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Mesophilic Hydrogen Production by *Clostridium butyricum* strain TM-9A, an Alkaline-Tolerant Dark Fermentative Bacterium

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ABSTRACT

The present study reports hydrogen production potential by an alkaline-tolerant bacterium *Clostridium butyricum* strain TM-9A isolated from an estuarine river sediment sample and identified on the basis of 16S rRNA gene sequencing. Different process parameters such as initial pH, temperature and NaCl concentration affected the hydrogen production potential and growth of TM-9A strain in batch dark fermentation experiments. Glucose (10 g L^{-1}) was used as substrate for an optimization study. TM-9A strain was able to tolerate up to 16 g L^{-1} of NaCl. Strain TM-9A produced maximum hydrogen, 57.8 mmol L^{-1}, at an initial pH 8 under mesophilic conditions, i.e. in the absence of NaCl. Acetic and butyric acid were the major soluble metabolites detected at 12.32 and 11.61 mmol L^{-1}, respectively. Hydrogen yield was 2.0–2.1 mol H_2/mol glucose. Furthermore, the strain was also evaluated for its ability to utilize different carbohydrate-rich substrates like corn syrup (25.74 mmol L^{-1}), molasses (23.44 mmol L^{-1}) and starch (43.29 mmol L^{-1}), sucrose (31.45 mmol L^{-1}), and cellulose (4.16 mmol L^{-1}), respectively for hydrogen production.

Keywords: biohydrogen, carbohydrate, dark fermentation, substrates, volatile fatty acids
Abbreviations: FID, flame ionization detector; **GC**, gas chromatography; **OD**, optical density; **RID**, refractive index detector; **TCD**, thermal conductivity detector; **VFA**, volatile fatty acid

INTRODUCTION

Global warming and depletion of non-renewable resources require development of sustainable energy production (Seppala 2011). Therefore, renewable energy resources have received considerable attention due to the depletion of fossil fuel and environmental pollution (Kim *et al*. 2009). Furthermore, extensive combustion of fossil fuel is leading to negative impacts on the environment because of the emission of harmful pollutants. In this context, it is necessary to explore for eco-friendly alternative renewable energy sources. Hydrogen is considered to be an ideal future energy carrier as it has a high energy content of 122 KJ g^{-1}. It produces only water when it is combusted as a fuel or converted to electricity (Kim *et al*. 2009).

Traditionally, hydrogen can be produced by thermo-chemical or electrochemical processes. However, biological hydrogen production routes are more eco-friendly and less energy intensive than thermo-chemical and electrochemical processes. Dark fermentative hydrogen production has several advantages as it can produce hydrogen in the absence of light from diverse substrates, including organic wastes. Currently, the dominant cost element in fermentative hydrogen production is the substrate (Ren *et al*. 2010). Therefore, hydrogen production from cheap and renewable sources or substrates using dark fermentation appears to be the most attractive method for hydrogen production. Although the rate of dark fermentative hydrogen production is faster than photosynthetic, it still requires further improvement for commercial exploitation (Zhao 2012). Further more renewable energy sources, such as cellulose, lignocellulose or starch containing biomass constitute abundant, inexpensive and reliable raw materials for bio-hydrogen production and offer considerable advantages (Argun *et al*. 2008; Kotay and Das 2008).

Microorganisms which can produce hydrogen are anaerobes includes photosynthetic and non-photosynthetic anaerobic bacteria (Nath and Das 2004; Schutz *et al*. 2004). Microorganisms which are known to produce hydrogen through a dark fermentation route include species of *Clostridium*, *Enterobacter*, *Bacillus* and *Thermoanaerobacterium* (Das and Veziroglu 2001; Kadpan and Kargi 2006; Kotay and Das 2007). Hydrogen production through dark fermentation of biomass or carbohydrate-based substrates (Ciranna *et al*. 2012) present a more feasible and promising route for hydrogen production because of their higher production efficiency, stability, simple control requirements, lower operating costs and no requirement of light as energy source (Hallenbeck and Benemann 2002; Levin *et al*. 2004; Wang and Wan 2008).

Among a large number of microbial species, pure cultures of the genera *Enterobacter*, *Bacillus*, and *Clostridium* are the most efficient producers of hydrogen (Nandi and Sengupta 1998; Kaushik *et al*. 2006). However, earlier studies on fermentative hydrogen production paid more attention to *Clostridium* sp. as one of the key hydrogen producers (Jo *et al*. 2007). Several species of the genus *Clostridium*, namely *C. butyricum* (Yokoi *et al*. 1998; Chen *et al*. 2005), *C. acetobutylicum* (Chin *et al*. 2003), *C. saccharoperbutylacetonicum* (Ferchichi *et al*. 2005) and *C. pasteurianum* (Lin and Lay 2004; Liu and Shen 2004) are reported to produce hydrogen from different substrates (Hawkes *et al*. 2007). *Clostridium* species are spore-forming anaerobic organisms capable of converting hexose to hydrogen with a yield of 4 mol of H_2/mol hexose, which is higher than the hydrogen yield of other dark fermentative bacteria such as *Enterobacter* sp. (Kapdan and Kargi 2006). Reports are also available on the use of pure or mixed cultures for biohydrogen production using sugars or complex substrates such as organic wastes (Mohan *et al*. 2007). However, glucose is

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the most preferred carbon source for dark fermentation processes, which predominantly produces acetic acid and/or butyric acid and hydrogen gas, according to the following reactions (Classen *et al*. 1999; Kaushik *et al*. 2006).

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (1)$$

$$C_6H_{12}O \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \quad (2)$$

From the above reactions, it is evident that a maximum of 4 mol H_2/mol glucose could be obtained with acetic acid as the end product, and 2 mol H_2/mol of glucose is obtained with butyrate as the fermentation end product. Thus, the highest theoretical yields of hydrogen are associated with acetate as the fermentation end product. However, in practice, high hydrogen yields are associated with a mixture of acetate and butyrate fermentation products (mixed fermentation pathway), and low hydrogen yields are associated with propionate and reduced end-products such as alcohols and lactic acid (Levin *et al*. 2004).

The optimization of fermentation conditions, particularly nutritional and environmental parameters are of primary importance for bioprocess development and improved hydrogen production. Dark fermentative hydrogen production can be affected by parameters such as initial pH, temperature, type of substrate, etc. Therefore, it is important to optimize the fermentation conditions for dark fermentative bacteria to improve hydrogen production. There are many reports available on the isolation of hydrogen-producing microbes from various sources, whereas only a few reports are available on the isolation of hydrogen-producing microbes from river sediments (Zuo *et al*. 2005). In this paper we report the isolation and identification of a hydrogen-producing bacterium from estuarine river sediment samples contaminated with high organic material. Investigations were also made to evaluate the effect and optimization of different process parameters such as initial medium pH, temperature, salinity, and different carbon sources on fermentative hydrogen production.

MATERIALS AND METHODS

Environmental sample

The estuarine sediment sample was collected from a river located in Mangalore, India [14° 41′ N 74° 40′ E]. The sampling site was highly contaminated with high organic pollutants or contents. The sample was transferred to the laboratory and stored below 10°C for further experiments. Before proceeding with enrichment, the sediment sample was heated at 90°C for 1 h to eliminate non-spore forming microbes and to inactivate the hydrogen consuming methanogenic microflora as per the method described earlier (Chen *et al*. 2005; Zuo *et al*. 2005; Singh *et al*. 2010).

Chemicals and reagents

Analytical grade chemicals (boric acid, magnesium chloride, dipotassium hydrogen ortho-phosphaste, zinc sulphate, citric acid, sodium hydroxide, D-glucose, magnesium chloride, manganous sulphate monohydrate, cuprous chloride, iron chloride, and analytical grade hydrochloric acid) were purchased from the Thermo Fischer Scientific (Mumbai, India). Analytical grade starch soluble, nitriloacetic acid, agar powder, cobaltous chloride, cellulose, and bacteriological grade peptone, yeast extract powder were purchased from Himedia Laboratories (Mumbai, India). Analytical grade purified potassium dihydrogen phosphate, and pure sodium molybdate were purchased from the Merck Specialities (Mumbai, India). Analytical grade nickel chloride and aluminium potassium sulphate were purchased from Qualigens Fine Chemicals (Mumbai, India). Folic acid, riboflavin, and para-amino-benzoic acid (PABA) were purchased from the Central Drug House (New Delhi, India). Molasses was obtained from Dhampur Sugar Mills (New Delhi, India).

Anaerobic medium preparation

BSH (Basal solution for hydrogen production) medium (Wang *et al*. 2007) consisted of: peptone (4 g L^{-1}); yeast extract (2 g L^{-1}); NaCl (2 g L^{-1}); K_2HPO_4 (1.5 g L^{-1}); KH_2PO_4 (1.5 g L^{-1}); 10 mL of each trace element and vitamin solution was used for enrichment, isolation of strain TM-9A and for hydrogen production study. The trace element solution was composed of: $MnO_4 \cdot 7H_2O$ (10 mg L^{-1}); $ZnSO_4 \cdot 7H_2O$ (50 mg L^{-1}); H_3BO_3 (10 mg L^{-1}); $N(CH_2COOH)_3$ (4500 mg L^{-1}); $CaCl_2 \cdot 2H_20$ (10 mg L^{-1}); Na_2MoO_4 (10 mg L^{-1}); $CoCl_2 \cdot 6H_2O$ (200 mg L^{-1}); $ALK[SO_4]_2$ (10 mg L^{-1}); $MgCl \cdot 6H_2$ (200 mg L^{-1}); $FeCl_3$ (100 mg L^{-1}); $CuCl_2 \cdot 6H_2O$ (50 mg L^{-1}). The vitamin solution was composed of: riboflavin (25 mg L^{-1}); citric acid (20 mg L^{-1}); folic acid (10 mg L^{-1}); and para-amino-benzoic acid (10 mg L^{-1}). BSH medium was supplemented with glucose (10 g L^{-1}) as the carbon source. The initial pH of the medium was adjusted to pH 7.2 ± 0.2 with 1N NaOH or 1N HCl at room temperature before autoclaving the medium. BSH medium was heated and spurged under a continuous stream of O_2-free N_2 gas (Laser gas, Hariyana, India) for 45 min. A 30 mL aliquot of medium was anaerobically dispensed into 67 mL of serum bottles pre-spurged with N_2 gas. The serum bottles were then closed with a butyl rubber septum and sealed with an aluminum cap and autoclaved for 15 min at 121°C at 15 lbs. Prior to inoculation, 10% sterilized glucose stock solution was added to the medium at a final concentration of 10 g L^{-1} with a sterile disposable syringe.

Enrichment, isolation and strain identification

The pre-treated sediment sample was dissolved in sterile normal saline (0.90% w/v) prepared in double distilled water. Then 1-2 mL of dissolved sample was inoculated into liquid medium with the help of a sterile disposable syringe, as described above. All the culture bottles were incubated at 37°C for 2-3 days depending on growth. Sub-culturing was done in the same medium after 3 days of incubation at 37°C to maintain an enriched culture. Furthermore, isolation and purification was performed by employing roll-tube method (Hungate 1969) using BSH medium solidified with 1.4% agar. Enriched cultures were serially diluted and 50 µl of serially diluted cultures (10^{-5} and 10^{-7} dilutions) were spread on anaerobically prepared roll tubes as described above. The roll tubes were incubated at 37°C for 2-3 days until single isolated colonies appeared on the agar medium. Single colonies obtained were picked with a sterile loop and transferred to liquid BSH medium (as explained above) and incubated under the same conditions as described above for enrichment. This procedure was repeated three times to ensure the purity of the strain. The purity of the isolate was routinely checked by Gram staining under a light microscope (BX41TF, Olympus, Tokyo, Japan). Among the isolates the strain with highest hydrogen production activity was selected for further study. Phenotyphic characterization of the isolate was performed under a light microscope (BX41TF, Olympus). The Gram type of the strain was studied using a Gram staining kit as per the protocol described by the manufacturer (Himedia Laboratories, Mumbai, India). RNA-free genomic DNA was extracted from cells with a standard method (Sambrook *et al*. 2001). Purified genomic DNA was used as template for the approximate 1.5 Kb size of 16S rDNA gene amplification by using a set of universal primers; 27F (5′-AGATTGATCMTGGCTAGGGA-3′) and 1492R (5′-TACGGY TACCTTGTTACGCTT-3′) as described (Lane 1991). PCR was performed in a DNA thermal cycler (MJ Research, USA). The amplified PCR product after analysis on agarose gel (1.2%) electrophoresis, was purified using a gel purification kit as per the manufacturer's instruction (QIAgen gel extraction kit, Valencia, Canada). Sequencing was done using the DyeDeoxy Terminator Cycle sequencing Kit (Applied Biosystems, UK) as per the manufacturer's instructions with an automatic DNA sequencer (Model 300; Applied Biosystems, USA). Nucleotide sequence was analysed using Finch TV software (Geospiza, Seattle). To identify the bacterial isolate, the gene sequence obtained was compared with reference sequences as available in the NCBI database, using the Basic Local Alignment Search Tool (Altschul *et al*. 1997).

Hydrogen production by *C. butyricum* TM-9A strain in batch fermentation

TM-9A strain was cultured in BSH medium for individual batch fermentation experiments. The batch experiments were set up in a 67 mL serum bottle containing 10 mL of liquid BSH medium and incubated at 37°C for 24 h. Anaerobic condition was maintained by spurging the medium with O_2-free nitrogen gas. Effect of process parameters such as initial medium pH (5 to 9 at an interval of 0.5), temperature (25, 30, 37, 47, 50 and 55°C) and NaCl concentration (0, 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 g L^{-1}) (w/v) on dark fermentative hydrogen production and growth by TM-9A, was investigated. Carbon sources; sucrose (10 g L^{-1}), starch (10 g L^{-1}), cellulose (10 g L^{-1}), corn syrup (10 g L^{-1}) and molasses (10 g L^{-1}), were separately investigated as substrates for hydrogen production. After incubation and growth, 0.5 mL of headspace gas samples was analysed by gas chromatography for hydrogen gas production. All the experiments were performed in triplicate, and mean value and standard deviation was calculated as described next in the text (http://easycalculation.com/statistics/standard-deviation.php).

Analytical methods

Bacterial growth density in individual batch experiments was determined by monitoring optical absorbance at 600_{nm} using a spectrophotometer (Hitachi U-2000 UV spectrophotometer, Tokyo, Japan). Headspace gas composition in the serum bottle was analyzed by gas chromatography (GC 6890N, Agilent Technologies, USA) equipped with thermal conductivity detector (TCD) and HP PLOTQ column (dimension 15 m × 53 µm × 40 µm). Nitrogen gas was used as the carrier gas as described in our previous study (Jayasinghearachchi *et al*. 2010). Volatile fatty acids were quantified with GC 6890N (Agilent Technologies, USA) equipped with the flame ionization detector (FID) connected to DB-WAXetr column (30 m × 530 µm × 1 µm), as described in our previous study (Jayasinghearachchi *et al*. 2010). High Performance Liquid Chromatography (HPLC, Agilent Technologies 1100 series, USA) equipped with Sugar- PAK.1 (Waters, Massachusetts, USA) column and refractive index detector (RID) was used for analysis of residual unutilized sugars and ethanol production (Jayasinghearachchi *et al*. 2010). Water was used as mobile phase at a flow rate of 0.6 ml/min.

Experimental design and statistical analyses

Standard deviation is widely used measure of variability in data set. A low standard deviation indicates that the data points tend to be very close to the mean, whereas high standard deviation indicates that the data points are spread out over a large range of values. In this study all the experiments were performed in triplicates for each parameter. To detect the significance difference and confidence in readings of each experimental set; data was statistically analyzed by calculating the mean and standard deviation. Easycalculation.com online tool was used to calculate the mean and standard deviation (http://easycalculation.com/statistics/standard-deviation.php).

RESULTS AND DISCUSSION

Identification and characterization of the hydrogen producing strains

TM-9A strain was isolated from the sample collected from estuarine river sediment, contaminated with high organic contents. The isolate grew well under strict anaerobic condition. Morphologically strain was found to be rod shaped bacterium. The cell ends were round in shape and spore forming. The cells were found to be gram positive in nature. 16S rRNA gene sequence analysis of strain revealed maximum homology towards *C. butyricum* ATCC 19398^T (99.75%) and *C. butyricum* DSM 523^T (syn. *C. kainanto*) (99%). Thus, TM-9A isolate was identified as a new strain within the species *C. butyricum* based on morphological and 16S rRNA gene sequence analysis (**Table 1**). TM-9A 16S rRNA gene sequence was deposited in the GenBank under the accession number FR734079.

Table 1 Best matches in GenBank database to the 16S rRNA gene sequence analysis of strain TM-9A.

Accession no.	Best BLAST match	Max. identity (%)	e-value
AB687551	*C. butyricum*	99	0.0
AB595129	*C. butyricum*	99	0.0
DQ831126	*C. butyricum* strain W4	99	0.0
AJ002592	*C. butyricum* MW8	99	0.0
X77834	*C. butyricum* DSM 523^T	99	0.0
AB075768	*C. butyricum* ATCC 19398^T	99	0.0

Table 2 Distribution of the acetate and butyrate (mean) during the hydrogen production (mean ± Standard Deviation, S.D) by *C. butyricum* TM-9A from various substrates under mesophilic condition.

Substrates *	Hydrogen[a]	OD[b]	VFAs[c]	
			Acetate	Butyrate
Starch	43.29 ± 1.96	3.812	8.94	6.53
Sucrose	31.45 ± 1.37	2.365	8.01	5.16
Corn syrup	25.74 ± 0.981	1.7786	8.16	3.83
Molasses	23.44 ± 1.25	-	6.76	5.47
Cellulose	4.16 ± 0.241	-	2.8	0.69

*Substrates (10 g L^{-1}); [a] hydrogen production; mmol L^{-1}; [b] optical density at 600_{nm}; [c] VFA mmol L^{-1}.

Effect of the initial pH on hydrogen production

Hydrogen fermentation pathways are sensitive to pH (Craven 1988). Previous studies reported that control of pH was crucial parameter for increased hydrogen production. Under unfavourable pH, hydrogen fermentation process shifts to solvent production pathway (Temudo *et al.* 2007) and prolongs the lag phase (Cheng *et al*. 2002; Liang 2003). Dark fermentative hydrogen production by TM-9A strain from glucose fermentation was observed to be pH dependent (**Fig. 1**). It was interesting to note that when initial pH was increased from 5.5 to 8.0, there was significant increase in total hydrogen production from 13.01 to 56.23 mmol L^{-1} (**Fig. 1**). Further, increase in initial pH from 8.0 to 9.0 resulted in slight decrease of hydrogen production (**Fig. 1**). Optimum hydrogen production (56.23 mmol L^{-1}) was obtained at pH 8.0. These results demonstrate that TM-9A strain could grow and produce hydrogen under moderately alkaline pH condition and has practical importance to produce hydrogen from wastes under alkaline conditions. Hydrogen production occurred in batch fermentation experiments till 30 h (data not shown). Further, incubation beyond 30 h did not increase the hydrogen production. The hydrogen production was accompanied with the production of acetic acid and butyric acid as the major soluble end products. These results demonstrate that TM-9A strain followed mixed fermentation pathway during fermentative hydrogen production from glucose. It was observed that acetic acid production was comparatively lower than butyric acid (**Table 2**).

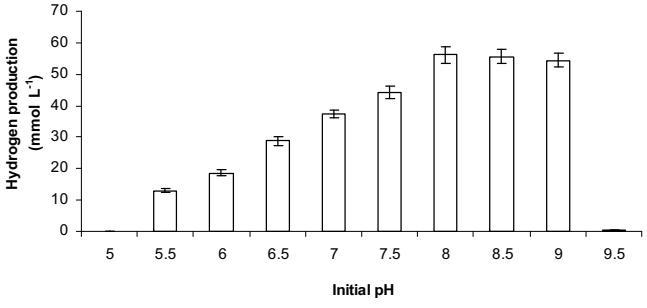

Fig. 1 Effect of different initial pH on fermentative hydrogen production (mean ± Standard Deviation, S.D.) from glucose by *C. butyricum* strain TM-9A from glucose fermentation.

Propionic acid and ethanol production was not detected during hydrogen production. These soluble end products known to inhibit hydrogen production and lower the total yield.

Hydrogen production efficiency of TM-9A strain is significantly higher than hydrogen production efficiency of *C. butyricum* CGS5 (88.1 mL L^{-1}) from rice husk hydrolyzate (Lo *et al*. (2008).

Influence of temperature on hydrogen production

Temperature affects hydrogen producing bacterial activities and hydrogen production rate (van Groenestijn *et al*. 2002; Nath *et al*. 2006). Dark fermentative hydrogen production can be operated at different temperatures: mesophilic (25-40°C), thermophilic (40-65°C), extreme thermophilic (65-80°C) or hyperthermophilic (>80°C) (Levin *et al*. 2004). Accordingly, batch experiments were carried out at various temperatures ranging from; 25, 30, 37, 47 and 50°C, to investigate their effect on hydrogen production performance of TM-9A strain. It was observed that TM-9A strain could produce hydrogen at 25, 30 and 37°C (**Fig. 2**). However, optimum hydrogen production was obtained at 37°C. It was interesting to note that significant amount of the hydrogen production also recorded at the lower temperature; 25 and 30°C (**Fig. 2**). However, hydrogen production was not detected at moderately thermohpilic conditions (47 and 50°C) (**Fig. 2**). These results are comparable and consistent with previously reported studies concerning hydrogen production from mesophilic microorganism (Mu *et al*. 2006; Wang and Wan 2008a; Akutsu *et al*. 2009).

Effect of NaCl concentration on hydrogen production

Salt is essential for cells, composed primarily of sodium chloride (Jayasinghearachchi *et al*. 2010). This study investigated the effect of different concentration of sodium chloride on growth and hydrogen production by TM-9A strain. Different batch experiments were carried out at various concentrations of NaCl ranging from 0, 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 g L^{-1} respectively. It was interesting to note that sodium chloride (NaCl) was not essential for growth and hydrogen production by TM-9A. TM-9A strain could produce hydrogen in the absence of NaCl. However, it was observed that gradual increase in NaCl concentration from 2 g L^{-1} to 20 g L^{-1} in the medium, adversely affected the growth and hydrogen production potential of TM-9A. With the increase in salinity percentage from 0 to 2%, hydrogen production decreased from 57.80 to 17.69 mmol L^{-1}, respectively during glucose fermentation under mesophilic conditions (**Fig. 3**). However, it was interesting to note that TM-9A strain could tolerate NaCl concentration up to 20 g L^{-1} and produce considerable hydrogen (**Fig. 3**). This property of strain to produce hydrogen under high salinity is of great importance and TM-9A strain can be explored further for waste treatment and hydrogen production from marine organic waste.

Dark fermentative hydrogen production from different carbon sources

It is important for hydrogen producing bacteria to use various types of substrates, especially complex carbohydrates (Thong *et al*. 2008). Hydrogen production performance of fermentative bacteria is known to be critically influenced by different carbon sources. In this study six different types of carbon based substrate; glucose, sucrose, starch, cellulose, corn syrup, and molasses, were selected and evaluated for hydrogen production by employing TM-9A strain.

TM-9A could efficiently utilize starch, sucrose, corn syrup and molasses for hydrogen production (**Fig. 4**). Starch is a major composition of agricultural by-products or crops such as corn, rice, potato, sweet potato, etc. (Lin *et al*. 2008). Previous studies have been conducted on starch-fermenting bacteria, such as *C. butyricum* and *E. aerogenes*, for fermentative hydrogen production (Yokoi *et al*. 1998). TM-9A strain could efficiently utilize and produce considerable hydrogen from starch (43.29 mmol L^{-1}), sucrose (31.45 mmol L^{-1}), corn syrup (25.74 mmol L^{-1}), and molasses (23.44 mmol L^{-1}) (**Fig. 4**). But, the property of using starch by TM-9A strain for hydrogen production is of great importance as starch being the major constituents in the corn syrup and/or molasses. These results demonstrate that TM-9A can be further explored for hydrogen production from other organic wastes. The strain could not grow on cellulose and hence no significant hydrogen production was obtained (**Fig. 4**). It was considered that cellulose is not suitable substrate for hydrogen production by TM-9A.

During the hydrogen fermentation pathway from different carbon sources, acetate and butyrate were detected as major soluble fermentation end products (**Table 2**). The results clearly demonstrate that hydrogen production by TM-9A probably occurred through mixed fermentation pathway (acetate and butyrate fermentation pathway).

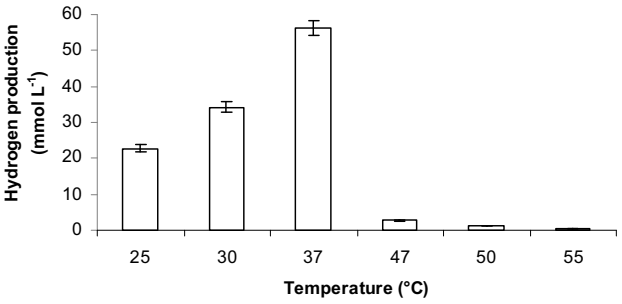

Fig. 2 Influence of temperature on hydrogen production (mean ± Standard Deviation, S.D.) by *C. butyricum* TM-9A from glucose fermentation.

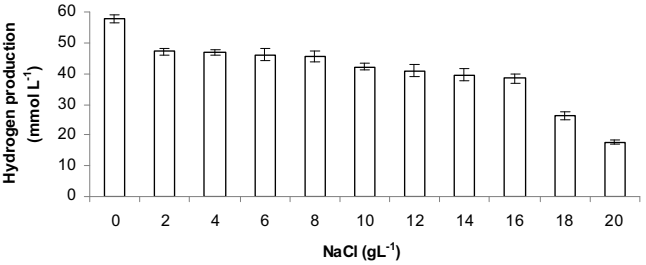

Fig. 3 Hydrogen production (mean ± Standard Deviation, S.D.) under different concentration of salt (NaCl) by *C. butyricum* TM-9A under mesophilic condition.

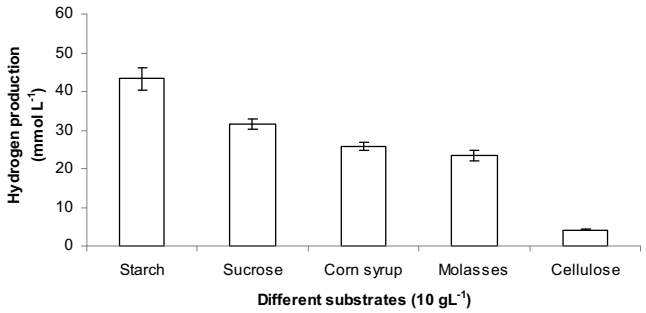

Fig. 4 Hydrogen production (mean ± standard deviation, S.D.) from the different carbon sources by *C. butyricum* TM-9A under mesophilic condition.

CONCLUSIONS

This study reports the hydrogen production potential of a newly isolated alkaline tolerant dark fermentative strain *C.*

butyricum TM-9A, (FR734079) isolated from estuarine river sediment samples. Morphologically the strain was rod shaped; spore forming gram positive bacterium. The strain was identified based on morphology and 16S rRNA gene sequence analysis. *C. butyricum* TM-9A produced maximum hydrogen of 58.8 mmol L^{-1} at initial pH 8 from glucose (g L^{-1}) fermentation under mesophilic conditions. This strain could tolerate wide range of NaCl concentrations from 2 g L^{-1} to 16 g L^{-1} and could produce considerable hydrogen at high salinity. However, it was interesting to note that NaCl was not essential for growth and hydrogen production. In addition to glucose, TM-9A strain could effectively utilize sucrose, starch, molasses, and corn syrup as substrates for hydrogen production. However, highest hydrogen production was observed from glucose as compared to other substrates investigated in this study. This study provides useful information for the use of abundant renewable energy resources like corn syrup, molasses etc. for dark fermentative hydrogen production as a clean fuel.

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