BIODEGRADATION OF WINERY WASTEWATER

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my original work and has not previously been submitted in its entirety or in part at any university for a degree.

Signature: _____ Date: _____

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SUMMARY

Large volumes of wastewater are generated annually during the grape harvest season from various processing and cleaning operations at wineries, distilleries and other wine-related industries. South African regulatory bodies dictate that wastewater should have a pH of 5.5 to 7.5 and a chemical oxygen demand (COD) lower than 75 mg/L. However, winery wastewater has a typical pH of 4 to 5 and a COD varying between 2 000 and 12 000 mg/L. Urban wineries channel the wastewater to local sewage treatment facilities and are often heavily fined for exceeding governmental requirements. Rural wineries usually have little or no treatment operations for their wastewater and it is often irrigated onto crops, which may result in environmental pollution and contamination of underground water resources.

Various criteria are important in choosing a wastewater treatment system, such as an eco-friendly process that is flexible to withstand various concentration loads and characteristics, requiring low capital and operating costs, minimal personal attention and do not require too much land. In this study, a large variation in COD, pH and chemical composition of the winery wastewater was observed that could be related to varying factors such as the harvest load, operational procedures and grape variety. Wastewater from destemming and pressing operations contained higher concentrations of glucose, fructose and malic acid, which originated from the grape berries. The fermentable sugars (glucose and fructose) contributed to almost half of the COD with a smaller contribution from ethanol and acetic acid. The low pH can be ascribed to relative high concentrations of organic acids in the wastewater.

The efficacy of biological treatment systems depends strongly on the ability of microorganisms to form biofilm communities that are able to degrade the organic compounds in the wastewater. Preliminary identification of microorganisms that naturally occur in winery wastewater indicated the presence of various bacterial and yeast species that could be effective in the biological treatment of the wastewater. When evaluated as pure cultures under aerobic conditions, some of the yeast isolates effectively reduced the COD of a synthetic wastewater, whereas the bacterial isolates were ineffective. The most effective yeast isolates were identified as *Pichia rhodanensis*, *Kloeckera apiculata*, *Candida krusei* and *Saccharomyces cerevisiae*.

Our search for cost-effective biological treatment systems led to the evaluation of a Rotating Biological Contactor (RBC) for the treatment of winery wastewater. The RBC was evaluated on a laboratory scale with 10% (v/v) diluted grape juice and inoculated with a mixed microbial community isolated from winery wastewater. The results showed a reduction in the COD that improved with an extended retention time. Evaluation of the RBC on-site at a local winery during the harvest season resulted on average in a 41% decrease in COD and an increase of 0,75 pH units.

RFLP analysis of the biofilm communities within the RBC confirmed a population shift in both the bacterial and fungal species during the evaluation period. The most dominant yeast isolates were identified with 18S rDNA sequencing as *Saccharomyces cerevisiae, Candida intermedia, Hanseniaspora uvarum* and *Pichia membranifaciens*. All these species are naturally associated with grapes and/or water and with the exception of *Hanseniaspora uvarum*, they are able to form either simple or elaborate pseudohyphae.

OPSOMMING

Groot hoeveelhede afloopwater word jaarliks gedurende die druiwe-oestyd deur verskeie prosessering- en skoonmaakoperasies deur wynkelders, distilleer- en ander wynverwante industrieë gegenereer. Suid-Afrikaanse beheerliggame vereis dat afloopwater ‘n pH van 5.5 tot 7.5 en ‘n chemiese suurstofbehoefte (COD) van minder as 75 mg/l moet hê. Kelderafloopwater het egter gewoonlik ‘n pH van 4 tot 5 en ‘n COD van 2 000 tot 12 000 mg/L. Stedelike wynkelders voer die afloopwater na ń plaaslike rioolsuiweringsaanleg wat dikwels tot swaar boetes vir oortreding van die wetlike vereistes lei. Plattelandse wynkelders het gewoonlik min of geen behandelingsprosesse vir hul afloopwater nie en gebruik die water dikwels vir gewasbesproeiing, wat tot omgewingsbesoedeling en kontaminasie van ondergrondse waterbronne kan lei.

Verskeie kriteria is belangrik in die keuse van ‘n waterbehandelingstelsel, byvoorbeeld ‘n omgewingsvriendelike proses wat verskillende konsentrasieladings en samestellings kan hanteer, ‘n lae kapitaal- en bedryfskoste en minimale persoonlike aandag vereis en min ruimte benodig. Hierdie studie het getoon dat kelderafloopwater ‘n groot variasie in COD, pH en chemiese samestelling het wat met wisselende faktore soos die oeslading, operasionele prosesse en selfs die druifkultivar verband kan hou. Afloopwater van ontstingeling- en parsoperasies het hoër konsentrasies glukose, fruktose en appelsuur wat van die druiwekorrels afkomstig is. Die fermenteerbare suikers (glukose en fruktose) dra tot amper 50% van die COD by, met ‘n kleiner bydrae deur etanol en asynsuur. Die lae pH kan grootliks aan organiese sure in die afloopwater toegeskryf word.

Die effektiwiteit van biologiese behandelingstelsels steun sterk op die vermoë van mikro-organismes om biofilmgemeenskappe te vorm wat die organiese verbindings in die afloopwater kan afbreek. Voorlopige identifikasie van mikro-organismes wat natuurlik in wynafloopwater voorkom, het die teenwoordigheid van verskeie bakteriese en gisspesies aangedui. Evaluering van hierdie isolate onder aërobiese toestande het getoon dat sommige van die gis-isolate die COD van ‘n sintetiese afloopwater effektief kon verlaag, terwyl die bakteriese isolate oneffektief was. Die mees effektiewe gis-isolate is as *Pichia rhodanensis*, *Kloeckera apiculata*, *Candida krusei* en *Saccharomyces cerevisiae* geïdentifiseer.

Ons soektog na ‘n koste-effektiewe biologiese behandelingsisteem het tot die evaluering van ‘n ‘Rotating Biological Contactor’ (RBC) vir die behandeling van afloopwater gelei. Die RBC is op laboratoriumskaal met 10% (v/v) verdunde druiwesap geëvalueer en met ‘n gemengde mikrobiese gemeenskap wat uit afloopwater geïsoleer is, innokuleer. Die resultate het ‘n verlaging in die COD getoon wat met ‘n langer retensietyd verbeter het. Evaluering van die RBC by ‘n plaaslike wynkelder gedurende die oesseisoen het gemiddeld ‘n verlaging van 41% in die COD en ‘n verhoging van 0,75 pH eenhede getoon.

RPLP analise van die biofilmgemeenskappe in die RBC het ‘n bevolkingsverskuiwing in beide die bakteriese en swamspesies aangetoon. Die mees dominante gisspesies is met 18S rDNA volgordebepaling as *Saccharomyces cerevisiae, Candida intermedia, Hanseniaspora uvarum* en *Pichia membranifaciens* geïdentifiseer. Al hierdie spesies word gewoonlik met druiwe en/of water geassosieer en is, met die uitsondering van *Hanseniaspora uvarum*, in staat om òf eenvoudige òf komplekse pseudohife te vorm.

BIOGRAPHICAL SKETCH

Lida Malandra was born in Johannesburg, South Africa, on the 14th of December 1975. She matriculated from Holy Rosary High School in 1993 and obtained a B.Sc. degree with Microbiology and Genetics as majors in December 1997 from the University of Stellenbosch. She completed the Hons. B.Sc. degree in Microbiology in 1998 and enrolled for her M.Sc. degree in Microbiology in 2000 at Stellenbosch University.

Lida contributed to the following research articles and conference presentations during her studies:

Publications in International Journals

Malandra L., Wolfaardt G.M. and Bloom M. 2002. Evaluating a rotating biological contactor for winery wastewater treatment. Manuscript submitted to *Water Research*.

International Conferences

- M. Viljoen-Bloom, L. Malandra and G.M. Wolfaardt. 2000. Biological treatment of winery effluents. 2nd International Viticulture and Enology Congress, Cape Town.
- L. Malandra, G.M. Wolfaardt and M. Viljoen-Bloom. 2001. Biological treatment of winery effluent. Ninth International Symposium on Microbial Ecology, Amsterdam.

Local Conferences

- L. Malandra, G.M. Wolfaardt and M. Viljoen-Bloom. 2001. Biodegradation of winery effluents. South African Society for Enology and Viticulture Congress, Somerset West.
- L Malandra, G.M. Wolfaardt and M. Viljoen-Bloom. 2002. Yeast biofilms in winery effluent treatment. The South African Society of Microbiology 11th Biennial Congress, Bloemfontein.

CONTENTS

INTRODUCTION

Jan van Riebeeck planted the first vineyard in Cape Town in 1655 and at present, the Western Cape wine industry accounts for over 90% of South Africa's wine production. France leads the international market (22%), Italy is second (20%), Spain is third (13,6%) and South Africa is eighth with 3% (http://www.wosa.co.za/overview.asp). About 950 million liters of wine are produced annually, which is a major contributor to the South African economy.

Excellent technology is an important prerequisite for global competitiveness, and the wine industry in South Africa is currently undergoing a renaissance to establish itself in the international market. In the past, economic sanctions caused a lag in the technological advancement that has been most prevalent in viticulture and winemaking processes. With the new political dispensation and open economy, South Africa is in the process of dynamic change. For this reason, the Wine Industry Network of Expertise and Technology (WINETECH) was established in 1995 to ensure the production of top quality wines and products using 'clean and green' technologies. One of its focus areas is the management of winery waste and by-products.

The problems experienced with winery wastewater are largely seasonal, with the largest quantities of wash-water being generated during the harvest season when the washing of machinery, tanks and floors produces a high pollution load. The harvest season lasts approximately 110 days during which a typical Western Cape winery handles around 10 000 tons of grapes. It is possible for wineries to produce as much as five kilolitres of wastewater per ton of grapes processed, depending on the extent of wash-water and storm-water that is allowed to enter the wastewater stream. The COD (is the oxidation of organic compounds to smaller sub units such as carbon dioxide and water) of winery wastewater peaks during the harvest period due to the grape pressing. There are secondary peaks, but not as drastic, when wine is re-filtered and purified, and when the tanks are rinsed after fermentation is completed.

Water in the industrial sector has many applications such as an ingredient together with other raw materials in the finished product, cleansing agents, coolant, a source of heat using

steam, solvents, as well as for personal consumption or service. Water that leaves the industry as wastewater may be discharged either into municipal sewers or into watercourses. Both methods are subject to certain restrictions as stated in Section 39 of the South African National Water Act, 1998 (Act No. 36 of 1998). Municipalities regularly sample wastewater from industries to determine the quality of the water and impose charges payable according to the quality of the wastewater that is discharged. Chemical Oxygen Demand (COD) and Biological Oxygen Demand (BOD) are used to measure the pollution load of winery wastewaters. Wineries pose an environmental risk to water resources when the inappropriate disposal of residual solids and wastewater occurs. Discharging high organic loads into the environment triggers deoxygenation which leads to depletion of dissolved oxygen.

Water is one of our most valuable resources, but water quality and environment related problems are not simply a technical question; the more difficult questions are often political, institutional and social. The speed at which eco-designed systems progress is dependent on several factors, including financial resources, the severity of the pollution problem, current investments in sewage systems and finally political decisions. There are many treatment systems available for the degradation of winery wastewater, but many of these are too expensive, inefficient or inappropriate. It is, therefore, necessary to search for alternative systems that will be efficient as well as cost-effective.

AIMS AND OBJECTIVES OF THIS STUDY

The aim of this study was to evaluate the efficacy of a Rotating Biological Contactor (RBC) for the treatment of winery wastewater. The specific objectives were to:

1. Characterize winery wastewater by means of chemical analyses; and to determine the components of the wastewater that are responsible for the high COD and acid pH;
2. Evaluate naturally occurring microorganisms for their ability to degrade winery wastewater both aerobically and anaerobically;
3. Investigate biofilm formation by naturally occurring microorganisms in winery wastewater;
4. Evaluate the RBC on laboratory scale and on-site at a winery; and
5. Use modern molecular methods to study population dynamics within the biofilm community that formed on the RBC discs and to identify the dominant yeast species.

CHAPTER 1

LITERATURE REVIEW

1.1 WINERY WASTEWATER

Wine production involves various steps, including the crushing of grapes, straining of skins and seeds, storage, clarification and maturation of the young wine. Winery wastewater consists mainly of organic materials and their salts, soluble proteins and carbohydrates, as well as various inorganic compounds, solids and dissolved material, which have a tendency to acidify and ferment (Ronquest and Britz, 1999). The solid particles consist of pomace, which are the skins of the grapes, stalks, and potassium bitartrate crystals that have formed before the wine has been chilled for storage. The semi-liquid lees consist of yeast cells, pulp particles, tartrates, etc., as well as the clarification sludge from the tanks and filters. The liquid fraction is the wastewater from the crushers, tanks, pipelines, pumps, presses and floors, and cooling water from the cooling systems.

Winery wastewater has a moderate salinity and the inorganic composition is characterised by a high potassium concentration together with all the other elements found in wine (Na, Ca, Mg, Fe, SO_2, etc.), but without toxic metals (Bernet *et al*., 1996). The pH of winery wastewater typically range between 4 and 5, with an average COD of 2 000 – 2 500 g/L COD per ton of grapes. During the high peak periods, the COD may range between 4 000 to 11 000 mg/L.

1.1.1 WINERY WASTEWATER MANAGEMENT

Various factors play a role in the design and operation of any treatment system. These factors include the position of the winery relative to densely populated areas, the quantity of grapes being pressed and the availability of land. It is essential for wineries and other related industries to implement good waste management systems to prevent the unnecessary loss of raw materials and valuable water resources. There are four key elements to waste management, i.e. waste segregation, water conservation, waste minimisation and resource recovery and upgrading.

Waste Segregation

Effluent of different organic loads, pH, concentration and temperature, such as storm water versus winery effluent, should be separated. This facilitates easier and more effective

handling of the wastewater. The pollution load of winery wastewater is proportional to the total organic load, which includes the solid material, i.e. skins, stalks and pips, as well as finely suspended material, lees and dissolved nutrients such as carbohydrates and fruit juices. One grape skin and one grape pip typically have COD's of 200 mg/L and 900 mg/L, respectively. The first and most important step is therefore to remove as much of this organic load as possible. The simplest and easiest is to sieve the wastewater, thereby removing the solid particles. The finer material can be sedimented.

Water Conservation

Only the very essential amount of water should be used to reduce the volume of water and to concentrate the pollutant. The basic guidelines are to use water wisely, keep the solid waste separated from the liquid waste, use high pressure hoses to clean cellar floors and tanks, use the correct amount of detergents that require minimum rinsing, recycle water for cooling or other simple operations, and use automatic controls to regulate the volume, temperature and pressure of water.

Waste Minimisation

Waste minimisation implies that raw materials are to be used more efficiently. Effective housekeeping involves the prevention of unnecessary wastes such as leaks and spillage. Process modifications, such as the use of compressed air during washing operations, and the use of automatic controls (e.g. to prevent overflows) can result in improved treatment practises.

Resource Recovery and Upgrading

Valuable resources can be recovered in the form of energy or useful materials. This is possible by the recycling of recovered materials, by-product recovery and upgrading the components separated from the wastes to high valuable secondary products (Mardikar and Niranjan, 1995). An example in the wine industry is the use of grape seed extracts to produce grape seed oil for health food and gourmet groceries. Grape pomace (mostly the skins and pips) can also be ploughed back into vineyard soils as a natural source of nitrogen and phosphate.

1.1.2 TRADITIONAL WINERY WASTEWATER TREATMENT PROCESSES

There are a number of chemical, physical and biological treatment systems employed by the wine industry for the treatment of winery wastewater. Biological purification or evaporation by irrigation (Benitez *et al*., 1999) has been suggested for the South African wine industry. However, long-term irrigation of crops or grasslands with winery wastewater can alter the soil quality due to chemical reactions during the oxidation process, resulting in an increase of total dissolved solids, alkalinity and hardness of up to 200 mg/L. It would therefore be beneficial to have a primary treatment system that degrades the wastewater to a quality that will not lead to environmental deterioration when used for irrigation.

The principles and relevant considerations regarding land application and a few biological systems currently used in the wine industry will be discussed in the following sections. The technical detail of the biological systems will be discussed in more detail in Section 1.3.

Land application

Land application provides an efficient means of recycling valuable water together with the wastewater's nutrient and organic components (Abu-Zeid, 1998). Irrigation of winery wastewater onto crops or grass fields is common practice at many South African wineries. There are many factors to consider before the water may be used in this type of application (Bertranou *et al*., 1987). When used for irrigation, the nature of the pasture or crop needs to be reviewed. Properties of the soil, climate conditions and the quality and quantity of the wastewater all play a role. The operational life of the application site is usually determined by the phosphorus absorption capacity and salt accumulation of the site.

The long-term application of winery wastewater could damage the soil. It is therefore necessary to find soil with the following characteristics: (1) soil structure that permits air flow and water penetration; (2) sufficient drainage or artificial drainage; (3) adequate capacity to retain water between successive irrigations; (4) sufficient quantities of nutrients for plant growth; (5) moderate pH; (6) no salinity problems; and (7) ample soil depth to allow for root development. Soil that is not adequate for irrigation is that of poorly structured clays, shallow soils with rock and gravel, swamps that cannot be drained, soils with poor drainage, soils with a high salt content and low permeability, and coarse silica

sand soils. The application rate is further limited by the hydraulic load, nutrient load, salt load and COD of the wastewater (Bitton, 1994).

To minimise surface runoff and soil erosion, wastewater cannot be used on land adjacent to streams and watercourses, land subject to flooding, waterlogged or saline land, rocky, slaking and erodible land, or highly permeable soil types. The equipment used to spray the winery wastewater should be either the low trajectory, large droplet equipment or drip irrigation equipment. Drip irrigation is a better option where the wastewater is low in suspended solids. It is advisable to use frequent short irrigations to minimise the risk of concentrating the salts and decreasing the permeability through the destruction of soil structure.

Activated Sludge Reactors

Activated sludge reactors are the most popular of the treatment systems available for winery wastewater. The process is based on the aerobic degradation of wastewater by mixing and stirring wastewater with recycled sludge, which is microbiologically very active, followed by the separation of the mineralized wastewater and sludge. Sludge from winery wastewater has a low content of heavy metals, organic matter and N, P, and K, making it suitable for compost production and agricultural use. The performance of the system is dependent on the operational conditions and design that affects the biological and hydraulic behaviour of the system (Chudoba and Pujol, 1996). It was found that a higher biomass concentration improves the performance of the system. It is possible to increase the microbial content by the addition of mineral material that facilitates sludge floc formation.

Activated sludge reactors are flexible enough to handle organic and hydraulic variations (Fumi *et al*., 1995). However, it is a rather costly treatment system, with its success dependent on the recirculation of activated sludge, settling tank capacity and sufficient aeration.

Upflow Anaerobic Sludge Blanket (UASB)

This is the most effective anaerobic wastewater treatment system for winery effluent. Wastewater is fed from the bottom of the reactor and is degraded as it passes through the sludge blanket covered by a layer of active bacterial flocs (Bitton, 1994). Factors such as

temperature, pH, sludge loading rates and essential nutrients affect the rate of floc formation. The organic material in the wastewater is converted to cell biomass and a biogas rich in methane is produced. Baffles in the reactor separate the gas and solids.

The UASB reactor is a less costly treatment from an energy point of view, as the biogas produced can be used to cover the energy required by the system (Benitez *et al*., 1999). Other advantages are the small amounts of sludge and biomass that are produced (Ronquest and Britz, 1999). However, problems may be experienced due to the high phenolic content of wine distillery wastewater that gives a prohibitive and antibacterial quality to the wastewater (Benitez *et al*., 1999).

Aerobic Sequencing Batch Reactors

This system uses microbial granules in a sequence of aerobic reactors. Wastewater is pumped into a primary settling tank for the removal of large solid particles, and is then pumped to a storage tank. From this storage tank wastewater is transferred to the aerobic treatment tank, which is equipped with fine bubble diffusers, a blower and an evacuation pump. The critical parameters are similar to those of the UASB, in that it is dependent on the effective recirculation of the granules and a higher biomass concentration improves the performance of the system. This system is well suited for small wineries with limited manpower and money, and requires minimum management and maintenance. The microorganisms are stable as granules and the buffer volume protects the reactor from toxic overloads such as bactericides (Torrijos and Moletta, 1997).

Constructed Wetlands

Constructed wetlands are becoming a more popular option for the final stage of winery effluent treatment. Different aquatic plants and their microorganisms are used to degrade the organic matter in the wastewater. This system uses a variety of floating, emerged and submerged plants. As with batch reactors, the treatment of winery wastewater is dependent on the retention time within the system. The success of a wetland is also dependent on the hydraulic parameters, such as the flow velocity and dispersion coefficient (Grismer *et al*., 2001).

1.2 BIODEGRADATION

1.2.1 WHAT IS BIODEGRADATION?

Biodegradation refers to the biological transformation of an organic chemical to smaller subunits such as CO_2 and H_2O. Organic compounds containing oxygen in the form of hydroxyl or carboxyl groups serve as a food source for many animals and microorganisms. Energy yielding metabolism is a result of oxidation reactions where electrons from hydrogen molecules are transferred to oxidized pyridine nucleotides (NAD^+ and $NADP^+$) resulting in reduced forms of pyridine ($NADP^+$ and NADPH). These molecules provide energy for biosynthetic reactions or transfer electrons to electron transport chains where ADP is phosphorylated to form high-energy bonds of ATP (Bitton, 1994). Unusual food sources such as alkanes and related ring structures are not subject to dehydrogenation reactions that are characteristic of many biological oxidations. Bacteria have the unique biochemical ability to catalyze oxidations of these alkanes using molecular oxygen. The terminal carbon of an alkane is oxidized and a fatty acid is formed which can then be metabolized by β-oxidation. When a hydroxyl group is inserted into a ring structure, further oxidations break the ring structure and produce fragments that can enter the normal catabolic pathways.

For biodegradation to occur, a suitable organism must be present, the opportunity must exist for enzymes to be produced, and the environmental conditions should be favourable for enzymatically-catalyzed reactions to occur at a significant rate. There are four major techniques to enhance biodegradation of wastewater: a) stimulation of the naturally occurring microorganisms; b) inoculating with microorganisms which have specific biotransforming abilities; c) addition of immobilized enzymes; and d) use of plants (phytoremediation) to remove and/or transform pollutants (Thassitou and Arvanitoyannis, 2001).

Complete degradation of a compound may require sequential metabolism by two or more organisms. These close associations are referred to as consortia, syntrophic and synergistic associations and communities (Atlas, 1997). Individual interactions in a complex community may reveal little about their behaviour in a complex community, as they may be simultaneously involved in various other interactions. These interactions are also in a constant state of flux based on environmental changes. Microbial ecosystems therefore

often lack long-term stability and are continually adapting. Members of the community can be referred to as dominants, associates and incidentals. Dominants are those species having a dominant role in the community, with associates being the species dependant on the dominants for survival. Incidentals are those species that are not influenced by either the dominants or the associates. Microbial interactions can also be classified as positive and negative effects. Positive effects can be mutualistic, commensalistic or neutral, whilst negative effects include parasitism, predation or competition.

Biodegradation with mixed communities has distinct advantages over pure cultures. A major advantage is the biodegradative capacity, both quantitatively and qualitatively. The community may be more resilient to harmful and toxic substances, since an organism that can detoxify them would most likely be present. Primary utilisers are capable of metabolizing the major carbon and energy sources in the system, while the secondary organisms rely on the utilization of products that are released by the primary utilisers. These interactions in the biofilm contribute to the homeostasis in the community (Marsh and Bowden, 2000).

1.2.2 EVALUATING BIODEGRADABILITY

Biodegradation is calculated by measuring the Biochemical Oxygen Demand (BOD) or the Chemical Oxygen Demand (COD). BOD measures the amount of oxygen used by the microorganisms during the oxidation of organic material found in the wastewater. This entails that the biodegradable organic material is oxidised to CO_2 and H_2O, using molecular oxygen as the electron acceptor. COD is based on the principle that most organic materials will be oxidised to CO_2 and H_2O by strong oxidising agents under acid conditions (Benefield and Randall, 1980).

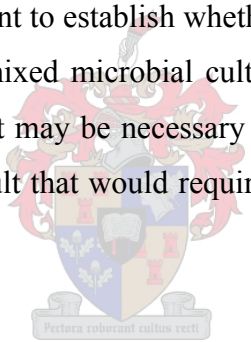
A multi-tiered approach could be useful to evaluate the biodegradation of a specific compound or mixture of compounds under either aerobic or anaerobic conditions. There are basic requirements that should be met when deciding on a treatment system. These include the presence of microorganisms that are able to thrive in the system and effectively degrade the wastewater. In order for the microorganism to survive, the conditions need to be conducive for biodegradation of the test compound. Other factors such as the concentration

of the test compound are also important, as they may be toxic to the microorganism. The chemical characteristics of the wastewater are also significant to determine its volatility, adsorptability and solubility (Grady, 1985). Whilst most biodegradation testing can be done aerobically, it may be beneficial to also use anaerobic methods.

Aerobic Biodegradation Testing

Tier I: Screening Tests

An initial screening test could indicate the ability of a compound to be degraded, as well as its possible toxicity towards microorganisms. One method of evaluating the biodegradation of a compound is to measure the COD. Positive results, i.e. a decrease in the COD, would indicate that a compound is readily biodegraded. The COD could also be used to measure the rate of substrate removal. For biodegradable compounds, it is necessary to run batch growth rate studies in shaker flasks, using various concentrations and seeding with the appropriate cultures. It is important to establish whether a compound is likely to exhibit any toxic or inhibitory effects on a mixed microbial culture. If the screen test gives negative results due to inhibitory factors, it may be necessary to repeat the test using other cultures. Toxicity would be a negative result that would require a different strategy in Tier II testing (EPA, 1991).



Tier II: Acclimation and Enrichment

Activated sludge units and continuous flow units are used to fulfil the requirements set in Tier I testing. The effectiveness of the reactor should be measured after at least three months. Mineralization of the compounds, measured by the carbon-oxygen removal, is confirmation of biodegradation. Specific analyses are performed to ensure that the compound has indeed been degraded by the microorganisms and has not disappeared due to abiotic factors, such as adsorption onto solid surfaces or evaporation of volatile compounds. After biodegradation, a final round of testing should be done on the compound to ensure that it has not simply altered and therefore can not be detected, but indeed has been degraded (Stover and Kincannon, 1983).

Precise growth rates can be obtained using continuous flow units. The initial inocula should be obtained from varied sources that have been exposed to the compound to ensure maximal diversity in the microbial community. The preliminary concentration, rate of increase and

the final concentration of the test compound in the reactor should be consistent with any inhibitory characteristics observed in Tier I.

Tier III: Assessment of Degree of Biodegradation

Numerous tests can be used to determine the percentage of compound degradation and to compile removal curves. It can be assumed that the compound has been completely mineralized if 50% of the carbon ends up as CO_2, with less than 40% in the microbial cells and 10% or less as intermediates. Mineralization refers to the microbial breakdown of organic materials to inorganic products (Prescott *et al*., 1996).

Tier IV: Kinetics of Biodegradation

If results from the previous three Tiers show positive results for biodegradation, it may be useful to determine the kinetics of biodegradation. Careful consideration should be given to the need for kinetic studies as they require a large amount of effort.

Anaerobic Biodegradation Testing

Tier I: Screening Tests

The focus of Tier I screening is to determine the rate of methane gas production under anaerobic conditions. It is possible to predict the approximate quantity of methane gas produced from a known organic substrate if it were completely mineralized. The measurement of gas produced in excess of the control is used to measure the degree of mineralization. This is done using the serum bottle tests based on the Hungate technique (Wolfe, 1999), which evaluates the ability of single organic compounds to serve as the sole carbon and energy source for mixed anaerobic cultures to produce carbon dioxide and methane.

Actively digesting sludge is used to seed the system at the same time that toxicity is evaluated through the effect of various concentrations of the test compound on the rate of gas produced from a mixture of acetic and propionic acids. Acetic acid is used as a substrate to test the sensitivity of acetic-utilising methanogenic bacteria, and propionic acid is used to estimate the impact of both hydrogen forming acidogenic and hydrogen-utilising

methanogenic bacteria (Jawed and Tare, 1999). These Tier I screening assays should be run for at least eight weeks.

Tier II: Acclimation and Enrichment

Tier II testing is similar to that of aerobic systems where gradual increases in the concentrations of the test compound are applied in a multi-component substrate using a continuous culture reactor operated at a specific growth rate. The increases in feed concentrations are only made in response to removal of the compound, which is revealed by specific analysis of the reactor wastewater.

Complete mineralization of many organic molecules (except e.g., acetic and formic acids) in an anaerobic reactor requires the actions of several interacting populations within the microbial community. This suggests that the correct multi-component substrate used in Tier II testing is the one in which non-methanogenic bacteria normally act. The correct basal food would be primary sewage sludge, which can be collected and frozen in smaller aliquots. It is wise to use semi-continuous reactors due to the inconsistency of raw primary sludge (Parkin *et al*., 1983).

Successful completion of Tier II testing is dependant on the degree of biodegradation that is obtained. The low specific growth rates require an operational period of 4 – 6 months. Anaerobic cultures are sensitive to stress and it is therefore beneficial to run control reactors.

Tier III: Assessment of the Degree of Biodegradation

Anaerobic sludge is much more complex in nature, therefore the assessment of biodegradation is more difficult. When testing for mineralization performed by the microorganisms, a repetition of Tier I serum bottle tests using acclimated sludge are used. Sterile controls used during Tier I testing give an indication of the importance of degradation by microorganisms. Radioactive-labelled compounds, combined with the measuring of the evolution of labelled gasses, can also be useful when testing for biodegradation.

Tier IV: Kinetics of Biodegradation

Kinetic data is very difficult to obtain in methanogenic communities because of the interacting population. To determine the kinetics of individual compounds degraded under anaerobic conditions, it is necessary to operate several acclimated semi-flow reactors at various growth rates and correlate their residual substrate concentrations with each other.

The results obtained from these biodegradation studies are dependent on the treatment system used and the microorganisms present in the system. There are numerous well-established wastewater treatment systems that range from simple clarification ponds to advanced technological equipment that require skilled operators (Sangodoyin, 1995). Some of these treatment systems will be discussed in more detail in the following sections.

1.3 BIOREACTORS USED FOR WASTEWATER TREATMENT

Wastewater bioreactors are classified as aerobic or anaerobic and the choice of the specific reactor is dependent on the wastewater that has to be treated. In general, bioreactors contain microorganisms that act as biocatalysts that degrade the organic components of the wastewater. Microorganisms are able to adapt according to the changes in the wastewater composition, which in turn is dependent on various industrial processes (Bramucci and Nagarajan, 2000).

1.3.1 BIOLOGICAL TREATMENT SYSTEMS

There are different degrees of wastewater treatment that can be obtained with the use of either conventional or natural systems (Abu-Zeid, 1998). Natural systems are cost-effective and less complicated in maintenance and operation. Wastewater treatment in conventional systems may be subject to preliminary, primary, secondary and tertiary treatment, depending on the nature of the wastewater and requirements regarding the final product. Preliminary treatment involves the removal of coarse solids and grit and includes operations such as pre-aeration, flocculation, odour control and chemical treatments (Mardikar and Niranjan, 1995). Primary treatment is for the most part a lowering in the biochemical demand (BOD). Sedimentation is the most economical way of removing settleable organic and inorganic solids. Any floating materials can be removed by skimming, but this is an energy intensive process.

The degree of secondary treatment is dependant on the laws stated by regulatory bodies as to the permissible pH and COD levels, as well as parameters such as the removal of organic matter and, in some instances, phosphorus and nitrogen. Secondary treatments include activated sludge, anaerobic digestion, aerobic digestion, oxidising lagoons and biological filters. Tertiary treatment is done when high quality water is required and the processes include activated carbon absorption, reverse osmosis, nitrification, denitrification, chlorination, ammonia stripping, coagulation-sedimentation and selective ion exchange (Abu-Zeid, 1998).

Bioreactor treatment systems are based on microorganisms that have specific nutritional requirements. The primary sources of carbon, nitrogen and energy originate from the wastewater as large complex molecules, such as polysaccharides, glycoproteins and lipids. Microbial communities with complementary enzyme profiles are able to break down these complex molecules into simpler ones (Marsh and Bowden, 2000).

Wastewater characterisation is a global term as it is either expressed as the organic mass or as the energy available for heterotrophic and autotrophic organisms. Microorganisms that are able to convert inorganic and organic compounds to energy are called chemotrophic. Those able to obtain carbon from organic matter are called heterotrophic, whereas autotrophic microorganisms are able to obtain carbon from carbon dioxide. The presence or absence of oxygen is also an important factor in cell metabolism. Obligate aerobes require the presence of molecular oxygen for metabolism to occur. In an environment devoid of molecular oxygen, obligate anaerobes obtain their oxygen in non-molecular form from chemical compounds (Prescott *et al*., 1996). Facultative anaerobes are able to grow in the presence or absence of oxygen, but prefer an aerobic environment as there is a greater energy yield from aerobic metabolism. Based on these requirements, microorganisms used in bioreactor systems for wastewater treatment can be classified as follows:

Aerobic Chemoheterotrophs

Organic matter + O_2 ⟶ energy + O_2 + H_2O
+ other by-products (e.g. NH_3) + cell mass

Major microorganisms involved in this process are *Clostridium*, *Bacteriodes*, *Peptostreptococcus*, *Peptococcus*, *Eubacterium* and *Lactobacillus*.

Anaerobic Chemoheterotrophs

Acid formers

Organic matter ⟶ energy + organic acids + CO_2
+ other by-products + cell mass

Acetogenic reactions are governed primarily by *Syntrophomonas*, *Syntrophobacter* and *Acetobacterium*.

Methane formers

Organic acids $\longrightarrow$ energy + methane + CO_2
+ other by-products + cell mass

Acetate can be converted to methane by *Methanosarcina* and *Methanosaeta* that are both primary acetate consumers (Fig. 1). Other methanogens include *Methanobacterium*, *Methanothermobacter* and *Methanobrevibacter* (Sekiguchi *et al*., 2001).

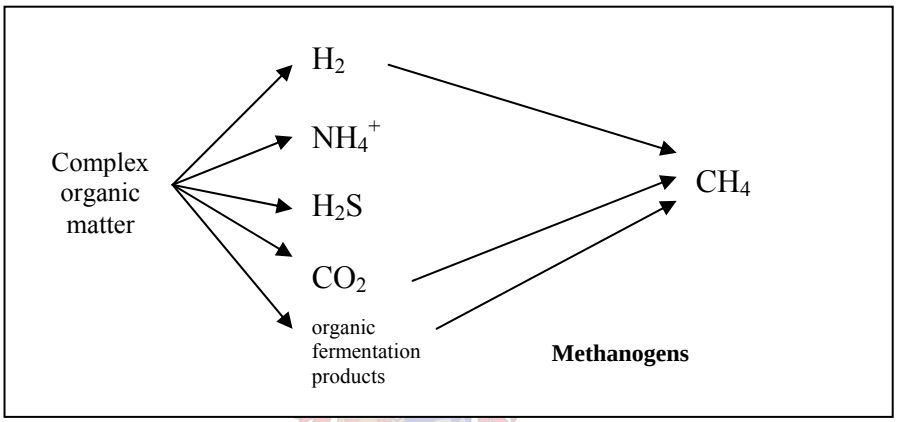


Fig. 1. Anaerobic carbon use by methanogens to produce methane gas via fermentation (Prescott *et al*., 1996).

Aerobic Chemo-autotrophs

Nitrifying bacteria

$2NH_4^+ + 3O_2 \longrightarrow$ energy + $2NO_2^- + 4H^+ + 2H_2O$

$2NO_2^- + O_2 \longrightarrow$ energy + $2NO_3^-$

Nitrosomonas and *Nitrosococcus* are key organisms in the first step of nitrification, whilst *Nitrobacter* plays a key role in the second step (Silyn-Roberts and Lewis, 2001). In acid environments, heterotrophic nitrifying bacteria and fungi are present.

The key to a successful biological reactor system is retaining the microbial population while allowing for degradation and discharge of reaction products. A number of systems have been developed to retain the microbes in a bioreactor. One of these is the use of immobilised cells that perform multi-enzyme reactions as do free cells, but they are present in higher biomass concentrations, resulting in faster reaction and processing times.

Attachment to a surface is dependent on the natural adhesion ability of the microorganisms, and may be helped by the use of specific support media. To retain cells in the system, semi-permeable barriers may be used, or aggregation and floc formation can allow cells to be retained in the system. A stirred tank reactor may cause damage to immobilised cells due to shear forces.

1.3.2 AEROBIC TREATMENTS

Aerobic digestion of wastewater involves the addition of air or oxygen to the wastewater for the chemo-autotrophic and chemoheterotrophic microorganisms to degrade the organic substrates (Fig. 2). Aerobic digestion of organic matter usually results in an increase in biomass. There are many aerobic treatment systems available and the overall advantages include low capital costs, easy operation, stable endproducts and the production of odourless sludge. The disadvantages are the high consumption of energy needed to supply the oxygen, and the production of endproducts (such as sludge) with low dewatering capacity (Bitton, 1994).

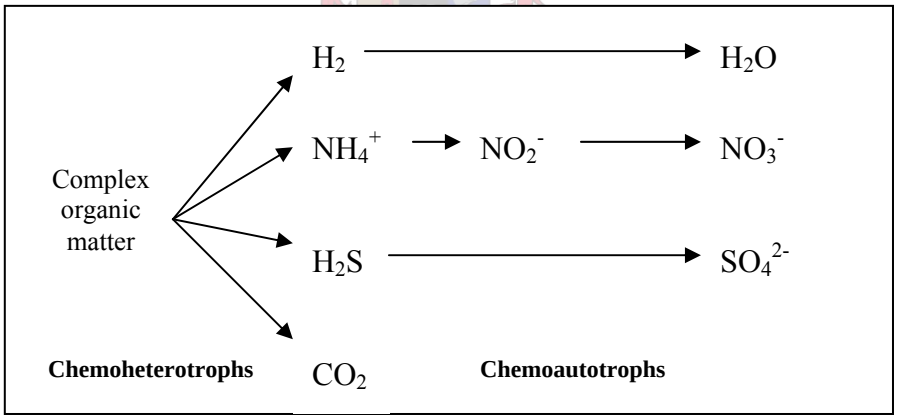


Fig. 2. Different routes for oxidation of organic matter by microorganisms during aerobic degradation. The organic matter is reduced by the primary utilisers (chemoheterotrophs) and used by the secondary utilisers (chemo-autotrophs) (Prescott *et al*., 1996).

Trickling Bed Filter

This is the most basic wastewater treatment system where the wastewater is sprayed over a bed of rocks covered with a biofilm layer. The wastewater running over the rocks is degraded by the biofilm community. Most sewage facilities use this as a secondary aerobic

treatment step. This requires extremely large aeration basins and is a time-consuming treatment process (Prescott *et al*., 1996).

Biofilm Fluidised Bed (BFB) Reactor

The wastewater is pumped through a bed of small particles, such as sand, at a velocity sufficient to cause fluidization. The particles provide a surface for attached biological growth (Borja *et al*., 1993) and some systems use granular activated carbon (GAC) as the carrier particle. These GAC particles have additional benefits as they show greater removal of slowly degradable or recalcitrant compounds that are concentrated on the carbon surface (Sutton and Mishra, 1994). The wastewater is recirculated (Fig. 3) from the reactor to an oxygenator where oxygen is bubbled through the wastewater. The biolayered particles are kept suspended by the upward flow of water, which also controls the thickness of the biolayer due to shear (Heijnen *et al*., 1989). The recycling of wastewater has advantages as it can help to neutralise the pH of incoming wastewater, reduce the effect of toxic biodegradable compounds, minimise the effect of shock loading and compensate for variability in the influent flow rate (Jeris, 1983).

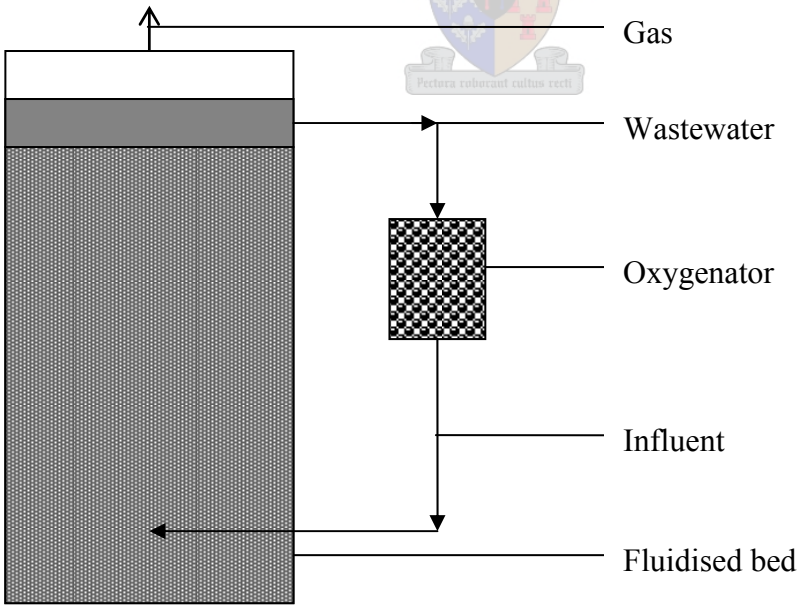


Fig. 3. Biofilm Fluidised Bed Reactor (Nicollela *et al.*, 2000b).

The BFB system uses carrier particles to which the biofilm attaches, thus eliminating most solid/liquid separation problems. This also allows for high biomass concentrations that result in smaller reactor volumes. Particles can move within the reactor and any changes in their physical characteristics (e.g. density, size, etc.) also affect the biomass distribution in the reactor. Particles with the thickest biofilm tend to float to the top of the bed, therefore the success factor for the thick biofilm is quite low, especially at the top of the bed. Low substrate removal rates are observed when the mean cell residence time (MCRT) is too short and a thinner biofilm forms. The thinner biofilm is less effective due to its limited removal rate (Hermanowicz and Cheng *et al*., 1990).

The BFB system is also used anaerobically where no oxygen is pumped into the reactor. The advantages of using this system anaerobically include low energy consumption, reduced solid formation and potential energy recovery from methane gas. Other design modifications include the use of the system as a down flow fluidised bed reactor (Garcia-Calderon *et al.,* 1998), or to use a filter to retain the anaerobic biomass independently of the wastewater flow rate (Berardino *et al*., 2000).

Biofilm Airlift Suspension (BAS) Reactor

BAS reactors, more commonly named 'Circox reactors' (Frijters *et al*., 1997), consist of two connected sections called a riser and a downcomer. There are many different variations, including internal and external loop reactors. However, the principle is the same: gas is released at the bottom and moves upward to the riser section. In the internal airlift loop reactors, the air is recirculated through the downcomer section to provide aeration for the whole reactor. The movement of air drives the wastewater to circulate between these two sections, mixing both the liquid and solid particles (Obradovic *et al*, 1994). If the velocity is sufficient, the small particles will be suspended and recirculated with the wastewater.

The liquid circulation rate is dependent on the reactor proportions, gas supply rate and the amount of solids present. The circulation speed then determines the mixing, gas hold up and solids suspension (Heijnen *et al*., 1997). Disadvantages of this system are high surplus biomass production, short sludge age and the need for land space to install the reactors and settlers (Tijhuis *et al*., 1994).

Aerobic Sequencing Batch (ASB) Reactor

Granular sludge is formed in a sequencing batch reactor and can be easily separated from the liquid. This dense floc-like sludge has good settleability and is easily maintained in the reactor (Dangcong *et al*., 1999). With a higher biomass concentration, the activity in the reactor is higher, the treatment is more efficient with better performance (Chudoba and Pujol, 1996). Successful application of this reactor requires sufficient air supply, limited rising velocity in the settling tank, recirculation of returned sludge and adequate waste sludge capacity. Advantages of this system are the low capital costs and moderate operating costs, which makes it a well-suited system for the biodegradation of winery wastewater (Torrijos and Moletta, 1997).

Membrane Aerated Biofilm Reactors (MABR)

This aerobic treatment diffuses oxygen through a gas permeable membrane (Fig. 4) into the biofilm for the oxidation of pollutants on the biofilm side of the membrane. This reactor can be in a shell and tube or a plate and frame configuration (Casey *et al*., 1999). The tubular or flat membranes are made from hydrophobic, porous polypropylene and the dense film type is made of silicone. It could also be a composite type where a porous membrane is coated with a thin film of dense material. The membrane lumen is either open or closed in the case of tubular membranes.

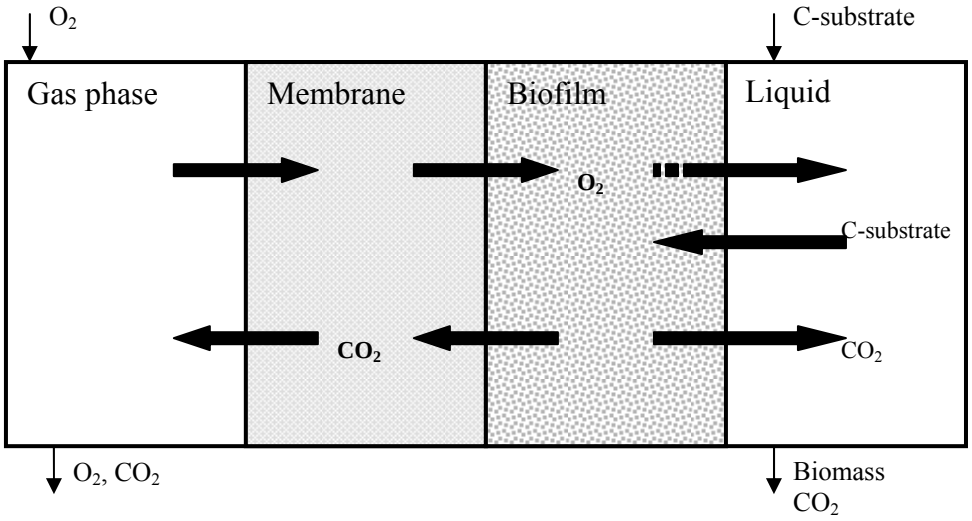


Fig. 4. Membrane Aerated Biofilm Reactor (Casey *et al*., 1999).

The membrane-attached biofilms have two limiting reaction substrates, i.e. carbon substrates and oxygen, supplied from opposite sides of the biofilm. It is possible to have aerobic/anaerobic or aerobic/facultative anaerobic type reactions in mixed culture biofilms due to the biofilm thickness which creates different active layers (Casey *et al*., 1999). These layers create anoxic regions where sulphate reducing bacteria and even methanogenic bacteria can exist.

Ozonation

Ozone is an effective oxidant and disinfectant for water treatment. It can improve particle flocculation through different mechanisms, or wastewater biodegradability by removing compounds that are either inhibitory or toxic to the cells. When used as an oxidising agent in wastewater treatment, the level of biodegradation is dependent on the pH of the wastewater (Beltran *et al*., 2001). At a low pH, ozone will react with the compound's functional groups by electrophilic, nucleophilic and dipolar addition reactions. At basic pH, ozone decomposes to generate hydroxyl radicals which react with the organic and inorganic compounds in the wastewater. When added during acidic periods, the ozone will strip off the carbonates as carbon dioxide, thereby, increasing the degradability of the wastewater.

Jet-Looped Activated Sludge Reactor (JLR)

This system consists of a column that contains activated sludge, with a central tube and a cylindrical degassing tank. The wastewater is pumped through an ejector nozzle where the air has been drawn into the liquid through an air tube (Petruccioli *et al*., 2002). The aerated wastewater passes from the column to the degassing tank from where it is recycled through the nozzle into the column. The degassing tank is connected to a settling tank for the displaced treated wastewater.

Rotating Biological Contactor (RBC)

There has been renewed interest in biofilm systems for the oxidation of carbonaceous wastewater since the middle of the 20th century (Rodgers, 1999). RBC's have been used extensively for single stage carbon removal and for separate stages in a series for COD removal. Various parameters such as turbulence, disc rotation speed, hydraulic retention time, organic matter and recirculation all play an important role (Gupta and Gupta, 1999).

The RBC system allows for the establishment of biofilms under near steady-state conditions, by providing a well mixed liquid phase, turbulent flow and constant shear forces.

The basic design (Fig. 5A) consists of a number of discs that are positioned on a shaft, which is turned by a motor so that the discs remain at a right angle to the flow of the wastewater. The discs are positioned with about 40% of the discs remaining submerged at all times. The rotation of the discs creates a process of alternating absorption of pollutants and exposure to air to provide sufficient amounts of oxygen and nutrients for microbial growth. The rotational speed should not exceed 10 revolutions per minute to prevent shear of the biofilm (Senior, 1992). When the biofilm becomes too thick, it simply sloughs off and settles at the base of the reactor.

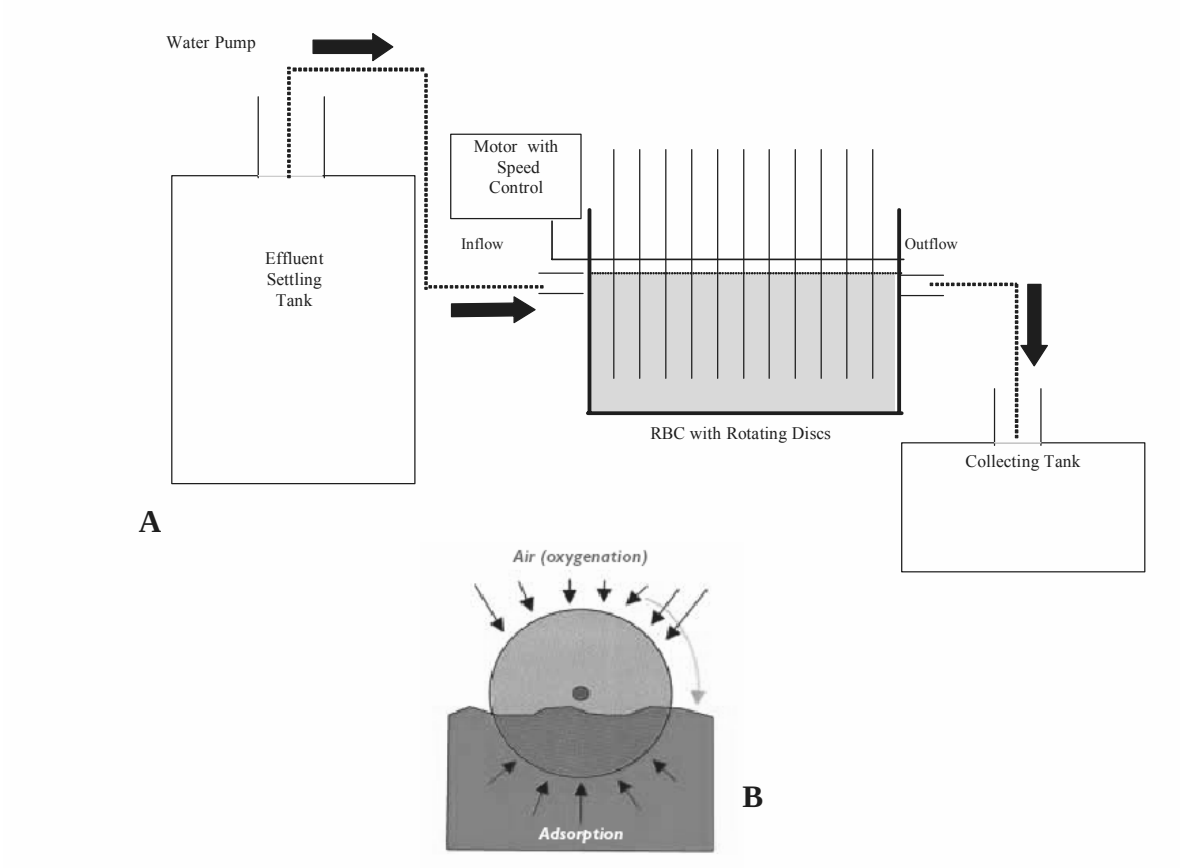


Fig. 5. A) Wastewater is pumped into the RBC and the rotating discs are covered with a biofilm layer that degrades the organic material in the wastewater. B) At least 40% of the surface of the rotating discs remains submerged in the wastewater, whilst the circulation provides sufficient aeration for the microorganism.

Inexpensive polystyrene discs can be used instead of plastic discs. Microbial biofilms form on these surfaces, and are able to metabolise organic matter, trace elements, etc. It is possible to have fully or partially submerged RBC biofilms for the treatment of wastewater to allow for an aerobic or anaerobic system. Partially submerged discs (Fig. 5B) allow for the aeration of the microbial biofilm and the oxygen is either supplied from the air or from the dissolved oxygen in the liquid.

The bulk of the microbial film on the RBC discs consists of bacteria, protozoa, metazoa, etc. (Nahid *et al*., 2001). The key factor in the RBC's performance is to maintain biofilm stability. It is therefore important to know the physical properties, composition and activity of the biofilm (Teixeira and Oliveira, 2001). An aerobic RBC system was used for the simultaneous carbon removal and denitrification using *Thiosphaera pantotropha,* which is both a heterotrophic nitrifier and aerobic denitrifier in a mixed bacterial biofilm (Gupta and Gupta, 2001).

Advantages of the RBC system include a more compact treatment plant and the degree of treatment is not dependent on a final sludge separation. The disadvantages are the rotational speed limitation and the need for backwashing. The system requires minimal energy consumption as no large air pumps are needed. It is simple to operate with little maintenance costs. Furthermore, the biofilm makes the system less sensitive to organic load variations and toxins. There have been many reports of operational failures associated with RBC's. However (Mba *et al*., 1999), most of the design inadequacies have been investigated and dealt with, resulting in RBC's designed to last for an operational life of twenty years.

1.3.3 ANAEROBIC TREATMENTS

Anaerobic digestion is the microbial fermentation of organic matter to CO_2 and methane gas. The production of methane gas is therefore directly proportional to biodegradable substrate assimilation (Sales *et al*., 1989). Different anaerobic microbial populations degrade the organic pollutants in multiple degradation steps such as hydrolysis/fermentation, acetogenesis and methanogenesis (Liu *et al*., 2002). The microbes include fermentative bacteria, acetogenic bacteria and methanogens, which form a syntrophic relation. Anaerobic systems are advantageous in that (1) there is less sludge produced, (2) CO_2 is used as the

electron acceptor, thus cutting the cost of oxygen addition, (3) methane that is produced can be used in other processes and (4) anaerobic systems are able to withstand high loading rates. The disadvantages of the aerobic process is that it is a slower process, very sensitive to toxic substances and has a long start-up time (Bitton, 1994).

Upflow Anaerobic Sludge Blanket (UASB)

The UASB was developed as a high rate biological treatment system in 1972 (Cheng *et al.,* 1990). It is based on the use of granules (1 – 4 mm) that form slowly into multi-layered biofilm structures (Tartakovsky and Guiot, 1997). The reactor is usually seeded with pelletised sludge and allowed to acclimatise to the conditions (e.g. pH and COD) in the reactor before the wastewater is pumped through for treatment (Moosburger *et al*., 1992). Wastewater enters the reactor from the bottom through the inlet system (Fig. 6) and is passed upward through the dense anaerobic sludge bed. The overlying sludge blanket encompasses about 70% of the reactor volume (Farmer *et al.,* 1989). Soluble COD is readily converted to biogas, rich in methane, and an upward circulation of water and gasborne sludge is established. The gas is removed at the settler section of the reactor and the dense granules settle back into the sludge blanket, creating a downward circulation. This continuous convection creates an effective wastewater to sludge contact. The reactor design has a highly active biomass concentration that can cope with high loading rates, resulting in shorter retention times. A major problem is the accumulation of suspended solids that reduce the reactor capacity.

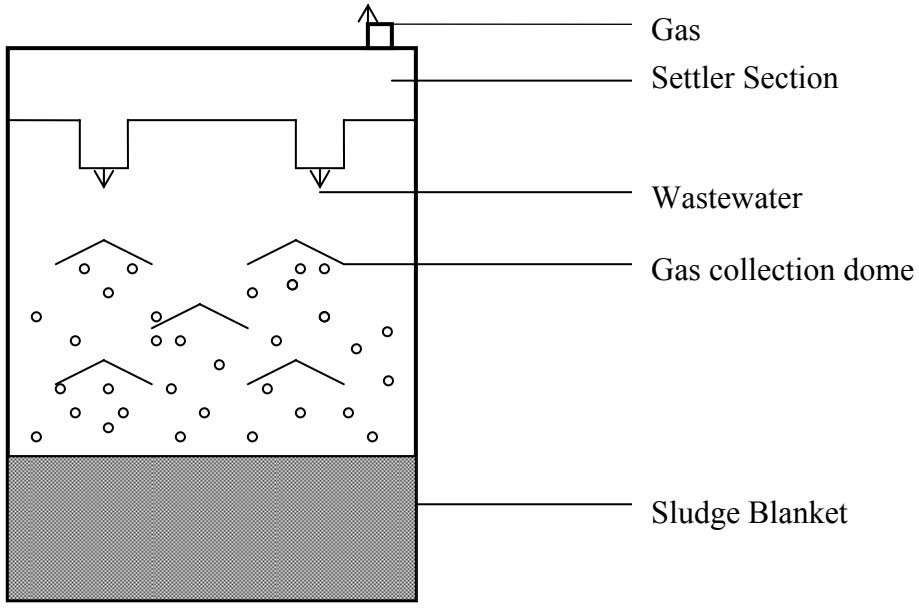


Fig. 6. Upflow Anaerobic Sludge Blanket (Nicollela *et al.,* 2000b).

The UASB is one of the most economic and effective anaerobic methods of biodegradation (Rajczyk, 1993). Yeast cells are readily incorporated into the flocs (Goodwin *et al.*, 2001) and granule formation also requires a large amount of filamentous organisms (e.g. *Methanothrix* spp). The key to the success of this reactor is maintaining the biomass solids in the reactor, as it is the single most important variable. The biogas is of significant economical value, as it can be combusted in a boiler room to produce steam for other processes (Çiftçi and Öztürk, 1995). The anaerobic treatment of wastewater has been applied at the Stellenbosch sewage works since 1974. The system has two UASB's and three clarifiers and the biogas produced is used to run the boilers (Ross, 1989).

Expanded Granular Sludge Blanket (EGSB)

This reactor is able to treat chemical, biochemical and biotechnological industry wastewaters. The wastewater enters the system at the bottom of the reactor via the influent distribution system (Fig. 7). Wastewater is pushed through the sludge bed, where the organic material is converted to biogas (Zoutberg and Frankin, 1996). The mixture of sludge, water and biogas is separated by the three-phase separator at the top of the reactor. Purified wastewater and biogas are discharged at the top of the reactor.

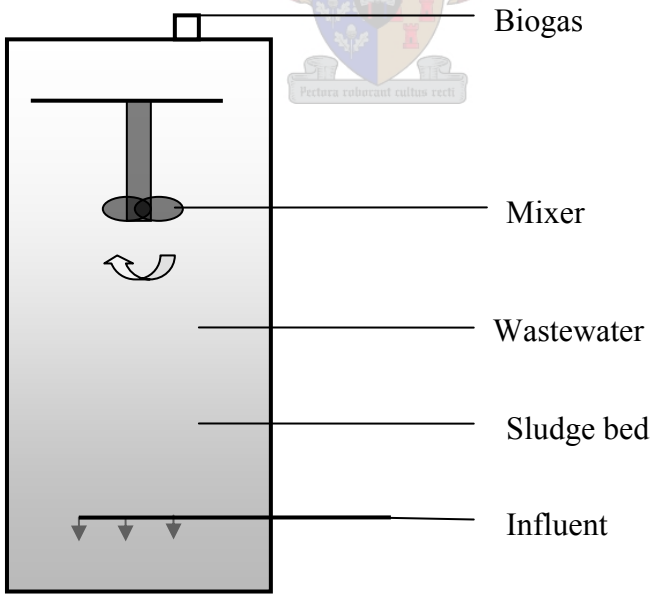


Fig. 7. Expanded Granular Sludge Bed Reactor (Nicollela *et al.,* 2000b).

This method is perceived as an ultra high rate UASB that consists of two major components, i.e. the settlers at the top of the tank and the feed distribution at the bottom of the tank. Biomass is present in a granular form and the upflow velocities (10 m/h^{-1}) for the liquid can be operated as ultra high loaded anaerobic reactors. At the top part of the reactor, biomass particles grow bigger due to milder shear conditions.

Disadvantages of this system are a long recovery time after an upset condition, and the system has no buffering capacity to shockloads of toxicants and COD (Frankin *et al*., 1992). The advantage of this closed system is the reduced odour emissions.

Biofilm Airlift Suspension (BAS)

Anaerobic airlift reactors are used for the reduction of sulphates (Nicollela *et al*., 2000a). Airlift reactors (Fig. 8) consist of two connected sections, namely a riser and a downcomer. Different variations are possible, but the principle operation is the same for all of them. The influent is pumped into the bottom, moves upwards and exits at the riser section. In internal-loop airlift reactors, air may recirculate through the downcomer section and provides an upward flow throughout the reactor.

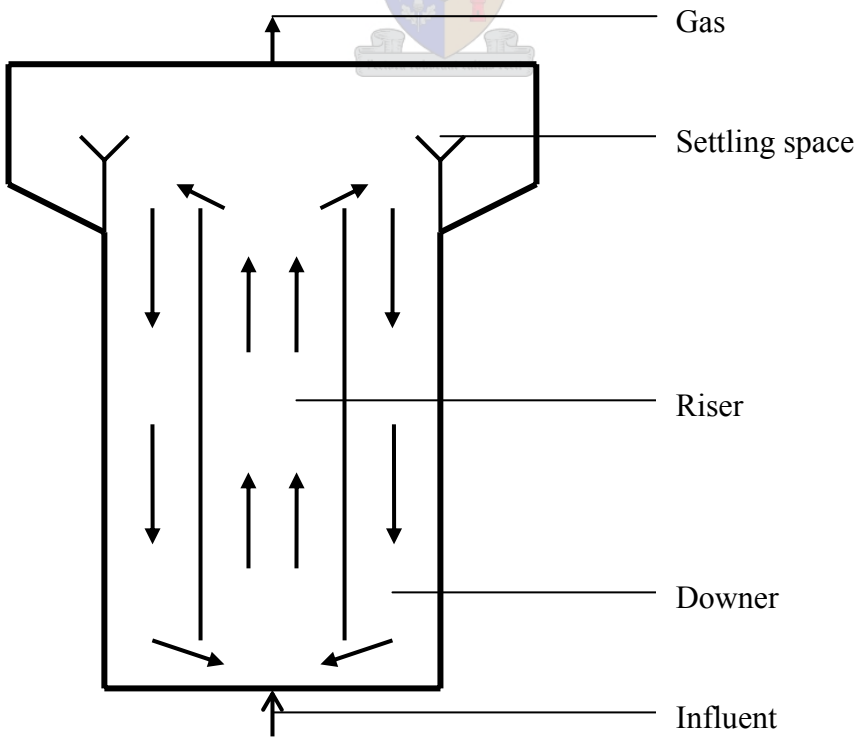


Fig. 8. Biofilm Airlift Suspension (Nicollela *et al.*, 2000b).

The difference in density between riser and downcomer drives the liquid to circulate between the two sections. When the velocity is sufficient, it allows small particles to be suspended and recirculate allowing for thorough mixing. This gas circulation is induced by recirculating the gas produced by the biochemical reactions.

Anaerobic Digestion Ultrafiltration (ADUF)

The reactor consists of two main units: an anaerobic digester and an external ultrafiltration membrane. This membrane-assisted process eliminates the sludge concentration and retention problems associated with conventional systems (Ross, 1989). In the ADUF process, the permeate is the final wastewater and the sludge concentrate contains the bacteria that are recycled back into the digester with minimal decrease in activity and temperature. Anaerobic digestion breaks down the organics that would normally foul the filter membranes and the filter membranes ensure biomass retention.

Advantages of the ADUF system are that it operates at neutral pH, at lower pressures and temperatures than other systems, and needs a minimal cleaning regime which all help to maintain the membranes and support structures.

Internal Circulation Reactors (ICR)

This reactor consists of two UASB-like compartments (Fig. 9) on top of each other, one highly loaded and the other lightly loaded.

The bottom reactor consists of an expanded granular sludge bed where most of the COD is converted to biogas (Nicollela *et al*, 2000b). The biogas is collected by the lower level phase separator and is used to generate a gas lift to carry the water and sludge upward via the riser pipe to the gas-liquid separator on the top of the reactor. Biogases can then escape and leave the system. The sludge-water is directed downwards to the bottom of the reactor via the concentric downer pipe, resulting in an internal circulation flow. The wastewater from the first compartment is post-treated in the second lightly loaded compartment, where the remaining COD is removed (Driessen and Yspeert, 1999).

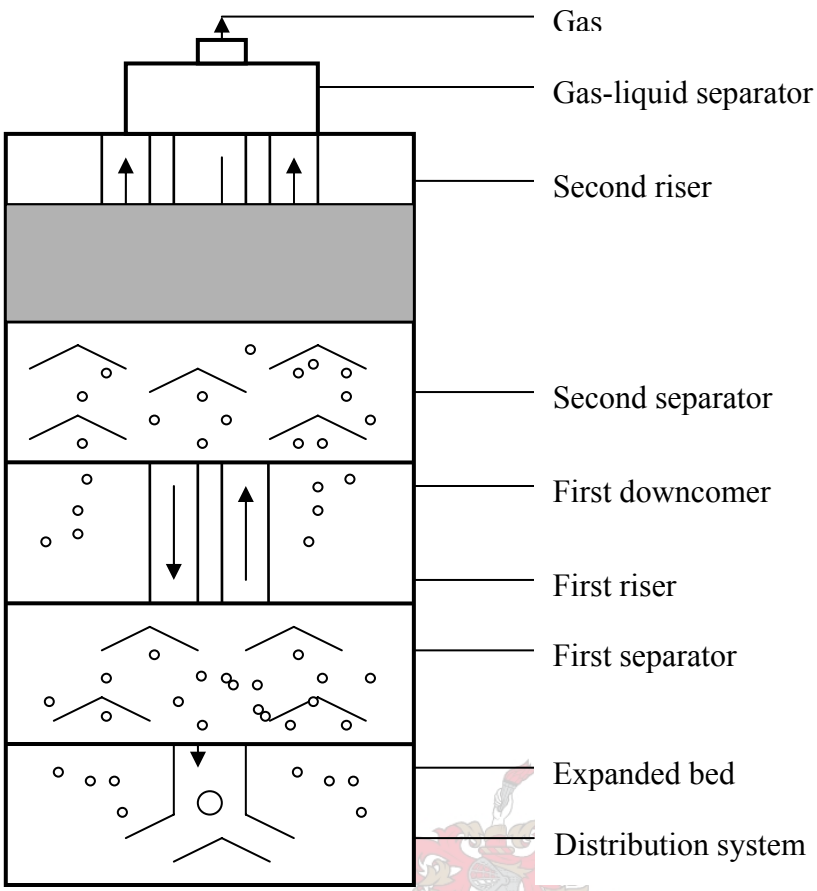


Fig. 9. Internal Circulation Reactor (Nicollela *et al.*, 2000b).

1.4 BIOFILMS

Biofilm refers to the biologically active matrix of cells (living and dead) and extracellular substances (including cell debris) in association with a solid surface. Alternatively, it can be described as a functional consortium of many different microbes, often from various lineages (bacteria, yeast, fungi, moulds, algae, protozoa, nematodes, etc.) attached to a surface and embedded in extracellular polymeric substances (EPS) produced by the microbes (Kumar and Anand, 1998). These biofilms have collective properties that are greater than the sum of the individuals, and are subject to continual dynamic change in community structure, composition and expression (McBain *et al*., 2000).

Microbial cell aggregates such as flocs and biofilms are of interest in reactor systems as they assist in the cell-liquid separation by sedimentation or filtration. The word 'floc' refers to groupings of free cells and colonies of cells. A biofilm can be a pure culture of cells or a complex community of cells and cellular products that forms readily as large granules or grow attached to static solid surfaces or on suspended carriers. Biosorption is the transport of organic matter from the wastewater to the flocs where it remains for a short time. This is a relatively fast phenomenon where equilibrium may be achieved in only a few minutes or hours. It is thought that biosorption may be a result of electrostatic or hydrophobic interactions depending on the nature of the organic matter (Guellil *et al*., 2001). Biosorption is dependent on the microbial community present in the floc.

Microbial aggregates, be it in the form of granules, biofilm or flocs, have two distinct phases that feature three major consequences:

1) Biomass retention is used to improve the reactor conversion capacity, as the biodegradation is limited to the amount of biomass present.
2) The substrates (carbon, oxygen, etc.) need to be transported through the biofilm to the microbial cells to be consumed. The penetration depth of substrates in the biofilm depends on the porosity of the biofilm, substrate concentration in the bulk fluid and reaction rate in the biofilm.
3) A multi-species biofilm leads to a layered structure which may have the fast growing microbes on the outside and the slow growers on the inside. This would protect the slow growers from being detached by shear forces. This can be seen where fast

growing heterotrophic bacteria are found on the outer layer of the biofilm where the nutrient and re-colonisation rates are high. In contrast, the slow growing nitrifying bacteria are found deeper inside the biofilm (Nogueira *et al*., 2002).

Various biological wastewater treatment processes (Nicollela *et al*., 2000b) are based on the use of three types of microbial aggregates: static biofilms (e.g. trickling filters), particulate biofilms (e.g. upflow anaerobic sludge blanket reactors) and flocs (e.g. in activated sludge processes). Biofilms are also used extensively in environmental biotechnology where large volumes of dilute aqueous solutions have to be treated, and natural mixed populations of biofilms are used. The advantage of biofilms is that the process can be operated at a high biomass concentration, without the need for settlers or recirculation.

1.4.1 BIOFILM FORMATION

Biofilms will form on virtually any submerged surface if organic matter is present. Nutrients found on the surfaces usually form a conditioning film that triggers the biofilm formation. The transport of microorganisms to a substratum surface can be by different mechanisms such as Brownian movement, gravitation, diffusion, convection or they may simply be in suspension already (Bos *et al*., 1999). Bacteria are able to sense their proximity to a surface by releasing protons and signalling molecules as they move through the bulk fluid. These molecules diffuse radially away from the floating cell if not adjacent to any surface. Higher concentrations of either the protons or the signalling molecules will be found on the bacterial side that is nearer to the surface. This allows the cells to sense the surface as diffusion becomes limited on that side.

Adherence to a surface is influenced by the nature of the surface, charge of the surface and the concentration and type of nutrients in the conditioning film (Apilanez *et al*., 1998). Wild bacterial strains form a thick layer of extracellular polymeric substances (EPS) that surrounds each cell. There may be fimbriae that protrude from the cells to form an effective part of the cell surface. The fimbriae are used for specific adhesions to tissue surfaces, whereas the EPS is used for non-specific adhesion to inert surfaces. Firm adhesion to surfaces requires the interaction of the elastic polymers of the EPS layer.

Bacterial cells may first roll across the surface before settling in one place to initiate adhesion (Fig. 10) with the help of van der Waals and electrostatic forces. Cells may also roll away from already adhered cells to form a monolayer of cells. Other species may congregate in certain areas to form microcolonies. Once the cell is positioned on the surfaces, it will engage in the active process of adhesion and biofilm formation. The cells upregulate specific adhesion genes to synthesize exopolysaccharide material to 'cement' the cell to the surface. This is achieved by interfacial forces such as dipole-dipole, dipole-induced dipole, ion-dipole, ionic, hydrogen bonds and hydrophobic forces. The irreversibly attached cells grow and divide by using the nutrients in the conditioning film and surrounding fluid.

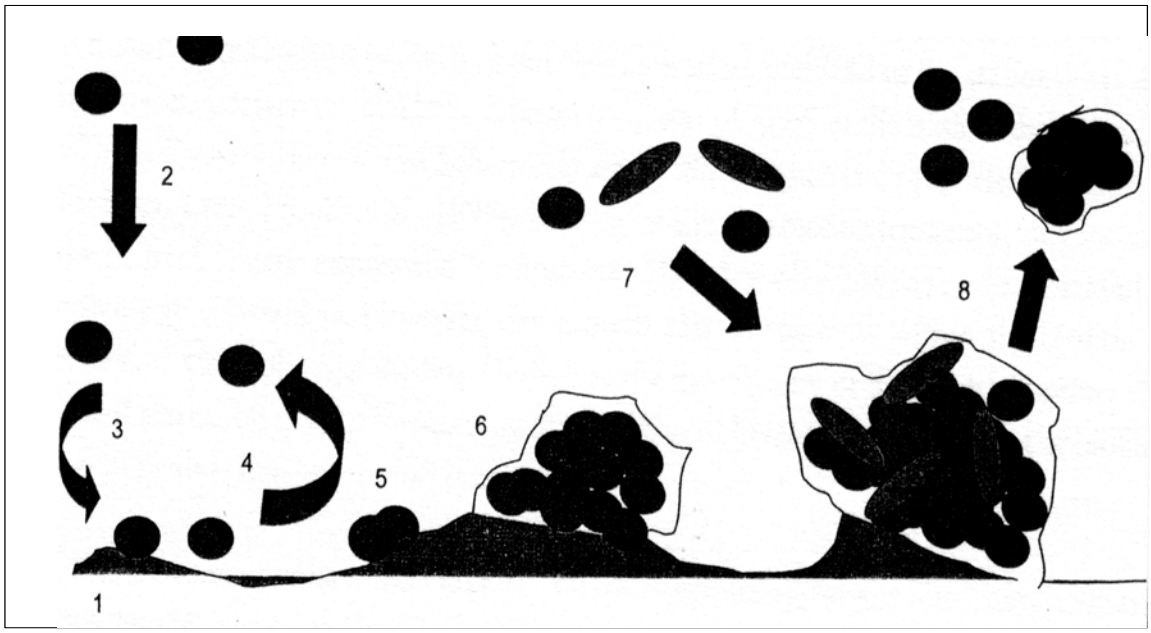


Fig. 10. Biofilm formation. 1, preconditioning; 2, transport of cells to substratum; 3, reversible adsorption; 4, desorption; 5, irreversible adsorption; 6, growth and EPS production; 7, attachment by other microorganisms and 8, detachment (Moore *et al*., 2000).

1.4.2 BIOFILM ARCHITECTURE

The structure of biofilms has been documented and evaluated using a variety of microscopic methods (light microscopy, electron microscopy, atomic force microscopy and confocal scanning laser microscopy (CSLM)); physio-chemical (micro-electrodes), and molecular biology (e.g. fluorescent *in situ* hybridisation (FISH) probes) (Wimpenny *et al*., 2000). There have been increasingly more complex mathematical models to explain the structure

and interactions in biofilms (Hermanowicz, 2001). Some of these techniques will be discussed later (Section 1.5).

A microcolony is the basic structural unit of the biofilm that consist of a matrix enclosed community of bacterial and other cells of one or more species. The matrix material appears most dense at the core of the microcolony. The microcolony has a mushroom-like shape (Fig. 11) with most of the cells in the 'crown' with few cells in the stalk. There are water channels (Xavier *et al*., 2000) through the microcolony architecture which allows solutes complete access to the water channel systems aided by the convective flow of the fluid (Costerton, 1999). In biofilms where there is a low substrate loading, or if it consists of slow growers, the protuberances are likely to erode creating a smooth biofilm. If the shear rate is low, it will lead to the growth of heterogeneous porous structures that will then lead to sloughing of the biofilm (Loodsdrecht *et al*., 1995).

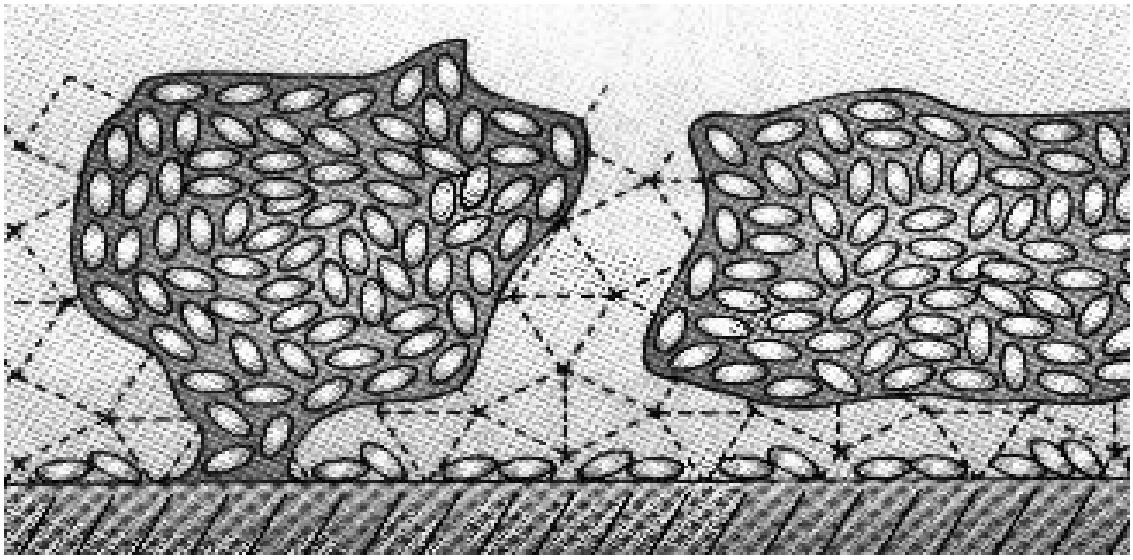


Fig. 11. A mature biofilm with the classic 'mushroom-shape', water channels, cell aggregates and interstitial pores (Prescott *et al*., 1996).

Substrates, nutrients, inhibitors and electron acceptors move from the bulk phase through a liquid boundary layer into the biofilm. The biofilm density, age, filaments and even electrostatic interactions influence diffusion in biofilms. Oxygen concentration, which is essential in the oxidation/reduction reactions of aerobic respiration, varies within the structure of the biofilm and with the cell utilisation rate (Bishop *et al*., 1995). When looking

at aerobic multi-species biofilm communities in granular activated carbon (GAC), the biofilm is a discontinuous multi-layer structure (Massol-Deya *et al*., 1995).

1.4.3 DETACHMENT AND DISPERSAL OF BIOFILMS

In order to survive and colonise new niches, it is necessary for the biofilm to detach and disperse. Microorganisms at the base of the biofilm have the advantage of being protected from being easily detached, unlike those found at the surface of the biofilm (Morgenroth and Wilderer, 2000). There are four steps in the detachment process: 1) abrasion (caused by the collision of biofilm support particles, e.g. during backwashing); 2) sloughing (detachment of large particles of biofilm biomass, which may be a result of fluid dynamics and shear effects); 3) erosion and 4) predator grazing. In biofilm reactors, detachment will be due to a combination of these processes, although one mechanism may be more dominant depending on the reactor type.

Factors influencing the detachment may be due to the environment within the biofilm or within the bulk fluid. Physical properties such as liquid velocity and shear stress at the biofilm-liquid interface influence the erosion of a biofilm. Chemical factors such as changes in the nutrient availability can also cause detachment. Electrochemical properties such as the need for protons for lactose transport in the cell could cause the extracellular matrix to expand or contract and thereby lose its cohesive properties. Other work has shown that cells change during the growth cycle and this may also affect the detachment rates. Biological factors such as the release of surface protein releasing enzyme (SPRE) produced by *Streptococcus mutans*, release proteins from the cell surface which brings about detachment. *Pseudomonas aeruginosa* biofilms produce alginate that assists in the attachment of cells to a surface. However, they also produce alginate lyase which degrades the polysaccharide and induces sloughing (Moore *et al*., 2000).

Abrasive detachment is particularly important in the water industry as it may affect the performance of fluidised-bed reactors. Biofilms form on inert material that collide and rub against each other in the reactor. The rate of biofilm detachment may be influenced by biofilm thickness, shear stress, growth rate and density, and leads to a constant biofilm thickness.

1.4.4 IMPORTANCE OF MICROBIAL COMMUNITIES

Most wastewater treatment systems rely on the action of complex microbial communities that are best suited to survive their environment. The thorough knowledge of the structure and function of these communities is essential for plant optimisation (Amann *et al*., 1998). However, there is very little information regarding the ecological relevance of the community structure for function of the system (Dahllöf, 2002). Complete biodegradation of a compound often requires the metabolism of two or more organisms as no single specie within the culture contains the complete genetic complement of the whole culture. Microbial community members are in constant interaction with each other and they can be divided into six types of metabolic interactions, i.e. neutralism, commensalisms, mutualism, amensalism, predation or competition (Stams, 1994). Amensalism is a significant factor in the composition of the community. Bacteria are able to produce antagonistic compounds, such as bacteriocins and lantibiotics, which are harmful to other microorganism and give them a competitive edge (Marsh and Bowden, 2000).

Microbial ecosystems lack long-term stability and are continually adapting. Symbiotic relations are beneficial to one or more species found in the community and these interactions enable the microbes to modify their local environment, making it more favourable for growth. Colonisation resistance is the phenomenon where antagonistic factors prevent other microbes from colonising established communities. Gene transfer can be seen where virulence factors such as toxins or antibiotic resistance are transferred between species. There is also evidence of plasmid DNA transfer. Mixed microbial communities have distinct advantages as the biodegradative capacity of a community is much greater, both quantitatively and qualitatively. Resistance to toxic substances is greater because of an increased probability that the microorganism with the ability to detoxify the toxicants is present (Cowan *et al*., 2000).

Dominants are those species having a dominant impact on the community while associates are dependent on the dominants for survival. Incidentals are those species that are indifferent to the actions of the dominants and the associates. Primary utilisers are those species capable of metabolising the sole or major carbon energy substrate provided to the system. The secondary organisms rely on the utilisation of products released by the primary

utlisers. The sequential breakdown of complex molecules creates a food web by the primary and secondary utilisers that contributes to the homeostasis of the community.

Anaerobic microbes are able to survive toxic aerobic environments by using one of two strategies. Firstly, by close association with aerobic-consuming microbes, the environmental levels of oxygen are sufficiently reduced to enable them to detoxify the low levels of oxygen with a range of protective enzyme systems. Secondly, protozoa have the ability to produce hydrogen during the oxidation of pyruvate to acetate and carbon dioxide, and they are able to survive low level oxygen environments. This is achieved by using the oxygen as the electron acceptor, creating methanogenic environments where methanogens can be found on the surface of ciliates. Communities are able to survive extreme pH conditions by the surface associated community. Bacteria are also able to regulate their local pH by upregulating genes involved in either acid or base production.

Microbial associations are seen as an important approach to modern environmental biotechnology (van der Merwe and Britz, 1994). The microbial progression is important to the community development (Fig. 12), with the co-adhesion and metabolism creating conditions conducive to microbial growth (Marsh and Bowden, 2000). Microbial communities respond to both internal signals and external environmental stimuli and are flexible enough to adapt to their population interactions and survive. 'Water channels' provide the organisation needed for signalling between cells, the transport of genetic material, nutrients and gases. The combined effort results in the degradation of substrates and unwanted molecules, as well as adaptation to and survival of the environmental stresses.

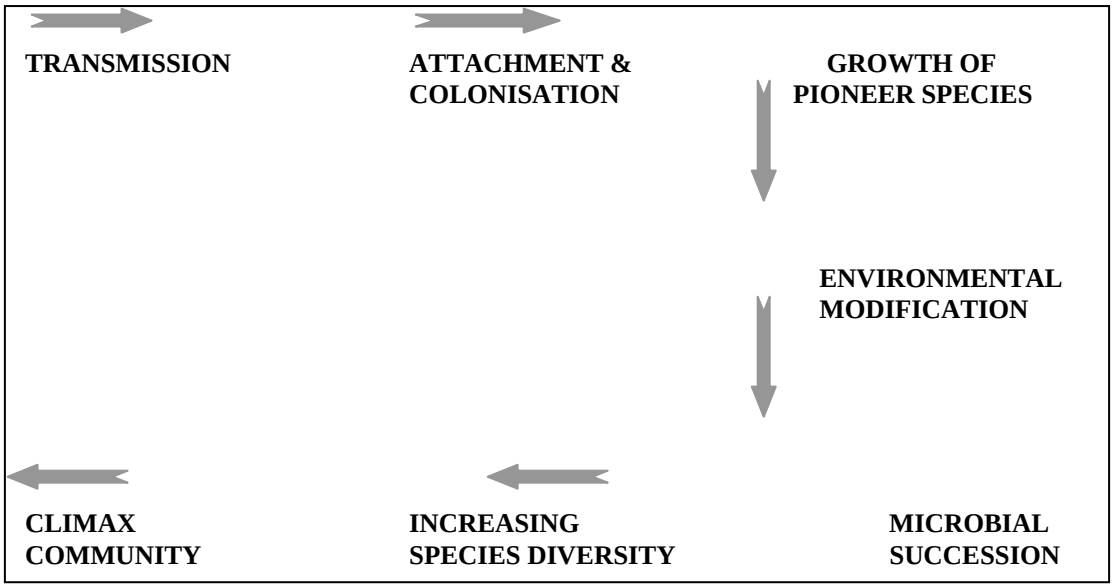


Fig. 12. Ecological stages in the development of microbial communities (Marsh and Bowden 2000).

1.4.5 MIXED SPECIES IN ANAEROBIC ENVIRONMENTS

Anaerobic bacteria in methanogenic environments have mutualistic interactions between fermenting, acetogenic and methanogenic bacteria caused by inter-species hydrogen and formate transfer (Zellner *et al*., 1997). Man-made methanogenic habitats are high-rate anoxic bioreactors where organic compounds are removed in a relatively short period of time. Examples of these high rate reactors are UASB's and fluidised bed reactors that incorporate the syntrophic relationship between fermentative bacteria, acetogenic bacteria and methanogens (Liu *et al*., 2002).

In methanogenic environments, organic compounds are degraded in the absence of inorganic electron acceptors (oxygen, nitrate, sulphur, etc.). This means that only fermentation and respiration processes with protons or bicarbonate as electron acceptors are possible. Organic compounds are completely mineralized to carbon dioxide and methane (Lange and Birgitte, 2001).

Methanogenic bacteria are limited in the substrates which they are able to degrade. These substrates include H_2/CO_2, formate, acetate, methanol, ethanol, isopropanol, methylated amines, methylated sulphur compounds and pyruvate. Some methanogens can only use one substrate at a time, whilst others are somewhat more versatile. Due to this restricted

metabolism of methanogens, organic compounds are degraded by associations of fermenting, acetogenic and methanogenic bacteria (Lange and Birgitte, 2001). Complex organic molecules such as butyrate, propionate and ethanol are fermented to acetate that is degraded to methane and carbon dioxide by mesophilic and thermophilic *Methanosarcina* and *Methanothrix* species.

The need for product removal results in an obligately syntrophic (Sekiguchi *et al*., 2001) growth of acetogenic and methanogenic bacteria, which occurs at a minimum of what is thermodynamically possible. *Methanosarcina* and *Methanothrix* species differ in both physiology and morphology. *Methanosarcina* sp. show fastest growth at high acetate concentrations and are easily enriched at high acetate concentrations. However, *Methanothrix* has a higher affinity for acetate and are most abundant in environments with a low acetate concentration, e.g. in methanogenic bioreactors.

During interspecies electron transfer, hydrogen and formate have to diffuse from the producing to the consuming organism. The flux of hydrogen and formate is directly dependent on the surface area of the producing bacterium, the diffusion constant of hydrogen and formate, the concentration difference between the producing and the consuming organism, and the distance between the organisms. The diffusion distance between producing and consuming organisms is largely determined by the biomass density of the clustering of cells. The maximum difference in concentration between the producing and the consuming bacterium is determined by the thermodynamical borders of the conversion carried out by these organisms. These thermodynamical borders refer to non-growing conditions; if the organisms have to conserve metabolic energy for growth, the difference in concentration is smaller.

1.4.6 ROLE OF EPS

EPS is the key components for the aggregation of microorganisms in biofilms, flocs and sludge. They are composed of polysaccharides, proteins, nucleic acids, lipids and other biological macromolecules that are excreted outside the cell. EPS provide a highly hydrated gel matrix in which microbial cells can establish stable biofilms. Cohesion and adhesion as well as morphology, structure, biological function and other properties such as mechanical

stability, diffusion, sorption and optical properties of microbial aggregates are determined by the EPS matrix. The EPS will vary in yield, composition and properties in response to environmental conditions and nutrient availability (Moore *et al*., 2000). The composition of the EPS varies with the species present and environmental conditions, and results in a variety of biopolymers with different structures and charges (Fig. 13). The polyanionic and hydrated nature of the EPS means they can act as an ion exchange matrix (Wimpenny *et al*., 2000).

The protection of biofilm organisms against biocides is also attributed to EPS for two reasons: 1) the antimicrobial agents must diffuse through the EPS matrix before it is in contact with the microbes; and 2) biofilm microbes grow at a slower rate, minimizing the intake of anti-microbials by the microbes (Donlan, 2001).

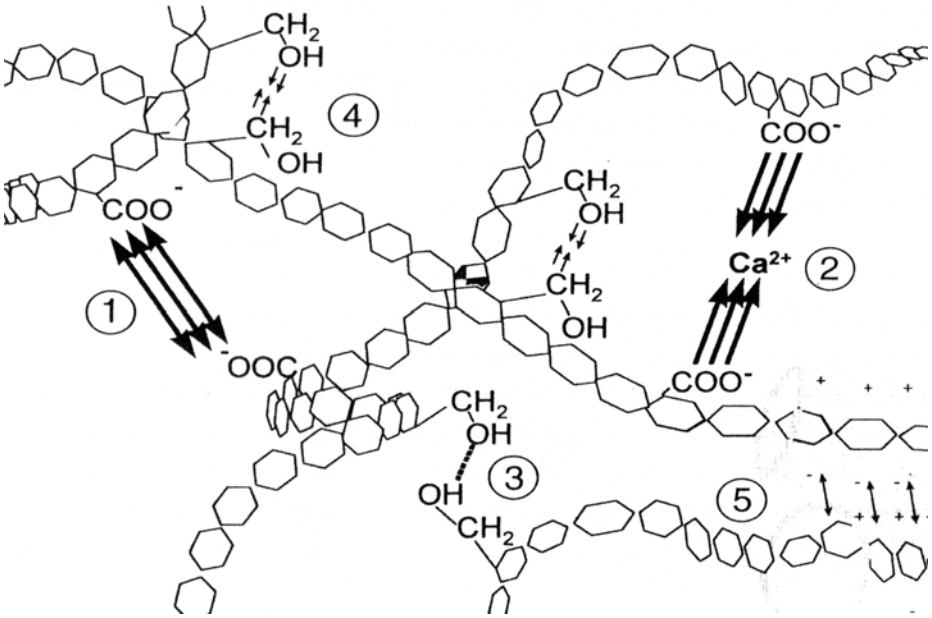


Fig.13. Interactions between EPS molecules: 1, Repulsion of two carboxylic groups; 2, attraction of two carboxylic groups by a divalent cation; 3, hydrogen bond; 4, electrostatic interaction; 5, dispersion forces (Flemming *et al*., 2000).

The EPS matrix allows phase separation in biofiltration and is also important for the degradation of particulate material which is of great importance for the purification processes in surface waters and for waste water treatment. Biofiltration refers to the separation of immobilised microorganisms and the treated wastewater that flows past them (Cohen, 2000). Treatment systems where the microorganisms are attached to a surface

allow for a higher metabolic activity; the slimy EPS traps the particulate matter and concentrates it around the biofilm. Both the trickle filter system and the RBC are examples of biofiltration.

The extracellular polymers bridge the bacterial cells together and bind granules. The composition of the wastewater influences granule formation and upflow systems also favour their creation. The granule size increases until there is a nutrient limitation in the centre of the granule. Once this happens, the core of the granule begins to decay, making them susceptible to shear forces. This in turn lessens the density and settling abilities causing washouts. These reactions happen until a dynamic equilibrium is reached within the granule (Morgenroth *et al*., 1997).

The activated sludge process is based on the formation of flocs containing microbial populations. Biosorption is described as the transfer of organic matter from the wastewater to activated sludge followed by a retention time in the floc (Guellil *et al*., 2001). It is a preliminary step to the hydrolysis of colloids by the microbes with the final assimilation in the microbes. Biosorption occurs due to electrostatic or hydrophobic interactions, depending on the wastewater content. The effectiveness of these systems is subject to good solid and liquid separation in a clarifier, which in turn depends on the biological and physiochemical properties of the floc. The floc is a heterogenous combination of flocs and filamentous microorganisms, as well as organic and metabolic excretions (Stams, 1994). These microbial cells produce the extracellular polymeric substances which lead to floc formation by agglomeration of microbes. EPS provides a large enough surface area per unit volume for microorganism binding and have a significant influence on activated sludge floc settling. The EPS coating of the floc may provide sites of attraction for some organic and inorganic compounds. Population dynamics, floc former/filament competition and substrate concentration often affect the EPS production in the micro-environment of the bulk liquid in the reactor, influencing the sludge settleability and the wastewater quality (Sponza, 2002). When EPS is not involved, there is little flocculation as the floc-forming bacteria are unable to interact with each other.

1.4.7 FUNGAL AND YEAST BIOFILMS

Very little attention has been paid to fungal and yeast biofilms and most investigations focused on their role in the medical environment. There is an increase in infections related to implants, endotracheal tubes, pace-makers and various types of catheters and other medically related surfaces that are colonised by *Candida sp.* These devices offer the surfaces required for biofilm formation (Ramage *et al.,* 2001). Of specific concern is that the sessile cells are phenotypically different from their planktonic counterparts, with increased resistance to antimicrobial agents and protection from host defences (Velazquez *et al.,* 2001). Biofilm formation by *Candida albicans* is characterised by the initial adherence of yeast cells, followed by germination and microcolony formation, filamentation, monolayer development, proliferation and maturation. The mature biofilm has the typical biofilm structure which consists of a dense network of yeast cells and hyphal elements.

Fungi in biofilm matrixes are due to spore deposition and hyphae found in suspension (Doggett, 2000). Initial spore attachment is obtained in two ways, i.e. passive attachment, when spores are entrapped or adhered to surfaces with the aid of appendages; or active attachment when the spore is stimulated into the production of adhesive mucilage (Jones, 1994). Once a spore has attached itself to a surface and conditions are favourable, the spore germinates with germ tubes enveloped by a hyphal sheath. The germ tube extends to form a hypha, thus securing their attachment for the colonisation of the substratum.

Until recently, it was unclear as to how yeasts were able to attach themselves to surfaces. Studies with *Saccharomyces cerevisiae* found that the gene *FLO11* is used as a target for both the MAP kinase and cAMP pathways (Rupp *et al.,* 1999). These pathways are necessary for the transformation of round yeast cells to form multicellular, invasive pseudohyphae. *FLO11* is responsible for the cell surface glycoprotein and can be found in the cell wall for cell-cell adhesion and for the integrity of pseudohyphal filaments (Reynolds and Fink, 2001). In *C. albicans,* the gene *INT1* has been linked to the virulence factor of the yeast (Stephenson, 2001). The gene encodes the surface protein Int1p and plays a role in both attachment and filamentous growth of the fungus (Asleson *et al*., 2000).

Fungi and yeast play an important role in the biodegradation of material and are readily found in natural biofilm communities (Elvers *et al*., 1998). Some work has been done using

support materials to grow pure culture fungal biofilms to be used in carbon degradation (Lhomme and Roux, 1992). Once a support is present for use by the fungus, e.g. *Rhizopus arrhizus*, it was better able to degrade the carbon medium. The yeasts *C. krusei*, *S. chevalierie* and *S. rouxii* have been used to successfully degrade olive mill wastewater. This wastewater contains large amounts of reducing sugars and organic acids that were both readily degraded by the yeasts when aerated in shaker flasks (Gharsallah, 1993). The most common interaction between yeast and bacteria has been seen in the oral cavity between *C. albicans* and oral streptococci (Millsap *et al*., 1998). Silicone rubber voice prostheses are rapidly colonised by bacteria, mainly streptococci, and a variety of yeast species and it is thought that the bacteria act as stimuli for the attachment of yeast species.

In a recent study using an aerobic jet-looped activated sludge reactor for the degradation of winery wastewater, yeast-like fungi were found in the liquid and biofilm. *Trichosporon capitatum* and *Geotrichum peniculatum* could be seen in their hyphal forms that formed communities with other microbes such as *Pseudomonas*, metazoa microbes and the yeast *S. cerevisiae* (Petruccioli *et al*., 2002).

A thorough knowledge of the structure and function of microbial communities and biofilms is required to understand how these communities interact and function within a treatment system. There are a number of classical techniques for monitoring biofilm communities, as well as more sophisticated processes that provide in-depth knowledge into the intricacies of microbial associations.

1.5 TECHNIQUES FOR STUDYING BIOFILMS

There are various analytical and investigative methods that are used to study species composition, architecture and functional properties within biofilms. Our knowledge of microbial diversity in complex, mixed biofilm communities has greatly increased due to the techniques currently available. We are now able to apply these methods for advanced mathematical modelling and numerical simulation of biological, chemical and physical processes in biofilms and bioreactors. Biofilms in bioreactors are composed of a diverse number of cells that is the result of the wastewater influent, environmental factors such as pH and temperature, as well as the reactor conditions. Other important factors include the mean solids retention time and hydraulic retention time distribution, oxygen and wastewater concentrations. To fully understand wastewater treatment processes, it is necessary to bridge the gap between science and engineering. The following sections will describe some of the methods available for studying biofilm communities.

1.5.1 CHEMICAL AND PHYSICAL TECHNIQUES

The most simple techniques vary from direct microscopic counting of cells with the use of dyes such as acridine orange or the use of the 'most probable number' (MPN) method to give an estimate of the amount of coliforms present in the wastewater (Yuan and Blackall, 2002). The agar plate technique is an old approach for isolating pure cultures, followed by physiological characterisation of the isolates. The microorganisms are classified and identified according to colony morphology, colour, cell size, staining reactions, etc. Although selective agar media can be used, this is unsuitable for the exact quantification of microorganisms (Amann *et al*., 1998). When isolating various species form a mixed community, it is possible to use specific antibiotics to isolate only yeast (streptomycin inhibits bacterial growth) or bacteria (nystatin inhibits fungal growth).

Microsensors are used to study the chemical concentration gradients and microbial activity in biofilms. These devices can measure a number of substances including O_2, CH_4, CO_2, H_2S, etc. Needle-shaped sensors with a tip size of 1 – 20 μm are used in amperometric sensors to measure the currents based on the oxidation and reduction of a substrate. Electric potential in biofilms is measured by the charge separation in potentiometric sensors.

Microsensors are small enough not to disturb the biofilm structure, and still measure the concentration gradients directly. For example, it may be necessary to determine the distribution of ammonia and nitrite oxidisers, but without information regarding the concentration of NO_2^-, NO_3^- and NH_4^+, no substantial conclusions can be derived.

1.5.2 MICROSCOPY AND IMAGE ANALYSIS

Pederson devices are designed for the study of undisturbed biofilms that form on microscope glass slides or coupons under controlled conditions (Cloete and Jacobs, 2001). This device contains a number of glass slides or coupons that can be removed and replaced as necessary. Wastewater is allowed to flow freely over these surface areas causing biofilms to form directly onto the slides, which are removed and stained for microscope analyses.

Simple light microscopy can be used to visualise a particular problem such as sludge bulking, where filamentous organisms that cause this phenomenon can be seen. However, when monitoring the biofilm community for intricate processes such as nitrification, it cannot be done with a simple light microscope. The most used microscopy is the confocal scanning laser microscopy (CSLM) that allows one to look at the sample in its natural hydrated state. The mode of action involves the excitation of a fluorophore (e.g. acridine orange, DAPI or other fluorescent dyes that are able to bind to the nucleic acids inside the cell) by a focused laser beam. Using image processing, all two dimensional images can be stacked to give a three dimensional picture (Wimpenny *et al*., 2000).

The use of CSLM and microsensors has revealed pores and channels in the biofilm that allow convective transport. CSLM combined with FISH (see Section 1.5.3) is an excellent approach to investigate microbial community structure and architecture. CLSM is especially useful when looking at thick samples and there are software packages available that allow for three-dimensional reconstructions of biofilm structures.

1.5.3 MOLECULAR METHODS

Numerous molecular methods are currently available to study individual and mixed microbial communities within biofilms, and more are developed as improved technologies

become available. The efficacy of these methods depends largely on the quality of the reagents and optics, as well as the technical skills of the investigator. Novel approaches enable researchers to discover the secrets held within these dynamic microcosms.

16S and 25S rDNA

It is possible to extract DNA from microorganisms and identify them using the Genebank database (www.ncbi.nlm.gov) (Dahllöf, 2002). There are more than 15 000 16S rDNA sequences that can be used to identify microorganisms by using phylum and even subclass specific probes. The 16S and 23S rDNA genes code for the rRNA molecules required for protein synthesis. They are approximately 1500 to 3000 base pairs long and contain both highly variable and conserved regions. By using specific primers, it is possible to amplify specific DNA regions using the polymerase chain reaction (PCR).

It is possible to extract DNA from environmental samples without the need to culture microorganisms (Stapleton *et al*., 1998). PCR is highly sensitive and can detect as few as ten copies of the DNA in a complex mixture if the DNA is 'clean' (Power *et al*., 1998). The sequences can be cloned and maintained in *Escherichia coli* to serve as host to obtain multiple copies of the DNA (Wilderer *et al*., 2002). Based on the 16S rDNA sequences, phylogenetic trees are compiled and used to classify bacteria using their 16S rDNA. Similarly, 18S rRNA sequences can be used for the identification of yeast and fungi using the methods mentioned above (Cappa and Cocconcelli, 2001).

Random Fragment Length Polymorphisms (RFLP's)

RFLP's can be used to identify individual strains within species by comparing the length of randomly amplified bacterial 16S rDNA or 18S rDNA fragments (Moeseneder *et al*, 1999). RFLP's make use of high-resolution poly-acrylamide gel electrophoresis to separate the fluorescently labelled DNA fragments for size determination. The fragment pattern of isolates from a mixed community can be compared to that of known species to identify specific strains. Alternatively, the technique can be used to identify similar strains within a community (comparing patterns from pure isolates).

DNA/RNA Fingerprinting

DNA/RNA fingerprinting methods use denaturing gradient gel electrophoresis (DGGE) to investigate microbial communities, which is an alternative to the 16S rRNA gene libraries. It is also based on the amplification of specific nucleic acid fragments that do not exceed 500bp. DNA fragments of equal length are separated in DGGE polyacrylamide gels based on their different sequences (Giraffa and Neviani, 2001). This produces unique gel bands dependent on the 16S rRNA genes, and is useful when working with microbial communities. DGGE is used to study the microbial community complexity, to observe population shifts and to follow expression of relevant genes in the environment. This is also a powerful technique to show microbial diversity in a wastewater community. Gel bands of specific size can be removed, eluted and then cloned to be used as FISH probes. Similarly, single-strand conformation polymorphisms (SSCP) with 400bp fragments are used to fingerprint regions of the 16S rRNA gene. SSCP is based on the differential intra-molecular folding of single-stranded DNA secondary structure (Giraffa and Neviani, 2001).

Dot-Blot Hybridisation

Dot-blot hybridisation is used for the quantification of specific rRNA sequences. Radioactively labelled probes are used to measure the relative abundance of a specific rRNA compared to the total rRNA pool measured by a universal probe. This enables many samples to be measured in a short time, but due to the instability of rRNA, exact measurements are rare (Amann *et al*., 1998).

Fluorescent *In Situ* Hybridisation (FISH)

FISH is used to quantitatively target rRNA present in the active cells for which the oligo probe has been designed. Specific FISH rRNA-targeted oligonucleotide probes can be used to verify the presence of specific microorganisms *in situ*. There are probes available for the detection of bacteria, as well as yeast and fungi (Moter and Göbel, 2000). To target the active members in the community, rRNA (for organism identification) and mRNA (for the detection of gene expression) is used instead of DNA (present extracellularly and in inactive and dead cells).

There are four main steps in the FISH protocol, namely the fixation of the sample, hybridisation, washing steps to remove any excess probes, and detection of the labelled

probe by microscopy or flow cytometry (Amann *et al*., 2001). Probes are between 15 and 30 nucleotides long and can be designed from sequences available in the Genbank database. The 5'-end is covalently linked to a single fluorescent dye molecule called a fluorophor. By using different fluorophors, two or more microorganisms may be detected at different excitation and emission maxima. These fluorescent probes are safer than radioactive labelled probes, do not require additional detection steps and offer better resolution.

Cell-Specific Antibodies

Cell-specific antibodies can be used to detect specific cells within a biofilm community. The immunological detection and measurement of enzymes specific for pollutant degradation in protein extracts from samples, can provide information as to the protein abundance and therefore the expression levels of a specific group of microorganisms (Power *et al*., 1998).

1.5.4 MATHEMATICAL MODELLING

There is an abundance of literature discussing mathematical modelling of biofilm structure and function. These models use a system of algebraic or differential equations to get an insight into the 'laws' of biofilm existence. The steps used in the model development include identification of crucial quantities and their interactions; defining the numerical algorithms and the corresponding code to simulate those most crucial; identification and calibration of the model parameters with initial and/or boundary conditions; and validation of the model with experimental data. Environmental bioreactors have been claimed to be the hardest field to simulate (Wilderer *et al*., 2002). There are many interacting parameters to consider, including physical (fluid dynamics, convective transport and detachment), chemical (metabolism and reactions), and biological parameters (communities and competition/cooperation within them). There is also a range of scales with respect to time and space, and different scales may require different mathematical methods for their description.

1.6 CONCLUSION

Water must be seen as a running cost in any industry, and should be used optimally to minimize purchase costs, and so reduce the product cost, to increase the profit margin. Industries have to pay attention to any new processes that may increase the concentration of biodegradable material in the wastewater. To execute efficient systems of ecological engineering, it is necessary for engineers to follow a holistic approach in their efforts to find new systems or retrofit existing systems (Guterstam and Etnier, 1996). Various treatment processes can be used to recycle and reuse valuable water that has both commercial and environmental benefits. As the worldwide demand for water and environmental awareness grow, recycling and conservation of water will become an absolute necessity.

The COD of winery wastewater is largely determined by the biodegradable organic content, whereas the organic acids contribute to the low pH. There are many aerobic and anaerobic biological treatment systems available for the degradation of winery effluent. In general, these treatment systems tend to be efficient and environmentally friendly, but each has its specific requirements and levels of efficacy. The common feature of these systems is their dependence on microorganisms, whether they are free flowing, aggregated into flocs or immobilised microbial biofilms.

There is no one single technique that can provide all the information required for a comprehensive understanding of the diversity within a microbial community or biofilm. It is beneficial to focus on the biofilm community structure and function within a treatment system to help explain the dynamics of the total community. By using the various techniques available, our understanding of the community structure and function will lead to the improvement of treatment system design, performance and management. It will also reveal how microorganisms adapt to their environment in a particular plant design and operation.

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CHAPTER 2

RESEARCH RESULTS

Evaluating a Rotating Biological Contactor for Winery Wastewater Treatment

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EVALUATING A ROTATING BIOLOGICAL CONTACTOR FOR WINERY WASTEWATER TREATMENT

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Abstract – Winery wastewaters are characterised by large seasonal fluctuations in volume and composition and are often discarded with little or no treatment. During the harvest season, the wastewater has a considerably high COD and low pH that does not adhere to the legal limits set by regulatory bodies. Sugars and ethanol present in the wastewater are the largest contributors to the high COD, whereas various organic acids contribute to the low pH. A Rotating Biological Contactor (RBC) was evaluated for the treatment of winery wastewaters using a natural occurring microbial community. Extensive biofilms developed on the RBC discs and contained a number of yeast and bacterial species that displayed a dynamic population shift during the evaluation period. The RBC was able to reduce the COD of winery wastewater by an average of 41% when operating at a retention time of approximately 1 hour.

Key words – Rotating Biological Contactor, biofilms, winery wastewater

2.1 INTRODUCTION

Wineries, distilleries and other grape processing industries annually generate large volumes of wastewater. This mainly originates from various washing operations during the crushing and pressing of grapes, as well as rinsing of fermentation tanks, barrels and other equipment or surfaces (Petruccioli *et al.,* 2000*)*. In general, regulatory bodies dictate that wastewater should have a pH of 5.5 to 7.5 and the chemical oxygen demand (COD) should not exceed 75 mg/l (South African Water Act no. 36, 1998) before discharge to the environment. However, winery wastewater typically has a pH of 3 to 4, with a COD of 800 to 12 800 mg/l (Petruccioli *et al.,* 2002). In general, wineries in urban areas channel the wastewater to local sewage treatment facilities resulting in heavy penalties due to the acid pH and high COD. In contrast, rural wineries have very little or no treatment operations for wastewater, which is usually discarded into reservoirs, streams or rivulets, or irrigated onto grass fields. Any of these operations can result in environmental pollution and contamination of underground water resources.

Several criteria should be considered when deciding on a treatment system for winery wastewater. These include an eco-friendly process that is flexible enough to handle various concentration loads and characteristics; low capital and operating costs; the system should require minimal personal attention and not occupy too much land, and the desired degree of degradation should be achieved without a need for dilution with water. A number of biological systems have been evaluated for winery wastewaters, such as anaerobic digesters (Daffonchio *et al*., 1998) and activated sludge reactors (Petruccioli *et al.,* 2002), which are very efficient in COD removal, but require long retention times.

A number of biological reactors systems have been developed for the treatment of industrial wastewaters, including various aerobic and anaerobic systems. It is interesting to note that previously 'hard to degrade' wastewaters are now being successfully degraded by

various yeasts and fungal species. For example, pyrene degradation has been obtained with the use of *Mucor racemosus* and *Phialophora alba* (Ravelet *et al.,* 2000) and a host of other micromycetes, whereas biphenyl is degraded with the ascomycetous yeast *Debaromyces vanrijiae* (Lange *et al.,* 1998). Biodegradation of phenol-containing wastewaters was achieved using *Trichosporon cutaneum* (Godjevargova *et al.,* 1998) and even *Candida nitrativorans* (Bastos *et al.,* 1997). Silage wastewater is an extremely recalcitrant pollutant; it is highly acidic and causes rapid deoxygenation of water. Several yeast strains isolated from silage wastewater were evaluated for the degradation of the wastewater and it was found that *C. utilis* and the filamentous yeast *Galactomyces geotrichum* were successful (Arnold *et al.,* 2000). Highly concentrated wastewater from the food industry has also been treated with *C. utilis* in a batch or continuous stirred reactor (Elmaleh *et al.,* 1999).

The Rotating Biological Contactor (RBC) is potentially suitable for the biological treatment of winery wastewater. The system is based on a microbial biofilm that develops on the surface of discs mounted onto a horizontal shaft with at least 40% of the discs submerged in the wastewater (Mba *et al*., 1999). Rotation of the shaft results in alternating contact of the discs with the wastewater and air that allows for the aerobic growth of the microorganisms on the surface of the discs (Nahid *et al.*, 2001). Various operating parameters can be controlled, such as disc rotation speed, recirculation (Gupta and Gupta, 1999) and hydraulic retention time (Costley and Wallis, 2001). RBC's are easy to operate, has a short start-up time, requires little maintenance and is easily oxygenated with little sloughing of biomass.

Biofilms can be defined as complex structures consisting of cells and cellular products, including extracellular polymers, which either form large, dense granules or grow attached on a static solid surface (static biofilms) or on suspended carriers (particle supported biofilms) (Nicolella *et al.,* 2000). Some of the advantages of biofilm reactors are their compact design and the degree of treatment is less dependent on final sludge separation. The system uses a

multi-species biofilm where organisms with the highest growth rates are found on the outside of the biofilm, and slower growing organisms are found on the inside. The slow growers are protected from shear forces and are therefore less likely to be lost through detachment and being washed out.

The efficacy of biological treatment systems often depends on the ability of the microorganisms to form biofilm communities that are able to degrade the organic compounds present in the wastewater. Microorganisms such as yeast and other fungi generally associated with grapes will most likely also be present in winery wastewater and could be useful in the degradation of the organic matter in the wastewater. The objectives of this study were therefore to evaluate a biofilm reactor for the biological treatment of winery wastewater and to investigate to what extent yeast species are incorporated into the biofilm.

2.2 METHODS AND MATERIALS

Chemical analysis of winery wastewater

Wastewater samples were obtained from different locations at wineries in the Stellenbosch region of South Africa. All solid particles were removed by centrifugation (5 min at 10 000 rev/min). The wastewater was filter-sterilized using a 0.22 μm filter (Osmonics) and the type and relative concentration of esters and higher alcohols in the samples were determined by gas chromatography. High performance liquid chromatography (HPLC) was used to measure the levels of monosaccharides and organic acids and the COD was determined with the Thermoreaktor TR300 and Spectroquant Nova 60 (Merck). Based on the chemical composition, a synthetic wastewater was devised to represent the major components present in winery wastewater (Table 1). Pure solutions of most of the constituents were also prepared and analysed for their respective contribution to the COD.

Table 1. Chemical composition of synthetic wastewater and the COD contributions of the respective constituents

Components	Concentration in synthetic wastewater	COD contribution of pure solution (mg/l)
Synthetic effluent		**8 800**
Glucose	1.8 g/l	2100
Fructose	1.8 g/l	2000
Citric acid	1m g/l	1
Tartaric acid	2m g/l	4
Malic acid	2 mg/l	2
Lactic acid	2 mg/l	3
Propanol	1.24 mg/l	4
Butanol	1 mg/l	4
i-Amyl alcohol	3,8 mg/l	11
Acetic acid	250 mg/l	260
Ethanol	10 mg/l	300
Ethyl acetate	4 mg/l	
Propionic acid	8 mg/l	
Valeric acid	1 mg/l	
Hexanoic acid	0.5 mg/l	
Octanoic acid	0.7 mg/l	
YNB	1.7 g/l	
NH_4SO_4	5 g/l	

pH adjusted to 4.0 with NaOH

Isolation and enumeration of micro-organisms present in the winery wastewater

A dilution series of the winery wastewater was prepared in FSO solution (0.85% NaCl) and plated onto plate count agar (PCA) plates containing 8.625 ml/l Nystatin (Velázquez *et al.,* 2000), and onto malt extract agar (MEA) plates containing 20 mg/l Streptomycin. The plates were incubated at 30°C for 48 hours and screened for bacterial growth on the PCA-nystatin plates or for fungal growth on the MEA-streptomycin plates. Pure cultures were isolated on the respective plates and preliminary identification of the isolates was done using standard morphological and physiological tests (Harley *et al.,* 1993). Further identification of yeast isolates was done by the Central Buro voor Schimmelcultures (Delft, The Netherlands).

Biofilm formation by natural occurring microorganisms in winery wastewater

Holders with microscope slides were immersed in winery wastewater at different sampling points, i.e. inside a collecting tank after pressing, inside a settling tank and at the outflow from the settling tank. The biofilms on the microscope slides were stained with acridine orange (Sigma-Aldrich) and viewed under an epifluorescent microscope (Nikon Eclipse E400).

Evaluation of biofilm communities and pure isolates to degrade synthetic wastewater

The biofilms were aseptically scraped from the microscope slides and 1 ml of the respective mixed communities was inoculated into 100 ml aliquots of synthetic wastewater. These cultures were incubated aerobically (shaking at 150 rpm) at 30°C for 24, 48 or 120 hours, after which the cells were removed by centrifugation (5 min at 10 000 rev/min) and the supernatant filter sterilized for COD analysis.

The previously isolated pure yeast isolates were inoculated into 10 ml synthetic wastewater at 2 x 10^4 cells/ml, and the bacterial isolates were inoculated to a final OD_{600} of 0.1. The cultures were incubated on a rotary wheel at 30°C and the COD and pH were determined after 24, 48 and 120 hours. Isolates that reduced the COD of the synthetic wastewater were evaluated further in 250 ml flasks containing 100 ml synthetic wastewater and incubated at 30°C under either aerobic conditions (shaking at 150 rpm) or with minimum aeration (non-shaking cultures sealed with parafilm). Samples were taken after 24, 48 and 120 hours; the cells were removed by centrifugation and the supernatant filter sterilized for COD analysis.

Design and evaluation of RBC

A laboratory-scale RBC was designed using a stainless steel trough with a capacity of 4.2 l (Fig. 1) with 16 polystyrene discs (20 cm in diameter) rotating at approximately 6 rev/min. Different substrates were evaluated as potential wastewater to be used in the laboratory scale RBC, i.e. grape juice, dry white wine and semi-sweet wine diluted to 10% (v/v). Flasks containing 100 ml of the filter-sterilised substrate was inoculated with 2 ml of a mixed community biofilm and incubated at 30°C shaking at 120 rev/min; the COD was measured after 24 and 36 hours.

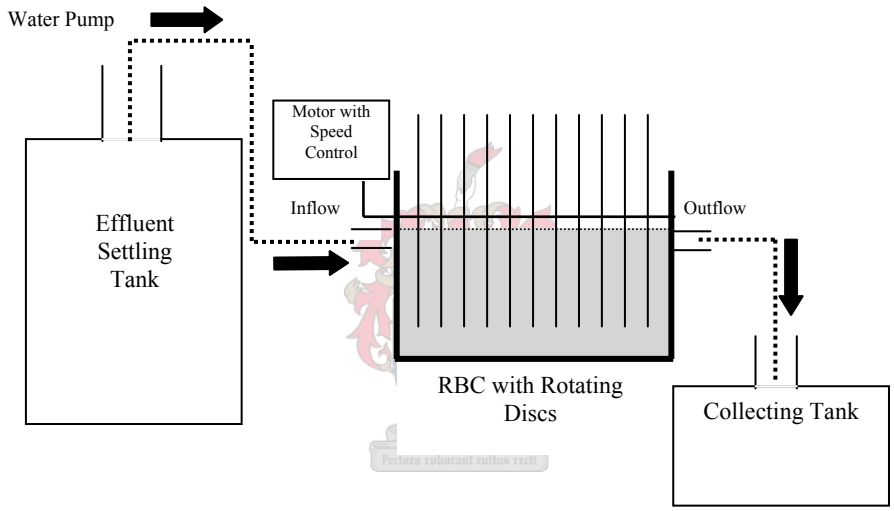

Fig. 1. Schematic representation of the set-up for the small-scale evaluation of the RBC.

A laboratory evaluation of the RBC was subsequently done using a 10% (v/v) solution of grape juice as the substrate, inoculated with a mixed biofilm community. The COD of the inflow and outflow wastewater was measured regularly, while the retention time varied between 58 hours and 6.4 hours. The RBC was subsequently evaluated on-site at a winery during the 2001-harvest. Winery wastewater was pumped into the RBC after the excess grape skins and seeds had been removed and the system operated at hydraulic retention times varying between 0.35 and 1.4 hours.

Genomic DNA isolation

Biofilm sludge from the RBC discs was collected at various time intervals, squeezed dry in a Mira cloth and frozen with liquid nitrogen. A portion of the frozen biofilm biomass was broken off and ground with a mortal and pestle together with a heaped spatula of sterilised sand and liquid nitrogen. Approximately 100 µl was mixed with 1 ml Extraction Buffer (100 mM Tris-HCl, (pH 8), 100 mM EDTA, 100 mM Na_2HPO_4, 1.5 M NaCl, 1% CTAB) and 200 µl glass beads (425-600 µm). Gram-positive cells were broken with three freeze-thaw cycles (-70°C/+65°C); followed by the addition of 200 µl TE Buffer and 200 µl PCI were added and the samples were vortexed for 3 min to break the fungal cells. The samples were centrifuged for 5 min at 13 000 rev/min. Five µl 20 mg/ml proteinase K and 10 µl RNAse A were added to the supernatant and incubated at 37°C for 30 min, shaking at 225 rev/min. To this, 150 µl 20% SDS was added and the mixture was incubated at 65°C for 2 hours with gentle mixing every 20 min. The mixture was centrifuged at 13 000 rev/min for 10 min and standard procedures (Ausubel *et al*., 1994) were used for chloroform/iso-amylalcohol extraction and precipitation of genomic DNA. The DNA pellet was dissolved in 150 µl TE and the genomic DNA was purified using the High Pure PCR Template Preparation Kit (Roche Molecular Biochemicals).

Concurrently, a dilution series of the mixed biofilm community was plated onto PCA-Nystatin and MEA-Streptomycin plates, incubated at 30°C for 48 hours and screened for bacterial or fungal growth on the respective plates. Pure cultures were isolated from the respective plates and subjected to genomic DNA isolation using the DNA isolation kit for cells and tissues (Roche Molecular Biochemicals).

PCR amplification and RFLP's

Random Fragment Length Polymorphism Analysis (RFLP) of the ribosomal DNA was used to determine the dominant isolates in the biofilm as well as population shifts within the biofilm over time. Universal primers for either the bacterial 16S rDNA (5'CCG GAT CCG TCG ACG TGC CAG CXG CCG CGG TAA3' and 5'CCA AGC TTC TAG ACG GXT ACC TTG TTA CGACTT3') or yeast 18S rDNA (nu-SSU-0817-5' ATT GCA ATG C($^{C}_{T}$)C TAT CCG CA and nu-SSU-1536-3' TTA GCA TGG AAT AAT ($^{A}_{G}$)($^{A}_{G}$)A ATA GGA) (Borneman and Hartin, 2000) were used for PCR amplification with TaKaRa Ex Taq (TaKaRa Biomedicals) using standard procedures. Amplification of the yeast rDNA was done with denaturing (95°C for 5 min), 35 cycles of annealing (94°C, 30 sec), elongation (58°C for 1 min) and denaturing (72°C for 90 sec), followed by a final denaturing step of 72°C for 5 min. A slightly different programme was used for the amplification of the bacteria rDNA: denaturing (94°C for 1 min), 25 cycles of annealing (94°C for 30 sec), elongation (55°C for 30 sec) and denaturing (72°C for 90 sec), followed by a final denaturing step (72°C for 2 min). The PCR products were digested with *Alu*I, *Hae*III, *Hpa*II, *Msp*I, *Taq*I or *Sau*3A (Roche Molecular Biochemicals) and separated on a 1.2% agarose gel.

Identification of dominant yeast species

The universal PCR primers were also used for the identification of the yeast isolates and to establish the phylogenetic relationship between them. The amplified yeast rDNA fragments from the pure yeast isolates dominating in the mixed biofilm were cloned using the p-GEM®-T Easy Vector System (Promega). The DNA sequences of the fragments were determined using standard procedures and analysed with NCBI Blast software (www.ncbi.nlm.nih.gov).

2.3 RESULTS AND DISCUSSION

Chemical composition of winery wastewater

Analysis of the winery wastewater at various point in the treatment process at a local winery suggested that the existing treatment operations are not effective to decrease the COD (Table 2); the COD of the outflow from an anaerobic stainless steel settling tank was significantly higher than the COD of the inflow.

Table 2. COD of winery wastewater at different stages of existing treatment operations

Day		Collection tank	Settling tank - inflow	Settling tank - outflow
1	**COD**	3220	3030	6315
	pH	3.78	3.04	4.72
7	**COD**	2075	1830	4435
	pH	5.12	4.99	4.83
14	**COD**	1690	1500	3795
	pH	6.95	10.21	4.83
35	**COD**	3580	3655	4795
	pH	5.19	4.60	4.76

The chemical analyses of winery wastewater (Table 3) indicated a large variation in COD, pH and chemical composition. The wastewater generated by the destemming and pressing operations contained higher concentrations of glucose, fructose and malic acid, which originated from the grape berries themselves. The considerable variation in the chemical composition can be ascribed to different varieties of grapes, harvest load, operation procedures, etc.

Table 3. Chemical composition of winery wastewater samples taken from various wineries[a]

	1	2	3	4	5	6	7	8	9
COD[b]	**4000**	**1600**	**1280**	**5120**	**5760**	**4800**	**2880**	**5280**	**320**
pH	4.23	4.50	4.35	nd	nd	4.86	3.93	3.98	5.17
Maltose (g/l)	0	0	0	0	2.22	0	0	0	0
Glucose (g/l)	1.80	0	0.92	1.60	1.47	1.17	0	1.12	0
Fructose (g/l)	1.53	0.73	0.47	1.16	1.25	1.19	0	0.94	0.49
Citric Acid	0.170	0.190	0	0	0.598	0.218	0	0	0
Tartaric Acid	0.218	0	0.123	0.346	1.34	0.324	1.66	0.276	0
Malic Acid	0.110	0	0	0	0	0	0	0	0
Lactic Acid	0	0	0	0	0	0.150	0.129	0	0
Total polyphenols	8.5	0	0	5.1	27.2	25.6	0	5.1	0
Ethyl Acetate	2.81	1.89	10.7	2.82	20.3	42.6	2.03	3.28	2.47
Metanol	0	0	0	0	0	0	0	0	15.0
Ethyl butyrate	0	0	0	0	0.433	0	0	0	0
Propanol	0.403	0.369	0	0.648	1.47	1.68	0	1.01	0
i-Butanol	0.773	0.329	0	0.308	1.35	0.997	0.148	0.606	0
i-Amyl Acetate	0	0	0	0	0.391	0	0	0	0
n-Butanol	0	0	0	0	0.180	0.822	0	0.360	0
i-Amyl Alcohol	1.37	1.41	0.150	1.74	4.72	3.45	0.5	3.05	0.561
Hexanol	0	0	0	0.320	0	0	0	0.416	0
Acetic Acid	239	27.2	70.3	26.5	132	663	491	78.5	0
Propionic Acid	7.01	1.46	6.19	2.83	15.9	67.1	4.02	13.6	0
i-Butyric Acid	0.595	0	0.181	0.223	0.817	1.65	0.496	1.09	0
n-Butyric Acid	4.27	1.69	0.478	2.18	7.23	66.8	2.58	8.76	0.493
Diethyl Succinate	0.535	0.140	0.146	0.164	0.579	1.36	0.945	0.354	0
n-Valeric Acid	0.747	0.314	0.137	0.762	1.16	8.11	0.164	3.54	0
Hexanoic Acid	0.571	0.324	0.244	0.355	0.242	4.78	0.185	1.48	0.134
2-Phenylethanol	3.24	3.54	3.33	3.26	3.49	0.4	3.28	0.446	4.50
Octanoic Acid	1.04	0.249	0.501	0.196	0.258	0.944	0.572	1.77	0.158
Decanoic Acid	5.0	0.769	2.52	0.850	1.12	3.64	3.45	7.04	0.583

[a] The different locations represent the outflow from (1) pressing operations; (2, 3) washing of crusher and press; (4) inflow and (5) outflow from a holding tank; (6) settling tank and (7-9) combined outflow from different wineries.

[b] All concentrations are given as mg/l unless indicated otherwise.

nd, not determined.

Zero values for Hexylacetate, Ethyl-lactate, 2-Phenyl-ethylacetate, *i*-Valeric Acid

The availability of wastewater with known and invariable characteristics is crucial for experimental purposes when comparing different variables, such as the effect of hydraulic retention times. The development of a synthetic wastewater (Table 1) provided a defined substrate with known composition that could be used for comparative analysis of different treatment variables. The COD of representing concentrations of the various synthetic

effluent constituents indicated that the fermentable sugars (glucose and fructose) contributed almost half of the COD. Ethanol and acetic acid contributed to a lesser extent to the COD.

Biodegradation of winery wastewater by natural occurring biofilm communities and pure isolates

If a biofilm-based treatment system is to be considered for the treatment of winery wastewater, it is necessary to determine whether the natural occurring microorganisms are able to produce biofilms while reducing the COD of the wastewater. Microscopic analysis of the slides suspended in wastewater streams showed thick biofilms on the surface of the slides, containing a large number of yeast and filamentous cells (Fig. 2). It was reported that filamentous microorganisms play an important role in biofilms as they maintain the yeast concentrations within their intertwined mycelial-like structures which also act as a backbone for yeast and bacterial attachment (Madoni *et al.*, 2000).

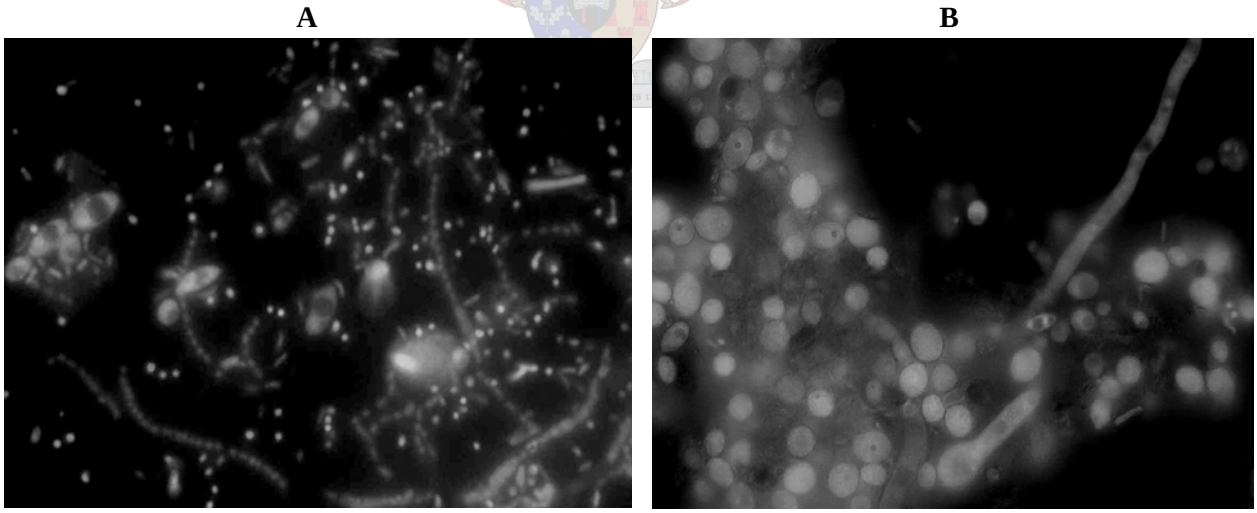

Fig. 2. Microscope photographs showing (A) mixture of yeast and bacteria, and (B) pseudohyphal growth of yeast.

Under aerobic conditions, the mixed biofilm communities isolated from the microscope slides reduced the COD of the synthetic wastewater by varying degrees (Fig. 3). The biofilm

community isolated from Settling Tank C was the most effective, reducing the COD by 62% within 72 hours. These results suggest that the natural occurring microorganisms were able to form a stable biofilm and also reduce the COD, i.e. utilise organic compounds that are susceptible to oxidation.

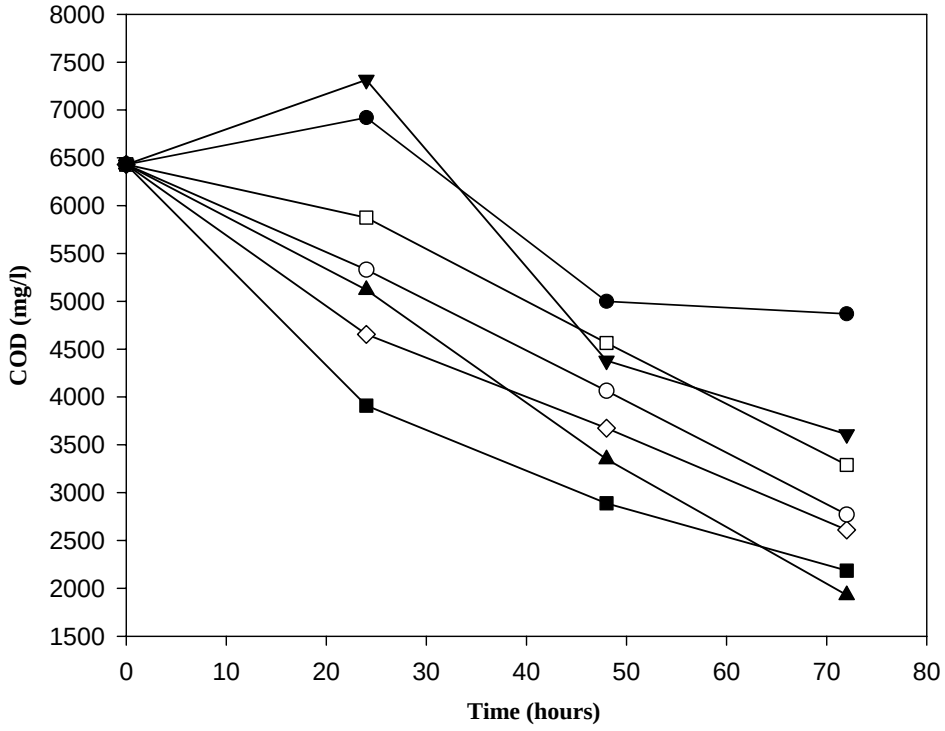

Fig. 3. Effect of mixed biofilm populations isolated from different locations on the COD of synthetic wastewater: (■) settling tank A; (◊) collecting tank A; (□) collecting tank B; (○) settling tank C; (▲) settling tank C; (▼) collecting tank C, and (●) control.

Plate counts of the various bacterial and yeast isolates indicated 4 x 10^3 CFU yeast cells and 1.64 x 10^6 CFU bacterial cells. Preliminary identification of the isolates indicated ten bacterial isolates and ten yeast isolates (Table 4). When evaluated in synthetic wastewater, the bacterial isolates were ineffective with only two isolates showing a decrease in COD after 48 hours. The yeast isolates were more effective in reducing the COD of the wastewater, e.g. MEA5 reduced the COD by 77% after 48 hours. Yeast isolates that best degraded the

synthetic wastewater were identified by the CBS as *Pichia rhodanensis* (MEA1), *Kloeckera apiculata* (MEA4), *Candida krusei* (MEA5) and *Saccharomyces cerevisiae* (MEA9).

Table 4. Gram reaction and cell morphology of pure isolates and their effect on the COD of synthetic wastewater

Organism	Preliminary Identification	COD of synthetic wastewater	
		0 hours	48 hours
		10 300	
PCA1	Bacterial, Gram– bacilli		11 680
PCA2	Bacterial, Gram– bacilli		nd
PCA3	Bacterial, Gram+ cocci		11 250
PCA4	Bacterial, Gram– bacilli		9 310
PCA5	Bacterial, Gram+ cocci		10 565
PCA6	Bacterial, Gram+ cocci		10 660
PCA7	Bacterial, Gram+ cocci		11 105
PCA8	Bacterial, Gram+ cocci		11 070
PCA9	Bacterial, Gram– bacilli		nd
PCA10	Bacterial, Gram– bacilli		5 900
		11 500	
MEA1*	Yeast, oval/rod		nd
MEA2	Yeast, oval/rod		3 855
MEA3	Yeast, rods		2 600
MEA4*	Yeast, irregular		5 925
MEA5*	Yeast, oval/rods		2 710
MEA6	Yeast, rods		4 305
MEA7	Yeast, rods		7 775
MEA8	Yeast, rods		nd
MEA9*	Yeast, irregular		3 430
MEA10	Yeast, oval		nd

*Identified by CBS as *Pichia rhodanensis* (MEA1), *Kloeckera apiculata* (MEA4), *Candida krusei* (MEA5) and *Saccharomyces cerevisiae* (MEA9).

nd, not determined

The yeast isolates MEA4, MEA5 and MEA9 were evaluated further in synthetic wastewater under aerated and non-aerated conditions (Table 5). Under aerated conditions, isolate MEA5 was able to reduce the COD by 95% within 24 hours. MEA4 and MEA9 were also able to reduce the COD by 19% and 38%, respectively after 24 hours, and by 46% and

69%, respectively after 48 hours under aerated conditions. The yeast isolates were less effective under non-aerated conditions, although more than 60% of the COD was reduced after 120 hours by all three isolates.

Table 5. Effect of pure yeast isolates on the COD of synthetic wastewater under aerated and non-aerated conditions

Time	**No inoculum**		**MEA4**		**MEA5**		**MEA9**	
(hours)	Aerated	Non-Aerated	Aerated	Non-Aerated	Aerated	Non-Aerated	Aerated	Non-Aerated
0	5200	4850						
24	5120	4800	1760	3600	240	2640	1760	2800
48	4160	4160	800	3120	24	2160	400	800
120	4480	4640	640	1600	1120	1760	640	1440

Laboratory and on-site evaluation

Although the synthetic wastewater represented the chemical composition of winery wastewater to some extent, we also evaluated diluted grape juice and white wine as a possible source of large volumes of wastewater for the laboratory evaluation of the RBC. A dilution of 10% (v/v) grape juice (free of preservatives and added sugars) proved to be very effective (Table 6), whereas the semi-sweet and dry white wines, treated with SO_2, resulted in less satisfactory results.

Table 6. Evaluation of different substrates for RBC using a biofilm community as inoculum

Substrates	COD		
	0 hours	24 hours (% Degradation)	36 hours (% Degradation)
Grape Juice			
No inoculum	16 740	18 624	24 050
Biofilm		11 620 (37.6%)	9 780 (59.5%)
Late Harvest			
No inoculum	19 000	22 450	27 550
Biofilm		20 460 (8.8%)	16 240 (41%)
Dry White			
No inoculum	18 450	20 330	27 950
Biofilm		16 020 (21%)	14 420 (48%)

Laboratory-scale evaluations of the RBC using 10% (v/v) grape juice and the mixed biofilm from Settling Tank C were done to determine the utility of the RBC for the treatment of winery wastewater. The results (Table 7) showed a reduction in the COD of the outflow that improved with a longer retention time. However, with a retention time of only 7 hours, a 44% decrease in the COD of the outflow was still observed.

Table 7. Laboratory scale evaluation of COD reduction in RBC with 10% (v/v) grape juice

Retention Time (hours)	Inflow COD	Outflow COD	% Degradation
58	8165	2890	65%
32	6800	3090	55%
17	11380	3995	65%
15.5	8410	4030	52%
13.7	9405	3725	60%
10	8800	4900	44%
7	9600	5402	44%
6.4	10450	6400	39%

Evaluation of the RBC on-site at a local winery indicated an average decrease of 41% in the COD of the outflow relative to the inflow with a retention time of approx. 1 hour (Fig. 4). Furthermore, the pH was increased by an average of 0.74 pH units. Sloughing of the biofilm occurred around Day 19, resulting in inadequate COD reduction towards Day 22. Weak degradation was also observed when the retention time was too short (e.g. Day 23 a retention time of only 0.6 hours was used). The RBC, therefore, proved to be an effective treatment method for the reduction of COD and acidity of winery wastewaters. The results also suggest that the configuration used in this study allowed sufficient aeration for the biofilms, but the rotation speed should not be too high to avoid excessive sloughing.

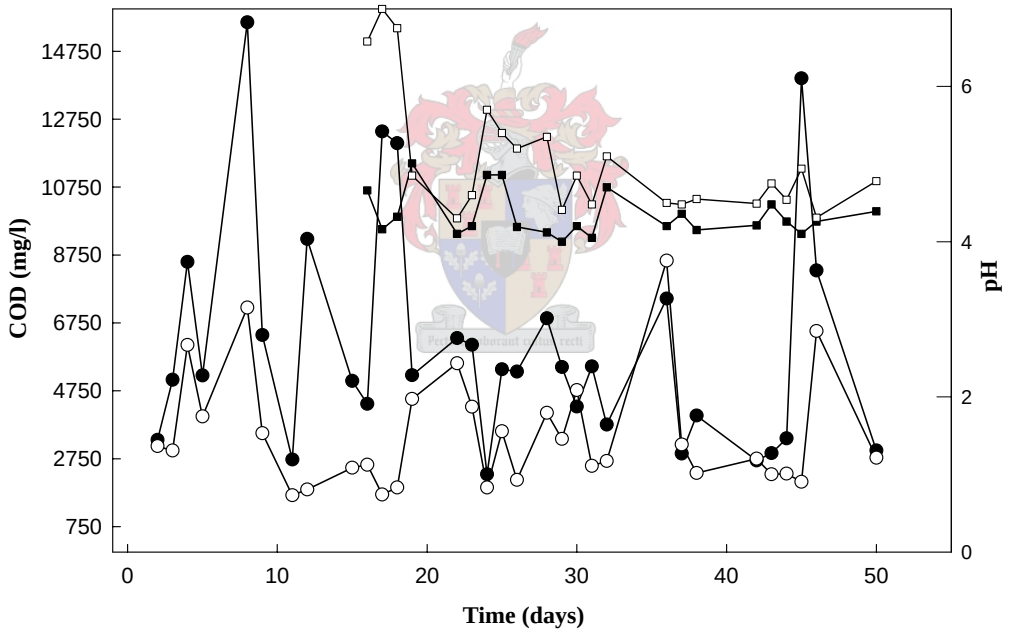

Fig. 4. COD values for RBC evaluated on-site with fresh winery wastewater and natural occurring biofilm population. (●) COD of inflow; (○) of outflow, (■) pH of inflow, and (□), pH of outflow.

Population shifts

Since biofilm communities are dynamic and thus constantly changing to adapt to their environment, it was important to determine whether there were dominant species in the

biofilm throughout the evaluation period. Three time intervals were used to determine a shift in the population within the biofilm. Plate counts of the mixed biofilm population indicated 4×10^6 CFU yeast and 2×10^7 CFU bacteria for Day 1; 7.2×10^7 CFU yeast and 2.4×10^9 CFU bacteria for Day 42; and 4.7×10^5 CFU yeast and 1.84×10^6 CFU bacteria for Day 63. After Day 45, very little grapes were processed at the winery and, therefore, a limited amount of fresh nutrients and organisms were introduced into the RBC, resulting in a decrease in the population numbers.

The RFLP analysis (Fig. 5) also indicated a shift in the microbial population from Day 1 to Day 63; notable changes in banding patterns (indicated by white boxes) were observed for the different time intervals for both bacteria and yeast.

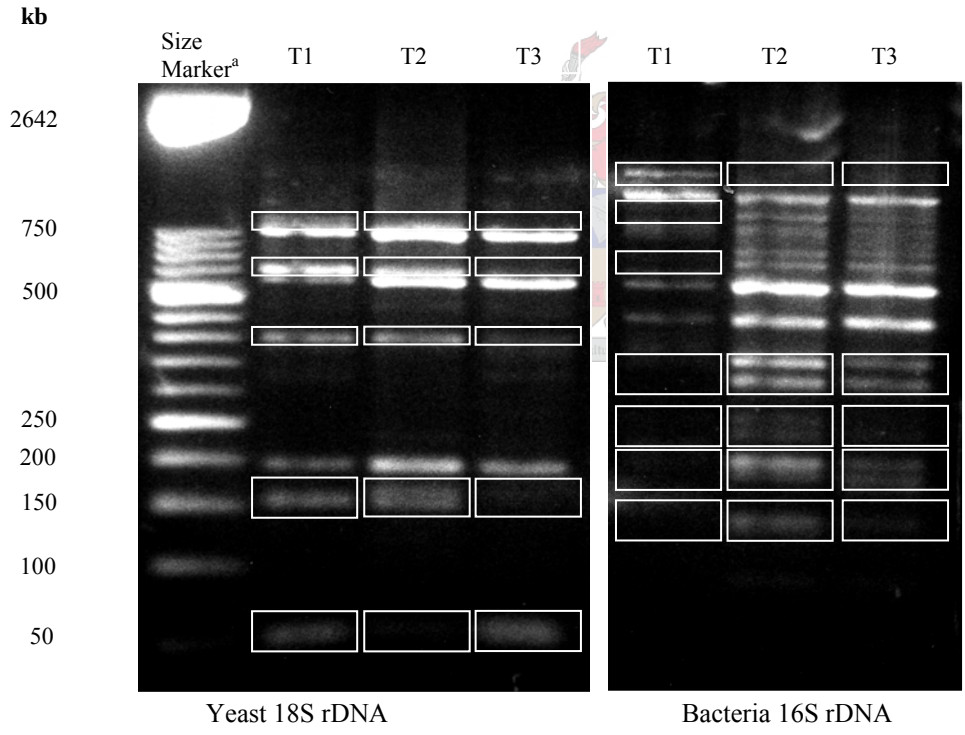

Fig. 5. RFLPs on genomic DNA isolated from the biofilm at different time intervals: T1, Day 1; T2, Day 49; and T3, Day 63. Internal fragments of the yeast 18S and bacterial 16S rDNA were PCR amplified using universal primers and the products were digested with TaqI for analysis by gel electrophoresis. (Results for other restriction digests are not shown).
[a] Molecular Size Marker XIII (Roche Molecular Biochemicals)

The most dominant yeast isolates identified with the rDNA sequences (Fig. 6) were *S. cerevisiae, C. intermedia, Hanseniaspora uvarum* and *P. membranifaciens*. All these species are naturally associated with grapes and/or water. With the exception of *H. uvarum*, all the yeast isolates are able to form either simple or elaborate pseudohyphae.

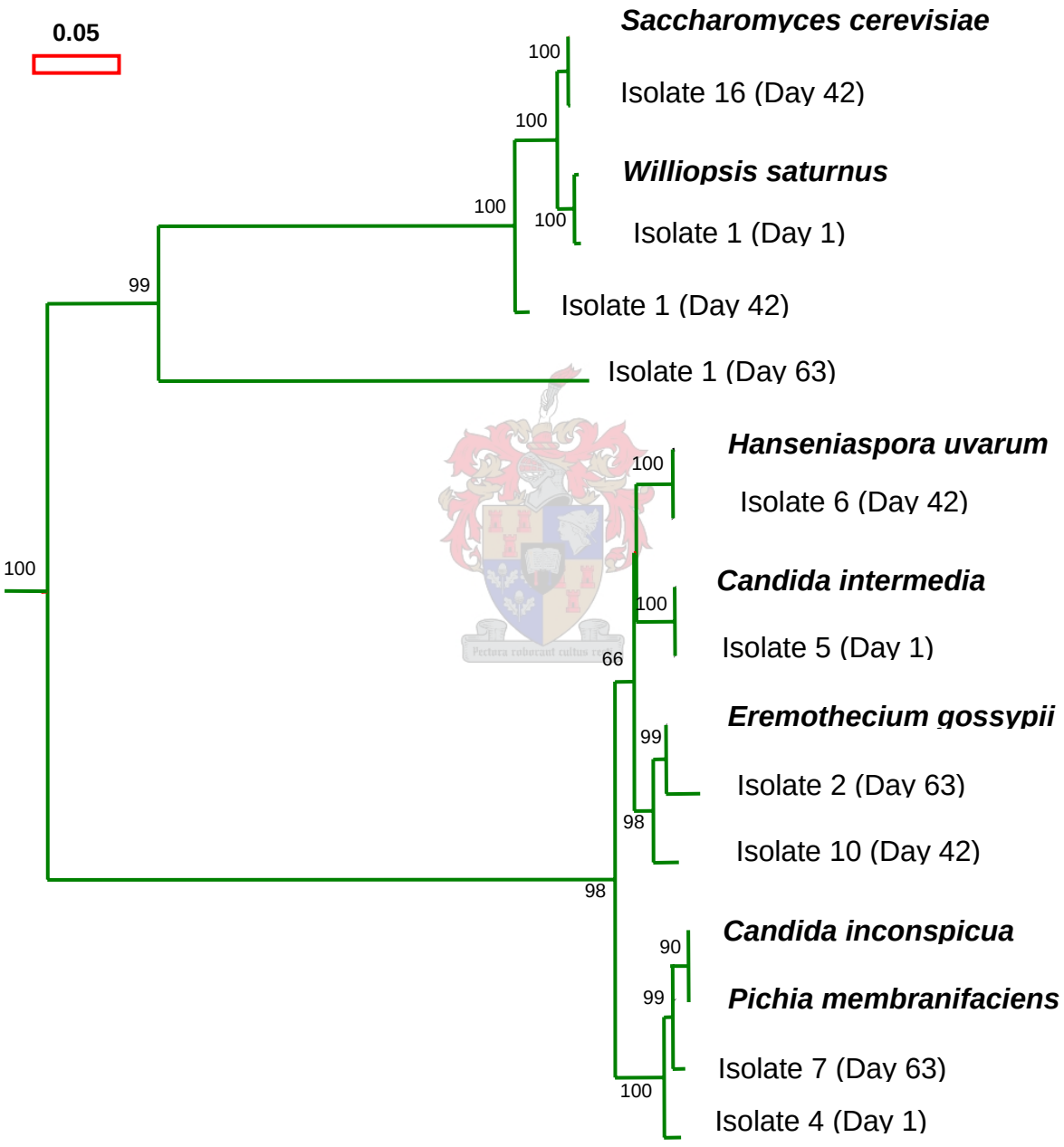

Fig. 6. Phylogenetic tree of yeast isolates from the mixed biofilm.

2.4 CONCLUSION

The chemical analyses of winery wastewater indicated that the relatively high concentration of sugars contribute largely to a high COD, whereas the organic acids play a more prominent role in the acidity of the wastewater. The RBC was an effective biological system for lowering the COD of winery wastewaters, providing sufficient aeration for the biofilm, requiring low maintenance and being cost-effective. The efficiency of the RBC (41% reduction in COD) is perhaps not comparable to that obtained with anaerobic digesters (Daffonchio *et al*., 1998) or activated sludge reactors (Petruccioli *et al.,* 2002), but in view of the very short retention time of 1 hour, the RBC could provide an effective system to address the peaks of high COD and acidity experienced during the harvest season.

Given the seasonal fluctuations in wastewaters discarded by wineries, the RBC could, therefore, be an effective primary treatment system to lower the COD to more acceptable levels for treatment by constructed wetlands or other biological, chemical or physiochemical processes. Noteworthy is the large number of yeast isolates within the biofilms that formed under the conditions associated with winery wastewaters. Although bacterial biofilms have been well represented, very little information is available on the role and dynamics of yeasts within biofilms. This study identified changes in the biofilm composition suggested that the yeast population in the biofilm was dynamic and able to adapt to their changing environment.

In view of these and other emerging information on the efficacy of yeast for the treatment of industrial wastewater, it is important to better understand the function and dynamics of yeast biofilms under industrial conditions. Future research will, therefore, focus on the yeast isolates found in the winery effluent, their survival and relative concentrations in the RBC biofilm under industrial conditions, as well as fundamental information regarding their role in the biofilm.

2.5 ACKNOWLEDGEMENTS

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