Chapter 21
Dark Fermentative Hydrogen Production from Lignocellulosic Agro-waste by a Newly Isolated Bacteria *Staphylococcus Epidermidis* B-6

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21.1 Introduction

Air pollution, exhaustion of fossil fuel reservoir and global warming have led to the relentless investigation for sustainable alternative fuels (Nagarajan et al. 2017). So, the production and use of non-carbonaceous fuels are getting much more attention nowadays. To elaborate, bio-based energy is a tenable and propitious replacement for unsustainable sources of energy; this can substitute and fortify against a catastrophe in the energy supply and the ever-increasing demand. Lately, hydrogen (H_2) gas has gained worldwide heed and is a promising future fuel (Anwar et al. 2019; Kumar et al. 2019b). H_2 is a conceivable multifaceted energy currency that could convert the utilization of non-renewable fossil fuels due to its an elevated yield per unit mass of energy (~122 kJ/g), which is 2.75 times substantial than the yield from hydrocarbon fuels (Mohan and Pandey 2019). Furthermore, upon ignition H_2 produces water (H_2O) after combining with O_2, the only externality of the process. It is an evidently commendatory denouement for greenhouse gases (GHG) emissions. Discretely, H_2 is more alike electrical energy and hence, an eminent solution (Onaran and Argun 2019). Currently, molecular H_2 is fundamentally generated from fossil fuels via the process of steam reforming of methane (CH_4) and/or natural gas. The global produce of H_2 presently surpasses at the rate of 1 billion m^3 per day, out of which around 48% is manufactured from 30% from oil, natural gas, 18% from coal

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and rest 4% is generated from splitting water through electrolysis (vikaspedia). When amalgamated with the steam reforming process, refined H_2 is produced attained via water gas shift reaction process, a crucial industrial process utilized peculiarly for manufacturing ammonia (Singh et al. 2019). There are many more thermochemical procedures/techniques obtainable for the manufacturing of H_2 that comprise autothermal reforming, catalytic oxidation, thermal decomposition, pyrolysis and steam gasification, etc. (Devasahayam and Strezov 2018; Bu et al. 2019; Waheed et al. 2016; Kouhi and Shams 2019). Nonetheless, the generation of H_2 established on natural fossil fuel reservoirs augment the discharge of harmful GHGs. In this context, biologically produced hydrogen which is a carbon-neutral, sustainable and eco-friendly technique is contemplated as the most propitious candidate. It possesses high energy, is clean in nature and can be obtained from a broad range of inexhaustible feedstock (Kotay and Das 2008; Winter 2009; Chong et al. 2009).

Considering the aspect of biomass conversion to energy, dark fermentative method of H_2 generation has been delineated to have huge prospective and more economic over other physicochemical methods (Bundhoo 2019). Lignocellulosic waste biomasses are promising raw materials for biofuel production due to high carbohydrate percentage (Taherzadeh and Karimi 2008; Nissilä et al. 2014). However, the sugar polymer cellulose and hemicelluloses in lignocellulosic biomass remain bound in compact form with lignin, which restrict them from easy microbial degradation. A few fungi are capable of solubilizing lignin, and some bacterial species can degrade cellulose, but the process takes a very long time (Lee 1997; Lynd et al. 2002; Kumar et al. 2008, Kumar et al. 2019a). Thus, these substances require proper pre-treatment prior to fermentative hydrogen production. Xylose shares a major fraction (35–45%) of total sugar yield from hydrolysis of lignocellulosic materials (Lavarack et al. 2002). Extensive research has been done on H_2 production via fermentation from substrates such as glucose and sucrose. However, due to inefficiency of microbes for xylose utilization, there are a very few reports (Li et al. 2010; Cheng et al. 2012; Chenxi et al. 2013; Wu et al. 2014; An et al. 2014; Poladyan et al. 2018; López-Aguado et al. 2018; Zhao et al. 2019; Kongjan et al. 2019) on H_2 production from xylose using a pure culture of microbes. Hence, for complete utilization of sugar released from lignocellulosic material, it is important to isolate bacterial species with efficiency to convert xylose to H_2. Thus, our current work is based on the isolation and identification of new microbial (bacteria) species having the potential to utilize xylose for fermentative H_2 production. Parameters that are crucial for fermentation was also studied to ascertain the optimum parameters for maximal H_2 yield and high production rate. This study also investigates the feasibility of the isolated bacterium for H_2 production from acid hydrolysed rice straw under batch culture condition.

21.2 Materials and Methods

21.2.1 Isolation of H_2-Producing Bacteria

The bacterial strain for H_2 production was isolated from the soil sample collected from the outskirt of Kaziranga national park of North East India. For isolation of bacterial strain, 1 g of the soil sample was homogenized with 0.85% NaCl (w/v) followed by several-fold dilution. 100 uL of the diluted sample was then spread on nutrient agar plates and kept at 37 °C for 24 h. Several bacterial colonies differing by colony morphology were obtained and were subsequently maintained as a pure culture for screening of H_2 production ability from xylose.

21.2.2 Screening for H_2 Production from Xylose and Culture Condition

The preliminary screening of bacterial strains for H_2 production from xylose was done under batch culture condition. The medium used was GM-2 (Yeast extract—1.0 g/L, K_2HPO_4 —1.0 g/L, $MgSO_4.7H_2O$—0.5 g/L and $FeSO_4.7H_2O$—1 mg/L) with slight modification (Patel et al. 2014). The experiments were conducted in 125 mL BOD bottle under anaerobic condition. 100 mL of the medium supplemented with 5 g/L xylose was inoculated with 2% (v/v) of culture (1 O.D at 600 nm). After bacterial inoculation, the bottles were made airtight and initial anaerobic condition was established by flushing N_2 gas. The bottles were then kept at 37 °C and evolved biogas was collected by water displacement method under acidic water. The potential strain with maximum biogas production ability was selected for this study and was named as strain B-6.

21.2.3 Identification of the Bacterial Strain B-6

For identification of the bacterial strain B-6, 16S rRNA gene sequencing was done by using universal primers, 27F (5′AGAGTTTGATCCTGGCTCAG3′) and 1492R (5′GGTTACCTTGTTACGACTT3′). The amplified product was purified by using PCR purification kit (Qiagen) and sent for sequencing (Eurofins Genomics India Pvt. Ltd., India). The obtained sequence was subjected to BLAST in National Centre of Biotechnology information (NCBI) BLAST search tool and the phylogenetic tree was constructed using the neighbour-joining method with MEGA 5.2 software.

21.2.4 Optimization of Culture Condition

The parameters crucial for fermentative H_2 production including initial pH, nitrogen source and substrate concentration were optimized under batch culture condition. The effect of pH was studied by adjusting the initial pH of the fermentation medium with the pH range of 5–9 with incremental step of 1. The fermentation was carried out at 37 °C with xylose concentration 10 g/L. The effect of nitrogen source on H_2 production was studied by fermentation medium GM-2 amended with inorganic (ammonium sulphate, ammonium chloride) and organic (yeast extract, peptone) nitrogen sources at a concentration of 1.0 g/L. The initial pH was 7.0, fermentation temperature 37 °C and xylose concentration 10 g/L. The effect of xylose concentration on fermentation was studied by varying the initial xylose load from 5–50 g/L under optimum pH, and nitrogen source at 37 °C.

21.2.5 H_2 Production with Different Carbon Source

The ability of the selected strain for utilization of other carbon sources were studied at optimum conditions (pH–7, N_2 source–yeast extract and incubation temperature –37 °C). The different carbon sources used were glucose, fructose, sucrose, mannose, lactose (10 g/L) and glycerol (10 mL/L).

21.2.6 Acid Hydrolysis of Rice Straw

Acid hydrolysis of the hemicellulose fraction of the rice straw was conducted by treating the dry rice straw (1%, 2%, 3%, 5% and 7%, w/v) with diluted H_2SO_4 (0.5%, v/v) at 121 °C for one hour in autoclave. After hydrolysis, the hydrolysate was filtered through a thin cloth to remove the solid fraction. Over-liming of the hydrolysate was done by adding $Ca(OH)_2$ with frequent stirring and the final pH adjusted to 10. The resulting precipitate was removed by centrifugation at 1500 rpm for 15 min. The supernatant was then re-acidified by lowering the pH to 7 and again centrifuged. The final supernatant thus obtained was then used for fermentative H_2 production (Nigam 2000).

21.2.7 Analytical Methods

The amount of biogas evolved during fermentation was measured by the water displacement method. The gas components were analysed by gas chromatograph (Nucon GC5765, India) equipped with Porapak-Q and molecular sieve columns

using a thermal conductivity detector (Nath et al. 2015). Argon was used as carrier gas with a flow rate of 20 mL/min, and temperature of oven, injector and detector was set to 60, 80 and 110 °C, respectively. The xylose concentration was measured by DNS method (Miller 1959).

21.3 Results and Discussion

21.3.1 Strain Identification and Phylogenetic Analysis

The sequenced 16S rRNA gene of the strain B-6 was aligned with gene bank NCBI (http://blast.ncbi.nlm.nih.gov) using BLAST program. A phylogenetic tree was constructed by neighbour-joining method with MEGA 5.2 software (Fig. 21.1). The tree indicates that the strain B-6 belonged to the genus *Staphylococcus* and showed maximum similarity with *Staphylococcus epidermidis* strain NBRC100911. Thus, the strain was identified and named as *Staphylococcus epidermidis* B-6. The gene sequence was also submitted to the NCBI gene bank with an accession number KT072716.

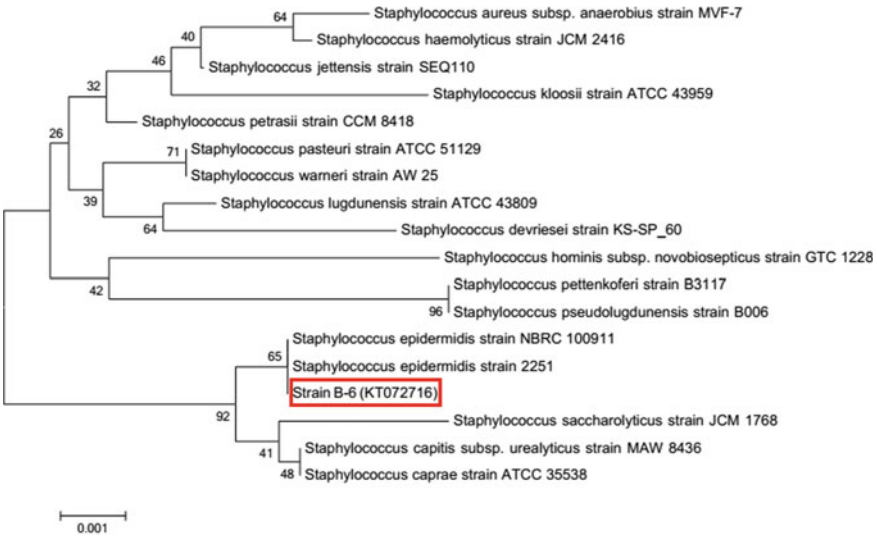

Fig. 21.1 Neighbour-joining tree showing the phylogenetic relationship of the isolated strain B-6 and related species based on 16S rRNA gene

21.3.2 *Effect of Various Nitrogen Sources on H_2 Production*

The effect of various nitrogen sources on H_2 production by *Staphylococcus epidermidis* B-6 is shown in Fig. 21.2a–c. A significant change in H_2 production was observed by changing the source of nitrogen in the fermentation medium. The maximum H_2 yield of 1.55 mol H_2/mol xylose was observed using yeast extract with the highest bacterial growth and 98% of xylose consumption (Fig. 21.2a, b). This is due to the fact that yeast extract is a complex nitrogen source comprising of peptides and amino acids, which can be easily taken up by the bacterium during fermentation and directly incorporated into proteins or transformed into other cellular nitrogenous constituents (Large 1986; Ferchichi et al. 2005). On the other hand, when inorganic

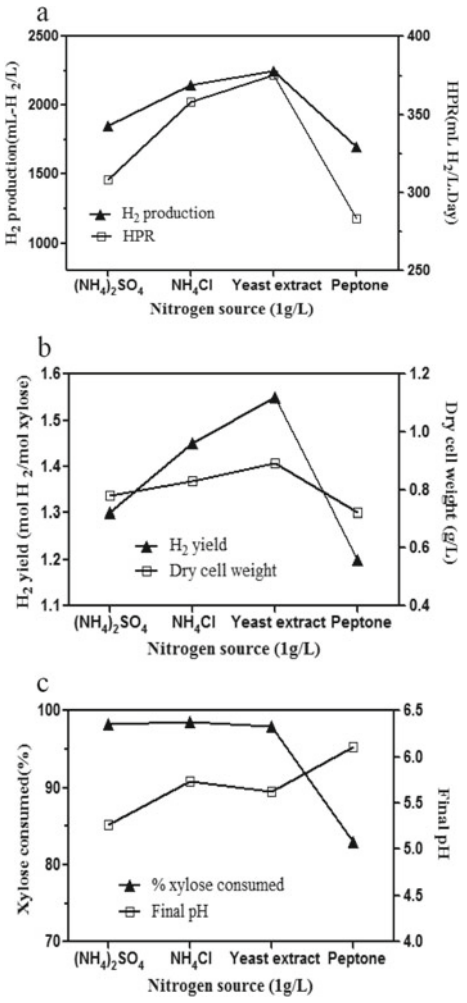

Fig. 21.2 Effect of N_2 source on H_2 production performance of *Staphylococcus epidermidis* B-6, **a** volume and rate of H_2 production, **b** H_2 yield and bacterial growth and **c** xylose degradation rate and final pH value

nitrogen sources are used, the cells have to spend more energy in synthesizing amino acids, and as a result, they spend a longer period of lag phase and decrease in H_2 yield (An et al. 2014). The results suggest that yeast extract as a better nitrogen source for maximum H_2 production with high production rate and bacterial growth. However, the other cheap nitrogen sources like ammonium chloride and ammonium sulphate also showed relatively higher production of H_2 (Fig. 21.2a). Thus, this can be beneficial for industrial-scale H_2 production by using cheap inorganic nitrogen sources instead of expensive organic nitrogen sources.

21.3.3 Effect of Initial pH on H_2 Production

The effect of initial pH on H_2 production by *Staphylococcus epidermidis* B-6 was investigated at pH 5–9 (with an interval of pH 1). The result (Fig. 21.3a–c) showed that the initial pH of the medium is an important factor in the bacterial growth and H_2 production process. The yield of H_2 and cell growth was increased significantly by increasing the initial pH from 5 to 7 and then decreased by further increasing the pH (Fig. 21.3b). The rate of hydrogen production (Fig. 21.3a) and xylose degradation (Fig. 21.3c) also showed a similar trend with cell growth and H_2 yield as shown in Fig. 21.3b. The maximum H_2 yield is 1.6 mol H_2/mol xylose with hydrogen production rate of 342 mL H_2/L. Day was observed at pH 7. The cell growth and xylose consumption were also observed maximum at this pH. The H_2 yield and cell growth were very low at pH 5 and bellow that no growth and H_2 evolution was observed. This can be the fact that, at high concentration of H^+ ion environment, the cell's ability to maintain internal pH get destabilized, consequently intracellular ATP level drops and inhibiting xylose uptake (Nigam et al. 1985; Xu et al. 2010). However, the strain B-6 was found to produce H_2 above 1 mol/mol xylose within the pH range of 6–9 and very little or no gas production was observed by further increasing the pH. This can be the fact that at higher pH range, the activity of the key enzyme [Fe − Fe] hydrogenase gets decreased and the changing direction of metabolic pathway from acidogenesis to solventogenesis results into low H_2 production (Zhu and Yang 2004; Gadhe et al. 2013).

21.3.4 Effect of Initial Xylose Concentration on H_2 Production

The initial substrate load usually plays a crucial role in cell growth and H_2 production. Figure 21.4a–c shows the various effects of initial xylose concentration on fermentation. An increase in the H_2 yield from 1.4 to 1.6 mol H_2/mol xylose was observed by increasing the xylose concentration from 5 to 10 g/L and was then decreased by further increasing the concentration. Though the volume of H_2 production (Fig. 21.4a)

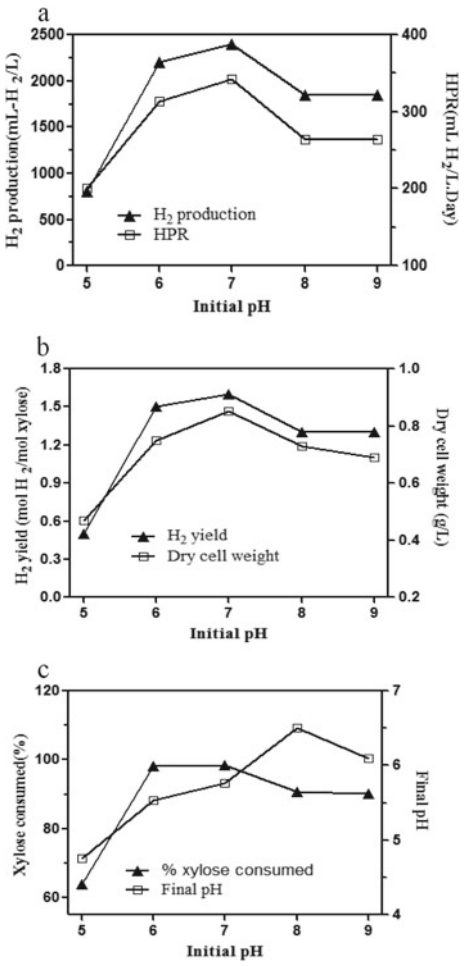

Fig. 21.3 Effect of initial pH on H_2 production performance of *Staphylococcus epidermidis* B-6, **a** volume and rate of H_2 production, **b** H_2 yield and bacterial growth and **c** xylose degradation rate and final pH value

was relatively higher by increasing xylose concentration above 10 g/L, but the rate of production was found gradually decreases with H_2 yield and xylose consumption rate (Fig. 21.4b, c). However, cell growth was observed maximum at 20 g/L xylose concentrations and was then decreased above this concentration. This is due to the fact that at higher substrate concentration, the yield is decreased by inhibitory effect of substrates (Lin and Cheng 2006). Another reason may be that at higher substrate concentration, the carbon flux is directed more towards the production of reduced by-products like organic acids and alcohols (Chittibabu et al. 2006). The undissociated organic acids get accumulated in the fermentation broth with higher substrate concentration, which would leak into the cell and decrease the pH of the intracellular environment. As a consequence, the cell growth and H_2 yield get inhibited (Akutsu et al. 2009).

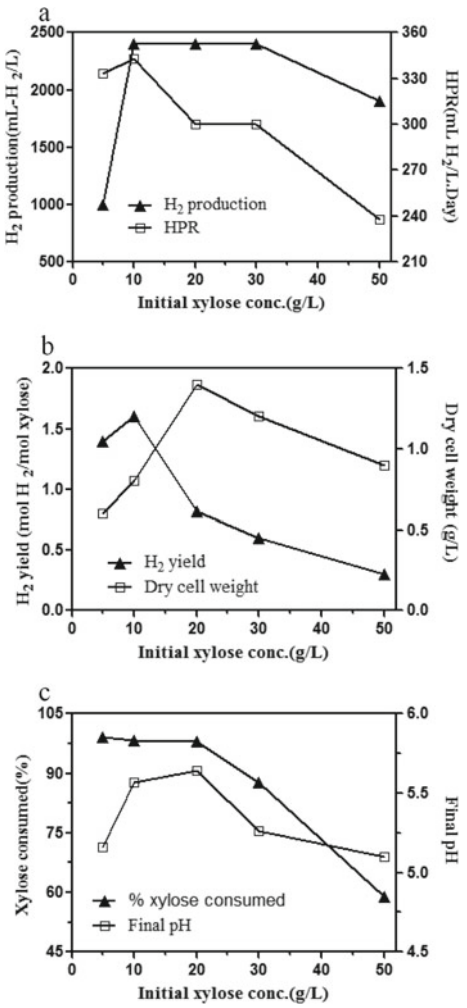

Fig. 21.4 Effect of xylose concentration on H_2 production performance of *Staphylococcus epidermidis* B-6, **a** volume and rate of H_2 production, **b** H_2 yield and bacterial growth and **c** xylose degradation rate and final pH value

21.3.5 Utilization of Different Carbon Sources

It is important for H_2 producing bacterial strain to have the ability to use various carbon sources for better utilization of complex waste biomass. A variety of carbon sources have been reported for fermentative H_2 production. Therefore, different carbon sources were fed to evaluate their effect on H_2 production by *Staphylococcus epidermidis* B-6. The hydrogen production data (Fig. 21.5) showed that the bacterium can utilize diverse carbon sources. H_2 production was observed with lactose (305 mL H_2/g), maltose (280 mL H_2/g), xylose (240 mL H_2/g), fructose (180 mL H_2/g), glycerol (120 mL H_2/g) and glucose (70 mL H_2/g). Thus, the ability of the strain B-6 to utilize a wide variety of carbon sources for H_2 production would be

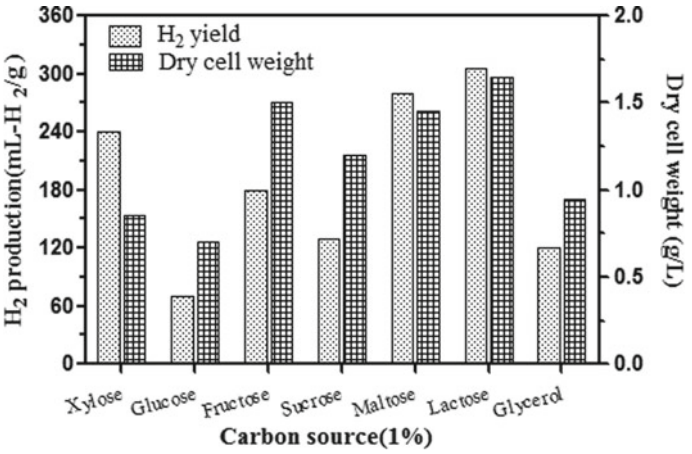

Fig. 21.5 H_2 production performance of *Staphylococcus epidermidis* B-6 by using different substrates

of great impact on waste management and converting the waste biomass to energy (Kapdan and Kargi 2006; Chong et al. 2009).

21.3.6 Production of H_2 from Rice Straw Hydrolysate

The lignocellulosic hydrolysate obtained from wood, agricultural waste by-product and crop contains a major fraction of xylose (Lavarack et al. 2002). As the strain *Staphylococcus epidermidis* B-6 was found potential in utilizing xylose for H_2 production, thus its feasibility to produce H_2 from acid hydrolysate of rice straw was examined. For this, the different concentration of dried rice straw was treated with diluted H_2SO_4 and the hydrolysate was used for batch fermentation. The maximum H_2 yield of 30 L/kg rice straw was observed with hydrolysate prepared by treating 1% (w/v) rice straw and the yield was decreased by further increasing the rice straw concentration (Fig. 21.6). Diluted acid was used for the hydrolysis process (0.5% v/v). Increasing the acid concentration in acid hydrolysis could provide a strong or complete reaction for hydrolysis, yielding more hydrolysed product (Chong et al. 2004). However, at higher acid concentration, the conversion of sugars to various inhibitory compounds takes place, which retard the cell growth. Furfural is one such compound, which is generated as a degradation product from xylose at higher H_2SO_4 concentration (Aguilar et al. 2002). The results suggest that the strain can be used for large scale H_2 production and can help on the way of complete utilization of lignocellulosic waste hydrolysate. This will be a great benefit for the conversion of waste into energy and reduce the waste generation (Fig. 21.7; Table 21.1).

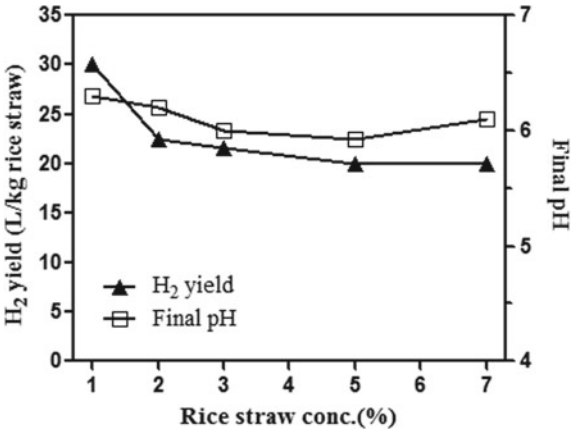

Fig. 21.6 Yield of H_2 and final pH value at different concentration of rice straw hydrolysate by *Staphylococcus epidermidis* B-6

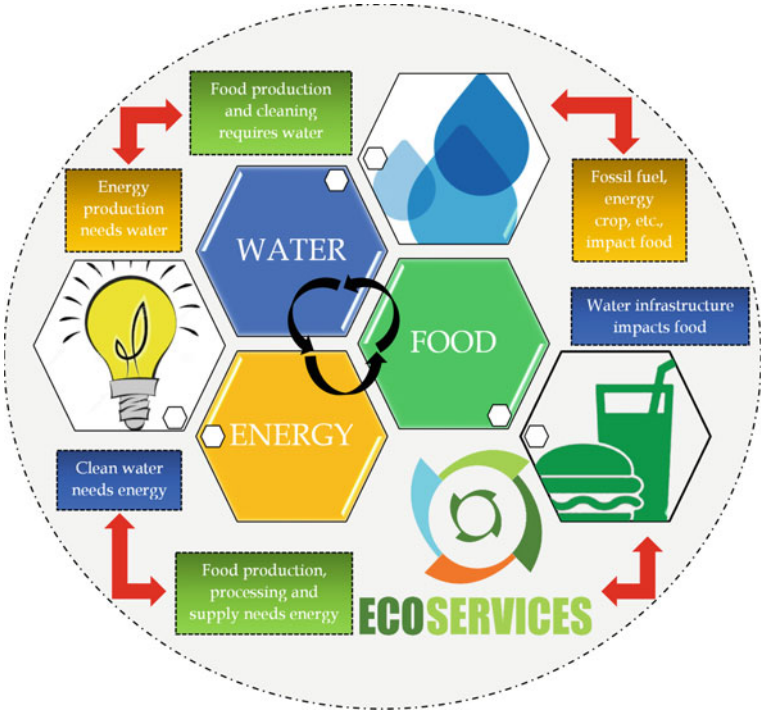

Fig. 21.7 Food–Energy–Water ecosystem services nexus web

Table 21.1 Comparison of H_2 yield from various process utilizing different substrate

Substrate	Treatment	H_2 yield	References
Food waste	Taihu Algae (Ultrasonic pre-treatment)	31.42 mLH_2/g-VS	Xu et al. (2019)
Digested sludge	*Laminaria japonica* (Microwave irradiation)	15.8 mLH_2/g-VS	Yin et al.(2019)
Corn stover, wheat straw, rice straw, Corncob, sorghum stalk	Simultaneous saccharification and fermentation	80.09, 62.49, 95.21, 102.62 and 81.94 mL/g TS	Li et al. (2018)
	Asynchronous hydrolysis and fermentation	66.44, 62.86, 86.31, 90.03 and 77.36 mL/g TS	
Rice husk	Anaerobic granular sludge	320.6 mL/g biomass	Gonzales and Kim (2017)
Sugarcane top	White rot fungus *Pleurotus pulmonarium* MTCC 1805	77.2 mL/g-VS	Kumari and Das (2016)
Cassava residue	Microwave-heated acid pre-treatment (1% v/v H2SO4, 135 C, 15 min) þ Enzymatic hydrolysis	106.2 mL/g-VS	Cheng et al. (2015)
Anaerobic sludge	*Chlorella vulgaris* (pre-treatment with Onozuka R-10 Enzyme)	39 mLH_2/g-VS	Wieczorek et al. (2014)
Oil palm empty fruit bunch hydrolysate	Acid/heat pre-treatment (6% w/v H2SO4, 120 C, 15 min)	1.98 moL H_2/mol xylose	Chong et al. (2013)
Rice straw hydrolysate	*Clostridium butyricum* CGS5	0.76 moL H_2/mol xylose	Lo et al. (2010)
Corn stover	*Thermoanaerobacterium thermosaccharolyticum* W16	2.24 moL H_2/mol sugar	Cao et al. (2009)
Wheat straw hydrolysate	Thermophilic mixed culture	178.0 mL/g sugars	Kaparaju et al. (2009)

21.4 Conclusions

Biohydrogen production by dark fermentative microbes is a sustainable solution to manage lignocellulosic wastes which are hard to solubilize. *Staphylococcus epidermidis* B-6 was used for the first time to produce bio-H_2 from xylose and rice straw. The highest H_2 yield obtained was 1.6 mol H_2/mol and 30 L/kg from xylose and rice straw respectively. The results suggested that the strain could be used for complete utilization of lignocellulosic waste hydrolysate and production of H_2 on a large scale. This will be a great benefit for the conversion of waste into energy and provide an economic technology to manage generated waste.

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