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Bioenergetics of lactate vs. acetate outside TCA enhanced the hydrogen evolution levels in two newly isolated strains of the photosynthetic bacterium *Rhodopseudomonas*

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Abstract: Two local hydrogen-evolving strains of purple nonsulfur bacteria have been isolated, characterized, and identified as *Rhodopseudomonas* sp. TUT (strains Rh1 and Rh2). Lactate followed by succinate and malate supported the highest amounts of H_2 production, growth (O.D.660_{nm}, proteins and bacteriochlorphyll contents), nitrogenase activity, and uptake hydrogenase; the least of which was acetate. Alginate-immobilized cells evolved higher hydrogen amounts than free cell counterparts. Rh1 was more productive than Rh2 at all circumstances. Lactate-dependent hydrogen evolution was more than twice that of acetate, due to ATP productivity (2/−1, respectively), which is limiting to the nitrogenase activity. The preference of lactate over other acids indicates the feasibility of using these two strains in hydrogen production from dairy wastewater.

Keywords: hydrogen evolution; immobilization; organic acids; *Rhodopseudomonas*.

1 Introduction

Phototrophic H_2 production has the advantage of high theoretical substrate conversion efficiency and mineralization of organic substrates, e.g. [1]. Many agricultural, food and industrial waste effluents such as cheese wastewater [2], palm mill oil effluents [3], sugar processing effluents [4], corn wastes [5], and orange peel [6] can be used as substrates for bacterial growth because they contain biodegradable compounds. Fang and Liu [7] observed that the specific hydrogen production rate can vary depending on both the waste type used as substrate and various operational parameters, such as pH, temperature, and nutrient supply. Elsamadony [8] reported hydrogen production from organic fraction of municipal solid waste via dry anaerobic digestion. Purple nonsulfur bacteria are the most promising group of microorganisms as compared with other microbial systems, not least due to high substrate to product conversion yields [9]. Their capability to synchronize electrons generated from both oxidizing organic substrates and light absorption makes them superior over the oxygenic photosynthetic organisms (cyanobacteria and green algae) due to oxygen hindrance of the process [10, 11].

In Egypt, with different phytogeographical habitats varying from subtropical to temperate, studies carried out on photosynthetic bacteria are scarce. All water bodies and waterlogged soils in such regions, certainly harbor unlimited population of bacterial genera and species, including photosynthetic bacteria with all different groups (sulfur, nonsulfur purple and green sulfur bacteria). However, only few and sporadic publications were available to the authors. Abdel-Malek and Truper [12] isolated and identified the salinity tolerant *Ectothiorhodospira abdelmalikai*. Shoreit and his collaborators published a number of works on isolating and studying some physiological activities of purple nonsulfur and purple sulfur [13].

This article was planned to isolate and characterize the hydrogen-evolving purple nonsulfur bacteria from various habitats in Egypt. Besides, it includes identifying

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the most efficient carbon substrate (lactate, malate, succinate, or acetate) in supporting bacterial growth and hydrogen evolution. Therefore, a catabolic pathway comparing lactate and acetate (highest and lowest efficient carbon sources) at hydrogen evolution conditions, i.e. anaerobic vs. aerobic, is postulated.

2 Materials and methods

2.1 Isolation and identification of bacteria

The two strains were isolated from organic-rich water ditch and waterlogged plants' rhizosphere growing at Assiut University, Egypt. After purification of isolates on RÄH media, preliminary characterization was carried out according to the methods described in Bergey's Manual [14, 15]. Diagnostic media of general use was prepared as recommended by Harrigan and Mecance [16]. Isolates were phenotypically examined (Gram stain, motility, morphological and biochemical characteristics). Biochemical tests applied for characterization the isolates were catalase activity [17] and utilization of different carbon sources [18].

2.1.1 Scanning electron microscopy and photography

Cell samples were dehydrated, dried in Gold Sputter Apparatus, the samples were evenly gold coated at a thickness of 15 nm by JEOL JSM 5300 Lv scanning electron microscope; the cells were then examined at 15 kv and photographed.

2.1.2 Genotypic identification

Identification of our isolates was conducted on the bases of 16s rRNA base sequence analysis after extracting the total genomic DNA [19], followed by PCR amplification of the 16s rRNA gene. The PCR product was sequenced using DNA sequencing Services in Sigma Company, South Korea. The nucleotide sequence data was aligned using the ClustalW and a multiple sequence alignment, molecular phylogenetic analyses, and the phylogenetic tree were carried out by the DNASTAR Lasergene (V.7.1) program. For nomenclature of the strains, the *Rhodopseudomonas* sp. strain Rh1 and Rh2 have been used as they have unique sequencing not similar to any known species, but are very close to *R. faecalis* and *Rhodopseudomonas* sp. TUT. The Phylogenic tree is given in Figure (S4).

2.2 Analytical methods

2.2.1 Turbidity (O.D.)

For fast assessment of growth, O.D.$_{660nm}$, was followed every 24 h over a period of 9 days spectrophotometrically (Thermoscientific, double beam spectrophotometer, Evolution 160, UV-VIS, Germany). All the experiments presented in this article were started by a constant inoculum giving a final absorbance of 0.2 A.

2.3 Assessment of bacteriochlorophyll

Bacteriochlorophyll was detected using the spectrophotometeric method recommended by Drews [20]. A known volume (0.5 ml) of bacterial cells was extracted with 5-ml methanol and the extinction was measured against a blank of methanol at two wavelengths (660 and 770–772 nm). The concentration of bacteriochlorophyll *a* was calculated using the absorption coefficient of 46.1 l/g·cm [21].

2.3.1 Determination of soluble protein contents

Soluble protein contents were estimated in water extracts according to the method of Lowry et al. [22].

2.3.2 Estimation of H_2 produced

The volume of evolved hydrogen gas was estimated in a graduated glass cylinder filled with water and inverted in a water jar. Hydrogen evolution bottles contained nutrient cocktail (70 ml phosphate buffer, 70 ml early log phase bacteria, with different concentrations of lactate and completed with H_2O to 700 ml). The bottles were, then, stoppered and stirred (as long as hydrogen was evolving) at light emulation of 100–300 lux and a temperature of 30 °C. The gas produced was passed over saturated NaOH solution to absorb carbon dioxide. Molecular hydrogen was then confirmed by TCD-GC (Thermal Conductivity Detector Gas Chromatography, Thermo Scientific, Germany) and the carrier gas was nitrogen gas [23].

2.3.3 Assessment of nitrogenase activity

It was assessed by the acetylene reduction technique according to Hardy et al. [24] using gas chromatography

FID-GC (Flam Ionization Detector Gas Chromatography, Thermo Scientific, Germany).

2.3.4 Assessment of uptake hydrogenase activity (Hup)

The hydrogenase uptake activity (Hup) has been assayed in a mixture containing methylene blue flushed with hydrogen gas [25].

2.4 Immobilization

Alginate beads (0.2 and 0.4 mm diameter) were used for cell immobilization to enhance growth and hydrogen evolution by the examined bacteria. Log phase cells of bacteria (300 mg/ml at 3–4 days old) were used to form beads such as in [26].

2.5 Statistical analysis

Statistical analysis of the data was conducted using ANOVA one-way test (analysis of variance) by SPSS program version 16.0 and Dunken; values were determined at 0.01 levels.

3 Results

3.1 Description of the studied strains

Rh1 and Rh2 are motile purple nonsulfur bacteria (PNSB), Gram-negative rods (0.3–0.5 × 0.6–3.2 and 0.4–0.6 × 0.7–2.7 µm for Rh1 and Rh2, respectively). Optimal temperature for their growth was 30 °C; optimal pH was 6.5–7.0; tweens 80 was hydrolyzed, but casein was not; H_2S was not produced (Table S1). They are both reducing acetylene, i.e. fixing nitrogen. They reproduce by budding and their buds were very clear at the two isolates (Figure S1a,b). Their color varies from light red in Rh1 (Figure S2a) to dark red in Rh2 (Figure S2b). Such color is ascribed to Chl. *a* and carotenoids but not to Chl. *b* (Figure S3).

The phylogenetic tree shows the genetic relationship among the different representative strains (Figure S4). Identification of these two isolates according to DNA analysis and 16s rRNA revealed that they are affiliated to the genus *Rhodopseudomonas* sp. TUT by similarity of 97% and 95 % for Rh1 and Rh2, respectively. Comparison with other known *Rhodopseudomonas* spp. (Table S2) indicated certain differences, which suggested that these isolates could be designated as new *Rhodopseudomonas* strains.

3.2 Growth of the studied strains

Although these two isolates belonged to the same species, they exhibited some differences in their physiological activities. Rh1 and Rh2 exhibited no preferential characteristics of growth at the three examined nitrogen forms (ammonia, nitrate, or glutamate); subsequently, ammonia was solely provided to these strains as the original nitrogen source in RÄH medium (data not shown). However, the two strains displayed varied responses to the carbon sources provided. A maximum O.D., soluble proteins and B. Chl. was obtained at 40 mM of all organic acids; higher concentration of 50 mM significantly suppressed the bacterial growth. Preference of the different organic acids was in the following order: lactate > succinate ≥ malate > acetate (Figures 1 and S5a,b).

3.3 Nitrogenase, hydrogenase activity, and accumulated hydrogen gas

Nitrogenase activity was highly enhanced in Rh1 compared with Rh2 (Table 1). Nitrogenase activity of *Rhodopseudomonas* sp. TUT (Rh1) exhibited almost the same pattern of growth; the highest N_2-ase activity was obtained at 40 mM lactate (Figure S6) whereas the least activity has been displayed at acetate (Table 1). Malate and succinate induced closely similar rates of activity in-between lactate and acetate. Hup activity has also been gradually enhanced by increasing the concentration of organic acids (Figure S7), showing maximum rates of activity also

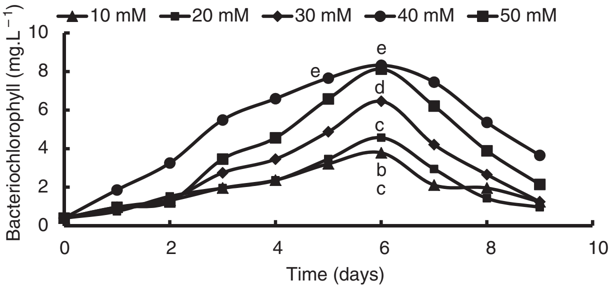

Figure 1: Growth (bacteriochlorophyll) of the PNS (*Rhodopseudomonas* sp. TUT, Rh1) at different concentrations of lactate. Values were recorded at 24-h intervals throughout the growth period of 9 days on RÄH medium at 30 °C ± 1 under light intensity provided by a tungsten lamp (3000 lux).

Table 1: Maximum values of Rh1 (1) and Rh2 (2) recorded at the sixth day of growth on different organic acids at 30 °C in the light.

	Malate	**Succinate**	**Lactate**	**Acetate**	**Acetate/lactate (%)**
O.D.1	4.56f	2.236e	3.995e	2.214e	55.42
O.D.2	2.85bc	2.987c	3.325c	2.45b	73.68
Protein 1 (mg/ml)	69.50e	68.59e	78.56f	45.36e	57.74
Protein 2 (mg/ml)	44.69c	45.23c	54.82d	35.69b	65.10
B.Chl. 1 (mg/ml)	8.23f	5.124f	8.325f	4.92e	59.14
B.Chl.2 (mg/ml)	4.13d	4.26d	4.82de	2.51b	52.16
N_2-ase 1 (nm·mg protein^{-1}·h^{-1})	2215f	1658f	2700d	1150d	42.59
N_2-ase 2 (nm·mg protein^{-1}·h^{-1})	1050bc	1305d	1658e	845b	50.97
Hup 1 (nm·mg protein^{-1}·min^{-1})	48.56f	38.56e	52.36f	26.56d	50.73
Hup 2 (nm·mg protein^{-1}·min^{-1})	38.56bc	41.23c	49.36d	30.45b	61.69
Accumulated H_2 1 (ml/culture)	1450cd	1630d	1955e	850b	42.46
Accumulated H_2 2 (ml/culture)	955d	950d	1400e	610b	43.57
Accumulated H_2 1 + alginate (ml/culture)	1930d	1870d	2450e	1300c	53.06
Accumulated H_2 2 + alginate (ml/culture)	1350de	1350de	1950e	830c	42.56

Letters next to values represent significance between the two strains at *p* 0.01%.

at 40 mM lactate relative to malate, succinate, and acetate (Table 1).

The accumulated hydrogen of Rh1 exhibited levels slightly different from the regular order of preference in growth, i.e. at lactate and succinate values of evolved hydrogen was closely similar but at acetate, it was significantly less than 50% that at lactate (Figure 2). Such amounts of hydrogen were 1955, 1630, 1450, and 850 ml/culture at lactate, succinate, malate, and acetate; respectively (Table 1), while the second isolate Rh2 exhibited less amounts (1400, 955, 950, and 610 ml/culture).

All the studied parameters (growth, nitrogenase activity, and hup activity) of the two organisms at the different organic acids exhibited their highest levels at the sixth day of growth (end of the log phase and onset of decline phase). Alginate immobilization intensified growth (as protein contents, nitrogenase, and hup activity) of Rh1 and Rh2 by the same proportions (Figure S8a–f) and accumulated hydrogen (Figure 3). Figure (S9) shows hydrogen evolution evolved upon utilizing domestic whey, the source of lactate; Rh1 evolved 250 ml, whereas Rh2 evolved 150 ml.

Table 1 summarizes a comparison between the two strains at the different organic acids. Rh1 shows higher level of activities than Rh2 and lactate was significantly the most efficient organic acid than the other organic acids.

4 Discussion

Two strains belonging to the purple nonsulfur bacterium *Rhodopseudomonas* sp. TUT were locally isolated,

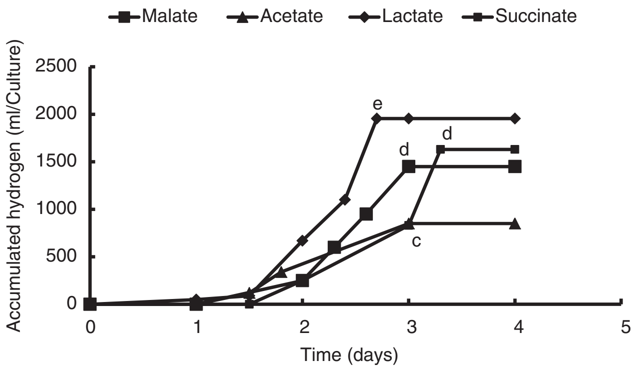

Figure 2: Accumulated hydrogen of the PNS Rh1 (*Rhodopseudomonas* sp. TUT) under malate, acetate, lactate and succinate as different C-forms.

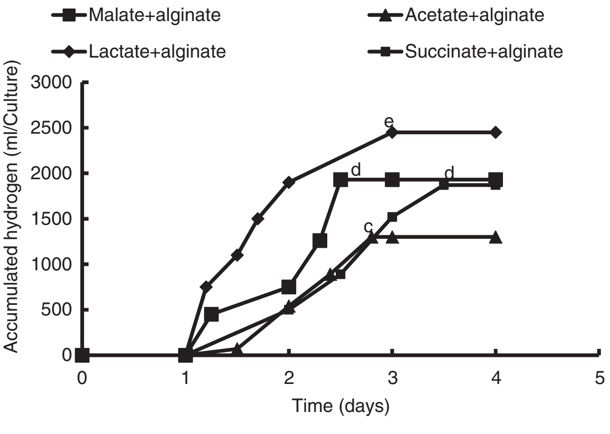

Figure 3: Accumulated hydrogen of the PNS Rh1 (*Rhodopseudomonas* sp. TUT) immobilized in Na-alginate at malate, acetate, lactate, or succinate.

identified, characterized, and referred to as Rh1 and Rh2 on phyllogenetic and molecular bases. In both strains, hydrogen produced from lactate was much more efficient than malate and succinate; the least was observed at acetate (less than 50% that of lactate). In accordance with this, the wild type *Rhodobacter sphaeroides* ATCC 17023 was reported to produce 2.31 ml H_2/ml culture using 30 mM lactate, 2.00 ml H_2/ml culture using 30 mM malate, 1.62 ml H_2 /ml culture using 30 mM glucose, and no hydrogen using 30 mM of acetate [27]. In addition, *Rhodopseudomonas palustris* and other microbial strains preferably used lactate [28]. Preference of lactate utilization is a privilege for the cells expressing lactate dehydrogenase (LDH); the key enzyme in lactate oxidation.

The literature usually focused on the bioeneregetics of organic acids inside the tricarboxylic acid cycle (Krebs cycle) only. For example, lactate (upon oxidation to pyruvate) and malate could easily enter the TCA cycle to meet the energy needs of the cell for hydrogen production [29]. However, TCA is an aerobic process, which should not (or minimally if any) proceed at the anaerobic conditions of hydrogen evolution. Therefore, we present here a postulated bioenergetic mechanism for releasing energy anaerobically from lactate outside (and excluding) the TCA (Figure 4). This figure is composed of two sections: one is anaerobic (lactate oxidation) and the other is aerobic (acetate, malate, or succinate oxidation), i.e. it shows the relevance of lactate at anaerobic conditions to supplement carbon and energy for hydrogen evolution while acetate, malate, or succinate cannot be oxidized in Krebs (TCA) cycle, since it proceeds only aerobically conditions. In bacteria expressing the LDH enzyme, lactate oxidation produces 2 moles NADH/mole lactate (= 6ATP) before being incorporated as acetyl CoA to TCA (one from lactate oxidation into pyruvate by LDH and the other NADH molecule from pyruvate oxidation into acetyl CoA by pyruvate dehydrogenase). However, the enzymatic conversion of acetate to acetyl CoA consumes 1 ATP to AMP (mole/mole) by acetyl CoA synthetase. Therefore, hydrogen evolution fluctuates in concomitance with ATP availability from the different substrate (lactate, malate, succinate, or acetate). Liu [30] reviewed lactate and pyruvate by lactic acid bacteria. In purple nonsulfur bacteria (PNSB), hydrogen evolution is exclusively a nitrogenase activity [31, 32]. As other hydrogen evolving purple nonsulfur bacteria, hydrogen evolution of the herein-studied bacteria was exclusively a nitrogenase activity. Since the nitrogenase-catalyzed hydrogen evolution is ATP dependent, the transfer of an electron through nitrogenase is accompanied by the hydrolysis of two ATPs. Therefore, to evolve one H_2 molecule by nitrogenase, four ATPs are required. The six ATPs produced from lactate oxidation fulfils the energy needs of nitrogenase. In this context, the inherent hydrogenase activity of the nitrogenase enzyme was proposed to be a "safety valve" for the adjustment of reducing power of the cell. However, in vitro hydrogen production using *Azotobacter vinelandii* showed that at least 25% of the electrons are used to reduce protons to molecular hydrogen even in the nitrogen pressure of 50 Atm [33], i.e. hydrogen is produced independent on nitrogen fixation and hydrogen evolution precedes N_2 fixation. Hydrogen acts as electron donor via the uptake hydrogenases, which catalyzes the conversion of molecular hydrogen into protons and electrons. Electrons are transferred via ubiquinone (UQ) to the photosystem (PS) where they are repeatedly energized by light and cycled through the photosynthetic electron transport chain to produce a proton gradient, which is used to produce ATP. The photoautotrophic mode of growth potentially consumes regenerating energy from reducing equivalents for CO_2 reduction [34].

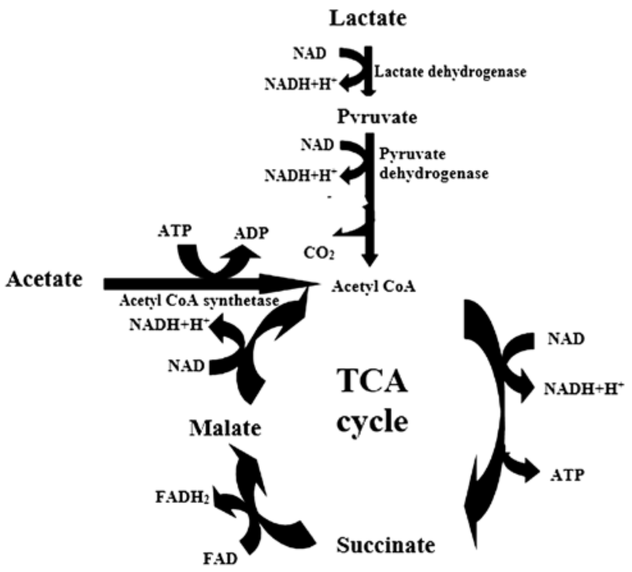

Figure 4: A postulated pathway of lactate, pyruvate, acetate, malate, and succinate under conditions of hydrogen evolution (anaerobic) in the purple nonsulfur bacteria (Rh1 and Rh2). This figure is composed of two sections: one is anaerobic (lactate oxidation) and the other is aerobic (acetate, malate, or succinate oxidation), i.e. it shows the relevance of lactate at anaerobic conditions to supplement carbon and energy for hydrogen evolution while acetate, malate, or succinate cannot be oxidized in Krebs (TCA) cycle that proceeds only at aerobic conditions.

Despite the above discussion, numerous other bacteria utilize acetate more efficiently than these under study. Within this context, the removal of lactate from the feeding media of *Rhodobacter capsulatus* did not influence hydrogen productivity significantly [35]. Instead, acetate and glutamate were prior for hydrogen production and bacterial growth in this strain. Acetate (10–20 mM) is a substrate that typically supports robust and photoheterotrophic growth of PNSB [36]. Acetate entering the TCA cycle at the level of acetyl-CoA needs to use side pathways where C4 acids such as malate and succinate. Different PNSB employ glyoxylate, citramalate, and ethylmalonyl-CoA pathways to utilize acetate [37]. The highest conversion (72.8%), hydrogen evolved (269 ml H_2 per vessel), hydrogen production rate (25 ml $H_2^{-1} \cdot h^{-1}$). However, our strains of *Rhodopseudomonas* sp. also showed better performance with malate at 40 mM, which is once again in a good agreement with the same authors [38].

The preference of lactate over acetate specifically indicates the feasibility of using these two strains in hydrogen production from dairy industry wastewater. Rh1 and Rh2 evolved volumes of 250 and 150 ml hydrogen when substituting whey for the organic acids. Lower hydrogen amounts would be ascribed to a probable less nutrients contained in whey than in RÄH medium, supplemented with organic acids and thus less energy availability for nitrogenase activity to evolve hydrogen molecules. Although such lower amounts of hydrogen indicate less nutritional value of whey than the organic acid-enriched media, whey would be utilized for hydrogen production instead of being a waste. Furthermore, whey might be supplemented with essential nutrients for more feasible cost/benefit ratio.

Alginate-immobilized cells (0.4 mm), under identical experimental regime, evolved almost one and half times higher H_2 than their respective free cell counterparts. Growth (O.D.660$_{nm}$, proteins and bacteriochlorphyll contents), nitrogenase activity, uptake hydrogenase (Hup) activity responded to the organic acids at the same order of preference as hydrogen evolution (lactate > succinate ≥ malate > acetate). Alginate immobilization induced higher amount of hydrogen in malate followed by lactate and succinate [26, 39]. Mathews and Wang [40] showed mixed acid fermentation for biohydrogen production in *E. coli* by the hydrogen-evolving formate–hydrogen lyase (FHL) complex.

5 Conclusions

- The strains under study are able to evolve hydrogen from dairy wastewater as they exhibited the highest efficiency in the presence of lactate; the efficiency might be further enhanced by enriching the whey with nutrient supplements.
- Alginate immobilization was also stimulatory to hydrogen evolution.

Compliance with Ethical Standards: The work complies with the Ethical Standards.

Conflict of interest statement: All authors declare that they have no conflict of interest.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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