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UTILIZATION OF LACTIC ACID RICH MEDIUM AND CASSAVA STARCH WASTEWATER FOR BIOLOGICAL HYDROGEN PRODUCTION

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ABSTRACT: H_2 production from lactic acid rich medium and H_2 production from cassava starch wastewater by isolated bacteria were investigated. The isolation of H_2 producing bacteria from heat treated sludge was carried out. The H_2 production from lactic acid rich medium which used 1.5 g/L sodium acetate, 1.5 g/L sodium lactate and 1 g/L sucrose as carbon sources. The effects of inoculums size of 10, 20, 30 and 40 % (v/v) and incubation temperature of 35, 45 and 55ºC using cassava starch wastewater as the substrate on H_2 production by isolation strains were investigated. Results showed 6 isolated strains i.e. isolation code of MK01to MK06 indicated the H_2 production capability when cultured in lactic acid rich medium. However, 3 isolated strains i.e. MK01, MK04, and MK06 were preferred to use as seed inoculums for further experiments depend on a high H_2 production capacity obtained. Results indicated that the optimal condition of H_2 production from cassava starch wastewater was 30% (v/v) inoculum size of MK06 under 35ºC of incubation temperature. The maximum H_2 production potential, H_2 yield and H_2 production rate of 4.20 L H_2/L medium, 1.47 L H_2/g substrate consumed, and 14.00 mL H_2/L/h was obtained. Identification and characterization of MK06 were investigated using 16S rRNA gene sequence analysis followed by phylogenetic analysis. The 100% similarity of 16S rDNA compare with closely related species was indicated *Klebsiella pneumoniae* subsp. *pneumoniae* DSM 30104(T).

Keywords: Hydrogen production, Lactic acid rich medium, Cassava starch wastewater

1. INTRODUCTION

H_2 producing bacteria such Clostridiaceae can ferment the carbohydrates in agricultural wastewater to H_2, CO_2, and volatile organic acids – mainly acetic and butyric acids. However, these bacteria are not able to further break down the organic acids to H_2 due to the positive Gibbs free energy of the reaction [1]. The genus *Clostridium* i.e. *Clostridium butyricum*, *Clostridium diolis*, and *C. beijerinckii* have been reported their ability to produce hydrogen from a mixture of acetic acid and lactic acid [2]. A mole ratio of depletion of acetic acid to lactic acid of 1:2 could be obtained from the biological H_2 production from a mixture of acetic and lactic acids by *C. diolis* JPCC H-3 [2]. Cassava starch wastewater is an industrial residue composed mainly of lactic acid bacteria with excess of the genera *Lactobacillus*, and organic acids [3]. The cassava starch wastewater is excluded, and it is returned an effluent with high chemical oxygen demand [4]. However, this residue presents a motivating component for prospective use in innovative products. The cassava starch wastewater has a low pH and it is an organic acid rich wastewater [4]. The major organic acids include lactic acid, acetic acid, butyric acid, and other components include starch residual. In dark fermentative H_2 production, the presence of lactic acid as a metabolite during H_2 production is frequently regarded as a sign of lower H_2 production [5]-[7]. Lactic acid has been rarely studied as substrate for H_2 production and despite reports of CH_4 production from lactate [7], no considerable H_2 production was reported when lactate was used as the individual substrate [2].

Although the previous report on H_2 production from mixture of lactic acid and acetic acid by *Clostridium diolis* showed the promise on efficient H_2 production process in batch scale but the information on the H_2 production from lactic acid rich wastewater are still lacking. Therefore, this research aims to isolate H_2 producing bacteria which capable to use lactic acid as substrate for H_2 production. The effects of inoculum size and incubation temperature on H_2 production parameters were investigated. Means of H_2 production parameters which indicated the high efficiency of H_2 producing strain were selected to produce H_2 from cassava starch wastewater. The results from this study provided high efficiency of hydrogen producing strain and the optimum condition could improve hydrogen yield from lactic acid rich wastewater.

2. MATERIALS AND METHODS

2.1 Anaerobic Sludge

Sludge of an up flow anaerobic sludge blanket (UASB) was pretreated by the dry heating method under 105°C for 2 h for suppressing the methanogen. After heating, the UASB sludge was dispersed into an aqueous suspension by vortexing it in PBS buffer (pH 7.2, 10 mM Na_2HPO_4, and 0.13% NaCl) for 5 min. The suspension was diluted serially, plated on NB medium (pH 6.8, 0.3% beef extract, and 0.5% peptone) and incubated at 30°C under anaerobic conditions. After incubating for 24-36 h, colonies were selected randomly and transferred to fresh NB plates.

2.2 H_2 Production Confirmation

Each isolated bacterium obtained was used as seed inoculums for the H_2 production confirmation experiment. The experiment was accompanied in 25 mL serum flasks with a working volume of 15 mL. The H_2 production medium was contained 10% (v/v) inoculum. The H_2 production capability of isolated bacteria was performed by cultured in synthetic medium (M solution) [8]. Serum flasks were flushed with nitrogen gas to eliminate oxygen in headspace and to generate the anaerobic condition then incubated at room temperature. After 24 h, the total gas volume was measured by releasing the pressure in serum flasks using the wetted glass syringe.

2.3 H_2 Production from Lactic Acid Rich Medium by Isolated Bacteria

Isolated colonies indicated the H_2 production capacity obtained from 2.2 were cultured in a synthetic medium (M solution) [8]. H_2 production ability of all isolated bacteria was conducted in the same size of serum flasks with a liquid volume of 70 mL and 50 mL of headspace volume. The H_2 production capability of isolated bacteria was accomplished by cultured in lactic acid rich medium (M solution contained sodium acetate, 1.5 g/L sodium lactate and 1 g/L sucrose as carbon sources). The 10% (v/v) inoculum was inoculated into 120 mL serum flasks then incubated at room temperature and activated in an orbital shaker with a rotation speed of 120 rpm. The biogas volume was continuously measured and collected by gas-tight syringe for biogas composition analysis.

2.4 H_2 Production from Cassava Starch Wastewater by Isolated Bacteria

Isolated strains showed the high H_2 production capacity was enriched in lactic acid rich medium for 48 h under anaerobic condition before used as seed inoculums. The cassava starch wastewater used as sole substrate for H_2 production consisted of 2.78 g/L starch, 4.59 g/L volatile fatty acid i.e. 0.89 g/L acetic acid, 1.33 g/L lactic acid, 1.09 g/L propionic acid and 1.28 g/L butyric acid. The variation inoculums size of 10, 20, 30 and 40 % (v/v) was adjusted then the effect of incubation temperature at 35, 45 and 55 ºC on H_2 production from cassava starch wastewater by isolated bacteria was investigated. The difference inoculums size was inoculated into cassava starch wastewater in 25 mL serum flasks with a liquid volume of 70 mL under adjusted initial pH of 5.5. Serum flasks were flushed with N_2 to remove O_2 in the headspace and to create an anaerobic condition. The bottles were incubated at room temperature and operated in an orbital shaker with a rotation speed of 120 rpm to provide better contact among substrates. After 24 h, the total gas volume was measured by releasing the pressure in the bottles using wetted glass syringe. The 3 mL of biogas produced was collected into another serum bottle then measured the H_2 concentration by gas chromatography analysis.

2.5 Analytical Method and Kinetics Model

H_2 content was measured using a gas chromatograph under the same analytical condition as [9]. Helium was used as the carrier gas at a flow rate of 25 mL/min. H_2 production was calculated from the headspace measurement of gas composition and the total volume of H_2 produced, at each time interval, using the mass balance equation [9].

$$V_{H,i}= V_{H,i-1}+C_{H,i}(V_{G,i} - V_{G,i-1})+V_{H,0}(C_{H,i} - C_{H,i-1})$$

Where $V_{H,i}$ and $V_{H,i-1}$ are the cumulative hydrogen gas volumes at the current ($_i$) and previous time interval ($_{i-1}$), respectively; $V_{G,i}$ and $V_{G,i-1}$ are total biogas volume at the current and previous time interval; $C_{H,i}$ and $C_{H,i-1}$ are the fraction of hydrogen gas in the headspace at the current and previous time interval; V_H is the volume of the headspace of serum bottles. A modified Gompertz equation was used for adequate H_2 production profiles [9]. Total sugar, volatile fatty acid (VFAs) and dry cell weight (DW) are measured according to the procedures described in standard methods [10].

$$H=Pexp\{-exp[(R_me/P)(\lambda-t)+1]\}$$

Where H represents the cumulative volume of hydrogen produced (mL), P_S the hydrogen production potential (mL), R_m the maximum production rate (mL/h), λ the lag-phase time (h), t the incubation time (h), and e is 2.718281828.

Parameters (P_S, R_m and λ) were estimated using the solver function in Microsoft Excel version 5.0 (Microsoft, Inc.).

ANOVA was used for selecting forecasting H_2 producing strain isolate based on comparison of means between forecasting results; that is for testing a significant difference between group means [11]. When differences between groups exist, a post hoc test then conducted to identify which group differs from the others. In this paper, Duncan multiple range tests were used.

2.6 16S rDNA Sequencing

2.6.1 PCR amplification of 16S rDNA

DNA templates for PCR amplification were prepared by using "Genomic DNA mini kit (Blood/culture cell)" (Geneaid Biotech Ltd., Taiwan). DNA coding for 16S rRNA regions was amplified by means of PCR with Taq polymerase, as described by [12**]-[**13]. A PCR product for sequencing 16S rDNA regions was prepared by using the following two primers, 20F (5'-GAG TTT GAT CCT GGC TCA G-3', positions 9-27 on 16S rDNA by the *E. coli* numbering system and 1500R (5'-GTT ACC TTG TTA CGA CTT-3', position 1509-1492 on 16S rDNA by the *E. coli* numbering system [14]. The PCR amplification was carried out with DNA Engine Dyad® Thermal Cycler (Bio-Rad Laboratories). One hundred µl of a reaction mixture contained 15-20 ng of template DNA, 2.0 µm each of the two primers, 2.5 units of Taq polymerase, 2.0 mM $MgCl_2$, 0.2 mM dNTP and 10 µl of 10xTaq buffer, pH 8.8, containing $(NH_4)_2SO_4$, which was comprised of 750 mM Tris-HCl, 200 mM $(NH_4)_2SO_4$ and 0.1% Tween 20. The PCR amplification was programmed to carry out an initial denaturation step at 94ºC for 3 min, 25 cycles of denaturation at 94ºC for 1 min, annealing at 50ºC for 1 min and elongation at 72ºC for 2 min, followed by a final amplification step at 72ºC for 3 min. The PCR product was analyzed by 0.8% **(**w/v**)** agarose gel electrophoresis and purified with a GenepHlowTM Gel/PCR Kit (Geneaid Biotech Ltd., Taiwan). The purified PCR product was stored at -20ºC for further step.

2.6.2 Direct sequencing of 16S rDNA

Direct sequencing of the single-banded and purified PCR products (ca. 1500 bases, on 16S rDNA by the *E. coli* numbering system [14] was carried out. Then, sequencing of the purified PCR products was performed on an ABI Prism® 3730XL DNA Sequence (Applied Biosystems, Foster City, California, USA) by sequencing service provider. The two primers 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') or 800R (5'-TAC CAG GGT ATC TAA TCC-3') and 518F (5'-CCA GCA GCC GCG GTA ATA CG-3') or 1492R (5'-TAC GGY TAC CTT GTT ACG ACT T-3') for single strand 16S rDNA sequencing, and 4 primers of 27F, 518F, 800R and 1492R for double strands 16S rDNA sequencing were used 3 sequence analyses**.** The nucleotide sequences obtained from all primers were assembled using the Cap contig assembly program, an accessory application in BioEdit (Biological sequence alignment editor) Program. The identification of phylogenetic neighbors was initially carried out by the BLASTN [15] program against the database containing type strains with validly published prokaryotic names [16]. The top thirty sequences with the highest scores were then selected for the calculation of pairwise sequence similarity using global alignment algorithm, which was implemented at the EzTaxon-e server [16-17]. The DNA sequences determined and obtained from databases were aligned with a program CLUSTAL X (version 1.8) [18] in BioEdit Program [19]. Alignment gaps and unidentified bases were eliminated. The evolutionary distances were computed using the Maximum Composite Likelihood method [20]. Phylogenetic trees were constructed by the neighbor-joining method [21]. The robustness for individual branches was appraised by 100 replications bootstrapping [22] with the program MEGA version 6.0 [23].

3. RESULTS AND DISCUSSION

3.1 H_2 Production from Lactic Acid Rich Medium by Isolated Bacteria

The 6 colonies isolated from heat-treated sludge (isolation code MK01-MK06) were selected as the H_2 producing bacteria depend on H_2 production capability obtained. They were subsequently characterized by gram-staining, optical microscopy, and the colony shape. Results showed the negative gram, circular form and smooth surface of colonies with the different colonies edge and cell size. Based on the microscopic observation, each colony was characterized by rod shape and endospore formation. The H_2 fermentation of isolated strains was performed in batch experiments under anaerobic conditions and analyzed the H_2 production by the gas chromatography. The cumulative H_2 profile of isolated bacteria was shown in Fig. 1. It was found that each isolate obtained showed the H_2 production capacities when cultured under an adjusted condition. H_2 production from lactic acid rich medium by isolated bacteria was performed in batch fermentation. The inoculum was cultivated in the serum bottle and transferred anaerobically during the late-exponential phase by a sterile hypodermic disposable syringe into another serum bottle containing a lactic acid rich medium. The lactic acid rich medium using 1.5 g/L sodium acetate, 1.5 g/L sodium lactate and 1 g/L sucrose as

carbon sources was prepared for H_2 production test for each isolate. Table 1 showed that all the isolates produced the amounts of H_2 until 120 h of incubation time. The highest amounts of the H_2 production potential and H_2 yield (170.18 mL/L and 3.98 mL/g DW/L) obtained from MK01.

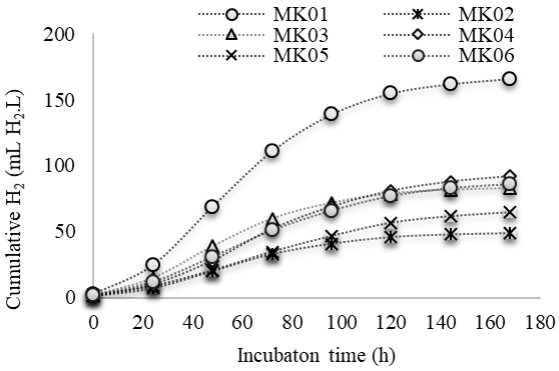

Fig. 1 Cumulative H_2 profile of all isolated bacteria.

The summarizes the batch fermentation results and volatile fatty acids produced with the new isolates when 1.5 g/L sodium acetate, 1.5 g/L sodium lactate and 1 g/L sucrose were used as carbon sources (Table 2).

Table 1 H_2 production from lactic acid rich medium by isolated bacteria.

Isolation code	H_2 production			H_2 yield* (mL/g DW.L)
	*P*s (mL/L)	R_m (mL/L.h)	λ (h)	
MK01	170.18[a]	0.44[d]	21.78[a]	3.98[a]
MK02	50.58[f]	0.58[c]	12.96[c]	0.92[c]
MK03	84.95[d]	1.05[a]	10.72[d]	1.95[bc]
MK04	96.99[b]	0.94[b]	16.31[b]	2.27[b]
MK05	70.98[e]	0.51[c]	16.02[b]	1.63[bc]
MK06	90.90[c]	0.85[b]	11.55[c]	2.09[b]

*H_2 yield (milliliter H_2 per gram of cell dry weight per liter of medium)

High lactate and acetate of 1.5 g/L and a relatively low glucose concentration of 5 g/L were employed in this experiment because the medium was performed as lactic acid rich medium. The increase in total volatile fatty acid produced was obtained from almost isolated strain.

3.2 Factors Affecting on H_2 Production from Cassava Starch Wastewater by Isolated Bacteria

The study of the effects of inoculums size on H_2 production from cassava starch wastewater by isolated bacteria was performed in batch experiment using 25 mL serum bottles. Three isolated strains i.e. MK01, MK04, and MK06 were preferred to use as seed inoculums depend on high H_2 production capacity (Table 2). The H_2 production capability of isolated bacteria was performed by adjusted initial pH of 5.5. The initial concentration of starch in cassava starch wastewater was 4.63 g/L and total volatile fatty acids including acetic acid, lactic acid, propionic acid, and butyric acid were also used as carbon source to produce H_2.

Table 3 indicated that increasing inoculums size of MK01 was improved biogas and H_2 production including content, *P*s and yield. The maximum *P*s and H_2 yield of MK01 was 836.8 mL/L and 255.9 mL H_2/g substrate consumed obtained from 40% (v/v) of inoculums size. On the other hand, biogas and H_2 production were suppressed by the increasing in inoculums size of MK04. The maximum *P*s and H_2 yield of MK04 was 804.2 mL/L and 175.2 mL H_2/g substrate consumed obtained from 10% (v/v) of inoculums size. However, the optimum of inoculums size in this experiment was 30% (v/v) of MK06 caused the highest *P*s and H_2 yield of 1033.3 mL/L and 467.5 mL H_2/g substrate consumed. Results suggested that *P*s obtained from MK06 was 5 and 6.6 folds greater than MK01 and MK04, respectively. In addition, optimum H_2 yield of MK06 obtained caused 1.3 and 2.7 folds greater than MK01 and MK04, respectively (Table 3).

Table 2 Summarizes the batch fermentation results of H_2 production from lactic acid rich medium by isolated bacteria.

Isolation code	Dry cell weight (g/L)		TVFAs* (g/L)		Volatile fatty acid composition (g/L)							
					Acetic acid		Lactic acid		Propionic acid		Butyric acid	
	Init.	final	Init.	final	Init.	final	Init.	final	Init.	final	Init.	final
MK01	13.3	11.9	5.0	11.2	1.0	2.2	1.4	3.2	1.2	2.7	1.4	3.2
MK02	14.8	14.7	5.0	14.3	1.0	2.8	1.4	4.1	1.2	3.4	1.4	4.0
MK03	13.6	12.8	6.5	15.9	1.2	3.1	1.9	4.6	1.6	3.8	1.8	4.5
MK04	12.6	10.7	5.0	14.3	1.0	2.8	1.4	4.1	1.2	3.4	1.4	4.0
MK05	13.3	11.9	6.5	12.8	1.2	2.5	1.9	3.7	1.6	3.0	1.8	3.6
MK06	13.4	10.8	11.2	14.3	2.1	2.8	3.2	4.1	2.7	3.4	3.2	4.0

*TVFAs is total volatile fatty acids; Init. is in the initial of fermentation; Final is in the final of fermentation.

Results suggested that MK01 has a higher ranging of starch consumed than MK04 and MK06. The optimum condition for substrate consumption was under 30% (v/v) inoculums size of MK01 that illustrated 99. 54% substrate consumption efficiency. Results also noteworthy that the low substrate consumption efficiency of MK04 and MK06 might be indicated the different of H_2 producing bacteria and starch might adversely affect the biodegradability.

Table 3 Effects of inoculums size on H_2 production from cassava starch wastewater.

Isolation code	inoculums size (% v/v)	H_2 Production		
		Content (% v/v)	*Ps* (mL/L)	Yield (mL H_2 /g substrate consumed)
MK01	10	2.71^{d}	207.86^{d}	70.94^{d}
	20	0.30^{fg}	15.66^{j}	3.38^{gh}
	30	0.32^{fg}	18.25^{i}	4.25^{fg}
	40	10.50^{b}	836.85^{b}	255.92^{b}
MK04	10	9.88^{c}	804.23^{c}	175.21^{c}
	20	0.70^{ef}	44.94^{f}	12.15^{e}
	30	0.89^{e}	48.41^{e}	13.45^{e}
	40	0.55^{ef}	27.55^{g}	11.34^{e}
MK06	10	0.37^{efg}	20.57^{h}	7.27^{f}
	20	-	-	-
	30	11.38^{a}	1033.30^{a}	467.56^{a}
	40	-	-	-

Kapdan and Kargi [24] reported that many agricultural and food industry wastes contain starch may adversely affect the biodegradability. Starch containing solid wastes is easier to process for carbohydrates and H_2 formation. Starch can be hydrolyzed to glucose and maltose by acid or enzymatic hydrolysis followed by conversion of carbohydrates to organic acids and then to H_2 [24]. However, other organic components in cassava starch wastewater could be used as the substrates to produce H_2 by isolated H_2 producing bacteria in this study.

The effect of incubation temperature on H_2 production from cassava starch wastewater by isolated bacteria was investigated after the optimum inoculums size obtained. Table 3 indicated the optimum of inoculums size was 30% (v/v) of MK06. H_2 producing bacteria strain MK06 was used as seed inoculums at the inoculums size of 30% (v/v) for H_2 production from cassava starch wastewater under various incubation temperatures of 35, 45 and 55ºC. Table 4 showed the effect of incubation temperature on H_2 production from cassava starch wastewater by H_2 producing bacteria strain MK06 at the optimum inoculum size (30% v/v). Results illustrated that the maximum cumulative H_2, yield and production rate of 4.20 L H_2/L medium, 1.47 L H_2/g substrate consumed, and 14.00 mL H_2/L/h were obtained under incubation temperature of 35ºC.

Table 4 Effects of incubation temperatures on H_2 production from cassava starch wastewater by MK06.

Incubation temp.	H_2 Production by MK06 at 10% (v/v) of inoculums size		
	Ps (L H_2/L medium)	Yield (L H_2 /g substrate consumed)	H_2 production rate (mL H_2/L.h)
35°C	4.20^{a}	1.47^{a}	14.00^{a}
45°C	2.89^{c}	0.99^{c}	9.30^{b}
55°C	3.78^{b}	1.26^{b}	12.70^{a}

3.3 16S rDNA and Phylogenetic Analysis of Isolated H_2 Producing Bacteria

Isolation of new H_2 producing bacteria with high capacity or unique properties in H_2 production was investigated using 16S rRNA gene sequence analysis. H_2 producing bacteria strain MK06 was preferred for the identification of the new isolates via sequencing of the full 16S rDNA followed by phylogenetic analysis. Results showed the 100% similarity of 16S rDNA compares with closely related species was indicated *Klebsiella pneumoniae* subsp. *pneumoniae* DSM 30104(T) reported by Schroeter (1886) and Trevisan (1887). In order to better understand the process, the microbial identification was investigated and linked to the H_2 production performance. The used method for microbial characterization in H_2 production is 16S rRNA based DGGE combined with sequencing and similarity-based phylogenetic analysis [25]. The DNA sequences determined and obtained from databases were aligned with a program CLUSTAL X (version 1. 8) [18] in BioEdit Program [19] . Alignment gaps and unidentified bases were eliminated. The evolutionary distances were computed using the Maximum Composite Likelihood method [20] . Phylogenetic trees of 16S rRNA genes were constructed by the neighbor-joining method of [21] which shown in Fig. 2. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [19]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method [21] and are in the units of the

number of base substitutions per site. The analysis involved 11 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1246 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [20].

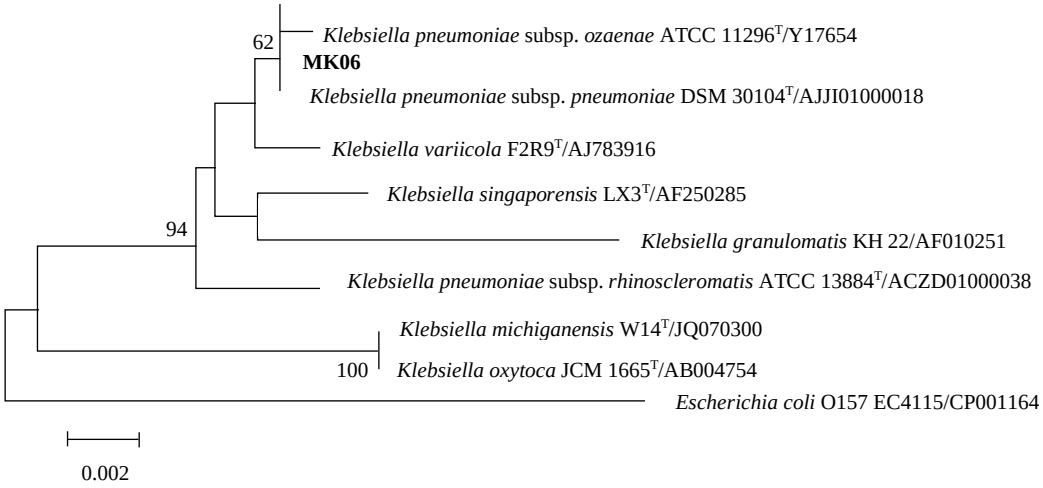

Fig. 2 The phylogenetic relationships based on 16S rDNA.

4. CONCLUSION

The 6 isolated bacteria i.e. isolation code of MK01-06 indicated the H_2 production capability when cultured in lactic acid rich medium. MK01, MK04, and MK06 were selected to use as seed inoculum for H_2 production from cassava starch wastewater which consisted of starch and volatile fatty acid i.e. lactic acid and acetic acid in composition. The optimal condition of H_2 production from cassava starch wastewater was 30% (v/v) of MK06 as inoculum under 35ºC which obtained the maximum *P*s, H_2 yield and H_2 production rate of 4.20 L H_2/L medium, 1.47 L H_2/g substrate consumed, and 14.00 mL H_2/L/h, respectively. Identification and characterization of MK06 indicated that 100% similarity of 16S rDNA compares with closely related species was indicated *Klebsiella pneumoniae* subsp. *pneumoniae* DSM 30104(T).

5. ACKNOWLEDGMENTS

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6. REFERENCES

[1] Baghchehsaraee B., Nakhla G., Karamanev D., and Margaritis A., Effect of extrinsic lactic acid on fermentative hydrogen production, International Journal of Hydrogen Energy, Vol. 34, Issue 6, 2009, pp. 2573-2579.

[2] Matsumoto M., and Nishimura Y., Hydrogen production by fermentation using acetic acid and lactic acid, Journal of Bioscience and Bioengineering, Vol. 103, Issue 3, 2007, pp. 236-241.

[3] Avancini S.R.P, Faccin G.L., Vieira M.A., Rovaris A.A., Rossana Podestá, Ricardo Tramonte, de Souza N.M.A., and Amante Edna R., Cassava starch fermentation wastewater: Characterization and preliminary toxicological studies, Food and Chemical Toxicology, Vol. 45, Issue 11, 2007, pp. 2273-2278.

[4] Pereira J.M., Sena Aquino A.C.M., Oliveira D.C., Francisco G.R.A., Barreto P.L.M., and Amante E.R., Ciencia Rural, Vol. 46, Issue 4, 2016, pp. 732-738.

[5] Saraphirom P., and Reungsang A., Biological hydrogen production from sweet sorghum syrup by mixed cultures using an anaerobic sequencing batch reactor (ASBR), International Journal of Hydrogen Energy, Vol. 36, Issue 14, 2011, pp. 8765 – 8773.

[6] Baghchehsaraee B., Nakhla G., Karamanev D., Margaritis A., and Reid G., The effect of heat pretreatment temperature on fermentative hydrogen production using mixed cultures, International Journal of Hydrogen Energy, Vol. 33, Issue 15, 2008, pp. 4064–4073.

[6] Noike T., Takabatake H., Mizuno O., and Ohba M., Inhibition of hydrogen fermentation of organic wastes by lactic acid bacteria, International Journal of Hydrogen Energy, Vol. 27, Issue 11-12, 2002, pp. 1367-1371.

[7] Nishio N., and Nakashimada Y., High rate production of hydrogen/methane from various substrates and wastes, Advances in Biochemical Engineering/Biotechnology, Vol. 90, 2004, pp. 63-87.

[8] Shiratori H., Sasaya K., Ohiwa H., Ikeno H., Ayame S., Kataoka N., Miya N., Beppu T., and Ueda K., Clostridium clariflavum sp. nov. and Clostridium caenicola sp. nov., moderately thermophilic, cellulose-/cellobiose-digesting bacteria isolated from methanogenic sludge, International Journal of Systematic and Evolutionary Microbiology, Vol. 59, Issue 7, 2009, pp. 1764–1770.

[9] Saraphirom P., and Reungsang A., Optimization of biohydrogen production from sweet sorghum syrup using statistical methods, International Journal of Hydrogen Energy, Vol. 35, Issue 24, 2010, pp. 13435-13444.

[10] A.P.H.A., Stand Methods for the Examination of Water and Wastewater, 19th ed. Washington DC: American Public Health Association, 1995, pp.1-541.

[11] Permanasari A. E., Rambli D. R. A., and Dominic P. D. D., Forecasting method selection using ANOVA and Duncan multiple range tests on time series dataset, 2010 International Symposium on Information Technology, Kuala Lumpur, 2010, pp. 941-945.

[12] Yamada Y., Katsura K., Kawasaki H., Widyastuti Y., Saono S., Seki T., UchimuraT., and Komagata K., Asaia bogorensis gen. nov., sp. nov., an unusual acetic acid bacterium in the α-Proteobacteria, International Journal of Systematic and Evolutionary Microbiology, Vol. 50, 2000, pp. 823–829.

[13] Katsura K., Kawasaki H., Potacharoen W., Saono S., Seki T., Yamada Y., Uchimura T., and Komagata K., Asaia siamensis sp. nov., an acetic acid bacterium in the α-Proteobacteria, International Journal of Systematic and Evolutionary Microbiology, Vol. 51, 2000, pp. 559–563.

[14] Klappenbach J.A., Saxman P.R., Cole J.R., and Schmidt T.M., rrndb: Ribosomal RNA operon copy number database. Nucleic Acids Research, Vol. 29, Issue 1, 2001, pp. 181-184.

[15] Jones D.T., and Swindells M.B., Getting the most from PSI–BLAST. TRENDS in Biochemical Sciences, Vol. 27, Issue 3, 2002, pp. 161-164.

[16] Kim O.S., Cho Y.J., Lee K., Yoon S.H., Kim M., Na H., Park S.C., Jeon Y.S., Lee J.H., Yi H., Won S., and Chun J.S., Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. International Journal of Systematic and Evolutionary Microbiology, Vol. 25, 2012, pp. 716–721.

[17] EzBioCloud, Search EzBioCloud Database. 2016. Available from: http://eztaxon-e.ezbiocloud.net

[18] Larkin M.A., Blackshields G., Brown N.P., Chenna R., McGettigan P.A., McWilliam H., Valentin F., Wallace I.M., Wilm1 A., Lopez R., Thompson J.D., Gibson T.J. and Higgins D.G., Sequence analysis Clustal W and Clustal X version 2.0. Bioinformatic Application Note, Vol. 23, Issue 21, 2007, pp. 2947–2948.

[19] Hall T., BioEdit: An important software for molecular biology, GERF Bulletin of Biosciences, Vol. 2, Issue 1, 2011, pp. 60-61.

[20] Tamura K., Nei M., and Kumar S., Prospects for inferring very large phylogenies by using the neighbor-joining method, Proceedings of the National Academy of Sciences (USA), 2004, pp. 11030-11035.

[21] Segata N., Börnigen D., Morgan X. C., and Huttenhower C., PhyloPhlAn is a new method for improved phylogenetic and taxonomic placement of microbes. Nature Communication, 2013, DOI: 10.1038/ncomms3304, pp.1-22.

[22] Shimodaira H., and Hasegawa M., CONSEL: for assessing the confidence of phylogenetic tree selection. Bioinformatic Application Note, Vol. 17, Issue 12, 2001, pp. 1246–1247.

[23] Tamura K., Stecher G., Peterson D., Filipski A., and Kumar S., MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0, Molecular Biology and Evolution, Vol. 30, Issue 12, 2013, pp. 2725–2729

[24] Kapdan I.K., and Kargi F., Review Bio-hydrogen production from waste materials. Enzyme and Microbial Technology, Vol. 38, Issue 5, 2006, pp. 569-582

[25] Guo X.M., Trably E., Latrille E., Carrère H., and Steyer J.P., Hydrogen production from agricultural waste by dark fermentation: a review. International Journal of Hydrogen Energy, Vol. 35, Issue 19, 2010, pp. 10660-10673.