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BIOSENSORS

Shewanella baltica in a Microbial Fuel Cell for Sensing of Biological Oxygen Demand (BOD) of Wastewater

Nga Phuong Dang[a], Chris Petrich[a], Dorina Pasztor[b], György Schneider[b], and Rajnish Kaur Calay[c]

[a]Department of Cold Climate Technology, SINTEF Narvik, Narvik, Norway; [b]Department of Medical Microbiology and Immunology, University of Pécs, Medical School, Pécs, Hungary; [c]UiT – The Arctic University of Norway, Tromso, Norway

ABSTRACT

Geobacter and *Shewanella* are the most characterized electroactive bacteria genera. Unlike genus *Geobacter* that is strictly anaerobic, *Shewanella* can grow under both oxic and anoxic environments and is capable of metabolizing a wider substrate range. In the present study, the use of strain *Shewanella baltica* 20 in MFC-based biosensor for BOD monitoring is reported. The *S. baltica* strain 20 was isolated from river sediment in Hungary. The bacterium could form a biofilm, oxidized glucose, and transferred electrons to produce current when they were enriched on the anode in an air cathode microbial fuel cell (MFC). The tested MFC system demonstrated linearity in the current response to glucose from 50 to 300 mg/L. The electrical efficiency was determined from the polarization curve using the method of Varying Circuit Resistance (VCR) with an external load probed from 10 Ω to 220 kΩ. The maximum power production was 1.2 mW/m^2 at an external load of 40 kΩ. Studying the effect of external resistors on the MFC performance showed that the MFC reached higher saturation for 500 mg/L of glucose at lower resistances of 100 and 470 Ω, in comparison to 300 mg/L at 1000 Ω. The results show that the response of the MFC can potentially be tuned by adjusting the external load. Our preliminary study suggests that *Shewanella baltica* strain 20 may be used for online monitoring of BOD (biological oxygen demand) in wastewater.

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KEYWORDS
Biological oxygen demand (BOD); biosensor; microbial fuel cell; wastewater; water quality

Introduction

BOD (biological oxygen demand) is an index of the degradable organic compounds present in water and is used to quantify the degree of organic contamination in a water system. This parameter has been traditional estimated with the BOD5 test. This test, however, has a serious limitation of requiring 5 days of incubation and is therefore not suitable for real-time monitoring where rapid feedback is required. Current methods for toxicity evaluation of toxicants in the water stream are based on fishes, invertebrates,

CONTACT Nga Phuong Dang nga.dang@sintef.no Department of Cold Climate Technology, SINTEF Narvik, Narvik 8504, Norway

diatoms, or microorgansisms which have been reviewed by Hassan et al. (2016). Those methods can be time consuming (up to several weeks), and require detection of the signal from organisms, complicating the measurement.

In recent years, microbial fuel cells (MFC) have been shown to be a potential alternative to the BOD5 test for real-time and on-site measurement of the biodegradable organic carbon content as well as a toxicant indicator in the water (Elmekawy et al. 2018; Jiang et al. 2018; Olias and Di Lorenzo 2021; Sun et al. 2015). In an MFC, exoelectrogenic bacteria are immobilized on the anode to generate electrical energy by oxidizing organic matter in the water and transferring electrons to an electron acceptor outside of their cells. The current generated by the bacteria directly correlates to the metabolic activity at the anode as well as the degradable organic content in the water stream. Any disturbance on the bacterial metabolism and their growth causes a change in the current output. Toxicants from the feeding water can also affect the output current of the MFC. Heavy metals such as copper, chromium, nickel, zinc, and arsenic, were among the tested toxicants using MFC (Do et al. 2022; Khan et al. 2020; Safwat et al. 2023).

Generally MFC-based biosensors use mixed bacterial cultures because of the higher biomass density and viability, which can result in better sensitivity for BOD in comparison to pure strains (Zou et al. 2019). However, Yi et al. (2018) reported higher sensitivity of *Shewanella loihica* to cadmium, while comparing it with mixed cultures, because of the lower extracellular polymeric substances, poorer ability to secrete protein under toxic shocks and looser biofilm structure of the pure-culture approach. This study give a new perspective in MFC-based biosensor development.

The most studied models of exoelectrogenic bacteria are members of the *Geobacter* and *Shewanella* genus. *Geobacter* are strictly anaerobic, known for forming biofims on the anode and using conductive pili to transfer electrons (Virdis et al. 2014), while genus *Shewanella* are facultative (can grow under oxic and anoxic condition), and can transfer electron through direct contact with the anode surface (Koch and Harnisch 2016) or through self-synthesized mediators (Marsili et al. 2008). Members of the genus *Shewanella* those have the capacity of transfer electrons and forming biofilms include: *S. amazonensis*, *S. decolorationis*, *S. japonica*, *S. loihica*, *S. oneidensis*, *S. putrefaciens* (Castellano-Hinojosa et al. 2022) and *S. baltica* (Cao et al. 2009; Das and Calay 2022).

The strain *S baltica* 20, was isolated from sediments from the Hungarian section of the Danube River and selected based on the exoelectrogenic potential and biofilm formation capacity. The strain was tested for its ability of producing current while oxidizing organic pollutants for wastewater treatment (Banerjee, Calay, and Das 2023; Das and Calay 2022). However, the application of *Shewanella* in MFC for power production is not promising since they produced less power in comparison to MFC using mixed bacterial cultures (Zou et al. 2019). Practical applications that only require very small currents such as biosensors may be a better choice. The aim of the present study is to investigate the use of *S. baltica* 20 in a small air cathode MFC system for online-monitoring of degradable organic carbon content in wastewater. The use of *S. baltica* in biosensor application has not been reported thus far.

The reasons for selecting a small air cathode MFC over bigger design are: an improvement of the mass transfer in small MFC, leading to a more reliable sensor;

better oxygen supply at the air cathode can improve the system operation, enhancing the detection range; shorter response time, because of short distance from anode to cathode; fabrication and assembly of small devices are more precise and less expensive; and scaling up of small-scale MFC is more practical as it is easier to connect or stack multiple smaller unit than larger units (Parkhey et al. 2019).

Materials and methods

Culture media

Luria broth and M9 minimal media were used for inoculating and operating the MFC. Luria broth (LB) medium purchased from Sigma-Aldrich (Missouri, USA) was used for the preparation of enrichment medium according to the manufacturer's intructions. M9 minimal medium (MM9) consisted of (g/L): $Na_2HPO_4{\cdot}2H_2O$: 8.5, KH_2PO_4: 3, NH_4Cl: 1, NaCl: 0.5, $CaCl_2{\cdot}2H_2O$: 0.015, $MgSO_4{\cdot}7H_2O$: 0.5, $FeCl_3{\cdot}6H_2O$ 0.05, and supplemented with 1 mL of vitamin and trace-element solution which were prepared according to the 141 methanogen medium (DSMZ). Tryptic soy broth (TSB) was used for both biofilm formation and other tests with *Shewanella baltica* strain 20. TSB was composed of (g/L): casein: 17, soya peptone:3, NaCl: 5, K_2HPO_4: 2.5, dextrose: 2.5. All media were autoclaved at 121 °C and 1 bar for 15 min before use.

Shewanella baltica strain 20

The *S. baltica* strain 20 was chosen from a mud isolate bacterium collection (Schneider et al. 2023), due to its electrogenic potential. The electrogenic potential was tested using two methods, reduction assay with azodye Reagent Black 5 (RB5) and with the tungsten nanorod described earlier (Yuan et al. 2014). The RB5 reduction assay is an agar plate-based assay for a 1000 fold-concentrated stock solution of RB5 reagent (Sigma, Germany) that was mixed with freshly autoclaved tryptic soy agar (TSA), to the final concentration of 0.1 g/L. After the agar became solid, bacteria were spread onto the plates and incubated anaerobically at 30 °C. Yellowish discoloration around the colonies appeared after two days of incubation, indicated the reducing potential of bacteria.

For the tungsten nanorod assay, tungsten trioxide (WO_3) was synthetized from 0.825 g of $Na_2WO_4{\cdot}H_2O$ (Sigma-Merck, Darmstadt, Germany) and 0.290 g of NaCl in 20 mL of deionized water at acidic pH in a teflon coated autoclave at 180 °C for 16 h as described in the literature (Yuan et al. 2014). The prepared nanorods were washed with deionized water and dried. A suspension containing 5 g/L of tungsten nanorods was prepared in TSB. A volume of 80 μL of TSB was added to 100 μL bacterium suspension (10^8 CFU/mL) in each well of a 96 well-plate. Each well was covered with 80 μL paraffin oil and incubated at 30 °C. Bluish discoloration of the solution in the well indicated the electrogenic potential of the isolate.

Biofilm formation

Biofilm formation capacity of the investigated *S. baltica* 20 was investigated as described in the literature (Horváth et al. 2023). The strain was cultured overnight in TSB at

30 °C and 120 rpm. Twenty microliters of the bacterial culture and 180 µL TSB were transferred to each well in 96-well plate (Sarstedt 83.3924, Germany) that were incubated at 23 °C and 30 °C for 4 days. No fresh medium was added during the incubation time. The biofilm was revealed and quantified by crystal violet staining everyday (Xu et al. 2016). The planktonic cells were removed by gentle washing the wells with phosphate buffer saline (PBS). The adhered cells in the wells were fixed with 2% (v/v) formalin in PBS for 2 min and air-dried. Fixed cells were stained with 0.13% (w/w) crystal violet for 20 min and washed with PBS three times. Bounded crystal violet which stained the biofilm was solubilized in 1% (w/w) SDS (1% SDS in 50% ethanol and 50% PBS). After 2 h, the solutions in each well which contained the solubilized crystal violet were subjected to OD measurement at 595 nm with a FLUOstar Optima Microplate Reader (BMG Labtech, Ortenberg, Germany).

Scanning electron microscopy

To visualize the formed biofilm on the surface of anodic material, scanning electron microscopy (SEM) was performed. For that purpose, circular carbon cloth pieces with a diameter of 12 mm were incubated in the suspensions of *S. baltica* 20 in TSB medium for three days. The TSB was changed daily. After three days, the carbon felt pieces were treated with 2.5% (v/v) glutaraldehyde for 2 h at 4 °C to fix the surface-adhered bacteria. The sample was dehydrated using ethanol at concentrations of 50, 80, and 96% (v/v). For each step, 30 min incubation was applied. Before SEM imaging, the sample was air dried and coated with gold by using a Jeol JFC-1300 auto fine coater (Jeol, Tokyo, Japan). Different magnifications were applied to visualize bacteria which adhered on the surfaces of the carbon fibers. Images were captured with a Jeol JSM-IT500HR (Jeol, Tokyo, Japan) SEM using secondary electron mode. The accelerating voltage was set to 5 kV while the probe current was 45 kV.

Heavy metal resistance test

Cadmium, manganese, zinc, coban, copper and nickel ($CdSO_4$, $MnSO_4{\cdot}H_2O$, $ZnCl_2$, $CoSO_4{\cdot}7H_2O$, $CuSO_4{\cdot}5H_2O$, $NiCl_2{\cdot}6H_2O$) were used in these tests. Stock solution of each metal at 1 M were prepared and autoclaved. A series of two-fold dilution of each metal was conducted to the final concentration of 0.1 µM from the stock solution and the LB medium in 96-well plates. Bacterium cultured in LB medium at 30 °C for overnight (OD_{600nm} of 0.92–0.94) was added to the LB at a concentration of 2% (v/v). The LB medium was used for diluting the heavy metal solutions. The 96-well plates were incubated at 30 °C for 72 h. Inhibition of bacterial growth in the wells were confirmed by no growth after speading 50 µl of the cultures on LB agar plates.

Microbial fuel cell and operating conditions

A small-design MFC was constructed according to Di Lorenzo et al. (2014) with some modifications. The electrodes were 20 mm × 20 mm in size and separated by 5 mm. Carbon fiber electrode tissue from the University of Reading, UK, was used for the anode and cathode. All components of the MFC were glued together.

A fresh 200 mL of LB medium containing 5% (v/v) of bacterial innoculum was used as a feeding solution for the enrichment of the anode. The bacterial innoculum was prepared by innoculating strain *S. baltica* 20 in LB broth at 30 °C for 24 h. The feeding solution was freshly prepared every day for two days and continuously recirculated through the MFC at a flow rate of 0.1 mL min^{-1}. The cell was operated at open circuit potential during the enrichment time of two days until achieving a stable potential of 730 mV. Susequently, the M9 minimal medium with glucose (0.3 g l^{-1}) as substrate was used instead of the LB medium with no bacteria addition. An external resistive load of 1 kΩ was applied and the potential difference was monitored with a Campbel Scientific CR1000X data logger. The current was calculated from potential difference and external resistance. The produced current increased over one week and became stable. After achieving a stable current, the MFC was supplied continously with the fresh MM9 with glucose as the substrate, and under a closed circuit with an external load of 1 kΩ, glucose was added at concentrations from 10 to 600 mg L^{-1}.

Glucose determination

The glucose concentration in the inlet and outlet of the MFCs were determined using an enzymatic method, GAHK 20 kit (Sigma Aldrich) following the manufacturer's instruction to establish the consumption rate.

Electrochemical measurements

The output power of an MFC is affected by its internal resistance (Helder et al. 2012; Kim, Chang, and Gadd 2007; Rahimnejad et al. 2015). Under working conditions, internal resistance in a fuel cell can be divided into charge-transfer resistance, ohmic resistance, and mass-transfer resistance (He et al. 2006; Khan and Iqbal 2005). The electrical efficiency of the stabilized MFCs was determined from the polarization curve using the varying circuit resistance (VCR) method. The MFC was successively connected to external loads (R_{load}) from 10 Ω to 220 kΩ (stabilization time between each change was 6 to 10 min) and recording the respective cell voltage. Both internal resistance and open circuit voltage were fitted to the polarization curve (voltage-versus-current graph) based on the average of the voltage readings at the respective external loads, and to the replicates individually. The expected potential difference (V), current density (I_A), and power density (P_A) were calculated as functions of external load.

Results and discussions

Biofilm formation and heavy metal resistance

Biofilm formation

Bacterial adherence and biofilm formation is a crucial aspect of proper MFC function as it assures direct contact between the biotic and abiotic parts of MFCs (Schneider et al. 2016). Biofilm forming capacity of the *S. baltica* strain 20 was first investigated in a 96 well plate format and revealed that biofilm forming capacity of this strain was more stable at 23 °C during the four day test period (Figure 1). At this temperature, no

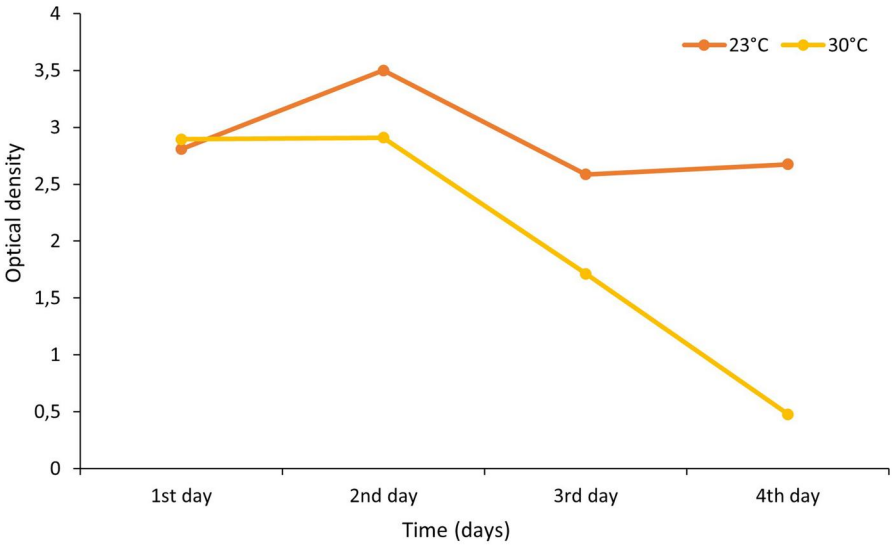

Figure 1. Comparison of the biofilm formation capacity of the *Shewanella baltica* strain 20 on the surface of the 96 well tissue culture plates at 23 °C and 30 °C. Quantities of the formed biomass in this static system were revealed with crystal violet staining on the first, second, third and fourth days of incubation. The values are depicted graphically.

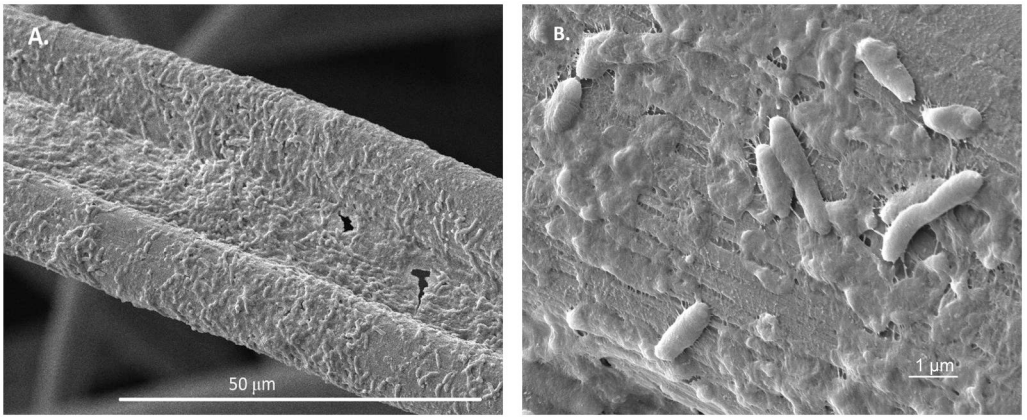

Figure 2. Established biofilm of the *Shewanella baltica* strain 20 on the surfaces of the filaments of the carbon felt. Scanning electron microscope images at (a) 500× with a 50 µm scale bar and (b) 1000× with a 1 µm scale bar.

significant loss was revealed among the staining intensities of the 1-, 2-, 3-, and 4 d incubations. The result was, however, to the contrary at 30 °C, the intensities of the stained biomass has been faded by the fourth day of incubation. This system was actually static in the sense that the medium was not changed in the wells during the four days incubation.

Since growth and the metabolic activity of the bacterial strain was higher at 30 °C than 23 °C, available nutrients were used up faster, and the bacterium culture went old sooner, which affected the structure and the stability of the previously formed biofilm. For this reason, another strategy was applied when the bacterial culture was grown onto

Table 1. Minimal inhibition concentrations of metals against *Shewanella baltica* 20.

Heavy metal concentration (mM)					
Cd	Mn	Zn	Co	Cu	Ni
0.06	15.62	3.9	7.8	3.9	7.8

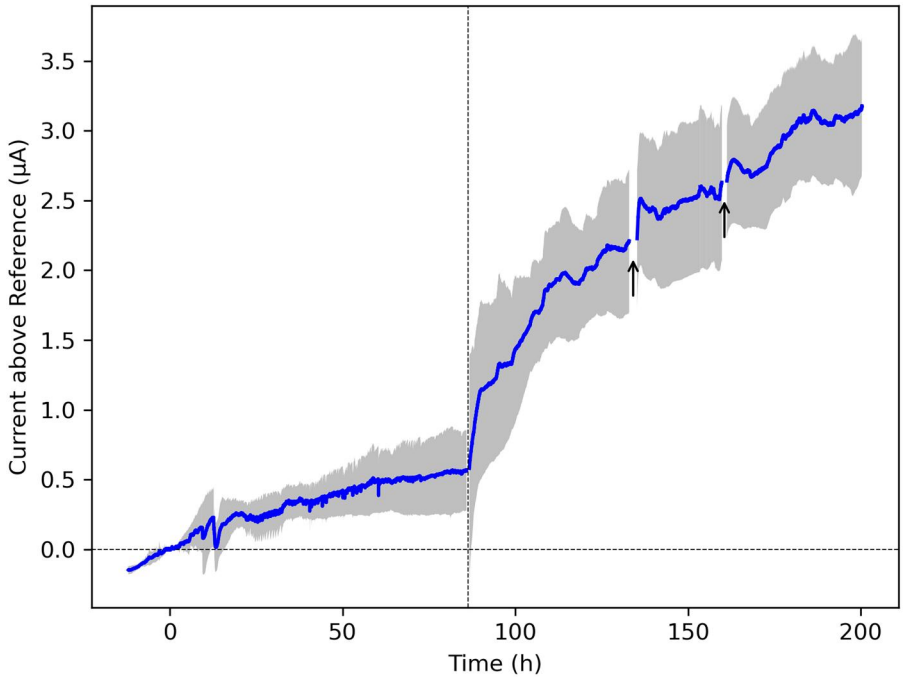

Figure 3. Evolution of microbial fuel cell (MFC) during startup. The MFC was fed with luria bertani broth and 5% of bacterial culture at 0.1 mL min^{-1} in an open circuit for 48 h followed by feeding with MM9 which was supplemented with glucose (0.3 g L^{-1}) and an external resistance of 1 kΩ. The dotted line at 86 h indicates the start of feeding MFC with a glucose concentration of 0.5 g L^{-1}. Arrows indicate times of the addition of new feeding solution ($n = 3$).

the surface of the carbon felt filaments. During the 3 day incubation, the medium was changed daily with induced enhanced bacterial growth and supported the biofilm fomation on the surface of the carbonized filaments (Figure 2(a)). The formation of a firm but not very thick biofilm was revealed in three days. It was also observed that the nanowire formation of *S. baltica* has started which assures electrical contact between the biotic and abiotic players of the anodic area (Figure 2(b)). These nanowires were recently reported from other *Shewanella* species like *S. oneidensis* (Pirbadian et al. 2014) and bacteria having the capacity to reduce different metals (Gorby, Beveridge, and Wiley 2005).

Heavy metal resistance

Since *S. baltica* 20 was isolated from sediments of the Danube River, where the bacterium may be exposed to heavy metals, the suseptibility of *S. baltica* 20 toward heavy metals is important if we plan to use the strain for wastewater monitoring. The results

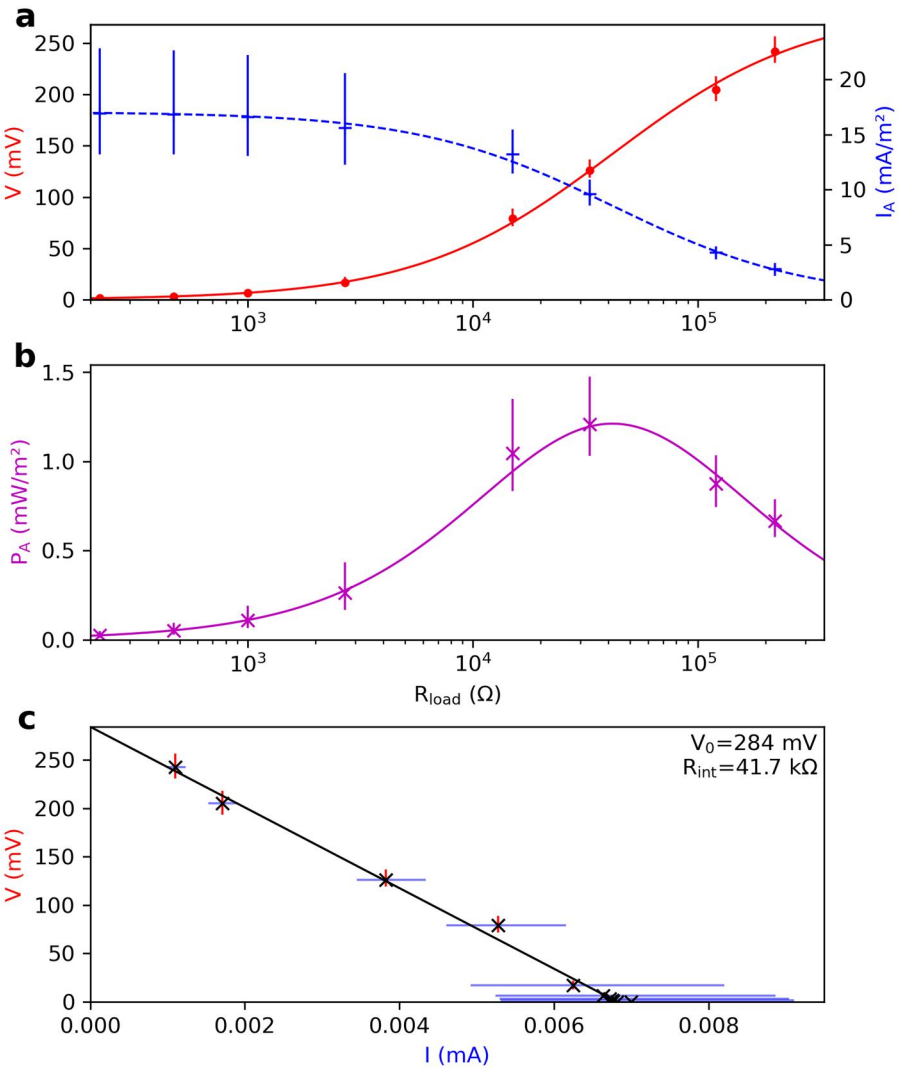

Figure 4. Results of varying circuit resistance measurements of the microbial fuel cells with external loads, r_{load}, between 220 Ω and 220 kΩ. (a) Average of the measured potential difference (dots) and derived current density (pluses) as functions of load resistance, r_{load}, (b) Power density, P_A as a function of load resistance, r_{load}. (c) Potential difference versus current. Bars indicate the voltage range observed in three replicates and corresponding ranges for current and power. Lines are calculated from the fitted internal resistance, r_{int}, and open circuit voltage, V_0. Note that (c) shows current rather than current density.

of heavy metal tests show that *S. baltica* 20 had the highest susceptibility to Cd and the lowest susceptibility to Mn. Among the tested metals, the susceptibility of the strain followed the order Cd > Cu/Zn > Co/Ni > Mn, while the strain exhibited equal susceptibility to Cu and Zn (3.9 mM), and Co and Ni (7.8 mM) (Table 1). This result suggest that *S. baltica* 20 may be used in MFC for sensing of cadmium or copper since it showed tolerance of those metals.

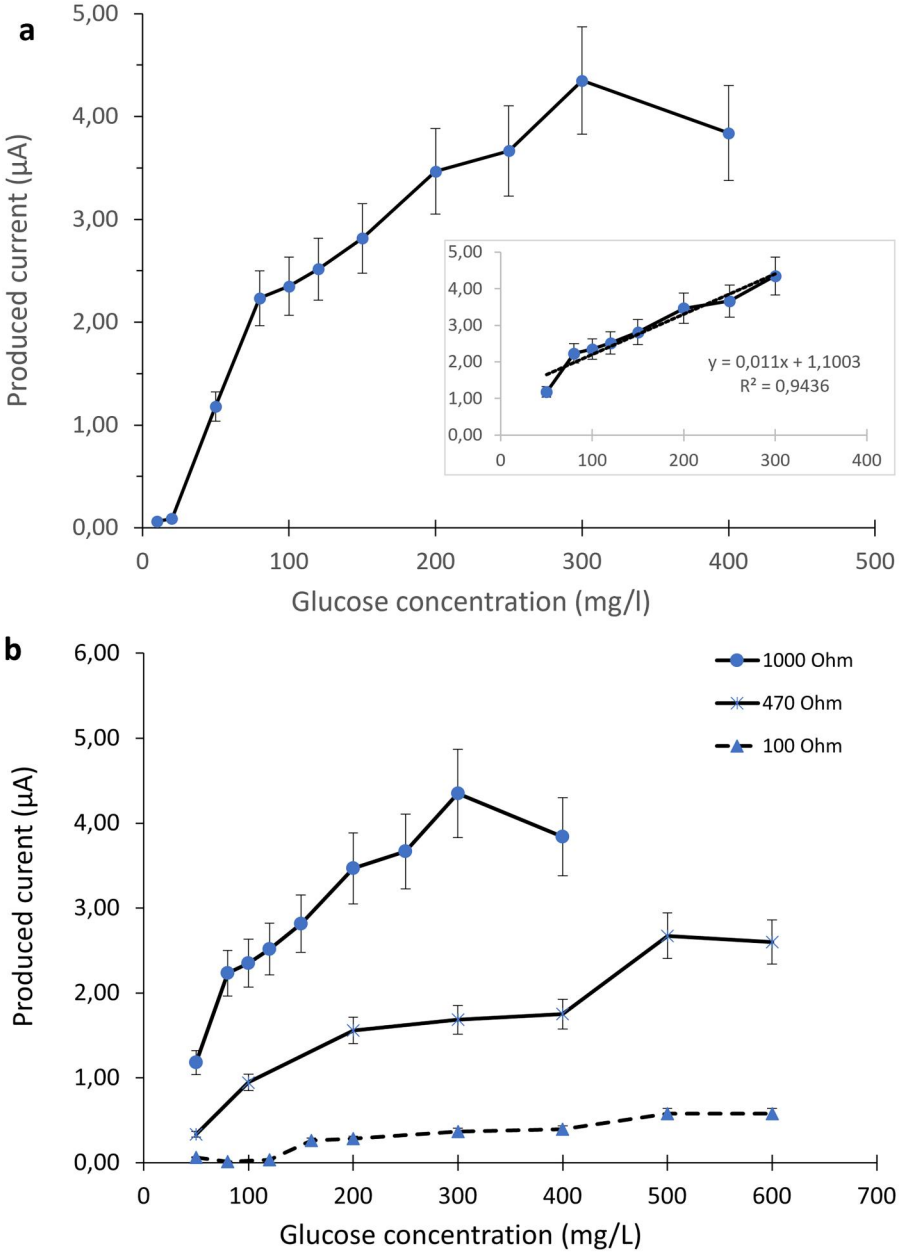

Figure 5. Influence of the substrate concentration and external load on the output current of microbial fuel cell (MFC) using *Shewanella baltica* 20. (a) The output current in response to change of glucose concentration (10–400 mg L^{-1}) in the inlet. (b) Influence of external loads 100, 470 and 1000 ohm on output current of MFCs which was fed with glucose concentrations from 50 to 600 mg L^{-1} ($n = 3$).

MFC performance

The anode of the MFC was enriched with the *S. baltica* 20 by feeding the MFC with LB broth and 5% (v/v) of the bacterial culture at 0.1 mL l^{-1} for 48h with a stable open

Table 2. Comparison of microbial fuel cell (MFC)-type BOD sensors using a single carbon source.

MFC type	Anodic chamber volume (cm^3)	Substrate	Culture	Linear range (ppm)	Reference
Single chamber	2	Glucose	Single culture	54–320	This study
Single chamber	2	Acetate	Mixed cultures	3–164	Di Lorenzo et al. (2014)
Single chamber	12.6	Glucose	Mixed cultures	50–350	Di Lorenzo et al. (2009)
Two chamber	20	Glucose	Mixed cultures	10–100	Chang et al. (2004)
Two chamber	14	Lactate	Single or Mixed culture	0–45	Yi et al. (2018)
Two chamber	300	Glucose	Mixed cultures	100–300	Do et al. (2020)

circuit potential of 730 ± 19 mV. Subsequently, the MFC was fed with MM9 and glucose with no bacteria addition. An external load of 1 kΩ was applied to the MFC. The startup process during the first 9 days of the MFC is shown in Figure 3.

After achieving a stable current, the effect of the electrical load on the performance of the MFC was investigated. Figure 4(a) shows that the maximum current density of 17 mA/m^2 was obtained for external loads of 1 kΩ or lower. The achieved power density was within 10% of its maximum value of 1.2 mW/m^2 from 20 to 80 kΩ (Figure 4(b)). The internal resistance was determined to be 42 kΩ (Figure 4(c)) with a range from 32 to 51 kΩ for individual replicates. The fitted open-circuit potential V_0 was 284 ± 10 mV.

Response to glucose and external load

The MFC enriched with *Shewanella baltica* 20 at the anode was tested as a sensor for the biodegradable organic carbon content in water. The test involved monitoring the produced current generated by MFC while feeding with a medium containing glucose at concentrations from 10 to 400 mg L^{-1} (Figure 5(a)). The output current exhibited a linear correlation to the glucose concentration from 50 to 300 mg L^{-1}. This linearity range is the non saturated fuel condition of the MFC. When the glucose concentration was above 300 mg L^{-1}, the produced current was no longer affected by the glucose concentration. In other words, increasing glucose concentration above 300 mg L^{-1} did not result in any increase of the output current. Since glucose is the only organic carbon in the tested water, the glucose concentration can theoretically be converted into BOD by multiplying the glucose concentration (50-300 mg L^{-1}) by 1.07, so the linearity range of BOD would be 54-320 mg L^{-1} (54-320 ppm). The response range in this case is comparable with some of the studied single-chamber systems (Table 2), but wider in comparison to the two-chamber systems due to greater oxygen supplied at the cathode. Further study of the MFC system with other organic substrates, mixed substrates, response time, and reproducibility of the system are essential for moving forward with practical applications.

The external load has a significant influence on the MFC performance as discussed above. Therefore, the influence of external load on the MFC performance under variation of glucose level in the inlet solution was investigated. External resistances of 100, 470 and 1000 Ω were applied to the MFCs and tested with different glucose concentrations (50–600 mg L^{-1}) to investigate the produced current (Figure 5(b)). The MFC with external resistance of 100 and 470 reached saturation at 500 mg L^{-1} of glucose, while the MFC with higher external resistance, 1 kΩ reached saturation at 300 mg L^{-1} of

glucose, much lower compared to the low external load MFCs (Figure 5). The result suggests that by applying low external resistance, we could potentially increase the response range of the MFC.

Conclusions

Species of genus *Shewanella* have been demonstrated for exoelectrogenic potential and use in treatment of wastewater. However, this is the first report to investigate *S. baltica* in MFC for biosensor application. The strain *S. baltica* 20, is able to form biofilm, utilize glucose and converting it into a current in an air cathode MFC. The MFC produced an open-circuit voltage of 280 mV, and a maximum current density of 17 mA/m^2 with a peak power production of 1.2 mW/m^2.

Our preliminary study suggests that the small-scale air cathode which was enriched with strain *S. baltica* 20 can be used in monitoring of glucose in water at the BOD values from 54 to 320 mg/L. The response of the MFC can be tuned by applying different external loads on the MFC. However, investigation of the response time and sensitivity of the system toward complex organic substrates is still needed. In addition, testing how the system responds with wastewater will also be necessary to evaluate the sensitivity of *S. baltica* 20 toward other fluctuating parameters such as pH, salinity and toxic chemicals.

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Disclosure statement

No potential conflict of interest was reported by the author(s)

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