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Optimization of Microbial Adsorption (Biofilm Creation) on Activated Carbon by Surface Modification of Substrate

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ABSTRACT

Conversion of biomass to useful bio-products is a lengthy and often inefficient process. Research has looked at the conversion of cellulose to ethanol by means of termite bacterial consortium in fluidized bed bioreactor, where the bacteria are attached as biofilm to an activated carbon or cellulose support. High conversion rates were achieved in this reactor and the process was fairly robust and flexible. However establishing the biofilm on the activated carbon was a lengthy process in terms of the time it took the bacteria to attach to the various substrates (Activated Carbon and Cellulose) and form flocculants. The formation of such flocculants substantially increases the reaction surface and hence should optimize the production of ethanol.

Many physical, chemical, and biological interactions facilitate the attachment of bacteria to surfaces. It was in this study that the electrostatic attraction was investigated. The understanding of the physical modifications of surface charge was chosen to be investigated in order to understand the ideal conditions to propagate and increase biofilm creation. Bacteria carry a negative surface charge and hence for increased attraction, the surface charge of the substrate should be modified to be positive. This research, performed as batch processes, has shown that with the correct surface charge modifications of the substrate the electrostatic attraction forces between the surface and the bacteria are maximized. As a result of the strong electrostatic attraction forces between the two surfaces the bacteria adsorbs and attaches to the substrate quicker and creates a biofilm on the surface.

Prior to the attachment investigation it was important to attempt to understand the bacteria consortium within the termite gut.. The “worker” termites collect the food and feed off the soil whilst building their mound.. The bacteria found within the termite are in line with cellulose degradation, which can be manipulated for biofuels production.

This study aimed to investigate a series of procedures of charge manipulation to the surfaces of both the substrate and bacteria in order to see the influence of electrostatic interaction on biofilm creation. It was seen that when only the activated carbons surface charge was modified to have a positive net charge the attachment of bacteria was most prominent. In addition it was proven that after being charged to a pH of 5 (4.28×10^4 Bacteria/mm^2 Activated Carbon), 6 (3.90×10^4

Bacteria/mm^2 Activated Carbon) and 7 (1.58 x 10^5 Bacteria/mm^2 Activated Carbon) were achieved. The greatest attachment was seen when the activated carbon was charged to a pH of 7. This can be explained as the optimal positive pH the activated carbon should be charged to if its pH_{PZC} is 9.7. If the pH is dropped lower than 7 although the surface becomes more positive, the surface actually approaches the surface charge of bacteria and the electrostatic interaction dissipates.

In an effort to use the optimal pH modification to create a biofilm on the surface of the activated carbon time experiments were performed. After 13 days biofilms on the surface of the activated carbon surfaces were seen. The bacteria attach to surface of the activated carbon and produce Extracellular Polymers Substance (EPS) fluid which then causes other bacteria to attach to them resulting in the formation of Biofilms.

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NOMENCLATURE

ABBREVIATION	EXPLANATION
A (DNA)	Adenine
A.C	Activated Carbon
BLAST	Basic Local Alignment Search Tool
C (DNA)	Cytosine
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
EPS	Extracellular Polymer Substances
ESEM	Environmental Scanning Electron Microscope
FBBR	Fluidized Bed Biofilm Reactor
G (DNA)	Guanine
HNO_3	Nitric Acid
H^+	Hydronium Ion
ITS	Internal transcribed spacer
LPS	Lipopolysaccharides
NaOH	Sodium Hydroxide
OH^-	Hydroxide Ion
PCR	Polymerase chain reaction
pH_{PZC}	pH point of zero charge
PVAM	polyvinyl amine
RNA	Ribonucleic acid
SEM	Scanning Electron Microscope
T (DNA)	Thymine
TAE	Tris-acetate-EDTA

UNIT OF MEASURE	EXPLANATION
g	Gram
l	Litre
mol	mole
°C	Degree Celsius
sec	Second
µl	microliter
rpm	Revolutions per minute

DECLARATION

I declare that this Thesis is my own, unaided work. It is being submitted for the Degree of Master in Chemical Engineering. It has not been submitted before for any degree or examination at any other University.

_____

(Signature of candidate)

_____day of _____20_____

DEDICATION

To my angel who stood by me through hardship.

Thank you for being my strength

HYPOTHESIS AND RATIONALE

HYPOTHESIS

Surface modification of the support matrix improves and increases the attachment of the bacteria resulting in an increased biofilm formation.

RATIONALE

The main objective of this research is to optimize the attachment of bacteria found in termite hindgut to activated carbon, which in turn will be used to produce bio products from the decomposition of biomass. The project will entail several steps and will develop as the research progresses.

The first objective was to identify and fully understand the various species of bacteria found within the termite gut. These bacteria once identified and isolated will be investigated as to their functioning in the degradation of cellulose. Termites were selected for this application as they have a unique ability to digest lignocellulosic biomass. The termite's digestive process takes advantage of a complex mixture of enzymes from symbiotic bacteria, primitive single-celled organisms residing in its stomach (gut fauna), as well as enzymes originating from the termites themselves to degrade cellulose and produce biofuels.

The secondary objective of this research was be to establish how these modifications of the surface charge on the activated carbon will help in biofilm creation. It was desired that this hypothesis would prove to improve the rate of biofilm creation.

- Biofilm creation has previously proved to be a lengthy process. It is therefore desired to optimize this process by enhancing the rates of adsorption of bacteria onto the activated carbon within the reactor. These continuous experiments will be performed to establish whether the hypothesis made above will prove to be successful.

It has been proposed that in order to enhance the adsorption of bacteria onto activated carbon surface the electrostatic forces between the two surfaces should be maximized. Hence the carbon surface should be modified to carry a net positive charge as the bacteria are typically negatively charged (Dastgheib, Karanfil and Cheng 2004). This will be done by increasing the H^+ ions found on the surface of the activated carbon, hence increasing its positive nature.

Chapter 1

LITERATURE REVIEW

1 LITERATURE REVIEW

The study of biofilms and their attachment to surfaces is of extreme importance as the study of alternative energy sources progresses. The following literature review outlines the relevant research that was required to gain a comprehensive understanding of this field of study. The review is set out as follows; Understanding lignocellulose degradation for ethanol production, biofuel research, biofuels that can be generated from cellulose, a clearer understanding of activated carbon as a suitable substrate for biofilm creation, a careful review of the steps and constituents in biofilms and finally the investigation of termite bacteria.

1.1 LIGNOCELLULOSE DEGRADATION

Biomass, which can be considered as the mass of organic material from all biological material, has become of interest as a source of energy. A huge variety of biomass is available on this planet and there is great potential for the conversion of them into bio products (Howard, Abotsi et al. 2003). Lignocellulose has been identified as the most abundant biomass on earth. It has proven to be difficult to convert this biomass into bio products and hence interest has shifted to the natural systems they occur in, in order to further our understanding of the decomposition of lignocelluloses (Ohkuma 2003). In section 1.3 the decomposition of lignocelluloses in the termite hind-gut is investigated as a potential bacteria source for this complicated conversion.

1.2 BIOFUELS FROM CELLULOSE

Cellulose based biofuels have been touted as the future source of bioenergy. Cellulosic conversion of biomass has however shown to be more complicated than the conversion of corn ethanol. This is due to the complexity of the biomass material. Nonetheless the advantages of using biomass have been shown to be extremely beneficial.

Cellulose-based biofuels have been thought to have considerable advantage in the current search for alternative fuel sources. Foremost is the fact that cellulosic biomasses are particularly abundant throughout the world. Attention has been directed towards biomass-to-biofuel conversion as a result of the ever depleting fuel sources available around the world. The difficulty of using biomass is its complex chemical composition. Biofuel production from plants requires the depolymerisation of the large carbohydrate polymers cellulose and hemicellulose. It is known that these polymers need enzymatic help to break down the ligands and other molecules in order to make the simple

sugars. This inability and inefficiency for breaking down the cellulose is the limiting factor for the bioethanol industry (Tartar et al. 2009).

Two methods for lignocellulosic conversion to biofuels are of interest- biochemical conversion and thermochemical conversion. The biochemical conversion process has been compared to the process that is used to produce ethanol from corn starch. The plants cellulose is broken down into sugars which are then fermented into liquid fuel. The process has been divided into four steps. First the feedstock is pre-treated in order to separate the cellulose from the lignin. Following pre-treatment enzymes break down the complex chains of sugars into more simple sugars which are then fermented into liquid fuel. The final stage is then the distillation of the fuel into a pure useable energy source.

The thermochemical conversion uses heat to decompose the feedstock into fuel. There are two types of thermochemical processes namely gasification and pyrolysis. Gasification is an anaerobic process where the biomass is partially combusted and converted into syngas, and is then cleaned. Fischer-Tropsch process is then used to convert the clean syngas into liquid fuel. Pyrolysis is the partial combustion of biomass to produce a bio-oil (similar to crude oil), which is then refined into biofuels.

Termites are arthropods that efficiently digest and survive on a lignocellulose diet. Damage to homes around the world is often caused by these wood eating insects, yet they are vital in the balance of the ecosystem. Recently it is for this ability to break down lignocellulose into useable energy that they have gained some publicity. Their digestion of lignocellulose is due to the complex set of microbes that reside in their hindgut (Tartar et al. 2009).

1.3 Activated Carbon Substrate

Activated carbons have been recognized as the most commonly used porous adsorbents (El-Sayed, Bandosz 2004). It has been shown that the adsorption of microorganisms on to activated carbon can create a biofilm around the carbon surface. This adsorption is primarily due to the adsorptive properties of carbon, the porous structure of the carbon particles, and the presence of various functional groups on the surface shown in Figure 1.1. (Yin, Aroua et al. 2007).

In recent years efforts have been made to improve this granular media to enhance its potential for absorbing microbial particles. Activated carbons have been shown to be able to remove certain waterborne bacteria from potable water supply systems. Microorganisms attach to activated carbon particles by means of strong Lifshitz - van der Waals forces (Busscher, Dijkstra et al. 2006). The activated Carbon is often negatively charged and is faced with typically negatively charged microorganisms such as bacteria (Pal, Joardar et al. 2008). Once there has been a charge reversal of the activated carbon, the electrostatic attraction between the negatively charged microbial cells and positively modified carbon particles will be strong (Busscher, Dijkstra et al. 2008). Few attempts have been made in the modification of the carbon surface charge yet those that have attempted this have shown significant results (Pal, Joardar et al. 2008).

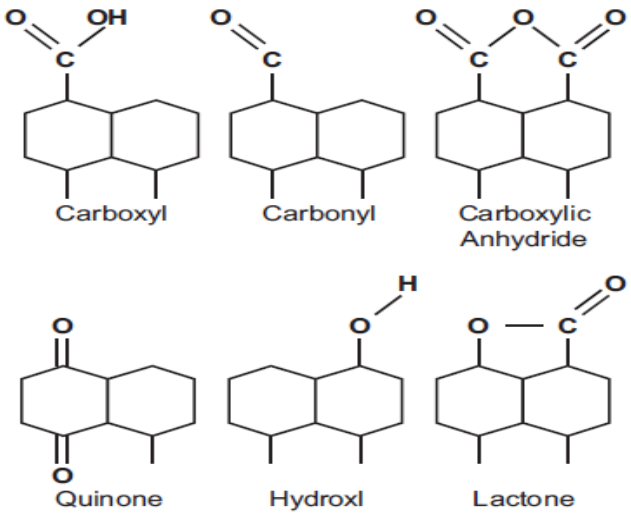


Figure 1.1: Functional groups found on the surface of activated carbon

1.4 BIOFILM

1.4.1 General

A biofilm can be defined as microbial cells which attach firmly to almost any surface submerged in an aquatic environment. The immobilized cells grow, reproduce, and produce extracellular polymers which frequently extend from the cell forming a tangled matrix of fibres which provide structure (Characklis, Wilderer 1989).

Advancement in biofilm research was relatively slow for a large part of this century. It wasn't until researchers became aware of the universal association of microorganisms with surfaces or with each

other that a sudden interest was taken (Characklis, Wilderer 1989). Research and understanding of the complexity of biofilms has progressed at an alarming rate. Biofilms are extremely intricate and offer many possibilities for advancement in nature and technology. The aim is to manipulate these natural systems for technological and ecological advancement (Characklis, Wilderer 1989).

They can be found at almost any surface exposed to water irrespective of the prevailing trophic state. Biofilms accumulating in a river bed or on suspended particles in rivers, lakes, and in the marine environment are often considered advantageous since they contribute largely to the removal of contaminants from water. In sewage treatment biofilms remove organic and inorganic pollutants (Characklis, Wilderer 1989).

It is a complex multi-step process which leads to the formation of a microbial community adhered to the surface (Bos, Van Der Mei et al. 1999). The speed and affinity with which bacteria bind to the artificial surface are influenced by the physical characteristics of both the substrate material and the bacterial cell surface (Cramton, Gotz 2004). Biofilms sometimes occur as uniform coverage over the surface of the substrate yet in other instances the biofilm occurs as a patchy layer.

Biofilms play an important role in nature and in recent biochemical technology. They can be found at almost any surface exposed to water irrespective of the prevailing trophic state. Biofilms accumulating in a river bed or on suspended particles in rivers, lakes, and in the marine environment are often considered advantageous since they contribute largely to the removal of contaminants from water. In sewage treatment biofilms remove organic and inorganic pollutants (Characklis, Wilderer 1989). A biofilm can be defined as a substratum with microorganisms attached. It is a complex multi-step process which leads to the formation of a microbial community adhered to the surface (Bos, Van Der Mei et al. 1999). The speed and affinity with which bacteria bind to the artificial surface are influenced by the physical characteristics of both the substrate material and the bacterial cell surface (Cramton, Gotz 2004).

It is extremely important to note that despite the fact that biofilm structure has been extensively studied, at present few standardized methods are available for quantification. Many studies have based their comparisons of biofilm creation largely on qualitative results, relying heavily on visual interpretations of the biofilm images (Characklis, Wilderer 1989).

Biofilms have been shown to be of extreme use in the removal of heavy metals. The structure of the bacteria surface which is made up of acidic functional groups like carboxyl, phosphoryl and amino groups are responsible for the bacterium great affinity to react.

1.4.2 Biofilm Development

The development of a biofilm can usually be characterised by five sequential phases (Renner 2011).

The first stage of biofilm creation is the attachment of the bacteria to the surface of the substrate. Organic molecules found within the medium may accumulate at the substratum, resulting in a "conditioned" substratum. Microbial cells are transported from the bulk water to the conditioned substratum (Characklis, Wilderer 1989). The bacteria are seen initially to attach to surfaces reversibly. In this step, bacteria use a selection of extracellular organelles and proteins, that are part of their anatomy, for sensing and attaching to surfaces, including flagella, pili, fimbriae, curly fibres, and outer membrane proteins (Renner 2011). A fraction of the reversibly attached cells will be seen to desorb back into the bulk medium.

The second stage is the irreversible attachment of the bacteria onto the substrate (Renner, 2011). Some of the reversibly attached cells become logged on the surface permanently and hence are irreversible. The bacteria secrete a substance called extracellular polymeric substance (EPS) that consists of DNA, proteins, lipids, and lipopolysaccharides. This substance facilitates adhesion between cells and surfaces and acts as the adhesion/'glue' that holds the bacteria together (Characklis, Wilderer 1989) (Renner 2011). Cells and other particulate matter will tend to attach to the biofilm, thereby increasing the thickness and biofilm accumulation (Characklis, Wilderer 1989).

The third stage involves the multiplication of the adhered bacteria (Characklis, Wilderer 1989). Cells adsorbed on surfaces replicate and grow into micro-colonies (Renner 2011). The attached bacteria secrete EPS and the bacteria are seen to be contained in a layer of hydrogel, which forms a physical barrier between the community and the extracellular environment (Renner 2011).

The fourth stage is the growth of these microbial communities. The community grows into a three-dimensional structure and matures into a biofilm as cells replicate and the EPS accumulates (Characklis, Wilderer 1989). With the additional excretion of EPS and the growth the strength and resilience of the biofilm is noticed in these final stages (Renner 2011).

The final stage is seen when some cells detach from regions of the biofim and disperse into the bulk fluid. The detached bacteria are seen to then adsorb on other surfaces and form biofilms here. This step is important for propagation (Renner 2011).

1.4.3 Physical and Chemical Properties of Biofilms

Biofilms are composed of two major constituents; bacterial cells and the Extracellular Polymer Substances (EPS). A biofilm can be defined as an organic polymer gel with living microorganisms trapped in it (Characklis, Wilderer 1989). Biofilms are extremely complex in nature and little is known about the extracellular polymers that make up the biofilm. The polymers will appear as a highly hydrated capsule attached to the cell or as a viscous soluble slime. The physical properties of these polymers are integral in the further understanding of biofilms as they contribute directly to the physical and physiological behaviour of biofilms. For this investigation it is important to look at these polymers as they play a part during the initial interaction between a bacterial cell and the substratum. Figure 1 below shows that attachment of bacteria to a carbon surface. It can be clearly observed that the bacteria are attached to one another by a form of extracellular polymer. This EPS, which resembles “slime”, links the bacteria and forms part of the biofilm. Figure 1.2 shows a biofilm seen under an SEM, what is interesting is that the biofilm is actually predominantly composed of the EPS rather than cells (Donlan 2001).

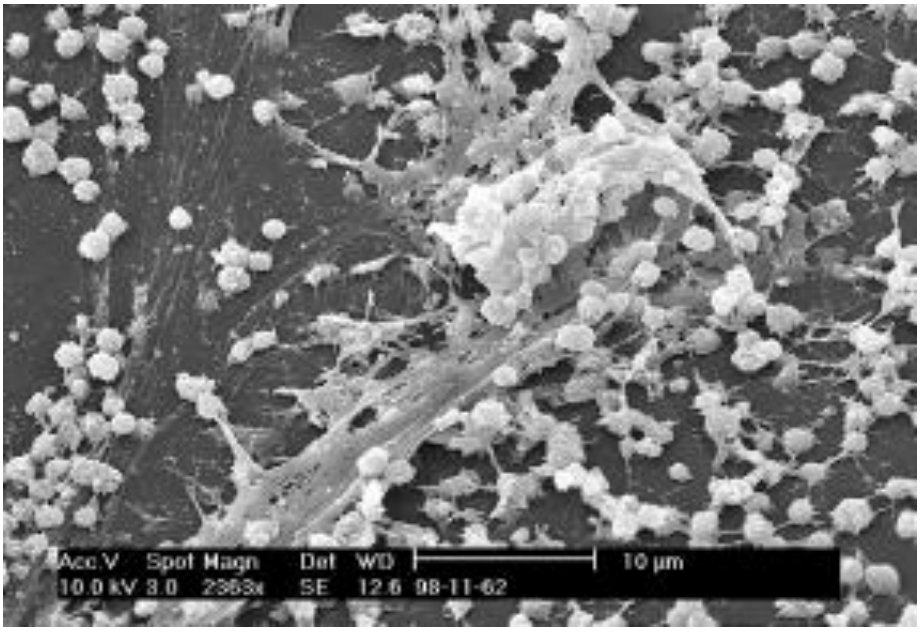


Figure 1.2: Scanning electron micrograph of a Staphyloccus biofilm on the inner surface of a needless connector.

It is therefore very important in the understanding of biofilms that these polymers are studied and understood. The integrity of a biofilm can be largely attributed to these polymers. The interest in these polymers has been quite lax overall, yet Mikkelsen et al. (1985), successfully studied the physical properties of some biopolymers. Theoretical studies (Marshall and Cruickshank, 1973) and electron microscopy studies suggest that the polymers associated with bacteria assist with the binding of bacterial cells to the surface of the substrate.

1.4.3.1 The Microbial Cell

In this section an in depth analysis of the microbial group and its function in a biofilm are explored. The adhesive nature of bacteria is primarily due to the features found on the outer membrane of the cell. These include pili, flagella, proteins and lipopolysaccharides (LPS's) (Characklis, Wilderer 1989).

1.4.3.2 Interfacial Transfer

The adsorption of bacterial cells onto the substratum is defined as an interfacial transfer process (Characklis, Wilderer 1989). Interfacial transfer differs from transport as it describes the transfer of material between the compartments whereas the latter describes the transport of material within compartments. This is true as the bacteria leave the bulk liquid (compartment 1) and attach to the substratum (compartment 2). This type of transport suggests the existence of a two stage adsorption process, reversible and irreversible adsorption.

1.4.3.3 Transport of Microbial Cells

When a clean substratum is immersed in a medium containing microbes, the initial attachment of cells on the surface is controlled by the transport of the cells from the bulk medium to the surface. The mass transport is influenced strongly by the mixing in the bulk fluid. The correct agitation will ensure optimal initial attachment as mass transport is largely influenced by mixing (Characklis, Wilderer 1989).

Reversible Adsorption

Reversible adsorption describes the very weak interaction forces between the cells and the substratum, such that the cell often exhibits Brownian motion. This adsorption involves the long range weak London Van-der Waals forces. Irreversible adsorption is indicative of a more permanent

bonding of the cells to the substratum. Irreversible adsorption is characterised by a high heat of adsorption and a one way chemical bond. The typical types of bonding that occur are namely the very strong interaction forms of dipole-dipole, dipole induced dipole, ion dipole interactions, hydrogen bonds and hydrophobic interactions. There are generally two theories that explain the reversible adsorption theory: the DLVO theory and the "wettability" theory (Characklis, Wilderer 1989).

DLVO (Double Layer) Theory

This theory involves both the electrostatic forces and the London-van der Waals forces, where the following equation describes the interaction.

$$E_{Interaction}=E_{LVDW}+E_{Electrostatic}$$

The theory suggests that the interaction takes place at two possible positions; the primary minimum (at small separations) and the secondary minimum (at larger separations). At some point between these two minima repulsive forces are at a maximum. The DLVO theory was developed for abiotic (non-living) colloidal particles which differ from viable microbial cells in that the latter have continuous energy flux through their boundaries, resulting in a pH gradient and proton motive force across the cell wall. The DLVO theory requires substantial modifications in order to clearly describe the attachment. It has been shown to predict adsorption well that occurs at long to medium ranges. It is unlikely that cells are able to approach a substratum closely enough to overcome the maximum repulsive forces found in the middle of the primary and secondary minimum (G. Muyzer, EC. De Waal, 1993).

1.4.3.4 Electromagnetic Effects

Lim et al. (2008) experimented with mixed populations and observed some interesting biofilm phenomena that have biofilm phenomena. The substrate flux to the biofilm prior to any substrate transient was on the order 2.2 x 105lactate molecules per cell per second. The substrate flux is coupled to a proton flux. Bacterial cell surface molecules contain a variety of functional groups; they can exhibit both repulsive and attractive interactions with the substrate. These electromagnetic forces will mediate bacterial adhesion; the adsorption of bacteria on to the surface of a substrate has often been described as the balance between the attractive and repulsive physiochemical properties of both the cell and the substrate.

Electrostatic Interactions

The electrostatic interactions are of interest in this investigation. Electrostatic potential can be measured by electrokinetic models such as electrophoresis and zeta potential. Electrostatic forces are due to charged groups found on the surface of the bacteria such as phosphates, lipopolysaccharides or carboxyls (Characklis, Wilderer 1989).

1.5 ELECTROSTATIC INVESTIGATION

As has been researched in the previous sections it is important to look at the electrostatic interactions that govern attachment. The interactions between the bacterial cell wall and surfaces (including other cell walls) are primarily influenced by interfacial electrostatic (e.g., repulsion, attraction) and van der Waals forces (Renner 2011). Most bacterial genera have a net negative charge as determined by zeta-potential measurements. Bacteria attach rapidly and tightly to positively charged surfaces, and electrostatic repulsion destabilizes cell contact with negatively charged surfaces (Renner 2011).

Busscher et al. (2006) modified the surface charge of a wood-based activated carbon by coating its surface with polyvinyl amine (PVAM). The coated carbon was shown to have 10% absorbed nitrogen at its surface, which was a result of the ammonium groups in PVAM and therefore yielded a positive surface charge. They were able to establish that the highest adsorption was achieved by the positively charged carbon and the most negatively charged bacteria.

Most of the research has dealt with the manipulation of the point of zero charge pH_{PZC}. The pH_{PZC} describes the condition when the electrical charge density on a surface is zero (Menéndez et al. 2005). The pH_{PZC} on an activated carbon depends on the chemical and electronic properties of the functional groups on its surface Song et al. (2010).

Moreno-Castilla et al. (2003) made use of this concept when they went about immobilizing Escherichiae coli (E.coli) onto activated carbon. E.coli is negatively charged with a pH_{PZC} value of 3. Bacteria are known to grow in a solution of pH 7, hence with a pH_{PZC} of 3 a base solution is added resulting in the surface area becoming more negative. While activated carbon was determined to have a pH_{PZC} of 8.6, when its pH was reduced to 7 it resulted in the surface area becoming more positive. They concluded that the interaction between the positively charged carbon and the

negatively charged bacteria would enhance the adsorption due to this electrostatic attractiveness (Moreno-Castilla et al., 2003).

According to Quintelas et al. (2010) the pH_{PZC} of activated carbon is described as the value at which surfaces carry no net electrical charges. The surfaces are positive below the pH_{PZC} and negative above pH_{PZC}.

According to Bolster et al. (2001) who performed experiments to determine the effect surface coatings, grain size and ionic strength have on the attachment of bacteria to sand surfaces, a positive surface will increase the attachment. When the surface is coated with metal-oxyhydroxide in circumneutral pH solutions the surface becomes positive. The electrostatic attraction substantially increases and the maximum amount of bacteria attached.

1.6 BACTERIA

1.6.1 Bacteria General

Understanding the growth of bacteria was integral for this study. When preparing the bacteria and experimenting with attachment it is important to understand the bacteria in terms of growth and physicality.

Gram-negative and Gram-positive bacteria are two types of bacteria which can be distinguished according to their cell wall structure. In Gram-negative bacteria the peptidoglycan layer is around 2 to 6 nm, whereas Gram-positive bacteria present a thicker peptidoglycan layer around 20 to 80nm (Lebleu, Roques et al. 2009). Microbial growth can be described by four phases. When a fresh medium is inoculated there will be an initial period of slow or no growth followed by rapid growth, then a levelling off and finally a decline in the viable population. These four phases have been labelled as the lag phase; the logarithmic phase; the stationary phase; the phase of decline or death (Characklis, Wilderer 1989).

1.6.1.1 The Lag Phase

Initially when the fresh medium is inoculated, the bacterium does not follow a doubling growth rate. In actual fact the population remains unchanged for the duration of this phase. The cells are not dormant during this period, they are in fact extremely active as they expand and begin to synthesize new cellular constituents. In this period the cell are adapting and adjusting to their new

environment, and are actively metabolizing. As a result of this there will be a lag in cell division, hence the name "lag" phase. At the end of the lag phase cell division commences (Characklis, Wilderer 1989).

1.6.1.2 The Logarithmic Phase

During this period the cells divide steadily over time. Their rate of division will be directly controlled by their physiological capabilities in the growth medium they inhabit. The growth of cells during this period is exponential; plotting the logarithm of the number of cells versus time will result in a straight line. The growth rate is maximised during this period (Characklis, Wilderer 1989).

1.6.1.3 The Stationary Phase

The logarithmic phase will begin to subside after a couple of hours. The growth rate therefore stops increasing. In Figure 1 the stationary phase shows a straight line with a zero gradient. The trend towards the completion of growth can be attributed to a number of factors namely the exhaustion of nutrients that sustain growth or the production of toxics that inhibit growth. The population tends to remain constant for this period which is either the result of cessation of growth or the balancing between the production rate of new cells and the equivalent death rate of old cells (Characklis, Wilderer 1989).

1.6.1.4 The Death Phase

Following the stationary phase, the bacteria exhibit a death phase. This is as a result of the bacterium depleting as a result of many factors. Generally the bacteria tend to die as a result of depleting nutrients within the medium. The death phase occurs when the death rate of cells exceeds the generation rate of cells (Characklis, Wilderer 1989).

The progression of cell numbers in batch experiment also known as "bacterial growth curve"

1.7 TERMITE BACTERIA

The termite is associated with cellulose digestion as they thrive on lignocellulolytic materials such as bark, wood and plant material. Termites are often regarded as pests yet in tropical and subtropical regions termites play a significant role in the degradation of organic matter and digestion of cellulose containing materials (Kuhnigk, Borst et al. 1994). Termites and their complex hindgut

microbiota are able to convert wood lignocellulose into hydrogen and other products used to fuel their metabolisms. Termites will eat wood fibre and the protozoans found in the hindgut will degrade the cellulose into sugars such as glucose. These sugars get supplied to the bacteria. The bacteria will fix atmospheric nitrogen and synthesize amino acids and vitamins whilst using the sugars supplied as a source of energy (Kuhnigk, Borst et al. 1994).

In the past attention was focused mainly on the protozoa as they were considered the main digestion mechanism of cellulose yet gradually there has been a progressive interest in trying to elucidate the role of bacteria within the hindgut of the termite (Ramin et al., 2009)(Schultz, Breznak 1978).

The cellulases, produced by termites, are the primary contributor to cellulose digestion. As was mentioned previously since there was inadequate understanding of the bacteria within the termite hindgut, it was assumed that the intestinal flagellates were responsible for the production of the cellulases. It has been shown that almost 75% of termites do not contain cellulolytic flagellates (Tokuda, Watanabe 2007). According to Tokuda and Watanabe (2007) when treating the termite with antibiotics the cellulase activity was significantly reduced in the hindgut as compared with the normal functioning of the hindgut. This observation gave quite a clear implication of the presence of bacteria and its involvement in the production of cellulase.

Brune, Friedrich (2000) determined that the degradation of lignocelluloses proceeds in two stages. The first stage consists of hydrolysis of the cellulose while the second stage involves the fermentation of the depolymerisation products into short chain fatty acids.

The lack of information about the bacteria in the hindgut was the main objective of Ramin et al (2009), who attempted to isolate and identify cellulolytic bacteria. They were able to isolate various bacterium within the gut. Some were shown to be able to take part in the first stage of cellulose degradation and others in the second stage. In this study five species of cellulolytic bacteria were isolated from the gut of the lower termite and identified as B. cereus Razmin A, E. cloacae Razmin C, E. aerogenes Razmin B, C. Kwangyangense Cb and Acinetobacter Raminalimon. Each strain of bacteria was shown to take part in either the first or second stage of lignocelluloses degradation. (Ramin et al 2009).

According to Ohkuma, head of the Japan Collection of Microorganisms RIKEN BioResource Center; "Humans are using only a part of the capabilities of microorganisms," continues Ohkuma. "Currently, only single microorganisms and some enzymes produced by microorganisms are being used in practice. Although combinations of two or more microorganisms are used for some purposes, including wastewater purification, the mechanisms involved in their interactions remain unknown. Like the gut microorganisms in termites, microorganisms with a wide range of different capabilities gather and interact with each other everywhere in nature, and build symbiotic systems of extremely high efficiency. If we can understand the mechanisms involved, we might be able to make better use of the excellent capabilities of microorganisms."

Symbiotic mechanism in gut microorganisms in the termite intestine

Fractions of wood eaten by termites are taken by protozoans, which decompose the cellulose component of the wood into sugar (glucose) and supply it to bacteria. The bacteria fix atmospheric nitrogen and synthesize amino acids and vitamins using the sugar as a source of energy, and supply the products to the termites and protozoans. The bacteria also produce acetic acid and hydrogen from the sugar; the protozoans produce acetic acid, hydrogen and carbon dioxide from the sugar. Other bacteria in the termite gut produce acetic acid from the hydrogen and carbon dioxide. The acetic acid thus produced serves as a source of energy for the termites

1.7.2 Molecular Study of Termite Bacteria

There is still a very minimal understanding of the microbiota within the hindgut of the termite and the process of lignocelluloses degradation (Warnecke, Luginbühl et al. 2007). The hindgut of lower termites has been shown to contain a microbiota of both protozoa and bacteria (Schultz and Breznak, 1978). The protozoa inhabiting the gut of a termite and their role in termite nutrition have been well described, however very little is known about the bacterial gut flora in termites. The lack of knowledge is a direct result of the difficulty in culturing bacteria from natural communities. It is important to note that only 1-10% of global bacterial species are actually artificially culturable. This is due to the selectivity of growth media and conditions making culture dependent ecological microbiological techniques increasingly outdated. Therefore the preferred choice for monitoring changes in microbial communities should be DNA/genome based.

In recent years differentiation between microorganisms, without their cultured isolation, can be performed with several molecular techniques. Polymerase chain reaction (PCR) based techniques

are becoming increasingly popular for ecological research ranging from diagnostic work to genome fingerprinting and probing. This is a revolutionary method that was founded by Kary Mullis in the 1980s. PCR is based on a series of thermal cycling steps that allow exponential amplification of a target portion of the genomic DNA. The 16S rDNA sequence is a gene encoding small subunit ribosomal RNA. This gene contains conserved sequences of DNA common to all bacteria and divergent sequences unique to each species of bacteria (Universal Bacteria Fact Sheet).

PCR uses the ability of DNA polymerase to synthesize new strands of DNA complementary to the template strand. Once the DNA has been amplified, the PCR product is then stained with eithidium bromide and visualized by electrophoresis on an agarose gel. If bacterial DNA is identified, it can then be directly sequenced to speciate the bacteria (Universal Bacteria Fact Sheet). Gene sequences for various bacteria are known and as such the amplified sequence can then be compared to these templates for bacterial strain identification (Universal Bacteria Fact Sheet). This technique is usually applied to assay environmental samples due to its ability to detect relatively small numbers of specific species of microorganisms without requiring cell culture.

The determination of bacterial species is performed in the following manner; PCR product is electrophoresed through a polyacrylamide gel containing a linear DNA-denaturing gradient. The resulting band pattern on the gel forms a genetic "fingerprint" of the entire community being examined shown in Figure 1.3. The 16S rRNA is the most common gene used to give an overall indication of the species composition of a sample since they can easily be compared with others from a gene database (Universal Bacteria Fact Sheet).

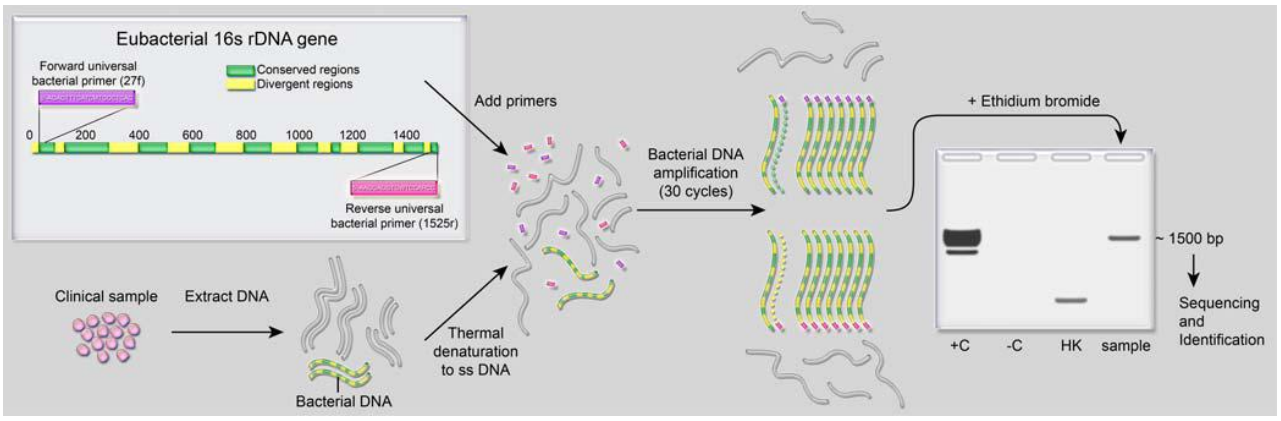


Figure 1.3: The 16S rDNA PCR Assay (Universal Bacteria Fact Sheet)

As a result in this study we will be working with a consortium of bacteria isolated from the termite hindgut. The consortium will contain all bacteria required for the two stages needed in lignocelluloses degradation.

Chapter 2

EXPERIMETAL AND METHODOLOGY

2 EXPERIMENTAL AND METHODOLOGY

The following chapter outlines the experiments that were performed in an attempt to optimize and speed up microbial attachment to activated carbon surface so as to shorten the start-up time required in a Fluidized Bed Biofilm Reactor (FBBR). This study was divided into the following sections;

- Identify and determination the bacteria found in the hindgut of local termites.
- Control experiments of microbial attachment to carbon substrate (These experiments were performed prior to any carbon charge modifications, in order to act as a control and reference of comparison).
- Surface modifications to optimise and increase attachment.
- Time dependent experimentation for biofilm creation. Using the optimised procedure for attachment see how long biofilm creation will take.

2.1 IDENTIFICATION OF BACTERIA FOUND IN A LOCAL TERMITE HINDGUT

2.1.1 Introduction

The hindgut of lower termites has been shown to contain a microbiota of both protozoa and bacteria. However, because most of these microorganisms are not yet cultured, their identities and nature cannot be examined in detail. For this reason, the symbiotic mechanisms between termites and microorganisms still remain unclear (Ohkuma, 2009).

Prior to the attachment investigation it was important to attempt to understand the bacteria consortium within the termite gut. Termites collected from a termite mound found in Kyalami, North of Johannesburg were sent to the University of Pretoria for a Microbial Molecular Study. The termite gut was isolated and the bacteria processed, allowed for DNA sequencing. DNA sequencing allowed for the identification of bacteria found within the termite's hindgut. With this information it is then possible to further the understanding of cellulose degradation within the termite gut (Surridge 2011).

2.1.2 Materials and Methodology for the Investigation of bacteria found within the Termite Gut

2.1.2.1 Sample Collection

Live termites were maintained until DNA could be extracted within 24 hours. They were sterilised in 10% sodium hypochlorite solution for 5 minutes and then in 70% ethanol for 5 minutes prior to DNA extraction.

2.1.2.2 DNA Extraction

Total DNA extraction was performed at the Department of Plant Production and Soil Science, University of Pretoria, South Africa (Surridge 2011). This was achieved using the Nucleospin soil DNA extraction kit. The DNA concentration was determined and samples were kept at -20°C for further analysis.

2.1.2.3 16S rDNA amplification by PCR

A portion of bacterial gene 16S ribosomal DNA was amplified, for Denaturing gradient gel electrophoresis (DGGE), by means of PCR using the K and M target Primes set;

K: 5'ATT-ACC-GCG-GCT-GCT-GG3'

M: 5'CGC-CCG-CCG-CGC-GCG-GCG-GGC-GGG-GCG-GGG-GCA-CGG-GGG-GAG-AGT-TTG-ATC-CTG-GCT-CAG3'

Each PCR tube contained a total volume of 20 µL 10.8 µl sterile nuclease-free water, 7 µl GoTaq PCR ready mix (Whitehead scientific), 1 µl primer K (50 µm), 1 µl primer M (50 µm), 1 µl template DNA (27 ng/µl). The DNA amplification was performed in a PCR thermal cycler using the following programme: 10 min at 95°C, 35 cycles of 30 sec at 94°C, 30 sec at 58°C and 1 min at 72°C, and then held at 4°C. PCR product was analysed on a 1.5% TAE agarose gel.

2.1.2.4 Partial ITS gene PCR

A screening of eukaryotic diversity was carried out on a portion of the internal transcribed spacer (ITS) gene sequence of the DNA from each sample and subjected to PCR using the primer set:

ITS3: 5'CGC-CCG-CCG-CGC-GCG-GCG-GGC-GGG-GCG-GGG-GCA-CGG-GGG-GCA-TCG-ATG-AAG-AAC-GCA-GC3'

ITS4: 5'TAC-TTG-AAG-GAA-CCC-TTA-CC3'

A reaction with no template DNA was included as a negative control. Each PCR tube contained a total volume of 20 µl- 7.5 µL sterile milliQ water, 10 µl Promega Green PCR Readymix®, 1 µl primer K (10 ρm), 1 µl primer M (10 ρm), 1 µl template DNA (ca. 27 ng/µl). DNA amplification was performed in a PCR thermal cycler using the following programme: 1 min at 92°C, 30 cycles of 1 min at 92°C, 1 min at 55°C and 1 min at 72°C, followed by 5 min at 72°C, and then held at 4°C. PCR products were analysed on a 1.5% TAE agarose gel.

2.1.2.5 Denaturing Gradient Gel electrophoresis (DGGE)

PCR product was subjected to DGGE according to the method described by G. Muyzer, EC. De Waal (1993). Ten microlites (ca. 250 ng) of 16S rDNA PCR product was loaded per lane onto 40-60% denaturing gradient gels. Gels were run at 70 V for 17 h at a constant temperature of 60°C.

2.1.2.6 DGGE band sequencing

Sequencing the DGGE bands from the gel using the K and M primers above, provided tentative species identification. These bands were out sourced to Inqaba Biotec (Pretoria, South Africa) for upPCR (re-amplification) and sequencing. Each sequence was subjected to a Basic Local Alignment Search Tool (BLAST) analysis on the GenBank database and matching hits were selected for alignment. Sequences were aligned using Clustal X (Lim et al., 2008) and inserted gaps were treated as missing data. Ambiguously aligned regions were excluded from the data set before analysis.

Phylogenetic analysis was based on parsimony using PAUP 4.0b8 (Phylogenetic Analysis Using Parsimony). Heuristic searches were done with random addition of sequences (1000 replicates), tree bisection-reconnection (TBR), branch swapping, MULPAR-effective and MaxTrees set to auto-increase. Phylogenetic signal in the data sets was assessed by evaluating tree length distributions over 100 randomly generated trees. The consistency (CI) and retention (RI) indecis were determined for all data sets. Phylogenetic trees of sequences were rooted with *E.coli* as out group to the remaining taxa. Bootsrap analyses were conducted, retaining groups with 70% consistency, to determine confidence in branching points (1000 replicate) for the most parsimonious trees generated.

2.2 MICROBIAL ATTACHMENT TO CARBON SUBSTRATE

2.2.1 Introduction

The primary aim of this research is to establish the main aspects that determine the adsorption of termite bacteria (consortium) onto activated carbon. With this we are attempting to increase attachment of bacteria on activated carbon in a short period of time. Research has indicated that modification of the surface charge on activated carbon to that opposite of the bacteria can prove to increase the electrostatic attraction and hence improve the adsorption potential. Batch experiments were performed to establish whether this hypothesis proved to be successful. The focus was to increase attachment and minimise time of attachment. The following section outlines the procedures taken to investigate the attachment of bacteria to the surface of activated carbon.

2.2.2 Experimental and Procedure

2.2.2.1 Determination of pH_{PZC} of activated carbon

Determination of the pH_{PZC} of activated carbon was performed as follows. Four sealed conical flasks were prepared with 50 ml of 0.01 mol/l NaCl solution. The pH_{PZC} of each flask was then adjusted to values of 2.7, 6.0, 8.0 and 10.7 by using 0.1 mol/l of HCl (To increase the acidic characteristic by addition of H^+) and NaOH (To increase basic characteristic by addition of OH^-). Once the four flasks were successfully prepared 0.15 g of activated carbon was added to each of the flasks. The flasks were subsequently tightly sealed to avoid the adverse effects of CO_2 contamination. The flasks were then placed in an incubator at 25°C where they were shaken at 100 rpm for 48 h. After removing the flasks, the pH of the activated carbon were measured (pH_{FINAL}.). A straight line was then plotted of $pH_{INITIAL}$ vs. pH_{FINAL}. The point at which this line crossed the line $pH_{INITIAL} = pH_{FINAL}$ is then taken as your pH_{PZC}.

2.2.2.2 Bacteria Preparation

Prior to bacteria preparation, all equipment was sterilized in an autoclave for 20 min at 120°C. This included 4 100 ml flasks, 1 large conical flask, mortar and pestle, tweezers, spatula and 100 ml distilled water. The fume hood was then wiped down with 70% ethanol, to eliminate any

contamination, and the procedures that followed were done alongside a flame, to ensure a complete sterile working environment. Bacterial growth media (200 ml) was prepared by adding yeast (0.4 g) to distilled water (160 ml) followed by stock solution (40 ml) (Appendix A). The 200 ml solution was split equally into the 4 flasks. At this stage the inoculation was being prepared. Worker termites (15), identified as Trinervitermes, were individually dipped into ethanol and then distilled water for 20s each. The termites were then placed in the pestle and mortar and homogenized to extract midgut bacteria as well as bacteria that may contaminate the remainder of the termite. The homogenate mixture was then added to 3 of the flasks containing growth media. The fourth flask was not inoculated so as to act as a control for bacteria growth determination. The flasks were then placed in the incubator at 30°C with shaking at 100 rpm until bacterial growth became dense.

2.2.2.3 Modification of activated carbon surface charge

Ion exchange testing on surface of activated carbon

In order to test and ensure ion exchange on the surface of the activated carbon the following was performed. Three 100 ml flasks containing 50 ml sterilised distilled water were altered to different pH's. The pH were altered to a pH of 4.5, 6.0 and 7.0 using 0.1 mol/l Nitric Acid to obtain positive charge (H^+) and 0.1 mol/l Sodium Hydroxide to increase negative charge (OH^-). Activated carbon (0.6 g) was submerged in each of the flasks and left for ten minutes. The pH of the flasks was then measured to observe the changes in ion content of the water.

2.2.2.4 Bacteria were immobilized onto the surface of activated carbon

Control: No charge manipulation

It is important that a control experiment was performed. This is done to determine if the effects of charge modification. For this experiment 0.6 g of activated carbon was placed in 20 ml bacterial solution. The solution was placed in the incubator at 30°C with shaking at 100 rpm and left for 4 days. Activated carbon samples were removed for Scanning Electron Microscope (SEM: ESEM FEI QUANTA) viewing.

Experiment 1: (Charge modification of bacterial surface adapted from Busscher et al. 2006)

The first experiment performed was adopted from Busscher et al. (2006). In this experiment, bacteria were grown as explained in Section 2.2.2.2. The bacteria were centrifuged at 10,000 rpm for 20 min and collected at the bottom of the centrifuge tube. After centrifugation the solid bacteria was washed with 3x distilled water solutions that had been modified to pH's of 4.5, 6 and 7. The pH of these sterile water solutions were altered using 0.1 mol/l Nitric Acid and 0.1 mol/l Sodium Hydroxide. The bacteria were washed adequately to ensure the charge modification. The aliquots of bacteria were then poured into the remaining three solutions (each 20 ml) respectively. Activated carbon (0.6 g) was then placed into each flask containing bacteria. These flasks were placed in an incubator (Shaking at 100 rpm) at 30°C and bacteria attachment was allowed to occur over 4 days and then viewed under SEM.

Experiment 2: (Charge modification after bacteria inoculation)

An additional experiment was used to try increase the attachment of bacteria to the surface of activated carbon. Activated carbon (0.6 g) was added to 20 ml of bacterial culture solution once the bacteria had grown. In this experiment, only after the activated carbon had been added to the bacterial solution was charge modification performed. The bacterial solutions containing activated carbon were adjusted to 3 different pH values (5, 6 and 7). The pH of these activated carbon and bacteria solutions were altered using 0.1 mol/l Nitric Acid and 0.1 mol/l Sodium Hydroxide. The 3 flasks were placed in an incubator (Shaking at 100 rpm) at 30°C and bacteria attachment was allowed to occur over 4 days and then viewed under SEM.

Experiment 3: (Charge modification of activated carbon only)

The final experiment focused on charging the activated carbons surface before bringing it into contact with the bacteria. In order to manipulate the surface charge on the surface of the activated carbon the following procedure was employed. Sterilized water was altered to the desired pH of the activated carbon. The pH was altered using 0.1 mol/l Nitric Acid and 0.1 mol/l Sodium Hydroxide. For this experiment 3x pH values were evaluated at pH 4.5, 6 and 7. Activated carbon (0.6 g for each pH) was washed in the modified sterile water for 20 min to ensure the alteration of the surface charge. Upon the completion of charge modification 20 ml of bacteria culture solution (pH 7.23) was added to the conical flasks each containing charged activated carbon. The 3 flasks were placed in an

incubator (Shaking at 100 rpm) at 30°C and bacteria attachment was allowed to occur over 4 days and then viewed under SEM.

2.3 Time Driven Experiments for Biofilm Creation

2.3.1 Introduction

A procedure was outlined and proved to show the best attachment for charged activated carbon at a pH of 5, 6 and 7. These pH values were taken as they were below the pH_{PZC}. It was decided to investigate how attachment is affected if the activated carbon is charged to its pH_{PZC}. In addition to this, the experiments would be run for different time intervals. This would allow for the determination of the rate of attachment over time.

Experiments were performed again at three different pH values of 4.0, 7.2 and 9.6. A. This experiment looked at a larger spectrum of pH's which included the activated carbons pH_{PZC} of 9.6. Here it was crucial to see how attachment would be affected when the activated carbon was charged to its pH_{PZC}.

For a greater understanding of the attachment over time activated carbon samples were taken at 24 h and after 13 days. These samples were then viewed under SEM.

2.3.2 Experimentation

Three flasks of sterilised water were charged to a pH of 4.00, 7.21 and 9.6 using 0.1 mol/l Sodium Hydroxide to increase alkalinity and 0.1 mol/l Nitric Acid to increase acidity. Activated carbon (0.6 g for each pH) was washed in the modified sterile water for 20 min to ensure the alteration of the surface charge. Upon the completion of charge modification 20 ml of bacteria culture solution (pH 7.23) was added to the conical flasks each containing charged activated carbon. The 3 flasks were placed in an incubator (Shaking at 100 rpm) at 30°C and bacteria attachment was allowed to occur. Activated carbon samples were removed from each flask after 24 h and 13 days of incubation.

2.4 ESEM (Environmental Scanning Electron Microscope) Sample Preparation

In order to view the attachment of bacteria to the surface of the activated carbon samples of activated carbon were prepared for ESEM. Four granules were removed from each and rinsed with sterile distilled water. The granules were first fixed in 1 ml of 3% aqueous glutaraldehyde at room

temperature overnight. After fixation, they were rinsed with sterile distilled water once and dehydrated in graded ethanol series (20, 30, 40, 50, 60, 70, 80, 90. 95 and 100%) at 10 min intervals. They were then subjected to critical-point drying, mounted on ESEM stubs and finally coated with normal gold/palladium for viewing on an ESEM FEI QUANTA.

Over ten pictures of each sample were taken, in order to capture enough data for an estimation of the attached bacteria to be made. Images were taken at various magnifications for comparative investigations.

2.5 QUANTIFICATION METHODS OF ATTACHMENT

2.5.1 Qualitative analysis

In order to view the attachment of bacteria to the surface of the activated carbon samples of activated carbon were prepared for SEM (Scanning Electron Microscope) as described in Section 2.4.

Over ten pictures of each sample were taken, in order to capture enough data for an estimation of the attached bacteria to be made. Images were taken at various magnifications for comparative investigations. The bacteria on the surface of these images were manually counted. The values obtained were extrapolated to represent the attachment on an area of 1 mm.

Chapter 3

RESULTS

3 RESULTS

In the following section all the results obtained from experimentation have been presented (Surridge 2011), as indicated in DNA Extraction. These results were presented in the report from Surridge (2011).

3.1 BACTERIAL DNA ANALYSIS

There is still a very minimal understanding of the microbiota within the hindgut of the termite and the process of lignocelluloses degradation (Warnecke et al., 2007). It has been observed that biofuels manufactured from cereal crops are set to be a future source of energy, however this would immediately cause the inflation of food prices which is detrimental to rural communities around the world. It has thus been proposed that focus be reverted to another reliable and abundant source, cellulose. Cellulose can potentially be a great source of energy provided it can be broken down into its valuable sugars. As a result investigations are under way all over the world to produce biofuels by degrading the cellulose contained in plant waste, such as wood chips, thinned wood and rice straw. However, the technology currently available for this purpose consumes a great deal of energy.

By turning to nature and natural bio-systems, one organism in particular has shown to obtain nutrients by degrading cellulose with extremely high efficiency: termites. These insects exhibit astonishingly high reproduction rates while feeding on wood or soil alone. Termites are capable of efficiently degrading cellulose into its sugars which can then be used to manufacture biofuels and hydrogen.

The key to this efficiency in the production of nutrients is the complex system of a hundred kinds of symbiotic microorganisms living in the termite gut. The following section outlines the results of the DNA testing performed on the termite gut.

3.1.1 DNA extraction

Genomic Bacterial DNA was extracted successfully as shown in Figure 3.1. DNA fragment was analysed on a 1.5% TAE Agarose gel shown below in Figure 3.1 .



Figure 3.1: 1.5% TAE agarose gel showing high-quality, clean genomic DNA extracted from the sample (size marker on the left) KEY: M- Molecular Marker, 1- Termite DNA

The sample was done at the University of Pretoria (Surridge 2011) and the marker sizes were not included in the final report presented.

3.1.2 PCR

PCR of prokaryotes was successful at yielding a ca. 510 base pair (bp) PCR product on a 1.5% TAE agarose gel shown below.

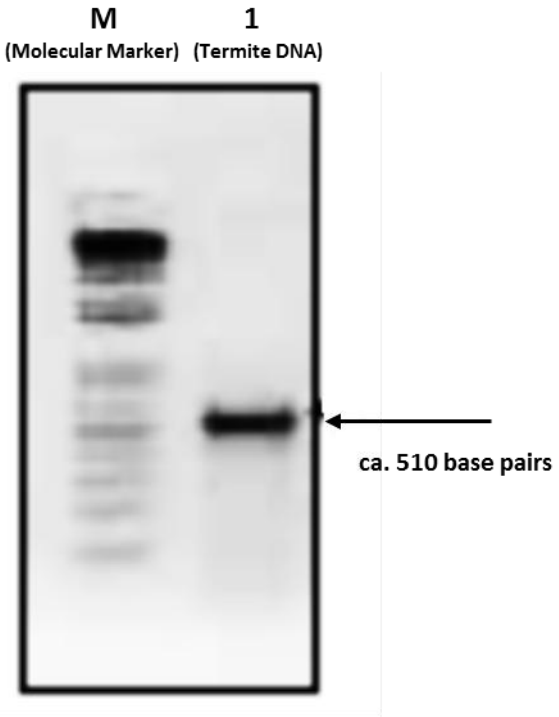


Figure 3.2: 1.5% TAE Agarose gel, 1-showing bacterial (partial 16S) PCR product from 16S bacterial gene amplification. KEY: M- Molecular Marker, 1- Termite DNA PCR Fragments

The sample was done at the University of Pretoria (Surridge 2011) and the marker sizes were not included in the final report presented.

3.1.3 DGGE

PCR fragment was loaded onto a DGGE gel. Multiple bands were produced that formed a fingerprint in the lane Figure 3.3. The DGGE gel shows fingerprints of the bacterial community within every sample. Each “band” within the fingerprint represents a unique bacterial species and can be identified by DNA sequencing. PCR product is separated according to base pair sequence differences to determine community richness and diversity of microorganisms based on these fingerprints.

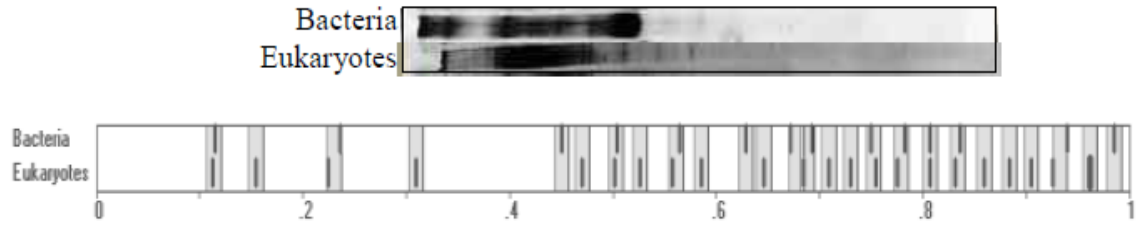


Figure 3.3: DGGE gel showing species diversity of bacteria and eukaryotes from termite samples, run at 40-60% denaturants. PCR product is separated according to base-pair sequence differences to determine community richness and diversity of microorganisms based on these fingerprints. (Dominant bands: bacteria 14, eukaryotes 22). The top strand is the actual DGGE band and the one below is a representative of the actual.

3.1.4 DNA sequencing

The final step in this study entailed tentative identification of bacterial species excised from the DGGE gel. These bands were sent to Inqaba Biotec (Surridge 2011). for reamplification and sequencing of the partial 16S ribosomal gene region. The table below shows the tentative identification of the bands cut from the DGGE gel. Each band shows the percentage similarity of the sequenced partial 16S gene to those in the GenBank BLAST database. This gives a tentative identification of the microorganisms present in the sample.

3.2 MICROBIAL ANALYSIS

A parsimony based phylogram was drawn from the sequence alignments attained after BLAST and sequence results were compared. The phylogram is a representation of how closely related the organisms are to each other seen in Figure 3.4. This illustrates that the individuals within each group/clade show closest similarity to the organisms identified in that clade. These clades are supported by “bootstrap values”, which are essentially statistical calculations of the amount of support each branch forming a clade has. For example the red clade shows that there is a 100% support that DGGE bands 26 and 27 are very closely related to *Rhodobacter* sp; The yellow clade shows 100% support for DGGE bands 119 and 120 being in the genus *Geobacillus*; The blue clade shows that there is a 71% support for DGGE band 159 being *Ralstonia* sp.

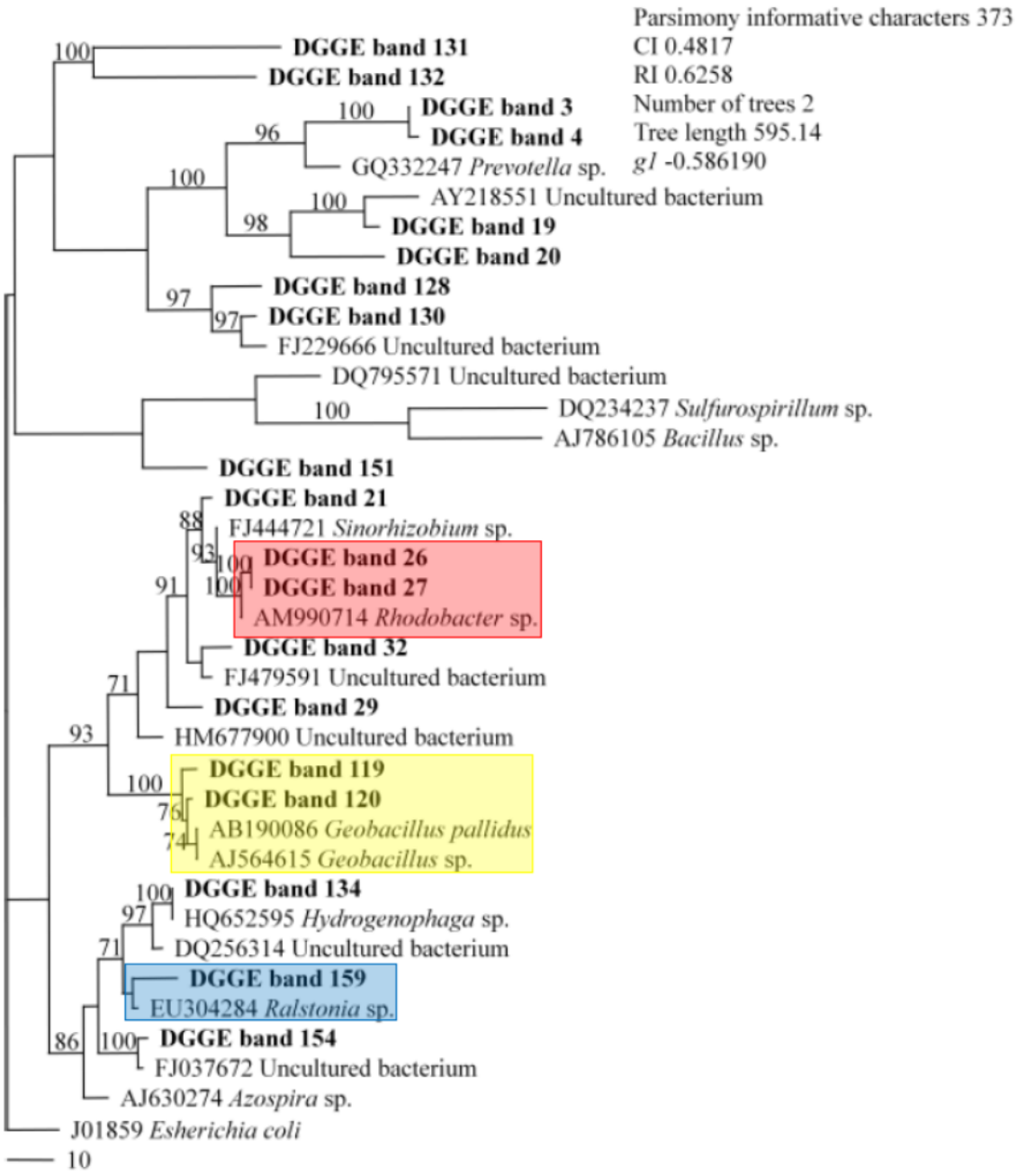
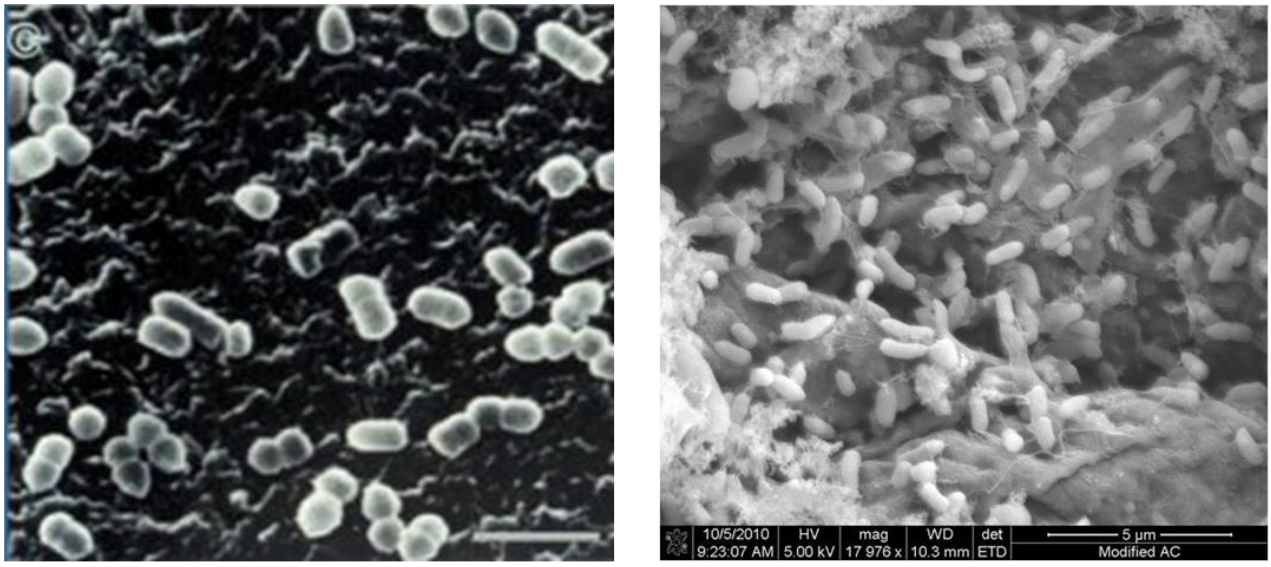


Figure 3.4: Phylogram of DGGE bands sequenced for tentative identification of bacteria found in termites

In Figure 3.5 we see a typical *Prevotella* sp. Bacterias seen under SEM (left). The picture alongside is one of the photographs taken under SEM during this investigation. The rod shapes of the bacteria are common in both.



A. Prevotella sp. Bacterium

B. Termite bacterium under SEM found attached to activated carbon after inoculation

Figure 3.5: Comparison of rod shaped bacteria (Prevotella sp.) with bacterium from the termite hindgut

Table 3.1 below presents the bacterial identification of each of the DGGE bands isolated. (APPENDIX B details the entire DNA analysis of the termite bacteria)

Table 3.1: Tentative identification and associated literature of each DGGE band sequenced according to BLAST results from NCBI GenBank database

Seq. no.	Accession number	% match	Associated Literature
3	GQ332247 Uncultured *Prevotella* sp.	82	(Adolphe et al.)
4	GQ332247 Uncultured *Prevotella* sp.	81	(Adolphe et al.)
19	AY218551 Uncultured bacterium	91	(Zhang et al.)
20	AJ630274 Uncultured *Azospira* sp.	73	(Kim et al. 2006)
21	FJ444721 Uncultured *Sinorhizobium* sp.	93	(Zhang et al. 2011)
26	AM990714 *Rhodobacter* sp.	96	(Contador, Interrtaglia & Lebaron)
27	AM990714 *Rhodobacter* sp.	95	(Contador, Interrtaglia & Lebaron)
29	HM677900 Uncultured bacterium	88	(Yergeau et al.)
32	FJ479591 Uncultured bacterium	93	(Youssef et al. 2009)
119	AB190086 *Geobacillus* pallidus	88	
120	AJ564615 Uncultured *Geobacillus* sp.	98	
121	JF825503 Uncultured *Geobacillus* sp.	90	(Wang)

128	FJ229666 Uncultured bacterium	89	(Gaidos et al. 2008)
130	DQ234237 Uncultured *Sulfurospirillum* sp.	91	
131	DQ256314 Uncultured bacterium	77	(Gihring, Moser & Onstott)
132	AJ786105 *Bacillus* sp.	83	(Kopke et al. 2005)
134	HQ652595 *Hydrogenophaga* sp.	98	(Lu, An & Guo)
151	DQ795571 Uncultured bacterium	88	(Ley et al. 2006)
154	FJ037672 Uncultured bacterium	96	
159	EU304284 *Ralstonia* sp.	88	(Quenmeneur et al. 2008)

The table that follows is supplementary to Table 3.1 and gives a description of each of the bacteria species identified.

Sequence #	Accession Number	% Match	Bacteria Description
3	**GQ332247** Uncultured *Prevotella* sp.[1]	82	Prevotella sp. are amoung the most numerous microbes culturable from the rumen and hind-gut of cattle and sheep, where they help the breakdown of protein and carbohydrate foods.
4	**GQ332247** Uncultured *Prevotella* sp.[2]	81	Same as above
19	**AY218551** Uncultured Bacterium[3]	91	-
20	**AJ630274** Uncultured *Azospira* sp.[4]	73	The genus Azospira accommodates a lineage of nitrogen-fixing bacteria. This bacteria has been associated with Kallar grass. Features include the Gram-negative reaction, heterotrophic metabolism, curved rods with cells ranging from 0.4-0.6 um in width and from 1.1 to 2.5 um in length. (Bae, et al. 2007)
21	**FJ444721** Uncultured *Sinorhizobium* sp.[5]	93	Sinorhizobium is a genus of nitrogen fixing bacteria (rhizobia). Typical soil bacteria. (Fraysse, et al. 2002)
26	**AM990714** *Rhodobacter* sp.[6]	96	Rhodobacter Sphaeroides is a purple nonsulfur photosynthetic bacterium that is considered a possible source of H2 production
27	**AM990714** *Rhodobacter* sp.[7]	95	Same as above
29	**HM677900** Uncultured Bacterium[8]	88	-
32	**FJ479591** Uncultured Bacterium[9]	93	-
119	**AB190086** *Geobacillus pallidus*[10]	88	
120	**AJ564615** Uncultured *Geobacillus* sp.[11]	98	Geobacillus sp. is rod-shaped Gram positive, thermophilic bacteria. These bacteria have drawn interest for their potential in biotechnology applications as sources of thermostable enzymes. (Sakaff, et al. 2012)
121	**JF825503** Uncultured *Geobacillus* sp.[12]	90	Same as above
128	**FJ229666** Uncultured Bacterium[13]	89	-
130	**DQ234237** Uncultured *Sulfurospirrillum* sp.[14]	91	-
131	**DQ256314** Uncultured Bacterium[15]	77	-
132	**AJ786105** *Bacillus* sp.[16]	83	
134	**HQ652595** *Hydrogenophaga* sp.[17]	98	Hydrogenophaga is a gram-negative, non-spore-forming, rod shaped bacteria. Gross optimally at pH values of 7-8 and at 30-37°C.
151	**DQ795571** Uncultured Bacterium[18]	88	-
154	**FJ037672** Uncultured Bacterium[19]	96	-
159	**EU304284** *Ralstonia* sp.[20]	88	Ralstonia spp. are gram negative bacteria found in the environment, primarily in water, soil, and on plants. The organism grows readily on media routinely used by clinical microbiology laboratories (ie. Agar)

3.2.1.1 Point of zero charge of activated carbon

The graph below represents the pH_{PZC} of the activated carbon. The pH_{PZC} of the activated carbon was found to be 9.6 (Figure 3.6). According to literature,which has the pH_{PZC} calculated as 10.3, this value corresponds well to the average pH_{PZC} of activated carbon.

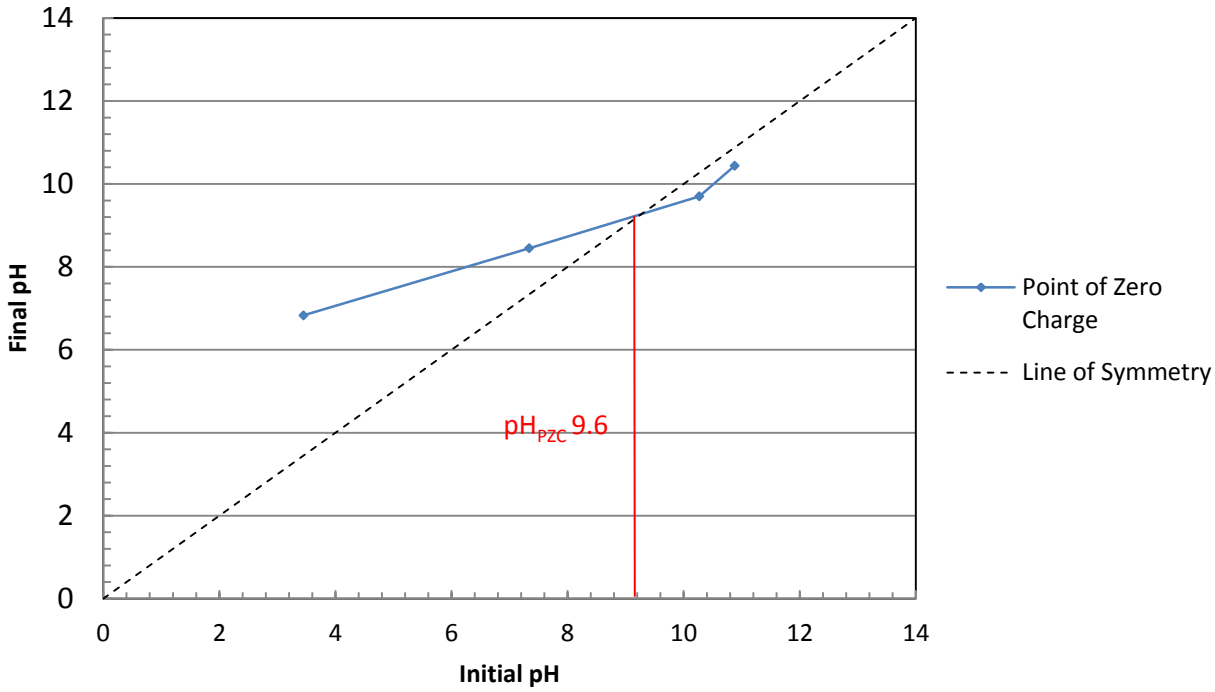


Figure 3.6: The point of zero charge of the activated carbon surface

The graph represents the $pH_{INITIAL}$ vs. pH_{FINAL} of the activated carbon. The point of intersection with the line $pH_{INITIAL} = pH_{FINAL}$ is the pH_{PZC}.

3.2.2 Ion exchange on the surface of activated carbon

The following table presents the variation of pH of the three solutions of sterilized water that have activated carbon.

Table 3.2: pH change of three solutions after the addition of activated carbon

INITIAL pH	FINAL pH	H+ Ions Removed (M)
5	**7.09**	9.92×10^{-6}
6.3	**7.07**	4.16×10^{-7}
7.1	**7.41**	4.1×10^{-8}

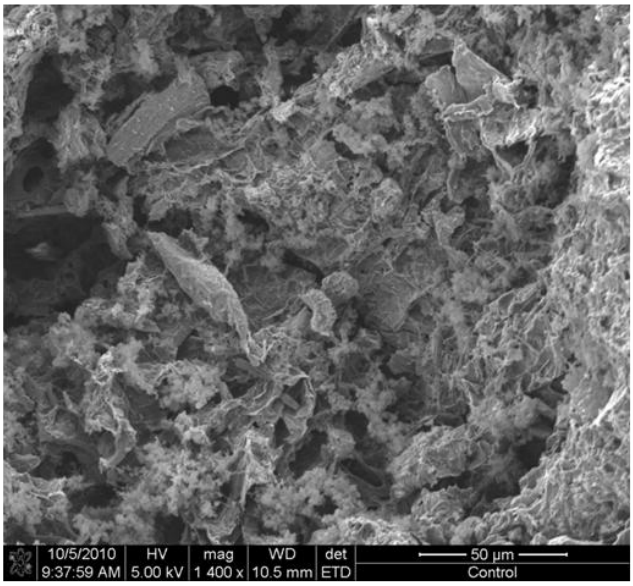
In Table 3.2 the change in pH of 3 solutions containing activated carbon, and their relative pH after 10 minutes is shown. The last column represents the amount of ions removed from solution and attached to the activated carbon surface.

It can be seen from the above that as the activated carbon remains in the charged solutions, H^+ ions are removed from the solution and attach to the surface of the activated carbon. This can be seen in the drastic change in solution pH (The pH of the solution that the activated carbon was submerged in). The solution pH increases its basic characteristic as it increases hence implying that it's losing H^+.

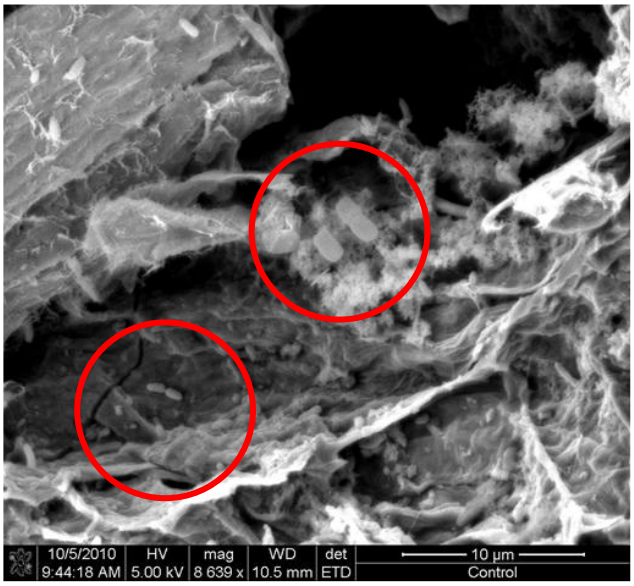
3.3 Control Experiment (No modifications performed to the substrate or bacteria)

Prior to any charge modifications a control experiment was performed. Activated carbon (0.6 g) was placed in a 20 ml solution of bacterial solution. Neither the bacteria nor the solution were manipulated in any way. This was done as a control experiment. It shows the degree of microbial adsorption onto a non-charged substratum. In Figure 3.7, it can be seen that there is minimal attachment of bacteria onto the surface of the activated carbon. There are a few bacteria attached to the surface of the activated carbon.

Control SEM Images



A: No Charge Modification Magnification 1400x



B: No Charge Modification Magnification 8639x

Figure 3.7: SEM images of the activated carbon surface. No charge modification was done and as a result this acts as a control.

3.4 SEM Images from Bacteria Surface Modification (exposed to pH's 5, 6, and 7).

In Experiment 1: (Charge modification of bacterial surface adapted from Busscher et al. 2006) the bacteria were centrifuged and washed with 3 different solutions. Once they were washed adequately the aliquots of bacteria were placed in the three charged solutions respectively. 0.6 g of activated carbon was then placed in 20 ml of each inoculated solution. The three solutions were then incubated for 4 days. The SEM images shown below show the attachment on the surface of the activated carbon after 4 days.

In Figure 3.8, it can be seen that attachment is inconsistent and minimal for the three samples. The most attachment was found on the substratum that had been in contact with bacteria charged to a pH of 7. In Figure 3.8 image F, there is a significant amount of attachment of bacteria to the surface of the activated carbon. This was not seen in any of the other samples.

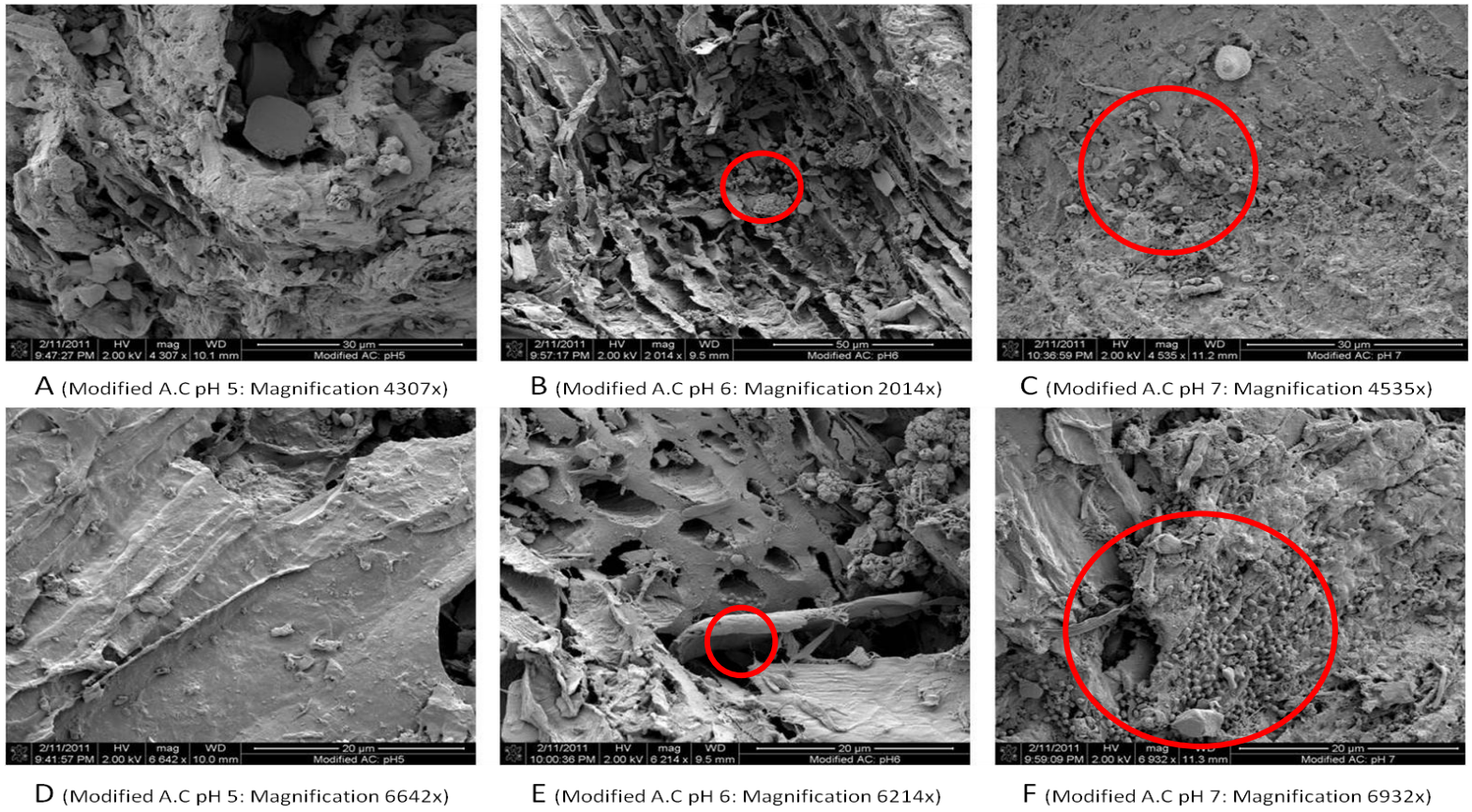


Figure 3.8: SEM images for experiment 1. Images A to C show the surfaces of the activated carbon, for pH of 5, 6 and 7 at magnifications of <5000. Images D to F show a closer view of the surface, magnification > 6000

3.4.1 Bacteria Quantification of Experiment 1: (Charge modification of bacterial surface adapted from Busscher et al. 2006)

The following graph shows the average amount of bacteria attached per mm^2 on the surface of activated carbon. At each of the pH's that the activated carbon was charged to the corresponding value of bacteria attachment is shown. The activated carbon with the most attachment is irrefutably that charged to a pH 7. There is a significant difference in the amount attached to that of pH 7 and that of the lower pH's.

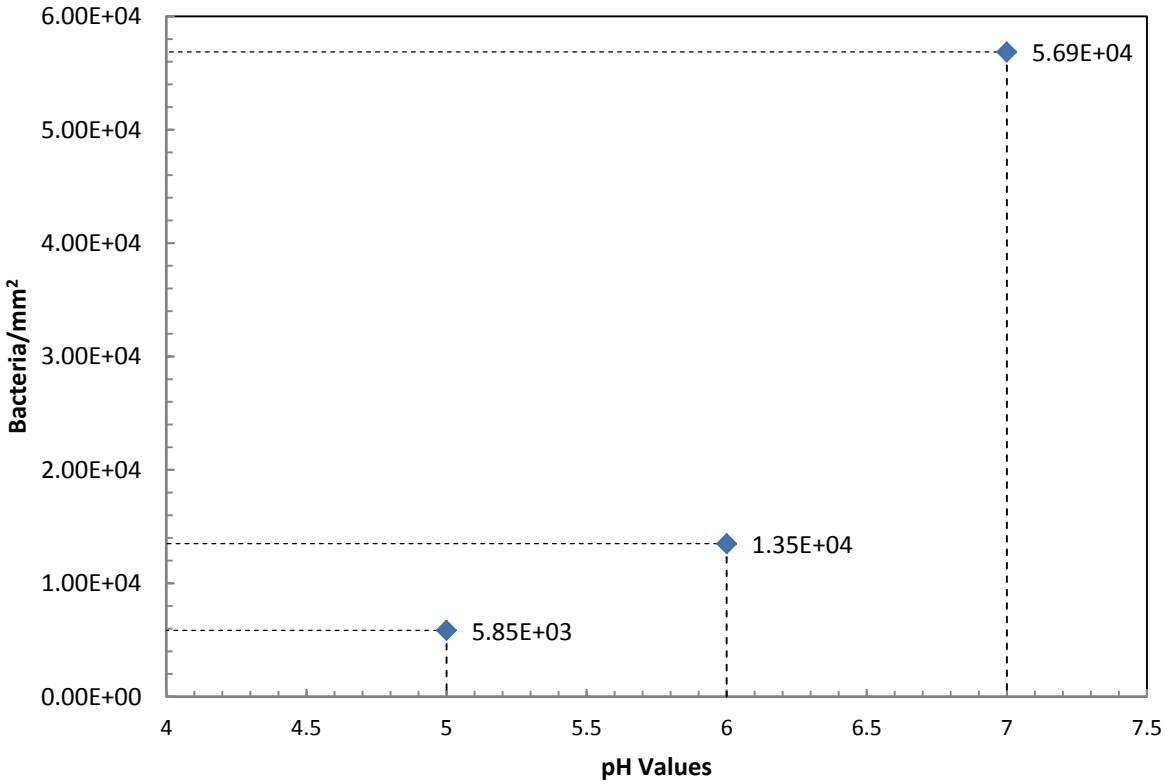


Figure 3.9: Quantification of bacteria attached to the surface of substratum.

3.5 SEM IMAGES OF EXPERIMENT 2: (CHARGE MODIFICATION AFTER BACTERIA INOCULATION).

According to Experiment 2: (Charge modification after bacteria inoculation), The samples were left for incubation for 4 days, upon which three activated carbon particles were removed and prepared for SEM analysis. In Figure 3.10, it is seen that there is a substantial increase in bacteria present on the surface of the activated carbon in all three samples as compared to Figure 3.8. There is negligible attachment at pH 5, however at a pH of 6 and 7 there is a definite amount of attachment. The beginnings of biofilm creation can be seen in images C and F.

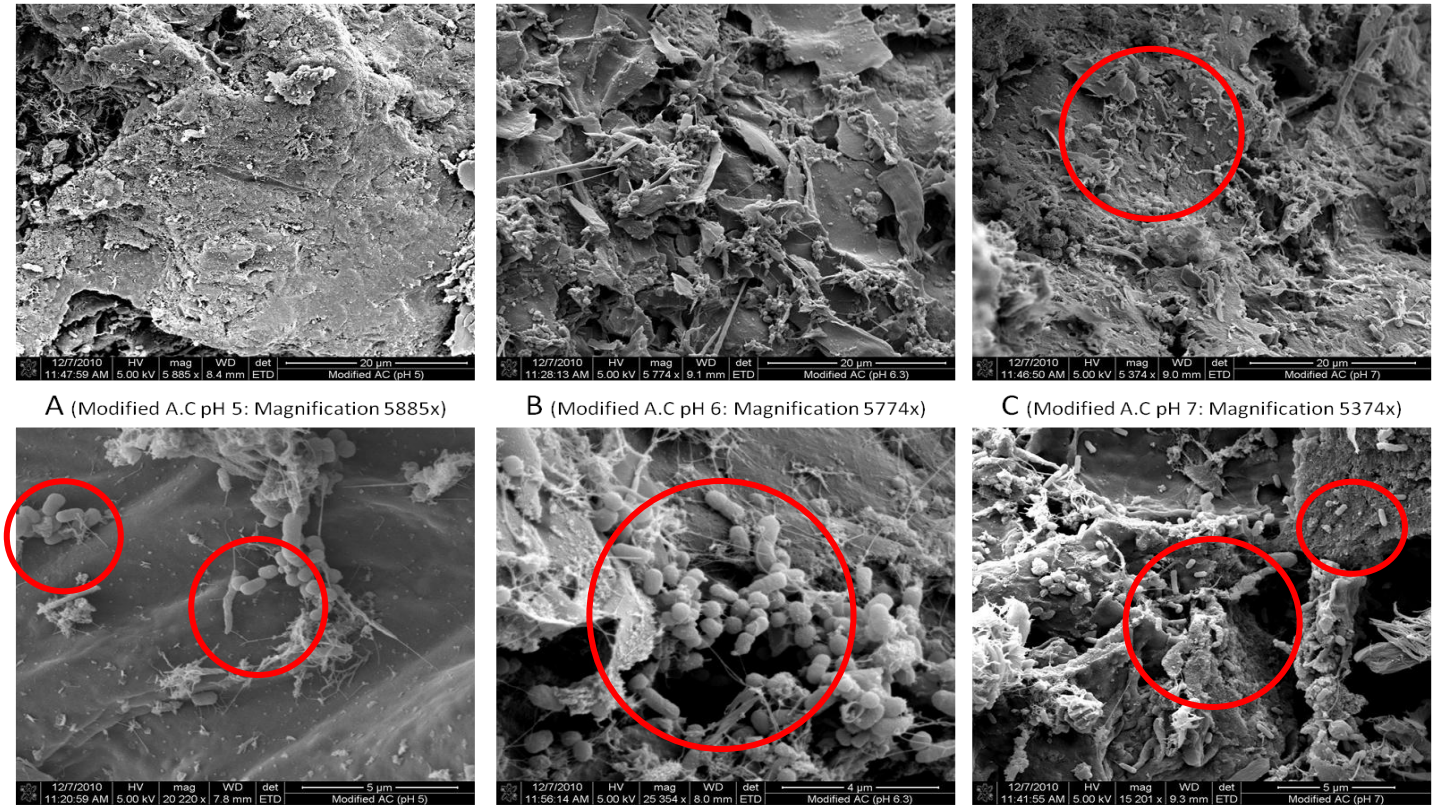


Figure 3.10: SEM images for experiment 2. Images A to C show the surfaces of the activated carbon, for pH of 5, 6 and 7 at magnifications of <6000. Images D to F show a closer view of the surface, magnification > 15000

3.5.1 Bacteria Quantification of Experiment 2: (Charge modification after bacteria inoculation)

The following graph shows the average amount of bacteria attached per mm^2 on the surface of activated carbon. At each of the pH's that the activated carbon was charged to the corresponding value of bacteria attachment is shown. The activated carbon with the most attachment is by far that charged to a pH 7. There is a significant difference in the amount attached to that of pH 7 and that of the lower pH's. In addition there is a definite improvement in attachment at each pH value with respect to Experiment 1: (Charge modification of bacterial surface adapted from Busscher et al. 2006).

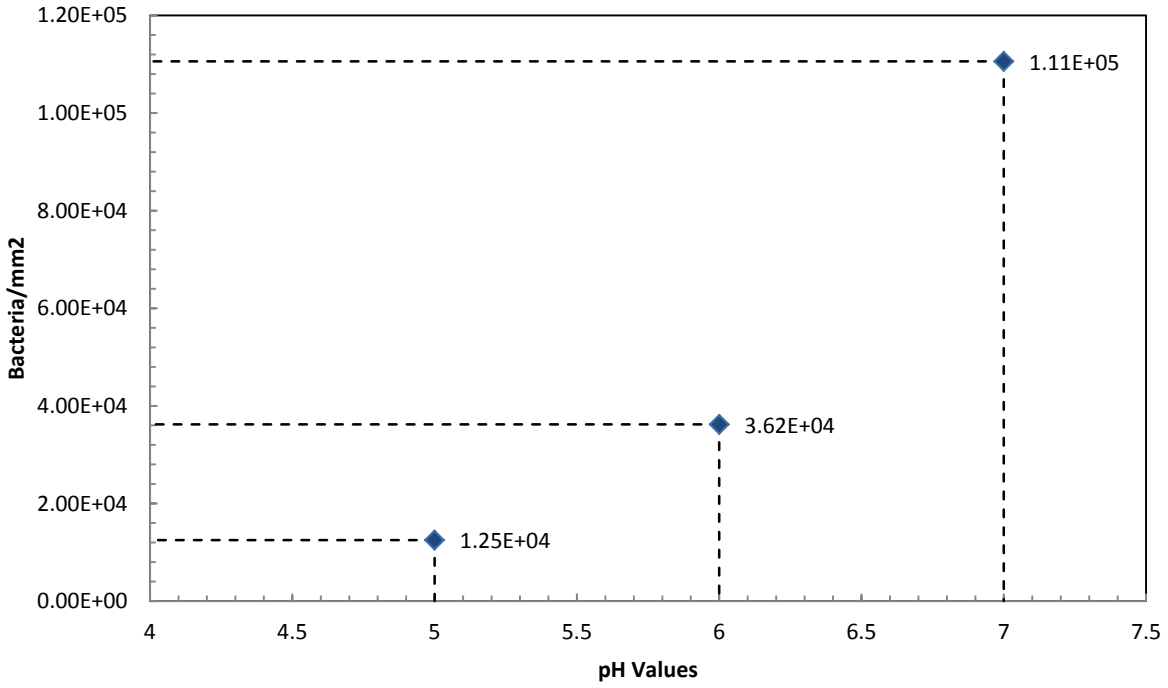


Figure 3.11: Quantification of bacteria attached to the surface of substratum.

3.6 SEM IMAGES OF EXPERIMENT 3: (CHARGE MODIFICATION OF ACTIVATED CARBON ONLY)

As mentioned in Section: Experiment 3: (Charge modification of activated carbon only), the activated carbon was charged separate before bacteria is introduced. The samples were left for incubation for a period of 4 days, upon which a few activated carbon particles were removed and prepared for SEM analysis. As is clearly observed in the SEM photographs below, the greatest amount of attachment is seen when the activated carbon was charged to a pH 7. The SEM images show both low and high magnifications. The low magnification allows for a broader view of the surface of the activated carbon. It is useful to use these images to get a clear idea of the attachment on the surface.

Upon inspection of the three surfaces biofilm formation is seen on the surface of the sample charged to a pH of 7.

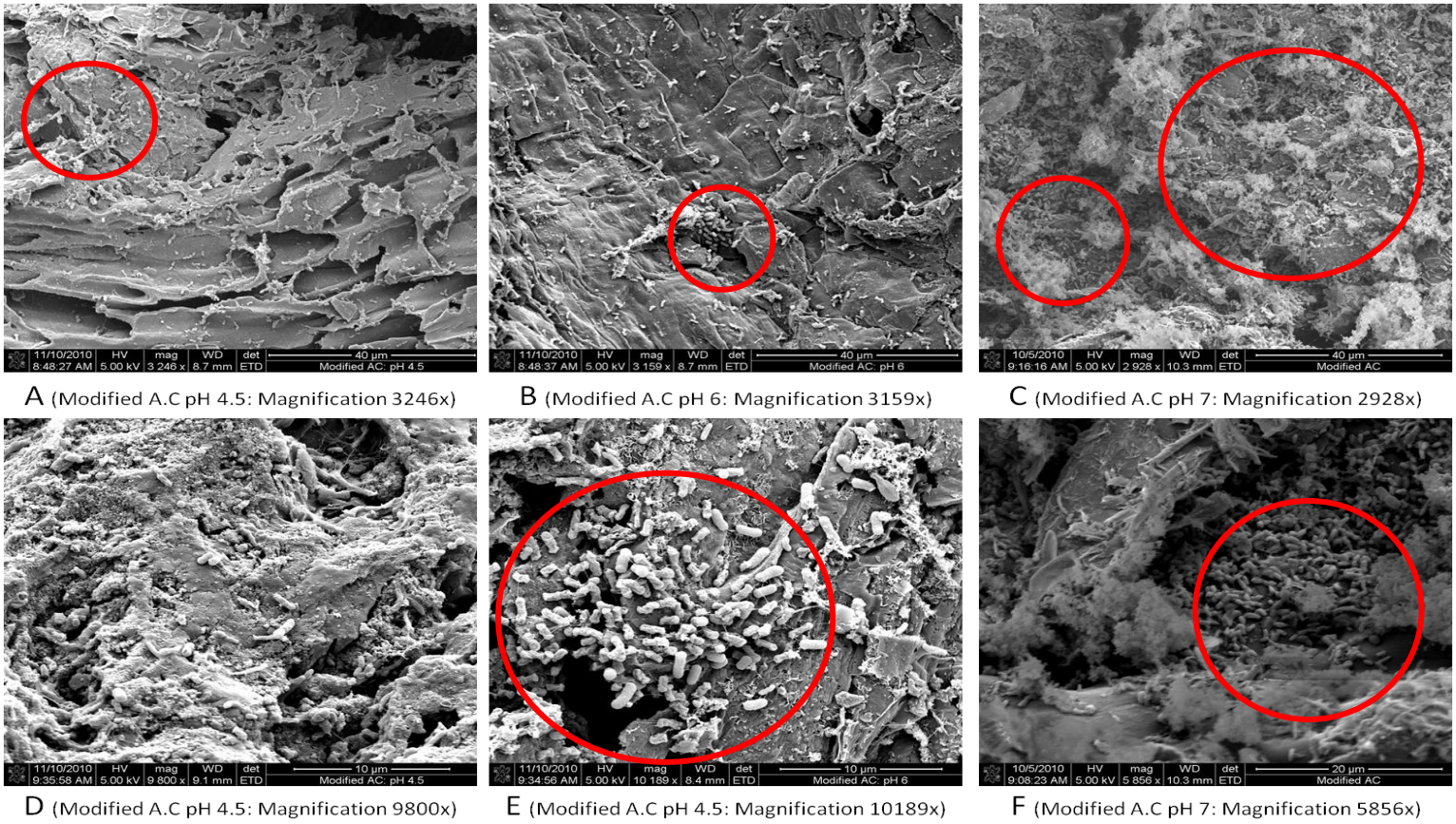


Figure 3.12: SEM images for experiment 3. Images A to C show the surfaces of the activated carbon, for pH of 5, 6 and 7 at magnifications of < 4000. Images D to F show a closer view of the surface, magnification > 5000

3.6.1 Bacteria Quantification of Experiment 3: (Charge modification of activated carbon only) after 4 days.

The following graph shows the average amount of bacteria attached per mm^2 on the surface of activated carbon. At each of the pH's that the activated carbon was charged to the corresponding value of bacteria attachment is shown. The attachment of bacteria per mm^2 is greatest for the activated carbon charged to a pH of 7. There is a 303% increase in the attachment of the bacteria when the activated carbon is charged to pH of 7 compared to when it was charged to the other pH's. Further at a pH of 7, Experiment 3 has shown the greatest attachment.

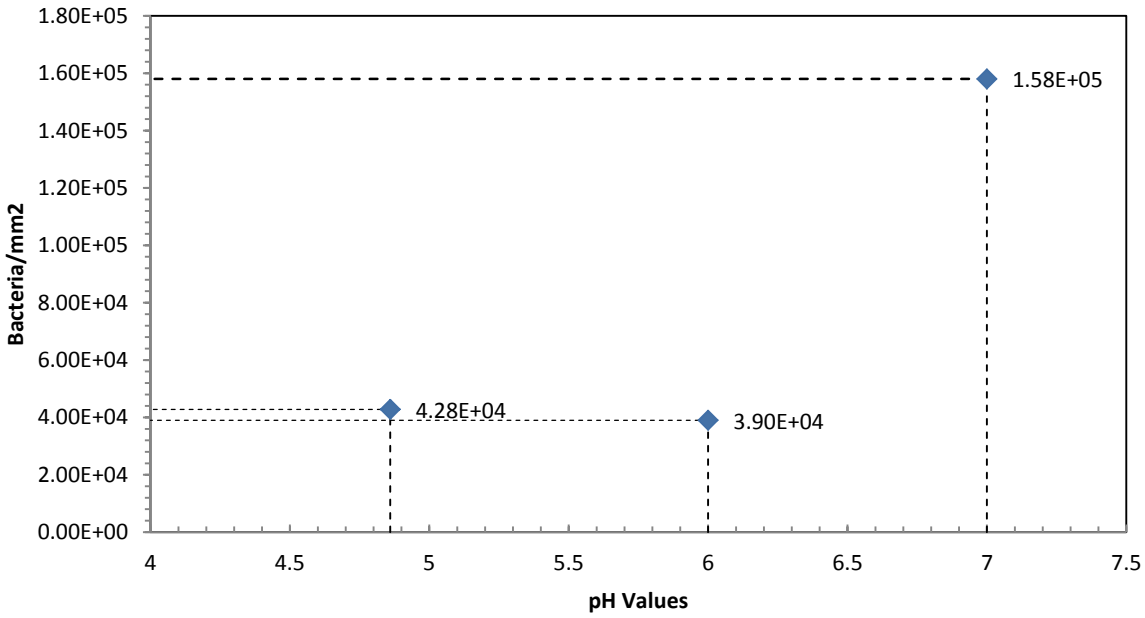


Figure 3.13: Quantification of bacteria attached to the surface of substratum.

3.7 RESULTS OF TIME OPTIMISATION

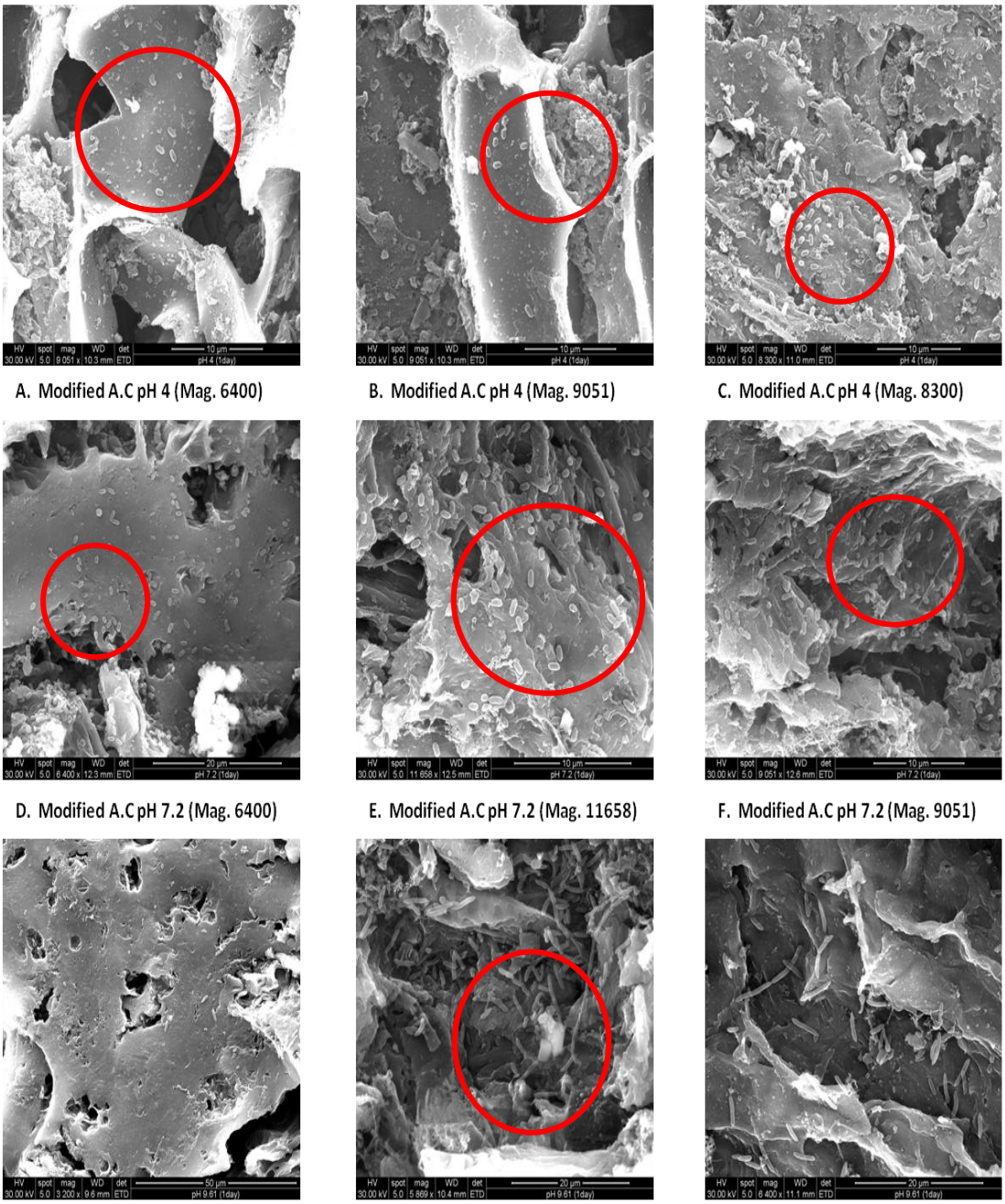
3.7.1 Attachment after 1 day

The images seen below represent the samples taken after three days of incubation. It can be seen that all three samples show an attachment of bacteria to their surface. However it was again the activated carbon charged to a pH of 7.2 that presented the most consistent attachment of bacteria on its surface. It was noticed that when the activated carbon was charged to a pH of 9.6 the bacteria tended not to attach directly on to the surface of the activated carbon but form a chain of bacteria with one bacterium attached to the surface and the rest to each other.

3.7.2 Attachment after 13 days

The images seen below were taken after the samples had been incubating for 13 days. There is a common trend seen in all three samples, namely the formation of clusters of bacteria floating on the surface of the activated carbon. The bacteria seem to have been drawn to one another where they almost stick to each other forming theses bunches. Bacteria are known to form Extracellular Polymeric Substance (EPS) fluid which causes bacteria to attach to each other. This is the early stages of biofilm formation.

In the experiments conducted previously there was a clear indication as to the degree of bacteria attachment on the surface for each of the pH's. In these images biofilms have been created on all the surfaces. There are numerous bacterial clusters on all the charged surfaces.



A. Modified A.C pH 4 (Mag. 6400)

B. Modified A.C pH 4 (Mag. 9051)

C. Modified A.C pH 4 (Mag. 8300)

D. Modified A.C pH 7.2 (Mag. 6400)

E. Modified A.C pH 7.2 (Mag. 11658)

F. Modified A.C pH 7.2 (Mag. 9051)

G. Modified A.C pH 9.61 (Mag. 3200)

H. Modified A.C pH 9.61 (Mag. 5869)

I. Modified A.C pH 9.61 (Mag. 6400)

Figure 3.14: SEM images for experiment 3, samples taken after 24 h of incubation. Images A to C show the surfaces of the activated carbon, for pH of 4 at magnifications of 6400 to 9051. Images D to F show the surfaces of the activated carbon, for pH of 7 at magnifications of 6400 to 11658. Images G to I show the surfaces of the activated carbon for a pH of 9.61 at magnifications of 3200 to 6400

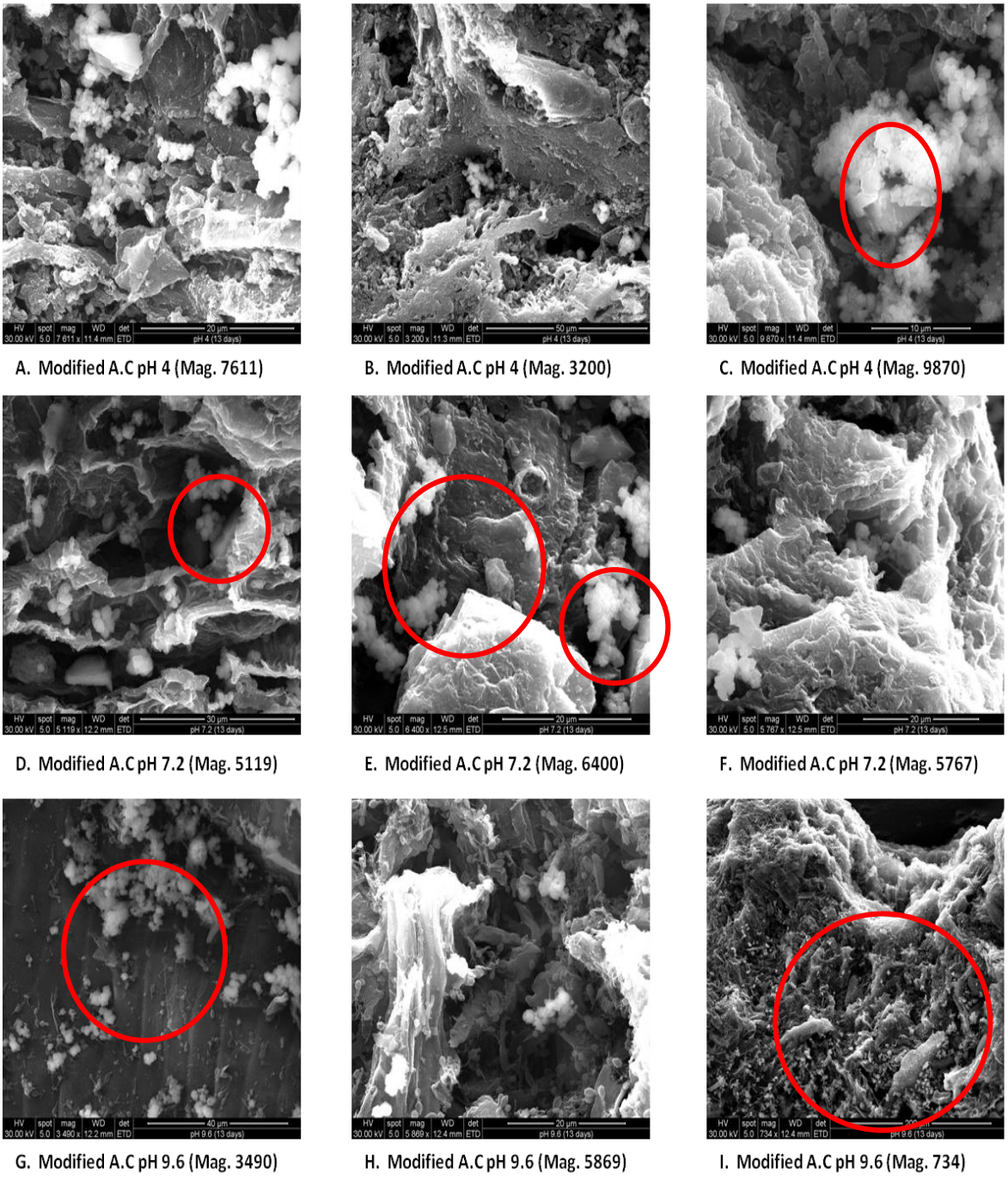


Figure 3.15: SEM images for experiment 3, samples taken after 13 days of incubation. Images A to C show the surfaces of the activated carbon, for pH of 4 at magnifications of 3200 to 9870. Images D to F show the surfaces of the activated carbon, for pH of 7 at magnifications of 5119 to 6400. Images G to I show the surfaces of the activated carbon for a pH of 9.61 at magnifications of 3200 to 6400. Red Circles in this figure represent biofilms.

Chapter 4

DISCUSSION, CONCLUSION AND FUTURE WORKS

4 DISCUSSION, CONCLUSION AND FUTURE WORKS

4.1 DISCUSSION

4.1.1 Discussion and Recommendations for DNA sampling

Literatures regarding the species found in the samples suggest that most species identified would be found in soil environments. The bacteria identified show that there are species associated with:

- Rhizosphere
- Grease Traps
- Avian Droppings
- Arsenite Oxidation
- Compost
- Subsurface Soil Water

The bacteria found in the hindgut of the termite coincide with the environment they inhabit. The "worker" termites collect the food and feed of the soil whilst building their mound. It is therefore clear that the bacteria within their gut would come primarily from the food they ingest. Formosan Termites eat wood and fabric made from plants. When they eat dead trees, these termites help the environment and make space for new plant life.

4.1.2 Charging the activated carbon

It was shown from experiments performed on the activated carbon, that when the activated carbon is placed in a charged solution it will pick up the ions floating in the solution. It was shown in Table 3.2 that the activated carbon will pick up H^+ . This reinforces the idea that the surface charge of the activated carbon can be manipulated by placing the carbon in a charged solution.

4.1.3 pH_{PZC} of activated carbon

The point of zero charge or pH_{PZC} has been explained as the pH at which the surface of the activated carbon will be neutral (zero charge). This point implies that if activated carbon is placed in a solution of its pH_{PZC} it will have no charge on its surface. This theory allows for the modification of the surface

charge based on where its pH_{PZC} lies on the pH scale. The pH_{PZC} of activated carbon was found to be pH of 9.6. Therefore if the activated carbon is placed in a solution with a pH lower than 9.6 its surface will become more positive (Figure 4.1). The inverse, if the carbon is placed in a solution with a pH higher than 9.6, it will increase the surface's negative character. This theory formed the basis for the charge modification of the activated carbon. Since it was desired that the surface of the activated carbon be more positive experiments 1-3 were conducted so as to determine whether making the surface positive would have a greater attachment effect for the bacteria.

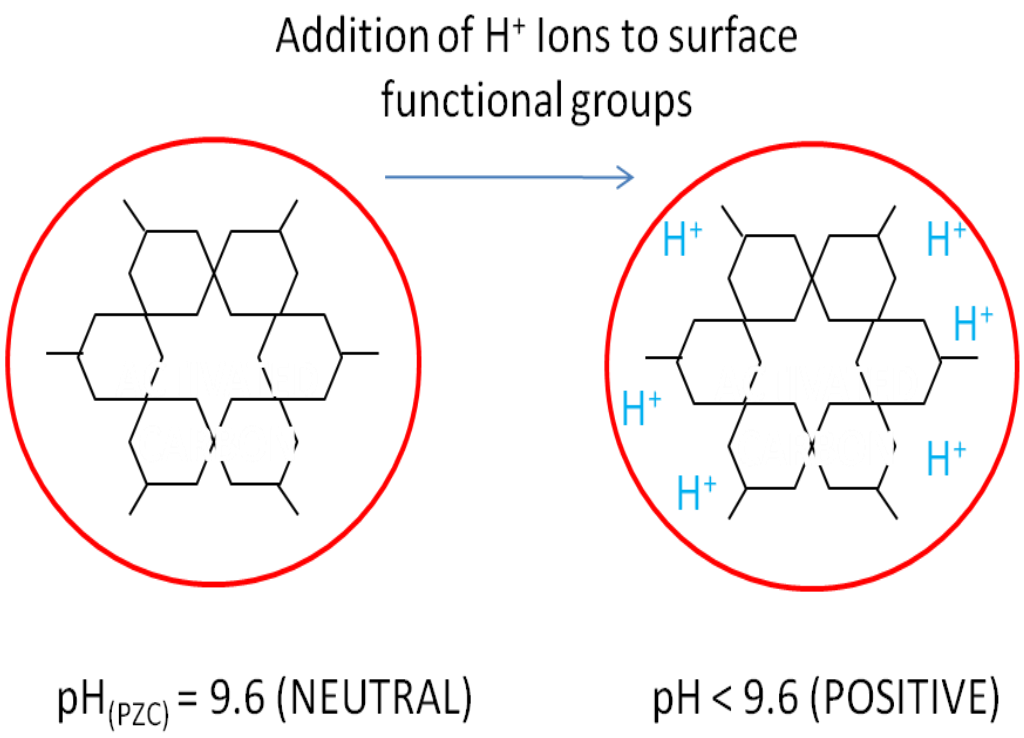


Figure 4.1: Schematic representation of the surface functional groups on activated carbon. At a pH of less than the pH_{PZC} implies the attachment of H^+ ions as seen above.

4.1.4 Attachment To the Uncharged Substrate

It was seen that the attachment of bacteria to the uncharged activated carbon substrate was minimal and showed no sign of biofilm formation. The implication of this coincided with the hypothesis that without charge modification to the surface the attachment of bacteria is minimal and sluggish.

4.1.5 Attachment experiment 1-3

4.1.5.1 Experiment 1: (Bacteria only was charged)

Experiment 1 was adapted from Busscher et al., 2006. From the results it can be seen that the attachment of bacteria to the surface is unimpressive. The attachment was minimal and showed no surface coverage or indication of film creation.

When comparing the three samples it can be observed that the bacteria showed preference to attach to the surface charged to a pH of 7. Large clusters of bacteria are seen to attach to the surface of the carbon when it was charged to a pH of 7 as opposed to the other lower charged surfaces. The quantitative estimations of bacteria/mm^2 show that the bacteria prefer to attach to the surface of the activated carbon charged to pH 7.

These results led to the hypothesis that the procedure may be improved for better attachment. It had been assumed that the issue for the lack of attachment is the competition for H^+ ions. When the bacteria is initially washed in the charged solution and then placed in the charged solutions the activated carbon possibly does not receive enough ions, as these are taken up by the bacteria. The bacteria will become more negative whilst the carbon remains uncharged. It is believed that there is a competition for these ions and the bacteria may be capturing them before allowing the activated carbon to be charged sufficiently. The procedure was thus modified in an attempt to allow for the surface modification of the activated carbon to be improved.

4.1.5.2 Experiment 2 (The mixture of bacteria and activated carbon was charged)

Experiment 2 was performed in an attempt to allow for more H^+ ions to attach to the surface of the activated carbon and allow for the surface to become more positive. Once the activated carbon was placed in the growing bacterial solution, the charge of the pH of the solution was modified. This allowed for there to be equal competition for ions, and would increase the probability for the activated carbon to take up H^+ ions.

It can be seen that the attachment showed drastic improvements. The attachment is significantly more observable and prominent on the surface. In comparing Experiment 2 with Experiment 1 it can be seen that the modifications to the procedure did assist in the improved attachment. It can be concluded that there is a competition for ions and that the more the activated carbon is charged the better the attachment. It can further be observed that the most attachment was seen at a pH 7. This

is consistent with Experiment 1. These results led to the final experiment being developed. This experiment charges the activated carbon prior to it being placed in the bacterial solution.

4.1.5.3 Experiment 3 (Activated carbon charged prior to bacteria inoculation)

Experiment 3 offers a direct charging of the surface of the activated carbon's surface. The activated carbon is charged for half an hour separately and then once charged placed within the bacterial solution.

The results from this procedure show a significant improvement in the attachment of bacteria to the activated carbon surface. In all the samples there is a definite success in the attachment of bacteria to the surface and the creation of a "film". When comparing the three samples it is clear that the attachment is most prominent when the surface had been charged to a pH of 7. At this pH there is an overwhelming amount of bacteria attached to the surface almost covering the entire surface. This attachment also shows a good sign of biofilm creation.

4.1.6 The optimal pH

It was clearly observed throughout all three experiments that the pH of 7 in each of the procedures showed the greatest attachment. It has been assumed that the lower pH's although being more positive in surface charge than that of pH 7, are too close to the pH of the bacteria. If this is the case the bacteria and activated carbon tend to have the same pH and as such will not be attracted. It has been theorised that when the pH's become too similar, the electrostatic attraction begins to dissipate, hence the lower attachment. It is therefore important that although the charge on the activated carbon is positive it should not be too low as it will affect the attraction.

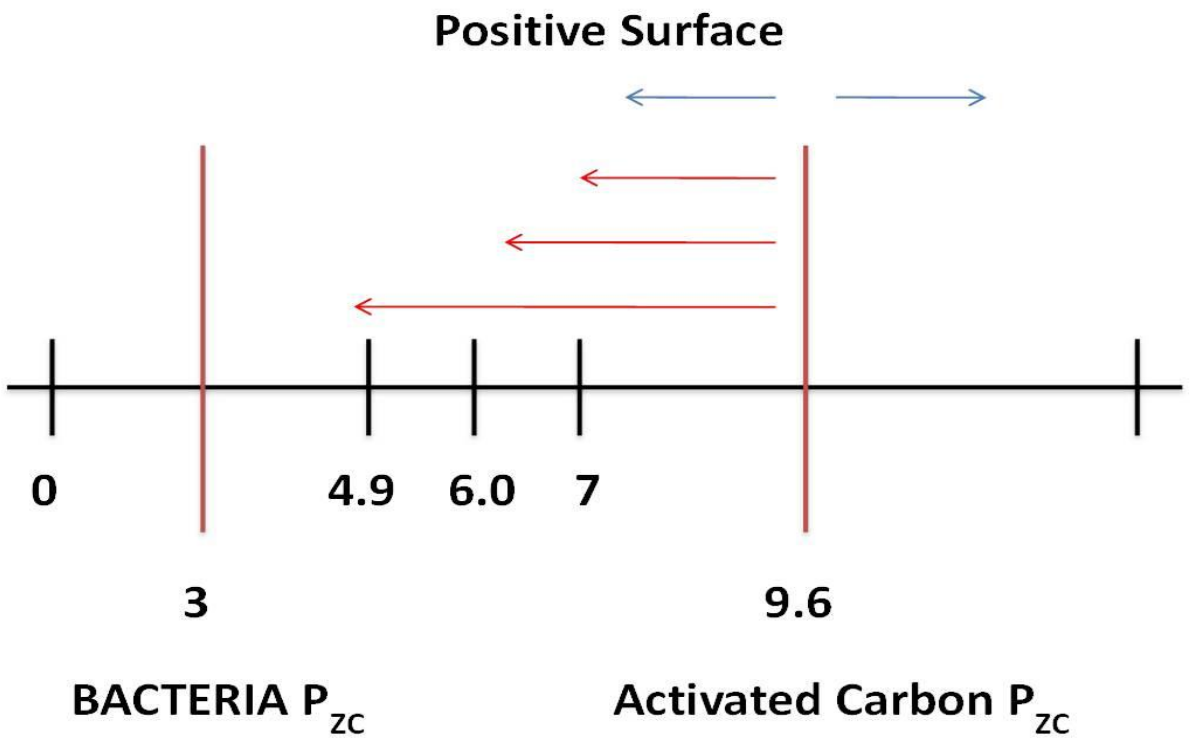


Figure 4.2: Schematic representation of charge modification

4.1.7 Attachment Time

4.1.7.1 Attachment after 1 day

After 1 day there is significant microbial attachment to the surface of the activated carbon. A strange occurrence happened on the surface of the activated carbon charged to a pH of 9.6. When comparing this sample with the others

4.1.7.2 Attachment after 13 days

As was seen from the SEM images the bacteria were into formed clusters on the surface of the activated carbon after 13 days. These clusters indicate that the bacteria begin to attach to one another. This type of biofilm is unlike the type of biofilm expected which is a thick layer of bacteria aligned on the surface of the activated carbon. A similar type of biofilm was observed by Campanac et al., 2002 who observed a cluster formation for *S.aureus* and a total overlap of cells for *P.aeruginosa* on the surface of Tygon tubes. As can be seen from the images below there is a similar configuration of biofilm

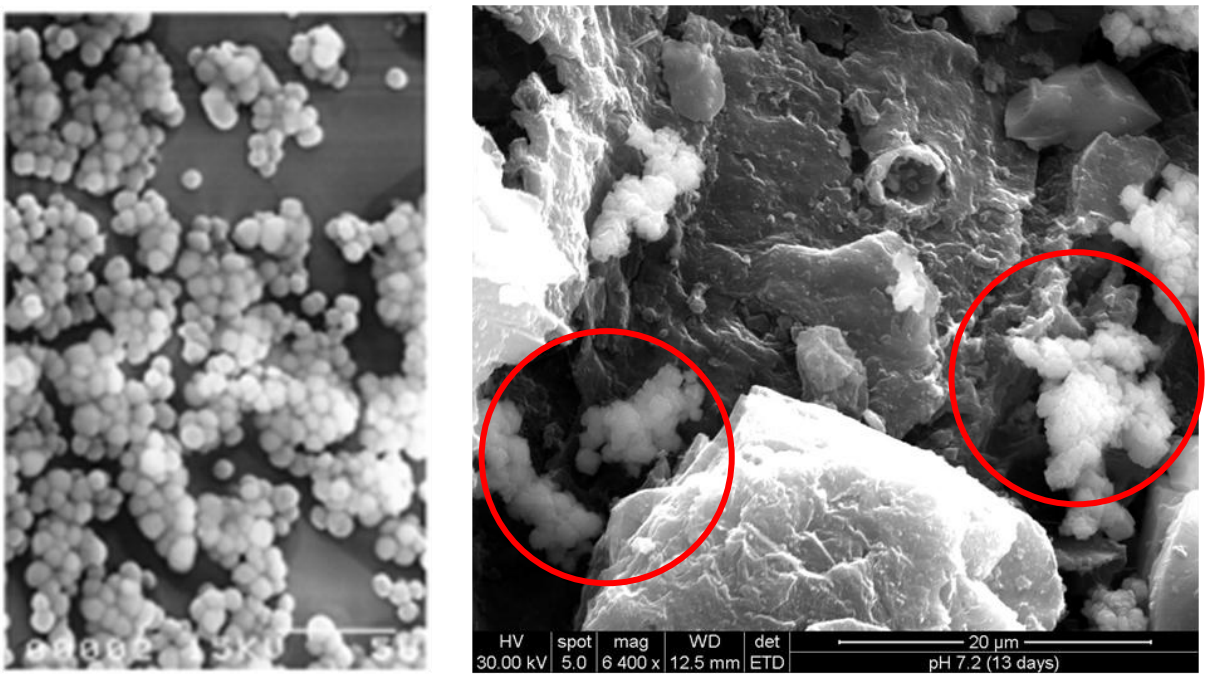


Figure 3: Biofilm comparison. A: Biofilm of Campanac et al., 2002, B: Biofilm observed after 13 days at pH 7.2

It is therefore clear that a biofilm was created after 13 days. However since the biofilms were now cluster based no comparison could be made as to which pH modified substrate had a more robust biofilm.

4.2 CONCLUSION

The main focus of this investigation was to develop a procedure that would optimise the rate of adsorption of bacteria to the surface of the activated carbon and the creation of biofilms. The use of ion exchange principals was thought to be a good method in trying to achieve this.

Bacterial interaction with activated carbon is primarily influenced by their surface charge. In this case the bacteria and activated carbon both have a negative surface charge, and as such the electrostatic adsorption is inhibited.

It was observed that the procedure taken from literature, Experiment 1: (Charge modification of bacterial surface adapted from Busscher et al. 2006), proved to be insufficient in its ability to ensure attachment. The reason for this lack of attachment was presumed to be a result of the unequal competition for H^+ ions in the bacterial and activated carbon solution (as only the bacteria were charged, and then once charged faced with activated carbon). The activated carbon was not being charged sufficiently and hence lacked a strong positive attraction strength.

The procedure was modified to improve the charge strength of the activated carbon. In the intermediate procedure (Experiment 2: (Charge modification after bacteria inoculation)) when there was equal competition for the H^+ there was a significant improvement in the attachment of the bacteria to the surface of the activated carbon. It was however, the final procedure (Experiment 3: (Charge modification of activated carbon only)), where the activated carbon was charged separate to the bacteria that saw the best results. Undoubtedly the attachment was consistent and showed signs of biofilm creation.

It was further observed that throughout all three procedures, it was when the solution/activated carbon charged to a pH of 7 were the most successful. Although with decreasing the pH the activated carbon becomes increasingly positive, as a result the surface charge became too close to the pH_{PZC} of the bacteria. This results in the bacteria and activated carbon having the same pH and as such loosing attraction.

In an effort to use the optimal pH modification to create a biofilm on the surface of the activated carbon time experiments were performed. After 13 days biofilms on the surface of the activated carbon surfaces were seen in all experiments. The bacteria attached to the surface of the activated

carbon and produced extracellular polymeric substance (EPS) which allow other bacteria to stick to them.

The experiment performed; where the activated carbon was charged separate to the bacteria produced the greatest attachment of bacteria onto the substrate. The attachment was consistent and showed signs of biofilm creation. It can therefore be concluded that the there is a proven approach that optimizes the microbial adsorption on activated carbon by the surface modification of the substrate.

4.3 FUTURE WORK

The future of this research will be to establish how the modifications and optimal conditions established in this work will behave in a fluidized bed reactor (Walker, 1999).

Conditions for isolation anaerobic bacteria and the use of an anaerobic culture camber are required to make sure all bacteria are isolated and identified.

Flock creation, in a continuous process has previously proved to be an even lengthier and difficult to control process. It is therefore desired to optimize this process by enhancing the rates of adsorption of bacteria onto the activated carbon within the reactor. These continuous experiments should be performed to establish whether the work done in this investigation holds true in a continuous reactor.

It is proposed then that the conversion of biomass to ethanol be optimized in the fluidized bioreactor. If the biofilm creation proves to be successful and the bacteria attaches quickly and in large amounts to the substrate, the rate of bioproducts converted should be measured and the process will potentially be optimized.

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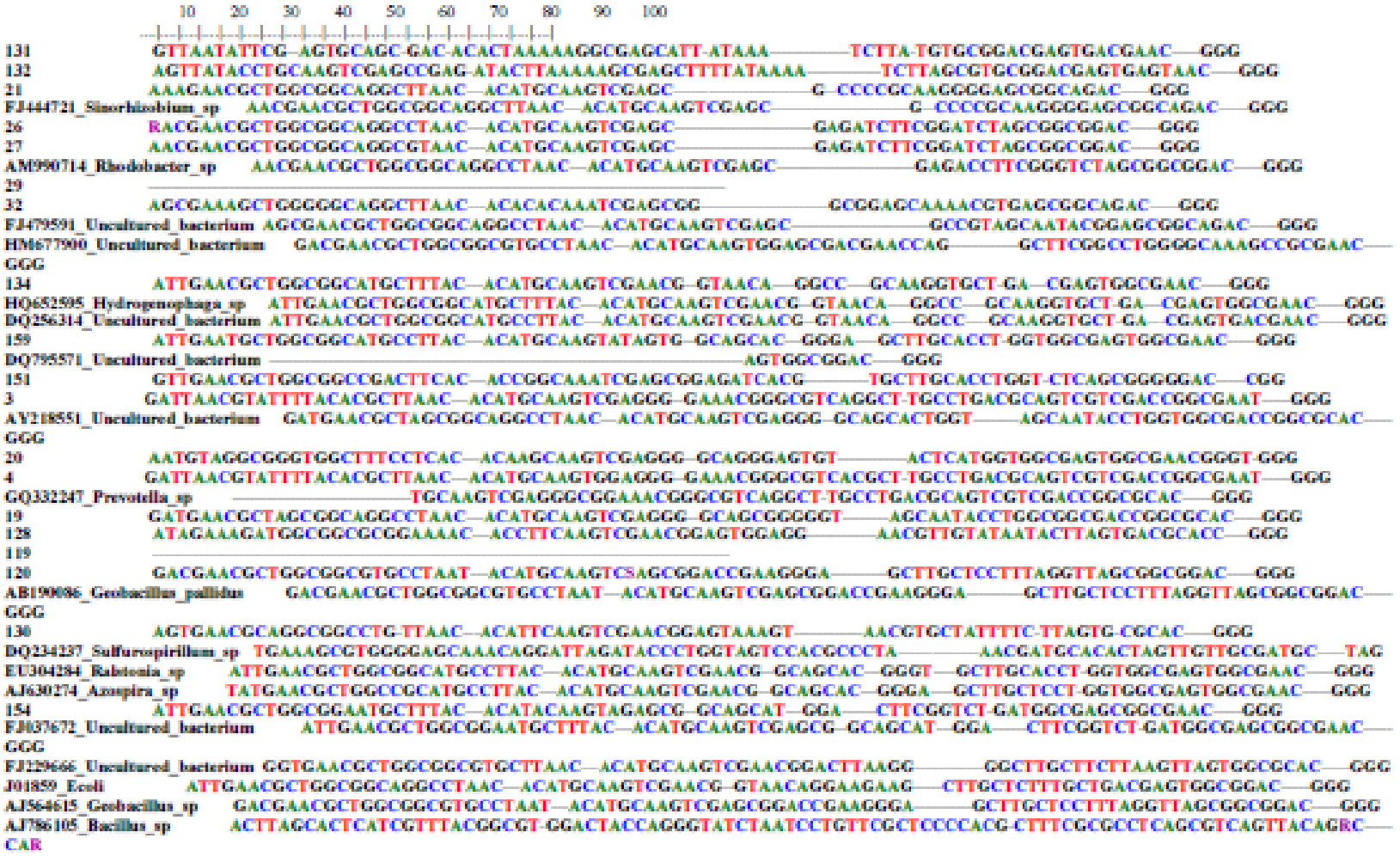
APPENDIX A: BACTERIA PREPERATION

In order to make 200ml of media the first step was to prepare the yeast extract. 2g/l of yeast extract is the amount used in order to prepare a litre of media, as such only 0.4g of yeast extract was required for the desired 200ml. This was placed in the large conical flask. Following this, the stock solution was prepared. It is necessary that the stock solution be prepared in a 1:5 ratio of stock solution to water. As such 40ml of stock solution was prepared in the conical flask and then 160ml of distilled water was added. The 200ml solution was then split into the four flasks.

APPENDIX B: MICROBIOLOGY

The following Appendix gives the detailed DNA analysis of the bacteria that was isolated from the local termite hindgut.



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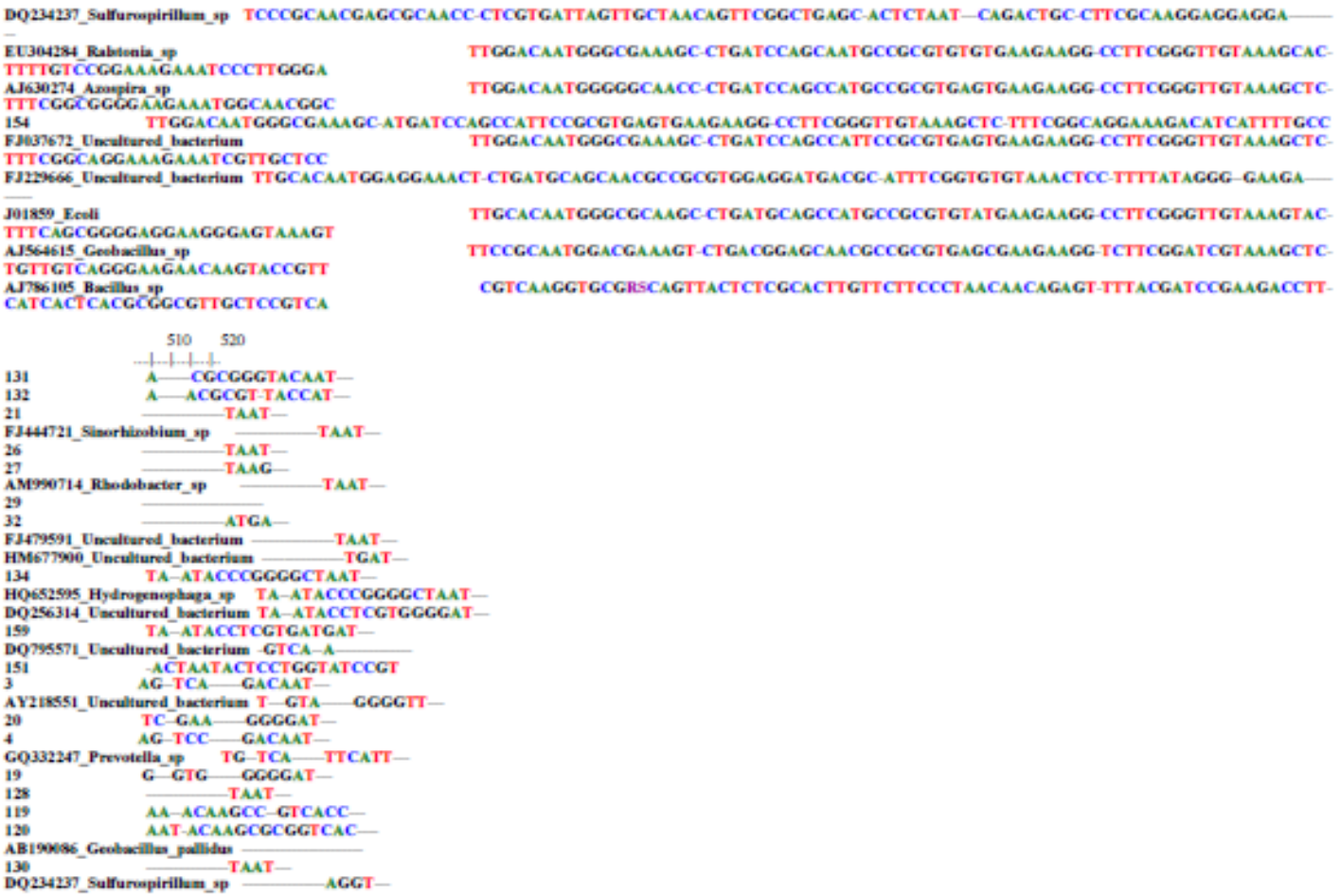
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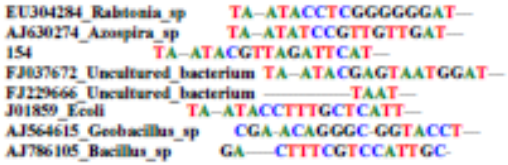
HQ652595_Hydrogenophaga_sp TA—ATACCCGGGGCTAAT—
DQ256314_Uncultured_bacterium TA—ATACCTCGTGGGGAT—
159 TA—ATACCTCGTGATGAT—
DQ795571_Uncultured_bacterium GTCA—A—
151 ACTAATACTCCTGGTATCCGT
3 AG—TCA—GACAAT—
AY218551_Uncultured_bacterium T—GTA—GGGGTT—
20 TC—GAA—GGGGAT—
4 AG—TCC—GACAAT—
GQ332247_Prevotella_sp TG—TCA—TTCATT—
19 G—GTG—GGGGAT—
128 —TAAT—
119 AA—ACAAGCC—GTCACC—
120 AAT-ACAAGCGCGGTCAC—
AB190086_Geobacillus_pallidus
130 —TAAT—
DQ234237_Sulfurospirillum_sp —AGGT—


EU304284_Rabtonia_sp TA--ATACCTCGGGGGGAT--
AJ630274_Azospira_sp TA--ATATCCGTTGTTGAT--
154 TA--ATACGTTAGATTCAT--
FJ037672_Uncultured_bacterium TA--ATACGAGTAATGGAT--
FJ229666_Uncultured_bacterium ----------TAAT--
J01859_Ecoli TA--ATACCTTTGCTCATT--
AJ564615_Geobacillus_sp CGA-ACAGGGC-GGTACCT--
AJ786105_Bacillus_sp GA----CTTTCGTCCATTGC-

APPENDIX C: SEM IMAGES

The following figure shows a magnified SEM image of bacteria attached to the surface of activated carbon that has been modified to a pH of 7.

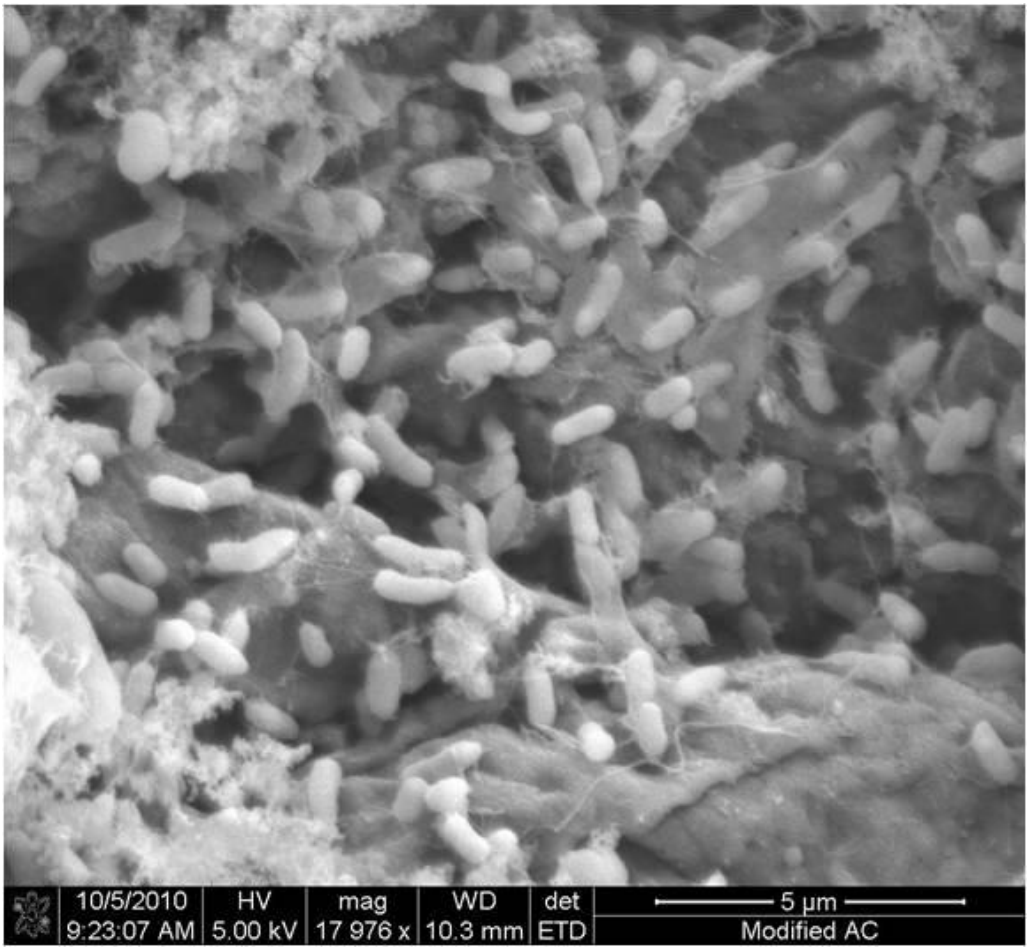


Figure C.1: Modified activated carbon charged to a pH of 7. Magnification 17976

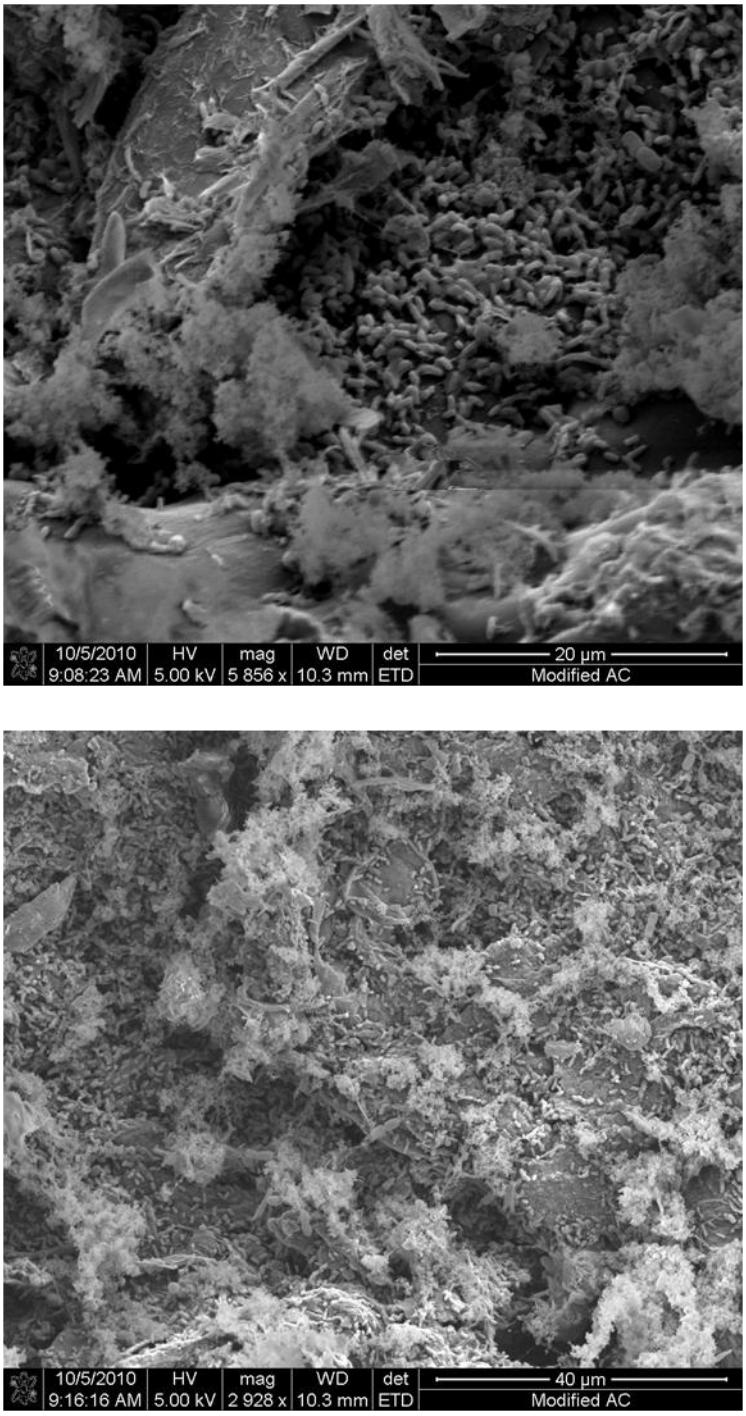


Figure C.2: Modified activated carbon charged to a pH of 7. Magnification 3000 to 6000

EXPERIMENT 1: (CHARGE MODIFICATION OF BACTERIAL SURFACE ADAPTED FROM BUSSCHER ET AL. 2006),SEM IMAGES.

After centrifugation the solid bacteria was washed with 3 different solutions of pH 4.5, 6 and 7. The pH of these sterile water solutions were altered using 0.1mol/l Nitric Acid and 0.1 mol/l Sodium Hydroxide. Once they were washed adequately the aliquots of bacteria were poured into the three solutions (each 20 ml) respectively. Activated carbon (0.6 g) was then placed in of each inoculated solution. The three solutions were then incubated for 4 days and later viewed under SEM.

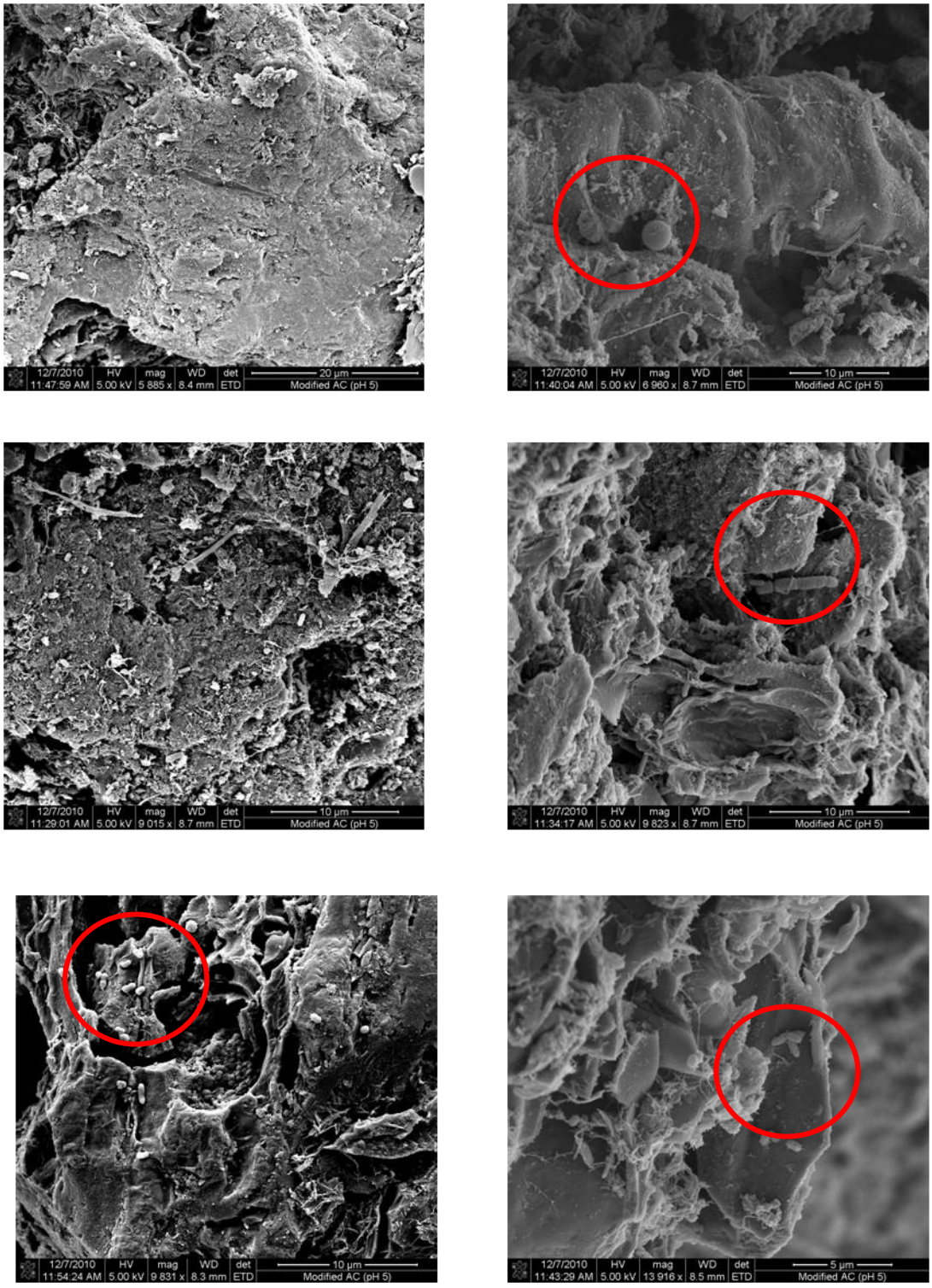


Figure C.3: Modified activated carbon pH 5 (Experiment 1)

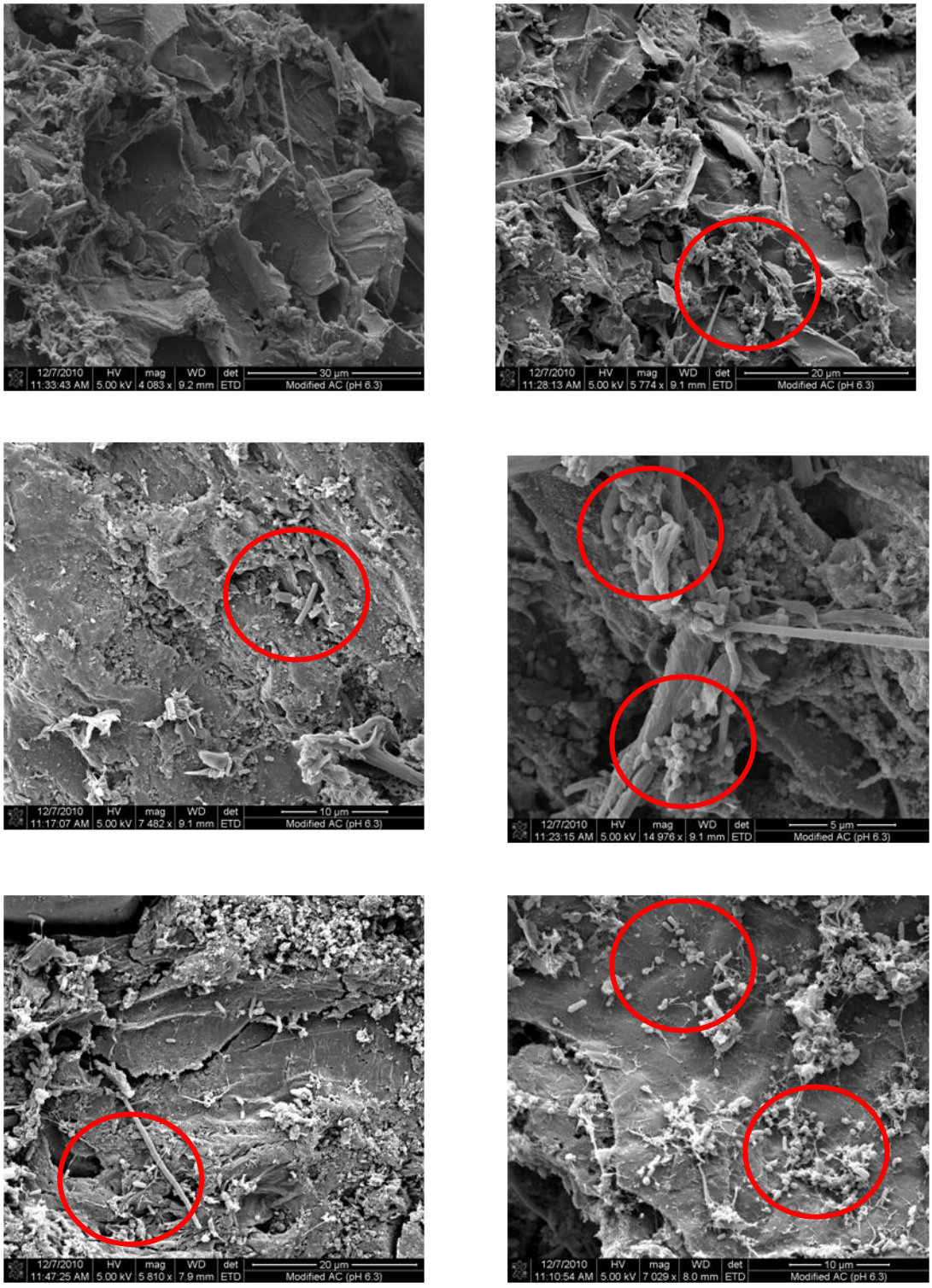


Figure C.4: Modified activated carbon pH 6.3 (Experiment 1)

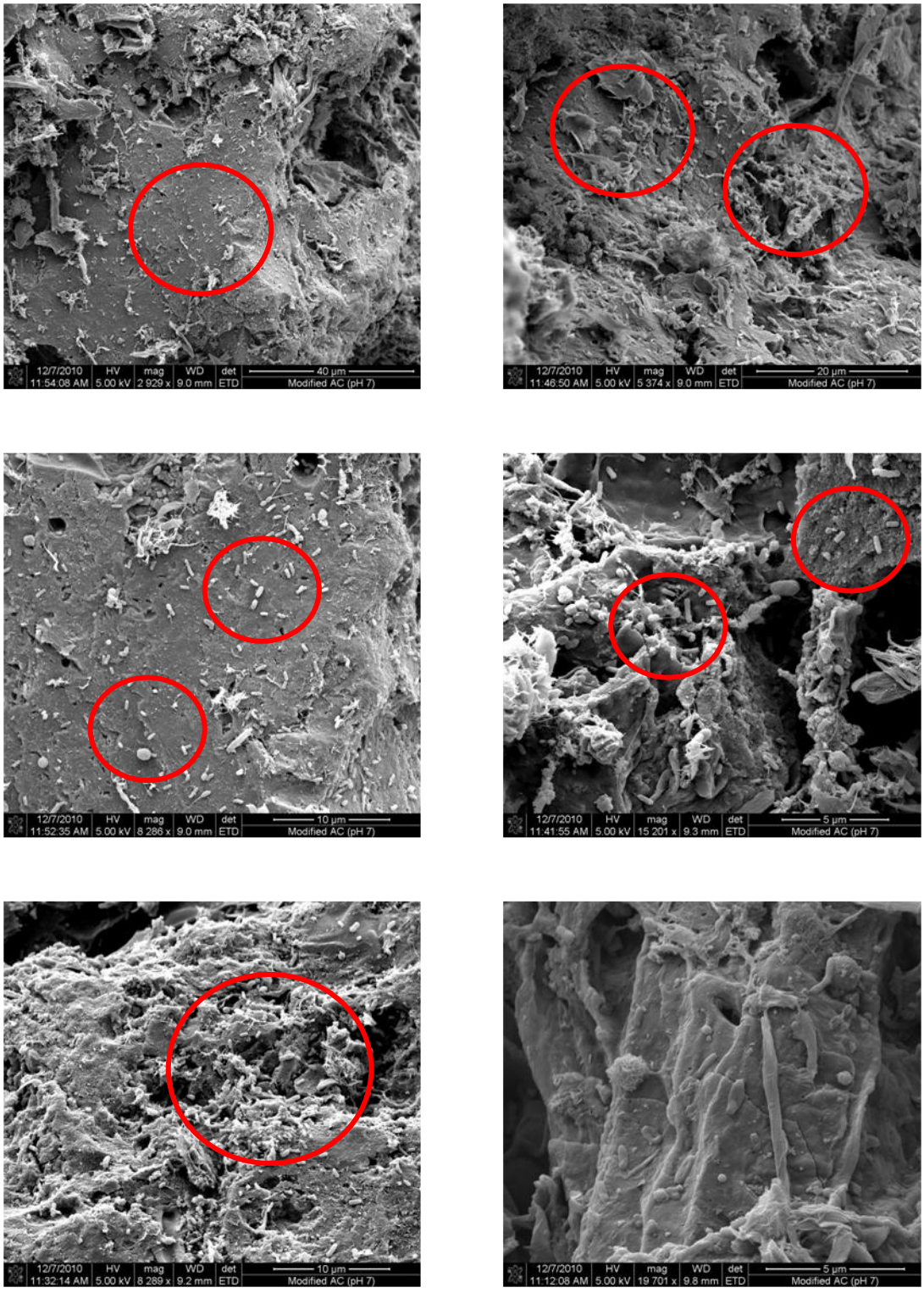


Figure C.5: Modified activated carbon pH 7 (Experiment 1)

EXPERIMENT 2: (CHARGE MODIFICATION AFTER BACTERIA INOCULATION), SEM IMAGES.

Experiment 2 as described previously was performed as follows. After the substratum was added to bacterial solutions, the solutions were adjusted to 3 different pH values (5, 6 and 7). The 3 flasks were then placed in an incubator at 30°C with shaking at 100 rpm, specimens were then taken for analysis after 4 days. The following figures show the SEM images that were taken for the specimens in this experiment.

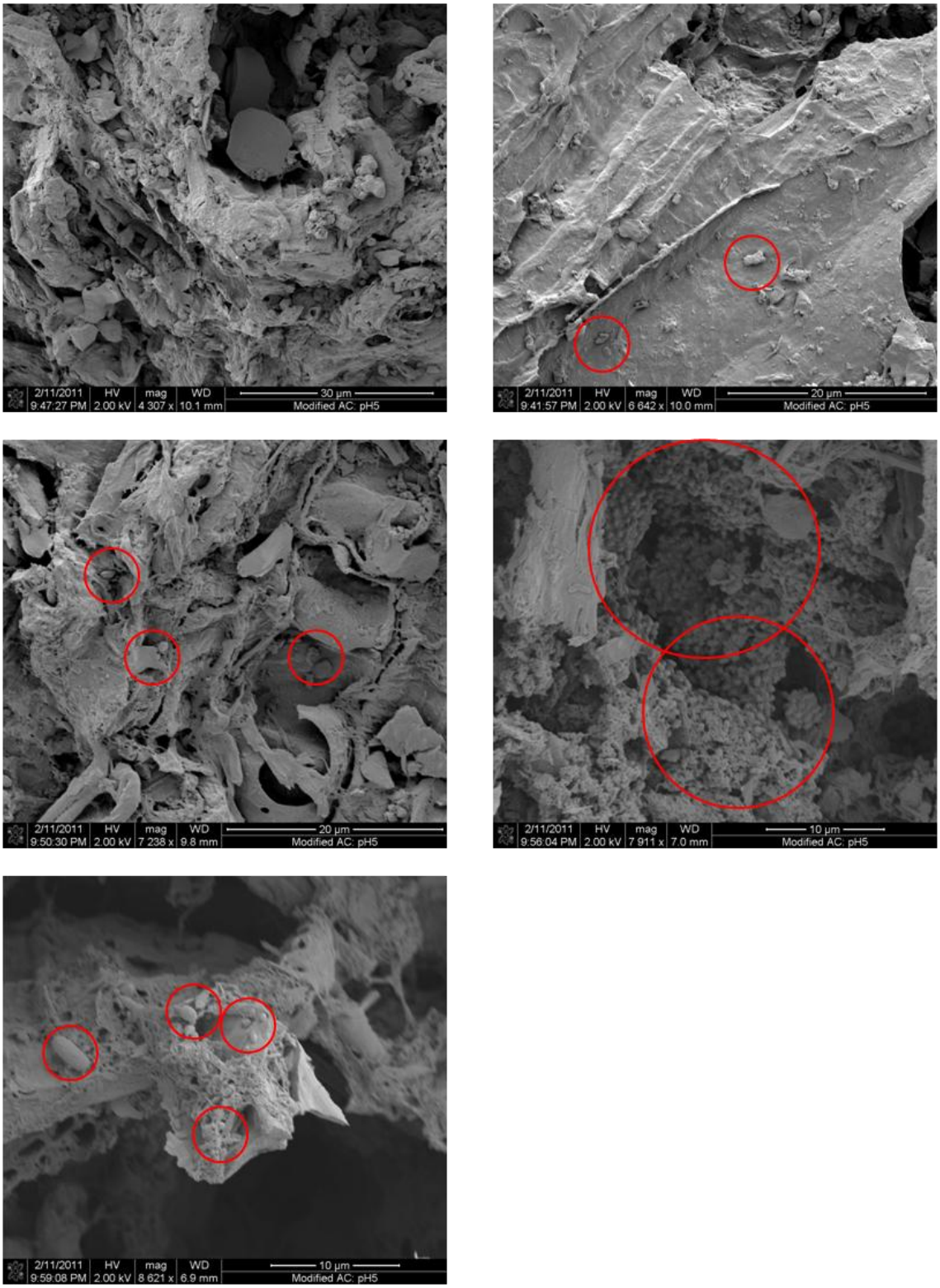


Figure C.6: Modified activated carbon charged to a pH 5 (Experiment 2)

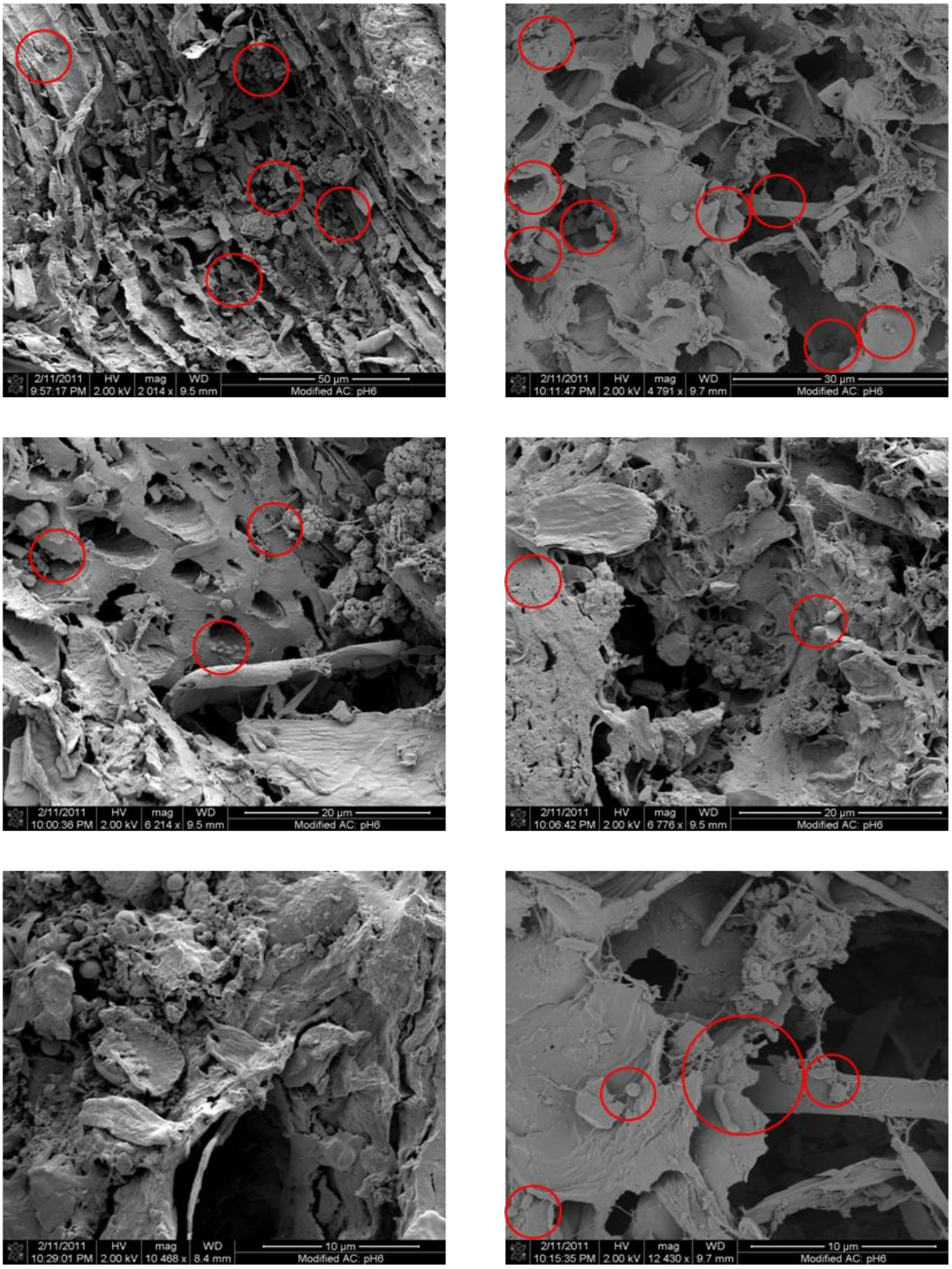


Figure C.7: Modified activated carbon charged to pH 6 (Experiment 2)

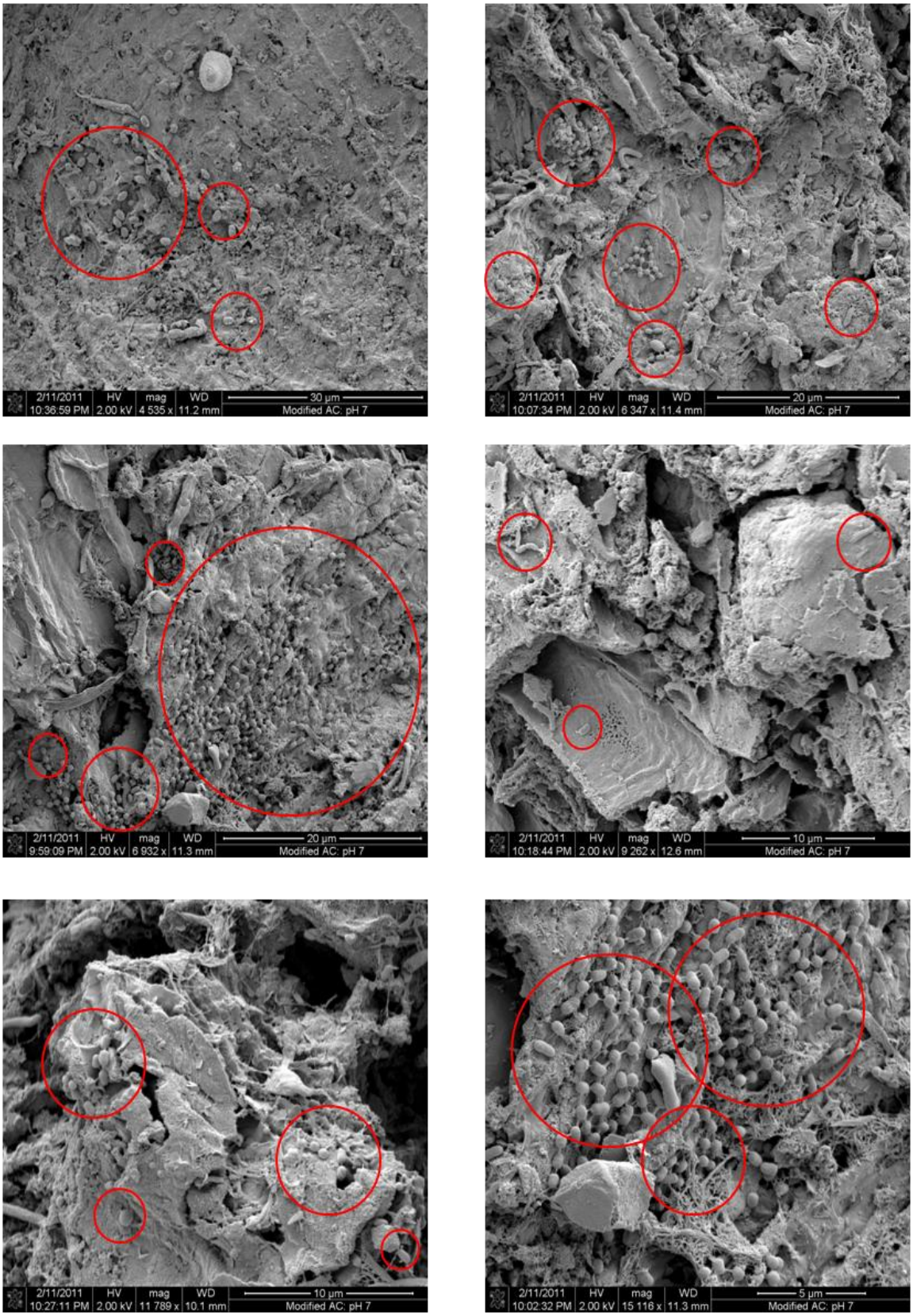


Figure C.8: Modified activated carbon charged to pH 7 (Experiment 2)

EXPERIMENT 3: (CHARGE MODIFICATION OF ACTIVATED CARBON ONLY), SEM IMAGES.

Sterilized water was altered to the desired pH of the activated carbon. The pH was altered using 0.1 mol/l Nitric Acid and 0.1 mol/l Sodium Hydroxide. Activated carbon (0.6 g for each pH) was washed in the modified sterile water for 20 min to ensure the alteration of the surface charge. Upon the completion of charge modification 20 ml of bacteria culture solution (pH 7.2) was added to the conical flasks each containing charged activated carbon. The following figures show the SEM images that were taken for the specimens in this experiment.

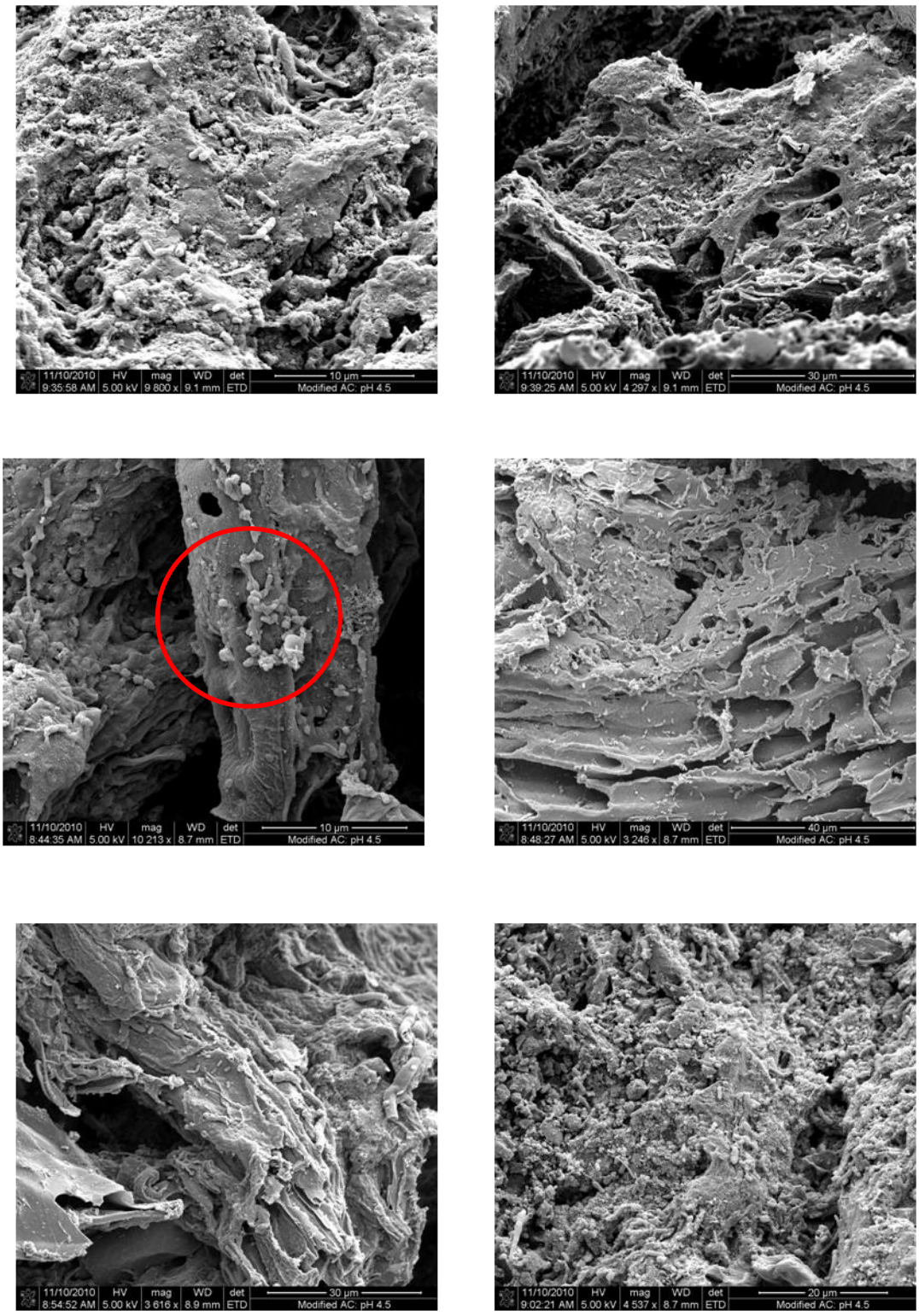


Figure C.9: Modified activated carbon charged to pH 4.86 (Experiment 3)

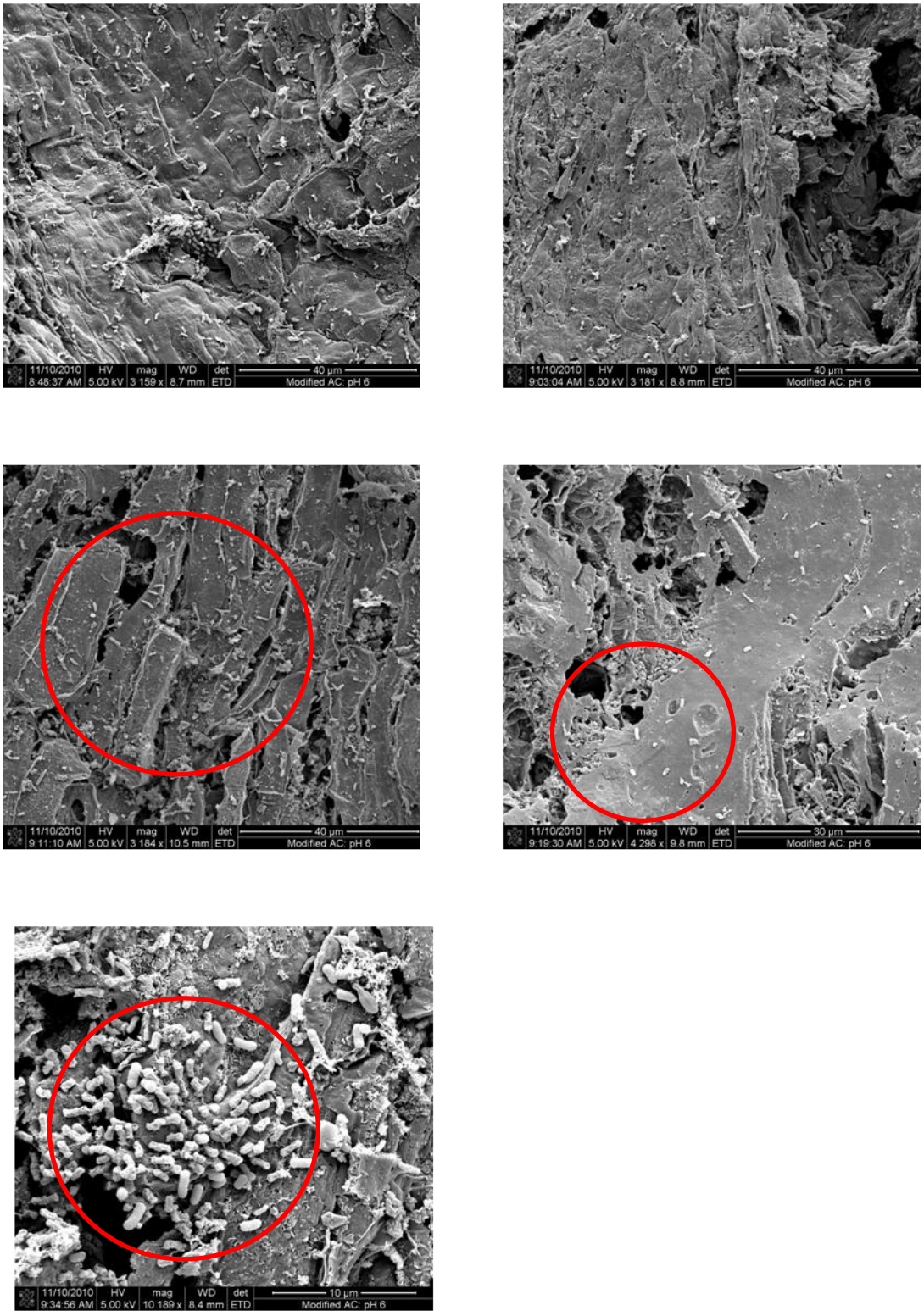


Figure C.10: Modified activated carbon charged to pH 6 (Experiment 3)

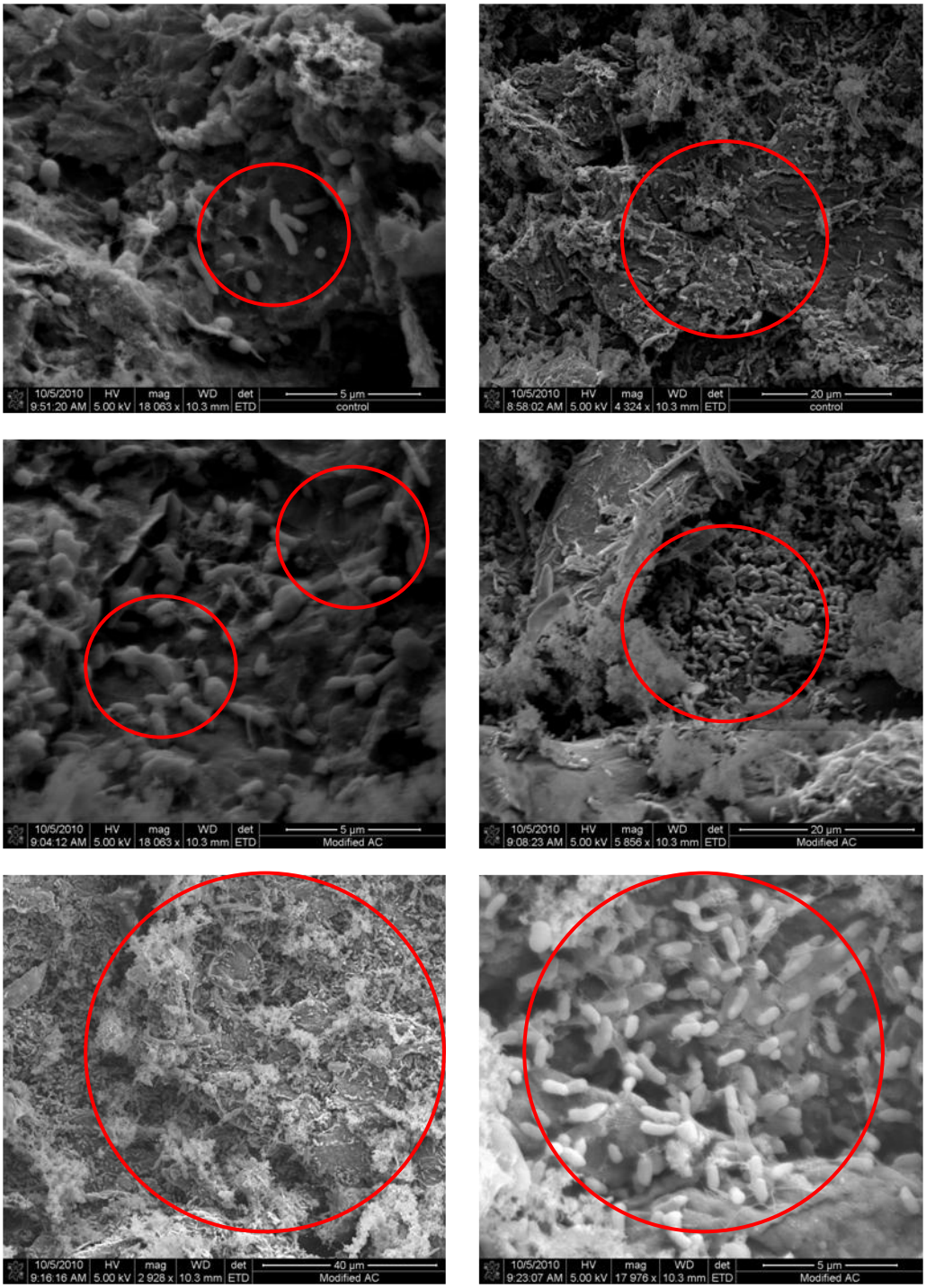


Figure C.11: Modified activated carbon charged to pH 7 (Experiment 3)