Isolation and Characterisation of Lipolytic Bacteria and Investigation of their ability to Degrade Fats, Oils and Grease in Grain Distillery Wastewater

by
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DECLARATION

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ABSTRACT

The large volumes of effluent water generated by distillery industries is an issue of great concern as it contains pollutants that must be treated according to environmental legislation. It has been reported that grain distillery wastewater (GDWW) is high in fats, oils and greases (FOG) that can be reduced by treating with suitable microorganisms. The objective of this study was to investigate the biodegradability of FOG in GDWW. This was done by isolating lipolytic bacteria from soil, which was situated close to the GDWW treatment plant at a distillery in Wellington, South Africa. These isolates were screened for lipolytic activity on various fat substrates. Secondly, the most desirable isolates were subjected to batch biodegradation trials using GDWW as substrate and tested for their ability to biodegrade FOG. Each of the four isolates, *Pseudomonas fluorescens (1), Pseudomonas luteola (2), Stenotrophomonas maltophilia (3)* and *Bacillus licheniformis (4)* were screened on three types of media: Difco™ Spirit Blue Agar with Tributyrin (SBA-Tri); Victoria Blue B Agar with Cotton Seed Oil (VBB-CSO); and Victoria Blue B Agar with GDWW (VBB-GDWW) at different temperatures (25°C, 30°C, 37°C and 50°C) to determine optimal enzyme activity for lipolysis. Lipolysis was taken as positive when growth of dark blue colonies was formed or by the formation of a clear zone around the colony. Lipolysis was observed at all the aforementioned temperatures for *P. fluorescens*, *P. luteola* and *S. maltophilia*. *Bacillus licheniformis* failed to show any lipolytic activity at 50°C on the SBA-Tri. A decrease in lipolytic (clear) zone was observed at an increase in temperature from 25°C to 37°C for *P. fluorescens*. When VBB-GDWW was used as lipid substrate, isolates failed to indicate any clear zone of lipolysis, however, growth was present for all isolates in the form of a dark blue zone around colonies, which were also positive for lipolytic activity.

Three lipolytic bacteria (*P. luteola, S. maltophilia,* and *B. licheniformis*) isolated from the above study were subjected to GDWW of various FOG concentrations (70 – 211 $mg.L^{-1}$). These isolates were allowed to acclimatise to GDWW during a batch biodegradation period (18 – 21 d) at 37°C. *Bacillus licheniformis* showed the highest FOG reduction of 83% after 18 d exposure. All the strains showed that an initial acclimatisation phase improved the biodegradation of the FOG. A fatty acid profile was obtained for each batch biodegradation trial after the acclimatisation phase. It was found that these strains either biodegraded the fatty acids (FAs) or, as in the case of *P. luteola,* formed myristic and pentadecyclic acids from free FAs. The formation of FAs may have occurred through a

process of inter-esterification. It was also found that certain precursors such as palmitoleic acid might be formed under aerobic or anaerobic conditions.

In this study it was shown that biodegradation of FOG can be improved by an initial acclimatisation period. Single cultures with the desirable properties can be used to lower the FOG in GDWW and need not be used in mixed cultures that could produce inhibitory components that would otherwise upset the biodegradation activity of isolates present. *Bacillus licheniformis* could be used as a FOG-degrading isolate during the treatment of wastewaters high in FOG. However, future studies should focus on bioaugmenting the FOG degrading bacteria from this study with other strains to monitor its activity and ensure survival and activity in larger scale studies.

UITTREKSEL

Die groot volumes afloopwater wat opgelewer word deur die distilleer-industrie is ‘n kwessie wat groot kommer wek aangesien dit groot hoeveelhede besoedelende stowwe bevat. Daarom moet dit, volgens omgewingsverwante wetgewing, behandel word. Daar is voorheen gerapporteer dat graandistillerings-afloopwater (GDAW) hoog is in vette, olies en ghries (VOG) en dat hierdie VOG verminder kan word deur die GDAW te behandel met toepaslike mikroörganismes. Die oorhoofse doelstelling van hierdie studie was om die bio-afbreekbaarheid van die VOG in GDAW te ondersoek. Dit is eerstens gedoen deur lipolitiese bakterieë uit grond wat naby ‘n graandistillerings-aanleg (Wellington, Suid-Afrika) geleë is, te isoleer. Verskeie vetsubstrate is gebruik om hierdie isolate vir lipolitiese aktiwiteit te toets. Tweedens is die verkose isolate getoets vir lipolitiese aktiwiteit deur gebruik te maak van lot-bio-afbreekbaarheidsmetode. Tydens hierdie metode is GDAW as substraat gebruik en die verskillende bakterieë se vermoë om VOG af te breek is getoets. Om die optimale ensiemaktiwiteit vir lipolise van elk van die vier isolate nl. *Pseudomonas fluorescens (1), Pseudomonas luteola (2), Stenotrophomonas maltophilia (3)* en *Bacillus licheniformis (4)*, vas te stel, is elk getoets op drie verkillende media: “Difco™ Spirit Blue Agar” met Tributirien (SBA-Tri); “Victoria Blue B Agar” met Katoensaadolie (VBB-KSO); en “Victoria Blue B Agar” met GDAW (VBB-GDAW) teen verskillende temperature (25°C, 30°C, 37°C en 50°C). Indien donker-blou kolonies gevorm is of ‘n deursigbare sone rondom ‘n kolonie waargeneem is, is lipolise as “positief” beskou. Lipolise is waargeneem teen alle voorafgenoemde temperature vir *P. fluorescens*, *P. luteola* en *S. maltophilia*. *Bacillus licheniformis* het nie lipolitiese aktiwiteit getoon teen 50°C op SBA-Tri. ‘n Afname in die deursigbare sone is waargeneem teenoor ‘n toename in temperatuur vanaf 25°C tot 37°C vir *P. fluorescens*. In die geval van VBB-GDAW as lipiedsubstraat, het isolate geen deursigbare sone vir lipolise getoon nie. Daar was egter ‘n donker-blou sone rondom kolonies teenwoordig, wat ook positief is vir lipolitiese aktiwiteit.

Drie lipolitiese bakterieë (*P. luteola, S. maltophilia,* and *B. licheniformis*) is geïsoleer uit bogenoemde studie en is aan inkubasie in GDAW teen verksillende VOG-konsentrasies (70 – 211 $mg.L^{-1}$) blootgestel. Hierdie isolate is toegelaat om te akklimatiseer tot die GDAW tydens ‘n lot-bio-afbreekbaarheidstydperk (18 – 21 d) teen 37°C. *Bacillus licheniformis* het die hoogste VOG-afname van 83% na 18 d blootstelling getoon. Alle bakterieë het getoon dat ‘n aanvanklike akklimatiserings-tydperk die bio-afbreekbaarheid van die VOG verbeter. ‘n Vetsuur-profiel is verkry vir elk van die lot-bio-

afbreekbaarheidstoetse na die akklimatiserings-fase. Daar is bevind dat hierdie bakterieë óf die vetsure afgebreek het óf, soos in die geval van *P. luteola,* miristiese en pentadesikliese sure, vanaf vry-vetsure, gevorm het. Die vorming van vetsure is moontlik as gevolg van die proses van inter-esterifikasie. Dit is verder bevind dat sekere voorlopers, soos palmitoë-oleïensuur, gevorm kan word onder aërobies of anaërobiese toestande.

In hierdie studie is getoon dan die bio-afbreekbaarheid van VOG verbeter kan word deur ‘n aanvanklike akklimatiserings-tydperk toe te pas. Enkel-kulture met die verkose eienskappe kan gebruik word om die VOG in GDAW te verminder. Gemengde kulture, wat inhiberende komponente produseer wat moontlik die bio-afbreekbaarheids proses negatief kan beïnvloed, hoef dus nie gebruik te word nie. *Bacillus licheniformis* kan gebruik word as ‘n VOG-afbrekende isolaat tydens die behandeling van afloopwater wat hoog in VOG is. Verdere studies moet egter fokus op die samevoeging van VOG-afbrekende bakterieë vanuit hierdie studie asook ander bakterieë om die aktiwiteit daarvan te monitor en sodoende oorlewing en aktiwteit op ‘n groter skaal te verseker.

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CONTENTS

Language and style used in this thesis are in accordance with the requirements of the *International Journal of Food Science and Technology*. This thesis represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters has, therefore, been unavoidable.

CHAPTER 1

INTRODUCTION

In South Africa there are many factors which influence the availability for water. These factors, amongst others include climate, economic growth as well as living standards (Adewumi *et al*., 2010). The scarcity of water in South Africa brings about a challenge in providing the general population with a basic amount of water and sanitation. South Africa has an annual rainfall of approximately 500 mm, which is below the world average of 860 mm (Friedrich *et al*., 2009). The west coast experiences the most aridity with many of the regions showing total annual rainfalls of 70 mm or less (Olivier & Rautenbach, 2002). The country has an unreliable rainfall pattern (Kahinda *et al*., 2007) and for this reason it may be necessary to investigate the potential for developing treatment methods that will ensure water supplies that are more reliable in terms of availability and potability.

The South African distillery industry contributes an important role in the South African economy. Statistics indicate that production of the total wine industry averages between 900 and 1 000 million litres of wine produced between 2009 and 2010 (SAWIS, 2010). Wine production is also known to increase as the demand for wine and spirits-related beverages increases (Musee *et al*., 2007). South African distilleries are highly polluting in terms of effluent water produced. Owing to the upsurge in demand for wine and spirits-related beverages, it is inevitable that wastewater generated via the alcohol distilleries will lead to large volumes of high strength distillery wastewater being produced (Musee *et al*., 2007). In South Africa, distillery wastewater sampled from various distilleries in the Western Cape, have been found to have chemical oxygen demands (COD) of between 30 000 and 70 000 $mg.L^{-1}$ and very low pH values of between 3 and 4 (Wolmarans & Villiers, 2002; Musee *et al*., 2007).

Wine distillery wastewater (WDWW) as well as grain distillery wastewater (GDWW) is produced by a distillery in Wellington, Western Cape (Laubscher *et al*., 2001). The WDWW generated are characterised as having high CODs (10 000 – 60 000 $mg.L^{-1}$), and a very acidic pH (3.0 - 4.0). The total suspended solids (TSS) content is also very high (± 4 900 $mg.L^{-1}$) (Laubscher *et al*., 2001). Similarly, GDWW has been characterised with high COD (20 000 – 30 000 $mg.L^{-1}$) acidic pH, (3.4 – 3.5) and lower TSS (900 – 1 600 $mg.L^{-1}$) (Gie, 2007). From the above figures indicated, distilling industries will be detrimental to the environment if the wastewater is discarded into existing water reservoirs or water

catchment systems. It is therefore imperative that wastewater of this kind be treated to ensure safety of the environment and the potable water supplies available (Mohana *et al*., 2009; Sheridan *et al*., 2011).

Agriculturally based industries have made extensive use of upflow anaerobic sludge blanket (UASB) systems (Britz *et al*., 1999). The UASB system has also been considered as a feasible treatment option for seasonal fruit-processing (Sigge *et al*., 2006) and distillery wastewater (DWW) treatments owing to its high operational efficiency, cost effectiveness and low equipment maintenance (Laubscher *et al*., 2001; Uzal *et al*., 2003; Gao *et al*., 2007; Pant & Adholeya, 2007). A key factor contributing to the efficiency of the UASB system is the microbial diversity present (Yuan *et al*., 2008). Literature reports that many operational problems have been associated with the treatment of GDWW. Treatment problems amongst others include scum layer formation and encapsulation of biomass by long chain fatty acids (LCFA) (Laubscher *et al*., 2001; Gie, 2007). These operational problems lead to sludge flotation and consequent granule washout, ineffective mass transfer of nutrient assimilation, which results in an overall decrease in the anaerobic digestion process.

Microbial bioaugmentation is also a technique used by selecting microbes that will aid in the biodegradation efficiency of particular compounds present in wastewaters with a specific composition. One study evaluated the effectiveness of fats, oils and greases (FOG) biodegradability using a lipase producing *Pseudomonas* sp. strain D2D3. This strain resulted in FOG removal efficiencies of 94.5 and 94.4% for olive oil and animal fat, respectively, while safflower oil was the lowest at 62% (Shon *et al*., 2002). Another study used bacterial mixed cultures for the treatment of bakery wastewater with a high content of FOG. During a 7 day treatment period with a single strain culture, there was 73 - 88% removal efficiency (Bhumibhamon *et al*., 2002). It was also reported that better degradation efficiencies were achieved with single strain cultures and in this case, the strains were identified as members of the genera *Acinetobacter*, *Bacillus* and *Pseudomonas* (Bhumibhamon *et al*., 2002). Literature also states that certain *Bacillus* strains can produce lipolytic enzymes that are beneficial to the treatment of wastewaters high in fat and protein content (Sangeetha *et al*., 2010). In another study cultures specific for degradation of wastewater high in FOG were investigated for the efficiency in grease removal (Wakelin & Forster, 1997). This study showed that a mixed-culture of selected microbes that had been acclimatised to the wastewater conditions had a FOG removal efficiency of > 90%.

The objective of this study was to investigate the biodegradability of fats, oils and greases (FOG) in grain distillery wastewater (GDWW). This will be initiated by firstly isolating lipolytic bacteria from a grain distillery environment and screening the isolates for lipolytic or esterase activity on various fat substrates. Secondly, to subject grain distillery wastewater (GDWW), at specific FOG concentrations, to the bacterial strains previously isolated (Chapter 3 of this thesis). These included *Stenotrophomonas maltophilia (1), Pseudomonas luteola (2), and Bacillus licheniformis (3)* strains. An acclimatization phase will also be initiated in order to determine which strains are capable of biodegrading the FOG in the shortest possible time. A fatty acid profile will then be determined for the individual strains to elucidate which fatty acids are utilised. Strains that show promising FOG biodegradation results can therefore be used as a pre-treatment option prior to anaerobic digestion to try and facilitate reactor operational efficiency treating GDWW.

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CHAPTER 2

LITERATURE REVIEW

Background on Water Crisis

An issue of great concern is the scarcity of freshwater with more than one billion people in developing nations lacking access to safe drinking water (Ridoutt *et al*., 2009). For the last century, there has been continual pressure on freshwater resources and the problem is intensifying rapidly (Verstraete *et al*., 2009) owing to population growth, continuing economic development, climate change, the degradation of various ecosystems that are considered critical for human life and the anticipated requirements of new bio-fuel crops. In addition, the scarcity of freshwater is of compelling concern to the agricultural food sector, which is the dominant user of global freshwater resources with a consumption figure of approximately 85% (Ridoutt *et al*., 2009).

Approximately 85% of the African continent is comprised of large river basins shared amongst several countries (Ashton, 2002). When compared to the rest of the world, water resource distribution in Africa is exceedingly variable and water supplies are unequally distributed in both geographical extent and time. The severity of droughts and floods has increased over the past 30 years due to climate change. Large areas of the African continent have been subjected to and continue to experience a series of prolonged and extreme droughts; frequently these droughts have been “broken” or “relieved” by equally extreme flooding events (Ashton, 2002). The International Panel on Climate Change stated that within the next 20 to 30 years, 25 African countries might experience water scarcity or water stress (Anon, 1999).

Water Availability and Use in South Africa

South Africa measures approximately 1 600 km from north to south, as well as from east to west and covers an area of 1.22 million km^2. The average annual rainfall is 495 mm. This volume ranges from less than 100 mm per year in the western deserts to roughly 1 200 mm per year in the eastern part of the country. Only 35% of the country has a precipitation of 500 mm or more, while 44% has a precipitation of 200 - 500 mm and 21%

has a precipitation of less than 200 mm. Owing to these figures, 65% of the country does not receive adequate rainfall to ensure successful rain-fed crop production and is instead used as grazing land. Crops thus grown in this area are grown under irrigation (Anon., 2005).

The South African water resources are currently allocated to 19 Water Management Areas, while most of the country's water requirements arise from surface water. Approximately 320 major dams provide surface water to the country and account for a total capacity of more than 3.2 X 10^6 m^3, which is roughly 66% of the total mean annual runoff of about 4.9 X 10^3 m^3 per annum. This includes about 4.8 X 10^8 m^3 per annum draining from Lesotho into South Africa and an additional 5 X 10^8 m^3 per annum draining from Swaziland to South Africa (Anon., 2007). In 2000, the total water consumed was approximated at 12.87 x 10^9 m^3 and was divided amongst six sectors. The water consumption percentage was dominated by the agricultural sector at 62%, followed by urban, mining and industry, rural, afforestation, and power generation sectors consuming 23, 6, 4, 3 and 2%, respectively (Anon., 2005). Water is thus a limiting factor owing to issues regarding the apportioning of water resources among different sectors. It is expected that South African water resources will decline markedly in the years to follow. The reason for decline being that the ratio of runoff to rainfall is amongst the lowest of any populated region of the world (Oberholster *et al*., 2008). South Africa alone has an estimated 1.3 million hectares of irrigated land to cover both commercial and subsistence agriculture. In effect, 60% of the groundwater, surface water and recycled water is consumed via irrigated agriculture and contributes to almost 30% of total agricultural production (Yokwe, 2009).

A few of the regional issues on water use and pollution are that certain areas, particularly the northern and eastern inland and coastal regions of South Africa are dominated by irrigation. However, pollution of these water sources are as a result of mining industries and thus place pressure on the availability of high quality water (Anon., 2001). It is thus imperative that the management of South Africa's water quality and availability is essential, as predictions are that the demand for water will outstrip its supply by 2025 (Oberholster *et al*., 2008).

Pollution of Water Resources and Possible Solutions to Water Crisis

In order to face the problem one has to consider the cause of the problem. As the income levels of the population increase so do the negative environmental externalities (Blignaut &

De Wit, 2004). The former can simply be explained by the fact that more income chases more goods implying that more materials are transformed. These materials eventually find their way back to nature, but not necessarily in the desired composition, but merely in the form of an input to a landfill, which is considered waste. The waste often ends up in our water reservoirs and therefore makes the water unsafe for usage.

Waste reduction is an effective practice on a global and local scale, in order to prevent mass pollution in the air and water resources. In recent years, Japan has had a demand for the reduction and effective utilisation of food waste (Komemoto *et al*., 2009). Their current means of municipal waste treatment, including food waste, is done via incineration. The remaining ash is dumped in landfills. In Denmark, there has been a higher demand for meat than in the past. This has led to an increase in the quantity of organic by-products from slaughterhouses (Hejnfelt & Angelidaki, 2009). Producing ethanol from agricultural by-products that can be utilised as an alternative fuel, has attracted much interest on a global scale, owing to the limitation on non-renewable energy resources (Pant & Adholeya, 2007). During sugar production, the most common raw material for fermentation industries such as bakers' yeast and ethanol production is molasses. Molasses, being a by-product, has the advantage of serving as a suitable fermentable product because of its low cost and high sugar content (Liang *et al*., 2009*;* Biswas *et al*., 2009). Water catchment can be seen as a feasible strategy for water harvesting, whereby run-off water can be collected from roofs or ground surfaces (Agromisa, 1997).

Wastewater generation is an inevitable phenomenon on a global scale. Municipal and industrial waste as well as carbon dioxide emissions are increasing and thus negatively impact the environment (Blignaut & de Wit, 2004). Food industries thus also produce large amounts of waste. Examples of such companies amongst others are beer industries, slaughterhouses, dairy industries and fat refineries (Cavaleiro *et al*., 2007; Cirne *et al*., 2007). The treatment of waste for fuel production while simultaneously recycling nutrients, is considered a sustainable phase (Singh & Prerna, 2009).

The South African wine industry produces large volumes of wastewater throughout the year most of which originates from cellar cooling as well as floor and equipment washdown (Ronquest & Britz, 1999). Effluent generated from the wine industries usually have a high organic content and thus cannot be directly discharged into the environment. Various treatment options for the wastewater should be considered to allow the disposal of large amounts of distillery effluent in an environmentally friendly manner.

Minimising waste is a central aspect to any industry, as it not only reduces the consumption of potable water but also decreases the volume of wastewater generated (Melamane *et al*., 2007). Treatment options may be physical, chemical and physicochemical methods. Although water saving techniques are employed by farmers and industries (Blignaut *et al*., 2009), the minimisation of water use is a critical factor and in order to address the problem, treatment of industrial effluent water should be seen as a possible solution.

The Role of Whisky on a Global and Local Scale

Typically, whisky production takes place as follows: Firstly, barley is steeped in water, allowed to germinate, and then dried to give malt. The malt is then milled to break open the husks and mixed with water in the mash tun, where enzymatic action occurs. The sugars are then fermented by action of yeast to give wash containing 6 – 7% ethanol by volume. Batch distillation then produces a distillate, which contains 20% ethanol by volume. Further distillation takes place after which the still is matured in wooden casks, prior to dilution, bottling and sale (Goodwin *et al*., 2001). The production of grain whisky takes place via column distillation, whereby the fermented mash of maize meal (± 9% ethyl alcohol/volume), called “wort” is used as raw material to generate the wastewater referred to as grain wastewater (Laubscher *et al*., 2001). Once the grain effluent is obtained from the still, it enters into a decanting process. Here the solids are separated from the water. The solids as well as the water are collected in separate tanks. A contractor purchases the solids while the grain wastewater produced is collected via a tanker.

An issue of concern is the high Chemical Oxygen Demand (COD) values within all these wastewaters produced. Typically the wastewater generated has an acidic pH of 3 - 4, a high organic content with COD values ranging from 10 - 50 $g.L^{-1}$ (depending on the wastewater composition), and low suspended solid and nutrient concentration (Heredia *et al*., 2005). It is therefore of cardinal importance that the most efficient treatment method be initiated before subsequent disposal of water into the environment. The discharge of the distillery wastewater into the environment causes serious problems as it contains a high organic load. Comparison between grain wastewater and wine wastewater indicates that grain wastewater has a higher COD than wine wastewater (Table 1). The Total Kjeldahl nitrogen (TKN), Phosphorus (P) and total soluble solids (TSS) also vary considerably between the wastewaters before and after treatment.

Table 1. Characteristics of wine wastewater and grain wastewater (Laubscher *et al*., 2001)

Influent wastewater characteristics used in laboratory scale UASB systems				
Wastewater type	**COD (mg.L^{-1})**	**TKN (mgN.L^{-1})**	**P (mgP.L^{-1})**	**TSS (mg.L^{-1})**
Wine wastewater before treatment	~60 000	~650	~200	150 - 49 760
Wine wastewater after centrifuging	20 000 - 30 000	300 - 350	180 - 200	100 - 500
Grain wastewater after decanting and centrifuging-unsettled	25 000 - 30 000	170 - 180	270 - 300	>1 000
Grain wastewater after decanting and centrifuging and settling	20 000 - 25 000	170 - 180	270 - 300	<1 000

The effluent generated by alcohol distilleries is referred to as distillery spent wash. Whisky production generates large quantities of effluent. Normally for every litre (L) of alcohol produced, 8 - 15 L of effluent is generated (Mohana *et al*., 2009) and for every litre of grain whisky produced, 16 – 21 L of effluent water is generated (Tokuda *et al*., 1998). On average, a distillery produces approximately 2.6 billion L of grain wastewater annually. In 2002, there were 285 distilleries in India producing 2.7 x 10^9 L of alcohol, thus generating 4 x 10^{10} L of wastewater per year (Ramana *et al*., 2002). There are an estimated 319 distilleries producing 3.25 billion litres of alcohol and generating 40.4 billion litres of wastewater annually in India alone (Pant & Adholeya, 2007). According to their Ministry of Environment and Forests, alcohol distilleries are rated at the top of 'Red Category Industries' (Mohana *et al*., 2009). In the UK whisky is recognised as one of the most economically important food and beverage exports, with more than a hundred active distilleries primarily in Scotland (Goodwin *et al*., 2001).

According to Surujlal *et al.* (2004), industries producing effluents must comply too, the technologically achievable levels, before disposal of wastewater into the environment.

These technologies will not only benefit the environment, but will also be cost-effective (Mohana *et al*., 2009).

A typical South African distillery industry can produce approximately 120 000 L of grain distillery wastewater (GDWW) per month which is likely to increase over the next few years (Bester, 2009). This increase is mainly attributed to the increase in demand for spirits and spirits-related beverages. There was an increase in domestic sales of natural wine between the periods of November 2009 to October 2010 by 1.6% to 299 million litres. The export of natural wines decreased by 3.3% during the same period while both bulk and packaged wines showed a negative growth trend (SAWIS, 2010).

The concentration of components present in grain and wine wastewaters differs for various distilleries globally (Tables 1, 2, 3, 4 and 5). The representative characteristics of the distiller's grains wastewater in rural wineries situated in China are presented in Table 2. These rural wineries generate large amounts of distiller's grain wastewater, which are often discarded directly into the environment without any treatment due to little or no treatment equipment (Gao *et al*., 2007). Raw distillery effluent characteristics obtained from a plant specialising in producing neutral spirits in the Western Cape area of South Africa using wine grapes as feedstock is reported in Table 3. Table 4 shows the average composition of the raw grain distillery wastewater for 15 samples from five batches and it was found that the composition of the batches varied noticeably, owing to daily and seasonal variations. Table 5 indicates the composition of different wine distillery wastewater (WDWW) streams, during the pre- and post- ozonation treatment of a constructed wetland system. An upflow anaerobic sludge blanket (UASB) reactor treating GDWW was investigated for a period of 420 days, whereby COD removal efficiencies from 80 – 97.3% had been achieved under mesophylic conditions (Gao *et al*., 2007). The development of granules during this study showed excellent methanogenic activity by using sucrose and acetate as substrates. In another study, post-treatment of distillery wastewater using aerobic techniques further improved the COD removal from 88.7 to 96.5%. This was achieved by using aerobically activated sludge (Musee *et al*., 2007). The latter study proves that sequential treatment of distillery wastewater using UASB followed by aerobically activated sludge can reduce the COD to be in accordance with requirements for effluent discharge as stipulated according to legislation (Musee *et al*., 2007). Malt whisky distillery wastewater was sequentially (anaerobically/aerobically) treated with and without supplementation. Values obtained for COD and BOD were 99.5 and 98.1% respectively (Uzal *et al*., 2003).

Table 2. Typical composition of grain distiller's wastewater obtained from rural wineries in China (Gao *et al*., 2007)

Component	**Component range ($mg.L^{-1}$)**
COD	16 500 - 22 520
Volatile fatty acids	3 000 - 3 600
Suspended solids	250 – 770
Volatile suspended solids	190 – 640
Total nitrogen	120 – 150
Total phosphorus	15 – 18
pH	3.3 - 4.3

Table 3. Effluent characteristics from a distillery producing grape feedstock (Musee *et al*., 2007)

Component	**Component range ($mg.L^{-1}$)**
COD	35 667 - 42 183
Total dissolved solids	10 184 - 16 123
Total Kjeldahl nitrogen	560 – 834
Ammonia	80 – 120
Phosphorus	177 – 215
pH	3.5 - 4

Table 4. Average composition of the raw GDWW batches used in a study (Gie, 2007)

Component	**Component range ($mg.L^{-1}$)**
COD_{total}	20 007 – 26 069
Fats, oils and greases	1 978 – 2 324
Total solids	13 915 – 18 395
Total suspended solids	908 – 1 612
Volatile suspended solids	812 – 1 560
Phosphorus	624 – 880
pH	3.4 – 3.5

Table 5. Wine distillery wastewater composition. (Green, 2007)

Component	Component range ($mg.L^{-1}$)
COD_{total}	12 609 – 22 150
Total solids	11 680
Total suspended solids	1 430
Total Volatile suspended solids	1 300
Phosphates	254
pH	4.52 – 4.68

Legislation

Currently the Department of Water Affairs (DWA) is responsible for the National Water Act (Act 36 of 1998) as well as the Water Services Act (No. 108 of 1997), thus DWA monitors the state of pollution from water resources in South Africa (DWA, 2004). Industries have to adhere to strict regulations as laid out by the legislation concerning effluent discharge standards (Republic of South Africa, 1998a). The ordinance of a new law referred to as polluter-pays-principle has been established whereby, the polluter pays for the treatment and disposal of the waste generated (Republic of South Africa, 1998a; Republic of South Africa, 1998b).

The controlling legislation that provides protection, development and utilisation of the water resources in South Africa is the National Water Act, 1998 (Act 36 of 1998). The National Water Act states that it is of cardinal importance that effluents be purified by the user to specified standards and the subsequent disposal thereof should take place in a manner, which will allow its reuse. The minister periodically prescribes compulsory national standards relating to quality of water that is discharged into the environment. The strict limitations and tariffs prescribed are used to promote or achieve water conservation (Anon., 1997). The respective industry or individual, in control of their water use, must adhere to the measures as laid out by the legislation. These measures state that the business should comply with any prescribed waste standard or management practice; contain or prevent the movement of pollutants and effectively eliminate any source of pollution (Republic of South Africa, 1998b).

To allow irrigation of wastewater, the General Authorisation stipulates that certain criteria should be met (Tables 6, 7 and 8).

Table 6. Parameters to be met for the irrigation of 50 m^3 of wastewater (Van Schoor, 2005)

Measured parameter	**Value**
pH	6 – 9
Electrical conductivity	<200 $mS.m^{-1}$
Faecal coliforms	<100 000 $cfu.100\ mL^{-1}$
Sodium adsorption ratio (SAR)	<5
COD	<5000 $mg.L^{-1}$

Table 7. Parameters to be met for the irrigation of 500 m^3 of wastewater (Van Schoor, 2005)

Measured parameter	**Value**
pH	6 - 9
Electrical conductivity	<200 $mS.m^{-1}$
Faecal coliforms	<100 000 $cfu.100\ mL^{-1}$
Sodium adsorption ratio (SAR)	<5
COD	<400 $mg.L^{-1}$ If 400 – 5 000 $mg.L^{-1}$ present, then may not irrigate >50 m^3

Table 8. Parameters to be met for the irrigation of 2000 m^3 of wastewater (Van Schoor, 2005)

Measured parameter	Value
pH	5.5 - 9.5
Electrical conductivity	<150 $mS.m^{-1}$
Faecal coliforms	<1 000 $cfu.mL^{-1}$
Ammonia	<3 $mg.L^{-1}$
Nitrogen	<15 $mg.L^{-1}$
Chlorine	<0.25 $mg.L^{-1}$
Suspended solids (SS)	<25 $mg.L^{-1}$
Ortho-phosphate	<10 $mg.L^{-1}$
Fluoride	<1 $mg.L^{-1}$
Soaps, oils and grease	<2.5 $mg.L^{-1}$
COD	<75 $mg.L^{-1}$

Treatment Technologies for the Treatment of Distillery Wastewater

In order to overcome the many disadvantages associated with treating effluent, continual research and development has led to the improvement in treatment processes of distillery wastewater. Treatment methods include adsorption (Mane *et al*., 2006), flocculation as well as coagulation (Migo *et al*., 1997) and oxidation processes includes Fenton's oxidation (Dwyer *et al*., 2008), ozonation (Sreethawong & Shavadej, 2008), electrochemical oxidation utilising electrodes and electrolytes (Prasad & Srivastava, 2009).

Pre-treatment methods are also used to enhance the overall anaerobic digestion process through alteration of the chemical and physical properties (Carrère *et al*., 2010). These alterations contribute to enhancing the hydrolysis process prior to the main digestion process. It is a process that can be incorporated into the classical wastewater treatment plant at different locations (Fig. 1). According to Carrère *et al*. (2010), there is an increased need to assess and review various treatment methods in terms of cost, efficiency and performance. Furthermore the article explains how chemical and mechanical treatment methods can contribute to enhancing the overall anaerobic sludge biodegradability. Examples of various wastewater treatment methods are explained in more detail below.

Physicochemical

The use of adsorption processes on activated carbon (AC) has been widely accepted owing to its extended surface area, increased adsorption capacity, microporous structure and high degree of surface responsiveness. AC assists in the removal of colour and specific organic pollutants in distillery effluents (Satyawali & Balakrishnan, 2008).

Coagulation and flocculation is a process whereby the forces that keep colloids apart, are neutralised. This then causes the association of particles to form flocs. Larger flocs are then removed by sedimentation, flotation, filtration or straining (Anon., 2010). A research study was conducted on the treatment of WDWW using an integrated Fenton-coagulation/flocculation process. The study investigated two steps, namely a Fenton process and a coagulation/flocculation step, whereby they used a theoretical expression to calculate the optimal molar ratio concentration. They evaluated the optimal concentration of $[H_2O_2]$:$[FeCl^{2+}]$ to be 15 mol/mol to achieve a removal efficiency of 74% COD (Heredia *et al*., 2005). Other studies were also conducted on pre-treatment by precipitation (Rusten *et al*., 1990; Rusten *et al*., 1993; Ødegaard, 1995).

Physicochemical methods (Heredia *et al*., 2005; Moletta, 2005; Melamane *et al*., 2007) have been developed and employed to treat lipid-rich wastewaters preceding the main treatment process, which is often of a biological nature.

Supercritical water oxidation has been applied to municipal excess sludge as well as distillery wastewater of molasses (Goto *et al*., 1998).

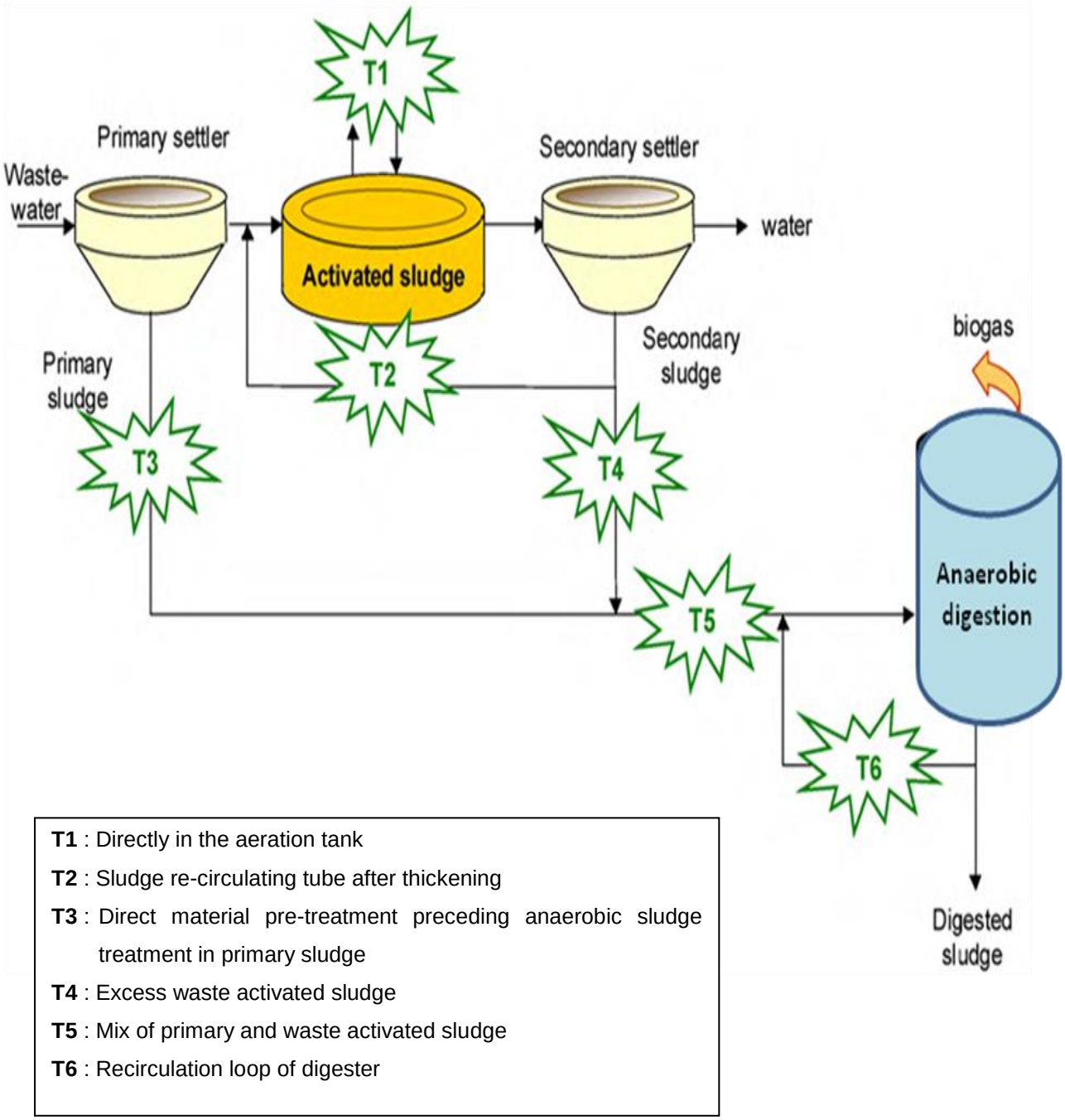

Figure 1. Classical wastewater treatment plant illustrating the various locations where pre-treatment may be incorporated (Carrère et al., 2010).

This is an environmentally attractive technology where organic materials are converted to carbon dioxide, water and N_2. Another study stated that distillery wastewaters are refractory to UV radiation, but when oxidation is applied with hydrogen peroxide, it leads to different COD reductions, which indicated that the process is mainly due to free radicals (Beltrán *et al*., 1997). Although distillery wastewaters of more than 3 000 $mgO_2.L^{-1}$

are refractory to advanced oxidation, reductions of COD for these wastewaters can only be as high as 38% when the initial COD is 850 $mgO_2.L^{-1}$ and the initial concentration of hydrogen peroxide is 0.1 M (Beltrán *et al*., 1997).

Ozone is a potent oxidant that is able to oxidise organic matter, carbon dioxide and water. The ozonation process also removes more than 99% of the microorganisms present. The cost involved in operating an ozone generator is high and precautions should be taken when handling toxic 0_3 (Stuatz, 2009). A study showed that the use of ozonation combined with conventional aerobic treatment methods can reduce the overall COD of distillery wastewater. The latter study used ozone mainly in the pre-treatment and post-treatment stage. The integrated technique (ozone-aerobic oxidation-ozone) achieved an approximate 79% COD reduction as opposed to a 34.9% reduction for non-ozonated sample over the treatment periods studied (Sangave *et al*., 2007). Another study showed that the ozonation of a pre-treated aerobic effluent, revealed an increase in the substrate removal in this ozonation stage from 16 – 21.5% COD (Benitez *et al*., 2000). A laboratory-scale UASB reactor combined with ozonation improved the degradation efficiency of diluted WDWW (Gie, 2007). When treated solely with an UASB reactor, WDWW (COD = 4 000 $mg.L^{-1}$) showed a reduction in COD of 92%. A combination of UASB and pre- or post-ozonation, showed a COD reduction of 94 and 96%, respectively (Gie, 2007).

Electrochemical oxidation is the process by which organic particles are oxidised with simultaneous oxygen evolution, using an appropriate electrode material (Comninellis, 1994). This type of treatment has been applied to distillery wastewater. An experiment was initiated to study the effects electrochemical oxidation has on the colour and COD of distillery wastewater (Piya-areetham *et al*., 2006). The results showed that titanium anodes had a higher potential for the treatment of distillery wastewater. The addition of additives, H_2O_2 and NaCl, promotes the reduction of COD and colour in wastewater by approximately 89.62 and 92.24%, respectively (Piya-areetham *et al*., 2006). Treatment of distillery wastewater using aluminum as electrode has also been studied (Krishna *et al*., 2010).

Biological Treatment

One of the most effective methods for the treatment of highly polluted industrial wastewater is biological treatment. Agro-industrial wastewaters including distillery plants, can either be treated via aerobic or anaerobic systems (Pant & Adholeya, 2007). Many biological wastewater reactors have been established and successfully implemented in the treatment of distillery wastewater and include enzymatic pre-treatment, microbial or a

combination thereof (Goodwin & Stuart, 1994; Tokuda *et al*., 1998; Akunna & Clark 2000; Goodwin *et al*., 2001; Uzal *et al*., 2003; Gao *et al*., 2007; Musee *et al*., 2007).

Stabilisation ponds include lagoons or oxidation ponds, which are holding basins where waste stabilisation and pathogen die-off occurs via natural processes (Droste, 1997). Important overviews of the vital interactions that occur during waste stabilisation in ponds are discussed by Stottmeister *et al.*, (2003).

Constructed wetlands are efficient wastewater treatment technologies and are low cost, easily operated and maintained (Kivaisi, 2001). Plants as well as microorganisms play a crucial role in biodegrading the contaminants within a constructed wetland. A previous study investigated the performance of a sub-surface flow constructed wetland, treating winery wastewater. Average removal efficiencies of 98% for COD and 97% total suspended solids were achieved. The system also rated effective at neutralising the high-strength acidic wastewater (Shepherd *et al*., 2001). The effectiveness of ozonation on a constructed wetland system, treating WDWW (COD = 7 000 $mg.L^{-1}$) showed an overall COD reduction of 84% (Green, 2007). The study also concluded that ozone as a pre-treatment system improved the overall efficiency of the wetland system.

Various anaerobic digesters are and have been implemented in the treatment of distillery effluent. A study investigated the feasibility of an UASB reactor to treat malt distillery wastewater without any mineral source except that used for pH adjustment. They found that the alkalinity increased, the sludge that developed was flocculent but did not form compact granules and the system became upset when fed with undiluted substrate (Goodwin *et al*., 2001). An anaerobic baffled reactor system also proved to be a worthy treatment option for a high strength distillery wastewater, containing up to 85 000 $mg.L^{-1}$ COD. It had a hydraulic retention time (HRT) of 20 d and an organic loading rate (OLR) of 4.28 g COD per day. The entire process showed reactor stability and achieved a removal efficiency of 98% (Bozadzhiev *et al*., 2007). COD values of up to 30 000 $mg.L^{-1}$ was treated via a UASB reactor, and removal efficiencies, remained on average above 90% (Wolmarans & Villiers, 2002).

Bacterial enzymes and fungi have been extensively studied for their ability to degrade fatty wastewaters from food industries (Wakelin & Forster, 1997) because of their enzyme specificity for substrate types. Enzymes are proteinaceous molecules that catalyse biochemical reactions. Two types of enzyme systems exist, namely endoenzymes and exoenzymes. Endoenzymes are produced intracellularly by the bacteria. These function to catalytically degrade soluble substrate within the cell. Exoenzymes on the other hand are also produced intracellularly, but are released to the external slime layer

surrounding the bacterial cell where the insoluble matter is attached. Here the insoluble matter is degraded to soluble substrate after which it is assimilated by the bacteria to be further degraded via endoenzymes (Gerardi, 2003).

In a study conducted on the bio-treatment of high fat and oil wastewater by lipase producing microorganisms, it was found that the removal of fat and oil and COD were 73 – 88% and 81 - 99% during a 7 day treatment process (Bhumibhamon *et al*., 2002). The lipase producing isolates were *Acinetobacter* sp. (KUL8), *Bacillus* sp. (KUL39) and *Pseudomonas* sp. (KLB1). The latter mentioned isolates were also said to have different degradation efficiencies due to the different lipases produced and their specificity for the substrate during catalytic reactions. It can therefore be explained that lipases belong to a group of enzymes referred to as enantiospecific enzymes that catalyse the hydrolysis of triacylglycerols to diacylglycerols, monoacylglycerols, fatty acids and glycerol at the interface between the aqueous and lipid phases. An essential limiting factor of lipases is a shortage thereof having the specific required processing characteristics to execute the desired actions during commercial applications (Sharma *et al*., 2001).

All bacteria have the ability to produce endoenzymes, but only certain bacteria can produce exoenzymes. It is not possible for a bacterium to produce all the exoenzymes that are necessary to degrade the large variety of particulate and colloidal substrates that sludge and wastewater consists of. As a result, a larger bacterial consortium, with each member producing the proper endoenzymes and exoenzymes are required to degrade the vast amount of substrates present (Gerardi, 2003). It should also be taken into consideration that the activity of lipolysis increases in direct proportion to the surface area until the enzyme concentration becomes a limiting factor (Alford & Steinle, 1967).

In a previous study, arable soil was experimentally contaminated with diesel oil at 5 mg hydrocarbons per gram of soil dry weight over a period of 116 days. Soil lipase activity, hydrocarbon degradation by indigenous soil microorganisms and the number of oil-degrading microorganisms in unfertilised and fertilised soil was used as a tool to monitor decontamination of the oil-contaminated soil. They found an increase in hydrocarbon utilisers coincided with a high biodegradation activity, stating a quick adaption process of indigenous microorganisms (Margesin *et al*., 1999). Studies include the use of commercial supplements concentrated with bacteria thus investigating their ability to degrade FOG (Brooksbank *et al*., 2007), the isolation of bacterial species from oil and grease contaminated industrial wastewater and their ability to degrade FOG (El-Bestawy *et al*., 2005) as well as the genetic engineering and bioremediation technology of microorganisms for improved contamination biodegradation (Gentry *et al*., 2004).

Anaerobic Digestion

The technology of anaerobic digestion (AD) should always be part of the list of process options for an industrial wastewater treatment, because it is considered energy saving and minimises sludge disposal costs (Brito *et al*., 1997). In modern day society, the wastewater treatment via AD, is considered to be state-of-the art technology (Ramirez *et al*., 2009).

The biochemical process by which high-moisture waste biomass is converted to bioenergy is known as AD. Anaerobic digestion is therefore the bacterial biodegradation of organic wastes or green crops in the absence of oxygen with the subsequent production of biogas rich in methane gas (Kim *et al*., 2010). Anaerobic digestion can be used to convert high-moisture organic wastes to methane, which serves as a substitute for fossil fuels.

During wastewater treatment, it is sometimes necessary to improve the digestion of slurry-like wastewaters by enzymatic pre-treatment. So is the case in a study conducted on the addition of enzymes to pre-treat intact yeast during AD of distillery wastewater, which improved biodegradation, by 87% (Mallick *et al*., 2010). The treatment of alcohol distillery effluents via upflow anaerobic sludge bed has proven to be a feasible method for the degradation of long chain fatty acids. Many anaerobic wastewater treatment studies conducted have focused their attention mainly on the anaerobic biodegradation with respect to dairy industries and oil refineries. According to Cammarota & Freire (2006), the milk industry in Brazil generated 21 x 10^9 L of milk alone in 2002, which resulted in approximately 84 x 10^9 L of post-production and processing effluent, from which more than 90% did not receive any type of treatment. Not so many studies have been conducted concerning GDWW.

In the past, it was seen as more economically attractive to utilise the malt distillery wastewater as cattle-feed as a disposal route (Goodwin & Stuart, 1994). However, the increase in demand for spirits related products and expansion of distillery industries on a global scale has led to the development of alternative methods of treatment. Larger anaerobic-treatment plants have been constructed to deal with increasing distillery wastewater volumes (Pant & Adholeya, 2007).

Conventional methods employed by the slaughterhouse and dairy industry for wastewater treatment includes the reuse of either blood by slaughterhouses and in the case of dairy industries, cheese whey (Cammarota & Freire 2006).

Microbiology of the AD process

Industrial wastewater consists of organic matter in the forms of carbohydrates, fats and proteins. Anaerobic digesters, biologically degrade approximately 80% of the influent organic waste (Gerardi, 2003). There are a series of bacterial events that account for this digestion process and these processes result in the breakdown of complex organic compounds to simplistic organic as well as inorganic compounds (Weng & Jeris 1976; Ramirez *et al*., 2009).

The methanisation process has been resolved through engineering aspects; however, the microbiology involved, still remains an issue of concern (Bories & Raynal, 1988). Methane fermentation takes place naturally in many ecosystems such as river mud, lake sediments, sewage, marshes and rice paddies (Schink, 1997). The layer of water acts like a blanket, thereby excluding oxygen and thus promotes the growth of anaerobes. The bacterial species involved can further be divided into three main groups, namely 1) strict aerobes, 2) facultative anaerobes and 3) anaerobes, inclusive the methane forming bacteria. These bacteria are classified as being unicellular gram-variable, strict anaerobes and non-endospore forming. Many methanogenic species that have been studied in pure cultures, are strictly anaerobic thereby growing only under conditions of oxygen-depleted environments and in the presence of a reducing agent. Most species require hydrogen and carbon dioxide for methanogenesis and growth. They grow best in a pH range of 6.4 to 7.4. The cell wall is of such unique chemical composition making it sensitive to toxicity from several fatty acids (Gerardi, 2003). Methanogenic bacteria, however, cannot solely obtain the simple compounds required to fulfil their process. They require these compounds to be supplied by other anaerobes present in methane fermentation.

Strict aerobes can only survive in environments with oxygen present, where they actively degrade substrate. They perform well in the presence of free molecular oxygen fulfilling a significant role in the waste degradation process. Strict anaerobes specifically prefer oxygen depleted environments and will therefore die within an oxygenated anaerobic digester. Facultative anaerobes are active in both oxygenated and oxygen depleted environments. When oxygen is available, it will be utilised for enzymatic activity as well as the degradation of wastes (Gerardi, 2003). The above-mentioned microbial groups play a distinctive role in various stages of the anaerobic process within a reactor.

It would be desirable to select for those species, which have the most desirable properties for the performance of a required function, especially since selecting for species with ‘better’ properties will contribute to improving the performance of a treatment system (Yuan *et al*., 2008).

The stages of anaerobic digestion

The AD process is classified into two distinct stages namely, acidification and gasification whereby the acidification stage is further categorised into three main stages involved in AD. These stages are initiated in sequence followed one after the other to allow for the digestion process and subsequent methane production (Komemoto *et al*., 2009; Hwang *et al*., 2010). The acidification stages are as follows: hydrolysis, acidogenesis, acetogenesis; followed by the gasification stage, methanogenesis (Fig. 2).

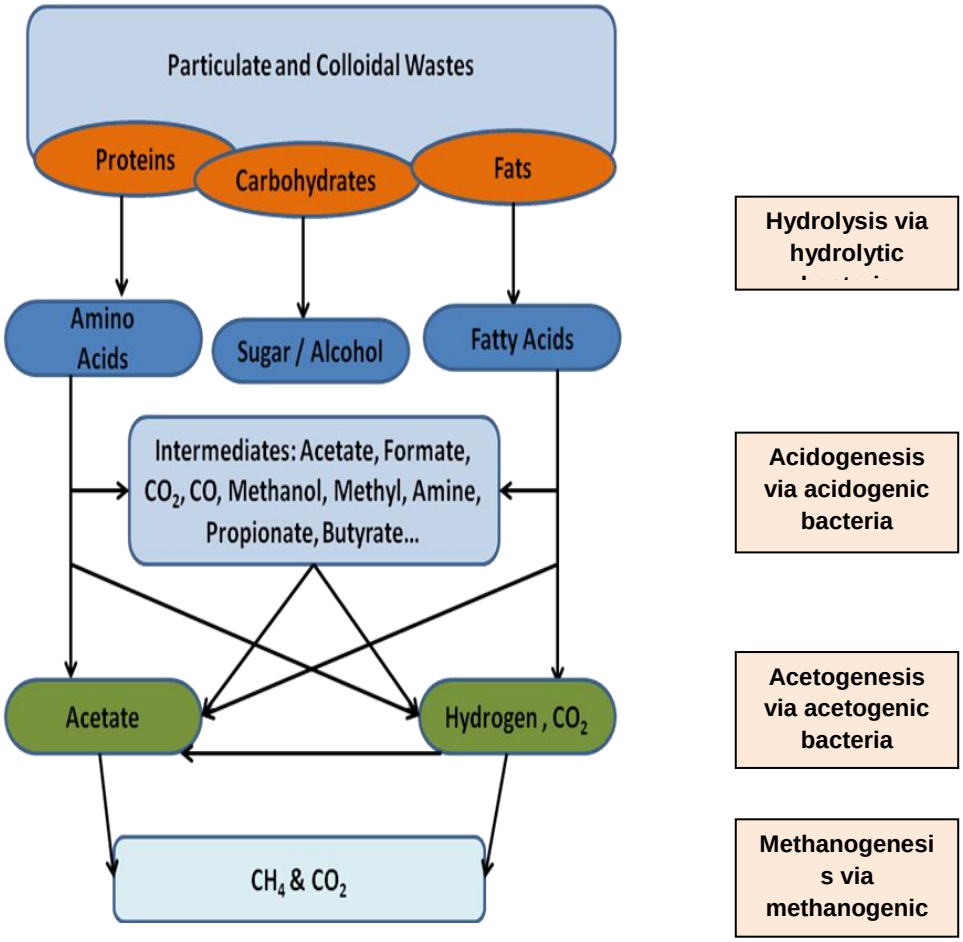

Figure 2. The four main stages of AD depicted above are initiated in a step-wise process.

Stage 1: Hydrolysis

Methanogens and acetogens are not capable of directly utilising the large complex polymeric compounds (Anderson *et al.,* 2003). This first stage thus involves the biodegradation of complex substrates namely carbohydrates, proteins and fats to their respective simpler compounds namely simple sugars, amino acids and peptides as well as shorter chain fatty acids. The acid forming bacteria consisting of both acidogenic (organic acid formers) as well as acetogenic bacteria (acetate forming bacteria) are primarily

responsible for the formation of the monomers from complex compounds (Anderson *et al*., 2003). The entire process relies on biodegradation of the complex compounds via hydrolytic enzymes that are extracellularly secreted by a consortium of microorganisms (Weng & Jeris, 1976). During this stage, it is essential to know that no organic waste stabilisation occurs and that the organic compounds are only converted to a form that can be assimilated by the bacterial population (Parkin & Owen, 1986). Stabilisation of organic compounds cannot occur unless the hydrolysis stage is functioning properly. For example, the inhibition of lipid hydrolysis may occur by product accumulation due to the particularity of lipases (Cirne *et al*., 2007). The hydrolysis stage can thus become a limiting step for the entire anaerobic process consequently inhibiting the stages to follow.

Stage 2: Acidogenesis

The facultative anaerobes and anaerobes are a diverse population, which during the acid-forming stage degrade the soluble compounds produced during the hydrolysis process. Various components are produced such as volatile fatty acids (VFA) which include propionic, butyric and valeric acid and of most importance is acetate (Alvarez, 2003; Gerardi, 2003). During the first stage, the acetate produced cannot be utilised directly by methanogens as energy can only be obtained following the reduction thereof (Gerardi, 2003). The VFA's produced has a chain length larger than that of acetate. The latter therefore must be further catabolised to allow utilisation by the methanogens (Anderson *et al*., 2003).

Stage 3: Acetogenesis

The VFA's which have been formed in the acidogenic phase, are further catabolised to carbon dioxide and hydrogen. In order for acetate production to occur, the hydrogen and carbon dioxide produced during acidogenesis are utilised as energy sources by a group of bacteria known as hydrogen-consuming acetogenic bacteria (Anderson *et al*., 2003; Alvarez, 2003; Gerardi, 2003). The conversion of short chain fatty acids such as propionic and butyric acids and alcohols into acetate, H_2 and CO_2 is only possible if a low H_2 pressure is maintained (Schmidt & Ahring, 1996).

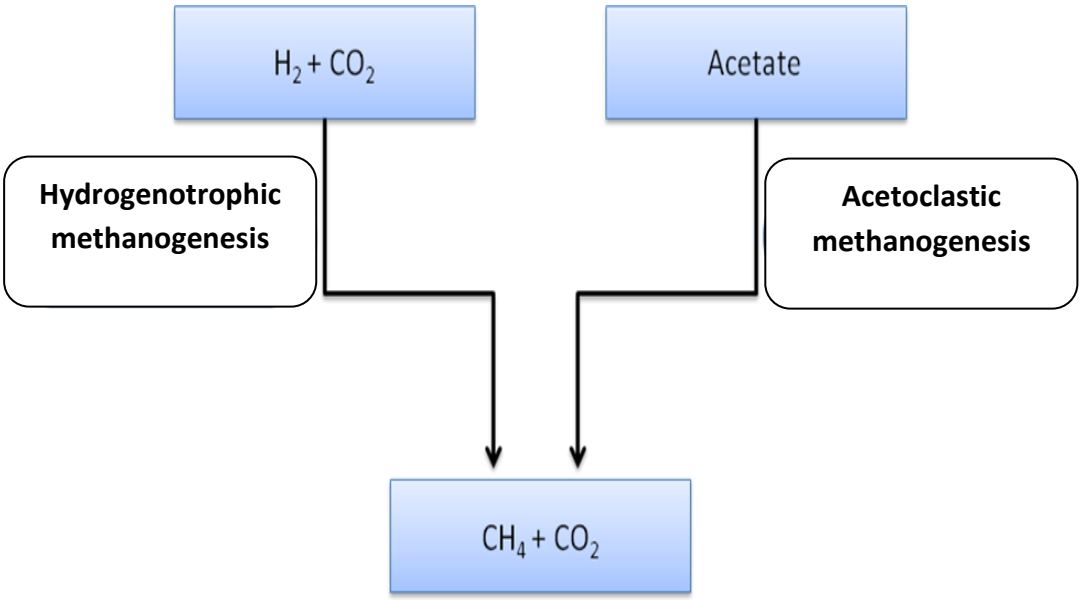

Figure 3. The two pathways of methanogenesis. Reduction of CO_2 (Hydrogenotrophic pathway) and oxidation of CO group to provide electrons for reduction of methyl group (Acetoclastic pathway).

Stage 4: Methanogenesis

The final phase of AD occurs when acetic acid is converted to methane (Fig. 3) and separated from the sludge mixture as methane is poorly soluble in water, inert under anaerobic conditions and able to escape from the anaerobic environment (Gerardi, 2003). This is known as the waste stabilisation phase (Parkin & Owen, 1986). Methane can be produced via two different pathways (Fig. 3). Hydrogen is utilised via hydrogenotrophic methanogenesis according to reaction 1.

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \quad \Delta G^{\circ\prime} = -131.0\ kJ.m^{-1}\ (1)\ (Schink, 1997)$$

Hydrolytic as well as acetotrophic bacteria produce H_2, which is thermodynamically unfavourable. This requires hydrogenotrophic methanogens to maintain low H_2 concentrations in order for methanogenesis to proceed efficiently (Ferry, 2010). Many H_2-utilising methanogens can also use formate as an electron donor for the reduction of CO_2 to CH_4 (Demirel & Scherer, 2008). During acetoclastic methanogenesis, CH_4 is formed from the methyl group via cleavage of acetate by methyltrophic methanogens, thus initiated by reaction 2.

$$CH_3COO^- + H_2O \rightarrow HCO_3^- + CH_4 \quad \Delta G^{\circ\prime} = -31.0\ kJ.m^{-1}\ (2)\ (Anderson\ \textit{et al.}, 2003)$$

Carbon dioxide is formed by the carboxyl group by acetotrophic methanogens. Most methanogens accept H_2 as an energy source and therefore consumes it rapidly during the reduction of CO_2 to methane (Parkin & Owen, 1986). Two distinct bacterial groups exist namely, acetoclastic methanogens and the H_2-consuming methanogens. Generally, acetate is utilised by *Methanosarcina* and *Methanosaeta* (Sekiguchi *et al*., 2001). Owing to its faster growth rate, *Methanosarcina* favours higher concentrations of acetate. For example, in a previous study the dynamics of methanogenic population were investigated in AD of solid waste and biosolids (Griffin *et al*., 2000). Mesophylic anaerobic sewage sludge and cattle manure were inoculated into two laboratory-scale anaerobic continuously mixed reactors. It was observed that *Methanosaeta* species decreased as the acetate concentration increased. *Methanosarcina* species however increased in numbers with a corresponding acetate concentration increase and form irregular cell clumps, which apparently provides protection against harmful chemical agents (Demirel & Scherer, 2008). *Methanosaeta* often found in high rate (biofilm) systems can only assimilate acetate as its sole energy source whereas *Methanosarcina* often found in solids digesters are also capable of utilising methanol, methylamines and sometimes hydrogen and carbon dioxide. Owing to the nature of the system, it is recommended that a single group of aceticlastic methanogens be used with different kinetic and inhibitory parameters (Bastone *et al.*, 2002).

It is of fundamental importance that the digestion process proceeds efficiently. Inhibition of the first stage will limit the amount of substrate available for the second and third stages resulting in the methane production decreasing. Inhibiting the third stage will cause the accumulation of the acids in the second stage (Gerardi, 2003). This results because acetate-producing organisms release H_2 during fermentation and if methanogenic bacteria do not consume the H_2 as fast as it is produced, it will result in the accumulation of organic acids in the second stage. For example the anaerobic systems treating wastewaters rich in vegetable oil must be designed in such a way to allow for both slow degradation of long chain fatty acids (LCFA) and potential inhibition by LCFA (Saatci *et al*., 2003). The third stage experiences inhibition mainly because of acids accumulating, which causes loss of alkalinity and subsequent decrease in pH due to an increase in H^+ (Gerardi, 2003).

It is important that the microbial consortium be in an environment that is suited for their growth, proliferation and activity within the anaerobic reactor system. They are highly dependent on the substrate, product concentration including the environmental parameters amongst others such as pH, alkalinity and temperature (Alvarez, 2003).

The hydrogen partial pressure plays a vital role in the methanogenic process. The degradation of organic compounds can only proceed if the H_2 partial pressure is kept low, i.e. < 10^{-4} atm. A low H_2 partial pressure will therefore ensure that fermentation products other than CO_2 and CH_4 do not accumulate (Gerardi, 2003).

By making use of modern molecular techniques, which complement traditional cultivation-dependant, and microscopic identification techniques involving non-destructive *in situ* analysis, a better understanding of the microbial ecology was derived (Merkel *et al*., 1999).

Introduction to the UASB system

For the treatment of high strength wastewaters, it is beneficial to undergo a biological treatment such as using an UASB reactor. Lettinga and his colleagues were responsible for the design and application of the UASB system in the late 1970's (Tawfik *et al*., 2005). Ever since the invention of the UASB system, it has gained extensive popularity and has been successfully applied in the brewery, distillery, food as well as paper and pulp industry (Laubscher *et al*., 2001; Loperena *et al*., 2006; El-Gohary *et al*., 2009; Stoica *et al*., 2009). This particular system is categorised as a high-rate anaerobic wastewater treatment system and is said to be one of the most preferred designs for the treatment of distillery wastewaters globally (Pant & Aldoleya, 2007; Mohana *et al*., 2009). The key feature to the success of the UASB is highly dependent on the microbial consortium that is established as a granular bed (Khademi *et al*., 2009). The granules are characteristically highly flocculated and well settled.

Operational design and mechanism of action

The UASB consists of two principle components, a sludge-bed and a 3-phase separator (Fig. 4). The sludge bed consists of bacterial granules, which are formed and remain intact owing to the up-flow velocity in the UASB reactor (Britz *et al*., 1999). The 3-phase separator separates the gas and liquid portion from the solids. This essentially allows the gas and liquid phases to leave the system separately (Colussi *et al*., 2009).

When the untreated influent wastewater enters the reactor, it flows in an upward motion (Wood *et al*., 2009) through the sludge bed thus causing gentle agitation and mixing of the granules, in effect allowing exposure to continual nutrient supply (Britz *et al*., 1999). The sludge bed is responsible for 80 - 90% of the degradation of the influent

substrate. During the hydraulic turbulence, sludge particles and microbial biomass together with biogas production, form a suspension referred to as a “sludge blanket”. The solids are separated from the gas phase via the 3-phase separator. To allow collection of gas, a 3-phase internal settler is directed towards a gas outlet (Rajeshwari *et al*., 2000).

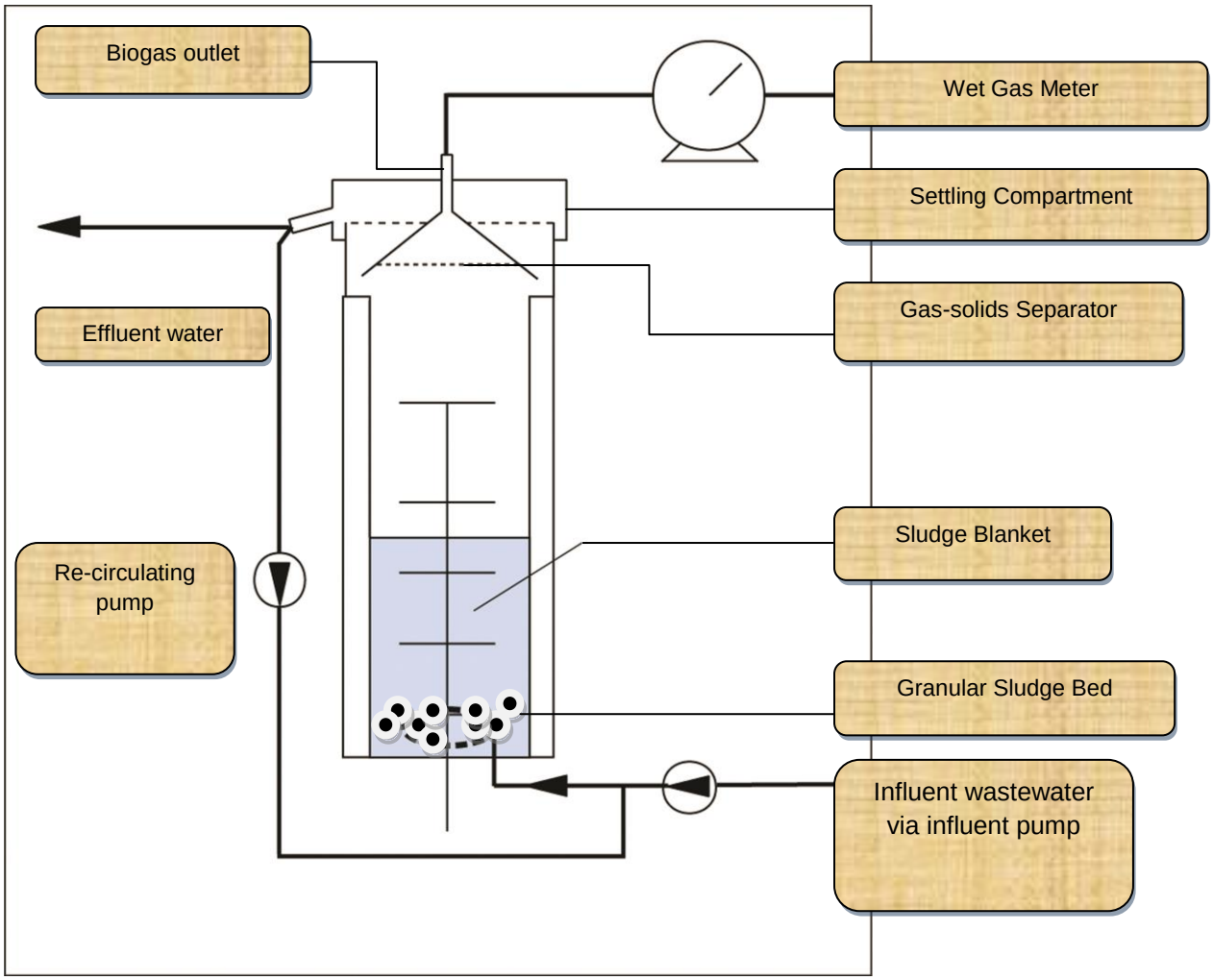

Figure 4. Schematic representation of a simple UASB reactor (Lettinga *et al*., 2001).

The advantages and disadvantages in the application of an UASB reactor

The UASB reactor is employed by many industries on a global scale owing to its ability to treat high strength wastewaters that are concentrated in organic pollutants, its high operational efficiency and cost-effective advantage (Chinnaraj & Rao, 2006; Carrère *et al.*, 2009). Anaerobic treatment has advantages over that of aerobic treatment because a large portion (>60%) of the organic fraction is converted to sludge during aerobic treatment whereas during anaerobic treatment, the organic fraction (<10%) in wastewater is mainly

converted to CO_2 and CH_4. This type of system produces the highest amount of biomethane, which is a valuable source of energy (Els *et al*., 2005; Pant & Adholeya, 2007). The accumulation of sludge during aerobic treatment also gives rise to increased waste disposal costs. Other problems associated with aerobic treatment are the generation of foul odours and high operating costs (Ross, 1989) owing to the oxygen rich aeration process.

The high-rate anaerobic reactor has the following advantages as mentioned by Lyberatos & Skiadas (1999). They operate effectively at high solids retention times (SRT) and very low hydraulic retention times (HRT). The HRT of UASB's and other high-rate anaerobic digesters are hours rather than days (Wood *et al*., 2009). The development of an anaerobic system that allows for the separation of HRT from SRT, allows slower growing anaerobic bacteria to remain within the reactor independently of the flow of wastewater (Akunna & Clark, 2000). There is no complexity in the design and they are characterised by efficient mass and heat transfer. The production of biogas secures good mixing characteristics and the UASB system is robust to disturbances. Apart from the above-mentioned, a UASB system offers useful energy recovery (Pant & Adholeya, 2007) from the biogas produced instead of energy consumption, and can practically be applied anywhere on various scales. Vast amounts of land are required for treatment options such as anaerobic lagooning and constructed wetlands as opposed to an UASB system where less land is required for system construction (Mai, 2006).

Treatment of food processing wastewaters

Many studies have been conducted on lipid biodegradation within an UASB reactor (Cammarota & Freire, 2006; Cavaleiro *et al*., 2007; Jeganathan *et al*., 2007; Cavaleiro *et al*., 2008). Grain whisky production generates a high-strength wastewater concentrated in fats, oils and greases (FOG) and therefore contributes to a high COD. Anaerobic digestion via UASB has therefore been regarded as a more feasible and most preferred treatment option for GDWW.

Amongst other constituents within food products, fats and oils form an integral part. The positioning and attachment of a fatty acid to the glycerol backbone, its degree of unsaturation and the length of the fatty acid chain greatly influence the sensory and nutritional value as well as the physical properties of the triglyceride (Sharma *et al*., 2001). The beer and distillery industries, along with slaughterhouses and dairy industries, edible oil and fat refineries are fundamental contributors to lipid wastewater production (Li *et al*.,

2002). A considerable amount of fatty waste is produced by numerous agro-industries, with particular emphasis on the food industry (Battimelli *et al*., 2009).

Meat Industry

During a study, the biodegradation of slaughterhouse by-products, mesophylic operational conditions was seen as more feasible than thermophilic conditions. Results showed that co-digestion of 5% pork by-products blended with pig manure at 37°C showed a 40% higher methane production compared to digestion of solely manure (Hejnfelt & Angelidaki, 2009).

The slaughterhouses and meat packing industries produce a considerable amount of lipid-rich waste. Various processing steps give rise to the generation of abattoir wastewater such as the cleansing of animals, bleeding-out, skinning, cleaning of slaughtered animals as well as cleaning rooms. The effluent generated contains high amounts of biodegradable organic matter, which includes soluble and insoluble fractions. Colloidal and suspended matter in the form of cellulose, fat and protein forms part of the insoluble fraction (Gannoun *et al*., 2009). A significant problem is caused by the high-suspended solid content within abattoir wastewater. This problem slows down the degradation rate and may also result in scum layer production (Hejnfelt & Angelidaki, 2009). This lipid waste causes flotation foams containing 60 – 65% lipid on a dry basis (Li *et al*., 2002). The total fat content within raw abattoir wastewater ranges from between 40 – 410 $mg.L^{-1}$ (Gannoun *et al*., 2009).

Dairy Industry

Although the dairy industry is considered to be an important economic sector, it poses a high pollution potential when the recovery of proteins, lipids and lactose is not performed (Leal *et al*., 2006). The major components of milk-fat include palmitic, oleic, myristic and stearic acids. These LCFA are produced under anaerobic conditions when fats and oils are hydrolysed and cause potential inhibition of microbial activities (Lalman & Bagley, 2000). A study conducted by Leal *et al.* (2006) stated that oleic acid concentrations above 30 $mg.L^{-1}$ brought about inhibition to acetate degradation; however, stearic acid up to a concentration of 100 $mg.L^{-1}$ did not inhibit acetoclastic methanogenesis. During cheese manufacturing, high quantities of whey and milk permeate present a foremost disposal problem and consist of <2.5 $g.L^{-1}$ fat (Wang *et al*., 2009).

Olive oil mill industry

Olive oil extraction is a mechanical process that produces a dark-coloured pigment, referred to as olive-mill wastewater (Brozzoli *et al*., 2009). The disposal of wastewater derived from olive oil extraction is one major environmental problem (Brozolli *et al*., 2009; Koutrouli *et al*., 2009). A study conducted by Koutrouli *et al.* (2009), proved that a two-stage AD of olive pulp is a reliable, stable and effective process for energy recovery in terms of methane and hydrogen production. In a study conducted on the fats and oils contained in palm oil wastewater, removal efficiencies of up to 80% COD and fats were achieved using bacteria isolated from soil rich in fats and oils (Bhumibhamon *et al*., 2002). In one study, the biodegradation of olive oil and the treatment of lipid-rich wool scouring wastewater under thermophilic conditions were investigated using an isolated strain, *Bacillus thermoleovorans* IHI-91. Olive oil was used as a model substrate, whereby more than 90% thereof was reduced in a time period of 2 hours. This strain was subjected to wool-scouring wastewater in a continuously operated laboratory-scale stirred-tank reactor. Although lipid degradation rates were high with up to 500 $mg.L^{-1}.h^{-1}$, removal, was only 20-25% at a residence time of 10h. Severe inhibition took place when the concentration of olive oil was increased to 4.0 $g.L^{-1}$ (Becker *et al*., 1999).

Lipid degradation during anaerobic digestion

The conversion of lipids to biogas has been considered to be very difficult. The lipids can be anaerobically hydrolysed by extracellular lipases secreted by microbial species. This hydrolysis step releases LCFA and glycerol into the sludge mixture. Glycerol is easily biodegraded to form VFA (Battimelli *et al*., 2009; Li *et al*., 2002) as opposed to LCFA, which is initially adsorbed to the surface of the microorganisms before being biodegraded via β-oxidation. The penultimate step is the production of acetate and hydrogen followed by their conversion to biogas, of which the main constituents are methane and carbon dioxide (Fig. 5). Lipid biodegradation is initiated and maintained by a symbiotic interaction between two metabolically different types of bacteria, which are highly dependent on each other for degradation of a specific substrate (Amani *et al*., 2010). There exists a syntrophic relationship between the fatty-acid oxidizing microbes, hydrogen-consuming methanogens, and acetate-consuming methanogens and without this complex food web, biodegradation of particulate or colloidal wastes cannot effectively occur (Sekiguchi *et al*., 2001).

Lipids are classified as substances of biological origin that are soluble in organic solvents such as chloroform and methanol, but only sparingly if at all soluble in water.

Triacylglycerols are fats and oils most abundant in plants and animals. The latter mentioned class of lipids are a highly efficient form of energy storage. This can simply be explained by the fact that fats are less oxidised than are carbohydrates or proteins and for this reason yield significantly more energy upon oxidation (Voet & Voet, 2004). Moreover, the oxidation metabolism of fats yields twice the energy of an equal amount of dry carbohydrate or protein (Table 7). According to Mendes *et al.* (2006), lipid hydrolysis can be considered as a limiting step for the biogas generation and organic removal during the AD process.

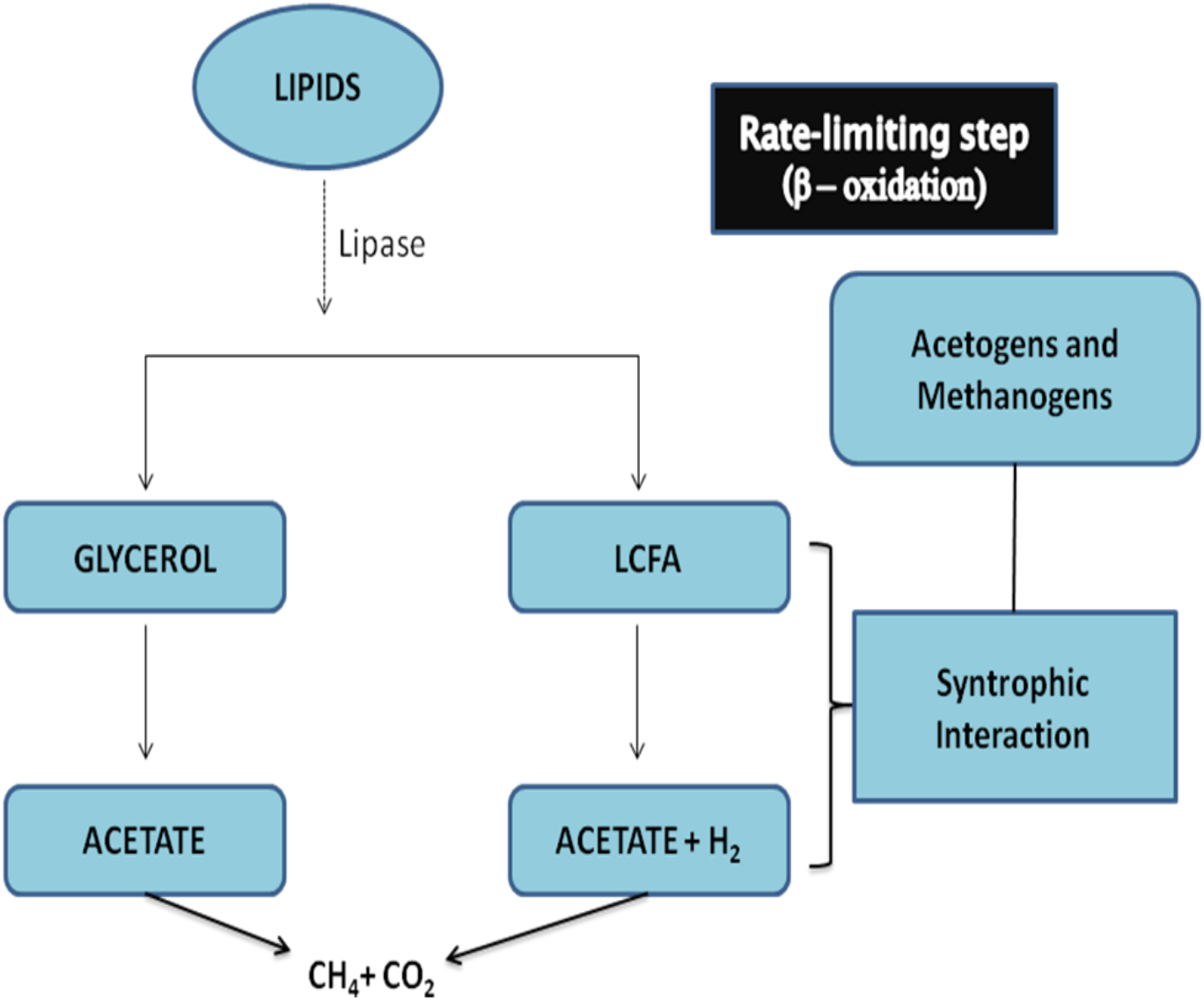

Figure 5. Schematic representation of lipid biodegradation and the rate limiting step namely, β-oxidation.

Table 7. Metabolic energy values for carbohydrates, fats and proteins expressed in kilojoules per gram of dry weight (Voet & Voet, 2004)

Constituent	**ΔH (kJ.g^{-1} dry weight)**
Carbohydrate	16
Fat	37
Protein	17

In order to exemplify an example of a syntrophic interaction between H_2-producing acetogenic bacteria and the H_2-consuming methanogens, the stoichiometry is shown in Table 8.

As can be derived from Table 8, reactions A and B are thermodynamically unfavourable. These reactions therefore require the utilisation of H_2 (SUM A + C and SUM B + C) by methanogenic bacteria for the reaction to proceed.

Table 8. Proposed stoichiometric reactions involved in the catabolism of propionate and butyrate by H_2-producing acetogenic bacteria in pure culture and in syntrophic association with H_2-utilising methanogens (Bryant, 1979)

Reaction	**Stoichiometry**	**ΔG°′ (kJ.mol^{-1})**
A. Propionate-catabolising acetogenic bacterium	$CH_3CH_2COO^- + 3H_2O \leftrightarrow CH_3COO^- + HCO_3^- + H^+ + 3H_2$	+76
B. Butyrate-catabolising acetogenic bacterium	$CH_3CH_2CH_2COO^- + 2H_2O \leftrightarrow 2CH_3COO^- + H^+ + 2H_2$	+48
C.H_2-utilising methanogenic bacterium	$HC_3O^- + 4H_2 + H^+ \leftrightarrow CH_4 + 3H_2O$	-135
SUM A + C. Syntrophic association	$4CH_3CH_2COO^- + 3H_2O \leftrightarrow 4CH_3COO^- + HCO_3^- + H^+ + 3CH_4$	-102
SUM B + C. Syntrophic association	$2CH_3CH_2CH_2COO^- + HCO_3^- + H_2O\leftrightarrow 4CH_3COO^- + CH_4 + H^+$	-39

The syntrophic interaction thus is essential for the growth of methanogenic bacteria and the maintenance and catabolism of substrate in order to maintain a very low H_2 partial pressure (Bryant, 1979; Schink, 1997). The thermodynamics regarding anaerobic metabolism and wastewater anaerobic fundamentals are well documented in literature (Parkin & Owen, 1986; Schink, 1997; Jackson & McInerney, 2002; Kotsyurbenko, 2005).

Alvarez (2003) gives a more detailed description of some key AD reactions. It has been hypothesised that the conversion of more complex compounds, present more difficult degradation problems, since more intricate biological reactions are required to allow substrate conversion to methane (Azbar *et al*., 2001).

Problems and positive aspects of LCFA degradation

Linoleic (C18:2), oleic (C18:1) and stearic acids (C18:0) are among the most abundant and common LCFAs in vegetable oils (Lalman & Bagley, 2000) and can therefore cause significant negative bioreactor operational inefficiency if not treated correctly. Long chain fatty acids resulting from the hydrolysis of fats in lipid-containing wastewaters pose a problem in that they cause inhibition and sludge flotation and washout (Salvador, 2007). Other problems, which result in low overall performance of the treatment system, are unpleasant odours, continual foaming, blockages in grease taps and partial and/or low degradation of oil and grease. The persistent accumulation of fats and oils essentially causes severe problems in piping systems, pumping stations as well as in wastewater treatment plants (Mahlobo, 2008). This problem is primarily linked to the accumulation of LCFA on the microbial consortium by mechanisms of adsorption, precipitation and entrapment (Cavaleiro *et al*., 2007).

Literature also mentions that wastewater containing a considerable amount of fat, such as dairy wastewater, makes the AD process complicated leading to scum layer formation at the surface of the reactor. The adsorption of these fats to the microbial population also hinders mass transfer, which prevents the effective assimilation of nutrients by the microbial population thus decreasing the methanogenic activity (Cirne *et al*., 2007; Cavaleiro *et al.*, 2008). A previous study showed that sludge encapsulated with oleic acid exhibited a lower methane yield after a lag phase of about 50 hours as opposed to an unencapsulated sludge that showed a significant methane yield. This suggests, that the intimate contact with LCFA accumulated biomass creates a barrier that hinders the transfer of substrates and products such as biogas, hence the lower methane yield of the oleic-encapsulated biomass (Pereira *et al*., 2005). Another study conducted research on the anaerobic treatment of olive oil mill effluent, using a two-reactor system with partial phase separation. The study proved that no stearic acid was detected, thus indicating that the saturation of LCFAs (oleic to stearic) was the rate-limiting step in the first part of beta-oxidation (stearic to palmitic) (Beccari *et al*., 1998). The conclusion was also drawn that saturated fatty acids (SFAs) are less inhibitory than unsaturated LCFAs as the formation of

palmitic acid from oleic acid to a greater extent, prevented lipid inhibition on methane production in the second reactor. (Beccari *et al*., 1998).

Figure 6 below depicts the three main ways by which LCFA accumulation can occur either by means of, (a) precipitation via ions during the hydrolysis step, (b) adsorption of LCFAs to the microbial strain or (c) entrapment of LCFAs to the sludge. To investigate the adsorption of LCFAs to the granular sludge in an UASB reactor, a study was conducted to characterise the biosorption of LCFAs to active and inactive granules (Hwu *et al.,* 1998). The mixture of LCFAs showed faster adsorption to granules than just a single fatty acid such as oleic acid. This study also concluded that sludge flotation resulted from the LCFA loading rate rather than the concentration. Therefore the higher the loading rate, the more prominent flotation was (Hwu *et al*., 1998). To investigate the process of LCFA precipitation, previous observations showed that palmitic acid formed precipitated flocs between sludge. However, when oleic acid was fed to the system, there was a conversion with the palmitic acid and the accumulated palmitic acid adsorbed onto the sludge or entrapped as flocculent aggregates (Pereira *et al*., 2005).

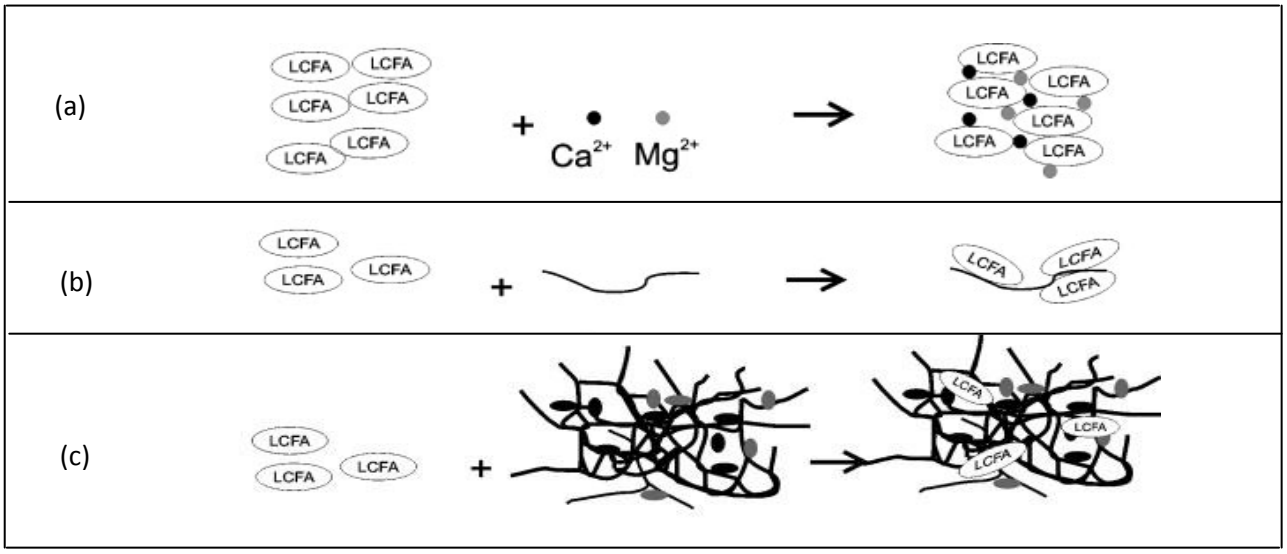

Figure 6. Schematic representation of three main mechanisms of biomass-associated LCFA accumulation: (a) precipitation, (b) adsorption and (c) entrapment (Pereira *et al*., 2005).

Aside from the negative reactor problems caused by FOG components, there are also potential positive aspects regarding FOG. In order to exemplify the possibility of LCFA as an attractive source for methane production is as follows. Consider the following equation below for the complete oxidation of 1 g glucose.

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O$$
$$180 \qquad 192$$

The COD of glucose is 192 g O_2/180 g which yields 1.067 g COD. At standard temperature and pressure (STP) 1 g COD produces 350 mL CH_4, while at 35°C = 395 mL CH_4. Therefore 1.067 g COD at 35°C theoretically yields 422 mL CH_4. The equation for the complete oxidation of 1 g of palmitic acid (saturated LCFA, C16:0) is shown below.

$$CH_3(CH_2)14COOH + 23O_2 \rightarrow 16H_2O + 16CO_2$$
$$256 \qquad 736$$

The COD for palmitic acid is calculated in a similar manner as for glucose, thus 736 g O_2/256 g palmitic acid yields 2.88 g COD. Therefore the COD at 35°C for palmitic acid theoretically yields 1133.65 mL CH_4.

Lipids are therefore attractive substrates for anaerobic co-digestion and digestion owing to the higher methane yield obtained when compared to carbohydrates (Cirne *et al*., 2007). Operational problems however restrict the potential for increased methane yield from lipid-rich wastewater. Inhibition by LCFA may complicate the degradation process, but adaptation may also occur. This is possible if a well-developed process is functional to readily degrade feeds with a lipid-rich content. This is possible because efficient LCFA degradation by an acclimatised culture will be able to remove LCFA as fast as they are released during the hydrolysis of lipids. However, importantly, acclimatisation is necessary to avoid high transient concentrations of LCFA (Batstone *et al.*, 2002). Alternative methods such as pre-treatment steps and physical methods before biodigestion have been implemented to try and overcome these operational limitations. The use of enzymes such as lipases facilitates the hydrolysis of triacylglycerols to LCFAs and glycerol. Previous studies were done to demonstrate the efficacy of enzymes on oily wastewater (Mendes *et al.*, 2006; Bochmann *et al.*, 2007; Jeganathan *et al.*, 2007). Jeganathan et al. (2007) concluded that the use of immobilised lipase increased the biodegradability of high strength oily wastewater from 45 to 65%. Mendes *et al*. (2006) showed that the use of a low-cost commercially available lipase preparation in pre-treated assays had a higher reaction rate than that of the crude wastewater assays. This was confirmed by the increased biogas production and the higher COD and colour removal.

Bioaugmentation as Treatment Technology

Bioaugmentation is defined as the process which attempts to improve the efficiency of treatment through increasing the diversity and/or activity via incorporation of either selected naturally occurring or genetically modified microorganisms to the wastewater treatment system (Vogel, 1996; Romero & Ferrer, 1999; Pepper *et al*., 2002; Perelo, 2010). The microorganisms are isolated from special sites where natural selection has already favoured microbes suited to unfavourable conditions (Gray, 2010). A key factor associated with the effective operation of a wastewater treatment plant, is dependent on the microorganisms present. Many treatment plants cannot always respond rapidly to a failing wastewater treatment system in order to produce the desired effluent characteristics (Romero & Ferrer, 1999).

When a newly isolated microbial strain is introduced to a natural consortium, a commensal or mutualistic relationship should be established and thus bioaugmentation is required to achieve this relationship. This relationship can be achieved by degradation of substances by the newly introduced strain that would otherwise be toxic to the consortium (Lanthier *et al*., 2002). A study evaluated the effect of bioaugmenting the AD of biosolids with a patent-pending commercial product (Duran *et al*., 2006). The product contained selected strains of bacteria from the genera *Bacillus*, *Pseudomonas*, and *Actinomycetes* along with ancillary organic compounds. The study proved that bioaugmenting with the commercial product improved methanogenesis and odour control under the study conditions (Duran *et al*., 2006). In another study, the biodegradation potential of oily sludge by pure and mixed bacterial cultures was investigated (Cerqueira *et al*., 2011). It was found that bacterial consortium showed an oily sludge degradation capacity, reducing 90.7% of the aliphatic fraction and 51.8% of the aromatic fraction, as well as biosurfactant production capacity, achieving 39.4% reduction of surface tension of the culture medium and an emulsifying activity of 55.1% (Cerqueira *et al*., 2011). Transiently overloaded digesters were bioaugmented with a propionate-degrading enrichment culture in an effort to decrease recovery time (Tale *et al*., 2011). Bioaugmenting with the propionate-degrading culture was a success in that there was a decrease in the recovery time following an organic overload. Biogas and methane production rates returned to pre-upset values more quickly when bioaugmentation was enforced. One should always be aware of problems that could arise during bioaugmentation. These include ensuring the survival of the organism as well as its activity is essential. Inhibition can be as a result of redox, pH, toxic contaminates or absence of key substrates (Perelo, 2010).

Some key factors for the application of bioaugmentation are (Gray, 2010):

- Improving removal efficiencies with regard to biological oxygen demand (BOD) and chemical oxygen demand;
- Improving the removal of recalcitrant or other substances which may cause operational problems;
- Restarting or commission of treatment plants;
- Reducing instability during process which is often caused by fluctuations in organic loading;
- Reduce the accumulation of scum and sludge from aerobic and anaerobic digesters to ensure stabilisation.

Several reasons have been suggested why bioaugmentation cannot occur effectively in nature. Some of the reasons are; substrate concentration may be too low to support growth (Goldstein *et al*., 1985), too few organisms may be present to have a significant effect on substrate change (McClure *et al*., 1991) and competition amongst organisms causes growth inhibition (Goldstein *et al*., 1985).

Biogas as Renewable Energy Source

Biogas production is the final product of AD of wastewater. Of great importance and economic value is the methane produced. Methane emissions into the atmosphere has a direct negative impact as the methane global warming potential can be evaluated to be that of a 100 year time span which is 25 times greater than that of carbon dioxide. It can further be emphasised that 1 kg of methane is equivalent to 25 kg of carbon dioxide (Calabrò, 2009). The methane fermentation process has been adopted globally. It can be used either alone or in combination with other processes for the stabilisation and disposal of biomass wastes which includes domestic, municipal, agricultural and industrial wastes and wastewaters. The amount of organic material, the BOD as well as pathogenic organisms present in the wastes is reduced during digestion (Colussi *et al*., 2009). Methane recovered can thus be used as a renewable source of energy (Lyberatos & Skiadas, 1999; Battimelli *et al*., 2009; Luste *et al*., 2009). Production of methane during fermentation has been opted for as an energy-saving wastewater treatment method specifically owing to the energy and oil crisis.

Previous studies have shown that there is a significant increase in methane yield, from 25 to 50 m^3 biogas/m^3 cattle waste, upon the addition of fish oil (for a total concentration of 5%) to a manure digester (Cirne *et al.*, 2007). It is therefore advantageous to apply methanisation as an alternative to waste incineration as it leads to the reduction of organic matter content as well as the production of biogas that primarily consists of methane. One can also say that not only is the removal of FOG from wastewater beneficial, but the addition thereof can have advantages. According to Battimelli *et al*. (2009), direct methanisation of pure fats is a complex process, solely because fats are insoluble in water and biodegradability is slow. Amongst other positive aspects regarding methane production mentioned earlier, the hydrolysis step reduces the coagulation of the lipid spheres, thereby maintaining a large lipid-water interface (Cirne *et al.*, 2007).

In a study conducted for the AD of lipid-rich waste, there was a methane recovery of 93% for 31% lipid (w/w COD basis). This was achieved by increasing the concentration of lipid from 5% to 47% (w/w) (Cirne *et al.*, 2007). Strong inhibition by the addition of lipase was observed. It was said that although the enzyme enhanced hydrolysis, there was a consequent accumulation of intermediates that led to the inhibition of degradation steps. The major obstacle to methane production was the long chain fatty acids.

The benefit of adding lipids to a digester to enhance the methane production is a promising approach which should be better explored. It is therefore imperative to continue to advance the knowledge in the degradation process of these wastes because food processing wastewater industries are generating more waste rich in fatty material owing to the growing needs of the population. All this fatty waste produced therefore has to be disposed of in an environmentally friendly manner and a feasible treatment method would be waste-to-energy treatment such as AD. Not only would the waste entering our landfill or water streams be reduced, but also the amount of methane released into the atmosphere from sewage streams and landfill sites.

There exists a considerable potential in biogas systems at a regional level owing to its positive economic, environmental and social impacts. Regional energy systems allow for individuals, organisations and communities to establish secure, local energy supply (Murto *et al.*, 2007). Business opportunities are generated together with the biogas establishment (Murto *et al.*, 2007).

Conclusions

The Distillery industry uses a substantial amount of water to operate various industrial

processes. Distillery production is said to increase in the years to follow as the demand for alcohol beverages is increasing (Bester, 2009). Subsequently this growth in production would also increase the usage of potable water leading to a vast amount of effluent water that is generated. This is regarded as hazardous to the environment owing to the very high COD content (Heredia *et al.*, 2005). These industries have to adhere to strict discharge standards and therefore must have the wastewater treated before being discharged into the environment (Surujlal *et al.,* 2004). To prevent damage to the natural ecosystems and avoid penalties being issued to the alcohol distillery industries via legislative organisations (Republic of South Africa, 1998a; Republic of South Africa, 1998b), various wastewater treatment technologies have been designed, considered and implemented (Mohana *et al*., 2009).

Grain whisky production generates effluent water that is high in FOG. It has been well documented that FOG and LCFA can cause serious problems during wastewater treatment processes (Mendes *et* al., 2006; Salvador, 2007). These problematic situations includes amongst others, mass transfer deficiency of microorganisms in digesters, leading to a decrease in biodegradation efficiency, sludge flotation and subsequent biomass washout (Pereira *et al.*, 2005). UASB has also been regarded as a more feasible and most preferred treatment option for high strength wastewaters such as those rich in FOG.

Anaerobic digestion shows good reliability for winery wastewater as it has shown to have a removal yield of 90 – 95% COD (Moletta, 2005). It is considered to be energy-saving, minimises sludge disposal costs and known to be a state-of-the-art technology (Brito *et al*., 1997; Ramirez *et al*., 2009).

The microbial population in a water treatment system has an important function. Therefore, by isolating and incorporating lipase producing microbial species into an UASB system will be advantageous for the performance of the system fed with lipid-rich wastewater. Lipases that are commercially available are also expensive, so culturing of lipid degrading microbial species on a laboratory scale would also be less costly. These lipolytic species can be employed either in a pre-treatment or augmented into the anaerobic digesters, to increase the methane yield. This could provide a valuable renewable energy source to offset the rising electricity prices, while also contributing to cleaner wastewater discharges and the possibility of water re-use. The knowledge gained using the techniques will help to identify a diverse microbial population required to operate efficiently in an anaerobic system treating a specific substrate type.

In order to identify unique microbial populations specific for a substrate type, more research has to be focused on the individual bacterial strains with emphasis on lipase

production and specificity for substrate. Although lipid-degrading microorganisms have already been successfully acclimatised to wastewater types, it does not necessarily mean that those same microorganisms will behave similarly to wastewater of a different composition. The choice of a bacterial inoculum therefore becomes increasingly important when treating a specific wastewater type as internal and external environmental conditions will have an effect on the performance of the biological treatment system.

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CHAPTER 3

ISOLATION, CHARACTERISATION AND FATS, OILS AND GREASE BIODEGRADABILITY BY BACTERIAL ISOLATES FROM A GRAIN DISTILLERY ENVIRONMENT

Summary

Four isolates had the ability to utilise the fats present on the solid media and were morphologically, phenotypically and biochemically characterised as being *Pseudomonas fluorescens, Pseudomonas luteola, Stenotrophomonas maltophilia,* and *Bacillus licheniformis*. These bacterial strains were tested for the ability to grow and utilise tributyrin, cotton seed oil (CSO) as well as fats, oil and grease (FOG) present in Grain Distillery Wastewater (GDWW). The isolates were also subjected to three temperature ranges namely, optimum growth temperature (50°C and 37°C), optimum enzyme activity temperature (25°C, 30°C and 37°C) and mesophylic temperature range (37°C). Lipolysis was prominent at all temperature ranges for each respective isolate except for *B. licheniformis* that had no lipolytic activity at 50°C, when using tributyrin as lipid source on Spirit Blue Agar (SBA). *Pseudomonas fluorescens* showed a decrease in halo diameter as the incubation temperature increased from 25°C to 37°C. *B. licheniformis* grown on Victoria Blue B (VBB) together with CSO as lipid substrate, formed deep blue colonies against a blue background at its optimum enzyme temperature of 37°C which is positive for lipolysis. When GDWW was utilised as lipid source, growth was present for all isolates at the predetermined temperature ranges; however there was no indication of lipolysis although colonies formed were dark blue. It could be said that using GDWW as lipid source does not form a turbid fat-emulsion agar and that the effectiveness of lipolytic visual observation will thus depend on a clear zone forming against a turbid background. The visual observation of lipid clearing on agar plates does not establish which fatty acids are biodegraded in the grain whisky wastewater for growth on VBB (GDWW) plates. For this study it was therefore concluded that when scanning for lipolytic activity, it is important to use SBA (Tributyrin) and VBB (CSO) in conjunction with VBB (GDWW) because VBB (GDWW) cannot distinctively give conclusive results for lipolytic activity on solid media.

Introduction

It has been clearly stated through research that wastewaters of various food industries are complex in composition and thus highly polluted (Ross, 1989; Melamane *et al*., 2007; Hejnfelt & Angelidaki, 2009; Liang *et al*., 2009; Wang *et al*., 2009). Distillery wastewater is characterised as being acidic (pH of 3 – 4), having a high chemical oxygen demand (COD) of 10 000 – 50 000 $mg.L^{-1}$ and low nutrient capacity (Heredia *et al*., 2005). Grain distillery wastewater (GDWW) in particular has a COD of 25 000 – 30 000 $mg.L^{-1}$ which can cause operational problems during treatment, especially with an increased FOG content (Laubscher *et al*., 2001). One problem observed during GDWW treatment was scum layer accumulation. This was a direct result of GDWW and not distillery wastewaters in general (Laubscher *et al*., 2001). For this reason, treatment through various methods, of the effluent generated is imperative to ensure minimal water pollution (Brito *et al*., 1997; Chan *et al*., 2009; Fermoso *et al*., 2009).

Biological wastewater treatment, whether aerobic (Loperena *et al*., 2006), anaerobic (Gao *et al*., 2007) or a combination (Sangave *et al*., 2007) thereof has been considered as a more feasible application when treating high or low strength industrial wastewaters, as opposed to chemical pre-treatments such as ozonation that often requires expensive equipment. The use of enzymes is advantageous, but requires economical considerations in order to be applied on an industrial scale (Pant & Adholeya, 2007). Anaerobic digestion for instance is cost effective and energy saving, as the biodegradation of wastewater can lead to generation of biogas which can be utilised as a renewable energy source. Aerobic systems can also be beneficial when combined with chemical treatments (Yoshida *et al*., 2009; Iaconi *et al*., 2010). The integration of anaerobic-aerobic bioreactors with a stacked configuration in treating high strength wastewaters is considered beneficial because of low costs involved, minimal space requirements and high COD removal efficiencies in excess of 83% (Chan *et al*., 2009).

Of cardinal importance is the microbial consortium. Extensive research has been applied to try and optimise the microbial community in systems that treat fatty wastewaters. The reason for this is that there have been many problems associated with their biological treatment including long chain fatty acid (LCFA) adsorption to the surface of microbial granules which subsequently leads to mass transfer problems, sludge flotation and washout of biomass and precipitation of fats by divalent ions (Pereira *et al*., 2005; Cavaleiro *et al*., 2007; Cirne *et al*., 2007). Loperena *et al*. (2006) compared the aerobic fat biodegradation potential and growth characteristics of commercial (commercially mixed

culture of microbes designed to biodegrade FOG of plant and animal source) and native (activated sludge from a pond treating dairy wastewater) inoculums. Both inocula showed a removal efficiency of 78% for milk fat. It was concluded that the selection of an inoculum or microbial population specifically for bioaugmentation of wastewaters being treated in bioreactors, requires knowledge on their biodegradation capabilities and tolerance to various other compounds present (Loperena *et al*., 2006).

The presence of FOG in wastewaters therefore may lead to many detrimental issues during the treatment of GDWW. Numerous microorganisms that have specific lipase activity have been isolated and used to treat wastewater containing FOG (Shigematsu *et al*., 2006; Matsuoka *et al*., 2009). An example of this was the performance of a mesophylic upflow anaerobic sludge blanket reactor (UASB) treating distillery grains wastewater and was evaluated as having COD removal efficiencies of up to 97.3% (Gao *et al*., 2007).

During a study, ozonation was applied as a post and pre-aerobic step for the treatment of distillery wastewater. The combined pretreatment of the effluent resulted in the enhancement of the subsequent biological oxidation step. The combined process achieved about 79% COD reduction compared to that of a non-ozonated sample where the system achieved a 34.9% COD reduction (Sangave *et al*., 2007). In a separate study the impact of GDWW on UASB granules was monitored and it was found that the GDWW lipid content was between 374 to 479 $mg.L^{-1}$ (Gie, 2007). The same study proved that granules treating wine distillery wastewater (WDWW) became encapsulated in a lipid layer after 24 days of exposure to diluted GDWW (COD = 4 000 $mg.L^{-1}$). From the above mentioned studies done, it can be deduced that various treatment methods can be used that contribute to the efficiency of a wastewater treatment (WWT) process, specifically GDWW as mentioned previously.

The objective of this study was to isolate lipid degrading bacteria from a grain distillery industry environment that anaerobically treats GDWW. These isolates were purified and then characterised biochemically in terms of their ability to degrade various fat substrates. Fat substrates were simple and complex in composition. For the initial isolation step, a triglyceride (tributyrin) was used to identify which microbes showed lipolytic/esterase activity on the solid media. The isolates that showed lipolytic/esterase activity was then also tested for their ability to utilise fats (cotton seed oil and GDWW FOG) that were more complex in composition in order to observe which isolates exhibit lipolytic activity therefore utilising primarily longer chain fatty acids (LCFA) on solid media.

Materials and methods

Isolation of lipolytic bacteria

Lipid degrading bacteria were isolated from soil obtained from a local distillery industry located in Wellington, South Africa. The soil source was a manure heap situated approximately 10 meters away from the GDWW anaerobic treatment facility. The reason for sampling this site was because the manure was collected from around the WWT facility where leaching of soil occurred via GDWW reactor piping. Because environmental stress may enhance mutation rates (Morgan, 2005), there is a possibility that microorganisms sampled from the manure heap may have adapted to the conditions of the GDWW. This may occur owing to the leaching thereof into the heap. If this adaption process did occur, there may be lipolytic species present that could be of aid to the WWT for GDWW rich in FOG owing to the constant leaching of GDWW to the soil. Furthermore, sampling from the manure heap would yield more diversity with respect to microbial populations because of decaying plant and animal excreta present.

A serial dilution was prepared by using 2 g of soil sample in sterile saline solution (0.86% NaCl) and thoroughly mixed. 100 µL from each dilution was then applied for both spread and pour-plates using Difco ^{TM} Spirit Blue Agar. The plates were incubated at 35°C for 24 to 48 hours.

Screening for lipolytic activity

Three types of media namely Difco ^{TM} Spirit Blue Agar with Tributyrin (SBA-Tri), Victoria Blue B Agar with Cotton Seed Oil (VBB-CSO) and Victoria Blue B Agar with Grain Distillery Wastewater (VBB-GDWW), were prepared for the isolation and purification of lipid degrading bacteria. Spirit Blue and Victoria Blue dyes served as indicators of lipolysis while the tributyrin, cotton seed oil (CSO) and GDWW are utilised as main source of lipids. The composition (per litre) for SBA is: 10 g of pancreatic digest of casein; 5 g yeast extract; 20 g agar; and 0.15 g Spirit Blue. Once the SBA had been autoclaved, it was allowed to cool to 40° - 50°C before aseptically adding 30 mL of Difco ^{TM} Tributyrin (3% lipase reagent).

The composition of agar together with the respective lipid sources was prepared according to a modified Davis & Ewing (1964) method (Table 1). The lipid substrate for VBB-CSO agar plates was prepared by mixing 1 mL of Tween 80 in 400 mL warm distilled water, after which 100 mL of CSO was added. This mixture was agitated vigorously to emulsify. The VBB-GDWW agar was prepared in the same manner as VBB-CSO agar,

except that the lipid substrate used was GDWW. It was not necessary to mix the GDWW with Tween 80 in 400 mL warm water as the GDWW was readily dispersed throughout the agar medium.

The reason for the choice of the different agar and lipid source combinations was to establish which combination would show the best visual result in terms of lipolytic activity on solid media. Using CSO and tributyrin as lipid sources are known to yield visual observation for esterase/lipolytic activity. FOG in GDWW will then be used as a fat substrate as well and then compared to the visual results obtained from the latter fat substrates to establish whether it may also be used as a visual indication of esterase/lipolytic activity on solid media.

Table 1. Composition of the different agar media and lipid substrates

SBA-Tri (1 000 mL)	VBB-CSO (750 mL)	VBB-GDWW (750 mL)
10 g pancreatic digest of casein	7.5 g peptone	7.5 g peptone
5 g yeast extract	2.25 g yeast extract	2.25 g yeast extract
20 g agar	3.75 sodium chloride	3.75 sodium chloride
0.15 g Spirit Blue	15 g agar	15 g agar
30 mL Tributyrin	75 mL VBB (0.5:750)	75 mL VBB (0.5:750)
	37.5 mL CSO	37.5 mL GDWW

Colonies that were surrounded by a clear zone on SBA-Tri or those that formed a dark blue precipitate on VBB-CSO were picked and purified on SBA containing Difco ™ Lipase Reagent as lipid source. Pure cultures were then transferred to 10 mL Nutrient Broth (NB) and incubated at 35°C for 24 h. The composition (per litre) of the NB was: 1 g meat extract; 2 g yeast extract; 5 g peptone; and 8 g NaCl. Purified strains were then frozen at -80°C in a storage medium (500 µL of 80 % sterile glycerol and 500 µL culture suspension). An overview of the purification and preliminary characterisation is shown in Figure 1.

Strain characterisation

The isolates with possible lipolytic activity were characterised in terms of their catalase and oxidase reactions, Gram stain and morphology, oxidative/fermentative reaction according

to Hugh and Leifson (1953) as well as API 20NE and/or 50CHB data profiles (Biomérieux® sa 69280 Marcy l' Etoile-France).

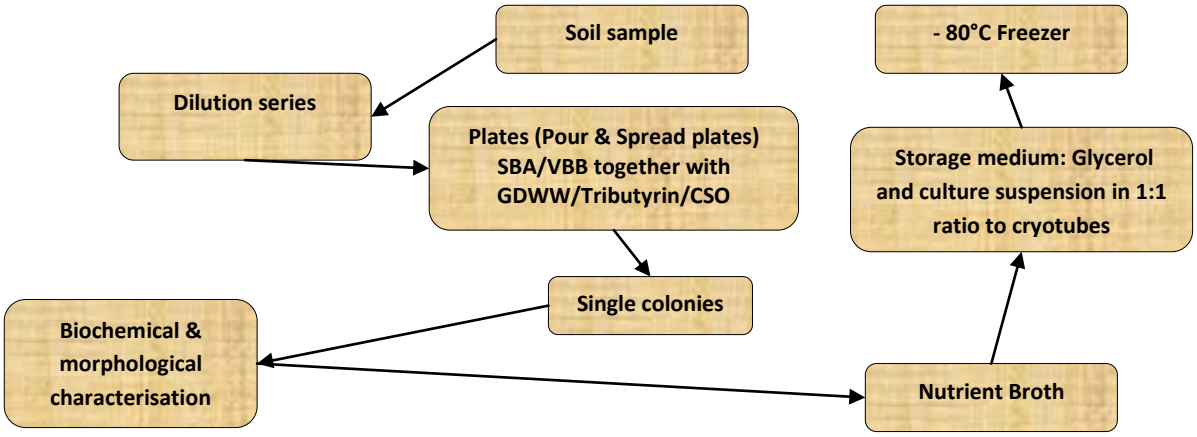

Figure 1. Overview of the isolation, purification and preliminary characterisation for lipid degrading bacteria.

Furthermore after strain identification, the isolates were tested for their ability to grow and utilise the lipid substrates at pre-determined temperatures (25°, 30°, 37° and 50°C). These temperatures were chosen on basis of literature that states varying optimum growth and enzyme temperatures (Williams *et al*., 1990; Markossian *et al*., 2000; Heylen *et al*., 2007; Senthilkumar & Selvakumar, 2008). The strains were each subjected to the following media and lipid sources namely SBA (Tributyrin), VBB (CSO) and VBB (GDWW). The plates containing the respective isolate for each medium, was incubated in triplicate at their optimum growth, mesophylic and optimum enzyme activity temperatures for 3 days. Temperature ranges were chosen to determine at which temperature enzymatic activity was the strongest.

Results and discussion

Screening for lipase activity

Colonies from SBA that showed a clear zone or a dark blue precipitate were isolated and then re-streaked to ensure culture purity. Tributyrin is the simplest of fats and therefore when metabolised, lipolysis can readily be detected by the formation of a clear zone around the colony in a turbid fat-emulsion agar. The presence of a dark blue precipitate indicates partial hydrolysis of the fat present. As mentioned by Alford & Steinle (1967), tributyrin is generally insoluble, but when metabolised, the hydrolysis products, monobutyrin and dibutyrin are water solublised to form the clear zones around the colony as shown in Fig 2. A recommendation was also made by Starr (1941) for the use of SBA

as the preferred medium for the detection of lipolytic bacterial species. Several bacteria, mould and yeast species were cultured on SBA (Starr, 1941; Fryer *et al*., 1966). The authors recommended this medium as the preferred culture medium, owing to its non-inhibitory effect on growth or lipolytic capability as well as not giving false positive tests by non-lipolytic microorganisms (Starr, 1941; Fryer *et al*., 1966).

In the current study, lipolytic colonies were also grown on VBB agar using CSO as the lipid substrate instead of tributyrin. The reason for this was that tributyrin is not sufficient to indicate true lipolytic activity as hydrolysis can also be catalysed by esterases (Salleh *et al.,* 2006).

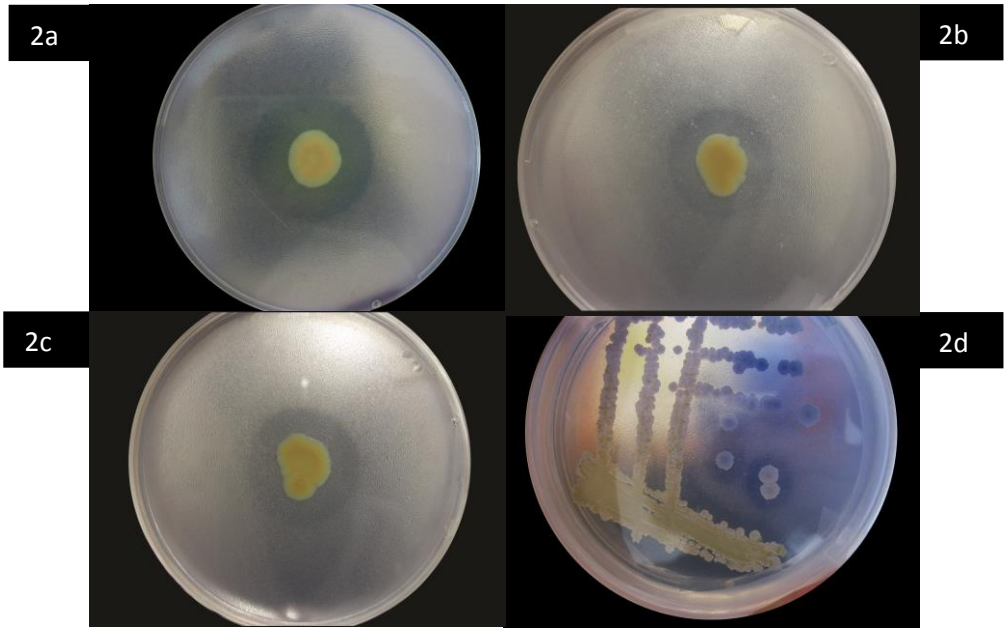

Figure 2. The four photographs (2a-d) depicts the clear zone formed around the colony. The clear zone formed against a turbid medium due to the hydrolysis of tributyrin which is indicative of lipolytic/esterase activity.

Colony morphology for the isolates is shown in Fig 3. Strains 3a, b and d have circular colonies, while 3c is irregular in shape. VBB shows an intense blue colour upon the reduction of the pH due to hydrolysis (Salleh *et al.,* 2006). For this purpose, CSO was used on the basis of possessing a more complex fatty acid profile (Table 2) as opposed to tributyrin which is a triglyceride.

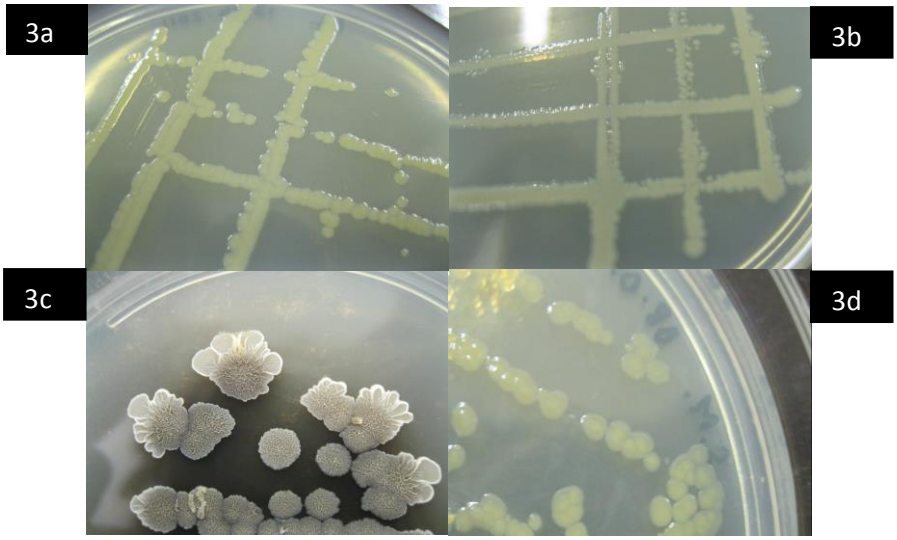

Figure 3. Colony morphology is depicted for each respective isolate on nutrient agar (3a – 3d).

Table 2. The composition of cottonseed oil indicating the methyl ester type and the respective percentage composition thereof, used during this study (Anon., 2008)

Methyl Ester type	(% Composition by mass)
C16:0	6.0
C18:0	3.0
C18:1	35.0
C18:2	50.0
C18:3	3.0
C20:0	3.0

The cottonseed oil conforms to the requirements of American Oil Chemicals Society (AOCS) Method Ce 1-62

Cottonseed oil also consists of the main LCFAs found in cereal grains and grain products namely palmitic, oleic and stearic acids (Becker, 2007). These LCFAs are known to be problematic during fatty acid wastewater treatment but also useful for methane production during anaerobic digestion (Li *et al*., 2002; Kim *et al*., 2004; Pereira *et al*., 2005; Palatsi *et al*., 2009). In this study there was a distinct blue zone that formed in the medium below colonies and/or around the outskirts of the colonies (Fig 4). These results are also in accordance with a previous study conducted by Alford & Steinle (1967), whereby triglycerides namely tributyrin, trihexanoin, trioctanoin, triolein, lard, butter oil, CSO, olive oil and corn oil were examined to determine their suitability as effective lipid substrates. The results showed a variation in the background colour in medium as well as in the lipolytic zones observed (Alford & Steinle, 1967). An indication of lipolysis yields a clear

zone surrounding the colony on a turbid medium while zones from fermentation producing acids retain the blue colour (Alford & Steinle, 1967; Jones & Richards, 1952). Therefore, no medium was supplemented with a carbohydrate source as this would probably yield false positive results (Shelley *et al*., 1987).

Tributyrin is insoluble; nonetheless when it is hydrolysed to butyric acid which is soluble, it tends to form a diffuse blue zone rendering tributyrin as a mere presumptive test (Fryer *et al*., 1966) for use as substrate during lipolytic studies. If, however, any monobutyrin or dibutyrin is formed, both being soluble to slightly soluble, a clear zone of hydrolysis will surround the colony as seen in Figs 2 a-d and 4. As part of this study, VBB agar was also combined with GDWW as lipid substrate. The reason for the use of GDWW as fat source, was that it resembles a substrate high in FOG as opposed to tributyrin which is chemically synthesised and not of natural origin and thus easily hydrolysed. Although bacterial isolates showed good growth on this medium, no distinct lipolytic activity could be identified. This could be due to the fact that GDWW does not form a turbid fat-emulsion agar and that the effectiveness of lipolytic visual observation depends on a clear zone forming against a turbid background. The good growth can also be attributed to the utilisation of other components 'other than the fatty acids' within the GDWW. The pH of GDWW added was 3.4 – 3.6.

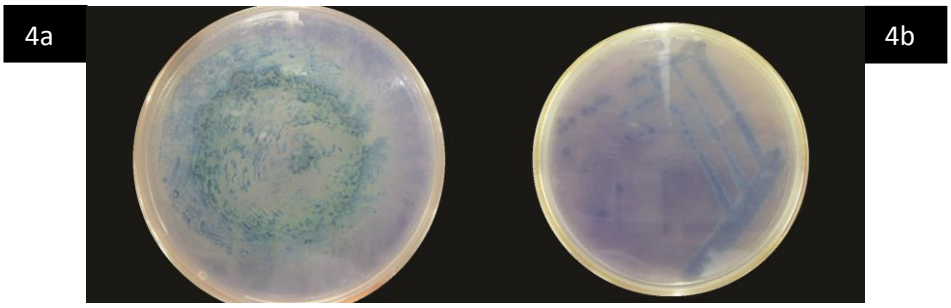

Figure 4. Two isolates (4a) and (4b) grown on VBB (CSO). Distinct blue zones formed below the colonies and/or around the outskirts of the colonies, against a blue background.

Morphological, phenotypical and biochemical characterisation

The four isolates (1-4) that showed visual clearing on the agar plates were morphologically and biochemically characterised (Table 3). Isolates 1, 2 and 3 were almost similar in structure with only morphological differences. One main structural difference between isolates 1 and 2 is that isolate 1 is short rods, while isolate 2 was small, narrow rods. Isolate 4 was the only Gram positive isolate and had distinctly short thick rods. It was also easily characterised as opposed to the other isolates by possession of a very prominent central endospore.

In the current study only one isolate was positive for oxidase activity but all 4 isolates were positive for catalase. These isolates were also grown in NB with isolate 3 forming a slimy pellicle layer at the top of the medium. This would suggest that this bacterium is an obligate aerobe as opposed to the other more facultative isolates which showed growth throughout the NB. In this case it could be that they are facultative. By using the method proposed by Hugh & Leifson (1953), the isolates were characterised as either oxidative or fermentative. Isolate 2 was observed and found to be the only fermentative bacterium.

The isolates were also phenotypically characterised on nutrient agar (Table 4). Isolates 1, 2 and 3 were similar in morphology, colony margin and pigmentation, whereas isolate 1 had a slightly convex elevation. Isolate 4 was observed as irregular in form and had an erose margin. It was also opaque and dull in appearance and formed a dark brown discolouration on the NA after 5 days at room temperature. This could be as a result of its metabolic activity upon assimilation of nutrients present, such as the organic nitrogen source present in the medium (Nakamura, 1989). Biochemical characterisation was done using the API 20NE for isolate 1, 2 and 3 and 50CHB identification system for isolate 4 (Tables 5 and 6). The identification was then generated using the Apiweb™ software V7.0 (Table 7).

Table 3. The morphological and biochemical characteristics for the four unidentified bacterial isolates

Isolate	Gram stain	Morphology	Endospore	[a]	[b]	Growth in [c]	[d] O/F (Open tube - aerobic)	O/F (Closed tube - anaerobic)	Reaction	Acid/ Alk
1	-	Short rods Single, double chains	No	+	+	Murky	+	-	Oxidative	Alk
2	-	Very small rods Single, double chains	No	+	-	Murky	+	+	Fermentative	Acid
3	-	Thin, small rods Long chains (3 & more cells per chain)	No	+	-	Murky Slimy layer top of medium	+	-	Oxidative	Alk
4	+	Short, thick rods Single cells	Endospore (Central)	+	-	Slightly murky	+	-	Oxidative	Alk

[a] catalase activity; [b] oxidase activity; [c] Nutrient Broth; [d] Oxidative/Fermentative test (Hugh & Leifson, 1953)

Table 4. Phenotypical characteristics of the four isolates when grown on NA

Isolate	1	2	3	4
Form of colony	Circular	Circular	Circular	Irregular
Elevation	Convex	Flat	Flat	Flat
Margin	Entire	Entire	Entire	Erose
Pigmentation	No	No	No	No
Texture, optical properties & Appearance	Smooth, Transparent and Shiny	Smooth, Transparent and Shiny	Smooth, Transparent and Shiny	Smooth, Opaque and Dull

Table 5. Biochemical characteristics of isolates using the API 20NE identification kit

Test		Isolate		
		1	2	3
NO_3		-	+	+
Indole production		-	-	-
Glucose acidification		-	-	-
Arginine dihydrolase		+	+	-
Urease		+	+	-
Esculin hydrolysis		+	+	+
Gelatine hydrolysis		-	+	+
PNPG		-	+	+
Fermentation of:	Glucose	+	+	+
	Arabinose	+	+	-
	Mannose	+	+	+
	Mannitol	+	+	-
	N-acetyl-glucosamine	+	+	+
	Maltose	-	+	+
	Gluconate	+	+	-
	Caprate	+	-	-
	Adipate	-	-	-
	Malate	+	+	+
	Citrate	+	+	+
	Phenyl-acetate	-	-	-
Oxidase		+	-	-

Table 6. Biochemical characteristics of isolate 4 using the API 50CHB identification kit

Test Strip 0 - 19	Result	Test Strip 20 - 39	Result	Test Strip 40 - 61	Result
CONTROL	+	Methyl-αD-mannopyranoside	-	D-turanose	+
Glycerol	+	Methyl-αD-glucopyranoside	+	D-lyxose	-
Erythritol	-	N-acetylglucosamine	-	D-tagatose	-
D-arabinose	-	Amygdalin	+	D-fucose	-
L-arabinose	+	Arbutin	+	L-fucose	-
D-ribose	+	Esculin Ferric citrate	+	D-arabitol	-
D-xylose	+	Salicin	+	L-Arabitol	-
L-xylose	-	D-cellobiose	+	Potassium gluconate	-
D-adonitol	-	D-maltose	+	Potassium 2-ketogluconate	-
Methyl-βB Xylopyranoside	-	D-lactose (bovine origin)	-	Potassium 5-ketogluconate	-
D-galactose	+	D-melibiose	+	ONPG	+
D-glucose	+	D-saccharose (sucrose)	+	Arginine dihydrolase	-
D-fructose	+	D-trehalose	+	Lysine decarboxylase	-
D-mannose	+	Inulin	+	Orthinine decarboxylase	-
L-sorbose	+	D-melezitose	-	Citrate utilisation	-
L-rhamnose	+	D-raffinose	+	H_2S production	-
Dulcitol	-	Amidon (Starch)	+	Urease	-
Inositol	+	Glycogen	+	Trytophane desaminase	-
D-mannitol	+	Xylitol	-	Indole production	-
D-sorbitol	+	Gentiobiose	+	Acetoin production	+
				Gelatinase	+
				NIT	-

Table 7. Identification using the API 20NE and 50CHB data System, of the strains from soil sampled close to the grain distillery wastewater treatment facility

Strain	Significant taxa	Percentage identification
1	*Pseudomonas fluorescens*	99.9
2	*Pseudomonas luteola*	99.9
3	*Stenotrophomonas maltophilia*	99.9
4	*Bacillus licheniformis*	87.7

Strains 1, 2, 3 and 4 were respectively identified as *Pseudomonas fluorescens, Pseudomonas luteola, Stenotrophomonas maltophilia* and *Bacillus licheniformis*.

Pseudomonas fluorescens (Table 8) showed a decrease in halo diameter as the incubation temperature increases. When tributyrin was used as lipid source the halo diameter after an incubation period of 48 h was as follows: 2 mm at 37°C, 3 mm at 30°C and 5 mm at 25°C. The optimum growth of the organism occurred at 25°C. VBB containing CSO as lipid source produced dark blue zones at 30 and 25°C which is positive for lipolysis. There was no indication of lipolysis where GDWW was used as lipid source. From Table 8 it can be deduced that the optimum enzyme activity of the organism occurred at 25°C as the halo diameter observed was the largest of the three temperatures. The possible reason for no dark blue zones forming at 37°C could be that this temperature is unfavourable for optimal enzyme activity.

P. luteola (Table 9) showed growth but, no indication of lipolysis on VBB (GDWW) at any of the temperatures, but VBB-CSO shows blue colony formation at 30°C which is indicative of lipolytic activity. Growth was observed at 37°C using CSO as lipid substrate, but lipolysis was absent as no blue colonies were formed. Utilisation of SBA-Tri had a distinct clear zone around colonies at 30° and 37°C.

Table 8. The growth of the *P. fluorescens* isolate (1) on media at mesophyllic and optimum growth temperatures and temperature for optimum enzyme activity

	Growth Temperature								
	Mesophyllic (37°C)			**Optimum Growth** (30°C)			**Optimum enzyme** (25°C)		
	Growth	**Lipolysis**	**Halo (mm)**	**Growth**	**Lipolysis**	**Halo (mm)**	**Growth**	**Lipolysis**	**Halo (mm)**
SBA (Tributyrin)	+	+	2	+	+	3	+	+	5
VBB (GDWW)	+	-		+	-		+	-	
VBB (CSO)	+	-		+	+		+	+	

(+) Growth/lipolysis observed; (-) Growth/lipolysis absent

Table 9. The growth of the *P. luteola* isolate (2) on media at mesophyllic and optimum growth temperature and temperature for optimum enzyme activity

	Growth Temperature					
	Mesophyllic (37°C)		**Optimum Growth** (30°C)		**Optimum enzyme** (30°C)	
	Growth	**Lipolysis**	**Growth**	**Lipolysis**	**Growth**	**Lipolysis**
SBA (Tributyrin)	+	+	+	+	+	+
VBB (GDWW)	+	-	+	-	+	-
VBB (CSO)	+	-	+	+	+	+

(+) Growth/lipolysis observed; (-) Growth/lipolysis absent

The *S. maltophilia* isolate (3) (Table 10) showed no indication of lipolysis on VBB-GDWW at 37°C and 30°C. At 30°C, using CSO as lipid substrate, the organism showed no sign of lipolysis. This could possibly be due to the inability of the isolate to biodegrade the fatty acids at the respective temperature. As lipolysis was shown at 37°C using VBB-CSO, the assumption could be made that this temperature provides optimum lipolytic activity for growth of the isolate on solid media. A previous study by Heylen *et al*. (2007) focused on the isolation of nitrate-reducing bacteria in soil, where a few strains showing lipolytic activity. These included *S. maltophilia* LMG 958 which hydrolysed Tween 80.

B. licheniformis (Table 11) grown on VBB-CSO was positive for lipolytic activity at 37°C. A clear zone surrounding the colony was visible using SBA-Tributyrin therefore indicating lipolytic activity. This organism showed good growth even at 50°C as well as at its optimum enzyme activity temperature of 37°C. However, no clear lipolysis was observed at 50°C as the agar plates at this temperature dried out and caused the blue colour of the media to fade, preventing clear lipolytic results being visualised.

A recommendation can, therefore, be made that when trying to establish lipolysis at high temperatures such as 50°C, 1 cm thick agar plates should rather be prepared. This could prevent the dehydration of media. This would also prevent the blue dye from disappearing, making it possible to establish whether or not lipolysis had occurred.

Table 10. The growth of the *S. maltophilia* isolate (3) on media at mesophylic temperature, optimum growth temperature and optimum enzyme activity temperature

	S. maltophilia					
	Mesophyllic (37°C)		**Optimum Growth** (30°C)		**Optimum enzyme** (30°C)	
	Growth	**Lipolysis**	**Growth**	**Lipolysis**	**Growth**	**Lipolysis**
SBA (Tributyrin)	+	+	+	+	+	+
VBB (GDWW)	+	-	+	-	+	-
VBB (CSO)	[a]+	+	+	-	+	-

(+) Growth/lipolysis observed; (-) Growth/lipolysis absent; (a) Faded blue colonies

Table 11. The growth of the *B. licheniformis* isolate (4) on media at mesophyllic growth temperature and optimum enzyme activity temperature

	B. licheniformis					
	Mesophyllic (37°C)		**Optimum Growth** (50°C)		**Optimum enzyme** (37°C)	
	Growth	**Lipolysis**	**Growth**	**Lipolysis**	**Growth**	**Lipolysis**
SBA (Tributyrin)	+	+	+	-	+	+
VBB (GDWW)	+	-	+	-	+	-
VBB (CSO)	+	[b]+	+	-	+	[b]+

(+) Growth/lipolysis observed; (-) Growth/lipolysis absent; (b) Deep blue colonies formed on blue background

Conclusions

Many microorganisms that have lipase activity have been isolated and characterised to treat wastewater containing fats, oils and grease (FOG) (Shigematsu *et al*., 2006; Matsuoka *et al*., 2009). During this study, by initiating several biochemical, chemical and microbiological techniques, four bacterial species from a manure heap situated close to a GDWW treatment facility were successfully isolates and characterised by showing visible lipase/esterase activity on SBA-Tri. These isolates were then subjected to VBB with GDWW as lipid source. Growth was present, but did not give a distinct indication of lipolytic activity, although colonies formed were dark blue with discolouration of dye around the growth. The growth was attributed to the assimilation of other components in the wastewater. The isolates were also subjected to VBB with CSO as lipid substrate whereby lipolytic activity was indicated by formation of distinct blue zones around or below the

respective colonies. No lipolysis was shown in the case of the *B. licheniformis* (50°C), *S. maltophilia* (37°C), *P. luteola* (37°C) and *P. fluorescens* (37°C) isolate at the respective temperatures indicated. By subjecting the isolates to different temperatures as well as media and fatty acid substrate combinations, information was obtained about their ability to utilise these lipid substrates and to visually observe the degree of lipolytic activity at a specific temperature. The *B. licheniformis* isolate failed to show any lipolytic activity at 50°C as result of the agar being dehydrated and the blue colour fading thus making it impossible to get a clear distinction of lipolytic activity. A recommendation can be made to pour the plates thicker and sealing the plates which would then facilitate reading the thermophilic incubation temperature. From this study it was found that the optimal temperature for *S. maltophilia* for lipolysis was visibly noted on VBB-CSO to be 30°C. The reason for this could be that at this temperature, enzymatic activity is more optimal. Although the *P. fluorescens, P. luteola, S. maltophilia* and *B. licheniformis* strains show lipolytic/esterase activity, the visual observation of lipid clearing on agar plates was not sufficient to establish which fatty acids were biodegraded in the grain distillery wastewater (GDWW) for growth on Victoria Blue B with GDWW as substrate (VBB-GDWW). For this study it was therefore concluded that when scanning for lipolytic activity, it is important to use SBA-Tri and VBB-CSO in conjunction with VBB-GDWW. Hydrolysis of lipids using the SBA-Tri combination, yields a clear zone of lipolysis and VBB-CSO yields dark blue colonies against a blue agar background. Although VBB-GDWW does not form a blue colony or yield a clear zone of lipolysis, bacterial growth using this agar media combination was present. This growth indicates that other components within the GDWW are possibly being utilised other than the FOG. This may be possible as GDWW has a complex composition. Furthermore, the use of GDWW as lipid source showed no signs of inhibition toward the growth of lipolytic strains. It is thus possible that these strains can possibly be used as bioaugmention in anaerobic digestors to assist the biodegradation process during the treatment of GDWW so as to minimise the COD load. It is important that the isolates be subjected to more definitive tests in terms of varying FOG concentrations in the GDWW especially during batch fermentation studies. When the batch fermentation process is complete, a fatty acid profile must also be established to determine specifically which LCFAs are being biodegraded. The results can then be used to see which isolate is capable of hydrolysing which specific LCFA. This information can then be beneficial in reducing specific LCFAs that are problematic in the treatment of fatty wastewaters such as GDWW.

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CHAPTER 4

BATCH BIODEGRADATION OF FATS, OILS AND GREASE IN GRAIN DISTILLERY WASTEWATER BY BIOAUGMENTING WITH SELECTED BACTERIAL STRAINS

SUMMARY

Three isolates *Stenotrophomonas maltophilia (1), Pseudomonas luteola (2)* and *Bacillus licheniformis (3)* were subjected to grain distillery wastewater (GDWW) and monitored in batch fermentation for their ability to biodegrade the fats, oil and grease (FOG). The *S. maltophilia* and *P. luteola* strains gave a total FOG reduction of 63% and 50% over an 18 and 21 d batch fermentation period, respectively. The *B. licheniformis,* however, resulted in a total FOG reduction of 83% after 18 d exposure to GDWW. The use of this strain was therefore recommended for GDWW treatment and may even be suitable for the treatment of other fatty wastewaters. All strains showed that biodegradation of GDWW could be improved by an initial acclimatization phase. Acclimatized strains were also subjected to GDWW and the fermentation samples quantified for long chain fatty acids (LCFA). This gave an indication of the strains metabolic behaviour in terms of LCFA utilised and formed when subjected to GDWW. This information is of value when selecting a suitable strain for GDWW wastewater treatment.

INTRODUCTION

The high-strength effluent wastewater produced by local distilleries in South Africa is an issue of concern. The chemical oxygen demand (COD) values may vary for the effluent depending upon the organic and/or recalcitrant products present. Wine distillery wastewater is high in phenols and heavy metals (Bustamante *et al*., 2005; Gie, 2007; Green, 2007; Musee *et al*., 2007) and has to undergo treatment, while grain distillery wastewaters are high in fats, oils and grease (FOG), leading to lower bioreactor operational efficiency during anaerobic digestion post-treatment (Gie, 2007). Owing to the recent upsurge in the demand for alcohol beverages and spirits-related products, their production has also contributed to an increase in wastewater production (Musee *et al*, 2007). For every litre of alcohol produced, 8 - 15 L of wastewater is generated (Mohana *et al*, 2009) and for every litre of grain whisky produced, 16 – 21 L of effluent water is

generated (Tokuda *et al*, 1998). On average the South African wine industry produces 140 – 160 million L of distilling wine each year (Anon., 2008). Distillery industries are therefore forced by legislation to consider and apply wastewater treatment techniques or be faced with the consequence of high penalties.

Over the years anaerobic digestion has gained popularity and a great deal of research has focused on anaerobic digestion as a feasible treatment option for distillery wastewater as well as other food and industrial wastewaters (Saatci *et al.*, 2003; Moletta, 2005; Wang *et al.*, 2009; Hwang *et al.*, 2010). Biological treatment whether aerobic, anaerobic, physicochemical or a combination thereof can be seen as a possible treatment option for the treatment of distillery wastewater (Goodwin & Stuart, 1994; Heredia *et al.*, 2005; Pant & Adholeya, 2007; Mohana *et al.*, 2009; Mallik *et al*., 2010).

The microbial ecology is a complex process, consisting of several bacterial groups all initiating reactions in a coordinated manner to convert organic compounds to carbon dioxide and methane (Anderson *et al*., 2003). A great deal of research has focused on optimising the sludge population and therefore selecting for the most desirable species for a specific function (Yuan *et al.*, 2008). A study also investigated a method for detecting dominant microbial population sizes within reactor sludge (Liu *et al.*, 2009) which could be important for controlling the growth of unwanted bacterial species.

Bioaugmentation is a treatment method where indigenously isolated or genetically modified bacteria are used to inoculate bioreactors treating wastewater or to treat hazardous industrial wastes to enhance the removal of undesirable compounds (Goldstein *et al.*, 1985; Limbergen *et al.*, 1998; Gentry *et al.*, 2004; Singer *et al.*, 2005). In a study in 2010, it was concluded that bioaugmenting anaerobic sludge treating oleate wastewater with *Syntrophomonas zehnderi* yielded a higher methane yield than found in the absence of this microorganism (Cavaleiro *et al.*, 2010). Bioaugmentation has also been used to treat pulp and paper mill industry wastewaters (Yu & Mohn, 2002) as well as for the treatment of dairy wastewater which is high in fat. It was concluded that the periodic addition of microorganisms may be needed to achieve a high performance treatment (Loperena *et al.*, 2006). Nonetheless, it may be that long chain fatty acid (LCFA) biodegradation relies on the addition of LCFA-degrading strains to the anaerobic sludge. This can contribute to a faster bioreactor start-up phase or to enhance the treatment of disrupted treatment systems, especially with the LCFA accumulating when adhesion occurs on the anaerobic biomass (Cavaleiro *et al.*, 2010).

The aim of this research was to subject grain distillery wastewater (GDWW), at specific FOG concentrations, to the three bacterial strains previously isolated (Chapter 3 of this thesis). These included *Stenotrophomonas maltophilia (1), Pseudomonas luteola (2) and Bacillus licheniformis (3)* strains. An acclimatization phase was initiated in order to determine which strains were capable of biodegrading the FOG in the shortest possible time. A fatty acid profile was determined for the individual strains to elucidate which fatty acids are utilised. Strains that show promising FOG biodegradation results can therefore be used as a pre-treatment option prior to anaerobic digestion to try and facilitate reactor operational efficiency treating GDWW.

MATERIALS AND METHODS

Bacterial growth curves

The batch biodegradation phase was done to determine which of the three strains (*S. maltophilia, P. luteola and B. licheniformis*) were able to adapt to the GDWW and biodegrade the FOG successfully. Growth curves of each strain were established and used to determine the concentration required when inoculating GDWW during batch biodegradation.

Nutrient broth (NB) (Merck) was prepared and inoculated with each of the respective isolates. This was initiated by monitoring the growth of isolates spectrophotometrically (Spectronic® 20 Genesys™) at an optical density (OD) of 500 nm and at 30 min intervals for 6 h. Viable cell counts were monitored by preparing a dilution series of 10^{-1} to 10^{-12} and using the pour plate technique with Nutrient Agar (NA) (Merck) as growth medium.

Statistical Analysis

IFR Microfit© (V1.0) is a computer application designed to analyse microbial growth data. Viable cell counts monitored spectrophotometrically in triplicate during the bacterial growth curve establishment for each isolate, was entered into an excel spread-sheet as logarithm to base 10 of the actual counts. These log cfu.mL^{-1} values were then transferred to Microfit© to statistically obtain information based on the Baranyi Growth model. The information was graphically represented and information regarding the initial bacterial cell density (N_0), lag time (t-lag) and doubling time (t-d) was statistically generated.

Inoculum preparation

Each isolate was streaked onto NA and incubated at 37°C for 24 h to ensure that a pure culture was being used. Single colonies grown in NB until an initial cell concentration of 1 x 10^{12} cells.mL^{-1} was obtained using the respective growth curve. One millilitre of each cell suspension was transferred to separate pre-treated GDWW sample batches for the biodegradation phase.

GDWW (substrate) preparation

Raw untreated GDWW obtained from Distell, Wellington, South Africa was pre-treated to try and lower the FOG concentration. This was achieved by flocculating 1 500 mL of raw GDWW with a pre-determined amount of $FeCl_3$ and then centrifuging (Beckman Coulter TJ-25 centrifuge) 10 000 rpm for 10 min at 4°C. Two distinct layers were formed after centrifugation with a clear supernatant layer and a solid pellet at the bottom of the centrifuge tube. This was concentrated FOG and total soluble solids (TSS). The solid pellet was discarded and the supernatant collected and used as substrate in the subsequent batch studies after being diluted with distilled water in a 1:4.5 ratio. The pH was adjusted to 7.2 – 7.3 using sulphuric acid (H_2SO_4:H_2O = 1:1). A final volume of 200 mL (44 mL pre-treated GDWW and 156 mL dH_2O) was prepared in 250 mL Schott bottles. The samples were sterilised before the addition of the respective isolate. Each sample contained only the pre-treated GDWW and the isolate of interest. No other trace minerals were added so as to ensure that the isolate present can, if possible, only utilise components in the GDWW as their primary nutrient sources.

FOG extraction

The Hexane Extractable Gravimetric method (APHA, 1998) was used for determination of the FOG concentration. A 50.0 g sample of pre-treated GDWW was acidified to pH = 2 with (H_2SO_4:H_2O = 1:1). The acidified sample and 100 mL ethanol ($\geq$99.8%), 20 mL n-hexane fraction (Merck) and 20 mL of diethyl ether (Merck) were added to a 500 mL separating funnel. The contents were shaken vigorously for 2 min and allowed to stand for 10 min to ensure separation of the water and solvent layer. Each layer was collected in a separate container. Two grams of anhydrous sodium sulphate was added to the container. The water layer was then re-treated by the addition of 20 mL n-hexane and 20 mL diethyl ether for further extraction of oils and this repeated three times. The cumulative FOG-containing solvent layer was added to a clean and previously weighed 250 mL round

bottom flask. The sample was then attached to the distillation apparatus (Büchi Rotavapor R-114) and the solvent evaporated at 60°C. The flask was then allowed to cool in a glass desiccator, gravimetrically measured, and quantified to give $mg.L^{-1}$ FOG.

Growth activity

During the batch biodegradation phase, three triplicate samples were prepared namely: P = *P. luteola*, S = *S. maltophilia* and B = *B. licheniformis* (Fig. 1,2 & 3). Each 250 mL bottle containing 200 mL pre-treated GDWW and the respective isolate (1 x 10^{12} $cells.mL^{-1}$) was incubated at 37°C on a shaker at 125 rpm. Growth activity was monitored for all samples by allowing the isolates in inoculation 1 for sample P (P1), inoculation 1 for sample S (S1) and inoculation 1 for sample B (B1) from each sample to utilise the GDWW. This implies biodegradation for a period of 5 d whereby each triplicate batch of P, S and B was sampled daily during these 5 d and to quantitatively determine bacterial counts in the GDWW on NA. This will give us an indication of cell viability when exposed to GDWW.

Isolate incubation protocol

When screening for lipolytic activity (Chapter 3), it was seen that *P. luteola and S. maltophilia* (td = 34 and 42 min) were slower growing isolates as opposed to *B. licheniformis* that grew faster (td = 28 min). For this reason, when exposed to GDWW for a period of 5 d, a different incubation protocol for each isolate was established.

It was decided that *P. luteola* and *S. maltophilia* be subjected to an incubation period of 3 d, while *B. licheniformis* to a 5 day incubation period. This was initiated after the initial 5 day incubation period in the case of *P. luteola* and *S. maltophilia* while for *B. licheniformis*, it was done during the 5 day growth activity monitoring period due to its faster growth rate.

Batch biodegradation phase

Sample P inoculated with *P. luteola* was allowed to reach a cell concentration of 1 x 10^{12} $cells.mL^{-1}$, before 1 mL was inoculated into P1 (Fig. 1). Within a 9 day biodegradation period, 1 mL of P1 was transferred after 24 h to a fresh batch of pre-treated GDWW and this was repeated for 7 days. P7 was incubated for 3 d before being sampled to determine the FOG content. P7 was used to inoculate P8 (1 mL) on day 10. P8 was incubated for 24 h. One millilitre of P8 was transferred to P9 and incubated for 3 days and then sampled to determine the FOG concentration. P10 was inoculated with 1 mL of P9 and incubated for

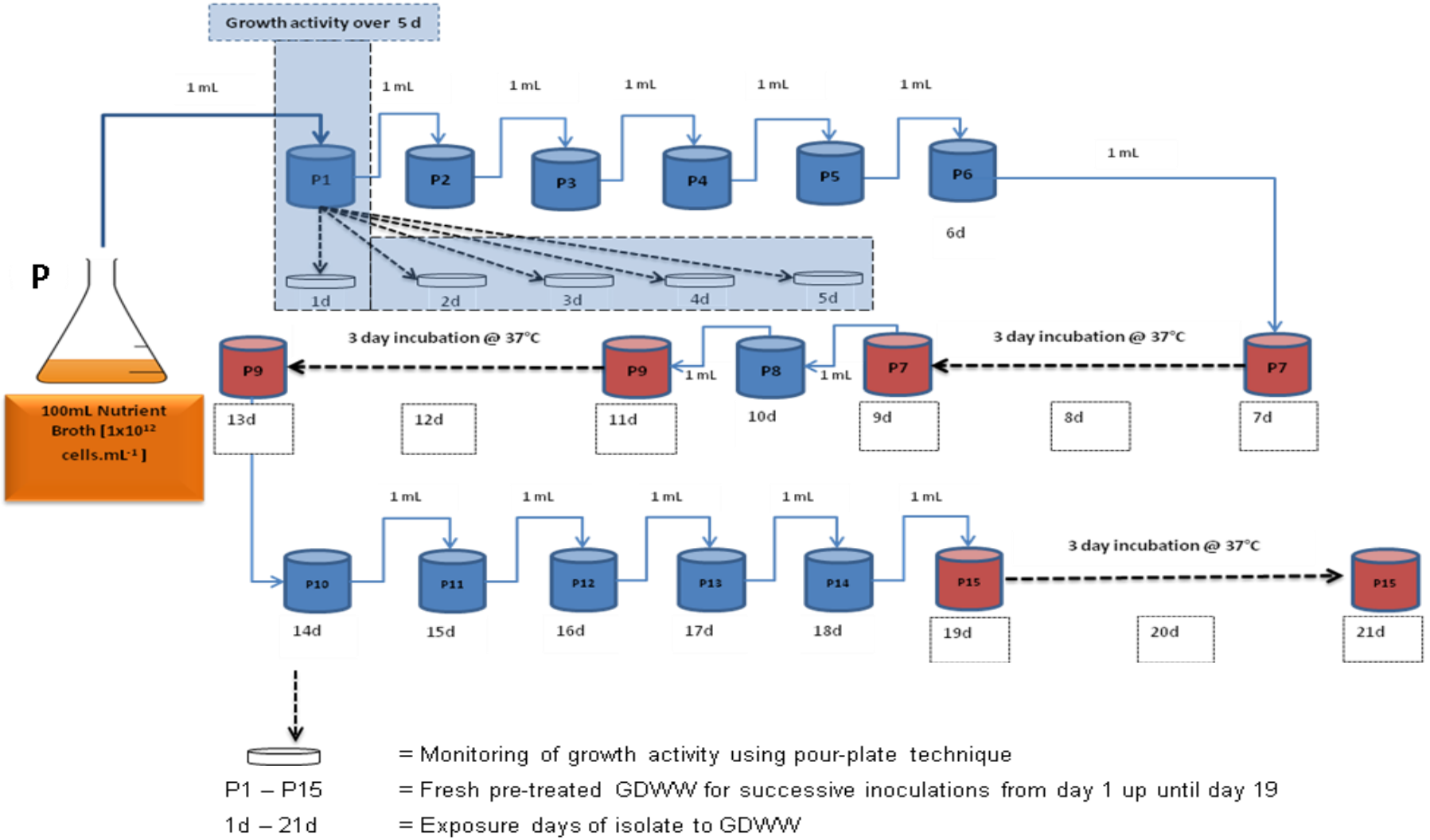


Figure 1. Sample P (*P. luteola*) included 15 one millilitre inoculations across a period of 21 d. Growth activity was monitored for 5 consecutive days by plating from the same sample (P1). Six FOG concentrations were measured at days 7, 9, 11, 13, 19 and 21 to monitor FOG reduction.

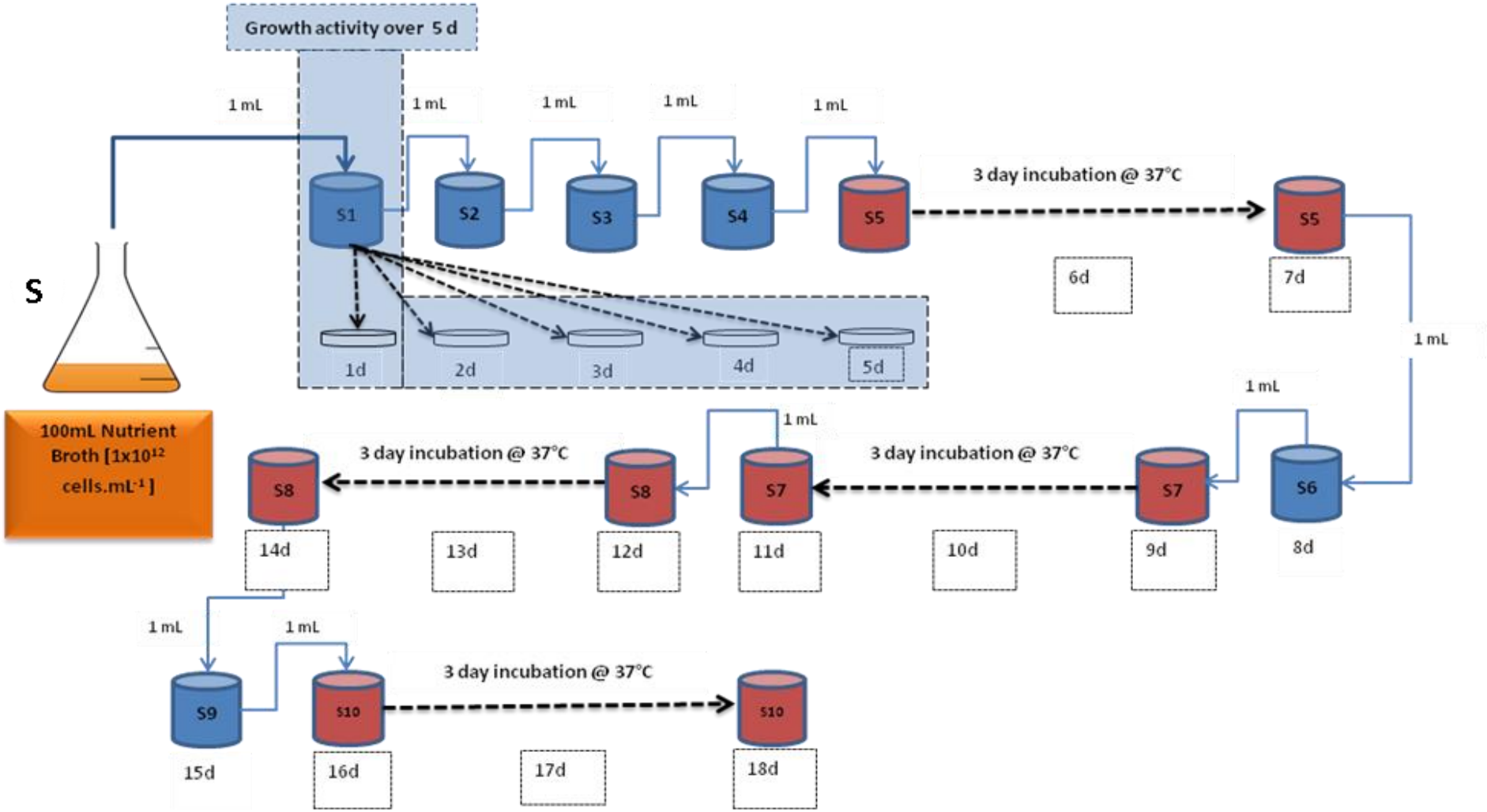
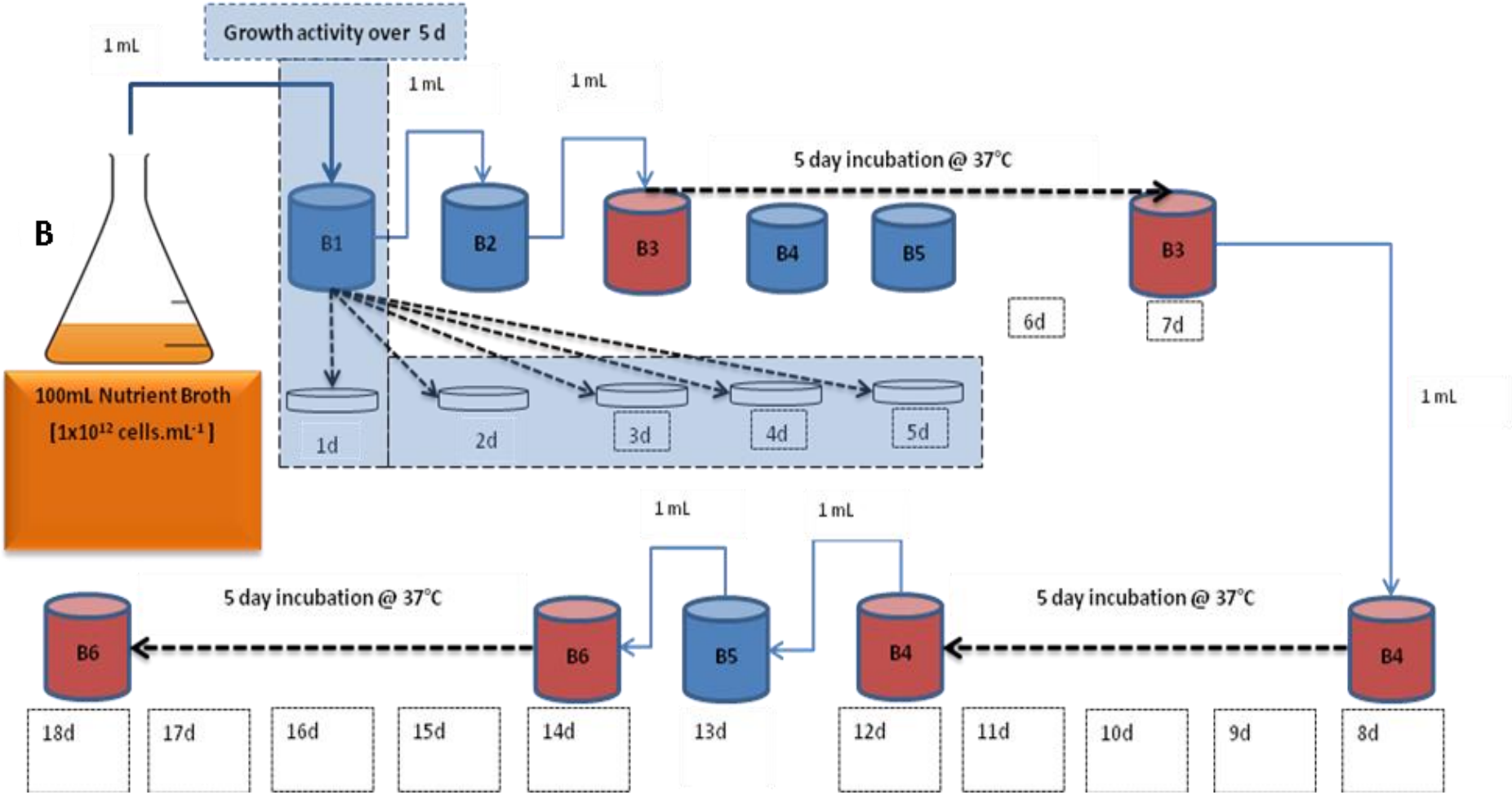


Figure 2. Sample S (*S. maltophilia*) included 10 one millilitre inoculations across a period of 18 d. Growth activity was monitored for 5 consecutive days by plating from the same sample (S1). Eight FOG concentrations were measured at days 5, 7, 9, 11, 12, 14, 16 and 18 to monitor FOG reduction.



= Monitoring of growth activity using pour-plate technique

B1 – B6 = Fresh pre-treated GDWW for successive inoculations from day 1 up until da

1d – 18d = Exposure days of isolate to GDWW

Figure 3. Sample B (*B. licheniformis*) included 6 one millilitre inoculations across a period of 18 d. Growth activity was monitored for 5 consecutive days by plating from the same sample (S1). Six FOG concentrations were measured at days 3, 7, 8, 12, 14 and 18 to monitor FOG reduction.

24 h. P11 to P14 was treated similarly as P10. P15 received 1 mL of P14 and was incubated for 3 d before determining a FOG concentration on the final day (21) (Fig. 1).

Sample S was inoculated with *S. maltophilia* and allowed to reach a cell concentration of 1 x 10^{12} cells.mL^{-1}, before 1 mL was inoculated into S1 (Fig. 2). One millilitre of S1 was transferred after a 24 h incubation period to a fresh batch of pre-treated GDWW, namely S2, within a 7 day biodegradation period. This was repeated for S3, S4 as well as S5, which underwent a 3 d incubation period. Following the 3 d incubation period of S5, the isolate at this stage has had a 7 day exposure period to GDWW and a FOG concentration was determined (At this stage, a FOG concentration for S1 was also determined). After the incubation period, 1 mL of S5 was transferred to S6 and allowed to incubate for 24 h after which 1 mL of S6 was transferred to S7. S7 was incubated for 3 d after which a FOG concentration was determined. One millilitre of S7 was transferred to S8 and incubated for 3 d, and a FOG concentration determined. 1 mL of S8 was transferred to S9 and incubated for 24 h after which 1 mL of S9 was transferred to S10. S10 was incubated for 3 d and a FOG concentration determined. The entire batch fermentation phase lasted 18 d (Fig. 2).

Sample B was inoculated with *B. licheniformis* and allowed to reach a cell concentration of 1 x 10^{12} cells.mL^{-1}, before 1 mL thereof was inoculated into B1 (Fig. 3). Within a 7 day biodegradation period, a FOG concentration was obtained for B1 and 1 mL of B1 was transferred after a 24 h incubation period to a fresh batch of pre-treated GDWW, namely B2. B3 was incubated for 5 d before being sampled to determine the FOG concentration. B4 was obtained by transferring 1 mL of B3 to B4 and allowed to incubate for 5 d before determining a FOG concentration. B5 received 1 mL of B4 and then incubated for 24 h after which 1 mL of B5 was transferred to B6. B6 was incubated for 5 d before determining a FOG concentration. Samples were also taken on days 12 and 18. The entire batch fermentation phase lasted 18 d (Fig. 3).

Fatty Acid Analysis

Upon completion of the batch biodegradation phase, the acclimatized isolates were stored at 4°C in a medium containing 200 mL fresh pre-treated GDWW until required for fatty acid analysis. The acclimatized samples were re-inoculated into fresh pre-treated GDWW and incubated for 4 days at 37°C on a shaker set to a speed of 125 rpm prior to fatty acid analysis. After 4 days, the samples were removed, centrifuged at 5 000 rpm for 2 min to remove microbial cells and the fatty acid analysis was done in triplicate on the supernatant

(Folch *et al.,*1956). Control samples were prepared for each sample batch by using 200 mL fresh pre-treated GDWW (excluding the isolate) and treated under the same conditions for the duration of the acclimatization period.

The gas chromatograph used was a Thermo Finnigan Focus GC (Thermo Electron S.p.A., Strada Rivoltana, 20090 Rodana, Milan, Italy). GC detection conditions were column: BPX70; 60m x 0.25 mm ID; 0.25 µm (SGE International Pty Ltd, 7 Argent Place, Ringwood, Victoria 3134, Australia), initial temperature: 140°C; 5 min, Rate 1: 4°C.min^{-1}; final temperature: 240°C; detector: 260°C; injector: 220°C; split: 100:1; carrier: Hydrogen gas at 30 mL.min^{-1} and injection volume: 1 µL run time for 35 min.

RESULTS AND DISCUSSION

Growth curves

Growth curves were established to determine the cell concentration required for inoculating the respective samples before batch fermentation. In figure 4 the growth curves (excluding the lag phase) are shown. By using IFR MicroFit (V1.0), statistical information regarding the t-lag and the t-d was obtained (Table 1). The aforementioned parameters will be discussed at a later stage.

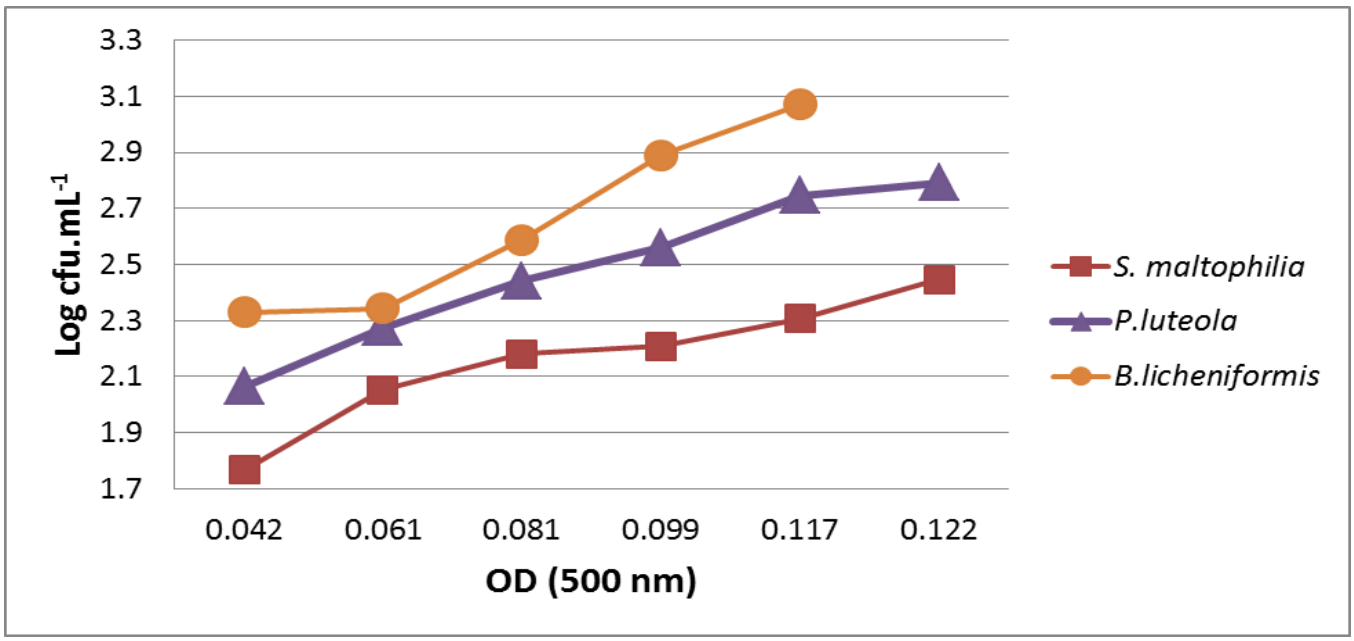


Figure 4. Growth curves for the respective isolates, namely *P. luteola*, *S. maltophilia* and *B. licheniformis* after an incubation period of 6 h at T= 36°C ±2°C.

Table 1. Statistical data obtained was the N_0, t-lag and t-d for the respective isolates *P .luteola, B. licheniformis* and *S .maltophilia* using IFR Microfit (V1.0)

Isolate	N_0 (Log cfu.mL^{-1})	t-lag (minutes)	t-d (minutes)
P. luteola	0.42	13.86	34.03
S. maltophilia	0.89	52.78	42.22
B. licheniformis	0.77	130.31	28.18

Batch biodegradation

Sample A (*P. luteola*) had an 'initially FOG concentration' (FOGi) of 72 mg.L^{-1} on day 1. P1 was followed by successive inoculations up until P7. Day 7 (P7) was allowed to incubate for 3 d at 37°C which resulted in the first 9 d of exposure to the GDWW and a FOG concentration was determined. A 'final FOG concentration' (FOGf) of 71 mg.L^{-1} was obtained and this indicated that even after a 9 day acclimatization period, no reduction in FOG had occurred. Day 10 (P8) received 1 mL of P7 and was incubated for 24 h after which 1 mL of P8 was transferred to day 11 (P9) and incubated for 3 d. The initial FOG concentration of P9 was 66 mg.L^{-1}. Following the 3 d incubation period, P9 had a FOG concentration of 51 mg.L^{-1}. The decrease from 66 to 51 mg.L^{-1} is minimal even after the isolate has now been exposed to pre-treated GDWW for 13 d. Due to this minimal decrease, the isolate was exposed to a longer period of acclimatization in order to have a more pronounced effect in terms of FOG biodegradation and thus 1 mL of P9 was transferred to day 14 (P10). The latter step was repeated up until P15. The initial FOG concentration of day 19 (P15) was 42 mg.L^{-1}. After an incubation period of 3 d, P15 had a FOG concentration of 36 mg.L^{-1}.

Although a reduction in FOG concentration was obtained and considering it was possible to initiate this trial on an industrial scale, it would not be very practical to have an acclimatisation period of 21 d and achieve such a small reduction. The small reduction in FOG can also be explained by the growth of *P. luteola* in pre-treated GDWW observed during growth activity period of 5 days during batch biodegradation (Fig. 5). Growth peaked between the first two days of incubation from 1.9 x 10^9 to 2.80 x 10^9 cfu.mL^{-1}. The sharp increase could be due to the consumption of components within the wastewater, other than the FOG present. The components present could be too complex in nature to degrade, thus inhibiting the release of lipolytic enzymes. Distillery wastewater is rich in components such as soluble proteins, organic acids, glycerol and carbohydrates, inorganic compounds and phenolic compounds (Uzal *et al.,*

2003; Gie, 2007; Mallik *et al.,* 2010). Some of these components are said to resist decomposition and therefore slow down the biodegradation process during wastewater treatment. On days 3 and 4, there was a decrease in cell concentration and this remained constant on day 5 at 2.1 x 10^9 cfu.mL^{-1}. Growth was monitored after day 5, but not depicted on figure 5 as there was no further growth thereafter whereby the value remained at 2.1 x 10^9 cfu.mL^{-1}. This decrease in cell number followed by a plateau could be as a result of nutrients that were depleted within the first 2 days. Cellular growth therefore reached a stabilized period and would have either remained in that dormant state until supplemented with a fresh nutrient source such as GDWW or eventually have reached a death phase. Similar results were obtained by a previous study whereby the microbial load peaked within the first 3 d after which it decreased as the food debris was utilised (Odeyemi *et al*., 2011). The above-mentioned reasons may therefore explain the slight decrease in FOG over the 21d batch biodegradation period.

Sample B (*S. maltophilia*) had a starting FOG concentration of 104 mg.L^{-1} (FOGi) on day 1. After S1 had undergone an incubation period for 7 d, it was sampled and had a FOGf of 95 mg.L^{-1}. This drop in FOG concentration can be explained by the biodegradation action of the isolate present. Indeed this is not a very large decrease in the FOG content over a period of 7 d, but signifies that activity may be present due to the release of enzymes. S5 initially had a FOGi concentration of 104 mg.L^{-1} and after 3 d of incubation, day 7 (S5) had a FOGf concentration of 60 mg.L^{-1}. The concentrations obtained by S1 (during its 7 day incubation period) and S5 are comparable in that both these samples were exposed to pre-treated GDWW for a total of 7 d (Fig. 6). The key difference is that S5 was exposed to an acclimatisation phase, by adapting to its environment and thereby had a lower FOG concentration. One could speculate that the higher the cell concentration, the more effective biodegradation occurs. The aforementioned could simply be explained by the fact that if more cells are present, the faster biodegradation will proceed. S6 then received 1 mL of S5 after which 1 mL of S6 was transferred to S7. S7 was incubated for 3 d. At this stage, S7 has been exposed to pre-treated GDWW for a period of 11 d and had a FOG concentration of 54 mg.L^{-1}. It was assumed that this low concentration may be contributed to the increase in cell concentration which could possibly have had a positive effect on the biodegradation activity. S8 was incubated for 3 d and after 14 d of exposure to pre-treated GDWW, a FOG concentration of 66 mg.L^{-1} was obtained. Furthermore, after 18 d of exposure to pre-treated GDWW a FOG concentration of 68 mg.L^{-1} was obtained. No further biodegradation

occurred as concentrations remained in the vicinity of 60 – 68 mg.L^{-1}. An average value for the aforementioned concentrations (60 – 68 mg.L^{-1}), was taken to represent the decrease in FOG concentration during biodegradation. It was assumed that the drop in concentration from the initial 104 mg.L^{-1} to the average value of 65 mg.L^{-1} may be attributed to the increase in cellular concentration which could possibly have had a positive effect on the biodegradation activity due to the release of enzymes.

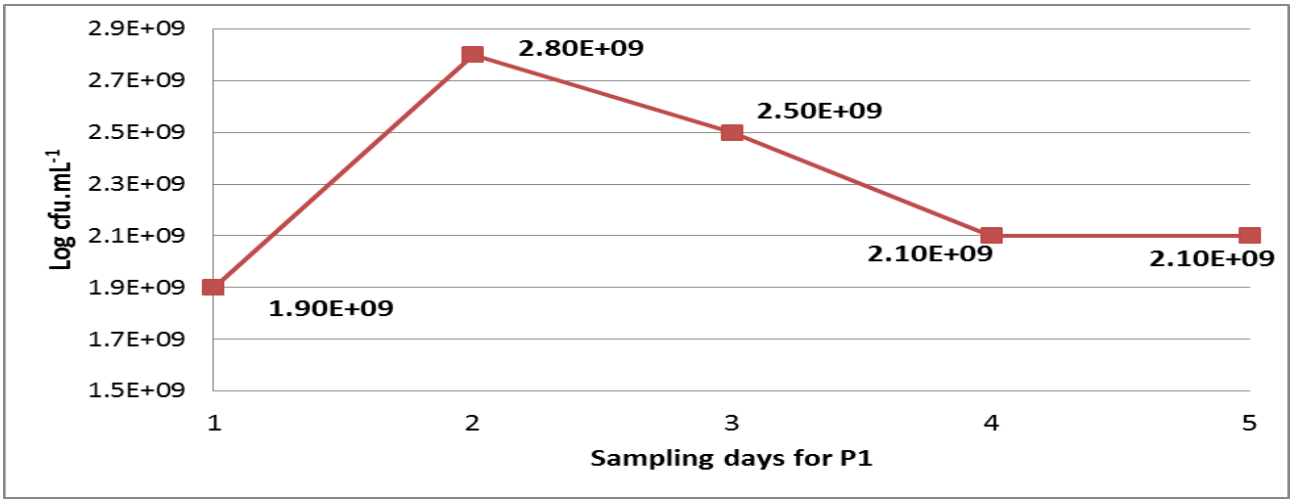


Figure 5. Growth of *P. luteola* in pre-treated GDWW sampled from P1 each day, for a period of 5 d.

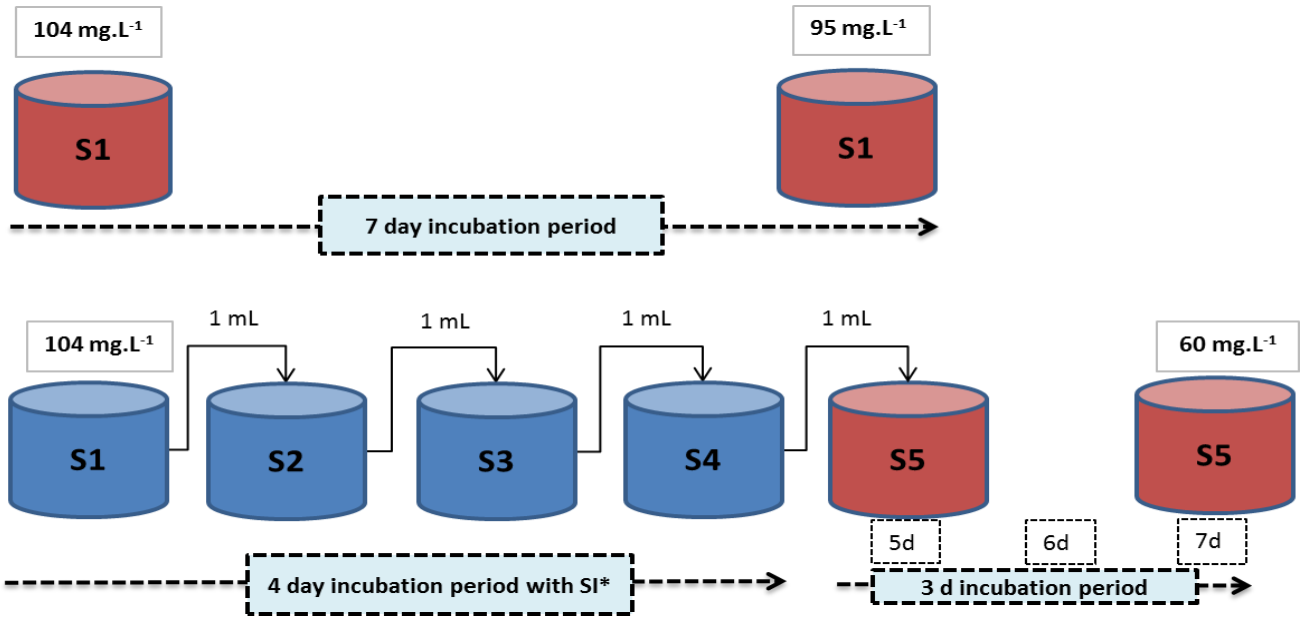


Figure 6. Batch biodegradation of S1 (non-acclimatised) and S5 (acclimatised) from sample B, whereby S1 was exposed to a 7 d incubation period without any successive inoculations (SI*). S5 received SI* for the first 4 d prior to being incubated for a further 3 d. S5 thus had an initial acclimatisation period for the first 4 d.

The latter information can also be represented by a graph which shows the increase in viable cells over a period of 5 d when exposed to pre-treated GDWW as growth media (Fig. 7). There is a decrease in FOG concentration and the period of

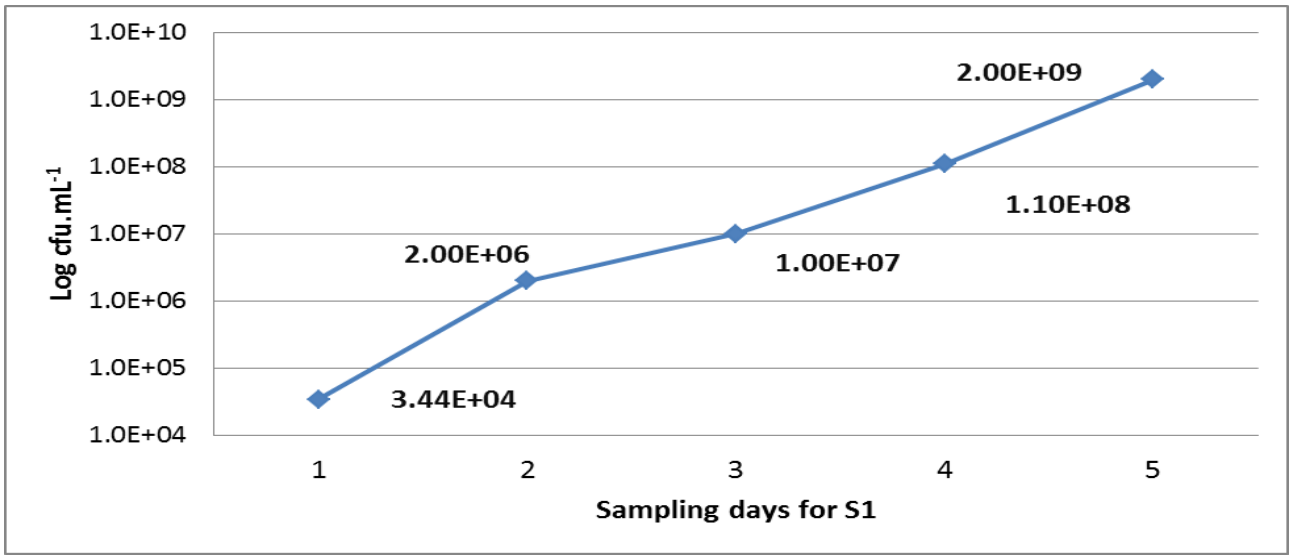


Figure 7. Growth of *S. maltophilia* in pre-treated GDWW sampled from S1 each day, for a period of 5 d.

acclimitasation has proven there is increased activity during biodegradation of FOG if one compares 95 mg.L^{-1} obtained in S1 which had no adaption period, to that of S5 that had a concentration of 60 mg.L^{-1}, which had a period of adaption (Fig. 6).

Sample C (*B. licheniformis*) had a FOGi concentration of 211 mg.L^{-1}. After an incubation period of 7 d, B1 was sampled and had a FOGf concentration of 93.8 mg.L^{-1} (Fig. 8). This drop in FOG concentration is the most promising obtained within a 7 day incubation period compared to the other 2 isolates. B3 was sampled after an incubation period of 5 d. In total, B3 also had a 7 day exposure to pre-treated GDWW and had a FOG concentration of 89.82 mg.L^{-1}. B4 then received 1 mL of B3 and was then incubated for 5 d. Day 12 (B4) had a FOG concentration of 32 mg.L^{-1} and day 18 (B6) a concentration of 36 mg.L^{-1}. A FOG reduction of 55% was experienced during the first 7 d of incubation without any acclimatisation phase. After an acclimatisation period of 12 d there was a noticeable decrease in FOG concentration. At this stage the isolate had been exposed to pre-treated GDWW for a total of 12 d. Of importance to note is that this isolate was subjected to 5 day periods of incubation as opposed to the previous 2 isolates which only received 3 d before a FOG reading was obtained. The reason for this is that during the growth curve establishment, *B. licheniformis* showed the fastest growth in terms of t-d with

a value of 28 minutes. The t-d values for *P. luteola* and *S. maltophilia* were 34 and 42 min, respectively. It was assumed that because of this shorter t-d, the isolate would be more effective in the wastewater treatment process before fresh feed is given. FOG biodegradation would be more efficient as more FOG is broken down in a shorter period of time give at a faster rate of cellular growth. This faster biodegradation can also be as a result of the increased cell number, brought about via successive inoculations during the acclimatisation phase.

It is interesting to note that *P. luteola and B. licheniformis*, when subjected to pre-treated GDWW, reached a stationary phase of growth at approximately 2.0 x 10^9 and 2.8 x 10^8 cfu.mL^{-1} when monitored over a 5 day period, while *S. maltophilia* continued to increase in cell concentration (1.84 x 10^9 cfu.mL^{-1}). This could possibly be due to changes in environmental conditions such as substrate accumulation, depletion of nutrient source or cellular saturation within the solution. FOG concentrations at days 12 (B4) and 18 (B6) were 32 and 36 mg.L^{-1}, respectively. These two values are very close in proximity (Standard Deviation = 2.82) and it was assumed that the isolate may have been inhibited by the accumulation of biodegradation products formed.

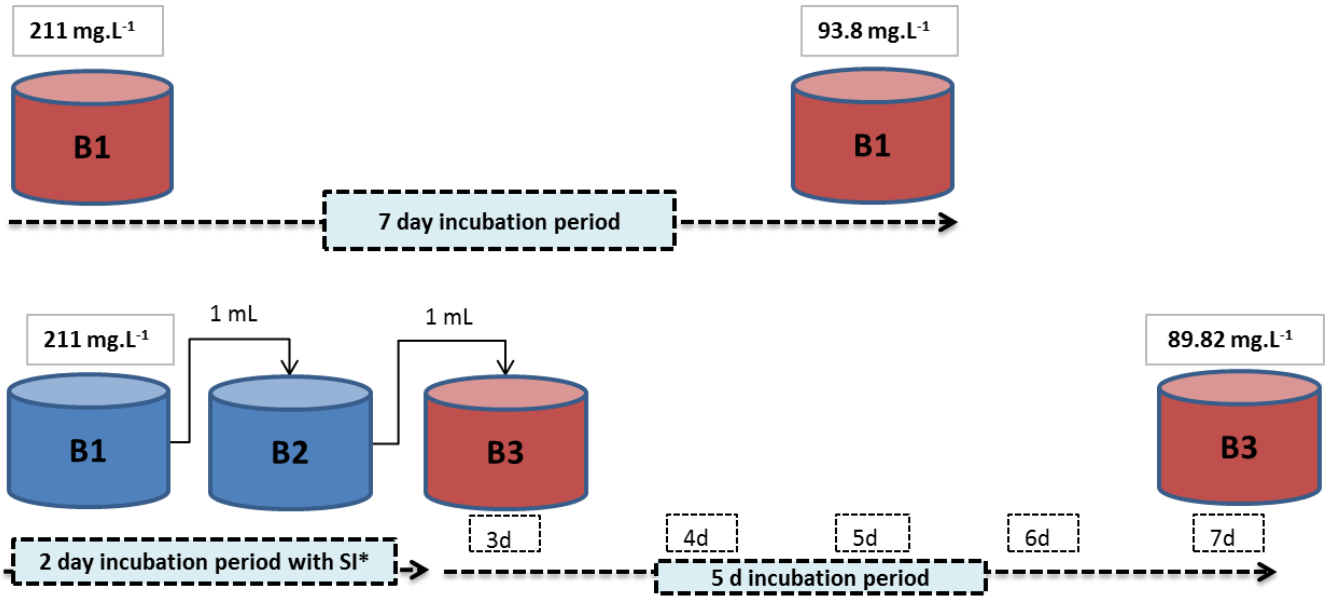


Figure 8. Batch biodegradation of B1 (non-acclimatised) and B3 (acclimatised) from sample C, whereby B1 experienced a 7 d incubation period without any successive inoculations (SI*). B3 received SI* for the first 2 d prior to being incubated for a further 5 d. B3 thus had an initial acclimatisation period for the first 2 d.

The average for these two values was taken in order to calculate the FOG reduction. This was an 83% reduction in FOG. If, however, there was an accumulation of shorter chain fatty acids such as organic acids, the pH would have decreased. This, however, was the case as the pH dropped from 7.4 to 6.6 within the first 12 d of biodegradation after which it increased to 7.3 on day 18, thus indicating a buffering effect. This buffering effect could be the response of the microorganism to pH stress by dissociating the molecule(s) that may be inhibitory to the biodegradation process. In addition to this, an increase in CO_2 may lower the pH. Furthermore, the high moisture content may retard the diffusion of CO_2 to the atmosphere, which may explain for the initial drop in pH. This shows that an initial acclimatisation phase does assist in optimising the biodegradation phase as B1 was incubated for 7 d without any addition of fresh pre-treated GDWW. The increased growth rate can also be seen by the increase in cell number within the first two days (Fig. 9).

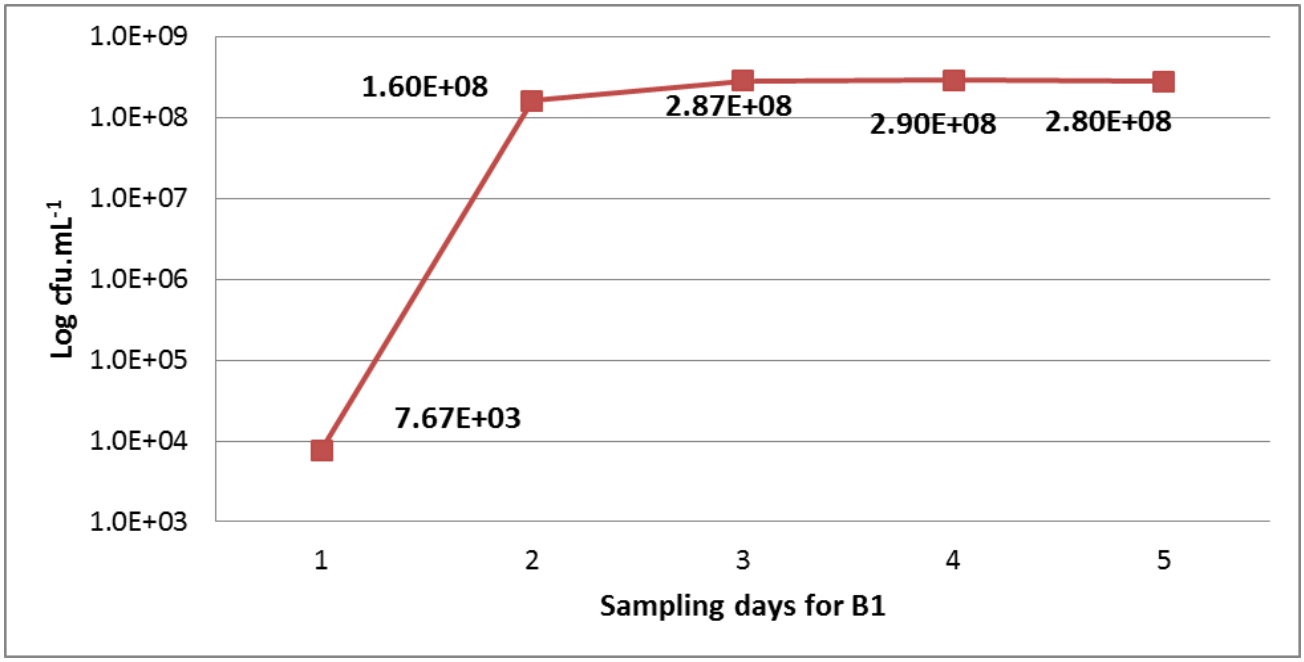
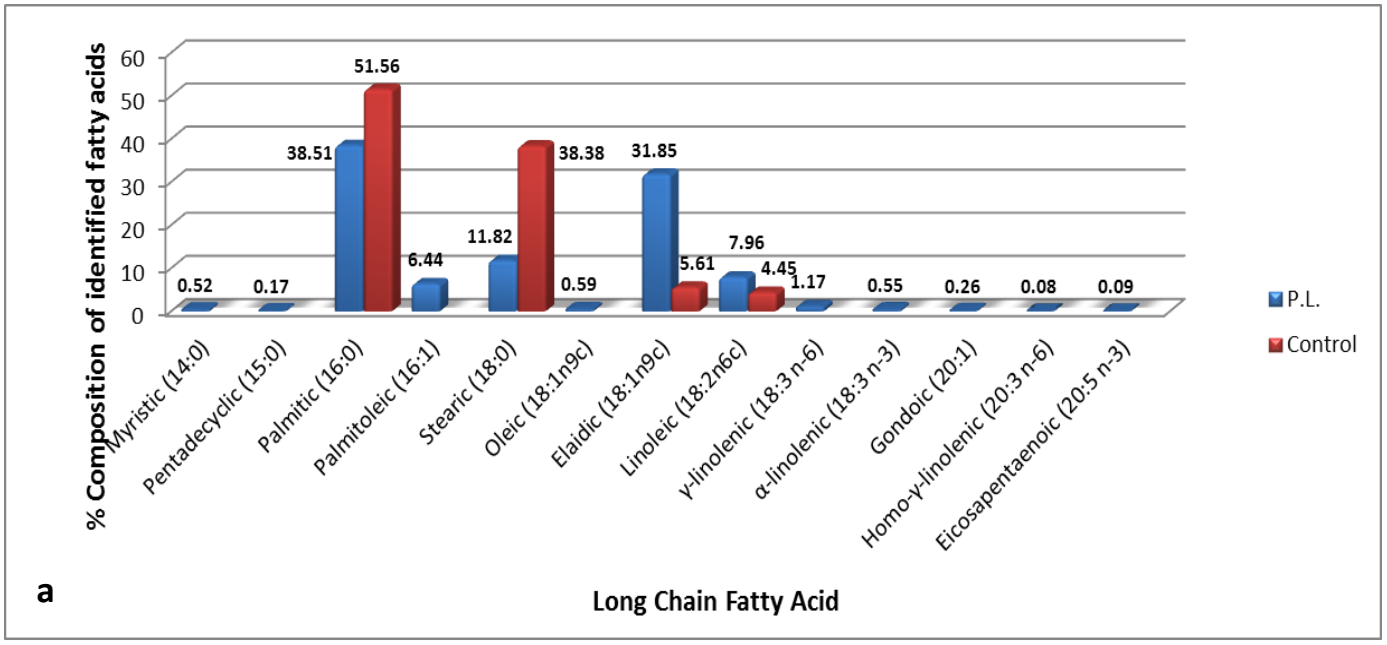


Figure 9. Growth of *B. licheniformis* in pre-treated GDWW sampled from B1 each day, for a period of 5 d.

Fatty acid profile

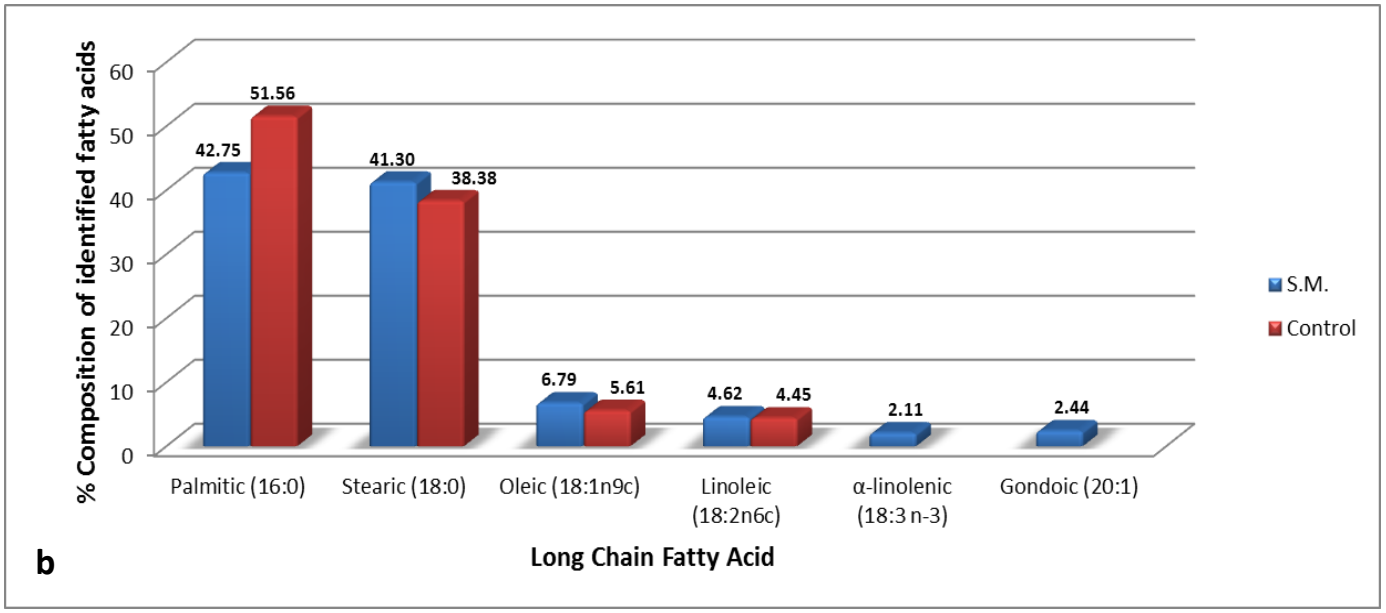
Prior to the fatty acid analysis, acclimatised isolates were transferred to fresh pre-treated GDWW and allowed to initiate biodegradation for 4 d at a temperature of 37°C on a shaker (speed set to 125 RPM). Figures 10 a, b and c respectively shows the fatty acid profile

obtained during the biodegradation of GDWW by *P. luteola, S. maltophilia and B. licheniformis* and after 4 days incubation. The values represented are the fatty acids detected within the respective samples.



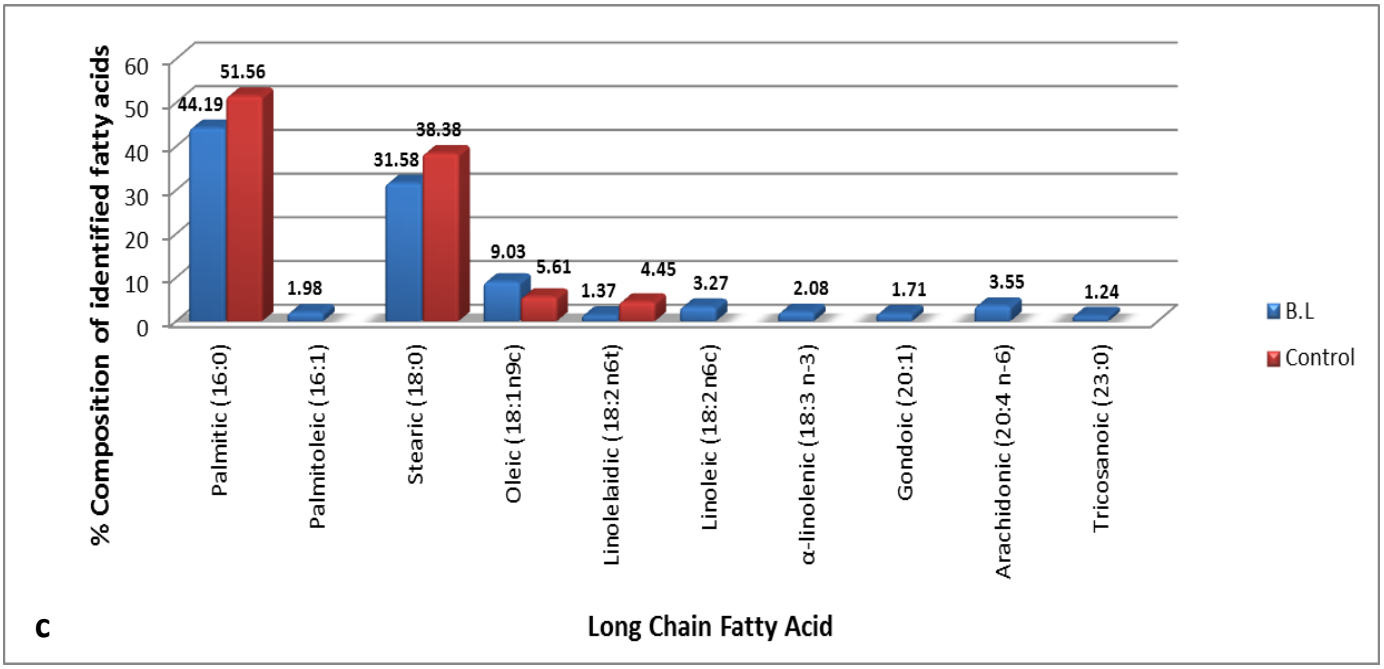
P.L = Inoculated sample after biodegradation phase (*P. luteola present)*

Control = Uninoculated sample before biodegradation phase (no strain present)



S.M = Inoculated sample after biodegradation phase (S*. maltophilia present)*

Control = Uninoculated sample before biodegradation (no strain present)



B.L = Inoculated sample after biodegradation phase (*B. licheniformis present)*

Control = Uninoculated sample before biodegradation (no strain present)

Figure 10. Fatty acid profile analysis representing the percentage composition of fatty acids after GDWW biodegradation for acclimatised bacteria *P. luteola (a), S. maltophilia (b)* and *B. licheniformis (c)*.

In the case of *P. luteola*, palmitic acid (C16:0) decreased from 51.56 to 38.51% while stearic acid (C18:0) decreased from 38.38 to 11.82%. Formation of palmitoleic (C16:1), oleic (C18:1n9c), elaidic (C18:1n9t) as well as linoleic acid derivatives (α- and γ-linolenic acid) also occurred. The free fatty acids (FFA) are very small in concentration (Fig. 10a). According to another study, by using an isolated strain, *Pseudomonas* sp. D2D3, FOG removal was 41% higher than the naturally occurring strain (Kim *et al.*, 2004).

According to literature inter-esterification is catalysed by either non-specific or specific lipases (Macrae, 1983). Specific lipases will catalyse reactions by which free fatty acids (FFA) actually exchanges with acyl groups of existing triglycerides to produce newly formed triglycerides, thus if a FA specific lipase is excreted by a bacterial isolate, it may target a specific FA in the mixture therefore synthesising other FA that were not previously present in the control as noticed in this study.

Another study investigated the biodegradation of oil-rich wastewater by treatment with microbes (Odeyemi *et al.*, 2011). It was said that the production of FA is more

prominent during aerobic conditions. This could also explain for the formation of FA during thus study.

B. licheniformis results show a decrease in C16:0 from 51.56 to 44.19%. Formation of C16:1 by FFA also occurred (1.98%). This value is minimal when compared to *P. luteola* (6.44%). There was a decrease in C18:0 from 38.38 to 31.58%. Furthermore, other polyunsaturated fatty acids (PUFA) were formed (Fig. 10c), while *S.maltophilia* showed a decrease in C16:0 from 51.56 to 42.75%. The biosynthesis of other PUFA such as C18:0, C18:1n9c and C18:2n6c was minimal (Fig. 10b). Another study done on monitoring of lipolysis in milk by FFA production, reported that *B. licheniformis* species have significant lipolytic activity in comparison to other *Bacillus* strains (Janštová *et al.,* 2006). This may possibly explain as to why in this study LCFAs are formed especially by *P. luteola* and *B. licheniformis* due to the release of enzymes. Odeyemi *et al*. (2011) showed an increase in the rate of microbial growth correlated to that of an increase in the rate of biodegradation of oil into FA. In this study, results were similar even though Odeyemi *et al*. (2011) monitored their biodegradation of oil wastewater using a mixed culture of bacteria, whereby in this study, single strains were used. It may be that by using a diverse bacterial population, a larger group of extracellular microbial lipases may have been excreted to aid in digestion of lipid materials (Macrae, 1983).

Lipases may also be specific for the fat it may catalyse (Macrae, 1983) and therefore act on more FA present in the wastewater (Bhumibhamon *et al*., 2002; Odeyemi, 2011). Interesterification as explained by Macrae. (1983) may also be as a result of the formation of other FA during the batch fermentation process in this study. By analysing the fatty acid profile of the GDWW, the individual strains behaviour in terms of FA formed and broken down could be determined. This information could be of value to a GDWW / WW treatment plant where the accumulation of a certain FA could cause operational problems. For instance, *P. luteola* may aid in the reduction of stearic acid whereas S. maltophilia would cause even more upset to the biodegradation process by increasing the stearic acid.

A previous study conducted by Lalman & Bagley (2000), showed the anaerobic degradation and inhibitory effects of linoleic acid (C18:2n6c). Some of the LCFA by-products that were formed during C18:2n6c biodegradation were C18:1n9c, C16:1 and C16:0. This study similarly showed an increase in the percentage of C18:1n9c. This may be due to the dehydrogenation of C18:0 to form the unsaturated C18:1n9c as in the case of *P. luteola* (Fig. 10a). Also the C16:1 that was formed under aerobic conditions is in

contrast to another study (Lalman & Bagley, 2000) whereby it was formed as a β-oxidation product of C18:1n9c. Also the increase was not necessarily brought about by the biodegradation of C18:2n6c only as in the case of the study done by Lalman & Bagley (2000), but by other FA in the GDWW as well. Although C18:0 acid was not detected by Lalman & Bagley (2000), however, in this study the C18:0 decreased in percentage for *P. luteola* and *B. licheniformis*, but increased in the case of *S. maltophilia*. It can also be said that the β-oxidation process anaerobically biodegrades LCFA such as saturated C18 to C16:0 and other shorter chain fatty acids (SCFA) until their final conversion to acetic acid (Lalman & Bagley, 2000).

It should be considered that these strains were only subjected to a 4 d incubation period during this study and that *P. luteola* gave the highest percentage reduction in this period for C18:0 as well C16:0. If, however, *B. lichniformis* or *S. maltophilia* were subjected to longer incubation, they could have possibly had a better reduction for certain FA during biodegradation thereof. It can thus be concluded from the aforementioned, that *P. luteola* had a faster adaption period in releasing the enzymes for the reduction achieved. C18:0 and C16:0 are known to be problematic during GDWW treatment and a strain such as *P. luteola* can assist in reducing these problematic FAs.

Conclusions

Wastewaters from various food industries differ in composition. During this study it was established how microorganisms (*S. maltophilia, P. luteola and B. licheniformis*) respond to grain distillery wastewater as their nutrient source. The LCFA content was quantified which were either biodegraded or caused inhibition to the biodegradation process. During batch biodegradation, *P. luteola* and *S. maltophilia* took 21 d and 18 d, respectively, to reduce the FOG concentration by 50 and 35%. The strains showed that an acclimatization period improved the biodegradation of GDWW. *Bacillus licheniformis* showed the best biodegradation after being exposed to GDWW for a 5d period, showing a 55% FOG reduction even without an acclimatization phase. The highest FOG reduction obtained by the acclimatized strain was between days 12 and 18 (83% FOG reduction).

The LCFAs quantified during this study has led to a better understanding of how *P. luteola, S. maltophilia* and *B. licheniformis* behaves when subjected to a more complex and natural wastewater FOG source (GDWW) rather than the use of a synthetic source (Tributyrin).

During the study it was also found that under aerobic incubation, the strains either biodegraded the FA, as in the case of *P. luteola* which produced myristic and pentadecyclic acids, or formed free FA. The formation of FA may have occurred through inter-esterification. It was also found that certain precursors such as palmitoleic acid may be formed under aerobic or anaerobic conditions. If a strain is to be inoculated for FOG wastewater treatment then a recommendation may be made to use *B. licheniformis* as a suitable fat-degrading microbe. Although one can isolate specific bacteria to biodegrade FOG, this study shows that it is difficult to degrade certain LCFA such as C16:0, C18:0, C18:1 and C18:2. Due to the results obtained in this study, more research has to be focused on single strain identification and their ability to adapt to wastewaters of specific composition as many commercially enzymes available that are targeted at industrial wastewaters high in FOG, are expensive.

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CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

GDWW in South Africa and associated problems during treatment

The agricultural industry in South Africa is diverse and its arable land available, accounts for approximately 53% of the entire country's surface. The South African population is growing as much as 2% each year and predicted to reach a population size of 82 million by the year 2035 (Scotcher, 2009). Owing to this increase in population size, it is inevitable that food production industries will be doubling their production and this will lead to an increase in wastewater production as well.

Wastewater varies in composition depending on the type of agricultural industry producing it, be it a papermill and pulp, abattoir and slaughterhouse or distillery industries, to mention but a few (Li *et al*., 2002; Battimelli *et al*., 2009). The South African grain distillery industry alone produces approximately 120 000 L of grain distillery wastewater (GDWW) monthly. This type of wastewater (WW) has an acidic pH of 3 - 4 and a high organic content with chemical oxygen demand (COD) values ranging from 10 – 50 $g.L^{-1}$. GDWW is also high in fats, oils and grease (FOG), which causes operational problems during its treatment. Amongst other problems, FOG hinders anaerobic digestion, which decreases the biodegradation efficiency and thus disrupts the entire treatment process. According to strict discharge standards as laid down by the South African legislation, wastewater should be treated to acceptable COD levels before being discharged into the environment.

Many treatment options have been proposed and are implemented as treatment methods during GDWW treatment. Amongst other treatment options, this includes physicochemical processes such as adsorption and flocculation as well as coagulation. Biological treatment such as anaerobic digestion has been seen as a preferred method of treatment as it is energy saving and minimises disposal costs.

Isolation and characterisation of lipid degrading bacteria

In this study, lipid-degrading bacteria was successfully isolated and characterised from a grain distillery environment using various agar media at different temperature ranges. The

four bacterial species *Pseudomonas luteola (1), Pseudomonas fluorescens (2), Stenotrophomonas maltophilia (3)* and *Bacillus licheniformis (4)* showed lipase/esterase activity on Spirit Blue Agar, which contained tributyrin (SBA-Tri) as lipid source. GDWW was used as lipid source together with Victoria Blue B (VBB-GDWW), which showed microbial growth, but failed to give a distinct indication of lipolytic activity. Using cottonseed oil as lipid source, lipolytic activity was indicated by formation of distinct blue zones around or below the respective colonies.

From this study, it was concluded that although VBB-GDWW does not yield a blue colony or clear zones of lipolysis, bacterial growth was prominent. This indicates that other components other that FOG, within the GDWW may have been utilised. The use of GDWW as lipid source had no inhibitory effects towards the growth of strains. The study also concluded the possibility of using these strains to assist the biodegradation process during the anaerobic treatment of GDWW and thus minimising the COD.

Batch biodegradation of FOG by bioaugmenting with bacterial strains

This study conducted LCFA quantification on GDWW biodegraded by specific strains. It has therefore investigated the behaviour of these isolates when subjected to this particular type of wastewater. The information obtained from the FA profile gives an idea of the FA biodegraded or synthesised by the individual strains when subjected to GDWW. It is beneficial to know which FA are metabolised by which organism(s) as this gives an idea of the microorganisms metabolic pathway.

Three bacterial strains isolated from a grain distillery environment namely, *P. luteola, S. maltophilia and B. licheniformis* were bioaugmented to GDWW and thus monitored in batch fermentation for their ability to biodegrade FOG. All strains successfully showed that biodegradation of GDWW are improved by an initial acclimatisation phase. *Stenotrophomonas maltophilia* and *P. luteola* had a total FOG reduction of 63% and 50% respectively over an 18 and 21 d batch fermentation period. *Bacillus licheniformis* however had a total FOG reduction of 83% after 18 d exposure to GDWW. This strain therefore had the highest FOG reduction of the three strains tested. A recommendation may be made to use *B. licheniformis* as a suitable fat-degrading microbe during FOG wastewater treatment.

The GDWW was also treated with the acclimatised strains and then quantified for long chain fatty acids (LCFA). The quantification of LCFA in this study has led to a better

understanding of how *P. luteola, S. maltophilia* and *B. licheniformis* behaves when subjected to a FOG source such as GDWW and not using a synthetic FOG medium. It was found that under aerobic incubation, the strains either initiated biodegradation to produce shorter chain fatty acids (SCFA) from LCFA, as in the case of *P. luteola*, which produced myristic and pentadecyclic acids, or formed FA.

A process called 'inter-esterification' may have also occurred whereby certain enzyme systems produced by the strain may result in the synthesis of another FA. It was also found that certain precursors such as palmitoleic acid might be formed under aerobic or anaerobic conditions. On a positive note, the research, obtained gives an indication of the strains metabolic behaviour in terms of LCFA utilised and formed when subjected to GDWW. This information can therefore be beneficial when choosing a suitable strain for wastewater treatment.

Concluding remarks

The isolation and characterisation of lipolytic species from a GDWW treatment environment was successfully done and found to utilise various lipid substrates on solid media through visual analysis.

Acclimatising the lipolytic isolates to GDWW improved the biodegradation of FOG. *Bacillus licheniformis*, which is a facultative anaerobe, gave the highest decrease in FOG concentration, with a total reduction of 83%. It was also found that by using low concentrations of FOG (70 – 211 $mg.L^{-1}$) in GDWW, does not inhibit biodegradation during batch fermentation.

Long chain fatty analysis showed that FOG was either biodegraded to shorter chains fatty acids or formed through a process of inter-esterification, depending on the type of enzyme system released by the respective isolate. Although lengthened biodegradation periods (18 – 21 d) were experienced, results from this study shows the ability of individual bacterial species to biodegrade FOG in GDWW without any additional nutrient source. The information obtained from the fatty acid profiles can be used to see which fatty acids are either biodegraded or formed thus making it possible to choose the most desirable isolate for FOG wastewater treatment.

Future research should investigate the lipolytic enzyme systems of these bacterial isolates. These enzymes could be useful to the industry for FOG wastewater treatment.

Bioaugmenting the lipolytic isolates from this study with other isolates can be done to monitor their survival when subjected to an industrial wastewater treatment level.

References

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