



# PRODUCTION HYDROGEN FROM *Nostoc* sp. TISTR 8872 BIOMASS: APPLICATION OF PHOTOSYNTHETIC FERMENTATION BY *Rhodopseudomonas* sp. TISTR 1953.

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## Abstract


Hydrogen is an abundant, clean and renewable energy source, and is therefore an optimal fuel for the development of a future hydrogen economy. Alternative biological processes can be practically applied to produce hydrogen. This study investigated hydrogen production from algal biomass by a three-step process; first, photosynthetic CO<sub>2</sub> fixation into starch by a cyanobacterium, *Nostoc* sp. TISTR 8872; second, dark anaerobic fermentation of biomass by 5 strains of anaerobic bacteria to produce organic acids; and third, conversion of organic acids to hydrogen by *Rhodopseudomonas* sp. TISTR 1953. The cyanobacterial strain *Nostoc* sp. TISTR 8872 produced 0.30±0.00 g (DW)/L of biomass with 30.66±0.58% of accumulated starch during photosynthetic growth in BG-11 media at 28±1°C under illumination at 60 μmol photon/m<sup>2</sup>/s on an orbital shaker for 20 days. The algal biomass was concentrated, and then converted into organic acids (360.52±87.67 mM of malic acid, 1,338.49±153.95 mM of acetic acid, 3,341.37±194.45 mM of citric acid and 21.18±11.27 mM of butyric acid) by *Lactobacillus brevis* subsp. *brevis* TISTR 868 in anaerobic fermentation for 48 h. A photosynthetic bacterial strain, *Rhodopseudomonas* sp. TISTR 1953, produced hydrogen at a rate of 10.38±0.53 ml/l/h. The results indicate that *Nostoc* sp. TISTR 8872 is a suitable stain for algal biomass production to produce hydrogen by a three-step microbial hydrogen-producing system.

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**Keywords:** Hydrogen production, biomass, starch, *Nostoc* sp. TISTR 8872

## Introduction

Today fossil fuels are the world's most important energy source, meeting about 80% of present demand. Combustion of fossil fuels is causing global climate change, mainly due to the emission of pollutants like CO<sub>x</sub>, NO<sub>x</sub>, SO<sub>x</sub>, C<sub>x</sub>H<sub>x</sub>, soot, ash, droplets of tars and other organic compounds, which are released into the atmosphere (Das and Veziroglu 2001). The development of new energy sources and devices will emerge more aggressively to address world energy demand and environmental concerns. At present, increasing environmental pollution, mainly due to rapid industrialization and urbanization, is of great concern to the world. Interest has increasingly focused on clean energy alternatives to meet the remarkably growth in energy demand. It has been reported that hydrogen energy is an optimal fuel for the future, mainly due to its high conversion efficiency, recyclability and nonpolluting nature. There are many ways of producing hydrogen from water, including electrochemical,



thermochemical and photobiological processes (Benemann 1974, Das and Veziroglu 2001, 2008; Kapdan and Kargi 2006). Biological hydrogen production processes have been found to be more environmentally friendly and less energy intensive compared to thermochemical and electrochemical processes (Das and Veziroglu 2001). Hydrogen can be produced by a photobiological water splitting reaction. Some bacteria and green algae are capable of biologically evolving hydrogen under certain conditions (Gaffon and Rubin 1942; Weissman and Benemann 1977). Among the various biological hydrogen production technologies, direct photolysis of H<sub>2</sub>O to evolve H<sub>2</sub> and O<sub>2</sub> by photosynthetic green algae is an ideal and classical hydrogen production technology. Nevertheless, there is still a major obstacle to overcome, because O<sub>2</sub> produced by photosynthesis during this reaction inhibits the hydrogenase enzyme that is responsible for the hydrogen production (Kim *et al.* 2006).

However, there is a potential alternative method for hydrogen production by a three-step microbial hydrogen-producing system. The first step is starch production by microalgae. The second is starch fermentation by hydrolyzing lactic bacterium to convert starch produced by microalgae to lactic acid, which is a suitable substrate for hydrogen production by photosynthetic bacteria in the third and final step (Ike *et al.* 1997b; Kawaguchi *et al.* 2001; Kim *et al.* 2006; Rodjaroen *et al.* 2011).

In this study, cyanobacterial biomass; *Nostoc* sp. TISTR 8872, were used to convert algal starch to organic acids by anaerobic bacteria. Subsequently, direct hydrogen production from organic acids by a photosynthetic bacterium, *Rhodospseudomonas* sp. TISTR 1953, was investigated.


## Methodology

### Microorganisms and growth condition

The cyanobacterial strain, *Nostoc* sp. TISTR 8872 was obtained from the Thailand Institute of Scientific and Technological Research (TISTR). Algal cells, at an initial concentration of 0.20 at OD<sub>1000</sub>, were grown in 250 ml of BG-11 medium (Rodjaroen *et al.* 2011) under an illumination of 60 μmol photon m<sup>-2</sup>s<sup>-1</sup> from cool white fluorescence lamps with a cycle of 12 h dark and 12 h light (Guan *et al.* 2004), and shaken at 150 rpm on an orbital shaker at 28±1°C (Kawaguchi *et al.* 2001; Kim *et al.* 2006). Five strains of anaerobic bacteria, obtained from TISTR, were used in this experiment; namely, *L. casei* TISTR 390, *L. brevis* subsp. *brevis* TISTR 860, *L. brevis* subsp. *brevis* TISTR 868, *L. pentosus* TISTR 920 and *L. amylovorus* TISTR 1110. Most of those anaerobic bacterial strains were grown in MRS broth (ATCC, 1989) and anaerobically incubated at 37°C in an incubator for a period of 48 h. Cells used for inoculation were harvested during the logarithmic growth phase based on an optical density at 600 nm (OD<sub>600</sub>) of ca. 0.2 (Zhang *et al.* 2006). For the photosynthetic bacterial strain, *Rhodospseudomonas* sp. TISTR 1953 was made available from the TISTR. Cells were stored at -80°C and grown in yeast broth, and then inoculated photoheterotrophically in a 250 ml flask under illumination at 100 μmol photon m<sup>-2</sup>s<sup>-1</sup> using halogen lamps with a cycle of 12 h dark and 12 h light, and shaken at 150 rpm in a water bath on an orbital shaker at 37±1°C for 24 h (Rodjaroen 2010).

### Organic acids fermentation of algal biomass

Algal cells were harvested by centrifugation (6,300g, 10 min) and a dense biomass prepared with a concentration up to 10 times the original algal culture. Five strains of anaerobic bacteria were cultured until the logarithmic growth phase and were harvested by centrifugation (6,300g, 10 min). The cells were washed with 0.085% saline solution and



added to 50 ml of concentrated algal biomass. Sample bottles, inoculated with *L. brevis* subsp. *brevis* TISTR 868, were anaerobically incubated at 37°C in anaerobic jars for a period of 48 h.

After anaerobic fermentation of the algal biomass, the fermentate was harvested by centrifugation at 10,000 g for 15 min to separate the supernatant and algal fermentate. The supernatant was collected to produce hydrogen by photosynthetic bacteria, and its organic acid concentration was determined using High Pressure Liquid Chromatography. The algal fermentate was washed five times in distilled water, freeze-dried and its dry weight and starch accumulation measured after algal hydrolysis.

#### Hydrogen production from fermentate of algal biomass

After the anaerobic fermentation of the algal biomass, the fermentate was centrifuged and 10% yeast broth was added to the supernatant. Sixty ml ( $OD_{600nm}=0.5$ ) of actively growing photosynthetic bacterium, *Rhodospseudomonas* sp. TISTR 1953, in a cell suspension culture in the logarithmic growth phase was harvested by centrifugation (6,300g, 10 min) and added to the reconstituted supernatant in a 600 ml glass bottle, which was then capped with a rubber stopper and purged with argon gas to remove oxygen from the bottle. The glass bottle was incubated at 37°C under illumination at 8 klux by halogen lamps (Kim *et al.* 2006).

Culture bottles were closed with rubber stoppers tightly fitted with a syringe for efflux of gases from the culture. A rubber tubule was attached tightly to each syringe for gas collection. The rubber tubule conducted gas to an upside-down cylinder filled with water and immersed in a beaker filled with water. Gas produced by the culture eventually accumulated in the inverted cylinder by displacing an equal volume of water, and was measured from the graduated divisions of the cylinder until no gas was released, as described by Guan *et al.* 2004.

Supernatant was collected every 4 h until hydrogen production was stopped and the organic acid concentration, pH and growth curve of photosynthetic bacteria was investigated.

#### Analysis

Algal growth curves were monitored by spectrophotometer. The strains were sampled every 2 days for determination of cell growth by a spectrophotometer at  $OD_{1000}$ . Algae were harvested at stationary growth stages. After 20 days of cultivation, algal biomass was determined as total dry weight. Starch accumulation was analyzed for glucose content using Kochert's method (1978) after hydrolysis in 2M  $HClO_4$  for 2 h at 100°C, using glucose (VWR International Ltd., England) as a standard. Percent dry weight was calculated as a percentage of starch per algal biomass dried weight. The concentrations of organic acids (malic, lactic, acetic and citric acid) were analyzed using High Pressure Liquid Chromatography (Hewlett Packard series 1100, USA.) fitted with an Platinum EPS C<sub>18</sub> organic acid analysis column (250 mm x 4.6 mm, 5  $\mu$ m) using 0.05 M  $KH_2PO_4$  as the mobile phase at pH 2.4 with a photodiode array detector and 1 ml/min flow rate. Butyrate was analyzed with a Gas Chromatograph (Agilent Technologies series 6890N, USA) equipped with a CP 58 Wax (FFAP) (30 m x 0.25 mm ID, 0.25  $\mu$ m) column using helium as a carrier gas and a FID detector. Hydrogen production was analyzed using a Gas Chromatograph (Agilent Technologies series 6890N, USA) equipped with a molecular sieve Plot Q (30 m x 0.25 mm ID, 0.25  $\mu$ m) column using a thermal conductivity detector with argon as a carrier gas, at flow rate 1 ml/min and using hydrogen (TIG public company Ltd.) as a standard.

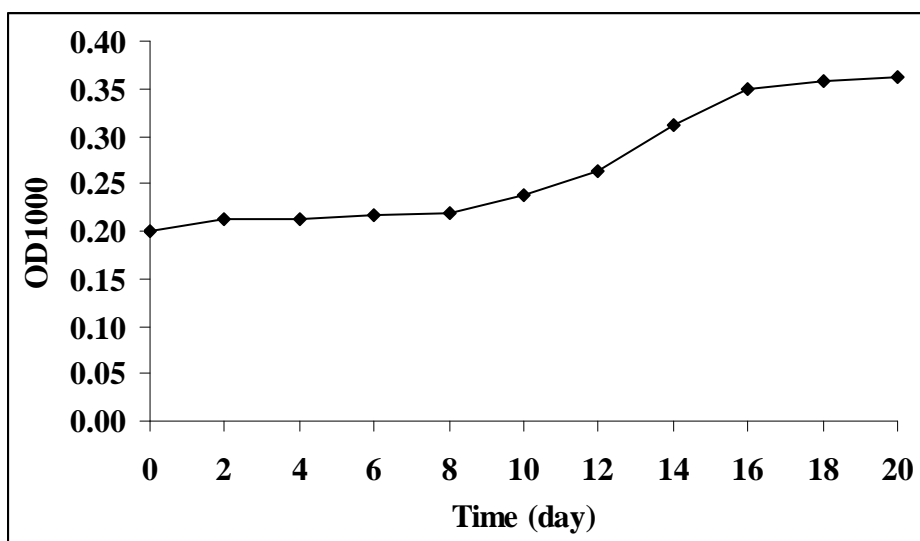
The means of dry biomass, starch accumulation and organic acid production from triplicate tests of algae were compared by a one-way ANOVA, separating the significantly different

mean values with by means of Tukey's multiple comparison tests (Zar 1996) using SPSS for Windows version 12.0.

## Results

### Algal growth, biomass production and starch accumulation

The time courses of growth of cyanobacterial strain, *Nostoc* sp. TISTR 8872 was investigated every 2 days until the growth rate decreased. Fig. 1 shows the growth curves of *Nostoc* sp. TISTR 8872 cultivated in BG-11 medium over 20 days. *Nostoc* sp. TISTR 8872 growth rate was increasing from initiation to eight days, followed by rapid growth until sixteen days. Subsequently, algal cells were in the stationary growth phase until 20 days, when algal cells were harvested to determine total dry weight and starch accumulation. Biomass production and starch accumulation of *Nostoc* sp. TISTR 8872 were  $0.30 \pm 0.00$  g dried weight (DW)/L and  $30.66 \pm 0.58\%$ , respectively.



**Figure 1** Growth curve of *Nostoc* sp. TISTR 8872 cultivated in BG-11 medium for 20 days.

### Organic acids fermentation of algal biomass

The results of anaerobic fermentation (Table 1) showed that the highest concentrations of low molecular weight organic acids were produced from *Nostoc* sp. TISTR 8872 biomass when fermented with *L. brevis* subsp. *brevis* TISTR 868. In that fermentation, organic acids such as malic acid, acetic acid, citric acid and butyric acid were produced with concentrations of  $360.52 \pm 87.67$ ,  $1,338.49 \pm 153.95$ ,  $3,341.37 \pm 194.45$  and  $21.18 \pm 11.27$  mM, respectively (Table 1). It was obvious that acetic acid and citric acid were the major organic acids produced by anaerobic fermentation with *L. brevis* subsp. *brevis* TISTR 868 and were significantly higher than with other species. Lactic acid was not produced by any anaerobic bacteria under the anaerobic conditions.

**Table 1** Production of organic acids from concentrated algal *Nostoc* sp. TISTR. 8872 biomass using various anaerobic bacteria strains during 48 h of anaerobic fermentation.

| Anaerobic bacteria strains                      | Organic acids (mM)         |                        |                              |                              |                           |
|---|----------------------------|------------------------|------------------------------|------------------------------|---------------------------|
|   | Malic acid                 | Lactic acid            | Acetic acid                  | Citric acid                  | Butyric acid              |
| <i>L. casei</i> TISTR 390                       | 361.94±88.81 <sup>b</sup>  | 0.00±0.00 <sup>a</sup> | 669.33±3.18 <sup>a</sup>     | 1,843.97±409.27 <sup>b</sup> | 14.89±10.14 <sup>a</sup>  |
| <i>L. brevis</i> subsp. <i>brevis</i> TISTR 860 | 192.47±16.33 <sup>ab</sup> | 0.00±0.00 <sup>a</sup> | 573.29± 7.14 <sup>a</sup>    | 1,703.51±243.71 <sup>b</sup> | 24.33±24.33 <sup>a</sup>  |
| <i>L. brevis</i> subsp. <i>brevis</i> TISTR 868 | 360.52±87.67 <sup>b</sup>  | 0.00±0.00 <sup>a</sup> | 1,338.49±153.95 <sup>b</sup> | 3,341.37±194.45 <sup>c</sup> | 21.18±11.27 <sup>a</sup>  |
| <i>L. pentosus</i> TISTR 920                    | 128.04±16.65 <sup>ab</sup> | 0.00±0.00 <sup>a</sup> | 534.86±92.07 <sup>a</sup>    | 267.60±36.35 <sup>a</sup>    | 49.05±20.64 <sup>a</sup>  |
| <i>L. amylovorus</i> TISTR 1110                 | 67.89±34.95 <sup>a</sup>   | 0.00±0.00 <sup>a</sup> | 657.53±151.65 <sup>a</sup>   | 206.63±112.74 <sup>a</sup>   | 121.68±28.17 <sup>b</sup> |

**Note:** Means in the column followed by the same letters are not significantly different as determined by means of Tukey's multiple comparison tests ( $\alpha=0.05$ ).

#### Algal fermentation

The initial concentration of *Nostoc* sp. TISTR 8872 algal biomass was 0.30±0.00 g DW/L, while that of the concentrated algal biomass was 10.26±0.31 g DW/L. The starch accumulations of the initial biomass and concentrated biomass were 30.66±0.58% and 38.47±0.72% respectively. After 48 h fermentation, the mean values ranged between 1.91±0.13-3.10±0.05 g DW/L for biomass production and 9.54±1.97-19.57±1.23% for starch accumulation. The starch in algal biomass fermented by *L. amylovorus* TISTR 1110 was degraded slowly, with only 49.13±3.20 % hydrolyzed in 48 h, allowing the fermentation to continue. In contrast, 75.20±5.12 % of starch in algal biomass fermented by *L. casei* TISTR 390 was rapidly hydrolyzed in 48 h to produce organic acids. However, there were no significant differences between *L. casei* TISTR 390, *L. pentosus* TISTR 920 and *L. brevis* subsp. *brevis* TISTR 868 in the hydrolysis of starch ( $p>0.05$ ) (Table 2).

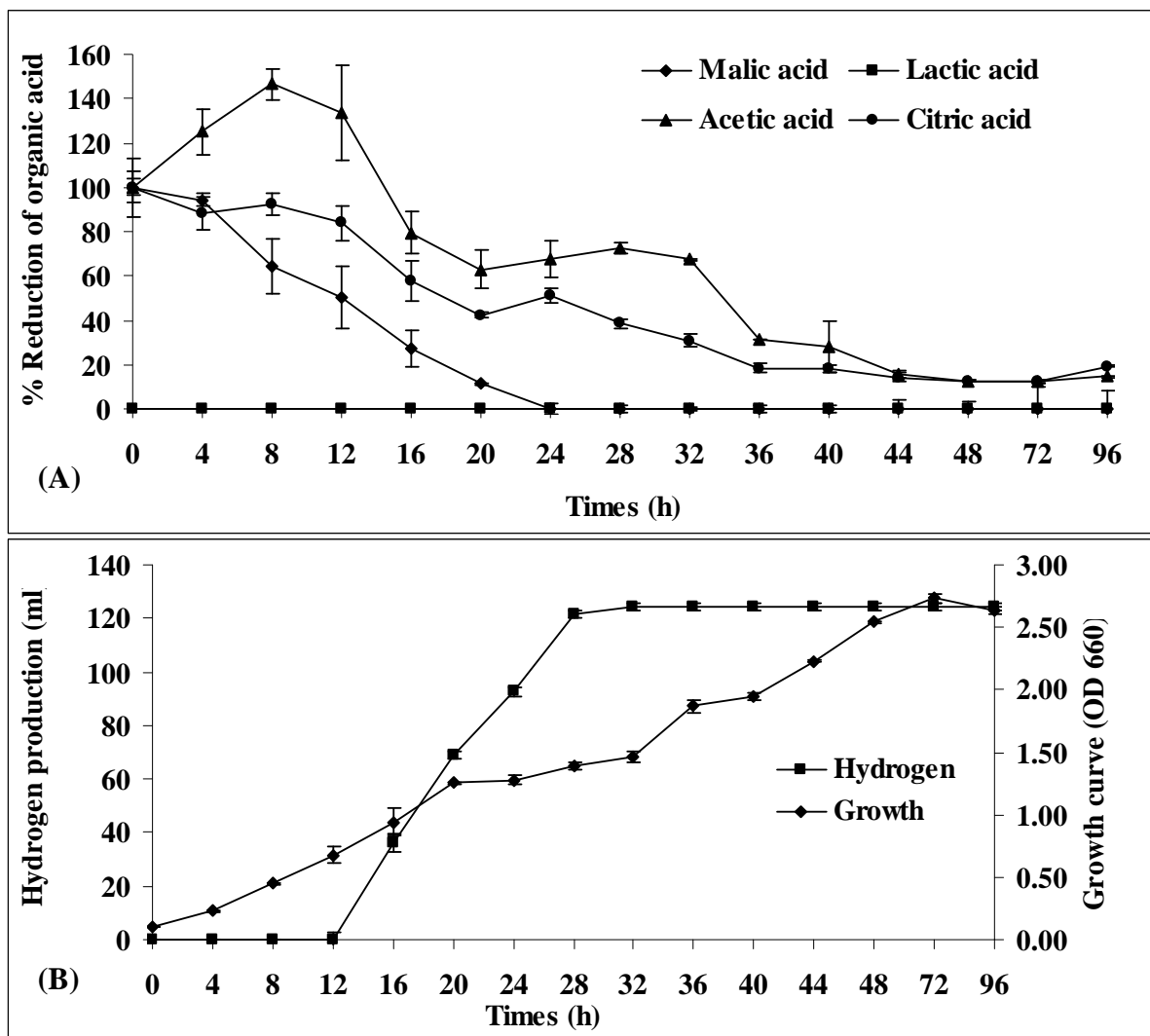
**Table 2** Algal biomass, starch accumulation and hydrolysis of algal biomass of *Nostoc* sp. TISTR 8872 after fermentation by various anaerobic bacteria strains during 48 h.

| Bacterial Strains                               | Algal biomass (Mean±SE; gDW/L) |                         |                             | % Starch (Mean±SE)      |                          |                          |
|---|--------------------------------|-------------------------|-----------------------------|-------------------------|--------------------------|--------------------------|
|   | Concentrate                    | After fermented         | Hydrolysis of algal biomass | Concentrate             | After fermented          | Hydrolysis of            |
|   | (10 fold)                      | 48 h                    | (%)                         | (10 fold)               | 48 h                     | starch (%)               |
| <i>Lactobacillus casei</i> TISTR 390            | 10.26±0.31 <sup>a</sup>        | 2.34±0.19 <sup>ab</sup> | 77.17±1.87 <sup>bc</sup>    | 38.47±0.72 <sup>a</sup> | 9.54±1.97 <sup>a</sup>   | 75.20±5.12 <sup>c</sup>  |
| <i>L. brevis</i> subsp. <i>brevis</i> TISTR 860 | 10.26±0.31 <sup>a</sup>        | 1.98±0.04 <sup>a</sup>  | 80.69±0.41 <sup>c</sup>     | 38.47±0.72 <sup>a</sup> | 16.71±0.54 <sup>bc</sup> | 56.56±1.41 <sup>ab</sup> |
| <i>L. brevis</i> subsp. <i>brevis</i> TISTR 868 | 10.26±0.31 <sup>a</sup>        | 3.10±0.05 <sup>c</sup>  | 69.80±0.51 <sup>a</sup>     | 38.47±0.72 <sup>a</sup> | 11.37±0.97 <sup>ab</sup> | 70.43±2.52 <sup>bc</sup> |
| <i>L. pentosus</i> TISTR 920                    | 10.26±0.31 <sup>a</sup>        | 1.91±0.13 <sup>a</sup>  | 81.41±1.25 <sup>c</sup>     | 38.47±0.72 <sup>a</sup> | 12.72±1.36 <sup>ab</sup> | 66.94±3.53 <sup>bc</sup> |
| <i>L. amylovorus</i> TISTR 1110                 | 10.26±0.31 <sup>a</sup>        | 2.86±0.88 <sup>bc</sup> | 72.09±0.87 <sup>ab</sup>    | 38.47±0.72 <sup>a</sup> | 19.57±1.23 <sup>c</sup>  | 49.13±3.20 <sup>a</sup>  |

**Note:** Means in the column followed by the same letters are not significantly different as determined by means of Tukey's multiple comparison tests ( $\alpha=0.05$ ).

### Hydrogen production from fermentate of algal biomass

The experimental results showed that *Rhodopseudomonas* sp. TISTR 1953 consumed organic acids and evolved hydrogen at a rate of  $10.38 \pm 0.53$  ml/L/h where the total of hydrogen collected (about 124.50 ml) was observed after 96 h in culture initially grown in concentrated supernatant with 10 % yeast broth. Hydrogen gas was produced during the period of 12-32 hours of the experiment. The time course of growth of *Rhodopseudomonas* sp. TISTR 1953 (Fig. 2A) showed that cell growth rapidly increased till 20 h and stabilized from 20-32 h. After 72 h, cell growth decreased. The rates of organic acid consumption and hydrogen production were compared with incubation times. The conversion efficiency of acetic acid and citric acid to hydrogen gas was the highest among the organic acids examined (Fig. 2B). Malic acid was completely used up after 24 h.



**Figure 2** Time courses of hydrogen production (A) and organic acids reduction (B) from supernatant of algal *Nostoc* sp. TISTR 8872 biomass during photo-fermentation by photosynthetic *Rhodopseudomonas* sp. TISTR 1953.



## Discussion

### Algal biomass and starch accumulation from green *Nostoc* sp. TISTR 8872

The result in this study showed that the cyanobacteria *Nostoc* sp. TISTR 8872 produced high biomass and starch. Thus, it was suitable for hydrogen production in a three-step microbial hydrogen-producing system. Starch accumulation by *Nostoc* is higher than by green algae because it is a nitrogen-fixing filamentous cyanobacteria which produces macroscopic or microscopic colonies with polysaccharides or glycoconjugates (Dodds *et al.*, 1995). Miyamoto (1997) and Spolaore *et al.* (2006) reported that most green algal strains accumulate starch between 4-26% , while cyanobacteria, *S. fusiformis* produces starch up to 56.7% (Rafiqul *et al.* 2003). Huang *et al.* (1998) found that *N. commune* Vaucher, *N. flagelliforme* and *N. sphaeroides* produced monosaccharides; glucose, xylose, and galactose, with an approximate ratio of 2:1:1. That is one reason why *Nostoc* sp. TISTR 8872 accumulates higher starch than green algae. Furthermore, *Nostoc* sp. TISTR 8872 is easily harvested by plankton net filtration. For those two reasons, that algal species was selected for further studies, although its growth rate is slower than green algae.


### Anaerobic fermentation of algal biomass

Ike *et al.* (1997a) reported that organic acid production from anaerobic fermentation of algal biomass depended on the anaerobic bacterial strain. This study of anaerobic fermentation of concentrated *Nostoc* sp. TISTR 8872 algal biomass by 5 strains of anaerobic bacteria, showed that *L. brevis* subsp. *brevis* TISTR 868 gave the highest acetic acid and citric acid content compared to other strains. *Nostoc* sp. TISTR 8872 is a nitrogen-fixing filamentous cyanobacteria, which has the highest percentage of starch due to its mucous sheet and the polysaccharides or glycoconjugates content of its cells (Huang *et al.* 1998). It seems likely that different anaerobic bacterial strains have different efficiencies of algal cell fermentation. Yamada and Sakagui (1981) reported that most *Chlorella* strains are very rigid microalgae and generally undigestible. Some *Chlorella* strains have a trilaminar outer layer containing sporopollenin, a polymer of carotenoides that is extraordinarily resistant to enzymatic attack, while *Dunaliella* is a unicellular green alga without a cell wall (Borowitzka and Borowitzka 1989), and the cells are easily broken. The structure of the cell wall is one reason why *Nostoc* sp. TISTR 8872 biomass gave good organic acid yield (acetic acid and citric acid) after the fermentation by *L. brevis* subsp. *brevis* TISTR 868.

The effective fermentation period of concentrated *Nostoc* sp. TISTR 8872 biomass by *L. brevis* subsp. *brevis* TISTR 868 under anaerobic conditions was observed to be 48 h from the beginning of fermentation. Similarly, Kim *et al.* (2006) reported that fermentation of *C. reinhardtii* biomass by *C. butyrium* gave the highest organic acid production at 48 h. Bacterial fermentation and subsequent organic acid production is highly active, because it is at a stationary phase of growth (Ogunbanwo *et al.* 2003; Tango and Ghaly 1999).

### Hydrogen production from algal biomass fermentation by photosynthetic bacteria

*Rhodospseudomonas* sp. TISTR 1953 produced hydrogen at a rate of 10.38±0.53 ml/l/h from supernatant of *Nostoc* sp. TISTR 8872 biomass fermented by *L. brevis* subsp. *brevis* TISTR 868. That hydrogen production rate is two times higher than that reported by Ngamjarearnwong (2004) who used raw starch as an electron donor for *R. gelatinosus* SB24, *R. sphaeroides* SB46/1 and co-culture of the two, and produced hydrogen at a rate of 8.83, 0.00 and 5.43 ml/l/h, respectively. Those results indicated that supernatant from algal starch is more effective for hydrogen production than raw starch. There are many supported reasons why hydrogen production yield depends on organic acid and photosynthetic bacterial strains.



A hydrogen production rate of 0.01 l/l/h from malic acid 15 mM by *R. sphaeroides* O.U. 001 was reported by Eroglu *et al.* (1999). Barbosa *et al.* (2001) reported production of 25.2 ml/l/h, 10.7 ml/l/h, 7.6 ml/l/h and 1.1 ml/l/h by *Rhodopseudomonas* from acetic acid 22 mM, lactic acid 50 mM, butyric acid 27 mM and malic acid 15 mM, respectively.

## Conclusion

Cyanobacterial strain, *Nostoc* sp. TISTR 8872 was cultivated in optimal conditions, harvested of algal cells by centrifugation and concentrated 10 folds from original cell concentration. Then, the concentrated algal biomasses were converted into organic acids by anaerobic fermentation with 4 strains of anaerobic bacteria. In which the concentrated algal biomasses were hydrolyzed to 360.52±87.67, 1,338.49±153.95, 3,341.37±194.45 and 21.18±11.27 mM of malic acid, acetic acid, citric acid and butyric acid, respectively by *L. brevis* subsp. *brevis* TISTR 868 under dark anaerobic fermentation at 37°C for 48 h which gave the highest citric acid production. After that, fermentation products were centrifuged and the supernatant was harvested. The supernatant added with 10% yeast broth was the most suitable for photosynthetic bacterial growth in anaerobic condition, under illumination at 37°C for 96 h. Photosynthetic bacterial strain, *Rhodopseudomonas* TISTR 1953 gave hydrogen production rate of 10.38±0.53 ml/l/h. These results indicated that algal biomass was the best raw material for hydrogen production by a three-step microbial hydrogen producing system.


## Acknowledgement


The author was thankful to the Ministry of Energy, Thailand for the financial support to carry out this research work.

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