RSC Advances

PAPER

View Article Online
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Cite this: *RSC Adv.*, 2019, **9**, 22957

# Effects and mechanism of riboflavin on the growth of *Alcaligenes faecalis* under bias conditions†

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Received 29th May 2019
Accepted 8th July 2019

DOI: 10.1039/c9ra04066h

rsc.li/rsc-advances

Some microorganisms can utilize photoelectrons and electrode electrons. Exogenous electrons generate enough energy for growth, and electron shuttles may accelerate this process. This research data supported photoelectron-responsive microorganism *Alcaligenes faecalis* was effected by the growth metabolism due to bias and electron shuttle riboflavin (RF) with an adaptive screening voltage under oligotrophic conditions. A slight change was observed in the redox property of RF. RF played the role of an electron shuttle. Microbial extracellular metabolites could bind additional nicotinamide adenine dinucleotide (NADH) species with RF. The intracellular protein content in the group of RF–Bias was 1.94, 1.93 and 4.02 times higher than those in the RF, bias and control groups, respectively, while the corresponding intracellular contents of humus were 1.10, 0.93 and 1.42 times higher. The content of CoA in RF–Bias, RF and bias increased to 116.0%, 108.5% and 103.8%, respectively. The organic acids of the RF–Bias group in the Krebs cycle are more advanced than those of other groups. Overall, in the Krebs cycle, RF and bias facilitated the growth and metabolism of *A. faecalis*. Finally, a mechanism was proposed, showing that the electron transfer chain and the Krebs cycle are stimulated by RF and bias.



## 1 Introduction

Both photosynthetic and non-photosynthetic organisms can use solar energy either directly or indirectly for growth.[1] Photosynthetic organisms directly utilize solar energy, while non-photosynthetic organisms can grow and metabolize by utilizing photoelectrons. They are produced by semiconductor minerals under light irradiation. In this process, electronic transitions occur and produce photoelectron–hole pairs.[2] Photoelectrons can affect the structure of the microbial community and promote the growth of heterotrophic microorganisms as well as the production of metabolites and energy.[4,5] To some certain extent, the photoelectrons generated by semiconductor minerals can reduce nitrates.[6] The growth and metabolism of the non-photosynthetic microorganism *Alcaligenes faecalis* screened from soils is promoted and responsible for photoelectrons.[7] The interaction between electrons and microorganisms is mainly based on electrons.

Up till now, the efficiency of the microbial utilization of photoelectrons has not been satisfactory.[3] To investigate the photoelectron cycle of microbes, external electrons are used to simulate photoelectrons. At a suitable electrode potential, microorganisms can grow by 2.5–3 times.[35,36] Even under the photocatalytic electrons, *Acidithiobacillus ferrooxidans* can grow by 12 times.[37]

The microbial extracellular electron transfer pathways are discussed below: (1) microorganisms can remain in direct contact with solid surfaces or form agglomerates with electron acceptors; however, only a few microorganisms can follow this mechanism.[8–10] (2) Additional energy is required in long-range electron transfer, which is mediated by conductive pili or flagella synthesized by the microorganisms themselves.[11] (3) Microorganisms use "wires" as conductive materials for electron transfer.[12–14] (4) Electron transfer can be conducted by small molecules with redox properties and electron mediators naturally occurring or manually synthesized. These small molecules are called electron shuttles (ESs). This process reduces a large amount of energy consumption and the limitation of direct contact.[8,15,16]

ESs are electronic carriers,[17,18] and their use is one of the important ways to mediate extracellular electron transfer. Flavin,[8] melanin,[19] sulfur compounds and humus[15] are common ESs. Some ESs interact with cells across membranes,[20] while other ESs directly transfer electrons from the structure.[21]

According to new data, a large number of researchers have applied ESs to control environmental pollution such as that

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/c9ra04066h

caused by uranium and the reduction of Fe and dichromate.[22,24–29] In addition, electron transfer can promote microbial metabolism.[30] In anaerobic microbial communities, organic matter can be degraded.[31] Hence, the production of methane by microorganisms can play the role of ES to accelerate the degradation process.[13]

Riboflavin (RF) is one of the isoalloxazines, and its first and fifth nitrogen atoms have a conjugated double bond (Fig. 1). Because of the conjugated double bond, RF can be easily replaced by a hydrogen atom and easily dehydrogenated after reduction; thus, RF has reversible redox characteristics. The addition of RF enables the sulfate-reducing bacteria to increase the pitting depth of 304 stainless steel across the cell membrane.[23] First, the extracellular ESs of flavin mononucleotide (FMN) and RF are identified, which are also involved in the metabolism of microbial cells.[55] However, the role of ESs in the process where non-photosynthetic microorganisms utilize electrode electrons is not clear.

In this research, *A. faecalis* was used as a model of non-photosynthetic microorganism treated in oligotrophic conditions with or without an RF medium under bias conditions. The data of the ES role in the process were investigated: *A. faecalis* utilized electrode electrons through growth and metabolism. Then, the mechanism of RF and electrons in promoting *A. faecalis* was predicted. At the molecular level, this research provides theoretical possibilities in the interactions among electron shuttle, bias and microorganisms.

## 2 Materials and methods

### 2.1 Source of bacteria

*Alcaligenes faecalis* was isolated and identified by Laboratory of Microbial-Mineral/Nuclear Interactions, College of Life Science and Engineering, Southwest University of Science and Technology.

### 2.2 Materials and reagents

Medium: 1% LB medium, 10 mg $L^{-1}$ riboflavin (RF, Sigma, 97.5%).

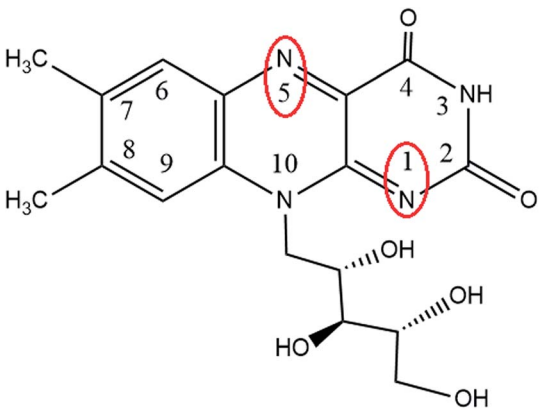


Fig. 1 Chemical structure of RF.

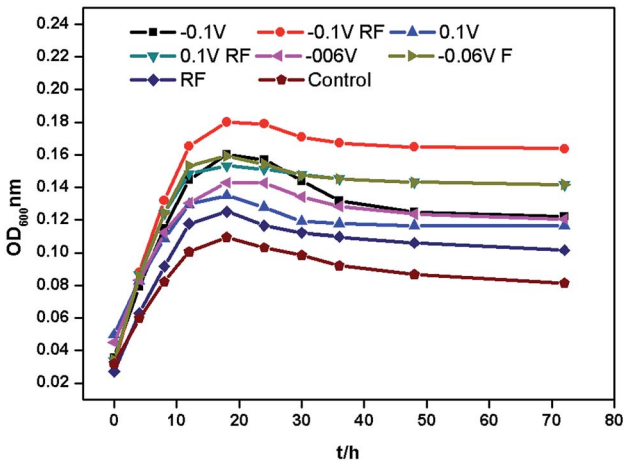


Fig. 2 Growth curve of *A. faecalis* cultured under different conditions.

Main instruments: field emission scanning electron microscope (SEM, UItra 55 Carl Zeiss), CHI660E Electrochemical Workstation (Shanghai Chenhua Instrument Co., Ltd.), F-7000 Fluorometer (Hitachi, JPN), U-3900 UV-Vis Spectrophotometer (Hitachi, JPN), high-performance liquid chromatograph (HPLC, Jiangsu Hanbang Technology Co., Ltd.), *etc.*

### 2.3 Methods

**2.3.1 Bias condition screening.** A control group was set in incubation with *A. faecalis* in oligotrophic conditions with or without RF media at −0.1 V, −0.06 V, 0 V and 0.1 V bias. The absorbance of samples at 0, 4, 8, 12, 18, 24, 30, 36, 48, and 72 h was observed by a UV spectrophotometer (wavelength of 600 nm). The optimum bias growth curve was screened.

**2.3.2 Morphological changes.** The 48 h culture of *A. faecalis* was centrifuged and washed with stroke-physiological saline solution. It was uniformly coated on cover slips and air-dried. The cover slips were immersed in 2.5% glutaraldehyde solution every time for 10 h and eluted with 70%, 80%, 90%, 95% and 100% ethanol gradients for 20 min. After air drying, the cell morphology was observed *via* SEM.

**2.3.3 The redox property of RF.** RF (10 mg $L^{-1}$) was dispensed with 0.5 mol $L^{-1}$ $Na_2SO_4$ and oxidation was performed using $N_2$ for 20 min.[32] The absorbance peak of the redox standard RF was obtained *via* cyclic voltammetry (CV) using a CHI660E electrochemical equipment. The CV and differential pulse voltammetry (DPV) results were compared before and after incubation with *A. faecalis*.

**2.3.4 Analysis of metabolites.** After 18 h incubation, *A. faecalis* was centrifuged. The supernatant and residue were tested through three-dimensional fluorescence for analysing fractional metabolites. The specification of the three-dimensional fluorescence instrument was as follows: wavelengths of 200–600 nm; speed of 12 000 nm $min^{-1}$; slit of 10 nm; step size of 10 nm; and PMT voltage of 700 V.

**2.3.5 The content of CoA.** The enzyme-linked immunosorbent assay kit was used (Shanghai MLBIO Biotechnology Co. Ltd).

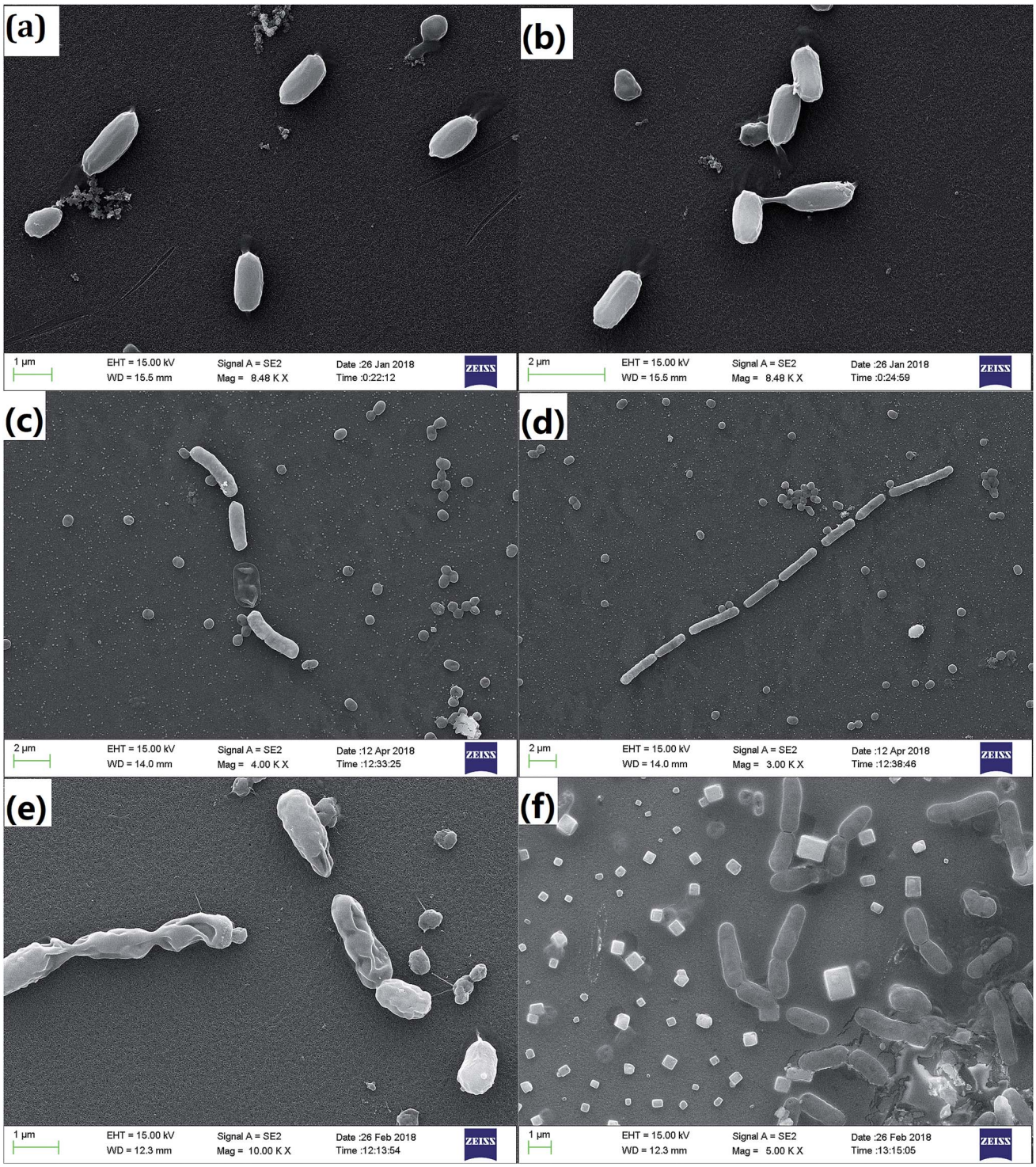


Fig. 3 The morphology of *A. faecalis* incubated in different conditions ((a) control, (b) RF, (c) −0.1 V, (d) −0.06 V, (e) 0.1 V, and (f) RF −0.1 V).

**2.3.6 The content of organic acids.** Each sample was broken by ultrasonication. HPLC was used for the Krebs cycle analyses. The specification of HPLC was as follows: Agilent's ZORBAX Eplipse XDB-C18 column (4.6 × 150 nm); mobile phases were 97% of 0.06 mol $L^{-1}$ disodium hydrogen phosphate solution and 3% methanol solution; column temperature was 35 °C; flow rate was 0.6 mL $min^{-1}$; injection volume was 20 μL; and UV detector wavelength was 205 nm.[33,34]

# 3 Results and discussion

## 3.1 Bias condition screening

Fig. 2 shows the growth curves of *A. faecalis* cultured under different conditions. They were almost the same in the growth cycle. The adaptation time period was about 0–4 h, the logarithmic time period was about 4–18 h, and the stable time period was about 18–32 h. The decomposition rate of *A. faecalis*

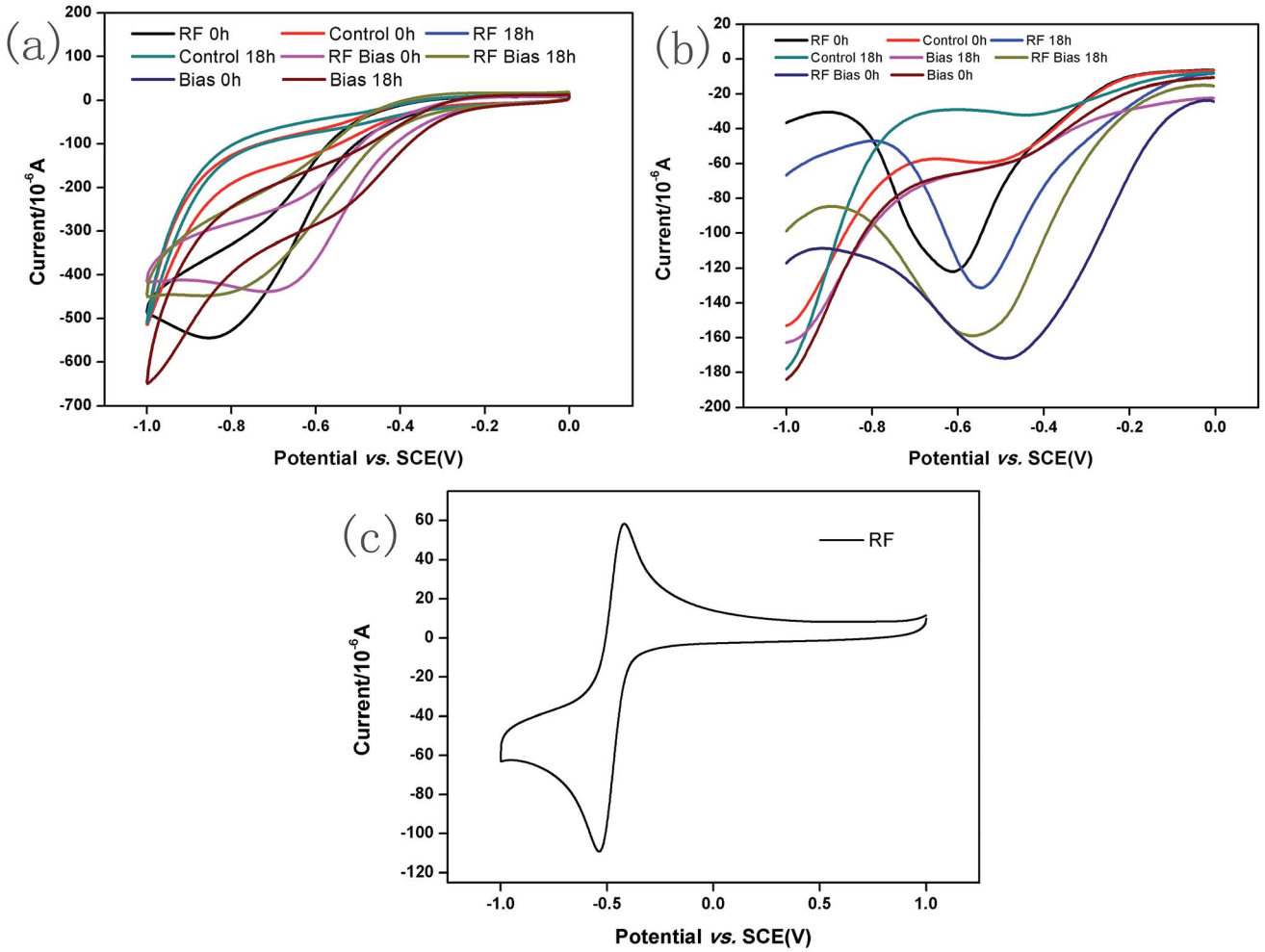


Fig. 4 CVs of systems before and after incubating *A. faecalis* (a); DPVs of systems before and after incubating *A. faecalis* (b); CV of RF at 10 mg $L^{-1}$ (c).

can be delayed by photoelectrons, while bias and RF have no significant effect on the decomposition rate.[6] Both bias and RF promoted the growth of *A. faecalis*, but the effect of bias was more productive. This is related to the results reported by Wang *et al.* (2009).[38] Moreover, negative bias promoted better growth of *A. faecalis* than positive bias. When cultured for 18 h, RF showed the lowest effect on the growth of *A. faecalis*, which was about 1.16 times greater than that of the control group. In contrast, at −0.1 V, RF showed the most potent effect on the growth of *A. faecalis*, which was about 1.70 times higher than that for the control group. Thereby, −0.1 V bias was used in this research.

### 3.2 Morphological changes

Different electrode potentials can change the morphology and generation time of microorganisms.[39,40] *A. faecalis* was observed using SEM (Fig. 3). Although RF accelerated extracellular electron transfer, *A. faecalis* exhibited normal size of 0.7–1.0 μm. However, bias caused a significant change, and the size of *A. faecalis* was longer than 1.0 μm. This is related to the results reported by Yu *et al.* (2012).[41] After the effects of bias, *A. faecalis* exhibited a slender shape.

### 3.3 The redox property of RF analysis

The CV of 5 mg $L^{-1}$ RF is shown in Fig. 4c with an oxidation peak of approximately −0.4 V and a reduction peak of approximately −0.6 V. The results of CV and DPV measurements are shown in Fig. 4a and b, respectively, before and after incubating *A. faecalis*. At 0 h, the CVs for the RF groups show redox peaks. The DPV results showed the reduction peak of −0.6 V. According to standard reduction peak, in these conditions, it was indicated that RF could undergo redox reactions. After 18 h of culture, the redox peak positions of the RF groups did not change, but the reduction peak positions of DPVs showed slight shifts. This might be because the microbial metabolites were more vigorous after culturing. Compared with the result at 0 h, the DPVs without RF groups showed reduction peaks in the same position as that of RF, which may be secreted by *A. faecalis* itself. The amount of RF secreted by *A. faecalis* was negligible[8] compared with the amount of manually added RF. RF was not consumed and played the role of an electron shuttle. FMN and

 

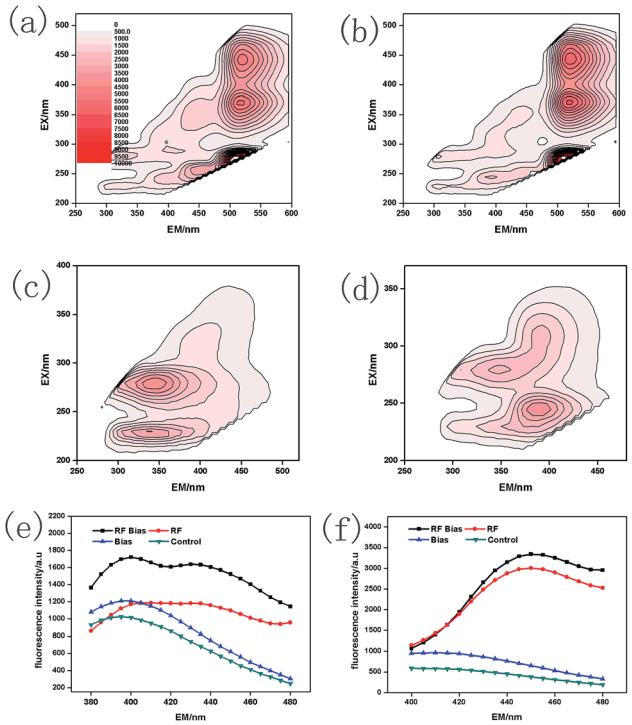


Fig. 5 Three-dimensional fluorescence analysis of extracellular metabolites ((a) RF–Bias; (b) RF; (c) Bias; (d) control); the contents of extracellular humic substance (EX = 320 nm, (e)) and NADH (EX = 350 nm, (f)).

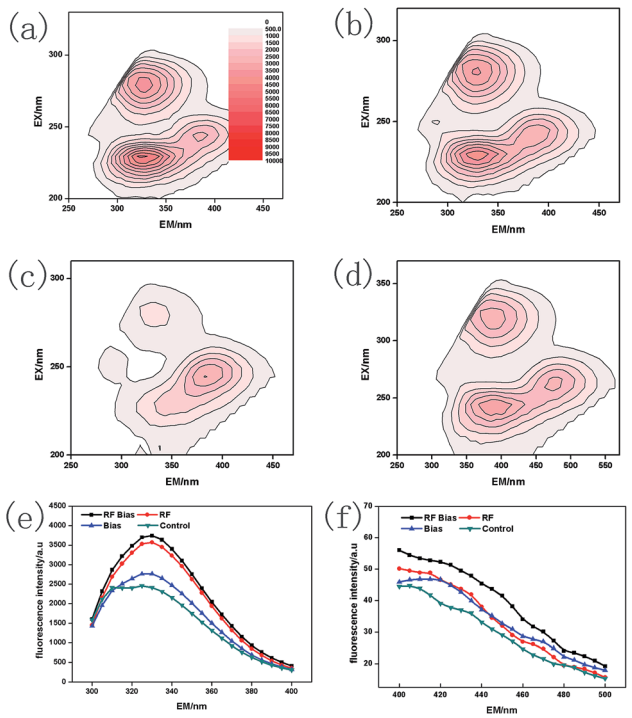


Fig. 6 Three-dimensional fluorescence analysis of intracellular metabolites ((a) RF–Bias; (b) RF; (c) Bias; (d) control); the contents of intracellular protein (EX = 280 nm, (e)) and humic substance (EX = 320 nm, (f)).

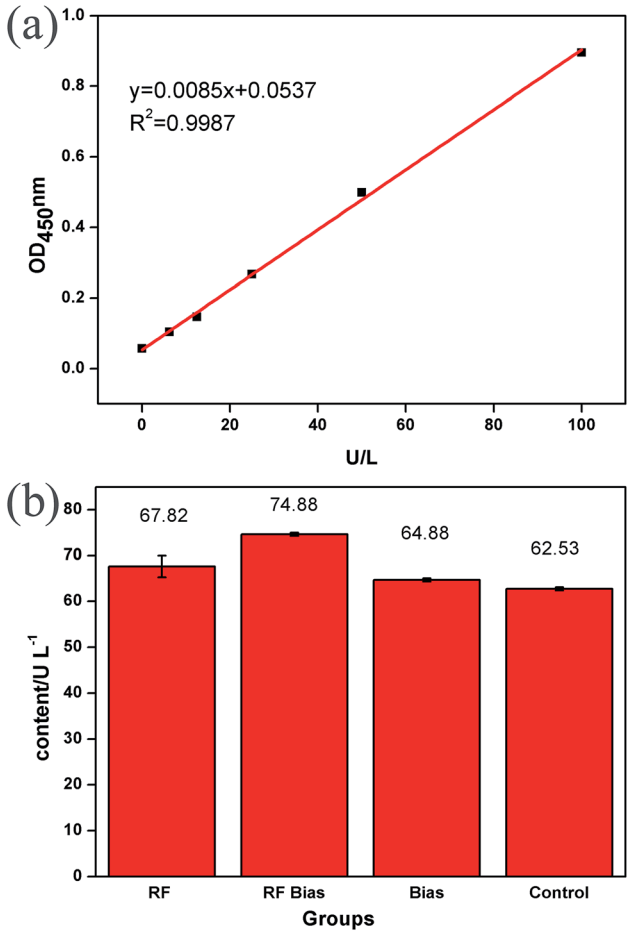


Fig. 7 Standard CoA content curve (a); intracellular CoA content (b).

RF secreted by *Shewanella* can accelerate the microbial reduction of ferrites as an electron shuttle, and RF has the potential to deliver electrons.[8,42]

### 3.4 Analysis of three-dimensional fluorescence

Microorganisms secrete maximum amounts of metabolites such as proteins, humic substances and RF when they are producing or gaining energy.[43] The results for the extracellular metabolites of *A. faecalis* are shown in Fig. 5. Furthermore, RF ($\lambda_{EX/EM}$ = 450/520 nm, 375/520 nm, and 267/520 nm)[44] can be shown in Fig. 5a and b. The UV humic-like ($\lambda_{EX/EM}$ = 225 nm/410 nm)[46,47] and humic substances ($\lambda_{EX/EM}$ = 320–390 nm/400–480 nm)[45] can be represented in Fig. 5. The UV humic-like was mainly high molecular weight humus.[48] RF also significantly promoted the formation of nicotinamide adenine dinucleotide (NADH) ($\lambda_{EX/EM}$ = 350 nm/450 nm) (Fig. 5f). The process of glycolysis and the Krebs cycle in NADH could be promoted by RF and bias. NADH is capable of interconversion with nicotinamide adenine dinucleotide ($NAD^+$) through oxidative phosphorylation. $NAD^+$ does not have fluorescence properties and can simultaneously produce ATP.[49] However, under anaerobic conditions, the oxidation process is inhibited and NADH will be accumulated.[50] Bias directly provides

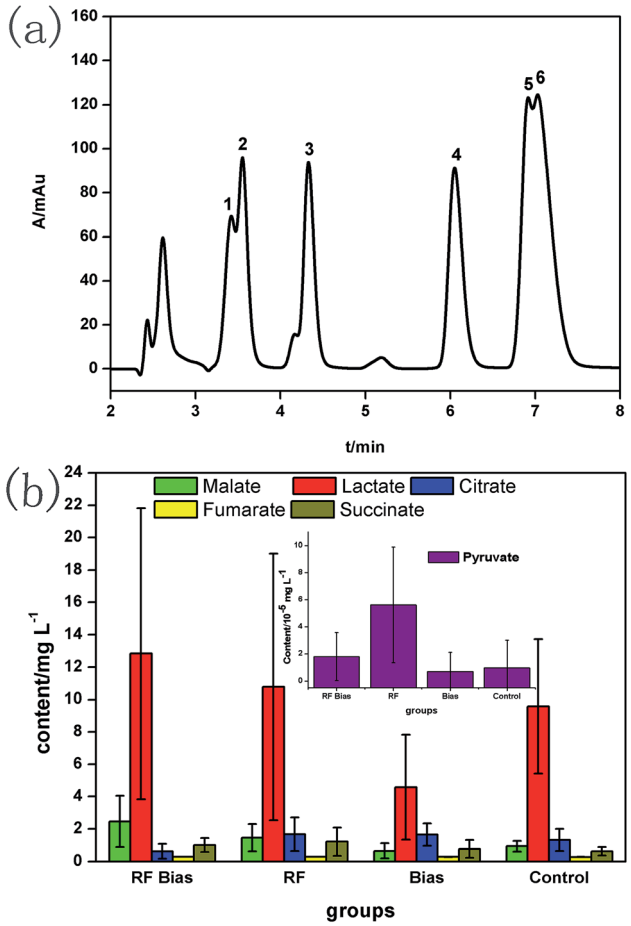


Fig. 8 Standard organic acids (a) ((1) pyruvate; (2) malate; (3) lactate; (4) citrate; (5) succinate; (6) fumarate); the content of intracellular organic acids (b).

Table 1 The standard curve of organic acids

| Organic acid | Regression equation | Related parameters |
|---|---|---|
| Malate | $y = 0.0246x - 0.0366$ | 0.9978 |
| Fumarate | $y = 2.7373x - 7.7253$ | 0.9902 |
| Citrate | $y = 0.0354x - 0.0112$ | 0.9970 |
| Succinate | $y = 0.0181x - 0.0426$ | 0.9915 |
| Lactate | $y = 0.0131x - 0.0215$ | 0.9994 |
| Pyruvate | $y = 248.35x + 0.0187$ | 0.9999 |

electrons to the oxidation process, while RF transfers them.[51] The process of electron transfer accelerated by the conversion of NADH to $NAD^+$ generated more ATP molecules. Then, the processes of glycolysis and oxidative phosphorylation were also promoted. RF and bias could indirectly promote the growth and metabolism of *A. faecalis*.

The results for *A. faecalis* intracellular metabolites are shown in Fig. 6. The value of RF–Bias was the highest in the intracellular protein, which was 1.94, 1.73 and 4.02 times higher than those of the RF, Bias and control groups, respectively. Compared with the results for other groups, the contents of

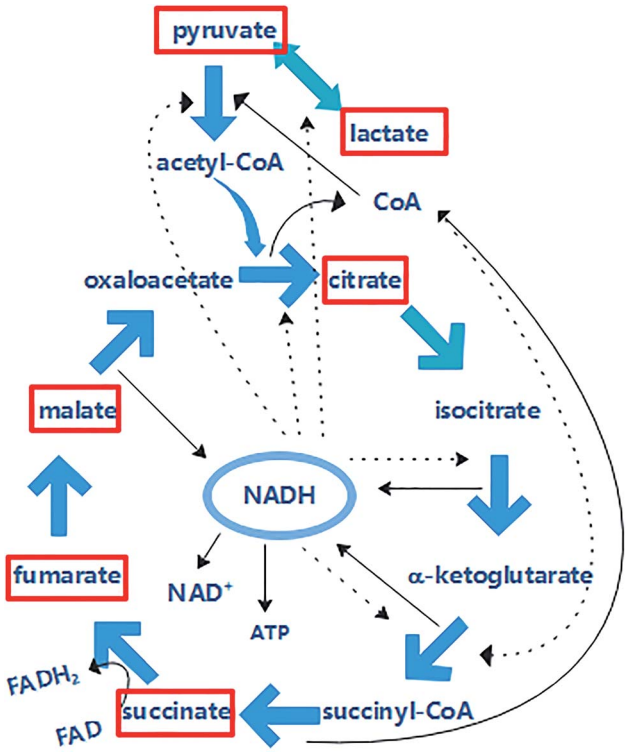


Fig. 9 The relationship among NADH, CoA and the Krebs cycle (the solid line represents the generation and the dotted line represents the participation in the regulation).

intracellular humus were 1.10, 0.93 and 1.42 times higher than those of the RF, Bias and control groups, respectively. Microbial intracellular substances mainly include enzymes and genetic materials. The main components are proteins, which help improve the growth and metabolism of bacteria. In addition, *A. faecalis* will secrete humus itself, which also acts as an electron shuttle.[15,52]

Bias could promote the redox reaction process and metabolism of microorganisms. The processes of RF might promote the glycolysis of *A. faecalis*, the Krebs cycle and oxidative phosphorylation electron transfer.

### 3.5 The content of CoA analysis

In an intracellular cycle, more than seventy enzyme reactions are followed by the CoA cofactor, including the synthesis of amino acids and fatty acids, pyruvate metabolism and decomposition of sugars. CoA provides 90% of energy for life, and it can be acetylated to acetyl-CoA by pyruvate (Fig. 9), which is the promoter of the Krebs cycle. The content of CoA is shown in Fig. 7b. It was found that the CoA activity was enhanced. In RF–Bias, the content of CoA increased by 116%, 108.5% and 103.8%, which boosted the process. Fig. 9 shows the relationship between CoA and the Krebs cycle. The Krebs cycle can produce and consume CoA. Oxaloacetate and acetyl-CoA synthesize citrate under the catalysis of citrate synthase; succinyl-CoA produces succinate under the action of succinyl-CoA synthetase. CoA can be accumulated by both processes.

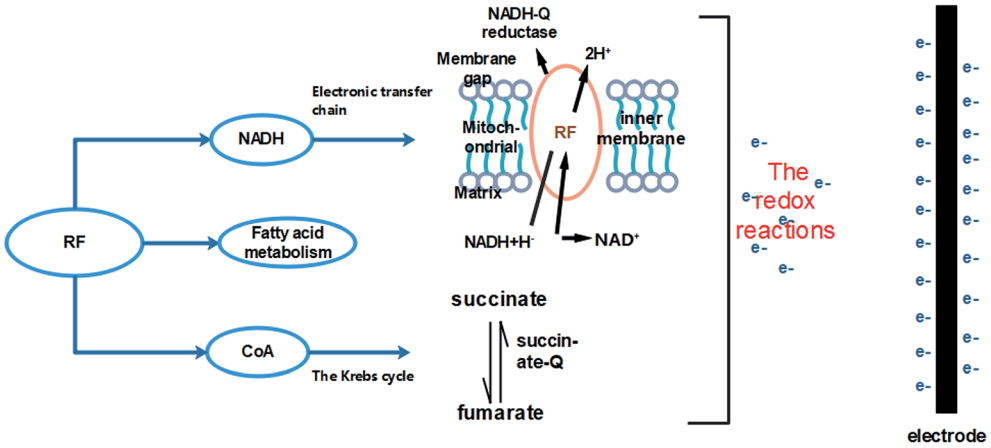


Fig. 10 The possible pathways where RF and bias generate the metabolism of *A. faecalis*.

Succinyl-CoA formed by α-ketoglutarate can be regulated by CoA. Therefore, the promotion of microbial metabolism by RF might be related to the Krebs cycle.

### 3.6 Analysis of organic acid content connected with the Krebs cycle

The Krebs cycle is the key of metabolism. Metabolites such as glycolysis and fatty acids enter the Krebs cycle through acetyl-CoA and are decomposed and supplied to the electron transfer chain to form ATP. Electrons are transferred to $O_2$ *via* the respiratory chain and react with ATP during microbial energy metabolism.[11] This process involves the participation of the Krebs cycle, NADH/$NAD^+$ and CoA. Fig. 9 shows the relationship between NADH and the Krebs cycle. The Krebs cycle can produce NADH, which in turn can be involved in the regulation of the Krebs cycle. NADH can be obtained by the conversion of isocitrate to α-ketoglutarate, α-ketoglutarate to succinyl-CoA and malate to oxaloacetate. NADH can be involved in the regulation of the interconversions of pyruvate to lactate, pyruvate to acetyl CoA, oxaloacetate to citric acid, isocitrate to α-ketoglutarate and α-ketoglutarate to succinyl-CoA. NADH accumulated in these processes enters the respiratory chain oxidative phosphorylation process, producing $NAD^+$ and ATP.

Fig. 8a shows the analysis of standard pyruvate, malate, lactate, citrate, fumarate and succinate. The six organic acids could be effectively analysed, and there was a good relationship between different concentrations of organic acids (Table 1). The content of intracellular organic acids in *A. faecalis* is shown in Fig. 8b. Similarly, the contents of CoA and NADH increased in RF–Bias. The content of citrate in each group did not have much difference for consuming. Electrons can participate in the reduction of fumarate to succinate in *Geobacter sulfurreducens*.[53] Hence, RF can promote the conversion of succinate to fumarate. Despite this, NADH can regulate lactate dehydrogenase. Consequently, the content of lactate with RF was significantly higher than that of others. In *A. faecalis*, the RF relationship in the Krebs cycle due to the increase in CoA, NADH and RF regulates the content of organic acids.

### 3.7 The possible mechanism of electron shuttles in microorganisms

RF and bias affected the growth and metabolism of *A. faecalis*. The possible mechanisms are as follows (Fig. 10). One way involves promoting and affecting the process of electronic transmission chain. The flavin electron shuttle has two forms: (i) oxidized and (ii) reduced. In microorganisms, the function of flavin electron shuttle is to transfer hydrogen during the redox process. Flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) are the main forms of RF. FMN is a prosthetic group of electron transfer chain-enzyme complex I (NADH-Q reductase); FAD is a prosthetic group of enzyme complex II (succinate-Q reductase). NADH-Q reductase promotes electrons across the mitochondrial inner membrane to the mitochondrial space, oxidizing NADH and reducing FMN to deliver electrons.[54] Succinate-Q reductase is a protein complex in the mitochondria and succinate dehydrogenase promotes the conversion of succinate to fumarate.[54] Electron transfer and intracellular redox reactions were promoted.

The promotion of the Krebs cycle is another way. RF and bias increased the content of CoA and related organic acids in *A. faecalis*. The process of the Krebs cycle should be promoted, and bias can provide electrons to the Krebs cycle.

## 4 Conclusions

Under oligotrophic conditions, both bias and RF promoted the growth and metabolism of *A. faecalis*. In terms of accelerating electron transfer, bias provided additional electrons to *A. faecalis*, while RF acted as a mediator. The growth curves indicated that RF and bias did not affect the growth cycle. At different potential voltages, bias would change the morphology of *A. faecalis* but not RF would. Through the three-dimensional fluorescence analysis of metabolites, *A. faecalis* synthesised more humus and NADH with RF, and the latter was closely related to microbial glycolysis and the Krebs cycle. This was confirmed by the content of CoA. Furthermore, these results corresponded to the content of organic acids related to the Krebs cycle. The Krebs cycle ought to be the means by which RF

and bias promoted *A. faecalis*. RF and bias support the promotion collectively. More research needs to be conducted to obtain better results at the molecular level for the growth and metabolism of non-photosynthetic microorganisms, which are directly influenced by electron shuttles.

## Conflicts of interest

There are no conflicts to declare.

## Acknowledgements

The authors thank the National Key R&D Program of China (2016YFC0502204), National Basic Research Program of China (973 Program: 2014CB846003), National Nature Science Foundation of China (Grant number: 41272371, 41502316), and Longshan Academic Talent Research Supporting Program of SWUST (18LZX507). The authors also thank Dr Toquier Azam for language help.

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