Research Article

Synthetic *Klebsiella pneumoniae-Shewanella oneidensis* consortium enables glycerol-fed high-performance microbial fuel cells†

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Abstract

Microbial fuel cell (MFC) is an eco-friendly bio-electrochemical system that uses microorganism as biocatalyst to convert biomass into electricity. Glycerol, as a waste in the biodiesel refinery processes, is an appealing substrate for MFC. Nevertheless, glycerol cannot be utilized as carbon source by well-known exoelectrogens such as *Shewanella oneidensis*. Herein, to generate electricity by rapidly harnessing glycerol, we rationally constructed a *Klebsiella pneumoniae-Shewanella oneidensis* microbial consortium to efficiently harvest electricity from glycerol, in which *K. pneumoniae* converted glycerol into lactate, fed to *S. oneidensis* as carbon source and electron donor. To improve electricity output, we systematically engineered the consortium in terms of carbon flux distribution and efficiency of extracellular electron transfer (EET). To direct more carbon flux to lactate biosynthesis in *K. pneumoniae*, we eliminated the ethanol pathway by knocking out the alcohol dehydrogenase gene (*adhE*), and enhanced lactate biosynthesis by heterologously expressing a lactate dehydrogenase gene (*ldhD*) from *Lactobacillus bulgaricus* and a lactate transporter gene (*lldP*) from *Escherichia coli*. To facilitate EET between *S. oneidensis* and anode surfaces, a biosynthetic flavins pathway from *Bacillus subtilis* was introduced into *S. oneidensis*. We further optimized the glycerol concentration, thus *S. oneidensis* could be continuously fed with lactate synthesized from *K. pneumoniae* at a constant rate. Our glycerol-fed MFC generated a maximum power density of 19.9 mW/m^2, significantly higher than that of the wild-type consortium. This work suggested that engineering microbial consortia is an efficient strategy to expand the spectrum of usable carbon sources and promote electricity power production in MFCs.

Abbreviations:

MFCs, microbial fuel cells**; EET,** extracellular electron transfer**;** ***adhE*****,** alcohol dehydrogenase gene; ***ldhD***, D-lactate dehydrogenase gene**;** ***lldP*****,** L-lactate transporter gene**; PPP,** pentose phosphate pathway**; TCA**, tricarboxylic acid cycle**; GTP**, guanosine 5-phosphate**; R5P,** ribulose 5-phosphate**; FAD,** flavin adenine dinucleotide**; FMN,** flavin mononucleotide**; NADH/NAD$^+$,** nicotinamide adenine dinucleotide**; CV,** cyclic voltammetry**; LSV,** linear sweep voltammetry**; OCPs,** open circuit potentials**.**

1 Introduction

Microbial electrochemical systems had been widely used in many applications, including organic wastes treatment and electricity production by microbial fuel cells (MFCs) [1-3], hydrogen production by microbial electrolysis cells [4], seawater desalination by microbial desalination cells [5], value-added chemicals production from CO_2 bioreduction by microbial electrosynthesis [6]. In particular, MFC, as a promising technology, offered many possibilities of harvesting electricity from various organic wastes [7, 8], for example, food wastes, human feces, industrial stubborn or toxic wastes, marine putrefaction and lignocellulose, *etc.* [9, 10], in which organic substrates were broken down by microbial metabolism and the derived electrons were transferred from exoelectrogens to anode surfaces via contact-based extracellular electron transfer (EET) or shuttles-mediated EET pathways for bioelectricity production [9, 11-13]. A great deal of efforts had been put into elucidating the fundamental mechanisms of EET [14, 15], and optimizing the efficiency of MFCs' electricity generation from the perspective of synthetic biology [16-18], electrode materials, environmental biotechnology and bio-electrochemical reactors [19].

Among the organic wastes, glycerol is regarded as an "industrial waste product" generated as inevitable byproduct during biodiesel refinery processes [20-22]. Converting the waste glycerol to green and sustainable electricity power by MFCs thus received increased attention in recent few years [23]. Nevertheless, glycerol could not be utilized by most exoelectrogens such as *Shewanella* and *Geobacter* as carbon source, owing to lack of proper glycerol transporters in these exoelectrogens [24, 25].

To utilize glycerol as carbon source in MFC for electricity generation, two strategies were studied. The first strategy was to genetically engineer *Shewanella oneidensis* to directly use glycerol. Fynn et al. [25] incorporated a glycerol uptake pathway into *S. oneidensis* MR-1 by introducing genes of *glpF*, *glpK*, *glpD* and *tpiA*, which realized direct glycerol utilization by *S. oneidensis*. Whereas, such engineered *S. oneidensis* consumed glycerol at a rather low rate, and generated a relatively low output power density (~2 mW/m^2). Another strategy was to use mixed cultures to de-

rive electricity from glycerol in MFCs [26, 27]. Reiche et al. [27] used an undefined mixed culture in MFCs with glycerol as carbon source and electron donor for power generation, resulting in a maximum power density of 11.7 mW/m^2. However, such undefined microbial mixture was hard to be engineered to further improve its power generation, since the complicated mechanisms of the microbial metabolic interactions and EET among fermenters and exoelectrogens were not elucidated. In another study, Kim et al. [24] built a well-defined microbial consortium including wild-type *K. pneumoniae* and wild-type *S. oneidensis*, which could generate a maximum power density of 2.15 mW/m^2.

For the purpose of further promoting bioelectricity production efficiency from glycerol to enable practical applications, we herein adopted the strategy of ‘‘division-of-labor’’ to design a rationally engineered microbial consortium with recombinant *K. pneumoniae-S. oneidensis*, in which metabolic interaction modes between the fermenter (*K. pneumoniae*) and the exoelectrogen (*S. oneidensis*) were enhanced to achieve a higher efficient conversion of glycerol to electricity (**Fig. 1**) [25, 28-32]. We rationally engineered the consortium strains by redirecting carbon flux distribution of the fermenter and improving EET of the exoelectrogen, respectively. To redirect more carbon flux into lactate biosynthesis, the ethanol biosynthesis pathway of *K. pneumoniae* was eliminated by knocking out the native alcohol dehydrogenase gene (*adhE*). To further redirect more carbon flux to lactate biosynthesis and balance the intracellular redox state of *K. pneumoniae*, the lactate dehydrogenase encoded by gene *ldhD* from *Lactobacillus bulgaricus* [33] was incorporated into *K. pneumoniae.* In addition, for reducing the transport resistance of hydrophilic lactate across the hydrophobic cell membrane, the lactate transporter encoded by gene *lldP* from *Escherichia coli* [33] was heterologously expressed in *K. pneumoniae.* On the other hand, to achieve higher EET efficiency of *S. oneidensis*, a synthetic flavins biosynthesis pathway from *Bacillus subtilis* was incorporated in *S. oneidensis* for producing abundant flavins as the electron shuttle [34]. Such systematical and rational design for both the fermenter and the exoelectrogen enabled more glycerol to be efficiently converted to electricity output. As a result, the engineered microbial consortium

($ET_{K.pneumoniae}$-$ET_{S.oneidensis}$) generated a maximum power density of 19.9 mW/m^2 in MFC, which was significantly higher than that of the wild-type consortium (8.1 mW/m^2). This work suggested that engineering microbial consortia is an efficient strategy to expand the spectrum of usable carbon sources and enhance the electricity power generation in MFCs.

2 Materials and methods

2.1 *In vitro* gene synthesis

The *ldhD* gene encoding the lactate dehydrogenase originated from *Lactobacillus bulgaricus* ATCC11842 (GenBank no. 103422405), and the *lldP* gene encoding the lactate transporter originated from *Escherichia coli* K-12 strain MG1655 (GenBank no. 1790031) were identified in the NCBI database (**Table S1**). Subsequently, the gene codon sequences were optimized for *K. pneumoniae* in Java codon adaption tool (JCAT) to prevent blocked translation by excluding the rare codons of tRNAs [33, 35]. In particular, the restriction enzyme sites of *BamH*I and *Kpn*I were avoided in the codon optimized sequence. Then, the DNA fragments of *ldhD* and *lldP* were flanked by restriction enzyme sites *BamH*I and *Kpn*I, and ligated into vector pUC18K digested by the same restriction enzyme sites to obtain reconstructed plasmid pUC18K-*ldhD-lldP*. The designed gene sequences were synthesized *in vitro*, verified by Sanger sequencing (AuGCT, China). No specific explains, all plasmids used in this study were stocked in the host strain Trans T1.

2.2 Strain construction and culture condition

The KG1-A^- strain was genetically engineered by knocking out the *adhE* gene encoding alcohol dehydrogenase following a previously established procedure [36]. The recombinant *K. pneumoniae* strain KG1-A^--L^+ ($ET_{K.pneumoniae}$) was constructed by electro-transferring, introducing the plasmid pUC18K-*ldhD-lldP* into KG1-A^-. In this electro-transferring process, pulser voltage selected 1.8 kV and 2 mm electroporate chamber was used. The flavin genes (*rib*ADEHC) from *Bacillus subtilis* were inte-

grated into the vector pYYDT to build an expression plasmid pYYDT-C5 by following our previous work [34], which subsequently transformed into the plasmid donor strain *E. coli* WM3064 (auxotroph, 100 μg/ml 2,6-diaminopimelic acid (DPA) was added for growth). Thus, the engineered *Shewanella* C5 ($ET_{S.oneidensis}$) was constructed by conjugation with the *E. coli* WM3064 strain, which subsequently transformed pYYDT-C5 into *S. oneidensis* MR-1

The *K. pneumoniae* was cultured in LB (Luria-Bertani) broth, shaking at 37°C with 200 rpm overnight, and *S. oneidensis* was cultured in the same condition except for 30°C instead of 37°C. Then, 3% inoculation amount of *K. pneumoniae* suspension was transferred to M9 minimal medium (pH 7.5) (**Table S2**) supplemented with 100 mM glycerol for amplification culture, while the same inoculation amount of *S. oneidensis* suspension was transferred to LB broth (pH 7.5). Whenever needed, 50 μg/ml kanamycin and 0.2 mM IPTG were supplemented to the culture medium for plasmid maintenance. All stains and plasmids used in this study are listed in **Table 1.**

2.3 MFC setup

The dual-chamber MFCs (H-type) applied in this study were separated by Nafion 117 membrane (DuPont Inc., USA), which was pre-treated in 1 M HCl overnight and washed in sterile distilled water before MFC setup. Carbon cloth was used as the electrodes for both anode (2.5 cm × 2.5 cm) and cathode (2.5 cm × 3 cm). The anolyte consisted of M9 buffer supplemented with 100 mM glycerol and 5% (v/v) LB broth. The catholyte was consisted of 50 mM $K_3[Fe(CN)_6]$ in 50 mM KH_2PO_4 and 50 mM K_2HPO_4 solution. A 2 kΩ external resistor was connected into the external circuit of MFCs, and the output voltages were recorded across the external loading resistor with a digital multimeter (DT9205A).

In this MFC, the microbial consortium was composed of $ET_{K.pneumoniae}$ and $ET_{S.oneidensis}$, while the control group consisted of $WT_{K.\ pneumoniae}$ and $WT_{S.\ oneidensis}$. After around 10 hours' culture, the amplification cultured cells, which grew up to OD_{600}=2.0, were subsequently centrifuged and collected at 4°C, 6000 rpm for 10 min to remove the supernatant. Then, the cell pellets were all dispersed into the anode

chambers (140 mL working volume). To ensure consistent culture condition for different MFCs, 50 μg/ml kanamycin and 0.2 mM IPTG were supplemented in anolytes whenever needed. All MFCs were incubated in a 30°C incubator and each group was tripled for parallel experiments. The OD_{600} was measured by an ultraviolet and visible spectrophotometer (TU-180, Beijing, China).

2.4 Electrochemical analysis

Before electrochemical analysis, MFC was in an open-circuit condition for 1-2 h when it reached the highest output voltage. All electrochemical experiments were conducted using a CHI1000C multichannel potentiostat (CH Instrument, Shanghai, China). Cyclic voltammetry (CV) analysis with a scan rate of 1 mV/s was conducted on a three-electrode mode, where the anode as the working electrode, the cathode as the counter electrode, and the Ag/AgCl (vs. SHE) as reference electrode.

Linear sweep voltammetry (LSV) analysis with a slow scan rate (0.1 mV/s) was conducted on a two-electrode mode, where the anode performed as the working electrode and the cathode as the reference as well as the counter electrode to obtain the polarization curves to estimate the maximum power density (the potential decreased from the open circuit potential (OCP) to around -0.3V). The current density and power density obtained by the above procedure were analyzed by following equations [37]:

$$I = V/(R \times S) \quad (1)$$

$$P = V \times I \quad (2)$$

V---- output voltage; R---- external resistance;

I----current density; P----power density;

S---- anode carbon cloth area

2.5 Quantification of metabolites

The metabolites in the anolytes were analyzed by a high-performance liquid chromatography (HPLC) system, which was equipped with a refractive index detector. All sample supernatants and standard solutions were pre-treated by a 0.22 μm filter and

diluted prior to HPLC assay. Analysis of glycerol, lactate and ethanol were separated on an Aminex HPX-87H ion-exchange column (Bio-Rad) operating at 45°C with sulfuric acid (5 mM) as the mobile phase (0.6 ml/min), and detection was performed with the Waters 2414 refractive index detector.

For the quantification of riboflavin, the samples in the MFC supernatant were firstly centrifuged (35,000 rpm for 20 min) and filtered (0.22 μm), then, the eluted medium was detected by a liquid chromatograph-tandem mass spectrometer (LC-MS) (Agilent LCMS-1290-6460) in a positive ion mode by a Waters XBridge C8 column (2.1 mm×100 mm; particle size: 3.5 μm).

2.6 Biofilms characterization

To measure the composition of *K. pneumoniae* and *S. oneidensis* and the change of consortium structure during the MFC operation process, both anolyte and anode carbon cloth were assayed by colony forming unit (CFU) counting (**Fig. S1**). The samples of anolyte and anode biofilms at different time were prepared by the following procedure. The samples were washed by PBS (phosphate buffered saline) for three times. Then, a series of diluted cultures (10^5-, 10^6-,10^7-,10^8- and 10^9-folds dilution) were spread onto the LB agar plate, respectively, and incubated for 36 h at 30°C. The colony of *S. oneidensis* was dark orange, while the colony of *K. pneumoniae* was white. Thus, the CFU of the two bacteria could be differentiated and counted.

3 Results

3.1 Engineering *K. pneumoniae* for lactate production and *S. oneidensis* for flavins biosynthesis

K. pneumoniae could produce a certain proportion of lactate, which utilized glycerol as carbon source. However, a considerable amount of ethanol was concurrently produced to compete carbon source with lactate, and such ethanol biosynthesis pathway was the prime process to consume NADH generated from the glycolysis of *K. pneumoniae*[36]. Therefore, to direct more glycerol to lactate biosynthesis, we blocked the

ethanol biosynthesis pathway by knocking out the native alcohol dehydrogenase gene (*adhE*), which encoding the critical alcohol dehydrogenase (ADH) in response for ethanol production in *K. pneumoniae*. Thus, the ethanol biosynthesis pathway was completely interrupted in *K. pneumoniae* by knocking out the *adhE* gene.

However, such interruption of ethanol biosynthesis would result in accumulating abundant reducing equivalent (NADH), causing intracellular redox out-of-balance, which would inhibit cell viability. To this end, we heterologously expressed the lactate dehydrogenase (LDH) encoded by *ldhD* gene from *L. bulgaricus*. LDH catalyzed the reduction of pyruvate to form lactate, in this process, the accumulated NADH was oxidized to NAD^+ to sustain intracellular redox balance. Furthermore, a lactate transporter encoded by *lldP* gene from *E. coli* for transporting lactate extracellularly by using proton motive force [34], was introduced in *K. pneumoniae* to improve lactate production, since the hydrophobic cell membrane is the main barrier for the secretion of hydrophilic lactate. Thus, the introduced lactate biosynthesis pathway would not only balance the intracellular redox state, but also redirect more carbon flux to lactate biosynthesis. Upon deleting the *adhE* gene and overexpressing the *ldhD* and *lldP* genes, the content level of metabolites (i.e., ethanol and lactate) in engineered *K. pneumoniae* was changed dramatically. Compared with the $WT_{K.pneumoniae}$ strain, the $ET_{K.pneumoniae}$ strain incorporated lactate biosynthesis pathway produced a significant amount of lactate without ethanol **(Fig. 2a)**.

In addition, electrons generated from oxidation of organic wastes by these exoelectrogens could transfer to anodes via contact-based EET pathways mediated by *c*-type cytochromes [38] and diffusible electron shuttles-mediated EET pathways [39, 40]. Among them, the electron shuttle-mediated electron transfer was proven to dominate the EET of *S. oneidensis* [41]. Herein, to facilitate the electricity generation of this consortium, we further heterologously expressed a synthetic flavins biosynthesis pathway from *Bacillus subtilis* in *S. oneidensis*. As a result, the flavins produced by the $ET_{S.oneidensis}$ (*S. oneidensis* C5) increased by 7.9 times compared with the $WT_{S.oneidensis}$ (*S. oneidensis* MR-1) (from 0.68 ± 0.13 μM to 5.36 ± 0.42 μM). **(Fig. 2b)**.

3.2 Design and optimize *K. pneumoniae*-*S. oneidensis* co-culture for glycerol-fed MFC

To enable the co-culture of *K. pneumoniae* and *S. oneidensis* in a common growth medium, a serial of orthogonal experiments were conducted to determine a prior seeding ratio of *K. pneumoniae* and *S. oneidensis*. The result demonstrated the final seeding OD_{600} ratio between *K. pneumoniae* and *S. oneidensis* being 1:10 (diluted to 0.05: 0.5 from the initial seeding OD_{600}=2.0 of each microorganism) would enable a maximum MFC electricity generation (**Fig. S2**).

Secondly, to produce lactate at a constant rate by *K. pneumoniae* to feed *S. oneidensis* as carbon source and electron donor for maximizing the MFC power generation, we optimized the initial feed-in glycerol concentration based on this seeding ratio. Three different concentration gradients of glycerol (including 50 mM, 100 mM and 150 mM in a common growth medium) were chosen to determine the optimal glycerol concentration for our recombinant strains. The results showed 100 mM feed-in glycerol resulted in a highest MFC output voltage (234 ± 5 mV), while 50 mM and 150 mM feed-in glycerol produced lower MFC output voltage, 147 ± 4 mV and 59 ± 5 mV, respectively **(Fig. 3)**.To expound the underlying reason, we measured lactate concentration sampled from the anolyte after 24 hours' cultivation by HPLC **(Fig. 3d)**. The data indicated that 150 mM feed-in glycerol could lead to a higher level accumulation of lactate in MFC **(Fig. 3c)**, however, the electricity power output of the MFC in such condition was not the optimum because such rapidly accumulated lactate resulting in an acidic media (pH~5), in which the power generation capability of *S. onedensis* would be reduced due to inhibited growth activity and flavins biosynthesis of *S. oneidensis* in the acidic condition revealed by our previous studies [19, 42]. When the feed-in glycerol concentration was adjusted to 50 mM in MFC, the voltage output maintained at a low level all the way to the end of MFC operations **(Fig. 3a)** because the substrate could be exhausted rapidly, leading to a shortage of lactate to feed *S. oneidensis*. In contrast, the recombinant consortium enabled higher and longer voltage output under 100 mM feed-in glycerol concentration than other glycerol concentrations. It may be explained that the lactate produced by $ET_{K.pneumoniae}$ remained in

an approximate constant level of 0.5 mM, suggesting *S. oneidensis* could be fed with lactate timely (**Fig. 3d**). Thus, the fermenter *K. pneumoniae* could continuously supply carbon source (i.e., lactate) to the exoelectrogen, *S. oneidensis*, and the lactate converted from glycerol was not too high to show cellular toxicity to *S. oneidensis*. Thus, the synergistically metabolite cooperation between the fermenter and the exoelectrogen enormously facilitated carbon metabolism and extracellular electron transfer in MFC, resulting in an optimal electricity generation.

Overall, upon optimization of the seeding ratio of the microorganisms and the feed-in glycerol concentration, we obtained an optimal carbon flux distribution and extracellular electron transfer efficiency for electricity generation.

3.3 MFC performance and bio-electrochemical analysis

To further investigate the EET efficiency of these rationally designed microbial consortia in MFCs, bio-electrochemical analysis were conducted. The cyclic voltammetry (CV) (at a scan rate of 1 mV/s) was applied to reveal the redox reaction kinetics at the interfaces of cells and anode electrodes. From **Figure 4b**, we found both the flavins-mediated and direct-contact-based EET mechanisms took effect [43], especially, the typical redox peaks of flavins in the CV curves around -0.4 V (vs. AgCl) demonstrated that flavins-mediated extracellular electron transfer was the dominating mechanism for bioelectricity production of *S. oneidensis*.

To study the performance of the MFC inoculated with these consortia, linear sweep voltammetry (LSV) was used to get the polarization curves at a low scan rate (0.1 mV/s) from their open circuit potentials (OCPs). As shown in **Figure 4c**, the power output curves (output voltage vs. current density) and the polarization curves (power density vs. current density), that were obtained by varying load resistances to demonstrate the dependence of voltage and power on the current, helped to further investigate the bioelectricity generation capability of the MFC with the recombinant consortium. Specially, the slope of the linear part of the $ET_{K.pneumoniae}$-$ET_{S.oneidensis}$ co-culture in the polarization curve was smaller than that of the $WT_{K.pneumoniae}$-$WT_{S.oneidensis}$ co-culture, implying relatively smaller internal resistance

of the MFC inoculated with $ET_{K.pneumoniae}$-$ET_{S.oneidensis}$ (**Fig. 4c**). Finally, the maximum power densities of the MFCs inoculated with the $ET_{K.pneumonia}$-$ET_{S.oneidensis}$ and $WT_{K.pneumoniae}$-$WT_{S.oneidensis}$ MFCs were obtained by multiplying the current with its corresponding voltage (19.9 mW/m^2 and 8.17 mW/m^2, respectively).

In addition, we found that not only the $ET_{K.pneumoniae}$ shown the superior performance of glycerol consumption than $WT_{K.pneumoniae}$ in a pure strain MFC, but also the consumption rate of glycerol in the consortium MFC was much faster than that inoculated the sole *K. pneumoniae* MFC. And the elevated EET of *S. oneidensis* (the exoelectrogen) could further enhance glycerol consumption by *K. pneumoniae* (the fermenter) **(Fig. 5)**. The result indicated the EET of exoelectrogen could enhance the glycerol consumption of the fermenter.

4 Discussion

To efficiently harvest electricity from glycerol, we rationally constructed a well-defined synergistic microbial consortium including *K. pneumoniae* (the fermenter) and *S. oneidensis* (the exoelectrogen). *K. pneumoniae* could convert glycerol into lactate with high efficiency, which in turn be continuously used as carbon source and electron donor by *S. oneidensis*. To promote electricity generation of the microbial consortium in MFCs, we rationally engineered the composite strains in the consortium by redirecting carbon flux distribution toward lactate biosynthesis in *K. pneumoniae*, and enhancing flavins-mediated EET efficiency of *S. oneidensis*, respectively. To obtain higher and more stable electricity output, the feed-in glycerol concentration was optimized. With 100 mM glycerol, the lactate produced by the recombinant *K. pneumoniae* could continuously feed the recombinant *S. oneidensis* at a constant level until the exhaustion of glycerol, which enabled a highest bioelectricity generation (19.9 mW/m^2) in glycerol-fed *Shewanella*-based MFCs.

In order to convert glycerol (and many other organic substrates) to electricity, exoelectrogen (such as *Shewanella, Geobacter*) should be used. However, due to lack of the transporters of glycerol (and many other sugars), *S. oneidensis* could not use

glycerol. One way is to construct a “fermenter-exoelectrogen microbial consortium” to convert glycerol to electricity. The fermenter *K. pneumonia* could convert glycerol to lactate, which is the favorite carbon source for *S. oneidensis*. As shown in Figure. S1, a portion of energy from glycerol was used for the growth of *K. pneumonia* in order to reach certain cell density to produce sufficient lactate to feed *S. oneidensis* for power generation. Although a proportion of glycerol was used for the cell growth of the fermenter, the entire synthetic microbial consortia acquired the capability of converting various organics into electricity. This work suggested that engineering microbial consortia is an efficient strategy to expand the spectrum of usable carbon sources of exoelectrogens and to promote electricity power generation of MFCs.

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Conflict of interest

The authors declare no financial or commercial conflict of interest.

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Table 1. Strains and plasmids used in this study

Strains or plasmids	Feature(s)	Source or reference
Strains		
K. pneumoniae		
KG1 ($WT_{K.pneumoniae}$)	Parent strain	Our lab
KG1-A^-	KG1(Δ*adh*E)	This study
KG1-A^--L^+($ET_{K.pneumoniae}$)	Carrying plasmid pUC18K-*ldhD-lldP*	This study
S. oneidensis		
MR-1($WT_{S.oneidensis}$)	Parent strain	Our lab
C5($ET_{S.oneidensis}$)	Carrying pYYDT -C5	Our lab[34]
E. coli		
Trans T1	F-80(lacZ)ΔM15ΔlacX74hsdR(rk-, mk +)ΔrecA1398endA1tonA	Transgen Biotech
WM3064	A dap auxtotroph *E. coli*	Our lab
Plasmids		
pUC18K	Kam^r, pUC ori, P_{lac}, MCS	Our lab
pUC18K-*ldhD-lldP*	Plasmid with *ldhD* and *lldP* gene inserted	This study
pYYDT	5.9kb; Km^r; *lacZ*	Our lab[34]
pYYDT -C5	Plasmid with the *ribA, ribD, ribE, ribH* and *ribE* gene inserted	Our lab[34]

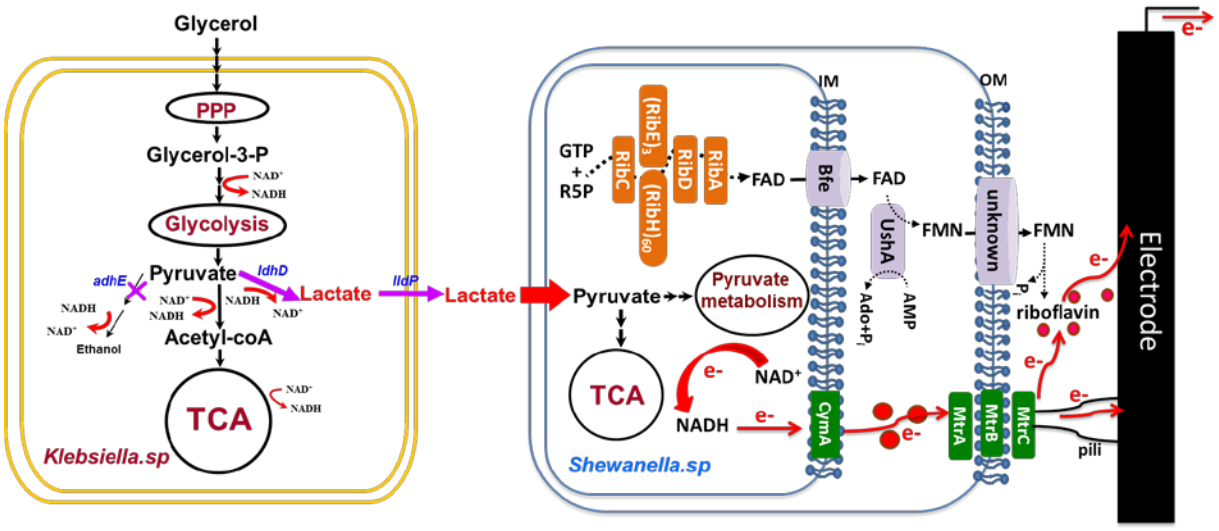

Figure 1. Schematic of the metabolic interaction of the recombinant *K. pneumoniae* ($ET_{K.pneumoniae}$)-*S. oneidensis* ($ET_{S.oneidensis}$) consortium. Lactate was produced by $ET_{K.pneumoniae}$ from glycerol, which in turn be used by $ET_{S.oneidensis}$ as carbon source and electron donor to produce electricity in MFCs. To direct more carbon flux to lactate biosynthesis in *K. pneumoniae*, the *adhE* gene was knocked out, and the *ldhD* gene (from *L. bulgaricus*) and the *lldP* gene (from *E. coli*) were exogenously introduced in *K. pneumoniae*. Furthermore, to facilitate EET between *S. oneidensis* and anode surfaces, a biosynthetic flavins biosynthesis gene cluster ribADEHC (originated from *B. subtilis*) was introduced into *S. oneidensis* for expressing the synthetic flavins pathway. The synthetic flavins worked as electron shuttles to promote the extracellular electron transfer between $ET_{S.oneidensis}$ and the anode surfaces.

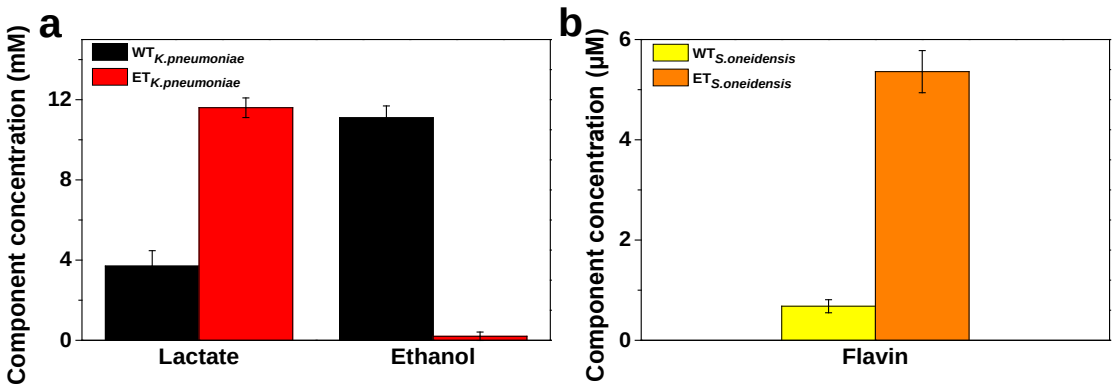

Figure 2. Quantification of metabolites of $ET_{K.pneumoniae}$ and $ET_{S.oneidensis}$. The metabolites concentration in the fermentation media (*K. pneumoniae* was inoculated in the anolyte with 75 mM glycerol, and *S. oneidensis* was cultured with 20 mM of sodium L-lactate instead of glycerol as the sole carbon source) was quantified by HPLC after 24th hours' fermentation. (a) The concentration of lactate produced by $ET_{K.pneumoniae}$ increased by 2.1 times (from 3.70 ± 0.77 mM to 11.60 ± 0.49 mM); the ethanol level produced by $ET_{K.pneumoniae}$ decreased by 98% (from 11.1 ± 0.19 mM to 0.2 ± 0.21 mM). (b) The concentration of flavins obtained from $ET_{S.oneidensis}$ increased ~7.9 times (from 0.68 ± 0.13 μM to 5.36 ± 0.42 μM) compared with the wild-type strain ($WT_{S.oneidensis}$). Statistics were calculated from three independent replicates of experiments.

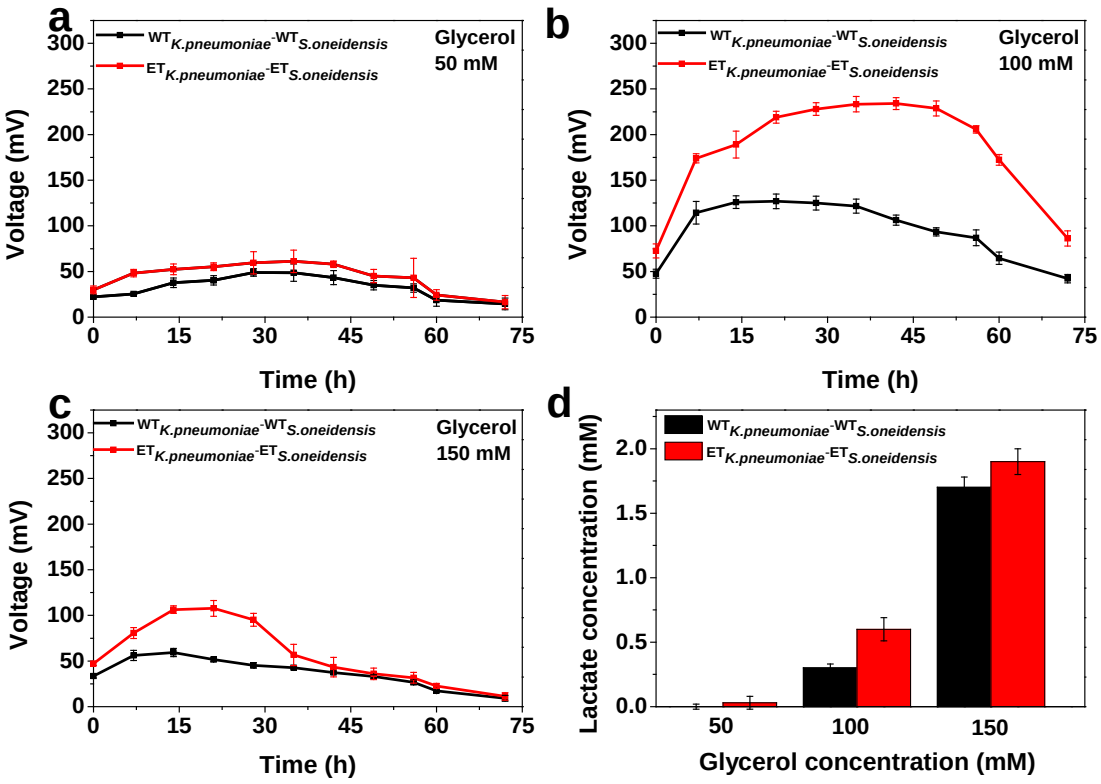

Figure 3. Optimizing glycerol concentration of the *K.pneumoniae-S.oneidensis* consortium to maximize power output in MFCs. The MFC voltage output of $WT_{K.pneumoniae}$-$WT_{S.oneidensis}$ and $ET_{K.pneumoniae}$-$ET_{S.oneidensis}$ consortium with different concentrations of feed-in glycerol. (a), (b) and (c) corresponded to feed-in glycerol concentration of 50, 100 and 150 mM, respectively. (d) Analysis of lactate level at 24th hour under different feed-in glycerol concentrations.

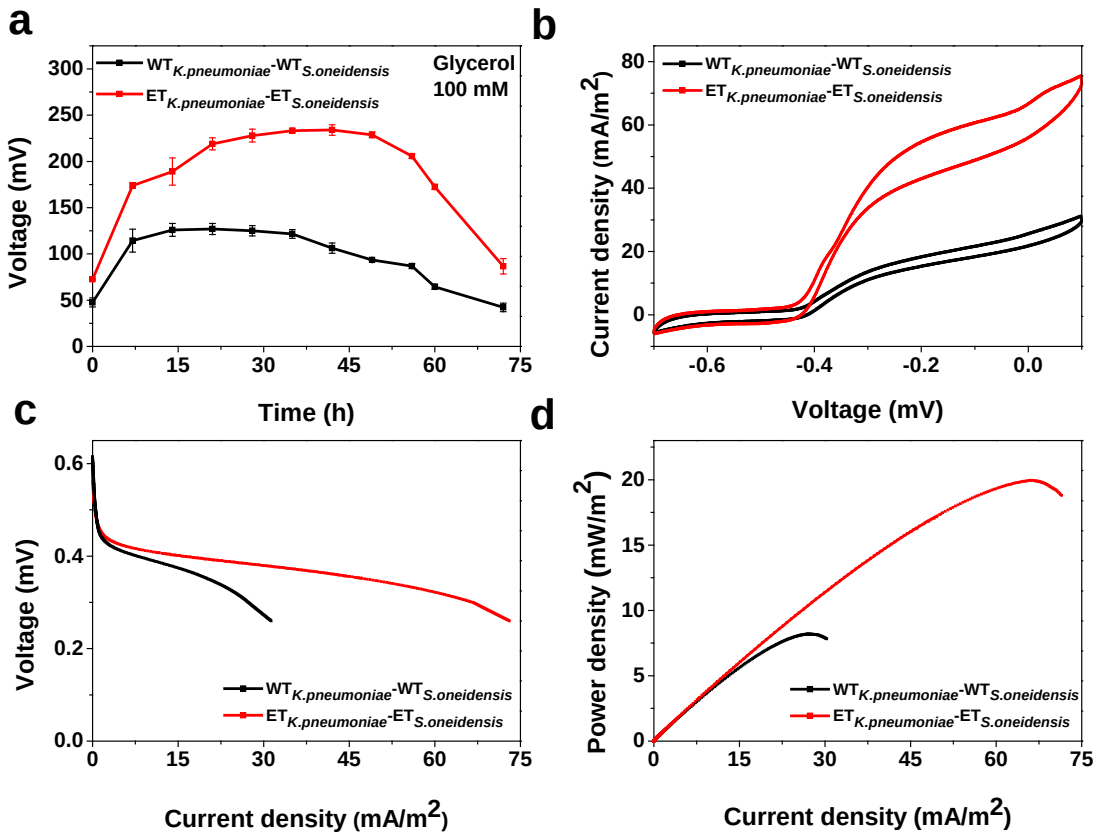

Figure 4. Electrochemical performances analysis of microbial consortium in MFCs. (a) MFC output voltage profiles produced by $WT_{K.pneumoniae}$-$WT_{S.oneidensis}$ and $ET_{K.pneumoniae}$-$ET_{S.oneidensis}$ under 100 mM glycerol feedstock anolyte. (b) Turnover cyclic voltammetry (CV) curves at a scan rate of 1 mV/s. (c) MFC polarization curves obtained by linear sweep voltammetry (LSV) with a slow scan rate of 0.1 mV/s. (d) MFC power density output curves calculated on the basis of the corresponding polarization curves.

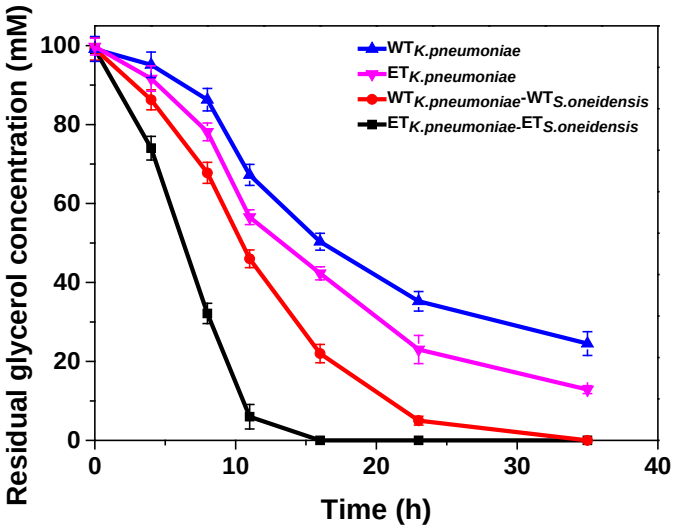

Figure 5. Glycerol consumption versus cultivation time by different combinations of strains. The blue, pink, red and black lines corresponded to different recombinants, $WT_{K.pneumoniae}$, $ET_{S.oneidensis}$, $WT_{K.pneumoniae}$-$WT_{S.oneidensis}$ and $ET_{K.pneumoniae}$-$ET_{S.oneidensis}$, respectively.