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Chidiebere Millicent Igwebuike

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Par

Chidiebere Millicent IGWEBUIKE

**Experimental study on the potential of different
feedstock on second generation bioethanol production: setting a methodology**

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Rapporteurs avant soutenance :

Ramla Gheith Associate Professor (HDR), ENIM/ Monastir
Talib Dbouk Professor, Université de Rouen Normandie

Composition du Jury :

President : Abdeltif Amrane Professor, Université de Rennes
Examiners : Ramla Gheith Associate Professor (HDR), ENIM/ Monastir
Talib Dbouk Professor, Université de Rouen Normandie

Thesis director : Sary Awad Professor, IMT Atlantique
Thesis co-director : Yves Andres Professor, IMT Atlantique

Preface

This research is a doctoral project carried out at IMT-Atlantique, GEPEA UMR CNRS 6144, Nantes, France. It is the original property of Chidiebere Millicent Igwebuike. This project was funded by the Petroleum Technology Development Fund (PTDF), Nigeria, and the funding was managed by Campus France. The timeline of this project was from January 2020 to December 2023. I was the principal investigator, in charge of all significant tasks, including concept development, data gathering, experimentation, data analysis, and manuscript production. Prof. Sary Awad was the thesis director/supervisor, and Prof. Yves Andrès was the co-director. They contributed fully to all phases of the project, particularly the concept generation, proofreading, and editing of the thesis and accepted manuscripts.

The dissertation is divided into two sections; the first provides an overview of the setting in which the outcomes of this doctorate were obtained, and the second section contains the items listed below.

List of scientific publications

C. M. Igwebuike, S. Awad, Y. Andrès (2022). Bioethanol production from dilute acid hydrolysis of cassava peels, sugar beet pulp, and green macro-algae (*Ulva lactuca*). *Biofuels* (*under review*).

List of oral flash communications in international conferences

Chidiebere Millicent Igwebuike, Sary Awad, Yves Andrès. Optimization of total soluble carbohydrate production from acid hydrolysis of lignocellulosic biomass including macroalgae, Paper 214. 9th International Conference on Engineering for Waste and Biomass Valorisation, June 27-30, 2022 Copenhagen, Denmark.

List of oral communications in a seminar

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List of video poster presentation

Igwebuike Chidiebere Millicent, Priadi Hasbi, Awad Sary, Villot Audrey, Purianto Widodo, Andrès Yves. 2nd generation bioethanol production from food processing by-products. Renewable energy and resources conference, the challenges of tomorrow: engineering territories 1st - 2nd and 6th April 2021, France.

This dissertation is dedicated to every member of my family, especially to my spouse (Benson Igwebuike Ilor), my children (Andres Chukwuemeke Igwebuike and Jesse Ifeanyichukwu Igwebuike), and my parents (Mr. & Mrs. Chukwunweike Washington Okoh), to whom I owe a great deal that can never be returned.

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Résumé en Français

Les combustibles fossiles constituent depuis près de deux siècles la source essentielle d'énergie de nos sociétés et ont façonné nos activités quotidiennes. Cependant, au fil des années, l'attention qui lui a été accordée a commencé à diminuer en raison de son épuisement rapide et de ces impacts environnementaux. Les combustibles fossiles sont le résultat d'un processus naturel qui implique une transformation chimique très lente de la biomasse enfouie il y a des millions d'années. Actuellement, l'accent est mis vers l'utilisation de sources renouvelables d'énergie, tant dans les pays développés que dans les pays en développement. Cela est dû à la croissance constante de la population et de l'industrialisation, au déclin des réserves connues, à l'incertitude croissante des approvisionnements en pétrole en raison de la demande croissante et au lien inéquivoque établi entre le changement climatique et les gaz à effet de serre majoritairement produits lors de l'utilisation de combustibles fossiles. Dans le but de satisfaire la demande mondiale d'énergie, de réduire la consommation de combustibles fossiles, de réduire les émissions de dioxyde de carbone (CO₂), et maintenir ou développer les activités agricoles, les gouvernements du monde entier encouragent l'exploitation des ressources et des énergies renouvelables telles que la biomasse, l'éolien, le solaire et l'hydroélectricité. Parmi les sources d'énergie alternatives disponibles, les biocarburants se distinguent par leur compatibilité générale avec les carburants liquides de transport existants. Ils sont considérés comme une alternative viable aux combustibles fossiles. Pour produire du bioéthanol, un biocarburant liquide incontournable dans le mix actuel, la biomasse végétale des déchets lignocellulosiques pourraient être exploités. Il s'agit d'une matière première de deuxième génération et elle est considérée comme la source d'énergie la plus abondante, la plus renouvelable et la moins coûteuse pour la production de bioéthanol, en raison de sa composition élevée en holocellulose. Cette biomasse se distingue par le fait qu'elle n'entre pas en compétition avec le marché alimentaire. La lignocellulose représente plus de 90% de la production mondiale de biomasse végétale. Cela représente environ 200 milliards de tonnes par an. Pour tirer des bénéfices supplémentaires, par exemple de la biomasse utilisée dans les bioraffineries, il est essentiel d'utiliser pleinement ses composants. Cependant, le manque de connaissances sur l'importance des déchets agricoles, l'insuffisance du financement pour l'achat des installations/équipements nécessaires et le manque de savoir-faire technique ou de compétences pour la production de biocarburants sont les principales causes de l'élimination incessante des déchets agricoles (tels que les pelures de manioc, bagasse de canne à sucre, paille de maïs, etc.) dans l'environnement, notamment dans les zones rurales des pays en

développement où les activités agricoles sont prédominantes et où ces déchets sont généralement riches en composants de cellulose et d'hémicellulose qui peuvent être décomposés en composants plus simples pour la production de carburants utiles. Les différentes activités pratiquées par l'homme pour l'élimination de ces déchets ont provoqué la détérioration de l'environnement, perdant ainsi sa valeur esthétique. Cela pourrait également entraîner plusieurs problèmes de santé. La conversion de ces déchets en carburants utiles comme le bioéthanol peut contribuer à réduire la présence de ces polluants environnementaux dans notre environnement et également à répondre à la demande énergétique de la population. Malgré les avantages inhérents à l'utilisation de la biomasse lignocellulosique pour la production de bioéthanol, il existe un inconvénient dû à la présence de lignine dans la paroi cellulaire qui confère au matériau son caractère récalcitrant et le rend peu dégradable. En conséquence, ces matériaux doivent être soumis à différents processus de traitement pour libérer des sucres fermentescibles. Ce travail vise à valoriser la biomasse des déchets lignocellulosiques d'origine locale en bioéthanol en choisissant un ensemble de matières premières représentatives, en tenant compte de leur disponibilité, de leur composition et des opportunités qu'elles peuvent offrir pour la mise à l'échelle et l'industrialisation. Chaque matière première choisie sera caractérisée et différentes possibilités de prétraitement seront appliquées pour maximiser son potentiel de production de sucre. Ensuite, sur la base des résultats du prétraitement, les micro-organismes appropriés seront choisis pour leur fermentation éthylique.

Dans ce premier chapitre, une revue détaillée de la littérature sur la biomasse et la bioénergie, en mettant l'accent sur la structure chimique et la composition des matériaux de biomasse lignocellulosiques (Tableau 1) tels que la cellulose, l'hémicellulose et la lignine, a été présentée. Le chapitre présente et cite différents travaux de recherche issus de la littérature axés sur la transformation et la production de biocarburants primaires et secondaires classés en trois différentes générations (Tableau 2). En outre, le chapitre présente et explique l'importance de la production continue de biocarburants comme le bioéthanol et le biodiesel en tant que biosources énergétiques, en mettant l'accent sur l'augmentation de la production globale entre 2000 et 2019. Différentes données statistiques liées à la production mondiale de bioéthanol par pays ont été présentées. En outre, les différents processus impliqués dans la production de bioéthanol à partir de biomasse lignocellulosique, tels que les prétraitements physicochimiques, biologiques et mécaniques, l'hydrolyse, la fermentation et la distillation, en mettant l'accent sur les avantages et les inconvénients de chaque processus, ont été expliqués (Tableau 3). Soutenu par des références tirées de la littérature, le chapitre explique en outre

comment la concentration d'acide, la température et le temps sont des facteurs importants qui déterminent la formation d'inhibiteurs dans le traitement de l'hémicellulose (Tableaux 5, 6 et 7). En outre, le chapitre explique les mécanismes de fermentation du sucre et les facteurs en plus de la distillation. Certaines législations de l'UE qui soutiennent la production de biocarburants avancés conformément à la directive sur les énergies renouvelables pour les objectifs et les stratégies définies entre 2021 et 2030 ont également été présentées, avec quelques exemples de différents projets industriels ayant été développés dans ce contexte. Enfin, le chapitre présente les trois différents types de biomasse de déchets lignocellulosiques (pelures de manioc, pulpe de betterave sucrière et *Ulva lactuca*), qui seront utilisés dans cette recherche, en abordant leur potentiel important dans la production de bioéthanol de deuxième génération.

Le deuxième chapitre décrit les matériaux utilisés comme matière première et les méthodes utilisées pour leur conversion en biocarburant. Le chapitre décrit la collecte (localisation géographique) et les procédures de préparation moulues des pelures de manioc, de la pulpe de betterave sucrière et des *Ulva lactuca*. Ici, deux matériaux, l'amidon et la cellulose, ont été utilisés comme matériaux de référence. Les processus de caractérisation des matériaux de biomasse par analyse élémentaire, analyse thermogravimétrique (TGA), analyse de l'humidité et de la teneur en cendres, extraction et mesure de la lignine, détermination des glucides solubles, quantification des sucres réducteurs et extraction de la pectine ont été expliqués. En outre, le chapitre décrit la technique de microscopie électronique à balayage (MEB) utilisée pour tester les transformations structurelles des matériaux de biomasse bruts et dilués traités à l'acide. Il a également présenté et expliqué la méthode Plackett-Burman Design (PBD) utilisée pour quantifier les facteurs qui ont un impact important sur les rendements au cours du processus d'hydrolyse acide. Les références utilisées pour le choix des niveaux des différents paramètres ont été obtenues à partir de la littérature et d'expériences réalisées précédemment (Tableau 10). Le chapitre présente ensuite et décrit le processus d'hydrolyse à l'acide dilué utilisée, y compris les réactifs, les mesures de précaution, la dilution et les formulations de rendement en glucides. Il décrit également le processus de fermentation détaillant la préparation des milieux de croissance, de la solution minimale, des milieux solides et de l'inoculum, ainsi que la manière dont les glucides fermentescibles ont été produits dans des conditions de fonctionnement optimisées. Ensuite, la méthodologie d'évaluation de la concentration en levures dans les échantillons de fermentation (formulation mathématique) et le processus de quantification du bioéthanol produit via différents équipements de laboratoire

ont été détaillés. Une conclusion générale de ce chapitre a ensuite été présentée en s'intéressant à la production de bioéthanol à partir de biomasse lignocellulosique (pelures de manioc, pulpe de betterave sucrière et *Ulva lactuca*) comme une solution prometteuse pour la future production d'énergie durable, en mettant l'accent sur l'importance de la sélection initiale des matériaux et des méthodes appropriés dans les processus de prétraitement, d'hydrolyse et de fermentation.

Le troisième chapitre a fourni une évaluation complète des propriétés des différents matériaux de biomasse utilisés et de leur impact sur la production de bioéthanol. Le chapitre présente et discute des processus de mesure des caractéristiques des matières premières, notamment la composition chimique, la composition élémentaire en carbone, hydrogène, oxygène, soufre et azote, ainsi que les différentes quantités de glucides, de cellulose et d'hémicellulose, à l'aide de différentes techniques d'analyse. À cela s'ajoutent les teneurs en cendres et en humidité ainsi que la composition structurale et non structurale des différentes biomasses. Le chapitre a également examiné l'impact des propriétés morphologiques des différentes biomasses sur le processus de conversion. Le chapitre conclut que la biomasse étudiée contient des teneurs élevées en polysaccharides qui lui confèrent un potentiel intéressant pour la production de bioéthanol et que la composition de chaque matière première peut varier en fonction du type de biomasse, des conditions de croissance, de la méthode de récolte et des conditions de stockage. De plus, la disponibilité du sucre, la présence d'inhibiteurs et la composition globale de la matière première jouent tous un rôle dans la détermination de son aptitude à la fermentation et à la production d'éthanol. Le chapitre se termine en soulignant l'importance de recherches et de développements supplémentaires nécessaires pour approfondir nos connaissances sur la faisabilité économique de l'utilisation de ces biomatériaux comme source de sucre pour la fermentation et la production d'éthanol à grande échelle.

Le quatrième, et dernier, chapitre, étudie les processus de saccharification et de fermentation des matières végétales de biomasse en bioéthanol. Le chapitre a examiné différents scénarios de conditions opératoires pour l'hydrolyse optimale de la biomasse en glucides simples pour la production de bioéthanol. Il a présenté la mesure et la quantification du rendement en glucides (CY) et en glucides totaux solubles (TSC) des différents matériaux de biomasse utilisés. Ceci a été étayé par des études d'analyse de régression basées sur une modélisation d'équations numériques qui prennent en compte l'impact de différents paramètres tels que la charge solide, la concentration en acide, le temps et la température. Le chapitre présente et discute en outre les résultats sous forme de courbes de niveau pour les glucides

solubles totaux (TSC) et le rendement en glucides (CY) de l'amidon, de la cellulose, des écorces de manioc, de la pulpe de betterave sucrière et *Ulva lactuca* en fonction du temps, de la température, de la concentration d'acide et du pourcentage de charge solide. Le chapitre présente également une comparaison de l'hydrolyse de la biomasse avec différents matériaux de référence rapportés dans la littérature et discute des différentes conditions opératoires optimales pour des processus améliorés de saccharification et de fermentation et une production optimale de bioéthanol. Le chapitre se termine par une conclusion claire sur l'efficacité de l'hydrolyse à l'aide d'acide dilué dans la production de grandes quantités de glucides solubles. En conclusion générale de ces travaux, la biomasse est une source d'énergie renouvelable et durable qui peut être utilisée pour diverses applications, notamment la production de biocarburants tels que le bioéthanol. L'examen des différentes générations de biocarburants révèle que l'utilisation de biocarburants avancés dérivés de matières premières de deuxième et troisième génération est à la fois plus respectueuse de l'environnement et économiquement plus avantageuse que l'utilisation de biocarburants produits à partir de biomasse de première génération. Il a été démontré que la récalcitrance de la biomasse lignocellulosique la soumet à des étapes de prétraitement afin que le matériau devienne plus réceptif aux agents catalytiques. En outre, il a été noté que bien que le prétraitement biologique et l'hydrolyse enzymatique de la biomasse soient des processus bénéfiques pour l'environnement, ils nécessitent plus d'étapes et de temps et peuvent être coûteux, par exemple lors de l'utilisation d'organismes génétiquement modifiés (OGM). Les voies de fermentation des pentoses et hexoses ont également été mises en évidence; il est important de noter que dans une combinaison de sucres, les microbes consomment en premier les hexoses (glucose) avant de se tourner vers les hexoses. Plusieurs initiatives sur la production avancée de bioéthanol ont également été présentées dans cette étude. La législation de l'UE soutient le développement de la production de bioéthanol de deuxième génération, qui utilise des matières premières non alimentaires et peut contribuer à réduire la dépendance aux combustibles fossiles. Cependant, la production à grande échelle de bioéthanol à partir de biomasse lignocellulosique reste un défi en raison de la complexité du processus et de la nécessité de technologies rentables et efficaces. La biomasse lignocellulosique est un groupe complexe et diversifié de matières végétales composées de cellulose, d'hémicellulose et de lignine. La cellulose et l'hémicellulose sont les principales sources de sucre pour la production de bioéthanol, tandis que la lignine sert de composant structurel et de barrière pour les processus de saccharification et de fermentation. La structure et la composition de la biomasse lignocellulosique affectent grandement l'efficacité de la production de bioéthanol et peuvent varier considérablement en fonction de la

source de biomasse. En raison de cette complexité, il est important de bien caractériser et comprendre la structure et la composition d'une biomasse lignocellulosique spécifique avant de tenter de la convertir en bioéthanol. La biomasse lignocellulosique, comme les pelures de manioc, la pulpe de betterave sucrière et *Ulva lactuca*, ont été identifiés comme matières premières potentielles pour la production de bioéthanol en raison de leur composition élevée en polysaccharides. Chaque matière première avait des compositions différentes en raison des variations du type de biomasse, des facteurs environnementaux, de la technique de récolte et des conditions de stockage. L'adéquation d'une matière première pour la fermentation et la production d'éthanol dépend de plusieurs facteurs, notamment la disponibilité du sucre, la présence d'inhibiteurs et la composition globale de la matière première. Pour comprendre pleinement la viabilité et les aspects économiques de l'utilisation de ces matériaux comme source de sucre pour la fermentation et la fabrication à grande échelle d'éthanol, des études et des tests supplémentaires sont nécessaires. L'hydrolyse acide est une méthode de prétraitement courante utilisée pour convertir la biomasse lignocellulosique en sucres pouvant être fermentés en bioéthanol. Les paramètres importants impliqués dans l'hydrolyse des différentes biomasses n'étaient pas exactement les mêmes, ce qui pourrait être dû à des différences dans les facteurs structurels de la biomasse, tels que la taille des particules, la taille des pores, le volume des pores, la surface spécifique, la cristallinité de la cellulose et le degré de polymérisation, ainsi que des facteurs chimiques, tels que la composition et la teneur en lignine, hémicelluloses et groupes acétyles. La réduction de la production de sucre et d'éthanol à partir des pelures de manioc a été un succès. L'hydrolyse et la fermentation séparées avec inoculation séquentielle de *Saccharomyces cerevisiae* et *Scheffersomyces stipite*, a conduit à une production maximale d'éthanol de $28,62 \pm 3,93$ g/L à partir des pelures de manioc. Cela révèle ainsi qu'il a un grand potentiel pour être utilisé dans la production d'éthanol à grande échelle. Cependant, les rendements en sucre et en éthanol ($3,36 \pm 0,04$ g/L, obtenus par fermentation avec *Saccharomyces cerevisiae* et *Scheffersomyces stipite*) à partir de pulpe de betterave sucrière hydrolysée à l'acide et *Ulva lactuca* ($7,69 \pm 0,20$ g/L, obtenu par fermentation avec *Neurospora intermédiaire*) suggèrent une optimisation plus poussée du processus et une exploration approfondie de la teneur en arabinose de la pulpe de betterave sucrière en tant que sucre dominant dans la biomasse. Les organismes de fermentation et les inhibiteurs étaient également des facteurs importants à prendre en compte dans la production de bioéthanol. Les conditions opératoires utilisées pour l'extraction de la pectine de la pulpe de betterave sucrière ont été appliquées aux pelures de manioc pour produire des glucides simples. L'examen physique de l'hydrolysats obtenu a révélé sa nature visqueuse et la teneur en éthanol obtenue était faible, ce

qui indique que les pelures de manioc nécessitent des niveaux de conditions opératoires plus élevés. Parmi les deux micro-organismes C_6 utilisant le sucre examinés, qui étaient de la levure de boulangerie achetée à l'épicerie locale et *S. cerevisiae* isolée des résidus de vin, cette dernière a été retenue en raison de ses meilleures performances même si les deux souches ont donné des résultats relativement proches. L'utilisation de micro-organismes appropriés et le contrôle des inhibiteurs peuvent contribuer à améliorer l'efficacité du processus de fermentation et à augmenter le rendement final en bioéthanol. Cependant, dans cette étude, les micro-organismes utilisés ne semblent pas être à l'origine du faible rendement en éthanol de la pulpe de betterave sucrière et *Ulva lactuca* car les micro-organismes étaient capables de consommer tous les sucres simples présents dans le milieu et étendaient même leur consommation aux dimères/oligomères. D'une manière plus générale, la production de bioéthanol à partir de biomasse lignocellulosique est un domaine de recherche prometteur susceptible de réduire la dépendance aux combustibles fossiles et de contribuer à un avenir énergétique durable. Cependant, des recherches et développements supplémentaires sont nécessaires pour améliorer l'efficacité et la rentabilité du processus et pour surmonter les défis associés à la production à grande échelle.

En perspective, des suggestions ont été faites pour améliorer l'efficacité et la rentabilité de la production de bioéthanol à partir de pulpe de betterave sucrière et *Ulva lactuca* qui a présenté certains défis, notamment:

- a. **Bioconversion:** Des recherches supplémentaires sont nécessaires pour une hydrolyse efficace de ces matières premières tout en tenant compte du coût des catalyseurs d'hydrolyse, des étapes, de la durée de production et de la sécurité environnementale.
- b. **Développement de nouvelles méthodes de prétraitement:** Prétraitement de la pulpe de betterave sucrière et *Ulva lactuca* est essentiel pour améliorer la libération des sucres pour la fermentation. Une nouvelle méthode de prétraitement devrait être explorée. Par exemple, les catalyseurs hétérogènes (solides) devraient être étudiés et testés de manière approfondie pour maximiser la saccharification de ces matières premières, car ils sont réputés respectueux de l'environnement, faciles à séparer des produits liquides et réutilisables.
- c. **Adaptation de la souche:** La souche *N. intermedia* doit être acclimatée à la biomasse neutralisée (*U. lactuca* et pulpe de betterave sucrière) des hydrolysats acides par transfert et croissance séquentiels de cellules dans des milieux contenant les hydrolysats. Cette approche devrait utiliser le micro-organisme de chaque expérience

comme inoculant pour la suivante. Le cycle doit être répété jusqu'à 16 fois avant la récolte finale et la culture sur plaques PDA (Potato dextrose agar). Ce faisant, la production d'éthanol avec la souche adaptée devrait être supérieure à celle de l'éthanol produit avec la souche mère (souche non adaptée).

- d. **Plages de pH de test:** Différentes plages de pH de fermentation doivent être testées lors de la fermentation de *U. lactuca* hydrolysée avec *N. intermedia* pour parvenir à un pH optimisé.
- e. **Explorer les autres sucres pentoses:** La fermentation de l'arabinose du composant hémicellulose de la pulpe de betterave sucrière pour la production de bioéthanol devrait être étudiée en profondeur puisque la pulpe de betterave sucrière le contient en forte concentration, mais étant un composant relativement faible dans d'autres biomasses, sa fermentation n'a pas fait l'objet de recherches approfondies.

Introduction

Fossil fuel has been introduced years back into our daily activities as an energy source. However, as the year went by the attention given to it began to wane due to its fast depletion and environmental concerns. Fossil fuel is a result of a natural process that involves a very slow chemical transformation of the remains of dead organisms buried millions of years ago. Examples of fossil fuels include; coal – polycyclic aromatic compounds, natural gas – methane, oil shale – Type I kerogen, and petroleum – aromatic hydrocarbons, paraffin, cycloparaffins (Sato, 1991). They are fuels obtained from ancient biomass. Currently, emphasis has shifted to the use of renewable sources of energy both in the developed and developing countries of the world. This is due to the steady growth in population and industrialization (Cherubini and Str, 2011), the decline in known reserves, the rising uncertainty of petroleum supplies as a result of increasing demand, and concerns over climate change (greenhouse gas emissions and global warming) which is linked to the use of fossil fuels (Saini et al., 2015). In a bid to satisfy the world demand for energy, lower fossil fuel consumption, reduce the emissions of CO₂, and maintain or develop agricultural activities (by utilizing bio-resources for energy, food, and material), governments across the world are encouraging the exploitation of renewable resources and energies such as biomass, wind, solar and hydroelectricity (Lange and Solutions, 2007).

Among the available alternative energy sources, biofuels stand out in their general compatibility with existing liquid transport fuels. It is considered a viable alternative to fossil-based fuels. Although fossil fuels and fuels obtained from fresh biomass are composed of the same elements (hydrogen and carbon), fossil fuels are regarded as non-renewable because it take a very long time to create (Holechek et al., 2022). Therefore, the rate at which it is consumed is higher than the rate it is produced. The development of biofuels has several interests which include: considerable environmentally-friendly potential, they represent a CO₂ cycle in combustion, they are easily available from common biomass sources, they are biodegradable and contribute to sustainability and also, they promote rural economies that produce crops used for biofuels (Balat, 2007), (Puppán, 2002). Carbon dioxide, present in the atmosphere and water from the earth reacts in the photosynthetic process to form sugars that constitute the building blocks of biomass. The energy from the sun which propels photosynthesis is stored in the chemical bonds of the plant's structural components. When this energy stored in the chemical bonds is extracted by effective burning of the biomass, oxygen present in the atmosphere, then reacts with the carbon in plants to form carbon dioxide and

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water. It is a cyclic process since carbon dioxide becomes available to produce new plant biomass (Figure 1). Unlike fossil fuels which are locked up away deep in the ground, and do not have an effect on the earth's atmosphere except when burned, plant biomass releases the majority of its chemical compounds back into the atmosphere when they decay. Reports have it that carbon dioxide is the most abundant greenhouse gas in the atmosphere. Others include methane, nitrous oxide, and F-gases (Figure 2). There are different methods of generating energy from biomass viz; (a) biological processing – this is a natural process of energy generation that could either be by fermentation or anaerobic digestion to produce useful liquid or gaseous fuel. (b) thermochemical processing which includes liquefaction, pyrolysis, and gasification processes, and (c) direct combustion of biomass (McKendry, 2002).

To produce bioethanol, a type of liquid biofuel, lignocellulosic waste plant biomass could be exploited and examples include cassava peels, sugar beet pulp, and *Ulva lactuca*. It is a second-generation feedstock and it is regarded as the most abundantly available, renewable, and inexpensive energy source for the production of bioethanol (Rajendran et al., 2018), owing to its high holocellulose composition (Isroi et al., 2012). This biomass stands out because they do not serve as food for human consumption. Hence, there is no competition in the food market as in the case of first-generation biomass (e.g. sugarcane and maize). Lignocellulose accounts for more than 90% of worldwide plant biomass production. It amounts to approximately 200 billion tons per year (Saini et al., 2015). To obtain extra benefits, for instance, from feedstock biomass used in biorefineries, it is essential to make complete use of its components (Ree and Jong, 2019).

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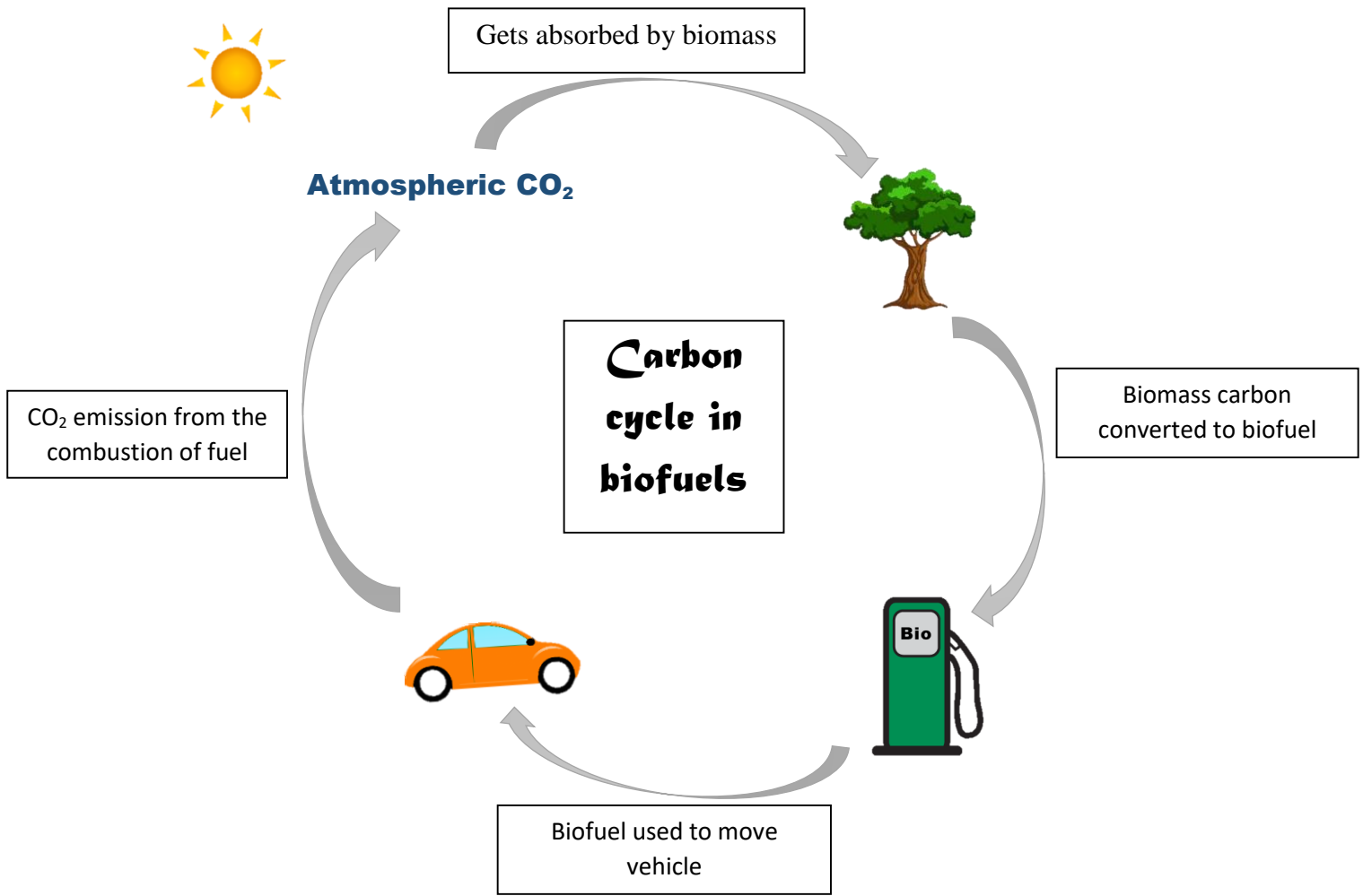


Figure 1. An illustration of the carbon cycle in biofuel production.

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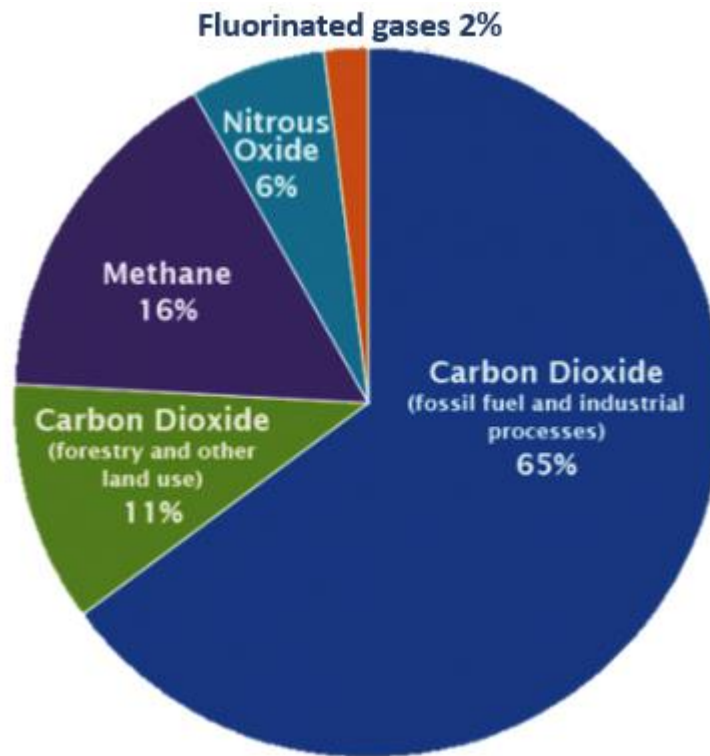


Figure 2. Global greenhouse gas emission by gas type (IPCC, 2014).

Statement of the problem

Lack of knowledge about the importance of agricultural wastes, inadequate finance for the purchase of needed facilities/equipment, and lack of technical know-how or skills for biofuel production are the major causes of incessant disposal of agricultural waste (such as cassava peels, sugarcane bagasse, corn straw, etc.) in the environment, especially in the rural areas of the developing countries where agricultural activities are prevalent and these waste are usually rich in cellulose and hemicellulose components that can be broken down into simpler components for the production of useful fuels. The various activities practiced by humans in the disposal of these wastes have caused the deterioration of the environment thus losing its aesthetic value. It could also cause several health challenges. The conversion of these wastes to useful fuels like bioethanol can help to reduce the occurrence of these environmental pollutants in our environment and also meet the energy demand of the populace. Despite the advantages inherent in the utilization of lignocellulosic biomass for bioethanol production, there exists a drawback due to the presence of lignin in the cell wall which confers upon the material its recalcitrance nature and renders it not easily degradable. As a result of this, such material needs to be subjected to different treatment processes to release fermentable sugars.

Introduction

Objective of study

This work aims at the valorization of locally sourced lignocellulosic waste biomass into bioethanol by choosing a set of representative feedstock, taking into consideration their availability, their composition, and the opportunities that they can open for scaling-up and industrialization. Each chosen feedstock will be characterized and different pretreatment possibilities will be applied in order to maximize its sugar production potential. Then, based on pretreatment results, appropriate microorganisms will be chosen for their fermentation to bioethanol.

Scope of work

Chapter 1 offers a bibliographic study and it is divided into two parts; the first part is a conceptual framework focused on biomass and bioenergy, the structure and composition of lignocellulosic biomass, biofuel classification, bioethanol as an energy source, bioethanol production processes, different pretreatment and hydrolysis techniques, inhibitory product formation, fermentation strategies/process, the microorganisms used for fermentation, and distillation. Also covered in this part is legislation in support of advanced biofuel and industrial-scale projects on advanced bioethanol/applications. The second part of this chapter covers the potential of second-generation bioethanol production from the representative feedstock. Chapter 2 presents the materials and methods for the realization of findings. Chapter 3 covers the characterization of chosen feedstock and the findings/observations from biomass characterization and lastly, Chapter 4 presents the saccharification and fermentation of samples and discusses with obtained results of previous works.

Chapter 1: Literature Review

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1.1 Biomass and bioenergy

For ages, humans have relied on the use of traditional bioenergy. Over 85 percent of biomass energy is being utilized as solid fuels for heating, cooking, and lighting at present but with low efficiency. Fuelwood and charcoal categorized as traditional bioenergy which only provides heat is said to dominate the consumption of bioenergy, especially in the developing world where about 95 percent of national energy consumption depends on biomass. This biomass will keep on being an essential source of bioenergy in many parts of the world. Up till now, wood fuels stand as the main source of bioenergy across different regions as it offer energy security services for large divisions of society. Modern bioenergy depends on efficient conversion technologies for domestic use and utilization at both industrial scales and for small businesses. Biomass could be processed into more convenient energy carriers such as liquid fuels (biodiesel, bioethanol, bio-oil), solid fuels (wood chips, firewood, charcoal, briquettes, pellets), gaseous fuels (hydrogen, synthesis gas, biogas) or heat directly from the production process (FAO/GBEP, 2007). The combustion of biomass is regarded as a carbon-neutral process since the carbon dioxide emitted has been absorbed by the plants from the atmosphere beforehand. So far, organic wastes and residues are the major biomass sources nevertheless energy crops such as poplar, willow, and eucalyptus are gaining significance and market share. Biomass resources comprise: wood wastes from industry and forestry, agricultural residues, residues from paper and food industries, animal manure, dedicated energy crops, sewage sludge, municipal green wastes, starch crops (wheat, corn) sugar crops (beet, sugarcane, sorghum), oil crops (oilseed rape, soy, sunflower, palm oil, jatropha) and grasses (*Miscanthus*) (Europe and Etp, 2007).

At present, biomass constitutes only a small fraction of the total carbon use despite often being applied as raw material and a source of energy. Its use is limited to large-scale production of bioethanol and to low-volume products. In the coming years there is expected to be a continual shift from the present fossil-based to a future bio-based carbon economy thus causing a gradual effect on all process industries. There will be a constant transition to more complex bio-renewable feedstock such as algae, agricultural residues, green plants, industrial wastes, or wood, and eventually, the bio-based products will replace the petrochemical product tree. This transitioning is not to be regarded as a threat but as a chance to redesign the industrial value chain from renewable material sources to new products and for this to be achieved, the

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rich molecular structure of renewable biomaterials is to be greatly exploited (Marquardt et al., 2010).

1.2 Structure and composition of lignocellulosic biomass

Lignocellulosic biomass is majorly composed of three polymers which are cellulose, hemicellulose, and lignin including a lesser amount of protein, pectin, ash, and extractives. The proportion of these components can differ from one biomass species to another. For instance, hardwoods have more amounts of cellulose, while leaves and wheat straws have more hemicellulose. The proportion of these components is also, not often the same within a single plant species due to the stage of growth, age, and certain other conditions. These polymers are linked with each other in a hetero-matrix to different degrees and varying relative composition depending on the type, species, and source of the biomass material (Bajpai, 2016). The relative amounts of cellulose, hemicellulose, and lignin are among others, important determining factors in identifying the suitability of plant species for use as energy crops (McKendry, 2002). Lignocellulosic materials as an alternative to fossil resources have great potential to generate second-generation biofuels including bio-sourced materials and chemicals without negatively impacting the world's food security. However, the main drawback of these materials' valorization is its recalcitrance to enzymatic hydrolysis due to the heterogeneous multi-scale structure of plant cell walls. The factors affecting the recalcitrance of these materials are strongly interconnected and not easily dissociated. These factors can be classified into chemical factors (composition and content in lignin, hemicelluloses, acetyl groups) and structural factors (cellulose crystallinity, cellulose specific surface area, degree of polymerization, pore size, and volume) (Zoghلامي and Paës, 2019). The structural composition of different lignocellulosic biomass is given in Table 1.

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Table 1. Compositions of selected lignocellulosic biomass.

Biomass	Cellulose (%)	Hemicellulose (%)	Lignin (%)	References
Oak Wood	49.3	25.9	21.7	(Lee et al., 1999)
Sugar beet pulp	20.71	14.98	3.96	(El-gendy et al., 2015)
Sugarcane bagasse	50	25	25	(Rath et al., 2011)
Rice straw	34.6	27.7	17.6	(Kandanelli et al., 2018)
Rice husk	33.4	22.1	22.8	(Kandanelli et al., 2018)
Wheat straw	33.5	24.6	19	(Kandanelli et al., 2018)
Oil palm empty fruit bunches	39.13	23.04	34.37	(Isroi et al., 2012)
Corn cobs	22.1	9.6	6.0	(Chongkhong and Tongurai, 2018)
Banana rachis	26.1	11.2	10.8	(Guerrero et al., 2018)
Banana pseudostem	20.1	9.6	10.1	(Guerrero et al., 2018)
Cassava peels	9.05	7.50	9.16	(Aruwajoye et al., 2017)
Tygra hemp	50.82	27.79	14.68	(Wawro et al., 2019)
Groundnut shell	35.7	18.7	30.2	(Ramgopal, 2016)
Corn stover	36.1	21.4	17.2	(Öhgren et al., 2007)
Poplar	42.34	15.23	25.40	(Liu et al., 2016)
Waste from urban greening	22.96	6.86	22.73	(Raud et al., 2014)
Spring leaves	21.06	6.00	27.74	(Raud et al., 2014)
Autumn leaves	14.54	8.45	11.16	(Raud et al., 2014)
Jerusalem artichoke	25.99	4.50	5.70	(Tutt and Olt, 2011)
Energy grass	37.85	27.33	9.65	(Tutt and Olt, 2011)
Sunflower	34.06	5.18	7.72	(Tutt and Olt, 2011)
Silage	39.27	25.96	9.02	(Tutt and Olt, 2011)
<i>Miscanthus saccharifloris</i>	42.00	30.15	7.00	(Tutt and Olt, 2011)
Reed	49.40	31.50	8.74	(Tutt and Olt, 2011)

1.2.1 Cellulose

Cellulose is the most abundant fraction in lignocellulosic materials and represents about 40 – 50% of the biomass by weight. It is a polymer of glucose, comprising linear chains of (1,4)-D-glucopyranose units (Figure 3), whereby the units are linked C₁ - C₄ oxygen bridges with the removal of water, in the β-configuration (β-glycosidic bonds), with an average molecular weight of around 100,000 (McKendry, 2002). The number of units (glucose) that make up the polymer i.e. the degree of polymerization is above 10,000 in native/unaltered wood while in highly bleached kraft pulps, it is less than 1000. Cellulose in appearance is a white

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solid material that may exist either in crystalline or amorphous states. Cotton fibers are an example of a very pure natural form of cellulose, containing over 93% of this polysaccharide. As for wood, cellulose is about 50 – 70% crystalline and forms the backbone structure of the wood fiber. The crystalline state of cellulose confers its ability to be resistant to chemical attack and degradation. The high strength of cellulose fibers is a consequence of the hydrogen bonding that exists between cellulose molecules (Bajpai, 2018). The degree of crystallinity of Eucalyptus was reported to be 29.9% - 34.2% (Kong et al., 2017). The thermal stability of cellulose is known to primarily depend on its crystallite size, degree of crystallinity (crystallinity index), and degree of polymerization (Poletto et al., 2012),(Kim et al., 2010),(Nada et al., 2000). Cellulose has an empirical formula of $C_6H_{10}O_5$ and is a polyanhydride of glucose ($C_6H_{12}O_6$). The two extreme glucose molecules of a cellulose material consist of two different end groups: one of them consists of a reducing hemiacetal group in the C_1 position and so, it is called the reducing end-group, while the other consists of an additional secondary hydroxyl group in the C_4 position and therefore, called the non-reducing end-group (Alam et al., 2014). Cellulose is a compound that is insoluble in water and can be biodegraded by enzymes produced by fungi and bacteria (Tutt and Olt, 2011).

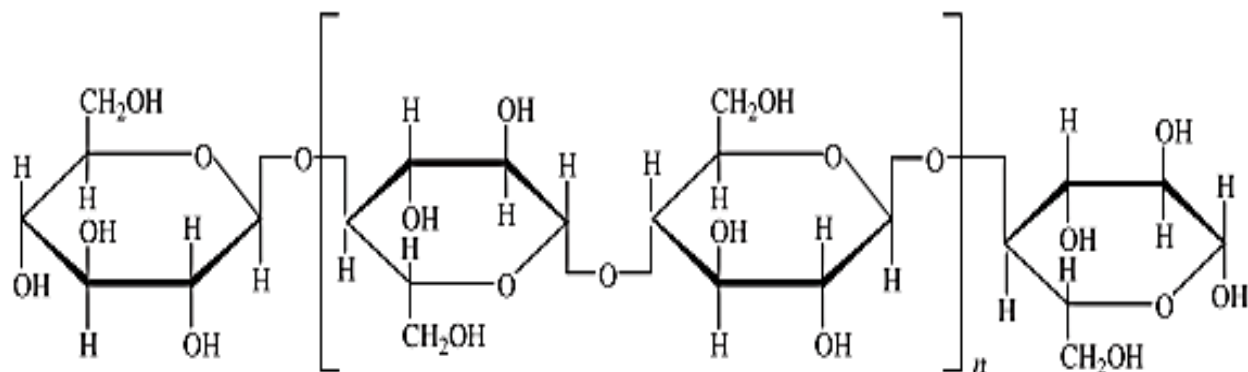


Figure 3. Cellulose structure (Alam et al., 2014).

1.2.2 Hemicellulose

After cellulose, hemicellulose ranks as the second topmost largest carbohydrate in the world. Daily production per person was estimated to be about 20 kg and about 45,000 million tons are produced on a yearly basis. The plant cell wall has a composite structure that can be related to that of fiberglass resins; the cellulose is situated within the hemicellulose matrix where it serves as support (providing cell rigidity and strength) just like glass fibers while the lignin binds the whole structure together (Holtzapple, 2003). Hemicellulose can be categorized into four general classes of structurally different cell wall polysaccharide types: xylans,

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mannans, β -glucans with mixed linkages, and xyloglucans. They exist in structural variations differing in side-chain types, distribution, localization, and/or types and distribution of glycoside linkages in the macro-molecular backbone (Kang et al., 2014). Lignin and cellulose of plant cell walls are closely interpenetrated by a mixture of polysaccharides known as hemicellulose (Alam et al., 2014). In appearance, hemicelluloses are also white solid materials but they are hardly crystalline or fibrous in nature; they make up some of the flesh that helps fill out the fiber (Bajpai, 2018). The hemicellulose fraction represents 20 – 40% of the biomass by weight. It is a mixture of polysaccharides that comprises mostly sugars like glucose, xylose, arabinose, mannose, methylglucuronic, and galacturonic acids. Hemicellulose is a heterogeneously branched polysaccharide (Figure 4) that binds tightly, though non-covalently, to each cellulose microfibril surface. Unlike cellulose, hemicellulose consists primarily of xylose and other five-carbon sugars, with an average molecular weight of <30,000 (McKendry, 2002). In plants, two distinct hemicelluloses are present which include the neutral and acidic hemicelluloses. A higher amount of uronic acids are found in the acidic hemicelluloses than the neutral hemicelluloses. Microorganisms of the colon are able to partially ferment hemicellulose thus producing some volatile fatty acids (Huffman, 2003). Hemicelluloses are condensation polymers that have a molecule of water eliminated with each linkage. Every one of the monosaccharides that form the hemicellulose possesses the D-configuration and occurs in the six-member pyranoside forms, with the exception of arabinose, which possesses the L-configuration and occurs as a five-member furanoside. They are short-chain polymers in comparison with cellulose and the degree of polymerization is about 100 - 200 sugar units per hemicellulose molecule (Bajpai, 2018). The structural diversities of hemicelluloses present amounts of possibilities for specific physical, chemical, and enzymic modifications (Kang et al., 2014). Compared to cellulose, hemicelluloses are very much soluble and liable i.e. they are more predisposed to chemical attack and degradation. Hemicelluloses with low molecular weight are soluble in dilute alkali at higher temperatures (Bajpai, 2018) and are readily hydrolyzed to pentose and hexose with some uronic acids (Alam et al., 2014).

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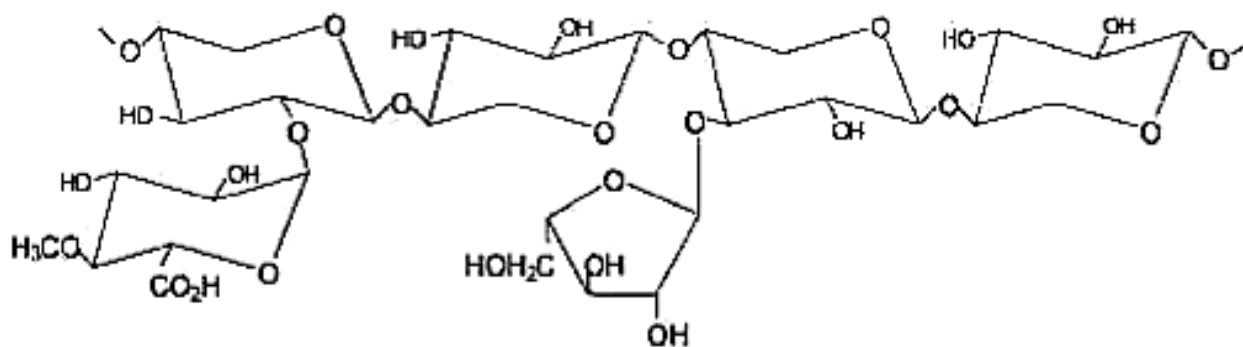


Figure 4. Hemicellulose structure (Walker, 2012).

1.2.3 Lignin

Lignin refers to a group of amorphous, high molecular weight (over 10,000), cross-linked, chemically related complex polymer compounds that form an essential part of the secondary plant cell wall. It is rich in aromatic subunits and relatively hydrophobic (Figure 5). It hinders the free access of cellulolytic enzymes due to its cross-linkages with other components of the cell wall. It has a very slow rate of decomposition but contributes a significant aspect to the materials that form humus (Tutt and Olt, 2011). It is thought that the building blocks of lignin are a three-carbon chain attached to rings of six carbon atoms known as phenyl-propane. These have either zero, one, or two methoxyl groups attached to the rings thus resulting in three structures, designated I, II, and III, respectively. The proportion of each structure depends on the polymer source, for example, structure I occurs in plants like grasses, structure II occurs in the wood of conifers, and structure III occurs in deciduous wood (McKendry, 2002). Lignin has an amorphous, three-dimensional structure. In the case of wood, for example, its molecular weight is very high and the degree of polymerization is difficult to measure due to the heterogeneous nature of the material. Lignin is regarded as the binder or adhesive in wood that holds the fibers together. It is highly present in the middle lamella and its removal during chemical pulping allows the fibers to be easily separated. The softening temperature (the glass transition temperature) is about 130 – 150°C. The glass transition temperature decreases slightly with moisture. Furthermore, lignin comprises of three basic monomers: *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol. All three lignin monomers are found in straws and grasses. Coniferyl alcohol is found in softwoods (gymnosperms e.g. cycads and conifers), while both coniferyl alcohol (50 – 75%) and sinapyl alcohol (25 – 50%) are present in hardwoods (dicotyledonous angiosperms) (Bajpai, 2018).

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Lignin represents the most stable component of biomass (Brunner, 2014). It possesses qualities that make it increasingly significant in many industrial applications since it is non-toxic and extremely versatile in performance (Zhang et al., 2008). Its conversion is of huge significance for the development of processes that employ renewable resources (Brunner, 2014). The conversion of lignin to higher-value fuel additives could considerably improve the competitiveness of bio-refinery technology (Zhang et al., 2008). The evaluation of its applications is based on its chemical composition. Thermal and hydrothermal decomposition of lignin occurs over a broad range of temperatures as a result of its complex structure and composition. Its composition is not the same for different plant sources and pretreatment processes. It is difficult to recover live lignin because the lignin structure gets modified during the recovery process (Brunner, 2014).

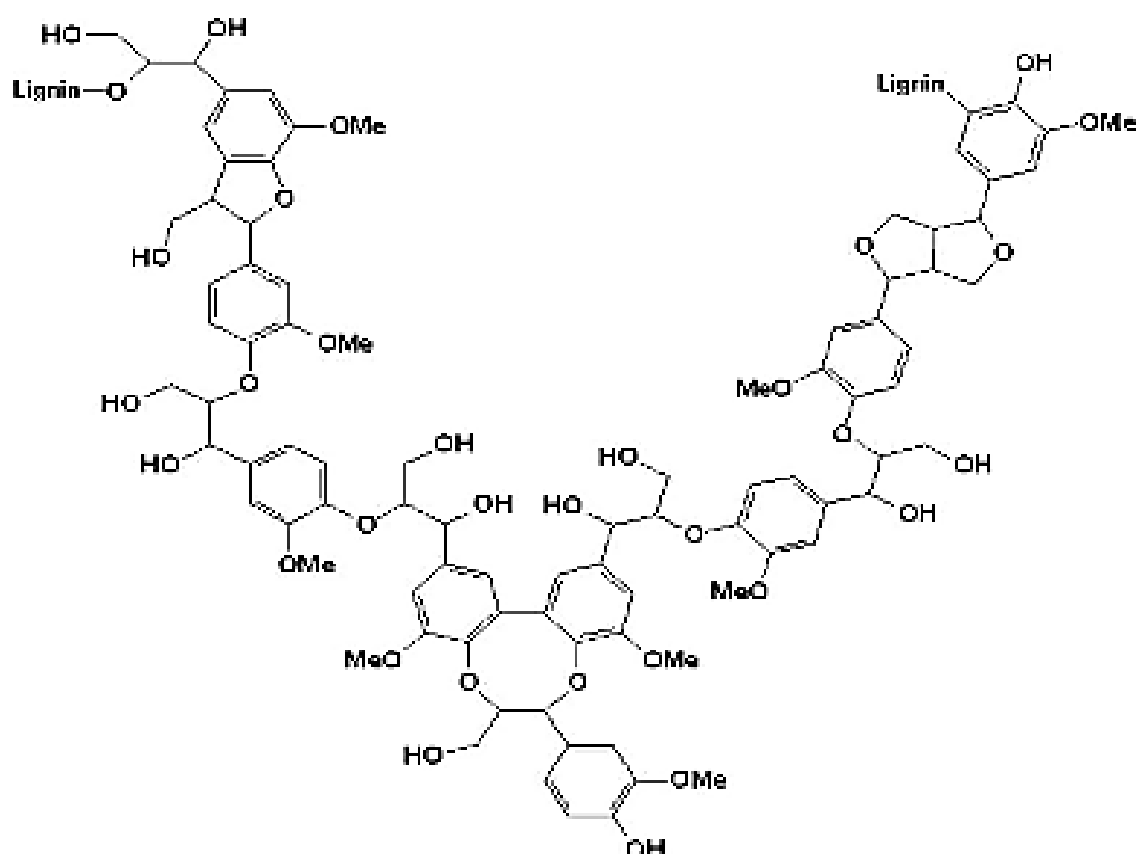


Figure 5. Lignin structure (Walker, 2012).

1.3 Groups of biofuels

Depending on the processing before utilization, biofuels can be classified into primary and secondary biofuels:

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1.3.1 Primary biofuels

Primary biofuels are used in their unprocessed form i.e. the organic material is used in its natural form, as harvested. Examples of such primary biofuels include; forest residues, pellets, wood chips, firewood, agricultural crops, and animal fats. They are primarily used for cooking, heating, agricultural needs, and the production of electricity in small and large-scale industrial applications. It is common in developing countries. Primary biofuels are also referred to as traditional biomass. The field of application of this biofuel is small, it does not require processing resource expenses. Energy derived from traditional biomass was about 9% of all energy consumed globally for the year 2013 (Fao.org, 2008; Meneses et al., 2017; Nigam and Singh, 2011; *Renewables 2015 Global Status Report*, n.d.; Voloshin et al., 2016).

1.3.2 Secondary biofuels

Secondary biofuels are processed materials. They are produced by processing biomass (the primary biofuel). Secondary biofuels can be in the form of solids (charcoal), liquids (bio-oil, ethanol, biodiesel), or gases (hydrogen, biogas, synthesis gas) which can be used in a wider range of applications such as high-temperature industrial processes, and transport to substitute fossil fuel. Based on the type of raw materials, the historical sequence of the fuel's appearance on the world energy market, and the processing technology employed in production, secondary biofuels can further be divided into three generations: first-generation biofuels, second-generation biofuels, and third-generation biofuels (Meneses et al., 2017), (Fao.org, 2008), (Nigam and Singh, 2011), (Voloshin et al., 2016). Table 2. gives some examples of different sources of secondary biofuels including benefits and issues associated with the distinct biofuel generations.

Table 2. Benefits and issues associated with secondary biofuels.

Secondary biofuels	Examples (biomass)	Benefits	Issues
First-generation	<ul style="list-style-type: none">• Corn• Sugarcane• Sugar beets• Wheat• Barley• Soybean• Palm oil• Vegetable oil and fats	<ul style="list-style-type: none">• Enhance energy, social, and economic security• Eco-friendly	<ul style="list-style-type: none">• Impact on food security, land-use, biodiversity, and carbon balances• High production cost• Partly blended with petroleum-based fuels

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Second-generation	<ul style="list-style-type: none"> • Sugarcane bagasse • Sugar beets pulp • Cassava peels • Corn Straw • Wheat straw • Switchgrass • Softwood • Hardwood 	<ul style="list-style-type: none"> • Lower impact on food sector. • Lower cost of feedstock. • Enhance energy, social, and economic security. • Better land-use efficiency. • Eco-friendly 	<ul style="list-style-type: none"> • Recalcitrance of cell walls • May incur high cost of production • Infancy stage of cell wall polysaccharide biosynthetic machinery and it's regulation
Third-generation	<ul style="list-style-type: none"> • Microbes • Seaweeds • Microalgae (Spirogyra, <i>Chlorococcum sp.</i>, <i>Undaria pinnatifida</i>, <i>Schizochytrium sp.</i>, <i>Chlorella vulgaris</i>, <i>Dunaliella tertiolecta</i>, <i>Nannochloropsis sp.</i> e.t.c) 	<ul style="list-style-type: none"> • Mitigates greenhouse gases • Higher yields • Shorter harvesting cycle (1 – 10 days) • High lipid content • Rapid growth rate • Reduced land-use • Higher CO₂ tolerance 	<ul style="list-style-type: none"> • Algae require large amounts of water, phosphorus, and nitrogen to grow • Algae biomass requires dewatering before extraction of lipids. • Oil from algae tends to be more volatile (unsaturated), especially at high temperatures, and hence, more likely to degrade. • Higher cost of cultivation • Higher energy consumption during harvesting

1.3.2.1 First-generation biofuels

First-generation biofuels are fuels that have been produced from biomass that is generally edible for example corn and sugarcane. Sugar beets, barley, potato wastes, and whey are also some of the marginal feedstock that is used or considered to produce first-generation bioethanol (Lee and Lavoie, 2013). From the environmental and economic outlook, sugarcane is an ideal feedstock for the production of ethanol but it is limited to certain regions due to soil

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and weather conditions (Naqvi and Yan, 2015). First-generation biofuels can help enhance domestic energy security and can offer some CO₂ benefits (Naik et al., 2010). However, the major concern about these biofuels is their inefficiency and sustainability since the viability of the production of such biofuel is questionable as a result of the conflict with food supply (Patil et al., 2008), including sourcing of feedstock, land use, and the impact it may have on biodiversity. Nowadays, first-generation biofuel production is commercial with an annual production of about 50 billion liters. Such biofuels (first generation) like bioethanol, biodiesel, and biogas are categorized by their ability to be blended with petroleum-based fuels, combusted in existing internal combustion engines, and distributed through existing infrastructure, or by their use in existing alternative vehicle technology such as natural gas vehicles or flexible-fuel vehicle (FFVs) (Naik et al., 2010). First-generation biofuels compete with food and so accrue high costs of production. Certain crops and foodstuffs have become expensive due to the fast expansion of global biofuel production from sugar, grain, and oilseed crops hence, with these drawbacks, there is, therefore, a need for the search for non-edible biomass for biofuel production (Nigam and Singh, 2011).

1.3.2.2 Second-generation biofuels

Second-generation biofuels are produced from lignocellulosic materials such as corn straw, wheat straw, sugarcane bagasse, sugar beet pulp, cassava peels, and switchgrass including softwood and hardwood. They rely on the use of biomass that is not suitable for being used as food (non-edible biomass). Second-generation biofuels comprise either plants that are mainly grown for energy production i.e. bioenergy crops on marginal lands (lands which are unsuitable for food production) or non-edible parts of crops and forest trees which should be processed efficiently for bioenergy by improving on existing technology (Aro, 2016). Lignocellulose which makes up the cell walls of plant biomass is divided into three main components which include: cellulose (30-50%), hemicellulose (10-40%), and lignin (5-20%) (McKendry, 2002). Different authors have certain values for all of these components, however, the extraction process of a particular component, particularly cellulose is somewhat difficult (Voloshin et al., 2016). The development of second-generation biofuels is generally seen as a sustainable response to the rising controversy surrounding first-generation biofuels (Mohr and Raman, 2015). The potential for bioethanol production will be influenced by the chemical composition of the organic compounds involved (Meneses et al., 2017). Second-generation liquid biofuels are commonly produced by two different methods which include the biological or thermochemical processing from lignocellulosic agricultural biomass. The main benefit of

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producing second-generation biofuels from inedible feedstock is that it curtails the direct food versus fuel competition connected with first-generation biofuels. Furthermore, in comparison with first-generation biofuels, second-generation biofuels are associated with an increase in land use efficiency (Nigam and Singh, 2011). According to Markus *et al.* (Pauly and Keegstra, 2008), the cell walls of plant biomass represent one of the most abundant renewable resources on earth. At present, only 2% of this biomass is utilized by man in spite of its abundance. This calls for the need to research the feasibility of utilizing plant cell walls in the production of inexpensive biofuels. The major drawback in the use of lignocellulosic materials is the recalcitrance of cell walls to degrade efficiently into simple fermentable sugars. The addition of wall structure-altering agents or the manipulation of the wall polysaccharide biosynthetic machinery should make tailoring of wall composition and architecture possible in order to improve sugar yields for biofuel production. However, the main challenge is that the study of biosynthetic machinery and its regulation is still in its early stage.

1.3.2.3 Third-generation biofuels

Third-generation biofuels commonly refer to biofuels produced from algal biomass (Bajpai, 2019). Microalgae biomass as a candidate for biofuel production is becoming popular owing to its rapid growth rate, high lipid, and starch content, ease of cultivation (Alam *et al.*, 2015), low land usage, and high carbon dioxide absorption (Bajpai, 2019). It offers a potential solution to one of the pressing issues faced by modern societies today (the development of renewable energy for transportation) owing to its high surface biomass productivity, ability to grow on marginal lands, and efficient conversion of solar energy to chemical energy (Li-Beisson and Peltier, 2013). Algae has the ability to produce higher yields with lesser input resources than other biomass, hence, it is separately classified from second-generation biofuels. In terms of the potential of fuel production, as regards quantity or diversity, no feedstock can contest with algae. The two attributes of algae with respect to the diversity of fuel it can produce include the following: (i) It produces oil that can be easily refined into diesel or certain gasoline components and secondly (ii) It can be genetically manipulated to produce fuels like ethanol, butanol, diesel, and gasoline. Algae is able to produce outstanding yields and has produced about 9000 gallons of biofuel per acre, this is about 10-fold what the best traditional feedstock has been known to produce. There has been a suggestion by those who work closely with algae that yields as high as 20,000 gallons per acre are achievable. Notwithstanding, algae biomass has some drawbacks and one of these drawbacks is that it requires large volumes of water, phosphorus, and nitrogen to grow even when grown in wastewater. Also, biofuels produced

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from algae tend to be less stable than those produced from other sources. The reason for this is that the oil present in algae tends to be highly unsaturated, especially at high temperatures, and hence, more liable to degrade (Bajpai, 2019). Furthermore, microalgae biofuels are not yet commercially sustainable. There are still challenges as regards the improvement of microalgae strains and cultivation technologies (Li-Beisson and Peltier, 2013).

1.4 Bioethanol as an energy source

In recent years, there has been an increase in the global production and use of biofuels for example from 18.2 billion liters, and 60.6 billion liters to 162 billion liters in 2000, 2007, and 2019, respectively with about 85 % of this being bioethanol (Bac-To-Fuel, n.d.; Saini et al., 2015). Bioethanol can be produced from a variety of cheap substrates and it is reported to be one of the important and most widely used liquid biofuels worldwide, especially in transportation (Igwebuike et al., 2021). Depending on the feedstock used, it has been estimated that bioethanol is able to lower the emission of greenhouse gases by approximately 30 - 85 % compared to gasoline (Fulton, 2004; Saini et al., 2015). Bioethanol and biodiesel are the two most common biofuels. While bioethanol is produced by the fermentation of biomass rich in carbohydrates, biodiesel can be produced from animal fats, vegetable oils, algae, or recycled cooking greases as shown in Figure 6. Ethanol can be used as a fuel additive and biodiesel can be used either in its pure form or as a diesel additive to fuel a vehicle (Puppán, 2002).

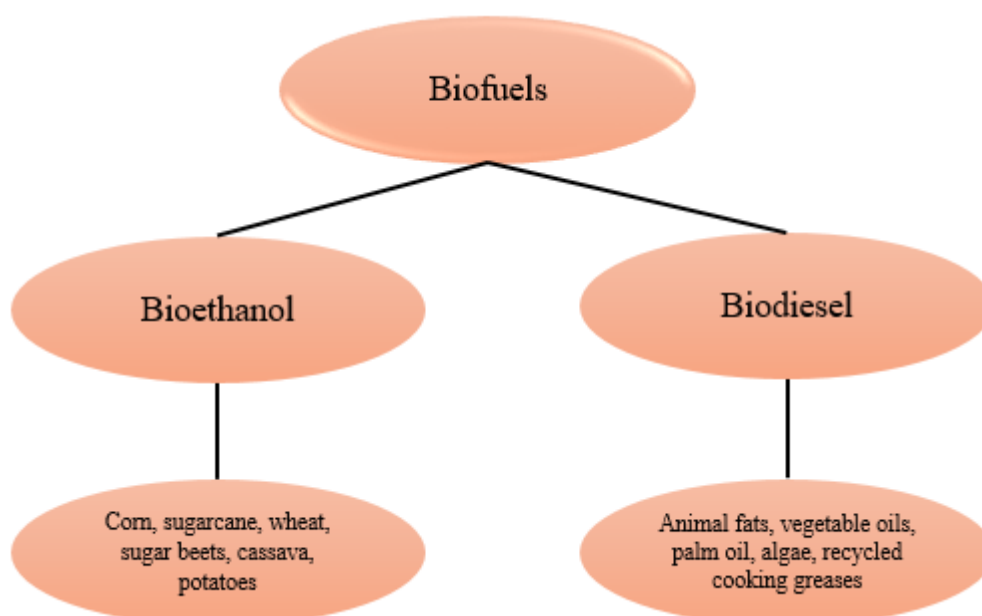


Figure 6. Biomass used for the production of common biofuels (bioethanol and biodiesel).

Ethanol has been described as being perhaps the oldest product obtained through traditional biotechnology (Neelakandan et al., 2009). The use of ethanol as a fuel for motors

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can be traced back to the days of the Model T. The first set of people to identify that the abundant sugars and starches present in plant biomass could be cheaply and easily converted to renewable biofuel were Henry Ford and Alexander Graham Bell. In the year 2016, the United States was the highest producer of ethanol worldwide, producing almost 60% of global production, Figure 7 (Dinneen, 2017), and this position is maintained to date.

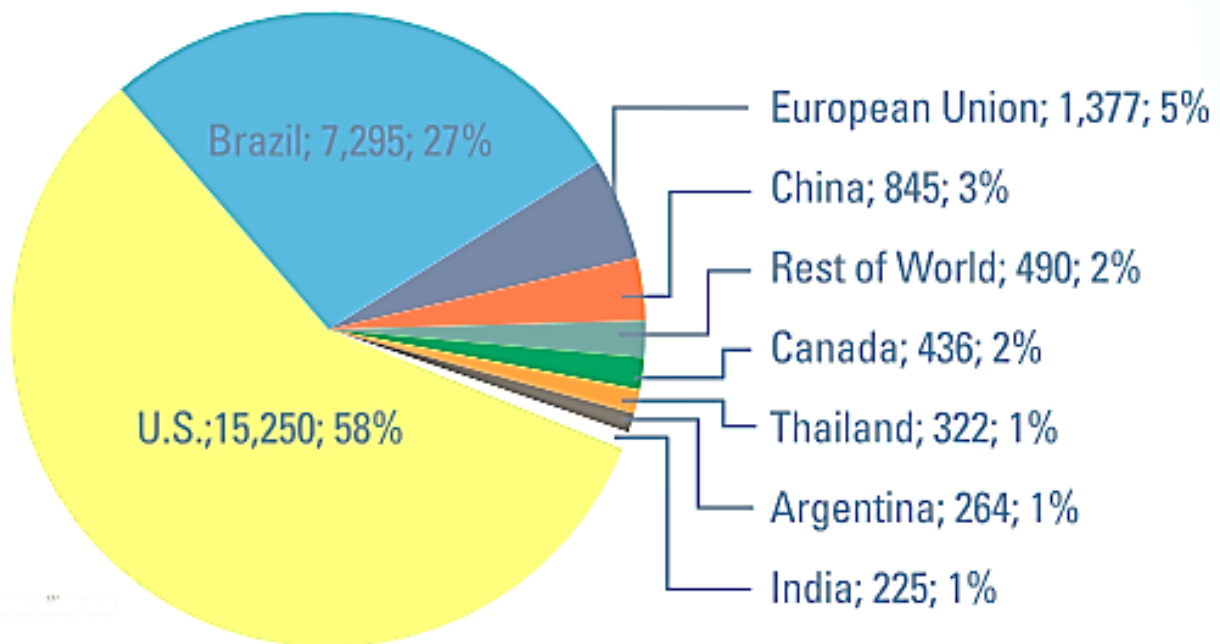


Figure 7. Global bioethanol production by country, million gallons, share of global production, 2016 (Dinneen, 2017).

Bioethanol finds application in the transportation sector, beverage and pharmaceutical industries, and electricity generation. Residues from bioethanol production could be used to produce thermal energy, valuable chemicals, and fertilizers. Bioethanol is a possible alternative to fossil-based transportation fuels because it has broader flammability limits, higher octane number, higher heat of vaporization, and higher flame speeds, and these characteristics allow for shorter burn time, higher compression ratio, and leaner burn engine which ultimately results to advantage in theoretical efficiency over gasoline in an internal combustion engine (Balat, 2007; Rozenfelde et al., 2017). Mixing ethanol with petrol for transportation boosts the performance of the latter. It also enhances fuel combustion in vehicles thus lowering the release of unburned hydrocarbons, carbon monoxide, and carcinogens. Nevertheless, the combustion of ethanol also leads to a heightened reaction with nitrogen in the atmosphere which can cause

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a marginal increase in nitrogen oxide gases. Compared to petrol, ethanol contains only trace amounts of sulfur. So, when ethanol is mixed with petrol, it will help to lower the fuel's sulfur content and hence, reduce the emissions of sulfur oxide which is a major component of acid rain and a carcinogen (Fao.org, 2008).

A combination of sugar beets and wheat is generally used in the production of EU bioethanol. It has been projected that bioethanol production in the EU probably has a greater potential than biodiesel; this is coming from the estimated abundant supplies and production potential for sugar beets and cereals but the cost of production of EU biofuels consequence of high-priced internal feedstock compared to fossil fuels remains a major barrier to the market-based expansion of EU biofuel production especially for bioethanol (Schnepf, 2006). At present, France is a front-runner in the EU's attempt to enhance the use of ethanol, accounting for 2% of global production, primarily from wheat and sugar beet (Balat, 2007; Dufey, 2006). Thereby making France the top producer of fuel ethanol in the European Union. Over 1.2 billion liters of output were expected to be produced in the nation in 2022, an increase of almost 4% from the year before. In contrast, it was anticipated that Germany's ethanol production would total 759 million liters in 2022. In the EU, Germany consumes more ethanol than any other country, with France coming in second (Sönnichsen, 2022). The three types of feedstock used in the production of bioethanol include: sucrose-containing feedstock such as sweet sorghum, sugar beet, sugarcane; starch-based feedstock such as maize, wheat, barley and lignocellulosic biomass like straw, grasses, and wood (Kumar NV et al., 2006; Rozenfelde et al., 2017).

1.5 Processes involved in bioethanol production from lignocellulosic biomass

The recalcitrance of plant cell walls due to their complex nature poses some challenges with the use of lignocellulosic biomass for the obtainment of maximum ethanol yield. Therefore, in order to facilitate ethanol productivity and lower production costs, lignocellulosic materials are subjected to different stages of ethanol production processes which include effective pretreatment processes, hydrolysis, and fermentation including distillation to separate ethanol produced from co-products (Himmel and Picataggio, 2009) (Figure 8).

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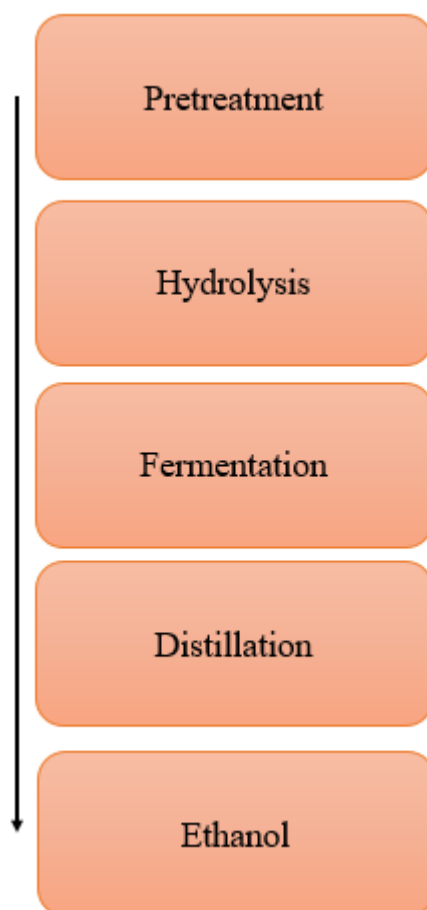


Figure 8. Stepwise process of bioethanol production from lignocellulosic biomass.

1.5.1 Pretreatment

Pretreatment is an essential stage in bioethanol production from lignocellulosic plant biomass as it aims at altering the complex structure of the material by breaking down the lignin seal to solubilize hemicellulose, reduce the crystallinity and increase the porosity of the material, so as to enhance the accessibility of hydrolyzing agents (enzymes or chemicals) which break down cellulose polymers into simple fermentable sugars (Mosier et al., 2005). An ideal pretreatment should be inexpensive, effectively de-lignify substrate materials, prevent the loss or deterioration of carbohydrates, produce high sugar yield, and prevent the formation of sugar degradation products (Sun and Cheng, 2002). Certain plant biomass such as cassava contains high cyanide content (Tivana, 2012) which could affect enzymes and microorganisms and ultimately lead to low production of reducing sugars. Hence, Mohammed *et al.*, demonstrated that 24 h of soaking and 120 min of boiling pretreatment condition is able to reduce the cyanide content in cassava peel waste and improve the total recovery of carbohydrates (Abdullahi et al., 2014).

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In a bid to overcome the challenges inherent to the use of lignocellulosic materials, there has been a shift in pretreatment procedures starting from chemicals and heating methods to biological methods (Hassan et al., 2018) but there is still not yet a satisfactory result from the different pretreatment methods adopted so far in terms of technology for industrial large scale production, cost-effectiveness, and production of a lower amount of inhibitory products (Kucharska et al., 2018). Several pretreatment processes for lignocellulosic biomass have been proposed and practicalized. They include physical, chemical, physicochemical, and biological pretreatment processes as shown in Figure 9. The objectives, advantages, and disadvantages of these different pretreatment processes are outlined in Table 3.

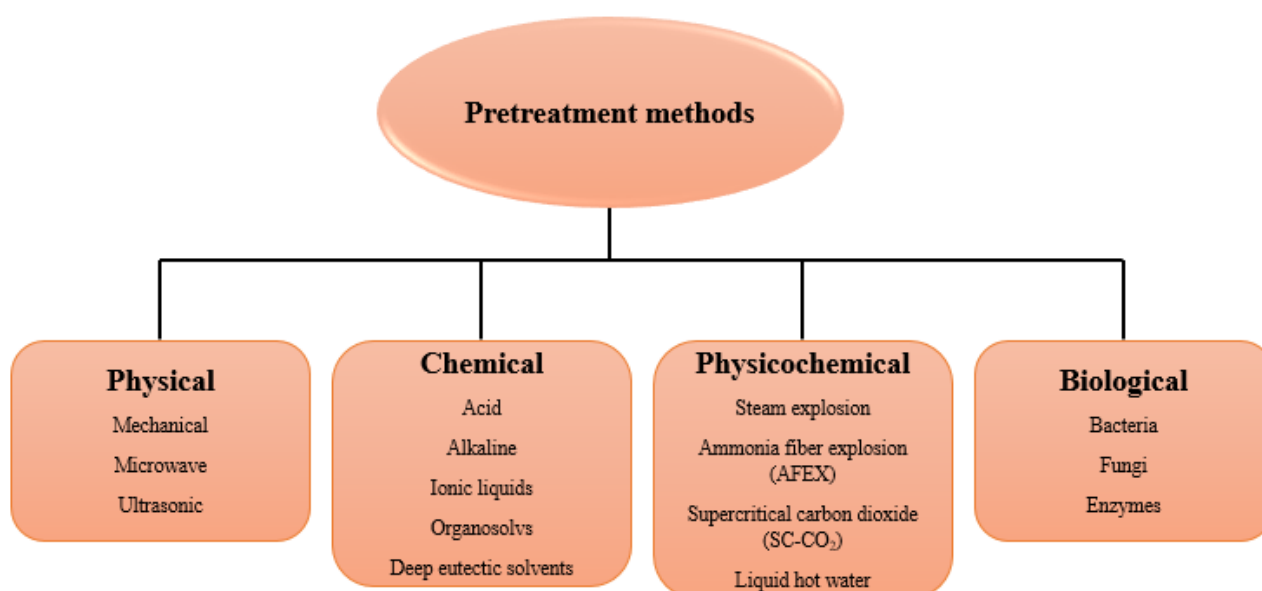


Figure 9. Different pretreatment processes.

1.5.1.1 Physical pretreatment method

This is usually carried out before any other pretreatment method. It aims at reducing the size of lignocellulosic biomass in order to enhance the hydrolysis process. The physical pretreatment methods involve different approaches such as mechanical (grinding and milling), extrusion, and irradiation (microwave, ultrasonic) pretreatment methods. Of these methods, the mechanical approach is the most commonly used method for plant biomass (Amin et al., 2017). With physical pretreatment, the consumption of energy in woody biomass is higher compared to agricultural residues or herbaceous materials. For instance, energy consumption for switchgrass and corn stover has been reported to be 27.6 kWh/metric ton and 11.0 kWh/metric

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ton respectively and those for pine chips and poplar are 118.5 kWh/metric ton and 85.4 kWh/metric ton respectively (Rajendran et al., 2018).

1.5.1.1.1 Mechanical pretreatment

Mechanical pretreatment procedure enhances an efficient bioconversion of biomass to biofuels, ensures appropriate particle sizes, particle distribution, and densification, and improves enzymatic accessibility. However, this method of pretreatment is uneconomical due to its high energy demand and operating costs (Barakat et al., 2014). In order to reduce the energy demand associated with mechanical pretreatment, Zhu *et al.* suggested chemical treatment of biomass before size reduction which is an entirely different approach from what has been reported in the reviewed literature. According to them, their proposed approach has some advantages, like; minimizing the resources required for separating solids from liquids since after pretreatment the plant material remains as chips, avoiding the use of mixing operations that are energy-consuming during pretreatment of pulverized (fiberized) materials and reducing the thermal energy involved in heating up water during pretreatment by minimizing the liquid-to-biomass ratio; compared to fiberized materials which are porous and hydrophilic in nature, wood chips take in a lesser amount of water (Zhu et al., 2010). Size-reduction of materials increases the specific area of biomass, decreases the crystallinity of materials, and also reduces the degree of polymerization (Kucharska et al., 2018; Sun and Cheng, 2002).

1.5.1.1.2 Extrusion

Extrusion involves subjecting lignocellulosic biomass to a combination of heating, shearing, and mixing in a specially designed apparatus called an extruder which eventually results in both physical and chemical modification of biomass. It is convenient and cost-effective (Kumar et al., 2017). Certain parameters such as reaction time, barrel temperature, pressure, screw configuration and speed, particle size, and biomass dry matter affect extrusion pretreatment. Nevertheless, an optimized process with increased efficiency can be achieved by modifying these parameters (Capolupo and Faraco, 2016). Chinnadurai and Kasiviswanathan, pretreated corn stover in a single screw extruder and reported maximum glucose of 75%, xylose 49%, and 61% combined sugar recovery at a barrel temperature of 125°C and screw speed of 75 rpm which was when a combination of cellulase and β -glucosidase was employed at a ratio of 1:4 during hydrolysis, resulting in maximum glucose, xylose, and combined sugar recovery which were 1.96, 1.70, and 2.0 times higher than the control sample (Karunanithy and Muthukumarappan, 2010). Works have also been done using a combination of the extrusion

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process and other pretreatment methods such as ionic liquids. For instance, to enhance the enzymatic saccharification of *Salix gracilistyla* Miq., Song *et al.*, investigated the effect of twin-screw extrusion process by employing 1-ethyl-3-methylimidazolium acetate and its co-solvent, dimethyl sulfoxide. They noticed an increase in the water-soluble fraction and a high disruption of the cellulose crystalline structure (which resulted in an increase in saccharification yield) with an increasing amount of 1-ethyl-3-methylimidazolium acetate in co-solvent and extrusion temperature and reducing screw rotating speed and solid loading. Maximum yield of glucose 99.0% and xylose 99.5% were recorded from the pretreated biomass with only 1-ethyl-3-methylimidazolium acetate at an extrusion temperature of 160°C, 15% solid loading, and screw rotating speed of 5 rpm (Han *et al.*, 2020). Ayla *et al.* also examined the use of a twin-screw extruder as a reactor for continuous pretreatment of sugarcane bagasse at high loadings in an ionic liquid, 1-ethyl-3-methylimidazolium acetate. They reported that the process permitted effective pretreatment of the bagasse at loadings of 25 wt.% for 8 min at 140°C, yielding over 90% glucose after 24 h of enzymatic saccharification of the pretreated biomass. In addition, they discovered that using the extruder as a pretreatment reactor reduced crystallinity and increased the specific surface area by over 100-fold. Compared to batch processes, the extrusion process can be performed continuously and is applicable in the industrial-scale processing of biomass, permitting greater concentrations of reactant and throughputs, higher mixing rates, and more uniform products (Da Silva *et al.*, 2013).

1.5.1.1.3 Microwave and ultrasonic pretreatment

Interests have been drawn to the use of microwave and ultrasonic pretreatment methods. Microwave pretreatment has benefits linked with dielectric heating effects. Such benefit entails rapid and efficient heating in a controlled setting, an increase in processing rates, and short reaction time. Nevertheless, lack of the essential knowledge on the mechanism of microwave heating of materials has stalled its growth in industrial applications for bioenergy generation (Kostas *et al.*, 2017). Ultrasonic pretreatment is able to disrupt the structure of the cell wall, cause a decrease in the degree of polymerization, and increase the specific surface area of biomass thus increasing the biodegradability of material (Zheng *et al.*, 2014). However, these two pretreatment processes are still very marginal and immature. They are still under study.

1.5.1.2 Chemical pretreatment method

Chemical pretreatment relies on chemical reactions for the alteration/modification of lignocellulosic biomass structure. Acid and alkaline are commonly used catalysts for chemical

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pretreatments. Other chemical pretreatment method includes the use of ionic liquids, organosolv (organic solvent), and deep eutectic solvent processes.

1.5.1.2.1 Acid pretreatment

The pretreatment of lignocellulosic biomass with acid causes the disruption of the material matrix by cleaving the glucosidic bonds, thus, resulting in the transformation of polysaccharides into oligomeric and monomeric sugars. The acid pretreatment process can be categorized into wet or dry pretreatment processes. However, wet acid pretreatment is the most commonly adopted technique for hemicellulose disruption of plant biomass. This pretreatment could either be concentrated acid pretreatment or dilute acid pretreatment, depending on the concentration of acid in the acid solution (Solarte-Toro et al., 2019). HCl and H₂SO₄ are examples of acid that has been commonly used for pretreatment. Others include HNO₃, H₃PO₄, and organic acids (maleic acid, oxalic acid, etc.). It solubilizes lignin alongside hemicellulose and reduces the crystallinity of cellulose thus rendering it porous for enzymatic access. The use of concentrated acid for the generation of biofuels is not very encouraging because it causes the corrosion of equipment, production of a very high amount of degradation products, requires neutralization, and it also has acid recovery issues thus rendering it an expensive catalyst to be used for pretreatment. Therefore, the use of dilute acid seems to be more promising in the industrial-scale application as its use for the treatment of lignocellulosic biomass has been broadly researched. Dilute acid could be used on biomass materials at a lower temperature but for a longer time duration or at a higher temperature for a lower time duration. It is advantageous in that it helps in the release of hemicellulose sugars but depending on the pretreatment conditions, the sugars could be further degraded into inhibitor products like aliphatic acids, furfural, 5-hydroxymethylfurfural (HMF) and aromatic lignin which interfere with the overall yield of biofuels (Tomás-Pejó et al., 2011). Hence several works of literature have suggested neutralization and detoxification measures prior to fermentation as a means to reduce the inhibitory effects of these by-products.

1.5.1.2.2 Alkaline pretreatment

Alkaline represents another chemical catalyst for the pretreatment of lignocellulosic biomass. It is a very attractive approach because it involves a non-complicated process. In comparison to acid pretreatment, it involves a milder reaction condition and does not have equipment corrosion and chemical recovery issues. It effectively/selectively removes lignin alongside a very minimal portion of hemicellulose. So, it can be said to be an ideal chemical for the conservation of carbohydrates. It also encourages enzymatic hydrolysis by increasing

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the porosity and surface area of biomass including altering crystallinity and degree of polymerization. (Kim et al., 2016). In the course of pretreatment of biomass with alkaline, the foremost reaction involves the dissolution of the amorphous components (lignin and hemicellulose) and saponification (de-esterification of intermolecular ester bonds) which causes swelling of the biomass and renders it more open to microbial and enzymatic activities (Xu and Sun, 2016),(Wen et al., 2011). KOH, NaOH, CaO, Ca(OH)₂, Mg(OH)₂, and NH₄OH are examples of alkaline used for the pretreatment of biomass but KOH and NaOH are expensive chemicals and recovery is low due to the formation of some salts such as soaps from fatty acids and as such, they can be substituted by lesser expensive chemicals like slaked lime (Ca(OH)₂) or quick lime CaO) which performs a similar function and allows for easier recovery of calcium salts after reaction with CO₂ (Martínez-Merino et al., 2013). Alkaline pretreatment (lime) is not as effective on woody biomass as it is on herbaceous materials or agricultural residues when set under the same process condition. This is due to the higher amount of lignin present in the woods (Yang and Wyman, 2008). The composition of feedstock biomass to a large extent determines the alkaline pretreatment conditions it should be subjected to. However, pretreatment temperature, time, biomass loading, alkali type, and concentration are essential factors that have impacts on biomass pretreatment. It is therefore important to consider developing a pretreatment approach that employs low alkali concentration and low temperature for economic reasons and in order to have more relevance on the industrial scale (Sindhu et al., 2015).

Chen *et al.*, who worked on the parameters of alkaline pretreatment of corn stover reported NaOH loading to be the most significant variable for enzymatic digestibility and that 0.08 g NaOH/g biomass was needed to achieve 70% glucan conversion and therefore the efficiency of pretreatment depended on the ratio of alkaline to solid loading (Chen et al., 2015). Kim and Han carried out alkaline pretreatment on rice straw and obtained 252.62 g/kg maximum glucose yield under optimal pretreatment conditions of 2.96% NaOH concentration, 81.8°C reaction temperature, and 56.7 min pretreatment time (Kim and Han, 2012). The alkali pretreatment conditions applied by Sindhu *et al.* for sugarcane tops were 15% w/w biomass loading, 3% NaOH, and 60 min pretreatment time at 121°C autoclave temperature (Sindhu et al., 2014).

1.5.1.2.3 Ionic liquid pretreatment

Ionic liquids are regarded as nonvolatile chemicals that could be a good substitute for volatile organic compounds. They are perceived as a useful and effective solvent for

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lignocellulosic materials. They find applications in different areas such as in the production of biofuels, chemicals, and material characterization. They are able to dissolve each component of plant cell wall including the whole biomass cell wall under mild circumstances causing only minor changes in the initial chemical structure and hence, they are regarded as non-degradative solvents. Ionic liquids consist of anions and cations. The anions are both organic and inorganic ions while the cations are basically organic and include pyridinium, imidazolium, alkylated phosphonium, aliphatic ammonium, and sulfonium ions. Ionic liquids are beneficial since they are non-toxic, non-corrosive, recoverable, and reusable but are expensive chemicals (Yoo et al., 2017).

Nafiseh *et al.*, pretreated rice straw with 1-ethyl-3-methylimidazolium acetate at 120°C, 5 h, and under atmospheric pressure. Then, saccharification was performed at 45°C and 72 h with 20 FPU cellulase and 30 IU β -glucosidase per gram of cellulose. An increase in glucose yield from 25.7% for the untreated biomass to more than 75% for the treated biomass was observed and also, ethanol yield by simultaneous saccharification and fermentation with *Saccharomyces cerevisiae* was observed to be 35.6% for the untreated biomass and 79.7% for the treated one. Furthermore, a more open and accessible structure and a decrease in crystallinity were also observed with the pretreated straw (Pooornejad et al., 2014).

1.5.1.2.4 Organosolv pretreatment

Organosolv is quite an interesting method for the separation of both cellulose and almost pure lignin from biomass materials. It is considered very useful in the biorefinery for the production of both lignin and bioethanol in the same process. Organic solvents include methanol, ethanol, formic acid, acetic acid, acetone, phenol, or glycerol (Borand and Karaosmanoğlu, 2018). Salapa *et al.*, in their experiment on organosolv pretreatment of wheat straw in the presence of sulfuric acid and using commercial cellulase for enzymatic digestibility of the pretreated material, reported that pretreatment with ethanol at a temperature of 180°C and duration of 40 min amounted to the highest cellulose conversion of 89% and ethanol yield 67% of the theoretical yield. It was also shown that pretreatment with diethylene glycol at a temperature of 160°C and a duration of 40 min gave a maximum ethanol yield of 65% and pulp yield of 51% (Salapa et al., 2017).

1.5.1.2.5 Deep eutectic solvent pretreatment

Deep eutectic solvents (DESs) consist of mixtures of two or more components with a freezing point much below the melting point for any of the original mixture components i.e. they are composed of two or more components and each of these components has melting point

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that is above that of the deep eutectic solvent and so, they are called eutectics (Poole, 2019). They comprise a combination of hydrogen bond acceptor and hydrogen bond donor with a specific molar ratio, hence, causing melting point depression of the solvent (Dwamena, 2019). The most common compound used in the preparation of deep eutectic solvents includes phosphonium, cholinium, or tetraalkylammonium halide salts mixed with urea, carboxylic acid, ethylene glycol, or different kinds of carbohydrates (Poole, 2019). DESs have some drawbacks such as high viscosity, a certain amount of volatility, low to moderate stability, high hygroscopicity, and flammability (Chen and Mu, 2021).

1.5.1.3 Physicochemical pretreatment methods

This is a combination of both physical and chemical pretreatment processes. This type of pretreatment includes; steam explosion, ammonia fiber explosion (AFEX), supercritical carbon dioxide (SC-CO₂), and liquid hot water pretreatment.

1.5.1.3.1 Fiber explosion

Fiber explosion includes steam explosion, AFEX, and SC-CO₂. Steam explosion involves the use of both mechanical forces and chemical processes on biomass. During this pretreatment method, the biomass is first submitted to high-pressure saturated steam ranging from 0.69 MPa to 4.83 MPa at 160 – 120°C for a few seconds to minutes to allow for biomass intake of water molecules. This is then followed by a quick release of pressure and steam which causes an explosive discharge of water molecules. Such swift release of pressure and steam to atmospheric conditions results in the explosion of biomass into smaller pieces (split fibers), disrupting the structure of the material. The high temperature and pressure cause the cleavage of hemicellulose-lignin bond and also the breakdown of glycosidic bonds present in hemicellulose and cellulose thus resulting in lignin transformation, hemicellulose degradation and improvement of the chances of cellulose hydrolysis (Baruah et al., 2018),(Chen and Liu, 2015). This method of pretreatment is also referred to as autohydrolysis because, in the course of the treatment, the breakdown of hemicellulose into simple sugars produces acetic acid which drives further hydrolysis of hemicellulose. Low pH values and high temperatures stimulate the production of this acetic acid from acetyl groups which are linked to hemicellulose (Chen and Liu, 2015).

Ammonia fiber explosion (AFEX) is a pretreatment procedure that employs a combination of both physical (high pressure and temperature) and chemical (ammonia) processes to attain efficient pretreatment. AFEX pretreatment increases the surface area of biomass in order to promote enzyme hydrolysis. It also causes partial depolymerization of

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hemicellulose, enhances cellulose decrystallization, and decreases the recalcitrance of lignin. Optimal enzymatic hydrolysis of AFEX-treated corn stover resulted in about 98% theoretical glucose yield. There are some advantages associated with the use of AFEX pretreatment method viz. ammonia remaining on the pretreated material serves as a nutrient for microbial growth, not demanding a washing step during the process enhances high solid loading hydrolysis, and also, there is the benefit of almost-complete recovery of the ammonia chemical pretreatment (Balan et al., 2009).

Supercritical carbon-dioxide pretreatment (SC-CO₂) process involves the use of carbon dioxide (CO₂) to treat lignocellulosic materials. CO₂ is cheap though this procedure requires high pressure. It is regarded as a green solvent since it is non-toxic. Also, it's non-flammable and easy to extract after the explosion (Rajendran et al., 2018). Various parameters such as temperature, time, pressure, the ratio of dry biomass to CO₂, moisture, and pressure release gradient influence the effectiveness of SC-CO₂ explosion pretreatment of different feedstock (Gu et al., 2013).

1.5.1.3.2 Liquid hot water pretreatment (LHW)

Liquid hot water pretreatment (LHW) is similar to the steam-explosion process. However, LHW utilizes water at high temperatures and pressure (of about 5 MPa) instead of steam to retain its liquid form so as to enhance fragmentation and separation of the biomass material. The temperature involved with this process could vary from 160°C to 240°C for a time duration of a few minutes to an hour with temperature controlling the types of sugar formation and time controlling the amount of sugar formation (Brodeur et al., 2011). LHW does not require the sudden release of pressure as practicable in the steam explosion process and the application of pressure is to avoid water evaporation (Baruah et al., 2018). Controlled-pH LHW pretreatment of biomass promotes enzymatic digestibility by removing hemicellulose thus enabling hydrolysis enzymes to gain more access to the cellulose component. The hemicellulose being removed gets dissolved in the liquid phase of the pretreated biomass as oligosaccharides. There is only little production of simple fermentable sugars during LHW pretreatment. This pretreatment process is usually done by cooking the biomass material in process water at a temperature range of 160°C - 190°C and a pH range of 4 - 7 (Kim et al., 2009). There is less chemical usage with this procedure.

1.5.1.4 Biological pretreatment methods

Biological pretreatment is a mild and eco-friendly pretreatment method that involves the use of enzyme-producing microorganisms (fungi or bacteria) to disrupt/break down the

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cross-linked structures in lignocellulosic materials. Fungi are known to be the most effective and suitable microorganisms for the degradation of lignin, hemicellulose, and cellulose. Examples of commonly used fungi include white-rot fungi, brown-rot fungi, and soft-rot fungi with white-rot fungi reported as the most effective. Brown-rot fungi attack cellulose while white-rot and soft-rot fungi attack both lignin and cellulose (Ghasemzadeh et al., 2017). Some crude enzymes (lignin peroxidases, laccases, Manganese peroxidase, etc.), microbial consortium, and bacteria have also been used to disrupt plant cell walls. However, in comparison to the chemical and physical pretreatment methods, this method is slow and not very efficient (Velmurugan et al., 2020). Pretreatment with microbes involves a solid-state fermentation procedure during which microorganisms grow on biomass material and selectively remove lignin and at times, hemicellulose while cellulose is most often not affected (Dalena et al., 2017).

The objectives, including the advantages and disadvantages of the different pretreatment methods, are given in Table 3.

Table 3. Advantages and disadvantages of the various pretreatment methods.

Pretreatment methods	Objectives	Advantages	Disadvantages	References
Physical	To reduce biomass size and decrease crystallinity.	Green pretreatment (rarely forms inhibitory product), improves hydrolysis rate	Energy-intensive, not economically viable, unable to remove/ alter lignin	(Aslanzadeh et al., 2014; Kumar et al., 2009)
Chemical	To break down/solubilize/remove lignin and hemicellulose and increase surface area.	Enzymatic hydrolysis might not be necessary (acid hydrolyzes lignocellulosic materials to simple sugars	Corrosion of equipment, Expensive, non-selective, requires high temperatures, chemical recovery issues, requires neutralization, fermentation inhibitor problem.	(Peral, 2016; Rozenfelde et al., 2017)

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Physicochemical	To alter lignin, degrade hemicellulose, reduce cellulose crystallinity, increase the surface area of biomass	Less use of chemicals, requires less energy compared to the mechanical method, high sugar recovery, limited environmental impact, low cost.	Unfinished disruption of lignin-carbohydrate matrix	(Kang et al., 2014; Pielhop et al., 2016)
Biological	To disrupt plant cell walls, selectively remove lignin and degrade hemicellulose.	Mild and eco-friendly, low energy requirement, no formation of inhibitor byproduct.	Relatively slow process, expensive (e.g. GMOs)	(Dalena et al., 2017; Velmurugan et al., 2020)

1.5.2 Hydrolysis

After the pretreatment process, the next stage is the hydrolysis procedure. Hydrolysis is a chemical process that involves the use (addition) of water to break down polymers (e.g. cellulose) into monomers (e.g. glucose). It is a chemical reaction that breaks the chemical bonds that exist between two substances and releases energy in which case, one molecule of a substance receives H^+ ion while the other molecule gets OH^- group. Hydrolysis is needed to get simple fermentable sugars. Acids (HCl , H_2SO_4 , etc.) and enzymes are commonly used catalysts in the hydrolysis of biomass.

1.5.2.1 Acid hydrolysis

This is an expatiation of the acid pretreatment method since acid pretreatment and acid hydrolysis are used interchangeably. Acid hydrolysis can be achieved by inorganic acids (liquid acid catalyst or solid acid catalyst) or organic acids. Liquid acid hydrolysis is of two types viz. dilute and concentrated, each of which possesses unique characteristics on biomass. Two reactions are involved with the use of dilute acid hydrolysis. One involves the conversion of cellulose to sugar while the other involves the conversion of sugar to chemicals and many of these chemicals act as growth inhibitors to fermenting organisms. Concentrated acid hydrolysis which consists of about 70% acid content operates at low temperatures ($37.8^\circ C$) and pressure. However, dilute acid is the most preferable in terms of economics and effect on biomass (Shahbazi and Zhang, 2010). Braide *et al.* pretreated 50g of corncobs, cornstalk, cornhusk, sugarcane bagasse, and sugarcane bark, each in 500 ml of 5% H_2SO_4 at $121^\circ C$ and for 15 min. (Braide et al., 2016) while Kanlaya and Jirasak carried out hydrolysis on 1.5% w/v Cassava

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peel using 0.1 M H₂SO₄, a temperature of 135°C, 15 lb/inch² pressure, and time duration of 90 min to obtain 66.28% yield of reducing sugars. Similar conditions were applied to obtain a 63.29% yield of reducing sugars when 0.025 M HCl was used for the hydrolysis of the biomass (Yoonan and Kongkiattikajorn, 2004). Sometimes, pretreatments such as steam pretreatment can be carried out prior to acid hydrolysis. For example, Berhe and Sahu performed steam pretreatment on sugarcane bagasse before hydrolysis with dilute sulfuric acid. Acid hydrolysis was carried out in an autoclave, temperatures between 80 and 100°C, and time between 30 to 60 min were tested (Berhe and Sahu, 2017). Chandel *et al.* reported 30.29 g/L total reducing sugar yields alongside some inhibitory byproducts when sugarcane bagasse was treated with 2.5% v/v HCl. They also reported the following: 75.8% and 63.4% reduction in total phenolics, and furans respectively on the treatment of acid hydrolysate with anion-exchange-resin, 57.5% and 38.7% reduction in total phenolics and furans, respectively on the treatment of hydrolysate with activated charcoal, 35.87% and 45.8% reduction in phenolics and furans, respectively on overliming, and 77.5% reduction in total phenolics on treatment with laccase while furans and acetic acid content in the hydrolysate were unaffected (Chandel *et al.*, 2007).

Solid (heterogeneous) acid catalysts have in recent years, experienced an increase in their use for cellulose hydrolysis into glucose. Examples of these catalysts include H-form zeolites, functionalized silica, immobilized ionic liquids, metal oxides, supported metals, acid resins, heteropoly acids, carbonaceous acids, and magnetic acids (Hu *et al.*, 2015). Wang *et al.* employed silica as a catalyst for the selective hydrolysis of cellulose into glucose. They obtained 73.3% cellulose conversion and glucose yield as much as 50% at 160°C, 12 hr. reaction time, under hydrothermal conditions in the absence of hydrogen gas. It was also reported that the silica catalyst presented a higher catalytic activity than oxides like ZrO₂, TiO₂, and Al₂O₃ synthesized through the same method (evaporation-induced self-assembly method) (Wang *et al.*, 2012). Ayumu *et al.* worked on solid acid catalysts such as H-form zeolite catalyst, sulfated, and sulfonated catalysts for the selective hydrolysis of cellulose and discovered that the sulfonated activated-carbon catalyst exhibited a considerably high glucose yield and this was accredited to high hydrothermal stability and effective catalytic property due to the strong acid sites of SO₃H functional groups and hydrophobic planes (Onda *et al.*, 2008).

Some of the advantages of heterogeneous catalysts are that they can easily be removed from a reaction mixture through a process like filtration. This is important for industrial manufacturing processes since it makes expensive catalysts simple and efficiently recoverable and reusable (Boundless, n.d.). They are also environmentally friendly

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and possess good thermal stability (Farnetti et al., n.d.). Heterogeneous catalysts also have some disadvantages; when the catalyst's surface has been entirely covered by reactant molecules, the reaction cannot continue until the products have left the surface and some area has once again become available for a fresh batch of reactant molecules to adsorb or attach. This explains why the rate-limiting stage in a heterogeneously catalyzed process is frequently the adsorption step (Boundless, n.d.). A heterogeneous catalyst is less active and selective compared to a homogeneous catalyst due to the possession of multiple active sites (Farnetti et al., n.d.). Also, solid catalysts have lower conversions than homogeneous catalysts and necessitate more extreme reaction conditions to provide the same conversions (Ramos et al., 2019).

Organic acids e.g. acetic acid, which is generally regarded as weak acids that do not dissociate completely in water have likewise, been examined for the hydrolysis potential of biomass. For example, Kanlaya and Jirasak achieved a 30.36% yield of reducing sugars when 0.25 M acetic acid was used to hydrolyze cassava peels at 135°C for 90 min (Yoonan and Kongkiattikajorn, 2004).

1.5.2.2 Enzymatic hydrolysis

Cellulases is a class of enzyme used to catalyze the hydrolysis of cellulose. They are produced by bacteria or fungi. However, there is more interest in the use of cellulase produced by fungi than the ones produced by bacteria. This is because most cellulase-producing bacteria are anaerobes that have a very low growth rate. The release of monomeric sugars from cellulose requires the action of three groups of enzymes viz. endoglucanases, exoglucanases (cellobiohydrolases), and β -glucosidases (Bhat and Bhat, 1997; Rozenfelde et al., 2017).

- a. **Endoglucanase:** This is one of the enzymes of cellulose deconstruction that acts by splitting the polymer i.e. the cellulose long chains into shorter molecules (which could be oligosaccharides or smaller polysaccharides units).
- b. **Exoglucanase:** This other group of enzymes frees/releases cellobiose (which is a disaccharide) either from the non-reducing end or the reducing end,
- c. **β -glucosidase** splits cellobiose and other short-chain cello-oligosaccharides into monomer units (glucose).

Other enzymes that have been used for the hydrolysis of plant biomass include xylanases that hydrolyze the major component of hemicellulose (xylan) and amylases for the digestion of starch etc. (Godoy et al., 2018). Xylan is a heterogenous/complex compound with a backbone consisting of β -1,4 linked xylosyl residues, and so, the xylanolytic enzymes

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generally consist of a collection of enzymes such as endoxylanase, β -xylosidase, α -glucuronidase, α -arabinofuranosidase, and acetylxyylan esterase (Verma and Satyanarayana, 2012) that act in collaboration to convert xylan into sugars (Belancic et al., 1995). Nevertheless, xylanases (endoxyylanases) are the most crucial since they are directly involved in cleaving glycosidic bonds and in releasing short xylo-oligosaccharides (Verma and Satyanarayana, 2012). Figure 10 and 11 gives a schematic description of the different enzymatic activities on cellulose and hemicellulose, respectively.

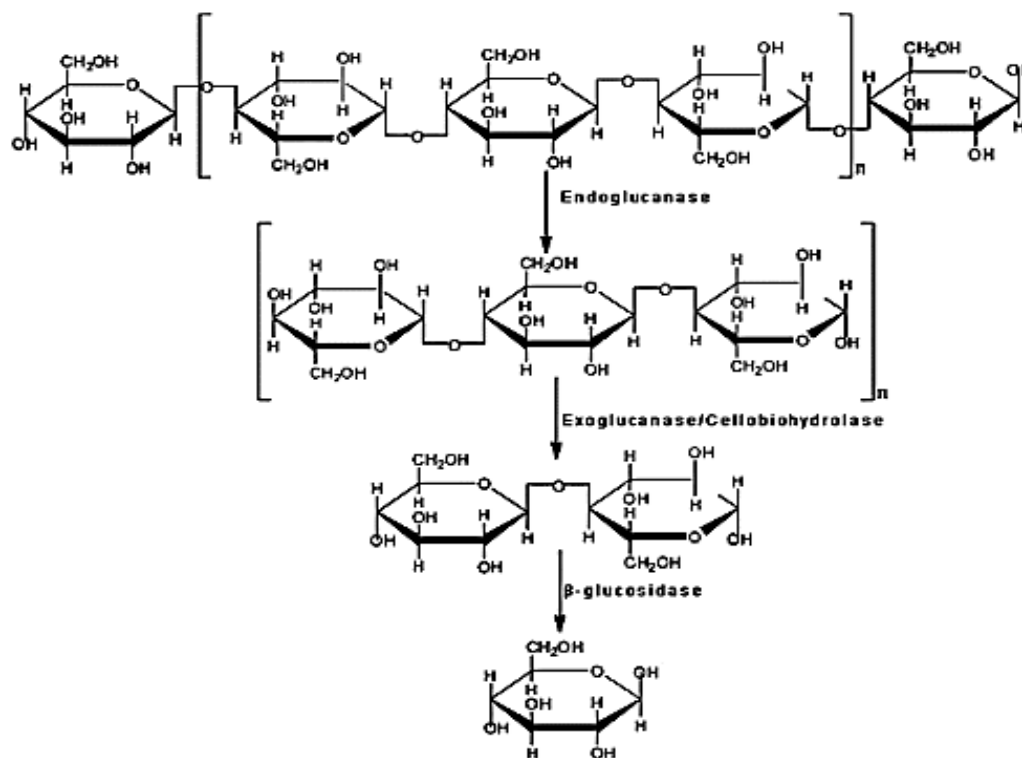


Figure 10. Enzymatic conversion of cellulose-polymer to glucose-monomer (Godoy et al., 2018).

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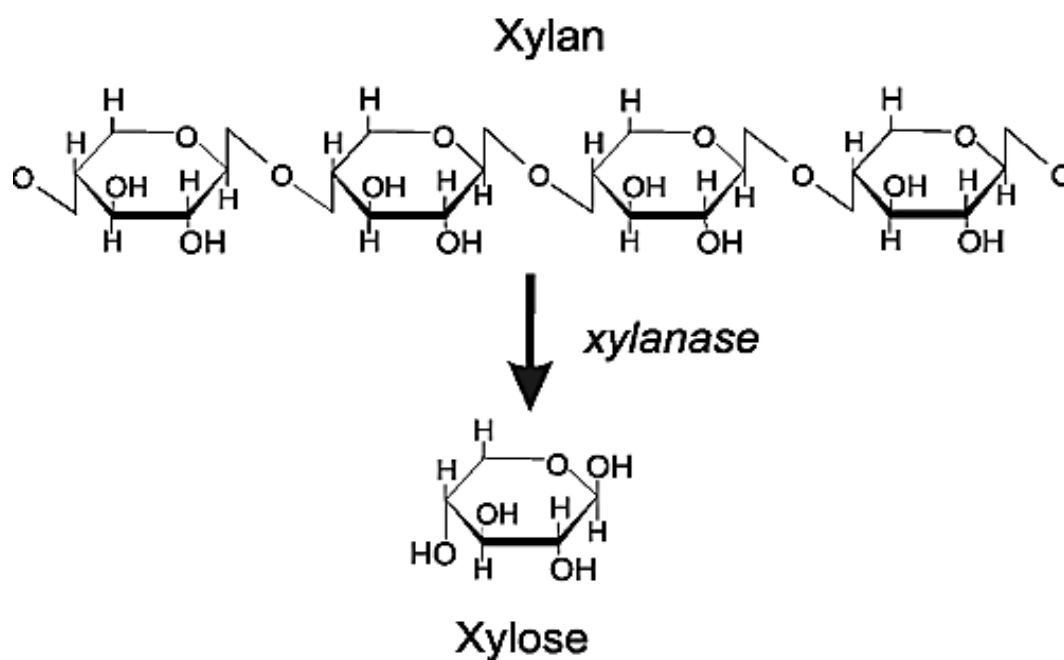


Figure 11. Enzymatic hydrolysis of xylan (Held, 2012).

There are differences in characteristics that exist with the use of acid hydrolysis versus enzymatic hydrolysis and these are outlined in Table 4.

Table 4. Differences between acid hydrolysis and enzymatic hydrolysis (Rozenfelde et al., 2017),(Ghasemzadeh et al., 2017).

Acid hydrolysis	Enzymatic hydrolysis
Corrosive	Non-corrosive
No specificity (selectivity)	More specific
Requires high process temperature (100°C – 160°C)	Operates in low/milder conditions (44°C – 50°C, pH 4.8)
Inhibitor formation issues	No inhibitor byproduct issues
Relatively low yield	Relatively high yield
In some instances after hydrolysis, requires neutralization with chemicals which could be expensive (e.g. NaOH, KOH)	Initial high cost of enzymes. No neutralization needed
Not sensitive to operating conditions	Sensitive to operating conditions
Do not require genetic modification	Could necessitate the genetic modification of enzyme-producing organisms to improve hydrolysis
Non-environmentally friendly	More eco-friendly
Faster process (in minutes)	Takes longer process time (in hours)

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1.6 Sugar degradation products/Fermentation inhibitors

The treatment of hemicellulose by dilute acid results in the formation of toxic compounds like furfural from pentose degradation and soluble aromatic aldehydes from lignin. On a weight basis, aromatic aldehydes are twice as toxic as HMF or furfural (Zaldivar et al., 1999). HMF is produced from the dehydration/degradation of hexose sugars during acid pretreatment or hydrolysis. These compounds inhibit the fermentation process which is needed to produce valuable ethanol from sugar by entering the nucleus of the cell and getting attached to the replicating DNA thus lowering microbial metabolism, reproduction, and enzymatic activities. Acid concentration, temperature, and time are important factors that determine the formation of inhibitors. High acid concentration and low temperature provide an optimum operating condition for acid hydrolysis of potato peels as shown in Table 5 (Lenihan et al., 2010). In addition to the above, the degradation of sugars also results in the formation of carboxylic acids such as acetic, propionic, formic, and lactic acids (Bensah and Mensah, 2019). The further degradation of furans gives rise to the formation of levulinic acid and formic acid and again, the contamination of substrate by microbes can lead to the formation of different acids such as lactic acid (van der Pol et al., 2015). The threshold of inhibition depends on the strain of microorganism and inhibitor tested (Pereira et al., 2016; van der Pol et al., 2014) (Tables 6 and 7). In the measurement of acid toxicity, pH is an essential parameter to be considered. The concentration of HMF beyond 8 g/L and the concentration of furfural beyond 5 g/L hinder the growth of all microbial strains. Notwithstanding, the concentration of furans at 1 to 2 g/L is lethal to the growth of some strains. Also, the growth rate can be inhibited at 15 g/L acetic acid concentration and 10 g/L formic acid concentration but these concentrations have not been detected to cause a severe inhibitory effect on productivity (van der Pol et al., 2014). Besides individual toxicity of compounds, it is also important to take cognizance of the cocktail effect of inhibitor products since the combined effect could elevate the toxicity of compounds. For instance, the interaction between acetic acid and furfural caused a negative effect as a reduction in ethanol yield, specific growth rate, and biomass yield of *S. cerevisiae* was observed (Palmqvist et al., 1999). Furthermore, a more negative result was observed with the interaction of 2 g per liter furfural and 2 g per liter HMF than with 4 g per liter HMF and 4 g per liter furfural acting separately (Taherzadeh et al., 2000). Several measures can be taken to reduce the effect of fermentation inhibitors viz. substrate concentration including salts and produced ethanol should be below the threshold tolerance of the microorganism involved, minimizing/ preventing the use of procedures (e.g. chemicals) that lead to inhibitor formation at the time of pretreatment, *in-situ* detoxification by microorganism used in fermentation, and

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modification of organisms either through microbial adaptation or genetic engineering (Taherzadeh and Karimi, 2011).

Table 5. Effects of acid concentration, temperature, and time on inhibitor formation (Lenihan et al., 2010).

Acid conc. (% w/w)	Temperature (° C)	Time (min)	Sugar yield(g /100 g biomass)	Inhibitor conc. (g/100 g biomass)	Ratio (inhibitor:sugar) (%)
5.0	135	30	26.32	0.6	2.25
5.0	150	15	25.97	2.2	8.4
10	135	8	55.2	1.1	1.9
10	150	8	46.4	1.91	4.1

Table 6. Growth (% of the control) of glucose and xylose-fermenting microorganisms in the presence of inhibitors (Adapted from (Delgenes et al., 1996)).

Inhibitors	Concentration (g/L)	<i>S. cerevisiae</i>	<i>Z. mobilis</i>	<i>P. stipitis</i>	<i>C. shehatae</i>
Furaldehyde	0.5	53	82	75	81
	1	19	81	53	62
	2	10	44	1	9.7
Acetate	5	79	76	63	96
	10	52	44	64	84
	15	56	26	64	79
Hydroxymethylfuraldehyde	1	35	51	95	92
	3	17	69	31	32
	5	11	33	1.4	8
Vanillin	0.5	49	62	12	67
	1	14	37	0.7	9
	2	9	12	1.4	1.6
Hydroxybenzaldehyde	0.5	75	16	57	60
	0.75	47	8	30	23
	1.5	13	8	0	0.8
Syringaldehyde	0.2	100	82	72	89
	0.75	39	72	38	45
	1.5	19	60	3.6	5

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Table 7. Concentration (g/L) of inhibitors at which growth of microbes is completely hindered; σ_i (%) represents standard errors of the estimates. (Adapted from (Pereira et al., 2016)).

Inhibitors	<i>S. cerevisiae</i>	σ_i (%)	<i>E. coli</i>	σ_i (%)	<i>B. subtilis</i>	σ_i (%)
HMF	2.2	18.0	2.2	20.1	1.9	15.7
Syringaldehyde	2.5	8.2	2.7	13.7	2.0	6.0
Vanillin	1.08	22.9	2.2	12.0	1.84	18.3
2-Butanone	45.0	11.4	17.8	14.4	31.0	9.1
2-Butanol	36.0	12.6	21.0	6.5	20.0	18.7
Methyl propionate	23.0	11.6	13.68	13.4	21.0	6.0
Ethyl acetate	22.0	19.6	19.0	12.6	30.0	14.6

1.6.1 Fermentation

Fermentation is a metabolic process that involves the breakdown of a substance into a simpler one by microorganisms such as bacteria, fungi, or yeast. In ethylic fermentation, it is a chemical process by which simple sugars are broken down anaerobically into ethanol.

1.6.1.1 Industrial fermentation technology for ethanol production

a. **Batch fermentation:** This is also referred to as a ‘closed system’, and is the most common and simplest method for producing ethanol. In this method, fermentation is carried out in separate batches. The fermenter is first loaded with the substrate after which, the microorganisms are added and left to ferment the substrate. Byproducts accumulate which continuously changes the culture environment. The products are removed at the end of the fermentation process and the fermenter is cleaned and sterilized in preparation for the next round. The microbes in the fermenter show three distinct growth phases viz. lag, log (exponential), and stationary phases as shown in Figure 12. In the lag phase, microbes are metabolically active but not dividing (growth is slow since it is the phase where microbes adapt to the new environment). The log phase is the period at which the organism is dividing/reproducing at an exponential rate. During the stationary growth phase, growth gets to a plateau; there is an accumulation of by-products as nutrients get exhausted which causes microbial growth to slow down. At this stage, the number of dying cells becomes equal to the number of dividing cells. The culture is usually harvested at the stationary phase, beyond which, it enters the death phase which is characterized by an exponential reduction in the viable cell density. The batch fermentation method has some advantages such as less labor demand, ease of operation, low investment cost, quick and easy control method, complete sterilization, and less risk of contamination (Yang and Sha, 2019).

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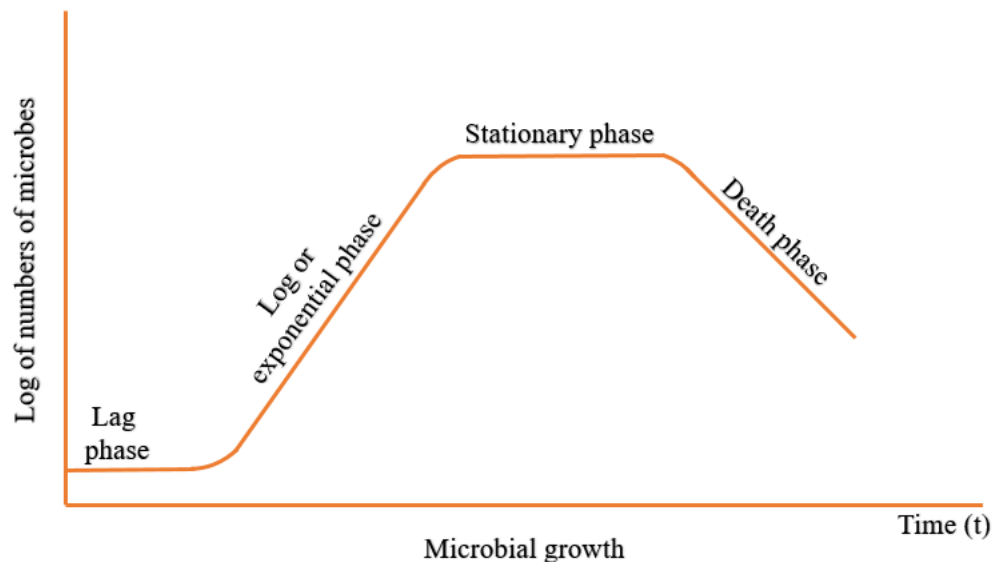


Figure 12. Microbial growth phases.

b. **Fed-batch fermentation:** This is an improved version of the batch fermentation process. Here, the feeds containing substrate, culture medium, and other vital nutrients are loaded into the fermenter after which, cultured microorganisms are introduced and left to ferment the substrate. The feed solution is continuously introduced into the fermenter on an incremental basis throughout the fermentation process without the removal of the products formed. The products are only removed/extracted at the end of each fermentation process. The amount of working volume is a limiting factor in this process (Yang and Sha, 2019).

c. **Semi-continuous fermentation:** This is sometimes referred to as either repeated fed-batch fermentation or a combination of some features notable in the batch and continuous fermentation process. Here, the feed solution is loaded into the fermenter at a constant interval, and the products formed are removed intermittently (not regularly). This process usually requires fixed volume i.e. the volume of fermented (used) medium removed from the fermenter is usually replaced by an equal volume of fresh feeds, and at a constant time interval. This practice could help to maintain the growth of microbes for some time as they get to feed on freshly provided nutrients which replace the already exhausted ones and also, the intermittent removal of formed products could prevent the fermenting organisms from quickly transiting into the inactive/death phase hence, an increase in product yield could be achieved. This process allows for an extended fermentation time and the cycle is not usually terminated until a decline in productivity is detected (Zohri et al., 2017).

d. **Continuous fermentation:** As the name implies, the feed solution is continuously loaded into the fermenting vessel, and the products formed are constantly removed/extracted.

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This allows for a longer fermentation time; the cycle is not interrupted as notable in the batch fermentation process. The growth of microorganisms is therefore maintained for a long time in the fermenting vessel due to the fresh nutrient supply and the regular removal of products whose accumulation has been reported to be detrimental to fermenting microorganisms. Hence, this process results in higher productivity (Ishizaki and Hasumi, 2013).

1.6.1.2 Microorganisms for sugar fermentation

1.6.1.2.1 Microorganisms for pentose sugar fermentation

Pentose sugars such as xylose and arabinose have been regarded as ‘un-fermentable sugars’. From a broad perspective, this could be correct since most microorganisms such as yeast, fungi, and bacteria are unable to effectively utilize pentose sugars. Nevertheless, there exist certain strains that play a significant role in nature’s economy that have been reported to be capable of breaking down these five-carbon sugars (B. Fred, W.H. Peterson, A. Davenport, 1920). Figure 13 and 14 shows the xylose and glucose fermentation pathway in the different fermentation organisms, respectively.

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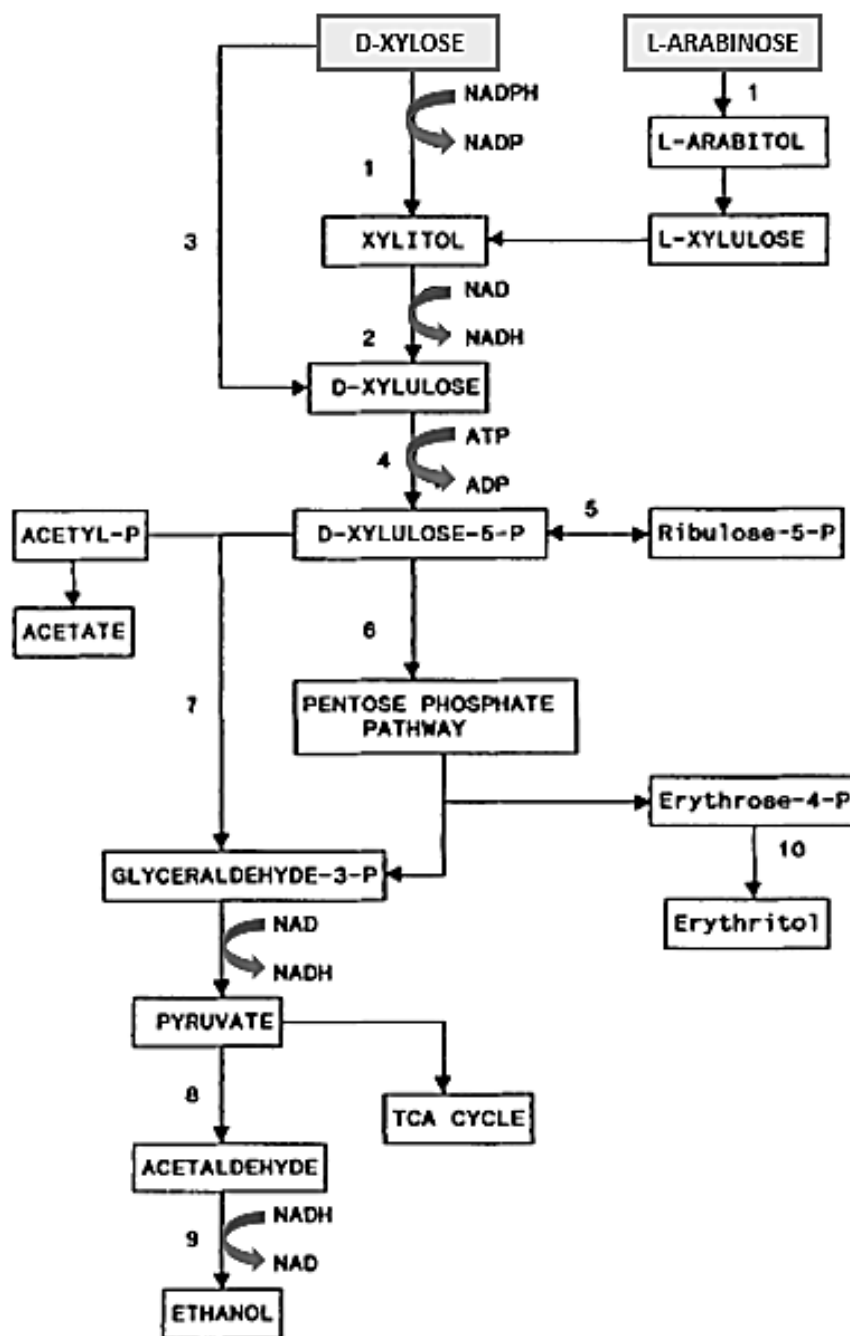


Figure 13. Pentose fermentation pathway by yeast (Adapted from (Mishra, A. Singh, 1993)). The enzymes that catalyze the reactions are represented in numbers (1) Aldose reductase, (2) xylitol dehydrogenase, (3) xylose isomerase, (4) xylulose kinase, (5) phosphoketopentose epimerase, (6) transaldolase and transketolase, (7) phosphoketolase, (8) pyruvate decarboxylase, (9) alcohol dehydrogenase, (10) erythritol dehydrogenase.

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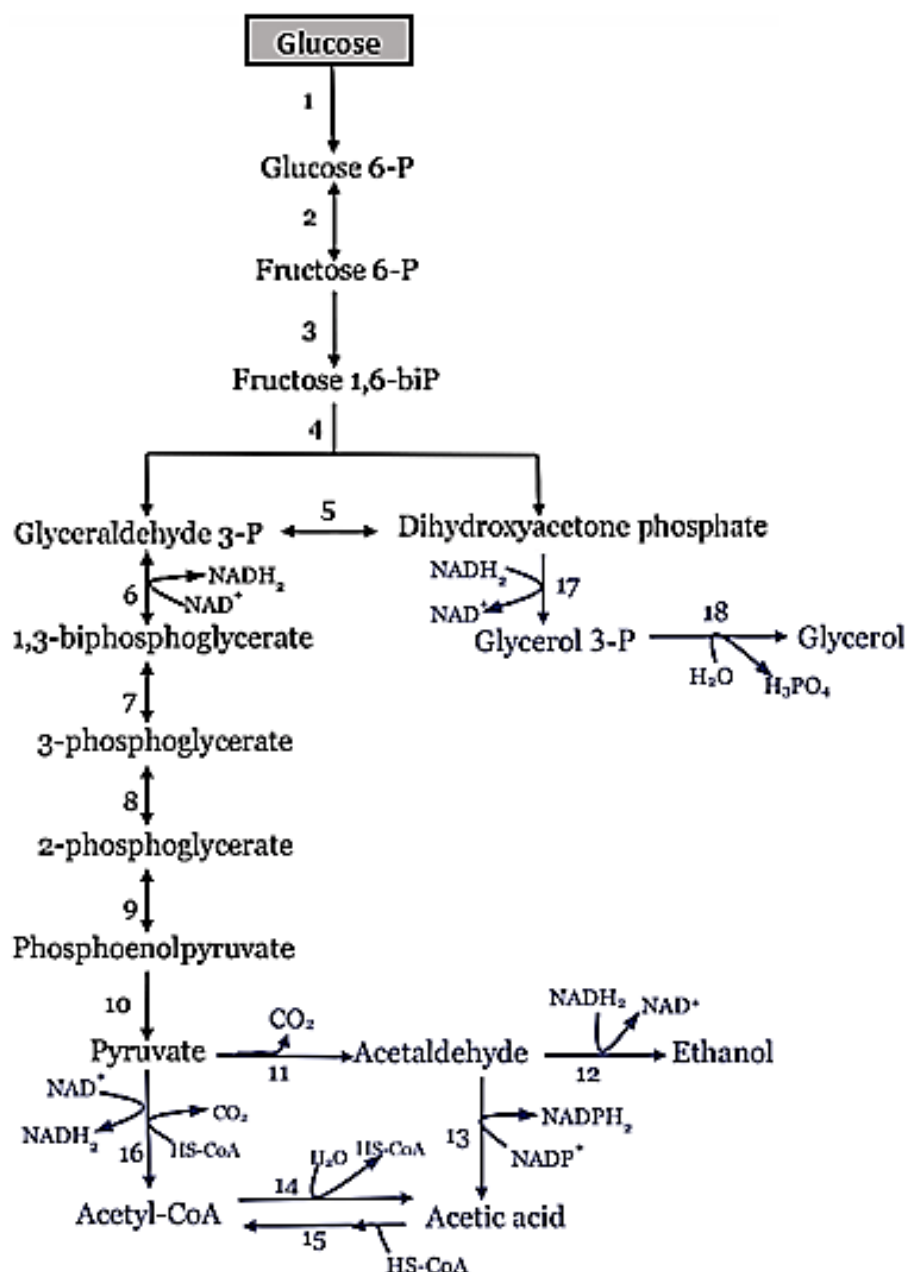


Figure 14. Hexose fermentation by yeast. The enzymes that catalyze the reaction are represented with numbers (Adapted from (Desiderio Estela Escalante, 2019)) (1) hexokinase, (2) phosphoglucose isomerase, (3) phosphofruktokinase, (4) fructose 1,6- bisphosphate aldolase, (5) triosephosphate isomerase; (6) glyceraldehyde 3-phosphate dehydrogenase, (7) phosphoglycerate kinase, (8) phosphoglycerate mutase, (9) enolase, (10) pyruvate kinase, (11) pyruvate decarboxylase, (12) alcohol dehydrogenase, (13) aldehyde dehydrogenase (14) acetyl-CoA hydrolase, (15) acetylCoA synthetase, (16) pyruvate dehydrogenase, (17) glycerol 3-P dehydrogenase, (18) glycerol 3-phosphatase.

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a. Bacteria

The majority of filamentous fungi and yeast are unable to ferment pentose sugars anaerobically but, bacteria are able to convert xylose to ethanol under anaerobic fermentation (Chandel et al., 2011). Xylose-fermenting bacteria comprises both native and genetically modified strains. During xylose fermentation, bacteria do not form xylitol instead, it uses its enzyme 'xylose isomerase' to convert xylose directly to xylulose, and xylulose is then converted to ethanol through the pentose phosphate pathway (PPP), and the Embden-Meyerhof-Parnas pathway (Singla et al., 2012). Examples of pentose-fermenting mesophilic bacteria include *Aerobacter hydrophila*, *E. coli*, *Clostridium acetobutylicum*, *Bacillus polymyxa*, *B. macerans*, and *Klebsiella pneumonia* (Mishra, A. Singh, 1993). Thermophilic anaerobic bacteria have been suggested as promising candidates for the conversion of pentose sugars to ethanol. Some of the species that have been studied include *Thermoanaerobacter ethanolicus*, *T. brockii*, *T. thermohydrosulfuricus*, *Thermoanaerobacterium thermosaccharolyticum*, and *Thermoanaerobacterium saccharolyticum* B6A (Sommer et al., 2004). The benefit of utilizing bacteria e.g. *E. coli* ATCC 11303 (pLOI297) for ethanol production is that the process does not need aeration to achieve high productivity but the downside is the high possibility of contamination since it functions at higher pH, another disadvantage is its high sensitivity to ethanol inhibition, and loss of productivity due to plasmid instability in the course of prolonged operation. Successful large-scale application of bacteria in fermentation is not very certain as compared to yeast (Mc Millan, 1993).

b. Yeast

Yeast is a common and suitable organism for the production of ethanol from sugars. This microorganism has been reported to act favorably in the fermentation of hexose sugars compared to pentose sugars (Saloheimo et al., 2007). However, certain strains such as *C. shehatae*, *Kluveromyces marxianus*, *P. tannophilus*, and *P. stipitis* have been evaluated for their ethanol production potential. Several other species of yeast that are able to utilize the five-carbon sugar (xylose) include *Clavispora sp.*, *Schizosaccharomyces sp.*, and *Brettanomyces sp.* Also included are *Debaromyces species* such as *D. nepalensis* and *D. polymorpha*, and *Candida species* like *C. blankii*, *C. tenuis*, *C. utilis*, *C. solani*, *C. tropicalis*, *C. parapsilosis*, and *C. friedrichii* (Mishra, A. Singh, 1993). Most yeasts are incapable of fermenting xylose directly so, they ferment/utilize xylulose which is an isomer of xylose. The bacteria enzyme, 'xylose isomerase' can catalyze the interconversion of xylose and xylulose (isomerization) which is achieved in a single step. Whereas, yeast utilizes xylose reductase to reduce xylose to xylitol

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and then makes use of xylitol dehydrogenase to convert xylitol to xylulose. Species of *Candida*, *Kluyveromyces*, *Brettanomyces*, *Torulasporea*, *Pachysolen*, *Saccharomyces*, *Hansenula*, and *Schizosaccharomyces* have been recognized as the best ethanol-producing yeast from xylulose (Skoog and Hahn-Hägerdal, 1988). Nutrient medium composition, temperature, aeration rate, and pH are some of the factors that affect xylose-fermenting yeast performance. Some of the benefits associated with the utilization of yeast e.g. *P.stipitis* for the conversion of xylose is that it has high selectivity for ethanol production unlike bacteria and fungi which form coproducts with ethanol, it is also relatively tolerant to ethanol and low pH; properties which reduce the risk of bacterial contamination. However, the drawback of this organism (xylose-fermenting yeast) is that it requires a little amount of oxygen (≤ 2 mMol/L-h) to realize high conversion efficiency; it is relatively easy to achieve micro-aeration on the laboratory scale but this is not easy to achieve in the industrial scale. Another downside of xylose-utilizing yeast is that it presents low volumetric productivities when compared to those obtained with bacteria or glucose-fermenting yeast (Mc Millan, 1993). Compared to *S. cerevisiae*, yeast that utilizes pentose sugars are poorly tolerant of ethanol, inhibitor products, and pH and these attributes can result in low ethanol yield (Hahn-Hägerdal et al., 2007),(Hahn-Hägerdal et al., 1994).

c. Filamentous fungi

Xylose conversion by fungi has not been extensively studied as it is with xylose fermentation by bacteria and yeast (Mc Millan, 1993). Filamentous fungi such as *Neurospora crassa*, *Mucor sp.*, *Fusarium oxysporum*, *Monilia sp.*, and *Paecilomyces sp.* have been known to have pentose sugars fermentation potential. One good thing about the Fungal process is that it has the capacity to grow on natural plant material which is usually absent in yeast-based processes. Nonetheless, the fungal system is associated with properties that make its application in ethanol production unpleasant which are low volumetric production, the longer time it takes for fermentation (4 days to 8 days), a small amount of oxygen requirement, the high viscosity of fermentation broth, growth in large clumps instead of as dispersed single cells, a coproduction of acetic acid alongside ethanol as a major end-product which ultimately leads to reduced ethanol formation, and low tolerance to substrate and product (Mishra, A. Singh, 1993).

An examination of bacterial, yeast, and fungal xylose fermentation, in general, is presented in Table 8.

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Table 8. Overall evaluation of xylose fermentation by bacteria, yeast, and fungi (Adapted from (Skoog and Hahn-Hägerdal, 1988)).

	1 st stage	2 nd stage	3 rd stage
Organism	Xylose to xylulose-5-P	Xylulose-5-P to pyruvate	Pyruvate to the final product(s)
Bacteria	Isomerization	Pentose phosphate + EMP pathway	Ethanol + mixed acids Ethanol + 2,3-butanediol Ethanol + acetone butanol
Yeasts	Oxidation-reduction	Pentose phosphate + EMP pathway	Ethanol
Fungi	Oxidation-reduction	Pentose phosphate + EMP pathway	Ethanol Acetic and lactic acids

1.6.1.2.2 Microorganisms for hexose sugar fermentation

The species of microorganisms that are able to ferment hexose (e.g. glucose) are more than those that have pentose-fermentation potential. Usually, microorganisms e.g. *E. coli* utilize glucose first until it gets exhausted during co-fermentation involving the mixture of sugars, before converting pentose sugars e.g. xylose and arabinose to ethanol. This sequential use of sugars can result in an incomplete or delayed consumption of secondary sugars which in turn leads to a decrease in yield and productivity (Fernández-Sandoval et al., 2019). A wide range of microorganisms such as bacteria e.g. *E. coli*, *Zymomonas mobilis*, *Aerobacter hydrophila*, *Clostridium acetobutylicum*, *Klebsiella pneumonia*, *Bacillus polymyxa*, *B. macerans*, *Thermoanaerobacter ethanolicus*, *Clostridium thermosulfurogenes*, *C. thermocellum*, *C. thermosaccharolyticum*, *C. thermohydrosulfuricum*, fungi e.g. *Mucor indicus*, *Neurospora crassa*, *Fusarium oxysporum*, *Monilia sp.*, *Paecilomyces sp.*, and yeast e.g. *S. cerevisiae*, *Pichia stipitis*, *P. angophorae*, *Candida shehatae*, *Kluyveromyces fragilis*, *K. marxianus*, *Pachysolen tannophilus*, etc. have been used in the fermentation of hexose sugars (Mishra, A. Singh, 1993). But of all microbes that have been employed in the production of ethanol from plant biomass, *S. cerevisiae* is the most famous and commonly used in industrial-scale applications due to its high tolerance to ethanol, a wide range of pH, high productivity, and its ability to ferment a wide range of sugars (Mohd Azhar et al., 2017). Pyruvate is the first stage in the alcoholic fermentation pathway and it is obtained by yeast (*S. cerevisiae*) through the Embden–Meyerhoff, and Parnas (EMP) pathway, and as for bacteria (*Zymomonas*), it is

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formed through the Entner–Doudoroff (ED) pathway. The next stage involves the decarboxylation of pyruvate to acetaldehyde; this reaction is catalyzed by the enzyme called pyruvate decarboxylase. The redox balance of alcoholic fermentation is realized via the reproduction of NAD^+ when acetaldehyde is reduced to ethanol by the enzyme alcohol dehydrogenase. Alcoholic fermentation produces 1 mol of ATP through the ED pathway or 2 mol of ATP through the EMP pathway for each mol of glucose oxidized (Ciani et al., 2008).

1.6.2 Distillation

Distillation is the process of purifying a liquid by separating the components of the liquid mixtures through heating/boiling and condensation. It is an effective purification technique that employs the differences in volatilities of constituents in a mixture (Onuki et al., 2008). Ethanol and water are soluble in each other and so, distillation is required for the separation and concentration of ethanol from the fermentation broth. It is not possible to obtain 100% purity through simple distillation because of the azeotrope between water and ethanol; there is a strong hydrogen bond that exists between water and ethanol which causes water to be attached to ethanol as it pulls out when heated and therefore, about 95% of ethanol can be recovered through this process which finds relevance in the solvent, chemical, cosmetic, and pharmaceutical industries (Aslanzadeh et al., 2014). To obtain 99.9% ethanol i.e. anhydrous ethanol, further drying of ethanol or a dehydration step is required (Meneses et al., 2017). Irrespective of the product being recovered, distillation is an energy-intensive technique, it is expensive and consumes approximately 40% of the total energy used in the chemical and petroleum refining industries (Pribic et al., 2006). Notwithstanding, since distillation remains the main separation technique in process industries, it, therefore, becomes important to enhance its energy efficiency, particularly when applied in the separation of azeotropic mixtures. To this end, several special separation methods have been employed such as liquid-liquid extraction, azeotropic distillation, pressure swing distillation, extractive distillation, pervaporation using membrane, salt addition, adsorption, etc. (Iqbal and Ahmad, 2015)

1.7 EU legislation supporting advanced biofuels

A 10% minimum goal for renewable energy used in the transportation sector was outlined in the 2009 EU Energy and Climate Change Package and was to be met by all EU member states in their respective nations by the year 2020. For the years 2010 to 2020, the Renewable Energy Directive (RED) outlined specific objectives and requirements for the transportation sector. The Renewable Energy Directive II (REDII) for 2021 – 2030 was approved by the European Union in 2018 (“Report Name: Biofuel Mandates in the EU by

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Member State - 2022,” 2022). They included a minimum of 32% target of renewable energy consumption across all sectors and a reduction of greenhouse gases of at least 40%. Transportation should be decarbonized as a top priority going forward because, in comparison to an 18% fall or more in all other sectors, greenhouse gas emissions in the European transportation sector have decreased by only 3.8% since 2008. The EU has encouraged the development of advanced biofuels, which are made from non-food feedstock, as well as conventional biofuels, which are based on food, through the use of directives and national laws. The EU Renewable Energy Directive (RED), which was passed in 2009, stipulated that by 2020, 10% of the energy utilized in the transportation sector must originate from renewable sources (Giuntoli, 2018a).

The EU Indirect Land Use Change (ILUC) directive changed the RED in 2015 by placing a 7% cap on the amount that food/feed-based biofuels might contribute to the RES-transport target. A non-binding 0.5% goal for advanced biofuels in 2020 was included by the ILUC directive as an additional measure to encourage the use of biofuels made from non-food feedstock and wastes (Giuntoli, 2018b). By agreeing to modify the Renewable Energy Directive (REDII) in June 2018, the EU Commission, Parliament, and Council established a target of 14% renewable energy sources - transportation energy and a sub-target of 3.5% for advanced biofuels by 2030. The capping of traditional food-based biofuels to a maximum of 7% of each member state's 2020 level signifies a target of at least 7% for non-food-based/advanced fuels (Giuntoli, 2018a). Advanced biofuels can be double-counted towards the 3.5% target and the 14% target (Giuntoli, 2018b).

1.8 Industrial-scale projects on advanced bioethanol/applications

Several initiatives on the development of renewable fuels (bioethanol) from second-generation (2G) plant biomass have been implemented and most of these technologies follow certain process sequences such as pretreatment of raw biomass, production of biocatalysts (enzymes/fermentation microbes e.g. yeast), simultaneous saccharification and fermentation (SSF) of feedstock or simultaneous saccharification and co-fermentation (SSCF) or separate hydrolysis and fermentation (SHF). For example:

a. Futurol™ technology in France which aims at the production of bioethanol from 2G raw materials. This technology enables the generation of 55,000 tons (or 70 million liters of ethanol) of bioethanol from lignocellulosic plant biomass like energy crops e.g. Silvergrass (*Miscanthus*), agricultural residues, and wood residues. The operation of this technology basically involves the steam-explosion of raw material, on-site production of biocatalysts

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(enzymes and yeasts resistant to inhibitors, particularly acetic acid), enzymatic hydrolysis and co-fermentation (SSCF) of five-carbon and six-carbon sugars which occur all together in one unit, and the recovery of products such as 2G ethanol, lignin, and stillage (Garriga, 2020; Marignan et al., 2020; Projet-futurol, 2020).

b. Sunliquid® technology/plant is an industrial/large-scale commercial project owned and developed by Clariant. The technology is aimed at the production of bioethanol from agricultural residues and it is to allow the production of about 50,000 tons of bioethanol on a yearly basis from 250,000 tons of wheat as well as other cereal straw obtained locally. This plant is located in Southwestern Romania. Meanwhile, Clariant has a demonstration plant that is capable of producing about 1,000 tons of bioethanol from about 4,500 tons of wheat straw, corn stover, or other feedstock biomass located in Straubing, Germany (Renewables, 2019). The Sunliquid® process of ethanol production involves the chopping of feedstock into smaller sizes, steam explosion pretreatment, enzymatic hydrolysis, the simultaneous fermentation with yeast of both C₅ and C₆ sugars, and ethanol recovery. Vinasse is also a by-product formed that can be routed to organic fertilizer applications (Sunliquid®, 2017).

c. The major products of **Domsjö Fabriker** located in Sweden include cellulose, lignin, and bioethanol which are sourced from trees (forest). Other biorefinery products like carbon dioxide are also produced which is further processed into carbonic acid, mainly for industrial purposes (“Domsjö Fabriker,” 2019). Domsjö process utilizes spruce and pine biomass (about 1.6 million cubic meters annually). The process starts with the debarking and chipping of the timber logs, then, it is fed into the digester alongside cooking chemicals. The bark is combusted to generate energy in the form of steam. The cellulose is washed after cooking and then bleached in a closed-loop bleaching plant after which, it is dried. Hemicellulose and lignin get dissolved in the process of cooking and this dissolved hemicellulose undergoes fermentation and distillation to produce bioethanol while lignin which gets refined in the course of cooking are dried and packaged and the cooking chemicals in the recovery boilers are then recycled and the steam obtained serves as an energy that is used for other processes (Winter, 2018).

d. The collective efforts of partners from Europe and Brazil have led to the initiation of the **PROETHANOL2G project** (ProEthanol2G, 2015). The main emphasis of the PROETHANOL2G project is on the combination of both the biological and engineering processes for bioethanol production from the most typical European (wheat straw) and Brazilian (sugarcane bagasse and straw) plant biomass (Gírio and Fonseca, 2015). The

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production process includes pretreatment and enzymatic hydrolysis to convert molecules into sugars, followed by fermentation with recombinant yeast strain of the sugar solution and distillation (Carvalho, 2014; ProEthanol2G, 2015).

e. **BALI™ Biorefinery Demo** is a technology operated by Borregaard and based in Sarpsborg, Norway. It aims at producing lignin and sugars from different biomass such as spruce, bagasse, willow, and straw. This technology converts lignocellulosic biomass (agricultural, wood, and forestry waste) to sugars which in turn could be used in the production of advanced biofuel (bioethanol), and the lignin component of the material becomes advanced performance biochemical. The plant has been able to process over 800 tons of biomass (Bredal, 2016). This demo plant has a processing capacity of 1 to 2 MT per day of biomass (Robek, 2017). BALI™ plant process involves chemical (sulfite) pretreatment, enzymatic hydrolysis with commercial enzymes, and some fermentation type (conventional fermentation of C₆ sugars, aerobic fermentation, or chemical conversion of C₅ sugars, as well as chemical modification of lignin) (Gregg et al., 2017). During the chemical pretreatment and fractionation of biomass, the lignin is rendered water-soluble and this makes it possible to easily separate lignin from the cellulose fraction at the early stage (Rødsrud et al., 2012).

f. **Chempolis**, a biorefining technology company in collaboration with Finnish energy company Fortum, and Indian NRL (Numaligarh Refinery Limited) established a joint venture for the construction of a bamboo biorefinery in Northeast India. On a yearly basis, the biorefinery is expected to use about 300,000 tons of bamboo and bioethanol of about 60 million liters, furfural of 19,000 tons, acetic acid of 11,000 tons, and 144-gigawatt hours of green energy is expected to be generated by the plant, yearly. The central processes of the commercial-scale biorefinery are built on Chempolis's patented formicobio™ technology ("Chempolis Seals Deal for Indian Biorefinery," 2018). The formicobio™ technology concept for the production of ethanol from non-food biomass includes the selective dissolution of the major components of biomass excluding cellulose by biosolvent under low temperature and pressure, purification of cellulose by washing with water, hydrolysis of cellulose to glucose with by enzyme, fermentation of simple sugar to bioethanol, and separation of ethanol. The process allows for the recycling and reuse of vaporized biosolvent. The obtained lignin-rich biofuel is combusted to produce steam and electricity for the biorefinery. Valuable co-products such as acetic acid and furfural formed in the course of delignification and evaporation are also recovered (Anttila, 2019).

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g. In Italy, **Versalis** (Eni's chemical company) reported the decision to put into operation all plants at the Crescentino biorefinery complex in the first half of 2020. The plant makes use of PROESA[®] proprietary technology (Ferrari, 2020; Milanese, 2020). The PROESA[™] technology involves characterization of energy crops, steam pretreatment, enzymatic hydrolysis and co-fermentation (SSCF), and valorization of secondary streams and co-products (Pescarolo, 2013). The industrial demonstration ethanol plant has a capacity of 40,000 tons of bioethanol per annum, beginning from more than 200,000 tons of raw materials on a dry mass basis (Piero, 2018). The Crescentino plant utilizes agricultural waste such as rice straw, wheat straw, and energy crops e.g. *Arundo donax* (giant cane). The lignin can be used to generate about 13 MW of electricity (Biochemtex, 2013).

h. **ARPA-E** (Advanced Research Projects Agency-Energy) and a program referred to as **MARINER** (Macroalgae Research Inspiring Novel Energy Resources) are working on how seaweed (macroalgae) farms could be used as a source of renewable energy. MARINER is creating the tools to commence the building of seaweed farms ("Research and development in the seaweed industry," 2020). MARINER projects aim at facilitating the United States to be a world leader in the production of marine biomass for applicability in significant areas like biofuel production. The technology consists of integrated cultivation and harvesting systems, advanced component technologies, computational modeling tools, aquatic monitoring tools, and advanced breeding and genetic tools. According to ARPA-E, it is estimated that the US has a favorable condition and geography to generate more than 500 million dry metric tons of macroalgae per annum and these production volumes could amount to approximately 2.7 quadrillion BTUs (quads) of energy (liquid fuel) and about 10% of the country's yearly transportation energy demand (von Keitz, 2017).

i. **TATA project**, an Indian infrastructure company support the EBP (Ethanol blended petrol) program of the country. TATA project got an order from BPCL (Bharat Petroleum Corporation Limited) concerning its project on second-generation bioethanol in India. Agro-based biomass such as rice straw will be employed as the design feedstock, while maize stalk will be used as the check-case. The production capacity projected for the bioethanol plant is 100,000 liters per day (TATA, 2020).

j. **LIGNOFLAG**, funded by the EU which has Clariant as its project coordinator, aims at optimizing efficiency while raising the production capacity of the technology/plant to about 60,000 tons of ethanol on an annual basis. The project also seeks to promote sustainable

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development of the production process that involves the utilization of co-products for the generation of clean energy and fertilizers for the soil (“Project Overview,” 2020).

k. IOCL's (Indian Oil Corporation Limited) 2G Ethanol Bio-Refinery at Panipat Haryana is built on Praj's technology for turning rice straw into feedstock for ethanol generation. The technology operation involves acid and steam explosion pretreatment, enzymatic hydrolysis, co-fermentation with GMO (Genetically Modified Organisms) type yeast, distillation, and dehydration. The 2G Ethanol Bio-Refinery is able to process 30 million liters of ethanol from 200,000 tonnes of rice straw per annum (Equity Bulls, 2022; Praj Industries Ltd, 2018).

l. AustroCel's bioethanol plant under construction in Hallein, Austria, will be provided with Valmet's automation technology. The bioethanol obtained from the plant is generated from waste materials gotten from the adjacent viscose pulp mill via the fermenting and distilling wood sugar process. The technology operation involves sulphite pulping/digestion of wood chips, and fermentation of sulphite spent liquor (SSL) with yeast. The generation of 30 million liters of advanced bioethanol annually could result from retrofitting. The biofuel is to be added to petrol, thus substituting approximately 1% of the yearly petrol consumption in Austria and saving about 50,000 tons of CO₂ per annum (Bacovsky et al., 2021; Dugandzic, 2020; Mäki et al., 2021; Valmet, 2020).

From the above, it can be deduced that the reviewed technologies employed steam pretreatment or chemical pretreatment, enzymatic hydrolysis, and wild or recombinant yeast for the fermentation of sugars.

1.9 Representative lignocellulosic waste biomass and their potential for second-generation bioethanol production

Representative lignocellulosic waste biomass that has the potential to be used as an energy source in the production of renewable bioethanol due to the possession of high holocellulose components include; cassava peels, sugar beet pulp, and the macroalgae, *Ulva lactuca*.

a. Cassava peels

Cassava with the scientific name *Manihot esculenta* is a perennial shrub that is largely cultivated vegetatively from stem cutting as an annual crop in tropical and subtropical areas for its edible root. It is a staple crop that belongs to the family Euphorbiaceae. Cassava consists of a starchy tuberous root which serves as an important source of carbohydrates. It is the world's sixth most essential crop and is cultivated in different parts of Africa, Asia, and Latin American

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countries (Bayitse et al., 2015). It is native to South America and was first introduced into Africa in the Congo Basin by the early Portuguese explorers in the late 16th century (“Cassava Development In Nigeria- A Country Case Study towards a Global Strategy for Cassava Development,” 2005). It serves as the main raw material in different industries in tropical regions and has been used to produce starch, and ethanol, including calories, sweeteners, and amino acids (Apiwatanapiwat et al., 2011). Cassava can also be processed into products like chips, flour, bread, ‘garri’, etc. which are of high local demand and are of export potential. Cassava contains nutrients that are of health benefits and are also an important source of phosphorus, iron, potassium, calcium, and manganese (Reddy, 2018). Cassava is tolerant to drought and can be cultivated in regions with tentative rainfall patterns that are not favourable for the growth of some other crops. Nigeria is the world’s highest producer of cassava (Otekunrin and Sawicka, 2019). It is a major staple food in almost every part of the country. As of 2018, cassava production quantity in Nigeria was estimated to be 59,475,202 tonnes as shown in Figure 15 (“Food and Agriculture Organization of the United Nations (FAO),” 2020). Cassava peels are the major by-product obtained from the processing of cassava tuberous roots for consumption by man. A mature root of cassava consists of three distinctive sections viz. the central vascular core, the cortex (flesh), and the phelloderm (peels). The peels are usually 1 – 4 mm thick and may form 10 – 12% of the total dry matter of the root. Cassava peels serve as a feedstuff for animals (Yoonan and Kongkiattikajorn, 2004). The production process of extracting starch from cassava root mainly comprises peeling, grinding/crushing, extraction, and drying. Two outer layers (coverings) are removed from the tuberous root during the peeling process and these are the thin brown outer layer and a thicker leathery parenchymatous inner layer. It has been evaluated that about 100 kg of cassava processed generates approximately 6 kg of cassava peels as waste (Sivamani and Baskar, 2015). Cassava peels consist of a high amount of hydrogen cyanide which is considered to be a toxic compound and can be removed to make it appropriate as animal feeds by drying the peels in the sun (Archibong et al., 2016). These peels generated from cassava processing are high and not optimally used; either they are used as animal feed or disposed of as waste (Witantri et al., 2017). The processing of cassava generates an enormous amount of cassava peels which are regarded as wastes and pose health and environmental risks (Amole et al., 2019). An increase in the problem associated with the disposal of this waste is expected due to the rise in the industrial production of cassava products like flour and ‘garri’. In fact, in some parts of Nigeria where cassava processing factories are situated, non-treatment and the improper disposal of cassava waste (letting it rot or waste burning) could lead to a kind of social rift between the hosting community and the factory

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management. A cassava processing unit with an installed capacity of processing 100 metric tonnes of cassava tubers per day could produce a total of 47 metric tonnes of fresh waste or 8.05 metric tonnes of dry weight which is of course enormous and could cause a degradation effect in the environment when left untreated (Aro et al., 2010). Cassava peels contain high organic and moisture content and the continuous disposal of this waste could render vegetation around it to be unproductive, cause a foul odour, pollute the air, and render the environment aesthetically unpleasing. Air polluted by such waste when inhaled could infect and cause disease in man and animals which may manifest in the long run (Archibong et al., 2016). Nevertheless, they are rich in cellulosic materials that serve as feedstock for bioethanol production. The utilization of cassava peels as a raw material for the production of biofuel is of great benefit since it can lead to the creation of wealth from waste, improve waste management, and enhance a clean environment. To obtain sugars from cassava peels for conversion to bioethanol, the cellulosic material is usually subjected to some processes (Egboosiuba et al., 2014). The potential of ethanol production from cassava peels has not received much attention (Adesanya et al., 2007).

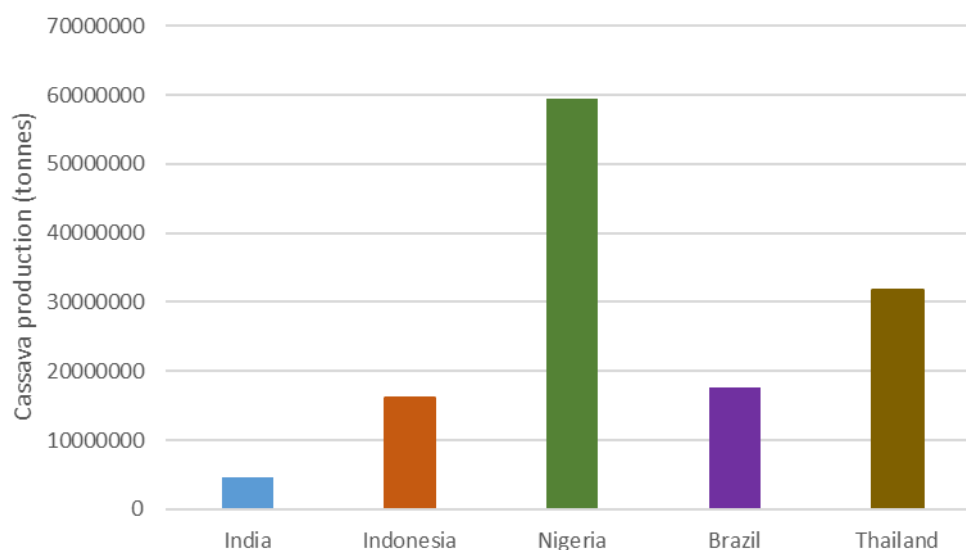


Figure 15. Cassava production in selected countries (“Food and Agriculture Organization of the United Nations (FAO),” 2020).

b. Sugar beet pulp (SBP)

Sugar beet (*Beta vulgaris*) is a member of the *Amaranthaceae* family, it is a biennial plant i.e. it completes its life cycle in two years; primary growth occurs in the first year when the plant develops its vegetative structures (stem, roots, and leaves) and then, after a period of dormancy for the winter/colder period at the end of the first year, the plant flowers in the second

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year. Sugar beet is tolerant to salt stress, frost, and water stress (Mall et al., 2021). It consists of a tapering, white, and fleshy root system with a flat crown and contains a high concentration of sucrose. It also comprises rosettes of leaves. Sugar beet produces sugar via photosynthesis in the leaves which is afterward stored in the root. It is one of the major global sugar crops. It is widely cultivated in temperate climates. It is made up of about 18% sugar, 75% water, and almost 5% cell walls. The European Union is one of the global leaders in sugar production and beet sugar makes up nearly all sugar produced in Europe. Sugar beet provides approximately 20% of the world's demand for sugar (Berlowska et al., 2018). As of 2018/2019, approximately 1,133 kilotons of sugar were produced from sugar beets in the United Kingdom with France being the highest sugar producer in Europe as the country produced about 5,286 kilotons of sugar from sugar beets which was 4.7 times the amount produced in the UK as displayed on the chart in Figure 16 (Coppola, 2020). Each year, about 20 million tons of sugar beet pulp is produced in Europe. The pulps only differ in terms of dry mass content. Dried pulp usually contains 87% - 92% d.m., pressed SBP 18% - 30% d.m., and wet pulp 6% - 12% d.m. Wet pulp possesses low dry matter content and this signifies that it is to some extent fluid in nature, and hence presents handling and storage challenges (Berlowska et al., 2018).

Sugar beet pulp is regarded as an industrial waste of relatively low value which is abundantly available and a potential candidate feedstock for bioethanol production due to its high hemicellulose, cellulose, and pectin content. It is the fibrous by-product obtained after the extraction of beet sugar from commercially cultivated sugar beets. SBP consists of 75 - 80% polysaccharides on a dry weight basis, comprising 30% hemicellulose, 22 - 24% cellulose microfibrils, and 25% pectin. It can serve as feed for livestock owing to its high energy and digestible fiber content (Vucurovic and Razmovski, 2012). For each ton of sugar beet after sucrose extraction, there is a potential yield of 170 kg of wet sugar beet pulp and 830 kg of sugar juice (Rezicó et al., 2013). SBP processing usually involves drying, pelletizing, and transporting which is of course energy-intensive. Hence, the need for the beet sugar industry to search for a way to add value to SBP through a process that does not necessitate drying (Zheng et al., 2012). In relation to hemicellulose and pectin content, SBP has lower cellulose content and this impedes its end use for instance in paper production. The combustion of SBP for the production of heat and power is unfavorable due to its low dry-matter content, nevertheless, its high sugar and low lignin content make it an attractive feedstock for the production of biofuel (bioethanol) and chemicals (El-gendy et al., 2015). SBP polymers must be broken down into monomeric carbohydrates to obtain bioethanol and the simple

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carbohydrates SBP contains after polymer reduction are primarily arabinose, glucose, galacturonic acid, as well as a lesser amount of other sugars (Sutton and Peterson, 2001). The arabinan content of the hemicellulose component of beet pulp is higher than xylan and only a little research has been carried out on arabinose fermentation for bioethanol since it is a relatively small component in the hydrolysate of most other biomass (Rorick et al., 2011). The pectin content of SBP could readily be hydrolyzed into galacturonic acid and cellulose hydrolysis is enhanced when pectin is removed (Rorick et al., 2009). At present, SBP is of relatively low economic value as it is principally used as feed for livestock. Hence, the need to explore ways to improve its economic value (Leijdekkers, 2015).

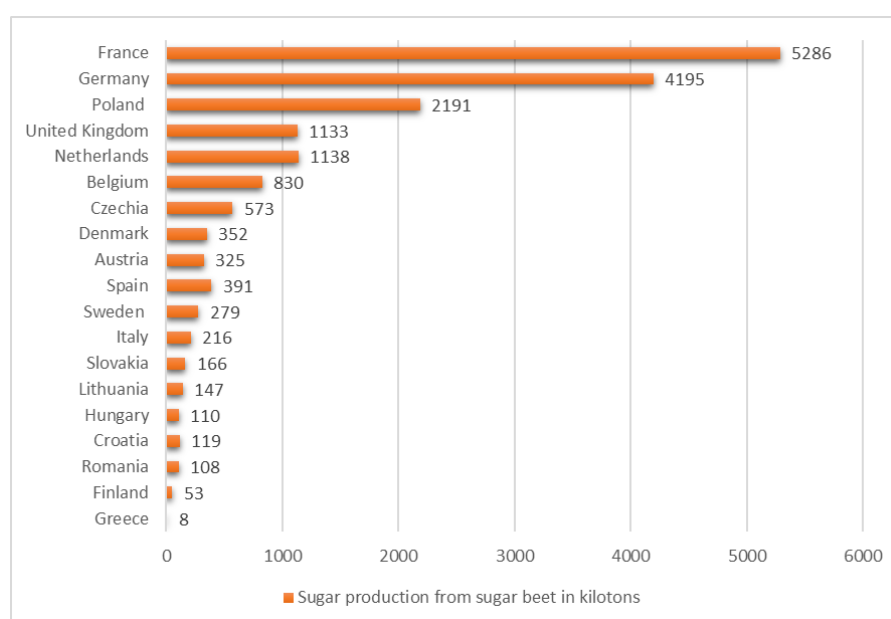


Figure 16. 2018/2019 sugar production from sugar beet in selected EU countries (Coppola, 2020).

c. Macroalgae (*Ulva lactuca*)

Algae consist of more than 100,000 genetically varied strains that possess various distinctive properties that could be advantageous for the production of algal biofuels and other bioproducts. It has been estimated by some researchers that algae could be 10 or 100 times more productive than traditional energy crops (“Advanced Algal Systems,” n.d.). Algae do not have to compete for arable land and also, it presents a high growth rate and contains high lipid content. Algae has the advantage of being able to grow in every season and in different water bodies such as seawater, freshwater, brackish water, lakes, and wastewater. They can also grow in deserts and marginal lands (Özçimen and İnan, 2015). Due to the fast growth rate and development exhibited by algae, it is expected to be able to meet the demand for bioethanol production more than other terrestrial plants (Kim et al., 2017). There are certain negative

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outcomes of algae which includes green tides in estuaries, pond slime in garden ponds, and algal bloom i.e. a rapid growth or increase in certain algal population in an aquatic environment such as freshwater or marine waters which is usually evidenced by water discoloration resulting from their pigments. This leads to the production of toxins, causing the death of aquatic life and rendering aquatic animals e.g. shellfish unfit for human consumption. Several human activities cause this nutrient pollution (surplus nitrogen and phosphorus) of water bodies which ultimately drives the wild growth of the biomass hence resulting in what is known as algal bloom (Raven and Giordano, 2014). Table 9 gives the chemical composition of representative macroalgae. Green seaweeds (Chlorophyta) have the pigments chlorophyll a and b, which is responsible for the green colour of the algae (Özçimen et al., 2012). Green seaweeds have been traditionally employed in different parts of the world in the human diet. However, in spite of their use, it has not been thoroughly researched, and though, they have potential but they do not have key industrial applications when compared to brown and red seaweeds. The green seaweed, *U. lactuca* is a candidate feedstock for bioethanol production. However, to harness its full potential, there is a need for appropriate technologies for cultivation, harvesting, and conversion (Schultz-jensen et al., 2013). There have been reports on the production of biodiesel from microalgae, however, reports on the production of bioethanol from macroalgae are few. It is therefore necessary to identify the bioethanol production potentials of this biomass (Khambhaty et al., 2012).

Table 9. Chemical composition of representative macroalgae (Adapted from (Kim et al., 2011)).

Components (%)	Red algae <i>Gelidium amansii</i>	Green algae <i>Ulva lactuca</i>	Brown algae <i>Laminaria japonica</i>	Brown algae <i>Sargassum fulvellum</i>
Carbohydrates	77.2	54.3	51.9	39.6
Proteins	13.1	20.6	14.8	13.0
Lipids	1.1	6.2	1.8	1.4
Ash	8.6	18.9	31.5	46.0

1.10 Challenges and gaps in knowledge

Large-scale commercial bioethanol production from lignocellulosic biomass is yet to be implemented because the total production cost based on the present technology is high due to the cellulose's crystalline structure and the physical barrier that the recalcitrant lignin creates around it which results in a low biomass conversion (El-Tayeb et al., 2012). Bioethanol from lignocellulosic biomass usually involves several steps, thus making it a non-simple procedure.

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Also, bioethanol production has been associated with the use of polluting chemicals and expensive enzymes (Qian, 2013). For example, to obtain fermentable sugars, enzymatic catalysis has been successfully developed and widely used either as a two-step process requiring acid or alkaline pretreatment or just in the saccharification of biomass. This makes biomass saccharification a relatively costly and time-consuming process. Several works have been done to improve biomass saccharification and/or ethanol production efficiency; Onyelucheya *et al.* reported a maximum glucose concentration of 2.22 mg/ml and xylose concentration of 33 mg/ml after 7.5 wt% and 10 wt% phosphoric acid hydrolysis of 10% cassava peels, respectively (Elechi *et al.*, 2016). Rorick *et al.* observed ethanol yield of 0.34 g/g sugar from the simultaneous saccharification and fermentation of sugar beet pulp involving *S. cerevisiae*, pectinases, cellulases, cellobiases, and then a second fermentation employing *Escherichia coli* KO11 (Rorick *et al.*, 2011). Nitin *et al.* reported a fermentation efficiency of 88.2% with *S. cerevisiae* after pre-heat treatment and enzyme hydrolysis of *Ulva fasciata* (Trivedi *et al.*, 2015).

In addition to the above, genetically modified fermentation organisms have been employed in the fermentation of reducing sugars to obtain bioethanol just as it is in the case of PROETHANOL2G project (ProEthanol2G, 2015) and IOCL's 2G Ethanol Bio-Refinery which we saw earlier. Genetically modifying organisms is costly. Furthermore, in wine production, for instance, wine can be affected by changes that increase glycerol production in yeast or by the deletion of the ALD-6 gene; by increasing glycerol production in yeast, ethanol production is reduced while acetic acid generation is suppressed by deleting the ALD-6 gene. Both of these modifications alter the sensory quality of wine made from the GM organism in comparison to the host strain (UC Davis, 2018). There are also environmental, social, and economic concerns associated with the use of GM organisms (Garcia-Yi *et al.*, 2014).

Since enzymes are expensive and require an extended time to achieve a maximum outcome and also, since concentrated acids are associated with corrosion of equipment and production of inhibitory products such as 5-hydroxymethylfurfural (HMF), furfural, acetic, formic, levulinic acids and phenolic compounds (Oktaviani *et al.*, 2019), a catalyst that could serve as a compromise between these two catalysts is therefore required.

1.11 Conclusion

The different generations of biofuels have been reviewed and it is obvious that the utilization of advanced biofuels obtained from second-generation and third-generation feedstock is more sustainable and cost-effective compared to biofuel generated from first-

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generation biomass. The processes involved in bioethanol production have been discussed; it was shown that the recalcitrance of lignocellulosic biomass compels it to undergo pretreatment steps to make the material more amenable to catalysis agents. It was also observed that though biological pretreatment and enzymatic hydrolysis of biomass are environmentally-friendly, it takes longer process time and steps, and could accrue high cost for example, in the case of genetically modified organisms (GMOs). Also highlighted are the pentose and hexose sugar fermentation pathways; here, it is worth noting that in a mixture of sugars, microorganisms consume hexose sugar (glucose) first. It is only after this sugar has been exhausted that it seeks pentose sugars. Furthermore, this study showed that the legislation in the EU supports the development of second-generation bioethanol production. It also observed the presentation of different projects on advanced bioethanol production and its applications and the potentials of using cassava peels, sugar beet pulp, and *Ulva lactuca* which are representative feedstock in ethanol production. The pursuit remains on the search for the most optimal condition and technology for sustainable high ethanol yield with minimal cost.

1.12 Specific objectives of the study

The objectives of this study include (i) characterization and application of different pretreatment possibilities to maximize simple carbohydrate production potential from chosen representative feedstock biomass while utilizing dilute acid as the sole hydrolysis agent, under the effect of temperature, and (ii) production of bioethanol from optimized saccharification step with the help of fermentation microorganisms such as *Saccharomyces cerevisiae* and *Scheffersomyces stipitis* formerly known as *Pichia stipitis*.

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This chapter describes the fundamental processes used for biomass (cassava peels, sugar beet pulp, and *Ulva lactuca* including reference materials; starch and cellulose) collection and preparation, it also presents the elemental compositional analysis of biomass, the chemical composition of biomass, material characterization by Scanning Electron Microscopy (SEM), screening and process optimization with Plackett-Burman design, dilute acid hydrolysis for soluble carbohydrate recovery, including preparation of the inoculum, fermentation for ethanol production, and bioethanol analysis procedure by GC/FID.

2.1 Biomass collection and preparation

2.1.1 Cassava peels

Cassava peels obtained by the mechanical peeling of the tuberous roots were collected from various cassava processing companies in Benin, a West African country (Figure 17) where cassava is the main meal. The peels were washed to remove debris from the surface, dried under the sun, and then in a Memmert UE 500 Lab Oven (Figure 18) at 105°C for 24 hours, and grounded using an IKA MF 10 model grinder. This was then collected in an air-tight container and stored at room temperature for analysis.

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Figure 17. Country where cassava peels were obtained (Adapted from (Law et al., 2022)).

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Figure 18. Memmert UE 500 Lab Oven

A pictorial view of cassava tuberous roots, its peels before and after drying, and after processing into powdered form is represented in Figure 19. The peels were dark-brown in colour when fresh and after sun-drying became light-brown.

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(a)



(b)



(c)



(d)



(e)

Figure 19. (a) Cassava root as uprooted (b) peeled cassava (c) fresh cassava peels (d) sundried cassava peels (e) milled cassava peels.

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2.1.2 Sugar beet pulp

Sugar beet pulp was provided by Lyven located in Cagny, France (see Figure 20). It came in a dried powdered form and was subjected to oven-drying at 105°C before being collected in an air-tight container and stored at room temperature for analysis.



Figure 20. Map of Cagny in France (Adapted from ("Présentation de Cagny," n.d.)).

A pictorial view of sugar beet, dried sugar beet pulp, and milled pulp is presented in Figure 21. The root system is tapering, white, and fleshy.

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Figure 21. (a) sugar beets (Michail, 2017) (b) dried sugar beet pulp (“Product - Beet pulp,” n.d.) (c) milled sugar beet pulp.

2.1.3 *Ulva lactuca*

The green macroalgae, *Ulva lactuca* was gathered near La Turballe, France (see Figure 22) and cleaned to remove salt and debris with tap water. It was then first air-dried before oven-drying at 105°C for a duration of over 24 hours, after which, it was processed to pass through a 2mm grille using an IKA MF 10 model grinder. In the end, it was collected in an airtight container to be analyzed.

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Figure 22. Map of La Turballe in France (Adapted from("Présentation de La Turballe," n.d.)).

A pictorial presentation of *Ulva lactuca* in its natural habitat and *Ulva lactuca* after drying and milling is given in Figure 23. They are marine organisms and are more closely related to higher plants as regards their photosynthetic pigments, cell walls, and energy storage form (both store carbohydrates as starch). They possess suitable substrates for attachment and can as well be seen floating in the open sea. Macroalgae is able to absorb carbon dioxide in the form of bi-carbonate directly from the seawater which is slightly alkaline and so, its photosynthetic efficiency can be about 7%, which is considerably greater than the typical 2% efficiency for terrestrial biomass leading to a greater rate of primary production (Schultz-jensen et al., 2013).

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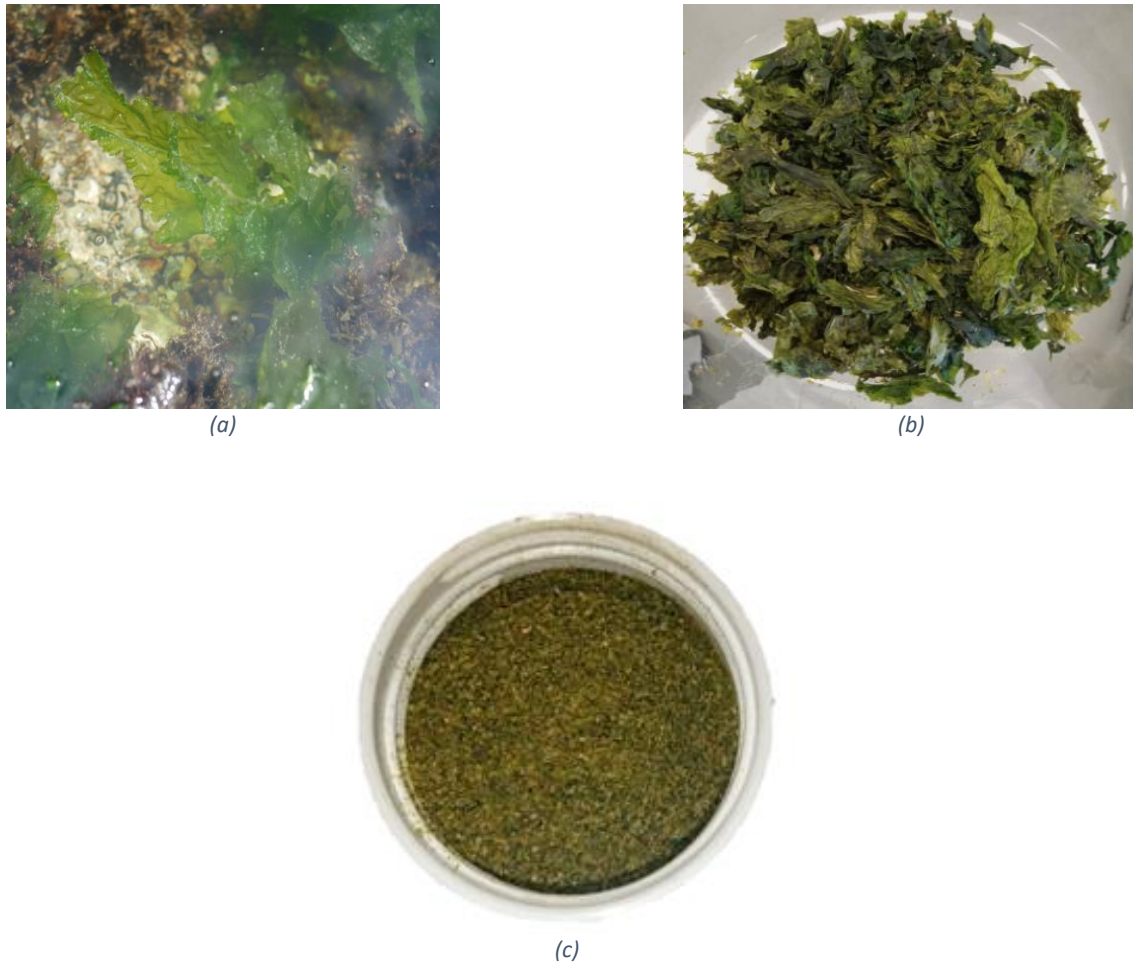


Figure 23. (a) Fresh *Ulva lactuca* (b) dried *Ulva lactuca* (c) milled *Ulva lactuca*

2.1.4 Reference materials (starch and cellulose)

The reference materials used in this study were starch and cellulose (Figures 24 and 26).

- a. **Starch:** Starch was purchased from a grocery in Nantes, France. All green plants produce starch; a white, granular organic substance. Starch is a powder that is soft, white, flavorless, and insoluble in cold water, alcohol, or other solvents. The starch molecule has the chemical formula $(C_6H_{10}O_5)_n$. Glucose monomers are linked in α 1,4 linkages to form the polysaccharide known as starch (Petruzzello, 2022). Figure 25 gives an illustration of the chemical structure of starch.

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Figure 24. Starch powder

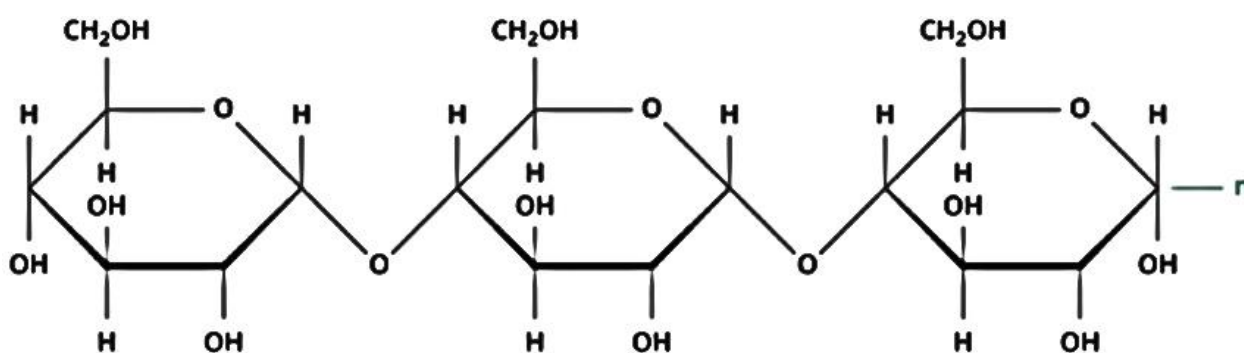


Figure 25. Starch molecule (Othman et al., 2018).

- b. Cellulose:** Cellulose was purchased from Sigma-Aldrich. Cellulose is a crystalline substance that has a powdery, white look. It is insoluble in water but soluble in organic solvents. It is also tasteless and odourless (Singh, 2021). More information on cellulose has been provided in the bibliographic chapter of this study.

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Figure 26. Cellulose powder.

2.2 Characterization of materials

2.2.1 Elemental analysis of biomass

The elemental analysis of samples was achieved using a Thermo Finnigan Flash EA 1112 Series CHNS-O Analyser (Figure 27). This analysis method is based on complete oxidation of samples. It enables the quick measurement of the content of carbon (C), hydrogen (H), nitrogen (N), sulfur (S) and oxygen (O) present in organic samples and other types of material based on the total combustion of the analytical sample at 1050°C, under a stream of oxygen, and under pressure. Carbon dioxide, water, nitrogen dioxide, and sulfur dioxide are produced through the transformation of carbon, hydrogen, nitrogen, and sulfur, respectively. These compounds are separated using column chromatography, and their measurements are made using a thermal conductivity detector (TCD). By subtracting the compound's ash contents and CHNS components, the oxygen content was determined.

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Figure 27. Thermo Finnigan Flash EA 1112 Series CHNS-O Analyser.

2.2.2 Thermogravimetric analysis (TGA)

Thermogravimetric analysis (TGA) was used to determine the hemicellulose and cellulose contents of the samples using SETSYS Evolution 16/18 (SETARAM) (Figure 28). The method entails determining the change in mass of a sample over time for a specific temperature profile. Studies have it that hemicellulose degrades at a temperature range of 220 – 315 °C while cellulose degrades at a temperature range of 300 – 400 °C (Waters et al., 2017). The sample was heated from ambient temperature to various temperatures; 100°C for 30 min. (eliminating moisture), 250°C for 180 min. (hemicellulose determination), and 300°C for 180 min. (cellulose determination). All tests were performed under 20 mL/min nitrogen flow rate.

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Figure 28. SETSYS Evolution 16/18 (SETARAM)

2.2.3 Moisture and ash content analysis

Moisture contents of the biomass can be determined after drying the weighed samples in an oven at 105°C to a constant weight based on the standardized method (Sluiter et al., 2008). For the determination of ash contents, cassava peels, sugar beet pulp, and *Ulva lactuca*, oven dried at 105°C were weighed and transferred to porcelain crucibles in order to remove the volatile/organic contents of the samples through oxidation. To get the composition of ash in each sample, the following procedures were employed:

- a. For organic matter removal, the samples were heated to 550°C in a muffle furnace (Nabertherm® FOU 012 n•1) (Figure 29) for a duration of 4 hours. The furnace temperature profile was set to rise from room temperature to 550°C within a period of 60 minutes and then, the temperature was sustained for 4 hours.

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Figure 29. Muffle furnace (Nabertherm® FOU 012 n#1)

- b. Once the set time got elapsed, the heating was stopped and the furnace temperature was allowed to gradually drop down to room temperature before weighing.
- c. Ash contents were calculated using Eq. 1 which is the percentage of residual samples after combustion to oven-dried samples (at 105°C) used.

A representation of ash samples of cassava peels, sugar beet pulp and *Ulva lactuca*, is shown in Figure 30.

$$\text{Ash (\%)} = \frac{W_3 - W_2}{W_1 - W_2} \times 100 \quad (1)$$

Where W_1 = Initial weight of oven-dried sample (at 105°C) + weight of crucible

W_2 = Weight of crucible

W_3 = Weight of sample after combustion (at 550°C) + weight of crucible

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Figure 30. (a) Cassava peels ash (b) Sugar beet pulp ash (c) *Ulva lactuca* ash.

2.2.4 Lignin extraction and measurement

The Klason lignin method for analyzing lignin content is dependent on the acid hydrolysis of the water-insoluble fraction. 250 mg of each sample were measured into 100mL Pyrex bottles. 3 mL of 12 M sulfuric acid was introduced into the samples in the bottles and stirred with the aid of a glass rod to encourage even distribution of acid in the samples and uniform hydrolysis. The bottles were then placed in a water bath set at 30 °C and incubated for 30 min. Once the 30-minute hydrolysis time elapsed, the bottles were taken out of the water bath, and to each bottle, 80 mL of deionized water was added, and the bottles were capped. The samples were mixed by inverting the bottles several times to prevent phase separation between

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acid layers of high and low concentration and then, they were autoclaved at 105°C for 1 hour. After which, the contents were vacuum-filtered while still warm to obtain the insoluble material which was further washed with hot water before subjecting to drying at 105°C for 16 hours. To evaluate the lignin content, the weight after incineration (ash content) was subtracted from the weight of the residue that was insoluble in sulfuric acid (Gomes et al., 2011).

2.2.5 Soluble carbohydrates and reducing sugar determination

a. Carbohydrate content determination: To obtain the total amount of carbohydrates present in each biomass, the Dubois *et al.* method (Dubois et al., 1956) was adopted. Firstly, 50 mg of each sample were weighed into several test tubes followed by the addition of 5 mL of different solution of acid (HCl) concentrations, in the order, 0.25 M, 0.5 M, 1 M, and 2.5 M. After which, the test tubes were taken to a preheated water bath and hydrolysis was allowed to take place at 100°C (at standard atmospheric pressure) for 3 to 6 hours. A preparation of D-glucose solutions (10, 25, 50, 75, and 100 µg/ml) was made which was used to create a standard calibration curve used in calculating the carbohydrate contents of each biomass sample. The standard linear equation generated was $y = 0.0125x$ with $R^2 = 0.9991$, where y represents absorbance, x represents total carbohydrates and R^2 is the linear regression correlation coefficient (Figure 31). The best carbohydrate content obtained from the tested conditions was selected and presented.

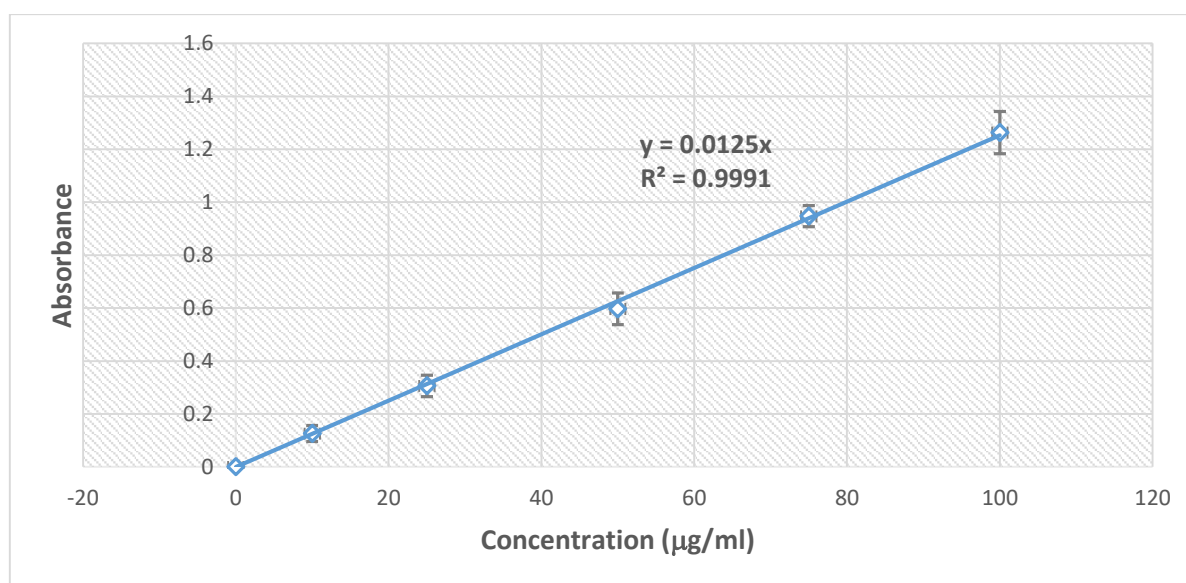


Figure 31. A standard curve with D-glucose.

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c. Reducing sugar determination by HPLC: The quantification of reducing sugars began with weighing approximately 5 g of the hydrolysate using a high-precision scale (Adventurer Pro AV264, Ohaus Corporation, Pine Brook, NJ, USA). This hydrolysate was then mixed with 30 mL of water. To this mixture, 5 mL of Carrez I reagent (a solution of potassium hexacyanoferrate(II) trihydrate in deionized water at a concentration of 150 g/L), 5 mL of Carrez II reagent (a solution of zinc sulfate heptahydrate in deionized water at a concentration of 300 g/L), and an additional 60 mL of water were added. The resulting solution was homogenized using a magnetic stirrer and subsequently filtered through a folded filter (Whatman no. 41 ashless) and a 0.45 μm pore size membrane filter before injection into the HPLC system.

For the HPLC analysis, an amino HPLC column Supelcosil LC-NH₂-NP (Supelco, USA) was utilized. Sugars were separated under isocratic conditions, with the column maintained at 30°C, using a mobile phase consisting of 90% acetonitrile and 10% ultrapure water, flowing at a rate of 1 mL/min. The sample injection volume was 5 μL . Sugar detection was performed using a refractive index detector. Sugars are quantified based on data obtained from the injection of standard solutions at concentrations as fully described in (Diler et al., 2015) and (Diler et al., 2021).

b. Reducing sugar determination by Megazyme kits: The reducing sugars (D-glucose and D-xylose) present in the hydrolyzed samples were evaluated by making use of Megazyme assay kits (Megazyme, 2018), (Megazyme, 2020), (Danielson et al., 2010). The Megazyme assay kit for glucose measurement consists of three different bottles; the first bottle contains the GOPOD reagent buffer, the second bottle contains the GOPOD reagent enzymes, and the third bottle contains the D-glucose standard solution. For the preparation of reagent solutions for glucose measurement, GOPOD reagent buffer was diluted with distilled water to 1 liter called solution 1, this was followed by the dissolution of GOPOD reagent enzymes in about 20 mL of solution 1 which was then quantitatively transferred into the bottle containing the remaining solution 1. After which, the bottle was covered with aluminum foil to protect it from light and was labeled GOPOD reagent i.e. Glucose determination reagent. For the glucose assay procedure, 3 mL of GOPOD reagent and 0.1 mL of distilled water were used to prepare the blank solution in a test tube, 3 mL GOPOD reagent and 0.1 mL D-glucose standard were used as the standard solution, and 3 mL GOPOD reagent and 0.1 mL samples were used. The test tubes were then incubated at 40-50°C for 20 min. After which, the absorbance was read at a

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wavelength of 510 nm against the blank. To obtain the concentration of glucose, Eq. 2 was utilized.

The Megazyme assay kit for xylose assay consists of 5 bottles; the first bottle is the buffer, the second bottle consists of NAD⁺ & ATP, the third bottle contains the hexokinase suspension, the fourth bottle is a solution of XDH/XMR, and the fifth bottle contains D-xylose standard solution. The content of the second bottle was dissolved in 21 mL of distilled water while the rest were used as supplied. For the xylose assay procedure, 2.10 mL distilled water, 0.4 mL buffer, 0.4 mL NAD⁺/ATP, and 0.02 mL hexokinase were used to prepare the blank solution in a cuvette, while 2 mL distilled water, 0.1 mL sample, 0.4 mL buffer, 0.4 mL NAD⁺/ATP, and 0.02 mL hexokinase were used for samples. The cuvettes were mixed and the absorbance of the solutions was read at a wavelength of 340 nm after 5 min and recorded as A₁ for both the blank and samples. The reaction was made to commence by the addition of 0.05 mL XDH/XMR into the cuvette with the blank and those with the sample. After mixing and leaving for approximately 6 min to complete the reaction, the absorbance was read and recorded as A₂. To obtain xylose concentration, Eq. 3 was employed.

$$\begin{aligned} & \text{Glucose concentration } (\mu\text{g}/0.1 \text{ mL}) \\ &= \frac{\Delta A_{\text{Sample}}}{\Delta A_{D\text{-Glucose standard } (100 \mu\text{g})}} \times 100 \end{aligned} \quad (2)$$

$$\text{Xylose concentration (g/L)} = \frac{V \times MW}{\varepsilon \times d \times v} \times \Delta A_{D\text{-Xylose}} \quad (3)$$

Where V = final volume
MW = molecular weight of D-xylose
 ε = extinction coefficient of NADH at 340 nm
d = light path
v = sample volume

2.2.6 Pectin extraction and measurement

In order to quantify pectin present in sugar beet pulp, the following procedures were followed:

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- a. 20 grams of sugar beet pulp powder was weighed into a 1000 mL Erlenmeyer flask.
- b. To the above, 500 mL of 0.05 N hydrochloric acid was added.
- c. The flask was placed in a water bath set at 90°C for 90 minutes for pectin extraction to take place under stirring conditions.
- d. After extraction, the mixture was filtered to obtain the filtrate.
- e. The obtained filtrate was evaporated to half the volume and cooled.
- f. Ethanol was added to the filtrate in the ratio 1:1 (v/v) and precipitation was allowed to take place for 24 hours.
- g. The precipitate was filtered with a filter cloth and washed with ethanol (96%) to remove impurities. It was then dried in the oven at 50°C to constant weight and ground.

Figure 32 gives an image of pectin during precipitation and after washing and filtration. Eq. 4 was used to calculate the amount of pectin produced.



(a)



(b)

Figure 32. (a) Precipitation of pectin (b) washed and filtered pectin

$$\text{Pectin(\%)} = \frac{\text{Weight of pectin (g)}}{\text{Dry weight of sugar beet pulp}} \times 100 \quad (4)$$

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2.2.7 Scanning Electron Microscopy (SEM)

SEM was used to examine the structural transformations of raw and dilute acid-treated biomass. This investigation was achieved using a Scanning electron microscope JEOL JSM 5800LV situated in IMN lab, Nantes Université, France. Samples were carefully mounted on the surface of the stubs with tabs, and since the samples were non-conductive, they were first metallized by coating with platinum before examination. Information on the elemental composition of the samples was also obtained during SEM analysis using Energy Dispersive X-ray Spectrometer (EDX) system.

2.2.8 Screening and process optimization with Plackett-Burman design

The Plackett-Burman design (PBD), a fractional factorial design, was used in this study to evaluate the factors that have a significant influence on yield upon acid hydrolysis. It is based on a linear first-order regression model as shown in Eq. 5. PBD is used to screen more factors with a reduced number of trials (Singh and Bishnoi, 2012).

$$Y = \beta_0 + \sum_{i=1}^n \beta_i X_i \quad (5)$$

Where Y represents the response i.e. total soluble carbohydrate and carbohydrate yield, β_0 and β_i represent intercept and linear coefficients, respectively, X_i is the independent factor, n and i are the total number of factors and factor numbers, respectively. The four factors examined include; solid loading, acid concentration, time, and temperature. The matrix design and data analysis were carried out using Minitab 19 software. Each factor was evaluated at high (+1) and low (-1) levels. Solid loading was varied from 8% to 10%, acid concentration was varied from 0.25 M to 1 M, the temperature was varied from 110°C to 130°C, while time was varied from 5 min to 60 min as shown in Table 10. Twelve experimental runs were conducted with two repetitions at the center points making it a total of fourteen runs. Based on the result obtained from PBD, a full factorial design was developed to examine two-factor interactions through the transformation of PBD (Scibilia, 2014). Analysis of variance (ANOVA) was used to determine significance, with a 95% confidence level ($p < 0.05$).

Table 10. The levels of independent parameters used in PBD.

	Parameters	Unit	Low level (-1)**	Zero level (0)**	High level (+1)**
A	Solid loading	% (w/v)	8	9	10
B	Acid concentration	M	0.25	0.625	1.0

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C	Temperature	°C	110	120	130
D	Time*	min	5	32.5	60

*Time, 32.5 minutes was approximated to 33 min in the autoclave.

**Choice of levels was based on previously conducted experiments and literature.

2.2.9 Dilute acid hydrolysis

2.2.9.1 Reagents

A brief introduction of the chemicals used in the hydrolysis of biomass and soluble carbohydrates assay is given below:

- a. **Hydrochloric acid (HCl):** Hydrochloric acid also called muriatic acid is a colourless liquid solution with a characteristic pungent smell. It consists of a gas called hydrogen chloride that has been dissolved in water. It is considered a strong acid. It is heavier than air, colorless to slightly yellow, corrosive, and nonflammable when it is at normal temperature. Hydrogen chloride produces thick, caustic fumes that are white when exposed to air (“Hydrochloric Acid,” 2022).

Hydrochloric acid used in this study has a purity of 32%, a molecular mass of 36.46 g/mol, a density of 1.17 g/ml, and a molar concentration of 10.27 M/L and was used as a catalyst for the hydrolysis of raw biomass.

- b. **Sulphuric acid (H₂SO₄):** Sulphuric acid also known as oil of vitriol and could be spelled as sulfuric acid is a colourless, odourless, and oily liquid that is very corrosive. It dissolves in water with the discharge of heat. On contact, it will scorch wood and the majority of other organic materials, although it is unlikely to start a fire (“Sulfuric Acid,” 2022).

The purity of H₂SO₄ utilized for carbohydrate assay was 96% with a molecular weight of 98.08 g/mol and a density of 1.840 g/mL (“Sulfuric Acid,” n.d.).

- c. **Phenol:** Phenols are organic hydrocarbon molecules that have a hydroxyl group linked directly to an aromatic hydrocarbon component such as the benzene ring. Phenolic compounds are another name for phenols. Phenols have the chemical formula C₆H₅O₆. Phenols are synthesized both naturally and artificially (“Physical And Chemical Properties Of Phenol,” n.d.). Pure phenol is a white, crystalline substance with a disinfectant-like smell. Because it immediately results in white skin blistering, it must be handled with extreme caution. Frequently, the crystals are discolored and somewhat moist (“Physical Properties of Phenol,” 2022). Phenol has a molecular weight of 94.11 g/mol. It is combustible (can catch fire) and has a flash point of 79.4°C (“Phenol,” 2022).

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For the carbohydrate assay, a 5% phenol solution was prepared by dissolving 5 g of phenol in 100 ml of distilled water.

2.2.9.2 Precautionary measures

Since the above-mentioned chemicals are corrosive, toxic, or constitute health hazards, several mandatory precautionary measures were taken such as:

- a. The putting on of lab coats
- b. The use of gloves
- c. The use of safety glasses
- d. The pouring of acid into the water instead of the other way around, and
- e. The manipulation of chemicals in the fume hood

2.2.9.3 Procedure

The grounded samples were loaded in 250 ml Pyrex bottles followed by the addition of the solution of different acid (HCl) concentrations. The different acid concentrations were prepared by making use of the formula in Eq. 6

$$C_1V_1 = C_2V_2 \quad (6)$$

Where C_1 = the initial concentration of acid

V_1 = the initial volume of acid

C_2 = the final concentration of acid

and

V_2 = the final volume of acid

The required quantity of the original concentration was measured using a graduated cylindrical flask and diluted with distilled water until the required concentration was achieved before introducing it into the 250 ml Pyrex bottles containing the grounded samples.

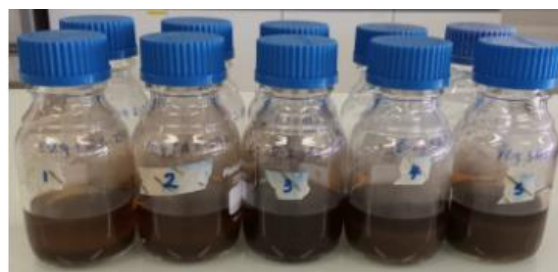
The solutions were then placed in an autoclave (see Figure 33 (a)) for incubation at different residence times and temperatures. This was performed to facilitate the breakdown of complex carbohydrates into simpler ones. At the end of hydrolysis, the samples were taken out of the autoclave and allowed to cool down to room temperature (Figure 33 (b)). The upper liquid layer of the sample called the supernatant/hydrolysate was used to test the amount of released soluble carbohydrates that was after sample dilution. 1 ml of phenol was added to the diluted supernatant followed by 5 ml of sulphuric acid thus causing the samples to show up an orange-

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yellow colour (Figure 34 (a)). The absorbance of the samples was then read off at a wavelength of 487 nm using an Orion Aquamate 7000 ultraviolet-visible spectrophotometer (Figure 34 (b)). Calibration solutions with D-glucose were prepared which consisted of concentrations of 10, 25, 50, 75, and 100 $\mu\text{g/ml}$. Based on these concentrations, a standard calibration curve was plotted and used to calculate the total soluble carbohydrates and carbohydrate yield. The standard linear equation generated was $y = 0.0126x$ with $R^2 = 0.9978$, where y represents absorbance, x represents total soluble carbohydrate and R^2 is the linear regression correlation coefficient (see Figure 35). Carbohydrate yield/biomass conversion was calculated using Eq. 7.

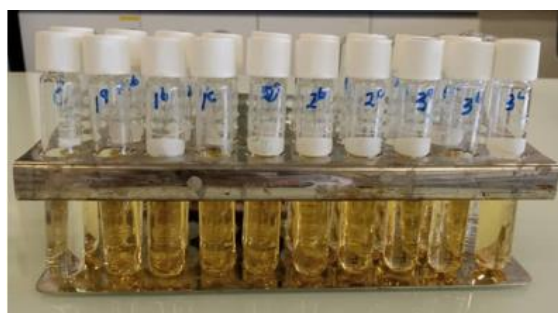


(a)



(b)

Figure 33. (a) Autoclave verticl 180 liters INOX - Brand: SMI – Used (b) Acid hydrolyzed biomass.



(a)



(b)

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Figure 34. (a) Soluble carbohydrate determination by Dubois method (b) Thermo Scientific™ Orion™ AquaMate 7000 Vis Spectrophotometer.

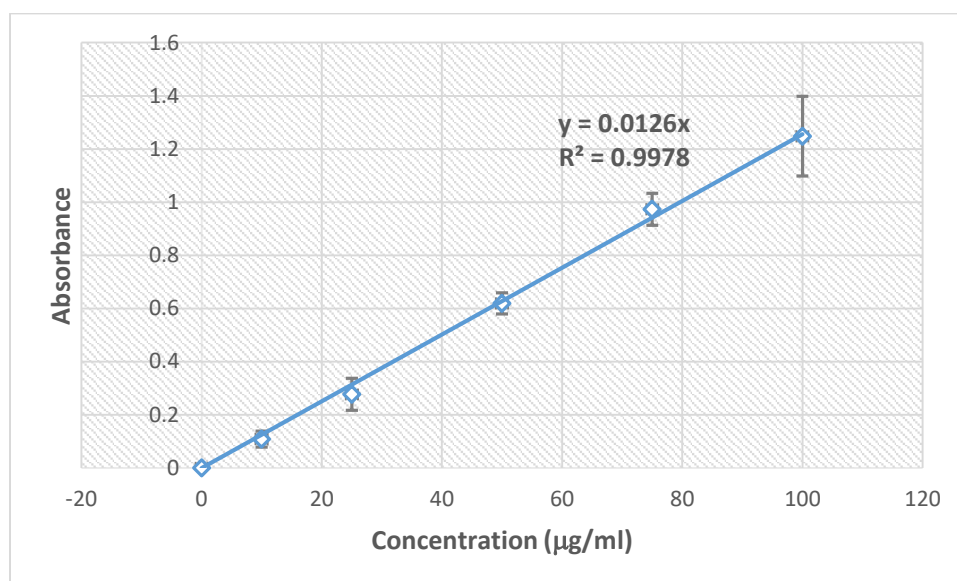


Figure 35. D-glucose standard curve.

Carbohydrate yield

$$= \frac{\text{Total soluble carbohydrates produced (g)}}{\text{Dried biomass(g)}} \times 100 \quad (7)$$

2.2.10 Fermentation

2.2.10.1 Preparation of growth media

The growth media solutions were prepared by making use of a 0.1 mg precision balance. Before taking the weight of any sample, the calibration of the balance is usually checked once every day to prevent measurement errors. Two weights are generally employed which were plotted on a graph and not required to exceed a certain limit. After this, the weights of the samples were taken.

2.2.10.1.1 Minimal solution preparation

In a 1000 mL Pyrex bottle, a 250 mL minimal solution containing 10 g of glucose or xylose, 0.5 g of KH_2PO_4 , 0.25 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25 g of $(\text{NH}_4)_2\text{SO}_4$, and 0.25 g of yeast extract was made and capped (see Table 11 for a clear presentation). This was agitated to ensure a homogeneous combination of the contents. The solution was then placed in a metallic basket and the cap/cover was loosened a bit to be able to let out high pressure before being placed in the autoclave. The lid of the autoclave was closed and screwed tightly in a clockwise direction to prevent pressure escape. The water valve was checked to ensure steady water flow which

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was followed by the closing of the two evaporation valves. The temperature was set to 120°C and time was set to 20 minutes before starting the sterilization process. At the end of sterilization, the solution was brought down and allowed to cool down to room temperature.

Table 11. Composition of minimal solution.

Minimal solution with D-glucose	Concentration (g/L)	Minimal solution with D-xylose	Concentration (g/L)
Glucose	40.0	Xylose	40.0
KH ₂ PO ₄	2.0	KH ₂ PO ₄	2.0
MgSO ₄ ·7H ₂ O	1.0	MgSO ₄ ·7H ₂ O	1.0
(NH ₄) ₂ SO ₄	1.0	(NH ₄) ₂ SO ₄	1.0
Yeast extract	1.0	Yeast extract	1.0

2.2.10.1.2 Solid media preparation

a. Preparation of YEPD (Yeast extract peptone dextrose medium) agar plate

In a 1000 mL Pyrex bottle, a 500 mL solution containing 5 g of glucose or xylose, 1.5 g of yeast extract, 2.5 g of peptone, 7.5 g of Agar, and 1.5 g of malt extract was prepared and capped (see Table 12 for a clear presentation). The solution was agitated to guarantee a uniform mixture of the content and placed in an autoclave half-closed for sterilization at 120°C for 20 minutes. When the cycle was complete, the solution was taken out of the autoclave and allowed some time to cool a bit before being taken to the fume hood and poured into several sterilized Petri dishes. The solution was allowed to solidify in the fume hood before being taken out and kept inverted (to keep water from dropping onto the surface of the agar due to condensation which could cause potential contamination) on a clean table surface ready to be used.

b. Preparation of PDA (Potato dextrose agar) plates

PDA was prepared using 1000 ml infusion from potatoes, 20 g glucose, and 15 g agar. The potato infusion was obtained by boiling 200 g of scrubbed and sliced potatoes in 1000 ml of water for 1 hour. After which, it was passed through a fine sieve and then taken to the fume hood where it was poured into several sterilized Petri dishes and allowed to solidify before being kept inverted on a clean table surface.

Table 12. Composition of solid media.

Solid media with D-glucose	Concentration (g/L)	Solid media with D-xylose	Concentration (g/L)
----------------------------	---------------------	---------------------------	---------------------

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Glucose	10	Xylose	10
Yeast extract	3	Yeast extract	3
Peptone	5	Peptone	5
Agar	15	Agar	15
Malt extract	3	Malt extract	3

2.2.10.2 Preparation of the inoculum

In a 100 mL Erlenmeyer flask containing a 30 mL minimal solution, *Saccharomyces cerevisiae* was pre-cultured. *The S. cerevisiae* was initially isolated from wine residue. It was isolated for its capacity to withstand stressful conditions during the fermentation processes (Eleutherio et al., 2019) and also to produce a high amount of bioethanol as a byproduct through the use of various saccharides as its only source of carbon and energy (Abo-State et al., 2013). Pre-culture was accomplished by aseptically collecting two colonies previously cultured in the YEPD (Yeast extract peptone dextrose medium) agar plate and inoculating them into the minimal solution using a sterile loop. The Erlenmeyer flask was capped with a ball of sterile cotton wool and aluminum foil before being placed in a 30°C incubator with a magnetic stirrer and left overnight. This pre-cultured sample was then used to streak a freshly prepared YEPD agar plate. The yeast *Scheffersomyces stipitis* DSM 3651 purchased from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH came lyophilized. It was isolated from insect larvae. This yeast was pre-cultured in a minimal solution containing xylose in place of glucose and then streaked on an agar plate also prepared with xylose in place of glucose. After 2 days, a colony was collected from the plate and placed in a tube containing 200 µl of 0.01 M sodium chloride (NaCl). This was mixed with a sterile loop and used to streak another agar plate. It was then ready to be used for the inoculation of biomass hydrolysate. *Neurospora intermedia* DSM 1265 purchased from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH also came lyophilized and a total of 900 µl of 0.01 M MgSO₄·7H₂O was used to wake it up before preparing pre-cultures on PDA (Potato dextrose agar) plates with 100 µl of the solution.

2.2.10.3 Fermentation of soluble carbohydrates

Fermentable carbohydrates were produced from optimized conditions. However, the pH of the hydrolysates was low and for the optimal functioning of the fermentation microorganisms, there was a need to bring up their pH. Hence, the pH of the hydrolysate of the 3 biomass was adjusted to 6 with NaOH pellets (which were first dissolved in distilled water before being utilized) while stirring gently with a magnetic stirrer. 100 ml of these hydrolysates

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were supplemented with 1g of yeast extract and 1g of peptone. 3 colonies of *S. cerevisiae* were inoculated into 10% of the nutrient-supplemented hydrolysates contained in Erlenmeyer flasks and plugged with a ball of sterile cotton wool and aluminum foil and then pre-cultured overnight. The pre-cultured samples were introduced into 200 mL Pyrex bottles containing 90 mL of the fermentation media, capped with a cover fitted with a septum, and then placed in an incubator at 30°C while stirring at 300 rpm (see Figure 36). 3 mL of samples were withdrawn at a time with a sterile syringe and filtered with a Thermo Scientific Nalgene Syringe Filter 0.2 µm SFCA before ethanol and residual sugar analysis. Plate counts were also carried out. After the C₆ sugars became completely exhausted, the media was centrifuged for 10 minutes at an RCF of 2090+g to recover *S. cerevisiae*. The media was again supplemented with nutrients. 3 colonies of *Scheffersomyces stipitis* DSM 3651 were directly inoculated into the C₆ sugar-exhausted media and monitored for bioethanol production.

For fermentation with *Neurospora intermedia* DSM 1265, the pH of the biomass hydrolysates was first adjusted to 6 after which, a sterile loop was used to inoculate a loopful of the fungi into each nutrient-supplemented (10 g/L-yeast extracts and 10 g/L-peptone) hydrolysates. The bottles were then capped and placed in an incubator at 30°C under stirring conditions of 300 rpm just as in the case of the yeasts.



Figure 36. Sequential fermentation with *Saccharomyces cerevisiae* and *Scheffersomyces stipitis*.

2.2.10.4 Plate counts of yeast in fermentation samples

The concentration of yeasts in the fermentation samples was evaluated by making use of the following procedures:

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- a. 900 μl of 0.1 M Magnesium sulphate (MgSO_4) dilution solution was pipetted into five labeled test tubes.
- b. A 10-fold dilution was performed by pipetting 100 μl of the sample into the 900 μl dilution solution in the first test tube. This was then agitated using a vortex machine before drawing off 100 μl of it into the second test tube. The same procedure was repeated from the second test tube to the third test tube until the last test tube.
- c. 100 μl of each dilution were sprayed on their respective agar plate and spread for even and complete distribution of the samples over the agar surface with the help of small beads.
- d. The agar plates were then wrapped in an aluminum foil, labeled, and placed in an incubator with a temperature set at 30°C.
- e. After 48 hours, the agar plates were examined for yeast growth, and yeast concentration was calculated using Eq. 8.

$$\begin{aligned} \text{Yeast concentration } \left(\frac{\text{CFU}}{\text{mL}} \right) \\ = \frac{\text{Number of colonies on the petri dish} \times \text{dilution factor}}{\text{spray volume}} \end{aligned} \quad (8)$$

2.2.11 Bioethanol analysis by GC/FID

Bioethanol quantification was performed using Perkin Elmer Clarus 500 GC (Figure 37) equipped with FID (flame ionization detector) and chromatographic column CPWAX 52CB (25 m x 0.25 mm). Helium (He) was used as carrier gas. The injection volume was 1 μl . A split injection mode of 30:1 was used and the injector temperature was 200°C. The oven temperature was 40°C which was held constant for 4 min, this then goes up to 200°C at a rate of 45°C/min and remains constant for a total run time of 9.56 min. The FID temperature was 250°C while the H_2 flow and air flows were 45 mL/min and 450 mL/min, respectively. A standard calibration curve with ethanol and acetonitrile (the chosen internal standard) was used to calculate ethanol concentration. The standard linear equation generated was $y = 0.01508x - 0.0191$ with $R^2 = 0.9981$, where $y = \text{ethanol area}/\text{acetonitrile area}$ and $x = (\text{the ratio of ethanol to acetonitrile area} + \text{the intercept})/\text{the slope}$. MilliQ water was used for all sample dilutions. Ethanol and acetonitrile were usually detected around 3.47 min and 4.57 min, respectively. The

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potential of bioethanol production from simple sugars follows the process in Eq. 9. Ethanol yield and fermentation efficiency were worked out following Eq. 10 and 11, respectively.



Figure 37. Perkin Elmer Clarus 500 GC.



$$\begin{aligned} \text{Ethanol yield (} gg^{-1} \text{)} \\ &= \frac{\text{Ethanol concentration (} gL^{-1} \text{)}}{\text{Biomass concentration (} gL^{-1} \text{)}} \end{aligned} \quad (10)$$

$$\begin{aligned} \text{Fermentation efficiency (\%)} \\ &= \frac{\text{Ethanol concentration (} gL^{-1} \text{)}}{\text{Fermentable sugars (} gL^{-1} \text{)} \times 0.51} \\ &\times 100 \end{aligned} \quad (11)$$

Where 0.51 is a constant for the theoretical yield of ethanol from sugars (glucose and xylose).

2.3 Conclusion

Bioethanol production from lignocellulosic biomass such as cassava peels, sugar beet pulp, and *Ulva lactuca* is a promising solution for sustainable energy production. The use of acid hydrolysis for the conversion of biomass into sugars for fermentation is an already known method, however, there are still challenges to be overcome in order to increase the sugar and

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ethanol yield. To achieve this, it is important to carefully select the appropriate materials and methods for pretreatment, hydrolysis, and fermentation. This includes the use of appropriate catalysts, microorganisms, and other high-yield determining factors such as solid loading, temperature, and time to optimize the conversion process. Additionally, the characterization of the biomass is crucial in order to understand its structure and composition and to identify potential inhibitors. In a nutshell, the development and implementation of effective materials and methods for bioethanol production, along with support from legislation and continued research, will help to make bioethanol a viable and sustainable energy source.

Chapter 3: Characterization of Materials

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This chapter of the thesis is dedicated to the characterization of biomass for ethanol production. The production of ethanol from biomass is a promising alternative to fossil fuels, as it is renewable, sustainable, and has a lower carbon footprint. However, the efficiency of ethanol production depends on the composition and properties of the biomass feedstock. Therefore, understanding the physical, chemical, and structural properties of biomass is essential for optimizing the conversion process and achieving high yields of ethanol.

In this chapter, we will discuss the characteristics found for the different feedstock using different analyzing techniques; we will discuss the composition of biomass, including the elemental composition, amounts of carbohydrates, cellulose, hemicellulose, and other compounds, we will also examine the morphological properties of biomass and their impact on the conversion process.

Our goal is to provide an understanding of the properties of biomass and how they affect ethanol production. By understanding the properties of biomass, we can optimize the conversion process and achieve high yields of ethanol. This chapter provides a foundation for the research and experimentation presented in the following chapter, where we will investigate the conversion of biomass into ethanol in more detail.

3.1 Elemental composition of biomass

One of the key characteristics of using biomass is its elemental makeup. The elemental analysis gives helpful details about the elements that make up biomass. The lack of understanding of the properties of biomass is an obstacle to using it as a source of energy. The features of biomass usually differ not only between species but even within a single species. The surroundings and the weather could make a significant variation from one location to another. Hence, characterizing the elemental composition of biomass is important for bioethanol production because it can provide valuable information about the composition and structure of the biomass, which can in turn help optimize the bioethanol production process (Guangul et al., 2012).

The result of the elemental and chemical composition of the three biomass is given in Table 13. The amount of carbon and hydrogen in a fuel determines its heat content, or how much energy is released when it is burned. When C and H combine with oxygen during combustion, heat is released. The amount of CO₂ released during the combustion of fuel depends on the fuel's carbon

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content. High oxygen and nitrogen content, on the other hand, lowers the calorific value and thus lowers the energy potential of the fuel material. Furthermore, low nitrogen and sulfur contents in biomass are preferred because these components contribute more to environmental degradation (Silva et al., 2019). Therefore, the key elements that are important to consider when characterizing the elemental composition of biomass for bioethanol production, include:

- a. Carbon:** Carbon is the primary component of all organic compounds, including cellulose, hemicellulose, and lignin, which are the main components of most biomass feedstock. The carbon content of the biomass can help to determine the theoretical maximum yield of ethanol that can be produced from the feedstock. Of the 3 biomass, sugar beet pulp contained the highest carbon content which is followed closely by cassava peels. The percentage of carbon present in cassava peels is similar to those obtained by (Tovar et al., 2015) and slightly lower than those reported by (Veiga et al., 2016) and (Fonseca et al., 2018). The carbon content of sugar beet pulp obtained in this study was close to that observed by (Slezak et al., 2020) and (Çakan et al., 2019) but a little different from what was observed by (Brachi et al., 2017). The carbon content observed in *Ulva lactuca* was close to that observed by (Mayala et al., 2022) and (Korzen et al., 2015). High carbon-nitrogen ratios indicate that less nitrogen will be released into the environment after the combustion of biomass. The combustion process that leads to the release of carbon dioxide (CO₂) into the atmosphere is affected by factors external to the process. The release rate is primarily influenced by the combustible material's temperature and humidity as well as the carbon-nitrogen ratio of the biomass. The lower the relationship, the more rapid the release of CO₂ into the atmosphere during combustion, which is undesirable because this gas is one of the factors that amplify the greenhouse effect (Silva et al., 2019).
- b. Hydrogen:** Hydrogen is an important element in the bioethanol production process, but not in the same way as carbon and oxygen. During the fermentation process, the hydrogen atoms in the sugars are not consumed by the microorganisms, instead, it is used in the metabolism of yeast and bacteria (Teng et al., 2019). The hydrogen content of the biomass can help to evaluate the theoretical maximum yield of ethanol that can be produced from the feedstock. It is worth noting that hydrogen is also an important byproduct of bioethanol production. It can be produced by the catalytic reforming of ethanol, a process that involves the conversion of ethanol into hydrogen and carbon dioxide. This hydrogen can be used as a clean and renewable fuel for various

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applications such as fuel cells, transportation, and power generation (Leal et al., 2008). Cassava peels, sugar beet pulp, and *Ulva lactuca* were composed of practically the same amount of hydrogen content. The hydrogen content of cassava peels was closer to that reported by (Tovar et al., 2015) and (Veiga et al., 2016) than what (Fonseca et al., 2018) observed. The hydrogen contents observed in sugar beet pulp and *Ulva lactuca* were comparable to what was observed in the literature (Brachi et al., 2017; Çakan et al., 2019; Slezak et al., 2020) and (Korzen et al., 2015; Mayala et al., 2022), respectively.

- c. **Oxygen:** Oxygen is also an important component of all organic compounds and is a key element in the chemical reactions that take place during bioethanol production. Knowing the oxygen content of the biomass can help to determine the theoretical maximum yield of ethanol that can be produced from the feedstock. Because an increase in oxygen content and a decrease in hydrogen or carbon content tend to reduce the calorific value of biomass, it is not desirable for biomass to have a high oxygen content when it is meant for energy use. Oxygen, however, is a crucial component for promoting material combustion (Silva et al., 2019). In bioethanol production, oxygen plays a key role in the fermentation process. It is required for fermentation microorganisms to carry out the process of respiration, which generates the energy needed for the fermentation process. Without sufficient oxygen, the fermentation process will be slowed down or halted. Additionally, the amount of oxygen present in the biomass feedstock can affect the overall yield of ethanol that can be produced. High levels of oxygen can lead to the formation of other byproducts, such as acetic acid, which can inhibit the growth of yeast and bacteria and reduce the overall yield of ethanol (Aguilar Uscanga et al., 2003). Therefore, monitoring the oxygen content of the biomass feedstock and controlling the oxygen levels during the fermentation process is important to optimize the bioethanol production process. Moreover, oxygen is also present in the final product ethanol, it is therefore, very important to remove the oxygen from the product to avoid oxidation of ethanol and to ensure the stability of the final product. The oxygen content calculated for the 3 biomass was comparable. However, sugar beet pulp was found to contain the highest amount. The oxygen content observed for cassava peels was related to what was presented in the literature (Fonseca et al., 2018; Veiga et al., 2016). For sugar beet pulp, the oxygen content reported by (Brachi et al., 2017) was closer to what was observed in this study

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compared to that reported by (Çakan et al., 2019). The oxygen content observed in *Ulva lactuca* was higher than what was recorded by (Mayala et al., 2022).

- d. Sulphur:** Sulphur is a minor component of most biomass feedstock and its presence in the feedstock is not considered to have a significant impact on the bioethanol production process. Sulphur is present in biomass in the form of sulphur-containing compounds such as sulfates, sulfides, and thiols. These compounds can be present in small amounts in the feedstock, but they are not typically considered to be major limiting factors in the bioethanol production process (Marcinkowska and Jeleń, 2022). The Sulphur content of cassava peels and sugar beet pulp was below the quantification limit. (Fonseca et al., 2018; Tovar et al., 2015; Veiga et al., 2016) recorded a low amount of Sulphur for cassava peels. The composition of Sulphur recorded for *Ulva lactuca* in this study was comparable to that obtained by (Korzen et al., 2015) for *Ulva rigida*. Sulphur compounds can have a negative impact on the fermentation process if they are present in high concentrations. They can inhibit the growth of microorganisms, which can reduce the rate of fermentation, and affect the overall yield and quality of the bioethanol (Nieves et al., 2011; Sun et al., 2016). Sulphur compounds can also contribute to the formation of unwanted byproducts such as hydrogen Sulphide, which can have a negative impact on the quality of the final product. For example in wine production, H_2S is capable of reducing the sensory quality of the wine (De Guidi et al., 2021). The release of Sulphur dioxide (SO_2) into the atmosphere is one of the effects of burning biomass for energy which is of great concern because it has a direct or indirect negative impact on the environment (Dadile et al., 2020). SO_2 is a pollutant that can lead to the formation of acid rain. When SO_2 reacts with airborne water, it creates Sulphurous acid (H_2SO_3), which subsequently combines with oxygen to create sulfuric acid (H_2SO_4) (“Environment and Climate Change,” n.d.). Additionally, Sulphur dioxide can negatively impact human health. It can contribute to respiratory issues like bronchitis, cause irritation to the nose and throat, and could also lead to asthma episodes, phlegm, coughing, and wheezing (Vale, 2012). When there is a high concentration of Sulphur, it can lead to sulphation, which releases Cl. It can result in the creation of components, $FeCl_2$ and $ZnCl_2$ which cause corrosion in boilers (Telmo et al., 2010). Sugar beet pulp and cassava peels have promising

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prospects for bioenergy due to their low Sulphur content which makes them compatible with the environment.

- e. **Nitrogen:** Nitrogen is also a minor component of most biomass feedstock and its presence in the feedstock is not typically considered to have a significant impact on the bioethanol production process (Varela et al., 2004); after carbon molecules, nitrogen compounds are the nutrients that yeasts absorb most during the fermentation of alcohol. They have an impact on the growth and metabolism of yeasts, on the proper progression of fermentation, and on the formation of volatile chemicals (Roca-Mesa et al., 2020). Nitrogen content in biomass parts can vary greatly, both in terms of its concentration and the different kinds of nitrogen molecules that are present (Guo et al., 2022). Additionally, only a small portion of these nitrogen molecules, referred to as YAN (yeast assimilable nitrogen), are processed by yeast. Amino acids and ammonia are YAN sources in *S. cerevisiae*. There are two types of YAN: preferred nitrogen sources and non-preferred nitrogen sources. Ammonia, glutamine, and asparagine are the preferred nitrogen sources for yeast growth. However, when non-preferred sources like proline and urea are the sole nitrogen sources available, minimal growth occurs. The most frequent reason for sluggish or stalled fermentation is nitrogen deficiencies (Varela et al., 2004). To prevent this issue with fermentation, biomass hydrolysates are occasionally given supplementary nutrients, usually ammonium salts (Roca-Mesa et al., 2020). Nevertheless, (Gutiérrez et al., 2012) demonstrated that several commercial *S. cerevisiae* strains had various nitrogen requirements and suggested using supplementation with organic nitrogen sources. In addition, nitrogen compounds can have a negative impact on the fermentation process if they are present in excess. They can inhibit the growth of microorganisms, which can reduce the rate of fermentation, and affect the overall yield and quality of the bioethanol (Martínez-Moreno et al., 2012).

Furthermore, since nitrogen compounds present in fuel are a major factor in the liberation of nitrogen oxides (NO_x) during the combustion of biomass, the amount of nitrogen in biomass is important (Kunle, 2019). NO_x is also another main pollutant that can cause the formation of acid rain. Nitric acid (HNO₃) is formed when NO_x and water react. Acid rain can cause harm to land and water environments and also to materials and objects made by man, like buildings (“Environment and Climate Change,” n.d.). *Ulva lactuca* had the highest nitrogen content of the 3 biomass and its nitrogen content was close to that observed by (Mayala et al., 2022) than that recorded

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for *Ulva rigida* (Korzen et al., 2015). The nitrogen content of cassava peels was close to the nitrogen content recorded by (Tovar et al., 2015) and (Veiga et al., 2016) compared to that of (Fonseca et al., 2018). For sugar beet pulp, the nitrogen content was near that of (Brachi et al., 2017) and (Slezak et al., 2020) compared to that of (Çakan et al., 2019).

The hydrogen, nitrogen, and Sulphur content of all the biomass were lower than their carbon contents which is logical for organic matters.

Table 13. Elemental composition of biomass in literature.

Elements (%)	<i>Ulva lactuca</i> ¹	<i>Ulva lactuca</i> ²	<i>Ulva rigida</i> ³	Sugar beet pulp ⁴	Sugar beet pulp ⁵	Sugar beet pulp ⁶	Cassava peels ⁷	Cassava waste ⁸	Cassava peels ⁹
Carbon	23.9	30.9	28.1	45.7	40.47	40.50	39.96	44.12	47.21
Hydrogen	4.6	4.3	5.5	6.8	5.84	5.66	3.98	6.44	7.74
Oxygen	32.0	34.9	--	46.7	--	51.74	--	48.62	43.70
Sulphur	--	--	2.3	--	--	--	0.12 (ppm)	<0.2	0.04
Nitrogen	1.5	2.2	4.5	1.0	0.77	2.08	0.26	0.81	1.35

^{1&2}(Mayala et al., 2022); ³(Korzen et al., 2015); ⁴(Brachi et al., 2017); ⁵(Slezak et al., 2020); ⁶(Çakan et al., 2019); ⁷(Tovar et al., 2015); ⁸(Veiga et al., 2016); ⁹(Fonseca et al., 2018).

Table 14. Elemental composition of biomass in this study.

Elements	<i>Ulva lactuca</i> (%)	Sugar beet pulp (%)	Cassava peels (%)
Carbon	29.3 ± 0.2	39.8 ± 0.6	37.5 ± 0.3
Hydrogen	5.3 ± 0.1	5.4 ± 0.1	5.4 ± 0.1
Oxygen	40.1 ± 0.8	48.7 ± 0.5	46.1 ± 0.6
Sulfur	1.9 ± 0.4	< LQ *	<LQ*
Nitrogen	1.7 ± 0.0	1.1 ± 0.1	0.5 ± 0.1

* = Below quantification limit, % Oxygen was calculated.

3.2 Higher heating value (HHV) determination

The higher heating value of the samples was evaluated based on the elemental compositions by using the experimental correlation (Eq. 12) by (Channiwala and Parikh, 2002), (Brachi et al., 2017).

$$HHV = (35.5 \times \%C + 142.3 \times \%H - 15.4 \times \%O - 14.5 \times \%N) \times 10^{-2} \quad (12)$$

Where C = carbon

H = hydrogen

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O = oxygen

N = Nitrogen

The high heating value (or gross calorific value) of a fuel is the amount of energy generated when it burns completely per unit mass or per unit volume of the fuel. The high heating value refers to the situation in which water condenses out of the combustion products. This high heating value also denoted by the energy density of each biomass, shown in Table 15, was determined from the elemental composition using an empirical formula and presented in MJ/kg (Cavalaglio et al., 2020). The high heating value of lignocellulosic biomass increases as lignin content increases; lignin content and high heating value have a strong correlation (Cavalaglio et al., 2020; Demirbas, 2002). Biomass with a higher heating value will generally be more desirable for combustion as it will release more energy per unit of mass. An HHV range of 11 to 14 MJ/kg is considered to be in the middle range for biomass and may be considered a good source for combustion (Brachi et al., 2017; Janas and Zawadzka, 2017; Sidikat et al., 2021). However, it is important to note that HHV may vary depending on the type of biomass and the method of analysis used. In addition, the HHV of a biomass species may also vary depending on the moisture content and composition of the biomass (Alyson et al., 2018; Janas and Zawadzka, 2017).

Table 15. High heating value (MJ/Kg).

Biomass	High heating value
Cassava peels	13.82
Sugar beet pulp	14.15
<i>Ulva lactuca</i>	11.52

3.3 SEM and EDX analysis of raw samples

The SEM image of raw biomass in Figure 38 displays a continuous and ordered surface presentation. Figure 38a, reveals that cassava peels contain a lot of starch granules and fibers (mostly consisting of cellulose, hemicellulose, and lignin) (An et al., 2022; Leticia et al., 2017). The rounded structures are the starch granules while the structures surrounding the granules are the fibers (Rubio et al., 2018). No starch granules were observed in Figures 38b and 38c (sugar beet pulp and *Ulva*, respectively). The non-visible starch granules of *Ulva lactuca* may be due to it being harvested very early and not having enough time to make starch (Cai et al., 2021).

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The x-axis of an X-ray spectrum is the energy in keV, and the y-axis is the number of counts. The EDX spectra for raw cassava peels, sugar beet pulp, and *Ulva lactuca* are given in Figure 39. Peaks of carbon, oxygen, sodium, magnesium, aluminum, silicon, phosphorus, Sulphur, chlorine, potassium, and calcium are noticeable in the spectrum for all biomass. Titanium was detected unexpectedly for cassava peels. However, the strong peaks for cassava peels are carbon, oxygen, aluminum, silicon, and potassium. The strong peaks for sugar beet pulp are carbon, oxygen, and calcium while for *Ulva lactuca* the strong peaks are carbon, oxygen, magnesium, Sulphur, chlorine, and potassium. In all, *Ulva lactuca* has the highest Sulphur count which agrees with the results of the biomass elemental composition presented in Table 14. The silicon element observed in cassava peels and *Ulva lactuca* must have been attached to the biomass from their natural environment. (Birch et al., 2014) demonstrated that maintaining high quantities of magnesium and calcium ratio will continue to stimulate ethanol fermentation and increase the sugar consumption rate. Nevertheless, when their ratio is extremely high, it could impact negatively fermentation performance which is an indication of an inhibitory effect on yeast consumption of sugar and production of ethanol. Potassium also is a natural stimulator of yeast fermentation under normal circumstances (Pandey et al., 2014).

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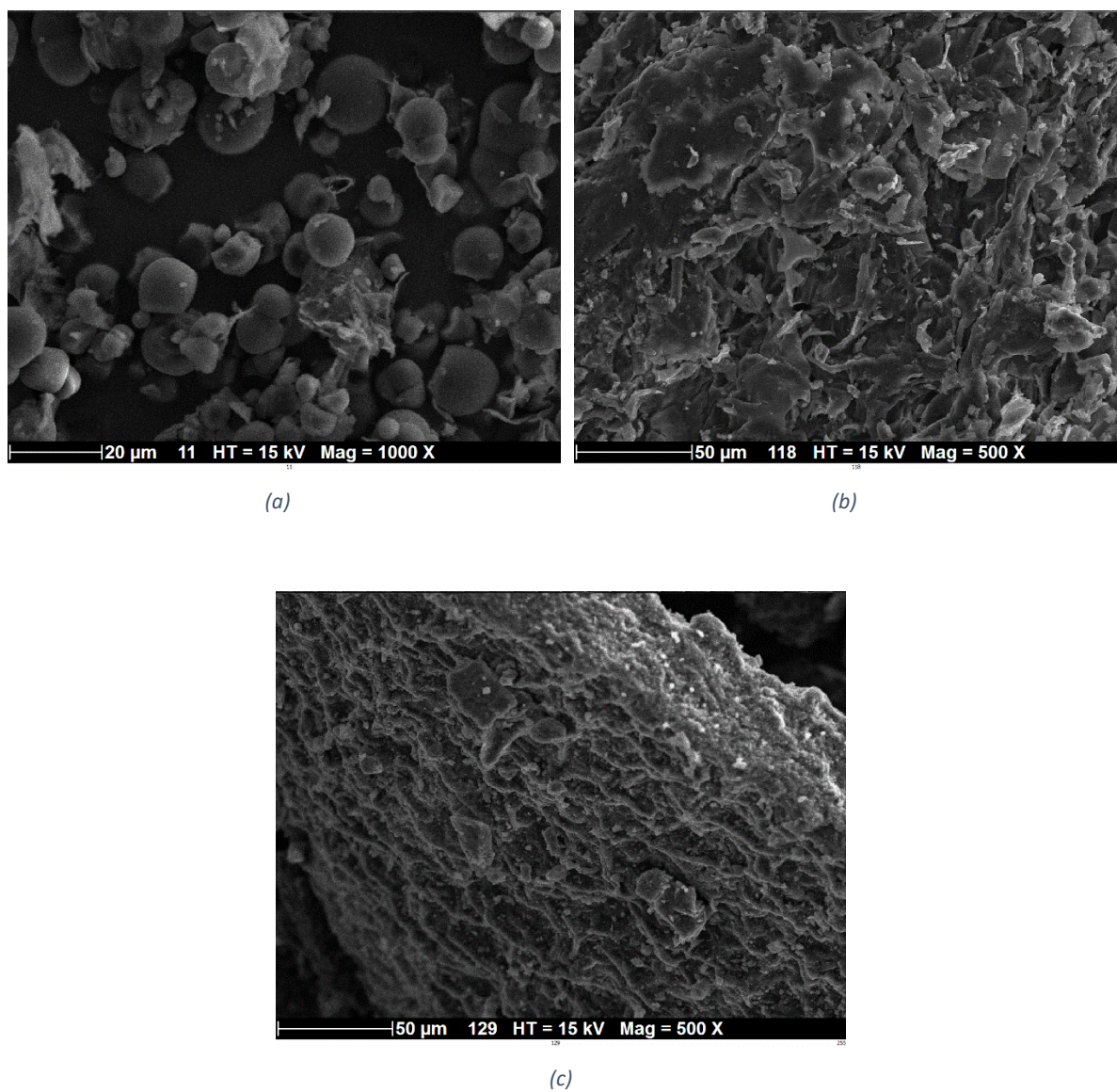


Figure 38. Scanning electron micrographs (SEM) of raw biomass (a) cassava peels (b) sugar beet pulp (c) *Ulva lactuca*.

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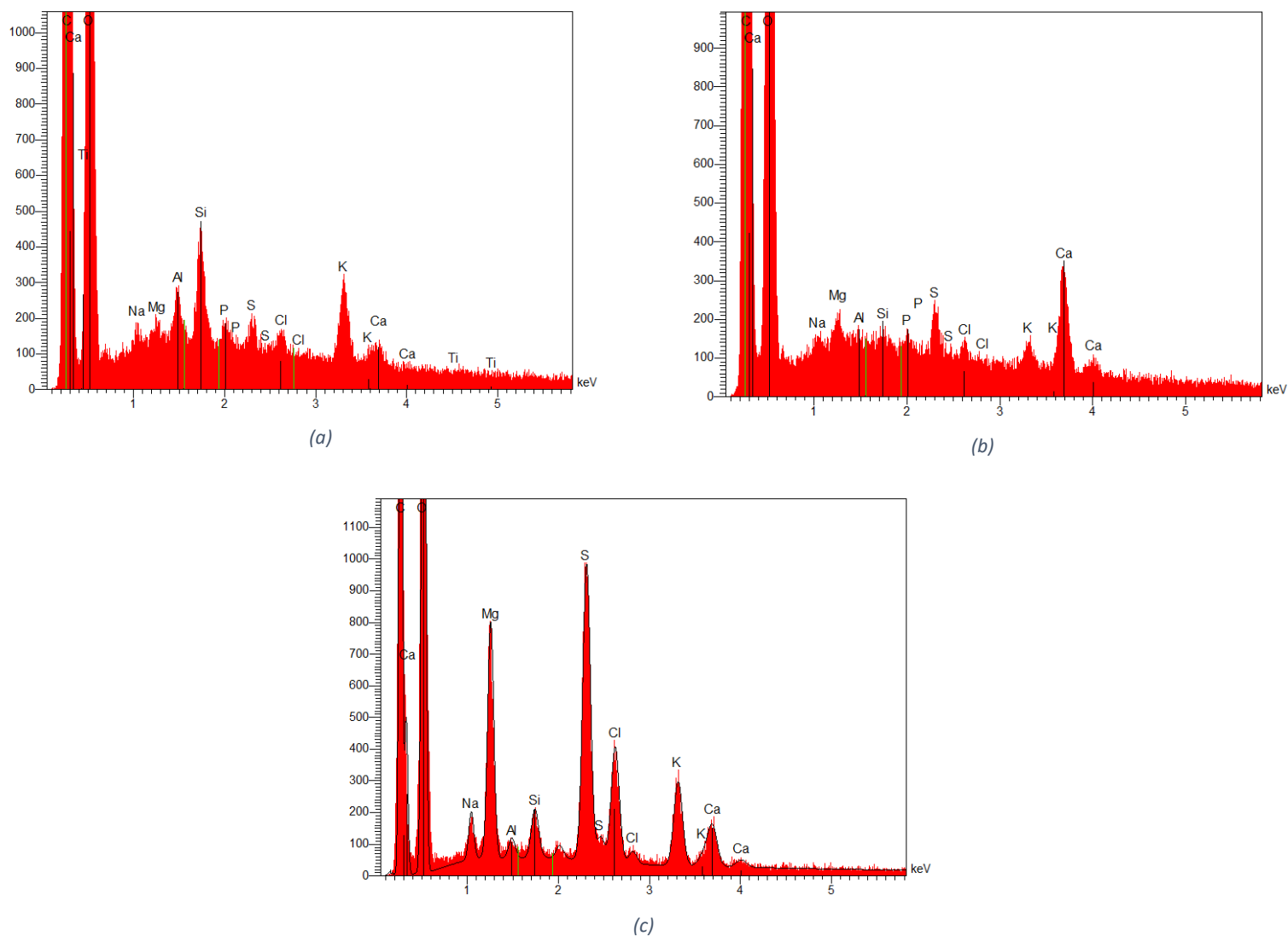


Figure 39. EDX of raw biomass (a) cassava peels (b) sugar beet pulp (c) *Ulva lactuca*.

3.4 Carbohydrate content of biomass

To determine the conditions that will yield the highest carbohydrate from the biomass, a preparation of different acid concentrations was made while employing parameters such as 1% solid loading, temperature of 100°C, and duration of 3 hours & 6 hours. The carbohydrate content of the biomass obtained after 3 hours and 6 hours of hydrolysis time using dilute HCl under atmospheric pressure are presented in Tables 16 and 17.

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Table 16. Carbohydrate content after 3 hrs. hydrolysis time.

Biomass	Acid concentration (M)	Carbohydrate content (%)
Cassava peels	0.25	82
	0.5	81
	1.0	81
	2.5	48
Sugar beet pulp	0.25	47
	0.5	53
	1.0	47
	2.5	53
<i>Ulva lactuca</i>	0.25	31
	0.5	39
	1.0	38
	2.5	36

Table 17. Carbohydrate content after 6 hrs. hydrolysis time.

Biomass	Acid concentration (M)	Carbohydrate content (%)
Cassava peels	0.25	82
	0.5	79
	1.0	76
	2.5	80
Sugar beet pulp	0.25	26
	0.5	29
	1.0	31
	2.5	38
<i>Ulva lactuca</i>	0.25	45
	0.5	44
	1.0	34
	2.5	33

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Maximum carbohydrate content of cassava peels was obtained using 0.25M HCl for 3 hrs. For sugar beet pulp, maximum carbohydrate content was achieved by employing 0.5M HCl for 3 hrs., and for *Ulva lactuca*, maximum carbohydrate content was realized with 0.25M HCl for 6 hrs. Overall, the highest carbohydrate contents of all the biomass were achieved at lower acid concentrations. Hence, for further experiments, acid concentration above 1.0 M was dropped.

3.5 Chemical compositional analysis

The result of the chemical composition of the three biomass is given in Table 18. The chemical composition of lignocellulosic materials differs between species (Naik et al., 2017).

3.5.1 Structural and non-structural composition of biomass

The amount of cellulose and hemicellulose presented by the biomass feedstock is an indication that they are potential materials for bioethanol production. The three substrates exhibited higher hemicellulose content compared to their cellulose content. However, the highest amount was found in cassava peels. Sugar beet pulp possessed the highest cellulose content, followed by cassava peels while *Ulva lactuca* presented the least cellulose content. The total carbohydrate contents evaluated by the Dubois method were 82%, 53%, and 45% for cassava peels, sugar beet pulp, and *Ulva lactuca*, respectively. These were comparable to those obtained by the TGA method. The decomposition temperature of starch (280 - 320°C) (Palm et al., 2020) overlaps more with hemicellulose (220 - 315°C) than cellulose (300 - 400°C) (Waters et al., 2017). This makes it difficult to quantify starch separately in TGA. Nonetheless, from the literature, cassava peels contain a high amount of starch content, about 29.84% (Pooja and Padmaja, 2015). (Korzen et al., 2015) reported the starch content of *Ulva rigida* to be approximately 8% while (Tsubaki et al., 2014) reported a 13% starch content of *Ulva meridionalis*. The lignin content of cassava peels was higher than the rest of the biomass. Sugar beet pulp and *Ulva* had a very close amount of lignin. The pectin content of sugar beet pulp was found to be 21%. (Phatak et al., 1988) reported pectin yield of 19.53% while (Zieminski et al., 2018) reported pectin content of 24.2% from sugar beet pulp. All values were calculated based on the dry weight of the biomass.

Table 18. Chemical composition of cassava peels, sugar beet pulp, and *Ulva lactuca*.

Feedstock (%)	Carbohydrate contents	Cellulose	Hemicellulose + starch	Lignin	Ash	References
Cassava peels	-	40.5	21.4	11.7	-	(Widiarto et al., 2019)
Cassava peels	-	9.71	32.36	16.89	11.38	(Nanssou et al., 2016)
Cassava peels	-	9.05	7.50	9.16	4.66	(Aruwajoye et al., 2017)

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Cassava peels	82	14.26	57.16	13.69	10.3	This study
Sugar beet pulp	-	30.0	26.8	4.1	1.28	(Zieminski et al., 2018)
Sugar beet pulp	-	22 - 30	24 - 32	3 - 7	3.4 – 3.9	(Simion et al., 2012)
Sugar beet pulp	53	21.0	32.6	7.95	4.6	This study
<i>Ulva lactuca</i>	-	45.07	18.24	7.02	2.97	(Kusmiyati et al., 2020)
<i>Ulva lactuca</i>	-	6.0	12.2	9.8	29.1	(Trinh et al., 2013)
<i>Ulva lactuca</i>	-	9.13	20.60	1.56	19.59	(Yaich et al., 2011)
<i>Ulva lactuca</i>	45	7.68	38.26	7.67	21.7	This study

(Widiarto et al., 2019) cellulose and hemicellulose portion was examined by TAPPI 203 cm-99 technique, while the lignin content was determined as Klason lignin. (Nanssou et al., 2016) utilized Van Soest and Robertson method, ash content was evaluated by Sluiter *et al* method. (Aruwajoye et al., 2017) employed Sluiter *et al* method. (Zieminski et al., 2018) quantified cellulose gravimetrically after lignin and hemicelluloses solubilization in nitric acid and sodium hydroxide, hemicellulose was determined by Ermakov method and Miller method, lignin by Sluiter *et al* method. (Simion et al., 2012) analyzed the chemical composition by HPLC. (Kusmiyati et al., 2020) determined cellulose, hemicellulose, and lignin contents by Chesson-Datta method. (Trinh et al., 2013) evaluated structural carbohydrate + Klason lignin by Sluiter *et al* method. (Yaich et al., 2011) employed Van Soest, Robertson, and Lewis method to evaluate cellulose, hemicellulose, and lignin while ash content was obtained by incineration in a muffle furnace at 550°C, 16 h.

The cellulose and hemicellulose contents of cassava peels were closer to those obtained by Van Soest and Robertson method (Nanssou et al., 2016), and the lignin content was similar to those obtained by the Klason lignin method (Widiarto et al., 2019). The structural composition of sugar beet pulp was close to that analyzed by HPLC (Simion et al., 2012). The cellulose and hemicellulose contents of *Ulva lactuca* were close to those achieved by Van Soest, Robertson, and Lewis method (Yaich et al., 2011), and the lignin content was close to that obtained by the Chesson-Datta method (Kusmiyati et al., 2020).

3.6 Ash content

Ulva lactuca had the highest ash content followed by cassava peels while sugar beet pulp had the least ash content. The large amount of ash content possessed by *Ulva* was not so surprising since it was harvested from the seaside and so, contains a high amount of minerals such as sodium, potassium, and chloride (Bikker et al., 2016). This agrees with the result from the EDX analysis of *Ulva*. Ash is an inorganic byproduct of the combustion of combustible materials. When using biomass for energy purposes, the ash content is an important factor that must be taken into consideration. The calorific value of biomass is negatively impacted by increased ash content (Borges et al., 2016). The ash produced during biomass conversion does not have to come completely from the biomass. Some contaminants or tiny particles of rubbish are frequently collected during the handling and collection of biomass and combined with the actual biomass (Amer and Elwardany, 2020). Ash content is therefore a measurement of the

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fuel's inorganic impurities, which might include sand, nickel, aluminum, silicon, sodium, and vanadium and can lead to a variety of issues. Sodium and vanadium, which combine to generate sodium sulphate and vanadium pentoxide, are the most problematic inorganic impurities because they melt and stick to engine parts, especially exhaust valves (Woodyard, 2009). It is strongly advised that the samples being used for characterization and analysis be washed and screened beforehand to make sure they accurately represent the examined biomass. Large-scale enterprises will not be able to use these screening or cleaning procedures since it would take too much time and money to quickly sort through the massive biomass bulk. In large industrial plants, these contaminants must thus be considered as a downside because they will somewhat lower the quality of the output fuel (Amer and Elwardany, 2020). Ash-related issues such as slagging, fouling, and surface corrosion should be investigated since they can lower facility efficiency, capacity, and availability, which raises power costs. The ash from biomass can be used as a building material and as fertilizer for agricultural and forestry lands. It is vital to thoroughly understand the nature of the ash before using it as fertilizer and building material because the composition of biomass ash changes based on the biomass species, growing conditions, and energy conversion method (Guangul et al., 2012). The ash content of cassava peels was closer to those obtained by Sluiter *et al* method (Nanssou et al., 2016). The ash content of sugar beet pulp was close to that reported by (Simion et al., 2012) while the ash content of *Ulva* was close to that achieved by incineration at 550°C for 16 h (Yaich et al., 2011).

3.7 Moisture content

Also included in the chemical composition of biomass is the moisture content. Because all plants require a significant amount of water to survive, biomasses are always characterized by high moisture content. Due to the presence of hydroxyl (-OH) groups, which create hydrogen bonds to hold onto extra water, raw biomass easily absorbs moisture (Basu, 2013). The fibrous nature of raw biomass makes it hygroscopic, meaning that it can still readily absorb moisture even after drying. This essentially means that if raw biomass is needed as a source of energy, it must be precisely dried before use because it cannot be kept dry throughout storage. When handling and using biomass, moisture is not good. Moisture content is crucial for determining how much it will cost to transport raw biomass. Hemicellulose, cellulose, and lignin are the three primary constituents of biomass; they all absorb moisture in a similar order, from higher to lower (Amer and Elwardany, 2020). The moisture content measures the amount of water in the biomass and is given as a percentage of the weight of the raw biomass feedstock.

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A high amount of moisture results in a much lower rate of carbon burn (Grandesso et al., 2011). Table 19 gives the moisture contents of the biomass under study. (Veiga et al., 2016) observed a moisture content of 9.62% in cassava waste while (Fonseca et al., 2018) observed a moisture content of 7.29% in cassava peels. (Slezak et al., 2020) observed a moisture content of 3.64% in sugar beet pulp while (Mayala et al., 2022) reported the moisture content of *Ulva lactuca* collected from Pointe Indienne and Matombi to be 6.5% and 11.9%, respectively.

Table 19. Moisture content of samples.

Feedstock (%)	Moisture content
Cassava peels	4.33
Sugar beet pulp	7.34
<i>Ulva lactuca</i>	5.00

3.8 Conclusion

The characterization of cassava peels, sugar beet pulp, and *Ulva lactuca* is an important step in understanding their potential for use as a source of sugar for fermentation and ethanol production. The 3 biomass feedstock contains high polysaccharide contents (14.26% cellulose and 57.16% hemicellulose + starch, 21.0% cellulose and 32.6% hemicellulose + starch, 7.68% cellulose and 38.26% hemicellulose + starch, for cassava peels, sugar beet pulp, and *Ulva lactuca*, respectively) that make them have interesting potentials for bioethanol production. The composition of each feedstock varies depending on the biomass type, growing conditions, harvesting method, and storage conditions. The availability of sugar, the presence of inhibitors, and the overall composition of the feedstock all play a role in determining its suitability for fermentation and ethanol production. More research and experimentation are needed to fully understand the feasibility and economics of using these materials as a source of sugar for fermentation and ethanol production on a large scale. The high heating value of these feedstocks can also be important in determining their potential for use in combustion as a source of energy. However, the HHV alone does not determine the suitability of biomass for combustion, other factors such as moisture content and ash content will also affect the combustion performance.

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In this chapter, we will examine acid saccharification and fermentation of biomass as a means of converting plant-based materials into biofuels such as ethanol. We will begin with the analysis of acid saccharification of biomass by employing the design of experiments including the various factors that can affect the efficiency of the process such as solid loading, acid concentration, temperature, and time. We will then present the results obtained from the fermentation of simple sugars from optimized saccharification points using the microorganisms; yeasts (*Saccharomyces cerevisiae* and *Scheffersomyces stipitis*) and fungus (*Neurospora intermedia*). The chapter will conclude with a discussion of the observations made from acid saccharification and fermentation, as well as potential strategies for overcoming encountered challenges in order to improve the efficiency and scalability of the process.

4.1 Biomass dilute acid hydrolysis

The hydrolysis of biomass was performed to realize simple carbohydrates for bioethanol production. The goal of this work is to examine the best conditions to obtain an optimized response.

4.1.1 Carbohydrate production from pectin-extracted sugar beet pulp versus non-extracted sugar beet pulp

To determine between the pectin-extracted sugar beet pulp and the non-extracted (raw) sugar beet pulp which could yield maximum carbohydrate, they were both subjected to the same hydrolysis condition (8% solid loading, 0.25 M HCl, 110°C, 60 min). The pectin-extracted sugar beet pulp produced total soluble carbohydrate and carbohydrate yield of 11.13 ± 1.25 g/L and 14%, while the non-extracted sugar beet pulp produced 24.4 ± 0.71 g/L and 30%, respectively. Hence, non-extracted sugar beet pulp was employed for further experiments. Meanwhile, the filtrate produced during pectin extraction contained a total soluble carbohydrate of 14.08 ± 1.98 g/L and a carbohydrate yield of 35%. Thus, this could imply that the conditions necessary for the extraction of pectin could also be utilized in the release of soluble carbohydrates from biomass. For this reason, the conditions were tested on cassava peels as will be seen later in subsection 4.5.1.

4.1.2 Pareto charts from PBD

The significant effects of variables from PBD can be viewed graphically in a Pareto chart, Figures 40 to 49. A Pareto chart represents individual variables in a bar graph in decreasing order of their frequency and cumulative impact. The red line on the Pareto chart is called the reference

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line for statistical significance and it depends on the significance level designated by alpha (α) = 0.05 or 0.1, such that any variable with bars above the red line has a statistically significant effect and bars below the red line have no significant effect on the response. The Pareto concept is based on the 80/20 rule. It consists of two categories of data classification viz. the “vital few” which is 80% and covers a large sum of cumulative percentage of occurrences, and the “trivial many” which covers the remaining 20% of occurrences (Silvestri et al., 2021). Thus, we could say that the reference line of statistical significance for each biomass could differ based on the frequency of each tested variable and the cumulative frequency of occurrence.

The experimental runs for the different biomass are given in Tables 20 to 24 for starch, cellulose, cassava peels, sugar beet pulp, and *Ulva lactuca*, respectively. The regression equations are given in Eqs. 13 – 22. Table 25 is a summary table of the significant variables.

Table 20. Design of experiment, total soluble carbohydrates, and carbohydrate yield of starch.

Runs	%	M	°C	Min	St TSC	St CY
1	10	0.25	130	5	100	1
2	8	0.25	110	60	78	0.95
3	8	1	130	5	79.5	0.97
4	9	0.625	120	32.5	87.4	0.96
5	10	1	110	60	84.9	0.84
6	10	1	130	5	97	0.97
7	10	0.25	130	60	90.5	0.9
8	10	1	110	60	83	0.82
9	9	0.625	120	32.5	88.7	0.97
10	10	0.25	110	5	97	0.96
11	8	1	110	5	76	0.93
12	8	0.25	130	60	73.4	0.89
13	8	1	130	60	60	0.72
14	8	0.25	110	5	81	0.98

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% = solid loading, M = acid concentration, °C = temperature, Min = time, TSC = total soluble carbohydrate (g/L), CY = carbohydrate yield (g/g), St = starch.

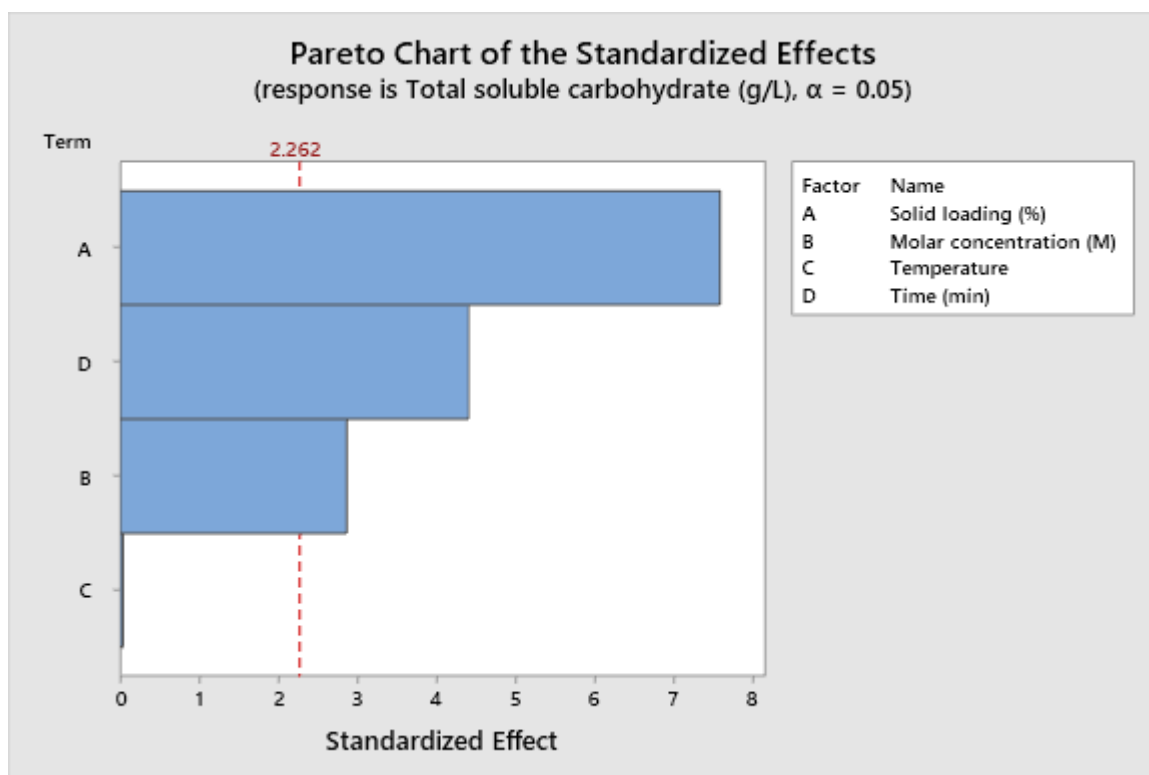


Figure 40. Pareto chart for TSC of starch

Regression equation of total soluble carbohydrate

$$= 6.9 + 9.68 \text{ Solid loading} - 8.78 \text{ Acid concentration} + 0.004 \text{ Temperature} - 0.1839 \text{ Time} \quad (13)$$

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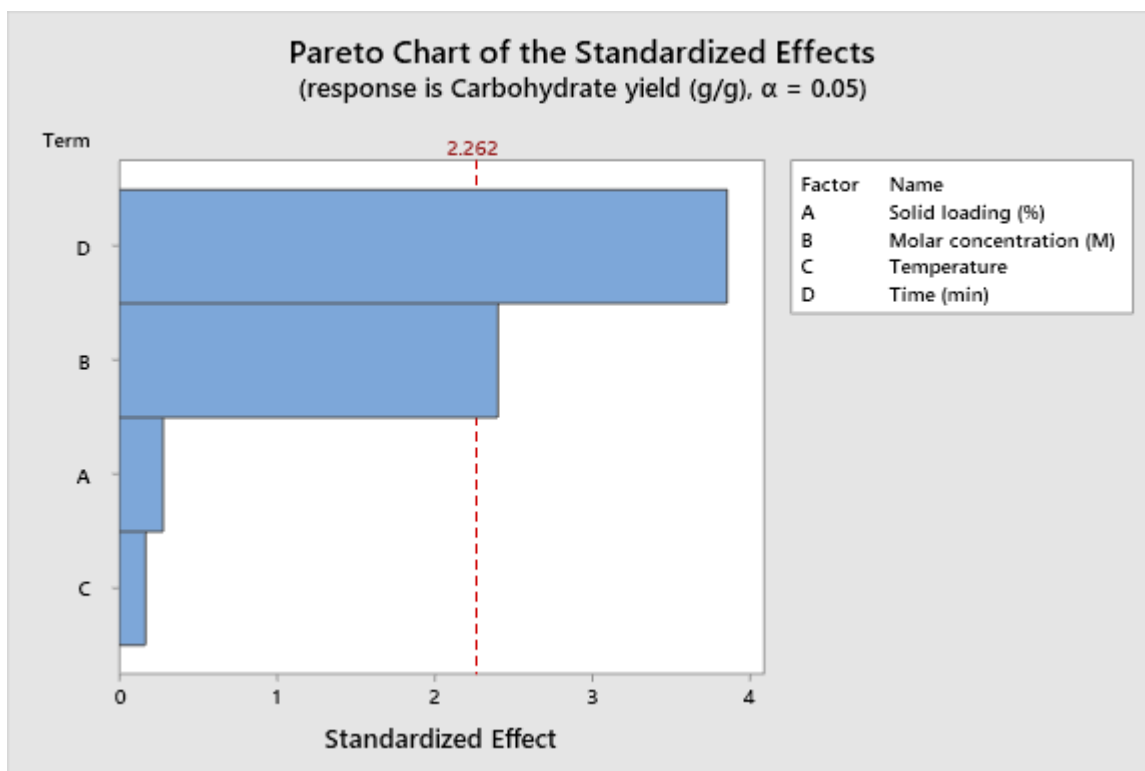


Figure 41. Pareto chart for CY of starch

Regression equation of carbohydrate yield

$$= 1.034 + 0.0046 \text{ Solid loading} - 0.0956 \text{ Acid concentration} - 0.00025 \text{ Temperature} - 0.002091 \text{ Time} \quad (14)$$

Table 21. Design of experiment, total soluble carbohydrates, and carbohydrate yield of cellulose.

Runs	%	M	°C	Min	Ce TSC	Ce CY
1	10	0.25	130	5	4	0.04
2	8	0.25	110	60	2	0.02
3	8	1	130	5	10	0.11
4	9	0.625	120	32.5	7	0.07
5	10	1	110	60	7.3	0.07
6	10	1	130	5	11	0.1
7	10	0.25	130	60	10	0.09

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8	10	1	110	60	7.1	0.07
9	9	0.625	120	32.5	7.3	0.07
10	10	0.25	110	5	0.4	0.004
11	8	1	110	5	2	0.02
12	8	0.25	130	60	9	0.1
13	8	1	130	60	19	0.23
14	8	0.25	110	5	0.4	0.005

% = solid loading, M = acid concentration, °C = temperature, Min = time, TSC = total soluble carbohydrate (g/L), CY = carbohydrate yield (g/g), Ce = cellulose.

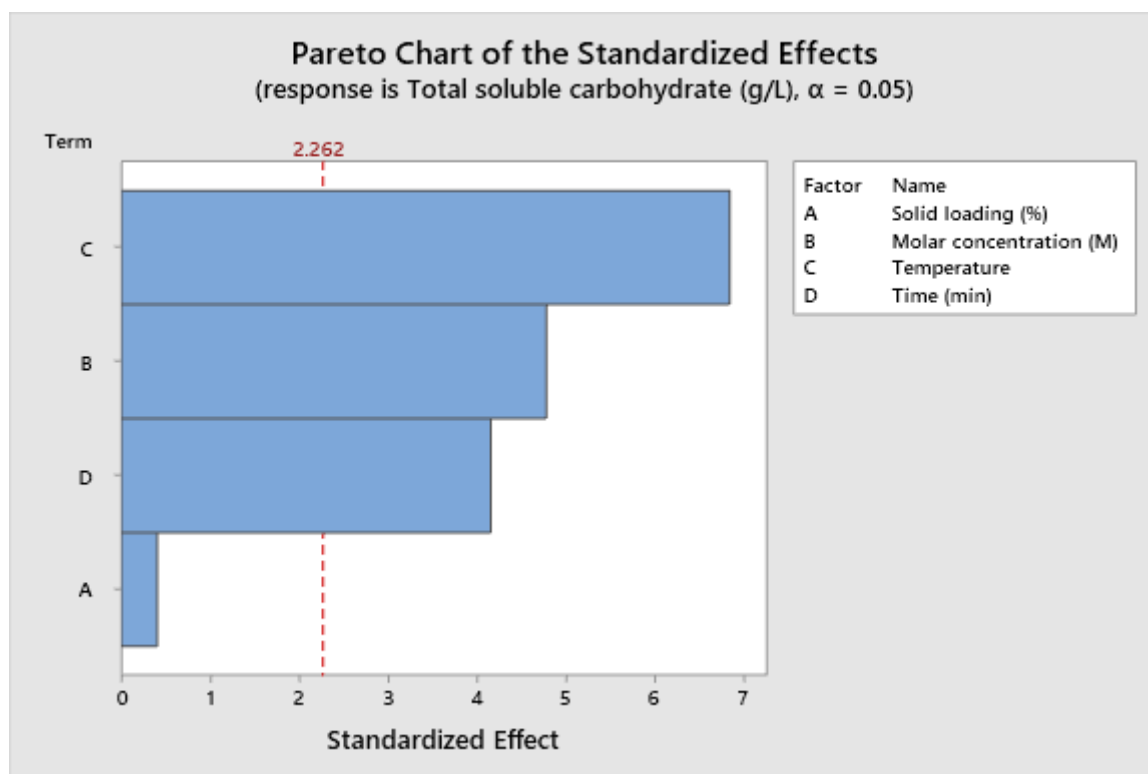


Figure 42. Pareto chart for TSC of cellulose

Regression equation of total soluble carbohydrate

$$= -41.59 - 0.241 \text{ Solid loading} + 6.80 \text{ Acid concentration} + 0.3650 \text{ Temperature} + 0.0806 \text{ Time} \quad (15)$$

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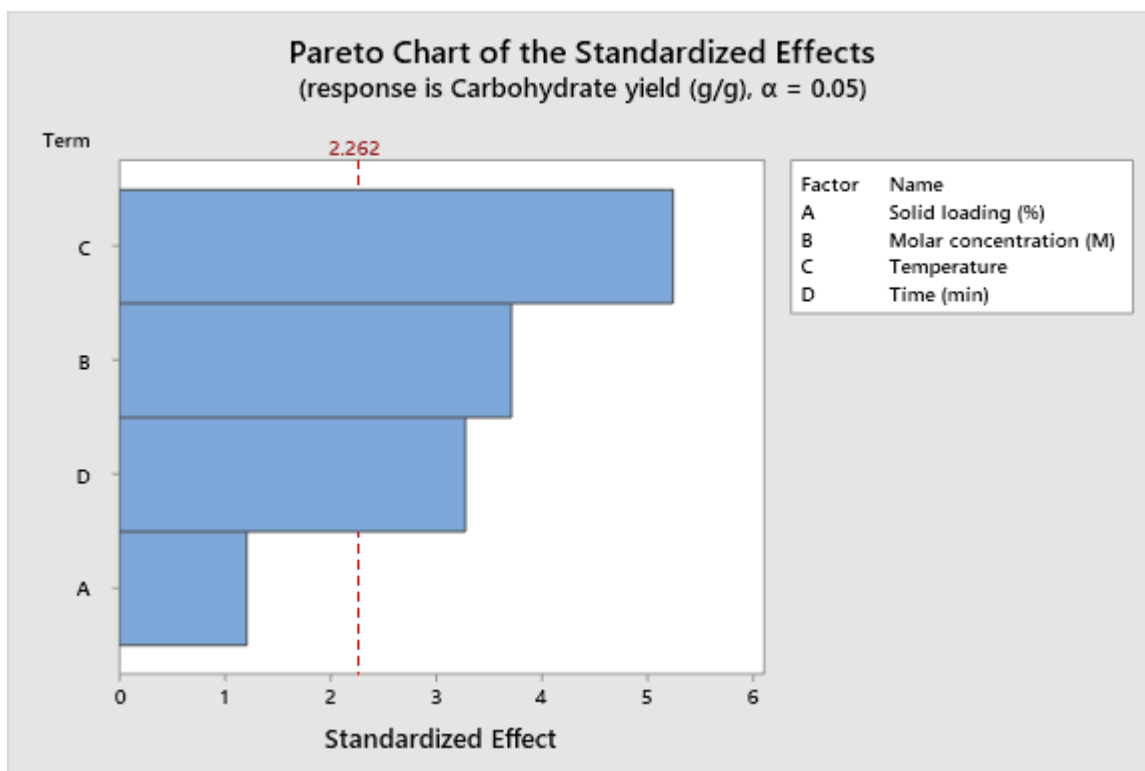


Figure 43. Pareto chart for CY of cellulose

Regression equation of carbohydrate yield

$$= -0.393 - 0.01028 \text{ Solid loading} + 0.0758 \text{ Acid concentration} + 0.004008 \text{ Temperature} + 0.000912 \text{ Time} \quad (16)$$

Table 22. Design of experiment, total soluble carbohydrates, and carbohydrate yield of cassava peels.

Runs	%	M	°C	Min	Ca TSC	Ca CY
1	10	0.25	130	5	73	0.73
2	8	0.25	110	60	61	0.74
3	8	1	130	5	50	0.61
4	9	0.625	120	32.5	65.2	0.71
5	10	1	110	60	68.3	0.68
6	10	1	130	5	66	0.65
7	10	0.25	130	60	77	0.77

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8	10	1	110	60	67.6	0.67
9	9	0.625	120	32.5	66	0.72
10	10	0.25	110	5	72	0.72
11	8	1	110	5	56	0.67
12	8	0.25	130	60	61	0.73
13	8	1	130	60	54.5	0.66
14	8	0.25	110	5	57	0.69

% = solid loading, M = acid concentration, °C = temperature, Min = time, TSC = total soluble carbohydrate (g/L), CY = carbohydrate yield (g/g), Ca = cassava peels.

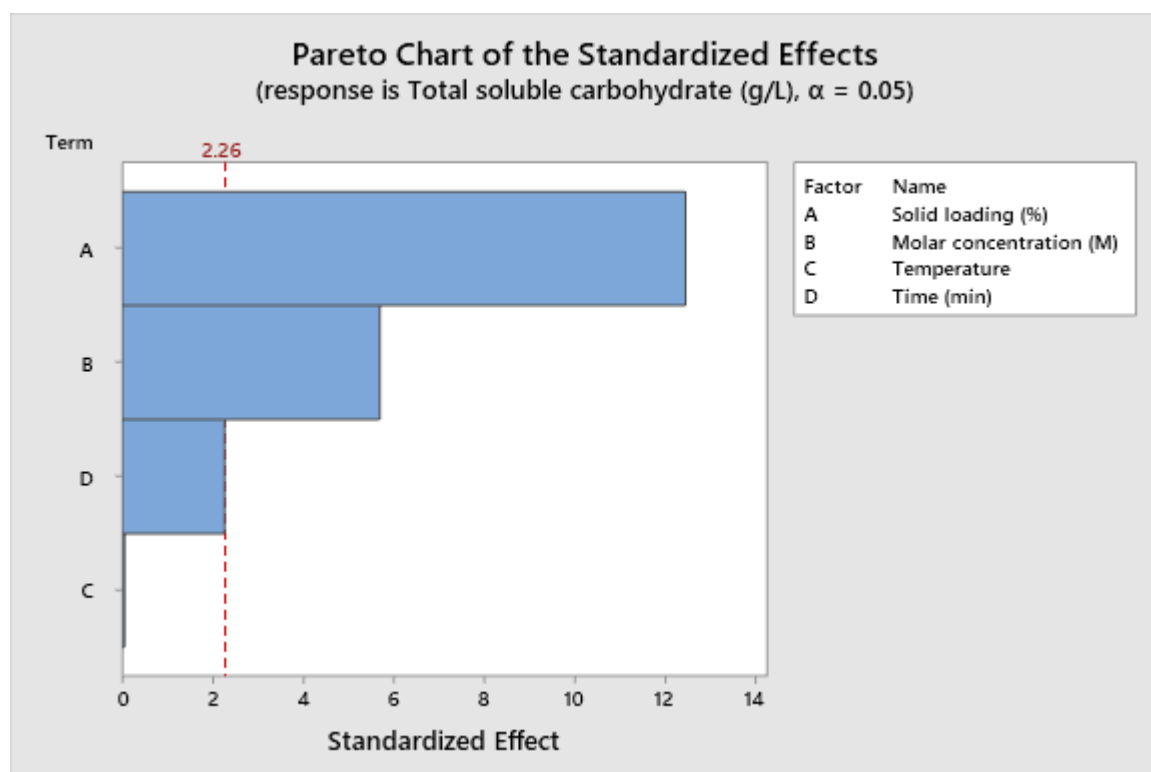


Figure 44. Pareto chart for TSC of cassava peels

Regression equation of total soluble carbohydrate

$$= -2.97 + 7.815 \text{ Solid loading} - 8.58 \text{ Acid concentration} - 0.0033 \text{ Temperature} + 0.0467 \text{ Time} \quad (17)$$

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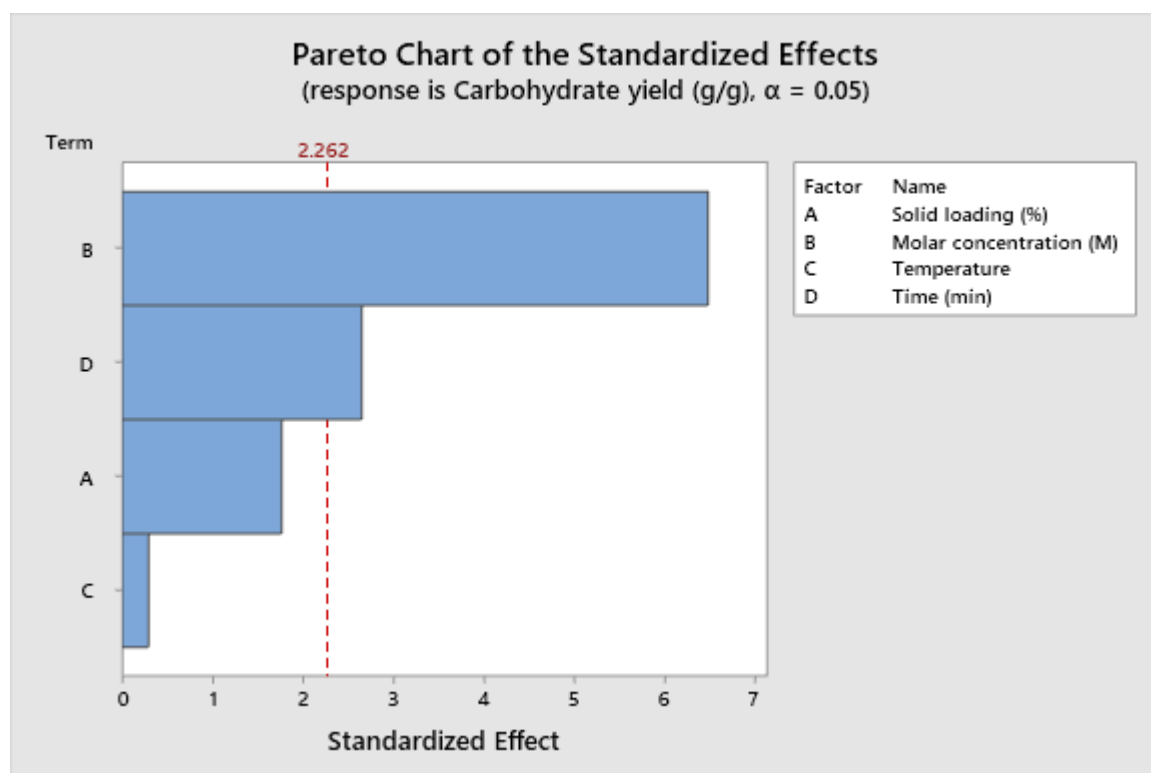


Figure 45. Pareto chart for CY of cassava peels

Regression equation of carbohydrate yield

$$= 0.6587 + 0.01111 \text{ Solid loading} - 0.0978 \text{ Acid concentration} - 0.000167 \text{ Temperature} + 0.000545 \text{ Time} \quad (18)$$

Table 23. Design of experiment, total soluble carbohydrates, and carbohydrate yield of sugar beet pulp.

Runs	%	M	°C	Min	Sb TSC	Sb CY
1	10	0.25	130	5	33.4	0.33
2	8	0.25	110	60	29	0.35
3	8	1	130	5	28	0.34
4	9	0.625	120	32.5	33	0.36
5	10	1	110	60	34.4	0.34
6	10	1	130	5	32.2	0.32
7	10	0.25	130	60	37	0.36

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8	10	1	110	60	35.8	0.35
9	9	0.625	120	32.5	30	0.32
10	10	0.25	110	5	29.3	0.29
11	8	1	110	5	24.13	0.29
12	8	0.25	130	60	31	0.37
13	8	1	130	60	28	0.33
14	8	0.25	110	5	25	0.30

% = solid loading, M = acid concentration, °C = temperature, Min = time, TSC = total soluble carbohydrate (g/L), CY = carbohydrate yield (g/g), Sb = sugar beet pulp.

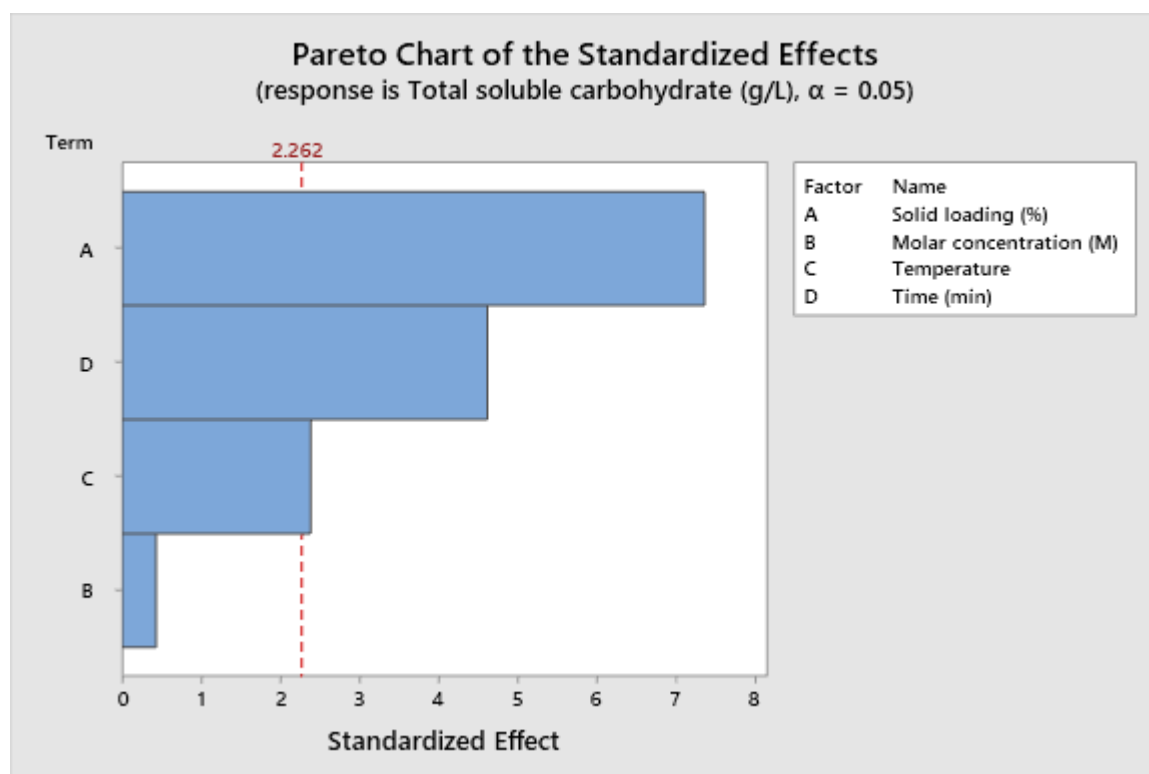


Figure 46. Pareto chart for TSC of sugar beet pulp

$$\begin{aligned}
 \text{Regression equation of total soluble carbohydrate} \\
 = -14.37 + 3.423 \text{ Solid loading} - 0.48 \text{ Acid concentration} \\
 + 0.0998 \text{ Temperature} + 0.0702 \text{ Time} \quad (19)
 \end{aligned}$$

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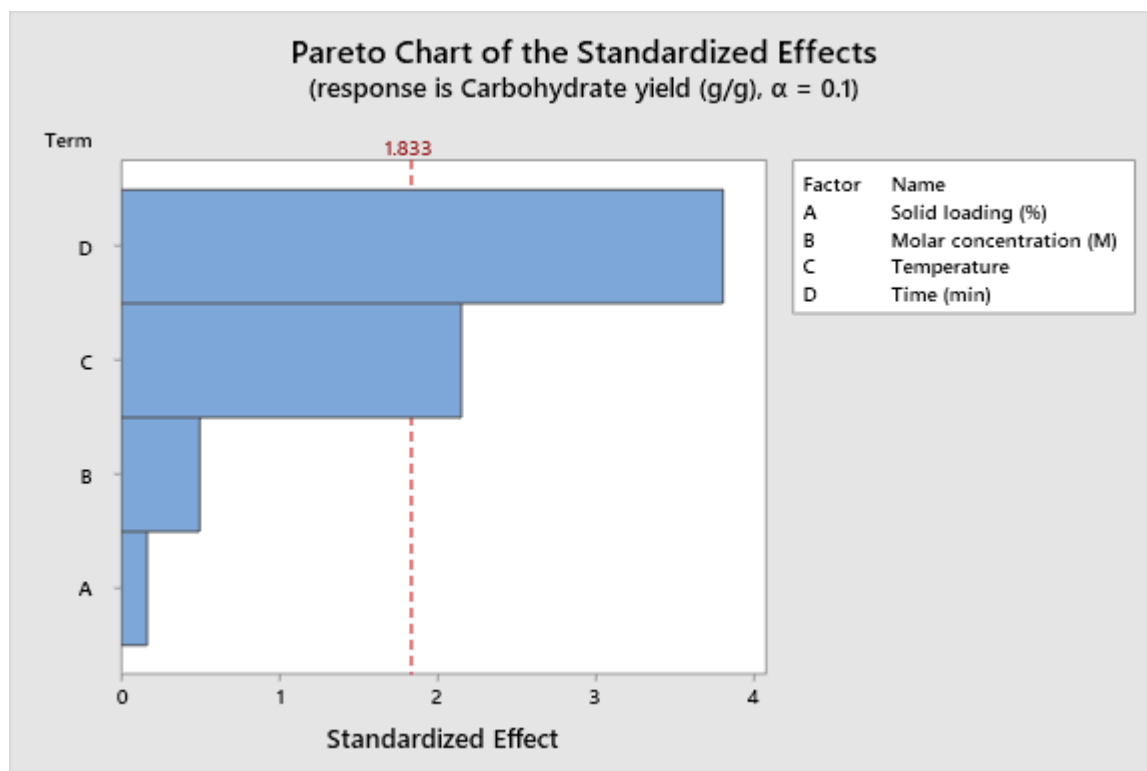


Figure 47. Pareto chart for CY of sugar beet pulp

Regression equation of carbohydrate yield

$$= 0.1752 + 0.00093 \text{ Solid loading} - 0.0067 \text{ Acid concentration} + 0.001083 \text{ Temperature} + 0.000697 \text{ Time} \quad (20)$$

Table 24. Design of experiment, total soluble carbohydrates, and carbohydrate yield of *Ulva lactuca*.

Runs	%	M	°C	Min	UI TSC	UI CY
1	10	0.25	130	5	33	0.32
2	8	0.25	110	60	31.8	0.38
3	8	1	130	5	27.8	0.34
4	9	0.625	120	32.5	32	0.36
5	10	1	110	60	40.6	0.40
6	10	1	130	5	36	0.36
7	10	0.25	130	60	34	0.33

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8	10	1	110	60	41	0.40
9	9	0.625	120	32.5	32.9	0.36
10	10	0.25	110	5	33	0.32
11	8	1	110	5	28	0.33
12	8	0.25	130	60	27.6	0.33
13	8	1	130	60	24	0.28
14	8	0.25	110	5	25.6	0.31

% = solid loading, M = acid concentration, °C = temperature, Min = time, TSC = total soluble carbohydrate (g/L), CY = carbohydrate yield (g/g), Ul = *Ulva lactuca*.

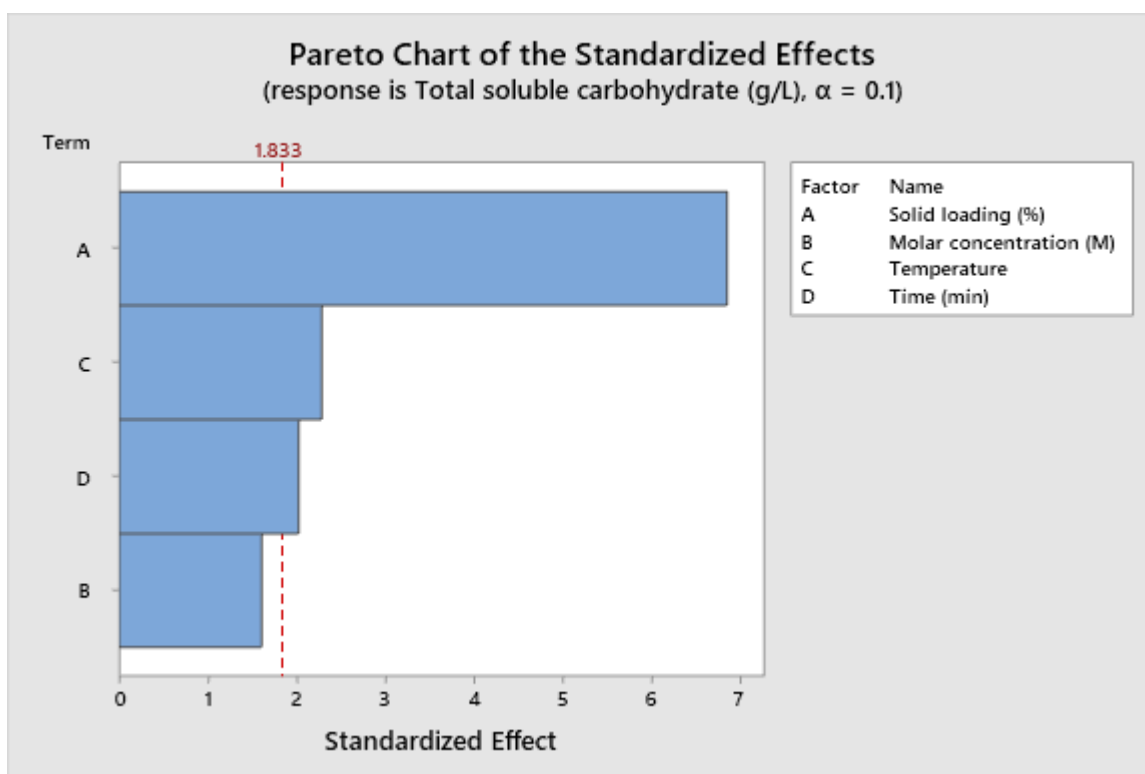


Figure 48. Pareto chart for TSC of *Ulva lactuca*

Regression equation of total soluble carbohydrate

$$= 1.8 + 4.889 \text{ Solid loading} + 2.76 \text{ Acid concentration} - 0.1467 \text{ Temperature} + 0.0473 \text{ Time} \quad (21)$$

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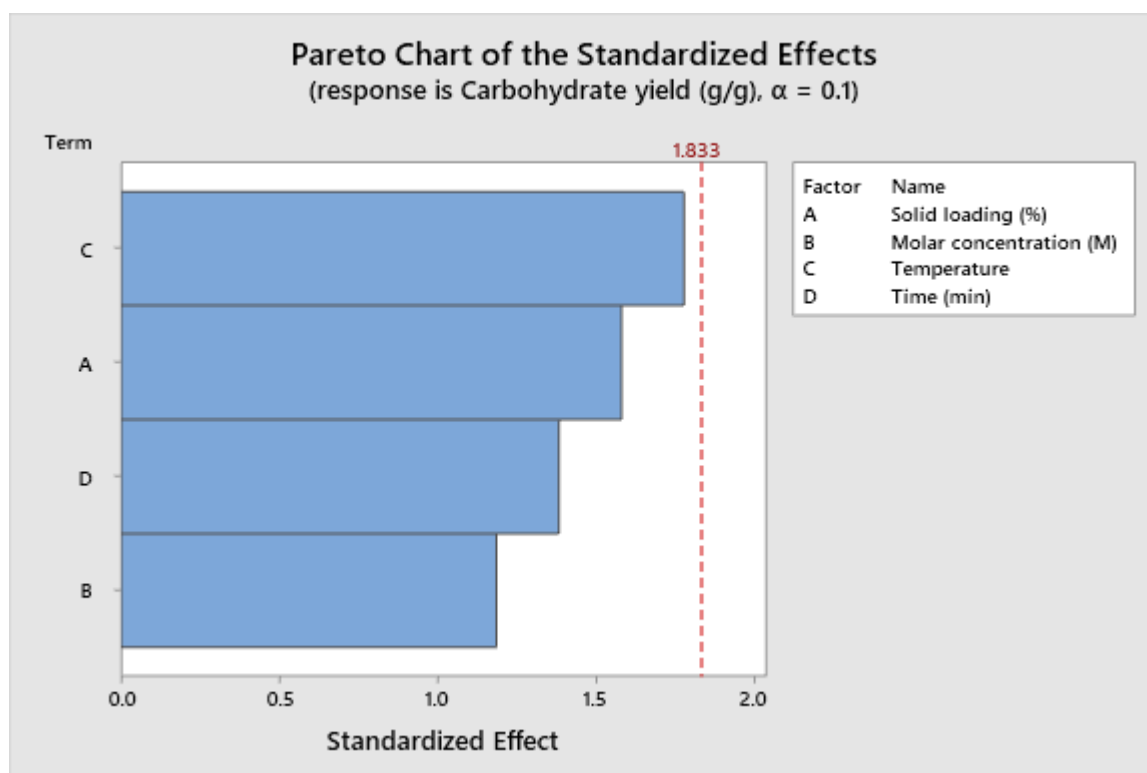


Figure 49. Pareto chart for CY of *Ulva lactuca*

Regression equation of carbohydrate yield

$$= 0.359 + 0.01481 \text{ Solid loading} + 0.0267 \text{ Acid concentration} - 0.001500 \text{ Temperature} + 0.000424 \text{ Time} \quad (22)$$

Table 25. Significant variables from PBD of the different feedstock

Feedstock	Significant variables
Starch (TSC)	Solid loading, time, acid concentration
Starch (CY)	Time, acid concentration
Cellulose (TSC)	Temperature, acid concentration, time
Cellulose (CY)	Temperature, acid concentration, time
Cassava peels (TSC)	Solid loading, acid concentration, time
Cassava peels (CY)	Acid concentration, time
Sugar beet pulp (TSC)	Solid loading, time, temperature
Sugar beet pulp (CY)	Time, temperature
<i>Ulva lactuca</i> (TSC)	Solid loading, temperature, time
<i>Ulva lactuca</i> (CY)	--

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4.1.3 Full factorial design of experiments

The analysis of variance from full factorial DOE for the total soluble carbohydrate and carbohydrate yield of the different materials (starch, cellulose, cassava peels, sugar beet pulp, and *Ulva lactuca*) are given in Tables 26 to 35. The regression equations are given in Eqs. 23 – 32.

Table 26. Analysis of variance for total soluble carbohydrates of starch.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	6	1418.59	236.432	23.40	0.000
Linear	3	1395.68	465.226	46.05	0.000
Solid loading (%)	1	950.04	950.042	94.04	0.000
Acid concentration (M)	1	121.50	121.500	12.03	0.010
Time (min)	1	324.14	324.135	32.09	0.001
2-Way Interactions	3	71.51	23.837	2.36	0.158
Solid loading (%) * Acid concentration (M)	1	19.80	19.802	1.96	0.204
Solid loading (%) * Time (min)	1	0.67	0.667	0.07	0.805
Acid concentration (M) * Time (min)	1	51.04	51.042	5.05	0.059
Error	7	70.72	10.102		
Lack-of-Fit	2	46.86	23.431	4.91	0.066
Pure Error	5	23.85	4.771		
Total	13	1489.31			

$$\begin{aligned}
 & \text{Regression equation of total soluble carbohydrate} \\
 & = 22.4 + 7.63 \text{ Solid loading} - 38.8 \text{ Acid concentration} - 0.160 \text{ Time} \\
 & + 4.04 \text{ Solid loading} * \text{Acid concentration} + 0.0101 \text{ Solid loading} * \text{Time} \\
 & - 0.2121 \text{ Acid concentration} \\
 & * \text{Time}
 \end{aligned} \tag{23}$$

Table 27. Analysis of variance for carbohydrate yield of starch.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	3	0.062092	0.020697	11.98	0.001
Linear	2	0.055083	0.027542	15.94	0.001
Acid concentration (M)	1	0.015408	0.015408	8.92	0.014
Time (min)	1	0.039675	0.039675	22.96	0.001
2-Way Interactions	1	0.007008	0.007008	4.06	0.072
Acid concentration (M) * Time (min)	1	0.007008	0.007008	4.06	0.072
Error	10	0.017280	0.001728		
Lack-of-Fit	1	0.005030	0.005030	3.70	0.087
Pure Error	9	0.012250	0.001361		
Total	13	0.079371			

$$\begin{aligned}
 & \text{Regression equation of carbohydrate yield} \\
 & = 0.9986 - 0.0194 \text{ Acid concentration} - 0.000626 \text{ Time} \\
 & - 0.00234 \text{ Acid concentration} \\
 & * \text{Time}
 \end{aligned} \tag{24}$$

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Table 28. Analysis of variance for total soluble carbohydrates of cellulose.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	6	326.870	54.478	298.09	0.00000
Linear	3	323.073	107.691	589.26	0.00000
Acid concentration (M)	1	86.640	86.640	474.08	0.00000
Temperature (°C)	1	164.327	164.327	899.16	0.00000
Time (min)	1	72.107	72.107	394.55	0.00000
2-Way Interactions	3	30.007	10.002	54.73	0.00003
Acid concentration (M)*Temperature (°C)	1	14.107	14.107	77.19	0.00005
Acid concentration (M)*Time (min)	1	7.260	7.260	39.73	0.00040
Temperature (°C)*Time (min)	1	8.640	8.640	47.28	0.00024
Error	7	1.279	0.183		
Lack-of-Fit	2	0.214	0.107	0.50	0.63235
Pure Error	5	1.065	0.213		
Total	13	328.149			

$$\begin{aligned}
 \text{Regression equation of total soluble carbohydrate} = & -10.64 - \\
 & 31.80 \text{ Acid concentration} + 0.0945 \text{ Temperature} - 0.3482 \text{ Time} + \\
 & 0.3067 \text{ Acid concentration} * \text{Temperature} + 0.0800 \text{ Acid concentration} * \text{Time} + \\
 & 0.003273 \text{ Temperature} * \\
 & \text{Time} \qquad \qquad \qquad (25)
 \end{aligned}$$

Table 29. Analysis of variance for carbohydrate yield of cellulose.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	6	0.043540	0.007257	161.34	0.00000
Linear	3	0.042857	0.014286	317.62	0.00000
Acid concentration (M)	1	0.011748	0.011748	261.21	0.00000
Temperature (°C)	1	0.021063	0.021063	468.31	0.00000
Time (min)	1	0.010045	0.010045	223.34	0.00000
2-Way Interactions	3	0.007020	0.002340	52.03	0.00004
Acid concentration (M)*Temperature (°C)	1	0.003015	0.003015	67.04	0.00008
Acid concentration (M)*Time (min)	1	0.001820	0.001820	40.47	0.00038
Temperature (°C)*Time (min)	1	0.002185	0.002185	48.58	0.00022
Error	7	0.000315	0.000045		
Lack-of-Fit	2	0.000214	0.000107	5.33	0.05757
Pure Error	5	0.000101	0.000020		
Total	13	0.043855			

$$\begin{aligned}
 \text{Regression equation of carbohydrate yield} \\
 = & 0.0115 - 0.4907 \text{ Acid concentration} - 0.000050 \text{ Temperature} \\
 & - 0.005921 \text{ Time} + 0.004483 \text{ Acid concentration} * \text{Temperature} \\
 & + 0.001267 \text{ Acid concentration} * \text{Time} + 0.000052 \text{ Temperature} \\
 & * \text{Time} \qquad \qquad \qquad (26)
 \end{aligned}$$

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Table 30. Analysis of variance for total soluble carbohydrates of cassava peels.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	6	746.191	124.365	33.73	0.000
Linear	3	696.601	232.200	62.98	0.000
Solid loading (%)	1	559.700	559.700	151.81	0.000
Acid concentration (M)	1	113.100	113.100	30.68	0.001
Time (min)	1	23.800	23.800	6.46	0.039
2-Way Interactions	3	8.651	2.884	0.78	0.541
Solid loading (%) * Acid concentration (M)	1	4.250	4.250	1.15	0.319
Solid loading (%) * Time (min)	1	0.150	0.150	0.04	0.846
Acid concentration (M) * Time (min)	1	4.250	4.250	1.15	0.319
Error	7	25.809	3.687		
Lack-of-Fit	2	6.744	3.372	0.88	0.469
Pure Error	5	19.065	3.813		
Total	13	772.000			

Regression equation of total soluble carbohydrate

$$\begin{aligned}
 &= -16.1 + 9.06 \text{ Solid loading} + 10.3 \text{ Acid concentration} + 0.049 \text{ Time} \\
 &- 1.87 \text{ Solid loading} * \text{Acid concentration} + 0.0048 \text{ Solid loading} * \text{Time} \\
 &- 0.0612 \text{ Acid concentration} \\
 &* \text{Time}
 \end{aligned}
 \tag{27}$$

Table 31. Analysis of variance for carbohydrate yield of cassava peels.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	3	0.018867	0.006289	13.51	0.001
Linear	2	0.018833	0.009417	20.23	0.000
Acid concentration (M)	1	0.016133	0.016133	34.66	0.000
Time (min)	1	0.002700	0.002700	5.80	0.037
2-Way Interactions	1	0.000033	0.000033	0.07	0.794
Acid concentration (M) * Time (min)	1	0.000033	0.000033	0.07	0.794
Error	10	0.004655	0.000465		
Lack-of-Fit	1	0.000805	0.000805	1.88	0.203
Pure Error	9	0.003850	0.000428		
Total	13	0.023521			

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Regression equation of carbohydrate yield

$$= 0.7365 - 0.0925 \text{ Acid concentration} + 0.000646 \text{ Time} \\ - 0.000162 \text{ Acid concentration} * \text{Time} \quad (28)$$

Table 32. Analysis of variance for total soluble carbohydrates of sugar beet pulp.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	6	177.115	29.5192	16.20	0.001
Linear	3	146.049	48.6830	26.71	0.000
Solid loading (%)	1	89.205	89.2047	48.95	0.000
Temperature (°C)	1	14.524	14.5237	7.97	0.026
Time (min)	1	42.321	42.3207	23.22	0.002
2-Way Interactions	3	6.539	2.1798	1.20	0.379
Solid loading (%) * Temperature (°C)	1	0.358	0.3577	0.20	0.671
Solid loading (%) * Time (min)	1	2.754	2.7540	1.51	0.259
Temperature (°C) * Time (min)	1	3.428	3.4277	1.88	0.213
Error	7	12.756	1.8223		
Lack-of-Fit	2	1.678	0.8390	0.38	0.703
Pure Error	5	11.078	2.2157		
Total	13	189.872			

Regression equation of total soluble carbohydrate

$$= 5.4 + 0.10 \text{ Solid loading} - 0.001 \text{ Temperature} + 0.133 \text{ Time} \\ + 0.0203 \text{ Solid loading} * \text{Temperature} + 0.0205 \text{ Solid loading} * \text{Time} \\ - 0.00206 \text{ Temperature} \\ * \text{Time} \quad (29)$$

Table 33. Analysis of variance for carbohydrate yield of sugar beet pulp.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	3	0.006492	0.002164	10.09	0.002
Linear	2	0.005817	0.002908	13.56	0.001
Temperature (°C)	1	0.001408	0.001408	6.57	0.028
Time (min)	1	0.004408	0.004408	20.56	0.001
2-Way Interactions	1	0.000675	0.000675	3.15	0.106
Temperature (°C) * Time (min)	1	0.000675	0.000675	3.15	0.106
Error	10	0.002144	0.000214		
Lack-of-Fit	1	0.000144	0.000144	0.65	0.442
Pure Error	9	0.002000	0.000222		
Total	13	0.008636			

Regression equation of carbohydrate yield

$$= 0.0731 + 0.001970 \text{ Temperature} + 0.00397 \text{ Time} \\ - 0.000027 \text{ Temperature} \\ * \text{Time} \quad (30)$$

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Table 34. Analysis of variance for total soluble carbohydrates of *Ulva lactuca*.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	6	320.525	53.421	24.59	0.000
Linear	3	186.365	62.122	28.59	0.000
Solid loading (%)	1	151.002	151.002	69.49	0.000
Temperature (°C)	1	17.682	17.682	8.14	0.025
Time (min)	1	17.682	17.682	8.14	0.025
2-Way Interactions	3	42.112	14.037	6.46	0.020
Solid loading (%)*Temperature (°C)	1	0.015	0.015	0.01	0.936
Solid loading (%)*Time (min)	1	3.082	3.082	1.42	0.273
Temperature*Time (min)	1	39.015	39.015	17.96	0.004
Error	7	15.210	2.173		
Lack-of-Fit	2	0.865	0.432	0.15	0.864
Pure Error	5	14.345	2.869		
Total	13	335.735			

Regression equation of total soluble carbohydrate

$$\begin{aligned}
 &= -17.4 + 3.97 \text{ Solid loading} + 0.135 \text{ Temperature} + 0.684 \text{ Time} \\
 &- 0.0042 \text{ Solid loading} * \text{Temperature} + 0.0217 \text{ Solid loading} * \text{Time} \\
 &- 0.00695 \text{ Temperature} * \text{Time}
 \end{aligned}
 \tag{31}$$

Table 35. Analysis of variance for carbohydrate yield of *Ulva lactuca*.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	6	0.012512	0.002085	5.16	0.025
Linear	3	0.003679	0.001226	3.03	0.102
Solid loading (%)	1	0.000337	0.000337	0.83	0.391
Temperature (°C)	1	0.001838	0.001838	4.54	0.070
Time (min)	1	0.001504	0.001504	3.72	0.095
2-Way Interactions	3	0.006046	0.002015	4.98	0.037
Solid loading (%)*Temperature (°C)	1	0.000004	0.000004	0.01	0.922
Solid loading (%)*Time (min)	1	0.000338	0.000338	0.83	0.391
Temperature (°C)*Time (min)	1	0.005704	0.005704	14.11	0.007
Error	7	0.002830	0.000404		
Lack-of-Fit	2	0.000580	0.000290	0.64	0.563
Pure Error	5	0.002250	0.000450		
Total	13	0.015343			

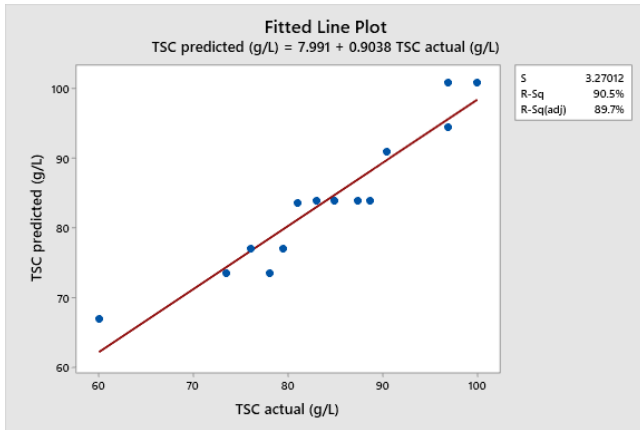
Regression equation of carbohydrate yield

$$\begin{aligned}
 &= 0.246 - 0.0095 \text{ Solid loading} + 0.00079 \text{ Temperature} \\
 &+ 0.00845 \text{ Time} + 0.000069 \text{ Solid loading} * \text{Temperature} \\
 &+ 0.000227 \text{ Solid loading} * \text{Time} - 0.000084 \text{ Temperature} \\
 &* \text{Time}
 \end{aligned}
 \tag{32}$$

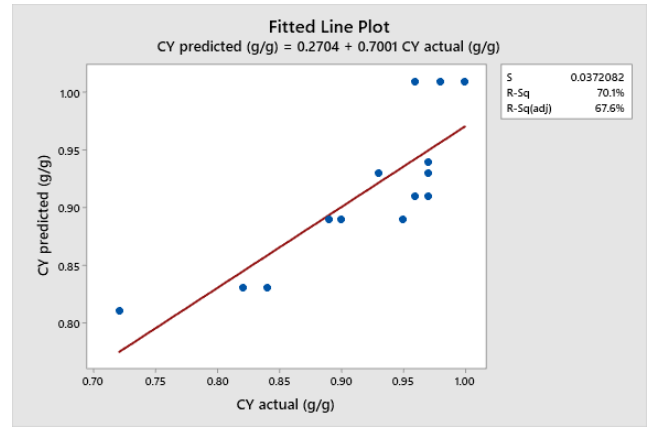
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Figure 50, gives the relationship between the predicted and actual total soluble carbohydrates and carbohydrate yield of the different biomass. A stronger correlation between the predicted and actual was observed for TSC compared to CY as represented by their respective R^2 values. The mathematical model fits the results obtained with cassava peels more than it does for other feedstock.

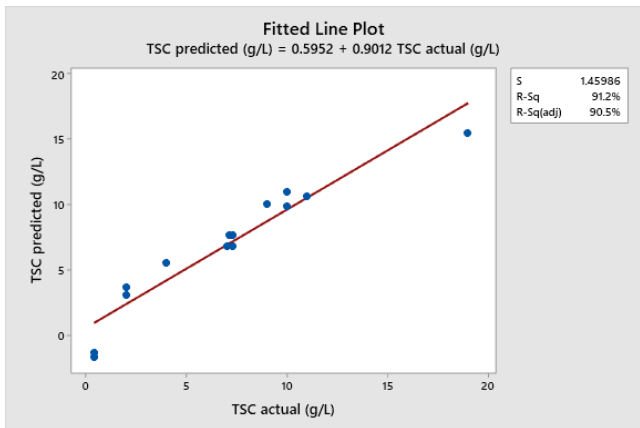
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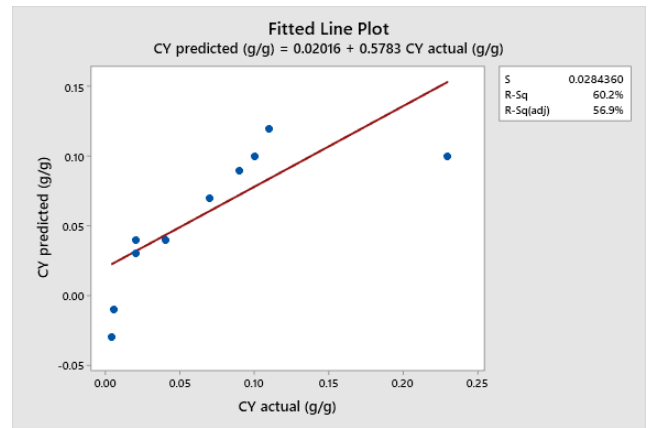
(a) TSC of starch



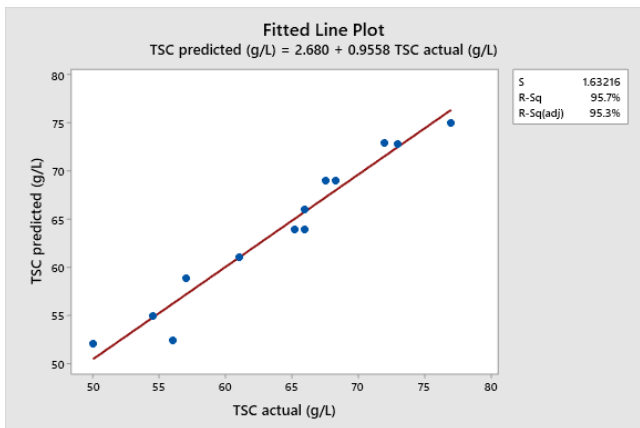
(b) CY of starch



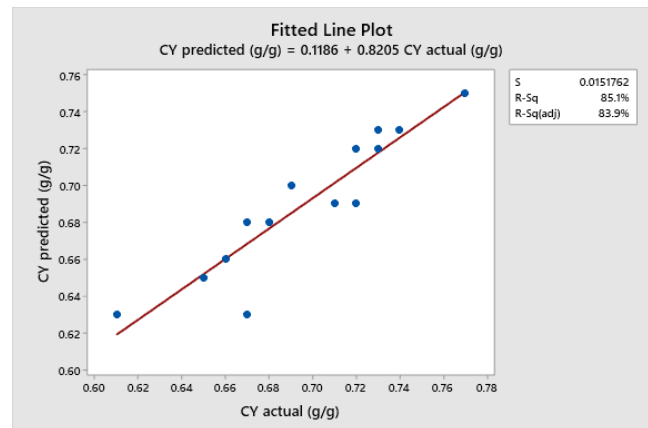
(c) TSC of cellulose



(d) CY of cellulose



(e) TSC of cassava peels



(f) CY of cassava peels

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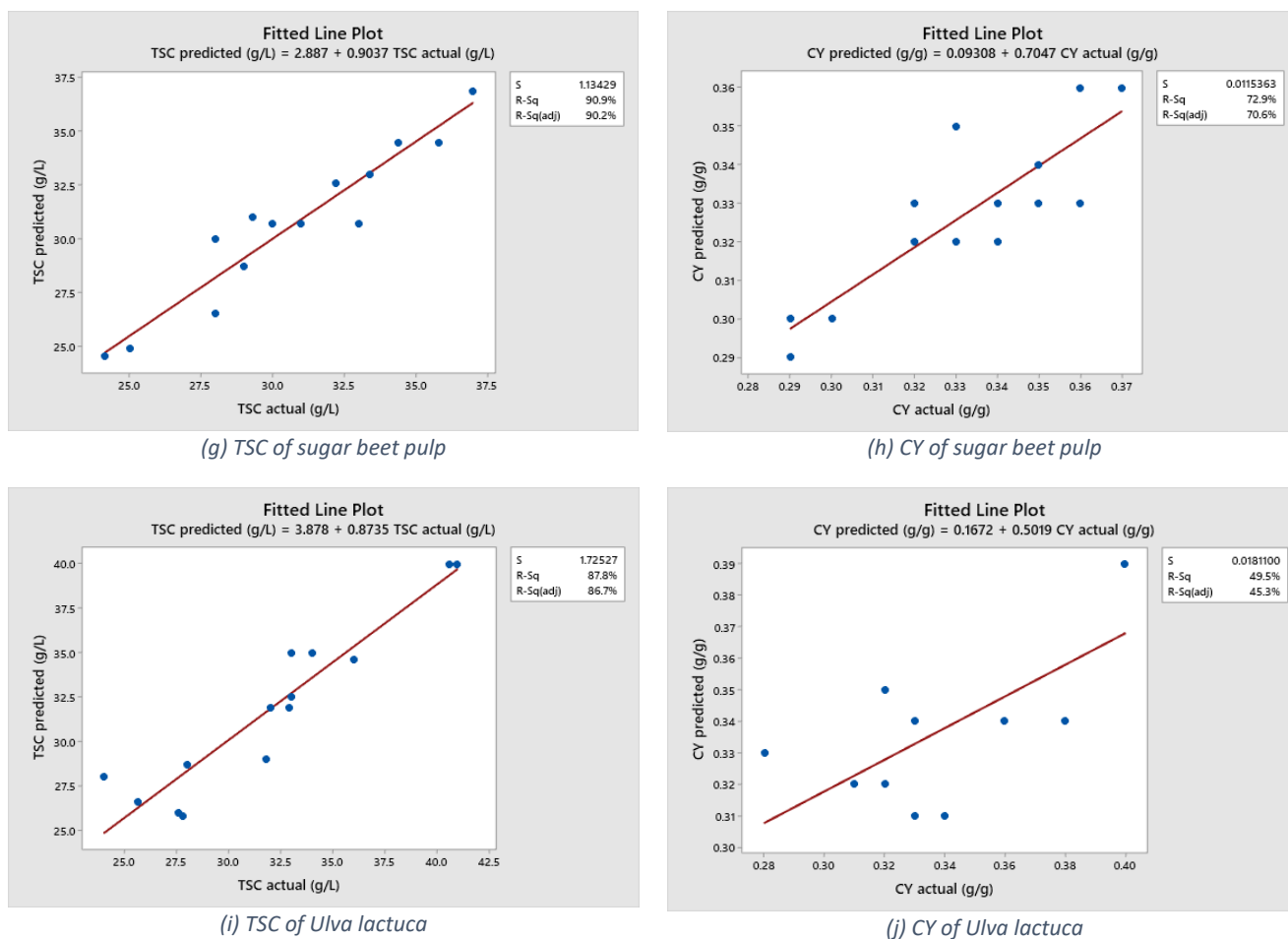


Figure 50. Comparison between the predicted and actual total soluble carbohydrate (TSC) and carbohydrate yield (CY) of the different feedstock.

Plackett-Burman design was transformed to a full factorial DOE based on the significant parameters obtained from the former. However, for the carbohydrate yields of *Ulva lactuca*, no significant parameter was recorded. Hence, 3 factors considered to be most significant among the insignificant parameters were selected and transformed into a full factorial design. The confidence level used for PBD was 95% for all biomass, except *U. lactuca* and the carbohydrate yield of sugar beet pulp (here 90% confidence level was employed). Nevertheless, for the full factorial DOE, a 95 % confidence level was used for all biomass.

For starch; statistical analysis showed that solid loading, time, and acid concentration were the significant parameters for total soluble carbohydrate realization while acid concentration and time were significant for carbohydrate yield. The R^2 value was 95.25% for total soluble carbohydrates and 78.23% for carbohydrate yield. The regression model described by the equations was significant. Solid loading, the interaction between solid loading and acid concentration, and also the interaction between solid loading and time had positive effects on total soluble carbohydrates while acid concentration, time, and the interaction between acid

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concentration and time had negative effects on total soluble carbohydrates. For the yield, it is observed that both acid concentration, time, and the interaction between acid concentration and time had negative effects. The contour plot (see Figure 51) which is a graphical representative of parameter interactions on response showed that at constant time, the highest TSC was achieved at the maximum level of solid loading and minimum level of acid concentration. The same could be said also for a plot between solid loading and time when acid concentration was held constant. Highest TSC was also detected at the lowest level of acid concentration and time while holding solid loading constant. Nevertheless, interactions between parameters were not statistically significant. Hence, we could say that the first-order model was statistically significant for the recovery of TSC. In terms of carbohydrate yield, it could be seen that acid concentration and time have some level of correlation.

For cellulose; temperature, acid concentration, time, the interactions between acid concentration and temperature, between temperature and time, and between acid concentration and time were the significant parameters for TSC. The same could also be said for CY. The coefficient of determination was 99.61% for TSC and 99.28% for CY. The regression model described by the equations was significant. Temperature, interactions between (acid concentration and temperature, acid concentration and time, temperature and time) affected TSC positively while acid concentration and time had negative effects on TSC. However, for CY, acid concentration, temperature, and time had negative effects while the interactions of parameters had the same effects as described for TSC. Results from contour plots (see Figure 52) showed that acid concentration and temperature have a strong correlation in terms of TSC production. This is also true for acid concentration and time, including temperature and time. The same explanation applies to CY.

For cassava peels, the significant parameters for TSC were solid loading, acid concentration, and time with an R^2 value of 96.66%. The interactions of parameters were not significant; this implies that the first-order model explains well the effectiveness of acid hydrolysis for TSC recovery. The same could be said for CY recovery with an R^2 value of 80.21%. However, for CY, only acid concentration and time were the significant parameters. Solid loading, acid concentration, and time showed positive effects on TSC recovery. While acid concentration influenced CY negatively, time influenced it positively. From the contour plots (see Figure 53), the highest TSC was observed at the maximum level of solid loading and minimum level of acid concentration at a constant time. At constant acid concentration, the highest TSC was achieved at maximum levels of solid loading and time. When solid loading

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was held constant, the highest TSC recovery was noticeable at the maximum level of time and minimum level of acid concentration. For CY recovery, the highest was achieved at the maximum level of time and minimum level of acid concentration.

For sugar beet pulp, the significant parameters for TSC recovery were solid loading, time, and temperature which could also be said to be well described by the first-order model with an R^2 value of 93.28%. The same could also be said for CY recovery with an R^2 value of 75.17% but for CY the significant parameters are time and temperature. From the regression equation, solid loading and time influence TSC positively while temperature influences it negatively. Temperature and time both have positive effects on CY. Results from contour plots (see Figure 54) revealed that solid loading and temperature have strong correlations on TSC recovery when the time was held constant, the highest TSC was achieved at maximum levels. The same also applies to solid loading and time when the temperature was held constant and also to temperature and time at constant solid loading. The highest carbohydrate yield was as well, observed at the maximum levels of temperature and time.

For *U. lactuca* the statistically significant parameters for obtaining TSC are solid loading, the interaction between temperature and time, temperature, and time with an R^2 value of 95.47%. Solid loading, temperature, and time have positive effects on TSC while the interaction between temperature and time poses a negative influence. The interaction between temperature and time was found to be the only significant parameter on CY with an R^2 value of 81.55% and this has a significant negative influence on the response. The contour plot (in Figure 55) showed that at constant time, the highest TSC was obtained at the maximum level of solid loading and minimum level of temperature whereas, at a constant temperature, the highest TSC was noticeable at the maximum levels of solid loading and time. At constant solid loading, it was noticeable at the maximum level of time and minimum level of temperature. Highest CY was also, observed at the maximum level of time and minimum level of temperature when solid loading was held constant.

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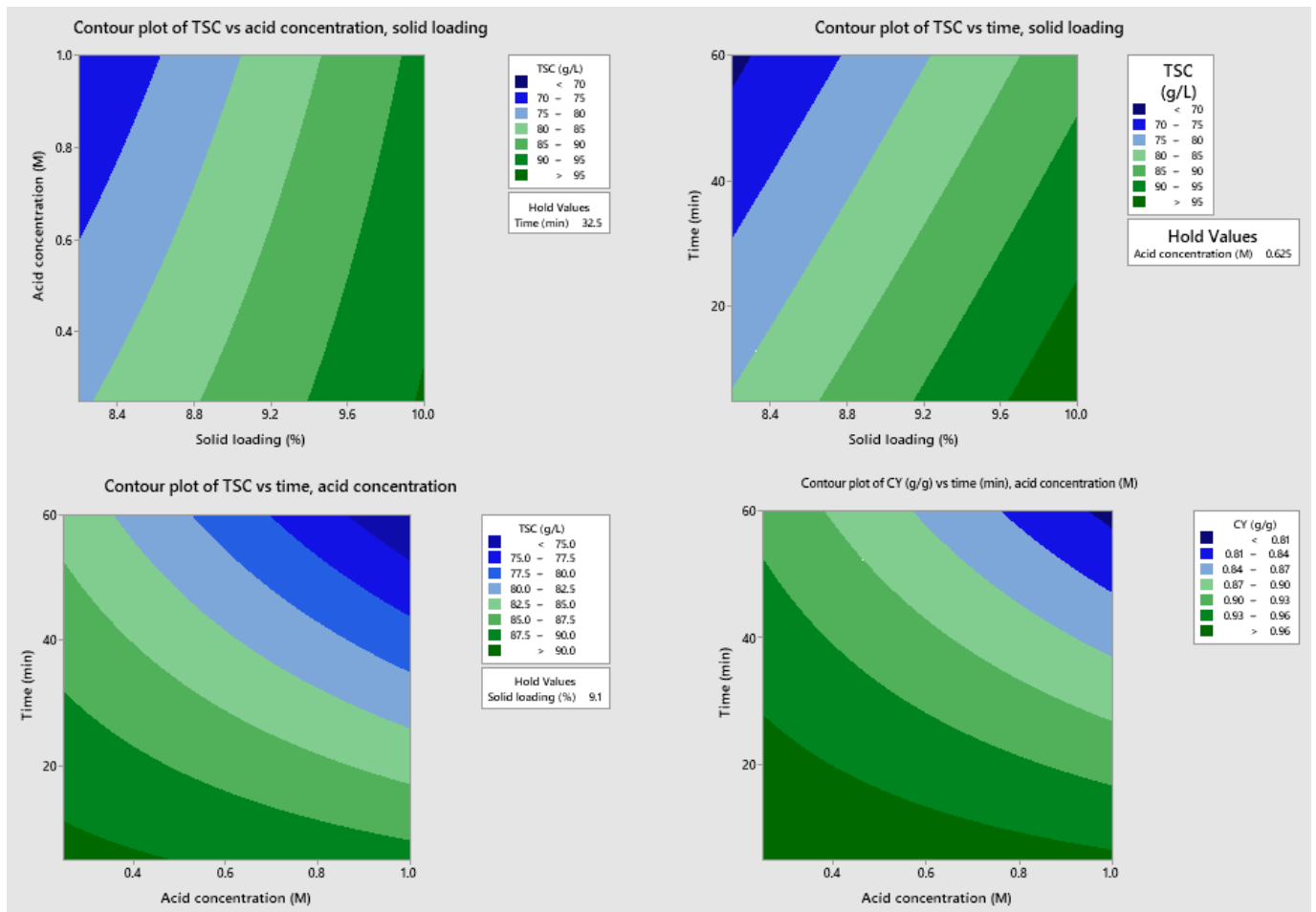


Figure 51. Contour plots for total soluble carbohydrate and carbohydrate yield of starch.

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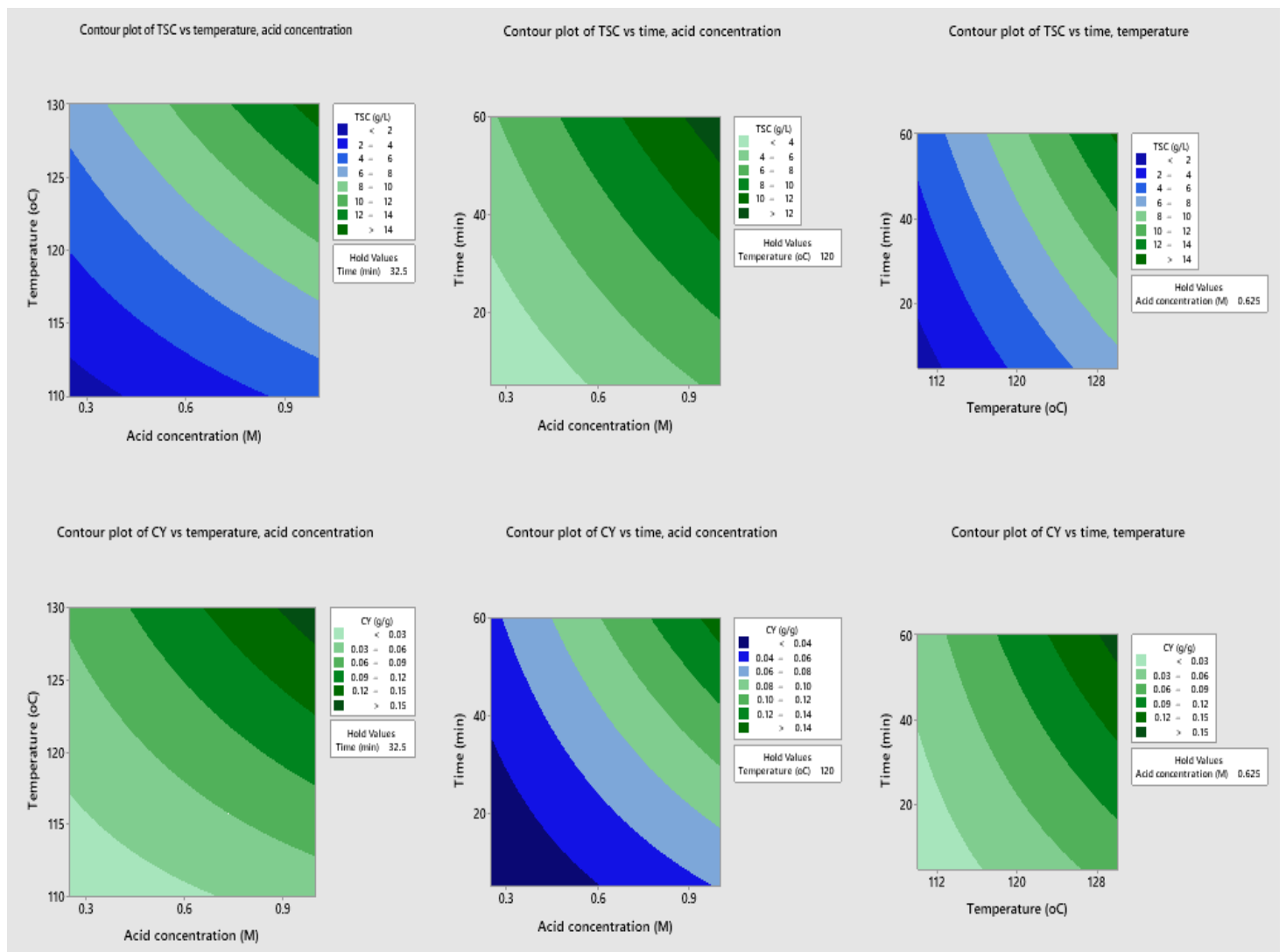


Figure 52. Contour plots for total soluble carbohydrate and carbohydrate yield of cellulose.

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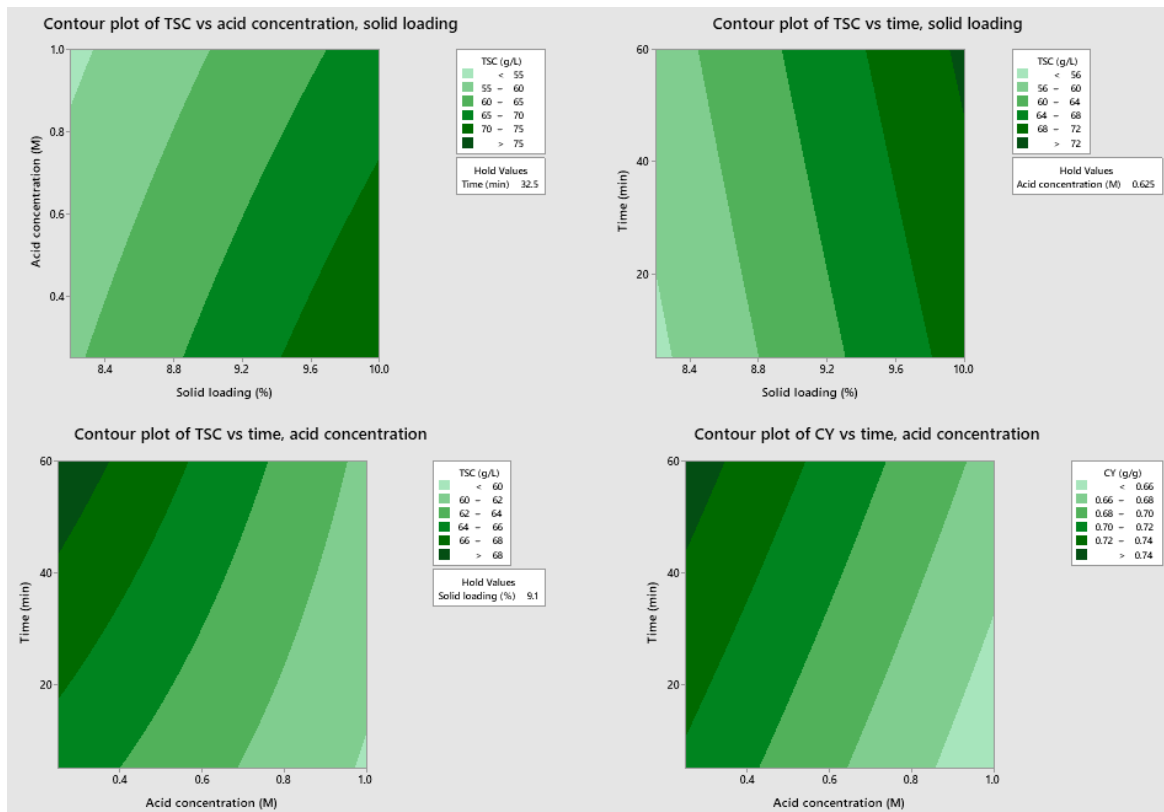


Figure 53. Contour plots for total soluble carbohydrate and carbohydrate yield of cassava peels.

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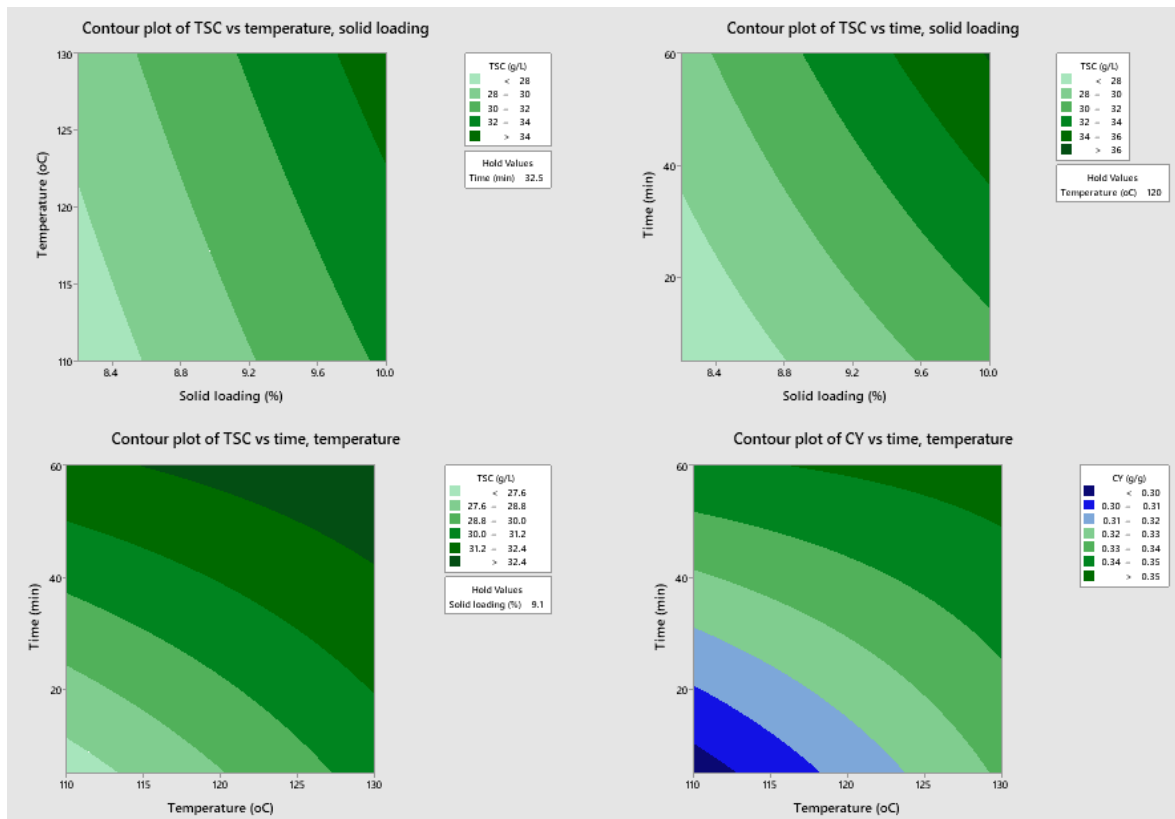


Figure 54. Contour plots for total soluble carbohydrate and carbohydrate yield of sugar beet pulp.

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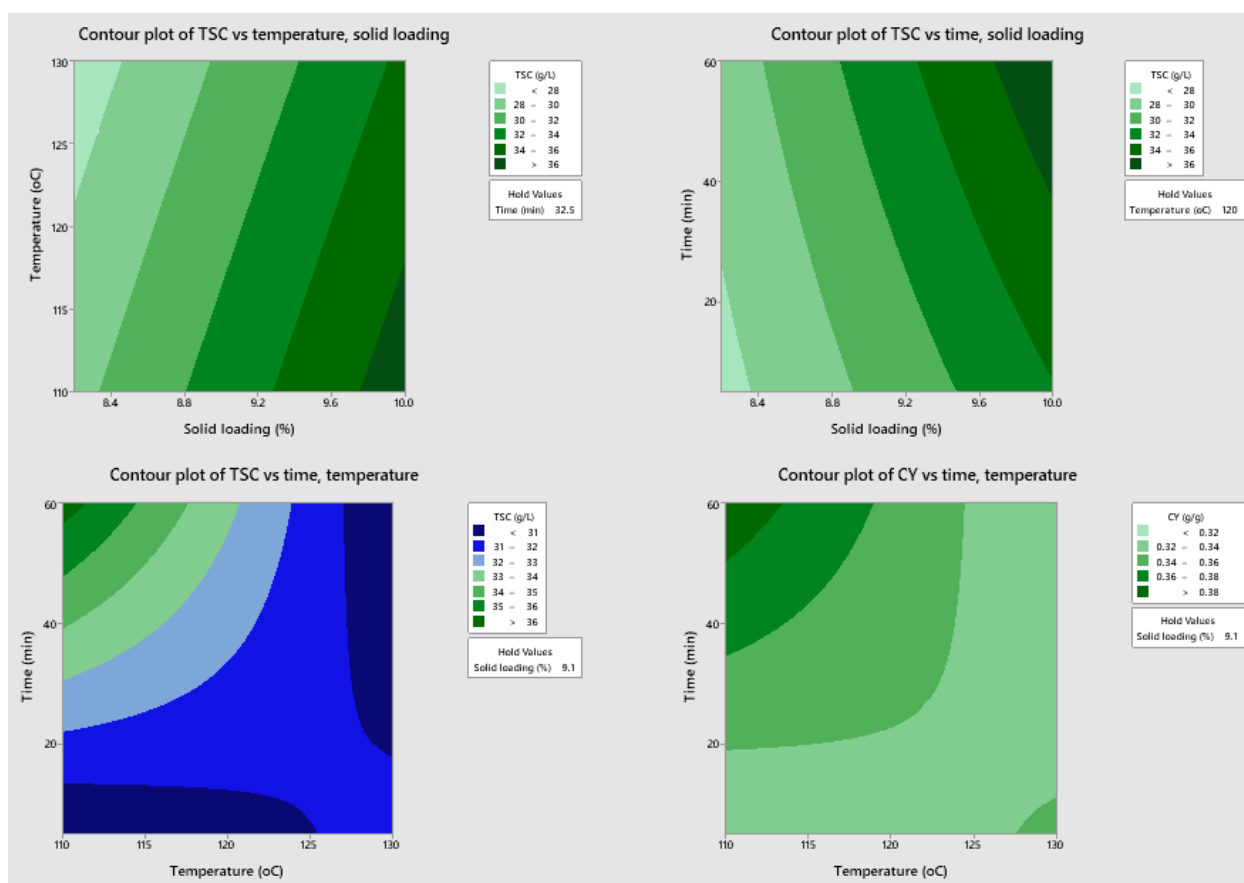


Figure 55. Contour plots for total soluble carbohydrate and carbohydrate yield of *Ulva lactuca*.

The model utilized in this investigation was significant, according to the ANOVA tables.

Carbohydrate yield at low substrate concentration was high when compared to that at high substrate concentration but statistically, the difference was not significant. The energy balance and economic sustainability of bioethanol production are dependent on ensuring consistently high concentrations of substrate all through the conversion process from lignocellulosic waste to ethanol (Kristensen et al., 2009). It is beneficial to employ high solid loading during acid hydrolysis of lignocellulosic biomass as it results in the high production of total soluble carbohydrates. Nevertheless, literature has it that the low carbohydrate yield is usually associated with a higher concentration of substrates (Irfan et al., 2016). This is not a favorable outcome because it negates the benefits of utilizing high substrate loading (high concentration of soluble carbohydrates, production cost reduction, amongst others). The exact reason for low carbohydrate yield at high solid loading is currently not fully identified (Jørgensen et al., 2007). Speculations have it that it could be due to mass transfer constraints and perhaps other factors connected to the higher insoluble solids concentration (Appiahnkansah et al., 2019). The effect of solid loading on yield was not significant based on the range

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we changed the biomass to water ratio; we are not certain if it applies to a wider range. Richen Lin *et al.* carried out hydrothermal acid hydrolysis of grass silage while utilizing 1.0% v/v dilute sulfuric acid at a time of 20 min. They studied the temperature range between 100 to 180°C and concluded according to the first-order model that increasing temperature favors hydrolysis at lower temperatures between 100 – 140°C, but favors further degradation at higher temperatures between 140 – 180°C (Lin *et al.*, 2020). The application of thermal energy in the acid hydrolysis of biomass permits improved access of acid catalysts in the presence of water to the interior structures of cellulosic biomass during treatment. The structure of cellulose evolves into crystalline and amorphous regions as a result of the arrangement of molecules. In cellulose, the amorphous region is more reactive than the crystalline region. The acid solution penetrates the interior structure of cellulose at varying temperatures based on its crystallinity, causing it to transition from crystalline to amorphous. The crystalline to amorphous domain conversion of cellulose results in accelerated oligomer formation rates which eventually leads to the production of fermentable carbohydrates (Paksung *et al.*, 2020). The significant parameters of the 3 different biomass were not exactly the same. The factors that influence the hydrolysis of biomass are intricately linked and not easily separated. They include biomass structural factors, such as particle size, pore size, pore volume, specific surface area, the crystallinity of cellulose, and degree of polymerization, as well as, chemical factors, such as lignin, hemicelluloses, and acetyl groups composition and content (Melati *et al.*, 2019; Zoghلامي and Paës, 2019). Outside of the biomass's optimum conditions, they produce relatively low soluble carbohydrates or high carbohydrates, some of which get degraded to furfural and hydroxymethylfurfural (HMF).

4.2 SEM and EDX analysis of treated biomass microstructural morphology

SEM was also used to assess the morphological modifications of treated (acid-hydrolyzed) biomass. Scanning electron micrographs of the treated biomass are shown in Figure 56. In general, the structure of the raw biomass is at first more whole and compact until it is subjected to acid hydrolysis. The introduction of an acid catalyst disrupts the component structure, creates more pore space, and increases the surface area of the material thus enabling the catalyst to gain more entrance to the carbohydrate components which can lead to an improved release of simple carbohydrates (Har *et al.*, 2013). Different biomass presents different surface areas. The higher the surface area of a substrate, the higher the possibility of an acid solution being properly soaked in. This could also determine the levels of other parameters, to be used such as biomass-to-water ratio, time, and temperature. Hence, the

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difference in the structural and chemical properties of biomass is close to being responsible for the difference in the specific levels of hydrolysis parameter requirements noticeable in our tested biomass. The amounts of soluble carbohydrates released can be determined not only by the contents of the structural components but also by the contents of the non-structural components such as starch. In Figure 56a, abrasion and fiber disruption are visible in the treated cassava peels. This could be a result of the hemicellulose solubilization and lignin breakdown following dilute acid treatment (Dong et al., 2019). Figure 56b shows the collapse of parenchymal tissues and fiber distortion of sugar beet pulp, revealing the vascular bundles. This could be attributed to pectin removal following treatment. The vascular bundles are noticeably cracked and eroded which could be an acid hydrolysis effect (Abou-Elseoud et al., 2021; Hassan et al., 2021; Zheng et al., 2017). Figure 56c illustrates the breakdown and removal of external cell walls of *Ulva lactuca* after acid treatment thereby increasing cell wall porosity and enhancing its surface area (Abdel et al., 2020; Greetham et al., 2020). Deposits of minerals are visible on the treated surface of *Ulva lactuca*.

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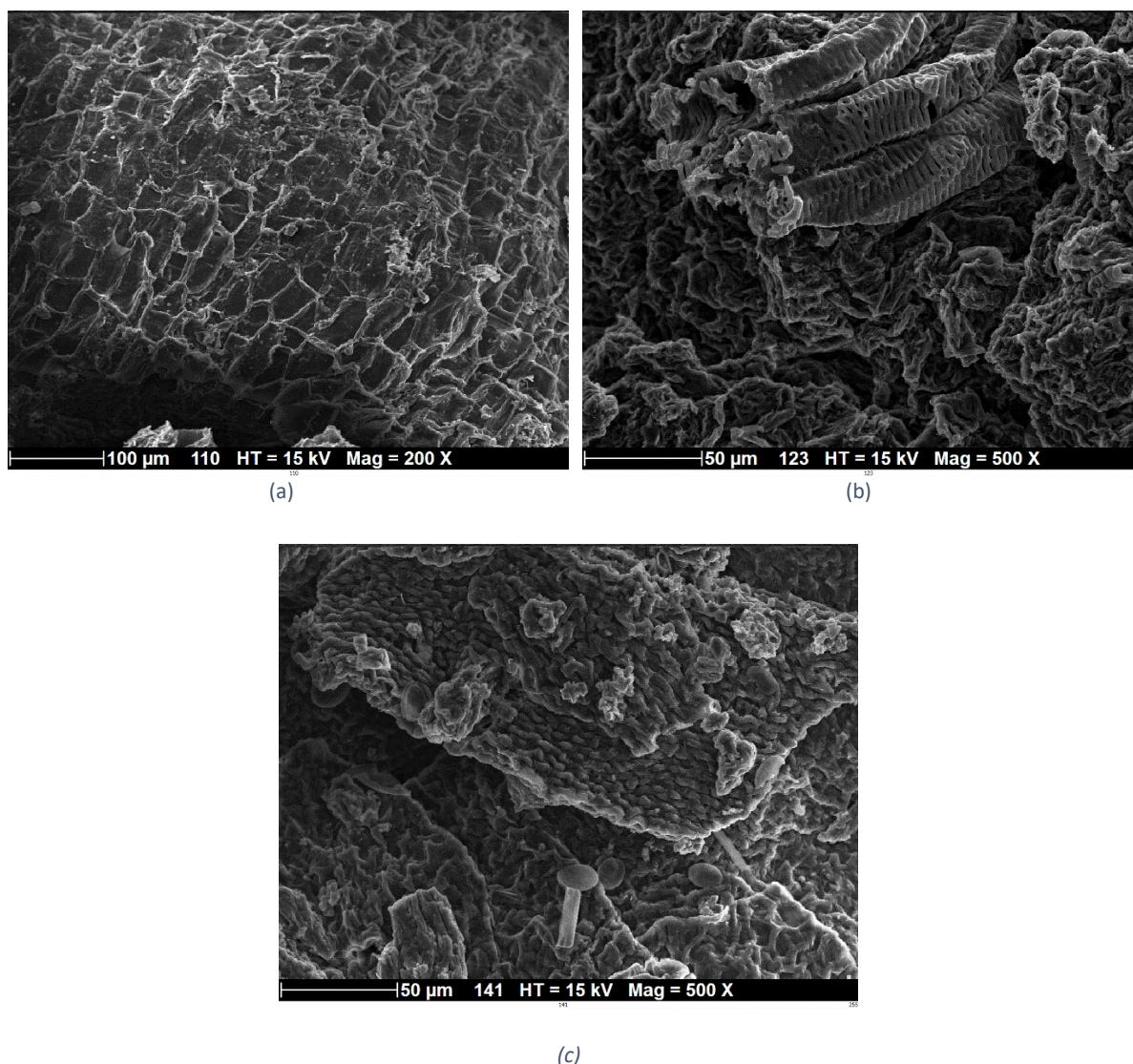


Figure 56. Scanning electron micrographs (SEM) of treated biomass (a) cassava peels (b) sugar beet pulp (c) *Ulva lactuca*.

From the EDX analysis of treated biomass (see Figure 57), an increase in counts of aluminum, silicon, and chlorine and a decrease in potassium was observed for cassava peels. A decrease in magnesium, sulphur, calcium and an increase in chlorine was observed for sugar beet pulp. For *Ulva lactuca*, a decrease in sodium, magnesium, Sulphur, chlorine, potassium, and an increase in silicon and calcium were observed. The increase in chlorine content of cassava peels and sugar beet pulp comes from biomass treatment with hydrochloric acid. On the contrary, *Ulva lactuca* as a sea organism contains high chlorine content in its raw form which decreased after treatment (Kumar et al., 2021; Mouritsen, 2013). (Bikker et al., 2016), reported the chlorine content of *Ulva lactuca* as a macro mineral to be 9.6 g.

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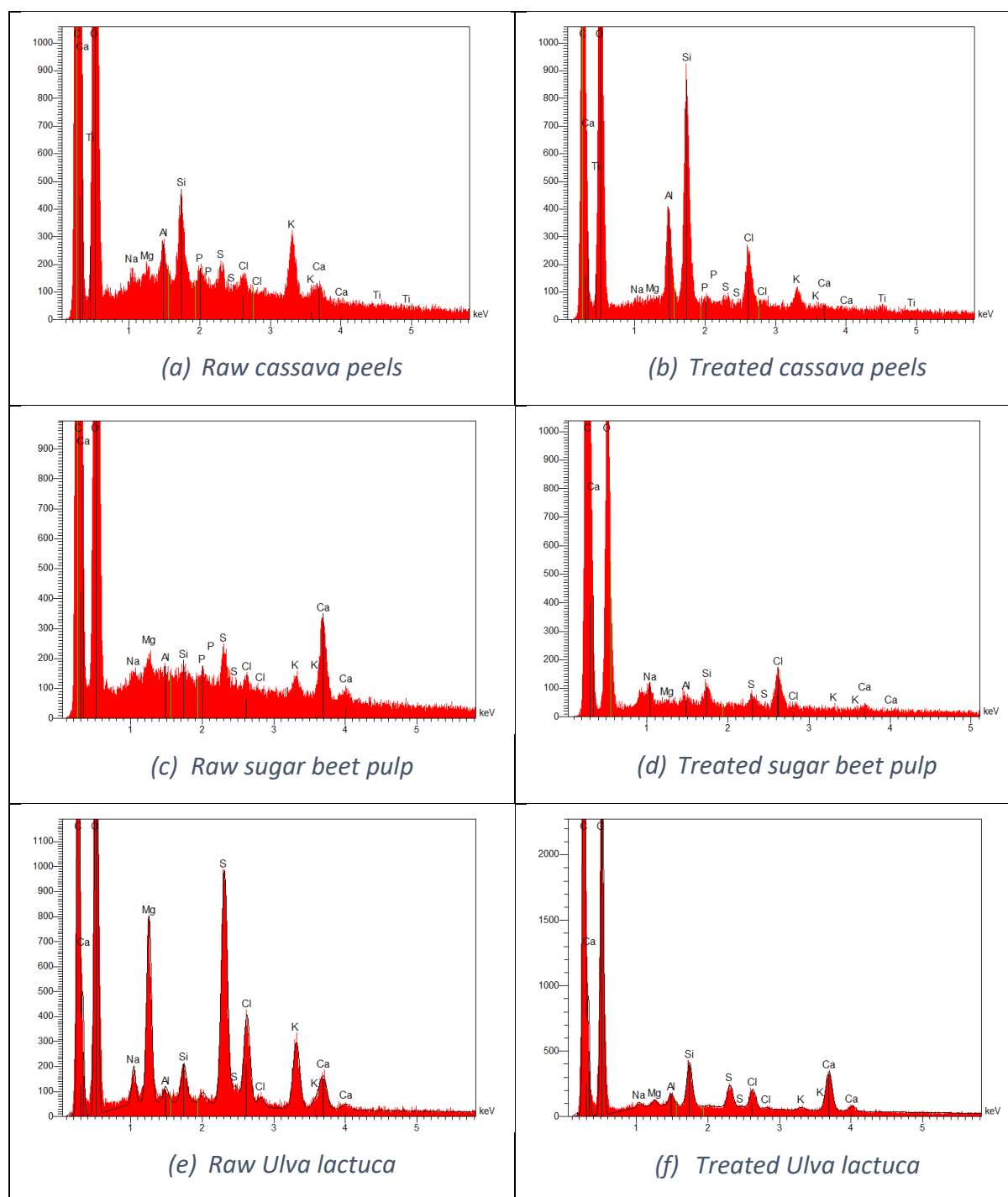


Figure 57. EDX of raw and treated biomass

4.3 Comparison of biomass hydrolysis to reference materials

Cassava peels, sugar beet pulp, and *Ulva* tend to behave more like starch than cellulose in terms of significant parameters (biomass to water ratio) and other conditions necessary to achieve the highest saccharification. While cellulose necessitates the most extreme conditions, the rest of the biomass requires subtler conditions. This could be explained by the compositional analysis of the biomass. Cellulose possesses a high degree of crystallinity and

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this makes it impossible for it to dissolve in water. The greater the degree of crystallinity in cellulose materials, the greater the mechanical properties (Kuutti, 2013). On the other hand, starch is regarded as semi-crystalline i.e. it has both amorphous and crystalline regions. It usually occurs in discrete granules (Song et al., 2017) and is composed of the polysaccharides amylose and amylopectin. Untreated starch is soluble in water when subjected to heat consequence of the starch gelatinization process and this causes the granules to swell and burst thus losing their native semi-crystalline structure, and eventually, the smaller amylose molecules begin to leach from the granule, generating a network that traps water and increases the viscosity of the mixture. In contrast to amylopectin (which is usually the major component of all starches), amylose is not soluble in water (Kuutti, 2013). Literature has proven that gelatinized starch is more prone to degradation (Crowther, 2012). Hemicellulose is in general amorphous in nature owing to the numerous side groups that are attached to their heteropolysaccharide configuration and so, depolymerizes rapidly compared to cellulose which is more crystalline and also, the pentose sugar from hemicellulose reacts more easily than the hexose sugars. Thus, the possession of high starch content (in cassava peels) and high hemicellulose content (in the 3 biomass) which could easily be acted on by acid solution made the biomass under study behave more like the reference starch than cellulose.

4.4 Optimized points generation and further model validation

Different optimized points were generated for cassava peels, sugar beet pulp, and *Ulva lactuca*. However, to come up with a common optimized point for the 3 different biomass, significant and non-significant parameters were put into consideration. For example, the generated optimized points for cassava peels were 10% solid loading, 0.25 M acid concentration, and 60 min time. The temperature variation was not significant so we set it to 110°C. For sugar beet pulp, 10% solid loading, 130°C temperature, and 60 min time were generated. The temperature was less significant among the significant parameters; so, we set it to 110°C also, acid concentration was non-significant; so, it was set to 0.25 M. For *Ulva lactuca*, 10% solid loading, a temperature of 110°C, and 60 min time were the optimized points generated. The acid concentration variation was not significant; so, it was set to 0.25 M. Therefore, the resultant optimized points tested to further validate this model were 10% solid loading, 0.25 M acid concentration, 110°C temperature, and 60 min time for the different biomass. TSC of 77 g/L and CY of 0.75 g/g, TSC of 35.3 g/L and CY of 0.35 g/g, TSC of 40.7 g/L, and CY of 0.40 g/g were predicted for cassava peels, sugar beet pulp, and *Ulva lactuca*, respectively. The actual experiment gave TSC of 76 g/L and CY of 0.76 g/g for cassava peels,

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TSC of 35.2 g/L and CY of 0.35 g/g for sugar beet pulp, and TSC of 36 g/L and CY of 0.36 g/g for *U. lactuca* (see Table 36). The actual yield based on the carbohydrate contents of cassava peels, sugar beet pulp, and *U. lactuca* were 93%, 66%, and 80%, respectively.

Table 36. Results from optimized points

Feedstock	Predicted TSC (g/L)	Experimental TSC (g/L)	Predicted CY (g/g)	Experimental CY (g/g)
Cassava peels	77	76	0.75	0.76
Sugar beet pulp	35.3	35.2	0.35	0.35
<i>Ulva lactuca</i>	40.7	36	0.40	0.36

4.5 Fermentation

Fermentation involves the chemical breakdown of organic matter such as glucose and xylose by microorganisms e.g. yeast, bacteria, or fungi to produce alcohol or acid.

4.5.1 Screening of hexose sugar yeast for the fermentation of simple carbohydrate

Besides solid loading (10%), the operating conditions (0.05 N HCl, 90°C, 90 min) used in the production of pectin from sugar beet pulp under stirring condition, was used in the hydrolysis of cassava peels to produce hydrolysate needed for the screening of *S. cerevisiae*. The total soluble carbohydrate obtained was 57 g/L and carbohydrate yield was 57%. The resulting hydrolysate was found to be viscous. However, fermentation of a portion of the hydrolysate was performed with baker's yeast (labeled without additive) purchased from a local grocery and streaked on an agar plate while the second portion was performed with the cultured *S. cerevisiae* isolated from wine residue. The highest ethanol production using baker's yeast was 1.8 g/L while 2.0 g/L was obtained with *S. cerevisiae* within 24 hrs. Hence, for further experimentation, baker's yeast was dropped and *S. cerevisiae* from wine residue was utilized. The low amounts of ethanol produced from the hydrolysate of cassava peels could be ascribed to the viscous nature of the hydrolysate which is an indication that the pectin extraction conditions were not sufficient in releasing enough simple carbohydrates for fermentation by the screened yeasts.

4.5.2 Fermentation of biomass hydrolysate

The optimized parameters were used to produce the reducing sugars for fermentation. After the saccharification of 100 g/L of each biomass, the hydrolysate of cassava peels contained total soluble carbohydrates of 76 g/L, reducing sugars of 51.46 g/L from HPLC analysis and 50.04 g/L from enzymatic kits analysis, while the hydrolysate of sugar beet pulp

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contained total soluble carbohydrates of 35.2 g/L, reducing sugars of 24.97 g/L from HPLC analysis and 5.02 g/L from enzymatic kits analysis, and the hydrolysate of *Ulva lactuca* contained total soluble carbohydrates of 36 g/L, reducing sugars of 8.13 g/L from HPLC analysis and 6.03 g/L from enzymatic kits analysis (Table 37). The difference observed between the values of total soluble carbohydrates assayed by Dubois method and the values of reducing sugars assayed by both HPLC and the enzymatic kits are because Dubois method takes into account both reducing and non-reducing sugars; the HPLC was used to quantify reducing sugars present in the hydrolysate, while the enzymatic kits were used to measure specific simple sugars (glucose and xylose) which are reducing sugars and oligomers were not taken into consideration. Xylose was detected by Megazyme kit but not by HPLC analysis in the hydrolysate of cassava peels and sugar beet pulp; this could be because Megazyme kit might be more sensitive since it is designed specifically for the detection of xylose, allowing it to detect lower levels of xylose that could be missed by HPLC. In addition, they employ different methodologies for the detection of xylose. Variations in the sample preparation, extraction, and analytical procedures can lead to differences in the results.

Table 37. Reducing sugar concentrations by HPLC and Enzymatic Kits

Reducing sugar concentrations by HPLC							
Feedstock	Rhamnose	Xylose	Arabinose	Fructose	Glucose	Maltose	Total reducing sugars
Cassava peels (g/L)	--	--	1.27 ± 0.03	1.22 ± 0.03	42.8 ± 0.2	6.17 ± 0.23	51.46 ± 0.49
Sugar beet pulp (g/L)	0.80 ± 0.03	--	16.8 ± 0.2	2.28 ± 0.07	4.57 ± 0.05	0.52 ± 0.10	24.97 ± 0.45
<i>Ulva lactuca</i> (g/L)	2.37 ± 0.08	1.61 ± 0.18	--	0.24 ± 0.08	2.47 ± 0.13	1.44 ± 0.15	8.13 ± 0.62
Reducing sugar concentrations by Enzymatic Kits							
Feedstock	Xylose		Glucose		Total reducing sugars		
Cassava peels (g/L)	0.84 ± 0.02		49.2 ± 2.0		50.04 ± 2.02		
Sugar beet pulp (g/L)	1.76 ± 0.52		3.26 ± 0.04		5.02 ± 0.56		
<i>Ulva lactuca</i> (g/L)	1.81 ± 0.55		4.22 ± 0.08		6.03 ± 0.63		

Ethanol fermentation by *S. cerevisiae*, *S. stipitis*, and *N. intermedia* are given in Tables 38 - 40. Maximum ethanol production achieved from cassava peels hydrolysate was 28.62 ±

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3.93 g/L after a day of fermentation with *S. cerevisiae* and 3 days of fermentation with *S. stipitis*. Maximum ethanol production obtained with *N. intermedia* from cassava peels hydrolysate was 28.59 ± 0.41 g/L after 4 days of fermentation. These are higher than what was achieved by (Worfa et al., 2017) who reported the highest ethanol yield of 19.36 % w/w dry biomass by fermenting with *S. cerevisiae* following a combination of *Pleurotus ostreatus* and *Aspergillus niger* fungal hydrolysis. For sugar beet pulp, the maximum ethanol production obtained was 3.36 ± 0.04 g/L after a day of fermentation with *S. cerevisiae* and 3 days of fermentation with *S. stipitis*. Maximum ethanol production from sugar beet pulp hydrolysate fermentation with *N. intermedia* was 3.0 ± 0.23 g/L after 4 days. In comparison, (Berłowska et al., 2020) after enzymatic hydrolysis, achieved an ethanol yield of 9.9 ± 0.4 kg from 100 kg of sugar beet biomass by the fermentation of hexose and pentose sugars done sequentially with a co-culture of the yeasts, *S. cerevisiae*, and *P. stipitis*. The variation in ethanol yield could be due to the use of saccharose in addition to sugars released from structural carbohydrates of sugar beet root in their work. *Ulva lactuca* produced highest ethanol concentration of 3.71 ± 0.07 g/L after a day of fermentation with *S. cerevisiae* and 3 days of fermentation with *S. stipitis*. Upon the fermentation of *Ulva lactuca* hydrolysate with *N. intermedia*, maximum ethanol concentration of 7.69 ± 0.20 g/L was achieved after 5 days. (Cioroiu et al., 2018) reported an ethanol yield of 0.0234 g/g dry matter with *S. cerevisiae* following enzymatic hydrolysis of *Ulva lactuca* while (Kumar et al., 2013) assessed the large-scale bioethanol production from algal biomass, *Gracilaria verrucosa* to be 3.8 kg/100 kg dry weight following enzymatic hydrolysis of the biomass. (Ahou et al., 2020) carried out wet oxidation pretreatment with sulphuric acid under operating conditions of 130°C, 75 min, and pH 1 on cassava peels and *Ulva lactuca* and reported pretreatment efficiencies of 63% and 95% respectively, and upon fermentation with *Neurospora intermedia*, produced 0.31 g/L and 0.26 g/L of ethanol from 6 g/L of cassava peels and *Ulva lactuca*, respectively.

At the end of hydrolysate fermentation by the different microorganisms, the residual reducing sugar concentration was zero (0).

Table 38. Ethanol production from fermentation by *S. cerevisiae*

Feedstock	Glucose (enzymatic kits)	5 hrs.	24 hrs.	30 hrs.
Cassava peels (g/L)	49.2 ± 2.00	5.13 ± 0.07	26.94 ± 3.21	24.83 ± 0.33
Sugar beet pulp (g/L)	3.26 ± 0.03	1.17 ± 0.01	1.74 ± 0.07	1.58 ± 0.05
<i>Ulva lactuca</i> (g/L)	4.22 ± 0.08	1.58 ± 0.05	2.04 ± 0.08	1.86 ± 0.03

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Table 39. Ethanol production from fermentation by *S. stipitis*

Feedstock	Xylose (enzymatic kits)	1 st day	2 nd day	3 rd day	4 th day
Cassava peels (g/L)	0.84 ± 0.02	0.37 ± 0.02	0.56 ± 0.03	1.68 ± 0.72	1.52 ± 0.09
Sugar beet pulp (g/L)	1.76 ± 0.53	0.28 ± 0.01	0.35 ± 0.05	1.62 ± 0.03	1.48 ± 0.07
<i>Ulva lactuca</i> (g/L)	1.81 ± 0.55	0.52 ± 0.04	1.10 ± 0.07	1.67 ± 0.01	1.48 ± 0.09

Table 40. Ethanol production from fermentation by *N. intermedia*

Feedstock	Glucose & xylose (enzymatic kits)	1 st day	2 nd day	3 rd day	4 th day	5 th day	6 th day
Cassava peel (g/L)	50.04 ± 2.02	10.20 ± 0.38	20.98 ± 0.35	25.78 ± 0.20	28.59 ± 0.41	28.52 ± 0.16	26.42 ± 0.10
Sugar beet pulp (g/L)	5.02 ± 0.56	1.39 ± 0.07	2.15 ± 0.04	2.77 ± 0.001	3.0 ± 0.23	2.98 ± 0.14	2.12 ± 0.08
<i>Ulva lactuca</i> (g/L)	6.03 ± 0.63	1.70 ± 0.03	2.40 ± 0.01	5.19 ± 0.15	6.91 ± 1.19	7.69 ± 0.20	6.75 ± 0.04

From the above, cassava peels and sugar beet pulp produced highest ethanol of 28.62 g/L and 3.36 g/L, respectively with *S. cerevisiae* and *S. stipitis* while *Ulva lactuca* produced highest ethanol of 7.69 g/L with *N. intermedia*. The fermentation efficiency in relation to total soluble carbohydrates was 74%, 18.8%, and 42% from cassava peels, sugar beet pulp, and *Ulva lactuca*, respectively. In relation to HPLC analysis, the fermentation efficiency was over 100% for cassava peels and *Ulva lactuca* while for sugar beet pulp, the fermentation efficiency was 26.4%. This low yield in sugar beet pulp is due to the inability of the employed microorganisms to utilize arabinose which is the dominant sugar in the hydrolysate of the biomass. In certain microorganisms, this high concentration of arabinose can interfere with the uptake and metabolism of glucose and xylose, resulting in reduced utilization of these sugars for fermentation. From the sugars obtained with enzymatic kits analysis, the fermentation efficiency was found to be over 100% for cassava peels and the rest of the biomass. In a control experiment having only monomers (fermentation with an equal mixture of D-glucose and D-xylose), (Table 41), the fermentation efficiency did not exceed 100%; 97% was obtained from the fermentation of D-glucose with *S. cerevisiae* under 24 hrs. and 93% was obtained from the fermentation of D-xylose with *S. stipitis* within 5 days. Hence, the observed results from the biomass hydrolysates could be due to the presence of lots of dimers such as maltose or in some

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cases sucrose (as well known as saccharose) or oligomers such as cellobiose in the supernatants of the biomass as accounted for by their respective amount of total soluble carbohydrates.

Table 41. Fermentation efficiency of D-glucose and D-xylose

	Sugar concentration (g/L)	Ethanol concentration (g/L)	Fermentation efficiency (%)	Day achieved
D-glucose	20	9.95	97	24 hrs.
D-xylose	20	9.56	93	5 days

S. cerevisiae is able to ferment saccharose to ethanol but it is not able to assimilate cellobiose because it lacks the cellobiose transporter and β -glucosidase required for the hydrolysis of cellobiose to glucose, a monomeric sugar (Nanssou et al., 2016). *S. cerevisiae* utilizes a proton symport mechanism for transporting maltose, while glucose is transported via facilitated diffusion (Weusthuis et al., 1993). *S. cerevisiae* cells are known to possess an extracellular invertase (β -D-fructosidase) that catalyzes the hydrolysis of sucrose into glucose and fructose. These monosaccharides are then taken up by hexose transporters and metabolized through glycolysis within the cell to produce ethanol (Badotti et al., 2008). The hydrolysates of the lignocellulosic biomass also contain xylose but since *S. cerevisiae* lacks the early metabolic route that transforms xylose to xylulose and because its metabolism of xylulose is poor, it is unable to utilize xylose. Hence, *S. stipitis* was inoculated into the medium (Ha et al., 2011; Zheng et al., 2020). *S. stipitis* has the ability to ferment xylose, and other sugars such as glucose, galactose, mannose, and cellobiose as well as xylan and mannan oligomers (Jeffries and Van Vleet, 2009). *S. stipitis* usually, assimilates xylose via the pentose-phosphate metabolic pathway (Hsu et al., 2016). *N. intermedia*, on the other hand, can produce and secrete enzymes that act on the oligomers present in biomass hydrolysate (Shahryari et al., 2019). This action enables the release of monosaccharides such as glucose and xylose which can then be further metabolized by the fungus leading to the production of bioethanol. This could thus, be the reason behind the improved bioethanol yield noticeable in the hydrolysate of *Ulva lactuca* in relation to the yield observed with yeasts.

Plate counts were performed at the end of fermentation and it was found that the cells of *S. cerevisiae* grew on average from 1.0×10^6 CFU/mL to 3.1×10^7 CFU/mL while the cells of *S. stipitis* grew on average from 1.0×10^6 CFU/mL to 5.0×10^7 CFU/mL. An overview of hydrolysate fermentation is given in Table 42.

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Table 42. Hydrolysate fermentation

Fermentation of hydrolysate with <i>Saccharomyces cerevisiae</i> and <i>Scheffersomyces stipitis</i>							
Feedstock	Total soluble carbohydrates (g/L)	Total reducing sugars by HPLC (g/L)	Total reducing sugars by Enzymatic Kit (g/L)	Ethanol concentration (g/L) with <i>S. cerevisiae</i>	Ethanol concentration (g/L) with <i>S. stipitis</i>	Total ethanol concentration (g/L)	Ethanol yield (g/g dry biomass)
Cassava peels	76	51.46 ± 0.49	50.04 ± 2.02	26.94 ± 3.21	1.68 ± 0.72	28.62 ± 3.93	0.286
Sugar beet pulp	35	24.97 ± 0.45	5.02 ± 0.56	1.74 ± 0.07	1.62 ± 0.03	3.36 ± 0.04	0.033
<i>Ulva lactuca</i>	36	8.13 ± 0.62	6.03 ± 0.63	2.04 ± 0.08	1.67 ± 0.01	3.71 ± 0.07	0.037
Fermentation of hydrolysate with <i>Neurospora intermedia</i>							
Feedstock	Total soluble carbohydrates (g/L)	Total reducing sugars by HPLC (g/L)	Total reducing sugars by Enzymatic Kit (g/L)	Ethanol concentration (g/L)		Ethanol yield (g/g dry biomass)	
Cassava peels	76	51.46 ± 0.49	50.04 ± 2.02	28.59 ± 0.41		0.286	
Sugar beet pulp	35	24.97 ± 0.45	5.02 ± 0.56	3.0 ± 0.23		0.03	
<i>Ulva lactuca</i>	36	8.13 ± 0.62	6.03 ± 0.63	7.69 ± 0.20		0.076	

To check if the yeasts perform better at higher sugar concentrations, the sugar concentration of the biomass supernatants was boosted with 5 g/L of D-glucose and 5 g/L of D-xylose. This led to maximum ethanol production from 26.94 ± 3.21 g/L to 33.19 ± 3.705 g/L, 1.74 ± 0.07 g/L to 5.315 ± 0.035 g/L, and 2.04 ± 0.08 g/L to 5.49 ± 0.042 g/L of the glucose-boosted supernatants of cassava peels, sugar beet pulp, and *U. lactuca*, respectively after 24 hrs. using *S. cerevisiae* as the fermenting microorganism. Also, maximum ethanol production from 28.62 ± 3.93 to 31.09 ± 3.196 g/L, 3.36 ± 0.04 to 5.84 ± 0.55 g/L, and 3.71 ± 0.07 to 6.15 ± 0.16 g/L were observed after 3 days in the xylose-boosted supernatants of

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cassava peels, sugar beet pulp, and *U. lactuca*, respectively with *S. stipitis* as the fermenting yeast after glucose fermentation with *S. cerevisiae*. The addition of D-glucose encouraged more bioethanol production from the supernatants of the biomass with a greater enhancement noticeable in cassava peels. This could be because glucose is a preferred carbon source for *S. cerevisiae* (Kayikci and Nielsen, 2015) and its metabolism must have brought about a revitalization of the yeast that encouraged further digestion of dimers. This cannot be said for D-xylose-boosted supernatants. However, this assay is an indication that ethanol production was not hindered by fermentation inhibitors.

In a nutshell, the proliferation of the yeast cells in the fermentation media is a demonstration that the yeasts were able to assimilate the sugars in the hydrolysates of acid-hydrolyzed biomass feedstock and metabolize them to ethanol.

4.6 Conclusion

The purpose of this study was to test various variables and maximize carbohydrate extraction from biomass, utilizing dilute acid as a catalyst and then fermenting the hydrolysates for bioethanol production. The results of this study showed that acid hydrolysis was effective in producing large amounts of soluble carbohydrates. The actual values of the optimized points were close to the predicted values. Separate hydrolysis and fermentation with sequential inoculation of *S. cerevisiae* and *S. stipitis*, led to maximum ethanol production of 28.62 ± 3.93 g/L from cassava peels and 3.36 ± 0.04 g/L from sugar beet pulp, while the use of the fungus, *N. intermedia* enabled *Ulva lactuca* to achieve maximum ethanol concentration of 7.69 ± 0.20 g/L. This shows that the studied biomasses are potential candidates for the production of second-generation bioethanol. Nevertheless, for optimum ethanol yield of sugar beet pulp, the arabinose content of the biomass should be explored as it is the major sugar in the biomass and being a relatively small component in other biomass, its fermentation has not been properly researched. Also, the ethanol yield of *Ulva lactuca* using *N. intermedia* as the fermentation microorganism can be improved by testing different fermentation pH ranges. In general, since it is evident that in addition to monomers the hydrolysates of these biomasses contain dimers or oligomers which might not be preferably metabolized by the fermentation microorganism, further research is required to achieve efficient hydrolysis while taking the cost of hydrolysis catalysts, steps, length of production, and environmental safety into consideration; a heterogeneous (solid) catalyst should be studied and extensively tested to maximize the saccharification of these feedstocks since it is reported to be environmentally friendly, easily separated from liquid products, and reusable.

General conclusion

Biomass is a renewable and sustainable source of energy that can be used for a variety of applications, including the production of biofuels such as bioethanol.

Reviewing the various biofuel generations reveals that using advanced biofuels derived from second and third-generation feedstock is both more environmentally friendly and economically advantageous than using biofuel produced from first-generation biomass. It was demonstrated that the recalcitrance of lignocellulosic biomass makes it be subjected to pretreatment stages so that the material will become more receptive to catalytic agents. Additionally, it was noted that although biological pretreatment and enzymatic hydrolysis of biomass are environmentally beneficial processes, they require more steps and time and can be expensive, an example is when using genetically modified organisms (GMOs). The pentose and hexose sugar fermentation pathways were also highlighted; it is important to note that in a combination of sugars, microbes consume hexose sugar (glucose) first. It only utilizes pentose sugars after hexose sugar has been consumed. The presentation of several initiatives on advanced bioethanol production was also observed in this study. Legislation in the EU supports the development of second-generation bioethanol production, which utilizes non-food feedstock and can help to reduce dependence on fossil fuels. However, large-scale production of bioethanol from lignocellulosic biomass is still a challenge due to the complexity of the process and the need for cost-effective and efficient technologies.

Lignocellulosic biomass is a complex and diverse group of plant materials that are composed of cellulose, hemicellulose, and lignin. Cellulose and hemicellulose are the main sources of sugar for bioethanol production, while lignin serves as a structural component and barrier for saccharification and fermentation processes. The structure and composition of lignocellulosic biomass greatly affect the efficiency of bioethanol production and can vary greatly depending on the source of the biomass. Due to this complexity, it is important to thoroughly characterize and understand the structure and composition of specific lignocellulosic biomass before attempting to convert it into bioethanol. Lignocellulosic biomass, such as cassava peels, sugar beet pulp, and *Ulva lactuca*, have been identified as potential feedstocks for bioethanol production due to their high polysaccharide composition. Each feedstock had different compositions due to variations in biomass type, environmental factors, harvesting technique, and storage conditions. The suitability of a feedstock for fermentation and the production of ethanol depends on several factors, including the availability of sugar, the presence of

General conclusion

inhibitors, and the overall composition of the feedstock. To completely comprehend the viability and economics of employing these materials as a source of sugar for fermentation and the large-scale manufacturing of ethanol, more study and testing are required. The possibility for these materials to be used in combustion as an energy source can also be influenced by their high heating value. However, other parameters like moisture and ash contents, also affect the combustion performance, so the HHV alone does not define the acceptability of biomass for burning.

Acid hydrolysis is a common pretreatment method used to convert lignocellulosic biomass into sugars that can be fermented into bioethanol. The significant parameters involved in the hydrolysis of the different biomass were not exactly the same which could be due to differences in biomass structural factors, such as particle size, pore size, pore volume, specific surface area, the crystallinity of cellulose, and degree of polymerization, as well as, chemical factors, such as lignin, hemicelluloses, and acetyl groups composition and content. Reducing sugar and ethanol production from cassava peels was a success and the feedstock revealed that it has great potential to be used in the large-scale production of ethanol. However, the sugar and ethanol yields from acid-hydrolyzed sugar beet pulp and *Ulva lactuca* suggest further optimization of the process and keen exploration of the arabinose content of sugar beet pulp as the dominant sugar in the biomass.

Fermentation organisms and inhibitors are also important factors to consider in bioethanol production. The operating conditions used in the extraction of pectin from sugar beet pulp were employed on cassava peels to produce simple carbohydrates. Physical examination of the obtained hydrolysate revealed its viscous nature and the ethanol obtained was low which was an indication that cassava peels needed higher levels of operating conditions. Of the two C₆ sugar-utilizing microorganisms screened, which were baker's yeast bought from the local grocery and *S. cerevisiae* isolated from wine residue, the latter was adopted due to its better performance even though both of the two strains gave relatively close results. The use of appropriate microorganisms and the control of inhibitors can help to improve the efficiency of the fermentation process and increase the final bioethanol yield. However, in this study, the microorganisms used didn't seem to be the cause of low ethanol yield from sugar beet pulp and *Ulva lactuca* as the microorganisms were able to consume all the simple sugars present in the medium and even extended their consumption to dimers/oligomers.

General conclusion

On a more general note, bioethanol production from lignocellulosic biomass is a promising area of research with the potential to reduce dependence on fossil fuels and contribute to a sustainable energy future. However, further research and development are required to improve the efficiency and cost-effectiveness of the process and to overcome the challenges associated with large-scale production.

Perspectives

We have discussed the potential of lignocellulosic biomass (cassava peels, sugar beet pulp, and *Ulva lactuca*) as a source of sugar for bioethanol production. However, sugar beet pulp and *Ulva lactuca* presented some challenges that need to be addressed in order to improve the efficiency and cost-effectiveness of the process.

- a. Bioconversion: Further research is required in this field for efficient hydrolysis of these feedstock materials while taking the cost of hydrolysis catalysts, steps, length of production, and environmental safety into consideration.
- b. Development of new pretreatment methods: Pretreatment of sugar beet pulp and *Ulva lactuca* is essential to improve the release of sugars for fermentation. A new pretreatment method should be explored. For example, heterogeneous (solid) catalysts should be studied and extensively tested to maximize the saccharification of these feedstocks since it is reported to be environmentally friendly, easily separated from liquid products, and reusable.
- c. Strain adaptation: The strain *N. intermedia* should be acclimated to neutralized biomass (*U. lactuca* and sugar beet pulp) acid hydrolysates through a sequential transfer and growth of cells in media containing the hydrolysates. This approach should employ the microorganism from each experiment as the inoculant for the subsequent one. The cycle should be repeated up to 16 times before final harvest and cultivation on PDA (Potato dextrose agar) plates. By so doing, ethanol production with the adapted strain is expected to be higher than ethanol produced with the parent strain (non-adapted strain).
- d. Testing pH ranges: Different fermentation pH ranges should be tested while carrying out fermentation of *U. lactuca* hydrolysate with *N. intermedia* to come up with an optimized pH.
- e. Exploring the other pentose sugar: Arabinose fermentation of the hemicellulose component of sugar beet pulp for bioethanol production should be studied in depth since sugar beet pulp contains it in high concentration but being a relatively small component in other biomass, its fermentation has not been properly researched on.

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Titre : Étude expérimentale sur le potentiel de différents matière première pour la production de bioéthanol de seconde génération : définition d'une méthodologie

Mots clés : Déchets de biomasse, hydrolyse acide diluée, glucides, fermentation, bioéthanol

Résumé : Les biocarburants sont des ressources énergétiques propres et renouvelables qui suscitent une attention croissante en tant que remplacement potentiel des carburants non renouvelables à base de pétrole. Elle est ainsi appelée car elle est issue d'une biomasse qui peut être soit d'origine animale, soit appartenir à l'une des trois générations de biomasse végétale (cultures agricoles, matières lignocellulosiques ou algues). Différentes méthodes et techniques ont été testées par les scientifiques et chercheurs dans ce domaine, et les conditions les plus optimales ont été adoptées pour la génération de biocarburants à partir de la biomasse. Cela a finalement conduit à une intensification ultérieure des procédures et à la création d'usines/bioraffineries pilotes, de démonstration et à grande échelle dans certaines régions du monde. Néanmoins, cette biomasse n'a pas été pleinement exploitée dans de nombreuses régions du monde pour la production de biocarburants, en particulier dans les pays en développement, et de ce fait, peu ou pas d'importance a été accordée aux résidus et déchets agricoles et forestiers à cet égard. Il reste donc encore beaucoup à faire pour remplacer en grande partie les combustibles fossiles par des biocarburants issus de la biomasse. La biomasse des déchets lignocellulosiques tels que les pelures de manioc, la pulpe de betterave sucrière et *Ulva lactuca* sont des matériaux appropriés pour la synthèse du bioéthanol. Leurs compositions sont présentées dans cette étude.

Les glucides solubles totaux et le rendement en glucides ont été évalués par hydrolyse acide diluée de la biomasse et de matériaux de référence (amidon et cellulose) dans différentes conditions. Pour explorer les interactions à deux facteurs, le plan d'expériences Plackett-Burman a été utilisé dans le criblage statistique des variables, puis transformé en un plan entièrement factoriel basé sur les variables significatives. Selon les résultats expérimentaux, l'hydrolyse acide diluée peut produire une quantité considérable de glucides solubles. La qualité de l'ajustement du modèle a été évaluée à l'aide du coefficient de détermination. Les rendements optimisés en glucides de la biomasse étaient de 0,76 g/g d'écorces de manioc, 0,35 g/g de pulpe de betterave sucrière et 0,36 g/g d'*Ulva lactuca*. Les valeurs réelles des points optimisés étaient proches des valeurs prédites. La production de bioéthanol à partir de conditions d'hydrolyse optimisées en utilisant *Saccharomyces cerevisiae* et *Scheffersomyces stipitis* DSM 3651 a conduit à un rendement maximal en éthanol de 0,286 g/g à partir de pelures de manioc et de 0,033 g/g à partir de pulpe de betterave sucrière, tandis que l'utilisation de *Neurospora intermedia* DSM 1265 a conduit à un rendement maximal en éthanol de 0,076 g/g d'*Ulva lactuca*. Par conséquent, les écorces de manioc, la pulpe de betterave sucrière et *Ulva lactuca* sont considérées comme des matières premières précieuses pour la production de bioéthanol de deuxième génération, les écorces de manioc ayant le potentiel le plus élevé.

Title : Experimental study on the potential of different feedstock on second generation bioethanol production: setting a methodology

Keywords : Waste biomass, dilute acid hydrolysis, carbohydrates, fermentation, bioethanol

Abstract : Biofuels are clean and renewable energy resources that are gaining increased attention as a potential replacement for non-renewable petroleum-based fuels. It is so-called because it is derived from biomass which could either be animal-based or belong to any of the three generations of plant biomass (agricultural crops, lignocellulosic materials, or algae). Different methods and techniques have been tested by scientists and researchers in this field, and the most optimal conditions adopted for the generation of biofuels from biomass. This has ultimately led to subsequent scale-up of procedures and the establishment of a pilot, demo, and large-scale plants/biorefineries in some regions of the world. Nevertheless, this biomass has not been fully exploited in many parts of the world for biofuel production, especially in developing countries, and as such little or no relevance has been attached to agricultural and forest residues and waste in this regard. Hence, there is still much work to be done in largely replacing fossil fuels with biofuels from biomass. Lignocellulosic waste biomass such as cassava peels, sugar beet pulp, and *Ulva lactuca* are suitable materials for bioethanol synthesis. Their compositions are presented in this study.

Total soluble carbohydrates and carbohydrate yield were evaluated using dilute acid hydrolysis of biomass and reference materials (starch and cellulose) under different conditions. To explore two-factor interactions, the Plackett-Burman design of experiments was used in the statistical screening of variables and then transformed to a full-factorial design based on the significant variables. According to the experimental findings, dilute acid hydrolysis can yield a considerable amount of soluble carbohydrates. The goodness of fit of the model was evaluated using the coefficient of determination. The optimized carbohydrate yields of the biomass were 0.76 g/g cassava peels, 0.35 g/g sugar beet pulp, and 0.36 g/g *Ulva lactuca*. The actual values of the optimized points were close to the predicted values. Bioethanol production from optimized hydrolysis conditions using *Saccharomyces cerevisiae* and *Scheffersomyces stipitis* DSM 3651, led to a maximum ethanol yield of 0.286 g/g from cassava peels and 0.033 g/g from sugar beet pulp while using *Neurospora intermedia* DSM 1265 led to a maximum ethanol yield of 0.076 g/g from *Ulva lactuca*. Hence, cassava peels, sugar beet pulp, and *Ulva lactuca* are considered valuable feedstock for the production of second-generation bioethanol with cassava peels having the highest potential.