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Clostridium carboxidivorans' Surface Characterization Using Contact Angle Measurement (CAM)

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In order to develop and optimize a syngas fermentation using a hollow fiber membrane bioreactor (HFMB), this study proposes to characterize the surface of *Clostridium carboxidivorans*, a strictly anaerobic and gram-positive bacterium capable of producing biofuels and other chemicals. The choice for using syngas as substrate arises from the necessity to give a better destination to urban and industrial residues, which are destined to landfills. In a HFMB, a *Clostridium carboxidivorans* biofilm can be used for the purpose of enhancing cell density in the system, which is normally a problem for anaerobe bacterium; and the membrane can increase the mass transfer coefficient, a bottleneck in this process due to the gas-liquid mass transfer. The knowledge of the interactions between cells and surfaces, cell hydrophobicity and/or hydrophilicity, and how to enhance the force balance between electrostatic and van der Waals interactions, could enhance cell adhesion, biofilm formation and cell interaction with substrate. The Contact Angle Measurement (CAM) showed that *C. carboxidivorans* have a total surface tension (γ^{TOT}) of 56.0 mJ m^{-2}, and its components: Lifshitz-van der Waals (γ^{LW}) of 24.3 mJ m^{-2} and Lewis acid-base (γ^{AB}) of 31.7 mJ m^{-2}. Also, the free energy of interaction between cells and water (ΔG_{mwm}) was 9.6 mJ m^{-2} and the free energy of interaction between cells and hexadecane when immersed in water (ΔG_{mwh}) was -14.7 mJ m^{-2}. With these values, it was possible to determine that *Clostridium carboxidivorans* presents a hydrophilic surface character but is also capable of being attracted by hydrophobic molecules, liquids and surfaces and capable of interacting with them when immersed in water. Moreover, *Clostridium carboxidivorans* presented an electron-donor character, which already appeared in previous studies but was confirmed with this test. Therefore, this strain, when immersed in an aqueous culture medium, has an affinity to a hydrophobic membrane, which would favor its adhesion.

1. Introduction

Urban and industrial wastes represent a complex problem for the environment, especially with the constant progress and population growth in certain countries. In order to reduce the residue accumulation and therefore decrease the need to project and implement landfills, this biomass can have a different destination.
Currently, three pathways are used and discussed in order to obtain biofuels from biomass. Simple sugar can be converted to ethanol through fermentation when lignocellulosic biomass is pretreated using enzymes or alkaline chemicals. Ethanol can also be obtained by a Fischer-Tropsch process through syngas, which can be produced via biomass gasification at high temperatures (750-800°C). A third way is to combine a biochemical and a thermochemical process by using all components in the lignocellulosic biomass through gasification and fermenting the resulting syngas (Orgill et al., 2013; Latif et al., 2014; Mohammadi et al., 2011). Some anaerobic microorganisms can convert syngas into important multi-carbon chemicals like biofuels (ethanol), acetate, butyrate, butanol and 2,3-butanediol (Latif et al., 2014).
However, one of the bottlenecks of synthesis gas fermentation is the gas-liquid mass transfer coefficient. Therefore, so far many articles have described the use of a hollow fiber membrane bioreactor (HFMB) to enhance the mass transfer coefficient and the carbon monoxide consumption in order to enhance ethanol production and optimize cell concentration (Shen et al., 2014; Yasin et al., 2014). In a HFMB, a *Clostridium*

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carboxidivorans biofilm can be used for the purpose of enhancing cell density in the system, which is normally a problem for anaerobe bacterium.

Characklis and Marshall (1990) described a biofilm formation in eight steps starting with a conditional layer, which is the foundation for biofilm and is composed of many organic and inorganic molecules. Subsequently, free-cell bacteria colonize the surface due to interactions with the substrate on this conditional layer. These interactions are explained by the DLVO (Derjaguin-Landau-Verwey-Overbreek) theory, a balance between steric interaction, van der Waals forces (attractive) and electrostatic interactions (repulsive).

In order to choose the best membrane surface possible for this fermentation, a surface characterization test was conducted intending to elucidate the bacterium surface tension and affinity. The Contact Angle Measurement (CAM) test consists of dropping a liquid at room temperature using three different solvents, two polar and one apolar. The test was conducted in similar conditions to Amaral et al. (2006), while the equations and theory were acquire in van Oss (1995) article.

2. Materials and Methods

2.1 Microorganism

Clostridium carboxidivorans (DSMZ 15243) obtained from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures is a gram-positive bacterium, which can produce ethanol, acetate, butyrate and butanol. The optimum range for pH and temperature are respectively 5.0-7.0 and 37-40 ºC.

2.2 Media and Culture Conditions

The media used for bacterium growth (ATCC 2713) consisted of tryptone (10.0 g/L), peptone from gelatin, pancreatic digestion (10.0 g/L), yeast extract (5.0 g/L), glucose (1.0 g/L), sodium chloride (5.0 g/L), L-arginine (1.0 g/L), sodium pyruvate (1.0 g/L), menadione (0.0005 g/L) and hemin (0.005 g/L). One hundred millilitres serum flasks containing 50 mL of ATCC 2713 and syngas were inoculated (5 mL) and incubated in shaker for 24 h, 37 ºC and 150 rpm. At the following day, 5 mL of ATCC 2713 concentrated media were added as well as more syngas and the serum flasks returned to shaker at the same conditions as before. The ATCC 2713 concentrated media has the same constituents as ATCC 2713 but 10 times more concentrated.

2.3 Synthesis Gas

The syngas used in this experiment was obtained from White Martins Gases Industriais Ltda., and is composed of hydrogen gas (40%), carbon monoxide (25%), carbon dioxide (10%), nitrogen gas (10%) and methane (11%).

2.4 Contact Angle Measurement (CAM)

The Contact Angle Measurement (CAM) test consists of dropping a liquid on a surface in order to measure the interfacial tension between the solid and this liquid, which can be water, polar or apolar solvents. The drop will have an angle with the surface, which will indicate the nature of the interaction between solid and solvent by estimating the surface interfacial tension. For this experiment three different solvents must be choose, being two of these polar solvents (van Oss, 1995).

In order to obtain a solid surface, the samples were prepared using a cellulose triacetate filter with pore diameter of 0.22 µm from Millipore Corporation. One hundred millilitres of media containing *Clostridium carboxidivorans* biomass were filtrated. To establish the moisture content, the filter containing the biofilm were placed in Petri dishes containing 1% w/v agar in water with 10% v/v glycerol until the samples were measured in a tensiometer Krus DSA 100 (Amaral, 2007; Amaral et al., 2006) represented in Figure 1. The test was performed under room temperature using three different liquids: water, formamide and diiodemethane, which superficial tensions are disclosed in Table 1. Water, alongside dichloromethane, was used in a pre-CAM test conducted in the same conditions as stated before except no concentrated media was added to ATCC 2713. The hexadecane data present in Table 1 was used to estimate the free energy of interaction between cells and hexadecane when immersed in water, ΔG_{mwh}.

Table 1: Liquids' superficial tension components used in Contact Angle Measurement (CAM) test. ([a]Rijnaarts et al., 1995; [b]Bellon-Fontaine et al., 1996)

Liquid	γ^{TOT} (mJ m^{-2})	γ^{LW} (mJ m^{-2})	γ^{AB} (mJ m^{-2})	γ^{+} (mJ m^{-2})	γ^{-} (mJ m^{-2})
Water[a]	72.8	21.8	51.0	25.5	25.5
Formamide[a]	58.0	39.0	19.0	2.3	39.6
Diiodomethane[a]	50.8	50.8	0	0	0
Hexadecane[b]	27.7	27.7	0	0	0

It was possible to estimate the Lifshitz-van der Waals (γ^{LW}) component and Lewis acid-base (γ^{AB}) positive (γ^{+}) and negative (γ^{-}) components by optimising Young-Good-Girifalco-Fowkes equation (Eq. 1) (van Oss, 1995) using least squares and interior point algorithm in MATLAB. The superficial tensions are described for "l" and "s", which stand for liquid (solvent) and solid (biofilm and membrane), respectively. Both γ^{LW} and γ^{AB} components result in the total superficial tension, γ^{TOT}, according to van Oss (1995) (Eq. 2). In Eq. 3, the acid-base component, γ^{AB}, is obtained through its positive and negative components.

$$(1 + cos\theta)\gamma = 2(\sqrt{\gamma_l^{LW}\gamma_s^{LW}} + \sqrt{\gamma_l^{+}\gamma_s^{-}} + \sqrt{\gamma_l^{-}\gamma_s^{+}} \quad (1)$$

$$\gamma^{TOT} = \gamma^{LW} + \gamma^{AB} \quad (2)$$

$$\gamma^{AB} = 2\sqrt{\gamma^{+}\gamma^{-}} \quad (3)$$

Moreover, it is possible to calculate the free energy of interaction between water and cells through Eq.4, as discussed in van Oss (1995) article as ΔG_{mwm}, which m stand for microorganism and w for the aqueous system. van Oss (1995) also rearranged Eq. 4 to determine the free energy of interaction between different molecules immersed in water by using Dupré equation. Therefore, it is possible to calculate the free energy of interaction between a microorganism (m) and a solvent (s) immersed in water (w), ΔG_{mws}, by Eq. 5.

$$\Delta G_{mwm} = -2\left(\sqrt{\gamma_m^{LW}} - \sqrt{\gamma_w^{LW}}\right)^2 - 4(\sqrt{\gamma_m^{+}\gamma_m^{-}} + \sqrt{\gamma_w^{+}\gamma_w^{-}} - \sqrt{\gamma_m^{+}\gamma_w^{-}} - \sqrt{\gamma_m^{-}\gamma_w^{+}} \quad (4)$$

$$\Delta G_{mws} = -2\left(\sqrt{\gamma_m^{LW}} - \sqrt{\gamma_w^{LW}}\right)\left(\sqrt{\gamma_w^{LW}} - \sqrt{\gamma_s^{LW}}\right) + 2(\sqrt{\gamma_m^{+}\gamma_w^{-}} + \sqrt{\gamma_s^{+}\gamma_w^{-}} + \sqrt{\gamma_m^{-}\gamma_w^{+}} + \sqrt{\gamma_s^{-}\gamma_w^{+}} - 2\sqrt{\gamma_w^{+}\gamma_w^{-}} - \sqrt{\gamma_m^{+}\gamma_s^{-}} - \sqrt{\gamma_m^{-}\gamma_s^{+}} \quad (5)$$

(a) (b)

Figure 1: Tensiometer Krus DSA 100 for Contact Angle Measurement, with the membrane containing the biofilm in position right below the needle through which the liquid will drop (a) and the Biomass filtration system (b).

3. Results and Discussion

As discussed in van Oss (1995), the hydrophobic attraction between molecules when immersed in water is mainly due to the hydrogen-bounding free energy of cohesion from water molecules. In this way, the free energy of interaction between molecules immersed in water, ΔG_{iwi}, can be expressed in terms of the interfacial tension between the molecules and water, γ_{iw}. The interfacial tension can be calculated from the surface tension components, which one of them consist of an apolar component, Lifshitz-van der Waals (γ^{LW}), and a polar component, Lewis acid-base (γ^{AB}), which in turn comprehend a positive parameter, γ^{+}, representing the electron-acceptor character of this polar surface tension component; and a negative parameter, γ^{-}, which represents an electron-donor parameter. In order to obtain the interfacial tension between a solid and a liquid, a contact angle measurement can be made using three different liquids, usually two of them polar and one apolar.

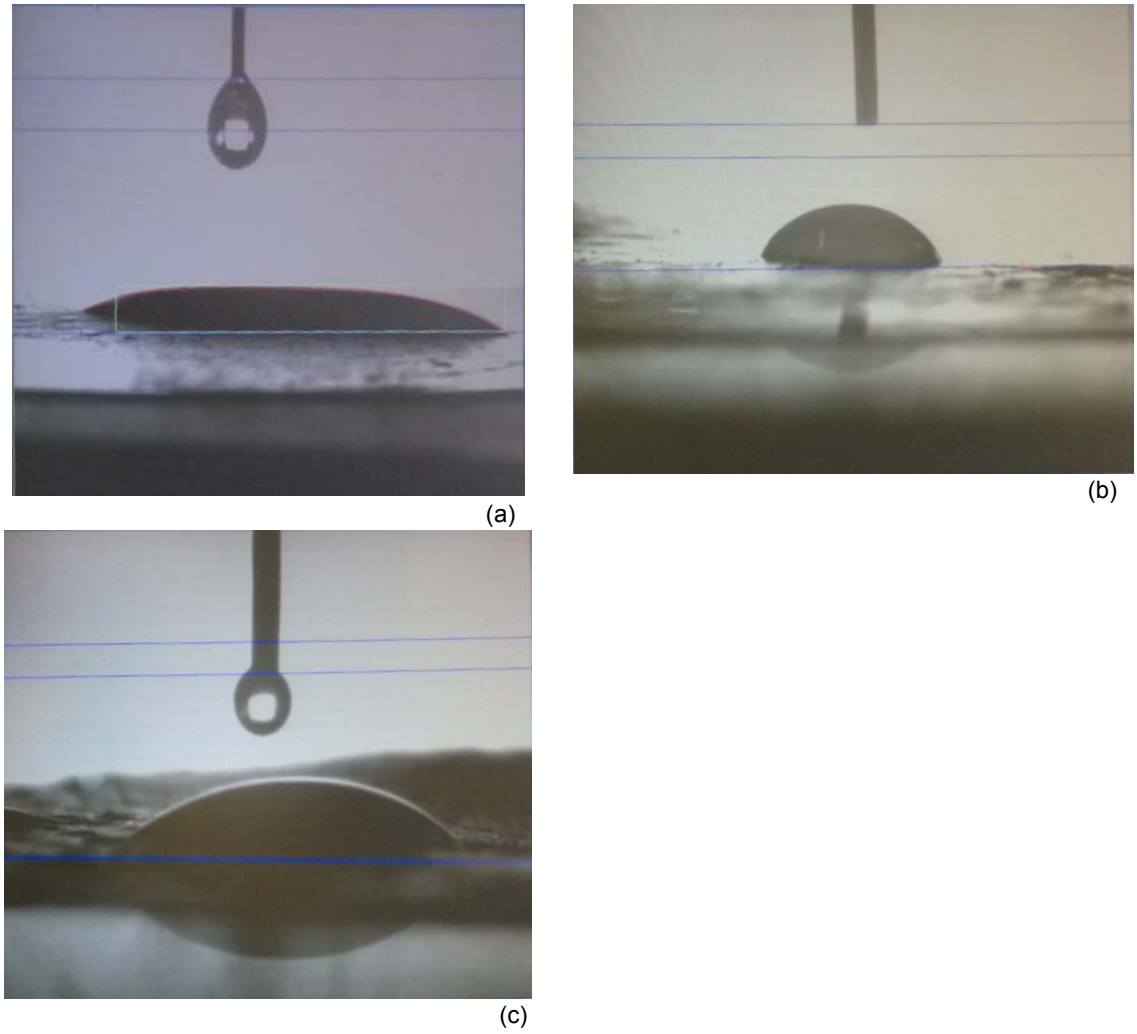

Figure 2: Drop aspect using sessile drop technique in Contact Angle Measurement (CAM) test to estimate the superficial tension and free energy between water and microorganisms using the liquids formamide (a), diiodomethane (b) and water (c).

A pre-CAM test using dichloromethane and water as liquids proved to be inconclusive for the first test because the liquid vanished as soon as it touched the biofilm, so it was impossible to measure a contact angle. On the other hand, water presented an area of 29.05 mm², 1.092 mm height and a final angle of 50.5°. However, this was considered an initial test, because water passed through the membrane to the base of the tensiometer due to the membrane surface hydrophilicity and the poor biofilm thickness, which was solved afterwards by adding concentrated ATCC 2713 media to the culture in order to enhance biomass production.
When the test was conducted for water, formamide and diiodomethane, it was possible to obtain three definitive angles, displayed in Table 2. The drops are illustrated in Figure 2, where it is possible to visualize the affinity between water, formamide and diiodomethane and the *Clostridium carboxidivorans* biofilm. The higher the angle between the drop and the surface, the lower the affinity between the biofilm surface and the liquid. In this way, as a first impression, *Clostridium carboxidivorans* has low affinity for diiodomethane, which is an apolar solvent.

Table 2: Contact angles between Clostridium carboxidivorans *and water, formamide and diiodomethane.*

	Water	Formamide	Diiodomethane
Contact Angle	33.3 ± 1.2	18.7 ± 2.1	67.4 ± 0.3

According to Rijnaarts et al. (1995a), it is possible to assume that *Clostridium carboxidivorans* has an intermediately hydrophobic character, as the contact angle between water and *Clostridium carboxidivorans*

cells is between 20º and 50º. Using the contact angles measured to optimise Eq. 1 using least squares through interior point algorithm, it is possible to estimate γ^{LW}, γ^{+}, and γ^{-} and to calculate the Lewis acid-base component through Eq. 3 and the total superficial tension through Eq. 2. These results, the free energy of interaction between water and microorganism and the free energy of interaction between microorganism and hexadecane when immersed in water, are displayed in Table 3.

Table 3: Clostridium carboxidivorans *biofilm surface tension components: γ^{LW}, γ^{AB}, γ^{+}, γ^{-}, γ^{TOT}, free energy of interaction between water and microorganism, ΔG_{mwm}; and free energy of interaction between microorganisms (m), hexadecane (h) immersed in water (w).*

	γ^{TOT} (mJ m^{-2})	γ^{LW} (mJ m^{-2})	γ^{AB} (mJ m^{-2})	γ^{+} (mJ m^{-2})	γ^{-} (mJ m^{-2})	ΔG_{mwm} (mJ m^{-2})	ΔG_{mwh} (mJ m^{-2})
Clostridium carboxidivorans	56.0	24.3	31.7	6.9	36.6	9.6	-14.7

The value for ΔG_{mwm} is positive, which indicates a certain affinity between this bacterium cells surface and water. As stated in Amaral et al. (2006) and van Oss (1995), a negative free energy of interaction between water and cells would indicate that bacterium cells would agglomerate in water, which is not normally observed in *Clostridium carboxidivorans*, except in certain conditions of stress. Moreover, according to van Oss (1995) definition, surfaces, compounds, cells and particles which have a $\Delta G_{mwm} < 0$ are hydrophobic, while hydrophilic cells, compounds, surfaces and particles show $\Delta G_{mwm} \geq 0$. Therefore, *Clostridium carboxidivorans* surface shows a hydrophilic character since Contact Angle Measurement test only evaluate the cell surface hydrophobicity, not considering physiochemical and structural factors related to bacterium adhesion (Amaral et al., 2006).

Rijnaarts et al. (1995b) concluded that DLVO interactions as well as steric interactions are both important concerning microorganism adhesion to both anthropic and natural environments. Bacterial cells normally have negatively charged surface, and increasing this negative charge surface would increase the repulsive electrostatic interaction. In Table 2 it is noted that the Lewis acid-base negative component (γ^{-} = 36.6 mJ m^{-2}) is higher than the positive component (γ^{+} = 6.9 mJ m^{-2}), which indicates that *Clostridium carboxidivorans* surface has an electron-donor character. This results confirm previous studies from Coelho et al (2015), which stated that *Clostridium carboxidivorans* cell surface is 13.7% hydrophobic, with 30.1% electron-donor character (EDC) and none electron-acceptor character.

Coelho et al. (2015) acquire these results through a MATS test, which consisted of mixing growing cells resuspended in phosphate buffer with four different solvents: hexadecane, hexane, chloroform and ethyl ether. The hydrophobicity was determined by the percentage of the cell adhesion to hexadecane (13.7%), while the EDC was determined by the cell adhesion to ethyl ether minus to hexane (30.1%). The electron-acceptor character (EAC) was determined by the cell adhesion to chloroform minus that to hexadecane (negative value). Although apparently inconsistent, this 13.7% hydrophobicity is also proved in this experiment. In fact, it is possible to say that *Clostridium carboxidivorans* cells are attracted to hexadecane, due to the negative free energy of interaction between cells and hexadecane when immersed in water. This shows that the attraction between the microorganism and hexadecane is thermodynamically favourable when they are immersed in water. As stated before, van Oss (1995) discussed that the hydrophobic attraction when molecules are immersed in water occur mainly because of the free energy of cohesion from the water hydrogen-bounding.

Although Contact Angle Measurement is undoubtedly a great method to measure surface hydrophobicity, the Microbial Adhesion To Solvents had a crucial role in determining the electron-donor character and showing some hydrophobicity, which explain why there is some affinity with diiodomethane in this work and a huge affinity with hexane (Coelho et al, 2015).

4. Conclusion

This work succeeds in expanding and confirming the surface characterisation for *Clostridium carboxidivorans* started in previous work (Coelho et al., 2015). With van Oss (1995) definition, it was possible to determine that *Clostridium carboxidivorans* is hydrophilic. However, the bacterium surface has a certain affinity with hydrophobic liquids, molecules and surfaces, as stated in MATS test previously and as it can be observed through the negative value for the free energy of interaction between microorganisms and hexadecane when immersed in water, ΔG_{mwh}. Also, the Contact Angle Measurement confirmed that *Clostridium carboxidivorans* surface has an electron-donor character. Therefore, since *Clostridium carboxidivorans* is hydrophilic and has affinity for hydrophobic molecules and surfaces when immersed in aqueous medium, these bacteria cells can adhere to the hydrophobic membrane, used for syngas fermentation.

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