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REVIEW

Mini review: hydrogen and ethanol co-production from waste materials via microbial fermentation

Chiu-Shyan Soo[1] · Wai-Sum Yap[2] · Wei-Min Hon[3] · Lai-Yee Phang[1]

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Abstract The simultaneous production of hydrogen and ethanol by microorganisms from waste materials in a bioreactor system would establish cost-effective and time-saving biofuel production. This review aims to present the current status of fermentation processes producing hydrogen accompanied by ethanol as a co-product. We outlined the microbes used and their fundamental pathways for hydrogen and ethanol fermentation. Moreover, we discussed the exploitation of renewable and sustainable waste materials as promising feedstock and the limitations encountered. The low substrate bioconversion rate in hydrogen and ethanol co-production is regarded as the primary constraint towards the development of large scale applications. Thus, microbes with an enhanced capability have been generated via genetic manipulation to diminish the inefficiency of substrate consumption. In this review, other potential approaches to improve the performance of co-production through fermentation were also elaborated. This review will be a useful guide for the future development of hydrogen and ethanol co-production using waste materials.

Keywords Hydrogen · Ethanol · Co-production · Waste · Microbial fermentation

✉ Lai-Yee Phang
phanglaiyee@upm.edu.my

[1] Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

[2] Faculty of Applied Sciences, UCSI University, 56000 Cheras, Kuala Lumpur, Malaysia

[3] KDU University College, Damansara Jaya, 47400 Petaling Jaya, Selangor, Malaysia

Introduction

The current human lifestyle is heavily dependent on the fossil fuels coal, petroleum, and natural gas. The high consumption rate of fossil fuels has raised concern among people that the availability of fossil fuels will decline eventually. Even though the oil supply surpassed the global demand which leads to the fall in oil prices, the constant fear of oil price fluctuations has driven the world to seek for renewable energy substitutes. Biofuels such as hydrogen and ethanol can serve as potential clean energy sources to replace fossil fuel. To date, corn, wheat and sugar cane have been used to generate biofuels such as ethanol; however, the process is associated with negative impacts on biodiversity, land use and competition as food crops. Biofuels derived from algae represent one option to overcome these issues. However, despite their high yield, algae have an enormous requirement for water, nitrogen and phosphorus for growth; thus, these factors are a major shortcoming in the development of large scale biofuel production from algae (Naik et al. 2010).

Hydrogen is a common element that contains a high energy content. The hydrogen molecule is considered to be a promising energy carrier with the characteristics of zero pollutant emission and superior energy conversion efficiency compared to the fossil fuels currently consumed. Because hydrogen is rarely present in its molecular form, numerous methods have been developed to produce hydrogen gas as the raw material for chemicals industry, hydrogenation of fats and oils in food industry, production of electronic devices, processing steel and desulfurization and reformulation of gasoline in refineries (Kapdan and Kargi 2006). These methods include water-electrolysis (Lin et al. 2012), reforming of natural gas (Bang et al. 2013), gasification of coal and biomass (Huang and Dincer

2014), high temperature decomposition (Pinilla et al. 2011), and biological approaches (Chaubey et al. 2013; Ntaikou et al. 2010).

Biological hydrogen or biohydrogen can be produced via a photobiological approach or anaerobic fermentation. Production of biohydrogen using anaerobic fermentation from carbohydrate-rich waste materials is an effective method that utilizes simple technology (Chaubey et al. 2013; Ntaikou et al. 2010). Hence, hydrogen fermentation is being widely studied, and the optimization of the process has been intensively investigated. Several products accumulate at the end of the fermentation process, such as organic acids and alcohol solvents. Aside from hydrogen as the major product, these soluble end products could be used for commercial applications instead of being discharged as wastes. The formation of ethanol in either smaller or significant amounts has been observed to occur simultaneously in most of the hydrogen fermentation techniques. Therefore, the ethanol co-produced during hydrogen fermentation can serve as the ideal substitute for gasoline and fuel additives in vehicles (Balat and Balat 2009; Suhaimi et al. 2012). Subsequently, hydrogen and ethanol co-produced during microbial fermentation could be used as potential alternative fuels that produce lesser pollutants; these products could be produced economically compared to other advanced biofuels such as fatty acids esters, bioalkanes, and biodiesel (Brouwer 2010; Mekhilef et al. 2011; Zhang 2011).

The utilization of various waste materials for hydrogen and ethanol co-production has been a hot research topic since Ito et al. (2005) first demonstrated hydrogen and ethanol co-production using biodiesel waste. Waste material is considered the most viable solution for the production of biofuels due to its sustainability. Additionally, biofuel production from waste materials is environmental friendly and advantageously reduces the greenhouse gas effect. Biodegradable waste materials such as biomass, municipal solid waste and household food waste have been widely utilized as the feedstock in biodiesel, biobutanol and biomethane production (Frigon and Guiot 2010; Talebian-Kiakalaieh et al. 2013; Tashiro and Sonomoto 2010).

The co-production of hydrogen and ethanol is more beneficial in terms of cost and time savings compared to fermentation focusing on either hydrogen or ethanol production alone (Murarka et al. 2008; Yoshida et al. 2006). Hydrogen and ethanol co-production has been performed at a laboratory scale using serum bottles (Lay et al. 2012; Reungsang et al. 2013; Varrone et al. 2012), feed batch bioreactor (Ito et al. 2005), anaerobic sequencing batch reactor (Intanoo et al. 2014), and continuous stirred tank reactor (12.5 l) (Han et al. 2011). However, in order to establish pilot scale production, limitations such as low substrate consumption, low complex substrate degradation and vast byproduct formation are always encountered. Thus, substantial research is still required to eliminate these constraints in order to develop large scale fermenters for successful commercial applications.

The fundamental understanding of hydrogen and ethanol co-production via anaerobic fermentation is essential to overcoming these barriers, especially for the processes utilizing waste materials as the substrate. Relevant literature in this area is in high demand. This mini review presents an overview of the current knowledge about the bioprocesses involved in the co-production of hydrogen and ethanol as biofuels. Up-to-date information on anaerobic fermentation will be discussed, including the type of microorganisms used, the different carbon sources of waste materials and the fermentation strategies involved.

Biochemical pathway of hydrogen and ethanol production

Hydrogen and ethanol co-production via biological fermentation is generally achieved by certain microorganisms under anaerobic conditions which degrade the carbon source to generate the desired products. Organic substances such as carbohydrates and sugars are broken down by microorganisms to produce metabolic energy for growth (Cai et al. 2011a). Then, the substrate is converted into pyruvate, the central metabolic intermediate that is converted into different end products depending on the characteristics of the microorganisms and the metabolic pathways involved. The microbial fermentation process can be further categorized into the following groups: homolactic fermentation (Romero-Garcia et al. 2009), mixed acid fermentation (Rachman et al. 1997), butanediol fermentation (Ji et al. 2009), butyric acid fermentation (Zhang et al. 2009), and propionic acid fermentation (Feng et al. 2010). Microorganisms that tend to undergo mixed acid fermentation or butyric acid fermentation are the most likely to metabolize carbon sources to generate end products including hydrogen and ethanol. These microorganisms are generally categorized as facultative anaerobes and strict anaerobes based on their oxygen requirements.

Metabolic properties of facultative anaerobes

Facultative anaerobes can survive both in the presence or absence of oxygen. Although these microorganisms tolerate aerobic conditions well, oxygen is not necessary for their growth. The characteristic of uninhibited growth in the presence of oxygen promotes the application of facultative anaerobes in hydrogen and ethanol co-production. Many facultative anaerobes are able to co-produce

hydrogen and ethanol, such as *Klebsiella* sp. (Wu et al. 2011), *Enterobacter aerogenes* (Ito et al. 2005; Jitrwung and Yargeau 2011; Reungsang et al. 2013; Sakai and Yagishita 2007) and *Escherichia coli* (Chaudhary et al. 2011; Hu and Wood 2010; Murarka et al. 2008; Yoshida et al. 2006). Examples of the utilization of facultative anaerobes in hydrogen and ethanol fermentation are provided in Table 1. It is noteworthy that facultative anaerobes lack the butyrate formation pathway (Fig. 1). Facultative anaerobes undergo mixed acid fermentation under anaerobic conditions, producing lactate, succinate, ethanol, acetate, carbon dioxide and hydrogen. Lactate, a useful component for polylactic acid based plastic materials, is generated from pyruvate with lactate dehydrogenase as the catalyst (Fig. 1) (Abdel-Rahman et al. 2013). The production of lactate could inhibit the co-production of hydrogen and ethanol as the lactate synthesis pathway shares the same precursor which is pyruvate.

As illustrated in Fig. 1, hydrogen production is associated with the conversion of pyruvate to acetyl-CoA and formate, which occurs via pathways mediated by pyruvate:formate lyase. Then, the formate is converted into carbon dioxide and hydrogen via the formate hydrogen lyase system, a membrane protein consisting of a formate dehydrogenase, hydrogenase and electron transfer mediators (Maeda et al. 2012; Manish et al. 2007). Conversely, the acetyl-CoA produced is converted into either acetate as the end product or acetaldehyde, which will subsequently be used to generate ethanol. In this pathway, 1 mol of pyruvate will generate 1 mol of hydrogen and 1 mol of ethanol. The engineered *E. coli* SY03 achieved the closest to the theoretical yield, when 1.02 mol hydrogen and 1.01 mol ethanol were simultaneously generated from each mole of glycerol under fermentation using 10 g/l of glycerol at pH 6.3 (Shams Yazdani and Gonzalez 2008). Under similar conditions, *Enterobacter aerogenes* HU-101 produced 0.89 mol H_2/mol glycerol and 0.86 mol EtOH/mol glycerol. Maru et al. (2012) reported that *Enterobacter* spH1 was able to produce 0.85 mol hydrogen and 0.96 mol ethanol per mol glycerol when a glycerol concentration of 20 g/l was used.

Metabolic properties of strict anaerobes

Strict anaerobes neither require oxygen to grow nor tolerate oxygen due to the absence of certain enzymes such as catalase, peroxidase and superoxidase dismutase that are required to degrade toxic oxygen derivatives. Strict anaerobes such as *Clostridium* spp. are common species used in butyric acid fermentation or butanol-acetone fermentation to produce end products such as butyrate, acetate, carbon dioxide, hydrogen, butanol, acetone, isopropanol, and ethanol. For instance, *C. acetobutylicum*, *C. beijerinckii*, *C. saccharoperbutylacetonicum*, and *C. saccharobutylicum* are well-known acetone-butanol-ethanol (ABE) producers (Huang et al. 2010; Lee et al. 2008; Papoutsakis 2008). The formation of hydrogen by strict anaerobes occurs via pathways mediated by pyruvate:-ferredoxin oxidoreductase, where pyruvate is converted into carbon dioxide and acetyl-CoA. The oxidative decarboxylation of pyruvate to carbon dioxide and acetyl-CoA is accompanied by the reduction of oxidized ferredoxin, leading to hydrogen evolution with the aid of ferredoxin-dependent hydrogenase (Carere et al. 2008a). Acetyl-CoA is converted into acetoacetyl-CoA and butyryl-CoA; these 3 CoA-derivatives are the main intermediates that direct the carbon flow to acid and ABE production (Jones and Woods 1986). Acidogenesis occurs when acetate is produced from acetyl-CoA, while butyrate is produced from butyryl-CoA. A high hydrogen yield is associated with a high acetate to butyrate ratio, possibly due to the inhibition of hydrogen production by the excess of reduced nicotinamide adenine dinucleotide (NADH) consumption during butyrate synthesis. The accumulation of acidic products during acidogenesis decreases the environmental pH and triggers metabolism by switching to solventogenesis to retain the pH. Solventogenesis occurs during stationary growth phase when acetone is produced from acetoacetyl-

Table 1 Potential facultative anaerobes in co-production of hydrogen and ethanol

Strain	[Glycerol] (g/l)	pH	T (°C)	Hydrogen yield	Ethanol yield	Source
Enterobacter aerogenes HU-101	10	6.3	37	0.89 mo/mol glycerol	0.86 mol/mol glycerol	Shams Yazdani and Gonzalez (2008)
Escherichia coli SY03	10	6.3	37	1.02 mol/mol glycerol	1.01 mol/mol glycerol	Shams Yazdani and Gonzalez (2008)
Escherichia coli HW2	10	6.3	37	21 μmol/mg protein	2.1 mg/mg protein	Hu and Wood (2010)
Escherichia coli	20	6.3	37	1.40 mmol/l	0.32 g/g glycerol	Chaudhary et al. (2011)
Klebsiella sp. HE1	50	6	35	0.345 mol/mol glycerol	0.49 mol/mol glycerol	Wu et al. (2011)
Enterobacter spH1	20		37	0.85 mol/mol glycerol	0.96 mol/mol glycerol	Maru et al. (2012)

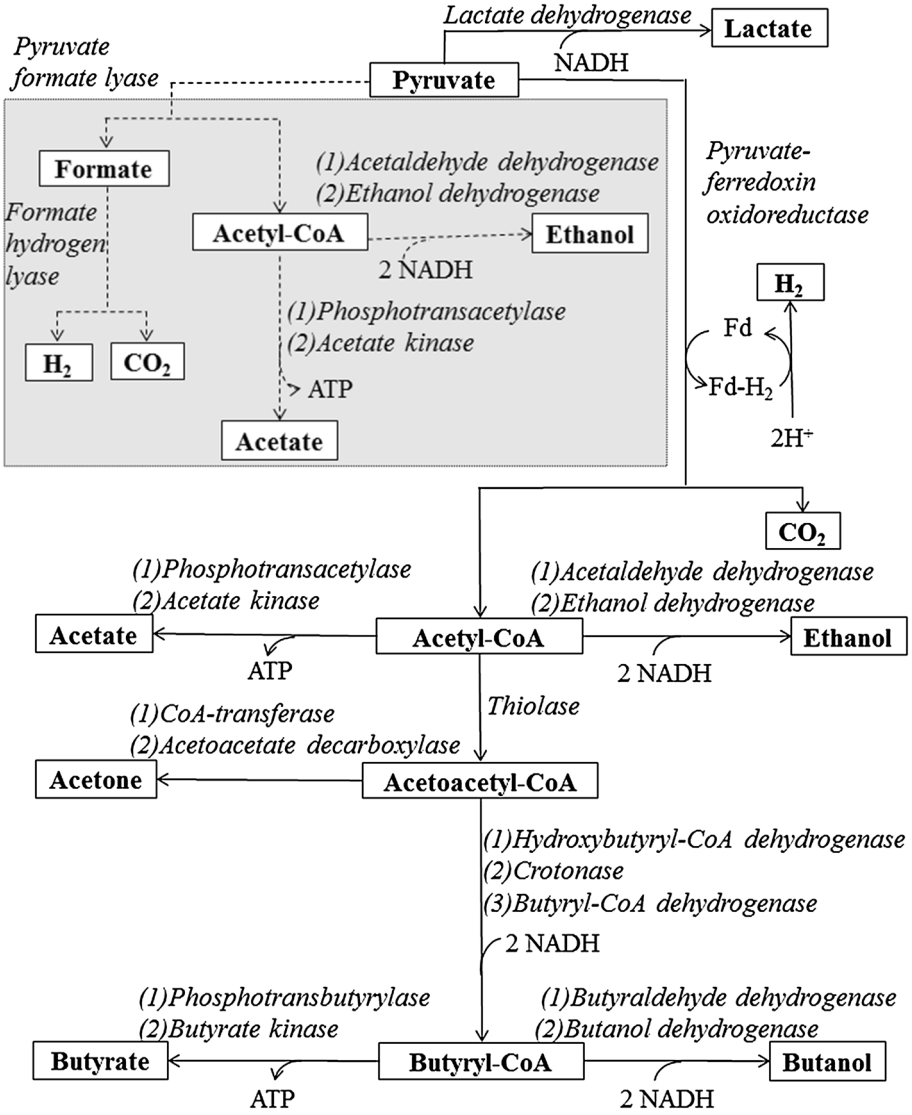

Fig. 1 Hydrogen and ethanol synthesis pathways under anaerobic condition via pyruvate formate lyase (*dashed line*) and pyruvate-ferredoxin oxidoreductase (*solid line*) (Adapted from Ref. Clomburg and Gonzalez 2013)

CoA, butanol is produced from butyryl-CoA and ethanol is produced from acetyl-CoA through several reduction reactions (Cai et al. 2011a; Lam and Lee 2010; Lehmann and Lütke-Eversloh 2011). Ramachandran et al. (2011) demonstrated the potential of hydrogen and ethanol production by *Clostridium* sp. strain URNW by achieving a total volumetric hydrogen production of 14.2 mmol/l culture and total ethanol production of 0.4 mmol/l culture from 2 g cellubiose/l.

Limited information was available on hydrogen and ethanol co-production by strict anaerobes until the recent characterization of the thermophilic microorganisms. Thermophiles could provide an outstanding yield of hydrogen and ethanol in comparison with mesophilic strict anaerobes. Thermophiles such as *Thermoanaerobacterium* have an advantage due to their survival capability at high temperatures. Moreover, they tend to grow at a faster rate and are able to degrade a wide spectrum of substrates, such as agricultural residues and other complex lignocellulosic wastes (Orlygsson et al. 2010). Because thermophiles produce less undesired fermentative end products, they are considered to be potential hydrogen and ethanol co-producing microorganisms. The stoichiometry of hydrogen production was favored at higher temperatures leading to higher hydrogen yield (Orlygsson et al. 2010). The production of byproduct such as lactate could be diminished in thermophiles, as the precursor, pyruvate are more likely converted into hydrogen and ethanol instead of lactate. Zhao et al. (2009) achieved a co-production yield of 1.58 mol hydrogen and 0.9 mol ethanol per mol glucose under fermentation by a mixed culture of thermophiles using 2 g/l glucose as the substrate. Koskinen et al. (2008) reported that anaerobic thermophilic isolate AK15 was able to produce 1.1–1.9 mol hydrogen and 0.6–0.8 mol ethanol

per mol glucose. *Thermoanaerobacterium* AK_{54} isolated by Sigurbjornsdottir and Orlygsson (2012) yielded 0.08 mol hydrogen and 1.35 mol ethanol per mol glucose. Nevertheless, the requirement for strict anaerobic conditions and the energy needed to maintain thermophilic fermentation systems have limited the development of large scale fermentation using strict anaerobes and thermophiles.

Feedstock for hydrogen and ethanol co-production

The substrate is regarded as one of the main components in fermentation because it supplies the nutrition for microbial growth. Most previous studies have used glycerol as the foremost carbon source in hydrogen and ethanol co-production (shown in Table 1). To date, there is no report of the conversion of glycerol into hydrogen and ethanol by strict anaerobes. Therefore, glycerol fermentation is generally demonstrated by facultative anaerobes (Table 1). The glycerol metabolic pathway of strict anaerobes has been reported to be incompatible with hydrogen and ethanol co-production due to its production of 1,3-propanediol as its major product (Saint-Amans et al. 2001). In contrast, glycerol degradation by facultative anaerobes generates mainly hydrogen and ethanol, theoretically yielding 1 mol of hydrogen and 1 mol of ethanol based on the equation: $C_3H_8O_3 \rightarrow C_2H_5OH + H_2 + CO_2$ (Chaudhary et al. 2011; Nwachukwu et al. 2012). Under anaerobic conditions, glycerol is converted into dihydroxyacetone phosphate and subsequently phosphoenolpyruvate (PEP), thereby leading to pyruvate synthesis (Fig. 2a). In this pathway, 2 mol of NADH are produced that would be recycled or oxidized during the ethanol synthesis pathway. This recycling process is vital for the maintenance of the intracellular redox balance for cell viability (Murarka et al. 2008). In this process, ethanol production is favored over acetate and lactate production to maintain the balanced ratio of NAD^+: NADH. However, the production of hydrogen in this pathway does not restrict ethanol production and vice versa because both products are produced independently. Hence, this pathway serves as a perfect foundation for the co-production of hydrogen and ethanol using glycerol as a substrate by the facultative anaerobes that have been popularized in recent years.

Glucose, the most commonly used carbohydrate for mixed acid fermentation systems, serves as another potential substrate candidate for hydrogen and ethanol co-production. Glucose is dissimilated through the Embden-Meyerhof pathway (Garrett and Grisham 2013) to produce 2 mol of pyruvate that eventually yield hydrogen and ethanol (Fig. 2b). Theoretically, two moles of hydrogen and one mole of ethanol could be generated from 1 mol of glucose: $C_6H_{12}O_6 + H_2O \rightarrow C_2H_5OH + CH_3COOH +$

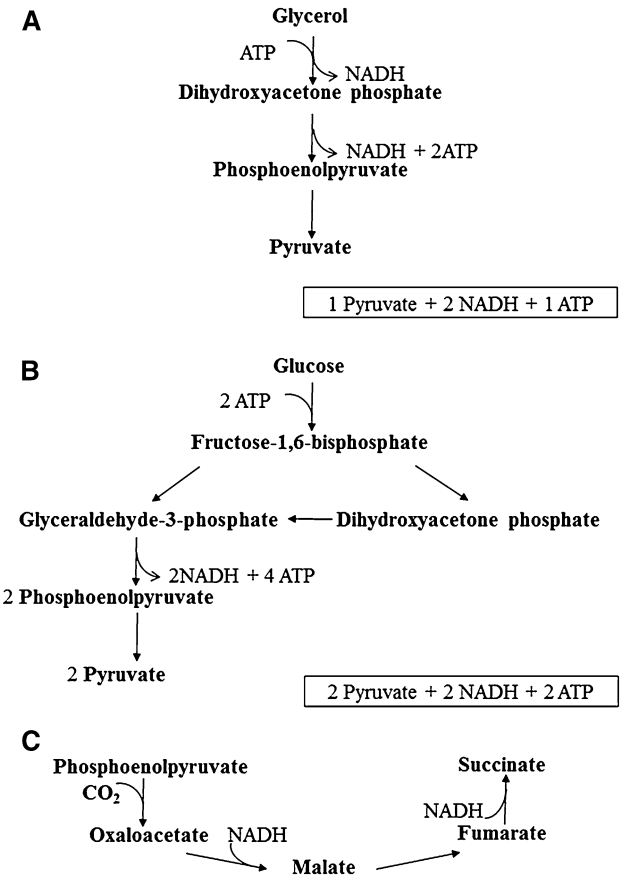

Fig. 2 **a** Glycerol and **b** glucose metabolic pathways during hydrogen and ethanol fermentation with **c** succinate synthesis pathways (Adapted from Refs. Clomburg and Gonzalez 2013; Förster and Gescher 2014)

$2H_2 + 2CO_2$ (Hwang et al. 2004). In this pathway, the conversion of glucose to pyruvate also produces 2 mol of NADH, but the NADH produced is insufficient for the 2 mol of pyruvate to generate an equal mole of ethanol as was described for glycerol degradation. Instead, acetate would be produced as a byproduct. Therefore, the ethanol to hydrogen yield ratio is generally lower when glucose is used as a substrate. Regardless of the type of substrate used, succinate and lactate synthesis could be potential competing pathways when NADH oxidations are involved. Succinate, a valuable product that is commonly used as flavoring agent in food industry, could be generated from PEP through a series of pathways (Fig. 2c) (Förster and Gescher 2014). The conversion of PEP to oxaloacetate catalyzed by PEP carboxylase is initiated at high level of CO_2. The oxaloacetate is then converted into malate, followed by the conversion of malate into fumarate, and eventually yielding succinate (Song and Lee 2006). Two moles of NADH are required per mole of succinate produced, whereas 1 mol of NADH is required per mole of lactate produced (Abdel-Rahman et al. 2013; Cheng et al.

2012). Therefore, the fermentation end product is highly dependent on the substrate and microorganisms.

Similar to glycerol, glucose and other simple sugars can be easily degraded; thus, they have been widely used as classical substrates for fermentation. However, it is not practical to utilize these substances because they are expensive for large scale production (Masset et al. 2012). The cost of raw material is the crucial factor for the scaling up of hydrogen and ethanol co-production during the fermentation process. Alternatively, waste materials could be considered as promising feedstock due to their high chemical oxygen demand (COD) value, negligible price and abundance. Therefore, utilizing waste materials derived from industry to generate valuable products is not only beneficial for commercial purposes but is also advantageous for environmental considerations. Previous studies have demonstrated that various waste materials can be utilized for hydrogen and ethanol co-production, including crude glycerol, molasses, sweet potato starch residues, tofu processing wastewater, cheese whey, and lignocellulose residues. These waste materials will be further discussed below.

Crude glycerol from biodiesel processing plants

Crude glycerol, which is a byproduct of biodiesel fuel production, is the most commonly studied substrate for hydrogen and ethanol co-production using waste materials. Crude glycerol is produced from biodiesel via the transesterification of vegetable oils or animal fats (Murarka et al. 2008). Due to the rapid growth of the biodiesel industry, a surplus amount of glycerol has been generated, resulting in the collapse of glycerol prices. As a result, excess glycerol is dumped into the waste stream. Instead of being disposed as waste, the conversion of low-cost crude glycerol into a higher value product could represent a better approach. Glycerol decomposition by conventional chemical or physical methods may require high purification costs to ensure that the crude glycerol is free from contaminants such as water, methanol, soap and oil. However, there is no requirement for the pretreatment and purification of crude glycerol for conversion into hydrogen and ethanol (Varrone et al. 2012). Furthermore, the glycerol metabolic pathway in facultative anaerobes tends to produce ethanol instead of acetate, lactate and butyrate during fermentation, indicating that this process produces fewer byproducts compared to other simple sugars. This feature alone could explain why crude glycerol is a promising feedstock for the co-production of hydrogen and ethanol.

Table 2 provides examples of fermentation systems that have demonstrated hydrogen and ethanol co-production using crude glycerol as the feedstock. Ito et al. (2005) was believed to be the first research team to elucidate the process of hydrogen and ethanol co-production from biodiesel wastes, thereby achieving the maximum hydrogen productivity of 63 mmol/l/h and ethanol yield of 0.85 mol/mol glycerol using *Enterobacter aerogenes* HU-101. Subsequently, the biodegradation of crude glycerol into hydrogen and ethanol by *Enterobacter aerogenes* was reported by Jitrwung and Yargeau (2011) and Reungsang et al. (2013). To date, Varrone et al. (2012) achieved the highest conversion efficiency, approaching a hydrogen yield of 0.96 mol and ethanol yield of 1 mol from each mol of glycerol with a starting concentration of 15 g/l crude glycerol.

There are some factors to be considered in hydrogen and ethanol co-production using crude glycerol. First, efficient glycerol utilization is important to ensure high performance hydrogen and ethanol production. Using a synthetic medium with the addition of yeast extract, tryptone and supplements such as nitrate and sulfate could supply organic nitrogen to promote cell growth and thus increase the glycerol consumption rate. Ito et al. (2005) reported that glycerol was completely consumed after 24 h of fermentation when biodiesel wastes were supplemented with nitrogen sources. In contrast, glycerol was not completely consumed even after 48 h when biodiesel wastes containing glycerol were used without nitrogen supplementation. Additionally, the glycerol consumption rate can be improved using an electrochemical reactor with thionine as an exogenous electron mediator (Sakai and Yagishita 2007). Ito et al. (2005), Jitrwung and Yargeau (2011), Varrone et al. (2012), Reungsang et al. (2013) and Chookaew et al. (2014) reported the concentrations of 10, 21, 15, 31 and 11 g/l, respectively, as the optimum crude glycerol concentrations used for co-production. The discrepancies in optimum concentrations are most likely due to variations in the compositions of the crude glycerol and differences in the microorganisms and fermentation modes used.

A high concentration of crude glycerol could affect the yield of hydrogen and ethanol. One explanation is that the presence of a high salt content in biodiesel wastewater induced toxic effects on the microorganisms. In contrast, an increase in the glycerol concentration over the optimum level might cause the disruption of intracellular osmotic pressure and result in cell damage (Reungsang et al. 2013). Moreover, Ito et al. (2005) stated that the fermentation process performed at high glycerol concentrations tended to produce metabolites other than hydrogen and ethanol (e.g., lactate). As the glycerol concentration in biodiesel waste increased from 1.7 to 25 g/l, the hydrogen and ethanol yield decreased from 1.12 mol H_2/mol glycerol and 0.96 mol EtOH/mol glycerol to 0.71 mol H_2/mol glycerol and 0.56 mol EtOH/mol glycerol, respectively; at the same time, the lactate yield increased from a non-detectable amount to 0.17 mol/mol glycerol. Pure glycerol had the

Table 2 Co-production of hydrogen and ethanol using crude glycerol

[Glycerol]	Culture	pH	T (°C)	Hydrogen yield (mol/mol glycerol)	Hydrogen production rate	Ethanol yield (mol/mol glycerol)	Source
110 mM	*Enterobacter aerogenes* HU-101	6.8	37	–	63 mmol/l/h	0.8	Ito et al. (2005)
21 g/l	*Enterobacter aerogenes* ATCC 35029	No data	37	0.95	–	0.79	Jitrwung and Yargeau (2011)
15 g/l	Wastewater sludge	8	38	0.96	2.2 l/l/day	1.0	Varrone et al. (2012)
31 g/l	*Enterobacter aerogenes* KKU-S1	8.14	37	0.12	0.24 mmol/l/h	0.83	Reungsang et al. (2013)
11.14 g/l	*Klebsiella* sp. TR17	8	40	0.26	–	0.58	Chookaew et al. (2014)

same effect as the biodiesel waste; the hydrogen and ethanol yield decreased from 1.05 mol H_2/mol glycerol and 1.00 mol EtOH/mol glycerol to 0.82 mol H_2/mol glycerol and 0.80 mol EtOH/mol glycerol, respectively, when the concentration of pure glycerol increased from 5.0 to 25 g/l. The performance using biodiesel waste would be much lower compared with the hydrogen and ethanol yield from pure glycerol at the same concentration due to the high salinity in the waste and the presence of contaminants that may cause inhibition (Ito et al. 2005).

Sugar-based byproducts from the food processing and manufacturing industry

Industrial waste and wastewaters from food processing are appealing feedstock for hydrogen and ethanol co-production. For instance, molasses may be one of the least inexpensive potential raw materials when the substrate cost and production efficiency are taken into account. Molasses, which is a sugar-based waste that contains mainly sucrose, glucose and fructose, is being produced in abundance from the sugar cane and sugar beet refining industries. Molasses also contains a large amount of organic nitrogen sources that are biochemically accessible to the fermentation bacteria (Wang and Jin 2009). The high amount of essential vitamins and salts in molasses are believed to accelerate bacterial growth, resulting in higher hydrogen yields and production rates (Wang and Jin 2009). Therefore, molasses has an added advantage over glucose apart from its cheap price. Guo et al. (2008) obtained a hydrogen yield of 3.47 mol/mol sucrose with ethanol as the major dissolved fermentation product from molasses fermentation. Han et al. (2012) demonstrated simultaneous hydrogen and ethanol production from molasses with immobilized sludge, and achieved a maximum hydrogen production rate of 12.4 mmol/h/l and maximum ethanol production rate of 20.27 mmol/h/l.

In addition to molasses, wastewater discharged from sweet potato starch manufacturers contains large quantities of starch residues that serve as desirable carbon sources for hydrogen and ethanol co-production. The components of sweet potato starch residues include starch, cellulose, hemicellulose, ash, moisture and other substances. Sweet potato starch residues have previously been utilized for citric acid fermentation (Yokoi et al. 2001). Because citric acid production has been decreasing over recent years, the bioconversion of starch residues into other profitable alternatives is greatly encouraged. The nitrogen-rich organic waste from sweet potato starch-manufacturing companies could serve as a potential feedstock for hydrogen and ethanol co-production. Recently, Lay et al. (2012) demonstrated hydrogen and ethanol co-production through sweet potato fermentation. A maximum cumulative hydrogen production of 97–120 mmol H_2/l and maximum ethanol concentration of 3.754–5.811 g/l were achieved with the addition of an external seed such as sewage sludge or cow dung (Chu et al. 2012; Lay et al. 2012). Although sweet potato residue is a starch-based waste, its long polysaccharides and fiber content might extend the period of biodegradability and affect the product yield. Pretreatment may be required to overcome this problem.

Another promising sugar-based byproduct is wastewater from tofu processing. Lay et al. (2013) evaluated the feasibility of using tofu processing wastewater in anaerobic fermentation for onsite energy recovery. The authors successfully obtained a maximum total energy production of 485 J/g COD from both hydrogen and ethanol. This indicated the potential of using tofu processing wastewater in hydrogen and ethanol co-production. A large amount of wastewater is generated during the production of tofu from soybeans. The wastewater has a high organic content and includes reducing sugars, sucrose, starch and volatile fatty acids. The high protein characteristic of wastewaters may affect hydrogen production (Lay et al. 2013); during the fermentation process the proteins would be converted into

ammonia, resulting in a basic condition that is unfavorable for hydrogen production. Moreover, calcium sulfate (an ingredient added during tofu production) would result in sulfate accumulation in the wastewaters and could inhibit hydrogen production. Thus, the removal of ammonium and sulfate is crucial in the fermentation system using tofu processing wastewater to produce hydrogen and ethanol.

Cheese whey, the main byproduct of cheese manufacturing in the dairy industry, is another attractive feedstock for the fermentation process to produce hydrogen and ethanol. Cheese whey contains milk fat, trace minerals, salts and vitamins. Its high organic content makes the discharge of cheese whey an environmental concern (Azbar et al. 2009). Lactose, the major component in cheese whey dry extract, is hydrolyzed by the β-galactosidase enzyme to produce glucose and galactose. Similar to glucose, galactose can be converted into hydrogen and ethanol, and galactose degradation has been reported to result in a higher hydrogen yield (Rosales-Colunga et al. 2013). Rosales-Colunga et al. (2013) evaluated fermentation using a mixture of glucose and galactose and achieved a hydrogen yield of 1.02 mol H_2/mol hexose. This yield was relatively higher than the hydrogen yield of fermentation using glucose as the sole substrate (0.3 mol H_2/mol glucose). Ferreira Rosa et al. (2014) demonstrated glucose fermentation, cheese whey fermentation and co-fermentation of glucose and cheese whey using sludge from a poultry slaughterhouse. When 5 g COD/l of substrate was utilized, cheese whey fermentation achieved the highest hydrogen yield (1.9 mmol H_2/g COD), with ethanol (1.6 mmol/g COD) as the dominant soluble metabolic product. Moreover, the co-fermentation of cheese whey and glucose using sludge from a poultry slaughterhouse resulted in yields of 1.7 mmol H_2/g COD and 3.45 mmol EtOH/g COD, respectively, indicating that the substrate mixture improved ethanol production but not hydrogen production.

Lignocellulosic residues

Cellulosic biomass derived from agricultural residues, forestry residues and industrial wastes are rich in cellulose, hemicelluloses and lignin (Chong et al. 2009). Cellulose is a linear polysaccharide polymer composed of D-glucose units linked by β-(1 → 4)-glycosidic bonds. Cellulose is the primary structural components of plant cell wall and presents mostly in crystalline form that is highly resistant to hydrolysis. Hemicellulose, on the other hand is a heteropolymers comprised of cellulose and short branches consisting other hexose and pentose sugars such as xylose and arabinose. In contrary to cellulose, hemicellulose is more susceptible to acid, base or enzymatic degradation. As for lignin, it is a complex polymer of phenolic monomers and present in amorphous structure. Lignin has a high resistance to enzymatic hydrolysis and forms a protective barrier of cellulose as well as hemicellulose (Brodeur et al. 2011; Kumar et al. 2009). In the industry, the lignocellulosic biomass that remain after the harvesting and processing of crops are preferred for biofuel production compared to the use of energy crops based on economic and environmental factors. Cellulosic waste is the largest renewable source of hexose and pentose sugars that could be used as the potential feedstock for hydrogen and ethanol fermentation. Xylose, the major pentose sugar present in lignocellulosic hydrolysate, was utilized in combined hydrogen and ethanol production using a thermophilic mixed culture by Zhao et al. (2010). The highest yield of 1.41 mol H_2/mol xylose and 0.81 mol EtOH/mol xylose was obtained under the optimized condition. Distiller grains are a cellulose-rich industrial waste generated in abundance. Chuang et al. (2012) evaluated the feasibility of bioenergy production from distiller grains using mixed microflora from a cow dung seed under thermophilic conditions, yielding 41 J/g substrate; the total bioenergy comprised 21 and 79 % from hydrogen and ethanol, respectively. On the other hand, fermentation process using lignocellulosic residues at a higher concentration has the potential of yielding higher products. Nevertheless, this is only practical below the threshold level. According to Manikkandan et al. (2013), the highest hydrogen and ethanol yield from bagasse hydrolysate was obtained at a concentration of 1.5 % (w/v) (glucose equivalent), whereas a lower yield was reported at bagasse hydrolysate concentrations above 1.5 % (w/v).

The lignocellulosic materials are hardly degraded into simple sugars during fermentation process attributed to their complex structures. Conventional cellulose fermentation for hydrogen and ethanol production requires several steps: pretreatment, enzymatic hydrolysis of lignocellulose or saccharification, followed by fermentation. This process is named separate saccharification and fermentation (SHF). Currently, cellulosic bioethanol is generated by simultaneous saccharification and fermentation (SSF), where enzymatic hydrolysis is combined with fermentation. In a study by Zhao et al. (2013), SSF was conducted using cornstalks pretreated with fungi for hydrogen production. The advantage of using SSF over SHF is the prevention of cellulase inhibition by hydrolysis products such as glucose because they can be fermented instantly. However, the optimum temperature for enzymatic hydrolysis may not be compatible with fermentation. The cellulose hydrolysis generally occurs optimally at 50 °C, whereas fermentation is performed at mesophilic condition. Hence, there is a possibility that the hydrolysis occurs at lower rate in SSF compared to SHF (Rana et al. 2014). Intanoo et al. (2014) investigated the thermophilic production of hydrogen using

alcohol wastewater which contained a large quantity of cassava chips added with untreated lignocellulosic residues. Their results showed that the thermophiles exhibited a strong ability to degrade cellulose and hemicellulose but not lignin. Pretreatment is required for the use of lignocellulosic residues to enhance the fermentation system for both SHF and SSF. Without pretreatment, lignin shields cellulose and hemicellulose from being hydrolyzed into sugars that serve as the substrate. The pretreatment process separates lignin and hemicellulose and subsequently alters the structure of cellulose to non-crystalline form that is more accessible for hydrolysis (Kumar et al. 2009). Besides improving lignocellulosic biomass degradation to maximize desired product, there are some conditions in the pretreatment process to be considered in order to make the fermentation viable. The pretreatment process has to be cost-effective, and it is essential to get rid of carbohydrate loss and formation of byproducts which could be inhibitory. The pretreatment methods include physical, chemical (acidic and basic treatment), physiochemical, and biological treatment using cellulolytic microorganisms such as *Clostridium* sp. (Brodeur et al. 2011; Kumar et al. 2009). Alternatively, consolidated bioprocessing production (CBP) has been developed; in this process, cellulase production, enzymatic saccharification and fermentation can be accomplished in a single step by cellulolytic microorganisms. Hence, CBP is an attractive alternative due to its simpler operation, lower energy input and costs and higher conversion efficiency (Carere et al. 2008b); hydrogen and ethanol co-production via CBP using lignocellulose and agricultural hemp residues, respectively, has been demonstrated (Agbor et al. 2014; Ho et al. 2011).

Challenges for improving hydrogen and ethanol co-production

Hydrogen and ethanol co-production have been intensively studied using pure cultures and mixed cultures on various substrates, especially glycerol and glucose in the form of either pure substrates or waste materials. Pure cultures have received much attention among researchers because a single strain of facultative anaerobe exhibited the feasibility to convert glycerol into hydrogen and ethanol. Both facultative anaerobes and strict anaerobes possess the potential to co-produce hydrogen and ethanol. However, under dissimilar circumstances where different substrate types are involved, the potential end products and theoretical yield achieved differ (summarized in Table 3). Pure cultures have been a popular option mainly due to the easier manipulation of metabolism. However, one drawback of fermentation using pure cultures is the requirement for aseptic conditions to prevent contamination. In contrast, most of the large scale fermentation processes utilize mixed cultures for several reasons. In addition to the simple operation and easy control, systems using mixed cultures generally require no medium sterilization. Hence, the overall cost of fermentation is reduced. Moreover, the mixed cultures derived from a variety of natural resources and wastes allow for a broader selection of feedstock (Ntaikou et al. 2010). The use of an appropriate mixed culture could contribute to fermentation using complex substrates through syntrophic mechanisms, and the metabolic interactions among microorganisms could counteract the inhibitory effects of toxic compounds. However, the possibility of the microbial diversity switching to undesired microorganisms such as methanogens, homoacetogens and lactic acid bacteria could eventually have a negative effect. Pretreatment such as heat-treatment, aeration, acid and base treatment is a strategy to minimize this possibility. Hydrogen producing microorganisms generally have the characteristic of forming endospore and survive under harsh condition such as heat treatment at 121 °C for 20 min, whereas most hydrogen consuming microorganisms such as methanogens could not survive under this condition (Kotay and Das 2009; Ren et al. 2008). Although the pretreatment enriches hydrogen producing microorganism in mixed cultures, the capital cost is also increased due to the pretreatment process, which is a disadvantage.

Several fermentation strategies, such as the continuous packed-bed reactor and the continuous stirred tank reactor, have been implemented for higher performance compared to batch mode. However, problems are still encountered despite the different fermentation systems used, including complex feedstock characteristics and substrate inhibition. The presence of contaminants may also have a detrimental effect on the conversion efficiency of waste materials to hydrogen and ethanol. The waste-containing medium can create extreme conditions that are undesirable for microbial growth. To date, only a limited number of hydrogen and ethanol co-producing microorganisms have been identified that can adapt to the harsh environment of the waste-containing medium. In order to establish the fermentation system to become commercially competitive, some other technical challenges are present. The research and development with respect to optimizing the bioreactor designs to enhance production rates and yields is still in demand. Hydrogen purification and storage is a primary concern as hydrogen is produced accompanied with CO_2. The integrated bioreactor system such as installment of poly(-dimethyl siloxane) membrane to fermenters is prerequisite to efficiently separate hydrogen from gaseous mixture (Bakonyi et al. 2015; Levin and Chahine 2009). Issues related to waste material collection and transportation to centralized biorefineries still exist. For instance, harvesting lignocellulosic biomass requires large machinery and

Table 3 Comparison of hydrogen and ethanol co-production using facultative anaerobes and strict anaerobes

Strain	Facultative anaerobes	Strict anaerobes	References
Substrate type	Glycerol	Common sugar mainly glucose	Chaudhary et al. (2011), Hwang et al. (2004) and Nwachukwu et al. (2012)
Potential end products	Hydrogen, ethanol, acetate, formate	Hydrogen, ethanol, acetate, butyrate	
Theoretical yield	1 mol hydrogen and 1 mol ethanol per mol glycerol	2 mol hydrogen and 1 mol ethanol per mol glucose	
Fermentation strategy	Continuous pack-bed reactor; continuous stirred tank reactor; cell immobilization; statistical optimisation of medium components	Continuous-flow bioreactor	Chookaew et al. (2014), Ito et al. (2005), Koskinen et al. (2008) and Sivagurunathan et al. (2014)
Molecular strategy	Disruption of succinate and acetate synthesis Glycerol dehydrogenase and dihydroxyacetone kinase gene-overexpressions	Disruption of butyrate or butanol formation pathway	Cai et al. (2011b), Hu and Wood (2010) and Shams Yazdani and Gonzalez (2008)

enormous fuel for transportation. It is also vital to ensure that the harvested biomass is free from soil contamination and moisture, for storage purpose. The texture variance, seasonal availability, moisture content, distance from the harvest site to biorefinery, mode of transportation, availability of infrastructure and on-site technology are needed to be taken into consideration as all those mentioned above have a remarkable impact on the cost of biomass harvesting (Balan 2014).

The environmental concern remains another challenge for hydrogen and ethanol co-production from waste materials. The bioconversion of feedstock into biofuels utilize tremendous water in all processing steps. Moreover, the processing steps also emit pollutants into the atmosphere and create noise pollution. It is important to reduce the capital cost, minimize water consumption and detrimental impacts of energy production on the environment. Expertise in agronomy, biomass logistics, biomass conversion, engineering studies, economics and environmental science are required to assess their practical implementation (Balan 2014).

Strategies for fermentation and strain improvement

Over the years, researchers and scientists investigated approaches to promote the current stage of hydrogen and ethanol co-production towards the advanced platform required for industrial applications. Several methods have been established that could overcome the problems encountered using waste materials. The immobilization technique has been suggested as an application to improve the efficiency of fermentation systems using wastes. Cell immobilization has an added advantage over free cells, where the substrate threshold level and harsh environment of the waste-containing medium would not be the critical limitations. Sivagurunathan et al. (2014) investigated beverage wastewater fermentation by *E. coli* XL1-Blue immobilized in calcium alginate beads at the mesophilic temperature. In their study, the highest hydrogen yield of 1.65 mol/mol substrate and ethanol yield of 1.13 mol/mol substrate were achieved using 5 g/l of beverage wastewater. In the same study, when 20 g/l of beverage wastewater was used, 1.13 mol/mol substrate and 1.33 mol/mol substrate were obtained for hydrogen and ethanol, respectively. The result suggested that the immobilization of *E. coli* demonstrated better product co-production at lower concentrations of wastewater. The yield obtained for both hydrogen and ethanol co-production was comparable to the thermophilic fermentation system, thereby demonstrating the advantage of using cell immobilization.

Although mixed cultures or co-cultures might be advantageous in the biodegradation of waste materials, it is believed that hydrogen and ethanol co-production from wastes could be enhanced to a greater extent using a genetically engineered strain. Because glucose is the major building block of most waste components, the bioconversion of glucose into hydrogen and ethanol by *E. coli* could be improved by disrupting the lactate synthesis pathway (Yoshida et al. 2006). Overexpression of the genes encoding for substrate uptake (i.e., the cellulolytic enzyme) is another approach that may diminish the inefficient substrate conversion and low substrate tolerance. However, difficulties have been encountered in designing recombinant systems, and to date, very few reports are available in the literature (Lambertz et al. 2014). In contrast, the overexpression of the genes encoding glycerol dehydrogenase and dihydroxyacetone kinase have been reported to accelerate glycerol utilization by improving the conversion of glycerol to glycolytic intermediates, and thus increasing the cell growth rate. As a result, higher substrate utilization was observed,

leading to increased production of hydrogen and ethanol (Hu and Wood 2010; Shams Yazdani and Gonzalez 2008).

The enhancement of strain performance by blocking byproduct formation using molecular approaches could be an effective way to improve fermentation efficiency using pure cultures. In a study by Shams Yazdani and Gonzalez (2008), engineered *E. coli* with disrupted genes encoding fumarate reductase (*frd*A) and phosphotransacetylase (*pta*) produced only insignificant amounts of succinate and acetate. Butanol is another major byproduct produced during fermentation processes leading to hydrogen and ethanol production using strict anaerobes. In the pathway of strict anaerobes, both the synthesis of ethanol and butanol consume NADH, the reduced electron carrier that contributes to hydrogen production (Tashiro and Sonomoto 2010). Cai et al. (2011b) demonstrated that the inactivation of *hbd*, the gene encoding the β-hydroxybutyryl-CoA dehydrogenase in *C. butyricum,* resulted in a significant increase in either ethanol or hydrogen production.

Alternatively, the activation of the pathway directing carbon flux towards the desired product is considered to be the most straightforward and effective way to improve yield and productivity (i.e., the overexpression of genes encoding pyruvate formate lyase (*pfl*) and hydrogenase). Asanuma and Hino (2002) showed that the formate-to-lactate ratio was increased by *pfl* gene overexpression in *Streptococcus bovis*. Homologous expression of the [FeFe] hydrogenase gene in *C. tyrobutyricum* JM1 reported by Jo et al. (2010) resulted in a 1.7-fold and 1.5-fold increase in hydrogenase activity and hydrogen yield, respectively, compared to the wild type. *Enterobacter cloacae* IIT-BT 08 with an overexpressed [FeFe] hydrogenase gene achieved a 1.3-fold increase in hydrogenase activity under fermentation using cheese whey (Khanna et al. 2011). The introduction of a foreign hydrogenase has been considered as another option to enhance hydrogen production. However, successful heterologous expression of a hydrogenase gene remains a challenge due to problems involving the complicated technique and insufficient knowledge of hydrogenase maturation (Kuchenreuther et al. 2010).

Apart from using genetic manipulations to construct a strain with multiple carbon source consumption capabilities, statistical optimization of the medium formulation for complex waste degradation could be used to determine the chemicals that have the most significant contribution. For example, NH_4NO_3 was added during the fermentation of crude glycerol to provide an additional nitrogen source, resulting in increased ethanol production, whereas the addition of $FeSO_4$ provided iron and oxygen to the cell and enhanced hydrogen and ethanol co-production (Jitrwung and Yargeau 2011). According to Chookaew et al. (2014), KH_2PO_4 and NH_4Cl are responsible for the buffer capacity and nitrogen source, respectively, and hence had a significant effect on hydrogen and ethanol production from crude glycerol by thermotolerant *Klebsiella* sp. TR17. The results from the Plackett-Burman design indicated that the optimum medium components were 11.14 g/l of crude glycerol, 2.74 g/l of KH_2PO_4 and 6.03 g/l of NH_4Cl; the maximum simultaneous hydrogen and ethanol yield expected to be achieved using this optimized media were 0.27 mol H_2/mol glycerol and 0.63 mol EtOH/mol glycerol, respectively.

Because the mixtures of simple sugars and organic wastes such as glucose and cheese whey showed encouraging results as reported by Ferreira Rosa et al. (2014), co-fermentation using a different combination of carbon sources should be further studied. To date, the strategies to improve the substrate conversion efficiency are too ineffective to accomplish hydrogen and ethanol co-production using waste materials in industrial applications. Hence, extensive investigation into relevant research is in demand. In the future, genetic modification in combination with the regulation of fermentation parameters could represent a promising step for microbial fermentation using various waste materials as feedstock, and make the co-production of hydrogen and ethanol in a commercial setting feasible.

Conclusion

A hydrogen and ethanol co-production system is considered a venture for the development of future fuels. In this review, some of the potential microorganisms that contribute to the metabolic pathways by consuming carbon sources to yield both hydrogen and ethanol under the fermentation conditions have been identified. A variety of potential feedstock derived from waste materials that could be utilized as the substrate also have been elucidated. However, the inefficient bioconversion rate and low product yield are major limitations of fermentation using these feedstock. A combination of fermentation strategies and molecular approaches could be a promising avenue to overcome these limitations. Because the development of hydrogen and ethanol co-production is still in the infancy stage, substantial research on improvements is necessary to establish a commercially viable large scale production with high performance and efficiency.

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