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Bioethanol as an alternative fuels: A review on production strategies and technique for analysis

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ABSTRACT

Bioethanol production represents an alternative source of energy that also helps minimize greenhouse gas effects. Currently, the focus of advanced technologies for bioethanol production is on the conversion of lignocellulosic biomass into renewable energy for transportation as it offers a low cost of investment and non-pollution bioprocesses. However, the utilization of lignocellulosic biomass has several challenges, including the high cost of pretreatment, the recalcitrant nature of the biomass and the requirement for robust microbes to ferment various types of sugars. Informations on the subject were achieved through a literature search using various electronic databases such as Google Scholar, ScienceDirect, Scopus, and others. From literature findings, few strategies such as separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), simultaneous saccharification and co-fermentation (SSCF), and consolidated bioprocessing (CBP); were found established to overcome these challenges, ultimately increasing the effectiveness of the bioconversion process and minimizing the overall cost of production. CBP was found to be the most promising strategy as direct production of ethanol from pretreated corn cob yielded 11.1 g/L ethanol without the addition of external hydrolytic catalyst. Various analytical techniques are commonly used to quantify bioethanol in a sample, and these methods were theoretically analyzed in relation to established theories. Currently, gas chromatography is known to be the most effective approach with limits of detection typically around 0.099 mg/mL, demonstrating excellent linearity and recovery rates between 91% and 109%. This paper aims to highlight the efficiency of every strategy involved in the bioconversion process and provide insights into every suitable analytical technique that can be employed to ensure the sustainability of biofuel by allowing researchers to improve the productivity and quality of bioethanol, thus promoting its role as a feasible alternative fuel.

1. Introduction

With the depletion of oil reserves due to the high consumption of fuels by transportation and industry, the demand for alternative fuels from renewable resources has increased over the years [1]. One of the suggested alternative fuels for transportation is bioethanol, as this biofuel can reduce greenhouse gas emissions by up to 75% compared with fossil fuels [2]. Bioethanol can be derived from various sources, such as wheat, corn, lignocellulose agro waste, genetically modified crops or even microalgae [3].

According to the 7th Sustainable Development Goal of the United Nations (SDG: Affordable and Clean Energy), to maintain food prices and supply, the transition toward second-, third- and fourth-generation biofuels is the best strategy, as this transition helps reduce adverse impacts on the environment, mitigate climate change and provide affordable and clean energy for all [4]. Lignocellulose based resources are considered suitable for producing bioethanol, as they are largely available in terms of quantities at very low prices, which is advantageous over the use of food-based feedstocks, as they reduce competition with the food supply [5]. The biomass components are influenced by several factors and are reasonably stable: 35%–50% cellulose, 20%–40% hemicellulose and 15%–30% lignin [6]. The most common approach in conversion technologies is biochemical conversion, which is performed in sequential phases: pretreatment, enzymatic hydrolysis and fermentation [7]. Pretreatment is the most crucial step to improve the efficiency of enzymatic saccharification process and minimize the inhibitory effect of lignin by removing parts of lignin content to increase the accessibility of substrate surface area to the hydrolytic enzymes such as cellulase [8]. The next stage is enzymatic hydrolysis, in which fermentable monosaccharides are released from the lignocellulosic structure of cellulose

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and hemicellulose [9]. After enzymatic hydrolysis, the fermentable sugars are ready to be converted into various fermented products, such as bioethanol. Both conversion processes (enzymatic hydrolysis and fermentation) can be performed via several strategies and at different levels of integration. The main strategies involved are simultaneous saccharification and fermentation (SSF), separate hydrolysis and fermentation (SHF), simultaneous saccharification and cofermentation (SSCF) and consolidated bioprocessing (CBP). These conversion strategies can be accomplished under different modes of operation, such as continuous mode, batch mode and fed batch mode [10]. The accuracy and precision of ethanol measurement are particularly necessary in a wide range of biotechnological processes and industries, such as pharmaceutical, cosmetics, food and alcohol beverages. Several analytical techniques can be applied to quantify the alcohol content to ensure that the highest standard of quality is based on physical (hydrometry, densitometry, pycnometry and refractometry), chemical (dichromate assay), spectrometric and chromatographic analyses. The official method for the quantification of ethanol in alcoholic beverages is pycnometry, which requires a well-balanced and controlled temperature, and this method was designed by the Association of Official Analytical Chemists (AOAC) [11]. The long and slow process of ethanol fermentation, especially on industrial scale normally takes 48–72 h to complete, hence, requires an instant quantitative method which is suitable to monitor the quality of ethanol produced and rapidly verifying the on-going process of fermentation [12]. Existing instruments available for qualitative and quantitative measurement for ethanol analysis not only have high accuracy and precision, but also complex, expensive and require trained personnel to operate the instruments [13]. Hence, this review aims to deliver comprehensive information on the types of feedstocks used for every bioethanol generations, deeper understanding on every strategy available for bioconversion process to improve the production of bioethanol, and offering critical insights on the properties of analytical technique to ensure the quality of bioethanol, thus contributing towards the advancements in bioethanol technology and also promoting the sustainability of biofuels in the global energy landscape.

2. Methodology

Information and analytical data on the subject were achieved through a literature search conducted for publications using various electronic databases such as Google Scholar, Scopus, ScienceDirect, PubMed and others. The date range of the publications selected for this review was from 2019 to 2024; this range was chosen to make sure the information gathered is validated and updated. The keywords ‘bioethanol production’, ‘bioconversion process’, ‘lignocellulosic biomass’ and ‘analytical techniques’ were used as primary searches; combined using Boolean operators “OR” and “AND”. The inclusion/exclusion criteria were used as a guideline during the selection process to make sure inclusion in this review comprises of only relevant studies and related to the theoretical basis of the selected keywords used in the search engine. While studies that do not align with the objectives of this review were excluded from the selection. The article choice was made by only considering English-language publications. The information from any publication focusing on current generations of bioethanol production, strategies for bioconversion process and techniques for bioethanol analysis were gathered and then analyzed by summarizing the advantages, weaknesses, the main features and key aspects on each method of analytical processes. The methodology of this study emphasizes theoretical analysis, which strengthens the quality of research by providing structure, fostering critical evaluation, guiding methodological choices, and facilitating new insights into bioethanol production processes. Integrating a theoretical perspective enhances the rigor of the review by establishing a foundation for evaluating existing studies. This approach also enables researchers to critically assess how well previous research aligns with established theories, thereby identifying gaps and inconsistencies in literature [14].

3. Type of feedstock for bioethanol production

Bioethanol production can be classified into three generations on the basis of the types of feedstocks used during the bioconversion process: (i) first-generation bioethanol, developed from food-based crops; (ii) second-generation bioethanol, developed from lignocellulosic biomass or municipal solid wastes; and (iii) third-generation bioethanol, which utilizes CO_2 as feedstock.

3.1. First generation of bioethanol

According to Renewable Fuel Association, leading countries such as United States and Brazil are known to be the main contributor for bioethanol production with an estimation of 13.9 billion gallons (53%) and 8.08 billion gallons (31%), respectively, but the total number of productions has been decreased to around 2.9 billion gallons due to the COVID-19 crisis [15]. The first generation of bioethanol (1G) has increased the value of biofuel in terms of cost and energy efficiency as well as reducing greenhouse gas (GHG) emissions [16,17]. Typically, this generation of bioethanol originates from the fermentation of biomass with high content of sugar (i.e. sugar beet and sugar cane) and/or starch (i.e. grain sorghum, barley, corn, wheat and rhizome) [18]. However, the production of 1G bioethanol has influenced the socioeconomic aspects of biorefinery and is considered unethical as it competes with the food supply, arable lands and natural resource input; creating a food versus fuel scenario [19].

Fig. 1 shows there are over 200 corn-based bioethanol plants in the United States that produces 15 billion gallons of bioethanol compared to 10 cellulose-based bioethanol plants, which can only produce approximately a half-billion gallons per year [21]. To meet half of the transport fuel necessity, the US biofuel industry highly depends on the utilization of energy crops which demand large areas, estimated around 24% of the total cropland for agricultural activities [22,23]. Growing concern over food security due to the increasing use of arable land for cultivation of energy crops as feedstock has led to the development of bioethanol from lignocellulosic biomass known as second-generation bioethanol (2G).

3.2. Second generation of bioethanol

Growing concern over food security due to the increasing use of arable land for cultivation of energy crops as a feedstock and impact on biodiversity has led to the development of bioethanol from lignocellulosic biomass (i.e. municipal, agricultural, forest and industrial wastes) known as second-generation bioethanol (2G). These feedstocks are inexpensive, have no issue with food sustainability and require no extra land. Globally, approximately 181.5 billion tons of lignocellulosic biomass are produced in a year but only 8.2 billion tons are currently utilized [24]. Lignocellulosic biomass (LCB), such as forest residues which mostly are woody residues, agricultural waste (paddy straw, corn stover, sugarcane bagasse, and wheat straw) and perennial grasses, is suitable for producing 2G biofuels [25].

Agricultural wastes are rich in lignocellulose materials and contain mainly cellulose, hemicellulose, extractives and lignin as seen in Table 1. The components of LCB (refer Fig. 2) primarily consists of cellulose (40%–60%), hemicellulose (20%–40%) and lignin (10%–25%), as well as small fraction of extractive, ash and protein [36]. The technology applied for this generation allows the cellulose and hemicellulose fractions of a plant to be separated from lignin to allow the fermentation of cellulose and hemicellulose into cellulosic ethanol [37,38]. Second-generation of bioethanol has become a favorable fuel owing to its clean combustion properties, low toxicity and volatility; making this generation of bioethanol suitable to address the environmental and social issues [39]. However, 2G bioethanol requires careful evaluation in the aspect of energy consumption, impact on the environment, maintenance involves throughout the process and is

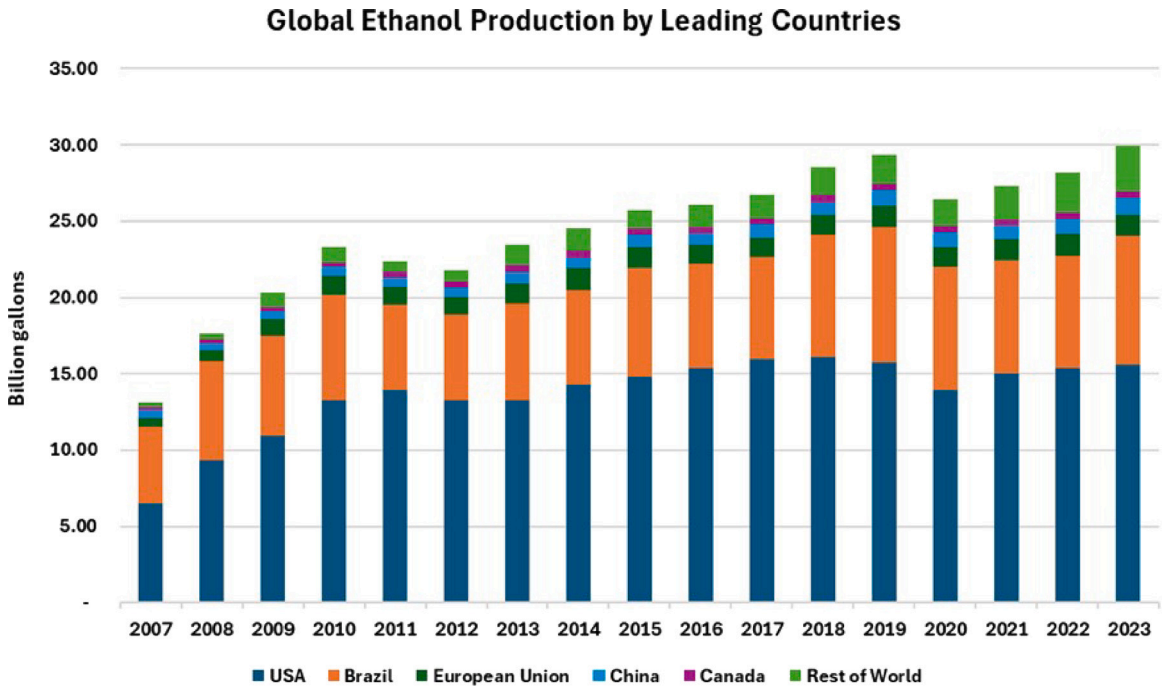

Fig. 1. The production of 1G bioethanol by top leading countries in 2023 [20].

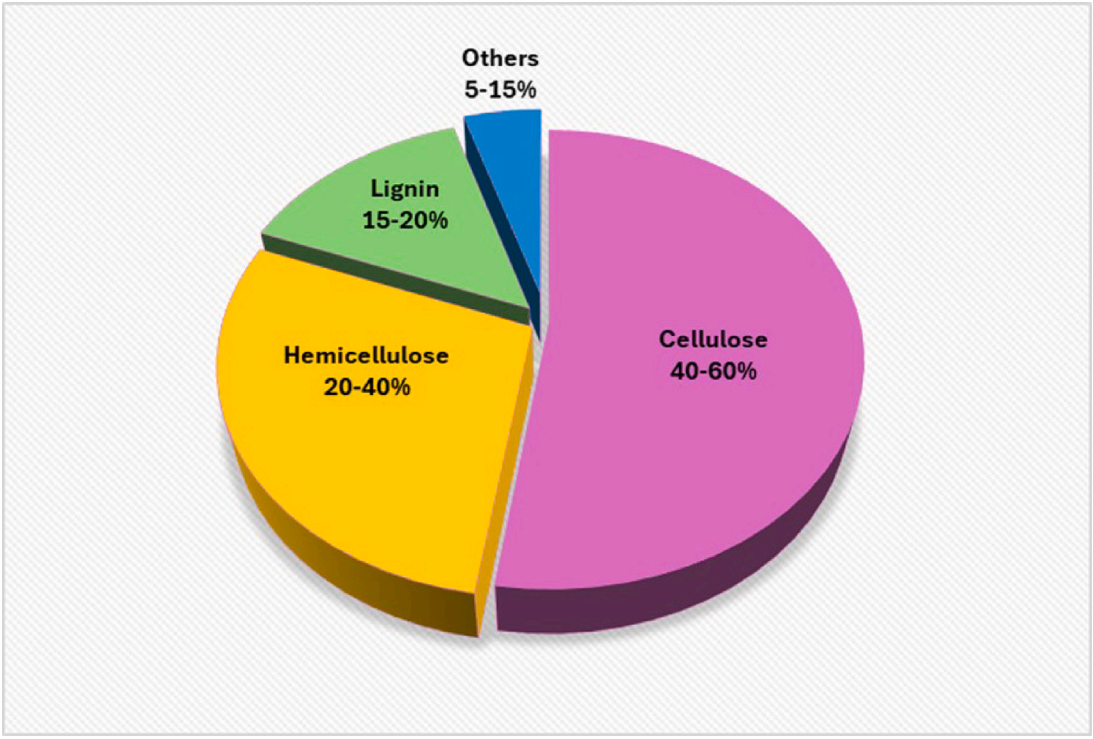

Fig. 2. The primary components in lignocellulosic biomass [26].

limited by the growing production costs as the substrates need to be pretreated before the fermentation process can take place [40]. Besides, the biological limitations in the 2G bioethanol often result in more diluted ethanol products compared to 1G bioethanol. This generation struggles to obtain the minimum amount of 40 g/L or more in a standalone process while 1G bioethanol can produce up to 80–115 g/L from fermentation broth [17,41]. Hence, modifications have been made in the development of third-generation biofuels to compensate for 2G biofuels.

3.3. Third generation of bioethanol

Third generation of bioethanol (3G) utilizes microorganisms as feedstock while fourth generation of bioethanol (4G) involves genetically modified microorganisms to attain a desirable amount of hydrogen to carbon (HC) output and create artificial carbon sink to minimize or eliminate carbon emissions [55]. However, these last two generations of bioethanol are still in early stage of development. Typically, third-generation biofuels (3G) are derived from algal biomass owing to

Table 1
The composition of lignocellulosic biomass for the production of 2G biofuels.

LCB Materials	Cellulose (%)	Hemicellulose (%)	Lignin (%)	References
Banana stem	30.08	27.8	6.1	[27]
Banana leaf	39.2	22.4	16.5	[27]
Corn stover	40.7	31.1	11.7	[28]
Empty fruit bunch	46.5	33.8	32.5	[29]
Hazelnut shell	30.0	35.0	32.0	[30]
Sugarcane molasses	38.4	24.3	26.0	[31]
Sweet sorghum	37.7	28.1	21.5	[32]
Switchgrass	39.4	20.3	21.2	[33]
Rice straw	24.0	27.8	13.5	[34]
Wheat straw	45.4	36.5	21.6	[35]

their properties, such as high starch and lipid contents, rapid growth rates, ease of cultivation, high carbon dioxide absorption and low land usage [56]. Red algae, green algae, brown algae, cyanobacteria (blue–green algae) and diatoms are recognized as the major groups in microalgae for bioethanol feedstocks. Among these, cyanobacteria are the most efficient group for manufacturing 3G bioethanol [57]. Fast-growing algae are usually known to have low oil content while high-lipid algae species have slower growth rate, hence, it is important to select the correct species with high biomass and lipid concentrations to ensure the efficiency of 3G bioethanol production [58]. Most importantly, productive cultivation of algae requires a direct supply of CO_2 which can be obtained from atmospheric carbon capture or industrial emitters for photosynthesis and eventually for bioethanol production with negative carbon footprint [23]. An example of microalgae species such as *Chlorella* sp. is considered as possible microalgal feedstock for bioethanol production, where this species was capable to generate up to 11 g/L of bioethanol using hydrolytic enzymes (i.e. α-amylase and glucoamylase) [51]. Besides, studies also reveal that macroalgae has better potential to produce high amounts of bioethanol by utilizing only 0.43 g/g substrate compared to land feedstock and consume less time of production [59]. A comparison between different feedstock utilized for the development of every generation of bioethanol can be observed in Table 2.

3.4. Fourth generation of bioethanol

Fourth-generation biofuels (4G) are produced from bioengineered algae (i.e. *Bacillariophyceae* (diatoms), *Eustigmatophyte*, *Chlorophyceae*, and *Chrysophyceae*) with the ability to capture carbon in their leaves and branches as a feedstock for biofuel production [60]. The genetic modification in microalgae intended to enhance certain traits in microalgae and optimized features to improve the production of bioethanol such as improvised photosynthesis and carbon fixation, increase the synthesis of lipid and ability to utilize diverse sugar especially pentoses and hexoses [61]. This generation of bioethanol includes an emerging field in which the direct conversion of solar energy into biofuel via designed microorganisms based on new synthetic techniques [62]. However, the development of 4G bioethanol has a negative impact on the environment because the release of transgenic agents can disrupt the ecological balance of a habitat [40].

3.5. The future of bioethanol generation

Currently, four generations of biofuels have been developed, but their availability in the world's market relies on the support of the government to provide tax credit incentives and energy subsidies [63]. Every generation of biofuels have their advantages and disadvantages from many aspects (Fig. 3). The future is expecting the development of the next generation of biofuels to compensate for the weaknesses of the previous generations, which constitute the fifth generation of biofuels (5G). This generation is expected to utilize knowledge of synthetic biology, such as genome editing, genetic engineering and direct evolution, to increase productivity and output in the production of biofuels [64]. The most promising option is the use of bioengineered plants with few modifications, such as destruction of lignin enzymes, increasing the level of polysaccharides or the growth of plant biomass and removing polysaccharides from the plant cell wall [65]. The development of bioengineered crops as feedstocks with modifications to the structure of the plant cell wall can potentially reduce the cost of pretreatment and hydrolysis processes [66]. In addition, altering the lignin profile via targeted lignin biosynthetic genes using the use of new technology can accelerate the overall biomass process [67]. In addition, metabolic engineering and genome editing techniques also help in the development of specific designed crops that can produce high-quality biocrude that is rich in alkenes and ketones to produce biofuels [63]. This type of feedstock offers significant potential in the future development of biofuels by improving sugar yields, enhancing biomass characteristics and enabling cultivation under stress conditions. However, overcoming the public perception of bioengineered plants as energy crops, especially concerning food security and impacts on the environment, is important before considering the potential of 5G biofuels.

4. Strategies for bioconversion

The recent fluctuation of fossil fuel prices and complete shift towards developing green energies have contributed to a new approach in biomass conversion techniques [68]. Significant studies have classified 4 important process configurations for a more effective bioconversion process for bioethanol production such as separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), simultaneous saccharification and cofermentation (SSCF), and consolidated bioprocessing (CBP) [69]. Normally, the process of substrate saccharification and ethanol fermentation are achieved separately but can also be accomplished efficiently by performing both processes in the same reactor, thus eliminating the possibility of inhibitory sugars accumulation, avoid inhibition of the end-product and reduce the risk of contamination in the medium [70].

4.1. Separate hydrolysis and fermentation (SHF)

This process configuration involves a traditional strategy in which the substrate is subjected to saccharification process and followed by fermentation of glucose to ethanol in separate reactors [71]. The strategy of separating the saccharification process from fermentation is to allow the enzymes to operate at high temperature while operating the fermentation of ethanol at a moderate temperature for optimum performance and increase the productivity of ethanol [72]. This process is typically the most preferable method because it allows flexibility in choosing the hydrolysis process and provides optimized conditions for enzymes, catalysts or even fermenting microorganisms in both saccharification and fermentation processes [73,74]. However, this approach results in low efficiency of ethanol production due to the time delay between sugar release and fermentation as high sugar concentrations can inhibit the performance of the enzymes [75]. Besides, there are several major drawbacks for SHF including high-cost of operations caused by having two separate reactors, enzyme inhibition by end-products, possible microbial contamination owing to long hydrolysis period and sterilizing them in a large-scale process remains challenging [76,77]. A study by [78], shows the process of saccharification involving 1.0% NaOH-pretreated rice straw using 200 FPU/mL extracted crude enzyme from *Aspergillus fumigatus* releasing 22.15 g/L reducing sugars in 20 h and resulted in 9.45 g/L ethanol during fermentation process with *Saccharomyces tanninophilus*. Besides, there is also a comparison study between SHF and SSF approaches using *S. cerevisiae*; which indicated SHF as a better method with a higher ethanol concentration (48.72 g/L) as compared to SSF method which was able to produce 29.59 g/L after 48 h, but SHF is still unfavorable due to having longer total time for bioethanol production [79].

Table 2
Comparative analysis between feedstocks used for different generations of biofuels production.

Generation of biofuels	Feedstocks	Pretreatment	Fermentation conditions	Maximum ethanol concentrations (g/L)	References
1G	Barley	Gelatinized with boiled distilled water (36%, w/v)	37 °C, 72 h	78.48	[18]
	Sugarcane	Autoclaved at 115 °C for 20 min	30 °C, 33 h	81.59	[42]
	Sweet sorghum	Milled using extractor and heated to increase solid content	30 °C, 60 h	113.3	[43]
	Wheat	Gelatinized with boiled distilled water (36%, w/v) at 70 °C for 10 min	37 °C, 168 h	72.1	[44]
2G	Corn stover	Steam + sodium carbonate (Na_2CO_3)	37 °C, 72 h	76.8	[45]
	Onion waste	Mechanical + autoclaving	86 h, 30 °C, pH 5	30.56	[46]
	Palm wood	3% sulfuric acid (H_2SO_4), 3% nitric acid and 3% phosphoric acid separately with solid to liquid ratio of 1:10 (w/v) + 2% sodium hydroxide (NaOH)	45 °C, 84 h, pH 5	22.90	[47]
	Rice straw	N-methylmorpholine N-oxide-(NMMO) and phosphoric acid (H_3PO_4) pretreatment	45 °C, 72 h	63.4	[48]
	Sugarcane molasses	Pretreated with 99.8% H_2SO_4	30 °C, 72 h, pH 5.5	56	[49]
	Wheat straw	NaOH/hydrogen peroxide (H_2O_2) for pretreatment	37 °C, 96 h	31.1	[50]
3G	Microalgae (*Chlorella* sp.)	Pretreated with 1.5% H_2SO_4 combined with α-amylase and glucoamylase	117 °C, 20 min	11	[51]
	Dried seaweed (*Gracilaria* sp.)	0.1M H_2SO_4	45 °C, 18 h, pH 4.5	3.84	[52]
	Spirulina (*Arthrospira platensis*)	2% NaOH for 3 days of pretreatment time	35 °C, 96 h	35.52	[53]
	Bloom-forming cyanobacteria (*Microcystis* sp.)	0.5N H_2SO_4 for 4 h at temperature 120 °C	30 °C, 43.6 h	18.57	[54]

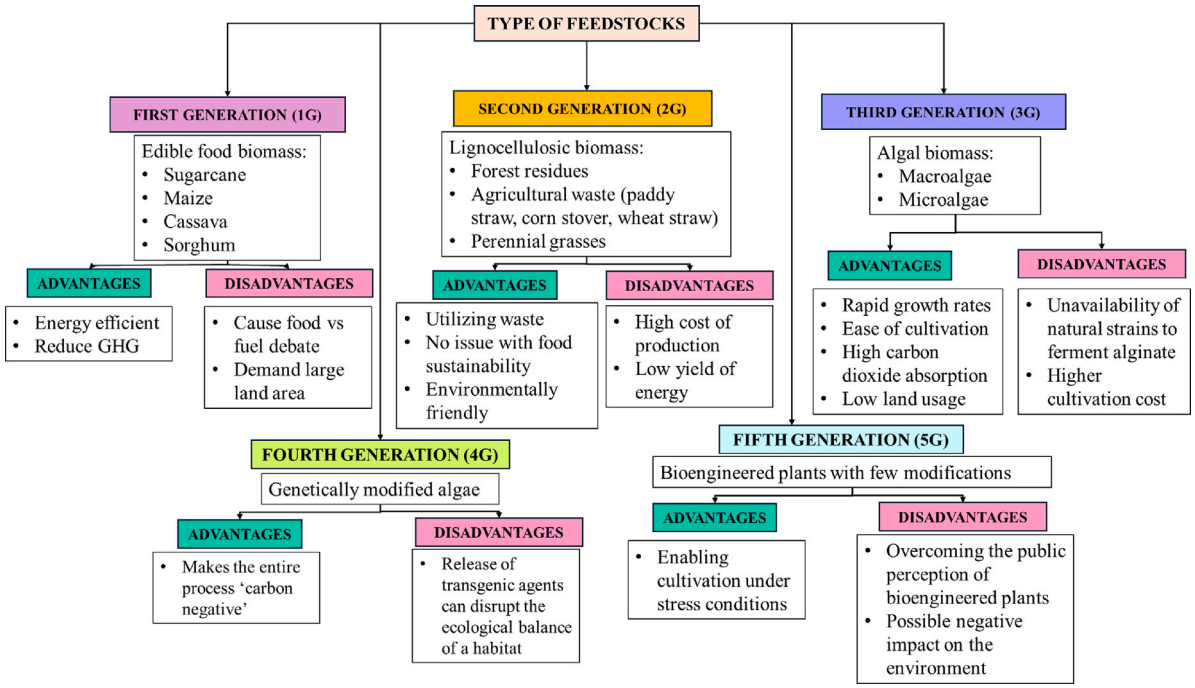

Fig. 3. The advantages and disadvantages of every generation of bioethanol production.

4.2. Simultaneous saccharification and fermentation (SSF) process

Simultaneous saccharification and fermentation (SSF) is the first bioprocess that integrated both hydrolysis and fermentation into a single vessel and has overcome the inhibitory compounds from enzymatic hydrolysis, thus increased cost efficiency and minimizes the residence time during bioethanol production [80]. Owing to these advantages, SSF has been broadly studied for the production of butanol and ethanol from lignocellulosic waste materials [81]. Alternatively, SSF is considered as a promising microbial technology, but several problems encountered during the process such as difficulty to find compromised optimal temperature range between saccharification (50 °C) and fermentation (28–37 °C), and simultaneously monitor several parameters of bioprocess [82]. Moreover, a slower rate of hydrolysis may result in lower sugar concentrations compared to SHF configuration [83]. Another drawback of this bioprocess is that most fermentative microorganisms, such as *S. cerevisiae*, are not capable to ferment xylose efficiently since glucose have a stronger affinity compared to xylose in the microbial transport system, thus glucose concentration must be lowered to allow more absorption of xylose [84].

4.3. Simultaneous saccharification and co-fermentation (SSCF)

Another bioconversion process that can produce bioethanol is simultaneous saccharification and cofermentation (SSCF), which is also similar to the SSF approach. In this method, the saccharification process of the feedstock and the fermentation of both pentose and hexose sugars from hemicellulose and cellulose, respectively, are simultaneously performed in a single step [85]. SSCF offers several advantages including the combination of saccharification and fermentation for various feedstocks which influences the overall efficiency of cost, time and resources. Besides, the ability of SSCF to co-ferment sugars as they produced during saccharification has increased the efficiency of the overall conversion process [86]. A great ratio of xylose to glucose can be maintained throughout the process as glucose is utilized rapidly, which reduces the competitive inhibition from glucose and enhances the utilization of xylose [87]. Furthermore, the lower enzyme load, shorter process duration, utilization of hexose and pentose sugars make this process have advantages over SHF and SSF process. Still, there are few concerns similar to SSF such as finding appropriate optimize conditions to accommodate saccharification and fermentation in a single vessel, inhibition cause by ethanol and increased water insoluble solids (WIS) [88]. Hence, it is important to achieve a compromise temperature by separating SSCF into two different stages of prehydrolysis: (1) high temperatures for cellulolytic enzymes and (2) microbial processes at low temperatures [89].

4.4. Consolidated bioprocessing (CBP)

Consolidated bioprocessing (CBP) provides another sustainable strategy by integrating all steps simultaneously (i.e. enzyme production, saccharification and fermentation) in a single reactor by utilizing either single culture of genetically modified microorganism or a consortium of microorganisms [90,91]. An ideal and suitable organism for CBP in the production of bioethanol from lignocellulosic materials must have essential features such as the ability to secrete lignocellulose-degrading enzymes, able to perform hydrolysis on biomass and fermentation of ethanol [92]. However, to date, no natural isolates have been discovered to perform CBP in conversion process [93]. Filamentous fungi such as basidiomycetes and zygomycetes have potential to be a good candidate for CBP due to their ability to naturally grow on solid biomass, penetrates deeper into the substrate with their hyphae and convert the substrate into ethanol in a single step bioprocess [94]. There are few fungal species that have been deliberated among researchers as possible CBP candidates include *Trichoderma reesei*, *Aspergillus* spp. and *Fusarium* spp., but these strains are not naturally ethanologenic (able to produce ethanol), and all research is still in an early stage. Since most cellulosic fungi are an aerobic microorganism, they struggle to survive in anaerobic condition during fermentation [95].

A study by [96] indicates the potential of *T. asperellum* B1581 as a single culture for bioethanol production in CBP with the volume of ethanol produced, 0.94 g/L. Genetically modified ethanologenic strains like *S. cerevisiae* are required to secrete enzymes for saccharification of materials and fermentation of resulting sugars to ethanol, simultaneously [97]. According to [98], recombinant *S. cerevisiae* ER T12 was used as a host to improve the productivity of ethanol during fermentation process and variant ER T12.7 displayed significant improvements with 36% higher ethanol yield than the parental strain. Another modification in CBP is the integration of biodelignification features, which offers better opportunities for ethanol production. Reports found only basidiomycetes such as *Trametes versicolor*, *T. hirsuta* and *Phlebia* MG-60 were able to directly produce ethanol from non-pretreated lignocellulosic materials [99]. These consolidation strategies have the potential to enhance the efficiency of production and reduce the overall cost compared to methods by SHF [100].

4.5. Ideal bioconversion strategy

The ideal bioconversion strategy involves various saccharification and fermentation processes used in cellulose-based ethanol production, such as SHF, SSF, SSCF and CBP (Fig. 4). The SSF approach has been shown to produce more bioethanol than the SHF process does, but this strategy is limited by its inability to provide compromised conditions for both saccharification and fermentation processes [112,113]. In addition, this bioconversion process produces low amounts of ethanol, as SSF fails to fully utilize the pentose sugars [114]. To overcome this problem, SSCF has been established in which the cofermentation of hexoses and pentoses can be carried out simultaneously. While both SSF and SSCF demonstrated the same ability to produce high yields of bioethanol, SSCF tends to be more favorable because of its potential to ferment various types of sugar simultaneously. All configuration of bioprocesses can be clearly compared in Table 3.

Although the biorefinery industry has been operating for long periods of time, finding a suitable and robust microorganism for an efficient bioconversion process is still active study field [115]. The ideal strain should be able to utilize various types of sugars (i.e. pentose and hexose) released from lignocellulosic feedstock and survive inhibitory compounds that are produced during pretreatment of biomass [116]. According to most of the literature, the ideal bioconversion approach is known as consolidated bioprocessing (CBP), where this strategy can reduce the production cost of cellulase, eliminate the requirement of separate hydrolysis enzymes and requires less utility than SSF or SHF [111,117]. CBP technologies developed using biosynthetic or cellulolytic microorganisms (i.e., microbial cocultures) exhibit significant potential as alternative pathways for bioethanol production. There are several positive reports on the direct conversion of biomass into bioethanol by a single microorganism. Owing to the prominent nature of *S. cerevisiae* in the fermentation of ethanol, this specific yeast has been genetically manipulated to meet the industrial demands of CBP for ethanol production [118]. Recently, the bioconversion of starch-based plant biomass in a CBP system (incubated at 30 °C for 192 h) using recombinant *S. cerevisiae* ER T12 and M2n T1 strains yielded 89.35 g/L and 98.13 g/L ethanol with high carbon conversion rates of 87% and 94%, respectively [119,120]. A study by [121], shows the process of CBP using recombinant strain*S. cerevisiae* AC14, in medium containing solid fraction of pretreated sugarcane bagasse with good ethanol productivity (4.46 g/L/h). Genetic modifications on *S. cerevisiae* allow direct production of ethanol from hemicellulosic liquor (non-detoxified) derived from pretreated corn cob, to achieved 11.1 g/L ethanol concentration, without the addition of external hydrolytic catalyst [122]. These results represent the ability of CBP to achieve a significant ethanol yield, but the specific yield can vary according to the microorganism used or the type of feedstock chosen for the bioconversion process. While CBP offers a promising platform to overcome the high cost of bioethanol production, this process is still under development for application in the biorefinery industry.

5. Techniques for bioethanol analysis

Owing to the large scale of production, the determination of the ethanol (CH_3CH_2OH) content in the alcoholic beverage industry has become a key parameter, as one failure will cause a very large loss of expenses. Hence, a fast and reliable quantitative method is needed to monitor the progress of ethanol production with the flexibility to adjust the current and real-time fermentation methods up to the optimum operating schemes. An ideal method to address this purpose should be simple, rapid, cost-effective, highly accurate, highly repeatable and highly sensitive. Moreover, methods that require inexpensive equipment/instruments are generally preferred [12]. The typical method used to determine the volume of alcohol is the measurement based on the density of the sample using pycnometer, hydrostatic balance, frequency oscillator and referencing it to the International Alcoholometric

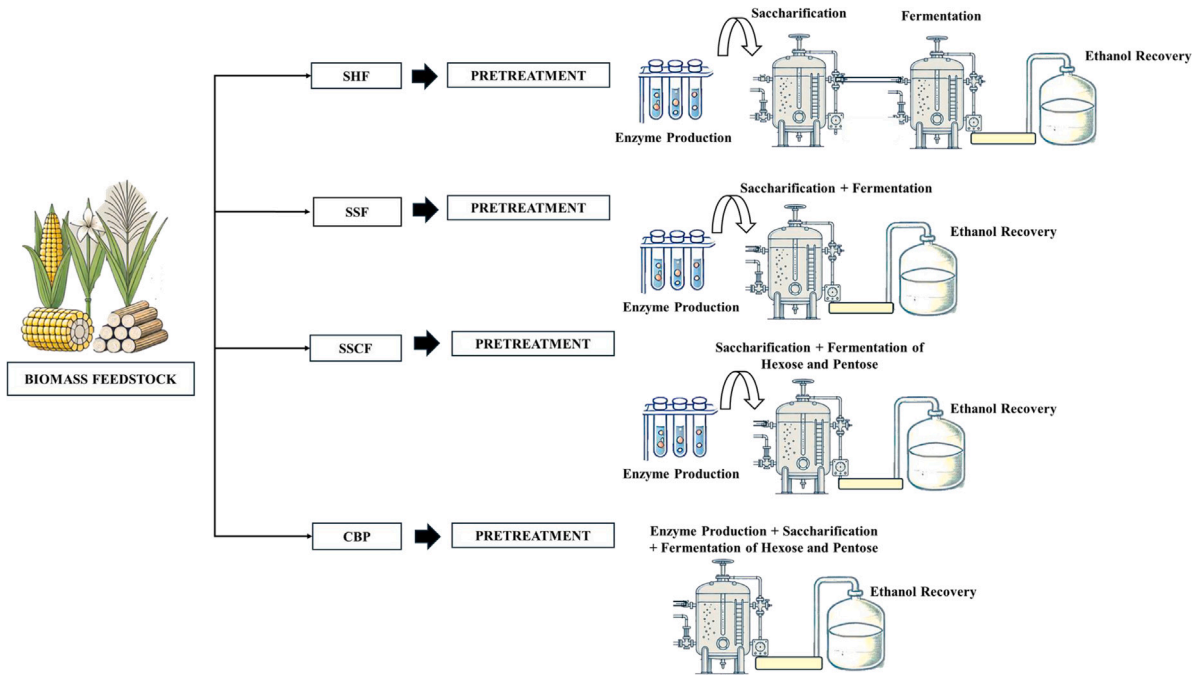

Fig. 4. Summary of the process involved in every bioconversion strategy for the production of bioethanol.

Table 3
Strategies of the bioconversion process for bioethanol production.

Strategies	Process Involved	Advantages	Disadvantages	Maximum Ethanol Yield (g/L)	References
Separate Hydrolysis and Fermentation (SHF)	Enzymatic hydrolysis and fermentation are performed sequential order.	SHF demands a smaller quantity of cellulolytic enzymes compared to SSF. Low risk of contamination as saccharified liquid with fermentable sugar can be sterilized.	The accumulation of glucose and cellobiose initiate partial inhibition to occur and influences the conversion process of biomass to glucose.	44.7	[101–103]
Simultaneous Saccharification and Fermentation (SSF)	Enzymatic hydrolysis and fermentation are performed simultaneously in the same vessel.	Minimizing inhibition on feedstock and cost of production. Instantaneous utilization of resulting sugars, reduced time of the process and improved the productivity. Manage to produce higher ethanol concentration than SHF.	Since the optimal temperature of enzymatic hydrolysis is higher than the fermentation temperature, finding an optimum and equilibrium point is necessary to ensure the process works efficiently	77	[74,81,104,105]
Simultaneous Saccharification and Co-Fermentation (SSCF)	Simultaneous enzymatic hydrolysis of glucose and xylose with the co-fermentation of xylose and glucose in a same vessel.	Low cost of production. Constantly eliminates the end-product inhibition that can reduce the cellulases or β–glucosidases. Short time of processing, high productivity as it avoids the end-product inhibition and low risk of contamination.	Temperature has become the limiting factor of this process as the ideal temperature for saccharification process is 50 °C while microbial process take place is 30-37 °C.	68.7	[85,106–108]
Consolidated Bioprocessing (CBP)	All processes such as production of enzyme, saccharification and fermentation are achieved in a single step process by the same lignocellulolytic microbes.	Minimizes capital investment, simplified total reactions, improves the efficiency of hydrolysis and reduces the risk of contamination.	Cellulolytic microorganisms have lacked in the metabolic pathways for desired products.	37	[109–111]

Tables, which provide the strength of alcohol by volume corresponding to the density measurement [123]. Currently, there are several complex analytical techniques for the determination and quantification of the resulting sugars generated by saccharification processes, such as gas chromatography (GC), high-performance liquid chromatography (HPLC), and enzymatic methods.

5.1. Colorimetric enzymatic assay

Although enzymatic methods are typically applied for the determination of a single sugar type, the most common method used for ethanol analysis is the calorimetric enzymatic assay. The method is based on the oxidation of ethanol catalyzed by the alcohol dehydrogenase enzyme. Two important parameters in the process of ethanol fermentation, (1) reducing sugar concentration and (2) total amount of alcohol, provide information on the optimization process used to increase the quality and yield of the products. Although chromatographic techniques have been more popular and widely used for the determination of ethanol, owing to their high cost of operation and expensive instruments, the quantification of ethanol via the titer acidic dichromate method has become the most convenient and applicable method in most laboratories [124].

5.1.1. Potassium dichromate method

The effect of ethanol can be determined via the use of oxidizing reagents such as potassium dichromate. The oxidation of ethanol by potassium dichromate reagents has been commonly applied to quantify the amount of ethanol in alcoholic beverages, blood plasma and even breathalyzers. Ethanol reacts with dichromate, where hexavalent chromium and Cr^{6+} are reduced to Cr^{3+} and ethanol is oxidized into acetic acid, making the orange color of the solution green [125]. Spectrometric analysis using dichromate as reagents for ethanol oxidation have integrated strong advances to analytical procedures, particularly in terms of analytical productivity, simplicity, practicality, cost-effective instrumentation and reduces waste generation [126]. However, this approach requires harmful and toxic reagents such as cerium (IV) and chromium (VI) along with extremely acidic conditions and longer reaction times [127]. In addition, this approach fails to determine the concentration of ethanol accurately, as a variety of side products and reducing sugars present in fermentation broth can also respond to potassium dichromate [128].

5.1.2. Ethanol assay kit

Several modern approaches are available to monitor the fermentation of ethanol and control the quality of product yield for the success of commercial products. One of the most commercially available Ethanol Assay Kit is manufactured by Megazyme company (catalogue no. K-ETOH) includes all essential components that are necessary for the analysis. The quantification process is based on ethanol oxidation to acetaldehyde by alcohol dehydrogenase (ADH) and continues with the oxidation of acetaldehyde by ADH by converting NAD^+ to NADH [129]. There is also another commercial enzymatic test kit, Enzytec™ Liquid Ethanol used to determine the concentration of ethanol in alcohol-free beer, fruit juices, kombucha and vegetable juices up to 0.5% alcohol by volume (ABV) which is equivalent to 3.95 g/L. The kit includes all the essential reagents prepared in the form of ready-to-use and was approved as AOAC Official Method^{SM} 2017.07 First Action in September 2017 [130]. The assay kit possesses high enzyme specificity that enables it to perform complex analysis with little interference and avoid complication during sample preparation, which on contrary may happen during chromatographic coelution. Although enzymatic assays are favorable due to short period of time for the sample analysis, the expensive cost for each sample compared to traditional laboratory-based method weigh the scale in favor of the latter [131].

5.2. Separation technique

Analytical methods can be classified into instrumental and classical methods, in which the classical approach is divided into 3 main types which are: (a) separation of analyte (i.e. extraction, distillation, precipitation and filtration), (b) qualitative analysis (i.e. boiling point, freezing point, color, odor, density, reactivity and refractive index) and (c) quantitative analysis (i.e. gravimetric analysis and volumetric analysis) [132]. After the fermentation of alcohol, the major components left in the broth are ethanol and total water, thus, separation is necessary to recover and purify ethanol from the broth through distillation process [133]. Unfortunately, the distillation process demands an enormous amount of energy along with high capital cost that does not align with the principals of circular economy and SDG [134]. Instrumental methods are divided into four main types: (a) chromatographic methods, (b) spectrophotometric methods, (c) electrochemical methods and (d) other techniques. These approaches normally require instrumentation that utilizes low analyte concentrations (less than 0.001) and focuses on the physico-chemical properties of the analyte such as absorption or emission of electromagnetic radiation, electrical conductivity of the analyte and separation properties [135].

5.3. Chromatographic techniques

Chromatographic method integrates separation techniques in a two-phase system: (a) stationary phase (absorbed mixture of components and separate in the next phase) and (b) mobile phase (migration of components can be observed) [136]. There are few analytical techniques using chromatography such as gas chromatography (GC) and high-performance liquid chromatography (HPLC) (refer Fig. 5). Today, GC is practically used as a measuring instrument to determine qualitative and quantitative compositions of volatile organic components (VOCs) using several internal/external standard calibrations that are responsible for the excellence quality of alcoholic products [137].

5.3.1. Gas chromatography

Gas chromatography (GC) is an instrument used to separate volatile or semivolatile liquids and gases in relation to their affinity for a stationary phase (liquid thin or layer of polymer on a solid). The volatile compound from an injection port is carried by a gaseous mobile phase to a column with stationary phase and finally to an amdetector [138]. The operation of GC depends on sample volatilization in the heated injector, followed by the separation of component mixtures in a prepared column, which allows the components to be vaporized without decomposing [139]. There are four main techniques in capillary GC to vaporized sample and transfer it onto the analytical column: (a) direct, (b) on-column, (c) split and (d) splitless injections. The common technique used are split and splitless injections [140]. Combination between two or more dimensions creates a robust platform for instrumental analysis by providing various types of information and offers great reliability in the identification and quantification process [141]. Generally, two-dimensional gas chromatography (2D-GC) is applied to identify each of the complex compounds in a sample, as it specifies a much greater resolution of chromatography than conventional GC methods do [142]. Mass spectrometry (MS), flame ionization (FID) and thermal conductivity are the most widely detectors used in GC. The common analytical technique for alcohol detection is GC-FID as this technique provides rapid speed of detection, excellent reliability and simplicity in sample preparation or extraction [143]. Although FID is not capable of performing the identification of compounds, this detector is known for high sensitivity, good retention times, easy to operate and has wide linear range of detection; making FID as a great detector for determination of alcoholic concentration [144]. A GC–MS instrument is used to identify small and trace organic compounds, whereas a GC–FID instrument is commonly applied to determine major compounds [2]. The application of MS allows specific detection of ions from ionized analytes, hence, results in high quantification accuracy of target components by comparing the ion mass to library data [145]. The development of standard operating procedures can be time-consuming and require extensive preparation. Typical GC-FID techniques demand for a long period of analytical run time and long columns to allow excellent chromatographic separation, hence, avoiding co-elutions to happen [146]. The analysis time of ethanol in olive oils using GC-FID and GC–MS requires approximately 40 min [147].

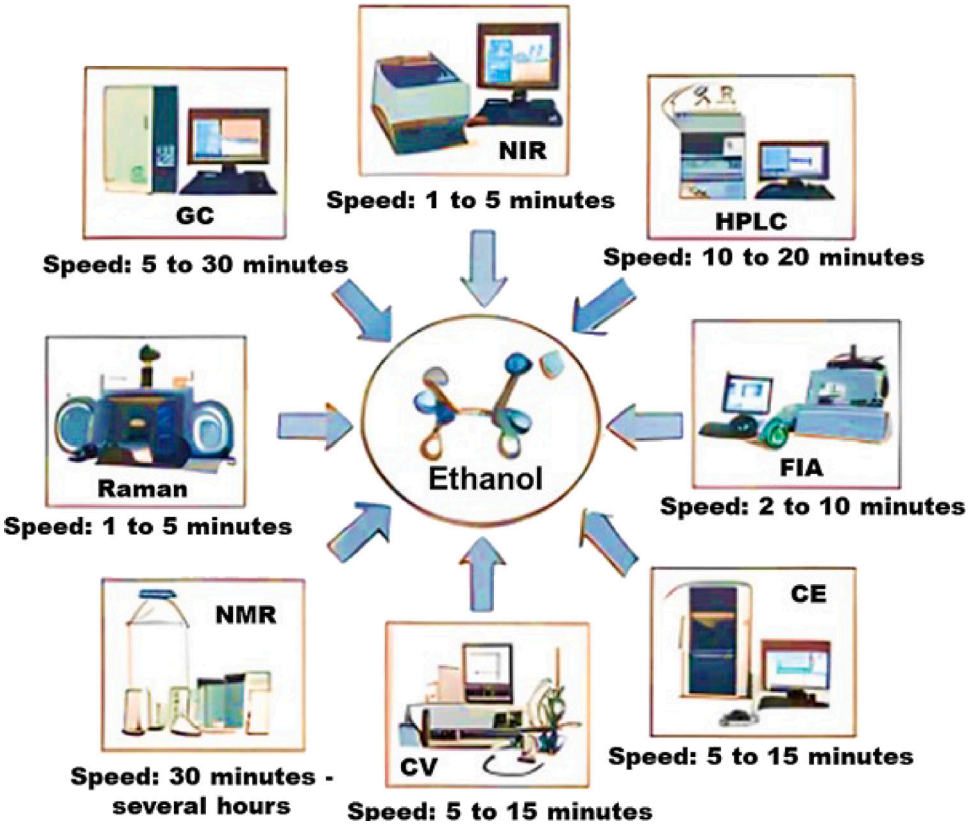

Fig. 5. Several analytical techniques with varying analysis times are used to determine the ethanol content in the sample.

Another major drawback of this technique is the reliance on a unique retention period of analytes for the identification process, as it can lead to misclassification or miscalculation if the peak measured is not a pure compound [148].

5.3.2. High-performance liquid chromatography (HPLC)

High-performance liquid chromatography (HPLC) is an analytical technique with the ability to control the quality of natural products and allows the identification of specific compounds in the medium. Normally, GC is used to conduct the quantification of acetone, ethanol, butyric acids and acetic, while HPLC is frequently applied for the quantification of sugars (i.e. glucose, arabinose and xylose) concentrations [149]. This analytical technique can be coupled with several detectors and become the most employed methods due to their wide range of application, cost-efficiency and robustness [150]. With the utilization of UV and mass detectors, HPLC can split and discover any hazardous compounds, active compounds or even contaminants in the products [151]. One of the common detectors used by HPLC is tandem mass spectrometry (HPLC-MS/MS), which enhances several features such as speed, high sensitivity, selectivity and improves the efficiency for detection [152]. By operating both instruments simultaneously, the principal cost and time needed to complete an analysis can be increased. In addition, this technique can be laborious and against the philosophy of green chemistry but can still be avoided by choosing fewer toxic solvents to reduce the generation of chemical waste [153]. Hence, many alternative protocols have been intensively developed to overcome these limitations.

5.4. Spectroscopic techniques

Analytical techniques such as MS, NIR and NMR are suitable for the immediate detection of ethanol and provide alternative methods with various detection specificities and sensitivities, but few studies have shown that the quantification of ethanol via direct injection in aqueous matrices has lower sensitivity.

5.4.1. Near-infrared (NIR) spectroscopy

Near-infrared spectroscopy (NIR) provides physical and chemical information related to the vibrational nature of molecular bonds such as N-H, C-H, and O-H bonds [154]. Over the years, the choice of screening method has been based on infrared spectroscopy (IR) technologies, which can identify up to 10 analytes, such as total sugars, ethanol, propanol and methanol, in a single assay [155]. This approach has become one of the popular choices of analysis over other contemporary analytical techniques as it offers rapid measurement with little or no sample preparation, the non-invasive and non-destructive properties, low cost of operations and portability of the spectrometers [156]. The NIR spectrum has been frequently used in several studies to evaluate the quality of food and beverages as well as characterizing each sample corresponds to their molecular bonds from its chemical composition [157]. Moreover, NIR spectroscopy has been successfully applied by the biorefinery industry for the assessment on the properties and quality of biofuel as well as inline monitoring for transesterification reactions [158]. The selection of wavelengths is important in multivariate calibration analysis because it can remove interfering and uninformative variables in the spectra to achieve greater performance in model prediction and enhance interpretability [159]. However, IRs have several limitations, such as poor sensitivity and overlapping signals, inability to perform direct analysis, indirect methods of calibration based on the model of partial squares regression, and low sensitivity for detecting analytes such as 5-(hydroxymethyl) furfural (HMF) and acetaldehyde, which typically occur at relatively low concentrations [160].

5.4.2. Nuclear magnetic resonance (NMR)

Compared with gas chromatography (GC) and high-performance liquid chromatography (HPLC), nuclear magnetic resonance (NMR) is considered among the unpopular options for quantitative analysis, especially in the characterization of biofuels [161]. NMR allows the

detection and quantification of intermediate reactions as well as non-invasive product molecules in a fixed-time manner with very high chemical specificity [162,163]. In addition, one of the advantages of NMR is the simplicity of the method in terms of sample preparation and speed and the need for a low volume of solvent; however, it also has several disadvantages, such as the high cost of instrumentation and maintenance costs, including the use of personal or chemical materials (liquid nitrogen and helium) used for the cooling of the superconductor magnet [164].

5.4.3. Raman spectroscopy

With the rapid development of chemometrics, the use of molecular spectroscopy (i.e. Raman spectrometry and NIR) techniques in various industries has also increased over the years [165]. Raman spectroscopy is another non-destructive technology that eliminates the need for chemical reagents and has become the most suitable candidate because of its flexibility in various samples and because the spectra are not limited to water. In addition, the interface of a sample can be determined via an immersion probe to directly monitor the culture and composition changes in the bioreactor while completing qualitative and quantitative analysis [166]. This analytical technique relies on the inelastic scattering of incident light via molecules, which involves changes in the polarization of the irradiated molecule and photon emission as well as the delivery of information on the vibrational energy bonds of the molecule [167]. A good polarization of nonpolar bonds leads to intensive Raman bands, whereas chemical bonds with a high dipole moment normally result in poor polarization, which causes a decline in Raman activity [168]. Raman spectroscopy provides useful insights into molecular structure and enables both qualitative and quantitative measurements on the substance as well as producing better and sharper peaks compared to NIR while maintaining minimum interaction with water [169]. However, even with these advantages, Raman spectroscopy is normally considered for qualitative analysis rather than a quantitative technique, and the application of a standard is compulsory for quantification analysis via Raman spectroscopy [170]. Besides, the scattering intensity in Raman is rather moderate, thus causing difficulties in determining the presence of analytes at low concentration in the fermentation broth [171]. The utilization of laser waves within visible light range results in excitation of fluorescence; leading to the interference with the Raman signal and cause sample decomposition due to the high intensity of excitation radiation [172].

5.5. Sensor technologies

Biosensors are classified according to the type of transducer, such as piezoelectric biosensors, optical biosensors, electrochemical biosensors, thermal biosensors or natural biological components. Currently, typical biosensors, such as electrochemical sensors, are popular because of their high sensitivity, rapid detection and low detection limits [173]. This device represents a breakthrough in scientific research, as it relies on the reactions of specific biochemicals involving immune systems, isolated enzymes, cells, tissues, or organelles during the thermal, optical or electrical signal detection of chemical compounds [174]. Some electrochemical instruments are portable, which makes it easy to monitor the environment [175]. Electrochemical detection is very sensitive towards electroactive molecules and provides selective detection as various molecules can be oxidized and reduced at different potentials [176]. Ethanol can be quantified and determined electrochemically as it is known as an electroactive molecule with relatively high theoretical energy density (approximately 8.10 kWh/kg), better efficiency in theoretical fuel cell (97%), and great number of electron transfer per ethanol molecule [177,178].

5.5.1. Electrochemical biosensors

Electrochemical biosensors have a wide range of applications, such as food safety, clinical diagnostics and environmental monitoring, and are adapted to detect a variety of substances depending on the type of bioreceptor applied. These biosensors are classified according to the type of signal obtained by transduction, such as current intensity or conductance changes and potential differences [179]. There are various electrochemical techniques, such as voltammetry, potentiometry and impedance spectroscopy. However, the response time varies depending on the type of transduction and design of the mechanism applied.

5.5.2. Cyclic voltammetry

Voltammetry is known to have great reliability, high detection speed, good sensitivity and accuracy, making it as one of the most applied electrochemical techniques in biosensors technology. Not only does it offer an efficient way to study the reaction mechanisms of an analyte in the electrochemical field but can also measure the parameters of the sample using various voltametric techniques [180]. There are many voltametric techniques which can be applied based on the type of voltage sweep such as square-wave voltammetry, cyclic voltammetry and differential pulse voltammetry [181]. Cyclic voltammetry (CV) is undeniably the most widely utilized electrochemical technique for quantification analysis. The efficiency of this technique relies on the simplicity of the application and its capacity to offer insights into intrinsic aspects such as kinetics of the interfacial electron, thermodynamics and chemical mechanism [182]. During analysis, a triangular voltage wave sweeps between two limits (cyclic form), producing peaks that represent the events of redox reactions [183]. Measurement of CV are normally performed with three-terminal cell arrangement made up of a working electrode (WE), a counter electrode (CE) and a reference electrode (RE); which this approach is used to determine the current response to a sweeping voltage potential when measuring the analyte using working electrode [184]. This technique provides various ranges of potentials for the working electrode to enhance oxidation or reduction of the analyte and develops a corresponding response for the current to allow quantification of the analyte [185]. However, this system is restricted by its sensitivity towards redox-active substances, but the range of application can be improved by altering the surface of the working electrode with different materials to enhance their selectivity and sensitivity for various compounds [66].

5.5.3. Enzyme-based biosensors

The application of enzyme-based biosensors for the quantification of ethanol in samples offers specificity and simple step treatment. These analytical devices utilize an enzyme as a bioreceptor or coupled tightly to a physical transducer to deliver a continuous or discrete digital/optical signal that is proportional to the amount of analyte in the sample [186]. Alcohol oxidase (AOx, EC 1.1.3.13) and alcohol dehydrogenase (ADH, EC 1.1.1.1) from microorganisms such as *S. cerevisiae*, are enzymes that are widely employed for the application of alcohol biosensor [187]. Biosensors based on ADH use the enzyme as a catalyst for the oxidation of ethanol to acetaldehyde and results in NAD^+ reduced to NADH, therefore monitored by amperometrical [188]. While this enzyme provide advantages such as high selectivity towards ethanol without relying on oxygen, it faces with other challenges including the enzyme instability and dependency on the constant recovery of NAD^+ in the assay [189]. Even with a variety of studies involving the application of alcohol biosensors using enzymes, further analysis of the influence of enzymes on alcohol chain structure, temperature, strain, pH, operational conditions, membrane materials and electrodes of designed biosensors is still lacking [190].

5.5.4. Capillary electrophoresis

Capillary electrophoresis (CE) is a technique employed to separate the molecular charges that are present in solution/medium on the basis of electrophoretic mobility [191]. This approach includes the separation that happens inside a thin silica capillary occupied by an electrolyte solution which allows the analyte to move through the capillary and the occurrence of separation due to the differences between the amplitude of electro-osmotic flow (EOF) and the electrophoretic mobility of the analytes [192]. There are many approaches in CE which have led to positive results such as Micellar ElectroKinetic Chromatography (MEKC), Capillary Zone Electrophoresis, Non-Aqueous Capillary Electrophoresis and MicroEmulsion Electrokinetic Chromatography [193]. This technique can be considered a complementary technique to high-performance ion chromatography because of its promising nature, such as low instrumentation costs, the small volume of sample and reagent used, short period of analysis, portability, potential for miniaturization and outstanding separation productivity [194]. Compared with that of GC, the major advantages of this analysis are the simplicity of the sample preparation process, and the high performance of separation compared with liquid chromatography (LC) [195]. Most importantly, CE has reduced the collection of sample workload, which the sample required (< 1mL) is significantly less than other techniques [196]. However, CE is known to have poor sensitivity in UV detection. According to Beer's law, the absorbance of an analyte is directly associated with the path length and concentration, which is equivalent to the inner diameter of a capillary [197]. The sensitivity of the CE-UV concentration using a typical 50 μm inner diameter capillary can be poorer than that of LC paired with a UV detector

5.6. Flow injection analysis

Flow injection analysis (FIA) is an automated method and frequently used for chemical analysis. The sample is analyzed by injecting it into a flow carrier solution before arriving to a detector [198]. This method is favorable because of its practicality in coupling with various detectors, such as UV–VIS spectrometers, amperometers, Rayleigh light scattering or fluorescence spectrometers. Owing to its advantages, such as rapid analysis; simple operation; low consumption of samples and reagents; and high accuracy, this method has been largely applied in food tests and biochemical, agronomical, industrial and environmental assays [199]. The FIA systems integrate techniques from laboratory routines to enhance the analytical output and reduce the risk of contamination as well as the analyte losses [200]. In comparison to the voltametric technique, the main advantages of this amperometric approach include the capacity to simultaneously analyze different samples, real-time monitoring and reduces the contact time between the sample and the surface of the electrode to minimize the absorption effects [201].

5.7. Comparison between analytical techniques

To measure the concentration of bioethanol across different fields and applications (i.e., fuel production, alcoholic beverage and fermentation processes), various analytical techniques can be employed. A detailed comparison of several prominent methods, such as gas chromatography (GC), high-performance liquid chromatography (HPLC), biosensors, capillary electrophoresis (CE), calorimetric assays, flow injection analysis (FIA), near-infrared spectroscopy (NIR), nuclear magnetic resonance (NMR), and Raman spectroscopy, is shown in Table 4. Despite the various advanced analytical techniques available, such as enzymatic analysis and chromatographic techniques, owing to their high operational costs, the titration of acidic dichromate has been considered a popular option, as it is inexpensive and available in most biofuel laboratories and wineries. The self-colorimetric method demonstrated limits of detection and quantification of 0.17% and 0.56%, respectively, with a high correlation coefficient ($R^2 = 0.999$), indicating excellent precision and reliability [124]. Although regression analysis by [216] between the enzymatic assay and HS/GC yielded a linear relationship described by the equation y=0.966x + 2.084 with a correlation coefficient $R^2 = 0.9987$, indicating strong agreement between the two methods, enzymatic assays have disadvantages such as low ethanol specificity and high tendency for cross-reactivity with toxic alcohols like methanol and LDH interferences.

The choice of analytical techniques for determining bioethanol concentration depends on the specific requirements of the analysis, including sensitivity, specificity, speed, complexity, and cost. Gas chromatography and HPLC are preferred for their high sensitivity and specificity, whereas biosensors and NIR offer rapid and real-time analysis. Capillary electrophoresis provides a fast alternative, while NMR and Raman spectroscopy offer detailed structural information but at higher costs and complexity. A comparison study using LF-NMR indicated that quantification based on the ethanol CH2 group signal is reliable and comparable to GC methods, offering advantages in speed and precision for ethanol concentrations between 80%–100%, but the quantification based on the CH3 group signal was found to be less accurate [226]. A research study by [227] tested reliable methods for quantifying ethanol concentrations in hand sanitizers using NIR. The study demonstrated the effectiveness of partial least squares (PLS), achieving a prediction error of 1.83%. NIR is preferred for its rapid result performance, but the presence of "false correlations" between spectral variables and properties makes it less favorable compared to the precision and reliability of GC analysis [159].

Analysis using HPLC with refractive index detection (HPLC-RI) was compared with traditional GC via paired t-test, showing no significant statistical differences, confirming the reliability of the HPLC method with recovery rates ranged from 98.6% to 103.2% [228]. However, GC typically provides faster analysis times than HPLC for volatile compounds like ethanol as rapid separation capabilities of GC columns can result in shorter run times, making it more efficient for high-throughput analysis scenarios [155]. Each method has unique advantages and limitations, making it essential to select the appropriate technique on the basis of the context of the analysis. Since thousands of analyses are performed every day to ensure the quality of the ethanol produced and its safe use, maintaining analytical techniques/methods and developing a new technique to improve the overall analytical process are important. Currently, GC is known to be the most effective approach for analyzing organic solvents such as ethanol [229]. This technique provides great information on the amount of ethanol and is able to detect ethanol at very minimal levels. The HS-GC-FID method validated by [230] demonstrated high selectivity and precision, with an estimated expanded uncertainty of 2%, indicating its reliability for characterizing ethanol in reference materials. Moreover, this analytical technique is considered as the gold standard for ethanol analysis due to its high specificity and sensitivity. It can detect low concentrations of ethanol with limits of detection typically around 0.099 mg/mL, demonstrating excellent linearity and recovery rates between 91% and 109% [231]. Owing to its high potential for output, high resolution, specificity, sensitivity, reproducibility and accuracy, this analysis garners major interest worldwide [232]. These advantages make it an ideal analytical technique in both research and industrial applications for bioethanol production.

6. Conclusion

Biofuels, especially bioethanol, have appeared to be promising alternative renewable energy sources that can reduce the emission of greenhouse gas, burn cleanly and improve the performance of automobile engines. However, the production of second-generation (2G) bioethanol faces a few challenges that need to be overcome before it becomes a sustainable source of energy. The key challenges for this production include the accessibility and cost of feedstock, limitations in technology, impacts on the environment and the feasibility of the economy. Seizing

Table 4
Analytical techniques were applied to determine the amount of bioethanol produced in the bioconversion process.

Analytical Techniques	Reliability and accuracy	Speed	Advantages	Disadvantages	References
Biosensors (Enzyme-Based)	Have low detection limit and high sensitivity.	Fast analysis.	Simple build up, portable and easy to use in a wide range of fields or applications. Cost-effective, high sensitivity, and do not require trained personnel to operate the instrument.	Great loss of enzyme caused by their interaction with the surface of electrode; reducing the lifespan of biosensor to only 2–4 weeks.	[173,202–204]
Capillary Electrophoresis	Manage complicated samples with great resolution but poor sensitivity due to small volume of detection.	Complete within a few seconds/minutes.	Demands only small injection volumes and this might be crucial if there is only limited volume of sample. Analyzing a broad-spectrum compound ranging from small to large molecules and ions.	Poor reproducibility and the high cost of instruments.	[205–207]
Calorimetric Assay	Poor ethanol specificity.	Processed swiftly by simple step of mixing reagents.	This technique is simple, rapid response and affordable.	Tedious sample preparation and demands for large sample volume. Poor accuracy, low reproducibility and minimal stability of enzyme–substrate.	[208–210]
Cyclic Voltametric	Naturally, sensitive, highly selective, good specificity and low detection limits.	Rapid response time and able to simultaneously determine the analytes.	Produce very low noise; lead to reproducible data with great specificity and sensitivity.	The presence of background noise limits the sensitivity as it can mask the Faradic current.	[184,211,212]
Flow Injection Analysis	High analytical frequency, accuracy and precision.	Rapid response time with great reproducibility.	Simple method with low cost of operation, high rate of sampling, able to be automated, low risk of contamination, utilizing modest amounts of samples and reagents.	Demands for an automated diluted system to perform in the minimal linear range	[213–215]
Gas Chromatography	Great resolution, high sensitivity and ethanol specificity (detect other alcohol such as isopropanol, and methanol).	In comparison to HPLC, GC respond in a shorter amount of time.	Have very high resolution, selectivity, good sensitivity, great accuracy and precision, as well as wide dynamic concentration range. Can be coupled with detectors such as Flame Ionization Detector (FID) and mass spectrometry.	The requirement for analyte to be thermally stable and volatile has limits the range of compounds that can be determined by the instrument.	[139,216,217]
High Performance Liquid Chromatography (HPLC)	Offers a very accurate technique for identification of specified chemical components in prepared sample.	Slower than GC.	Able to analyze volatile and nonvolatile compounds including carbohydrates, amino acids and drugs. Can be coupled with UV/VIS, fluorescence and mass spectrometry.	Involves sample derivatization prior analysis and this process can be time-consuming, hence, affecting the accuracy and reliability of the results. Requires expensive organic materials, power supply and regular maintenance. Reliability of HPLC pump depends on the mobile phase, purity of the sample and the usage of right procedures.	[155,218]
Near-Infrared Spectroscopy (NIR)	Restricted sensitivity for detection of ethanol concentration.	Rapid analysis.	Nondestructive analysis, which is eco-friendly, has low cost of production and does not require sample derivatization. Provide an excellent depth of information and stability.	Time-consuming and require expensive tools.	[219]

(*continued on next page*)

these challenges requires recent technological development, especially to improve the bioconversion process using lignocellulosic biomass and develop suitable analytical techniques for monitoring bioethanol concentration. The ideal bioconversion approach is known as CBP as this strategy requires less preparation steps and instruments compared to other bioprocess configurations such as SHF, SSF and SSCF, thus reducing the overall cost of production. Besides, many studies have shown the capacity of CBP to reach significant amount of ethanol

Table 4 (*continued*).

Analytical Techniques	Reliability and accuracy	Speed	Advantages	Disadvantages	References
Nuclear Magnetic Resonance (NMR)	Low resolution of spectrum, overlapping signals, and restricted sensitivity.	Rapid analysis	Great capacity for reproducibility and able to recognize all components in a mixture without any pretreatment or prior separation. Offers simplified process of sample extraction and automation of protocols.	Hard to detect metabolites at minimal concentration, hence requires solvent suppression method to enhance the resolution for improved metabolite recognition.	[220–222]
Raman Spectroscopy	Narrow bandwidth and better spectral resolution. Great sensitivity and specificity to variety chemical components.	Simple technique and fast analysis.	Requires no sample preparation, simple measurement, no interference from water and CO_2, produces fast and nondestructive analysis and less contact with the sample.	The Raman signal is weak for detection of minimal concentration of substances. Easily influenced by optical system, for example, the signal of the sample can be affected by the fluorescence scattering and the fluorescence impurities may conceal the Raman spectrum.	[223–225]

yield, but the specific yield of production depends heavily on the type of feedstock and microorganism used during the bioconversion process. A study investigated the performance of CBP in direct production of ethanol from pretreated corn cob, achieving a yield of 11.1 g/L without the need for an external hydrolytic catalyst, while the maximum ethanol yield from CBP can reach up to 37 g/L. With all the benefits offered by CBP, the process is still under development for application in the biorefinery industry. Progressive research in this area will facilitate the development of more sustainable, efficient and cost-effective bioprocess, contributing to the transformation of renewable energy. The integration of analytical techniques is also important to enhance the production of bioethanol. With support from literature, GC is known to be the most effective approach for analyzing ethanol as this technique offers excellent detection of ethanol with high resolution, sensitivity, accuracy and reproducibility. This analysis can detect low ethanol concentrations with a detection limit of >0.099 mg/mL, demonstrating excellent linearity and recovery rates ranging from 91% to 109%. These advantages have turned GC to be the gold standard of analytical technique in both industrial and research applications for the production of bioethanol.

CRediT authorship contribution statement

Mona Fatin Syazwanee Mohamed Ghazali: Writing – review & editing, Writing – original draft, Investigation, Funding acquisition, Conceptualization. **Muskhazli Mustafa:** Writing – review & editing, Validation, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data used in this article has been credited to the authors.

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