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Cellulosic biobutanol by Clostridia: Challenges and improvements

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ABSTRACT

The gradual shift of transportation fuels from oil based fuels to alternative fuel resources and worldwide demand for energy has been the impetus for research to produce alcohol biofuels from renewable resources which focus on utilizing simple sugars from lignocellulosic biomass, the largest known renewable carbohydrate source as an alternative. Currently, the usage of bioethanol and biodiesel do not cover an increasing demand for biofuels. Hence, there is an extensive need for advanced biofuels with superior fuel properties. Biobutanol is regarded to be an excellent biofuel compared to bioethanol in terms of energy density and hygroscopicity, could be produced through acetone-butanol-ethanol (ABE) fermentation process. Even though the ABE fermentation is one of the oldest large-scale fermentation processes, biobutanol yield by anaerobic fermentation remains sub-optimal. For sustainable industrial scale of biobutanol production, a number of obstacles need to be addressed including choice of feedstock, low product yield, product toxicity to strain, multiple end-products and downstream processing of alcohol mixtures plus the metabolic engineering for improvement of fermentation process and products. Studies on the kinetic and physiological models for fermentation using lignocellulosic biomass provide useful information for process optimization. Simultaneous saccharification and fermentation (SSF) with *in-situ* product removal techniques have been developed to improve production economics due to the lower biobutanol yield in the fermentation broth. The present review is attempting to provide an overall outlook on the discoveries and strategies that are being developed for biobutanol production from lignocellulosic biomass.

1. Introduction

Global warming, climate change, unstability of petrol price, depletion of petroleum reservoir and severe environmental pollution due to consumption of fossil fuel for energy generation are among the recent world crisis [1]. These situations happened due to the increase in the number of human population that subsequently contribute to the increase in energy demand for industrial activities, transportations and households. Utilisation of fossil fuel has been reported as the major contributor to the increment of carbon dioxide percentage in the atmosphere [2] that brings to the rise of the global mean temperature and cause many environmental problems [3]. Due to the rapid increase of human population, there is an urgent need for scientists to find alternative energy source to solve our energy problems. The alternative energy should be renewable, clean, environmental friendly and has potential for future energy development.

Biofuel produced from biomass is one of the potential alternative energy. At present, the largest commercial biofuel produced worldwide is bioethanol, particularly in Brazil. Since the 1970s, Brazil has implemented a bioethanol fuel program which has allowed the country to become the world's second largest producer of bioethanol (after the United States) and the world's largest exporter [4]. This bioethanol produced from corn and sugar cane, which create concern on worlds food supply. Therefore, in recent studies the production of bioethanol has been switched from food source to non-food source like lignocellulosic biomass. Lignocellulosic biomass is a plant material composed of lignin, cellulose and hemicellulose in its cell wall structure. The cellulose and hemicellulose can be digested into sugar monomers [5], which can be subsequently used as substrate for fermentation. Besides, lignocellulosic biomass also abundantly available, mainly generated from agricultural and forestry activities, considered as a waste and need to be treated before discharge to environment [6]. Due to these advantages, research on utilisation of lignocellulosic biomass as fermentation feedstock for bioethanol has grown rapidly over the years.

Abbreviations: **ABE**, acetone-butanol-ethanol; **HMF**, Hydroxymethyl furfural; **PTS**, Phosphoenolpyruvate (PEP)-dependent phosphotransferase system; **SHF**, Separate hydrolysis and fermentation; **SHFR**, Separate hydrolysis and fermentation with *in-situ* recovery; **SSF,**, Simultaneous saccharification and fermentation; **SSFR**, Simultaneous saccharification and fermentation with *in-situ* recovery; **CBP**, Consolidated bioprocessing; **SF**, Severity factor; **PKC**, Palm kernel cake

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Unfortunately, bioethanol has lower energy content, more volatile and more corrosive as compared to gasoline. Thus, bioethanol cannot be directly used in cars engine and distribution system without modification. Therefore, alternative biofuel that is better than bioethanol has been searched. Out of many types of biofuels available (biodiesel, biohydrogen, biomethanol and bioethanol), biobutanol has almost similar characteristic to gasoline, overcome major problems posses in other type of biofuel [7,8]. Biobutanol can be produced through fermentation route by a microorganism, usually *Clostridium* spp. The process known as acetone-butanol-ethanol (ABE) fermentation since this microorganism produces acetone, butanol and ethanol as a solvent in its metabolic pathway [9]. Besides solvent, *Clostridium* sp. also produces acids (acetic and butyric acid) and gases (hydrogen and carbon dioxide) [10].

Although biobutanol production has been studied for many years ago, it is still unviable to compete with fossil fuel due to high production cost and impractical process for commercial production in industrial sector [11]. The cost could be reduced by employing free raw material like lignocellulosic biomass, however, there are still several major drawbacks that contribute to the limitation of cellulosic biobutanol production such as (1) multiple processing steps (from pretreatment until recovery process), (2) low sugar concentration produced from lignocellulosic biomass, (3) presence of inhibitors, (4) strain capability and (5) multiple end products that lead to low biobutanol concentration, yield and productivity. These limitations subsequently contribute to inefficient biobutanol recovery and make the whole process yet unviable for commercial production. Thus, this review paper will discuss on the ABE fermentation current progress by Clostridia that focus on the challenges and improvements of this process to produce cellulosic biobutanol as biofuel.

2. Biobutanol characteristics and applications

The production of biobutanol has been started during the late 19th century. It is the second largest fermentation in the world after bioethanol production during World War II. In 1945, it was reported that two-third of the industrially used biobutanol was produced through fermentation in the United States. However, the production of biobutanol has become non-competitive in the 1960s due to the increase of feedstock and cheaper petrochemical products [9]. At present, the biobutanol production through biological process is back to tackle the industrial attention due to the depleting resources of the non-renewable fossil fuel.

Butanol (CAS No: 71-36-3) is a primary alcohol with a structure of $C_4H_{10}O$. It is also known as butyl alcohol, *n*-butanol and butan-1-ol, a straight-chain isomer ending with an alcohol functional group –OH. The molecular weight of butanol is 74.12 g/mol. Butanol is a colourless liquid with a distinct odour and it is completely miscible with organic solvents and partly miscible with water [9]. Butanol properties include a boiling point of 177.7 °C, a melting point of −89.3 °C, an ignition temperature of 35 °C, a flash point of 365 °C, a density of 0.8098 g/mL at 20 °C, critical pressure of 48.8 hPa and critical temperature of 287 °C.

Biobutanol has a great potential in the global market to replace bioethanol as the leader in the biofuel market. The global market demand for biobutanol accounted around USD 7.0–8.4 billion with the expansion over 3% per year [12]. It has been estimated about 4.5–5.4 million tonnes of petrol-butanol are produced every year through the chemical synthesis from petroleum [13]. As a fuel blend stock, biobutanol has the potential to meet a demand of 122 million tonnes per year by 2020. The biofuel blend opportunity for biobutanol alone exceeds USD 80 billion and the overall biofuel opportunity for biobutanol is USD 700 billion. The current butanol price is around USD 4.00 per gallon equivalent to about USD 1.05 per litre estimated in 2013 [14].

Chemically synthesis butanol is widely used for manufacturing variety of products in many industries, where half of the butanol production is used in the latex industry [12]. The primary use of butanol is as a chemical intermediate in the production of other chemicals such as butyl acrylate, methacrylate and plastics. It is also being used as a solvent in the production of glycol ethers and butyl acetate for manufacturing of paint, lacquer, dyes, vegetable oil and waxes [8]. Besides, it is also being used as a solvent in the production of hormones, antibiotics and vitamins. Butanol also acts as a swelling agent for coating fabric in the textile industry and as an ingredient in eye make-up, lipstick and foundations in cosmetics industry. Recently, butanol also shows as a potential alternative fuel for transportation [7].

Biobutanol is a better option as biofuel compared to bioethanol due to its physico-chemical properties. Biobutanol receiving renewed interest because it can directly replace the use of gasoline or can be used as a fuel additive [9]. It has almost similar characteristics to the gasoline that make it a better candidate as our future energy supply. Compared to other alcoholic biofuels (bioethanol and biomethanol), biobutanol has a higher heating value, higher energy content which is 110 kBtu/gal and 25% more energy than bioethanol. These properties can reduce the fuel consumption and better mileage could be obtained. Biobutanol allegedly can be burned directly in existing gasoline engines. It is also can be used either in its pure form or in a mixture with gasoline at any concentration while bioethanol can be mixed only up to 85% [11]. This is due to the fact that the oxygen content in biobutanol is lower than in bioethanol. In addition, biobutanol can also decrease particle number concentration and emissions compared to when using gasoline only [15]. Biobutanol is feasible to be used directly, hence will not require any modifications to the existing engine system [7,8], and can be shipped and distributed through existing pipelines and filling stations [7].

With higher prices of petrol fuel and concern on the environmental problems, several groups are attempting to increase the biobutanol yield by improving the process involved in order to improve its competitiveness. Two large companies (British Petroleum and DuPont) developed several plans to convert an existing bioethanol plant for biobutanol production as soon as the technology is available [16]. There are a number of companies that are working obstinately towards the production of biobutanol from lignocellulosic biomass [17]. Since the fermentation substrate is an important factor that influencing the cost of biobutanol production, it is necessarily relevant to use inexpensive agricultural residues and wastes as fermentation feedstock [18].

3. Lignocellulosic biomass

Lignocellulosic biomass is a plant-based material composed of lignin, cellulose and hemicellulose. This class of biomass includes wood and fibrous materials from organic sources, agricultural wastes, organic municipal wastes and organic industrial wastes [19]. On average, lignocellulosic biomass composed of 38–50% of cellulose, 23–32% of hemicellulose and 15–25% of lignin. Cellulose is physically associated with hemicellulose, and physically and chemically associated with lignin [20]. The individual cellulose molecules are linked together to form elementary microfibrils, in which aggregated by intermolecular hydrogen bonding into larger subunits called fibrils. The microfibrils contain alternating phases of highly ordered (crystalline) and randomly oriented (amorphous) cellulose embedded in a matrix of hemicellulose. The cellulose and hemicellulose fractions are covered in an amorphous layer of lignin [21]. The presence of lignin and hemicellulose makes the access of cellulase enzymes to cellulose becomes difficult, thus reducing the efficiency of the hydrolysis process. The ratio of cellulose, hemicellulose and lignin within the polymer varies between different plants, wood tissues and cell wall layers [5].

The major factor to be considered in utilizing lignocellulosic biomass for biofuel production is the yield of sugar that could be obtained from the hydrolysis process. Sugar yield depends on the type

Table 1
Chemical composition of untreated lignocellulosic biomass that has been reported for biobutanol production.

Lignocellulosic biomass	Lignocellulosic composition (%)			[a]Potential sugar (%)	References
	Cellulose	Hemicellulose	Lignin		
Sago pith residue	58	23	9	81	[25]
Corn cob	45	33	15	78	[26]
King grass	37	34	6	71	[27]
Rice straw	48	16	7	64	[28]
Sugarcane baggase	42	22	20	64	[29]
Switch grass	36	28	20	64	[30]
Oil palm empty fruit bunch	39	21	19	60	[31]
Pinewood	39	24	20	59	[32]
Corn stover	31.0	20.1	25.4	51	[33]
Oil palm decanter cake	22	4	31	26	[34]
Corn stalk	32–41	23–38	11–21	55–79	[35]

[a] Potential sugar (%) = cellulose (%) + hemicellulose (%)

of substrate, pretreatment and hydrolysis process. Structural arrangement of lignocellulosic biomass (tough or soft wood, size and fibrous structure) and chemical composition (potential sugar) are important criteria to be considered. These criteria contribute to the success of pretreatment and hydrolysis process to produce sufficient sugar yield. For example, harsh pretreatment like superheated steam could yield higher sugar from hardwood as compared to softwood [22,23]. Similar situation occurred in biological pretreatment by brown-rot fungi on hardwood *Eucalyptus urophylla* (Ep) and softwood *Pinus massoniana* (Mp), where hardwood Ep showed higher carbohydrate lose than softwood Mp [24]. Besides, potential sugar composition (hemicellulose+cellulose) and/or starch (for some type of lignocellulosic biomass containing starch) significantly affect the hydrolysis yield. Table 1 shows the chemical composition of untreated lignocellulosic biomass that has been reported for biobutanol production. Lignocellulosic biomass with low potential sugar and high lignin content is not considerable as fermentation substrate since it's required extreme pretreatment to remove and/or to alter lignin composition hence generate low sugar yield.

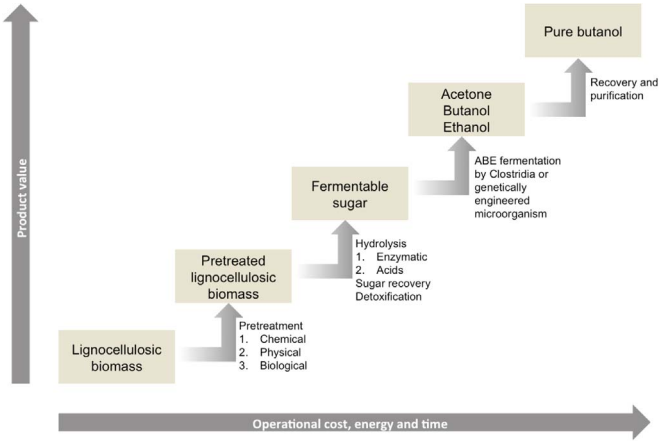

Fig. 1. Schematic diagram of the general processes involved in converting lignocellulosic biomass into biobutanol. (1) Pretreatment (2) Hydrolysis, sugar recovery and detoxification (3) Acetone-butanol-ethanol (ABE) fermentation by Clostridia or genetically engineered microorganism and (4) Biobutanol recovery and purification.

4. Challenges in cellulosic biobutanol production

4.1. Multiple processing steps

The economic goals for biobutanol production are depending on the cost of fermentation feedstock which must be available at low price [1]. Although lignocellulosic biomass has a good potential, this feedstock has to undergo multiple steps to liberate the sugars from its heteropolymer fibre (cellulose and hemicellulose). These processes include (1) pretreatment to alter and/or to remove lignin and/or structural arrangement of lignocellulosic biomass, (2) hydrolysis to produce fermentable sugar, sugar recovery and detoxification (3) ABE fermentation and (4) product recovery as shown in Fig. 1. As compared to other fermentation feedstocks like commercial glucose, sugarcane and starch biomass, lignocellulosic biomass required extra processes which are pretreatment, hydrolysis and detoxification for sugar production before this sugar can be used for fermentation to produce biobutanol. Although the product value increase through the processes, these multiple processing steps contributed to extra cost of labour, energy, equipment, time and productivity for the whole conversion of lignocellulosic biomass into biobutanol (Fig. 1).

4.1.1. Pretreatment, hydrolysis and detoxification

Pretreatment of lignocellulosic biomass is a crucial step in cellulosic biobutanol production. The cellulose and hemicellulose are tightly linked with lignin, a protective structure presence in lignocellulosic biomass. Lignin starts decomposed at temperature higher than 220 °C, while hemicellulose at 180 °C and cellulose at 280 °C [36]. A high denaturation temperature needs high energy input, makes the pretreatment become a tedious process. It is considered as one of the most expensive processing steps and the challenges of this process has been widely reported [6,37–40]. An effective pretreatment of lignocellulosic biomass should have a number of features that include [6]:

i. High recovery of all carbohydrates in the system by preserving the cellulose and hemicellulose composition.
ii. High digestibility of the cellulose and hemicellulose in the subsequent enzymatic or acid hydrolysis.
iii. The hydrolysed pretreated lignocellulosic biomass should be possible to ferment and not toxic to culture.
iv. Pretreated residues should have low energy demand or can be presented in a way so that the energy can be reused in other process steps as secondary heat.
v. Cost effective for capital and operational.

Selection of appropriate biomass pretreatment is essential to avoid excessive degradation of cellulose and hemicellulose polymer, while at the same time altering the lignin structure so that the internal cellulose and hemicellulose can be efficiently degraded during the hydrolysis. At present, none of the pretreatment method has been considered as the preeminent by considering all the factors for effective pretreatment method, especially on the operational cost, which become the most crucial factor for commercial viability. Hence, more research efforts are required to improve the pretreatment for lignocellulosic biomass.

Fermentable sugar is the primary precursor for microorganism to

synthesise various bioproducts through fermentation process. Hydrolysis of lignocellulosic biomass into fermentable sugar contributes additional challenge for cellulosic biobutanol production. This process can be done using either acid or a group of enzymes called cellulase. Acid hydrolysis process is cheaper and faster than enzymatic hydrolysis. However, the acid itself is not environmental friendly and toxic to the cells [41]. Although the acid hydrolysis process seems to be possible for cellulosic biobutanol production as reported by Qureshi et al. [42], a process called detoxification is necessary to remove the toxic presence in the acid hydrolysate which contributes additional step in converting lignocellulosic biomass into biobutanol.

The inhibitors are classified into three categories: week acids, furan derivatives and phenolic compound, produced from the hydrolysis of lignocellulosic biomass. These inhibitors can be removed from sugar hydrolysate by several strategies including physical/physicochemical, biological, chemical and other new innovation on horizons as described by Chandel et al. [43]. The effect of microbial inhibitors are proportionally increase with severity factor (SF). SF has been proposed by Overend and Chornet in 1987 to facilitate the reaction condition of time and temperature of pretreatment process into single variable and express in Eq. (1) [44]:

$$SF = log_{10}\left[t \times \frac{\exp(T_H - T_R)}{14.75}\right] \quad (1)$$

Where t is time in min, T_H is hydrolysis temperature in °C and T_R is reference temperature, most often 100 °C. Level of toxicity in ABE fermentation depends on many factors such as product inhibition, pH, type of strain, physicochemical condition, presence of oxygen and many others . Inhibitors presence in the acid hydrolysate is the principal factor that causes significant reduction in biobutanol yield. Type of inhibitors and its concentrations are varied depending on biomass composition and pretreatment. Formic acid, soluble and insoluble lignin, salts and hemicellulose degradation products such as syringaldehyde, furfural, hydroxymethyl furfural (HMF), acetic, ferulic, glucuronic, *p*-coumaric acids, and phenolic compounds can be found in acid and pretreated biomass [45,46]. The *p*-coumaric acid and ferulic acid demonstrated inhibitory effect with the concentration as low as 0.3 g/L [46].

On the other hand, enzymatic hydrolysis using cellulase is a biological process, environmental friendly and not toxic to biobutanol fermentation. Complete hydrolysis of cellulose and hemicellulose required mixture of enzymes; endoglucanase, exoglucanase, β-glucosidase, xylanase and other cellulase components, hence those enzymes act differently based on the structure of lignocellulosic biomass [47]. Enzymatic hydrolysis also produces trace of inhibitory compounds such as ferullic, acetic and glucoronic acids [48]. The detoxification process usually is not necessary. However, simple and oligomeric phenolics derived from lignin of pretreated biomass possess inhibition effect on cellulolytic enzymes, thus hinder hydrolysis process. The oligomeric phenolics have stronger impact on enzymatic reaction compared to simple phenolics with different mechanism. Simple phenolic compounds have high tendency to adsorb onto the cellulosic substrate. On the other hand, the oligomeric compounds bind to the cellulase and consequently generate complexes that inactivate the cellulase [49]. Besides, cellulolytic enzymes are expensive and add about 50% of the production cost, which become a drawback to the whole biobutanol production process [50]. The contribution of cellulase cost in lignocellulosic degradation for cellulosic biofuel production like biobutanol continues to be a much debated topic [50]. In addition, the public information on the cost of enzyme is limited [51,52]. However, the highly demanding on cellulase for various industries had brought the research and development to improve the efficiency of cellulase production in many ways.

4.1.2. ABE fermentation by Clostridia

ABE fermentation is an anaerobic fermentation that utilises carbon source to produce solvents (acetone, butanol and ethanol) and its by-products including organic acids (butryric and acetic acid) and gases (hydrogen and carbon dioxide) through the aid of anaerobes, commonly the Clostridia species. It has been discovered by Pasteur in 1861 [53]. After more than half a century, Schardinger reported the production of acetone through ABE fermentation in 1905. The work continued in 1911 through the culture isolation done by Fernbach for the fermentation of potatoes to produce biobutanol. Then, between 1912 and 1914, Chaim Weizman discovered the first *Clostridium acetobutylicum* isolates. This strain has the capability to utilise a variety of starchy substances and produces a much better yield of biobutanol and acetone compared to the original cultures of Fernbach and being industrialized during 1940s to 1950s [54]. This industrial scale used large batch fermentation ranging from 200,000 to 800,000 L capacity. They used mostly corn mash and sugarcane molasses as substrate with highest biobutanol concentration obtained at that time was 16 g/L. However, this industry faced the uneconomical production due to the product toxicity to the culture growth, large production volume with low yield when using diluted sugar, as well as the discovery of a new cheaper fuel resource after World War II which is petroleum [18].

The metabolic pathways of Clostridia consist of two distinct characteristic phases namely acidogenesis and solventogenesis [55]. The acidogenic process occurs at the initial growth phase (usually within the first 24 h) while the solventogenic process occurs at the stationary phase of the microbial growth (usually after 48 h) [31]. During the acidogenic phase, the rapid growth of the microorganism produces cells, biohydrogen, carbon dioxide, butyric acid and acetic acid. This stage indicate by a drop of pH due to the rapid secretion of butyric and acetic acid into the medium [56]. These acids mainly produced within 24 h of the fermentation [31]. As the pH drops even further, metabolism shifts from acidogenic phase to the solventogenic phase. During the solventogenic phase, the acids produced from acidogenic phase are reassimilated into the cells and subsequently increase the pH value. The carbon source is continuously consumed and converted into acetone, butanol and ethanol.

4.1.3. Biobutanol recovery and purification

The production of cellulosic biobutanol by Clostridia faces several challenges including difficulties in recovery process. A low biobutanol yield less than 25% (w/w) and biobutanol concentration less than 20 g/L in fermentation broth has made the recovery process become uneconomical [57]. The energy required for separation of biobutanol from fermentation broth is higher than energy required for petrol-butanol production. It was estimated between 8 and 24 kJ/g of biobutanol were required for recovery process using adsorption-desorption, gas stripping, extraction, pervaporation and conventional distillation, which significantly contributes to high operational cost [58]. Among all the recovery methods, gas stripping is the simplest method, has low energy input, cheaper set up and does not harm to the culture. However, recovery yield from gas stripping is relatively low, which then required continuous purification that leads to intensive energy input. This method also needs continuous supply of nitrogen gas which add to the operational cost [59]. Besides, biobutanol also possess the azeotropic properties which cause the recovery process through distillation become an inefficient method [10]. Therefore, novel strategies should be discovered to overcome these challenges.

4.2. Products inhibition

4.2.1. Acid inhibition

The biphasic metabolism of *C. acetobutylicum* from an acidogenic followed by solventogenic phase are responsible for the ABE fermentation. The production of undissociated butyric acid by *C. acetobutylicum*

showed linear interaction with the specific production of biobutanol, suggesting the butyrate as the main factor influencing the biobutanol fermentation [60]. However, the surplus amount of acetic and butyric acid produced by *C. acetobutylicum* at its maximum growth rate cause the failure of transition from acidogenic to solventogenic phase [61], hence lead to the toxicity effect [62]. The growth of cells in the medium with uncontrolled pH cause the inhibition of solvent production, or the phenomenon called "acid crash" [63]. The high concentration of butyric acid produced as compared to acetic acid can be toxic that bring to the premature termination of fermentation process [64].

In other findings, Qureshi et al. [65] speculated that acetic acid production did not affect solventogenesis and biobutanol production from wheat straw hydrolysate. The results showed that the high level of acetic acid (5.9 g/L) as compared to butyric acid (1.2 g/L) is not the main factor contributing to the production of biobutanol by *C. beijerinckii*. It was also reported that the lactic acid and acetic acid produced by *C. pasteurianum* from glycerol up to 16 g/L and 10 g/L, respectively did not significantly affect the biobutanol production [66]. Apparently, Wang et al. [63] reported that instead of acetate and butyrate, formic acid is known to be able to trigger the "acid crash" phenomenon.

It has been known that pH control is one of the crucial steps for optimizing the biobutanol production. However, there are still unclear explanations regarding the suitable pH for the production of biobutanol and at what pH level the accumulation of acid and "acid crash" phenomenon will start to occur. According to Maddox et al. [62], the pH value less than 5.0 was able to activate the "acid crash" due to insufficient production of acids, hence bring to the termination of solventogenesis. For that reason, the determination of initial pH in the ABE fermentation is critically important to ensure the switch of acidogenic to solventogenic phase can take place. Qureshi et al. [42] reported that the initial pH near 6.8 in P2 medium, adjusted with 1 M NaOH for biobutanol production by *C. beijerinckii* showed reduction to pH 5.0–5.5 during the fermentation, which then able to activate the solventogenesis, hence resulted in the high production of biobutanol.

4.2.2. Butanol toxicity

Attempts have been made to overcome the butanol toxicity problem as it is considered as the major challenge in the ABE fermentation. The incomplete utilisation of glucose by the culture cells were reported which resulting from the butanol toxicity; hence lead to lower productivity and reduction of biobutanol recovery [67]. The main drawback on the ABE fermentation is the ability of the culture cells to survive in the high solvent concentration. According to Ezeji et al. [68], the ability of the solventogenic bacteria to trigger the adaptation mechanisms is crucial to ensure the toxicity of biobutanol on the cell membrane and enzymes involved can be prevented, hence conserve the functionality of the cell. The transportation of solute including sugars into the cell cytoplasm by the relatively low GC-content, Gram positive bacteria including solventogenic *Clostridium* through the phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS) is a critical way for further metabolism and later on for the synthesis of ATP for the cell growth. The higher productivity of biobutanol rely on the competency of the solventogenic Clostridia for the uptake of sugar and its accumulation in the cytoplasm, before the conversion of sugar into pyruvate and finally reduction into ABE. Therefore, the disruption in the glycolysis will cause the conflicts on the cell growth, hence lowering the productivity of biobutanol [69].

According to Mitchell et al. [70], the PTS is composed of two types of proteins; membrane-bound and soluble proteins, which important in the sugar uptake and pyruvate generation. Commonly, Enzyme I and HPr are the two cytoplasmic proteins function for the PTS, which involved in the uptake and the phosphorylation of the substrates. The reduction in the PTS activity recorded in the cell extract of *C. beijerinckii* BA101 consist of glucose, fructose and glucitol indicates the irregularity of the PTS proteins [71]. The study conducted on the PTS system of *C. acetobutylicum* NCIB 8502 showed the presence of both Enzymes I and HPr in the cytoplasmic fraction of the cell. However, the presence of Enzyme I and III^{Glc} also have been observed in the membrane fractions, which indicate the association of the PTS proteins with the cell membrane. Therefore, the high concentration of biobutanol will direct or indirectly affect the cell membrane fluidity, which influence the transportation of sugar by the PTS system, hence resulted in the reduction of pyruvate formation and finally interruption in the glycolytic pathway [68].

Qureshi et al. [72] reported that the biobutanol concentration between 15 and 20 g/L is toxic to the culture cells, thus inhibit the glucose consumption by the fermenting microorganisms. Similar findings has been reported by Ezeji et al. [48], which showed the inhibition of cell growth in the reactor started to occur at 16 g/L of biobutanol. However, the level of biobutanol toxicity is depending on the strains used, which commonly can tolerate at 1.0–1.5% (v/v) of biobutanol concentration. *C. tyrobutyricum* ATCC 55025 was able to stand up at 1.5% (v/v) biobutanol with relatively 30–50% of growth rate reduction, meanwhile *C. beijerinckii* ATCC 55025 showed an approximately 85% of growth rate reduction at the same biobutanol concentration [73]. The effects of butanol toxicity to the microorganism lead to the low concentration of ABE in the culture broth which is around 1.5% (v/v), as compared to ethanol fermentation which can reach more than 15% (v/v), 10 times higher than biobutanol [67]. The butanol toxicity phenomenon causes the ABE fermentation and recovery tougher and more challenging as compared to the ethanol production [67]; hence increase the recovery cost [48].

4.3. Strain capability

Numbers of Clostridia have been employed for the production of biobutanol. They are rod–shaped, strictly anaerobes, Gram positive and spore-forming when introduce to non–favourable conditions [9]. Among the solventogenic Clostridia, *C. acetobutylicum* is the most commonly used for ABE production. This species was first deposited by Weyer and Rettger as strain W which is now known as ATCC 824 [74]. In 2001, the genome of *C. acetobutylicum* has been completely sequenced by Nölling et al. [75]. The complete genome sequence helps researchers to find out the gene regulation in ABE production as well as improving the metabolic pathways for biobutanol production. *Clostridium* spp. like *C. beijerinckii, C. saccharobutylicum* and *C. saccharoperbutylacetonicum* are also among the most vigorously studied for ABE fermentation [76] with higher levels of biobutanol was produced. *C. saccharobutylicum* was originated from *C. acetobutylicum* [type strain N1-4 (HMT) and ATCC 27021^T] and *C. saccharoperbutylicum* [type strain DSM 13864^T and ATCC BAA-117^T] after amended by Keis et al. [77]. Previous works showed the biobutanol production ranging from 0.30 to 18.01 g/L using these varieties of microorganisms. These Clostridia have the capability to consume both pentose and hexose sugars [54]. Complex nitrogen content such as yeast extract, meat extract and peptone are required for good cell growth and biobutanol production [78].

Despite of having biobutanol toxicity and acid crash inhibition phenomenon, Clostridia also has limitation to substrate concentration. It was reported that the sugar concentration higher than 100 g/L would decrease the biobutanol yield and productivity [79]. The optimal sugar concentration introduced to Clostridia for biobutanol production was at 60–90 g/L depending on the strain and sugar source. This situation will limit the strain to produce higher biobutanol concentration in the ABE fermentation system.

4.4. Multiple end products

Major end products of ABE fermentation are classified into three categories; solvents (butanol acetone, ethanol), organic acids (acetic and butyric acid) and by-product gases (carbon dioxide and biohydro-

gen) [80]. Typically, the maximum total amount of solvents in ABE fermentation by *Clostridium* species is less than 20 g/L with the ratio of butanol: acetone: ethanol is 6:3:1 [9]. The average biobutanol concentration could be obtained is below 15 g/L. The mass ratio of biobutanol over total solvents is about 60–70%, and below 30% over the feedstock [81]. This also indicates that the bacterial cells are only capable to tolerate up to 20 g/L of solvent concentration only with average sugar consumption approximately 60 g/L [64], mainly due to solvent toxicity [82]. The sugar provided in the fermentation will be used for formation of other by-products instead of being strictly converted into biobutanol. Low biobutanol yield increase energy consumption for recovery and purification process and subsequently increase the whole operational cost [81]. The distillation process accounts about 20% of the total operational cost [83], but could be significantly reduced if the biobutanol concentration can be increased from 13 g/L to 19 g/L [81].

5. Improvements

Many improvement strategies have been developed for enhancement of cellulosic biobutanol production, including strain development, pretreatment of lignocellulosic biomass, hydrolysis, detoxification, ABE fermentation and recovery processes. Every single strategy that could improve the challenges associated with cellulosic biobutanol production will be intensively described in the following sub-section.

5.1. Strain Improvement

Genetic engineering is a powerful tool that can be used to improve the capability of strains for biobutanol production. This approach could enhance solvent production, reduce biobutanol toxicity, increase biobutanol ratio, allow strain to grow in a complex cellulosic substrates and develop new strain as host for biobutanol production [84]. Besides that, development of molecular biology and genetic tools brings to the understanding of the genes and metabolism involved in the ABE fermentation. Up to date, many effective and efficient molecular approaches have been proposed and developed for enhancement of biobutanol production by engineering the metabolic regulatory system of biobutanol producers, either by overexpressing genes associated with butanol formation, altering metabolic enzyme activities and pathway or knocking out genes associated with formation of by-products.

In order to achieve these aims, metabolic engineering of butanol pathway derived from Clostridia is the most frequently reported approach. The *adhE1* mutant of *C. acetobutylicum* ATCC 824, defective in alcohol/aldehyde dehydrogenases 1 developed by Cooksley et al. [85] produced very little solvents, demonstrating the significance of this particular gene in the solvents production. Similar finding has been reported by Bhandiwad et al. [86], whereby no butanol has been produced following deletion of *adhE*, suggesting diversion of carbon flow into lactic acid pathway. However, the overexpression of *adhE2* from *C. acetobutylicum* ATCC 824 in *C. tyrobutyricum* ATCC 25755 was able to enhance biobutanol production up to 1.1 g/L, caused by conversion of butyryl-CoA to butanol [73]. In addition, elimination of by-products, such as acetone is desired to enhance the production of biobutanol. Disruption of acetoacetate decarboxylase gene (*adc*) in *C. acetobutylicum* EA 2018 was able to reduce the acetone production, hence increase the butanol ratio from 70% to 80.05% [87]. A role of *adc* gene in acetone synthesis pathway was proved by Gong et al. [88] where the absence of this gene in *C. tetanomorphum* results in production of butanol without acetone. Meanwhile, a different approach to minimize or suppress the production of acetone was reported through disruption of acetone metabolic pathway by converting acetone to isopropanol, whereby isopropanol can be used as a fuel additive [89,90].

Furthermore, given the difficulties in performing genetic manipulations in Clostridia due to its complex metabolic characteristics, *Escherichia coli* and *Saccharomyces cerevisiae* have been widely used as hosts for overexpression of genes involved in butanol production pathway. Sakuragi et al. [91] demonstrated the introduction of butanol pathway together with elimination of glycerol production pathway in *S. cerevisiae*, also with addition of trans-enoyl-CoA reductase in the engineered pathway enhanced the butanol production to yield 1.41 g/L. A different study by Krivoruchko et al. [92] showed an enhancement of butanol production in engineered *S. cerevisiae* by improving cytosolic acetyl-CoA supply, which act as the precursor metabolite for 1-butanol biosynthesis. Besides that, an engineered butanol biosynthetic pathway in *E. coli* was reported able to enhance biobutanol production. Atsumi et al. [93] performed the overexpression of *adhA* gene encoding alcohol dehydrogenase I derived from *Lactococcus lactis* to enhance biobutanol production by increasing activity of *adhA* towards isobutaraldehyde. Halloum et al. [94] suggested that alcohol dehydrogenase from *L. brevis* LB19 can be used as a potential enzyme for pathway engineering of the novel alcohol biofuel. Due to potential of biobutanol as chemical feedstock and advanced biofuel, a variety of genetic engineering approaches are being studied to enhance its production and increase resistance to butanol toxicity. Other examples of successful studies are shown in Table 2.

5.2. Substrate pretreatment and medium formulation

Pretreatment is a crucial step to convert lignocellulosic biomass into sugar prior to ABE fermentation. Numerous studies and improvement strategies have been widely reported with the aim to improve the hydrolysis performance and subsequently increase the sugar yield. Out of three major options (chemical, biological and physical pretreatment), chemical pretreatment showed significantly more efficient than biological and physical pretreatment. It is a fast and cheap process. Chemical pretreatment using 12% NaOH yielded 69% sugar while pretreatment using 85% concentrated phosphoric acid yielded 50% sugar [103]. Certain substrate required only 70% concentrated phosphoric acid to produce the same sugar yield [104]. Alkaline pretreatment using 2% NaOH combined with hydrothermal using autoclave on oil palm empty fruit bunch fibre yielded 70% sugar [31]. ABE fermentation using the sugar obtained from this pretreatment yielded approximately 0.10–0.15 $g/g_{substrate}$ of biobutanol. These chemical pretreatment processes require between 3–6 h. However, due to environmental factors when using chemical, dilute acid or alkaline pretreatment and/or alternative chemical-free pretreatment are preferable. Dilute alkaline pretreatment for ABE fermentation produced significantly comparable yield of sugar and biobutanol as has been previously reported [29,35,105] using various types of substrate and fermentation mode as shown in Table 3. It was also reported that acetone pretreatment could produce about 36 g/L sugar with 94% hydrolysis yield and subsequently fermented to 11.4 g/L ABE [106]. Many researchers used sulphuric acid pretreatment along with steam explosion, hydrothermal, ammonia and NaOH pretreatment methods [44]. A superior lignocellulosic biomass like sago pith residue that produced comparable yield of sugar without pretreatment prior to hydrolysis has also been reported [25,107].

Along with pretreatment, improvement on detoxification process has also been done for optimal biobutanol production. Shukor et al. [108] studied on the detoxification effect of acid hydrolysate of palm kernel cake (PKC) where polymeric adsorbent resin (XAD-4) employed in this work successfully removed 77.42% of HMF and 50% furfural. Besides removing inhibitor compounds, detoxification also helps to enhance sugar recovery from PKC hydrolysate and subsequently improves the biobutanol yield. However, there are several biobutanol strains are not affected by the presence of the inhibitors. These inhibitor tolerant bacteria provide a great potential to be introduced in the biomass hydrolysate. Marchal et al. [109] investigated on the effect of acidic and enzymatic hydrolysates towards biobutanol produc-

Table 2
Biobutanol production by genetically engineered microorganism.

Microorganism	Genetically engineered strategies	Improvement remarks	Biobutanol production (g/L)	References
Clostridium acetobutylicum	Knock-out of histidine kinase	Enhanced *n*-butanol tolerance and production	21.00	[95]
Clostridium acetobutylicum	Double overexpression of 6-phosphofructokinase (pfkA) and pyruvate kinase (pykA) genes	Enhanced butanol production by increasing the intracellular concentrations of ATP and NADH; enhanced resistance to butanol toxicity	19.12	[96]
Clostridium tyrobutyricum	Overexpression of extracellular α-glucosidases (*aglu1*, *aglu11*) from *C. acetobutylicum* in *C. tyrobutyricum*	Enhanced *n*-butanol production by catalyzing the hydrolysis of α−1,4-glycosidic bonds in maltose and starch from the non-reducing end	17.20	[97]
Clostridium tyrobutyricum	Overexpression of xylose proton-symporter (*xylT*), xylose isomerase (*xylA*), and xylulokinase (*xylB*) from *C. acetobutylicum*, co-expressed with aldehyde/alcohol dehydrogenase (adhE2) in *C. tyrobutyricum*	Enhanced butanol production from lignocellulosic biomass through co-utilisation of glucose and xylose by engineered strain	15.70	[98]
Escherichia coli	Random mutagenesis of global transcription factor cyclic AMP receptor protein (CRP) of *E. coli*	Improved *n* -butanol tolerance	9.70	[99]
Escherichia coli	Engineered the metabolite nodes in the central metabolism of *E. coli*	Enhanced *n*-butanol production by enhancing pyruvate decarboxylation in the oxidative pathway; blocked pathway for G6P isomerization to force the glycolytic flux through the pentose phosphate pathway; increased NADH level	6.10	[100]
Saccharomyces cerevisiae	Optimization of synergistic pathway (the endogenous threonine pathway and the introduced citramalate pathway); overexpression of KDC-like and ADH enzymes to push the flux from α-ketovalerate to *n*-butanol	Enhanced *n*-butanol production	0.84	[101]
Saccharomyces cerevisiae	Deletion of aldehyde dehydrogenase (*ALD6*) and valine synthesis (*BAT1*); expression of a constitutively active form of Leu3 transcriptional activator; overexpressed genes involved in isobutanol production; overexpressed leucine biosynthetic genes	Enhanced 3-methyl−1-butanol production by eliminating competing pathways, increasing transcription of endogenous genes in valine and leucine biosynthetic pathways	0.77	[102]

tion in batch culture by *Clostridium acetobutylicum*. The acid hydrolysate of corn fibre exhibited low yield of biobutanol compared to enzymatic hydrolysate (0.35 g/g). Severe pretreatments liberated inhibitors that will hinder not only for bacteria growth in biobutanol fermentation but also enzymatic saccharification. Qureshi et al. [65] showed that detoxification of alkaline peroxide of pretreated wheat straw using electrodialysis with the subsequent enzymatic hydrolysis enhanced biobutanol production up to 10-fold.

Despite other improvement strategies mentioned above, limitation of nitrogen and access of carbon in the fermentation medium will also trigger the cell to produce more ABE than acids [56,110]. A suitable medium formulation could enhance biobutanol production. The study showed that glucose, tryptone, yeast extract, peptone, K_2HPO_4, Na_2CO_3, and $MgSO_4$ had significantly affecting the biobutanol production [111], however, depending on the source of sugar hydrolysate and bacterial strains [34]. P2 medium was introduced by Monot et al. [78] which later has widely used by many researchers for biobutanol production by Clostridia. A 60 g/L of glucose has been found as the most suitable concentration for biobutanol production, which was tested on *C. beijerinckii* 8052, BA101 [112], *C. acetobutylicum* B3 [113], ATCC 824 [78] and several other strains. A higher sugar concentration than 60 g/L could inhibit the cell growth. Besides, the used of methyl viologen as electron carrier was initially reported by Tashiro et al. [114] that proved the addition of 0.1 mg/L of methyl viologen enhanced the biobutanol yield from 0.577 to 0.671 mol/mol of glucose. Methyl viologen could divert the electron flow away from H_2 production and reinforce the NAD(P)H supply, which increased biobutanol yield up to 37.8% by severely diminished acetone production.

5.3. Integrated fermentation and recovery technology

The conventional ABE fermentation using fermentable sugar from pretreated lignocellulosic hydrolysate involves many steps and processes, which contributed to additional cost for apparatus, material, time and labour. Combination or integration several steps together could improve the total yield ($g/g_{raw\ substrate}$), productivity and reduce the operational cost [117]. Fig. 2 (modified from Jouzani and Taherzadeh [118]) shows several possible integrated fermentation and recovery strategies that could overcome several problems associated with cellulosic biobutanol production.

5.3.1. Simultaneous saccharification and fermentation

Simultaneous saccharification and fermentation (SSF) is a process where saccharification and ABE fermentation conducted simultaneously in the same vessel and at the same time. This process can be done by adding the cellulase (to perform the saccharification) and inoculum (to perform the ABE fermentation) into a flask containing pretreated lignocellulosic biomass (carbon source) [117]. In a usual practise using separate saccharification and fermentation (SHF), the saccharification is conducted to produce fermentable sugar before this sugar is being recovered for ABE fermentation (Fig. 2). The SSF process could reduce the number of step in cellulosic biobutanol production and therefore could possibly reduce the overall operational cost. It could also produces higher biobutanol yield and productivity, and prevent glucose inhibition to the β-glucosidase in the cellulase system due to immediate glucose consumption by microorganism during SSF [117].

Unfortunately, only few studies had been reported on the SSF for biobutanol production. Qureshi et al. [119] reported on the production of 13.12 g/L of ABE from wheat straw through SHF as compared to 11.93 g/L using SSF, with only 9% difference. The sugar released from these two processes was 41.9 g/L and 25.6 g/L, respectively, become a major concern to improve the SSF process. All the sugar was consumed and no inhibition effect was observed during fermentation although the system containing remaining un-hydrolysed lignocellulosic material. A

Table 3
Pretreatment of lignocellulosic biomass for biobutanol production by *Clostridium* sp.

Substrates	Pretreatment strategies	Hydrolysis and detoxification	Sugar recovery/initial sugar conc. (g/L)	Hydrolysis yield (%)	Biobutanol production			References
					Conc. (g/L)	Yield (g/$g_{total\ sugar}$)	Prod. (g/L/h)	
Barley straw	• Milled to 1–2 mm • 1% sulphuric acid pretreatment, autoclaved at 121 °C for 1 h	• Commercial enzymes; cellulase 4.5 U/mL, β-glucosidase 2.3 U/mL, xylanse 59 U/mL • Detoxification using overliming method [115]	42.2 Concentrated/diluted to 60	NA	7.5	0.12	0.27	[72]
Corn stalk	• 2% NaOH, heating at 100–120 °C for 1 h	• Commercial cellulase: 40 FPU/$g_{substrate}$ • No detoxification	32–40	NA	7.5–9.4	0.23–0.24	NA	[35]
Corn stover	• 2% NaOH followed by ionic liquid [Bmim][Cl], can be recycled at least 10 times. Heating at 130 °C for 2 h	• Commercial cellulase: 30 FPU/$g_{substrate}$ • No detoxification	18.7	NA	7.9	0.21	0.11	[116]
Oil palm decanter cake	• 1% NaOH pretreatment, autoclaved at 121 °C for 20 min	• In-house crude cellulase • No detoxification	4.4 Concentrated to 70	69	6.0	0.11	0.08	[34]
Oil palm empty fruit bunch	• 2% NaOH, 4 h soaked, 15 min autoclaved	• Commercial cellulase: 5.0 u/mL β-glucosidase • No detoxification	31.6 Diluted to 25.0	NA	1.9	0.10	0.02	[117]
Palm kernel cake	• Grinding and sieving to 600 μm • Liquid hot water	• Commercial enzyme; (cellulase: 14.3 FPU, β-glucosidase: 59.0 IU, mannase 158.9 IU)/$g_{substrate}$ • No detoxification	98	98	4.2	0.08	0.04	[108]
Sago pith residue	• No pretreatment	• In-house crude cellulase: 23.4 IU β-glucosidase • No detoxification	21 Concentrated to 30	NA	5.4	0.18 g/$g_{glucose}$	0.08	[25]
Sugarcane baggase	• Ground to 40 mesh particle size • 1% NaOH, 60 °C, 3 d	• In-house crude cellulase: 5 mL/L • No detoxification	61.3 Concentrated to 80	98	7.7	0.09	NA	[29]
Sweet sorghum bagasse	• 50% acetone solution, heating at 180 °C for 1 h	• Commercial cellulase: 25 FPU/$g_{substrate}$ • No detoxification	36.3	94	7.1	0.20	0.25	[106]
Switch grass	• Ground and sieved to 2 mm	• Commercial cellulase at 50 FPU/$g_{substrate}$	80	58	0.99	0.07	0.01	[30]
	• Hydrothermolysis in 1 L parr reactor, 200 °C, 10 min, 500 rpm	• No detoxification • Commercial cellulase: 50 FPU/$g_{substrate}$ • Detoxification using 10% rod shape activated carbon	80	58	11.0	0.20	0.15	[30]

NA – Not available
Conc. – Concentration

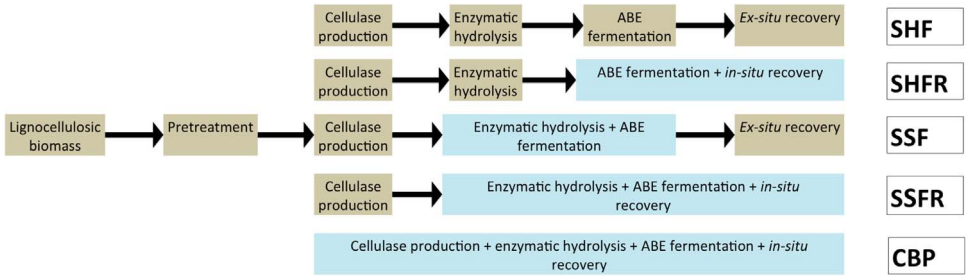

Fig. 2. Bioprocessing strategies for production of cellulosic biobutanol. SHF: Separate hydrolysis and fermentation, SHFR: Separate hydrolysis and fermentation with *in-situ* recovery, SSF: Simultaneous saccharification and fermentation, SSFR: Simultaneous saccharification and fermentation with *in-situ* recovery and CBP: Consolidated bioprocessing.

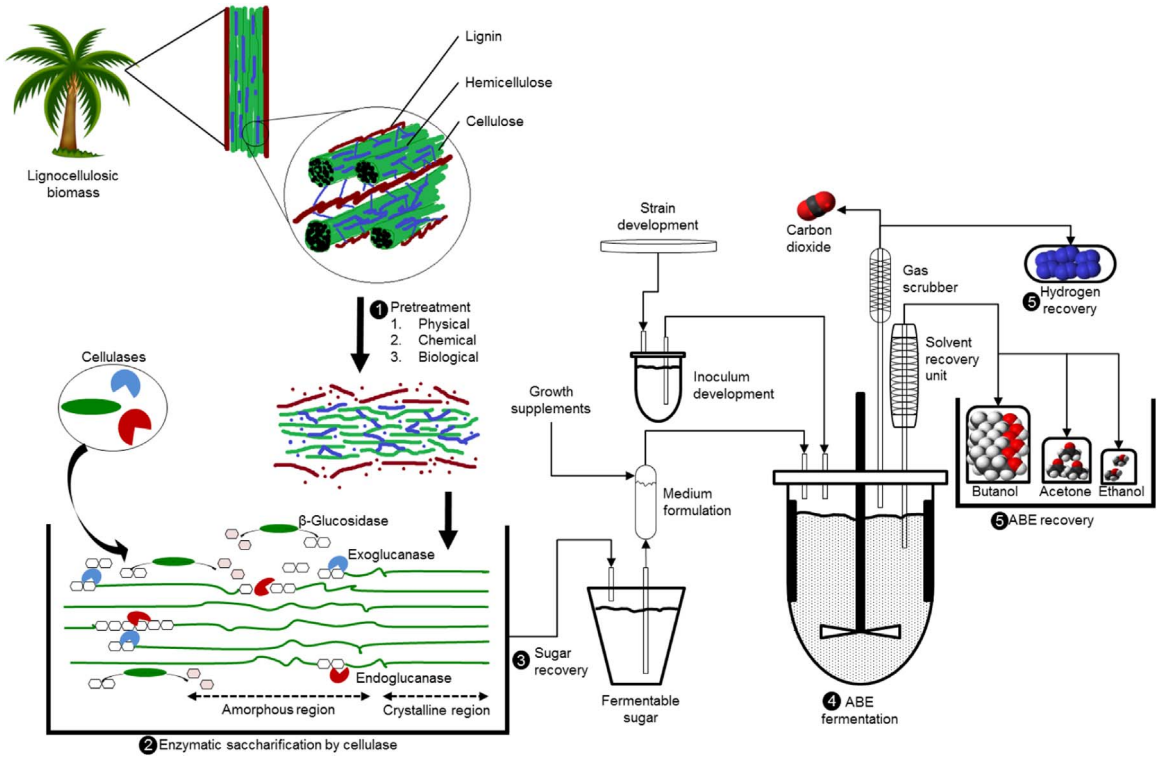

Fig. 3. Schematic diagram of the processes involved in acetone-butanol-ethanol (ABE) fermentation using lignocellulosic biomass as substrate. (1) Pretreatment to remove or to alter the lignin structure (2) Enzymatic saccharification by cellulase to produce fermentable sugar [127] (3) Sugar recovery and detoxification process (4) ABE fermentation by Clostridia or genetically engineered microorganisms and (5) *In-situ* product recovery for ABE and biohydrogen. Process (2, 3, 4 and/or 5) can be combined together for consolidate bioprocessing technology.

100% of ABE conversion rate was obtained by Sasaki et al. [120], using 6% of wood chip as substrate through SSF. Their study obtained 13.41 g/L of ABE as compared to 15.27 g/L of ABE obtained through SHF, with 12% difference. Ibrahim et al. [117] obtained higher ABE (4.45 g/L) through SSF as compared to only 2.51 g/L of ABE through SHF, using oil palm empty fruit bunch (OPEFB) as substrate. SSF using overlimed pretreated corn stover produced 14.2 g/L ABE as compared to 13.31 g/L ABE obtained through SHS [121], showing the potential of SSF to be implemented for biobutanol production.

The major challenge considering in SSF process is optimal temperature condition difference for cellulase activity (45–60 °C) *versus* ABE fermentation condition (30–37 °C) [117,118]. The enzymatic hydrolysis usually performed at lower temperature than its optimal condition to meet the ABE fermentation condition, since Clostridia prohibit at temperature higher than 40 °C. This process could affect the hydrolysis performance and subsequently increase enzyme utilisation. Up to date, there is no report on thermotolerance bacterial strain for SSF at high temperature for biobutanol production although many studies have been widely reported for SSF bioethanol production. However, a process called non-isothermal simultaneous saccharification and fermentation (NSSF) [118] was introduced to overcome this problem that showed more advantageous than SSF.

5.3.2. *In-situ* recovery and purification

In-situ product recovery technique has been well accepted as a promising technique to reduce the butanol toxicity. It was reported that the biobutanol concentration in the fermentation broth could be increased by up to 2% by conducting this technique. Several *in-situ* recovery techniques have been investigated and keep on improving for higher recovery yield, low energy input and cost effective. In recent reports, Wiehn et al. [122] recovered about 81% of biobutanol using *in-situ* expanded bed adsorption. This technique increased up to 2.3-fold of the biobutanol concentration in the system as compared to conventional batch culture. Permeating-heating gas stripping has only able to extract the mixture of ABE with biobutanol percentage of 15% (w/v) and concentrated up to 70% (w/v) using phase separation [113]. Two-stage *in-situ* gas stripping has been introduced by Xue et al. [59] to enhance biobutanol fermentation and reduce the recovery energy consumption. The process has concentrated the biobutanol from 175.6 g/L in the first stage to 420.3 g/L of biobutanol after the second recovery stage. Another technique is vacuum recovery that has been developed for ethanol recovery from fermentation beer [123] and

tested for integrated *in-situ* recovery of ABE from fermentation broth [67]. This recovery process resulted in a reduction of fermentation time, complete utilisation of glucose, greater cell growth, and more concentrated product stream than SHF without recovery [124].

Combination of these *in-situ* recovery strategies with suitable mode of ABE fermentation may also enhance the biobutanol formation. For example, the biobutanol recovery using two-stage gas stripping coupled with fed-batch fermentation has increased the biobutanol concentration up to 48.5 g/L after first stage gas stripping, with highly concentrated condensate as higher as 515.3 g/L biobutanol could be obtained after applying the second stage gas stripping [125]. *In-situ* recovery has not limited to biobutanol separation only, but has also been designed and tested for continuous co-recovery of biohydrogen and biobutanol. A process design known as dual immobilization reactor and continuous recovery (DIRCR) by Ramey and Yang [126] described the co-recovery of biohydrogen and biobutanol in one system. The SHF with *in-situ* recovery of biobutanol and biohydrogen is also illustrated in Fig. 3.

5.3.3. Simultaneous saccharification and fermentation with in-situ recovery

Integration of *in-situ* recovery with SSF (namely SSFR as referring to Fig. 2) into single operation could also be performed. The process has been conducted by Qureshi et al. [121] that obtained 20.79 g/L ABE through SSFR, 32% higher than SSF alone that produced 14.20 g/L ABE. The process also showed that sugar level and product concentration does not inhibit the cell metabolism, thus the culture produced ABE vigorously than SSF without *in-situ* recovery. Another attempt was done by Qureshi et al. [128] who had suggested that the combination of all three processes in an integrated system (SSFR) appears to be the most attractive for economic reasons as compared to SSF alone or SHF with *in-situ* recovery due to process complexity. In that particular studies, it showed that the microorganism was not interfere by the recovery process, yet performed better than SHF. However, the substrate should be partially hydrolysed to reduce viscosity and improve dissolvability. The successful studies of integrated bioprocessing technology for biobutanol production are summarized in Table 4. This summary showed that the integration process is possible to be conducted with higher ABE titer, productivity and yield as compared to single operational process. However, the process required further improvement in terms of significant model simulation and optimization together with economic and feasibility studies for industrial application. It should be noted, there is no report on the CBP for biobutanol production although this technology has been recently reported for bioethanol production.

5.3.4. Consolidated bioprocessing

In the effort to reduce the number of steps for cellulosic biobutanol production, consolidated bioprocessing (CBP) has been introduced to combine all the major processes into single operation system as shown in Fig. 2. However, CBP has only been investigated increasingly in recent years for cellulosic bioethanol production. This recent technology showed a promising fermentation approach that could overcome the cost constrain for cellulosic biofuel production [129]. CPB could be conducted by using a single microorganism or microorganism consortia that able to produce cellulase, conduct the hydrolysis and ABE fermentation in a single step. An *in-situ* recovery process could also been integrated with the system to improve the whole process into single run. Despite of reducing number of step and operation cost for apparatus, materials and time, there are several other advantages that have been discussed for CBP on biofuel production such as reduction on the cost of purified enzyme and prevent substrate/product inhibition [118]. The pretreatment process also could be possible to be integrated together into single operation system if effective microorganism could be found to remove lignin simultaneously with hydrolysis and ABE fermentation process. However, combining all processes into single operating unit might reduce the biobutanol titer due to competition among biological process and increase process complexity that will risk the whole production operational process.

5.3.5. Co-production from ABE fermentation

The ABE fermentation also faced the problem of multiple by–products formation during the fermentation (acids, ABE and gases) which contributed to difficulties in purification and recovery process [76] especially for biobutanol. The acids produced in the system ceased the cells metabolic pathway and thus decreasing the ABE production [62]. Studies on the controlling of the amount of acids produced during the ABE fermentation were conducted in order to improve the ABE production. Since the biobutanol production in ABE fermentation depending on the acids accumulation in the system, Li et al. [130] studied on the possibility of using the co-culture *C. beijerinckii* (producing biobutanol) and *C. tyrobutyricum* (producing acids) in a

Table 4
Integrated bioprocessing technology for biobutanol production by *Clostridium* spp.

Substrate	Microorganism	ABE production				Reference
		Conc. (g/L)	Productivity (g/L/h)	Yield (g/g $_{susbtrate}$)	Recovered ABE (g/L)	
Separate hydrolysis and fermentation (SHF)						
Glucose	*C. acetobutylicum* JB200	25.5	0.48	0.32	–	[59]
Wood chip *Quercus acutissimaas*	*C. acetobutylicum* NBRC 13948	15.29	0.13	0.31	–	[120]
Corn fibre	*C. beijerinckii* BA101	9.3	0.10	0.39	–	[42]
Sago pith residue	*C. acetobutylicum* ATCC 824	4.22	0.06	0.20	–	[25]
Oil palm empty fruit bunch	*C. acetobutylicum* ATCC 824	2.51	0.03	0.13	–	[117]
Separate hydrolysis and fermentation with *in-situ* recovery (SHFR)						
Corn stover	*C. beijerinckii* P260	50.14	0.70	0.43	127.57	[72]
Barley straw	*C. beijerinckii* P260	47.20	0.60	0.42	135.15	[72]
Glucose	*C. acetobutylicum* JB200	31.8	0.66	0.40	532.3	[59]
Simultaneous saccharification and fermentation (SSF)						
Wheat straw	*C. beijerinckii* P260	17.92	0.19	0.40	–	[119]
Corn stover	*C. beijerinckii* P260	14.20	0.22	0.30	–	[121]
Wood chip *Quercus acutissimaas*	*C. acetobutylicum* NBRC 13948	13.41	0.09	0.27	–	[120]
Oil palm empty fruit bunch	*C. acetobutylicum* ATCC 824	4.45	0.05	0.19	–	[117]
Simultaneous saccharification and fermentation with *in-situ* recovery (SSFR)						
Wheat straw	*C. beijerinckii* P260	21.42	0.31	0.41	–	[119]
Corn stover	*C. beijerinckii* P260	20.79	0.34	0.39	175.6	[121]

system. The acids produced will trigger the formation of ABE and thus improve the biobutanol production. The same process has been done by Ramey and Yang [126] that implemented a combining process on butyric acid production by *C. butyricum* in acidic reactor with biobutanol production by *C. acetobutylicum* in solventogenic reactor using lignocellulosic biomass as substrate. The processes were conducted up to 150 L capacity using stirred tank bioreactor.

Besides acids and solvents production in a single system, a study on biohydrogen production from ABE fermentation has also been conducted. Ibrahim et al. [31] have reported on ABE production by *C. butyricum* EB6 although this strain has been found as a good biohydrogen producer [131]. An SSF process for biobutanol and biohydrogen production from oil palm empty fruit bunch has been introduced by Ibrahim et al. [117] with biobutanol concentration of 2.8 g/L and biohydrogen 2.8 L/L. Rajagopalan et al. [132] optimized the SSF co-production of biobutanol and biohydrogen by *Clostridium* strain BOH3 with biobutanol and biohydrogen concentration 13.5 g/L and 4.4 L/L, respectively. Besides, removing the biohydrogen from the fermentation reactor also enhance the biobutanol production. Yerushalmi et al. [133] reported that biohydrogen partial pressure in the fermenter head space may contribute to low biobutanol yield. However, Chen et al. [134] recently reported that the slight pressure has no obvious effect on the performance of ABE fermentation. With this regards, recovery system for dual products (hydrogen and biobutanol) should be developed.

Since ABE fermentation by Clostridia also producing biohydrogen from its metabolic pathway, an integrated system to recover biohydrogen and biobutanol could be advantageous. Rajagopalan et al. [135] has reported on the biohydrogen and biobutanol production by *Clostridium* strain BOH3 using xylan as a substrate. This strain has the capability to express xylanase production for a direct conversion of xylan into biohydrogen (12.7 L/L) and biobutanol (14.8 g/L). A patent by Claassen et al. [136] described the methods for combined production of biohydrogen and biobutanol from biomass. The methods comprising the steps of fermenting biomass to obtain butanol in a first reaction mixture; removing the butanol and hydrogen from the first reaction mixture to obtain effluent; and using the effluent as a substrate in a second reaction mixture in a process using low substrate concentrations.

5.3.6. Other fermentation modes

Fermentation mode plays an important role in the successful of biobutanol production. Batch fermentation was normally used for ABE production due to the simple operation and low risk of contamination [9]. However, low productivity of ABE in batch fermentation occurred due to product inhibition. Thus, fed–batch fermentation was applied to increase cell mass together with the use of immobilization system [137]. Several attempts have been tested on ABE fermentation using batch, fed-batch and continuous mode. A comparison investigation on the performance of batch, fed-batch and continuous mode by Li et al. [130] showed that fed-batch fermentation is not suggested for solvent production. In that particular study, batch fermentation produced higher yield of biobutanol, while the continuous mode was preferred in terms of biobutanol yield and productivity. However, fed-batch mode is preferable to produce higher biobutanol concentration than using batch and continuous mode, a main factor for high recovery and purification yield.

Passive immobilization technique for the production of ABE by *C. saccharoperbutylacetonicum* N–14 increased the yield by 88.37% [138]. Biofilm reactor for biobutanol production by *C. acetobutylicum* immobilized on fibrous matrix increased the biobutanol tolerance and subsequently improved the ABE productivity by 4–6-fold and 3–5-fold, respectively, as compared to traditional batch fermentation [58]. Highest biobutanol titer in batch fermentation was 15.6 g/L with highest productivity was 1.88 g/L/h as reported by Liu et al. [139]. They implied immobilization of the cells by adsorption onto a fibrous matrix with addition of methyl viologen. The large fermentation scale for ABE production was investigated by Ramey and Yang [126] using 150 L bioreactor. In addition, a cell recycle using continuous bioreactor was attempted to enhance the cell yield and productivity, which able to improve the ABE production [18].

6. Summary and future outlooks

Production of renewable alcoholic biofuel like biobutanol is a good approach to overcome our reliance on limited fossil fuel for transportation. Among other alcoholic biofuels, biobutanol shows the best candidate as future alternative fuel to replace gasoline. This is because biobutanol has similar characteristics with gasoline and it can be used in the presence engine and pipeline system without any modification. However, producing butanol through biological route like ABE fermentation by *Clostridium* sp. faces many challenges. One of the major problems is this ABE fermentation produces multiple by-products (gasses, acids and ABE) which limits the yield of biobutanol production. Various approaches have been considered by many researchers including manipulating genetic material and modifying the fermentation condition to enhance the biobutanol yield and concentration. Many *Clostridium* spp. have been employed to find the most superior strain and until now *C. beijerinckii* has been found as the most productive strain while *C. acetobutylicum* is the most widely studied. In addition, various types of substrates have been tested to produce butanol through ABE fermentation. It was proved that *Clostridium* sp. is able to consume both pentose and hexose sugars. Thus, ABE fermentation now is not limited only to glucose–based medium but can also be carried out using fermentable sugars obtained from lignocellulosic biomass. This situation provides some advantages to ABE fermentation since lignocellulosic biomass is cheap. However, utilizing lignocellulosic biomass as substrate requires multiple process with low sugar concentration to initiate the biobutanol production. This material must be pretreated to remove lignin before it can be hydrolysed into fermentable sugars. Enzymatic hydrolysis of lignocellulosic biomass by cellulase is costly because the cellulase itself is very expensive. In addition, cellulase action on lignocellulosic biomass is complicated and depending on many factors. One of them is synergistic effect because cellulase consisted of three types of cellulase components, which are endoglucanase, exoglucanase and ß–glucosidase. These cellulase components act differently on different substrate makes the comparison among lignocellulosic biomass exhibit complexity. In addition, numerous steps in converting lignocellulosic biomass into biobutanol are not economically viable, and thus the biobutanol production is still far away to be commercialized. Extensive and progressive research effort should be conducted in order to produce biobutanol from lignocellulosic biomass at high concentration, yield and productivity, using cheap substrate, efficient pretreatment process with low cost and has less steps and processes, creating the whole cellulosic biobutanol processes feasible for industrial production.

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