

QCM Viscometer for Bioremediation and Microbial Activity Monitoring

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Abstract—A quartz crystal microbalance has been used to monitor the polymer production of a bacterial population in liquid medium. The increasing amount of produced polymer corresponds to an increase in the viscosity of the liquid, which is directly measurable as the fluid contacts the surface of the quartz crystal in the sensor system. This procedure is being developed as a novel method for measuring microbial polymer production and growth of an environmental isolate obtained from river sediment contaminated with petroleum hydrocarbons. This measurement technique may be used to monitor growth characteristics of unknown anaerobic bacteria when used in conjunction with other currently employed microbiological test methods, such as spectrophotometry, to measure turbidity.

In the presence of glucose, a novel, strictly anaerobic bacterial isolate, designated strain JEL-1, produces a viscous, as yet unidentified, polymer. In defined minimal media containing amino acids and glucose under a nitrogen gas atmosphere, copious quantities of this polymer are produced. This research investigates the corresponding increase in quantity of the polymer produced by JEL-1 as well as the polymer production rate in a controlled liquid medium.

I. INTRODUCTION

BIOREMEDIATION utilizes microbial processes to degrade environmental pollutants to nontoxic compounds. This process has been used in applications such as oil spill cleanups. Analytical devices to monitor pollutants in a liquid environment are commercially available and employ several different technologies. These devices utilize optical as well as electrical techniques to achieve characterization of biological properties in a liquid medium [1]. Optical techniques include fluorescence intensity detection and laser based optical density (OD) measurements. Some electrical techniques involve capacitor value changes due to oil and water separation at the surface of a test liquid. Many of these current methods require sample collection at remote sites and skilled technical personnel to analyze the collected material. The use of the quartz crystal microbalance (QCM) for liquid property monitoring could complement currently incorporated measurement techniques. The QCM also has the advantage of characterizing liquid properties, including viscosity, by way of a shift in the resonant frequency of the device. This easily interpreted frequency shift

parameter eliminates the necessity of complex data interpretation of samples by trained technicians.

The aim of this study was to show that the QCM is a unique and effective method to monitor a biological growth process to aid in characterizing unknown bacterial populations. As a viscometer, this high shear rate device could be used in conjunction with low shear rate laboratory viscosity measurement techniques that currently include mechanical (rotational), capillary, falling ball, and other gravity or positive displacement methods.

II. DESCRIPTION OF BACTERIAL STRAIN

An unusual bacterial population, designated strain JEL-1, was isolated from a reductively dechlorinating enrichment culture derived from anaerobic sediments of the King Salmon River, Alaska. The sediments were contaminated with petroleum hydrocarbons from a nearby refinery. Microscopic, physiological, and phylogenetic examinations of strain JEL-1 revealed that this was indeed a novel isolate. Strain JEL-1 exhibited a distinct morphology, and grew in irregularly sized nearly spherical cells that were connected by an undescribed matrix. Sometimes, filaments were seen holding the cells in unusual arrangements. Small cells appeared to “bud” from the ends of some filaments, or at other times from large round cells. Other cells were grouped together in a three-dimensional array, held together by an unseen gelatinous matrix. Although strain JEL-1 was a pure bacterial culture, the cells appeared highly pleiomorphic. A phase contrast micrograph of JEL-1 is shown in Fig. 1.

The novel bacteria were sensitive to oxygen and grew only in the absence of air. Under anaerobic conditions, strain JEL-1 multiplied under variety of defined laboratory conditions, ranging from near starvation (where only the basic requirements for growth are supplied) to those conditions where abundant nutrients are given in the form of predigested soy and milk proteins and glucose. Under conditions where the bacteria are supplied amino acids (digested protein) and sugars (glucose, fructose, maltose), the bacteria produced a thick gelatinous polymer. Polymer production could be visualized easily by shaking the growth vessels.

Initially, it was crucial to classify this microorganism. By knowing its nearest relative, it might allow educated guesses as to the metabolic potential of this novel bacterial population. To find out what type of microbe we had in hand, we determined the DNA sequence of a molecule used universally to classify living organisms. The 16S ribosomal RNA molecule is a component of the ribosome, the protein making machinery of a bacterial cell. The results were astonishing, in that the novel microbe appeared to be most closely related to the spirochetes, a group of

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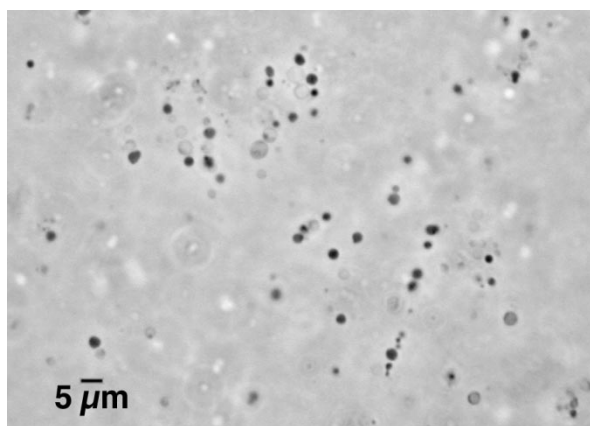


Fig. 1. Phase contrast micrograph 1000x of JEL-1 in polymer matrix.

organisms that are all related by their unique corkscrew-shaped cell morphology and their anaerobic lifestyle. Spirochetes are an important group of bacteria, both medically and ecologically. Several members of the group cause disease, for instance, *Borrelia burgdorferi* causes Lyme's disease and *Treponema pallidum* is the causative agent of syphilis. Some spirochetes are found as symbionts in the digestive tracts of animals such as cows and termites. Free living spirochetes have been found in sediments and in wastewater treatment plants. However, despite diverse means of making a living, universally spirochetes have a spiral shape, at least when they are growing and reproducing. In medical literature, it is well documented that when spirochetes encounter unfavorable environments, like in the presence of oxygen or during nutrient starvation, they ball up and form so called spherical bodies. These spherical bodies vaguely resemble the newly discovered strain JEL-1. However, when the medically relevant spirochetes become round, it indicates that the bacteria are dying, and have become incapable of multiplying. Strain JEL-1, on the other hand, grows and reproduces only in the round state, and has never been observed to produce a spiral morphology under any of the laboratory conditions that have been tested so far.

A few additional bacterial isolates similar to JEL-1 have been obtained from different locations throughout the U.S., although strain JEL-1 is the only one that produces such large quantities of this viscous polymer. Their widespread distribution, and the fact that these novel organisms often are found in association with bacteria that degrade priority pollutants, makes them interesting from ecological, biotechnological, and bioremediation perspectives.

III. BIOLOGICAL MATERIALS AND METHODS

While the exact nature of the biopolymer produced by JEL-1 is still under investigation, we wanted to determine whether the formation of the polymer correlated with the growth of the bacterial strain. We measured bacterial growth using the OD, or absorbance, of the growing culture at a wavelength of 600 nm. An increase in bacterial numbers is observed as increased light scattering which is recorded as the absorbance using a Varian Cary 3E UV Visible spectrophotometer (Palo Alto, CA). The density of the solution was measured by determining the mass of 50

μl of culture fluid on a balance. The increase in viscosity was measured using a QCM microbalance, as described below. As a comparison with the QCM, samples were periodically tested for viscosity using a Haake RS-75 rheometer with a DG41-T1 sensor (Karlsruhe, Germany). A growth medium that had not received an inoculum, as well as deionized water, were used as blanks and quality control measures.

Bacterial cultures were grown in Tryptic Soy Broth (27.5 g/L) (Sigma), supplemented with glucose (7.5 g/L) and yeast extract (1 g/L). The medium was boiled under a nitrogen stream to remove oxygen, and after cooling, 1-mM dithiothreitol (DTT) was added to reduce the solution. The pH was adjusted to 7.2 and 100 ml of medium was dispensed into 160-ml bottles under a constant stream of nitrogen gas (Hungate technique). A butyl rubber stopper and aluminum crimp made an airtight seal. The medium was sterilized in an autoclave (30 min, 121 °C). Using a sterile hypodermic needle and syringe, 1 ml of a pregrown JEL-1 culture was used to inoculate the fresh medium. All cultures were incubated without shaking at room temperature in the dark. To sample the growing cultures, 3 ml were withdrawn aseptically using a syringe and 1 ml was used to determine the OD, 1 ml to determine the viscosity (QCM), and the remainder was used for density determination. When the cultures no longer increased in OD and the viscosity remained unchanged for two consecutive days, the experiment was concluded.

IV. EXPERIMENTAL SETUP AND TEST PROCEDURE

The experimental QCM setup consisted of a Maxtek TM-400 plating monitor, a crystal controlled oscillator, a liquid flow cell, and a single AT-cut 10 MHz polished crystal. The AT-cut crystal operates in thickness shear mode (TSM), which is most advantageous in liquid phase tests where the density and mass of the liquid can cause motion normal to the crystal surface to be muted. The surface of the crystal was affixed with polished gold electrodes that facilitated electrical connection to the oscillator. The oscillator, flow cell, and crystals were supplied by International Crystal Manufacturing (ICM).

The crystal was connected to the oscillator and mounted in an acrylic flow cell. The flow cell was used to secure the crystal in place while physically and electrically isolating each side of the device. This allowed the introduction of a liquid test medium to one surface of the crystal, via a 1-mL static chamber, while keeping the other side exposed to air. The TM-400 was used as a counter to monitor the frequency of the oscillator and PC-based software provided by Maxtek permitted this frequency information to be periodically recorded on a desktop PC via the RS-232 serial port. A block diagram of the measurement test setup, including a side view (scale exaggerated) of the crystal and flow cell, is shown in Fig. 2.

The tests were performed on a semi-daily basis to monitor any growth of the bacterial population in the test liquid. The same crystal was used throughout the course of the experiment to eliminate measurement inaccuracies attributable to inherent physical variations in different crystals. Water, blank medium, and three replicates of the inoculated medium (JEL-1.1, JEL-1.2, and JEL-1.3) were tested separately by transferring 350–400 μL of each solution into the 1-mL reservoir of the

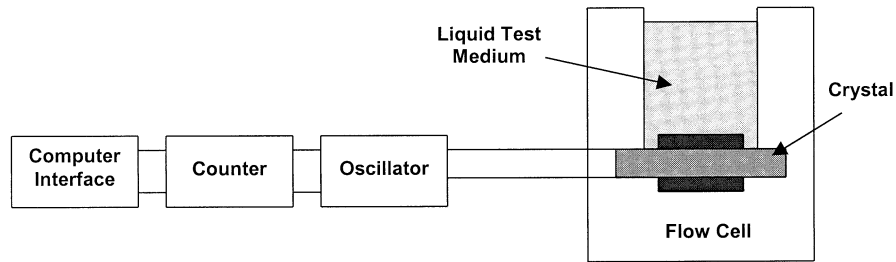


Fig. 2. Block diagram of measurement test setup.

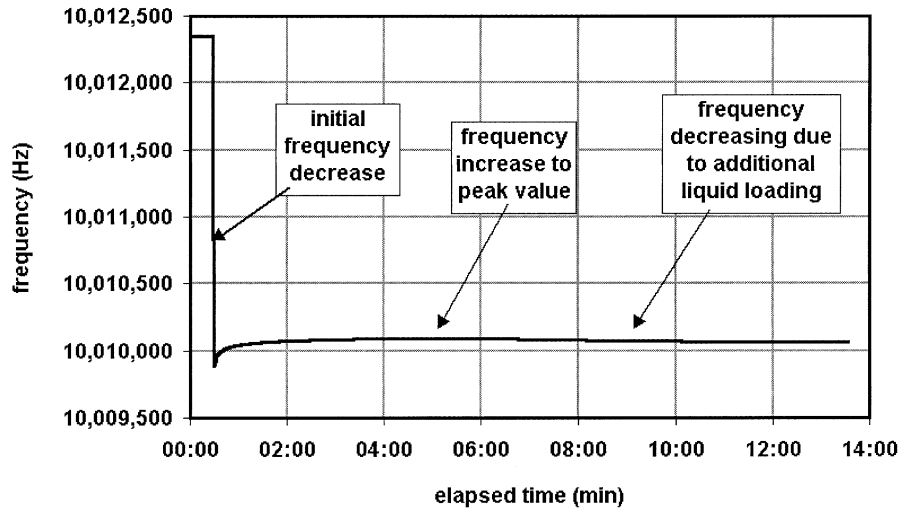


Fig. 3. Frequency shift trends upon introduction of liquid to QCM.

flow cell using a 3-ml hypodermic syringe, supplied by Becton, Dickinson, and Company (BD). Liquid volumes were chosen so that the distance between the face of the crystal and the top surface of the liquid would be much greater than the liquid decay length for a TSM crystal operating at 10 MHz. By greatly exceeding the decay length, approximately $0.15 \mu\text{m}$ at the 10-MHz operating frequency, the energy entrained into the liquid by the shearing motion of the crystal would be sufficiently muted before reaching the surface of the liquid. This eliminated the possibility of erroneous results due to energy reflections at the liquid/air interface. The liquid was introduced into the top of the open flow cell reservoir with a nontipped syringe. This method was utilized to prevent shearing of the polymer in the solution that might have occurred if the liquid was forced through a needle tip of small radial proportions. Also, previous system validation experiments using specific glycerol/water mixtures were conducted to determine volumes that facilitated rapid oscillator stability and accuracy after introduction of the test fluid.

Prior to introducing the liquid to be tested, the surface of the crystal was cleaned with isopropyl alcohol and carefully dried. This procedure was performed with the crystal operating in the oscillator and exposed to air while monitoring the resonant frequency of the system. This method ensured the removal of any substance remaining on the surface of the crystal from the preceding test, as the cleaning process was repeated until a specific (steady) nominal frequency was reached. During testing, the resonant frequency was monitored on the Maxtek

TM-400 and recorded at intervals of 30 points/min on the desktop PC.

Each test followed the same general trend, in which the system exhibited a rapid frequency decrease at the initial liquid introduction and was followed by a gradual frequency increase to a peak value. This was followed by a gradual and steady frequency decrease, which was attributed to nonviscous liquid loading effects as detailed in [2]. A plot illustrating this trend is shown in Fig. 3 for one of the tests conducted using deionized water. This frequency shift behavior was typical for all of the blank and inoculated liquid media tests as well as multiple water tests.

The value of the peak frequency after the initial transient drop was determined to be the most accurate and repeatable representation of the viscous properties of the liquid before any subsequent loading effects were observed. In light of these observations, the peak values of each subsequent measurement were used to track the growth progression of the viscous substance. The physical and electrical characteristics of the test system were factored out by normalizing all JEL-1 readings to measurements of the blank liquid taken during the same test period.

Viscosity readings of deionized water were also conducted prior to each daily set of tests to ensure that the system was functioning properly and to extract data with which to normalize subsequent tests of the blank media. Three water tests were run and the system operation was validated each day for consecutive readings that fell within 2% of the measured average viscosity. These readings were used to achieve an absolute cali-

bration of the system so that the blank media readings could be validated by separate rheometer tests. By assuming a viscosity of 1.0 centipoise (cP) for water, the blank measurements were normalized by dividing the uncalibrated viscosity measurement for this medium by the readings taken for water. Also, density measurements of each of the test liquids were taken each test day so that the viscosity information could be extracted from the frequency shift information.

By employing these methods, any errors attributable to electrical offsets in the test equipment or physical inconsistencies in the crystal, such as surface roughness or density, were factored out allowing the relative viscosity information to be extracted.

The theoretical viscosity measurement resolution for the samples with this test setup was approximately 1.5×10^{-5} cP at the test frequency of 10 MHz and measured viscosity levels ranging from 10 to 16 cP. In practice, repeatable results were achieved down to resolutions of 5×10^{-3} cP, which corresponded to a minimum detectable frequency shift of approximately ± 5 Hz at the 10 MHz operating frequency.

V. EXPERIMENTAL RESULTS

Each inoculated solution, as well as the blank, was tested three times during each QCM viscosity measurement session. The average of the three tests was used to calculate the property changes of the test liquid, while the minimum and maximum readings were analyzed as an additional validation component to characterize sample deviation.

Three independent replicate solutions inoculated with JEL-1 were measured at regular intervals from the day of the initial introduction of the bacteria until a peak viscosity was reached about four weeks later. These measurements showed an increase in the amount of produced polymer until the liquid reached and maintained a maximally viscous state.

The measurements of the blank and inoculated media were normalized against regular measurements of deionized water. As detailed in [3], the change in resonant frequency for a fundamental mode TSM resonator with a Newtonian liquid applied to the crystal surface is

$$\Delta f_s \cong -f_s^3 \left(\frac{\rho\eta}{\pi\mu_q\rho_q} \right)^{\frac{1}{2}} \quad (1)$$

where f_s is the resonant frequency, ρ and η are the density and viscosity of the contacting liquid, and μ_q and ρ_q are the shear stiffness and mass density of the quartz crystal. The values for the shear stiffness and mass density for quartz are given by [3] as

$$\begin{aligned} \mu_q &= 2.95 \times 10^{11} \text{ dyne/cm}^2 \\ \rho_q &= 2.65 \text{ g/cm}^3 \end{aligned} \quad (2)$$

Fig. 4 details the progression of the three replicate JEL-1 cultures over a period of 28 days. This plot shows the extracted viscosity values as referenced to readings for the blank media taken on the same day.

Note that the liquids all approached a maximum value toward the end of the experimental period.

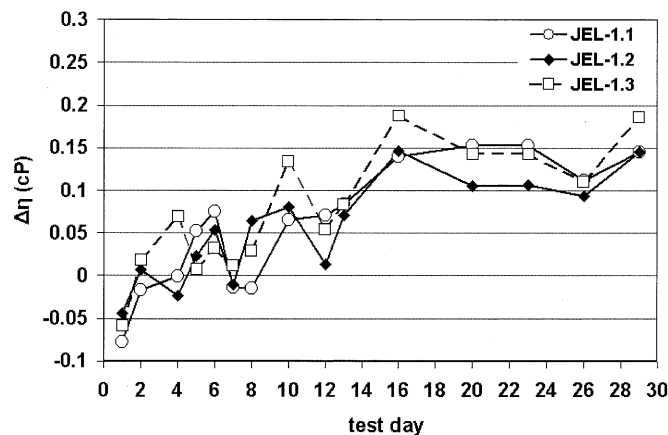


Fig. 4. Comparative viscosity changes for all three inoculated media measured with the QCM.

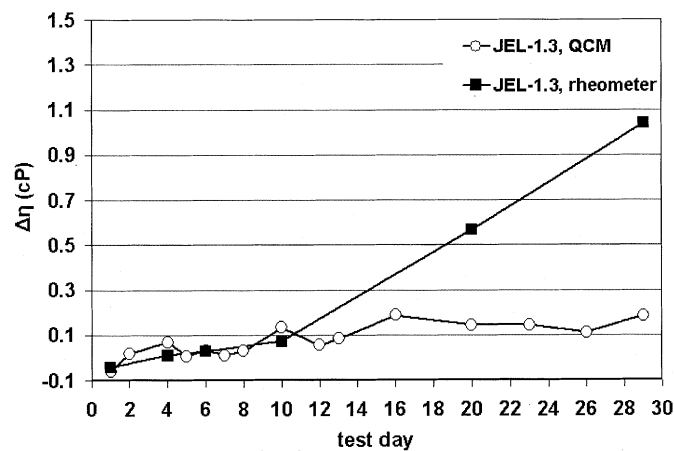


Fig. 5. Comparative viscosity changes for JEL-1.3 measured with QCM and rheometer.

The QCM readings of the JEL-1.3 culture as compared with measurements taken with the Haake rheometer are shown in Fig. 5.

The early stages of the experiment, up to day 10, display good agreement between the QCM and rheometer measurement techniques. However, from day 16 until the completion of the experiment, the readings using the rheometer increase greatly while the QCM readings level off. It is assumed that the fluid becomes non-Newtonian in nature in the latter stages of the experiment, which indicates that the calculation for absolute viscosity given in (1) is only valid up until approximately 0.2 cP with this particular microbe.

The QCM readings of the JEL-1.3 inoculum compared to OD measurements are shown in Fig. 6. These results show the OD measurements increasing rapidly in the latter stages of the test (after day 16) in a similar manner to the rheometer measurements, while the QCM has leveled off between 0.1–0.2 cP.

Bacterial growth occurred when polymer production began. Growth paused for four days at day 12, as polymer was produced. Growth resumed on day 16, and the resulting polymer production could no longer be detected using the QCM (Fig. 6). The resulting polymer induced a significant viscosity increase as determined using the rheometer (Fig. 5).

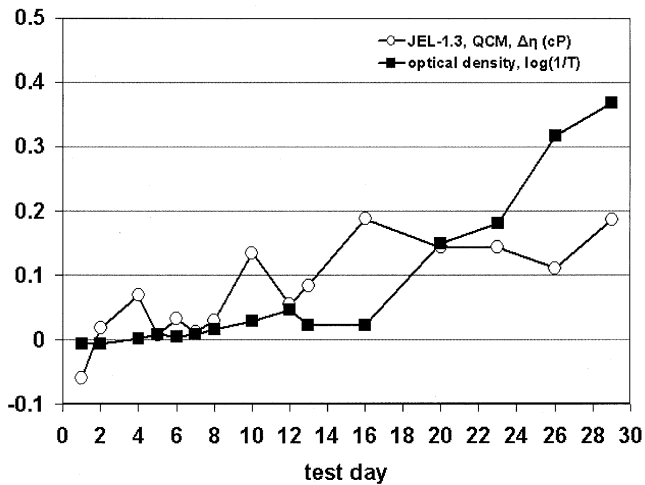


Fig. 6. Comparative growth changes for JEL-1.3 measured with QCM and 600-nm OD measurement.

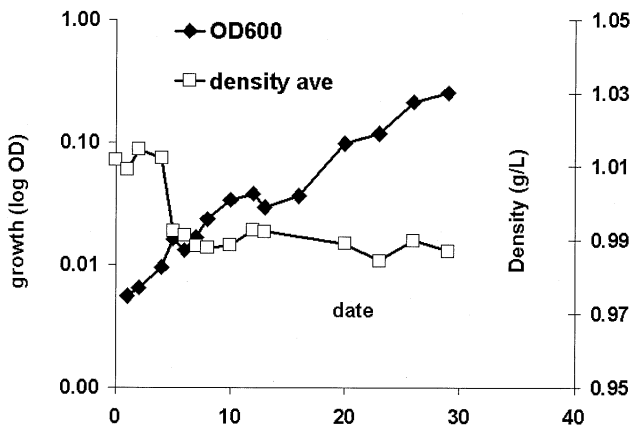


Fig. 7. Growth of strain JEL-1 (average of three replicates) with a decrease in the density of the growth media after day four.

Interestingly, we noticed a slight decrease in the viscosity of the inoculated solution relative to the uninoculated control the day after inoculation. The decreased viscosity was transient, and by the second day, the viscosity measurements increased to equal the blank. On day four, the density of the inoculated solution decreased from 1.012 g/l (equal to the blank) to 0.989 g/L (Fig. 7). No increase in viscosity was observed. By day five, the viscosity began to increase, and the solution became visibly thicker, which correlated with an increase in viscosity measured on the QCM. Microbial growth, as evidenced by an increase in OD, continued even after the QCM readings had reached a plateau.

VI. DISCUSSION

There were some inherent difficulties in measuring growth of JEL-1 using OD. Initially, it was expected that bacterial growth could be accurately monitored using the spectrophotometer. This is a standard microbiological technique that is frequently used to calculate the rate at which a bacterial population doubles in size when growing uniformly in liquid medium. Strain JEL-1, however, is a very unique organism, and the

polymer that it produces hampered accurate readings of light scattering. For example, as the bacteria grew and produced the polymer, the bacterial cells clumped together in separate parts of the matrix. These growth characteristics were influenced by incubation conditions. Repeated shaking of the cultures distributed the bacteria more evenly in the bottles, while cultures left without agitation for several weeks grew in a much more heterogeneous manner.

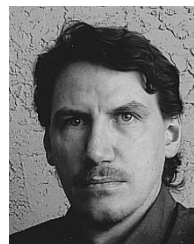
The QCM proved very valuable in measuring the increase in viscosity, and demonstrating that strain JEL-1 produces a viscous biopolymer. The QCM method for viscosity measurement has advantages over the traditional rheometer. For instance, the rheometer required 6 ml of sample, as compared with 0.25 to 0.50 ml used for the QCM. Smaller sample sizes result in less disturbance of the bacterial culture during growth and allowed for more samplings. Furthermore, the QCM is portable, which would allow for it to be used on site (e.g., a bioreactor).

As the amount of polymer increases in the growth medium, the liquid appears to take on non-Newtonian properties. This is evident in the differences between the rheometer and QCM readings as more polymer is produced and also visibly evident through observation of the cell clumping noted previously. Noting this, the QCM appears to be most useful in the accurate measurement of the initial growth stages in this particular application. Current efforts are focused on expanding the QCM technique for these types of polymers possibly by comparing measurements to previously recorded reference QCM data for particular microbial progressions.

While we have demonstrated the successful application of the QCM to polymer formation by a novel bacterial population, it may also be useful in other applications, such as monitoring polymer degradation resulting in a decreased viscosity of a solution. The method's relative ease makes it a promising tool for measuring microbial activity, whenever changes in viscosity occur.

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