

Flavins contained in yeast extract are exploited for anodic electron transfer by *Lactococcus lactis*

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Abstract

Cyclic voltammograms of yeast extract-containing medium exhibit a clear redox peak around -0.4 V vs. Ag|AgCl. Fermentative bacterium *Lactococcus lactis* was hereby shown to exploit this redox compound for extracellular electron transfer towards a graphite anode using glucose as an electron donor. High performance liquid chromatography revealed that this may be a flavin-type compound. The ability of *L. lactis* to exploit exogenous flavins for anodic glucose oxidation was confirmed by tests where flavin-type compounds were supplied to the bacterium in well defined media. Based on its mid-point potential, riboflavin can be regarded as a near-optimal mediator for microbially catalyzed anodic electron transfer. Riboflavin derivative flavin mononucleotide (FMN) was also exploited by *L. lactis* as a redox shuttle, unlike flavin adenine dinucleotide (FAD), possibly due to the absence of a specific transporter for the latter. The use of yeast extract in microbial fuel cell media is herein discouraged based on the related unwanted artificial addition of redox mediators which may distort experimental results.

Keywords: exogenous mediator, extracellular electron transfer, microbial fuel cell, riboflavin

1. Introduction

The purpose of this work is to demonstrate the possibility of flavin-type compounds in yeast extract to work as redox mediators for bacterial extracellular electron transfer towards graphite anodes. Extracellular electron transfer by bacteria has been studied by many researchers due to its various applications, such as direct conversion of organic substances to electricity in microbial fuel cells [1] and the possibility to selectively determine fermentation end-products by means of anodic potential control [2]. Different mechanisms of electron transfer from bacteria to electrodes have been proposed, including soluble redox mediators [3], nanowires [4], and membrane associated electron transfer [5]. Soluble redox mediators can be endogenous or exogenous. As we have previously reported [6], endogenous and exogenous ACNQ (2-amino-3-carboxy-1,4-naphthoquinone) can accelerate electron transfer for *Lactococcus lactis*; *Propionibacterium freudenreichii* ET-3 produces and secretes DHNA (1,4-dihydroxy-2-naphthoic acid) and exploits it for extracellular electron transfer [2]. In a recent study by Marsili et al. [7], *Shewanella oneidensis* was shown to secrete flavin-type compounds, which are used by the bacterium as soluble redox mediators. That work also revealed that flavin can work as a redox mediator at very low concentrations (approximately 250 nM). In a recent study by von Canstein et al. [8], several kinds of bacteria, including *Shewanella* sp., *Pseudomonas* sp. and *E. coli* were shown to secrete flavins in similar quantities. However, *Pseudomonas* and *E. coli* do not utilize the produced riboflavin for extracellular electron transfer, suggesting that the rapid shuttling of flavins through the cell membrane is a somewhat specialized process. In this article, we show that flavin-type compounds contained in yeast extract are exploited by fermentative bacterium *L. lactis* as redox mediators for extracellular electron transfer to graphite electrodes during anodic oxidation of glucose. Yeast extract is one of the most important components for microbial growth media and it is routinely used as a source of key nutrients in many studies in the field of microbiology. We found that riboflavin in yeast extract can accelerate anodic electron transfer by *L. lactis*. As a consequence, yeast extract should be avoided in any study aimed at elucidating the mechanisms of electron transfer in exoelectrogenic bacteria.

2. Materials and Methods

2.1 Preparation of Microorganisms

Growth medium contained 5 g of glucose, 5 g of polypeptone, 5 g of yeast extract, 1 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ dissolved in 1 L of distilled water and was utilized to pre-culture *Lactococcus lactis* NBRC 12007 and *Propionibacterium freudenreichii* ET-3 under anaerobic conditions without shaking. After incubation at 37 °C, the cells were harvested by centrifugation at 5,000 g and 4 °C for 10 min. After washing twice in physiological saline solution, the cells were re-suspended in sterile phosphate buffer (pH 7, 0.1 M) and immediately injected into the bioelectrochemical reactors.

2.2 Bioelectrochemical Reactor

The electrochemical bioreactor was constructed as previously described [2]. In the anode chamber, the growth medium (10 g L⁻¹ of glucose, 5 g L⁻¹ of yeast extract, 5 g L⁻¹ of polypeptone, 1 g L⁻¹ of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ added to a 0.1 M phosphate buffer solution, pH 7) was used unless otherwise indicated. The anode liquid volume was 0.1 L. After injection of *L. lactis* to such medium, chronoamperometric monitoring (+0.4 V vs. Ag/AgCl) was carried out using a potentiostat (HA-151A, Hokuto Denko, Tokyo, Japan).

2.3 Cyclic Voltammetry

Cyclic voltammetry was carried out using an electrochemical analyzer (CHI611C, ALS/CH Instruments, USA) with the electrochemical bioreactor described above at a scan rate of 1 mV s⁻¹ under quiescent conditions. All of experiments using microorganisms were carried out when the catalytic current reached a maximum value and was stable, as observed during chronoamperometry.

2.4 HPLC Detection

Flavin-type compounds contained in yeast extract were detected by a HPLC system (10ATVP, Shimadzu, Kyoto) with an ODS reversed phase column (Shimadzu, Kyoto). The mobile phase was initially a 0.1% aqueous solution of tetrafluoroacetic acid (TFA). The flow rate for elution was 1 mL min⁻¹ at 40 °C. After 10 minutes at constant flow, a linear gradient of a solution containing 0.1% TFA in acetonitrile was added to the eluent at a concentration increasing from 0 to 80% in 60 minutes. The spectrophotometer (SPD-M10AVPR, Shimadzu, Kyoto) was used for flavin-type-compound detection at wavelengths of 350 nm and 440 nm. All chemicals for the standards were of HPLC grade and used without further purification.

3. Results and discussion

Medium which contains yeast extract displayed clear electrochemical activity as revealed by a reversible redox peak around -0.4 V vs. Ag|AgCl in the cyclic voltammogram (Fig. 1). Moreover, a remarkable catalytic current with an onset at -0.4 V vs. Ag|AgCl was observed after *L. lactis* was added to the medium (Fig. 1). This catalytic current was not observed in a similar experiment where *L. lactis* was not provided with yeast extract (Fig. 2) and thus can be explained with the utilization by *L. lactis* of a soluble redox compound which must be present in yeast extract. The cyclic voltammogram of Fig. 2 also exhibits a catalytic current, but with a different onset at approximately -0.1 V vs. Ag|AgCl. This result confirms previous findings that *L. lactis* also produces its own redox compounds and uses them as electron transfer mediators for anodic glucose oxidation [6]. However, the higher potential at which the onset of the catalytic current is observed reveals a different electron transfer pathway in this case. These results show that *L. lactis* can transfer electrons to electrodes through both exogenous and endogenous redox compounds.

The occurrence of non-biological catalytic current producing mechanisms such as electrochemical oxidation of excreted metabolites could be excluded as no catalytic current could be observed with centrifuged cell growth medium supernatant.

Figure 1

Figure 2

In order to identify the redox compounds in yeast extract, HPLC detection was carried out. The chromatogram shows that a compound in yeast extract was eluted at the same retention time and exhibiting the same spectra as riboflavin (data not shown). The concentration of this compound was estimated by peak analysis to be approximately 0.5 μ M. The standard redox potential of riboflavin is -0.43 V vs. Ag|AgCl, and this matches well the redox peaks observed in Fig. 1.

To ensure that riboflavin can indeed work as a redox mediator for *L. lactis* in the same way as the redox compound found in yeast extract, cyclic voltammetry was carried out using a medium which contained 0.1 M phosphate buffer (pH 7) and riboflavin (1 μ M). Upon addition of *L. lactis* and glucose, a clear catalytic current was observed (Fig. 3), proving that riboflavin does mediate the electron transfer from *L. lactis* to the electrode. The catalytic current in this case was smaller than with yeast extract despite the greater

riboflavin concentration. This may be explained by the greater metabolic activity of the bacterium in the presence of a more complete nutrient medium such as one that contains yeast extract.

When flavin-related compounds FAD (flavin adenine dinucleotide) or flavin mononucleotide (FMN) were added to the bioelectrochemical cell at the concentration of 1 μM , FMN induced a catalytic current, whereas FAD did not, as revealed by the chronoamperometric results shown in Fig. 4. The different behavior of *L. lactis* with different flavins may be ascribed to the selective binding of specific flavins to the flavin transporter in the cell membrane. *L. lactis* riboflavin transporter RibU was indeed previously shown to selectively bind riboflavin and FMN, but not FAD [9].

Figure 3

Figure 4

To establish whether the ability to exploit flavins for extracellular electron transfer is widespread among bacteria, cyclic voltammetry was carried out using flavin in the presence of other two electrochemically active species of bacteria, *Escherichia coli* and *Propionibacterium freudenreichii*, but no catalytic current was observed with these microbes, indicating that the ability to exploit flavins may be restricted to few microorganisms. This result is consistent with the previous finding that out of the three species tested, only *L. lactis* is normally a riboflavin consumer [10] and is thus geared with a fast riboflavin transport system to enhance flavin uptake from the medium at even very small concentrations [11]. *E. coli* and *P. freudenreichii* are instead normally riboflavin producers [10, 12], with *E. coli* having been shown to lack a dedicated transport system for this vitamin, with concentrations greater than 0.7 mM required for diffusive uptake. The presence of a flavin transport system may thus reasonably be ascribed as the cause of the ability of *L. lactis* to rapidly shuttle flavins in and out of the cells at concentrations in the order of 1 μM .

4. Conclusions

This work revealed that riboflavin and other flavin-type compounds contained in yeast extract are exploited as exogenous redox mediators for microbial extracellular electron transfer. In particular, this is the first report to show that flavin contained in yeast extract works as an exogenous mediator for fermentative bacterium *L. lactis*. As yeast extract containing medium is often used as a nutrient source in microbial fuel cell studies,

exogenous redox mediators may be often unintentionally added to the media, thus affecting the results. The use of yeast extract as a source of nutrients in microbial fuel cell media is thus strongly discouraged, and all previous results obtained in the presence of yeast extract should be re-analysed in light of this finding.

Flavins are suitable redox mediators in microbial fuel cells as their potential is just slightly higher than that of organic electron donors (-0.4 V compared to -0.6 V vs. Ag|AgCl for the latter). The microbially catalyzed anodic oxidation of organics happens via NADH oxidation (the redox potential of NADH is -0.52 V vs. Ag|AgCl). If flavin is used as a redox mediator, the loss of potential from NADH to the electrode is reduced compared to other redox mediators (such as quinones or cytochromes), because the redox potential of flavin is close to that of NADH, at approximately -0.4 V vs. Ag|AgCl. In light of this consideration, flavin can be regarded as a near-optimal mediator for microbially catalyzed anodic electron transfer.

This study shows that not all electrochemically active bacteria can utilize flavin for electron transfer. According to recent studies, some electrochemically active bacteria have no ability to exploit flavin for extracellular electron transfer even though they can produce and secrete flavin compounds. The knowledge of which bacterial species are capable of flavin utilization is of great importance for the selection of cultures for microbial fuel cell anodes.

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Figure captions

Figure 1. Cyclic voltammograms (1 mV s^{-1}) of yeast extract containing (5 g L^{-1}) medium, in the absence (dashed line) and presence (solid line) of *L. lactis* and glucose (5 g L^{-1}). A redox compound present in yeast extract is utilized by the bacterium for extracellular electron transfer. Liquid volume: 0.1 L .

Figure 2. Cyclic voltammograms (1 mV s^{-1}) of a *L. lactis* bioanode without added yeast extract, in the presence (solid line) and absence (dashed line) of glucose (5 g L^{-1}). *L. lactis* utilizes self excreted mediators for extracellular electron transfer [6]. Liquid volume: 0.1 L .

Figure 3. Cyclic voltammograms of a *L. lactis* bioanode, with $1 \text{ }\mu\text{M}$ riboflavin in 0.1 phosphate buffer (pH 7) in the absence (dashed line) and presence (solid line) of 5 g L^{-1} glucose. Liquid volume: 0.1 L .

Figure 4. Chronoamperometry ($E = -0.1 \text{ V vs. Ag/AgCl}$) of *L. lactis* bio-anode in phosphate buffer (0.1 M , pH 7) in the presence of glucose. The bioanodes were supplemented with $1 \text{ }\mu\text{M}$ of riboflavin (solid line), FMN (dashed line), FAD (dotted line). The dash-dotted line displays a control with no added flavins. Liquid volume: 0.1 L .