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RESEARCH ARTICLE

Acinetobacter junii AH4-A Potential Strain for Bio-hydrogen Production from Dairy Industry Anaerobic Sludge

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Abstract

The present study aims to enhance the efficiency of anaerobic sludge microorganisms to produce hydrogen (H_2) through various pre-treatment methods. The various pre-treatment methods such as base, acid, chloroform, heat shock, freezing and thawing have enabled to isolate acidogenic bacteria with higher bio H_2 producing activity in an anoxic environment. From these various treatments, bacteria were isolated and screened for bio H_2 capabilities. Among the one bacterial strain, AH4 strain showed maximum cumulative H_2 production and Hydrogen Yield (HY) using 100% diary anaerobic sludge. AH4 strain was identified as *Acinetobacter junii* using 16S rRNA gene sequence and used for further experimental analysis. Biohydrogen productions of *Acinetobacter junii* were measured at different experimental setup such as various pH levels (5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0) and different substrate concentration (10 - 100%) of dairy anaerobic sludge substrate. At pH 7.5 and 60% substrate concentration, the strain AH4 *Acinetobacter junii* displayed the maximum cumulative H_2 production of 945.7 ml/L and H_2 yield 1.35 mol H_2/mol glucose. Based on our results, we concluded that *Acinetobacter* junii can be used as a promising bio agent for hydrogen production on a large scale using dairy anaerobic sludge as substrate.

Key words: Bio hydrogen, Dairy industry anaerobic sludge, Pretreatment, Optimization, Batch experiment.

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INTRODUCTION

Hydrogen (H_2) gas is the lightest element and highly combustible gas with faster diffusibility. H_2 is greatly attributed as future fuel owing to its combustion energy of 120 MJ/kg and a heat capacity of 14.4 kJ/kg K [1]. Being friendly, it is used in different chemical process industries especially in automobile industries to reduce pollution as its only product is water and does not discharge CO_2 or any other deleterious pollutants, making H_2 a justifiable replacement for the declining fossil fuels[2,3]. H_2 is generated from both renewable and non-renewable sources, either by biological or physicochemical methods[4]. Karthic and Joseph (2012), a survey on 2008 reported that the total annual H_2 fuel production was 368 trillion cubic meters which mostly from various industries such as chemical industry (40%), oil refineries (40%) and other huge variety of processes (20%) and, is growing exponentially (12% annually) at presently and will be contributing to a total energy market of 10% by 2025[5]. H_2 can also be produced by biological processes especially with aid of microorganisms providing a feasible means for the viable supply of H_2 with low pollution and high efficiency[6]. Species of anaerobe such as *Clostridium butyricum* [7], *Enterobacter asburiae* [8], facultative anaerobe *Escherichia coli*[9],aerobes *Bacillus coagulans*[10] plays dominant role in fermentative H_2 production. Microbial population from mixed anaerobic and other multiple sources (soil, sediment, compost, aerobic and anaerobic sludge) have been considered for significant H_2 production by dark fermentation[11]. From an engineering point of view, production of H_2 by mixed cultures is often preferred because it is economical and ease of control for the use of organic wastes as feedstock[12]. In another view as a microbiologist, the exploration to find the target specific strain with high activity of producing H_2 is highly inevitable.

Energy tapping from waste has gained more attraction in the past decade. H_2 generation from domestic and industrial waste water pioneered with great interest on consideration of the environment as well as economy. Many effluents from various industries have been exploited for H_2 energy recovery using microorganisms as prime source[13]. Dairy industries are one of the sectors which dispenses various dairy products (yogurt, cheese, butter, milk, ice cream etc.) ascribing as major source of wastewater generation. Dairy industry wastewater consists of 99% organic substances which are biodegradable[14]. The incomplete degradation of organic fraction in such sludge process cause foul odour and serious health effect to the environment, human, animals and insects[15]. Therefore, a cost-effective biological treatment process with efficient degradation potential is strongly recommended for management of huge quantum of dairy industry wastewater.

Deploying microbes towards the treatment of wastewater to increase the productivity and to enhance the overall treatment efficiency is a better practice to minimise the energy spent over process. The increase growth of bacteria and their H_2 evolving activity can be enhanced by pre-treatment of sludge in anaerobic condition[16].Three main criteria of microbial H_2 production include (i) the material selection with a bacterial population of interest; (ii) enrichment and (iii) acclimatize bacteria to specific substrates. Pre-treatment strategies for enhancing H_2 producing microorganisms include heat-shock, load-shock, acid treatment, base treatment, aeration, freezing along with thawing, chloroform, and using iodopropane[17–19]. Several studies reported that pretreatment methods are highly responsible for the elevated H_2-production on using targeted strains.To enhance the H_2 generation the pretreatment of the anaerobic inoculum, and the hydrolysis reduces the impact of rate[15,20]. Therefore, a pre-treatment of seed sludge is necessary to repress H_2 consuming bacteria and preserve the H_2 producing bacteria from a mixed culture system. Reports suggested that currently heat shock pre-treatment method has been used successively and obtained highest H_2 production rate (HPR)[21,22]. Thong *et al*, obtained a maximum H_2 production yield of 1.96 mol H_2/mol hexose with the application of load- shock treatment and resulted with a HPR of 11.2 mmol H_2/ (L^{-h}). Mu *et al*. (2007) reported heat-shock pre-treatment has produced high H_2 production, among the three pre-treatment methods studied. The present investigation aims to isolates H_2 producing bacteria from dairy industry anaerobic sludge by applying various pre-treatments that enable to enhanced production of H_2. In addition, the effects of pH

and substrate concentration were analyzed and optimized for the H_2 production along with the treatment of dairy anaerobic sludge.

MATERIALS AND METHODS

Collection of sample

The anaerobic sludge was collected in sterile decanters from the local dairy industry (Aavin) located in Madurai, Tamil Nadu, India. Aseptically collected sludge was transported immediately to the laboratory and stored at 4˚C until further use. The important physico and chemical characteristics (pH, Conductivity, Salinity, volatile fatty acids (VFA), alkalinity, chemical oxygen demand (COD), total solids (TS), total suspended solid (TSS), volatile suspended solid (VSS) , Alkalinity of the dairy industry anaerobic sludge were analysed in accordance to APHA[23]. In addition, estimation of glucose concentration after experimentation was determined by DNS calorimetric method as glucose used as stranded [24]. Total protein concentration was measured by Lowry method [25] with bovine serum albumin as standard.

Pre-treatment methods for isolation of enhanced H_2 producing microorganisms

The collected anaerobic sludge was exposed to various pre-treatment methods namely heat shock, treatment with acid, base, chloroform, aeration, freezing and thawing. These pre-treatment methods were performed with procedures as described. According to Wang and Wan (2008) during heat shock treatment, the sludge was boiled at 100˚C for 15 min and incubated at 37°C for 24 h [26]. The acid pre-treatment was conducted by adjusting the pH at 3.0 of the sludge to by adding 1M HCl and incubated at 37°C for 24 h[27]. Similarly, the other treatments such as the base, aeration and chloroform pre-treatments were followed as Wang and Wan [26]. In base pre-treatment, the pH of sludge was adjusted to 12 with 1M NaOH and incubated at 37°C for 24 h. The aeration treatment was carried out by aerating the sludge completely with air for 24 h. The chloroform pre-treatment was prepared by adding chloroform to the sludge at a concentration of 2% and incubated at 37°C for 24 h. Freezing and thawing pretreatment was conducted by freezing the sludge at -20°C for 24 h and afterward thawing it in a water shower at 37°C until it achieved room temperature[28]. The sludge without any pre-treatment was maintained as control for comparison. After the pretreatment, the pre-treated anaerobic sludge was used for isolation of bio H_2 producing microorganisms.

Isolation of bacterial strain from pre-treated dairy anaerobic sludge

The bio H_2 producing bacteria were isolated from the pre-treated dairy anaerobic sludge samples. The medium used for isolation and cultivation of strains grown on Thioglycolate Agar (TGA) medium and was prepared as follows; 15 g/L pancreatic digest of casein, 5.5 g/L dextrose, 5 g/L yeast extract, 2.5 g/L sodium chloride, 0.5 g/L sodium thioglycolate, 0.5 g/L L-cystine, 1 mg/L resazurin, 75 g/L agar with final pH 7[29]. The medium was prepared and sterilized at 121˚C for 15 min. successively, 100 µl of the pre-treated anaerobic sludge samples were plated individually on TGA agar plate and incubated overnight in an anaerobic jar at 37°C for 24 hrs. After that, the different colonies obtained in the plates were picked up individually and streaking was done on TGA plate. A single colony from well grown plate was taken and aseptically streaked over the fresh TGA agar plate to get pure culture for further study.

Screening and analysis of bio H_2 production in serum bottle fermentation

The obtained pure culture of isolates were screened for production of H_2 potential in batch fermentation. Working volume of 50 ml of 100% dairy sludge in 100ml Serum bottles were sterilised, after that 1ml of bacterial culture was inoculated for the evolution of H_2 production capability. Each bottle was purged with pure nitrogen gas for 5 min to generate an anaerobic environment inside the bottle. Mouth of each serum bottles was sealed with a rubber stopper and crimped aluminium caps using manual crimper. The complete experimental setup was in shaking incubator for 48 h in 120 rpm at 37°C. After 48 h, the H_2 gas production were confirmed by hungate technique using aseptic glass syringe[30]. Each experiment was repeated thrice.

The composition of gas evolved was analysed using Gas chromatography (SHIMADZU GC-2014, Japan) was equipped with a thermal conductivity detector (TCD) and stainless-steel column packed with Porapak Q (80/100 mesh). The injection port, column oven and detector were

operated at 100°C, 80°C and 150°C respectively. Nitrogen was used as the carrier gas at a flow rate of 20 ml/min[29]. The samples were injected using a 2 ml airtight gas syringe under the operating conditions mentioned above.

Identification of the selected isolate and microscopic examination

Based on screening, the higher bio H_2 production bacterial isolate was selected. The selected isolate was identified using 16S rRNA gene sequencing[31] and a phylogenetic tree was constructed using MEGA5.0 Software.

For microscopic observation, the bacteria culture was centrifuged at 8000rpm for 10min at 4°C. Then the pellet was resuspended with 0.8% saline solution. A loopful of cell suspension was placed on glass slide, heat fixed, washed with sterile disH_2O and followed by gram staining method. After complete air drying, the slide were observed under the trinocular microscopic (LABOMED, Inc).

In addition, the cell suspension were fixed on glass slide for overnight at 40°C with 2.5%, glutaraldehyde in phosphate buffer solution (PBS) at pH 7.4. After incubation, the slides were washed with PBS for three times and followed by dehydrated in series of ethanol (20%, 40%, 60%, 80%, & 100%) for 10 min interval and dried at room temperature in desiccator. After coating with pt-pd using a sputter coating (TESCAN, VEGA3, and CZ) for 30min, the bacterial cells were observed under scanning electron microscope (SEM).

Optimization of bio H_2 production

The optimization study of bio H_2 production (substrate concentration and pH) was carried out utilizing dairy anaerobic sludge with the selected bacterial strains. Serum bottles (100 ml) containing 10% to 100% concentration of dairy sludge at various pH (5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0) were prepared and sterilized. Each bottle were aseptically inoculated and incubated as described earlier. After 48 h incubation in shaker, the gas volume and composition were analysed as mentioned before. The various physic chemical parameters (pH, Conductivity, Salinity, BOD, COD, VFA, TSS, TS, Glucose and Protein) were analysed as described earlier. All the experiments were done in triplicates.

The cumulative bio H_2 production profile from batch fermentation was calculated by modified Gompertz equation (Eq.1) [29]

$$H(t) = P.exp\left\{-exp\left[\frac{R_m.e}{P}(\lambda - t) + 1\right]\right\}$$

...Eq. 1

Where H (t): cumulative volume of H_2 production (ml) at time t, l: the time of the lag phase (h), P: H_2 production potential (ml), R_m: maximum H_2 production rate (mL L^{-1} h^{-1}), and e: exponential constant 2.71828. Cumulative H_2 production (mL/L) was obtained by using Gompertz equation (Eq.1). Hydrogen Yield (HY) (mol H_2 /mol glucose) was calculated as the total molar amount of H_2 divided by molar amount of consumed glucose (as reducing sugar). The total molar amount of H_2 (mol/l) was calculated using ideal gas law; total molar amount of H_2 (mol/l) = Cumulative H_2 production (L) divided by RT. Where, R = 0.0821 atm K^{-1} mol^{-1} and T=310 K.

The COD removal efficiency (COD_R) was calculated using Eq. (2).

$$COD_R = \frac{(C_i - C_f)}{C_i} \times 100\%$$

...Eq. 2

where C_i represents the initial COD concentration (mg/L) and C_f denotes the final COD concentration (mg/L) in the batch experiment.

RESULTS AND DISCUSSION

Physico-chemical characteristics of raw dairy industry wastewater

The Physico-chemical characterization of the collected dairy industry anaerobic sludge was analysed and results were shown in Table 1. Dairy industry anaerobic sludge have rich source of organic matter substance. This useful form of energy could be converted into potential H_2 energy source[32]. Table 1 show that the main component had many organic substances with low molecular weight, proteins, carbohydrates and Volatile Fatty Acids (VFA); which was consumed by microorganisms to convert bio H_2.

Pretreatments, isolation, screening and identification of H_2 producing microorganisms

In the present investigation, the pre-treated dairy industry anaerobic sludge (Aavin) was used for isolation and identification of potential strain (i.e. bio H_2 producing microorganisms). By heat shock treatment among the five isolates, two isolates only had the efficiency to produce biogas. In base treatment among three bacterial

Table 1. Physicochemical Characteristics of the Dairy Industry anaerobic sludge

Parameters	Results
Colour	Black
Temperature (°C)	37.8±0.3
pH	7.5±0.3
Conductivity (µS)	3.18±0.03
Salinity (ppm)	1.67±0.02
Total solids (mg/L)	2415.3±3
Total dissolved solids (ppm)	2222.3±3
Total suspended solids (mg/L)	288±2
Alkalinity (mg/L)	21.6±4
Volatile fatty acids (mg/L)	22.80±4
COD (mg/L)	1153.3±3
BOD (mg/L)	472.6±2
Protein (g/L)	32.8±0.2
Glucose (g/L)	26.8±0.6

isolates one bacterium is responsible for biogas production. In chloroform treatment among the four bacterial isolates, one bacteria isolate was involved in biogas production. In other treatment methods such as acid treatment, aeration, freezing and thawing used, there was no biogas production evident by single bacteria out of five. As a result, only 4 out of 17 isolates were capable of producing H_2 within 48 h and they were designated as AH2, AH4 (Aavin/Heat), AB2 (Aavin/Base) and AC3 (Aavin/Chloroform). However, the amount of bio H_2 gas produced by each isolates were varied as shown in Table 2. The gas volume and gas composition were analysed. Among the four isolates, the isolate AH4 produced maximum amount of H_2 gas production (560 mL/L) and cumulative hydrogen production (441.3 ml/L) respectivily utilizing dairy anaerobic sludge. As the AH4 isolate produced higher amount of H_2 gas, it was selected for further studies.

Results from the gas analysis showed that the biogas produced from the anaerobic fermentation contains only detectable bio H_2 gas. The effect of different pre-treatment methods on the cumulative H_2 production in batch tests (Table 2). The results indicated that the H_2 production process stopped within 48 hrs for the entire test. No methane was observed in all the experiments. This was also reported by other scientists[27,33]. The progress of cumulative H_2 production in the batch test was described as by the modified Gompertz model[36]. In this study, the results showed the cumulative H_2 production obtained from each batch test was used to in the modified Gompertz model[34] using software Origin 7.5 and the coefficient (R^2) of all the regression was 0.943. Thus, the results from this study indicated that the modified Gompertz model could be used to describe the progress of cumulative H_2 production in the batch tests.

Bacteria such as *Clostridium* sp and *Enterobacter* sp could produce H_2 during fermentation of glucose. However, spores of *Clostridium* sp (obligate bacteria) might survive in harsh environment but some homoacetogens (obligate anaerobic bacteria) failed to survive to anaerobic environment as O_2 hindered to survive. Therefore, appropriate seed sludge pre-treatment is essential to conserve the activity of the H_2-producing bacteria and suppress homoacetogens which ultimately increase the yield of H_2 and maximum H_2 production rate. This result proposed that all the pretreatment could successfully obtain H_2 producing microorganisms, which can use glucose as a substrate. Among all the four pre-treatment techniques, heatshock pre-treatment was the best pre-treatment strategy. In the present study, the isolate AH4 showed maximal HY of 0.63

Table 2. Effect of various pre-treatment methods on biohydrogen production utilizing dairy industry anaerobic sludge. P - The hydrogen production potential, R_m - Maximum hydrogen production rate, $H_{(t)}$- Cumulative H_2 Production, HY – Hydrogen Yield

Pre-treatment method	Isolates	P (ml/L)	R_m (mL L^{-1} h^{-1})	H (t) (ml/L)	HY(molH_2/mol glucose)
Heat shock	AH2	200	4.16	157.6	0.23
Heat shock	AH4	560	11.6	441.3	0.62
Base	AB2	320	6.66	252.2	0.30
Chloroform	AC3	400	8.33	315.2	0.24

Table 3. Hydrogen production during optimization studies by *Acintobacter junii* (AH4). $H_{(t)}$ – Cumulative H_2 Production; HY – Hydrogen Yield * % of H_2 calculated based on GC analysis

Substrate conc. (%)	pH	Gas evolved (ml/L)	COD_R (%)	% of H_2 evolved*	H_2 evolved (ml/L)	R_m (mL L^{-1} h^{-1})	$H_{(t)}$ (ml/L)	HY(mol H_2/mol glucose)
20	5	240	52.38	100	240.00	3.94	189.1	0.27
40	5	860	47.94	50.99	438.51	7.20	345.6	0.29
30	5.5	320	28.57	53.02	169.66	2.79	133.7	0.19
40	5.5	420	44.69	82.31	345.70	5.68	272.4	0.55
80	5.5	1100	51.41	65.74	723.14	11.87	569.9	0.81
70	6	540	68.28	74.77	403.76	6.63	318.2	0.45
100	6	440	88.90	100	440.00	7.22	346.7	0.49
10	6.5	240	52.17	100	240.00	3.94	189.1	0.13
10	7	480	57.39	100	480.00	7.88	378.3	0.54
50	7	560	55.63	75.75	424.20	6.97	334.3	0.47
70	7	560	84.14	100	560.00	9.19	441.3	0.62
10	7.5	600	44.35	52.95	317.70	5.22	250.3	0.35
50	7.5	800	43.48	61.2	489.60	8.04	385.8	0.45
60	7.5	1200	58.38	100	1200.00	19.70	945.7	1.35
80	7.5	280	37.53	100	280.00	4.60	220.6	0.20
90	7.5	560	47.59	75.5	422.80	6.94	333.2	0.26
30	8	660	73.03	77.62	512.29	8.41	403.7	0.29
40	8	860	44.47	100	860.00	14.12	677.7	0.44
50	8	640	77.82	100	640.00	10.51	504.4	0.30

*% of H_2 concentration calculated based on Gas chromatography

mol H_2/mol glucose which was higher than other isolates (Table 2).

The bacteria strain AH4 was identified by gene sequence of 16S rRNA. The partial sequence of the selected higher bio H_2 producing bacteria (AH4) 16S rRNA gene was determined. The 16S rRNA gene sequence was aligned using the Blast program (http://www.ncbi.nlm. nih.gov. BLAST). The 16S rRNA gene sequence showed the 99% similarity with that of *Acintobacter junii*. Subsequently, a phylogenetic tree was constructed by MEGA 5.0 (Figure 1). From the results of 16S rRNA, the strain AH4 belong to the species of *Acintobacter junii* and designated as *Acintobacter junii* AH4 (Accession number: KR809375). The gram staining method reveals the organism as

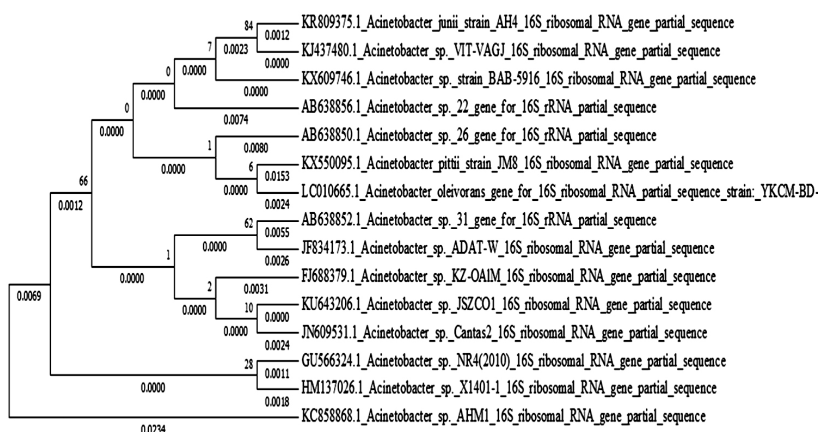

Fig. 1. Phylogenetic tree showing the relationships between strain *Acintobacter junii* AH4 and related species

gram positive the morphology of AH4 strain obtained from SEM image is shown (Figure 2).

Optimization of bio H_2 production

Fermentative H_2 production is influenced by two factors (pH and substrate concentration). Optimization of bio H_2 production with substrate (industry anaerobic sludge) of different concentration (10 to 100%) and pH (5 to 8) by *Acintobacter junii* cultures is shown in (Table 3). At pH 7.5, the maximum amount of cumulative H_2 production (945.7 ml/L) and maximum HY (1.35 mol H_2/mol glucose) was observed 60% substrate concentration under mesophilic condition at a COD removal effiviency of the isolates is 58.38%. It was reported that the pH range 7.5 was found to be the highest H_2 production condition[35].The results of other reports are compared with our results as shown in (Table 4). Therefore, *Acintobacter junii* AH4 is a potential bacterial strain that can be used for efficient H_2 production utilizing anaerobic sludge in the large scale under mesophilic condition.

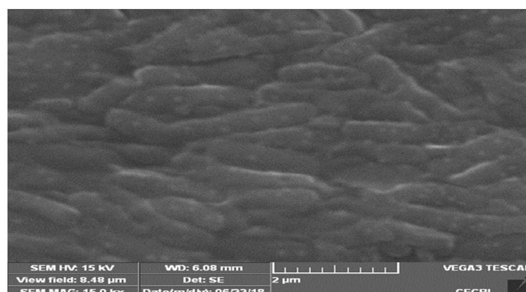

Fig. 2. SEM image of *Acintobacter junii AH4*

Table 4. Comparison of fermentative hydrogen production

Substrate used	Organisms	Condition (Temp / pH)	Pre-treatment conditions	Hydrogen Yield	Reference
OPEFB*	Clostridium butyricum KBH1	37°C/pH 9	Hydrothermal on industrial scale	1.21 mol H2/mol pentose	[36]
OPEFB*	Oil palm sludge	37°C/pH 5.5	120 °C, 15 min with 6% (w/v) H_2SO_4	1.98 mol H2/mol xylose	[37]
Oil palm Trunk	Hot spring Sediment	60°C/pH 6.3	Microwave at 450 W, 7.50 min with 1.56% (w/v) H_2SO_4	0.71 mol H2/mol sugar	[38]
Sugarcane Biogases	Clostridium Butyricum	37°C/pH 5.5	121 °C, 60 min with 0.5% (v/w) H_2SO_4	1.53 mol H2/mol glucose	[39]
Rice straw	Seed sludge	45°C/pH 6.5	150°C, 60 min with 3% (acid/biomass) H_2SO_4	0.844 mol H2/mol glucose	[40]
Biscuit Industry waste	Bacillus subtilis	37°C/pH 6.5	Heat - acid (100°C-2h; pH 3-24h)	0.87 mol H2/mol glucose	[29]
Dairy anaerobic sludge	Acintobacter junii AH4	37°C/pH 7.5	100°C, 15 min	1.35 mol H2/mol glucose	This study

*OPEFB- oil palm empty fruit bunch

CONCLUSION

The present study concluded that the efficient H_2 producing bacteria can be enriched by heat shock pre-treatment method directly from the anaerobic sludge of the dairy industry. It was found to be the most effective method for enriching H_2-producing bacteria and suppressing H_2 consuming bacteria. Here, we isolated *Acinetobacter junii*

AH4 after heat shock-treatement which exhibited highest cumulative H_2 production and HY. Similarly, the optimum pH was found to be at 7.5 with 60% substrate concentration for higher bio H_2 production. Finally, *Acinetobacter junii* can be used for large scale H_2 production utilizing dairy waste water.

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