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**THE PRODUCTION OF BIOETHANOL FROM DIARY CATTLE'S
WASTE/MANURE USING FERMENTATION BY SACCHAROMY-
CES CEREVISIAE**

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ABSTRACT

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<p>The worldwide need for biofuels, specifically bioethanol, is consistently increasing due to significant growth in population and industrial development need for energy. Starch and sugar base bioethanol faces competition as a source of food and fodder for animals and humans, popular crops such as sugarcane, corn, and cassava fall short of meeting the worldwide requirement for ethanol production. There is currently growing interest in using animal manure as the primary source of biomass for producing bioethanol. Cattle excrement is plentiful, easily replenished, and cost effective. To achieve successful removal of lignin from lignocellulosic materials, it is necessary to use a pretreatment method that boosts the production of sugars that can be easily fermented after hydrolysis. This enhancement in the overall process is crucial. Technological progress and the presence of cow manure as a viable substitute feedstock are responsible for the emergence of fermentation and saccharification. This fermentation technique helps decrease the overall expenses associated with the production of ethanol from glucose and various other types of sugars. This method of bioconversion includes pretreatment, hydrolysis, fermentation and dehydration of biomass feedstocks to generate ethanol. Ethanol production from cow manure and this thesis describes the technology readily available for this purpose.</p>		

<p>Key words Acid pretreatment, bioethanol, cow manure, saccharomyces cerevisiae, simultaneous saccharification and fermentation</p>

CONCEPT DEFINITIONS

E100

Renewable biofuel which is 100% ethanol

ETBE

Ethyl tertiary-butyl ether

AD

Anaerobic digestion

DM

Diary manure

SSF

Simultaneous saccharification and fermentations

DNA

Deoxyribonucleic acid

S. cerevisiae

Saccharomyces cerevisiae

ABSTRACT

CONCEPT DEFINITIONS

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1 INTRODUCTION

dung or manure is mostly digested grass produced by herbivorous dairy cattle animals, and depending on the cattle diet, grain, fruits, or vegetables. It is not just dropping as it consists of both faeces and urine, with tracks of hay, straw and grains. (Onwosi, Ozoegwu, Nwagu, Nwobodo, Eke, Igbokwe, Ugwuoji, Ugwuodo 2022, 2). It mainly comes from Forages, which are fibrous foods such as corn silage (chopped fermented whole corn plants), legumes (e.g., alfalfa, clover, vetch), and grasses consisting of lignocellulosic substances. Dairy cows look thin and lean with an angular shape, and might be thought of as under-fed cattle, but that seems to be their body shape. They have a large and pronounced udder with the ability to produce 30 - 37 liters of milk per day. Dairy cattle manure/faeces contain large fibres (cellulose, hemicellulose, and lignin), which can be transformed into other forms of sugars, such as mono sugars, to produce value-added products such as ethanol, organic acids, and enzymes. (Yue, Teater, MacLellan, Liu, Liao 2011, 1.) One of the most common agricultural wastes is cow manure, which is an ordinary type of lignocellulosic material. A 250 kg cow could produce more than 10 tons of manure annually which is a potential source of bioenergy. (Yan, Liu, Wang, Li, Li, Zhou, Qu, Li, Bao 2018, 2.)

Currently, bioethanol is the dominant biofuel globally, with the United States and Brazil being the primary producers and consumers of this renewable fuel. In 2017, the United States accounted for 50.6 % of the world's bioethanol production, chiefly utilized as a petrol additive at a rate of 10 %. In 2017, approximately 23.8 % of the world's bioethanol production came from Brazil. This bioethanol is mainly used in the fuel industry either as a pure energy (E100) or blended with petrol at a volume of 20-25 %. Additional countries such as Europe, China, and Canada have respective production volumes of 3.78 million m³, 1.1 million m³, along with 1.9 million m³. (Di Donato, Finore, Poli, Nicolaus, Lama 2019,1.)

Ethanol can be used as a fuel for transportation in four ways, these include using pure ethanol, a mixture of ethanol and water, a blend of ethanol and gasoline, and ethyl tert-butyl ether (ETBE). Using bioethanol in more recent engines has the potential to minimize the emission of carbon monoxide (CO) from vehicles, thereby enhancing air quality. (Akinyele & Bamgboye 2021,1.) In comparison to fossil fuels, it has the potential to significantly decrease greenhouse gas emissions by 30 to 85 % and help decrease the production of particulate matter in the air by approximately 50 % (Di Donato et al. 2019,1). Mixing bioethanol with gasoline will aid in reducing the heavy reliance on countries that

produce oil. Bioethanol is easily decomposable and significantly less harmful than non-renewable sources of energy. Ethanol has a higher latent heat of vaporization (855MJ / kg) as compared to petrol (293kJ / kg). Therefore, using ethanol instead of petrol led to a decreased octane rating (ranging from 80 to 100), which effectively prevented pre-ignition. The combustion process of ethanol produces a greater degree of combustion, which results in substantially lower hydrocarbon emissions than petrol. (Akinyele & Bamgboye 2021,1.)

In certain situations, it can be challenging to effectively handle and maintain manure and slurry because of the intensive production methods used within confined spaces. However, it is crucial to assess the viability of other alternatives in terms of energy efficiency, economic viability, and environmental sustainability, a financially viable approach to producing bioethanol from manure could offer a practical alternative for handling this material, benefiting both farmers' in-comes and decreasing greenhouse gas emissions. (Bona, Vecchiet, Silvestri, Fornasier, Guzzon 2011, 1.)The production of bioethanol from lignocellulosic biomass is complex and time-consuming because it includes several stages, including sourcing of raw material (biomass), transportation of the biomass, pretreatment, fermentation by saccharification, distillation, product (bioethanol), and by-product (methane and CO₂) management, along with other chemicals (sulfuric acid) necessary for the production process. (Broda, Yelle, Serwańska 2022, 2.)

This review aimed to highlight the possibility of utilizing cattle manure for the creation of bioethanol and its associated byproducts. To back this up, certain studies have investigated the use of cattle manure as a biomass and inoculum in anaerobic digestion (AD), considering various factors that influence the production of bioethanol with optimal quality and yield. The concluding and discussion section of this thesis work examines the difficulties presented, recommended potential remedies, and areas for future investigation in the field of bioethanol technology.

2 LITERATURE REVIEW

The availability, fermentability, carbohydrate content, and cost are the key factors to consider when producing biofuels. The biomass, which is widely available, is considered as the most plentiful biopolymer which is an eco-friendly and cost-effective resource and can be efficiently converted into chemicals and fuels in significant quantities. Moreover, biomass sources such as agricultural oil and sugar crops as well as their by-products, lignocellulosic materials including wood and wood waste, aquatic vegetation like algae and water weeds, municipal or industrial waste, and animal waste. (Kucharska, Rybarczyk, Hołowacz, Łukajtis, Glinka, Kamiński 2018, 20.) Bioethanol production can be classified into three primary feedstock types, first-generation bioethanol refers to sucrose and starch crops, such as cereals, corn, sugarcane, and similar plants. Second-generation bioethanol is obtained from lignocellulosic biomass and Third-generation bioethanol is produced by micro-algae. (Broda, Yelle & Serwańska 2022, 2.)

Ethanol is produced from sugar beets, sugarcane molasses, and sweet sorghum stalks. The main components of these substances are sugars that can be extracted, such as glucose, fructose, and sucrose. These sugars are then fermented to generate ethanol. Ethanol produce from starch-based feedstocks, such as corn, wheat, and cassava. Starch, which is a chain of glucose molecules, is converted into individual glucose molecules to undergo fermentation and produce ethanol. Lignocellulosic feedstocks consist of biodegradable components found in various products, as well as residues and leftovers from energy crops, agriculture, and wood. They are then transformed into ethanol through the processing of carbohydrate polymers. (Akinyele & Bamgboye 2021, 2–3.)

2.1 Lignocellulose biomass

Lignocellulose is considered the most abundant biomass, making it a potential replacement for fossil resources for chemical synthesis and fuel production (Shen & Sun 2021, 1). The competition within the food chain is intense because of the utilization of sugary or starchy resources such as corn and sugarcane. Furthermore, the utilization of sucrose and starch by itself falls short in satisfying the increasing demand for bioethanol. Moreover, cultivating these substances for energy purposes not only harms biodiversity but also potentially plays a role in deforestation, which would necessitate the expansion of agricultural land. As a result, there has been a shift in focus towards second generation technologies in

research because of worries regarding the potential harm to the food chain and biodiversity brought about by first generation bioethanol. In contrast, these technologies make use of crops that are not intended for food production or utilize different areas of crops, as well as the waste produced by wood-based or food-based industries. These sources are incredibly abundant and renewable in the natural world (Di Donato et al. 2019, 1). By utilizing Lignocellulose as a primary resource, pollution effectively decreases, as it eliminates the presence of potentially harmful wastes. In addition, the fact that second-generation bioethanol does not pollute materials used by humans and animals contributes to the promotion of sustainable development. (Mejía-Barajas, Alvarez-Navarrete, Saavedra-Molina, Campos-García, Valenzuela-Vázquez, Amaya-Delgado, Arellano-Plaza 2018, 3–4.)

2.2 Structure of lignocellulose

The three main components of lignocellulosic biomass are hemicellulose, cellulose, and lignin as shown in figure 1. The complex structure of these components is intertwined, and the presence of lignin restricts the ability to break down biomass for energy production by impeding the action of enzymes. The β -1,4 glycosidic bonds tightly connect the glucose disaccharides found in cellobiose. The presence of numerous hydroxyl groups in the cellulosic chains facilitates the creation of hydrogen bonds within these chains as well as with neighbouring chains. These bonds, along with van der Waals forces, interconnect the chains and contribute to the development of exceptionally strong microfibrils. (Dahunsi & Enyinnaya 2019, 3.)

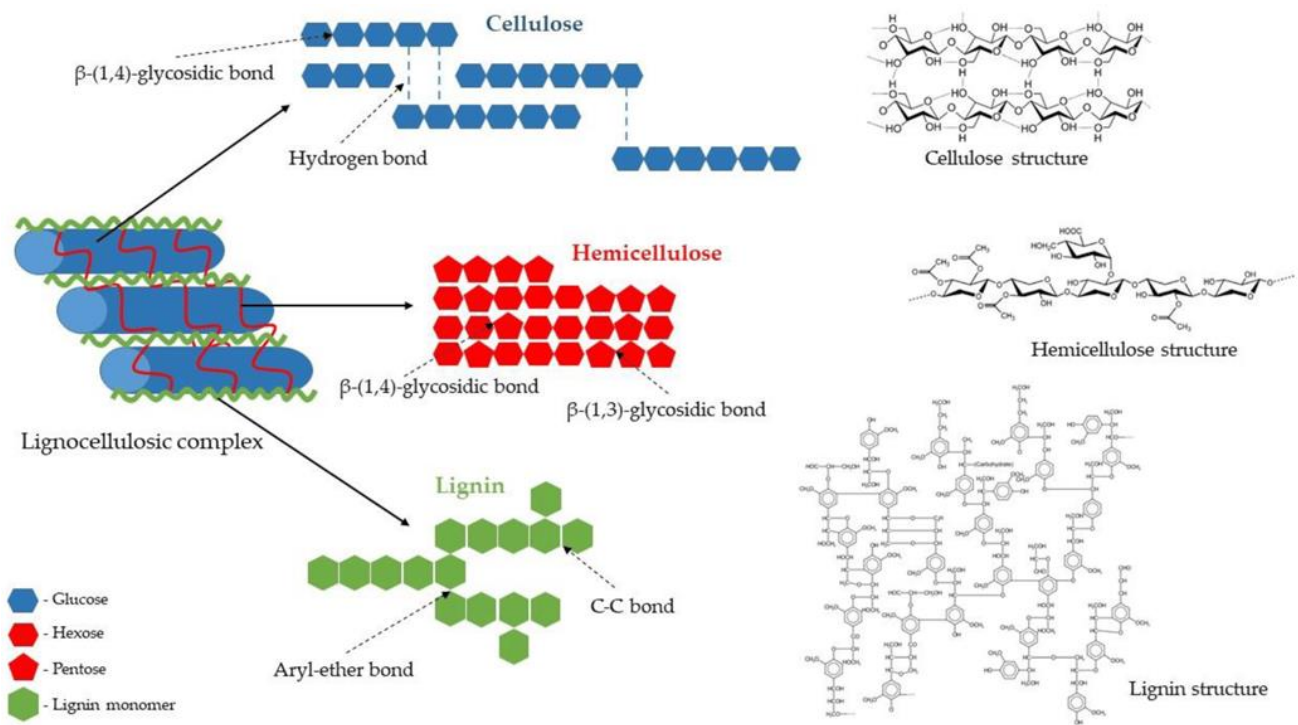


FIGURE 1. Lignocellulosic structure (Broda, Yelle & Serwańska 2022, 6).

Another quality that grants resistance to cellulose will be the various possible orientations of molecules along the fibres, leading to varying levels of crystallinity. These levels can be categorized into 2 regions: amorphous (with low crystallinity) and crystalline (with high crystallinity). The resistance of cellulose is linked to the presence of high levels of crystallinity. (Camargo et al. 2023, 4.) Hemicellulose is a diverse polymer composed of a wide variety of sugars, such as L - arabinose, D - galactose, D - glucose, D - mannose, and D - xylose. It exists in both branched and linear forms. The central structure of the hemicellulose chains can consist of either a single type of sugar (a combination or homopolymer) of various sugars (heteropolymer). Hemicelluloses have an amorphous molecular structure characterized by shorter chains that branch out from the primary chain molecules. This feature distinguishes them from cellulose, in addition to the variation in sugar units. The chemical characteristic of hemicellulose makes it easier to hydrolyse compared to cellulose. (Tran, Le, Mai, Nguyen 2019, 4.) The cell walls contained a rigid and strong structure called lignin, which was made up of a complex network of substituted aromatic compounds. This, along with celluloses and hemicelluloses, provided the cell walls with strength and rigidity. The aromatic framework consisted of three standard components, namely p-hydroxyphenyl, guaiacyl, and syringyl units. However, the number of methoxyl groups connected to the aromatic ring could vary, ranging from none to just one or two. In lignin,

mononuclear aromatic polymers are typically formed by linking a variety of structural units through' - O-4 aryl or carbon-carbon bonds. (Zhuang, Zhan, Song, He, Huang, Yin, Wu 2019, 11–12.)

The success of converting biomass into ethanol by way of bioconversion primarily depends on the amount of fermentable sugars obtained during the enzymatic hydrolysis of hemicellulose and cellulose which are structural polysaccharides. The primary factor that restricts the extent of cellulose breakdown is the physicochemical barrier and crystallinity structure created by the unique composition of lignocellulose that hinders the ability of hydrolytic enzymes to reach the polysaccharide chains. The high prevention of cellulolytic enzymes is attributed to the fact that biomass, being hydrophobic, acts as a physical obstacle that hinders water from reaching these enzymes. In addition to serving as a physical barrier against hydrolytic enzymes, lignin has the capability to bind with cellulases, resulting in a decrease in their ability to catalyse reactions. The enzymatic hydrolysis of lignocellulose is hindered by these phenomena, resulting in limited production of cellulosic ethanol. (Kłosowski & Mikulski 2023, 1.)

2.3 Composition of dairy cow manure

Dietary carbohydrates (Grass) contain 60 -80 % of the dry matter in dairy cow diets. Carbohydrates can be categorized into two groups: non-fibrous carbohydrates and fibrous carbohydrates. Fiber carbohydrates is good for the rumen, encourage the process of chewing cud, and increase the production of saliva, but contain lower energy than non-fibre carbohydrates, which include organic acids, sugars, starch, and basic detergent soluble fibre, containing higher energy but may decrease the pH in the rumen, potentially affecting the digestion of fibre and overall rumen well-being. Dairy cows possess a certain level of efficiency when it comes to breaking down and transforming nutrients into energy, even though they tend to be relatively efficient but still excrete 3.2 % of their body weight as undigested content in their waste. Grain and grain silage are among the excreted nutritive materials. (Harper 2008. 4.)

The primary element found in dairy manure (DM) is fibre, comprising cellulose, hemicellulose, and lignin. however, various factors, with animal breed and diet being the most significant, influence the chemical makeup of DM. figure 2 illustrates the higher quantities of cellulose, hemicelluloses, in addition to lignin that are in DM in comparison to other commonly used biomass sources. (Zhu, Wu, Pisutpaisal, Wang, Ma, Dai, Qin, Tan, Maeda, Xu 2021, 3.)

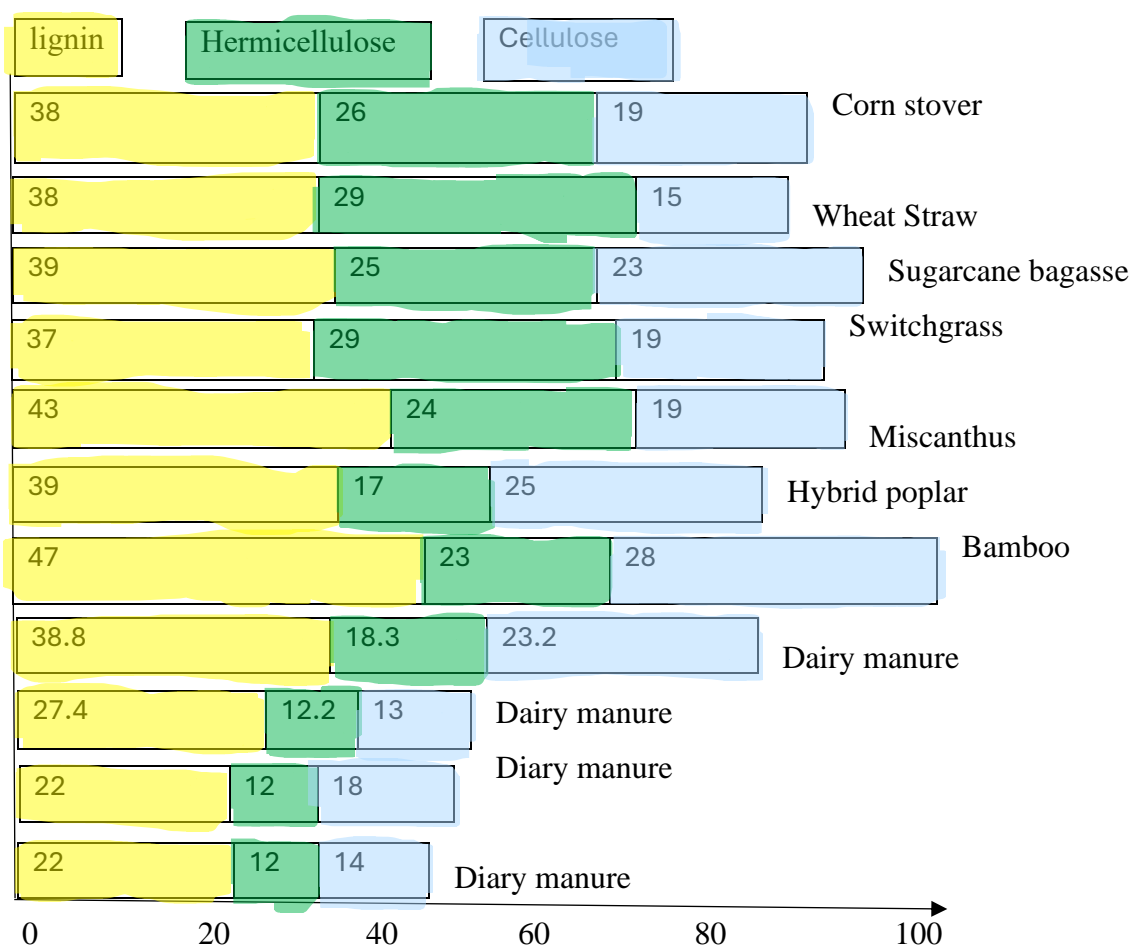


FIGURE 2. Dairy manure composition percentage by mass (Adapted from Zhu et al. 2021, 3).

According to Zhu et al. (2021, 3), DM has significantly higher levels of nitrogen (around 3.03 %), calcium (around 2.41 %), potassium (around 1.24 %), iron (around 0.38 %), sodium (around 1.33 %), zinc (around 2.41 %), manganese (around 0.66 %), copper (around 0.32 %), and cobalt when compared with various other biomass resources, such as bamboo, polar, miscanthus, switchgrass, straw. The higher amount of nitrogen found in the liquid portion of manure compared to the solid portion which can be isolated from fibres by using a sieve or by a straightforward process of pouring off. To reduce the manure's total solids (TS) concentration it is necessary to mix it with water, which leads to an expansion in the size of the reactor and an escalation in the pollution of the discharged liquid.

When hydrolysis occurs, especially through acid pretreatment, amino acids found in hydrolysed protein can interact with mono-sugars, resulting in a decrease in the quantity of sugars produced. This reaction is particularly noticeable with ammonia and peptides being form. Nevertheless, the total amount

of dry matter they possess is below 1 %. These essential nutrients are crucial for the growth and development of plants. Manure contains a diverse range of microorganisms including bacteria, archaea, fungi, and protozoa. Microorganisms can enhance nutrient availability and soil health by enhancing soil microorganisms. Furthermore, manure can potentially include antibiotics, hormones, and various medications used in the treatment of animals responsible for its production. The total nutrient content can also be influenced by these factors. (Saady, Rezaeitavabe, Ruiz Espinoza 2021, 3–5.)

3 PROCESS DESCRIPTION

Cow manure is processed to bioethanol through the following four major processes: pretreatment (consisting of solid/liquid separation, acid pretreatment), hydrolysis, fermentation, and distillation (Akinyele and Bamgboye 2021, 4). Bioethanol can only be generated from cellulose and hemicelluloses into valuable products, such as ethanol, carbon dioxide, methane, and fertilizer. The procedure involves first collecting waste manure that flows from the dairy cattle and storing it in a surge tank as a combination of solid manure, urine, and water in the form of a slurry mixture. Mixing animal waste in a sludge tank with acid (the slurry undergoes a preliminary treatment; this is done to decompose the intricate composition of manure and enhance the accessibility of cellulose). Sugar and microorganisms are added to the mixture, which moves to the anaerobic digester that is subjected to heat, resulting in the extraction of methane from the digester. Methane undergoes dehydration and compression for storage, or it can be utilized as a source of fuel to produce electrical energy. The remaining mixture of carbon dioxide and ethanol in the water-based solution is separated by distillation, allowing the recycle of water. The remaining solid organic matter is used for fertilizer production. (Harper, 2008, 3.) Fiber found in manure from dairy cows undergoes enzymatic hydrolysis within the digestive system. The initial treatment assisted in further breaking down this substance. This promotes hydrolysis at a later stage and prevents furfural formation. These preliminary treatments enhance the accessibility of lignocellulose, enabling it to undergo subsequent processes, such as bioethanol fermentation. (Akinyele and Bamgboye 2021, 5.)

3.1 Pretreatment and Hydrolysis

The main goal of pre-treating lignocellulosic biomass is to separate its components: cellulose, hemicellulose, and lignin. The aim is to eliminate lignin without giving up hemicellulose while also lowering the crystallinity of cellulose and improving its porosity. (Muktham, K. Bhargava, Bankupalli, S. Ball 2016, 12). According to Broda et al. (2022, 8) the pretreatment technique should be simple to perform, economically viable, and eco-friendly, generating minimal amounts of substances that impede progress and facilitating the full utilization of lignocellulosic biomass. This leads to a highly efficient production of bioethanol and effective handling of leftover lignin waste.

The pretreatment techniques can be categorized into four broad categories: biological, physio-chemical, chemical, and physical. Though not all biomasses have a standardized pretreatment method. Chemical pretreatment is mainly applied in the manufacturing process to convert the DM to bioethanol, since the complex organic polymer lignin can be dissolved in lignocellulose using acidic and basic pretreatments. Acidic solutions, such as HCl and H₂SO₄, are utilized as pretreatments, under different residence times (lasting for an hour), temperatures between 100° C and 290° C, and a pressure of 1.5 bar. Excessive amounts can be harmful and lead to the breakdown of cellulose and hemicellulose. Alkaline pretreatments, such as sodium hydroxide, reduce the carbohydrate loss caused by acidic pretreatment and assist in eliminating acetyl groups (Tran et al. 2019, 7.) Concentrated pretreatment requires a higher acid concentration of 10-30 %, whereas dilute acid pretreatment requires a lower acid concentration of 2-5% (Verardi, Bari, Ricca, Calabrò. 2012, 7.)

The main factors that have an impact on the method are the concentration of acid, the number of solids being loaded, the temperature, and the amount of time the materials stay in the system. Increasing the temperature enhances the efficiency of the process when diluted acids are employed. The primary impact of acid pretreatment is on polysaccharides with limited effects on lignin. Breaking down hemicelluloses and separating polysaccharide-lignin connections enhances the enzymatic accessibility of cellulose. (Broda, Yelle & Serwańska 2022, 9.) The main disadvantages of acid pretreatment are the expensive process of acid retrieval and the generation of substances that inhibit certain processes. In acid pretreatment, the inhibitory compounds produced in large quantities include phenolic, 5-hydroxymethylfurfural, aldehydes, and furfurals acids. (Aftab, Iqbal, Riaz, Karadag, Tabatabaei., 2019 3.) The above substances prevent the breakdown by microorganisms, so they need to be removed before hydrolysis. Inhibitors can be removed by either adsorbing them onto activated carbon or by reacting them to precipitate with calcium oxide (buffer solution). To undergo acid pretreatment, it is necessary to use reactors that can withstand corrosive, dangerous, and poisonous acids. Acid pretreatment is costly, resulting in high costs. A variety of types of reactors have been created, such as flow through, percolation, shrinking bed, counter current reactor, batch, and plug flow reactors. (Kucharska et al. 2018, 10.)

The breakdown of biomass through hydrolysis can be inhibited by presence of an acid from acid pretreatment therefore it is essential to neutralize the acid prior to the fermentation process of sugars. To improve the economic viability of acid pretreatment, the pH of the solution must be adjusted by neutralizing the pretreated lignocellulose prior to hydrolysis. The hydrolysis process enhances the enzymes performance by creating ideal circumstances, leading to higher effectiveness and productivity in

decomposing the lignocellulosic substance into sugars. (Aftab et al. 2019, 3.) Over-liming (calcium oxide) process results in the production of gypsum ($\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$) as a secondary product as shown in the equation (1) below, it led to a reduction of sugar content due to lime adsorption. Although the over-liming technique has its drawbacks due to calcium sulphate hydrates are highly insoluble which forms scales practically anywhere for calcium and sulphate are present in aqueous solutions. The end consequence is corrosion of reactor walls, impellers, and pumps, as well as clogged pipelines. What makes them so challenging is that they form even at low pH and can only be efficiently eliminated mechanically. (Adams & Papangelakis 2007, 1.)



Alkaline pretreatment lignin degradation and removal in dilute solutions of NaOH, KOH, Ca(OH)_2 or ammonia is typically accomplished to increase the availability of cellulose for enzymatic hydrolysis. The treatment causes the fibrous cellulose to swell, leading to increase the porosity of biomass. This is achieved by disrupting the connections between hemicellulose and other polymers. The procedure can be conducted for an extended duration under high temperatures or even for a brief period under increased temperatures. The technique offers multiple benefits, such as the ability to remove lignin selectively without losing carbohydrates, improving the porosity of the feedstock for enzymatic hydrolysis, and disinfecting the biomass. The main disadvantage is the extended response times, which can take up to 24 hours. (Broda, Yelle & Serwańska 2022, 9.)

enzymatic hydrolysis depends on the action of enzymes generated by specific fungi, such as *Trichoderma reesei*, and may be improved by the presence of metal elements, such as Ca (II) and Mg (II). Hydrolysis can be conducted either concurrently with fermentation or separately, the former approach is thought to be more effective when it comes to efficiency. (Achinas & Euverink 2016, 6.) The purpose of hydrolysis is to break down polysaccharides to monosaccharides found in the pretreated lignocellulosic biomass. Enzymes are primarily used to facilitate the breakdown (figure 3) of cellulose and hemicellulose (specifically xylan) through hydrolysis. Key factors that greatly impact the hydrolysis of biomass are solid loading, sugar concentration, enzyme quantity, shaking intensity, hydrolysis duration, inhibitor concentration, and the influence of different additives. (Broda et al. 2022, 13.)

Hydrolysis process is initiated and boosted using starter sugars during bioethanol production. This procedure aids in the transformation of sugars present in the feedstock into ethanol, enhancing the overall effectiveness of bioethanol production. (Mohd Azhar, Abdulla, Jambo, Marbawi, Gansau, Mohd Faik,

Rodrigues. 2017.) Hemicelluloses showed greater susceptibility to temperature they quickly transformed into polysaccharides with a reduced molecular weight. These polysaccharides included gluconoxylan, galactoglucan, and xyloglucans. Subsequently, the continuous breakdown of polysaccharides resulted in the production of various types of monosaccharide units. These included hexoses like galactose, mannose, and glucose, pentoses like xylose and arabinose, and different saccharides such as rhamnose and fructose. (Kucharska et al. 2018, 10.)

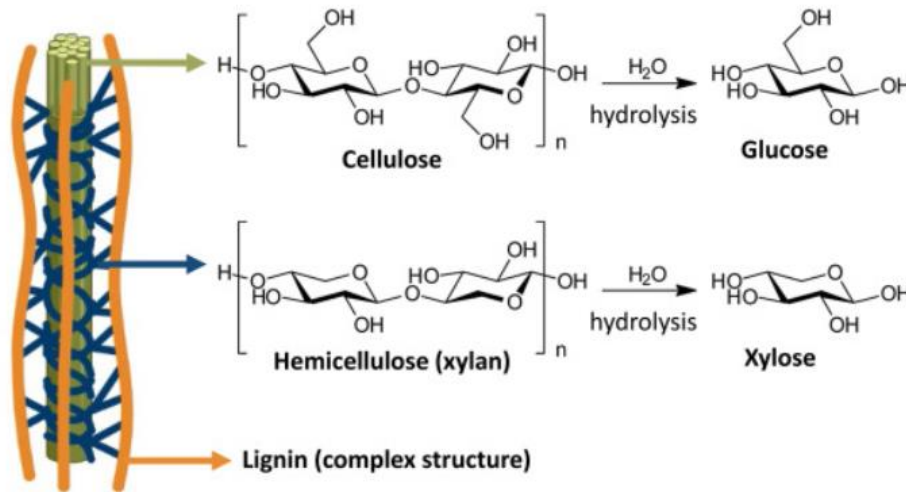


FIGURE 3. A simplified diagram monomers produced through the breakdown of hemicellulose and cellulose (Camargo et al. 2023, 5.)

3.2 Fermentation

Fermentation occurs when bacteria or yeast transform sugars such as glucose into ethanol, this process occurs in the absence of oxygen in an anaerobic digester. Different Fermentation techniques, comprising Separate Hydrolysis and fermentation (SHF), simultaneous saccharification and fermentations (SSF), simultaneous saccharification and co-fermentation (SSCF), and consolidated bioprocessing (CBP). (Akinyele & Bamgboye, 2021, 7.) The process of transforming naturally occurring carbohydrates made of glucose into bioethanol can be described by the following chemical equation (2).

Where carbohydrates from fibres in DM react with water in acid pretreatment to gives glucose which is being fermented by the microorganism's *saccharomyces cerevisiae* to produce ethanol and carbon dioxide.



(Meenakshisundaram, Fayeulle, Léonard, Ceballos, Liu, Pauss 2022, 3).

In SHF, lignocellulosic feedstock that has been treated beforehand is broken down into glucose, which is then converted into ethanol through fermentation. These processes occur in various bioreactors. The action occurs at the most favourable temperatures for the breakdown of molecules via hydrolysis and the conversion of substances through fermentation. In contrast, SSF combines the processes of fermentation and hydrolysis in a single bioreactor. The microorganism promptly utilizes the sugars produced through enzymatic hydrolysis, which helps decrease the inhibition of cellulase. The ideal temperature for SSF involves finding a balance between the optimal temperatures for fermentation and hydrolysis. (Bušić et al. 2018, 11.) The SSF process minimizes energy expenditure and subsequently decreases operating expenses while also improving efficiency by lowering the duration of each step. The cellulases in these phases exhibit higher levels of enzymatic activity, which helps avoid product inhibition. This occurs because yeasts metabolize sugars concurrently during the hydrolysis process. The SSF process generally yields higher ethanol yields than the SHF process, ranging from 13 to 30%. SSF exhibits significant efficiency at elevated temperatures and can rapidly generate substantial quantities of ethanol during fermentation. (Mejía-Barajas et al. 2018, 7.)

Various types of microorganisms can be utilized to convert glucose into ethanol through the process of fermentation, with *Saccharomyces cerevisiae* being the most utilized organism. *S. cerevisiae* can break down mono- and disaccharides such as sucrose, maltose, fructose, and glucose, but unable to convert pentoses such as xylose and arabinose. Cellulose and hemicellulose cannot be break down directly by *S. cerevisiae* to ethanol. To deal with this drawback, scientists employ either recombinant DNA technology or specific microorganisms that can ferment pentose sugars, such as *Pichia stipitis*, *Pachysolen tannophilus*, and *Candida shehatae*. The ethanol production rates of these microorganisms that ferment pentose tend to be at least 5 times lower compared to the ethanol production rates of *S. cerevisiae* when it ferments glucose. Additionally, these microorganisms exhibit a decrease in oxygen and ethanol tolerance by 2-4 times lower. (Bušić et al. 2018, 11.)

Saccharomyces cerevisiae is a cell fungus containing nuclear genomic DNA of 12068 kilobases (kb) distributed on 16 chromosomes, with the genome fully sequenced with approximately 6000 genes

whereby 5570 seems to be protein-encoding genes. It is a model organism that is accommodating for all standard studies. Unlike other model organisms such as *Escherichia coli* or *Caenorhabditis elegans*, *S. cerevisiae* is likewise the most valuable species for different manufacturing uses, this is partly due to its ability to make, accumulate, consume, this unique characteristics is derived from the Crabtree effect, whereby, *S. cerevisiae* does not react with respiratory mechanism to absorb saccharides to increase biomass growth in anaerobic environment, but instead uses pyruvate to create ethanol along with the other two carbon elements. This has the unfortunate consequence that *S. cerevisiae* makes and accumulates ethanol-toxic or inactive for most other microbial species that would compete with it for sugar compounds, thus avoiding competition with other microorganisms. (Parapouli, Vasileiadis, Afendra, Hatziloukas 2020, 1.)

Table 1 demonstrates *S. cerevisiae* is an appropriate yeast for converting sugar solution into bioethanol through fermentation. *Saccharomyces cerevisiae* is recognized as probably the most widely used yeast in the production of bioethanol, primarily because it can withstand high levels of ethanol and substances that can hinder the production process.

TABLE 1. Some commonly used microorganisms in the production of bioethanol (Adapted from Tran et al. 2019, 10)

Characteristics	Microorganism			
	E.coli	Z.mobilis	Saccharomyces cerevisiae	Pichia stipitis
D-Glucose fermentation	+	+	+	+
Other hexose utilization (D-galactose and D-mannose)	+	-	+	+
Pentose utilization (D-xylose and L-arabinose)	+	-	-	+
Direct hemicellulose utilization	-	-	-	w
Anaerobic fermentation	-	+	+	w
Mixed-product formation	+	w	w	w
High ethanol productivity (from glucose)	-	+	+	w
Ethanol tolerance	W	w	+	w
Tolerance to lignocellulose-derived inhibitors	W	w	+	w
Acidic pH range	-	-	+	w
+, positive; -, negative; w, weak				

Under anaerobic conditions, yeast transforms glucose into ethanol through the Embden-Meyerhof pathway, as shown in figure 4. In this process *S. cerevisiae*, glucose is broken down through the glycolytic pathway into pyruvate through a chain of reduction and oxidation of arabinose reductase (AR) and L-arabitol dehydrogenase (LAD) using the cofactors NAD(P)⁺/NAD(P)H. This breakdown is facilitated by hexokinases, which convert glucose into glucose-6-phosphate through phosphorylation. Phosphoglucose isomerase enzyme convert glucose-6-phosphate to fructose=6-phosphate, which is converted to fructose-1,6-biphosphate by phosphofructokinase(PFK). This compound can then be converted back and forth into glyceraldehydes-3-phosphate and dihydroxyacetone phosphate by yeast fructose bisphosphate isomerase (TPI), it can also be converted into pyruvate.

Initially, the enzyme 3-phosphate dehydrogenase (GDH) changes glyceraldehydes-3-phosphate into 1,3 diphosphoglycerate. Then, phosphoglycerate kinase (PGK) converts 1,3-diphosphoglycerate into 3-phosphoglycerate. Phosphoglycerate mutase (PGM) facilitates the transfer of the phosphate group from carbon 3 to carbon 2, enabling the dehydration process carried out by enolase (Eno). This leads to the formation of phosphoenolpyruvate. The pyruvate kinase (PK) transforms a molecule with a high amount of energy into pyruvate, which can take on various outcomes based on the environmental conditions of the microorganism. Under conditions with no oxygen, pyruvate is transformed through a decarboxylation process, resulting in the production of acetaldehyde and carbon dioxide. This transformation is facilitated by the enzyme pyruvate decarboxylase (PDC). Subsequently, acetaldehyde is further converted into ethanol through the activity of alcohol dehydrogenase (ADH). (Rastogi & Shrivastava 2017, 3–6.)

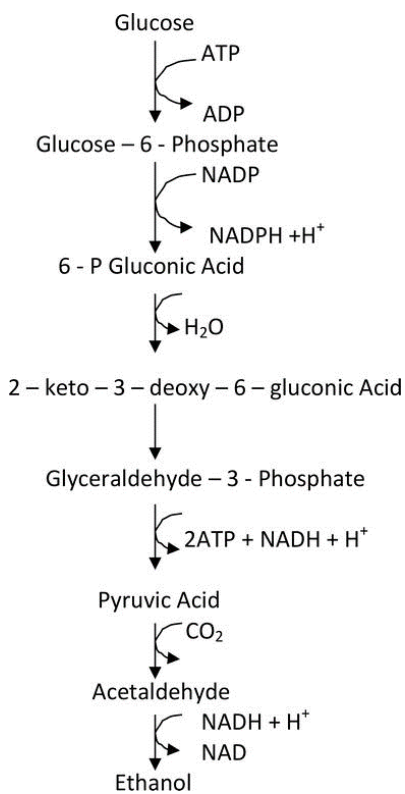


FIGURE 4. The EMP route for converting glucose into ethanol (Kanagasabai, Maruthai, Thangavelu 2019, 8)

3.3 Anaerobic digester

The key factors that determine the functioning of anaerobic digestion are the pH, temperature, organic loading rate, hydraulic retention time, and stress. The interaction of these parameters, either individually or in combination, can lead to the maximization of biogas or digestate yield and quality in anaerobic digestion. Selecting an anaerobic digestion facility is frequently the favoured approach to maximize its efficiency, depending upon absolute selection of these vital factors. Feedstock utilization can be classified into four main areas: substrate, inoculum, co-digestion, and pretreatment. The process conditions include pH, pressure, temperature, and reactor design. Reactor control involves managing hydraulic retention time (HRT) and organic loading rate (OLR). Inhibition can occur because of the presence of ammonia and volatile fatty acids (VFAs). (Sarker, Lamb, Hjelme, Lien 2019, 4.)

Microorganisms from two primary domains collaborate to carry out the process through coordinated actions. Microorganisms such as bacteria and archaea the groups of microorganisms are divided into 4 different levels, emulating the four phases of the process, and carrying out distinct duties. Therefore, organisms are classified according to the specific point in the anaerobic process when they carry out their functions. These organisms consist of hydrolytic bacteria, which are responsible for hydrolysis; acidogenic bacteria, which carry out acidogenesis; acetogenic bacteria, which perform acetogenesis; and finally, methanogens, which engage in methanogenesis (Methane production). The complexity of anaerobic digestion arises from the diverse range of microorganisms involved, their different needs for growth, and their specific capabilities to carry out their function. Maintaining a balance in different factors is essential for achieving an optimal design and successful implementation, as microorganisms are highly responsive to environmental and nutritional conditions.

The impact of temperature on biochemical processes and the development of microorganisms, as well as the relationships between different groups of microbes, is of great importance. The process of anaerobic digestion can be categorized into three different types based on the temperature: psychrophilic, mesophilic, as well as thermophilic microbial degradation. These types occur at temperatures below 30° C, between 30-40° C, and 50-60° C, respectively. All anaerobic digestion systems share the same fundamentals, irrespective of the operating temperature, feeding mode (continuous or batch) and feedstock (animal byproducts, food wastes and wastewater sludge). Therefore, the process is impacted by several factors such as the ratio of carbon to nitrogen (C/N), temperature, the rate at which organic material is added (OLR), the length of time the material is retained in the system (HRT), and the acidity or alkalinity of the environment (pH). (Manyi-Loh and Lues 2023, 7–8.) The capacity and speed at

which methane is produced from cattle manure are restricted when it is solely utilized, as it has a considerable proportion of materials that are resistant to decomposition and cannot be easily broken down, thereby impeding the overall process. (Bi, Hong, Yang, Yu, Fang, Bai, Liu, Gao, Yan, Wang, 2020, 1.) Methane produced in the anaerobic digesters is vacuum out into a storage tank by aid of a vacuum pump. The collected methane gas is first pass through a dryer/compressor for processing before transferred to the storage tank to be use or sale. The methane can be used to produce electrical energy by utilizing a generator as a source of fuel. (Harper 2008, 5.)

3.4 Distillation

Once sugars undergo fermentation, the resulting solution contains ethanol in the range of 2-12 %. This mixture undergoes distillation to create a mixture known as an azeotropic blend containing 96 % ethanol and 4 % water. Nevertheless, this combination is not appropriate for mixing with gasoline because it tends to separate when exposed to water, particularly under colder conditions. Additional dehydration was necessary to reduce the moisture level to below 1 %. One of the ways to accomplish this is by utilizing techniques like extractive distillation or molecular sieve drying. (Beschkov 2017, 13.) The fermentation mixture contains various impurities and a small quantity of ethanol, requiring the development of effective separation methods to eliminate them. The cutting-edge technique consists of a sequence of distillation columns, followed by a molecular sieve. (Amornraksa, Subsaipin, Simasatitkul, Assabumrungrat 2020, 5.)

Purified ethanol from the binary azeotrope ethanol-water requires two energy-intensive separation steps. The initial stage involved a typical distillation process that increased the concentration of ethanol. The process of cyclic distillation used for ethanol purification is a cost-effective and environmentally friendly option that requires minimal initial capital. In the second stage, ethanol underwent dehydration to acquire an anhydrous form (ethanol with concentrations exceeding the azeotropic composition). (Bušić et al. 2018, 12.) The filter material in the distillation column separates water from carbon dioxide and ethanol. Subsequently, the liquid form of ethanol and the gaseous state of carbon dioxide are directed into different containers for storage. Eventually, after extracting all liquid products from the digester, a concentrated organic solid substance is left behind. This material, which is abundant in nitrogen, is an ideal resource for fertilizer production. (Harper, 2008, 5.)

4 DISCUSSION

Production process from cow manure as shown in figure 5. Water is added with cow sludge feed (manure and urine). Insoluble solids are taken out of the feed by passing it through a solid/liquid separator. The mixture is transferred to the pretreatment stage (sludge tank) where it encounters a solution of dilute sulfuric acid. (Harper, 2008, 3.) The fibre present in manure in the sludge tank undergoes chemical pretreatment, which breaks it down into its individual components. This material is further broken down through the hydrolysis stage.

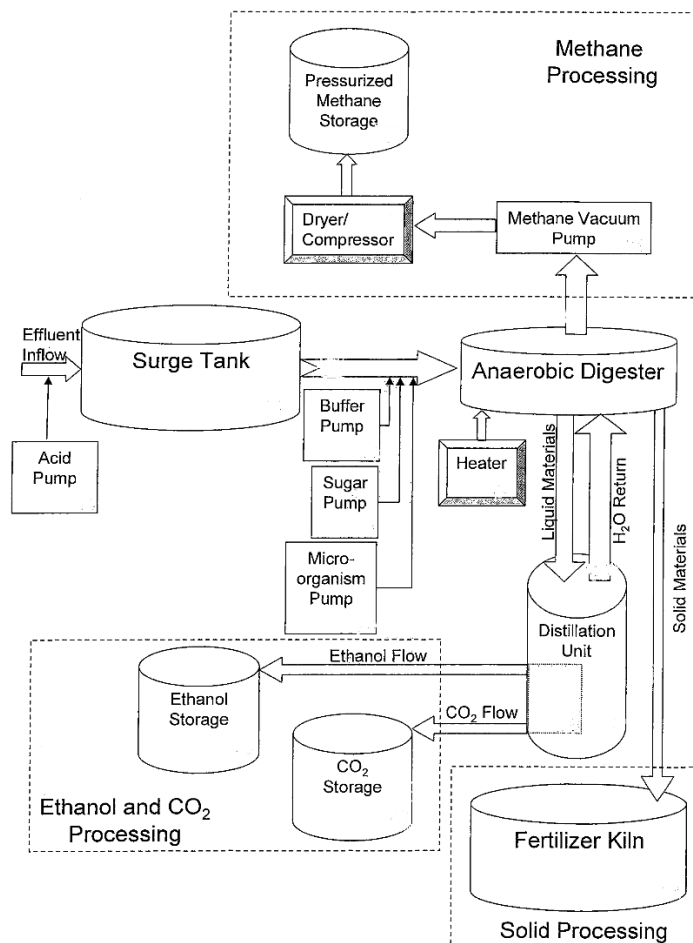


FIGURE 5. Bioethanol production process (adapted from Harper 2008, 2).

The feed is directed towards a detoxification process (buffer pump), stater sugar of 0.01% is being introduce in the hydrolysis stage and a microorganism (*saccharomyces cerevisiae*) via a microorganism pump. The buffer neutralizes any substances that may hinder fermentation. The mixture is transferred

and heated in an anaerobic digester. The mixture's temperature is preferably raised to between 28°C and 58°C. *Saccharomyces cerevisiae* microorganisms transform the mixture into a solution of ethanol, CO₂, water as liquid and methane as gas. Beer or broth is the name given to the liquid product that contains ethanol from fermentation. Methane is extracted as a gas from the anaerobic digester as by-product. Applying a vacuum to the anaerobic digester's methane outflow may help in methane extraction. The methane can be dried and compressed in a suitable storage tank or utilised as a fuel to generate energy via a generator. (Harper, 2008, 3.)

Aside from a small amount of ethanol, the fermentation mixture also has several impurities. As a result, it is crucial to use suitable separation techniques to separate these impurities from the mixture. Initially, the fermentation mixture is introduced into a Beer column. At the base, solid elements are separated and collected as sediment, while at the upper part, gaseous elements like dissolved CO₂ are extracted. During the 2nd distillation process, referred to as rectification column, the water and ethanol are separated, and the ethanol is concentrated to a point close to azeotrope. Hydrous ethanol is the product of these distillation processes, with a purity of approximately 95 % ethanol. Nevertheless, this level of purity falls short of the standard minimum requirement of 99 %, which is usually deemed satisfactory. The hydrous ethanol must be dehydrated to produce anhydrous ethanol, thereby requiring further purification. Molecular sieve is the traditional method for accomplishing this. (Beschkov 2017, 13.)

Feedlot cattle manure is commonly stored in dry pads or lagoons and then applied to soils through direct application or composting following traditional farm management methods. Nevertheless, the environmental health, and economic attractiveness of these practices are diminishing because of issues related to microbial and nutrient runoff, surface, and subsurface water contamination (eutrophication), soils with excessive nitrogen and phosphorous levels, unpleasant odours, and the production of greenhouse gases such as nitrous oxide and methane. The combination of livestock manure and agricultural residues in anaerobic digestion are becoming a favourable choice for cattle waste disposal. (Vancov et al. 2015, 1.)

The economic advantage of using DM diminishes as the distance for manure transportation increases, as it has lower fertility than commercial fertilizers. One suggestion for increasing the amount of animal manure is to implement deposit refund system. Under this system, farmers who provide manure to a biogas plant pay a deposit in euros per ton of manure supplied. This deposit would be fully refunded if they retrieved the same amount of digestate, which contains nitrogen and phosphorus, as these

nutrients have harmful effects on the environment. The refund would increase proportionally if the amount taken back exceeds the initial amount of nutrients, whereas the reverse will occur if the amount taken back is slightly less than the original amount. The deposit that is kept will be utilized to locate a farmer who can handle surplus manure at a biogas facility. Biogas facilities have the potential to act as local centres for nutrients, connecting the divide between availability and need for animal waste. They can also effectively distribute excess nutrients from farms in areas with a high concentration of livestock to farms with the capacity to utilize them. (Schröder & Latacz-Lohmann 2023, 2.)

Although current pretreatments improve the ability of lignocellulosic biomass to undergo hydrolysis and fermentation, none of them have been able to achieve the same level of efficiency as cattle. One possible explanation is that a few complex factors of the cattle process are often overlooked, like the animals regurgitating and chewing partially digested material for a long time. This practice offers various benefits, which includes a larger surface area for enzymes or microbes to break down the feed particles through tearing and separating, as well as the release of soluble nutrients from fermented cells. Hence, comprehending the cattle process can assist in converting lignocellulosic particle size reduction and enhancing surface area characteristics to produce essential components employed in the initial treatment of anaerobic digestion (AD). The innovative grinding head, inspired by the chewing principles of cattle, improves the reduction of particle size, and lowers the operating temperature in comparison to conventional grinders. (Bhujbal, Ghosh, Vijay, Singh 2022, 2.)

According to Zhuang et al. (2019, 12), there was a discovery of a reciprocal transformation between two primary monosaccharides (fructose and glucose), which is referred to as isomerization, these reactions resulted in the expansion of hydrothermal products. Aldehyde compounds such as pyruvaldehyde and acetaldehyde were formed by retro-aldol condensation by breaking C-C bonds. Under the influence of acid catalysis, the dehydration process led to the creation of organic acids and insoluble intermediate compounds, including furans and furfurals. Under hydrothermal conditions, various highly reactive intermediates can undergo intermolecular dehydration and ring condensation to produce cyclopentanone, furanone derivatives, as well as monocyclic aromatics. It is worth noting that hemicellulose, which is a type of carbohydrate, can transform into aromatic structures under these conditions. In addition, the increased stability of aromatic structures through the process of polymerization was seen as a significant factor in the eventual creation of aromatic clusters. As soon as the concentration of aromatics arrived at a certain level, the nucleation process began in the water phase, leading to a quick

buildup of clusters of aromatic compounds. As the duration of reaction time increased, aromatic clusters spread out and attached themselves to the surface.

5 CONCLUSION

Fuel issue can be solved using bioethanol. Renewable biomass pretreatment, cellulase production, co-fermentation of pentose and hexose sugars, and bioethanol separation and purification have all made notable advancements. However, bioethanol remains less economically viable than fossil fuels owing to production costs. The primary obstacle is to decrease the expenses associated with bioethanol production. Therefore, it is necessary to implement the biorefinery concept to fully utilize renewable resources and produce additional valuable byproducts. This approach would ultimately reduce the expenses associated with bioethanol production. This can lead to bioethanol becoming a more economically viable option than fossil fuel.

The pretreatment, fermentation and distillation process require high energy which increases the production cost of bioethanol, modification of pretreatment process which will requires lower temperature or at least reduce the duration of the pretreatment will decrease the bioethanol production cost. The operating time of the fermentation stage can be reduced using simultaneous hydrolysis and fermentation but the main disadvantage is finding a thermotolerant strain of yeast that can perform at its optimal and do fermentation at about 50°C.

Most vehicles engines can only run by blending bioethanol of about (20-25) % maximum, if higher percentage of blended bioethanol or pure bioethanol is to be use the engines of the vehicles needs to be modified, the flex fuel engines are engines which are being innovated to utilised various kinds of fuels and their combinations. Another way is to use biotechnological method to reduce the lignin content which will avoid the pretreatment processing. Overall, the biofuel holds great promise for future along with other renewable energies.

Cow manure base bioethanol is sustainable, considering their vast availability and difficulty in disposing without long term effect. Considering the effort made by government in the form of supporting the schemes, companies in Finland like Valio and St1 are setting up biorefinery plants for bioethanol production with a capacity of 1,000 GWh (1 terawatt-hour) by 2030.

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