Chapter 3

Simultaneous degradation of bad wine and electricity generation with the aid of the coexisting biocatalysts Acetobacter aceti and Gluconobacter roseus

ABSTRACT

This chapter describes the cooperative effect of the two biocatalysts Acetobacter aceti and Gluconobacter roseus for biodegradation as well as current generation. The electro activity of the biofilms of these two microorganisms was investigated by the bioelectrocatalytic oxidation of ethanol and glucose using cyclic voltammetry. Two chamber microbial fuel cells (MFCs) were constructed using single culture of Acetobacter aceti (A-MFC), and Gluconobacter roseus (G-MFC) and also using mixed culture (AG-MFC). Each MFC was fed with four different substrates viz., glucose, ethanol, acetate and bad wine. AG-MFC produced higher power density with glucose (1.05 W/m$^3$), ethanol (1.97 W/m$^3$), acetate (1.39 W/m$^3$) and bad wine (3.82 W/m$^3$). COD removal (94%) was maximum for acetate fed MFCs. Higher coulombic efficiency was obtained with bad wine (45%) as the fuel. This work provides the scope of using these biofuel cells in wineries for performing the dual duty of bad wine degradation along with current generation.
3.1. Introduction

During the last decade interest in microbial fuel cell has increased significantly because it produces electricity from the degradable organic matter by using microorganisms (Pant et al. 2009, Logan et al. 2007, Logan 2004, Lovley et al. 2006, Rabaey et al. 2005). In the microbial fuel cell the electrons obtained from substrate oxidation can be transferred onto anode via nanowires of the bacteria attached on anode surface (Wang et al. 2009, Reguera et al. 2005, Gorby et al. 2006), by endogenous electron transfer mediators (Rabaey et al. 2005) or through membrane-associated complexes (Kim et al. 2004). In the case of mixed cultures, acting as biocatalysts all these mechanisms may be found to be operative. Mixed-culture MFCs usually provide better electric performance compared to the pure-culture counterparts (Jung et al. 2007). This situation may be regarded as a tight alliance amongst the mutually dependent and challenging bacterial species contributing to the consortium aimed at the full fuel substrate degradation. First group of fermentation bacteria break complex molecules into energy-rich reduced metabolites suitable for the anaerobic respiration of a second bacterial group. Finally, some bacteria in the latter group are able to carry out an extra-cellular respiration when provided with a proper anode material, while the remaining ones take advantage of co-existing bacterial strains to enhance the metabolic breakdown of the complex molecules. The commonly used microorganisms in the MFC research for the construction of mediatorless microbial fuel cells include members of Shewanella, Rhodoferax, and Geobacter. Geobacter belongs to dissimilatory metal reducing microorganisms, which produce biologically useful energy in the form of
ATP during the dissimilatory reduction of metal oxides under anaerobic conditions in soils and sediments. The electrons are transferred to the final electron acceptor such as Fe$_2$O$_3$ mainly by a direct contact of mineral oxides and the metal reducing microorganisms (Lovley et al. 2004, Vargas et al. 1998). The anodic reaction in mediator-less MFCs are constructed with metal reducing bacteria belonging mainly to the families of *Shewanella*, *Rhodoferax*, and *Geobacter* is similar to that in this process because the anode acts as the final electron acceptor just like the solid mineral oxides. Though most of the mediator-less MFCs are operated with dissimilatory metal reducing microorganisms, few exceptions were reported with *Clostridium butyricum* (Oh et al. 2006, Park et al. 2001), *Hansenula anomala* (Prasad et al. 2007), *Clostridium sp.* (Prasad et al. 2006), *Glucanobacter roseus* and *Acetobacter aceti* (Karthikeyan et al. 2009) and *Candida melibiosica* 2491 (Hubenova et al. 2010). Another point of interest in the development of biofuel cells is the selection of substrates which influences the power production to a greater extent (Liu et al. 2009). A wide variety of substrates have been utilized to get power from MFC namely, acetate, glucose, lignocellulosic biomass, synthetic wastewater, brewery wastewater, starch processing wastewater, dye wastewater, landfill leachates, cellulose and chitin, inorganic and other substrates (Pant et al. 2009). It has been demonstrated in the chapter 2 the mixed culture of *acetobacter aceti* and *Glucanobacter roseus* can be used as biocatalysts in batch type mediatorless MFC (Karthikeyan et al. 2009). *Glucanobacter roseus* and *Acetobacter aceti* are responsible for spoilage of wine. These two genera are called as acetic acid bacteria i.e., they oxidize sugars, sugar alcohols, and ethanol with the production of acetic
acid as the major end product. It is reported that *Gluconobacter* sp., spoil the grape and *Acetobacter* sp., spoil the wine. Acetic acid bacteria are present at all stages of wine making, from the mature grape through winification to conservation (Joyeux et al. 1984). Low levels of *Acetobacter aceti* are present in the wine and they exhibited rapid proliferation on short exposure of the wine to air and caused significant increase in the concentration of acetic acid. Higher temperature of wine storage and higher wine pH favored the development and metabolism of these species. Free SO$_2$, used as a preservative of red wines, does not sufficiently protect against the metabolism of acetic acid bacteria. Such a wine loses its freshness and their usage creates wine allergy problem. The bad wine thus produced can be converted to useful electrical energy in MFCs. In this work we have shown the effect of power production and coulombic efficiency by the coexisting bacterial strains over a range of substrates like glucose, ethanol, acetate and bad wine. The direct electron transfer of the mixed culture biofilm in presence of the fuels glucose and ethanol is demonstrated by cyclic voltammetry.

3.2 Materials and Methods

3.2.1 Biofilm formation

In order to understand the electron transfer property of mixed culture, (*Acetobacter aceti* and *Gluconobacter roseus*) biofilms were formed by applying a constant anodic current density Galvanostatically (Busalmen et al. 2008). The biocatalysts (1:1 composition of mixed culture containing wet weight of 0.15 g *Acetobacter aceti* and 0.15 g *Gluconobacter roseus*) were suspended in the phosphate buffer (25 mL) containing 25 mM of glucose (6 g/L) in the three
electrode electrochemical cell. The GC working electrode (WE, 3 mm dia) is used for biofilm formation. Before the experiments the WE was polished using a polishing cloth and alumina powder. A platinum electrode was used as the counter electrode, and a normal calomel electrode (NCE) was used as the reference electrode. Buffers were purged with nitrogen gas for at least 30 min before the experiments, and a nitrogen environment was then kept above the solution in the cell to protect the solution from oxygen. A constant current of 50 µA was anodically applied for a period of 168 hrs. After that the GC was gently removed from the culture medium and washed with phosphate buffer (pH 7) to remove loosely held microorganisms on the electrode. Cyclic voltammetry was used to study the direct electron transfer of the biofilm. The cyclic voltammogram were recorded (PARSTAT) with a potential range from -1 V to 1 V with respect to NCE at a scan rate of 50 mV/s. The electrocatalytic oxidation of the biofilm was analyzed by the addition of various concentration glucose and ethanol. All experiments were performed at room temperature (28±2 °C).

3.2.2 MFC construction

A dual chamber microbial fuel cell was constructed, separated by nafion 117 membrane (Aldrich). Each chamber is made up of perspex sheet and each chamber has the volume of 125 mL. The anode is a piece of carbon felt (5 x 5 x 0.5 cm). Anolyte is phosphate buffer (100 mM). Graphite (5 x 5 x 0.5 cm) was used a cathode. 0.1 M K₄[Fe(CN)₆] in phosphate buffer was used as a catholyte. Acetobacter aceti (NCIM No. 2116) and Gluconobacter roseus (NCIM No. 2049) were procured from NCL, Pune, India. Sub-culturing was carried out using the following media
composition: Tryptone (1 g), yeast extract (1 g), glucose (1 g) and CaCO$_3$ (1 g) in 100 mL of distilled water. Twelve fuel cells were operated by different substrates (glucose, ethanol, acetate and bad wine) with these biocatalysts namely pure culture of *Acetobacter aceti* (A-MFC), pure culture of *Glunonobacter roseus* (G-MFC) and mixed culture (AG-MFC).

Each MFC was fed with four different substrates, 25 mM of glucose (5.8 ± 0.2 g/L of COD), Ethanol (2.1 ±0.2 g/L of COD), acetate (2 ± 0.2 g/L of COD) and also bad red wine (7.8 ±0.2 g/L of COD). All the MFCs were operated for a period of 72 hrs to compare the substrate oxidation under the operating external resistance. In order to evaluate the long time performance and the reproducibility of bad wine fed MFCs period of operation was extended to 144 hrs for one cycle. Three cycles of operation were carried out to check the reproducibility of results for the bad wine fed MFC (each cycle 144 hrs, for three cycle 432 hrs). During the operation the anode chamber was completely deaerated by N2 gas and the pH of the MFC was maintained at 6.4 -7.0 at 29± 2 °C.

3.2.3 Analysis and calculation

The voltage difference between two electrodes was measured across the fixed external resistance for every 5 or 10 min interval by using the data logger (Agilent acquisition 34970A data acquisition/switch unit). The data were collected automatically by a data acquisition program and a personal computer. Polarization tests were carried out by applying the variable resistance in the circuit and recording the resulting steady state voltage (Yazdi et al. 2011). Current (I) was calculated on the basis of Ohm's law (I = V/R), where V is voltage and R the applied
resistance and current density, \( i \) (A/m\(^3\)), was calculated using the formula, \( i = \frac{I}{v} \), where \( v \) is the volume of the anolyte (125x10\(^{-6}\) m\(^3\)). Power density, \( P \) (W/m\(^3\)), was calculated by multiplying the current by voltage and dividing with anolyte volume, \( P = \frac{IV}{v} \). It is understood that the Ohmic resistance (Internal resistance) of MFC collectively refers to resistance of electrodes, electrolytes and interconnections to electron and proton transport process. Ohmic resistance was calculated from the slope of polarization curve at the linear (ohmic) region (Fan et al. 2008, O’Hayre et al. 2006). Coulombic efficiency (CE) was calculated based on \( CE = \frac{C_e}{C_t} \times 100\% \), where \( C_e \) is the total coulombs calculated by integrating the current generated over the total time of operation, and \( C_t \) is the theoretical amount of coulombs available based on the measured COD removal in the MFC.

3.3 Results and Discussions

3.3.1 Direct bioelectrocatalysis

The electrochemical activity of the AG-biofilm on GC was observed by cyclic voltammetry after imposing a constant current density of (0.71 mA/cm\(^2\)) for 168 hrs. The biofilm exhibits redox peaks at 0.0716 V and -0.1098 V (vs. NCE). The presence of redox peak could indicate the presence of electroactive redox enzymes present in the biofilm itself. In order to visualize the bioelectrochemical oxidation of glucose directly through electroactive biofilm the voltammograms were recorded at pH 7 (Figure 1a).
The biofilm exhibits increasing catalytic oxidation current with the addition of the glucose. The oxidation of glucose occurs at two peaks, indicating that different electron transfer mechanisms are active at different potentials and also it favors electronic coupling of at least two sub units containing few heme c sites (Tkac et al. 2009). While increasing the concentration of glucose above 59mM no increase of oxidation current is observed which indicates saturation effect of the electroactive biofilm with glucose. The oxidation of glucose at pH 4.0 (Figure 1b) buffer medium shows a lower catalytic oxidation current compared to the currents observed at pH 7.0. An oxidation current of only 0.5 µA was observed for 71mM of glucose at pH 4.0 where a current of 1.7 µA was obtained for 59 mM of glucose at pH 7.0.
Figure 2. Background current subtracted voltammogram (at 50 mV) of biofilm for different additions of ethanol (mM) at pH 7 (a) and at pH 4 (b), insets: CV for biofilm

Figure 2 (a-b) shows the background current subtracted voltammogram of biofilm for different additions of ethanol under pH 7 and pH 4. In this case, the oxidation current increased nearly 4 times compared to that glucose oxidation (1.7 µA to 8 µA i.e., 25 µA/cm² to 113 µA/cm²) at pH7. The same observation could be observed at pH4 (0.5 µA to 4.07 µA i.e., 7.1 µA/cm² to 58 µA/cm², ≈ 8 times). It is inferred that the mixed culture *Acetobacter aceti* and *Gluconobacter roseus* has better catalytic/degradation effect on ethanol compared to glucose which is expected naturally. Under alkaline conditions the biofilm did not show any oxidation current with glucose and ethanol.

*Acetobacter aceti* cells can oxidize ethanol favourably due to the presence of membrane bound alcohol dehydrogenase (ADH) in the cells which acts as a mediator between the enzyme and anode of the fuel cell (Ikeda et al. 1997). *Gluconobacter roseus* will oxidize the ethanol and glucose due to the presence of
quinohemoprotein ADH and quinoprotein glucose dehydrogenase (GDH) in the bacterial cell membrane (Ikeda et al. 1992) which act as mediator between enzyme and anode of the fuel cell. Electrochemically active biofilm on the electrode can be formed by either under constant applied potential or current. In both cases biofilm formation was induced by the electron sink nature of the electrode surface which attracts the negative surface charges of the bacteria (Wang et al. 2009). It was recently found that in the case of Geobacter sulfurreducens, biofilm formation induced under OCV condition did not oxidize the acetate, though it exhibits redox behavior. Biofilm formed under applied potential (0.2 V or 0.4 V) showed the presence of surface confined redox species and (Katuri et al. 2010) and oxidized the acetate. These constraints are avoided by forming the biofilm under galvanostatic conditions.

**Performance of MFCs with various substrates**

**3.3.2 Electricity generation from glucose fed MFC**

Figure 3 shows the polarization curve of glucose fed MFCs with three types of biocatalysts. Maximum operating cell voltage (0.324V) was found at 900Ω for AG-MFC$_{glu}$ with a resulting power density of 1.05 W/m$^3$ (3.24 A/m$^3$) which is higher than the power generated by A-MFC$_{glu}$ and G-MFC$_{glu}$. It supports our previous experiments with a similar configuration and condition showing the better performance while using the mixed culture of Acetobacter aceti and Gluconobacter roseus MFC (AG-MFC) (Karthikeyan et al. 2009). The literature contains information about different types of MFCs with glucose using specific bacteria such as Escherichia coli (Qiao et al. 2008), Pseudomonas aeruginosa (Rabaey et al. 2004),
Rhodoferax ferrireducens (Chaudhuri et al. 2003) and Yeast Saccharomyces cerevisiae (Walker et al. 2006), Actinobacillus succinogenes (Park et al. 2000, Park et al. 1999a, 1999b). The direct comparison of these MFC outputs may not provide any conclusion (Pant et al. 2009) because of the variable parameters such as electrode designs, electrode materials, operating conditions, surface area, electrolyte conductivity, biocatalyst, units employed for power output etc…, that were used in these MFCs.

Figure 3. Polarization and Power generation of glucose \((C_6H_{12}O_6)\) based MFCs

3.3.3 Electricity generation from ethanol fed MFC

Figure 4 shows the polarization curve of ethanol fed MFCs with three types of biocatalysts. The Maximum operating cell voltage (0.384V) was found at 500Ω for AG-MFC\(_{et}\) with a power output of 1.97 W/m\(^3\) (5.12 A/m\(^3\)) which is higher than the power generated by A-MFC\(_{et}\) and G-MFC\(_{et}\). It should be noted that the optimum operating resistance of Ethanol fed MFCs has considerably decreased to 500 Ω when compared to Glucose fed MFCs which implies the rate of electron transfer in this fuel cell configuration is more when compared to glucose fed MFCs (MFCs\(_{glu}\)).
Further it can be added that ethanol is one of the metabolic pathway product during the glucose degradation and being a small molecule can be effectively degraded compared to glucose. Complete oxidation of 1 mole each fuel corresponds to 24 $e^-$/mol for glucose, 12 $e^-$/mol for ethanol and 8 $e^-$/mol for acetate.

### 3.3.4 Electricity generation from acetate fed MFC

Acetate is a simple molecule which has been extensively studied so far in electricity generation using electroactive bacteria (Bond et al. 2002). Due to its inertness towards alternative microbial conversions (fermentations and methanogenesis) at room temperature acetate can be used to benchmark MFC components (Aelterman 2009). More over acetate is the end product of several metabolic pathways (e.g., Entner-Doudoroff pathways for glucose metabolism) for higher order carbon sources (Biffinger et al. 2008).
Figure 5. Polarization and Power generation of Acetate (CH₃COONa) based MFCs

Figure 5 shows the polarization curve of acetate fed MFCs with three types of biocatalysts. The open circuit voltage (OCV) was found to be 0.687 V - 0.728 V and operating maximum steady cell voltage (0.417 V) was found at 900 Ω for AG-MFC_{act} generating a power density of 1.34 W/m³ (at 3.34 A/m³) which was comparable to the power generated by A-MFC_{act} and G-MFC_{act}. The optimum operating resistance (900 Ω) is relatively higher compared to the case of ethanol (Table 1). Though acetate involves only two carbon atoms, the electron transfer kinetics is not favourable when compared to ethanol.

3.3.5 Electricity generation from bad wine fed MFC

It is clear that these biocatalysts (Acebacter aceti and Gluconobacter roseus) can recover the electrons from the substrates such as glucose, ethanol and acetate. Since the biodegradation conditions are favourable for ethanol, the ability of the mixed culture for the degradation of bad wine has been evaluated.
Figure 6. Polarization and Power generation of bad wine based MFCs

Figure 6 shows the polarization curve of wine fed MFCs with three types of biocatalysts. The OCV was found to be between 0.790 V and 0.823 V. The maximum operating cell voltage of 0.535 V was determined at 600 Ω (R_ohm438 ± 47 Ω) for AG-MFCwn. As a result AG-MFCwn can generate a power density of 3.82 W/m$^3$ (7.13 A/m$^3$) which is higher than the power generated by A-MFCwn and G-MFCwn. The optimum operating resistance of the wine fed MFCs was higher when compared to ethanol fed MFCs and lower when compared to glucose fed MFCs.

Table 1 shows the fuel depletion and efficiency of MFC with optimal external resistance during 72 hrs. It indicates that higher COD removal (90-94%) was achieved with acetate fed MFCs and lower COD removal (59-41%) was accomplished with bad wine. The electron transfer yield is reflected in the values of coulombic efficiency. Glucose fed MFC shows lower efficiency (0.9-3.8%). Bad wine fed MFC exhibit a coulombic efficiency of (12-45%) with optimum current production of 4-7 A/m$^3$. 
Figure 7. Current and Voltage profile of A-MFC\textsubscript{wn} under 900\,\Omega resistor for a period of 432 hrs (1-3\textsuperscript{rd} cycle)

The optimum operating external resistance was found to be the same in all acetate fed MFCs and similar power output (0.9 to 1.3 W/m\textsuperscript{3}) was obtained in all the acetate fed MFCs. It indicates that acetate can be oxidized equally well in all fuel cell configuration (A, G, AG-MFC\textsubscript{act}). The lower coulombic efficiency and power density was achieved for glucose fed MFCs. It can be explained due to the fact that glucose is a fermentable substrate implying its consumption by diverse competing metabolisms such as fermentation and methanogenesis (Pant et al. 2009, Chae et al. 2009).

Figure 7, 8, 9 shows the current and voltage profile vs. time for A-MFC\textsubscript{wn}, G-MFC\textsubscript{wn} and AG-MFC\textsubscript{wn}. The current derived from wine fed MFC follows the order of AG-MFC\textsubscript{wn} (0.89 mA) > A-MGC\textsubscript{wn} (0.55 mA) > G-MFC\textsubscript{wn} (0.32 mA) and Figure 10 shows the power density vs. time at an optimum external resistance. It indicates AG-MFC\textsubscript{wn}
produces the maximum power density (3.5-3.9 W/m$^3$) in all cycles. Figures 11&12 shows the coulombic efficiency and COD removal in each cycle.

![Figure 8. Current and Voltage profile of G-MFC$_{wn}$ under 1400Ω resistor for a period of 432 hrs](image)

It is inferred that while increasing the operating period of bad wine fed MFC from 72 hrs-144 hrs the COD removal increased from 41 to 87.5% while the coulombic efficiency decreased from 45.2 to 41%. More recently researchers developed a power management system (PMS) that enables a Sediment Microbial fuel cell (SMFC) to operate a remote sensor consuming 2.5W of power (Donovan et al. 2011). They designed a custom PMS to microbial energy in capacitors and use the stored energy in short bursts using two DC/DC converters and a digital logic circuit to convert low-level power from a SMFC (maximum 0.38V*64mA=24mW and a average continuous power of 3.4mW) to 2.5W power for 5 seconds. Similar electronic circuitry can be developed to deliver power output of the order of watts from our MFCs.
Figure 9. Current and Voltage profile of AG-MFC\textsubscript{wn} under 600\,\Omega resistor for a period of 432 hrs (1-3\textsuperscript{rd} cycle)

In this study, AG-MFC\textsubscript{wn} shows a sustainable operating voltage of 0.535V with 0.89mA. Hence the power output will 470\pm5\,\mu W. This power will be 500 times higher than the power required to run a wall clock and which require only 8-10\,\mu W at minimum voltage of 1.3\pm0.2V.
Figure 10. Power profile for the three MFCs over a period of 432 hrs (1-3rd cycle)

Figure 11. Coulombic efficiency of wine fed MFCs for 1-3 cycles (each cycle 144 hrs)
Figure 12. COD removal of wine fed MFCs for 1-3 cycles (each cycle 144 hrs)

Figure 13. Laboratory set up of two bad wine fed MFC (AG-MFC\textsubscript{wn}) powering to run the wall clock and Cell Voltage with Time profile during the operation of wall clock
Table 1. COD removal and MFC power output 72 hrs.

<table>
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<th>MFCs</th>
<th>COD removal (±1%)</th>
<th>Coulombic efficiency (±%)</th>
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<th>Ohmic Resistance (Ω)</th>
<th>Steady state Current density (A/m³)</th>
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Note: glu-glucose, et-ethanol, act-acetate, wn- bad wine

Our laboratory experiment shows that AG-MFC<sub>wn</sub> can power a wall clock over a period of 160hrs with a sustainable power of 8µW. Two AG-MFC<sub>wn</sub> are connected in series to run the wall clock (Figure 13). The voltage drain with respect to time was plotted (Figure 13). The microbial energy available from bad wine can further be extended watt power application by specially designed electronic circuit consisting of current boosters and capacitors with an efficient power management system.
3.4 Conclusion

In conclusion mixed culture of Acetobacter aceti and Gluconobacter roseus can be used as biocatalyst in mediatorless microbial fuel cell for the degradation of compounds like glucose, ethanol acetate and bad wine. Based on the experimental results bad wine fed mixed culture fuel cell (AG-MFC$_{wn}$) generates a power density of $3.8 \pm 0.2$ W/m$^3$ with 45% coulombic efficiency. While increasing the period of operation (72 hrs to 144 hrs) bad wine fed AG-MFC$_{wn}$ exhibited a COD removal increase from 41% to 87.5%. Further coulombic efficiency decreased from 45.2% to 41%. Maximum sustainable power of $470\pm10\mu$W (0.535V with 0.89mA) can be obtained from one AG-MFC$_{wn}$. When two such cells are connected in series, the power output obtained is sufficient to run devices like wall clock normally run with 1.5V dry cell. The results indicate the scope of utilizing bad wine as a fuel for current generation in the presence of the biocatalysts Acetobacter aceti and Gluconobacter roseus and can be used as alternate energy source.