Sinusoidal CVD diamond micro-tools for the manufacture of microstructured surfaces used in bioremediation

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INTRODUCTION

In natural settings microbial cells often form multicellular aggregates on surfaces and at interfaces, commonly known as biofilms. The formation and preservation of these aggregates depends on the production of extracellular substances which, in this association, constitutes the extracellular matrix (ECM)[1,2] [1, 2]. Bioremediation of environmental contaminants often relies on the use of endogenous or exogenous microorganisms. These organisms are frequently found in biofilm communities. Biofilm systems are especially suitable for the *in situ* degradation of the fats, oils and grease (FOGs) (a multimillion pound cost to sewer operators [3][3]) due to their high microbial biomass and ability to immobilise compounds [4][4]. The adhesion of bacteria to the surface is one of the first stages in the development of a biofilm and is believed to be influenced by a number of microbiological, physical, chemical, and material-related parameters [5][5]. In particular, surface topography has been widely discussed as a factor influencing bacterial adhesion. R_a (arithmetic average of the absolute values) and R_t (range of the collected roughness data points) are the most commonly used amplitude roughness parameters. However R_a does not describe surface features such as "soft" or "sharp" topography or the presence of scratches or pores [6][6]. Over recent years, Atomic Force Microscopy (AFM) has proved to be a useful tool for the analysis of microbial systems and biointerfaces. High-resolution, three-dimensional AFM imaging of surfaces can provide topographical parameters in the nanometre range which can help elucidate the mechanism of microbial attachment to surfaces [7][7].

Single Point Diamond Turning (SPDT) has been used in combination with single crystal diamond tools manufactured using Focused Ion Beam (FIB) machining [8,9] [8,9] to create microstructured surfaces with features of selected profile and dimension [10] [10]. This is not achievable with photolithographic methods that can offer high level of miniaturization but are constrained to 2.5D shaped features. Cost effective manufacturing of large micro-structured polymer surfaces can be achieved by using microstructured patterned "drums" [11,12][11,12]. The process is based on the surface structuring of metal drums by SPDT. The structured drums are then replicated onto large polymer films by either UV embossing or extrusion methods. The obtained polymer microstructured films are compatible with the current fabrication technologies of sewer pipes and could potentially be lined into new pipes or retrofitted into existing pipework to modify their surface properties.

Diamond tools in which the diamond is synthetically produced by Chemical Vapour Deposition (CVD) to produce a polycrystalline material are cheaper than natural diamond tools and are also hardwearing when compared with other tool materials [13] [13], which adds to the economic advantage. The extension of FIB to machining polycrystalline diamond (PCD) aims to combine PCD's attributes of lower cost, superior wear and toughness relative to single crystal diamond. This paper reports on the novel work on FIB figuring of PCD- CVD diamond microtools and on their use in the fabrication of microstructured surfaces of complex topography for use in bioremediation.

The structured surfaces are here used to enhance the biofilm formation of *Bacillus sp.,* one of the most-used microorganisms in commercially available bioadditions for FOG degradation. The surface design strategy was to create features with dimensions comparable to the size of *Bacillus sp.* (3-10μm) with the aim of influencing the microbial adhesion [14][14].

EXPERIMENTAL PROCEDURE

A microtool with a sinusoidal profile was manufactured using Focussed Ion Beam (FIB) technology (FEI 200). The blank tool was a laser-shaped CVD polycrystalline diamond tool with a round nose (Contour Fine Tooling). The sinusoidal tool tip geometry was initially produced on a CAD model and then imported onto a FIB milling control/pattern design software package (Raith GmbH). The FIB milling was carried out in water-assisted conditions using a Ga ion beam at 30keV and currents of 0.15nA - 1nA. The milling sequence for the 3D sinusoidal shape was adjusted to avoid redeposition problems and to optimize the sidewall smoothness [15,16][15,16].

A second tool was used to produce trapezoidal microstructured surfaces. The tool was in this case a commercial single crystal diamond tool with a flat end of 3μm (Contour Fine Tooling).

The replication of both sinusoidal and trapezoidal tools was

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obtained by a turning "plunge" process. The tools were mounted onto a diamond turning machine (Nanotech 350 UPL – Moore Nanotechnology System). It features an aerostatic spindle and hydrostatic X and Z guideways that ensure precise and smooth positioning. The tools' patterns were replicated onto aluminium alloy discs (6061-T6) at an infeed rate of 0.006 mm/min and spindle speed of 1200rpm to create microstructures consisting of 50 concentric circles. White spirit was used as coolant.

All aluminum samples were diamond turned to obtain a flat, optically smooth surface of only a few nanometres R_a, prior to turning with the sinusoidal/trapezoidal diamond microtools.

The microtool replication strategy was the same for both sinusoidal and trapezoidal: firstly the tool was positioned at the centre of the work-piece. The setting along the Z axis was adjusted using a manual procedure (figure 1): two plastic shims (60 and 30µm) were used as controls to probe the distance between workpiece and tool. The tool was then advanced towards the work-piece with 1 µm steps until the shim would not fit in the gap. To guarantee the accuracy of the process a witness cut was performed using increments of 0.1 µm until the first swarf formed as a sign of contact.

The surface properties of the microstructured surfaces were studied using Atomic Force Microscopy (AFM - DME DualScope 200) and optical profilometry (Olympus 3100).

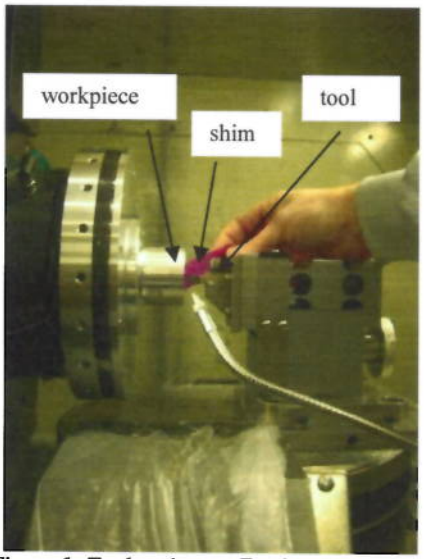

Figure 1: Tool setting on Z axis using a shim.

Commercially available microbial additions of *Bacillus sp.* (ACO Technologies Plc) at a concentration of 10^7 Colony Forming Unit (cfu)/ml were added to flasks containing 100ml of Nutrient Broth (Oxoid) prepared according to the manufacturer's instructions. Cultures were incubated at 37°C for 12 hours under gentle agitation (150rpm).

To determine the effect of substrate surface on the adhesion of *Bacillus sp.*, the structured surfaces were cleaned (ethanol, acetone and methanol) and then aseptically added to the microbial cultures in Nutrient Broth at approximately 10^7cfu/ml. Adhesion was allowed to take place under slow agitation at 37°C. The structured surfaces were analysed after one week and three weeks using a high-resolution scanning electron microscope equipped with a field emission gun filament (SFEG-SEM XL30 Philips).

RESULTS & DISCUSSION

The tip of the sinusoidal CVD diamond micro-tool fabricated using FIB technology is shown in figure 2. The amplitude and wavelength of the sinusoid were approximately 6 and 9µm respectively.

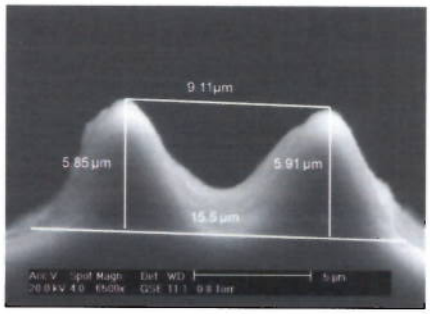

Figure 2: CVD diamond tool tip fabricated using FIB technology

A section of the surface obtained by the turning process using the sinusoidal tool is shown in figure 3.

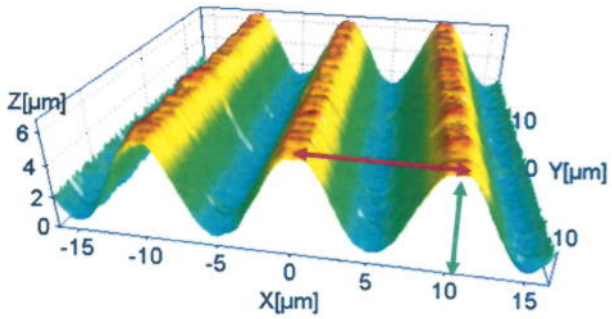

Figure 3: Profile of the sinusoidal surface determined using confocal microscopy. The amplitude and wavelength of the sinusoid are 5.6 ± 0.6 µm and 9.1 ± 0.6 µm.

For comparison purposes a second aluminium surface was manufactured similarly to the sinusoidal surface shown in figure 3 using a commercial single crystal diamond tool with a 3µm flat end. The resulting microstructure is shown in figure 4.

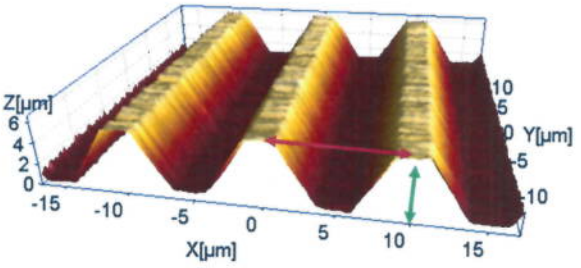

Figure 4: Profile of the trapezoidal surface determined using atomic force microscopy. The height and pitch of the structures are indicated by the arrows and are 4.7 ± 0.3 µm and 10.2 ± 0.4 µm respectively.

The AFM data obtained for both surfaces were analysed to extract the following surface roughness parameters:

- S_a (average roughness) and S_q (root mean square roughness); Sa and Sq were estimated after removal of the sinusoidal or trapezoidal geometry to allow estimation of the residual roughness left on the sideflanks, on the top and on the bottom of the machined grooves.
- S_{dr} (surfaces area ratio); This parameter is a measure of the increment of the interfacial surface area relative to the area of the projected (flat) x,y plane. In general the higher the roughness or the geometrical complexity of the surface the higher the value of Sdr.

The average values of S_a, S_q and S_{dr} are reported in table 1.

Table 1: Surface roughness parameters for the generated sinusoidal and trapezoidal microstructures

Surface parameter	Microstructure geometry			
	Sinusoidal		Trapezoidal	
	Average value	Standard deviation	Average value	Standard deviation
S_a^* (nm)	165	18	136	16
S_q^* (nm)	209	22	184	21
S_{dr} (%)	233	19	164	18

* residual surface roughness after filtering the micro-geometry

The biofilm growth was observed following incubation on both sinusoidal and trapezoidal microstructures. Figure 5 shows the clear preference of the biofilm (white agglomerates) for the microstructure (right) compared to the original smooth surface (left) of the aluminium samples.

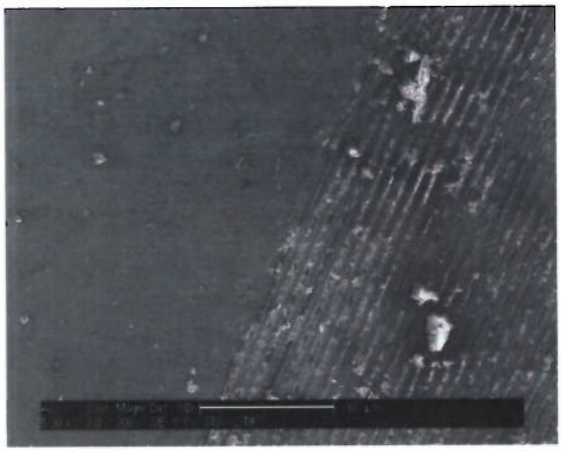

Figure 5: Biofilm formation on the structured surface following 1 week incubation

The microorganisms also showed a partial preference for the sinusoidal structure compared to the trapezoidal. Figure 6a illustrates the larger amount of surface biofilm observed on the sinusoidal microstructure (in white ellipses) compared to the few bacteria observed on the trapezoidal microstructure (figure 6b). This initial observation was confirmed from the analysis of the structured surfaces after 3 weeks incubation (Figures 7a and b). In the sample with the trapezoidal microstructure (figure 7b) the grooves are still partly visible (in the centre of the image) whereas the sample with the sinusoidal microstructure is completely covered by a thick biofilm layer.

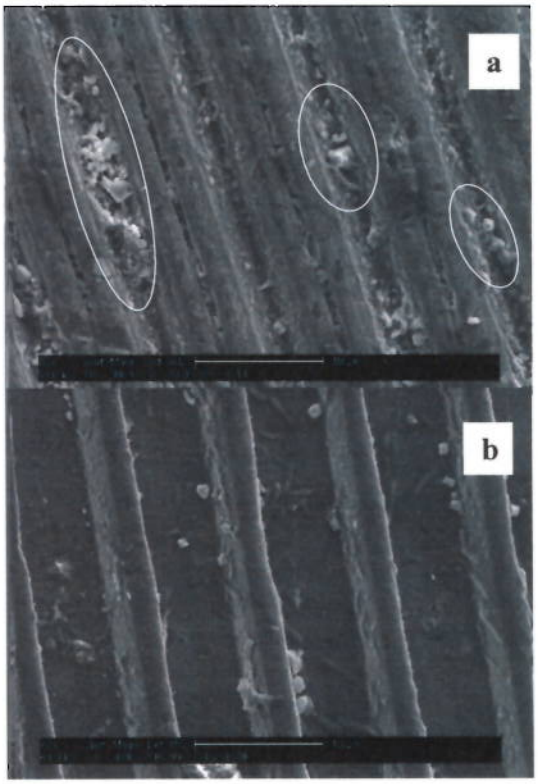

Figure 6: Biofilm formation on microstructured surfaces following 1 week incubation. a) sinusoidal and b) trapezoidal microstructure.

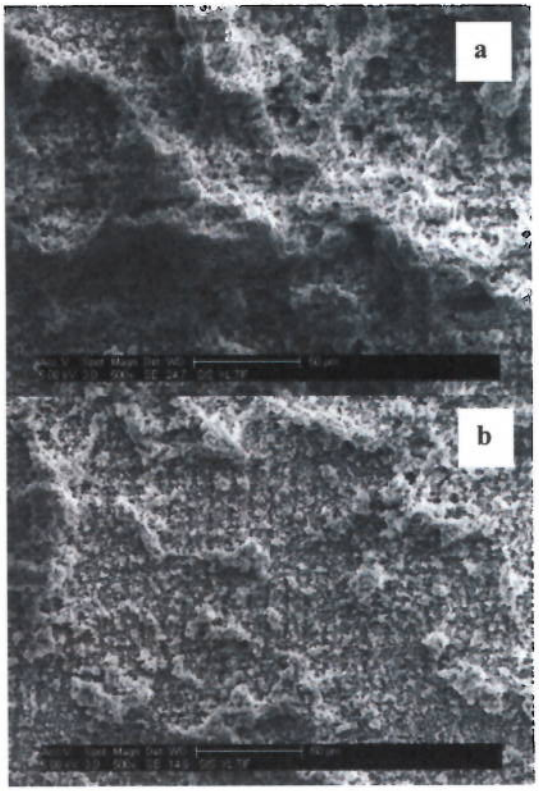

Figure 7: Biofilm formation on microstructured surfaces following 3 weeks incubation. a) sinusoidal and b) trapezoidal microstructure.

The topographical analysis of the fabricated surfaces provides some useful information to understand the preferential adhesion to the sinusoidal surface. The residual roughness (S_a and S_q, Table 1) is slightly higher in the case of the sinusoidal structures. As reported in literature [17][17], rough surfaces promote adhesion and bacterial growth. This is confirmed by the higher adhesion and growth found in the case of the sinusoidal structure. However this parameter cannot be considered determinant due to the relatively high standard deviations.

The S_{dr} parameter (Table 1) quantifies the actual 3D developed interface area as a percentage of an ideally flat surface. The S_{dr} for the sinusoidal structure is approximately 42% higher than the trapezoidal one. This suggests that the sinusoidal structure has a surface area in contact with the biological material which is higher than the trapezoidal surface. In other words the substrate with a sinusoidal microstructured surface provides a larger surface area for the bacteria attachment and growth which confirms the observed results.

In addition to the above mentioned differences in the quantity of observed biofilm colonies, changes were also observed in the length of the attached cells and in the amount of extracellular matrix (ECM) produced. The SFEG analysis of a limited sample of the attached cells reveals a cell average length of 1.8µm and 2.9µm for the cells grown on the sinusoidal and trapezoidal structures respectively (figure 8 and 9). Moreover figure 8 shows the presence of abundant ECM in the form of "slime" surrounding the bacteria (white contrast areas in the SFEG micrographs). This ECM is only present in very small amounts in the trapezoidal structure (figure 9). It has been reported that nano-structured topographies can lead to the production of different amounts of ECM. This, in turn, can lead to changes in cell morphology [18][18]. The results obtained from this work confirm that the surface topography affects the size of the *Bacillus sp.* We can speculate that the size of the *Bacillus sp.* is correlated to the amount of the ECM produced which in turn affects the strategies used by this microorganism to attach to the surface.

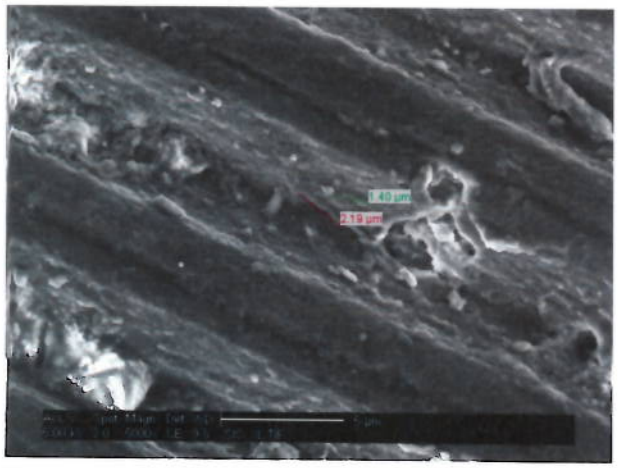

Figure 8: Biofilm formation on the sinusoidal surface following 1 week incubation. The average cell size is1.8µm

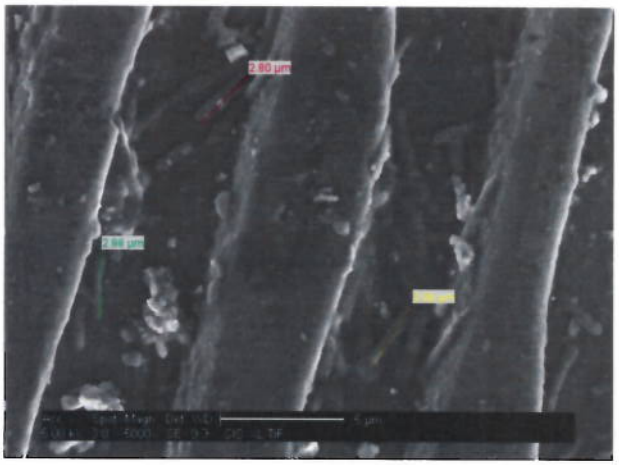

Figure 9: Biofilm formation on the trapezoidal surface following 1 week incubation. The average cell size is 2.9µm

CONCLUSIONS

FIB technology showed great potential in the fabrication of complex shapes in precision microtools made of CVD polycrystalline diamond. This is a hardwearing tool material but otherwise difficult to be ground and shaped into precision cutting tools. A sinusoidal shape was successfully produced and replicated onto aluminium substrates via a "plunging" single point diamond turning process.

The incubation of the aluminium samples confirm that microstructured surfaces have the potential to act as directors for the formation of biofilms of *Bacillus sp.* on metal surfaces.

In particular:

- Microstructured surfaces show a preferential cell adhesion compared to planar surfaces with optical finish
- Sinusoidal grooves show higher potential than trapezoidal grooves for cells attachment and biofilm growth
- The S_{dr} parameter is a good indicator for the available surface area, thus quantifying the potential for bacterial adhesion

Further work is currently ongoing to investigate the role of the material composition and mechanical properties (stiffness) in the biofilm formation in conjunction with the quantification of biofilm formed on different microstructured surfaces.

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