Faculty of Engineering and Science
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Physicochemical Characterisation and Flocculation Analysis of a Novel Cationic Chitosan-Like Bioflocculant from *Citrobacter Youngae* GTC 01314 to Improve Sludge Dewatering

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DECLARATION BY STUDENT

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgement has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

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Date: 9 February 2021

DECLARATION BY SUPERVISOR

I hereby declare that I have read this thesis and in my opinion this thesis is sufficient in terms of scope and quality for the Degree of Master of Philosophy (Chemical Engineering).

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ABSTRACT

Wastewater treatment often generates large amounts of sludge as a by-product, leading to the difficulties in managing and disposing the sludge which is acknowledged as one of the major issues faced globally. High-water content in the sludge is not only cause difficulties in sludge management, but also leads to serious environmental problems and public health risk. This is due to the application of chemicals in conventional wastewater treatment processes. In the past few years, bioflocculants derived from natural sources have emerged as promising substitutes for synthetic flocculants. Bacterial strains from the genus *Citrobacter* are reported to produce chitosan-like bioflocculants with strong flocculation activity, but the understanding of their characteristics and molecular interactions with suspended matter is still lacking. These bacterial strains are proven to be cultivated effectively in acetate medium which could be a cheaper alternative substrate. Furthermore, its existence in a readily soluble form could be advantageous over the natural chitosan derived from marine or fungi sources.

This study aims to investigate the flocculation behaviour of a novel bioflocculant (BF), namely, BF01314 produced by *Citrobacter youngae* in enhancing sludge dewaterability. Selected physicochemical characteristics in terms of sugar, protein and apparent chitosan content, ionic behaviour, functional groups and thermal degradation properties of the BF were studied. The flocculation behaviour of the BF was first examined in kaolin suspension under the effects of flocculant dosage, pH and temperature, and then extended to its application to a real activated sludge sample. The effects of flocculant dosage, pH, temperature, and flocculation speed and time, on sludge dewatering performance were investigated in terms of capillary suction time (CST), specific resistance to filtration (SRF), cake solids content (CSC), zeta potential, and flocculation activity. The performance of the BF was then compared with the ones of chitosan and cationic polymer MF 7861 to evaluate its efficiency. Fourier-transform infrared (FTIR) spectrum and microscopic images of the sludge before and after conditioning were analysed to elucidate the possible flocculation mechanisms.

From the characterisation studies, it is confirmed that BF01314 is a chitosan-like bioflocculant with an identical pattern of infrared spectrum to the one of chitosan. The

presence of hydroxyl, amino, and amide (C=O and C-N stretches) groups were observed at 3265 cm^{-1}, 1532 cm^{-1}, 1632 cm^{-1} and 1323 cm^{-1}, respectively. The BF solution contained 12.38% apparent chitosan and 5.34% sugar with no trace of protein detected. Upon the addition to kaolin suspension, BF01314 exhibited strong flocculation activity of >95% in a wide range of dosage between 4 and 120 mg-dry weight/L with the optimal dosage recorded at 15 mg-dry weight/L. The BF also revealed reasonably high pH stability (>98% activity) in acidic solution up to the pH below its isoelectric point (pH 8.25). High flocculation activity and thermostability were also observed over the temperature range between 25 to 95°C, which supported the observation of its polysaccharide backbone.

In sludge conditioning and dewatering experiment, BF01314 showed significant improvement of dewatering performance, comparable to the results obtained when using chitosan and MF 7861. The optimal performance was found at the flocculant dosage of 3.0 kg/t dry solids (CST: 8.1 s; SRF: 3.26×10^{10} m/kg; CSC: 25.67%; zeta potential: -4.86 mV) within the range tested. Similar to the kaolin flocculation experiment, the BF demonstrated good dewatering results between pH 2 to 8 and at a temperature range between 25 to 80°C. The flocculation kinetics of the BF was best fitted to pseudo-first order reaction in terms of turbidity removal. Electrostatic charge patching was proposed as one of the governing flocculation mechanisms of the BF based on the zeta potential results. Additionally, bridging was also proposed as the most-likely mechanism of the BF, supported with its high molecular weight (327kDa) and the results from the analysis of microscope images and infrared spectrum. In conclusion, it is postulated that BF01314 is a chitosan-like polysaccharide comprising of a glucosamine backbone with amino groups which can be protonated in acidic regime, similar to those of natural chitosan. The effectiveness of BF01314 in kaolin flocculation advocates its potential application in removal of suspended solids in water and wastewater treatment. The potential application of BF01314 in sludge conditioning and dewatering has been confirmed in this study. Nevertheless, future investigations are still necessary and strongly recommended to address the limitations of the present work and expand the scope of this study.

Keywords: bioflocculant, *Citrobacter*, sludge dewatering, flocculation, chitosan

NOMENCLATURE

Notation	Description
BF	Bioflocculant
WAS	Waste activated sludge
FA	Flocculation activity (%)
SRF	Specific resistance to filtration (m/kg)
CPAM	Cationic polyacrylamide
CST	Capillary suction time (s)
CSC	Cake solids content (%)
DS	Dry solids (g/L)
TS	Total solids (g/L)
TSS	Total suspended solids (g/L)
TDS	Total dissolved solids (g/L)
SVI	Sludge volume index
FTIR	Fourier transform infrared
GPC-MALLS	Gas permeation chromatography-multi angle laser light scattering
ESEM	Environmental scanning electron microscope
TGA	Thermogravimetric analysis
DTGA	Derivative thermogravimetric analysis

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CHAPTER 1

INTRODUCTION

1.1 Background

Natural based flocculants or bioflocculants (BFs) have received much attention in recent years due to its nontoxic and biodegradable properties, which offer important eco-friendly benefits. Studies have reported the practical application of natural polysaccharide BFs derived from biomass feedstocks, marine resources, and microorganisms, mainly in water and wastewater treatment. Polysaccharide BFs such as chitosan, starch, and pullulan have shown promising results in treating water and wastewater as an alternative to synthetic flocculants (Lichtfouse et al. 2019; Salehizadeh, Yan and Farnood 2018; Yang et al. 2016). Other authors also reported the potential use of microbial BF and bio-based flocculant (combined/grafted) in wastewater treatment (Guo and Wen 2020; Hassimi et al. 2020; Liu et al. 2020; Xu et al. 2018; Guo and Chen 2017; Kurade et al. 2016).

Wastewater treatment plant often generates large amounts of sewage sludge as a by-product, especially for urban treatment plants compared to industrial treatment plant (OECD 2015). This is because the primary sludge contains typically 98% of water content which leads to significant difficulties in sludge management and disposal (Evans 2016). Raw sludge commonly undergoes a series of pre-treatment processes, such as thickening, stabilisation, conditioning and dewatering to minimise pathogens, heavy metals, organic contaminants as well as to reduce bad odours and sludge volume (Peirce, Weiner and Vesilind 1998). Poor management and improper disposal of sludge may release these harmful materials into the soil at landfills (Lara et al. 2007; Mihelcic 2018). The constant increase of sludge production in global trend calls for a need to advance the existing technique of sludge dewatering with sustainable and environmental friendly methods (Rorat et al. 2019; Yang, Zhang and Wang 2015).

Sludge dewatering is a key pre-treatment process to increase the efficiency of the subsequent sludge treatment operations before disposal or further treatment for

beneficial uses as biosolids. Prior to dewatering, synthetic polymers are commonly applied in chemical conditioning to alter the sludge characteristics in order to facilitate water removal during the mechanical dewatering process. Cationic polyacrylamide (CPAM) has been extensively applied in wastewater and sludge treatment due to its good dewatering performance and cost-effectiveness (Zhou, Liu and Pan 2014). However, degraded polyacrylamide in the environment may release acrylamide monomer which has been reported to be potentially carcinogenic while residues from the application of aluminium-based flocculants have been argued to cause Alzheimer's disease (Polizzi et al. 2002; Rudén 2004; Campbell 2002). The effects of polyacrylamide degradation towards the environment and public health are still being discussed and remain unsolved (Okaiyeto et al. 2016; Huang et al. 2018; Xiong et al. 2018).

Today, there are growing appeals for application of microbial BFs in wastewater treatment and other industries owing to its high flocculation performance, simple cultivation process and relatively ease to source for (Rebah, Mnif and Siddeeg 2018; Shahadat et al. 2017; Al-Mamun, Hassan and Alam 2017). For example, bacterial strains of *Klebsiella pneumoniae*, *Bacillus pumilus*, *B. agaradhaerens*, *Streptomyces platensis*, *Rhodococcus erythropolis*, and *Pseudomonas aeruginosa* strain have been applied successfully in producing microbial BFs for wastewater treatment (Wang et al. 2020; Ngema, Basson and Maliehe 2020; Liu et al. 2019; Agunbiade, Pohl and Ashafa 2018; Guo, Liu, et al. 2018; Pathak et al. 2017). Many efforts have been explored to develop better bacterial strains with high flocculating potential for sustainable production of microbial BFs from cheaper substrates.

Previous researches have reported that chitin/chitosan-like BFs can be produced from bacterial strains in the genus *Citrobacter* (Kimura et al. 2013) and *Enterobacter* (Son, Hong and Lee 2007). Chitosan consists of reactive functional groups such as hydroxyl and amino groups, which are believed to greatly enhance the flocculation process through mechanisms like charge neutralisation and polymer bridging (Lichtfouse et al. 2019; Lapointe and Barbeau 2020). Chitosan has been substantially studied in various fields, mainly as effective flocculant/coagulant to remove wastewater pollutants and reduce turbidity in drinking water (Yang et al. 2020; Wang et al. 2020). However, the commercial production of chitosan from marine sources is challenging due to the

seasonal distribution and limited supply of crustaceans, in addition to the complex production processes in its practical production (Salehizadeh, Yan and Farnood 2018). Therefore, the application of chitin/chitosan-like bioflocculant secreted by microbial cultures in a soluble form could greatly simplify the production and downstream processes of chitosan (Takeo et al. 2018).

A high flocculated cation independent microbial BF (named BF04) produced from acetate and propionate by *Citrobacter* sp. TKF04, was first discovered to have a similar structure to chitin/chitosan using infrared spectroscopic analysis (Fujita et al. 2000). Acetate could be a cheap alternative substrate, comparable to glucose, when derived from thermal treatment and anaerobic digestion of organic wastes (Fujita et al. 2000). Similar findings were reported several years later for BFs produced from *Enterobacter* sp. BL-2 (Son et al. 2005) and *Citrobacter* sp. BL-4 (Kim et al. 2006). Subsequently, Kimura et al. (2013) reported that 21 out of 36 *Citrobacter* strains selected from different microbial culture collection centres showed the presence of flocculation activity in kaolin suspension, and some of them were found to have high activity. However, the physicochemical properties and the flocculation behaviour of their BFs have not been characterised yet. Effective flocculation demonstrated by many BFs is known to be a result of several simultaneous mechanisms influenced by many factors, including the characteristics of the polymer itself (Li et al. 2020). Hence, it is of significant interest to know the governing flocculation mechanisms in relation to the physicochemical characteristics of the BFs.

1.2 Problem statement

With rapid population growth and industrialisation leading to a spike in total number of wastewater treatment plants (WWTPs), the large amounts of sludge generated annually have become a global environmental issue of concern. Almost all types of sludge are difficult to manage and dispose due to high water content, which require more sustainable and effective sludge dewatering technologies to reduce the sludge volume. Various technologies such as ultrasound, microwave, alkali, and thermal treatment methods have been introduced to improve sludge dewaterability by disrupting the sludge cells to release the bound water trapped inside the cell structure. However, the disruption of sludge cells creates additional surfaces for water binding

which then requires further treatment to reduce the binding sites (Wei et al. 2018). Conventional method via chemical conditioning is widely recognised as one of the effective approaches in sludge treatment and has become an indispensable process prior to mechanical dewatering. Synthetic polymers and/or inorganic coagulants are still preferred in chemical conditioning by the industry today because of their cost-effectiveness even though it has been reported to cause potential environmental issues and health risks.

Many attempts have been made to search for potential replacement of synthetic flocculants through the utilisation of natural polymeric flocculants such as chitosan, but the commercial production of these flocculants remains challenging mainly because of the lack in sustainability of resources and efficacy of current processes. The use of microbial BFs could possibly address these bottlenecks since the cultivation processes are simpler than the current industrial processes, while many bacterial strains including those from the genus *Citrobacter* can be isolated from waste organic materials such as soil, sludge, and water. Besides, there are past and ongoing research efforts in developing better bacterial strains from cheaper substrates and in optimisation of the production of microbial BFs to reduce the production cost. The scaling-up of the process from flask culture to large-scale cultivation is also another area of ongoing research interest within this domain. *Citrobacter youngae* GTC 01314 was reported to be able to be cultivated effectively in a cheaper acetate medium, which brings the attention to the further investigation of this particular strain in addition to the claim by Kimura et al. (2013) on the potential of *Citrobacter* strains producing chitin/chitosan-like BFs.

1.3 Research gap

In recent years, there are emerging reports on the potential application of chitosan or chitosan-based flocculants for wastewater treatment (Lichtfouse et al. 2019; Wang et al. 2020; Yang et al. 2020) and for sludge dewatering enhancement (Chen et al. 2020; Liu et al. 2020; Zhang et al. 2019; Shi et al. 2019; Lichtfouse et al. 2019; Wang et al. 2016; Zemmouri, Mameri and Lounici 2015; Lau et al. 2015), mainly attributed to the unique functional groups of chitosan (hydroxyl, amide and amino groups) and its cationic behaviour when dissolved in acidic solutions. Further studies on the

application chitin/chitosan-like BF secreted by microbial cultures in a soluble form may offer greater production and processing advantages along with sustainable resources, as stated earlier (Takeo et al. 2018).

A number of studies on microbial BFs have been carried out using different types of bacterial strains (Busi et al. 2017; Kurade et al. 2016; Guo et al. 2017; Guo, Yu, et al. 2015; Guo and Ma 2015; Guo and Chen 2017; Guo, Chen, et al. 2018; Liu et al. 2014; Yang et al. 2012; Zhang, Xia and Zhang 2010); none of these strains was identified as chitin/chitosan-like BFs or produced from the genus *Citrobacter* strains. Limited studies have reported that several *Citrobacter* strains potentially secreted chitin/chitosan-like polysaccharides in acetate medium and few of them showed high flocculation ability in kaolin suspension including *Citrobacter youngae* (*C. youngae*) GTC 01314 (Kimura et al. 2013). However, there is no substantial evidence or study to support the claim of the chitin/chitosan-like structure of the biopolymer produced by this particular strain GTC 01314. To the author's knowledge, this biopolymer has not been investigated for its characteristics and flocculation behaviour at different environmental conditions such as pH and temperature. Hence, the present study is useful in knowledge contribution to the understanding and application of a novel cationic BF produced by this newly identified BF-producing bacterial strain *C. youngae* GTC 01314, which is the novelty of this research. Successful outcomes are expected to address the aforementioned potential environmental issues and health risks associated with the use of synthetic polymer flocculants through the replacement of microbial BFs, in general; and to propose a potential BF substitute for chitosan flocculant in order to justify further investigation into the scaling-up and the optimisation of the BF production, in particular.

1.4 Aim and objectives

In this study, a novel soluble cationic chitin/chitosan-like polysaccharide secreted by the strain GTC 01314 from *C. youngae* (named as BF01314) was selected for the physicochemical characterisation and flocculation analysis. The aim of this research is to investigate the effectiveness of BF01314 as a novel sludge dewatering agent, with the following specific objectives:

1. To determine the physicochemical characteristics of BF01314 using chemical assays, surface charge analysis, molecular weight determination, functional group analysis, and thermogravimetric analysis.
2. To evaluate the flocculation characteristics of BF01314 in kaolin suspension under the influences of flocculant dosage, pH, and temperature, and to elucidate its flocculation mechanisms from zeta potential and spectrometric analysis.
3. To examine the sludge dewatering performance of BF01314 in activated sludge suspension under the effects of flocculant dosage, pH, and temperature, and to compare its feasibility as dewatering agent with commercial chitosan and industrial cationic polymer.
4. To assess sludge flocculation kinetics using BF01314 at different flocculation speeds and times, followed by data fittings to simple kinetic models of zero, pseudo-first and pseudo-second orders.

1.5 Scope of the study

The scope of the study includes two major parts in investigating the feasibility of BF01314 as a novel sludge dewatering agent:

- **Part One: Evaluation of flocculation performance in kaolin suspension**

The flocculation performance of BF01314 was evaluated in a 10 mL synthetic kaolin suspension with a fixed concentration of 5 g/L. This concentration was assumed to be sufficient for the preliminary study of a novel flocculant before being tested in a real sludge suspension, according to most literature related to wastewater treatment. The effects of flocculant dosage, initial pH and temperature on the flocculation activity were assessed by spectrometric analysis. Ranges of bioflocculant dosage, pH and temperature selected for this study are 0.058–478 mg/L, pH 2–11, and 25–95°C, respectively. Zeta potential was also measured to provide an insight to the possible flocculation mechanisms.

- **Part Two: Assessment of dewaterability performance in real sludge**

The sludge conditioning and dewatering experiment was conducted in a batch experiment similar to jar test, in a 100 mL of sludge. The waste activated sludge (WAS)

was sourced from a domestic septic sludge treatment plant and used as received for all experiments in this study. The sample was obtained from the sludge holding tank, located after Sequencing Batch Reactor (SBR) and before polymer dosing and dewatering operation. Sludge dewatering performance was evaluated using standard filterability and dewaterability tests at laboratory scale, which are CST, SRF, CSC and zeta potential measurements. These tests are commonly reported in many published research articles on sludge dewatering. Three major environmental factors affecting sludge dewaterability were examined, namely the flocculant dosage (0.3–6.0 kg/t dry solids), pH (2–10), and temperature (10–100°C). The effects of flocculation speed (50–200 rpm) and time (1–7 minutes) were assessed for the percentage of flocculation activity to analyse the flocculation kinetics of BF01314. The type and rate of the reaction kinetics were also determined. The comparison of sludge dewatering performance was made between BF01314, chitosan and MF 7861, a synthetic cationic polymer sample from the industry. The sludge treated by BF01314 at optimal conditions of dosage, pH and temperature in present work was further examined using FTIR spectrum analysis and microscopic images.

1.6 Significance of the study

The significance of the study can be discussed in two aspects: theoretical contributions and practical implementations. The theoretical contributions can be seen from the addition to the knowledge repository of the current list of microbial BFs in literature as well as the understanding of the molecular interactions and governing flocculation mechanisms in relation to the physicochemical characteristics of the BFs, particularly for the BF produced by *C. youngae* GTC 01314.

Meanwhile from the aspect of practical implementations, this study is expected to provide supporting evidence of the potential application of microbial BFs in water and wastewater treatment processes, especially for sludge conditioning and dewatering. Potential application of the BF-treated sludge as a green energy source and biofertiliser after further processes is another practical aspect for extended study.

1.7 Thesis overview

This thesis consists of six main chapters as follows:

- **Chapter 1**: Summary of research background and problem statement, research gap, research objectives, scope and significance of the study in knowledge addition and practical application in water and wastewater treatment.
- **Chapter 2**: Literature review on wastewater sludge treatment processes, relevant sludge characterisation parameters, major flocculation mechanisms and factors affecting sludge conditioning and dewatering, and recent advance of application of microbial BFs in water and wastewater industries.
- **Chapter 3**: Research methodology presenting the materials, detailed experimental procedures and analytical methods adopted in this research study.
- **Chapter 4**: Analysis of flocculation performance and early insights of possible mechanisms determined by the characterisation of BF01314 and its flocculation behaviour in kaolin suspension under the effects of flocculant dosage, pH and temperature.
- **Chapter 5**: Evaluation of sludge dewaterability performance under the effects of flocculant dosage, pH, temperature, and by comparison study with other cationic flocculants, along with the analysis of flocculation kinetics, type of reaction kinetics, and proposed governing mechanisms of BF01314 in sludge conditioning.
- **Chapter 6**: Conclusion summarising all findings of the study and recommendations for future work related to this research area.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

This chapter provides literature review relevant to the research area. This includes an overview of sludge production and processing in wastewater treatment, sludge characterisation parameters, fundamental theory of coagulation-flocculation and its mechanism, different types of coagulants/flocculants, factors affecting the flocculation and sludge dewaterability, and a summary of the published reports on the application of microbial bioflocculants in the field of wastewater treatment.

2.2 Wastewater sludge processing

Wastewater treatment process generates semi-solid by-product known as sludge which requires further treatment before it can be safely disposed. Figure 2.1 shows a flow diagram of basic wastewater treatment processes including sludge treatment.

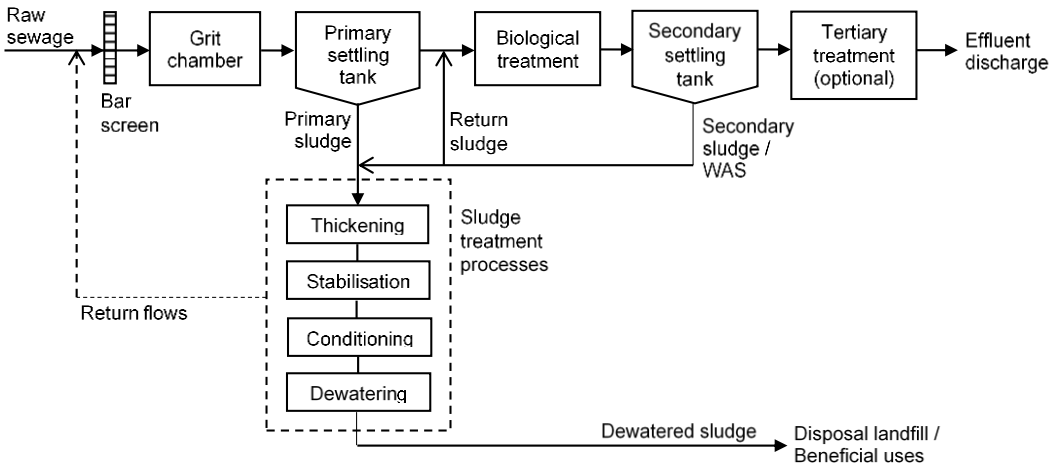

Figure 2.1: Typical flowchart of wastewater sludge treatment processes

Source: Figure adapted from Lau (2014, 14).

In general, raw wastewater undergoes a series of treatments; preliminary, primary and secondary to reduce contaminants and harmful substances. Preliminary treatment filters large floating particles and removes grit by mechanical means before passing through the sedimentation tank in primary treatment. The preliminary step is sometimes categorised under primary treatment, involving preliminary and pre-sedimentation steps which are essential to control the flow distribution from one process to another (EPA 2004). Secondary treatment is normally a biological process involving aerobic digestion which helps in removing about 90% of wastewater organic matter (EPA 2004). Tertiary treatment is optional and has been employed widely to further purify the wastewater for removal of specific inorganic compounds such as nitrogen and phosphorus (Sydney Water 2010).

The sludge produced from primary and secondary treatments are typically mixed and then treated through several processes. Thickening increases sludge concentration by removing free water content using gravitational flotation thickener (Peirce et al. 1998). Sludge stabilisation aims to minimise the pathogens and reduce bad odours either by addition of lime, aerobic or anaerobic digestion (Peirce et al. 1998). The last two steps are crucial to ensure the final products comply to environmental standards before disposal or beneficial use. The conditioning process enhances the performance of mechanical dewatering by altering the sludge characteristics either by chemical, physical or biological methods. Meanwhile, dewatering removes large amounts of water using mechanical equipment such as centrifuge and filter press. The additional step of drying is sometimes employed after dewatering, to further remove the remaining water content.

2.3 Type of sludge and its characteristics

Primary sludge and secondary sludge (the latter is also known as waste activated sludge) are two major types of sludge often reported in the literature. However, depending on the treatment process, other types or specific categories of sludge may be generated, varying in percentage of solids, colours, odours, and dewaterability characteristics as described in Table 2.1. Difficulties in sludge management and disposal is mainly due to the high-water content of the sludge which varies between 80% and 99%, and is typically reduced to 55% at most (Table 2.2)(Sperling and

Goncalves 2007). By determining the sludge characteristics, appropriate dewatering methods and type of conditioning agent can be selected.

Table 2.1: Type of sludge and its characteristics

Type of sludge	Percent. Solids (%)	Other characteristics
Raw primary	4 – 8	• Grey-brown; unpleasant odour • High concentration of pathogenic organisms • Poor dewaterability on drying beds
Anaerobic primary digested	6 – 10	• Black; musty; produces gas Good dewaterability on drying beds
Waste activated sludge (WAS)	0.5 – 1.5	• Yellow – brown; fluffy; little odour • Difficult to dewater • Highly active biologically
Mixed digested (primary + WAS)	2 – 4	• Black – brown; musty; produces gas • Difficult to dewater than digested primary
Aerobic digested	1 – 3	• Yellow – brown • Sometimes difficult to dewater • Biologically active
Waste alum	0.5 – 1.5	• Grey – yellow; odourless • Very difficult to dewater

Source: Table reproduced from Sanin et al. (2011, 6).

Table 2.2: Water content in different types of sludge

Type of sludge	Total solids (TS, %)	Water content* (%)
Raw primary	2.0 – 8.0	92 – 98
Digested primary	6.0 – 10.0	90 – 94
Raw activated (WAS)	0.5 – 1.5	≈ 99
Mixed (primary + WAS)	3.0 – 6.0	97 – 94
Digested primary + WAS	2.0 – 12.0	88 – 98

Source: Table adapted from Spinosa and Vesilind (2001, 10).

Note: * values calculated from percentage of TS.

2.4 Sludge characterisation and its dewaterability

2.4.1 Sludge characterisation parameters

Sludge characterisation can be divided according to their physical, chemical and biological properties. Solids content is one of the foremost basic parameters to be examined and is commonly quantified in terms of total solids (TS) and can be further divided into suspended solids (SS) and dissolved solids (DS). The fractal nature of sludge contributes to difficulties in the dewatering process. Therefore, the most prevalent characterisation parameters are commonly selected depending on the treatment process (Kopp and Dichtl 2001). In sludge conditioning and dewatering process, polymer dosage, particle size, capillary suction time (CST), water distribution, density, floc strength, and specific resistance to filtration (SRF) are among the parameters concerned. Other possible parameters are listed in Table 2.3.

Table 2.3: Sludge characterisation

Type of properties	**Characterisation parameters**
Physical properties	Solids content, sludge index, demand of conditioning agent, particle size and shape, specific gravity, settleability, odour, colour, capillary suction time (CST), water distribution, density, floc strength, resistance to filtration (SRF), rheology/viscosity, shear stability, and heat value.
Chemical properties	pH/alkalinity, fatty acids, and concentration of carbon, nitrogen, and phosphorus.
Biological properties	Pathogenic features, biological stability, and bulking.

Source: Table adapted from Spinosa and Vesilind (2001, 20) and Lau (2014, 15).

2.4.2 Sludge dewaterability

In laboratory scale experiments, filterability tests are commonly measured in terms of SRF and CST. Sludge with low resistance to filtration (normally $<10^{12}$ m/kg) and fast water discharge is easy to dewater using mechanical dewatering device. The higher the resistance of the sludge to filtration is, the more difficult it is to dewater. Therefore, the reduction of SRF and CST values obtained after sludge conditioning indicates the improvement in sludge dewaterability. Table 2.4 describes the level of difficulties of the sludge to be dewatered according to the typical ranges of SRF and water discharge velocity (CST/TSS).

Table 2.4: Sludge dewaterability with respect to typical ranges of SRF and water discharge velocity

Description	Ranges of standards	Reference
Easily dewaterable	SRF ≤ $10^{12.5}$, CST/SS ≤ 30 s/%	(Kopp and Dichtl 2001)
Averagely dewaterable	SRF ≈ $10^{12.5}$ – $10^{14.5}$, CST/SS ≈ 30 – 150 s/%	
Poorly dewaterable	SRF ≥ $10^{14.5}$, CST/SS ≤ 150 s/%	
Easier-to-dewater	SRF ranges from 10^{10} – 10^{11}	(Sanin, Clarkson and Vesilind 2011)
Difficult-to-dewater	SRF ranges from 10^{14} – 10^{15}	

Note: CST/TSS represents the water discharge velocity (time measured with respect to solids content).

Poor dewaterability by mechanical means often arises from high content of bound water trapped within the sludge structure. The selected dewatering processes vary in each industrial application, depending on the type of sludge, the polymer added and the purpose of dewatered products. Traditionally, sludge dewatering adopts a principle of natural evaporation and percolation with the use of mechanical devices such as filter press, centrifuge, drying beds and filtration to transform the sludge into biosolids with total solids contents of 10-40% (Amuda et al. 2008). Advanced methods include hybrid dewatering process which combines the mechanical dewatering device with other techniques such as thermal, ultrasonic, magnetic, microwave and electrical treatments (Mahmoud et al. 2013). Recent studies also demonstrated that more than one technique can be integrated with mechanical dewatering, for example, by varying the pressure to a desired condition in hydrothermal carbonisation (Gao et al. 2019) and the ultrahigh pressurised electro-dewatering methods (Rao et al. 2020). Dewatering is always preceded with chemical conditioning process to enhance sludge dewaterability which is viewed as one of the most effective methods (Mowla, Tran and Allen 2013).

2.5 Sludge conditioning

Sludge conditioning methods can be classified into physical, chemical, and biological methods. In cases where the usage of chemicals is of concern, physical conditioning methods such as thermal treatment, freeze-thaw, ultrasonic or microwave treatments are utilised. Heat treatment generates a product with solids content between 30-50% (ASCE 2000) while freeze-thaw can solubilise the organic matters from sludge matrix (Hu et al. 2011). However, the disruption of sludge cells resulted from most physical

conditioning treatments could lead to the production of fine particles and extra surfaces for water binding sites, which may require further treatment (Wei et al. 2018). Chemical conditioning involves the addition of chemical additives to alter sludge characteristics, while biological conditioning improves the dewatering ability by disintegrating the sludge organic matters (Amuda et al. 2008). Table 2.5 summarises the benefits and drawbacks of typical conditioning methods.

Table 2.5: Comparison of typical conditioning methods

Method	**Advantages**	**Drawbacks**
Inorganic coagulant conditioning (*Chemical*)	✓ Solutions can be kept for long-term storage	❖ Corrosive - require special equipment and operational procedure ❖ Increased production of sludge for disposal
Organic polymer conditioning (*Chemical*)	✓ Low maintenance and operational issues ✓ No reduction in calorific value – potentially used as fuel source for incineration	❖ Commercial products of liquid polymers have short shelf-life ❖ Non-dissolved dry polymer may cause clogging and scaling
Heat treatment (*Physical*)	✓ Eliminates problems associated with use of chemicals ✓ Reduces sludge bad odours ✓ Reduces amount of heat required for incineration	❖ High capital cost, maintenance, and operational skills requirement ❖ Equipment corrosion ❖ May require pre-treatment for recycled supernatant ❖ Sludge cell disruption creates additional surfaces for water binding
Enzyme conditioning (*Biological*)	✓ Reduces odours and chemical oxygen demand ✓ Suitable to treat wastewater from meat industry ✓ Less or no damage to biological treatment systems ✓ Low dosage requirement	❖ Requires specific nutrients such as humic acids and amino acids for enzyme culture ❖ Excessive fragmentation of sludge cells could deteriorate the sludge dewaterability performance

Source: Adopted from Amuda et al. (2008, 88), Andreoli (2007, 83–85) and Wei et al. (2018, 627).

In the past few years, the efficiency of combined conditioning methods or techniques in sludge treatment has become another area of research interest. These includes the treatment combinations of ultrasound and freezing (Carrasco 2013), enzyme treatment and chemical flocculation (Chen et al. 2015), and dual conditioning of biomass ash

and polyelectrolyte (Wójcik and Stachowicz 2019). Nevertheless, chemical conditioning remains preferable and is widely used as a pre-treatment prior to sludge dewatering due to its cost-effectiveness (Wei et al. 2018; Zhou et al. 2014). Chemical conditioning is also known as coagulation-flocculation process that involves the destabilisation of colloid particles (coagulation) and collision of these particles to agglomerate into flocs (flocculation) as a two-step process, as shown in Figure 2.2.

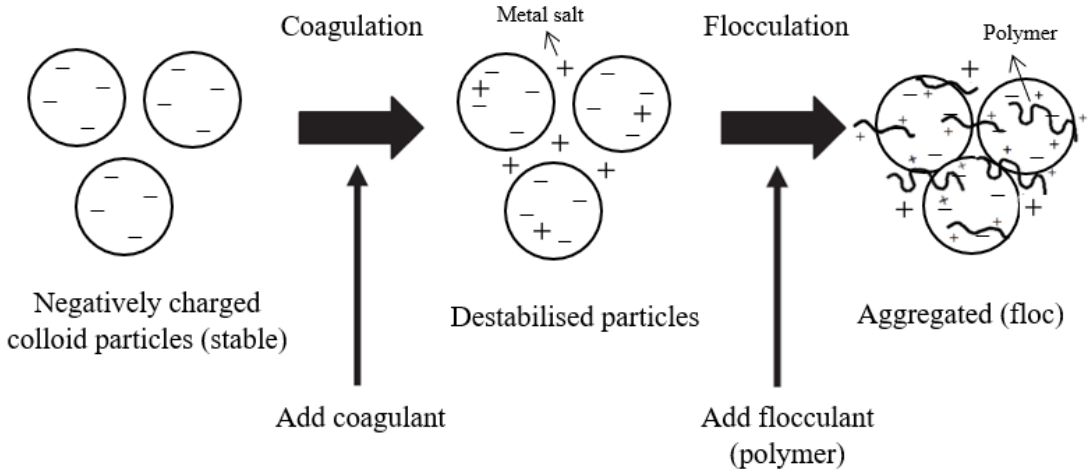

Figure 2.2: Coagulation-flocculation process

Adding inorganic coagulants into the colloidal suspension increases the ionic strength of the colloid surface charges leading to double layer compression and destabilisation, but this might not be sufficient to promote the flocculation effect (Gregory 2005). As a result, addition of polymeric flocculant is essential to form large flocs for fast and ease of sedimentation (Zhang et al. 2014). In most cases, conditioning agents can act as both coagulant and flocculant which explains why the term "coagulation" and "flocculation" have been used interchangeably. This process is called "direct flocculation" which involves the simultaneous process of destabilisation of colloidal particles and further adsorption onto the particles by bridging to form larger flocs (Chong 2012). Water-soluble ionic organic polymers can be utilised to accomplish direct flocculation without classical metal-based coagulants (Lichtfouse et al. 2019).

2.5.1 Type of coagulants/flocculants

Inorganic and organic materials are the two major types of coagulants/flocculants. In recent years, grafted flocculant or graft copolymer has been synthesised to enhance the

flocculation performance and reduce the utilisation of chemical conditioners. Figure 2.3 shows the classification of coagulants/flocculants with examples.

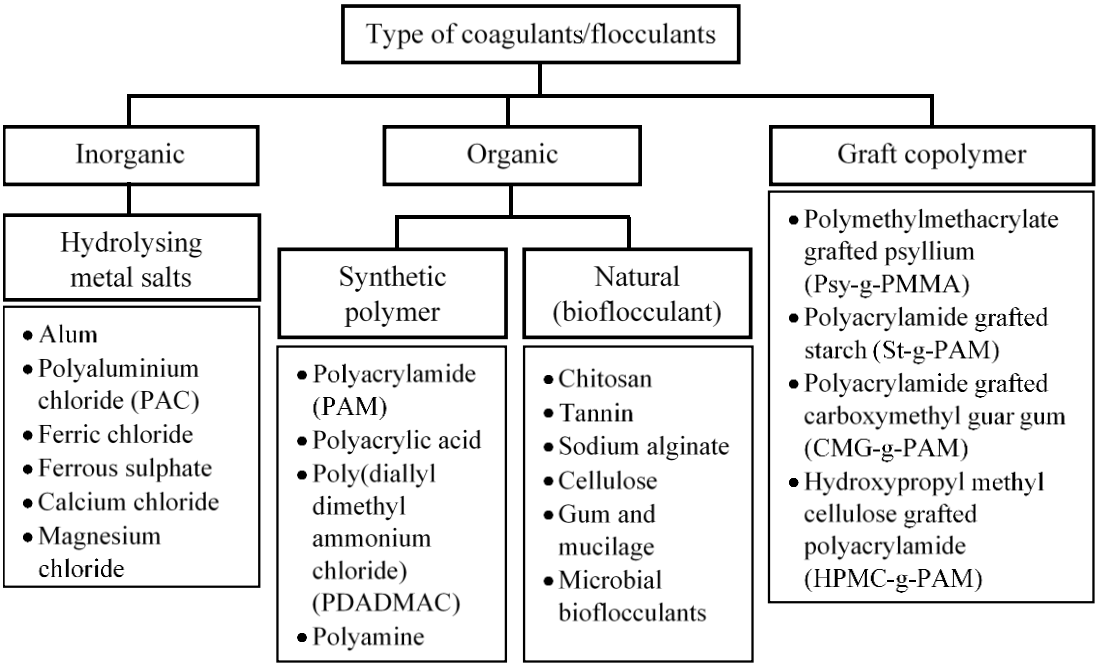

Figure 2.3: Type of coagulants/flocculants

Source: Data obtained from Salehizadeh et al. (2018, 92-119) and Lee et al. (2014, 489–508).

2.5.1.1 Inorganic coagulants/flocculants

Multivalent metal salts hydrolyse when added to the wastewater sludge and produce metal cations. Monovalent cation can be directly formed in one step of hydrolysis when the metal salt dissolves as shown in Equation 2.1.

$$KCl\ (s) \rightarrow K^{+}\ (aq) + Cl^{-}\ (aq) \qquad (2.1)$$

However, trivalent metal undergoes a series of hydrolysis stages as shown in Equations 2.2–2.5 (Gregory 2005). Each hydrolysis stage has different equilibrium constant which determines the solubility of each metal hydrolysis species in water.

$$Fe^{3+} + H_2O \rightleftharpoons Fe(OH)^{2+} + H^{+} \qquad (2.2)$$

$$Fe(OH)^{2+} + H_2O \rightleftharpoons Fe(OH)_2^{+} + H^{+} \qquad (2.3)$$

$$Fe(OH)_2^{+} + H_2O \rightleftharpoons Fe(OH)_3 + H^{+} \qquad (2.4)$$

$$Fe(OH)_3 + H_2O \rightleftharpoons Fe(OH)_4^{-} + H^{+} \qquad (2.5)$$

Typically, trivalent metal cations such as Fe(III) and Al(III) are preferable and said to effectively destabilise the negatively charged colloids. The uncharged metal salt, $Fe(OH)_3$ has very low solubility, and thus is likely to form precipitate rather than hydrolyse. The formation of precipitate plays an important role in the action of colloid entrapment, known as sweep-flocculation mechanism (Gregory 2005). A study by Niu et al. (2013) reported that $FeCl_3$ enhanced the sludge dewaterability more effectively than polyaluminium chloride (PAC) and high performance PAC (HPAC), with respect to flocculant dosage. Nevertheless, the sludge treated by PAC and HPAC resulted in larger flocs as compared to the flocs formed when treated with $FeCl_3$, which was probably due to the abundance of binding sites in polymeric species (Niu et al. 2013).

2.5.1.2 Organic coagulants/flocculants

Polymeric flocculants can be classified into non-ionic, anionic, cationic and ampholytic types. In conventional practice, organic polymeric flocculant is often coupled with inorganic metal coagulants to promote aggregation of destabilised colloid particles. Cationic polymer such as cationic polyacrylamide (CPAM) is commonly utilised as it can effectively flocculate the particles without the addition of inorganic coagulant. However, the potential release of monomer acrylamide from polyacrylamide degradation has raised significant concern as monomer acrylamide was reported to be carcinogenic (Rudén 2004).

Natural bioflocculants (BFs) derived from plants or animals are biodegradable and non-toxic. In a recent study by Zemmouri et al. (2015), it was reported that the sludge conditioned by chitosan showed comparable dewaterability performance to synthetic cationic polyelectrolyte Sedipur CF802 (Sed CF802). Chitosan is known as one of the most promising BFs, yet it suffers from many problems such as inconsistent level of deacetylation, protein contamination, seasonal limitation supply, laborious processes as well as generation of large amounts of chemical wastes (Salehizadeh et al. 2018). The chitosan production derived from crustacean shells through chemical deacetylation in industrial processing generally requires hot concentrated base or acid solution which could also contribute to high costs (Salehizadeh et al. 2018).

Microbial BFs have shown effective flocculation performance and dewaterability in recent studies. For instance, biogenic flocculant produced by *Acidithiobacillus ferrooxidans* was able to reduce the CST and SRF by 74% and 89% respectively in comparison to CPAM (Kurade et al. 2016). Novel BFs, M-C11 and MBF10 produced by *Klebsiella sp.* were able to exhibit high flocculation activity of about 92.4% and 86.5% respectively in kaolin suspension (Liu et al. 2014; Yang et al. 2012). Furthermore, previous studies showed that BFs produced by *Citrobacter* strains were able to be cultured in acetate medium with less production cost (Kimura et al. 2013; Fujita et al. 2000).

2.5.1.3 Grafted flocculants

Grafted flocculants attempt to improve the flocculation efficiency by combining the best properties of two polymers. The result of grafting also helps in minimising the amount of chemicals used. As a result, the level of toxicity is reduced as reported in chitosan-based flocculant (CMC-g-PDMC) (Yang et al. 2014). A water-soluble copolymer, starch-graft-poly(2-methacryloyloxyethyl) trimethyl ammonium chloride (STC-g-PDMC) shows relatively high performance in kaolin flocculation and better dewatering capability compared to polyacrylamide (Wang et al. 2013). The most recent literature by Liu et al. (2020) reported the superior performance of novel chitosan-based flocculants (CS-g-PAO) with amphiphilic structure in reducing sludge cake moisture content from 95% to 78%. Besides, a grafted flocculant named TMT^{-1}-g-PAM 2, produced from the strain *Bacillus pumilus* JX860616 and acrylamide was reported to be non-cytotoxic at concentration <200 µg/mL, and demonstrate longer shelf-life as compared to the natural BF without grafting (TMT^{-1}) (Ngema et al. 2020). However, the use of proper air-tight container and addition of stabilising agent are still required for chitosan and its derivatives to preserve the physicochemical properties of the polymer due to chitosan's degradation (Szymańska and Winnicka 2015).

2.5.2 Flocculation mechanisms and interactions

The addition of polymer to a colloidal suspension creates molecular interactions that leads to adsorption of many segments of polymer chains to the surface of colloid

particles (Figure 2.4), which further promotes the flocculation process (Gregory and Barany 2011). The interactions involved in adsorption may be hydrogen binding, hydrophobic interaction, ion binding, and electrostatic attraction (Gregory and Barany 2011).

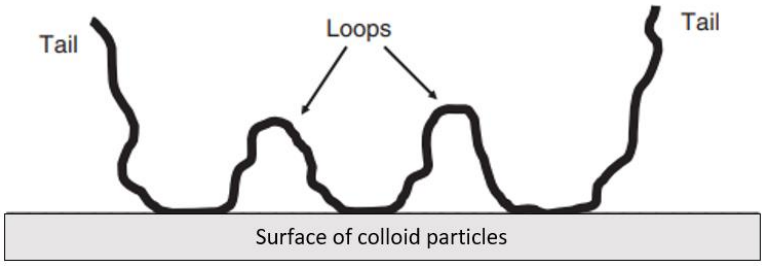

Figure 2.4: Adsorption of polymer segments (tails and loops) to the surface

Source: Figure reproduced from Gregory and Barany (2011, 3).

Although investigating the interactions at the molecular level is challenging, a few studies have been reported using various methods including zeta potential measurement, atomic force microscope imaging and spectroscopy (Wang, Zhang, et al. 2015; López-Maldonado, Oropeza-Guzmán and Ochoa-Terán 2014). The detailed interactions were able to be studied with the help of advanced technology such as molecular dynamic simulation (Liu et al. 2015). Results obtained from the above techniques could provide an insight to the molecular interactions and possible flocculation mechanisms involved. Sincero (2003) has listed four fundamental mechanisms for destabilisation of colloidal particles which include:

- electrical double layer (EDL) compression
- charge neutralisation
- entrapment in precipitate
- polymer bridging

2.5.2.1 Electrical double layer compression

Colloidal particle is surrounded by two layers known as stern layer and diffuse layer which consist of positive and negative ions, as shown in Figure 2.5. In a stable colloidal system, repulsive forces (V_R) dominate over a large distance from the surface of the particles while the attractive forces (V_A) attributed by van der Waals forces exhibit

strong interactions initially, but reduce significantly at a shorter range of distance (Sanin et al. 2011). As a result, $V_R > V_A$ leads to a positive resultant energy (repulsion). The theory of electrical double layer (EDL) compression can be explained through a huge reduction of net interaction energy to become positive (attraction) by adding the counterions (coagulants/flocculants) in bulk solution. This phenomenon occurs when the particle surface attracts more counterions and sufficiently compressed the double-layer of colloids.

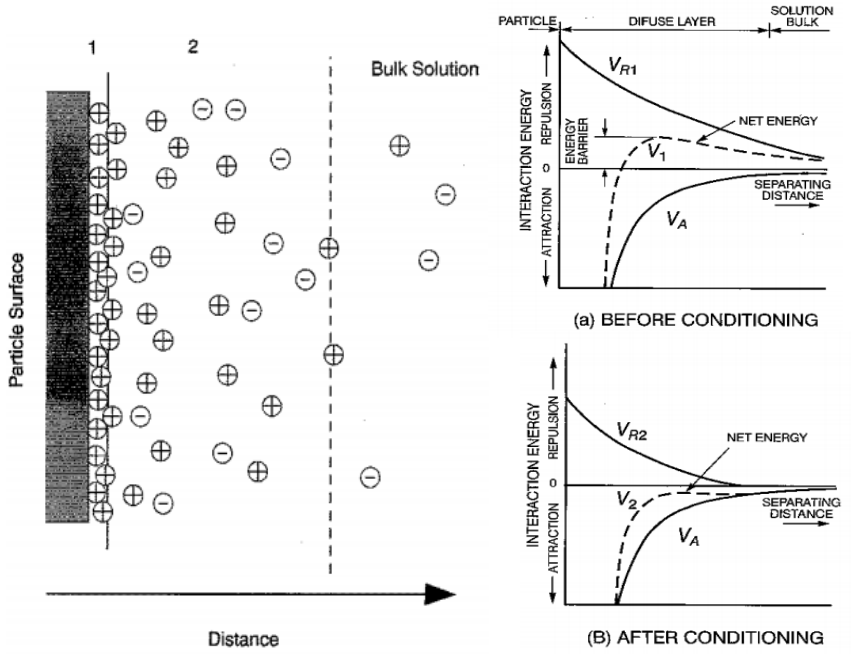

Figure 2.5: Fundamental theory of double layer compression. (1) stern layer; (2) diffuse layer. V_R and V_A represents repulsive and attractive forces respectively.

Source: Figure reproduced from Sanin et al. (2011, 252–253).

2.5.2.2 Charge neutralisation

Since many interactions require addition of the opposite charged ions to be accomplished, it is difficult to distinguish a solely charged neutralisation from other mechanisms. Charge neutralisation applies to the coagulant/flocculant with ability to adsorb directly to the particle surface and neutralise the colloid surface charges (Sincero 2003). This is common for cationic polyelectrolytes, in addition to causing a bridging effect.

2.5.2.3 Colloid entrapment, precipitation or sweeping

The entrapment of colloidal particles is a result of precipitation formed due to the characteristics of hydrolysed metal coagulants such as alum and ferric chloride when added to the system (Sincero 2003). Gregory (2005) used the term "sweeping" to better describe this process. Sweeping is thought to be more effective than simple charge neutralisation as it forms stronger and larger flocs because of the production of hydroxide precipitate which often occurs at higher dosages.

2.5.2.4 Polymer bridging

Bridging is very common in flocculation compared to coagulation due to the long chain of polymeric flocculants. For polyelectrolytes, charge neutralisation may co-exist. Like other mechanisms, agitation is necessary to initiate the collisions between particles. Depending on the characteristics of polymer, colloidal particles are adsorbed to the polymer surface by the following interactions (Gregory and Barany 2011; Gregory 2005).

- *electrostatic interaction*: due to opposite charges, mainly for cationic polyelectrolytes
- *hydrogen bonding*: due to presence of active functional groups such as amide and hydroxyl
- *hydrophobic interaction*: occurs in hydrophobic or non-polar section of polymer chains
- *ion binding*: adsorption on like-sign surfaces, commonly for anionic polyelectrolytes

2.5.2.5 Electrostatic charge patching

Another flocculation mechanism, namely "electrostatic patch effect" is also worth noting. A highly charged cationic polymer adsorbed onto a negative particle surface creates an uneven distribution of charges at the surfaces, forming positive "patch" leading to flocculation by interacting with other patches formed (Demir et al. 2020), as illustrated in Figure 2.6. This mechanism is likely to happen for low molecular

weight polymer with high charge density (Sharma, Dhuldhoya and Merchant 2006), and possibly at low polymer dosage under alkaline condition (Lin et al. 2008).

Figure 2.6 depicts four types of flocculation mechanisms reported in typical wastewater studies are charge neutralisation, sweeping, bridging, and charge patching (Suopajärvi 2015). However, polymer bridging and charge neutralisation were found in almost all published reports (Yang et al. 2012; Zhang et al. 2010; Guo, Yu, et al. 2015). To be exact, each fundamental mechanism such as charge neutralisation appears to be correlated with other mechanism. As a result, there will be two or more mechanisms responsible for a single type of coagulant/flocculant, depending on the nature of the polymer as well as environmental factors such as pH and temperature. Each mechanism is sometimes led to another mechanism, or more than one mechanism simultaneously occur, depending on the type of polymers added.

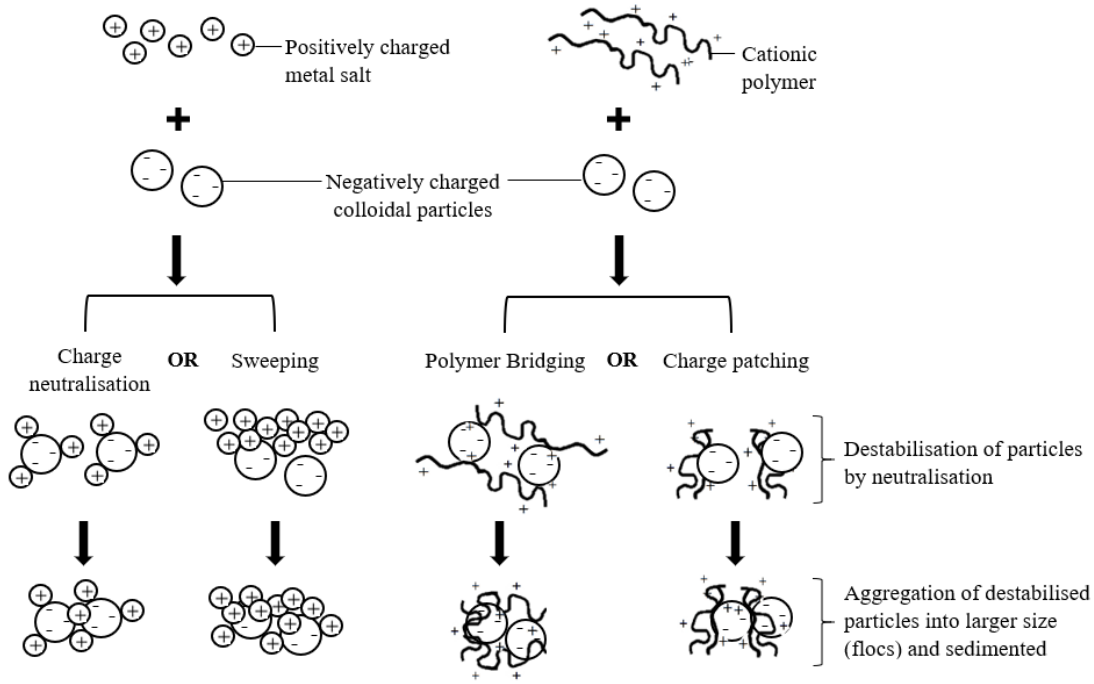

Figure 2.6: Schematic interpretation of typical flocculation mechanisms

Source: Figure adapted from Suopajärvi (2015, 22).

2.6 Factors affecting flocculation and dewaterability

2.6.1 Flocculant dosage and its nature

2.6.1.1 Effect of dosage

Optimum flocculation can be achieved when there is sufficient interaction between the flocculant and colloidal particles. At a dosage lower than optimum, the number of flocculant molecules adsorbed on colloid particle surfaces is not enough to cause effective flocculation whilst beyond optimum dosage, electrostatic repulsion between colloidal particles increases (Salehizadeh et al. 2018). In both cases, the aggregation of colloidal particles is unstable, resulting in re-stabilisation and charge reversal of the particles which inhibits the floc formation (Yuan et al. 2011; Salehizadeh et al. 2018). A novel BF produced from *Klebsiella* sp. ZZ-3 exhibited the highest activity (92%) in kaolin suspension at dosage of 0.126 mg (2.52 mg/L), but gradually declined as the dosage was further increased (Yin et al. 2014). There was also a significant decrease in the activity at a dosage below the optimum dosage (<0.126 mg) (Yin et al. 2014). Furthermore, the order of dosing also could affect the sludge dewaterability. Wang et al. (2018) reported that better sludge filterability and less sludge cake water content were attained when treated with coagulation-flocculation process in comparison to that in flocculation-coagulation process. In coagulation-flocculation system, the coagulant interacted completely with dispersed floc particles during rapid mixing to disrupt the sludge floc and release bound water content, and the destabilised floc particles were then bridged together upon addition of the flocculant during slow mixing (Wang et al. 2018). In contrast, the addition of the flocculant prior to coagulant caused the sludge particles to agglomerate into larger flocs and prevented some part of the inner flocs to function with the coagulant (Wang et al. 2018).

2.6.1.2 Effect of molecular weight

Variation in molecular weight accounts for the number of available binding sites primarily for polymeric flocculants. Several studies and reviews have shown that high flocculation activity were exhibited by polysaccharide BFs with the molecular weight range between $10^4 – 10^7$ Da (Yin et al. 2014; Guo and Chen 2017; Guo, Yu, et al. 2015; Salehizadeh et al. 2018). However, the boundary between the range of low, medium

and high molecular weight is rather ambiguous. Table 2.6 shows the proposed range of molecular weight of polymers from various sources.

Table 2.6: Different molecular weight range of polymers

Reference	Type of polymer	Molecular weight range* (kDa)			
		Low	Medium	High	Very high
EPA (1987)	Cationic polymer	100 – 200	200 – 1,000	1,000 – 4,000	-
Dentel (2001)	Cationic polymer	10 – 100	100 – 1,000	1,000 – 10,000	>10,000
Sigma-Aldrich (2019)	Biopolymer (chitosan)	50 – 190	190 – 310	310 – 375	>375
Lee, Robinson, and Chong (2014)	General synthetic polymer	1,000 – 3,000	3,000 – 6,000	10,000 – 15,000	>15,000

Note: * assume 1 g/mole ≈ 1 Da.

The range of molecular weight may vary according to the different chemical characteristics of polymer mainly on ionic nature, thus it is recommended that this be classified by charge type as well as charge amount (Dentel 2001). Based on Table 2.6, the ranges proposed by EPA (1987) and Dentel (2001) encompass the typical cationic polymers from two main categories which are the 'Mannich' polymers and acrylamide-based copolymers. Molecular weight range recorded in Sigma-Aldrich (2019) was based on cationic chitosan products only. Meanwhile, a much higher range of molecular weights compared to the others for general synthetic polymeric flocculants with any charges (amphoteric/anionic/cationic/non-ionic) was proposed by Lee et al. (2014).

2.6.1.3 Effect of ionic nature of flocculant

Zhou et al. (2014) reported that dose requirements decreased with increasing charge density of polymeric flocculant meanwhile the optimum dosage remained the same as the molecular weight decreased. Cationic polymers with high molecular weight are said to be more effective in bridging the particles (Gregory 2005). A review by Okaiyeto et al. (2016) emphasised that the ability of BF to adsorb on colloid surfaces is attributed to the presence of active functional groups which comprises of polar

(charged) and non-polar segments that creates hydrophilic/hydrophobic areas. Increase in hydrophobicity of a flocculant would be significant for the adsorption of organic pollutants and promote its aggregation (Okaiyeto et al. 2016; Yang et al. 2020). Charged groups such as carboxyl, hydroxyl, phenolic, phosphoric and sulfhydryl were also reported to be responsible for bridging and charge neutralisation during flocculation (Salehizadeh et al. 2018).

2.6.2 Effect of pH

Flocculation efficiency as well as sludge dewatering performance have been reported to be strongly dependent of the system pH as reported in literature. The system refers to the colloidal system where the flocculation process takes place such as wastewater and wastewater sludge. The bioflocculant ZZ-3 has shown that the highest flocculation activity (95%) at pH 5 in synthetic kaolin suspension (Yin et al. 2014). Meanwhile, bioflocculant PSB-2 extracted from biological sludge was able to maintain high activity (>96%) in kaolin suspension from pH 4 to 11 (Zhang et al. 2013). The effective flocculation performance of this BF in a wide pH range was believed to be attributed to the high solubility of its main components in the diluted hydrochloric acid (pH 1–3) utilised during the fractional precipitation and purification stage of the BF (Zhang et al. 2013).

Different pH dependencies of various flocculants were influenced by their properties such as surface charge and solubility (Wei et al. 2018). Electrostatic charge exhibited by the flocculant varies in different pH levels, causing changes towards surface electric property of the suspension which may increase or decrease the electrostatic repulsion between the flocculant particles and colloids (Pan, Shi and Zhang 2009). The literature review by Wei et al. (2018) shows that for water-soluble organic polymers, the flocculation performance of weak ionic flocculants is affected to a greater extent by the system pH in comparison with strong ionic and amphoteric polymers. For example, natural chitosan, a weak cationic polymer was able to dewater sludge under acidic conditions from pH 4–7 whereas cationic polyacrylamide (CPAM) and chitosan grafted with dimethyl diallyl ammonium chloride (chitosan-g-PDMDAAC) showed good dewatering performance at pH 2–9 (Wang et al. 2016). Zhang et al. (2010) reported that the SRF performance was greatly enhanced from 10^6 to 10^5 at pH 7.5

after treatment by TJ-F1 produced from *Proteus mirabilis* TJ-1. Therefore, initial pH adjustment of the suspension is sometimes required. However, polymeric flocculants often have a broad range of pH stability compared to the conventional inorganic coagulants, hence no pH adjustment is required (Chong 2012).

2.6.3 Effect of temperature

Temperature effects also plays an important role during flocculation and sludge dewaterability. This kind of effect can be viewed from two aspects – effect on the coagulant/flocculant and effect on the suspension. A majority of prior studies suggested that high thermal stability of flocculant was due to its polysaccharide backbone (Salehizadeh et al. 2018). BF produced from the *Rhodococcus erythropolis* strain was able to maintain high flocculation activity of >80% up to 120°C (Guo and Chen 2017). A similar observation was made for the BF, XMMBF by *Bacillus licheniformis* whereby >80% of activity was recorded across a wide temperature range between 10–85°C (Wang, Shen, et al. 2015). However, high temperatures may cause changes to characteristics of some colloidal systems. In trona suspension, the flocs formed at temperature beyond 45℃ was found to be unstable, leading to dispersion and expansion of the sediment solids volume (Lu et al. 2005).

Conversely, very high temperatures or tremendously low temperatures might be beneficial on sludge conditioning and dewatering application which explains the feasibility of physical heat treatment and freeze-thaw treatment. It was reported in literature that in the case of activated sludge, the freezing process causes water in sludge cells to expand and may disrupt the cells which leads to release of some organic materials (Örmeci and Aarne Vesilind 2001; Hu et al. 2011). However, alum sludge contains mainly inorganic compounds and less organic materials, thus insignificant change was observed throughout the process (Örmeci and Aarne Vesilind 2001). Similarly, the release of extracellular polymeric substance (EPS) from the sludge matrix at high temperature helps in floc formation by bridging, although it depends on the type of EPS released (Cao et al. 2020; Ye, Liu and Li 2014; Li and Yang 2007).

2.6.4 Effect of mixing speed and time

Mixing speed in coagulation-flocculation process can be divided into two parts which are fast and slow mixing. Fast mixing during initial stage is required to ensure a more uniform dispersion of coagulant/flocculant in the colloidal system (Teh et al. 2016). Kopp and Dichtl (2001) suggested that the optimum speed of fast mixing for floc formation was about 300 rpm within 30 seconds. A review by Teh et al. (2016) reported that fast mixing should have shorter time whereas slow mixing requires longer time to promote large floc formation and avoid floc breakage. However, mixing at a relatively longer time has insignificant effects on the stronger but smaller flocs because of low collision efficiency between the small flocs (Teh et al. 2016). Previous work by Aljuboori et al. (2015) found that the optimum speed for fast mixing (150–200 rpm) was achieved in 2 minutes and 5–7 minutes for slow mixing (30–80 rpm).

2.7 Advances of microbial bioflocculants in wastewater treatment

Several reviews have reported on the potential of microbial BF application in water and wastewater treatment (Rebah et al. 2018; Salehizadeh et al. 2018; Al-Mamun et al. 2017; Shahadat et al. 2017; Salehizadeh and Yan 2014). However, the outcome from the recent bibliometric study by Okaiyeto, Ekundayo, and Okoh (2020) reveals that published reports on the BF potentials in wastewater remediation are still lacking. Moreover, most of the studies were still in the stage of optimising the production yield by using different sources of cultivation medium or by varying the operational parameters such as initial pH, incubation temperature, type of cations, and agitation speed. In most studies using wastewater or sludge suspensions, the verification was generally made based on a single dosage without further investigation on the flocculation/dewaterability performance under the influence of major environmental factors such as pH and temperature, which serves as a research gap to be addressed.

Table 2.7 summarises the application of microbial BFs in water and wastewater treatment, including sludge conditioning and dewatering, for the past ten years. The most common BF-producing bacterial strains reported were from the genus *Bacillus* (Zhao et al. 2016; More et al. 2015; Giri et al. 2015; Guo, Zhang, et al. 2015; Guo, Lau, et al. 2015; Tang et al. 2014; Li et al. 2013; Hassimi et al. 2020; Vimala 2019; Abu Tawila et al. 2019; Liu et al. 2019; Joshi et al. 2019; Guo, Chen, et al. 2018; Busi

et al. 2017; Lin and Harichund 2011), followed by the genus *Klebsiella* (Liu et al. 2014; Liu et al. 2013; Yang et al. 2012; Nie et al. 2011) as the second most studied. Many authors reported BFs with high flocculation activity typically around 80-94% except Tang et al. (2014) and Aljuboori et al. (2015) that were able to achieve highest activity up to 98.4%. Meanwhile, BFs produced from *Bacillus salmalaya* (Abu Tawila et al. 2019) and *Streptomyces platensis* (Agunbiade et al. 2018) require the addition of metal cation to enhance its flocculation rate.

The variation in environmental conditions especially pH and temperature of the flocculation system could influence the flocculation activity of the BF. BFs with more than 20% protein exhibited declining flocculation activity upon heating at a temperature range between 50–100°C. For example, MBF-9 with 24.1% of protein content decreased its flocculation activity from 92.9% to <80% when heated at temperature of 65°C (Zhong et al. 2020). Meanwhile, MNXY1 with 26% of protein content reduced its flocculation activity about 30% after being heated at 100°C for 30–60 minutes and continued to decrease by 24% after 2 hours (Nie et al. 2011). Furthermore, optimum flocculation of most BFs were determined near neutral pH between 6.0 to 7.5, except for the BFs reported by Liu et al. (2019), Guo and Chen (2017) and Ugbenyen et al. (2015) where the BFs were found to be effectively flocculated in alkaline pH solutions between 7.0 to 12.0 attributed to their alkali-resistant characteristics.

As summarised in Table 2.7, none of these strains was identified as chitin/chitosan-like BFs or produced from the genus *Citrobacter* strains. Therefore, the outcome of this study will be the first report on the effects of pH and temperature on the flocculation performance as well as the practical implementation in sludge conditioning and dewatering using the BF produced from the *C. youngae* GTC 01314 strain.

728 Table 2.7: Advances of microbial bioflocculant in water and wastewater treatment for the past ten years (2010 – 2020)

Bioflocculant / bacterial strain	**Type of sludge / wastewater**	**Effective parameter range (opt. refers to optimum)**	**Remarks**	**Reference**
TJ-F1 produced from *Proteus mirabilis* TJ-1	Municipal sludge (93% domestic, 7% industrial)	Dosage: 0.17% (w/w) aided with $CaCl_2$ pH: 7 – 7.5 (neutral)	➢ Contained 63% of polysaccharides and 31% of protein. ➢ BF-treated sludge showed better dewaterability effects than commercial synthetic flocculant in terms of SRF and time to filter (TTF). ➢ Combined use with the synthetic flocculant was able to improve the dewaterability.	(Zhang et al. 2010)
Klebsiella pneumoniae strain NY1	Municipal wastewater	Using kaolin pH: strongly acidic (<5) Temperature: <100°C	➢ Composed of 66% of total sugar and 26% of protein. ➢ The activity slightly declined about 30% when heated at 100°C for 30-60 min but rapidly reduced to >50% after 2 h, indicating its low thermal stability due to significant protein content. ➢ Able to remove 72% of TSS, 11% of carbonaceous biological oxygen demand (CBOD) and 84% of COD.	(Nie et al. 2011)
Herbaspirillium sp. CH7, *Paenicibacillus* sp. CH11, *Bacillus* sp. CH15, and *Halomonas* sp.	Industrial wastewater effluents	-	➢ Removed heavy metals like Cr^{2+}, Ni^{2+}, Mn^{2+}, Fe^{2+} and Pb^{2+} significantly from Biavin blue dye compared to Whale dye and chemical effluents. ➢ Able to flocculate the bacterial population effectively in chemical effluent but less effective in dye effluents. ➢ Reduced 50-80% of the turbidity for all selected effluent samples.	(Lin and Harichund 2011)

Bioflocculant / bacterial strain	Type of sludge / wastewater	Effective parameter range (opt. refers to optimum)	Remarks	Reference
MBF10 produced from *Klebsiella* sp. strain N-10	Activated sludge	Dosage: 34 mg/L, 87% FA	➢ Dry solids and SRF were decreased by 34% and 69%. ➢ Combined use of MBF10 and $Al_2(SO_4)_3$ improved the dewaterability significantly by 84% and 63% of SRF and dry solids, respectively. ➢ Has MW about 800 kDa.	(Yang et al. 2012)
Paenibacillus elgii B69	Combined urban, agricultural and industry wastewater	Using kaolin pH: 3 – 11, >80% FA	➢ Main constituents were inferred to be polysaccharide due to negative reaction of Bradford assay. ➢ Effectively removed COD, turbidity and colour by 68%. 83% and 88% respectively. ➢ Heavy metals removal rate was higher for Al^{3+} (72%), followed by Pb^{2+} (60%), Cu^{2+} (53%) and Co^{2+} (49%). ➢ Has high MW of approximately 3.5 MDa based on standard calibration of dextran. ➢ Has great potential use in all kinds of wastewater given its broad range of pH stability.	(Li et al. 2013)
Paenibacillus mucilaginosus GIM1.16	Industrial wastewater	Dosage: 0.5 – 4 mg/L, >94% FA (opt. at 2 mg/L) pH: 3 – 9, >97% FA	➢ High percentage removal rates of COD and SS in different types of wastewater – biological product factory (83-92%), paper mill (70-88%) and garbage incineration plant (60-70%). ➢ Has MW ranges from 253 – 1,320 MDa.	(Tang et al. 2014)

Bioflocculant / bacterial strain	Type of sludge / wastewater	Effective parameter range (opt. refers to optimum)	Remarks	Reference
M-C11 produced by *Klebsiella pneumoniae*	Waste activated sludge (WAS)	Dosage: 3 mL (with $CaCl_2$) pH: 4 – 8 (opt. at 6) Culture temperature: 20 – 60°C.	➢ Composed of 91.2% sugar, 4.6% protein and 3.9% nucleic acids. ➢ FTIR confirmed the presence of carboxyl, hydroxyl, methoxyl and amino groups. ➢ Effectively reduced the SRF by 60%, the lowest compared to $Al_2(SO_4)_3$, PAC and CPAM. ➢ Optimum dosage was comparable to model prediction.	(Liu et al. 2014; Liu et al. 2013)
Paenibacillus polymyxa	Potato starch wastewater and sludge from secondary settling tank	Using sludge Dose: 1.5 g/L with $CaCl_2$ pH: 6.5 – 8.5 (opt. at 7.5)	➢ Composed of 96% of sugar and high MW of 1.16 MDa. ➢ At optimum conditions, dry solids increased about 58% while SRF decreased about 65%. ➢ BF dewatered effectively compared to inorganic coagulants and is comparable to PAC and PAM. ➢ COD and turbidity removal from wastewater achieved 52% and 82% respectively at pH 7.5 (dose: 30 mg/L).	(Guo, Zhang, et al. 2015; Guo, Lau, et al. 2015)
Mixed culture of marine bacteria (*Cobetia* sp. OAUIFE, *Bacillus* sp. MAYA, and *Bacillus* sp. Gilbert)	River, dairy, and brewery wastewaters	Using kaolin Dosage: 100 mg/L, 87% FA with $CaCl_2$ pH: 3 – 12, >75% FA (opt. at pH 12 with 96%)	➢ High turbidity reduction in all samples, better than alum and PAM in river wastewater. ➢ Effectively reduced the COD from dairy and river samples. ➢ Addition of multivalent cations resulted in >70% flocculation activity (FA) but inhibited by K^+ and Fe^{3+}. ➢ IR spectra revealed the presence of amino, hydroxyl and carboxyl.	(Ugbenyen et al. 2015)

Bioflocculant / bacterial strain	Type of sludge / wastewater	Effective parameter range (opt. refers to optimum)	Remarks	Reference
Bacillus subtilis F9	Drinking water	Using kaolin pH: 3 – 8 (opt. at 7) Temp.: 10 – 100°C (opt. at 40°C)	➢ The turbidity and COD of treated tap water were reduced by 91% and 58% respectively. ➢ Composed of 88.3% sugar and 10.1% protein. ➢ Has low MW of about 530 kDa.	(Giri et al. 2015)
Mixed culture of *Bacillus*, *Serratia* and *Yersinia* strains	River water, municipal and brewery wastewater	Dosage: 0.8 mg BF/gram kaolin, 91% FA Temp.: 4 – 60°C	➢ Zeta potential of the BF was negative. ➢ Flocculation was greatly enhanced with addition of Ca^{2+}, able to remove COD and turbidity in all samples. ➢ High removal efficiency of turbidity in river (94%), municipal (92%) and brewery wastewater (82%), comparable to commercial flocculant Magnafloc-155.	(More et al. 2015)
Bioflocculant IH-7 produced by fungus *Aspergillus flavus*	Different types of suspended solids	Using kaolin Dosage: 0.06 – 25 mg/L (opt. at 1) pH: 4 – 8 with 0.5 mg/L Temperature: 5 – 45°C Mixing: 150 rpm (2 min), 80 rpm (5 min)	➢ >93% FA was observed for flocculation of activated carbon and soil solids at 5 mg/L dosage while 95% FA was recorded for yeast flocculation at 30 mg/L dosage. ➢ Better flocculation performance than PAC in kaolin suspension and determined to be cation-independent flocculant. ➢ Has zeta potential value of +59.2 mV at pH 6.2.	(Aljuboori et al. 2015)

Bioflocculant / bacterial strain	Type of sludge / wastewater	Effective parameter range (opt. refers to optimum)	Remarks	Reference
Acidithiobacillus ferroxidans	Anaerobically digested (AD) sludge and chemically enhanced primary treatment (CEPT) sludge	(Biogenic flocculant treatment) pH: acidic (<6.79) Treatment time: 1 hour	➢ Rapidly decreased the treatment time compared to normal biological method-bioleaching (>48h). ➢ CST and SRF of treated AD sludge declined by 74% and 89% respectively, better than synthetic CPAM. ➢ After the application of filter press, sludge moisture content decreased by 30% while 28% of TSS, 14% of TDS, and 29% of COD were removed. ➢ AD sludge dewaterability improved with increasing Fe^{3+} concentration ➢ For CEPT sludge, the flocculant fraction obtained after filtration reduced the CST and flocculate better than the BF obtained from the culture without filtering.	(Kurade et al. 2016, 2014; Wong, Murugesan, Selvam, et al. 2016; Wong, Murugesan, Yu, et al. 2016)
Bacillus fusiformis CZ1003	Tannery wastewater	Dosage: 110 mg/L (added to 50 mL of wastewater sample)	➢ Able to remove total nitrogen, COD and colour from the wastewater although the removal rates were low, below 30%.	(Zhao et al. 2016)

Bioflocculant / bacterial strain	Type of sludge / wastewater	Effective parameter range (opt. refers to optimum)	Remarks	Reference
Rhodococcus erythropolis	Swine wastewater and sludge from secondary settling tank	Dosage: 20 mg/L, 94% FA with Ca^{2+} pH: 7 – 9 (opt. at 8)	➢ Removed about 48%, 44% and 76% of COD, ammonium and turbidity of swine wastewater at pH 8. ➢ At optimum conditions, dry solids and SRF reached 18-23% and 3.4 – 4.8 ($\times 10^{12}$ m/kg), better than $FeCl_3$ and $Al_2(SO_4)_3$ but poorer than PAC and PAM. ➢ BF composited with PAC showed significant improvements compared to single flocculant treatment. ➢ BF produced using alkaline-thermal (ALT) pre-treated sludge and rice stover hydrolysate have high MW of 421 kDa and 393 kDa, respectively. ➢ Increase in zeta potential of suspension when BF was added, indicating its negative charge.	(Guo and Ma 2015; Guo, Yang and Peng 2014; Guo, Yu, et al. 2015; Guo and Chen 2017)
Bacillus cereus SK	Wastewater sludge	Using kaolin Dosage: 12 mg, 83% FA with $CaCl_2$	➢ Identified as glycoprotein with presence of carboxyl, hydroxyl and amino groups. ➢ Non-toxic: up to the tested concentration (800 mg/mL). ➢ Good sludge volume index was obtained (31 mL/g).	(Busi et al. 2017)
BF-ADSW produced from *Bacillus subtilis*	Biological sludge from secondary settling tank	(Sludge) Dose: 15% dry solids pH: 6.5 – 8.5 (opt. at 7.5)	➢ Composed of 96% of sugar and has MW of 421 kDa. ➢ Zeta potential value was 13.2 mV. ➢ Dry solids and SRF were improved by 64% and 60%. ➢ Combined conditioning with PAC slightly enhanced the sludge dewaterability.	(Guo, Chen, et al. 2018)

Bioflocculant / bacterial strain	Type of sludge / wastewater	Effective parameter range (opt. refers to optimum)	Remarks	Reference
Streptomyces platensis strain HBUM174787	River and meat processing wastewater	Dosage: 200 mg/L Medium pH: 7	➢ Reduced 63.1% and 46.6% of COD, 84.3% and 75.6% of turbidity, and 60.2% and 72.8% of SS in river and wastewater, respectively. ➢ Flocculation was enhanced with the addition of multivalent cations except for Fe^{3+}. ➢ Showed comparable flocculation performance (95% FA) to PAM in kaolin suspension.	(Agunbiade et al. 2018)
Terrabacter sp. strain SFD 11	River, dairy, brewery, sewage, and meat processed wastewater	Dairy wastewater Dosage: 0.5 mg/mL	➢ Flocculate better in dairy wastewater than river but no flocculation detected in other wastewater samples. ➢ Presence of carboxyl, hydroxyl and amino groups could be responsible for flocculation. ➢ Higher removal of BOD, COD, turbidity, SS and nitrate content than conventional flocculants (dairy sample). ➢ Heavy metals removal was up to 77.7% (dairy sample).	(Mayowa Oladele et al. 2019)

Bioflocculant / bacterial strain	Type of sludge / wastewater	Effective parameter range (opt. refers to optimum)	Remarks	Reference
BFBI produced from *Bacillus licheniformis* strain NJ3	Different types of industrial wastewaters and sludge from alkaline chemical producing industry	Using kaolin Dosage: 100 mg/L (purified), 20 mg/L (crude) pH: 4 – 8 (better in acidic and neutral) Temperature: 30 – 90°C (opt. at 60°C)	➢ Cation-independent: showed high FA >84% without $CaCl_2$. Use of crude BF required low dosage thus could reduce the wastewater treatment cost. ➢ Increase of the net charge from -13.6 mV to -27.7 mV suggested the negative charge of BFBI. ➢ Percentage removal of turbidity, COD and oil from synthetic wastewater were much better than alum. ➢ Superior performance was observed in real wastewater, which reduced 53% TDS, 62% TSS, 54% turbidity, 26% COD. Ni, Pb and Se were completely removed while As, Cr, Cu and Fe removal was 62-93%. ➢ For pharmaceutical wastewater, no change in turbidity and TSS were observed. ➢ BF-treated sludge settled three times faster than alum. ➢ SVI result of BF-treated sludge (24 mL/g) was slightly poorer than alum (17 mL/g) but better in terms of TSS, TDS and turbidity reduction.	(Joshi et al. 2019)
Bacillus agaradhaerens C9	Mineral processing wastewater	Dosage: 9 mg/L added to 30L sample (pilot-scale) pH: >7	➢ Can be produced directly from unsterilised kitchen waste due to its alkali-resistant characteristics, which reduces the production cost. ➢ Divalent cations (Ca^{2+}, Zn^{2+} and Mg^{2+}) enhanced the flocculation but the BF alone was able to achieve reasonable flocculating rate of 92%.	(Liu et al. 2019)

Bioflocculant / bacterial strain	Type of sludge / wastewater	Effective parameter range (opt. refers to optimum)	Remarks	Reference
Alcaligenes faecalis HCB2	Coal mining wastewater	Dosage: 0.2 – 1.0 mg/mL, >78% FA (opt. at 0.8, 86%) pH: 5 – 12, >85% FA (opt. at 7, 93%) Thermal stability: up to 600°C with 27% weight loss	➢ 59%, 72% and 75% of removal rates were recorded for parameters BOD, COD and sulphur, respectively. ➢ Composed of 88.6% carbohydrates and 9.5% protein. ➢ IR spectrum revealed the presence of effective functional groups: hydroxyl, amide and amino groups. ➢ BF showed no significant cytotoxic effect. ➢ Zeta potential value of the BF was negative. Addition of K^+ increased the flocculation efficiency.	(Tsolanku, Albertus and Nkosinathi 2019)
Bioflocculant QZ-7 produced from *Bacillus salmalaya* 139SI	Industrial wastewater	Using kaolin Dosage: 2 mg/mL, 94% FA with $CaCl_2$ pH: 4 – 7 (opt. at 7) Thermal stability: up to 300°C with <25% weight loss	➢ Composed of polysaccharides, proteins and uronic acid. ➢ IR analysis indicated the presence of hydroxyl, amino and carboxyl groups. ➢ Has low quantity of sulphur which suggests its potential in heavy metal removal such as lead. ➢ Multivalent cations enhanced the flocculation except for Fe^{3+} which inhibited the activity significantly. ➢ Effectively removed heavy metals (As, Zn^{2+}, and Cu^{2+}) from the wastewater at 60 mg/L (pH 7 – 9).	(Abu Tawila et al. 2019)

Bioflocculant / bacterial strain	Type of sludge / wastewater	Effective parameter range (opt. refers to optimum)	Remarks	Reference
Bacillus subtilis	Municipal wastewater	Using kaolin Dosage: 50 mg/L pH: 7, 90% FA	➢ Composed of 64% total sugar and 18% protein. ➢ Trial experiment using 50 mg/L of the BF (with $CaCl_2$) confirmed its feasibility in treating real wastewater, reducing the amount of COD, SS and BOD.	(Vimala 2019)
Bacillus velezensis	Lake water	Dosage: 100 mg/L	➢ All BFs demonstrated their ability in reducing turbidity and colour from the lake water. ➢ Among all BFs, BioF-SME produced using sago mill effluent culture medium was the most effective but slightly lower than traditional alum flocculant.	(Hassimi et al. 2020)
MBF-9 *Diaphorobacter nitroreducens* R9	Pulping wastewater	Dosage: 150 – 750 mg/L (opt. at 750 with 93% FA) pH: 5 – 9 (opt. at 7) Temperature: 15 – 50°C, >80% FA (opt. at 25°C)	➢ Proteoglycan: 74% carbohydrate and 24% protein. ➢ Successfully reduced 96% turbidity, 80% COD, 60% lignin and 63% sugar from the wastewater at 831 mg/L. ➢ Has MW of 1.67 MDa and zeta potential of -58 mV.	(Zhong et al. 2020)

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2.8 Summary

This review presents the potential application of microbial BFs in enhancing flocculation activity as well as sludge dewaterability in water and wastewater treatment. Various operational parameters such as the flocculant dosage, pH and temperature strongly affect the flocculation performance and sludge dewaterability, in addition to the varying characteristics of suspension samples. Besides, the mixing speed and flocculation time, as well as the composition and the ionic nature of the flocculant contribute to various extents of flocculation activities. Typically, cationic flocculants with medium to high molecular weight are preferred to promote destabilisation of colloidal particles and bridging to the flocculant surface. In particular, microbial BFs with polysaccharide backbone are found more thermally stable compared to the BFs with protein content of more than 10%. The inherent biodegradable and non-toxic properties of BFs offer safer handling of the treated residues compared to the conventional synthetic polymeric flocculants. While ongoing research focuses in optimising the BF production yield with high flocculation performance at low cost for selected BF-producing strains, simultaneous work should be performed to validate the viability of the remaining potential BFs and to further investigate their optimum operational conditions using real wastewater and sludge before they can be practically applied at industrial scale.

BFs produced from *Citrobacter* strains have been studied continuously by the group of researchers from Japan and Korea due to the close resemblance of these BFs to chitin/chitosan as discussed earlier (Fujita et al. 2000; Fujita et al. 2001; Kim et al. 2006; Kim et al. 2012). Chitin/chitosan-like BFs were reported to be potentially distributed in the genus *Citrobacter*, exhibiting strong flocculation activity when grown in acetate medium (Kimura et al. 2013). Acetate could be a cheaper alternative substrate to glucose which is commonly used in BF production. The BF-producing strain *C. youngae* GTC 01314 used in the present work was found to grow effectively in acetate medium cultivation in previous research by Kimura et al. (2013). In addition to the ongoing research on the genetic study and the optimisation of the BF production yield, it is of high interest to investigate the physicochemical characteristics of the BF produced from this particular strain and to verify its practical implementation, in order to supplement the uncharted aspect of the previous work.

CHAPTER 3

MATERIALS AND METHODS

This chapter introduces the list of materials required and detailed experimental methods adopted to investigate the characteristics and flocculation behaviour of the bioflocculant (BF) produced from *Citrobacter youngae* (*C. youngae*) GTC 01314, named BF01314. Figure 3.1 simplifies the overall research methodology of this study.

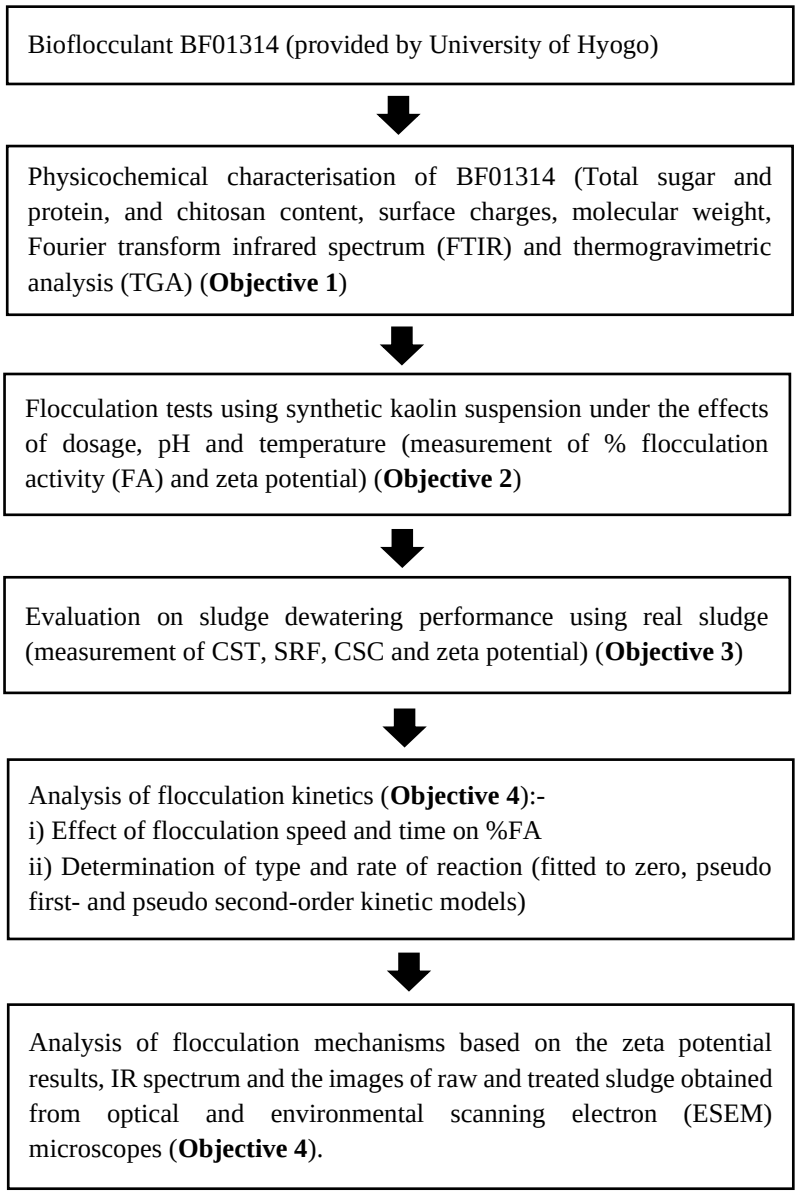

Figure 3.1: Research Methodology Flowchart

3.1 Materials

3.1.1 Bioflocculant samples (BF01314)

A concentrated solution of BF01314 sample used in this study (Figure 3.2) was obtained from Department of Applied Chemistry, Graduate School of Engineering, University of Hyogo, Japan. The sample was kept in a refrigerator at 4℃ and used as received in all flocculation and sludge conditioning experiments. A 20 mL aliquot of the sample was freeze-dried for 24 hours at -40℃ and 0.133 mbar to obtain the dry weight as calculated according to Equation 3.1. Dried BF was used for the analysis of molecular weight, Fourier transform infrared (FTIR) spectrum, thermal degradation properties, and microscopic image.

$$\text{Dry weight yield (g/L)} = [W_1 - W_0]/\text{Volume of BF solution used (L)} \quad (3.1)$$

where W_1 and W_0 are the weight of the freeze-dried residue with sample tube and the weight of empty tube in gram (g), respectively.

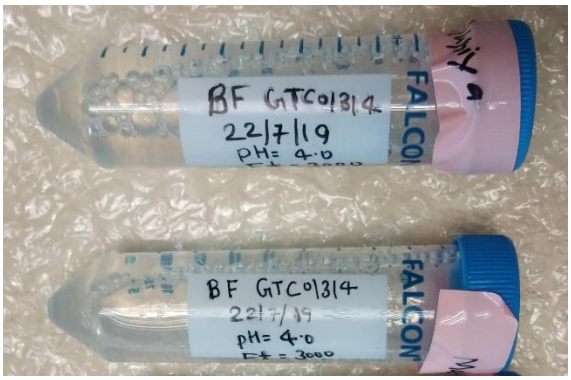

Figure 3.2: Bioflocculant BF01314 samples

This concentrated BF01314 solution sample was prepared from the cultivation of *C. youngae* GTC 01314 as follows. The bacterial strain *C. youngae* GTC 01314 was sourced from the Pathogenic Bacterial Genetic Resource Stock Centre, School of Medicine, Gifu University, Japan, and was grown in a modified acetate medium (MAM). Table 3.1 shows the composition of MAM, prepared in 1 L of distilled water at pH 7.2, which was sterilised by autoclaving at 120°C for 20 minutes prior to use. The strain was pre-cultivated overnight using 10 mL of Luria-Bertani (LB) medium (Sambrook and Russell 2001) on a rotary shaker (120 rpm, 37°C). The culture was then inoculated in 1 L of MAM at the concentration of 1% v/v, followed by 48-h

incubation on a rotary shaker (120 rpm, 30°C). The culture supernatant was obtained by removing the cells by centrifugation (10,000 rpm, 30 minutes, 4°C). The culture supernatant was then concentrated 40 times from 1 L to 25 mL using the Spectrum KRIIi tangential flow filtration system equipped with hollow fibre membrane modules (modified polyethersulfone membrane filter module (mPES), 20 cm^2, 100 kDa MWCO, Spectrum Labs, USA). The concentrated solution was diluted with 1 L of distilled water and again concentrated in the same manner to remove low molecular weight contaminants consisting of the medium ingredients (metal ions and unknown low molecular matters from peptone in MAM or yeast extract in LB medium) and cell-originated matters (by cell lysis or secreted from cells) which were lower than 100 kDa. The process of removing these contaminants is important to improve the purity of the product yield as the differences in the compositions could affect the flocculation characteristics of the BF (Diao et al. 2019). ProClin300 (Sigma-Aldrich) was added at a ratio of 1:5000 to the resulting concentrated BF01314 solution for preservation. The pH value of the BF solution samples used in all experiments was adjusted to 4 unless stated otherwise.

Table 3.1: Composition of Modified Acetate Medium (MAM)

Component	**Weight (g)**
Sodium acetate	10.0
$(NH_4)_2SO_4$	1.0
K_2HPO_4	1.0
Bacto peptone	0.3
$FeCl_3$	0.3
$MgSO_4$	0.3
NaCl	0.05
$CaCl_2$	0.05

3.1.2 Waste activated sludge (WAS)

Sludge samples were collected from Miri Septic Sludge Treatment Plant (SSTP) in Sarawak, Malaysia. This local treatment plant treats septic sludge collected within the city and its outskirts by aerobic digestion process (also known as activated sludge process) using Sequencing Batch Reactor (SBR). The effluent discharged from SBR is constantly monitored by the site personnel according to the effluent standards

namely Biochemical Oxygen Demand (BOD), Chemical Oxygen Demand (COD), and Suspended Solids (SS) to meet Malaysia's effluent quality of Standard A for discharge upstream of any raw water intake. The sludge sampling point for this study was located at the sludge holding tank after SBR, and before polymer dosing and dewatering operation, as illustrated in Figure 3.3. Commercial cationic polymer was added to the waste activated sludge (WAS) prior to mechanical dewatering. The dewatered sludge from the treatment plant was then transported to landfills for disposal (Sewerage Services Department Sarawak 2019). The sludge was collected and stored at 4°C (Figure 3.4).

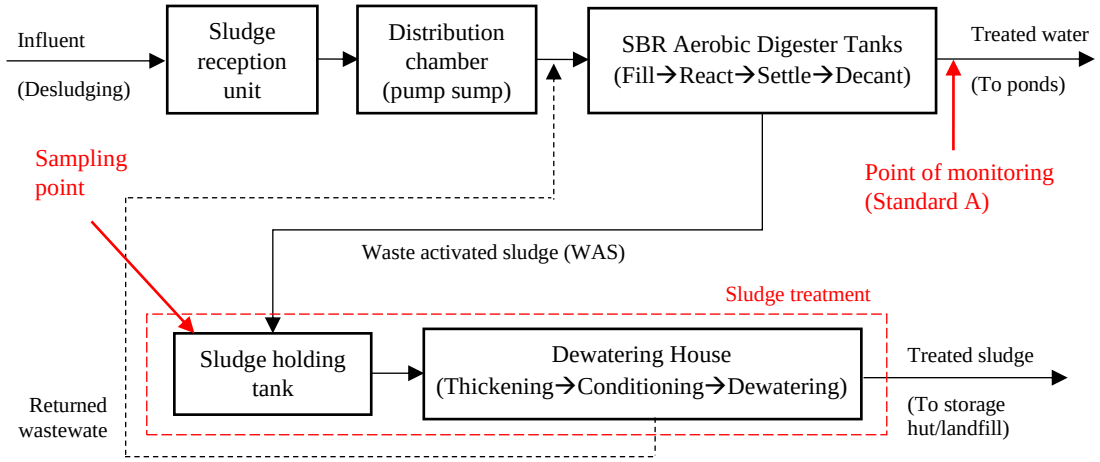

Figure 3.3: Miri SSTP overall sludge septic treatment flow diagram

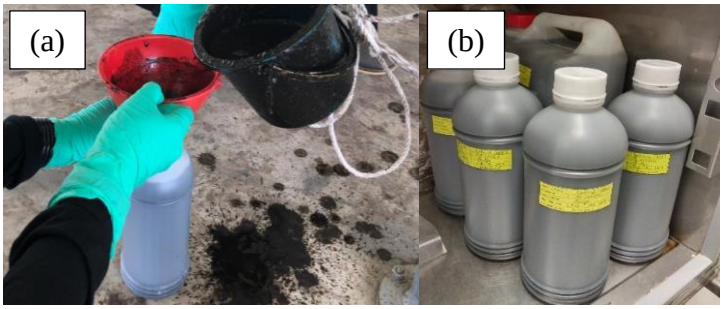

Figure 3.4: (a) Sludge collection (b) Sludge samples stored at 4℃

3.1.3 Commercial cationic flocculants

3.1.3.1 Natural chitosan

High molecular weight chitosan (310-375 kDa, 419419, Sigma-Aldrich, St. Louis, MO, USA) 419419 derived from chitin from crustacean shells was used as received to

compare its sludge dewatering performance with BF01314. The chitosan solution was freshly prepared according to the study by Zemmouri et al. (2011) with minor modifications. For comparison, the same concentration as BF01314 (4.78 g-dry weight/L) was used to prepare the chitosan solution. 0.478 g of chitosan powder was dissolved in a 100 mL of 1%v/v acetic acid solution with constant mixing on a magnetic plate stirrer (400 rpm, 30˚C) for 60 minutes or until no translucent particles were observed (SNF Floerger 2019). The solution was utilised within a week.

3.1.3.2 Synthetic cationic polymer

Synthetic cationic polymer MF 7861 was generously supplied by Miri SSTP and used as received. The polymer solution was prepared at the same concentration as BF01314 and chitosan by adding 0.478 g of polymer beads to 100 mL distilled water with constant mixing of 400 rpm at room temperature for 60 minutes or until fully dissolved (SNF Floerger 2019). The solution was utilised within a week.

3.2 Characterisation methods and analytical techniques of BF01314

3.2.1 Chemical analysis

Total sugar and protein contents in the prepared BF01314 sample were determined using the phenol sulphuric acid method (Sadasivam and Manickam 1996) and Bradford assay (Bradford 1976; Sigma-Aldrich 2019), respectively. The amount of apparent chitosan content in the sample was quantified according to Larionova et al. (2009) with minor modifications. All results were expressed in percentage of composition (sugar/protein/chitosan) in the BF using Equation 3.2.

$$\text{Composition in the BF sample}(\%) = [C_x/\text{Dry weight yield}] \times 100 \qquad (3.2)$$

where C_x represents the contents of sugar, protein, or apparent chitosan (g/L), obtained from the calibration curves of absorbance versus concentration using the standard solutions, namely glucose, bovine serum albumin, and chitosan, respectively.

3.2.1.1 Phenol Sulphuric Acid Method

All reagents and 0.1 mg/mL of D-(+)-glucose (G8270, Sigma-Aldrich, St. Louis, MO, USA) working standard solution were prepared prior to the analysis. A series of test tubes consisting of 0.0–0.1 mg/mL of glucose standard solution was prepared according to the volume ratio from Table 3.2. Concentrated sulphuric acid 95-97% was used as received. 5% phenol was prepared by dissolving 5 g of phenol crystals in 100 mL of distilled water.

Table 3.2: Volume of reagents and standard for total sugar determination

Test tube label	**0**	**1**	**2**	**3**	**4**	**5**	**BF**
Glucose conc. (mg/mL)	0.00	0.02	0.04	0.06	0.08	0.10	unknown
Vol. of std. solution (mL)	0.0	0.2	0.4	0.6	0.8	1.0	0.2
Vol. of distilled water (mL)	1.0	0.8	0.6	0.4	0.2	0.0	0.8

Under a fume hood, 1 mL of phenol reagent was added to Tube 0 followed by 5 mL of sulphuric acid, pipetted directly onto the liquid surface. The solution was then rapidly mixed on a vortex mixer for 20 seconds and allowed to stand for 10 minutes under the fume hood, and another 10 minutes in a water bath at room temperature. The solution was then gently mixed and transferred to a clean quartz cuvette to measure its absorbance at 490 nm using a LAMBDA Bio UV-VIS spectrophotometer (Perkin Elmer Waltham, MA, USA). The same procedure was repeated for Tube 1 to 5 and a tube containing BF01314 sample. The results obtained were used to plot a standard calibration curve to determine the corresponding amount of total sugar in the BF sample. All experiments were performed in triplicates.

3.2.1.2 Bradford Assay

Total protein in the BF sample was determined using the Bradford method (Bradford 1976), following the 3.1 mL assay protocol by Sigma-Aldrich (2019). A standard solution of 1 mg/mL bovine serum albumin (BSA) was prepared using phosphate buffered saline (PBS) (P4417, Sigma-Aldrich, St. Louis, MO, USA) as ‘diluent’. The same buffer solution was used to prepare a series of test tubes containing the BSA standard solution with concentration of 0.0 – 1.0 mg/mL according to the volume ratio from Table 3.3.

Table 3.3: Volume of reagent and standard for total protein determination

Test tube label	0	1	2	3	4	5	BF
BSA conc. (mg/mL)	0.0	0.2	0.4	0.6	0.8	1.0	unknown
Vol. of BSA solution (mL)	0.00	0.02	0.04	0.06	0.08	0.10	0.05
Vol. of PBS (mL)	0.10	0.08	0.06	0.04	0.02	0.00	0.05

For the test, 3 mL of the reagent solution was added to Tube 0 and the solution was vortexed for 30 seconds. The solution was then allowed to stand for 5 minutes at room temperature before transferring to a clean quartz cuvette to for absorbance measurement at 595 nm. The remaining Tube 1 to 5 and the tube containing BF sample was tested in the same manner. All readings were recorded within 60 minutes to maintain the stability of protein-dye complex in each solution while the measurements were recorded. A standard calibration curve was plotted using the recorded results to determine the corresponding amount of total protein in the BF sample. All experiments were performed in triplicates.

3.2.1.3 Colorimetric Assay of Chitosan

This approach was based on the derivation reaction of chitosan primary amino groups with a mixture of o-phthataldehyde and a thiol. The reaction resulted in the formation of isoindoles Figure 3.5 which can be monitored spectrophotometrically against the reference solution at the wavelength of 340 nm (Larionova et al. 2009).

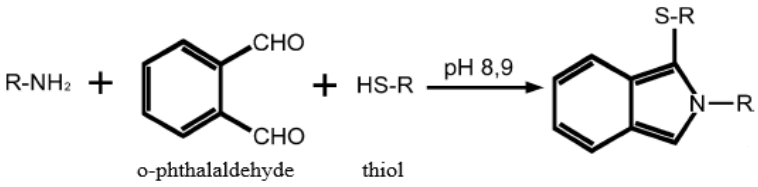

Figure 3.5: Formation of isoindole by o-phthalaldehyde and thiol reacted with primary amino groups

Source: Figure reproduced from Larionova et al (2009, 725).

A standard reagent solution was prepared prior to the colorimetric assay by adding each ethanol solution to the borate buffer with respective volumes: -

0.11M o-phthalaldehyde – dissolved in ethanol	200 µL
0.071M N-Acetyl-L-Cysteine (NAC) – dissolved in ethanol	200 µL
0.2M Borate buffer (pH 8.9)	4.6 mL

A series of standard solutions with the concentration of 0.0-0.1 mg/mL was prepared by dissolving the chitosan powder in 0.01M HCl accordingly. For each test, 500 µL of standard solution was mixed with 500 µL of reagent solution and its absorbance measured at 340 nm after 60 seconds reaction time. A calibration curve was then plotted. To determine the amount of corresponding chitosan in BF01314 from the calibration curve, the test was repeated by replacing the standard solution with the diluted BF sample (1:10 dilution with distilled water). All experiments were performed in triplicates.

3.2.2 Surface charge analysis

Ionic characteristic of polymeric flocculant can be characterised based on its surface charges commonly presented by zeta potential values (Guo, Yu, et al. 2015; Picos-Corrales et al. 2020). Zeta potential is the potential difference measured between two boundaries expressed in terms of volt or millivolt to characterise the electrical charge of a substance. The Malvern Zetasizer Nano ZS equipped with a multi-purpose titrator (MPT-2) connected to a computer, was used to measure the zeta potential of BF01314 at different pH levels from 2 to 12 and to further determine its isoelectric point (IEP). The titrants used were 0.5 M HCl, 0.25 M HCl and 0.5 M NaOH. Using the software provided, a measurement file for zeta potential with pH titration was created and all the instructions displayed were followed. The pH probe was calibrated, and all titrants were primed before taking the measurement. 15 mL of BF sample was filled in a sample tube with a small stirrer inside the tube and attached to the titrator. The sample was filled to a folded capillary cell (DTS 1070) placed inside the cell holder via a tube connecting the cell with MPT-2 (by clicking *Fill* button in the software). The measurement was then recorded and a copy of an isoelectric titration graph was obtained.

3.2.3 Molecular weight determination

Dried BF was sent out for external analysis to determine the molecular weight of BF01314 following the method of Hamzah, Talip, and Mahmud (2011). The average molecular weight of purified BF01314 was determined using Gel Permeation

Chromatography Multi Angle Laser Light Scattering (GPC-MALLS) system coupled with multiple detectors: an Agilent UV detector (k = 280 nm), an Optilab rEX differential refractive index (dRI) detector (k = 658 nm), and a Dawn Heleos MALLS detector (k = 661 nm) (Wyatt Technology, Santa Barbara, CA). The column was PL aquagel-OH MIXED-H 8 µm (Agilent Technologies, Santa Clara, CA). The mobile phase contained 0.2 M acetic acid and 0.1 M sodium acetate. Prior to injection, the samples (1.5 mg/mL) were dissolved in the mobile phase and filtered with 0.45-µm nylon syringe filter. The analysis of the sample was performed at room temperature (25°C) at a flow rate of 1 mL/min. The raw chromatogram obtained for BF01314 can be found in Appendix A.

3.2.4 Fourier-transform infrared (FTIR) spectroscopy

Cary 630 Fourier-transform infrared (FTIR) spectrometer (Agilent Technologies, Santa Clara, CA, USA) with attenuated total reflection (ATR) accessory was used to obtain the spectrum of BF01314 and chitosan as control for comparison. The analysis of infrared (IR) spectrum provides the information on the functional groups (Zhao et al. 2016) of BF01314 over a wavelength range of 650 to 4000 cm^{-1}, which was then compared with the one of chitosan. This technique uses a diamond crystal as the interface between the sample and the infrared energy. Figure 3.6 shows the main components of the ATR accessory. The sample was analysed with simple three steps prompted by the Agilent Microlab software. The diamond sampling window surface was cleaned with ethanol prior to the background scanning. Background scanning was measured without any sample on the sampling window (as reference). Once the background spectrum was collected, the dried BF sample was placed on the sampling window and turned the knob until the press tip was in contact with the sample.

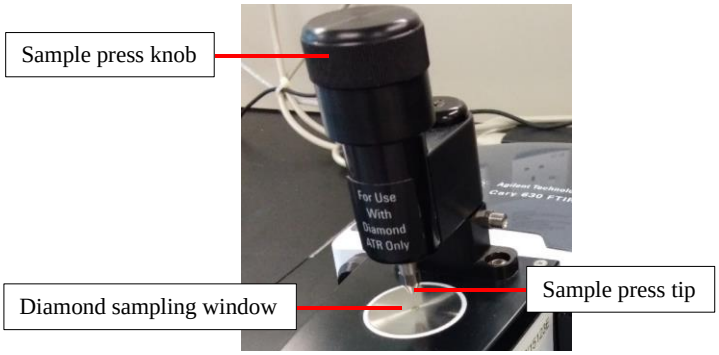

Figure 3.6: Cary 630 FTIR spectrometer Diamond Accessory sample press

3.2.5 Thermogravimetric analysis (TGA)

Thermal degradation study of BF01314 was carried out by heating 5 mg of the dried sample starting from room temperature (25±1°C) up to 800°C with a heating rate of 10°C/min at a N_2 gas flow rate of 20 mL/min using a thermogravimetric analyser TGA/DSC 1 (Mettler Toledo, OH, USA) according to the method of Pathak et al. (2017). These settings were inputted to a computer connected to the analyser using a STAR software (Mettler Toledo STAR Thermal Analysis system). 5 mg of the BF sample was weighed in a crucible and placed on a crucible holder inside the furnace, ready to be analysed (Figure 3.7). The temperature and weight measured during the analysis were displayed on a SmartSens terminal for ease of monitoring. The analysis was completed in approximately one hour and the plotted curve of sample weight against temperature was obtained. Similar procedures were repeated for chitosan and the result was compared with BF01314.

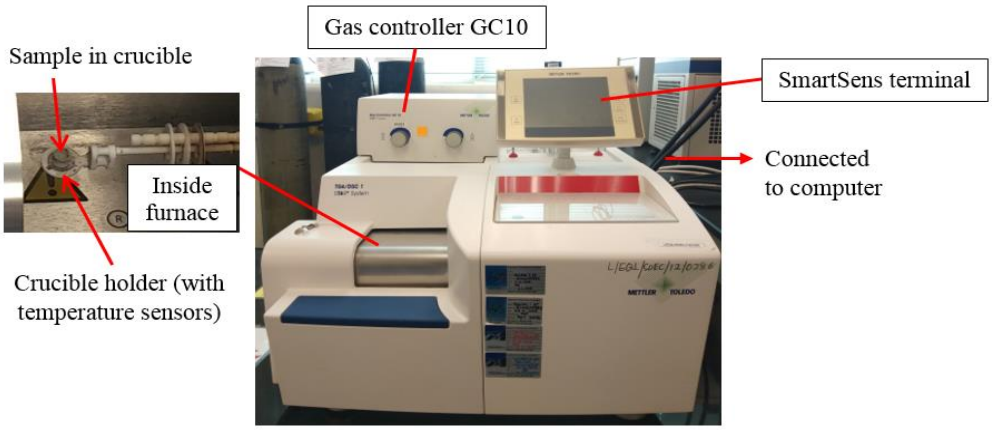

Figure 3.7: TGA analyser (TGA/DSC 1, Mettler Toledo)

3.3 Flocculation characterisation under various parametric conditions

3.3.1 Preparation of synthetic kaolin suspension

All flocculation tests were tested using kaolin clay suspension with a concentration of 5 g/L according to the method by Kimura et al. (2013). The suspension was freshly prepared prior to the test by adding 5 g of kaolin clay (K7375, Sigma-Aldrich, St. Louis, MO, USA) to 1 L of distilled water, and the mixture was rapidly stirred on a plate stirrer at 500-700 rpm for 30 minutes. The pH and zeta potential of the suspension were then measured.

3.3.2 Measurement of flocculation activity

The flocculation tests involved the measurement of flocculation activity under the effects of different experimental parameters – BF concentration, pH and temperature. All flocculation tests were conducted using the reported method by Kimura et al. (2013). 1 mL of the readily BF01314 concentrated sample or its diluted samples were added to 10 mL of 5 g/L of kaolin suspension using a 100-1000 µL micropipette. The mixture was then vigorously mixed for 30 seconds at 10-15 Hz and allowed to settle for 5 minutes. A small amount of the supernatant (upper part of the mixture) was transferred to a clean cuvette and its absorbance was measured at 550 nm using a LAMBDA Bio UV-VIS spectrophotometer (Perkin Elmer). For the sample with absorbance reading of more than 1.0, the sample supernatant was diluted, and final absorbance was multiplied by the dilution factor. The same procedure was repeated for control experiments by replacing the BF solution with 1 mL of distilled water. The flocculation activity was calculated using Equation 3.3 based on the average of the triplicate measurements.

$$\text{Flocculation Activity}\ (\%) = [A_0 - A]/A_0 \times 100 \qquad (3.3)$$

where A is the absorbance of the supernatant of BF-treated kaolin, and A_0 is the absorbance of the control sample.

Table 3.4 summarises the range of parameters tested which was adopted from a previous report by Fujita et al. (2000) with minor modifications.

Table 3.4: Range of parameters of flocculation tests

Parameter	Range tested	Constant variable
BF concentration	Dilution of 2^0 – 2^{13} (0.058 – 478 mg/L)	- Unadjusted pH (4.7±0.3) - Room temperature (25±1°C)
pH of suspension	2, 4, 6, 8, 9, 10, 11	- BF concentration of 2^5 dilution - Room temperature (25±1°C)
Temperature (℃)	Room temperature (25±1), 40, 60, 80, 95	- BF concentration of 2^5 dilution - Unadjusted pH (4.7±0.3)

3.3.2.1 Flocculation titre and effect of BF concentration

The experiment was conducted according to the "flocculation titre (Ft)" method introduced by Kimura et al. (2013) to detect the presence of flocculation activity with Ft≥1, whereby the strains with Ft>80 were considered as high activity strains. Ft is defined as the fold dilution of the culture supernatant to reduce the saturated flocculation activity by 50% (Kimura et al. 2013). Prior to all tests, a two-fold serial dilution of BF solution from zero to thirteenth dilution (2^0 to 2^n, where n = integer of 1 to 13) was prepared by diluting the BF with the same volume of distilled water as shown in Figure 3.8.

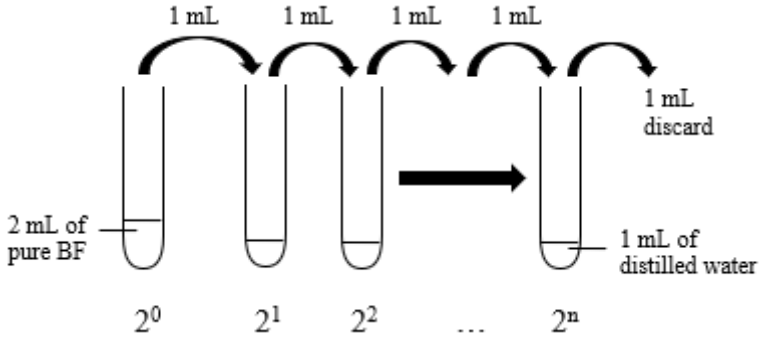

Figure 3.8: Preparation of two-fold serial dilution of BF solution

The result obtained using 1 mL of pure BF solution was taken as the result of dilution 2^0. The experiment was then repeated for each BF diluted solution. The results of flocculation activities were plotted against the dilution factor to determine Ft value at 50% activity from the curve. To analyse the effects of BF concentration, the result was expressed in mg BF per litre of kaolin suspension using Equation 3.4. Sample calculation was included in Appendix B. Both pH and zeta potential of the supernatant were also measured at the end of the experiment. All tests were performed in triplicates.

$$\text{BF concentration (mg/L)} = [W_B/V_S] \times [1/\text{dilution}] \quad (3.4)$$

where W_B and V_S are the BF yield presence in 1 mL of purified BF solution (mg) and volume of kaolin suspension used for each test (0.01 L).

3.3.2.2 Effect of suspension pH

The pH of kaolin suspension was adjusted using 0.5 M hydrochloric acid or 0.5 M sodium hydroxide to the desired pH in a small beaker prior to each flocculation test.

10 mL of the pH-adjusted kaolin suspension was then transferred to a clean test tube, added with 1 mL of fifth dilution of BF (2^5) and measured its flocculation activity using the same aforementioned procedure. A control experiment for each desired condition (pH) was repeated in the same manner using 1 mL of distilled water instead of BF. The zeta potential of the supernatant and equilibrium pH (final) were also determined. All tests were performed in triplicates.

3.3.2.3 Effect of temperature

A test tube containing 10 mL of kaolin suspension was heated to the desired temperature in a water bath set up with a beaker, on top of a plate stirrer. As the desired temperature was reached, 1 mL of fifth dilution of BF (2^5) was added to the kaolin suspension and rapidly mixed on a vortex mixer. The mixture was allowed to stand for 5 minutes and then transferred to a clean cuvette to measure its absorbance. Control experiment for each desired condition (temperature) was performed in the same manner using 1 mL of distilled water. Zeta potential of the supernatant was also determined.

3.4 Sludge conditioning and dewatering experiments

3.4.1 Sludge characterisation

3.4.1.1 pH

The pH of the sludge sample was measured with a portable pH meter (HACH sensION+ PH1) according to the standard operating procedure (HACH 2013). A pH probe was calibrated with three different buffer solutions at pH 4.01, 7.00 and 10.01 prior to the measurement. Before taking the first and subsequent measurements, the probe and electrode were rinsed with distilled water. It was ensured that the electrode was fully immersed during each measurement.

3.4.1.2 Total solids (TS)

Total solids content was determined by Standard Method 2540B (APHA 2012). A clean evaporating dish was prepared by heating it in the oven at 103-105℃ for at least

1 hour until it obtained constant weight. The dish was cooled in a desiccator until needed. The sample was stirred on a magnetic stirrer at 100–200 rpm for 30 minutes to ensure a homogenous mixture. While mixing, 10 mL of sludge sample was pipetted (from a point between the beaker wall and vortex) and transferred to the prepared dish and weighed. The sample was dried in the oven for approximately 3 hours until a constant weight was achieved or if it was observed to reach less than 4% changes of previous weight. The determination was repeated three times. The amount of TS was presented in g/L and percentage (%) using Equation 3.5 and 3.6, respectively.

$$\text{TS (g/L)} = (\text{W} - \text{W}_0)/\text{Volume of sample(L)} \quad (3.5)$$

$$\text{TS (\%)} = [(\text{W} - \text{W}_0)/(\text{W}_w - \text{W}_0)] \times 100 \quad (3.6)$$

where W, W_0 and W_w are the weight of dish with dried residue (g), the weight of empty prepared dish (g), and the weight of dish with wet sludge (g).

3.4.1.3 Total Suspended Solids (TSS)

Total suspended solids (TSS) content was determined by Standard Method 2540D (APHA 2012). A glass fibre filter disk was prepared by washing it in three successive 20 ml of reagent-grade water, and before commencing to suction process using a simple set up of lab filtration apparatus to remove the traces of water. The washing was discarded whilst the disk was transferred to an aluminium weighing dish and dried in the oven at 103–105℃. The drying was repeated until a constant weight of disk was obtained. The disk was cooled in a desiccator until needed. The sample was stirred on a magnetic stirrer at 100–200 rpm for 30 minutes to ensure a homogenous mixture. While mixing, 5 mL of sludge sample was pipetted (from a point between the beaker wall and vortex) and transferred to the prepared disk to begin the filtering process. After 3 minutes of suction, the disk with solids retained on it was weighed and then dried in the oven for at least 1 hour until constant weight was achieved or if it achieved less than 4% changes of previous weight. The filtrate was kept for the determination of total dissolved solids (TDS). The determination was repeated at least twice. The amount of TSS was calculated in the same manner as total solids.

3.4.1.4 Total Dissolved Solid (TDS)

Total dissolved solids content was determined by Standard Method 2540C (APHA 2012). A clean evaporating dish was prepared by heating it in the oven at 180℃ for at least 1 hour until achieving constant weight. The dish was cooled in a desiccator until needed. The filtrate from the determination of TSS was transferred to the prepared dish and weighed using an analytical balance. The determination was repeated at least twice. The amount of TDS was calculated in the same manner as total solids.

3.4.1.5 Capillary suction time

Capillary suction time (CST) test for the sludge follows the Standard Method 2710G (APHA 2012). The digital CST meter was switched on and reset prior to the test. A piece of chromatography paper was placed between the lower and upper test block with the rough side up, and the sludge reservoir was inserted into the test block as shown in Figure 3.9. The sludge sample was poured into the reservoir until it was full. The timer was automatically started as the liquid drawn into the paper reached the inner sensors and stopped when it reached the outer sensor. The measurement was repeated for at least three times.

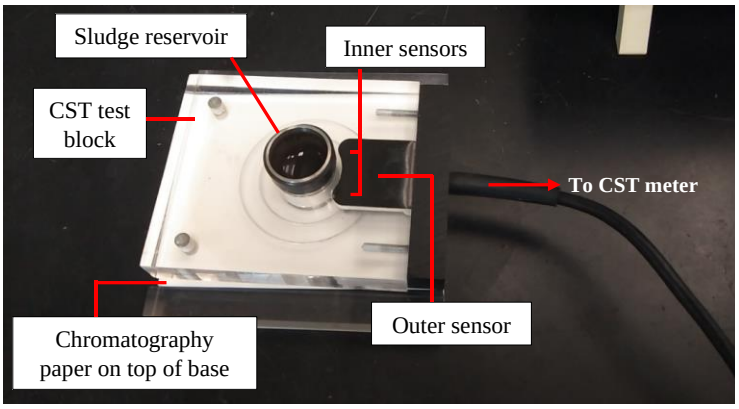

Figure 3.9: CST test block for sludge dewaterability analysis

3.4.1.6 Specific resistance to filtration and cake solids content

Specific resistance to filtration (SRF) was determined using an established method introduced by Coackley and Jones (1956), described in Sanin et al. (2011) with minor modifications to the experimental set up as shown in Figure 3.10.

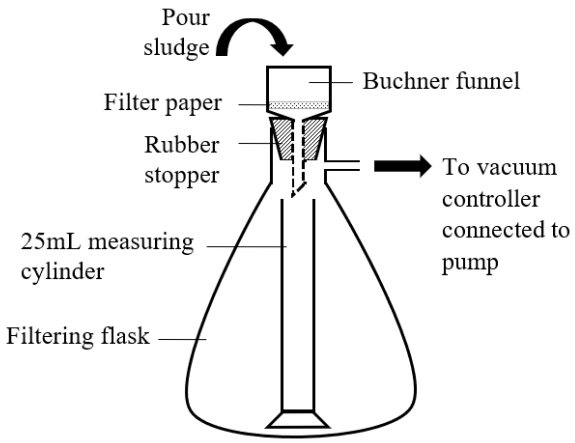

Figure 3.10: Experimental set up for SRF test of the sludge

A complete assembled 25-mL graduated cylinder inside a filtering flask with 50-mL Buchner funnel and stopper was connected to a vacuum pump via a hose equipped with a vacuum controller. The vacuum pressure was set to 50 kPa (Lau 2014). A constant volume of sludge was poured into the funnel (seated with filter paper) and the vacuum pressure was applied at timeframe of zero. The volume of filtrate was recorded as a function of time throughout the filtration process. The filtered sludge cake was then dried overnight in oven at 105°C and the weight of dry cake solids content (CSC) was calculated using Equation 3.7 (Lau 2014). A graph of inverse flux (time/filtrate volume, t/V) versus volume of filtrate (V) was constructed and the SRF value was determined using Equation 3.8.

$$\text{CSC}\ (\%) = [W_2 - W_0] \,/\, [W_1 - W_0] \tag{3.7}$$

where W_2, W_1 and W_0 are the weight of dried cake, the weight of wet filtered cake with filter paper, and the weight of filter paper, all in grams (g), respectively.

$$\text{SRF}\ (\text{m/kg}) = [2(\Delta P)A^2 b] \,/\, \mu w \tag{3.8}$$

where ΔP is the pressure difference set using the vacuum controller (N/m^2), A is the filtration area (m^2), μ is viscosity of filtrate ($N.s/m^2$) (assumed similar to water), w is the weight of dry cake per volume of filtrate (kg/m^3), and b is the straight-line slope of t/V versus V graph.

3.4.1.7 Zeta potential measurement

Zeta potential of the sludge supernatant was measured using the same instrument Malvern Zetasizer Nano ZS mentioned in 3.2.2, without the titrator. All procedure follows the instructions from the Easier Nano User Manual. To prevent any bubbles from forming inside the sample cell, the sample was injected into a folded capillary cell (DTS1070) using a syringe suspended upside-down, until half-filled. The sample cell was then inverted to an upright position, and the liquid sample was continued to be injected slowly until the cell electrodes were fully covered or it reached the MAX line marked at the cell. Both openings of the cell were closed using the caps provided together with the cell. The cell was then inserted into a cell holder inside the instrument with Malvern logo faces to the front (ensured not to touch the optical measurement area) and the sample was then ready for measurement.

3.4.2 Batch conditioning experiment

A batch conditioning experiment follows the method of Lau (2014) with minor modifications. The effects of BF dosage, suspension pH, and temperature, as well as flocculation speed and time were investigated in this experiment. The sludge was initially brought to room temperature and a series of 250-mL beaker consisting of 100 mL of well-mixed sludge was prepared. For all experiments, the sludge was slowly stirred on a plate stirrer at 100 rpm while dosing the flocculant to the sludge within 60 seconds with a 100-1000 µl micropipette. The sludge was rapidly mixed at 400 rpm for 60 seconds to promote coagulation followed by slow mixing at 100 rpm for 5 minutes to simulate the flocculation. CST test was performed immediately after the flocculation. After 10 minutes of settlement, sludge supernatant was collected for zeta potential measurement and flocculated sludge was tested for SRF and CSC.

For the pH effect experiment, the sludge pH was initially adjusted using 1 M hydrochloric acid or 1 M sodium hydroxide. For the effect of temperature study, the sludge temperature was brought from 4°C to the desired temperature and maintained using ice bath (for 10°C and 25°C), and by heating on a hot plate as shown in Figure 3.11 for 40, 60, 80 and 100°C. Table 3.5 summarises the range of parameters used, constant variables, and the measurements recorded to evaluate the sludge dewatering performance. The range tested was selected based on the flocculation behaviour of

BF01314 in kaolin and other related literatures with minor adjustments (Aljuboori et al. 2015).

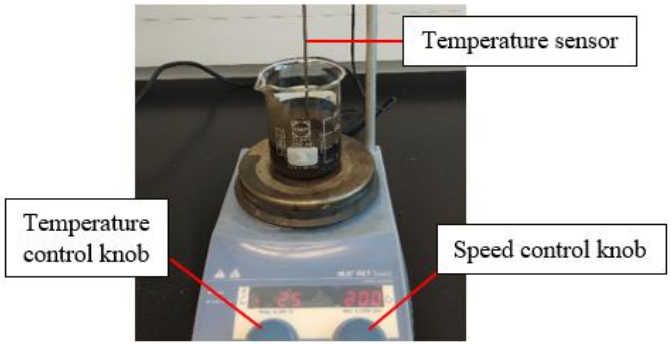

Figure 3.11: Set up for effect of temperature experiment

Table 3.5: Range of parameters tested for sludge conditioning and dewatering

Parameter	Range tested	Constant variable	Performance test
BF dosage	0.3, 0.5, 1.0, 1.5, 2.0, 3.0 and 6.0 kg/t dry solids (DS)	- Unadjusted pH (7±0.3) - Room temperature (25±1°C)	Specific resistance to filtration (SRF), Capillary suction time (CST), Cake solids content (CSC) and zeta potential
Sludge pH	2, 4, 6, 7, 8, and 10	- BF dosage of 1.0 kg/t DS - Room temperature (25±1°C)	
Temperature	10, 25, 40, 60, 80 and 100℃	- BF dosage of 1.0 kg/t DS - Unadjusted pH (7±0.3)	

3.5 Comparison with commercial flocculants

The sludge dewatering performance of BF01314 was compared to the commercial high molecular weight chitosan (HMWC) and synthetic cationic flocculant. Similar procedures of sludge conditioning and dewatering experiment in 3.4 was applied. For each type of flocculant, the dosage was varied from 0.5, 1, 2 and 3 g/kg dry solids (DS) (Zemmouri et al. 2015). The dewatering performance was evaluated for CST, SRF, CSC and zeta potential of the supernatant. Within the range tested, the sludge treated at optimal dosage of BF01314 was selected for further analysis of FTIR spectrum, and surface morphology using optical microscope and environmental scanning electron microscope (ESEM) to elucidate the molecular interactions between the BF and sludge particles.

3.6 Analysis of flocculation kinetics

There are two parts involved in this preliminary analysis of flocculation kinetics. In the first part, the kinetic behaviour of BF01314 was interpreted in terms of flocculation activity under the effects of mixing speed and time according to the methods reported by Lau (2014) and Aljuboori et al. (2015). In the second part, the flocculation kinetics was observed in terms of turbidity reduction against the sedimentation time. The type and rate of reaction were then determined based on the method of Bisht and Lal (2019).

Similar procedure was adopted according to the sludge conditioning method described in Section 3.5 with variation in flocculation speed and mixing time. Sludge conditioning experiment simulates a two-step mixing process– coagulation (first stage) and flocculation (second stage). In this study, the sludge conditioning process was studied at low, moderate and high mixing speeds of 50, 100 and 200 rpm, respectively, for the flocculation stage. At each flocculation speed, the experiment was repeated by varying the mixing time to 1, 2, 3, 5 and 7 minutes (Table 3.6). 2-mL aliquot of sludge supernatant was extracted after 5 minutes settling, and its absorbance was measured at 550 nm. A control experiment was conducted at each flocculation speed by replacing the BF with distilled water for the determination of flocculation activity using Equation 3.3 (see Section 3.3.2).

Table 3.6: Range of parameters for the study of effects of flocculation speed and time

Flocculation speed	Mixing time (min)	Constant variable	Performance test
Low – 50 rpm	1, 2, 3, 5 and 7	- BF dosage of 1.0 kg/t DS	Flocculation
Moderate – 100	1, 2, 3, 5 and 7	- Unadjusted pH (7±0.3)	activity (using
rpm		- Room temperature (25±1°C)	Equation 3.3 in
High – 200 rpm	1, 2, 3, 5 and 7	- Coagulation: 400 rpm, 60 s	Section 3.3.2)

The second part of the study was carried out to determine the kinetic model and rate of reaction of the sludge treated by BF01314 according to the method conducted by Bisht and Lal (2019) with modification. 1 L of well-mixed sludge was allowed to settle for 20 minutes and then the sample supernatant was collected for the flocculation test. The turbidity of the raw sludge supernatant was initially measured. 10 mL of the supernatant was pipetted into a clean test tube and added with 0.3 mL of BF01314 (3% BF dosage). The mixture was vortexed for 30 seconds and then transferred to a sample cell for turbidity measurements at 1-minute intervals, from 1 to 10 minutes. The

experiment was then repeated using 0.5 mL (5%) and 1.0 mL (10%) BF dosage. The experimental results were fitted to zero-, pseudo first- and pseudo second-order kinetic models to determine the rate and type of reaction according to Equations 3.9–3.11. Plots of turbidity in terms of N_t , $\ln(N_t)$, and $1/N_t$, against time t, were constructed accordingly for zero-, pseudo first- and pseudo second-order kinetic models. The rate of reaction was determined from the initial gradient of each plot.

Zero-order:	$N_t = -kt + N_0$	(3.9)
Pseudo first-order:	$\ln(N_t) = k_1 t + \ln(N_0)$	(3.10)
Pseudo second-order:	$1/N_t = k_2 t + 1/N_0$	(3.11)

Where k, k_1 and k_2 represent the zero-, pseudo first- and pseudo second-order rate constants, respectively. N_0 and N_t represent the initial turbidity and turbidity at time t.

Each plot was evaluated for its coefficient of determination (R^2), percentage of error, and residual plots (residual versus time), according to Equation 3.12 and 3.13. The fitted kinetic model with high R^2 value, low percentage of error and good residual plot (randomly scattered around x-axis) was selected as the best-fitted model.

Residual: Actual data of y-axis (y) – Predicted data of y-axis ($\hat{y}$)		(3.12)
Percentage of error (%):	$\lvert y - \hat{y} \rvert / y \times 100$	(3.13)

CHAPTER 4

PHYSICOCHEMICAL CHARACTERISATION AND FLOCCULATION ANALYSIS OF BF01314

In this chapter, the physicochemical characteristics of the bioflocculant BF01314 was analysed and presented in Section 4.1. Selected characterisation methods include total sugar, total protein, and total chitosan contents of BF01314, molecular weight, Fourier transform infrared (FTIR) analysis, surface charge determination and thermogravimetric analysis (TGA). Subsequently, flocculation performance of BF01314 in kaolin suspension at different bioflocculant (BF) concentration, pH and temperature conditions were thoroughly discussed in Section 4.2 in relation to its characteristics determined in Section 4.1. Significant findings on the flocculation analysis and charge interaction between the BF and kaolin clay particles were used to provide an insight to the preliminary interpretation of flocculation mechanisms of BF01314 in activated sludge sample in the next chapter.

4.1 Characteristics of BF01314

4.1.1 Total sugar, protein, and chitosan contents in BF01314

Figure 4.1 shows a calibration plot for sugar determination with a standard error estimated at 0.023. The total sugar content in BF01314 sample was approximately 255.47 mg/L after being corrected with a dilution factor of four. The equivalent percentage with respect to the BF dry weight (4.78 g/L) was 5.34%.

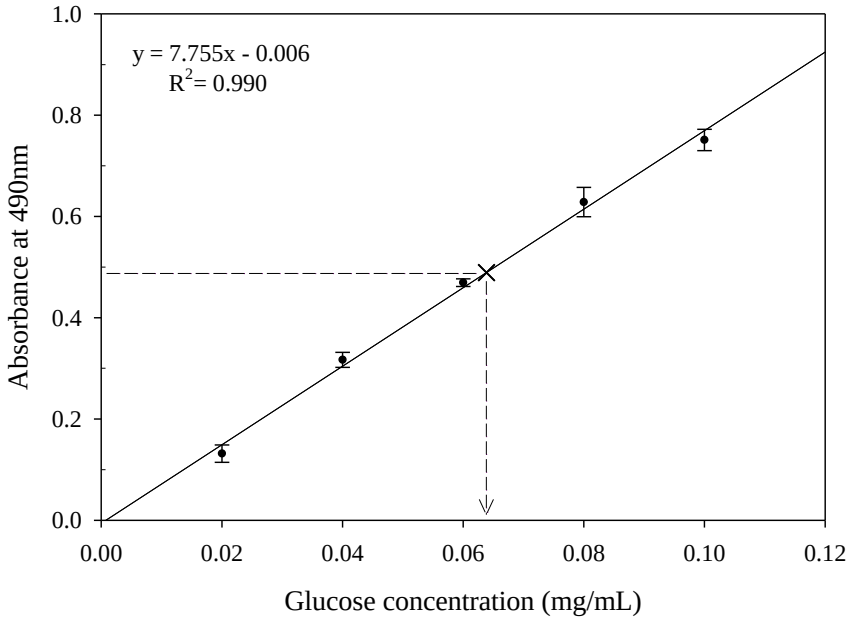

Figure 4.1: Standard curve of glucose concentration; (×) BF01314 sample. Error bar represents ±standard deviation (SD) of triplicates.

For the determination of total protein content, no trace of protein was detected in BF01314, indicated by low absorbance value <0.01 which is out of the range tested. Figure 4.2 shows the difference in colour of the BSA solutions over the range of 0.0–1.0 mg/L of reagent addition. The solutions contained with BSA (acted as protein standard) turned its colour from brown to blue upon addition with the reagents and darkens as the concentration of protein increased. However, the addition of reagent to BF01314 sample demonstrates the negative reaction of the sample whereby the colour of the solution remained unchanged, indicating the absence of protein. Unlike the BF04 produced from *Citrobacter* TKF04 as reported by Fujita et al. (2000), no precipitation was observed during the assay which confirmed the reliability of the result. This could indicate the absence or limited presence of incompatible substances such as detergent in the BF sample, which could interfere with the assay through precipitation and increase the absorbance reading as generally reported in protein quantitation (Cheng et al. 2016).

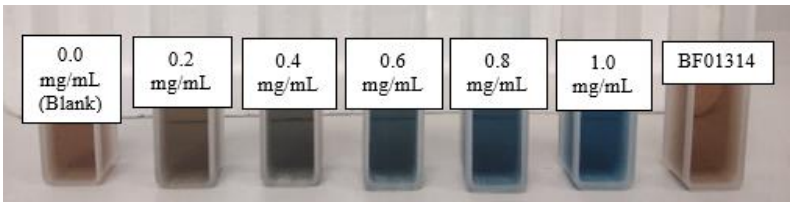

Figure 4.2: Protein determination by Bradford assay. The colour of the BF01314 solution remains unchanged after adding the reagent.

Figure 4.3 shows a calibration curve obtained using a standard commercial chitosan with R^2 value and a standard error of estimates of 0.996 and 0.021, respectively. The corrected apparent chitosan concentration in BF01314 sample was 591.93 mg/L, equivalent to 12.38%, which was unexpectedly much higher than the total sugar content in the sample.

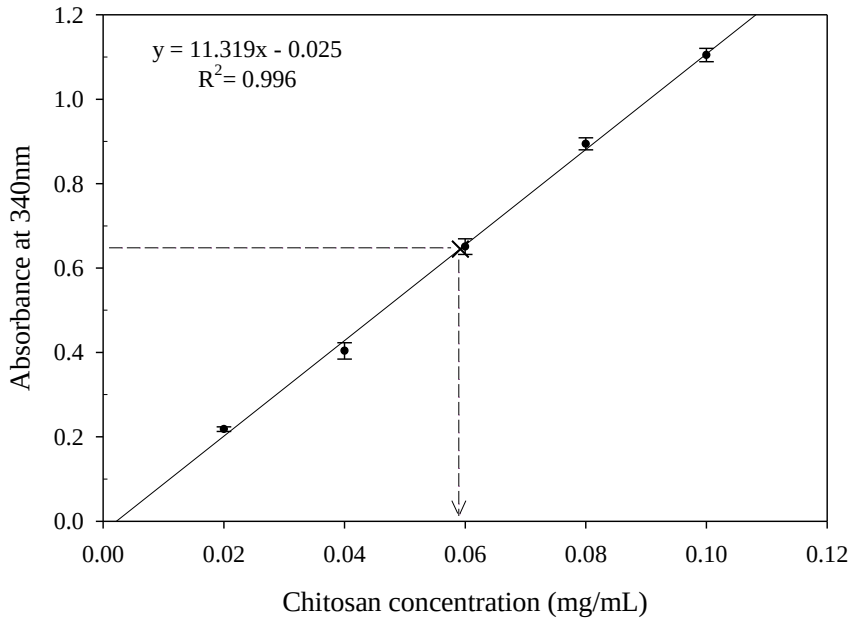

Figure 4.3: Standard curve of chitosan concentration; (×) BF01314 sample. Error bar represents ±SD of triplicates.

In the present study, the results revealed that chitosan content in BF01314 was higher compared to sugar content, which suggests the necessity of further characterisation studies on the elemental and chemical composition of the BF in future work. It is more likely that the large percentage difference between chitosan and sugar content obtained from the present work was attributed to the undesirable interference caused by other impurities present in the BF (Larionova et al. 2009). This could be attributed to the low molecular weight components in the BF originated from the culture medium as discussed in Section 3.1.1.

4.1.2 Molecular weight and FTIR analysis of BF01314

The weighted average of molecular weight (M_w) and number average of molecular weight (M_n) of this novel bioflocculant BF01314 were found to be 327 kDa and 187 kDa respectively, with polydispersity index (PDI) of 1.75. PDI characterises the

broadness of the BF's molecular weight distribution, defined statistically by the ratio of M_w to M_n. The notable higher value of M_w indicates the presence of significant amounts of high molecular weight fractions in BF01314 compared to low molecular weight fractions presented by M_n (Lapointe and Barbeau 2020; Meiczinger et al. 2005). The infrared (IR) spectrum of BF01314 was compared to the chitosan of equivalent molecular weight (310-375 kDa, >75% deacetylated) with typical chemical structure of partial deacetylated chitin as shown in Figure 4.4.

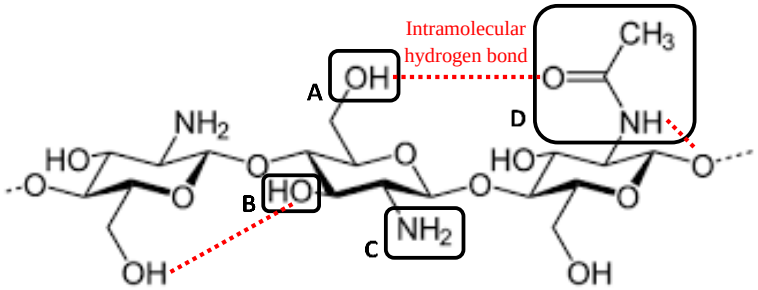

Figure 4.4: Typical structure of partial deacetylated chitin (chitosan). (A) primary hydroxyl; (B) secondary hydroxyl; (C) primary amino; (D) secondary amide.

Figure 4.5 demonstrates the similarity of BF01314 and chitosan spectra with almost similar region of characteristic bands as summarised in Table 4.1.

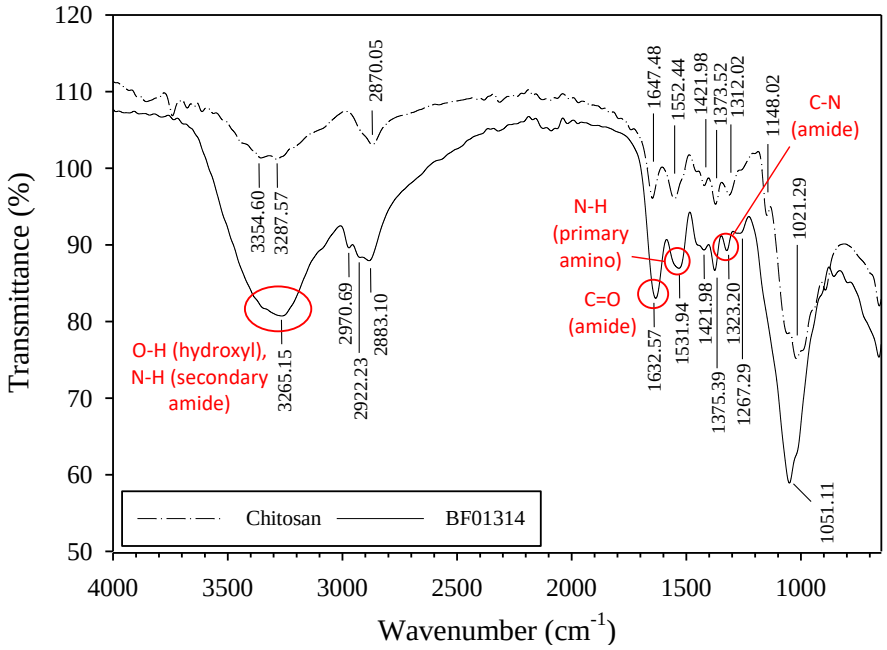

Figure 4.5: Functional groups of BF01314 in comparison to the chitosan of equivalent molecular weight

Table 4.1: Summary of characteristic bands of chitosan and BF01314

Functional group	Characteristic band (cm^{-1})		Roles description
	Chitosan	**BF01314**	
Hydroxyl and secondary amide	3354.60, 3287.57	3265.15	Due to O-H stretching resulted from the intermolecular and intramolecular hydrogen bonds of the BF particles. This band is most likely overlapped with N-H stretching from secondary amide. These groups may provide adsorption sites for bridging via hydrogen bonding.
Primary amino	1552.44	1531.94	Associated with N-H bending from primary amino group. Amino group can be protonated in aqueous acidic solution which then can attract the negative colloids via electrostatic interaction.
Amide	1647.48, 1312.02	1632.57, 1323.20	Due to the stretching of C=O and C-N which typically found in N-acetyl group. The oxygen from carbonyl (C=O) can also form hydrogen bond to promote flocculation.

Strong absorption bands between 3200–3400 cm^{-1} were attributed to N-H and O-H stretches resulted from intramolecular hydrogen bonds (Figure 4.4) as well as intermolecular interactions between the BF particles (Queiroz et al. 2014). The N-H stretching at around this characteristic band is commonly represents a secondary amide and sometimes can be overlapped with hydroxyl band (Menkiti et al. 2016), which most likely occurred in the BF01314 spectrum (Figure 4.5). These vibration bands may reflect the hydroxyl groups on the glucosamine rings. Primary hydroxyl denoted as A in Figure 4.4: Typical structure of partial deacetylated chitin (chitosan). (A) primary hydroxyl; (B) secondary hydroxyl; (C) primary amino; (D) secondary amide. Figure 4.4 is especially predicted to be reactive in providing adsorption sites for bridging via hydrogen bonding because of the reduced steric hindrance and high electronegativity of its oxygen (Okaiyeto et al. 2016; Yang et al. 2016). A corresponding N-H bending at 1531.94 cm^{-1} indicated the presence of primary amino groups (Queiroz et al. 2014; Anderson 2004). In aqueous acidic solutions, this amino group will be protonated and exhibit positive charge ($NH_2 \rightarrow NH_3^+$) (Jagadish et al. 2017), which increases the chance to adsorb the negative colloidal particles through an electrostatic interaction (Gregory and Barany 2011).

In the fingerprint region, the characteristic bands at 1647.48 and 1312.02 cm^{-1} in chitosan were assigned to C=O stretching and C-N stretching, respectively found in a typical N-acetyl group as a result of partial deacetylation of chitin to chitosan (Queiroz et al. 2014). BF01314 spectrum also produced similar characteristic bands of this typical N-acetyl group at 1632.57 and 1323.20 cm^{-1}. The bands between 2870–2980 cm^{-1} were most likely attributed to C-H stretches which are typically found in polysaccharides and are rarely useful (Queiroz et al. 2014; Ning, Ernst and Ning 2011). The remaining peaks between 1000-1400 cm^{-1} in the chitosan and BF's spectrum could be attributed to CH_2 bending, CH_3 symmetrical deformations, C-O stretching, and asymmetric stretching of C-O-C bridge which were commonly determined in a chitosan structure (Queiroz et al. 2014). All of the above discussions verify that BF01314 is a chitosan-like BF with the presence of functional groups of hydroxyl, amide and amino groups. Any shifting or change in intensity of a peak in sludge spectrum around the respective characteristic bands after conditioning may indicate an interaction between the sludge and these functional groups of the BF (Feki et al. 2020), which is further discussed in Section 5.7.

4.1.3 Surface charge analysis

Figure 4.6 shows an isoelectric titration plot of BF01314 over pH levels within a range of 2 and 12. The results indicate that the BF exhibited polycationic behaviour, ranging from +36.3 mV to 0 mV under acidic conditions and under a slightly alkaline region below the isoelectric point (IEP) at pH 8.25. This behaviour imitates a wide standard polyelectrolyte nature of chitosan in acidic aqueous solution as a result of the protonation of amino groups (Luo and Wang 2014).

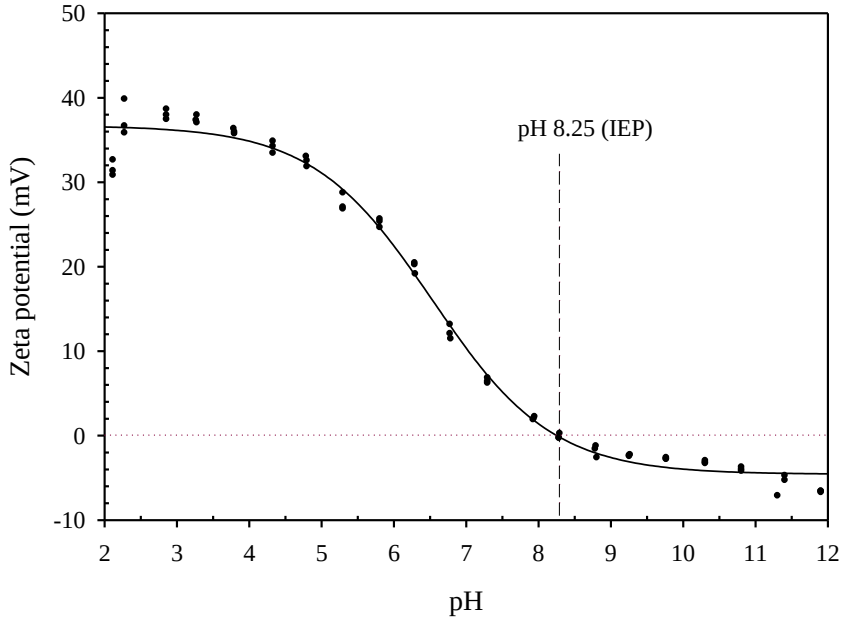

Figure 4.6: Isoelectric point (IEP) titration graph of BF01314

Figure 4.7 demonstrates the reversible reactions of primary amino groups of chitosan at low and high pH conditions. The protonation of amino groups ($-NH_2$) to $-NH_3^+$ formation could be induced by the abundance of H^+ and H_3O^+ ions in acidic solutions, leading to an increase of positive charge density (Jagadish et al. 2017; Costa et al. 2015). Studies have shown that the medium with pH 4 or below was able to protonate more than 90% of amino groups in chitosan (Zemmouri et al. 2011; M. Nomanbhay and Palanisamy 2005). Meanwhile, this amino group can also undergo deprotonation at high pH values, which may explain the negative surface charges of BF01314 at pH >8 (Wadhwa et al. 2009).

$$\underset{\text{At low pH (acidic)}}{\text{Chit-NH}_3^+} + H_2O \underset{\text{protonation}}{\overset{+\ H_3O^+}{\rightleftharpoons}} \underset{\text{Chitosan}}{\text{Chit-NH}_2} \underset{\text{deprotonation}}{\overset{+\ H_2O\ +\ OH^-}{\rightleftharpoons}} \underset{\text{At high pH (basic)}}{\text{Chit-NH}^-} + 2H_2O$$

Figure 4.7: Reversible reactions of chitosan primary amino group (denoted as Chit-NH_2) at low and high pH conditions.

IEP was defined as a characteristic property in which the zeta potential is zero and identical to the pH value at the point of zero charge (Alvarez-Silva et al. 2010; Swain et al. 2009). For a new biopolymer like BF01314, determination of IEP can be very useful in understanding its ionic behaviour before being considered for practical applications. It could also indicate the capacity of the biopolymer to adsorb the ions of

target pollutants, which was seldom reported in previous studies (Yazdani et al. 2017). Most recent and previous works revealed that the IEP values of natural chitosan also ranges between pH 7.5 to 8.6 depending on the electrolytic medium (Picos-Corrales et al. 2020; Swain et al. 2009). The combined effects of the polycationic behaviour and the presence of reactive functional groups in BF01314 are believed to promote strong flocculation activities in negatively charged colloidal systems, as a result of its chitosan-like properties.

4.1.4 Thermogravimetric analysis (TGA)

Thermal degradation study of chitosan and BF01314 was performed over temperatures ranging from 25–800°C and the results are plotted in Figure 4.8 and Figure 4.9, respectively. In the first thermal event, the maximum weight loss of both biopolymers was observed at about the same temperature indicating the point of highest rate of change on their respective weight loss curve. Chitosan lost 10% of its weight at the first derivative peak temperature of 77°C while BF01314 has higher percentage of weight loss (33%) than chitosan which was observed at a lower temperature of 69°C. This phenomenon was attributed to the evaporation of water molecules that are strongly bonded to the polymeric chains of the polymer by the hydroxyl and amino groups, which generally occurs below the temperature of 100°C (Grząbka-Zasadzińska, Amietszajew and Borysiak 2017; Pereira et al. 2013). In the present study, BF01314 is a water-soluble polymer and has a very high tendency to absorb moisture from the air in dried form therefore, high percentage of weight loss was recorded at this stage. As the temperature increased, chitosan and the BF remained at its constant weight (Chitosan: 90% of weight; BF01314: 70% of weight) up to the temperature of 265°C and 202°C respectively.

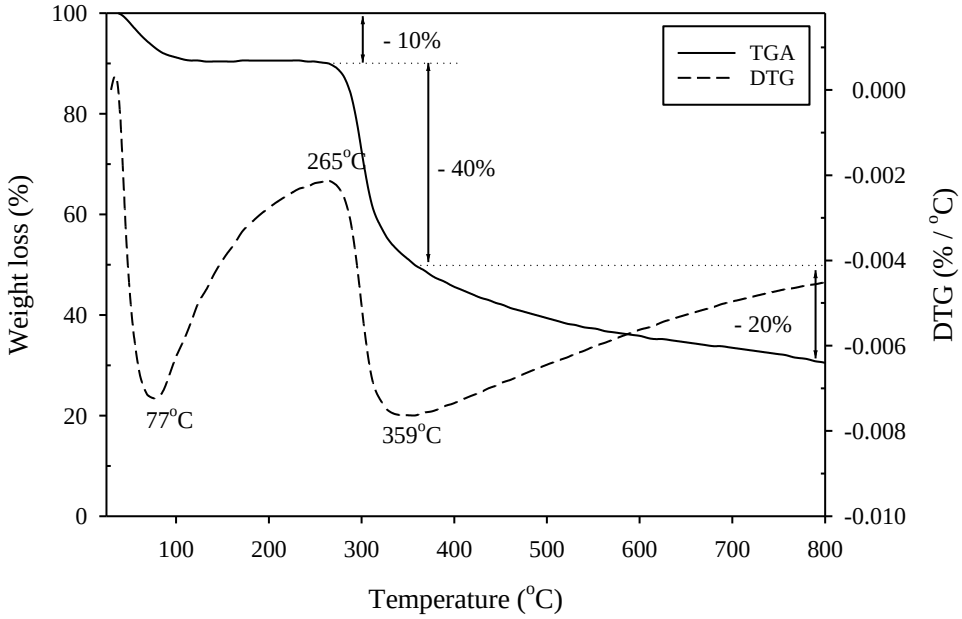

Figure 4.8: TGA/DTG curve of one of chitosan

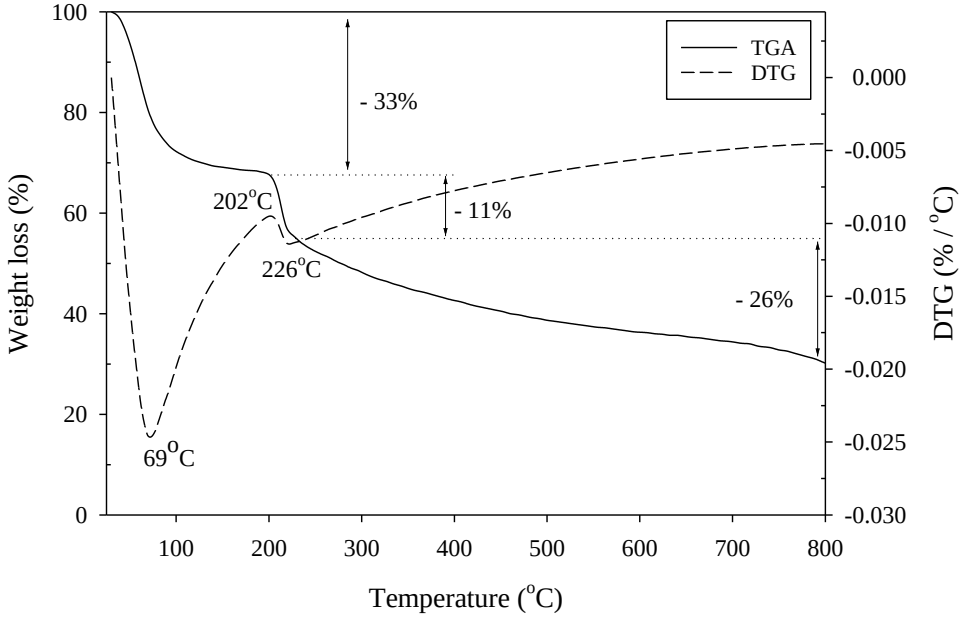

Figure 4.9: TGA/DTG curve of BF01314

In the second thermal event, the degradation of chitosan polymeric chains occurred within the range of 265–365°C, with the greatest rate of change at 359°C, resulting in 40% weight loss. On the other hand, BF01314 continued to lose much lower percentage of weight (11%) within a relatively lower range of 202–230°C, with the maximum at 226°C. This second stage of thermal decomposition occurred due to the breakdown of glycosidic bonds that linked the D-glucosamine (GlcN)/N-acetyl-D-glucosamine (GlcNAc) rings as shown in Figure 4.10 (Grząbka-Zasadzińska et al.

2017; Pereira et al. 2013). Based on the result in the present work, BF01314 may contain less glucosamines supported by the low apparent chitosan content of 12.38% found in the sample from the colorimetric assay in Section 4.1.1. It was reported that the C–O–C of glycosidic bonds stretched upon entering this decomposition stage and eventually separated the polyglucosamine chain (Pereira et al. 2013), leading to the formation of pyrazine compounds through a series of mechanisms from the pyrolysis of chitosan to the formation of α-amino carbonyls as the precursors of pyrazine compounds (Zeng et al. 2011). Hence, further analysis to determine the decomposition products of the BF are recommended for future work.

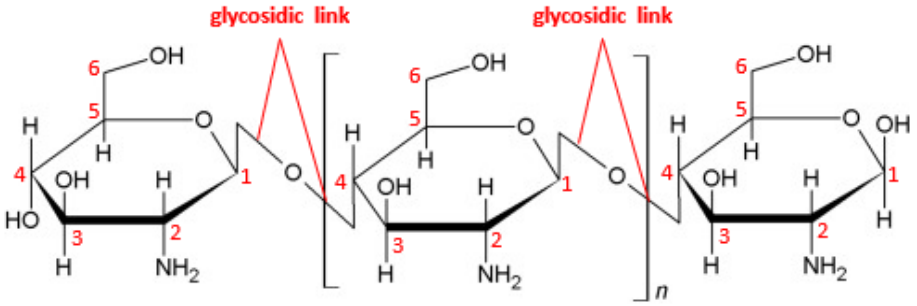

Figure 4.10: D-glucosamine linked with β-(1,4) glycosidic bonds in chitosan polymer. 1–6 shows the position of each carbon

At the end of the experiment, chitosan and BF01314 showed further weight loss of about 20% and 26% respectively, with the remaining percentage of residual mass of 30%, indicating incomplete decomposition of both biopolymers up to 800°C. The overall analysis reveals that BF01314 is thermally stable as it was able to withstand high temperatures of up to 202°C, slightly lower than that of chitosan (265°C) before it started to decompose.

4.2 Flocculation analysis of BF01314 using kaolin suspension

4.2.1 Effect of BF01314 concentration

Figure 4.11 shows a graph of flocculation activity of BF01314 measured to determine the 'flocculation titre (Ft)' value, defined as the fold dilution of the culture supernatant to reduce the saturated flocculation activity by 50%, whereby the BF-producing strains with Ft>80 were classified as high activity strains (Kimura et al. 2013). In this study,

the Ft values were determined within the first month of production (1 month) and after the tenth month (10 months) of storage at temperature of 4°C. The Ft value of BF01314 declined from 4390 ($2^{12.1}$) to 1663 ($2^{10.7}$) after about 10 months of storage at 4°C but sustained its strong flocculation activity of >95% between dilution 2 to 7. This indicates that BF01314 was stable up to the month tested (tenth) with the addition of ProClin300 as preservative, which is comparable to commercial liquid polymers with common storage periods between 6–12 months (Lara et al. 2007). Szymańska and Winnicka (2015) also reported the necessity of chitosan-based product to be stored at temperatures below 5°C for long-term stability.

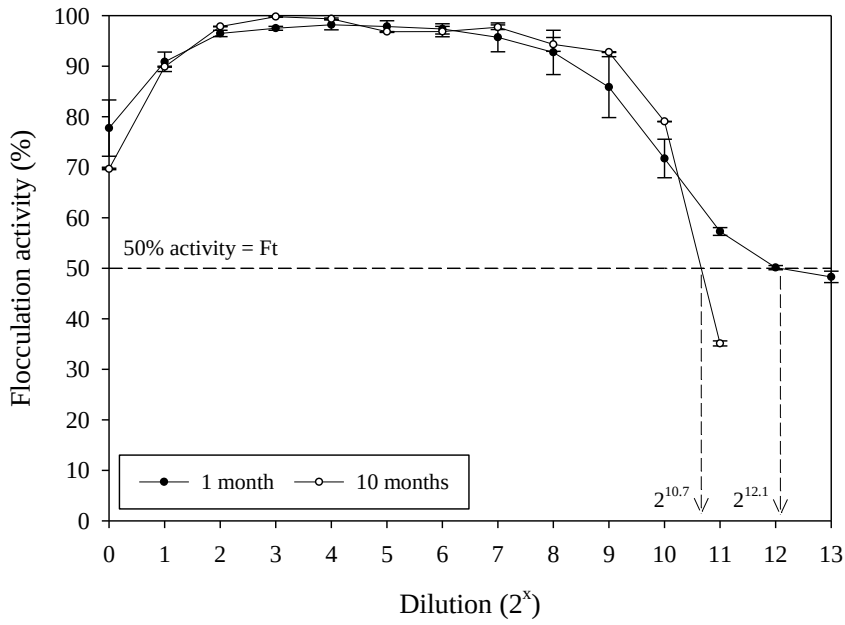

Figure 4.11: Measurement of 'flocculation titre (Ft)' of BF01314. Error bar represents ±SD of triplicates.

Figure 4.12 presents the plot of flocculation activity in kaolin suspension against the BF dosage expressed in terms of concentration instead of dilution for the BF (1 month), calculated using Equation 3.4 (see Appendix B.1 for sample calculation). The flocculation activity increased from 48.31% to a maximum of 98.21% as the BF concentration increased from 0.058 to 15 mg/L (optimum), and then retained its activity at >90% up to 239 mg/L. The flocculation activity reduced to 77.7% at the maximum of 478 mg/L within the range of concentration tested. A similar pattern was reported for BF04 from *Citrobacter* sp. TKF04 where the flocculation activity of >90% was reported for the range between 1 and 10 mg/L with a slightly reduced activity at 100 mg/L (Fujita et al. 2000). The increase of flocculation activity with the BF

concentration in the present work could be attributed to the increasing adsorption capacity due to the increase of biopolymers present in BF01314. At higher BF concentration, more biopolymers (and hence more adsorption sites) are available for the complex formation between the BF and sludge particles. Nevertheless, overdosing the BF resulted in decreasing flocculation activity as shown in Figure 4.12, causing re-stabilisation when the neutralised suspended particles were completely surrounded by excess BF biopolymers and again recharged causing the destabilised particles to become stable again. Longer time may also be required for all BF particles to be completely utilised and reached adsorption equilibrium (Nechita 2017). Furthermore, high molecular weight polymers appear to have higher maximum adsorption capacity (Lee and Schlautman 2015), which explains the capability of BF01314 exhibiting reasonably high flocculation activity of >80% even at low concentration of 1 mg/L.

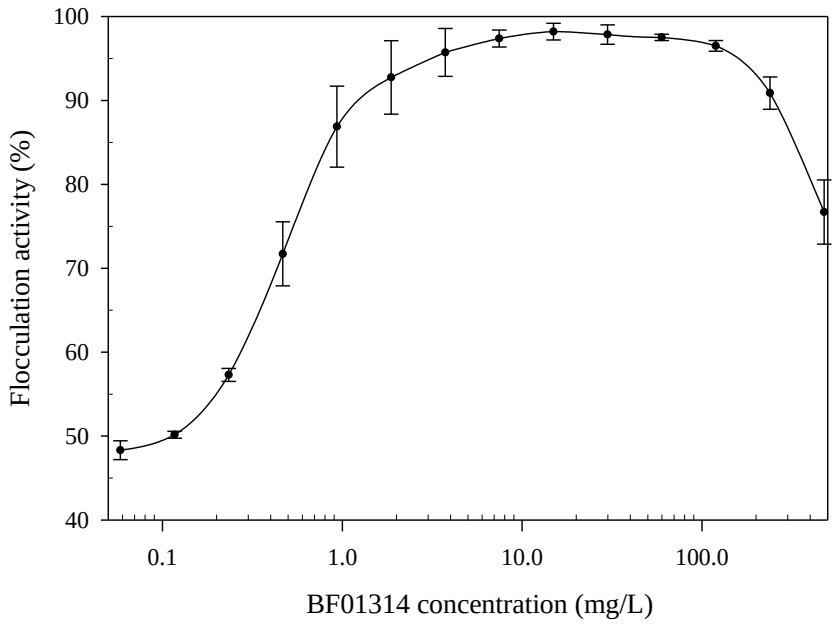

Figure 4.12: Effect of BF01314 concentration on flocculation activity for the BF (1 month). Error bar represents ±SD of triplicates.

Figure 4.13 shows the effect of BF concentration on the zeta potential of the suspension. The zeta potential values were barely affected by the concentration below 1 mg/L, and remained around -15.7±1.4 mV, compared with the initial zeta potential value (-13.2 mV) of the kaolin suspension. Further increase of BF concentration beyond 1 mg/L however resulted in a charge reversal from -14.3 mV at 0.93 mg/L to +8.3 mV at 1.87 mg/L.

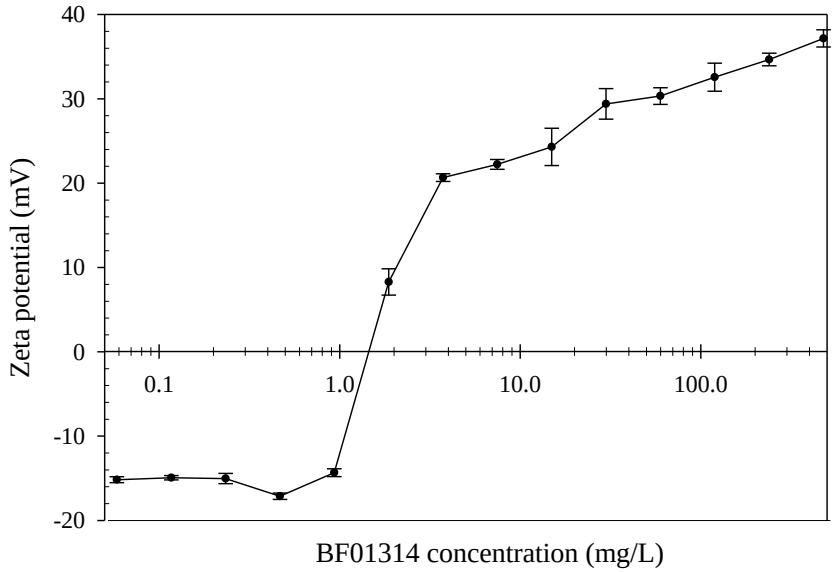

Figure 4.13: Effect of BF01314 concentration on zeta potential of the suspension for the BF (1 month). Error bar represents ±SD of triplicates.

At the concentration of 15 mg/L with the highest flocculation activity (98.21%), the zeta potential was +24.3 mV. The zeta potential value continued to increase to 34.7 mV with increasing BF concentration up to 239 mg/L without any significant negative overdose effect. Therefore, it can be concluded that charge neutralisation was attained between 0.93 and 1.87 mg/L with effective flocculation at around 90%. Beyond this point, BF01314 showed a reasonably broad "flocculation window" of >95% between 4 and 120 mg/L, with the apparent optimal concentration at around 15 mg/L, as shown in Figure 4.12, while the zeta potential values continued to deviate far from zero, without re-stabilisation effect.

Yang et al. (2016) and Yang et al. (2011) interpreted simple charge neutralisation as the predominant mechanism when the zeta potential value increases linearly with the flocculation rate and reaches zero at optimal flocculant concentration. Meanwhile charge patching has been described as incomplete neutralisation of unevenly distributed surface charges (Yang et al. 2016). Hence, charge patching may provide effective flocculation before the charge neutralisation is complete, resulting in non-zero zeta potential value at an optimal concentration (Hu et al. 2020; Yang et al. 2016; Liu et al. 2017). Nevertheless, for cationic polyelectrolytes like chitosan, simple charge neutralisation and electrostatic charge patching are hardly distinguishable as both mechanisms simultaneously increase the zeta potential of the suspension (Yang et al. 2016). Therefore, it is believed that both neutralisation mechanisms are dominant

at low BF concentration, as evidenced in the present study since the effective flocculation took place even before the apparent optimal concentration was attained at the zeta potential value of +24.3 mV.

In addition, the polymer bridging mechanism is speculated to prevail at high BF concentration due to the high molecular weight of BF01314. Lapointe and Barbeau (2020) pointed out that the higher chance of available dangling segments (loops and tails) of a polymer for bridging was translated by its higher molecular weight. The long chain conformation allows the biopolymers to adsorb onto the surface of the suspended particles with their loops and tails for effective bridging and flocculation, despite the net positive charge at the concentration above 1 mg/L, as shown in Figure 4.13. Lapointe and Barbeau (2020) also reported that chitosan with moderate-to-high molecular weight can neutralise the negatively charged colloid particles by cationic amino groups and simultaneously flocculate the particles via interparticle bridging, leading to an increase of floc size. Overall, these findings seem to be consistent with the results of the present study indicating that BF01314 is an effective cationic chitosan-like BF that demonstrates similar flocculation mechanisms in kaolin flocculation as discussed above.

4.2.2 Effect of suspension pH

Figure 4.14 presents the effect of suspension pH on the flocculation activity in kaolin flocculation when treated with the BF01314 sample at the optimal concentration of 15 mg/L. The results revealed good BF stability in a wide range of pH (from pH 2 to 8) with outstanding flocculation activity (>98%). The maximum flocculation activity of 99.75% was observed at pH 6, equivalent to the results of zeta potential at 0.77 mV as depicted in Figure 4.15. At <pH 6, the zeta potentials of BF-treated kaolin suspension were positive and high flocculation activities were exhibited. In contrast, the zeta potential value was reversed at >pH 6, including the IEP of the BF01314 sample (pH 8.25), eventually producing about similar charge with the untreated kaolin suspension.

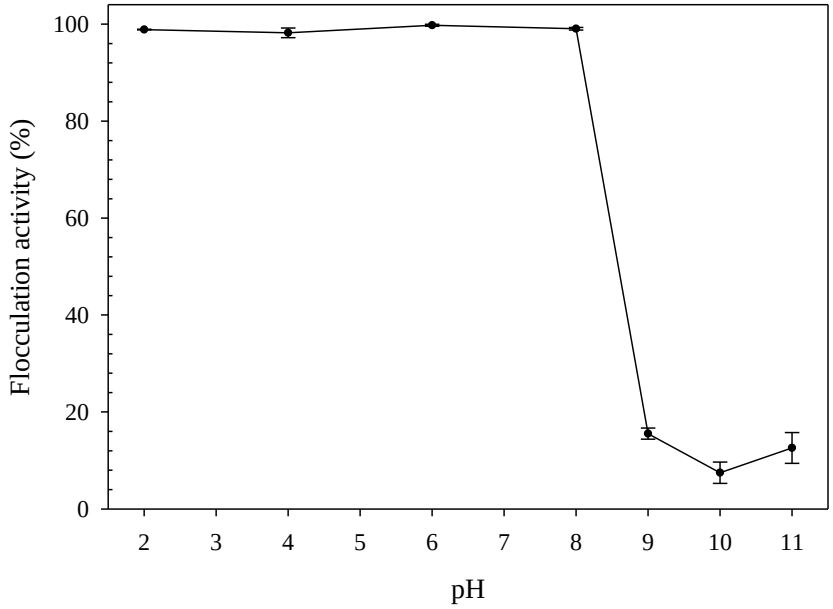

Figure 4.14: Effect of suspension pH on flocculation activity of BF01314. Error bar represents ±SD of triplicates.

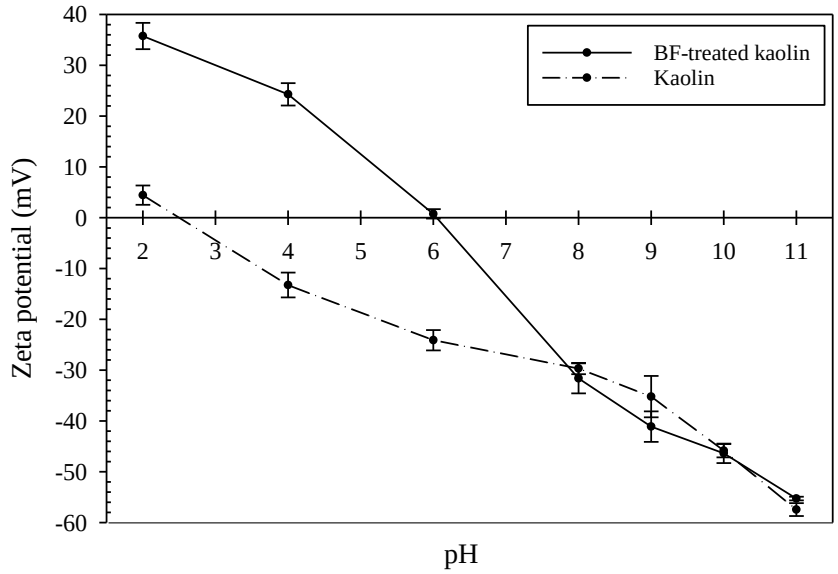

Figure 4.15: Effect of suspension pH on zeta potential. Error bar represents standard deviation of triplicate determinations.

Nevertheless, high flocculation activity was still observed even in some parts of the negative charge region (from pH 6 to 8). At around IEP, BF01314 loses the electrostatic potential to neutralise the negative surface charge of kaolin particles. This may prove the presence of different mechanisms apart from charge neutralisation that can promote effective interparticle bridging among kaolin particles with BF01314. On the other hand, a sharp decrease of flocculation activity was observed during the transition from pH 8 to 9 and above. This could be due to the excessive OH^- ions in

alkaline medium that competes with the anionic kaolin particles and hinders the complex formation between the BF and kaolin particles (Okaiyeto et al. 2016; Yazdani et al. 2017). Besides, the available bridging sites might be reduced as the amino groups on the BF deprotonate from $-NH_2$ to $-NH^-$ and reverses the BF charges to negative, under very alkaline conditions. Such change increases the electrostatic repulsion between the negatively charged kaolin particles and the BF (Pan et al. 2016; Wadhwa et al. 2009).

Overall, the analysis of flocculation activity along with the zeta potential measurement suggested that kaolin flocculation by BF01314 was mainly dependent on charge neutralisation with additional mechanisms such as polymer bridging governing at the pH values of 2 and 8, in view of the insignificant electrostatic interaction between the flocculant polymer and kaolin particles under these conditions (Lee et al. 2014; Eriksson, Alm and Stenius 1993). Both electrostatic interaction and polymer bridging could be significant at pH 4 whereas simple charge neutralisation seems to be dominant at pH 6 as shown in Figure 4.15, although there is no direct evidence. The poor flocculation activity at pH 9 and above may explain the absence of these two mechanisms.

4.2.3 Effect of temperature

Figure 4.16 reveals the effect of temperature on flocculation activity of BF01314 in kaolin suspension with 15 mg/L of the BF01314 sample. The BF contributed to high flocculation activities (>98%) from room temperature (25±1°C) to 95°C signifying strong thermostability of BF01314 which results in effective flocculation even at high temperatures up to 95°C. This is due to the polysaccharide structure of BF01314 indicated by FTIR and GPC-MALLS analyses, supported by the results of earlier thermal degradation study of BF01314 showing that the BF can resist higher temperature up to 202°C. Additional preliminary experiments at 10°C and 100°C revealed reasonably high flocculation activities of 96-98% when the BF was applied in kaolin suspension. This also indicates the potential to apply BF01314 in treating wastewater in cold climate which worth further investigation. Giri et al. (2015) and Aljuboori et al. (2015) have also reported similar findings for BFs produced from *Bacillus subtilis* F9 and *Aspergillus flavus* with minimum flocculation activities of 89%

and 93%, respectively, in temperature range of 5–100°C. On the other hand, a novel BF produced from *Diaphorobacter nitroreducens* R9 exhibited moderately low flocculation activity of <80% at 0–5°C (Zhong et al. 2020), which could be due to cold denaturation of its protein content (Yan et al. 2018).

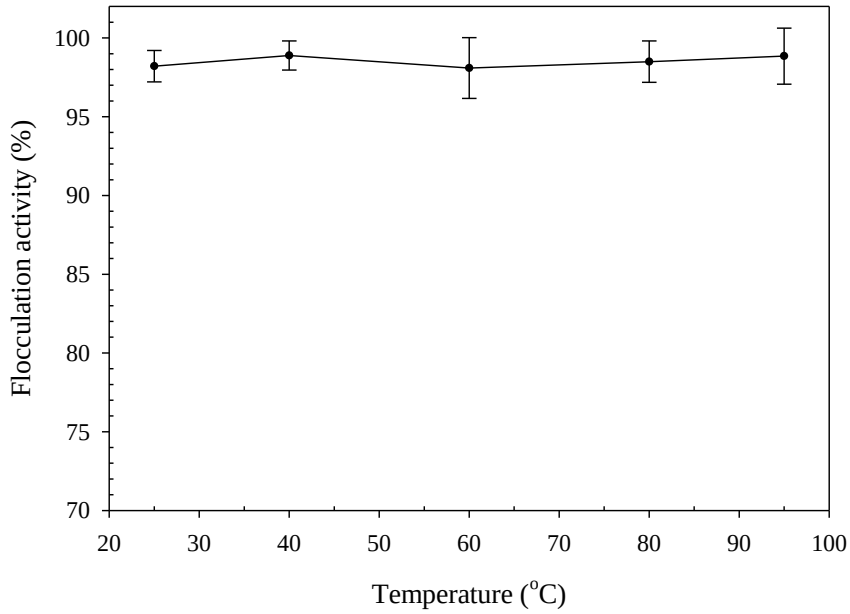

Figure 4.16: Effect of temperature on flocculation activity of BF01314. Error bar represents ±SD of triplicates.

Many BFs have also been reported thermally stable up to 100°C or more owing to their polysaccharide backbone such as the BFs produced from *B. Agaradhaerens*, *Paenibacillus polymyxa*, *Enterobacter* sp. and *Citrobacter* sp. TKF04 (Salehizadeh et al. 2018; Fujita et al. 2000). Meanwhile, BF with protein content such as MC11 produced from *Klebsiella* sp. showed a decrease in its flocculation activity at temperatures above 60℃ (Liu et al. 2014). This phenomenon was due to the simultaneous increase in kinetic energy of suspension particles along with protein denaturation as explained in Yadav et al. (2012).

Figure 4.17 shows the zeta potential of the BF-treated kaolin suspension as compared to the untreated suspension. The zeta potential value gradually decreased from +24.3 to +20.3 mV as the temperature increased from 25 to 60°C and became negative at 80°C and above. The kaolinite characteristic itself might be influenced by the temperature rise which causes the zeta potential value to decline (Rodríguez and Araujo 2006). This could be observed from the parallel trend in zeta potential change

for both untreated and BF-treated kaolin suspensions, decreasing gradually from 25 to 60°C but retaining the flocculation activity of >98%.

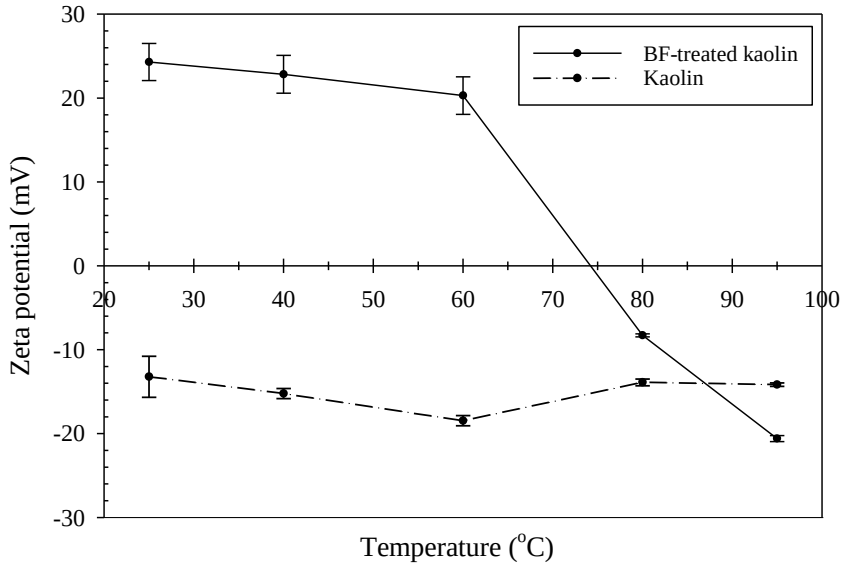

Figure 4.17: Effect of temperature on zeta potential of the suspension. Error bar represents ±SD of triplicates.

When the temperature increased from 60 to 80°C, the surface charge of the BF-treated kaolin suspension reverted to negative sign which coincided with the maximum weight loss of the BF probably due to the release of water molecules at 69°C as discussed in Section 4.1.4. This may diminish the protonation of amino groups in BF01314 leading to a net negatively charged kaolin suspension. Although weakening of adsorptive forces with increasing temperature has been reported by Pan et al. (2016), such weakening effect was not observed in the present kaolin flocculation with the BF as demonstrated by the high flocculation activity in the temperature range studied.

4.3 Summary

This chapter summarises all findings on the characterisation studies of the BF of *C. youngae* GTC 01314 (named as BF01314) and its flocculation analysis in kaolin suspension. Based on the results, it is confirmed that BF01314 is a polysaccharide-based polymer that resembles the chitosan with possible reactive functional groups of hydroxyl, amide, and amino groups. These characteristics are responsible for the high thermal stability as well as the polycationic behaviour of BF01314 in acidic and weak alkaline aqueous solutions, which are comparable to those of chitosan. When tested in

kaolin suspension, BF01314 exhibited high flocculation activity of more than 95% within the BF concentration range of 4–120 mg/L with no negative overdose effect. Its flocculation performance remained high either when subjected to heating up to 100°C or under acidic and weak alkaline pH below its IEP. Simple charge neutralisation, electrostatic charge patching, and polymer bridging are believed to be involved in promoting effective flocculation below the IEP to different extents. Overall, the findings suggest that BF01314 is a potential novel cationic BF with excellent flocculation performance over wide pH and temperature ranges attributed to its chitosan-like structure. Subsequently, further experimental work was conducted in order to identify its practicability in wastewater treatment focusing on sludge conditioning and dewatering application with detailed results and discussion as presented in Chapter 5.

CHAPTER 5

SLUDGE FLOCCULATION AND DEWATERING PERFORMANCE WITH BF01314

In this chapter, waste activated sludge (WAS) collected from a local sludge treatment plant was used to examine its flocculation and dewatering performance when BF01314 was applied as flocculant. Raw sludge characteristics are summarised in Section 5.1 as the control parameters when evaluating the dewatering efficiency. Preliminary findings on the effects of bioflocculant (BF) dosage, pH and temperature on sludge dewaterability were presented and compared with the commercial chitosan and synthetic cationic flocculant in Sections 5.2 to 5.5. Results on the flocculation kinetics of the BF evaluated from the study of the effects of flocculation speed and time as well as the determination of rate of reaction were also presented in Section 5.6. Subsequently, possible flocculation mechanisms were discussed and proposed based on all preliminary findings and further analysis of infrared spectrum and micrographs of the sludge before and after conditioning at selected conditions (Section 5.7).

5.1 Raw sludge characteristics

Table 5.1 shows the selected physicochemical characteristics of the raw WAS sample used for sludge conditioning and dewatering experiments in the present study in comparison with the typical activated sludge characteristics reported in literature. The capillary suction time (CST) values were found vastly different from each other due to different sources of the sludge. WAS sample in the study of Guo, Chen, et al. (2018) was collected from the secondary settling tank which is generally difficult to dewater because of very high water content of about 99%, hence high CST was recorded (Spinosa and Vesilind 2001). On the contrary, lower CST reported by Wong, Murugesan, Selvam, et al. (2016) could be attributed to the high salinity as the sludge sample was sourced from the sewage treatment plant that received seawater flushed sewage. Salt in high salinity sludge was reported to behave similarly to a chemical

coagulant (Lo, Lai and Chen 2001). As for the current study, the WAS sample was collected from a local treatment plant which receives sludge from domestic sources within the city and its outskirts. The plant utilises a sequencing batch reactor system (SBR) in the activated sludge process without primary and secondary settling tanks, such as those found in full scale wastewater treatment plants (WWTPs) (EPA 2000).

Although CST has been widely used to determine the optimum flocculant dosage, it is not suitable for comparison between the sludge sourced from different WWTPs (Gray 2015). In such cases, specific resistance to filtration (SRF) results are more appropriate to be used for comparing the dewatering performance (Gray 2015). The sludge in this study has a SRF value of $7.4\pm0.9 \times 10^{11}$ m/kg which is categorised as easy-to-dewater sludge whereas the other sources of sludge in Table 5.1 were classified as medium or slightly difficult to dewater sludge (Kopp and Dichtl 2001; Sanin et al. 2011; Vu Hien Phuong et al. 2016). SRF provides information about the resistance of sludge to withdraw the water through a porous medium either by vacuum or pressure whereby the higher the resistance is, the more difficult it is to dewater (Vu Hien Phuong et al. 2016).

Table 5.1: Characteristics of activated sludge in comparison to previous literature

Characteristic	**Present study**	**Zemmouri, Mameri, and Lounici (2015)**	**Wong et al. (2016)**	**Guo et al. (2018)**
pH	7.0±0.3	8.3	6.7	6.5
Total solid, TS (g/L) **(TS in %)**	29.1±2.0 (2.7 – 3.1)	NA (NA)	NA (2.1)	14.9 (NA)
Total suspended solid, TSS (g/L)	27.5±2.0	3.3	NA	NA
Total dissolved solid, TDS (g/L)	1.3±0.2	NA	NA	NA
Capillary suction time, CST (s)	22.0±1.0	48.0	12.6	132
Specific resistance to filtration, SRF (m/kg)	$7.4\pm0.9 \times 10^{11}$	6.8×10^{12}	1.0×10^{13}	11.3×10^{12}
Cake solids content (CSC), %	15.2±1.5	3.22	NA	12.1
Zeta potential (mV)	-15.5±1.3	NA	NA	-15.4

Sources: Data from Zemmouri et al. (2015); Wong, Murugesan, Selvam, et al. (2016); Guo, Chen, et al. (2018).

Note: NA is not available in the report.

5.2 Effect of BF01314 dosage

Figure 5.1 displays the effect of BF01314 dosage on the sludge dewaterability measured by CST at initial pH of 7.0±0.3 and at room temperature (25±1°C). The results indicated good dewaterability of the sludge treated by BF01314 (BF-treated sludge) with the reduction of the CST value from 22.0 (raw WAS) to less than 10.5 s for the range of BF dosages from 0.3 to 6.0 kg/t DS. Again, no overdose effect was observed. At the dosage above 1.0 kg/t DS, the CST value remained approximately the same between 7.7–8.7 s with the lowest value observed at 6.0 kg/t DS. According to Sanin et al. (2011), a CST value of about 8 s confirmed that the sludge were well-flocculated but could not provide useful information on the effectiveness of the flocculant, which is one of the limitations of CST test. Therefore, the test is often accompanied with other filterability and dewaterability test such as SRF.

The BF dosage was expressed in terms of kilograms of BF per ton of dry solids (kg/t DS) which is commonly applied in the industry in predicting the polymer demand (see Appendix B.2 for sample calculation). As shown in Figure 5.1, the BF-treated sludge demonstrated steady CST performance at the dosage between 0.3 to 6.0 kg/t DS. This finding agrees with the typical optimum dosage for various organic polymeric flocculants which are between 0.5–20 mg/g DS (equivalent to kg/t DS)(Wei et al. 2018). Furthermore, polymer dosage mainly for polyelectrolytes are generally not higher than 15 mg/g DS (Kamizela and Kowalczyk 2019). On the other hand, the recommended dosage of polymer for industrial sludge conditioning prior to mechanical dewatering also varies between 3.0–10.0 kg/t DS according to one of the polyacrylamide-based polymer manufacturers (SNF Floerger 2019).

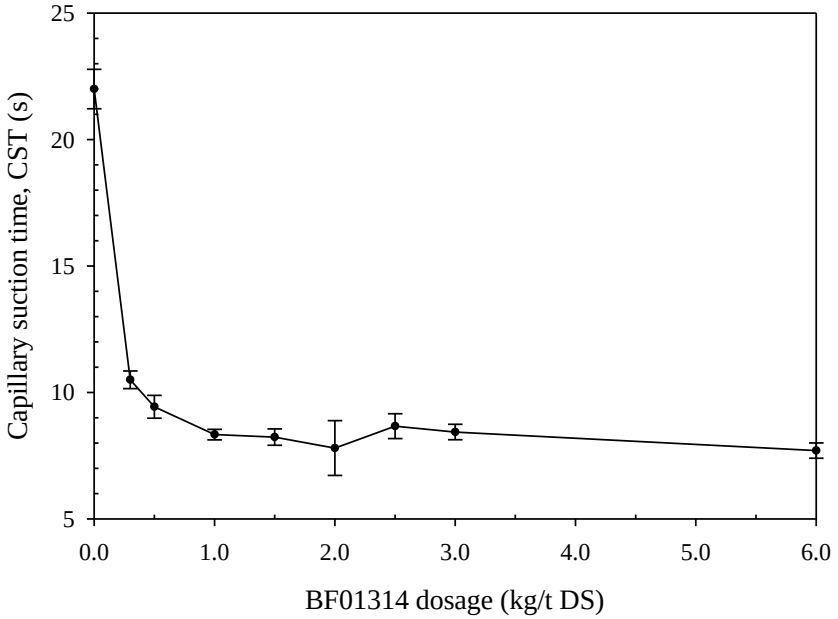

Figure 5.1: Effect of BF01314 dosage on CST of treated sludge. Error bar represents ±SD of triplicates.

Figure 5.2 combines the results of the effect of BF01314 dosage on the SRF and cake solids content (CSC). The SRF value was significantly reduced by one order from 7.42×10^{11} to 9.59×10^{10} m/kg while the CSC increased from 15.22 to 23.23%, at the dosage as low as 0.5 kg/t DS, indicating the efficacy of BF01314 in dewatering this type of activated sludge. Further increase of dosage to 6.0 kg/t DS improved the dewatering performance by reducing the SRF to 1.52×10^{10} m/kg, while increasing the CSC to 25.41%. The enhanced CSC results obtained in this study were in agreement with the typical standard values of CSC between 20–28% after polymer treatment for the sludge generated from a conventional digestion system (Minall, Smyth and Horan 2014; Rebah et al. 2018). However, both SRF and CSC showed insignificant enhancement after 3.0 kg/t DS within the range tested in the present work.

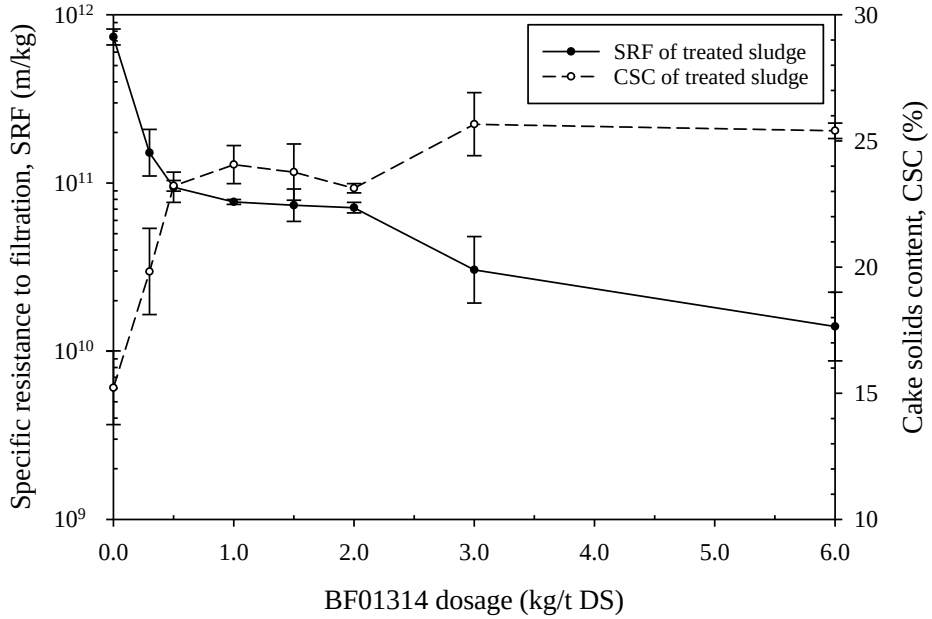

Figure 5.2: Effect of BF01314 dosage on SRF and CSC of treated sludge. Error bar represents ±SD of triplicates.

Figure 5.3 depicts the effect of BF01314 dosage on the zeta potential value of the sludge supernatant, which indicates the net surface charge of the sludge after adding the BF. The zeta potential value increased from –14.2 mV to –4.9 mV with increasing dosage from 0.5 to 2.0 kg/t DS. The value remained at -4.8±0.1 mV when the dosage increased further to 6.0 kg/t DS. The neutralisation effect is believed to take place between the positive charges of the BF molecules and the negatively charged sludge particles, as evident from the reduction of the negative zeta potential values (Ghernaout et al. 2015; Li et al. 2020). Subsequently, the repulsive forces between the particles may reduce, leading to the aggregation of the colloids and suspended solids by the attraction of van der Waals forces (Li et al. 2020).

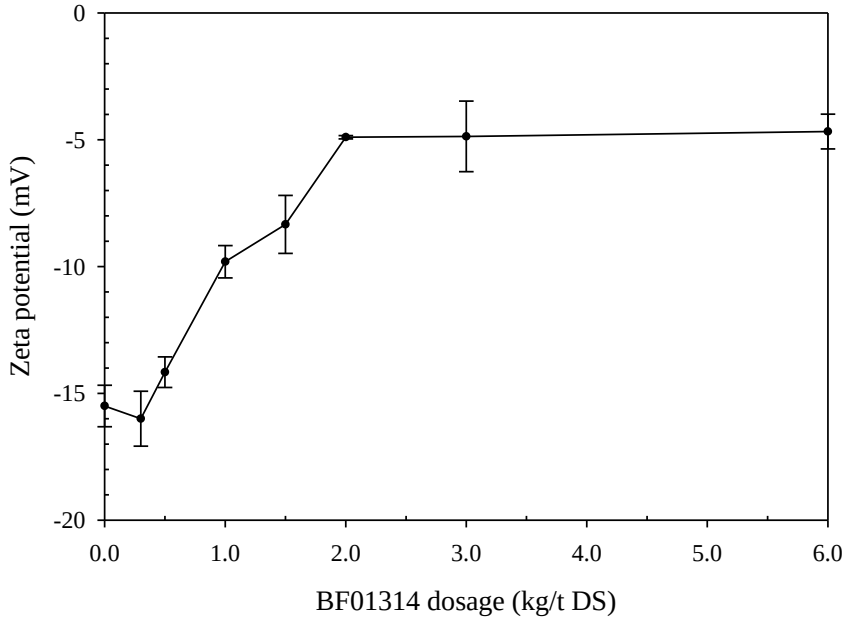

Figure 5.3: Effect of BF01314 dosage on zeta potential of the supernatant of treated sludge. Error bar represents ±SD of triplicates

Although the dewaterability was enhanced from 2.0 to 6.0 kg/t DS in terms of SRF and CSC, the measured zeta potential values were slightly far from zero charge when they reached a plateau region without net charge neutralisation or reversal. This phenomenon could not be explained by a simple charge neutralisation mechanism and as discussed in Section 4.2.1. The zeta potential close to zero commonly reflects the best flocculation effect, whilst the occurrence of charge reversal may cause the particles to be dispersed again (Li et al. 2020). The results obtained in the present work suggest that the flocs formed by BF01314 were successfully destabilised and aggregated, and were less likely to disperse again at higher dosages above 2.0 kg/t DS, within the range tested.

Figure 5.4 and Figure 5.5 compare the images of the top and side views of the sludge before and after it was conditioned with 3.0 and 6.0 kg/t DS. Through visual observation, a fluffier floc was detected when the sludge was conditioned with the BF at the dosage of 6.0 kg/t DS, suggesting the formation of larger fractal floc structures related to sludge floc properties such as size, strength and porosity, which were not examined in the present work and in most other dewaterability studies in literature due to the complexity and uncertainties in the measurement protocols. To date, correlation between floc size and dewaterability is not well understood and related research is still

ongoing, hence limited information is available (Sun et al. 2015; Smoczynski et al. 2019; Zheng et al. 2017; Zhou et al. 2020).

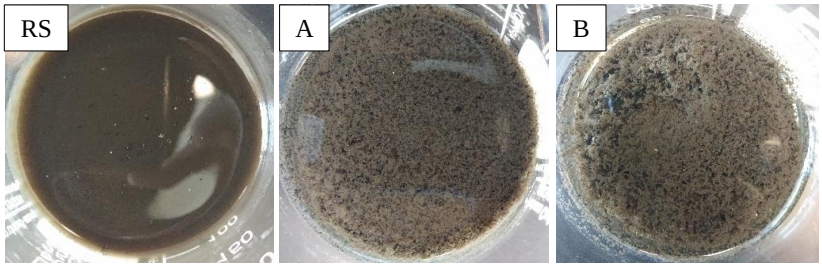

Figure 5.4: Images of sludge before and after treatment with BF01314 (top view). (RS) Raw sludge; (A) 3.0 kg/t DS; (B) 6.0 kg/t DS.

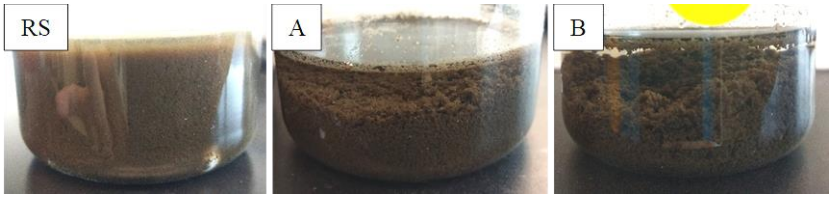

Figure 5.5: Images of sludge before and after treatment with BF01314 (side view). (RS) Raw sludge; (A) 3.0 kg/t DS; (B) 6.0 kg/t DS.

Based on the overall analysis of the dewatering performance in terms of CST, SRF, CSC and zeta potential, the feasibility of BF01314 in dewatering the real sludge sample is confirmed. Within the range of dosage tested, the optimal range of BF01341 dosages was determined to be between 3.0 and 6.0 kg/t DS. Nevertheless, all three dewatering parameters as well as zeta potential revealed less obvious or no improvement of sludge dewaterability when the dosage were doubled from 3.0 to 6.0 kg/t DS. Hence, 3.0 kg/t DS was selected as the optimum dosage in the present study. Comparable findings were reported on the optimum dosage of chitosan (2–3 kg/t DS) and MBF10 from *Klebsiella* sp. (6 kg/t DS) for sludge dewatering application (Zemmouri et al. 2015; Yang et al. 2012). The present experimental study ended with 6.0 kg/t DS of BF dosage without any observation of overdose effect.

5.3 Effect of sludge pH

As shown earlier in Table 5.1, the pH of the raw sludge was close to neutral, at pH 7.0±0.3 with a CST value of 22.0 s. Figure 5.6 reveals an increasing trend of CST values of the raw sludge from 12.8 to 35.9 s when pH increased from 2 to 10. As

compared to the untreated raw sludge, the BF-treated sludge using 1.0 kg/t DS of BF dosage was able to maintain its good dewatering performance with CST values recorded between 8.3 and 10.5 s as the pH increased from 2 to 7, similar to the CST value of distilled water (8–11 s) (Sanin et al. 2011; Peng, Ye and Li 2011). At pH 8, the CST value of the treated sludge increased slightly to 12.0 s and then increased sharply to 30.2 s at pH 10. The optimum pH condition for BF-treated sludge was determined to be between pH 6–7 based on the lowest CST values of 8.3–9.1 s.

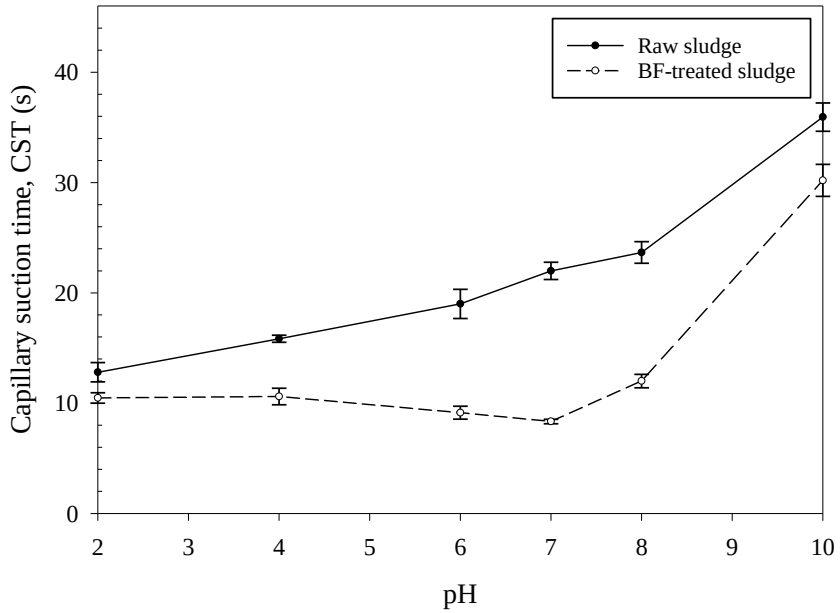

Figure 5.6: Effect of sludge pH on CST of raw and treated sludge. Error bar represents ±SD of triplicates.

Figure 5.7 shows a similar trend of SRF results as compared with CST results for both untreated and treated sludge samples. The SRF values of the raw sludge increased from 2.42×10^{11} to 7.66×10^{11} m/kg when the pH was increased from pH 2 to 8, followed by a greater extent of increase by one order (2.32×10^{12} m/kg) at pH 10. On the contrary, the SRF of BF-treated sludge remained constant between 1.31×10^{11} and 9.41×10^{10} m/kg between pH 2 to 8, indicating good dewaterability of the sludge at acidic, neutral, and weak alkaline conditions. Nevertheless, at a strong alkaline condition of pH 10, the dewatering performance of the BF-treated sludge deteriorated significantly indicated by the increase of SRF by one order (2.10×10^{12} m/kg), which was almost identical to the one without treatment. Figure 5.8 shows that clear supernatant was observed for the sludge treated at all range of pHs tested except pH 10, in agreement with the results of SRF and CST.

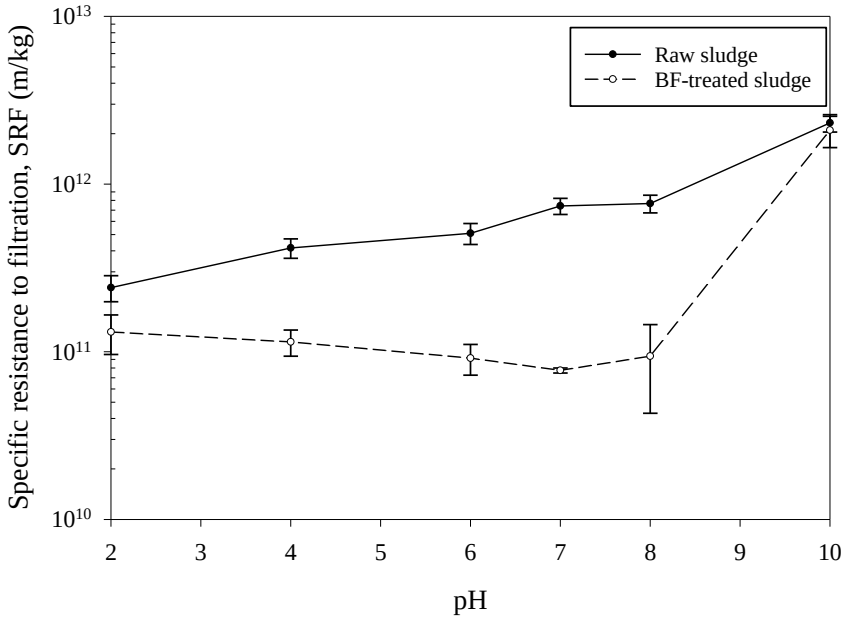

Figure 5.7: Effect of sludge pH on SRF of raw and treated sludge. Error bar represents ±SD of triplicates.

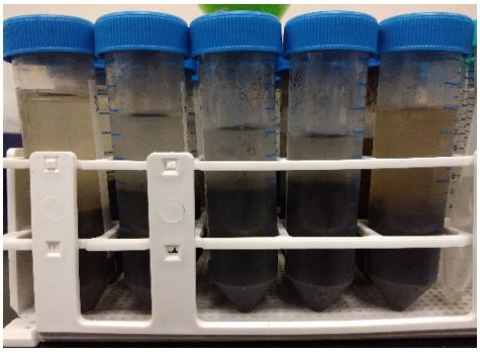

Figure 5.8: Turbidity appearance of sludge supernatant treated by 1.0 kg/t DS of BF01314 at different pH (from left: pH 2, 4, 6, 8 and 10).

Figure 5.9 reveals the CSC results of untreated and treated sludge obtained in the pH range of 2 to 10. The CSC of the raw sludge showed approximately the same values between 15.22 and 19.23% at all range of pHs tested. Meanwhile, the CSC values of the BF-treated sludge were significantly higher than those of the untreated sludge, recording 19.26 to 24.07% from pH 2 to 8 with the maximum CSC of 24.07% at pH 7. The CSC decreased to 17.12% when the pH increased to 10 as the dewatering performance worsened as indicated by the abovementioned SRF and CST results. Based on the overall SRF, CST and CSC results, it is deduced that BF01314 demonstrated a wide pH stability between 2 to 8 in sludge condition and dewatering with maximum performance at neutral pH 7.

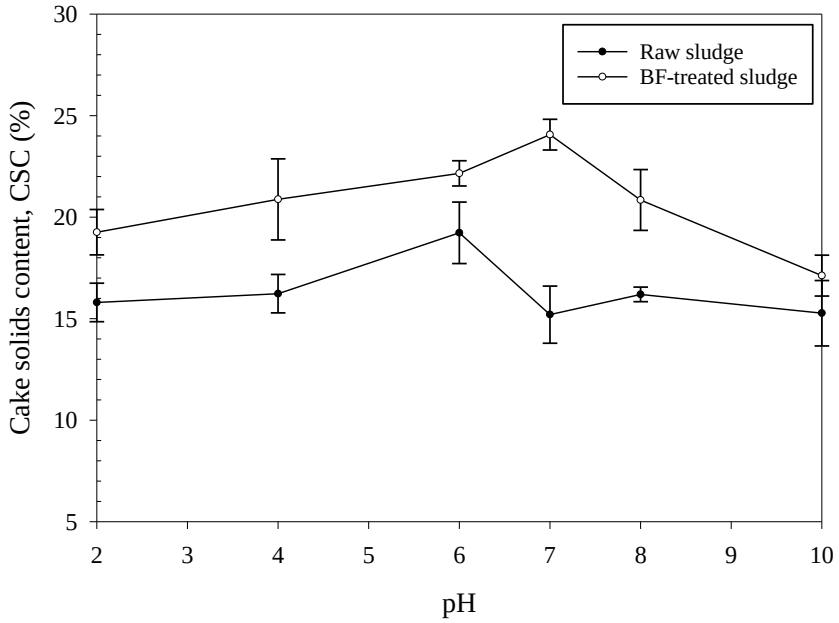

Figure 5.9: Effect of sludge pH on CSC of treated sludge. Error bar represents ±SD of triplicates.

Figure 5.10 demonstrates the results of zeta potential measured for the sludge treated by BF01314 at pH between 2 to 10 as compared to the untreated sludge at similar pH conditions. The zeta potential values decreased almost linearly as the pH increased from 2 to 10. Excess positively charged ions (H^+) in strong acid solution may lead to a stronger charge neutralisation effect and reduce the negative zeta potential values to almost zero charges (-2.3±0.7 mV) at pH 2 for both untreated and treated sludge samples (Cao et al. 2010). Under strong alkaline pH, higher concentration of OH^- ions interfered with the complex formation of the BF and sludge particles which resulted in poor flocculation and dewaterability (Okaiyeto et al. 2016). Consequently, the negatively charged sludge particles remained suspended, which increased the negative charges in the sludge suspension as shown in Figure 5.10.

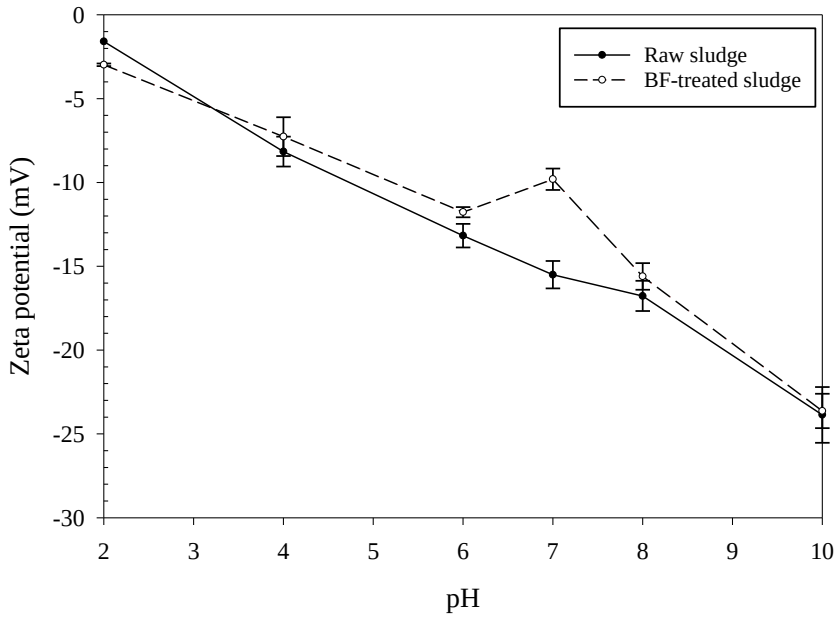

Figure 5.10: Effect of sludge pH on zeta potential of the supernatant of treated sludge. Error bar represents ±SD of triplicates.

Nevertheless, the sludge dewaterability as indicated by CST, SRF and CSC was less affected at pH 8 compared to pH 10, which was most likely attributed to the cationic behaviour of BF01314 below its IEP (pH 8.25) like natural chitosan (Picos-Corrales et al. 2020) (see Chapter 4, Section 4.1.3). The sludge with characteristic pH of 8.3 has also been reported to be dewatered effectively by the natural chitosan (Zemmouri et al. 2015). However, the close range of zeta potential values between the untreated and BF-treated sludge despite the reasonably good dewaterability results suggested that simple charge neutralisation may not be the primary flocculation mechanism in sludge treatment with BF01314 at the dosage of 1.0 kg/t DS.

5.4 Effect of temperature

Figure 5.11 demonstrates the effect of temperature on the CST values of the sludge treated by BF01314 as compared to the unconditioned sludge. The CST of the raw sludge was almost constant (21±1 s) at temperatures between 10 to 40°C but gradually decreased to 14.0 s as the temperature elevated from 60 to 100°C. On the contrary, the CST values of BF-treated sludge remained consistently in good dewaterability range of 6.7–10.6 s in a wide temperature range, from 10 to 100°C. At 10°C, the CST value increased slightly, which may be attributed to the drop of desorptivity (indicating

increase of water retention ability) as the sludge temperature decreased (Sawalha and Scholz 2012). As the temperature increased above 40°C, thermal energy caused the sludge network strength to be destructed, which was further enhanced by the release of some bound water from sludge floc during mixing (Abu-Orf and Örmeci 2005; Yen et al. 2002; Yeneneh et al. 2016). This may improve the dewaterability (CST reduced) with enhanced water removal from the sludge at high temperature.

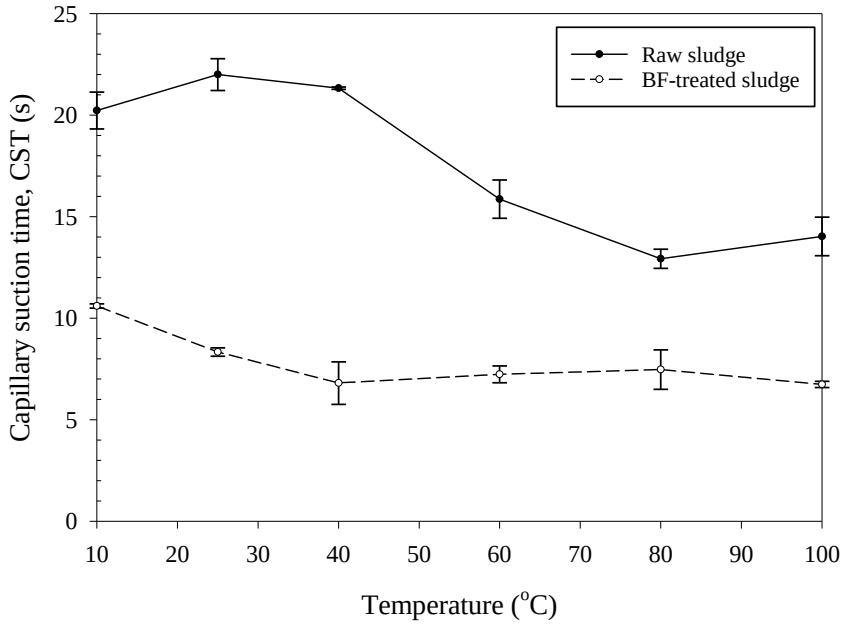

Figure 5.11: Effect of temperature on CST of raw and treated sludge. Error bar represents ±SD of triplicates.

On the other hand, the effect of temperature was less significant in terms of SRF. Figure 5.12 presents the SRF results of the raw and BF-treated sludge under the effect of temperature. For both cases, the SRF values remained almost constant over a wide temperature range of 10 to 100°C at 1.37±0.70 $\times 10^{12}$ m/kg (raw sludge) and 1.15±0.80 $\times 10^{11}$ m/kg (BF-treated sludge). Nevertheless, the slight increase in SRF value at 100°C may indicate the onset of sludge disintegration with the release of the negatively charged EPS at high temperature resulting in deteriorated sludge filterability (Wang et al. 2017). Similar observations were made for the CSC results of BF-treated sludge at temperatures between 10°C to 80°C (22.3±1.8%) as shown in Figure 5.13. The CSC value suddenly reduced to 17.8% at 100°C but all values remained higher than the CSC of unconditioned sludge over the temperature range tested, which indicated the significance of utilising BF01314 in enhancing the sludge dewaterability with notable thermal stability.

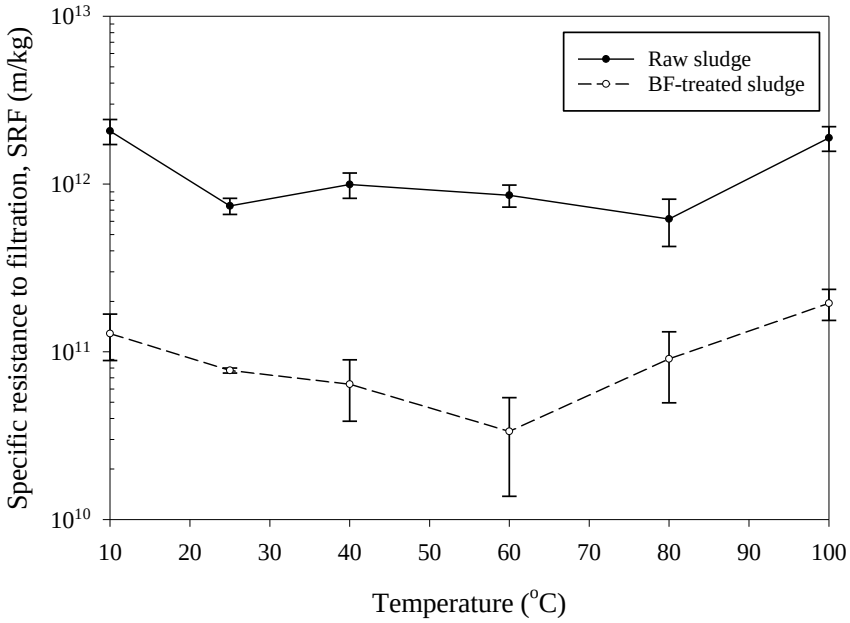

Figure 5.12: Effect of temperature on SRF of raw and treated sludge. Error bar represents ±SD of triplicates.

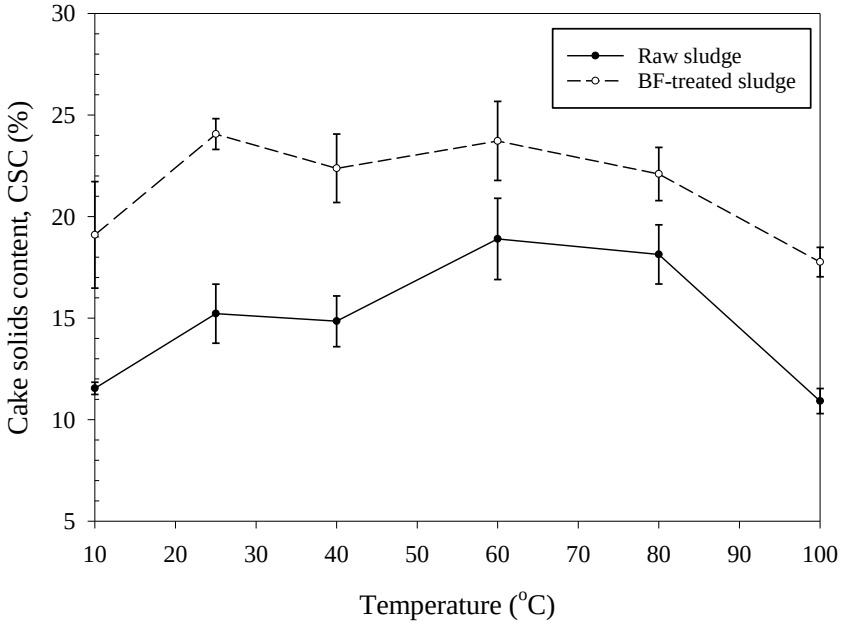

Figure 5.13: Effect of temperature on CSC of raw and treated sludge. Error bar represents ±SD of triplicates.

A temperature increase was reported to enhance the fluidity and dewaterability of sludge due to the release of extracellular polymeric substance (EPS) that helps in floc formation by bridging (Cao et al. 2020; Li and Yang 2007). However, large amounts of EPS were unfavourable as they reduced the CSC possibly due to water entrapment in the floc structure surrounded by the EPS, which may explain the decrease of CSC

at 100°C in Figure 5.13. Excess EPS could deteriorate the dewatering performance depending on the type of EPS released (Ye et al. 2014; Li and Yang 2007). Overall, the results suggested that BF01314 has high thermal stability owing to its polysaccharide backbone supported by earlier chemical analysis and thermal degradation results (Chapter 4, Section 4.1.1 and 4.1.4).

Figure 5.14 depicts a plot of zeta potential results against the change of temperature between 10 to 100°C. For both raw sludge and BF-treated sludge, the zeta potential results decreased (increasing negative values) with temperature rising from 25 to 60°C, but increased (decreasing negative values) from 60 to 100°C. However, the surface charges of the raw sludge appeared to be less affected by the temperature, with zeta potential results between -13.7 and -18.4mV. A similar trend of results was found in the study of the dissolution characteristics of sludge by Penghe et al. (2020), where the dissolution amount of soluble carbohydrates and soluble proteins in the sludge increased when heated from 50 to 70°C, but decreased slowly when further heated up to 90°C. These organic components are typically identified as major components in sludge EPS that carried negative surface charges (Ramesh, Lee and Hong 2006; Lin et al. 2020), which may explain the results obtained in Figure 5.14. Nevertheless, a sudden decrease in zeta potential value of the BF-treated sludge from 25°C to 10°C was unexplainable. Supplementary analysis is essential to understand the effect of cold conditions (10°C and below) on the BF-treated sludge behaviour. Overall, BF01314 showed promising results in enhancing sludge dewaterability over a broad temperature range from 25 to 80°C.

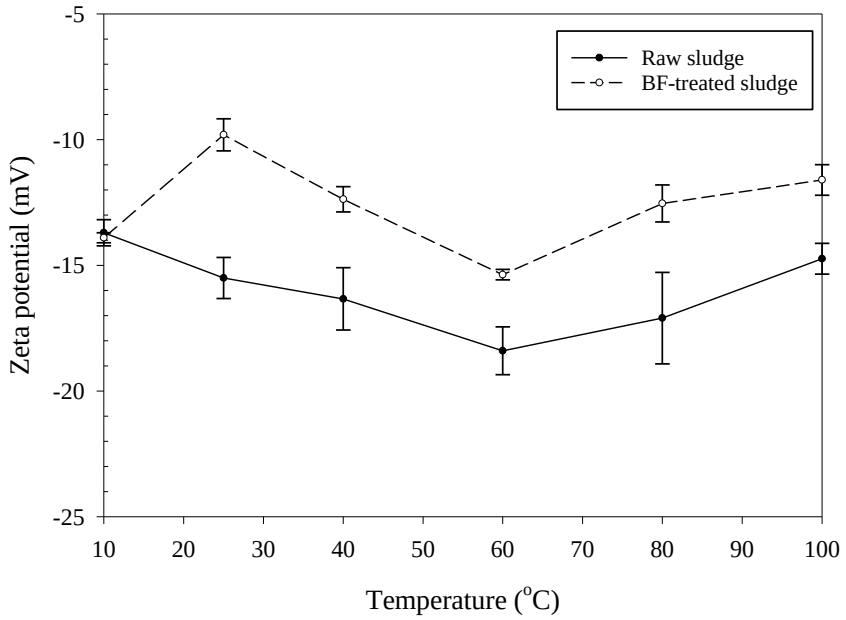

Figure 5.14: Effect of temperature on zeta potential of the supernatant of treated sludge. Error bar represents ±SD of triplicates.

5.5 Comparison of dewatering performance with other flocculants

The dewatering performance of BF01314 was compared to chitosan and industrial cationic polymer MF 7861 with their characteristics summarised in Table 5.2.

Table 5.2: Characteristic of flocculants used in this study

Characteristic	BF01314	Chitosan	MF 7861
pH	4.02	3.57	3.17
Zeta potential (mV) at 25°C	+33.0	+74.8	+68.7
Concentration (g/L)	4.78	4.78	4.78

Figure 5.15 compares the results obtained from the CST test of all three cationic flocculants at dosages ranging from 0.5–3.0 kg/t DS. BF01314 and chitosan demonstrated essentially similar dewatering performance over the dosage range tested with the lowest CST values (maximum dewaterability) of 7.8 and 7.6 s recorded at the same dosage of 2.0 kg/t DS, respectively. The synthetic flocculant, MF 7861 however showed the lowest CST value (7.3 s) at even lower dosage (1.0 kg/t DS), but the dewatering performance deteriorated as the dosage gradually increased, indicating the presence of significant overdose effect.

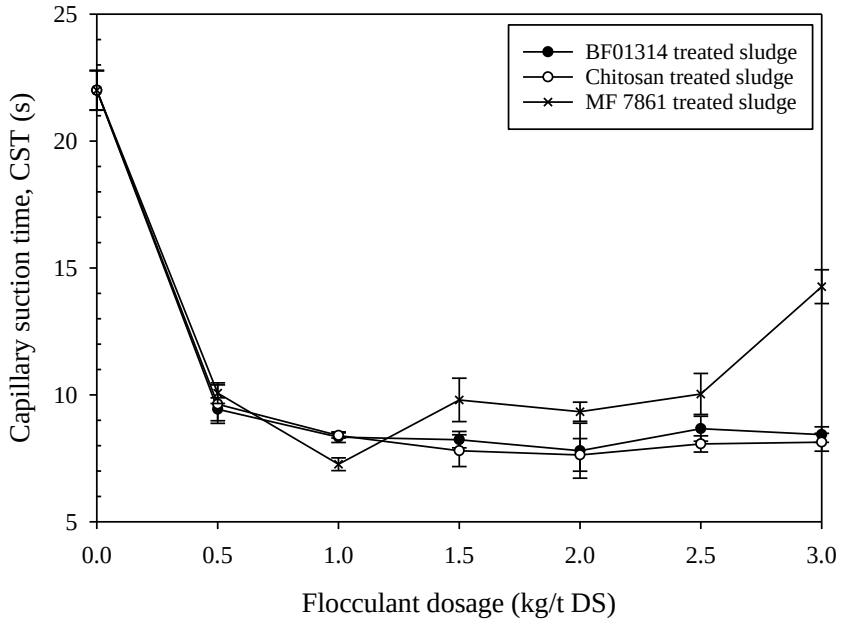

Figure 5.15: Sludge dewatering performance of BF01314 compared to chitosan and MF 7861 in terms of CST. Error bar represents ±SD of triplicates.

As shown in Figure 5.16, the SRF results for all the three flocculants were less distinctly different, considering the overlapping SD error bars. All the three flocculants reduced the SRF by one order upon addition to the sludge suspension over the dosage range studied. A distinct difference was only noted at the dosage of 2.0 kg/t DS where both chitosan and MF 7861 showed greater extent of reduction in SRF as compared with the BF.

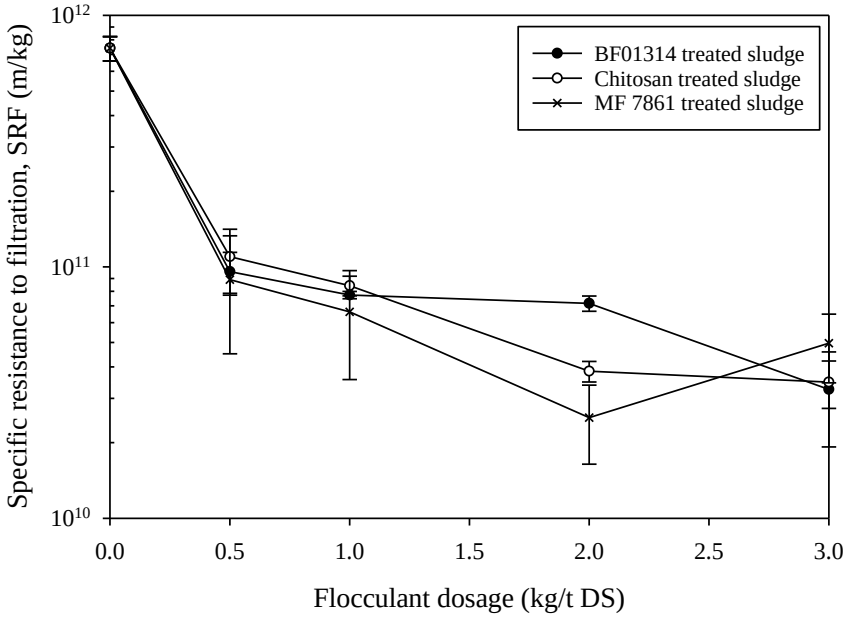

Figure 5.16: Sludge dewatering performance of BF01314 compared to chitosan and MF 7861 in terms of SRF. Error bar represents ±SD of triplicates.

Figure 5.17 and Figure 5.18 present the results of zeta potential of sludge supernatant and CSC for the sludge conditioned with BF01314 in comparison to chitosan and MF 7861. Again, the CSC values showed insignificant variation between 22–25% for all the three flocculants over the specified range of dosage although the change in zeta potential of the supernatant was noticeable at certain regions. As shown in Figure 5.18, the zeta potential of the sludge treated by MF 7861 was reversed at its optimum dosage of 1.0 kg/t DS (+2.7 mV) and remained in positive charge region with increasing dosage. The sludge treated by chitosan and BF01314 also reduced the negative charges but did not surpass the zero charge at their respective optimum dosage.

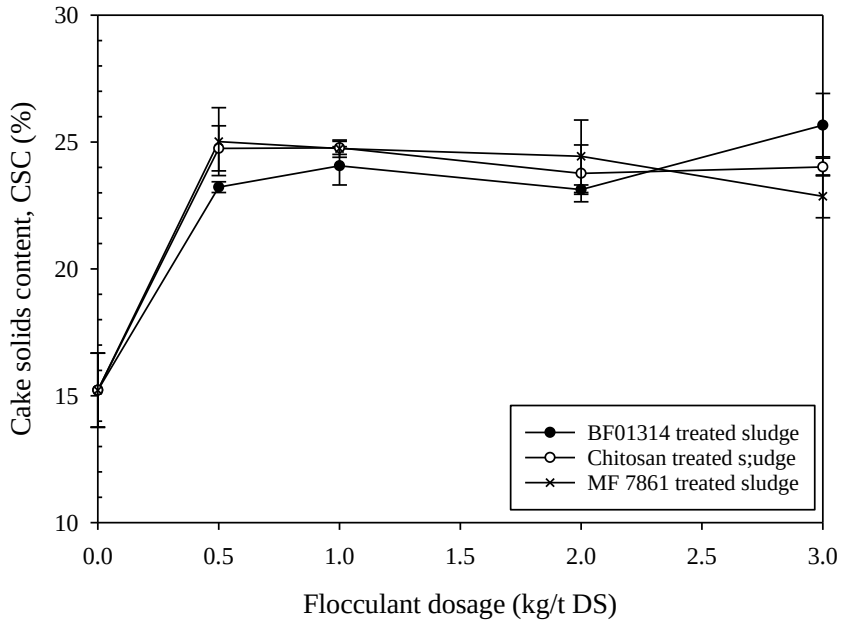

Figure 5.17: Sludge dewatering performance of BF01314 compared to chitosan and MF 7861 in terms of CSC. Error bar represents ±SD of triplicates.

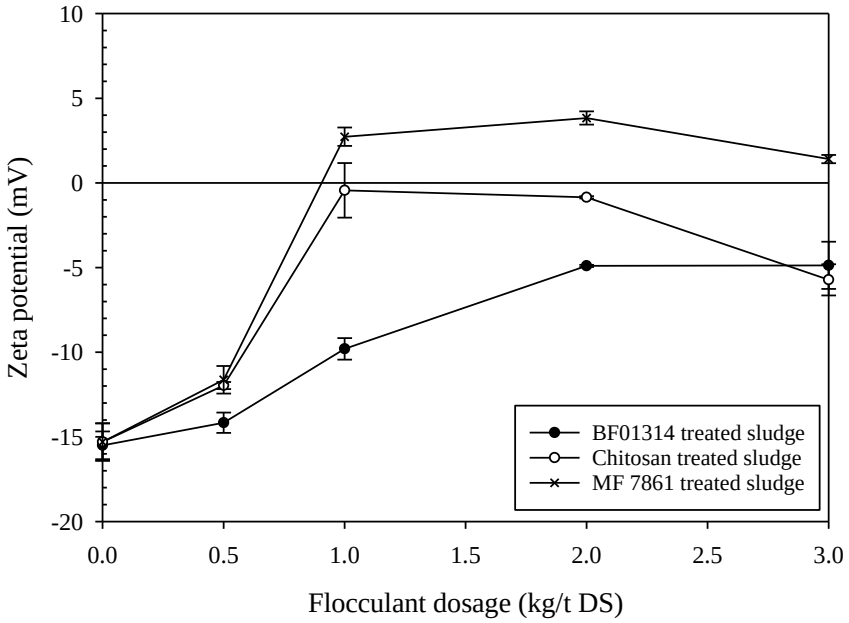

Figure 5.18: Zeta potential results of BF01314 compared to chitosan and MF 7861. Error bar represents ±SD of triplicates.

Within the specified range of dosage, the overdose effect was observed only for the synthetic polymer MF 7861 while both biopolymers (chitosan and BF01314) enhanced the dewatering performance without any significant overdose effect. For chitosan, it was reported that the optimum dosage was between 2.0–3.0 kg/t DS (Zemmouri et al. 2015), which was similar to the results obtained in this study. BF01314 may require a higher dosage to reduce the negative charges of the sludge particles due to its lower polysaccharide content (Section 4.1.1) that holds the functional groups responsible for effective flocculation (Salehizadeh et al. 2018). Lower surface charge of BF01314 which was approximately half of the one of chitosan may also contribute to a higher demand of polymer dosage (Table 5.2). The presence of sufficient positive charges in flocculants is very important to neutralise the negatively charged colloids in the sludge to further bridge the aggregated particles (Lichtfouse et al. 2019).

Additional separate tests were conducted for chitosan and BF01314 to observe any overdose effects by doubling the maximum dosage tested from 3.0 to 6.0 kg/t DS. For the chitosan-treated sludge, it was found that the zeta potential value barely passed the zero charge (+0.04 mV) while the increase in SRF and decrease in CSC indicated a decline in dewatering performance when the dosage doubled as shown in Table 5.3. However, for the BF-treated sludge, the SRF reduced to 1.52×10^{10} m/kg as the dosage doubled whereas the CST and CSC results were approximately the same. Interestingly,

the zeta potential value remained constant at about -4 to -5 mV between 3.0 and 6.0 kg/t DS, indicating that the mechanism governing the sludge flocculation by BF01314 was not mainly contributed by simple charge neutralisation, but most likely to be electrostatic charge patching (Yang et al. 2016), as discussed in Section 4.2.1 for kaolin flocculation.

Table 5.3: Comparison of dewatering enhancement at dosage 3.0 and 6.0 kg/t DS

Type of flocculant	Dosage (kg/t DS)	CST (s)	SRF (m/kg)	CSC (%)	Zeta potential (mV)
BF01314	3.0	8.1	3.26×10^{10}	25.67	-4.86
	6.0	7.7	1.52×10^{10}	25.41	-4.67
Chitosan	3.0	8.4	3.48×10^{10}	24.02	-5.72
	6.0	8.2	6.09×10^{10}	22.69	+0.04

5.6 Analysis of flocculation kinetics

5.6.1 Effects of flocculation speed and time on flocculation activity

In the present study, sludge conditioning experiments were carried out as a two-step processes simulated by a rapid mixing stage at 400 rpm (coagulation) followed by a slow mixing stage at 100 rpm (flocculation). Zhou, Liu, and Jia (2016) describes these two stages as a mixing stage (coagulation) and a reaction stage (flocculation) in which the former stage produces a very high speed of particle collision due to strong stirring, and then form flocs in the latter stage. Uncontrolled mixing conditions may affect the flocculation efficiency and floc growth due to insufficient or extremely high kinetic energy during the mixing (Marques and Ferreira Filho 2017). Floc formation is commonly initiated during the flocculation stage, aggregated into larger flocs and then removed by sedimentation. Therefore, the present study investigated the effects of flocculation speed and time on flocculation activity to determine the range of ideal mixing conditions for the flocculation stage. Figure 5.19 depicts the flocculation activity of BF01314 obtained using three different flocculation speeds (50, 100 and 200 rpm), each varied with mixing period from 1 to 7 minutes.

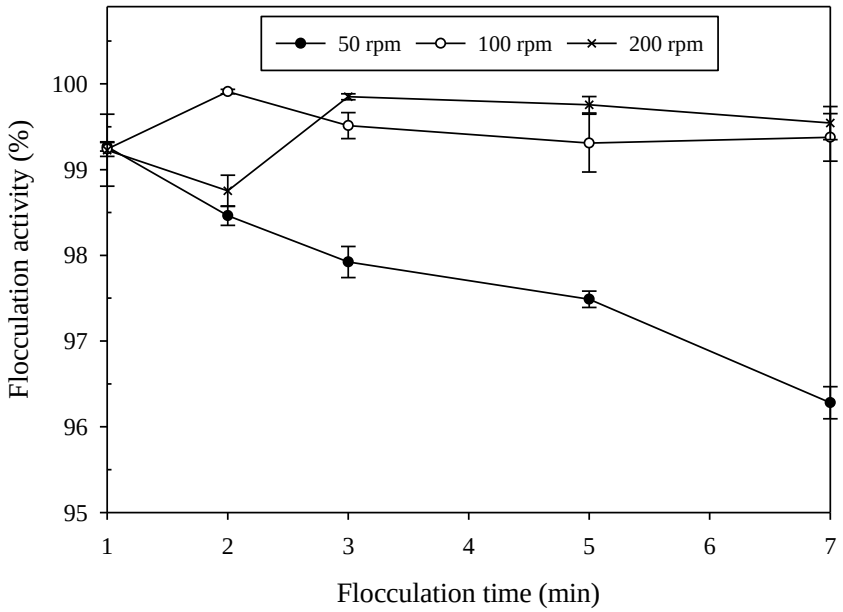

Figure 5.19: Effect of flocculation speed and time on flocculation activity of BF01314. Error bar represents ±SD of triplicates.

After 1-minute flocculation time, the flocculation activity was high (>99%) at all range of speeds tested. However, at low flocculation speed of 50 rpm, the activity continued to decline gradually from 99.27% to 96.28% with increasing flocculation time from 1 minute to 7 minutes. This could be due to the lack of particle collisions to induce the floc growth (Ayoub et al. 2014) and less adsorption efficiency (Zhou et al. 2016) during slow stirring, hence the flocculation activity declined as reflected in Figure 5.19. During the transition of rapid mixing speed (coagulation) to slow mixing speed (flocculation), the kinetic energy was slowing down gradually and started to induce the formation of flocs which may explain high activity within the first minute of flocculation time (Ayoub et al. 2014). However, the flocs could not continue to grow due to insufficient kinetic energy as a result of less collisions at low mixing speed, which then decreased the flocculation activity. At a moderate speed of 100 rpm, BF01314 was able to maintain high flocculation activities of >99.24% at all range of flocculation times tested, with the maximum activity of 99.91% at 2 minutes. A slightly higher percentage of activity was obtained using the high flocculation speed of 200 rpm, but required longer time before it became stable at above 3 minutes (>99.54%).

According to Zhou et al. (2016), flocculation kinetics mechanism can be understood as two processes comprising the (1) collision frequency (motion and approach of floc

particles) and (2) its efficiency (collision and bonding of the particles). As seen from Figure 5.19, the highest collision efficiency is believed to occur at the high mixing speed of 200 rpm, indicated by the highest plateau region of flocculation activity as compared to those obtained at lower speeds. Extremely high collision frequency is unfavourable as it can cause the breakage of readily formed flocs and create permanent residual particles that remain in the suspension (Marques and Ferreira Filho 2017), depending on the mixing duration (Ayoub et al. 2014). While the exact high collision frequency value which caused the floc breakage was not reported in literature, a mathematical model derived by Marques and Ferreira Filho (2017) could be useful to examine the presence of irreversible breakup process through the increased residual turbidity as the average velocity increased. On the other hand, floc breakage may also create primary particles which are able to re-flocculate, thus stronger flocs can be formed (Marques and Ferreira Filho 2017). Hence, a decrease in flocculation activity at 2 minutes for the high mixing speed of 200 rpm may indicate the breakage of some flocs, creating the primary particles which were able to re-flocculate upon prolonged mixing (Marques and Ferreira Filho 2017). Another possible reason could be due to the rapid dispersion and collisions of particles induced by a higher mixing speed. The resultant kinetic energy was still high during the transition from coagulation speed to flocculation speed, thus a relatively longer time was required for the particles to start forming the flocs (Ayoub et al. 2014).

Overall, the results suggest that the preferred range of flocculation speeds were determined to be between 100 to 200 rpm, with flocculation time ranging from 2 to 3 minutes. Similar results were reported for IH-7 where a maximum flocculation activity was recorded between 1 to 3 minutes, for mixing speeds of 150–200 rpm (Aljuboori et al. 2015). Extending the mixing duration more than 7 minutes or applying high speed above 200 rpm may lead to irreversible flocs breakage, and hence it was not investigated in this present work.

5.6.2 Fitting of zero-, pseudo first- and pseudo second-order reactions

The results obtained from the previous section (Section 5.6.1) prove that high collision frequency indicated by high mixing speed may result in high flocculation efficiency as described by Zhou et al. (2016). The second part of the analysis on flocculation

kinetics aims to determine the rate of reaction after the first mixing process (i.e., after the coagulation stage) where the floc formation was slowly initiated. Figure 5.20 reveals the results of turbidity reduction of the sludge before and after it was treated with BF01314 at different dosages over the sedimentation time. The turbidity reduction was obvious within the first ten minutes using BF dosage of 3% and 5%, decreasing from the initial turbidity N_0 of 398 NTU to <150 NU, but the reduction rate was slower at the high dosage of 10%. Hence, the results obtained between 1–10 minutes were used for the fitting of zero-, pseudo first- and pseudo second- order kinetic models in order to determine the rate of reaction of BF01314 in sludge flocculation.

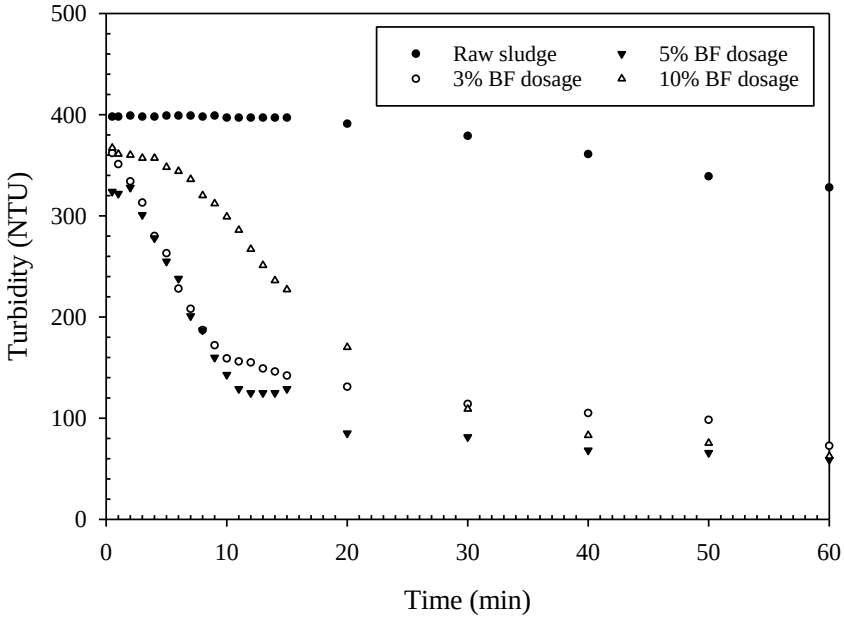

Figure 5.20: Turbidity reduction at different BF dosages over sedimentation time.

Figure 5.21 displays three turbidity reduction plots for the three BF dosages of 3%, 5% and 10%, within the first 1–10 minutes extracted from Figure 5.20 in order to evaluate the model fitting of the zero-order reaction. At the BF dosages of 3% and 5%, high turbidity removal of about 200 NTU was recorded. As the dosage doubled to 10%, the amount of turbidity removed was reduced significantly to <100 NTU, indicating a potential overdose effect which was not observed in batch conditioning experiments in Section 5.2. An optimum dosage for organic polymeric flocculants is typically between 0.5–20 kg/t DS (Wei et al. 2018) and less than 15 kg/t DS (Kamizela and Kowalczyk 2019). This may explain the overdose effect observed using 10% BF

dosage in this study, which is equivalent to 16.41 kg/t DS (see Appendix B.3 for sample calculation).

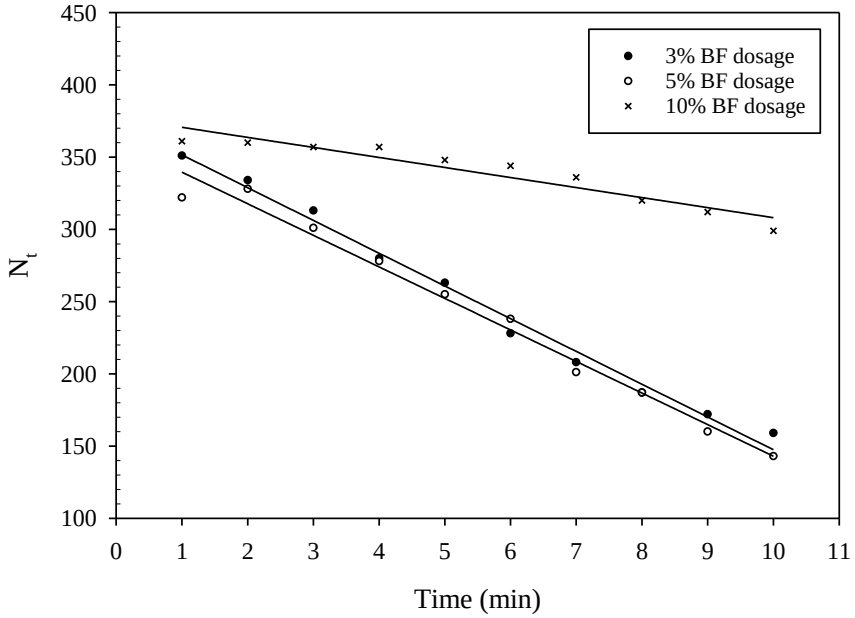

Figure 5.21: Zero-order kinetic model fitting of BF01314-induced sludge flocculation. (N_t represents the turbidity measured at time t. Solid lines symbolise the model data.)

Figure 5.22 and Figure 5.23 illustrates the fitting of the experimental results to pseudo first- and pseudo second-order kinetic reactions, respectively. All parameters obtained from the fittings of the three models are summarised in Table 5.4.

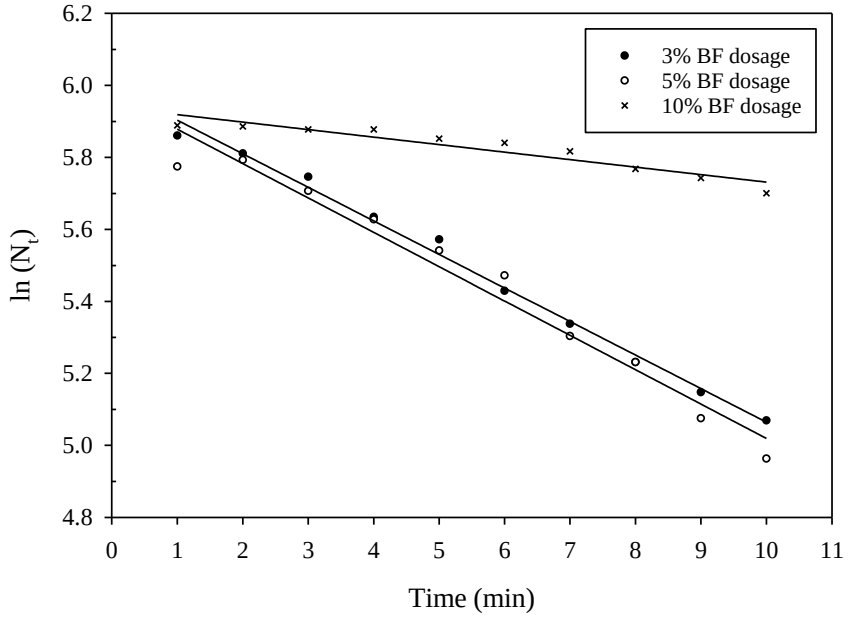

Figure 5.22: Pseudo first-order kinetic model fitting of BF01314-induced sludge flocculation.

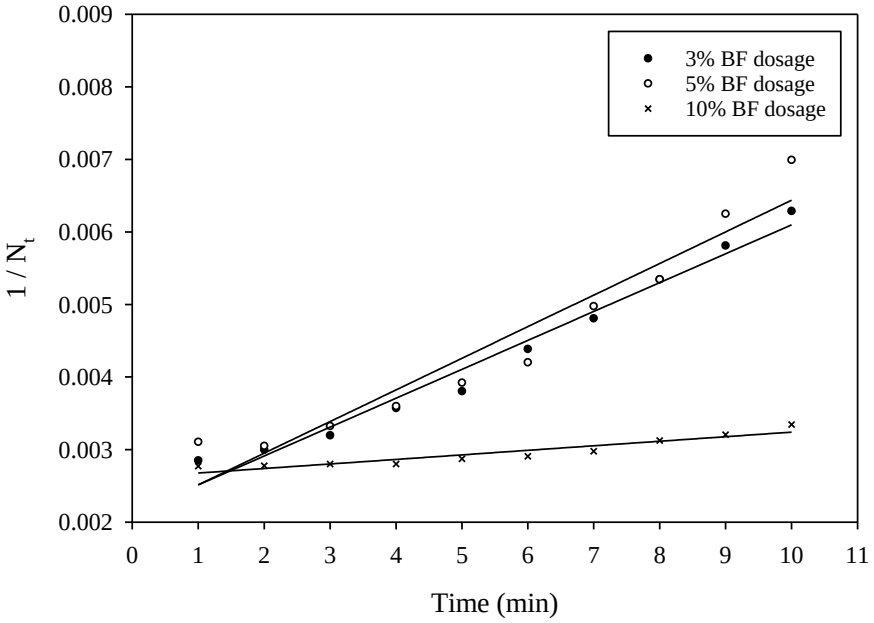

Figure 5.23: Pseudo second-order kinetic model fitting of BF01314-induced sludge flocculation.

Table 5.4: Parameters obtained from the model fitting of zero-, pseudo first- and pseudo second-order reactions

BF dosage	Zero-order		Pseudo-first order		Pseudo-second order	
	R^2	k (NTU.min^{-1})	R^2	k_1 (min^{-1})	R^2	k_2 (NTU^{-1}.min^{-1})
3%	0.990	22.685	0.993	0.093	0.976	3.985×10^{-4}
5%	0.985	21.848	0.968	0.095	0.928	4.360×10^{-4}
10%	0.909	6.945	0.898	0.021	0.886	6.266×10^{-5}

Note: k, k_1 and k_2 represents rate constants (rate of reactions) of zero, pseudo first- and pseudo second-order reactions obtained from the graphs, respectively.

The coefficient of determination (R^2) values of all kinetic models were close to each other. Zero-order plot has R^2 values ranging from 0.985 to 0.990, while pseudo first- and pseudo second-order give R^2 values between 0.898–0.993 and 0.886–0.976, respectively. Figure 5.24 compares the residual plots for zero- and pseudo first-order, whereby the data randomness suggests that the reaction of BF01314 was slightly better when fitted to pseudo first-order than zero-order at 3% BF dosage. With increasing BF dosage to 5% and 10%, the residual plots were not randomly distributed and formed a pattern of a curve (quadratic) which reduces the rate of reactions indicated by the rate constant values in Table 5.4. This 'curve' pattern was more obvious in the pseudo second-order residual plots as the dosage increases. At dosage of 10%, the residuals were all grouped in the negative region, indicating the poor randomness and unfitness of the data with this pseudo second-order kinetic model (Figure 5.25). The rate constant values (k, k_1 and k_2) for zero-order were the highest, ranging from 6.945–22.685 NTU.min^{-1}, followed by pseudo-first order (0.021–0.095 min^{-1}) and lastly pseudo second-order (6.266×10^{-5}–3.985×10^{-4} NTU.min^{-1}). P-values for all rate constants were highly significant <0.001. However, pseudo first-order reaction has the smallest average percentage errors of k_1 values for all dosages (0.32–0.79%) compared to k (6.08–16.51%) and k_2 (3.39–11.99%).

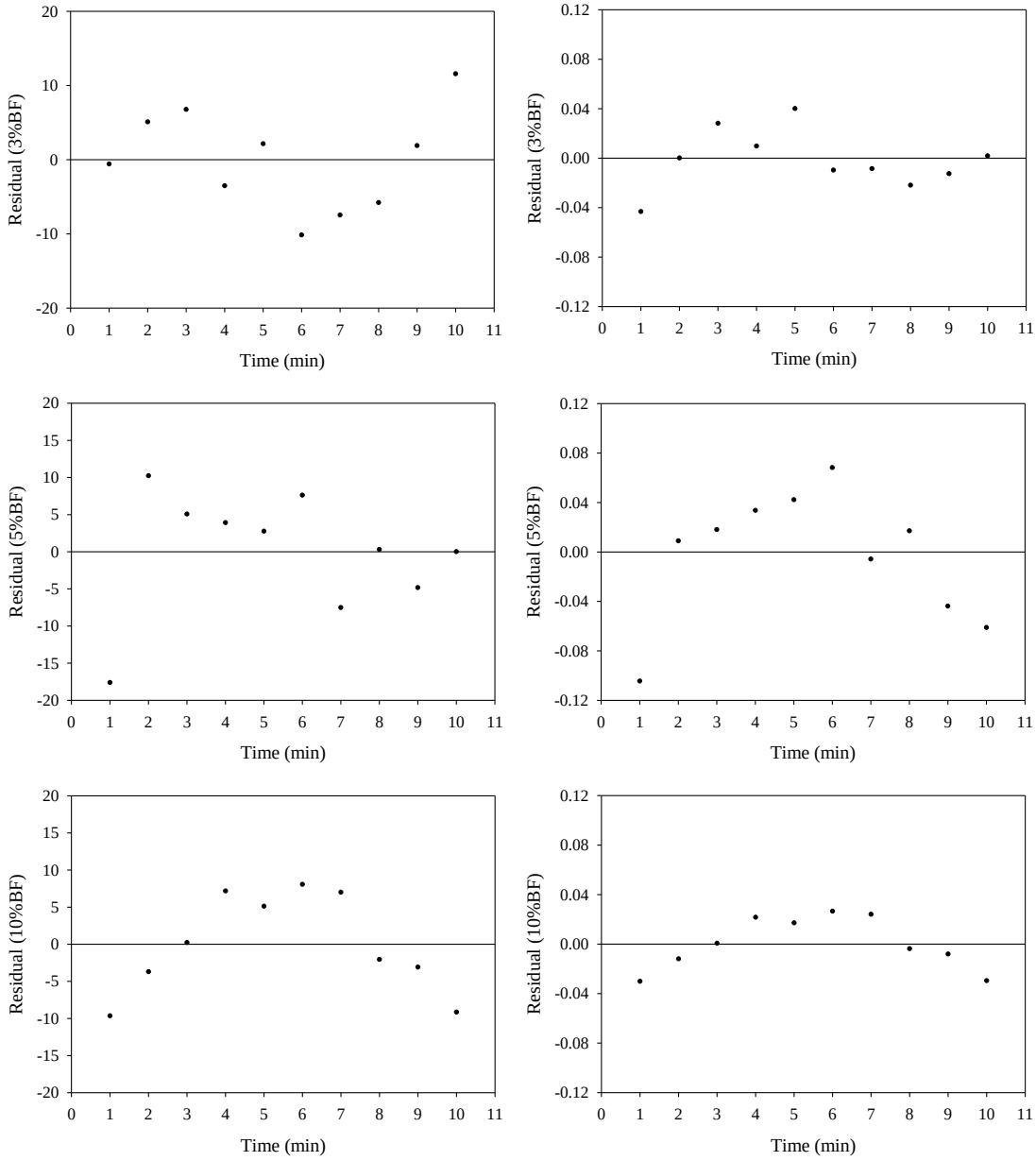

Figure 5.24: Residual plots of zero-order (left) and pseudo first-order (right) reactions at BF dosages of 3%, 5% and 10%.

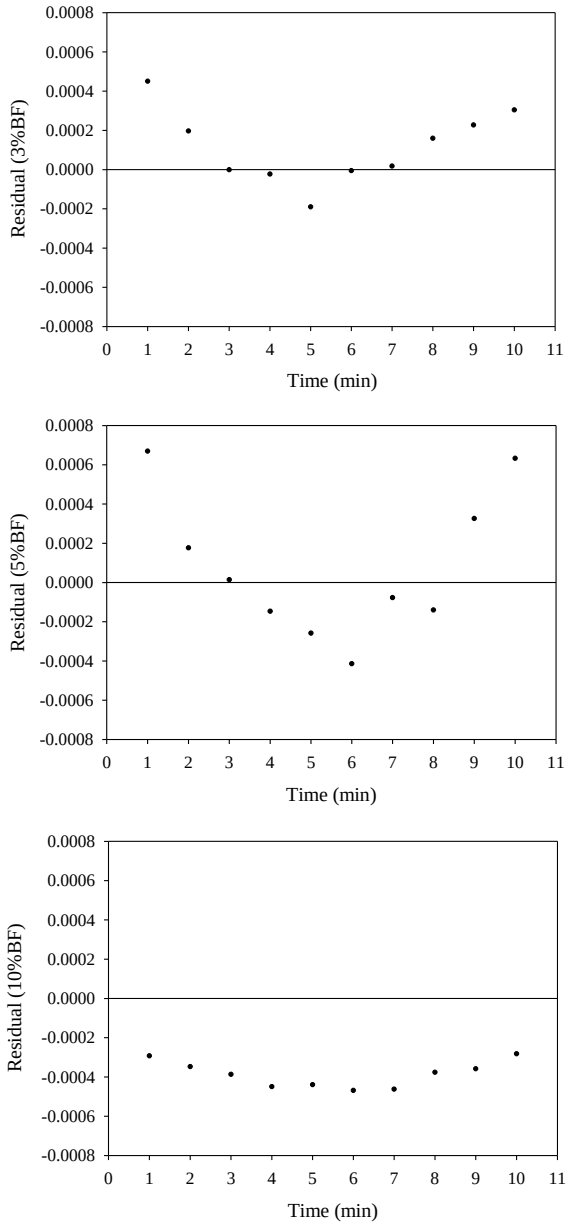

Figure 5.25: Residual plots for pseudo second-order reaction at BF dosages of 3%, 5% and 10%.

Based on the overall results, the reaction of BF01314 was best fitted to pseudo first-order kinetic model. The randomness of data and rate constant value may provide useful information in determining the range of optimum dosage of a polymer (Al-Sameraiy 2017). Within the range tested in the present work, 3% BF dosage (equivalent to 6.54 kg/t DS) was at the optimum with a rate constant of 0.093 min^{-1}, which was in agreement with the results obtained from previous batch conditioning experiment in Section 5.5 (3–6 kg/t DS).

5.7 FTIR analysis, surface morphology and possible mechanisms

A classical mechanism of any coagulant/flocculant is charge neutralisation and for polymeric flocculants, bridging is definitely involved in addition to charge neutralisation. Effective flocculation by polysaccharide-based flocculants were thought to be contributed by their unique functional groups such as carboxyl, hydroxyl, amino, and phosphate (Salehizadeh et al. 2018). In the present study, the BF contained hydroxyl, amide and amino groups as determined by FTIR analysis, which has been discussed in Chapter 4 (Section 4.1). Figure 5.26 reveals the shifted bands observed in the IR spectrum of the sludge before and after treatment with 3 kg/t DS of BF01314 and 2 kg/t DS of chitosan at their optimal dosages respectively.

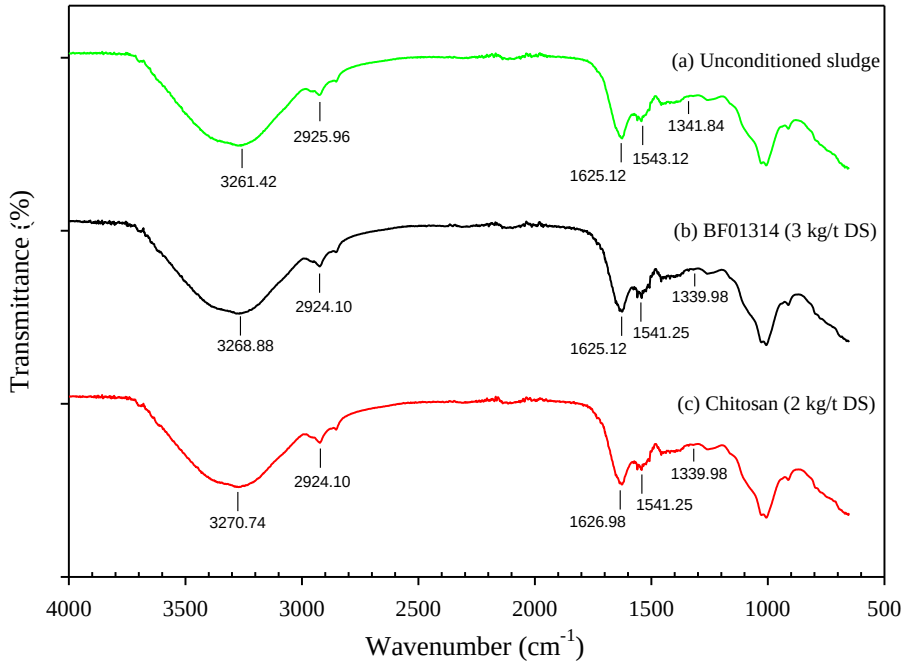

Figure 5.26: IR spectrum of the unconditioned sludge and the sludge conditioned by BF01314 and chitosan

A slight shift of the peak towards higher wavenumber from 3261 to 3268 cm^{-1} for BF01314, and to 3270 cm^{-1} for chitosan in Figure 5.26(b)-(c) were observed with increased intensity of about 1–3% as compared to the unconditioned sludge. An increase of intensity at this frequency (wavenumber) suggested an increase in the amount of hydrogen bonds, however the peak was shifted from low to high wavenumber which was opposite to the results in most published reports (Feki et al. 2020). A shifted band from 1543 to 1541 cm^{-1} in Figure 5.26(a)–(b) further supports the presumption of the interaction of amino groups of BF01314 with sludge particles.

The IR spectrum of the sludge treated by the BF and chitosan individually show the interaction of their amide group at the band of around 1339 cm^{-1} which was associated with C-N stretching. For chitosan, a shifted band was also observed at around 1625 cm^{-1} which was attributed to C=O stretching. The band at 2924 cm^{-1} in both treated sludge with increased intensity of 1–3% may indicate the adsorption of C-H bonds of the flocculant on the sludge after treatment (Kowalski et al. 2018). Figure 5.27 reveals the images of raw sludge, BF-treated sludge and BF01314, obtained from an optical microscope and an environmental scanning electron microscope (ESEM). The optical microscopic image of untreated sludge shows more dispersed sludge particles with smaller sizes (Figure 5.27(a)) compared to the treated sludge with aggregated flocs (Figure 5.27(b)). As compared to the ESEM image of raw or untreated sludge in Figure 5.27(c), it appears that the network-like sludge particles were adsorbed and bridged to the BF surfaces and flocculated together in Figure 5.27(d). The enlarged image of the BF-treated sludge in Figure 5.27(f) confirmed the existence of crystal-like particles (red circle) originally present in BF01314 as shown in Figure 5.27(e), while the other sludge particles seemed to be adsorbed to the smooth surfaces of the BF.

Similar SEM image was obtained for kaolin flocculation by IH-7 which indicated the adsorption of kaolin precipitates on its smooth surfaces (Aljuboori et al. 2015). In contrast, Shi et al. (2019) reported that the sludge treated by a grafted chitosan-based flocculant, carboxymethyl chitosan-graft-poly(acrylamide-methacryloxyethyltrimethyl ammonium chloride (CCPAD) showed a smoother surface as compared to the unconditioned sludge. This was explained by the increase of particles accumulation which caused denser and smoother structures (Shi et al. 2019). Newly-formed floc generally is less compact and has loose structure, which was easily broken apart and had low settleability when compared to the flocs formed after breakage (Nan et al. 2016). In-depth investigations on strength, size and structure of the flocs formed after treatment with BF01314 are encouraged for future work. Based on the analysis of both IR spectrum and microscopic images, it is believed that bridging mechanism are certainly involved in sludge flocculation by BF01314, in addition to high molecular weight.

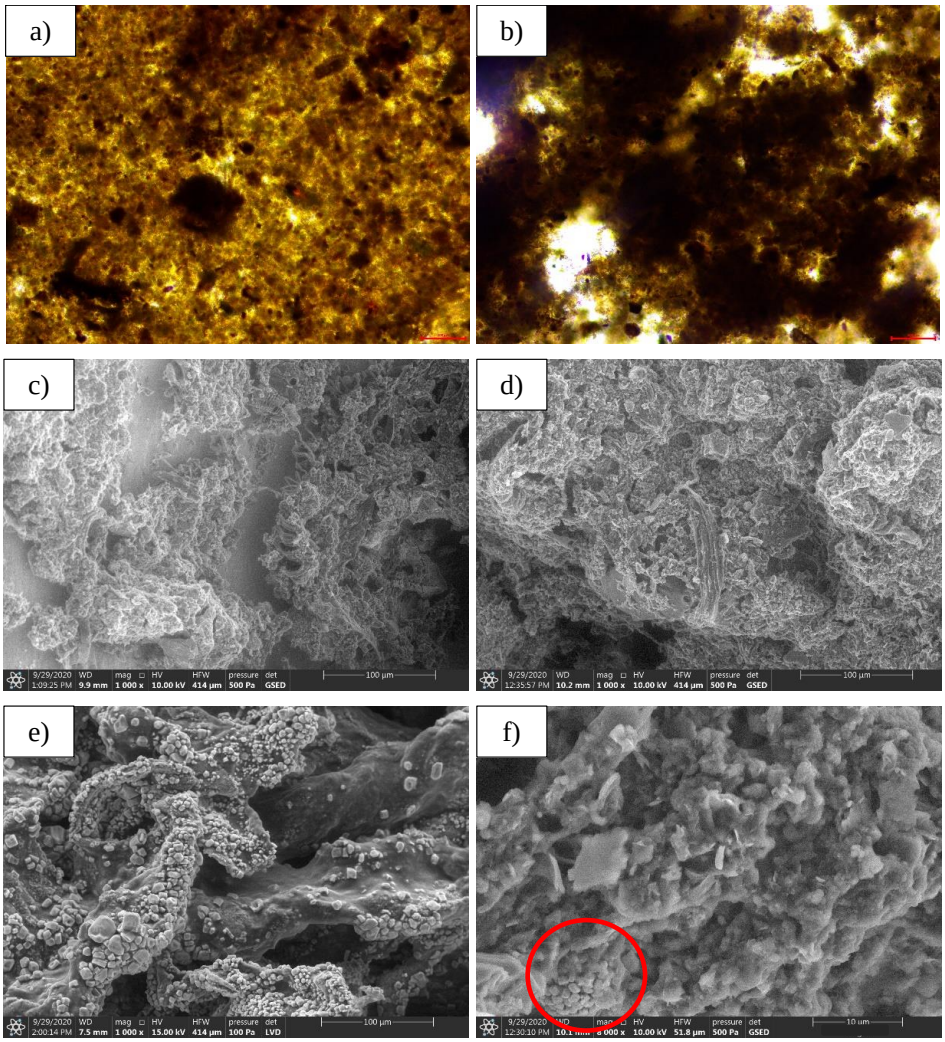

Figure 5.27: Optical microscope images of (a) raw sludge and (b) flocculated sludge. SEM images of (c) raw sludge, (d) BF-treated sludge, (e) BF01314 (1000x) and (f) BF01314 (8000x).

As observed in the change of zeta potential values of sludge treated by BF01314, chitosan and synthetic cationic polymer in Section 5.5, it was evident that the charge neutralisation process took place in sludge flocculation by all three flocculants. Nevertheless, the sludge surface charges treated by BF01314 reached a plateau before reaching the zero-charge point, from dosage of 2.0 to 6.0 kg/t DS. Similar observations were found in the earlier study of kaolin flocculation in Section 4.2.1 and this phenomenon was viewed as the dominating role of electrostatic patch mechanism instead of simple charge neutralisation (Ye et al. 2007). Significant surface charge difference between two oppositely charged ionic species, in this case, the highly charged cationic BF01314 polymer chains interacting with the lowly charged anionic sludge particles, may cause the formation of the electrostatic patches of unevenly

distributed charges (Salehizadeh et al. 2018). The resulting cationic patches attract the other negatively charged colloidal particles to be adsorbed to the charged polymer surfaces (Ye et al. 2007).

At high dosage, the negatively charged sludge particles might be entrapped by the high amount of positively charged BF polymers, forming a bulky precipitate, aggregated to form larger flocs and sedimented (Lichtfouse et al. 2019; Wang, Tang and Gregory 2002), which could explain the flocculation of sludge treated using 6.0 kg/t DS of BF01314 (see Figure 5.5(B)). This mechanism is known as 'sweeping', sometimes also knows as 'colloid entrapment' or precipitation charge neutralisation (PCN) (Lichtfouse et al. 2019; Ye et al. 2007; Sincero 2003). Lichtfouse et al. (2019) reported that chitosan was governed by sweeping mechanism through the formation of a bulky precipitate trapping the colloidal particles, which then self-sedimented or flocculated together. In conclusion, the overall results indicated that the flocculation of BF01314 was governed by a charge neutralisation process in the form of 'electrostatic patching' instead of simple charge neutralisation. The loops and tails of the adsorbed polymer chains in a patch may attach itself to the other oppositely charged particles from another patch, which eventually form a bridge (Salehizadeh et al. 2018), supported by the IR spectrum analysis and microscopic images. At high dosages of above 6.0 kg/t DS, sweeping may also present due to the formation of bulky precipitate of the 'patches'.

5.8 Summary

The dewatering performance of the sludge was evaluated through dewaterability tests of SRF, CST, CSC and zeta potential measurements before and after conditioned with BF01314. Based on the results, the optimum dosage of BF01314 was determined at 3.0 kg/t DS, comparable to that of chitosan but slightly higher than industrial cationic polymer MF 7861. The BF revealed its pH stability in acidic and weak alkaline solutions at a pH's range similar to the findings from earlier flocculation tests using kaolin suspension. Meanwhile, good dewatering performance of the BF-treated sludge was attained across a temperature range of 25–80°C. Based on the flocculation kinetic study, the flocculation activity of the BF remained high when using moderate (100 rpm) to high flocculation speeds (200 rpm) with minimum of 3 minutes mixing time.

The reaction of BF01314 with sludge particles was best fitted to pseudo first-order kinetics. Based on the zeta potential results, IR spectrum analysis, optical microscope and ESEM images, the flocculation of BF01314 was believed to be ruled by electrostatic charge patching, leading to the adsorption and bridging of the suspended particles. In addition, sweeping flocculation was predicted to occur at high dosage supported by the visual appearance of loose and fluffier flocs.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

In this study, a novel bioflocculant (BF) produced from *Citrobacter youngae* GTC 01314, named BF01314 was utilised to condition the activated sludge, which was sourced from a domestic septic sludge treatment plant. The BF was characterised, evaluated for its flocculation behaviour using kaolin suspension, and further examined for its effectiveness in enhancing sludge dewaterability using a real sludge suspension. The following conclusions are drawn based on the analysis and interpretation of the results acquired from the present work.

The results from the characterisation studies revealed that the BF01314 is a polysaccharide-based flocculant instead of glycoprotein, although the percentage of total sugar content was only 5.34% (0.255 g/L) with respect to the BF-dry weight (4.78 g/L). The apparent chitosan content was found to be higher than the total sugar content with a ratio of approximately 2:1. Nevertheless, the analysis of infrared (IR) spectrum indicated that BF01314 has a chitosan-like structure with hydroxyl, amide and amino groups. The analysis of surface charges showed that the BF is cationic under the acidic, neutral and weak alkaline pHs below its isoelectric point (IEP) at pH 8.25. The weighted average molecular weight of BF01314 was determined to be 327 kDa, which was considered as a high molecular weight biopolymer according to the molecular weight range of natural chitosan used in this study. The thermogravimetric analysis of BF01314 depicted that the BF was able to withstand high temperatures up to 202°C, which appeared to be slightly lower than chitosan (265°C) due to less polysaccharide content. Based on the results, it is confirmed that BF01314 is a cationic chitosan-like polysaccharide, with effective functional groups for the flocculation of suspended solids.

The flocculation characteristics or behaviour of BF01314 in kaolin suspension was further investigated under the influences of BF concentration, pH and temperature.

BF01314 demonstrated high flocculation activity of >95% in a broad flocculation window (4 to 120 mg/L) with optimum dosage at 15 mg/L. The BF also demonstrated excellent flocculation performance at a wide range of pH (2–8) and temperature (25–95°C) at its optimum dosage, in agreement with the results obtained from the thermogravimetric analysis and surface charge analysis. The zeta potential results suggested that electrostatic charge patching is the dominating flocculation mechanism over simple charge neutralisation, when effective flocculation was observed before the charge neutralisation is complete. In view of the high molecular weight of BF01314 on the other hand, the polymer bridging mechanism is speculated to prevail when there is less evidence of electrostatic interaction between the flocculant polymer and suspended particles, especially with the observation of high flocculation activity where the zeta potential value departed far from zero.

The sludge dewatering performance was evaluated in terms of capillary suction time (CST), specific resistance to filtration (SRF), cake solids content (CSC) and zeta potential measurements. The dewaterability of BF-treated sludge was greatly enhanced (CST of <10 s, SRF reduced by one order, and CSC of >23%) within the dosage range tested from as low as 0.5 kg/t DS to 6.0 kg/t DS, without any overdose effect. BF01314 also revealed good dewatering performance under acidic, neutral and slightly alkaline conditions (pH 2–8), and at a wide range of temperature (25–80°C), at the constant BF dosage of 1.0 kg/t DS, which were similar to its flocculation behaviours when tested in kaolin suspension. At the BF dosages between 2.0 to 6.0 kg/t DS, a plateau region of high flocculation activity was detected with negative zeta potential values, which further support the presence of electrostatic patching mechanism. When compared to chitosan and synthetic polymer MF 7861, similar results of CST, SRF and CSC were achieved by the BF01314-treated sludge but at a slightly higher demand of dosage (3.0 kg/t DS). The successful outcomes of this study confirmed the feasibility of BF01314 to improve sludge dewaterability, thus suggesting its promising results at a large-scale experiment. Nevertheless, the optimum conditions of dosage, pH, and temperature also depend on the type of sludge, which is not studied in this research. The optimal range obtained from the present work can be used as initial prediction or guidelines for optimisation study in future investigations.

The study of flocculation kinetics was carried out to examine the effects of flocculation speed and time on the flocculation activity, and to determine the type and rate of reaction of BF01314 in sludge suspension. An excellent flocculation performance (>99%) was attained at the flocculation speeds of 100 and 200 rpm, within 3–7 minutes of mixing. The results obtained from the subsequent experiment suggested that the reaction kinetic of BF01314 was best described with a pseudo first-order kinetic model.

The unconditioned sludge and sludge conditioned by BF01314 at dosage of 3.0 kg/t DS were selected for the analysis of infrared (IR) spectrum and surface morphology. The increase in intensity and shifting of the bands reflected on the IR spectrum indicate an increase in the amount of hydrogen bonds and the presence of interaction between the BF functional groups (amino and amide groups) with sludge particles. The images obtained from the optical microscope and the environmental scanning electron microscope (ESEM) reveal that the network-like sludge particles were adsorbed around the BF smooth surfaces and bridged together. Furthermore, BF01314 has high molecular weight which could provide more active sites for the adsorption and bridging with sludge particles. Based on the zeta potential results obtained from the parametric studies, IR spectrum analysis, and sludge micrographs, it is believed that the flocculation of the cationic chitosan-like BF01314 with high molecular weight was governed by electrostatic charge patching and bridging.

6.2 Recommendations for future work

The outcome of this research has confirmed the capability of BF01314 in enhancing the sludge dewaterability for easy-to-dewater activated sludge from domestic sources. The flocculation characteristics of BF01314 in both model and real suspensions were revealed with the preferred operational range for effective flocculation and dewatering performance (dosage, pH, temperature, flocculation speed and time). Comparison of the dewatering performance of BF01314 with some of the commercial flocculants was conducted. Lastly, detailed analysis was performed based on the overall findings to propose the possible flocculation mechanisms of BF01314. Hence, the following research directions are recommended for future work.

6.2.1 Characterisation study of BF01314 and sludge

Although this research includes the characterisation of BF01314, most of the results are qualitative. Elemental analysis and determination of chemical compound in BF01314 are recommended to support the current FTIR results. Other characterisation methods such as X-ray diffraction (XRD) can be performed to observe the changes between the untreated and treated sludge. The use of BFs has gained significant interest among the researchers due to its biodegradability and non-toxic properties. Therefore, it is highly suggested that the biodegradability test and toxicity assay of BF01314 should be conducted to validate the assumption. Moreover, in-depth characterisation of sludge before and after conditioning in terms of composition are proposed, to investigate the effects of other micropollutants (minerals and organics) on the efficiency of sludge flocculation. The final composition of the dewatered sludge cake may provide an indication of the applicability of the treated sludge as a green fertiliser.

6.2.2 Optimisation study and cost analysis

Optimum conditions are vital to ensure that maximum performance can be achieved at the lowest operational cost, or in other words, cost-effective. Optimisation study is proposed mainly on the dosage requirement using the response surface methodology (RSM) based on the optimal range attained in the present work. Initial investigation can be done using synthetic suspension to develop an empirical model. Subsequently, the validation process may be tested using a real suspension. Furthermore, cost analysis is required to assess its practicability in a large-scale production, compared to traditional chemical flocculants. In this case, pilot-scale experimental research is recommended prior to the analysis to ensure high precision results.

6.2.3 Application of BF01314 in different type of wastewater or sludge

With the outstanding flocculation characteristics revealed in this study, the BF01314 has the potential to treat other types of wastewaters. Based on its chitosan-like structure and ionic behaviour, it is possible for the BF to remove various wastewater pollutants, dye, heavy metals and organic matter. Different types of sludge ranging from easy-to-dewater sludge to difficult-to-dewater sludge can be utilised to further investigate the

efficiency of BF01314. Besides, wastewater or sludge from different industries may be sourced to further evaluate the flocculation performance of BF01314.

6.2.4 Adsorption and mechanism of BF01314 flocculation

In this study, the rate of reaction was determined along with the kinetic equilibrium equation. This information allowed the prediction of kinetic reaction of BF01314 in determining the dosage requirement but could not describe the adsorption capacity. A specific substrate of an interest may be selected to identify the appropriate adsorption isotherm based on the range of effective BF concentration obtained from kaolin flocculation experiment and kinetic test as initial indication. The outcome of the study may be useful in designing the batch system for even larger scales. For the study of flocculation mechanism, the analysis on floc strength and size could provide better evidence on the bridging and adsorption effects.

6.2.5 Pilot-scale experiment of sludge dewatering

Although BF01314 displayed its superior dewatering performance in the current study, the test has to be repeated in a larger scale experiment for further verification. This is because the dewatering parameters reported in literature including this research only suggests the capability of the polymer to condition and dewater the sludge. An effective dewatering process mostly relies on the efficiency of the dewatering equipment. Filtration dewatering equipment such as belt-filter press may be favoured in comparison to centrifugal dewatering, depending on the type of polymer or process requirements. Hence, further investigation along with close replicate of mechanical dewatering device used in industry is required for confirmation of the feasibility of BF01314 and for identification of potential associated operational issues.

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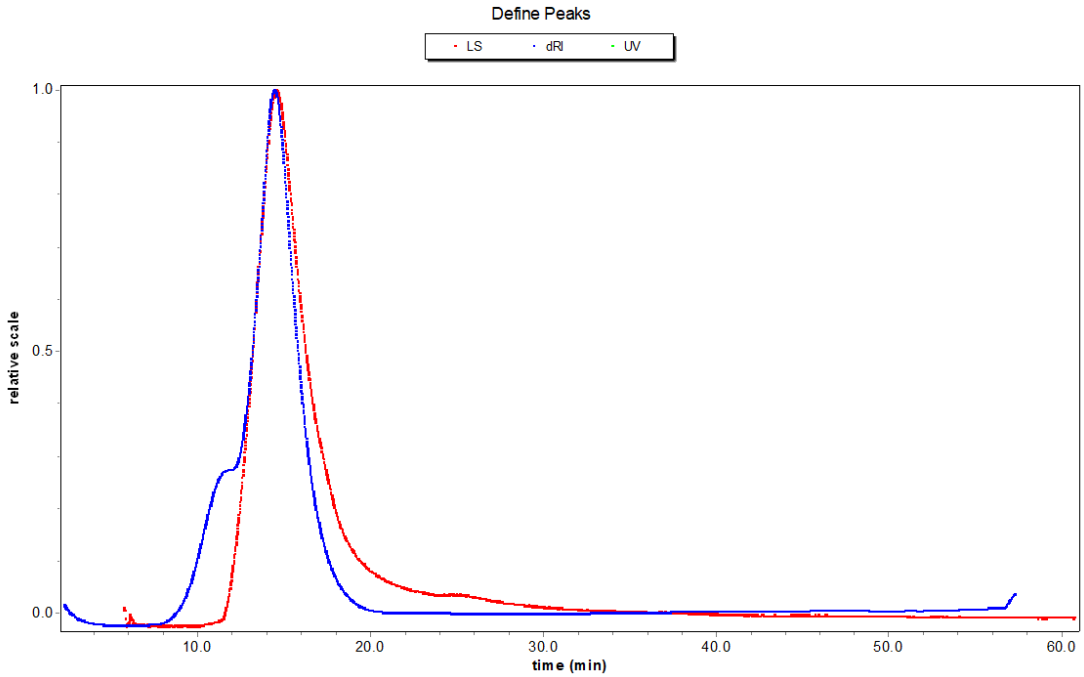
Every reasonable effort has been made to acknowledge the owners of copyright materials. I would be pleased to hear from any copyright owner who has been omitted or incorrectly acknowledged.

APPENDIX A

RAW DATA OF MOLECULAR WEIGHT DETERMINATION BY GPC-MALLS

A.1 Chromatogram of BF01314



A.2 Summary of the report

CONFIGURATION

Viscometer: n/a Dilution factor: n/a
Light scattering instrument: DAWN HELEOS Cell type: K5
Laser wavelength: 658.0 nm
Calibration constant: 6.6311e-5 1/(V cm)
RI Instrument: Optilab rEX UV Instrument: Generic UV instrument Solvent: 0.2M Acetic Acid/0.2M NaAc
Refractive index: 1.331 Flow rate: 0.800 mL/min

PROCESSING

Processing time: Monday October 14, 2019 12:47 PM Malay Peninsula Standard Time Collection time: Friday October 04, 2019 01:46 PM Malay Peninsula Standard Time Detectors used: 3 4 5 6 7 8 9 10 11 13 14 15 16 17 18 Concentration detector: RI Mass results fitting: none (fit degree: n/a) Radius results fitting: none (fit degree: n/a)

	Peak 1
Peak limits (min)	11.366 - 19.869
dn/dc (mL/g)	0.180
A_2 (mol mL/g^2)	0.000
UV ext. (mL/(g cm))	0.000
Model	Zimm
Fit degree	1
Eta Model	Huggins
Huggins Constant	0.0000
Kraemer Constant	0.0000
Injected mass (g)	0.0000
Calc. mass (g)	3.0822e-4
Mass Recovery	n/a
Mass Fraction	100.0000 %

Polydispersity	
Mw/Mn	1.746(13%)
Mz/Mn	2.075(17%)
Molar mass moments (g/mol)	
Mn	1.874e+5(12%)
Mp	2.652e+5(4%)
Mv	n/a
Mw	3.271e+5(5%)
Mz	3.888e+5(11%)
rms radius moments (nm)	
Rn	80.8(9%)
Rw	112.0(2%)
Rz	112.8(2%)

APPENDIX B

SAMPLE CALCULATION OF BIOFLOCCULANT CONCENTRATION AND DOSAGE

B.1 Calculation of BF dosage from dilution of 2^5

From Equation 3.4,

$$\text{BF dosage (mg/L)} = (W_B/V_S) \times (1/\text{dilution})$$

W_B = BF-dry weight yield presence in 1 mL of purified BF solution (mg) = 4.78 mg

V_S = volume of kaolin suspension used for each test (L) = 0.01 L

Dilution = 2^5

Hence,

$$\text{BF dosage} = (4.78/0.01) \times (1/2^5) = \mathbf{14.94\ mg/L}$$

B.2 Calculation of BF solution required for dosage of 1.0 kg/t DS

Total solids of raw sludge = 29.13 g/L

Total solids in 100 mL of sludge = 2.913 g dried solids (DS)

BF-dry weight in 1 mL = 4.78 mg

Step 1:

BF required in mg = BF dosage (mg/g DS) × total solids in 100 mL sludge (g DS)

= 1.0 mg/g DS × 2.913 g DS

= 2.913 mg

Step 2:

BF required in mL = BF required in mg / total BF-dry weight in 1 mL (mg)

= 2.913 mg / 4.78 mg

= **0.6094 mL**

B.3 Calculation of equivalent BF dosage from %BF to unit of kg/t DS

Total solids of raw sludge = 29.13 g/L

Total solids in 10 mL of sludge = 0.2913 g DS

BF-dry weight in 1 mL = 4.78 mg

Step 1 (example for 3% or 1.0 mL of BF dosage):

BF required in mg = BF required in mL × total BF-dry weight (mg/mL)

= 0.3 mL × 4.78 mg/mL

= 1.434 mg

Step 2:

BF dosage (mg/g DS) = BF required in mg / total solids in 100 mL sludge (g DS)

= 1.434 mg / 0.2913 g DS

= **6.54 mg/g DS (equivalent to kg/t DS)**

APPENDIX C

LETTER OF COPYRIGHT PERMISSION FOR REUSE

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Senior Lecturer
Curtin University Malaysia
CDT 250 98009 Miri, Sarawak

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The specific material / extract that I would like to use for the purposes of the thesis is **Figure 2.1 Wastewater and sludge processing flow diagram (page 14 of the thesis).**

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Position: Senior Lecturer, Curtin University Malaysia

Date: 15 June 2021

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acta.toimitus@oulu.fi <acta.toimitus@oulu.fi> Mon, Jun 21, 2021 at 6:04 PM
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APPENDIX D

ATTRIBUTION STATEMENT

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Project Title:	Spectrometric analysis of flocculation activity and molecular interactions of chitosan-like bioflocculant of Citrobacter strains to improve sludge dewatering (Master's thesis title: Physicochemical Characterisation and Flocculation Analysis of a Novel Cationic Chitosan-Like Bioflocculant from Citrobacter Youngae GTC 01314 to Improve Sludge Dewatering)		
Project identifier (if applicable)	Fundamental Research Grant Scheme (FRGS/1/2017/TK02/CURTIN/03/1)		
Principal Investigator	Lau Shiew Wei		
Other named investigator/s#	Masahiro Takeo	**Role**	Co-investigator (conception and design of the project, drafting of work and critical review, contribution of knowledge)
	Han Bing Chua		Co-investigator (conception and design of the project, drafting of work and critical review, contribution of knowledge)
	Tushar Kanti Sen		Co-investigator (Contribution of knowledge, critical review)
	Ha Ming Ang		Co-investigator (Contribution of knowledge, critical review)
Other researcher/s#	Nur Syahirah Mohamed Hatta	**Role**	Master student (Acquisition of research data, analysis and interpretation of research data, contribution of knowledge, critical review)
	Priyanka Baranwal		Acquisition of research data, analysis and interpretation of research data, contribution of knowledge
	Nabisab Mujawar Mubarak		Analysis and interpretation of research data, contribution of knowledge, critical review
	Mohammad Khalid		Acquisition of research data, analysis and interpretation of research data
	Danial Aminin Zairin		Acquisition of research data, analysis and interpretation of research data

Insert additional rows if required

2. Publications/Outputs – the intended outputs from the above research are identified below

Pub#	Description (e.g. method paper)	Publication Type (Conference, journal article etc)
1	Novel cationic chitosan-like bioflocculant from *Citrobacter youngae* GTC 01314 for the treatment of kaolin suspension and activated sludge	Journal article
2	Enhanced sludge dewaterability by a novel chitosan-like bioflocculant BF01314, its mechanism and flocculation kinetics	Journal article

Insert additional rows if required

3. Proposed order of authors

(add rows as required for additional authors and/or if authorship will differ between multiple outputs)

Pub*	Author**	Corresponding? Y/N
All	1. Nur Syahirah Mohamed Hatta	N
All	2. Shiew Wei Lau	Y
All	3. Masahiro Takeo	N
All	4. Han Bing Chua	N

1	5. Priyanka Baranwal	N
2	6. Tushar Kanti Sen	N
2	7. Ha Ming Ang	N
All	8. Nabisab Mujawar Mubarak	N
All	9. Mohammad Khalid	N
2	10. Danial Aminin Zairin	N
All	Named Acknowledgement: Ministry of Higher Education (FRGS/1/2017/TK02/CURTIN/03/1)	N

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2. Shiew Wei Lau		22/11/2020
3. Masahiro Takeo (University of Hyogo)		24/11/2020
4. Han Bing Chua		23/11/2020
5. Priyanka Baranwal		24/11/2020
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Publication 'in-press' (Chapter 4 and part of subchapter 5.2):

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Novel cationic chitosan-like bioflocculant from *Citrobacter youngae* GTC 01314 for the treatment of kaolin suspension and activated sludge

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ARTICLE INFO

Editor: Vitor J.P. Vilar

Keywords:
Citrobacter
Novel bioflocculant
Flocculation activity
Polysaccharide backbone

ABSTRACT

In this study, a novel soluble chitosan-like bioflocculant (BF), named BF01314, was prepared from the culture supernatant of *Citrobacter youngae* GTC 01314 for potential application in the treatment of kaolin suspension and activated sludge. The physicochemical characteristics of the BF and its flocculation behavior under the effects of BF concentration, pH and temperature in kaolin suspension were investigated. Results reveal that BF01314 exhibited a chitosan-like infrared spectrum with the presence of the characteristic hydroxyl, amide, and amino groups. The BF demonstrated strong flocculation activities of more than 95% in kaolin suspension within a broad "flocculation window" of BF concentrations (4–120 mg-dry weight/L), pH range of 2–8, and temperature range