reaction is thermodynamically only possible when an electron donor is available [273]. In addition, a comparative study of two-step fermentation with and without the addition of ethanol showed that the caproate yield increased significantly, reaching 0.27 mmol/mmol when ethanol was added to the fermentation, with a maximum productivity of 1.31 mmol/day including butyrate and hydrogen. In contrast, without ethanol, the caproate yield and maximum productivity were 0.18 mmol/mmol and 0.59 mmol/day, respectively. However, in the case of butyrate yield, the yield reduced from 0.26 mmol/mmol to 0.12 mmol/mmol when ethanol was added. This proved that ethanol is necessary to maximize the caproate yield as well as for the metabolic pathway to shift toward an increased amount of caproate. According to Grootscholten et al. [274], the addition of ethanol accelerates the chain elongation process in multiculture acidification reactors from municipal solid wastes of organic fraction. Furthermore, a higher ethanol/acetate ratio turned out to be more favorable for caproate production, while butyrate production was more favorable at lower ethanol/acetate ratios [247]. In addition, the hydrogen gas produced during two-step fermentation holds promise as a valuable substrate

for syngas fermentation, which consists of hydrogen, carbon dioxide, and carbon monoxide, with the aim of producing ethanol and feeding it back into the two-step fermentation process as needed for caproate production. This strategic use has the potential to improve the sustainability of the two-step fermentation process, enabling it to fulfill ethanol demand. Research has confirmed the ability of *Clostridium ljungdahlii* to perform syngas fermentation, leading to the production of ethanol [156]. Moreover, a deeper investigation should be conducted into optimizing the fermentation process of anaerobic fungi to maximize the amount of acetate and ethanol they produce from Enset fiber.

4.5 Conclusions

In this study caproate production from Enset fiber was realized in one-pot two-step fermentation using *N. cameroonii* and *C. kluyveri*. The results showed that *N. cameroonii* grew better on Enset fiber and produced more metabolites than with wheat straw. A successful method was developed to convert anaerobic fungi metabolites into caproate and butyrate. In addition, with this method, hydrogen production increased, and *C. kluyveri* growth was not inhibited by either *N. cameroonii* growth or accumulated organic compounds. Utilizing inexpensive raw materials for caproate production, such as Enset fiber, is an effective way to create a sustainable system and minimize environmental impacts. This novel approach provides valuable data for future research and industrial applications. However, further studies need to be done to maximize the yield and productivity of caproate production, such as optimizing fermentation parameters for one-pot two-step fermentation and conducting feasibility studies on the process to ensure that it can be applied to industrial processes.

5. Final Conclusions and Future Perspectives

An efficient way to establish a sustainable system and mitigate environmental impacts is to use low-cost feedstocks such as Enset biomass for biorefinery processes. The Enset plant is a potential food source for about 20 million Ethiopians. Enset biomass is a waste product of the traditional Ethiopian Enset food processing that is discarded in large amounts. In this thesis, the processing potential of Enset biomass for various biorefinery processes was investigated.

The carbon and hydrogen content of Enset biomass were comparable to that of typical agricultural lignocellulose biomasses, indicating its suitability for energy production. However, the calorific values of Enset biomass fell below the standard; therefore, Enset biomass could not be recommended for thermochemical processes. Enset biomass parts mainly consisted of 36–67% cellulose, 16–20% hemicelluloses, and less than 6.8% lignin. Enset fiber had the highest cellulose content among all parts, similar to banana and pineapple fiber. In addition, the Enset biomass had low levels of inhibitory compounds, making it a promising feedstock for bioconversion processes.

Enset biomass is a potential bioenergy source, however, it needs to be processed before ABE fermentation. This process includes pretreatment and enzymatic hydrolysis, which modify the structure of Enset biomass and releases simple sugars from the polysaccharides. The dilute alkali pretreatment method shows higher glucose production and faster hydrolysis rate compared to the dilute acid pretreatment method. Pretreatment of the Enset biomass with 2% (w/v) NaOH improved cellulose digestibility and degraded the hemicellulose content of the biomass. However, further research is needed to find the optimal alkaline concentration and to explore other pretreatment methods that are both cost-effective and environmentally friendly.

C. saccharoperbutylacetonicum utilized Enset biomass hydrolysate as a substrate for ABE fermentation without requiring additional detoxification or sugar supplementation. Comparable butanol production was achieved in Enset biomass hydrolysate compared to pure glucose as a substrate using SHF method. However, the strain on Enset biomass hydrolysate had a slower growth rate than pure glucose, which might be due to the preculture adaptation and substrate inhibition. In addition, we found that *C. saccharoperbutylacetonicum* consumed various sugars from Enset biomass hydrolysate, such as glucose, xylose, mannose, and galactose.

Furthermore, *C. saccharoperbutylacetonicum* grew and produced butanol and hydrogen from pretreated Enset fiber using SSF process within a temperature range of 28 to 37 °C. The production of hydrogen and butanol from affordable materials like Enset fiber is crucial for ensuring the sustainability of the biofuel industry. However, to achieve the highest yield for both products, the process parameters need to be separately optimized for each product. This is because the process parameters influence cell metabolism and can potentially shift the metabolic pathway in favour of hydrogen or butanol production. In a bottle experiment, a maximum butanol concentration of 11.36 g/L with a yield of 0.23 g/g and a productivity of 0.16 g $L^{-1}h^{-1}$, and a maximum hydrogen amount of 18.86 mmol with a yield of 168.99 mL/g-Enset fiber were achieved under different optimal parameters for each product. In addition, a scaled-up SSF process from pretreated Enset fiber was successfully established using a 2.5 L stirred tank bioreactor. By maintaining a slight overpressure inside the bioreactor and optimizing the process parameters, a comparable butanol yield was achieved as in the bottle experiment. However, the butanol productivity in the bioreactor experiment decreased. This was due to poor mass transfer between substrate and enzymes in the SSF process. To address this issue, the PSSF method was introduced, incorporating a 2–3 hours prehydrolysis before inoculation. This approach alleviated the mass transfer problem and enhanced the butanol concentration and productivity by 30% and 27%, respectively, compared to the SSF process. This study found that the PSSF process achieved the highest butanol concentration and yield compared to previous studies with different biomass and *Clostridial* strains. However, butanol productivity was lower than in a previous study using *C. saccharobutylicum* from corn stover at 37 °C [200]. More research is needed to boost butanol productivity from Enset fiber by using a strain that can tolerate high temperatures or cold-active cellulase. Furthermore, hydrogen production in the bioreactor experiment was maximized by operating at atmospheric pressure and optimum process conditions. Hydrogen production increased by 79.7% under atmospheric pressure and optimal process conditions in the bioreactor compared to the overpressure experiment at optimum conditions for butanol production. This is the first study to use this strain and substrate combination for the SSF process, and it contributes to the advancement of bioengineering. However, more research on 5L, 10L bioreactors as well as pilot-scale processes is essential to make the SSF process from Enset fiber viable for industrial-scale production.

Finally, this study successfully produced additional valuable products, thereby contributing to the expansion of the biorefinery product range derived from Enset biomass without the

need for pretreatment. A novel method was developed to produce caproate directly from Enset fiber without pretreatment or external enzymes in a one-pot two-step fermentation using anaerobic fungi (*N. cameroonii*) and *C. kluyveri*. In two step fermentation, *N. cameroonii* grew on untreated Enset fiber and produced acetate, succinate, ethanol, hydrogen, formate and lactate. After inoculation with *C. kluyveri*, caproate and butyrate were produced, and hydrogen production also increased compared to sole *N. cameroonii* fermentation. This innovative approach offers a sustainable and viable solution for producing biofuels and bioproducts from Enset biomass. One challenge in the two-step fermentation process was the requirement for ethanol as an electron donor. To overcome this challenge, ethanol was added to the culture during *C. kluyveri* inoculation, leading to enhanced caproate and hydrogen production, along with increased acetate consumption. To further optimize the biorefinery process, the two-step fermentation can be integrated into syngas fermentation, with the aim of producing ethanol and feeding it back into the two-step fermentation process as necessary for caproate production. This strategic integration holds the potential to enhance the sustainability of the two-step fermentation process. Moreover, further studies are required to maximize caproate production including the optimization of fermentation parameters for the two-step fermentation.

In summary, Enset biomass can be efficiently utilized for biorefinery processes through various bioconversion methods, including ABE fermentation, anaerobic fungi fermentation, and chain elongation processes, with or without treatment. However, it is important to assess the economic and environmental impacts of Enset biomass valorisation for the biorefinery process using methods such as techno-economic analysis, life-cycle assessment, and social-impact assessment. This thesis plays a crucial role in advancing a circular bioeconomy and a sustainable energy system by providing valuable insights for future research and industrial applications.

List of References

1. Jong, E. de; Higson, A.; Walsh, P.; Wellisch, M. Biobased Chemicals - Value Added Products from Biorefineries. *A Rep. Prep. IEA Bioenergy-Task* **2011**, 36. https://www.ieabioenergy.com/blog/publications/bio-based-chemicals-value-added-products-from-biorefineries/

2. Calvo-Flores, F.G.; Martin-Martinez, F.J. Biorefineries: Achievements and challenges for a bio-based economy. *Front. Chem.* **2022**, *10*, 1–23, doi:10.3389/fchem.2022.973417.

3. Chowdhury, R.; Ghosh, S.; Debnath, B.; Manna, D. Indian agro-wastes for 2G biorefineries: Strategic decision on conversion processes. *Green Energy Technol.* **2018**, *0*, 353–373, doi:10.1007/978-981-10-7188-1_16.

4. Kumar, B.; Bhardwaj, N.; Agrawal, K.; Chaturvedi, V.; Verma, P. Current perspective on pretreatment technologies using lignocellulosic biomass: An emerging biorefinery concept. *Fuel Process. Technol.* **2020**, *199*, doi:10.1016/j.fuproc.2019.106244.

5. Zhu, L.D.; Hiltunen, E. Application of livestock waste compost to cultivate microalgae for bioproducts production: A feasible framework. *Renew. Sustain. Energy Rev.* **2016**, *54*, 1285–1290, doi:10.1016/j.rser.2015.10.093.

6. Guerrero, A.B.; Ballesteros, I.; Ballesteros, M. The potential of agricultural banana waste for bioethanol production. *Fuel* **2018**, *213*, 176–185, doi:10.1016/j.fuel.2017.10.105.

7. Srirangan, K.; Akawi, L.; Moo-Young, M.; Chou, C.P. Towards sustainable production of clean energy carriers from biomass resources. *Appl. Energy* **2012**, *100*, 172–186, doi:10.1016/j.apenergy.2012.05.012.

8. Cherubini, F. The biorefinery concept: Using biomass instead of oil for producing energy and chemicals. *Energy Convers. Manag.* **2010**, *51*, 1412–1421, doi:10.1016/j.enconman.2010.01.015.

9. Ndaba, B.; Chiyanzu, I.; Marx, S. N-Butanol derived from biochemical and chemical routes: A review. *Biotechnol. Reports* **2015**, *8*, 1–9, doi:10.1016/j.btre.2015.08.001.

10. ECETOC *JACC report of n-Butanol (CAS No. 71- 3 6-3)*; 2003;

11. Kujawska, A.; Kujawski, J.; Bryjak, M.; Kujawski, W. ABE fermentation products

recovery methods - A review. *Renew. Sustain. Energy Rev.* **2015**, *48*, 648–661, doi:10.1016/j.rser.2015.04.028.

12. Michael, K.; Steffi, N.; Peter, D. The Past, Present, and Future of Biofuels – Biobutanol as Promising Alternative. In *Intech*; 2016; Vol. 11, p. 13 doi: 10.5772/20113.

13. Liu, H.; Wang, G.; Zhang, J. The Promising Fuel-Biobutanol. In *Intech*; 2013; Vol. 11, p. 25 doi: 10.5772/52535.

14. Richa, Kothari Anita, Singh Naveen Kumar, A. *Biomass, Bioenergy & Bioeconomy*; 2022; ISBN 978-981-19-2912-0.

15. Abo, B.O.; Gao, M.; Wang, Y.; Wu, C.; Wang, Q.; Ma, H. Production of butanol from biomass: recent advances and future prospects. *Environ. Sci. Pollut. Res.* **2019**, *26*, 20164–20182, doi:10.1007/s11356-019-05437-y.

16. Guo, Y.; Liu, Y.; Guan, M.; Tang, H.; Wang, Z.; Lin, L.; Pang, H. Production of butanol from lignocellulosic biomass: recent advances, challenges, and prospects. *RSC Adv.* **2022**, *12*, 18848–18863, doi:10.1039/d1ra09396g.

17. Momirlan, M.; Veziroglu, T.N. The properties of hydrogen as fuel tomorrow in sustainable energy system for a cleaner planet. *Int. J. Hydrogen Energy* **2005**, *30*, 795–802, doi:10.1016/j.ijhydene.2004.10.011.

18. Ramachandran, R.; Menon, R.K. An overview of industrial uses of hydrogen. *Int. J. Hydrogen Energy* **1998**, *23*, 593–598, doi:10.1016/s0360-3199(97)00112-2.

19. Fayaz, H.; Saidur, R.; Razali, N.; Anuar, F.S.; Saleman, A.R.; Islam, M.R. An overview of hydrogen as a vehicle fuel. *Renew. Sustain. Energy Rev.* **2012**, *16*, 5511–5528, doi:10.1016/j.rser.2012.06.012.

20. Jain, I.P. Hydrogen the fuel for 21st century. *Int. J. Hydrogen Energy* **2009**, *34*, 7368–7378, doi:10.1016/j.ijhydene.2009.05.093.

21. Singh, V.; Yadav, S.; Sen, R.; Das, D. Concomitant hydrogen and butanol production via co-digestion of organic wastewater and nitrogenous residues. *Int. J. Hydrogen Energy* **2020**, *45*, 24477–24490, doi:10.1016/j.ijhydene.2020.06.282.

22. Holladay, J.D.; Hu, J.; King, D.L.; Wang, Y. An overview of hydrogen production technologies. *Catal. Today* **2009**, *139*, 244–260, doi:10.1016/j.cattod.2008.08.039.

23. Dincer, I.; Acar, C. Review and evaluation of hydrogen production methods for better sustainability. *Int. J. Hydrogen Energy* **2014**, *40*, 11094–11111, doi:10.1016/j.ijhydene.2014.12.035.

24. Villafán-Vidales, H.I.; Arancibia-Bulnes, C.A.; Riveros-Rosas, D.; Romero-Paredes, H.; Estrada, C.A. An overview of the solar thermochemical processes for hydrogen and syngas production: Reactors, and facilities. *Renew. Sustain. Energy Rev.* **2017**, *75*, 894–908, doi:10.1016/j.rser.2016.11.070.

25. Ramprakash, B.; Lindblad, P.; Eaton-Rye, J.J.; Incharoensakdi, A. Current strategies and future perspectives in biological hydrogen production: A review. *Renew. Sustain. Energy Rev.* **2022**, *168*, 112773, doi:10.1016/j.rser.2022.112773.

26. Yin, Y.; Zhang, Y.; Karakashev, D.B.; Wang, J.; Angelidaki, I. Biological caproate production by *Clostridium kluyveri* from ethanol and acetate as carbon sources. *Bioresour. Technol.* **2017**, *241*, 638–644, doi:10.1016/j.biortech.2017.05.184.

27. Chen, W.S.; Strik, D.P.B.T.B.; Buisman, C.J.N.; Kroeze, C. Production of Caproic Acid from Mixed Organic Waste: An Environmental Life Cycle Perspective. *Environ. Sci. Technol.* **2017**, *51*, 7159–7168, doi:10.1021/acs.est.6b06220.

28. Otten, J.K.; Zou, Y.; Papoutsakis, E.T. The potential of caproate (hexanoate) production using *Clostridium kluyveri* syntrophic cocultures with *Clostridium acetobutylicum* or *Clostridium saccharolyticum*. *Front. Bioeng. Biotechnol.* **2022**, *10*, 1–14, doi:10.3389/fbioe.2022.965614.

29. Nzeteu, C.O.; Trego, A.C.; Abram, F.; O'Flaherty, V. Reproducible, high-yielding, biological caproate production from food waste using a single-phase anaerobic reactor system. *Biotechnol. Biofuels* **2018**, *11*, 1–14, doi:10.1186/s13068-018-1101-4.

30. Steinbusch, K.J.J.; Hamelers, H.V.M.; Plugge, C.M.; Buisman, C.J.N. Biological formation of caproate and caprylate from acetate: Fuel and chemical production from low grade biomass. *Energy Environ. Sci.* **2011**, *4*, 216–224, doi:10.1039/c0ee00282h.

31. Cavalcante, W. de A.; Leitão, R.C.; Gehring, T.A.; Angenent, L.T.; Santaella, S.T. Anaerobic fermentation for n-caproic acid production: A review. *Process Biochem.* **2017**, *54*, 106–119, doi:10.1016/j.procbio.2016.12.024.

32. Isikgor, F.H.; Becer, C.R. Lignocellulosic biomass: a sustainable platform for the production of bio-based chemicals and polymers. *Polym. Chem.* **2015**, *6*, 4497–4559,

doi:10.1039/c5py00263j.

33. Cao, G.; Sheng, Y. Biobutanol Production from Lignocellulosic Biomass: Prospective and Challenges. *J. Bioremediation Biodegrad.* **2016**, *7*, doi:10.4172/2155-6199.1000363.

34. Okolie, J.A.; Nanda, S.; Dalai, A.K.; Kozinski, J.A. Chemistry and Specialty Industrial Applications of Lignocellulosic Biomass. *Waste and Biomass Valorization* **2021**, *12*, 2145–2169, doi:10.1007/s12649-020-01123-0.

35. Scheller, H.V.; Ulvskov, P. Hemicelluloses. *Annu. Rev. Plant Biol.* **2010**, *61*, 263–289, doi:10.1146/annurev-arplant-042809-112315.

36. Katahira, R.; Elder, T.J.; Beckham, G.T. A Brief Introduction to Lignin Structure. *RSC Energy Environ. Ser.* **2018**, *2018-Janua*, 1–20, doi:10.1039/9781788010351-00001.

37. Zoghlami, A.; Paës, G. Lignocellulosic Biomass: Understanding Recalcitrance and Predicting Hydrolysis. *Front. Chem.* **2019**, *7*, doi:10.3389/fchem.2019.00874.

38. Chaturvedi, T.; Torres, A.I.; Stephanopoulos, G.; Thomsen, M.H.; Schmidt, J.E. Developing Process Designs for Biorefineries—Definitions, Categories, and Unit Operations. *Energies* **2020**, *13*, 22, doi:10.3390/en13061493.

39. Jatoi, A.S.; Abbasi, S.A.; Hashmi, Z.; Shah, A.K.; Alam, M.S.; Bhatti, Z.A.; Maitlo, G.; Hussain, S.; Khandro, G.A.; Usto, M.A.; et al. Recent trends and future perspectives of lignocellulose biomass for biofuel production: a comprehensive review. *Biomass Convers. Biorefinery* **2023**, *13*, 6457–6469, doi:10.1007/s13399-021-01853-8.

40. Mankar, A.R.; Pandey, A.; Modak, A.; Pant, K.K. Pretreatment of lignocellulosic biomass: A review on recent advances. *Bioresour. Technol.* **2021**, *334*, 125235, doi:10.1016/j.biortech.2021.125235.

41. Seid, N.; Griesheimer, P.; Neumann, A. Investigating the Processing Potential of Ethiopian Agricultural Residue Enset/*Ensete ventricosum* for Biobutanol Production. *Bioengineering* **2022**, *9*, 1–14, doi:10.3390/bioengineering9040133.

42. Bhatnagar, R.; Gupta, G.; Yadav, S. A Review on Composition and Properties of Banana Fibers. *Int. J. Sci. Eng. Res.* **2015**, *6*, 49–52, ISSN 2229-5518.

43. Kim, H.; Ahn, Y.; Kwak, S.Y. Comparing the influence of acetate and chloride anions on the structure of ionic liquid pretreated lignocellulosic biomass. *Biomass and Bioenergy* **2016**, *93*, 243–253, doi:10.1016/j.biombioe.2016.07.022.

44. Kim, D. Physico-chemical conversion of lignocellulose: Inhibitor effects and detoxification strategies: A mini review. *Molecules* **2018**, *23*, doi:10.3390/molecules23020309.

45. Martín, C.; Klinke, H.B.; Thomsen, A.B. Wet oxidation as a pretreatment method for enhancing the enzymatic convertibility of sugarcane bagasse. *Enzyme Microb. Technol.* **2007**, *40*, 426–432, doi:10.1016/j.enzmictec.2006.07.015.

46. Westphal, E. *Agricultural systems in Ethiopia*; Centre for Agricultural Publishing and Documentation Wageningen 1975;

47. Brandt, S.; Spring, A.; Hiebsch, C.; McCabe, J.T.; Tabogie, E.; Wolde-Michael, G.; Yntiso, G.; Shigeta, M.; Tesfaye, S. *The " Tree Against Hunger " Enset-Based Agricultural Systems in Ethiopia*; 1997;

48. Birmeta, G.; Nybom, H.; Bekele, E. Distinction between wild and cultivated enset (*Ensete ventricosum*) gene pools in Ethiopia using RAPD markers. *Hereditas* **2004**, *140*, 139–148, doi:10.1111/j.1601-5223.2004.01792.x.

49. Berhanu, H.; Kiflie, Z.; Miranda, I.; Lourenço, A.; Ferreira, J.; Feleke, S.; Yimam, A.; Pereira, H. Characterization of crop residues from false banana /*ensete ventricosum*/ in Ethiopia in view of a full-resource valorization. *PLoS One* **2018**, *13*, 1–21, doi:10.1371/journal.pone.0199422.

50. Tsegayei, A.; Struik, P.C. Enset (*Ensete ventricosum* (Welw .) Cheesman) kocho yield under different crop establishment methods as compared to yields of other carbohydrate-rich food crops. *NJAS - Wageningen J. Life Sci.* **2001**, *49*, 81–94, doi:10.1016/S1573-5214(01)80017-8.

51. Fekadu, D.; Ledin, I. Weight and chemical composition of the plant parts of enset and the intake and degradability of enset by cattle. *Livest. Prod. Sci.* **1997**, *49*, 249–257, doi:10.1016/S0301-6226(97)00047-X

52. Nurfeta, A.; Eik, L.O.; Adugna Tolera; Sundstøl, F. Chemical composition and in sacco dry matter degradability of different morphological fractions of 10 enset (*Ensete ventricosum*) varieties. *Anim. Feed Sci. Technol.* **2008**, *146*, 55–73,

doi:10.1016/j.anifeedsci.2007.12.003.

53. Haile, A.T.; Kovi, M.R.; Johnsen, S.S.; Tesfaye, B.; Hvoslef-Eide, T.; Rognli, O.A. Genetic diversity, population structure and selection signatures in Enset (*Ensete ventricosum*, (Welw.) Cheesman), an underutilized and key food security crop in Ethiopia. *Genet. Resour. Crop Evol.* **2023**, doi:10.1007/s10722-023-01683-9.

54. Dalle, G.; Daba, D. Diversity and uses of enset [*Ensete ventricosum* (Welw.) Cheesman] varieties in Angacha district, Southern Ethiopia: call for taxonomic identifications and conservation. *Genet. Resour. Crop Evol.* **2021**, *68*, 485–498, doi:10.1007/s10722-020-00998-1.

55. Central Statistical Agency *The Federal Democratic Republic of Ethiopia Report on Area and Production of Majr Crops*; 2020; Vol. I;

56. Zerfu, A.; Gebre, S.L.; Berecha, G.; Getahun, K. Assessment of spatial distribution of enset plant diversity and enset bacteria wilt using geostatistical techniques in Yem special district, Southern Ethiopia. *Environ. Syst. Res.* **2018**, *7*, doi:10.1186/s40068-018-0126-9.

57. Koch, O.; Mengesha, W.A.; Pironon, S.; Pagella, T.; Ondo, I.; Rosa, I.; Wilkin, P.; Borrell, J.S. Modelling potential range expansion of an underutilised food security crop in Sub-Saharan Africa. *Environ. Res. Lett.* **2022**, *17*, doi:10.1088/1748-9326/ac40b2.

58. Sirany, T.; Tadele, E.; Hibistu, T.; Kefalew, A.; Reta, H. Economic Viability and Use Dynamics of the Enset Food System in Ethiopia: Its Implications for Food Security. *Adv. Agric.* **2022**, *2022*, doi:10.1155/2022/5741528.

59. Fanta, S.W.; Neela, S. A review on nutritional profile of the food from enset: A staple diet for more than 25 per cent population in Ethiopia. *Nutr. Food Sci.* **2019**, *49*, 824–843, doi:10.1108/NFS-11-2018-0306.

60. James S., B.; Goodwin, M.; Guy, B.; Kim, J.; Sebsebe, D.; Abebe M., W.; Dawd, G.; Ermias, L.; Zemede, A.; Wilkin, P. Enset-based agricultural systems in Ethiopia A systematic review of production. *Plants People Planet* **2019**, doi: 10.1002/ppp3.10084.

61. Hunduma, T.; Ashenafi, M. Effect of altitude on microbial succession during traditional Enset (*Ensete Ventricosum*) fermentation. *Int. J. Food, Nutr. Public Heal.*

Vol. 4, No. 1, **2011.**

62. Gizachew, T.; Abdeta, T.; Abdo, H. Development and Evaluation of Enset Fermenting Box. *Int. J. Eng. Res. Technol.* **2019**, *V8*, 667–676, doi:10.17577/ijertv8is100334.

63. Seid, N.; Ochsenreither, K.; Neumann, A. Caproate production from Enset fiber in one-pot two-step fermentation using anaerobic fungi (*Neocallimastix cameroonii* strain G341) and *Clostridium kluyveri* DSM 555. *Microb. Cell Fact.* **2023**, *22*, 1–12, doi:10.1186/s12934-023-02224-w.

64. Seid, N.; Wießner, L.; Aliyu, H.; Neumann, A. Stirring the Hydrogen and Butanol production from Enset Fiber via Simultaneous Saccharification and Fermentation (SSF) process. **2024**, 1–31, doi:10.21203/rs.3.rs-3836946/v1.

65. Lamore Erebo, D. Evaluation of Bioethanol Production from Enset (*Ensete ventricosum* (Welw.) Cheesman) Processing Waste and Leaf Using *Saccharomyces cerevisiae*. *Bioprocess Eng.* **2021**, *5*, 49, doi:10.11648/j.be.20210502.13.

66. Berhanu, H.; Kiflie, Z.; Neiva, D.; Gominho, J.; Feleke, S.; Yimam, A.; Pereira, H. Optimization of ethanol-alkali delignification of false banana (*Ensete ventricosum*) fibers for pulp production using response surface methodology. *Ind. Crops Prod.* **2018**, *126*, 426–433, doi:10.1016/j.indcrop.2018.08.093.

67. Temesgen, A.G.; Turşucular, Ö.F.; Eren, R.; Aykut, Y. Potential of Ethiopian Enset Fiber for Textile Application. In Proceedings of the International fiber and polymer research symposium 24-25 January 2020; 2020; pp. 63–66.

68. Tenaye, T.; Mohammed, S.A.; Jabasingh, S.A. Sustainable synthesis and characterization of Enset cellulose nanocrystals (E-CNp) from *Enset ventricosum* biomass and its application in the fabrication of Enset cellulose nanocomposite (E-CNc). *Biomass Convers. Biorefinery* **2022**, doi:10.1007/s13399-022-02682-z.

69. Esayas, E.; C Agon Ass, E.; Assefa, S. Development of Wall Construction Material Stabilized with Enset Vegetable Fibers for Rural Housing Units. *Am. J. Civ. Eng. Archit.* **2018**, *6*, 54–62, doi:10.12691/ajcea-6-2-2.

70. Wondimu, A.; Molla, F.; Dinda, S.C.; Gebre-Samuel, N.; Tadese, E.T. Literature Review on Enset Starch: Physico-Chemical Properties and Pharmaceutical Applications. *J. Drug Deliv. Ther.* **2014**, *4*, 1–6, doi:10.22270/jddt.v4i3.822.

71. Hirose, R.; Tezuka, Y.; Kondo, T.; Hirao, K.; Hatta, T.; Nemoto, S.; Saio, K.; Takahashi, S.; Kainuma, K. Characteristic Physico-chemical Properties and Potential Uses of Enset (*Ensete ventricosum*) Starch: Comparative Studies with Starches of Potato, Sago and Corn. *J. Appl. Glycosci.* **2010**, *57*, 185–192, doi:10.5458/jag.57.185.

72. Agrawal, R.; Verma, A.; Singhania, R.R.; Varjani, S.; Di Dong, C.; Kumar Patel, A. Current understanding of the inhibition factors and their mechanism of action for the lignocellulosic biomass hydrolysis. *Bioresour. Technol.* **2021**, *332*, doi:10.1016/j.biortech.2021.125042.

73. Baruah, J.; Nath, B.K.; Sharma, R.; Kumar, S.; Deka, R.C.; Baruah, D.C.; Kalita, E. Recent trends in the pretreatment of lignocellulosic biomass for value-added products. *Front. Energy Res.* **2018**, *6*, 1–19, doi:10.3389/fenrg.2018.00141.

74. Rodionova, M. V.; Bozieva, A.M.; Zharmukhamedov, S.K.; Leong, Y.K.; Chi-Wei Lan, J.; Veziroglu, A.; Veziroglu, T.N.; Tomo, T.; Chang, J.S.; Allakhverdiev, S.I. A comprehensive review on lignocellulosic biomass biorefinery for sustainable biofuel production. *Int. J. Hydrogen Energy* **2022**, *47*, 1481–1498, doi:10.1016/j.ijhydene.2021.10.122.

75. Jönsson, L.J.; Martín, C. Pretreatment of lignocellulose: Formation of inhibitory by-products and strategies for minimizing their effects. *Bioresour. Technol.* **2016**, *199*, 103–112, doi:10.1016/j.biortech.2015.10.009.

76. Tramontina, R.; Brenelli, L.B.; Sodré, V.; Franco Cairo, J.P.; Travália, B.M.; Egawa, V.Y.; Goldbeck, R.; Squina, F.M. Enzymatic removal of inhibitory compounds from lignocellulosic hydrolysates for biomass to bioproducts applications. *World J. Microbiol. Biotechnol.* **2020**, *36*, 1–11, doi:10.1007/s11274-020-02942-y.

77. Das, N.; Jena, P.K.; Padhi, D.; Kumar Mohanty, M.; Sahoo, G. A comprehensive review of characterization, pretreatment and its applications on different lignocellulosic biomass for bioethanol production. *Biomass Convers. Biorefinery* **2023**, *13*, 1503–1527, doi:10.1007/s13399-021-01294-3.

78. Tian, S.Q.; Zhao, R.Y.; Chen, Z.C. Review of the pretreatment and bioconversion of lignocellulosic biomass from wheat straw materials. *Renew. Sustain. Energy Rev.* **2018**, *91*, 483–489, doi:10.1016/j.rser.2018.03.113.

79. Shirkavand, E.; Baroutian, S.; Gapes, D.J.; Young, B.R. Combination of fungal and

physicochemical processes for lignocellulosic biomass pretreatment - A review. *Renew. Sustain. Energy Rev.* **2016**, *54*, 217–234, doi:10.1016/j.rser.2015.10.003.

80. Hernández-Beltrán, J.U.; Hernández-De Lira, I.; Cruz-Santos, M.; Saucedo-Luevanos, A.; Hernández-Terán, F.; Balagurusamy, N. Insight into Pretreatment Methods of Lignocellulosic Biomass to Increase Biogas Yield: Current State, Challenges, and Opportunities. *Appl. Sci.* **2019**, *9*, 3721, doi:10.3390/app9183721.

81. B. R. Prasad; Padhi, R.K.; Ghosh, G. A review on key pretreatment approaches for lignocellulosic biomass to produce biofuel and value - added products. *Int. J. Environ. Sci. Technol.* **2023**, *20*, 6929–6944, doi:10.1007/s13762-022-04252-2.

82. Sharma, H.K.; Xu, C.; Qin, W. Biological Pretreatment of Lignocellulosic Biomass for Biofuels and Bioproducts: An Overview. *Waste and Biomass Valorization* **2019**, *10*, 235–251, doi:10.1007/s12649-017-0059-y.

83. Saini, J.K.; Himanshu; Hemansi; Kaur, A.; Mathur, A. Strategies to enhance enzymatic hydrolysis of lignocellulosic biomass for biorefinery applications: A review. *Bioresour. Technol.* **2022**, *360*, 127517, doi:10.1016/j.biortech.2022.127517.

84. Zhen, F.; Richard L., S.J.; Xinhua, Q. *Production of Platform Chemicals from Sustainable Resources*; **2017**, doi:10.1007/978-981-10-4172-3.

85. Wright, J.D.; Wyman, C.E.; Grohmann, K. Simultaneous saccharification and fermentation of lignocellulose - Process evaluation. *Appl. Biochem. Biotechnol.* **1988**, *18*, 75–90, doi:10.1007/BF02930818.

86. Hamid, S.B.A.; Islam, M.M.; Das, R. Cellulase biocatalysis: key influencing factors and mode of action. *Cellulose* **2015**, *22*, 2157–2182, doi:10.1007/s10570-015-0672-5.

87. Amit, K.; Nakachew, M.; Yilkal, B.; Mukesh, Y. A review of factors affecting enzymatic hydrolysis of pretreated lignocellulosic Biomass. *Res. J. Chem. Environ.* **2018**, *22*, 62–67.

88. Yang, B.; Dai, Z.; Ding, S.Y.; Wyman, C.E. Enzymatic hydrolysis of cellulosic biomass. *Biofuels* **2011**, *2*, 421–449, doi:10.4155/bfs.11.116.

89. Van Dyk, J.S.; Pletschke, B.I. A review of lignocellulose bioconversion using enzymatic hydrolysis and synergistic cooperation between enzymes-Factors affecting enzymes, conversion and synergy. *Biotechnol. Adv.* **2012**, *30*, 1458–1480,

doi:10.1016/j.biotechadv.2012.03.002.

90. Mansfield, S.D.; Mooney, C.; Saddler, J.N. Substrate and enzyme characteristics that limit cellulose hydrolysis. *Biotechnol. Prog.* **1999**, *15*, 804–816, doi:10.1021/bp9900864.

91. Adams, M.R. The birth of modern industrial microbiology: The acetone-butanol fermentation. *Int. J. Hist. Eng. Technol.* **2017**, *87*, 81–95, doi:10.1080/17581206.2017.1329970.

92. Birgen, C.; Dürre, P.; Preisig, H.A.; Wentzel, A. Butanol production from lignocellulosic biomass: Revisiting fermentation performance indicators with exploratory data analysis. *Biotechnol. Biofuels* **2019**, *12*, 1–15, doi:10.1186/s13068-019-1508-6.

93. Moon, H.G.; Jang, Y.S.; Cho, C.; Lee, J.; Binkley, R.; Lee, S.Y. One hundred years of *Clostridial* butanol fermentation. *FEMS Microbiol. Lett.* **2016**, *363*, doi:10.1093/femsle/fnw001.

94. Lin, Z.; Cong, W.; Zhang, J. Biobutanol Production from Acetone–Butanol–Ethanol Fermentation: Developments and Prospects. *Fermentation* **2023**, *9*, 1–17, doi:10.3390/fermentation9090847.

95. Jones, D.T.; Woods, D.R. Acetone-butanol fermentation revisited. *Microbiol. Rev.* **1986**, *50*, 484–524, doi:10.1128/mmbr.50.4.484-524.1986.

96. Hevroni, B.L.; Moraïs, S.; Ben-David, Y.; Morag, E.; Bayer, E.A. Minimalistic Cellulosome of the Butanologenic Bacterium *Clostridium saccharoperbutylacetonicum*. *Appl. Environ. Sci.* **2020**, *11*, 1–17, doi:10.1128/mbio.00443-20.

97. Grosse-Honebrink, A.; Little, G.T.; Bean, Z.; Heldt, D.; Cornock, R.H.M.; Winzer, K.; Minton, N.P.; Green, E.; Zhang, Y. Development of *Clostridium saccharoperbutylacetonicum* as a Whole Cell Biocatalyst for Production of Chirally Pure (R)-1,3-Butanediol. *Front. Bioeng. Biotechnol.* **2021**, *9*, 1–13, doi:10.3389/fbioe.2021.659895.

98. Nasr, N.; Gupta, M.; Hafez, H.; El Naggar, M.H.; Nakhla, G. Mono- and co-substrate utilization kinetics using mono- and co-culture of *Clostridium beijerinckii* and *Clostridium saccharoperbutylacetonicum*. *Bioresour. Technol.* **2017**, *241*, 152–160,

doi:10.1016/j.biortech.2017.05.086.

99. Baba, S. ichi; Tashiro, Y.; Shinto, H.; Sonomoto, K. Development of high-speed and highly efficient butanol production systems from butyric acid with high density of living cells of *Clostridium saccharoperbutylacetonicum*. *J. Biotechnol.* **2012**, *157*, 605–612, doi:10.1016/j.jbiotec.2011.06.004.

100. Ivarsson, M.; Schnürer, A.; Bengtson, S.; Neubeck, A. Anaerobic fungi: A potential source of biological H_2 in the oceanic crust. *Front. Microbiol.* **2016**, *7*, 1–8, doi:10.3389/fmicb.2016.00674.

101. Stabel, M.; Schweitzer, T.; Haack, K.; Gorenflo, P.; Aliyu, H.; Ochsenreither, K. Isolation and biochemical characterization of six anaerobic fungal strains from zoo animal feces. *Microorganisms* **2021**, *9*, doi:10.3390/microorganisms9081655.

102. Marvin-Sikkema, F.D.; Lahpor, G.A.; Kraak, M.N.; Gottschal, J.C.; Prins, R.A. Characterization of an anaerobic fungus from llama faeces. *J. Gen. Microbiol.* **1992**, *138*, 2235–2241, doi:10.1099/00221287-138-10-2235.

103. Gruninger, R.J.; Puniya, A.K.; Callaghan, T.M.; Edwards, J.E.; Youssef, N.; Dagar, S.S.; Fliegerova, K.; Griffith, G.W.; Forster, R.; Tsang, A.; Anaerobic fungi (phylum *Neocallimastigomycota*): Advances in understanding their taxonomy, life cycle, ecology, role and biotechnological potential. *FEMS Microbiol. Ecol.* **2014**, *90*, 1–17, doi:10.1111/1574-6941.12383.

104. Ma, J.; Zhong, P.; Li, Y.; Sun, Z.; Sun, X.; Aung, M.; Hao, L.; Cheng, Y.; Zhu, W. Hydrogenosome, Pairing Anaerobic Fungi and H_2-Utilizing Microorganisms Based on Metabolic Ties to Facilitate Biomass Utilization. *J. Fungi* **2022**, *8*, doi:10.3390/jof8040338.

105. Roghair, M.; Liu, Y.; Strik, D.P.B.T.B.; Weusthuis, R.A.; Bruins, M.E.; Buisman, C.J.N. Development of an effective chain elongation process from acidified food waste and ethanol into n-Caproate. *Front. Bioeng. Biotechnol.* **2018**, *6*, 1–11, doi:10.3389/fbioe.2018.00050.

106. Roghair, M.; Liu, Y.; Adiatma, J.C.; Weusthuis, R.A.; Bruins, M.E.; Buisman, C.J.N.; Strik, D.P.B.T.B. Effect of n-Caproate Concentration on Chain Elongation and Competing Processes. *ACS Sustain. Chem. Eng.* **2018**, *6*, 7499–7506, doi:10.1021/acssuschemeng.8b00200.

107. Stabel, M.; Haack, K.; Lübbert, H.; Greif, M.; Gorenflo, P.; Aliyu, H.; Ochsenreither, K. Metabolic shift towards increased biohydrogen production during dark fermentation in the anaerobic fungus *Neocallimastix cameroonii* G341. *Biotechnol. Biofuels Bioprod.* **2022**, *15*, 1–16, doi:10.1186/s13068-022-02193-z.

108. Candry, P.; Van Daele, T.; Denis, K.; Amerlinck, Y.; Andersen, S.J.; Ganigué, R.; Arends, J.B.A.; Nopens, I.; Rabaey, K. A novel high-throughput method for kinetic characterisation of anaerobic bioproduction strains applied to *Clostridium kluyveri*. *Sci. Rep.* **2018**, *8*, 1–13, doi:10.1038/s41598-018-27594-9.

109. Candry, P.; Carvajal-Arroyo, J.M.; Pratt, S.; Sousa, J.; Akyol, Ç.; Fatone, F.; Ganigué, R. Anaerobic fermentation technologies for the production of chemical building blocks and bio-based products from wastewater. In *Resource Recovery from Water*; 2022; pp. 159–195 ISBN 9781780409566.

110. Amid, A. *Recombinant Enzymes— From Basic Science to Commercialization*; Springer International Publishing Switzerland, 2015; ISBN 9783319123967.

111. Li, Z.; Lu, H.; Zhang, Z.; Liu, B. Study on Scale-Up of Anaerobic Fermentation Mixing with Different Solid Content. *Fermentation* **2023**, *9*, doi:10.3390/fermentation9040375.

112. Lee, J.M. *Biochemical engineering*; Prentice-hall international series in the physical and chemical engineering sciences, 1st Edition, 1992.

113. Xu, S.; Bowers, J.; Seamans, T.C.; Nyberg, G. Bioreactor Scale-Up. In *John Wiley & Sons, Inc.*; 2018; pp. 1–35 ISBN 0471238961.

114. Ruocco, G. *Introduction to transport phenomena modeling: A multiphysics, general equation-based approach*; 2018; ISBN 9783319668222.

115. Treybal, R.E. *Mass-Transfer Operations*; McGraw-Hill Book Company, Vol. 4; 1981.

116. Sun, C.; Meng, X.; Sun, F.; Zhang, J.; Tu, M.; Chang, J.S.; Reungsang, A.; Xia, A.; Ragauskas, A.J. *Advances and perspectives on mass transfer and enzymatic hydrolysis in the enzyme-mediated lignocellulosic biorefinery: A review*; 2023; Vol. 62; ISBN 0734975022001.

117. Zhang, H.; Chen, L.; Lu, M.; Li, J.; Han, L. A novel film-pore-surface diffusion model to explain the enhanced enzyme adsorption of corn stover pretreated by ultrafine

grinding. *Biotechnol. Biofuels* **2016**, *9*, 1–13, doi:10.1186/s13068-016-0602-2.

118. Valgepea, K.; De Souza Pinto Lemgruber, R.; Abdalla, T.; Binos, S.; Takemori, N.; Takemori, A.; Tanaka, Y.; Tappel, R.; Köpke, M.; Simpson, S.D.; et al. H2 drives metabolic rearrangements in gas-fermenting *Clostridium autoethanogenum*. *Biotechnol. Biofuels* **2018**, *11*, 1–15, doi:10.1186/s13068-018-1052-9.

119. Boyadjiev, C. Mass Transfer Theories. In *Theoretical Chemical Engineering: Modeling and Simulation*; 2010; pp. 1–594 ISBN 9783642107771.

120. Morsi, B.I.; Basha, O.M. Mass Transfer in Multiphase Systems. In *INTECH*; 2016; Vol. 11, p. 13, doi:10.5772/60516.

121. Noorman, H.J. Basic Biotechnology. In *Cambridge University Press*; 2006; pp. 201–218 ISBN 9780511802409.

122. Pauss, A.; Andre, G.; Perrier, M.; Guiot, S.R. Liquid-to-Gas mass transfer in anaerobic processes: Inevitable transfer limitations of methane and hydrogen in the biomethanation process. *Appl. Environ. Microbiol.* **1990**, *56*, 1636–1644, doi:10.1128/aem.56.6.1636-1644.1990.

123. Afolalu, S.A.; Yusuf, O.O.; Abioye, A.A.; Emetere, M.E.; Ongbali, S.O.; Samuel, O.D. Biofuel; A Sustainable Renewable Source of Energy-A Review. *IOP Conf. Ser. Earth Environ. Sci.* **2021**, *665*, doi:10.1088/1755-1315/665/1/012040.

124. Malode, S.J.; Prabhu, K.K.; Mascarenhas, R.J.; Shetti, N.P.; Aminabhavi, T.M. Recent advances and viability in biofuel production. *Energy Convers. Manag. X* **2021**, *10*, 100070, doi:10.1016/j.ecmx.2020.100070.

125. Negash, M.; Swinnen, J.F.M. Biofuels and food security: Micro-evidence from Ethiopia. *Energy Policy* **2013**, *61*, 963–976, doi:10.1016/j.enpol.2013.06.031.

126. Guta, D.D. Assessment of Biomass Fuel Resource Potential And Utilization in Ethiopia : Sourcing Strategies for Renewable Energies. *Int. J. Renew. ENERGY Res.* **2012**, *2*.

127. Berhanu, M.; Jabasingh, S.A.; Kifile, Z. Expanding sustenance in Ethiopia based on renewable energy resources – A comprehensive review. *Renew. Sustain. Energy Rev.* **2017**, *75*, 1035–1045, doi:10.1016/j.rser.2016.11.082.

128. Chandel, A.K.; Garlapati, V.K.; Jeevan Kumar, S.P.; Hans, M.; Singh, A.K.; Kumar,

S. The role of renewable chemicals and biofuels in building a bioeconomy. *Biofuels, Bioprod. Biorefining* **2020**, *14*, 830–844, doi:10.1002/bbb.2104.

129. Li, H.G.; Luo, W.; Gu, Q.Y.; Wang, Q.; Hu, W.J.; Yu, X. Bin Acetone, butanol, and ethanol production from cane molasses using *Clostridium beijerinckii* mutant obtained by combined low-energy ion beam implantation and N-methyl-N-nitro-N-nitrosoguanidine induction. *Bioresour. Technol.* **2013**, *137*, 254–260, doi:10.1016/j.biortech.2013.03.084.

130. Savaliya, M.L.; Dhorajiya, B.D.; Dholakiya, B.Z. Recent advancement in production of liquid biofuels from renewable resources: A review; 2015; Vol. 41, doi:10.1007/s11164-017-3177-z.

131. Hönig, V.; Kotek, M.; Mařík, J. Use of butanol as a fuel for internal combustion engines. *Agron. Res.* **2014**, *12*, 333–340.

132. Cheng, H.H.; Whang, L.M.; Chan, K.C.; Chung, M.C.; Wu, S.H.; Liu, C.P.; Tien, S.Y.; Chen, S.Y.; Chang, J.S.; Lee, W.J. Biological butanol production from microalgae-based biodiesel residues by *Clostridium acetobutylicum. Bioresour. Technol.* **2015**, *184*, 379–385, doi:10.1016/j.biortech.2014.11.017.

133. Qureshi, N.; Saha, B.C.; Dien, B.; Hector, R.E.; Cotta, M.A. Production of butanol (a biofuel) from agricultural residues: Part I - Use of barley straw hydrolysate. *Biomass and Bioenergy* **2010**, *34*, 559–565, doi:10.1016/j.biombioe.2009.12.024.

134. Ruan, Z.; Zanotti, M.; Archer, S.; Liao, W.; Liu, Y. Oleaginous fungal lipid fermentation on combined acid- and alkali-pretreated corn stover hydrolysate for advanced biofuel production. *Bioresour. Technol.* **2014**, *163*, 12–17, doi:10.1016/j.biortech.2014.03.095.

135. Singhania, R.R.; Saini, J.K.; Saini, R.; Adsul, M.; Mathur, A.; Gupta, R.; Tuli, D.K. Bioethanol production from wheat straw via enzymatic route employing penicillium janthinellum cellulases. *Bioresour. Technol.* **2014**, *169*, 490–495, doi:10.1016/j.biortech.2014.07.011.

136. Dagnino, E.P.; Chamorro, E.R.; Romano, S.D.; Felissia, F.E.; Area, M.C. Optimization of the acid pretreatment of rice hulls to obtain fermentable sugars for bioethanol production. *Ind. Crops Prod.* **2013**, *42*, 363–368, doi:10.1016/j.indcrop.2012.06.019.

137. Laser, M.; Schulman, D.; Allen, S.G.; Lichwa, J.; Antal, M.J.; Lynd, L.R. A comparison of liquid hot water and steam pretreatments of sugar cane bagasse for bioconversion to ethanol. *Bioresour. Technol.* **2002**, *81*, 33–44, doi:10.1016/S0960-8524(01)00103-1.

138. Baral, N.R.; Shah, A. Microbial inhibitors : formation and effects on acetone-butanol-ethanol fermentation of lignocellulosic biomass. *Appl Microbiol Biotechnol* **2014**, 9151–9172, doi:10.1007/s00253-014-6106-8.

139. Shahzadi, T.; Mehmood, S.; Irshad, M.; Anwar, Z.; Afroz, A.; Zeeshan, N.; Rashid, U.; Sughra, K. Advances in lignocellulosic biotechnology: A brief review on lignocellulosic biomass and cellulases. *Adv. Biosci. Biotechnol.* **2014**, *05*, 246–251, doi:10.4236/abb.2014.53031.

140. Mohammed, B.; Gabel, M.; Karlsson, L.M. Nutritive values of the drought tolerant food and fodder crop enset. **2013**, *8*, 2326–2333, doi:10.5897/AJAR12.1296.

141. Borrell, J.S.; Biswas, M.K.; Goodwin, M.; Blomme, G.; Schwarzacher, T.; Heslop-Harrison, J.S.; Wendawek, A.M.; Berhanu, A.; Kallow, S.; Janssens, S.; et al. Enset in Ethiopia: A poorly characterized but resilient starch staple. *Ann. Bot.* **2019**, *123*, 747–766, doi:10.1093/aob/mcy214.

142. Acero, M.; Mukasa, S.B.; Baguma, Y. Morphotypes, distribution and uses of false banana in Uganda. *African Crop Sci. J.* **2018**, *26*, 575, doi:10.4314/acsj.v26i4.10.

143. Hunduma, T.; Mogessie, A. Traditional Enset (*Ensete ventricosum*) Processing Techniques in Some Parts of West Shewa Zone, Ethiopia. *J. Agric. Dev.* **2011**, *2*, 37–57.

144. Erebo, D.L. Evaluation of Bioethanol Production from Enset (*Ensete ventricosum* (Welw .) Cheesman) Processing Waste and Leaf Using *Saccharomyces cerevisiae*. *Sci. Publ. Gr.* **2021**, *5*, 49–55, doi:10.11648/j.be.20210502.13.

145. Wang, X.; Zhang, Z.; Wang, Y.; Wang, Y. Improvement of acetone – butanol – ethanol (ABE) production from switchgrass pretreated with a radio frequency-assisted heating process. *Fuel* **2016**, *182*, 166–173, doi:10.1016/j.fuel.2016.05.108.

146. ANKOM Technology, Determining acid detergent lignin in beakers; 2020;

147. Ankom Technology, Neutral detergent fiber in feeds - filter bag technique *(for A2000 and A2000I)*; 2011;

148. ANKOM Technology, Acid Detergent Fiber in Feeds - Filter Bag Technique *(for A200 and A200I)*; 2017;

149. Van Soest, P.J.; Robertson, J.B.; Lewis, B.A. Methods for Dietary Fiber, Neutral Detergent Fiber, and Nonstarch Polysaccharides in Relation to Animal Nutrition. *J. Dairy Sci.* **1991**, *74*, 3583–3597, doi:10.3168/jds.S0022-0302(91)78551-2.

150. Hindrichsen, I.K.; Kreuzer, M.; Madsen, J.; Bach Knudsen, K.E. Fiber and lignin analysis in concentrate, forage, and feces: Detergent versus enzymatic-chemical method. *J. Dairy Sci.* **2006**, *89*, 2168–2176, doi:10.3168/jds.S0022-0302(06)72287-1.

151. Sluiter, A.; Hames, B.; Ruiz, R.; Scarlata, C.; Sluiter, J.; Templeton, D. Determination of Sugars , Byproducts , and Degradation Products in Liquid Fraction Process Samples; 2008;

152. Gao, K.; Boiano, S.; Marzocchella, A.; Rehmann, L. Cellulosic butanol production from alkali-pretreated switchgrass (*Panicum virgatum*) and phragmites (*Phragmites australis*). *Bioresour. Technol.* **2014**, *174*, 176–181, doi:10.1016/j.biortech.2014.09.152.

153. Sluiter, A.; Hames, B.; Hyman, D.; Payne, C.; Ruiz, R.; Scarlata, C.; Sluiter, J.; Templeton, D.; Wolfe, J. Determination of Total Solids in Biomass and Total Dissolved Solids in Liquid Process Samples; 2008;

154. Procentese, A.; Raganati, F.; Olivieri, G.; Elena Russo, M.; Marzocchella, A. Pre-treatment and enzymatic hydrolysis of lettuce residues as feedstock for bio-butanol production. *Biomass and Bioenergy* **2017**, *96*, 172–179, doi:10.1016/j.biombioe.2016.11.015.

155. Wang, P.; Chen, Y.M.; Wang, Y.; Lee, Y.Y.; Zong, W.; Taylor, S.; McDonald, T.; Wang, Y. Towards comprehensive lignocellulosic biomass utilization for bioenergy production: Efficient biobutanol production from acetic acid pretreated switchgrass with *Clostridium saccharoperbutylacetonicum* N1-4. *Appl. Energy* **2019**, *236*, 551–559, doi:10.1016/j.apenergy.2018.12.011.

156. Infantes, A.; Kugel, M.; Neumann, A. Evaluation of media components and process parameters in a sensitive and robust fed-batch syngas fermentation system with *clostridium ljungdahlii*. *Fermentation* **2020**, *6*, doi:10.3390/fermentation6020061.

157. Ujor, V.; Bharathidasan, A.K.; Cornish, K.; Ezeji, T.C. Evaluation of industrial dairy waste (milk dust powder) for acetone-butanol-ethanol production by solventogenic *Clostridium* species. *Springerplus* **2014**, *3*, 1–10, doi:10.1186/2193-1801-3-387.

158. Yao, D.; Dong, S.; Wang, P.; Chen, T.; Wang, J.; Yue, Z.B.; Wang, Y. Robustness of *Clostridium saccharoperbutylacetonicum* for acetone-butanol-ethanol production: Effects of lignocellulosic sugars and inhibitors. *Fuel* **2017**, *208*, 549–557, doi:10.1016/j.fuel.2017.07.004.

159. Baleeiro, F.C.F.; Kleinsteuber, S.; Sträuber, H. Hydrogen as a Co-electron Donor for Chain Elongation With Complex Communities. *Front. Bioeng. Biotechnol.* **2021**, *9*, 1–15, doi:10.3389/fbioe.2021.650631.

160. Ochsenreither, K.; Fischer, C.; Neumann, A.; Syldatk, C. Process characterization and influence of alternative carbon sources and carbon-to-nitrogen ratio on organic acid production by *Aspergillus oryzae* DSM1863. *Appl. Microbiol. Biotechnol.* **2014**, *98*, 5449–5460, doi:10.1007/s00253-014-5614-x.

161. Neumann, A.; Scholz-Muramatsu, H.; Diekert, G. Tetrachloroethene metabolism of Dehalospirillum multivorans. *Arch. Microbiol.* **1994**, *162*, 295–301, doi:10.1007/s002030050141.

162. García, R.; Pizarro, C.; Lavín, A.G.; Bueno, J.L. Characterization of Spanish biomass wastes for energy use. *Bioresour. Technol.* **2012**, *103*, 249–258, doi:10.1016/j.biortech.2011.10.004.

163. García, R.; Pizarro, C.; Lavín, A.G.; Bueno, J.L. Spanish biofuels heating value estimation . Part I : Ultimate analysis data. *Fuel* **2014**, *117*, 1130–1138, doi:10.1016/j.fuel.2013.08.048.

164. Naik, S.; Goud, V. V.; Rout, P.K.; Jacobson, K.; Dalai, A.K. Characterization of Canadian biomass for alternative renewable biofuel. *Renew. Energy* **2010**, *35*, 1624–1631, doi:10.1016/j.renene.2009.08.033.

165. Ribas, E.; Fernandes, K.; Marangoni, C.; Souza, O.; Sellin, N. Thermochemical characterization of banana leaves as a potential energy source. *ENERGY Convers. Manag.* **2013**, *75*, 603–608, doi:10.1016/j.enconman.2013.08.008.

166. Raj, T.; Kapoor, M.; Gaur, R.; Christopher, J.; Lamba, B.; Tuli, D.K.; Kumar, R. Physical and Chemical Characterization of Various Indian Agriculture Residues for

Biofuels Production. *Energy and Fuels* **2015**, *29,3111-31*, doi:10.1021/ef5027373.

167. Librenti, E., Ceotto, E., & Di Candilo, M. Biomass characteristics and energy contents of dedicated lignocellulosic crops. In Proceedings of the Biomass characteristics and energy contents of dedicated lignocellulosic crops lignocellulosic crops; CRA-CIN, Research Center for Industrial Crops: Bologna, Italy, 2018; p. 9.

168. Sohni, S.; Norulaini, N.A.N.; Hashim, R.; Khan, S.B.; Fadhullah, W.; Mohd Omar, A.K. Physicochemical characterization of Malaysian crop and agro-industrial biomass residues as renewable energy resources. *Ind. Crops Prod.* **2018**, *111*, 642–650, doi:10.1016/j.indcrop.2017.11.031.

169. Diyana, Z.N.; Jumaidin, R.; Selamat, M.Z.; Alamjuri, R.H.; Yusof, F.A. Extraction and Characterization of Natural Cellulosic Fiber from *Pandanus amaryllifolius* Leaves. *Polymers (Basel).* **2021**, *13*, 16, doi:10.3390/polym13234171.

170. Adapa, P.; Tabil, L.; Schoenau, G. Compaction characteristics of barley , canola , oat and wheat straw. *Biosyst. Eng.* **2009**, *104*, 335–344, doi:10.1016/j.biosystemseng.2009.06.022.

171. Mani, S.; Tabil, L.G.; Sokhansanj, S. Effects of compressive force , particle size and moisture content on mechanical properties of biomass pellets from grasses. *Biomass and Bioenergy* **2008**, *30*, 648–654, doi:10.1016/j.biombioe.2005.01.004.

172. Guimarães, J.L.; Frollini, E.; Silva, C.G.; Wypych, F.; Satyanarayana, K.G. Characterization of banana , sugarcane bagasse and sponge gourd fibers of Brazil. *Ind. Crop. Prod. J.* **2009**, *30*, 407–415, doi:10.1016/j.indcrop.2009.07.013.

173. Melbi Mahardika , Hairul Abral, Anwar Kasim, S.A. and M.A. Production of Nanocellulose from Pineapple Leaf Fibers via High-Shear Homogenization and Ultrasonication. *fibers* **2018**, *6*, 1–12, doi:10.3390/fib6020028.

174. Anwar, Z.; Gulfraz, M.; Irshad, M. Agro-industrial lignocellulosic biomass a key to unlock the future bio-energy : A brief review. *J. Radiat. Res. Appl. Sci.* **2014**, *7*, 163–173, doi:10.1016/j.jrras.2014.02.003.

175. Raud, M.; Kikas, T.; Sippula, O.; Shurpali, N.J. Potentials and challenges in lignocellulosic biofuel production technology. *Renew. Sustain. Energy Rev.* **2019**, *111*, 44–56, doi:10.1016/j.rser.2019.05.020.

176. Nanda, S.; Mohanty, P.; Pant, K.K.; Naik, S.; Kozinski, J.A.; Dalai, A.K.

Characterization of North American Lignocellulosic Biomass and Biochars in Terms of their Candidacy for Alternate Renewable Fuels. *Bioenergy Res.* **2013**, *6*, 663–677, doi:10.1007/s12155-012-9281-4.

177. Rasmussen, H.; Sørensen, H.R.; Meyer, A.S. Formation of degradation compounds from lignocellulosic biomass in the biorefinery: Sugar reaction mechanisms. *Carbohydr. Res.* **2014**, *385*, 45–57, doi:10.1016/j.carres.2013.08.029.

178. Haldar, D.; Sen, D.; Gayen, K. A review on the production of fermentable sugars from lignocellulosic biomass through conventional and enzymatic route - a comparison. *Int. J. Green Energy* **2016**, *13*, 1232–1253, doi:10.1080/15435075.2016.1181075.

179. Zhu, L.; O'Dwyer, J.P.; Chang, V.S.; Granda, C.B.; Holtzapple, M.T. Structural features affecting biomass enzymatic digestibility. *Bioresour. Technol.* **2008**, *99*, 3817–3828, doi:10.1016/j.biortech.2007.07.033.

180. Lorenci, A.; José, C.; Neto, D.; Porto, L.; Vandenberghe, D.S.; Pedro, D.; Neto, D.C.; Cristine, A.; Sydney, N.; Alberto, L.; et al. Lignocellulosic biomass: Acid and alkaline pretreatments and their effects on biomass recalcitrance – Conventional processing and recent advances. *Bioresour. Technol.* **2020**, *304*, 122848, doi:10.1016/j.biortech.2020.122848.

181. Gupta, A.; Prakash, J. Sustainable bio-ethanol production from agro-residues : A review. *Renew. Sustain. Energy Rev.* **2015**, *41*, 550–567, doi:10.1016/j.rser.2014.08.032.

182. Gao, K.; Rehmann, L. ABE fermentation from enzymatic hydrolysate of NaOH-pretreated corncobs. *Biomass and Bioenergy* **2014**, *66*, 110–115, doi:10.1016/j.biombioe.2014.03.002.

183. Cho, D.H.; Shin, S.J.; Kim, Y.H. Effects of acetic and formic acid on ABE production by *clostridium acetobutylicum* and *clostridium beijerinckii*. *Biotechnol. Bioprocess Eng.* **2012**, *17*, 270–275, doi:10.1007/s12257-011-0498-4.

184. Antranikian, G.; Friese, C.; Quentmeier, A.; Hippe, H.; Gottschalk, G. Distribution of the ability for citrate utilization amongst *Clostridia*. *Arch. Microbiol.* **1984**, *138*, 179–182, doi:10.1007/BF00402115.

185. Kihara, T.; Noguchi, T.; Tashiro, Y.; Sakai, K.; Sonomoto, K. Highly efficient continuous acetone–butanol–ethanol production from mixed sugars without carbon

catabolite repression. *Bioresour. Technol. Reports* **2019**, *7*, doi:10.1016/j.biteb.2019.03.017.

186. Rajak, J. A Preliminary Review on Impact of Climate change and our Environment with Reference to Global Warming. *Int. J. Environ. Sci.* **2021**, *10*, 11–14, ISSN: 2277-1948.

187. Michael, B.; Maria, A.T. Global Climate Litigation Report: 2023 Status Review; 2023, https://www.unep.org/resources/report/global-climate-litigation-report-2023-status-review.

188. International Energy Agency (IEA) *Global CO2 emissions rebounded to their highest level in history in 2021*, https://www.iea.org/reports/co2-emissions-in-2022.

189. Midhun Prasad, K.; Somasundaram, M.; Anand, B.; Shankar, R.; Rajkumar, P. A Review on Pyrolysis of Agro-waste and Plastic Waste into Biofuels: Shifting to Bio-based Economy. *Bioenergy Res.* **2023**, *16*, 1438–1466, doi:10.1007/s12155-023-10565-y.

190. Yadav, S.; Singh, V.; Mahata, C.; Das, D. Optimization for simultaneous enhancement of biobutanol and biohydrogen production. *Int. J. Hydrogen Energy* **2021**, *46*, 3726–3741, doi:10.1016/j.ijhydene.2020.10.267.

191. Goria, K.; Singh, H.M.; Singh, A.; Kothari, R.; Tyagi, V. V. Insights into biohydrogen production from algal biomass: Challenges, recent advancements and future directions. *Int. J. Hydrogen Energy* **2023**, doi:10.1016/j.ijhydene.2023.03.174.

192. Rajagopalan, G.; He, J.; Yang, K.L. One-pot fermentation of agricultural residues to produce butanol and hydrogen by *Clostridium strain* BOH_3. *Renew. Energy* **2016**, *85*, 1127–1134, doi:10.1016/j.renene.2015.07.051.

193. Sarkar, Begum and Yaser, D. Enhanced biobutanol production using novel *clostridial* fusants in simultaneous saccharifi cation and fermentation of green renewable agriculture residues. *Biofuels, Bioprod. Biorefining* **2014**, *8*, 743, doi:10.1002/BBB.

194. Valles, A.; Álvarez-Hornos, F.J.; Martínez-Soria, V.; Marzal, P.; Gabaldón, C. Comparison of simultaneous saccharification and fermentation and separate hydrolysis and fermentation processes for butanol production from rice straw. *Fuel* **2020**, *282*, doi:10.1016/j.fuel.2020.118831.

195. Kumar, M.; Goyal, Y.; Sarkar, A.; Gayen, K. Comparative economic assessment of

ABE fermentation based on cellulosic and non-cellulosic feedstocks. *Appl. Energy* **2012**, *93*, 193–204, doi:10.1016/j.apenergy.2011.12.079.

196. Zheng, J.; Tashiro, Y.; Wang, Q.; Sonomoto, K. Recent advances to improve fermentative butanol production: Genetic engineering and fermentation technology. *J. Biosci. Bioeng.* **2015**, *119*, 1–9, doi:10.1016/j.jbiosc.2014.05.023.

197. Cheng, C.L.; Lo, Y.C.; Lee, K.S.; Lee, D.J.; Lin, C.Y.; Chang, J.S. Biohydrogen production from lignocellulosic feedstock. *Bioresour. Technol.* **2011**, *102*, 8514–8523, doi:10.1016/j.biortech.2011.04.059.

198. Wu, Y.; Wang, Z.; Ma, X.; Xue, C. High temperature simultaneous saccharification and fermentation of corn stover for efficient butanol production by a thermotolerant *Clostridium acetobutylicum*. *Process Biochem.* **2021**, *100*, 20–25, doi:10.1016/j.procbio.2020.09.026.

199. Chen, C.; Wang, L.; Xiao, G.; Liu, Y.; Xiao, Z.; Deng, Q.; Yao, P. Continuous acetone-butanol-ethanol (ABE) fermentation and gas production under slight pressure in a membrane bioreactor. *Bioresour. Technol.* **2014**, *163*, 6–11, doi:10.1016/j.biortech.2014.04.004.

200. Dong, J.J.; Ding, J.C.; Zhang, Y.; Ma, L.; Xu, G.C.; Han, R.Z.; Ni, Y. Simultaneous saccharification and fermentation of dilute alkaline-pretreated corn stover for enhanced butanol production by *Clostridium saccharobutylicum* DSM 13864. *FEMS Microbiol. Lett.* **2016**, *363*, 1–6, doi:10.1093/femsle/fnw003.

201. Fatehi, P. Recent advancements in various steps of ethanol, butanol, and isobutanol productions from woody materials. *Biotechnol. Prog.* **2013**, *29*, 297–310, doi:10.1002/btpr.1688.

202. Guan, W.; Shi, S.; Tu, M.; Lee, Y.Y. Acetone-butanol-ethanol production from Kraft paper mill sludge by simultaneous saccharification and fermentation. *Bioresour. Technol.* **2016**, *200*, 713–721, doi:10.1016/j.biortech.2015.10.102.

203. Qi, G.; Huang, D.; Wang, J.; Shen, Y.; Gao, X. Enhanced butanol production from ammonium sulfite pretreated wheat straw by separate hydrolysis and fermentation and simultaneous saccharification and fermentation. *Sustain. Energy Technol. Assessments* **2019**, *36*, 100549, doi:10.1016/j.seta.2019.100549.

204. Salleh, M.S.M.; Ibrahim, M.F.; Roslan, A.M.; Abd-Aziz, S. Improved Biobutanol

Production in 2-L Simultaneous Saccharification and Fermentation with Delayed Yeast Extract Feeding and in-situ Recovery. *Sci. Rep.* **2019**, *9*, 1–9, doi:10.1038/s41598-019-43718-1.

205. Cheng, C.; Bao, T.; Yang, S.T. Engineering *Clostridium* for improved solvent production: recent progress and perspective. *Appl. Microbiol. Biotechnol.* **2019**, *103*, 5549–5566, doi:10.1007/s00253-019-09916-7.

206. Md Razali, N.A.A.; Ibrahim, M.F.; Bahrin, E.K.; Abd-Aziz, S. Optimisation of simultaneous saccharification and fermentation (SSF) for biobutanol production using pretreated oil palm empty fruit bunch. *Molecules* **2018**, *23*, doi:10.3390/molecules23081944.

207. Singh, V.; Singh, H.; Das, D. Optimization of the medium composition for the improvement of hydrogen and butanol production using *Clostridium saccharoperbutylacetonicum* DSM 14923. *Int. J. Hydrogen Energy* **2019**, *44*, 26905–26919, doi:10.1016/j.ijhydene.2019.08.125.

208. Alalayah, W.M.; Kalil, M.S.; Kadhum, A.A.H.; Jahim, J.M.; Alauj, N.M. Hydrogen production using *Clostridium saccharoperbutylacetonicum* N1-4 (ATCC 13564). *Int. J. Hydrogen Energy* **2008**, *33*, 7392–7396, doi:10.1016/j.ijhydene.2008.09.066.

209. Wu, H.; Wang, C.; Chen, P.; He, A.Y.; Xing, F.X.; Kong, X.P.; Jiang, M. Effects of pH and ferrous iron on the coproduction of butanol and hydrogen by *Clostridium beijerinckii* IB4. *Int. J. Hydrogen Energy* **2017**, *42*, 6547–6555, doi:10.1016/j.ijhydene.2017.02.094.

210. Nakayama, S.; Bando, Y.; Ohnishi, A.; Kadokura, T.; Nakazato, A. Decreased hydrogen production leads to selective butanol production in co-cultures of *Clostridium thermocellum* and *Clostridium saccharoperbutylacetonicum* strain N1-4. *J. Biosci. Bioeng.* **2013**, *115*, 173–175, doi:10.1016/j.jbiosc.2012.08.020.

211. Yerushalmi, L.; Volesky, B.; Szczesny, T. Effect of increased hydrogen partial pressure on the acetone-butanol fermentation by *Clostridium acetobutylicum*. *Appl. Microbiol. Biotechnol.* **1985**, *22*, 103–107, doi:10.1007/BF00250028.

212. Olofsson, K.; Bertilsson, M.; Lidén, G. A short review on SSF – an interesting process option for ethanol production from lignocellulosic feedstocks. **2008**, *14*, 1–14, doi:10.1186/1754-6834-1-7.

213. Balsan, G.; Astolfi, V.; Benazzi, T.; Meireles, M.A.A.; Maugeri, F.; Di Luccio, M.; Dal Prá, V.; Mossi, A.J.; Treichel, H.; Mazutti, M.A. Characterization of a commercial cellulase for hydrolysis of agroindustrial substrates. *Bioprocess Biosyst. Eng.* **2012**, *35*, 1229–1237, doi:10.1007/s00449-012-0710-8.

214. Öhgren, K.; Bura, R.; Saddler, J.; Zacchi, G. Effect of hemicellulose and lignin removal on enzymatic hydrolysis of steam pretreated corn stover. *Bioresour. Technol.* **2007**, *98*, 2503–2510, doi:10.1016/j.biortech.2006.09.003.

215. Dos Santos, A.C.; Ximenes, E.; Kim, Y.; Ladisch, M.R. Lignin–Enzyme Interactions in the Hydrolysis of Lignocellulosic Biomass. *Trends Biotechnol.* **2019**, *37*, 518–531, doi:10.1016/j.tibtech.2018.10.010.

216. Qureshi, N.; Singh, V.; Liu, S.; Ezeji, T.C.; Saha, B.C.; Cotta, M.A. Process integration for simultaneous saccharification, fermentation, and recovery (SSFR): Production of butanol from corn stover using *Clostridium beijerinckii* P260. *Bioresour. Technol.* **2014**, *154*, 222–228, doi:10.1016/j.biortech.2013.11.080.

217. Al-Shorgani, N.K.N.; Shukor, H.; Abdeshahian, P.; Mohd Nazir, M.Y.; Kalil, M.S.; Hamid, A.A.; Wan Yusoff, W.M. Process optimization of butanol production by *Clostridium saccharoperbutylacetonicum* N1-4 (ATCC 13564) using palm oil mill effluent in acetone-butanol-ethanol fermentation. *Biocatal. Agric. Biotechnol.* **2015**, *4*, 244–249, doi:10.1016/j.bcab.2015.02.004.

218. Kumar Ramamoorthy, N.; Sambavi, T.R.; Renganathan, S. A study on cellulase production from a mixture of lignocellulosic wastes. *Process Biochem.* **2019**, *83*, 148–158, doi:10.1016/j.procbio.2019.05.006.

219. Oberoi, H.S.; Vadlani, P. V.; Saida, L.; Bansal, S.; Hughes, J.D. Ethanol production from banana peels using statistically optimized simultaneous saccharification and fermentation process. *Waste Manag.* **2011**, *31*, 1576–1584, doi:10.1016/j.wasman.2011.02.007.

220. He, C.R.; Kuo, Y.Y.; Li, S.Y. Lignocellulosic butanol production from Napier grass using semi-simultaneous saccharification fermentation. *Bioresour. Technol.* **2017**, *231*, 101–108, doi:10.1016/j.biortech.2017.01.039.

221. Brosseau, J.D.; Yan, J. -Y; Lo, K.V. The relationship between hydrogen gas and butanol production by *Clostridium saccharoperbutylacetonicum*. *Biotechnol. Bioeng.*

1986, *28*, 305–310, doi:10.1002/bit.260280302.

222. Stein, U.H.; Abbasi-Hosseini, M.; Kain, J.; Fuchs, W.; Bochmann, G. Influence of gas-release strategies on the production of biohydrogen and biobutanol in ABE fermentation. *Biofuels* **2022**, *13*, 9–15, doi:10.1080/17597269.2019.1619246.

223. Lee, S.Y.; Park, J.H.; Jang, S.H.; Nielsen, L.K.; Kim, J.; Jung, K.S. Fermentative butanol production by C*lostridia*. *Biotechnol. Bioeng.* **2008**, *101*, 209–228, doi:10.1002/bit.22003.

224. Yang, X.; Tu, M.; Xie, R.; Adhikari, S.; Tong, Z. A comparison of three pH control methods for revealing effects of undissociated butyric acid on specific butanol production rate in batch fermentation of *Clostridium acetobutylicum*. *AMB Express* **2013**, *3*, 1–8, doi:10.1186/2191-0855-3-3.

225. Cebreiros, F.; Ferrari, M.D.; Lareo, C. Cellulose hydrolysis and IBE fermentation of eucalyptus sawdust for enhanced biobutanol production by *Clostridium beijerinckii* DSM 6423. *Ind. Crops Prod.* **2019**, *134*, 50–61, doi:10.1016/j.indcrop.2019.03.059.

226. Weiss, N.D.; Felby, C.; Thygesen, L.G. Enzymatic hydrolysis is limited by biomass-water interactions at high-solids: Improved performance through substrate modifications. *Biotechnol. Biofuels* **2019**, *12*, 1–13, doi:10.1186/s13068-018-1339-x.

227. Wingren, A.; Galbe, M.; Zacchi, G. Techno-economic evaluation of producing ethanol from softwood: Comparison of SSF and SHF and identification of bottlenecks. *Biotechnol. Prog.* **2003**, *19*, 1109–1117, doi:10.1021/bp0340180.

228. Pratto, B.; Chandgude, V.; de Sousa, R.; Cruz, A.J.G.; Bankar, S. Biobutanol production from sugarcane straw: Defining optimal biomass loading for improved ABE fermentation. *Ind. Crops Prod.* **2020**, *148*, doi:10.1016/j.indcrop.2020.112265.

229. Zhao, T.; Tashiro, Y.; Sonomoto, K. Smart fermentation engineering for butanol production: designed biomass and consolidated bioprocessing systems. *Appl. Microbiol. Biotechnol.* **2019**, *103*, 9359–9371, doi:10.1007/s00253-019-10198-2.

230. Wang, Y. Bin; Gao, C.; Zheng, Z.; Liu, F.M.; Zang, J.Y.; Miao, J.L. Immobilization of cold-active cellulase from antarctic bacterium and its use for kelp cellulose ethanol fermentation. *BioResources* **2015**, *10*, 1757–1772, doi:10.15376/biores.10.1.1757-1772.

231. Ahmed, S.F.; Rafa, N.; Mofijur, M.; Badruddin, I.A.; Inayat, A.; Ali, M.S.; Farrok,

O.; Yunus Khan, T.M. Biohydrogen Production From Biomass Sources: Metabolic Pathways and Economic Analysis. *Front. Energy Res.* **2021**, *9*, 1–13, doi:10.3389/fenrg.2021.753878.

232. Dahman, Y.; Syed, K.; Begum, S.; Roy, P.; Mohtasebi, B. Biofuels: Their characteristics and analysis; Elsevier Ltd, 2019; ISBN 9780081024263.

233. Li, J.; Zhang, Y.; Shi, S.; Tu, M. Effect of residual extractable lignin on acetone-butanol-ethanol production in SHF and SSF processes. *Biotechnol. Biofuels* **2020**, *13*, 1–12, doi:10.1186/s13068-020-01710-2.

234. Ferchichi, M.; Crabbe, E.; Hintz, W.; Gil, G.H.; Almadidy, A. Influence of culture parameters on biological hydrogen production by *Clostridium saccharoperbutylacetonicum* ATCC 27021. *World J. Microbiol. Biotechnol.* **2005**, *21*, 855–862, doi:10.1007/s11274-004-5972-0.

235. Vamsi Krishna, K.; Bharathi, N.; George Shiju, S.; Alagesan Paari, K.; Malaviya, A. An updated review on advancement in fermentative production strategies for biobutanol using *Clostridium* spp.; Springer Berlin Heidelberg, 2022; Vol. 29; ISBN 1135602220637.

236. Foulquier, C.; Rivière, A.; Heulot, M.; Dos Reis, S.; Perdu, C.; Girbal, L.; Pinault, M.; Dusséaux, S.; Yoo, M.; Soucaille, P.; et al. Molecular characterization of the missing electron pathways for butanol synthesis in *Clostridium acetobutylicum*. *Nat. Commun.* **2022**, *13*, 1–11, doi:10.1038/s41467-022-32269-1.

237. Li, D.; Chen, H. Biological hydrogen production from steam-exploded straw by simultaneous saccharification and fermentation. *Int. J. Hydrogen Energy* **2007**, *32*, 1742–1748, doi:10.1016/j.ijhydene.2006.12.011.

238. Fernando, S.; Adhikari, S.; Chandrapal, C.; Murali, N. Biorefineries: Current status, challenges, and future direction. *Energy and Fuels* **2006**, *20*, 1727–1737, doi:10.1021/ef060097w.

239. Sekoai, P.T.; Ghimire, A.; Ezeokoli, O.T.; Rao, S.; Ngan, W.Y.; Habimana, O.; Yao, Y.; Yang, P.; Yiu Fung, A.H.; Yoro, K.O.; et al. Valorization of volatile fatty acids from the dark fermentation waste Streams-A promising pathway for a biorefinery concept. *Renew. Sustain. Energy Rev.* **2021**, *143*, 110971, doi:10.1016/j.rser.2021.110971.

240. Ranganathan, A.; Smith, O.P.; Youssef, N.H.; Struchtemeyer, C.G.; Atiyeh, H.K.; Elshahed, M.S. Utilizing anaerobic fungi for two-stage sugar extraction and biofuel production from lignocellulosic biomass. *Front. Microbiol.* **2017**, *8*, 1–10, doi:10.3389/fmicb.2017.00635.

241. Jin, W.; Cheng, Y.F.; Mao, S.Y.; Zhu, W.Y. Isolation of natural cultures of anaerobic fungi and indigenously associated methanogens from herbivores and their bioconversion of lignocellulosic materials to methane. *Bioresour. Technol.* **2011**, *102*, 7925–7931, doi:10.1016/j.biortech.2011.06.026.

242. Hess, M.; Paul, S.S.; Puniya, A.K.; van der Giezen, M.; Shaw, C.; Edwards, J.E.; Fliegerová, K. Anaerobic Fungi: Past, Present, and Future. *Front. Microbiol.* **2020**, *11*, 1–18, doi:10.3389/fmicb.2020.584893.

243. Arslan, D.; Steinbusch, K.J.J.; Diels, L.; Hamelers, H.V.M.; Strik, D.P.B.T.B.; Buisman, C.J.N.; De Wever, H. Selective short-chain carboxylates production: A review of control mechanisms to direct mixed culture fermentations. *Crit. Rev. Environ. Sci. Technol.* **2016**, *46*, 592–634, doi:10.1080/10643389.2016.1145959.

244. Grootscholten, T.I.M.; Steinbusch, K.J.J.; Hamelers, H.V.M.; Buisman, C.J.N. Chain elongation of acetate and ethanol in an upflow anaerobic filter for high rate MCFA production. *Bioresour. Technol.* **2013**, *135*, 440–445, doi:10.1016/j.biortech.2012.10.165.

245. Duber, A.; Jaroszynski, L.; Zagrodnik, R.; Chwialkowska, J.; Juzwa, W.; Ciesielski, S.; Oleskowicz-Popiel, P. Exploiting the real wastewater potential for resource recovery-: N -caproate production from acid whey. *Green Chem.* **2018**, *20*, 3790–3803, doi:10.1039/c8gc01759j.

246. Kenealy, W.R.; Waselefsky, D.M. Studies on the substrate range of *Clostridium kluyveri*; the use of propanol and succinate. *Arch. Microbiol.* **1985**, *141*, 187–194, doi:10.1007/BF00408056.

247. Angenent, L.T.; Richter, H.; Buckel, W.; Spirito, C.M.; Steinbusch, K.J.J.; Plugge, C.M.; Strik, D.P.B.T.B.; Grootscholten, T.I.M.; Buisman, C.J.N.; Hamelers, H.V.M. Chain Elongation with Reactor Microbiomes: Open-Culture Biotechnology to Produce Biochemicals. *Environ. Sci. Technol.* **2016**, *50*, 2796–2810, doi:10.1021/acs.est.5b04847.

248. Callaghan, T.M.; Podmirseg, S.M.; Hohlweck, D.; Edwards, J.E.; Puniya, A.K.; Dagar, S.S.; Griffith, G.W. Buwchfawromyces eastonii gen. nov., sp. nov.: A new anaerobic fungus (*Neocallimastigomycota*) isolated from buffalo faeces. *MycoKeys* **2015**, *9*, 11–28, doi:10.3897/mycokeys.9.9032.

249. Theodorou, M.K.; Davies, D.R.; Nielsen, B.B.; Lawrence, M.I.G.; Trinci, A.P.J. Determination of growth of anaerobic fungi on soluble and cellulosic substrates using a pressure transducer. *Microbiology* **1995**, *141*, 671–678, doi:10.1099/13500872-141-3-671.

250. Bauchop, T. Biology of gut anaerobic fungi. *BioSystems* **1989**, *23*, 53–64, doi:10.1016/0303-2647(89)90008-7.

251. Borneman, W.S.; Akin, D.E.; Ljungdahl, L.G. Fermentation products and plant cell wall-degrading enzymes produced by monocentric and polycentric anaerobic ruminal fungi. *Appl. Environ. Microbiol.* **1989**, *55*, 1066–1073, doi:10.1128/aem.55.5.1066-1073.1989.

252. Del Río, J.C.; Rencoret, J.; Prinsen, P.; Martínez, Á.T.; Gutiérrez, A.; Ralph, J. Structural characterization of wheat straw lignin. Evidence for a novel monomer in grasses. *Cons. Super. Investig. Científicas* **2013**.

253. Geoffrey L. R. Gordon, M.W.P. Degradation and utilization of cellulose and straw by three different anaerobic fungi from the ovine rumen. *Appl. Environ. Microbiol.* **1989**, *55*, 1703–1710, doi:10.1128/aem.55.7.1703-1710.1989.

254. Sijtsma, L.; Tan, B. Degradation of perennial ryegrass leaf and stem cell walls by the anaerobic fungus *Neocallimastix* sp. strain CS3b. *Appl. Environ. Microbiol.* **1996**, *62*, 1437–1440, doi:10.1128/aem.62.4.1437-1440.1996.

255. Akin, D.E.; Benner, R. Degradation of polysaccharides and lignin by ruminal bacteria and fungi. *Appl. Environ. Microbiol.* **1988**, *54*, 1117–1125, doi:10.1128/aem.54.5.1117-1125.1988.

256. Joblin, K.N.; Naylor, G.E. Fermentation of woods by rumen anaerobic fungi. *FEMS Microbiol. Lett.* **1989**, *65*, 119–122, doi:10.1111/j.1574-6968.1989.tb03608.x.

257. Theodorou, M.K.; Longland, A.C.; Dhanoa, M.S.; Lowe, S.E.; Trinci, A.P.J. Growth of *Neocallimastix* sp. Strain R1 on Italian Ryegrass Hay: Removal of Neutral Sugars from Plant Cell Walls . *Appl. Environ. Microbiol.* **1989**, *55*, 1363–1367,

doi:10.1128/aem.55.6.1363-1367.1989.

258. Sijtsma, L.; Tan, B. Degradation and utilization of grass cell walls by anaerobic fungi isolated from yak, llama and sheep. *Anim. Feed Sci. Technol.* **1993**, *44*, 221–236, doi:10.1016/0377-8401(93)90049-P.

259. Zhu, W.Y.; Theodorou, M.K.; Longland, A.C.; Nielsen, B.B.; Dijkstra, J.; Trinci, A.P.J. Growth and survival of anaerobic fungi in batch and continuous-flow cultures. *Anaerobe* **1996**, *2*, 29–37, doi:10.1006/anae.1996.0004.

260. Kumar, M.; Gayen, K. Developments in biobutanol production : New insights. *Appl. Energy* **2012**, *88*, 1999–2012, doi:10.1016/j.apenergy.2010.12.055.

261. Baleeiro, F.C.F.; Kleinsteuber, S.; Neumann, A.; Sträuber, H. Syngas-aided anaerobic fermentation for medium-chain carboxylate and alcohol production: the case for microbial communities. *Appl. Microbiol. Biotechnol.* **2019**, *103*, 8689–8709, doi:10.1007/s00253-019-10086-9.

262. Benito-Vaquerizo, S.; Diender, M.; Parera Olm, I.; Martins dos Santos, V.A.P.; Schaap, P.J.; Sousa, D.Z.; Suarez-Diez, M. Modeling a co-culture of *Clostridium autoethanogenum* and *Clostridium kluyveri* to increase syngas conversion to medium-chain fatty-acids. *Comput. Struct. Biotechnol. J.* **2020**, *18*, 3255–3266, doi:10.1016/j.csbj.2020.10.003.

263. Fernández-Blanco, C.; Veiga, M.C.; Kennes, C. Efficient production of n-caproate from syngas by a co-culture of *Clostridium aceticum* and *Clostridium kluyveri*. *J. Environ. Manage.* **2022**, *302*, doi:10.1016/j.jenvman.2021.113992.

264. Ganigué, R.; Sánchez-Paredes, P.; Bañeras, L.; Colprim, J. Low fermentation pH is a trigger to alcohol production, but a killer to chain elongation. *Front. Microbiol.* **2016**, *7*, 1–11, doi:10.3389/fmicb.2016.00702.

265. Gildemyn, S.; Molitor, B.; Usack, J.G.; Nguyen, M.; Rabaey, K.; Angenent, L.T. Upgrading syngas fermentation effluent using *Clostridium kluyveri* in a continuous fermentation. *Biotechnol. Biofuels* **2017**, *10*, 1–15, doi:10.1186/s13068-017-0764-6.

266. Li, Y.; Hou, Z.; Shi, Q.; Cheng, Y.; Zhu, W. Methane Production From Different Parts of Corn Stover via a Simple Co-culture of an Anaerobic Fungus and Methanogen. *Front. Bioeng. Biotechnol.* **2020**, *8*, 1–9, doi:10.3389/fbioe.2020.00314.

267. Dollhofer, V.; Callaghan, T.M.; Griffith, G.W.; Lebuhn, M.; Bauer, J. Presence and

transcriptional activity of anaerobic fungi in agricultural biogas plants. *Bioresour. Technol.* **2017**, *235*, 131–139, doi:10.1016/j.biortech.2017.03.116.

268. Yanli, C. Development and application of co-culture for ethanol production by co-fermentation of glucose and xylose: A systematic review. *J. Ind. Microbiol. Biotechnol.* **2011**, *38*, 581–597, doi:10.1007/s10295-010-0894-3.

269. Johannes Hackstein, Scott Baker, Jaap van Hellemond, A.T. Hydrogenosomes of Anaerobic Chytrids: An AlternativeWay to Adapt to Anaerobic Environments Johannes. *Microbiol. Monogr.* **2006**, doi:10.1007/7171.

270. R. K. Thauer, K. Jungermann, H. Henninger, J. Wenning, and K.D. The Energy Metabolism of *Clostridium kluyveri* R. *Eur. J. Biochem.* **1968**, *4*, 173–180, doi: 10.1111/j.1432-1033.1968.tb00189.x.

271. Spirito, C.M.; Richter, H.; Rabaey, K.; Stams, A.J.M.; Angenent, L.T. Chain elongation in anaerobic reactor microbiomes to recover resources from waste. *Curr. Opin. Biotechnol.* **2014**, *27*, 115–122, doi:10.1016/j.copbio.2014.01.003.

272. Ding, H.B.; Tan, G.Y.A.; Wang, J.Y. Caproate formation in mixed-culture fermentative hydrogen production. *Bioresour. Technol.* **2010**, *101*, 9550–9559, doi:10.1016/j.biortech.2010.07.056.

273. Zhang, C.; Yang, L.; Huo, S.; Su, Y.; Zhang, Y. Optimization of the Cell Immobilization-Based Chain-Elongation Process for Efficient n-Caproate Production. *ACS Sustain. Chem. Eng.* **2021**, *9*, 4014–4023, doi:10.1021/acssuschemeng.0c07281.

274. Grootscholten, T.I.M.; Kinsky dal Borgo, F.; Hamelers, H.V.M.; Buisman, C.J.N. Promoting chain elongation in mixed culture acidification reactors by addition of ethanol. *Biomass and Bioenergy* **2013**, *48*, 10–16, doi:10.1016/j.biombioe.2012.11.019.

List of Figures

Figure 1. Lignocellulosic biomass structure. 7

Figure 2. Morphology of Enset plant 9

..... 13

Figure 3. Potential of Enset plant for integrated biorefineries. 13

Figure 4. Degradation products from lignocellulosic biomass. 15

Figure 5. Metabolic pathway of *C. saccharoperbutylacetonicum* 20

Figure 6. Main metabolites in one-pot two-step fermentation process using *N. cameroonii* and *C. Kluyveri*. 23

Figure 7. Mass transfer paths in the SSF process 27

Figure 8. Two-film theory for gas-liquid mass transfer 28

Figure 9. Morphology of Enset biomass based on traditional Enset food processing 35

Figure 10. Biobutanol production process from mixed Enset waste 38

Figure 11. Glucose produced from enzymatic hydrolysis of Enset biomass samples with 5% (dry weight) solid loading, pretreated using different pretreatment methods: (A) alkali pretreatment method; (B) acid pretreatment method. 43

Figure 12. Comparison of the glucose yield after 72 h of enzymatic hydrolysis for acid- and alkali-pretreated Enset biomass samples. 45

Figure 13. Growth profiles of *C. saccharoperbutylacetonicum* DSM 14923 during fermentation of mixed Enset waste hydrolysate and pure glucose (40 g/L) as a control. 46

Figure 14. ABE fermentation using *C. saccharoperbutylacetonicum* DSM 14923 from different carbon source: (A) enzymatic hydrolysates of mixed Enset waste, pretreated with 2% (*w/w*) NaOH; (B) control medium with 40 g/L glucose. 47

Figure 15. Validation experiment at 5% substrate loading, 16 FPU/g cellulase loading, and 100 rpm (A) SSF process, (B) saccharification process. 59

Figure 16. Metabolites in the SSF process from Enset fiber with (A) pH-controlled and (B) pH-uncontrolled 61

Figure 17. Metabolites in the PSSF process at high substrate loading with Enset fiber. 62

Figure 18. Effect of temperature on hydrogen production in the SSF process. 63

Figure 19. Effect of initial pH values on hydrogen production in the SSF process from Enset fiber (A) pH profile and (B) hydrogen production. 64

Figure 20. Effect of gas release strategy in the SSF process from Enset fiber (A) hydrogen partial pressure (B) hydrogen production. 65

Figure 21. Metabolites in the SSF process from the Enset fiber SSF process at atmospheric pressure. 67

Figure 22. Caproate production from Enset fiber in two-step fermentation using *N. cameroonii* and *C. Kluyveri* 82

Figure 23. Gas production and pressure generated during *N. cameroonii* growth on 0.25 g (A) Enset fiber or (B) wheat straw. 84

Figure 24. Soluble products of *N. cameroonii* growth on 0.25 g (A) Enset fiber, (B) wheat straw (C) mol percentage of all metabolites. 85

Figure 25. Pressure value measured at different substrate loading with Enset fiber during *N. cameroonii* growth at the end of fermentation. 86

Figure 26. Fermentation products of *N. cameroonii* growth on different substrate loading of Enset fiber at the end of fermentation. 87

Figure 27. Fermentation products (A) one-pot two-step fermentation using Enset fiber without additional carbon source (B) *C. kluyveri* fermentation using acetate and ethanol as a carbon source. 88

Figure 28. Metabolites from one-pot two-step fermentation using Enset fiber and with the addition of 2.18 mmol ethanol. 89

List of Tables

Table 1. Properties of fuels 3

Table 2. Chemical compositions of lignocellulosic biomass from a variety of sources. 8

Table 3. Proximate and elemental analysis results for Enset biomass parts. 40

Table 4. Lignocellulosic composition of Enset biomass parts. 41

Table 5. Sugars and degradation products in liquid hydrolysate of Enset biomass parts after two-step acid hydrolysis. 42

Table 6. Effect of agitation speed on butanol production in the SSF process and glucose production in the saccharification process in bottles 56

Table 7. Effect of cellulase loading on butanol production in the SSF process and glucose production in the saccharification process in bottles 57

Table 8. Effect of substrate loading on biobutanol production in the SSF process and glucose production in the saccharification process in bottles 58

Table 9. Comparison of butanol production from different biomass using the SSF process in bottle experiments 71

Table 10. Carbon balance for *N. cameroonii* growth on Enset fiber, and two-step fermentation using *N. cameroonii* and *C. kluyveri* on Enset fiber 90

Table 11. Carbon conversion efficiency for *N. cameroonii* growth on different substrate loading of Enset fiber 93

List of Abbreviations

ABE: Acetone-butanol-ethanol

ADF: Acid detergent fiber

ADL: Acid detergent lignin

AFEX: Ammonia fiber explosion

AK: Acetate kinase

BADH: Butyraldehyde dehydrogenase

BDH: Butanol dehydrogenase.

BK: Butyrate kinase

CAZymes: Carbohydrate-active enzymes

CoA: Coenzyme A

CoAT: CoA transferase

EF: Enset fiber

EMP: Emden-Meyerhof-Parnas

FdOR: Ferredoxin oxidoreductase

Fdox: Oxidized ferredoxin

Fd-red: Reduced ferredoxin

FID: Flame ionization detector

Fr: Froude number

GC: Gas chromatography

HMF: 5-Hydroxymethylfurfural

HPLC: High performance liquid chromatography

IEA: International energy agency

L: Leaf

LC: Lower corm

LIS: Lower inflorescence stalk

LS1: Leaf sheath-1

LS2: Leaf sheath-2

LS3: Leaf sheath-3

LS4: Leaf sheath-4

LSP: Leaf sheath peel

M: Midrib

MCC: Medium-chain carboxylates

NDF: Neutral detergent fiber

Np: Power number

OD: Optical density

PFO: Pyruvate-ferredoxin oxidoreductase

PLOT: Porous layer open tubular

PSSF: Prehydrolysis simultaneous saccharification and fermentation

PTA: Phosphotransacetylase

PTB: Phosphotransbutyrylase

Re: Reynolds number

Rnf: Ferredoxin-NAD reductase complex

SHF: Separate hydrolysis and fermentation

SSF: Simultaneous saccharification and fermentation

UC: Upper corm

UIS: Upper inflorescence stalk

WCOT: Wall coated open tubular

Appendix A

Table A1. Monomeric sugars and degradation products in liquid hydrolysate. Enset biomass parts after pretreated with different methods (**A**) alkali pretreatment (2% (*w/v*) NaOH); (**B**) acid pretreatment (2% (*v/v*) H_2SO_4).

(a) alkali pretreatment (2% (*w/v*) NaOH)				
Analysis % (*w/w*)	**Leaf sheath peel**	**Enset fiber**	**Midrib**	**Mixed Enset waste**
Cellobiose	0.04 ± 0.01	0	0.03 ± 0.00	0.07 ± 0.00
Glucose	0.83 ± 0.04	0.36 ± 0.03	1.44 ± 0.12	0.37 ± 0.01
Arabinose	0	0	0.05 ± 0.00	0
Other sugar (xylose, mannose, and galactose)	0.28 ± 0.02	0.17 ± 0.01	0.45 ± 0.06	0.28 ± 0.01
Formic acid	0.84 ± 0.16	0.59 ± 0.00	1.38 ± 0.04	1.03 ± 0.14
Acetic acid	5.41 ± 0.38	7.82 ± 0.01	11.01± 0.56	11.47 ± 0.50
(b) acid pretreatment (2% (*v/v*) H_2SO_4)				
Analysis % (w/w)	**Leaf sheath peel**	**Enset fiber**	**Midrib**	**Mixed Enset waste**
Cellobiose	0.88 ± 0.77	2.09 ± 0.02	1.28 ± 0.02	1.32 ± 0.03
Glucose	10.46 ± 1.27	2.33 ± 0.13	4.71 ± 0.12	7.82 ± 0.20
Arabinose	1.07 ± 0.20	0.38 ± 0.03	3.66 ± 0.09	2.58 ± 0.05
Other sugar (xylose, mannose, and galactose)	7.37 ± 0.39	9.38 ± 0.04	9.33 ± 0.17	7.15 ± 0.12
Formic acid	0	0	0.38 ± 0.05	0.27 ± 0.02
Acetic acid	4.46 ± 0.54	5.95 ± 0.09	7.72 ± 0.87	8.25 ± 0.27

Appendix B

Table B1. Effect of temperature on hydrogen and butanol production in the SSF process from Enset fiber in bottles [a, b,c]

Temp. (°C)	Final glucose concentration (g/L)	Butanol concentration (g/L)	Butanol yield (g/g)	Butanol productivity (g/(L h))	Hydrogen (mmol)	Hydrogen yield (mL/g)	Maximum hydrogen production rate (mmol/h)
30	2.69 ± 0.98	11.25 ± 0.05	0.23	0.17	12.03 ± 0.23	107.79	0.35
35	0.50 ± 0.04	10.59 ± 0.10	0.21	0.15	14.67 ± 0.13	131.44	0.61
37	5.86 ± 0.81	8.30 ± 0.39	0.17	0.12	14.90 ± 0.13	133.50	0.45

[a] All calculations accounted for 2.5 g Enset fiber in 50 mL medium at 72 hours fermentation period; [b] Conversion of mmol to mL of hydrogen was carried out in accordance with the ideal gas law under standard temperature and pressure conditions.; [c] Values are means from triplicate bottles.

Table B2. Effect of initial pH on hydrogen and butanol production in the SSF process from Enset fiber in bottles [a, b,c]

Initial pH	Final glucose concentration (g/L)	Butanol concentration (g/L)	Butanol yield (g/g)	Butanol productivity (g/(L h))	Hydrogen (mmol)	Hydrogen yield (mL/g)	Maximum hydrogen production rate (mmol/h)
5.0	38.00 ± 0.45	0.24 ± 0.20	0.005	0.003	1.74 ± 0.16	15.59	0.10
6.0	5.36 ± 0.62	8.70 ± 0.13	0.174	0.121	15.48 ± 0.34	138.70	0.39
7.0	5.76 ± 0.03	8.74 ± 0.26	0.175	0.121	15.54 ± 0.30	139.24	0.50
8.0	3.5 ± 0.66	9.36 ± 0.20	0.187	0.130	15.52 ± 0.07	139.06	0.53
9.0	30.39 ± 1.46	1.81 ± 0.70	0.036	0.025	5.33 ± 0.87	47.76	0.32

[a] All calculations accounted for 2.5 g Enset fiber in 50 mL medium at 72 hours fermentation period; [b] Conversion of mmol to mL of hydrogen was carried out in accordance with the ideal gas law under standard temperature and pressure conditions.; [c] Values are means from triplicate bottles.

Table B3. Effect of hydrogen partial pressure on hydrogen and biobutanol production in the SSF process from Enset fiber in bottles [a, b,c]

Gas release strategy	Final glucose concentration (g/L)	Butanol concentration (g/L)	Hydrogen (mmol)	Hydrogen yield (mL/g)	Maximum hydrogen production rate (mmol/h)
Without gas release	3.50 ± 0.66	9.36 ± 0.20	15.52 ± 0.07	139.06	0.53
Gas release to 1-L bottle	2.40 ± 0.45	9.23 ± 0.93	17.24 ± 1.59	154.47	0.41
Gas release to 2-L bottle	0.19 ± 0.08	9.65 ± 0.17	18.86 ± 0.81	168.99	0.59

[a] All calculations accounted for 2.5 g Enset fiber in 50 mL medium at 72 hours fermentation period; [b] Conversion of mmol to mL of hydrogen was carried out in accordance with the ideal gas law under standard temperature and pressure conditions.; [c] Values are means from triplicate bottles.

Appendix C

Table C1. Carbon balance calculation and conversion factor for *N. cameroonii* growth on Enset fiber

Compound	Chemical formula	Molecular weight (g/mol)	mol Carbon/mol	Mole of Compound (mmol)	Carbon per compound (mmol)
Intial Substrates					
Enset fiber					8.58[a]
Ethanol	C_2H_6O	46.07	2	0.64	1.28[b]
CO_2	CO_2	44.01	1	8.06	8.06
				Sum of carbon in substrate	17.92
Final Products					
Acetate	$C_2H_4O_2$	60.05	2	1.11	2.22
Ethanol	C_2H_6O	46.07	2	0.71	1.43
Succinate	$C_4H_6O_4$	118.09	4	0.08	0.32
Lactate	$C_3H_6O_3$	90.08	3	0.17	0.49
Formate	CH_2O_2	46.03	1	1.34	1.34
CO_2	CO_2	44.01	1	9.66	9.66
				Sum of carbon in product	15.47
				Carbon recovery (%)[c]	86.33

[a] The mole of carbon in Enset fiber was calculated from 0.25g Enset fiber and carbon content of 41.2 %(w/w): [b] The amount of ethanol came from the hemin solution in the medium; [c] Carbon recovery (%) = (Sum of carbon in products/ Sum of carbon in substrate) × 100 %

Table C2. Output from ANOVA analysis in OriginPro 2021; the pressure developed by *N. cameroonii* on different substrate loading of Enset fiber

ANOVAOneWay (14/06/2023 08:04:22)

Descriptive Statistics

	N Analysis	N Missing	Mean	Standard Deviation	SE of Mean
0.5%	3	0	1.57667	0.01528	0.00882
1%	3	0	1.72	0	0
3%	2	1	1.63	0.04243	0.03
5%	3	0	1.64667	0.00577	0.00333
7%	3	0	1.53333	0.04933	0.02848

One Way ANOVA

Overall ANOVA

	DF	Sum of Squares	Mean Square	F Value	Prob>F
Model	4	0.06049	0.01512	18.90402	2.09592E-4
Error	9	0.0072	8E-4		
Total	13	0.06769			

Null Hypothesis: The means of all levels are equal.
Alternative Hypothesis: The means of one or more levels are different.
At the 0.05 level, the population means are significantly different.

Fit Statistics

	R-Square	Coeff Var	Root MSE	Data Mean
	0.89364	0.01745	0.02828	1.62071

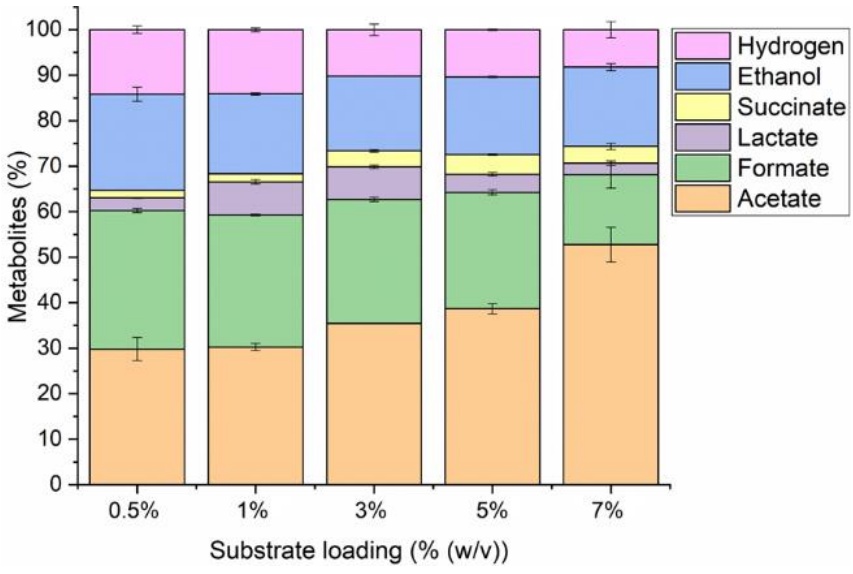

Figure C1. Percentage of all metabolites for *N. cameroonii* growth on different substrate loading of Enset fiber at the end of fermentation. All values are means from triplicate bottles.