Cape Peninsula University of Technology

BIOFLOCCULANT DISSOLVED AIR FLOTATION SYSTEM FOR THE REDUCTION OF SUSPENDED SOLIDS-LIPIDS-PROTEINACEOUS MATTER FROM POULTRY SLAUGHTERHOUSE WASTEWATER

By

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DECLARATION

I, **Cynthia Dlangamandla**, 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 the intellectual property, methodologies and mathematical models used in this thesis and those that are published in scientific journals were derived by the first author of the published manuscript. The intellectual property of others was acknowledged by appropriate references. The contribution of co-authors, for conference and published manuscripts, were in a training capacity (Ms S A Dyantyi, in-service training), research assistance (Ms Y P Mpentshu) and supervisory capacity (Mr M Basitere and Prof. S K O Ntwampe).

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Signed **Date**

ABSTRACT

Poultry slaughterhouse wastewater (PSW) contains organic matter that can be degraded by microorganisms. Such matter can further be used by the microbial community as a nutrient source for growth. Moreover, this type of wastewater also contains a high quantity of particulate matter, lipids and proteins, including antimicrobial compounds such as triclosan (TCS) and trichlorocarbanilide (TCC) used during cleaning and sanitising of processing facilities. Lipids and particulate matter lead to clogging of pipes and fouling of diffusers in the wastewater treatment plants (WWTPs). To overcome this problem, a pre-treatment system such as a dissolved air flotation system (DAFs) in which synthetic flocculants are used, is commonly used prior to the biological treatment of the wastewater. Synthetic flocculants add to the environmental burden associated with the use of synthetic compounds, particularly when these compounds are used in WWTPs. This study focused on the reduction of suspended solids, lipids and proteinaceous matter using a bioflocculant- supported DAF for the treatment of PSW.

To bioprospect bioflocculant producing organisms, six bacterial strains were initially isolated from the PSW. These isolates were cultured on two different types of liquid media, namely, synthetic and biological media. Bioflocculant activity was quantified using kaolin clay (4 g/L). These microorganisms produced bioflocculants under different conditions, such as, pH and temperature. Two isolates were identified and they were able to produce bioflocculants with higher flocculation activity using different media. These isolates were identified as *Comamonas aquatica* (BF-3) and *Bacillus* sp. BF-2. The highest flocculation activity achieved was 93.84% at 32.96 ˚C and pH 6.5 for *C. aquatica*, and 88.3% at 40.03 ˚C and pH 6.5 for *Bacillus* sp. BF-2, respectively, when these isolates were cultured in synthetic medium.

The Fourier Transform Infrared Spectroscopy (FTIR) analysis for the bioflocculants produced by *C. aquatica* (BF-3) showed the presence of hydroxyl, carboxyl, and amine functional groups, which was an indication that the bioflocculants were protein constituents; whereas the FTIR analysis for bioflocculants produced by *Bacillus* sp. BF-2 portrayed the presence of hydroxyl, carbonyl, alkane and alkyl halide functional groups, which showed that the bioflocculants produced by this isolate were polysaccharides constituents. When these isolates were cultured in the biological medium, the highest flocculation activity achieved was 56.9% and 46.8% at 34 ˚C and pH 4 for BF-3 and BF-2, respectively. These two isolates were further assessed for their antimicrobial resistance, that is, to produce bioflocculants even in the presence of TCS and TCC at different concentrations, that is, 18 mg/L (min), and 55 mg/L (max), respectively.

The *C. aquatica* and *Bacillus* sp. (BF-2) isolates were both able to produce bioflocculants with a high activity at 32 ˚C and pH 6.5 even in the presence of TCS and TCC. *Bacillus* sp. BF-2 was able to tolerate TCS even at 55 mg/L, producing bioflocculants with 92% activity and 16.9% activity at 55 mg TCC /L. Similarly, the *C. aquatica* produced bioflocculants with 92% activity in the presence of 55 mg TCS/L; and a 49.5% flocculation activity was achieved in the presence of 55 mg TCC/L. The isolates were further assessed, for antimicrobial activity using an agar well diffusion method, for their ability to grow in the presence of other microorganisms found in the PSW. When isolates were added into the wells individually, there were distinct zones of clearance which were narrow; however, when a mixed culture of the two isolates was added into the wells, the zones of clearance were wider, showing that these isolates had antimicrobial activity against other microorganisms present in the PSW. Hence, both isolates were determined to be suitable candidates to utilise in the bioflocculant-supported DAF system.

A DAF was subsequently designed to operate at a flow rate of 0.11 mL PSW/min and an hydraulic retention time (HRT) of 12 days, with sparging to create microscopic bubbles using air diffusers. A 2% (v/v) (bioflocculant: PSW) strategy was used for the DAF to reduce total suspended solids (TSS), lipids and proteins in the PSW, by supplementing the bioflocculants produced and the co-culture directly into the DAF. The bioflocculant-supported DAF (BioDAF) was able to reduce 91% TSS, 79% proteins and 93% lipids when the DAF was operating at steady state, in comparison with a chemical DAF (ChemDAF) operated using 2% (v/v) alum that was able to reduce 84% TSS, 71% proteins and 92% lipids.

It was concluded that the BioDAF system worked efficiently for the removal of suspended solids, lipids and proteins, achieving better results than when alum was used. A cost-benefit analysis should be assessed to conclusively encourage the use of bioflocculants in DAF systems. This study was the first study to report on a BioDAF, a system considered to be environmentally benign for the pre-treatment of PSW.

DEDICATION

I would like to dedicate this thesis to God, the creator and my loving parents

Sylvia Dlangamandla (my late mom)

and

Nceba Londa

You are my love, my life, source of my strength, the reason I wake up every day and work hard. You made me realise that nothing is impossible with God.

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RESEARCH OUTPUTS

Peer-reviewed publications

Dlangamandla, C., Dyantyi, S.A., Mpentshu, Y.P., Ntwampe, S.K.O. & Basitere, M. 2016. Optimisation of bioflocculant production by a biofilm-forming microorganism from poultry slaughterhouse wastewater for use in poultry wastewater treatment. *Water Science & Technology*, 73(8):1963-1968. (DOI: 10.2166/wst.2016.047).

Basitere, M., Williams, Y., Sheldon, M.S., Ntwampe, S.K.O., De Jager, D. & **Dlangamandla, C.** 2016. Performance of an expanded granular sludge bed (EGSB) reactor coupled with anoxic and aerobic bioreactors for treating poultry slaughterhouse wastewater. *Water Practice and Technology*, 11(1):86-92. (DOI: 10.2166/wpt.2016.013).

Poster presentations

International Water Association (IWA) 7th Eastern European Young Water Professionals Conference (17 to 19 September 2015). Title: Bioflocculant production by biofilm forming microorganisms from poultry wastewater for use in poultry wastewater treatment. Authors: **Dlangamandla, C**., Ntwampe, S.K.O. & Basitere, M.

International Water Association (IWA) 4th South African Biennial Young Water Professionals (YWP) and First African IWA YWP Conference (16–18 November 2015). Title: Triclosan, trichlorocarbanilide-resistant microorganisms and their ability to produce bioflocculants for the removal of lipids and suspended solids from poultry wastewater. Authors: **Dlangamandla, C.** & Ntwampe, S.K.O.

LAYOUT OF THESIS

The primary aim of this thesis was to isolate and identify microorganisms from PSW that were capable of producing sufficient quantities of bioflocculants with high flocculation activity as well as to characterise the bioflocculants as well as to utilise them to enhance a bench-scale DAF system for the removal of lipids, suspended solids including proteins contained in the PSW.

This thesis comprises the following chapters:

Chapter 1: This chapter provides the background on the PSW, bioflocculants, synthetic flocculants and dissolved air flotation systems (DAFs). It also contains the research problem statement, hypothesis, aims and objectives, significance and delineation of the study.

Chapter 2: This chapter comprises additional information on the characteristics of the PSW, as well as South African legislation for PSW discharge standards. It discusses PSW treatment systems as well as different types of pre-treatment systems, the application of a dissolved air flotation system, flocculation, bioflocculants and chemical flocculants.

Chapter 3: This chapter focuses on the materials and methods used in this study to isolate and identify microorganisms that are able to produce bioflocculants with a high flocculation activity. Furthermore, this chapter lists materials and equipment used to quantify flocculation activity, including optimisation of bioflocculant-production systems. Moreover, it lists methods and materials used in this study to design bench-scale DAFs, including the determination of the reduction of suspended solids, proteins, and fats, oil and grease (FOG) from the PSW.

Chapter 4: This chapter contains results and discussion of the experiments carried out to achieve the aims and objectives of the study.

Chapter 5: This chapter comprises the overall conclusions and recommendations for future research studies.

The literature that was consulted in this study is listed in the references section.

Furthermore, the primary research outputs emanating from this study is attached as an appendix to the thesis.

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LIST OF SYMBOLS

Nomenclature

Symbol	Description	Units
X_o	Feed solid concentration	mg/L
C_a	Soluble air concentration at one	mg/L
R	Recirculation ratio	-
P	Gauge pressure	Kg/ms^2
f	Pressurisation system efficiency	-
A	Optical density of the control	nm
B	Optical density of the sample	nm
$sCOD$	Soluble chemical oxygen demand	mg/L
$tCOD$	Total chemical oxygen demand	mg/L
μ_g	Specific growth rate	h^{-1}
R^2	Coefficient of determination	-
X_i	Coded independent variables	-
Y	Flocculation activity	(%)

GLOSSARY

Abbreviation	Description
ANOVA	Analysis of variance
BOD	Biochemical oxygen demand
CCD	Central composite design
COD	Chemical oxygen demand
DAF	Dissolved air flotation
DAFs	Dissolved air flotation system
EC	Electrocoagulation
FOG	Fats, oil and grease
FTIR	Fourier transform infrared spectroscopy
HRT	Hydraulic retention time
O&G	Oil and grease
PCR	Polymerase chain reaction
PSW	Poultry slaughterhouse wastewater
rDNA	Ribosomal deoxyribonucleic acid
rpm	Revolutions per minute (rev/min)
rRNA	Ribosomal ribonucleic acid
RSM	Response surface methodology
SS	Suspended solids
sCOD	Soluble chemical oxygen demand
tCOD	Total chemical oxygen demand
TCC	Trichlorocarbanilide (also known as triclocarban)
TCS	Triclosan
TDS	Total dissolved solids
TSS	Total suspended solids
WWTPs	Wastewater treatment plant

CHAPTER 1

INTRODUCTION

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INTRODUCTION

1.1 General background

Wastewater has socio-economic issues, as there are costs associated with its disposal as per government regulations. Furthermore, strict government regulations for local municipalities only permit the disposal of low-contaminant concentrations into local rivers (Rout, 2013). This is a problem that makes wastewater treatment critical prior to its being released into receiving rivers. Different types of wastewater treatments, such as chemical, physical and biological, have been used to reduce the concentration of contaminants in wastewater, in order to reduce environmental impacts and health risks. Biological treatment of the wastewater uses enzymatic and other metabolic functions in appropriate microorganisms to degrade harmful water pollutants into less toxic by-products. These microorganisms may also be able to detoxify the wastewater by using their metabolic extracellular by-products.

Physical wastewater treatment systems include dissolved air flotation systems (DAFs), which use flotation of suspended solids and other particles to the water surface by attaching them to the surface of tiny bubbles that accelerate upwards in the system. DAFs pneumatically separate these solids from the wastewater, after which the solids can then be removed by skimming (Zouboulis & Avranas, 2000). DAFs can be improved by the use of bioflocculants that facilitate and enhance the attachment of suspended solids (SS), such as lipids and proteins, in wastewater. Bioflocculants are polymers that are synthesised by microorganisms during their developmental stages (Deng et al., 2003). These flocculants promote flocculation by forming bridges between themselves and particulate matter, resulting in the aggregation of suspended particles into flocs.

Generally, when the suspended particles are flocculated into larger flocs, the resultant wastewater is clarified, reducing its turbidity. When bioflocculants are used with a DAFs, they can improve the formation of flocs so that the flocs can move to the surface of the wastewater easily, and can then be removed physically. Numerous synthetic flocculants have been used; however, they impose ecological risks, as some are carcinogenic (Deng et al., 2003).

Bioflocculants can be produced by a variety of microorganisms found in many habitats. They are cost effective and environmentally friendly (Deng et al., 2003).

This study focused on the production of bioflocculants by using microorganisms isolated from PSW for use in DAFs, that is, as a pre-treatment step, prior to the biological treatment of the wastewater. These flocculants were incorporated with DAF to remove lipids and protein as well as suspended solids from the PSW. The presence of synthetic antimicrobial compounds, suspended solids, lipids and proteinaceous matter in this PSW necessitated a pre-treatment of the wastewater prior to its biological treatment, as indicated by Basitere et al. (2016).

1.2 Research problem

Fats, oil and grease (FOG), including proteins from food-processing industries and households, constitute a major problem for wastewater treatment plants (WWTPs), as it is difficult to treat such wastewater by using conventional biological treatment methods that in many cases, FOG result in the fouling and reduced performance of the treatment system. Furthermore, FOG become solidified at low temperatures, resulting in the clogging of pipes and reduced transfer of nutrients required to sustain microbial growth, which effectively renders biological treatment systems inefficient. This culminates in increased operational costs. In aerobic treatment systems, FOG decrease the rate at which dissolved oxygen is transferred, while in anaerobic treatment systems, they elevate the quantity of actinomycetes, *Nocardia amarae* causing scum formation, including foaming, which leads to poor sludge activity (Jeganathan et al., 2006). Poultry slaughterhouses produce wastewater containing lipids, proteins, phosphorous, nitrogen, blood and other organic matter (Chávez et al., 2005). Hence, industries that generate waste materials from a biological origin are often amenable to the application of bio-based processes for their remediation, although it is difficult to treat this kind of wastewater in bioreactors since it contains high quantities of FOG (Del Nery et al., 2007). Therefore, there is a need to design a dissolved air flotation system (DAFs) in which bioflocculants are used for the flocculation of FOG, including protein and other suspended solids, prior to the biological treatment of the wastewater.

1.3 Hypothesis

Using a DAFs with bioflocculants can efficiently reduce suspended solids, FOG and proteinaceous matter from poultry PSW.

1.4 Research questions

The following questions were posed to develop and conceptualise the aims and objectives of the research:

- Can microorganisms from PSW produce bioflocculants with suitable activity for application in the designed DAFs for the reduction of FOG in PSW?
- What type of bioflocculants will the isolates produce, that is, will they be polysaccharides or protein based?
- Will the PSW be suitable as a medium to develop an inoculum to be used in the DAFs?
- Since PSW contains antimicrobial compounds used in sanitary operations of slaughterhouses, will the isolates be resistant to these compounds?
- Furthermore, will the isolates producing flocculants with a higher flocculation activity be able to perform this function even in the presence of antimicrobial compounds?
- Will the integrity of the produced bioflocculants be maintained during the DAF operation?
- Will there be significant differences in the DAFs when bioflocculants are used rather than chemical flocculants?

1.5 Research aims and objectives

This study was divided into four phases: Phase 1 (Aim 1): to isolate and identify microorganisms with bioflocculant production potential; furthermore, to characterise the bioflocculants produced by the isolates; moreover, to optimise the bioreactor conditions for the production of these bioflocculants by using a response surface methodology (RSM). Phase 2 (Aim 2): To reassess the ability of the isolated microorganisms to produce bioflocculants in the wastewater from which they were isolated and optimise the bioreactor conditions using RSM. Phase 3 (Aim 3) constituted the evaluation of the microbial resistance of the microorganisms isolated in Phase 1 to triclosan (TCS) and trichlorocarbanilide (TCC) and their ability to produce bioflocculants in the presence of these contaminants as well as in the presence of the PSW microbial community. Phase 4 (Aim 4): to design a bench-scale bioflocculant-supported DAFs and assess its efficiency in removing SS, proteins and FOG from the PSW. The phases and their individual objectives are highlighted below:

Phase 1: Aim 1: To isolate and identify microorganisms with bioflocculant-production potential; furthermore to characterise the bioflocculants that were produced by the isolates; moreover, to optimise the bioreactor conditions for the production of bioflocculants from the isolates, producing flocculants with a higher activity by using response surface methodology (RSM), in which synthetic nutrient media were used.

Objective 1: Isolation and identification of microorganisms from the PSW.

Objective 2: Assess whether these isolates can produce bioflocculant, using (4 g/L) kaolin clay to quantify bioflocculation activity.

Objective 3: Choosing highly competitive isolates over the other isolates for optimisation of bioflocculant production by RSM.

Objective 4: To determine the constituents of the produced bioflocculants using FTIR.

Phase 2: Aim 2: The aim of this part of the study was to produce bioflocculants by using PSW (biological medium), as it is nutrient rich, subsequent to optimisation using RSM as done in Phase 1 of the study, by using isolates deemed appropriate for bioflocculant production (Phase 1: Aim 1). The objectives therefore were:

Objective 1: To culture the isolates in a biological medium and assess the isolates' ability to produce bioflocculants with higher activity by using kaolin clay (4 g/L).

Objective 2: To optimise the bioreactor production conditions using RSM.

Phase 3: Aim 3: Evaluation of the microbial resistance of the isolates to TCS and TCC including their ability to produce bioflocculants in the presence of these contaminants (antimicrobial compounds).

Objective 1: To assess whether the isolates are able to produce bioflocculants in the presence of antimicrobial agents normally found in sanitation chemicals used in PSW (TCC and TCS).

Objective 2: To comparatively analyse the growth of the microorganisms in the presence of TCC and TCS.

Objective 3: To perform an antimicrobial activity test to assess whether the chosen isolates can dominate other species in the PSW by inhibiting their growth when used individually or as co-cultures.

Phase 4: Aim 4: To design a bench-scale bioflocculant-supported DAFs and assess its efficiency in removing suspended solids, proteins and FOG from PSW. The objectives of this part of the study were primarily to:

Objective 1: Evaluate the efficiency of the bioflocculant-supported DAFs in the removal of TSS, protein and FOG from PSW.

Objective 2: Compare the efficiency of the bioflocculant DAFs with those in which chemical flocculants are used, including a conventional DAFs (control), in order to come to a definite

conclusion as to whether the use of bioflocculants is an appropriate strategy for a PSW pre-treatment process to reduce TSS, protein and FOG.

1.6 Significance of study

Physical, chemical and biological treatments have been applied to PSW for the removal of pollutants. Although these treatments have been used, FOG remains a significant problem in biological treatment systems where activated sludge is used. These solids (FOG) build-up and clog piping systems, resulting in system failure. Cleaning of clogged piping systems increases operational cost in WWTPs. This necessitates the implementation of a pre-treatment system that can assist in the reduction of FOG, as well as of proteins. Although, DAFs with synthetic flocculants have been used in pre-treatment systems, the use of chemicals is deemed harmful to the environment owing to residual chemicals containing sludge formed during such pre-treatment of the wastewater. This is the first study to assess a DAFs from this perspective.

1.7 Delineation of study

This study did not quantify the residual sugar content and other organic compounds in the wastewater used. Similarly, microbial and flocculation kinetics were not studied, that is, the system was not modelled. Furthermore, DAF optimisation, including the economic feasibility of the proposed system, was also not studied. All these studies are to be researched in subsequent studies.

CHAPTER 2

LITERATURE REVIEW

CHAPTER 2
LITERATURE REVIEW

2.1 Introduction: Wastewater

According to the South African water act, no. 36 of 1998, section 21 (f and h), wastewater can be defined as any water that contains waste or water that has been in contact with waste material. Furthermore, wastewater can also be defined as any industrial discharge that contains 10% (v/v) of the industrial water that is discharged in the process of manufacturing goods. In general terms, wastewater is the untreated water generated from domestic, commercial, institutional and industrial areas. This wastewater is made up of 99.9% water and 0.1% solids (dissolved and suspended solids) (Pescod, 1992). In attempting to treat this water, most or all suspended solids must be removed. This water is characterised by a foul odour which smells like fish (amines), rotten eggs (sulphides, ethyl and methyl), decayed cabbage (organic sulphides), rotten cabbage (skatole/3-methylindole) and faecal matter (Rabah, 2010). It is also characterised by a temperature averaging 16 °C, with minimal dissolved oxygen in warm water compared with cold water. At ambient temperature, microbial growth proliferation is prevalent. Additionally, wastewater also contains volatile solids, protein and FOG, which contribute to high concentrations of biochemical oxygen demand (BOD) and chemical oxygen demand (COD). Other constituents include nitrogen and phosphorous compounds which are the main causes of eutrophication, a process that results in ecological degradation in rivers and marine environments (Lapointe et al., 2015). Industrial wastewater is generated from different industries; hence its characteristics differ from one industry to another.

2.2 Composition of poultry slaughterhouse wastewater (PSW)

The most significant aspect of biotechnological applications in wastewater treatment plants is the reduction of environmental pollution. Wastewater raises socio-economic issues as its disposal costs escalate often and strict governmental regulations permit only wastewater with low contaminant concentrations to be discharged into freshwater streams (Rout, 2013). This is a problem that makes industrial wastewater treatment critical and costly. PSW contributes hugely to environmental pollution problems; it endangers human, animal and aquatic life if it is discharged untreated into clean water bodies (Yetilmezsoy & Sakar, 2008). The major constituents of this wastewater are suspended solids (SS), proteins, lipids, fats, faecal material, blood and carcass debris, including antimicrobial compounds used to clean the utensils and facilities for processing carcasses. Additionally, it contains high nutrient levels as well as inorganic materials such as nitrogen and phosphorus compounds (Chávez et al., 2005; Lo et al., 2005; Plumber et al., 2012).

This wastewater also comprises different types of microorganisms, which may be pathogenic or non-pathogenic. Microorganisms that survive the cleaning of utensils and surface areas are considered resistant to the antimicrobial agents used, which end up in the wastewater. To reduce pathogens in poultry products, large volumes of water and disinfectants are utilised (Avula et al., 2009). The BOD and COD for PSW are considerably higher than those observed for grey and black water generated from domestic anthropogenic activities (Rajakumar et al., 2011). These properties make PSW appropriate for biological treatment (De Nardi et al., 2008).

There are two types of antimicrobials mostly found in antimicrobial cleaning chemicals, namely triclosan (5-chloro-2-[2.4-dichloro-phenoxy]-phenol) (TCS) and (3, 4, 4-trichlorocarbanilide) (TCC). These two compounds are used as antimicrobials in cosmetics, detergents, and deodorants, etc. (Ying et al., 2007). Both these compounds are highly hydrophobic, with microorganisms that are found in PSW being either TCS or TCC resistant or tolerant. TCS and TCC are co-pollutants, with an octanol-water coefficient of (log K_{ow}) 5.0 (Balmer et al., 2004). They are largely soluble in fats; hence, they can easily be mobilised in wastewater containing high quantities of FOG. When they enter a cell, they inactivate the enzymes required by the cell to survive, thereby inhibiting the cell growth that results in cell lysis (Halden & Pauli, 2005). Owing to their similar structure, it is assumed that both TCS and TCC have a similar mechanism of deactivating microorganisms. These compounds are highly toxic to humans and other animals in the environment. Additionally, TCC is more persistent in the environment when compared with TCS. It was recently reported that triclosan could be degraded by the microorganisms under aerobic conditions (Chu & Metcalfe, 2007). Some microorganisms develop mechanisms to protect themselves against these contaminants so that they are able to survive, particularly when their concentrations are low. Hence, microorganisms that are able to resist TCS and TCC can be utilised to remediate PSW, using direct methods of remediation (bioremediation) and indirect methods such as the use of DAFs, for FOG reduction in WWTPs influent.

Suspended solids are colloids that are negatively charged. They repel one another; hence, they are free floating in wastewater (Laurent, 1995), whereas lipids are organic molecules (such as oil, fats, lipids grease, fatty acids, and waxes), all which contain neither a negative nor a positive charge. They are said to be non-polar, hydrophobic and only soluble in non-polar substances. Water is denser than most lipids; hence, these lipids float in the wastewater. The presence of these molecules in PSW results in the inefficient operation of biological

treatment processes since they reduce the molecular biomass transfer of essential nutrients and also cause foam formation; thus, they need to be removed prior to the primary treatment stage in WWTP (Chipasa & Mędrzycka, 2006).

As previously mentioned, in aerobic treatments, oil and grease decrease the rate at which dissolved oxygen is transferred, while in anaerobic treatment systems, lipids elevate the quantity of actinomycetes *Nocardia amarae* causing scum formation including foaming, which leads to poor sludge activity, therefore decreasing the treatment efficiency of the WWTP (Jeganathan et al., 2006). There is a great need for the removal of particulates and organic matter from the poultry processing plant wastewater prior to both the treatment and discharge of such wastewater to other water bodies.

2.3 Constituents of the poultry slaughterhouse wastewater collected

The PSW used in this study was collected from a slaughterhouse located in the Western Cape province, South Africa. The characteristics of the wastewater are summarised in Table 2.1, which lists averaged values of parameters that were quantified. All measurements were performed using Standard Methods (APHA, 2005). The PSW was characterised by high BOD_5 (1250 mg/L), FOG (406 mg/L), and TSS (413 mg/L), including proteins (70 mg/L) as indicated in Table 2.1. Furthermore, Table 2.2 lists PSW from several studies used for comparative analyses during the duration of this study.

Table 2.1: Characteristics of the poultry slaughterhouse wastewater from an industrial slaughterhouse in the Western Cape, South Africa

Parameter	Unit	Poultry slaughterhouse wastewater	
		Range	Average
pH	-	6.4-6.32	6.88
tCOD	mg/L	1829-1900	1865
sCOD	mg/L	948-1500	1224
BOD_5	mg/L	-	1250
Ammonia	mg/L	50-70	120
TP	mg/L	15.6-20	17.8
FOG	mg/L	380-416	406
TDS	mg/L	378-622	500
TSS	mg/L	346-480	413
Soluble proteins	mg/L	56-84	70

Table 2.2: Characteristics of poultry slaughterhouse wastewater from literature reviewed

Parameter	Unit	Poultry slaughterhouse waste water	
		Range	Average
pH	-	6.5-7.0	6.75
TCOD	mg/L	750-1890	1320
SCOD	mg/L	595-1526	972
BOD_5	mg/L	750-1890	1320
Ammonia	mg/L	20-68	44
TP	mg/L	33-128	80.5
FOG	mg/L	1800-1500	1650
TDS	mg/L	372-936	654
TSS	mg/L	300-950	625
Soluble proteins	mg/L	0-368	184
References			
Basitere et al., 2016, Debik & Coskun, 2009; Del Nery et al., 2007; Kobya et al., 2006; Rajakumar et al., 2011.			

2.4 Local wastewater legislation

According to the National Water Act No. 36 of 1998 (South African Department of Water Affairs, 1998), abattoir wastewater can be described as wastewater that contains contaminants such as blood, urine, faeces, fats, animal trimmings, and carcass stomach contents. The discharge of such wastewater in South Africa is regulated under the National Water Act, No. 36 (1998) and aimed at protecting the quality of raw water other than seawater. Water discharge permission can be obtained through the use of water-use licences (Section 21 f of the Act). Section 19 of the Act is also important as it lists remedial actions aimed at pollution prevention. To protect fresh water sources from toxic spillages, Section 20 of the Act is also useful. Procedures to prevent and remediate the wastewater to meet the discharged standards are documented in a water use authorisation process, but for these processes to be put in place, Sections 19 and 20 of the Water Act have to be applied.

Furthermore, the South African National Water Act, No. 36 of 1998 recommends the remediation of water so that wastewater can be recycled, re-used and discharged into municipal WWTPs, provided the municipal discharge standards are met. The wastewater generated by poultry slaughterhouses is highly contaminated. It is recommended that a wastewater treatment system be used, so that the treated water can be recycled or discharged only if it meets the prescribed discharge standards (South African Department of Water and Sanitation, 2001). This means that wastewater can be discharged to a municipal sewer after partial treatment only if the municipal by-law standards are met, that is, COD 5000mg/L, SS 1000mg/L, FOG 400 mg/L and pH 5.5-12 (City of Cape Town, 2006). The wastewater discharged into the municipal sewers must meet these standards to avoid high

discharge fee rates. In South Africa, abattoirs mostly discharge their wastewater into municipal sewers, but the discharge cost escalates based on the quality of the untreated wastewater from the processing facility. The slaughterhouses in most cases do not meet the wastewater municipal by-law discharge standards since their wastewater has a high content of FOG and suspended solids. *In-situ* remediation processes are then required to decrease the above-mentioned constituents. Therefore, treatment processes and standards have to be implemented, while these processes have to be both environmentally friendly and cost effective.

2.5 Poultry slaughterhouse wastewater treatment process

PSW treatment systems consist of four consecutive steps and all of these steps are crucial in the wastewater treatment process. In the pre-treatment steps, suspended solids are reduced, that is, the primary step removes suspended particulate matter, reducing the BOD by 20 to 30%. The secondary step involves the removal of other organic matter which enhances the BOD reduction by 90 to 95%, thus eliminating toxic pollutants. In aerobic treatment systems, dissolved organic matter is consumed to generate biomass and carbon dioxide. When such a system operates under optimum conditions, the microorganisms are able to form biofilms, but under minimal dissolved oxygen conditions, the microorganisms' ability to form biofilms is reduced, resulting in the bulking of sludge. Tertiary treatment systems are not usually applied, because these processes are expensive. They are only applied when the water to be recycled is highly contaminated and/or it is to be used for human consumption. For most cases, the tertiary treatment systems reduce nitrogen and phosphorous, which are the main constituents of eutrophication (Cloern et al., 2007). This study focused mainly on the pre-treatment stage, that is, DAFs aided by bioflocculants. Figure 2.1 illustrates a process used in the treatment of PSW.

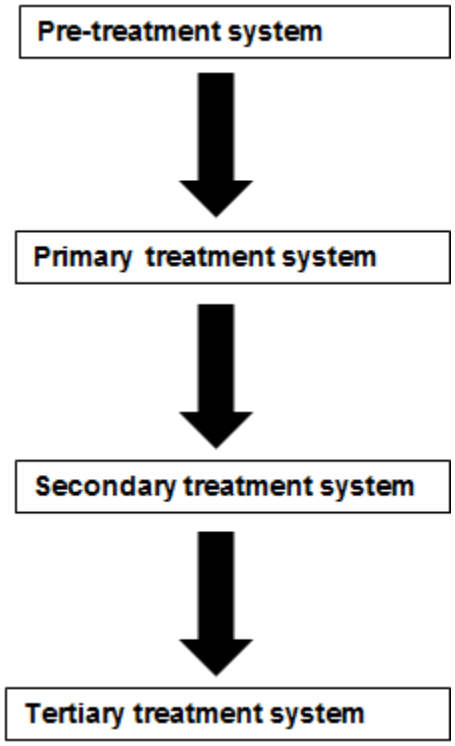

Figure 2.1: Poultry slaughterhouse wastewater treatment process

2.6 Pre-treatment in wastewater treatment plants

In most WWTPs, pre-treatment systems are used to remove suspended solids. In PSW, this process is particularly significant as it is used for the removal of FOG, and proteins, as well as suspended solids. Pre-treatment systems generally used include screening, flotation, settling tanks, and equaliser catch basins. Screens are responsible for the removal of solids such as feathers, hair and carcass debris resulting from the slaughtering process. These screens have strainers that are classified as fine and coarse strainers. The coarse screen strainer aperture size ranges from 6 to 25mm, while that of the fine strainer screen is 6mm in diameter. The coarse screen is usually used prior to the application of a fine screen. Coarse screens remove larger solids, while the fine screen is used to remove finer solids. There are different types of screens, namely, static screens, rotary drum screens, brushes, and vibrating screens. Catch basins and settling tanks are used to remove FOG, with denser suspended solids settling by gravity. Solids heavier than water sinks to the bottom of the settling tank, while grease floats to the surface of the wastewater subsequent to removal by skimmers. These pre-treatment systems reduce suspended solids by 50 to 70% (Mittal, 2006).

2.7 Flocculation

Flocculation is a process whereby mixing is applied and microflocs move closer together, forming macroflocs. The particle size increases as more microflocs bind, forming macroflocs.

As the microflocs come together to form macroflocs, they adsorb to other suspended solids, leading to the formation of larger flocs (Li et al., 2014). The size of the flocs is increased as pneumatic mixing is applied, while increased collisions between microflocs further facilitate the adherence of these particles to one another. This process can be enhanced through the addition of polymers such as flocculants. These flocculants will form bridges between the flocculants and the solids, increasing the surface tension, which in turn increases the floc size. This process requires and necessitates a suitable process control strategy, particularly as the mixing rate influences floc formation. This is because when high pressure is applied to the flocs, they may disintegrate, rendering the process ineffective. If pneumatic mixing is properly applied, the water becomes clear (Sharma et al., 2006).

2.8 Flocculants

There are three types of flocculants, namely, organic, inorganic and naturally occurring flocculants. Organic flocculants are polymeric chemicals such as polyacrylamide derivatives and polyethylene amine (Xia et al., 2008). Organic flocculants are biodegradable, meaning they are not harmful to the environment. Inorganic flocculants are chemical compounds such as aluminium sulphate and polyaluminium chloride; they are efficient when they are used in high concentrations (Xia et al., 2008). The addition of these chemicals at higher concentration leads to high quantities of non-biodegradable residue. These flocculants are susceptible to changes in wastewater pH (Brostow et al., 2009; Lee et al., 2014). On the other hand, synthetic chemical flocculants result in some health concerns for humans and animals, for example, aluminium-based flocculants have been determined to cause Alzheimer's disease, while acrylamide monomers have been determined to cause neurological and carcinogenic diseases (Xia et al., 2008). Naturally occurring flocculants are biological, mostly from yeast, bacteria, and fungi. In most cases, they are deemed to be environmentally friendly and are known as bioflocculants (Tang et al., 2014).

2.8.1 Organic flocculants

2.8.1.1 Synthetic flocculants

Organic flocculants are used in many industries for the removal of suspended particulate matter and organic material in wastewater (Ji et al., 2010). These flocculants have a competitive advantage of being efficient, even when they are added in lower concentrations (mg/L range). Organic flocculants are divided into two groups, namely, synthetic and naturally occurring flocculants (Dao et al., 2016). Synthetic flocculants are composed of monomers such as acrylamide, acrylic acid, diallyldimethylammonium chloride (DADMAC), as well as styrene (Sharma et al., 2006). They include polymers such as polyacrylamide (PAA),

polyethylene amine, and poly DADMAC (Singh et al., 2000). These polymers have a high molecular weight and contain multiple charges, thus making them efficient and economical (Lee et al., 2014). For example polyacrylamide reduces sludge bulking, it is pH insensitive, and can dissolve in water as well as in organic solvents (Wong et al., 2006; Huang et al., 2014). The constituents of the PAA are not biodegradable and they have been determined to be harmful to humans due to their carcinogenic and neurotoxicity properties (Wang et al., 2014; Roselet et al., 2015).

2.8.1.2 Naturally organic flocculants

These flocculants are derived from starch, cellulose, natural gums, mucilages and their derivatives. They are also polymers that are derived from monomers and they are largely characterised by the monomers from which they are constituted (Sharma et al., 2006). They have a high molecular weight, are non-toxic, biodegradable and comparatively effective when compared with synthetic flocculants (Zeng et al., 2008). One example of these flocculants is chitosan, which is a cationic polysaccharide that is derived from alkaline deacylation of chitin found in shellfish. It is soluble in water even at low pH, as well as in organic solvents. Furthermore, it is highly charged, has a high electrostatic potential and has high adsorption strength (Lee et al., 2014). It also eliminates bulking of sludge, although it can aggregate onto smaller suspended solids, limiting the formation of macroflocs when used in large quantities (Renault et al., 2009). It is also effective in lower doses. Another example of a naturally occurring organic flocculant is cellulose. Cellulose is a polysaccharide found in agricultural waste. This renders it economical and environmentally friendly. This polysaccharide has been found to be effective; it is also largely biodegradable (Kono & Kusumoto, 2014).

2.8.2 Inorganic flocculants

Inorganic flocculants are multivalent metal salts such as aluminium and iron salts. In order for them to be efficient, they are required in large quantities; when used in WWTP (on a large scale) they add to the operational costs of such facilities. They cause bulking of sludge, and they are sensitive to both pH and temperature. Additionally, they can only be used in certain systems, as they cannot remove finer colloidal particles from the wastewater (Sharma et al., 2006). Other examples include polyaluminium chloride (PAC), aluminium chloride, aluminium sulphate, and alum. For iron salts, ferric chloride and ferrous sulphates are included (Lee et al., 2014). Mostly, they carry a positive charge. When they are added to wastewater, they attach to the negatively charged suspended solids and form microflocs that can easily sediment (Suopajärvi et al., 2013). The major disadvantage of these flocculants is that the aluminium that the PAC is derived from can persist in the treated water, especially if the

effluent is discharged into river water used for irrigation and drinking, which can contribute to human health problems (Banks et al., 2006). Hence, it is important to design environmentally friendly processes in which inorganic salts are not used. It is therefore important to assess whether bioflocculants can be used.

2.9 Bioflocculants

Bioflocculants are safe, biodegradable polymers produced by a variety of microorganisms. They are shear, temperature and pH sensitive. They can be produced from organic waste, further contributing to the reduction of pollution as most organic waste is discarded into landfills (Bolto, 2005). These flocculants are produced by bacteria, fungi and algae as by-products of cellular growth. These microorganisms seem to produce extracellular polymeric substances such as polysaccharide chains, polypeptides, polyglycans and proteins, which have flocculation activity.

They are environmentally safe, although hampered by the uneconomical processes for their production, reducing their appeal for use in wastewater treatment processes. They are biocompatible with other biological processes because of their non-hazardous properties (Li et al., 2009). These polymers can be used in several industries, such as wastewater treatment, food processing, and cosmetics. These flocculants can be used to enhance the efficiency of DAFs for PSW treatment.

2.9.1 Bioflocculant properties

Bioflocculants are extracellular polymeric substances that possess flocculation properties. These polymers have been employed in a variety of industries, such as drinking-water purification and wastewater treatment, including fermentations. These flocculants have an advantage over synthetic flocculants, as they contain properties that make them highly efficient and mostly preferable. They have functional groups such as hydroxyl, carboxyl and amine functional groups (Wang et al., 2011). These functional groups are important for adsorption. The carboxyl group is negatively charged and acts as a binding site for metal ions, including suspended solids (Okaiyeto et al., 2016). The amino and carboxylic groups contain a strong attractive force that overcomes repulsion forces and neutralises the positive charge of any suspended solids or metal ions present in the wastewater to be treated (Yue et al., 2006). Adsorption efficiency is dependent on the number of carboxyl and hydroxyl groups, that is, functional sites present in the bioflocculants. Furthermore, bioflocculants contain glycoprotein groups that contain adsorption abilities compared with those that contain

protein functional groups, which provide an assortment of attachment sites compared with proteins (Okaiyeto et al., 2016).

In a controlled environment, bacteria increase owing to nutrient availability. However, in the absence of nutrients, secondary metabolites such as bioflocculants (glycoproteins and proteins) are produced as the microorganisms try to mobilise nutrients (More et al., 2014). When these secondary metabolites are utilised in wastewater treatment, they deactivate deflocculation enzymes (Sheng et al., 2010), and the microorganisms present in the wastewater utilise some of the deflocculation enzymes as a nutrient source; hence, their accumulation is reduced, effectively improving the flocculation of suspended solids.

The hydrophobicity and hydrophilicity of a flocculant is vital as the hydrophobic site acts as an attachment site for organic contaminants. Both sites are important because they greatly influence flocculation activity. Overall, in WWTP, the flocculation activity of these bioflocculants is influenced by many factors, such as dosage and presence of cations, as well as pH and temperature.

2.9.2 Bioflocculation mechanism

Bioflocculation mechanisms are not yet well understood but can be defined by using two mechanisms, namely, bridging and charge neutralisation (Wang et al., 2011). In a biological system, the bioflocculants form flocs that are similar to fibres, in a manner that facilitates the adsorption of particles, thus moving them together to form fibre looking aggregates (Li et al., 2008). The efficiency of the bridging mechanism depends on the molecular weight and the net charge of the bioflocculant, the type of suspended particulate matter, and the ionic strength of the suspension, as well as mixing (Yuan et al., 2011). Bioflocculants with a high molecular weight contain numerous functional groups, thereby having multiple attachment sites resulting in efficient bridging (Zhang et al., 2010). The suspended solids attaches to the active binding site of the bioflocculant while the non binding site of the bioflocculant is situated in the wastewater (Yim et al., 2007). If the biopolymer is available in abundance in the wastewater, it destabilises the repulsive forces of the particulate matter by surface saturation (Li et al., 2008).

In wastewater, particles move continuously owing to Brownian motion and electrostatic repulsion forces that are larger than Van der Waals attraction forces; this makes it impossible for the particles to attach to one another and settle. For the particles to settle, a bioflocculant that contains a negative charge has to be added to the wastewater to neutralise the charge of the suspended particles (Salehizadeh & Shojaosadati, 2001; Lachhwani, 2005).

2.10 Chemical removal of suspended solids and lipids: Chemical coagulation

In this process, a coagulant is added into the wastewater to remove suspended solids and lipids. The coagulation mechanism is to destabilise particle matter charges. Coagulants usually have opposite charges to the suspended solids present in the wastewater (De Nardi et al., 2008). When the charge is neutralised, the suspended solids present in the wastewater agglomerate, forming larger particles called macroflocs, which lead to the wastewater becoming clear. If the water does not become clear, this means that the charges of the particles are not neutralised; hence, the wastewater will remain turbid, which will then require the addition of a higher dosage of the coagulants. In this process, mixing speed is also important to enhance particle collision as inadequate mixing may result in an incomplete particle collision process.

2.11 Physical removal of suspended solids, lipids and proteinaceous matter

2.11.1 Settling tank

The effluent is introduced to the settling tank at a slow velocity. The larger particles settle to the bottom of the tank by gravity, whereas the smaller particles are decanted into the liquid overflow. The settling tank process has a hydraulic retention time of at least two hours on average. This method is used to physically reduce suspended solids and clarify the wastewater prior to it being biologically treated. The suspended solids that settle to the bottom of the settling tank form a granular biomass that must be recovered frequently to prevent the formation of larger-sized solid clumps. These granules can then be used to further process the wastewater. Settling tanks, however, are inefficient in the removal of small suspended solids (Spellman, 2010).

2.11.2 Sieves or screens

Sieves or screens are used to separate solids from liquids, whereby the solids are separated according to their size. This method can be employed for solid sizes of up to 50µm and not for finer particles smaller than this (Richardson et al., 2002). If finer particles are prevalent in wastewater, the screens may be clogged and be inefficient. There are two types of screens, coarse and fine screens. The finer screens are made of a woven wire cloth, while the coarser screens are made of perforated plates. Industrial screens are used in series or in a parallel rod; they have circular or square apertures. The coarse screen may be washed continuously by high velocity water, which keeps the screen free from particles that can attach to the screen

while removing finer particles, which will flow with the washing water stream. For this to happen, pressure is sometimes applied.

It is important that the finer screens vibrate to declog finer particulate matter from the screens and to increase the efficiency of the system. These sieves and screens play a critical role in PSW treatment systems as a physical pre-treatment system, prior to the wastewater entering the primary biological treatment system. These screens remove feathers and other bigger solids that are present in the wastewater, with secondary screens removing other larger particles that may cause clogging in the system (Kiepper, 2009).

The screening method can be easily operated and it is an economical way to remove solids. There are three types of screens used in PSW treatment, namely, rotary, shaker, and bar screens. They can be classified according to the aperture size of the openings on the screen. Fine screens have apertures of 1500 to 6000 µm, whereas very fine screens have apertures of between 250 and 1500 µm. There are also micro screens that have aperture sizes of less than 250 µm (Vesilind, 2003). When fine and very fine screens are used, finer suspended solids can still persist in the wastewater; hence, 53 µm micro-sieves can be used as reported by Kiepper (2009). In this study, TSS was reduced by 27 to 37% by using micro screens. Such a process is labour intensive because the sieves have to be shaken to remove solids and to reduce clogging.

2.11.3 Sedimentation of coarse and fine suspended solids

Sedimentation is a method whereby suspended solids and wastewater are separated by gravity according to the size and density of the solids (Richardson et al., 2002). The primary aim of this method is to reduce the turbidity of the wastewater (Spagni, 2012). An efficient sedimentation process is often required to maintain WWTP efficiency. This method cannot be used for the separation of emulsions or any material that does not settle. It cannot also be used for very dense materials that settle rapidly.

2.11.4 Filtration

Filtration is a physical process for the separation of solids and fluids by applying pressure to a barrier with pores for the fluid to pass through, while the solids that are bigger than the pore size are retained. The fluid that passes through the filter is called a filtrate. The filtrate may be contaminated with solids smaller than the pore size of the filter. The main purpose of filtration is to remove suspended solids with hydrophobic matter such as FOG, thereby

reducing the efficiency of the filter. There is a difference between sieves and filters. Sieves only contain one layer that retains bigger particulate matter, whereas filters are mostly made up of multiple membrane layers or cartridges that have different pore sizes between 0.1 and 0.5µm (Gupta et al., 2012). The disadvantage of this method is that solids retained in the filter may form a filter cake that clogs the filter, resulting in the inefficiency of the operation; hence, filters have to be cleaned frequently.

This method is used in wastewater treatment in collaboration with other wastewater treatment systems. There are different types of filtration systems, such as ultra-filtration (0.01 to 0.1µm), nano-filtration, micro-filtration (0.1 to 10µm), etc. (Bialas et al., 2014). The type of membranes used in filtration can also be used to remove proteins. Yordanov (2010) used ultrafiltration for the removal of suspended solids as well as fats, achieving 98 to 99% removal rates. Multiple filtration systems (ultra-filtration in combination with micro-filtration) can be used in a combination system for the recovery of high-value protein constituents with removal rates of 84% (Bialas et al. 2014). In a study by Lo et al. (2005), an ultrafiltration system made of polysulfone was used to retain protein, which resulted in membrane fouling. This technology is expensive and the operational energy requirements are high; therefore, it is not feasible for a developing country, such as South Africa.

2.11.5 Dissolved air flotation (DAF)

Flotation is a physical process used to separate liquids and suspended particulate matter smaller than 40µm in size, for example, FOG etc. (Zoubulis & Avranas, 2000). The efficiency of this technique relies on the type of material to be separated, and so hydrophobic materials are the ideal constituents to be separated by using this technique (Hanotu et al., 2012). Overall, it is used to separate only the material that cannot sediment to the bottom of a bioreactor and/or settling tank. Flotation can be operated in three ways: as dispersed air flotation, electrolytic flotation or dissolved air flotation. For separation of FOG and suspended solids, micro-bubbles are required; electrolytic flotation requires a costly electric rectifier to generate micro-bubbles, whereas dispersed air flotation generates larger bubbles of 1mm; hence, this study focused only on dissolved air flotation, which does not require costly capital including operational expenses and can generate the micro-bubbles required for effective suspended solids separation (Pandey et al., 2014).

DAF is a reactor whereby saturated air pressure is dissolved into the wastewater containing the particulates to be separated, and micro-bubbles are created. The micro-bubbles act as attachment sites whereby the suspended solids adhere to the bubble so that they float to the

wastewater surface subsequent to being skimmed off mechanically (Yap et al., 2014). For DAF, bubble size is crucial, and the required bubble size should be smaller than 100µm, i.e. micro-bubbles (Rodrigues & Rubio, 2007). These types of bubbles rise more easily compared with larger bubbles that cause hydraulic disturbances a, reducing micro-flocs collisions required for flocculation. To produce these types of bubbles, saturation pressure pumped into the system must be suitable for the purpose (Alemayehu, 2010).

The concentration of the bubbles also has an effect on the collision of the suspended particles and bubble agglomeration. Pressure applied into the system is directly proportional to bubble concentration and size, as long as the pressure is above the required threshold. The bubble size, diameter and agglomeration will remain constant in the system by using an appropriate diffuser. Generally, to saturate the entire wastewater present in the system cannot be achieved at a pressure below 350 kPa. Full bubble saturation can only be achieved at a pressure above 355 kPa (Al-Shamrani et al., 2002). However, this is not always the case because in this study the PSW was fully saturated and micro bubbles were generated at a pressure of 6 kPa.

2.11.5.1 Conventional dissolved air flotation (DAF)

This type of DAF is operated with water and pressurised air only. There are no supporting flocculants added to the system. This type of DAF is mostly used in wastewater containing low-density particles. The microscopic bubbles in DAF attach to the solids, elevating the solids' buoyancy so that they rise to the water surface where they are removed physically (Behin & Bahrami, 2012). Hydrophobic materials are suited to separation, using this system. On a bench scale, the air used must also be pressurised and supplied directly to the flotation tank; however, on a pilot scale, a small portion of the wastewater can be recycled using semi-saturated air prior to its entering the tank: a technique used to improve the efficiency of the system. This portion of water is then combined with the rest of the influent prior to its entering the flotation tank (Bondelind, 2016).

DAFs design relies highly on the rate of sparging, as well as the quantity of solids present in the wastewater to be treated. This is called the air-solid ratio. This is determined by quantifying the ratio of the force of air introduced and the weight of solids in the unprocessed wastewater. The relationship between the air-solid ratio (A:S) can be calculated using Eq. 2.1:

$$\mathrm{A}:S = RC_a/X_O[\left(\frac{10.3+P}{10.3}\right)f - 1] \quad , \qquad (2.1)$$

Where A:S = air-solid ratio Kg air released/ Kg of dried solids applied,

X_o = feed-solid concentration (mg/L),

C_a = soluble air concentration at one atmosphere pressure (mg/L),

R = recirculation ratio,

P = gauge pressure (Kg/ cm^2) Pa, and

f = pressurisation system efficiency assumed to be 0.82.

In a study conducted by Mittal (2006), a conventional DAF was also determined to reduce tCOD by 35%.

2.12 Physico-chemical removal of suspended solids and lipids

2.12.1 Electrocoagulation

Suspended solids and lipids can also be removed by electrocoagulation (EC). This process is more efficient when the wastewater has high conductivity. Since PSW has a relatively high conductivity, this characteristic will make it amenable to EC treatment (Bayar et al., 2011). This process involves multiple reactions that occur simultaneously. The anodes are made up of aluminium or iron with the generation of hydrogen gas occurring in the cathode. The cathode can either be made of the same material as the anode, or can be made of stainless steel. In the anode, the metal ions are driven to the water surface, and in the cathode, water is hydrolysed into hydrogen gas and hydroxyl (OH) groups (Arroyo et al., 2009). The electrons flow freely from the cathode to the anode. This destabilises the surface charges on suspended solids and FOG. The metal ion form complexes with the OH groups, forming large flocs that include metal residues and other contaminants. Additionally, suspended solids as well as FOG present in the wastewater can be reduced owing to the destabilisation of the charges that will reduce the adherence to the wall of the bioreactor. Finally, the hydrogen gas produced assists in pneumatically lifting the flocs to the wastewater surface.

The following reactions, Eq. 2.2 and 2.3, simply describe EC reactions whereby metals (Al^{3+}) are deposited on the anode, while in the cathode hydrogen gas is generated (Guzmán et al., 2012):

Anode: $Al_{(s)} \rightarrow {Al^{3+}}_{(s)} + 3e^-$ (2.2)

Cathode: $3H_2O + 3e^- \rightarrow 1.5H_{2(g)} + 3{OH^-}_{(aq)}$ (2.3)

Monomeric species such as $Al(OH)^{2+}$, $Al(OH)_2^{+}$ and polymeric species such as $Al_6(OH)_{15}^{3+}$, $Al_7(OH)_{17}^{4+}$, $Al_8(OH)_{20}^{4+}$, are formed during the EC process. The aluminium hydroxide flocs act as adsorbents for other pollutants and thus eliminate them from the wastewater. During EC, minimal chemicals are added to the wastewater; hence, salt concentration in the treated wastewater does not escalate, in comparison with other processes whereby salts and chemicals are added to the wastewater. This results in a stable metallic sludge, compared with the sludge generated by chemical precipitation. Additionally, EC requires simple equipment and can be operated at smaller retention times, which simplify the operation. These characteristics contribute to reduced operational costs (Meunier et al., 2006). This process has previously been employed to treat wastewater from numerous industries, including wastewater from poultry slaughterhouses. In a study by Kobya et al. (2006), where an aluminium anode was used, 93 and 98% reductions in COD and FOG respectively, were achieved at pH 5–6, using a current density of 150 A/m^2 for 25 min.

Bayramoğlu et al. (2006) performed a similar study whereby two sacrificial anodes were compared, using COD removal efficiency as well as FOG removal as a basis for comparison at a current density of 150 A/m^2 at an initial pH of 3 for 25 min. These anodes were Al^{3+} and Fe^{3+} electrodes. The Al^{3+} electrode was determined to be more efficient, removing 93% of COD, whereas the Fe^{3+} electrode was more efficient in removing FOG, achieving results averaged at up to 98% (Bayramoğlu et al., 2006). In another study, where a mild steel electrode was used with a system operating at a lower current density of $0.3A/m^2$, comparable results were obtained after 60 to 90 min of treatment. For this, the highest efficiency achieved was only when the aluminium electrode was used as the anode with BOD removal being 86%, while COD removal was found to be 99%. Overall, the reduction of suspended solids and the improvement in the turbidity of the wastewater being treated was found to be 89% and 90%, respectively (Asselin et al., 2008).

Like many other treatment methods, this treatment also has its shortcomings, particularly when a low electrical current is used, as it culminates in low efficiencies, resulting in increased treatment periods. It also generates minute quantity of flocs. However, when the electrical current is high, the system functions optimally as a result of coagulation being rapid, with more flocs being formed. Also, as the high-energy consumption escalates, the system will not be economical (Bazrafshan et al., 2012). The electrode in the EC system also deteriorates owing to anode oxidation; therefore, it requires frequent replacement. Sometimes a thin layer may form on the electrodes, reducing gas production. Similarly, if the wastewater to be treated has a low conductivity, this system will not function optimally to treat wastewater that contains FOG.

2.12.2 Chemical dissolved air flotation

The major disadvantage of this type of DAF is related as to how to select an appropriate coagulant that will meet the operational requirements. The most significant part in chemical DAFs is to find a flocculant or coagulant that is easy to store and prepare, and which does not generate a residue. The efficiency of a DAF system depends on the characteristics of the wastewater to be treated. Although chemical flocculants enhance flotation, environmental implications have to be considered. The literature reviewed revealed that when a chemical DAF is utilised as a pre-treatment system, 63 to 95% of FOG can be removed (Massé & Masse, 2000). Chemical DAF can on average, remove up to 95% of total suspended solids, and the resultant residue is classified as an environmental contaminant. A variety of chemical coalescence agents have also been employed to enhance the efficiency of this system. These chemicals include alum and iron salts (Sahu & Chaudhari, 2013) which produce residue and add to the environmental burden caused by metallic ions, and end up in the receiving freshwater sources.

Inorganic polymers, such as polyaluminium chloride, and as well as other inorganic polymers, have also been used. Hence, inorganic polymers seem to be more advantageous than those of organic origin. When it comes to environmental health considerations, organic flocculants are less harmful compared with the toxic inorganic flocculants. Advantages related to inorganic salts are related to the wide range of pH in which they can be used. Similarly, they can function at low temperatures while producing a minute quantity of residue and have lower sludge dewaterability (Aguilar et al., 2002, 2005).

There are two types of organic polymers, namely, anionic and cationic polymers. Cationic polymers are positively charged; hence, they function optimally when the suspended particles to be removed are negatively charged as they neutralise the charge of the suspended solids. Both anionic and non-ionic polymers can be combined with cationic polymers as well as with inorganic salts to improve flocculation efficiency, thus enhancing the removal of suspended solids and FOG. When the above-mentioned chemicals are mixed, they can be added to the wastewater in lower doses, reducing the operational costs of the system. But when the wastewater to be treated contains high COD, it is advisable to add salt-based flocculants for the primary treatment stage, that is, DAFs, which can further increase the operational costs of WWTP.

De Nardi et al. (2008) used a chemically supported DAF, where 24 mg Al^{3+}/L PAC in combination with 1.5 mg/L anionic polymer were used in a PSW pre-treatment system having pressurised air. The system performed poorly, achieving only 15% suspended solids' removal from the wastewater, while only 8% of the FOG was removed. Furthermore, COD reduction of 6 to 22% as well as total phosphorus removal, was between 4 to 34%. In a study by Mittal (2006), only 67% tCOD and sCOD were removed by chemical DAFs. This highlights some of the deficiencies encountered when using a chemical DAFs.

2.13 Biological pre-treatment system: Bioflocculant supported dissolved air flotation

Bioflocculants can be added into a DAF tank to improve flocculation, whereby up to 10% (v/v) of the bioflocculant-producing culture supplemented as a mono or consortium of bioflocculant-producing microorganisms (grown over 48 to 90 h). The cultures and/or bioflocculants can be added to the wastewater entering the DAF tank. Since the suspended solids are negatively charged, they will repel one another. When the bioflocculants that contain opposite electrical charges are added to the DAF, they will adhere to the surface of the suspended solids, thus allowing them to attach to one another, forming flocs (Laurent, 1995). The microflocs attach to each other and form macroflocs, which grow larger and become heavier, resulting in their gravitational sedimentation at the bottom of the DAF. The buoyant particles, that is, smaller flocs, will float to the surface of the wastewater owing to upward movement of the micro-bubbles that are formed and to which other flocs adhere (Edzwald, 2007; Bahadori et al., 2013). Overall, there are minimal studies focusing on bioflocculant-supported DAF, herein referred to as BioDAFs.

2.14 Application of the dissolved air flotation (DAF) without additives

The dissolved air flotation technique has been used in a variety of industries to treat and clarify wastewater by removing pollutants such as FOG, proteins and other suspended solids from wastewaters. It has been used as a pre-treatment system for reducing the organic load in biological wastewater treatment systems. Applications of this method are such that the following can be achieved: thickening of bio-solids, water recovery and reuse, solids removal and solids-liquid separation (Rodrigues & Rubio, 2007). Numerous industries apply DAFs for these applications, particularly in the following industries: food processing, petroleum, pulp and paper, plastic recycling, and metal plating (Ross et al., 2000). Like all other physical separation methods, dissolved air flotation has some advantages and disadvantages, particularly when flocculation agents are not supplemented or added to such a system.

2.14.1 Advantages

A dissolved air flotation system can produce better water quality; it can be operated at high loading rates, using small facilities which have a small footprint; it can be started quickly and it is easy to operate and monitor; it thickens and produces large quantities of sludge including algae, resulting in excellent algae removal efficiencies (Zabel, 1985). Additionally, the separation process is rapid compared with that of sedimentation clarifiers; and the capital costs of a DAF unit are lower than those of sedimentation clarifiers (Wang et al., 2010).

2.14.2 Disadvantages

A dissolved air flotation process is generally not suited to treating highly turbid water; it is mechanically more complex than conventional gravity clarifiers and the DAF basin requires a protective cover to protect the floating layer from being removed by the sparging (Zabel, 1985). DAF consumes more energy than gravity clarifiers owing to the sparging requirements needed to provide air to the wastewater (Ross and Valentine, 2008). DAFs also require flocculants to assist in the removal of smaller colloidal particles. Hence, it is necessary to improve this system so that inexpensive flocculants can be used. If bioflocculants with a high flocculation activity are used, a decrease in the hydraulic retention time (HRT) of the system can be achieved, which will reduce energy requirements.

2.15 Biological removal of suspended solids and FOG from poultry slaughterhouse wastewater

Most PSW treatment systems use physical and physic-chemical treatment systems as their pre-primary treatment systems; hence, not much attention has been given to biological processes for the removal of both FOG and total suspended solids. This does not mean that biological processes are inefficient or uneconomical. This provides an opportunity for many scientists to research and to try to augment a physical separation process using products from biological processes. Hence, this study focused on a biologically augmented pre-treatment system i.e. Bio-DAFs. Biological systems are usually cheaper to operate compared with chemical and physical processes. This is because in biological systems, the microorganisms used can be isolated directly from the wastewater itself, rather than by purchasing expensive isolates and chemicals that might not be functional owing to the characteristics and pH of the wastewater, as well as temperature.

The microorganisms isolated directly from the wastewater have the competitive advantage of surviving in the same wastewater being treated. For wastewater containing usable nutrients,

the microorganisms are able to use some of the nutrients in the wastewater as their carbon and nitrogen source for growth, subsequent to biodegrading the contaminants, resulting in the wastewater's being in a less toxic state. Furthermore, the proliferation of microorganisms in such wastewater can enhance the isolates' ability to produce both their intracellular and extracellular polymeric products, such as bioflocculants, which are important in solid-liquid separation processes.

CHAPTER 3

MATERIALS AND METHODS

CHAPTER 3
MATERIALS AND METHODS

3.1 Phase 1 experiments

3.1.1 Microbial isolation and identification

The isolates were collected, using sterile swabs, from a poultry slaughterhouse wastewater discharge trough belonging to a commercial producer of poultry products (Cape Town, South Africa). Furthermore, biofilm samples were collected by scraping the biofilm attached to the discharge point. Nutrient agar plates were used to cultivate numerous isolates, using a streak plating technique, and the inoculated agar was incubated at 30 °C overnight, subsequent to reculturing onto freshly prepared nutrient agar plates for isolate purification. Agar plates with pure colonies were then stored at 4 °C and recultured when other experiments were conducted. DNA extraction was done using appropriate extraction kits (Promega) and the 16S rRNA was amplified using a forward primer 27F 50-AGAGTTTGATCATGGCTCAG-30 and a reverse primer 1492R 50-TACGGTTACCTTGTACGACTT-30. The polymerase chain reaction (PCR) thermocycler program was used, with the initial phase being at 94 °C for 2 min, while the denaturation step was at 95 °C for 40 s, subsequent to annealing and extension at 55 °C for 30 s and 72 °C for 1 min, respectively. The PCR products were sequenced at Stellenbosch University (Western Cape, South Africa). The 16S rRNA sequences obtained were compared with the readily available sequences in the Gene Bank (US National Library of Medicine Basic Local Alignment Search Tool [BLAST]) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The *C. aquatica* and *Bacillus* sp. BF-2 isolates obtained were deemed appropriate for use in this study and were similar to *C. aquatica* strain LMG 92370 (93%) and to *Bacillus* sp. gene accession number GQ344805.1 (96%), respectively.

3.2 Phase 1 and 2 experiments

3.2.1 Bioflocculant production and inoculum preparation media

The bioflocculant production medium, which was also used for inoculum preparation for the isolates, comprised (per 100 mL): peptone (0.5 g), $(NH_4)_2SO_4$ (0.2 g), yeast extract (0.1 g), $CaCl_2$ (0.7 g), NaCl (0.01 g), $MgSO_4.7H_2O$ (0.02 g), K_2HPO_4 (0.1 g) and glucose (0.1 g). This medium was used for Phase 1 of the experiments. The only difference between Phase 1 and Phase 2 experiments was that the medium used for Phase 2 was sterile PSW, which was filtered using 0.22 µm filter papers. The pH of the production medium was adjusted to various pH values ranging from 2.9 to 10, using 1 M NaOH/ 1 M HCL.

3.2.2 Experimental set-up: inoculum development and bioreactor set-up

Isolates growing on nutrient agar plates were inoculated into 50 mL of the medium contained in a 250 mL conical flask and the flask was incubated in a rotary shaker (121 rpm) at 30 °C for inoculum preparation. After 24 h of the inoculum cultivation, the pH of the inoculum broth was adjusted to a value at which the experiment was conducted, after which 10 mL was used as a seed culture to inoculate 90 mL of the bioflocculant production medium, making a 100 mL fermentation culture in 250 mL conical flasks. The 250 mL conical flask bioreactors were then incubated in a temperature-controlled shaking incubator (121 rpm), at different temperatures varying from 32.9 to 40 °C for 10 h for bioflocculant production, with sampling at 2 h intervals. These samples were used to quantify microbial growth by measuring the optical density of the fermentation broth using Jenway 7305 spectrophotometer at 660 nm. Thereafter, the sample broth was centrifuged at 4,000g for 30 min to sediment the cells and to recover the supernatant subsequently used for flocculation activity analyses.

3.2.3 Response surface methodology (RSM)

RSM was used to determine the optimum pH and temperature for the bioflocculant production process, using the isolates. The experiments were conducted in duplicate and the experiments were repeated for each run, with flocculation activity being used as the output variable. Design-Expert® software version 10 was used to generate conditions for experiments conducted. A two-factor, five-level, central composite design with 13 experiments was performed. A *t*-test was performed to assess statistical significance of the regression coefficients used in the quadratic model employed to predict bioflocculant production, thus activity, while a Fisher test (F) was used to ascertain the veracity of the model acquired to describe optimal process conditions for bioflocculation production. The coefficient of determination (R^2) was calculated to evaluate the adequacy of the model.

3.2.4 Partial determination of flocculation activity

Using a suspension of kaolin clay (4 g/L), flocculation activity was quantified according to the method described by Kurane et al. (1994) and Li et al. (2008) with minor modifications. A volume (3 mL) of a 1% (w/v) $CaCl_2$ solution and 2 mL of the sample were added to 100 mL of the kaolin suspension in a 250 mL conical flask, followed by swirling (5 s). This mixture was then allowed to stand for 5 min. The optical density of the clarified solution was measured using a UV/VIS spectrophotometer at 550 nm. The flocculation activity was determined as: flocculation activity (%) = {(A–B)/A}*100, where *A* and *B* were the optical density of the control and samples from different experiments, respectively.

3.2.5 Bioflocculant recovery and functional group quantification

The isolates were recultured using the aforementioned flocculation production media, followed by an extended incubation period, that is, 27 h at 121 rpm, optimum temperature and pH. After fermentation, the culture was centrifuged at 8,000 rpm for 15 min, collecting the supernatant for further processing. Cold ethanol (4 °C) was added to the supernatant, using a ratio of 2:1 (v/v), that is, alcohol: supernatant, followed by swirling the mixture to form a precipitate which was recovered using a bench-top centrifuge operated at 8,000 rpm for 3 min. This process was repeated three times to obtain significant quantities of the precipitate from each sample. The precipitate was rinsed using distilled water, and dialysed against distilled water overnight. Thereafter, the precipitate was vacuum dried. The recovered dried precipitate was analysed using a Fourier transform infrared spectrophotometer (Brucker Vector 33). A mass of 1mg of the dried bioflocculant sample was mixed with KBr, forming a pellet, which was pressed to form a disc, prior to analysis.

3.3 Phase 3 experiments

3.3.1 Triclosan (TCS), trichlorocarbanilide (TCC) resistant tests

The synthetic bioflocculant production media for the isolates were prepared as in Section 3.2.1. The media had a pH of 6.5. Additionally, TCS and TCC were added to the media to achieve concentrations of 18 and 55 mg/L. Since both TCS and TCC are hydrophobic, they were initially dissolved in 70% ethanol and 99% methanol, respectively. These two antimicrobial agents were added to bioflocculant production media, which was followed by autoclaving the media at 121 °C for 15 min to vaporise the alcohols. Thereafter, a Jones oxidation for primary and secondary alcohols method (Bowden et al., 1946) was used to determine whether the alcohols were totally removed from the media, and the results showed that the alcohols were not present. The media were then adjusted to a pH of 6.5 prior to cooling at room temperature. For inoculum preparation, the organisms were inoculated into 50 mL of media incubated in a rotary shaker operated at 121 rpm at a temperature of 32 °C for 24 h. The bioflocculant-producing media were inoculated with 1% (v/v) of the previously grown culture. The conical flasks were then incubated in the shaking incubator operated at 121 rpm at a temperature of 32 °C for 5 days for bioflocculant production, with sampling at 24 h intervals. Bacterial growth was determined using the spread plate technique after a series of dilutions, and the cell concentration was quantified as CFU/mL. The sample broth was centrifuged at 4000 g for 30 min, in order to sediment the cells to recover the supernatant used for the analysis of flocculation activity (Section 3.2.4).

3.3.2 Antimicrobial test against other microorganisms in PSW: agar well diffusion

Since both isolates were to be used in the PSW treatment system, an antimicrobial test was performed to see whether or not these isolates would survive in the presence of the microbial community present in the PSW. An agar well diffusion method was used (Boyanova et al., 2005). Mueller-Hinton agar plates were inoculated with the PSW; wells (6 to 8mm) were drilled using sterilised sterile swab holders. A 48 h culture of both *Bacillus* sp. BF-2 and *C. aquatica* was used as antagonistic microorganisms. As per the literature, 20 to 100 µL of the 48 h culture can be used to inoculate into the agar wells (Balouri et al., 2016). However, in this study only 10 µL of the isolates, both individually and as co-cultures, were added into the wells. The plates were prepared in duplicates, with each of the plates containing either a single isolate or a co-culture. These plates were incubated at 33 °C overnight, an optimum growth temperature for the isolates determined, using RSM. The isolate extracts and/ or extracellular by-products were expected to diffuse into the agar and prevent microbial growth of other microorganisms in the PSW and the clear zones of inhibition were measured (Appendix C).

3.4 Phase 4 experiments

3.4.1 Dissolved air flotation system design and operation

A dissolved air flotation system was designed (Fig 3.1). The DAF tank was designed to have a 2 L capacity. A Resun air pump (Ac 9906) was used to pump air into the DAF tank using silicone tubing. The silicon tubing that transfers air into the DAF tank was connected to four air diffusers to provide sufficient micro-bubble formation in the system. The air was pumped at 16000 Pa. PSW was pumped from the feed tank into the system using a Gilson® mini plus evolution pump at 0.11 mL/min and at an HRT of 12 days. The system contained skimmers to skim FOG, and proteins including suspended solids floating on the surface of the wastewater. The suspended solids skimmed off were washed down the DAF tank's side by gravity, using four openings in the system that collected spillages from the overflow. The effluent was pumped out of the tank using a Watson-Marlow 502 peristaltic pump at the same rate as the influent to a sampling container.

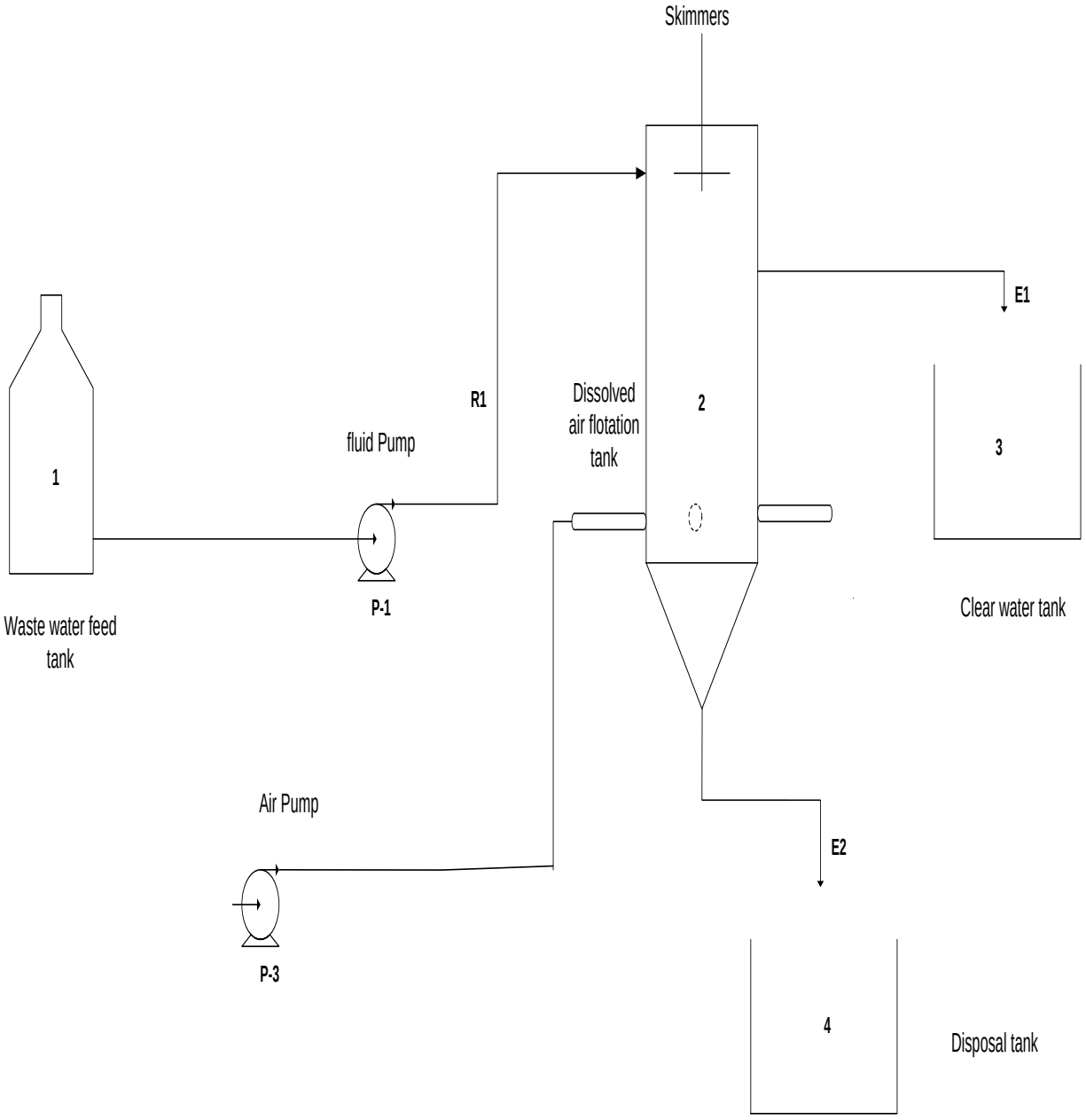

Figure 3.1: Schematic diagram of DAFs operational set-up

Table 3.1: Dissolved air flotation systems (reactor) specifications

Dimensions	Specification
Material:	Polyvinyl chloride (PVC)
Sparging pressure:	16000 Pa
Tank diameter:	16 cm
Tank length:	29.5 cm
Cone length:	11.5 cm

3.5 Sample collection for the dissolved air flotation

A volume (50 L) of the PSW was collected from a poultry slaughterhouse once every 2 weeks, using sterile 25 L polypropylene bottles. The wastewater was analysed for pH, temperature, total dissolved solids (TDS), total suspended solids (TSS), turbidity, FOG, protein concentration, tCOD and sCOD concentration. Prior to the addition of the wastewater to the DAFs, bioflocculants were produced, screened and characterised. For every 2 L of the collected wastewater, 20% (v/v) of the culture of bioflocculants produced by the isolates *C. aquatica* and *Bacillus* sp. BF-2 grown over 48 h was added to the PSW after which the air diffusers were switched on. The micro-bubbles created by the high pressure applied by sparging into the system elevated the solids to the PSW surface, where they were skimmed off by stainless-steel skimmers agitated at 66 rev/min using a Dragon Laboratory OS20-S agitator (Gulas et al., 1978; Zabel, 1985). The samples were collected periodically at 48 h intervals from the DAF system and analysed immediately.

3.6 Analytical methods

The total suspended solids (TSS) were measured using the ESS method 340.2 (Appendix D 3). The total dissolved solids (TDS) were analysed with the PSCTestr 35 multi- parameter (Wirsam Scientific, Malaysia). The turbidity of the samples was quantified by using a Turbidimeter TN-100 (Wirsam Scientific, Indonesia). The chemical oxygen demand (COD)(tCOD and sCOD) was quantified using Merck solutions: A (1.14679.0495) and B (1.14680.0495). A Merck Spectroquant® NOVA 60 was used to measure the COD concentrations (Appendix C1 and 2). The COD test is an indirect measure of organic compounds in wastewater, which operates on the basis that the strong oxidising agent (potassium dichromate) fully oxidises the organic matter in the presence of a 50% inorganic acid solution (sulphuric acid). FOG were analysed at the scientific services laboratory according to American Public Health Association (APHA) (2005) standards. Similarly, protein concentration was quantified using the Bradford's assay (Appendix E). Furthermore, the primary basis for the Bradford's assay is to detect the presence and concentration of proteins in a solution based on the absorbance shift, using a dye which changes colour from reddish to blue when proteins are present in a solution and the protein concentration was quantified at a wavelength of 595 nm using a SpextraMax microtiter plate reader (Molecular Devices, USA).

CHAPTER 4

RESULTS AND DISCUSSION

CHAPTER 4
RESULTS AND DISCUSSION

This chapter is divided into four sections for the different phases of the study:

- **Phase 1: Aim 1:** To isolate and identify microorganisms with bioflocculant-production potential; furthermore to characterise the bioflocculants produced by the isolates; moreover, to optimise the bioreactor conditions for the production of bioflocculants from the isolates producing flocculants with a higher activity using response surface methodology (RSM), in which synthetic nutrient medium was used.
- **Phase 2: Aim 2**: The aim of this part of the study was to produce bioflocculants by using PSW (a biological medium) as it is nutrient rich, subsequent to optimisation using RSM as done in Phase 1 of the study, using isolates deemed appropriate for bioflocculant production (Phase 1: Aim 1).
- **Phase 3: Aim 3:** The aim of this phase of the study was to evaluate the resistance of the microbial isolates to TCS and TCC including their ability to produce bioflocculants in the presence of these contaminants.
- **Phase 4: Aim 4:** The aim of this part of the study was to design a bench-scale bioflocculant supported DAFs and assesses its efficiency in removing suspended solids, proteins and FOG from PSW.

4.1 Phase 1: Isolation and identification of bioflocculant-producing isolates as well as characterisation of the bioflocculants produced by these isolates

4.1.1 Introduction

Flocculation is a process whereby mixing is applied and microflocs coalesce to form visible macroflocs. As the size of the flocs increases, they agglomerate to other suspended solids, leading to the formation of larger flocs (Li et al., 2014). During this process, suspended solids can be removed from the wastewater, thus the water becomes clear (Sharma et al., 2006). For flocculation to occur, flocculants must be introduced into the water to be treated. There are different types of flocculants, namely inorganic, naturally occurring, organic, and bioflocculants. Synthetic flocculants are used frequently in comparison with bioflocculants (Humudat et al., 2014). Some of the synthetic flocculants were found to be toxic to the environment, humans and animals. For example, alum, an inorganic flocculant, has been directly linked to Alzheimer's disease, while acrylamide monomers have been determined to cause neurological and carcinogenic diseases (Xia et al., 2008). Some organic flocculants are biodegradable, thus have minimal impact on environmental health. However, their usage in high concentrations leads to large quantities of residue, which creates additional problems

with regard to the disposal of such residue. Additionally, their functionality can be susceptible to changes in pH and temperature (Brostow et al., 2009).

Bioflocculants are safe biodegradable polymers produced by a variety of microorganisms. Therefore, they are shear, temperature and pH-sensitive. They can be produced from organic waste, further contributing to the reduction of pollution, as most organic waste is discarded into landfills, particularly in South Africa (Santos et al., 2013). These flocculants are produced by bacteria, fungi and algae as bio-products of cellular growth. They are environmentally safe, although their production is hampered by uneconomical processes designed to produce them, reducing their appeal for use in wastewater treatment processes. Furthermore, they are biocompatible with biological processes, because of their biodegradability and non-hazardous properties (Li et al., 2009).

4.1.2 Aim and objectives

The aim of this part of the study was to isolate and identify microorganisms with bioflocculant-production potential; furthermore, to characterise the bioflocculants produced by the isolates; moreover, to optimise the bioreactor conditions for the production of bioflocculants from the isolate-producing flocculants with a higher activity using response surface methodology (RSM), in which synthetic nutrient medium was used. This aim was achieved by the following objectives:

- Isolating and identifying microorganisms from the poultry slaughterhouse wastewater.
- Assessing whether these isolates could produce bioflocculant, using (4 g/L) kaolin clay to quantify bioflocculation activity.
- Choosing isolates that were highly competitive over the other isolates for optimisation of bioflocculant production by RSM.
- Determining the constituents of the produced bioflocculants by using FTIR.

4.1.3 Microbial isolation and identification

Of the microorganisms isolated from the slaughterhouse trough, only two microorganisms, that is, *C. aquatica* (BF-3) and *Bacillus* sp. (BF-2), were selected for bioflocculant production, as these isolates showed potential for the production of bioflocculants at moderate pH compared with the other isolates. The *C. aquatica* isolate morphology was rod-like, mucoid and white in colour. When it was viewed under a light microscope, its Gram test showed that it was a gram-negative coccus, whereas the *Bacillus* sp. BF-2 Gram reaction test, showed that it was a gram-positive rod and its morphology was mucoid and yellowish in colour

(Appendix A 1). Biochemical tests were performed prior to identifying the isolates, using 16S rRNA sequencing (Appendix B 1 and B 2). The 16S rRNA of the isolate BF-3 was sequenced and the results proved this strain was 93% similar to the *C. aquatica* strain LMG2370. The 16S rRNA of the strain BF-2 proved it was 96% similar to *Bacillus* sp. gene accession number GQ344805.1. Fig. 4.1 (a) and (b) illustrate the genomic DNA and PCR products of *C. aquatica* (BF-3). Fig. 4.1 (c) and (d) show genomic DNA and PCR gel products of *Bacillus* sp. BF-2, respectively (Appendix A 2). These isolates were utilised to produce bioflocculants under different conditions (Table 4.1)

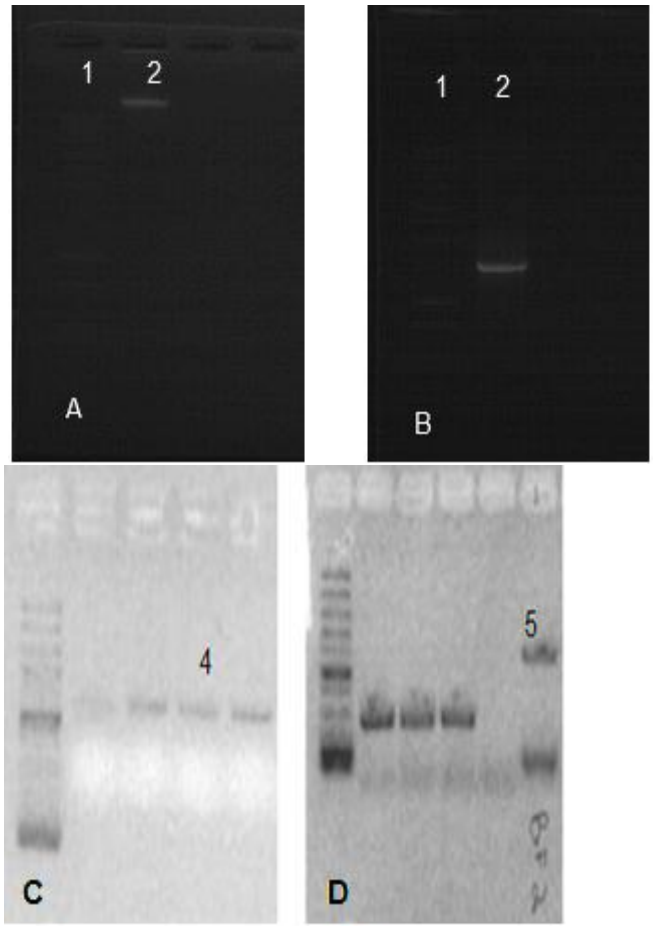

Figure 0.1: (A) Lane 1: molecular weight marker. Lane 2: Genomic DNA of *C. aquatica*. (B) PCR product - Lane: 2 *C. aquatica.* (C) Lane 4: Genomic DNA of *Bacillus* sp. BF-2 (D) Lane 5 PCR product of *Bacillus* sp. BF-2

4.1.4 Bioflocculants production, optimisation and characterisation

Two independent variables, temperature (range 34 to 39 °C) and pH (range 4 to 9), were evaluated to determine their effect on flocculation activity for bioflocculants produced from these isolates. The results depicted that moderate pH (6.5) and temperature lower than 34 °C, specifically 32.9 °C, had a positive effect on bioflocculant production by *C. aquatica*, which significantly influenced flocculation activity in a positive way. In general terms, as the

temperature increased, flocculation activity decreased; an observation similar to when the production medium was slightly acidic (pH 4) or alkaline (pH 9). Furthermore, the only temperature that was favourable for *Bacillus* sp. BF-2 to produce bioflocculants was 40 °C at a moderate pH (6.5). At temperatures (33–39°C), flocculation activity deteriorated. At an acidic pH (4) and alkaline pH (9), flocculation activity decreased gradually. Tables 4.2 and 4.3 enlist the ANOVA of the quadratic model used to simulate bioflocculant production, for *C. aquatica* and *Bacillus* sp. BF-2, respectively. Two independent variables, temperature (range 34 to 39 °C) and pH (range 4 to 9), were evaluated to determine their effect on flocculation activity.

Table 0.1: Experimental design matrix of independent variables, temperature (A) and pH (B) (*C. aquatica* and *Bacillus* sp. BF-2)

Run	Temperature (°C) (A)	pH (B)	*C. aquatica* Flocculation activity (%)	*Bacillus* sp. BF-*2* Flocculation activity (%)
1	39.0	9.0	28.4	23.2
2	36.5	6.5	90.9	32.2
3	36.5	6.5	90.9	32.2
4	32.9	6.5	93.8	6.60
5	34.0	9.0	68.4	46.8
6	36.5	6.5	90.9	32.2
7	36.5	2.9	0.0	38.9
8	36.5	6.5	90.9	32.2
9	34.0	4.0	73.4	0.07
10	40.0	6.5	67.0	88.3
11	39.0	4.0	18.2	0
12	36.5	6.5	90.9	32.2
13	36.5	10.0	85.2	33.5

The analysis of variance (ANOVA) for the quadratic model used to estimate bioflocculant production for *C. aquatica*, Eq. 4.1, indicated the suitability of the model to predict bioflocculant production, with the determination coefficient (R^2) of ~0.8. The significance of individual parameters in the quadratic model was determined using $p < 0.05$, with several parameters having a lesser influence in the prediction of bioflocculant production. Table 4.3 indicates that B, AB, and A^2 have negligible influence, thus Eq. 4.1 was reduced to Eq. 4.2.

$$Y = -1633.4 + 92.6A + 41.6B + 0.6AB - 1.4A^2 - 4.4B^2 \quad (4.1)$$

$$Y = -1633.4 + 92.6A - 4.4B^2 \quad (4.2)$$

Table 0.2: Analysis of variance (ANOVA) of the quadratic model parameters used to estimate bioflocculation production by isolate *C. aquatica*

Source	Sum of Squares	Degree of Freedom	Mean Square	*F* value	Prob > *F*
Model	9801.51	5	1960.30	5.52	0.0224
A	2216.36	1	2216.36	6.24	0.0411
B	1973.89	1	1973.89	5.56	0.0505
AB	58.98	1	58.98	0.17	0.6958
A^2	543.78	1	543.78	1.53	0.2558
B^2	5359.37	1	5359.37	15.10	0.0060
Residual	2485.28	7	355.04	-	-
Lack of Fit	2485.28	3	828.43	-	-

$R^2 = 0.7977$

Furthermore, the ANOVA for the quadratic model used to estimate bioflocculant production by the *Bacillus* sp. BF-2 (Eq. 4.3) indicated the unsuitability of the model to predict bioflocculant production, $R^2 \sim 0.3$. The significant parameters in the quadratic model were indicated by $p < 0.05$, and parameters that had a value higher than that were rendered insignificant in the quadratic model. Table 4.3 indicates that all the parameters were negligible; hence the model was unsuitable for the prediction bioflocculant production.

$$Y = 32.20 + 11.48A + 7.79B - 5.88AB + 1.55A^2 - 4.08B^2 \quad (4.3)$$

For this model, *Y* represents flocculation activity of the bioflocculants produced by the isolate *Bacillus* sp. BF-2, while *A* and *B* represent temperature and pH, respectively

Table 0.3: Analysis of variance (ANOVA) of the quadratic model parameters used to estimate bioflocculation production by isolate *Bacillus* sp. BF-2

Source	Sum of Squares	Degree of Freedom	Mean Square	*F* value	Prob > *F*
Model	1824.75	5	364.95	0.59	0.7120
A	1055.04	1	1055.04	1.70	0.2341
B	485.06	1	485.06	0.78	0.4066
AB	138.42	1	138.42	0.22	0.6516
A^2	16.67	1	16.67	0.027	0.8746
B^2	115.62	1	115.62	0.19	0.6794
Residual	4356.26	7	622.32	-	-
Lack of Fit	4356.26	3	1452.09	-	-

$R^2 = 0.2952$

RSM has been used to optimise bioprocess systems with the primary objective to optimise the bioreactor conditions so that the best response is obtained (Bezerra et al., 2008). There can be more than one response, which implies that a strategy must be put in place so that more than one response can be optimised (Oehlert, 2000; Ren et al., 2013). Fig. 4.2(a) shows the relationship between pH and temperature for the production of bioflocculants. The optimal production of bioflocculants was obtained at pH 6.5 and a temperature of 32.9 °C, with a flocculation activity of 93.8%. Bioflocculant production was clearly affected by temperature and pH. Xia et al. (2008) reported that the production rate of bioflocculants could be affected by many factors, such as agitation, temperature, pH of the production media, nitrogen and carbon sources, as well as other constituents of the growth media. Aljuboori et al. (2013) used *Aspergillus flavus* for bioflocculant production, achieving 91.6% flocculation activity, results which are similar to those achieved by the *C. aquatica* used in this study. Temperature and pH, which were both favourable for microbial growth, did not correspond to high bioflocculant production. While under normal circumstances, bioflocculants are produced during microbial growth (Buthelezi et al., 2010), flocculation activity was affected by changes in temperature and pH; hence, at an acidic pH, there was little or minimal flocculation activity, while at very alkaline pH and at high temperature, flocculation activity decreased.

Fig. 4.2 (b) shows the effect of temperature and pH on microbial growth rate during bioflocculant production, focusing on the specific growth rate of the isolate. When *C. aquatica* was grown at lower pH, the growth rate was reduced to 0.066 h^{-1} (Run 7), which affected the flocculation activity (Figures 4.2a and 4.2b). Additionally, a high flocculation activity was

observed for Run 4, while the corresponding growth rate was not the highest observed for the experiments, meaning that a high specific growth rate did not necessarily correspond to a high flocculation activity, a phenomenon which should be investigated further.

Fig. 4.2 (c) depicts the relationship between pH and temperature for the production of bioflocculants by the *Bacillus* sp. BF-2. Optimum production was obtained at a moderate pH of 6.5 and a high temperature of 40 °C with flocculation activity of 88.3%. This flocculation activity was lower compared with that of other flocculants produced by *Bacillus mucilaginosus*, which had 99.6% folocculation activity using kaolin clay (Deng et al., 2003). The *Bacillus* sp. BF-2 isolate flocculant production was affected by both temperature and pH. At a temperature between 32.9 and 39 °C, it produced minute quantities of bioflocculants, while at an acidic pH (4) and 39 °C, it produced minimal bioflocculants.

Fig. 4.2 (d) illustrates the specific growth rate of the microorganism that produced the flocculants. Similar to the *C. aquatica* cultures, the flocculation activity did not always correspond to the growth rate of the microorganism; at higher flocculation activity of 88.3%, the specific growth rate that was obtained was 0.86 h^{-1}, which was lower compared with the highest specific growth rate of 1.36 h^{-1} achieved, whereby flocculation activity of 46.8% was obtained. This meant that the bioflocculants were not always produced during the growth phase of BF-2, that is, they were also produced during the death phase as well, an indication that some bioflocculants might have been intracellular polymeric substances.

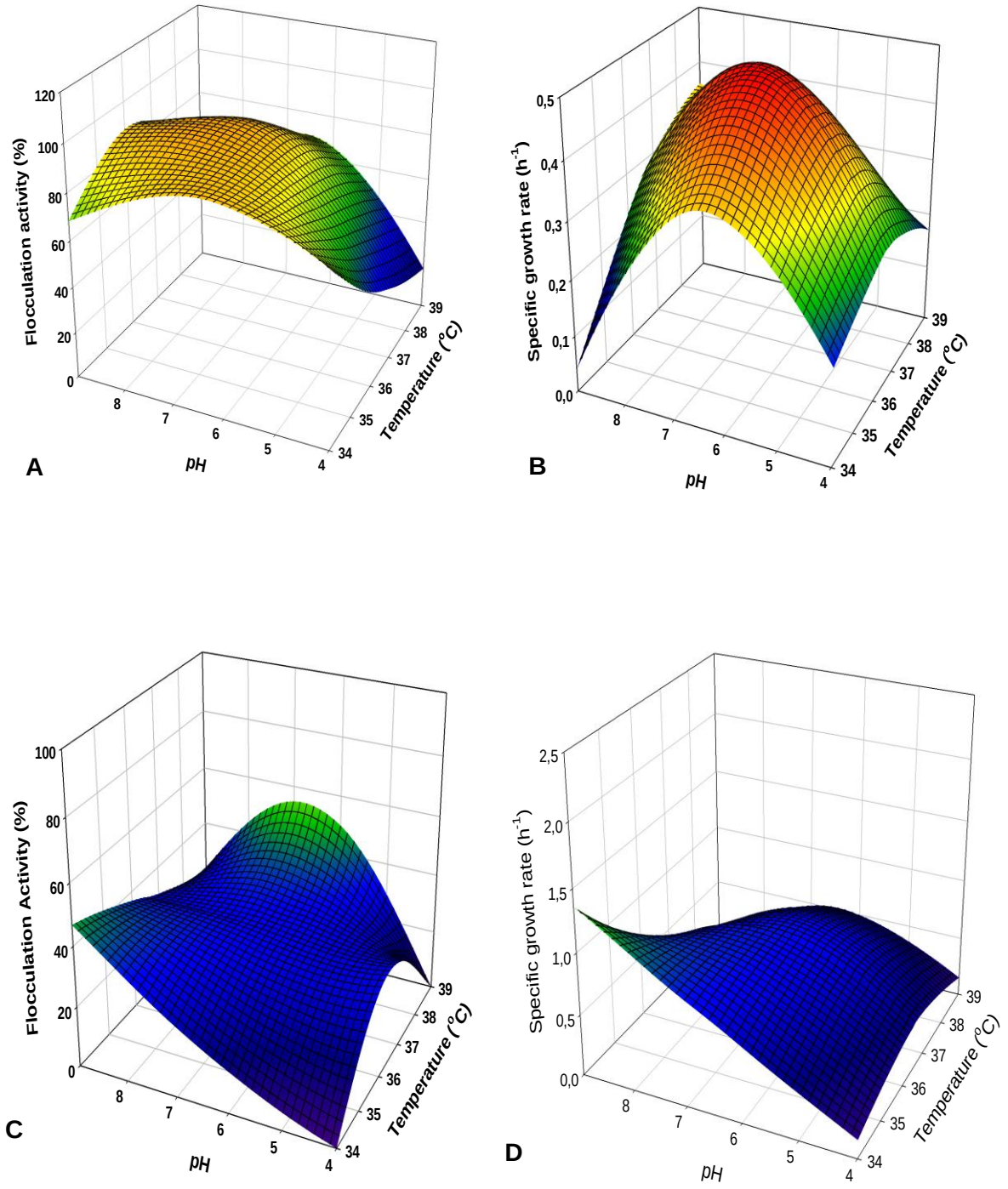

Figure 0.2: A representative graph showing the interaction of pH and temperature on: (A) and (C) flocculation activity of *C. aquatica* and *Bacillus* sp. BF-2, (B) and (D) the specific growth rate achieved for each of the experiments by *C. aquatica* and *Bacillus* sp. BF-2, respectively

The Fourier transform infrared spectrum (FTIR) of the purified bioflocculants that were produced by *C. aquatica*, highlighted on the spectrogram (Fig. 4.3), indicated several

functional groups similar to those observed for proteins. Several peaks were observed at 3,447, 1,654, 1,405, and 1,113 cm^{-1}, representing hydroxyl, carboxyl, alkane and amine functional groups, respectively. It was thus postulated that the bioflocculants under assessment are pH and temperature sensitive, resulting in their inability to facilitate flocculation activity at high temperatures and extremes of pH, as some of the functional groups might be sensitive to these adverse conditions. Temperature can limit the effectiveness of the bioflocculants when used for suspended solid removal from PSW, as such wastewater was previously determined to have a pH range of 6.5 to 8 (Basitere et al., 2016), with the lowest pH (6.5) of PSW indicated as having a positive effect on bioflocculation production.

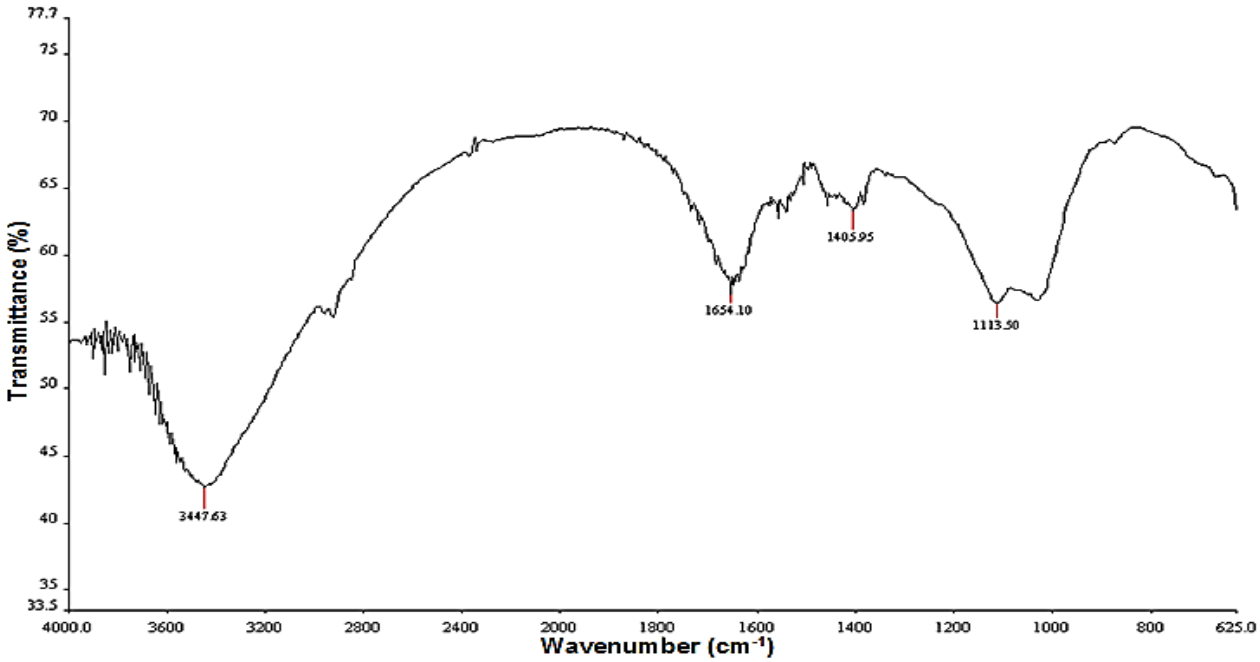

Figure 0.3: Fourier transform infrared spectrum (FTIR) spectrogram for the bioflocculants produced by the *C. aquatica* used in this study

Additionally, the FTIR of the purified bioflocculants from the *Bacillus* sp. BF-2 had several functional groups similar to those observed for polysaccharides. Several peaks were observed at 2962.62, 1657.48, 1470.55, 1363.93, and 796.12 cm^{-1}, indicating hydroxyl, carbonyl, alkane and alkyl halide functional groups, respectively; which were indicative of a polysaccharide-based bio-product. It was observed that the flocculant was tolerant to high temperatures, but was pH sensitive. This was because some of the functional groups were known to be pH sensitive, resulting in the isolate being unable to produce flocculants at low temperatures and moderate pH. pH sensitivity of the microorganism could limit its effectiveness when used as the primary isolate for flocculant production in PSW treatment systems.

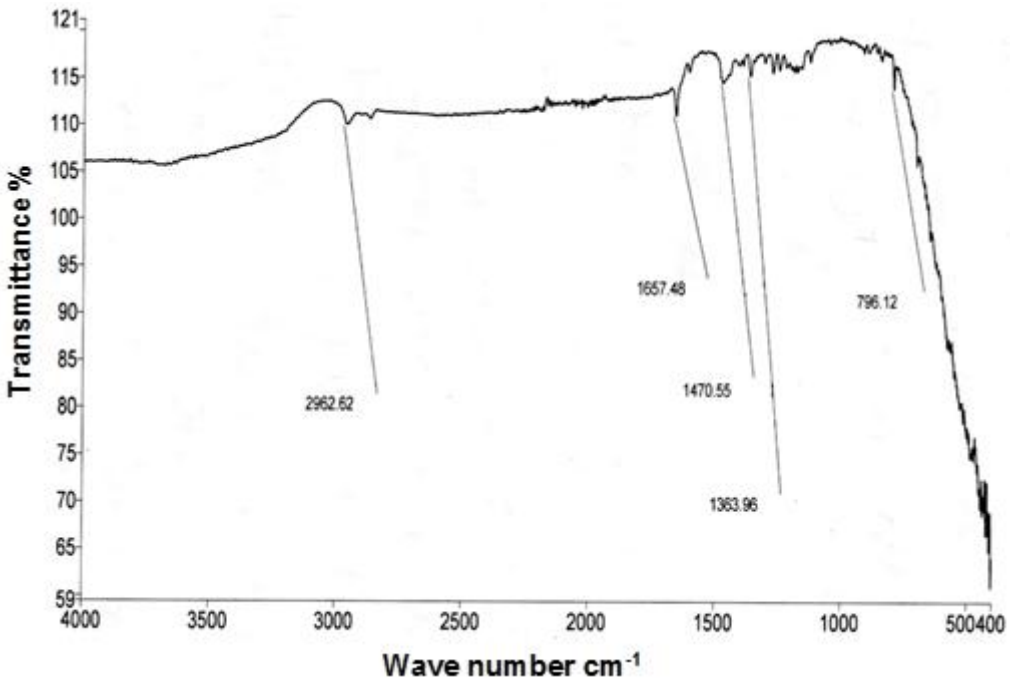

Figure 0.4: FTIR spectrogram for the bioflocculants produced by the *Bacillus* sp. BF-2 used in this study

4.1.5 Summary

PSW contains nutrients that are sufficient for microbial growth; moreover, the wastewater has microorganisms that can be harnessed to perform specific functions. Additionally, these microorganisms can grow either in planktonic (free floating) mode or sessile (attached) mode. This part of the study focused on the optimisation of bioflocculant production by quantifying flocculation activity determined using kaolin clay (4 g/L), using isolates obtained from the PSW. Subsequent to their identification and characterisation, six bacterial strains were initially isolated from the poultry slaughterhouse wastewater. Although all the isolated microorganisms produced bioflocculants under different conditions, that is, pH and temperature, the isolates that produced bioflocculants with a higher flocculation activity were isolate BF-3, identified to be *C. aquatica* and BF-2, a *Bacillus* sp. that achieved a flocculation activity of 93.8% at 32.9 °C, pH 6.5 and flocculation activity of 88.3% at 40 °C, pH 6.5, respectively. The FTIR analysis of the bioflocculants produced by the isolate *C. aquatica* showed the presence of hydroxyl, carboxyl, alkane and amine functional groups, an indication that the bioflocculants were protein constituents. The FTIR for bioflocculant-produced isolate *Bacillus* sp. BF-2 showed the presence of hydroxyl, carbonyl, alkane and alkyl halide functional groups, illustrating that the bioflocculants produced by this isolate were polysaccharide constituents. Overall, this suggested that the isolates might be suited to be co-cultured to improve their effectiveness in a bioflocculant-supported DAFs.

4.2 Phase 2: Assessing the poultry slaughterhouse wastewater isolates for their ability to grow and produce bioflocculants in the environment from which they were isolated

4.2.1 Introduction

PSW contains a high quantity of organic matter that can be broken down by the microorganism for energy and as a nutrient source. The PSW has a temperature of between 20 and 30 °C, which makes it an optimal microbial growth environment (Kundu et al., 2013). When the macro and micronutrients are present in such an environment, the microorganisms acquire energy to grow and reproduce as well as be enzymatically active. In a suitable habitat, microorganisms can break down the toxic wastewater constituents by using their metabolic functions. The PSW also contains contaminants that can make the environment unfavourable for microbial growth; however, under such conditions, microorganisms use their ability to adapt to the environment to grow and reproduce. Hence, when they are cultured in the PSW, they are able to flourish and produce the bioflocculants required for the purpose of this study. Additionally, the microorganisms can grow and produce bioflocculants even under stressful culture conditions with limited nutrients; moreover, unfavourable culture conditions seem to enhance bioflocculant production (Zulkeflee et al., 2016).

4.2.2 Aim and objectives

The aim of this phase of the study was to produce bioflocculants, using PSW (a biological medium), as it is nutrient rich, subsequent to optimisation using RSM as done in Phase 1 of the study, using isolates deemed appropriate for bioflocculant production (Phase 1: Aim 1). The objectives therefore were to:

- culture the isolates in a biological medium and assess the isolates' ability to produce bioflocculants with higher activity, using (4g/L) kaolin clay; and
- optimise the bioreactor production conditions using RSM.

Since *C. aquatica* and *Bacillus* sp. BF-2 bioflocculants were to be used for FOG removal from the PSW, both isolates had to be assessed whether they could grow in the wastewater being pre-treated. The PSW was sterilised using 0.2µm filter papers. This was done to remove other microorganisms in the PSW such as to avoid the production of bioflocculants by the microbial community present in the PSW. The experimental design matrix for the experiments is shown in Table 4.4.

Since, bioflocculants are secondary metabolites produced by microorganisms; their production can be affected by many parameters, such as micro and macromolecules in the nutrient medium, including pH, temperature, as well as the dissolved oxygen (Li et al., 2009). When the PSW was used, it was observed that the wastewater on its own contained contaminants that limited both microbial growth and bioflocculants production. When both isolates were grown under the same culture conditions, using a synthetic medium, they performed better in the synthetic medium than when the PSW was used. This was largely attributed to the limited nutrient availability in the wastewater, as it was different from the synthetic medium nutrient constituents, meaning that the culturing media, including conditions, were unfavourable. Overall, *C. aquatica* (Table 4.4) performed better in this medium (PSW) compared with *Bacillus* sp. BF-2.

Table 0.4: Experimental design matrix of independent variables, temperature (A) and pH (B) for *C. aquatica* and *Bacillus* sp. BF-2

Run	Temperature (°C) (A)	pH (B)	*C. aquatica* Flocculation activity (%)	*Bacillus* sp. BF-2 Flocculation activity (%)
1	39.0	9.0	35.6	16.5
2	36.5	6.5	34.6	24.8
3	36.5	6.5	34.6	24.8
4	32.9	6.5	0.0	0.0
5	34.0	9.0	14.4	0.0
6	36.5	6.5	34.6	24.8
7	36.5	2.9	25.6	0.0
8	36.5	6.5	34.6	24.8
9	34.0	4.0	56.9	43.8
10	40.0	6.5	53.9	4.84
11	39.0	4.0	11.8	8.29
12	36.5	6.5	34.6	24.8
13	36.5	10.0	24.2	35.9

The highest flocculation activity in this medium was achieved at a temperature of 34 °C, pH 4 (Run 9), while for the *Bacillus* sp. BF-2, the highest bioflocculant production was at 34 °C and pH 4. There was minimal flocculation activity at 32.96 and pH 6.5 for both isolates, conditions suitable when synthetic medium was used. For Run 10, where *Bacillus* sp. BF-2 isolate had the highest flocculation activity in synthetic medium, there was only flocculation activity of 4.84%, which was much lower than the 88.3% achieved using synthetic medium. This was directly attributed to the limited nutrients present in the PSW.

The ANOVA for the quadratic model used to estimate bioflocculant production (Eq. 4.4), indicated that the model was not suitable to predict bioflocculant production ($R^2 \sim 0.5$). The significant parameters in the quadratic model were indicated by $p < 0.05$, whereas parameters

that had minimal influence on the model were neglected and did not have an influence on the quadratic model. Eq. 4.5 indicates that the parameter that had a significant contribution was AB; therefore Eq. 4.4 was reduced to Eq. 4.5:

$$Y = 34.60 + 6.56A - 2.58\,B + 16.58\,AB - 2.87A^2 - 3.91B^2 \quad (4.4)$$

$$Y = 34.60 + 16.58\,AB \quad (4.5)$$

This model was similar to that used to described bioflocculation production by the *Bacillus* sp. BF-2 isolate (Table 4.6 Eq 4.6 and 4.7).

Table 0.5: Analysis of variance (ANOVA) of the quadratic model parameters used to estimate bioflocculation production by isolate *C. aquatica*

Source	Sum of Squares	Degree of Freedom	Mean Square	*F* value	Probe > *F*
Model	1642.08	5	328.42	1.74	0.2430
A	343.87	1	343.87	1.83	0.2187
B	53.10	1	53.10	0.28	0.6119
AB	1099.25	1	1099.25	5.84	0.0464
A^2	57.23	1	57.23	0.30	0.5986
B^2	106.52	1	106.52	0.57	0.4766
Residual	1318.48	7	188.35	-	-
Lack of Fit	1318.48	3	439.49	-	-

$R^2 = 0.5547$

$$Y = 24.80 - 1.56A + 171\,B + 12.91AB - 9.42A^2 - 1.86B^2 \quad (4.6)$$

$$Y = 24.80 + 12.91AB \quad (4.7)$$

Table 0.6: Analysis of variance (ANOVA) of the quadratic model parameters used to estimate bioflocculation production by isolate *Bacillus* sp. BF-2

Source	Sum of Squares	Degree of Freedom	Mean Square	*F* value	Prob > *F*
Model	1330.47	5	266.09	1.69	0.2544
A	19.58	1	19.58	0.12	0.7347
B	23.46	1	23.46	0.15	0.7109
AB	667.19	1	667.19	4.24	0.0785
A^2	617.46	1	617.46	3.92	0.0881
B^2	23.97	1	23.97	0.15	0.7079
Residual	1101.42	7	157.35	-	-
Lack of Fit	1101.42	3	367.14	-	-

$R^2 = 0.5471$

Fig. 4.5 (a) illustrates that the contaminants in the wastewater affected the bioflocculation production by *C. aquatica* drastically, as the flocculation activity of this isolate decreased from a maximum of 93.8% at a pH 6.5, 32.96 °C achieved in a synthetic medium to the maximum of 56.9% at an acidic pH 4 and 34 °C. This was assumed to be due to the lack of adaptation by the microorganism, to the unfavourable environment. The PSW contains a high quantity of salts and other contaminants that can decrease flocculation production.

Furthermore, the growth rate of the microorganism decreased gradually at alkaline pH. Additionally, the growth rate of the microorganism was directly associated with flocculation activity in this case, a phenomenon not observed when synthetic medium was used. Flocculation activity can either be directly proportional to growth, or it can be growth independent (More et al., 2014). Since the wastewater contains other contaminants, the rate of cell lysis might have been high, reducing bioflocculant production, and the bioflocculants that were present in the medium might have been utilised by viable microorganisms as previously indicated by More et al. (2014). Additionally, the presence of bioflocculant-degrading enzymes might have been high, as the production of these enzymes is activated when the nutrients in the medium used are low.

Fig 4.5 (b) depicts the specific growth rate of *C. aquatica.* The highest specific growth rate was observed at run 9 was 0.4 h^{-1} which corresponded with the highest flocculation activity of 56.9 at pH 4 and 34 °C. This meant that the flocculation activity was influenced by the growth rate

of the microorganism in the biological medium. For run 9 at pH 6.5 and 32.9 °C, the growth rate was 0.1 h^{-1} but the flocculation activity was minute. This showed that flocculation activity can depend on microbial growth under certain conditions with researchers suggesting that this might not be the case (More et al., 2014).

Fig.4.5 (c) shows the flocculation activity from *Bacillus* sp. BF-2 cultures was also determined to be low. The salinity of the wastewater was approximately 372.5 mg/L. If salinity of the PSW is high, dissolved oxygen in the wastewater depreciates. The *Bacillus* sp. BF-2 is an aerobe/facultative anaerobe; it requires dissolved oxygen to grow and to be able to produce bioflocculants. The highest flocculation activity (Fig. 4.5 c) obtained was 43.8%. The absence of bioflocculant production did not mean that the microorganism was non-viable, but was rather an indication of an unfavourable environment (Zulkeflee et al., 2016).

Fig. 4.5 (d) depicts the specific growth rate of *Bacillus* sp. BF-2. As noted previously, the specific growth rate did not correlate with the flocculation activity obtained. The specific growth rate for the highest flocculation activity was lower than the flocculation activity obtained when the growth rate was higher. This was in accordance with the observation of More et al. (2014), that flocculation activity is not always dependent on microbial growth. This meant that flocculation production can also occur even in the absence of higher microbial growth, where cells in the medium lyse, owing to nutrient deprivation and high toxicant levels. In certain cases biomass releases intracellular materials to the surrounding medium because of cellular autolysis. This means that bioflocculants are not always extracellular polymeric structures/exopolymeric substances. They can be embedded intracellularly and can be released by dead or non-viable cells.

The highest flocculation activity achieved in biological medium was 56.9% for *C. aquatica* and 43.8% for *Bacillus* sp. BF-2; this proved that these microorganisms can produce sufficient bioflocculants, even when growing in PSW, which suggested the isolates are suitable for use in a PSW-treating DAF system. Although, salinity and other PSW parameters are majorly influential in limiting microbial growth, bactericidal TCS and TCC, which are present in most PSW, were determined to be major contributors to microbial growth limitations. A toxicity experiment therefore had to be carried out to prove whether the isolates selected because of their bioflocculant-production capabilities were capable of producing bioflocculants in the presence of TCS and TCC, which was the next phase of the study. It was also necessary to ascertain the dominance of the isolates in the presence of other organisms in PSW to be treated, a task which could be achieved by using the agar well diffusion test.

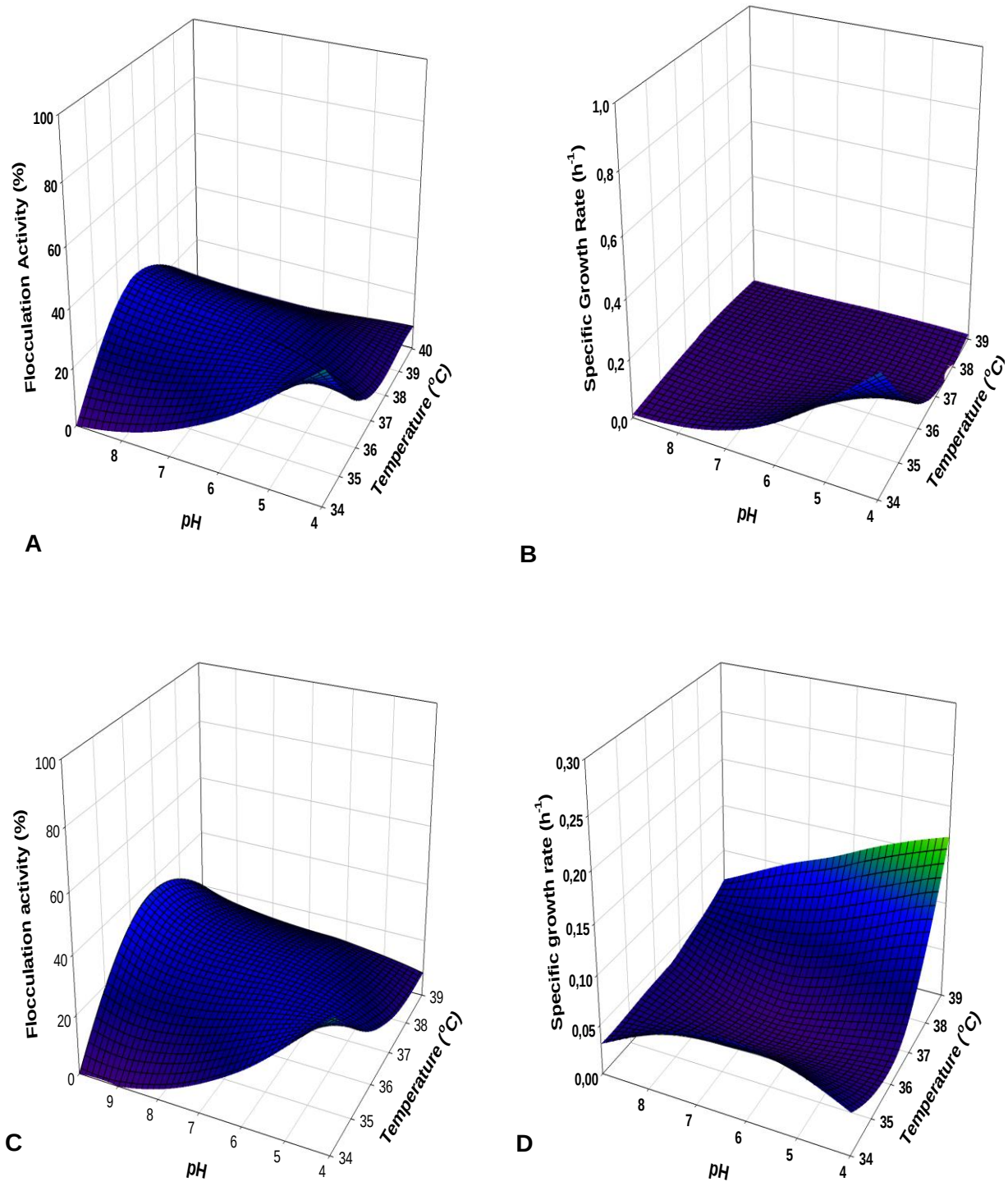

Figure 0.5: A representative graph showing the influence of pH and temperature on: (A) and (C) flocculation activity of *C. aquatica* including *Bacillus* sp. BF-2, (B) and (D) the specific growth rate achieved for each of the experiments for *C. aquatica* and *Bacillus sp.* BF-2, respectively, using PSW as a growth/production medium

4.2.3 Summary

PSW contains nutrients and contaminants that may be utilised by the microorganism for growth and survival. This part of the study mainly focused on the optimisation of bioflocculant production by *C. aquatica* and *Bacillus* sp. BF-2, cultured in the PSW for bioflocculant production under varying conditions. These two isolates had the potential to produce bioflocculants with sufficient flocculation activity when they were cultured in PSW, with the isolate *C. aquatica* cultures achieving a flocculation activity of 57% at 34 °C and pH 4, while the cultures of the isolate *Bacillus* sp. BF-2 achieved a flocculation activity of 44% at 34 °C and pH 4. It was concluded that, *Bacillus* sp. BF-2 and *C. aquatica*, were able to produce bioflocculants even if they were cultured under a stressful environment. The results depicted that the optimum conditions could be adapted for use in a DAFs, although the isolates' susceptibility to TCS and TCC had to be investigated.

4.3 Phase 3: Triclosan-, trichlorocarbanilide-resistant microorganisms and their ability to produce bioflocculants for use in poultry slaughterhouse wastewater treatment

4.3.1 Introduction

Triclosan (TCS) (5-chloro-2-(2,4-dichlorophenoxy)-phenol) and trichlorocarbanilide (3,4,4-trichlorocarbanilide) (TCC) are bactericidal agents used in soaps, toothpaste and cleaning detergents (Coogan et al., 2007). These products contain 0.1 to 2% (w/v) TCC and TCS, but surface wastewater contains 9 ng/L to 7 µg/L for both contaminants (Zhao et al., 2010). These two co-contaminants are highly insoluble in water but their trace concentrations have been detected in wastewater treatment plants (Cha & Cupples, 2010). They are toxic to humans and animals and they dissolve in fats. Once they enter the human body, they inhibit some cellular functions (Halden & Pauli, 2005; Venkatesan et al., 2012). Recent studies have shown that these contaminants are prevalent in wastewater, surface water, sediment, human serum, and wild life. TCC is more persistent in the environment when compared with TCS. TCS can be degraded by the microorganisms under aerobic conditions (Chu & Metcalfe, 2007).

This part of the study focused on the ability of the microorganisms to produce bioflocculants in the presence of TCS and TCC, since both of these antimicrobial chemicals are present in PSW. TCS is a co-pollutant with TCC, and both of these chemicals are very toxic to the environment, while the latter is more persistent in the environment when compared with TCS. Hence, microorganisms that are able to resist TCS and TCC, and are able to produce bioflocculants in the presence of such co-toxicants, would perform better in a DAF PSW pre-

treatment system. Similarly, the isolates must be able to have antagonistic characteristics towards other organisms in the PSW, in order to sustain bioflocculation in a DAF system.

4.3.2 Aim and objectives

The aim of this part of the study was to evaluate the microbial resistance of the isolates, *C.aquatica* as well as *Bacillus* sp. BF-2 to TCS and TCC and their ability to produce bioflocculants in the presence of these contaminants. The objectives therefore were to:

- assess whether the isolates were able to produce bioflocculants in the presence of antimicrobial agents normally found in sanitation chemicals used in PSW (TCC and TCS);
- analyse and compare the growth of the microorganisms in the presence of TCC and TCS; and
- perform an antimicrobial activity test to assess whether the chosen isolates could dominate other species in the PSW by inhibiting their growth when used individually or as co-cultures.

4.3.3 Bioflocculants production in the presence of (TCS) and (TCC)

The microorganisms used in this study were *Bacillus* sp. BF-2 and *C. aquatica*, isolated from the PSW. Since these two isolates were to be used in PSW contaminated with TCS and TCC, further studies were required to determine if these organisms were able to survive under conditions in which high concentrations of TCS and TCC were prevalent, particularly as these toxicants were present in the PSW that was to be pre-treated using a DAF system.

Fig. 4.6 illustrates the flocculation activity of bioflocculants from *Bacillus* sp. (BF-2) and *C. aquatica* in the presence of TCS and TCC at the temperature of 32 ˚C and pH 6.5 conditions that were previously determined to be optimal for bioflocculant production using synthetic media. In Fig. 4.6 (a) and (b), the highest flocculation activity was achieved on day 5, and it was observed to be 92% for both isolates, respectively. Fig. 4.6 (c) also indicates the highest flocculation activity, 16.9%, achieved on day 3 at a concentration of 55 mg/L, which was in the presence of TCC; the flocculation activity obtained on day 3 decreased to 15.6% on day 5. This was because the isolate was affected by the length of exposure of the strain *Bacillus* sp. BF-2 to the contaminant. In Fig. 4.6 (d), the highest flocculation activity achieved at a concentration of 55 mg TCC/L (on day 5) was 49.5%, showing that the isolate *C. aquatica* was more resistant to TCC compared with the *Bacillus* sp. BF-2. Both these isolates were able to produce flocculants in the presence of both contaminants. Yu et al. (2015) suggested that a stressful environment enhances bioflocculant production, although a variety of microorganisms can respond differently in such conditions, particularly in the presence of TCS and TCC.

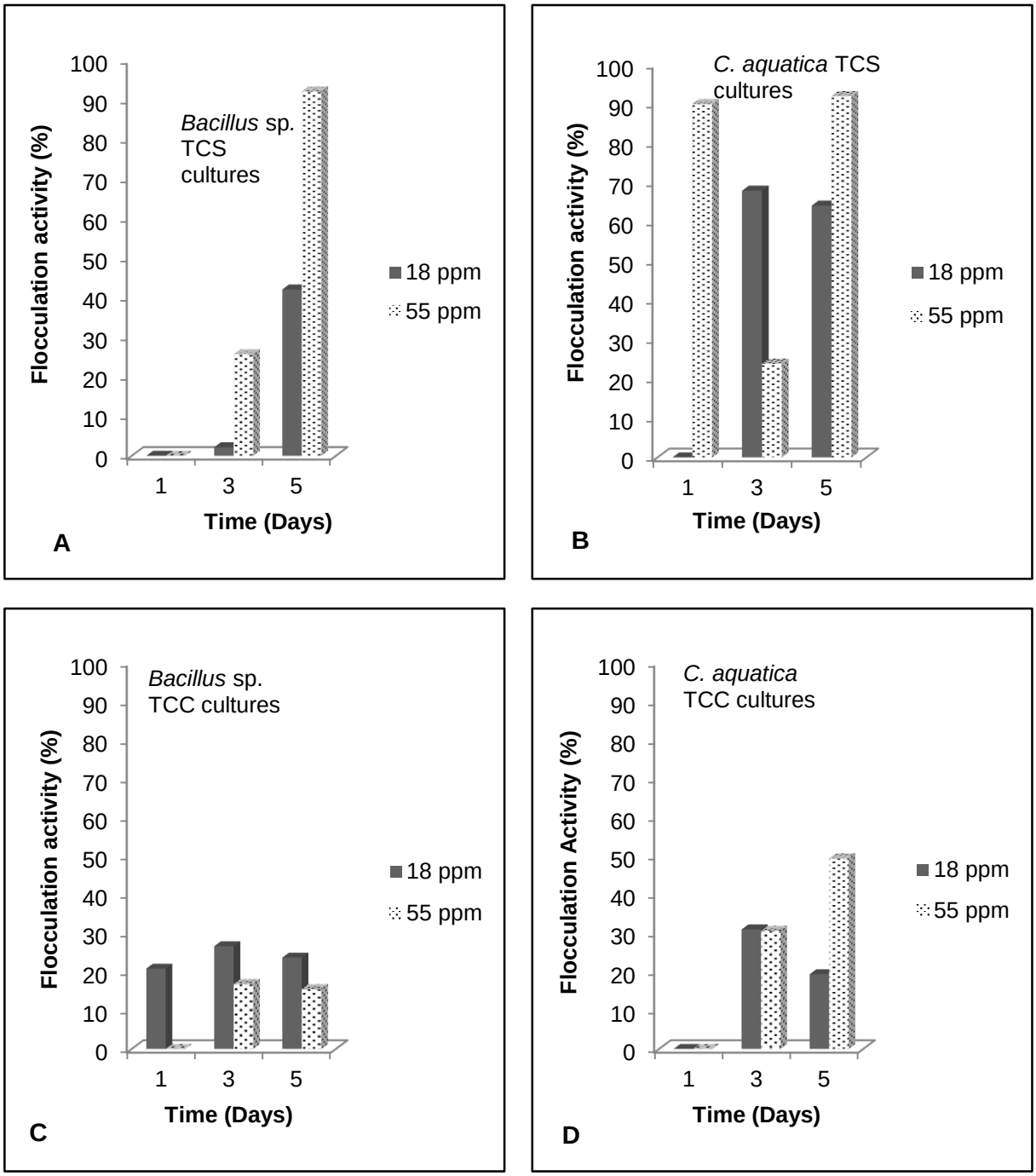

Figure 0.6: A representative graph showing flocculation activity of *Bacillus* sp. BF-2 in the presence of TCS at different concentrations of 18 ppm and 55 ppm (B) Flocculation activity of *C. aquatica.* In the presence of TCC at the aforementioned concentration (C) and (D) represent flocculation activity of the aforementioned species respectively in the presence of TCC at 18 ppm and 55 ppm

4.3.4 Microbial growth in the presence of the antimicrobials TCS and TCC

Fig. 4.7 represents the microbial growth profile (in CFU/mL) of *Bacillus* sp. BF-2 and *C. aquatica*. In Fig. 4.7 (a), the highest cell growth concentration was 280 x 10^6 CFU/mL for

Bacillus sp. BF-2 isolates growth was directly proportional to the flocculation activity of 92% at a concentration of 55 mg/L TCS. Fig. 4.7 (b) shows that the cell concentration was 246 x 10^6 CFU/mL for *C. aquatica.* In Fig. 4.7 (c), the highest growth was achieved on day 1, which was (100 x 10^6 CFU/mL). Fig. 4.7 (d) demonstrates minimal growth on day 3, with the flocculation activity being as low as 30.8%. This depicts that the flocculation activity of these microorganisms did not always correspond to the microbial growth concentration, suggesting that the extracellular accumulation of bioflocculants in the growth might have been due to autolysis, not biosynthesis (Kurane et al., 1986) because of cellular inhibition by TCS and TCC.

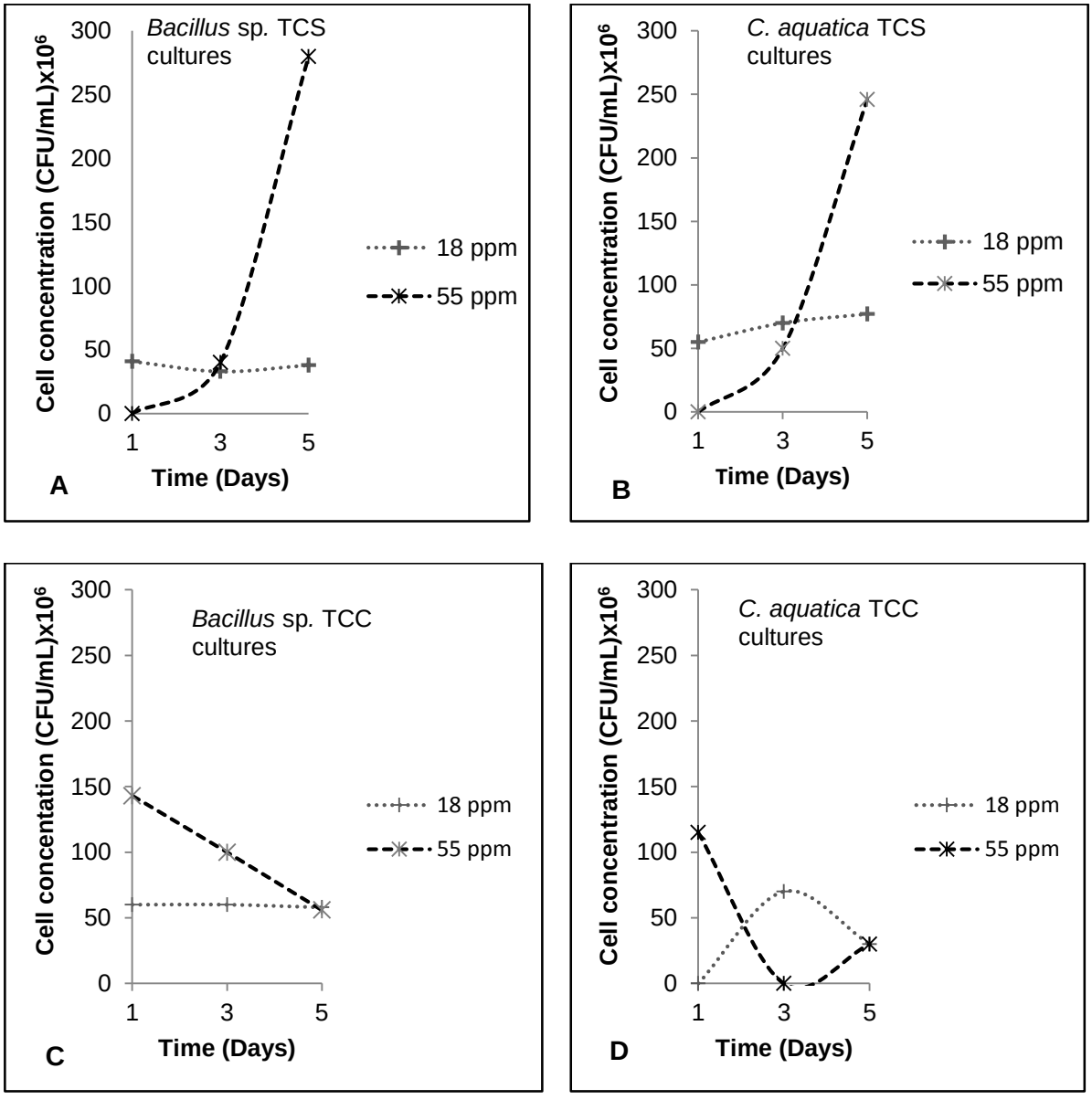

Figure 0.7: Graphical illustration of microbial cell concentration in CFU/mL in the presence of TCS and TCC at concentrations of 18 mg/L and 55 mg/L. (A) and (C) represent *Bacillus* sp. (BF-2) cell concentration in the presence of 18 mg/L TCS and TCC

respectively. (A) and (D) represents *C. aquatica* cell concentration in 18 mg/L TCS and TCC.

4.3.5 Antimicrobial tests against other microorganisms in PSW

Parameters that must be considered in a wastewater treatment system, is the makeup of the wastewater, competition between species in the wastewater and the species that are introduced into the wastewater to perform a certain function. The microorganisms have been tested for their ability to survive under extreme conditions and toxicants (such as TCS and TCC), which they have to either tolerate or degrade (Al-Gheethi et al., 2015). In this section, the microbial community present in the wastewater was cultured in the presence of the bioflocculant-producing microorganisms to observe whether the selected isolates were able to survive in the presence of the PSW microbial community.

The antimicrobial test was carried out using an agar well diffusion test (Appendix C). When both isolates were cultured as a co-culture, the clear zones were averaged at 10 mm, while for *C. aquatica* alone the clear zones of inhibition were merely 2 mm, with those of the *Bacillus* sp. BF-2 being 1 mm. The plates showed a higher clearance zone in which co-cultures were used, with individual isolates contributing much lower inhibition activity against microorganisms in PSW. Resistant bacteria survive in the presence of other microorganisms by destruction, neutralisation and inactivation. This study showed that these isolates better survived in the presence of the PSW organisms in co-cultures. This means that the isolates were growing competitively when compared with the other strains present in the PSW, which suggested that they could be able to survive in a DAF system as co-cultures in order to produce bioflocculants.

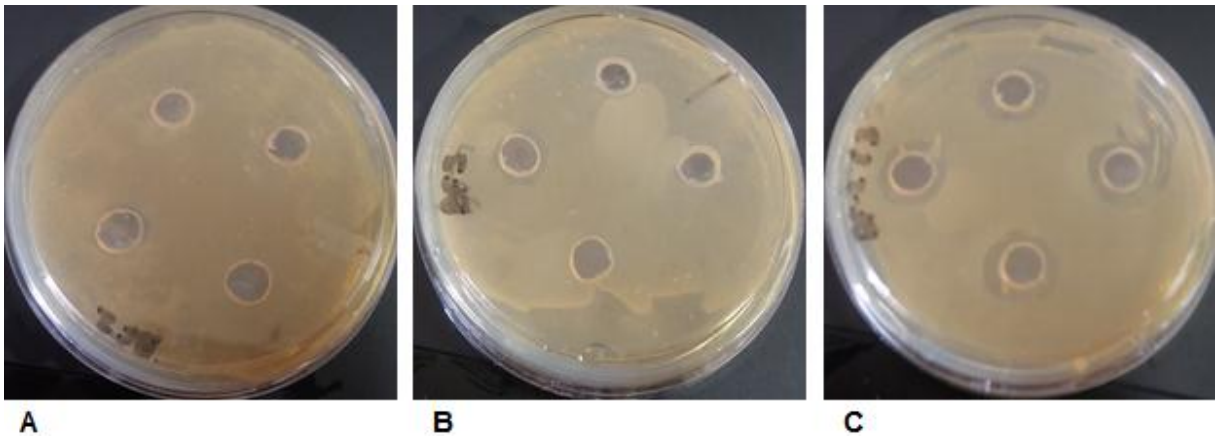

Figure 0.8 Agar well diffusion plates (A) *Bacillus* sp. BF-2 as an antibiotic (B*) C. aquatica* as an antibiotic (C) *Bacillus* sp. BF-2 and *C. aquatica* well used as antibiotics

4.3.6 Summary

PSW contains a high quantity of particulate matter, FOG, and proteins, including antimicrobial compounds such as TCS and TCC. To overcome this problem, pre-treatment using synthetic flocculants in DAFs is commonly used prior to treatment of the wastewater. Synthetic flocculants add to the environmental burden; therefore, the use of bioflocculants is desirable. Additionally, isolates used for bioflocculant production for PSW pre-treatment must not only be TCS and TCC resistant, they must also dominate other microorganisms in the PSW. Since the microorganisms that have the ability to produce bioflocculants were directly isolated from the PSW, their compatibility was thoroughly assessed. The isolates *C. aquatica* and *Bacillus* sp. BF-2 isolated from PSW were able to produce flocculants with suitable activity at pH 6.5 and 32 ˚C in the presence of TCS and TCC at concentrations of 18 and 55 mg/L. The maximum flocculation activity in the presence of 55 mg TCC/L and TCS was 49.5% and 92%, respectively. When an agar well diffusion plate method was performed, it proved that both isolates were antagonistic to other organisms in the PSW. This ensured their dominance when used in a DAF system; however, only when they were co-cultured.

4.4 Phase 4: Design of an efficient bench-scale dissolved air flotation system for the removal of suspended solids, lipids and protein matter from poultry slaughterhouse wastewater

4.4.1 Introduction

DAF is a wastewater treatment technique employed in the separation of low-density solids from wastewater (Al-Shamrani et al., 2002). This technique is highly dependent on the suitability of the sparging system used in the DAF. If sufficient pressure is applied to the diffusers, microscopic bubbles are formed when the wastewater is pumped into the flotation cell. The micro-bubbles generated in the system can be harnessed for the removal of FOG, suspended solids and biomass (Amaral Filho et al., 2016). The effectiveness of this system can be attained when the size of the bubble is suitable when compared with the size of the particles that have to be separated; furthermore, compatibility of the surface charges for both the suspended particles and the micro-bubbles can also influence the DAFs operational efficiency (Han et al., 2007). The quantity of the micro-bubbles generated, including their size, is dependent on the pressure applied to the air diffusers.

The addition of flocculants into the DAF can also enhance the efficiency of the system. In most cases, flocculation-flotation methods involve the addition of chemical flocculants into the system. Chemical flocculants such as aluminium sulfate and ferric chloride, as well as polyacrylamide, have been determined to be highly efficient. However, they are expensive, non-biodegradable, and can have a harmful effect on the environment; moreover, they have

been determined to be toxic to humans (Wang et al., 2011). These considerations prompted researchers to assess alternative solutions with minimal impact on the environment. Hence, bioflocculants have received much attention lately. These polymers are biodegradable, environmentally friendly and they do not pose a risk to human health. This part of the study mainly focused on the application of bioflocculants produced by isolates from the PSW, for their use in DAFs for the removal of suspended solids, FOG and protein material from the PSW.

4.4.2 Aim and objectives

The aim of this phase of the study was to design a bench-scale bioflocculant supported DAFs and assess its efficiency in removing suspended solids, proteins and FOG from PSW. The objectives for this part of the study were primarily to:

- evaluate the efficiency of the bioflocculant-supported DAFs in the removal of TSS, protein and FOG from PSW; and
- compare the efficiency of the bioflocculant DAF to those in which chemical flocculants are used, including a conventional DAF (control), to come to a definite conclusion whether the use of bioflocculants was an appropriate strategy for a PSW pre-treatment process, that is, to reduce TSS, protein and FOG.

4.4.3 DAF efficiency

In order to achieve the above aim, DAFs was designed to minimise clogging when the wastewater is fed to the treatment system and to reduce diffuser fouling. The DAF system was operated using bioflocculants and a chemical flocculant, and conventional DAFs was used for control studies, that is, without flocculant supplementation, to observe which system had a competitive advantage. Three states were observed, namely, unsteady state (from day 0 to 2), transition to steady state (day 3), and steady state (day 4 to 10). Therefore, the DAF efficiency was quantified during the steady state.

4.4.4 Removal of total suspended solids by the dissolved air flotation system

A total suspended solid (TSS) removal method is a familiar technique utilised to determine particulates that cannot be filtered using conventional filters. These solids are usually bigger than 2 µm, whereas for total dissolved solids (TDS), smaller particulate matter less than 2 µm is quantified (Anon, 2014). TSS are made up of organic and inorganic matter present in the wastewater. This parameter is important when observing water quality and it is indirectly correlated to turbidity. When a high quantity of suspended solids is present in the wastewater,

the water becomes turbid. By description, turbidity is the determination of light scattering in the water sample (Hannouche et al., 2011); hence, the turbidity of the PSW can be directly affected by both suspended and dissolved solids.

Fig. 4.9(a) illustrates the removal efficiency of TSS by bioflocculant-supported and chemical-supported DAF (BioDAF and ChemDAF) at steady and unsteady state. When observing BioDAF at unsteady steady it reduced 7% and 56.5% TSS on day 0 and day 2, respectively. When observing the steady state, the average TSS removal for the BioDAF was 91%. This was attributed to the microbial isolates introduced into the system – an indication of their competitiveness compared with other microorganisms present in the PSW. A 48 h acclimatisation period for the isolates was required for the BioDAF system. Such an operational strategy can be ensured when the isolates are cultured for 48 h prior to the initiation of the PSW inflow, thus enabling the production of active bioflocculants. Overall, the isolates selected and used in the BioDAF were able to produce bioflocculants, thus modifying the surface charge of the suspended solids, which enabled macroflocs to be formed. This was followed by using stainless-steel skimmers (Dragon Laboratory OS20-S agitators) at 66 rev/min, to skim the flocculated solids.

For the chemical flocculant-supported DAF (ChemDAF), 2% (w/v) alum [$KAl(SO_4)_2.12H_2O$] was used, which was added to the PSW, resulting in the successful suspension of TSS in the wastewater, a phenomenon also attributed to the interaction of negatively charged suspended matter and the positively charged metal ions which neutralised the negatively charged suspended particles, forming flocculatable flocs in the PSW. The buoyancy of the particles (assumed to have been encapsulated by a film of FOG) was hypothesised to have been assisted by FOG presence in the PSW, which resulted in the increased removal efficiency of the suspended solids so that the smaller flocs attached to larger flocs rising to the surface of the wastewater at a higher velocity. Owing to the immediate availability of surface charge alteration constituents in the ChemDAF, in comparison with the BioDAF, which needed a 48 h acclimatisation, the ChemDAF at unsteady state reduced 71 to 77% of the TSS. During the unsteady state, the ChemDAF was more efficient as compared with the BioDAF. Under steady state, the average TSS removal achieved by the ChemDAF was 84% in comparison with the 91% achieved by the BioDAF. Overall, the average TSS removal achieved by the ChemDAF used in this study was higher than the 34% removal of the TSS obtained by Amuda and Alade (2006), using alum as a flocculant in a jar test, where the DAF dose was 2 mg/L of alum – a concentration similar to that used in this study.

In control studies, a conventional DAF (ConvDAF) system, without chemical or bioflocculant supplementation, also resulted in reduced TSS in the PSW. At the steady state, this system removed an average TSS of 33%, which is lower than the TSS removed by both a chemical and bioflocculant-supported DAF system (Fig. 4.9 – additional information).

In both the ChemDAF and BioDAF, TDS and turbidity reduction were also assessed. At steady state, the BioDAF achieved a TDS reduction of 76%, including turbidity reduction of 98%, while the ChemDAF only obtained a reduction of 22% TDS and 97% turbidity; whereas the ConvDAF only achieved a reduction of 26% and 97% TDS and turbidity, respectively – a result attributed to the direct influence of flocculant supplementation in the DAFs. In the BioDAF, there was a conventional correlation between TSS, TDS and turbidity. As more TSS was removed, turbidity improved, while TDS was reduced. The low TDS removal in the ConvDAF system was attributed to the absence of flocculants, with the micro-bubbles being unable to facilitate the attachment of particulate matter as a consequence of FOG presence.

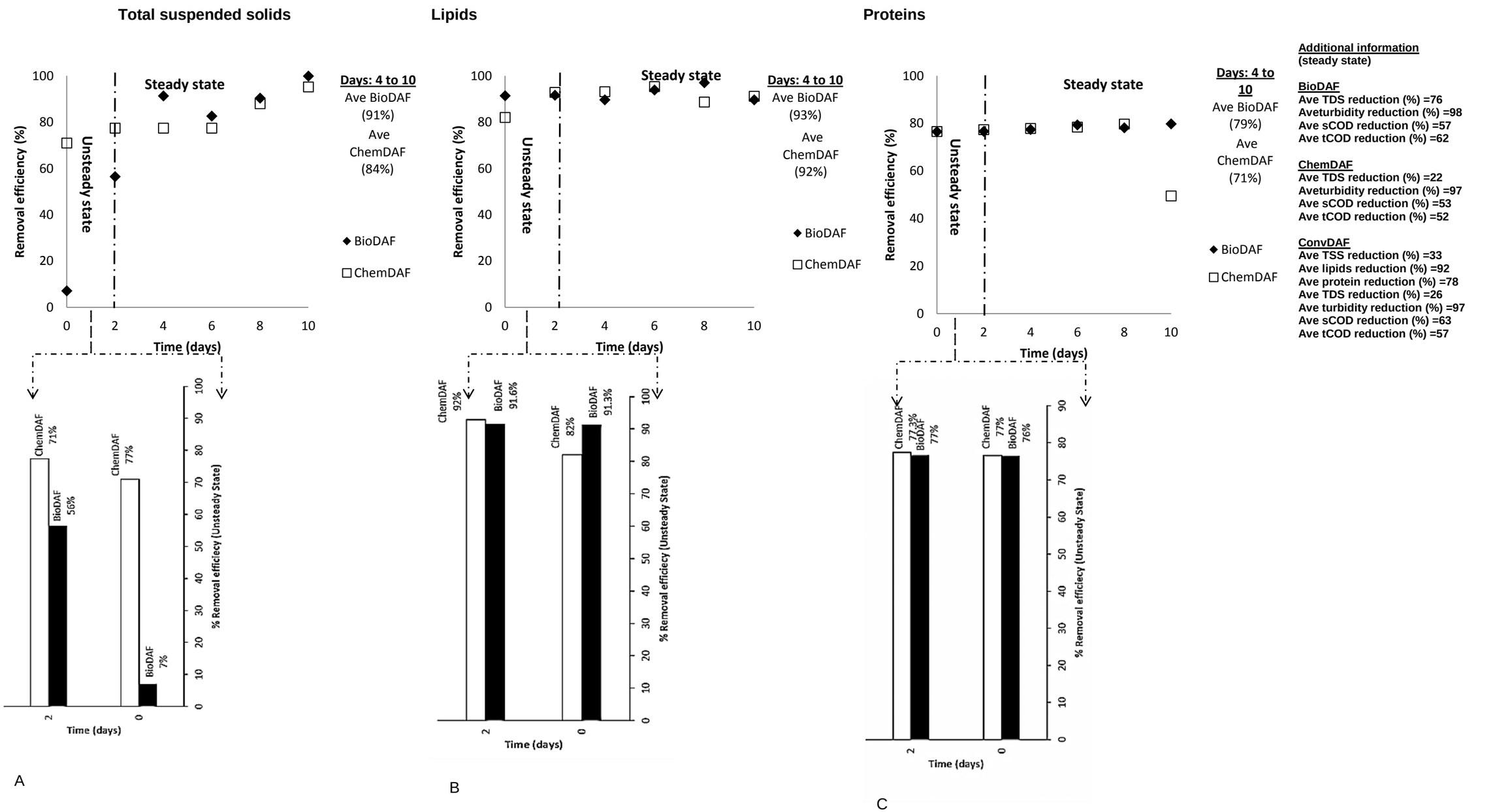

Figure 0.9 Profile of the percentage removal (%) TSS (A), lipids (B) and protein (C) of a bioflocculant and chemical supported DAF system

As the objective of this part of the study was to assess the removal of TSS in the PSW, the resultant effluent was determined to be in accordance with the South African by-law discharge standards of TSS (<1000 mg/L). Overall, a higher TSS removal obtained in this study was higher when compared with the 37% TSS removal efficiency achieved in a conventional DAF (Del Nery et al., 2007). Similarly, in a study by De Nardi et al. (2011), the efficiency of a DAF system supported by a cationic polymer as a flocculant for the treatment of PSW with similar characteristics as the PSW used in this study, only 65% TSS reduction was achieved which is lower than the 91% TSS removal obtained using the BioDAF.

4.4.5 Removal of lipids by dissolved air flotation system

Lipids can be defined as oils, grease, fats and long chains of fatty acids. These lipids are the constituents of wastewater, mainly from the food-processing industry. Their presence in wastewater as organic material, decreases dissolved oxygen (DO) of the wastewater and enhances levels of BOD and COD (Chipasa & Mędrzycka, 2006). In biological wastewater treatment systems, lipids are degraded by an enzyme called lipase (Andersson, 1980). During the unsteady state, the removal of lipids in the BioDAF was between 91.3 to 91.6%, compared with 82% to 92% achieved by the ChemDAF. During the steady state operation of the system, the average lipids' removal efficiency slightly increased to 93% for BioDAF when compared with the 92% obtained by both ChemDAF and ConvDAF systems. FOG contains a carboxyl group that is negatively charged; hence, for flocculation to occur, it requires an oppositely charged flocculant (Vance & Vance, 2008). As the BioDAF contained bioflocculants from a co-culture, with different functional groups, the bioflocculants produced, particularly those produced by *C. aquatica* (protein constituents), had functional groups such as amino (positively charged) as well as carboxyl (negatively charged), while the other bioflocculants produced by *Bacillus* sp. BF-2 were negatively charged; hence, the FOG could attach to both the positive site of the *C. aquatica* bioflocculants and the negatively charged BF-2 flocculants, which could improve floc formation.

Generally, *Bacillus* sp. produces lipases which can break down FOG which in turn influences the removal of lipids in the BioDAF; hence, there was higher reduction of FOG in the BioDAF as compared with the ChemDAF and ConvDAF (Eggert *et al*., 2003; Dlangamandla et al., 2016). Similarly, the ChemDAF contained a positively charged flocculant; hence, the flocculation of the lipids was high although slightly lower than that of the BioDAF. It was previously determined that the BioDAF can remove a high quantity of lipids compared with the ConvDAF, particularly when the separation of FOG is facilitated by the supplementation of bioflocculants into a DAF system, with the literature indicating that such supplementation

can result in O&G removal of between 63 to 99% and 80 to 99% for fat (De Nardi et al., 2008). This study proved that the BioDAF can remove lipids more efficiently than the ChemDAF.

4.4.6 Protein reduction by dissolved air flotation

Proteins are known as the basic components of life. They are composed of nitrogen, hydrogen and oxygen. They are present in wastewater as soluble microbial products (SMPs), and proteolytic enzymes produced by the microorganisms during their metabolic processes. The enzymes are produced by the microorganism extracellularly to degrade organic matter present in the wastewater so that the cells can utilise it as a nutrient source. They are major components of Total Organic Carbon (TOC) and Total Organic Matter (TOM). High nitrogen to carbon ratio indicates the presence of proteins in the wastewater (Westgate, 2009). The nitrogen and carbon concentration of the PSW used in this study were 211 and 546 mg/L respectively, which resembled the similar characteristics observed in studies carried out by Basitere et al., 2016; Bustillo-Lecompte et al., 2016 . These soluble proteins can be removed by coagulation and flocculation; however, in wastewater that contains FOG, a DAF system is also required. The proteins that are recovered by a flocculation-flotation system used to treat the wastewater cannot be reused, as the flocculants that attach to the proteins change their structure, which in turn changes their function (Bialas et al., 2014).

In this study, soluble proteins were quantified to assess whether the system designed could effectively reduce total protein concentration prior to the wastewater's being biologically treated. The untreated influent contained 70 mg/L of soluble proteins. Fig. 4.9(c) illustrates the protein concentration in the PSW prior to treatment using BioDAF, ChemDAF and ConvDAF. At unsteady state, the BioDAF removed 76 to 77%, with similar removal rates observed for the ChemDAF that removed 77% to 77.3%. An improvement to 79% (BioDAF) was achieved during the steady state, whereas 71% of proteins were removed by ChemDAF, with the ConvDAF removing 78% of soluble proteins. The ChemDAF achieved the lowest protein removal compared with the BioDAF and ConvDAF. Protein charge is dependent on the pH of the solution; it can carry a net positive or a net negative charge. In this part of the study, the BioDAF removed high protein concentration as compared with the ChemDAF; this was because bioflocculants contain different functional groups to which the protein could be attached, resulting in efficient flocculation; whereas the ChemDAF contained a flocculant that carried a single charge.

4.4.7 Overall wastewater quality improvement

Chemical oxygen demand (COD) is the method used to quantify soluble and particulate organic matter in wastewater. This parameter is measured by using potassium dichromate that is a strong oxidising chemical to oxidise organic matter in an acidic solution to water and carbon dioxide (Appendix D 1 and D 2) (Yao *et al*., 2014). High COD concentration in wastewater means there is high quantity of oxidisable organic matter that leads to deterioration of dissolved oxygen in the wastewater, further creating an anaerobic environment which endangers aquatic life. Soluble COD determines the biodegradable part of COD, while total COD determines the non-biodegradable portion in the wastewater.

The BioDAF reduced 62% tCOD as well as 57% sCOD, whereas the ChemDAF reduced only 52% tCOD including 53% sCOD. The ConvDAF reduced 57% as well as 63% of tCOD and sCOD, respectively. The BioDAF was operated at high pressure and slower flow rate that in turn increased the residence time and mixing (Hami et al., 2007) – an operational strategy suited to effective COD reduction. Flocculation activity is affected by time and mixing, that is, when the residence time was increased, the microorganisms inoculated into the wastewater were exposed to more mixing; hence, this system was effective for COD reduction. This data depicted that aerobic bacteria inoculated into the wastewater were capable of degrading these organic contaminants (Magnaye et al., 2009). In the ChemDAF, these results depicted that there was high tCOD removal compared with sCOD removal in the system. Since this system was a chemical system, it achieved the highest removal of the non-biodegradable COD. In the ConvDAF system, sCOD reduction was higher compared with that of the bioflocculant-supported DAF. The tCOD reduction of the ConvDAF and ChemDAF was lower compared with the tCOD reduction achieved by the bioflocculant-supported DAF – a phenomenon which needs further exploration.

4.4.8 Summary

The use of a dissolved air flotation (DAF) system has been described. However, this technology has also been criticised, because in order for it to operate efficiently for the removal of suspended solids and FOG, it requires the addition of flocculants, which are expensive and harmful to the environment as well as to humans. This part of study focused on the design and efficiency of a bioflocculant-supported DAFs, compared with a chemical and conventional DAF for the removal of TSS, lipids and protein material. The results showed that the BioDAF can remove up to 91% TSS, 93% lipids and 79% protein, while the ChemDAF only removed 84% TSS and 92% lipids, including 71% protein. Therefore, it was proved that the bioflocculant dissolved air flotation system, that is, the BioDAF, was more efficient than

the chemical dissolved air flotation system (ChemDAF) and the conventional DAF (ConvDAF) in the removal of suspended solids, lipids and protein material present in the PSW.

CHAPTER 5

CONCLUSIONS

AND

RECOMMENDATIONS

CHAPTER 5
CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The isolated microorganisms produced bioflocculants with high flocculation activity when using bioreactor conditions optimised by RSM. The highest flocculation activity obtained for *C. aquatica* and *Bacillus* sp. BF-2 was 93.8% at 32.9 °C and pH 6.5 as well as flocculation activity of 88.3% at 40 °C at pH 6.5, respectively. Further studies were carried out using the same RSM conditions; the culturing mediu was then changed into sterile poultry slaughterhouse wastewater to prove that these bioflocculants were suitable for use in a dissolved air flotation system for the pre-treatment of poultry PSW, achieving bioflocculant activity of 57% at 34 °C, pH 4 for *C. aquatica*, with *Bacillus* sp. BF-2 achieving a flocculation activity of 44% at 34 °C and pH 4. Since TCS and TCC are used in poultry slaughterhouses, the wastewater from such facilities also contains these antimicrobial compounds; hence, the isolated species were further tested for resistance and their ability to produce flocculants in the presence of 18 and 55 mg/L of TCS and TCC. *Bacillus* sp. BF-2 was able to tolerate triclosan and achieved up to 92% flocculation activity at a concentration of 55 mg/L TCS and 16.9% at a concentration 55 mg/L TCC. Similarly, the *C. aquatica* achieved the highest flocculation activity of 92% in the presence of 55 mg/L TCS and 45.9 % in the presence 55 mg/L TCC.

Subsequently, a two-litre DAF system was designed to be supplemented with bioflocculants. The bioflocculants were added directly into the DAF. The BioDAF removed up to 91% TSS, 93% lipids and 79% protein, while the ChemDAF, supplemented with 2% alum, could only remove 84% TSS and 92% lipids, including 71% proteins. It was concluded that at steady state, the BioDAF was more efficient in the removal of suspended solids, lipids and proteins compared with both the ChemDAF and conventional DAF (ConvDAF) without flocculant supplementation.

5.2 Recommendations for future studies

For future studies on bioflocculants, the flocculants could be further characterised beyond identifying the functional groups only, that is, to identify the type of proteins, glycoproteins, their zeta potential reduction abilities and polysaccharides produced as flocculants by the microorganisms. Furthermore, bioflocculant kinetics and modelling of the DAF system are also recommended. It is further recommended that the bioflocculant dissolved air flotation be operated at a lower HRT to sustain its efficiency when it is operated at a shorter HRT.

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APPENDICES

Appendix A: Microbial isolation and identification

Appendix A 1: Gram staining procedure

- Use an inoculating loop to pick up small amount of the liquid culture and transfer it into the glass slide, make a smear on a glass slide, if the microbial culture is transferred from and agar slant or petri dish, first add a loop fool of sterile distilled water into the glass slide then transfer a small amount of the microbial culture into the glass slide then mix it with the water to make a smear, and heat fix the smear over a Bunsen burner.
- Add crystal violet on the fixed culture and allow it to stand for 60 sec.
- Rinse the slide with running tap water.
- Add iodine into the fixed culture on the glass slide, allow it to stand for 60 seconds, rinse the slide under running tap water.
- Add a few drops of acetone to the fixed culture.
- Rinse it off immediately with running tap water.
- Add safranin and allow it to stand for 60 seconds.
- Rinse with running tap water and blot dry.

Appendix A 2: DNA extraction

1. Add 1ml of an overnight culture to a 1.5ml microcentrifuge tube.
2. Centrifuge at 13,000x g for 2 minutes. Discard the supernatant.
3. Resuspend the cells into 480µl of 50mM EDTA.
4. Add 120µl of lytic enzyme(s) and gently to mix it by pipetting up and down.
5. Incubate at 37 °C for 60 minutes. Centrifuge for 2 min at 13,000x g and discard the supernatant.
6. Add 600µl of Nuclei Lysis buffer solution.
7. Incubate at 80 °C for 5 minutes to lyse the microbial cells, then cool to room temperature.
8. Add 3µl of RNase solution to the lysate. Invert the tube 2 to 5 times to mix.
9. Incubate at 37 °C for 60 minutes and cool the sample at room temperature.
10. Add 200µl of Protein Precipitation Solution to the RNase-treated cell lysate, and vortex.
11. Incubate the sample on ice for 5 minu.
12. Centrifuge at 13,000x g for 3 min.
13. Transfer the supernatant containing DNA into 1.5 ml centrifuge that contains 600µl isopropanol at room temperature and discard the pellet.

14. Gently invert the centrifuge tube until thread-like DNA strand appears.
15. Centrifuge at 13,000 to 16,000 x g for 2 minutes. Discard the supernatant and let the pellet air dry for 10 to 15 min.
16. Add 70% ethanol and invert to wash the DNA pellet.
17. Centrifuge at 13,000 to 16,000x g for 2 minutes.
18. Carefully aspirate the ethanol. Drain the tube on clean absorbent paper and allow the pellet to dry for 10 to 15 minutes.
19. Dissolve the pellet by adding 100µl DNA Rehydration Solution and incubate the mixture in a water bath at 65 °C for an hour. Periodically mix the solution by tapping the tube. Incubate the DNA solution at room temperature or at 4 °C

Appendix: B Biochemical Tests

Bacillus sp. BF-2

Biochemical Details																	
1	BXYL	+	3	LysA	+	4	AspA	-	5	LeuA	+	7	PheA	+	8	ProA	-
9	BGAL	+	10	PyrA	+	11	AGAL	+	12	AlaA	+	13	TyrA	+	14	BNAG	-
15	APPA	-	18	CDEX	-	19	dGAL	+	21	GLYG	-	22	INO	+	24	MdG	-
25	ELLM	-	26	MdX	-	27	AMAN	-	29	MTE	+	30	GlyA	+	31	dMAN	+
32	dMNE	+	34	dMLZ	-	36	NAG	+	37	PLE	+	39	IRHA	+	41	BGLU	+
43	BMAN	+	44	PHC	-	45	PVATE	+	46	AGLU	-	47	dTAG	-	48	dTRE	+
50	INU	-	53	dGLU	+	54	dRIB	+	56	PSCNa	-	58	NaCl 6.5%	+	59	KAN	+
60	OLD	+	61	ESC	+	62	TTZ	+	63	POLYB_R	-						

Comamonas aquatica

Biochemical Details																	
1	BXYL	+	3	LysA	+	4	AspA	-	5	LeuA	+	7	PheA	+	8	ProA	-
9	BGAL	+	10	PyrA	+	11	AGAL	+	12	AlaA	+	13	TyrA	+	14	BNAG	-
15	APPA	-	18	CDEX	-	19	dGAL	+	21	GLYG	-	22	INO	+	24	MdG	-
25	ELLM	-	26	MdX	-	27	AMAN	-	29	MTE	+	30	GlyA	+	31	dMAN	+
32	dMNE	+	34	dMLZ	-	36	NAG	+	37	PLE	+	39	IRHA	+	41	BGLU	+
43	BMAN	+	44	PHC	-	45	PVATE	+	46	AGLU	-	47	dTAG	-	48	dTRE	+
50	INU	-	53	dGLU	+	54	dRIB	+	56	PSCNa	(+)	58	NaCl 6.5%	+	59	KAN	+
60	OLD	+	61	ESC	+	62	TTZ	+	63	POLYB_R	+						

Appendix: C Agar well diffusion

- Innoculate 250 mL of nutrient broth with 1 loopful of an overnight culture.
- Store the inoculated broth at 33 °C and shaking speed of 121rpm for 48 h.
- Mueller-Hinton Agar plates were prepared and raw poultry slaughterhouse wastewater was spread on the agar plates.
- Four wells (6 to 8 mm) were drilled in the plate, using an autoclaved sterile swab cover.
- Add 10 µl of the cultured microorganisms into the wells.

- Store the plates were stored at 33 °C for 24 h and assess the plates for clear zones.
- Measure the clear zones using a ruler.

Appendix D: Analytical procedures

Appendix D 1: Total COD analysis

- Switch on the Spectroquant® thermoreactor to the pre-set setting of 148 °C for two hours and let the thermoreactor heat to the desired temperature. (This will take approx. 10 min.)
- Place approximately 100ml distilled water into a 250ml beaker.
- When using COD solution A and B for a range of 500 to 10000 mg/L.
- Pipette 2.2mL of COD solution A into a cell using P5000 pipette.
- Pipette 1.8mL COD solution B into the cells with COD solution A was pipetted in using P5000.
- Using a new pipette, tip pipette 1ml of the sample into the cells sing P1000.
- Tightly attach the screw cap to the cells.
- Vigorously mix the cell with a shaker.
- Heat cells in the thermoreactor at 148 °C for 2 hours.
- Carefully remove the cells after 2 hours and place them in a test tube rack to cool. (Do not cool with cold water.)
- Wait 10 minutes, place them in the shaker again and leave in the test tube rack to cool at room temperature. (Cooling time is at least 30 minutes.)
- Place the cells in the Nova 60 for COD reading.
- When using the Nova 60, make sure that the indicator line on the cell lines up with the indicator line on the Nova 60. But when using COD solution for 500 to 10000 mg/L, put in the code 0.24. When using COD solution for 100 to 1500mg/L put in the code 0.23.
- When using COD solution A and B for a range of 100 to 1500mg/L
- This procedure is exactly the same as for COD solution A and B for 500 to 10000 mg/L with the exception of:
- Add 0.30mL of COD solution A into the cell using P1000.
- Pipette 2.30mL COD solution B into the cell using P5000.
- Add 3mL of the sample into the cell that contains solution A and B using P5000 pipette.

Appendix D 2: Soluble COD Analysis

- The Büchner funnel is placed into a 500ml suction flask which is connected to a vacuum pump.
- Place the glass microfibre filter disc, 5.5 cm, without organic binder, Whatman type GF/F (0.7 Fm) inside the Büchner funnel.
- Filter the raw sample using the vacuum pump.
- The filtered sample is then used to run a COD test.
- The procedure for the COD test is the same as for the total COD test; the difference is that only filtered samples are used.

Appendix D 3: Total suspended solids

Apparatus

- Glass microfibre filter discs, 5.5cm, without organic binder, Whatman type GF/F (0.7 Fm).
- Disposal aluminium dishes
- Tweezers
- Suction flask, 1000ml
- 47mm glass microanalysis filter holder (funnel, clamp and base).
- Drying oven for operation 103 to 105 °C.
- Muffle furnace for operation at 550± 50 °C.
- Desiccator.
- Analytical balance, capable of weighing 0.1mg, an RS232C interface and personal computer.
- Milli-Q® reagent grade water (ASTM Type-1 water), Millipore Corp., Bedford, MA.

Procedure for total suspended solids

- Insert the glass-fibre filter disc: onto the base and a clamp funnel. While vacuum is applied, wash the filter disc with three successive 20mL volumes of Milli-Q® water. Remove all traces of water by applying the vacuum after the water has passed through. Remove the funnel from the base and place filter on the aluminium dish and ignite in the muffle furnace at 550± 50 °C for 30 min. Rewash the filter with an additional three successive 20mL volumes of Milli-Q® water, and dry it in an oven at 103 to 105 °C for 1 h. When, remove the dish from the oven, desiccate and weigh.
- Select the sample volume (200mL max) that will yield no more than 200mg of total suspended solids.
- Place the filter between the base and clamp, and add a small volume of Milli-Q® water so that the filter attaches to the base, remove the water from the base.

- Shake the sample vigorously, then transfer the sample into the filter paper. Remove water by applying vacuum even after the water has passed through.
- Remove the filter from the base and dry for 1 hour at 103 to 105 °C. Cool in the desiccator and weigh.

Calculation for total suspended solids

TSS (mg/L) = (A-B) x 1000/C

Where A = Weight of filter and dish+ residue in mg.

B = Weight of filter and dish in mg.

C = Volume of sample filtered in mL.

APPENDIX E: Bradford's method

- 10 mg of crystal bovine serum albumin (BSA) was dissolved into 10 mM of 10 mL sodium phosphate. The standard curve was made from the stock.
- 50 µL of each of the standard samples containing a known concentration of BSA were added into the 96 wells microtiter plate (in duplicates).
- 150 µL of the Bradford reagent was added to each well that contains the standard sample and left to stand for 2 minutes.
- The absorbance of the standards was quantified using an Anthos Xenthal 1100 microtiter plate reader at an absorbance of 595 nm.
- A standard curve was constructed using the absorbance of the standard versus their concentration.
- A standard curve was then drawn for the diluted unknown protein concentration sample (1:10).
- The concentration was estimated using the regression line.