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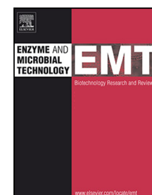

Microbial surface displaying formate dehydrogenase and its application in optical detection of formate

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In the present work, NAD^+-dependent formate dehydrogenase (FDH), encoded by *fdh* gene from *Candida boidinii* was successfully displayed on *Escherichia coli* cell surface using ice nucleation protein (INP) from *Pseudomonas borealis* DL7 as an anchoring protein. Localization of matlose binding protein (MBP)-INP-FDH fusion protein on the *E. coli* cell surface was characterized by SDS-PAGE and enzymatic activity assay. FDH activity was monitored through the oxidation of formate catalyzed by cell-surface-displayed FDH with its cofactor NAD^+, and the production of NADH can be detected spectrometrically at 340 nm. After induction for 24 h in Luria-Bertani medium containing isopropyl-β-D-thiogalactopyranoside, over 80% of MBP-INP-FDH fusion protein present on the surface of *E. coli* cells. The cell-surface-displayed FDH showed optimal temperature of 50 °C and optimal pH of 9.0. Additionally, the cell-surface-displayed FDH retained its original enzymatic activity after incubation at 4 °C for one month with the half-life of 17 days at 40 °C and 38 h at 50 °C. The FDH activity could be inhibited to different extents by some transition metal ions and anions. Moreover, the *E. coli* cells expressing FDH showed different tolerance to solvents. The recombinant whole cell exhibited high formate specificity. Finally, the *E. coli* cell expressing FDH was used to assay formate with a wide linear range of 5–700 μM and a low limit of detection of 2 μM. It is anticipated that the genetically engineered cells may have a broad application in biosensors, biofuels and cofactor regeneration system.

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1. Introduction

We are in an age that the energy crisis is becoming more critical than ever. The development and utilization of biomass energy is an effective way to solve the above problems. In recent years, lignocellulosic materials have been used as raw material to produce bioethanol [1]. However, Due to the complexity of their structures, lignocelluloses should be firstly hydrolyzed to monosaccharides by acids or enzymes pretreatment for the fermentation step [2]. Among these approaches, hydrolysis with enzyme is considered as a comparatively matured pretreatment approach. However, a large amount of fermentation inhibitors such as organic acids, furfural and phenolic compounds are produced from pretreatment [3], which could inhibit the subsequent hydrolysis and fermentation process [4]. It has been reported that organic acids could inhibit fermentation by reducing biomass formation [3]. Formic acid and acetic acid are the most common organic acids present in lignocellulosic hydrolysates [3]. Besides, formic acid is applied extensively in the fields of manufacturing, medicine and food industry. Formic acid is also an effective treatment against warts [5] and can be made as a preservative and antibacterial agent in foodstuffs or livestock feed. On the other hand, the concentrated formic acid is corrosive to the skin and chronic formic acid exposure may cause a skin allergy and kidney damage. Considering the potential hazards of formic acid in fermentation industry and our daily life, an effective analytical method for determination of formic acid is of great importance. To date, various analytical methods have been proposed for the detection of formic acid, including high-performance liquid chromatography (HPLC) [6] and gas chromatography [7,8]. However, these methods experienced challenges, including time consumption, troublesome operations, lower selectivity and expensive instrumentation. Additionally, in the analysis of formic acid by HPLC and gas chromatography, formic acid must be converted into its derivatives prior to analysis. Thus,

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a simple, reliable and efficient detection method based on NAD^+-dependent formate dehydrogenase (FDH) for detection of formic acid or formate appears to be an attractive approach to solve these issues. And the results obtained through enzymatic method correlated well with those values measured by above methods [9,10]. Nevertheless, the use of free enzyme could be limited by laborious operations and high cost for isolation and purification of intracellular expressed enzymes, as well as the poor stability of the enzyme. Thus, a novel strategy should be developed to produce FDH with excellent activity and stability at low cost.

Microbial cell surface display has been effectively used as a tool for the expression of heterologous proteins or peptides of interest on the surface of microbes by fusing them with a suitable anchoring motif [11,12]. Compared with intracellular expressed proteins, surface-expressed proteins can directly interact with substrates without crossing the membrane barrier, thereby simplifying the protein isolation and purification process. Moreover, it could enhance the stability of the displayed proteins. Endowing intact cells with new features, this approach has emerged as a biotechnology for a wide range of potential applications, such as screening [13], biosensors [14,15], biofuels [16,17], bioremediation [18], and vaccine development [19].

FDH was classified into several types, including NAD^+-dependent FDH (EC 1.2.1.2) [20], FDH (cytochrome) (EC 1.2.2.1) [21], FDH (acceptor) (EC 1.1.99.33) [22], $NADP^+$-dependent FDH (EC 1.2.1.43) [23], FDH (cytochrome-c-553) (EC 1.2.2.3) [24], FDH (coenzyme F420) (EC 1.2.99.9) [25] and nitrate inducible FDH (EC 1.1.5.6) [26]. To date, genes of FDH have been identified in many methylotrophic bacteria including *Pseudomonas* sp. 101 [27], *Paracoccus* sp. 12-A [28] and *Bacillus* sp. F1 [29], as well as yeasts including *Candida methylica* [30] and *Candida boidinii* [31]. The amino acid sequences of these FDHs originated from different organisms reveal strong similarity, for which there is above 80% identity within bacteria, yeast, fungi and plants. These FDHs, composing of two identical subunits without metal ions and prosthetic groups in the active center, belong to the superfamily of D-specific 2-hydroxy acid dehydrogenases. With NAD^+ as its cofactor, FDH could catalyze the irreversible oxidation of formate ion to carbon dioxide [32], which is shown in Eq. (1) .

$$HCOO^- + NAD^+ \rightarrow CO_2 + NADH \quad (1)$$

The N-terminal domain of ice nucleation protein (INP) from *Pseudomonas borealis* DL7 has been successfully served as an anchoring motif for expression of proteins on the microbial surface in our laboratory, and the INP display system has been proved to improve the enzymatic activity and stability [33]. So far, xylose dehydrogenase-[34], glucose dehydrogenase-[14], organophosphorus hydrolase- [35,36], glutamate dehydrogenase- cell surface display system [37,38] have been successfully employed for the sensitive and selective detection of xylose, glucose, *p*-nitrophenol substituted organophosphates and glutamate, separately. In the present work, an expression system for displaying FDH from *C. boidinii* on the surface of *Escherichia coli* with N-terminal domain of INP from *P. borealis* DL7 as an anchoring motif was constructed. Further, the characteristics of the recombinant *E. coli* cells were systematically studied, including enzymatic activity, stability, substrate specificity, effects of various ions on enzyme activity and organic solvents on the stability of cultures expressing fusion proteins. And finally, the bacteria displaying FDH was applied for selective, sensitive and rapid detection of formate.

2. Materials and methods

2.1. Strains, plasmids and culture conditions

pMAL-c2x, kindly provided by Dr. Jianping Cai (Department of Avian Diseases, Lanzhou Veterinary Research Institute, China), was used as the parent vector for the construction of recombinant vector. Plasmid harboring *fdh* gene from *C. boidinii* was kindly gifted by Dr. Christopher M. Cheatum (Department of Chemistry, University of Iowa, USA). Plasmid pTInaPb-N harboring INP-coding *inaPb* gene from *P. borealis* DL7 was constructed on the basis of pET28a in our laboratory. *E. coli* DH5α was the host strain used for plasmid amplification during the construction of expression vectors. *E. coli* transetta (DE3) was used as host for expression of recombinant protein. *E. coli* strains carrying recombinant plasmids were cultured in Luria-Bertani (LB) medium at 200 rpm and 37 °C. Cultures of *E. coli* DH5α containing recombinant plasmids were supplemented with 50 μg/mL ampicillin, and cultures of *E. coli* transetta (DE3) were supplemented with 50 μg/mL ampicillin and 34 μg/mL chloramphenicol.

2.2. Construction and expression of inaPb- fdh gene fusions

To introduce the *C. boidinii fdh* gene into the plasmid, the coding sequence was amplified using specific primers FDH-*Bam*-F (5′-CGGGATCCATGAAGATTGTCTTAGTTC-3′) and FDH-*Sal*-R (5′-ACGCGTCGACCTATTTCTTATCGTGTTTAC-3′), the PCR-amplified and purified product was cleaved with *Bam*HI and *Sal*I and then inserted into plasmid pMAL-c2x cleaved with the same enzymes. For the construction of MBP (matlose binding protein)-INP-FDH fusion, plasmid pTInaPb-N was used as a template for PCR with primers INP-*Eco*-F (GGAATTCATGAACGATGACAAAGTTTTGGTC) and INP-*Bam*-R (CGGGATCCCACCGCTGTCTCCAGCGTTTG) to generate N-terminal domain of *inaPb* encoding fragment. The resulting PCR fragments and plasmid pMAL-c2x-Fdh harboring *fdh* gene were digested with *Eco*RI and *Bam*HI and ligated at the corresponding sites. The resultant plasmid which was designated as pMAL-c2x-Inp-Fdh, was initially transformed into *E. coli* DH5α for selection of positive clones and then transferred to *E. coli* transetta (DE3) for the expression of recombinant proteins. Colonies of *E. coli* transetta (DE3) harboring the recombinant plasmids were inoculated into LB medium containing ampicillin and chloramphenicol and cultivated by rotary shaking at 200 rpm until absorbance at 600 nm (OD_{600}) reached 0.6 at 37 °C. MBP-INP-FDH fusion protein was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) in the medium.

2.3. FDH activity assay

The recombinant *E. coli* strains were harvested by centrifugation at 6000 rpm for 5 min, washed three times using 50 mM glycine-NaOH buffer (pH 9.0) and re-suspended with the same buffer after 24 h of IPTG induction. FDH activities of whole cells and cellular fractions with sodium formate as a substrate were measured spectrophotometrically at 340 nm by monitoring the formation of NADH over a time period of 10 min. The assay mixture was thermostated to 50 °C in the presence of 60 mM sodium formate, 2 mM NAD^+ and whole cells (OD_{600} = 1.0) and then removed cells by centrifugation at 12,000 rpm for 1 min. One unit of FDH activity was defined as 1 μmol NADH produced per minute per OD_{600} whole cells at pH 9.0 and 50 °C.

2.4. Cell fractionation and SDS-PAGE analysis

E. coli strains expressing MBP-INP-FDH fusion protein were harvested by centrifugation at 6000 rpm for 5 min after induction

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