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Characterization of a Hydrogen evolving strain of *Rhodopseudomonas sphaeroides*

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수소생성 *Rhodopseudomonas sphaeroides* 의 特性

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Many photosynthetic bacteria capable of hydrogen production were isolated from samples of mud flats of paddy field collected in Kim Hae and Dae Jeo. A strain 230 was selected for the high capability of hydrogen evolution. As the results of examination in physiological, morghological and cultural characteristics, the strain 230 was identified as *Rhodopseudomonas sphaeroides*.

Photosynthetic bacteria ubiquitously distributed. They can be isolated from natural waters, soils, sewages, and related environments (Medigan, 1984). They have been implicated as significant contributors to the total fixed nitrogen of rice paddies in certain areas of the world (Kobayashi and Haque, 1971). They can use light energy to produce the ATP required for biosynthesis. The reducing power is derived from the oxidation of organic substances or inorganic compounds other than water, and consequently, O_2 is not produced. Photosynthetic bacteria grown with N_2 or certain amino acids produce large quantities of molecular hydrogen when illuminated anaerobically in the presence of accessory electron donors (Ormerod et al., 1961). Hydrogen evolution has been suspected to be mediated by nitrogenase (Colbeau et al., 1980 Kim et al., 1980). For this reasons, photosynthetic bacteria were isolated from mud flats of paddy field in Kim Hae and Dae Jeo and identified.

MATERIAL AND METHODS

Bacterial Strains

The strains were isolated from mud flats of paddy field in Kim Hae and Dae Jeo in Korea.

Media

The mineral media contained: Basal salt solution 100ml; KH_2PO_4, 0.4g: K_2HPO_4, 0.6g per liter distilled water. Basal salt solution contained (per litter distilled water): $MgSO_4 \cdot 7H_2O$, 2.0g; $CaCl_2 \cdot 2H_2O$, 0.75g; $FeSO_4 \cdot 7H_2O$, 0.118g; EDTA, 0.2g; $MnSO_4 \cdot 4H_2O$, 0.21g; H_3BO_3, 0.28mg; $Cu(NO_3)_2 \cdot 3H_2O$, 0.004mg; $ZnSO_4 \cdot 7H_2O$, 0.024mg; $Na_2MoO_4 \cdot 2H_2O$, 0.075mg. The complete media were added with 30mM sodium succinate, 7mM glutamate and 2% (w/v) yeast extract. The pH was adjusted to 6.8 with 1M KOH prior to autoclaving. When solid media were required, agar was added to 1.5-2.0% (w/v).

Enrichment and Isolation

Enrichment was performed in screw-capped

bottles completely filled with complex medium and transferred several times (Malik and Schlegel, 1980). Isolation and purification were achieved by serial dilution and repeat restreaking on to complete agar medium. Incubation were performed at 30C with incandescent lamps, light intensity of 6,000 lux.

Selection of strains

The strain 230 was selected for the high capability of hydrogen evolution. Hydrogen production rate was determined by Warburg manometer (B. Braun V166) (Hanus et al., 1980).

Identification

Physiological and cultural characteristics of the isolated strain 230 were examined according to the "Manual of Methods for General Bacteriology" (Gerhardt et al., 1981).

Cell mass determinations

Bacterial concentrations were measured either by determining absorbance at 660nm or dry weight of washed cell with 10mM K-Na phosphate buffer (pH 6.8). The washed cells were dried in preweighed aluminum cups at 105C for 6 hrs.

Absorption spectrum

The absorption spectra of whole cells and bacteriochlorophylls were obtained by suspending in about 60% (w/v) sucrose and by extracting into aceton-methanol (7: 2) mixture, respectively (Eckersley and Dow, 1980) Carotenoids from cells in the early stationary growth phase were extracted according to the procedure of Kim's (1982) and seperated by thin-layer chromatography (TLC) on silica gel 60G.

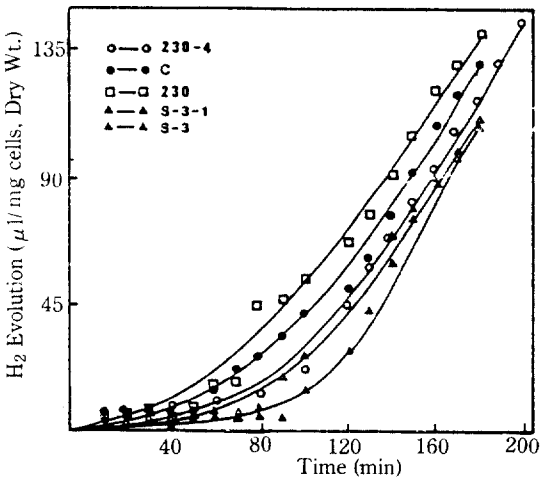

Fig. 1. *The hydrogen evolution rate by various isolated strains.*

RESULTS AND DISCUSSIONS

The strain 230 was the highest hydrogen producer among the isolates (Fig. 1). The morphological, physiological and cultural characteristics of isolated strain 230 were described in Table 1. The isolated strain 230 was rod-form, gram-negative, and motile. As the cells were cultured anaerobically under the light, the colour of cultures was first dirty greenish brown, later dark brown. The cells cultured in the presence of air were distinctly red. Under the dark condition, the colour did not appear. The strain 230 had catalase and hydrogenase and could not liquefy gelatin and not hydrolyze starch. The strain 230

Table 1. *Morphological, physiological and cultural characteristics of the isolated strain 230.*

Items	Characteristics
Morphology	
shape	rod
motility	motile
Gram staining	negative
Flagella staining	positive
Growth	
anaerobic, light	positive
anaerobic, dark	positive
aerobic, light	positive
aerobic, dark	positive
Pigment	
anaerobic, light	dark brown
anaerobic, dark	none
aerobic, light	red
aerobic, dark	none
Bacteriochlorophyll of living cell	a (850, 805, 590, 375nm)
Carotenoid of living cell grown under anaerobic, light	group II
Gelatin liquefaction	negative
Starch hydrolysis	negative
Catalase	positive
Hydrogenase	positive

Table 2. *Utilization of reduced sulfur compounds of the isolated strain 230.*

Sulfur Compound	Concentration (%)	Cell Growth (μg cells, dry weight/ml)
Control (no sulfur)	0	160
Thiosulfate	0.02	40
	0.05	23
Thioglycolate	0.02	55
	0.05	50
Sulfide	0.02	55
	0.05	53

grew anaerobically under the light as a photoheterotroph on various orgarnic carbon sources and as a fermentative anaerobe, in darkness. They also grew aerobically in darkness and light. Table 2 showed the utilization of sulfur compounds by isolated strain 230. The growth of isolated strain 230 was reduced at 0.02% of thiosulfate, thioglycolate, and sodium sulfide. The isolated strain 230 belonged to the family Rhodospillaceae. As the shape of cells was rod and didn't form filaments, the cells belonged to genus *Rhodopseudomonas*. The utilization of electron donors and organic compounds of isolated strain 230 was shown in Table 3. The cells cultured in the minimal medium containing 7 mM glutamate and 30 mM various electron donors. Malate, succinate, lactate, glycerol, and tartrate were utilized well, but benzoate and propionate were not. In order to know the requirement of growth factors, the isolated strain 230 was cultured in the vaious vitamines instead of yeast extract-containing minimal medium. The cells grew better in the presence of biotin, thiamine, and nicotinic acid. Therefore, this strain required at least these three compounds to grow (Table 4). The absorption spectrum of whole cells (Fig. 2a) showed the absorption maxima characteristic of bacteriochlorophyll a at 850, 805, 590, and 375nm with carotenoid absorption maxima at 475 and 510nm. The extracted photopigments with

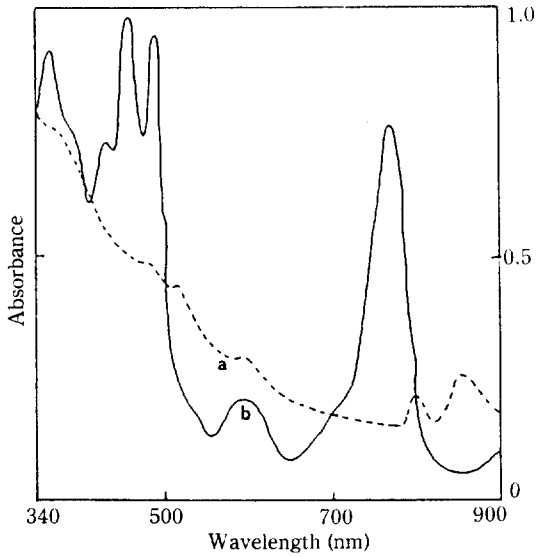

Fig. 2. *The absorption spectra of whole cells of and extraceted photopigments from R. sphaeroides 230 grown anaerobically under the light.*

Table 3. *Utilization of electron donors and organic compounds of isolated strain 230.*

Carbon or Electron donor	Cell Growth (μg cells, dry weight/ml)
Control	91
L-arginine	482
L-asparagine	732
Benzoate	62
Casamino acid	724
Citrate	168
Ethanol	624
Fructose	134
Glucose	139
Glutamate	615
Glycerol	782
Lactate	807
Malate	832
Maltose	134
Mannitol	153
Methanol	573
Propionate	91
Pyruvate	115
Sorbitol	153
Succinate	907
Tartrate	757
Sulfide	57
Thiosulfate	53

Table 4. *Effects of vitamine and yeast extract on the growth of the isolated strain 230.*

Vitamine	Concentration (%)	Cell growth (μg cells, dry weight/ml)
Control (Vitamine free)	1mg/l	23
Pantothenic acid	200μg/l	31
P-aminobenzoic acid	1mg/l	48
Nicotinic acid	1mg/l	187
Thiamine	1mg/l	125
Biotin	10μg/l	232
Biotin+Pantothenic acid		207
Biotin+P-aminobenzoic acid		168
Biotin+Thiamine		240
Biotin+Nicotinic acid		407
Biotin+Thiamine+pantothenic acid		257
Biotin+Thiamine+P-aminobenzoic acid		187
Biotin+Thiamine+Nicotinic acid		498
Yeast Extract		910

methanol-aceton (7: 2) mixture showed the absorption maxima at 770, 488, 458, 430, and 364 nm (Fig. 2b). The carotenoids were extracted with benzene and ethyl ether (7: 3) mixture after extracting the bacteriochlorophylls from the cells which was cultured anaerobically under the light in the complete medium for one day. The extracted carotenoids were separated into three

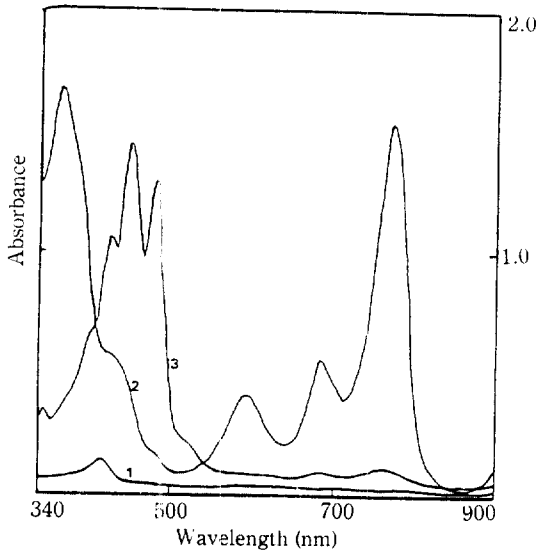

Fig. 3. *Absorption spectra of the pigment components isolated by TLC after extraction with acetone-methanol mixture.*

bands on the TLC plate. The colour of two major bands and minor one was orange, bluish green, and green with the Rf value of 0.94, 0.87, and 0.02 respectively. The absorption spectra of two major bands, orange and bluish green, were shown in Fig. 3. These two major bands are like to be group II carotenoid including spheroidene (450, 428nm) and spheroidenone (484nm (平山修, 1976). From the above results, the isolated strain 230 was identified as *Rhodopseudomonas shpaeroides* according to Bergy (1974) and Truper et al. (1978).

적 요

김해 대저 근교의 양지바른 수계 혐기층에서 수소 생성능을 가진 광합성 세균을 분리하였다. 그 중 수소 생성능이 가장 우수한 균주 230을 선택하여, 여러가지 형태적, 생리적 특성과 배양상의 특징을 조사한 결과, 균주 230은 그람 음성이고, 막대형으로 운동성이 있었으며, benzoate와 propionate는 진자공여체로 이용하지 못하고, 황화합물은 전혀 이용하지 못하였으며, biotin, thiamine, nicotinic acid를 growth factor로 요구하고, 박테리오 클로필 a와 group II 카로티노이드계색소를 가지고 있었으므로 ***Rhodopseudomonas sphaeroides***로 동정되었다.

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