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Review

Bio-hydrogen production by different operational modes of dark and photo-fermentation: An overview

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ABSTRACT

This article overviews reported studies on bio-hydrogen production from different raw materials by dark and photo-fermentations operated with different modes. Sequential and combined dark and photo-fermentations operated in batch, continuous and fed-batch modes were compared. Operating conditions and modes resulting in the highest hydrogen yield and formation rate were revealed. Relative advantages of sequential and combined dark and photo-fermentations were discussed. Sequential fermentation was found to be preferable due to high H_2 yields and productivities. High cell density fed-batch culture with controlled feeding and simultaneous product removal was concluded to be the most suitable operation mode at the optimum environmental conditions.

1. Introduction

Hydrogen (H_2) is considered to be one of the most promising fuels of the future [1] due to high energy content (122 kJ/g) as compared to hydrocarbon fuels [2–4]. Hydrogen gas is also a clean fuel with no CO_x, SO_x and NO_x emissions. Besides, H_2 is an important energy carrier and can be used in fuel cells for generation of electricity [5,6]. However, hydrogen gas is not readily available in nature like fossil fuels and natural gas, but can be produced from renewable materials such as biomass [2,7] and water [3]. Hydrogen gas production technologies has gained special attention during the last fifty years due to the increasing energy demand, rapid consumption of non-renewable fossil fuel reserves and hydrocarbon fuel based atmospheric emissions [7–9].

Steam reforming of natural gas and water electrolysis are the most commonly used processes for H_2 gas production [1]. Due to energy intensive nature of those processes more energy efficient H_2 production methods are searched for [6]. Hydrogen gas production from renewable resources (e.g. biomass) and carbohydrate rich waste materials by bio-processes offers distinct advantages over energy intensive methods used [2,10]. Major drawbacks in bio-hydrogen production are low yields and productivities requiring large reactor volumes and long residence times [2,11].

Main bio-hydrogen production processes are direct/indirect bio-photolysis, dark and photo-fermentations [1,6,10,11]. A brief comparison of those processes is depicted in Fig. 1. Bio-photolysis of water under sunlight is considered as the cleanest approach for bio-hydrogen production. However, low

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H_2 gas productivity [12], strict light requirement and oxygen inhibition are the main problems in bio-photolysis of water [2,12]. Fermentative hydrogen gas production from carbohydrates is a much faster process than bio-photolysis [8,10,13] with volatile fatty acids (VFAs) and H_2 gas formation [14,15]. However VFAs need to be fermented for further H_2 gas production [16,17]. Photo-fermentative bacteria have the ability to use VFAs for H_2 production under light irradiation [2,6,13] for which dark fermentation effluent can be used as feedstock [14,15,18–20]. Hydrogen gas formation can be enhanced by integration of dark and photo-fermentations in the form of sequential or combined fermentations [8,14,15,18–21]. Both the dark and photo-fermentations can be realized by using suspended and immobilized-cell reactors operating in batch, continuous and fed-batch modes. Major mechanisms for bio-hydrogen production by dark and photo-fermentations have been elucidated. However, development of an effective bio-hydrogen production process at industrial scale is still a challenge. Recent reviews published on fermentative hydrogen gas production summarized studies using different raw materials, bacterial cultures, operating conditions (T, pH, ORP) and pre-treatment methods [1–3,6,10,11,13]. The reported rates and the yields of fermentative hydrogen gas production were not high enough to make the process economically viable. The most suitable raw materials, pre-treatment methods, bacterial cultures, operating conditions, cultivation types, operating modes and processing schemes are yet to be determined for an effective and economically viable fermentative hydrogen production process.

Operating modes of bioreactors affect the rate and the yield of hydrogen gas formation during dark and photo-fermentations by affecting the biomass, substrate and product concentrations in the reactor. None of the published review articles considered the effects of operating modes and processing schemes as factors affecting hydrogen production rate and the yield. Therefore, the main objective of this study is to overview the reported studies on bio-hydrogen production by different operating modes. Sequential and combined dark and photo-fermentations operated in batch, continuous and fed-batch mode were compared in terms of hydrogen gas formation rates and yields. Some conclusions were drawn to identify the most suitable cultivation type (suspended or immobilized cultures) and operation mode (batch, continuous, fed-batch) for effective bio-hydrogen production.

2. Dark fermentation

A wide variety of heterotrophic bacteria have the ability to ferment carbohydrates under anaerobic conditions to produce H_2 gas, volatile fatty acids (VFAs) and CO_2 [8,22–24]. In general, spore forming *Clostridium* species, facultative *Enterobacter* sp, *Bacillus* sp [2,13,25], some thermophilic bacteria [22,24,26,27] and anaerobic acidogenic sludge [2,22,28–30] are the most widely used cultures for this purpose. Hydrogenase is the key enzyme catalyzing molecular H_2 formation by combining protons and electrons in dark fermentation [31,32]. Usually, monosaccharides are main carbon sources [22,33–35] which can be generated by acid or enzymatic hydrolysis of polysaccharides like starch or cellulose [23].

Dark fermentative conversion of glucose to H_2, acetic acid and CO_2 is presented in (Eq. (1) [10]). Negative free energy indicates that the reaction proceeds toward product formation spontaneously with no external energy requirement. Theoretically, a maximum of 4 mol of H_2 can be produced per mole glucose when acetic acid is the only VFA product. However, lower yields are obtained in practice since part of the glucose is used for microbial growth and maintenance [34]. Butyric acid formation is accompanied with formation of 2 mol of H_2 per mole of glucose and propionic acid formation consumes 1 mol of H_2 per mole of propionic acid [36,37]. Lactic acid and ethanol fermentations do not result in H_2 formation or consumption. When both acetic and butyric acids are produced in dark fermentation of glucose, theoretically 2.5 mol H_2 is formed per mole glucose [38].

$$C_6H_{12}O_6 + 2\,H_2O \rightarrow 2\,CH_3COOH + 4\,H_2 + 2\,CO_2 \quad \Delta G^o = -206\ \text{kJ} \tag{1}$$

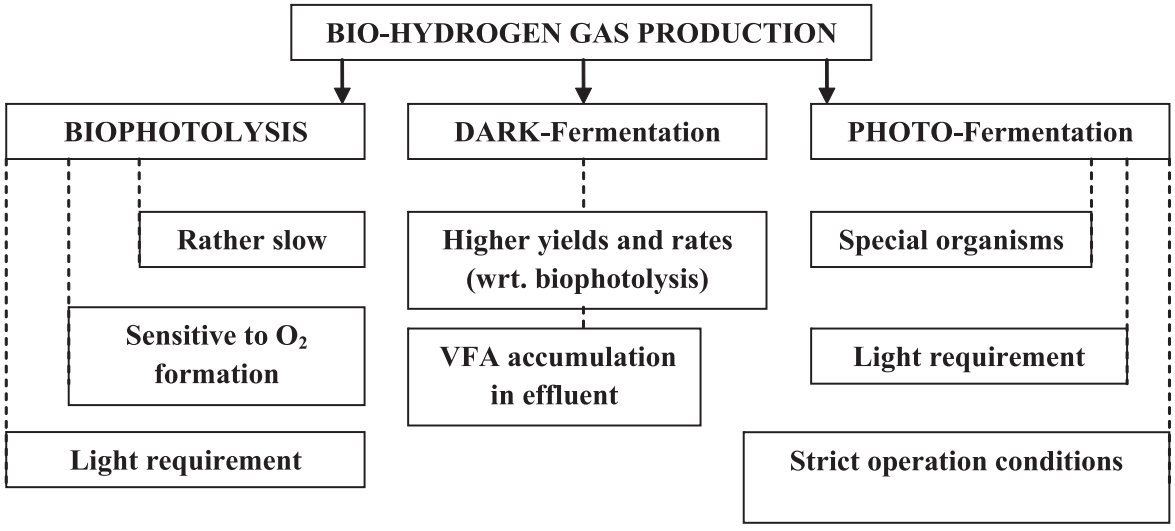

Fig. 1 – Comparison of bio-hydrogen production by algal and bacterial bioprocesses.

Presence of H_2 consumers such as homoacetogens, methanogens [22,39,40], nitrate and sulfate reducing bacteria [7,22] in mixed non-sterile cultures are other factors reducing the hydrogen yield [22,39,41]. Some seed culture pre-treatment methods like heat shock, acid or alkaline treatment, sonication, freezing and thawing, chloroform, sodium 2-bromoethansulfonate or 2-bromoethansulfonic acid (BESA), iodopropane treatments [22,42,43] and control of operation conditions (e.g. acidic pH and short HRT) [7] can be used to eliminate H_2 consuming bacteria to some extent.

Other important factors affecting the performance of dark fermentation are operating conditions such as pH and temperature [7,22,24]. Hydrogen gas production occurs at acidogenic stage of anaerobic metabolism [2,22]. Therefore pH values between 5.5 and 6.5 were reported to be the optimal [2,44,45]. Low pH values (pH = 4.5) were stated to result in solvent formation since *Clostridium acetobutylicum*, *Clostridium butylicum*, and *Clostridium beijerinkii* have the ability to produce ethanol, butanol and acetone at low pH ranges reducing H_2 formation [46,47]. On the contrary, Zhao et al. [48] recently proposed operation of dark fermentation at alkaline pH of 10 to avoid formation of propionic acid and effective inhibition of acetogenic bacteria responsible for H_2 consumption when waste activated sludge was used as the substrate. Therefore, pH control at the optimum level is of great importance for effective H_2 gas formation in dark fermentation. Dark fermentation can be realized under mesophilic (25–40 °C), thermophilic (40–65 °C) or hyper-thermophilic (>80 °C) conditions [11]. Mesophilic temperature range has been used more widely as compared to thermophilic temperature [26] due to less energy requirement [27]. However, thermophilic operation was reported to yield higher H_2 yields due to limited growth of H_2 consumers and faster metabolic activity of bacteria [26]. Optimum oxidation–reduction potentials (ORP) for *Clostridium* sp. vary between −200 and −250 mV [49]. ORP values out of this range might result in sub-optimal conditions for H_2 producing bacteria.

Nitrogen (N), phosphorous (P), Iron (Fe) and sulfur (S) are required nutrients playing crucial roles in H_2 production by dark fermentation [24]. Nutritional requirements of acidogenic bacteria depend on the type of bacteria and the experimental conditions. Various studies were reported in literature investigating the optimal C/N and C/P ratios in dark fermentation [33,50]. Optimum ratios ranged between 11.4/1 and 200/1 for COD/N and 73/1 and 970/1 for COD/P ratio, respectively [24].

Hydrogen gas formation rate and yield are the two most important criteria used in selecting the most suitable bacteria for fermentative H_2 production. The yield is defined as the amount of H_2 produced per amount of substrate consumed (mol H_2/mol glucose) [51,52]. Hydrogen gas production rate (HPR) is defined as the amount of H_2 (ml) produced per unit time and per unit reactor volume (volumetric rate) or per unit biomass (specific rate) [52].

2.1. Batch dark fermentation

Most of the dark fermentation studies for hydrogen gas production were realized by batch operation where different types of bacteria and substrates were used. Batch fermentations are usually subject to substrate and product inhibitions yielding low hydrogen gas productivities. The reported batch studies were mainly aimed to identify conditions enhancing H_2 production by substrate and culture selections and adjustment of operation parameters. Due to the excessive number of articles on dark fermentative H_2 production, only striking reports are summarized below.

H_2 formation yields are usually between 1.5 and 2.5 mol H_2 mol^{-1} glucose in most of the dark fermentation studies. However, H_2 yields between 3 and 4 mol H_2 mol^{-1} glucose have been reported recently. One of those studies was done by Ntaikou et al. [53] where *Ruminococcus albus* was used as inoculum culture for H_2 production from sweet sorghum biomass. *R. albus* was stated as promising culture able to ferment hexoses and pentoses as well as cellulose and hemicelluloses present in sorghum biomass. Hydrogen formation yield of 3.15 mol H_2 mol^{-1} glucose was reported when 3 g L^{-1} glucose equivalent sorghum stalks was used as substrate in batch experiments.

Vegetable waste supplemented with sewage was used by Mohanakrishna et al. [54] for H_2 production by dark fermentation. A high H_2 yield of 25.8 ± 0.6 mol H_2 kg COD was obtained with a substrate concentration of 4.8 kg COD m^{-3} when vegetable waste was supplemented with sewage. About 65% of total TVFA concentration consisted of acetic acid resulting in high H_2 yields. High concentrations of TVFAs formed at high substrate concentrations resulted in lower pH values adversely effecting H_2 gas formation.

Hydrolyzates produced from steam explosion of corn stover were used by Datar et al. [47] for H_2 production by batch dark fermentation. Heat pre-treated anaerobic sludge was used as inoculum culture with major products of H_2, CO_2, acetic and butyric acids. Fermentation of corn stover acid hydrolyzate (20%, v/v) followed by steam explosion resulted in the highest H_2 yield and rate of 3.0 mol H_2 mol^{-1} glucose and 275 mL H_2 L^{-1} h^{-1}, respectively. The anaerobic sludge was unable to ferment lignocellulosic solids without acid hydrolysis unless supplemented with cellulase enzymes. pH control at pH = 5.5 was reported to be necessary for high H_2 gas yields. Effects of glucose concentration on H_2 formation rate were also investigated by the same authors [47]. The rate increased from 191.25 to 905 mL H_2 L^{-1} h^{-1} when glucose concentration was increased from 3 to 25 g L^{-1}. The same trend was also stated by Lee et al. [55] who was able to increase the H_2 formation rate from 534 to 1119 mL H_2 L^{-1} h^{-1} by increasing the initial starch concentration from 8 to 24 g L^{-1}. Further increases of the substrate concentration resulted in decreases in the rate due to accumulation of inhibitory VFAs in the medium [47,55].

Hydrogen gas production by thermophilic–anaerobic fermentation was reported to be more effective than mesophilic fermentation [56,57]. In this context, Zeidan and van Niel [56] recently reported high H_2 yields close to the theoretical yield of 4 mol H_2 mol^{-1} glucose. A co-culture of *Caldicellulosiruptor kristjanssonii* and *Caldicellulosiruptor saccharolyticus* resulted in a yield of 3.8 ± 0.2 mol H_2 mol^{-1} glucose [58]. In another study of the same author, *Caldicellulosiruptor owensensis* strain OLT (DSM 13100) was used in anaerobic–thermophilic fermentation using glucose (10 g L^{-1}) supplemented with a rich vitamin solution. The highest hydrogen yield was reported to be 3.8 ± 0.1 mol H_2 mol^{-1} glucose with a formation rate of 15 mmol H_2 L^{-1} h^{-1}. pH control and N_2 gas flushing to reduce

hydrogen gas partial pressure were reported to be important factors resulting in high H_2 yields [56].

Product inhibition on H_2 gas formation in dark fermentation was investigated by increasing acetate concentration in fermentation medium [59]. Heat pre-treated anaerobic sludge was used for the fermentation of sucrose containing synthetic wastewater (25 g COD L^{-1}) with pH control at 5.5. Acetate was externally added to the fermentation media. The highest yield (1.06 ± 0.05 mol H_2 mol^{-1} glucose) and SHPR (74.2 ± 1.2 mL H_2 g^{-1} VSS h^{-1}) were obtained in control flasks where no external acetate was added. Almost 50% of the substrate was converted to VFAs consisting of 60% butyric acid. Increases in acetate concentration gradually decreased the rate and the yield of H_2 formation. A non-competitive product inhibition model was used to describe acetate inhibition.

Chen et al. [60] operated a fermenter in sequencing batch mode for H_2 production from carbohydrate rich organic wastewater. Effects of pH (4.9, 5.5, 6.1, and 6.7), and cyclic duration (4, 6, and 8 h) were investigated by using pre-treated anaerobic sludge. The highest H_2 formation yield of 2.53 mol H_2 mol^{-1} sucrose was obtained at pH 4.9, HRT = 16 h, and with the feed COD of 25 g COD L^{-1} using 4 h cycles. High VFA accumulations were reported to lower the pH of fermentation medium. pH and durations of the cycles were reported to affect H_2 gas production by sequencing batch operation.

Argun et al. [33] investigated the effects of C/N and C/P ratios on H_2 gas formation rate and yield by dark fermentation of wheat powder solution (WPS). Nitrogen and phosphorous were externally added to the fermentation media in the desired concentrations. The highest yield (281 ml H_2 g^{-1} starch) and SHPR (98 ml H_2 g^{-1} biomass h^{-1}) were obtained at C/N/P ratio of 100/0.5/0.1 (ww^{-1} w^{-1}).

Hydrogen gas can be produced from various resources through dark fermentation by the utilization of both sterile and non-sterile inoculum cultures. Notable yields were reported when complex and pure carbon sources were used. Yields around 3 mol H_2 mol^{-1} glucose were reported at low glucose concentrations (<5 g L^{-1}) resulting in low VFA formation with no considerable product inhibition [47,53,61]. At higher initial carbohydrate concentrations above 10 g L^{-1}, H_2 formation yields were around 1.0 to 2.0 mol H_2 mol^{-1} glucose which is probably due to the VFA inhibition. On the other hand, H_2 formation rate had an increasing trend with increasing initial substrate concentration up to 20 g glucose L^{-1} followed with a decrease at higher substrate concentrations [47,55,62]. Hydrogen formation yields and rates need to be further improved for effective hydrogen gas production in batch dark fermentation.

2.2. *Continuous dark fermentation*

Although most of the dark fermentation studies were performed by batch operation, continuous operations were also reported using different substrates and microbial consortia. Continuous operation provides constant product quality, production rate and the yield with high productivities at the steady-state as compared to batch fermentation. Suspended and immobilized cultures were used by employing continuous stirred tank reactors (CSTRs) and upflow anaerobic sludge blanket reactors (UASB) for bio-hydrogen production.

Reported H_2 yields in continuous dark fermentation are between 0.58 mol H_2 mol^{-1} glucose [63] and 2.80 mol H_2 mol^{-1} glucose [64] which are lower than the highest yields obtained in batch studies. In a study performed by Ren et al. [65] continuous H_2 production from diluted molasses was realized in a flocculated activated sludge reactor. High cell density was maintained in order to achieve faster conversions at low HRTs. The reactor was reported to provide a maximum yield value of 26.13 mol H_2 kg^{-1} COD at 27.58 kg COD m^{-3} d^{-1} loading rate and HRT of 6 h. The highest SHPR (31.25 mL H_2 g^{-1} ML VSS h^{-1}) and VHPR (232.08 mL H_2 L^{-1} h^{-1}) were obtained with 68.21 kg COD m^{-3} d^{-1} loading rate at HRT of 4 h. By using the ratio of 1.066 g COD/g glucose [66] the highest yield can be calculated as 4.55 mol H_2 mol^{-1} glucose which is quite higher than the theoretical yield of 4 mol H_2 mol^{-1} glucose. This is probably due to the contribution of different carbon sources for H_2 production rather than glucose.

In another study, hydrogen gas was produced from cheese processing wastewater by anaerobic–thermophilic fermentation using a CSTR [67]. Heat pre-treated anaerobic sludge was used as the inoculum culture. Effects of HRT and substrate loading on the rate and yield of H_2 gas formation were investigated. The yield was 22 mmol H_2 g^{-1} COD corresponding to 590 mL H_2 g^{-1} glucose (55 °C, 1 atm) with a mean H_2 formation rate of 62.5 mL H_2 L^{-1} h^{-1} at 13.42 kg COD m^{-3} d^{-1} loading rate at HRT = 3.5 days and T = 55 °C. Fluctuating patterns of H_2 formation rates, COD removal efficiencies and total biogas production were reported.

Krupp and Widmann [38] produced H_2 gas from waste sugar media using a 30 L CSTR inoculated with anaerobic sludge. H_2 yield of 1.78 mol H_2 mol^{-1} glucose was obtained at HRT = 15 h with a substrate loading rate of 14 kg VS m^{-3} d^{-1}. pH control around 5.5 was reported to be an important factor yielding high H_2 gas formation.

Effects of HRT (10, 5.0, 2.5, 1 h) and glucose loading rate (0.5–18.9 g COD h^{-1}) on the rate and yield of H_2 gas formation were examined by Van Ginkel and Logan [64] using heat shocked agricultural soil as inoculum in dark continuous fermentation [64]. The most favorable operating conditions of 2.5 g L^{-1} feed glucose, 6 kg glucose m^{-3} d^{-1} loading rate, and HRT = 10 h were reported to result in a yield of 2.8 mol H_2 mol^{-1} glucose and H_2 formation rate of 81 mL H_2 L^{-1} h^{-1}, respectively. High feed glucose concentrations at low HRT levels resulted in flocculation adversely effecting H_2 formation.

Continuous operation was found to be more advantageous for immobilized-cell systems. An immobilized-cell fluidized-bed bioreactor was used by Zhang et al. [68] for continuous H_2 production from glucose where pre-treated anaerobic sludge was immobilized in the reactor yielding biomass concentrations between 34 and 37 g VSS L^{-1}. The highest H_2 yield (1.7 mol H_2 mol^{-1} glucose) and formation rate (7600 mL L^{-1} h^{-1}) were obtained with a feed glucose of 10 g L^{-1}, organic loading rate of 960 g glucose L^{-1} d^{-1}, at HRT = 0.25 h, pH = 5.5 and T = 37 °C. Stable biogas production with nearly 45% H_2 content was achieved for 50 days.

Another study reporting high H_2 formation rate by continuous dark fermentation from fructose (20 g COD L^{-1}, 480 kg COD m^{-3} d^{-1}) was reported by Lee et al. [69] where

a membrane cell recycle reactor (MCR) along with a CSTR fermenter was used. MCR provided high cell concentrations in the fermentor allowing operation at high dilution rates. The highest yield (1.36 mol H_2 mol^{-1} hexose) and H_2 formation rate (2750 mL H_2 L^{-1} h^{-1}) were obtained at HRT = 1 h, pH = 6.7 and $T = 35$ °C.

Cheng et al. [70] studied continuous H_2 production using agitated granular sludge bed reactor (AGSB) fed with starch (20 g L^{-1}, 960 kg starch m^{-3} d^{-1}). Anaerobic sludge immobilized on powdered activated carbon was used as inoculum. Effects of pH (5.5 and 6) and HRT (12, 2, 1, 0.5 h) on H_2 gas formation were investigated in continuous dark fermentation. The highest yield (5.06 mmol H_2 g^{-1} starch = 0.83 mol H_2 mol^{-1} glucose) with the lowest rate (700 mL H_2 L^{-1} h^{-1}) was obtained at HRT of 12 h. The lowest yield (2.74 mmol H_2 g^{-1} starch = 0.45 mol H_2 mol^{-1} glucose) with the highest rate (1770 mL H_2 L^{-1} h^{-1}) occurred at HRT of 0.5 h. Presence of *Clostridium* sp. and *Bifidobacterium* sp. in the microbial consortia was reported to play crucial role in H_2 gas formation.

As presented above, continuous operation yielded much higher H_2 formation rates (up to 7600 mL H_2 L^{-1} h^{-1}) as compared to batch systems (max. 1119 mL H_2 L^{-1} h^{-1}) with relatively lower yields (<3 mol H_2 mol^{-1} glucose). Considerable differences were noticed in H_2 production performances of continuous suspended (CSTR) and immobilized-cell reactors. High biomass concentrations in immobilized-cell reactors provided considerable advantages over CSTR such as high substrate loading rates (960 kg glucose m^{-3} d^{-1} vs. 68.21 kg glucose m^{-3} d^{-1}), low HRTs (0.25 h vs. HRT > 10 h) with extremely high H_2 formation rates (7600 mL H_2 L^{-1} h^{-1} vs. 233 mL H_2 L^{-1} h^{-1}). On the contrary, suspended cell CSTRs provided higher yields (2.8 mol H_2 mol^{-1} glucose vs. 1.78 mol H_2 mol^{-1} glucose) at low substrate feeding concentrations (<5 g glucose L^{-1}) when compared to immobilized systems. CSTRs could not tolerate high substrate loadings at low HRTs < 10 h due to rapid biomass flocculation adversely affecting H_2 production performance [64]. Immobilized-cell reactors seem to be more advantageous than suspended cell CSTR in terms of high H_2 productivities. However, lower H_2 yield obtained in immobilized-cell reactors is an important disadvantage. Neither continuous suspended cell, nor immobilized systems were found to be capable of providing both high H_2 yield and high rates under high substrate loading rates and low HRT levels. High cell density fed-batch cultures may overcome the shortcomings of continuous suspended and immobilized-cell systems.

2.3. *Fed-batch dark fermentation*

High cell density fed-batch operation has considerable advantages as compared to batch and continuous operation and is usually used to overcome substrate/product and toxic compound inhibitions encountered at high substrate concentrations. Fed-batch operation is also used to adjust the metabolic rates by adjusting the feed flow rate and composition. The substrate solution is added with a rate sufficient to support the bacterial community and to eliminate the substrate and product inhibitions with no effluent removal. When the substrate consumption rate is equal to the feeding rate the reactor substrate concentration reaches a low quasi steady-state level. When the reactor is full, the contents are settled, the supernatant containing the products are removed and another cycle of fed-batch operation is started. Part of the settled bacteria may also be removed in order to adjust the cells retention time. Despite considerable advantages, fed-batch operation has not been used widely in Hydrogen gas production by dark fermentation. Few studies on the subject matter are summarized below.

Chin et al. [71] reported H_2 production by fed-batch operation with an extremely high feed glucose concentration (500 g L^{-1}). Constant H_2 formation yield of 2 mol H_2 mol^{-1} glucose with a rate of 930 mL H_2 h^{-1} was reported for long term fed-batch operation. Prevention of VFA accumulation during dark fermentation was recommended for more efficient H_2 production.

In another study Kargi and Pamukoglu [72] investigated H_2 production from boiled waste wheat powder (WP) by fed-batch operation. Effects of substrate loading rate on the rate and the yield of H_2 gas formation were investigated. The highest yield (3.1 mol H_2 mol^{-1} glucose) and rate (36 mL H_2 h^{-1}) were reported with 20 g L^{-1} feed WP concentration with loading rate of 4 g WP d^{-1}.

Yokoi et al. [73] performed H_2 production experiments from sweet potato starch by using repeated fed-batch operation. Pure culture of *Clostridium butyricum* and co-culture of *C. butyricum* and *Enterobacter aerogenes* were used to compare H_2 production capabilities in the presence of 0.1% polypeptone as nitrogen source. Co-culture of *C. butyricum* and *E. aerogenes* was found to be more effective than pure culture of *C. butyricum*. A stable and high H_2 yield between 2.3 and 2.4 mol H_2 mol^{-1} glucose was obtained up to 2.0% feed starch concentration. A yield of 2.7 mol H_2 mol^{-1} glucose was reported by Yokoi et al. [74] using a similar co-culture approach through repeated fed-batch operation in the presence of corn steep liquor as nitrogen source.

3. Photo-fermentation

Photosynthetic non-sulfur (PNS) bacteria have the ability to convert VFAs to H_2 and CO_2 under anoxygenic conditions [2,6,13,75]. PNS bacteria also have the ability to use carbon sources like glucose, sucrose, succinate rather than VFA for H_2 production [76–78]. The most widely known PNS bacteria used in photo-fermentative H_2 production are *Rhodobacter sphaeroides* O.U001, *Rhodobacter capsulatus*, *R. sphaeroides*-RV, *Rhodobacter sulfidophilus*, *Rhodopseudomonas palustris* and *Rhodospirillum rubrum* [79]. Both hydrogenase and nitrogenase enzymes were detected in PNS bacteria [6,8]. However, nitrogenase is the main enzyme responsible in molecular H_2 production under anoxygenic conditions [8,80]. Eq. (2) [10,81] presents H_2 gas formation from acetic acid by photo-fermentation. Due to positive free energy change, the reaction is not spontaneous requiring external energy input in the form of light which could be provided as artificial or solar light source [18,21,82]. As presented in Eq. (2) [10,81], theoretically 4 mol of H_2 can be produced from 1 mol of acetic acid when acetic acid is the only VFA present in fermentation medium.

$$CH_3COOH + 2\,H_2O \rightarrow 4\,H_2 + 2\,CO_2 \qquad \Delta G^{o} = +104\ \text{kJ}, \tag{2}$$

Strict control of environmental conditions is essential for efficient H_2 production by photo-fermentation [83–85]. Optimal pH and temperature ranges were reported to be 6.8–7.5 and 31–36 °C, respectively [79]. Suitable wavelength and light intensities for photo-fermentation were reported to be between 400 and 1000 nm [80,86], 6 and 10 klux, respectively [79]. Fe and Mo are known to be the most important cofactors required by the nitrogenase enzymes in H_2 production [87]. The limited use of nitrogen source is of special importance since even 20 μM of ammonia was reported to cause inhibition on the nitrogenase enzyme responsible for H_2 formation [80]. Malate/glutamate ratio larger than 1 is recommended for effective H_2 formation by photo-fermentation [80]. Optimum VFA concentrations were reported to be between 1800 and 2500 mg L^{-1} [88–90]. Major problems in photo-fermentation were reported to be lack of preferred C-sources such as malate and lactate, non-uniform light distribution through fermentation broth and metabolic shift from H_2 production to PHB synthesis [79,80,91].

H_2 production performance of PNS bacteria is evaluated on the basis of the H_2 yield and the light efficiency [86]. The yield coefficient is the ratio of the amount of produced H_2 to the consumed carbon source. The light efficiency denotes the ratio of generated H_2 energy to the supplied light energy. Depending on the carbon source, H_2 formation yields up to 80% of the theoretical yield were reported in literature [86,92–94]. Light conversion efficiencies varied between 0.2 and 9.3% [80,86]. A light efficiency of 10% for PNS bacteria corresponds to the theoretical maximum of photochemical efficiency [86,95]. As presented in Eq. (3) [86] the light efficiency could be increased by reducing the supplied light energy to the reactor. Light efficiencies at around 10% level were obtained only at low light intensities and low H_2 rates which is dependent on the fraction of energy absorbed by the bacteria and the energy loss during the several steps of excitation and electron transfers [86]. Dasgupta et al. [8] proposed genetic strain development by modification of light-harvesting antenna complexes responsible for capturing solar energy, reduction in pigment content of bacteria, improvement in the nitrogenase enzyme efficiency and reduction of uptake hydrogenase enzymes. Convenient reactor geometry and light distribution are the key factors for efficient conversion of solar energy to H_2 [79,80,96].

$$\text{Light efficiency}\ (\%) = \frac{H_2\ \text{production rate} \times H_2\ \text{energy content}}{\text{absorbed light energy}} \quad (3)$$

Design of photo-bioreactors enabling efficient H_2 production is still a challenge [96]. Light distribution inside photo-bioreactors constitutes the most important parameter effecting H_2 production rate [86]. Thus, optimization of light distribution with high reactor surface area was reported as an essential factor to enhance the light efficiency in photo-fermentation [8,86]. Operating parameters also affect the photo-fermentation process efficiency. The concept of net energy ratio (NER) is used to determine the process efficiency which is the ratio of total energy produced to the energy required for plant operations like mixing, pumping, aeration and cooling [97]. The objective is to obtain NER > 1 by improving the light conversion efficiency to H_2 [8].

$$\text{NER} = \frac{\sum \text{Energy Produced (biomass/hydrogen)}}{\sum \text{Energy input (mixing, aeration, pumping, cooling, etc.)}} \quad (4)$$

Immobilization of PNS bacteria on solid matrix was reported to yield higher H_2 formation rates than the suspended culturing [13]. H_2 formation rates between 3600 and 3800 mL H_2 L^{-1} h^{-1} were expected by using immobilized PNS bacteria on porous glass [98]. The expected rate was calculated by scaling up the small scale experimental results of 3.6–3.8 mL H_2 mL^{-1} h^{-1} and is not guaranteed at production scale.

3.1 Batch photo-fermentation

Batch photo-fermentative hydrogen gas production from different substrates has been studied extensively during the last ten years. Most of the studies were carried out by using pure carbon sources in sterile and nutrient rich fermentation media investigating optimum operating conditions, culture selections, nutrient concentrations and inhibitory conditions. Indoor photo-fermentation experiments exceeded the outdoor experiments utilizing solar irradiation as light source. Reported yields and rates were usually inconsistent in units causing difficulties for performance comparisons. Therefore, the H_2 yields were reported as the percentage of the theoretical yield in this article. Some striking studies on the subject matter are summarized below.

Fang et al. [77] investigated photo-fermentative H_2 production from glucose by using *R. sphaeroides* as bio-catalyst. Nitrogen and light sources were Na-glutamate and tungsten lamp with a light intensity of 135 W m^{-2}, respectively. Almost all glucose was fermented to H_2, VFA and CO_2. The yield and rate of H_2 formation were 110 mL H_2 g^{-1} glucose (6.6% of the theoretical maximum according to Eq. (7)) and 80.42 mL H_2 L^{-1} h^{-1}, respectively. Residual acetic acid in the media was about 540 mg L^{-1} indicating VFA accumulation to some extent.

Hydrogen gas was produced from starch by a new strain called *Rubrivivax gelatinosus* which was reported to be capable of utilizing a wide range of carbon sources [99]. Initial starch and glutamate concentrations were 5.4 g L^{-1} and 1 g L^{-1}, respectively. Tungsten lamps were used as the light source with 105 W m^{-2} light intensity. Hydrogen gas formation rate of 829 mL H_2 g^{-1} h^{-1} was obtained after a long lag phase of 870 h. The strain was not able to convert acetate, propionate and butyrate to H_2. The highest yield and rate were reported to be 1.1 mol H_2 mol^{-1} glucose (9.2% of the theoretical maximum) and 12.1 mL H_2 L^{-1} h^{-1}, respectively [99].

Tao et al. [100] used a newly isolated PNS strain named ZX-5 for batch H_2 production by photo-fermentation. Strain ZX-5 was reported to produce H_2 most efficiently from butyrate (118 mL H_2 L^{-1} h^{-1}) as compared to succinate, lactate, malate, acetate, pyruvate, valerate, isobutyrate, xylose, fructose, maltose, sucrose, propionate, D-mannitol and glucose. The optimum conditions for ZX-5 were reported as, 4–5 klux light intensity with tungsten lamp, initial pH of 6–9, and 7 mM L-glutamate as a nitrogen source. ZX-5 strain was also used for H_2 production from succinate wastewater, from effluents of dark fermentation of kitchen waste. A yield of 500 ml H_2 g^{-1} COD and COD removals over 80% were obtained when succinate wastewater was used. Total VFA concentration in the effluent was less than 0.001% after photo-fermentation. The ZX-5 strain was reported as a potential H_2 producer from wastewaters utilizing wide range of carbon sources.

Basak and Das [92] operated an annular photo-bioreactor in order to enhance H_2 production through more efficient light penetration. DL-Malate (2.01 g L^{-1}) was fermented by *R. sphaeroides* OU 001 at indoor conditions. Overall maximum yield of 4.5 ± 0.05 mol H_2 mol^{-1}DL-malic acid, VHPR of 6.5 mL H_2 L^{-1} h^{-1} were reported with initial pH of 6.8, $T = 32$ °C and 15 W m^{-2} light intensity using tungsten lamps. The reported H_2 yield corresponds to 75% of the theoretical yield which is 6 mol H_2/mol malate [81].

A temperature controlled flat-plate solar bioreactor (8 L) was used by Eroğlu et al. [101] for batch H_2 production from malate, lactate, acetate and olive mill wastewater (OMW). *R. sphaeroides* OU 001 was used as the inoculum culture and experiments were carried out at outdoor conditions. The highest H_2 production rate (10 mL H_2 L^{-1} h^{-1}) was obtained when malate was used as the sole carbon source. Use of acetate as substrate resulted in high PHB accumulation with low H_2 production. Nearly 11.4 L H_2 was produced per liter OMW with a rate of 3 mL H_2 L^{-1} h^{-1} within 9 days at outdoor conditions with an average sunlight intensity of 405–428 W m^{-2} when 4% OMW and 2 mM Na-glutamate were used.

Acid-hydrolyzed wheat starch was subjected to photo-fermentation using three different *Rhodobacter* species. *R. sphaeroides*-RV yielded the highest H_2 production yield (1.23 mol H_2 mol^{-1} glucose = 1.02% of the theoretical maximum) and rate (46 mL H_2 g^{-1} biomass h^{-1}) among the other strains tested. Hydrogen gas formation increased with total sugar concentration up to 8.5 g L^{-1} and the optimum was found to be 5 g L^{-1} resulting in the highest rate and the yield [102].

3.2. Continuous photo-fermentation

There is limited number of continuous culture studies on photo-fermentative H_2 production. Most studies on this subject are realized by sequential dark and photo-fermentation which are summarized later in this article. Hydrogen yields and rates varied in continuous photo-fermentation depending on the substrate, inoculum culture and experimental conditions. Yields up to 80% of the theoretical yield were reported depending on the substrate used [93]. Reported H_2 formation rates varied between 1.12 and 165.9 mL H_2 L^{-1} h^{-1} [78,83]. Long HRTs from 25 h [87] to 120 h [103] were required indicating slow conversion of VFAs to H_2 and CO_2 by the PNS bacteria.

Najafpour et al. [104] conducted continuous hydrogen gas production experiments from carbon monoxide (CO) using *R. rubrum*. A 2 L fermenter was continuously fed with acetate (4 g L^{-1}) for cell growth and CO (14 mL min^{-1}) which was further used for H_2 gas production. The reactor was operated at pH = 6.5, HRT = 55.5 h with 4 klux illumination by tungsten lamp. In photo-fermentation CO was oxidized to CO_2 while water was reduced to H_2 by *R. rubrum*. Hydrogen gas was produced with a rate of 397.5 mL H_2 g^{-1} h^{-1} and 80% of the theoretical yield, respectively.

Fermentation of lactic acid by *R. sphaeroides*-RV was realized in a two stage chemostat for H_2 production [93]. The first reactor was used for nitrogen removal and for bacterial growth while the second reactor was for H_2 production. The feed lactic acid, ammonium and yeast extract concentrations were 100 mM, 4.7 mM and 0.5 g L^{-1}, respectively. Illumination was provided by tungsten lamps (10 klux). HRTs were 10 h and 20 h for the first and the second reactors, respectively. Hydrogen gas formation yield and SHPR were 4.8 mol H_2 mol^{-1} HLa and 75 mL H_2 g^{-1} h^{-1}, respectively. Increasing nitrogen concentration adversely effected H_2 formation due to inhibition of the nitrogenase enzyme.

Fascetti et al. [87] investigated continuous H_2 production from dark fermentation effluent (DFE) of source selected municipal solid wastes. *R. sphaeroides*-RV was used in photo-fermentation of DFE containing 4.1 g L^{-1} acetic acid, 0.38 g L^{-1} lactic acid and 59 mg L^{-1} total nitrogen as carbon and nitrogen sources, respectively. The photo-bioreactor (1 L) was operated at HRT = 25 h and was illuminated with 10 klux tungsten lamp to yield the highest SHPR of 100 mL H_2 g^{-1} h^{-1}.

Continuous H_2 production in a pneumatic flat panel photo-bioreactor was performed by using *Rhodobacter pseudomonas* as an inoculum culture [105]. The reactor was first operated in batch mode followed by continuous operation at HRT = 28.5 h, 175 W m^{-2} illumination, 30 °C and pH = 6.8–7.0. Mixing was provided by recirculating argon gas through the reactor. However, no H_2 production was observed due to high residual NH_4 levels (>4 mM) from batch operation and high acetate concentrations. VFA and ammonium concentrations were recommended to be below certain limits (VFA < 2500 mg L^{-1}, NH_4 < 2 mM) for effective photo-fermentation. CO_2 was identified as an essential nutrient for growth of PNS bacteria and should not be removed by continuous gas sparging [105].

Studies for continuous photo-fermentation have been realized mostly by suspended cultures rather then using immobilized cells. High H_2 gas formation rate of 1300 mL H_2 L^{-1} h^{-1} with 75% substrate conversion efficiency was reported in a study where *R. sphaeroides*-RV was immobilized on porous glass indicating an advantage over suspended culture [106].

3.3. Fed-batch photo-fermentation

As stated in Section 2.3, fed-batch operation for H_2 production offer distinct advantages. However there is limited number of fed-batch photo-fermentation studies reported in literature. *Rhodopseudomonas faecalis* strain RLD-53 was used for bio-hydrogen production from acetate by using fed-batch operation with H_2 yields nearly 80% of the theoretical yield [107]. The reactor was illuminated by 4 klux incandescent lamp source. pH and temperature were controlled at 7.0 and 35 °C, respectively.

Initial acetate and Na-glutamate concentrations were 50 mM and 10 mM, respectively. Acetate was injected to the fermenter below certain limits. The highest yield and volumetric rate of H_2 gas production were 3.17 mol H_2 mol^{-1} acetate and 37.2 mL H_2 L^{-1} h^{-1}, respectively. Control of the feed acetate concentration and pH was identified as important factors effecting fed-batch H_2 production.

Boran et al. [108] operated a 80 L pilot scale photo-bioreactor in fed-batch operation mode during winter time in Ankara–Turkey. The experiments were performed at outdoor conditions in a greenhouse. The system was fed with 40 mM acetic acid and 2 mM glutamate containing feed solution. *Rhodobacter capsulatus* was used as the inoculum culture. The system resulted in an overall yield of 0.6 mol H_2 mol^{-1} acetic acid with a productivity of 7.7 mL H_2 L^{-1} h^{-1}. The highest light conversion efficiency was reported as 1%.

4. Sequential dark and photo-fermentations

As presented above dark fermentative H_2 production suffers from low H_2 yields due to accumulation of VFAs in the medium [94]. However, the VFA fermentation capability of PNS bacteria provides a unique opportunity for valorization of dark fermentation effluent (DFE) as a substrate for photo-fermentation [14,15,18–20,83]. When dark and photo-fermentations were operated simultaneously, the maximum theoretical H_2 yield would increase to 12 mol H_2 mol^{-1} glucose when acetic acid is the sole product of the dark fermentation [18,19,83]. The DFE has to meet certain conditions for effective photo-fermentation such as TVFA and NH_4^+ concentrations must be below 2500 mg L^{-1} and 40 mg L^{-1}, respectively [83,84,110–113]. Dilution, ammonium stripping, centrifugation and sterilization of DFE have been used as pre-treatment steps to reduce TVFA and NH_4^+ below certain limits [21,84]. Residual glucose in the DFE is also a problem since a shift from glucose to VFA fermentation by the PNS bacteria takes a long time resulting in low H_2 gas productivities [109]. Therefore, DFE has to be ammonia and glucose deficient with desirable VFA concentration (<2500 mg L^{-1}) for effective H_2 gas production by photo-fermentation [84]. Bio-process schemes for bio-hydrogen production from biomass by sequential dark and photo fermentations are described in Fig. 2. Acid hydrolysis of biomass can be used before fermentations (Fig. 2a) or bio-hydrolysis step can be incorporated into dark fermentation (Fig. 2b). Direct photo-fermentation of carbohydrates derived from acid hydrolysis of biomass is also possible (Fig. 2c). Pre-treatment of DFE prior photo-fermentation and neutralization after acid hydrolysis are important steps to be considered.

Sequential dark and photo-fermentation of glucose can be represented by the following reactions [10] when acetic acid is the only VFA produced.

Dark fermentation:

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2\,CH_3COOH + 4H_2 + 2\,CO_2 \qquad \Delta G^o = -206\ kJ \tag{5}$$

Photo-fermentation:

$$2\,CH_3COOH + 4H_2O \rightarrow 8H_2 + 4\,CO_2 \qquad \Delta G^o = 104.6 \times 2 = 209.2\ kJ \tag{6}$$

Sequential or combined dark and photo-fermentation (overall reaction):

$$C_6H_{12}O_6 + 6H_2O \rightarrow 12\,H_2 + 6\,CO_2 \qquad \Delta G^o = 3.2\ kJ \tag{7}$$

As presented in Eqs. (5) and (6), the overall maximum theoretical yield in sequential fermentation is 12 mol H_2 mol^{-1} glucose when acetic acid is the only VFA. However, real yields are much lower than that due to formation of a mixture of VFAs and utilization of part of the substrate for growth, maintenance and PHB formation [21,84]. An overall

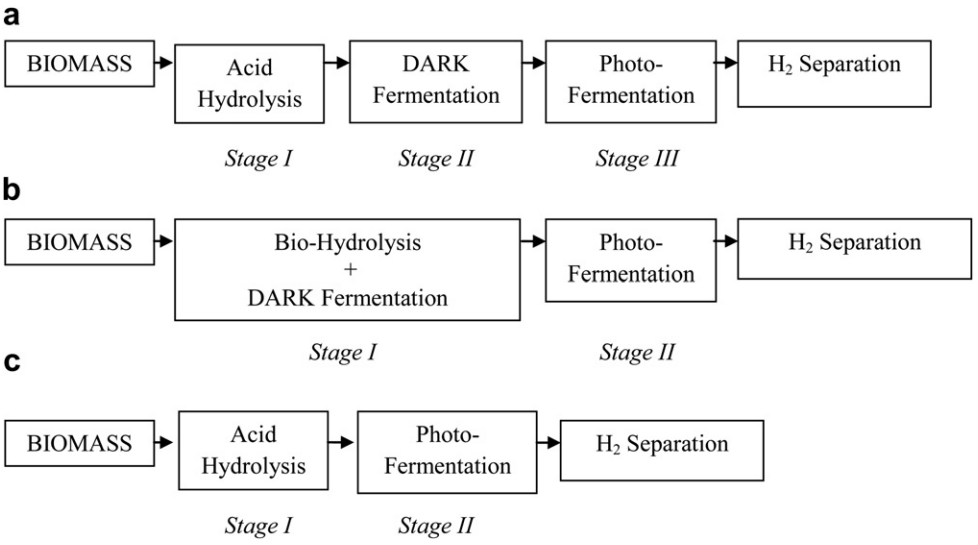

Fig. 2 – Bio-hydrogen production from biomass by sequential dark and photo-fermentation. (a). Three step process: Acid hydrolysis followed by dark and photo fermentations. (b) Two-step process: Bio-hydrolysis with simultaneous dark fermentation followed by photo fermentation. (c) Two-step process: acid hydrolysis followed by photo fermentation.

Table 1 – Yields and rates of bio-hydrogen production from different substrates by sequential dark and photo fermentations.

Substrate	DF MO	PF MO	Yield of DF (mol H_2 mol^{-1} glucose)	Yield of PF (mol H_2 mol^{-1} glucose)	VHPR of DF (mL H_2 L^{-1} h^{-1})	VHPR of PF (mL H_2 L^{-1} h^{-1})	DF op. mode	PF op. mode	Refs.
Hydrolyzed cassava starch (25 g L^{-1})	Pre-heated activated sludge	*Rhodopseudomonas palustris*	2.00	0.86	262.4	16.4	Batch	Batch	[110]
Sugar beet molasses (15 g sucrose L^{-1})	*Caldicellulosiruptor saccharolyticus*	hup_mutant *of R. capsulatus*	2.10	4.75	200	34.04	Batch	Batch	[111]
Ground wheat solution (6.7 g starch L^{-1})	Anaerobic sludge	*Rhodobacter* sp. RV	1.87	2.68	69.26[a]	8.72[a]	Batch	Batch	[21]
Cassava starch (10.4 g L^{-1})	Anaerobic mixed bacteria (mainly *Clostridium* species)	Mixed photosynthetic bacteria (mainly *Rhodobacter palustris*)	2.53	3.54	334.8	21.10	Batch	Batch (immobilized)	[112]
Glucose (20 g L^{-1})	*Clostridium saccarolyticus*	*Rhodobacter sphaeroides*	–	4.8[a]	–	29.31	–	Batch	[14]
Sucrose (17.81 g L^{-1})	*Clostridium pasteurianum CH4*	*Rhodopseudomonas palustris* WP3-5	1.90	3.22	–	21.20	Batch	Batch	[18]
Sucrose (17.81 g L^{-1})	*Clostridium butyricum* CGS5	*Rhodopseudomonas palustris* WP3-5	0.49 3.28	2.24 2.53	266.0 667.3	12.61 6.5	Batch Batch	Batch Continuous (HRT = 12h)	[15]
Sweet potato starch (10 g L^{-1})	Mixed culture of *Clostridium butyricum* and *Enterobacter aerogenes* HO-39	*Rhodobacter sphaeroides* M-19	2.4	4.6	–	–	Repeated batch	Repeated batch	[73]
Sweet potato starch (10 g L^{-1})	Mixed culture of *Clostridium butyricum* and *Enterobacter aerogenes* HO-39	*Rhodobacter sphaeroides* M-19	2.7	4.5	–	–	Repeated batch	Repeated batch	[74]
Waste activated sludge (10.1 g COD L^{-1})	*Clostridium beijerinkii*	*Rhodobacter sphaeroides*	–	160.5 (mL H_2 g^{-1} TVFA)	–	4.16	Continuous (HRT = 3d)	Continuous (HRT = 5 d)	[103]
Ground wheat (10 g L^{-1})	*Anaerobic sludge*	*Rhodobacter sphaeroides* (NRRL-B1727)	–	0.45 (mol H_2 mol^{-1} acetate)	–	1.125	Continuous (HRT = 12 h)	Continuous (HRT = 72 h)	[83]

DF: Dark Fermentation; PF: Photo Fermentation; MO: Microorganism; Op: Operation.
a Calculated according to the data given in the relevant article.

yield of at least 8 mol H_2 mol^{-1} glucose is aimed for an economically viable process [18].

Hydrogen gas production studies from waste and pure carbon sources by sequential dark and photo-fermentation using different operational modes have been reported in the literature. Results of striking studies are summarized in Table 1. The reported overall yields are considerably higher than single-stage dark or photo-fermentation. However, H_2 formation rates in sequential fermentation are lower (<700 mL H_2 L^{-1} h^{-1}) than those of dark fermentation alone. PNS bacteria require long HRT levels (up to 5 days) for efficient conversion of VFAs to H_2 in continuous mode of operation. The highest H_2 productions were obtained using repeated-batch operation mode where the substrate concentrations varied between 5 and 25 g glucose L^{-1}.

The highest H_2 formation yield of 7.2 mol H_2 mol^{-1} glucose was reported by Yokoi et al. [74] where medium containing 10 g L^{-1} sweet potato starch was fermented by sequential dark and photo-fermentation operated in fed-batch mode. Co-culture of *E. aerogenes* HO-39 and *Clostridium butyricum* was used in dark fermentation producing H_2 and VFAs. *R. sphaeroides* efficiently converted VFAs present in DFE by photo-fermentation. Individual yields of repeated-batch dark and photo-fermentations were 2.7 mol H_2 mol^{-1} glucose and 4.5 mol H_2 mol^{-1} glucose, respectively. Addition of $Na_2MoO_4 \cdot 2H_2O$ and EDTA was stated to be crucial in enhancing photo-fermentation yield. Similar approach was used in another study and total yield of 7.0 mol H_2 mol^{-1} glucose was obtained from sweet potato starch in sequential dark and photo-fermentation [73].

Ozgur et al. [111] investigated H_2 production from sugar beet molasses in sequential dark and photo-fermentations both of which were operated batchwise. Strains used as inoculum in the first and second fermenters were thermophile *C. saccharolyticus* and PNS bacteria, respectively. Dark fermentation was operated with initial sucrose concentration of 15 g L^{-1} at 70 °C and pH = 6.9 ± 0.1 resulting in a yield of 2.1 mol H_2 mol^{-1} glucose. The dark fermentation effluent was centrifuged, diluted and sterilized prior to PNS bacteria inoculation. Photo-fermentation took place at 30–33 °C, pH = 6.7 and with illumination at 150–200 W m^{-2} using tungsten lamp. Phosphate buffer, Fe and Mo source were added to the second stage to enhance H_2 production in photo-fermentation. The highest H_2 yield was 4.75 mol H_2 mol^{-1} glucose by the PNS strain hup_mutant of *R. capsulatus*. The overall yield of dark and photo-fermentations was reported to be 6.85 mol H_2 mol^{-1}.

In another study H_2 was produced from waste wheat starch by sequential dark and photo-fermentation by Argun et al. [21]. The substrate was first fermented by dark fermentation with an initial starch concentration of 6.7 g L^{-1} for about 70 h. The DFE was subjected to NH_4–N removal, centrifugation and dilution process in order to obtain suitable initial TVFA and NH_4–N concentrations for photo-fermentation. About 60% of initial TVFA (2.1 g L^{-1}) was fermented by *R. sphaeroides*-RV at 5 klux illumination with halogen lamp. H_2 formation yield of dark and photo-fermentations was 1.87 and 2.68 mol H_2 mol^{-1} glucose, respectively.

Su et al. [113] performed two stage dark and photo-fermentation in order to enhance H_2 production from glucose. *C. butyricum* and *R. palustris* were used as dark and photo-fermentative bacteria, respectively. Glucose (20 g L^{-1}) was fermented to H_2, CO_2 and VFA by dark fermentation and the resulting acetate and butyrate containing DFE was subjected to photo-fermentation after centrifugation, filtration and dilution. Contributions of dark and photo-fermentative yields were 1.32 mol H_2 mol^{-1} glucose and 4.16 mol H_2 mol^{-1} glucose, respectively resulting in a total yield of 5.48 mol H_2 mol^{-1} glucose. Hydrogen gas production from glucose in dark fermentation (100 mL H_2 L^{-1} h^{-1}) was faster than that of the photo-fermentation (26.9 mL H_2 L^{-1} h^{-1}). More than 90% of VFA was fermented during photo-fermentation with a light conversion efficiency of 2.96% [113].

Simultaneous H_2 gas production along with COD removal was studied by Chen et al. [52] using sequential dark and photo-fermentation of sucrose. Dark fermentation took place at sucrose concentration of 20 g L^{-1}, pH = 7, T = 32 °C using *Clostridium pasteurianum* CH_4. The resulting effluent was centrifuged (13,000 rpm), diluted and inoculated with *R. palustris* (0.875 g L^{-1}) for continuous photo-fermentation at 32 °C, pH = 7.1 and 95 W m^{-2} illumination using tungsten lamps. Dark and photo-fermentation yields were reported to be 1.9 and 3.11 mol H_2 mol^{-1} glucose, respectively. Photo-fermentation yield was increased to 5.10 mol H_2 mol^{-1} glucose by illumination using optical fibers (95 W m^{-2}) with 2.0% (w/v) of clay carriers. A yield of 5.11 mol H_2 mol^{-1} glucose was maintained for 10 days when continuous photo-bioreactor was operated at HRT = 96 h.

A three step integrated system consisting of starch hydrolysis, dark fermentation and photo-fermentation was performed by Lo et al. [114]. Starch feedstock (35 g L^{-1}) was first subjected to hydrolysis by *Caldimonas taiwanensis* On1 and the resulting sugar rich (17 g L^{-1}) hydrolysate was fed continuously from a storage tank to the sequential dark and photo-fermentation reactors. Dark fermentation was operated at pH = 5.8–6.0, 37 °C and HRT = 12 h. The photo-bioreactor was fed from another storage tank containing pre-treated dark fermentation effluent (3800 mg L^{-1} HAc and HBu) and was operated at 35 °C, 100 W m^{-2} irradiation, pH 7.0, HRT = 48 h. *R. palustris* WP3-5 was used as inoculum culture in photo-fermentation. The overall yield of dark and photo-fermentations was 16.1 mmol H_2 g^{-1} COD or 3.1 mol H_2 mol^{-1} glucose with COD removal efficiency of 54.3%.

In general, sequential dark and photo-fermentations improved H_2 yields to more than 5 mol H_2 mol^{-1} glucose. However, H_2 gas production by photo-fermentation was slow

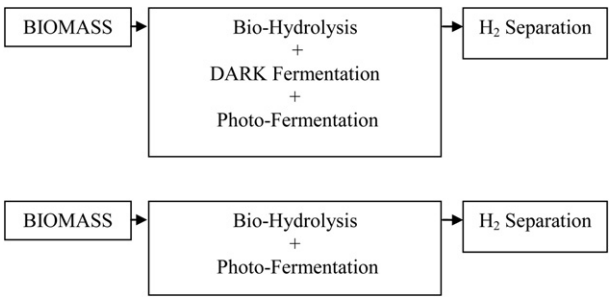

Fig. 3 – Bio-hydrogen production from biomass by combined dark and photo fermentation. (a) Combined bio-hydrolysis, dark and photo fermentations. (b) Combined bio-hydrolysis and photo fermentation by special bacteria.

Table 2 – Yields and rates of bio-hydrogen production from different substrates by combined dark and photo fermentations.

Inoculum cultures	Substrate	Light source	Light intensity	VHPR (mL H_2 L^{-1} h^{-1})	Yield (mol H_2 mol^{-1} glucose)	Op. Mode	Refs.
Clostridium butyricum DSM 10702 and *Rhodobacter sphaeroides* DSM 158	Glucose (5 g L^{-1})	Tungsten	135 W m^{-2}	–	0.86	Batch	[77]
Clostridium butyricum and *Rhodopseudomonas faecalis* RLD-53	Glucose (9 g L^{-1})	Incandescent	4 klux	20.83	1.98	Batch	[120]
Clostridium acidisoli and *Rhodobacter sphaeroides*	Sucrose (11.43 g L^{-1})	–	4 klux	–	5.08	Batch	[121]
Anaerobic sludge and Rhodobacter sphaeroid NRLL-1727	Ground wheat starch (12.8 g L^{-1})	Tungsten and Halogen	6 ± 0.5 klux	1.40[a]	0.36	Batch	[119]
Anaerobic sludge and *Rhodobacter* sp. RV	Ground wheat starch (4.3 g L^{-1})	Halogen	270 W m^{-2}	6.70[a]	1.45	Batch	[109]
Anaerobic sludge and mixture of *Rhodobacter* sp (NRRL B-1727) *Rhodobacter* sp (DSMZ-158) *Rhodopseudomonas palustris* (DSMZ-127) *Rhodobacter* sp. RV	Ground wheat starch (4.1 g L^{-1})	Fluorescent	9.5 klux	3.86[a]	1.16	Batch	[34]
Anaerobic sludge and mixture of *Rhodobacter* sp (NRRL B-1727) *Rhodobacter* sp (DSMZ-158) *Rhodopseudomonas palustris* (DSMZ-127) *Rhodobacter* sp. RV	Ground wheat starch (3.9 g L^{-1})	Fluorescent	9.5 klux	7.12[a]	1.03	Batch	[122]
Lactobacillus delbrueckii NBRC13953 and *Rhodobacter sphaeroides*-RV	Glucose (4.5 g L^{-1})	Halogen	0.19 μEinstein m^{-2} s^{-1}	41.27[a]	7.1	Batch (Immobilized culture)	[123]
Clostridium butyricum and Rhodopseudomonas faecalis RLD-53	Glucose (6 g L^{-1})	Incandescent	8 klux	33.85	4.13	Batch (Immobilized)	[124]
Ethanoligenens harbinense B49 and *Rhodopseudomonas faecalis* RLD-53	Glucose (6 g L^{-1})	Incandescent	4 klux	17.2	3.1	Batch (Immobilized)	[125]
Clostridium butyricum and *Rhodobacter* sp. M-19	Starch (50 g L^{-1})	Incandescent	5 klux	16.50[a]	6.6	Repeated Fed-Batch	[126]
Anaerobic sludge and mixture of *Rhodobacter* sp (NRRL B-1727) *Rhodobacter* sp (DSMZ-158) *Rhodopseudomonas palustris* (DSMZ-127) *Rhodobacter* sp-RV	Wheat powder solution (20 g dm^{-3})	Halogen	5 ± 0.5 klux	5.5 (mL H_2 h^{-1})	0.43	Fed-Batch	[116]
Anaerobic sludge and *Rhodobacter* sp (NRRL B-1727)	Ground wheat powder (10 g L^{-1})	Fluorescent	5 klux	18.1 (mL H_2 h^{-1})	1.32	Fed-Batch	[118]
Clostridium beijerinkii DSM-791 and *Rhodobacter* sp. RV	Ground wheat starch (5.0 g L^{-1})	Halogen and Fluorescent	10 klux	5.95	0.6	Continuous	[117]

a Calculated according to the data given in the relevant article.

reducing H_2 productivity. Pre-treatment of the DFE prior photo-fermentation has been used in almost all studies. Operation modes providing high cell density and high substrate loadings such as immobilized-cell reactors and fed-batch operations have not been investigated in details.

5. Combined dark and photo-fermentation

Simultaneous dark and photo-fermentation can be realized in a single reactor where VFAs produced by dark fermentation are converted to H_2 and CO_2 by photo-fermentation. Theoretically, 12 mol of H_2 per mole glucose can be obtained by combined dark and photo-fermentations (Eq. (7)). In order to realize the same conversion thermochemically, 600 °C and 34.5 MPa pressure need to be provided [115]. However, the same conversion can be realized at room temperature and atmospheric pressure [2] by combined fermentation with a slower rate [13]. A typical process scheme for bio-hydrogen production by combined dark and photo-fermentations is described in Fig. 3. Bacterial hydrolysis of biomass and dark-photo fermentations can be realized in the same reactor (Fig. 3a). Alternatively, hydrolysis and photo-fermentations can be realized by some special photo-fermentative bacteria with starch hydrolysis capability (Fig. 3b).

Typical combined fermentation proceeds at initial glucose concentration of less than 5 g L^{-1}, at pH = 7, T = 30 °C with supplementation of Fe (II) and Mo, using a proper biomass ratio of PNS/DF bacteria and a suitable dark/light illumination cycle [109,116]. The major problem in combined fermentation is the fact that PNS bacteria are capable of fermenting carbohydrates along with dark fermentation bacteria producing VFAs. Once PNS bacteria are adapted to carbohydrate fermentation, it takes a long lag time time to switch the metabolism for VFA fermentation [117]. In other words, the growth and product formation by the PNS bacteria behave like 'diauxic growth and substrate utilization' utilizing glucose first and VFAs later with some lag time in between [118]. This behavior causes accumulation of VFAs in the medium resulting in inhibition of dark fermentation and PNS bacteria [119]. Simultaneous removal of VFAs from the medium and utilization of low carbohydrate concentrations may partially overcome this problem. Operating conditions in combined fermentation was recommended to be closer to that of the photo-fermentation (pH = 7–7.5, ORP = −150 mV, 30 °C) rather than dark fermentation since PNS bacteria are known to be more sensitive to changes in environmental conditions [117]. For effective VFA fermentation by combined continuous fermentation, HRTs more than 6 days were advised by Argun et al. [117].

Combined fermentation has not been studied as extensively as sequential dark and photo-fermentations. The reported studies on the subject matter are summarized in Table 2. Hydrogen yields are considerably higher than the single-stage dark or photo-fermentation. However, H_2 formation rates are extremely lower than single-stage dark fermentation (<35 mL H_2 L^{-1} h^{-1}). Most combined fermentation studies were carried out by suspended and immobilized cultures in batch operation mode. No information was provided about light conversion efficiencies in any of the reviewed articles. Repeated fed-batch operation enabled use of higher feed substrate concentrations up to 50 g starch L^{-1} as compared to conventional batch operations usually operated with 5 g substrate L^{-1}.

Among the combined fermentation studies, the highest yield (7.1 mol H_2 mol^{-1} glucose) was reported by Asada et al. [123] where a co-culture of *Lactobacillus delbrueckii* NBRC 13953 and *R. sphaeroides*-RV were used. The experiments were performed in 200 mL roux bottles with initial glucose concentration of 4.5 g L^{-1} at pH = 6.8, 30 °C and 0.19 μEinstein m^{-2} s^{-1} illumination by halogen lamps. Acetate and lactate were the major VFAs formed. Optimum optical density ratio of *Lactobacillus delbrueckii* to *R. sphaeroides*-RV resulting in highest H_2 formation was reported to be 1/5.

Statistical experiment design methods were used for enhancing H_2 production through combined dark and photo-fermentation from sucrose under 4 klux light illumination [121]. Important factors were first screened by Plackett–Burman design followed by a central composite design for determination of optimum levels of the selected parameters. The optimal substrate concentration, initial pH, and inoculum ratio of used *C. acidisoli* to *R. sphaeroides* strains were 11.43 g L^{-1} sucrose, pH = 7.13, and C/R = 0.83. At the optimal operating conditions, the highest H_2 yield was reported to be 10.16 mol H_2 mol^{-1} sucrose corresponding to 5.08 mol H_2 mol^{-1} hexose.

Hydrogen gas was produced from starch by combined fermentations operated in fed-batch mode [126]. *Rhodobacter* sp. M-19 and *C. butyricum* were used as inoculum cultures with an initial biomass ratio of R/C = 10/1. The fermenter containing 1 g L^{-1} starch was fed with 1 mL of 50 g L^{-1} starch solution at 24 h intervals for four times. pH, temperature and light intensity were pH = 6.8, T = 30 °C and 5 klux, respectively. The system resulted in a high yield of 6.6 mol H_2 mol^{-1} glucose.

Argun et al. [117] investigated continuous combined dark and light fermentation in a hybrid annular bioreactor. Effects of HRT on H_2 formation yield and rate were studied. *Clostridium beijerinkii* DSM 791 and *R. sphaeroides*-RV were used as microbial strains with a biomass ratio of 1/3.9. Boiled waste ground wheat containing 5 g L^{-1} wheat starch was used as feed substrate with different loading rates depending on the HRT. The system was operated under 10 klux illumination with halogen and fluorescent lamps. pH and temperature were kept around 7–7.5 and 32 ± 2 °C, respectively. Hydrogen yields, SHPR and VHPR at steady-state were 0.6 mol H_2 mol^{-1} glucose, 9.16 mL H_2 g^{-1} h^{-1}, 5.95 mL H_2 L^{-1} h^{-1}, respectively. VFA accumulation and sub-optimal conditions for dark and light fermentation were identified as major problems resulting in low H_2 formation rates and yields.

6. Conclusions

Recent experimental studies on bio-hydrogen production by different operational modes of dark and photo-fermentation were reviewed in this article. The objective was to identify the most suitable operation mode resulting in the highest H_2 formation rate and the yield. Theoretically, high substrate loading rates, cell concentration and dilution rate should be

used in order to enhance the yield and rate of H_2 production. However, due to some biological constraints such as substrate and product inhibitions, substrate feeding and product removal should be applied periodically. Just like the type of bacteria, substrate and experimental conditions, mode of the operation also affects performance of bio-hydrogen formation. An ideal process scheme for fermentative H_2 production is expected to provide a cheap, simple, and robust operation resulting in both high H_2 formation rate and the yield.

Most widely used batch operations suffer from high initial substrate and final product concentrations causing inhibitions. Continuous operation provides constant quality product when operated at steady-state; however, the performance would be determined by the HRT (or dilution rate). Slow feeding of substrate and continuous removal of products are effective methods used to overcome substrate and product inhibitions. So far, in-situ product removal technologies have been applied for fermentations to remove products such as alcohols and organic acids. However, no effective simultaneous product removal studies were reported on fermentative H_2 production where accumulation of products like VFAs, solvents and H_2 gas cause severe product inhibitions on the bacteria.

Among four types of fermentation approaches reviewed in this article, single-stage continuous dark fermentation provided the highest H_2 formation rate (7.5 L H_2 L^{-1} h^{-1}) at an extremely low HRT of 0.25 h when immobilized reactors were operated in continuous mode. However, H_2 yields could not exceed 3 mol H_2 mol^{-1} glucose in continuous operation. High H_2 yields were possible by batch operation at low initial substrate concentrations (<5 g glucose L^{-1}). In this context, fed-batch operation provides considerable advantages resulting in high yields (nearly 3 mol H_2 mol^{-1} glucose) and rates at high feed substrate concentrations. Further improvements in dark fermentation can be achieved by using the following approaches.

a. Development of anaerobic bacterial strains producing mainly acetic and butyric acids with no lactic, propionic acids and alcohol production through metabolic and genetic engineering techniques.
b. Elimination of H_2 consuming homoacetogens from the inoculum and fermentation media by proper pre-treatment methods.
c. Elimination of substrate and product inhibitions by slow addition of substrate and continuous removal of products (VFAs and H_2 gas)
d. Minimization of nutritional needs of bacteria and optimization of environmental conditions (T, pH, ORP).

Photo-fermentation of DFE is usually used for H_2 gas production from VFAs by using PNS bacteria (mainly *Rhodobacter* sp). Photo-fermentation is more problematic than dark fermentation due to requirements for strict control of environmental conditions, unusual nutritional requirements (glutamate, Fe, Mo, V, vitamins) and uniform light intensities. PNS bacteria are more sensitive to environmental changes. The rate of H_2 gas production by *Rhodobacter* sp is also quite slower (max. 165.9 mL H_2 L^{-1} h^{-1}) than that of dark fermentation. Nearly 80% of the theoretical yields were reported under low light intensities and low H_2 formation rates. PNS bacteria cannot tolerate VFAs and NH_4^+ above 2500 mg L^{-1} and 40 mg L^{-1}, respectively due to substrate inhibition. These limitations reduce H_2 gas productivity in batch fermentation. Slight improvements in H_2 yields and rates were reported when continuous suspended cultures were used instead of batch operations in photo-fermentation. However, immobilization of PNS bacteria on solid matrices such as on porous glass was reported to yield extremely high rates of H_2 formation (3600–3800 mL H_2 L^{-1} h^{-1}) as compared to suspended cultures. Studies on fed-batch and repeated-batch photo-fermentations are limited in literature. Those operations were mainly used in sequential dark and photo-fermentations. Hydrogen gas yields in fed-batch photo-fermentation studies were up to 80% of the theoretical yield with low H_2 formation rates. High cell density fed-batch operation with slow and controlled addition of substrate offers advantages over batch and continuous operations. Photo-fermentation yields may be improved by using the following approaches.

a. Development of new strains of photo-fermentative bacteria with improved metabolic capabilities to tolerate high VFA and NH_4–N concentrations.
b. Use of mixed culture of *Rhodobacter* sp with capabilities of fermenting different VFAs with high H_2 yields.
c. Elimination of substrate and product inhibitions by slow addition of substrate (VFA) and continuous removal of products (H_2 gas).
d. Minimization of nutritional needs of the bacteria and optimization of environmental conditions (T, pH, ORP).
e. Development of new technologies for more effective illumination and light distribution (e.g. fiber optics, nanoparticles).

The most suitable operation mode providing both high H_2 formation yields and rates seems to be the high cell density fed-batch culture with controlled feeding and environmental conditions. Considering the difficulties in controlling the environmental conditions (T, pH, ORP, substrate/product concentrations) for immobilized-cell reactors due to heterogeneous nature of such reactors, high cell density suspended cultures should be preferred.

Dark and photo-fermentations can be used in sequential (consecutive operation) or combined (simultaneous operation) forms to improve H_2 formation yields to the economical level of 8 mol H_2 mol^{-1} glucose. However, yields at this level have not been reported yet. Sequential fermentation scheme requires some pre-treatment of the dark fermentation effluent (DFE). Removal of dark fermentation bacteria by filtration or centrifugation, adjustment of VFA and NH_4–N concentrations by dilution or removal, addition of some extra nutrients (Mo, Fe, V) and adjustment of pH and ORP to desired level would be required for DFE before feeding to photo-fermentation. Therefore, some other membrane processes such as ultrafiltration or reverse osmosis may also be required to remove some undesirable compounds from DFE. Sequential fermentation requires larger fermenter volumes and separation/pre-treatment units in between the two stages.

In combined fermentation process both dark and photo-fermentations take place in the same reactor. Due to

differences in optimum environmental conditions and nutritional requirements of dark and photo-fermentative bacteria it is difficult to operate the reactor under optimum conditions. However, operation of combined fermentation at pH = 7 ± 0.2, T = 30 °C, ORP = −150 ± 50 mV, DO = 0, light intensity = 10 klux may be a good compromise for both bacteria. The conditions are adjusted to be nearly optimum for photo-fermentative bacteria, in order to avoid VFA accumulation in the medium. Combined fermentation may seem to be more advantageous than sequential fermentation due to reduction in fermenter volumes and simpler operation. However, H_2 gas yields and productivities in sequential fermentation were reported to be higher than that of the combined fermentation due to long lag times between the dark and light fermentations in combined fermentation scheme. Adverse interactions and different nutritional needs of different bacteria also cause low H_2 yields and formation rates in combined fermentations. For the aforementioned reasons sequential fermentation should be preferred over combined fermentation.

Fermentative route of H_2 production from carbohydrate rich renewable sources such as biomass or waste materials is a promising approach provided that the rate and the yields of H_2 formation were improved to economically feasible levels and large scale operations were developed.

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