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Directional regulation of the metabolic heterogeneity in anaerobic mixed culture to enhance fermentative hydrogen production by adaptive laboratory evolution

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ABSTRACT

In this study, an adaptive laboratory evolution strategy was originally developed to enhance fermentative hydrogen production by directionally regulating the metabolic heterogeneity in anaerobic mixed culture. The results indicated that the co-introduction of 4-methylpyrazole and oxamate could redistribute the metabolic flux to butyrate-type hydrogen fermentation. Subsequently, a synergistic evolutionary pressure, combining exogenous butyrate stress with 4-methylpyrazole and oxamate, was employed to evolve hydrogen-producing mixed culture with continuous fermentation system. The metabolic engineering strategy could directionally regulate the metabolic heterogeneity through efficiently shaping powerful butyrate-type hydrogen-producing community, by which evolved culture acquired a significantly improved hydrogen yield and productivity. Furthermore, compared with original culture, evolved culture possessed much higher activities of pyruvate-ferredoxin oxidoreductase and hydrogenase but a much lower ferredoxin-NAD^+ oxidoreductase activity, and these enzymatic evolutionary mechanisms were crucially important for the enhanced hydrogen fermentation.

Introduction

Biological hydrogen, as a green renewable energy carrier, has recently become one of the highlights in energy and environment researches [1,2]. Compared with pure culture based biotechnology, fermentative hydrogen production via anaerobic mixed culture is more advocated for some practical reasons such as wide substrate spectrum, no sterilization requirement and better adaptive capacity, which is cost-effective and has a great potential for continuous production [3]. The mixed culture biotechnology is now primarily based

Abbreviations: ALE, adaptive laboratory evolution; POR, pyruvate-ferredoxin oxidoreductase; FNO, ferredoxin-NAD^+ oxidoreductase; Fd^{2-}_{red}, reduced ferredoxin; VSS, volatile suspended solids; HRT, hydraulic retention time.

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on the selection and enrichment of hydrogen producers by manipulating reactor operation conditions and/or by pretreating undefined natural inoculum [4]. Nevertheless, since different species of hydrogen producers, with discrepant physiological and biochemical behaviors, should possess their respective metabolic properties, the microbial diversity of mixed culture can result in significant metabolic heterogeneity during the fermentation process [5,6]. The heterogeneity limits the overall hydrogen yield and productivity by decreasing product specificity and inducing process instability, and it cannot be effectively controlled by fermentation condition optimization, especially under high organic loads [7,8]. Hence, how to regulate the metabolic heterogeneity is of crucial importance to enhance the hydrogen productivity of mixed culture. However, few relevant researches have been reported until now.

According to the stoichiometric calculations of the metabolic pathways involved in hydrogen fermentation (Fig. S1), a high hydrogen yield should be accompanied by the most production of butyrate and acetate, while the formations of alcohol, lactate and propionate can cause a low hydrogen yield (Table S1) [9]. Namely, the metabolic heterogeneity of mixed culture also affects the distribution of liquid products, and its directional regulation to improve the biosynthesis of butyrate and acetate is very crucial for enhancing hydrogen production. Adaptive laboratory evolution (ALE) employs a specified selection pressure as driving force for the directional selection of producers with desired phenotypes [10,11]. The metabolic engineering strategy has been proven highly effective for improving stress tolerance, activating metabolic latent pathways and improving product formation [12,13]. Unlike that of pure culture, the ALE of mixed culture should be a biological process for inter- and intra-species directional selection, by which the metabolic heterogeneity can be regulated for a desired function through restructuring microbial community. However, the ALE strategy for bio-hydrogen fermentation is now challenging due to the lack of developed evolutionary schemes.

In this study, an ALE strategy for enhancing fermentative hydrogen production by directionally regulating the metabolic heterogeneity in anaerobic mixed culture was originally reported. As 4-methylpyrazole and oxamate are respectively considered as the specific inhibitors against alcohol dehydrogenase and lactate dehydrogenase, the effects of the two inhibitors on mixed culture hydrogen fermentation were first evaluated. Subsequently, a synergistic evolutionary pressure, combining exogenous butyrate stress with 4-methylpyrazole and oxamate, was employed to evolve hydrogen-producing mixed culture in continuous fermentation system. After the adaptive evolution process, the fermentation performances under high organic load were compared between original culture and evolved culture, and the enzymatic mechanisms for the significantly enhanced hydrogen production were further investigated.

Materials and methods

Inoculum and culture medium

Anaerobic sludge from a full-scale anaerobic digestion reactor (Jiangsu Clean Environmental Technology Co. Ltd., Suzhou, China) was baked at 100 °C for 1 h to harvest spore-forming clostridial hydrogen producers and to kill methanogens. The heat-pretreated mixed culture was used as inoculum (original culture) after a 12 h enrichment cultivation. The culture medium used in this study contained glucose, NH_4Cl, KH_2PO_4, and $FeCl_2{\cdot}4H_2O$ with a glucose:N:P:Fe ratio of 500:5:1:0.5 (g/g), and was simultaneously supplied with the following nutrients (mg/L): $MgSO_4{\cdot}7H_2O$ 200; NaCl 20; $CaCl_2{\cdot}2H_2O$ 10; L-cysteine 10; $Na_2MoO_4{\cdot}4H_2O$ 0.5; $MnSO_4{\cdot}7H_2O$ 0.5; H_3BO_3 0.5; $ZnCl_2$ 0.5; $CuCl_2$ 0.5; $CoCl_2{\cdot}2H_2O$ 0.5; pyridoxal 0.05; riboflavin 0.05; nicotinic acid 0.05.

Effects of 4-methylpyrazole and oxamate on hydrogen-producing mixed culture

Each reactor (500 mL) contained 300 mL of culture medium (18 g/L glucose), where different doses of 4-methylpyrazole and oxamate were respectively added. The solution was buffered with 0.1 M 2-(N-morpholino)ethanesulfonic acid and the initial pH was adjusted to 6.8. After inoculation with an initial volatile suspended solids (VSS) concentration of 2.0 g/L, all the reactors were purged with N_2 gas for 10 min, and then were anaerobically operated at 36 °C and a stirring speed of 120 rpm. The amounts of gaseous and liquid products were measured after 36 h of fermentation.

Adaptive laboratory evolution for hydrogen-producing mixed culture

According to the results from the above experiments, a certain amount of oxamate and 4-methylpyrazole were added into culture medium (18 g/L glucose) for the adaptive evolution. After inoculation and purging with N_2 gas, a continuously stirred tank reactor (5 L working volume) constantly operated at 36 °C, pH 5.8 and a stirring speed of 120 rpm was first initiated in a batch mode, and the continuous fermentation with an 8 h hydraulic retention time (HRT) was not carried out until hydrogen production was detected. The evolution procedure was started by introducing 50 mM exogenous butyrate stress in the feed. Subsequently, the stress was gradually elevated based on the reactor steady-state establishment. Finally, the introduced butyrate stress reached up to 250 mM, by which evolved culture was acquired.

Fermentation performance analysis

Batch tests were performed to compare the fermentation performances between original culture and evolved culture in 5 L bioreactors at 36 °C. The initial VSS and glucose concentrations were respectively 2.0 g/L and 40 g/L. After inoculation and purging with N_2 gas, the reactors were operated at constant pH of 6.0 and a stirring speed of 120 rpm. The fermentation mixtures were periodically sampled to measure critical enzyme activities. The amounts of gaseous and liquid products were determined after 36 h of fermentation.

Enzyme activity assays

The collected fermentation mixture (25 mL) was washed and re-suspended in 10 mL of 0.1 M Tris buffer containing 10 mM