



EFFICACY OF FLUIDISED BED BIOFILM BIOREACTOR
IN BIOREMEDIATING DAIRY WASTEWATER

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DECLARATION

I declare that this is my own, unaided work It is being submitted for the degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

Shamini Deva

_____ Day of _____, 2010

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ABSTRACT

Industrial effluents such as that from dairy factories are known to cause major pollution in water bodies which the effluent is discharged into. Nitrogen and phosphorous levels increase and cause eutrophication which results in the death of aquatic life, as well as causing disease in terrestrial animals, and humans. Dairy effluent is rich in organic wastes, and have variable pH due to fluctuations caused by sanitation chemicals. This study served to use an aerobic fluidised bed biofilm bioreactor to treat a synthetic dairy wastewater by utilizing 2 different types of consortia as a bioaugmentation tool. Dairy spoilage isolates were isolated from dairy products, and identified using 16S rDNA identification. Isolates were then evaluated for proteolytic and lipolytic ability using milk agar and lipolytic agar. Isolates exhibiting both proteolytic and lipolytic ability were evaluated for its effect in a synthetic dairy wastewater in flask cultures, each isolate both independently and in combination. Two consortia were chosen which degraded the synthetic medium most efficiently. One consortium contained a Gram-negative and Gram-positive bacteria, while the other consortium contained Gram-positive only bacteria. An appropriate carrier material was then chosen from 5 different types, a 4mm pellet carrier sourced from coal. The aerobic fluidized bed biofilm bioreactor was then set-up, and bioaugmentation was used to degrade the total organic carbon in the synthetic wastewater using first the Gram-positive consortium, then the Gram-positive and –negative consortium, while the pH and flow rate was varied to simulate real dairy wastewaters. Total organic carbon reduction was evaluated, as well as attached and planktonic growth in the bioreactor. The Gram-positive and –negative consortium was successful in depleting TOC in the synthetic wastewater, while the other consortium although degrading TOC, did not degrade it completely. Biofilm growth was sustained when both consortia were used. Each experiment was done in triplicate and statistically evaluated using multiple variable analysis ($P < 0.05$).

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In Memory of my father,

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CHAPTER 1

Introduction

1.1 LITERATURE REVIEW

Waste effluent from all industrial sectors is a major environmental problem, causing eutrophication, pollution, and disease in both humans and animals. Diseases in humans such as methaemoglobinaemia in infants, and interference with the cardiac function in adults are some of the diseases associated with eutrophication (Zayed & Winter, 1998). The bioremediation of industrial effluents is a large sector of research in the recent years. Bioremediation involves the use of microorganisms to transform waste effluents to a cleaner state, which does not pollute the environment. Using biological means of remediation is preferred since costs are lower, and complete mineralization is possible (Jiang *et al*, 2002). Several bioremediation processes are being used currently to treat food processing wastes worldwide (Thassito & Arvanitoyannis, 2001). Although these systems are successful, current economic recession conditions, and interest in environmental conservation, is causing research to divert to those systems which are most economically sound, and those which cause the least harm to the environment.

1.1.1 Eutrophication and pollution caused by wastewaters from the food industry

Eutrophication is broadly defined as “high aquatic biological productivity resulting from the increased input of either nutrients or organic matter” (Tusseau-Vuillemin, 2001). It is a process in which nitrogen and phosphorous concentrations are increased in water bodies due to waste disposal, which results in growth of algal blooms and death of organisms living in the water (Danelwich *et al*, 1998; Tripodo *et al*, 2004; Carta-Escobar *et al*, 2004). Industrial effluents contain several soluble organic compounds. The majority of these compounds are those produced by microorganisms from substrate metabolism, and was not initially present in the influent. It is referred to as soluble microbial products (SMPs). In anaerobic systems, levels of SMPs are lower than in aerobic systems, where it exhibits toxicity. Granular activated carbon (GAC) has been shown to be

the most effective in removing SMPs (Barker, 1999). It is these phosphorous and nitrogen compounds, as well as some organic compounds which contribute largely to eutrophication.

Food processing wastewaters, including dairy processing wastewater, have high concentrations of organic materials, nitrogen concentrations, and high chemical oxygen demand (COD), and biological oxygen demand (BOD) (Cammarota & Freire, 2006). The food industry plays a major role in the eutrophication caused by its wastewaters, however most of the nitrogen and phosphorous present in the wastewater is from the sanitizers and detergents used for cleaning processes (Danelwich *et al*, 1998; Souza *et al*, 2004). Ammonia is one of the common constituents of these sanitisers, and is highly toxic to aquatic life. At high temperatures it results in a high pH in water bodies due to increased photosynthesis, resulting in the death of these aquatic organisms (Craggs *et al*, 2003). Additionally, constituents of dairy wastewaters affect livestock if untreated, causing mastitis in cows, as well as other diseases.

1.1.2. Composition of dairy industry wastes

The main organic waste constituents of dairy effluent are milk and milk products that have been lost in the process, containing the natural milk solids: fat, lactose and protein (Carawan *et al*, 1979; Environmental protection authority, 1997). Dairy wastewater is rich in biodegradable organic constituents, and contain high levels of fats and proteins, which when degraded produce glycerol and long chain fatty acids (LCFAs), which are known to inhibit certain microorganisms (Carta-Escobar *et al*, 2004). Cleaning products add to the effluent waste, and these include alkali detergents such as caustic soda and soda ash; and acid detergents such as muriatic, sulphuric, phosphoric, nitric and acetic acid (Bremer *et al*, 2006). These detergents are also composed of surfactants, phosphate and calcium sequestering compounds (Environmental protection authority, 1997). Acids and surfactants determine biological oxygen demand (BOD). The sanitizers include

chlorine compounds, iodine compounds, quaternary ammonium compounds, and acids. Inorganic constituents include phosphates from the cleaning products; chlorine from the detergents and sanitizing products; and nitrogen from wetting agents (Environmental protection authority, 1997).

The lipid constituent of dairy waste is considered the main problem affecting anaerobic digestion of dairy effluent. Lipids form 60% of the COD of dairy waste and thus determine the efficacy of the bioreactor, since the LCFAs may inhibit the anaerobic biocatalysts (Cammarota & Freire, 2006; Haridas, 2005). The fats may also cause operational problems with the reactors due to the formation of filamentous organisms through reduced oxygen transfer rates (Cammarota & Freire, 2006).

1.1.3. Dairy bacteriology

The groups of bacteria typically found in dairy industries are lactic acid bacteria, psychrotrophic Gram-negative bacteria, Gram-positive spore formers, and *Salmonella*. Raw milk contains pathogenic bacteria such as: *Escherichia coli* 0157:H7, *Streptococci agalactiae*, *Pseudomonas aeruginosa*, *Listeria monocytogenes*, *Salmonella typhimurium*, and *Staphylococcus aureus*. Other pathogens which may enter the milk through infected cows are *Coxiella burnetti*, *Mycobacterium bovis*, *Mycobacterium paratuberculosis*, *Yersinia*, and *Campylobacter*. However after pasteurization, the bacterial content is low in the effluent, but Gram-negative rods may occur post-pasteurisation as a result of spoilage or post-pasteurisation contamination, while Gram-positive bacteria suggest that spores survived heat treatment (Robinson, 1985; Jayarao, 1999). Viruses include Hepatitis A, and Polio type 1, while protozoans found in dairy wastes may include *Giardia*, and *Cryptosporidium parvum* (Pell, 1997).

1.1.4. Process of digestion

1.1.4.1. Anaerobic and aerobic digestion

Anaerobic digestion is the hydrolysis of organic material by a microbial consortium living in an environment with no oxygen. This produces sugars, fatty acids, and amino acids. Carbon dioxide, methane, hydrogen, and ammonia may be produced as by-products (Fig 1.1). Anaerobic digestion converts organic nitrogen compounds to ammonia; sulfur compounds to hydrogen sulfide; phosphorus to orthophosphates; and calcium, magnesium, and sodium to a variety of salts (Burke, 2001). The bacteria allowing this hydrolysis act as biocatalysts, and their presence determines the efficacy of the bioreactors. Three groups of bacteria exist in an anaerobic system, the first is that which ferments the waste and so performs hydrolysis and acidogenesis. The second group is the acetogenic bacteria, and the third group is the methanogenic bacteria (Kansal *et al*, 1998).

The activity of these bacteria in the fluidized bed bioreactor seems to be greatest for acetogenic and methanogenic bacteria in the central region of the bed, and for the bioconversion of milk, high acetic acid yields are achieved by combining cells of *Clostridium thermolacticum*, *Moorella thermoautotrophica*, and *Methanothermobacter thermoautotrophicus* (Angenent *et al*, 2004). These combined species are also less sensitive than single cultures and so can adapt to the fluctuations of wastewater characteristics. Aerobic pretreatment enhances the anaerobic digestion up to 2.5- 4.5 times (Mantzavinos & Kalogerakis, 2005). Less research has been conducted on aerobic processes alone, however, recent advances show aerobic processes may be more favourable than slower anaerobic processes (Abdulgader *et al*, 2007).

Aerobic digestion is the degradation of nutrients by bacteria in the presence of oxygen which produces an end-product of carbon dioxide (Fig 1.2). Aerobic digestion occurs much more efficiently at high temperatures compared to temperatures in the mesophilic range, however can still be achievable at lower temperatures. The 2 types of aerobic treatment include: suspended growth processes, and attached growth processes (Abdulgader *et al*, 2007). In this study we chose the latter. There are many advantages to aerobic digestion compared to anaerobic digestion, such as its ease of operation, lower equipment costs, the lower safety hazard level when cleaning, and its production of gases which are not

explosive digester gases (Water Environment Federation, 2007). Aerobic digestion is also much faster than anaerobic digestion and requires a shorter hydraulic retention time. These factors, along with the renewed interest in aerobic systems for bioremediation, led to the choice of aerobic bioreactor in this study.

1.4.2 Parameters for digestion in anaerobic and aerobic systems:

- pH

The pH is usually kept slightly above neutral pH (7.2- 7.6). When volatile fatty acids (VFAs) accumulate due to methanogenesis not occurring, the pH drops (Holland *et al*, 1987). Some microorganisms are not adapt to living in acidic conditions, and some in alkaline conditions. The death of organisms which are used as a tool in bioremediation would result in unsuccessful remediation of pollutants.

- Hydraulic Retention Time (HRT)

The HRT determines the amount of time available for biofilm growth, and conversion into biogas. The amount of days the waste effluent remains in the tank is called the HRT, and this is equal to the volume of the tank divided by the daily flow (Burke, 2001). HRT is usually 1-2 hours once biofilm formation has developed (Stronach *et al*, 1986). A shorter HRT allows for a smaller reactor, and more favoured economics, without causing biomass washout (Dugba & Zhang, 1999).

- Substrate for biofilm attachment

Porous materials promote biofilm formation (Stronach *et al*, 1986). Colonisation of bacteria onto these substrates depends on the size of the particles and degree of porosity (Anderson *et al*, 1994). Pore size should be 1-5 times the dimension of the microorganism (Anderson *et al*, 1994). When a surface is available for attachment in a reactor, and if it exposed to

fluid flow containing organic molecules, nutrients, and microbes, biofilm formation is initiated. Granular activated carbon (GAC) is known to retain 3-10 times more attached biomass, and biomass is accumulated at a higher rate than with other surfaces, and thus will be used in this study.

- Other parameters include temperature which is important for the survival of certain microbial species, amount and duration of aeration is important for aerobic systems, while the removal of gases in anaerobic processes are essential as well.

1.1.5 Types of digestors used in the food industry

Reactors can be run in batch mode or continuous mode. Batch mode means the system is given a fixed amount of substrate. Concentrations of the products will vary at certain times, and for aerobic digestion carbon dioxide should be removed from the system. Additionally, the pH can be adjusted. The advantage of batch mode is that the growth period is shorter, allowing much less contamination, and cheaper costs since less volume of inoculua is needed than a continuous system. A disadvantage is the time constraints for cleaning and sterilisation between each feed.

In a continuous system, the inoculum is continuously fed into the reactor. This offers a higher degree of control compared to the batch system, and products are more reproducible. However washout can cause loss of optimum growth (Williams, 2002). In industry digestors are typically cylindrical and made of reinforced concrete, and are closed by a fixed or floating lid. They usually carry volumes larger than 4500m³.

1.1.5.1 Anaerobic digesters

Anaerobic reactors have been in use since the 1880's (Langwaldt & Puhakka, 2000). There exists 3 types of anaerobic systems: (1) Batch systems in which

there is a separation between the first phase and second phase, wherein the first phase acidogenesis occurs faster than methanogenesis, and the second phase where the acids are transformed into biogas (Bouallagui *et al*, 2005); (2) Continuous one-stage systems, which are characterised by an acid-forming phase followed by methanogenesis (Bouallagui *et al*, 2005); and (3) Continuous two-stage systems, which separates the acid and methanogenesis phase in two separate reactors (Chen, 1984; Bouallagui *et al*, 2005). Various anaerobic bioreactors are used in industry, and these include:

- Upflow anaerobic sludge blanket (UASB) bioreactor:

Sludge granules are used in this reactor for high residence time, and at the same time proves to be economical. USAB has no bed media, and has previously been used to treat low-strength wastewater (Fig 1.3) (Nicolella *et al*, 2000). UASB been used in distilleries, tanneries, and food processing units (Kansal *et al*, 1998). However, it has a long start-up period and requires a sufficient amount of sludge for start-up (Cordoba *et al*, 1995).

- Inverse fluidised bed

Floating reactors are fluidised by a downflow current of liquid gas bubbles generating downward liquid motion and bed expansion (Buffière *et al*, 2000). Thus expansion also occurs downwards (Fig 1.4).

- Covered lagoon

Solids are broken down more slowly in lagoons because of the warm temperature in which it operates. The methane biogas which is produced, is trapped by a cover over the lagoon (Wright, 2001).

- Fixed film

The reactor has an inert biomass support. By immobilizing the bacteria, biomass is not loss through washout of slower growing cells, and thus

larger volumes of effluent can be treated (Wilkie, 2003). Biogas from this system can be used as a direct energy source or converted to electricity

- Expanded Bed Sludge Blanket reactor (EGSB)

Upflow velocity of effluent causes fluidization. The granules, biogas, and effluent are separated in a three-phase separator which occurs at the top of the reactor, this allows a higher hydraulic load than in USB reactors. It has been used to treat effluent from the biotechnological, chemical, and biochemical industries. It has been successfully operational for the food, chemical, and pharmaceutical industries internationally (Nicolella et al, 2000) (Fig1.5).

1.1.5.2. Aerobic Digesters

Aerobic digestion is characterised by its rapid inactivation of pathogenic microorganisms, high substrate degradation, and low amount of sludge production (Lasik *et al*, 2002). The aerobic bioreactors used currently are:

- Stirred-tank reactors

Air enters the system from the bottom, and agitator system causes mixing. Substrate is continuously added to the system and waste continuously removed (Williams, 2002).

- Airlift reactor systems

Air is pumped into the system from the bottom causing bubbles, and degassed liquid flows downward. The biggest advantage is the large volumes the system can withstand, and the biggest disadvantage is the cost of the system (Williams, 2002).

- Airlift external-loop reactors

This is used during batch phase only. Air enters through the bottom of the reactor. These reactors exhibit good mass and heat transfer as well as low shear rates (Gavrilescu & Tudose, 1997). However, studies suggest

disadvantages during gas sparging, whereby cells are damaged, and biofilms are thinned due to shear forces (Loh & Liu, 2001)

- Trickling Filter

This system is based on the concept of allowing water to trickle through the support medium by gravity. This aerobic system acquires air naturally (Fig 1.6). Support media for this system include pumice, slag, crushed rock, and plastic fills (Langwaldt & Puhakka, 2000). It has limited reactor capacity, so is able to treat large volumes of effluent with low substrate concentration (Nicolella *et al*, 2000). Trickling filters are however, susceptible to environmental stresses and clogging problems (Resmi & Gopalkrishna, 2004).

- Upflow Fixed-Film Reactor

The principle of this system is the growth of biomass in a submerged packaging (Fig 1.7). The biomass obtains nutrient from the upflowing water, and air sparging is used to make the system aerobic. This system is mostly used for bioremediation of groundwater which has been contaminated (Langwaldt & Puhakka, 2000; Moharikar *et al*, 2005).

- Rotating Biological Contactors

Biomass is allowed to grow on rotating discs which are partially immersed in water, and partially in contact with the atmosphere (Fig 1.8). Efficacy of this system is lower than in an upflow fixed-film reactor (Langwaldt & Puhakka, 2000; Moharikar *et al*, 2005).

- Internal Circulation reactor

This reactor has two reactor compartments, one for granular sludge, and one for the gas-liquid separator (Fig 1.9). This system is utilized efficiently for brewery waste effluent in the Netherlands (Nicolella *et al*, 2000).

- Upflow fluidised bed bioreactor

Large amounts of nitrifying bacteria can be retained due to a larger gas-liquid interface. Lower aeration is needed compared to other aerobic bioreactors (Tsuneda *et al*, 2003).

- Activated Sludge process

The sludge and effluent are separated by a mechanical clarifier, and an aeration basin. Air is produced by air blowers or aeration nozzles (Fig 1.10). This system however, has a low toxic organic removal capacity and the system cannot be optimized, making it a less preferred method of bioremediation (Chipasa & Mędrzycka, 2006; Langwaldt & Puhakka, 2000). It also exhibits problems such as sludge bulking, and the requirement of recycling, and regular monitoring (Resmi & Gopalkrishna, 2004).

- Biofilm airlift Suspension (BAS) reactor

Air enters the system and circulates the gas liquid and biofilm through the reactor (Fig 1.11). The system was initially used as an aerobic pretreatment of anaerobic sludge, but later introduced as a three phase independent aerobic system (Nicolella *et al*, 2000). The liquid velocity in

the reactor can be adjusted to optimize bed expansion (Nicolella *et al*, 2000).

- Fluidised bed bioreactor

Biomass is allowed to grow on a granular support medium such as activated carbon. This system relies on high recirculation rates of the effluent, to dilute the concentration to a level which is non-toxic for bacteria. Natural aeration or artificial aeration from air blowers is used. Those fluidised bed bioreactors with carbon as a source have the advantage of a more rapid microbial growth. The fluidised bed system can be used both aerobically and anaerobically (Langwaldt & Puhakka, 2000). However it is much more efficient when used as an aerobic system (Rajasimman & Karthikeyan, 2007). This bioreactor has been used for the aerobic remediation of starch wastewaters, with optimal COD removal found at 48hours HRT, and 94% COD removal achieved (Rajasimman & Karthikeyan, 2007).

1.1.6. Advantages of Fluidised bed bioreactors (FBBRs)

Fluidised bed bioreactors immobilize the biomass on small, porous fluidised particles. FBBRs operate most efficiently at reduced HRT's and increased recycling (Kargi & Karapinar, 1997). Fluidisation also overcomes operating problems such as bed clogging, minimized solid production, high pressure drop, low hydraulic loss, better hydraulic circulation, poor mixing and oxygen transfer, which are commonly encountered in fixed media treatment systems such as trickling filters (Koran *et al*, 2001; Nicolella *et al*, 2000; Pala, 2001). It can be operated at a higher fluidisation capacity than the UASB (Nicolella *et al*, 2000). In the FBBR, particles are in full contact with the liquid phase providing larger surface area for nutrient transfer and utilisation as compared to trickling filters. The maximum loading rate in fluidised bed bioreactors exceeds that of plug flow

reactors, high rate trickling reactors, activated sludge processes, and oxidation ditch processes (Pala, 2001). It also combines the most efficient features of the 2 most common treatment methods applied in industry, the trickling filter and activated sludge (Kargi & Karapinar, 1997). FBBR's allow for suspended as well as attached or immobilized growth, thus enhancing the treatment of wastewaters.

Koran *et al*, (2001) recommended an aerobic fluidized bed reactor to remove residual phenol intermediates in the anaerobic reactor effluent, demonstrating the need for aerobic digestion even when using anaerobic systems. FBBR's are capable of treating both low and high strength waste effluents, and has resistance to altered operating conditions (Pala, 2001). Economically it is much preferred system since the amount and size of reactors are reduced, the cost is reduced (Kansal *et al*, 1998). The FBBR gives greater microbial concentration than rotating disc filter, and trickling filters. All the above mentioned advantages made it the preferred system to be used in this study.

1.1.7. Current systems used for dairy wastewater remediation

Waste stabilisation ponds (WSP) are being used in New Zealand. The system includes an anaerobic pond, and facultative pond. Other WS ponds include: Advanced pond system which consists of 4 ponds: Advanced Facultative Pond (AFP), High Rate Pond (HRP), Alga settling pond (ASP), and Maturation Pond (MP). The ASP gives much more efficient treatment compared to the conventional WSP system (Craggs *et al*, 2003). However, these systems are mostly used for animal dairy wastewaters (Olguín, 2003).

Cyanobacteria or blue-green algae, have been used in anaerobic lagoons to bioremediate wastewater from the dairy industry. Nitrogen removal has shown to be rapid with options for the nitrogen to be recycled (Lincoln *et al*, 1996). The genus *Spirulina* is one of the most utilized cyanobacteria used in wastewater

treatment (Olguín, 2003). Strains with good potential for wastewater treatment include *Rhodobacter sphaeroides*, *Chlorella sorokiniana*, *Spirulina platensis*, *Botryococcus braunii* (Olguín, 2003).

Constructed wetlands are being used on dairy sites in the USA, however nitrogen removal isn't sufficient enough, and this system needs to be optimised (Newman *et al*, 2000).

A relatively new system first tested in Thailand and Japan, utilizes multi-soil layers to treat the dairy wastewater aerobically and anaerobically. The aerobic layers consist of zeolite or perlite, and the anaerobic layers consist of soil mixture blocks. These layers alternate, and the system was found to be cost efficient yet effective, and simple to operate (Pattnaik *et al*, 2007).

1.1.8. Bioaugmentation

Bioaugmentation is a biological tool used in the bioremediation of industrial effluents. It is defined as “The technique for improvement of the capacity of a contaminated matrix (soil or other biotope) to remove pollution by the introduction of specific competent strains or consortia of microorganisms” (Fantroussi & Agathos, 2005). By isolating organisms from the environment in which pollutants originate, appropriate consortia can be combined in order to bioremediate polluted areas (Fantroussi & Agathos, 2005; Loperena *et al*, 2009). These areas can include soils, water bodies, as well as industrial effluents- the sector in which we were most interested. Bioaugmentation has previously been reported as a successful tool for the bioremediation of industrial effluents (Dabert *et al*, 2005; Stephonson & Stephonson, 1992). Different ways of using bioaugmentation include its use in encapsulation, immobilization, or in combination with a surfactant (Fantroussi & Agathos, 2005). The use of bioaugmentation as a tool in the bioremediation of dairy wastewater could be very useful if an appropriate consortium is found, and that is what this study aimed to achieve.

1.1.9 Aerobic granulation

Recent studies have shown the advantage of aerobic granules in the degradation of industrial effluents. These granules are defined as densely packed microbial consortia which perform different roles in the degradation of waste effluents (Liu & Tay, 2004). These granules are usually initiated during microbial self adhesion. Aerobic granules may be important in aiding the degradation of wastes, and it is this characteristic which will be evaluated in the degradation of total organic carbon in this study.

1.1.10 Conclusion

Eutrophication caused by the various sectors of the food industry is on the rise. The development of algal blooms cause nitrification, and destroys much of the aquatic wildlife in water bodies, which surround the industries where waste effluent is released. The constituents of the dairy industry contribute largely to the eutrophication in water bodies surrounding the dairy factories. Improvement in treatment mechanisms of waste effluent of the dairy industry, will allow a significant reduction in environmental pollution and disease within animal, humans, and even plant populations. The advancement of biotechnology has brought with it the utilisation of bioreactors to treat these waste effluents with the use of biological organisms instead of chemicals. Industrial wastewater bioreactors will not only remediate the environment, but may also be used to observe the effects of bioaugmentation and the bioenzymes produced from mixed consortia which are bioaugmented, so that novel chemicals and polymers can be produced (Bramucci & Nagarajan, 2000). This study will serve to highlight the optimum conditions of the aerobic FBBR which will be used to treat dairy wastewater on a pilot-scale laboratory bioreactor.

Aerobic digestion has many advantages, and the use of fluidised bed bioreactors may prove beneficial with regards to costs, bed expansion, and hydraulic retention

time. The fluidised bed bioreactor can be used to treat waste aerobically, or anaerobically, making it flexible for optimization of parameters needed for efficient reduction of total organic carbon load in the wastewaters. Aerobic treatment is already being used in various sectors of the food industry, including the winery sector, for the treatment of olive mill wastewater, brewery wastewater, for the treatment of starch wastewater, and many others. The method is efficient, simple and cost effective, making it a viable method for industries in South Africa, and internationally. Aerobic treatment has also been found to further enhance anaerobic treatment, and many industries use a two-phase bioremediation system to further enhance treatment.

Biofilm-producing bacteria are used in these studies to rapidly breakdown the waste effluent. These bacteria are from a broad category of genera. In this study dairy spoilage bacteria with enhanced biofilm-forming capacity will be isolated, as well as enhanced lipolytic and proteolytic ability. Various combinations of these isolates were mixed together to simulate bioaugmentation in order to create optimum conditions for biofilm formation, and protein and lipid degradation.

The FBBR used in this study will be optimized for efficient operation parameters, and will be tested on a pilot-scale to simulate field conditions of dairy waste effluent. The reduction in total organic load will reflect the efficiency of the system, and the effect of different pH levels due to the constant fluctuating nature of dairy wastewater.

1.2 MOTIVATION

Large dairy factories dispose of their waste effluent into municipal sewers, however some still dispose by means of land irrigation, and disposal in water bodies. This poses a threat to surface and ground water. Since South Africa is a major water user, water should be reused in boilers and cooling systems where it does not affect the bacteriological and chemical quality of foods (Strydom *et al*, 2001). Treatment processes currently used, are unreliable, and capital insecure. The use of FBBR's may prove more beneficial than current systems.

The dairy industry in South Africa consumes approximately $4.5 \times 10^6 \text{ m}^3$ water per annum. 75%- 95% of the water intake volume is discharged as effluent milk, and milk products have exceptionally high COD values $\sim 3800 \text{ mg/l}$ (Strydom *et al*, 2001). These volumes require immediate treatment to avoid eutrophication. Focusing on the dairy industry would allow a large sector of the food industry to be remediated.

Current technologies used for bioremediation are not cost effective and convenient. Optimisation of the fluidised bed bioreactor system may prove more beneficial to the dairy industry and South Africa. It has already proven to be cost efficient, even with the expense of aeration. This pilot study will reflect the efficacy and parameters to be optimised, before it can be tested on a full-scale.

Very little is known about the microflora associated with aerobic digestion, especially with regards to food waste. This project will serve to broaden the knowledge of the microorganisms associated with the dairy industry wastewaters, and the appropriate consortia used to develop optimum bioremediation conditions.

The evaluation of optimum flow rates under varying pHs will be used to observe changes in consortia, or changes in reduction of total organic carbon load of the system. These varying pHs will provide deeper insight into “real” dairy wastewater, the pH of which is constantly fluctuating.

The advance in biotechnology for the dairy industry of South Africa will prove beneficial for the future, and will have a great impact on the economy and environment, while allowing a deeper understanding of the aerobic digestion of dairy wastes using an FBBR.

1.3 OBJECTIVE

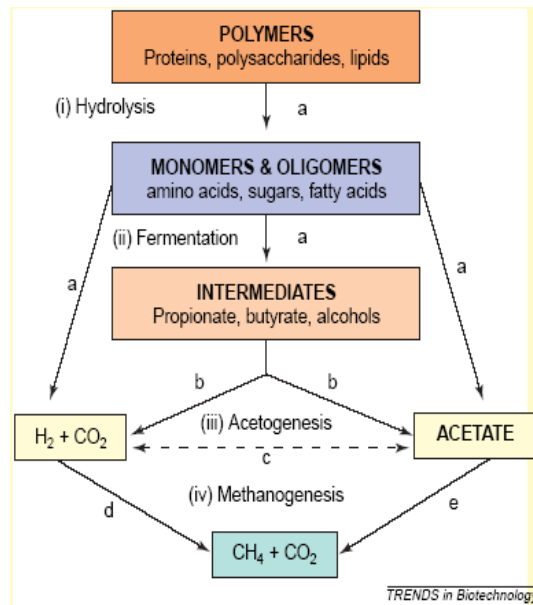
To evaluate efficacy of lab-scale aerobic FBBR on simulated dairy wastewaters using defined cultures, and the analyses of optimum conditions for bioremediation of dairy industry wastewaters.

1.4 AIMS

- 1.4.1 Selection of optimal activated carbon carrier granules, for attachment and growth of bacterial consortia
- 1.4.2 Isolation of dairy spoilage isolates from dairy products, as well as dairy manufacturing processes, and the identification of these isolates by 16S rDNA sequence analysis.
- 1.4.3 Evaluation of most efficient combination of isolates for the degradation of the synthetic milk medium.
- 1.4.4 Set-up and sterilisation of lab-scale aerobic FBBR
- 1.4.5 Development of a synthetic dairy wastewater to be used as growth medium and to simulate dairy wastewater
- 1.4.6 Operation of aerobic FBBR in batch mode, by varying pH and flow rates in order to simulate acid and alkaline shock loads from sanitisers during cleaning-in-place procedures, and to evaluate its effect on biofilm growth and total organic carbon (TOC) degradation
- 1.4.7 Comparison of biofilm development in aerobic FBBR between 2 defined cultures isolated from dairy industry, by counting attached populations as well as planktonic populations

- 1.4.8 Observation of the morphology of attached consortia by scanning electron microscopy
- 1.4.9 Evaluation of TOC content before and after treatment in the FBBR
- 1.4.10 Evaluation of most efficient consortium to be used as a tool in bioaugmentation of dairy wastewater
- 1.4.11 Obtain optimum flow rate and pH for efficient bioremediation and aerobic granulation

a



b

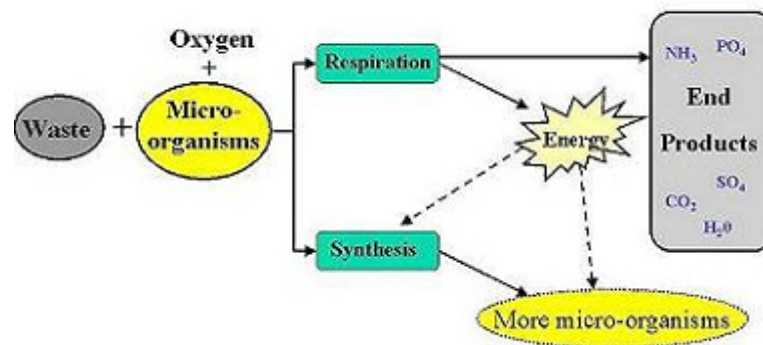


Figure 1.1: (a) Process of anaerobic digestion (Angenent, 2004), and (b) aerobic digestion (Forest Encyclopedia Network)

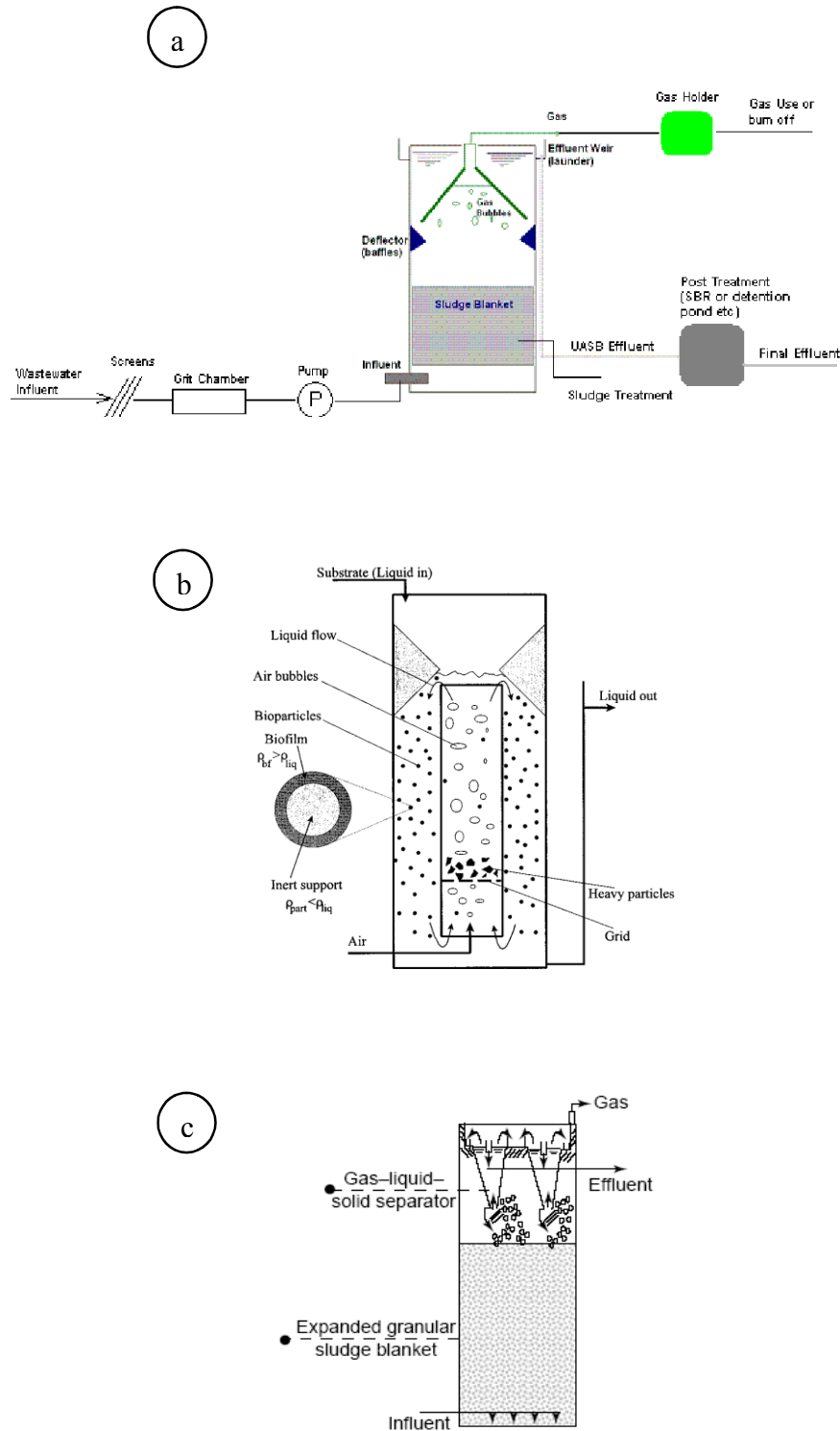


Figure 1.2: Anaerobic digestion systems: (a) Upflow anaerobic sludge blanket reactor (Wastewater Engineering, 2010); (b) Inverse fluidized bed (Kryst & Karamanev, 2001); and (c) Expanded Bed Sludge Blanket reactor (Nicolella, 2000)

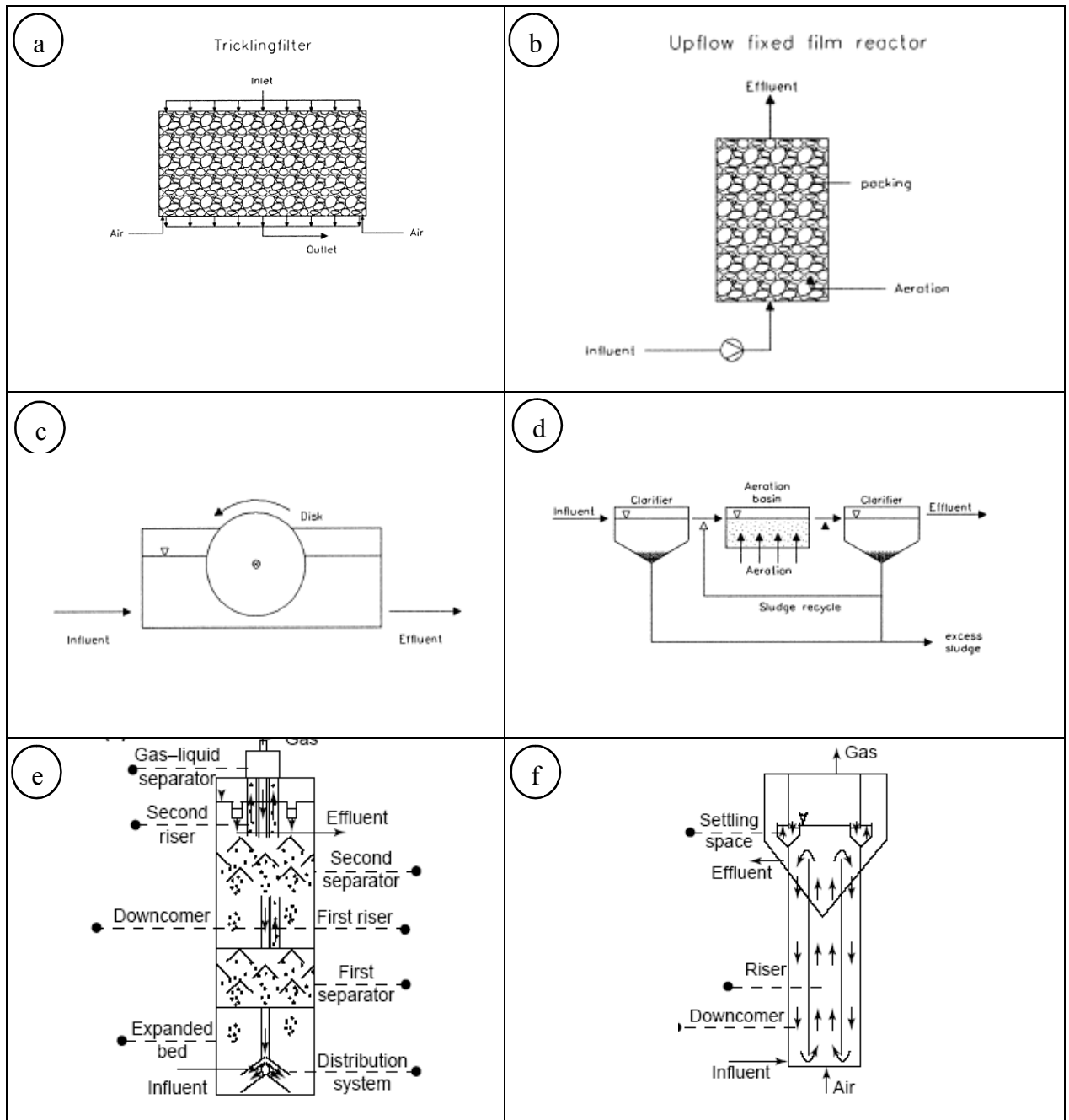


Figure 1.3: Aerobic treatment systems used in industry: (a) Trickling Filter reactor (Langwaldt & Puhakka, 2000); (b) Upflow Fixed-Film Reactor (Langwaldt & Puhakka, 2000); (c) Rotating Biological Contactors (Langwaldt & Puhakka, 2000); (d) Internal Circulation reactor; (e) Activated sludge process; and (f) Biofilm airlift Suspension (BAS) reactor (Nicolella, 2000)

CHAPTER 2

**Pilot Study: Selection of optimal carrier granules and
biofilm consortia for biodegradation of dairy
wastewater**

ABSTRACT

Fluidised bed biofilm bioreactors are novel methods for bioremediation of various types of wastewaters, with the high-strength wastewaters from food industries contributing a major sector. Carrier particles for biofilm development are of utmost importance in these bioreactor systems, as well as the appropriate type or consortium of biodegrading bacteria. In this study, 5 types of activated carbon as carrier particles for biofilms were tested, including: 4mm activated carbon pellets, 3mm activated carbon pellets, and granular carbon (12x40mm) sourced from coal, and granular carbon (8x16mm and 12x30mm) sourced from coconut shell. *Bacillus subtilis* EL39 was chosen as a model bacterium for biofilm formation on the carrier particles. Counts of attached *Bacillus subtilis* EL39 cells on the various carrier particles were determined in conjunction with corresponding planktonic populations *in vitro*. Results from attached and planktonic counts proved that the 4mm activated coal pellet was the most advantageous for the bioreactor system in this study, as it displayed the greatest attachment (5.5 log cfu/ml) as well as the least detachment from the carrier according to plate counts and scanning electron micrographs of this carrier. Bacteria with both proteolytic and lipolytic ability were then isolated from raw milk, raw cheese whey, and alkaline wash solutions used for cleaning-in-place in South African dairy factories. The isolates were identified by 16S rDNA analysis, and included: *Bacillus pumilus* 137 (EU847736), *Bacillus subtilis* C1 (EU860286), *Bacillus pumilus* G1 (EU860287), *Bacillus amyloliquefaciens* G2 (EU 860288), *Bacillus licheniformis* G3 (EU860289), *Bacillus pumilus* G5 (EU860290), *Bacillus cereus* S111 (EU847736), *Bacillus thuringiensis* V1 (FJ235080), *Bacillus cereus* V2 (FJ235081), *Bacillus thuringiensis* V3 (FJ235082), and *Pseudomonas aeruginosa* P1 (EU860291). This study demonstrated an optimal degrading Gram-positive and –negative binary consortium to be *Bacillus subtilis* C1 and *Pseudomonas aeruginosa* P1, and an optimal degrading mixed Gram-positive consortium to be *Bacillus subtilis* C1; *Bacillus amyloliquefaciens* G2; and *Bacillus pumilus* G5 based on how rapidly these consortia degraded a 50% synthetic milk medium.

INTRODUCTION

Dairy wastewaters are high in organic matter such as fat, protein and lactose and thus require remediation. Bioremediation is a common method used in recent times due to the small carbon footprints it creates. Current systems used for dairy wastewater bioremediation include aeration, trickling filtration, and activated sludge (Manyele *et al*, 2008). Aerobic treatments using a fluidised bed bioreactor has been very limited due to the assumption that aeration is costly. However, its high efficiency in chemical oxygen demand (COD) removal makes it a viable option (Arojo *et al*, 2004; McGarvey *et al*, 2007; Adav *et al*, 2008).

The main organic waste constituents of dairy effluent are milk and milk products that have been lost in the process, containing milk solids, fat, lactose and protein (Carawan *et al*, 1979 & EPA, 1997). The lipid constituent of dairy waste is considered the main factor affecting anaerobic digestion of dairy effluent. Lipids form 60% of the COD of dairy waste, and thus determine the efficiency of the bioreactor.

Lipids are also of interest because of the production of methane in elevated amounts (Cavaleiro & Alves, 2001). The degradation of lipids results in long chain fatty acids, which are converted into acetate and hydrogen. The production of long chain fatty acids however, inhibit the activity of methanogenic bacteria, thus slowing down anaerobic systems (Cavaleiro & Alves, 2004). Utilisation of aerobic bacteria may enhance lipid degradation without inhibition of important bacterial enzymes.

Proteolytic and lipolytic enzymatic activity is made easier because of pasteurisation of dairy products, whereby the membrane surrounding fat globules are agitated, thus making the fat molecules more accessible to the enzymes (Aaku, *et al*, 2004). Wastewater treatment is therefore made much simpler in this aspect, and the efficacy of added lipolytic and proteolytic microorganisms in degrading this wastewater can only be enhanced.

Microorganisms secrete many different types of hydrolyzing enzymes, such as the proteolytic and lipolytic enzymes. There are 3 classes of lipolytic enzymes: carboxylesterases, true lipases, and phospholipases (Arpigny & Jaeger, 1999). These enzymes release fatty acids and glycerol, which give the characteristic unwanted flavours and odours, characteristic of spoiled foods.

Proteolytic activities are illustrated by the enzymes trypsin, and chymotrypsin produced by bacteria (Aaku *et al*, 2004). Both of these enzymes are thermostable endopeptidases, belonging to a class of serine proteases, the product of which results in a bitter taste in milk. Microbial proteases are used for the functioning of the cell such as cell growth and differentiation (Gupta *et al*, 2002). It is the most important class of hydrolytic enzymes, with most exhibiting a certain commercial value. Proteases are important for the biodegradation of dairy wastewater as proteins form a major part thereof. *Bacillus spp* synthesise many of these various proteolytic and lipolytic enzymes, most of which are maximally produced in the late exponential and early stationary phases of growth (Chen *et al*, 2004). *Bacillus* and *Pseudomonas* enzymes are known to work optimally in alkaline environments, (Gilbert, 1993; Svendsen *et al*, 1995). It is thus predicted that optimal biofilm growth and biodegradation should occur between pH 7 and 10, and poor growth at pHs less than pH 6. The combination of these bacteria which produce the proteolytic and lipolytic enzymes should prove to be a good degrading consortium for use in the biotechnological industry, as well as applied at industrial level at dairy factories, to control the amounts of organic carbon reaching the natural water systems, and causing eutrophication.

The type of carrier of support material for these microorganisms is vital in FBBR's as it affects the performance of the bioreactor (Arnaiz *et al*, 2006). The attachment of cells onto carrier material is determined by many factors, some being the hydrophobicity or surface chemistry, porosity, pore size distribution, and surface area of the carrier (Qureshi *et al*, 2005). Granular activated carbon

encompasses these traits and is used in the bioremediation of industrial wastewaters, drinking water, and various other instances (Camper *et al*, 1986).

The aims of this study were to find the optimal carrier particle (GAC) as support substrate, and to develop appropriate binary and mixed consortia to be used as inoculums in the synthetic medium for the FBBR, to demonstrate bioremediation capability.

MATERIALS AND METHODS

Bacillus subtilis EL39 as a model bacterium for biofilm formation

Bacillus subtilis is a Gram-positive rod. It is a well recognised and researched organism, as well as an important model organism. It is a common spoilage organism of dairy products due to its biofilm development in production pipelines, as well as on dairy processing equipment (Flint *et al*, 1997). The various enzymes produced by *Bacillus spp.* are already widely used in many commercial sectors. These include heavy metal bioremediation of soil, oil recovery, biocontrol, and biotechnological applications (El-Safey & Abdul-Raouf, 2004; Al-Ajlani, *et al*, 2007). The use of this organism in this study for its biofilm-forming capability, is essential for the determination of an appropriate support substrate. With this organism being widely studied, the type and rapidity of growth can be well understood.

Physical properties of carrier granules

The 5 types of carriers investigated are represented in Fig 2.1 and include:

Envirocarb AP3-60 (3mm) and 4mm Envirocarb AP4-60 (Carrier I and V), are both pellet-shaped carbon carriers which are produced from coal, and are mostly used for air and gas purification. The porous coal is produced from a high

temperature steam activation which develops a larger surface area for microbial attachment. It has a high loading capacity for organic carbons, and low outlet of emissions.

Aquacarb 208EA sized 12x40 (Carrier II) is a coal-based granular activated carbon, used for purification of liquids, and has many industrial applications. The adsorption capacity allows for effective removal of organic constituents, taste and odour. Excellent resistance to abrasion, makes it commonly used for potable water treatment, groundwater remediation, and industrial applications where removal of organics is required.

Goldcarb 207C sized 8x16 and 12x30 (Carrier III and IV) is a coconut-based granular activated carbon, which is used in the recovery of gold from cyanide solution. It is manufactured from particular grades of coconut shell, and has rapid adsorption kinetics.

Biofilm development on carrier particles

Bacillus subtilis EL39 was inoculated into 50ml Nutrient Broth (NB) (Biolab; Midrand, South Africa), and incubated overnight at 30°C. From the overnight culture, 200µl was used to inoculate 5 flasks each containing 5g of 1 type of 4mm activated coal pellets (I), granular coal (12x40) (II), granular carbon (8x16) (III), granular carbon (12x30) (IV), or 3mm activated coal pellets (V), and 100ml NB. Flasks were incubated for 24h at 30°C and 1500rpm on an orbital shaker. After 24h, 1g of activated carbon was removed from the NB to determine attached cell populations, by rinsing in sterile dH₂O, and shaking by hand for 10min, in 20g glass beads and 10ml sterile Quarter-strength Ringers Solution (Oxoid Ltd, Basingstoke, Hampshire, England) (Lindsay & von Holy, 1997), to dislodge the biofilm. The solution was left to stand for 10min at room temperature to allow for cell recovery, followed by serial dilutions and plated in duplicate onto Nutrient

Agar (NA) (Biolab, Midrand, South Africa), using the droplet plate technique (Lindsay *et al*, 1998). To enumerate planktonic cells, 1ml of nutrient broth was removed from the 24h culture, and serial dilutions prepared before plating onto NA. This procedure was repeated in triplicate and results analysed by multifactor ANOVA using Statgraphics v15.2 (Centurion) using a 95% confidence interval.

Scanning electron microscopy

From each extracted activated carbon, 0.5g was rinsed in sterile dH₂O, and fixed in 3% gluteraldehyde for 18h at room temperature. Dehydration was then performed by a graded ethanol series, as described by Lindsay and von Holy, (1999), (20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 95% for 10min each, and 100% overnight). Each sample was then subjected to critical point drying, after which it was mounted and coated with thin carbon and normal gold or palladium. Samples were viewed on the Joel 840 Scanning Electron Microscope (Jsm840, Jeol Ltd, Tokyo, Japan).

Isolation of potential dairy biodegrading bacteria

Raw milk samples, and cheese whey samples were incubated at 25°C, 30°C, and 37°C for 18h, 24h, 72h, 1 week and 2 weeks. At each time interval, a serial dilution was prepared of each sample, and plated on Tryptone Soy Agar (TSA) (Biolab; Midrand, South Africa). The plates were incubated overnight, and colonies selected for further identification. After preliminary identification by Gram staining, pure colonies were plated onto milk agar [5g skim milk powder, 1g Bacteriological Agar (BA) (Biolab; Midrand, South Africa), 50ml sterile dH₂O] (Lindsay *et al*, 2000a), to determine proteolytic ability, or onto lipolytic agar [12g BA- Biolab, 5g peptone, 3g yeast extract, 50g unsalted butterfat, 60mg Victoria Blue stain, 1l sterile dH₂O] to determine lipolytic ability (Lindsay *et al*, 2000a). Plates were incubated at 25°C for 48h. Proteolysis was indicated by clear zones on milk agar around the colonies after flooding with 10% acetic acid. Blue

zones around and within bacterial colonies on lipolytic agar indicated lipolysis. Ten strains which exhibited both proteolysis and lipolysis were subjected to 16S rDNA for further identification (Christison *et al*, 2007). The positive control for proteolysis and lipolysis was *Bacillus cereus* DL5 (AF363441), and negative control was *Lactobacillus brevis* (EU169994) (Fig 2.2).

Identification of potential dairy bioremediation bacteria by 16S rDNA sequencing

DNA from 10 isolates was extracted, and amplified as previously described by Christison *et al*, (2007).

To extract the DNA, 40µl filtered distilled water, 20µl filtered chloroform, and 1 colony of an isolate were mixed, and gently tapped. It was placed on ice for 20min, and spun down at 12000rpm for 5min at room temperature. The supernatant was used as DNA template for PCR reactions. The supernatant was used in combination with the following primers: Bac 27F (5'- AGA GTT TGA TCM TGG CTC AG-3'), and reverse primer, U1392R (5'-ACG GGCGGT GTG TRC-3'), and 2X PCR master mix (Fermentas Life Sciences, Burlington, Ontario, Canada; www.fermentas.com). PCR amplification was performed at temperature cycles described in Christison *et al*, (2007). Sequences were analysed by BLAST (<http://www.ncbi.nlm.nih.gov/GenBank/>) against 16S rDNA sequences from GenBank, and a rooted phylogenetic tree constructed using DNAMAN version 4 (Lynnon Biosoft, Montreal, Quebec, Canada).

Selection of the most efficient proteolytic and lipolytic isolates

Isolates were rated according to how rapidly they grew in, and visibly changed a synthetic milk medium consisting of an autoclaved solution of 65g full-cream milk, diluted with 1l dH₂O. A loopful of colony of each of the 10 positive strains were inoculated into 75ml of the synthetic milk medium, and incubated at 30°C (1500rpm). Changes in colour of the medium, was observed at 24h and 48h. In

addition, populations of the isolated dairy bioremediation bacteria were also tested in a similar manner. To develop an appropriate binary consortium, the 10 positive proteolytic and lipolytic isolates were inoculated in pairs into the synthetic medium, and incubated at 30°C at 1500rpm. The most rapidly degrading binary culture was chosen as the inoculum for the binary consortium run of the bioreactor. Similarly, several populations of the chosen dairy spoilage isolates were combined and incubated at 30°C at 1500rpm on an orbital shaker. As with the binary consortium, the most rapidly degrading mixed consortium was chosen as inoculum for the mixed consortium run of the bioreactor.

RESULTS AND DISCUSSION

Biofilm development on carrier particles

Cell counts on the 4mm activated coal pellet was the highest overall (5log cfu/ml), and therefore was most efficient for attachment (Fig 2.3). The attached cell counts were indicative of much more biofilm attachment on carrier I compared to the other 4 carrier types. The amount and size of pores which allow for attachment of bacteria, seemed to be influenced by the source of carrier material, with those carriers derived from fruit, being more porous than that derived from coal. Figure 2.3 showed that higher counts were obtained on the granular coal (II), than on both types of granular carbon carriers (III, IV) although no significant difference was observed ($P < 0.05$). The texture or the adsorption properties of the coal may have had an effect on attachment of cells, causing the coal carrier to be more favoured for attachment than the coconut-sourced carbon carriers. The size and distribution of pores also appeared to have played a role in attachment of cells. Daud & Ali (2004), reported a greater amount of macropores on coal carriers compared to carriers derived from coconut or other fruit, which contains micropores. When comparing the larger amount of attachment on coal carriers in this study, it's plausible that these macropores had a positive influence on the growth of bacteria. However a statistically significant difference ($P < 0.05$) between all 5 carriers was not observed. The absence of significant difference (P

< 0.05) between attached and suspended cell counts indicated equal attachment and detachment on granule IV, preventing the biofilm bed from growing. A greater detachment of cells from carriers III and IV, than attachment indicated the poor absorption on these carriers. These carbon carriers (III and IV), are produced from coconut. Previous studies have proven these support materials made from coconut to be advantageous for attachment because of the greater surface area on support materials made from fruit, as a result of the amount of micropores (Aygün *et al*, 2003; Daud & Ali, 2004). However, the coconut-based support substrates in this study contradicted these reports, and were not observed to be favourable for attachment. Biomass adhesion on the carrier I, the 4mm coal carrier, proved to be the highest. The larger surface area and porosity allowed it to be more favourable than the other three types of carriers. The large pore size on this carrier provided a larger surface for the growth of *B. subtilis* EL39, surface chemistry, and the vast amount of crevices, due to its rough texture all played a role in the increased amount of attached cells.

Scanning electron microscopy

Sparse attachment of cells was observed by scanning electron micrographs on carrier IV, the 3mm coal pellet, while on carrier I, the 4mm coal pellet, much greater amount of attached cells could be observed (Fig 2.4a). Given the rough texture of the 4mm pellet and larger surface area compared to the other carriers, growth was expected to be pronounced. Barnes *et al*, (1999) suggested that surface roughness played a role in adhesion of cells onto support material conditioned with milk, as it offered protection from shear forces, and increased the surface area of the carrier. The 3mm pellet, although only slightly smaller in size, was much smoother in texture, offering no crevices for better adsorption. The other 3 carriers supported growth, although not as vast as the 4mm pellet (Fig 2.4b-e). The granular carriers (II, III, and IV) although having a much smaller surface area than the pellets, showed greater attachment counts than the 3mm pellet. This could be attributed to surface texture, where again it is proven that the

smooth surface of the 3mm pellet was disadvantageous for attachment of cells, regardless of its large surface area.

There was no significant difference ($P < 0.05$) between attachment of cells on each of the 5 types of activated carbon carriers, and also no significant difference between the attached and planktonic cell counts of each of the 5 carriers. This indicated that statistically, either one of the carriers could be used, however based on higher cell counts on TSA plates, and the greater attachment observed on the carrier I in SEM images, carrier I was most appropriate for this study. Carrier I (4mm coal pellet) was therefore chosen to serve as support material in the aerobic FBBR used in chapter 3.

Identification of proteolytic and lipolytic dairy spoilage bacteria by 16S rDNA

The 10 selected isolates showing positive proteolysis and lipolysis, were identified as: *Bacillus (B.) pumilus* 137 (EU847736), *B. subtilis* C1 (EU860286), *B. pumilus* G1 (EU860287), *B. amyloliquefaciens* G2 (EU860288), *B. licheniformis* G3 (EU860289), *B. pumilus* G5 (EU860290), *B. cereus* S111, *B. thuringiensis* V1 (FJ235080), *B. cereus* V2 (FJ235081), *B. thuringiensis* V3 (FJ235082), and *Pseudomonas (P.) aeruginosa* P1 (EU860291)) (Table 2.1). A Gram-positive and Gram-negative phylogenetic rooted tree was constructed (Fig 2.4a, b) to identify genetic similarity between other organisms.

The *Bacillus* and *Pseudomonas* species are commonly found in spoiled dairy products (Aaku *et al*, 2004), so it is not unexpected that the dominant species found in all the various dairy product isolations, was the *Bacillus* species: *B. pumilus* 137, *B. subtilis* C1, *B. pumilus* G1, *B. amyloliquefaciens* G2, *B. licheniformis* G3, *B. pumilus* G5, and *Pseudomonas aeruginosa* P1.

The phylogenetic analysis of the Gram-positive isolates, indicated that *B. pumilus* 137 (EU847736), *B. pumilus* G1 (EU860287), and *B. pumilus* G5 (EU860290), were 99% genetically similar, and 90% related to *Bacillus pumilus* CT10 (EU660362), and *Bacillus pumilus* SS-02 (EU624442) which were both isolated from coral reefs. The coral being an ideal place for biofilm attachment with its porous structure. *B. subtilis* C1 and *B. amyloliquefaciens* G2 were 90% related to *Bacillus amyloliquefaciens* W30-21 (AB300814) and *Bacillus subtilis* ZJ06 (EU266071), which are fermentation related organisms, indicating the relation of these to degrading microorganisms.

Phylogenetic analysis of the Gram-negative isolate *P. aeruginosa* P1 indicated that it was 90% genetically similar to *Pseudomonas aeruginosa* CS1CO (DQ304683), which was previously used for bioremediation in an aerobic fluidised bed bioreactor, suggesting the use of *P. aeruginosa* P1 in bioaugmentation in aerobic FBBR's may be advantageous. It was also distantly related to *Buttiauxella agrestis* HS-39 (DQ440549), which is a β -galactosidase producer, essential in milk degradation. Both of these relations prove *P. aeruginosa* P1 may be useful in the biodegradation of dairy wastewaters, because of its degrading enzymes.

The use of *Bacillus subtilis* EL39 as a model organism, proved beneficial since majority of the isolates identified by 16S rDNA were from the *Bacillus spp.* The most efficient binary consortium consisted of a *Bacillus subtilis*, while the mixed consortium consisted of 3 *Bacillus* isolates, proving this species to be highly efficient in degradation of dairy waste.

Selection of most efficient proteolysing and lipolysing isolates

A medium characteristic of undergoing proteolysis turns a clear yellow colour from its original opaque white (Kohlmann *et al*, 1990). Enright *et al*, (1999),

reported a translucent colour in UHT milk after 28 days of incubation due to proteolysis. Our study observed similar characteristics in samples undergoing proteolysis. It also develops a creamy white precipitate settled at the bottom of the flask. A control is shown in Fig 2.6a, compared to 5 of the most rapidly proteolytic and lipolytic isolates and the effect it had on the synthetic medium (Fig 2.6b-f). The 5 selected isolates were *B. subtilis* C1 (EU860286), *B. amyloliquefaciens* G2 (EU 860288), *B. licheniformis* G3 (EU860289), *B. pumilus* G5 (EU860290), and *P. aeruginosa* P1 (EU860291).

Selection of binary and mixed consortium

The binary pair which degraded the synthetic medium most rapidly, was the combination of *P. aeruginosa* P1, and *B. subtilis* C1. The control synthetic medium (Fig 2.7a) was opaque white, and the change in colour to a more yellow colour was indicative of protein degradation (Kohlmann *et al*, 1990). The binary combination chosen degraded the medium within 24h, and turned it into a clear yellow medium, with a white precipitation at the top layer (Fig 2.7b). Other pairs in combinations did not degrade the synthetic medium as rapidly, and as efficiently as this combination.

For the mixed consortium, *B. subtilis* C1, *B. pumilus* G5, and *B. amyloliquefaciens* G2 degraded the synthetic medium most efficiently (Fig 2.7c). This *Bacillus spp* consortium will prove useful for the reduction of contamination in the bioreactor, due to the antimicrobial agents it produces (Foglar *et al*, 2005). Literature reports many studies suggesting the effectiveness of mixed cultures which have been efficient in reducing nitrate, BOD, and COD (Carta *et al*, 1999; Foglar *et al*, 2005), thus the combination of these three *Bacillus* species may demonstrate enhanced degrading capabilities. The types of enzymes present in each *Bacillus spp* used in this study are important for the biodegradation of the synthetic milk medium, as well as for genetic engineering, should the need arise

to produce these enzymes commercially for use in FBBR's. The enzymes secreted are listed below:

B. amyloliquefaciens contains extracellular enzymes such as α -amylase, isoamylase, mannanase, xylanase, metal protease, serine protease, alkaline phosphatase, and ribonuclease (Priest, 1977). All these are important enzymes for the degradation of proteins.

B. subtilis contains α -amylase, arabinase, cellulase, dextranase, levansucrase, maltase, pectate lyase, xylanase, aminopeptidase, esterase, metal protease, serine protease, β -lactamase, alkaline phosphatase, ribonuclease, 3-nucleotidase, and 5-nucleotidase (Priest, 1977). Commercial applications of these enzymes include: cosmetics, pharmaceuticals, detergents, food, alcohol, baking, brewing, feed, leather, and photographic waste (Gupta *et al*, 2002). All of these characteristics make it optimal for degradation for both proteins and lipids.

B. pumilus has cellulase, lichenase, serine protease, and ribonuclease (Priest, 1977). As part of the mixed consortium, this organism will be a degrader of mainly proteins in the synthetic medium.

B. licheniformis contains α -amylase, aminopeptidase, serine protease, and β -lactamase. The enzymes have commercial applications in silk degumming, denture cleaners, detergents, and food (Priest, 1977; Gupta *et al*, 2002), proving this organism to be beneficial for protein degradation in waste, however will not be used in this study as it was less efficient in degrading the synthetic dairy medium, than then the *Bacillus spp* chosen for the 2 consortia.

B. cereus contains β -amylase, metal protease, β -lactamase, β -1,3-glucanase, alkaline phosphatase, ribonuclease, 5-nucleotidase, and phospholipase-C (Priest, 1977; Lindsay *et al*, 2000a), which would be valuable for the degradation of both fats, and proteins. However since this is a potentially pathogenic organism (Christiansson *et al*, 1989), its industrial use would prove to be a safety hazard, and has thus been eliminated from both of our consortia.

Pseudomonas aeruginosa is important in many industries for its lipolytic activity, which causes spoilage of milk due to the affect of its lipases (Gilbert, 1993). Phospholipases are the main type of lipolytic enzyme found in *Pseudomonas aeruginosa*. The combination of *P. aeruginosa* P1 and *B. subtilis* C1 was most effective for degradation as a binary pair, and the strong lipolytic activity of the phospholipases *P. aeruginosa* P1 secretes, combined with the strong lipolytic and proteolytic activity of *B. subtilis* should enhance the degradation of total organic carbon. The *B. subtilis* C1 and *P. aeruginosa* P1 binary combination was not unexpected, since *B. subtilis* is known to have elevated proteolysing properties compared to other species (Mayda *et al*, 1985; Bendicho *et al*, 2003), and *P. aeruginosa* has elevated lipolysing effects (Svendson *et al*, 1995). The combination of the 2 relevant degrading enzymes will be essential for biodegradation of the wastewater, especially since both organisms have lipolytic ability and lipids are the most problematic in wastewater degradation (Janczukowicz, *et al*, 2007). The lipolytic enzymes from these 2 bacteria are 2 of the most studied along with *Staphylococcus* (Arpigny *et al*, 1999), so upscaling the production of these enzymes for commercial use, and understanding its biochemical nature will not be problematic. Furthermore, *B. subtilis* has a strong proteolytic ability, and is highly specific, making it a much more potent degrader of proteins (Mayda *et al*, 1985).

The compatibility of these organisms to form biofilm in unison, is important, and in order for isolates to grow and proliferate well with each other is dependant on

many factors, and with biofilms one of these factors is the quorum sensing between the cells. The type of quorum sensing utilised between the binary Gram-negative and Gram-positive pair, was most likely the AI-2 system which is widely used between all cells (Parsek & Greenburg, 2005). Quorum sensing in *P. aeruginosa* has shown to be important to biofilm formation in relation to the types of biofilm formed, being either structured or flat (Parsek & Greenburg, 2005). The addition of *P. aeruginosa* P1 to this binary consortium is an obvious contributor to the rapid degradation of the synthetic wastewater. It may also be that the presence of one of the species in the binary consortium is promoting the attachment of the other, and this could be why they are so efficient in degrading as a pair rather than independantly (Geesey *et al*, 1992). Coaggregation occurring between the cells also results in the appropriate attachment of a population of cells, thus enhancing the rate of biofilm formation (Geesey *et al*, 1992).

However, the *Bacillus* consortium chosen for the mixed consortium run of the bioreactor is characteristic of coaggregation, and the micrograph in Fig 4c is a good example of the kinds of “bridges” they build between each other to allow biofilm formation. *B. subtilis*, *B. licheniformis* and *B. pumilus* are known to behave differently during attached and planktonic phase, where they produced much more antimicrobial compounds as well as certain pigments in attached phase which were not as apparent in planktonic phase (Morikawa, 2006). The production of compounds using a consortium of one type of species such as *Bacillus*, may prove significant for the biodegradation of the wastewater. The type of quorum sensing used in this consortium would be a peptide-based signaling system, such as that found in Gram-positive species (Parsek & Greenburg, 2005). Bioaugmentation of different types of bacteria is often used in the degradation of industrial wastes, and these quorum signals aid in the effective degradation of wastes, by allowing the bacteria to work in unison. Studies have shown bioaugmentation to be advantageous in the treatment of dairy wastewater (Loperena *et al*, 2007), and the bioaugmentation of the isolates chosen in this

study for the binary and mixed consortia may prove beneficial in the industrial degradation of wastewater.

The type of support material, and combination of each of the binary and mixed consortia, should elevate degradation of the synthetic dairy wastewater, while allowing growth of these consortia to proliferate, and possibly form aerobic granules in the bioreactor, increasing degradation even further.

CONCLUSION

- The 4mm coal carrier carbon pellets (Carrier I) exhibited the highest attachment compared to the 4 other granular carbon carriers.
- A binary consortium consisting of 2 different species: *Bacillus subtilis* C1, and *Pseudomonas aeruginosa* P1, was most efficient in degradation of the synthetic milk wastewater medium.
- 3 *Bacillus* species worked proficiently in degradation of the synthetic waste medium, to develop the mixed consortium. These were: *B. subtilis* C1, *B. pumilus* G5, and *B. amyloliquefaciens* G2.

Table 2.1: Source, identity, and proteolytic and lipolytic ability of dairy spoilage isolates

Isolate	Source	Gram	Lipolytic	Proteolytic	16S rDNA identification	Accession number
137	Alkaline wash	+	+	+	<i>Bacillus pumilus</i> S137	(EU847736)
C1	Raw cheese whey	+	+	+	<i>Bacillus subtilis</i> C1	(EU860286)
G1	Gouda cheese	+	+	+	<i>Bacillus pumilus</i> G1	(EU860287)
G2	Gouda cheese	+	+	+	<i>Bacillus amyloliquefaciens</i> G2	(EU860288)
G3	Gouda cheese	+	+	+	<i>Bacillus licheniformis</i> G3	(EU860289)
G5	Gouda cheese	+	+	+	<i>Bacillus pumilus</i> G5	(EU860290)
P1	Raw unpasteurised milk	-	+	+	<i>Pseudomonas aeruginosa</i> P1	(EU860291)
V1	Pasteurised vanilla milk	+	+	+	<i>Bacillus thuringiensis</i> V1	(FJ235080)
V2	Pasteurised vanilla milk	+	+	+	<i>Bacillus cereus</i> V2	(FJ235081)
V3	Pasteurised vanilla milk	+	+	+	<i>Bacillus thuringiensis</i> V3	(FJ235082)

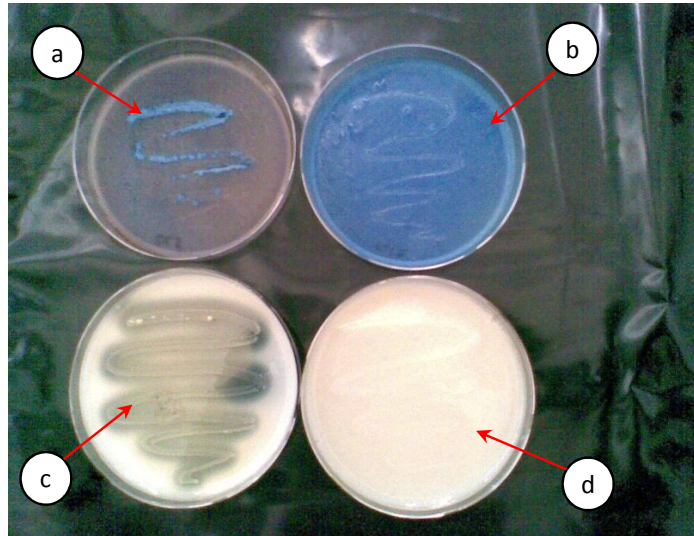


Figure 2.1: Butterfat agar plates (A, B), and milk agar plates (C, D), showing positive proteolysis (C) and lipolysis (A), induced by the control strain *Bacillus cereus* DL5; compared to negative lipolysis (B), and proteolysis (D), induced by the control strain *Lactobacillus brevis*

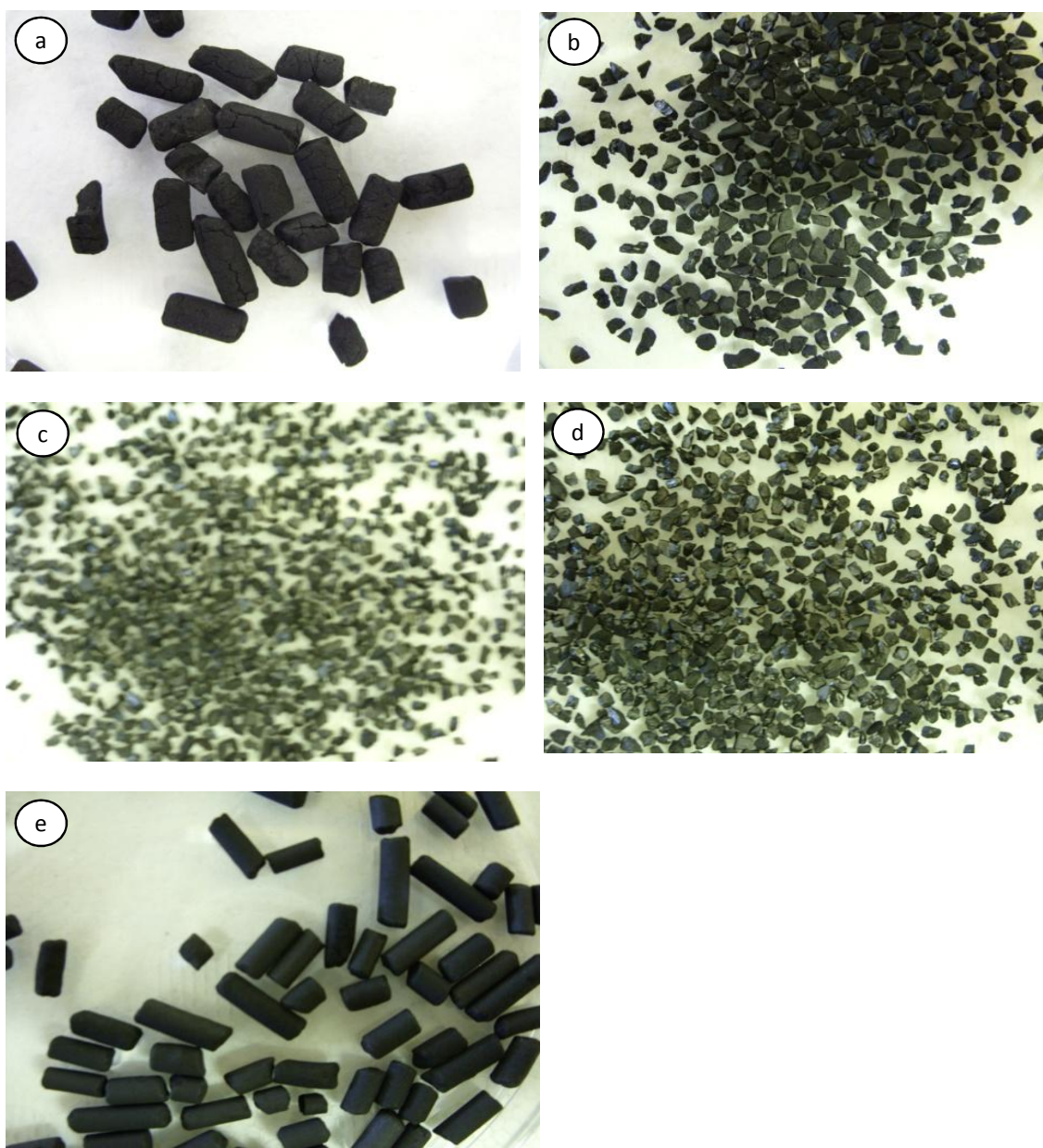


Figure 2.2: Activated carbon carrier granules used to determine the most efficient for biofilm attachment. The 5 types used in this study included 3 types originating from coal the 4mm pellet activated coal (*a*), granular coal (12x40) (*b*), and the 3mm pelleted activated coal (*e*). Two carriers originated from coconut shell extracts: granular carbon (8x16) (*c*), and granular carbon (12x30) (*d*)

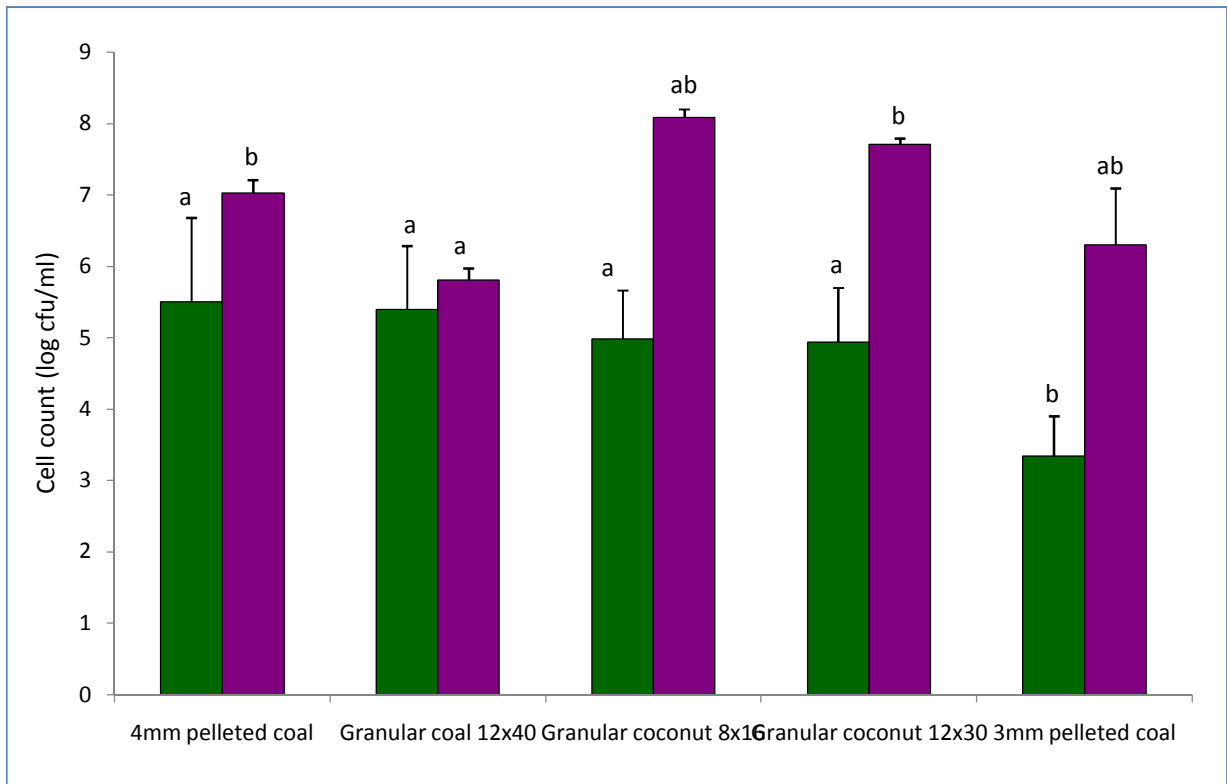


Figure 2.3: Attached ■ and planktonic ■ cell counts (log cfu/ml) of *B. subtilis* EL39 on each of the 5 types of activated carbon carriers, after 24h incubation at 30°C and 1500rpm. superscripts indicate statistically significant differences ($P < 0.05$)

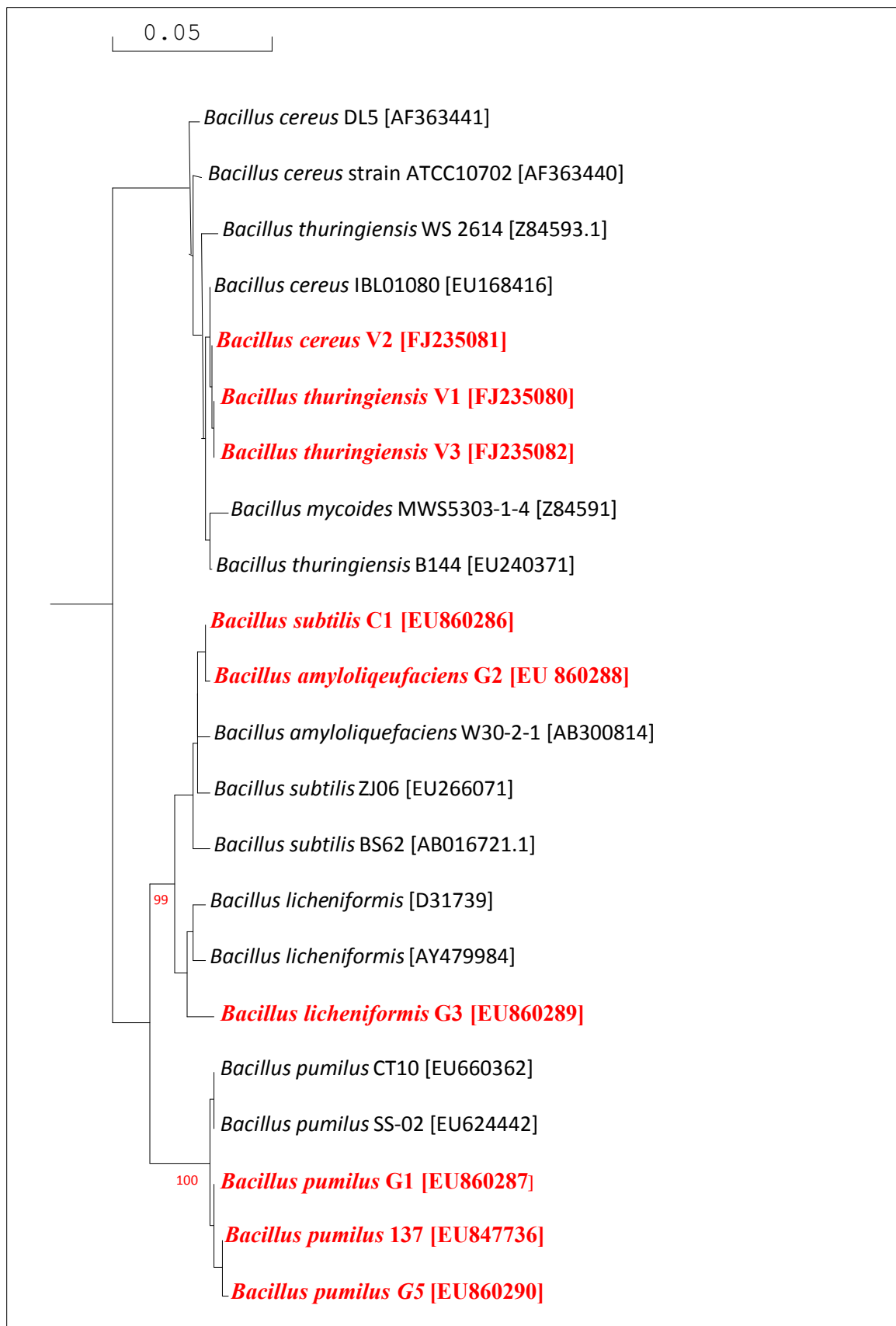


Figure 2.4a: Phylogenetic relationship of the members of the genus *Bacillus* compared to 10 dairy-associated *Bacillus* isolates, as revealed by 16S rDNA gene sequence analysis. Relationship are presented as an unrooted tree with branch lengths proportional to the estimated genetic distances between strains. The values at the branch nodes indicate bootstrap values (%)

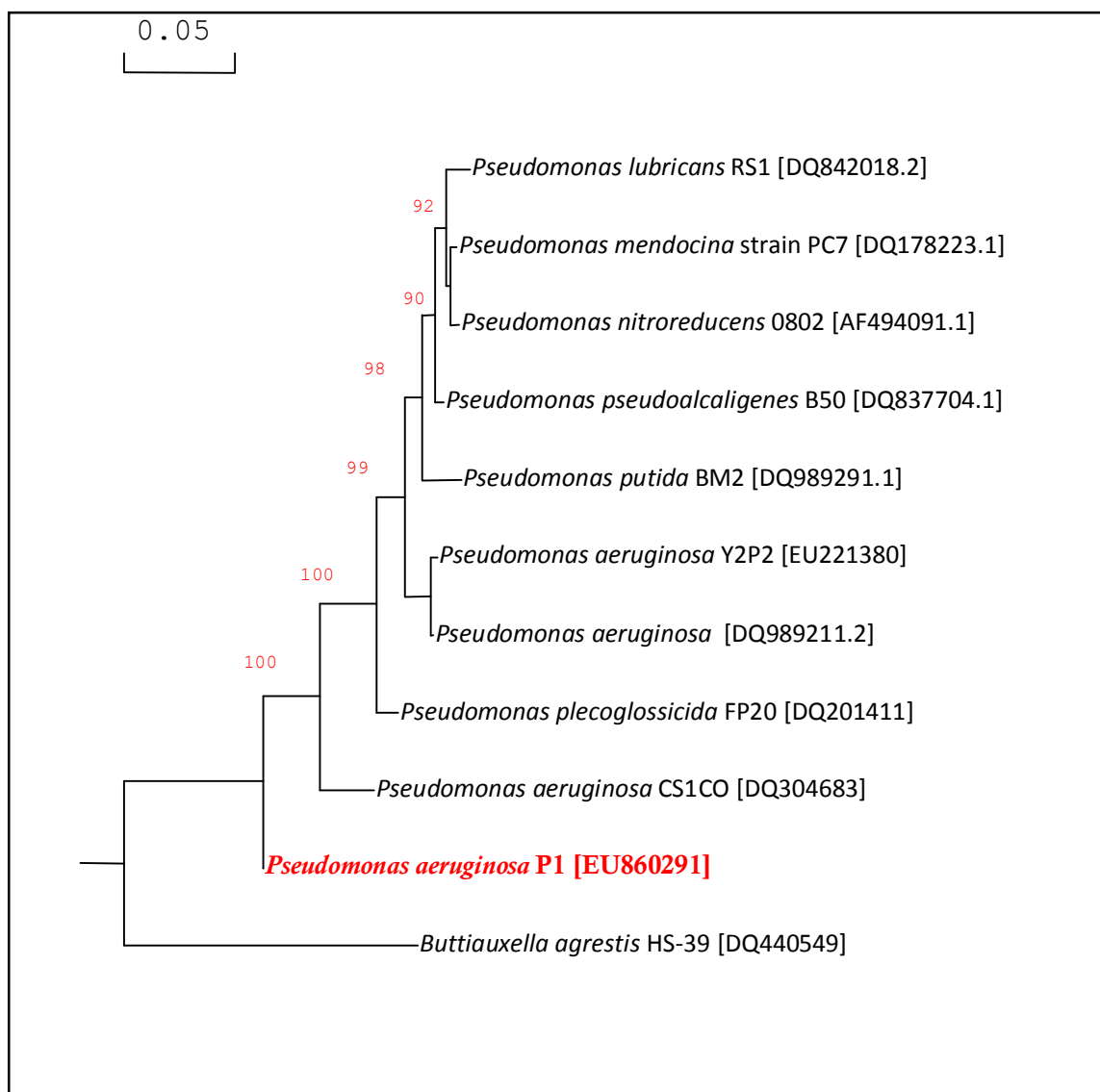


Figure 2.4b: Phylogenetic relationship of the members of the genus *Pseudomonas* compared to 1 dairy-associated *Pseudomonas* isolate, as revealed by 16S rDNA gene sequence analysis. Relationships are presented as an unrooted tree with branch lengths proportional to the estimated genetic distances between strains. The values at the branch nodes indicate bootstrap values (%)

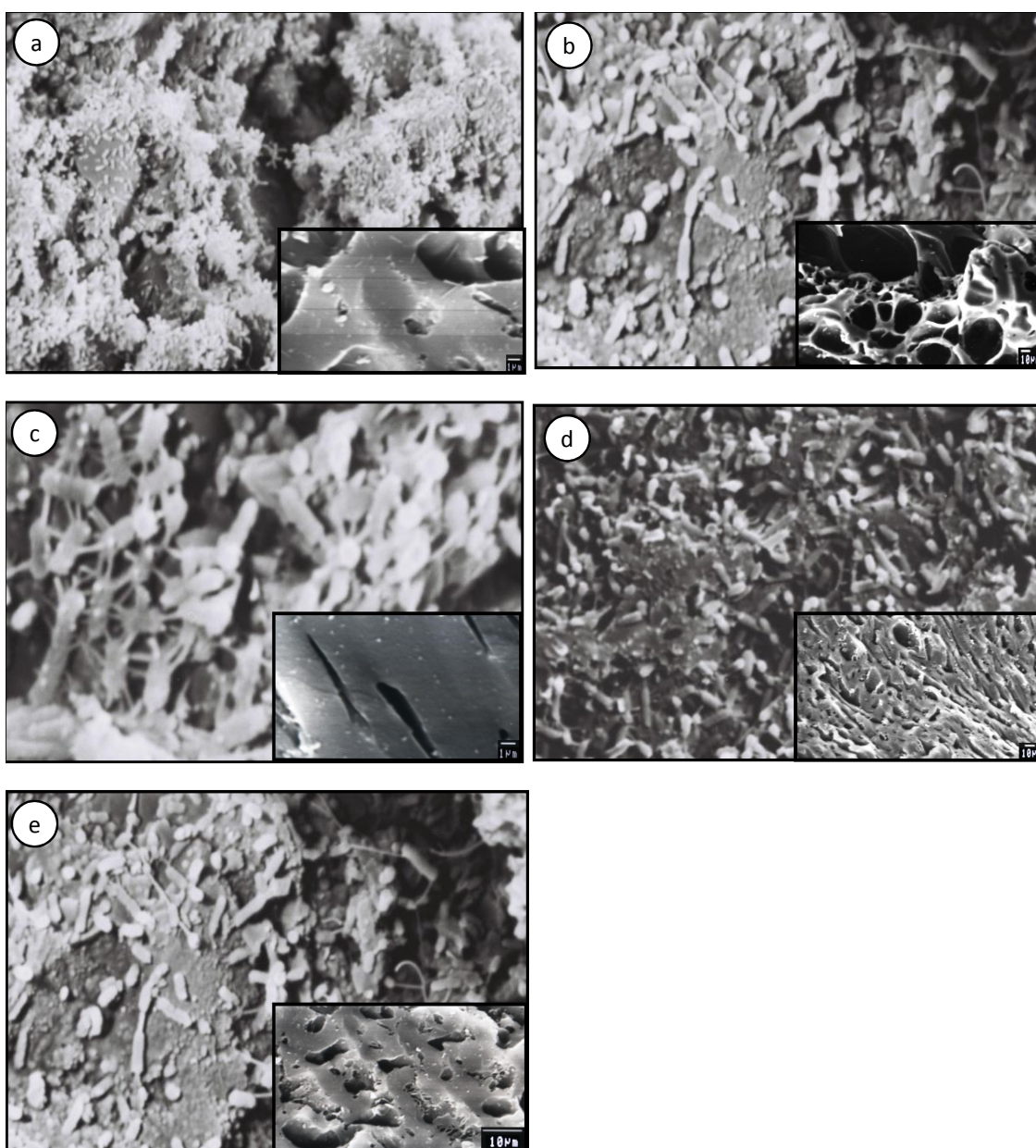


Figure 2.5: Scanning electron micrographs showing the robust attachment of *Bacillus subtilis* EL39 on the 4mm activated coal pellet (a); Sparse attachment on granular coal (12x40) (b), granular carbon (8x16) (c), granular carbon (12x30) (d); and the least attachment on the 3mm activated coal pellet (e). A Scanning electron micrograph of a control uncolonised surface of each carrier is inserted in each image.

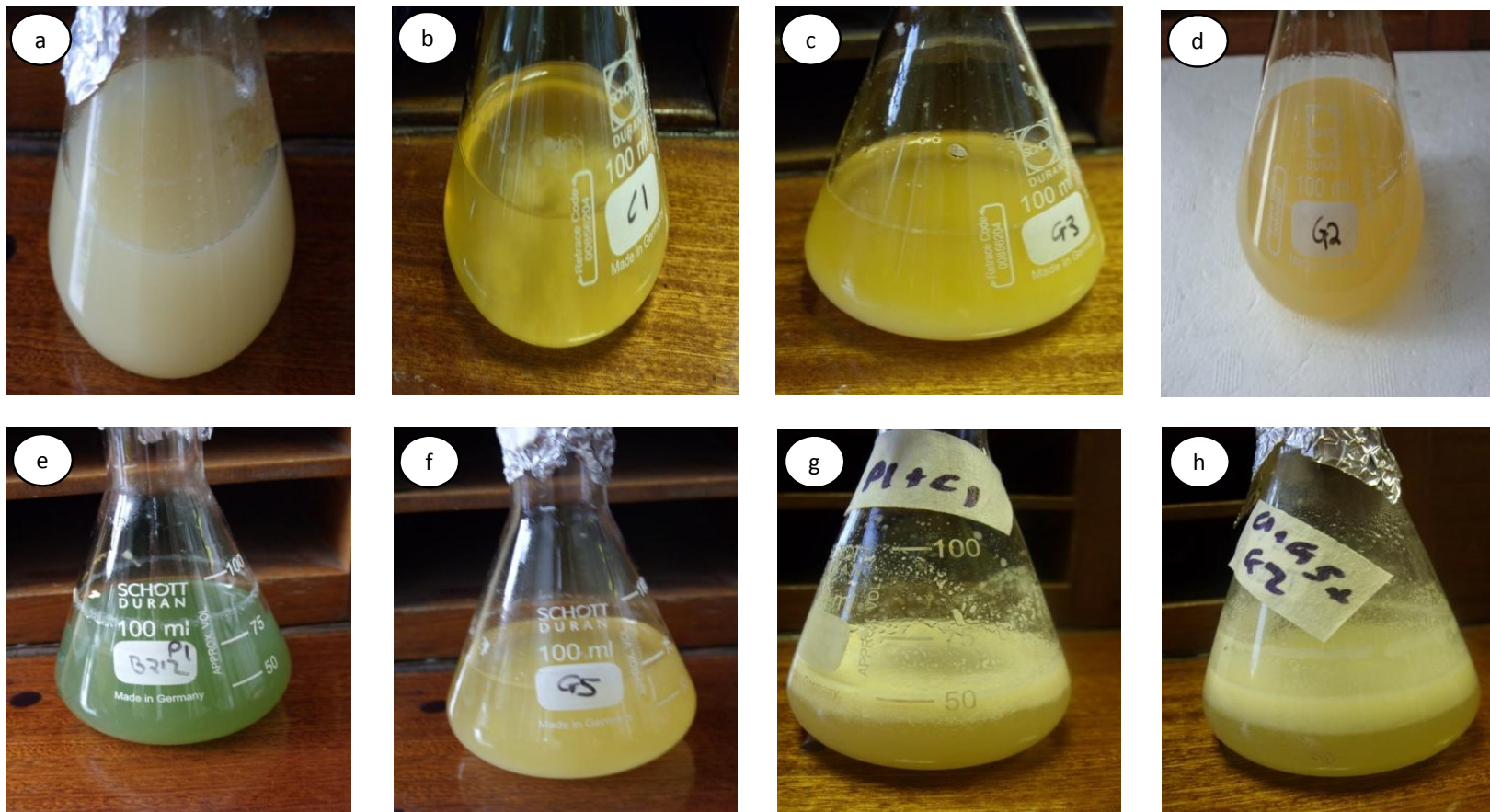


Figure 2.6: Colour changes in synthetic medium after inoculation with *Bacillus subtilis* C1 (EU860286) (b), *Bacillus amyloliquefaciens* G2 (EU 860288) (c), *Bacillus licheniformis* G3 (EU860289) (d), *Pseudomonas aeruginosa* P1 (EU860291) (e), and *Bacillus pumilus* G5 (EU860290) (f), in comparison to the Uninoculated Control (50% synthetic milk medium) (a). The binary consortium (g) which degraded most rapidly consists of *Bacillus subtilis* C1 (EU860286) and *Pseudomonas aeruginosa* P1 (EU860291), and similarly the mixed consortium (h) consisted of *Bacillus subtilis* C1 (EU860286), *Bacillus amyloliquefaciens* G2 (EU 860288), and *Bacillus pumilus* G5 (EU860290)

CHAPTER 3

Degradation of total organic carbon content in synthetic dairy wastewater, using a Gram-positive mixed consortium, and a Gram-positive and –negative mixed consortium, in an aerobic fluidised bed biofilm bioreactor, under simulated dairy processing flow rates and pH conditions

ABSTRACT

This study evaluated the efficacy of 2 different consortia of different bacterial populations in the bioremediation of dairy wastewater using a fluidised bed biofilm bioreactor (FBBR). One consortium consisted of *Bacillus* strains only [*Bacillus subtilis* C1 (EU 860286), *Bacillus pumilus* G5 (EU 860290), and *Bacillus amyloliquefaciens* G2 (EU 860288)], and the other contained *Bacillus subtilis* C1 (EU860286) and *Pseudomonas aeruginosa* P1 (EU860291). The efficacies of the different attached populations in the FBBR were evaluated for the degradation of total organic carbon (TOC), by assessing the reaction of each consortium to pH and flow rate fluctuations. An FBBR was utilised in batch mode, to aerobically induce biofilm growth and degrade TOC in the synthetic wastewater medium. Acid and alkaline shock was imposed on the synthetic medium by the addition of sodium hydroxide and orthophosphoric acid, to simulate CIP procedures in dairy factories, and growth under these conditions was evaluated in relation to TOC degradation. Every 3 days, carrier carbon, and synthetic wastewater was sampled to evaluate levels of TOC, as well as attached and planktonic growth of bacteria. Scanning electron micrographs were used to visualise colonisation on the carrier carbon (GAC) sampled from the FBBR. Aerobic granulation was thereafter initiated to evaluate its effect on the degradation of TOC. This study suggested that bioaugmentation using bacteria from 2 different genera, has a significant effect on the degradation of TOC in synthetic dairy wastewater, by reducing the TOC concentration content to 0 mg/l after 60 days, while building a considerable biomass in batch mode. By contrast, the consortium containing only Gram-positive *Bacillus* strains resulted in TOC degradation from 1.128 mg/L to 0.757 mg/L within 60 days. By converting the FBBR from batch to continuous mode, the study proposed an increase in biofilm, and aerobic granulation. Both of these further enhance the degradation of TOC, and possibly at a faster rate than with biofilm only. TOC may be depleted after only 18 days, when utilising the Gram-positive and –negative mixed consortium.

INTRODUCTION

Releasing dairy wastewaters into rivers and streams results in eutrophication and death of many aquatic organisms, and also pollutes the drinking water systems, making it harmful to humans, and other animals (Pattnaik *et al*, 2007). To reduce environmental contamination and health hazards, various anaerobic and aerobic, biological systems have been used to correctly dispose of industrial wastewaters, and are currently used in industry. However, although used in industry in the form of trickling filters, aerated lagoons, and activated sludge processes (Carta-Escobar *et al*, 2004), aerobic biological systems have been less researched or problematic, and as a result are mostly used as an additional step to an anaerobic system.

The type of aerobic systems currently used in industry are functional and contrary to common belief, are cost efficient (Gavrilescu & Macoveanu, 1999; Ndegwa *et al*, 2007). The types of aerobic biological reactors range from stirred tank reactors, to trickling filters, rotating disc filters, and upflow fluidised bed bioreactors, including aerobic ponds and lagoons (Craggs *et al*, 2003). Bioreactors featuring attached microorganisms on a carrier particle are more efficient than other types of reactors, and offer higher organic loading rates (Abdulgader *et al*, 2007). The most commonly used is the FBBR.

FBBR's are capable of treating both low and high strength waste effluents, and has resistance to altered operating conditions (Pala, 2001). Economically it is the more preferred system since the amount of reactors required are reduced, which reduces the cost (Kansal *et al*, 1998). FBBR's give greater microbial concentration than rotating disc filter and trickling filters. Additionally, particles are in full contact with the liquid phase, providing larger surface area for nutrient transfer and utilization as compared to trickling filters. The maximum loading rate in fluidised bed bioreactors exceeds that of plug flow reactors, high rate trickling reactors, activated sludge processes, and oxidation ditch processes (Pala, 2001). The FBBR also tolerates varying operational conditions (Pala, 2001), smaller

equipment size, and lower hydraulic retention time (Souza, *et al*, 2004), making it the preferred reactor system in this study.

The constantly fluctuating nature of dairy wastewaters in terms of varying pH (Lindsay *et al*, 2000a) resulting from cleaning-in-place (CIP) procedures, and the fatty and proteinaceous constituents from the dairy products, makes it difficult to degrade the total organic carbon content (Janczukowicz *et al*, 2007; Loperena *et al*, 2006; Donkin, 1997; Brewer *et al*, 1999). Biological treatment of dairy wastewaters have included the use of green algae or phycoremediation (Lincoln *et al*, 1996); solid-state fermentation (Couto & Sanromán, 2006); anaerobic filter reactors, sequential batch reactors (Omil *et al*, 2003); vascular aquatic macrophytes (Tripathi & Upadhyay, 2003); and various other methods, which have mostly been successful in vitro. With many treatment options incorporating multiple treatments (Yu & Fang, 2002; Xiao *et al*, 2007), as well as chemical pretreatments (Dyrset *et al*, 1998; Sarkar *et al*, 2006), which do not prove economical.

Studies have found that the attachment of bacteria to support media in these reactors which allow the biodegradation of toxic wastes, do not favour single strains of microorganisms, which are known to be inefficient in degrading dairy wastes. While bioaugmentation using multiple strains for wastewaters which are high in fats, is more efficient and commonly used (Chipasa & Mędrzycka, 2006; Cooper & Atkinson, 1981; Loperena *et al*, 2009). A mixture of bacteria secreting essential enzymes needed for the degradation of specific constituents can prove more efficient than a single strain alone. Mixed cultures are known to have strong adhesion characteristics, and form layers of biofilm more commonly than pure cultures (Cooper & Atkinson, 1981; Van Loosdrecht & Heijnen, 1993). The addition of external bacteria with enhanced degradation capacities is known to increase the efficacy and performance of bioreactor systems (Loperena *et al*, 2009). In a recent study in this laboratory, an aerobic FBBR was used to degrade

sodium benzoate with a consortium of Gram-negative bacteria, and continuous and batch operating modes were compared. Results showed that batch mode was more efficient in degradation of sodium benzoate than continuous mode (Lindsay *et al*, 2008). This study therefore chose to evaluate the batch mode of operation in bioremediation of dairy wastewater using an aerobic FBBR.

This current study adopted 2 different approaches. One approach used a Gram-positive only mixed consortium, and the other used a binary Gram-positive and Gram-negative mixed consortium. Each served to use bioaugmentation as a means of degrading TOC in a dairy synthetic medium, with the use of an aerobic FBBR. The binary consortium used in this study was one exhibiting high synergistic capabilities, and included *Bacillus subtilis* C1 (EU860286) and *Pseudomonas aeruginosa* P1 (EU860291). It was found that the degrading capacities of the pair were high in single culture, but even higher in combination (Chapter 2). This was found to be in accordance with a study by Loperena *et al* (2009) who suggested that these 2 species be combined as an inoculum for the treatment of dairy wastewaters, because of their enhanced ability to degrade fats and proteins. *Pseudomonas* has also been found to be predominant as a dairy spoilage microorganism, proving its proteolytic and lipolytic degradation capacities (Nörnberg *et al*, 2010). Dual-species biofilms containing *Pseudomonas spp.*, have previously been found to demonstrate good interactions when exposed to organic compounds, thus this study served to evaluate its efficacy in a binary species consortium (Simões *et al*, 2008). However, it should be noted that dairy wastewaters favour Gram-positive over Gram-negative bacteria in its treatment (Donkin, 1997), and it is this finding together with the known resilience of *Bacillus spp* in bioreactors, which made it a well-suited species of choice for this study. This is also in accordance with the pilot-study conducted in Chapter 2, in which the *Bacillus* mixed-species consortium was proven highly efficient in the degradation of a synthetic milk medium. *Bacillus spp* are widely known for the use of enzymes for industrial purposes (Morikawa, 2006; Moon & Parulekar, 2004; Balows *et al*, 1992), and has been used successfully in bioreactors as

inoculum for the bioremediation of industrial wastewaters (Noeth *et al*, 1988, Lim *et al*, 2001; Choi *et al*, 2002). The enzymes of *Bacillus* are also commonly associated with the spoilage of dairy products, making the proteolytic and lipolytic enzymes of this microorganism ideal for dairy wastewaters.

The efficacy of an aerobic FBBR using 2 different mixed microbial consortia, would further enhance knowledge on the bioremediation of dairy wastewaters, so as to understand how to further minimise environmental contamination, and health risks associated with dairy wastewater discharges.

MATERIALS AND METHODS

Bioreactor set-up, and sterilisation:

The design and structure of the FBBR used in this study is depicted in Fig 3.1a and Fig 3.1b. The FBBR was constructed from acrylic, with a length 102cm, volume of 6.5l, and an inner diameter of 11cm for the fluidisation of GAC. A KNF Laboport air pump was used to aerate the system, while a Watson-Marlow 505 Du peristaltic pump was used to recycle the synthetic medium through the reactor. Tygon tubing (8mm diameter, and 1.6mm wall diameter) was used to connect both air, and peristaltic pump to the FBBR, and the synthetic medium reservoir. Synthetic medium was fed through the bottom of the reactor, recycled through the top, and back into the main reservoir of the medium.

Before start-up, the FBBR was sterilised with 500ppm hypochlorite, rinsed once with neutralising buffer (Difco), and 3 times with sterile distilled water (Lindsay *et al*, 2008). Granular activated carbon pellets (GAC) sourced from coal, 4mm in size, were measured to 2kg and sterilised by rinsing 3 times with distilled water and autoclaving, before adding to the FBBR.

Synthetic medium preparation:

A 10% synthetic milk medium was prepared by diluting 39g full cream powder milk in 3l of distilled water. This was adjusted to pH 7, and 3l of this medium used in the FBBR each time.

Conditioning:

To initiate the hydrophobic attachment and colonisation of bacteria onto the carbon carrier, the GAC was conditioned with uninoculated synthetic milk medium by recycling into the FBBR at a flow rate of 2 ml/min, over 6 days. Conditioning of surfaces is considered as the first step in the development of biofilms, as higher nutrient conditions at surfaces have been found to positively influence microbial attachment (Hood & Zottola, 1997; van Loosdrecht *et al*, 1990).

Inoculum preparation for mixed Gram-positive consortium:

Bacillus subtilis C1, *Bacillus pumilus* G5, and *Bacillus amyloliquefaciens* G2, were grown overnight in 50ml tryptone soy broth (TSB) at 30°C, on an orbital shaker at 1500rpm.

Inoculum preparation for mixed Gram-positive and Gram-negative consortium:

Pseudomonas aeruginosa P1, and *Bacillus subtilis* C1 were grown overnight in 50ml tryptone soy broth (TSB) at 30°C, on an orbital shaker at 1500rpm.

Inoculation:

The prepared mixed Gram-positive and –negative inoculum (15ml) was added to 3l of the synthetic milk medium, and allowed to incubate for 1h at room

temperature before being fed into the FBBR, at a flow rate of 2 ml/min. This allowed the bacteria in the TSB inoculum to acclimate to the synthetic milk medium.

Bioreactor operation and flow rate:

The flow diagram in Fig 3.2 illustrates the operation of the FBBR. After inoculation, the FBBR was operated in batch mode for a total of 54 days. An uninoculated control bioreactor was also operated for 54 days for comparison. Medium was replaced with inoculated fresh stock every 6 days. The flow rate in the reactor was 2 ml/min. This represented bioaugmentation of dairy wastewater at neutral pH.

pH variation studies:

Acid and alkaline shocks are common in dairy processing wastewaters due to the cleaning-in-place (CIP) regimes imposed during processing. Caustic soda wash and nitric acid rinses are common CIP methods employed (Lindsay *et al*, 2000a; Parkar *et al*, 2004; Vlkova *et al*, 2008). Thus, similar pH changes were made to the synthetic dairy wastewater used in this study to simulate this process, in order to evaluate the effect it has on the growth of binary-species biofilm, as well as its effect on the degradation of TOC.

Following 6 days at pH 7, the medium was replaced with synthetic medium and adjusted to a pH of 10, using sodium hydroxide. The reactor was then run in a similar manner as described for pH 7 for 6 days. This represented the bioaugmentation of dairy wastewater under conditions of alkaline shock. Following 6 days at pH 10, the medium was replaced with synthetic medium and adjusted to a pH of 4 with orthophosphoric acid. The reactor was then run in a

similar manner as described for pH 7 and 10 for 6 days. This represented bioaugmentation under conditions of acid shock.

Flow rate variation studies:

The above procedure of bioaugmentation under neutral, alkaline, or acidic conditions, was then repeated with different flow rates. These included 8.5 ml/min, and 170 ml/min.

Initiation of granulation in the FBBR:

At the end of each experimental procedure described above, the FBBR was operated for an extra 12 days, at its optimal pH and flow rate (pH 10, with a flow rate of 2 ml/min), to observe the effect granules have against the degradation of TOC. Samples were removed every 3rd day to evaluate the level of TOC in the FBBR. Aerobic granules are defined as self-immobilised microorganisms, dense in multiple species of bacteria, each playing a particular role in degradation of toxic wastewater (Liu & Tay, 2004). Aerobic granules withstand high-strength organic wastewater, and shock loadings (Li & Liu, 2005), characteristic of the wastewaters discharged from dairy factories. Compared to biofilms, aerobic granules are self-aggregated without the use of a carrier, and 3-dimensional (Li & Liu, 2005), allowing a larger interface for nutrient absorbance. These granules have been shown to be more efficient in reducing organic pollutants in industrial wastewaters, and produce a better quality effluent than other biological means of remediation (Adav *et al*, 2008).

All Gram-positive and –negative mixed consortium experiments were repeated in triplicate. Following these, the FBBR was inoculated with a mixed consortium of Gram-positive isolates only in a similar manner and the bioreactor system run as described previously.

Attached and planktonic cell counts:

Bulk fluid and GAC was sampled from the reactor every 3 days, allowing samples to be recovered on the 3rd and 6th day of each pH at each flow rate. Synthetic medium, and carrier material from the FBBR was removed, and attached and planktonic growth determined, using the method of Lindsay & von Holy, (1999). After plating onto tryptone soy agar using the droplet plate technique (Lindsay & von Holy, 1999; Herigstad *et al*, 2001), plates were incubated at 30°C overnight, and colonies counted.

Scanning electron microscopy:

From each sample of carbon carrier pellets, 1g was rinsed in sterile dH₂O, and fixed in 3% gluteraldehyde for 18h at room temperature. Dehydration was then performed by a graded ethanol series, as described by Lindsay and von Holy (1999), (20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 95% for 10min each, and 100% overnight). Each sample was then subjected to critical point drying, after which it was mounted and coated with thin carbon and normal gold-palladium sputter. Samples were viewed on the Joel 840 Scanning Electron Microscope (Jsm840, Jeol Ltd, Tokyo, Japan), at 20kv, and varying working distances.

Determination of total organic carbon (TOC) degradation

Every 6 days, before the medium was replaced, bulk fluid from the FBBR was collected, and used to determine the level of TOC present in the system. This was compared to the TOC levels of the uninoculated control. To evaluate TOC, a Hach TOC kit was used. Ten ml of bulk fluid from the FBBR, and 400µl of buffer (pH 2) was combined, and stirred for 10min at moderate speed. 300µl of this sample was then transferred to a vial containing deionised water. Hach TOC persulphate powder was added to the vial, and then an ampule. This was placed in a heating block (Hach DRB200) at 105°C for 2h. The vial was left to cool for 1h,

after which absorbance and concentration readings were taken at a wave range of 430- 598nm using a spectrophotometer (BOECO S-20). Following the Hach operation manual, samples were evaluated at 430- and 598nm, and the wave-range used to study any alteration in pattern in each sample. Additionally, a Hach TOC standard solution (1000mg/L C) was run each time to compare to the sample for accuracy.

Statistical analysis

Means and standard deviations were calculated between attached and planktonic counts, and TOC data for triplicate runs. Multiple variable analysis using Statgraphics v15.2 (Centurion), was used to analyse data at the 95% confidence level. Statistically significant differences were determined between the attached and planktonic counts, and between TOC degradation for all pH's and flow rates.

RESULTS

Part 1: Gram-positive mixed consortium evaluation

Bioaugmentation at a flow rate of 2 ml/min and at neutral, acid and alkaline pH:

Attached and planktonic cell counts

At this slowest flow rate, conditions seemed to positively affect growth of attached cell populations with attached counts increasing an overall 2log cfu/ml. Planktonic cells however remained steady overall at 7.5log cfu/ml. At pH 7, after conditioning and inoculation, attached counts increased from 0 to 7log cfu/ml, while planktonic counts decreased by 1log cfu/ml to 7log cfu/ml. The alkaline shock imposed on the system, caused an increase in attached counts (7.6log cfu/ml), and a further decrease in planktonic counts (7log cfu/ml). At pH 4, the acid shock generated a slight increase in attached and planktonic cell populations (Fig 3.3 a, b).

Microscopy

The SEM image in Fig 3.4a showed the uncolonised control surface, displayed the topography of the 4mm activated carbon carrier, and revealed the pores and crevices on the surface of the GAC. At pH 7 rod-shaped cells of *B. subtilis*, *B. amyloliquefacies*, and *B. pumilus*, were visible on the surface of the GAC, and what is thought to be EPS extending from one of the cells to the surface of the GAC upon which it was attached (Fig 3.5b). Further colonisation was then observed at pH 10, with many more cells observed to be attached to the surface, and strands of EPS connecting cells to one another (Fig 3.5b). In Fig 4.4f at pH 4, clusters of cells were found in the SEM images, and many cells were observed to be covering the entire surface of the GAC. By the end of the 2 ml/min run, a large amount of EPS was observed between cells.

Total organic carbon degradation

This study considered 2 wavelengths in the spectrophotometric evaluation of TOC degradation, as did most studies that previously evaluated the degradation of TOC in industrial wastewaters (Nataraja, *et al*; 2006). During this flow rate the total TOC degradation was 0.734 mg/l. The degradation associated with pH 10 was highest (0.905 mg/l), and most significant ($P < 0.05$), however at the acid shock and neutral pH, degradation was less (0.734 mg/l and 0.463 mg/l respectively). All the peaks in wavelength curves observed in Fig 3.9a were rounded, which was indicative of the large amount of TOC still present in the synthetic medium, however, all pHs exhibited a significant decrease ($P < 0.05$) in TOC from the control.

Bioaugmentation at a flow rate of 8.5 ml/min and at neutral, acid and alkaline pH:

Attached and planktonic counts

The conversion to a higher flow rate proved to be unfavourable, by causing a decrease in both attached and planktonic counts. At neutral pH, both attached and planktonic cell populations decreased considerably, while at alkaline pH (pH 10) attached counts continued decreasing, and planktonic showed an initial increase, followed by a decrease in counts. When exposed to acidic conditions, cells of both attached and planktonic nature, decreased considerably (3log cfu/ml, and 2log cfu/ml respectively) (Fig 3.3 c, d). Fig 4.3 illustrates the sparse cell distribution on the GAC due to a decrease in counts at the acidic run of flow rate 8.5 ml/min.

Microscopy

At pH 7, cells in SEM images appeared to have proliferated, and thick strands of EPS could be found connecting, and crossing over cells (Fig 3.6a), as well as what is thought to be milk soils were observed between, and on cells (Fig 3.6b). At pH 10 thick intertwining strands of EPS could be observed between and around clusters of cells. The development of thicker biofilm was noticeable as cells and EPS matrices were found covering pores at pH 10 in Fig 3.6c, and then in Fig 3.6d, the entire surface was covered in a layer of biofilm, although further growth did not appear to have been promoted. During the acid shock from orthophosphoric acid, less cells, and milk soils were visible (Fig 3.6e), and less extensive matrices networks were visible on the GAC surface. EPS strands also appeared less thick than previously (Fig 3.6f). Oliveria *et al*, (1994) stated that bacteria suppress EPS production at low pHs, thus the decrease in amount of EPS can be accounted for.

Total organic carbon degradation

pH 7 was found to be most efficient in degradation of TOC at this flow rate, degrading TOC by 0.793 mg/l, and showed the most significant decrease in TOC ($P < 0.05$). Overall TOC was degraded by 0.774 mg/l at this flow rate. pH 10 was least efficient degrading TOC by only 0.691 mg/l, however, still showed a significant decrease in TOC ($P < 0.05$). pH 4 degraded TOC significantly to 0.354 mg/l. The wave-range peaks observed in Fig 3.9b are sharper than that observed at 2 ml/min, indicative of less TOC in the synthetic medium at this flow rate compared to 2 ml/min.

Bioaugmentation at a flow rate of 170 ml/min and at neutral, acid and alkaline pH

Attached and planktonic counts

The highest flow rate showed an unexpected gradual increase in both attached and planktonic cell counts. Attached cells increased by 1log cfu/ml overall, and planktonic cells, by 2log cfu/ml. At pH 7 both attached and planktonic counts increased. At pH 10, while attached counts increased further (0.5log cfu/ml), planktonic cell counts decreased slightly (0.4log cfu/ml). Attached cells at pH 4 increased by 0.3log cfu/ml, and planktonic counts by 2log cfu/ml (Fig 3.3e, f).

Microscopy

When the flow rate increased, and synthetic medium returned to neutral conditions, clusters of cells were again observed on the surface of the GAC, with much more production of EPS noticeable from the cells, which was evident from the network of EPS and thick appearance of biofilm coverage in the SEM images (Fig 3.7a). From Fig 3.7b, clusters of cells were again evident, and biofilm pores appeared to be developed to allow the transfer of air and nutrients to deeper layers of the biofilm. During alkaline conditions, strands of networking EPS were

observed covering the entire surface of the GAC (Fig 3.7c), but towards the end of the alkaline run, the thick covering of the EPS diminished, and sparse attached cells observed to be covering the GAC (Fig 3.7d). At pH 4 attached cells and EPS content were found to decrease, and no clusters of cells visualised on any part of the surface of GAC (Fig 3.7e, f).

Total organic carbon degradation

The fastest flow rate decreased TOC to 0.292 mg/l, with pH 10 being most efficient, and showing the greatest significant decrease ($P < 0.05$) in TOC by 0.9 mg/l. pH 4 was least efficient and not significant in degrading TOC, to yield a TOC level of 0.836 mg/l in the synthetic medium. pH 7 decreased TOC to 0.422 mg/l. Fig 3.9c illustrates the sharp peak achieved at pH 10.

Initiation of granulation in the FBBR:

Aerobic granules were not successfully initiated by this Gram-positive only consortium. When the FBBR was switched to optimal operating conditions, overall attached counts remained steady at approximately 6.5log cfu/ml, and planktonic counts decreased by 2.8log cfu/ml. Attached counts were observed to increase again on day 57 by 0.8log cfu/ml, while planktonic counts decreased by 0.8log cfu/ml, after the acid shock imposed during the 170 ml/min run. On day 60, counts decreased by 1log cfu/ml in both attached and planktonic counts. SEM images revealed a slight increase in coverage of the GAC, as well as an increase in appearance of molecules though to be milk soils (Fig 3.8a, b). TOC was decreased significantly ($P < 0.05$) to 0.403 mg/l on day 60. Day 63 showed an increase of 0.5log cfu/ml in attached counts, while planktonic cell population remained steady at 4.8log cfu/ml. On the last day of operation, day 66, both attached and planktonic cell populations decreased significantly ($P < 0.05$) by 0.2log cfu/ml (Fig 3.3g, h), and SEM micrographs are evident of the decrease of attached cell content, although traces of EPS and milk soils were still observable

(Fig 3.8c, d). TOC was significantly ($P < 0.05$) degraded to 0.371 by day 66 (Fig 3.10 and Fig 3.11a, b).

pH variation studies:

pH 7 showed the greatest increase in attached counts at 8.5 ml/min, as well as the greatest reduction of TOC at the same flow rate. pH 7 had the highest planktonic counts at 8.5 ml/min and 170 ml/min. pH 10 exhibited the greatest increase in attached counts at 170 ml/min, and the biggest reduction of TOC at 2 ml/min, and 170 ml/min. At pH 4, attached counts were highest at 2 ml/min, but lowest at 8.5; and 170 ml/min. Planktonic counts were lowest at 8.5 ml/min for pH 4. TOC degradation at this acidic pH, was lowest at 170 ml/min, while degradation at 2; and 8.5 ml/min was second highest. During the optimal run of the FBBR, pH 10 exhibited the highest attached counts on day 57, while highest TOC degradation was on the last day of operation, day 66.

Part 2: Gram-positive and Gram-negative mixed consortium evaluation

Bioaugmentation at a flow rate of 2 ml/min and at neutral, acid and alkaline pH:

Attached and planktonic cell counts

Under neutral conditions, cells of both bacterial strains attached to the GAC and growth increased from $<1 \log$ cfu/ml to $8 \log$ cfu/ml. When the pH of the growth medium was changed to pH 10, bacterial growth increased by a further $1 \log$ cfu/ml. By contrast, cell growth decreased when exposed to orthophosphoric acid at pH 4 (Fig 3.12a). Planktonic cell growth decreased significantly from an initial $8 \log$ cfu/ml count at pH 7 and 10 ($P < 0.05$) (Fig 3.12 b), however, during the acid shock (pH 4), planktonic counts increased significantly by $2 \log$ cfu/ml ($P < 0.05$), due to the detachment of attached cells from the GAC caused by exposure to the orthophosphoric acid. For attached counts, this the lowest flow rate exhibited the most significant differences ($P < 0.05$). Significant differences between the 3rd day

after feeding, and 6th day after feeding when starvation ensued, were apparent at pH 7 and 10, while pH 4 showed no significance between the 3rd and 6th day of sampling. Overall, all 3 pHs showed significant decreases of TOC compared to the uninoculated control.

Microscopy

Scanning electron micrographs confirmed colonisation of the GAC by both *B. Subtilis* C1, and *P. aeruginosa* P1 on the GAC coal carrier pellet. After colonisation was initiated, clusters of cells were found covering the GAC, web-like material which is thought to be EPS (extrapolysaccharide substances) was then seen, and finally EPS matrices were visualised layering the GAC. Short rod-shaped cells were observed to be attached by web-like material resembling fimbriae, extending from the cell surface, and colonisation initiated on the carrier carbon on day 3 of pH 7 (Fig 3.14b), in comparison to the control GAC which exhibited no colonisation (Fig 3.14a). Further colonisation was observed on day 6 of the neutral run, albeit starvation conditions. On day 9 at pH 10, the secretion of EPS became visible between rod cells (Fig 3.14c), and at day 12, clusters of cells were seen covering the entire surface, with the development of biofilm pores (Fig 3.14d). During the acid shock, cells decrease by 4log cfu/ml within the first 3 days of acid exposure (Fig 3.14e), and after 6 days of acid exposure, cells continued to decrease, however EPS was still visible on the surviving cells (Fig 3.14f).

Total organic carbon degradation

Overall TOC was degraded from the initial 1.281 mg/l to 0.903 mg/l at this flow rate. TOC was significantly decreased ($P < 0.05$) from the control at pH 7 and 10 (Fig 3.18a).

Bioaugmentation at a flow rate of 8.5 ml/min and at neutral, acid and alkaline pH:

Attached and planktonic counts

Further growth was not promoted in both attached or planktonic cells (Fig 3.12c, d), and no significant increase in growth occurred at this flow rate ($P < 0.05$). Attached growth remained steady, while planktonic cells increased overall by 3log cfu/ml, indicating the cells adjustment to the increased flow rate.

Microscopy

Clusters of cells were observed on the carrier at pH 7 (Fig 3.15a, b), with the development of more extensive network of EPS, following loss of cells after the acid shock during the previous flow rate of 2 ml/min. During the alkaline shock, cells continued to proliferate, and EPS matrices were clearly visible from 10 μ m (Fig 3.15c, d). Possibly due to the increased shearing from a higher flow rate, the biofilm remained intact at pH 4 (Fig 3.15e, f), and as a result attached counts remained stable at this flow rate. Additionally it is also known that EPS production continues as growth continues even during stationery phase, and thus the observation of EPS at this flow rate is not unusual (Oliveira *et al*, 1994).

Total organic carbon degradation

This flow rate, yielded different results, whereby the overall TOC content increased slightly from that of the lower flow rate, instead of further reduction (Fig 3.18b). pH 7 yielded slightly decreased TOC results from the control (0.003 mg/l), while pH 10, and 4 decreased TOC (0.553 mg/l), however, not reducing to the low levels achieved at 2 ml/min. TOC was significantly decreased ($P < 0.05$) from the control at pH 7 and 4.

Bioaugmentation at a flow rate of 170 ml/min and at neutral, acid and alkaline pH

Attached and planktonic counts

Less biofilm mass was recorded at this the highest flow rate, due to the high flow rate and inability of the cells to remain attached or initiate attachment. In addition, planktonic populations also did not increase. The fastest flow rate yielded a decrease in attached counts at each pH (Fig 3.12e). Planktonic counts at 170 ml/min remained fairly steady at 7log cfu/ml (Fig 3.12f).

Microscopy

Counts began to decrease at pH 7 (Fig 3.16a, b) and pH 10 (Fig 3.16c, d) due to the high flow rate. A breakdown of EPS was noticeable, and more individual cells were observed than previously due to the absence of the web-like cover of the EPS. Then at the acid shock, cells began adapting to the high flow rate as well as the acidic pH, and EPS began to increase again to develop thicker biofilm (Fig 3.16e, f).

Total organic carbon degradation

TOC degradation continued even though cell counts decreased, and was reduced by 0.687 mg/l. At the highest flow rate (170 ml/min), pH 7 and 4 yielded a decrease, while the alkaline shock was not as efficient in decreasing the TOC, however the sharper peaks accounted for less organic carbon (Fig 3.18c). TOC was significantly decreased ($P < 0.05$) from the control at pH 10 and 4.

Initiation of granulation in the FBBR:

Aerobic granule formation was successfully initiated after 3 days of optimal operation. The FBBR was operated for 12 more days at optimum flow rate (2 ml/min) and pH (10), and yielded an overall increase of 3log cfu/ml in attached growth. Aerobic granules were visibly developed during this optimal operation of the FBBR (Fig 3.17e). Figure 3.17f provides a microscopic visualisation of the aerobic granule, and the thick EPS which enclosed the cells. Cells could clearly be seen proliferating, and EPS matrices networking throughout the GAC, already after only 3 days of operation of the optimum run (Fig 3.17a, b). After 9 days of this optimal run, clusters of cells were visible, completely covering the GAC. During this time, counts steadily increased, only decreasing at points when starvation ensued, but growth increased an overall 2 log cfu/ml (Fig 3.12g, h). TOC was degraded completely after only 6 days of optimal operation. The very sharp peak at 540nm, indicated the absence of TOC at 60 days (Fig 3.19). The complete degradation of TOC from 1.284 mg/l to 0 mg/l was significant ($P < 0.05$), and reproducible (Fig 3.20b).

From beginning to end of the reactor operation cycle of 66 days, attached growth had increased to 9 log cfu/ml, while planktonic cell counts remained steady throughout the cycle (7 log cfu/ml) (Fig 3.13), demonstrating the capability of the aerobic FBBR to maintain biofilm growth, and aerobic granules, while still depleting TOC in the system to 0 mg/l after 66 days in batch mode of operation (Fig 3.20a,b).

pH 4 showed the most TOC degradation overall at both 430nm and 598nm. pH 10 exhibited the greatest significant difference ($P < 0.05$) at all flow rates, and was more significant when compared to each of pH 7 and 4. The acid and alkaline shock imposed on the system still allowed for growth of cells, proliferation of biofilm, and degradation of TOC.

pH variation studies:

Results were then evaluated according to which pH reduced organic carbon most efficiently, and which would be accumulating TOC. At pH 7, the highest flow rate (170 ml/min) was most efficient in synthetic wastewater, degrading TOC to 0.597 mg/l (Fig 3.18c). 8.5 ml/min was the least efficient flow rate for wastewater at pH 7, allowing TOC to accumulate, rather than degrade (Fig 3.18b). The trend exhibited by the flow rate of 8.5 ml/min was unusual, as it allowed TOC to be accumulated to 1.281 mg/l at pH 7. It also displayed the most significance compared to the control.

During the alkaline shock (pH10), TOC was reduced by 0.584 mg/l. The slowest flow rate was most efficient in degrading synthetic wastewater to this concentration (Fig 3.18a). However the fastest flow rate was least efficient, degrading TOC levels by only 0.224 mg/l (Fig 3.18c). Additionally the sharp peak at 540nm for 170 ml/min, usually indicating little organic carbon content, did not correlate with the high TOC concentrations at 430nm (Fig 3.18c). Visibly, more growth was found in the bioreactor at the slowest flow rate (2 ml/min), and most indication of granule formation was observed at this flow rate. This flow rate also showed most significance difference from the control, and was used with optimal pH of 10, to initiate thicker biofilm bed growth, and granule formation, to observe its effect on the further degradation of TOC fed into the system. The acid shock (pH 4) revealed similar results to the alkaline runs. The most efficient flow rate was the slowest (2 ml/min) reducing TOC by 0.903 mg/l (Fig 3.18a), suggesting that this pH was optimal for TOC degradation, and the least efficient being 8.5 ml/min, reducing TOC by 0.553 mg/l (Fig 3.18b). Degradation during acid shock was significantly lower ($P < 0.05$) from the control, at 8.5 ml/min, and 170 ml/min.

Comparison between efficacy of Gram-positive only, and Gram-positive and –negative mixed consortia

Attached and planktonic counts at 2; 8.5; and 170 ml/min

The Gram-positive *Bacillus* consortium exhibited a trend of increasing growth at 2 ml/min while the Gram-positive, and –negative binary consortium showed fluctuating growth during this flow rate. At 8.5 ml/min, attached counts of the Gram-positive and –negative consortium remained steady, however, the Gram-positive consortium displayed an almost 4log cfu/ml decrease in attached growth. By contrast, at the highest flow rate (170 ml/min), the Gram-positive *Bacillus* consortium showed a gradual increase in growth, while the binary consortium exhibited a gradual decrease in attached cell population counts. Interestingly, when the FBBR switched to optimal operating conditions (pH 10 and 2 ml/min), the binary consortium showed an increasing attached cell population count compared to the steady-state of the Gram-positive consortium (Fig 3.21). However, these observed differences in attached counts between the reactors containing the 2 types of consortia were not significantly different from each other ($P < 0.05$). Overall, planktonic cell growth remained steady for the Gram-positive, and –negative binary consortium, while the mixed Gram-positive consortium decreased by 3 log cfu/ml. There was also no significant difference between the planktonic cell populations for reactors containing both types of consortia ($P < 0.05$).

Microscopy

At 2 ml/min, similar growth patterns were observed between the 2 consortia except at pH 4, where the binary consortium exhibited less EPS compared to the Gram-positive consortium. Scanning electron micrographs at 8.5 ml/min showed much more EPS production in the binary consortium compared to the Gram-positive only consortium, where many cells were exposed and not completely covered by EPS. At 170 ml/min growth was similar at pH 7 and 4, however at pH 10 much more growth was observed for the Gram-positive consortium. The

optimal mode of operation of the FBBR showed a lot more EPS produced for the binary consortium. However, it was interesting to note that what is thought to be milk soils, were more visible during Gram-positive bioaugmentation than during Gram-positive and -negative bioaugmentation (Fig 3.21).

Total organic carbon degradation

The binary Gram-positive and –negative consortium depleted TOC completely, compared to the Gram-positive only consortium which degraded TOC only to 0.607 mg/l, and there was a statistically significant difference in TOC degradation between the 2 consortia ($P < 0.05$). By comparing the amount of TOC degradation between both consortia at 598nm, the Gram-positive only consortium revealed erratic degradation of TOC, and overall, TOC was not fully degraded. However, with the binary consortium, TOC was proven to have completely degraded (Fig 3.22).

Initiation of granulation in the FBBR

The optimal run of the FBBR induced granule formation in the binary Gram-positive and –negative consortium run of the FBBR, but not with the Gram-positive only mixed consortium. TOC degradation when aerobic granulation was initiated was greater than when no granulation occurred. TOC was significantly decreased to 0 mg/l during the granulation phase of bioreactor using a Gram-positive and –negative mixed consortium, however was not significantly decreased when using a Gram-positive mixed consortium for bioaugmentation.

pH variation studies

At 2 ml/min and pH 4, TOC was optimally degraded in the binary consortium, whereas at pH 7 TOC was optimally degraded for the Gram-positive mixed consortium. When the flow rate was increased to 8.5 ml/min, pH 4 was again

optimal for TOC degradation in the binary consortium, whereas pH 10 was optimal for the Gram-positive mixed consortium. The highest flow rate caused a great reduction in TOC at pH 7 for the binary consortium and pH 4 for the mixed gram-positive consortium. Attached cell counts were greatest at pH 10 during 2 ml/min and 8.5 ml/min (8.71 log cfu/ml and 7.97 log cfu/ml), and at pH 4 during 170 ml/min (7.37 log cfu/ml) for the binary consortium. Attached cell counts were highest at pH 4 during 2 ml/min (8.15 log cfu/ml); pH 7 during 8.5 ml/min (8.37 log cfu/ml); and at pH 10 for 170 ml/min (6.72 log cfu/ml) for the Gram-positive consortium.

DISCUSSION

Part 1: Gram-positive mixed consortium evaluation

Bioaugmentation at a flow rate of 2 ml/min and at neutral, acid and alkaline pH:

Attached and planktonic cell counts and microscopy

The slow flow rate at pH 7 allowed the initiation of colonisation of the consortium on the GAC, which was observed by both attached counts, as well as SEM images. The web-like material thought to be EPS seen extending from either side of one cell, is thought to have promoted conditioning of the surface (Oliveira *et al*, 1994) in order to allow further colonisation of the surface of the GAC. The reduction in planktonic counts allowed the assumption that, since cells were beginning to attach, much less cells would then be in suspension. SEM images illustrated the production of EPS-like material extending from cells to the GAC surface, as well as to other cells. EPS surrounds cells in biofilms (Costerton, 1999). It consists of exopolysaccharides which mediate both cohesion and adhesion of cells, and assist in maintaining the structural integrity of the biofilm matrix (Liu & Tay, 2002). During the alkaline shock, attached counts continued to increase, and it is known that *Bacillus spp.* grow optimally during alkaline conditions (Lindsay *et al*, 2000a). SEM images revealed the increase in population of attached cells, especially within the pores of

the GAC, where it would be initially easier to form networks of EPS. The web-like material of EPS can clearly be seen at this pH. The decrease in planktonic cell population may be accountable due to the further attachment of cells to the GAC, thus reducing suspended cell counts. The acid shock did not negatively influence growth as predicted, but in fact increased growth, in both attached and planktonic cell populations, and the clusters of cells found in SEM micrographs indicated the ability of cells to continue growing in this acidic environment. EPS production also did not cease, and overall the slowest flow rate seemed to be favourable at all pHs. Slow flow rate conditions are known to influence growth of cells, due to the ability of cells to easily attach to surfaces when shear force is low. Thicker biofilm has been observed in environments where shear force is low (Qureshi *et al*, 2005).

Total organic carbon degradation

The consumption of TOC by the mixed consortium at this flow rate was high at alkaline pH, but lower during neutral and acidic conditions. The low degradation associated with pH 7 may be due to the cells initiating attachment and colonisation, and not being able to consume TOC in large amounts due to the lesser amount of cells available, and thus less enzyme production. During acidic conditions, enzymes involved in TOC degradation may have been inhibited by the pH, and thus although cells were allowed to grow, the inhibition of these degrading enzymes, such as protease and lipase may have affected the total degradation of TOC. It has been found that the rounded peaks on wave-range curves for TOC, indicate a high level of TOC in the sample (Deflandre *et al*, 2001). This was in accordance with this flow rate therefore, although degradation occurred, TOC concentration was still high during the first stage (2 ml/min) of operation of the FBBR.

Bioaugmentation at a flow rate of 8.5 ml/min and at neutral, acid and alkaline pH:

Attached and planktonic counts and microscopy

Increasing the flow negatively influenced further growth of the mixed-consortium. The decrease in both attached and planktonic populations may have been the cause of the increased flow disturbing the initial attachment from the previous flow rate. The mixed-consortium had become adapted to the slow flow rate conditions (2 ml/min), and the sudden variation in flow rate disturbed that equilibrium. At neutral pH, cells initially continued to grow, but towards the end of the neutral pH run, a decrease in counts was observed. Increased shear or flow rate has previously been found to cause detachment of cells, and this must be the cause of the sudden decrease in attached cells at this flow rate (Hunt *et al*, 2004). The lack of sufficient nutrients by the end of the pH 7 run, may also have caused the detachment of cells since attached cells detach during nutrient starvation (Hunt *et al*, 2004). During alkaline conditions, attached and planktonic cells continued to decrease, although alkaline condition is known to be optimum for growth of *Bacillus spp.* SEM micrographs however, still showed many clusters of cells, and thick strands of intertwining EPS covering the surface of the GAC, indicating the resistance of the biofilm. During the acid shock at this flow rate, counts of cells both attached and planktonic markedly decreased. The decrease in amount of clusters of cells, and EPS can be visualised in the SEM micrographs, where parts of the surface topography of the GAC can be seen. This indicated the detachment of cells at pH 4 possibly due to interactions of orthophosphoric acid with the cell membrane of the bacteria thereby preventing production of enzymes, or its interference with production of proteolytic and lipolytic enzymes itself (Lindsay *et al*, 2000b).

Total organic carbon degradation

pH 7 was the most efficient in allowing the mixed-consortium to consume TOC. The enzymes responsible for degradation of TOC may have been more efficient at

this neutral pH, as the detachment of cells had not yet begun following the increase in flow rate, and thus the amount of enzymes being produced was still much higher than after detachment occurred. pH 10 was not as efficient degrading TOC, due to the detachment of cells and reduced amount of enzymes available for degradation. Lindsay *et al*, (2000a) found that *B. subtilis*, and *B. pumilus* produced lipolytic enzymes at pH 7 and 10, and only weak lipolysis at pH 4. This corresponded with results at this flow rate, where pH 7 and 10 showed most TOC degradation and pH 4 the least. Flemming *et al* (2007), reported the retention of extracellular proteins such as lipases by alginate in the EPS, thus the EPS observed in SEM micrographs at pH 7 and 10 may have played a role in the retention of lipase which assisted in the degradation of TOC. At pH 4 the degradation may have increased due to the adaptation of surviving cells to the acidic pH, and production of small amounts of protease, and lipase. Additionally, the proteins involved in EPS production such as alginate, may have increased the stability of the TOC degrading enzymes under this acidic pH (Flemming & Wingender, 2001). Findings by Resmi & Gopalkrishna, (2004), revealed that the efficiency of removal of organic compounds in dairy wastewater increased with hydraulic retention time, and decreased with hydraulic flow rate, which correlates with findings in this study, whereby this increased flow rate of 8.5 ml/min reduced TOC degradation.

Bioaugmentation at a flow rate of 170 ml/min and at neutral, acid and alkaline pH

Attached and planktonic counts and microscopy

By the highest flow rate, the mixed consortium seemed to have adapted to the constant change in flow rate and the pH variation, as attached cells were found to have increased. SEM images confirmed attachment of cells, and production of vast amounts of EPS on the entire surface of the GAC. Planktonic cells were found to decrease due to the attachment of cells onto the GAC, as previously found at the slowest flow rate. Counts increased at neutral, acid and alkaline pHs

although the flow rate had increased, allowing the assumption that the mixed-consortium, over time adapts to the variation in flow rate and pH, and with continued operation, may allow attached and planktonic counts to stabilise. Additionally, SEM images showed copious amounts of biofilm on the GAC at each pH, reinforcing the idea that biofilm formation and thickness could be further increased regardless of an increase in flow rate. It is also likely, that *Bacillus* spores survived this increase in flow rate, as previously reported by Gentil *et al*, (2010), where *Bacillus* spores survived high shear stress. Slow bacterial growth as well as low pH, is known to enhance EPS growth, and the vast amounts of EPS found in SEM images may be a result of the slow rate of growth due to adaptation to the new flow rate, as well as the acidic pH of 4 during the acid shock of this run (Donlan, 2002; Li & Liu, 2008).

Total organic carbon degradation

pH 10 was most efficient in degradation of TOC at the highest flow rate. This pH is known to be optimal for *Bacillus spp.* showing the potential for growth even at a faster flow rate. The sharper peaks are indicative of the lesser amount of TOC present in the synthetic medium at all pHs in comparison to the more rounded curve of the control peak, which showed represented the larger amount of TOC present (Deflandre & Gague, 2001).

Optimal operation of FBBR

Attached counts remained steady during this run, while planktonic counts decreased, which allowed for the development of more biofilm, however not for the production of aerobic granules as previously assumed. Greater amounts of biofilm could be seen in the SEM images, as well as what is thought to be milk soils attached to the cells, and EPS. Nutrients and molecules from the surrounding medium are known to be found in the biofilm matrix (Donlan, 2002). The mixed consortium of *Bacillus spp.* was not efficient in producing aerobic granules, it

may be that the cohesive forces in producing an aerobic granule were weaker when compared to biofilm production.

pH variation studies

pH 7 showed the greatest increase in attached counts at 8.5 ml/min, as well as the greatest reduction of TOC at the same flow rate. pH 7 had the highest planktonic counts at 8.5 ml/min and 170 ml/min. pH 10 exhibited the greatest increase in attached counts at 170 ml/min, and the biggest reduction of TOC at 2 ml/min, and 170 ml/min. At pH 4, attached counts were highest at 2 ml/min. Planktonic counts were lowest at 8.5 ml/min for pH 4. TOC degradation at this acidic pH, was lowest at 170 ml/min. During the optimum run of the FBBR, pH 10 exhibited the highest attached counts on day 54, while highest TOC degradation was on the last day of operation, day 66. The cell wall synthesising systems which are involved in enzyme and polymer production in *Bacillus spp.* have been reported to be affected by pH, explaining the low levels of degradation at pH 4 in this study (Lindsay *et al*, 2000a). This part of operation of the FBBR, using the *Bacillus spp.* consortium was run during the winter months, and it has been previously found that the stability of proteolytic and lipolytic enzymes decrease as temperature decreases (Nörnberg *et al*, 2010), which may be a possible explanation for the low TOC degradation during this mode of bioaugmentation. Newman *et al*, (2000) also reported reduced degradation of BOD and COD in winter months. The batch mode of operation resulted in starvation of the bacterial consortium at certain points, however EPS, and biofilm mass was maintained. Li & Liu, (2008), reported an increase in amount of EPS production during nutrient starvation conditions, which correlated with this study, indicating the batch mode of operation was advantageous in allowing for a sustainable biofilm.

Part 2: Gram-positive and Gram-negative mixed consortium evaluation

Bioaugmentation at a flow rate of 2 ml/min and at neutral, acid and alkaline pH:

Attached and planktonic cell counts and microscopy

The start-up flow rate was slow to allow for attachment of the cells which increased from 0log cfu/ml to 8log cfu/ml, after only 3 days at pH 7. A study by Oliveria *et al*, (1994), showed the pH preference of a *Pseudomonas fluorescens* isolate to attach to a surface and produce EPS was under neutral conditions. This may be similar for *P. aeruginosa* P1, which together with *B. subtilis* C1 attached at this neutral pH. The same study also states that this is the preferable pH for attachment and production of EPS in most bacterial species, which may explain the large increase in attached counts at this pH, since both species of bacteria in the binary consortium would attach. SEM micrographs confirmed attachment of cells to the GAC, with cells observed inside the pores of the GAC, and what is thought to be EPS was seen. EPS plays a role in the attachment of cells to support media, and gives structural integrity for the formation of biofilm (Costerton, 1999). By the 9th day of batch mode at pH 10, clusters of cells with biofilm pores to allow for nutrient flow, were also observed on the surface of the GAC, as attached counts increased. The decrease of planktonic cell populations at pH 7 and 10, was due the attachment of cells onto the GAC. Detachment of cells from the GAC also occurred at the pH 4, after 12 days, which resulted in an increase in planktonic cell counts. Similarly, SEM images showed that visible clusters of cells diminished from the surface area of the GAC, however some web-like strands of EPS was still visible, indicating the resistance of the EPS secreted from the cells. As described in a study by Wulff *et al*. (2008), the pH of the surrounding medium in which cells are attached, affects the transcription of genes involved in biofilm formation, maintenance, and repair. In addition, unfavourable growth environments such as starvation, is a well known trigger for detachment of *Pseudomonas* biofilms (Hunt *et al*, 2004). The growth medium at acidic pH may have represented an unfavourable growth environment for the 2 bacteria used in this study, which may have resulted in cell detachment, and hence a slower degradation rate of the TOC. Our findings correlate with that of Wulff *et al*, (2008), who suggested that lower pH may inhibit biofilm growth.

Total organic carbon degradation

During the start-up flow rate, initiation of colonisation onto the GAC particles, resulted in the degradation of TOC to be very low, since bacterial counts were low. The wave-shift in peak observed at pH 7 at 2 ml/min, is usually indicative of a change in chemical structure. At the alkaline shock, attached counts increased by 1.5 log cfu/ml. pH 10 had the least sharp peak, indicating the highest levels of TOC, which was consistent with data at 430nm, since lower wavelengths show increased sensitivity (Deflandre & Gagne, 2001). The pH 4 curve had the sharpest peak showing the least amount of TOC (Deflandre & Gagne, 2001). Attached growth proliferated at this pH during the slow flow rate. At the acid shock, although attached counts were low, the most degradation occurred. The suspended cells may have been responsible for consuming the TOC, considering planktonic cells reached 8.5log cfu/ml. This was in accordance with a study by Lindsay *et al*, (2000a) who observed that extracellular enzymes are secreted in both attached and planktonic phases by dairy strains of *Bacillus* and *Pseudomonas*. Protease and lipase activity may have also have been inhibited at acid pH, as it has previously been found that the activity of both these enzymes is inhibited in *Bacillus spp* under acidic conditions (Lindsay *et al*, 2000a). In general, the activity of serine-protease enzymes are inhibited by acid conditions (Gupta *et al*, 2002), therefore, low attached counts combined with the acid pH of the medium, may have contributed to the poor degradation of TOC at this flow rate.

Bioaugmentation at a flow rate of 8.5 ml/min and at neutral, acid and alkaline pH:

Attached and planktonic cell counts and microscopy

When the flow rate was increased to 8.5 ml/min after 18 days, attached counts decreased at first but remained practically constant after 6 days of the flow rate. A

neutral pH is known to result in high amounts of EPS formation in *Pseudomonas* (Oliveira *et al*, 1994) and this correlated to our study, whereby, EPS matrices were visibly formed on the GAC, forming extensive networks across the GAC. Although the flow rate was higher, the distribution of nutrients to attached cells was still achievable, and degradation of TOC took place, although at a steadier pace, compared to the lowest flow rate. This could be that the proliferation of growth of a biofilm, was less likely, due to insufficient nutrients being accessible to the cells. Previous studies conclude that *Bacillus* spores, when attached to surfaces, may germinate, and Lindsay *et al*, (2006) showed that this happens in both favourable, and unfavourable conditions, with *Bacillus subtilis* 168. This germination allows for biofilm formation even under nutrient deficient conditions, and this may account for the development of biofilm, even under the nutrient-limited conditions found in batch mode this flow rate. It has also been previously found that *Bacillus* spores adhere to surfaces more easily than vegetative cells, allowing germination of these spores on the surface of the GAC when favourable conditions return (Rönnner *et al*, 1990). Additionally, *Pseudomonas spp* are also known to accelerate EPS development during initial stages of starvation due to activation of the *algC* gene (Vandevivere & Kirchman, 1993). With both *B. subtilis*, and *P. aeruginosa* capable of producing biofilm under starvation conditions, the clusters of cells and extensive EPS matrices visualised in the SEM micrographs can be accounted for.

Total organic carbon degradation

After detachment of cells in acid shock from the previous flow rate, attached and planktonic cell counts decreased slightly due to the increased flow rate to 8.5 ml/min at pH 7, forcing TOC degradation to be the weakest at this flow rate. The accumulation of TOC at pH 7 may be attributed to the sudden increase in flow, making it more difficult for absorption of TOC by the binary-species consortium, showing the inefficiency of the consortium to quickly adapt to the sudden increase in flow rate, and hence allowing TOC to accumulate. During pH 10 and 4, TOC degradation continued, although counts remained fairly similar. The increasing

flow rate did not allow biofilm formation to proliferate, however still allowed the degradation of TOC, allowing the belief that the binary consortium was still adapting to the higher flow rate, and as such could only absorb enough nutrients for survival.

Bioaugmentation at a flow rate of 170 ml/min and at neutral, acid and alkaline pH:

Attached and planktonic cell counts and microscopy

The highest flow rate (170 ml/min) encouraged detachment, with attached counts decreasing rapidly, and planktonic cells remaining the same overall. The SEM micrographs show less clusters of cells compared to previous flow rates, which is indicative of the detachment or shearing taking place during the high flow rate, and the inability of cells to attach to the GAC. The detachment of cells after an overall steady state is the result of shearing caused by the increased flow rate, this has been observed in drinking water distribution systems, which like dairy factories, also have variable flow rates, and have been shown to cause the detachment of biofilms (Telgmann *et al*, 2004).

Total organic carbon degradation

The highest flow rate caused counts to decrease drastically and TOC degradation to be very low at all pHs. The decrease could be attributed to the higher shear forces, causing a decrease in attached cells, by allowing the migration of the cells to the inner parts of the biofilm, or into the pores of the GAC, causing TOC degradation to be low (Di Iaconi *et al*, 2005). Overall if left as the final mode of operation, TOC would have only degraded 0.03 mg/l, however, the conversion to optimal mode of operation after this flow rate, allowed the depletion of TOC.

Optimal operation of FBBR

The optimum pH and flow rate was determined, and used to stimulate aerobic granule formation, to decrease TOC content in the synthetic wastewater. TOC was completely depleted, and attached growth was greatly increased. A final attached growth of almost 9 log cfu/ml was achieved. Although thickness of the biofilm bed did not increase greatly, cells involved in the formation of aerobic granules instead of biofilm formation could account for this. Planktonic cells decreased slightly overall, possibly due to the attachment of cells to the carrier, and the formation of aerobic granules. The transcription of genes involved in aggregation and biofilm formation, seem to increase with the increase of pH of a medium (Wulff *et al*, 2008), which correlates to the findings in this study of pH 10 being optimum for biofilm, and aerobic granule formation. However, a possible explanation for granulation not increasing in size, as quickly as other systems, could be the types of strains used for bioaugmentation in this study (Ivanov *et al*, 2006). Additionally, we predict that a change in operation mode from batch to a continuous feed, may allow the granules to increase in size, and provide an increased surface area for the consumption of TOC. The EPS formed in the previous run of 170 ml/min may have assisted in the development of aerobic granules, by bridging the cells and compacting them into aggregates (Adav, *et al*; 2008). The use of aerobic granules may be vital in allowing continual degradation, even during acid, and alkaline shock, and the addition of selected bacteria with enhanced floc-forming ability would positively enhance the development of further granulation (Ivanov *et al*, 2006). This optimal flow rate may be used as a constant flow rate on dairy wastewater premises, by altering specific factories' needs and volumes of wastewater removal, as a means of more efficient removal or bioremediation of the wastewaters (Brewer *et al*, 1999). Therefore industrial upscale is possible utilising the optimal pH, and flow rate.

pH variation studies

It was evident that, at pH 4, attached cells degraded TOC most efficiently at both 8.5 ml/min and 170 ml/min as evidenced at 598nm, while at pH 10, attached cells degraded the synthetic medium most efficiently at 2 ml/min.

In summary, after 66 days of operation, biofilm bed interestingly growth did not necessarily correlate with TOC degradation. When attached counts were higher, TOC degradation was not always higher than when attached or planktonic counts were lower. However at day 60 of the cycle, TOC was degraded completely, and on day 66 attached, and planktonic counts increased while TOC remained at 0 mg/l. This implied that biofilm bed growth, if at the optimum pH and flow rate, will increase attached growth, and planktonic cell growth will increase proportionally, as will the growth of aerobic granules. The long period of time taken for the TOC to be reduced to zero may be attributed to biocatalytic processes needing a long period of time, and large surface areas, to develop efficiently in the fluidized bed biofilm bioreactor (Metzdorf *et al*, 1985). However, once a biofilm is established, little time is required to deplete TOC levels. Overall bioaugmentation by the Gram-positive and Gram-negative binary species consortium was successful in reducing TOC levels while maintaining biofilm bed growth, and inducing aerobic granule formation. Furthermore, the batch mode system used to operate the FBBR should not be seen as a downfall, considering this study proved the depletion of TOC using this method. Previous studies have shown that attached bacterial cells may exhibit enhanced nutrient status under nutrient limitation (Lindsay *et al*, 2006), and that biofilm density increases under decreasing substrate loading (Horn & Morgenroth, 2006). Another study showed that EPS formation is accelerated in species of *Pseudomonas* under starvation conditions (Vandevivere & Kirchman, 1993), proving the viability of this study even under batch mode of operation.

Comparing degradation within the reactors containing 2 different consortia types

Attached and planktonic counts at 2; 8.5; and 170 ml/min

The trend of steadily increasing growth at 2 ml/min, by the *Bacillus* only mixed consortium is as a result of the ability of these 3 species to adapt to the medium and its conditions fairly quickly. All 3 of these *Bacillus* species have previously been isolated from dairy processing lines where they are common spoilage microorganisms of dairy products (Nörnberg *et al*, 2010). The source of these particular isolates were also from dairy products, therefore their adaptation to the synthetic medium was not unusual, and allowed it to grow steadily, even through starvation conditions characteristic of batch mode of operation. The erratic growth exhibited by the binary Gram-positive and –negative consortium, may be due to the starvation caused by batch mode, since counts increased when fresh medium was recycled through the reactor. The slow flow rate may have favoured the *Bacillus* species more than the combined *B. subtilis* and *P. aeruginosa* consortium as it *Bacillus* may have been more favoured for attachment. When the flow rate was increased attached cells remained steady for both consortia, possibly due to the steady-state achieved from the equal attachment and detachment of cells after an increase in flow rate. The highest flow rate interestingly allowed an increase in cells for the Gram-positive consortium, again showing the resilience of *Bacillus* species to varying conditions, while a decrease was observed for the mixed Gram-positive and –negative consortium, most probably as a result of the inability of cells to attach to the carrier at a higher flow rate. *Bacillus* spores are also known to attach to carrier particles, and germinate when conditions are favourable (Lindsay *et al*, 2000a, Rönnner *et al*, 1990). Although no significant difference was found between attached counts of the 2 consortia, the difference in TOC degradation as a result of attached cell populations depicted the distinct difference between the consortia. Planktonic cell counts remained steady for both consortia, and decreased only when an increase in attached cells occurred while it increased when detachment occurred due to increased hydraulic shear forces.

Microscopy

During the lowest flow rate the growth patterns between 2 consortia were similar except during acid shock, where the Gram-positive consortium showed resistance to the orthophosphoric acid, and EPS was not broken down to the extent observed in the Gram-positive and –negative consortium SEM micrographs. The increase to flow rate 8.5 ml/min showed contrast in cells from the Gram-positive and –negative consortium which showed resistance to increased shear forces, compared to the Gram-positive consortium which showed a breakdown in EPS due to increased shear. At 170 ml/min similar patterns of attached cell growth were observed at pH 7 and 4 for the 2 consortia, but at pH 10 the Gram-positive consortium displayed more growth. *Bacillus* species are known to thrive in alkaline environments and hence this is not unusual (Lindsay *et al*, 2000a). However, it is known that aerobic granule formation is influenced by the content and amount of EPS available (Liu & Tay, 2004), thus it is interesting to observe that no aerobic granules were formed from bioaugmentation of the Gram-positive consortium, although this consortium showed more EPS content in SEM images than the Gram-positive and –negative consortium which did produce aerobic granules. The observation of what is thought to be milk soils during the bioaugmentation of Gram-positive species may be absent from the other consortium as a result of the greater absorption of nutrients by the binary consortium, compared with the Gram-positive consortium.

Total organic carbon degradation

A greater amount of TOC was degraded by the binary consortium. The combination of degrading enzymes secreted by this pair greatly enhanced TOC degradation compared to the mixed Gram-positive consortium which overall showed no significant decrease in TOC levels. The binary pair may have produced increased levels of degrading enzymes compared to the mixed Gram-positive consortium where the secretion of enzymes was evident, however they

were possibly not secreted in large enough amounts to cause a significant decrease in TOC. Lipolytic enzymes from *P. aeruginosa* are used commercially for degradation of industrial wastewaters, so it is not unusual that the binary consortium exhibited strong proteolytic and lipolytic abilities (Camarota & Freire, 2006).

Initiation of granulation in the FBBR

Initiation of granulation was successful for the binary consortium but not for the mixed Gram-positive consortium. Furthermore granulation successfully depleted TOC levels, while the absence of granules in the bioaugmentation using a Gram-positive consortium caused TOC levels to remain the same. Aerobic granules have been previously used to degrade toxic components in industrial wastewaters, and this was no exception for the binary pair. The optimal run of the FBBR induced granule formation in the binary Gram-positive and –negative consortium run of the FBBR, but not with the Gram-positive only mixed consortium. This finding led us to believe that the production of aerobic granules in the reactor may cut down the time required for complete TOC depletion to occur. These findings contrasted with those of Liu & Tay (2004), who stated the requirement of high shear forces for the development of aerobic granules, whereas this study showed the preference of a slow flow rate for aerobic granulation. Additionally, it was found that aerobic granules form commonly under starvation conditions, since cells become more hydrophobic when under stress of starvation (Liu & Tay, 2004), thus the granules formed by using batch mode of operation in this FBBR was not unusual.

pH variation studies

From attached counts and SEM micrographs, it is clear that the Gram-positive consortium adapted well to acid and alkaline shocks at all flow rates, compared to the binary pair, where an observable decrease in counts and attached cells

occurred during acid shock. pH 7 allowed the growth of both consortia, but did not enhance growth beyond what was expected for both consortia. The alkaline shock (pH 10) allowed increased growth for both consortia, as alkaline conditions are known to be optimal for growth of certain *Bacillus spp.* Lindsay *et al.*, (2000a) reported enhanced attachment of 3 *Bacillus* strains used in the study at alkaline pH, and of these 3 species, 2 were utilised in this study (*B. subtilis*, and *B. pumilus*). Overall the Gram-positive consortium maintained growth at all pH's and flow rates. However, this was not similar for the binary consortium, which did show an increasing adaptation to the acid and alkaline shocks by increasing after any variation in flow rate, and by further increasing the degradation of TOC.

CONCLUSIONS

The operation of the FBBR in batch mode was successfully used to deplete total organic carbon when a binary consortium of both Gram-positive and –negative *spp.* was utilised for bioaugmentation. However the use of a Gram-positive *Bacillus* consortium did not reduce TOC as expected. The combination of degrading enzymes from *Bacillus subtilis* C1 and *Pseudomonas aeruginosa* P1 was more effective for proteolysis and lipolysis of fats and proteins in the synthetic dairy wastewater. The enhanced lipolytic ability of *P. aeruginosa* P1 combined with the enhanced proteolytic and lipolytic ability of *B. subtilis* C1, seemed to enhance the degradation of TOC. The strong ability of enzymes from these species to degrade TOC is beneficial for dairy wastewaters, as it is the proteins and lipids, which are of environmental concern. Biofilm bed growth was maintained when using both consortia, but was more abundant, and developed much faster during bioaugmentation using a Gram-positive mixed consortium. The combination of *Bacillus* species, all of which produce abundant amounts of EPS allowed the increased production of the observed EPS. Aerobic granules known to enhance TOC degradation was only formed when using a binary consortium of *Bacillus subtilis* C1 and *Pseudomonas aeruginosa* P1. TOC was depleted during optimal operation while in the presence of aerobic granules. It is thus reasonable to presume that the production of aerobic granules enhances the

degradation of TOC. In summary, the aerobic FBBR using a mixed-consortium consisting of *Bacillus subtilis* C1; *Bacillus pumilus* G5; and *Bacillus amyloliquefaciens* G2, although having the capability to produce biofilm and degrade TOC, didn't achieve the required depletion of TOC, and aerobic granules were not formed to further enhance degradation. These aerobic granules formed by the binary consortium can be applied industrially, since it is now known that this pair is capable of developing aerobic granules and thus large-scale treatment plants can easily be developed. The combination of the mixed *Bacillus spp* consortium did not correlate to Chapter 2, where it was found that these 3 in combination, accelerated degradation of fats and proteins in the synthetic medium. However, the utilisation of a Gram-positive and –negative binary consortium did successfully deplete TOC, although biofilm growth was not as abundant compared to the Gram-positive consortium. This finding proves the efficacy of a Gram-positive and –negative binary consortium in the degradation of TOC, which can be used to prevent discharge of toxic wastewater into natural water bodies thereby preventing eutrophication and other harmful implications. The economic implications will not be a downfall although aeration is needed, since costs will be saved from enzyme production, because of the industrial availability of these enzymes from both *B. subtilis* and *P. aeruginosa*. Temperature control was also not needed, thus costs of heaters and coolers would be saved. The ecological benefits are clear since the prevention of eutrophication would be apparent as well as the prevention of death of animals and humans on consumption of water from water bodies where dairy factories have discharged its wastewaters.

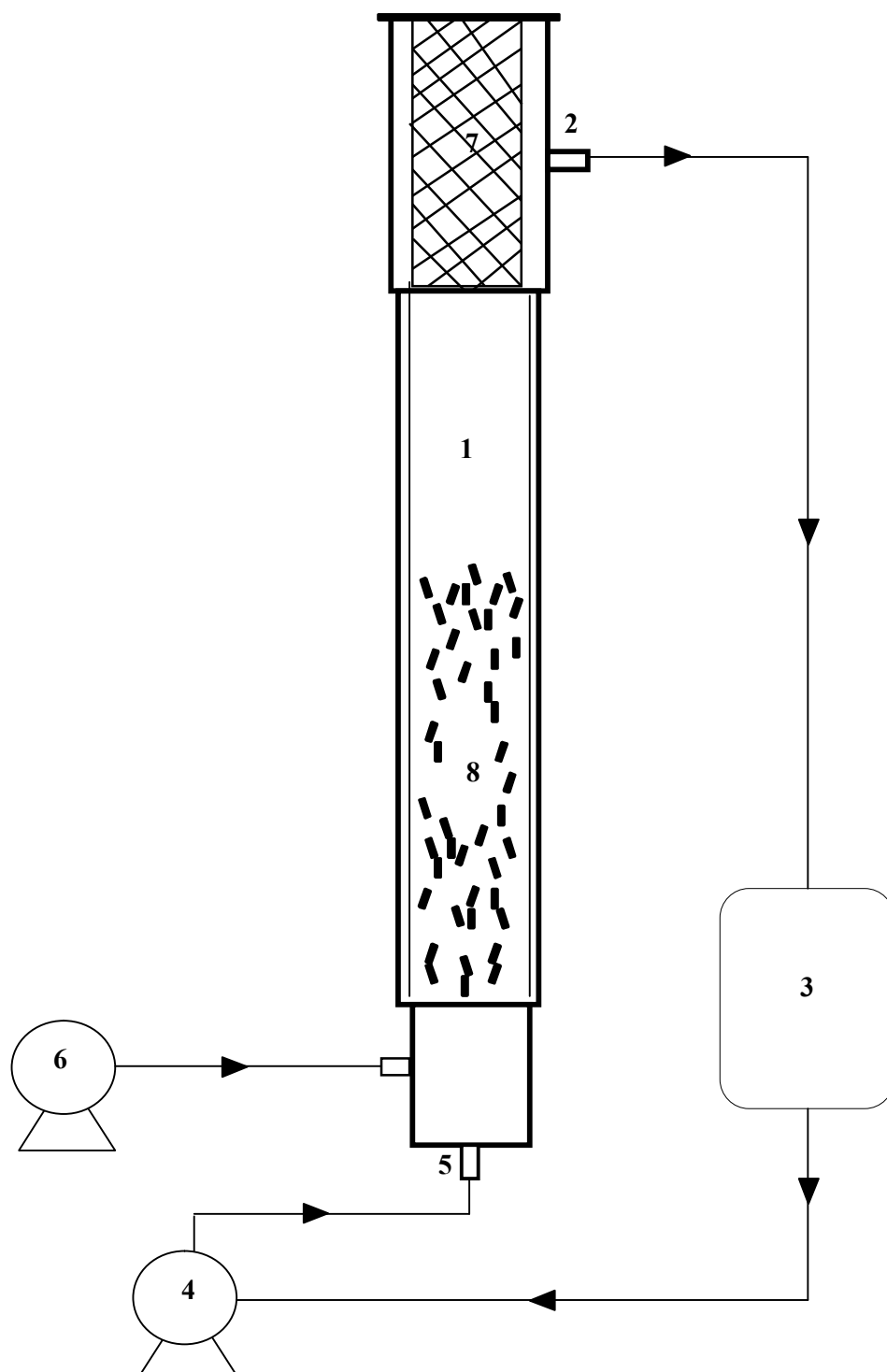


Figure 3.1a: Schematic diagram of aerobic fluidised bed biofilm bioreactor used in this study. 1: FBBR column; 2: outlet; 3: 10% synthetic medium reservoir; 4: peristaltic pump; 5: inlet; 6: air pump; 7: sieve; and 8: 4mm GAC pellets



Figure 3.1b: Visual set-up of FBBR

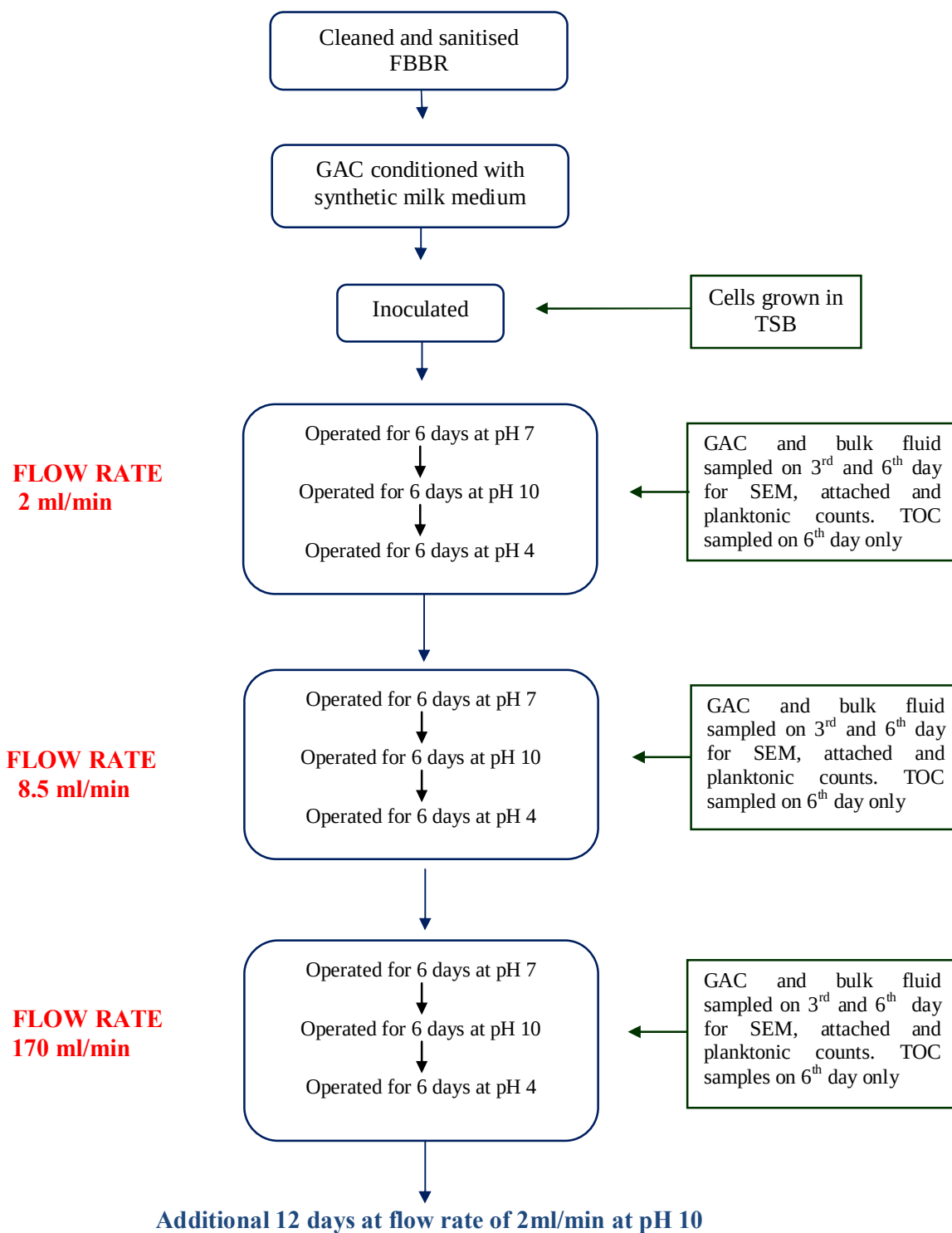


Figure 3.2: Flow diagram illustrating the methodology used to operate the fluidised bed biofilm reactor during this study

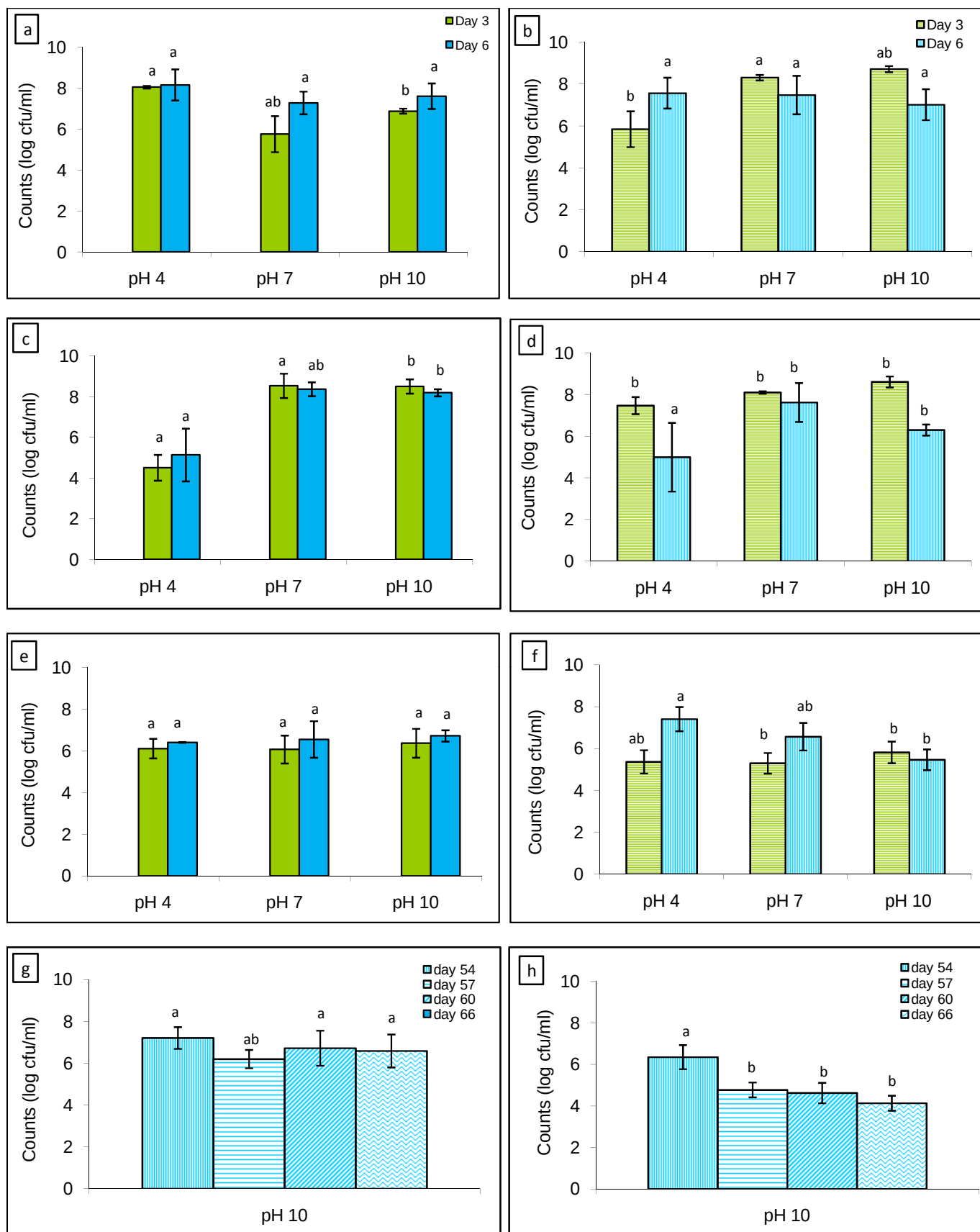


Figure 3.3: Counts of attached cell population of Gram-positive mixed-consortium in aerobic FBBR on 3rd and 6th day of sampling at 2 ml/min (a); 8.5 ml/min (c); and 170 ml/min (e) , and planktonic cell counts at 2 ml/min (b); 8.5 ml/min (d); and 170 ml/min (f) . Attached (g) and planktonic (h) counts during optimal run of FBBR.

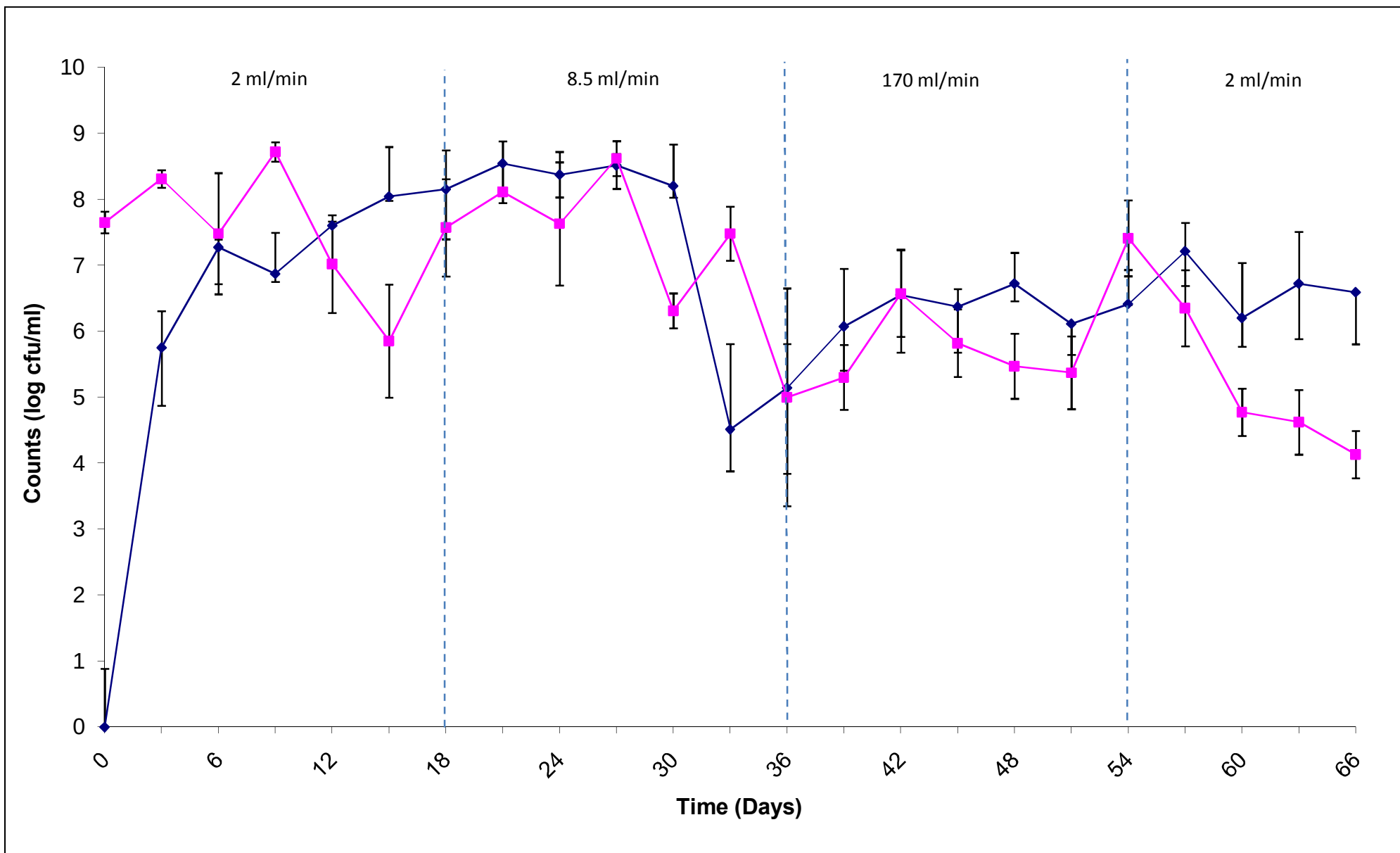


Figure 3.4: Total attached and planktonic cell growth of Gram-positive mixed-consortium over 66 days in aerobic FBRR

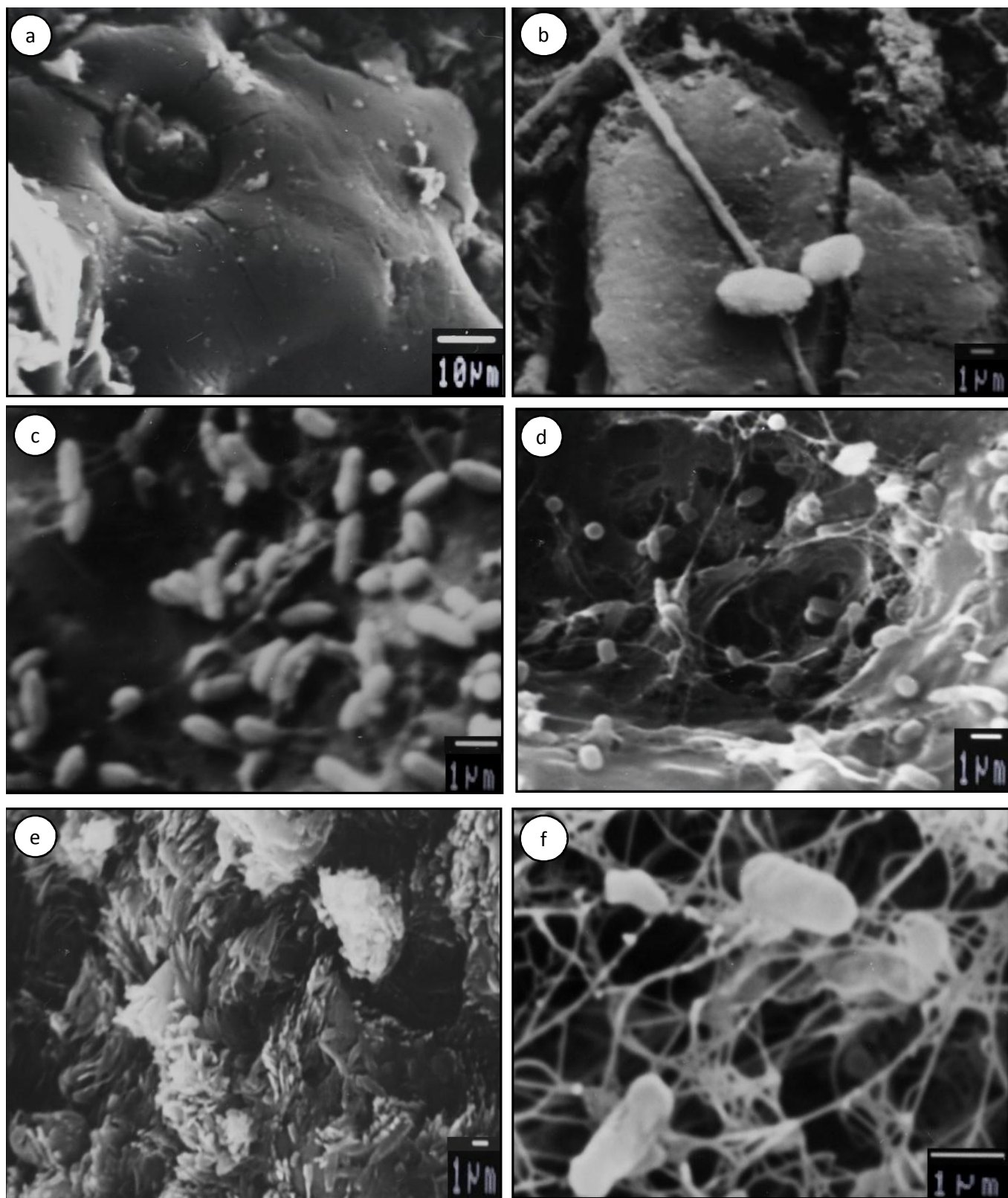


Figure 3.5: Scanning electron micrographs of Gram-positive mixed-consortium on control GAC (a) and at 2ml/min and (b) pH 7 day 6; (c) pH 10 day 3; (d) pH 10 day 6; (e) pH 4 day 3; (f) pH 4 day 6

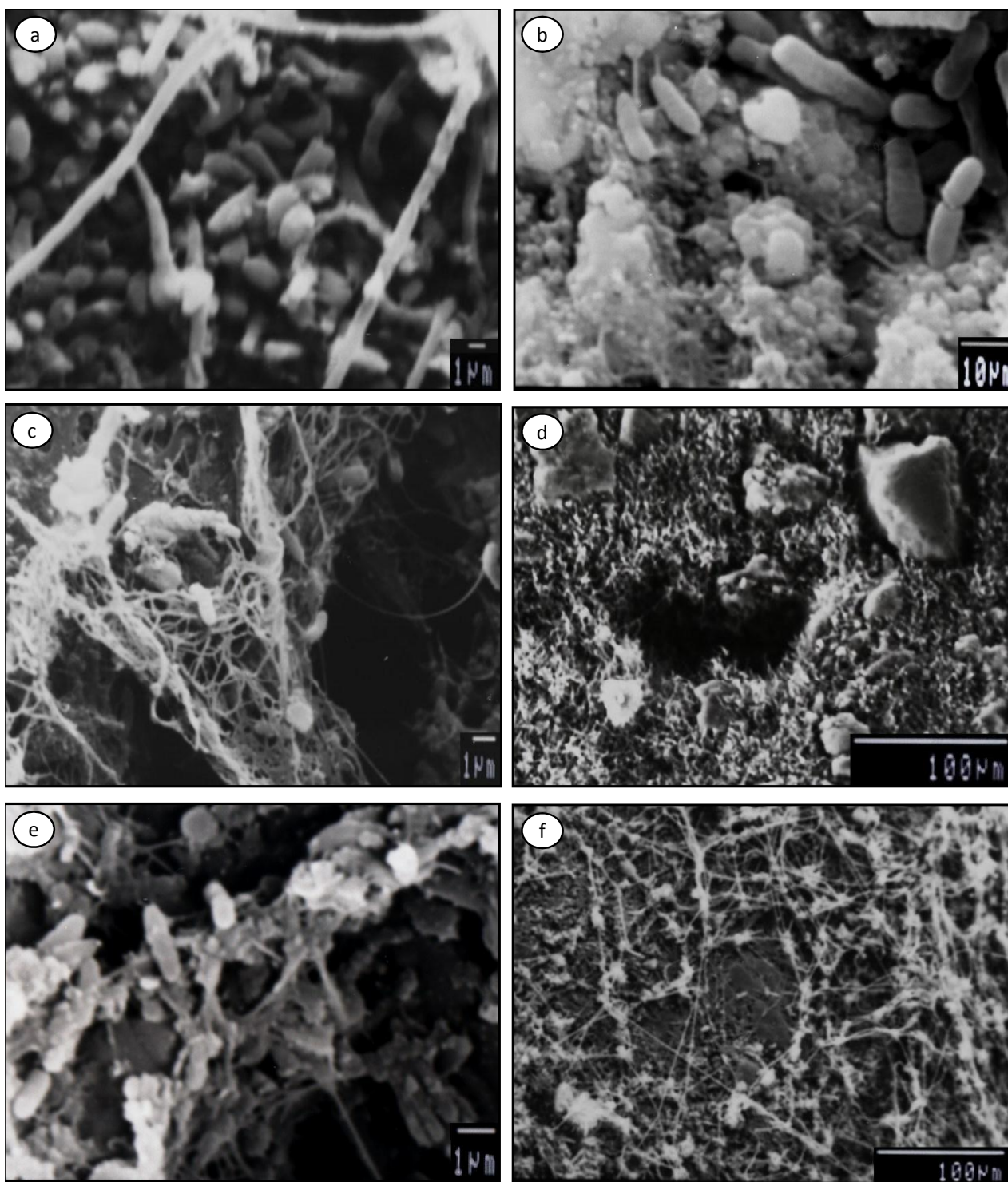


Figure 3.6: Scanning electron micrographs of Gram-positive mixed-consortium on GAC at 8.5 ml/min and (a) pH 7 day 3; (b) pH 7 day 6; (c) pH 10 day 3; (d) pH 10 day 6; (e) pH 4 day 3; (f) pH 4 day 6

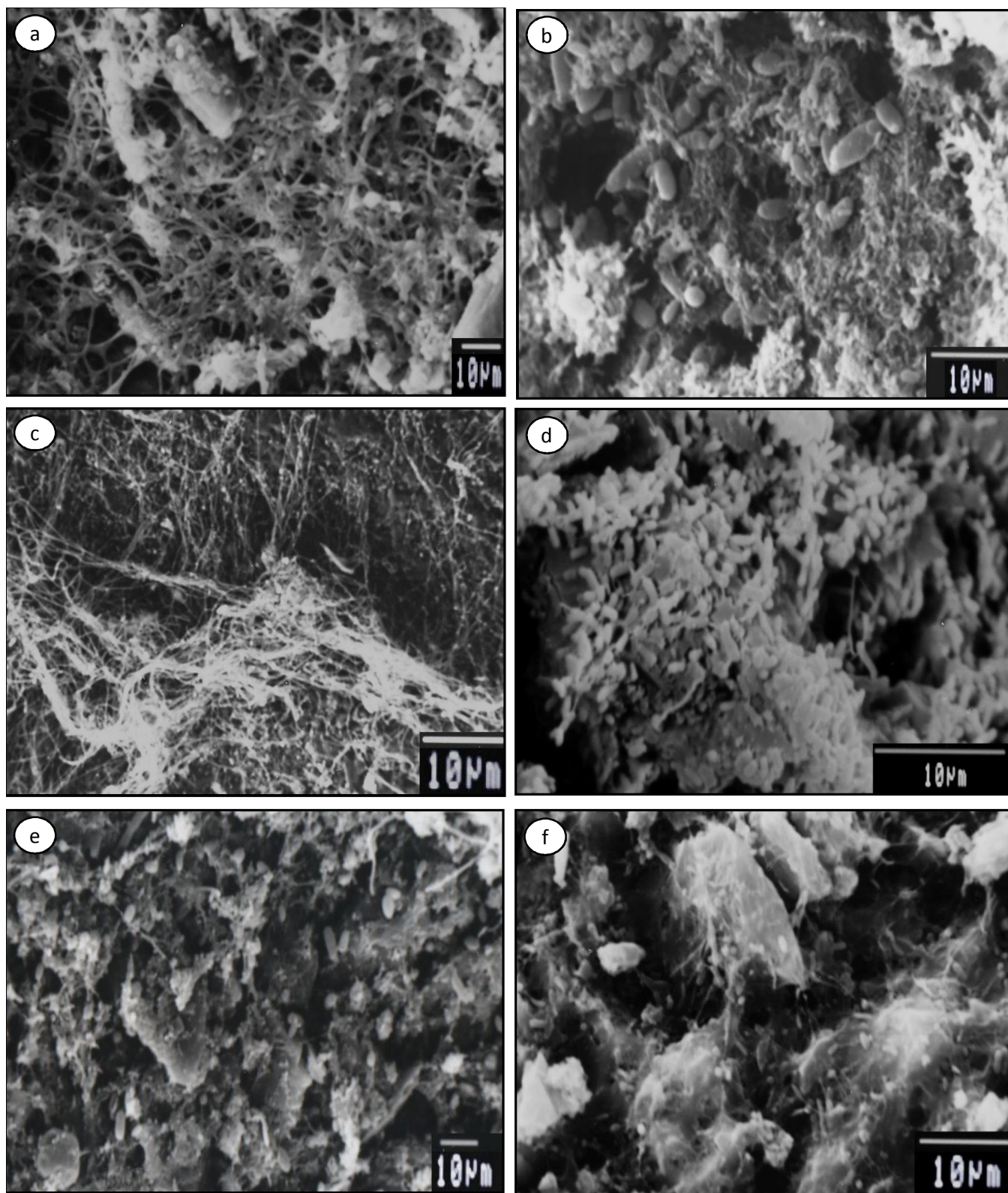


Figure 3.7: Scanning electron micrographs of Gram-positive mixed-consortium on GAC at 170 ml/min and (a) pH 7 day 3; (b) pH 7 day 6; (c) pH 10 day 3; (d) pH 10 day 6; (e) pH 4 day 3; (f) pH 4 day 6

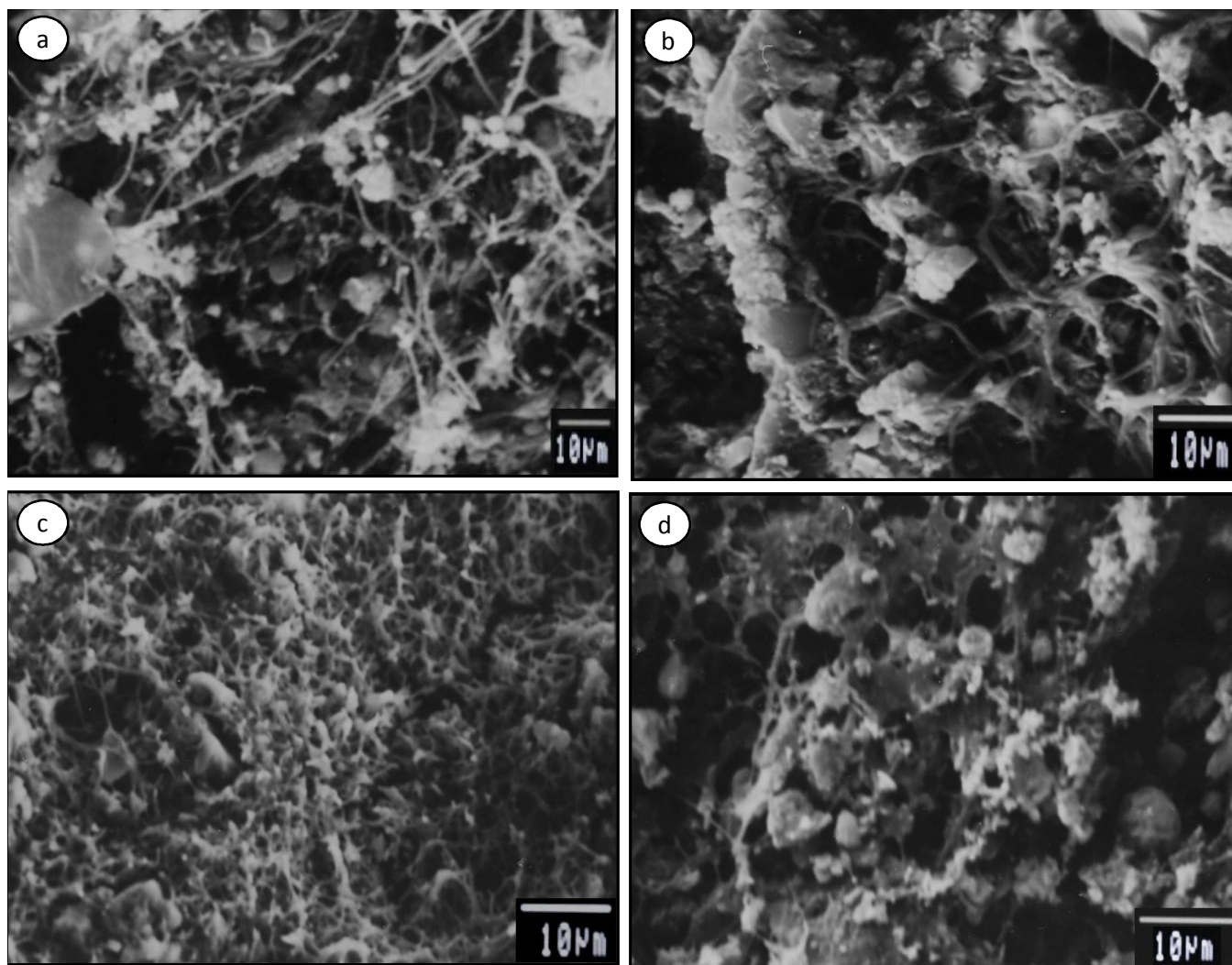


Figure 3.8: Scanning electron micrographs of Gram-positive mixed-consortium on GAC at during optimal mode of operation of FBBR of 2 ml/min and pH 10 on (a) day 57; (b) day 60; (c) day 63; and (d) day 66

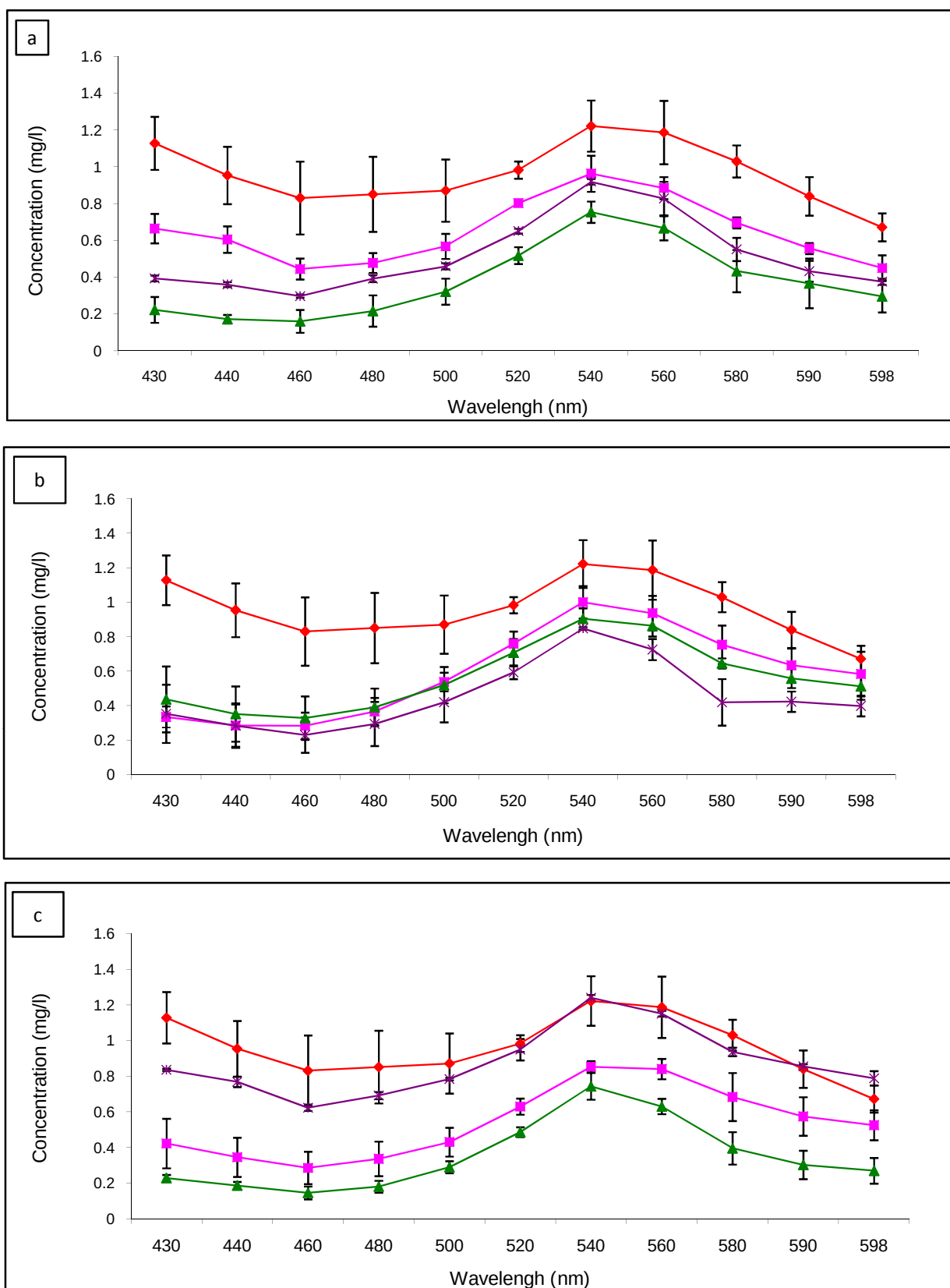


Figure 3.9: TOC degradation for pH 4 (—*), 7 (—■), and 10 (—▲) at flow rate 2 ml/min (a); 8.5 ml/min (b), and 170 ml/min (c) compared to control (—♦) for Gram-positive mixed consortium

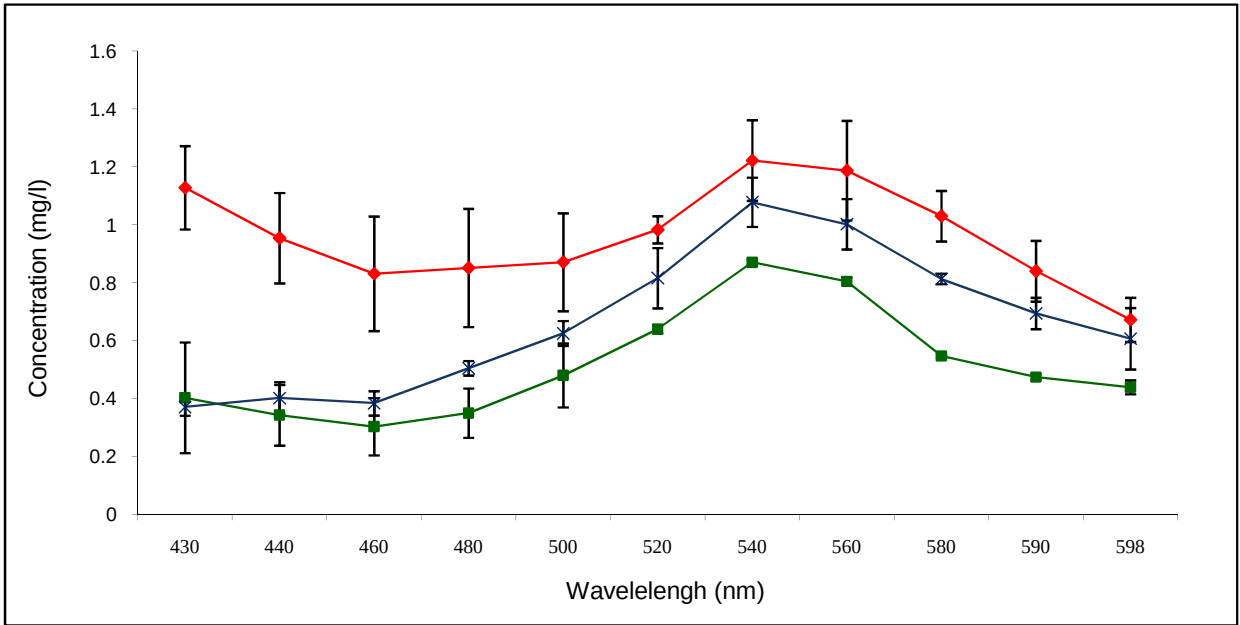


Figure 3.10: TOC degradation during optimal operation of pH 10 and 2 ml/min, on day 60
■ and day 66 * compared to control ◆ for Gram-positive mixed consortium

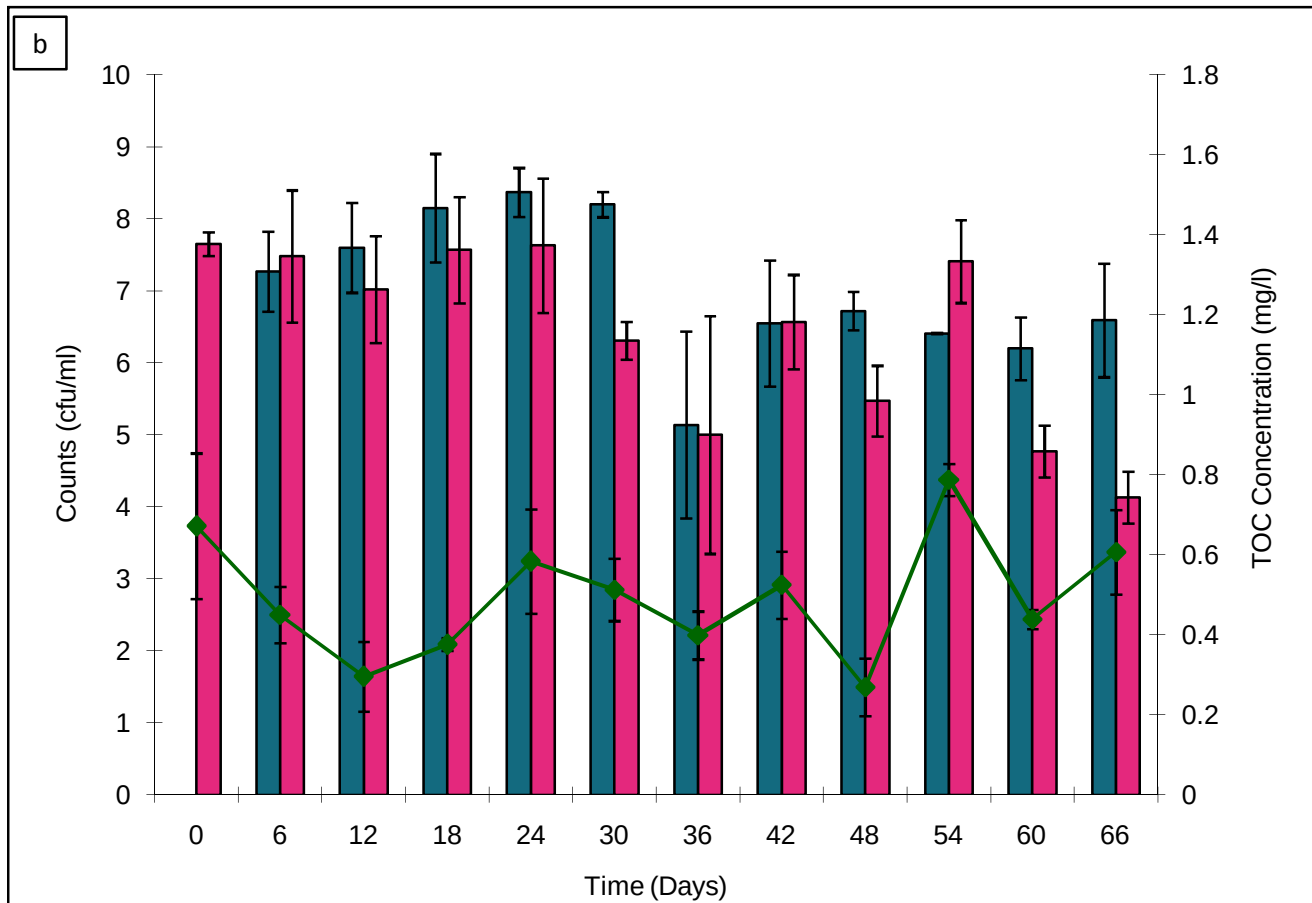
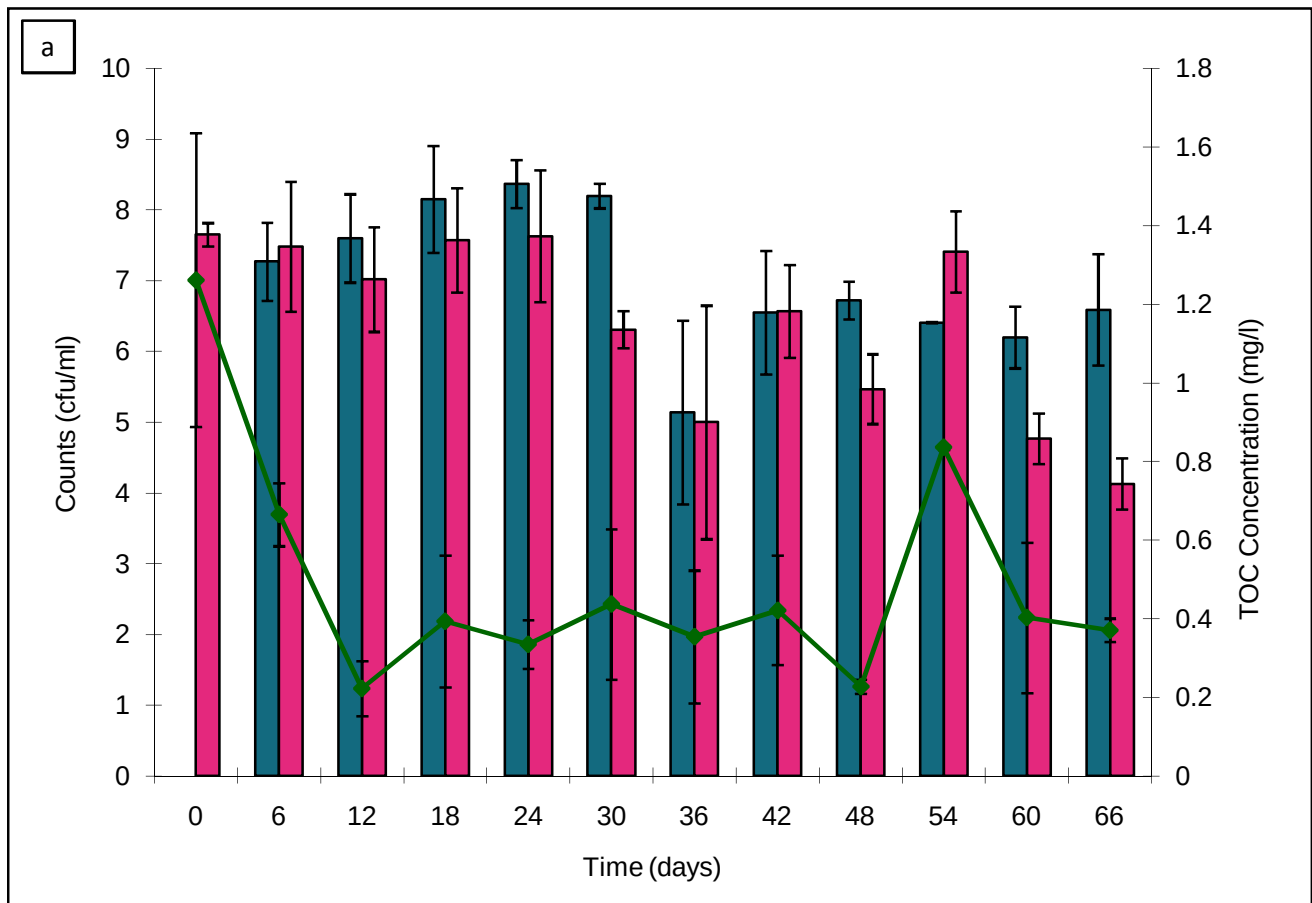


Figure 3.11: TOC degradation ◆ vs attached ■ and planktonic ■ cell counts of Gram-positive mixed consortium over time in aerobic FBFR

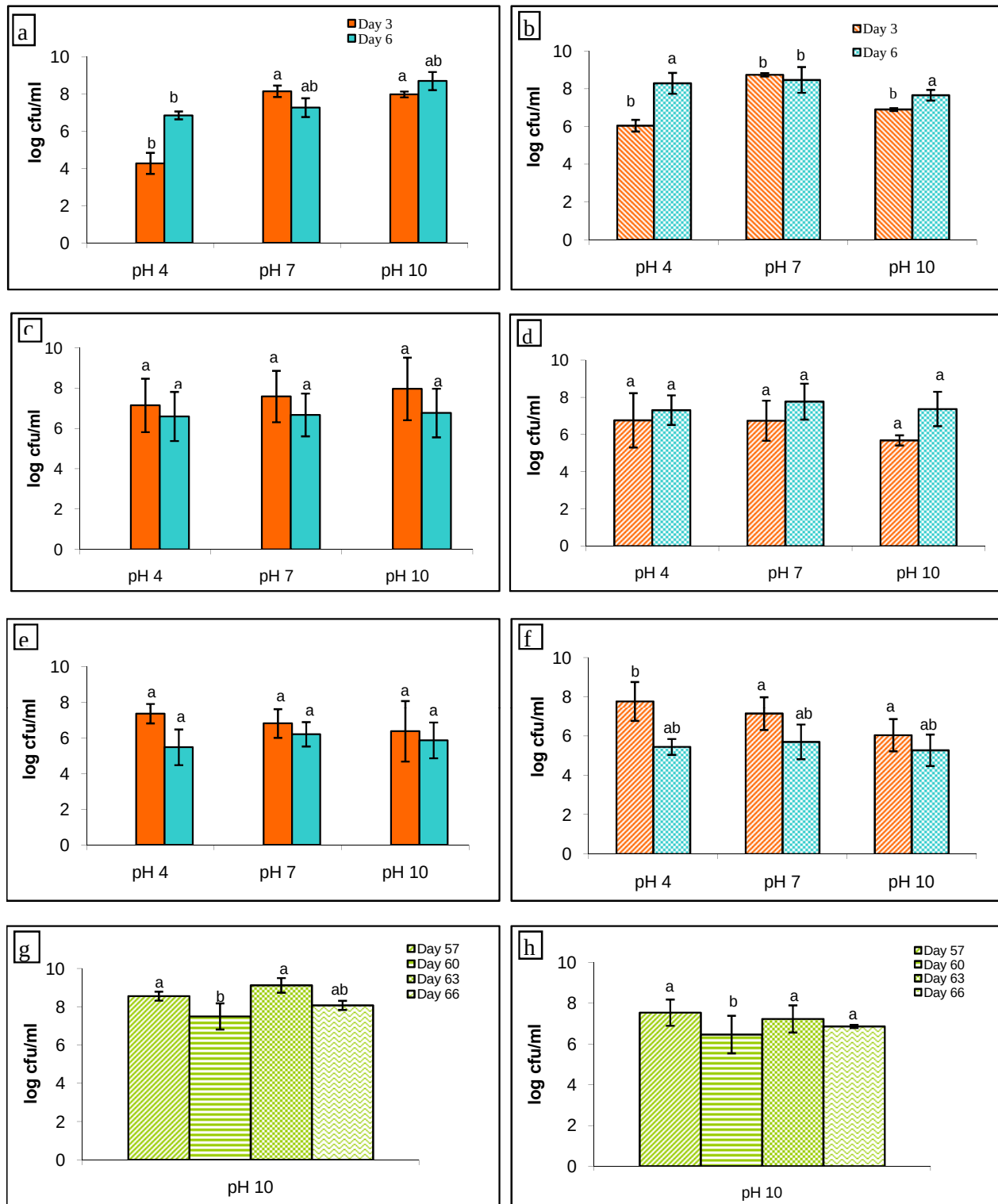


Figure 3.12: Gram-positive and -negative binary consortium attached cell counts at flow rates: 2, 8.5, and 170ml/min consecutively (a,c,e). Planktonic cell counts at flow rates: 2; 8.5, and 170ml/min consecutively (b,d,f). Binary consortium run at optimal flow rate and pH of 2ml/min and pH 10: attached cell counts (g), and planktonic cell counts (h).

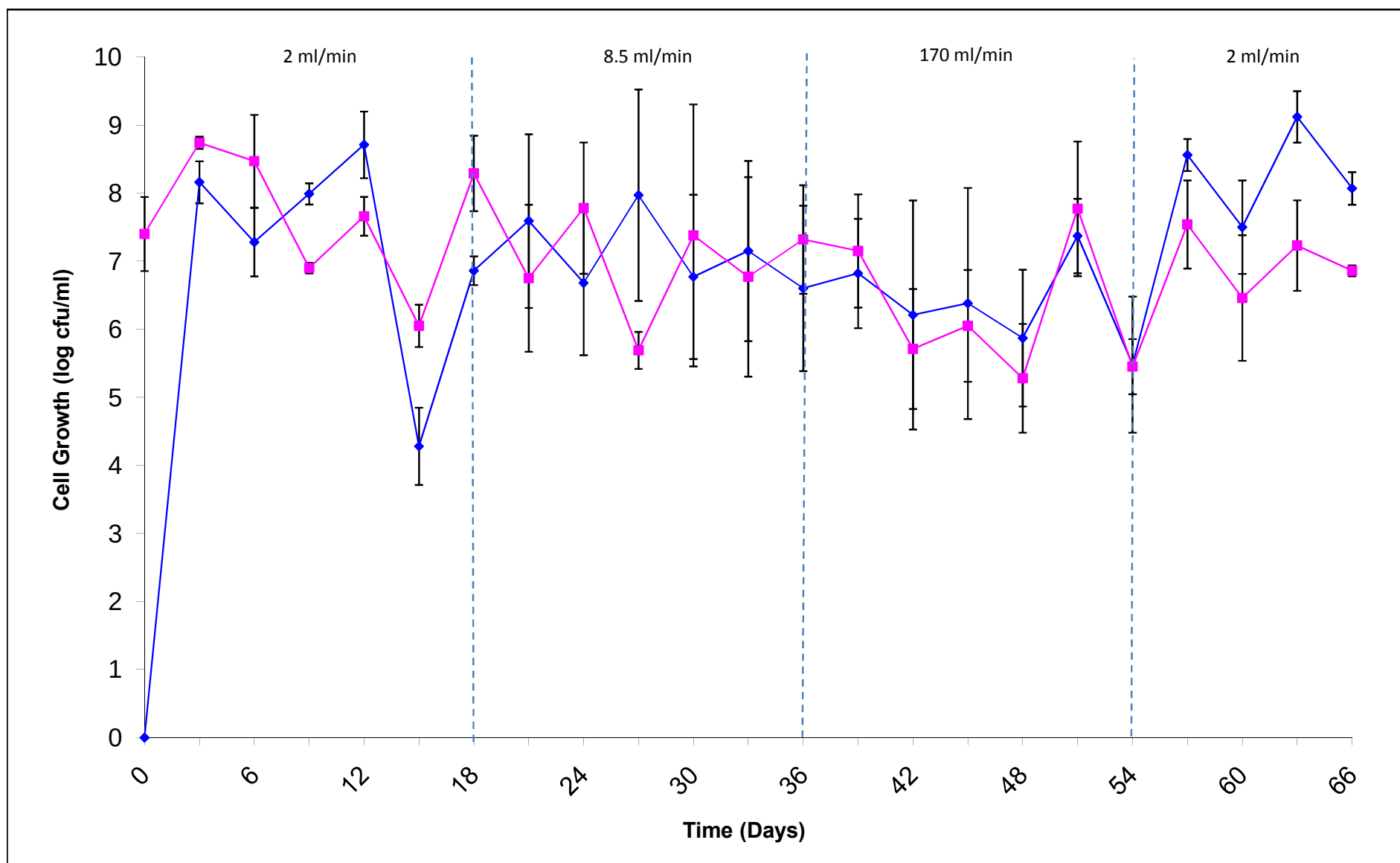


Figure 3.13: Attached ◆ and planktonic ■ cell counts of Gram-positive and -negative binary consortium over 66 days of operation of FBRR

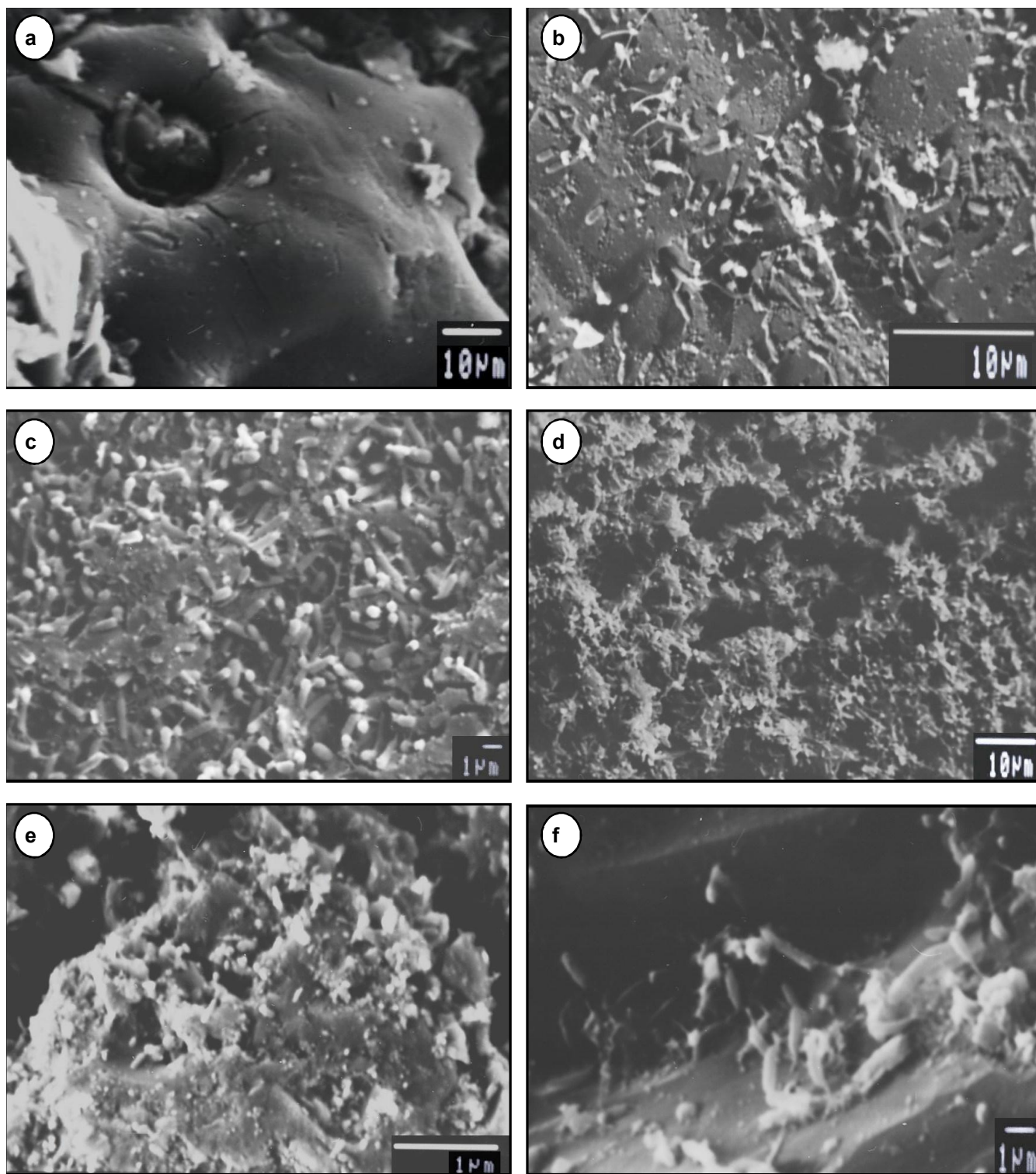


Figure 3.14: Scanning electron micrographs of Gram-positive and -negative binary consortium for control and experimental GAC for flow rate 2ml.min at (a) control GAC; (b) pH 7 day 6; (c) pH 10 day 3; (d) pH 10 day 6; (e) pH 4 day 3; and (f) pH 4 day 6

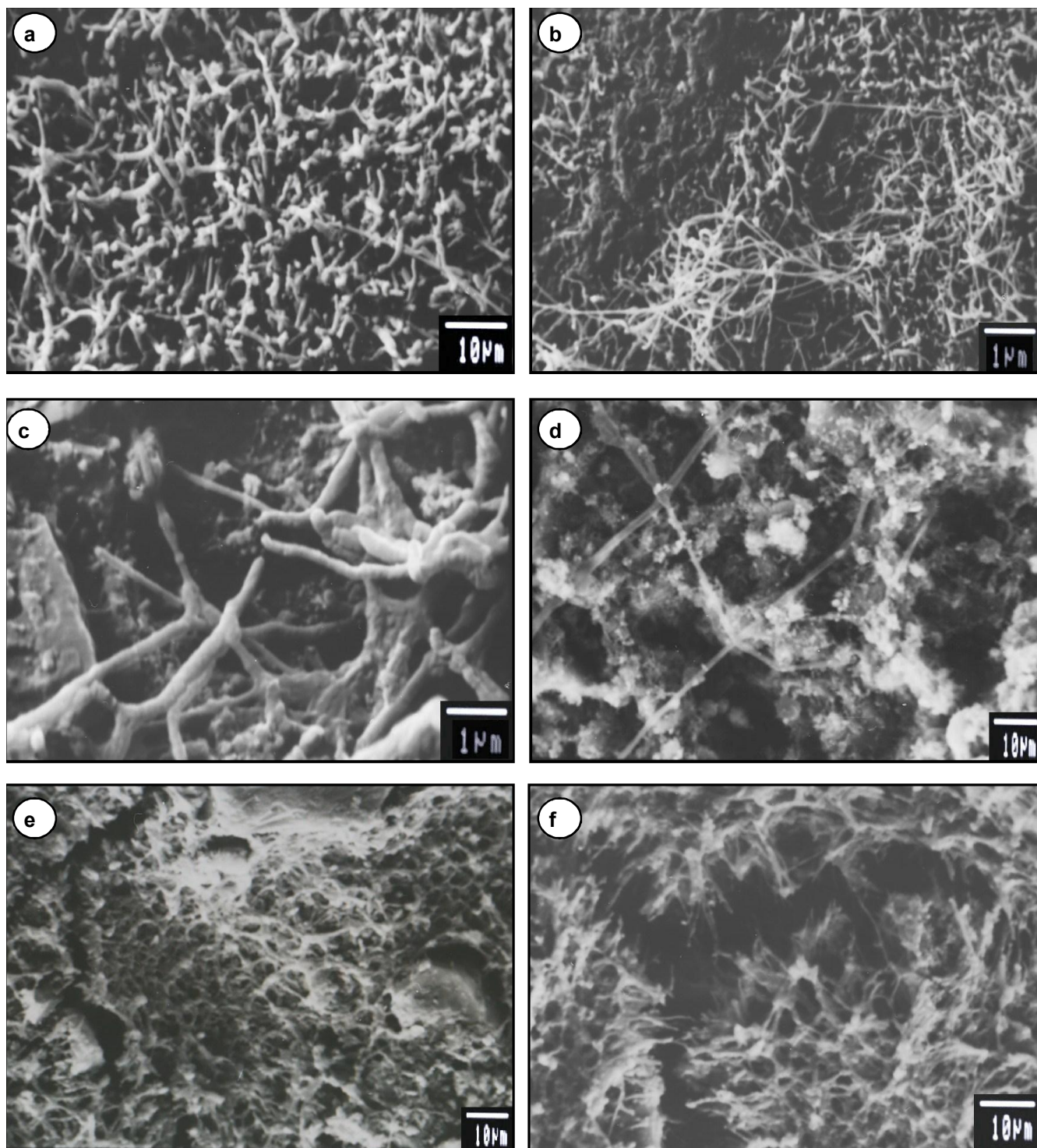


Figure 3.15: Scanning electron micrographs of Gram-positive and -negative binary consortium for experimental GAC for flow rate 8.5ml/min at (a) pH 7 day 3; (b) pH 7 day 6; (c) pH 10 day 3; (d) pH 10 day 6; (e) pH 4 day 3; and (f) pH 4 day 6

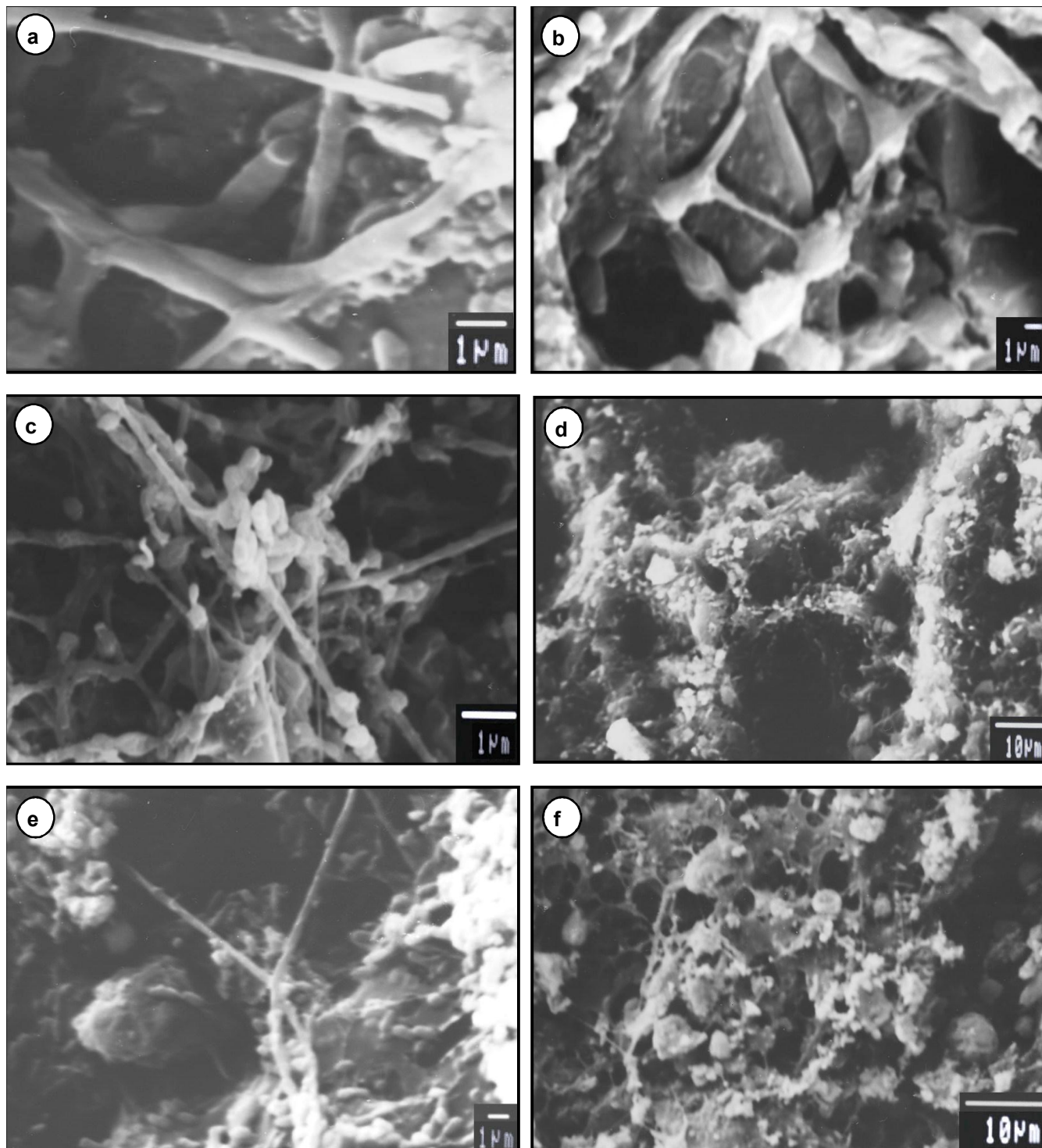


Figure 3.16: Scanning electron micrographs of Gram-positive and -negative binary consortium for experimental GAC for flow rate 170ml/min at (a) pH 7 day 3; (b) pH 7 day 6; (c) pH 10 day 3; (d) pH 10 day 6; (e) pH 4 day 3; and (f) pH 4 day 6

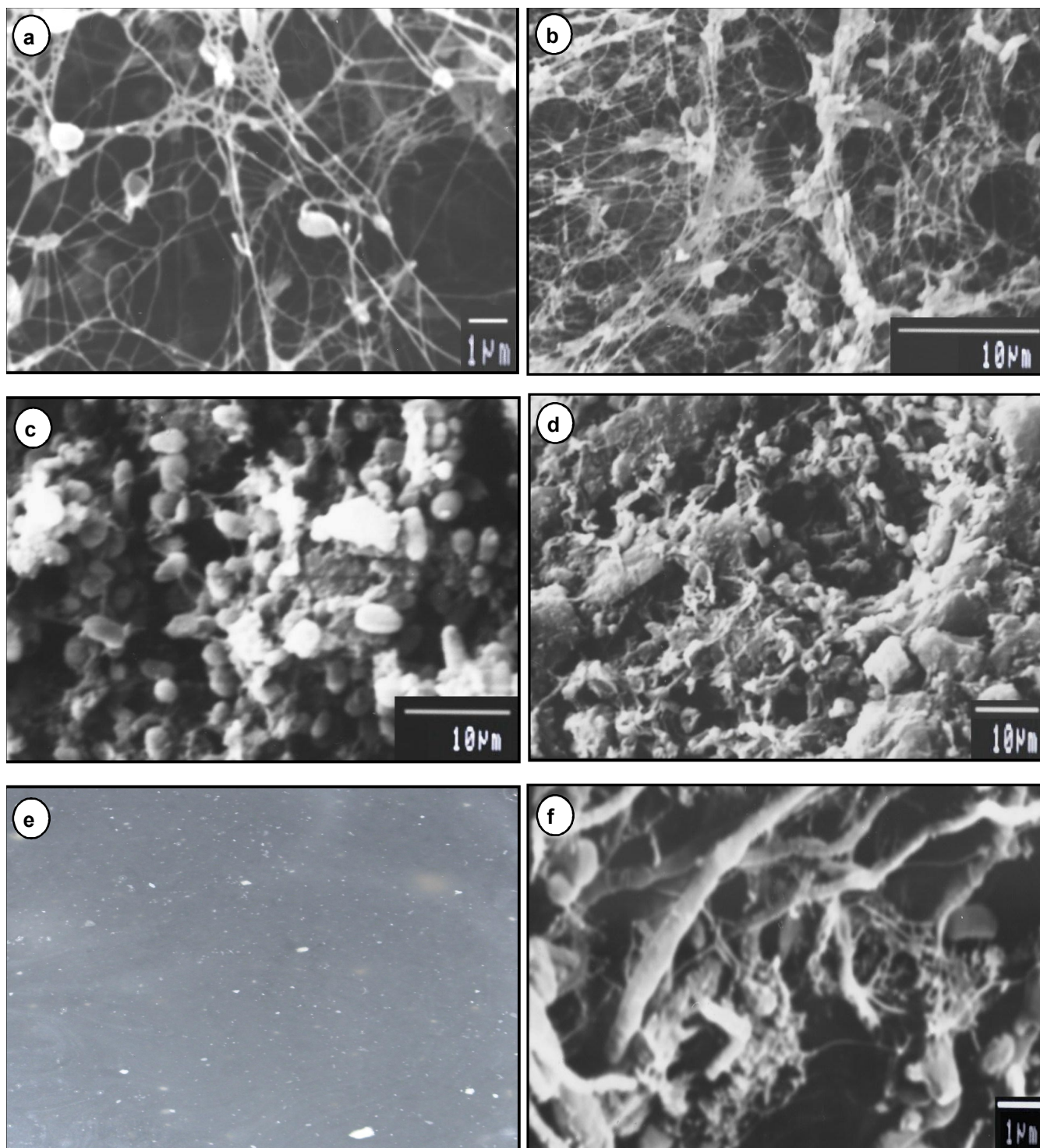


Figure 3.17: Scanning electron micrographs of Gram-positive and -negative binary consortium for experimental GAC for optimal flow rate 2ml/min at pH 10 (a) day 54; (b) day 57; (c) day 60; and (d) day 66. Aerobic granules were visible at this flow rate: (e) Morphology of granules in synthetic medium; and (f) SEM micrograph of aerobic granule

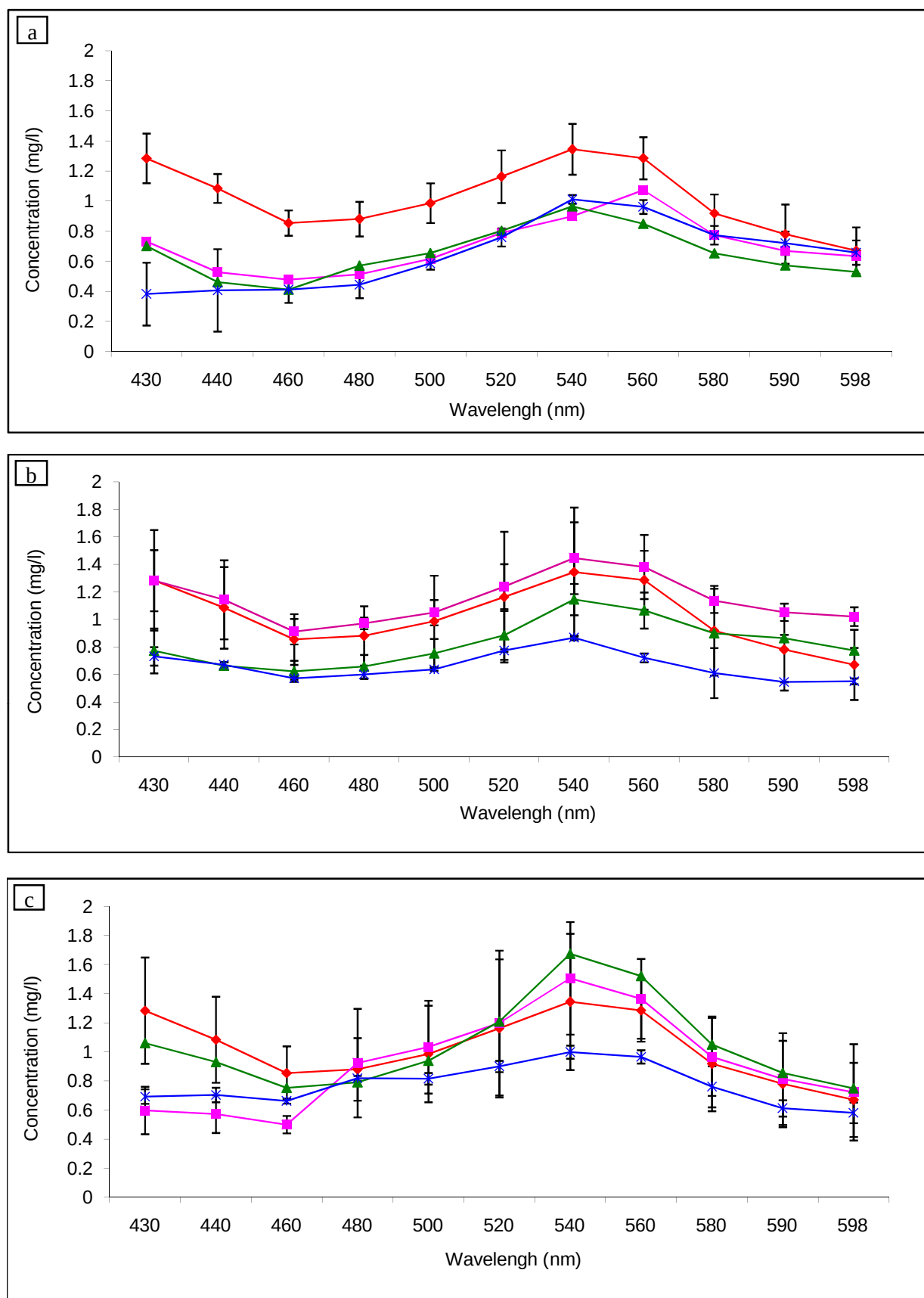


Figure 3.18: Total organic carbon degradation at pH 4 $\times$; 7 $\square$; and 10 $\triangle$; compared to control $\diamond$ at 2 ml/min (a); 8.5 ml/min (b); and 170 ml/min (c) for Gram-positive and -negative binary consortium

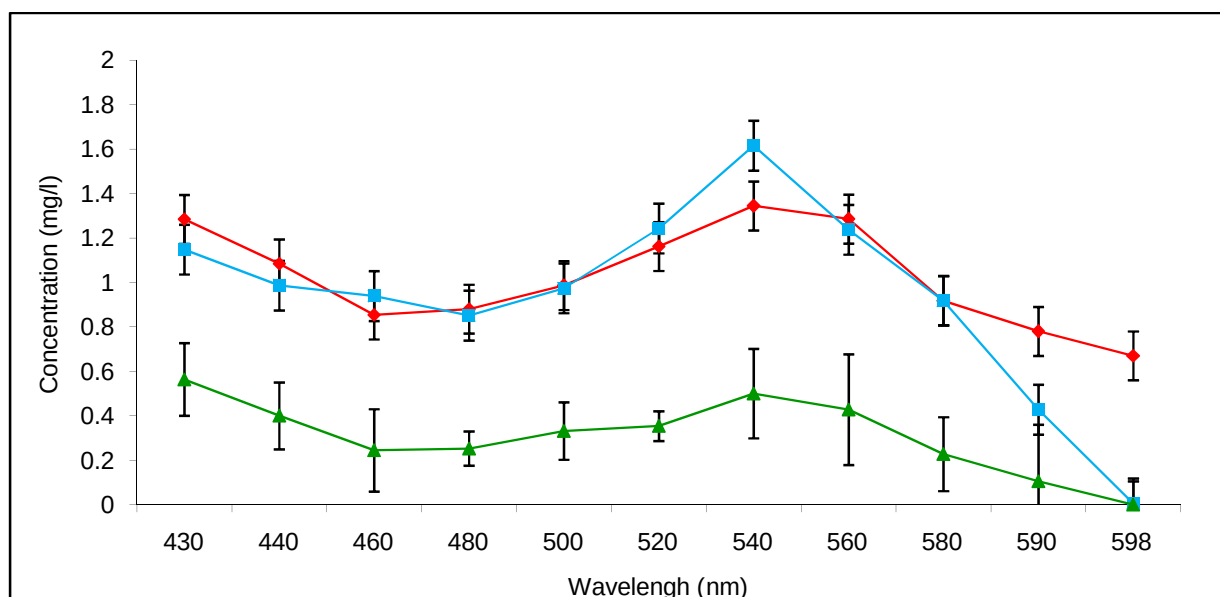


Figure 3.19: Total organic carbon degradation on day 60 —■— and day 66 —▲— of optimal mode of operation of FBFR compared to control —◆— for Gram-positive and -negative binary consortium

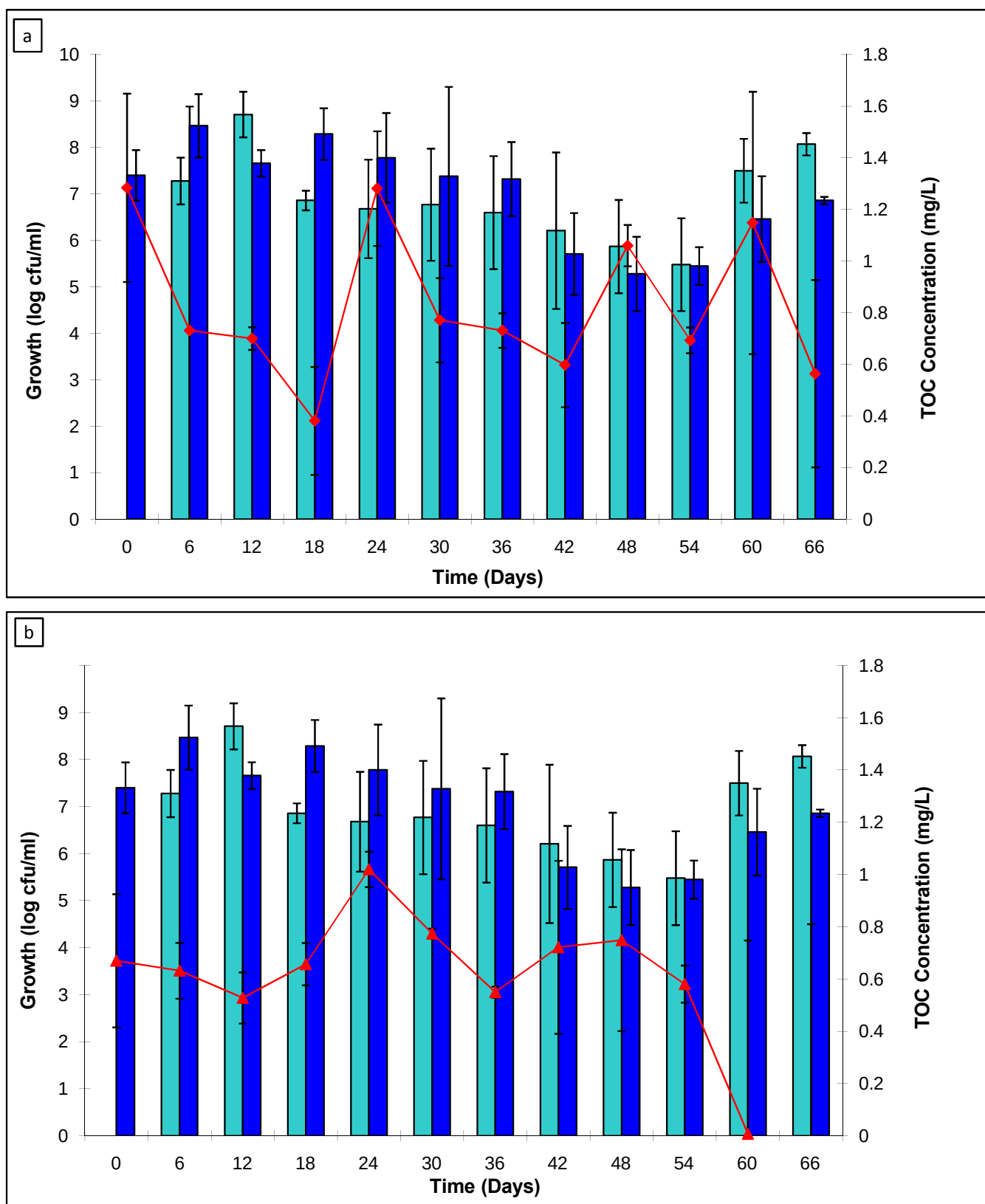


Figure 3.20: Total organic carbon ◆ vs Attached ■ and Planktonic ■ cell growth of binary consortium, over 66 days in batch mode FBBR, at (a) 430nm, and (b) 598nm for Gram-positive and -negative binary consortium

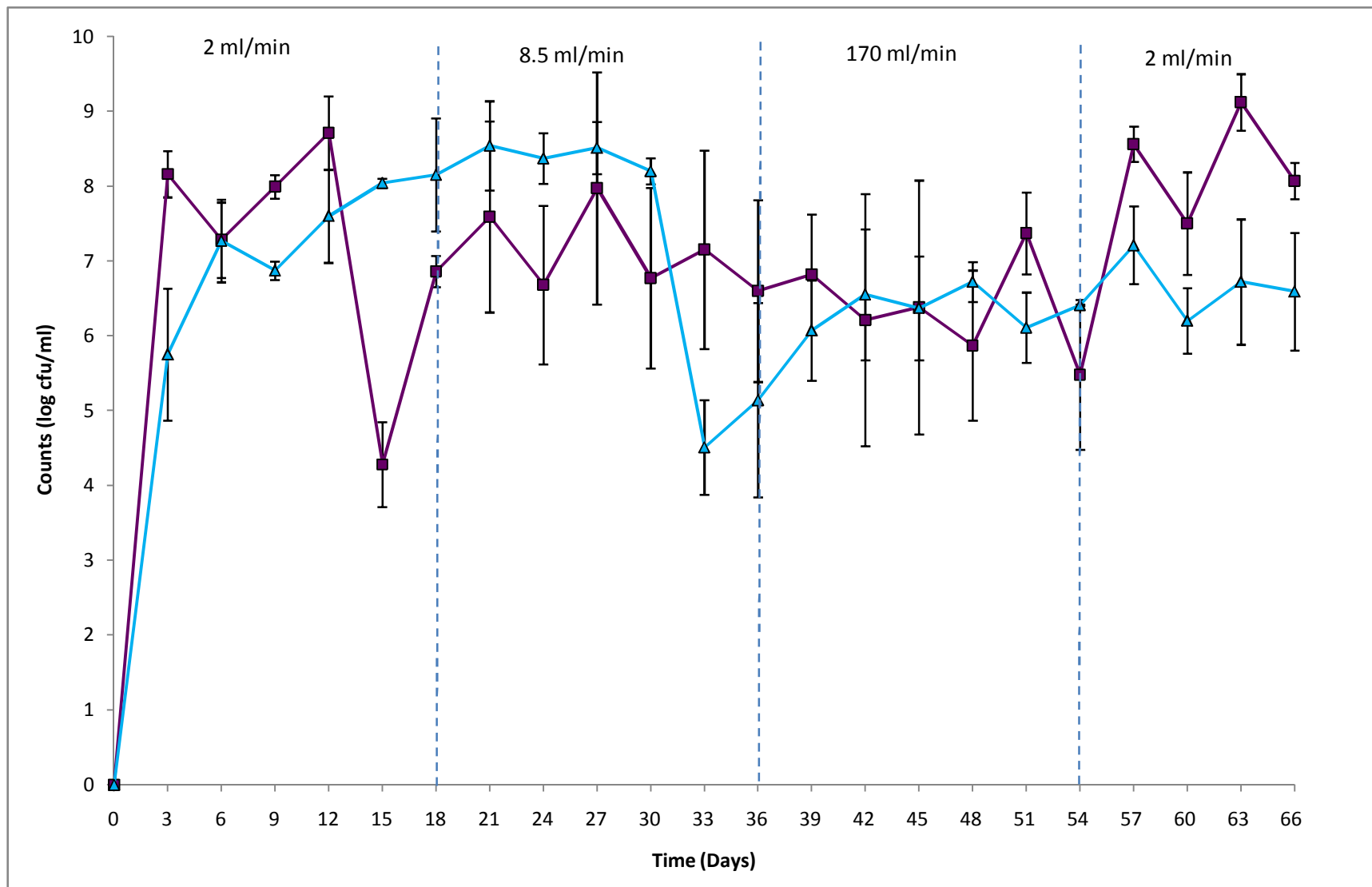


Figure 3.21: Comparison between attached counts of Gram-positive ▲, and Gram-positive and -negative ■ consortia over 66 days in FBRR

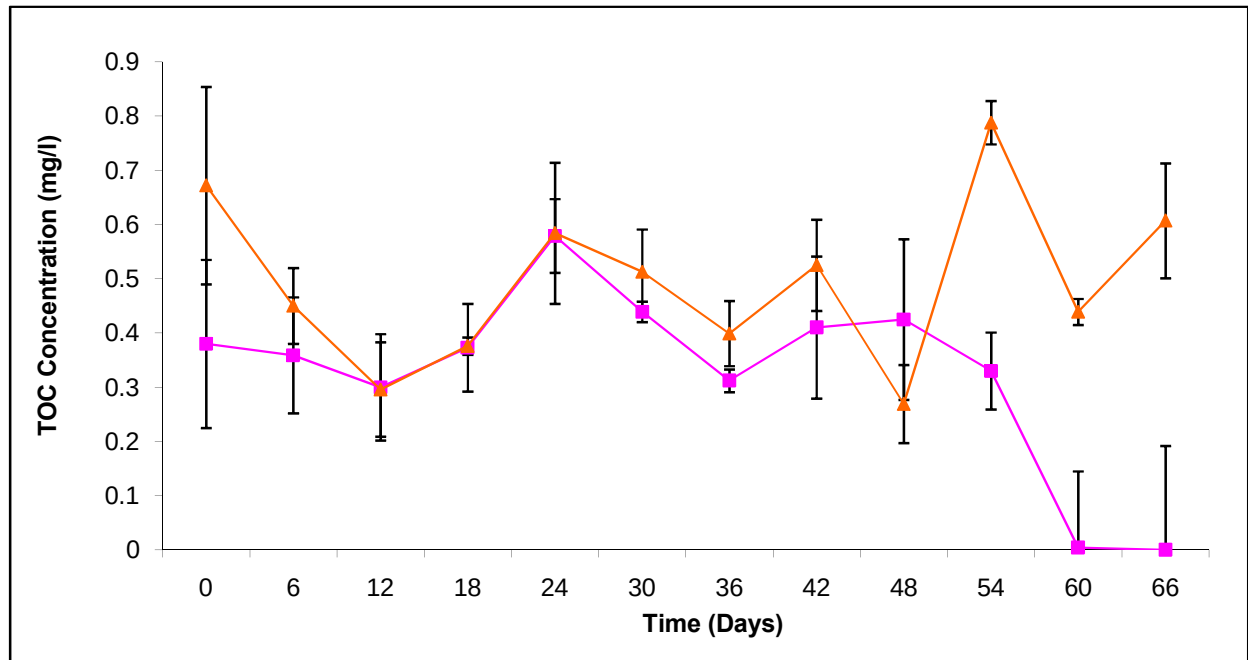


Figure 3.22: TOC degradation at 598nm for mixed Gram-positive consortium —▲— , and mixed Gram-negative and -positive consortium —■—

CHAPTER 4

SUMMARISING DISCUSSION AND CONCLUSION

Background

Dairy factories play an important role in the economy of all countries. The products from dairy factories are commonly purchased foods and are an important part of every person's staple diet. Milk especially is an important food, and regularly used in most households, offices, and other places as a commodity to coffee, tea, cereal, and as an ingredient in other foods. The necessity of these dairy products suggests that it will always be in demand, and will regularly be manufactured as large quantities are required. Dairy factories operate daily, with large quantities and varieties of products being produced. Products range from milk to sour milk, ice-cream, and cheese. Wastewater or effluent originates from cleaning the equipment and utensils used for manufacturing of these products. The wastewaters would thus consist of residues from these products, such as the milk fats and proteins (Janczukowicz *et al*, 2007). The effluent generated is of high volume, and thus may cause serious damage to the environment because of its toxic constituents

The CIP procedures conducted in dairy factories contribute largely to changing the composition and pH of wastewaters, and in doing so contribute to the pollution of receiving water bodies. The chemicals and detergents used for sanitation are rinsed off using water, and form part of the dairy wastewaters as well. Increased levels of nitrogen and phosphorous result in eutrophication by increasing anoxia in the receiving water body, and thus destroying aquatic life. Therefore, the wastewater effluents which contain CIP chemical residues contribute to the eutrophication of the water bodies it's discharged into.. In between sanitation rinses, pipelines and equipment are rinsed with water, thus the pH of the wastewater is constantly fluctuating since some dairy factories conduct

these CIP procedures a few times a week. The rinse or wastewater pHs can vary between pH 1 and 13 (Brião & Tavares, 2007). To reduce pollutants by treating these effluents, systems have to be developed which treat the effluents before it is discharged into the environment. The treatment option must be able to withstand the constantly varying pH, varying flow rates, and high volumes of effluent, as well as be sustainable.

In order to use biological means of remediation, the organisms used to remediate the waste compounds, need to be adaptable to fluctuating pHs ranging from acid to alkaline. If using bioaugmentation, it is important for cells to be able to not only survive but continue replicating, forming a sustainable biofilm, and utilising specific unwanted wastes from the wastewater. In utilising these compounds as nutrients, organisms must be able to deplete the unwanted compounds, so as to release a less toxic wastewater into receiving water bodies. If this is not achieved, this would result in the eutrophication of wastewaters. Nitrogen and phosphorous (in inorganic forms and organic forms) are the main compounds that cause eutrophication of the receiving waters. Eutrophication is the rapid addition of large amounts of unnatural compounds into water bodies (Tusseau-Vuillemin, 2001). This may increase photosynthesis, and oxygen depletion occurs in deeper levels of water bodies, a term known as anoxia (Tusseau-Vuilleman, 2001). This causes insufficient oxygen for fish and other forms of aquatic life. Furthermore, these rich phosphorous and nitric acid conditions, which are caused by polluting water from factories, promote the growth of blue-green algae (Lincoln *et al*, 1996). Blue-green algae in high concentrations, reduce growth of aquatic plant life, and also destruct the natural habitats of aquatic life in the water body. The products released in the water by some species of blue-green alga or cyanobacteria are known to be toxic to animals, humans and plant life (Tusseau-Vuilleman, 2001).

To prevent pollution, many treatment options are available and being used. However the development of a new systems or improvement of a current system could increase efficacy of removal of TOC from dairy wastewaters in a more cost efficient and ecologically friendly manner. Fluidised bed biofilm bioreactors (FBBR) use immobilised biomass to reduce harmful components in wastewaters by using it as a nutrient source. It has been successful in the removal of toxic components from several different sectors in the food industry. Aerobic FBBR's although being utilised have been less preferred than anaerobic systems. It has been used mainly as a pre-treatment step (Mantzavinos & Kalogerakis, 2005). However, the number of advantages of this system is increasing. The most current area of research is the development of aerobic granules. The characteristic of which is favourable for reducing contaminants or pollutants in industrial wastewaters.

Consequently this study served to evaluate the efficacy of an aerobic FBBR in the reduction of TOC, and development of aerobic granules to further enhance TOC, by using bioaugmentation of 2 different consortia.

Selection of optimal carrier granules and biofilm consortia for biodegradation of dairy wastewater (Chapter 2- Pilot study)

To use bioaugmentation, isolates must be chosen from the ecological niche of the organism to achieve optimal results. However, these isolates are often genetically modified to enhance degradation or included in a mixed consortium to achieve optimal results (Van Limbergen, 1998; Fantroussi & Agathos, 2005). Therefore, this study aimed to isolate dairy spoilage bacteria from sources such as raw milk, raw cheese whey, and variants of pasteurised milk, and then combine them in a mixed culture suitable for efficient degradation of dairy wastewater. Products were spoiled at varying temperatures in order to stimulate the activity of dairy spoilage microorganisms, and 16s rDNA was used to identify potential isolates. Of all the isolates found in these products, 90% of these were *Bacillus spp.* This

was not unusual, as *Bacillus* is the most common spoilage microorganism found in dairy products (Lindsay *et al*, 2002; Nörnberg *et al*, 2010). Some of these isolates were pathogenic in nature, such as *B. cereus*, thus it were not included in this study as it posed potential health hazards. All of the isolates found were closely related to bacteria originating from other fermentation sources. Gram-negative isolates are also common spoilage microorganisms in dairy products. In addition, the one isolate used, *Pseudomonas aeruginosa* P1 was isolated from raw milk and was closely related to *P. aeruginosa* CS1C0 isolate found in an aerobic FBBR utilised for the degradation of sodium benzoate. Furthermore, 16S rDNA sequencing showed that this isolate was also distantly related to *Buttiauxella agrestis* HS-39 which produces the β -galactosidase enzyme required for degradation of lactose in milk. This characteristic is important for the degradation of dairy wastes, since lactose is a major constituent of dairy wastewater (Thassito & Arvanitoyannis, 2001).

The strong lipolytic ability from *P. aeruginosa* P1 combined with the strong proteolytic ability of *B. subtilis* C1 could enhance efficacy of TOC removal from dairy wastewaters. These bacteria have certain proteolytic and lipolytic ability, and many studies describe the ability of *Bacillus* and *Pseudomonas* species to produce enzymes capably of breaking down unwanted protein and lipid compounds (Cammarota & Freire, 2006; Chen *et al*, 2003; Chen *et al*, 2004; Morikawa, 2006). The enzymes are produced and utilised industrially for various treatment systems, thus the efficacy of these enzymes are not questionable, and the ease of accessibility of these enzymes is increased due to its industrial production. However, for use in bioaugmentation, the correct combination of bacteria each of which have specific enzymatic activity, can be crucial for the degradation of industrial wastewaters. This study found that most isolates exhibited a certain level of proteolytic and lipolytic ability, and this was confirmed by the clear zones formed in agar after plating these isolates onto milk agar and lipolytic agar. When in combination, the most effective bacterial consortia was one containing only Gram-positive *Bacillus* isolates, and one containing a single species each of

Bacillus and *Pseudomonas*. All of these isolates are commonly associated with spoilage of dairy products (Becker *et al*, 1994), thus its isolation and effectiveness in the degradation of the dairy synthetic medium is not unusual. Increased efficacy of these consortia in the synthetic medium was observed in flask cultures and reported as consortia which degraded the medium efficiently, thus its efficacy in an aerobic FBBR could be evaluated for the same efficiency.

The type of carrier or granular activated carbon (GAC) used for the immobilisation of bacteria was important, since its properties would affect the attachment of cells (Arnaiz *et al*, 2006). Various factors affect attachment: hydrophobicity of cells, surface texture of carrier, and porosity. To select the most appropriate carbon carrier, 5 different types were evaluated. Each of the 5 types of carriers varied in terms of the surface area, porosity, and source from which it was produced. Carriers were tested at specific incubation temperatures and times, and attached and planktonic plate counts were taken. Attached cells and biofilm development was verified by scanning electron microscopy. By using scanning electron microscopy, the size and shape of cells could be confirmed, and amount of attached cell and biofilm coverage on the surface of the GAC observed. The most favoured carbon carrier was one exhibiting a large surface area, large pores, and originated from a coal source. The pores were reasonably distanced thereby allowing an increased surface area, where attachment could initiate in pores, as well as surfaces surrounding the pores. The large amount of visible crevices in this GAC, may have increased the attached counts and biofilm mass on the GAC. The ability of GAC to carry large amounts of biofilm mass is important for the formation of biofilm, which serves to immobilise bacteria and assist in the degradation of TOC.

In this chapter, potential bioaugmentation isolates and consortia were successfully isolated, identified and verified for their ability to utilise dairy wastes due to their

production of lipase and protease enzymes, and were also shown to be good biofilm formers on GAC, suitable for use in a FBBR.

Degradation of total organic carbon content in synthetic dairy wastewater, using a Gram-positive mixed consortium, and a Gram-positive and –negative mixed consortium, in an aerobic fluidised bed biofilm bioreactor, under simulated dairy processing flow rates and pH conditions (Chapter 3)

An aerobic FBBR was set-up and sterilised before operation. The synthetic medium was used to condition GAC, added to the FBBR, and then 2 different consortia were chosen for this particular study. One consortium containing only Gram-positive isolates, and the other containing 1 Gram-positive and 1 Gram-negative isolate. These consortia were used as a bioaugmentation tool in the degradation of TOC in an aerobic FBBR, operating at varying flow rates and pH levels. Sodium hydroxide and orthophosphoric acid were used to imitate the acid and alkaline shocks common in dairy factories due to CIP procedures.

The Gram-positive consortium was unsuccessful in the complete degradation of TOC from the synthetic medium, however still maintained biomass on the GAC. Although biofilm growth was resistant to acid and alkaline shocks as well as varying flow rates, the rate of removal of TOC was minimal when compared to the other consortium. The resilience of *Bacillus* species was ascertained during this run, and the ability of *Bacillus* strains to maintain growth during these highly variable conditions proved its durability as a species in bioreactors, and specifically for use in dairy wastewaters. It may be suggested that the minimal levels of TOC degradation by the *Bacillus*-only consortium, was due to the low levels of enzymes being secreted by the consortium. Even during optimal mode of operation, aerobic granules were not initiated to enhance TOC degradation. It is predicted that the addition of a Gram-negative isolate may accelerate degradation (Simões, 2008).

The Gram-positive and –negative consortium was successful in the development of biomass, as well as in the development of aerobic granules which aided in the depletion of TOC. Although fluctuating growth was observed when using this consortium, TOC degradation continued until it was depleted during the optimal mode of operation. Aerobic granules were also found to have formed during this optimal mode. When compared to the other consortium TOC depletion occurred when in the presence of aerobic granules. This allows the assumption that aerobic granules are an important factor in the degradation of TOC. Reasonable biofilm growth was observed and growth persisted even under nutrient-limited conditions. This may be possible due to the cannibalistic behaviour of *Bacillus subtilis*, which releases an enzyme which kills sister sporulating cells, allowing surviving cells to feed off the nutrients, during these starvation conditions in order to maintain growth (González-Pastor *et al*, 2003). Since *B. subtilis* spores attach more readily to surfaces than vegetative cells of the same species (Rönnner *et al*, 1990), it can be deduced that nutrients were readily available to surviving vegetative cells which were closer to the GAC surface, since cells near the liquid-biofilm interface were detaching due to extreme conditions, thus leaving cells in the form of spores on the surface with nutrients in order to survive. Additionally, it has also been reported that *B. subtilis* feeds of bacteria such as *Pseudomonas aeruginosa*; *Xanthomonas campestris*; and *Acinetobacter lwoffii*, preferring predation to cannibalism (Nandy *et al*, 2007). This may be true in this study, as *B. subtilis* C1 may have used *P. aeruginosa* P1 as a nutrient source during starvation conditions. *P. aeruginosa* is also known to produce a lyase which breaks down EPS during starvation conditions, allowing attached cells to detach, and find nutrients elsewhere, or use the EPS itself as nutrients (Qureshi *et al*, 2005). The use of this consortium should be considered for use in field after a full-scale experiment has been conducted.

Optimal parameters for growth and degradation appeared to be at the lowest flow rate (2 ml/min) and at an alkaline pH (pH 10). Alkaline pH is not an uncommon growth pH for *Bacillus* species, where growth often thrives, compared to other

bacteria (Lindsay *et al*, 2002). Ochieng *et al*, (2003) reported an increased power consumption when fluidisation was increased, proving the advantage of using a slow flow rate, as energy can be saved. It is also interesting to note that caustic soda has more of an impact on the pH of wastewater than the acid as it is used in larger quantities than the acid during CIP procedures (Danelwhich *et al*, 1998). Our finding of optimal pH of 10 for TOC degradation would thus operate well in real dairy wastewaters, and TOC degradation would most definitely occur at this more favourable pH, since the development of aerobic granules would be stimulated due to the larger amounts of alkaline detergents being released compared to acid detergents.

In this chapter, aerobic FBBR's were successfully set-up and operated using 2 different types of bacterial consortia. Simulated dairy wastewater was most efficiently bioremediated using a bacterial consortium comprising a Gram-positive *Bacillus subtilis* and Gram-negative *Pseudomonas aeruginosa* strain. The Gram-positive and Gram-negative binary-species consortium was able to colonise and proliferate on the GAC in the FBBR, as well as form aerobic granules, and completely metabolise the TOC in the system. Comparatively, a consortium comprising *Bacillus*-only strains did not remove the TOC associated with dairy wastewater as efficiently. Both types of consortia also survived varying fluctuations in acid and alkaline pH, simulating changes which may occur in real dairy wastewaters. From this work, the most efficient consortium to be used as a bioaugmentation tool in an FBBR for dairy wastewater remediation, proved to consist of a *Bacillus* and *Pseudomonas* binary consortium at a pH of 10 and a flow rate of 2 ml/min.

Significance of this study

The results of this study advances the limited knowledge on the utilisation of aerobic FBBR's for dairy wastewater bioremediation. The choice of GAC for immobilisation of bacteria, as well as choice of consortia utilised in

bioaugmentation, all play a role in the efficiency of TOC degradation. The correct type of microflora associated with aerobic degradation in an FBBR was determined to allow for efficient bioremediation of dairy wastewaters, and the success of consortia used in this study is suggested for use in a full-scale experiment. The development of aerobic granulation during batch mode of operation enhances knowledge about aerobic granules, the significance of which has only come to light in recent years.

The economic implications are wide. A reduced number of reactors are needed due to using the FBBR since immobilised bacteria on carrier particles are used, which reduces the surface area needed for bacteria to proliferate and utilise wastes. Although aeration is required constantly, the degradation of TOC is possibly in a shorter time than that of an anaerobic bioreactor, and aeration has recently been proven to be not as costly as in previous years. However, from a practical and industrial perspective, TOC degradation occurring in a shorter timeframe than that which occurred in this study (i.e. 60 days), would be needed for this type of bioremediation system to be economically viable. Degradation of TOC occurred regardless of temperature control, thus reactors can be run at room temperature, and in doing so can be more economically viable option in industry, by reducing the number of coolers or incinerators needed for temperature control. Anaerobic processes are known to increase ammonium concentrations, because of protein degradation, and as a result, a further aerobic degradation step is necessary to allow the safe discharge of wastewater into water bodies (Brewer *et al*, 1999). Eliminating this step would prove to be very economical, and time saving.

The findings of this study showed the optimal parameters of operation of an aerobic FBBR for dairy wastewater bioremediation. By using these parameters in industry, the prevention of pollution in natural water bodies may be preventable and in doing so aquatic life can be preserved, as well as the health of humans and animals coming into contact with these natural water bodies.

Future studies

- A further consideration may be to switch operation of the FBBR to continuous mode of operation. This may allow biomass and aerobic granules to increase in size, and hence, further increase the rate of degradation of TOC.
- By initiating granulation at the start of operation, the rate of TOC degradation may be increased, and the bioremediation may occur in a shorter timeframe
- Since the Gram-positive consortium showed more biomass than the Gram-positive and -negative consortium, the inability of the consortium to form aerobic granules may be due to the absence of *P. aeruginosa* P1. Adding this isolate to the Gram-positive only consortium may increase biomass and initiate aerobic granulation. The steadier growth observed by the Gram-positive consortium combined with the aerobic granule initiation by the Gram-positive and -negative consortium may enhance TOC degradation.
- Further isolation of bacteria can be conducted to find more bacteria capable of TOC degradation, as well as the evaluation of more combinations of bacteria for use in bioaugmentation. The addition of Gram-negative bacteria with strong proteolytic and lipolytic abilities may positively enhance a Gram-positive consortium. Nörnberg *et al*, (2010), found that majority of their isolates found in refrigerated raw milk were Gram-negative psychrotrophs. If raw milk were to be kept under refrigerated conditions, to allow for isolation, a greater amount of these bacteria may be found, and may aid in enhancing TOC degradation, since they have known proteolytic, and lipolytic enzymes.
- Taking into account that a synthetic medium was used, TOC levels would be slightly lower than under conditions in field. However other studies have used the same synthetic medium for lab-scale studies (Ramasamy *et*

al, 2000), and a future consideration would be to test wastewater directly from factories, to observe whether or not the same results can be achieved.

- The mode of operation when using bioaugmentation of the Gram-positive and –negative consortium should be tested on real dairy wastewater to evaluate its efficacy at dairy factories.

CHAPTER 5

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