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Synergistic enzymatic saccharification and fermentation of agar for biohydrogen production

Yi-Rui Wu [1], Mingming Zhang [1], Mingqi Zhong, Zhong Hu *

Department of Biology, Shantou University, Shantou, Guangdong 515063, China

HIGHLIGHTS

- The optimal condition for biohydrogen production from galactose was developed.
- Agarase AgaXa and NH852 were employed in the synergetic enzymatic saccharification.
- The agar hydrolysate was assessed for H_2 fermentation by *Enterobacter* sp. CN1.
- The platform of the bioconversion of agar biomass into biofuels was established.

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ABSTRACT

Nowadays, marine biomass is gradually considered as another utilizable material for the sustainable bioenergy development. In the present study, galactose, the main component of agar polysaccharide, was utilized for the biohydrogen production by *Enterobacter* sp. CN1. The highest hydrogen yield of 303.2 mL/g was obtained in the cultivation media containing 5.87 g/L of galactose, together with initial pH of 7.3 and incubation temperature of 36 °C, after the response surface methodology (RSM) analysis. After the saccharification process by the agarase (AgaXa) and neoagarobiose hydrolase (NH852), the agar hydrolysate obtained was further applied to generate biohydrogen by strain CN1. Under the synergistic enzymatic saccharification and fermentation process, the production of biohydrogen was obtained to be 5047 ± 228 mL/L from 50 g/L of agar, resulting in 3.86-fold higher than the control without enzymatic pretreatment.

1. Introduction

Currently, the depleting fossil fuel reserves and the continuous growing fuel demand has led to the exploration of the safe and environment-friendly alternatives (e.g., biofuels). Owing to its abundant production and non-polluting characteristics, biohydrogen is one of the ideal and potentially sustainable biofuels with bright future prospects (Mathews and Wang, 2009). Marine biomass, such as algal biomass, is considered as another renewable source for bioenergy conversion due to several advantages, including the high growth rate, the high carbohydrate content (Falter et al., 2015), and the low concentration or lack of lignin, which can strongly inhibit the attack from the enzymes or microorganisms to hydrolyze the polysaccharides within the biomass (Yanagisawa et al., 2013).

Agar, composed of agarose and agaropectin, is the main component in the cell walls of red algal biomass, and agarose is a linear polysaccharide, containing D-galactose and 3,6-anhydro-L-galactose (AHG) via the alternative α-1,3 and β-1,4 linkage (Yun et al., 2015). In the practice of converting such algal biomass into biofuels or other valuable biochemicals, the critical step is to decompose the substrate into fermentable oligo sugars or monosugars through the chemical liquefaction or enzymatic saccharification process (Naik et al., 2010). Kim et al. (2012) combined the enzymatic hydrolysis with acetic acid treatment to obtain monosugars from agarose for ethanol fermentation by *Saccharomyces cerevisiae*. Seo et al. (2016) also demonstrated that a two-stage enzymatic process without acid pretreatment was involved in converting the agarose into ethanol by *S. cerevisiae* KL17. *Enterobacter* species has the potential in the biohydrogen production using various fermentable sugars, such as glucose, xylose, sucrose, etc. (Lu et al., 2011; Mohanraj et al., 2014; Subudhi et al., 2013). However, there have been no reports on the H_2 fermentation by *Enterobacter* sp. using galactose, the major ingredients in agar polysaccharides,

* Corresponding author.
E-mail address: hzh@stu.edu.cn (Z. Hu).
[1] These authors contributed equally to this work.

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as the substrate so far. Moreover, the research on the biofuels production from agar biomass has been very limited, and even there has been no systematical investigation, including the galactose utilization enhancement, the agarase performance analysis as well as the synergistic process of saccharification and fermentation, on the biohydrogen production directly using agar as the substrate by *Enterobacter* species.

By simultaneously applying the agarase AgaXa and neoagarobiose hydrolase NH852, the agar can be decomposed into galactose and AHG, further converted to biohydrogen with the addition of a galactose-utilizing bacterial strain *Enterobacter* sp. CN1. Therefore, the objective of this study is to develop a feasible process on the synergy of the agarase saccharification and fermentation by strain CN1.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Enterobacter sp. CN1 was isolated from the previous study (Long et al., 2010) and maintained in the laboratory. The fermentation medium for biohydrogen generation by strain CN1 was prepared according to the method of Xin et al. (2014), and the fermentation process was carried out in 100 mL serum bottles sealed with butyl rubber stopper and aluminum seal caps, containing 50 mL of the fermentation medium with 2% of inoculum (v/v). The recombinant agarase (AgaXa) and neoagarobiose hydrolase (NH852) were obtained from the gene-engineered *E. coli* BL21, carrying the plasmid of pET-32a-*AgaXa* and pET-32a-*NH852*, respectively (Xie et al., 2013). The *E. coli* cells were cultivated in the Luria–Bertani (LB) medium added by 100 μg/mL ampicillin (Sigma-Aldrich, USA). All the cultivation was incubated in an orbital shaker with a rotation speed of 150 rpm unless otherwise stated.

2.2. Construction of optimal conditions for biohydrogen production using galactose

A 3-variable Box-Behnken Design (BBD) model was set up to optimize the key process parameters (Table S2), including the cultivation temperature, the initial pH as well as the concentration of galactose, to achieve the highest biohydrogen yield from galactose by strain CN1. 17 representative runs of experiments were generated via the Design Expert software (Version 7.0), and the yield of biohydrogen from each run was acquired within three independent data. By solving the regression equation, the optimal culture conditions of strain CN1 for the highest biohydrogen yield using galactose were attained, and the response surface contour plots was also built up.

2.3. Production of the recombinant AgaXa and NH852

The gene-engineered bacterial strains *E. coli* BL21 (DE3) and BL21 (Rosetta), respectively, carrying the plasmid pET-32a-*AgaXa* and pET-32a-*NH852*, were cultivated at 37 °C in LB medium supplemented with 100 μg/mL ampicillin. After adding the isopropyl-β-D-galactopyranoside (IPTG) to a final concentration of 0.1 mM for protein induction when the OD_{600nm} of the culture reached 0.6, the cells were incubated at 25 °C for 24 h, and collected via the centrifugation at 4 °C for 10 min. The cells were further ultrasonically lysed, and the lysates were applied to a Ni^{2+} affinity column (HiTrap SP, GE Healthcare) to obtain the purified recombinant protein AgaXa and NH852 according to the method of Wu et al. (2017).

2.4. Enzyme assay

The agarase AgaXa activity was measured by the release of the reducing sugar (D-galactose equivalent) using 3,5-dinitrosalicylic acid (DNS) method as described by Xie et al. (2013). One unit (U) of agarase was defined as the amounts of enzyme required to release 1 μmol of the reducing sugars per minute at the above same condition. Similarly, the activity of NH852 was measured by the release of *p*-nitrophenol (*p*NP) equivalent hydrolyzing *p*-nitrophenyl-β-D-galactopyranoside (*p*NPG). The absorbance was spectrophotometrically monitored at the wavelength of 400 nm, and one unit (U) of NH852 was defined as the amounts of enzyme required to release 1 μmol of *p*NP per minute at the above same condition.

2.5. Simultaneous enzymatic saccharification and fermentation process

To determine the optimal reaction system for enzymatic saccharification process, the substrate, agar (50 g/L), was prepared by autoclaving in the sealed serum bottle for 20 min at 121 °C alone, and the buffer and different amounts of enzymes (AgaXa and NH852) were aseptically injected using syringes through the 0.22 μm size filter membranes. The reaction was incubated at 40 °C with the agitation speed of 150 rpm for 8 h before adding the bacterial cells.

The strain *Enterobacter* sp. CN1 was introduced for biohydrogen fermentation from the agar hydrolysate. 2% of inocula together with the anaerobic culture medium was further injected to the serum bottle, and incubated at 37 °C under the agitation speed of 150 rpm. The pH value of the culture medium was manually maintained as the initial pH by adding 3 M NaOH in every 8 h during the fermentation process. At each time interval of cultivation, the hydrogen was collected and analyzed with a GC-2010 gas chromatograph (Shimadzu, Japan) equipped with a thermal conductivity detector (TCD) according the method of Rajagopalan et al. (2014), and the bacterial growth was monitored at the wavelength of 600 nm. 150 μL of culture medium was collected and centrifuged, and the supernatant was further used to determine the concentration of the generated reducing sugar as well as D-galactose by the DNS reagent and the Galactose Assay Kit (Abcam, UK), respectively. All the experiments were independently carried out in triplicates.

3. Results and discussion

3.1. Production of biohydrogen from galactose by Enterobacter sp. CN1

The statistical analysis of biohydrogen fermentation from galactose was performed via the Box-Behnken design using RSM. The effect of individual factors and their interaction on the biohydrogen yield was determined, and the model was established to obtain the optimal fermentation conditions (Table S2). A quadratic polynomial equation for identifying the relationship between the hydrogen yield and the selected variables was described as follows:

$$\begin{aligned} H_2\ \text{yield}\ (Y) = {} & -3547.54167 + 80.65556 \times A + 664.06667 \times B \\ & - 22.88222 \times C + 1.55556 \times A \times B - 0.57556 \times A \times C \\ & + 6.16000 \times B \times C - 1.22500 \times A^2 - 51.33333 \times B^2 \\ & - 0.27378 \times C^2 \end{aligned}$$

where A, B and C were indicated as the values of the temperature, the initial pH and the concentration of galactose, respectively.