

## Biohydrogen production by *Thermoanaerobacterium thermosaccharolyticum* KKU-ED1: Culture conditions optimization using mixed xylose/arabinose as substrate

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### Abstract

**Background:** Biological hydrogen production by microorganisms can be divided into two main categories *i.e.* photosynthetic organisms that produce hydrogen using light as energy source and anaerobic bacteria that produce hydrogen via dark fermentation. Dark fermentative hydrogen production by anaerobic bacteria has the advantages of a higher HPR without illumination and of the capability to convert various kinds of substrate.

**Results:** Thermophilic hydrogen producer was isolated from elephant dung and identified as *Thermoanaerobacterium thermosaccharolyticum* KKU-ED1 by 16S rRNA gene analysis, which was further used to produce hydrogen from mixed pentose sugar *i.e.*, xylose/arabinose. The optimum conditions for hydrogen production from mixed xylose/arabinose by KKU-ED1 were a 1:1 xylose/arabinose mixture at the total concentration of 5 g/L, initial pH of 6.5 and temperature of 55°C. Under the optimum conditions, hydrogen from sugar derived from acid-hydrolyzed sugarcane bagasse at a reducing sugar concentration were achieved. Soluble metabolite product (SMP) was predominantly acetic acid indicating the acetate-type fermentation.

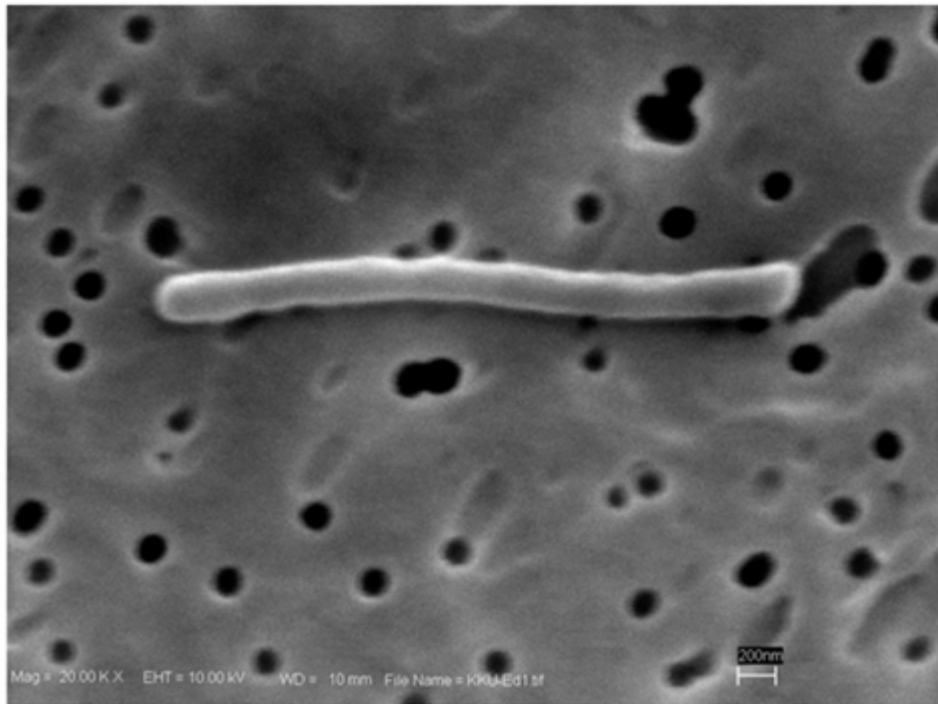
**Conclusions:** The strain KKU-ED1 appeared to be a suitable candidate for thermophilic fermentative hydrogen production from hemicellulosic fraction of lignocellulosic materials due to its ability to use various types of carbon sources.

**Keywords:** arabinose, biohydrogen, elephant dung, hydrolysate, thermophilic, xylose.

### INTRODUCTION

Hydrogen is considered to be the ultimate solution for clean and renewable energy due to its high energy content per unit (122 kJ/g) and non-polluting characteristics (water is the only by-product after its combustion) (Kim et al. 2009). Hydrogen can be produced via several methods, including electrolysis of water, thermo-catalytic reformation of hydrogen and biological processes (Kim et al. 2009). Biological hydrogen production by microorganisms can be divided into two main categories *i.e.* photosynthetic organisms that produce hydrogen using light as energy source and anaerobic bacteria that produce hydrogen via dark fermentation (Levin et al. 2004). Dark fermentative hydrogen production by anaerobic bacteria has the advantages of a higher HPR without illumination and of the capability to convert various kinds of substrate (Levin et al. 2004).

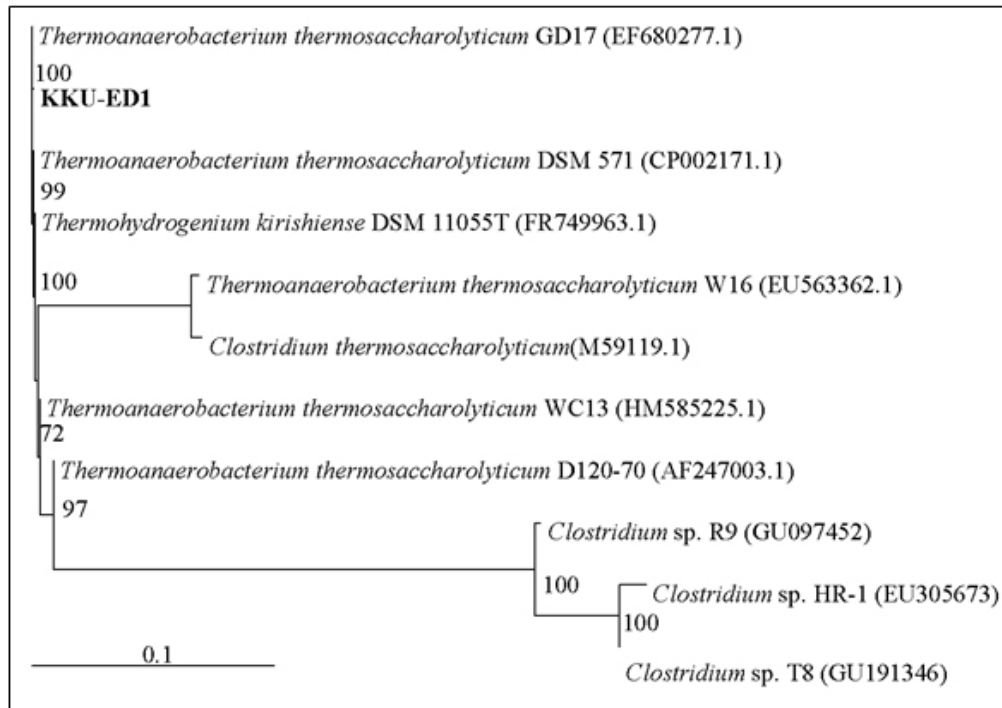
Lignocellulosic materials are receiving the attention to use as the substrate for hydrogen production due to its abundant and low/no cost. Lignocellulosic material can be fermented directly to hydrogen by some bacteria, but the rate is extremely slow due to the heterogeneity and crystallinity of the substrate (Datar et al. 2007). Therefore, the lignocellulosic materials have to be hydrolyzed into simple sugar before subsequently fermented to value products. Lignocellulosic material is composed of cellulose, hemicellulose and lignin. After the hydrolysis of cellulose, glucose is obtained, whereas the hydrolysis of hemicellulose, pentose sugars (xylose, arabinose) and hexose sugar (glucose) are obtained. Xylose and arabinose are the pentose sugars that have been used to produce hydrogen (Fardeau et al. 1996; Kadar et al. 2004; Danko et al. 2008; Ren et al. 2008; Li et al. 2010; Eriksen et al. 2011). However, the information on producing hydrogen from mixed xylose/arabinose is not as well described (Fangkum and Reungsang, 2011b). Therefore, an investigation into hydrogen production from mixed xylose/arabinose is needed in order to efficiently utilize the hemicellulosic fraction of lignocellulosic materials.



**Fig. 1** Scanning electron microscope imaged of *Thermoanaerobacterium thermosaccharolyticum* KKU-ED1.

The isolation and identification of highly efficient hydrogen producers are important for the fermentative hydrogen production process. A number of recent studies have reported on HY when arabinose and xylose were used as the carbon source by a variety of newly isolated bacteria. *Clostridium* sp. HR1, isolated from cow dung compost, was reported to be capable of generating a HY of 1.63 mol-H<sub>2</sub>/mol-xylose at a xylose concentration of 12 g/L (Xu et al. 2010). *C. butyricum* CGS 5 isolated from effluent sludge of a continuous dark fermentation bioreactor, gave a HY of 0.73 mol-H<sub>2</sub>/mol-xylose (Lo et al. 2008). A new marine *C. amygdalinum* strain C9 isolated from an offshore crude oil pipeline produced a HY of 1.78 mol-H<sub>2</sub>/mol-arabinose (Jayasinghearachchi et al. 2010). These reported strains are all mesophilic bacteria. However, this study has an attempt to use thermophilic microorganisms as the hydrogen producer due to their advantages including a high HPR at high temperatures, a high molar hydrogen yield by reducing the amount of by products and the ability to ferment lignocellulosic material (glucose, xylose and arabinose) (Sommer et al. 2004; O-Thong et al. 2008). Thermophilic hydrogen producer used in this research was isolated from the elephant dung. The elephant dung is expected to be abundant in cellulase-producing microorganisms as well as those that could utilize the hemicellulosic fraction of lignocellulose. This is due to the elephant's diet that is mainly plant materials. Thus, an investigation into hydrogen production from mixed xylose/arabinose by bacteria isolated from

elephant dung would provide the information towards the utilization of the hydrolysate fraction of lignocellulosic materials by this isolate.



**Fig. 2** Neighbour-joining tree showing the phylogenetic position of isolated strain, *Thermoanaerobacterium thermosaccharolyticum* KKU-ED1, based on 16S rRNA gene sequence. The numbers at nodes indicated that levels of bootstrap support percentages based on the neighbour-joining of 1000 replicates. The scale bar indicates 0.1 nucleotides substitution per nucleotide position.

The aim of this research was to isolate, characterize and identify a thermophilic fermentative bacterium from elephant dung. The optimum conditions for fermenting hydrogen production by the isolate in terms of initial pH, temperature and substrate concentration were investigated. The kinetic parameters for hydrogen production from mixed xylose/arabinose were identified. The possibility of using the sugarcane bagasse (SCB) hydrolysate which mainly contains pentose sugars (xylose and arabinose) to produce hydrogen under the optimum conditions was also explored.

## MATERIALS AND METHODS

### Isolation for hydrogen- producing bacteria from elephant dung

A hydrogen-producing bacterial strain was isolated from elephant dung. Enrichments were started by adding 21 g of wet heat-treated (100°C, 2 hrs) elephant dung to 70 mL of modified basic anaerobic (BA) medium (Table 1) in 120 mL serum vials that contained a 1:1 xylose/arabinose mixture at the total concentration of 5 g/L as carbon sources followed by adjustment pH to 6.5. The serum bottles were purged with nitrogen gas to create anaerobic condition. The enrichment culture was incubated at 55°C for 3 days. Every 3 days, 7 mL of the fermentation broth was transferred to 63 mL of a sterile modified BA medium containing of a 1:1 xylose/arabinose mixture at the total concentration of 5 g/L. After the three enrichment steps, the culture was then serially diluted in the same medium solidified by 0.3% (w/v) Gelrite (Kelco) and purged with nitrogen gas to create anaerobic condition. The cultures were incubated at 55°C for 3 days. Single colonies from each dilution series were inoculated into fresh BA medium and analyzed further for hydrogen production. The strain KKU-ED1 with the best performance among the isolates in terms of hydrogen production was selected to further characterize its hydrogen production.

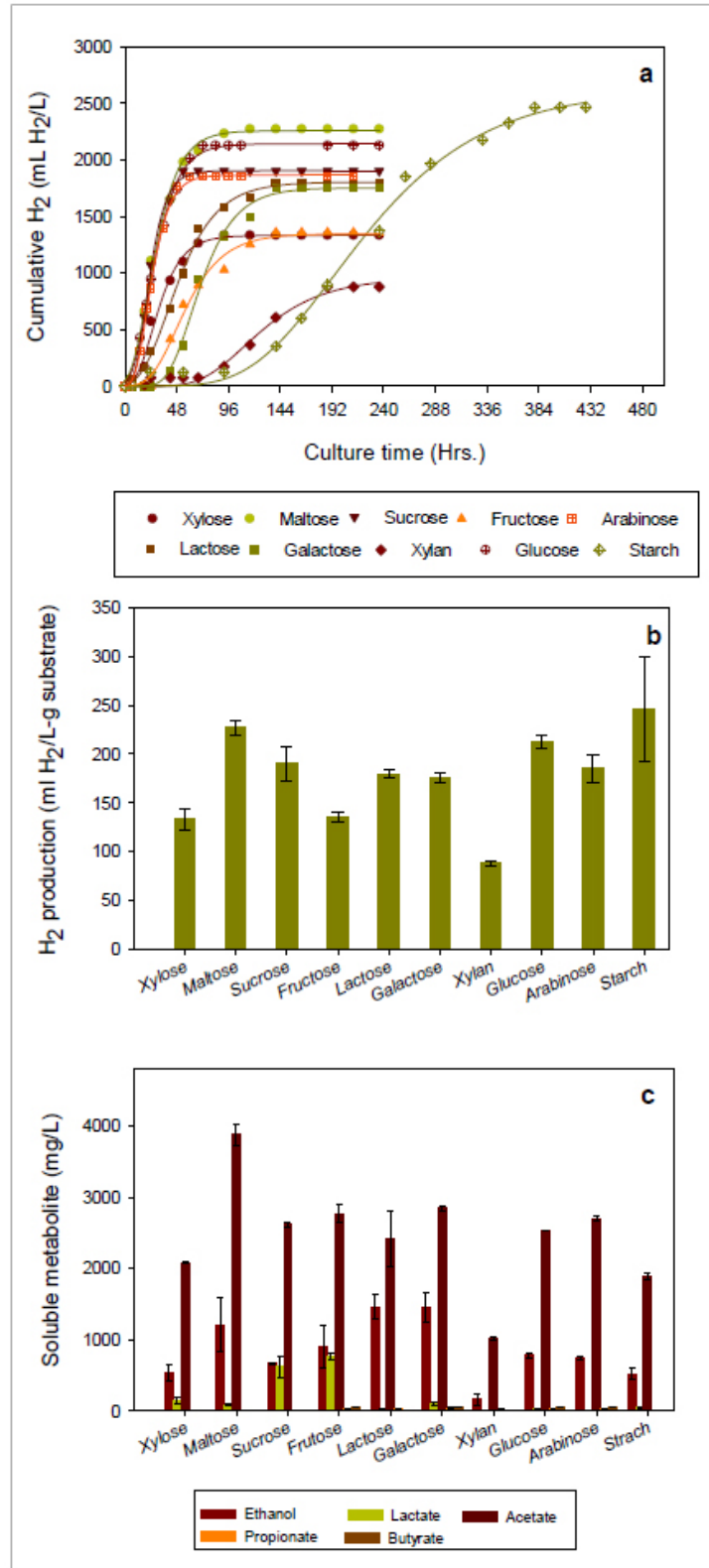


Fig. 3 Hydrogen production (a) cumulative hydrogen, (b) HY and (c) SMPs at different carbon source.

## Morphological analysis

The morphology and motility of cells at the exponential phase in modified BA medium were observed by CHS light microscopy (Olympus, Japan) and LEO-1455VP scanning electron microscopy (SEM) (EDAX<sup>®</sup>, USA). The sample preparation methods were modified from Pata et al. (2011). Briefly, the cell suspension was centrifuged at 8,000 rpm for 3 min and then the cell pellet was washed twice with 0.1 M phosphate buffer saline (PBS) (pH 7.2). Twenty  $\mu$ L of cell pellet in PBS were dropped onto nucleopore polycarbonate membranes (13 mm in size with a 0.2  $\mu$ m pore size) and waiting until the membrane was dry. The dry membrane with cells was soaked in a fixation solution consisting of 2.5% glutaraldehyde in 0.1 M PBS overnight at 4°C. The preparations were dehydrated by successive transfers in a series of 30, 50, 70, 90% ethanol solutions for 15 min and 100% ethanol solutions for 10 min. The dehydrated samples were transferred to the stub and coated with gold. Gram staining of isolated bacteria was performed by the Hucker method (Doestsch, 1981).

## Identification of the strain KKU-ED1

The genomic DNA of strain KKU-ED1 was extracted using a modified phenol chloroform isoamyl alcohol method (Sreela-or et al. 2011). The 16S rRNA gene was amplified by PCR using a pair of primers, PA19-38 (5'-AGAGTTTGATCCTGGCTCAG-3') and PH1541-1561 (5'-AAGGAGGTGATCCAGCCGCA-3'). The PCR products were purified using the QIAquick<sup>®</sup> PCR purification Kit (QIAGEN, USA) and sequenced using primers PA19-38 and PH1541-1561 with an ABI PRISM Big Terminator Cycle Sequencing Kit version 3.1 (Applied Biosystems, USA) in accordance with the manufacturer's instructions. The closest matches for partial 16S rRNA gene sequences were identified by database searches in GenBank using the BLAST (Altschul et al. 1990). Alignments for phylogenetic analysis were made by using CLUSTAL X (Thompson et al. 1994). A phylogenetic tree was then constructed using the neighbour-joining method (Saitou and Nei, 1987) with PHYLIP 3.69 (Felsenstein, 1993). Bootstrapping analysis (Felsenstein, 1985) for 1000 replicates was performed to estimate the confidence of tree topologies.

## Effect of carbon source on hydrogen production by KKU-ED1

Different carbon sources *i.e.*, glucose, xylose, maltose, sucrose, fructose, lactose, galactose, xylan, arabinose and starch at a concentration of 10 g/L were tested for hydrogen production. The experiment was conducted in 120 mL serum bottles with a working volume of 70 mL which contained 7 mL (10% v/v) of microbial suspension at the exponential phase ( $OD_{600} = 0.8-1.0$ ) and 63 mL of modified BA medium supplemented with a different carbon source at a concentration of 10 g/L. The initial pH was then adjusted to pH 6.5 by using 2 N HCl or 2 N NaOH. The serum bottles were purged with nitrogen gas to create an anaerobic condition and the serum bottles were incubated in an incubator at 55°C (BD 53, Binder, Germany). All treatments were conducted in four replicates.

## Effect of pH, temperature and substrate concentration on batch hydrogen fermentation from mixed xylose/arabinose by KKU-ED1

The batch fermentation studies were performed in a 120 mL serum bottle with a working volume of 70 mL containing of 7 mL (10% v/v) of the inoculums at the exponential phase ( $OD_{600} = 0.8-1.0$ ) and 63 mL of sterile modified BA medium supplemented with mixed xylose/arabinose as the carbon source. The initial pH was adjusted to the expected value by using 2 N HCl or 2 N NaOH. The serum bottles were purged with nitrogen gas to create an anaerobic condition and the serum bottles were incubated at the selected temperature. All treatments were conducted in four replicates.

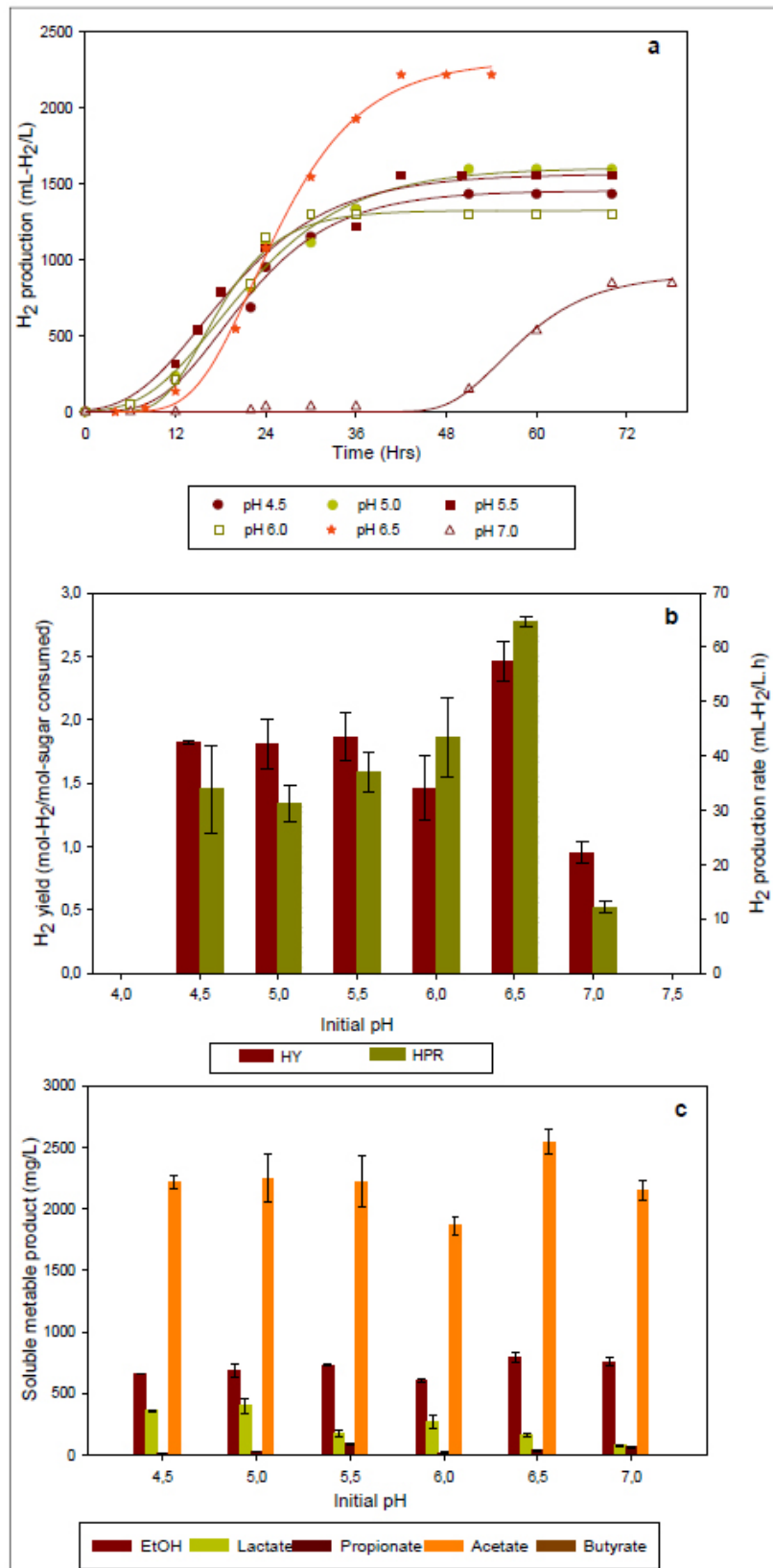


Fig. 4 Hydrogen production (a) cumulative hydrogen, (b) HY, HPR and (c) SMPs at different initial pH.

The effect of environmental factors *i.e.*, initial pH, temperature and substrate concentrations on hydrogen production by the strain KKU-ED1 at a thermophilic temperature were investigated in batch fermentation. The effect of initial pH (pH 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0) on hydrogen production was first examined at the initial total concentration of 1:1 xylose/arabinose mixture of 5 g/L and incubation temperature of 55°C. The obtained optimal pH which gave the maximum HPR was further used to study the effect of temperature (45, 50, 55, 60 and 65°C) on hydrogen production at the initial total concentration of 1:1 xylose/arabinose mixture of 5 g/L. After that, the obtained optimum initial pH and temperature were used to study the effect of substrate concentration on hydrogen production using 1:1 xylose/arabinose mixture at the total concentration of 3, 5, 7, 10, 15 and 20.0 g/L.

The growth kinetic analysis was performed on the batch hydrogen fermentation treatments using 1:1 xylose/arabinose mixture at the total concentration of 3, 5, 7 and 10 g/L. The specific growth rate ( $\mu$ ) was represented by the Malthus model (Equation 1) as follows:

$$\frac{dX}{dt} = \mu X \quad \text{[Equation 1]}$$

where  $\mu$  represents the specific growth rate ( $\text{h}^{-1}$ ),  $X$  represents the cell concentration (g/L) and  $t$  represents time (hrs). The maximum specific growth rate ( $\mu_{\max}$ ) and half-saturation constant ( $K_s$ ) were investigated with the Monod model (Equation 2).

$$\mu = \frac{\mu_{\max} S}{K_s + S} \quad \text{[Equation 2]}$$

where  $\mu$  denotes the specific growth rate ( $\text{h}^{-1}$ ),  $S$  denotes the substrate concentration (g/L),  $\mu_{\max}$  denotes the maximum specific growth rate ( $\text{h}^{-1}$ ) and  $K_s$  denotes the half-saturation constant (g/L). Lineweaver-Burk plots (Equation 3) were constructed to predict  $\mu_{\max}$  and  $K_s$  values.

$$\frac{1}{\mu} = \left( \frac{K_s}{\mu_{\max}} \times \frac{1}{S} \right) + \frac{1}{\mu_{\max}} \quad \text{[Equation 3]}$$

### Batch fermentation of hydrogen from SCB hydrolysate by strain KKU-ED1

**SCB hydrolysate preparation.** SCB was obtained from the local chipboard industry (Panel Plus Industry, Chaiyaphum, Thailand). The composition of SCB is 51.52% (w/w) cellulose, 23.49% (w/w) hemicellulose and 8.33% (w/w) lignin on a dry basis. The acid hydrolysis of SCB and inhibitor removal methods was conducted according to method previously described in our research (Fangkum and Reungsang, 2011a; Fangkum and Reungsang, 2011b). The obtained SCB hydrolysate contained (all in g/L) glucose, 1.46; xylose, 9.10; arabinose, 0.72; acetic acid, 1.30; and furfural, 0.22. The SCB hydrolysate was concentrated by heat and the resulting syrup had a reduced sugar concentration of 91 g/L. The hydrolysate syrup was diluted with distilled water to the designated concentrations before being used as the substrate.

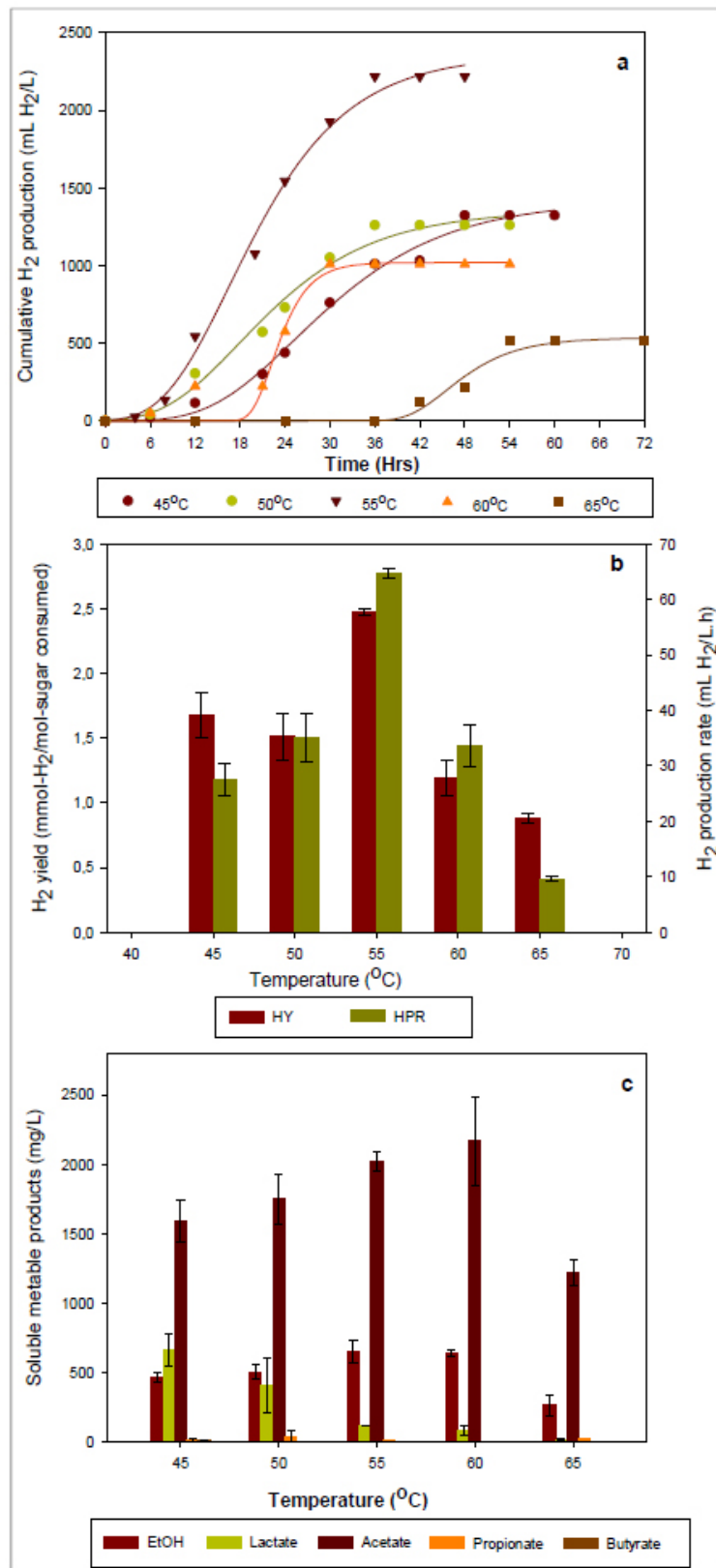


Fig. 5 Hydrogen production (a) cumulative hydrogen, (b) HY, HPR and (c) SMPs at different temperature.



**Hydrogen production from SCB hydrolysate.** Hydrogen production from SCB hydrolysate by KKU-ED1 was conducted under the optimum conditions obtained from the previous experiments. This was conducted in order to examine the possibility of hydrogen production from an actual hemicellulosic fraction of SCB by KKU-ED1. Hydrogen production was conducted in a similar manner to what described above but SCB hydrolysate at a final reducing sugar concentration of 5 g/L was used as the carbon source.

### Analytical methods

During fermentation, cell concentration was determined by measuring the absorbance ( $OD_{600}$ ) of samples at wavelength of 600 nm using the 6131 biophotometer (Eppendorf, Germany). The  $OD_{600}$  was transformed to the cell dry weight (DCW) concentration by the calibration of 1 unit of  $OD_{600}$  = 0.203 g DCW/L.

Preparation of liquid samples was conducted by centrifuged the fermentation broth at 10,000 rpm for 5 min, then the supernatants were filtered through a 0.2  $\mu$ m nylon membrane prior to analysis for sugar (glucose, xylose and arabinose) concentration by high performance liquid chromatography (HPLC) (Shimadzu LC-10AD) equipped with RI detector and Aminex HPX-87H column following method described by Fangkum and Reungsang (2011b).

For SMP analysis, the supernatants were acidified by 0.2 N oxalic acid and filtered through 0.2  $\mu$ m nylon membrane. The liquid samples were then analyzed by HPLC with Aminex HPX-87H column. UV detector was used for the analysis of acetic, butyric, lactic, and propionic acids. RI detector and Aminex HPX-87H column were used for analysis of ethanol and butanol. The HPLC conditions followed the method of Fangkum and Reungsang (2011b).

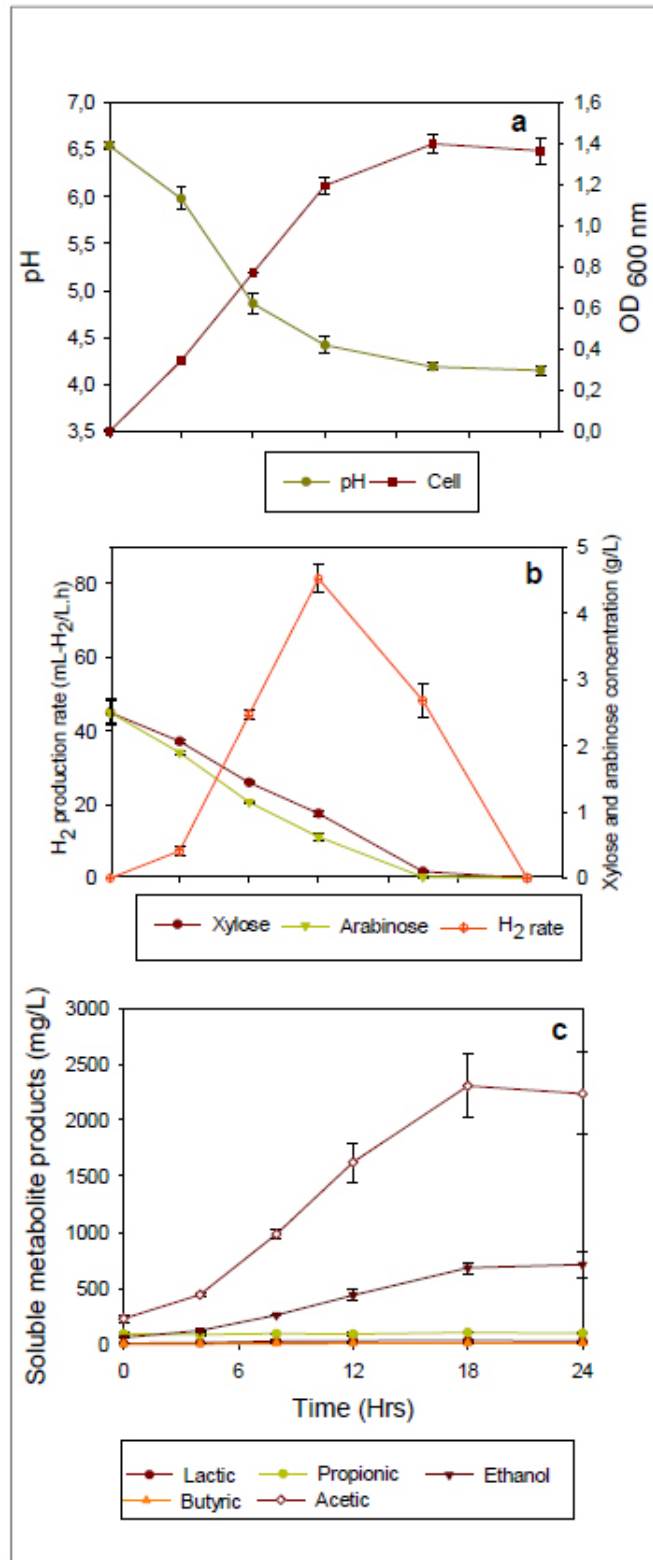
The biogas produced was collected at the interval time and the biogas composition was analyzed by a gas chromatograph (GC-2014, Shimadzu) equipped with a thermal conductivity detector (TCD) and 2 m stainless column packed with Shin carbon (50/80 mesh). The operational temperatures of the injection port, the column oven and the detector were 130, 120 and 140°C, respectively. Helium was used as the carrier gas at a flow rate of 25 mL/min. The volume of biogas was determined using wetted glass syringes with a capacity of 20-50 mL.

Hydrogen production was calculated from the headspace measurement of gas composition and the total volume of hydrogen produced at each time interval using the mass balance equation (Zheng and Yu, 2005). The HPR ( $\text{mL-H}_2/\text{L}\cdot\text{h}$ ) was calculated from the cumulative hydrogen gas volume divided by fermentation time (hour). HY was calculated as total molaric amount of hydrogen divided by the molar amount of consumed total sugar ( $\text{mol-H}_2/\text{mol-total sugar consumed}$ ). The total molar amount of hydrogen was calculated using the ideal gas law as molar hydrogen production ( $\text{mmol-H}_2/\text{L}$ ) = volumetric hydrogen production ( $\text{mL H}_2/\text{L}$ )/(RT), where  $R = 0.08205784 \text{ L atm/K mol}$  and  $T = 328 \text{ K}$  (55°C) (Zhang et al. 2006).

## RESULTS AND DISCUSSION

### Isolation and identification of strain KKU-ED1

Among the isolated colonies of hydrogen-producing bacteria, the strain KKU-ED1 had the ability to produce the highest hydrogen content (32%  $\text{H}_2$ , data not shown). Therefore, the KKU-ED1 was selected to use as hydrogen producer in this study. Morphology of the strain KKU-ED1 under SEM showed that the bacteria have a rod shape, with a length of 3.33  $\mu$ m and width of 0.23  $\mu$ m at a magnification of 20,000x (Figure 1). Cells were motile when observed under the light microscope (data not shown). Gram staining indicated that the strain KKU-ED1 is a Gram-positive bacteria.



**Fig. 6** Physiological characterization of *T. thermosaccharolyticum* KKKU-ED1 grown on 1:1 xylose/arabinose mixture medium at the total concentration of 5 g/L and inoculated with 10% v/v inoculum cells in exponential phase ( $OD_{600} = 0.8-1.0$ ). (a) Optical density of cells and pH variation, (b) cumulative hydrogen production, HPR and xylose, arabinose concentration during fermentation time and (c) SMPs.

The partial-length sequences of 16S rRNA gene (522 bp) were identified and deposited at GenBank under Accession number JN049814. A phylogenetic tree was constructed (Figure 2). The closest phylogenetic relative was *Thermoanaerobacterium thermosaccharolyticum* GD17 at a similarity of 100%. According to the results of 16S rRNA gene sequence examinations, the strain KKU-ED1 is in the genus *Thermoanaerobacterium*. The species of *Thermoanaerobacterium* spp. including *T. thermosaccharolyticum* are well-known hydrogen producer (Hoster et al. 2001; O-Thong et al. 2008; Ren et al. 2008).

### Effect of carbon source on hydrogen production

*T. thermosaccharolyticum* KKU-ED1 was able to assimilate and produce hydrogen from all carbon sources tested *i.e.*, glucose, xylose, arabinose, galactose, fructose, maltose, sucrose, lactose, xylan and starch at a fixed substrate concentration of 10 g/L (Figure 3). The highest hydrogen production of 246.31 mL-H<sub>2</sub>/g substrate (2463 mL-H<sub>2</sub>/L) was obtained when starch was used as the substrate while the lowest hydrogen production was obtained from xylan (87.65 mL-H<sub>2</sub>/g-substrate) (877 mL-H<sub>2</sub>/L) (Figure 3a and Figure 3b). The formation of SMP from different substrates at the end of fermentation was investigated. Acetate was found as the main by product followed by ethanol, propionate, lactate and butyrate, respectively (Figure 3c).

The ability of *T. thermosaccharolyticum* KKU-ED1 to produce hydrogen from different carbon sources implying that the strain KKU-ED1 has the potential of using mixed carbon sources including the mixture of sugars containing in the lignocellulosic hydrolysate.

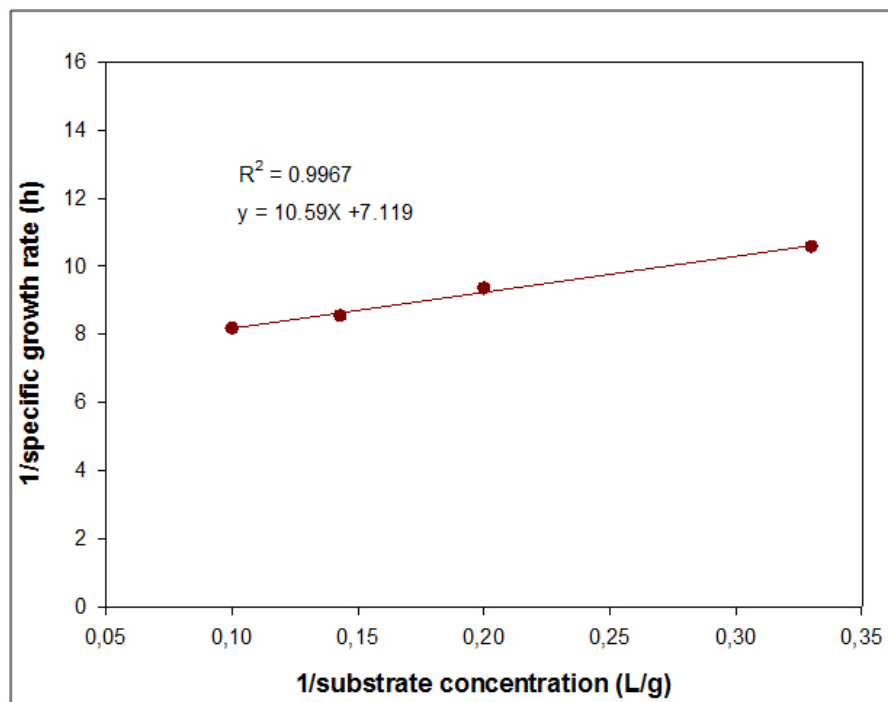


Fig. 7 Lineweaver-Burk plot for prediction of growth kinetic parameters at initial pH 6.5 and temperature of 55°C.

### Batch hydrogen fermentation from mixed xylose/arabinose by KKU-ED1

**Effect of initial pH on hydrogen production.** The initial pH had a strong effect on cumulative hydrogen production, HY and HPR. The cumulative hydrogen production and HY increased with an increase in the initial pH in the range of 4.5-6.5. The maximum cumulative hydrogen production, HY and HPR were obtained at the initial pH of 6.5. A further increase in the initial pH from 6.5 to 7.0 resulted in a low cumulative hydrogen production, HY and HPR (Figure 4a and 4b). The optimum initial

pH obtained from our batch experiment coincided with a study by Ren et al. (2008) who reported that HY yield of 2.0-2.2 mol-H<sub>2</sub>/mol-xylose was achieved at the optimum pH of 6.5.

Our results showed that acidic conditions were favourable for hydrogen production. The optimum initial pH was found to be 6.5. An increase in the initial pH above 6.5 led to a decrease in HY, HPR and cumulative hydrogen production. A lower hydrogen production at the initial pH below the optimum initial pH of 6.5 might be due to the denaturation of hydrogenase enzymes at a lower pH. Therefore, it can be concluded that too high or too low pH can result in a low hydrogen production due to inhibition of the activity of hydrogenase (Pandey et al. 2009).

The main SMP in the fermentation broth at different initial pH were acetate followed by ethanol, lactate, propionate and butyrate, respectively (Figure 4c). Therefore, hydrogen production from mixed xylose/arabinose by *T. thermosaccharolyticum* KKU-ED1 was carried out through acetate-type fermentation.

The results indicate that pH control at an optimum initial pH is necessary in order to obtain high hydrogen production from mixed xylose/arabinose by *T. thermosaccharolyticum* KKU-ED1.

**Table 1. Composition of BA medium stock modified from Angelidaki and Sanders (2004).**

Nutrients (g/L)	Usage (mL/L)
(A) NH <sub>4</sub> Cl, 100; NaCl, 10; MgCl <sub>2</sub> .6H <sub>2</sub> O, 10; CaCl <sub>2</sub> .2H <sub>2</sub> O, 5	10
(B) K <sub>2</sub> HPO <sub>4</sub> .3H <sub>2</sub> O, 200	2
(C) NaHCO <sub>3</sub> , 52	50
(D) FeCl <sub>2</sub> .4H <sub>2</sub> O, 2; H <sub>3</sub> BO <sub>3</sub> , 0.05; ZnCl <sub>2</sub> , 0.05; CuCl <sub>2</sub> .2H <sub>2</sub> O, 0.038; MnCl <sub>2</sub> .4H <sub>2</sub> O, 0.05; (NH <sub>4</sub> ) <sub>6</sub> Mo <sub>7</sub> O <sub>24</sub> .4H <sub>2</sub> O, 0.05; AlCl <sub>3</sub> , 0.05; CoCl <sub>2</sub> .6H <sub>2</sub> O, 0.05; NiCl <sub>2</sub> .6H <sub>2</sub> O, 0.092; ethylenediaminetetraacetate, 0.5; concentrated HCl, 1 ml; Na <sub>2</sub> SeO <sub>3</sub> .5H <sub>2</sub> O, 0.1	1
(E) Yeast extract, 100	10
(F) Na <sub>2</sub> S, 25	10

### Effect of temperature on hydrogen production

Figure 5 shows the progress of hydrogen production and SMP in the batch tests at the optimum initial pH 6.5 and different temperatures. The results showed that the cumulative hydrogen production from mixed xylose/arabinose by *T. thermosaccharolyticum* KKU-ED1 in batch tests increased with an increase in temperature from 45°C (1322 mL-H<sub>2</sub>/L) to 55°C (2216 mL-H<sub>2</sub>/L). A further increase in temperature from 55°C to 65°C resulted in a low hydrogen production (514 mL-H<sub>2</sub>/L) (Figure 5a). The profiles of HY and HPR were similar to those of cumulative hydrogen production (Figure 5b). The maximum HY and HPR of 2.46 mol-H<sub>2</sub>/mol-sugar consumed and 64.46 mL-H<sub>2</sub>/L.h, respectively, was obtained at the temperature of 55°C. In contrast, the temperature of 65°C gave the lowest HY and HPR (0.88 mol-H<sub>2</sub>/mol-sugar consumed and 9.51 mL-H<sub>2</sub>/L.h). The obtained optimum temperature from our batch experiment was similar to previously reports of Li et al. (2010) in which an optimum temperature for hydrogen production from xylose by *Thermoanaerobacterium* sp. SCUT27 was 55°C.

As for most enzymes, the reaction rate increases with temperature until a temperature is reached at which the enzyme is no longer stable. Above this point, the enzyme is denatured (Nigam, 2000). Thus, initially, hydrogen production was increased with an increase in temperature until 55°C: beyond this point, hydrogen production was retarded due to enzyme denaturation.

Acetate, ethanol, lactate, propionate and butyrate were detected as the SMP in the fermented broth (Figure 5c). The major SMP was acetate in all batch cultures which indicated acetate-type fermentation.

### Effect of substrate concentration on hydrogen production

Cumulative hydrogen production increased with an increase in total concentration of 1:1 xylose/arabinose mixture from 3 to 10 g/L (Table 2). A further increase in total concentration of 1:1 xylose/arabinose mixture from 10 to 20 g/L result in a lower cumulative hydrogen production. The HY decreased with an increase in total concentration of 1:1 xylose/arabinose mixture from 3 to 10 g/L while the HPR increased with an increase in total concentration of 1:1 xylose/arabinose mixture from 3 to 5 g/L (44.10 mL-H<sub>2</sub>/L.h to 64.46 mL-H<sub>2</sub>/L.h), and then HPR gradually decreased with an increase in total concentration of 1:1 xylose/arabinose mixture from 5 to 7 g/L. The maximum cumulative hydrogen production, HY and HPR were obtained at the total concentration of 1:1 xylose/arabinose mixture of 10 g/L. The total concentration of 1:1 xylose/arabinose mixture greater than 10 g/L resulted in a lower cumulative hydrogen production, HY and HPR. This might be the result of substrate inhibition (Kotay and Das, 2007) and end products inhibition (van Niel et al. 2003) which are formed during the fermentation process. A high initial substrate concentration could lead to an accumulation of organic products which probably results in an unfavourable thermodynamic state that prevents further substrate degradation (Rodriguez et al. 2006). In addition, an increase in the substrate concentration could increase the hydrogen production as well as the partial pressure of hydrogen in the head space of serum bottles. An increase in hydrogen partial pressure could switch the hydrogen production pathway to the solvent production pathway and that could lead to a decrease in hydrogen production (Fan et al. 2004).

The maximum cumulative hydrogen production of 3489 mL-H<sub>2</sub>/L was obtained at the total concentration of 1:1 xylose/arabinose mixture of 10 g/L, while the total concentration of 1:1 xylose/arabinose mixture of 3 g/L gave a maximum HY of 2.70 mol-H<sub>2</sub>/mol-sugar consumed. The highest HPR of 64.46 mL-H<sub>2</sub>/L.h was achieved at the total concentration of 1:1 xylose/arabinose mixture of 5 g/L. In general, the HY reflects the technical efficiency of fermentation, while the HPR reflects the economic efficiency (Gavala et al. 2006). In this study, the optimum total concentration of 1:1 xylose/arabinose mixture was chosen to be 5 g/L based on the maximum HPR obtained (Table 2).

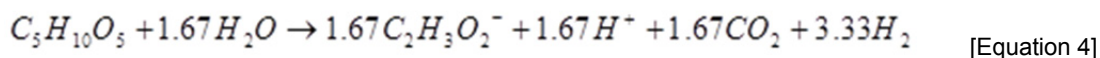
Therefore, the optimum conditions for hydrogen production from mixed xylose/arabinose by strain KKU-ED1 were an initial pH of 6.5, temperature of 55°C and the total concentration of 1:1 xylose/arabinose mixture of 5 g/L, which gave a HPR and HY of 64.46 mL-H<sub>2</sub>/L.h and 2.46 mol-H<sub>2</sub>/mol-sugar consumed, respectively.

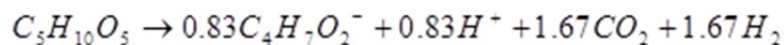
### SMP production

SMP released during hydrogen fermentation were acetate, ethanol, lactate, propionate and butyrate (Table 2). The SMP was increased with an increase in the mixed xylose/arabinose concentration. Acetate was presented as the main SMP and constituted more than 60% of the total end products which indicated that the fermentation process followed the acetate type fermentation pathway.

### Hydrogen fermentation from mixed xylose/arabinose under the obtained optimal condition

The profiles of pH, cell density, HPR, substrate concentration as well as SMP production during hydrogen production of mixed xylose/arabinose under the obtained optimal condition are shown in Figure 6. The pH decreased from 6.5 to 4.2 within 24 hrs (Figure 6a). Results indicated that the strain KKU-ED1 grew and produced hydrogen at the same time (Figure 6a and 6b). Hydrogen production continued for 24 hrs and the maximum HPR was obtained at 12 hrs (81.39 mL-H<sub>2</sub>/L.h) (Figure 6b). The concentration of xylose and arabinose was decreased with an increase in fermentation time (Figure 6b). Acetate was found as the main SMP followed by ethanol. A low amount of lactate, butyric, propionate and butanol were produced by this strain (Figure 6c). Theoretically, xylose and arabinose can be converted to hydrogen with a maximum yield of 3.33 mol-H<sub>2</sub>/mol-pentose when acetate is produced as the fermentation by product (Equation 4). Alternatively, xylose and arabinose can be converted into hydrogen by the butyrate pathway as shown in Equation 5 with a lower yield of 1.67 mol-H<sub>2</sub>/mol-pentose.





[Equation 5]

A HY of 2.46 mol-H<sub>2</sub>/mol-sugar consumed obtained under the optimal conditions in this study was lower than the theoretical yield. Some of xylose/arabinose might be converted to the biomass of KKU-ED1. Kim et al. (2006) reported that approximately 11% of the substrate, sucrose, is converted to microbial biomass. The HY of strain KKU-ED1 was compared with different reported strains that produce hydrogen from xylose and arabinose under thermophilic conditions. A HY of 2.19, 1.0, 0.96 and 3.2 mol-H<sub>2</sub>/mol-substrate was achieved from strain *T. thermosaccharolyticum* W16 (Ren et al. 2008), *Thermoanaerobacterium* sp. AK17 (Koskinen et al. 2008), *Thermoanaerobacterium* sp. SCUT27 (Li et al. 2010) and *Thermotoga maritime* (Eriksen et al. 2011), respectively. A HY obtained from KKU-ED1 was nearly equal to that of *T. thermosaccharolyticum* W16, which produced hydrogen from 10 g/L xylose at an initial pH 6.5 and a temperature of 60°C.

**Table 2. Hydrogen production from 1:1 xylose/arabinose mixture and SMPs at a fixed initial pH of 6.5 and temperature of 55°C under different substrate concentration.**

Total concentration of 1:1 xylose/arabinose mixture (g/L)	CHP(mL-H <sub>2</sub> /L)	HY(mol-H <sub>2</sub> /mol-sugar <sub>consumed</sub> )	HPR(mL-H <sub>2</sub> /L.h)	SMPs (mg/L)					
				Ethanol	Lactate	Acetate	Propionate	Butyrate	SMPs
3	1456	2.71 ± 0.00 <sup>d</sup>	44.10±0.03 <sup>a</sup>	372.22	142.91	1250.96	5.44	0.00	1771.53
5	2216	2.46±0.22 <sup>bcd</sup>	64.46±0.94 <sup>f</sup>	534.64	304.19	1785.00	2.45	3.40	2629.69
7	2116	2.29±0.03 <sup>abc</sup>	49.86±0.74 <sup>c</sup>	617.17	306.67	1831.88	29.57	2.14	2787.43
10	3489	2.53±0.20 <sup>cd</sup>	60.98±0.30 <sup>a</sup>	913.63	421.55	2454.65	11.04	3.99	3804.86
15	1949	1.96±0.04 <sup>a</sup>	53.25±1.24 <sup>d</sup>	712.30	650.71	1969.72	16.62	0.68	3350.03
20	2363	2.15±0.17 <sup>ab</sup>	46.17±0.24 <sup>b</sup>	814.56	664.40	2479.87	14.40	5.88	3979.12

### Kinetic analysis of cell growth of strain KKU-ED1

Growth kinetic analysis was conducted to provide kinetic information on cell growth and substrate consumption of strain KKU-ED1 which is valuable for bioreactor design and process scale-up for a fermentative hydrogen operation. The specific growth rate was determined by the slope of the growth curve during the exponential phase. The analysis was performed using the data from the batch hydrogen fermentation experiment with the total concentration of 1:1 xylose/arabinose mixture ranged between 3 and 10 g/L. The estimated parameters ( $\mu_{max}$  and  $K_s$ ) from the Lineweaver-Burk plots (Figure 7) were 0.14 h<sup>-1</sup> and 1.49 g/L, respectively. The  $\mu_{max}$  of *T. thermosaccharolyticum* KKU-ED1 was closed to *C. butyricum* CGS5 which had a  $\mu_{max}$  of 0.15 h<sup>-1</sup> when xylose was used as the carbon source (Lo et al. 2008). However, the value of  $\mu_{max}$  (0.31 h<sup>-1</sup>) of *T. thermosaccharolyticum* PSU-2 (Ren et al. 2008) and *C. pasteurianum* CH<sub>4</sub> (Lo et al. 2008) using sucrose as the carbon source was much higher than the  $\mu_{max}$  of *T. thermosaccharolyticum* KKU-ED1 on mixed xylose/arabinose.

### Hydrogen production from SCB hydrolysate by strain KKU ED-1

The optimum conditions (initial pH of 6.5, temperature of 55°C and sugar concentration of 5 g/L) were used to produce hydrogen from SCB hydrolysate by *T. thermosaccharolyticum* KKU-ED1. The syrup of SCB hydrolysate was diluted with distilled water to a reducing sugar concentration of 5 g/L before used as the substrate. The concentrations of each sugar in diluted SCB hydrolysate were 3.9 g/L xylose, 0.66 g/L glucose, 0.28 g/L arabinose and the concentrations of the inhibitors were 0.60 g/L acetic acid, 0.10 g/L furfural. The results reveal that the strain *T. thermosaccharolyticum* KKU-ED1 degraded xylose, glucose and arabinose in SCB hydrolysate to hydrogen, acetate, ethanol, lactate, butyrate and propionate. The cumulative hydrogen production, HY and HPR were 840 mL-H<sub>2</sub>/L, 1.12 mol-H<sub>2</sub>/mol-sugar consumed and 17.50 mL-H<sub>2</sub>/L.day (420 mL-H<sub>2</sub>/L.day), respectively (data not shown). The HY of this study was lower than that of our previous study (1.48 mol-H<sub>2</sub>/mol-sugar consumed) using SCB hydrolysate as the carbon source to produce hydrogen by mixed cultures in elephant dung (Fangkum

and Reungsang, 2011b). However, the HPR in this study (420 mL-H<sub>2</sub>/L.day) was superior over our previous results (176.77 mL-H<sub>2</sub>/L.day). This suggests that a pure culture could produce hydrogen at a higher rate than mixed cultures. For the SMP produced during hydrogen fermentation, acetate (1289.94 mg/L) was presented as the main SMP followed by ethanol (183.70 mg/L), lactate (99.88 mg/L), butyrate (36.90 mg/L) and propionate (32.85 mg/L) (data not shown). However, the HY using SCB hydrolysate as the carbon source was lower than the HY from using mixed xylose/arabinose as the carbon source (Table 3) which may due to the presence of inhibitors (acetic acid and furfural) in the SCB hydrolysate. Acetic acid can inhibit the microbial growth by entering the cell membrane and decrease intracellular pH which consequently affecting the metabolism of the microorganisms (van Zyl et al. 1991). Furfural can inhibit the growth of microorganisms by reducing enzymatic and biological activities, breaking down DNA and inhibiting protein and RNA synthesis (Liu et al. 2004).

**Table 3. Hydrogen production of different pure bacterial isolates.**

Microorganisms	pH, Temp (°C)	Substrate	Hydrogen yield	References
<i>T. thermosaccharolyticum</i> W16	6.5, 60	10 g/L xylose	2.1-2.2 mol-H <sub>2</sub> /mol-xylose	(Ren et al. 2008)
		10 g/L hydrolysate of corn stover	2.3-2.4 mol-H <sub>2</sub> /mol-xylose	
<i>Thermoanaerobacterium</i> sp. SCUT27	6.5, 55	10 g/L xylose	0.96 mol-H <sub>2</sub> /mol-xylose	(Li et al. 2010)
<i>Thermoanaerobacter finnii</i>	7.0, 60	3.4 g/L xylose	0.14 mol-H <sub>2</sub> /mol-xylose	(Fardeau et al. 1996)
<i>T. thermosaccharolyticum</i> KKU-ED1	6.5, 55	5 g/L 1:1 xylose/arabinose mixture	2.46 mol-H <sub>2</sub> /mol-sugar	This study
		5 g/L SCB hydrolysate	1.12 mol-H <sub>2</sub> /mol-sugar	

Hydrogen production by *T. thermosaccharolyticum* KKU-ED1 was compared with other literatures (Table 3). The HY of strain KKU-ED1 was higher than *Thermoanaerobacterium* sp. SCUT27 (Li et al. 2010) and *Thermoanaerobacter finnii* (Fardeau et al. 1996) which produced hydrogen from xylose at 55 and 60°C, respectively. It should be noted that an HY obtained from the strain KKU-ED1 was relatively close to those of strain W16 but the xylose concentration that W16 used was 10 g/L at initial pH 6.5 and temperature of 60°C. The results indicate that *T. thermosaccharolyticum* KKU-ED1 is a promising candidate for thermophilic fermentative hydrogen production from mixed xylose/arabinose with possible applications for hydrogen production from a hemicellulosic fraction (hydrolysate) of SCB.

## CONCLUDING REMARKS

This study investigated the use of a newly isolated thermophilic bacterium KKU-ED1 to produce hydrogen from mixed xylose/arabinose and SCB hydrolysate. The bacterium was isolated from an enriched culture of elephant dung, identified based on 16S rRNA gene sequences as *T. thermosaccharolyticum* and designated *T. thermosaccharolyticum* KKU-ED1. The optimum conditions for hydrogen production from mixed xylose/arabinose by *T. thermosaccharolyticum* KKU-ED1 were an initial pH of 6.5, temperature of 55°C and the total concentration of 1:1 xylose/arabinose mixture of 5 g/L, which resulted in a respective HY and HPR of 2.46 mol-H<sub>2</sub>/mol-sugar consumed and 64.47 mL-H<sub>2</sub>/L.h. The optimum conditions were then applied to produce hydrogen from SCB hydrolysate (reducing sugar concentration of 5 g/L) in which a HY of 1.12 mol-H<sub>2</sub>/mol-sugar consumed was obtained. The end product was predominantly acetate indicated the acetate-type fermentation. The strain KKU-ED1 appeared to be a suitable candidate for thermophilic fermentative hydrogen production from hemicellulosic fraction of lignocellulosic materials due to its ability to use various types of carbon sources.

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