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Review on biohydrogen production by dark fermentative bacteria using starch-containing waste as a substrate

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ABSTRACT

The need of energy has become a global issue that is challenging to the humanity due to its high demand, with fossil fuel is still being the main source of energy. However, fossil fuel is a non-renewable form of energy. The insufficiency of oil reservoir to fulfil overwhelming demands of energy will lead to the energy scarcity. Biohydrogen produced from waste has a potential to be an alternative energy for the future. Numerous papers have discussed biohydrogen process technology using several methods such as dark fermentation, photofermentation and integration of both methods. Unfortunately, there is still lack of papers addressing specifically on what is the most suitable bacteria for biohydrogen production. Dark fermentative bacteria are bacteria that need no light during fermentation. They are reported to be robust and have capability in consuming sludge and waste to synthesize biohydrogen. Therefore, this paper aims to discuss the correlation between dark fermentative bacteria as an agent, with hydrogen yield. The scope of this paper will focus on the performance and requirement of different species of dark fermentative bacteria using starchy waste as substrate.

1. Introduction

As an energy carrier for alternative fuel in the future, hydrogen is a clean energy because it produces water as a byproduct [1]. However, current hydrogen gas production is not eco-friendly as it is generated from fossil fuels through thermo-chemical processes, such as hydrocarbon reforming, coal gasification and partial oxidation of heavier hydrocarbons [2]. Thus, production of

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hydrogen by biological means attracts researchers due to its potential for inexhaustible, low-cost and renewable source of clean energy.

On the other hand, the expansion of industries also contributes to the environmental problems. Industries discharge chemicals and organic waste that needs to be treated in such a way that it will not harm ecosystem when it is released to the environment. The great challenge is how to utilize sludge or waste to become a useful and viable product. The main objective of this consideration is to reduce the health or environmental side effect as low as possible as well as maintaining the sustainability of raw material.

From this notion, many researchers nowadays focus on utilization of organic wastes as substrate for biohydrogen production. However not all wastes can be used to produce biohydrogen. Cost, availability, carbohydrate content and biodegradability should become the major criteria in choosing appropriate waste [3]. Some of the starchy wastes that have been used as biohydrogen substrate include wheat straw, barley straw, corn stalk and corn cob [4], tofu [5], olive oil wastewater [6], sugar refinery wastewater [7], bagasse and soy sauce wastewater [8].

Selection of hydrogen producing bacteria becomes critical since not all bacteria have the capability to utilize waste. Among wide range of species, dark fermentative bacteria is the most prominent bacteria that can produce biohydrogen from waste. Dark fermentative bacteria are usually classified based on oxygen sensitivity and temperature condition. In term of oxygen tolerance level, dark fermentative bacteria can be classified as obligate anaerobe, facultative anaerobe and aerobe. Based on the temperature condition, it is classified as mesophilic, thermophilic and extreme thermophilic. The objective of this paper is to investigate variety of bacteria that has the ability to synthesize bacteria from starchy waste. The systematic approach includes reviewing research on classification of dark fermentative bacteria as well as evaluating performance of strain in producing biohydrogen.

2. Dark Fermentation

Biohydrogen production method generally includes photolysis, photofermentation and dark fermentation. The first and second methods are able to convert solar energy into hydrogen. However, they are not preferred in practice due to low utilization efficiency of light and difficulties in designing photobioreactor which is caused by light shading effect [9]. Light shading effect is a phenomenon where the denser cell prevents the light to penetrate the reactor [10]. Moreover, compare to photosynthesis, dark fermentation shows significant performance when organic waste is used as a substrate. Therefore, generating biohydrogen from waste not only helps in getting biohydrogen but also helps in bioremediation of wastewater [11,12].

Dark fermentative bacteria do not require light to grow. During fermentation and the absence of oxygen, alcohol and organic acid are formed. Complex carbon is hydrolyzed into a simple carbon such as glucose. The glycolysis will convert glucose to pyruvate. In obligate anaerobe, pyruvate is oxidized to acetyl co-A. The oxidation triggers reduction of ferrodoxin. [FeFe]-hydrogenase will oxidize reduced ferrodoxin to produce hydrogen molecules. This process pathway is given by Eq. 1. In the case of facultative anaerobe such as *E. coli*, as given in Eq. 2, pyruvate will be oxidized into formate and acetyl co-A. Formate will be then converted to produce 2 moles of hydrogen and CO_2 [13].

$$Glucose \rightarrow Pyruvate \rightarrow Acetyl\ CoA \rightarrow Ferrodoxin \rightarrow H_2 \quad (1)$$

$$Glucose \rightarrow Pyruvate \rightarrow Acetyl\ CoA \rightarrow Formate \rightarrow H_2 + CO_2 \quad (2)$$

Dark fermentation is simpler compared to the photofermentation that carries out sophisticated mechanism. Variety of wastes can be used as the substrate regardless how darkish the color is, since it will not be affected by the penetration of light. Therefore in the large scale operations, extra cost to enlighten the process can be avoided. However, theoretically, only 2 and 4 molecules of hydrogen can be synthesized from the dark fermentation process [14]. Dark fermentative bacteria generate organic acids as metabolic products such as acetic acid and butyric acid. Notwithstanding, unlike photofermentation which is able to convert organic acids, only glucose and monosaccharide can be converted to hydrogen in dark fermentation.

3. Classification of Dark Fermentative Bacteria

Dark fermentation shows simpler mechanism and preparation as compared to photofermentation and hybrid process especially for large-scale production. Most of the dark adaptive microorganisms are usually found in those places where the oxygen is devoided, for example under the soil, waste water, lake, river, ocean, sludge and sewage, and inside the vegetable [15]. Table 1 summarizes the variety of bacterial cultures that are commonly used to produce hydrogen.

3.1 Oxygen Tolerance Level

3.1.1 Obligate anaerobe

Obligate anaerobic bacteria have no ability to synthesize oxygen-linked respiratory chain during metabolism [15]. In the presence of oxygen, the hydrogen carrier or reduced flavoprotein will bind with oxygen to produce hydrogen peroxide and superoxide radical [13]. The two compounds are toxic for bacteria. Aerobic bacteria have catalase and superoxide dismutase enzymes that can digest the toxin. In obligate anaerobes, yet there is no such enzyme found [16]. In further investigation, these enzymes have been identified in some obligate anaerobic bacteria, and the genes responsible to the enzyme have been characterized in their genome [17]. However, hydrogen peroxide and superoxide radicals are not the sole inhibitors to the cell. Full-bore oxygenation can cause serious reactive oxygen species (ROS) stress and central metabolic pathway disruption [18]. Even though oxygen can suppress bacterial life, it is still needed to support the growth. The growth of *Desulfovibro vulgaris* was not affected at 0.02 to 0.04% of oxygen concentration (0.24 to 0.48 µM), but was completely inhibited at 0.08% (0.49 µM) [19].

In other investigation, it is found that during succinate and acetate excretion of carbohydrate fermentation in obligate anaerobes, some of the pathways were blocked in the air [20]. Thus, instead of oxygen, obligate anaerobes utilize sulphate, nitrate, iron, manganese, mercury and carbon monoxide as the acceptor electrons. During fermentation, 4 mol of hydrogen per mol of glucose is produced by obligate aerobe [21].

Clostridium is strict anaerobes, gram-positive, rod shape bacteria which can be found in animal intestine, soil and sewage. Clostridium has successfully been isolated from sewage sludge which capable of producing hydrogen of 2.78 mol H_2/mol sucrose [22]. This indicates that this strain belongs to bacteria that can produce hydrogen effectively even in the acclimated anaerobic sludge. Clostridium needs minerals for growth support. The hydrogen production from sugarcane bagasse with supplementation of $CaCO_3$ by *Clostridium thermocellum* (*C. thermocellum*) was reported to be about 116.72% of increment from the control. The percentage proves that addition of $CaCO_3$ can improve metabolites during fermentation [23]. On the other hand, addition of 0.03 g/l $FeSO_4.7H_2O$ to the rice bran also helps *C. saccharoperbutylacetonicum* to produce a maximum yield of 3.29 mol H_2/mole substrate [24].

3.1.2 Facultative anaerobe

Facultative anaerobic bacteria can grow in the presence of oxygen by producing ATP. But they are rapidly switched from aerobe to anaerobe when oxygen is depleted, by then, they undergo fermentation. In industry, facultative anaerobe is more preferable than obligate aerobe due to the capability to survive even in the contamination of oxygen [15]. Most facultative anaerobe produces 2 mol of hydrogen per mol of glucose [21].

Among facultative bacteria, Enterobacter is the most stringent hydrogen producing bacteria. They are able to utilize starchy waste to produce hydrogen. It is reported that *Enterobacter cloacae* (*E. cloacae*) contains [Fe]-hydrogenase [25]. [Fe]-hydrogenase is one of the prominent enzyme that causes high rate of hydrogen synthesis [25]. Other than [Fe]-hydrogenase, [FeFe]-hydrogenase and [NiFe]-hydrogenase are also important for biohydrogen production [26]. In later investigation, it was found that *E. cloacae* follows Embden-Meyerhof-Parnas (EMP) pathway with 2,3 butanediol as major fermentative product [27].

Waste derived crude glycerol provides high carbon source. However, it still needs a lot of supplementation. The addition of 10 mg/l urea as nitrogen source increased the hydrogen yield by 38.57% to 116.41 mmol H_2/l media [28]. Urea provides nitrogen that is mostly essential for the growth of bacteria. Furthermore, other metals such as iron, magnesium and cuprum are also needed by bacteria. The addition of those metals to the distillery effluent waste have given a significant increment to 7.38 mol H_2/kg COD [11].

3.1.3 Aerobe

Hydrogenase is highly sensitive to oxygen. Most of the time, hydrogenase does not show any activities in the presence of oxygen. However, it was discovered that hydrogen production was catalyzed by *Alcaligenes eutrophus* (*A. eutrophus*) under aerobic condition. *A. eutrophus* is a strictly aerobic hydrogen-oxidizing bacterium. The hydrogenase of *A. eutrophus* has a high stability to oxygen so that this enzyme is capable of producing hydrogen from NADH by reducing methyl viologen [29]. While in other bacteria, it is hard to reduce methyl viologen under aerobic condition due to quick substrate oxidation [29]. In later investigation, it was revealed that *A. eutrophus* that was supplemented with nitrate can produce hydrogen under anaerobic condition [30]. During fermentation, nitrate had a role as the terminal acceptor electron [30]. Even though it was proven that aerobic bacteria can produce hydrogen under anaerobic condition, the question on which enzyme is responsible during hydrogen production in the restriction of oxygen is still open. Other than *A. eutrophus*, *Bacillus lichenformis* (*B. lichenformis*), the aerobic bacteria that was isolated from cattle dung, were also capable of producing hydrogen [31]. About 0.5 mol H_2/mol glucose was produced from the wheat grain waste under anaerobic condition by *B. lichenformis*.

3.2 Temperature Condition

3.2.1 Thermophile

Fermentation process by using thermophilic bacteria usually occurs in the range of 80-115^{o}C (optimum 80^{o}C) or little lower at 40-110^{o}C (optimum 60 ^{o}C) [15]. Studies have found that hydrogen production is an endothermic reaction which needs more heat in order to accelerate the chemical reaction [32]. The substrate from industrial waste can be used directly without the need of thermal sterilization. Since the temperature is very high, contamination by mesophilic or psycrophilic bacteria can be avoided.

The strains from species of Thermotoga, Thermoanaerobacter and Caldicellulosiruptor are the most common thermophilic hydrogen producing bacteria [50-52]. Recent investigation had successfully produced biohydrogen by Thermoanaerobacter GHL15 using lignocellulostic biomass; Whatman paper (cellulose), hemp (*Cannabis sativa*), newspaper, barley straw (*Hordeum vulgare*), and Timothy grass (*Phleum pratense*) [36]. The optimum temperature and pH were 65^{o}C and 6, respectively. At a lower partial pressure, the strain can produce a maximum 3.1 mol H_2/mol glucose. However, for lignocellulostic biomass, the highest hydrogen yield was obtained from Timothy grass at 7.6 mmol H_2/g grass [36]. This yield was quite low compared to pure glucose. It is concluded that the choice of chemical pretreatment method of lignocellulostic material is essential for maximizing the hydrogen yield.

Previously, there was a report on investigating the optimum condition for fermentative hydrogen production by *Thermoanaerobacter mathranii* (*T. mathranii*) strain A3N using starch as substrate [37]. At pH 9 and temperature of 70^{o}C, the hydrogen harvested was 17.91 mmol/g starch from 7.5 g/l starch. Under this condition, the hydrogen yield was nearly 78-80% of the theoretical yield. Interestingly, when the initial starch concentration was increased to 35 g/l, only 32.8% of starch can be converted to hydrogen. The finding is consistent with the previous study on biohydrogen production from Potato Steam Peels (PSP) waste using *Caldicellulosiruptor saccharolyticus* (*C. saccharolyticus*) and *Thermotoga neapolitana* (*T. neapolitana*) [38]. In this study, *T. neapolitana* is more sensitive to concentrated PSP hydrolysate. The strain produced maximum hydrogen at 10-14 g/l of glucose unit. When the substrate concentration in the growth medium was increased to 30-40 g/l of glucose units, efficient hydrogen production was no longer feasible.

However, this trend was not observed in *C. saccharolyticus* because the strain is more resistant to high substrate loading [38]. It was predicted that high initial substrate concentration has led to the accumulation of high biomass and organic metabolites, which probably has resulted in the unfavorable conditions for further hydrogen generation in a batch experimental mode [37].

3.2.2 Mesophile

Mesophilic bacteria are highly adapted at the temperature range of 10-50^{o}C (optimum at 37^{o}C) [15]. Among variety of mesophilic bacteria, strain from *Enterobacter sp*. and *Clostridium sp*. are the most prominent strains to produce biohydrogen. They are inoculated either in pure [39] or mix cultures [40]. Since it used mild operating temperature, mixed cultures of mesophilic bacteria is a suitable approach for commercial improvement of biohydrogen production.

3.2.3 Psycrophile

Psycrophilic bacteria have commercial interest due to their unique protein structure and thermal stability which can make them possible to survive in the harsh environmental condition at extremely low temperature [41]. They have been used widely in medicine, food technology and bioremediation of environment [42]. Psycrophilic bacteria can survive in the temperature range of 0-20^{o}C (optimum at 15^{o}C). Other paper investigated the performance of *Rahnella aquatilis* (*R. aquatilis*) to produce biohydrogen from cheesy whey waste [43]. At the temperature of 20^{o}C, 3087.57 ml H_2/g bacterial biomass is produced by *R. aquatilis*.

Today, there is still lack of interest on the research of biohydrogen production using this strain due to the complexity of conditions inside the bioreactor, especially if the operation takes place in a humid and hot area. However, another study has proven that *Geobacter psychrophilus* (*G. psychrophilus)* can produce significant amount of hydrogen (2.66 mol H_2/mol acetate) at the

temperature of 4-9^{o}C [44]. It was reported that such low operating temperature can inhibit methanogenic activity caused by *Methanobrevibacter arboriphilus* (*M. arboriphilus*) during fermentation [44]. Therefore, more hydrogen is produced compared to methane. Even though psycrophilic bacteria seems to be promising, it should be noted that extremely low temperature condition may cause reduction of enzyme activity, decrease of membrane fluidity, change of nutrient transport and waste products, decrease of transcription rate, translation and cell division, protein denaturation, and intracellular ice formation of the cell [15].

Table 1
Performance of diverse group of hydrogen producing microorganism during dark fermentation of various carbon source

No	Bacteria	Raw Material	Process parameters			Hydrogen Yield	Reference
			pH	*Temperature (^{o}C)*	*Inoculum size*		
	Obligate anaerobe						
1	*C. thermocellum*	Sugarcane bagasse (SCB)	6-7	55	10% v/v	4.89 mmol H_2/g SCB	[23]
2	*C. saccharoperbutylacetonicum* N1-4	Ricebran	6	30	n/a	3.37 mol H_2/ mol sugar consumed	[24]
3	*Clostridium sp.*	POME	6	37	0.1% v/v	27.09 ml H_2/g COD	[45]
4	*Clostridium pasteurianum*	Basal media	6.8	35	n/a	3,43 mol H_2/mol sucrose	[46]
	Facultative anaerobe						
5	*Escherichia coli*	Sago waste	6	40	30% v/v	2.22 mol H_2/mol glucose	[47]
6	*E. cloacae* IIT-BT 08	Distillery effluent	7.5	37	10% v/v	7.38 mol H_2/kg COD	[11]
7	*E. aerogenes*	Biodiesel-based glycerol	n/a	37	0.2% v/v	0.84 mol H_2/mol glycerol	[48]
8	*E. aerogenes* NRRL B 407	Crude glycerol	6	n/a	0.05% v/v	116.41 mmol H_2/l media	[28]
9	*K. pneumoniae*	Basal media	6	37	n/a	5363.8 ml H_2/l	[49]
10	*E. aerogenes*	Glucose	6.15	30	n/a	1.69 mol H_2/mol glucose	[50]
11	*E. cloacae* IIT-BT 08	Basal media	5.5-7.5	35	0.1% v/v	3.1 mmol H_2/mol glucose	[51]
12	Anaerobic mixed sludge	Basal media	7 & 8	35	0.25% v/v	236 ml H_2	[52]
13	*C. saccharoperbutylacetonicum*	Cheese whey	6	30	5% v/v	7.89 mmol H_2/g lactose	[53]
	Aerobe						
14	*B. lichenformis*	Wheat grain waste	6-8	38-40	0.01% v/v	0.5 mol H_2/mol glucose	[31]
	Thermophiles						
15	*Caldicellulosiruptor bescii*	Wastewater biosolid	7	78	2.5 g VS/L	4.4 mmol H_2/g VS_{added}	[34]
16	*Thermoanaerobacter* GHL15	Timothy grass	6	65	n/a	7.6 mmol H_2/g grass	[36]
17	*C. saccharolyticus*	Wheat straw	7	70	n/a	3.3 mol H_2/mol glucose	[54]
18	*T. mathranii*	Starch	9	70	0.5% v/v	17.91 mmol H_2/g starch	[37]
19	*C. saccharolyticus*	Barley straw	7	70	n/a	40 mmol H_2/l	[55]
20	*T. neapolitana*	Biodiesel manufacturing waste	7.5	70	10% v/v	2.73 mol H_2 mol/ $glycerol_{consumed}$	[56]
21	*Thermoanaerobacterium saccharolyticum, Thermoanaerobacterium thermosulfurigenes,* and *uncultured Thermoanaerobacterium sp*	Sago waste	6.5	60	0.1% v/v	422 mlbH_2/g starch	[35]
22	*C. saccharolyticus* and *T. neapolitana*	Potato steam peel	6.9	72 & 75	10% v/v	2.4-3.8 mol H_2/mol gluose	[38]
	Mesophiles						
23	*E. aerogenes* and *C. butyricum*	Apple pomace	6.5	37	15% v/v	26.07 mmol H_2/l	[40]

		hydrolysate					
24	*C. butyricum*	*Scenedesmus obliquus*	6.8	37	1% v/v	113.0 mL H_2/g VS_{alga}	[39]
25	*E. aerogenes*	*Scenedesmus obliquus*	6.8	30	10% v/v	57.6 mL H_2/g VS_{alga}	[39]
	Psychrophiles						
26	*Pseudomonas meridiana*	Glucose	6.5	25	n/a	0.51 mol H_2/mol glucose	[57]
27	*Devosia limi*	Glucose	6.5	25	n/a	0.8 mol H_2/mol glucose	[57]
28	*Flavobacterium limicola*	Glucose	6.5	25	n/a	0.74 mol H_2/mol glucose	[57]
29	*Actimicrobium antarcticum*	Glucose	6.5	25	n/a	0.52 mol H_2/mol glucose	[57]
30	*R. aquatilis*	Cheese whey	n/a	20	n/a	3087.57 ml H_2/g bacterial biomass	[43]
31	*G. psychrophilus*	Basal media	n/a	4-9	n/a	2.66 mol H_2/mol acetate	[44]

4. Raw Material

Microorganisms need carbon sources in order to produce hydrogen. In nature, the carbon is available in many forms. Simple sugar is one of the feedstock that is widely used. *Enterobacter aerogenes (E. aerogenes)* was able to consume monomeric sugars; glucose, arabinose, xylose, mannose, galactose, rhamnose during fermentation [58]. The conversion rate of simple sugar such as glucose, sucrose and xylose is quite high because simple sugar is readily digestible for bacteria [3]. Despite of prior evidence, the use of sole carbohydrate as the source of hydrogen synthesis is quite expensive [59]. Therefore more viable and renewable waste must be developed [59].

Biomass feedstock could enhance production of biohydrogen. In recent studies, starch based crops (corn and wheat) [60] and sugar containing crops (sugar cane, sweet shorgum, and sugar beet) [23], are mostly used for fermentation process (Table 1). However, the use of food crops for energy production will likely impossible because it can trigger the crisis of food shortage. In view of that, the second generation of cellulosic biomass such as barley straw, wheat straw, corn stalk and corn cob and sweet potato starch residue are employed for energy crops [4].

In recent years, there has been an increasing interest in waste as the feedstock. The main consideration of choosing waste as substrate compared to pure carbohydrate and biomass is due to the cost effectiveness and renewability. Waste material is more economical as well as environmentally friendly [12]. In addition, generating biohydrogen from waste not only helps in getting biohydrogen but also helps in bioremediation of wastewater [11]. Several aspects including the availability, cost, carbohydrate content and biodegradability must be considered in the selection of waste [3].There is variety of carbon sources from waste that has been used for biohydrogen production. Rice bran [24], crude glycerol [28], apple pomace [40], POME [45], and cheesy whey [53] are reported to yield significant amount of hydrogen during dark fermentation. Those wastes contain glucose which can be converted to hydrogen. Dark fermentative bacteria are not only able in producing hydrogen but also reducing toxin in wastewater [15].

E. aerogenes was able to produce 1.69 mol H_2/mol glucose using glucose as the sole substrate [50]. When using glycerol waste from biodiesel, the hydrogen yield produced by this strain was slightly lower which was 0.84 mol H_2/mol glycerol [48]. In other study, *C. pasteurianum* can produce 3.43 mol H_2/mol sucrose using basal media and sucrose as carbon source [46]. As compared to *C. thermocellum*, the hydrogen from sugarcane bagasse is 4.89 mmol H_2/g SCB [23]. Even though the yield of hydrogen from simple sugar is higher than those from waste, utilization of waste to produce biohydrogen should be considered. Therefore, in order to maximize hydrogen yield from

waste, optimization of biohydrogen process condition and pretreatment method must be taken into account.

5. Conclusion

As the global fuel hike is inevitable, it is essential to find other options which can substitute fossil fuel. Biohydrogen appears as the promising energy alternative which not only meets the demand of energy but also results in the clean environment.

Dark fermentation is more preferable method to produce biohydrogen from waste due to the simplicity of its mechanism. It does not use light as the source of energy. Dark fermentative bacteria are very robust. Based on oxygen tolerance level, they can be categorized as obligate ananerobe, facultative anaerobe and aerobe. While, based on the fermentation temperature, they are categorized as thermophile, mesophile and psycrophile. All of them has special characteristic that need different treatment conditions.

The research of raw material for biohydrogen production is improving from time to time. Initially, the carbon source is in the form of synthetic simple sugar which was then going to the biomass feedstock and then currently the starchy waste. Although there were many reports on the process technology in the wastewater, only few of them focusing on the pre-treatment process. Indeed, one of the challenges in the production of hydrogen from the waste is the pre-treatment [61]. Therefore, extensive and deep studies on the waste pre-treatment method are necessary to increase the effectiveness of using waste as one of the substrates to produce hydrogen.

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