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Molecular hydrogen production by a thermotolerant *Rubrivivax gelatinosus* using raw cassava starch as an electron donor

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ABSTRACT: Thirteen strains of phototrophic purple non-sulfur bacteria selected from 226 isolates showed the ability to digest raw cassava starch at elevated temperature, 40°C, under illuminated anaerobic conditions. A selected strain, designated as SB24, produced more amylolytic enzyme activities toward raw and cooked cassava starch, when grown in cooked starch than when grown in raw starch. SB24 showed photoproduction of molecular hydrogen using raw cassava starch as an electron donor, when incubated with illumination under anaerobic conditions at 40°C. In small scale (23 mL) culture, SB24 produced hydrogen from raw cassava starch after 20-24 h of cultivation, with a 3-fold higher rate of H_2 production and a 3-fold higher total accumulation of hydrogen at 72 h, as compared to when it used malate as an electron donor. In the larger scale reactor containing a 5.5 liter culture of SB24, hydrogen was produced at an earlier time using raw cassava starch than using malate. In addition, the highest rate of H_2 production (38.79 mL H_2 liter culture^{-1} h^{-1}) by SB24 with raw cassava starch was 7 times higher than that with malate, while the total volume of H_2 accumulated with raw cassava starch (4.61 liters of H_2 at 90 h) was almost two-fold higher than with malate. Raw starch from rice, sticky rice, corn and mungbean, could also be used as electron donors for H_2 production by SB24 at 40°C. From morphological and biochemical characteristics, comparison of 16S rDNA sequence, and comparative studies of the bacterial characteristics, SB24 was found to be a thermotolerant anoxygenic phototrophic purple non-sulfur bacterium, *Rubrivivax gelatinosus*.

KEYWORDS: Hydrogen production; Photosynthetic bacteria; *Rubrivivax gelatinosus*; raw cassava starch.

INTRODUCTION

Hydrogen is a clean and efficient fuel, which may be a potential substitute for fossil fuels[1,2]. Compared to fossil fuels, H_2 has the advantage of producing only water, without pollutants such as CO, CO_2 and C_2H_6.

Biological hydrogen production stands out as an environmentally harmLess process occurring under mild operating conditions[2,3]. Several microorganisms may be used for hydrogen production, such as phototrophic green and purple bacteria, cyanobacteria, algae and some fermentative bacteria. The phototrophic purple non-sulfur bacteria produce a high ratio of molecular hydrogen to carbon dioxide ranging from 98:2 to 85:15 by volume. They can also utilize organic substrates as electron donors for hydrogen production[4,5,6]. Hydrogen metabolism has been studied in various phototrophic purple non-sulfur bacteria, such as *Rhodospirillum rubrum*[4], *Rhodomicrobium vannielii*[5], *Rhodopseudomonas capsulata*[6], *Rhodopseudomonas palustris*[7], *Rhodopseudomonas sphaeroides* or *Rhodobacter sphaeroides*[2,8], *Rhodopseudomonas gelatinosa* and *Rhodocyclus gelatinosus*[9].

Various factors have been studied in relation to bacterial hydrogen production, including kinetics of production[2] and enzymes related to production[7], as well as the inhibitory effects of NH_4^+ on gas production[10] and the enzymes involved[10,11]. In addition, various attempts have been made to enhance hydrogen production by using proper cultivation conditions, such as nitrogen atmosphere[12], with various organic acids as electron donors[5,13,14,15,16,17]. Various agricultural or industrial wastes containing organic acids and sugar residues have also been used, for example corn steep liquor from the sugar industry or waste products from the tofu industry[,18,19,20,21].

In addition, various studies have been performed to search for thermotolerant strains of phototrophic purple non-sulfur bacteria, producing hydrogen at elevated temperatures, which may be suitable for outdoor cultivation using solar energy[14,15,16]. Moreover, investigations have been made on the design of suitable reactors and the role of light for the most efficient production of hydrogen [22, 23, 24, 25, 26].

In the present study, a thermotolerant phototrophic purple non-sulfur bacterium was selected from various strains isolated in Thailand. This bacterial strain showed good ability to digest raw starch, especially cassava starch, which is generally poorly digested by microbes. It also showed photoproduction of hydrogen under laboratory scale conditions, using raw starch from cassava and other sources. Study at a larger scale, using a 5.5 liter reactor, showed that raw cassava starch could be used as a substrate to donate electrons for the production of hydrogen.

Materials and Methods

Assay of Amylolytic Enzyme Activity toward Cooked Starch

Amylolytic enzyme activity toward 1% cooked cassava starch was determined by measuring reducing sugars liberated from the enzyme reaction at 40°C after 10 min. The reaction mixture contained an equal volume of the enzyme solution and 2% cooked cassava starch in 50 mM phosphate buffer, pH 6.8. The amount of reducing sugars formed was determined by the Nelson-Somogyi method [27], using maltose as a standard. One unit of amylolytic enzyme activity toward cooked starch (CS unit) was defined as the amount of the enzyme that liberated reducing sugars equivalent to 0.1 μmole of maltose in 10 min at 40°C.

Assay of Amylolytic Enzyme Activity toward Raw Cassava Starch

The reaction mixture containing equal volumes of enzyme solution and 2% raw cassava starch in 50 mM phosphate buffer, pH 6.8, was incubated at 40°C for 4 h with shaking. Then, the remaining starch granules were removed by centrifugation at 4°C. The amount of reducing sugars in the resulting supernatant was determined by Nelson-Somogyi method, using glucose as a standard. One unit of amylolytic enzyme activity toward raw cassava starch (RS unit) was defined as the amount of the enzyme that liberated reducing sugars equivalent to 0.1 μmole of glucose in 1 h at 40°C.

Hydrogen Production

For small scale photoproduction of molecular hydrogen in a 23 mL culture, one percent (w/v) raw starch solutions from cassava, rice, sticky rice, corn and mungbean were compared to 30 mM malic acid. For larger scale photoproduction, only raw cassava starch was used as the source of electron donor. The photoproduction of hydrogen was carried out in Ormerod's medium[4] with 5 mM L-glutamate as nitrogen source, pH 6.8, under 10 klux light and anaerobic conditions at 40°C. Cell suspensions were sampled at the period of time defined in each experiment. Cells were subjected to alkaline lysis for protein determination. The protein content, the amount of reducing sugars and pH were determined. For anaerobic conditions, the 23 mL culture was grown under a N_2 atmosphere in a 30 mL side arm tube, while larger scale culture was performed by placing the 5.5 liter-broth into a flat-design 6.0 liter polyvinyl reactor.

The amount of hydrogen produced was followed by water replacement. The gas produced was shown to be hydrogen by gas chromatography (Shimadzu GC 9A) on a molecular sieve 5A column using argon as the carrier gas. The rate of hydrogen production (mL H_2 liter culture^{-1} h^{-1}) was determined in the 23 mL culture from the amount of gas produced from the first hour in which H_2 was detected (*e.g.* 24 h of incubation) until 72 h of incubation. The specific activity of hydrogen production (mL H_2 mg protein^{-1}) was determined from the rate of H_2 production per mg protein of cells. The highest rate of H_2 production (mL H_2 liter culture^{-1} h^{-1}) was determined from the amount of accumulated hydrogen at the period of time that showed the highest slope.

Protein Determination

Protein content was determined by the Lowry method [28] using bovine serum albumin as a standard.

Identification, Molecular Taxonomy and Phylogenetic Studies

Morphological and biochemical characteristics

Morphological and biochemical characteristics were studied based on Bergey's Manual of Determinative Bacteriology 9th edition[29] and Bergey's Manual of Systematic Bacteriology, 2nd edition[30].

16S Ribosomal DNA Sequence Determination and Phylogenetic Analysis

DNA extraction: Cells at late log phase were collected from 10 μL malate-glutamate Ormerod's medium at 40°C and resuspended into 400 μL 0.01 M Tris-EDTA buffer, pH 8.0 (TE buffer). DNA was extracted by cell breakage with 5 mL of 20 μg mL^{-1} proteinase K, 20 mL of 10% SDS, and 4 μL of 100 mg mL^{-1} RNase A, followed by strong vortex mixing. After incubation at 37°C for 30 min, final concentrations of 0.7 M NaCl and 1% CTAB were added. The solution was incubated at 65°C for 10 min. DNA was purified by chloroform extraction, recovered by precipitation with isopropanol, and

Table 1. Primers used in this study [31]

Primer	Sequence (5' –3')	Purpose
27F	AGA GTT TGA TCM TGG CTC AG	PCR amplification of 16S rDNA
1389R	ACG GGC GGT GTG TAC AAG	PCR amplification of 16S rDNA
520F	CAG CMG CCG CGG TAA T(A/T)C	Sequencing of 16S rDNA
520R	G(A/T)A TTA CCG CGG C(G/T)G CTG	Sequencing of 16S rDNA
750R	TAC CAG GGT ATC TAA TCC	Sequencing of 16S rDNA
SP6	ATT TAG GTG ACA CTA TAG	Sequencing of 16S rDNA

washed with 70% ethanol. The purified chromosomal DNA was dissolved in TE buffer and kept at –20°C.

PCR amplification of 16S rDNA: The purified chromosomal DNA was used as the template. PCR was performed using an automated thermal cycler (Perkin Elmer, Applied Biosystems Division.) with an initial denaturation at 95°C for 1 min, followed by a total of 25 cycles of 15 sec DNA denaturation at 95°C, 30 sec DNA annealing at 50°C and 2 min DNA extension at 72°C. The final extension was performed at 72°C for 4 min. The forward primer was 27F (5'-AGAGTTTGATCMT GGCTCAG-3') and the reverse primer was 1389R (5'-ACGGGCGGTGTGTACAAG-3') (Table 1). The primers generated a nearly complete 16S DNA fragment (approx. 1.5 kb). The PCR product was generated with AmpliTaqGold™ DNA polymerase (Perkin Elmer, Applied Biosystems Division.) and further cloned using the pGEM-T Easy cloning kit (Promega, Madison, WI, USA)[31].

Sequencing of 16S rDNA: The sequencing reactions were performed with a Big Dye™ Terminator Cycler Sequencing Kit (Perkin Elmer, Applied Biosystems Division). Sequencing was carried out with an ABI Model 377 DNA Sequencer (Perkin Elmer, Applied Biosystems Division). The primers for sequencing are as listed in Table 1. The 16S rDNA sequences were analyzed and aligned with selected reference sequences obtained from the GenBank database by the Blast N program at NCBI web site using CLUSTAL X ver.1.8 and CAP3.

Multiple-sequence alignments of the nucleotide sequences of SB24-16S rDNA and those of other phototrophic non-sulfur bacteria was made using both the CLUSTAL X1.8 and MEGA2 programs. Then, phylogenetic trees were constructed by either the Neighbor-Joining, UPGMA, Minimum Evolution or Maximum Parsimony methods. To evaluate the robustness of branches in the inferred tree, the bootstrap resampling method of Felsenstein with 100 replicates was used.

Results

Digestibility of Raw and Cooked Cassava Starch by Isolates of Thermotolerant Phototrophic Purple Non-Sulfur Bacteria

From 226 isolates of anoxygenic phototrophic purple non-sulfur bacteria, 13 strains gave clear zones with diameters greater than 2 cm on raw cassava starch in Ormerod's agar under light-anaerobic conditions at 40°C using the iodine method. Another 43 strains gave clear zones with diameters between 1-2 cm and 170 strains gave clear zones with diameters less than 1 cm.

The 13 strains described were selected and confirmed to be anoxygenic phototrophic purple non-sulfur bacteria, which grew better at 40°C than at 30°C. Supernatants from six-day cultures, grown in 15 mL of Ormerod's liquid medium containing raw cassava starch, were assayed for their extracellular digestive activity against the raw starch, compared to *Rhodocyclus gelatinosus* strain T-20 (Fig. 1A). Another set of six-day cultures, grown in cooked cassava starch medium, were assayed for their digestive activities against cooked starch (Fig 1B). Strain SB52 gave the highest amylolytic enzyme activity against raw cassava starch and was 3 times more active than T-20 (Fig. 1A). Strains SB24 and SB55 showed two-fold higher activity against raw cassava starch than T-20. In terms of ability to digest cooked starch (Fig 1B), SB24 and WFR(II)3 showed 1.5 times higher activity than T-20, while SB52 also showed 1.3 times higher activity than T-20. On the other hand,

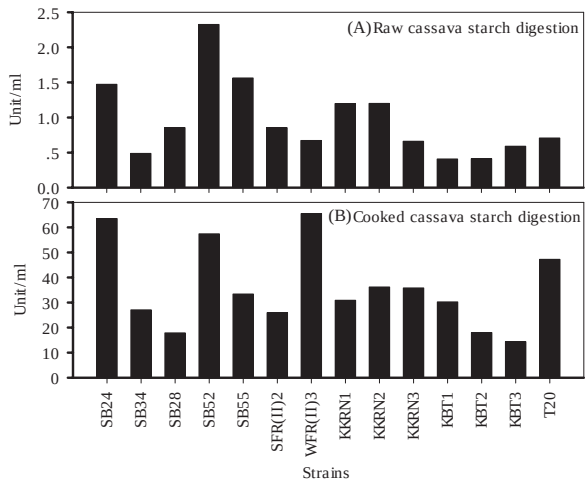

Fig 1. Amylolytic activities of cell free culture medium from 13 isolates, grown in Ormerod's medium containing raw or cooked cassava starch under light-anaerobic conditions at 40°C for 6 days. (A) Amylolytic activity against raw starch, from culture medium containing raw cassava starch. (B) Amylolytic activity against cooked starch, from culture medium containing cooked cassava starch. Results are compared to those of *Rhodocyclus gelatinosus* T-20.

SB55 had 1.4 fold less activity in digesting cooked starch than T-20.

Although SB52 gave the highest ability to digest raw cassava starch, it flocculated within 6 days when grown on raw starch. SB55 also showed flocculation with raw starch. Therefore, SB24 was selected for further studies on hydrogen production with raw cassava starch as the substrate.

Whether grown in raw or cooked cassava starch, SB24 exhibited amylolytic enzyme activities toward both raw cassava starch and cooked cassava starchHowever, when the amount of enzyme per mL of culture was compared after 6 days cultivation, cells grown in raw starch medium showed less amylolytic activities toward both raw and cooked cassava starch, compared to cells grown in cooked starch medium. Cells grown in cooked starch medium showed 2.9-fold higher amylolytic activity toward raw starch than cells grown in raw starch medium. Similar results were obtained with the cooked starch amylolytic activity. The cooked starch amylolytic activity obtained from cells grown in cooked starch medium was 1.2-fold higher than that obtained from cells grown in raw starch medium. Therefore, for study of amylolytic enzymes, growth of cells in medium with cooked starch would provide more amylolytic activity. This is because growth in cooked starch medium was faster than growth in raw starch mediumHowever, we decided to use raw starch as the source of electron donors for producing hydrogen, since this would save energy and time in cooking starch.

Hydrogen Production by SB24 in 23 mL Culture

For small scale hydrogen production in a 23 mL culture, SB24 started to produce hydrogen at 20-24 h of cultivation with 1% raw starch from various plants or with malic acid as electron donor, when grown under 10 klux light-anaerobic conditions at 40°C (Fig 2, Table 2). SB24 could digest all types of raw starch and continue to produce hydrogen for 72 h at 40°C (Fig 2). Hydrogen was produced in small amounts from raw rice starch after 20 h (Fig.2, Table 2), 4 h earlier than from other starches (cassava, sticky rice, corn, mungbean) or from malic acid. Accumulated H_2 within 72 h was the highest with raw rice starch (14 mL) while that with corn starch was the second best (12 mL) and 9-10 mL H_2 was obtained from the other starches (Table 2). With malate, only 3 mL H_2 could be produced by SB24 in the 72 h. Rates of hydrogen production (mL H_2 liter culture^{-1} h^{-1}), calculated from yield of gas from the first hour of H_2 detection until 72 h of cultivation, obtained with all types of raw starch were 3-4 times higher than that obtained with malic acid. The highest rates of hydrogen production were obtained with rice and corn starch were 10-11 mL H_2 liter culture^{-1}h^{-1}, while 8-9 mL H_2 liter culture^{-1}h^{-1} were obtained with cassava, mungbean and sticky rice, simultaneously, and only 3 mL H_2 Liter culture^{-1}h^{-1} with malic acid.

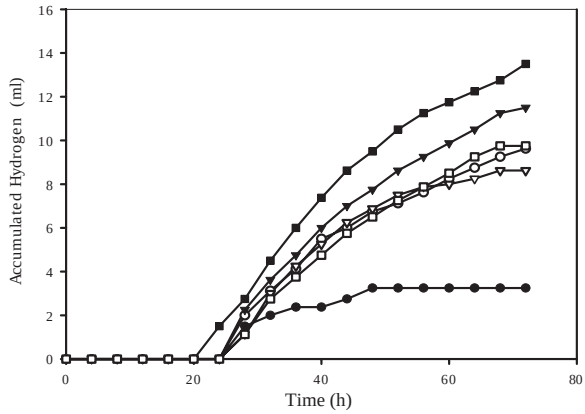

Fig 2. Hydrogen production from SB24 in a 23 mL reactor under anaerobic –illuminated conditions, with various raw starches or malic acid as electron donors. (■) rice starch, (▼) corn starch, (□) cassava starch, (○) mung bean starch, (▽) sticky rice starch, (●) malic acid.

Table 2. Production of Hydrogen by SB24 in 23 mL culture.

Source of electron donor	Total volume of hydrogen accumulated at 72 h(ml)	Time at which hydrogen production started (h)	Rate of hydrogen production* (ml H_2. litre culture^{-1}. h^{-1})	Protein of cells (mg.ml^{-1})	Specific activity of hydrogen production (ml H_2 mg protein $^{-1}$)
Raw cassava starch	9.75	24	8.83	0.26	1.63
Raw rice starch	13.5	20	11.29	0.60	0.98
Raw sticky rice starch	8.6	24	7.81	0.56	0.67
Raw corn starch	11.5	24	10.42	0.33	1.52
Raw mungbean starch	9.6	24	8.72	0.36	1.16
Malic acid	3.25	24	2.94	0.21	0.67

* Rate of hydrogen production was defined as the amount of H_2 produced per liter culture per hour during the time period extending from the time at which H_2 was first detected to 72 h of cultivation.

Interestingly, raw cassava starch appears to be a better electron donor for hydrogen production by SB24 compared to malic acid, which is commonly used by the purple non-sulfur bacteria. In addition, although the yield of H_2 accumulated at 72 h with raw cassava starch was less than that with rice and corn starch, the specific activity of hydrogen production was the highest with raw cassava starch (1.63 mL H_2 . mg protein^{-1}) and was 2.4 times higher than with malic acid (0.67 mL H_2 mg protein^{-1}). This suggests that raw cassava starch was the best source of electron donors for H_2 production by SB24, although, it was not the best carbon source for growth compared to other starches (Table 2).

Since large amounts of cassava are produced in Thailand and raw cassava starch is very cheap, the high specific activity of hydrogen production using raw cassava starch led us to use raw cassava starch for further studies of hydrogen production at a larger scale.

Hydrogen Production from SB24 in 5.5 Liter Culture

Raw cassava starch (10 g L^{-1}) was used as a source of electron donor on the semi-preparative scale using 5.5 liters Ormerod's medium in a 6 liter reactor under 10 klux light-anaerobic conditions at 40°C. Bacterial growth started at 20 h and gradually increased, reaching late logarithmic phase in 103 h (Fig 3). Photoproduction of hydrogen could be detected at 42 h in the early logarithmic growth phase, increased rapidly to yield 4.13 liters of H_2 accumulated at 78 h, and reached a maximum of 4.61 liters of H_2 accumulated at 90 h (Table 3, Fig 3). The highest rate of H_2 production was 38.79 mL H_2 liter culture^{-1}h^{-1} from 66 to 78 h (Table 3). Cell mass at 78 h was 130 mg.mL^{-1}. Therefore, SB24 could produce H_2 at the rate of 26 mL H_2 g DCW^{-1}h^{-1}However, the biomass of SB24 in the plastic reactor was difficult to be determined, because cells attached to the reactor surface at the logarithmic phase. This type of growth was mentioned by Kim *et al.*,[8] who studied hydrogen production by *Rhodopseudomonas sphaeroides* in Ormerod's medium, with DL-lactate as source of carbon and electron donor. Therefore, cell mass would tend to be underestimated, so that the specific rate of H_2 production in the larger scale reactor could not be determined.

Table 3. Production of hydrogen by SB24 in a 5.5 liter culture

Source of electron donor	Total volume of hydrogen produced (ml)	Starting time for hydrogen production (h)	Finishing time for hydrogen production (h)	Protein concentration at 166 hmg protein mL^{-1}	Rate of hydrogen production (mL H_2 liter culture^{-1} h^{-1}) during time period shown (h)
Raw cassava starch	4,610	42	90	0.306	38.79 (66-78)
malic acid	2,430	48	114	0.114	5.56 (48-66)

The level of reducing sugars in the culture rapidly increased from 31.5 μg. mL^{-1} at 24 h to 240 μg.mL^{-1} at 30 h, which is the logarithmic phase of growth Later, the reducing sugar level only increased slightly more, reaching a maximum of 332 μg.mL^{-1}. Thus, growth of SB24 appears to be accompanied by amylolytic enzyme digestion of raw cassava starch, releasing reducing sugars, which the cells assimilated to become electron donors for the hydrogen production.

The culture with 10 g L^{-1} raw cassava starch, after sterilization by irradiation, had an initial pH of 7.5. This increased slightly to pH 8.25 at the early logarithmic phase and to pH 8.57 at 30 h. Between 48 and 78 h,

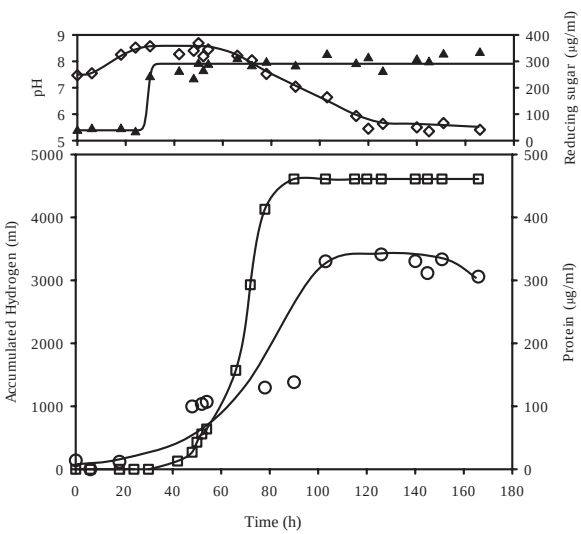

Fig 3. Hydrogen production, growth, pH and reducing sugar level during cultivation of SB24 in a 5.5 liter reactor, containing 5.5 liter culture medium, pH 6.8 with 1% raw cassava starch. Cells were grown under 10 klux light-anaerobic conditions at 40°C for 7 days. (□) accumulated hydrogen, (○) protein concentration in mg/ml, (▲) reducing sugars, and (◇) pH.

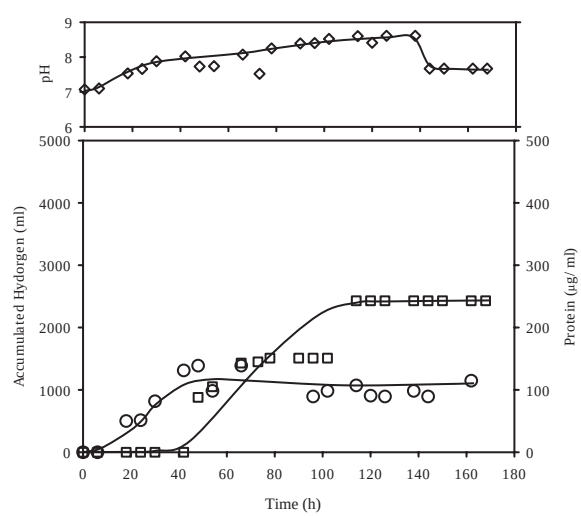

Fig 4. Hydrogen production, growth and pH during cultivation of SB24 in a 6.0 liter reactor, containing 5.5 liter culture medium, pH 6.8 with 30 mM malic acid as electron donor, under 10 klux light-anaerobic conditions at 40°C for 7 days. (□) accumulated hydrogen, (○) protein concentration in mg/ml (◇) pH.

when H_2 accumulation was rapid, the pH decreased gradually from 8.4 to 7.5. This suggests that the reducing sugars from digestion of raw starch were metabolized to organic acids, which were the actual electron donors for production of H_2. After 90 h, when H_2 production had stopped, reducing sugars produced from the excess starch continue to be metabolized to organic acids, which accumulate causing the pH to decrease further down to a final pH of 5.4 at the end of the experiment. Mizuno, *et al*., suggested that during the logarithmic phase of growth, protons from the organic acids produced are used for hydrogen production making the solution become alkaline, but when hydrogen production stops, protons are no longer being used, so the solution gradually becomes more acid[6].

Comparison of the two electron donors shows that SB24 started to grow faster in malic acid medium than in raw cassava starch medium (Fig 3 and Fig 4)However, raw starch was a better C-source for growth than malate, since raw starch yielded 2.7 times more biomass by the end of the logarithmic phase (Table 3). The yield of hydrogen accumulated with malate was 2.43 liters, which was 1.9 times less than the yield with raw cassava starch (Fig 4 and Table 3). In addition, the highest rate of H_2 production with raw cassava starch (38.8 mL H_2 liter culture$^{-1}h^{-1}$) was also 7 times greater than the highest rate of H_2 production with malate (5.56 mL H_2 liter culture$^{-1}h^{-1}$) (Table 3). The specific rate of hydrogen production per mg protein of cells in the reactor with raw starch was 0.424 mL H_2 mg protein$^{-1}h^{-1}$, which was higher when compared with that obtained with malate (0.114 mL H_2 mg protein$^{-1}h^{-1}$)

The morphological and biochemical characteristics of SB24 are summarized in Table 4. SB24 was a Gram negative bacterium, having a short rod shape and non-spore forming cells with a polar flagellum. It grew better at 40°C under light-anaerobic conditions than at 30°C. Biochemical characteristics showed that SB24 was an anoxygenic phototrophic purple non-sulfur bacterium, capable of photosynthesis using various organic compounds (*e.g.* malate, glutamate, lactate, succinate, glucose and fructose) as electron donors. Biotin and thiamine were necessary for growth. The bacteria could also liquefy gelatin. From its morphological and biochemical characteristics (Table 4), SB24 was similar to *Rhodocyclus gelatinosus* [29], except that SB24 could not use citrate and ethanol as an electron donors. From the data at this stage, we identified SB24 as *Rhodocyclus gelatinosus*, a thermotolerant phototrophic purple non-sulfur bacterium.

16S rDNA Analysis and Phylogenetic Studies of SB24

The nearly complete 16S rDNA sequence (1445 nucleotides) of SB24 was determined. The sequence was aligned and assembled using the CLUSTAL X 1.8 and CAP3 programs, simultaneously. Comparative taxonomy of the 16S rDNA sequence of SB24 was performed by comparison to sequences in the GenBank database using the BLAST N program at the NCBI website (www.ncbi.nlm.nih.gov/BLAST/). The 16S rDNA of SB24 was compared to that of *Rubrivivax gelatinosus* (current name of *Rhodocyclus*

Table 4. Morphological and biochemical characteristics of SB24 and other anoxygenic phototrophic purple non-sulfur bacteria from Bergey's Manual of Systematic Bacteriology, 9th edition[26]. C = coccus, P = polar flagella[8], R = rod, S = spirillum, SR = short rod.

Characteristics \ Strains		SB24	*Rhodocyclus gelatinosus*	*Rhodocyclus perpureus*	*Rhodocyclus tenuis*	*Rhodopseudomonas palustris*	*Rhodopseudomonas rutila*	*Rhodopseudomonas acidophila*	*Rhodobacter capsulatus*	*Rhodobacter sphaeroides*	*Rhodobacter sulfidophilus*
morphology		R	R	C	S	SR	R	C	SR	SR	R
flagella		P	P	-	P	P	P	P	P	P	P
electron donor	malate	+	+	+	+	+	+	+	+	+	+
	acetate	+	+	+	+	+	-	+	+	+	+
	aspartate	+	+	-	-	+/-	-	-	+/-	0	+/-
	benzoate	-	-	+	-	+	-	-	-	-	+
	butyrate	-	+/-	+	+	+	0	+/-	+	+	+
	citrate	-	+	-	-	+/-	+/-	+/-	+/-	+	-
	formate	-	+/-	-	-	+	-	+/-	+	-	+
	glutamate	+	+	-	-	+	+	-	+	+	+
	lactate	+	+	-	+	+	-	+	+	+	+
	propionate	-	+/-	-	+/-	+	+/-	+	+	+	+
	pyruvate	+	+	+	+	+	0	+	+	+	+
	succinate	+	+	-	+	+	+	+	+	+	+
	tartrate	-	+/-	-	-	-	-	+/-	-	+	-
	valerate	-	+	-	+	+	-	+	+	+	+
	glucose	+	+	-	-	+/-	-	+/-	+	+	+
	fructose	+	+	-	-	+/-	+	-	+	+	+/-
	mannose	-	+	-	-	+/-	0	0	-	+	+/-
	glycerol	-	-	-	-	+	+	+/-	-	+	+
	methanol	-	+/-	-	-	+/-	-	+/-	-	+/-	-
	ethanol	-	+	-	+/-	+/-	+	+	-	+	+/-
	mannitol	-	-	-	-	+/-	-	-	+/-	+	+/-
	sorbitol	-	-	-	0	-	0	0	+/-	+	+/-
	sulfide	-	-	-	-	+	0	-	+	+	+
	thiosulfate	-	-	-	-	+	-	-	-	-	+
gelatin liquefaction		+	+	-	-	-	-	-	-	-	-
vitamin	biotin	+	+	+	-	+/-	-	-	-	+	+
	thiamine	+	+	-	-	-	-	-	+	+	+
	p-aminobenzoic acid	-	-	+	-	+	-	-	-	-	+
	nicotinic acid	-	-	-	-	-	-	-	-	+	+

gelatinosus)[30], as shown in Fig. 5. The results showed 99% identity (1361 out of 1368 bases).

Phylogenetic trees constructed from multiple sequence alignment, with related rDNA by either the Neighbor Joining, UPGMA, Minimum Evolution or Maximum Parsimony metods showed that SB24 was most closely related to *Rubrivivax gelatinosus*. A typical phylogenetic tree for the relationship of SB24 16S rDNA to the 16S rDNA of other phototrophic bacteria and other hydrogen producing organisms is shown in Fig 6.

From morphological characteristics, biochemical

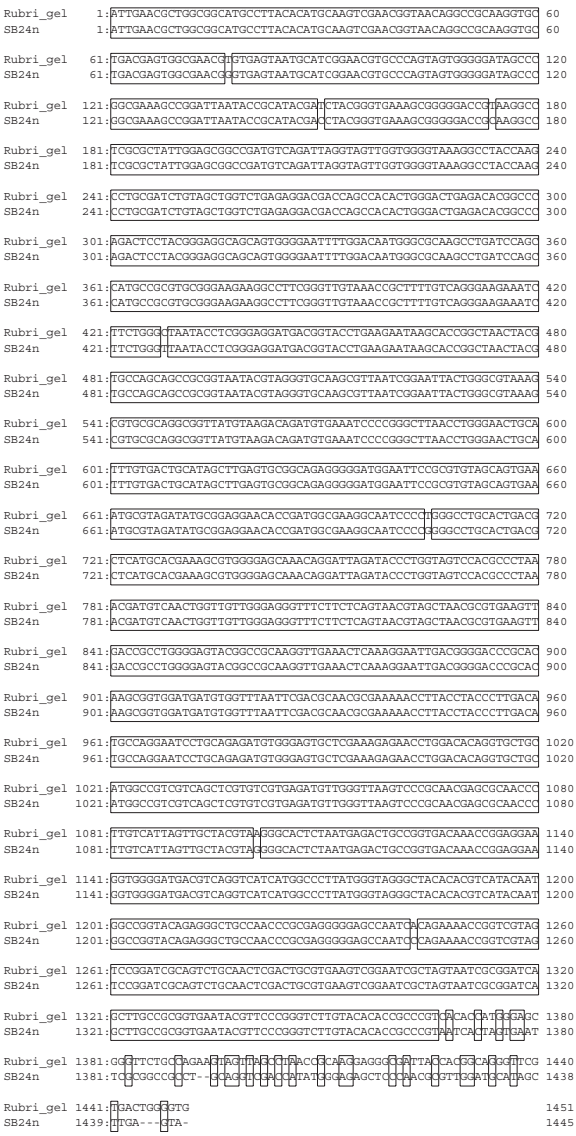

Fig 5. Comparison of 16S rDNA sequences from SB24 and *Rubrivivax gelatinosus*. BLAST analysis indicated 1361 identities from first 1368 bases (99%), with 2 gaps.

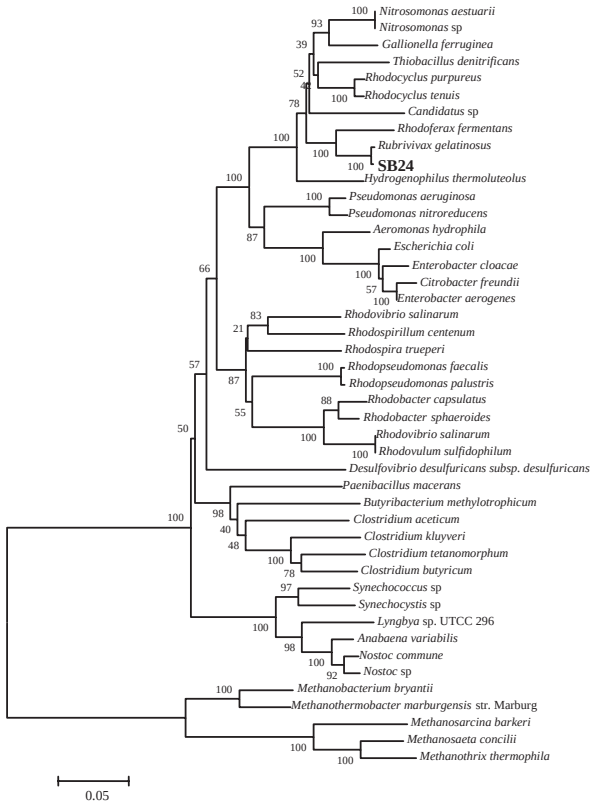

Fig 6. Phylogenetic relationship between SB24, other phototrophic purple non-sulfur bacteria and other hydrogen producing organisms, constructed by the Bootstrap percentages are written on internal branches, neighbor-joining method using the MEGA 2 program

characteristics, 16S rDNA molecular taxonomy, and capability for hydrogen photoproduction, it was concluded that SB24 was *Rubrivivax gelatinosus*, a thermotolerant phototrophic purple non-sulfur bacterium that could utilize raw starch as an electron donor to produce molecular hydrogen at an elevated temperature of 40°C.

Discussion

The biological photoproduction of molecular hydrogen, a clean fuel, was successfully demonstrated in laboratory scale using raw cassava starch as the substrate to provide electron donors. Cassava starch has been considered to be one of the most difficult starches for microorganisms to digest due to its complex structure. Raw starch granules are even more difficult to digest not only because of their structure, but also to low solubility in the aqueous phase, and especially in the case of cassava which contains cyanide, a toxic substance for cells, which still remains when no heating

has been done. Only 6% of the 226 isolates of purple non-sulfur bacteria showed the ability to digest raw cassava starch. Actually, only 3 strains were satisfactory according to their amylolytic activity against raw cassava starch. The selected strain, SB24, showed the ability to digest raw cassava starch at 40°C under anaerobic-illuminated conditions. When the ability of SB24 in raw cassava starch digestion was compared with that of *Rhodocyclus gelatinosus* T-20[9] under the same conditions, SB24 had 2 times higher capability. Not only raw starch from cassava but also that from rice, sticky rice, corn and mungbean could be digested by SB24, and become a source of electron donor in hydrogen production metabolism which occurred under anaerobic-illuminated conditions and at 40°C. The ability to grow and produce hydrogen at 40°C was a promising characteristic, since the strain could tolerate an elevated temperature when illuminated, which would result in a reduction in the cost of cooling the system.

Use of morphological and biochemical characteristics, molecular taxonomy based on 16S rDNA sequence, and comparative metabolic studies allowed the identification of SB24 as a thermotolerant phototrophic purple non-sulfur bacterium, *Rubrivivax gelatinosus*.

In 23 mL-scale of H_2 production, yields of H_2 accumulated by SB24 with all kinds of raw starch were higher than those obtained from malate, the commonly used electron donor [8,13,15,16]. The rates of H_2 production with the raw starch were 3-4 times higher than with malate. In this small scale, SB24 started to produce H_2 with all sources at almost the same time, 24 h, except it was 4 h sooner with raw rice starch. The accumulated hydrogen obtained at the 72 h with all of the raw starches was 3-4 times higher than that with malic acid. Raw rice and sticky rice starches were good sources for growth compared to cassava, though, the highest specific activity of H_2 production (1.63 mL H_2 mg protein $^{-1}$) was found with raw cassava starch. In conclusion, with raw cassava starch in the 23 mL culture, the yield of H_2 accumulated at 72 h was 9.75 mL and the rate of the H_2 production was 8.83 mL H_2 liter culture^{-1}h^{-1} with the specific activity of H_2 production equal to 1.63 mL H_2 mg protein^{-1}.

In the 5.5 liter-scale of H_2 production, raw cassava starch was effectively converted into an electron donor for SB24 to produce H_2. The photoproduction of H_2 started in the 42nd h, rapidly accumulated to 4 liter by the 78th h, and reached a maximum yield of 4.6 liters at 90 h. The highest rate of H_2 production by SB24 was 38.79 mL H_2 liter culture^{-1}h^{-1} or 26 mL H_2 g DCW^{-1}h^{-1}, which occurred between 66 and 78 h of cultivation. However, because cells attached to the reactor surface at the logarithmic phase and caused difficulty in sampling for biomass determination, we rather report the highest rate of H_2 production in mL H_2 liter culture^{-1} h^{-1}. From the results of H_2 production, pH and reducing sugars changes in the reactor, it could be concluded that the bacterium digested raw cassava starch into reducing sugars, which were assimilated to become electron donors for the hydrogen production metabolism. Ten g L^{-1} of raw cassava starch in the 5.5 liter culture could be converted into the accumulated H_2 with a volume of 4.6 liters and 350 μg protein mL^{-1} of gained cell mass. Moreover, SB24 could produce 2 times more H_2 with raw cassava starch than with malic acid.

Compared to some previous reports that mostly suggested the production of hydrogen by the phototrophic purple non-sulfur bacteria by using organic acids as electron donor, *Rhodobacter sphaeroides* O.U.001 was reported to produce H_2 lower than SB24 utilized raw cassava starch. *Rhodobacter sphaeroides* O.U.001 produced only 5.3-7.2 mL H_2 g DCW^{-1}h^{-1} when 30 mM malate was used [32,33] and even less, 2.4 mL H_2 g DCW^{-1}h^{-1} (or 10 mL H_2 liter culture^{-1}h^{-1}) when 15 mM malate was used[34]. As well, *Rhodopseudomonas* sp. and *Rhodopseudomonas palustris* with 15 mM either citrate or malate, as an electron donor yielded 1.1 and 5.8 mL H_2 liter culture^{-1}h^{-1}, respectively[35], which was also less than the yield obtained from SB24 in this report. Organic wastes, such as wastewater containing amino acids, organic acids and/or protein, from starch and tofu factories were also used as sources of electron donors. Wastewater from sugar refining was utilized by *Rhodobacter sphaeroides* O.U.001 to yield H_2 at the lower rate of 6 mL H_2 liter culture^{-1}h^{-1} [19]. Wastewater from a tofu factory was utilized by immobilized *Rhodobacter sphaeroides* RV cells and yielded H_2 at the rate of 15 mL H_2 liter culture^{-1}h^{-1}, which could be enhanced depending on the reactor system.[20,21] However, Hillmer and Gest [6] reported a higher yield of H_2 produced by *Rhodopseudomonas capsulatus* with 30 mM malate. *Rhodopseudomonas capsulatus* produced 90 mL H_2 g DCW^{-1}h^{-1} which was 3.5 times higher than SB24. However, organic acids are more expensive than the cassava starch. *Rhodopseudomonas sphaeroides* B5 was also reported to produce a very high yield of H_2 at the rate of 138 mL H_2 gDCW^{-1}h^{-1} with 30 mM malate in a 32 liter reactor under the sunlight[8]. There were many factors affecting the H_2 production metabolism, not only kinds and amount of electron donors but also light intensity, time of light illumination and size and configuration of the reactor. Though the yield of H_2 by SB24 utilizing cassava starch was not so high as the latter 2 reports, there are advantages of this system to be considered. The advantages of using cassava starch to organic acids as source for H_2 production was not only the production of H_2, a clean fuel, but also the use of a renewable agricultural resource, which has recently

been exported at a cheap price. Moreover, SB24 could utilize raw cassava starch, which resulted in the reduction of time and energy in production of solubilized starch. It is also noteworthy that photoproduction of H_2 by this bacterium could be performed at the elevated temperatureof 40°C, since the temperature in Thailand can be high. Therefore, hydrogen production may be feasible with solar energy rather than under illumination.

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