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Photofermentaion and lipid accumulation by *Rhodobacter* sp. KKU-PS1 using malic acid as a substrate

Thitirut Assawamongkholsiri [a], *Pensri Plangklang* [a], *Alissara Reungsang* [a,b,*]

[a] *Department of Biotechnology, Faculty of Technology, Khon Kaen University, Khon Kaen 40002, Thailand*
[b] *Research Group for Development of Microbial Hydrogen Production Process from Biomass, Khon Kaen University, Khon Kaen 40002, Thailand*

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ABSTRACT

Photofermentation and lipid accumulation by the purple non-sulfur photosynthetic bacterium, *Rhodobacter* sp. KKU-PS1, from malic acid was investigated in batch fermentation. Media compositions including malic acid concentration, glutamate concentration, amount of vitamin addition and Fe concentration were optimized by response surface methodology with central composite design in order to achieve a maximum hydrogen production rate (R_m). The predicted R_m of 11.7 mL H_2/L. h was obtained under optimal media compositions of 2.36 g/L malic acid, 224 mg/L glutamate, and 13.5 mL/L of vitamin solution addition along with 64 mg/L of Fe. The cellular lipid content in *Rhodobacter* sp. KKU-PS1 was checked at the end of photo-fermentative process. A nitrogen limited condition was suitable for lipid accumulation by *Rhodobacter* sp. KKU-PS1. A maximum lipid production of approximately 592 mg/L was obtained with 4.7 g/L malic acid, 159 mg/L glutamate, 21.7 mL/L of vitamin solution along with 330 mg/L Fe (equivalent to C/N ratio of 132). Triacylglycerol (TAG) was the major component of the lipid in which the predominant fatty acid was octadecanoic acid (C18:1) in the form of oleic acid (C18:1n-9). The physicochemical properties of biodiesel predicted from fatty acid methyl esters (FAMEs) compositions of lipid produced by *Rhodobacter* sp. KKU-PS1 under the suitable conditions indicated that the lipid produced by the strain KKU-PS1 has a fairly good potential for use as biodiesel.

Introduction

Environmental impacts on global warming, climate change and energy shortages with a consequent increase in energy prices emphasize the need of producing sustainable and renewable fuels [1,2]. For instance, bio-hydrogen has received much attention due to its high energy content and environmental friendliness [3]. Biological hydrogen production includes biophotolysis, dark fermentation and

* *Corresponding author*. Department of Biotechnology, Faculty of Technology, Khon Kaen University, Khon Kaen 40002, Thailand. Fax: +66 43 362 121.
E-mail address: alissara@kku.ac.th (A. Reungsang).
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photofermentation. Among these approaches, photofermentation by purple non-sulfur photosynthetic bacteria (PNSB) has become of great interest due to its high hydrogen yield (HY). PNSB can produce hydrogen from short chain organic acids such as malate [4], lactate [5] and butyrate [6]. Mixtures of organic acids, i.e. acetate, propionate, and butyrate [7], and volatile fatty acids (VFAs) contained in waste effluents had also been used by PNSB to generate bio-hydrogen [8,9].

A disadvantage of photofermentation is its low hydrogen production rate. Optimization of the medium composition was found to increase the rate of hydrogen production [10,11]. Malic acid is a favored carbon and energy source for hydrogen production by PNSB. This substrate is part of the tricarboxylic acid (TCA) cycle in which energy is gained from carbon metabolism to generate hydrogen [12]. Glutamate as nitrogen source in the fermentation media is an excellent amino acid for photo-fermentative hydrogen production and it can support cellular growth. Moderate concentrations of glutamate have minor effects on nitrogenase repression, while nitrogenase activity is completely inhibited by the formation of excessive NH_4^+, which can be released from amino acids [13]. Han et al. [14] reported that the ammonium sulphate concentration greater than 1.6 g/L was toxic to the microorganisms which resulted in a decrease in cumulative hydrogen volume. Additionally, nitrogen-starvation prevents cell proliferation hence decreasing the hydrogen production rate. PNSB require vitamins for carbohydrate, protein and lipid metabolism [15,16]. Some vitamins such as nicotinic acid are key electron carriers ($NADH^+$, NAD^+). Therefore, the absence of these vitamins in a photofermentation medium can lead to a decrease in the hydrogen production rate [16]. Furthermore, iron is an important cofactor for the synthesis of nitrogenase, which is an essential enzyme to reduce protons in the formation of molecular hydrogen [12]. Therefore, iron in the medium at a suitable concentration is believed to enhance the efficiency of photo-fermentative hydrogen production [6,14]. For these reasons, the concentrations of malic acid, glutamate, iron and vitamins in a hydrogen production medium need to be optimized in order to improve photohydrogen production efficiency and maximize the hydrogen production rate.

PNSB can effectively produce hydrogen under favorable growth conditions. Hydrogen production occurs during the early stationary phase of growth [17]. Long fermentation times in batch fermentation processes lead to decreased ferredoxin (electron carrier) activity, resulting in a reduction of the hydrogen production efficiency of PNSB. Consequently, metabolic pathways are directed towards lipid or polyhydroxyalkanoate production in the late stationary phase of growth [12,18]. Some PNSB such as *Rhodobacter sphaeroides* KD131 [2] and *Rhodopseudomonas palustris* 420L [19] are capable of accumulating more than 20% of their dry cell weight as lipids, in the form of fatty acids. The recovery of fatty acids from PNSB cells harvested at the end of photofermentation is useful in terms of biofuel production, since these fatty acids can potentially be used as a biodiesel source [19,20]. Previous researches reported hydrogen production from organic acids by PNSB [4,6,7]. However, there is very limited information on photofermentation and lipid accumulation from malic acid by *Rhodobacter* species.

In this study, photofermentation by *Rhodobacter* sp. KKU-PS1 was investigated. The individual and interactive effects of malic acid concentration, glutamate concentration, the amounts of vitamin solution and iron concentration on the maximum hydrogen production rate (R_m) were examined using response surface methodology (RSM) with central composite design (CCD). Lipid accumulation by *Rhodobacter* sp. KKU-PS1 was determined at the end of the photo-fermentative process. In addition, the potential of fatty acid methyl esters (FAMEs) of lipid produced by the strain KKU-PS1 for use as biodiesel was investigated. The results of this study would provide the useful information on the conditions favoring photofermentation and lipid accumulation by *Rhodobacter* sp. KKU-PS1 using malic acid as a substrate.

Materials and methods

Microorganism and culture conditions

Rhodobacter sp. KKU-PS1 (GenBank Accession No. KC478552) [21] was photo-anaerobically grown for 48 h in a basal medium containing 15 mM DL-malic acid and 3 mM sodium glutamate under previously reported optimal conditions for hydrogen production [21]. The seed cultures were harvested by centrifugation at 7000 rpm for 10 min and used as inoculum for bio-hydrogen and microbial oil production in further experiments.

Experimental design

In this study, RSM with CCD was applied to design experiments and to estimate the effects of four independent variables, including DL-malic acid concentration (X_1), sodium glutamate concentration (X_2), amount of vitamin solution addition (X_3), and Fe concentration (X_4). The response was the maximum hydrogen production rate (R_m). First, the experiment was designed using fractional factorial design in which the values of independent variables were varied at 5 levels, with $\pm\alpha$ as the axial points, ±1 as the factorial points and 0 as the center point. The symbols and coded levels of the independent variables are shown in Table 1 (Block 1). The levels of the independent variables used in the current study were set based on the previous reports of Chen et al. [6], Han et al. [14], Carlozzi et al. [19] and Carlozzi and Lambardi [22].

Bio-photohydrogen production

Bio-hydrogen production was conducted in 300 mL serum bottles with a working volume of 180 mL. The hydrogen production medium (HPM) contained (g/L): KH_2PO_4, 3.9; K_2HPO_4, 2.8; $MgSO_4.7H_2O$, 0.2; $CaCl_2.2H_2O$, 0.075; $Na_2Mo_4.2H_2O$, 0.01 and 1 mL/L stock solution of trace elements [23]. DL-malic acid and sodium glutamate were added to the HPM according to the experimental design (Table 2). The initial pH values of the media were adjusted to 7.0 by addition of NaOH pellets. The serum bottles were capped with rubber stoppers and aluminum caps before being sterilized in an autoclave at 121 °C for 15 min. After autoclaving and cooling, sterile Fe and vitamin solutions were aseptically added to the serum bottles