BIO-DELIPIDATION OF PRE-TREATED POULTRY SLAUGHTERHOUSE WASTEWATER BY ENZYMES FROM THE WASTEWATER ISOLATES

by

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DECLARATION

I, **Siyasanga Mbulawa**, declare that the contents of this thesis represent my own unaided work and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology. All intellectual properties, theories, and methodologies used in this thesis and published were derived solely by the candidate. Where appropriate, intellectual property of others is acknowledged by using appropriate references. The contribution of co-authors, for conferences and published manuscripts, was in a training capacity, research assistance, and supervising capacity, to meet the requirements for the degree award.

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ABSTRACT

Pre-treatment of wastewater such as that from poultry slaughterhouses, which contains fats, oil,and grease (FOG) is necessary prior to the primary biological treatment of the wastewater to meet legislated discharge standards and to prevent environmental pollution. Physico-chemical pre-treatment is often applied to remove FOG in poultry slaughterhouse wastewater (PSW) before biological treatment. These pre-treatment methods, in particular physical pre-treatment systems, use synthetic chemicals, known to cause environmental contamination challenges, with FOG being inefficiently removed in certain instances. Biological techniques such as bio-delipidation using enzymatic catalysis for the pre-treatment of FOG-laden PSW could enhance the efficiency of the downstream biological treatment processes. This research focused on further bio-delipidation of PSW pre-treated with a dissolved air flotation system (DAF) for FOG removal using microbial lipases from bacterial strains isolated from the PSW itself.

Bacterial strains (n = 2) isolated from the PSW and screened for their potential to produce lipases were found to have a higher bio-delipidation potential when compared to other isolates (n = 18). Both isolates were identified using 16s rRNA as *Bacillus* sp*.,* i.e. both *Bacillus cereus* AB1 (BF3) and CC-1 (B3O). These isolates were used to produce lipases, whereby are sponse surface methodology (RSM) was used to optimise pH (4-8) and temperature (30-60°C) as critical production conditions. achieving an optimum lipase production was achieved, with activity of 11.25 U/mL at 60°C, a pH of8 for BF3, and 15.50U/mL at 45°C and pH of 8.8 for B3O respectively, after 72 hours of bioreactor operation.

The enzymes produced from both isolates were partially purified using a Bio-Rad size exclusion chromatography column (Bio-Gel® P-60) prior to use in subsequent experiments. The presence and activity of lipase were further determined using *p*-nitrophenyl acetate (*p*-NPA) as a substrate with the functionality of the semi-purified enzymes being characterized by optimizing the conditions in which the enzymes were required to function. Lipase activity was enhanced by Mg^{2+} while Fe^{2+}, Na^{+}, K^{+}, Ca^{2+} were observed to have an inhibitory effect on the enzymes from both strains. Similarly, reduced stability of the lipases in organic solvents, namely toluene, methanol, and isopropanol, was also established. Additionally, detergents, Triclosan (TCS) (5-chloro-2-(2,4-dichlorophenoxy-phenol) and trichlorocarbonilide (3,4,4- trichlorocarbonilide)(TCC), usually found in PSW as antimicrobial and disinfectant agents to sanitise poultry product processing facilities, were used assess the activity of the enzyme in their presence at a concentration of 30% (v/v) (although these anti-microbial agents are used in minute quantities in cleaning products). The lipases from isolate

BF3 maintained an activity of 91.43% and 81.36% in the presence of TCS and TCC, while that of B3O enzyme had 85.32% and 73.91% acitivity, when compared to the reference (control) experiments.

The bio-delipidation efficacy was studied under varying pH and temperature conditions using DAF pre-treated PSW, observing a further removal efficiency of fatty acids from the protein-laden PSW at different pH and temperature. Bio-delipidation was found to be largely influenced by pH, as a pH below 7 and above 10 at 40°-45°C, calculated in the bio-delipidation efficiency reduction to below 50%. The temperature range mentioned, i.e 40°-45°C, had a positive effect on further deffating of the protein-rich DAF pre-treated PSW, as high removal efficiency was observed at this temperature range. This could be due to the characteristic of the enzymes used,or the formation of stable FOG agglomerates and/oremulsion.

Overall, a DAF effluent containing residual FOG and proteins was bio-delipidated effectively using enzymes from the PSW isolates, achieving further removal of FOG and proteins by 64.35% to 80.42%, culminating in tCOD reduction and reduced PSW turbidity, further resulting in improved wastewater quality characteristics meeting disposal standards. This study demonstrated that sequential DAF pre-treated PSW bio-delipidation has the potential to enhance the efficiency of downstream biological anaerobic treatment processes for PSW by further reducing residual FOG from a DAF system.

Keywords: Bio-delipidation; dissolved air flotation (DAF) system; fats, oil, and grease (FOG); Poultry Slaughterhouse Wastewater.

DEDICATION

I dedicate this thesis to my family for inspiring me to believe that I am capable of achieving clearly defined goals.

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LAYOUT OF THESIS

This thesis is divided into the following chapters:

Chapter 1: In this chapter, the background to this study is discussed. The research problem statement, hypothesis, aims and objectives, and significance and delineation of this study are also stated.

Chapter 2: This chapter explains the state of the art, with a literature review related to poultry slaughterhouse wastewater (PSW), the impact of fats, oil and grease (FOG) in PSW on the environment, and the biological treatment of PSW, including pre-treatment systems used for the removal of FOG in PSW. Furthermore, the bio-delipidation concept is discussed, focusing on appropriate bioprocess engineering concepts and biocatalysis for lipid/FOG conversion and/or removal in PSW.

Chapter 3: In this chapter, materials and methods used for the isolation and identification of microorganisms used for the production of enzymes of interest for the bio-delipidation of PSW from a pre-treatment system are explained. This includes quantitative methods associated with enzyme activity and analytical methods for water quality parameters.

Chapter 4: This chapter discusses results, their interpretation,and discussion based on phenomena observed from experiments.

Chapter 5: In this chapter general conclusions and recommendations for future research are listed.

Bibliography

A comprehensive list of references used to generate the thesis and to explain some of the theories contained herein is cataloged in this section using the Harvard method of referencing as per university guidelines.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS

Nomenclature

Symbol	Description	Units
g	Acceleration due to gravity	m/s^2
R^2	Correlation coefficient	-
U	Enzyme activity	µmol fatty acid/mL
d	Particle diameter	m
t	Time	minutes
V_c	Velocity of the particles	m/s

Greek symbols

μ	Absolute viscosity of fluid	Kg/(s.m)
ε	Extinction coefficient	$mM^{-1}\,cm^{-1}$
ρ	Fluid density	Kg/m^3
ρ_s	Particle density	Kg/m^3

Abbreviations	Description	
ANOVA	Analysis of variance	-
BSA	Bovine serum albumin	-
BOD	Biological oxygen demand	mg/L
COD	Chemical oxygen demand	mg/L
DAF	Dissolved air flotation	-
FOG	Fats, oil and grease	mg/L
p-NPA	p-nitrophenyl acetate	-
PSW	Poultry slaughterhouse wastewater	-
RSM	Response surface methodology	-
SS	Suspended solids	mg/L
TSS	Total suspended solids	mg/L
UV	Ultraviolet	-

GLOSSARY

Clarification of basic terms

Bio-delipidation: The removal/reduction of lipids from a liquified matrix facilitated by a biological process (*Oxford Dictionary of Biochemistry and Molecular Biology*, 2008).

Bioflocculants: Microbial flocculants composed of macromolecular substances that promote agglomeration of flocs during flocculation by forming larger flocs that can be removed (Zaki *et al*., 2011).

Delipidation: Removal or detachment of lipids by breakage of covalent bonds (*Oxford Dictionary of Biochemistry and Molecular Biology*, 2008).

Dissolved air flotation: A reactor system that uses pressurized air bubbles to allow attachment of suspended solids culminating in their separation, and thus flocculation, in wastewater (Al-Shamrani *et al*., 2002).

Lipids: Non-polar, hydrophobic organic molecules which areinsoluble in water, including fats, oil, grease and waxes and some steroids (Shuler & Kargi, 2002).

Protein: Polymeric complex organic macromolecules containing constituent chains of smaller subunits of amino acid monomers (Alberts *et al*., 2014).

CHAPTER 1

INTRODUCTION

INTRODUCTION

1.1 Background

Poultry slaughterhouse wastewater (PSW) pre-treatment is necessary prior to sequential biological treatment and the release of such treated wastewater into freshwater bodies. If not pre-treated, the PSW may contribute to the pollution of the environment, and therefore the contamination of fresh water sources, which can culminate in a negative impact on human health and aquatic life. The PSW contains a high concentration of lipids (fats, grease, oil and fatty acids) as a major component of organic matter (Commarota & Freire, 2006). Therefore, the implementation of pre-treatment process(es) is necessary as it is an important step in improving the wastewater quality prior to further treatment using anaerobic biological systems. Suitable pre-treatment systems have been developed for lipid-containing wastewater from slaughterhouses, with such techniques being applied prior to the use of primary and secondary treatment processes, such as anaerobic digesters for organic matter reduction, nitrification, and subsequent denitrification for total nitrogen reduction (Abdel-Rand *et al*., 2012).

Pre-treatment processes such as dissolved air flotation systems (DAFs) and grease traps are often applied (Tanikawa *et al*., 2016). However, complications can occur during their utilisation, contributing to the inefficient removal of lipids which will buildup in the sludge used in anaerobic processes, reducing their effectiveness to treat the PSW (Commarota & Freire, 2006). Overall, influent of improperly pre-treated high-lipid content PSW to downstream wastewater treatment processes may impede both aerobic and anaerobic downstream processes (Rigo *et al*., 2008). In an aerobic processes, a layer of lipids may be formed which will interrupt pollutant transformation by the bioremediating biomass,and also decrease access to dissolved oxygen (DO) in aerobic processes such as nitrification (Bustillo-Lecompte *et al*., 2016).

Furthermore, operational damage which culminates in process redundancies, due to solidified lipids at low temperatures during anaerobic treatment, has been reported in numerous studies for slaughterhouse wastewater (Commarota & Freire, 2006; Valladão *et al.,* 2007). Even after successful primary pre-treatment, further lipid removal might be required in a process which is environmentally benign. When a DAF system is utilised as a pre-treatment system, 60-85% of lipids can be removed (Massé & Masse, 2000), with the rest passing down to downstream processes. Clearly, the remaining lipids will thus accumulate in these downstream PSW bioremediation systems, which will effectively reduce the efficiency of such processes overtime. The use of alternative biological methods with current pre-treatment systems involving enzymes has been referred to as a promising

alternative for further FOG reduction in effluent from pre-treatment processes, a technique suitable for high lipid-containing wastewater such as PSW (Commarota & Freire, 2006; Valladão *et al*., 2007; Rigo *et al*., 2008).

Enzyme usage can sustainably provide a way for which residual FOG in high lipid-containing effluents, can be separated from protein-laden wastewater. These enzymes are from a variety of organisms that are responsible for catalysing a wide range of reactions, providing for catalytic conversions and destabilisation of bonds between proteins and lipids, and resulting in thefurther removal of unwanted FOG in wastewater.

1.2 Research problem

Physical and chemical pre-treatment systems are employed for the delipidation of protein-rich wastewater from poultry slaughterhouses prior to the biological treatment of such wastewater for the overall reduction of FOG, and therefore of total chemical oxygen demand (COD). However, these treatment techniques are capital intensive and contribute to the accumulation of toxicants in wastewater treatment processes as chemical compounds, e.g. synthetic chemical flocculants, are used for the removal of FOG. Biological techniques offer an innovative, cost-effective and environmentally benign alternative for the reduction of lipids contained in wastewater, such as that of poultry slaughterhouses. Bio-delipidation of PSW using biological methods driven via enzymatic catalysis is a promising alternative pre-treatment technique, although it has not been studied extensively. Recently, a bioflocculant supported dissolved air flotation system (DAF) was successfully used as a pre-treatment technique of PSW, whereby significant quantities of protein-including FOG were reduced in the PSW (Dlangamandla, 2016). However, minute quantities of FOG remained in the PSW, which could culminate in the reduction of the efficiency of downstream biological processes, as FOG would limit nutrient and pollutant transfer rates to organisms responsible for the bioremediation of the PSW. Therefore, a further polishing step is requiring, i.e., a post-DAF treatment system, prior to other downstream biological remediation processes. This study therefore focused on the development of an enzyme-facilitated bio-delipidation process for removal of residual FOG from a DAF effluent, using enzymes from organisms that were isolated from the pre-treated PSW.

1.3 Hypothesis

Some isolated organisms from PSW have the ability to produce biological agents which can further delipidise protein-rich PSW pre-treated using DAFs.

1.4 Research questions

The objectives of this study were based on the following questions:

- Will microorganisms isolated from the PSW be suitable to produce enzymes for application in the bio-delipidation of DAF pre-treated PSW?
- How will these microorganisms be induced into producing enzymes and/or bio-products that will demonstrate activitysuitable to facilitate bio-delipidation?
- Under what conditions will the bio-delipidation process be efficient?
- How will the performance of the bio-delipidation process be quantified?

1.5 Research aims and objectives

A broader aim of this study was to produce enzymes with bio-delipidation potential for use in PSW effluent pre-treated using DAFs. The research was achieved by the specific objectives as highlighted below:

- Isolation and characterization of microorganisms from PSW suitable to facilitate lipid reduction using their bio-products, i.e. enzymes by screen and monitor the isolated microorganisms' ability to produce required bio-delipidation enzymes and select microorganisms with a high potential to produce bio-products (enzymes) for bio-delipidation.
- Optimisation of reactor conditions to produce enzymes required for bio-delipidation employing Response Surface Methodology (RSM) and partially purify the lipases produced, for use in subsequent experiments characterization of the enzymes' functionality using environmental conditions in which they will be employed and to optimise the conditions in which the lipases can be used for a bio-delipidation system for maximum deffating of the protein-rich PSW, post DAF pre-treatment.
- Assessment of lipid removal from DAF pre-treated PSW for elucidation of optimum conditions by optimising bio-delipidation conditions of DAF pre-treat PSW, analysing PSW quality parameters pre- and post- bio-delipidation furthermore analysing bio-delipidation efficiency using FOG/lipid analysis and other water quality parameters.

1.6 Significance of the study

This study contributes to the efficacy and optimisation of FOG removal systems for protein-rich PSW, applying an environmentally benign bio-delipidation strategy as a further deffatting step of the PSW from DAFs, something which has never been attempted before. This led to the establishment of conditions for the operation of a bio-delipidation system, used in conjunction with a DAF prior to the PSW being treated in downstream systems containing

anaerobic biomass. This research therefore contributes to the knowledge that allows for the design of potential industrially important PSW pre-treatment systems that can be utilised to enhance slaughterhouse wastewater pre-treatment processes, especially for PSW from poultry slaughterhouses with varying production capacity.

1.7 Delineation

The following was not covered in this research:

- How the pre-treatment system, herein termed bio-delipidation, improves the functionality of primary/downstream biological anaerobic treatment processes.
- The effect and cost implications of the bio-delipidation process developed in industrial scale wastewater treatment plants.

CHAPTER 2

LITERATURE REVIEW

LITERATURE REVIEW

2.1 Introduction

Increased demand for meat products due to protein needs by the increasing world population, has also raised pollution concerns world-wide (Kundu *et al*., 2013; Davarnejad *et al*., 2017) and in South Africa (Basitere *et al*., 2017). This pollution results from waste and wastewater generated by activities employed in slaughterhouses (abattoirs) during the production of safe high-quality products for consumers (Chukwu *et al*., 2011). According to the South African Meat Safety Act (MSA), Act no. 40 of 2000, abattoirs are described as any facility producing meat products by slaughtering animals. This could include facilities involved in the slaughtering of poultry and other animals (Department of Agriculture and Rural Development, 2009). Direct discharge of raw or improperly treated wastewater from these slaughterhouses has been recognised to cause pollution and contamination of surface and groundwater bodies. Such pollution has a contributory effect on eutrophication, temperature changes and dissolved oxygen mass transfer limitations, which further leads to freshwater pollution, and thus the scarcity of freshwater for portable use(Bustillo-Lecompte *et al*., 2017), particularly in developing countries such as South Africa.

Slaughterhouses consume large volumes of freshwater, estimated to be 24% of all water used in the food and beverage industry, with 90% of the used water being generated as wastewater (Bustillo-Lecompte *et al*., 2016; Naderi *et al*., 2017). Freshwater is consumed for activities such as slaughtering, product processing, packaging, and cleaning of the slaughterhouse facilities. The wastewater generated typically contains high levels of fats, oil and grease (FOG), organic matter and nutrients, which are responsible for its high-strength characteristics (Kundu *et al*., 2013).

In South Africa, there are more than 470 slaughterhouses (which are expected to increase progressively in number due to an increase in poultry product demand), with potable water consumption being estimated at over 15-20 liters per bird slaughtered (Department of Agriculture and Rural Development, 2009; Basitere *et al*., 2017). South Africa's poultry slaughterhouses individually are estimated to produce over 65% of meat products due to the demand for white meat compared to other meats (Molapo, 2009). This clearly indicates that poultry slaughterhouses are significant contributors to wastewater generation, compared to other slaughterhouses.

Discharge of poultry slaughterhouse wastewater (PSW) into rivers usually culminates in the formation of a FOG layer on the water surface, decreasing the transfer of oxygen into the waterwhile limiting light penetration, which dilapidates the rivers and ecosystem, threatening the life of aquatic animals. To limit such ecological pollution, numerous treatment techniques

are employed. For these reasons, wastewater treatment has become crucial, while the need for regulatory standards for appropriate disposal and/or discharge has become stringent, making the improvement of the treatment technologies a necessity (Bayar *et al*., 2014; Bustillo-Lecompte *et al*., 2016).

2.2 Characteristics of poultry slaughterhouse wastewater (PSW)

Poultry slaughterhouse wastewater (PSW) is characterised by high concentration of lipids, proteins, suspended solids, biodegradable organic matter and other nutrients (Commarota & Freire, 2006). It is also characterised by high chemical oxygen demand (COD) and biochemical oxygen demand (BOD) compared to domestic sewage wastewater (Basitere *et al*., 2016). High levels of BOD and COD are due to the presence of numerous constituents, including carcass debris. This wastewater is also described as high-strength wastewater because of its high COD, protein and FOG content (Coskun *et al*., 2014; Harris *et al*., 2015). The composition of the wastewater varies according to the process used in the slaughterhouses, the number of birds slaughtered andthe chemical agents used during the sanitation of facilities (De Nardi *et al*., 2008; Jayathilakan *et al*., 2012).

The birds' gastric system material including blood, is a major contributor to PSW, which is responsible for pollution and contamination. Non-intestinal blood is another major pollutant due to its high contributory effect to the COD (Kundu *et al*., 2013).The presence of compounds such as nitrogen and phosphorus when discharged into fresh water bodies may lead to challenges such as eutrophication. Nitrogen and phosphorus are sourced from blood, urine and faeces, and from chemical compounds used in facility cleaning and sanitation processes (Kundu *et al*., 2013). Heavy metals and pharmaceuticals may be prevalent in PSW from veterinary pharmaceuticals including antibiotics (Bustillo-Lecompte & Mehrvar, 2017). Further, PSW may also contain non-pathogenic and pathogenic microorganisms that are not deactivated during slaughterhouse cleaning and sanitation processes (Dlangamandla, 2016).

Characteristics of raw PSW from local slaughterhouses in the Western Cape, South Africa, are summarised in Table 2.1. Additionally, PSW quality characteristics from previous studies are summarised in Table 2.2. PSW quality parameters must be analysed and monitored due to the variation of the wastewater quality being produced, as they are dependent on the species slaughtered and processes used in slaughterhouses. These analyses can include total organic carbon (TOC), biochemical oxygen demand (BOD), chemical oxygen demand (COD), total nitrogen (TN), total suspended solids (TSS) and total phosphorus (TP) among others (Bustillo-Lecompte & Mehrvar, 2017).

Table 2-1: Characteristics of raw poultry slaughterhouse wastewater from a poultry slaughterhouse in the Western Cape, South Africa (Dlangamandla, 2016; Basitere *et al*., 2017).

Parameters	Average values
pH	6.88
Total alkalinity (mg/L)	185.25
Total chemical oxygen demand (tCOD) (mg/L)	1865
Soluble chemical oxygen demand (sCOD) (mg/L)	1224
Biochemical oxygen demand (BOD_5) (mg/L)	1250
Ammonia (mg/L)	120
Total phosphorus (TP) (mg/L)	17.8
Fats, oil and grease (FOG) (mg/L)	406
Total dissolved solids (TDS) (mg/L)	500
Total suspended solids (TSS) (mg/L)	413
Soluble proteins (mg/L)	70

Table 2-2: General characteristics of poultry slaughterhouse wastewater from previous studies (Basitere *et al*., 2016; Cosmann *et al*., 2017).

Parameters	Average values
pH	6.46
Total alkalinity (mg/L)	185.25
Total chemical oxygen demand (tCOD) (mg/L)	1865
Soluble chemical oxygen demand (sCOD) (mg/L)	2400
Biochemical oxygen demand (BOD_5) (mg/L)	781
Ammonia (mg/L)	1260
Total phosphorus (TP) (mg/L)	47
Fats, oil and grease (FOG) (mg/L)	1600
Total dissolved solids (TDS) (mg/L)	585
Total suspended solids (TSS) (mg/L)	800
Soluble proteins (mg/L)	90

2.3 Poultry slaughterhouse wastewater (PSW) treatment

Due to environmental pollution and contamination potential associated with PSW, direct discharge of the raw PSW into the environment is impractical; therefore, adequate treatment and disposal of such wastewater is usually required. Wastewater treatment methods used in the treatment of municipal wastewater are often inadequate for the treatment of PSW;

methods which include the following steps: (1) pre-treatment, (2) primary, (3) secondary and sometimes (4) tertiary treatment systems should be implemented (Taskan *et al*., 2016).

A pre-treatment method involves the application of screens, settlers, catch basins, and flotation systems in order to reduce and separate floating suspended solids (SS)from slaughtered animals such as meat debris, feathers, bones, proteins and FOG. The solids are separated from the wastewater and are treated as solid waste. Solids of over 30mm diameter are separated by screens while those less than 0.5mm, are retained within the wastewater as they do not contribute significantly to clogging and fouling of equipment in wastewater treatment plants. Reduction and separation of solids can be up to 60%, and BOD can be reduced by 30 to 40%, particularly if SS is reduced by 50 to 70%.

Pre-treatment of the effluent is followed by primary treatment, in which the effluent organic load is reduced by anaerobic systems, which reduce the COD and BOD in particular. In secondary treatment systems, biological treatment by microorganisms is used to remove pathogens and harmful compounds. Over 90% of the toxic pollutants are removed from the wastewater by aerobic and consequently anoxic methods. For the tertiary system, membrane bioreactor technology is normally used, which results in further pollutant reduction.Tertiary treatment systems are highly efficient in removing suspended and dissolved solids. Removal of nutrients such as nitrogen or phosphorus species, which cannot be accomplished by biological treatment, can then be achieved using tertiary treatment technology. The use of tertiary treatment systems in the treatment of PSW, however,is limited due to their high capital and operational costs (Mittal *et al*., 2006). Generally, treatment of wastewater depends on the systems available and the standard of treatment required. Amongst the treatment systems available, each system has its own operational advantages and disadvantages (Rajkumar *et al*., 2011).

2.3.1 Biological treatment

Physical treatments used as primary pre-treatment systems do not properly treat PSW to a standard that complies with those set by regulatory agencies, at national government levels and local manucipility. For further treatment, anaerobic treatment processes, i.e. for organic matter biodegradation, are used for the reduction of the remaining COD and BOD, i.e. organic matter from pre-treatment systems. Biological treatment is normally used as a primary technology for reduction of COD/BOD through the biodegradation of organic matter using suitable microorganisms. Additionally, these processes are used to remove pathogens and other pollutants present in the wastewater. Biological processes include aerobic, anaerobic treatment systems or a combination of these processes (Bustillo-Lecompte & Mehrvar 2016, 2017; Hamawand *et al*., 2017).

2.3.1.1 Aerobic biological treatment

Aerobic systems utilise bacterial microorganisms with the ability to grow in the presence of dissolved oxygen to facilitate the removal of organic matter, thus COD/BOD reduction (Hamawand *et al*., 2017). Dissolved oxygen is required by these microorganisms for biomass generation in the wastewater to be treated (Mittal *et al*., 2006). The quantity of dissolved oxygen and treatment time required thus defines the hydraulic retention time of the treatment reactor which is dependent on the strength of the wastewater being treated.This makes aerobic treatment suitable after anaerobic treatment, which is usually used for high strength wastewater (Al-Mutairi *et al*., 2009). Application of aerobic treatment systems as a sole treatment technology for the removal of organic matter is impractical. For this reason, aerobic systems are often applied after physicochemical treatment as the last stage for the removal of residual nutrients and further reduction of COD/BOD in the wastewater (Bustillo-Lecompte & Mehrvar, 2016). The removal of nutrients such as ammonia and phosphorus is one of the vital functions of aerobic treatment systems.

The advantages of aerobic systems are minimal odour production and rapid biomass growth rates, and therefore the ability to handle fluctuating organic loading rates. However, these systems have high operational costs compared to anaerobic systems, owing to the maintenance and energy required for artificial dissolved oxygen supplementation using spargers. Furthermore, the operational costs are also high due to the process units used. which have a large foot print and require disposal of excess sludge produced. Typical aerobic treatment systems for PSW include rotating biological contactors (RBCs) and aerobic sequencing batch reactors (SBRs).

In RBCs, oxidation is utilised by facilitating air/bacteria contact. The RBC process mechanism for treatment includes wastewater contact with biologically active biomass for metabolism of organic matter and other pollutants, prior to the discharge of the treated wastewater to the environment. According to the literature reviewed,RBCs are inefficient in treating wastewater from meat processing plants compared to other aerobic treatment systems (Johns, 1995; *Li et al*., 2008; Zhan *et al*., 2009; Kundu *et al*., 2013; and Pan *et al*., 2014), On the other hand, aerobic SBRs consist of the following stages: (1) filling, (2) bioreaction, (3) settling and (4) decanting. During the filling stage, the bioreactor is fed with wastewater in the absence of air. Air is sparged and organic matter biodegradation occur in the second stage. There after, TSS are allowed to settle by ceasing homogenisation and sparging. As a result, clarification of the effluent ensues prior to discharge. Numerous studies, including those by Li *et al*.(2008), Zhan *et al*. (2009), Kundu *et al*. (2013) and Pan *et al*. (2014), report SBR to have one primary disadvantage, namely high energy intensity for sparging and fluid transportation requirements.

2.3.1.2 Anaerobic biological treatment

Anaerobic treatment is widely used for the treatment of PSW, i.e. high strength wastewater, due to its high effectiveness and other advantages, including high removal of COD, nitrogenous compounds, and other substrates (De Nardi *et al*., 2008; Bustillo-Lecompte *et al*., 2016, 2017). Furthermore, the high organic content of the PSW makes it suitable for treatment using anaerobic processes (Messè & Masse, 2000; Hamawand *et al*., 2017). During this treatment, different bacterial species are used to degrade organic compounds into CO_2 and CH_4 (biogas) in the absence of dissolved oxygen, while reducing COD/BOD (Bustillo-Lecompte *et al*., 2015). Compared to aerobic processes which are highly sensitive to fluctuations in temperature, pH and organic loading rates, anaerobic processes are suitable for PSW treatment (Mittal *et al*., 2006). The success of the anaerobic process depends on the physicochemical pre-treatment system used. Some typical anaerobic treatment systems are anaerobic lagoons (AL), anaerobic baffled reactors (ABRs), upflow anaerobic sludge bed (UASB) reactors, anaerobic sequencing batch reactors (ASBRs) and expanded granular sludge bed (EGSB)reactors (Basitere *et al*., 2016; 2017).

Anaerobic lagoon construction is appropriate where there are availability of land and suitable weather conditions. Typical ALs are constructed to be 3 up to 5m with a retentiontime of up to 10 days. The advantage of AL is high removal effectiveness for COD, BOD and TSS, with treatment efficiency of up to 97%. Major operational challenges are related to odour generation and unpredictable weather changes (Chipasa& Mędrzycka, 2006; Bustillo-Lecompte & Mehrvar 2015). A disadvantage of using such systems is its open-air characteristics which can lead to pollutant (nitrogen compound) air stripping, and the transportation of VOCs into the atmosphere, further contributing to environmental pollution. Generally, between these systems, the UASB is the most commonly used for the treatment of municipal and industrial wastewater due to its low operating expense and its efficacy in treating high strength industrial wastewater (Chernicharo, 2006). In UASB, granular sludge is used whereby bacteria form biomass granules. The wastewater influent enters at the bottom of the reactor, moves upward through the sludge blanket, interacts with the sludge or biomass, and then moves to the top of the reactor, where it is recovered.The effectiveness of operation is influenced by bacterial granule activities which are placed at the bottom of the digester. UASB has been demonstrated to have a high success rate of organic matter degradation and it is suitable for PSW treatment. Where failures do occur, they are attributed to high FOG concentration and difficulties in degrading proteins in the wastewater. Highest COD removal achievable using the UASB was determined to be up to 95%,in the case of wastewater containing polymeric fatty acids (Chipasa & Mędrzycka, 2006).

An EGSB also employs granular biomass for treatment of high-strength wastewater and is operated similarly to the UASB, with the difference being in operational parameters and reactor configuration. The EGSBs are constructed to handle a variety of particulate matter/FOG loading rates and are able to operate at high upflow velocities. This allows for improvement in sludge-wastewater contact. High treatment efficiency has been shown for EGSB compared to a UASB, due to its height to diameter ratio. The high height/diameter ratio permits the EGSB design to operate both at high loading rates and hydraulic retention time (HRT) (Rodríguez *et al*., 2017).

Even if anaerobic treatment processes are economically feasible, the effluent from such systems hardly complies with current legislated wastewater discharge standards. For PSW, this is due to its characteristics, which present a plethora of challenges including reduced stabilisation at varying organic loading rates. Thus, post-treatment for anaerobically treated effluents is necessary for the removal of residual organic matter and other nutrients (Bustillo-Lecompte & Mehrvar 2016; 2017).

2.4 Impact of fats, Oil and grease (FOG) from poultry slaughterhouse wastewater on biological treatment systems

High-strength wastewater rich in fat, oiland grease (FOG), with an example being PSW from poultry slaughterhouses, can cause various operational challenges if released to downstream biological treatment processes (Harris *et al*., 2015). In such treatment systems, lipids present in the wastewater are normally referred to as part of the organic matter that will be treated. In aerobic systems, dissolved oxygen required by the aerobic bacteria used is reduced. This deprives the bacterial consortia of dissolved oxygen, leading to the reduction in microbial activity (Chipasa & Medrzycka, 2006). This is due to the entrapment of a lipid layer around the biomass that encourages the development of filamentous microorganisms such as *Thiothrix, Sphaerotilusnatans, Beggiatoa, Nocardia and Microthrix* sp.,which are responsible for the build-up of scum and foam formation on the surface of the wastewater. Furthermore, biomass flocculation is promoted, resulting in bulking, biomass flotation and washout (Commarota & Freire, 2006). Therefore, in such systems, FOG and low dissolved oxygen lead to poorly treated water of quality that does not meet by-laws for discharge. Other methods of treatment for wastewater with high levels of FOG must therefore be investigated as an alternative.

Although anaerobic biological treatment provides advantages that mitigate influences of FOG, i.e. negative effects experienced due to clogging of pipes, wash-out of the sludge-bed and the formation of scum, adequate pre-treatment can mitigate such inadequacies more effectively. FOG presence is likely to limit the efficiency of the biological treatment by slowing the biodegradation process, thus reducing biogas generation which is an alternative source

of energy. Monitoring of this treatment is necessary to prevent accumulation of the FOG layer with in the system. Even if monitoring does work for a short period of time, this does not guarantee a prevention of process inhibition by the FOG.

From the literature reviewed with regard to advantageous anaerobic biological treatments, anaerobic lagoons (ALs) and UASBs, are considered suitable systems for treatments of wastewater with a high FOG content. ALs are able to operate properly without failing even when a large quantity of FOG is discharged into these systems. This is due to insufficient mixing that permits flotation of the FOG on the wastewater surface forming a FOG layer, preserving an anaerobic environment. This cannot be said of UASBs, for which granular sludge activity can be reduced with biomass washout being problematic. Furthermore, FOG accumulation on the surface of lagoons can reduce lagoon volume,resulting indead zones over time. This results in the reduction of AL efficiency (Harris *et al*., 2015).

In a UASB, two major operational challengesare attributed to FOG:flotation of the sludge granules and microbial inhibition; and acidification and accumulation of ammonia. Ammonia is produced from the degradation of complex/polymeric compounds containing molecular nitrogen. The combination of both anaerobic-aerobic biological process systems is there fore considered to be the most suitable alternative to achieve maximum treatment efficiency while reaching current discharge standards (Chan *et al*., 2009; Bustillo-Lecompte & Mehrvar 2016; 2017), as shown in Table 2.3. To mitigate FOG accumulation effects, by physical removal in combination with biocatalysis, for complete and/or partial degradation of FOG, lipolytic enzymes can be used (Commarota &Freire, 2006).

Table 2-3: Industrial wastewater discharge standards (City of Cape Town, 2006).

Parameters	**Average values**
pH	8.75
Total chemical oxygen demand (tCOD) (mg/L)	5000
Ammonia (mg/L)	5
Total phosphorus (TP) (mg/L)	25
Fats, oil and grease (FOG) (mg/L)	400
Total dissolved solids (TDS) (mg/L)	4000
Total suspended solids (TSS) (mg/L)	1000
Settle-able solids (mg/L) per 60 minutes	50
Total sulphate (SO4) (mg/L)	1500
Temperature (°C)	40

2.5 Lipolytic enzyme

2.5.1 Lipases

Lipases can be defined as triacylglycerol acylhydrolases which are an important member of the class of hydrolases with an ability to catalyse the hydrolysis of acylglycerols, long or short-chained fatty acids, glycerols, diglycerides and monoglycerides, thus destabilising the water-lipid interface, and leading to FOG separation from the wastewater (Ruiz *et al*., 2005). Additionally, lipases are capable of catalysing a variety of reactions such as transesterification, alcoholysis, acidolysis and esterification that exhibit stereo- and regio-selectivity (Reis *et al*., 2009). Lipases are abundant in nature and can be produced by a variety of plants and microorganisms (yeast, fungi and bacteria). These lipases are the most commonly used enzymes for biotechnological and bioprocessing applications (Treichel *et al*., 2010), and were selected to be suitable for FOG bio-delipidation for DAF pre-treated PSW. Microbial lipases are widely used due to their ability to facilitate biocatalysis under mild conditions, their high stability in organic solvents, and their ability to transform a high number of substrates, thus facilitating and sustaining conversion rates during biocatalysis reactions (Anobom *et al*., 2014).

The physiological functionality of lipases varies depending on their source. In plants, they are active in the energy reserve tissues and are activated during germination. In several microorganisms, these enzymes are involved in lipid metabolism, i.e. FOG digestion, absorption and reconstitution. Lipase-producing microorganisms have been isolated from industrial plants, soil and waste containing oils; therefore, it is prudent to hypothesise that some species within the PSW can produce lipases. Bacterial lipases are intracellular glycoproteins and extracellular lipoproteins. Bacterial lipases are the most commonly used due to properties such as versatility and ease of production in a bulk form (Sharma *et al*., 2011; Anobom *et al*., 2014). Bacteria translocate these enzymes by secretion into the surrounding environment via their membranes. A typical bacterial lipolytic enzyme secretion pathway is demonstrated in Fig. 2.1. Here the inner or cytoplasmic membrane in gram-positive bacteria is used to secrete the enzymes with the inner membrane performing a similar function in gram-negative bacteria. These processes are mediated through the Sec- and Tat-secretion pathway via cellular inner membranes. Secretion of the lipases from bacteria is largely influenced by nutrient medium components during production (Jeagar & Eggert, 2002). Compared to other factors such as pH, temperature, and dissolved oxygen including a suitable medium typically composed of a carbon source, lipid sources such as oil, fatty acids, triacylglycerol or a hydrolysable ester (Gupta *et al*., 2004) can facilitate higher production rates.

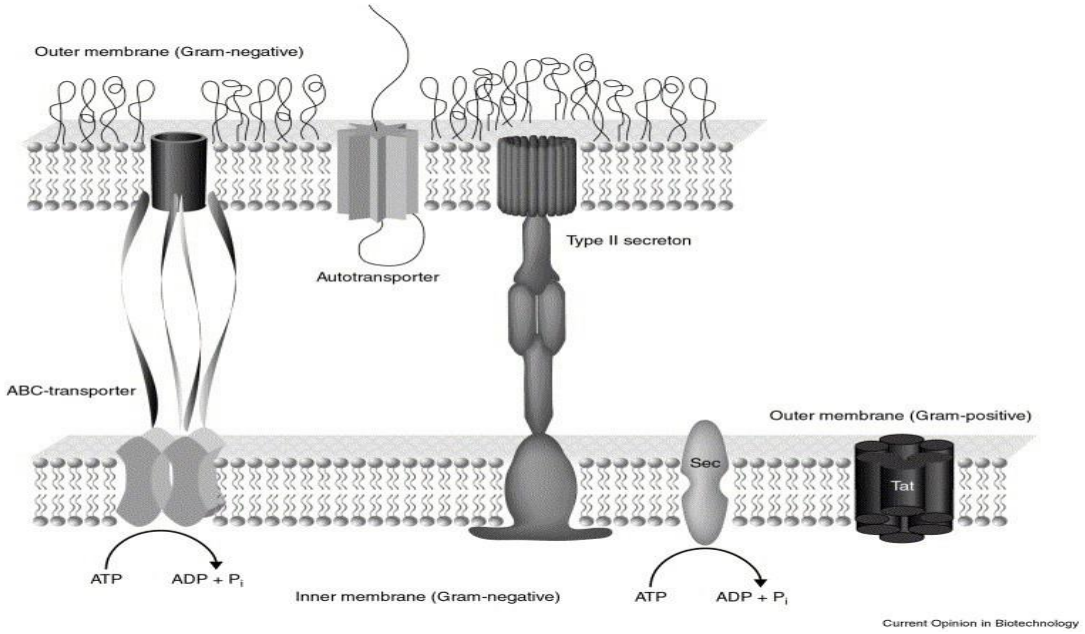


Figure 2-1:Typical pathway utilised by bacteria to secrete lipolytic enzymes (Jaegar and Eggert, 2002).

Presence of a lipidic carbon source is necessary for high lipase production; however, high lipase yields in the absence of oils or fats has also been reported (Treichel *et al*., 2010). Selection of a suitable nitrogen and micronutrient sources plays an important role in increasing microbial growth, as lipase production is biomass-dependent. Nitrogen sources were reported to influence increases in lipase activity in a study by Facchini *et al.*(2015), resulting in reduced production costs. However, organic nitrogen sources such as peptone and yeast extract are preferred, with research reports indicating maximum production of extracellular lipases when these constituents are used (Gupta *et al*., 2004; Treichel *et al*., 2010).

Generally, bacterial lipases show stability over a wide pH range, i.e. 4-11,and temperatures, i.e. 30-60°C. However, there are bacterial lipases reported to have an optimum temperature at low or a higher range, i.e. <30°C and >60°C (Treichel *et al*., 2010). The activity of bacterial lipases is also stimulated by the presence of calcium and magnesium ions, although they are inhibited by EDTA. Furthermore, fungal lipases have an optimum pH of 4-8, with some being able to function both under acidic and alkaline pH at an optimum temperature of 45-75°C (Sharma *et al*., 2011). As previously stated, these enzymes, from both bacterial and fungal species, are stable in organic solvents such as ethanol, methanol and acetone, while some of the organic solvents have a distinct inhibition effect on lipases from certain microorganisms, i.e. *Pseudomonas aeruginosa* lipases which are inhibited by acetone and those of *Bacillus* sp. which are inhibited by hexane (Gupta *et al*., 2004).

2.5.2 Lipase characteristics, structure and mechanism

Lipases are proteins with a molecular weight ranging from 16-670 kDa (Sharma *et al*., 2011). They are characterised to by α/β fold similar to other enzymes amongst the class of hydrolases such as proteases, esterases, dehalogenases and epoxide hydrolases. The α/β hydrolases fold is characterised by its β-sheet composed of eight β-strands with β2 antiparallel to other β strands, while β3 to β8 are inter-connected by a bundle of helices A and F packed at the centre of the β-sheet (Gupta *et al*., 2004). The α/β hydrolases active site is made up of a catalytic triad consisting of the following: (1) a nucleophilic (serine, cysteine and aspartic acid) group described as a serine residue, (2) a catalytic acidic (aspartic or glutamic acid) residue and (3) a histidine residue. An active site nucleophilic residue of lipases is found in the pentapeptide residue, forming a turn between β5 and a nucleophilic elbow. Nuecleophic residue is a conserved feature of the α/β hydrolases fold. A catalytic acid functional group is located in the loop found in the β6 or β7 strand connected to the catalytic histidine by a hydrogen bond. In the class of α/β hydrolases, lipases are the only enzymes with a glutamic acid present in the catalytic triad. Histidine is located in the loop after the β8 strand with the shape and length varying amongst the family members (Anobom *et al*., 2014) (see Fig. 2.2 for a lipase structure).

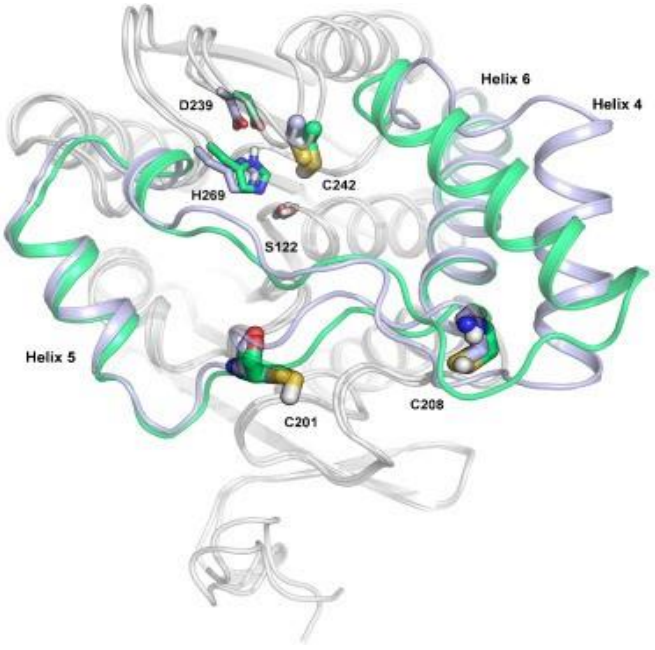


Figure 2-2: Lipase structure (Scalvin *et al*., 2016)

Lipases have a high activity on a wide range of substrates including stability without the requirement for additional cofactors in their biocatalysis reactions. These characteristics of lipases are responsible for the unique functionality of the lipid-water interface (Facchini *et al*., 2015). This unique feature is largely due to the activity of these enzymes on the lipid-water/oil-water interface,increasing the lipid-water interface destabilisation which effectively culminates in de-emulsification (Jegannathan *et al*., 2008), and thus bio-delipidation,as referred to in this thesis in page 28 to 29.

2.5.3 Reaction mechanism of lipases

Enzyme biocatalysis by lipases is initiated when the oxygen atom of the catalytic acid serine mechanically attaches to a carbon atom of the ester bond linked to the carbonyl group. This produces a tetrahedral intermediate generating a bond containing nitrogen atoms as a backbone to stabilise a negatively charged transitional state occurring in hydrolysis. Alcohol is released from an acyl-lipase complex hydrolysis, freeing fatty acids thus regenerating the enzyme. Active sites have been reported to be negatively charged at an optimal pH of 6 to10. After initiation of the ester cleavage, ionised carboxylic acid is released from the active site by electrostatic repulsion of the negatively charged carboxyl group. The negative electrostatic potential of the active site is mediated by the electrostatic mechanism. Lipases contain a Lid domain that allows access of the substrate to the catalytic center which is controlled by closed and open state conformations. In a closed state, a substrate cannot access the active site, making the enzyme inactive. During the opened state,theactive site is easily accessible by substrates (Anobom *et al*., 2014). Some biocatalysis reactions facilitated by lipases are listed below in Eq. 2.1- 2.6.

Hydrolysis: $$RCOOH + H_2O \rightarrow ROH + COOH \quad (2.1)$$

Esterification: $$RCOOH + ROH \rightarrow ROOR + H_2O \quad (2.2)$$

Acidolysis: $$ROOR + R^2COOH \rightarrow ROOH + R^2COOR^1 \quad (2.3)$$

Interesterification: $$RCOOR^2 + RCOOR^4 \rightarrow R^2COOR^2 + RCOOR^4 \quad (2.4)$$

Alcoholysis: $$ROOR^1 + R^2OH \rightarrow ROOR^2 + R^1OH \quad (2.5)$$

Aminolysis: $$ROOR^1 + R^2NH_2 + RONHR^2 + R^1OH \quad (2.6)$$

2.6 Bio-delipidationas a pre-treatment system for high-lipid wastewater

Lipase properties, i.e. high activity over a range of pH, temperature and selectivity, have made this group of enzymes suitable to be explored for numerous bioprocesses (Gupta *et al*., 2004). Overall, the understanding of the foldandinfluential environmental parametersfor the secretion of these enzymes is as important as the during large-scale production and application. Lipases have an extensive relevance and industrial applications in agrochemical production, food modification, detergent formulation, cosmetics, pharmaceuticals, and wastewater treatment,and particularly for pre-treatment stages of high-lipid containing wastewaters (Treichel *et al*., 2010; Anobom *et al*., 2014).

In a previous study reporting on the pre-treatment of high-lipid wastewaters, pancreatic lipases were observed to reduce the particle size of FOG in wastewater from pork processing facilities (Mendes *et al*., 2006). Similarly, the reduction of a high COD by lipases for dairy wastewater treatment was also reported (Leal *et al*., 2006; Mendes *et al*., 2006). Recently, lipase usage in wastewater pre-treatment has been found to transform organic matter, reducing the turbidity of the wastewater assessed, and reducing the hydrophobicity ofsuspended solids (Meng *et al*., 2017). Table 2.4 lists some applications of lipases.

Table 2-4: General application of lipases

Application	**References**
Lipase and lipolytic enzymes are involved in plant germination and lipid metabolism in microorganisms,	Alford *et al*. (1964); Feussner *et al*. (2001)
As minimizers for thermal degradations in the biochemical industry, facilitating hydrolysis, glycolysis, and alcoholism while saving energy,	Choudhury & Bhunia (2017)
As detergent enzymes in the detergent industry,	Liu *et al*. (2009)
Biocatalysts in the production of biodegradable compounds such as polyesters,	Kobayashi (2009)
Modification and breakdown of biomaterials for flavor development and processing of food in the food industry,	Aravindan*et al*. (2007)
Transesterification and catalysis of hydrolysis reaction in the pharmaceutical sector for synthesis of drugs,	Kanwar*et al*. (2014)
In the paper and pulp industry for the removal of wood triglycerides,	Bajpai, (1999); Demuner *et al*. (2011)

In biosensors, i.e. in pH/oxygen electrodes, in conjunction with glucose oxidases,	Ramani & Sekaran (2012)
As catalysts in biodiesel production as bio-catalysts,	Fan *et al*., (2012); Aguieiras *et al*. (2017)
For production of biosurfactants and aroma biomolecules for the cosmetic and fragrance industries.	Ansorge-Schumacher *et al*. (2013)
Used in the pre-treatment systems of anaerobic and certain aerobic waste processes for removal of FOG layers and to permit increased dissolved oxygen dissolution.	Rasit *et al*. (2015)

Despite lipase characteristics that have made them suitable for extensive applications in various industries, their high production costs have limited their use, especially for the hydrolysis of fats (Salihu *et al*., 2012). Limited application for production for lipid-rich wastewater treatment has been due to factors associated with procedures for their production, purification, and bioreactor operation, including stability and regeneration (Chipasa & Medrzycka, 2006; Salihu *et al*., 2012). Furthermore, availability of sufficient quantities of lipases for application in delipidation can be another limiting factor. Research to identify production conditions for improved excretion, and a search for new hyper productive strains, is thus required for improving lipase application in low-performance processes such as wastewater treatment.

2.7 Pre-treatment systems for FOG removal prior to biological treatment: a focus on PSW

A pre-treatment system for PSW is primarily used to reduce lipids, proteins and suspended solids (feathers and carcass debris) prior to biological treatment processes (Dlangamandla, 2016). This allows for the reduction of the organic load in wastewater destined for biological treatment (Mittal *et al*., 2006). Reduction or elimination of high levels of proteins and FOG is necessary to enable and facilitate high biological treatment efficiency. Overall, physical and physicochemical pre-treatment systems remain the most popular alternative for the removal of FOG in pre-treatment systems for poultry slaughterhouse wastewater(Mittal *et al*., 2006).

2.7.1 Physicochemical pre-treatment systems

2.7.1.1 Dissolved air flotation system (DAFs)

Dissolved air flotation is a suspended solids/water-separation method utilised for the removal of particulate matter, proteins and FOG by introducing air through sparging into the wastewater (Rajakumar *et al*., 2011). The DAF is the most commonly applied physicochemical pre-treatment system for the reduction of FOG and suspended solid

particles due it slow operational expenses, although, it is energy-intensive due to the sparging of air into the system. Major challenges associated with the operation of DAF systems, however,arise in relation to improper separation of solid particles due to insufficient floc formation (Bustillo-Lecompte & Mehrvar, 2016). The mechanism of operation is based on solid particle agglomeration which facilitates the transportation of these particles in wastewater to the surface, resulting in a layer that is constantly skimmed off. Reduction in COD of over 30 to 90%, and BOD of 70to 80%, is normally observed. DAF systems consist of components such as an air supply unit, wastewater pump, and/or a retention tank acting as a chamber for flotation (Srinivasan & Viraraghavan, 2009). The operation of this system is based on three phases:(1) gas transformation, i.e. air and water interaction in the retention tank;(2) gas precipitation, i.e. gas bubbles are formed in the dissolved state and(3) flotation associated with bubble supported SS agglomeration. The generation of bubbles, conditions under which bubbles are formed, and bubble-particle agglomeration, are amongst the most important factors in dictating the success of the DAF process (Parsons & Jefferson, 2006).

In a typical DAF system, air is sparged using the dissolved air technique into the wastewater under a high pressure, i.e. fed through the retention tank, to flocculate suspended solids and provide flotation capabilities to reduce FOG and proteins. This system operates according to Henry's Law, which states that increased pressure increases the solubility of agas in an aqueous solution. Dissolved air is allowed to pass into a retention tank containing the wastewater to be treated. Depressurisation increases bubble size as the bubbles rise through the chamber. The reduced pressure in the flotation chamber results in the production of initial tiny bubbles. These bubbles attach to the surface of FOG, pneumatically accelerating these upwards at a rate of 0.15 to 0.6m/min. This enables the FOG to be skimmed off from the surface of the wastewater by scrapers and skimming equipment (Wang *et al*., 2005).

Generally, the flotation of solids and lipids is achieved by the dispersed air, using electrolytic flotation or dissolved air flotation (Al-Shamrani *et al*., 2002). Pre-treated water is discharged or recovered from the bottom of the flotation chamber for subsequent primary treatment. Hydraulic retention time for the flotation chamber depends on the characteristics of the wastewater processed and the performance of the flotation unit. The efficiency of the DAF depends on the attachment of tiny bubbles to the FOG and solid particles to be removed. The bubble size plays an important role in the electrostatic attraction between the bubbles and the suspended solid particles. To improve the performance of a DAF system, sometimes organic chemicals, including polymeric flocculants, are supplemented to the flotation to reduce like charge repulsion in order to facilitate floc formation (Mittal *et al*., 2006).Fig. 2.3 illustrates procedures and process units used for pre-treating wastewater with a high concentration of FOG and suspended solids.

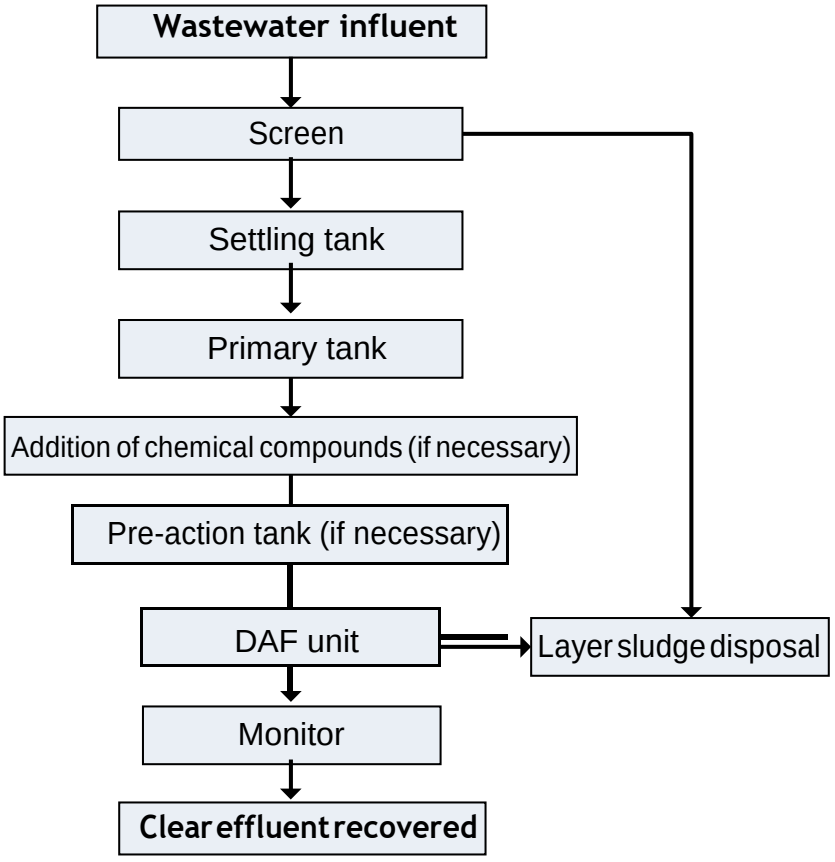


Figure 2-3: Typical DAF system operation.

2.7.1.2 Fat/grease traps and gravity separation methods

Fat/grease traps are also sometimes used as physical pre-treatment systems instead of DAFs. A grease trap causes agglomerated fats, grease and suspended solids to be separated from the wastewater by screening the solids prior to the wastewater being fed to other downstream processes, with plate separators being preferably used (Commarota & Freire, 2006). For gravity separation methods, a mechanism based on the Stokes' Law explaining the particulate matter ability to settle; i.e. a theory of separatability that is independent on wastewater depth in the settling tank, but dependent on the density and diameter of solids, and the viscosity of the wastewater. This method, which is preferred, is shown in Eq.2.7.

$$v_c = \frac{g(\rho_s - \rho)d^2}{18\mu} \quad (2.7)$$

Where:

V_c= velocity of the particles (m/s)
g = acceleration due to gravity (m/s^2)
ρ_s= particle density (Kg/m^3)
d = particle diameter (m)
μ = absolute viscosity of fluid (mPa.s)
ρ =fluid density (Kg/m^3)

2.7.1.3 Electrochemical methods

Electro-oxidation and coagulation are used as electrochemical methods after pre-treatment methods for reduction of residual FOG and TSS, which reduces COD and BOD for different types of wastewater, including PSW (Bazrfshan *et al.*, 2012). These electrochemical systems are used for removal of residual nutrients, metals, organic matter and the deactivation of pathogenic microorganisms present in the wastewater. Electrodes, usually those made of Fe, Al, TiO_2, Pt, and SnO_2, are used to facilitate electrochemical reactions in the wastewater. Fe and Al are the most commonly utilised electrodes due to their reliability and are considered less harmful to the environment. In these methods, the metal released, i.e.M^{+3} reacts with H^+ or OH^- ions in an acidic/alkaline medium, forming a hydroxidewhich attaches to suspended solids. These are mostly cationic, facilitating floc formation which is dependent on the pH and the strength of particle charges to be neutralised (Moussa *et al.*, 2017). Chemical reactions which take place at the electrodes for both Al (Eq. 2.8 to 2.10) and Fe (Eq. 2.11 to 2.13) electrodes are:

$\mathrm{Al} \leftrightarrow [\mathrm{Al}]^{3+}$ (anode) (2.8)

$3H_2O + 3e \leftrightarrow \frac{3}{2} H^+$ (cathode) (2.9)

An Al^{3+} and OH^- ion generated at the electrodes facilitates reactions in the wastewater, forming aluminum hydroxide(Eq. 2.10).

$[Al]^{3+} + 3OH^- \leftrightarrow [Al(CH)_3]$ (bulk) (2.10)

$Fe \leftrightarrow Fe^{3+}$ (anode) (2.11)

$4Fe^{2+} + 10H_2O \leftrightarrow 4Fe(OH)_3$(cathode) (2.12)

Similarly, Fe^{3+} and OH^- ions formed at the electrodes, facilitate reactions in the wastewater forming iron hydroxide.

$Fe^{2+} + 2OH^- \leftrightarrow Fe(OH)_2$ (bulk) (2.13)

The interaction of Al^{3+} (Eq. 2.10) and Fe^{3+}(Eq. 2.13) with OH^- results in hydroxides which neutralises charged suspended solids to form agglomerated flocs. The flocs formed are usually large, with their surface being able to absorb soluble organic matter and facilitate the entrapment of colloidal particles. In addition, when the agglomeration of these flocs occurs, it enhances the removal of suspended particle through flotation by hydrogen gas formed ,and/or sedimentation of high density flocs . A variety of advantages accrue to electrochemical methods compared to coagulation processes, including simplicity of operation, and reduced/minimal sludge production. However, the constant replacement of electrodes increases operational costs (Moussa *et al*., 2017).

Asseline *et al*. (2008) examined the effectiveness of using an electrocoagulation process in treating PSW prior to discharging it into a municipal sewer system. This was done using mild steel and an Al electrode arranged in a bipolar (BP) or a monopolar system at current intensity of 0.3A for 60 and 90 min treatments without differences respectively. The experiment under these conditions resulted in the removal of up to 86% BOD, 84% COD, 93% TSS, 94% turbidity and 99% FOG. Efficient decolorisation and clarification of the PSW were also reported. A comparable study by Kobya *et al*. (2006) similarly used an electrocoagulation process for PSW, resulting in high removal efficiency of COD reduction of 93 to 98% using an Al electrode, while achieving a maximum FOG removal using an Fe electrode.

2.7.1.4 Membrane technology

Membrane technology processes using membrane bioreactors (MBRs) have been used as yet another method for PSW treatment over conventional anaerobic processes. MBR processes are unlike anaerobic processes, which require secondary and tertiary treatment steps (Gürel & Büyükgüngör, 2011). Various membranes are used in MBR processes,i.e. ultrafiltration (UF), microfiltration (MF), reverse osmosis (RO) and nanofiltration (NF) membranes. During MBR operations, membrane separation by filtration through high pressure or vacuumis applied. Advantages of using MBR processes include high quality of the treated water, ease of operation and minimal sludge production compared to anaerobic sludge processes. However, biofouling due to FOG during MBR treatment of high-strength

PSW is a major challenge (Bustillo-Lecompte & Mehrvar, 2017). Biofouling can be described as the adhesion of biologically non-active and active agents including bacteria that grow and attach to the membranes, including their by-products, i.e. extracellular polymeric substances (EPS), including macromolecules that facilitate biofilm formation and attachment (Flemming *et al*., 2011; Misdan *et al*.,2015). Biofoulingis a major challenge due to its higher contribution to fouling of membranes, which further exacerbates the operational energy requirements for MBRs. This culminates in additional costs associated with chemical treatment used to clean the foulants from the membranes (Nguyen *et al*.,2012). Chemicals with biocide activity are used in MBRs to de-foul membranes. Provided they are not corrosive,they can be effective; however, there have been concerns related to the use of these chemicals because they reduce membrane lifespan and produce harmful by-products which are known toxicants to humans and the environment (Dobosz *et al*., 2015).

2.7.2 Additives to biological pre-treatment systems

In biological pre-treatment systems, methods that utilise by-products from microorganisms are used. These methods are considered suitable alternatives in FOG removal from lipid-rich wastewater. Use of enzymes and bioflocculants produced by microorganisms that can be isolated from the wastewater itself lessens the need for the use of chemical compounds,an approach which is preferred (Mendes *et al*., 2006; Duarte *et al*., 2015) to de-emulsify FOG that can be removed.

2.7.2.1 Biosurfactants as additives

The use of biosurfactants in pre-treatment systems for wastewater with a high FOG concentration such as PSW is also considered a suitable alternative. The use of these biosurfactants as additives in biological primary and secondary supported pre-treatment systems is favored in systems that incorporate downstream biological treatment, due to the benignity of the biomolecules used (Harris *et al*., 2015). Generally, biosurfactants are described as biological compounds with ionic, non-ionic, cationic or amphoteric functional groups importing suitable properties required for wastewater treatment. They form the primary components of detergents and soaps which facilitate emulsification,with biosurfactants with anionic properties being the most commonly used in detergent production (Zanoletti *et al*., 2017). In selecting an appropriate biosurfactant, the most important factors to be considered include its benignity and ability to lessen the ecological burden on the environment, as synthetic surfactants may accumulate and persist, further facilitating eutrophication, which can negatively affectaquaticlife.

Biosurfactants are produced by a variety of microorganisms (bacteria, yeast,and fungi) through extracellular excretion, with amphiphilic, i.e. hydrophobic or hydrophilic, properties

(Mulligan, 2005). Classification of biosurfactants is based on their structure and the microorganism of origin. They are classified as lipopeptides, glycolipids, phospholipids, polymeric fatty acids and neutral biomolecules (Fakruddin, 2012). Biosurfactants of varying molecular weight and chemical structure are classified by their ability to reduce water/wastewater surface tension in order for the insoluble organic matter of the water to be bioavailable for bioremediation (Yadav *et al*., 2016). Due to their advantages and the ability to reduce the surface tension of liquefied matrices, they are used in several industrial applications including bioremediation of wastewater.

In a study by Nakhla *et al*. (2003), biosurfactants were applied to evaluate a BOD-balance during the treatment of wastewater with ahigh FOG content in an anaerobic system, with COD biodegradation increasing. COD biodegradation increased by 60 to 74%. Removal of FOG was also noticed to have improved by 45 to 85%.

2.7.2.2 Bioflocculants as additives

Bioflocculants are biodegradable biopolymers produced by microorganisms such as bacteria, fungi,and algae during the cell growth phase. They are utilised as flocculation agents in flocculation processes for formation of flocs through colloid and particle aggregation. Bioflocculation is a flocculation process facilitated by macromolecules, i.e.bioflocculants, produced from microorganisms (Zaki *et al*., 2011).Bioflocculants facilitate flocculation by forming ties and/or charge neutralisation of particles leading to thea ggregation of suspended solid particles present in the wastewater. When large flocs are formed and then removed or settled. Additionally, bioflocculants are able to facilitate adsorption and entrapment of metal ions,making them a potential agent for removal of heavy metals (Liu *et al*., 2017). There are three types of flocculation agents; synthetic, inorganic and organic flocculants. Chemical flocculants are usually utilised for water and wastewater treatment in industrial applications due to their high flocculation activity. However, their use could result in environmental deterioration and human health-related issues (Aljuboori *et al*., 2015). Bioflocculants alone have received more attention as a suitable substitute for chemical and inorganic synthetic flocculants due to their environmental benefits, biodegradability,and nontoxicity.They have been successfully applied in industrial applications such as wastewater treatment, food,and beverage fermentations(Devasahayam, 2016).

Bioflocculants are composed of proteins, polysaccharides, glycoproteins, nucleic acids and macromolecular compounds (López *et al*., 2003). They can be extracted as metabolites during cell growth in the presence of a suitable nutrient media. They are often produced as biopolymers with a high molecular weight outside the growing cell. Sometimes natural flocculants from microorganisms are sometimes produced by inducement by chemical

compounds or by modification.Their effectiveness in different applications is affected by the functional groups attached to the carbohydrate structure of the exopolysaccharide that is influenced by bacterial growth conditions, i.e. production conditions (Devasahayam, 2016).

There are two main mechanical processes that are used to define the mechanism of the bioflocculant's functionality during bioflocculation and separation processes, namely, bridging and charge neutralisation as previously mentioned (Salehizadeh & Shojaosadati, 2001; Lian *et al*., 2008). Bridging occurs by the aggregation of bioflocculants with flocs, bridging threads, or forming fibers by adsorption. Bioflocculant macromolecules attach to colloidal particles, with the macromolecules' reactive sites forming attachments with the suspended solids surfaces, while unreactive sites expand into the solution. Adsorption of bioflocculants into the solution and surface segments occurs. This enables other free solid particles in the wastewater to attach,while the surface segment forms solid particle-bioflocculant aggregates which form a bridge. Formation of strong bonds occurs by segment attachment. A bridging mechanism of bioflocculants during bioflocculation depends on its molecular weight, polymeric structure and particle charge, ionic charge strength of the wastewater, and mixing (Salehizadeh & Shojaosadati, 2001). A bridging mechanism is more likely to occur with flocculants that have high molecular weight than those with a lower molecular weight. Furthermore, flocculants with a high molecular weight lead to larger flocs (He *et al*., 2010). During bridging,floc formation is then dependent on the mechanism which occurs when flocculant and particle interact, particularly when the constituents are oppositely charged. Attachment of particles and bioflocculants can occur through adsorption in cases whereby attractive forces are deactivated. Cationic bioflocculants can be adsorbed onto negatively charged solid particles, and thus flocculation is facilitated by the reduction of the negative charge on the particles (Sharma *et al*., 2006; Devasahayam, 2016).

In a recent study by Dlangamandla (2016), a DAF supplemented with bioflocculants was successfully used for the pre-treatment for PSW, whereby bioflocculants were added into the DAF with its efficiency being compared to a chemical supplemented DAF. Results showed bioflocculant supplemented DAF to have efficient removal of TSS (91%), lipids (93%), and proteins (79%), while a chemical supplemented DAF could only remove slightly reduced quantities of analysed parameters, i.e. TSS (84%), lipids (92%) and proteins (71%).

2.7.2.3 Other enzymatic pre-treatment methods

Enzymatic pre-treatment to improve hydrolysis of FOG enhances the performance of anaerobic processes. Hydrolysis using lipolytic or hydrolytic enzymes has been reported to improve the efficiency of treatment processes for wastewater from slaughterhouses, and that containing food waste with floating grease. However, numerous studies conducted on pre-

treatment of FOG using enzymatic methods only focused on feedstocks or enzyme pools for pre-treatment. Meng *et al*. (2017) investigated the enhancement of anaerobic digestion performance when liquefied extracts containing enzymatically treated crude lipids from food waste were treated with lipases to hydrolyse floatable grease from the food waste, therefore enhancing the digestibility of organic matter during anaerobic treatment. From the literature survey conducted, lipases were determined to be the dominantand suitable class of enzymes suitable for bio-delipidation, as discussed in Section 2.5. It was therefore hypothesised that lipases for pre-treatment systems treating wastewater rich in FOG – a process here after classified as biodelipidation – can effectively increase the further removal of residual FOG from other pre-treatment systems, such as DAFs.

2.8 Delipidation and biodelipidation of FOG-laden PSW

Delipidation is a method of defatting. In general, it is the removal of lipids or lipid groups such as fatty acidst, steroids,and polysaccharides from matrices in which they are present in minute quantities (Kraghhansen *et al*., 1993). Their removal occurs by breakage of covalent bonds of lipid groups from liquefied matrices. In biodelipidation, the removal or reduction of lipids from matrices is facilitated by abiological process via biological catalysis using macromolecules such as lipases. This could involve reactions where the covalent bonds attaching to lipids are broken or destabilised, with the lipids beinghydrolysed and/or semi-hydrolysed. Due to the high insolubility of lipids in liquid media,usually facilitated by their hydrophobicity, the application of suitable enzymes can thus further reduce the efficacy of the bondage mechanism which will result in easier delipidation procedures. Studies on delipidation focus on biological samples of plasma or serum, with minimal research being conducted on processes in wastewater treatment. The removal of lipids from protein-rich PSW is necessary prior to downstream processing of the wastewater, since proteins in biological mixtures from slaughtering will be made-up of triglycerides, phospholipids, and amphiphilic constituents. For example, lipid removal is a necessity to prevent fouling of downstream process in numerous industries. Normally during treatment of biological samples and/or wastewater containing organic matter, sophisticated systems are used to provide an environment for lipid removal through numerous methods including adsorption (Gardner, 1996).

Delipidation methods for defatting of organic matter containing matrices such as those that utilise charcoal, solvents,and detergents, do exist. Although these methods are effective in removal of fatty acids, challenges associated with their application occur, such as the contamination of the final product caused by adsorption of charcoal.Similarly, the use of solvents and detergents for delipidation may lead to persistence of chemicals as residue which may not be easily removed from a delipidised product and which may cause changes

in the properties of the final product. Therefore, the use of such a strategy will also be unsuitable for wastewater treatment (Sham & Knowles 1976; Osborne, 1986).

2.8.1 Quantifying biodelipidation in wastewater treatment post-DAF application

Delipidation methods and performance in removing lipids from matrices can be quantified by determining the lipid content of the samples before and after the delipidation procedure (Osborne, 1986). Kraghhansen *et al*.(1993) developed a delipidation method where removal of lipids including steroids and long-chain fatty acids from aqueous protein mixtures (2-10 mg) was achieved. Different albumin preparations were used as protein models to be delipidated. To determine the efficiency of the method, the fatty acid content of the protein model preparations was measured by monitoring delipidationusing palmitate or progesterone and enzymatic methods for quantifying fatty acids pre- and post-treatment. The method operated under predetermined conditions whereby hydroxyl alkoxypropyl dextran was used to extract lipids without protein denaturation. Test tubes with the suspension of the protein preparations and the dextran derivative, at pre-defined pH,were vortexed periodically, subsequent to separation of the purified protein and dextran mixture, which was done by applying the suspension to a column and allowing for the passage of the proteins. Additionally, the effect of the temperature and time on the delipidation process was determined. Removal efficiency results reported in the study averaged 97%.

In this study, the delipidation was facilitated by lipase producing organisms (bio-delipidation). Since the PSW contains protein matter with blood in which lipids are bound, constituting a minute quantity residual FOG content necessary to be removed, suitable procedures such as those developed by Kraghhansen *et al*. (1993) are required. Quantification of bio-delipidation as a post DAF treatment method to assess its efficacy can be done by determining lipid content reduction for the wastewater to be delipidised after the DAF pre-treatment, which was a focus of this study. Fig. 2.4 illustrates the location of a bio-delipidation unit designed in a PSW treatment plant.

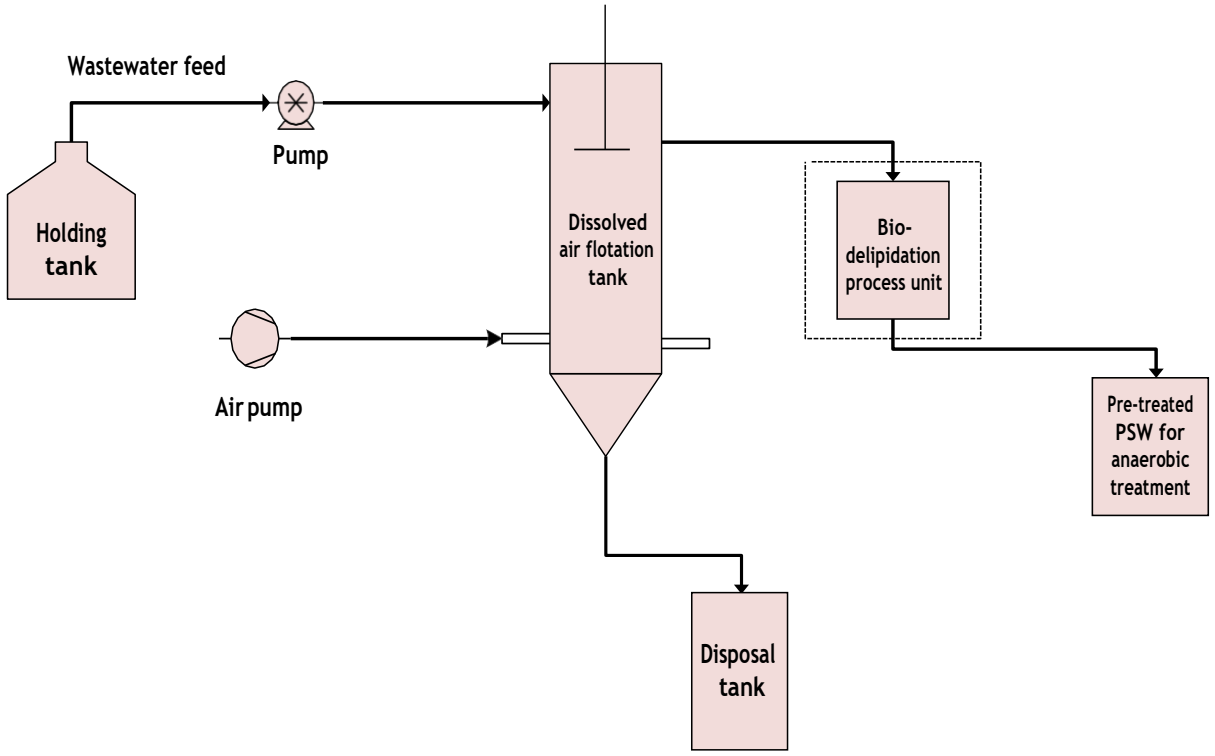


Figure 2-4: Schematic diagram of the DAF for pre-treatment system for PSW and a process unit for bio-delipidation.

CHAPTER 3

MATERIALS AND METHODS

MATERIALS AND METHODS

This chapter comprises detailed experimental procedures for all the specific objectives to achieve the study.

3.1 Phase 1: Materials and Methods

3.1.1 Isolation and identification of lipolytic bacteria

Sterile swabs were used to collect lipolytic bacteria from PSW and a local poultry slaughterhouse wastewater discharge point in Cape Town, South Africa. Nutrient broth with olive oil was used as an inducer and the cultures were maintained on nutrient agar by a streaking technique subsequent to incubation at 37°C for 48 h, with a regular sub-culturing strategy being implemented to obtain pure colonies. Pure cultures with high lipase activity were subjected to identification by morphologically studying each species under a microscope using a gram staining method, followed by further identification using 16S rRNA sequencing techniques. For 16S rRNA analyses, samples were sent to Inqaba Biotechnical Industries (Pretoria, South Africa) for sequencing, with the DNA from the isolates being obtained using a ZR Fungal/Bacterial DNA Kit™ (Zymo Research, USA). The 16S rRNA target region was amplified by DreamTaq™ DNA polymerase (Thermo Scientific™, SA) and the primers used were: (forward) 16S-27F 5'-AGAGTTTGATCMTGGCTC-3' and (reverse) 16S-1492R 5'-CGGTTACCTTACGACTT-3'. PCR products were gel extracted using a Zymoclean™ Gel DNA Recovery kit (Zymo Research, USA). This was followed by sequencing in the forward and reverse directions on an ABI PRISM™ 3500xl Genetic Analyser. Purified sequences were analysed using a CLC Main Workbench 7 and subjected to a Basic Alignment Search Tool (BLAST) to compare the sequence with those available on the NCBI-NIH database (National Centre for Biotechnical Information, n.d.) for identification of the microorganisms.

3.1.2 Screening of lipase production/activity on agar plates

Lipase activity of the isolated strains was determined using agar plate screening methods adapted from Ramnath (2017), using tributyrin medium containing (per litre): 2.5g peptone from casein, 3g yeast extract, and 12g nutrient agar. After autoclaving and cooling of the medium to 60°C,10 mL of tributyrin was added. A homogeneous mixture was maintained prior to pouring the media into Petri plates. A loopful of each strain was inoculated on the agar plates and incubated at 37°C for 48 h. Presence of lipase activity/production was monitored by a clear zone around the colonies. Microorganisms identified to be lipolytic, using the tributyrin medium screening method, were further screened using a Rhodamine B agar plate method. Rhodamine agar plates consisted of the following (per litre): 8g peptone, 4g yeast extract, 3g NaCl, and 20g nutrient agar. Rhodamine B dye (1mg/mL) was prepared

and filter-sterilised to make a 10 mL solution. After autoclaving and cooling of the agar medium to 60°C, 30 mLof olive oil and10 mL of the filter sterilised Rhodamine B solution were added. A loopful of each strain was inoculated on the agar plates and incubated at 37 °C for 48 h. UV irradiation (350nm) to assess clearing zones was used as an indicator of lipase activity.

3.2 Phase 2: Materials and Methods

3.2.1 Response surface methodology

Response surface methodology was employed to optimise the production of lipases from isolated microorganisms. Design-Expert® softwarev.6.0.8 (Stat-Ease, Inc, USA) was used for RSM analyses and for reactor parameter optimisation.

3.2.2 Lipase production

Post-screening of microorganisms producing lipases, isolates which were found to be the best producers were selected and inoculated in a fermentation medium for lipase production. The fermentation medium comprised (g/L): 5.0 g of peptone, 10 g yeast extract and 5.0 g NaCl supplemented with 10mL of filter-sterilized olive oil (Ramnath *et al*., 2017) to induce lipase production, as lipases are induced by the high concentration of triolein prevelent in olive oil. The pH of the medium was adjusted to various values, from 4 to 7 using NaOH/HCl, which was followed by incubation at 37 °C for 72 h at 121 rpm. Quantification of bacterial growth was performed by measuring absorbance at 660nm using a UV/Vis Jenway spectrophotometer, Cole Parmer, USA.

3.2.3 Partial purification to recover lipases

Culture broth was centrifuged at 7000 rpm at 4°C for 25 min. All supernatants were separated from the biomass pellet,with the pellet being resuspended in 50mMTris-HCl buffer (pH 8.0) by vortexing. In order to harvest intracellular lipases,a sonicator was used for the disruption of the cells constituting the pellet for 10min at 10s intervals to minimise overheating, with sonicated samples being put on ice (4°C) between sonication cycles. The mixture from the sonicator was centrifuged at 15000 rpm and 4°C for 25 min. Crude enzyme extracts in the form of supernantants from the centrifugation and sonication procedures were recorved and stored at 4°C prior to enzyme activity quantification in duplicates. The crude enzyme extracts with high enzyme activity were concentrated by loading on to Bio-Rad/Bio-Gel® P-60 size exclusion chromatography columns equilibrated with potassium phosphate buffer (50 mM pH 7.0) with the fractions (5 mL) collected and stored at 4°C for further use in this study.

3.3 Phase 3: Materials and Methods

3.3.1 Lipase activity determinations

Enzyme activity was quantified by using a titration method develped for olive oil hydrolysis (Venkateshwarlu & Reddy, 1993; Pallavi *et al*., 2017). The reaction mixture contained 2mLof olive oil as a substrate, 2 mL of the semi-purified enzyme, 5 mL of a 50 mM phosphate buffer (pH 7.0), and incubation at 37°C for 3 h. After incubation, the reaction was terminated by the addition of 10 mL analytical grade ethanol to the assay mixture. A few drops of phenolphthalein were used as an indicator by adding to the reaction mixture, in which free fatty acids were quantified by titration against 0.05M NaOH to an endpoint which was determined by the change of color to pink at pH 10.0.The control assay without the enzyme extracts, was titrated in the same manner and used as a blank.

The enzyme activity was determined by the following formula (Eq. 3.1) modified from Jaiswal et al. (2017).

$$(U) = \frac{N(NaOhforsample - Naohforblank) \times 1000}{M} \quad (3.1)$$

Where:
U = µmol fatty acid/mL,
N = Volume of the NaOH titrant used, and
M= Total volume of the reaction mixture used.

Additionally a aspectrophotometric method was used to measure lipase activity, whereby lipases contained in the culture supernatant werefurther assessed for hydrolysis of p-nitrophenyl acetate (p-NPA) into lauric acid and p-nitrophenol as described by Wongwatanapaiboon *et al*. (2016), with reaction assay mixtures containing 250µL of crude lipases (semi-purified sample) and 710µL of 50mM phosphate buffer (pH 7.0) including 40µL of 25mM p-NPA solutions, in reaction vessels which were allowed to stand for 10 min at 37 °C. The absorbance was measured at 410 nm using a UV/Vis spectrophotometer (Cole Parmer, USA).

The enzyme activity was determined by Eq. 3.2 as derived from Wongwatanapaiboon *et al*. (2016).

$$\frac{U}{ml} = \frac{\Delta A.V}{\varepsilon.t.v} \quad (3.2)$$

Where:

ΔA = the change in absorbance overtime,

V = the total volume of reaction mixture,

ε= the molar extension coefficient of the substrate in mM^{-1} cm^{-1},

t = the incubation time in minutes, and

v = the volume of the enzyme in there action mixture.

3.3.2 Determination of protein content

Bradford's assay (Bradford, 1976) was used to determine the total protein concentration of the PSW samples being biodelipidated. A 96-well microtitre plate containing 20µL of the samples was used per well (in duplicate), to which 180µL of Bradford's reagent was added. The reactions were allowed to stand for 2 min and the absorbance was measured at 450nm using an Anthos Xenyth1100 microtitre plate reader. Bovine serum albumin (BSA) was used for standards solution, with the preparation being by the dissolution of unsaturated BSA (10mg of crystalline BSA) in 10mL of 10mM sodium phosphate buffer. Various BSA concentrations were prepared as working standards (Appendix B). A standard curve was constructed by plotting the absorbance of the standards at various concentrations. The concentration of each sample was estimated by using a regression equation (y= 0.000x-0.006) from the standard curve.

3.3.3 Effect of pH and temperature on enzyme stability and thus activity

Optimum pH for functionality of the semi-purified enzymes was determined spectrophotometrically using a method modified from Edupuganti (2017) and Ramnath (2017). Different buffers (50mM) at different pH ranges such as citrate buffer (pH 3.0-5.0), Tris-HCl buffer (pH 6.0-7.0), K_2HPO_4-KH_2PO_4 (pH 8.0-9.0) and glycine-KOH buffer (pH10.0) were used as buffers for reaction mixtures. A typical assay reaction mixture contained 200µL of the semi-purified enzyme and 1800µL of the buffer and incubation at 37°C for 15 min. The enzyme activity was measured under similar conditions as described in 3.3.1. Temperature stability of the enzymes was studied at a pre-determined optimum pH, by incubating the assay mixture at different temperatures, i.e. 25, 45, 55, 65and 75°C in a 50mM phosphate buffer for 30 min, after which the samples were allowed to cool subsequent to enzyme activity quantification.

3.3.4 Effect of solvents and metallions on enzyme stability, and thus activity

The effect of solvents as inhibitors on enzyme stability was determined using solvents (isopropanol, 2-mercaptoethanol, acetone, methanol, ethanol, hexane, chloroformandtoluene) added to the crude enzyme sample, followed by incubation at 37°C

for 30 min. Enzyme activity was measured as described in 3.3.1.A reference experiment without the solvent was also used. The effect of metal ions on the lipases was also studied by incubating the enzymes in the presence of metal salts. Pre-incubation (30 min) in 2mM final concentration for each metallic salt was done at 37°C. Metal salts used were $CaCl_2$, KCl, $NaCl_2$, $MgSO_4$,and $Fe_2(SO_4)_3$.

3.3.5 Effect of detergents on enzyme stability

Presence of detergents in the wastewater (PSW) to be bio-delipidised may affect the enzyme activity, and thus functionality. In order to understand how enzyme activity is influence by the presence of key ingredients found in detergents, antimicrobial agents triclosan (TCS) (5-chloro-2-(2,4-dichlorophenoxy)-phenol) and trichlorocarbanilide(3,4,4-trichlorocarbanilide) (TCC), which are usually found in PSW, were used to determine their effect on the activity of lipases from selected isolates that were deemed to have lipase production capabilities, with activity assays being conducted as described in 3.3.1. Detergents such as EDTA and Tweens were also included in this part of thestudy. A reference experiment without the detergents, i.e. only those with crude enzymes, was prepared for comparative analysis,and analyzed for activity.

3.3.6 Bio-delipidation assessment

PSW samples were prepared by mixing PSW and crude enzyme extracts in 15 mL tubes using a vortex mixer, with the homogenised PSW/enzyme mixture being allowed to settle for 5 to 10 min, which culminated in separate zones being formed after enzyme treatment.PSW without an oily layer was used to prepare delipidation samples, in which 13.5 mL of PSW and 1.5 mL of semi-purified enzyme supernatants were mixed. A control was prepared with only the media that were used to ferment delipidation cultures. Lipid layer thickness differentiation was measured after preparing both biodelipidation samples and reference experiments.

3.3.6.1 Effect ofpH on bio-delipidation

To assess bio-delipidation at different pHs, actual fatty acid content before and after defatting was quantified. To determine the optimal pH for bio-delipidation,different delipidation reactions mixtures were used at pH values 3, 4, 5, 6, 7, 8, 9, 10 and 12, which were adjusted using NaOH and HCl. The delipidation reactions consisted of PSW and enzyme solutions. Fatty acid concentration before and after bio-delipidation was determined by protein concentration determinations using Bradford's (1976) method in a spectrophotometer with Bovine serum albumin (BSA) being used as a standard (Appendix B2). The residual percentage removal efficiency of fatty acids from PSW at various pH levels was determined

by titration using ethanol to dissolve oil and titrate with a strong base, and using phenolphthalein as an indicator,, in accordance with Eq. 3.3.

$$\frac{RA/[P]A}{RB/[P]B} \times 100\% \qquad 3.3$$

Where:

R and *P* stand for activity and protein concentration. *A* and *B* are for post and prior bio-delipidation.

3.3.6.2 Effect of temperature on bio-delipidation

The optimum temperature for bio-delipidation was also assessed at different temperatures. Tubes were agitated in a temperature-controlled waterbath at different temperatures (20to 55°C) for 6 h. All protein concentrations were quantified using Bradford's assay, with bovine serum albumin (BSA) as a standard. Fatty acid concentration was determined using a titration-enzymatic method for quantitative determination of fatty acids by titration. Ethanol was used to dissolve oil and titrated with a strong base using phenolphthalein as an indicator. The residual percentage removal efficiency of fatty acids removed from PSW at the various temperatures by bio-delipidation, by means of delipidation culture extracts from the isolated strains, was determined using Eq. 3.3.

3.4 Phase 4: Materials and Methods

3.4.1 Bio-delipidation of DAF pre-treated PSW

Poultry slaughterhouse wastewater (PSW) was collected from a poultry slaughterhouse in Cape Town, South Africa. Wastewater was stored at 4°C until use for primary pre-treatment using DAF systems. DAF systems were operated by dispersing pressurized dissolved air into the system, flocculating the lipids and solids from the PSW to the surface, prior to removalusing skimming equipmentat 60revs/min (Dlangamandla, 2016). The resultant primary pre-treated PSW was collected and analyzed for water quality tests,; thereafter, it was used for the bio-delipidation experiments. PSW was mixed at 121 rpm for 6 h, at optimum temperature and pH, in flasks in which bio-delipidation enzymes produced by each isolate were supplemented. The efficiency of the bio-delipidation was observed by quantifying PSW quality parameters pre- and post-bio-delipidation process.

Figure 3-1: Dissolved air flotation (DAF) used in this study.

3.4.2 Analytical tests and methods

PSW quality parameters analyzed included the following:protein content, lipids (FOG), total chemical oxygen demand (COD), total suspended solids (TSS), and pH. Prior to bio-delipidation of the DAF pre-treated PSW, lipases were produced from isolated strains AB1 (BF3) and CC-1 (B3O), semi-purified and used for the bio-delipidation. Quality parameters of the wastewater, i.e. collected samples from the DAF pre-treatment system, were analyzed before and after delipidation. Turbidity was measured using a Wirsam Scientific TN-100 turbidimeter. The total protein concentration content was measured using Bradford's assay as described in 3.3.2 (see also Appendix B2). To measureTSS, filter paper was weighed and used to filter PSW samples by placing the filter paper on a Buchner funnel connected to a vacuum pump. Weighed filter paper with SS residue from the PSW samples was transferred into a weighing dish. The filter papers were dried in the oven at 105°C for 1h, allowed to cool in desiccators and weighed again there after.TSS were computed using the following: TSS (mg/L) = (A-B) ×1000/C. Where, A = Weight of dried filter paper (mg) with SS, B = Weight of clean filter (mg), C = Volume of the sample filtered (mL).For confirmatory analyses, FOG were measured according to a standard method to minimise bias (American Public Health Association, 2005) at the City of Cape Town Scientific Service laboratory.

The analyses of tCOD was measured using a Merck Spectroquant® Nova 60.A spectroquant® themoreactor was heated up to 148°C for 2 h and allowed to stand before use. The COD solutions A and B (500-1000 mg/L) were used with 2.2 mL of solution A pipetted into a glass tube, followed by the addition of1.8 mL of COD solution B into the same glass tube. A volume (1 mL) of the sample was pipetted into the glass tube. Tubes were mixed and placed in a thermoreactor for 2 h for digestion at 148°C. These tubes were removed and allowed to cool for 10 min and mixed before placement into the Nova 60 spectroquantfor a COD reading, ensuring that each cell aligns with the indicator line for measurements.

CHAPTER 4

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

This chapter comprises of the following phases:

- **Phase 1 Aim 1:** Isolation and characterization of microorganisms from PSW suitable to facilitate lipid reduction using their bio-products, i.e. enzymes by screen and monitor the isolated microorganisms' ability to produce required bio-delipidation enzymes and select microorganisms with a high potential to produce bio-products (enzymes) for bio-delipidation

- **Phase 2 Aim 2:** Optimisation of reactor conditions to produce enzymes required for bio-delipidation employing Response Surface Methodology (RSM) and partially purify the lipases produced, for use in subsequent experiments

- **Phase 3 Aim 3:** Characterisation of enzyme activity using environmental conditions in which they will be employed and to optimise the conditions in which the lipases can be used for a bio-delipidation system for maximum deffating of the protein-rich PSW, post DAF pre-treatment.

- **Phase 4 Aim 4:** Assessment of lipid removal from DAF pre-treated PSW for elucidation of optimum conditions by optimising bio-delipidation conditions of DAF pre-treat PSW, analysing PSW quality parameters pre- and post- bio-delipidation furthermore analysing bio-delipidation efficiency using FOG/lipid analysis and other water quality parameters.

4.1 Phase 1 Aim 1: To isolate and characterize microorganisms from PSW which are suitable to facilitate lipid reduction using their bio-products, i.e. enzymes.

4.1.1 Introduction

Many qualitative assays have been documented as important for the identification of microorganisms with potential to produce lipases. However, due to the broad substrate specificity and divergence of reactions catalysed by lipases, it is a challenge to have well defined and specific assays of lipases (Gupta *et al*., 2003). These methods are usually used as first-hand alternatives for screening a wide range of microorganisms with a potential for suitable enzyme production prior to quantitative methods being used (Gupta *et al*., 2012).

Quantitative estimation of lipase activity to identify lipolytic microorganisms can be achieved using plate assays with agar medium supplemented with lipids as a carbon source. After inoculation and incubation of the microbial strains isolated, enzymes can be produced in the presence of lipids supplemented in a defined medium such that these lipids are hydrolysed with clear hydrolysed zones forming around microbial colonies. Agar plate assays with

tributyrin (tributyryl glycerol), is one of the most widely utilised assays for screening lipase-producing strains. However, a disadvantage of this method is that acidic metabolites produced by the lipolytic microorganisms could lead to acidification of the medium, giving false-positive results (Hasan *et al.*, 2009), as the tributyrin substrate is not only hydrolysed by lipases, but also by esterases. The use of agar supplemented with olive oil can be a suitable substitute for tributyrin, because olive oil contains high concentrations of triolein that could be utilised in the screening of lipase producing microorganisms, and because it is inexpensive (Gupta *et al*., 2003).

Kouker & Jaeger (1986) used a plate assay to screen lipase producing strains with a fluorescent dye, i.e. Rhodamine B, for the observation of a lipolysed zones caused by substrate hydrolysis; it forms an orange fluorescent clear zone around bacterial colony under UV light at 350nm. Disadvantages of this method include clear fluorescent zones that are only visible under UV light when high quantities of the enzyme of interest are found, and occasionally the non-fluorescent clear zone indicating hydrolysis of the olive oil is only be observable under visible light (Gupta et al., 2003). In this part of the study, the isolated strains were screened using plate assay methods, and strains of interest that were found to have potential lipase-producing potential were further experimented on.

4.1.2 Aims and Objectives

The aim of this phase of the study was to isolate and characterize microorganisms from PSW which are suitable to facilitate lipid reduction using their bio-products by screening and monitoring the isolated microorganisms' ability to produce required bio-delipidation enzymes and select microorganisms with a high potential to produce bio-products (enzymes) for bio-delipidation

Specific objectives of this part of the study were:

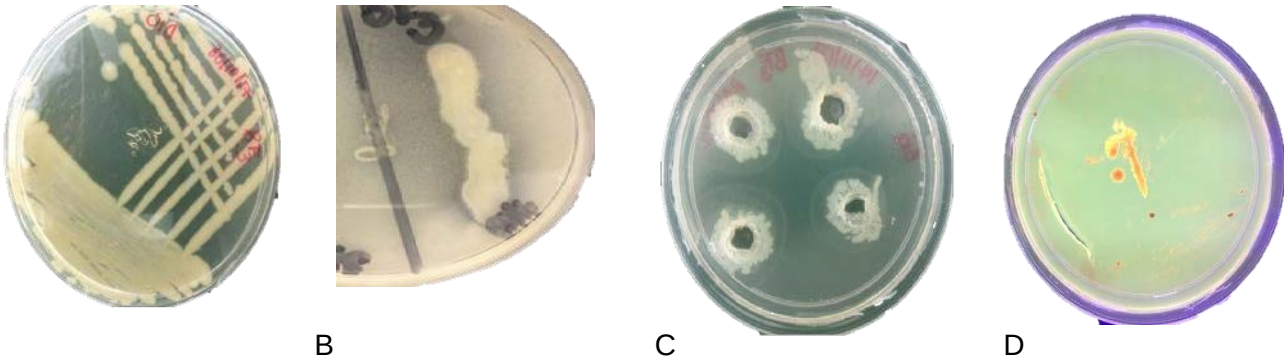
- To isolate and characterise lipase producing microorganisms,
- To screen and monitor the ability of isolated microorganisms to produce the delipidation enzymes required, in sufficient quantities, using tributyrin agar and rhodamine-oil tests, and
- To select microorganisms with a high potential to produce bio-products for the bio-delipidation unit.

4.1.3 Microbial screening and lipase production monitoring on agar plates

Pure culture samples obtained from poultry slaughterhouse wastewater (PSW), and from a discharge point at a local poultry slaughterhouse,were screened to isolate lipolytic strains using qualitative assay methods. Isolates (n = 20) were maintained on nutrient agar, following lipase activity screening procedures on agar plates supplemented with tributyrin. A number (n = 2)of isolates produced distinct zones of hydrolysis (clearance) as shown in Figure 4.1 (b) for strain BF3 and 4.2 (b) for strain B30. The isolates were further tested for lipase activity, specifically due to challenges with visualisation of the clear zones, as the agar clearance zone was slightly hydrolyzed, and so that the tributyrin hydrolysis could also be facilitated by esterases. Additional plate assay methods specific for lipase activity detection were used for further confirmation of lipase activity using agar supplemented with substrates, namely Tween 80 and olive oil, with Rhodamine B dye as an indicator. Screening of the lipase producing microorganisms was conducted using detergents such as tweens supplemented to the agar media. Lipase activity was then observed by salt precipitation caused by calcium deposition around the lipolytic microorganisms from fatty acid hydrolysis of tweens. The fatty acid-calcium complex was observed by insoluble crystals formed around the inoculated area. From the tweens assessed, tween 80 can be highly hydrolyzed by lipases and sparingly by esterases as it is comprised of oleic acid esters . In this part of the study, zones of calcium deposition in Tween 80 agar plates by the isolates were observed, i.e. around agar wells inoculated with the cell-free enzyme supernatants for both these strains as shown in Figure 4.1(c) for strain BF3 and 4.2 (c) for strain B30, thus confirming that both these isolates have the potential to produce the required lipases for bio-delipidation. Similarly the principle as sociated with the utilisation of the Rhodamine B dye method is based on the esterification reaction of long chain alcohols and fatty acids in the Rhodamine B agar plate media. Enzymes produced facilitate the formation of an ester and fluorescence of the Rhodamine B-fatty acid complex, which then becomes observable under UV light. The formation of the orange fluorescent on the plates as shown in Figure 4.1 (d) for strain BF3 and 4.2 (d) strain B30, indicated an esterification of free fatty acids for each of the lipase producing isolates.

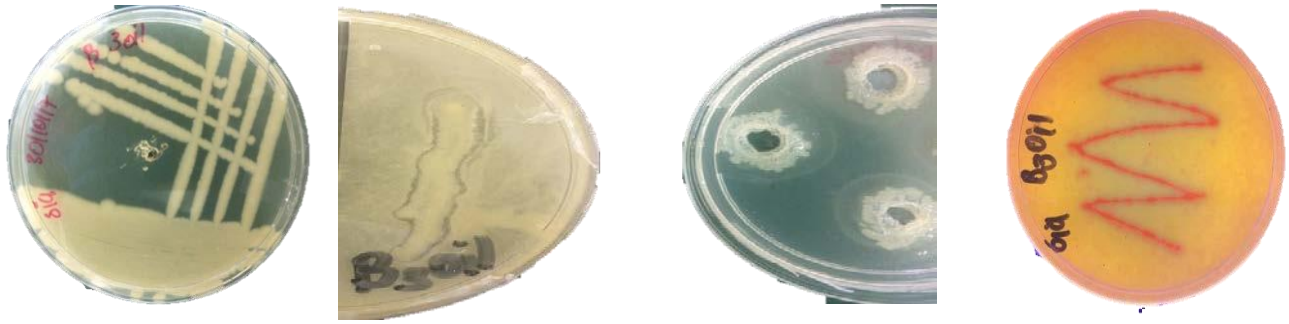
Few successes have been reported for lipase screening methods using agar plates (Kouker & Jaeger 1986; Gupta *et al*., 2012; Bakir *et al*., 2015; Ramnath *et al*., 2017). According to Kouker & Jaeger (1986) this could be due to the low concentration of the lipase enzyme produced by a single colony of the microorganism of interest, especially when Rhodamine B dye assay is used, with low concentrations of the enzyme produced by a colony making it a challenge to visualise orange fluorescence that indicates lipase activity under UV light. These methods are used for the general estimation of lipase activity; however, sensitive quantitative

methods (titration and colorimetric methods) can be used for effective lipase activity determinations, an approach adopted in this study.



A B C D

Figure 4-1:(a) BF3 growth on nutrient agar; (b) Tributyrin agar plate with hydrolysis zone by BF3; (c) Tween 80 agar plate with precipitation zone by BF3 cell free enzyme supernatant; (d) Olive oil rhodamine b dye showing orange fluorescence under UV irradiation.



A B C D

Figure 4-2: (a) B3O growth on nutrient agar; (b) Tributyrin agar showing hydrolysis zone by B3O; (c) Tween 80 agar precipitation by B3O cell free enzyme supernatant; (d) Olive oil rhodamine b dye showing an orange fluorescence under UV irradiation.

4.1.4 Isolation and identification

Microbial identification of the isolates that showed ahigh lipolytic potential was achieved by morphological identification and 16S rRNA sequencing. The microorganisms were subjected to gram staining for morphological characterisation, with both being gram positive,with a rod shape anda yellow color for B3O, and while BF3 was coccus. Identification methods using 16S rRNA sequencing confirmed the isolates to be both *Bacillus spp.*,most likely *Bacillus cereus*, with Genbank accession numbers CP023179.1 (B3O) and MF800922.1 (BF3).

4.2 Summary

In this part of the study, swaps collected from the poultry slaughterhouse discharge point and the PSW itself were used for isolating lipolytic microorganisms using molecular techniques. Two isolates were identified to be suitable lipolytic microorganisms, as determined by screening using simple lipase screening methods. Further identification by sequencing 16S rRNA encoding genes of the isolates confirmed both organisms to be *Bacillus spp.*, most likely *Bacillus cereus* strains AB1 (BF3) and CC-1 (B3O).The strains were further used in subsequent sections to produce lipase enzymes for bio-delipidation of protein-rich PSW.

4.3 Phase 2 Aim 2: To optimise reactor conditions for the production of enzymes required for bio-delipidation employing Response Surface Methodology (RSM) and partially purify the lipases produced, for use in subsequent experiments.

4.3.1 Introduction

Bacterial lipases have been reported as the most significant biocatalytic agents compared to those from plants and animals, due to the ability of microorganisms to grow rapidly, and their ease of modification at gene level (Damasio *et al*., 2013). Microbially-sourced lipases have also been considered important for biotechnological industrial applications due to their ability to catalyse reactions both in aqueous and non-aqueous systems.

Generally, lipases are commercially produced by various microorganisms including those from microbial genera Pseudomonas, Bacillus, Staphylococcus, Streptomyces, Penicillium, Aspergillus and Rhizopus (Faisal *et al.,* 2014). These are the most studied and reported lipases. These enzymes can be produced by submerged fermentation (SMF) and solid state fermentation (SSF) on an industrial scale whereby solid substrates such as agro-waste and oils can be used as inducers or substrates for lipase production.

Like any other microbially produced extracellular protein, lipase production is influenced by media composition before any other physicochemical factors such as pH and temperature are considered. The carbon source in the production media has been reported as the major influential component as it facilitates expression of lipase activity . Production is usually induced by the presence of lipids, including oils and triacylglycerols (Zarevúcka *et al*., 2012). Olive oil and glucose have long been reported to be responsible for lipase activity expression; however, glucose is also known to suppress lipase expression in other microorganisms such as in *Pseudomonas* sp. In this study, olive oil was used as a carbon source and to serve as an inducer for maximum lipase production.

In any bioprocess modelling and optimisation undertaking, physicochemical conditions are important to identify and optimise to improve the production system and enhance the product concentration while decreasing the production cost. Response surface methodology (RSM) is a statistical method utilized for modelling, analysis, and optimisation of production parameters for high-value biomolecules (Selvakumar *et al*., 2017). The optimum conditions for lipase enzyme production can be established by the determination of lipase activity. Generally, high lipase activity is achieved by changing one parameter while others are kept constant. However, with this method, optimum conditions are not easily identified and the relationship between variables is not easily indicated (Rani *et al*., 2015). In RSM, the relationship between responses and variables is indicated, and the effect of variables on the

responses is established. In this part of the study, RSM was used for experimental design for optimization of lipase production by the isolated strains focusing on process variables, 1) pH and 2) temperature, for maximum lipase activity. The use of RSM was necessary as it easily establishes optimum conditions for maximum lipase activity to allow for rapid production of the enzyme for the bio-delipidation process.

4.3.2 Aim and Objectives

The aim of this phase of the study was to produce lipase enzymes from the isolated organisms identified to be lipolytic microorganisms, and to optimize environmental variables for enzyme production using response surface methodology (RSM) for maximum lipase activity.

Specific objectives of this part of the study were:

- To prepare an inoculum for enzyme production in a suitable media supplemented with olive oil,
- To statistically optimise parameters for lipase production from PSW isolates employing RSM, and
- To partially purify the lipases produced.

4.3.2.1 Lipase production: Optimisation

The production of lipases can be influenced by many factors including initial pH, the temperature used, and the physiological characteristics of the microorganism, including incubation time (Faisal *et al*., 2014). In this part of the study, two parameters, pH (4 to 8) and temperature (30 to 60°C), were considered for the RSM design to optimize lipase production. An incubation period of 72h was used for production in all the experiments, as incubation period increases to more hours and days have been found to be unsuitable to produce lipases ata maximum rate. For example, maximum production of lipases by *Pseudomonas* sp. was at 48 to 96 h (Pabai *et al*., 1996; Dong *et al*, 1999; Ali *et al*.,2017). Optimum conditions were selected by assaying lipase activity by a titrimetric method using olive oil hydrolysis. A set of 13 experiments was used to ascertain optimum conditions for high lipase production by the isolated strains, B3O and BF2. All the experiments were conducted in duplicate and activity was measured according to the method developed. Table 4.1 lists lipase activity results from the RSM experimental design for both strains B30 and BF3.

Low pH was observed to have agreater impact on the production of the lipase for both strains B30 and BF3. Generally, the pH of the media has an ability to strongly influence bacterial cell performance and thus the transportation of nutrients and by-products across and/or through

the cell membrane, increasing the chances of maximum enzyme production. Maximal lipase production for both strains was at pH 6–8 and at 45–60°C.The conditions for *Bacillus cereus* strain AB1 (BF3) formaximum lipase activity was obtained at pH 8 under 60°C achieving maximal lipase activity of 11.25 U/mL, with optimised conditions for *Bacillus cereus* CC-1 (B30) being pH 8.83 at 45 °C, with an activity of 15.50 U/mL.

Table 4-1: Lipase activity with variable temperature and pH for B3O and BF3 strains obtained by RSM design

Run	Factor 1: pH	Factor 2: Temperature °C	B3O Enzyme activity (U/mL)	BF3 Enzyme activity (U/mL)
1	6	45	13.06	10.59
2	6	45	13.06	10.59
3	8.83	45	15.50	8.94
4	4	60	11	8.75
5	4	30	8.74	7.89
6	6	45	13.06	10.59
7	6	45	13.06	10.59
8	6	45	13.06	10.59
9	6	66.21	8.75	9.25
10	8	30	7.89	6.84
11	3.17	45	7.80	7.63
12	8	60	12.50	11.25
13	6	23.79	8.75	7.50

Table 4.2 presents astatistical analysis of variance (ANOVA) for the quadratic model (Eq. 4.1) used to predict lipase activity for isolate B3O. Variables, i.e. pH and temperature, were used to determine optimised lipase production conditions in cultures grown/incubated for 72h.A P-value< 0.05 was used for evaluating the significance of the model parameters. Parameters in the model which had P-values more than 0.05 were referred to as being insignificant for the model. It was observed that pH (A) played a major influential role when compared to temperature (B), and the product of pH and temperature (AB). The non-significant parameters were then deemed redundant, and the model was reduced to Eq. 4.2.

$$\text{B3O Lipase Activity (U/mL)} = 13.06 + 1.44*A + 0.86*B + 0.59*AB - 0.75*A^2 - 2.20*B^2 \quad (4.1)$$

$$\text{B3O Lipase Activity (U/mL)} = 13.06 + 1.44*A - 0.75*A^2 - 2.20*B^2 \quad (4.2)$$

Where: *A* is pH and *B* is temperature, respectively.

The model, as indicated by the correlation coefficient R^2 value, was sufficient to be a suitable response (activity) predictor (Abu *et al.,* 2017). The model for B3O lipase activity (U/mL) had a coefficient (R^2) of 75.5%,as shown by the statistical analysis of variance (ANOVA) data for the quadratic model.

Table 4-2: Statistical analysis of variance (ANOVA) data of quadratic model to predict lipase activity by B3O

Source	**Sum of Squares**	**Df**	**Mean square**	**F value**	**Prob> F**
Model	59.00	5	11.80	4.33	0.0408
A-pH	16.64	1	16.64	6.11	0.0427
B-Temperature	5.90	1	5.90	2.17	0.1845
AB	1.38	1	1.38	0.51	0.4995
A^2	3.88	1	3.88	1.43	0.2715
B^2	33.57	1	33.57	12.33	0.0098
Residual	19.06	7	2.72		
Lack of fit	19.06	3	6.35		
Pure Error	0.000	4	0.000		
Corr. total	78.06	12			

Std. Dev. = 1.65; R-Squared = 0.7558; Mean = 11.25; Adj R-Squared = 0.5814; C.V.% = 14.67; Pred R-Squared = 0.7365;Adeq Precision = 5.490.

a) Significant at $p< 0.05$

b) Not significant at $p>0.05$

Similarly, table 4.3 presents statistical analysis of variance (ANOVA) data of a quadratic model used to predict lipase activity for isolate BF3. The interdependance of parameters, i.e. pH (A) and temperature (B), was observed, with individual parameters in the model having p-values< 0.05. Lipase activity for BF3 could be modelled by Eq. 4.3, with a correlation coefficient (R^2) of 95%, which was indicative of the fitness of the model in predicting the experimental outcomes, and thus lipase production.

$$\text{BF3 Lipase Activity (U/mL)} = 10.59 + 0.41*A + 0.97*B + 0.89*AB - 1.06*A^2 - 1.02*B^2 \quad (4.3)$$

Table 4-3: Statistical analysis of variance (ANOVA) data of quadratic model to predict lipase activity by BF3

Source	Sum of Squares	Df	Mean square	F value	Prob> F
Model	25.38	5	5.08	28.53	0.0002
A-pH	1.36	1	1.36	7.66	0.0278
B-Temperature	7.50	1	7.50	42.14	0.0003
AB	3.15	1	3.15	17.71	0.0040
A^2	7.88	1	7.88	44.30	0.0003
B^2	7.23	1	7.23	40.63	0.0004
Residual	1.25	7	0.18		
Lack of fit	1.25	3	0.42		
Pure Error	0.000	4	0.000		
Corr total	26.63	12			

Std. Dev.= 0.42; R-Squared = 0.9532; Mean = 9.13; C.V.% = 4.53; Pred R-Squared = 0.6674;Adeq Precision= 12.951.

a) Significant at $p< 0.05$

b) Not significant at $p>0.05$

Graphical illustrations as shown in Fig. 4.3 and 4.4 were generated for analysis by studying the interaction of two parameters (pH and temperature) while maintaining the production time (72h) as an independent parameter. Lipases produced by *Bacillus cereus* strains AB1 (BF3) and CC-1 (B3O) in the culture media were subjected to partial purification using miniaturised fractionation. The purification involved centrifugation and sonication followed by fractionation usinga size exclusion chromatography column (Bio-Gel® P-60). The centrifuged and sonicated crude enzyme was used to measure lipase activity using *p*-nitrophenyl acetate (*p*-NPA) as a substrate. Lipase activity after purification for *Bacillus cereus* strains AB1 (BF3) and CC-1 (B3O) was quantified as 19.47 and 28.36 U/mL respectively. The slightly higher activity after purification indicated that purification of the enzyme was effective.

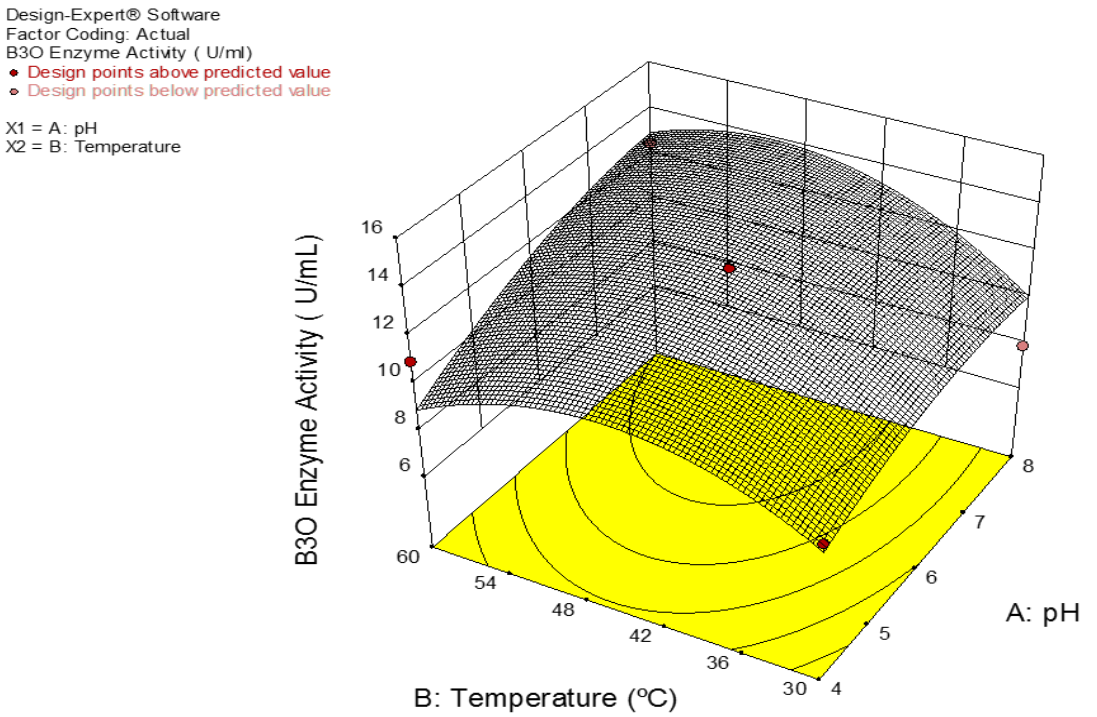


Figure 4-3: Response surface plot for showing the effect of parameters on lipase production for strain B3O.

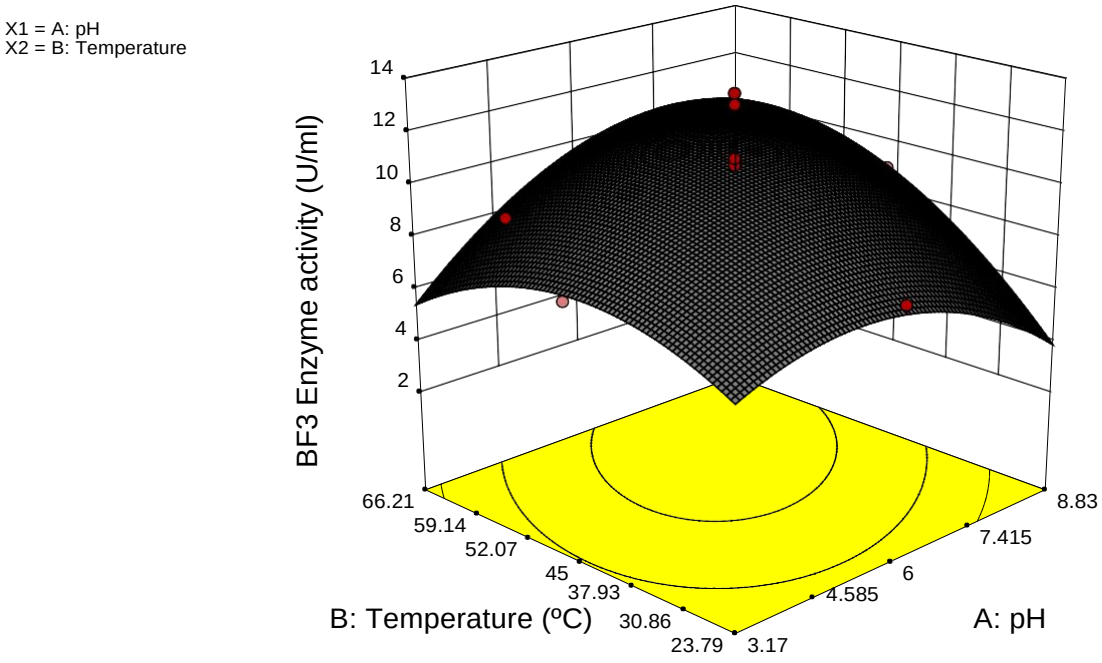


Figure 4-4: Response surface plots showing the effect of parameters on lipase production for strain BF3

4.4 Summary

In this part of the study, the enzyme production by the two isolated strains was optimised for maximal lipase activity. The bioreactor parameters for maximum activity were used to produce large quantities of lipases which were further purified and used for bio-delipidation. Lipases from both organisms, *Bacillus cereus* strains AB1 (BF3) and CC-1 (B3O), showed maximum activity of 11.25 U/mL when produced atpH 8 and 60°C,with15.50 U/mL being observed for CC-1 (B3O) at 8.83 and 45°C. Fractionation was used to semi-purify the enzymes, followed by determination of activity using *p*-nitrophenyl acetate (*p*-NPA) as a substrate, achieving 19.47 U/mL activity for *Bacillus cereus* strains AB1 (BF3) and 28.36 U/mL for *Bacillus cereus* strains CC-1 (B3O).

4.5 Phase 3 Aim 3: To characterise enzyme activity using biochemical assays and to optimise the conditions in which the lipases can be used for a bio-delipidation system for maximum deffating of the protein-rich PSW, post DAF pre-treatment.

4.5.1 Introduction

Interest in thermo- and alkaline-stable lipases is increasing rapidly due to the usability of these enzymes in extreme process conditions (Cherif *et al*., 2011). Optimal production conditions for lipases, such as pH and temperature, could be different for individual species, particularly when maximum activity and enzyme stability are to be achieved; therefore, it is important to assess the effect of these conditions on enzyme functionality in an environment in which the enzymes will be applied.

Some of the pollutants in PSW are heavy metals,and pharmaceuticals including antibiotics and detergents (Bustillo-Lecompte & Mehrvar, 2017). Substances such astriclosan (TCS) (5-chloro-2-(2,4-dichlorophenoxy-phenol) and trichlorocarbonilide (3,4,4- trichlorocarbonilide) (TCC) are anti-microbial agents used indetergents in slaughterhouses, due to their antimicrobial and disinfectant efficacy. Their presence in PSW has been established, and they are also known to be detrimental to human and environmental health (Bustillo-Lecompte & Mehrvar, 2017). The effect of these pollutants on the functionality and stability of the enzymes must be investigated, as all these contaminants could change the structure of the enzyme leading to loss or reduction of activity.

The activity of enzymes in the presence of TCS, TCC and metal ions as well as some solvents, was therefore assessed to understand their effect on the functionality of the enzymes, as these pollutants are found in PSW. Furthermore, assessment of environmental conditions in combination with the pollutants, which could affect bio-delipidation, should also

be investigated. This was done to understand the bio-delipidation conditions suitable for application in an industrial bio-delipidation unit.

4.5.2 Aim and Objectives

The aim of this phase of the study was to characterise the bacterial lipases under environmental conditions in which they will be employed.

Specific objectives of this part of the study were:

- To determine the effect of pH and temperature on the lipase activity from isolated microorganisms,
- To assess effects of solvents, detergents and metal ion inhibitors on the lipase activity, and
- To determine optimised conditions in which the lipases can be used for a bio-delipidation system for maximum deffating of the protein-rich PSW, post-DAF pre-treatment.

4.5.3 Effect of pH on enzyme stability and activity

The enzymes to be used in PSW delipidation must be stable and showhigh activity under varying and alkaline conditions. Effect of pH on the lipase produced by the PSW isolated strains was determined by assaying lipase activity at different pH with different buffers using *p*-nitrophenyl acetate as a substrate. The maximum lipase activity was observed to vary at different pH values i.e. pH 7 (9 U/mL), pH 8 (8.25 U/mL) and pH 9 (7.2 U/mL) for *Bacillus cereus* (BF3); and pH 6-10,7.9 U/mL, 9.3 U/mL, 2 U/mL, 12.72 U/mL, 8.2 U/mL and 8.75 U/mLfor*Bacillus cereus* (B30). These results suggested that *Bacillus cereus* (B30) lipases are resistant to pH changes as they retained a higher activity over a range of pH assessed; both strains produced lipases which function appropriately under an alkaline pH near neutral, with activity being reduced in pH below 7 and above 9 (Fig. 4.5). The enzyme activity at alkaline pH for both lipases showed their potential for application in alkaline conditions such as those observed for PSW (pH 6.46 to 6.88) and the resultant DAF pre-treated wastewater. In the literature reviewed, many lipases from *Bacillus* sp.were reported to have stability at alkaline conditions between pH 7 to 9 (Dutta *et al*., 2009), with the PSW having a pH range higher than 6.5 (Table 2.1 & 2.2).

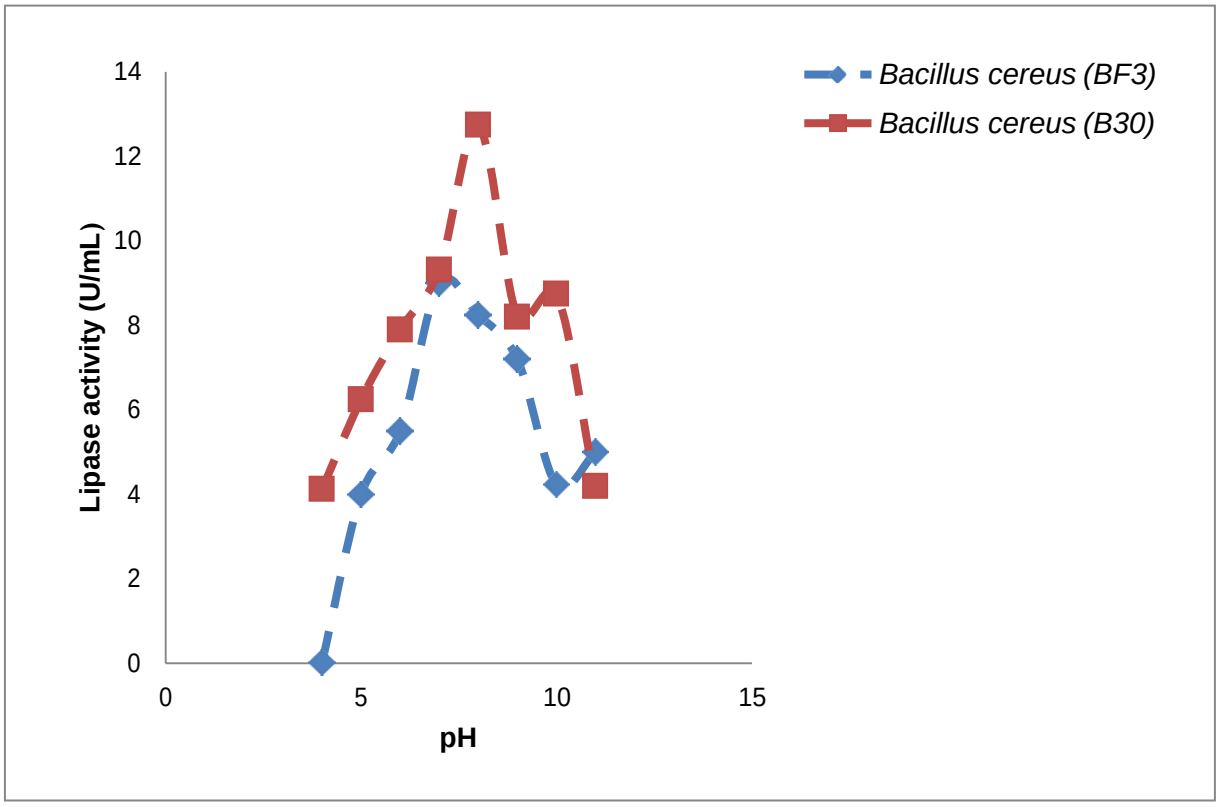


Figure 4-5: Effect of pH on enzyme activity for *Bacillus cereus* strains AB1 labelled BF3 and CC-1 labelled B3O.

4.5.4 Effect of temperature on enzyme stability and activity

Lipases from *Bacillus cereus* are generally thermostable (Sharma, 2014). The effect of temperature on the stability of lipases was carried-out at pH 7 for *Bacillus cereus* (BF3) and pH 8 for *Bacillus cereus* (B30) respectively. At these pH values, the enzymes were observed to have a high activity, with p-nitrophenyl acetate being used as a substrate at different temperatures. The enzymes from both strains retained their activity in temperatures from 40 to 45°C(Fig. 4.6). Generally, an optimum temperature facilitates enzyme-substrate binding, leading to high subtrate conversion rates. A decrease in activity was observed as the temperature was increased beyond 45 to 60°C, indicating that the enzymes used for bio-delipidation were not as stable at higher temperatures as those reported for other *Bacillus* sp.,which retained their activity at temperatures over 60°C (Lee *et al*., 2001).

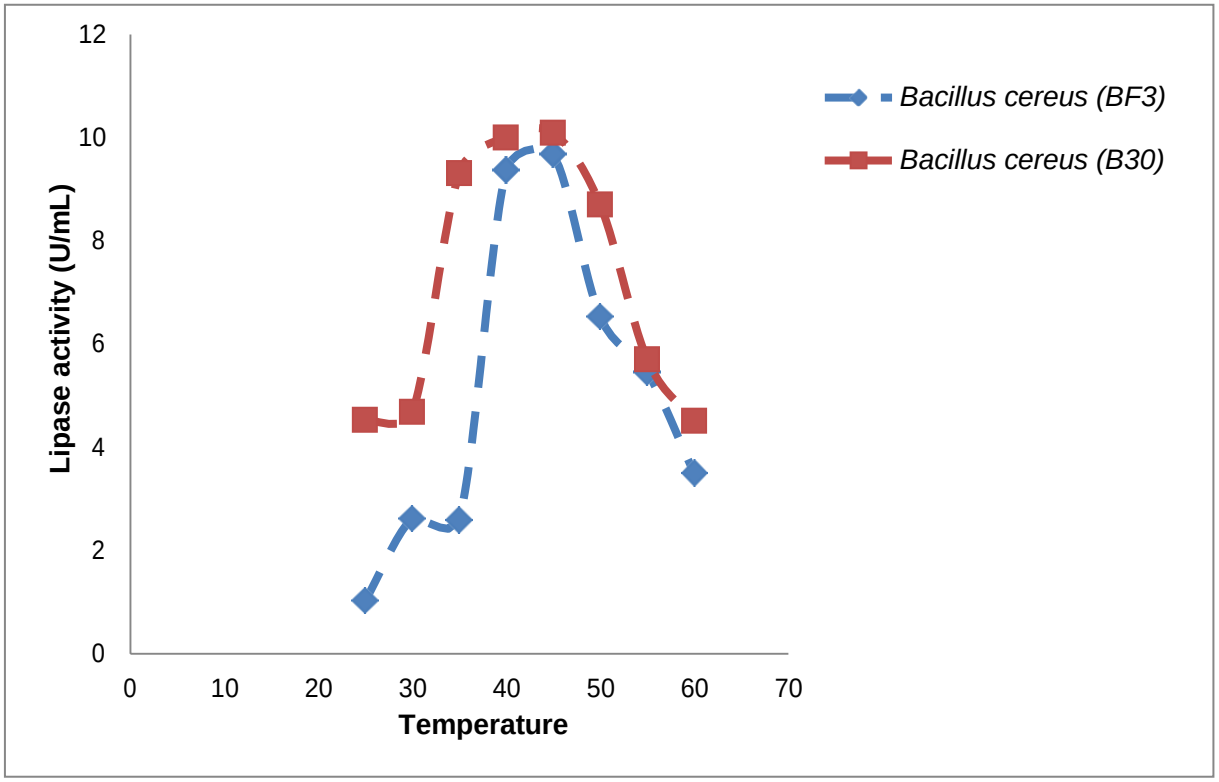


Figure 4-6: Effect of temperature on enzyme activity (lipase) from PSW strains *Bacillus cereus* strains AB1 labelled BF3 and CC-1 labelled B3O

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4.6 Effect of detergents including metal ions on enzyme stability and activity

Detergents and metalions are known to have an influence on the biological function of enzymes, by enhancing or inhibiting their activity via various mechanisms, such as acting as an electron donor or acceptor and forming complexes with limited reactivity (Sachan *et al*.,

2017). This study illustrates the effect of metal ions and detergents on enzyme activity from the lipases of the two isolates used. Lipase studies have reported that metal ions have a role to play in maintaining the stability of lipases (Kanderi *et al*., 2014), with Mg^{2+} being known to activate lipases, in particular, those from *Bacillus* sp., while Na^{+} and EDTA are usually reported to cause agreat inhibitory effect. In this study, lipases retained 95.01% and 89.94% of their activity in the presence of Mg^{2+} (1 mM), with the presence of 1mM EDTA inhibiting the activity of the enzymes; decreases of over 90% were observed for lipases from both strains. Furthermore, lipase activity for enzymes produced by both strains was inhibited by Na^{+}, Fe^{2+}, and K^{+}, with enzyme activity also being reduced by 90% (Table 4.4). Like any other enzyme, inhibition of lipase activity by these metal ions is caused by inhibition of the catalytic site (Dutta & Ray, 2009). Contradictorily, activity of lipases from both strains was also drastically inhibited by Ca^{+}, unlike lipases from numerous microorganisms, including those from *Bacillus* sp. that are Ca^{+} dependent.

The lipases from both strains retained their activity in the presence of Triclosan (TCS) (5-chloro-2-(2,4-dichlorophenoxy-phenol) and trichlorocarbonilide (3,4,4- trichlorocarbonilide) (TCC), which are components of antimicrobial agents and disinfectants used in slaughterhouses, and thus found in PSW. As shown in Table 4.4, the activity of both enzymes was not inhibited by the presence of either of these components; however, TCS had a higher inhibitory effect on the activity of lipases from the isolate B3O. Similarly, Dlangamandla (2016) observed that isolates *C. aquatic* and *Bacillus* sp. isolated from PSW were able to produce flocculants in the presence of TCS and TCC with flocculation activity of up to 92%, indicating resistance towards these components. These results suggested that the presence of these components in PSW as antimicrobial agents is not likely to influence the activity of the lipases during application, and that the microbial population in the PSW had adapted to the TCS and TCC.

Table 4-4: Effect of detergents on lipase activity of *Bacillus cereus* strains AB1 (BF3) and CC-1 (B3O)

Metal ion and detergent	Concentration (mM)	Relative activity (%) BF3	Relative activity (%) B3O
Control	0.0	100	100
$CaCl_2$	1.0	9.41	11.95
NaCl	1.0	3.70	8.83
KCl	1.0	7.33	12.03
$MgSO_4$	1.0	95.01	89.94
$FeSO_4$	1.0	2.65	4.48

EDTA	5.0	4.85	8.83
Triclosan	1.0	81.36	73.91
Trichlorocarbonilide	1.0	91.43	85.32

4.6.1.1 Effect of solvents on enzyme stability and activity

Organic solvents are known to support and maintain enzyme activity due to the ability of the solvents to convert the closed form of an enzyme into an open form, leading to stimulation of the enzyme's open conformation, and reaction sites (Tripath *et al*., 2014). Maintenance of the stability and activity is not only influenced by solvent properties, but by the characteristics of the enzyme (Borkar *et al*., 2009), including those produced by BF3 and B3O. Enzyme activity in the presence of organic solvents 2-mercaptoethanol, ethanol, hexane, isopropanol, methanol, and toluene, was observed by adding solvents to the enzyme solution subsequent to incubation for 30 min. The addition of acetoneand 2-Mercaptoethanol inhibited the activity by over 70% (Table 4.5) for lipases from both strains, when compared to other solvents. It has been reported that lipase from *Bacillus* sp.have a high stability in hydrophobic organic solvents, with activity being retained in 10 to 50 % (v/v) mixtures of alkanes, including toluene (Kumar *et al*., 2016).

Table 4-5: Effect of organic solvents on lipase activity of *Bacillus cereus* strains AB1 (BF3) and CC-1 (B3O)

Organic solvent	**Concentration (% v/v)**	**Relative activity (%) BF3**	**Relative activity (%) B3O**
Control	0.0	100	100
2-Mercaptoethanol	30	25.4	23.49
Acetone	30	15.56	26.72
Chloroform	30	61.77	61.63
Ethanol	30	71.20	71.05
Hexane	30	79.90	77.68
Isopropanol	30	85.14	85.97
Methanol	30	84.47	83.78
Toulene	30	88.70	90.00

4.6.1.2 Effect of pH on bio-delipidation

The assessment ofthe deffating of PSW under varying pH conditions was similarly investigated at pH values 4, 5, 6, 7, 8, 9, 10, 11 and 12 at 37°C. The bio-delipidation effect was investigated by determining the fatty acid content using an enzymatic hydrolysis method by titration. Ethanol was used to dissolve oil with a strong base of phenolphthalein solution,

which was it was used as an indicator. Fig. 4.7 represents the effect of pH on residual fatty acid removal efficiency from the DAF pre-treated PSW. Bio-delipidation removal efficiency was high at pH values between 7-10, with pH values below 7 and above 10 having removal efficiency of fatty acids below 50%. This was attributed to an acidic pH below 7, in which an emulsion was not easily formed compared to an alkaline pH, with the acidity reducing enzymatic functionality. Furthermore, due to the characteristics of the enzymes from both strains, i.e. their retainment of a higher activity under alkaline conditions, it was expected that fatty acid removal efficiency would be high.Shon *et al*. (2002) observed FOG removal efficiency to be high at pH 5-9 using bacterial bio-delipidation aliquots, concluding that the removal of FOG may not have been affected by the initial pH of the wastewater containing FOG, but by the pH of the aliquots containing bio-delipidation enzymes.

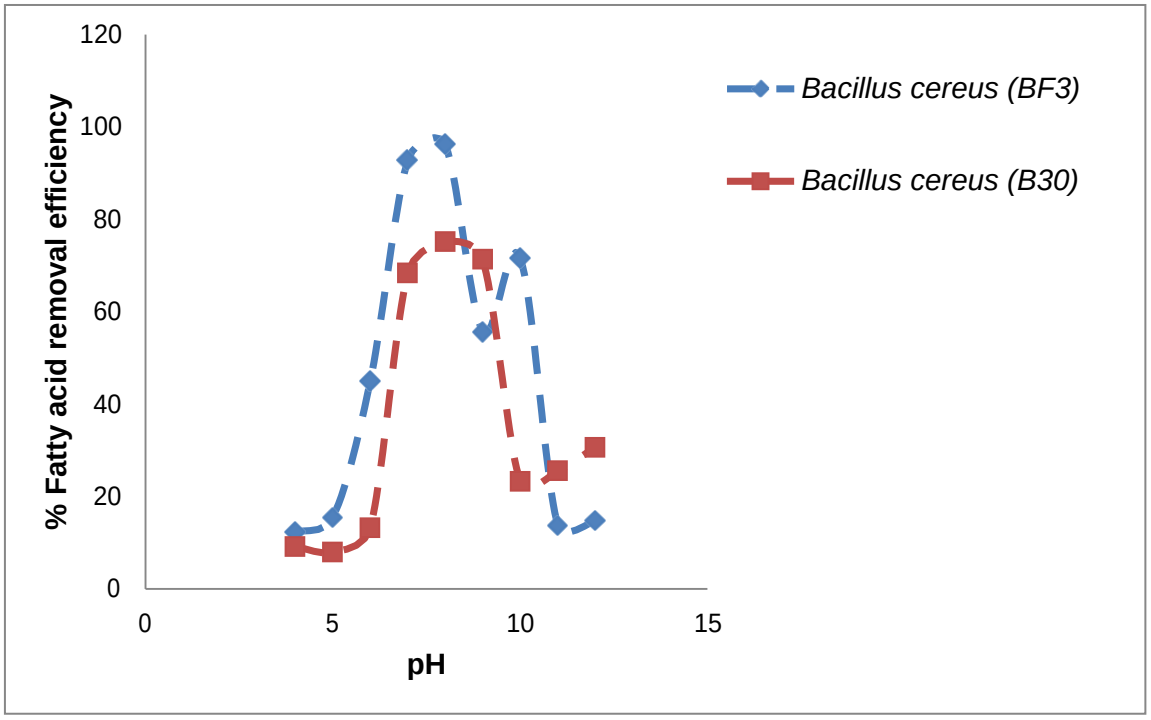


Figure 4-7: Fatty acid removal efficiency from PSW at various pHs by bio-delipidation bio-products of *Bacillus cereus* strains AB1 labelled BF3 and CC-1 labelled B3O.

4.6.1.3 Effect of temperature on bio-delipidation

The effect of temperature on PSW bio-delipidation by lipases from isolated organisms was carried out under optimum pH at different temperatures. As shown in Fig. 4.8, the bio-delipidation efficiency was highest at 45°C, which was attributed to the functionality and

retainment of activity by lipases of both isolates, previously found to be more stable and active at 40-45°C. Leal *et al*. (2006) reported rapid pre-treatment of slaughterhouse wastewater in a study in which enzymes were used, showing a delipidation rate determined to be high at 45°C. Similarly, Krach-Hansen *et al*.(1998), while developing a method for delipidation of proteins, reported delipidation was better at 40°C than lower or higher temperatures.

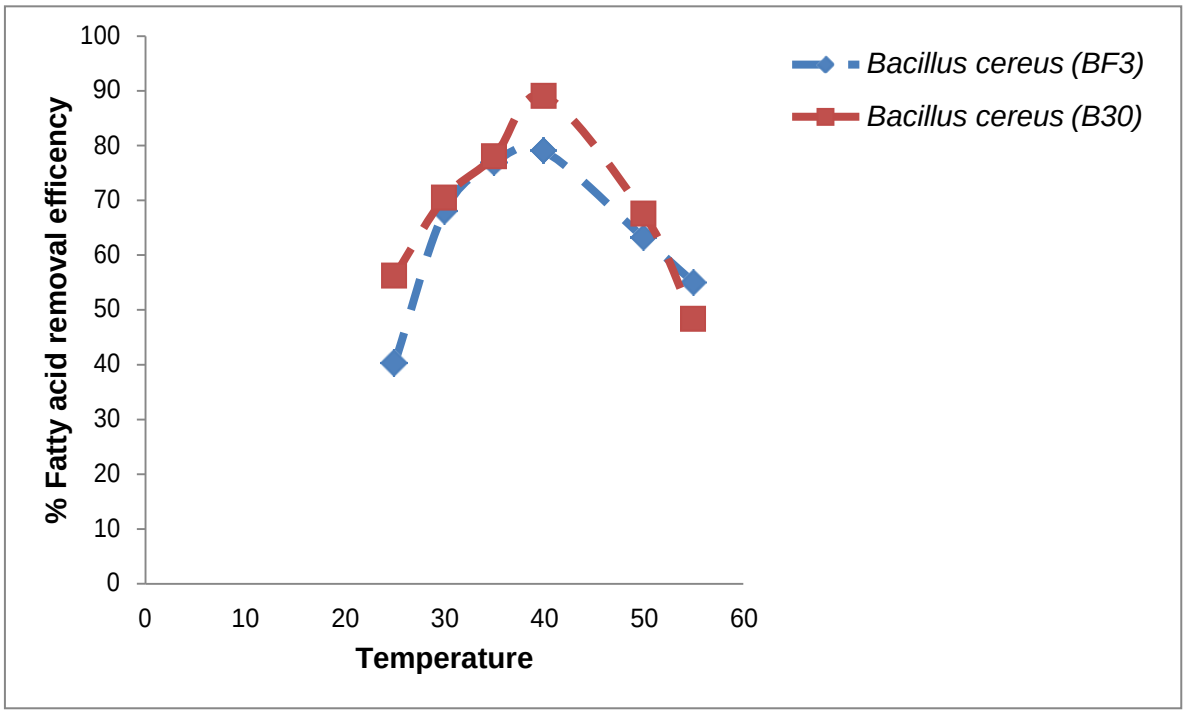


Figure 4-8: Removal efficiency of fatty acids from PSW at various temperatures by bio-delipidation bio-products (enzymes) from *Bacillus cereus* strains AB1 labelled BF3 and CC-1 labelled B3O.

4.7 Summary

This part of the study focused on the characterisation of the enzyme by determining optimum lipase activity and stability at different pH levels and temperatures, while determining the effect of inhibitors such as detergents, solvents and metal ions. Lipase enzymes from *Bacillus cereus* strains AB1 (BF3) and CC-1 (B3O) exhibited maximal activity and stability at pH 6-10 and at 40°C to 45°C. Activity was retained under 1 mM Mg^{2+}, with Fe^{2+}, Na^{+}, K^{+}, and Ca^{2+} being observed to have an inhibitory effect on the enzymes from both strains. Stability of the enzyme in organic solvents toluene, methanol and isopropanol was shown for bio-products from both strains. The enzymes from isolate BF3 retained an activity of 91.43% and 81.36% in the presence of TCS and TCC, while enzymes from isolate B3O retained 85.32% and 73.91% of activity under similar conditions. Bio-delipidation was determined to be influenced by pH, as pH below 7 and above 9 at 40-45°C culminated in a fatty acid removal efficiency below 50%.

4.8 Phase 4 Aim 4: To assess removal of lipids from DAF pre-treated PSW for elucidation of optimum conditions.

4.8.1 Introduction

Pre-treatment of PSW is necessary prior to biological treatment of the wastewater by anaerobic digestion, and as such, the reduction of FOG is required. PSW is classified as high strenghth wastewater, due to the FOG which contributes to the strength of the wastewater culminating in greasy wastewater with a potential to cause process challenges during biological treatment. Utilisation of effective pre-treatment processes for reduction of FOG may enhance biological treatments increasing efficiency of the processes while decreasing hydraulic retention time. Pre-treatment systems, including chemical supplemented DAF systems for removal of FOG, are widely utilised. However, DAF is capable of only removinglipids up to 60-98% (Messè & Masse, 2000) with synthetic chemicals being utilized to enhance the FOG removal efficiency, from such pre-treatment systems.

The use of a physico-chemical pre-treatment system including DAFs in combination with biological techniques such as bio-delipidation with lipases could result in the reduction of FOG while removing organic matter such as proteins in the PSW. In this part of the study, pre-treated PSW from a DAF system, is further bio-delipidised using lipases produced by organisms isolated from the PSW itself.

4.8.1.1 Aim and objectives

The aim of this phase of the study was to further pre-treat PSW from a DAF system by bio-delipidation and assess removal of FOG, proteins,and qunatity other wastewater quality parameters prior and post the bio-delipidation process.

Specific objectives of this part of the study were:

- To pre-treat the PSW by dissolved air flotation (DAF) system prior to bio-delipidation,
- To analyse pre-treated water quality parameters using standard methods (American Public Health Association, 2005), i.e. pre- and post- bio-delipidation, and
- To analyse the bio-delipidation efficiency by FOG/lipid analysis and other water quality parameters.

4.8.1.2 Primary pre-treatment by dissolved air flotation

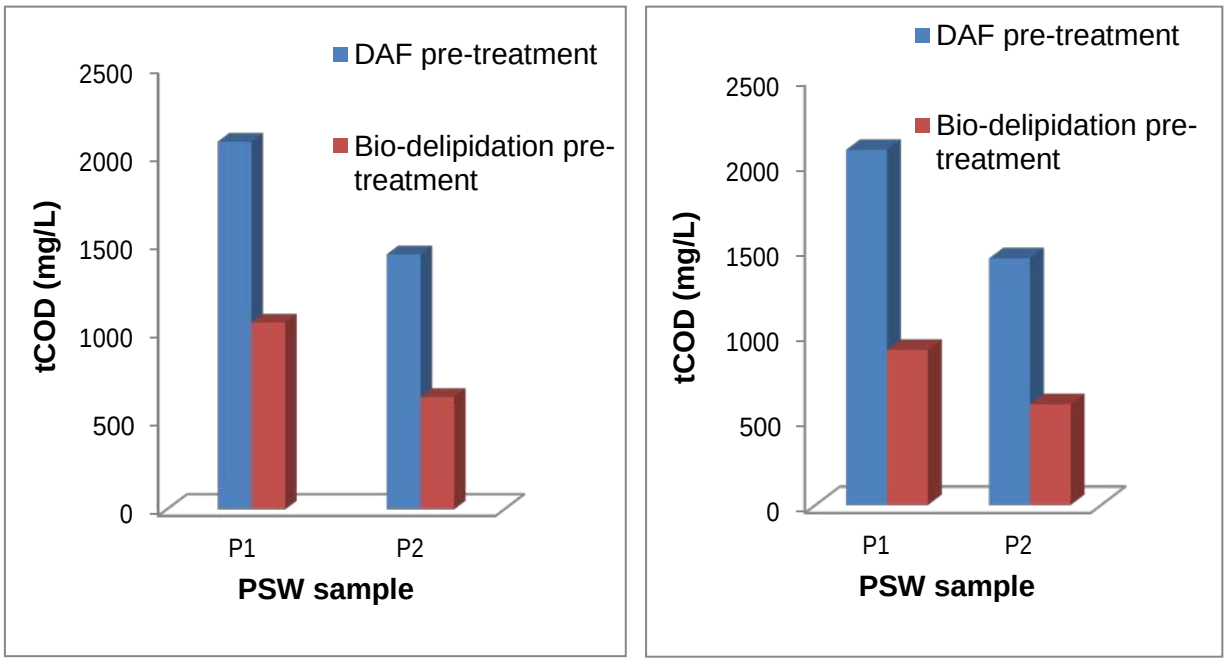
Primary pre-treatment of the PSW prior to bio-delipidation was achieved using a DAF system as shown in Fig. 4.9. Two samples of the PSW labeled P1 and P2 were collected from the

DAF system and analyzed for water quality parameters followed by bio-delipidation using lipases from the two isolated microbial strains which were found to produce enzymes with lipolytic properties. Results of the PSW parameters after DAF and bio-delipidisation pre-treatment are represented as P1 for the first experiment and P2 for the second experiment. Each experiment included a DAF and subsequent bio-delipidation pre-treatment.

4.8.1.2.1 Reduction of total chemical oxygen demand (tCOD) by bio-delipidation

PSW is characterised by high levels of chemical oxygen demand (COD) due to the presence of lipids and other organic matter, including carcass debris (Kundu *et al*., 2013). COD is used to measure the water quality by quantifying total organic matter composition, represented as the amount of oxygen needed to facilitate oxidation of oxidisable matter in the wastewater. Due to a high concentration of pollutants in PSW, such as proteins and other organic matter, residual organic matter might be disposed off into fresh water sources which could result in the depletion of oxygen in thesereceiving sources, further polluting the ecosystem (Van Schoor, 2005). Application of lipases has been reported to result in decreased organic matter, i.e. in reducing the COD of lipid-rich wastewater (Leal *at al*., 2006). In this part of the study, total chemical oxygen demand (tCOD) of DAF pre-treated and bio-delipidized PSW was measured to assess the efficiency of the bio-delipidation in reducing tCOD.

Figure 4.11 (a) and (b) represents removal of the tCOD from the PSW after DAF pre-treatment and bio-delipidation pre-treatment using lipases from the two isolated microbial strains Bacillus cereus AB1 (BF3) and Bacillus cereus CC-1 (B3O). The tCOD of raw PSW was measured prior to both pre-treatments and was assessed to be as high as 6500 mg/L. Primary pre-treatment of the PSW using the DAF was observed to reduce the tCOD by 68.0% (P1) with a further 77.7% (P2) reduction in the bio-delipidation unit. After DAF pre-treatment, bio-delipidation of PSW by lipases from Bacillus cereus AB1 (BF3) reduced tCOD by 49% (P1) and 55.7 (P2) while Bacillus cereus CC-1 (B3O) lipase was observed to reduce tCOD by 56.3% (P1) and 66.0% (P2) for the tCOD removal. The bio-delipidation was attributed to the supplementation of lipases from both strains which culminated in the removal of lipids (FOG) resulting in a reduction of organic matter. Valladão et al.(2011) observed enhanced reduction in COD by enzymatically pre-treated fatty wastewater compared to unpretreated wastewater, with better occurence of anaerobic reaction. Therefore, the pre-treatment of PSW with bio-delipidation and a DAF system could improve anaeronic biological treatment resulting in wastewater which is safer to discharge into the environment.

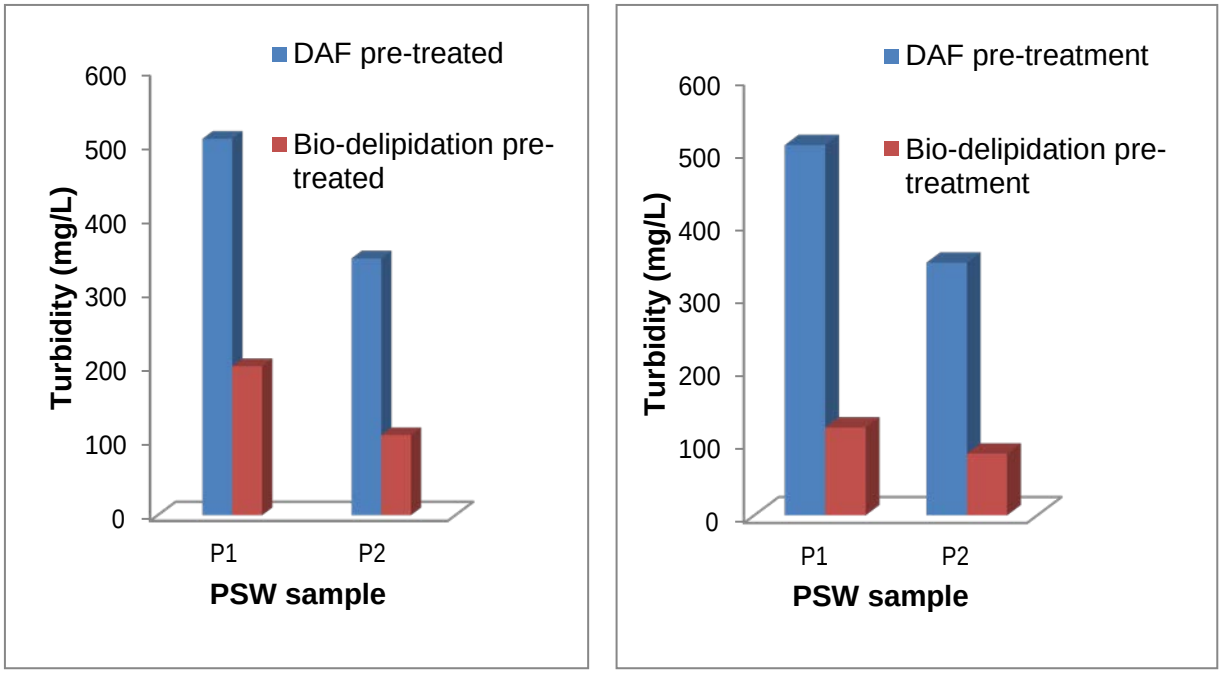


A *B. cereus* AB1 (BF3) **B** *B. cereus* CC-1 (B3O)

Figure 4-9: Reduction of tCOD of PSW after DAF pre-treatment (blue) and bio-delipidation pretreatment (red) for two experiments P1 and P2 (with different PSW) treated with lipases from (a) *Bacillus cereus*AB1 (BF3) and (b). *Bacillus cereus* CC-1 (B3O) Initial tCOD concentration was 6500 mg/L.

4.8.1.2.2 Reduction of turbidity by bio-delipidation from dissolved air flotation

Figure 4.12 (a) and (b) represents a reduction of turbidity for the PSW after DAF pre-treatment and bio-delipidation pretreatment using lipases from the two isolated microbial strains *Bacillus cereus* AB1 (BF3) and *Bacillus cereus* CC-1 (B3O). The untreated PSW was assessed to have turbidity up to 792 mg/L. Pre-treated PSW by the DAF was observed to have reduced turbidityof 35.85% (P1) and 56.1% (P2). Further bio-delipidation of the PSW by lipases reduced remaining turbidity effectively, with *Bacillus cereus* AB1 (BF3) aliquots reducing turbidity by 60.2% (P1) and 68.6% (P2), and *Bacillus cereus* CC-1 (B3O) lipase aliquots reducing turbidity by 76.18% (P1) and 75.5% (P2). Additionally, after bio-delipidation, the PSW was observed to be clearer compared to that supplied to the DAF system. Mendes *et al.*(2010) and Meng *et al*.(2017) observed similar results where the application of lipase enzyme pools in fatty wastewater reduced wastewater turbidity, resulting in clear wastewater.



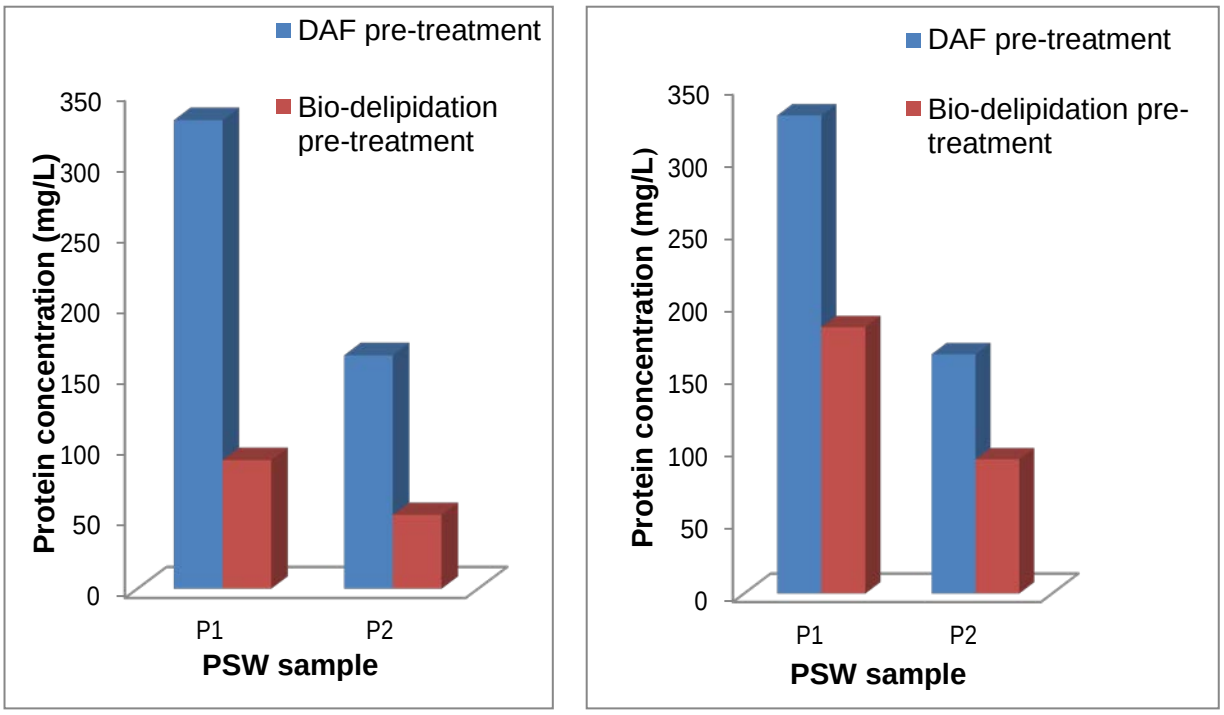
A *B. cereus* AB1 (BF3) **B** *B. cereus* CC-1 (B3O)

Figure 4-10: Reduction of turbidity of PSW after DAF pre-treatment (blue) and bio-delipidation pretreatment (red) for two experiments P1 and P2 (with different PSW for both experiments) treated with lipases from (a) *Bacillus cereus* AB1 (BF3) and (b) *Bacillus cereus* CC-1 (B3O). Initial turbididy of PSW was 792 mg/L.

4.8.1.2.3 Reduction of protein concentration by bio-delipidation

Blood and debri proteins are huge contributors to pollution in PSW, resulting in high levels of BODs and tCOD (Lo *et al*., 2005). In this part of a study, a standard curve using BSA concentrations as working standards was used for determination of total protein concentration of the untreated, DAF pre-treated, and bio-delipidized PSW. This was done to assess the efficiency of the bio-delipidation pretreatment in reducing protein concentration in the PSW. A standard curve with a suitable correlation coefficiant (R^2 = 0.989) was obtained using the BSA as a substrate to quantify protein concentration. Prior to DAF pre-treatment, raw untreated PSW was quantified to contain 800 mg/L of total protein concentration. In Figure 4.12, reduction of total protein concentration of PSW after DAF pre-treatment and bio-delipidation pre-treatment by lipase enzyme from *Bacillus cereus* CC-1 (B3O) and *Bacillus cereus* AB1 (BF3) indicates adequate protein removal efficiency. DAF system pre-treatment, and therefore removal of total protein concentration of the effluent, for P1and P2 was 58% and 79.3% respectively; residual protein being to the bio-delipidization unit in which lipases from the two isolated microbial strains were supplemented. Total protein removal, by the bio-delipidation system was observed to have been up to 72.32% (P1) and 68.1% (P2), while

Bacillus cereus AB1 (BF3) aliquotes only managing to remove protein by only 42.7% (P1) and 44% (P2) respectively.



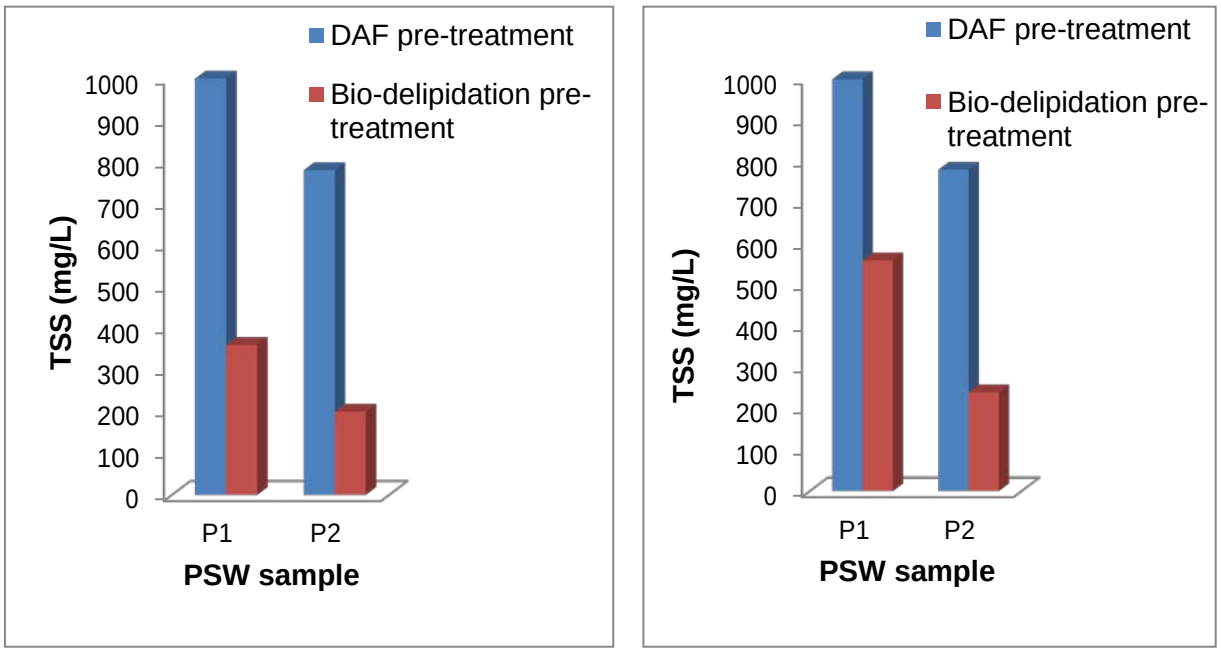
A *B. cereus* AB1 (BF3) **B** *B. cereus* CC-1 (B3O)

Figure 4-11: Reduction of total protein concentration after DAF pre-treatment (blue) and bio-delipidation pretreatment (red) for two experiments P1 and P2 (with different PSW) treated with lipase enzyme from (a) *Bacillus cereus* CC-1 (B3O) and (b) *Bacillus cereus* AB1 (BF3). Initial total protein concentration of PSW was 800 mg/L.

4.8.1.2.4 Reduction of total soluble solids by bio-delipidation

Major characteristics of PSW included total suspended solids (TSS) made up of floating, settleable, colloidal organic and some inorganic matter. These total suspended solids (TSS) are classified as being smaller than 0.2µm in size. Quantification of TSS prior to the discharge of the PSW into the environment is necessary, as TSS can result in higher levels of turbidity, which will have higher impact on the environment and aquatic life (Plumber &Krepper, 2011). In this part of the study, raw PSW, PSW from the DAF system, and PSW post bio-delipidation, were used to quantify total soluble solids, with each unit being assessed for performance adequacy by determining the efficiency of the bio-delipidation in reducing residual TSS from the DAF. Figure 4.13 (a) and (b) represent the removal of total soluble solids after DAF pre-treatment and bio-delipidation pretreatment using lipases from the two isolated microbial strains *Bacillus cereus* AB1 (BF3) and *Bacillus cereus*CC-1 (B3O). The TSS of the untreated PSW was quantified to be 2400 mg/L prior to pre-treatment. On the

first run of DAF systems, 58% of TSS was removed from P1 and 67.5% TSS was removed from P2. Removal of residual TSS by bio-delipidation using the isolates enzyme from BF3 resulted in 44% (P1) and 69.2% (P2) respectively, while the enzyme from B30 resulted in 64% (P1) and 74.3% (P2) respectively.



A *B. cereus* AB1 (BF3)

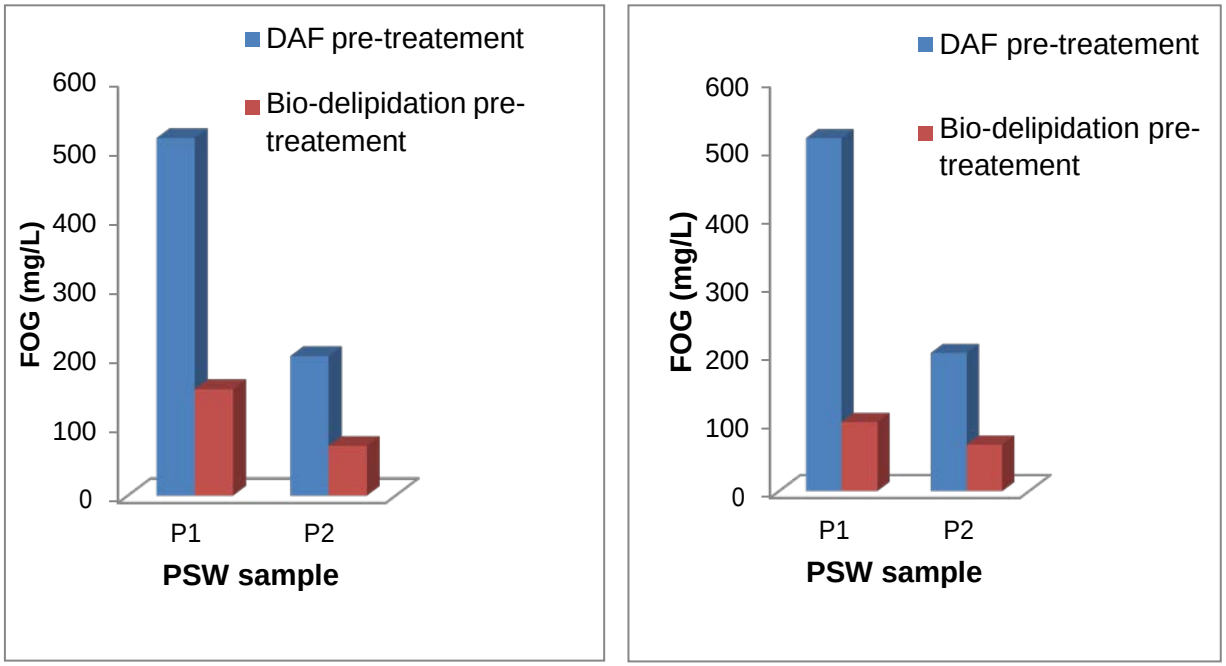
B *B. cereus*CC-1 (B3O)

Figure 4-12: Removal of TSS of PSW after DAF pre-treatment (blue) and bio-delipidation pretreatment (red) for two experiments P1 and P2 (with different PSW) treated with lipase enzyme from (a) *Bacillus cereus* AB1 (BF3) and (b) *Bacillus cereus* CC-1 (B3O). Initial TSS of PSW was 2400 mg/L.

4.8.1.2.5 Removal of residual FOG by bio-delipidation

As bio-delipidation involves deffating of wastewater protein solutions,FOG removal after DAF and bio-delipidation pretreatment was assessed to determine the efficiency of bio-delipidation in removing minute quantities of residual FOG remaining in the PSW, which can reduce the effeciency of downstream anaerobic biological processes. Prior to the DAF system preatment, the raw PSW had a FOG concentration of up to 1029 mg/L. Figure 4.14 (a) and (b) illustrate removal of FOG after DAF pre-treatment and bio-delipidation pre-treatment. Removal of FOG from the raw PSW by the DAF system was 49.8%(P1) and 80.37% (P2) respectively, with the remaining FOG not being properly removed by the DAF system. However, bio-delipidisation removed a futher 70.15% (P1) (first experiment) and 64.35% (P2) (second experiment) by lipases of *Bacillus cereus* CC-1 (B3O). Lipases from

Bacillus cereus AB1 (BF3) removed 80.42% (P1) and 66.35% (P2) of FOG remaining from the DAF pre-treatment system. A similar comparative study by Dlangamandla (2016) observed the reduction of FOG from a Bio-DAF supplemented with bioflocculants produced by a *Bacillus* sp. compared to a chemically supplemented DAF. This indicates that defogging using biological extracts to supplement a physical system can be effective. Similar studies using wastewater from slaughterhouses and dairy industries, have shown that lipase enzymes can reduce FOG to improve the effectiveness of downstream processes (Rosa *et al*., 2009; Mendes *et al*., 2010).



A *B. cereus* AB1 (BF3)

B *B. cereus*CC-1 (B3O)

Figure 4-13: Removal of FOG of PSW after DAF pre-treatment (blue) and bio-delipidation pretreatment (red) for two experiments P1 and P2 (with different PSW) treated with lipase from (a) *Bacillus cereus* CC-1 (B3O) and (b) *Bacillus cereus* AB1 (BF3). Initial FOG of PSW was 1029 mg/L.

4.9 Summary

This part of the study focused on the bio-delipidation of PSW from a DAF pre-treatment system by lipases from *Bacillus cereus* CC-1 (B3O) and *Bacillus cereus* AB1 (BF3). PSW parameters were measured after the PSW was pre-treated using a DAF system, with the same parameters measured after a bio-delipidation pre-treatment procedure, to assess any resulting improvement in wastewater quality. Bio-delipidation by enzymes from both strains further removed up to an average of 80.3%FOG, 72.3% proteins, 66.0% tCOD, 74.3% TSS,

and 76.2% turbidity, containment which remained in the PSW after DAF pre-treatment. This showed that bio-delipidation pre-treatment has the potential to further enhance the efficiency of physical pre-treatment systems for PSW.

CHAPTER 5

CONCLUSIONS

AND

RECOMMENDATIONS

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

In this research study, two lipolytic microorganisms were isolated from PSW to produce lipases for the bio-delipidation of protein-rich PSW. The isolates, identified using 16S rRNA sequencing, were determined to be *Bacillus* sp., most likely *Bacillus cereus,* named AB1 (BF3) and CC-1 (B3O). Optimum conditions for lipase production were identified using RSM, prior to their usage for the production of the enzymes (lipases) required for bio-delipidation.

Stability and retainnment of activity characteristics were studied for lipases of both strains, with high activity and stability being exhibited under alkaline pH conditions and at an optimum temperature of 45°C. The enzymes produced by both isolates were mostly unaffected by the presence of organic solvents and detergents found in PSW, as observed by their ability to retain 80 to 90% of activity in the presence of these synthetic compounds; however, some metal species exceeding 1Mm could reduce the activity of these enzymes.

Initially, raw PSW was pre-treated using a DAF system followed by bio-delipidation using lipase enzymes from the isolated lipolytic microorganisms. Efficiency of the bio-delipidation pre-treatment using lipase enzymes was observed by determining wastewater quality after DAF pre-treatment compared to before. Bio-delipidation utilising lipases from the isolated strains *Bacillus cereus* strains AB1 (BF3) and *Bacillus cereus* strains CC-1 (B3O) was found to have a removal efficiency of up to 64.3 to 80.4% of FOG, therefore, reducing the tCOD by 50 to 66% from the DAF pre-treated PSW. With regard to deffating of PSW by bio-delipidation, total protein concentration remaining from DAF system was reduced by 42.7% to 73.3% using enzymes from both isolated strains. An improved, clear color of the PSW after bio-delipidation was also observed. In this study, bio-delipidation as a biological technique for pre-treatment systems was therefore shown to have the potential to enhance downstream biological processes.

5.2 Recommendations

It is recommended as a topic of further study that bio-delipidation be applied in situations where pre-treatment of PSW is challenging, with further performance of downstream biological remediation processes being monitored to assess consequent improvement.

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APPENDICES

Appendix A: Isolation and identification

Appendix A1: Gram staining method

- A loop of sterile distilled water was transferred to the surface of the glass microscope slide then another loop foolof pure culture from an agar petri dish wasfixated into the glass slideto create a smear. Heating to preserve the smear was done.
- Crystal violet was applied as the primary stain and allowed to stand for 60 seconds to stain all the cells blue/purple.
- After 60 sec, the glass slide was rinsed under running tap water.
- The iodine solution was added to the glass slide and allowed to stand for 60 seconds for the formation of the crystal violet-iodine complex.
- A few drops of acetone for decolorization were added to the fixated smear.
- Rinsing followed immediately to prevent over-decolorization of the smear.
- Safranin was applied as the counter stain and allowed to stand for 60 seconds for staining of the decolorized smear.
- This was followed by rinsing under running tap water, after which it was blotted dry.

Appendix B:

Appendix B1: Buffers

50mM Citrate-phosphate buffer at pH 4.0-5.0

- The buffer solution was prepared by 7.10g Na_2HPO_4 and 11.50g of citric acid. The buffer was then adjusted to the desired pH using NaOH and HCl prior to bringing to a final volume of 1L.

Carbonate-bicarbonate buffer at pH 10.0

- 28.62g of $Na_2CO3.10H_2O$ and 8.40g of $NaHCO_3$**.** The buffer was then adjusted to the desired pH using NaOH and HCl prior to bringing to a final volume of 1 L.

50 mM Potassium phosphate buffer at pH 7.0, 10.0, 11.0, 12.0

- 50 mM phosphate buffer solution was prepared by adding 3.33g of K_2HPO_4 into 400mL of distilled water and 4.21g of KH_2PO_4 into 400mL distilled water. Equal volumes of both solutions were mixed and the buffer was then adjusted to the desired pH using NaOH and HCl prior to bringing to a final volume of 1 L.

50 mM Tris-HCl pH 6.0-7.0

- 8.06 g of the above solution was dissolved in 800ml of distilled water. The buffer was then adjusted to a pH of 8.0 using NaOH and HCl prior to bringing to a final volume of 1 L.

Appendix B2: Bradford's Method to determine protein concentration

Working standard for standard curve to determine protein concentration from the BSA was prepared as described in the table below:

Standard	Vol BSA	Vol 50Mm sodium phosphate pH 8	BSA conc µg/ml
Blank	0	100	0
1	10	90	100
2	20	80	200
3	40	60	400
4	60	40	600
5	80	20	800
6	100	0	1000

BSA Standard curve: