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A rheological approach to identify efficient biopolymer producing bacteria

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Abstract

This study investigates the relationship between collective motion and propulsion of bacterial consortia and their biopolymer production efficiency. Rheological tests were conducted for suspensions of two different methanotrophic bacterial consortia obtained after enrichment of sediment samples from mangrove sites in Brazil. We considered the linear viscoelasticity region and analyzed the values of storage and loss moduli as functions of days of cultivation, for different values of the volume fraction. The suspensions' rheological behaviors reflected the bacterial growth stage. We found that the formation of structures over time in some types of consortia can hinder the movement of bacteria in the search for nutrients. The change in complex viscosity of the two consortia followed a different and rich behavior that appears to be closely related to their capacity to capture methane. Our analysis showed a possible correlation between collective motion, viscosity reduction, and biopolymer production. The pieces of evidence from this study suggest that the efficiency of bacterial motion is directly related to biopolymer production, and this could facilitate the process of identifying the best consortium of biopolymer producing bacteria.

KEYWORDS

active matter, methanotrophs, polyhydroxybutyrate, polyhydroxyalkanoates, rheology

1 | INTRODUCTION

Natural or artificial systems that are out of thermodynamic equilibrium are called active matter. The particles contained in active matter are known for dissipating energy into their surroundings. This can lead to self-motility, growth, or replication of the particles. Living entities such as birds, fish, or bacteria intrinsically exist out of equilibrium by converting the chemical content of their food into some form of mechanical work (Doostmohammadi et al., 2018). Similarly, synthetic systems can be designed to perform work driven by energy from light or chemical gradients (Ladoux & Mege, 2017).

Active fluids include a wide range of self-propelled particles in fluid media such as swimming microorganisms (Schwarz-Linek et al., 2016), colloidal swimmers (Bricard et al., 2013; Palacci et al., 2010), and ATP-driven cytoskeletons (Sanchez et al., 2012; Schaller et al., 2010). Interesting phenomena arise from these nonequilibrium systems, including collective motion (Sokolov & Aranson, 2012; Wensink et al., 2012), fluctuations (Narayan et al., 2007; Zhang et al., 2010), and enhanced diffusion of passive particles (Mino et al., 2013; Peng et al., 2016; Wu & Libchaber, 2000). Among all these novel properties, the rheological response of active fluids presents arguably the most surprising phenomena (Saintillan, 2018).

Important examples of active matter are liquid suspensions of bacteria, very common in bioprocesses. Understanding the motion of bacteria and how it affects the bulk rheology of a suspension is extremely important for the understanding of their subproducts' production. Some bacteria are able to produce secondary metabolites such as biopolymers in response to environmental stress (Marjadi &

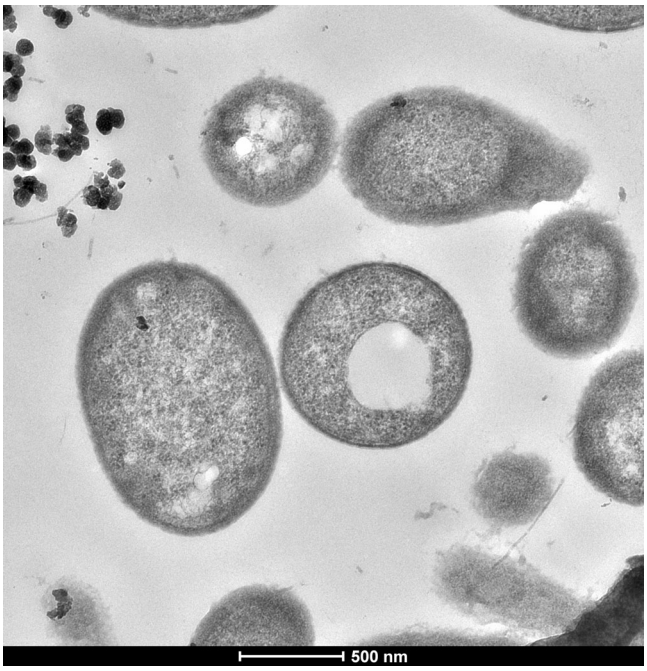

FIGURE 1 Photo from *Methylorubrum extorquens* with intracellular PHB obtained using transmission electron microscopy (TEM)

Dharaiya, 2011), as shown in Figure 1. Such microorganisms can be located in diverse ecological niches like coastal water body sediments, marine region, rhizospheric soil, water sediments, and sludge (Mohapatra et al., 2017a). Microbial biopolymers can be produced by chemical polymerization of monomers, which are in turn produced through fermentation. Endocellular biopolymers produced by microorganisms require specific nutrients and controlled environmental conditions, as they are biosynthesized as hydroxy-fatty-acids stored as lipid inclusions when carbon source is in abundance and nutrients like nitrogen, phosphorus, oxygen, or sulfur are limited (Mohapatra et al., 2017b).

Polyhydroxyalkanoates (PHAs) are a class of biodegradable polymers that can be produced from renewable sources as potential alternatives to oil-based plastics due to their similar physical properties. These biopolymers have a wide range of applications and, in addition to their biodegradability and eco-friendly manufacturing processes, can be produced through bio-refineries as part of integrated bioprocesses. In this context, polyhydroxybutyrate (PHB) is the best known biopolymer from the PHAs class (Raza et al., 2018) with some striking applications in the field of pharmacology (Villanova et al., 2010), veterinary (Costa et al., 2015), biomedicine (Ke et al., 2017; Lobler et al., 2002; Mosahebi et al., 2002) and agriculture (Alejandra et al., 2012).

Due to excessive fossil fuel production and consumption, the atmospheric concentrations of CH_4 have exceeded natural levels. In fact, methane is the second most important greenhouse gas with a global warming potential that can be 28 times higher than CO_2 (Stocker et al., 2013). This means that it is much more powerful at trapping heat in the atmosphere and causing harmful effects to several ecosystems and the environment. The rapid depletion of crude oil and the mounting adverse effects on the environment, accompanied by their increasing rates of consumption and the unrestrained production of oil-based plastics that end up polluting the environment due to inappropriate discard (Geyer et al., 2017), are driving forces for alternatives to petroleum mineral-based products and processes. This mitigation of greenhouse gas emissions through their conversion into high added value bioproducts is attracting considerable interest.

In this regard, biopolymers are important alternatives to non-sustainable products. However, the high production cost of biopolymers makes their diffusion in the market difficult, mainly because of the substrate used, which can account for up to 40% of the final product price (Choi & Lee, 1997), and make them more expensive than plastics derived from petrochemicals (Getachew & Woldesenbet, 2016). For this reason, finding an abundant and cheap feedstock for PHAs production has been the goal of many researchers over the years.

As explained, methylotrophic bacteria are widespread in the environment and are capable of consuming compounds like methanol, methane, or methylamine. Methane consumers are denominated as methanotrophs and they have the ability of PHB accumulation using methane as a carbon source (Chistoserdova, 2015), which could help lower the final price of PHB as CH_4 is a low-cost and abundant feedstock in the environment. Methane consumption by methanotrophs occurs through its oxidation into methanol by an enzyme MMO (methane monooxygenase); methanol can then be oxidized into formaldehyde, formate, and CO_2 that will be used for assimilation into biomass. There are mainly four groups of methanotrophs that differ from their carbon assimilation pathways: Gammaproteobacteria (also denominated as Type I or Type X) that assimilate formaldehyde through the ribulose monophosphate (RuMP) pathway, Alphaproteobacteria (Type II) that assimilate formate and CO_2 through the serine cycle and both Verrumicrobia and NC10 assimilate CO_2 through the Calvin-Bensen-Bassham (CBB) cycle pathway (Kalyuzhanaya et al., 2019; Meraz et al., 2020).

Recently, much effort has been committed to reducing the production cost of PHB by using strategies such as constructing engineered bacterial strains, optimizing fermentation and recovery processes (Gurieff & Lant, 2007; Yi et al., 1998). The present study aims to propose a new methodology that may allow the identification of the best bacterial consortium for PHB production through rheological tests, which extensively reduces the price of the analyses. Within the framework of these criteria, we want to understand how the collective motion, propulsion efficiency, and bulk rheological behavior affect the capture of methane by bacteria.

2 | METHODS

2.1 | Mixed microbial cultures

Methanotrophic bacteria (1SED and 2SUB consortia) were obtained after enrichment of sediment samples from mangrove sites

(coordinates: −23.91744, −46.21605 and −23.91358, −46.20936) in a methane atmosphere. Enrichment was performed by inoculating 1 g of sediment in 10 ml nitrate mineral salts (NMSs) culture medium into 25 ml serum bottles closed with crimp sealed butyl stoppers. The headspace was filled with a proportion of 1:4 methane in air. After 7 days, an aliquot of the supernatant was introduced into fresh new medium to discard any traces of organic matter and the methane atmosphere was replenished to ensure it was the only carbon source. This last step was repeated three more times to a sum of 42 days of enrichment. The bottles that presented optical density, which corresponds to cellular growth, were selected and submitted to PHB production experiments.

2.2 | PHB production experiments

Experiments performed to evaluate PHB production were carried out in two stages: the first aimed at maximum cellular growth and the second aimed at stimulating PHB production by nitrogen depletion. Nutrient deficiency in the presence of abundant carbon source is required for natural accumulation of PHB within bacterial cells. The culture medium used was adapted from NMS ATCC 1306, with the composition as follows (per liter): $MgSO_4{\cdot}7H_2O$, 1.0 g; $CaCl_2{\cdot}6H_2O$, 0.2 g; $NaNO_3$, 1.0 g; KH_2PO_4, 0.27 g; $Na_2HPO_4{\cdot}12H_2O$, 0.717 g; 2 ml of chelated iron solution (100 ml): $C_6H_8FeNO_7$, 0.1 g; Na_2EDTA, 0.2 g; HCl 0.3 ml; 1 ml of trace elements solution (1 L): EDTA, 500 mg; $FeSO_4{\cdot}7H_2O$, 200 mg; $ZnSO_4{\cdot}7H_2O$, 10 mg; $MnCl_2{\cdot}4H_2O$, 3 mg; H_3BO_3, 30 mg; $CoCl_2{\cdot}6H_2O$, 20 mg; $CuCl_2{\cdot}2H_2O$, 1 mg; $NiCl_2{\cdot}6H_2O$, 2 mg; $Na_2MoO_4{\cdot}2H_2O$, 3 mg. When nitrogen depletion was required, $NaNO_3$ was not included. The atmosphere used in the headspace was optimized according to Asenjo and Suk (1986) to ensure complete methane oxidation. Therefore, a proportion of 1:8 methane in air was used. Experiments were carried out in 250 ml Erlenmeyer flasks containing 50 ml NMS and bacterial inoculum to a final concentration of 0.1 (600 nm). These flasks were closed with rubber stoppers coupled with syringe needles and hoses to assist atmosphere renewal. They were incubated at 28°C and 200 rpm for 15 days with atmosphere replenishment every 48 h. On the seventh day of the experiment, cells were harvested by centrifugation at 7830 *g* for 10 min and transferred to N-free NMS to stimulate PHB production. At this point, the methane atmosphere was replenished every 24 h to avoid substrate limitation. PHB production was evaluated over time, and samples were taken at 96, 120, and and 192 h after nitrogen depletion. Cells were harvested and lyophilized to be analyzed by gas chromatography (GC) for biopolymer quantification and characterization.

2.3 | PHB quantification and characterization

PHB is produced and accumulated within bacterial cells. To quantify and characterize the biopolymer produced, lyophilized cells were first subjected to propanolysis following a protocol adapted from Riis and Mai (1988) and described by Gomez et al. (1996) to convert PHB into its propyl-esters allowing gas chromatography analysis. The GC analysis was performed with an Agilent 7890A equipped with an HP-5 capillary column and FID detector. Helium was used as the carrier gas at a flow rate of 0.8 ml/min. A column-temperature program was set to hold temperature at 100°C for 1 min and then increase it up to 210°C at a rate of 8°C/min, which was maintained for 15 min. About 1 μl of organic phase containing propyl esters was subjected to split injection (1:100) and benzoic acid was used as the internal standard.

2.4 | Rheological experiments

Amplitude sweep and oscillatory shear baseline tests were conducted in a controlled strain Anton Paar rotational rheometer MCR 301. A steel plate/plate geometry, with a diameter of 50 mm and a 700 μm gap (to ensure a good signal) was used for the measurements. For the amplitude sweep tests, the strain was varied from 0.2% to 20%. The oscillatory shear test to study the baseline was conducted for both frequencies $\omega = 10$ and $\omega = 1$ rad/s and $\gamma = 10\%$. To allow measuring down to the lowest viscosities, the small amplitude oscillatory shear tests were conducted in a controlled strain Anton Paar rotational rheometer MCR 502, with a double-gap measuring system. The double active shear area is created due to the inner cylinder and hollow bob, decreasing experimental errors. We measured the storage and loss moduli (G' and G'') versus the angular frequency ω in the linear regime, imposing 5% of strain. Essays were performed at 22°C to delay bacterial growth during measurements, using a Peltier system. All tests were performed at least three times for statistical analysis.

2.5 | Computer vision to determine the volume fraction

Using an Axio Scope A1 Carl Zeiss binocular microscope with a 40× objective lens, the bacterial suspensions were photographed throughout the cultivation process. The images were segmented into smaller versions of 400 × 250 pixels. A Python algorithm using the OpenCV library was written to separate bacteria from the image background. The analysis was made with several images allowing the calculation of the standard deviation. Following Chayes (1953), we considered that the proportion of the area of any section occupied by bacteria is an unbiased estimate of its relative volume. First, the median blur operation, an averaging method, was applied. The edges were processed while removing the noise. After that, an adaptive threshold algorithm was used. The algorithm determines the threshold for a pixel-based on a small region around it, so different thresholds are defined for other different areas of regions of the same image, considering the lighting variation. At this point, two morphological operations were used: dilation and erosion. The first consists of convolving the image with some kernel (horizontal, in this

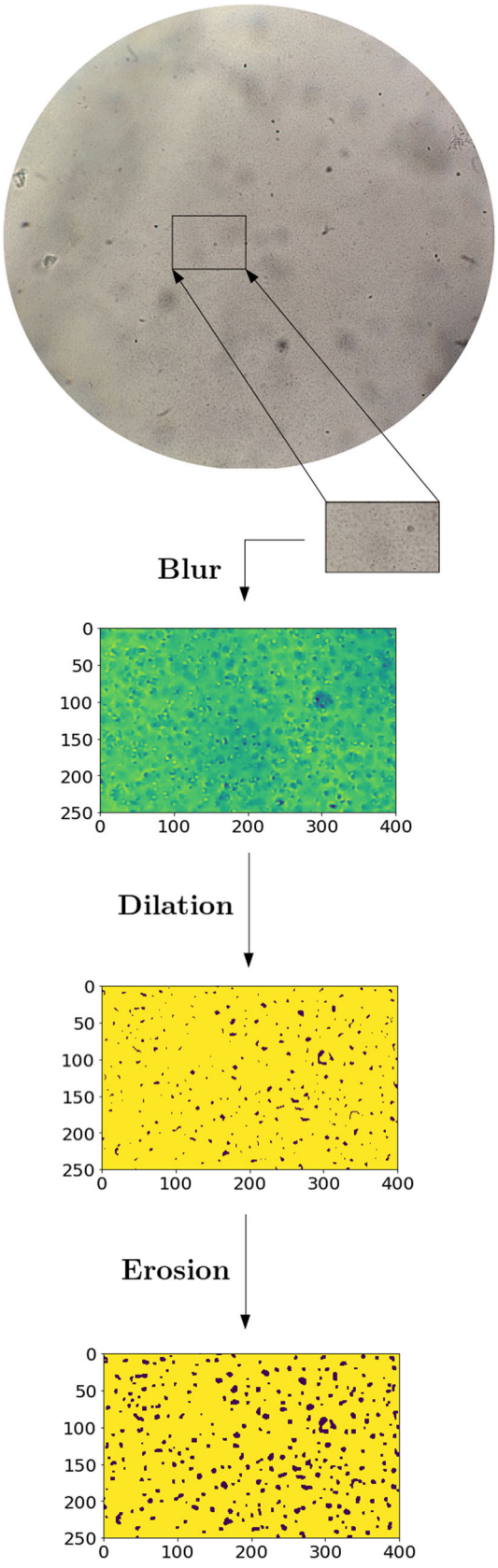

FIGURE 2 Schematic showing the blur, dilation, and erosion processes in one of the images obtained from the 1SED consortium [Color figure can be viewed at wileyonlinelibrary.com]

case). As the kernel is scanned over the image, we computed the maximal pixel value overlapped by the kernel. We replaced the image pixel in the anchor point position with that maximal value. After that, we used erosion to compute a local minimum over the area of the given kernel. In this case, the minimum pixel value overlapped by the kernel replaced the image pixel under the anchor point. Using both processes creates the morphological closing of the imaging, which is useful for filling small holes preserving the shape and size of the bacteria in the image. After that, the percentage amount of black pixels out of the pixels in total was calculated based on the defined threshold. The photo modification process is shown in Figure 2.

3 | RESULTS AND DISCUSSION

As mentioned, PHB accumulation occurs in a two-stage process. Nitrogen is an essential nutrient for bacterial cell duplication (Stein, 2018), so PHB accumulation under nitrogen deficiency is only stimulated when cells achieve maximum cellular growth. As PHB is stored as a lipid inclusion intracellularly, more cells imply more storage capacity for PHB granules. The accumulation was studied over time at 96, 120, and 192 h after nitrogen depletion. Each consortium showed a different accumulation capacity during the experiments as shown in Table 1. The 1SED consortium had the highest production at 120 h of accumulation, with 0.190 g of PHB per liter of suspension. The other consortium, 2SUB, reached its peak at 192 h of accumulation, with 0.124 g of PHB per liter of suspension. We concluded that the 1SED consortium had the best production performance, delivering more PHB than the 2SUB consortium and reaching its peak production in less time. Throughout the text, we will consider the production efficiency of 100% as the amount of PHB achieved in 120 h of accumulation of the 1SED consortium. In this sense, the 2SUB consortium obtained a comparative efficiency of 63.15%.

In an effort to understand the correlation between collective behavior, propulsion, and PHB production, we conducted several rheological experiments. The experimental protocol used included Small Amplitude Oscillatory Shear tests (SAOS). To perform SAOS, it is essential to determine the region of linear viscoelasticity. The linear viscoelastic region is that in which the storage modulus (G') and the loss modulus (G'') are independent of strain, and it was determined for higher concentrations from dynamic strain sweeps at a constant frequency. Considerable care must be taken regarding the torque during oscillation when dealing with active suspensions with low viscosity.

In the tests, an oscillatory shear was applied at a constant frequency of $\omega = 5$ rad/s in which the amplitude of deformation of the material was varied from $2\% \leq \gamma \leq 20\%$, as shown in Figure 3. This type of test gives information about the structural character of a sample. In both cases, the elastic behavior of the suspension was noticeable and $G' > G''$. We can also see that the 2SUB suspension presented a more significant variation in moduli values.

TABLE 1 Total PHB production in g/L evaluated over time, in hours, after nitrogen depletion for both consortia of bacteria

	1SED			2SUB		
Time (h)	PHB (g/L) (Experiment I)	PHB (g/L) (Experiment II)	Average	PHB (g/L) (Experiment I)	PHB (g/L) (Experiment II)	Average
96	0.099	0.044	0.07	0.053	0.071	0.06
120	0.224	0.136	0.19	0.105	0.120	0.12
192	0.111	0.136	0.14	0.124	0.119	0.14

Next, the viscometric moduli were measured as a function of time, aiming to identify any thixotropic or rheopetic behavior. Figure 4 shows G' and G'' for both 1SED ans 2SUB suspensions considering $\omega = 10$ and 1rad/s. It is known that a key factor in the rheological response of active suspensions, in addition to microswimmer transport properties, is the disturbance flows induced by individual particles (Saintillan, 2018). In the case of passive objects, a disturbance velocity only arises if an external force or flow field is imposed; this is unlike self-propelled particles, which drive fluid disturbances even in quiescent environments. In Figure 4 we observe that 2SUB does not show any stability regarding the measurements of loss modulus (G''), especially for the lower frequency ($\omega = 1$ rad/s). For the higher frequency ($\omega = 10$ rad/s), the instabilities are less noticeable, indicating a possible dependence on the flow. In this case, the disturbance flows induced by active particles can interact with external flows to modify the rheological response of their suspensions. These instabilities are likely to be also associated with the interaction between the microorganisms present in the 2SUB composite.

After those preliminary analyses, small amplitude oscillatory shear tests were performed. Considering the high-frequency movement of microorganisms, the focus of this analysis was in the low-frequency domain. Furthermore, at low frequencies, we can get information on how the sample behaves at rest so that the sample is characterized more precisely without extra effort or flow interaction.

The first analysis concerned the observable differences in viscosity and elastic modulus depending on the days of cultivation. There was a reasonable change in these conditions due not only to the volume fraction of the suspension but also to its interaction and activity. On the first day of cultivation, the 1SED consortium presented 1.44 ± 0.44% of bacteria. On the 15th day, the volume fraction was estimated at 9.47 ± 2.3%. The 2SUB consortium presented a volume fraction of 1.14 ± 0.35% on the first day and 14.67 ± 2.10% on the 15th day.

Before presenting the results of this analysis, it is important to remark that it is known that in the case of an extensile swimmer such as *E. coli*, the active stresslet opposes the passive flow-induced

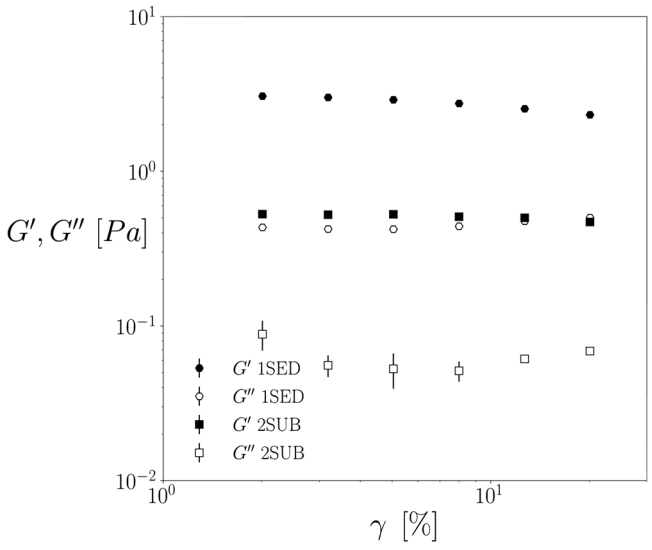

FIGURE 3 Amplitude sweep test for 1SED and 2SUB suspensions on the 8th day of cultivation for low constant frequencies ($\omega = 5$ rad/s). From the values of G' and G'', we have checked the linear viscoelasticity region with small deviations

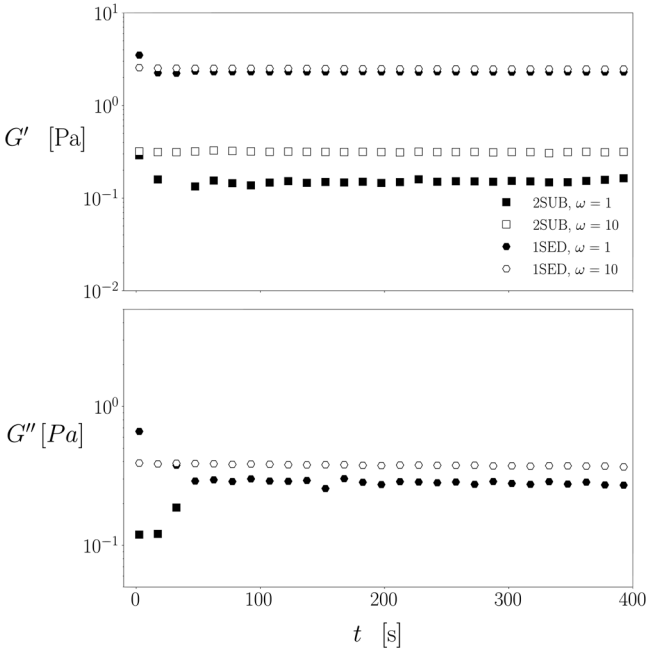

FIGURE 4 Viscometric moduli G' and G'' over time (in seconds) for different values of oscillation frequency. The values obtained for the 1SED consortium are represented by the black filled circles for $\omega = 1$ rad/s and hollow circles for $\omega = 10$ rad/s. For the 2SUB consortium, the moduli are represented by the black filled squares for $\omega = 1$ rad/s and hollow squared for $\omega = 10$ rad/s. All measurements were obtained using a constant shear rate of $\dot{\gamma} = 0.05$L/s

stresslet and drives a disturbance that tends to reinforce the applied flow, thus reducing the shear stress required to drive the flow and decreasing the effective viscosity of the system. The situation is reversed in the case of a contractile swimmer such as *Chlamydomonas reinhardtii*, which drives an active flow that opposes the applied shear and thus increases the effective viscosity (Saintillan, 2018).

Another critical point is that, at low shear rates, bacteria tend to maintain an irregular order leading to high zero shear viscosity. This is directly related to particle interactions and the restorative effects of active stresses. Besides this, there might be the formation of aggregates or networks. When the shear rate (or stresses) becomes high enough to overcome these effects, bacteria can rearrange and align in the flow direction. Moreover, there might be stretching in the particles and the breakdown of aggregates. As a consequence of these rearrangements, there is a decrease in bacterial interaction and an increase in free space between dispersed components, which both contribute to the drop in viscosity (Loisy et al., 2018).

Figure 5 shows the complex viscosity of both consortia measured on the first, the seventh, and the 15th day of cultivation, right before the accumulation period started. In both cases, the complex viscosity decreased as the volume fraction increased, which is expected from extensile swimmers. However, the reduction was more pronounced in the 1SED consortia, which showed an 84% decrease in $|\eta^*|(\omega \to 0)$, while 2SUB presented a 36.4% decrease. This suggests that the viscous dissipation in the flowing suspension is macroscopically balanced by the input of energy from swimming, thus allowing for a sustained flow without any applied torque. Usually, if active stresses dominate passive contributions from the flow, there is a linear decrease in viscosity as a function of volume fraction.

In addition, there is an unexpected behavior in the 2SUB consortium. Initially, there is an increase in complex viscosity during the exponential growth phase of the crop. It is possible that the type of bacteria found in this consortium did not move so incisively, obtaining food from the agitation itself. On the other hand, during the deprivation phase, there was a significant decrease in viscosity, probably related to the movement of bacteria. This change in behavior actually impairs PHB accumulation. For this reason, the rheological study of suspensions throughout the process can help to identify which consortia will be more efficient in terms of PHB accumulation even before the deprivation phase.

Figures 6 and 7 show the comparison between G' and G'' for consortia 2SUB and 1SED, respectively, as a function of frequency on Day 0 and Day 14. In Figure 6, which represents the consortium with the lowest PHB production, we found an inverse proportionality between G' and G'' as a function of time. Interestingly, the dispersed particles should float, due to their gel structure and active stress. At frequencies above 10 rad/s, there was an increase in the loss modulus, a behavior usually seen in gel-like structures of viscoelastic solids. The most striking result to emerge from the data, and that is clear in the graph at the bottom of Figure 6, was that the storage modulus did not present a significant decrease from Day 0 to Day 14, as opposed to what was observed for the viscosity (see Figure 5). This is directly related to the active stresses that have an elastic behavior and also the morphological characteristics of the bacteria that are primarily viscoelastic. No noteworthy differences between G' and G'' for $\omega \to 0$ were found on Day 14.

A further novel finding is related to the viscometric moduli of 1SED, the consortium with higher PHB production. Interestingly, the observed behavior is just the opposite when comparing the difference between G' and G'' as a function of days passed. For 1SED, the difference between G' and G'' increases when comparing the suspension on Day 0 and Day 14. In contrast, this difference decreases on the 2SUB consortium. In the case of 1SED, there was a slight decrease in the elastic moduli, but a reasonable reduction in the loss moduli, reiterating the decrease in viscosity observed previously.

It is important to state that the mechanical response and the rheological characteristics of the suspensions can be significantly different depending on the bacterial strain. It also depends on the

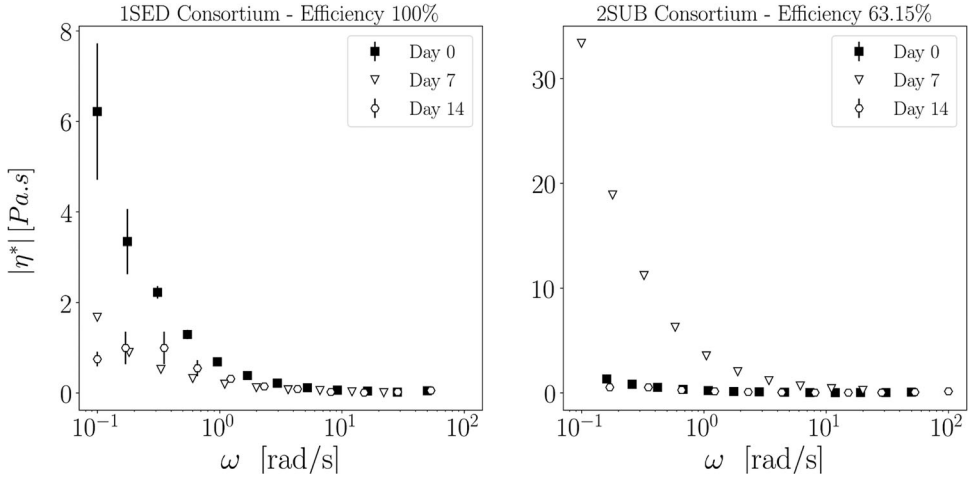

FIGURE 5 Complex viscosity, $|\eta^*|$ as a function of the frequency, ω for both consortia on different days of cultivation. The consortium 1SED presented an 84% decrease in viscosity after 15 days of cultivation, while 2SUB presented a 36.4% decrease. The efficiency of polyhydroxybutyrate (PHB) production is shown in the title with 1SED representing 100% of efficiency and 2SUB 63.15%

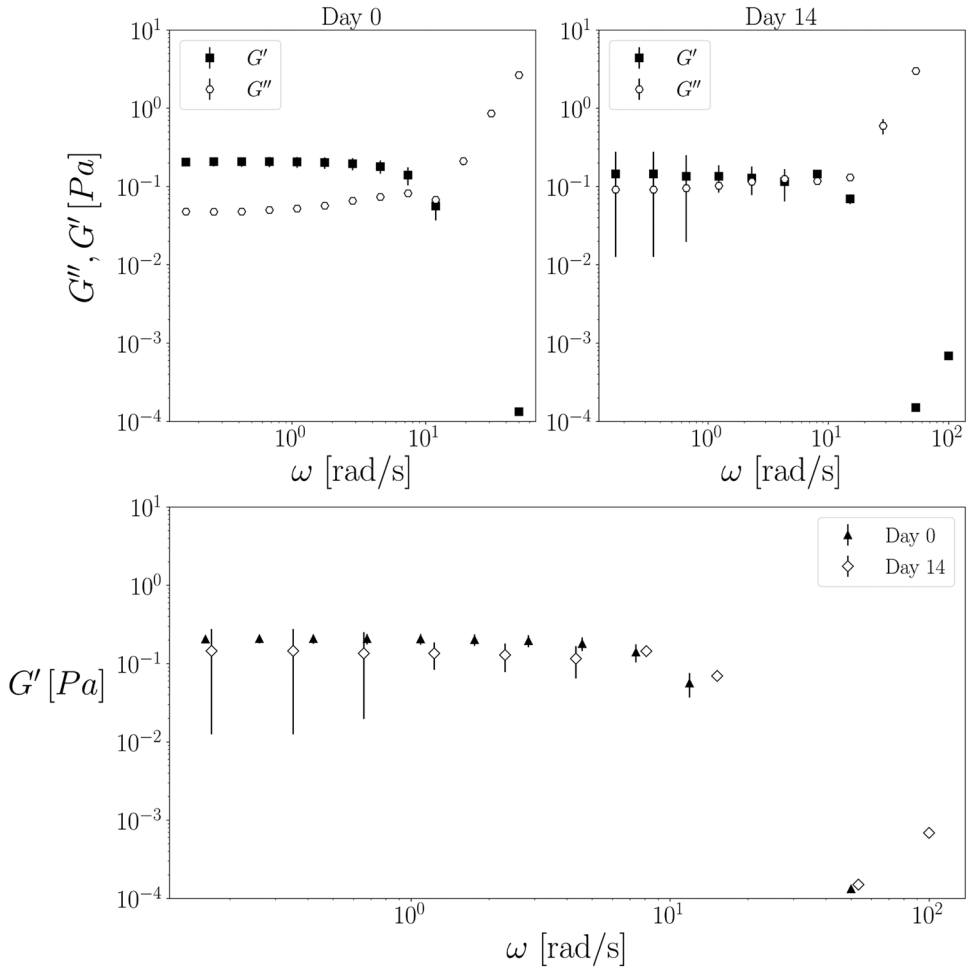

FIGURE 6 The three plots present the viscometric moduli for 2SUB consortia. On the top left plot, G' and G'' as a function of the frequency are presented. On the top right plot, the same measurements are shown for Day 14. On the bottom plot, the storage modulus is observed for the first and the 15th day of cultivation. This consortium presented a polyhydroxybutyrate (PHB) efficiency of 63.15%

particular environment and the external physical stimuli imposed on the system. However, identifying the bulk characteristics of consortia already found in nature facilitates and decreases the costs of processes. We have also compared the works of Alarfaj et al. (2015), who extracted PHB from *Bacillus thuringiensis* isolated from the mangrove, and Brar et al. (2007), who analyzed the rheology of *B. thuringiensis* hydrolyzed sludge. A reasonable difference in viscosity ($\eta(\dot{\gamma} \to 0)$) was observed during the different stages of cultivation, a behavior very similar to what we observed. Thus, we believe that the bulk behavior of suspensions should be similar for extensible microorganisms.

The results point to the likelihood that the production of biopolymers is directly related to the ability of bacteria to move. The elastic factor and the decrease in loss moduli could well be responsible for this result. The consortium with less prominent elastic behavior and smaller differences when comparing storage and loss moduli might indicate that the interaction between the bacteria decreases the propulsion efficiency of the individuals separately, which worsens the capture of the gases necessary for the subsequent production of biopolymers. It seems likely that a significant decrease in viscosity, observed in the 1SED, may also indicate an increase in active stress, which consequently indicates a more positive interaction of bacteria regarding propulsion.

These results correlate favorably with the work conducted by Ryan et al. (2011), who experimentally found a relationship between collective motion and viscosity reduction and further support the idea that this behavior is directly related to positional fluctuations that arise due to a large-scale organized motion of swimmers via an instability of the homogeneous state (Saintillan & Shelley, 2007).

These results could lead us to hypothesize that motion is directly related to nutrient absorption. As bacteria from 1SED absorb nutrients better, they would move more, leading to a feedback loop related to cell metabolism. However, though adenosine triphosphate, or ATP, is considered the primary energy source in cells, the flagellar motor is driven by a different energy source: the energy gained from the ion flux through the electrochemical potential across the bacterial cell membrane (Manson et al., 1977; Matsura et al., 1977). This electrical potential across the membrane, also called transmembrane voltage, is a source of free energy that enables cells to do chemical and mechanical work (Benarroch & Asally, 2020). In addition, Miller

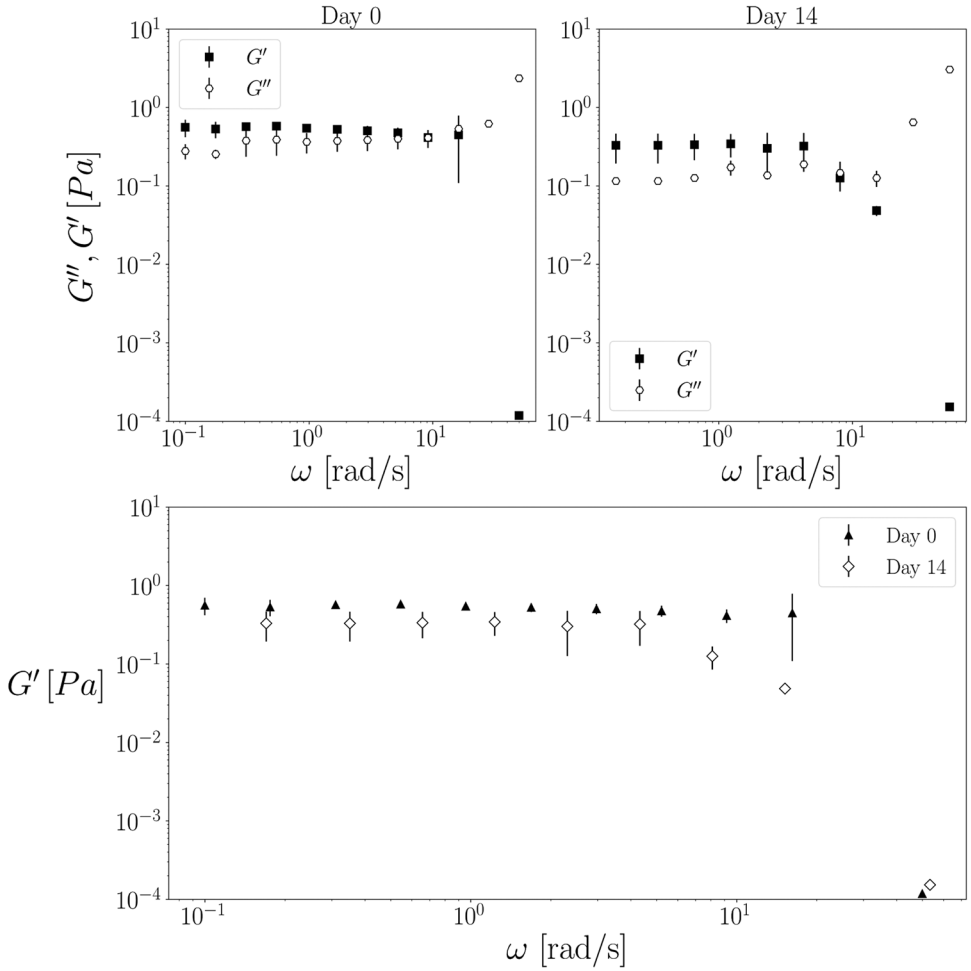

FIGURE 7 The three plots present the viscometric moduli for 1SED consortia. On the top left plot, G' and G'' as a function of the frequency are presented. On the top right plot, the same measurements are shown for day 14. On the bottom plot, the storage modulus is observed for the first and the 15th day of cultivation. This consortium presented a polyhydroxybutyrate (PHB) efficiency of 100%

and Koshland (1977) discovered that when the potential was sufficient for motility and constant over time, its absolute level did not affect the swimming behavior of the bacteria. For example, in the case of *Bacillus subtilis*, a change of the overall membrane potential is not required for chemotaxis. Thus, despite the highly exothermic nature of the aerobic methanotrophic growth process (El Abbadi & Criddle, 2019), we cannot consider that the consumption of methane during the growth of the bacteria directly affects their locomotion performance. On the other hand, the pieces of evidence from this study suggest that the efficiency of bacterial motion could be related to biopolymer production. In addition, we demonstrated that the use of rheological techniques is a new and promising methodology for identifying consortia with better locomotive performance and higher PHB production.

4 | CONCLUSIONS

In this paper, we experimentally analyzed the rheology of two different consortia of biopolymer producing bacteria. The conditions of linear viscoelasticity, complex viscosity, and elastic moduli were measured. The characterization of the production of biopolymers was also carried out to identify phenomenological correlations between the propulsion of the bacteria and the efficiency of gas consumption.

The findings of this study support the idea that consortia formed by bacteria with better propulsion efficiency or those in which the forming bacteria interact constructively, that is, generating a negative stresslet strength, may favor PHB accumulation. It seems likely that the rheology of these suspensions can direct the choice of the consortium that will have the highest PHB production. In general, considering both consortia compared between Day 0 and Day 14, the suspension with a greater decrease in complex viscosity shows greater collective behavior that is related to a greater capture of methane and further PHB production.

In addition, the increase in the difference between storage and loss moduli over the course of days has also been positively correlated with PHB production. Again, this increase in G' and G'' difference is directly related to the decrease in the loss modulus, despite the increase in volume fraction.

Our findings appear to show that by swimming together, specific bacteria can generate forces in the fluid that are large enough to counterbalance friction. This occurs in suspensions where bacteria are more active or interact better hydrodynamically. In that sense, they would be more efficient in capturing gases in the atmosphere and could produce more biopolymers. It is also important to note that this occurred concomitantly to the increase in the volume fraction of the bacteria, that is, despite the forecast of increased viscosity due to the concentration of microorganisms. Furthermore, we observed that, although the elastic modulus was independent of time in both samples, the loss modulus behavior was highly non-monotonic in 2SUB. This may represent the formation of aggregates or even a hydrodynamic interaction behavior that reduces the strength of the negative stresslet imposed by pusher-type microorganisms, such as bacteria.

We believe that the proposed framework could be usefully employed in the large scale production of PHB. Currently, the entire process of acquisition, accumulation, and, finally, measurement of PHB is time-consuming and expensive. In this sense, performing rheological analyses on the suspensions before the nitrogen depletion phase can decrease the production time and help in identifying the best bacteria strains. The great novelty of the rheological study during the process is that we might identify the best consortium and whether it will accumulate a reasonable amount of PHB before the accumulation phase, saving time and lab resources. As we have shown, it is possible that consortia with more significant motion during the entire growth and nutrient depletion phase produce more PHB. Thus, it is expected to reproduce these tests in different biomes, with consortia found in nature, and identify before the accumulation period start, that is, before the 15th, which one will produce the better results. Tests at the beginning or during the production period may indicate that bacteria from a specific consortium do not show good motility or that the bacteria are not interacting constructively. Thus, this new methodology can shorten the experiment period, as non-promising consortia would be discarded even before the accumulation period. Furthermore, how those bacteria move during the growth and accumulation process is extremely important to enhance their methane capture and allow improvements. The addition of macromolecules or nanomaterials can modify the movement of microorganisms and, thus, it is possible to also modify the accumulation of PHB. This is the first study in this sense, which starts filling an important gap in the literature.

The impact of bacterial interactions on the multispecies consortia properties is very complex to unravel. They may interact in synergetic or antagonistic ways, move through flagella, cilia, or body movements, and obtain energy from different sources. In this sense, a rheological analysis shows a new perspective for the identification of the mechanical characteristics of the suspension considering a direct correlation with the production of PHB. The analyses carried out in this paper and associations made between bacteria motion and PHB production can also give insights on procedures that could potentially increase PHB production efficiency. For example, the addition of polymers or nanoparticles could improve not only the movement of bacteria but also the mixture of methane in the suspension and the production of PHB.

Considering the sensitivity of collective motion concerning the system's geometry Theillard et al. (2016); Beppu et al. (2017), future work is required to establish whether the relationship reported in this study holds in the general case. More importantly, given the small sample size, caution must be exercised and the picture is thus still incomplete. We hope that further tests using different consortia will confirm our findings. To further our research, we are also studying algae consortia, mixtures of algae and bacteria, and other consortia of bacteria. We are confident that our research will serve as a base for future studies on the implications of the locomotive behavior of microorganisms that produce bioproducts and how the efficiency of this process can be improved.

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AUTHOR CONTRIBUTIONS

Sara Malvar: study conception and design, acquisition of rheological data, analysis and interpretation of data, drafting of the manuscript. Letícia O. B. Cardoso: study conception and design, acquisition of biological data, analysis and interpretation of data, drafting of the manuscript. Bruno Karolski: acquisition of biological data, critical revision. Elen A. Perpetuo: study conception and design, analysis and interpretation of data, critical revision. Bruno S. Carmo: study conception and design, analysis and interpretation of data, critical revision. Julio R. Meneghini: supervision, study conception, and design, analysis and interpretation of data, critical revision.

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