Cape Peninsula University of Technology

Production of a bioflocculant from marine *Stenotrophomonas maltophilia* and its application in wastewater treatment

By

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DECLARATION

I, **Aphiwe Tyikane** 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.

Signed  **Date** 13.02.2024

Abstract

The lack of access to safe drinking water and the need for an environmentally friendly approach has prompted bioflocculants to be promising alternatives to chemical flocculants in water and wastewater treatment. This study assessed the production of a bioflocculant from a marine environment and evaluated its application in wastewater treatment. The 16s rDNA nucleotide sequence analysis of isolate H7 revealed 99% similarity to *S. maltophilia* and was deposited in the GenBank with the accession number MT291866.1. A statistically based experimental design matrix for the bioflocculant production was explored using Plackett Burman (PB) to screen the significant factors and response surface methodology (RSM) coupled with central composite design (CCD) to optimise the production medium. PB revealed that glucose ($p < 0.0071$), yeast extract ($p < 0.0041$), K_2HPO_4 ($p < 0.0032$) and $(NH_4)_2SO_4$) ($p < 0.009$) exhibited a statistically significant influence on the production of the bioflocculant producing strain with the probability values less than 0.05. CCD demonstrated that glucose as a carbon source and yeast extract as a nitrogen source supported the maximum bioflocculant production from marine *S. maltophilia.* The optimal quantities of 16.25 g/l glucose, 1.61 g/l yeast extract, 1.1 g/l K_2HPO_4 and 3.5 g/l $(NH_4)_2SO_4$) achieved a maximum flocculation activity of 96.05% and 4 g yield of the purified bioflocculant. Fourier transform infrared (FTIR) spectroscopy revealed the presence of hydroxyl and carbonyl groups and sugar derivatives. This confirmed that a polysaccharide is the major backbone of bioflocculant produced by *S. maltophilia*. A Thermogravimetric analyser (TGA) indicated that the bioflocculant produced is thermostable as it retained 85% of the weight when heated up to 500 ℃. The scanning electron microscope (SEM) analysis revealed clumped sheath layers and an irregular pattern. The energy dispersive x-ray (EDX) analysis affirmed the presence of carbon, oxygen, magnesium, sulphur and potassium, (49.42), (34.23), (0.73), (7.78), (0.14) and (7.7) respectively. Compared to commercially available flocculants, the bioflocculant exhibited 84.5% flocculation activity (FA), while polyethyleneimine and polyacrylamide demonstrated 65.7% and 29.6% FA, respectively. The application of the produced purified bioflocculant was investigated by treating primary sludge from a wastewater conventional plant with a COD removal efficiency of 71.5%, the bioflocculant produced has the potential to serve as an alternative to traditional flocculants.

Keywords: *Bioflocculant, wastewater, marine environment, chemical flocculant, flocculation activity, COD, sludge, removal efficiency*

DEDICATION

My humble efforts are dedicated to my late parents and late great grandmother:

Nomvuyo Tyikana

"Without you, life has been challenging, but I have persevered and I am still okay. May this serve as a living tribute to your memory Mambathane"

Thobile Mabongo

"Your memory lives on as this tree blossoms and grows, till we meet again my dear father"

Great grandmother

"Mambhushwa, you have taught me respect, to be unique, determined, to believe in myself, to be fearless in the pursuit of what sets my soul on fire, and to always persevere. I am truly thankful and honoured to have been under your guidance from a tender age"

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List of tables

List of Figures

Table of Contents

Glossary

Abbreviations	Definitions
ANOVA	Analysis of Variance
AMSB	Antimicrobial-susceptible bacteria
AMRB	Antimicrobial-resistant bacteria
BAF	Biologically aerated filter
BBD	Box-Behnken Design
BLAST	Basic Local Alignment Search Tool
BOD	Biological oxygen demand
BSA	Bovine serum album
CBF	Compound bioflocculant
CCC	Circumscribed central composite
CCD	Central Composite Design
CCF	Face-cantered central composite
CCI	Inscribed central composite
CD	Charge density
COD	Chemical oxygen demand
DOE	Design of experiments
DNA	Deoxyribonucleic acid
DF	Degrees of freedom
DWS	Department of water and sanitation
EDTA	Ethyline diamine tetra acetic acid
EDX	Energy Dispersive X-ray analysis
EPS	Extracellular polymeric substances
EPSs	Exopolysaccharides

FA	Flocculation Activity
FTIR	Fourier Transform Infrared
ISP2	Implantation Serine Proteinase Gene
l	litres
MW	Molecular Weight
MS	Mean Square
NCBI	National Centre for Biotechnology Information
NF	Nanofiltration
nm	Nanometre
NOM	Natural Organic Matter
p	Probability
OD	Optical Density
PAC	Polyaluminium chloride
PB	Placket Burman
PBD	Placket Burman design
PBSD	Placket Burman Screening Design
PCR	Polymerase chain reaction
rDNA	Ribosomal deoxyribonucleic acid
RO	Reverse osmosis
rRNA	Ribosomal ribonucleic acid
RSM	Response Surface Methodology
SEM	Scanning Electron Microscope
SS	Sum of Squares
TEA	Techno-economic analysis
TGA	Thermogravimetric Analysis
UF	Ultrafiltration

WSA	Water service authorities
Y	Response (Bioflocculation activity)
YMA	Yeast malt agar
YME	Yeast Malt Extract
b_i	Linear coefficient
b_0	Model intercept (PB)
k	Number of variables involved
n	Number of factors
x_i	Level of the independent variable
α	Axial distance
δ_0	Intercept term (CCD)
δ_1, δ_2 And δ_3	Coefficients of linear terms
δ_{12}, δ_{13} And δ_{23}	Coefficients of interaction terms
δ_{11}, δ_{22} and δ_{33}	Coefficients of quadratic terms

Chapter 1

1. General Introduction

1.1. Introduction

Water is one of the critical constituents required to survive and thrive in carbon-based life forms. It occupies about 78% of the earth's surface and is a source of life and energy (Okaiyeto *et al.*, 2015). Access to safe potable water is considered a fundamental human right and a symbol of dignity, recognising its inherent value and significance (Sershen *et al.*, 2016). However, millions lack access to safe potable water due to the inevitable surge in urbanisation and everyday human activities that harm the environment, resulting in rising organic and inorganic pollutants contained in discharged wastewater (Sharma and Rout, 2011). South Africa has been identified as a water-scarce country as drought has been experienced on numerous occasions (Sershen *et al.*, 2016). Drought is one of the operational burning issues in the Eastern Cape at Gqeberha. Nevertheless, this is a worldwide challenge (Otieno and Ochieng, 2004; Pamla *et al.*, 2021). Released untreated or moderately treated wastewater accounts for numerous health-related issues in humans, the environment and aquatic life (Agunbiade *et al.*, 2017). Safe water and sanitation are essential for domestic, agricultural, industrial, and environmental purposes.

Environmental pollution is a substantial universal predicament; water pollution is among the most challenging environmental issues. It has become a worldwide difficulty to improve the quality of life in various communities (Yang *et al.*, 2012). Unwitting urbanisation and rapid population growth have contributed vastly to the unsafe state of water pollution and the current unhealthy environment. This growing urbanisation destroys river catchments, groundwater, industry, mining, and damming of rivers (Prasertsan *et al.*, 2006). The primary pollution sources arise from the discharge of domestic and agricultural wastes, untreated sanitary, and toxic industrial effluents. Subsequently, these pollutants in water bodies can be toxic to aquatic life and render them unsuitable as potable water sources for domestic usage (Okaiyeto *et al.*, 2015; Kurniawan *et al.*, 2020). Careful consideration has been taken into account regarding water and wastewater treatment to deliver safe water to the environment and aquatic forms (Bhatnagar and Sillanpää, 2010). According to Bhatnagar and Sillanpää (2010) and Sershen *et al.* (2016), numerous strict regulations have been initiated by various countries regarding the presence of contaminants in water to ensure proper treatment of domestic and agricultural wastewater, as well as industrial effluents before their discharge into different waterbodies (Okaiyeto *et al.*, 2015). In South Africa, the Department of Water and Sanitation (DWS) introduced Green Drop and Blue Drop programs to improve the performance and compliance of the water service authorities (WSAs) (Sershen *et al.*, 2016).

Various traditional and advanced technologies are usually adopted to remove colloidal particles from wastewater, such as coagulation-flocculation, filtration, ion exchange, oxidation processes, adsorption, solvent extraction, and electrolysis (Lee *et al.*, 2014). According to Okaiyeto *et al.* (2015), coagulation-flocculation is one of the most used solid-liquid separation methods to remove suspended solids and organic matter in wastewater. Flocculation is an essential process in wastewater treatment whereby

colloids, cells, and suspended material come out of suspension as flocs due to aggregation (Ayangbenro *et al.*, 2019). Flocculants are classified into three categories: organic flocculants, such as polyacrylamide derivatives; inorganic flocculants, such as ferric chloride; as well as naturally occurring flocculants, such as chitosan, sodium alginate, and bioflocculant (Agunbiade *et al.*, 2016). Inorganic and organic flocculants are chemical flocculants (Verma *et al.*, 2012). Chemical flocculants have high flocculating efficiency and are used to remove suspended solid particles or toxins in water and wastewater treatments as they have low production costs (Okaiyeto *et al.*, 2020). However, the downside of chemical flocculants is their potential to pose environmental and health hazards. For example, acrylamide is not only a potent carcinogen and neurotoxic to humans but is non-biodegradable, thus posing a health hazard to the environment (Giri *et al.*, 2015). Due to numerous alarming concerns regarding the usage of chemical flocculants, bioflocculants produced by microorganisms have been attracting more attention (Giri *et al.*, 2015).

Bioflocculants are natural macromolecules produced by living microorganisms, and they can flocculate particles like suspended solids, cells, and colloidal solids out of solution (Giri *et al.*, 2015). According to the literature, bacteria, algae, fungi, *Actinomycetes*, and *Stenotrophomonas maltophilia* have been reported as bioflocculant producers. These microbial flocculants (bioflocculants) are an alternative to chemical flocculants due to their comparable efficiencies (Agunbiade *et al.*, 2019). It has been documented that bioflocculant-producing microorganisms are majorly from the freshwater environment, marine sediment, activated sludge, soil, and brewery wastewater (Agunbiade *et al.*, 2018). However, there is a dearth of information on bioflocculants produced by *Stenotrophomonas maltophilia.* Hence, there is a need to isolate and screen for bioflocculant-producing strains from the marine environment and validate their potential application in wastewater treatment.

1.2. Motivation for the study

The availability of safe water is an essential source of life. Increasing population and industrialisation have put the water and sanitation department under pressure as the demand for water supply increases, leading to excessive wastewater (Joshi *et al.*, 2017). Water pollution is one of the most challenging issues globally and affects several disadvantaged communities' good quality of life (Joshi *et al.*, 2020). The primary source of water pollution is the discharge of domestic and agricultural wastes, untreated sanitary, and toxic industrial effluents (Li *et al.*, 2013; Crini *et al.*, 2018). Discharging effluents highly composed of organic and inorganic compounds may be toxic to the environment. The discharged effluents will directly affect the water bodies, such as oceans, rivers, lakes, and dams since they will have high oxygen demand for degradation (Syafalni *et al.*, 2012). Furthermore, microbes, fauna, and flora in the water bodies oxidise organic matter, thus, depleting the oxygen in the water quicker than the amount of oxygen that dissolves back into the water bodies from the air. Subsequently, the reduced oxygen availability may lead to aquatic life deaths and ecosystem imbalance (Agunbiade *et al.*, 2017). It is required that wastewater must be treated to mitigate these issues, as well as Green Drop's mandatory

prerequisite to discharge a good quality and safe effluent for downstream end users (Hassimi *et al.*, 2020). Notably, chemical flocculants are widely employed in wastewater, drinking water treatment, food, and fermentation industries and for downstream processing due to their high flocculating efficiency and cost-effectiveness (Lee *et al.*, 2014). However, the utilisation of chemical flocculants in wastewater treatment has drawn several health concerns. In addition, acrylamides are not biodegradable and consequently create an environmental nuisance (Okaiyeto *et al.*, 2015, 2016; Awolusi *et al.*, 2020a). The outlined inevitable drawbacks associated with chemical flocculants necessitate a search for alternative flocculants that are eco-friendly and safe. Thus, there is a need to screen for new microorganisms with high bioflocculant production potentials and develop novel methods to optimise culture conditions for better bioflocculant yields with improved flocculating activity.

1.3. Aim

This research aims to isolate and screen for bioflocculant-producing strains from the marine environment and validate their potential application in wastewater treatment.

1.4. Objectives

The following sets of research objectives are anticipated to achieve the research aim:

i. To screen the cultural marine bacterial strains isolated from the marine sediments for bioflocculant production using the Plackett-Burman (PB) design program.

ii. To optimise the critical media components using the response surface method (RSM) coupled with central composite design (CCD)

iii. To identify positive bioflocculant-producing organisms using 16S rDNA gene sequence analysis.

iv. To purify the bioflocculant compound(s) produced for further processing.

v. To validate the potential of the bioflocculant produced in the treatment of wastewater.

Chapter 2

2. Literature Review

2.1. Introduction

Water treatment is any process that enhances the quality of the constantly discharged effluents to render them safe for a specific end-use (Crini *et al.*, 2018). The end-use may involve drinking water, industrial water supply, irrigation, river flow maintenance, and water recreation, including safely returning to the environment (Bhatnagar and Sillanpää, 2010; Pamla *et al.*, 2021). Water pollution has become a significant predicament, a source of critical concern, and a priority for society and public authorities**.** Water pollution occurs when a substance that can negatively affect the characteristics of water is discharged into the water bodies (Crini and Lichtfouse, 2019). Subsequently, toxic substances may pose health concerns to humans, animals, their habitats, and the environment (Agunbiade *et al.*, 2019). There are various sources of water pollution, such as untreated industrial effluents and agricultural and domestic effluents. Water pollution demands constant efforts to put mitigation measures to protect water resources from pollutants (Crini and Lichtfouse, 2019). Wastewater treatment removes contaminants and undesirable components, reduces their concentration in the waste stream, and makes them suitable for release into the environment (Crini *et al.*, 2018).

As solid material decays, it uses up oxygen content in the river or lake, which is required by aquatic life (Dlamini *et al*., 2019). Therefore, this results in high oxygen demand, leading to fish, marine flora, and fauna dying. The removed solid particles are primarily organic but may also include inorganic solids. Wastewater treatment operations are subjected to the National Water Act 1998 (Act No. 36 of 1998). This Act recognises water as a scarce natural resource distributed unequally worldwide even though it belongs to everyone. It is a fundamental human right to access safe water (Ntombela *et al.*, 2016).

According to Sershen *et al.* (2016), in 2009, a legislative program known as Green Drop was implemented in South Africa to improve wastewater treatment plants' performance and compliance by discharging suitable quality effluent into the environment. When wastewater is polluted, a purification process is necessary to eliminate the toxins, and this happens in four stages: preliminary treatment, primary treatment, secondary treatment, and tertiary treatment (Hedaoo *et al.*, 2012). Conventional wastewater treatment consists of a physical, chemical, and biological process that enhances treatment in the outlined stages. Through the four steps of the wastewater treatment process, the removal of solids, including colloids, organic matter, nutrients, and soluble contaminants (metals, organics, and other contaminants) from effluent may be achieved (Crini and Lichtfouse, 2019).

2.2. Methods of wastewater treatment

The Water Services Act regulates the wastewater treatment industry, 1997 (Act 108 of 1997) (Edokpayi *et al.*, 2020). The act prescribes the legislative duty of municipalities as water-service authorities (WSA) to provide the rights of access to essential water supply and sanitation according to national standards and norms (Adewumi *et al*., 2010; Edokpayi *et al.*, 2020). Wastewater treatment industries often employ a combination of physical, chemical, and biological methods to achieve the desired level of contaminant removal (Crini and Lichtfouse, 2019). It is important to note that the selection and effectiveness of chemical methods depend on the specific pollutants present in the wastewater, the treatment goals, and the regulatory requirements. Also, proper handling and dosage of chemicals are essential to ensure effective treatment and prevent adverse environmental impacts (Pamla *et al*., 2021). The following section elaborates more on the methods of wastewater treatment used.

2.2.1. Physical treatment

Physical treatment of wastewater is the removal of emerging contaminants without changing the biochemical characteristics of the pollutants since there is no involvement of chemical or biological addition agents. Various physical methods are used in wastewater treatment to separate, remove, or transform the pollutants (Ahmed 2021). These physical methods include processes without gross chemical or biological changes, and strictly physical phenomena usually improve wastewater quality (Bhatnagar and Sillanpää, 2010). Some commonly adopted physical treatment methods comprise screening, grit removal, sedimentation, dissolved air flotation, filtration, oil-water separation, membrane processes and adsorption (De Sanctis *et al.*, 2016). These physical wastewater treatment methods are often combined with biological and chemical treatments to achieve efficient and comprehensive treatment. Selecting specific methods depends on the wastewater's characteristics and the treatment level required to meet regulatory standards or reuse purposes (Adewumi *et al*., 2010; Ntombela *et al.*, 2016). The main advantage of physical treatment methods is that they use simple equipment and can be adapted to several treatment formats (Crini and Lichtfouse, 2019).

2.2.2. Chemical treatment

The chemical treatment utilises chemical(s) to improve water quality. It is divided into various categories: chemical precipitation, neutralisation, adsorption, and disinfection (Cosa and Okoh, 2014). Chemical methods play a crucial role in treating wastewater and removing various contaminants. These methods involve using chemicals to facilitate the removal of pollutants through coagulation, flocculation, pH adjustment, oxidation, and other chemical reactions. Some standard chemical methods used in wastewater treatment include;

Coagulation and Flocculation: The coagulation process involves the use of chemical coagulants, such as aluminium sulphate (alum) or ferric chloride, which are added to wastewater to neutralise charges on suspended particles and destabilise them before distribution to the end-users (Verma *et al*., 2012).

As a result, smaller particles coagulate and form larger flocs, making it easier to separate them from the water during sedimentation or filtration. Aluminium salts, by far, are the most widely used coagulants in wastewater treatment (Ndabigengesere and Subba Narasiah, 1998; Ahmad *et al.*, 2005).

The **pH Adjustment** includes altering the pH of wastewater with pH-influencing chemicals such as lime and alum to improve the removal of certain contaminants. For example, adjusting the pH to alkaline conditions can precipitate heavy metals as metal hydroxides, aiding their removal. A study by (Gregor *et al.*, 1997) established this method by optimising the natural organic matter removal from low turbidity waters by controlled pH adjustment of aluminium coagulation, and they have observed that controlled pH maximised the soluble natural organic matter removal.

Chemical Precipitation: Specific chemical precipitants like iron salts and aluminium salts are added to wastewater to enhance the precipitation process of the dissolved contaminants as insoluble solids. This process is commonly used to remove heavy metals, phosphates, and anions such as fluoride, cyanide, and phosphate, as well as organic molecules such as the precipitation of phenols and aromatic amines by enzymes and detergents and oily emulsions by barium chloride (Nwodo *et al.*, 2014; Peng and Guo, 2020).

Chemical Oxidation: Oxidising agents like chlorine, hydrogen peroxide, ozone, and potassium permanganate break down organic compounds and other recalcitrant pollutants. Chemical oxidation helps degrade harmful substances and reduce their impact on the environment (Gregor *et al.*, 1997). A study by Ksibi (2006) indicated that using hydrogen peroxide as an oxidant effectively controls the organic load, offensive odour and foaminess in domestic wastewater treatment.

Adsorption: Activated carbon and other chemical adsorbents remove dissolved organic compounds, colour, and certain trace contaminants by attracting and binding them to their surfaces. Lu and Chiu (2006) investigated the adsorption of Zn^{2+} from water with purified carbon nanotubes. Their study reports that the carbon nanotubes were purified by sodium hypochlorite solutions and were employed as adsorbents to study the adsorption characteristics of zinc from water.

Ion Exchange: Ion exchange is a versatile separation process with the potential for broad applications in water pollution control. The process uses resins to exchange ions in the wastewater with ions of similar charge in the resin. This process is effective for removing dissolved salts, heavy metals, and some organic compounds and is applied in the purification of bioflocculants. A study by Jorgensen and Weatherly (2003) on ammonia removal from wastewater by ion exchange in the presence of organic contaminants revealed that organic compounds enhance the uptake of ammonium ions onto the ion exchangers. Al-Enezi *et al.* (2004) investigated ion-exchange extraction of heavy metals from wastewater sludge in another study. Their results showed high extraction efficiency, with almost 99% of heavy metals in wastewater effluents and sludge.

Disinfection: Chemical disinfectants, such as chlorine or chlorine dioxide, are added to the treated wastewater to kill harmful microorganisms before the water is discharged or reused. In the wastewater treatment industry, it is imperative to deactivate pathogenic microorganisms. Chlorine-based disinfection is used worldwide due to its high sterilisation spectrum, cost-effective, easy decomposition that leaves minimal residue and high inactivation efficiency (Azuma and Hayashi, 2021). It is noteworthy that chlorine is a strong oxidising chemical used to kill bacteria and slow down the decomposition rate of wastewater (Agunbiade *et al.*, 2019). Onsite chlorination is responsible for the effective disinfection of wastewater from a hospital study conducted by Azuma and Hayashi (2021). Their overall results indicated that chlorine disinfection effectively inactivated most antimicrobial-susceptible bacteria (AMSB) and antimicrobial-resistant bacteria (AMRB).

Chemical Neutralisation: The chemical neutralisation method is often applied to control the pH to the range required for the subsequent treatment. In some cases, wastewater may be acidic or alkaline due to industrial processes. The chemical neutralisation method is often considered due to being highly efficient and easy to control. HCl and NaOH are used as neutralisers in this method (Zhao and Chen, 2019)

2.2.3. Biological process

Biological processes are a fundamental component of wastewater treatment that effectively removes organic pollutants and some nutrients from wastewater. These processes rely on the activity of microorganisms, such as bacteria, fungi, protozoa and rotifers, to break down hazardous organic wastes using normal cellular processes to stable inorganic forms (Crini and Lichtfouse, 2019). The selection of a biological process depends on the specific wastewater characteristics, treatment goals, and regulatory requirements. Proper operation and maintenance are essential to ensure the efficiency and reliability of biological treatment systems. A biological process is a secondary stage in wastewater treatment that removes any leftover contaminants (Zhang *et al.*, 2013). Generally, biological treatment methods are divided into two processes, aerobic and anaerobic, based on the availability of dissolved oxygen (Buthelezi et al., 2010). These two terms are directly related to the type of bacteria or microorganisms involved in the degradation of organic impurities in each wastewater and the operating conditions of the bioreactor (Hedaoo *et al.*, 2012)**.** The aerobic treatment process occurs in the presence of oxygen, whereby microorganisms (aerobes) use available oxygen to absorb organic impurities and convert them into carbon dioxide, water, and biomass.

On the contrary, the anaerobic treatment process occurs in the absence of oxygen. The microorganisms (anaerobes) do not require oxygen to assimilate organic impurities (Hedaoo *et al.*, 2012). The final products of organic assimilation in anaerobic treatment are methane, carbon dioxide gas and biomass. Some of the common biological processes used in wastewater treatment include but are not limited to the activated sludge process, trickling filter, biological aerated filters, etc.

Activated Sludge Process: One of the most widely used biological processes, the activated sludge process involves mixing wastewater with a culture of microorganisms in aeration tanks. The microorganisms feed on the organic matter in the wastewater, breaking it down into carbon dioxide, water, and new microbial biomass. After aeration, the wastewater is separated from the activated sludge in a settling tank, and a portion of the sludge is recycled back to the aeration tank to maintain the microbial population. Activated sludge is also a good source for various bioflocculant-producing microorganisms. Tang *et al*. (2014) isolated *Enterobacter* sp. from activated sludge to produce a bioflocculant, while Li *et al.* (2010) also reported to have isolated *Agrobacterium* sp. M-503 from wastewater-activated sludge, and the bioflocculant yield reached 14.9 g/l.

Trickling Filters: In this process, wastewater is distributed over a bed of rocks or other media, allowing a thin film of microorganisms to grow on the surface. As the wastewater trickles through the media, the microorganisms remove organic matter through biological degradation. The treated wastewater then passes through a settling tank to separate the biomass from the effluent. A study by Kim *et al*. (2014) involved performance evaluation of a partially saturated vertical-flow constructed wetland with a trickling filter and chemical precipitation for domestic and winery wastewater treatment. In another study by Kornaros (2006), the effectiveness of a bio-trickling filter for treating wastewater from organic dyes was evaluated, and it was reported to be effective in removing COD by $60-70\%$ efficiency.

Biological Aerated Filters (BAF): BAF systems combine the principles of activated sludge and trickling filters. Wastewater passes through a filter media where a biofilm of microorganisms grows. Oxygen is supplied through aeration to support the microbial activity, and the biofilm breaks down organic matter as the water flows through the media. Hasan *et al.* (2009) conducted a review of the BAF process, focusing on designing an effective process for organic and inorganic contaminants removal in drinking water, particularly COD and Ammonia removal and according to their findings, the expected removal efficiency was within $80-90\%$. Another review by Dhokpande *et al*. (2014) confirmed that the BAF system could remove up to 90% COD and 99% nitrogen, respectively.

2.3. Technologies available for contaminant removal in wastewater

As mentioned in section 2.2, various wastewater contaminant removal technologies are developed and categorised broadly into conventional methods, traditional recovery processes and advanced treatment processes (Crini and Lichtfouse, 2019). The selection of a specific technology or method depends on factors such as the nature of contaminants, water quality standards, environmental regulations, and economic considerations (Zhang *et al.,* 2013). This study focuses on the conventional method of coagulation-flocculation process. Table 1 illustrates the classification of various technologies for contaminants and their processes.

Table 1: Classification of technologies for contaminants removal and their processes

Type of treatment	Method	Process	Technology Media	References
Physical	Screening	Mechanical separation of large debris and solids	Bar screens, rotary drum screens	Al-Enezi *et al*. 2004
	Sedimentation	Gravity settling of particles	Primary clarifiers, sedimentation tanks	
	Filtration	Passage of water through a porous medium to remove particles	Sand filters, multimedia filters, membrane filtration (e.g., ultrafiltration)	
Chemical	Coagulation and Flocculation	Addition of chemicals to induce the formation of flocs for easier removal	Coagulants (e.g., alum, ferric chloride), flocculants.	Crini and Lichtfouse, 2019
	Chemical Precipitation	Addition of chemicals to form precipitates for removal.	Lime precipitation, phosphate precipitation	
Biological	Activated sludge	Microbial digestion of organic matter in wastewater.	Aeration tanks, secondary clarifiers.	Tang *et al.*, 2014
	Bioreactors	Controlled environments for microbial treatment.	Moving bed bioreactors (MBBR), sequencing batch reactors (SBR).	
Advanced oxidation processes	Ozonation	Ozone reacts with contaminants to break them down.	Ozone generators, contact tanks	De Sanctis *et al.*, 2016
	Ultraviolet light radiation	Ultraviolet light generates reactive species for oxidation.	UV disinfection systems	
Membrane processes	Reverse osmosis (RO)	Semi-permeable membrane separates contaminants	RO units	Cosa and Okoh, 2014
	Ultrafiltration (UF) and Nanofiltration (NF)	Membrane filtration with different pore sizes	UF and NF systems	
Adsorption	Activated carbon adsorption	Contaminants adhere to the surface of activated carbon.	Activated carbon filters.	Crini and Lichtfouse, 2019
	Ion exchange	Exchange of ions between a solid phase and water.	Ion exchange resins	
Thermal	Incineration	Combustion of organic contaminants at high temperatures	Incinerators	
	Distillation	Vaporisation and condensation to separate water from contaminants	Distillation units	

2.4. Coagulation-Flocculation process in wastewater treatment

The stability and instability of suspended particles is one of the primary causes of attraction and repulsive forces such as electrostatic forces, Van der Waals forces, and Brownian movement. Coagulation is both a physical and a chemical process (Nadeem *et al*., 2020). Coagulants and particles interact to form aggregates that result in sedimentation. Suspended particles can deteriorate water quality. Thus, coagulation and flocculation processes are deemed crucial in wastewater treatment to overcome the forces stabilising the suspended particles by enabling the particles to collide and form flocs, resulting in clear water (Rebah *et al*., 2018). This process involves adding coagulants and flocculants to destabilise and aggregate contaminants, making them easier to remove during subsequent treatment steps (Agunbiade *et al*., 2016; Rebah *et al*., 2018).

- **Coagulation:** Coagulation is the first stage of the process, whereby particle destabilisation and charge neutralisation occur because of the addition of the positively charged ion of metal salt. The most commonly used coagulants metal salts include aluminium sulphate (alum), ferric chloride, and polyaluminum chloride (PAC). According to a study by Hu *et al.* (2006), PAC has been claimed to be superior to traditional coagulants such as $AlCl_3$ and Alum. When these coagulants are added to the wastewater, they dissociate into positively charged metal ions. These positively charged ions neutralise the negatively charged particles and colloids in the wastewater, causing them to come together and form larger particles called coagulates. A study by Gregor *et al*. (1997) optimised natural organic matter removal from low turbidity waters by controlled pH adjustment of aluminium coagulation.
- **Flocculation:** In the flocculation stage, a flocculant is added to the wastewater after coagulation. Flocculants are chemicals used to cluster colloidal particles, cells, and suspended solids into flocs of considerable size that can be removed effectively from the solution through sedimentation (Agunbiade *et al.,* 2019). Flocculation accumulates microbial cells to form flocs with other compounds present in the media (Dlamini *et al*., 2019). Flocculants are typically long-chain polymers that act as bridging agents, linking the coagulated particles to form visible flocs. The flocculation stage is vital in water and wastewater treatment technologies to eliminate organic and inorganic toxins (Okaiyeto *et al.*, 2015). Flocculants enhance solid and liquid separation in various industrial processes, including wastewater treatment (prior to sludge dewatering) and potable water purification (Sharma and Rout, 2011). Flocculation is a complicated process that involves various stages such as particle, bioflocculant mixing, attachment of bioflocculant molecules onto the particle surface, particle flocculation and floc breakup (Zhou *et al.*, 2017).

After the coagulation-flocculation stage, the wastewater enters the sedimentation basin, where the flocs settle to the bottom, forming a sludge (Okaiyeto *et al.*, 2015). The clarified water (effluent) is then separated from the settled solids and further treated through a tertiary treatment to achieve the desired

water quality standards (Maliehe *et al.*, 2022). The effectiveness of the coagulation-flocculation process depends on various factors such as the type, dosage, pH of the wastewater and the characteristics of the contaminants present. Proper control and optimisation of the process are essential to ensure efficient removal of pollutants and optimal wastewater treatment performance (Ahmad *et al*., 2005).

Coagulation-flocculation is often a crucial step in the treatment train, especially in conventional wastewater treatment plants, where it precedes secondary biological treatment and other advanced treatment processes. According to Kurniawan *et al.* (2022), the flocculation process depends on varying active compounds inside the bioflocculants, which corresponds to various mechanisms such as charge neutralisation, sweep coagulation involving colloid entrapment and double layer compression, bridging and patch flocculation, figure 1 demonstrates how each mechanism behave to enhance flocculation (Agunbiade *et al.*, 2017). Various studies detail the characteristics of bioflocculants based on their uniqueness, performances and mechanisms (Hasan *et al.*, 2012).

2.4.1. Coagulation - flocculation mechanisms

2.4.1.1. Charge neutralisation mechanism

The charge neutralisation mechanism is frequently implicated in the flocculation process by bioflocculants (Maliehe *et al.*, 2016). This mechanism occurs when the negative charges on the adsorption site are neutralised by a positively charged bioflocculant (Lee *et al*., 2014). This promotes the electrostatic interaction between positively charged bioflocculant and colloids. Thus, it causes attraction and charge neutralisation of the colloids' surfaces, resulting in flocs formation and reducing their electrical repulsion to one another (Aljuboori *et al.*, 2015). Macromolecules, including proteins, polysaccharides and various functional groups, are known to promote the process of charge neutralisation (Koul *et al.*, 2022). Figure 1 illustrates the charge neutralisation mechanism whereby the oppositely charged flocculants are mixed to neutralise the electrostatic repulsion to encourage the formation of flocs. As the surface of the colloidal particles is usually negatively charged, it is recommended to use inorganic flocculants to enhance the flocculation (Koul *et al.*, 2022). The functional groups such as hydroxyl and carboxyl can be ionised in the suspension, thus releasing a positively charged bioflocculant particle that will encourage the charge neutralisation (Abu Bakar *et al.*, 2021a). The success of this mechanism is dependent on the molecular weight, the charge of the flocculant, the ionic strength of the suspension, and the nature of mixing the flocculant process (Agunbiade *et al.*, 2017; Joshi *et al*., 2020). A study by Zhang *et al.* (2013) indicated that the flocculating mechanism investigation attributed that the sludge bioflocculant caused kaolin suspension instability by means of charge neutralisation, subsequently; aggregation of suspended particles was promoted by adsorption and bridge. Aljuboori *et al.* (2015) studied the flocculation behaviour and mechanism of bioflocculant produced by *Aspergillus flavus*, and their findings confirmed that the primary flocculation mechanism attributed was charge neutralisation. This was due to IH-7 being a

cation-independent bioflocculant with a positive charge that can neutralise and destabilise negatively charged particles via adsorption on the bioflocculant.

2.4.1.2. Sweep coagulation mechanism

The sweep coagulation mechanism is one of the most widely used mechanism for destabilising a turbid suspension as it involves an addition of a flocculant or coagulant to the wastewater (Abu Bakar *et al.*, 2021a). Figure 1 shows the process of a sweep coagulation mechanism. Sweep coagulation with colloid entrapment requires the addition of large portion of inorganic flocculants viz. aluminium hydroxide ($Al(OH)_3$) or ferric hydroxide ($Fe(OH)_3$) to water at a concentration that is significantly high enough to promote the precipitation of amorphous metal hydroxide, colloid particles can then be entrapped in these precipitates, subsequently separated from the clean water (Nan *et al.*, 2016). Destabilisation and transport are involved in this type of flocculation (Okoh, 2010; Suopajarvi, 2015). Gentle mixing usually enhances the process of sweep coagulation, which helps distribute the flocculant evenly and ensures thorough contact between the particles (Kurniawan *et al.*, 2022). The advantage associated with this mechanism is that, in low turbidity water, an addition of a large amount of precipitate particles increases the probability of contact with wastewater colloids and enhances floc growth (Nan *et al.*, 2016). Das *et al*. (2021) indicated that this method of coagulation was mainly independent of the chemical nature of the colloids, a characteristic that enhances its efficiency. When Li *et al.* (2014) investigated factors influencing coagulation performance and floc characteristics on compound bioflocculant and polyaluminum chloride in kaolin-humic acid coagulation, their study reported that more compact flocs were generated under alkaline conditions due to the sweeping effect of hydrolysed aluminium species. In another study conducted by Priya *et al.* (2018) on the effect of bioflocculants on the coagulation activity of alum for the removal of trihalomethane precursors from low turbid water, their result showed that the combination of alum and C. *tetragonoloba* was more efficient in reducing trihalomethane surrogates from chlorinated water as compared to M. *oleifera*. C. *tetragonoloba* elicited synchronised effects of sweep coagulation and particle bridging-adsorption, which eventually facilitated the efficient removal of hydrophobic fractions of natural organic matter (NOM).

2.4.1.3. Bridging mechanism

The bridging mechanism is a process whereby a long chain-like macromolecular flocculant with oppositely charged colloids comes together to form a bridge, thus promoting flocculation by neutralising the suspended particle's repulsion (Joshi *et al.*, 2020). High molecular-weight polymers with a low charge density adsorb on the surface so that long loops extend to the second (Joshi *et al.*, 2020). An essential requirement for the bridging mechanism is that there should be an unoccupied surface on a particle to attach segments of polymer chains already adsorbed on other particles. This permits polymers to interact or attach, forming a bridge between the colloids (Agunbiade *et al.*, 2016). In a study conducted by Ma *et al.* (2019), it was reported that polymer bridging was the best mechanism to enhance flocculation in the production of a bioflocculant from *Klebsiella* sp. OS-1 using wastewater

as the source. Furthermore, polymer bridging was reported to be the main mechanism during turbidity removal in isolation and characterisation of bioflocculant-producing bacteria from aquaculture effluent and its performance in treating high turbid water (Abu Bakar *et al.*, 2021a). In addition, another study by Li *et al.* (2014) on the effect of solution pH on the aid effect of compound bioflocculant (CBF) in dye wastewater treatment combined with widely used alum coagulants, their findings suggested that adsorption and bridging effect of CBF performed a positive role in dye wastewater treatment.

2.4.1.4. Electrostatic patch flocculation mechanism

Electrostatic patch flocculation is a widely used process in which a polyelectrolyte with a high charge density and a low molecular weight adsorbs on negatively charged surfaces with a low density of charged sites (Zhou and Franks, 2006). During the addition of oppositely charged particles, the electrostatic attraction is assumed to be the main driving force for adsorption and further mechanisms (Bache *et al.*, 1999; Zhou and Franks, 2006).Thus, the direct electrostatic attraction between oppositely charged patches promotes flocculation (Agunbiade *et al.*, 2016, 2017). Several methods may stimulate flocculation, including metals such as alum and ferric chloride, which are often utilised coagulants. When these metal ions are decanted into the water, they usually dissociate and induce flocculation through charge neutralisation (Kurniawan *et al.*, 2020a). A review on application of flocculants in wastewater treatment reported that flocs produced in the patch flocculation mechanism are not as strong as those produced in the bridging mechanism. However, they are stronger than flocs formed in the presence of metal salts or charge neutralisation (Lee *et al.*, 2014). Unlike the bridge mechanism, electrostatic patch flocculation is independent of particle concentration. Lin *et al.* (2008) studied the coagulation dynamics of fractal flocs caused by enmeshment and electrostatic patch mechanisms. Their findings showed that poly aluminium chloride (PAC) coagulation favoured electrostatic patch at the low dosage or charge neutralisation at the high dosage. Furthermore, Haasler *et al.* (2023) evaluated the flocculation mechanism and dewatering performance. Their observations highlighted that electrostatic patch flocculation was the favoured mechanism due to high charge density (CD) and low intrinsic viscosity of the biopolymers.

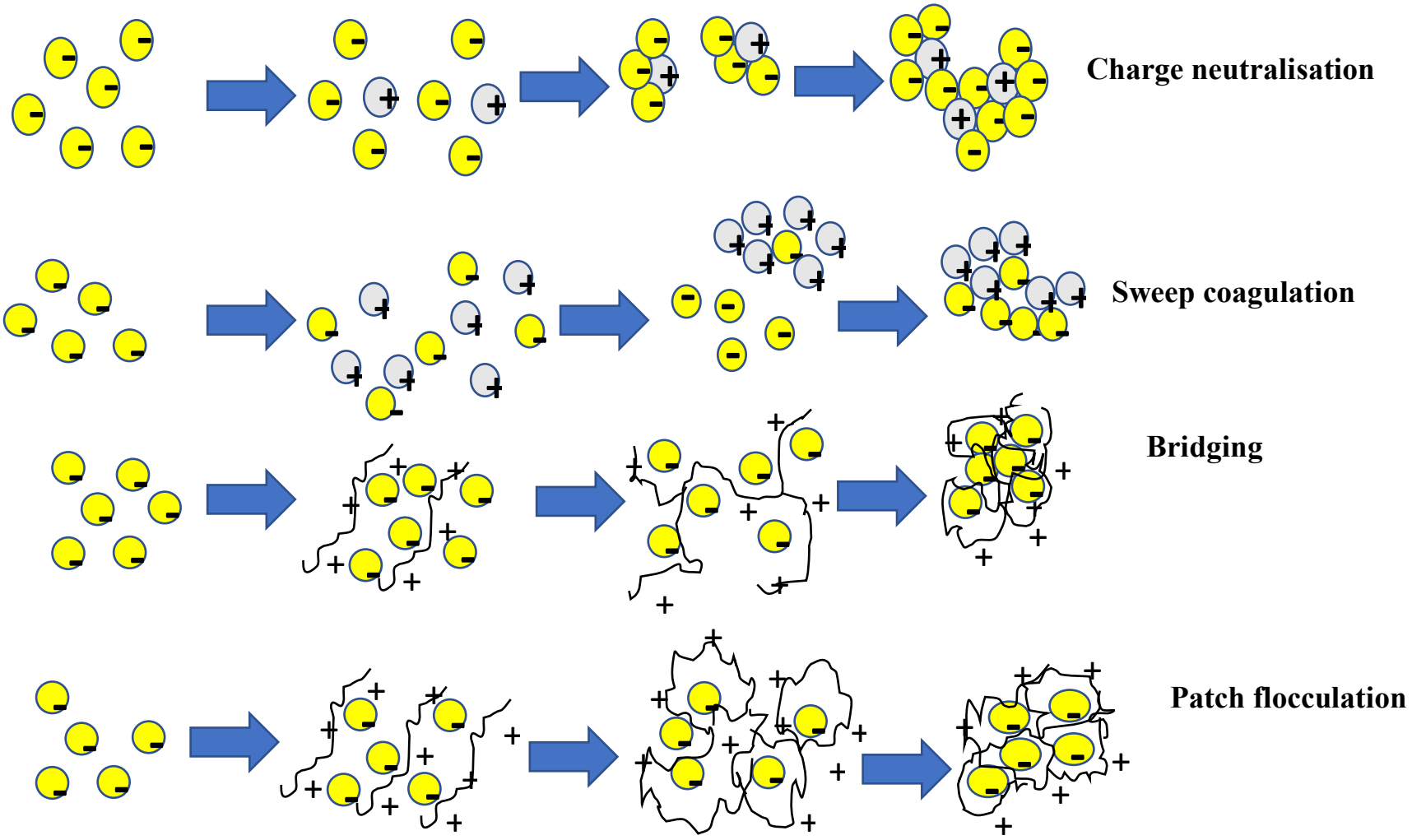

Figure 1: Coagulation-flocculation mechanisms (Okaiyeto *et al*., 2015)

2.5. Classification of flocculants

Flocculants are well established, readily available and are wildly used for various water and wastewater treatment (Okaiyeto *et al.,* 2015). Flocculants are divided into three categories viz. inorganic, organic and natural flocculants, as illustrated in figure 2 and table 2.

2.5.1. Chemical flocculants

Both inorganic and organic flocculants are referred to as chemical flocculants. Chemical flocculants are commonly used in water and wastewater treatment industries due to their advantages, that include high flocculating efficiencies, cost-effectiveness and commercial availability (Aljuboori *et al.,* 2015). However, the extent of their usage poses a dangerous concern to the environment, humans, and aquatic life (Agunbiade *et al.*, 2017, 2019). Chemical flocculants are associated with a few drawbacks due to their ability to generate secondary pollution, such as large volumes of toxic sludge, low biodegradability, and water pollution by metals (Salehizadeh *et al.,* 2018). The accumulation of alum content in the environment is implicated in causing neurological diseases. Polyacrylamide degraded monomers are carcinogenic and non-biodegradable, thus, toxic to downstream users (Joshi *et al.,* 2020).

2.5.2. Inorganic flocculants

According to literature, inorganic flocculants such as polyaluminium chloride (PAC), ferric chloride, aluminium sulphate and ferrous sulphates are commonly used in potable water treatments more than wastewater water treatment (Joshi *et al.*, 2017). The majority of suspended solid particles in wastewater are usually negatively charged, thus, the salts of these metals are ionised when added to wastewater, forming cationic charges that can attach to the negatively charged suspended particles (Lee *et al.,* 2014). Therefore, the flocculation mechanism associated with inorganic flocculants is charge neutralisation (Mubarak *et al.,* 2019). Compared to other inorganic flocculants, ferric chloride is cheaper, and only a small amount is required for treatment. Inorganic flocculants vary based on their small or high molecular weight (Mubarak *et al.,* 2019). Inorganic flocculants with small molecular weight are least utilised due to their poor flocculation efficiency (Salehizadeh and Shojaosadati, 2001), whereas, inorganic flocculants with high molecular weight are frequently used, their complex structure promotes an effective and high flocculation efficiency (Salehizadeh *et al.*, 2018). Inorganic flocculants are sensitive to pH changes and generate excess sludge in the environment. Consequently, metal ions from such sludge penetrating groundwater are of serious concern (Maćczak *et al.,* 2020). Liang *et al.* (2009) used ferric chloride as a coagulant to remove colour and chemical oxygen demand (COD) from molasses effluent. Their results indicated that under the optimum conditions, up to 86% of COD, and 96% colour removal efficiencies were achieved, and charge neutralisation was proposed as the predominant coagulation mechanism.

2.5.3. Organic flocculants

Organic flocculants are predominantly used in wastewater treatment to enhance the flocculation of suspended solids (Joshi *et al.,* 2020). The flocculation mechanism of organic flocculants is charge neutralisation (Mubarak *et al.,* 2019). Organic flocculants are either natural organic flocculants or synthetic organic flocculants. They are usually the preferred flocculants compared to inorganic due to being inert when pH varies, easy handling and high efficiency with low dosage (Sharma *et al.,* 2006). Synthetic organic flocculants are produced from a variety of monomers, including polyacrylamide, polyacrylic acid, diallyldimethyl ammonium chloride (DADMAC), and styrene sulphonic acid. In contrast, natural organic flocculants are produced from natural polymers such as starch, cellulose, natural gums and mucilages and their derivatives (Crini and Lichtfouse, 2019). Synthetic organic flocculants are the main flocculants used in industrial applications since they can produce large, dense, compact flocs that are stronger and have better settling characteristics than those obtained by coagulation. These organic flocculants are also easy to handle and immediately soluble in aqueous systems. However, they are associated with several environmental and health drawbacks (Sharma *et al.*, 2006; Crini and Lichtfouse, 2019). According to Mubarak *et al.* (2019), natural organic flocculants consist of *Moringa oleifeira* seed, while synthetic organic flocculants consist of polyelectrolytes. A study by Nwodo *et al.* (2014) confirmed that acrylamide and its derivatives have a high affinity for mammalian sperm cells, causing genetic damage with high efficiency during sperm cell development.

2.5.4. Natural flocculants

Natural flocculants are organic, biologically produced polymers such as chitosan, cellulose, sodium alginate, tannin, and microbial flocculants. Natural occurring flocculants are different, and they are classified based on their biological origins, for example, plant-based bioflocculants such as tannin, cellulose, and alginate (Xia *et al.*, 2008). Animal-based flocculants such as chitosan are produced from the deacetylation chitin, a natural polymer of major importance (Agunbiade *et al*., 2016). The main sources include two marine crustaceans, shrimps and crabs. Chitosan has unique characteristics among biopolymers, including the ability to act as a flocculant and a coagulant, owing to the presence of main amino groups, and it is a commercially attractive flocculant due to its higher nitrogen concentration than cellulose (Nadeem *et al.,* 2020). Chitosan is applied in various industries, including water and wastewater treatment because it carries advantages such as biodegradability and non-toxicity and is a renewable resource (Xia *et al.*, 2008). Sodium alginate is a water-soluble anionic polymer produced from the sodium salt of alginic acid with a molecular weight of approximately 500.000 (Okaiyeto *et al.,* 2015). Wu *et al.* (2012) examined its flocculating capability together with aluminium sulphate as the coagulant in the treatment of synthetic dye wastewater. They found that it exhibited strong flocculating rates of about 93.4% and 80.1% for maximum colour removal and COD reduction, respectively. Tannin is an anionic polymer reported to be a safer flocculant derived from secondary metabolites of vegetables, fruits, tree leaves and others (Okaiyeto *et al.,* 2015). Alcides *et al.* (2019) optimised the

coagulation/flocculation treatment of brewery wastewater with vegetable tannin as a flocculant. Their study reports that vegetable tannin substantially removed approximately 99% of turbidity and apparent colour. These naturally occurring flocculants from renewable biomass are widely used in water and wastewater treatment. However, they are reported to exert weak activity and application even though they are cheap and environmentally friendly as they pose no secondary pollution (Joshi *et al.*, 2017). This study focuses on microbial flocculant production from the marine environment.

2.5.5. Microbial flocculants (Bioflocculants)

In recent years, the demand for biopolymers for various industrial applications has influenced a vast interest in the research field pertaining to the production of Extracellular Polymeric Substances (EPS) (Okaiyeto *et al.*, 2016a). EPS carry complex properties such as long chain and high molecular weight (MW). Microbial flocculants are macromolecular substances produced by microorganisms such as fungi, bacteria, yeast, algae, and *Actinomycetes* are bioflocculant producers that have gained widespread attention in the biotechnology field (Agunbiade *et al.*, 2018). Most microbial flocculants are proteins, polysaccharides, DNA, cellulose and glycoprotein, produced by microorganisms during fermentation, and nucleic acids released primarily through cell lysis (El-Gaayda *et al.*, 2021). The production of microbial flocculants is highly affected by mainly physiochemical parameters, viz., media constituents and growth conditions (He *et al*., 2004). The nutritional constituents such as carbon source, nitrogen source, pH of the production medium, shaking speed, culture time, ionic strength, incubation temperature, metal ion and inoculum size are the primary factors extensively considered for bioflocculant production (Okaiyeto *et al.*, 2016b). Thus, these factors have been widely investigated due to their influence on bioflocculant yield (Bao-jun and Jiang-mei, 2012). Due to their advantages, these naturally occurring flocculants have gained increasing attention for water and wastewater treatment, and they are reported as promising alternatives to chemical flocculants (Okaiyeto *et al*., 2015). They are of great interest because they are safe and biodegradable, and the produced sludge of organic nature can be degraded by microbes and reused in agriculture to improve soil fertility (Yang *et al.*, 2009). Table 2 shows the benefits and limitations of flocculants.

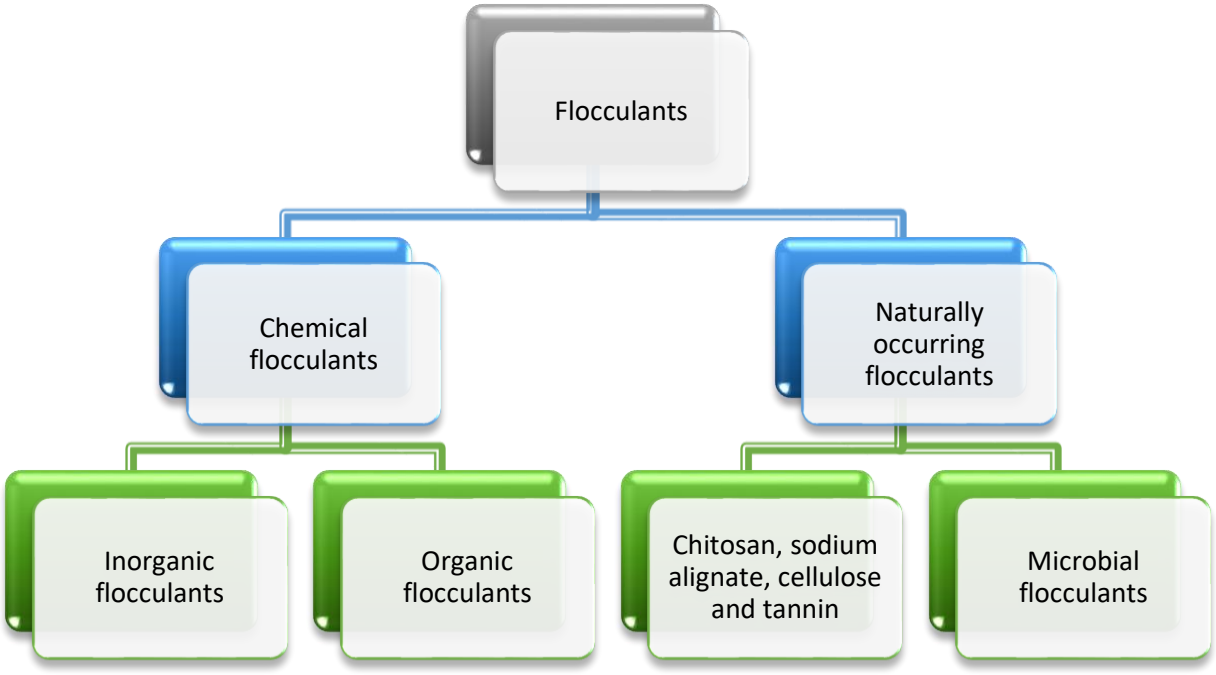

Figure 2: Categories of flocculants (Agunbiade *et al*., 2017, 2019)

Table 2: Benefits and limitations of flocculants

Flocculant type	**Main ingredients**	**Benefits**	**Limitations**	**References**
Inorganic	Aluminium salt polymers and iron salt polymers, ferric chloride	Cost effective Readily available	Secondary pollution Non-degradable	Li *et al.*, 2020
Organic	Poly acrylamide and their derivatives	Rapid flocculation, Less dosage, Easy to extricate	Secondary pollution Difficult to degrade	Liu *et al.*, 2021
Microbial	Microbes and their metabolites	Biodegradable, safe and effective	Environmental weakness High production costs	Zhong *et al.*, 2020

2.6. Bioflocculants overview and prospective application

Bioflocculation is an essential step in the treatment of wastewater which occurs when stable aggregates are formed by extracellular polymer substances (EPS) produced by various living cells to reduce turbidity, organic load and other pollutants (Buthelezi *et al.*, 2012; Alias *et al.*, 2022). Bioflocculants have emerged as promising alternative materials to replace traditional flocculants in recent years as the demand for environmentally friendly materials in wastewater treatment has increased (Lee *et al.*, 2015). Bioflocculants are biodegradable polymeric compounds whose degradation products are harmless to the ecosystem as they pose no secondary pollution (Okaiyeto *et al.*, 2020). Bioflocculant-producing microorganisms such as bacteria, *Actinomycetes*, algae, and fungi have been isolated and screened from various environments, including soil, river, marine sediments, salt pans, biological sludge, kombucha tea, and wastewaters (Agunbiade *et al.*, 2019; Ayangbenro *et al.*, 2019; Zhong *et al.*, 2020; Tsilo *et al.*, 2021). Some of these Bioflocculant-producing microorganisms are stipulated in table 3 and their requirements. The characteristics qualifying these microorganisms as bioflocculant producers are polysaccharides, protein polymers, and some functional groups, including hydroxyl and carboxyl groups (Kurniawan *et al.*, 2020). Polysaccharides, proteins, and some functional groups stimulate the process of adsorption, polymer bridging, and charge neutralisation (Hassimi *et al.*, 2020).

Furthermore, the marine environment is reported to be a good reservoir of microorganisms as it is a suitable growth environment for bacterial isolates with unique properties to function under adverse conditions such as pressure, salinity, low temperature, and low nutrient concentrations (Awolusi *et al.*, 2020; Joshi *et al.*, 2020). These properties affect microbial diversity in morphological, physiological, and metabolic adaptation. In contrast to those terrestrial microorganisms, they are designed to survive under extreme conditions. The compounds secreted by these bacteria have robust properties that can be important in various industrial processes (Joshi *et al.*, 2020). The discovery and application of microbial flocculants have been more than 100 years as Louis Pasteur first reported the flocculation process in microorganisms in 1876 while studying yeast (Tawila *et al.*, 2018). Since then, the study of microbial flocculants has attracted more attention recently, with many bioflocculant-producing microorganisms. When the bioflocculant is added to the water to remove toxin, the repulsive energy is between the particles is reduced primarily by the compression of the electric double layer (Joshi *et al.*, 2017). Charge neutralisation, adsorption bridging effect, and net capture style eventually form agglomeration (Joshi *et al.*, 2020).

Bioflocculants are charged biopolymers, and their mechanism includes bridge action, charge neutralisation, and rolling effect. Adopting biotechnological methods to produce bioflocculants solely depends on the possibility of using different microorganisms to synthesise extracellular substances with different compositions. The use of chemical flocculants in wastewater treatment is widely established, as they have advantages such as high flocculating efficiencies and cost-effectiveness. However, they are often associated with several drawbacks, such as being prone to cause Alzheimer's disease and

neurotoxicity (Ntozonke *et al.*, 2017). The performance efficiency of the produced bioflocculant is evaluated by measuring the bioflocculant's ability to remove chemical oxygen demand (COD), pigments, biological oxygen demand (BOD) and suspended solids (SS) (Salehizadeh and Shojaosadati, 2001).

Several reports in the literature outline the production of bioflocculants by various microorganisms, viz. *Chryseomonas luteola, S.platensis, Oceanobacillus, P.pseudoalcaligenes* and K. *terrigena* utilised in the removal of turbidity and bacterial load reduction from river waters (Syafalni *et al.*, 2012; Cosa and Okoh, 2014; Nwodo *et al.*, 2014; Abd El-Salam *et al.*, 2017; Joshi *et al.*, 2017; Agunbiade *et al*., 2018; Joshi *et al*., 2020; Mohammed and Dagang, 2020). The application of microbial flocculants has been vastly explored in the research field regarding treating different types of wastewater such as municipal, industrial, tannery, brewery, etc. They are also implicated in drinking water treatment, dye removal and sludge dewatering (Pathak *et al.*, 2014).

Generally, bioflocculants are ideal for treating potable water due to their biodegradable and functional nature (Awolusi *et al*., 2020). A study by Li *et al.* (2009) produced a bioflocculant from *Bacillus lichenformis* X14, which affirmed its application in low temperature drinking water treatment. The bioflocculant revealed a good flocculation performance of 99.2%. Furthermore, it demonstrated good industrial potential for the treatment of low temperature drinking water. Thus, the maximum removal efficiency of COD and turbidity were 61.2% and 95%, respectively. Another study by Buthelezi *et al.* (2012) reported that the application of a bioflocculant produced by indigenous bacterial isolates was evaluated in textile dye removal from wastewater. They found that bioflocculants produced from indigenous bacteria were very effective for decolourising different dyes tested. A maximum removal efficiency of 97.04% was attained.

Pathak *et al.* (2015) produced a non-cytotoxic bioflocculant from a bacterium utilising petroleum hydrocarbon source and validated its application in heavy metal removal. The bioflocculant was efficient in removing heavy metals such as $Ni^{+2}, Zn^{+2}, Cd^{+2}$ and Pb^{+2} with a removal efficiency of 76.14 ± 0.11; 62.69 ± 0.48; 53.22 ± 0.04; 47.64 ± 0.47 and $40.58 \pm 0.28\%$, respectively. In addition, a study conducted by Abu Tawila *et al.* (2019) revealed that a bioflocculant QZ-7 from *Bacillus salmalaya* 139SI was efficient for heavy metals removal from industrial wastewater.

Due to growing environmental awareness, the positive advantages of bioflocculants enable them to be the best candidates for wastewater treatment (Joshi *et al.*, 2020). Selepe *et al.* (2022) isolated marine bioflocculant-producing bacteria from *Ochrobactrum oryzae* and evaluated its removal efficiency on pollutants in wastewater. The flocculation efficiency of the bioflocculant was 92%, and the removal efficiency was 98% COD, 91% BOD and 86% sulphur. Therefore, their bioflocculant demonstrated a potential for pollutant removal from industrial wastewater. According to Zhang *et al.* (2013), compared to conventional chemical flocculants, bioflocculants revealed the best performance for the wastewater

treatment . It is worth noting that specific bioflocculants may have varying effectiveness and applications depending on their composition, source, and intended use.

Table 3: Microorganisms implicated in bioflocculant production and their requirements

Strain	pH (optimal level)	Metal ions	Thermo-stability (℃)	Flocculation activity (%)	References
Bacillus aryabhattai	2-10 (7)	Ca^{2+} independent	40-80	91	Abd El-Salam *et al.*, 2017
Bacillus samalaya	7	Ca^{2+} independent	20 – 80	83 – 92.6	Tawila *et al.*, 2018
***Klebsiella* sp.**	2.1	Stimulated by Mg^{2+}, Fe^{2+}, Zn^{2+}, K^{+} and inhibited by Ca^{2+}	Stable	76.2	Ma *et al.*, 2019
***Terrabacter* sp**	2 – 11 (8)	Ca^{2+}	30 – 50	85	Agunbiade *et al.*, 2019
Klebsiella pneumoniae	4-12 (4.44)	Ca^{2+} independent	30 – 60	81-85	Joshi *et al.*, 2017
***Citrobacter* sp.**	2 – 8 (6)	Cation independent	3 – 96	95	Joshi, Kumar and Mody, 2020
Bacillus velezensis	7	Ca^{2+}	20 – 100	92.3	Alias *et al.*, 2022
Pseudomonas aeruginosa	7	Ca^{2+}	40 – 100	80.5	Joshi, Kumar and Mody, 2020
Nocardiopsis sp.	7	Ca^{2+}	Stable	80.6	Rajivgandhi *et al.*, 2021
D. *nitroreducens*	8	Ca^{2+}, Mg^{2+} Na^{+}	5 - 90	95	

2.6.1. Extraction of bioflocculants

Extraction is the primary step in separating the desired bioflocculants from the raw materials. Extraction methods of bioflocculants vary depending on the source of the microorganism, the expected yield and the intended application considering that extraction methods for bioflocculants are unique (Guo *et al.*, 2018). Consequently, there are various methods associated with the extraction of bioflocculants that are classified as physical and chemical extraction methods. Physical extraction methods comprise centrifugation, filtration, sonication (ultrasound-assisted), and heating (hydrothermal and microwave-assisted). In contrast, chemical extraction methods include cation exchange resin, ethylene diamine tetra acetic acid (EDTA), alkaline/acid treatment, enzyme assisted, and solvent extraction (water, ethanol, salt solution, acid and alkali extraction) (Abu Bakar *et al.*, 2021). According to Siah (2017), the selection of extraction methods is critical in bioflocculant production process since it determines both yield and cost of production of the biolocculant. Water extraction, ethanol extraction, hydrothermal and microwave extraction, and centrifugation are some of the extraction methods commonly used in bio-flocculant production (Nwodo *et al.*, 2014). An ideal method for extraction is imperative for the analysis of physiochemical properties associated with the bioflocculant produced. Thus, this study will focus on solvent extraction (discussed in chapter three).

2.6.1.1. Solvent extraction method

The solvent extraction method is commonly used in the extraction of plant-based or microorganisms-based bioflocculants. It is often regarded as the main conventional extraction technique that uses one or a combination of two solvents (Abu Bakar *et al.*, 2021). This type of extraction is known as liquid-liquid extraction. It mainly uses water as the primary solvent followed by an organic solvent such as hexane, dichloro-methane of ethanol or chloroform and n-butyl alcohol as the secondary solvent (Schlesinger *et al.*, 2011).

This extraction process occurs in four stages, viz.

i. the solvent infiltrates into the matrix of the substance,
ii. the solvents dissolve the solutes,
iii. diffusion of the solute from the solid matrix and
iv. the collection of extracted solutes (Zhang *et al*., 2018)

The characteristics of the extraction solvent strongly influence the extraction efficiency, the quantity of the raw materials particles, the solvent-to-solid ratio, the extraction temperature and the extraction time (Zhang *et al*., 2018). Solvent extraction includes various methods such as water, ethanol, salt solution, and acid and alkali extraction. Abu Bakar *et al.* (2021) observed that the solvent extraction often uses water, ethanol, and salt solutions especially cetylpyridinium chloride solution (CPC) and sodium chloride (NaCl) as well as acid and alkali solvents such as hydrochloric acid (HCl) and sodium

hydroxide (NaOH). This study focuses on the ethanol extraction method that is further explained in chapter three.

2.6.1.2. Ethanol extraction method

Ethanol extraction methods are widely used since they have the ability to separate water and lipo-soluble components (Sun *et al.*, 2012). Compared to water extraction methods, recovering bioflocculants through ethanol extraction methods can extend the lifetime of the extraction solution. Tawila *et al.* (2018) reported that the ethanol extraction method was utilised in the extraction of a bioflocculant from *Bacillus salmalaya* 139SI-7. The fermented culture containing the bioflocculant was centrifuged for 15 min at 3500 rpm to separate pelleted bacterial cells. Subsequently, the extracted supernatant was mixed with one volume (v/v) of sterile distilled water, followed by centrifugation for 15 min at 3500 rpm to remove insoluble materials. The supernatant was mixed with two volumes of cold ethanol. The sample was thoroughly mixed with a stirrer and allowed to stand at 4℃ for 12 h. Subsequently, the precipitate was extracted, and the obtained crude polymer was dissolved in sterile distilled water for further processing. The maximum flocculating activity obtained by Tawila *et al.* (2018) was 83.3% for *B. salmalaya* strain 139SI-7. Furthermore, Agunbiade *et al.* (2017) also utilised an ethanol extraction method in their study to extract a bioflocculant from *Arthrobacter humicola*. They centrifuged culture broth at 8000 rpm for 30 min prior to mixing the supernatant with distilled water and then centrifuged for 15 min. Subsequently, two volumes of ethanol were later added to the supernatant, stirred and left to stand for 12 h at 4 ℃. The precipitate obtained was vacuum-dried to obtain crude bioflocculant. Agunbiade *et al.* (2017) reported that the maximum flocculating activity obtained in the study was 85%. In addition, Devi and Natarajan (2015) extracted a bioflocculant from *B.licheniformis* and *B.firmus* using two volumes of ethanol, methanol and acetone (1:2 v/v). Comparing the three organic solvents used for extraction, the highest bioflocculant yield attained was through ethanol extraction, followed by acetone then methanol. This further proves that comparative to other organic solvents, ethanol extraction is the best method when extracting bioflocculants from microorganisms, thus it was applied in this study.

2.6.2. Purification of bioflocculant

Purification is the final stage in producing a bioflocculant as it plays a major role in eliminating undesired substances from the extracted bioflocculant (Abu Bakar *et al.*, 2021). The purification process is usually carried out through lyophilisation, chromatography, and dialysis as summarised in Table 4. Zhang *et al.* (2013) considered the dialysis method for the purification of a bioflocculant from biological sludge. Double volume of ethanol was used to precipitate the crude bioflocculant, subsequently it was dissolved in 15 ml of hydrochloric acid and pH was adjusted to 7. The mixture was fed into dialysis bags, which were placed into a beaker, filled with flowing de-ionised water and the dialysis process lasted for 12 h. subsequently, the purified sludge bioflocculants was evaporated for 2 h for dryness to remove all water in vacuum by a rotary evaporator and vacuum drying overnight. Yang *et al.* (2017)

also used dialysis purification method coupled with lyophilisation. The bioflocculant was dialysed against de-ionised water overnight and then lyophilised to obtain purified bioflocculant produced from *Bacillus mucilaginosus* MY6-2. In another study, Li *et al.* (2010) used chromatography purification method, the crude bioflocculant was dissolved in distilled water and the active fraction was collected following a DEAE column chromatography and concentrated with PEG-2000. The purified active polysaccharide fraction was obtained using Sephacryl S-500 column chromatography, and 14.7 g/l yield was recovered. Ugbenyen and Okoh (2014), and Agunbiade *et al.* (2018) adopted the lyophilisation method to purify bioflocculants produced from a consortium of *Cobetia* and *Bacillus* species and *Streptomyces platensis* respectively. The process before lyophilisation entailed dissolving the crude bioflocculant into distilled water. Thereafter, a volume of chloroform and n-butyl alcohol (5:2 v/v) mixture was added. After stirring the mixture gradually, it was poured into a separating funnel and then left for 12 h at room temperature. After the supernatant was removed, two volumes of ethanol were again added to recover the precipitate, which was then lyophilised. Ugbenyen and Okoh (2014) attained a bioflocculant yield of 0.256 g/l while Agunbiade *et al.* (2018) attained a purified bioflocculant yield of 4.61 g/l. Okaiyeto *et al.* (2015) also used lyophilisation to purify a glycoprotein bioflocculant produced from *Bacillus toyonensis* strain AEMREG6. About 3.2 g/l of purified bioflocculant was attained. In the literature, lyophilisation purification method is commonly used compared to chromatography and dialysis due to lyophilisation process not entailing the use of heat which therefore ensures that materials remain un-degradable and maintain their stability in room temperature as well as prolonged shelf life (Abu Bakar *et al.*, 2021). Thus, lyophilisation was considered for the purification of a bioflocculant produced in this study.

Table 4: Different types of bioflocculant purification methods

Lyophilisation	Chromatography	Dialysis	References
• Dissolution of crude bioflocculant • Mixture with chloroform and • n-butyl alcohol • Stirring process • Separation process using a separation funnel • Addition of ethanol to the obtained pellet • Lyophilisation procedure	• pH adjusting • Utilisation of gigapite amphoteric column equipped with phosphate buffer • Purification • Resuspension into de-ionised water • Anion exchange chromatography • Elution	• Cold ethanol addition • pH adjusted to 8 • Continuous mixing • Storing overnight at 4℃ • Centrifugation • Washing and vacuum drying	Abu Bakar *et al.* (2021); Zhang *et al.* (2013); Agunbiade *et al.* (2018); Li *et al.* (2010)

2.6.3. Bioflocculant characterisation

Characterisation of a bioflocculant is mainly conducted to establish and understand it's physical, chemical and biological properties such as, surface morphology, structure, elemental compositions and the factors affecting it (Rajivgandhi *et al*., 2021). There are various techniques used to analyse the characteristics and these have been widely explored in previous studies. These techniques include Fourier Transform Infrared Spectroscopy (FTIR), Scanning Electron Microscopy (SEM), and Energy Dispersive X-ray Spectroscopy (EDX) (Okaiyeto *et al.*, 2015). FTIR is used to identify functional groups present in bioflocculants. Peaks in the spectrum correspond to specific vibrational modes of chemical bonds. FTIR helps determine the chemical composition of bioflocculants by identifying characteristic peaks associated with various organic functional groups (e.g., hydroxyl, carboxyl, amine, and phosphate groups) (Ugbenyen and Okoh, 2014). Furthermore, FTIR provides information about the molecular structure and conformation of bioflocculants, aiding in understanding their performance in flocculation processes. Chen *et al*. (2017) characterised a novel bioflocculant from a marine environment and determined its functional groups using FTIR spectra over a wavenumber range of $4000 - 500 cm^{-1}$. Their FTIR spectrum results showed the presence of hydroxyl, amide and carboxyl groups. FTIR also revealed the characteristic peaks for carbohydrates and amides further confirmed the bioflocculant produced by *Alteromonas* sp. CGMCC 10612 belonged to the glycoprotein group. Another study by Nwodo *et al*. (2013), explored the characterisation of an exopolymeric flocculant produced by a *Brachybacterium* sp and reported that FTIR showed the presence of carboxyl,

hydroxyl and amino groups, amongst others, typical for heteropolysaccharide and glycosaminoglycan polysaccharides.

SEM is a type of electron microscope that divulges the image of a sample by scanning it with a high-energy beam of electrons in a raster scan pattern. It allows for the visualisation of the morphology and surface characteristics of bioflocculants at a micro or nanoscale (Guo *et al*., 2013). In addition, SEM provides information on the size and shape of bioflocculant particles, which is crucial for understanding their flocculation behaviour. Furthermore, surface characteristics, such as roughness and texture, can be observed using SEM, providing insights into the physical properties of bioflocculants (Agunbiade *et al*., 2018). Similar to FTIR, SEM is widely used tool in the bioflocculant production research field. A study by Okaiyeto *et al*. (2015) confirmed that SEM revealed an amorphous structure for bioflocculant (MBF-UFH), produced by *Bacillus sp*. AEMREG7.

EDX is used to determine the elemental composition of bioflocculants by analysing the X-rays emitted when the sample is bombarded with electrons. EDX mapping can be employed to visualise the distribution of different elements within bioflocculant particles, helping to understand their elemental homogeneity. In addition, EDX can be used to identify and quantify impurities present in bioflocculants, contributing to the assessment of their purity and potential environmental impact (Arayes *et al.*, 2023). EDX has been widely explored in literature by various researchers such as (Okaiyeto *et al*., 2015; Agunbiade *et al*., 2018; Cosa *et al*., 2013). Apart from these techniques, the characterisation of a bioflocculant can also done by determining total protein content using bovine serum album (BSA). Sivasankar *et al*. (2020) reported this method on the bioflocculant produced by *Streptomycetes* sp. AUABF. Their results states that the yield, total sugar and protein contents of the bioflocculant were 4.94 g/l, 86.9% and 12.8%, respectively.

Thermogravimetric analysis (TGA) is a technique used in analytical chemistry to determine the changes in mass of a sample as a function of temperature or time. While TGA provides both qualitative and quantitative techniques, it can be complemented with other analytical methods for characterisation such as FTIR, SEM and EDX (Maliehe *et al.*, 2016). According to a study by Cosa *et al*. (2013), TGA exhibited a degradation temperature T_d of$\sim$140℃ with the flocculation efficiency of the bioflocculant at 86.2% compared with 82.6%, 74.5% and 70.9% for polyethylimine, ferric chloride and alum, respectively. The current study characterised the bioflocculant produced using FTIR, SEM, EDX, and TGA. The methods used are outlined in chapter 3.

2.6.4. Factors affecting bioflocculant production and flocculation activity

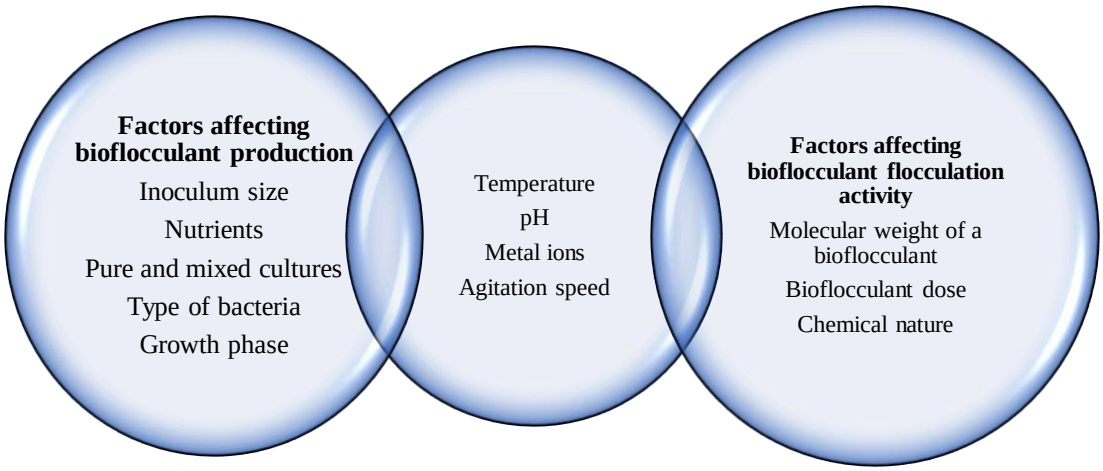

Figure 3: Factors affecting bioflocculant production and flocculation activity (Okaiyeto et al., 2016; Mohammed and Wan Dagang, 2019).

Bioflocculant production is highly influenced by culture medium compositions and several other physiochemical parameters (Okaiyeto *et al.*, 2016; Mohammed and Wan Dagang, 2019). Media constituents, growth conditions, and nutrient availability play a vital role in the cultivation stage of a bioflocculant (Rebah *et al*., 2018). According to literature, the effects of the nutritional components on the production of bioflocculants have been extensively studied, and the most important factors such as pH, carbon source, culture duration or incubation period, metal ions, production medium, agitation speed, nitrogen source, incubation temperature and inoculum size (Zhong *et al.*, 2020). The effect of pH and cations will not be established in this study as they have already been evaluated in the preceding study at BTech level, thus, previously obtained optimal pH level will be maintained in this study. Carbon and nitrogen sources play a significant role in stimulating the secretion of bioflocculants by microorganisms (Okaiyeto *et al.*, 2016). In the development process of a bioflocculant, these factors require optimisation to ensure high yields, productivity, and high flocculation efficiency (Agunbiade *et al*., 2016). Ren *et al.* (2013) optimised the growth medium to improve the flocculation rate of a compound bioflocculant **CBF-256** using a response surface methodology (RSM). They have reported that yeast extract, urea, glucose and soluble starch as carbon source and nitrogen source respectively, presented good 82.65 % flocculation results after optimisation, as well as the increase in bioflocculant yield from initial 2.31 g/l to 2.84 g/l. Furthermore, Zhou *et al.* (2017) reported that the optimisation of media constituents improved the novel glycoprotein production by *Streptomyces kanasenisi* as the

preferred millet medium contained soluble starch and yeast extract as carbon and nitrogen sources. RSM was used for further optimisation whereby a higher yield of 2.5 mg/l was attained. The growth condition varies on the type of organism screened for bioflocculant production. However, a suitable bioflocculant production medium would consist of glucose as a carbon source and yeast extract as a nitrogen source. This is confirmed by a study by Chouchane *et al.* (2018) which reported a novel thermally stable hetero-polysaccharide-based bioflocculant from hydrocarbonoclastic strain *Kocuria rosea* **BU22S**, glucose, peptone, and incubation time were identified as most significant factors which affected the bioflocculant production and a maximum of 4.72 g/l bioflocculant yield was attained. Furthermore, a small addition of phosphates and metal irons is essential (Mu *et al.*, 2019). During a bioflocculant production process, thermal stability is one of the vital parameters that aid the stability of the bioflocculant (Abd El-Salam *et al.*, 2017). Another study by Lian *et al.* (2008) evaluated the applications and mechanisms of a microbial flocculant produced by *Bacillus mucilaginosus* that was cultivated in a nitrogen-free medium. The bioflocculant was used to treat domestic, brewery and pharmaceutical wastewater, and ultimately, a good removal efficiency of 93.3% COD, 93.6% SS and 88.4% BOD was attained. Cations neutralise the negatively charged particles and form bridges between particles and polymers. Consequently, an increase in the accumulation of the molecular species at the surface will be rapid, thus promoting flocculation (Agunbiade *et al.*, 2019). The concentration dosage is one of the sensitive properties of a bioflocculant. Low or excess dosage concentration may lead to incomplete bridging mechanisms in a colloidal system (Joshi *et al.*, 2017). Furthermore, overloading the concentration dose of the bioflocculant changes the charge of the colloid solution and re-stabilise it. Both conditions may affect flocculating efficiency; thus, an optimum dose validation is essential before application (Joshi *et al.*, 2020). Furthermore, the high molecular weight of the bioflocculant provides more adsorption points, higher flocculation activity, and stronger bridging, resulting in low dosage requirements compared to low molecular weight bioflocculant (Joshi *et al*., 2020). The chemical composition of a bioflocculant defines the charge on its surface due to the functional groups that provide an adsorption environment for suspended particles. The number of functional groups in the molecular chain determines the efficiency of the bioflocculant, while its hydrophobic nature contributes to faster settling (Awolusi *et al.*, 2020).

2.6.5. Advantages of utilising bioflocculants

Given the increased demand for green environment and sustainable water treatment technologies, microbial flocculants have demonstrated positive advantages such as exhibiting secondary pollution, being environmentally friendly, reducing sludge production, reliable performance, minimal waste production, and producing potential sludge for sludge reuse (Kurniawan *et al.*, 2020), figure 4 displays a summary of these advantages. Various reports in the literature indicate that bioflocculants are suitable for utilisation in the treatment of drinking water and wastewater since they are environmentally friendly compared to chemical flocculants (Verma *et al.*, 2012; Crini and Lichtfouse, 2019; Dlamini *et al*., 2019;

Mohammed and Dagang, 2019; Xu, 2020; Abu Bakar *et al.*, 2021). According to Kurniawan *et al.* (2020), bioflocculants produce minimal to no harmful by-products and the environment degrades them naturally. Bisht and Lal (2019) reported that a bioflocculant produced by strain *Bacillus sp* exhibited a good flocculation activity of 99.8%. Another study by Tawila *et al.* (2018) on the bioflocculant produced by Bacillus salmalaya, 139SI-7 strain presented a flocculation activity of 83.6%. Current research explores other sources of bioflocculants production around the utilisation of waste, including food, agricultural and industrial waste. Consequently, this exercise may be beneficial to waste reduction (Kurniawan *et al.*, 2020). Using chemical flocculants, specifically aluminium salts, generate high volumes of non-biodegradable sludge (Okaiyeto *et al.*, 2016). On the contrary, using a bioflocculant results in up to 30% less sludge produced from treatment processes than using aluminium. According to Kurniawan *et al.* (2020), less sludge production benefits sludge handling sections. Bioflocculants leave no harmful residues on the treated effluent and pose no secondary environmental pollution. A study by Guo *et al.* (2015) indicated that bioflocculants had shown good performance in sludge dewatering. This sludge is highly biodegradable and may be further utilised as a soil fertiliser (Kurniawan *et al.*, 2020).

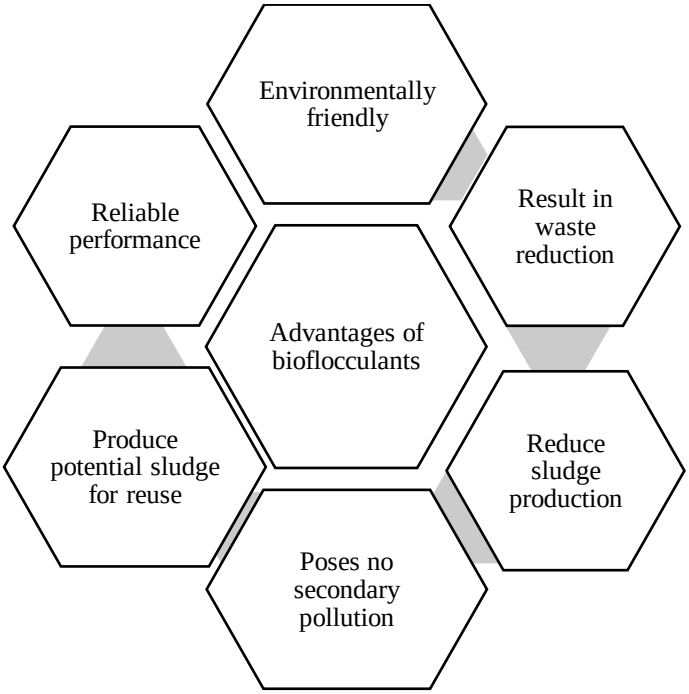

Figure 4: Advantages of utilising bioflocculants summary (Kurniawan *et al*., 2020)

2.7. Design of experiments (DOE)

Design Expert software is widely used for experimental design, modelling, optimisation and statistical analysis. It provides different types of programs, such as fractional factorial design, surface response, full factorial, mixing and custom designs (Usman *et al.*, 2021). Design of Experiments (DOE) is a powerful tool used for exploring new processes in terms of planning, designing, and analysing the process steps involved in designing a statistical experiment from which reliable, objective conclusions

may be drawn with the least number of laboratory testing (Ranga *et al.*, 2014). DOE analyses three different components of the process such as Factors (from independent variable to controlled variable), Levels (range of each factor) and Response (Output of the experiment). It is vital to incorporate simple and consistent statistical techniques into the experimental design process to obtain findings from the experiment that are statistically sound (Ruiz Espejo, 2006).

A designed experiment is a tool utilised for collecting data with characteristics that involve strategic testing, data analysis approach, simultaneous factor variability and scientific approach (Vanaja and Rani, 2007). Design of experiments benefits include determining the crucial variables, assisting with handling experimental error, reducing confounding effects, measuring interactions, allowing for a broad exploration of data and plotting graphs to describe the relationship between variables and illustrate at what level of variables provide the optimum product (Vanaja and Rani, 2007; Jin, 2016). Using statistical models measures the interrelationship between variables and screens a large number of variables to identify the significant ones. When the DOE method is applied in design, the functions are typically evaluated numerically. The numerical method does not have many errors and is a deterministic method (Anderson, 1997; Ranga *et al.*, 2014).

2.7.1. The general process of the DOE method involves

i. Define problems
ii. Defining the experiment objectives
iii. Selection of characteristic values
iv. Selection of factors and levels
v. Conducting experiments to study the factors in different combinations
vi. Analyse data
vii. Interpret the results
viii. Verify the interpreted results

Table 5: Description of terminologies utilised in DOE and Design models

Terminology	DOE/Design	Definition	References
Factor	DOE	Sources that influence the objective function and they can be altered to improve performance in a system	(Anderson, 1997; Ranga *et al.*, 2014)
Design variable	Design		
Level	DOE	Values that are contained by a factor or design variable	
Values of design variables	Design		
Characteristics	DOE	Responses in the system that can be maximised or minimised to obtain optimal product	(Vanaja and Rani, 2007; Jin, 2016)
Objective	Design		
Function	DOE/Design		

Various terms in DOE correspond to those in design and these are shown in table 5. DOE consist of three principles that involve randomisation, replication, and blocking, which are applied to reduce the experimental bias (Ruiz Espejo, 2006). Randomisation of experimental trials is applied on an unstable system to avoid obtaining insignificant data. Randomisation of the experiment enables averaging out the effects of noise factors that may be present in the system (Ranga *et al.*, 2014). Replication is a process of running experimental trials in a random sequence whereby the repetition of an entire experiment under varying conditions occurs. Replications consist of two properties whereby determining of experimental error occurs and a more precise estimate of the interaction effect is obtained. Blocking is a method of eradicating the effects of unnecessary discrepancies due to noise factors and enhances the efficiency of the experimental design (Anderson, 1997; Ruiz Espejo, 2006; Ranga *et al.*, 2014). At the initial stage of an experiment, there are numerous control factors to be studied to determine which of these have an impact on the response variable, a screening design method is necessary to achieve this (Anderson, 1997; Vanaja and Rani, 2007; Ranga *et al.*, 2014).

2.7.2. Screening designs

Screening design is an experimental design that is applied when numerous design parameters are examined to identify and select the variables consisting of highly significant impact on the process response (Ruiz Espejo, 2006). Screening design is of utmost importance since it advances reducing the number of parameters to be investigated further in a subsequent experiment as well as the number of experiments performed (Ranga *et al.*, 2014). Following the identification of the relevant parameters, experiments can be carried out with these parameters to investigate the nature of interactions between them using Placket Burman (PB), full or fractional factorial designs and response surface methods (Ruiz Espejo, 2006). Screening designs benefits entail

i. Encourages the improvement of the quality control process by establishing the upper and lower control limits of a variable to be investigated.
ii. Characteristics of the response are enhanced through a structural approach
iii. Allows for minimisation of the number of experiments while maximising the response
iv. Information attained can be utilised to further optimise a process

Screening design is usually carried out in a random sequence, and it is known as randomisation. Running the experimental tests randomly prevents the confounding of effects likely to occur when an experiment is run in a standard order (Vanaja and Rani, 2007). Randomisation is usually performed in replicates; meaning that each combination of factor levels in the design is run more than once. To determine pure error and statistical significance of the experimental results, replication is paramount (Vanaja and Rani, 2007).

2.7.2.1. Placket Burman screening design

Placket Burman (PB) design is the most widely used non-regular design that was developed by R.L. Placket and J.P. Burman in 1946 (Ranga *et al.*, 2014). It was developed to study the independence of specific measured quality from numerous independent factors, using a limited number of experiments; each factor takes L levels to reduce the variance of the estimations of these dependencies (Abd El-Salam *et al.*, 2017). The orthogonal arrays that Placket and Burman (PB) developed are effective for screening since they produce accurate estimates of the primary effects in the smallest design. A $'n + 1'$ run PB design can screen a variety of $'n'$ elements (Vanaja and Rani, 2007). Saturated PB designs are used to study $n - 1$ variables in 'n' experiments, presenting experimental designs for more than seven factors, in particular, $n \times 4$ experiments, meaning that the number of runs can be 8, 12, 16, 20, which are suitable for investigating up to 7, 11, 15, 19, and more factors accordingly (Vanaja and Rani, 2007).

In comparison to fractional factorial designs, a selection of two-level PB design is equivalent to fractional factorial design; however, to study 11 factors, PB design is used with 12 runs while the fractional factorial design will require 16 observations (Jin, 2016). Placket Burman design's primary benefit is the minimum number of observations required to determine the impact of a specific factor. The drawbacks in PB design include the aliasing pattern that is more complicated, and each main effect tend to be aliased with every two-way interaction excluding that effect; it is also challenging to evaluate the lack of fit, and first order effects could be confounded with interaction effects (Vanaja and Rani, 2007). According to Vanaja and Rani (2007), the use of PB design is beneficial for screening and further analysis of the significant factors is required to identify and estimate interaction terms. Figure 5 shows eight process steps associated with PB screening design. The critical steps in a screening design are selection of factors, defining their levels and responses that must be measured.

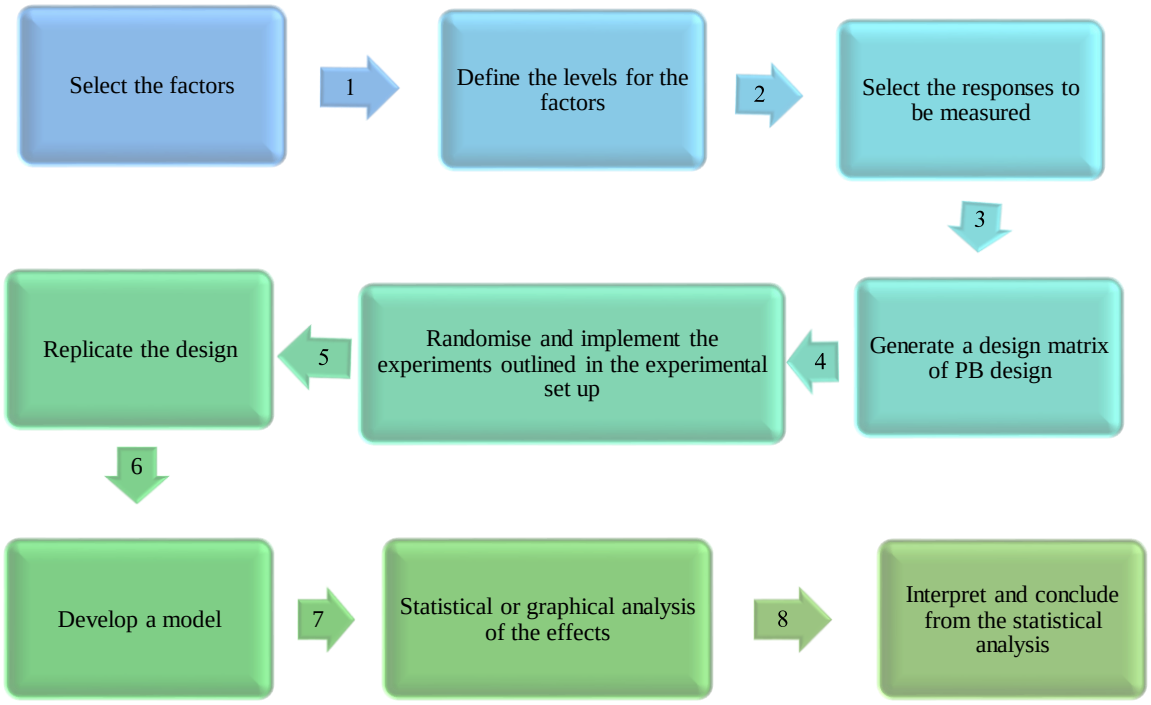

Figure 5: Screening process steps involved in PB design (Vanaja and Rani, 2007).

2.7.2.2. Advantages and limitations of Placket Burman (PB) design

Placket Burman design reveals information about an effect of a single-factor response and allows for efficient estimation of the main effects of all factors being explored (Vanaja and Rani, 2007). PB design offers reduced time, consumables, and human resources, thus, proving to be an excellent primary screening method for evaluating multiple variables (Bharati and Jigajini, 2021). Placket Burman designs are highly convenient when multiple factors are to be investigated. However, they consist of drawbacks as well. PB model is unable to define the interactions among factors hence it is mainly used for screening purposes (Palvannan and Sathishkumar, 2010).

2.8. Design matrix

A design matrix of an experiment, shown in table 6, is generated after the selection of factors and identifying their levels as stipulated by screening process steps in figure 5. PB screening design (PBSD) is mostly used to denote two-level fractional factorials although more levels are possible (Jin, 2016). Screening design enables the effective estimation of the main effects or interactions of every factor being investigated. The n factors are usually screened in $n + 1$ run, meaning that 11 factors can be examined with only 12 trials as shown in the PBSD matrix table 6. The element in the columns specifies the levels of the numerical factors, high levels are denoted by (+) and low levels are denoted by (-). With the screening design matrix in table 6, it can be verified that each factor is evaluated at six high and six low levels and the main factor can be verified that it is not confounded when the effects are determined (Vanaja and Rani, 2007). Modified PBSD was reported by Singh and Sahu (2019) when

developed the GDN-loaded liposome formulation by modified thin film hydration method, whereby an 11-factor PBD at two levels (high and low) was applied for the preliminary screening of the main effects of eleven variables.

Table 6: An example of a 12-run PB design matrix

Trial	A: A	B: B	C:C	D: D	E: E	F: F	G: G	H: H	J: J	K: K	L: L
1	1	-1	1	1	-1	1	1	1	-1	-1	-1
2	-1	1	1	1	-1	-1	-1	1	-1	1	1
3	-1	-1	1	-1	1	1	-1	1	1	1	-1
4	1	1	1	-1	-1	-1	1	-1	1	1	-1
5	-1	1	1	-1	1	1	1	-1	-1	-1	1
6	-1	-1	-1	1	-1	1	1	-1	1	1	1
7	1	1	-1	-1	-1	1	-1	1	1	-1	1
8	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1
9	1	-1	1	1	1	-1	-1	-1	1	-1	1
10	1	1	-1	1	1	1	-1	-1	-1	1	-1
11	1	-1	-1	-1	1	-1	1	1	-1	1	1
12	-1	1	-1	1	1	-1	1	1	1	-1	-1

2.8.1.1. Statistical analysis of research data

Statistical analysis assesses the accuracy of the results, provides additional description of the data and reveals the statistical significance of comparisons (Vanaja and Rani, 2007). Regression coefficients and analysis of variance is established through statistical analysis. Saturated PB designs are widely used for screening and are based on Hadamard matrices (Goupy, 2005). A first-degree polynomial model interprets PBDS results as shown in the equation

$$Y = b_o + \sum_{i=1}^{k} b_i x_i \qquad \text{Equation 1}$$

Where, Y is the response, b_0 is the model intercept, b_i is the linear coefficient, x_i is the level of the independent variable, and k is the number of variables involved (Abd El-Salam *et al.*, 2017). Plackett Burman designs are identified as saturated designs. Thus, the main effects would not reveal the standard errors while all the degrees of freedom are used to estimate the main factor effects (Vanaja and Rani, 2007). Subsequently, ANOVA is carried out to evaluate the significant factors affecting the dependent variable, including their interactive effects. ANOVA allows for the study of the sum of squares (SS) which is used to determine the factor main effects, F-ratios (F) as the mean square (MS) ratio effect, including mean square error. Probability (p) values indicate the significant factors affecting the decedent variable (Ruiz Espejo, 2006).

2.8.1.2. Response surface methodology (RSM)

Response surface methodology (RSM) was developed and introduced in 1950s by Box and Wilson (Kleijnen, 2008; de Oliveira *et al.*, 2019). RSM is the integration of the design and analysis of experiments, modelling techniques and optimisation methods in a solid approach that makes use of fewer experimental runs to obtain process improvements (Bhagwat *et al.*, 2015). Response surface methodologies incorporate all the independent variables analysis to mathematically fit the experimental data inputs investigated in the output's theoretical design (Hanrahan and Lu, 2006). Response surface methodologies generate a model equation to establish the relationship and interactions among multiple parameters using quantitative data (Usman *et al.*, 2021). RSM evaluate local gradients to search for the optimum conditions of the designed experiment, and the gradient estimates use a locally fitted first-order polynomials (Kleijnen, 2008). Response surface methodology findings are subsequent to a constructive regression analysis that investigates the relationship between the independent variable and controlled values (Usman *et al.*, 2021). The dependent variable can then be predicted based on the new values of the independent variables. The Central composite and Box-Behnken designs (BBD) are the two predominant designs utilised in response surface methodology, which incorporates statistical and regression analysis to build model equations that describe the modelling of the response surface and variable optimisation through a model equation (Hanrahan and Lu, 2006; Usman *et al.*, 2021). The current study only focuses on using central composite design (CCD) to optimise the process parameters that profoundly influence the model. Numerous researchers such as (Yang *et al.*, 2009; Ismail and

Nampoothiri, 2010; Ren *et al.*, 2013; Surendhiran and Vijay, 2013; Bhagwat *et al.*, 2015; Shakeel *et al.*, 2020; Masilan *et al.*, 2021) have shown interest in studying the response surface methodology and its application on various research topics.

Table 7: An overview of the integration interpretation of Response surface methodology (RSM)

RSM integration	Method	References
Design and analysis of the experiment	Data collection, identification of factors and interactions influencing the process	Bhagwat *et al.*, 2015
Modelling techniques	Define the relationship between independent and dependent variables statistically	Hanrahan and Lu, 2006
Optimisation process	Maximise or minimise data to improve the process	Usman *et al.*, 2021; Bhagwat *et al.* 2015

2.8.1.3. Central Composite design

A Box Wilson Central composite design is a test array that is specifically designed for Response surface methodology. In the response surface model, the central composite design is the most commonly used fractional factorial design (Hetzner *et al.*, 2014). The CCD structure consists of three components; the factorial (Cube) portion of at least resolution V, the star (axial) portion at a distance, α , from the design's centre along each axis and the centre point located at the centre of the design region (Chigbu and Ukaegbu, 2017). Resolution V is defined as a design in which main effects are not confounded with other main effects, two-factor interactions, three-factor interactions, however, two-factor interactions are confounded with three-factor interactions (Chigbu and Ukaegbu, 2017). With reference to figure 6, CCD is a two-level full or fractional factorial design with corner points (green dots), centre point (red dot) supplemented by a group of axial points (yellow dots) that allow for curvature (Hetzner *et al.*, 2014). The centre point is selected to obtain properties such as orthogonality when fitting quadratic polynomials, it is usually replicated to have a measurement of re-productivity and model lack of fit (Tarley *et al.*, 2009; Zolgharneina *et al.*, 2013). CCD enables the assessment of the main effect's parameters, 2^k factor interactions and quadratic effects. To implement a central composite design, at least two numerical inputs are required which includes α (axial distance) over three (-1, 0, +1) or five ($-\alpha$, -1, 0, +1,$+\alpha$) levels (Usman *et al.*, 2021). The axial distance value on CCD allows for determining the type of the design such as face-centered central composite (CCF) design, rotational, spherical, orthogonal quadratic and practical (Usman *et al.*, 2021). The correlation amongst a response and independent variables is acquired by fitting them into a second-order polynomial using a multiple regression program as depicted by equation 2.

$$Y = b_0 + \sum_{i=1}^{k} b_i x_i + \sum_{i=1}^{k} b_{ii} {x_{ii}}^2 + \sum_{i=1}^{k} \sum_{i=1}^{k} b_{ij} x_i x_j + \varepsilon \qquad \text{Equation 2}$$

$i \neq j$

Y represents a model response, b_0 is the intercept, k is the total number of independent factors, b is the coefficient values for linear, quadratic and interaction effects respectively, $x_i x_j$ depicts coded variables and ε is the random error (de Oliveira *et al.*, 2019). Central composite design is classified into three categories such as Circumscribed design (CCC), Inscribed design (ICC), and Face-centered design (FCC). This study only focused on the Circumscribed central composite design. Table 8 shows a detailed description of the three types of central composite design (Zhang and Baixiaofeng, 2009).

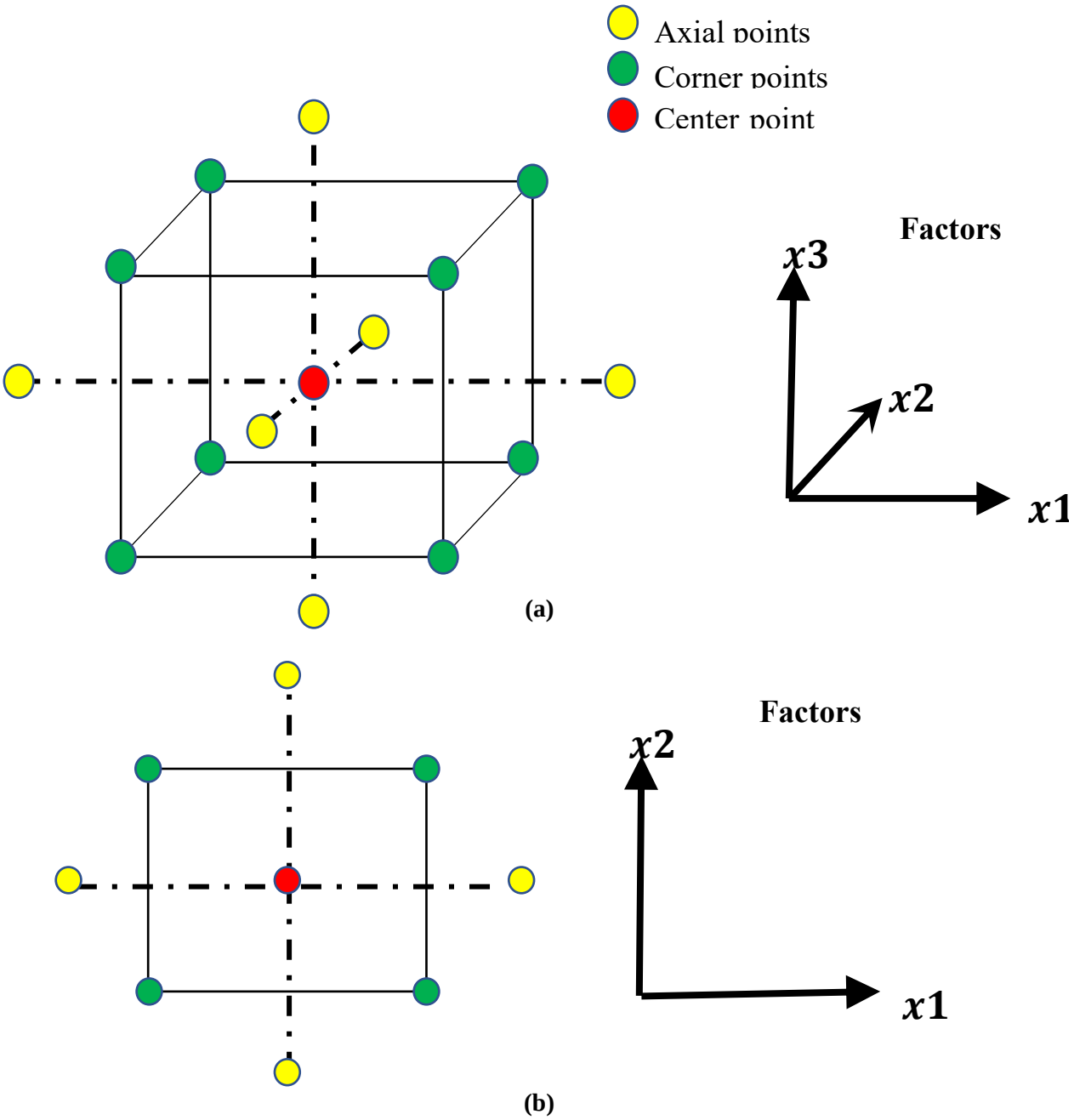

Figure 6: Generation of a Central composite design with (a) three factors ((x1, x2 and x3)) and (b) two factors (x1, x2) entailing a full factorial design (corner points) that are extended by axial points and a centre point (Zhang and Baixiaofeng, 2009).

Table 8: Three types of central composite design

Central Composite design type	Description	References
Circumscribed design (CCC)	This is the original form of CCD. The axial points are at equidistance (α) from the centre, depending on the design properties desired and number of factors in the design. Axial points define the level settings (high/low) for all the factors. These designs are either circular, spherical or hyper spherical in symmetry requiring five levels of each factor. This design can be rotated, and it explores the largest design region.	Usman *et al.* 2021
Inscribed design (CCI)	This design makes use of the factor settings as the axial points and forms full or fractional factorial design within the specified limits. It also requires five levels of each factor like CCC. This design explores the smallest design region and can be rotated.	Zhang and Baixiaofeng, 2009
Face centered (CCF)	Axial points are at the centre of each face of the factorial region, therefore $\alpha = \pm 1$. Unlike CCC and CCI, this design requires only 3 levels of each factor. CCF are non-rotatable designs	Zhang and Baixiaofeng, 2009

2.8.1.3.1. Advantages of Central composite design

- CCD helps with estimating the nonlinear characteristics of responses in the data set provided
- Optimum conditions are achieved with minimum number of experimental trials.
- Allows for curvature estimation in obtained continuous responses with minimum error
- CCD consists of rotatability and orthogonality properties
- CCD are extensively utilised in response surface modelling and optimisation (de Oliveira *et al.*, 2019)

2.8.1.3.2. Central composite design limitations

Central composite designs are not able to estimate individual interaction terms. The central-composite design generally necessitates a rigid pattern of data collection points, which may not correspond to human factors engineering study specifications. Each factor must have five levels. They must be arranged symmetrically around the centre at specific points on a scale that varies depending on the number of variables under consideration (Gannet, 2013; Zolgharneina *et al.*, 2013).

Chapter 3

3. Research Methodology

3.1. Collection and processing of samples

Soil and water samples were collected aseptically from West coast in Langebaan lagoon and Mossell Bay dam, sterile containers and airtight bottles were used to accomplish proper sampling. Samples were kept in a cooler box containing ice packs and transported aseptically into the laboratory for processing. Sample sites are shown in the South Africa map in figure 7.

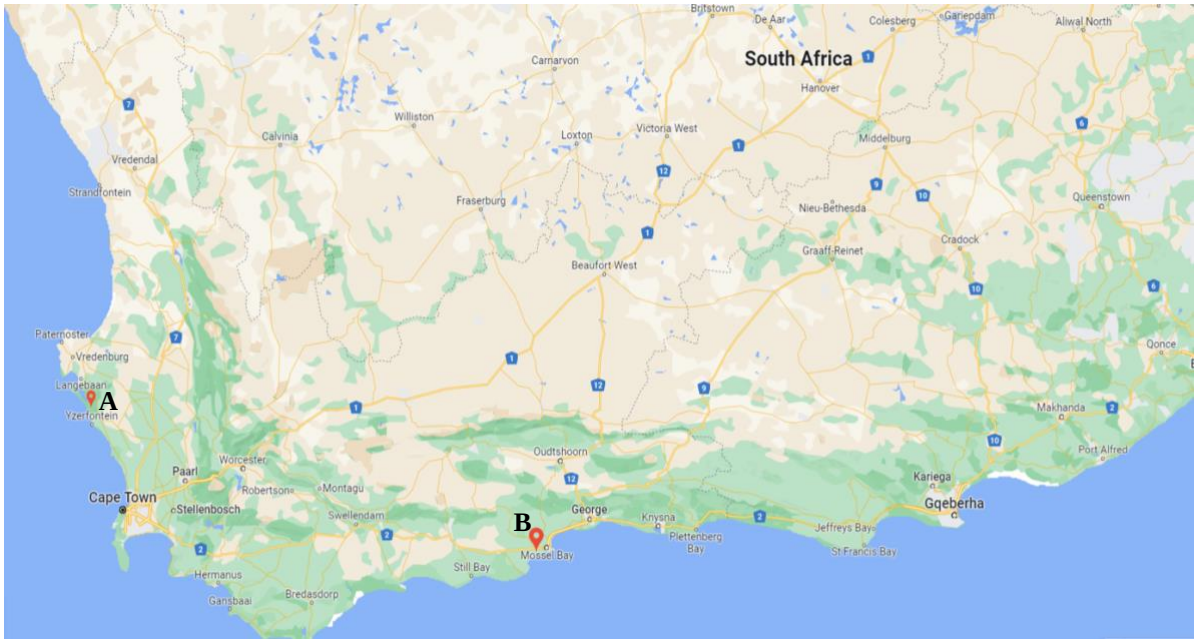

Figure 7: Sample locations: (A) Langebaan lagoon, (B) Mossel bay

3.2. Isolation and cultivation of bioflocculant-producing strain

Isolation was performed under a sterile laboratory condition at the Cape Peninsula University of Technology. Figure 8 shows a pictorial summary of the research methodology; about 5 g of each wet soil sample was air-dried at room temperature for three days. Serial dilutions were performed and cultivation of microorganisms from the processed soil sample was done according to the method described by Agunbiade *et al.* (2019) using Yeast Malt Extract (YME) agar supplemented with 50 mg/l cyclohexamide and 20 mg/l nalidixic acid to inhibit the growth of fungi and other bacteria respectively. About 1 g of the treated soil sample was weighed and decanted into a tube containing 10 ml of sterile distilled water, the mixture was thoroughly mixed at room temperature. Subsequently, an aliquot of 9 ml sterile distilled water was dispensed into six test tubes. Serial dilutions were carried out by transferring 1 ml from the stock solution to the second test tube with 9 ml of distilled water to make a dilution factor of 10^{-1} and this this was repeated until a dilution factor of 10^{-6} was achieved (Kurniawan *et al.*, 2021). After serial dilution, an aliquot (0.1 ml) of each sample was inoculated into the surface of the petri dishes containing solid YME agar isolation medium (Awolusi *et al.*, 2020b). The plates were incubated at 30 ℃ for 48 hours and after the incubation period; the distinct isolates were sub-cultured onto fresh yeast malt extract agar (YME) plates and further incubated at 30 ℃ for 3

to 5 days at pH 7. The pure cultures were maintained on slants containing yeast malt extract agar (YME) in 40% glycerol stock, and stored at 4 ℃ for further studies (Ismail and Nampoothiri, 2010).

3.3. Flocculation activity assay

Kaolin clay suspension was used as a test material in determining the flocculating efficiency of the isolated strains. 3 ml of $CaCl_2$ solution ($1\% w/v$) and 2 ml of culture supernatant was added into 95 ml of kaolin clay suspension (4 g/l in distilled water), stirred and allowed to settle for five minutes prior to taking an optical density (OD). A spectrophotometer was used at a wavelength of 550 nm to determine the optical density of the mixture; this technique was also used to determine the flocculation activity of the control whereby the cell free supernatant was replaced with 2 ml of culture medium. Flocculating activity (FA) was evaluated using Equation 3

$$FA(\%) = \frac{B-A}{B} \times 100 \qquad \text{Equation 3}$$

where A is the absorbance of the sample experiment and B is the absorbance of control at 550 nm, respectively. The strain that exhibited optimum flocculating activity was selected for further study.

3.4. Identification of a bioflocculant producing organism

The genomic DNA of the strain was extracted using the Quick DNA™ Fungal/Bacterial Miniprep kit (Zymo Research catalogue No. D6005). The universal primers 27F and 1492R shown in table 9 were used to amplify the 16S target region (Hashim *et al.*, 2019). The 16S target region was amplified using OneTaq® Quick-Load® 2X Master Mix (NEB, Catalogue No. M0486) with the universal primers presented on table 9. The PCR products were run on a gel and gel extracted with the Zymoclean™ Gel DNA Recovery Kit (Zymo Research, Catalogue No. D4001). The extracted fragments were sequenced in the forward and reverse direction (Nimagen, BrilliantDye™ Terminator Cycle Sequencing Kit V3.1, BRD3- 100/1000) and purified (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit™, Catalogue No. D4050). The purified fragments were analysed on the ABI 3500xl Genetic Analyser (Applied Biosystems, ThermoFisher Scientific) for each reaction for every sample. CLC Bio Main Workbench v7.6 was used to analyse the. ab1 files generated by the ABI 3500XL/ABI 3730XL Genetic Analyser and results were obtained by a BLAST search (NCBI) (Agunbiade *et al.*, 2016, 2019).

Table 9: 16S Primers sequences

Primer name	Target	Sequence (5' to 3')
16S- 27F	16S rDNA sequence	AGAGTTTGATCMTGGCTCAG
16S – 1429R	16S rDNA sequence	CGGTTACCTTGTTACGACTT

3.5. Experimental design matrix for Bioflocculation activity

Plackett-Burman (PB) design for screening

PB is a design tool developed for screening n factors specifically in $n + 1$ experimental studies (Tyssedal, 2014). In comparison to the conventional full factorial design, PB design notably reduces the number of experiments required to reach the set goal. Therefore, reducing the cost of resources in terms of labour and time. In this present study, eight independent medium variables were analysed in a PB design to identify the variables that presented great influence on the flocculation activity (Abd El-Salam *et al.*, 2017). These variables included glucose, $MgSO_4.7H_2O$, yeast extract, NaCl, KH_2PO_4, K_2HPO_4, $((NH_4)_2SO_4$ and urea. For the evaluation of each medium variable, 2 levels of concentration designated as $+1$ (high) and -1 (low) were chosen accordingly (Gong *et al.*, 2008; He *et al.*, 2009). Design expert version 12.0 (Stat-Ease Inc., Minneapolis, MN, USA) statistical software was applied for designing and developing the PB experimental matrix according to the first order regression as described in Equation 4.

$$Y = b_o + \sum_{i=1}^{k} b_i x_i \qquad \text{Equation 4}$$

Where, Y is the response, b_0 is the model intercept, b_i is the linear coefficient, x_i is the level of the independent variable and *k* is the number of variables involved. Each experiment was carried out in duplicate with the corresponding average flocculation activity made as the response variable. Regression analysis was used to identify the variables of great significance (i.e., at 95% level with $p < 0.05$) and these identified variables were subjected to further optimisation studies (Abd El-Salam *et al.*, 2017).

3.5.2. Central composite design (CCD) for optimisation

The influence of the most significant process variables identified by the PB design was further investigated using response surface methodology (RSM) coupled with CCD. These independent variables and their chosen ranges were based on variables that have higher effects on flocculation activity. Applying a fractional factorial design consisting of 5 levels and 3 factors, a total of 30 experimental conditions were generated (Awolusi *et al.*, 2020). The design matrix comprised 6 centre points and 6 axial points, with an axial distance (α) of ± 1.68 to transform the design to its orthogonal form (Okaiyeto *et al.*, 2015). To establish a relationship between the dependent variable and the independent variables, the flocculation activity was fit to a second-order regression model as shown in Equation 5.

$$Y = \delta_0 + \delta_1 A + \delta_2 B + \delta_3 C + \delta_{12} AB + \delta_{13} AC + \delta_{23} BC + \delta_{11} A^2 + \delta_{22} B^2 + \delta_{33} C^2 \qquad \text{Equation 5}$$

Where, Y is the bioflocculation activity (response), δ_0 is the intercept term; δ_1, δ_2 and δ_3 are coefficients of linear terms; δ_{12}, δ_{13} and δ_{23} are coefficients of interaction terms; δ_{11}, δ_{22} and δ_{33} are coefficients of quadratic terms respectively. Each experimental condition was conducted in duplicate, and the mean

flocculation activity was recorded for the corresponding response. Design-Expert version 12.0 (Stat-Ease Inc., Minneapolis, MN, USA) software was applied for the model and statistical data analysis.

3.6. Extraction and purification of bioflocculant

The different types of extraction methods are described in chapter two of this study. The extraction and purification of a bioflocculant was done according to the modified methods of (Chen *et al.*, 2002; Nontembiso *et al.*, 2011). After 72 h of fermentation (1 L flask) the culture broth was centrifuged at 8000 rpm for 30 minutes at room temperature to remove bacteria cells. One volume of sterile distilled water was added to the supernatant phase and centrifuged at 8000 rpm for 15 minutes to remove insoluble substances. Thereafter, two volumes of ethanol were added to the supernatant, stirred, and allowed to stand for 12 h at 4 ℃. The mixture was then centrifuged to extract the crude bioflocculant. The precipitate was vacuum-dried to obtain crude bioflocculant, and the crude product obtained was dissolved in water to yield a solution, to which one volume of a mixed solution of chloroform and n-butyl alcohol (5:2 v/v) was added. Subsequently, the mixture was stirred, poured into a separating funnel and allowed to stand for 12 h at room temperature (Barbarino and Lourenço, 2005). Finally, the supernatant was discarded, and two volumes of ethanol was added to recover the precipitate and then lyophilised to obtain a partially purified bioflocculant (Cosa and Okoh, 2014).

3.7. Characteristics of a bioflocculant

3.7.1. Fourier Transform Infrared Spectroscopy (FTIR)

The functional groups of the purified bioflocculant were analysed using Fourier Transform Infrared (FTIR) Spectrophotometer (Perkin Elmer System 2000, England). The bioflocculant was pulverised with potassium bromide salt at 25˚C and pressed into a pellet for FTIR spectroscopy at the frequency range of $4000 - 450\ \mathrm{cm}^{-1}$.

3.7.2. Thermogravimetric analysis (TGA) and thermal stability

Thermogravimetric analysis (TGA) measures the change in sample mass as a function of temperature. It determines the change in heat flux in a sample as the temperature varies. TGA of the purified bioflocculant was determined by using a thermo-analyser over a temperature range of 10 ℃ to 600 ℃ at a heating rate of 10 ℃ per min under a constant flow of nitrogen gas. The TGA percentage weight change was plotted on the Y-axis against the reference material temperature on the x-axis.

3.7.3. Scanning electron microscopy (SEM) and energy dispersive X-ray analysis (EDX)

A small amount of a purified bioflocculant was placed on an Aluminium SEM stub covered with carbon glue. The samples were carbon coated and the scanning electron micrograph of the bioflocculant was obtained using a Tescan MIRA3 RISE SEM. The analysis of elemental compositions of the purified bioflocculant was carried out in the Nova NanoSEM using an Oxford XMax $20\mathrm{mm}^2$ detector and INCA software.

3.8. Lab scale studies of a bioflocculant-producing strain in wastewater treatment

3.8.1. Determination of optimum bioflocculant dosage

This was done by measuring different concentrations of the purified powdered bioflocculant ranging from 0.2 mg/ml to 1.2 mg/ml Optimum dosage was achieved by mixing the purified bioflocculant with kaolin clay suspension and metal ions at 160 rpm for 3 minutes, followed by flocculation at 40 rpm for 2 minutes and settling for 5 minutes. Subsequently, 2 ml of the supernatant was gently withdrawn using a pipette and the optical density of the upper clarifying part was assayed using a spectrophotometer at 550 nm (Gong *et al.*, 2008; Agunbiade, Pohl and Ashafa, 2018).

3.8.2. Comparison of coagulation performance with purified bioflocculant and chemical flocculants

The flocculation efficiencies of the polyacrylamide, polyethyleneimine and the purified bioflocculant were evaluated following the standard flocculation assay protocol as described by (Ugbenyen and Okoh, 2014). Flocculants (chemically synthesised and microbial) were prepared at different concentrations and assessed against kaolin clay suspension with the addition of cations to stimulate flocculation using the Jar test method. The control experiment was also prepared the same way; however, the flocculants (microbial or chemically synthesised) was replaced with distilled water. Flocculation activity was assessed as reported in equation 3 above.

3.8.3. Wastewater treatment application

Wastewater sample was collected aseptically from a municipal wastewater treatment plant and analysed immediately upon arrival at the laboratory. An optimum concentration dose of the purified bioflocculant established through the jar test was found to be 1 mg/ml, this concentration was used for subsequent experiment. 1 mg/ml of the purified bioflocculant and 2 ml of $CaCl_2$ was added into 100 ml of wastewater sample. The mixture was agitated at 160 rpm for 2 minutes using a jar test followed by flocculation period at 40 rpm for 2 minutes, then allowed to settle for 5 minutes. The supernatant was then taken for the analysis of the OD and flocculation activity (Agunbiade, Pohl and Ashafa, 2018). To assay for chemical oxygen demand (COD), 2 ml of raw and treated samples were added into COD vials (150 mg/l). For the blank analyses, 2 ml of sterile distilled water was added to the COD vial. The caps were tightly closed, rinsed with water, and finally wiped with a clean paper towel. A TR420 Spectroquant was preheated up to 148 ℃ prior to inserting the COD vials for analysis. The COD vials were inserted in the digester, heated for 2 hours, then removed and allowed to cool down at room temperature for 30 minutes. Thereafter, the vials were analysed using a DR 900 spectrophotometer. The optical density obtained for the raw and treated sample was then used to determine the removal efficiency (Tawila *et al.*, 2018). The removal efficiency was calculated using equation 6.

$$RE(\%) = \frac{C_o - C}{C_o} \times 100 \qquad \text{Equation 6}$$

Where; C_o is the initial concentration value before the treatment and C is the concentration value after the flocculation treatment.

3.9. Statistical analysis

Using SPSS 16.0, the results were reported as means standard deviation of three replicates and subjected to one-way analysis of variance (ANOVA) followed by Duncan multiple range tests to find significant differences in all parameters.

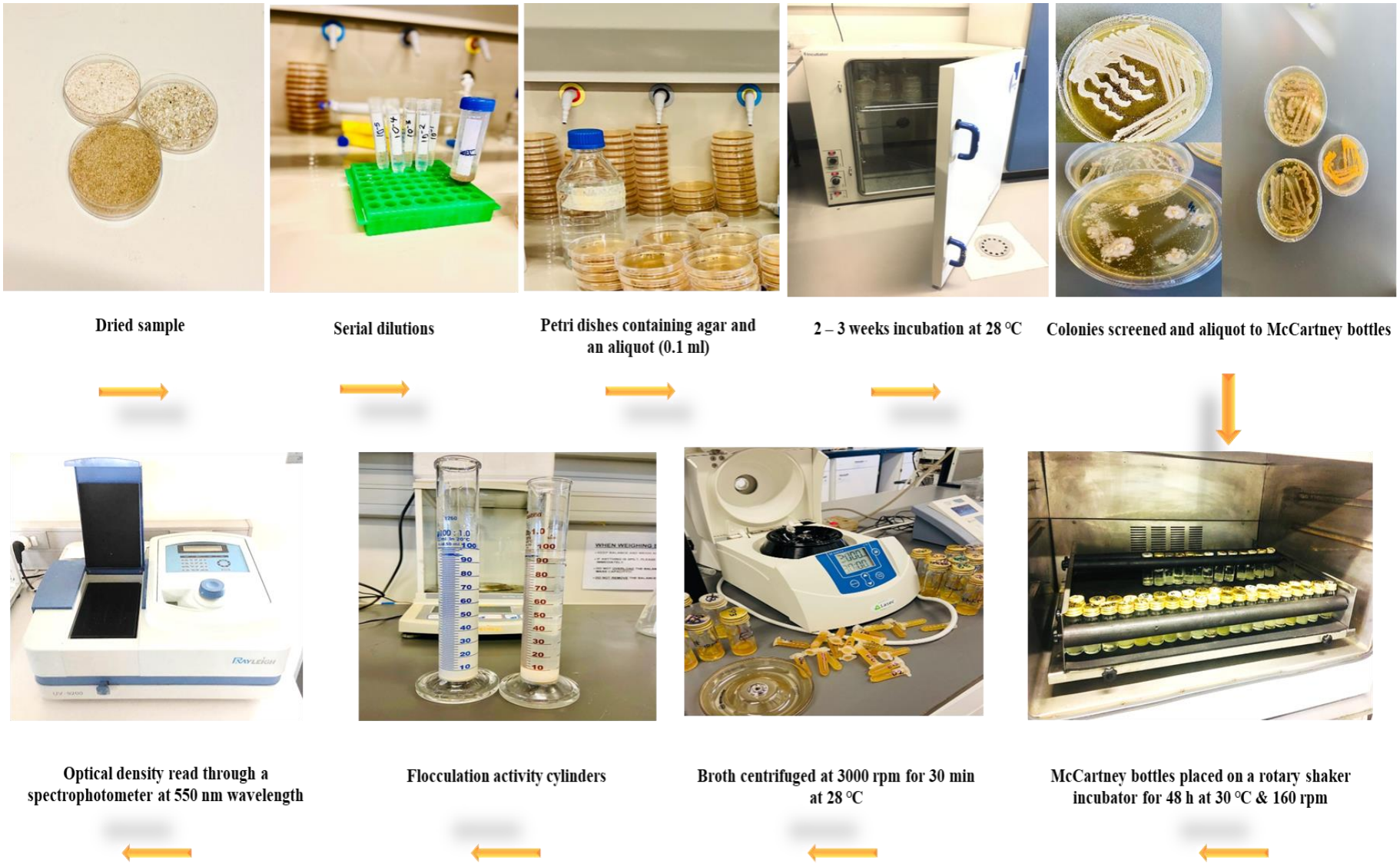

Figure 8: Schematic summary of the research methodology

Chapter 4

4. Results and Discussion

4.1. Isolation, screening, and identification of a bioflocculant-producing organism

Forty-four bacterial strains were isolated, plated on agar plates, screened and evaluated using kaolin clay suspension as a test material. **Table 10** shows that among the 44 isolates, only eight demonstrated a flocculation activity above 70%. While only three isolates presented the acceptable least flocculation activity from 60% to 65%. The highest flocculation activity of **88.3%** was observed with isolate **H7**. Thus, this isolate was selected for further assay. As shown in **Figure 9**, **H7** appeared non-pigmented, smooth, milky white and shiny. The 16S rDNA gene sequencing was used to confirm the identity of the positive strain that exhibited the highest flocculating efficiency against kaolin clay suspension. This yielded a product of the expected size (approximately 1.5 kb). Basic Local Alignment Search Tool (BLAST) analysis of the nucleotide sequence of the 16S rRNA revealed the bacteria to have 99% similarity to *Stenotrophomonas maltophilia* strain U1G52Y7N016. The sequence was deposited in the GenBank as *Stenotrophomonas maltophilia* (*S. maltophilia*) with accession number MT291866.1. *S.maltophilia* is an environmental global emerging Gram-negative bacteria belonging to the family xanthomonadaceae of γ-proteobacteria (Kang *et al.,* 2015). According to Chen *et al.* (2016), about 81.43% of flocculation activity was achieved by a bioflocculant produced by *Stenotrophomonas maltophilia* ZCC-06 from phenol-containing wastewater when tested against the removal of cadmium. Similarly, in another study by Zaidi *et al.* (2023), the bioflocculant-producing *Stenotrophomonas maltophilia* strain isolated from palm oil mill effluent revealed a flocculation activity of 95.29% when tested against kaolin clay suspension. Previous studies have reported bioflocculant-producing *S.maltophilia* from various sites, excluding marine environments. Thus, it was investigated in this study. According to our knowledge, this study is the first to report the production of a bioflocculant by *S. maltophilia* from a marine environment. As observed from other reports, bioflocculants produced from *S. maltophilia*, regardless of the habitat isolated from, have the potential to exhibit high flocculation efficiencies, thus, making them a great alternative to chemical flocculants.

Table 10: Flocculating activities of isolates screened from a marine environment

Isolate code	Flocculation activity (%)	Isolate code	Flocculation activity (%)
G6	24.9	D8	15.3
E12	81.5	E2	61.7
G3	18.0	E1	71.6
G1	25.2	D4	63.2
E5	39.3	H2	58.5
E3	45.5	**H7**	**88.30**
H3	51.5	D10	85.8
D1	20.2	G4	70.2
D3	14.1	E7	66.8
H4	52.3	E14	86.9
H6	65.8	H1	57.5
E10	15.7	BN2	38.1
D7	24.2	L9	84.4
D2	18.8	HN5	70.6
E11	12.1	JN13	58.3
D5	74.1	JN4	63.9
E4	58.3	DN5R	63.2
D9	36.5	HRN10	21.6
G7	66.0	G7	10.9
D6	44.6	GN16	58.3
H5	64.5	HNR3	56.1
G5	40.4	G2	41.8

Bold value = Highest flocculation activity

Figure 9: Picture of an organism producing a bioflocculant on an agar plate

4.2. Experimental design matrix for bioflocculation Activity

Design-Expert version 12.0 (Stat-Ease Inc., Minneapolis, MN, USA), a blend of mathematical and statistical methods, was used to evaluate the impacts of several independent variables on system response for the optimal production of a purified bioflocculant from marine *S. maltophilia*. Plackett Burman (PB) design and Response surface methodology (RSM), together with central composite design (CCD), were considered in this study (Okaiyeto *et al.*, 2015).

4.2.1. Plackett Burman (PB) screening significant factors

In this study, the Plackett Burman (PB) design matrix was utilised to screen for the most significant factors that influenced the production of a bioflocculant from marine *S. maltophilia*. **Table 11** displays coded variables in terms of high-level (+) and low-level (-) concentrations, which yielded the actual and predicted flocculation activity during the analysis. As shown in **Table 11**, trial 6, it is evident that high levels of glucose, NaCl, $(NH_4)_2SO_4$ yeast extract, K_2HPO_4, K_2HPO_4 and low levels of $MgSO_4.7H_2O$ and urea have led to the best bioflocculant production. The actual flocculation activity of a bioflocculant achieved in this trial was **88%** while the predicted was **86.75%**. **Table 12** displays the PB design regression analysis, approximately eight independent variables were evaluated, viz. glucose, yeast extract, urea, NaCl, $MgSO_4.7H_2O$, K_2HPO_4, $(NH_4)_2SO_4$ and KH_2PO_4. Statistically, the probability value ($p < 0.05$) reveals the significance of each independent variable within the design, the larger the magnitude of the t-value and smaller the p-value, the greater the significance and effect of the corresponding variable on the response (Fayez *et al.*, 2022), this is shown in **Table 12**. The p-value less than 0.05 was achieved by only four variables, viz. Glucose ($p < 0.0071$), yeast extract ($p < 0.0041$), K_2HPO_4 ($p < 0.0032$) and $(NH_4)_2SO_4$, ($p < 0.009$).

These variables demonstrated a statistically significant positive influence on the generation of the bioflocculant-producing strain, while the rest had a negative impact ($p > 0.05$). The notable effects displayed by glucose, K_2HPO_4 and $(NH_4)_2SO_4$ were most likely attributable to the necessity of these medium constituents for substantial cell growth. Correspondingly, $(NH_4)_2SO_4$ was reported to be amongst the most preferred nitrogen sources in the bioflocculant production by *Bacillus pumilus* strain (Makapela *et al.*, 2016). Preceding research also revealed that carbon-to-nitrogen (C/N) ratios carried out an extensive function in microbial metabolism, including converting fatty acids from heterotrophic *Chlorella vulgaris* (Liang *et al*., 2009) and promoting biological hydrogen production from *Clostridium pasteurianum* and *Clostridium tyrobutyricum* (Lin and Lay, 2004; Jo *et al.*, 2008). Furthermore, in the EPS production using *Azotobacter indicus* (Patil *et al*., 2010), inorganic salts such as potassium phosphate have been reported to significantly influence the physiological functions viz. cell growth, cell division and enzyme activity of an organism. Thus, dipotassium hydrogen sulphate may have positively influenced the metabolism of *S. maltophilia*. The significant variables identified to have a positive influence on the bioflocculant production in this study is corroborated by the findings reported by Abd El-Salam *et al.* (2017), where a bioflocculant produced by *Bacillus aryabhattai* PSK1 revealed that glucose, yeast extract, K_2HPO_4, KH_2PO_4, $MgSO_4$ and NaCl had significant influence during its production. Contrastingly, the findings reported by Agunbiade *et al.* (2022) indicated that yeast extract, $K_2HPO_4.3H_2O$, KH_2PO_4 revealed no significant influence on the bioflocculant produced by *Bacillus velezensis*. Identifying critical media components is vital in the experiment to enhance the flocculation efficiency of the bioflocculant produced. Based on these observations, the first-order regression analysis generated is shown by equation 7. The equation reveals the bioflocculant production from marine *S. maltophilia* production as a function of eight independent variables.

$$S.maltophilia\ MT291866.1 = 66.5 + 3.2\,V_1 - 0.66\,V_2 + 3.38\,V_3 - 0.33\,V_4 - 4.17\,V_5 + 1.17\,V_6 + 1.33\,V_7 + 6.33\,V_8$$

Equation 7

The analysis of variance (ANOVA) of the Plackett Burman design revealed that the model was highly significant as shown in **Table 13** that the p-value attained was **0.0046**. As evident from **Table 13**, the F-value of $\mathbf{46.38}$ obtained was high, thus, this implied that the model was significant and there was only a $\mathbf{0.46}\%$ chance that an F-value this large could occur due to noise. The correlation evaluation between significant variables is not possible by first order equation. Hence, a further investigation was conducted through a second-order model in response surface methodology (RSM) coupled with central composite design (CCD).

Table 11: Plackett Burman design for the screening of critical components

Trial	Coded variable levels								Flocculation activity (%)	
	V_1	V_2	V_3	V_4	V_5	V_6	V_7	V_8	**Actual**	**Predicted**
1	-1	1	1	1	-1	-1	1	1	60	58.92
2	1	1	-1	-1	-1	-1	1	1	70	71.08
3	1	-1	-1	1	1	-1	-1	1	69	69.08
4	-1	-1	-1	-1	-1	-1	-1	-1	57	55.75
5	-1	1	-1	-1	1	1	1	-1	74	74.08
6	**1**	**1**	**1**	**-1**	**1**	**1**	**-1**	**1**	**88**	**86.75**
7	-1	-1	-1	1	1	1	1	1	61	60.92
8	-1	1	1	1	1	-1	-1	-1	69	70.08
9	1	-1	1	1	-1	1	1	-1	54	53.92
10	-1	-1	1	-1	-1	1	-1	1	59	60.25
11	1	1	-1	1	-1	1	-1	-1	61	61.08
12	1	-1	1	-1	1	-1	1	-1	75	75.08

Table 12: The concentrations of independent variables at different levels and the regression analysis of the Plackett Burman design.

No	Variables	Concentration (g/l)		Estimate	t-value	p-value
		low level (-)	high level (+)			
V_1	Glucose	7.5	12	3.17	6.59	**0.0071**
V_2	NaCl	0.01	0.15	-0.6667	-1.39	0.2599
V_3	Yeast extract	0.15	1	3.83	7.96	**0.0041**
V_4	Urea	0.25	0.75	-0.3333	-0.69	0.5382
V_5	K_2HPO_4	3.5	7.5	4.17	8.67	**0.0032**
V_6	KH_2PO_4	1.5	4	1.17	2.43	0.0938
V_7	$MgSO_4.7H_2O$	0.2	0.5	1.33	2.76	0.0695
V_8	$(NH_4)_2SO_4$	0.1	0.45	6.33	13.16	**0.0009**

Table 13: Plackett Burman analysis of variance (ANOVA) for the fitted first-order regression

Source	Sum of Squares	DF	Mean Square	F-value	p-value	
Model	1030.67	8	128.83	**46.38**	**0.0046**	**Significant**
V_1-Glucose	120.33	1	120.33	433.2	0.0071	
V_2 - NaCl	5.33	1	5.33	1.92	0.2599	
V_3-Yeast extract	176.33	1	176.33	63.48	0.0041	
V_4-Urea	1.33	1	1.33	0.48	0.5382	
V_5-K2HPO4	208.33	1	208.33	7.	00.032	
V_6-KH2PO4	16.33	1	16.33	5.88	0.0938	
V_7MgSO4.7H2O	21.33	1	21.33	7.68	0.0695	
V_8-(NH4)2SO4	481.33	1	481.33	173.28	0.0009	
Residual	8.33	3	2.78			
Cor Total	1039	11				

4.2.2. Response surface methodology (RSM) coupled with Central composite deign (CCD)

Optimisation of the growth medium for the production of a bioflocculant from *S. maltophilia* was necessary to enhance an increase in the production yield. Based on the results obtained from PB design, a 3-factor-5 level central composite design (CCD) was carried out, as depicted in **Table 14**. To obtain an optimised medium for improved production of a bioflocculant from *S. maltophilia*, out of eight independent variables evaluated initially, only four variables from the experimental data were revealed to have a positive effect on the flocculation activity. These variables were further optimised through response surface methodology (RSM) coupled with CCD to maximise their impact on bioflocculant production. **Table 14** shows the design matrix of the experiment as well as the flocculation activity results of 30 evaluated trials using CCD analysis. The optimal conditions of 16.25 g/l glucose, 1.61 g/l yeast extract, 1.1 g/l K_2HPO_4 and 3.5 g/l $(NH_4)_2SO_4$) achieved a maximum flocculation activity of **96.05**% shown in bold in **Table 14** trial 17 and 4 g yield of the purified bioflocculant. Yeast extract, glucose, $(NH_4)_2SO_4$ and K_2HPO_4 formed an optimal nutrient environment for the cell growth in the production of the bioflocculant from marine *S. maltophilia*. The *S. maltophilia* microorganism utilised these components to grow, metabolise, and synthesise the bioflocculant while promoting the maximum flocculation activity. According to reports in literature, organic nitrogen sources are more suitable for the production of a bioflocculant (Cosa *et al.*, 2013). However, a complex nitrogen substrate consisting of yeast extract and $(NH_4)_2SO_4$ greatly increased the cell activity of the bioflocculant production in this study. Contrastingly, Hwang *et al.* (2003) and Nie *et al.* (2011) reported that organic nitrogen sources, with their nutrient richness, ease of absorption, and stimulus properties, have been proven to be more favourable and efficient in promoting the bioflocculant production. Furthermore, they are more absorbed by the cells than in the inorganic nitrogen sources. In another study, it was observed that the optimisation process for the production of exopolysaccharide (EPS) from *Chlamydomonas reinhardtii* resulted in a medium containing only four inorganic salts and a 1.6-fold enhancement in EPS yield from 382.5 to 628 mg/l (He *et al.*, 2013). The concentration levels (high and low) used to optimise the medium compositions are shown in regression analysis **Table 15**. The regression equation obtained from ANOVA proved that the bioflocculant produced by marine *S. maltophilia* is a function of four variables such as glucose, yeast extract, K_2HPO_4 and $(NH_4)_2SO_4$ as equation 8 shows there was a direct relationship between these variables.

The quadratic regression of the model was obtained as;

$$S.maltophilia = 3.26 + 13.14\,V_1 - 7.49\,V_3 - 0.87V_5 + 6.09\,V_8 - 0.25\,V_1V_3 - 0.04V_1V_5 + 0.15\,V_1V_8 + 0.55\,V_3V_5 + 2.2\,V_3V_8 - 0.52\,V_5V_8 - 0.45\,{V_1}^2 + 2.40\,{V_3}^2 + 0.13\,{V_5}^2 - 2.85\,{V_8}^2$$

Equation 8

Where V_1,V_3, V_5, V_8 were coded factors of glucose, yeast extract, K_2HPO_4 and $(NH_4)_2SO_4$, respectively.

The fitness of the model was confirmed by ANOVA of the regression model and the regression correlation coefficient $\mathbf{R^2}$ (Liu *et al.*, 2010). **Table 16** demonstrate the ANOVA quadratic regression of CCD analysis whereby it is revealed that the model was statistically significant at a probability level of 99% while the lack of fit probability value was $0.404 > 0.05$, indicating that lack of fit was not significant relative to pure error. Equation 8 displays a highly significant model ratio of variances (F-value) of $\mathbf{14.99}$ that implies the model is significant and there is only a 0.01% chance that model this large could be due to noise. The correlation coefficient obtained was $\mathbf{R^2}(\mathbf{0.9333})$, which implied that the model is reliable and can explain more than $\mathbf{93.3}\%$ of the total variations, the model could not explain only **6.7%** variations of a bioflocculant production from marine *S. maltophilia*. The **adjusted-**$\mathbf{R^2}(\mathbf{0.8710})$ is in reasonable agreement with the correlation coefficient as the difference is less than 0.2 indicating that the model has good significance. The adequate precision measures the signal-to-noise ratio. A ratio greater than four is desirable. Furthermore, the obtained adequate precision ratio obtained was **18.259** indicating that there is adequate signal. Hence, the model was acceptable for the prediction of bioflocculant production from *S. maltophilia*. The flexibility, efficiency, and statistical rigour of RSM make it a widely explored and invaluable tool in research and industry for process optimisation and system analysis. Various reports in literature are in agreement with the optimisation statistical results attained in this study, it has been documented that RSM coupled with CCD have promoted the maximum yield of various bioflocculants produced from marine *Bacillus subtilis* MSBN17 (Selvin 2012); *Chlorella* sp. HS2 (Kim *et al.*, 2019) and *Pseudosomonas* sp (Wang *et al.*, 2022).

Table 14: Optimisation of production media through Central Composite Design matrix

	Glucose (g/L)	**Yeast extract (g/L)**	**K_2HPO_4 (g/L)**	**$(NH_4)_2SO_4$ (g/L)**	**Flocculation activity (%)**	
Trial	V_1	V_3	V_5	V_8	Actual	Predicted
1	13.75	1.15	6.25	1.5	93.59	94.08
2	15	0.8	4.5	1.8	93.24	93.61
3	13.75	1.15	2.75	1.5	94.88	93.59
4	13.75	0.45	6.25	1.5	94.21	95.05
5	13.75	1.85	6.25	1.5	95.44	93.92
6	13.75	1.15	6.25	1.5	93.16	95.65
7	12.5	1.5	4.5	1.2	93.12	93.61
8	12.5	0.8	8	1.8	90.93	92.84
9	12.5	1.5	4.5	1.8	93.12	91.21
10	13.75	1.15	6.25	1.5	94.43	93.04
11	15	0.8	8	1.8	91.87	93.61
12	12.5	0.8	4.5	1.2	92.91	92.32
13	13.75	1.15	6.25	1.5	94.03	92.88
14	15	1.5	4.5	1.2	93.84	93.61
15	15	0.8	8	1.2	93.78	93.59
16	13.75	1.15	9.75	1.5	95.61	93.89
17	**16.25**	**1.61**	**1.1**	**3.5**	**96.05**	**95.77**
18	15	1.5	4.5	1.8	93.78	91.5
19	15	0.8	4.5	1.2	93.87	94.01
20	13.75	1.15	6.25	0.9	93.12	94.09
21	12.5	1.5	8	1.2	94.01	93.28
22	12.5	1.5	8	1.8	93.52	94.34
23	12.5	0.8	8	1.2	93.12	93.44
24	12.5	0.8	4.5	1.8	91.85	93.03
25	11.25	1.15	6.25	1.5	89.95	92.16
26	15	1.5	8	1.2	94.92	89.86
27	13.75	1.15	6.25	2.1	92.22	94.75
28	13.75	1.15	6.25	1.5	92.82	91.89
29	13.75	1.15	6.25	1.5	93.71	93.61
30	13.75	1.15	6.25	1.5	93.63	93.65

Table 15: Regression analysis showing critical components of a bioflocculant production from *S. maltophilia*

No	Variables	Concentration(g/l)		Estimate	t-value	p-value
		low level (-)	high level (+)			
V_1	Glucose	12.5	15	0.461	5.031	**0.0001**
V_3	Yeast extract	0.8	1.5	0.434	4.740	**0.0003**
V_5	K_2HPO_4	1.2	1.8	0.053	0.573	0.5751
V_8	$(NH_4)_2SO_4$	2.75	9.75	-0.349	-3.812	**0.0017**

Table 16: Analysis of variance (ANOVA) for the fitted quadratic polynomial model for optimisation of a bioflocculant producing strain

Source	DF	SS	MS	F-value	P-value
Model	**42.27**	**14**	**3.02**	**14.99**	**< 0.0001**
V_1-Glucose	5.1	1	5.1	25.3	**0.0001**
V_3-Yeast extract	4.52	1	4.52	22.46	**0.0003**
V_5- K_2HPO_4	0.0661	1	0.0661	0.3283	0.5751
V_8-$(NH_4)_2SO_4$	2.93	1	2.93	14.52	**0.0017**
V_1V_3	0.2025	1	0.2025	1.01	**0.032**
V_1V_5	0.1156	1	0.1156	0.5738	0.4605
V_1V_8	0.0484	1	0.0484	0.2402	0.6311
V_3V_5	1.84	1	1.84	9.11	**0.0086**
V_3V_8	0.8556	1	0.8556	4.25	0.0571
$V_5\ V_8$	1.2	1	1.2	5.95	**0.0276**
$V_1{}^2$	13.73	1	13.73	68.15	**< 0.0001**
$V_3{}^2$	2.37	1	2.37	11.75	**0.0037**
$V_5{}^2$	4.09	1	4.09	20.31	**0.0004**
$V_8{}^2$	1.8	1	1.8	8.94	**0.0092**
Residual	**3.02**	**15**	0.2015		
Lack of Fit	1.35	10	0.1346	0.4014	0.8972
Pure Error	1.68	5	0.3352		
Cor Total	**45.29**	29			

$R^2 = 0.9333$; $Adjusted\ R = 0.8710$; *Cor = Corrected, Bold values = Statistical significance*

4.2.3. Interaction of variables

Three-dimensional (3-D) response surface plots graphically represent the regression equation and are used to demonstrate interactions between the response and experimental levels of each variable, while the 2-D surface contour plots identify the type of interaction between the variables (Hazime *et al.*, 2013; Mohammed and Dagang, 2019). The saddle and elliptical nature of the contour plots indicate the significance of the interactions amongst the variables, while the concave and circular nature suggests the opposite (Murthy *et al.*, 2000). Therefore, the 3-D response surface and 2-D contour plots allow for visualisation of the optimum levels of each variable for maximum flocculation activity. **Figure 10 (a) - (f)** demonstrate the 3-D response surface plots interactions between independent variables. Each figure represents an interaction between two variables and flocculation activity while keeping all the other factors in range. **Figure 11 (a) – (f)** displayed the contour plots revealing the magnitude of the interactions between independent variables optimised in this study. The nature of the various surface plots in **Figure 10** and **11** allows for an interpretation of the level and quality of the interactions of the independent variables optimised in this study.

Figure 10 (a) shows the flocculation rate interaction between glucose with a concentration ranging from 11.25 to 16.25 g/l and $(NH_4)_2SO_4$ concentration from 0.1 to 2.9 g/l. Surprisingly, the interaction between these two variables was insignificant, and this is confirmed by ANOVA ($\mathbf{p = 0.6311}$) in **Table 16**. However, it is worth noting that individually, these variables demonstrated their statistical significance, indicating that they are suitable substrates for the maximum flocculation activity. Furthermore, **Figure 11 (a)** shows a circular contour plot that ultimately proves there was no significant interaction between these two variables. Similar observations were reported by Uppala *et al.* (2019) that an interaction between $(NH_4)_2SO_4$ and glucose showed no significance on the decolourisation of azo dye black 10B by *Kocuria Kristine* RC3. Similarly, **Figure 10 (b)** shows the flocculation rate interaction between glucose with a concentration from 10 to 17 g/l and K_2HPO_4 with a concentration from 2 to 7 g/l. The interaction between these variables is insignificant this is confirmed by ANOVA ($\mathbf{p = 0.4605}$) in **Table 16**, even though individually, glucose was found to be significant as shown in **Table 16** by ANOVA ($\mathbf{p = 0.0001}$).

The combination of these two variables appears to have a neutral effect on the flocculation activity. The contour plot shown in **Figure 11 (b)** further confirms minimal to no interaction between these variables. Contrastingly to the observations by Uppala *et al*. (2019) indicated that this interaction was significant and the phosphate governed the glucose concentration for the dye decolourisation by *Kocuria Kristine* RC3. **Figure 10 (c)** illustrates the flocculation rate interaction between glucose and yeast extract at a concentration ranging from 10 to 17 g/l and 0.5 to 1.5 g/l respectively. The interaction between these two variables was significant, indicating that the carbon to nitrogen (C/N) ratio played a vital role in the maximum flocculation activity achieved; ANOVA ($\mathbf{p = 0.032}$) also confirms this significance as displayed in **Table 16**.

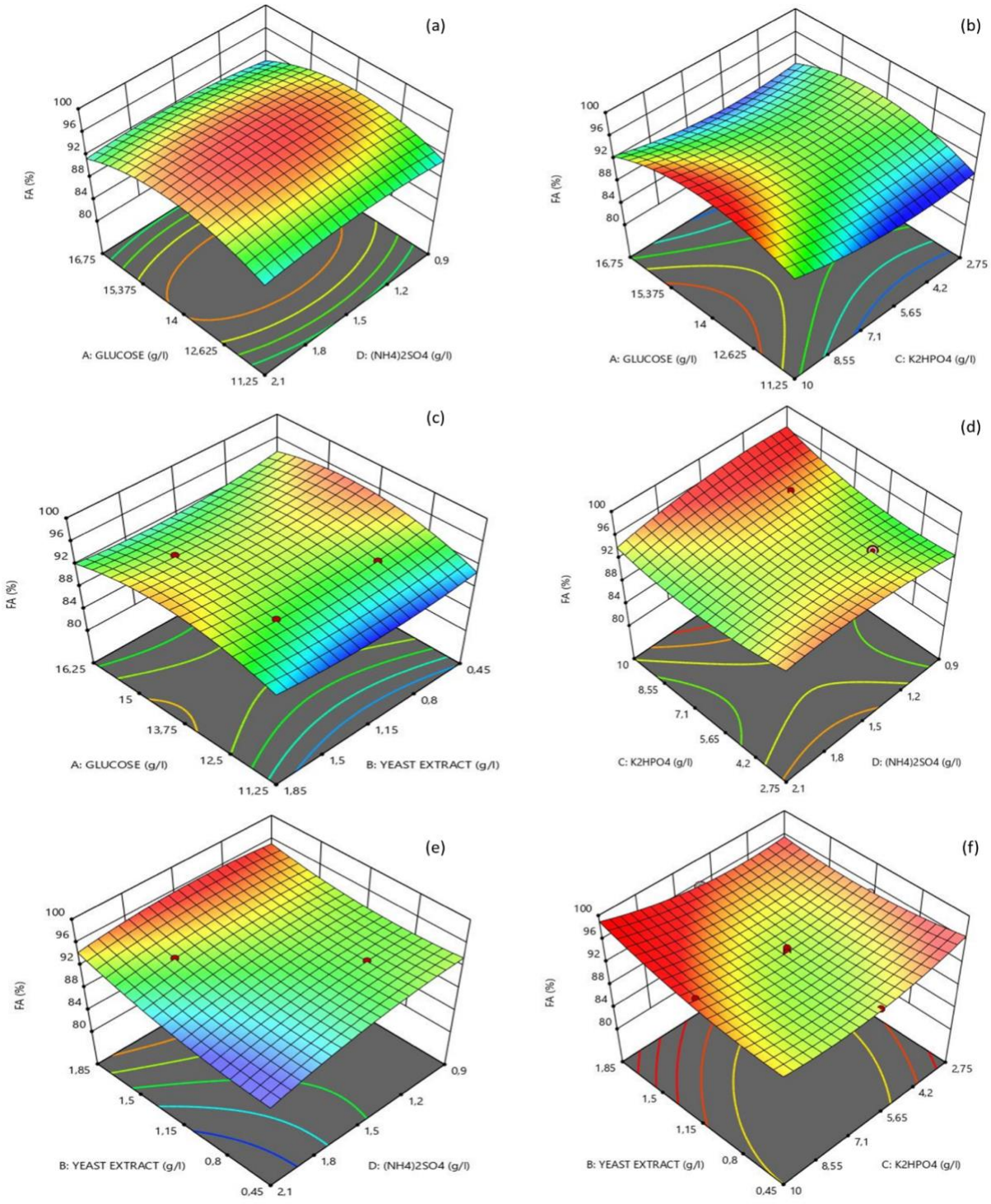

Figure 10: 3-D surface response plots interactions between; (a) (Glucose and $(NH_4)_2SO_4$); (b) (Glucose and K_2HPO_4); (c) (Glucose and Yeast extract); (d) (K_2HPO_4 and $NH_4)_2SO_4$); (e) ($(NH_4)_2SO_4$ and Yeast extract); (f) (Yeast extract and K_2HPO_4).

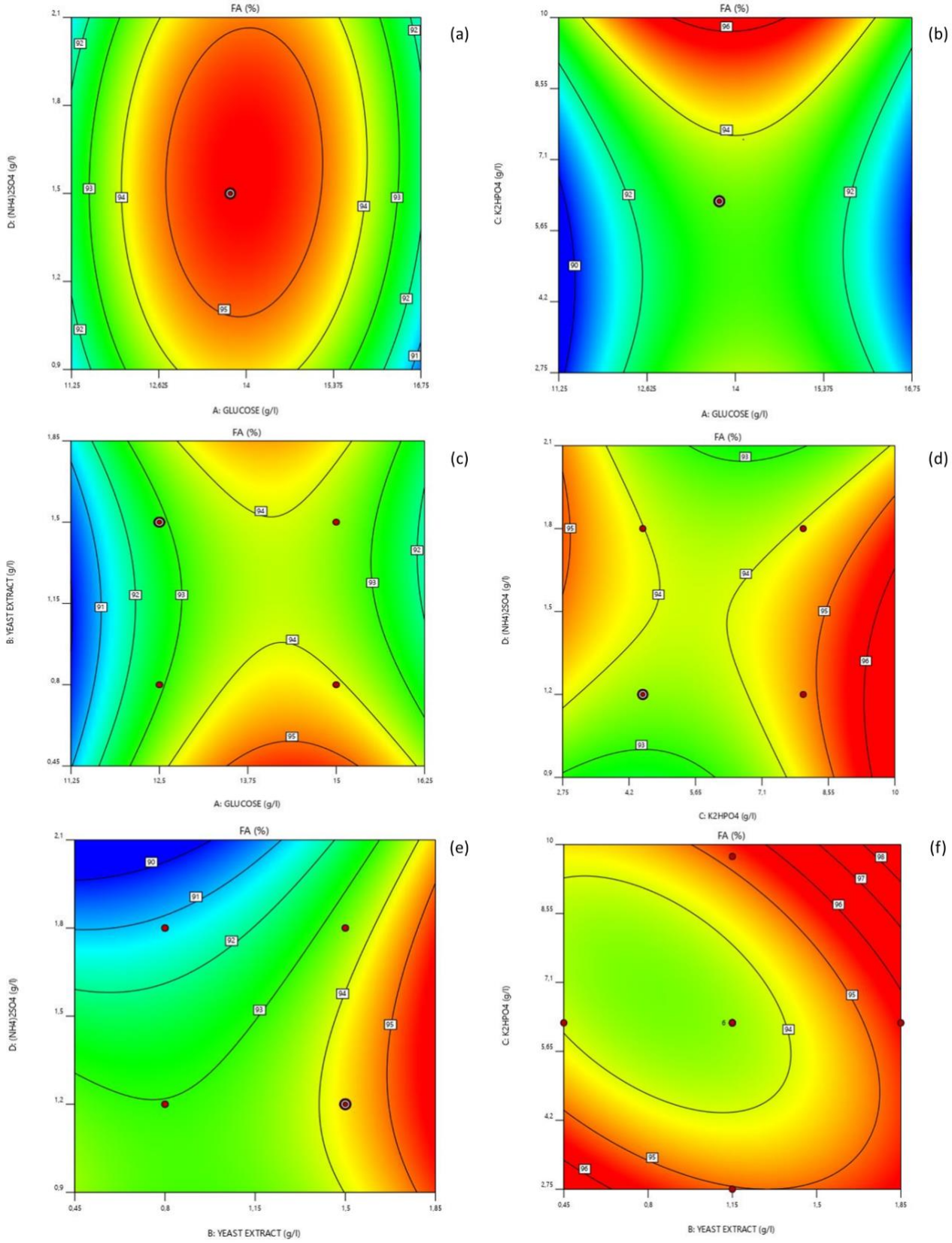

Figure 11: Contour maps of variable interactions of flocculation rate

Compared to the study conducted by (Tanyildizi *et al*., 2005), there was moderate interaction between these variables on *Actinorhodin* production by *Streptomyces coelicolor* A3(2). Furthermore, **Figure 11 (c)** illustrates the response surface contour plot with the elliptical shape, confirming the significant interaction between these variables. **Figure 10 (d)** depicts the flocculation rate interaction between $(NH_4)_2SO_4$ and K_2HPO_4 at different concentrations ranging from 0.5 to 3 g/l and 3.5 to 6.5 g/l, respectively. When paired, the interaction demonstrated by these two variables was significant; this observation was confirmed by ANOVA ($\mathbf{p = 0.0276}$) depicted in **Table 16**. However, individually, K_2HPO_4 revealed no significant impact as shown by ANOVA ($\mathbf{p = 0.5751}$) in **Table 16**. This shows that it did not contribute to the flocculation activity of the bioflocculant. Comparable findings pertaining to this interaction were observed by Uppala *et al*. (2019) stating that $(NH_4)_2SO_4$ concentration was slightly governed by K_2HPO_4 in the dye decolourisation.

Furthermore, **Figure 11 (d)** illustrates a paddle-shaped contour plot confirming the effective interaction between these variables. This indicates that the inorganic salt paired with nitrogen source forms a good nutritional substrate since they are both essential in the formation of cellular structure and metabolites (Nwodo and Okoh, 2014). Furthermore, **Figure 10 (e)** illustrates the flocculation rate interaction between yeast extract and $(NH_4)_2SO_4$ at various concentrations from 1 to 1.7 g/l and 0.1 to 2.7 g/l, respectively. The interaction between these variables was found insignificant as denoted by ANOVA ($\mathbf{p = 0.0571}$) shown in **Table 16**. It is evident that yeast extract and $(NH_4)_2SO_4$ are both nitrogen sources, thus demonstrated neutral interaction when paired and rather significant individually. Consequently, the surface contour plot displayed in **Figure 11 (e)** further confirmed there was no significant interaction between these variables. In addition, **Figure 10 (f)** shows the flocculation rate interaction between yeast extract and K_2HPO_4 at various concentrations from 0.45 to 1.8 g/l and 2.75 to 10 g/l, respectively. These observations are analogous to **Figure 10 (d)** observations, the interaction between these variables was significant and was confirmed by ANOVA ($\mathbf{p = 0.0086}$) shown in **Table 16**, while K_2HPO_4 individually shows no significance. Subsequently, the convex-shaped surface contour plot in Figure 11 (f) further confirmed that interaction existed between the two variables. The interaction observations reported in this study is congruent with those of He *et al.* (2009); Nwodo and Okoh, (2014); Meriem and Mahmoud, (2017).

4.3. Extraction and purification of a bioflocculant produced by S. *maltophilia* MT291866.1

The bioflocculant was extracted from marine S.*maltophilia* MT291866.1 using an ethanol extraction method. **Figure 12** illustrates the results of the extraction process of crude to purified bioflocculant. **Figure 12 (a)** shows a solid-liquid separation of crude bioflocculant, **Figure12 (b)** demonstrates a dried crude bioflocculant, the purification separation process is depicted by **Figure 12 (c),** and **Figure 12 (d)** shows a dried purified bioflocculant in powder form. The crude bioflocculant yield obtained before purification was $\mathbf{4.28\ g/l}$. Subsequently, the purification process was carried out by using a mixture of chloroform and n-butyl alcohol $(5{:}2\ \mathrm{v/v})$ and the purified bioflocculant yield recorded was **4 g/l**. Contrary to a study by Tsilo *et al.* (2022), the attained bioflocculant yield by S.*maltophilia* was high when compared to Pichia *kudriavzevii* MH545928.1 of 2.836 g/l and that of marine *Bacillus primilus* JX860618 which yielded a bioflocculant production of 2.4 g/l (Maliehe *et al.*, 2016). However, compared to other studies, the bioflocculant yield attained in this study was much lower. Chen *et al.* (2016) reported that a yield of 4.99 g/l was recorded for bioflocculant production by *Stenotrophomonas maltophilia* ZZC-06. In another study by Nie *et al.* (2021), a maximum bioflocculant yield of 9.53 g/l was attained, while Chen *et al.* (2017) reported that a maximum bioflocculant production of 11.18 g/l was achieved by *Alteromnas sp* CGMCC 10612 when the characterisation of a novel marine bacterium was assessed. It is worth to note that techniques employed during the bioflocculant extraction process plays a significant role in attaining high bioflocculant yields. Some methods are preferred, and various strains respond differently and according to their nutrition preference.

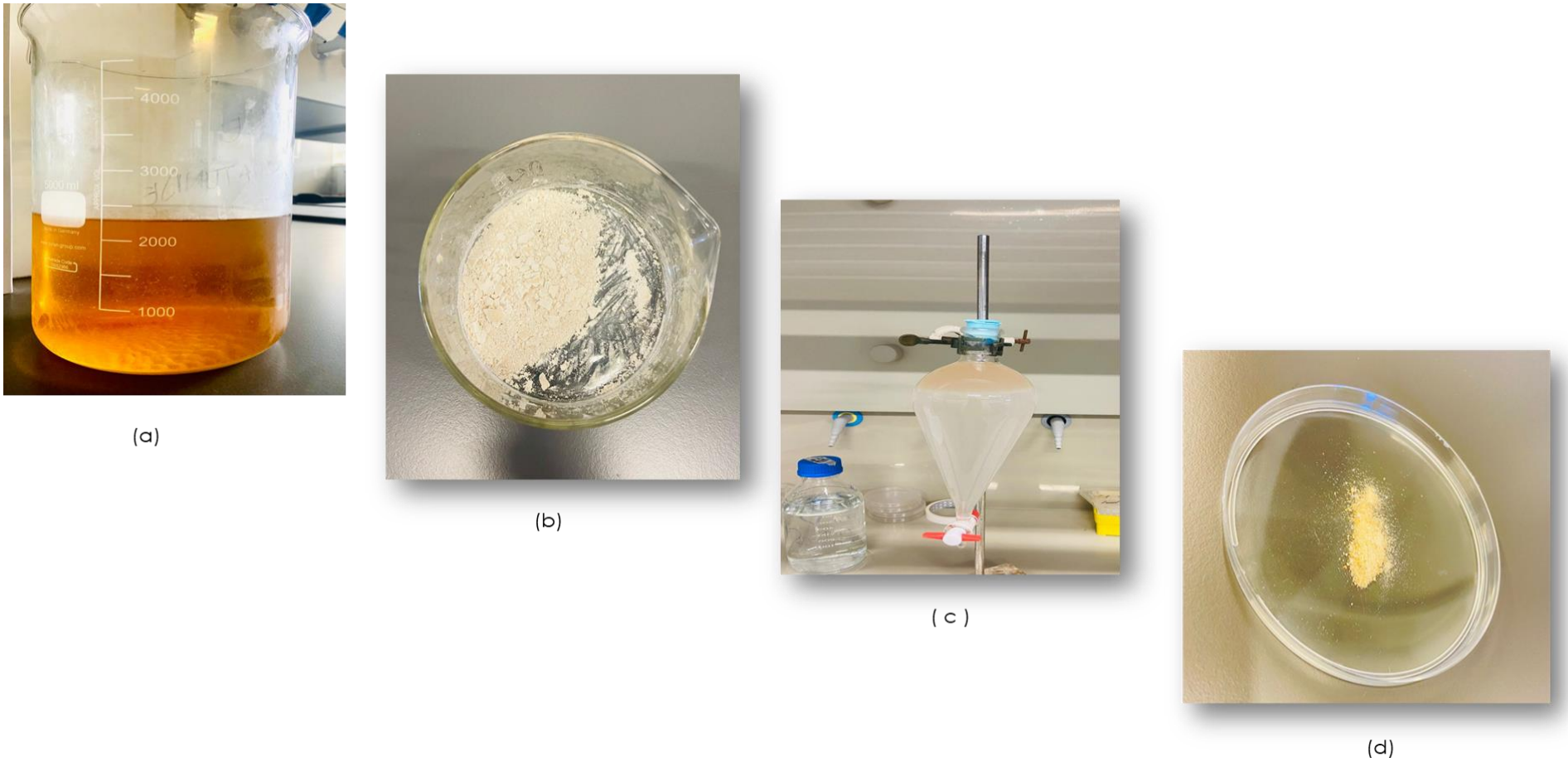

Figure 12: Extraction and purification of *S. maltophilia* (a) Extraction of a bioflocculant; (b) crude bioflocculant; (c) bioflocculant purification and (d) represents a yield of a purified bioflocculant.

4.4. Micrographic imaging and compositional characteristics

4.4.1. Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR Spectrogram of the purified bioflocculant produced by *S. maltophilia* exhibited various peaks from **4000** to **500 cm^{-1}** presented in **Figure 13**. FTIR spectroscopy was performed to establish the functional groups present in the bioflocculant produced by *S. maltophilia*. As illustrated in **Figure 13**, the first observed small band was at **3668 cm^{-1}**, followed by a broad stretching intense peak at **3370 cm^{-1}**, which indicates the presence of the stretching vibrations of hydroxyl and amine groups that are preferred in flocculation process. This was also observed by Abd El-Salam *et al.* (2017) when the purified PSK 1 biopolymer indicated the presence of hydroxyl and amino groups by attributing a broad stretching intense peak at 3425 cm^{-1}. The peak observed at **3120 cm^{-1}** suggests the presence of alkenyl C-H stretch. The small bend at **2440 cm^{-1}** is an indication of the presence of aliphatic C-H stretching, similar to the observations reported by Abd El-Salam *et al.* (2017) and Agunbiade *et al.* (2019). The peaks observed at **1685 cm^{-1}**, **1590 cm^{-1}** and **1466 cm^{-1}** indicates the presence of carbonyl groups stretching vibrations in amide group. The small band at **1247 cm^{-1}** and the intense stretching peak at **1020 cm^{-1}** confirms the presence of $C-O$ group. In addition, the stretching peak observed at **947 cm^{-1}** and **860 cm^{-1}** depicts the characteristics of $C-H$ group. The intense stretching peak at **525 cm^{-1}** indicates the presence of sugar derivatives. The FTIR analysis revealed the presence of hydroxyl and carbonyl groups as well as sugar derivatives. This confirms that the major backbone of bioflocculant produced by *S. maltophilia* is a polysaccharide. Similarly, a study reported by Li *et al.* (2017) indicated that the FTIR analysis of MBF-HG6 bioflocculant produced from *Oceanobacillus polygoni* displayed the function of carboxyl, hydroxyl and amino groups, suggesting that it is a polysaccharide. Furthermore, the functional groups exhibited by the bioflocculant produced by *S. maltophilia* are essential as they provide surface charges that serve as the active sites for binding suspended particles, thus, promoting aggregation in solutions (Luvuyo *et al.*, 2013). Our finding corroborates with the reports various reports in literature whereby the bacterial bioflocculant are majorly polysaccharide. A study by Guo *et al.* (2018) observed that the signals at 1352 cm^{-1} and 1234 cm^{-1} indicated symmetric CH bending and S=O stretching and the peaks ranging from 1200 cm^{-1} to 800 cm^{-1} meant the presence of sugar derivatives. Furthermore, Bisht and Lal. (2019) also reported that the major functional groups of BF-VB2 by strain *Bacillus* sp responsible for flocculation were hydroxyl, carboxyl, amines, and halides. thus this confirmed that the major backbone of BF-VB2 by strain *Bacillus* sp. was a polysaccharide.

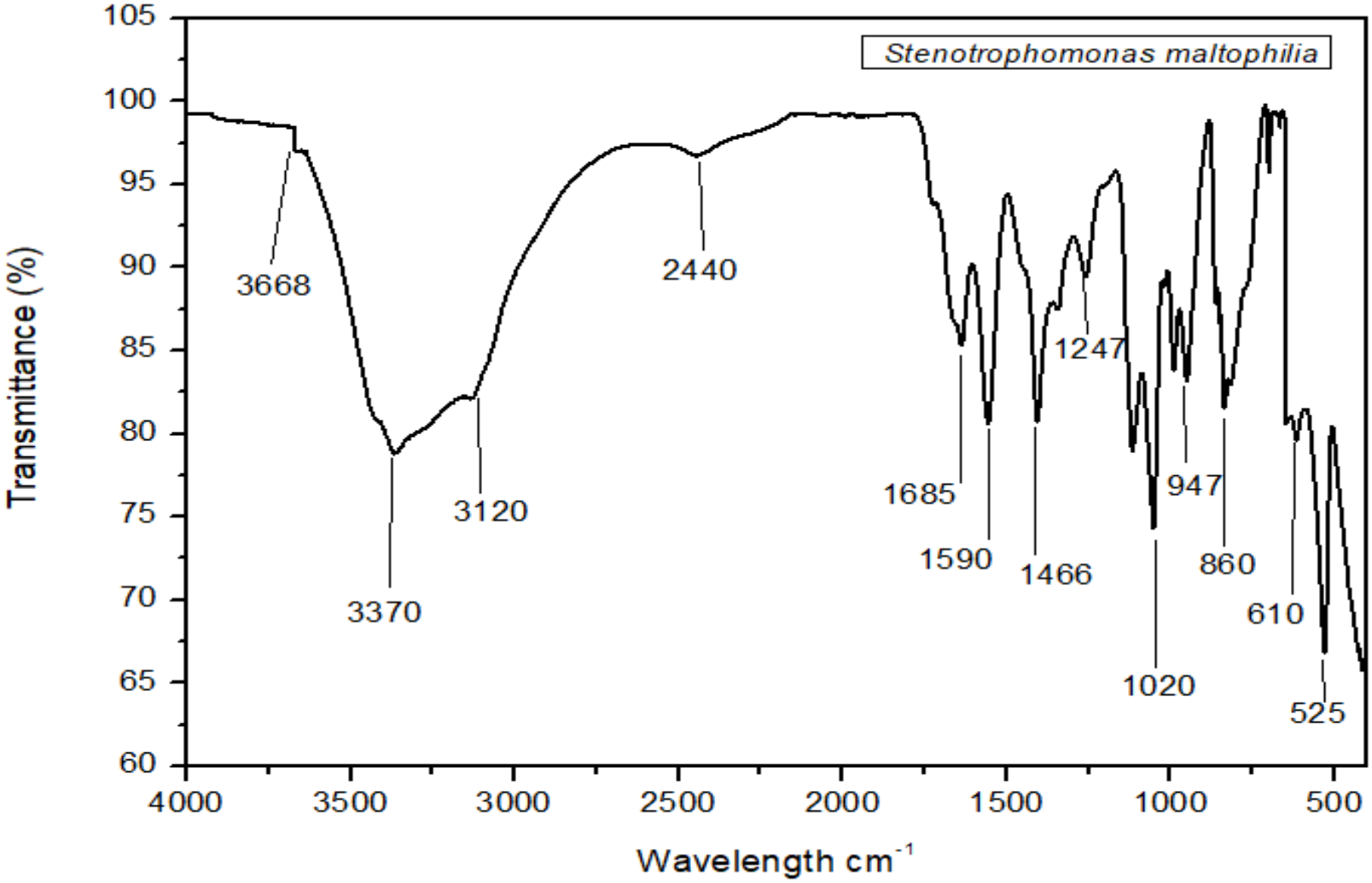

Figure 13: FTIR spectrum of purified *S. maltophilia*

4.4.2. Thermogravimetric Analysis (TGA)

A thermogravimetric analyser (TGA) was used to establish the pyrolysis profile of a purified bioflocculant produced from *S. maltophilia*. The bioflocculant TGA analysis results are displayed in **Figure 14**. The bioflocculant showed an initial weight loss of about 10% at a temperature range of **45** ℃ **to 80** ℃. This could be attributed to the moisture content present in the bioflocculant. The moisture content in the bioflocculant is owed to the presence of hydroxyl and amine groups in the purified bioflocculant. This finding is corroborated by Maliehe *et al.* (2016) who observed that the moisture content in TMT^{-1} from marine *Bacillus pumilus* JX860616 occurred due to the presence of carboxyl (in amide) and hydroxyl groups. In literature, it has been reported that high hydroxyl or carboxyl content leads to greater affinity of polysaccharides, thus, responsible for water molecules (Kumar *et al.*, 2004; Cosa *et al.*, 2013). The loss in material observed at about **220** ℃ could be the leftover organic material present. About **85%** of weight was maintained after heating the material at over **500** ℃. Notably, the pyrolysis profile of the bioflocculant produced by *S. maltophilia* confirmed the thermal stability of the test organism.

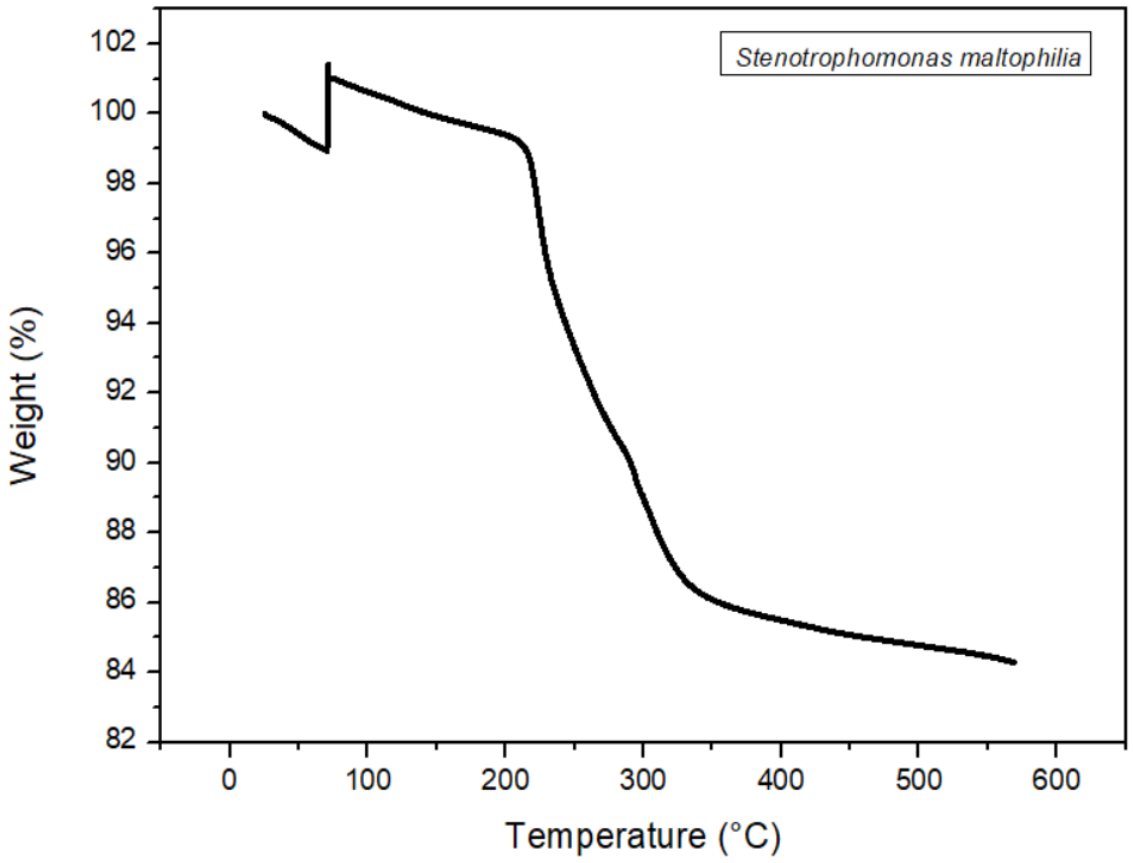

Figure 14: Effect of thermogravimetric analysis (TGA) of the purified bioflocculant produced by *S. maltophilia*

4.4.3. SEM and EDX analyses of the purified bioflocculant

The surface morphology structure and elemental compositions of the purified bioflocculant were analysed using SEM and EDX, respectively, the results obtained are presented in **Figure 15** and **Figure 16**. The image in **Figure 15 (a)** revealed that the purified bioflocculant produced by *S. maltophilia* consists of clumped sheath layers, crystalline rods and irregular pattern. The efficient flocculation exhibited by the test organism against kaolin clay suspension could be due to the structure of the purified bioflocculant produced. These findings are similar to those obtained by Arayes *et al.* (2023) which stated that a bioflocculant from marine *Actinomycete Norcardiopsis aegyptia* sp.nov had a porous crystal-linear-flute-like structure that may have contributed to its highest flocculation performance. Due to the interaction between the bioflocculant functional groups and the kaolin clay particles, flocs developed, which subsequently aggregated to form larger flocs, as depicted in **Figure 15 (b)**. Interestingly, the floc precipitated from the suspension due to gravity, confirming that bridging was necessary for flocculation. Agunbiade *et al.* (2017) observed a similar finding when evaluating the flocculation performance of a bioflocculant produced by *Arthrobacter humicola*.

As shown in **Figure 16**, the EDX analysis of the purified bioflocculant revealed its elemental composition in mass proportion (*%w/w*), affirming the presence of carbon, oxygen, magnesium, sulphur, phosphorus and potassium; **C (49.42), O (34.23), Mg (0.73), S (7.78) P (0.14)** and **K (7.7))** respectively. This observation agrees with that of Singh *et al.* (2011), indicating that elements such as C (38.48), O (55.71), Na (2.34), P (0.5), S (1.47), Ca (0.25), and Cl (1.24) were detected on the exopolysaccharide produced by *Bacillus licheniformis*. Furthermore, Okaiyeto *et al.* (2015) reported analogous cases with the bioflocculant MBF-UFH produced by *Bacillus sp*. AEMREG7, which was composed of the following elements in mass proportion (*%w/w*); C (17.21), N (6.66), O(40.04), Na (5.21), Mg (5.02), P (7.90), S (0.60),Cl(6.11), K (1.63) and Ca (9.63). In contrast to these observations Bisht and Lal (2019) reported that the elemental compositions of a novel bioflocculant BF-VB2 by strain *Bacillus* sp. in mass proportion (*%w/w*) was C (36.21); H (10.53); N (8.61) and O (19.28), there was no sulphur nor cations detected.

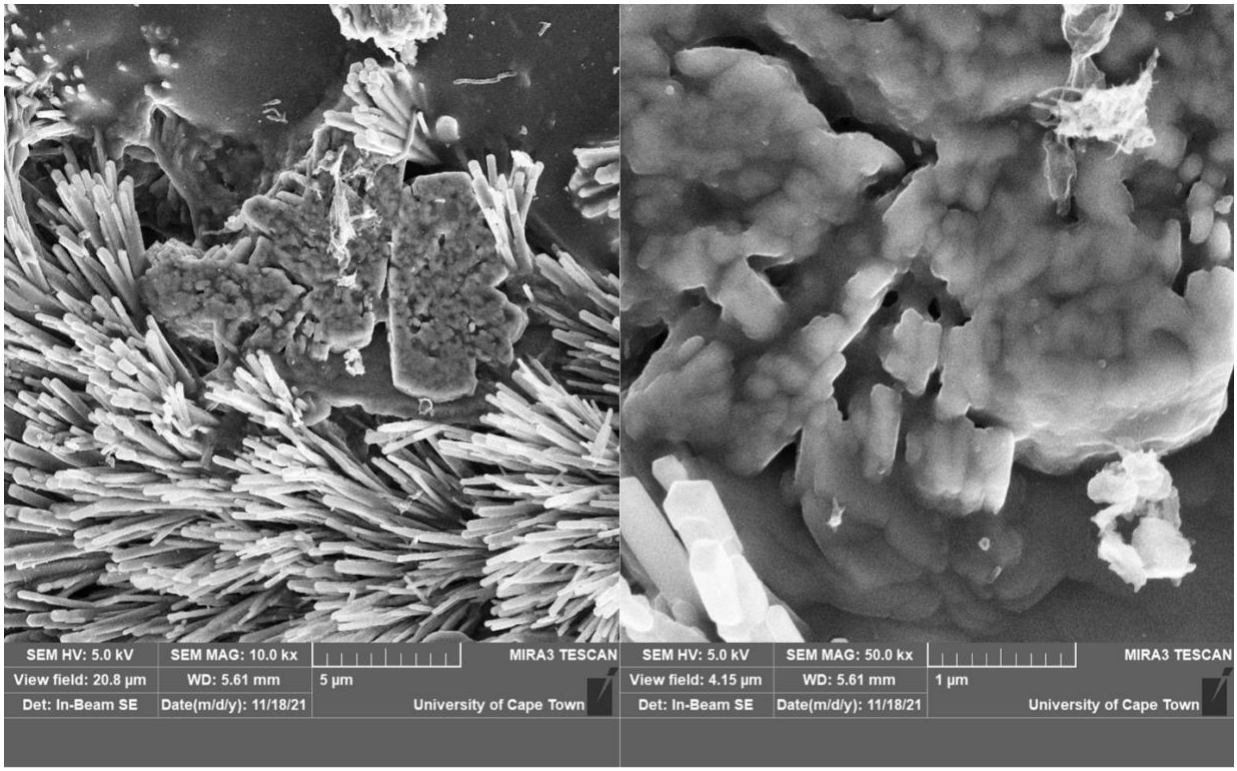

Figure 15: Scanning electron microscopy imaging of: (a) purified bioflocculant; (b) Kaolin clay flocculated by purified bioflocculant

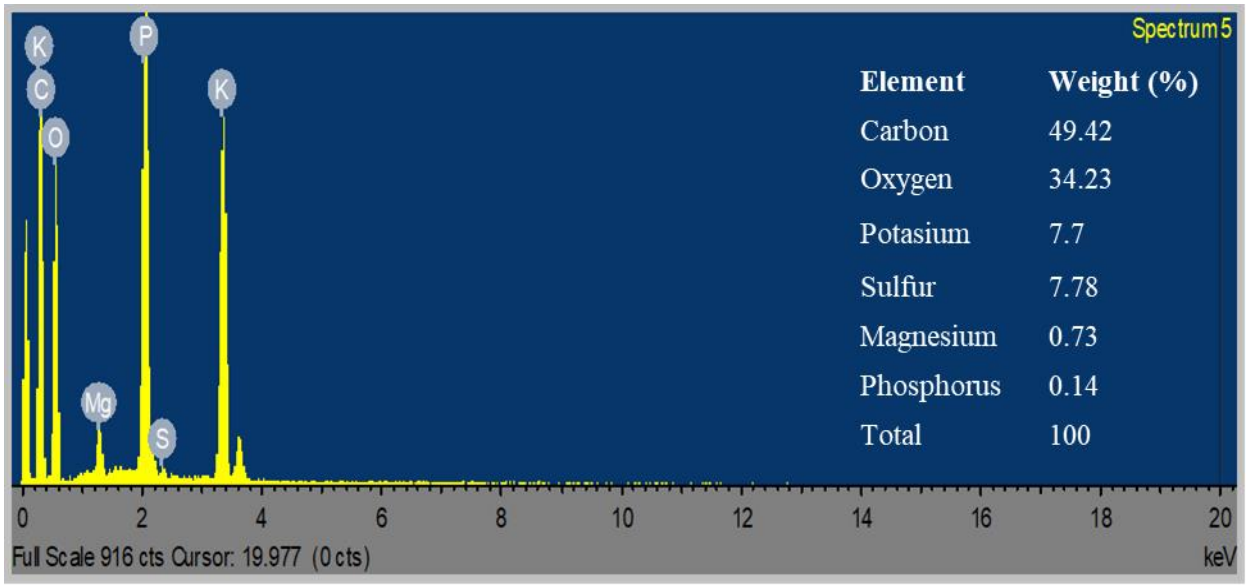

Figure 16: EDX elemental analysis of purified bioflocculant

4.5. Lab scale studies of a bioflocculant produced by *S. maltophilia* MT291866.1

4.5.1. Determination of optimum bioflocculant dosage

The bioflocculant optimal dosage was evaluated within a range of 0.2 to 1.2 mg/ml and the results are presented in **Figure 17**. It has been established in literature that overdosing or insufficient dose could result in weak flocculating activity of bioflocculant dosage during the process of coagulation (Agunbiade *et al*., 2018). Consequently, it is necessary to validate the optimum dosage in water and wastewater treatment to minimise the cost of production and prevent health-related concerns associated with higher usage of coagulants (Patel and Vashi, 2013). As shown in **Figure 17**, optimum flocculation activity of **91%** was observed when 1 mg/ml bioflocculant dosage at pH 7 used to flocculate kaolin clay suspension. Contrastingly, lower concentration of bioflocculant dosage ($0.2 - 0.8$ mg/ml) resulted in poor flocculation and further increase of bioflocculant dosage above 1.0 mg/ml resulted in poor flocculation, as shown in **Figure 17**. This variation in dosage could be due to the charge reversal and destabilisation of colloidal particle (Agunbiade *et al.*, 2017). In contrast to the present study, 90% flocculating efficiency was observed in the range of $0.3 - 1.2$ mg/l and an optimum value of 96.9% was achieved at the dosage of 1.2 mg/ml at optimum pH 5.5 and temperature of 15 ℃ by the bioflocculant produced by *C. daeguense* (W. J. Liu *et al.*, 2010). According to an earlier study Bisht and Lal (2019), the maximum flocculation efficiency of 99% was attained on BF-VB2 bioflocculant produced by *Bacillus* sp. at an optimum dosage of 0.2 mg/l. Comparing this observation to the current study, at the concentration dosage of 0.2 mg/l the flocculation efficiency attained by a bioflocculant produced from *S. maltophilia* was just above **75**%, as shown in **Figure 17** and this contrasts with their findings. However, the current findings are backed up by a study conducted by Agunbiade *et al.* (2017) on a bioflocculant produced by *Arthrobacter humicola*. They observed that a concentration range of 0.1 – 0.7 mg/ml revealed that the flocculation efficiency was weak which resulted in insufficient bridging, their optimum maximum dosage was 0.8 mg/ml which gave 89% flocculation efficiency. Contrary to all these findings outlined, Wang *et al.* (2011) reported that a bioflocculant produced from a mixed culture of *Rhizobium radiobacter* F2 and *Bacillus sphaeicus* F6 revealed a flocculation efficiency above 96.21% at 12 mg/ml. The high flocculation efficiency results obtained in this study at a low bioflocculant dosage of 1 mg/ml suggest that this bioflocculant may be beneficial to industrial-scale applications and further reduce treatment costs.

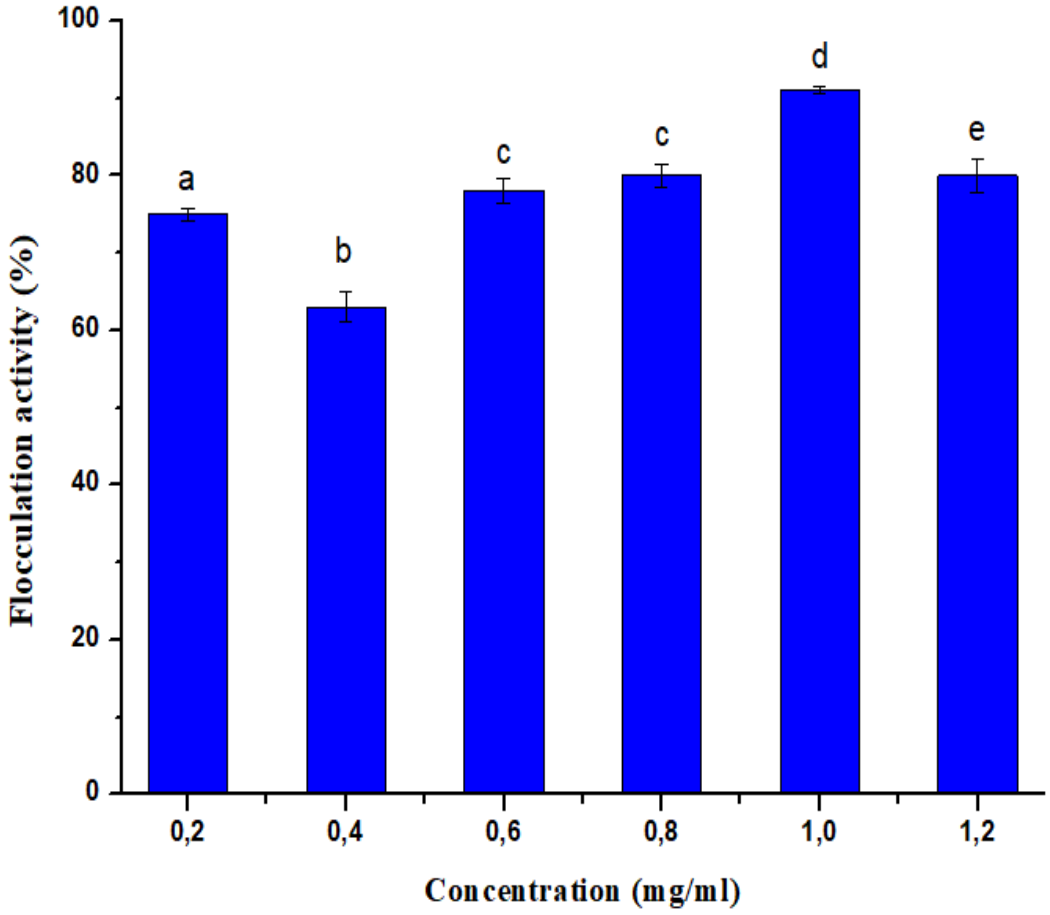

Figure 17: The effect of concentration dosage on bioflocculant produced by *S. maltophilia* H7

4.5.2. Comparative analysis of purified bioflocculant and chemical flocculants

4.5.2.1. Flocculation efficiency

A flocculant's molecular structure and charge play a crucial role in its flocculation efficiency (Okaiyeto *et al.*, 2016). Conventional flocculants, such as metal salts or synthetic polymers, are designed with specific chemical structures to target and interact with certain types of contaminants. Similarly, bioflocculants possess specific chemical structures derived from natural sources, which can affect their flocculation efficiency. The flocculation efficiency of *S. maltophilia* was compared to readily available flocculants such as polyethylenimine and polyacrylamide against kaolin clay suspension at concentration range from $0.2 - 1.2$ mg/ml; the results are presented in **Figure 18**. The results revealed that the purified bioflocculant from *S. maltophilia* was significantly efficient at an optimum concentration of 1.0 mg/ml when compared with 0.4 mg/ml, and 0.8 mg/ml for polyethylemine and Polyacrylamide respectively. The significant flocculating efficiency of **84. 5**% exhibited by the purified bioflocculant affirms its potential in biotechnological applications as a substitute for chemical flocculants implicated in various health related problems. In contrast to the obtained polyacrylamide flocculation efficiency of 29.6%, Okaiyeto *et al.* (2015) reported that polyacrylamide exhibited the

highest flocculating efficiency of 94.30% compared to the produced MBF-UF bioflocculant that presented 91.1%. This observation is also confirmed by a study conducted by Ugbenyen *et al.* (2012) which showed that polyacrylamide exhibited highest flocculation efficiency of 93.19% while the bioflocculant produced by a consortium of *Cobetia* and *Bacillus* gave 90% efficiency. Furthermore, in the current study, the maximum polyethylenimine flocculation efficiency was recorded as 65.7% while this is in contrast with findings by Ugbenyen and Okoh.(2014), they have reported that polyethylenimine revealed the least flocculation efficiency of 42.85%. In addition, Agunbiade *et al.* (2018) reported similar findings to the current study pertaining polyethylenimine that exhibited 86.95% flocculating efficiency. On the other hand, polyacrylamide exhibited high flocculation efficiency of 95.02%.

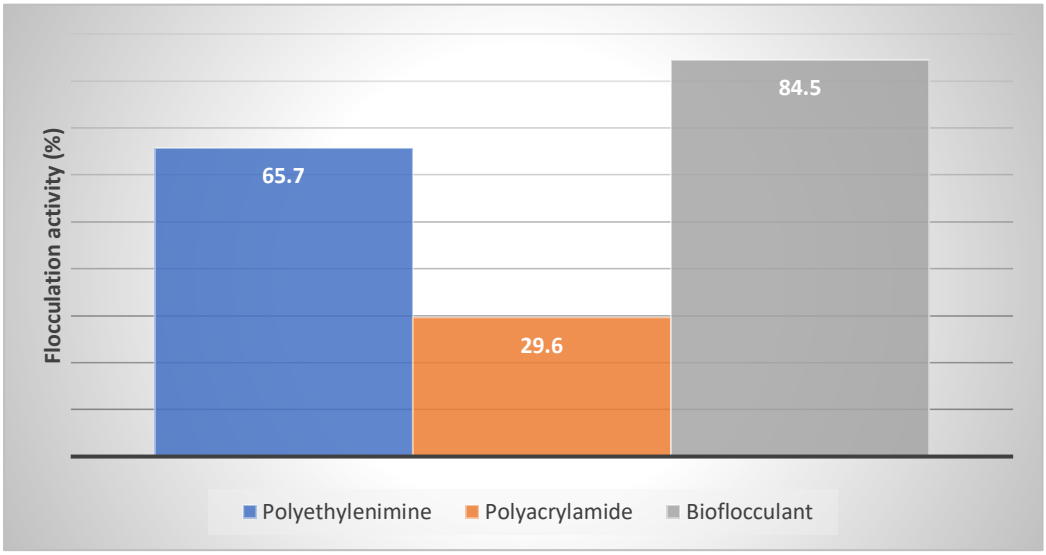

Figure 18: Flocculation activity Statistical chart

4.5.2.2. Acceptance of a bioflocculant produced from marine *S. maltophilia* by the public

Concerning issues outlined in this report associated with the use of chemical based flocculants, bioflocculants are emerging as an alternative technology solution to overcome public potential health and environmental hazards (Kurniawan *et al.*, 2020). On the basis that bioflocculants are biodegradable and pose no secondary pollution to the environment, the utilisation of a bioflocculant produced from *S. maltophilia* has proven to exhibit good flocculation capabilities compared to other chemical flocculants, therefore, it would be greatly considered in terms of public acceptance.

4.5.2.3. Availability of bioflocculant-producing resources

As indicated in this report, microorganisms from natural resources produce bioflocculants. These natural resources include rivers, marine sediments, algae, biological sludge, wastewater, etc. The abundance of these natural resources for bioflocculants is considered sufficient for further utilisation pertaining to bioflocculant production, and access is not limited (Kristianto, 2017). In the current study,

the bioflocculant was produced from marine environment soil sediment samples. As explained in research methodology of this report, the extraction methods of the bioflocculants are considered complex and this is making the availability of these flocculants limited as compared to chemical flocculants, which are readily available on the market to use (Kurniawan *et al.*, 2020).

4.5.2.4. Sludge generation, dewatering, disposal and potential resource recovery

Bioflocculants can form larger and denser flocs compared to existing conventional flocculants. Consequently, a smaller quantity of bioflocculant is required to achieve flocculation, resulting in reduced sludge volume. This can lead to cost savings in terms of sludge handling, transportation, and disposal (Kurniawan *et al.*, 2020). Conventional flocculants, such as metal salts or synthetic polymers, may leave chemical residues in the sludge after flocculation (Chew *et al.*, 2019). These residues can have potential environmental and health concerns, as outlined in this report. In contrast, bioflocculants are typically composed of natural substances with lower chemical residues, making the generated sludge safer for disposal or further treatment (Kominko *et al*., 2017; Kurniawan *et al.*, 2020). Bioflocculants have biodegradable advantages as compared to conventional flocculants. Thus, the utilisation of conventional flocculants such as alum produces an enormous amount of non-biodegradable sludge, while bioflocculants produce highly biodegradable sludge. In terms of dewatering, bioflocculants often exhibit improved dewatering characteristics compared to conventional flocculants. They can promote better water release from the sludge, leading to higher cake solids and reduced moisture content (Kominko *et al*., 2017). Accordingly, more efficient dewatering is attained, including lower energy consumption and reduced costs associated with sludge disposal. Unlike conventional flocculants, bioflocculants have the added advantage of being capable of binding with certain contaminants or nutrients in the wastewater. This opens opportunities for resource recovery from the generated sludge. For example, bioflocculant-bound heavy metals or organic matter can be recovered or recycled, contributing to a more sustainable approach to waste management (Ahmad *et al*., 2016; Kominko *et al*., 2017; Chew *et al.*, 2019).

4.6. Application of a bioflocculant produced from *S. maltophilia* H7

Bioflocculants are reported to have applications in various industries, as outlined in this study. Ugbenyen and Okoh, (2014) evaluated the application of a bioflocculant produced by the consortium in various wastewaters, such as brewery wastewater, dairy wastewater, and river wastewater, focusing on a few wastewater characteristics, including COD removal and flocculation activity. In this study, the application of the produced bioflocculant from marine *S. maltophilia* was investigated through wastewater from a city of Cape Town municipal wastewater treatment works. The wastewater was composed of domestic wastewater and industrial wastewater. The chemical properties of primary wastewater sludge before treatment and after treatment are shown in **Table 17**. Interestingly, it was observed that the purified bioflocculant produced by *S. maltophilia* could flocculate the municipal wastewater with efficiency, COD removal, and turbidity removal values of **81%**, **71.5%** and **75%**,

respectively. Bioflocculant dosage of 1 mg/ml has been well established in the literature that bioflocculant isolated from different sources has been implicated in wastewater and potable water treatment. Similarly, *Serratia ficaria* flocculated river water at an efficiency of 90.4% with COD and turbidity removal efficiencies of 87.1% and 84.2%, respectively (Gong *et al.*, 2008). In another study, maximum COD and turbidity removal of 61.2% and 95.6% were achieved when a bioflocculant produced by *Bacillus licheniformis* X14 was used to treat low-temperature drinking water (Li *et al.*, 2009). Accordingly, these results agree with previous studies Pathak *et al.* (2015) and Agunbiade *et al.* (2022), which reported that bioflocculants exhibited high-efficiency removal of contaminants and heavy metals in wastewater. Therefore, the performance of our test organism in this study affirmed its industrial application in wastewater treatment. In addition, bioflocculants applications have been implicated in sludge dewatering, where it has been demonstrated to be an effective conditioner in enhancing the dewater-ability of the sludge (Liu *et al.*, 2010; Guo and Ma, 2015); nutrient recovery, Pu *et al.* (2014) described that a bioflocculant produced from two strains of *Rhizopus* sp. was effective in the recovery of protein.

Table 17: Physiochemical characteristics of sewage wastewater before and after treatment with purified bioflocculant

Parameters	Wastewater BT	Wastewater AT	Units
pH	7.63	7.89	
Turbidity	136	34	**NTU**
COD	1623	462	**(mg/L)**
Flocculation activity		81	**(%)**
COD removal		71.5	**(%)**

BT is before treatment, and AT is after treatment; COD – Chemical oxygen demand

Chapter 5

5. Conclusion and Recommendations

5.1. Conclusion

This study explored a marine environment to search for a bioflocculant-producing strain. PB design was considered for screening and establishing significant independent variables, and RSM coupled with CCD was used to optimise these variables further. The screening design model revealed that glucose, yeast extract, $(NH_4)_2SO_4$ and K_2HPO_4, exhibited a statistically significant influence on the bioflocculant production as carbon, nitrogen and cation sources. At the optimum operating conditions of glucose 16.25 g/l, yeast extract 1.61 g/l, K_2HPO_4 1.1 g/l, and $(NH_4)_2SO_4$ 3.5 g/l, the statistical regression model predicted a flocculation activity of 95.97% and this was confirmed in the laboratory as $96.05\% \pm 0.02\%$ and a high bioflocculant yield of 4 g/l was attained. Marine *S. maltophilia* with GenBank accession number MT291866.1 was found to be a bioflocculant-producing bacterium. FTIR revealed the presence of functional groups such as hydroxyl group, carboxyl group and sugar derivatives. This affirms that the bioflocculant produced from *S. maltophilia* is a polysaccharide. The pyrolysis property of the bioflocculant proved its thermal stability as it retained 85% of its weight when heated to 500 ℃. SEM confirmed the biofloculant's morphological structure to be amorphous as it revealed clumped sheath layers, crystalline rods and irregular patterns. EDX affirmed the presence of carbon, magnesium, sulphur, potassium, oxygen and phosphorous. The purified bioflocculant was more effective at an optimal concentration of 1 g/l at pH 7. Compared to conventional flocculants such as polyethyleneimine and polyacrylamide, purified bioflocculants exhibited a high flocculation activity of 84.5%. This confirms its potential in biotechnological applications as an alternative to chemical flocculants, which have been linked to various health issues. The bioflocculant application in wastewater revealed that it could potentially remove chemical oxygen demand (COD) turbidity as it exhibited a high flocculation activity of 81%. Incorporating the statistical design and modelling to improve the growth medium and culture conditions may aid in lowering the overall production costs. The discovery of a bioflocculant produced from marine *S. maltophilia* will contribute to the body of knowledge, as this is the first study to report on *S. maltophilia* isolated from a marine environment.

5.2. Future work recommendations

The recommendations for future work are as follows:

- The bioflocculant production in this study was conducted on a laboratory scale as it is still at a research level, unlike chemical flocculants that are already well known and developed. Therefore, more research can be directed towards scaling up bioflocculant production to a pilot plant and further on an industrial scale.
- Considering that this study performed a solvent extraction method coupled with ethanol extraction yielded a 4.28 g crude bioflocculant, optimisation of extraction and purification techniques suitable for bioflocculant production may be explored in future for attaining a better yield on a pilot scale or industrial scale.
- The availability and cost-effectiveness of bioflocculants compared to conventional flocculants are mainly influenced by factors such as production costs, scalability, and market demand. Hence, a techno-economic (TEA) analysis for the bioflocculant produced in this study can be conducted.
- As the demand for sustainable and environmentally friendly alternatives continues to rise, the availability of bioflocculants is likely to grow as well. Thus, the feasibility demonstrated by the bioflocculant in the industrial wastewater treatment in this study may be justified in future work since the bioflocculant-producing strain (S. *maltophilia*) is reported for the first time from the marine environment in this study.
- Furthermore, for future studies, the application of the bioflocculant in the current study can be explored in the removal of metals from wastewater, nutrient recovery and treatment of river wastewater and tannery wastewater.

5.3. References

Abd El-Salam, A. E. *et al.* (2017) 'Isolation, characterisation, optimisation, immobilisation and batch fermentation of bioflocculant produced by Bacillus aryabhattai strain PSK1', *Journal of Genetic Engineering and Biotechnology*

Abu Bakar, S. N. H. *et al.* (2021a) 'A review of the production process of bacteria-based polymeric flocculants', *Journal of Water Process Engineering*.

Abu Bakar, S. N. H. *et al.* (2021b) 'A review of the production process of bacteria-based polymeric flocculants', *Journal of Water Process Engineering*

Abu Tawila, Z. M. M. *et al.* (2019) 'A novel efficient bioflocculant QZ-7 for the removal of heavy metals from industrial wastewater', *RSC Advances*. Royal Society of Chemistry

Adewumi, J. R., Ilemobade, A. A. and Van Zyl, J. E. (2010) 'Treated wastewater reuse in South Africa: Overview, potential and challenges', *Resources, Conservation and Recycling*.

Agunbiade, M. *et al.* (2018) 'Bioflocculant production from Streptomyces platensis and its potential for river and waste water treatment', *Brazilian Journal of Microbiology*. Sociedade Brasileira de Microbiologia

Agunbiade, M. *et al.* (2022) 'Bioflocculant produced by Bacillus velezensis and its potential application in brewery wastewater treatment', *Scientific Reports*. Nature Research,

Agunbiade, M. O. *et al.* (2016) 'A review of the application of biofloccualnts in wastewater treatment', *Polish Journal of Environmental Studies*

Agunbiade, M. O. *et al.* (2017) 'Flocculating performance of a bioflocculant produced by Arthrobacter humicola in sewage waste water treatment', *BMC Biotechnology*. BMC Biotechnology,

Agunbiade, M. O. *et al.* (2019) 'Evaluation of fresh water actinomycete bioflocculant and its biotechnological applications in wastewaters treatment and removal of heavy metals', *International Journal of Environmental Research and Public Health*

Agunbiade, M., Pohl, C. and Ashafa, O. (2018) 'Bioflocculant production from Streptomyces platensis and its potential for river and waste water treatment', *Brazilian Journal of Microbiology*. Sociedade Brasileira de Microbiologia

Ahmad, A. L. *et al.* (2005) 'Optimisation of coagulation-flocculation process for palm oil mill effluent using response surface methodology', *Environmental Science and Technology*

Ahmad, A. L., Ismail, S. and Bhatia, S. (2005) 'Optimisation of coagulation-flocculation process for palm oil mill effluent using response surface methodology', *Environmental Science and Technology*,

Ahmad, T., Ahmad, K. and Alam, M. (2016) 'Sustainable management of water treatment sludge through 3'R' concept', *Journal of Cleaner Production*.

Al-Enezi, G., Hamoda, M. F. and Fawzi, N. (2004) 'Ion Exchange Extraction of Heavy Metals from Wastewater Sludges', *Journal of Environmental Science and Health - Part A Toxic/Hazardous Substances and Environmental Engineering*

Alcides, T. J. *et al.* (2019) 'Optimisation of Coagulation/Flocculation Treatment of Brewery Wastewater Employing Organic Flocculant Based of Vegetable Tannin', *Water, Air, and Soil Pollution*

Alias, J. *et al.* (2022) 'Properties of bioflocculant-producing bacteria for high flocculating activity efficiency', *Environmental Technology & Innovation*.

Aljuboori, A. H. R. *et al.* (2015) 'Flocculation behavior and mechanism of bioflocculant produced by Aspergillus flavus', *Journal of Environmental Management*

Arayes, M. A. *et al.* (2023) 'Exopolysaccharide production from Alkalibacillus sp. w3: statistical optimisation and biological activity', *Biologia*. Springer Science and Business Media Deutschland

Awolusi, O. O. *et al.* (2020) 'Marine actinobacteria bioflocculant: A storehouse of unique biotechnological resources for wastewater treatment and other applications', *Applied Sciences (Switzerland)*,

Ayangbenro, A. S. *et al.* (2019) 'Bioflocculant production and heavy metal sorption by metal resistant bacterial isolates from gold mining soil', *Chemosphere*.

Azuma, T. and Hayashi, T. (2021) 'On-site chlorination responsible for effective disinfection of wastewater from hospital', *Science of the Total Environment*.

Bache, D. H. *et al.* (1999) *Sweep coagulation: structures, mechanisms and practice*.

Bao-jun, J. and Jiang-mei, Y. (2012) 'The Research Status and Development Trend of Microbial Flocculant', *Physics Procedia*

Bhagwat, P. K. *et al.* (2015) 'Statistical medium optimisation for the production of collagenolytic protease by Pseudomonas sp. SUK using response surface methodology', *Microbiology (Russian Federation)*,

Bharati, M. and Jigajini, S. (2021) 'Identification of Effective Factors for Immobilised Lipase Catalysed Biodiesel Production from Pongamia pinnata Seed Oil Using Plackett-Burman Design', *International Journal of Current Microbiology and Applied Sciences*,

Bhatnagar, A. and Sillanpää, M. (2010) 'Utilisation of agro-industrial and municipal waste materials as potential adsorbents for water treatment-A review', *Chemical Engineering Journal*,.

Bisht, V. and Lal, B. (2019) 'Exploration of performance kinetics and mechanism of action of a potential novel bioflocculant BF-VB2 on clay and dye wastewater flocculation', *Frontiers in Microbiology*,

Buthelezi, S. P. *et al.* (2012) 'Textile dye removal from wastewater effluents using bioflocculants produced by indigenous bacterial isolates', *Molecules*

Chai Siah, L. B. (2017) 'Extraction of Bio-Flocculant From Okra Using Hydrothermal and Microwave Extraction Methods Combined With a Techno-Economic Assessment Thesis Submitted in Fulfillment of the Requirements for the Degree of Doctor of Philosophy Department of Chemical and Env'.

Chen, H. *et al.* (2016) 'Removal of cadmium by bioflocculant produced by Stenotrophomonas maltophilia using phenol-containing wastewater', *Chemosphere*.

Chen, Z. *et al.* (2017) 'Characterisation of a novel bioflocculant from a marine bacterium and its application in dye wastewater treatment', *BMC Biotechnology*.

Chew, K. W. *et al.* (2019) 'Transformation of biomass waste into sustainable organic fertilisers', *Sustainability (Switzerland)*.

Chouchane, H. *et al.* (2018) 'A novel thermally stable heteropolysaccharide-based bioflocculant from hydrocarbonoclastic strain Kocuria rosea BU22S and its application in dye removal', *Environmental Technology (United Kingdom)*.

Cosa, S. *et al.* (2013) 'Characterisation of a thermostable polysaccharide bioflocculant produced by Virgibacillus species isolated from Algoa bay', *African Journal of Microbiology Research*. Academic Journals,

Cosa, S. and Okoh, A. (2014) 'Bioflocculant production by a consortium of two bacterial species and its potential application in industrial wastewater and river water treatment', *Polish Journal of Environmental Studies*,

Crini, G. and Lichtfouse, E. (2019) ‘Advantages and disadvantages of techniques used for wastewater treatment’, *Environmental Chemistry Letters*. Springer International Publishing,

Das, N., Ojha, N. and Mandal, S. K. (2021) ‘Wastewater treatment using plant-derived bioflocculants: Green chemistry approach for safe environment’, *Water Science and Technology*. IWA Publishing,

Dhokpande, S. R., Kulkarni, S. J. and DR Kaware, J. P. (2014) ‘A Review On Research On Application Of Trickling Filters In Removal Of Various Pollutants From Effluent

Edokpayi, J. N. *et al.* (2020) ‘Recent trends and national policies for water provision and wastewater treatment in South Africa’, in *Water Conservation and Wastewater Treatment in BRICS Nations: Technologies, Challenges, Strategies and Policies*..

El-Gaayda, J. *et al.* (2021) ‘Natural flocculants for the treatment of wastewaters containing dyes or heavy metals: A state-of-the-art review’, *Journal of Environmental Chemical Engineering*. Elsevier

Fayez, D. *et al.* (2022) ‘Carotegenic Virgibacillus halodenitrificans from Wadi El-Natrun Salt Lakes: Isolation, Optimisation, Characterisation and Biological Activities of Carotenoids’, *Biology*. MDPI,

Gannet (2013) ‘Central composite design’, (1), pp. 1–2.

Gong, W. X. *et al.* (2008) ‘Bioflocculant production by culture of Serratia ficaria and its application in wastewater treatment’, *Bioresource Technology*.

Gregor, J. E. *et al.* (1997) ‘Optimising natural organic matter removal from low turbidity waters by controlled pH adjustment of aluminium coagulation’, *Water Research*, 31(12), pp.

Guo, H. *et al.* (2018) ‘Bioflocculants’ production from a cellulase-free xylanase-producing Pseudomonas boreopolis G22 by degrading biomass and its application in cost-effective harvest of microalgae’, *Bioresource Technology*. Elsevier Ltd, 255, pp. 171–179

Guo, J. and Ma, J. (2015) ‘Bioflocculant from pre-treated sludge and its applications in sludge dewatering and swine wastewater pretreatment’, *Bioresource Technology*. Elsevier Ltd, 196, pp. 736–740.

Haasler, S. *et al.* (2023) ‘Synthetic and biopolymers for lake restoration – An evaluation of flocculation mechanism and dewatering performance’, *Journal of Environmental Management*. Academic Press,

Hasan, H. A. *et al.* (2009) ‘A review on the design criteria of biological aerated filter for COD, ammonia and manganese removal in drinking water treatment Direct Ethanol Fuel Cell View project Green-Treatment of Aquaculture Effluent View project’, 70(4), pp. 25–33.

Hasan, H. A. *et al.* (2012) ‘Isotherm equilibria of Mn2+ biosorption in drinking water treatment by locally isolated Bacillus species and sewage activated sludge’, *Journal of Environmental Management*,

Hazime, R. *et al.* (2013) ‘Optimisation of imazalil removal in the system UV/TiO2/K2S2O8 using a response surface methodology (RSM)’, *Applied Catalysis B: Environmental*,

He, J. *et al.* (2009) ‘Medium optimisation for the production of a novel bioflocculant from Halomonas sp. V3a′ using response surface methodology’, *Bioresource Technology*, 100(23),

He, J. *et al.* (2013) ‘Characterisation and optimisation of production of exopolysaccharide from Chlamydomonas reinhardtii’, *Carbohydrate Polymers*

He, N., Li, Y. and Chen, J. (2004) ‘Production of a novel polygalacturonic acid bioflocculant REA-11 by Corynebacterium glutamicum’, *Bioresource Technology*,

Hu, C. *et al.* (2006) ‘Coagulation behavior of aluminum salts in eutrophic water: Significance of Al13 species and pH control’, *Environmental Science and Technology*,

Hwang, H. J. *et al.* (2003) 'Production and characterisation of exopolysaccharides from submerged culture of Phellinus linteus KCTC 6190', *Enzyme and Microbial Technology*. Elsevier Inc.

Ismail, B. and Nampoothiri, K. M. (2010) 'Production, purification and structural characterisation of an exopolysaccharide produced by a probiotic Lactobacillus plantarum MTCC 9510', *Archives of Microbiology*,

Jo, J. H. *et al.* (2008) 'Biological hydrogen production by immobilised cells of Clostridium tyrobutyricum JM1 isolated from a food waste treatment process', *Bioresource Technology*,

Jorgensen, T. C. and Weatherley, L. R. (2003) 'Ammonia removal from wastewater by ion exchange in the presence of organic contaminants', *Water Research*,

Joshi, N. *et al.* (2017) 'Recycling of starch processing industrial wastewater as a sole nutrient source for the bioflocculant production', *Environmental Progress and Sustainable Energy*,

Joshi, N. *et al.* (2020) *Bioflocculated industrial wastewater for ameliorating bioflocculant production, Current Developments in Biotechnology and Bioengineering: Resource Recovery from Wastes*. Elsevier

Kang, X. M. *et al.* (2015) *Genome-wide identification of genes necessary for biofilm formation by nosocomial pathogen Stenotrophomonas maltophilia reveals that orphan response regulator FsnR is a critical modulator, Applied and Environmental Microbiology*.

Karthiga Devi, K. and Natarajan, K. A. (2015) 'Production and characterisation of bioflocculants for mineral processing applications', *International Journal of Mineral Processing*. Elsevier,

Kim, H. S. *et al.* (2019) 'Optimisation of heterotrophic cultivation of Chlorella sp. HS2 using screening, statistical assessment, and validation', *Scientific Reports*. Nature Research,

Kominko, H., Gorazda, K. and Wzorek, Z. (2017) 'The Possibility of Organo-Mineral Fertiliser Production from Sewage Sludge', *Waste and Biomass Valorisation*. Springer Netherlands,

Koul, B. *et al.* (2022) 'Application of Natural Coagulants in Water Treatment: A Sustainable Alternative to Chemicals', *Water (Switzerland)*. MDPI.

Kristianto, H. (2017) 'The Potency of Indonesia Native Plants as Natural Coagulant: a Mini Review', *Water Conservation Science and Engineering*. Springer Science and Business Media

Ksibi, M. (2006) 'Chemical oxidation with hydrogen peroxide for domestic wastewater treatment', *Chemical Engineering Journal*,

Kumar, C. G. *et al.* (2004) 'Purification and characterisation of an extracellular polysaccharide from haloalkalophilic Bacillus sp. I-450', *Enzyme and Microbial Technology*,

Kurniawan, S. B. *et al.* (2020a) 'Challenges and opportunities of biocoagulant/bioflocculant application for drinking water and wastewater treatment and its potential for sludge recovery', *International Journal of Environmental Research and Public Health*. MDPI AG,

Kurniawan, S. B. *et al.* (2020b) 'Challenges and opportunities of biocoagulant/bioflocculant application for drinking water and wastewater treatment and its potential for sludge recovery', *International Journal of Environmental Research and Public Health*,

Kurniawan, S. B. *et al.* (2022) 'What compound inside biocoagulants/bioflocculants is contributing the most to the coagulation and flocculation processes?', *Science of the Total Environment*,

Lee, C. S. *et al.* (2014) 'A review on application of flocculants in wastewater treatment', *Process Safety and Environmental Protection*. Institution of Chemical Engineers,

Lee, C. S. *et al.* (2015) 'Optimisation of extraction and sludge dewatering efficiencies of bio-flocculants extracted from Abelmoschus esculentus (okra)', *Journal of Environmental Management*.

Elsevier Ltd

Li, J. *et al.* (2017) 'Novel bioflocculant produced by salt-tolerant, alkaliphilic strain Oceanobacillus polygoni HG6 and its application in tannery wastewater treatment', *Bioscience, Biotechnology and Biochemistry*. Taylor & Francis.

Li, Q. *et al.* (2010) 'Isolation and characterisation of temperature and alkaline stable bioflocculant from Agrobacterium sp. M-503', *New Biotechnology*. Elsevier B.V.

Li, R. *et al.* (2014) 'Compound bioflocculant and polyaluminum chloride in kaolin-humic acid coagulation: Factors influencing coagulation performance and floc characteristics', *Bioresource Technology*. Elsevier Ltd.

Li, Z. *et al.* (2009) 'Production of a novel bioflocculant by Bacillus licheniformis X14 and its application to low temperature drinking water treatment', *Bioresource Technology*. Elsevier Ltd.

Lian, B. *et al.* (2008) 'Microbial flocculation by Bacillus mucilaginosus: Applications and mechanisms', *Bioresource Technology*,

Liang, Y. *et al.* (2009) 'Biomass and lipid productivities of Chlorella vulgaris under autotrophic, heterotrophic and mixotrophic growth conditions', *Biotechnology Letters*,

Lin, C. Y. and Lay, C. H. (2004) 'Carbon/nitrogen-ratio effect on fermentative hydrogen production by mixed microflora', *International Journal of Hydrogen Energy*,

Lin, J. L. *et al.* (2008) 'Coagulation dynamics of fractal flocs induced by enmeshment and electrostatic patch mechanisms', *Water Research*. Elsevier Ltd.

Liu, S. *et al.* (2010) 'Optimisation of the production of organic solvent-stable protease by Bacillus sphaericus DS11 with response surface methodology', *Bioresource Technology*.

Liu, W. J. *et al.* (2010) 'Production and characterisation of an intracellular bioflocculant by Chryseobacterium daeguense W6 cultured in low nutrition medium', *Bioresource Technology*. Elsevier Ltd.

Lu, C. and Chiu, H. (2006) 'Adsorption of zinc(II) from water with purified carbon nanotubes', *Chemical Engineering Science*.

Luvuyo, N. *et al.* (2013) *Studies on bioflocculant production by a mixed culture of Methylobacterium sp. Obi and Actinobacterium sp. Mayor*.

Ma, L. *et al.* (2019) 'Production of a bioflocculant from Klebsiella sp. OS-1 using brewery wastewater as a source', *Environmental Technology (United Kingdom)*,

Maćczak, P. *et al.* (2020) 'Recent achievements in polymer bio-based flocculants for water treatment', *Materials*. MDPI AG.

Makapela, B. *et al.* (2016) 'Assessment of bacillus pumilus isolated from fresh water milieu for bioflocculant production', *Applied Sciences (Switzerland)*. MDPI AG.

Maliehe, T. S. *et al.* (2016) 'African Journal of Biotechnology Production, characterisation and flocculation mechanism of bioflocculant TMT-1 from marine Bacillus pumilus JX860616',

Maliehe, T. S. *et al.* (2022) 'Isolation of a Marine Bacterium and Application of Its Bioflocculant in Wastewater Treatment', *Microbiology Research*. MDPI,

Masilan, K. *et al.* (2021) 'Valorisation of discarded industrial fish processing wastes for the extraction of gelatin to use as biodegradable fish bait matrix using RSM', *PeerJ Materials Science*,

Meriem, G. and Mahmoud, K. (2017) 'Optimisation of chitinase production by a new Streptomyces griseorubens C9 isolate using response surface methodology', *Annals of Microbiology*. Springer Verlag,

Mohammed, J. N. and Dagang, W. R. Z. W. (2019) 'Role of Cationisation in Bioflocculant Efficiency: a Review', *Environmental Processes*. Springer Basel.

Mohammed, J. N. and Wan Dagang, W. R. Z. (2019) 'Culture optimisation for production and characterisation of bioflocculant by Aspergillus flavus grown on chicken viscera hydrolysate', *World Journal of Microbiology and Biotechnology*. Springer Netherlands,

Mohammed, J. N. and Wan Dagang, W. R. Z. (2020) 'Implications for industrial application of bioflocculant demand alternatives to conventional media: Waste as a substitute', *Water Science and Technology*,

Mu, J. *et al.* (2019) 'Preparation and characterisation of a substitute for Ruditapes philippinarum conglutination mud as a natural bioflocculant', *Bioresource Technology*. Elsevier Ltd.

Mubarak, M. *et al.* (2019) 'Flocculation: An effective way to harvest microalgae for biodiesel production', *Journal of Environmental Chemical Engineering*. Elsevier Ltd,

Murthy, M. S. R. C. *et al.* (no date) *Statistical optimisation of lipase catalysed hydrolysis of methyloleate by response surface methodology*.

Nadeem, F. *et al.* (2020) *Advancements in natural bio-flocculants for water treatment, IJCBS*.

Nadeem, F., Khan, F. G. and Bashir, I. (2020) *Advancements in natural bio-flocculants for water treatment, IJCBS*.

Nan, J. *et al.* (2016) 'Breakage and regrowth of flocs formed by sweep coagulation using additional coagulant of poly aluminium chloride and non-ionic polyacrylamide', *Environmental Science and Pollution Research*. Springer Verlag.

Ndabigengesere, A. and Subba Narasiah, K. (1998) 'Quality of water treated by coagulation using Moringa oleifera seeds', *Water Research*.

Nie, M. *et al.* (2011) 'Production of a novel bioflocculant MNXY1 by Klebsiella pneumoniae strain NY1 and application in precipitation of cyanobacteria and municipal wastewater treatment', *Journal of Applied Microbiology*.

Nie, Y. *et al.* (2021) 'Aspergillus oryzae, a novel eco-friendly fungal bioflocculant for turbid drinking water treatment', *Separation and Purification Technology*. Elsevier B.V.

Ntombela, C. *et al.* (2016) 'A critical look at South Africa's green drop programme', *Water SA*

Ntozonke, N. *et al.* (2017) 'A marine bacterium, Bacillus sp. Isolated from the sediment samples of Algoa Bay in South Africa produces a polysaccharide-bioflocculant', *International Journal of Environmental Research and Public Health*. MDPI.

Nwodo, U. U. *et al.* (2014) 'Bioflocculant production by a consortium of Streptomyces and Cellulomonas species and media optimisation via surface response model', *Colloids and Surfaces B: Biointerfaces*. Elsevier B.V.

Nwodo, U. U. and Okoh, A. I. (2014) 'Mixed culture fermentation and media optimisation by response surface model: Streptomyces and brachybacterium species in bioflocculant production', *Molecules*. MDPI AG.

Okaiyeto, K., Nwodo, U. U., Leonard, M. V., *et al.* (2015) 'Bacillus toyonensis strain AEMREG6, a bacterium isolated from South African marine environment sediment samples produces a glycoprotein bioflocculant', *Molecules*.

Okaiyeto, K., Nwodo, U. U., Mabinya, L. V., *et al.* (2015) 'Characterisation of a bioflocculant (MBF-UFH) produced by bacillus sp. AEMREG7', *International Journal of Molecular Sciences*.

Okaiyeto, K., Nwodo, U. U., Mabinya, L. V., *et al.* (2016) 'Evaluation of flocculating performance of

a thermostable bioflocculant produced by marine Bacillus sp.', *Environmental Technology (United Kingdom)*. Taylor and Francis Ltd.

Okaiyeto, K., Nwodo, U. U., Okoli, S. A., *et al.* (2016a) 'Implications for public health demands alternatives to inorganic and synthetic flocculants: Bioflocculants as important candidates', *MicrobiologyOpen*. Blackwell Publishing Ltd.

Okaiyeto, K., Nwodo, U. U., Okoli, S. A., *et al.* (2016b) 'Implications for public health demands alternatives to inorganic and synthetic flocculants: Bioflocculants as important candidates', *MicrobiologyOpen*.

Okaiyeto, K. *et al.* (2020) 'Global research trends on bioflocculant potentials in wastewater remediation from 1990 to 2019 using a bibliometric approach', *Letters in Applied Microbiology*,

Okoh, S. A. I. (2010) 'Assessment of bioflocculant production by some marine bacteria isolated from the bottom sediment of algoa bay by Sekelwa Cosa Submitted in fulfillment of the requirements for the degree of MASTER ' S OF SCIENCE (MICROBIOLOGY) Department of Biochemistry',

Otieno, F. A. O. and Ochieng, G. M. M. (2004) 'Water management tools as a means of averting a possible water scarcity in South Africa by the year 2025', *Water SA*.

Pamla, A. *et al.* (2021) 'Persistent droughts and water scarcity: Households' perceptions and practices in Makhanda, South Africa'

Patel, H. and Vashi, R. T. (2013) 'Comparison of naturally prepared coagulants for removal of COD and color from textile wastewater', *Global Nest Journal*.

Pathak, M. *et al.* (2014) 'Application of bioflocculating property of Pseudomonas aeruginosa strain IASST201 in treatment of oil-field formation water', *Journal of Basic Microbiology*.

Pathak, M. *et al.* (2015) 'Production of a non-cytotoxic bioflocculant by a bacterium utilising a petroleum hydrocarbon source and its application in heavy metal removal', *RSC Advances*.

Patil, S. V. *et al.* (2010) 'Bioflocculant exopolysaccharide production by Azotobacter indicus using flower extract of Madhuca latifolia L', *Applied Biochemistry and Biotechnology*.

Peng, H. and Guo, J. (2020) 'Removal of chromium from wastewater by membrane filtration, chemical precipitation, ion exchange, adsorption electrocoagulation, electrochemical reduction, electrodialysis, electrodeionisation, photocatalysis and nanotechnology: a review', *Environmental Chemistry Letters*. Springer International Publishing,

Priya, T. *et al.* (2018) 'Effect of bioflocculants on the coagulation activity of alum for removal of trihalomethane precursors from low turbid water', *Journal of Environmental Sciences (China)*. Chinese Academy of Sciences,

Pu, S. yan *et al.* (2014) 'Preparation and application of a novel bioflocculant by two strains of Rhizopus sp. using potato starch wastewater as nutrilite', *Bioresource Technology*. Elsevier Ltd,

Rebah, F. Ben, Mnif, W. and Siddeeg, S. M. (2018) 'Microbial flocculants as an alternative to synthetic polymers for wastewater treatment: A review', *Symmetry*. MDPI AG.

Ren, D. *et al.* (2013) 'Medium optimisation to improve the flocculation rate of a novel compound bioflocculant, CBF-256, using response surface methodology and flocculation characters', *Bioscience, Biotechnology and Biochemistry*.

Salehizadeh, H. *et al.* (2018) 'Recent advances in polysaccharide bio-based flocculants', *Biotechnology Advances*. Elsevier Inc.

Salehizadeh, H. and Shojaosadati, S. A. (2001) 'Extracellular biopolymeric flocculants: Recent trends and biotechnological importance', *Biotechnology Advances*..

De Sanctis, M. *et al.* (2016) 'Integration of an innovative biological treatment with physical or chemical disinfection for wastewater reuse', *Science of the Total Environment*. Elsevier B.V.

Schlesinger, M. E. *et al.* (2011) 'Solvent Extraction', *Extractive Metallurgy of Copper*, (Chapter 15), pp. 323–347.

Selepe, T. N. *et al.* (2022) 'Isolation and Optimisation of Culture Conditions for a Marine Bioflocculant-Producing Bacterium and Application of Its Bioflocculant in Wastewater Treatment', *International Journal of Environmental Research and Public Health*. MDPI, 19(16).

Shakeel, S. *et al.* (2020) 'Application of Response Surface Methodology to Optimise Ammonia Volatilisation Determination by Enclosure Method', *Communications in Soil Science and Plant Analysis*. Taylor & Francis, pp. 2038–2044.

Sharma, B. R. *et al.* (2006) 'Flocculants - An ecofriendly approach', *Journal of Polymers and the Environment*.

Singh, I. and Sahu, A. K. (2019) 'A review on stone columns used for ground improvement of soft soil', *International Journal of Innovative Technology and Exploring Engineering*.

Singh, R. P. *et al.* (2011) 'Isolation and characterisation of exopolysaccharides from seaweed associated bacteria Bacillus licheniformis', *Carbohydrate Polymers*. Elsevier Ltd.

Sun, J. *et al.* (2012) 'Bioresource Technology Preparation and characteristics of bioflocculants from excess biological sludge', *Bioresource Technology*. Elsevier Ltd.

Suopajarvi, T. (2015) *Functionalised nanocelluloses in wastewater treatment applications*.

Surendhiran, D. and Vijay, M. (2013) 'Influence of bioflocculation parameters on harvesting Chlorella salina and its optimisation using response surface methodology', *Journal of Environmental Chemical Engineering*.

Syafalni, S. *et al.* (2012) 'Production of bioflocculant by Chryseomonas luteola and its application in dye wastewater treatment', *Modern Applied Science*.

Tang, W. *et al.* (2014) 'Production, characterisation, and flocculation mechanism of cation independent, pH tolerant, and thermally stable bioflocculant from Enterobacter sp. ETH-2'

Tanyildizi, M. S., Özer, D. and Elibol, M. (2005) 'Optimisation of α-amylase production by Bacillus sp. using response surface methodology', *Process Biochemistry*,

Tarley, C. R. T. *et al.* (2009) 'Chemometric tools in electroanalytical chemistry: Methods for optimisation based on factorial design and response surface methodology', *Microchemical Journal*. Elsevier B.V.

Tawila, Z. M. A. *et al.* (2018) 'Production and characterisation of a bioflocculant produced by bacillus salmalaya 139si-7 and its applications in wastewater treatment', *Molecules*,

Tsilo, P. H. *et al.* (2021) 'Isolation and Optimisation of Culture Conditions of a Bioflocculant-Producing Fungi from Kombucha Tea SCOBY', pp. 950–966.

Tsilo, P. H. *et al.* (2022) 'Production and Characterisation of a Bioflocculant from Pichia kudriavzevii MH545928.1 and Its Application in Wastewater Treatment', *International Journal of Environmental Research and Public Health*. MDPI.

Ugbenyen, A. *et al.* (2012) 'Thermostable bacterial bioflocculant produced by Cobetia spp. Isolated from Algoa Bay (South Africa)', *International Journal of Environmental Research and Public Health*. MDPI.

Ugbenyen, A. M. and Okoh, A. I. (2014) 'Characteristics of a bioflocculant produced by a consortium of Cobetia and Bacillus species and its application in the treatment of wastewaters', *Water SA*.

Uppala, R., Sundar, K. and Muthukumaran, A. (2019) 'Response surface methodology mediated optimisation of decolorisation of azo dye amido black 10B by Kocuria kristinae RC3', *International Journal of Environmental Science and Technology*. Center for Environmental and Energy Research and Studies.

Vanaja, K. and Rani, R. H. S. (2007) 'Design of experiments: Concept and applications of plackett burman design', *Clinical Research and Regulatory Affairs*.

Verma, A. K. *et al.* (2012) 'A review on chemical coagulation/flocculation technologies for removal of colour from textile wastewaters', *Journal of Environmental Management*. Elsevier Ltd.

Wang, H. *et al.* (2022) 'Constructed microalgal-bacterial symbiotic (MBS) system: Classification, performance, partnerships and perspectives', *Science of the Total Environment*. Elsevier B.V.

Wang, L. *et al.* (2011) 'Characterisation of a compound bioflocculant produced by mixed culture of Rhizobium radiobacter F2 and Bacillus sphaeicus F6', *World Journal of Microbiology and Biotechnology*.

Wu, C. *et al.* (2012) 'Coagulation performance and floc characteristics of aluminum sulfate using sodium alginate as coagulant aid for synthetic dying wastewater treatment', *Separation and Purification Technology*

Xia, S. *et al.* (2008) 'Production and characterisation of a bioflocculant by Proteus mirabilis TJ-1', *Bioresource Technology*

Yang, M. *et al.* (2017) 'Characterisation of an extracellular polysaccharide produced by Bacillus mucilaginosus MY6-2 and its application in metal biosorption', *Chemistry and Ecology*. Taylor & Francis.

Yang, Z. H. *et al.* (2009) 'Optimisation of flocculation conditions for kaolin suspension using the composite flocculant of MBFGA1 and PAC by response surface methodology', *Bioresource Technology*.

Zaidi, N. N., Rosmiza, W. and Dagang, Z. W. (2023), *Journal of Materials in Life Sciences*.

Zhang, Q. W., Lin, L. G. and Ye, W. C. (2018) 'Techniques for extraction and isolation of natural products: A comprehensive review', *Chinese Medicine (United Kingdom)*. BioMed Central,

Zhang, X. *et al.* (2013) 'Production and flocculating performance of sludge bioflocculant from biological sludge', *Bioresource Technology*. Elsevier Ltd.

Zhao, C. and Chen, W. (2019) 'A review for tannery wastewater treatment: some thoughts under stricter discharge requirements', *Environmental Science and Pollution Research*. Environmental Science and Pollution Research,

Zhong, C. *et al.* (2020) 'Production of a bioflocculant from ramie biodegumming wastewater using a biomass-degrading strain and its application in the treatment of pulping wastewater', *Chemosphere*.

Zhou, Y. *et al.* (2017) 'Optimisation of medium compositions to improve a novel glycoprotein production by Streptomyces kanasenisi ZX01', *AMB Express*. Springer Verlag.

Zhou, Y. and Franks, G. V (2006) 'Flocculation Mechanism Induced by Cationic Polymers Investigated by Light Scattering'.

Zolgharneina, J. *et al.* (2013) 'Comparative study of Box-Behnken, central composite, and Doehlert matrix for multivariate optimisation of Pb (II) adsorption onto Robinia tree leaves', *Journal of Chemometrics*.