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ORIGINAL ARTICLE

Biohydrogen production by locally isolated facultative bacterial species using the biomass of *Eichhornia crassipes*: effect of acid and alkali treatment

Jerry Mechery[1] · B. Biji[1] · Daniya M. Thomas[1] · V. P. Sylas[1]

[1] School of Environmental Sciences, Mahatma Gandhi University, Kottayam, Kerala 686560, India

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Abstract Hydrogen (H_2) produced from biological methods is a potential option to meet the growing clean energy needs. The present study aimed to produce biohydrogen by dark fermentation from nuisance aquatic weed, *Eichhornia crassipes,* using facultative anaerobic bacteria. A total of 12 bacterial strains were isolated from different wastewater sources and were screened for the potential of H_2 production using glucose as carbon source. Ten strains showed the H_2-producing potential and were identified up to the generic level by biochemical tests. Two strains with higher H_2 production were sequenced using PCR technique and identified as *Proteus mirabilis* and *Pseudomonas aeruginosa* and selected for the studies with *E. crassipes* as the substrate. It was found that *P. aeruginosa* could produce 19.54 ± 0.03% of H_2 from 2% acid (H_2SO_4) treated substrate which was comparatively higher than that of 4 and 8% treatments. *P. mirabilis* also yielded better results of 5.42 ± 0.02% H_2 f or 2% acid (H_2SO_4) treated substrate than 4 and 8% treatments. In total, 33.52 ± 0.04% of H_2 was produced by *P. aeruginosa* for the substrate treated with 2% alkali (NaOH). It was noted that with respect to *P. mirabilis* 4% alkali treated substrate yielded a higher percentage of H_2 (20.23 ± 0.03%) compared to the other two concentrations. The results indicate that alkali treated substrate produced comparatively higher amount of H_2 than that of acid treated substrates. Regarding efficiency, *P. aeruginosa* was found to be more competent than *P. mirabilis.*

✉ V. P. Sylas
mgubioenergy@gmail.com

Keywords Biohydrogen · Dark fermentation · *Pseudomonas aeruginosa* · *Proteus mirabilis* · *Eichhornia crassipes* · Acid and alkali treatment

1 Introduction

The extensive use of fossil fuels has depleted the limited resources, and the emissions have been behind the environmental issues like global warming, climate change and ozone depletion (Show et al. 2012; Suleman et al. 2015; Thomas et al. 2016). Hydrogen (H_2) is considered as a suitable alternative source of energy owing to its regenerative, carbon neutral and high energy yielding (122 kJ/g) property (Kapdan and Kargi 2006; Brown et al. 2007; Balat 2008; Das and Veziroglu 2008; Wang and Wan 2009; Suleman et al. 2015). However, presently about 96% of H_2 are produced from fossil fuels, which are again energy intensive, economically and environmentally not feasible (Momirlan and Veziroglu 2002; Nath and Das 2004; Ewan and Allen 2005; Mohanty et al. 2015). Hence, biological methods of H_2 production from renewable sources exhibit significant advantages as clean energy and less expensive (Ren et al. 2009; Guo et al. 2010; Pudukudy et al. 2014; Marc and Koohi-Fayegh 2016). The established biological methods are direct biophotolysis by green algae, indirect biophotolysis by cyanobacteria, photofermentation by photosynthetic bacteria and dark fermentation by anaerobic fermentative bacteria (Das and Veziroglu 2008; Oncel and Sukan 2011; Show et al. 2012; Hay et al. 2013).

Dark fermentation involves the conversion of organic substrates to H_2 along with butyric, lactic and acetic acid

by the action of anaerobic fermentative bacteria which has dual advantage of energy production and waste management (Nath and Das 2003; Ren et al. 2009; Hay et al. 2013). Bacteria belonging to varied groups can perform fermentative H_2 production (Reginatto and Antônio 2015). Strict anaerobe Clostridium and facultative anaerobes of the family Enterobacteriaceae are the well-established H_2-producing strains (Seol et al. 2008; Elsharnouby et al. 2013; Reginatto and Antônio 2015).

Eichhornia crassipes (Mart.) Solms (water hyacinth) is an aquatic weed, having invaded many water bodies, growing vigorously, blanketing the entire water surface and considered as one of the world's 100 worst invaders (Center et al. 1999; Cheng et al. 2010; Luque et al. 2013). Various mechanical, chemical and biological control methods have been implemented for the removal of this noxious weed (Parolin et al. 2012). The control measures adopted worldwide have exhibited varying extent of success, but complete eradication has not yet achieved (Ray et al. 2009; Patel 2012; Stubbs and Kennedy 2012). However, studies have reported that water hyacinth can be a good source of energy owing to the profuse growth and abundance (Mathur and Singh 2004; Gunnarsson and Petersen 2007; Mishima et al. 2008; Chuang et al. 2011; Das et al. 2016). The dry plant biomass mainly comprises of cellulose (18–31%), hemicellulose (18–43%) and lignin (7–26%) (Kumar et al. 2009; Bergier et al. 2012; Barua and Kalamdhad 2016). The high content of carbohydrate can be hydrolysed through acidic and alkaline treatment into fermentable sugars (Kumar et al. 2009; Aswathy et al. 2009; Barua and Kalamdhad 2016). Efforts done to tap the biomass as a suitable feedstock for the production of biofuels like biogas (Vivekanand et al. 2013; Barua and Kalamdhad 2016), bioethanol (Das et al. 2016; Shanab et al. 2017), biohydrogen (Cheng et al. 2010; Chuang et al. 2011; Lazaro et al. 2014) and biodiesel (Shanab et al. 2017) have been proved successful (Kumar et al. 2009; O'Sullivan et al. 2010; Sharma et al. 2016).

Kuttanad, an integral part of Vembanad-Kol Ramsar site, is also adversely affected by various nuisance invasive aquatic macrophytes. *E. crassipes* is one among the most problematic invasive in the area (Sylas 2010). Hence, the present study has attempted to produce H_2 from *E. crassipes* through dark fermentation in the laboratory condition using two locally isolated facultative bacterial species.

2 Materials and methods

2.1 Organism and growth conditions

The facultative anaerobic bacteria were isolated from different sources of wastewater such as fish market sewage, tapioca processing, coir retting pond and cow dung slurry. These samples were collected in sterilized containers from different locations of Kottayam district, Kerala, India, and brought to the laboratory. The samples (200 ml) were filtered through 2-mm sieve to remove larger particles, then heated in an oven at 100 °C for about 45 min and then cooled (Li and Fang 2007). Hundred millilitres of the heat-treated wastewater was inoculated into sterile nutrient broth and incubated at 37 °C in anaerobic condition for 3 days. Subsequently, from this broth, cultures were inoculated onto nutrient agar plates and incubated at 37 °C for 24 h. Later, the grown colonies were aseptically inoculated into nutrient broth for growth enhancement and used as the inoculums for the further experiments.

2.2 Screening test for bacteria with hydrogen production potential

The isolated bacteria were screened for the potential to produce H_2. For the screening test, the bacterial cultures were inoculated into nutrient medium containing glucose (1.5%) in sterilized distilled water in 250-ml Erlenmeyer flask having screw cap and a port for the gas collection. The final working volume was 200 ml (150 ml glucose solution, 40 ml nutrient medium and 10 ml bacterial inoculums) (Lay et al. 2013). The nutrient medium consisted of 3.77 g/l NH_4CO_3, 0.125 g/l K_2HPO_4, 2 g/l $NaHCO_3$, 0.005 g/l $CuSO_4$, 0.1 g/l $MgCl_2$, 0.015 g/l $MnSO_4$, 0.025 g/l $FeSO_4$ and 0.00125 g/l $CoCl_2$ (Fang and Liu 2002). The initial pH of the medium was maintained at 7, and the flasks were purged with CO_2 for 5 min and sealed tightly with Teflon tape. It was then kept in anaerobic condition at 37 °C for 7 days, and all the experiments were done in quadruplicate. After 7 days, the gas from the headspace of the container was collected with a gas-lock syringe and analysed in gas chromatograph.

2.3 Identification and characterization of bacteria

The bacterial strains having the potential of H_2 production were identified up to the generic level by biochemical characterization and gram staining (Barrow and Feltham 1974). Polymerase chain reaction (PCR) was performed for species-level identification of the two bacterial strains which showed higher H_2 production. The DNA sequences were searched for similarity using BLAST (basic local alignment search tool) and were submitted in the NCBI GenBank DNA database.

2.4 Substrate collection and pre-treatment

The *E. crassipes* plants were collected from Kuttanad wetland ecosystem, part of Vembanad-Kol Ramsar site, in Kerala, India. The collected plants were first washed with tap water and chopped into small pieces (2–3 cm) and sun-dried. The dried biomass was then finely powdered in

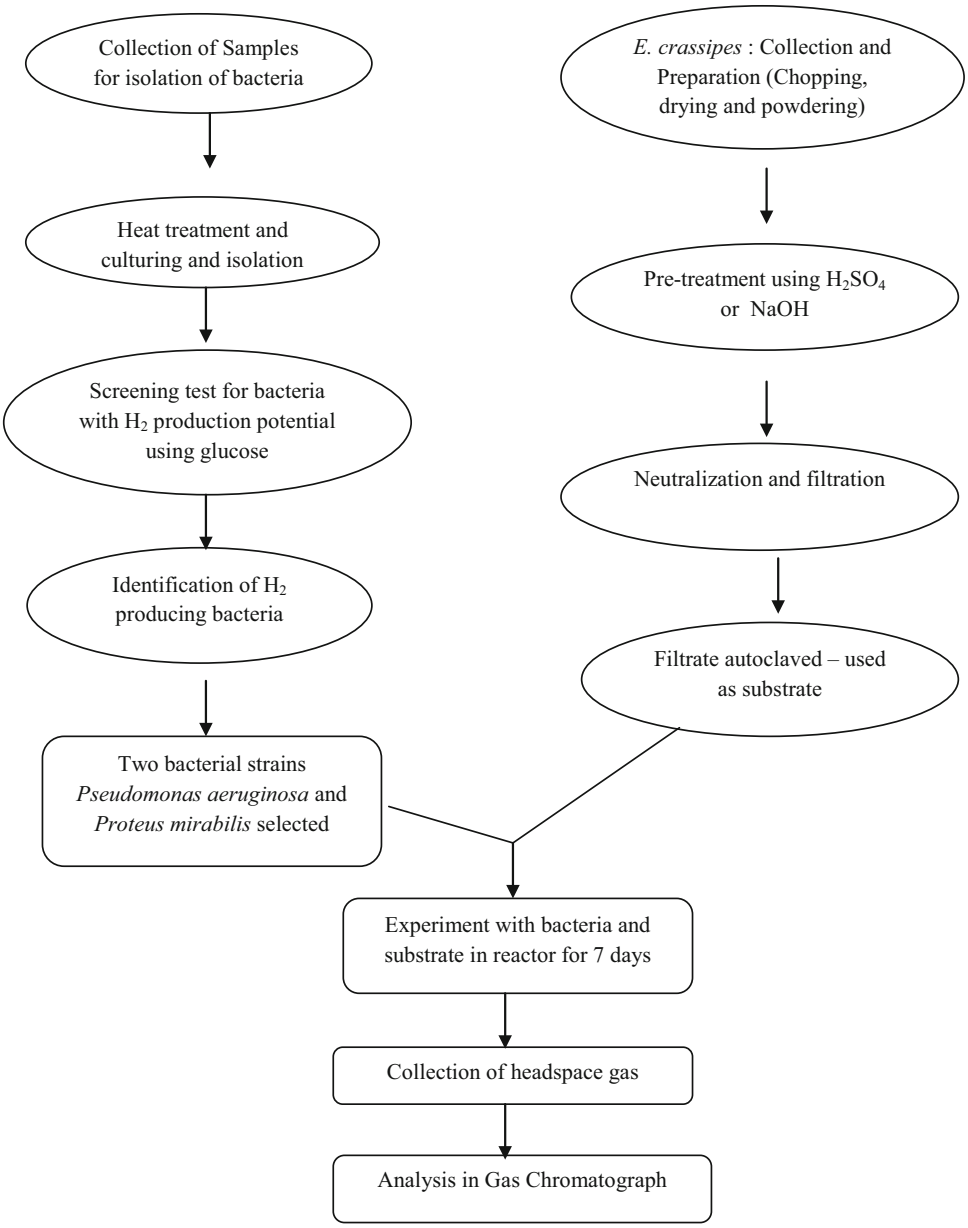

Fig. 1 Schematic representation of experimental procedure

a plant grinder and sieved through 0.25-micron mesh and stored in airtight containers at room temperature for further analysis. The physico-chemical properties of the biomass were analysed as per APHA (1998) [tests were done with hyacinth solution of 1 g dried biomass in 1 l of deionized water (Lay et al. 2013)]. The biomass was pre-treated with different concentrations (2, 4 and 8%) of sulphuric acid (H_2SO_4) as well as sodium hydroxide (NaOH) separately. Ten gram biomass was mixed with either H_2SO_4 or NaOH solution at a solid/liquid ratio of 1:15 and kept under steam treatment at 121 °C at 15 lbs. The pre-treated biomass was then neutralized with acid or alkali and then filtered using Whatman filter paper no: 1. The filtrate was finally sterilized and used as substrate for the experiments.

2.5 Experimental set-up

The two bacteria with higher H_2 production potential were selected for the tests with substrate. The experiments were performed in the similar manner as the screening test with glucose mentioned above in Sect. 2.2. The gas produced was analysed in gas chromatograph. The schematic representation of the entire work is shown in Fig. 1.

2.6 Analytical methods

The pH of the experiments was measured using a portable pH metre. Glucose content was determined using phenol sulphuric acid method (DuBois et al. 1956), and COD was estimated using open reflux method (APHA

Table 1 Hydrogen production from the isolated bacteria

Sl. no	Strain code	Identified bacterial genera	H_2 (ml)
1	HPB1	*Proteus*	5.15
2	HPB2	*Salmonella*	0.83
3	HPB3	*Providencia*	0.045
4	HPB4	*Klebsiella*	0.045
5	HPB5	*Providencia*	0.415
6	HPB6	*Klebsiella*	0.155
7	HPB7	*Klebsiella*	0.7
8	HPB8	*Salmonella*	0.305
9	HPB9	*Salmonella*	0.0
10	HPB10	*Pseudomonas*	9.94
11	HPB11	*Salmonella*	0.065
12	HPB12	*Providencia*	0.0

1998). The headspace gas was analysed in gas chromatograph (Nucon, India, Model 5700) equipped with thermal conductivity detector (TCD). Isothermal separation was done in a packed 2-m-long Porapak Q (80/100) mesh column. The operating temperature of injection port, the oven and the detector was set at 80, 60 and 100 °C, respectively. Nitrogen was used as the carrier gas at a flow rate of 20 ml/min.

3 Results and discussion

3.1 Bacteria: isolation, screening of H_2 production potential and identification

A total of 12 bacterial strains were obtained through the heat treatment and subsequent culturing and were named as HPB1-12. Among them, ten strains showed H_2 production in the screening test (Table 1). Two strains, HPB1 and HPB10, were found to produce higher percentage of hydrogen, and hence, they were selected for the further study of anaerobic fermentation with *E. crassipes*.

The identified bacterial strains belonged to five different genera, namely *Salmonella, Pseudomonas, Proteus, Klebsiella* and *Providencia*. The H_2-producing capability of *Salmonella* (Watanapokasin et al. 2009), *Pseudomonas* (Xie et al. 2008; Soniagandhi and Krishnaveni 2013), *Proteus* (Patel et al. 2010), *Klebsiella* (Costa et al. 2011) and *Providencia* has already been reported.

The DNA sequence data revealed the strains as HPB1 showing 100% similarity with *Proteus mirabilis* (Fig. 2) and HPB10 showing 100% similarity with *Pseudomonas aeruginosa* (Fig. 3). The accession numbers for the submitted sequence data in NCBI GenBank are *Proteus mirabilis* KY817361 and *Pseudomonas aeruginosa* KY817362.

3.2 Hydrogen production: effect of acid pre-treatment

The dried *E. crassipes* is composed of cellulose (22.3%), hemicellulose (39.8%) and lignin (20.6%). These complex organic compounds could not be degraded directly by the bacteria (Su et al. 2010). Direct conversion of raw substrate to H_2 is difficult due to complex nature of lignin hemicellulose–cellulose complex; hence, various pre-treatment methods were carried out to break down the complex structure into simpler fragments so as to enable the bacterial action (Singhal and Singh 2014). Acidic and alkaline pre-treatment helps in the breakdown of cellulose into simpler monomer units of glucose (Sun and Cheng 2002; Li and Fang 2007; Aswathy et al. 2009). Glucose is the carbon source on which microbial communities act upon to produce hydrogen through fermentation (Benemann 1996; Das and Veziroglu 2001; Levin et al. 2006; Guo et al. 2010). In the present study, both acid and alkali treatments (concentrations of 2, 4 and 8%) were employed for the

TGCAGTCGAGCGGTAACAGGAGAAAGCTTGCTTTCTTGCTGACGAGCGGCGGACGGTGAGTAATGTATGGGGATC
TGCCCGATAGAGGGGGATAACTACTGGAAACGGTGGCTAATACCGCATAATGTCTACGGACCAAAGCAGGGGCTC
TTCGGACCTTGCACTATCGGATGAACCCATATGGGATTAGCTAGTAGGTGGGGTAAAGGCTCACCTAGGCGACGAT
CTCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGT
GGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTAGGGTTGTAAAG
TACTTTCAGCGGGGAGGAAGGTGATAAGGTTAATACCCTTATCAATTGACGTTACCCGCAGAAGAAGCACCGGCT
AACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCA
GGCGGT

Fig. 2 Consensus sequence data of HPB1—*Proteus mirabilis*

TCGAGCGGATGAAGGGAGCTTGCTCCTGGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAG

TGGGGGATAACGTCCGGAAACGGGCGCTAATACCGCATACGTCCTGAGGGAGAAAGTGGGGGATCTTCGGACCTC

ACGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCGTAACTG

GTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATAT

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TTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTGACGTTACCAACAGAATAAGCACCGGCTAACTTCGTGC

CAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCAG

CAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTACTGAGCTAGAGTACGGTAGAGGG

TGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCGTGGCGAAGGCGACCACCTGGAC

TGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGA

TGTCGACTAGCCGTTGGGATCCTTGAGATCTTAGTGGCGCAGCTAACGCGATAAGTCGACCGCCTGGGGAGTACGG

CCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAAC

GCGAAGAACCTTACCTGGCCTTGACATGCTGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCAGACACA

GGTGCTGCATGGCTGTCGTCA

Fig. 3 Consensus sequence data of HPB10—*Pseudomonas aeruginosa*

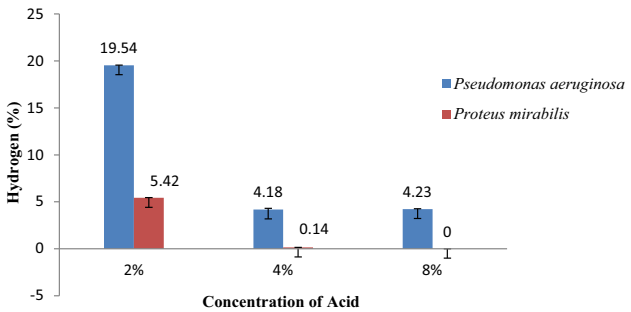

Fig. 4 H_2 production (%) with acid treatment substrates using the two bacteria

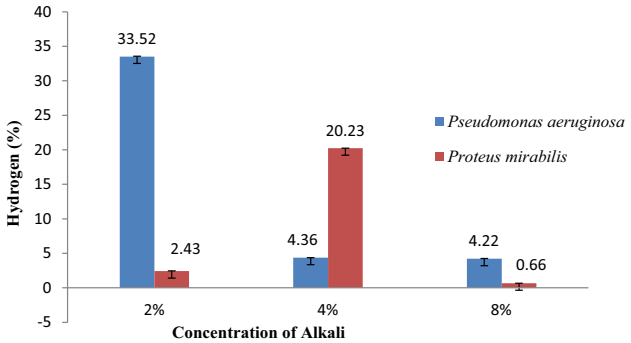

Fig. 5 H_2 production (%) with alkali treatment substrates using the two bacteria

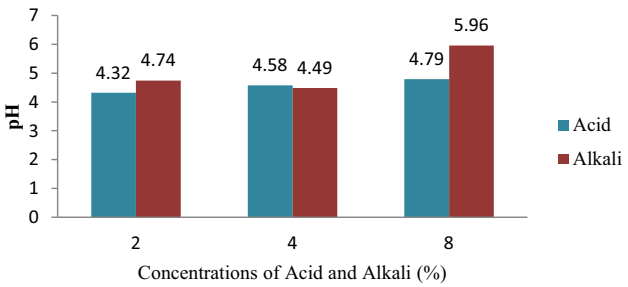

Fig. 6 Final pH of the solution with *P. aeruginosa*

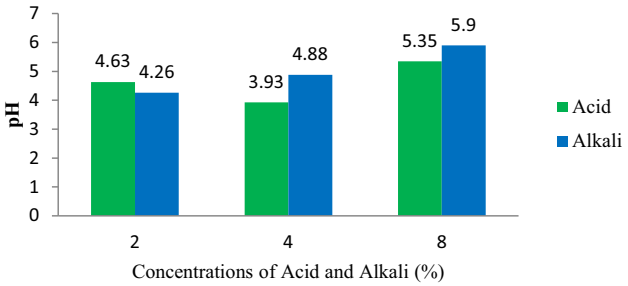

Fig. 7 Final pH of the solution with *P. mirabilis*

conversion of polysaccharides into simple sugars. Later, the isolated bacterial species, *P. aeruginosa* and *P. mirabilis,* were inoculated for the production of H_2 under anaerobic condition.

In acidic pre-treatment, the acid hydrolyses hemicelluloses into xylose (Cui et al. 2009; Thomsen et al. 2006).

Table 2 Percentage degradation of sugar

Concentration (%)		% degradation of sugar	
		P. aeruginosa	*P. mirabilis*
Acid	2	81.34	47.67
	4	48.79	21.13
	8	49.73	20.93
Alkali	2	87.56	33.82
	4	47.42	82.13
	8	48.93	24.79

Table 3 Percentage COD reduction

Concentration (%)		% COD reduction	
		P. aeruginosa	*P. mirabilis*
Acid	2	67.90	56.93
	4	63.45	50.21
	8	59.76	39.98
Alkali	2	71.34	47.23
	4	44.89	69.87
	8	56.76	37.54

The bacteria, *P. aeruginosa* and *P. mirabilis,* could produce H_2 using the pre-treated substrate (*E. crassipes)* under anaerobic fermentation. In the present study, the percentage of H_2 production with *P. aeruginosa* was found to be higher for the substrate treated with 2% acid (19.54 ± 0.03%) than that of 4 and 8% acid treated substrates (4.18 ± 0.13 and 4.23 ± 0.04% H_2, respectively) (Fig. 3). Likewise, for *P. mirabilis* also similar trend was observed, i.e. 2% acid treated substrate yielded better result (5.42 ± 0.02%) than 4 and 8% treated substrates (0.14 ± 0.02 and 0% H_2, respectively) (Fig. 4). In terms of H_2 yield, it was 9.77 ± 0.01, 2.09 ± 0.06 and 2.12 ± 0.02 ml H_2/g substrate, respectively, for 2, 4 and 8% acid treated substrates for *P. aeruginosa*. And for *P. mirabilis,* the yield was 2.71 ± 0.01, 0.07 ± 0.01 and 0 ml H_2/g substrate, respectively, for 2, 4 and 8% acid treated substrates.

The result showed that low acid concentration gave better yield. Similarly, the mild acid treatment of water hyacinth yields high ethanol production (Ma et al. 2010; Sathyanagalakshmi et al. 2011; Idrees et al. 2013). The increased acid concentration lowered the H_2 production due to the conversion of the available sugars to other compounds like xylose, acetic acid and furfural (Aguilar et al. 2002). Furfural and soluble lignin compounds generated during the acid hydrolysis are inhibitors of the fermentation and can even stop the fermentation (Ramos 2003).

3.3 Effect of alkali pre-treatment

Alkaline pre-treatment results in an increase in the internal surface by cellulose swelling, decrease in polymerization degree and structural alteration of lignin. The treatment also causes the crystallinity destruction of links between lignin and other polymers, causing the breakdown of lignin (Sun and Cheng 2002; Ibrahim et al. 2011; Yan et al. 2015). Alkaline pre-treatment changes the structures and properties of cellulosic fibres and improves the enzymatic digestibility of fibres (Zhu et al. 2004; Teater et al. 2011). The percentage of H_2 production for the substrate treated with 2% alkali with *P. aeruginosa* was 33.52 ± 0.04%, which is higher than that of 4 and 8% alkali treated substrates (4.36 ± 0.02 and 4.22 ± 0.03% H_2, respectively) (Fig. 5). The 4% alkali substrate yielded a higher percentage of H_2 compared to other two concentrations for *P. mirabilis.* The percentage of H_2 was 2.43 ± 0.02% for 2% alkali treated substrate and 20.23 ± 0.03% for 4% treated substrate and 0.66 ± 0.02% for 8% alkali treated substrate (Fig. 5). In terms of yield, it was 16.76 ± 0.02, 2.18 ± 0.01 and 2.11 ± 0.01 ml H_2/g substrate, respectively, for 2, 4 and 8% alkali treated substrates for *P. aeruginosa*. And for *P. mirabilis,* the yield was 1.21 ± 0.01, 10.11 ± 0.01 and 0.33 ± 0.01 ml H_2/g substrate, respectively, for 2, 4 and 8% alkali treated substrates.

A similar trend was obtained in other studies also; higher H_2 yields were obtained with alkaline pre-treated sludge (Cai et al. 2004). Alkaline pre-treatment enhances the enzymatic hydrolysis, thereby facilitating H_2 production (Su et al. 2010; Aswathy et al. 2009). During alkaline treatment, the H_2 produced was maintained and less consumed within the reactor; thus, the higher amount is present at the end of the experiments (Cai et al. 2004). Alkaline pre-treatment causes less sugar transformation (Antonopoulou and Lyberatos 2011; Ibrahim et al. 2011; Sills and Gossett 2011) making the sugars available for microbial action.

Presence of methane was not observed in any of the reactors. This might be the positive effect of heat pre-treatment of raw inoculums. The heat pre-treatment was done to eliminate H_2-consuming methanogens and selectively favour the growth of H_2 producers (Lin et al. 2006; Li and Fang 2007; Sivaramakrishna et al. 2014).

3.4 Process variables: pH, COD and glucose

At the end of the experiments, the pH dropped to acidic range in all the reactors for both the treatments (Figs. 6 and 7). This decrease is owed to the production of acidic intermediates such as volatile fatty acids (Alkaya and Demirer 2011), which was not quantified in the present

Table 4 Biohydrogen production from different substrates by dark fermentation

Substrate	Microorganism used	H_2 yield	References
Glucose	*Klebsiella oxytoca* HP1	1.0 mol H_2/mol substrate	Minnan et al. (2005)
Jackfruit peel	Cow dung slurry	55 ± 2% H_2	Vijayaraghavan et al. (2006)
Molasses	Mixed microbial culture	3.47 mol/mol substrate	Guo et al. (2008)
Wheat powder	*E. aerogenes*	545 ml H_2/g starch	Argun et al. (2009)
Water hyacinth	Anaerobic sludge	51.7 ml- H_2/g-TVS	Cheng et al. (2010)
Sugarcane bagasse	Elephant dung	0.84 mol H_2/mol total sugar	Fangkum and Reungsang (2011)
Cheese whey powder	Anaerobic seed sludge	1.03 mol/mol substrate	Kargi et al. (2012)
Pineapple waste	Anaerobic seed sludge	1.83 mol H_2/mol glucose	Reungsang and Sreela-or (2013)
Rice straw	*Clostridium pasteurianum*	0.44 mol H_2/mol T-sugar	Liu et al. (2013)
Water hyacinth	Pig slurry	13.65 ml/g feedstock	Lay et al. (2013)
Benincasa hispida	Mixed microbial culture	14 mmol H_2/mol sugar	Singhal and Singh (2014)
Vegetable waste	Seed microflora	2.2 mol/mol substrate	Marone et al. (2014)
Sugar beet juice	Anaerobic digested sludge	3.2 mol H_2/mol hexose	Dhar et al. (2015)
Waste peach pulp	Anaerobic sludge	123 ml H_2/g TOC	Argun and Dao (2016)
Raw cassava starch	Mixed microbial culture	1.72 mol H_2/mol glucose	Wang et al. (2017)
Water hyacinth	*P. aeruginosa*	16.76 ml H_2/g substrate	Present study
Water hyacinth	*P. mirabilis*	10.11 ml H_2/g substrate	Present study

study. Volatile fatty acid productions are interrelated with H_2 production (Prakasaham et al. 2009).

The substrate had an initial sugar content of 2.2 mg/g. The sugar degradation values ranged from 48.79 to 81.34% in acid treated substrates and 47.42–87.56% in alkali treated substrates for the bacteria *P. aeruginosa* (Table 2). For the bacteria *P. mirabilis,* in acid treated substrates, the values ranged from 20.93 to 47.67% and in alkali treated substrates the values ranged from 24.79 to 82.13%. The sugar degradation values are in accordance with the result of hydrogen production, and the higher degradation percentage was shown in the reactor which produced higher percentage of H_2. Pure glucose substrates show 100% sugar degradation efficiency (Kumar et al. 2013; Nasra et al. 2014), and the less degradation exhibited in the study may be due to the composite nature of the substrate (Kumar et al. 2013).

The initial chemical oxygen demand (COD) of the substrate was 390 mgO_2/l. The COD reduction percentage ranged between 59.76 and 67.90% for *P. aeruginosa* in acid treated substrate and 44.89 and 71.34% in alkali treated substrates (Table 3). The values ranged from 39.98 to 56.93% in acid treated substrate for *P. mirabilis,* and in alkali treated substrates it ranged from 37.54 to 69.87% (Table 3). From the overall results, the highest reduction percentage was observed in the 4% alkali treated substrate with *P. mirabilis,* which is in line with the result of hydrogen production. The COD removal efficiency of an organism is a characteristic feature determining its hydrogen production capability (Ramprakash and Muthukumar 2014). The general range of COD removal efficiencies in fermentative hydrogen production processes is between 20 and 40% (O-Thong et al. 2008). However, higher efficiencies have been exhibited with various other studies; COD removal of 71.8% was obtained during the fermentation of enzymatic hydrolysed rice mill wastewater (Ramprakash and Muthukumar 2014). About 75% efficiency was recorded for the fermentation of paper and pulp industry effluent with *Enterobacter aerogenes* (Lakshmidevi and Muthukumar 2010). In the fermentation of palm oil mill effluent, a COD removal efficiency of 63% was obtained which could enhance hydrogen production (O-Thong et al. 2008).

The results of the present study are compared with previous reports of biohydrogen production with different substrates by dark fermentation (Table 4).

4 Conclusion

The study succeeded in producing hydrogen gas from pre-treated aquatic weed *E.crassipes* through anaerobic fermentation. Out of the twelve bacteria isolated from different sources of wastewater, ten strains had the potential of producing hydrogen under anaerobic condition. These bacterial species belonged to five genera, namely *Salmonella, Pseudomonas, Proteus, Klebsiella* and *Providencia*. Of them, *Pseudomonas sp.* and *Proteus* sp. produced higher amount of hydrogen and hence were selected for the anaerobic experiments with acid and alkali pre-treated *E.*

crassipes. These two bacteria were sequenced through PCR technique to identify the species as *P. aeruginosa* and *P. mirabilis*. The results of the experimental analysis show that alkali treated substrate produced higher amount of hydrogen than that of acid treated substrates. Comparing the efficiency of the bacteria, *P. aeruginosa* was found to produce higher percentage of hydrogen.

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Compliance with ethical standards

Conflict of interest The authors have no conflict of interest in the present work.

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