IDENTIFICATION AND EVALUATION OF BIOFLOCCULANT FROM *Bacillus salmalaya* 139SI FOR ITS APPLICATION IN WASTEWATER TREATMENT

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FACULTY OF SCIENCE
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ABSTRACT

Bioflocculants are flocculating compounds produced by microorganisms during their growth and has recently received extensive consideration from researchers due to their biodegradable, non-toxicity and lack of secondary pollution from degradation intermediates characteristics. The production, optimization, and Characterisation of bioflocculant QZ-7 produced by a novel *Bacillus salmalaya* strain 139SI which was isolated from a private farm soil in Selangor, Malaysia, were determined. Meanwhile, the optimal culture condition for bioflocculant production was achieved after cultivation at 35.5 °C for 72 h at pH 7, with an inoculum size of 5% (v/v) and sucrose, glucose as carbon source and yeast extract, urea as nitrogen sources. A bioflocculant yield of 2.72 g was recovered from 1 L of broth culture, with maximum flocculating activity that was found to be 92.6%. Chemical analysis revealed that the pure bioflocculant QZ-7 consisted of 79.08% carbohydrates and 15.68% proteins. Infrared spectrometry analysis showed the presence of carboxyl (COO-), hydroxyl (-OH), and amino (-NH3) groups, which are typically from polysaccharides and proteins. The NMR spectroscopy analysis confirmed the result of FTIR, through the presence of functional groups of the QZ-7. Scanning electron microscopy (SEM) analysis showed that QZ-7 exhibited a clear crystalline brick-shaped structure. The average molecular weight of the bioflocculant QZ-7 was calculated to be 5.13×10^5Da. LC-MS analysis confirmed that QZ-7 was a glycoprotein compound detected at 741m/z–745m/z. Moreover, the presence of glucose at 182.96 m/z, rhamnose at 354.3m/z, and glucuronic acid at 212.8 m/z. SEM- EDX analysis indicated the existence of C, O, N, P and S in this macromolecule as 55.74%, 42.74%, 0.54%, 0.93%

and 0.06%, respectively. Thermogravimetric analysis (TGA) of the bioflocculant QZ-7 contained thermos-stable and thermo-labile molecules. Bioflocculant QZ-7 exhibited wide pH stability that ranged from 4 to 7, with a flocculation activity of more than 70%. In addition, QZ-7 was thermally stable and retained more than 80% of its flocculating efficiency after being heated at 60 °C for 30 min. The highest bioflocculating activity of 93.6% was obtained for Ca^{+2} at 2 mg/L of QZ-7 concentration at pH 7. The treatment of river water by purified bioflocculant QZ-7 showed high performance in the removal of turbidity, total suspended solids and COD. After treating the wastewater, the bioflocculant QZ-7 showed significant flocculating performance with a COD removal efficiency of 93%, whereas a BOD removal efficiency of 92.4% was observed in the *B. salmalaya* strain 139SI. In addition, results for the removal of heavy metals from industrial wastewater revealed that the bioflocculant QZ-7 was capable of removing the heavy metals. For example, the maximum adsorption of As (89.8 %), and Zn^{+2} (77.4 %), and Cu^{+2} (58.4%). Moreover, the bioflocculant QZ-7 had significant removal efficiency of different pharmaceutical compounds, such as Simvastatin (92.45%), Salbutamol (88.69%), Acetaminophen (69%), and Caffeine (66.52%). Furthermore, *B. salmalaya* 139SI strain and pure bioflocculant QZ-7 could synthesise AgNPs. Also, an antibacterial activity of the AgNPs was detected against test bacterial strains, such as *Escherichia coli* ATCC35401, *Salmonella enteritidis* ATCCBAA-711, *Staphylococcus aureus* ATCC2592 and *Pseudomonas aeruginosa*, as application of AgNPs.

Keywords: *Bacillus salmalaya* 139SI, Optimization, Production, Bioflocculant, Characterisation, Application.

PENGECAMAN DAN PENILAIAN BIOFLUKULAN DARI *Bacillus salmalaya* 139SI DAN APLIKASINYA DALAM RAWATAN AIR KUMBAHAN

ABSTRAK

Gumpalan bioflok adalah bahan yang dihasilkan oleh mikroorganisma semasa berlakunya proses tumbesaran dan sejak akhir ini telah mendapat perhatian khusus daripada para penyelidik berdasarkan sifat boleh urai, tidak toksik, dan tidak menyebabkan pencemaran kedua berlaku disebabkan proses penguraian. Penghasilan, pengoptimum, dan ciri gumpalan QZ-7 ditentukan oleh bakteria novel *Bacillus salmalaya* strain 139SI diambil dari tanah ladang milik persendirian di Selangor, Malaysia. Keadaan kultur optimum penghasilan gumpalan dicapai pada pH 7 ± 0.2, dengan saiz inokulum adalah 5% (v/v) dan sukrosa, glukosa sebagai sumber karbon dan ekstrak yis, urea sebagai sumber nitrogen selepas pengkulturan pada suhu 35.5 °C selama 72 jam. Hasil gumpalan adalah 2.72 g telah diperoleh daripada 1 L kultur broth, dengan kadar maksimum aktiviti gumpalan iaitu 92.6%. Analisa kimia mendedahkan bahawa gumpalan asli QZ-7 mengandungi 79.08% karbohidrat dan 15.68% protein. Analisa meggunakan spektrometri inframerah menunjukkan kehadiran kumpulan karboksi (COO-), hidroksi (-OH), dan amino (-NH3); yang mana tergolong dalam kumpulan polisakarida dan protein. Analisa NMR menggunakan hasil dari FTIR melalui kehadiran kelpelbagaian kumpulan QZ-7. Analisa menggunakan mikroskop electron (SEM) menunjukkan bahawa QZ-7 mempamerkan struktur berbentuk bata jernih. The average molecular weight of the bioflocculant QZ-7 was calculated to be 5.13×10^5Da. LC-MS analisa membuktikan biofluculant QZ-7 adalah kampaun glycoprotein dikesan pada 741m/z -745 m/z. manakala kehadiran glucose olikasan pada 203.1m/z, rhamnose 354.3m/z. olan glucuronic acid 189.1 m/z. Analisa menggunakan SEM-EDX pula menunjukkan kewujudan C, O, N, P dan S dalam makromolekul ini masing-masing adalah sebanyak 55.74%, 42.74%, 0.54%, 0.93% dan 0.06%. Analisa termogravimetrik (TGA) ke atas

gumpalan QZ-7 menunjukkan QZ-7 adalah molekul yang stabil haba dan ubahsuai haba. Gumpalan QZ-7 mempunyai kadar kestabilan pH adalah daripada skala 4 hingga 7, dengan 85% aktiviti gumpalan pada pH 7. Tambahan lagi, QZ-7 mempunyai haba yang stabil dan dapat dikekalkan melebihi 80% kecekapan gumpalan selepas dipanaskan pada suhu 80 °C selama 30 minit. Aktiviti gumpalan paling tinggi (93.6%) dapat dicapai oleh Ca^{+2} pada pH 7.0. Rawatan air sungai yang dilakukan oleh gumpalan QZ-7 menunjukkan kecekapan yang tinggi dalam mengurangkan kekeruhan, pepejal yang tergantung dan COD. Selepas merawat air sisa, gumpalan QZ-7 menunjukkan signifikasi antara kecekapan gumpalan dengan pengurangan COD sebanyak 93% dengan pengurangan BOD dalam bakteria *B. salmalaya* strain 139SI pula adalah sebanyak 92.4%. Nilai-nilai tersebut menunjukkan bahawa kecekapan aplikasi gumpalan QZ-7 dalam rawatan air sisa. Selain itu, keputusan mendedahkan bahawa gumpalan QZ-7 mempunyai kemampuan untuk menghapuskan logam berat. Di samping itu, hasil penyingkiran logam berat dari air sisa industri menunjukkan bahawa bioflocculant QZ-7 mampu mengeluarkan logam berat. Sebagai contoh, penjerapan maksimum As (89.8%), dan Zn^{+2} (77.4%), dan Cu^{+2} (58.4%). Selain itu, gumpalan juga menunjukkan kecekapan dalam menghapuskan partikel farmaseutikel seperti Simvastatin 92.45%, Salbutamol 88.69%, Acetaminophen 69%, Caffeine 66.52%. Tambahan lagi, gumpalan QZ-7 boleh mengsintesis AgNPs dan disalut dengan biopolymer yang akan menghasilkan daya tujahan antara partikel untuk menjauhkan diri antara satu sama lain dan dapat mengelakkan daripada perlekatan. Juga, didapati bahawa aplikasi sintesis AgNPs oleh gumpalan mampu melawan strain bakteria seperti *Escherichia coli* ATCC35401, *Salmonella enteritidis* ATCCBAA-711, *Staphylococcus aureus* ATCC2592 and *Pseudomonas aeruginosa*.

Kata Kunci: *Bacillus salmalaya* 139SI, Pengoptimuman, Pengeluaran, Bioflocculant, Pencirian, Permohonan.

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LIST OF SYMBOLS AND ABBREVIATIONS

˚C	:	Celsius
Da	:	Dalton
µg	:	Microgram
µL	:	Microliter
mg	:	milli gram
mM	:	mille Molar
M	:	Molar
PPT	:	Part Per Thousand
PPM	:	Parts Per Million
Pmol	:	Pico mol
AAS	:	Atomic Absorption Spectroscopy
AgNPs	:	Silver Nanoparticles
BOD	:	Biological Oxygen Demand
BSA	:	Bovine Serum Albumin
CHNS	:	Carbon, Hydrogen, Nitrogen, Sulfur
COD	:	Chemical Oxygen Demand
CPO	:	Crude Palm Oil
DOE	:	Design of Experiments
D_2O	:	Deuterium Oxide
EDA	:	Exploratory data analyse
EDXS	:	Energy Dispersion X-Ray Spectroscopy
EPS	:	Extracellular Substance
e.g	:	For example
et al	:	And others
EWW	:	Efluent waste water
FFB	:	Fresh Fruit Bunches
FTIR	:	Fourier-Transform Infrared Spectroscopy
H-NMR	:	Proton Nuclear Magnetic Resonance
HPGPC	:	High Performance Gel Permeation Chromatography
HPLC	:	High Performance Liquid Chromatography
ICPMS	:	Inductive Coupled Plasma Mass Spectrometry
IWW	:	Influent Waste Water

LC/MS	:	Liquid Chromatography Mass Spectrometry
MPOB	:	Malaysian Palm Oil Board
NMR	:	Nuclear Magnetic Resonance
NOM	:	Natural Organic matter
NTU	:	Nephelometric Turbidity Units
OD	:	Optical Density
PAA	:	Polyacrylamide
PAC	:	Poly-aluminium Chloride
PDADMA	:	Poly-Diallyl-Dimethyl Ammonium Chloride
PDMDAA	:	Poly-Dimethyl-Allyl Ammonium Chloride
PFC	:	Poly-Ferric Chloride
POME	:	Palm Oil Mill Effluent
PS	:	Polysaccharide
rpm	:	Revolution Per Minute
SEM	:	Scanning Electron Microscopy
SPE	:	Solid- Phase Extraction
i.e	:	That is
TDS	:	Total Dissolved Solids
TEM	:	Transmission Electron Microscopy
TGA	:	Thermo Gravimetric Analysis
TSS	:	Total Suspended Solids
UV Vis	:	Ultraviolet Visible
XRD	:	X-Ray Diffractometry

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CHAPTER 1: INTRODUCTION

1.1 General Overview

Environmental pollution has become one of the major universal problems with its associated weakening effects on economic development (Chen *et al.*, 2018; Pathak *et al.*, 2014). Water pollution is one of the most thought-provoking environmental concerns and has become a global stumbling block for improving the quality of life in many communities (Okaiyeto *et al.*, 2016). Water is a source of life and energy, even though many societies worldwide are suffering from the scarcity of safe water for drinking purposes (Rani *et al.*, 2013).

Innocent development and rapid growing in population have enormously given rise to the risky condition of water pollution and primary harmful environment (Prasertsan *et al.*, 2006). The main sources of these pollutions come from toxic industrial discharge and domestic wastewater effluents, agricultural wastes and untreated sanitary wastewater (Li *et al*., 2013). The existence of these contaminants in water bodies could be malicious to bio-aquatic life and has also reduced its incompatibility, such as drinkable water sources for domestic utilisation, and a consequence of pollution in aquatic environment is a life-threatening effect on healthful human life (Yang *et al.*, 2012). Water, soil, rice and wheat are contaminated with heavy metals by several sources, such as chemical plants, ceramic factories and plants of recycling materials, because they are heavily dispersed. Besides, advanced transportation and agriculture activities are also a crucial source of heavy metal contamination (Chen *et al.*, 2018).

Environmental pollution with all types of heavy metalhave conferred more consideration in current years because of their high toxicity, bioaccumulation and persistence (Chen *et al.*, 2018; Liu *et al.*, 2017). In recent years, heavy metal contamination has become progressively serious (Yu *et al.*, 2017; Ran *et al.*, 2016). The

essential assessment of health threats to the population is important. Therefore, any adversative effects can be circumvented to the maximum range even though the health threats of heavy metal have been vastly studied (Liu *et al.*, 2017; Cai *et al.*, 2015).

According to the WHO/UNICEF (2017) statement, about 70% to 80% of all diseases in developed countries are related to drinking polluted water, specifically among vulnerable people. When untreated wastewater is released into natural water bodies, it becomes poisonous to marine life and reduces the potable waters for utilisation. For instance, a consequence of these alarming water-borne diseases as well as an increase in the demand for non-toxic water for mutually municipal and industrial utilisation, more consideration was focused on water treatment. Therefore, it is very essential to evaluate water quality on a continuous basis (Yang *et al.*, 2012). Numerous stringent rules were initiated by many countries with respect to the occurrence of pollutants in water to safeguard appropriate treatment of industrial and domestic discharge as well as agricultural wastewater effluents, before their discharges into different water- bodies (Li *et al.*, 2013). Relatively, many water treatment technologies are available and these include coagulation/flocculation, filtration, ion exchange, oxidation processes, adsorption, solvent extraction and electrolysis (Low *et al.*, 2011; Ong *et al.*, 2012).

However, the major obstruction with some of these techniques is limited financial costs in their operations which tend to reduce their capability and effectiveness in application (Li *et al.*, 2013). On the other hand, coagulation/flocculation is the preferred process because it is less costly, effective, not labour-intensive, and requires less skilled personnel (Li *et al.*, 2013; Renault *et al.*, 2009). Moreover, one of the main advantages of the coagulation/flocculation method is the decolourisation of wastewater due to the elimination of dye molecules from the final discharges, and thus removing potential deterioration of dyes which can release toxic chemicals that contain aromatic compounds

(Aboulhassan *et al.*, 2006). Flocculants are naturally occurring organic macromolecules; they are substances with the ability to flocculate suspended solids, cells, and solid colloid particles (Bhunia *et al.*, 2012). Flocculants are widely applied for separation techniques, such as in drinking water purification, wastewater treatment, activated sludge dehydration, downstream processing, and food fermentation (Chen *et al.*, 2017). Shih and Van (2001), recognised that flocculation could be applied as a substitute to filtration and centrifugation for microbial cell separations from broth in food, pharmaceutical and beverage industries. Flocculation is an operative system that is normally applied in wastewater treatment to remove suspended solids and heavy metals (Deng *et al.*, 2003).

Flocculants are typically classified into three groups: organic synthetic flocculants such as polyethylene imine and polyacrylamide byproducts, inorganic flocculants including aluminium sulphate and polyaluminium chloride, and natural flocculants such as chitosan, sodium alginate and bioflocculants (Aljuboori *et al.*, 2013). Organic and inorganic flocculants are referred as chemical flocculants. These chemicals/ synthetic flocculants are commonly applied to remove organic matter from different wastewater because of their high flocculating efficiency and low cost (Zheng *et al.*, 2008). However, several neuropathological studies have showed a relation between residual aluminium in water and the pathogenesis of Alzheimer's disease and dementia (Banks *et al.*, 2006). Ferrite flocculants are also good flocculating agents except for causing an unpleasant metallic taste, malodour and being highly corrosive in nature (Li *et al.*, 2008). On the other hand, polyacrylamides are non- biodegradable, and the carcinogenicity property of the residual acrylamide monomers restricts their industrial applicability (Zhuang *et al.*, 2012). A number of cationic polymers are documented to constitute an important potential hazard to aquatic life, specifically fish and other aquatic lives. Also, the fish gill surfaces have negative charge to which cationic polyelectrolytes can eagerly bind due to electrostatic attraction (Murgatroyd *et al.*, 1996). This effect leads to blockage and

decrease in oxygen transport and produce mucous on the gill surfaces, thereby causing death of the fish which subsequently reduces the supply of healthy fish for human consumption (Narkis & Rebhun, 1975). Being outstanding to numerous problems that are related to the use of chemical flocculants, more attention is diverted to microbial flocculants (bioflocculants).

Bioflocculants are produced by many kinds of microorganisms, such as bacteria, algae and fungi which have been isolated from soil, water and activated sludge (Aljuboori *et al.*, 2013; Czemierska *et al.*, 2017; Tiwari *et al.*, 2015). Different microorganisms (i.e. bacteria, algae, fungi, and actinomycetes) can consume the nutritious substance in the culture medium to synthesise intracellular high molecular weight bioflocculant which can be excreted into fermented broth or on the bacterial cell surface as capsules possessing flocculant properties (Desouky *et al.*, 2008; Gao *et al.*, 2006). Lately, the use of microbial flocculants was activated as a solution to environmental problems that develop from the use of chemical flocculants (Li *et al.*, 2009). Bioflocculants are natural macromolecules produced by microorganisms through their growth, their use has received consideration due to their biodegradability, non-toxicity, safety and eco-friendly characteristics because they lack secondary pollution from intermediate degradation (Ntsangani *et al.*, 2017; Xia *et at.,* 2018). These macromolecules are composed of polysaccharides, protein, glycoproteins, lipids, nucleic acid, lectins and other polymers commonly present in lower concentrations (Giriet *et al.*, 2015; Tang *et al.*, 2014). Extracellular polymeric substance (EPS), are possessed primarily of sugars, protein, glycoprotein and nucleic acids. EPS can be used in processes that correspond to drinking water purification, wastewater treatment, food and fermentation processing as well as in pharmaceutical and cosmetic industries as stabilising, viscosifying and emulsifying agents (Czemierska *et al*., 2017; Zhong *et al*., 2018). Also, microbial polymers are important as antibacterial, antiviral, and anti-algal agents and as inducers of microbial accumulation and biofilm formation

(Zhong *et al.*, 2018). Distinct conventional flocculants, bioflocculants have the benefit of being biocompatible and environmentally friendly. Until now, they have been employed in several applications, for example, dye wastewater treatment, sludge dewatering and heavy metal removal (Wang *et al.*, 2015; Xia *et al.*, 2018). The flocculating efficiency of a bioflocculant depends uniquely on its chemical structure which can be related to high molecular weight and the types of functional groups present in the molecular chain of such biopolymers (Gao *et al.*, 2006). For example, Zhong *et al.*, (2018), found the novel bioflocculant MBF-15 which is an exo-polysaccharide produced by alkaliphilic, *Paenibacillus jamilae*. Czemierska *et al.* (2017) also, stated that the exopolymer from *Rhodococcus rhodochrous*, with a molecular weight of about $1.3x10^3$ KDa, constitutes polysaccharide (62.86%) and protein (10.36%). Nwodo *et al.* (2014) and Li *et al.* (2010) found that the biopolymers secreted by both *Agrobacterium* sp. M-503, and a consortium of *Cellulomonas* and *Streptomyces* species were predominantly composed of glycoproteins. Meanwhile the bioflocculants synthesised by *Bacillus* sp. Gilbert (Ugbenyen *et al.*, 2014) and *Halomonas* sp. AAD6 (Sam *et al.*, 2011) were composed of polysaccharides.

However, the production of bioflocculants is usually subjective by culture media constituents, such as carbon, nitrogen sources, salts as well as factors like pH and temperature of the medium, and aeration level (Lopez *et al.*, 2003; Salehizadeh & Shojaosadati, 2001). Several researchers have been investigated on the synthesis of bioflocculants from different microorganisms by utilising complex media for growth and optimal yields (Piyo *et al.,* 2011). For example, Cosa *et al*, (2011) found that glucose and peptone were utilised as carbon and nitrogen sources, respectively, with Fe^{+2} as an optimal salt for the production of bioflocculants by *Virgibacillus* sp. Rob. *Serratia ficaria* utilises beef extract, urea, lactose and Mg^{+2} or Ca^{+2} for bioflocculant production (Gong *et al.*, 2008), whereas *Enterobacter cloacae* WD7 favoured sucrose, yeast extract and Ca^{+2}

as the salt for bioflocculant production (Prasertsan *et al.*, 2006). Complex media utilisation renders the fermentation process to be economically expensive due to high nutrient costs, such as glucose, sucrose, peptone, yeast extract and salts. There is a need to reduce fermentation costs, whereas increasing bioflocculant yields demands the need for an alternative, but cost-effective, substrates (Fujita *et al.*, 2000). Agricultural industrial wastes, such as sugarcane molasses, fishmeal wastewater and corn-steep liquor could be utilised as an alternative substance for cost-effective production of bioflocculants (Zhuang *et al.*, 2012).The utilisation of organic wastes as substrates in the production media is ecologically promising, sustainable and economically acceptable. Liu *et al*. (2016) reported that *Pseudomonas veronii* L918 can transform the hydrolysate of peanut hull into bioflocculants and a harvest of 3.39 g/L bioflocculant MBF-L918 was reached after peanut hull was utilised as a carbon source. Zhou *et al.* (2003) and Huang *et al.* (2001) reported on the utilisation of some cost-effective substrates, such as soybean juice and fishmeal wastewater for bioflocculant production. For example, finding by Kurane *et al.* (1994) showed that two-thirds of the cultivation cost could be saved when yeast extract in the growth medium was replaced by different supplements, such as aquafarm wastewater, bean cake or cattle blood for microbial production of (NOC-1) bioflocculant. The presence of nutrient constituents in brewery wastewater makes it a potential substrate for bioflocculant synthesis by certain microorganisms (Chen *et al.*, 2003). Similarly, the brewery wastewater was used as a carbon source for bioflocculant production by multiple-microorganism consortia (Zhang *et al.*, 2007). High flocculating activity and bioflocculant yields were also achieved when *Serratia ficaria* and *Klebsiella mobilis* were cultivated in the presence of dairy wastewater (Gong *et al.*, 2008; Wang *et al.*, 2007). Likewise, the synthesis of a novel bioflocculant REA-11(polygalacturonic acid) by *Corynebacterium glutamicum* was optimally gained when sucrose and corn steep liquor were used as substrate (He *et al.*, 2004). In contrast, the production of an

exopolysaccharide bioflocculant by *Sorangium cellulosum* was observed in the presence of soluble starch, which is a cheaper substrate than glucose (Zhang *et al.*, 2002). Theconsumption of these cost-effective substrates for bioflocculant production creates a reasonable logic if production costs are to be contained. Furthermore, to reduce the production cost, different wastewaters were used as low-cost substrates, such as potato starch wastes as carbon source (Liu *et al.*, 2015b; Pu *et al.*, 2014), palm oil mill discharge (Aljuboori *et al.*, 2014; Wong *et al.*, 2012), dairy waste effluents (Wang *et al.*, 2007) and chromotropic acid wastes (Zhong *et al.*, 2014). In addition, agricultural wastes, such as corn stover, rice stover are rich in lignocelluloses, in which hydrolysates are applied as low-cost carbon source to produce bioflocculants (Guo *et al.*, 2015b; Wang *et al.*, 2013b). Bioflocculants are documented to have applications in wastewater treatment, for example, in the decolourisation of dyes in solutions (Deng *et al.*, 2005), inorganic solid suspensions e.g. aluminium oxide, bentonite, solid clay, and activated carbon (Yim *et al.*, 2007; Zhou *et al.*, 2003).

Currently, a significant progress is being made by green nanotechnology, in considering environmental benefits, time duration and cost efficiency. Biological synthesis of nanoparticles has a natural tendency to work on different scientific disciplines, including material science and biomedical application (Muthulakshmi *et al.*, 2017). The main advantages of the nanoparticles are that their increase in surface to volume ratio and biocompatibility. The most commonly used synthesis route for nanoparticles is by chemical and physical approach.

1.2 Research Problems

Industrial development has been established as a desirable choice, owing to its impact to economic growth. However, it has extensively increased the level of water contamination, particularly from industrial sources and this has become a crucial

environmental concern (Sakar *et al.*, 2006). Water pollution is one of the greatest stimulating issues that is globally distressing the fine quality of life in many countries. The main sources of water pollution are the effluents of untreated sanitary, noxious industrial discharge and domestic and agriculture wastes (Li *et al.*, 2013). The occurrence of contaminated water bodies can be antagonistic to marine life as well as rendering it inappropriate for domestic usage. As a consequence, an increase in waterborne diseases becomes predictable, striking more for non-toxic water for both metropolitan and industrial demands. Therefore, great consideration has been focused on water treatment; consequently, making it essential to estimate water quality on a constant base (Yang *et al.*, 2012).

Chemical flocculants are widely involved in drinking water and wastewater treatment, food and fermentation industries and also downstream processing due to their cost-effectiveness and high flocculation competence (You *et al.*, 2008). However, their wide practises have raised significant environmental and health concerns (Mabinya *et al.*, 2012). The use of aluminium as the coagulant in water treatment could cause a higher concentration of aluminium in the treated discharge than in raw water. Besides, aluminium residual in immoderate sludge formed throughout coagulation tends to accumulate in the environment (Ma *et al.*, 2016). For example, a number of studies have presented that aluminium salts are related to Alzheimer's disease (Bank *et al.*, 2006; Arezoo, 2002). In addition, polyacrylamide displays high flocculation activity, while the acrylamide monomer residuals are carcinogenic and neurotoxic to humans (Ruden, 2004; Polizzi *et al.,* 2002). Moreover, acrylamides are not biodegradable and consequently represent an environmental irritant (Lofrano *et al.*, 2013).These expected problems that are associated with chemical flocculants have imposed a search for different flocculants that are eco-friendly and non-toxic (Nwodo *et al.*, 2013).

On the other hand, bioflocculants are harmless, biodegradable, neither neurotoxic nor carcinogenic as compared to the synthetic flocculants used in many industrial processes (Li *et al.*, 2009; Liu *et al.*, 2010). However, the high costs associated with their production as well as the corresponding low yields are still the major problems that prevent their industrial usage (Gao *et al.,* 2006; Wang *et al.*, 2007). Therefore, there is a need to screen new microorganisms with high bioflocculant production capacities and also develop several strategies to optimise culture conditions in enhancing bioflocculant yields with improved flocculating activity (Li *et al.*, 2009; Ugbenyen *et al.*, 2012). Biosynthesis of silver nanoparticles by using bioflocculant is used in deactivating *Acidithiobacillus* bacteria which are involved in the generation of acid mine drainage (Natarajan. 2017).

1.3 Hypothesis

It is hypothesised that the bacterial bioflocculant produced by *Bacillus. salmalaya* 139SI can significantly reduce the COD, BOD, TSS and turbidity from surface wastewater and removing heavy metals and pharmaceuticals from wastewater effluent and nanoparticles synthesis.

1.4 Scope of Study

Different flocculants, such as organic high-polymer flocculants, inorganic flocculants and naturally occurring flocculants are applied in wastewater, industrial downstream processes and dredging. Meanwhile organic high-polymer is very effective, a few of them are not simply degraded in nature and some of the monomers obtained from synthetic polymers are deleterious to the human body. In recent years, to resolve these environmental problems, the use of microbial bioflocculants have been anticipated due to their biodegradability, non-toxicity, safe and environmentally friendly, including lack of secondary pollution from intermediate degradations (Ntsangani *et al.*, 2017; Xiang *et at.*, 2018).

1.5 Objectives of Study

The aim of this study is to produce a new bioflocculant from *Bacillus salmalaya* 139SI and apply it onsite in water and wastewater treatment. The specific objectives of this study include:

1. To determine the optimal medium compositions and cultural conditions for the production of bioflocculant by *B. salmalaya* 139SI strain to enhance the bioflocculant yield.
2. To extract, purify and characterise the bioflocculant compound produced by *B. salmalaya* 139SI.
3. To investigate the potential of bioflocculants produced by *B. salmalaya* 139SI in surface water and industrial wastewater treatment.
4. To determine the performance of the bioflocculant in synthesising nanoparticles.

1.6 Significance of Study

The importance and significance of study are:

1. Contribution in the investigation of *B. salmalaya* 139SI potential as a novel bioflocculant source and its application in water and wastewater treatment.
2. The test the common chemical flocculant substitute by using a new bioflocculant in water treatment to avoid toxic chemical sludge, achieve a lower treatment cost and green environment.
3. The empirical of synthesised nanoparticles as an antibacterial agent.

1.7 Thesis Structure

The thesis focuses on the production of a bioflocculant from a selected bacterium, which is *Bacillus salmalaya* strain 139SI, with flocculating potentiality. The scope of the thesis includes factors which affect the production of bioflocculant, extraction, purification and Characterisation of bioflocculant, as well as factors affecting bioflocculant performances and applications.

The following section depicts the flow and outline of the thesis.

Chapter 2 contains a general introduction to different types of flocculants and a review of relevant literature on bioflocculant production, Characterisation of the bioflocculant, bioflocculant performance and applications.

Chapter 3 explains the general material and methods, whereby the experimental research is described and discussed in detail.

Chapter 4 presents the results of the factors affecting the *Bacillus salmalaya* 139SI on bioflocculant production, Characterisation of the bioflocculant and factors affecting bioflocculant performance and bioflocculant applications in river water and wastewater, heavy metal removals and nanoparticle synthesis.

Chapter 5 presents on the overall results and discussions.

Chapter 6 includes the research summary and conclusion with some recommendations for using different methods in water and wastewater treatment and future studies.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

Implications for public health have demand alternatives to inorganic and synthetic flocculants. Bioflocculants have been chosen as important candidates. Water is one of the vital sources of life and energy, even though many societies worldwide are suffering from the scarcity of safe water for drinking purposes (Okaiyeto *et al.*, 2016). The bountiful capacity of water on Earth is the sole exclusive factor that distinguishes this planet from others. The significance of water to the existence of natural life is vital for human survival so that the search for water on other planets have grown to be the basic factor to recommend the manifestation of life (Rani *et al.*, 2013). Water occupies 78% of the planet's surface, and until now available water for human use is inadequate. In fact, it is an essential need for human development, health and welfare. Safe drinking water is a globally accepted human right (WHO, 2001) which is registered as one of the 10 targets in the Millennium Development Goals (MDGs), (Rout & Sharma, 2011). The property of water used by humans in certain communities could be considered as an important indicator of the human lifespan superiority surrounded by that environment. Water is extremely necessary for municipal, industrial, agricultural and environmental needs (Okaiyeto *et al.*, 2016). Still, pollutants in water will decrease its effectiveness in utilisation because the undesirable effect of water contamination is threatening humans and the environment.

Water pollution is one of the major thought- provoking environmental concerns and has become an international stumbling block for improving the quality of life in many communities (Chen *et al.*, 2018). Unintended development and rapid growth in population have massively given rise to water contamination risks and prevalent harmful environments (Prasertsan *et al.*, 2006; Okaiyeto *et al.*, 2016). The main sources of water

contamination are the effluent of agricultural and domestic waste, industrial toxic waste and untreated sanitary discharge (Li *et al.*, 2013). The occurrence of these contaminants in water bodies could remain malicious to aquatic biota lifespan and has reduced their inappropriate as drinkable water sources for domestic utilisation, and the consequent of aquatic environment pollution has a serious influence on healthful human life (Yang *et al.*, 2012).

According to WHO/UNICEF (2017), report around 70%–80% of all diseases in developed nation states are related to the consumption of polluted water, particularly amongst susceptible individuals. A report by the World Health Organisation (2015) found that 29% of the worldwide population has lacked safety in managing drinkable water services. 61% of the global population requires safely managed sanitation services. Meanwhile, raw wastewater effluent entering fresh water bodies, become poisonous to aquatic biota and return the unsafe water that is intended for drinking (Okaiyeto *et al.*, 2016). The consequences of this threat are increase in water borne diseases and an increase in the demand for non-hazardous water for public and industrial needs. The impact of this current situation caused an annual death of 361,000 children under five years old from diarrheal diseases due to poor sanitation, poor hygiene or polluted drinking water which corresponds to diseases like cholera, dysentery, hepatitis and typhoid (WHO/UNICEF, 2017).

Significant awareness was concentrated on water treatment; hence, its significant potential to assess water standard on a constant basis (Yang *et al.*, 2012). The most significant is the development of a new technological approach in water and wastewater treatment to contain a stringent environmental rule on the quality of the wastes that are discharged into water bodies (Wong *et al.*, 2006). Several states have established many strict regulations which consider the occurrence of water pollution, and confirm

appropriate treatment of local, industrial wastewater discharge and agricultural wastewater effluents before the effluent enter different water bodies (Bhatnagara & Sillanpaa, 2010; Li *et al.*, 2013).

2.2 Flocculation Process in Water Treatment

The majority of the water treatment stations should have raw water from the tank to enter the first chamber where flocculant substances are added. Then the water is transferred to a sedimentation pond where the flocculating procedure proceeds and suspended particles precipitate (Jarvis *et al.*, 2012). After this stage the clarified water passes through a filtration process preceeding to disinfect for distribution systems (Rong *et al.*, 2013). Coagulation and flocculation are the most important processes for the removal of particulates and natural organic matter in potable water treatment plants (Keeley *et al.*, 2016; Rong *et al.*, 2013).

Flocculants are naturally occurring organic macromolecules; they are substances that can flocculate suspended solids, cells, and solid colloid particles (Bhunia *et al.*, 2012). Flocculants are widely used in separation techniques, such as in drinking water purification, wastewater treatment, activated sludge dehydration, downstream processing, and food fermentation (Chen *et al.*, 2017). Flocculants also play an important role in flocculating and sludge dewatering processes for the removal of solids and reduction of sludge bulk in wastewater treatment (Lee *et al.*, 2015). Moreover, flocculants are applied as alternatives for filtration and centrifugation in the separation of microbial species from fermented food, pharmaceutical and beverage industres (Shih & Van, 2001),

Flocculation is an operative skill that is normally applied in wastewater treatment for removing a number of metal ions as well as suspended particles (Deng *et al.*, 2003). Bestowing to the bio-flocculation mechanism, the bioflocculants can be adsorbed onto the surface of suspended particles, should in adjacent narrowly enclosed to the suspended

particles and its requisite has the essential sufficient attractive force to control the electrostatic repulsive force (Wang *et al.*, 2011).

Furthermore, Lee *et al.* (2012) stated that the effective and prompt flocculation process was influenced by the size of particles, which implicated that the bigger size had a more rapid settling rate. Besides, the designated flocculants have a key effect on the flocculating process capabilities, accumulated particles potency and quantity as well as formation of strong bounds as the result of the flocculating mechanism (Zhang *et al.*, 2014). For instance, the flocculating competence and magnitude of the poly-electrolytebonds are better than that of ferric chloride.

Regardless of the flocculating process peak effectiveness in water treatment, as well as the main weakness of flocculation, it produces minor-flocs through flocculation at low temperature or produces fragile- flocs that can dissolve with the employment of a physical force (Lee *et al.*, 2014). Therefore, it is important to control these problems and expand the flocculating processes to improve its successful applications.

2.3 Classification of Flocculants

Flocculants are applied in various industrial processes, such as purification of drinking water, wastewater treatments, and dredging / downstream processes and in food fermentation industries (Chen *et al.*, 2017). Flocculants are commonly classified as organic, naturally occurring flocculants and inorganic flocculants, as shown in Figure 2.1.

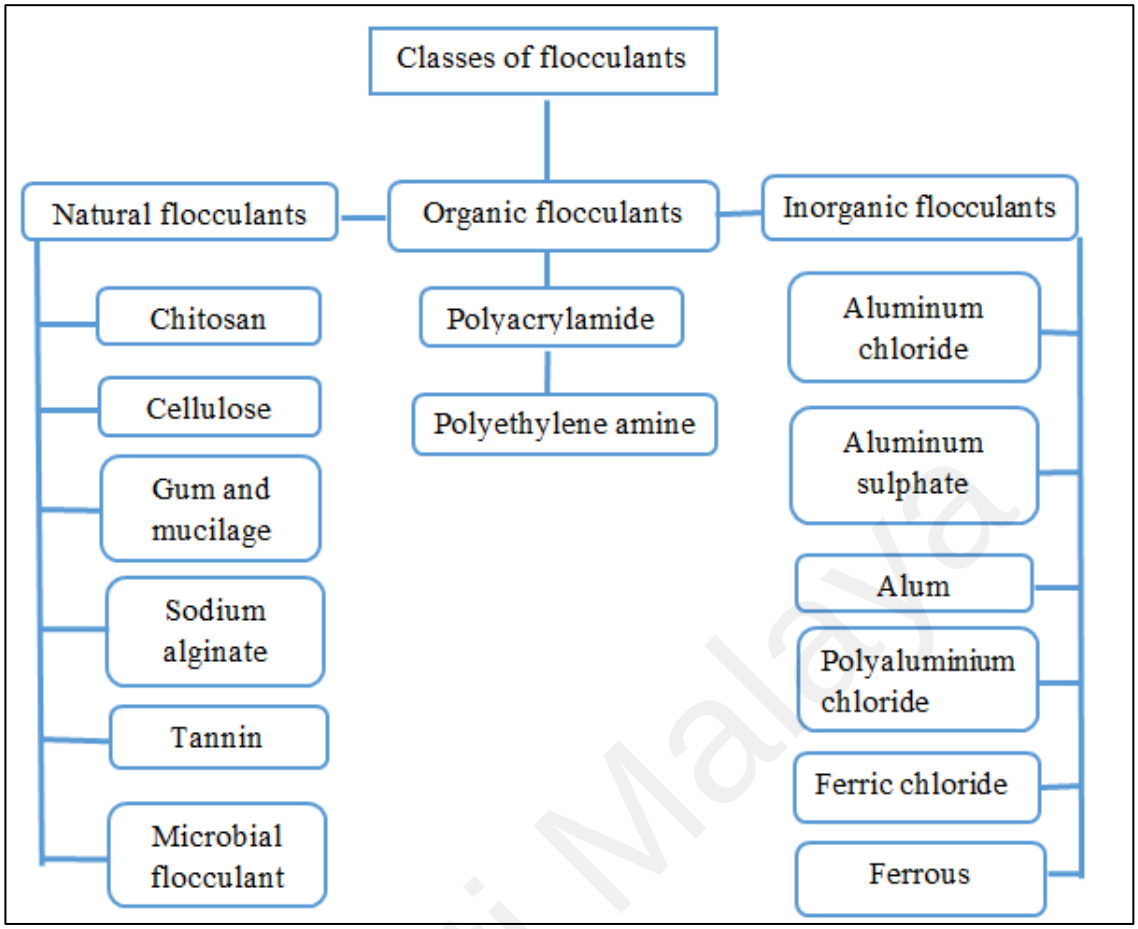


Figure 2.1: Classes of Flocculants.

2.3.1 Organic Flocculants

Organic flocculants are usually applied in several industrial processes as flocculating agents and they comprise of polyethylene amine, polyacrylamide (PAA) and poly-diallyl dimethyl ammonium chloride (PDADMAC) (Lee *et al.*, 2014). As reported by Moussas and Zoulboulis (2009), the acrylamide products are the main type of organic and artificial polymers that are widely applied as flocculation agents due to their cost-efficiency and competence. In nature acrylamide is a white crystalline and a moderately constant monomer that is greatly solvable in water and several organic solvents and it has no scent (Wong *et al.*, 2006). It has a multifunctional group that consists of an amide group and a vinyl carbon-carbon bond with an insufficient double bond that is susceptible to a wide range of chemical reactions (Girma *et al.*, 2005).

However, polyacrylamide (PAA) is a non-ionic polymer, which does not donate extra charges to the flocculating reaction, and thus has no influence on the capability of the inorganic cationic polymers (Okaiyeto *et al.*, 2016). Moreover, the usage of non-ionic organic polymers, such as polyacrylamide might prevail over the extent of the priorly revealed problem encountered with inorganic polymers (Moussas & Zoulboulis, 2009). Besides, these organic polymers are frequently the resultant from non-renewable raw materials or oil-based production (Suopajarvi *et al.*, 2013).

In addition, they generally have a large molecular weight, and contains various polyelectrolyte charges in their molecular sequence which increase their flocculation efficiency (Lee *et al.*, 2014). Huang *et al.* (2014) reported that the volume of sludge produced in wastewater treatment could be condensed by applying artificial polymers like polyacrylamide (PPA) which is not sensitive to pH.

A combination of polyferric sulphate (PFS) with polyacrylamide (PAA) must be present in an improved flocculation activity, while PAA is the excellent flocculating mediator. Synergetic of PAA with PFS would increase the molecular weight of polymers; hence, increasing its flocculation activities as compared to an inorganic polymer (Okaiyeto *et al.*, 2016). Yet, these monomers of the polyacrylamide are not simply decomposed, and thus poised an environmental hazard. In addition, these types of polymer have carcinogenic properties and neurotoxicity. Consequently, these related disadvantages have decreased their usage in many countries (Li *et al.*, 2008).

2.3.2 Inorganics Flocculants

Inorganic flocculants consist of, alum, aluminium chloride, aluminium sulphate polyaluminium chloride, ferrous sulphate and ferric chloride. Meanwhile, the majority of the suspended solids present in wastewater commonly display a negative charge, but

when these metals or salts are added to wastewater they can be ionised to form positive charges which could bind with the negatively charged suspended particles (Lee *et al.*, 2014). This type of interaction decreases the surface charge and formation of micro-floc which can create aggregates to form bigger flocs that are easily settled down in solution (Suopajarvi *et al*., 2013). Amongst these inorganic polymers, polyaluminium chlorides (PAC) are generally applied in drinking water and wastewater treatment. Actually, they are sensitive to pH, ineffective at low temperature, restricted to only limited separation systems and need a larger volume for its flocculating efficiency, and hence produce a bulk capacity of sludge which is activated in wastewater treatment plant systems (Lee *et al.,* 2014; Sharma *et al.*, 2006). Therefore, it is important to investigate the effective technologies that must be rational and operative in excessive sludge recycling, Likewise, many researches have designated that PAC includes aluminium which may spoil potable water and cause severe health complications to users (Banks *et al.*, 2006).

Latest biochemical, neuropathological and epidemiological research were identified as the potential effect of aluminium on the neurological system, as far as aluminium is anxious, the highest brain-health concern is Alzheimer's disease (Lee *et al.*, 2014). Moreover, inorganic flocculants such as ferric polysilicate was discovered to possess smaller molecular weight and flocculation effectiveness as compared to organic flocculants (Moussas & Zouboulis, 2008; Shi & Tang, 2006). Furthermore, despite the flocculation ability of improved ferric polysilicate over ferric sulphate, the addition of polysilicic acid, which have negative charge, can distress the destabilisation capability of the modified flocculants, in the meantime the positive charge on iron classes may be compromised (Moussas & Zouboulis, 2009).

Recently, the awareness of exploiting flocculating compounds in wastewater treatment has attracted more consideration as well as many labour force (Gao *et al.*, 2008). For

example, the greatest flocculation performance was obtained when 1.0 mg/L of PAC and 0.1 mg/L PDMDAAC were used as treatment dose. While the addition of PDMDAAC has improved the natural organic matter (NOM) on removal activities, particularly at low PAC dosages (Shen *et al.*, 2017). For example, the treatment of kaolin suspension or dye solution by using poly-ferric chloride and poly-dimethyldiallyl ammonium chloride (PFC, PDMDAAC), together revealed a better flocculation activity than that of using a single flocculant agent (Gao *et al.*, 2008). Besides, the use of this flocculant compound to the dye solution for textile manufacturing, produced a higher flocculation activity is achieved (Wang *et al.*, 2007). Gao *et al.* (2007) reported that flocculation efficiency was influenced by the proportion of organic flocculants used, as PFC-PDMDAAC which carries more cationic charges as compared to PFC. However, the reduced application of these polymers compound is because they are found merely to be effective in some specific sample treatments which have a higher cationic charge (Moussas & Zouboulis, 2009).

2.3.3 Naturally Occurring Flocculants

Microorganisms produce many polysaccharides that are classified according to their biological functions, such as intracellular storage polysaccharides, extracellular polysaccharides (e.g. alginate, cellulose, sphingan, xanthan) and, capsular polysaccharides (closely interconnected to the outer-surface). Moreover, many bacteria produce a wide-range of exo-polysaccharides which are synthesised by divergent biosynthesis pathways (Jochen *et al.*, 2015). The demand for natural polymers, for example, chitosan, collagen, gelatine and starch, for the treatment of acute and chronic wounds is increasing due to their biocompatibility and biodegradability properties (Zhong *et al.*, 2018).

2.3.3.1 Chitosan

Are hydroxyl (-OH) and amino groups (-NH_2). These two groups possess long-pair electrons that contribute an electron pair to vacant d-trajectories of metal ions; thus, chelating into a chemical compound complex (N-glucosamine) (Okaiyeto *et al.*, 2016). Furthermore, it is a bio-polymer acquired from shellfish sources, a relatively deacetylated polymer induced from the chitin alkaline deacetylation (Lee *et al.*, 2014). Defang *et al.* (2008) found that chitosan is a cationic polymer (polysaccharide) which functions as an artificial polymeric flocculant that could be used in flocculating organic matters in the water and wastewater treatment process ecause they are biodegradable products, environmentally safe and non-corrosive. Szygula *et al.* (2009), reported that chitosan is soluble in diluted organic solvents, while it is insoluble in concentrated organic solvents, as well as water. Chitosan also exists as a solvable cationic polymer, having intensity charge density at acidic pH (Rinaudo, 2006). When chitosan is dissolved in acidic medium, it can synthesise a neutralised amine group through an electrostatic attraction reaction that could eliminate different undesirable heavy metals such as Ag+, Al^{+3}, Ca^{+2}, Cd^{+2}, Cr^{+2}, Pb^{+2}, Hg^{+2} and Zn^{+2} that prevail in the wastewater (Defang *et al.*, 2008).

Chitosan has a strength electrostatic attraction and strong adsorption due to the presence of amino groups (-NH_2) in the molecular sequence which can protonate with H^+ in water to make cationic ammonia (NH^{+3}). As a result, it could coagulate small suspended particles into a large flocculate, which can be effectively removed by sedimentation out of a solution (Yang *et al.*, 2016). Chitosan was found to be very effective in the removal of the chemical oxygen demand (COD) and suspended solid substance (SS) in water treatment of contaminated water with organic materials (Bolto, 1995; Ishii *et al.*, 2008). Moreover, it has various benefits over the conventional polymeric flocculants that are commonly applied in water treatment. These advantages

comprise: a small dose requirement, quicker residue ingrate, high COD reducing ability, heavy metals and suspended solids. Likewise, it is employed to decrease the production of bulky sludge mass that is commonly produced by inorganic polymer and it does not produce any secondary pollution (Renault *et al.*, 2009). On account of high density of chitosan, it enhances to form larger flocs, which will increase the floc settling volume and decrease the duration of the sedimentation phase (Renault *et al.*, 2009). Even though chitosan is potent in water treatment, it is expensive, and hence its usage effectiveness increases in the overall treatment costs.

2.3.3.2 Sodium Alginate

Sodium alginate is a linear anionic polymer, which is water-soluble and is obtained from the sodium salt of alginic acid and possess a molecular weight of approximately 500,000 (Wu *et al.*, 2012). Sodium alginate was evaluated for its flocculation efficiency in combination with aluminium sulphate as coagulants in the fabric wastewater effluents with artificial dyes which revealed a rugged flocculation efficiency, reduction of colour removal of about 90% and COD of around 80% (Wu *et al.*, 2012). Moreover, sodium alginate was used as the coagulant facilitate which can lead to stronger and larger flocs which could enhance treatment efficiency of the coagulation process (Wang *et al.*, 2014).

2.3.3.3 Tannin

Tannin is an anionic organic substance that are are described as a harmless flocculant which can be practically applied as an alternative for frequent use of polymers in water treatment, owing to its safety to humans, biodegradability and environmental friendly (Ozacar & Sangil, 2000). Tannin is derived from the secondary metabolites of plant tissues, green leaves and fruits of vegetables (Beltran-Herediaa & Sanchez-Martin, 2009). Previously, many investigators have established the flocculation competency of

tannin in the removal of colloidal and suspended particles present in potable water after treatment, in addition to the removal of suspended particles from artificial wastewater and the removal of inks, dyes, and colours from industrial wastewater (Roussy *et al.*, 2005). Lee *et al.* (2014) indicated that the combination of tannin with aluminium sulphate as a coagulant to stabilise the negative charge of colloidal particles.

2.3.3.4 Cellulose

Cellulose is an insoluble material that is a major component of the plant cell wall and plant fibres and very abundant in natural polysaccharides acquired from agriculture wastes (Lee *et al.*, 2014). In current years, cellulose has been the theme of studies due to several applications in industries, such as paper, textiles and forestry products (Caciced *et al.*, 2016). Khiari *et al.* (2010) reported that the carboxymethyl-cellulose anionic sodium (CMCNa) is a classical sample of flocculants derived from cellulose, which are designated as environmentally friendly and is used as a composite with aluminium sulphate as a flocculant for the removal of turbidity in potable water. Despite the fact, cellulose is widely known as a plant product, while some bacteria have acquired consideration as a sustainable and alternative source of cellulose by using *Komagataeibacter xylinus* as the representative of bacterial cellulose producer (Caciced *et al.*, 2016).

Plant cellulose and bacterial cellulose have the same chemical formula, but bacterial cellulose has fine physical properties with respect to thermos-stability, mechanical stability, tensile strength, purity, crystal fibrous structure and bio-compatible substance (i.e. biologically non-toxic and no immune system response) (Cacicedo *et al.*, 2016; Zhang *et al.*, 2015). Moreover, Suopajarvi *et al.* (2013) described that an ionised dicarboxylic acid nano-cellulose (DCC) bioflocculant was obtained from cellulose, which

has a strength flocculation property in the prevalence of ferric sulphate in municipal wastewater treatment.

2.3.3.5 Bioflocculant

Recently, natural polymers requirement for different industrial uses havewere directed to an important development in the exopolysaccharide production (EPS). Typically, it is a composite with large molecular weight and has long-chain mix of polymers, consisting of branched repeating units of sugar or carbohydrate derivatives, such as glucose, galactose, fructose, and mannose which are synthesised and secreted by different growing microorganisms (Ismail & Namoothiri, 2010; Sheng *et al.*, 2010).

Microbial flocculation was first described in a yeast strain *levur casseeuse* by Louis Pasteur in 1876. Subsequent, a similar phenomenon was detected by Bordet (1899), in bacterial cultures; vast studies of flocculation mechanism by using Zoogloea- forming bacteria isolated by Butterfield (1935) from activated sludge presented a direct association between the cell aggregation and accumulation of extracellular bioflocculants. The involvement of extracellular produced compounds in microbial flocculation was further confirmed when Tenny and Stunn (1965) suggested that flocculation by microorganisms was mediated by polymers they excreted extracellularly.

According to Aljuboori *et al.* (2015), the use of cell-free supernatant, was surveyed for their ability to disrupt the spread of bacteria. So far, over hundreds of bioflocculant-producing microorganisms are reported in literature with the produced bioflocculants extensively characterised. Among the exopolymeric substance (EPS) discovered in the literature, those that have flocculation property are mainly concerned with the subjects of bioflocculation and this recommends their application in different industrial processes and water treatment. Due to the limits of these organic and inorganic polymers,

bioflocculants have acquired vast scientific considerations because of its extraordinary flocculating activities and biodegradability, non-secondary toxic waste, and their deteriorating intermediates are harmless for humans and their immediate environment (Mabinya *et al.*, 2012; Shahadat *et al.*, 2017).

The bioflocculants are biological macromolecules eco-friendly, and were successfully confirmed for wastewater treatment (Pathak *et al.*, 2017). Moreover, polysaccharides produced by *cyanobacteria* are considered as environmentally friendly, and illustrate an efficient ability to adsorp metals or inorganic substances. Therefore, the type of polysaccharides is suitable for formulating eco-friendly and functional bionanocomposites with different rigid materials (Okajima *et al.*, 2018). Therefore, it has become essential to isolate and detect novel bioflocculant producing microorganisms and determine procedures for their optimum production conditions to recover the bioflocculant yield or by using a consortium microbe to enhance the bioflocculant production (Okaiyeto *et al.*, 2013). Exopolysaccharides (EPS) are generally complex in nature with heterogeneous compounds. Their components, position and locality are dependent on numerous metabolic processes, for instance, modifications in logarithmic growth phase secretion, cell rupture due to cell death and releasing on the cell surface macromolecules, i.e. lipopolysaccharides (LPS) and outer membrane protein and their interaction with the instant environmental conditions (Cristina *et al.*, 2011). Abdel-Aziz *et al.* (2011) detected the definite enzymes that exist in clusters which regulate the exploitation of nutrient in culture media by microorganism. This was to synthesised bioflocculant with large molecular weight, which secreted or form a capsule on the surface of the microbial cell. EPSs are commonly named as exopolysaccharides due to their position; this is to distinguish them from other classes of polysaccharides that could be made by enclosed cells (Nwodo *et al.*, 2012).

For example, Morris and Harding, (2009) reported that naturally the capsular polysaccharides tremendously provoke the immune system (immunogenic), and might have altered their infrequent biological diversity as a mode of escaping the antibody response to develop/design a vaccine production. Furthermore, they act a major role in the adherence and penetration of the host cells. Exopolysaccharides are the best significant component of biological coagulates and are more effective on the deprivation of organic matters from wastewater, which also contain activated sludge and biofilms after treatment (Martin- Cereced *et al.*, 2001). Exopolysaccharides stimulate the formation of bioflocs by improving the relation between bacterial clusters, diverse bacterial strains, as both inorganic and organic particles. Moreover, their essential starring role is to clamp the microbial cells together (Sheng *et al.*, 2010). They are commonly occurring as a sheet on the surface of the microbial cell, and thus provide protection for the microbial cell against adversative environmental conditions, for example, temperature, oxygen tension, high osmotic pressure and noxious substance. Likewise, Nichols *et al.* (2005) reported that EPSs might contribute to the adsorption of heavy metals as well as avert desiccation under definite environmental conditions.

Recently, bioflocculants are produced by many kinds of microorganism, such as algae, actinomycetes, bacteria and fungi which were found and isolated from different habitats and marine environment (i.e. soil, water, activated sludge) which sustain a fertile biodiversity of microorganisms, remaining largely unexplored (Aljuboori *et al.*, 2013; Cosa & Okah, 2014; Czemierska *et al.*, 2017; Kumari *et al.*, 2014; Nwodo *et al.*, 2014; Okaiyeto *et al.*, 2014; Tiwari *et al.*, 2015).

2.4 Composition Analysis and Flocculating Potential of some Bioflocculants

Many researches have publicised that the majority of bioflocculants produced are either functional proteins or functional carbohydrates (Huang *et al.*, 2005), for example,

the EB-EPS bioflocculant secreted by *Enterobacter* sp. whereby Fourier-transform infrared (FTIR) spectrometry analysis of the purified EB-EPS showed the occurrence of carboxyl, hydroxyl and amide groups. Chemical analysis showed that the purified EB-EPS contain 88.7% w/w of carbohydrate and 11.3% w/w of protein (Muthulakshmi *et al.*, 2017). Muthulakshmi's *et al.* (2017), study showed and identified that the bioflocculant, named B1-EPS produced by *Bacillus* sp., comprise of carbohydrate (92.8% w/w) and protein (21.8% w/w). The bioflocculant PSK1 produced by *Bacillus aryabhattai* is mainly composed of glycoprotein which consist of glucose and rhamnose with some amino acids, such as arginine and phenylalanine. FTIR spectrum analysis of PSK1 indicated the presence of amino and hydroxyl groups (Ayat *et al.*, 2017). Czemierska *et al.* (2016), found that the bioflocculant secreted by *Rhodococcus opacus* contain polysaccharides (64.6% w/w) and protein (9.44% w/w) and the bioflocculant constituent sugars were glucose, galactose and mannose. Furthermore, the FTIR spectrometry examination of purified bioflocculant from *Bacillus pumilus* showed the manifestation of amino, hydroxyl and carboxyl groups and the bioflocculant composed of sugar (75.4%), protein (5.3%) and uronic acid (15.4%) (Makapela *et al.*, 2016). Moreover, novel bioflocculant have functional groups, i.e. hydroxyl, amino and carbonyl and its carbohydrate and protein content were estimated to be 57.12% and 31.7% protein, respectively, while the polysaccharide content comprised of four monosaccharides, i.e. D-xylose, D-fructose, maltose and mannose (Sun *et al.*, 2015). The bioflocculant secreted by *Bacillus* sp. was found to be water soluble and insoluble in organic solution, FTIR spectrum analysis showed that the manifestation of carboxyl, hydroxyl and carbohydrate derivatives in the polymers (Karthiga & Natarajan, 2015). Nwodo *et al.* (2014), found that the bioflocculant synthesised by a consortium species of *Actinobacteria* i.e. *Cellulomonas* and *Streptomyces*, FTIR spectrum analysis specified the manifestation of carboxyl, hydroxyl and amino groups, with molecular weight 494.81- 18,330.26 Da. The chemical analysis

of purified polymers also showed the presence of polysaccharides and protein and uronic acids. Yin *et al.* (2014) established that the novel bioflocculant synthesised by *klebsiella* sp. ZZ-3, revealed that its chemical analysis had carbohydrates (84.6%) and protein (6.1%) with relatively high molecular weight of 603-1820 kDa. The bioflocculant MBF-TG-1 produced by *klebsiella* sp. TG-1 primarily composed of polysaccharides (84.6%) and protein (11.2%) (Liu *et al.*, 2013). Gomaa E.Z. (2012) indicated that the bioflocculant from *Pseudomonas aeruginosa* ATCC-10145, composed of sugars (89% w/w) and protein (27% w/w) and the infrared spectrometry analysis revealed that the purified exopolymer comprised of hydroxyl, carboxyl, amino and sugar derivative groups.

He *et al.* (2010), indicated that the bioflocculant HBF-3 secreted by *Halomonas* sp. V3a was isolated from deep sea was composed of carbohydrates, comprising of neutral sugar (20.6% w/w) , uronic acid (7.6% w/w) , amino sugar (1.6% w/w) and sulphate group (5.3 % w/w) , and with molecular weight of 595KDa. Likewise, bioflocculant MBFA9 secreted by *B. mucilaginosus* composed of carbohydrates containing primarily of neutral sugar (47.4% w/w), uronic acid (19.1% w/w) and amino sugar (2.7% w/w) and the FTIR spectrometry analysis showed the occurrence of hydroxyl and carboxyl groups as the essential function moieties (Deng *et al.*, 2003). Feng and Xu (2008) found that the acidic bioflocculant synthesised by *Bacillus* sp. BF3-3 contained polysaccharides (66.1% w/w) and protein (29.3% w/w). The produced bioflocculant by *Aspergillus parasiticus* mainly composed of carbohydrate (76.6% w/w) and protein (21.6% w/w) with an average molecular weight of 3.2×10^5 Da (Deng *et al.*, 2005).

This type of bioflocculant display a high flocculating efficiency against kaolin clay suspension, and it has the capability to flocculate dye from synthetic suspension. Deng *et al.* (2005) described that the manifestation of amino, carboxyl, hydroxyl, and amide functional groups in the molecular sequence of bioflocculants were the major functional

moieties, which played an essential part in the flocculating processes of suspended particles because these functional groups were responsible for adsorption sites, whereby the suspended particles could be bound. The exopolymers secreted by *Bacillus* genus and isolated from a Qatari ecosystem, were applied against kaolin suspension, indicating a flocculating activity of about 85% at 20 mg/L concentration (Desouky *et al.*, 2008). The flocculating activity of the bioflocculant secreted by *B. mucilaginosus* by using kaolin clay suspension was found to be 99.6% at a concentrated dose of 0.1mg/L (Deng *et al.*, 2003). Similarly, bioflocculant secreted by *Vagococcus* sp. W31 mainly composed of polysaccharide, with molecular weight of 2×10^6 Da, with high flocculation activity in a wide-range of pH (7-11) at a prerequisite dosage of 25 mg/L (Gao *et al.*, 2006). Moreover, the bioflocculant derived from *Corynebacterium glutamicum* was found to be a polysaccharide as well as it revealed thermo-stability in an acidic range of the pH 30 to pH 6.5 (He *et al.*, 2004). The flocculation effectiveness of the bioflocculant was comparatively higher as compared to artificial flocculants. These characteristics designate its application in the decolourisation of molasses effluent waste. The FTIR spectrum analysis presented the manifestation of carboxyl and hydroxyl groups in the molecular sequence (He *et al.*, 2010).

However, some of these bioflocculant composed higher quantity of protein than polysaccharides. For example, the intracellular bioflocculant called MBF-W6 synthesised by *C. daequense* W6, is mostly composed by protein (32.4%) and polysaccharides (13.1%) (Liu *et al.*, 2010). The bioflocculant synthesised by any microorganisms are a pre-determining factor that affects its flocculation competence (Gao *et al.*, 2006).

2.5 The Functional Groups in Bioflocculant Molecule

The functional group in bioflocculant molecule plays very a significant role in the flocculation process. Therefore, studies on bioflocculant production and Characterisation

investigated the manifestation of functional groups in bioflocculant molecules to recognise and determine their flocculation performances and mechanism. For example, many studies exhibited the presence of carboxyl, hydroxyl, amino and methoxyl groups in bioflocculant molecules were identified to contribute to the flocculation of colloids, such as those detected in polyelectrolytes (Li *et al*., 2009; Salehizadah & Shojaosadati, 2001). According to Zheng *et al.* (2008) finding the presence of hydroxyl (OH), carboxyl (COOH) groups in the bioflocculant contents and OH^-, H^+, groups on the surface of the constituent part can perform hydrogen bonds when the bioflocculant chains are attached to the surface of particles.

2.6 Bioflocculation Mechanisms

Investigation on the flocculation mechanism of bioflocculants discovered the flocculation performance of bioflocculant in flocculation process. Many researches presented the components comprised of bioflocculants which regulated the flocculation mechanism and capability (He *et al.*, 2010; Zhang *et al.*, 2010). However, the main bioflocculation mechanism could be divided into: charge neutralisation and polymer bridging.

2.6.1 Charge Neutralisation

Charge neutralisation is one of the major bioflocculant mechanisms where the negative charge of impurities, either particles or colloids, are neutralised by positively charged bioflocculants (Lian *et al*., 2008). The occurrence of amino groups in bioflocculant molecules could be simply protonated and result in a positive charge (Deng *et al.*, 2005). Consequently, electrostatic interaction between the positively charged bioflocculant and negatively charged colloids might produce electron attraction and charged neutralisation of the colloid surface, controlling the formation of flocs and decrease the electrical attraction between them (Figure 2.2). Based on the above mechanism, this bioflocculant

was classified as cation-independent bioflocculant, including bioflocculants produced by *Klebsiella* sp. ZZ-3(Yin *et al.*, 2014), *Solibacillus silvestri* W01 (2013). *Pseudomonas aeruginosa* ATCC-10145 (Gomaa. 2012), *B. subtilis* (Bhunia *et al.*, 2012), *B. mojavensis strain* 32A (Elkady *et al.*, 2011), *Methylobacterium* sp. (Ntsaluba *et al.*, 2011), *B. licheniformis* (Shih *et al.*, 2010), *Chryseobacterium daeguense* W6 (Liu *et al.*, 2010), *B. mucilaginosus* (Lian *et al.*, 2008), *Klebsiella* sp. (Sheng *et al.*, 2006).

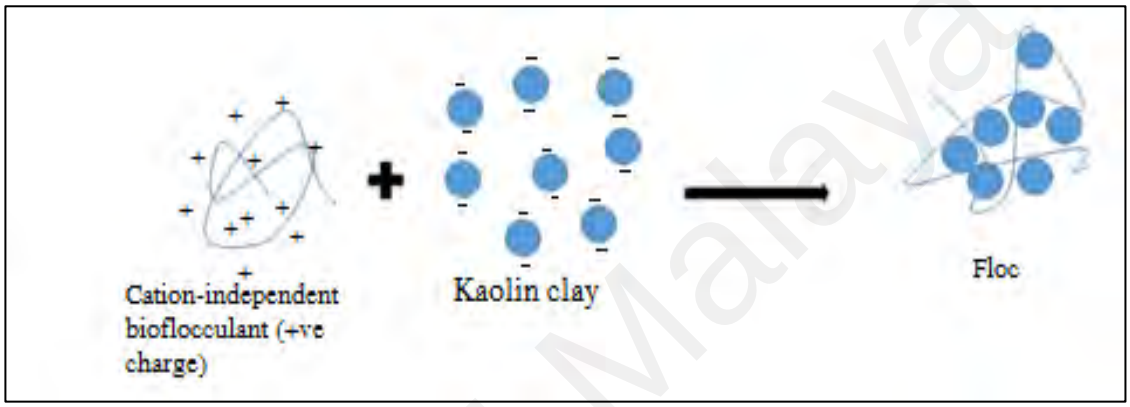


Figure 2.2: Charge Neutralisation Flocculation Mechanism (Reproduced with permission from the Library, Bhavnagr University, India)

2.6.2 Polymer Bridging

Biopolymer bridging suggested the flocs formed primarily by cation mediated bridges between the bioflocculant molecules and kaolin particles (Sobeck & Higgins, 2002). The cation could prompt the bioflocculation by neutralisation and destabilisation of residual negatively charged carboxyl groups of the bioflocculant, establishing bridges which bind kaolin particles to each other (Prasertsan *et al.*, 2006). Figure 2.3 shows the polymer bridging flocculation mechanism. Based on this mechanism, this type of bioflocculants is classified as cation-dependent bioflocculant. Many studies presented that bioflocculants are produced by *Actinomycete*, S*treptomyces* sp. hsn06 (Li *et al.*, 2017), *Bacillus* sp. AEMREG4 (Ntsangani *et al.*, 2017). *Bacillus* sp. (Okaiyeto *et al.*, 2016), *B. amyloliquefaciens* ABL19 (Ogunsade *et al.*, 2015), *Arthobacter* sp. B4 (Li *et al.*, 2014). *Klebsiella* sp. TG-1 (Liu *et al.*, 2013), *Klebsiella mobilis* (Wang *et al.*, 2007),

Enterobacter cloacae WD7 (Prasertsan *et al.*, 2006), *Enterobacter aerogenes* (Lu *et al.*, 2005), *Sorangium cellulossum* NUST06 (Zhang *et al.*, 2002b), *Enterobacter* sp. BY-29 (Yokoi, 1997), *Bacillus subtilis* (Yokoi *et al.*, 1996) were cation-dependent bioflocculants.

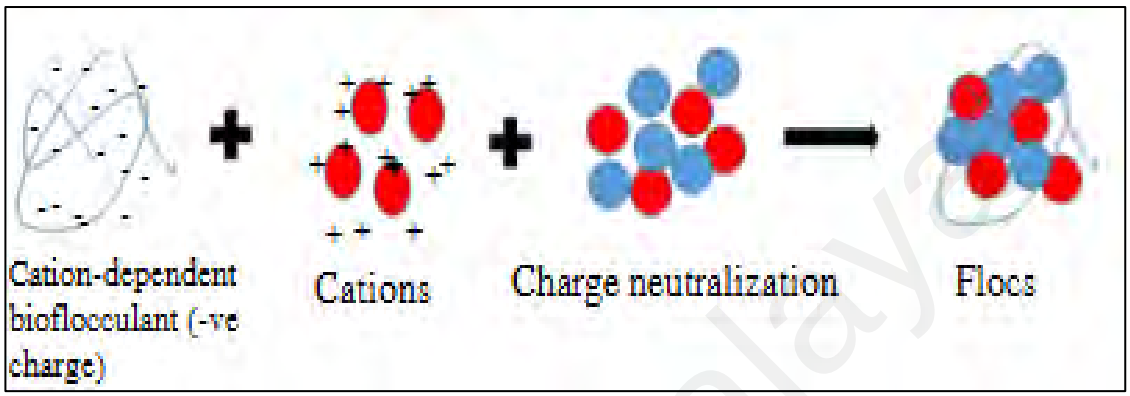


Figure 2.3: Bioflocculant Bridging Flocculation Mechanism (Reproduced with permission from the Library, Bhavnagr University, India)

2.7 Factors Affecting Bioflocculant Production

Bestowing to the presented literature study, the production of microbial bioflocculants was extremely affected by the composition of the production medium and some other physicochemical parameters (Fang *et al.*, 2013). Besides, these discoveries indicated that the bioflocculant production was affected by many factors that comprised of the culture medium ingredients in growth addition, environmental conditions (He *et al.*, 2004). The effects of the nutritional constituents have been widely examined for the production of bioflocculants (Abdel-Aziz *et al.*, 2011).

The influence of the essential factors, such as the initial pH of the production medium, carbon, nitrogen source, inoculum size, culture time, ionic strength, metal ion, shaking speed, and incubation temperature, greatly affect the bioflocculant production (He *et al.*, 2004). Remarkably, in the bioflocculants production, the microorganisms can consume uncommon carbon sources as nutrients besides the production of other bacterial

secondary metabolites. For example, *Rhodococcus* sp. is efficiently consumed by the alkanes in biosurfactant, to produce bioflocculants (Pathak *et al.*, 2017). Also, some studies illustrated that the potential of microbes to utilise petroleum hydrocarbon during its metabolic pathway to produce bioflocculants (Pathak *et al.*, 2015).

2.7.1 Effect of Carbon and Nitrogen Sources

Many researches have recognised the implication of carbon and nitrogen sources, which played a crucial role in improving the bioflocculant production by many microorganisms (Salehizadeh & Yan, 2014). For example, Makapela *et al.* (2016) found that the maltose was the sole carbon source, as well as nitrogen sources, such as urea, yeast extract and ammonium sulphate and initial pH 7, were favoured by *Bacillus pumilus* for bioflocculant production. Meanwhile, the best preferred carbon, nitrogen sources for consortium culture of *Bacillus sphaericus* F6 and *Rhizobium radiobacter* F2 were found to be glucose and urea for producing a novel bioflocculant CBF (Li *et al.*, 2016). Nwodo *et al.* (2014) found that the consortium of *Cellulomonas* and *Streptomyces* species for bioflocculant production, sucrose, peptone and magnesium chloride were favoured as carbon and nitrogen sources, giving flocculating activity of 91% and 82%, respectively. Lee *et al.* (2014), stated that *B. licheniformis* X14 favoured sucrose, starch and ethanol as the preferred carbon sources for the production of bioflocculant ZS-7, while ammonium chloride was the favoured choice as the nitrogen source. Sheng *et al.* (2006) found that maltose and urea are the most preferred carbon and nitrogen sources for bioflocculant production by *Klebsiella* sp. Cosa *et al.* (2013b) detected that tryptone and sodium carbonate were the best appropriated nitrogen source for *Oceanobacillus* sp. to produce bioflocculants. Tryptone was chosen as the organic nitrogen source for the bioflocculant production via *Chryseobacterium daeguense* W6 (Liu *et al.*, 2010). Whereas, the organic carbon sources, such as glucose, lactose, maltose, sucrose and xylitol are all appropriate

substrates for *Paenibacillus polymyxa* BY-28 to produce the bioflocculant (Gong *et al.*, 2011).

The novel bioflocculant REA-11 synthesised by *Corynebacteria glutamicum*, sucrose was utilised as a carbon source and composite nitrogen source containing corn steep liquor and urea as documented by (He *et al.*, 2004). Cosa *et al.* (2013a) reported that glucose was favoured as carbon source among other sources examined for the production of bioflocculant by *Virgibacillus* sp. Whereas, as presented glucose, glycerol, corn starch, and sucrose. were suitable substrates for *Aspergillus parasiticus* to produce bioflocculant, performing a good flocculating activity of over 80% at three days of fermentation (Deng *et al.*, 2005).The optimum carbon source for bioflocculant production by *Solibacillus silvestris* W01 was prevailed after maltose was used as the carbon source in the cultivation medium (Wan *et al.*, 2013).

For instance, *Sorangium cellulosum* utilised soluble starch as a carbon source to sustain the optimum bioflocculant production, while the addition of 3 g/L glucose as an enhancement, completely inhibited cell binary fusion and synthesis of the bioflocculant (Zhang *et al.*, 2002). But, the lactose, glucose, and fructose were not appropriate for *B. licheniformis* to produce bioflocculant, while the simultaneous occurrence of numerous carbon sources, such as citric acid, glutamic acid, and glycerol in the production fermentation media hadenhanced cell growth and bioflocculant yield (Shih *et al.*, 2010). While, bioflocculant yield was increased by the optimised strain of *Penicillium* sp. HHE-P in the fermented medium, consisted of glucose and yeast extract.

Glucose was the greatest preferable carbon source for bioflocculant production by most microorganisms, but the higher cost of glucose increased the production cost (Liu & Chen, 2010). For instance, while molasses was replaced by glucose, flocculation efficiency against kaolin suspension achieved over 90%, a strong significant indication

confirmation has revealed that different bacterial strains could exploit as either organic, inorganic nitrogen source or their mixture sources for producing bioflocculants (Gong *et al.*, 2008).

The production of exopolysaccharides by *Rhizobium radiobacter* SZ4S7S14 was optimised for bioflocculant yield enhancement, where the D-Mannose and yeast extract were utilised as carbon, nitrogen sources (Rasulov *et al.*, 2017). However, when peptone merged with inorganic sodium nitrate, it was found to be the most appropriate nitrogen source for *Aspergillus parasiticus* for bioflocculant synthesis (Deng *et al*., 2005).

However, when combined with $(NH_4)_2SO_4$, no bioflocculant was produced. Deng *et al.* (2005) found that the organic nitrogen sources, were preferred and had enhanced bioflocculant yield in many bacterial strains. For instance, when both beef extract and urea was utilised as a nitrogen source by the strain of S-14, it was found to be the most appropriate for the production of bioflocculants. Meanwhile, strain TJ-1 was successfully capable of consuming peptone, beef extract, and yeast extract as a nitrogen source. However, when the peptone was used as the sole organic nitrogen, the source was found to increase the bioflocculant yield and was the cheapest substrate (Xia *et al.*, 2008)., Cosa *et al.* (2013a) also examined the efficiency of a composite nitrogen source, containing of yeast extract, urea and $(NH_4)_2\ SO_4$ to enhance the optimum production of bioflocculant by *Virgibacillus* sp. Likewise, Gong *et al.* (2008) reported that the combined nitrogen source, such as beef extract and urea revealed a considerable enhancement in production of bioflocculant by *Serratia ficaria* among others that were examined.

Correspondingly, the bioflocculant was produced by mixing strains of *Acintobacter, Agrobacterium* and *Enterobacter* species, in which the medium were merged with a yeast extract complex and ammonium sulphate as the nitrogen source (Kurane & Matsuyama, 1994). The sodium nitrate and peptone were found to be the most favoured nitrogen

source among other sources examined by *A. parasiticus* for the production of bioflocculant (Deng *et al.*, 2005). Likewise, a study by Li *et al.* (2013) confirmed that the peptone was found to be the most preferable nitrogen source for the *Paenibacillus elgii* B69 to produce bioflocculant among other assessed sources.

Table 2.1: Optimum Culture Conditions, Chemical Compositions, Flocculating Activity and Yields of some Bioflocculant.

Microorganism	**Source**	**Carbon source**	**Nitrogen source**	**Chemical composition**	**Flocculating activity (%)**	**Yield**	**Reference**
Achromobacter xylosoxidans	Activated sludge	Sucrose	Yeast extract +urea	Glycoprotein	83.3	$5gL^{-1}$	Subudhi *et al.* (2016).
Aeromonas sp.	Activated sludge	Corn flour	Soyabean flour	Polysaccharide	49.34	NA	Li *et al.* (2008).
Aeromonas sp.	Activated sludge	Glucose	Peptone	Polysaccharide	92.4	2.25	Li *et al.* (2007).
Arthrobacter sp. Raats	Freshwater	Lactose	Urea	Glycoprotein	87.5	NA	Mabinya *et al.* (2012).
Aspergillus parasiticus	NA	Starch	Pepton+sodium nitrate	Glycoprotein	98.1	NA	Deng *et al.* (2005)
Aureobasidium pullulans	NA	Sucrose	$NaNO_3$	Polysaccharide	12.5	NA	Ravella *et al.* (2010).
Bacillus aryabhattai	Soil	Glucose	Yeast extract	Glycoprotein	92.8	-6g/l	Ayat *et al.* (2017).
Bacillus sp. AEMREG7	Marine	Glucose	Urea + yeast extract + $(NH_4)_2$ SO_4	Glycoprotein		1.6	Okaiyeto *et al.* (2015b).
Bacillus sp. AEMREGH	Thyume river	Starch	Yeast extract	Glycoprotein	76	NA	Nozipho *et al.* (2017).
Bacillus alvei NRC-14	Soil	Chitosan	Yeast extract	Polysaccharide	98	10	Abdel-Aziz *et al.* (2011).
Bacillus clautti	Brewery wastewater	Glucose	NA	Glycoprotein	88.67	NA	Adebayo, T.B & Adebami, G.E. (2014).
Bacillus sp. Gilbert	Marine	Sucrose	Ammonium chloride	Polysaccharide	91	NA	Piyo *et al.* (2011).

Table 2.1, continued.

Microorganism	Source	Carbon source	Nitrogen source	Chemical composition	Flocculating activity (%)	Yield	Reference
Bacillus licheniformis	Contaminated LB medium	Sucrose	Yeast extract + urea	Glycoprotein	700 U/ml	2.94	Xiong *et al.* (2011).
Bacillus licheniformis X14	Soil	Glucose	NH_4 Cl	Glycoprotein	99.2	NA	Li *et al.* (2009).
Bacillus sp.	Maya Marine	Glucose	Ammonium nitrate	Glycoprotein	95.6%	. NA	Ugbenyen & Okoh. (2013).
Bacillus subtilis	Soil	Cane molasses	Yeast extract	Polysaccharide	NA	4.92	Abdul-Razack *et al.* (2014).
Bacillus subtilis F9	Wastewater sludge	Sucrose	Peptone	Glycoprotein	NA	2.32	Giriet *et al.* (2015).
Bacillus toyonesis strainAEMREG6	Marine	Glucose	NH_4 NO_3	Glycoprotein	89.5	3.2	Okaiyeto *et al.* (2015a).
Bacillus velezensis 40B	Brackish water	Glucose	Yeast extract	Glycoprotein	99.9	3.54	Zaki *et al.* (2013).
Brachybacterium sp.	Freshwater	Maltose	Urea	Glycoprotein	91.2	NA	Nwodo *et al.* (2013a).
Brachybacterium sp.	Freshwater	Maltose	Urea	Glycoprotein	87.8	NA	Nwodo *et al.* (2013a).
Cellulomonas sp. Okoh	Freshwater	Glucose	$(NH_4)_2$ SO_4	Glycoprotein	86.3	4.47	Nwodo & Okoh. (2013).
Chryseobacterium daeguense W6	Backwashing sludge	Glucose	Trypetone	Glycoprotein	96.9	NA	Liu *et al.* (2010).
Citrobacter sp. TKF04	Soil	Propionic acid and acetic acid	Yeast extract	Glycoprotein	85	0.2	Fujita *et al.* (2000).

Table 2.1, continued.

Microorganism	Source	Carbon source	Nitrogen source	Chemical composition	Flocculating activity (%)	Yield	Reference
Cobetia sp. OAUIFE	Marine	Glucose	Urea+Yeast extract+$(NH_4)_2SO_4$	Glycoprotein	92.78	NA	Ugbenyen *et al.* (2012).
Coryneobacillus glutamicum NA	NA	Corn steep liquor	Urea	NA	520U/mL	NA	He *et al.* (2004).
Enterobacte aerogenes	Soil	Fructose+Glucose	Urea+Yeast extract + $(NH_4)_2$ SO_4	Glycoprotein	80	1.3	Lu *et al.* (2005).
Enterobacter cloacae WD7	Activated sludge	Glucose or sucrose	$(NH_4)_2$ SO_4	Polysaccharide	105	2.27	Prasertsan *et al.* (2006).
Halobacillus sp. Mvuyo	Marine water	Glucose	Ammonium chloride	Glycoprotein	93	0.34	Cosa *et al.* (2012).
Halomonas sp. Okoh	Marine	Glucose	Urea	Polysaccharide	95	NA	Mabinya *et al.* (2011).
Halomonas sp.	Marine sediment	Glucose	Urea	polysaccharide	95	NA	Mabinya *et al.* (2011).
Klebsiella mobilis	Soil	Dairy wastewater+ Ethanol		Polysaccharide	95.4	2.58	Wang *et al.* (2007).
Klebsiella pneumoniae	Human saliva	Glucose	Peptone	Glycoprotein	96.5	4.7	Luo *et al.* (2014).
Klebsiella pneumoniae	Sputum	Glucose	Urea + Yeast extract+$(NH_4)_2$ SO_4	Glycoprotein	98	NA	Zhao *et al.* (2013).
Klebsiella sp.	Activated sludge	Glucose	Yeast extract + Urea	Polysaccharide	86.5	3.52	Yang *et al.* (2012).
Klebsiella sp.	Activated sludge	Glucose	Yeast extract + Urea	Polysaccharide	86.5	1.8	Yang *et al.* (2012).

Table 2.1, continued.

Microorganism	Source	Carbon source	Nitrogen source	Chemical composition	Flocculating activity (%)	Yield	Reference
Klebsiella sp. TG-1	Wastewater	Sucrose	Beef extract	Glycoprotein	86.9	NA	Liu *et al.* (2013).
Klebsiella sp. ZZ-3	Sludge	Glucose	$NaNO_3$	Glycoprotein	92.6	0.126	Yin *et al.* (2014).
Methylobacterium sp.	Freshwater	Glucose	Urea+Yeast extract+$(NH_4)_2$ SO_4	Glycoprotein	95	8.203	Ntsaluba *et al.* (2013).
Methylobacterium sp.	Freshwater	Glucose	Peptone	Polysaccharide	72	NA	Ntsaluba *et al.*, (2011).
Micrococcus sp. Leo	Marine	Glucose	Urea+Yeast extract	Glycoprotein	87.5	0.738	Okaiyeto *et al.* (2014).
Paenibacillus elgii B69	Soil	Sucrose	Peptone+Yeast extract	Polysaccharide	87	25.63	Li *et al.* (2013).
Paenibacillus mucilaginosus	Soil	Sucrose	Yeast extract	Polysaccharide	97	NA	Tang *et al.* (2014).
Paenibacillus polymxya BY-28	Soil	Sucrose	Bean cake powder	Glycoprotein	99	NA	Gong *et al.* (2011).
Penicillium purpurogenum	NA	Glucose	Yeast extract	Polysaccharide	96	6.4	Liu &Cheng. (2010).
Rhodococcus erythropolis	Activated sludge	Livestock wastewater	NA	Glycoprotein	87.6	1.6	Peng *et al.* (2014).
Serratia ficaria	Soil	Lactose	Yeast extract	Polysaccharide	95.4	NA	Gong *et al.* (2008).
Solibacillus silvestris	Marine	Maltose	Yeast extract	Glycoprotein	90	1.7	Wan *et al.*, (2013).

Table 2.1, continued.

Microorganism	Source	Carbon source	Nitrogen source	Chemical composition	Flocculating activity (%)	Yield	Reference
Sorangium cellulosum	NA	Soluble starch	$NaNO_3$	Glycoprotein	96.6	17.5	Zhang *et al.* (2002).
Streptomyces and Brachybacterium species	Freshwater	Glucose	NH_4 NO_3	Polysaccharide	63.7	3.02	Nwodo & Okoh. (2014).
Virgibacillus sp.	Marine	Glucose	Urea+ $(NH_4)_2$ SO_4 +Yeast extract	Polysaccharide	91.8	2.43	Cosa *et al.* (2013a).
Vagococcus sp.	Wastewater	Glucose	Urea+Yeast extract+$(NH_4)_2$ SO_4	Polysaccharide	86.5	2.3	Gao *et al.* (2006).

2.7.2 Effect of Temperature

Temperature has a significant influence on cultivation and production of bioflocculant in microorganisms, as the enzymes in control of bioflocculant synthesis are activated at an optimal temperature (Li *et al.*, 2009; Zhang *et al.*, 2007). After the literature review, the study documented that the optimum temperature for the bioflocculant production differed at therange between 25 ˚C –37 ˚C (Salehizadeh & Shojaosadati, 2001). The optimal cultural temperature and shaking speed was 30˚C, at 140 rpm for 24 h, were favoured for the mixed culture of *Bacillus sphaericus* F6 and *Rhizobium radiobacter* F2 to produce novel bioflocculant CBF with flocculating activity reached 98.52% (Li *et al.*, 2016). Nakata and Kurane (1999) found that the bioflocculant synthesised by *Citrobacter* sp. TKF4 was cultured at 30 ˚C temperature, which had a crucial effect on bioflocculant production. Meanwhile the optimal enzymatic reactions were frequently achieved at optimal temperature for microbial growth. Giriet *et al.* (2015), confirmed that *Bacillus subtilis* F9 produced bioflocculants with tremendous flocculating activity at optimum temperature 40 ˚C.

2.7.3 Effect of Inoculum Size

The inoculum size among many physiological properties plays an important role in metabolism processes, as well as a substantial effect on microbial cell growth and the synthesis of organic compounds (Gong *et al.*, 2008). Consequently, small inoculum size might extend the stationary growth phase. However, larger inoculum size can extremely overlap the function of the microbial multiplication, and thus reduce the production of the bioflocculant (Li *et al*., 2009). For example, the inoculum size of 1% (v/v), was found to be the optimal size for X14 and support the adjustment of strain X1 for the production medium, reducing the logarithmic growth phase which activate the bioflocculant ZS-7

production (Li *et al.*, 2009). Also, 2% (v/v) inoculum volume of *Oceanobacillus* sp. Pinky was found to be the optimum value of production of bioflocculant (Cosa *et al.*, 2013b).

Similarly, the study showed that the best favourable inoculum volume was obtained at 2% (v/v) of *Micrococcus* sp. Leo for the bioflocculant production (Okaiyeto *et al.*, 2014). Besides, 3% (v/v) inoculum size of *Bacillus* sp. Gilbert, was favoured for bioflocculant production (Ugbenyen *et al.*, 2014). Moreover, the optimal culture condition, i.e. inoculum size of 4% (v/v) *Bacillus pumilus* strain ZAP028 was appropriate for the bioflocculant production (Makapela *et al.*, 2016). Similar result of Ntsangani *et al.* (2017), found that the optimum inoculum size of *Bacillus* sp. AEMREG4 was found to be 4% (v/v) for bioflocculant production and the flocculating activity achieved 80%. In addition, the optimum cultivation condition as inoculum size at 5% (v/v) was preferred by *Bacillus* sp. W7 for bioflocculant production (Okaiyeto *et al.*, 2016)

2.7.4 Effect of Initial pH

The initial pH is one of the parameters of the production medium that performed and the most significant role in bioflocculant production as well as flocculation activities (Zheng *et al.*, 2008). It also regulates the oxidation-reduction potential and electrical charge of the microbial cells, which may possibly affect the absorption of nutrients and enzymatic reaction in the fermentation medium (Salehizadeh & Shojaosadati, 2001).

The initial pH 7 of fermentation medium was found to be the optimal pH for bioflocculant production by *B. pumilus*, with high bioflocculating activity (Makapela *et al.*, 2016). For example, the optimised pH 7 was found to be the most favourable condition for bioflocculant production by *Halomonas* sp. Moreover, a similar study indicated that pH 7 was more appropriate for the production of bioflocculant by *Halobacillus* sp. Mvuyo (Cosa *et al.*, 2012; Mabinya *et al.*, 2011).

Moreover, Okaiyeto *et al.* (2016) found that the *Bacillus* sp. isolated from marine environment favoured a slightly acidic condition of fermentation medium (pH 6) for bioflocculant production and flocculating activity of 94.9 % was stretched at three days of cultivation. A different study showed that that *Aspergillus parasiticus* favoured acidic condition of fermentation medium for bioflocculant production (Deng *et al.*, 2003). Whereas, *Klebsiella* sp.TG-1 chose alkaline condition of pH 8 for the production of bioflocculant (Liu *et al.*, 2013). An extremely pH stable and more efficient bioflocculant was produced by *Rhodococcus erythropolis* by using a combination of pre-treated livestock and sludge wastewater as a cheap medium with the supplementation of Na_2HPO_4 (Peng *et al.*, 2014).

2.7.5 Effect of Agitation Speed on Bioflocculant Production

The shaking or agitation speed regulates dissolved oxygen concentration that stimulates enzymatic reactions and facilitates nutrient absorption (Lopez *et al.*, 2003). Optimum shaking speed for the bioflocculant production by *Bacillus licheniformis* X14 was found between 140 rpm–160 rpm. Even so, the change in shaking speed prerequisite for various microorganisms can be the effect on the different oxygen demands at various microbial growth phases (Li *et al.*, 2009).

Similarly, research indicated that the difference in oxygen demand depends on the growth stage of BPMs and found that the demand of oxygen in the early growth phase was low which then increased as the *Bacillus licheniformis* entered the exponential and stationary phases. In contrast, Xia *et al.* (2008) found that the production of bioflocculant by *Proteus mirabilis* was significantly decreased at shaking speeds of lower or higher than 130 rpm. This may due to the oxygen demand of *P. mirabilis* which was limited to the oxygen concentration at 130 rpm shaking speed. The highest yield of bioflocculant

produced by *Klebsiella mobilis* was 2.58 g/L at optimal shaking speed at 120 rpm (Wang *et al.*, 2007).

2.7.6 Effect of Metals on the Bioflocculant Production

The bioflocculant production is affected via the chemical nature of metal ions supplemented in the production medium (Li *et al.*, 2009). For example, the production of the bioflocculant by *Flavobacterium* sp. was enhanced with the addition of Ca^{+2}, Mn^{+2} and Ba^{+2}, respectively, but inhibited by the presence of Mg^{+2} (Gonzalez & Hu, 1991).The cations, such Na^{+}, Ca^{+2}, Mg^{+2} and Fe^{+2}, also had no effect on production of bioflocculant ZS-7, whereas Cu^{+2} extremely inhibited cell growth (Li *et al.*, 2009). For example, the production of bioflocculant by *Serratia ficaria* was activated by adding K^{+}, Ca^{+2}, Ba^{+2} and Fe^{+3} but was inhibited by the presence of Mg^{+2} and Cu^{+2} (Gong *et al.*, 2008).

Furthermore, the production of bioflocculants by *Proteus mirabilis* was influenced by the presence of Ca^{+2}, Mg^{+2} and Fe^{+3}, while the same study stated Na^{+}, K^{+} and Fe^{+2} have no evident correspondence with the production of bioflocculant, but Al^{+3} cation inhibits its production (Xia *et al.*, 2008). The cell growth and bioflocculant production of *Paecilomyces* sp. was induced by Ca^{+2}, while Cu^{+2} and Fe^{+2} ions were found to inhibit the cell growth (Tang *et al.*, 2014).

2.8 Factors Affecting Flocculation Efficiency of Bioflocculants

The flocculation performance of bioflocculants is influenced by chemical and physical factors, pH, bioflocculant concentration, temperature, metal ions and some other factors.

2.8.1 Effect of pH on Flocculating Efficiency

The flocculation performance of bioflocculants is commonly affected by the pH value of the suspended solution. Many studies investigated the effect of pH on the flocculating

efficiency of bioflocculants to determine the optimal range. Some study concluded, that at acidic pH, both bioflocculant and kaolin particles were conceivably adsorbed to hydrogen ions (H^+), which destabilised the formation of complexes between bioflocculant molecules and kaolin particles intermediated by Ca^{+2}. Likewise, hydroxide ions (OH^-) interfered with the coagulation of the bioflocculant molecules and kaolin particles at high alkaline pH values, causing lower flocculation efficiency (Prasertsan *et al.*, 2006).

For instance, the maximum flocculation efficiency of bioflocculant produced by *B. firms* and *B. licheniformis* was observed at optimum pH that ranged from 7–9 (Karthiga & Natarajan, 2015). Makapela *et al.* (2016) indicated that the bioflocculant from *B.pumilus* was found to be thermos-stable with maximum flocculating activity in a wide range of pH 3–pH11. The determined flocculating efficiency of PSK1 bioflocculant was documented at pH 2.0, 94.56% with kaolin clay suspension concentrated at therange of 5 to 9 g/L (Ayat *et al.*, 2017).

Moreover, the flocculating activity of the produced bioflocculant CBF-F26 by a consortium strain of *Bacillus sphaeicus* F6 and *Rhizobium radiobacter* F2 was found to be the highest at neutral pH and weak alkaline conditions (Wang *et al.*, 2011). Meanwhile, the highest flocculating efficiency of the bioflocculant produced by *Enterobacter cloacae* WD7 was at pH 6.0 (Prasertsan *et al.*, 2006). A similar finding revealed that the bioflocculant was produced by *Aspergillus parasiticus* at a range pH 5– pH 6. Meanwhile, a higher pH may reduce cell growth and bioflocculant production, as well as its flocculation efficiency.

However, at low pH it was found that fungus growth and production, were significantly enhanced, as well as increasing of the bioflocculant yield in the fermentation broth medium (Deng *et al.*, 2005). The bioflocculant from a novel strain SW-2 sustain its flocculating activity with kaolin suspension over pH range of 3–9 and 20 ˚C – 80˚C

(Zhong *et al.*, 2014). Li *et al.* (2010) found that the flocculating efficiency of the bioflocculant synthesised by *Agrobacterium* sp. M-503 was appropriate at range pH 7–pH12. Bioflocculant REA-11 produced by *Corynebacterium glutamicum* CCTCCM201005 was found quite thermo-stable at a moderate acidic range of pH 3–pH6.5 (He *et al.*, 2004). While, the flocculation activity of the biopolymer produced by *Gyrodinium impudicum* KG03 was identified to be suitable at a range of pH 3 – pH 6 with the optimal efficiency documented at pH 4 (Yim *et al.*, 2007). Besides, bioflocculant WF-1 secreted by *E. aerogenes*, was found to be stable and capable of flocculating the strong alkaline Trona suspension at a pH of over 13 efficiently (Lu *et al.*, 2005). While other bioflocculant showed their flocculating activity between pH 3 – pH 8, such as *B. licheniformis* CGMCC2876 (Xiong *et al.*, 2010). Moreover, the bioflocculant IH-7 synthesised by *Aspergillus flavus*, was found to have good flocculating activity and stable in a wide range of pH and temperature (Aljuboori *et al.*, 2013).

2.8.2 Effect of Bioflocculant Concentration on Flocculation Efficiency

Many studies found that the flocculating efficiency increased as the concentration of bioflocculants increased to the optimal level, and thus high concentration of bioflocculant might involve more neutralisation site, strong bridging and optimum flocculating efficiency than the bioflocculant with a lower concentration or low molecular weight (Li *et al.*, 2008; Zhang *et al.*, 2010).

For example, the flocculating efficiency of bioflocculant secreted by *Bacillus* sp. F19 was achieved at above 90% at concentration range of 1–20 mg/L at pH 3 and the maximum flocculating efficiency was reached at bioflocculant dosage of 2 mg/L (Zheng *et al.*, 2008). Meanwhile, the flocculating activity of bioflocculant from *Sorangium cellulosum* was found to require 20 mg/L – 100 mg/L of bioflocculant concentration to reach over 90% in the presence of cations. Whereas, the flocculating efficiency of

bioflocculant produced by *Pseudoalteromonas* sp. increased as the bioflocculant concentration increased from 2 mg/L – 10 mg/L, and the highest flocculating activity was achieved at 10 mg/L of bioflocculant (Li *et al.*, 2008). Moreover, the maximum flocculating efficiency of bioflocculant produced by *E. cloacae* WD7 was detected at a dosage of 2 mg/L (Prasertsan *et al.*, 2006). However, the effect of bioflocculant concentration on flocculating efficiency is dependent on the type and concentration of cations if the bioflocculant is cation-dependent. Therefore, because more concentration of cation-dependent bioflocculants contain more carboxyl groups that need high cation concentration to neutralise the charge of the bioflocculant and kaolin particles (Wu & Ye, 2007).

2.8.3 Effect of Temperature on Flocculating Efficiency

Many researches have deliberated that the chemical composition of bioflocculant has a crucial role in the thermo-stability of bioflocculants. Bioflocculants compose only peptide or protein in its structure, which is commonly sensitive to heat, but those components of polysaccharides are thermo-stable. For example, bioflocculant produced by *B. licheniformis* CGMCC 2876 was found to be very stable in a wide range of temperature of 4 ˚C to 80˚C and the flocculating activity kept over 90% was constant when the bioflocculant sample was heated for 30 min at 80 ˚C (Xiong *et al.*, 2010). The polysaccharides ofbioflocculant from *B. mucilaginosus* had flocculating activity of 85% to 89% at a temperature that ranged from 23 ˚C to 70˚C, while decreasing significantly over 70 ˚C to 90˚C (Lian *et al*., 2008). Also, the bioflocculant WF-1 (polysaccharides) secreted by *E. aerogenes*, had its flocculating activity decreased by up to 22% when the bioflocculant was exposed to heat for 15 min at 100˚C and decreased by 45% after 50 min (Lu *et al*., 2005).

Furthermore, Hetero-polysaccharide bioflocculant (pKr) synthesised by *Kocuria rosea* BU22S exhibited strong flocculating activity at wide range of temperature from 0 ˚C to 100˚C and pH 2 to pH 11 (Chouchane *et al.*, 2017). Bioflocculant secreted by *B. aryabhattai* strain PSK1 showed maximum flocculating activity of 92.8% at 50˚C, while it was found thermally stable up to 80˚C (Abd El-Salam *et al.*, 2017). Moreover, bioflocculant produced by *Bacillus* sp. was found to be thermostable with approximately 65.6% of residual flocculating activity reserved after heat exposure of the bioflocculant for 25 min at 100˚C (Ugbenyen *et al.*, 2013).

2.8.4 Effect of Metal Ions on Flocculating Efficiency

Cations perform a dynamic role in bio-flocculation processes, in which they can enhance the flocculation efficiency via neutralisation and stabilisation of the negative charge residual /net surface charge of the functional groups on the bioflocculant; hence, stimulating the formation of bridges between the bioflocculant and suspended particles (Wu & Ye, 2007). According to Wang *et al.* (2011), cations have an energetic role in enhancing the adsorption of suspended particles on bioflocculant in reducing the distance among them and expanding the electrostatic attraction between the suspended particles and the bioflocculant molecules.

Furthermore, Prasertsan *et al.* (2006) mentioned that the carboxylate functional groups present in the bioflocculant chain are involved in the adsorption of the cation ions, which can form complexes between kaolin particles and bioflocculants. Li *et al.* (2016) indicated that bioflocculant active components from *Shinella albus* xn-1 comprised accumulated double bonds and triple bonds, metal ions, temperature and algal pH exhibited high influences on the flocculation efficiency. However, highest flocculating activity reached 85.65%. The maximum flocculating efficiency of exopolymer R-202 was detected in the presence of 10 mM solutions of Ca^{+2}, Mg^{+2}, Fe^{+2} and Na^{+}, but this effect depended on the

type of ions and increased in the order - $Na^{+} < Ca^{+2} < Mg^{+2} < Fe^{+2}$. Meanwhile Fe^{+2} ions showed the highest flocculating activity (Czemierska *et al.*, 2017). Bioflocculant produced by *Bacillus* spp. UPMB13 activity was affected by monovalent and divalent cations synergistic, including Na^{+}, Ca^{+2}, and Mg^{+2}, while Fe^{+2} and Al^{+3}, causing inhibiting effects on flocculating activity. Divalent cations were revealed as the preferred cation source to improve flocculation efficiency (Zulkeflee *et al.*, 2012). The highest flocculating activity of the bioflocculant was produced by aquatic bacteria *Oceanobacillus*sp. Pinky, was revealed by using cations such as aluminium chloride and calcium chloride (Cosa *et al.*, 2013b).

When the bioflocculant is protein in nature, that is rich in amino acids, and contain carboxylate functional groups might provide negative charges of the suspended particles, which activates cations to enhance neutralisation, and create bridges between the bioflocculant and suspended particles (Cosa *et al.*, 2013a). The occurrence of extra functional groups, such as carboxyl groups on the bioflocculant molecule, can act as cation binding sites for the cations, and thus, by adding of these cations to a suspension might enlarge the floc size, causing better sedimentation (Li *et al.*, 2007). The flocculation competence of protein bioflocculants produced by *Alcaligenes cupidus* and *Rhodococcus erythropolis* revealed that it was more efficient to add Al^{+3} and Ca^{+2}, respectively (Banks *et al.*, 2006).

Moreover, the flocculating efficiency of the bioflocculant MBF3-3 secreted by *Bacillus* sp. was found to be more appropriate with the addition of the metals, such as K^{+}, Na^{+} Mg^{+2}, Ca^{+2} and Al^{+3} ions, but become suppressed by the addition of Fe^{+3} ions (Feng & Xu. (2008). A similar result was detected by Zheng *et al.* (2008) found that flocculation efficiency was totally inhibited by the addition of Fe^{+3} to the bioflocculant produced from *Bacillus* sp. F19 and kaolin suspension. Divergent to the above finding, the flocculation

efficiency of bioflocculant p-KG03 was enhanced with the addition of Fe^{+3} with analogous results displayed by the bioflocculant produced from *Enterobacter* sp. BY-29 (Wu & Ye, 2007). However, the novel bioflocculants produced by *Bacillus* sp. F19, *Citrobacter* sp. TKF04 and *Gyrodinium impudicum* KG30 did not require metal ions for their flocculation efficiency (Zheng *et al.*, 2008).

The flocculating activity of bioflocculant produced from haloalkalophilic *Bacillus* sp. was remarkably induced by the addition of divalent cations such as Ca^{+2}, Zn^{+2} and Cu^{+2}, respectively (Kumar *et al.*, 2004). While, flocculating competence of the bioflocculants from *Halomonas* sp. V3a was enhanced in the presence of Ca^{+2} at a wide range of pH 3–pH11, increasing in flocculating efficiency of above 80% against kaolin clay suspension (He *et al.*, 2010). Similarly, the flocculating activity of the bioflocculant secreted by *Serratia ficaria* achieved 95.4% in the presence of Ca^{+2} and Mg^{+2}, respectively, at pH 5–pH 7 range against kaolin suspension.

2.9 Cost-Effective Substrates for Bioflocculant Production

In recent years, bioflocculants have acquired vast scientific and biotechnological consideration because of their biodegradability, the non-hazardous nature of their decomposing products and prospect demand (Nwodo *et al.*, 2013). To reduce the production cost, challenges were engaged to search for lower-cost substrates. Some considerable determination to reduce the relative cost of producing bioflocculants at industrial large scale was to use economical substrates (Fujita *et al.*, 2000). Low-priced substrates were used for bioflocculant production (Zhuang *et al.*, 2012). For instance, activated sludge was applied as a raw substrate for the bioflocculant production (Guo *et al.*, 2014; Peng *et al.*, 2014). Moreover, different industrial wastes were utilised as a cheap carbon source substrate to reduce production cost, such as potato starch waste effluent (Pu *et al.*, 2014; Liu *et al.*, 2015b), palm oil mill effluent (Wong *et al.*, 2012; Aljuboori

et al., 2014), and brewery effluent wastewaters (Zhang *et al.*, 2007).The comparison in costs of commonly utilised substrates, such as glucose, sucrose, maltose, fructose and galactose, sustain an adverse impact on manufacture budgets, and thus confine the marketing potential of these bioflocculants (Mabinya *et al.*, 2012). Moreover, determinations have also concentrated on the isolation of new microbial species for novel bioflocculant production competences in exploiting economical substrates and improving the production medium ingredients and fermentation parameters condition that is required to enhance bioflocculant yield (Sathiyanarayanan *et al.*, 2013).

2.9.1 Molasses as Substrate

Molasses is a secondary product derived from sugar-cane manufacturing process that contains about 50% (w/w) of total carbohydrate, nitrogenous compounds and vitamins (Moosavi-Nasab *et al.*, 2010). Therefore, it has tremendous properties that can be utilised as a potential-substrate for microbial growth and bioflocculant production (Zhuang *et al.*, 2012). Besides, the sugar-cane molasses is a strong liquid with some distinctive properties, including, high concentration of biochemical oxygen demand (BOD) range of (40000-60000 mg/L) and chemical oxygen demand (COD) concentrations range (80000 mg/L –120000 mg/L), this effluent fluid requires treatment before discarding to avoid environmental contamination. The hydrolysates of agricultural wastes, rich in polysaccharides, were used to produce biological products (Zheng *et al.*, 2005). It supports the production of bioflocculants by *Klebsiella* sp. after neutralisation without the addition of any nutrient supplements, which makes the commercial production considerably cost effective. Many bioflocculant-producing microbes discovered previously exploited carbohydrate-rich compounds as an energy source and the major source of carbon (Ugbenyen *et al.*, 2012). For example, molasses is a low-cost substrate that can be recycled as an enriched medium through various microorganisms for exopolymer compounds production (He *et al.*, 2004). Similarly, molasses was used as a

production medium for *Penicillium* sp. HHE-P7 to produce bioflocculant, and its highest flocculating efficiency was achieved after three days of cultivation (Liu *et al.*, 2010). Sam *et al.* (2011), stated that pre-treated molasses was used as fermentation substrate in the bioflocculant production by halophilic bacteria. According to Mao *et al.* (2010) *Pseudomonas alcaligenes* PS-25 and *Pseudomonas fluorescens* C-2, were cultivated for three days in molasses substrate for bioflocculant production. However, the bioflocculant REA-11 from *Corynebacterium glutamicum* CCTCC M201005 was enhanced by the addition of glucose, sucrose and fructose, but sucrose was found to be more favoured as the sole carbon source, due to its low-cost and high bioflocculant yield (He *et al.*, 2004). Therefore, the capability of microorganisms to utilise sucrose may give rise to potentiality to consume molasses as a carbon source on large-scale production; hence, is promising for producing bioflocculant commercially.

2.9.2 Palm Oil Wastewater

The palm oil industry is one of the most important industries in Malaysia with a production of 20.7 million tonnes of crude palm oil (CPO) from an oil palm planted area of 5.39 million hectares in 2014 (Kamalrudin and Ramli, 2014). Although, such production has resulted in a large amount of palm oil mill effluent (POME), evaluated at approximately three times the quantity of CPO. One tonne of the fresh fruit bunches (FFB) processed, an approximate of 0.67 tonnes of POME are produced. Therefore, in 2014, about 67.28 million tonnes of POME were produced. POME is a highly polluted wastewater since it has high biological oxygen demand (BOD) and chemical oxygen demand (COD) (Nurul *et al.*, 2014). Also, POME has a variation of microbial classes as indicated by the BPM. Furthermore, palm oil biomass waste contains high organic components, such as cellulose, hemicellulose, lignin and extractive (Hashim *et al.*, 2011; Tsai *et al.*, 2009)

2.10 Application of Bioflocculant

Recently, the investigation of potentiality extracellular polymeric substance (EPS) exploitation was extremely amplified due to its various distinctive properties that recommend its possibilities, applications in many industrial processes (Elkady *et al.*, 2011). For example, EPS are applied in many industrial processes, due to their unique bio-physicochemical properties, such as, in the manufacturing of pharmaceuticals, cosmetics, detergents, food additives as thickening, emulsifying and stabilising agents, textiles, adhesives as well as brewing (Kunmani *et al.*, 2011; Mishra and Jha, 2013). In addition, EPS can function as antioxidant, natural immunomodulatory, a drug carrier agent in wastewater treatment, oil recovery, dredging, in different downstream processes, bioflocculant and heavy metal removal (Wang *et al.*, 2008).

Lin and Zhang, (2004) indicated that some of these biopolymers have been designated to have anti-inflammatory, anti-viral and anti-tumour properties, which function as inducers for colony stimulating factors and interferon. Among recognised bioflocculant, polysaccharides attract the consideration of scientists in flocculating processes, mostly in drinking water purification (Raza *et al.*, 2011). Bioflocculants are also widely applied in wastewater treatment, e.g., in the treatment of dye solutions and inorganic solid suspension, such as activated carbon, aluminium oxide, solid clay, and bentonite (Deng *et al.*, 2005; Shih *et al.*, 2001; Yim *et al.*, 2007). Sathiyanarayanan *et al.* (2013) reported that bioflocculant production rich in polysaccharide is not type specific, due to each microbial strain of the same species may be producing different types of carbohydrates in the fermentation medium accompanied by many biological functions. In addition, as polysaccharides have a hydroxyl group, with a hemiacetal reducing mechanism, with other capability and suitability that could have crucial roles in the reduction reactions (Mata *et al.*, 2009).

2.10.1 Treatment of Wastewater

2.10.1.1 Removal of COD and Suspended Solids from Wastewater

Several studies had proved the effectiveness of bioflocculants in the removal of COD, suspended solids, heavy metals and humic acids from wastewater discharged to streams, microorganisms, latex particles and separation of oil from oil-water suspension suspended solids, COD, humic acids, heavy metals from waste streams, microorganism, latex particles and separation of oil from oil-water suspension and fine coal procedures (Zemmouri *et al.*, 2011). The majority of the bioflocculant documented in literature have shown satisfaction in flocculating efficiency against kaolin suspension. However, most bioflocculant display different flocculation capabilities for further suspended materials in aqueous solution. Likewise, bioflocculant rich in carbohydrate showed an exceptional flocculation efficiency in removing suspended solids from starch wastewater (Deng *et al.*, 2003). The bioflocculant from *Aspergillus latus* has the potential to flocculate oil emulsion (Kurane *et al.*, 1991). Whereas, bioflocculant from *Paenibacillus elgii* B69 strain display a good flocculating capability in treating various wastewaters which comprise colour removal (88%), turbidity reduction (83%) and COD reduction (68%) (Li *et al.*, 2013). Also, the bioflocculant from *Serratia ficaria* was found to have significant flocculating activity against kaolin suspension as well as presenting high flocculation efficiency in different wastewater (Gong *et al.*, 2008). For example, river water was treated with bioflocculant SF-1 secreted by *Serratia ficaria*, and the flocculation efficiency removal has shown 90.4% of colour removal, 87.1% of COD reduction, and 84.2% of turbidity removal (Gong *et al.*, 2008). Similarly, bioflocculant SF-1 thatwas used to treat brewery wastewater, revealed efficiency in removing turbidity (91.8%), COD (80.7%) . Meanwhile for meat processing, wastewater, the turbidity reduction was 93.7%, COD removal was 76.3% and colour removal was 64.1% of soy sauce brewery waste-water (Gong *et al.*, 2008). The bioflocculant MBFA9 from *Bacillus mucilaginosus*

showed a high flocculating efficiency of suspended solids with a reduction percentage of 85.5% and 68.5% of COD (Deng *et al.*, 2003). Furthermore, bioflocculant MBF-6 secreted via *Klebsiella pneumoniae* YZ-6, which was isolated from human saliva, had been used for flocculating at various wastewaters, such as dairy, sugar, textile and the brewery industry effluents (Zhengshan *et al.*, 2014).The highest flocculating activity was detected in sugar effluent wastewater, whichexhibited maximum removal of COD (77.8%) and BOD (80.7%), while the reduction of suspended solids was about 78.6% (Zhengshan *et al.*, 2014). However, the bioflocculant produced by *Bacillus mucilaginosus* had a BOD reduction percentage of 42.3% and 74.6% of the COD for domestic wastewater, whereas the removal efficiency of the brewery wastewater standard was 77.4% of the BOD and 70.5% of COD. In addition, its removal efficiency of BOD and COD from pharmaceutical wastewater was 41.7% and 66.2%, respectively (Lian *et al.*, 2008).

2.10.1.2 Drug Removal

Pharmaceuticals are biologically active and persistent constituents, which are identified as a permanent threat to environmental safety (Santos *et al.*, 2010). Figure 2.4 showed.

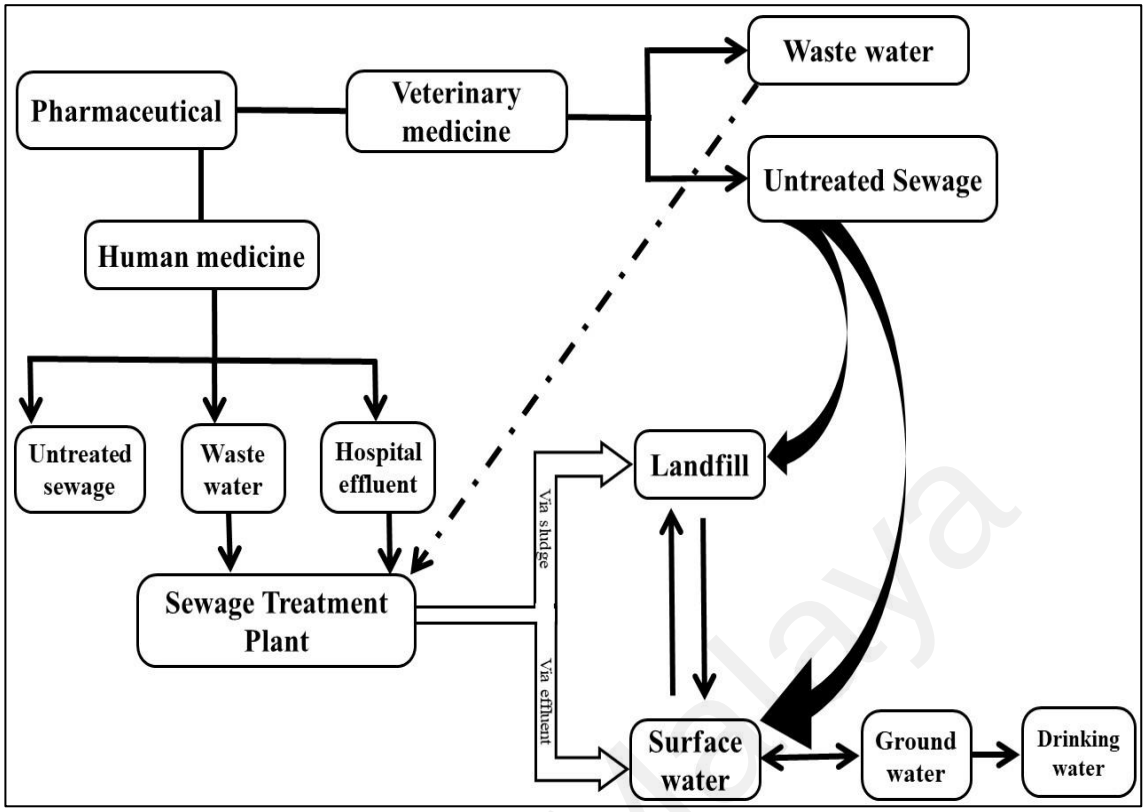


Figure 2.4: Representation for Source and the Fate of Pharmaceutical in the Environment (Reproduced with permission from Elsevire)

Pharmaceuticals are applied widely in human and veterinary medicine to prevent diseases and as well as growth promoters in livestock and agriculture such as fish farming. After utilisation, pharmaceuticals might be conjugates of sulphuric and glucuronic acid (Heberer *et al.*, 2002; Nikolaou *et al.*, 2007). The use of pharmaceuticals and personal care products is strongly increasing, and these are classified as organic micro-contaminants that are relatively recalcitrant and slowly bio-accumulating and produce permanent damage to the ecosystem in time (Xing *et al.*, 2013).

Jelic *et al.*, (2011) reported that some pharmaceuticals are residues in wastewater. This showed that even if it has a good reduction percentage level achieved in the aqueous phase, for example, comparison of influent streams and effluent wastewater concentration cannot imply degradation of similar degree. Generally, the removal of most of the substance is inadequate and the improvement of the wastewater treatment which can precede treatment of the produced sludge which is necessary to prevent the initiation of

these micro-contaminants in the environment (Jelic *et al.*, 2011). Similarly, some of the common pollutants, such as pesticides, cleansing agent, petroleum among other drugs, are constantly distributed, even at low extent, could influence by increasing the toxicity even without persistence estimate (Santos *et al.*, 2010). The most evident pathway for environmental pollution of pharmaceutical is by the untreated discharge of feaces and urine, while another anthropogenic mechanism must be expected. For example,

1. Household disposal, any topical formulation or unused medicine is discarded via the sink/toilet or through waste collection before they are actually reserved to landfill sites, where they might appear as a terrestrial ecosystem pollutant. Instead, they probably leak into adjacent water bodies (Bound *et al.*, 2005).
2. Diagnostic composites, such as X-ray contrast media are directly discarded in its natural form.
3. Metabolism post-ingestion, whereby many drugs are metabolised as the organism attempts to transform hydrophobic compound into more simple excreted polar residues (Timbrell. 2002).
4. Impacts due to anthropogenic activities, for example, sewage treatment plant sludge, which can convey non-suspected drugs and is commonly employed as a fertiliser on agricultural fields (Topp *et al.*, 2008).

Veterinary medicines are excreted in urine and faeces by animals before being spread onto land through feed application as fertilisers. Besides the potential for direct soil contamination, there is also a risk of heavy run-off. Therefore, possibly contaminate both the surrounding surface and groundwater (Kay *et al.*, 2005). Another important source of environmental contamination of pharmaceuticals is the effluents of pharmaceutical production plants (Larsson *et al.*, 2007). Removal efficiencies in WWTPs depend on various factors, such as composite physicochemical properties, the environment

conditions e.g. sunlight intensity and temperature and the type of treatment process applied, the operational conditions of the treatment process, such as temperature of operation, redox conditions, solids retention time and hydraulic retention time as well as the phase of the activated sludge used in the plant system (Castiglioni *et al.*, 2006; Suárez *et al.*, 2008; LeMinh *et al.*, 2010). So, removal efficiencies can vary significantly from plant to plant and within a plant at different time periods (Vieno *et al.*, 2007). Sulfamethoxazole is a medicine that is generally used in animal food and animal droppings that contaminate water bodies. The bioflocculant synthesised by *Klebsiella* sp. displayed significant adsorption ability for sulfamethoxazole in aqueous solution; under optimised conditions (Xing *et al.*, 2013).

2.10.1.3 Heavy Metal Removal

The heavy metal contamination is a serious environmental issue, and the use of bioflocculants was shown to perform a significant role in the removal of heavy metals from contaminated water. The bioflocculants possess various anionic groups that help in binding metal ions. The metal adsorption by bioflocculant depends on several factors, such as pH, initial metal concentration, temperature, bioflocculant dosage, charge density and type of conformation of polymer with adsorbed ions (Gomma, 2012; Lin & Harichund, 2011a, 2011b; Morillo *et al.*, 2006; Salehizadeh & Shojaosadati, 2003; Wang *et al.*, 2013b). Several industrial manufacturing procedures evolved in the discharge of heavy metals into aquatic ecosystems. This has been demanded suitable consideration due to the adverse effects of these heavy metals in the marine environment (Salehizadeh & Shojaosadati, 2003). Heavy metals, for example, arsenic, lead, mercury, copper, cadmium, nickel, zinc and chromium are the main noxious waste of fresh water reservoirs because of their hazard, non-biodegradable, and nature persistence (Azmi *et al.*, 2017; Kuniawan *et al.*, 2002). The development industries are the key source of heavy metals,

leading such chemicals into different environmental parts, including water, soil, air and biosphere. For example, sea foods and vegetables will easily absorb heavy metals due to their high solubility in the aquatic environment. Therefore, they can accumulate into aquatic biota and the human body by means of the food chain (Arezoo *et al.* 2017). Moreover, they can combine with sulfhydryl groups of protein, inhibiting the enzyme activity, the existences of accumulation of heavy metals in polluted ecosystem threaten both human and livestock health and merit distinctive responsiveness (Cobbing, 2008; Cristina *et al.*, 2011). Therefore, the strong affinity of arsenate to methanetheiol (mercaptan) groups present in living organism, for example, amino acids, peptides and proteins containing some enzymes, which can cause severe toxicity to humans (Altumn *et al.*, 2014). Copper frequently originates in high concentration near minefield and disposal, waste sites which originate from electroplating and metal finishing industries. Copperiedus causes itching and dermatisation, hand plane warts (keratinisation) and allergic contact dermatitis in human beings (AL- Asheh *et al.*, 2003). Aydin *et al*. (2008) reported that the inhalation of copper spray can cause lung cancer among unprotected personnel. On the other hand, lead is one of the most dangerous heavy metals since it gets into the human body. Zhu *et al.* (2008) indicated that upon its dissemination into the body, lead causes severe damages on the physiology of the organs. For instance, it can impair the erythrocytes and reduce their capability of oxygen transport to the biological human system. Also, its effect can harm the kidneys, hearing and nervous system. Grandjean, and Landrigan. (2014) demonstrated that lead compound is identified as an enzyme inhibitor and metabolic poison, and they showed that foetuses and young children are more subjected to poisonous lead ions that can threaten their health.

Cadmium is presented into the water from the various manufacturing processes such as: mining, metal plating, phosphate fertilizers, nickel batteries, composite industries, sewage sludge, dyes and stabilisers (Zhu *et al.*, 2008). The destructive effects of cadmium

comprise serious and chronic metabolic syndromes, such as emphysema, hypertension, renal damage, itai-itai disease and testicular atrophy. Therefore, it is necessary to remove heavy metal polluted wastewaters before they are discharged into the aquatic environment (Kurniawan. 2002).

Environmental system as abiotic methods, have been applied for the removal of heavy metals from polluted locations, for instance, activated carbon adsorption, reverse osmosis, chemical precipitation and ion exchange. The escalating catastrophe of heavy metal contamination of water, soil and some other sediment has setthe search for changes to remove these pollutants as priority. Many researches on heavy metal removal from wastewater and petroleum had highlighted the development of substance which can undergo improved selectivity and affinity and volume for targeted metals (Parirandeh *et al.*, 1998). The treatment of industrial wastewater effluent contaminated with noxious heavy metals is very important from the perspective of environmental contamination control (Guangyu & Thiruvenkatachari, 2003). The EPS produced by *B. subtilis* exhibited chromium (VI) adsorption of 48% (Chug *et al.*, 2016). The bioflocculant from *P. aeruginosa* showed the adsorption ability for Hg^{+} (89.09%) and Cu $^{+2}$ (87.39%) at a bioflocculant concentration of 20 mg/L; Cd $^{+2}$ (79.93%) and Pb $^{+2}$ (79.7%) at 40 mg/L and Zn^{+2} (80.5%) and Arsenate (72.92%) at 60 mg/L (Gomma, 2012). Many strains that were screened from *Pseudomanas* sp. *Paenibacillus* sp. and *Herbaspirillium* sp. have the capability and tolerate the adsorption of heavy metals. For example, the bioflocculants from *Pseudomonas* sp. showed that over 90% of Pb^{+2} and 78% of Hg^{+} was adsorbed. Another example, the efficient removal of bioflocculant from *Paenibacillus* sp. CH11 showed a significant removal of about 90% of Cd^{+2} (Lin & Harichund, 2011a). Similarly, a finding showed that the lead (Pb^{+2}) tolerant *Achromobacter* sp. revealed that it can tolerate up to 1500 mg/L of Pb^{+2}. It has revealed tremendous lead adsorption from effluent and water and showed 95% flocculating efficiency (Batta *et al.*, 2013) As reported by

Geddie and Sutherland (1993), bacteria producing compounds, such as cell wall components and extracellular polysaccharide (EPS) that can adsorb heavy metals and EPS has been recognised to show a significant role in monitoring heavy metal pollution in the sewage treatment processes. Ozdemir *et al.* (2003) reported that the EPS secreted by different microorganisms documented in the literature was found to be acidic polysaccharides with various carboxyl functional groups that carried negative charges that can adsorb the metal ions. The bioflocculant from *Bacillus firmus* can adsorb approximately 98.3% of Pb^{+2}, 74.9% of Cu^{+2} and 61.8% of Zn^{+2} from synthetic wastewater (Salehizadeh & Shojaosadati, 2003). Also, the maximum removal efficiency of bioflocculant secreted by *Paenibacillus validus* MP5 for heavy metals was revealedas Zn^{+2}(27%), Ni^{+2} (16%), Cd^{+2} (15%), Cr^{+2} (9%) and Pb^{+2} (7.5%) (Rawat & Rai, 2012). Meanwhile, the bioflocculant from *P. elgii* showed the highest removal efficiency for aluminium (Al^{+3}) at 72% and good removal efficiency of Pb^{+2} (60%), Cu^{+2} (53%) and of Co^{+2} (49%). Aluminium (Al^{+3}) has multi-valent ions, so enhanced affinity binding to the EPS. As indicated, since the bioflocculant have a strong capability for interaction with heavy metals, they are suggested as surface active agents for the removal turbidity, heavy metal and bacterial population from their industrial wastes (Lin & Harichund, 2012). Consequently, the investigation of novel technologies for the treatment of industrial sewage has become significant. Bioflocculant MBF4-13 has shown removal efficiency of dichromate ion ($Cr_2O_7^{-2}$) about 69.3% and the Nickel (Ni^{+2}) 19.2%, because the bioflocculant MBF4-13 primarily composed of polysaccharide that comprised hydroxyl groups in the molecular sequence which could simply form hydrogen bonds with $Cr_2O_7^{-2}$, which cause the highest removal efficiency of Ni^{+2} (Gao *et al.*, 2009). Moreover, the novel bioflocculant MBF-TG-1 from *Klebsiella* strain TG-1 has a flocculating activity of almost 86.9% for Trona suspension. Many designated researches had revealed that bioflocculant from *Paenibacillus* have the capability of removing heavy metals from

water (Mokaddem *et al.*, 2009; Rawat & Rai, 2012). Recently, studies have described that these bio-polymers display a significant potentiality to adsorb heavy metals because of the presence of functional groups, such as carboxyl, amino, hydroxyl, sulfhydryl, phosphoryl and phenolic groups (Chug *et al.*, 2016).

2.11 Synthesis of Nanoparticles

The area of nanotechnology research is emergent as a progressive technology, that is interdisciplinary with biology, chemistry, physics, material science and medicine. There are a huge number of chemicals, physical, biological and hybrid methods available to synthesise different types of nanoparticles (NPs) (Gudikandula & Maringanti, 2016). Meanwhile physical and chemical methods are more prevalent in the synthesis of nanoparticles, but the use of toxic chemicals greatly limits their biomedical applications, especially, in clinical fields. Therefore, biogenic synthesis of nanoparticles offers an attractive alternate to chemical synthesis methods. Various hazard free, eco-friendly methods of synthesis of silver nanoparticles are in operation (Gudikandula & Maringanti, 2016).

Silver nanoparticles have various applications, for instance, they can be used as spectral coating for solar energy absorption and intercalation material for electrical batteries, optical receptors, catalysts in chemical reaction, for bio-labelling and as antimicrobials (Mokhtari *et al*., 2009). Furthermore, synthesis of silver nanoparticles by *Pseudomonas fluorescens* was identified and the synergistic effect of the biosynthesised nanoparticles with common antibiotics concerning microbial drug resistance was detected (Marsili & Das, 2016). Maharani *et al.* (2016) detected the cytotoxicity effect of biosynthesised silver nanoparticles by *E. coli*. as well as their potential for anticancer application. Also, the silver nanoparticles are used to control mosquito larvae responsible for the transmission of dengue, malaria, Zika virus and other serious diseases. Moreover,

due to their distinguished antimicrobial competence, silver nanoparticles can be applied as free radical scavengers (Marsili & Das, 2016). In addition, the maximum antibacterial activity of silver nanoparticles synthesised by biological and chemical method was revealed in *Staphylococcus aureus* and *E. coli*. (Gudikandula & Maringanti, 2016). Raveendran *et al.* (2013) found that *Halomonas maura* produce a good extracellular acidic bioflocculant, comprising of glucose, galactose, mannose and glucuronic acid was applied to synthesise Mauran/Chitosan nanoparticles in the size range of between 30 nm and 200 nm. The bioflocculant contains polysaccharides are considered favourable applicants for the synthesising of nanoparticles and essential role as reducing and stabilising agents (Salehizadeh *et al.*, 2012; Raveendran *et al.*, 2013). The bioflocculant from *B. subtilis* was applied to synthesise silver nanoparticles in the size range of 60 nm with stability of 5 months (Sathiyanarayanan *et al.*, 2013). Zaki *et al.* (2014) found that the bioflocculant secreted by *B. mojavensis* strain 32A produced silver nanoparticles of 7 nm–72 nm size. Mokhtari *et al.* (2009), found that the bioflocculant synthesised by *Klebsiella pneumonia*, effectively synthesise consistently scattered silver nanoparticles with a uniform size and shape in the range of 1nm–6 nm with an average size of 3 nm.

2.12 Conclusion and Future Prospects

Synthetic chemical flocculants are effective at flocculating colloids and are broadly applied in several industrial processes. Because of their adverse health influence and the environmental hazards related with chemical flocculants, microbial flocculants have acquired enormous scientific and biotechnology planning due to their safety and biodegradable characteristics. Aquatic habitation, which provide a very rich biodiversity of marine microorganism remain under discovered for this determination, and thus bear tremendous potential as reservoirs of novel bioflocculant producing organisms. Meanwhile many bioflocculants were recounted in literature, their large-scale production

is quite insufficient by high production cost, low flocculation efficiency and low yield. Optimisation of culture medium components and fermentation condition parameters are the most significant approaches to increase on bioflocculant yield and flocculation efficiency. Still, the high cost of production media components, would make it very favourable to exploit cost effective substrate for their large scale production in bioflocculant industry. Moreover, the application of consortium microorganism for bioflocculants production might keep the enhanced flocculation efficiency and larger bioflocculant yields than pure strains is an essential. Moreover, some cation independent bioflocculants were recognised and reported, and thusregistered in literature. Consequently, more research studies are necessary to explore a novel cation independent bioflocculant with higher flocculation efficiency.

CHAPTER 3: MATERIALS AND METHODS

3.1 Experimental Design and Sampling Sites

The overall experimental design of the production of bioflocculant named QZ-7 from *B. salmalaya* 139SI, characterisation and application are illustrated in this chapter. Its flocculation performance and its application in surface water and industrial wastewater treatment and synthesis of nanoparticles are shown in Figure 3.1.

Influent and effluent wastewater samples was collected from a wastewater treatment plant as shown in Table 3.1, using a homemade glass sampler. Then, the samples were kept in clean 1Liter amber glass bottles. The bottles were stored in an icebox at 0˚C while the samples were transported to the laboratory. Then the samples were micro- filtered using 1.2 µm glass fiber filter (Whatman, Maidstone, UK). The filtered samples were preserved by addition of 1g/L of sodium azide to prevent microbial degradation (APHA 2005).

Table 3.1: Sampling Sites and their GPS Locations.

Sample source	**Place**	**GPS location**
River Water	Kajang	2°59'40"N 101°47'02"E
Ethanol mill wastewater	Perlis. Malaysia	6˚47’74’77”N 100˚26’0712”E
Sugar mill wastewater	MSM Perils SDN.BHD	5˚37’4302”N100390499”E
Rubber mill wastewater	Shorubber SDN.BHD Perils Malaysia	6˚44’07”N 100˚22’834”E
Hospital wastewater	University of Malaya	3°06'46"N 101°39'11"E

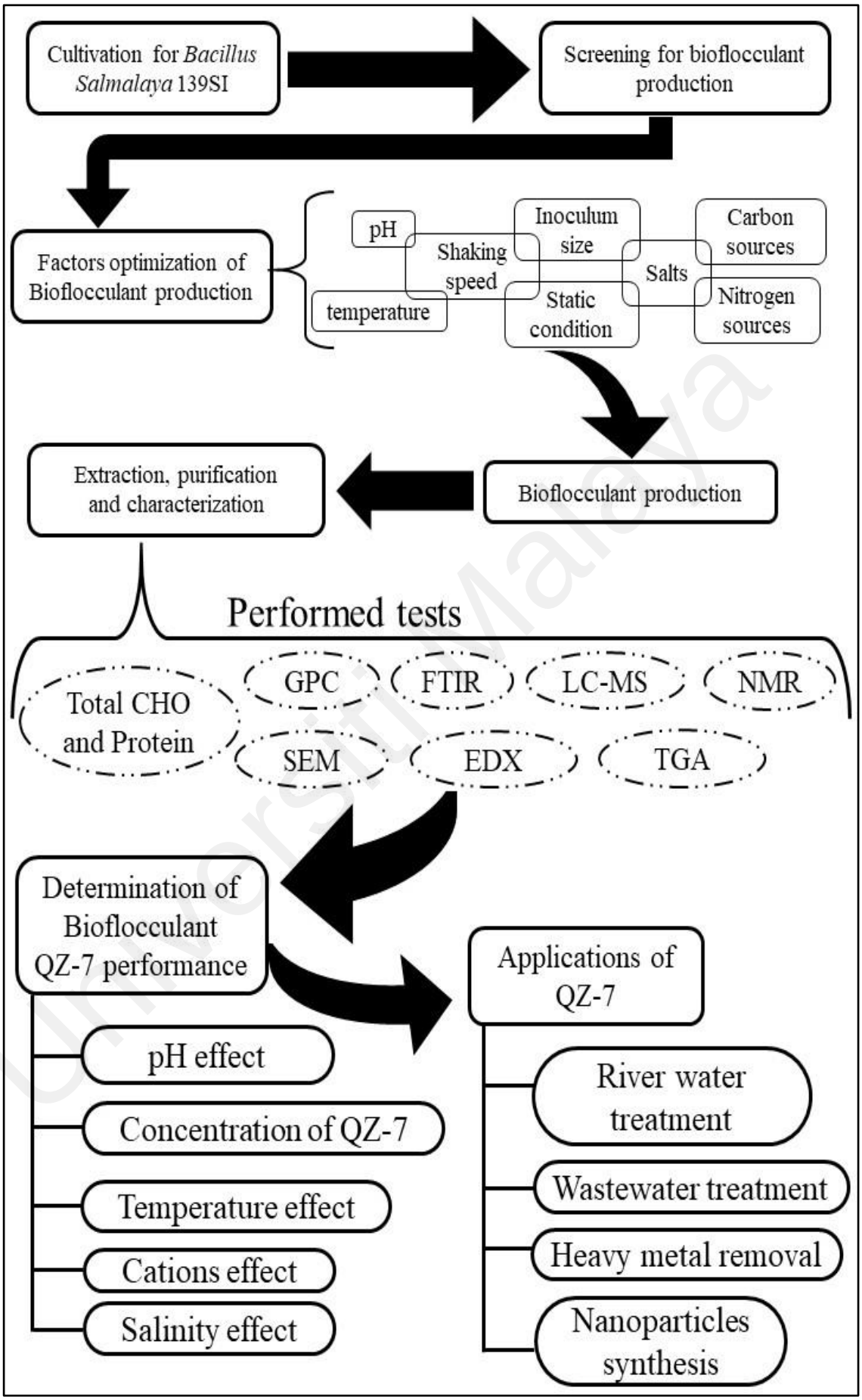


Figure 3.1: Overall experimental design.

3.2 Chemicals and Reagents

Chemical material used in this study are ammonium chloride, potassium carbonate, potassium dihydrogen phosphate anhydrous, magnesium sulphate, sodium chloride and sodium hydroxide (Friendemann Schmidt), arsenate, copper chloride, cadmium chloride, lead acetate, carbazole, yeast extract, zinc sulphate and Folin-Ciocaltens phenol reagent (Merk, Germany), Di potassium hydrogen orthophosphate anhydrous (Fisher, UK), glucose (Systerm), sucrose (UNIVAR, Australia), maltose, lactose and starch (Sigma), potassium dichromate (R & M Chemical, UK). The European Pharmacopoeia (EP) reference standard materials for mefenamic acid, ibuprofen, and perindopril as well as caffeine, diclofenac, simvastatin, and nifedipine were purchased from Sigma-Aldrich (Schnelldorf, Germany). The purity of all standards used in this study was ≥98%. The HPLC-grade ethanol, methanol and acetonitrile, formic acid, ammonium formate, acetic acid, and ammonium acetate (LiChrosolv) were supplied by Fisher Scientific (Loughborough, UK). The Ultra-pure water was prepared from a Milli-Q water purification system (MA, USA). All other reagents were of analytical grade.

For composition of the used Media, there are different types of media were used for screening and production of the produced bioflocculant. These media are cultivation media, seed media and production media**.**

1. The cultivation media for slant and subculture such as Brain Heart Infusion Agar (BHIA) has approximative formula g/L; brain heart infusion 6.0g, peptic digest of animal tissue 6.0g sodium chloride 5.0g, dextrose 3.0g, pancreatic digest of gelatine agar 14.4g, disodium phosphate 2.5g and agar 15.0g and the final pH 7.4 ± 0.2.

2. The seed medium has constituents in g/L as follows; glucose 10g (C source), yeast extract 1.5g and urea 1.5g (N source), KH_2PO_4 0.1g, NaCl 0.1g and $MgSO_4.7H_2O$ 0.2g. The pH was adjusted to 7.0 ± 0.2.
3. Production medium has components in g/L as follows; sucrose 20 g (C source), yeast extract 1.5g and urea 1.5g (N source), KH_2PO_4 0.1g, K_2HPO_4 0.1g, NaCl 0.1g and 0.2g $MgSO_4.7H_2O$ 0.2g. The initial pH was adjusted to 7.0 ± 0.2 (Xiong *et al.*, 2010).

3.3 Cultivation and Isolation of the Bacteria

The *B. salmalaya* strain 139SI was obtained from the Molecular Bacteriology and Toxicology laboratory at the University of Malaya. The bacterial isolate was originally isolated from soil samples in a private farm located at 2.99917 °N and 101.70778 ° E in Selangor, Malaysia. The bacterium was identified as *B. salmalaya* strain 139SI and deposited in Gen Bank KM0511837 (Ismail & Dadrasnia, 2015). The selected strain was streaked on blood agar medium plates and incubated at 37 °C for 18-24 h. The bacterial colonies were 2-3 mm in diameter, large and white-grey with a rough and irregular edge and shows strong β- haemolytic activities were obtained and sub cultured on slant tubes of brain heart infusion agar. The subcultures were incubated under aerobic conditions at 37°C for 24 h. This bacterial strain was consistently cultivated on nutrient agar and preserved in glycerol solution (20% w/v) suspended at −80 °C**.**

3.4 Screening for Bioflocculant-Producing Bacteria

Fifteen colonies from fresh culture of *B. salmalaya* strain 139SI were inoculated into 15 set of McCartney bottles each contains 10 mL seed medium. The pre- cultured bottles were incubated at 37 ˚C and shaken at 150 rpm for 24h. Subsequently, 2% of each bacterial culture broth was seeded into 100 mL of fermentation medium. The fermentation

bottles were kept incubated at 37 ˚C for 24 h with shaking at 150 rpm. For bacterial cell separation, the cell-free supernatant was harvested via centrifugation at 4000 rpm for 30 min (Yang *et al.*, 2015). The flocculating activity was measured in order to select the best strain to produce the bioflocculant.

3.5 Determination of Flocculating Activity

To select the best strain that produced bioflocculant**,** flocculating activity was determined from the cell-free supernatants. The flocculating activity was investigated using a suspension of kaolin clay. The suspension was prepared by mixing 4.0 g kaolin clay in 1.0 L of distilled water (Kurane & Nohata, 1994). A mixture of 95 mL of kaolin suspension with 3 mL of 1.0% calcium chloride ($CaCl_2$) solution and 2.0% (v/v) of cell-free supernatant was prepared. The mixed solution was vigorously agitated and left to settle at room temperature for 5 min. The optical density (OD_{550}) of the obtained clarified solutions was determined via spectrophotometric at 550 nm (UV-1700 spectrophotometry, SHIMADZU). A control sample was prepared in the same way, except the cell-free supernatant was replaced with unfermented broth media. The flocculating activity was calculated using the following expression (Kurane & Nohata, 1994):

$$\text{Flocculating activity (\%)} = \frac{\text{Ac}-\text{Bs}}{Ac} \times 100 \qquad (3.1)$$

Where A_c and B_s represent the OD of the control and real samples, respectively.

3.6 Optimisation of Cultural Conditions of *B. salmalaya* 139SI for Bioflocculant Production

The bioflocculant production is affected through many factors, such as the constituents of the culture medium and culture conditions (He *et al.*, 2004; Nakata & Kurane, 1999).

The effects of the key factors, such as cultivation time, initial pH of the production medium, carbon source, nitrogen source, cultivation temperature, shaking speed, static conditions, metal salts and inoculum size, were investigated in the optimisation of the culture condition of *B. salmalaya* 139SI strain for bioflocculant production. Then the flocculating activities which indicate the highest level of bioflocculant production was measured as mentioned in section 3.5. All the experiments were conducted in triplicate.

3.6.1 Effect of Temperature, Static Conditions and Shaking Speed on Bioflocculant Production

The *B. salmalaya* 139SI strain was inoculated into seed media and incubated at 37˚C on a shaker at 150 rpm for 24h. From fresh culture of 2% v/v, was inoculated into several sets of 200 ml bottles containing 50 ml of production medium, then incubated at different cultivation temperatures were investigated, i.e. 25, 30, 35, 40 and 45°C on shaking 150 rpm for 144 h. Also, the shaking speeds were investigated for different speeds such as, 100, 120, 140, 160,180, 200 and 220 rpm, respectively. While, for the static condition of the inoculated production medium bottles were kept in static condition (without aeration). The cell free supernatant was obtained by centrifuge at 4000 rpm for 30 min to separate the cells. Then the flocculating activity was checked as mentioned in section 3.5, using Equation 3.1.

3.6.2 Effect of pH

The effect of pH on the production medium was determined at different pH value ranging from 3-11 adjusted by using 1 N HCl and 1 N NaOH solutions as needed A fresh culture of 2% (v/v) *B. salmalaya* 139SI strain selected was inoculated into the prepared medium, incubated for 144h at 35.5°C, and shaken 160 rpm. The flocculating activity was examined using kaolin clay to check the optimal pH required for the bioflocculant

production as indicated above (Aljuboori *et al.*, 2013). The flocculating activity was checked, using Equation 3.1.

3.6.3 Effect of Inoculum Size

The influence of the inoculum volume for bioflocculant production by *B. salmalaya* 139SI strain was examined because different inoculum sizes exert certain effects on the bioflocculant production and cell mass growth. The inoculum sizes used were 0.1%, 0.5%, 1%, 2%, 5%, 10% and 15% (Aljuboori *et al.*, 2013), whereas, the cultivation condition obtained were applied and the samples kept at shaken 160 rpm, 35.5˚C and pH 7 for 144 h. The flocculating activities were checked, using Equation 3.1.

3.6.4 Effect of Cultivation Time on Bioflocculant Production

The cultivation condition was displays as pH 7, temperature 35.5˚C, shaking speed 160 rpm and inoculum size 5% v/v, for 144 h were applied. The flocculating activities were calculated, using Equation 3.1.

3.6.5 Effect of Carbon and Nitrogen Sources on Bioflocculant Production

Bioflocculant production by microorganisms is significantly influenced by the carbon and nitrogen sources (Xia *et al.*, 2008). These parameters were assessed on bioflocculant production according to the protocol of Lachhwani (2005). The production media were prepared in separate flasks. Fresh bacterial suspension of 5% (v/v) was inoculated into the prepared medium. The media were supplemented with 10 g/L of different carbon sources such as glucose, sucrose, maltose, lactose, starch and a mixture of glucose and sucrose. The cultivation condition was displays as pH 7, temperature 35.5˚C, shaking speed 160 rpm and incubated for 144 h were applied. To determine the influence of nitrogen source on bioflocculant production 1.5 g/L of each nitrogen sources was integrated into the fermentation medium in separate bottles, and similar cultivation

condition as above were applied. Flocculation activities were calculated according to Lachhwani (2005), using Equation 3.1.

3.6.6 Effect of Various Salts on Bioflocculant Production

To study the effect of different salts sources on bioflocculant production, were carried out by one at a time method where various metal sources such as calcium chloride ferric chloride, copper sulphate, potassium chloride, magnesium sulphate, aluminium sulphate, dipotassium phosphate, disodium phosphate and monopotassium phosphate were added to the fermentation medium at final concentration of 0.25g/L, cultivation condition was displays as pH 7, temperature 35.5˚C, shaking speed 160 rpm and incubated for 144 h were applied. Flocculating activity was determined according to Lachhwani (2005), using Equation 3.1.

3.6.7 Time Course Assay for Bioflocculant Production by *B. salmalaya* 139SI

Seed culture was prepared by inoculation 5% (v/v) bacterial suspension in 50 mL of enriched medium followed by overnight incubation at 35.5°C and 160 rpm. For optical density (OD600) test, sterile saline water was used to dilute the fermented broth to 0.1% (Cosa *et al*. 2012). In one litre of production medium, the optimized bacterial strain suspension was inoculated and incubated at 35.5 °C under shaking at 160 rpm for 144h. A 10 mL aliquot of the sample were taken periodically at timed intervals of 24 h, and 5 mL of the fermented broth was centrifuged. The obtained supernatant was used for determination of bioflocculant activity, according to Kurane and Nohata. (1994). The rate of bacterial growth was monitored by bacterial count using the standard plate method and the OD_{600}. In addition, the pH and flocculating activity were determined during the study.

3.6.8 Extraction and Purification of Bioflocculant

At the end of fermentation period, the culture was subjected centrifugation for 15 min at 3500 rpm in order to separate pelleted bacterial cells. The extracted supernatant was mixed with one volume (v/v) of sterile distilled water, followed by centrifugation for 15 min at 3500 rpm to remove indissoluble materials. Furthermore, the supernatant was mixed with two volumes of cold ethanol (1:2). The sample was thoroughly mixed with a stirrer and allowed to stand at 4°C for 12 h. Subsequently, the precipitate was extracted. The obtained crude polymer was dissolved in sterile distilled water. The solution sample was then mixed with chloroform and n-butyl-alcohol in proportion (5:2 ratios, v/v) with stirring and kept to stand at room temperature overnight. The upper surface portion was separated and subjected to centrifugation at 3500 rpm for 15 min to obtain a pure bioflocculant (Gao *et al.*, 2006). The purified supernatant was concentrated at 40 °C. To recover the precipitate, two volumes of ethanol were added. Finally, the precipitate was vacuumed, dried, and dissolved in deionized water to obtain a pure bioflocculant (2.72 g/L) (Cosa *et al.*, 2011), and coded as bioflocculant QZ-7.

3.7 Analysis and Characterisation for Purified Bioflocculant QZ-7

3.7.1 Phenol-Sulfuric Acid Method for Total Carbohydrates

The phenol-sulfuric acid is a colorimetric method used to determine the total carbohydrate concentration in a bioflocculant or in any other biochemical compounds. It detects all the types of carbohydrates such as monosaccharides, disaccharides, oligosaccharides and polysaccharides.

To measure the carbohydrate concentration a calibration curve of glucose was first prepared at six different concentrations, and the absorbance was measured using UV-vis spectrophotometer at 490 nm wavelength. 0.05 mL of phenol (80%) was added into

glucose standard and sample solution tubes and mixed well by a vortex. Then 5 ml of H_2SO_4 were added rapidly into each tube in order to get a good mixing and mixed again by vortex. All the tested tubes kept standing for 10 min and then placed in a 25˚C water for another 10 min before the absorbance reading (Chaplin & Kennedy, 1994). The concentration of total sugar in bioflocculant samples was calculated by the standard curve equation.

3.7.2 Bradford Method for Total protein

The Bradford is a colorimetric assay for total protein determination that implicates the binding of Coomassie Brilliant Blue to protein. This method is faster, involves less mixing step, heating is not required, and more stable colorimetric response than other protein assays.

Different concentrations of Bovine Serum Albumin (BSA) were prepared as the standard solution in order to get the protein standard curve. The unknowns' protein concentration samples were diluted to obtain between 10 µg -100 µg of protein. 5 mL of dye reagent was added into standard and sample tubes, then mixed well and allowed to stand for 5 min to complete the reaction. As a final point, the absorbance of the standard and sample were measured by spectrophotometer at 595 nm and the concentration of total protein in bioflocculant sample was calculated by the standard curve equation (Bradford, 1976).

3.7.3 Carbazole Assay for Uronic Acid

Uronic acid occurs in polysaccharides of Bacteria, fungi and plants cells. Carbazole assay is the fast method used for detecting and quantifying free and polymeric uronic acids according to Chaplin and Kennedy (1994).

Chemical reagents were prepared for the test including:

1. 0.95g of sodium tetraborate decahydrate dissolved in 2 mL of hot water and 98mL of ice-cold concentrated sulphuric acid was added carefully with stirring. Then the reagent kept in refrigerator
2. 125 mg of carbazole dissolved in 100 mL of absolutes ethanol.

Initially, different concentrations of D (+) Glucuronic acid were prepared by using distilled water as a standard solution. All the standard, samples and control (125 µL) cooled in ice bath, and 1.5 mL of an ice-cold reagent A was added with mixing in the ice bath. Then the mixture was heated at 100˚C for 10 min, followed by rapid cooling in the ice bath. 50µL o reagent B was added and mixed well and then the mixture was reheated at 100˚C for 15 min. Finally, the mixture was rapidly cooled to room temperature and the absorbance at 525 nm was obtained (Chaplin & Kennedy, 1994). The concentration of uronic acid in bioflocculant sample was calculated by the standard curve equation.

3.7.4 Fourier-Transform Infrared Spectroscopy (FTIR)

The purified bioflocculant QZ-7 was further subjected to Fourier-transform infrared (FTIR) spectroscopy (Perkin Elmer spectrum 400. USA). The purified bioflocculant QZ-7 was blended with potassium bromide (KBr) powder and compressed into disc to obtain translucent pellets for FTIR analysis. The background reference compound used was the pelleted form of potassium bromide. Infrared absorption spectra were recorded with PerkinElmer spectrum 400. The spectral resolution and wave number accuracy were 4000 - 400 cm^{-1} under ambient conditions (Rasulov *et al.,* 2016c; Ugbenyen & Okoh, 2013)

3.7.5 Nuclear Magnetic Resonance (NMR)

The proton NMR spectrum was used to inspect the manifestation of chemical composition in the bioflocculant. The column purified bioflocculant was prepared by

dissolving 5 mg into 1mL deuterium oxide (D_2O), and kept shaking it overnight at 50 x g. Then the solution was transferred into the NMR tube, capped and subjected to ^{1}H-MNR spectra were plotted in 0-20 ppm at (500 MHz) or (400MHz) Bruker Advance-2 t (NMR BCX 400MHz, Jeol. Japan)

3.7.6 Determination Molecular Weight of Purified Bioflocculant QZ-7

The molecular weight (MWs) of QZ-7 was determined by high- performance gel permeation chromatography (HPGPC) coupled to refractive index (RI detector, Shimadzu, Japan), with a TSK G4000PWx1 column operated at 40˚C. The column was calibrated by using dextran standards. The mobile phase was deionized- distilled (DDI) water at flow rate of 0.5 mL/min. Before injection, the sample was filtrated through 0.45µm filter (Chen *et al.*, 2017). The following regression equation was obtained

$$\text{Log (mass)} = K_1T + K_2 \quad (3.2)$$

Where mass (Da) and T (min) are the molecular mass and retention time of the samples, respectively, and K_1 and K_2 are constants.

3.7.7 Liquid Chromatography Mass Spectrometry (LC/MS)

To confirm the chemical composition of purified bioflocculant QZ-7 was determined by LC-MS analysis (Agilent Technology. Germany). For this, 10 mg of purified QZ-7 was completely dissolved in HPLC grade water and employed for LC-MS analysis (Nwodo *et al.*, 2014).

3.7.8 Scanning Electron Microscopy (SEM)

The SEM remarks was examined. Small amount of purified bioflocculant QZ-7 powder was spread and fixed on the iron stub. The fixed sample was scanned at 2kV using scanning electron microscopy (SEM, HITACHI- SU8220. Japan) (Rasulov *et al.,* 2017)

3.7.9 Energy Dispersion X-ray (EDX) Spectroscopy

The quantitative elemental analysis of the bioflocculant QZ-7 were conducted by fixing 5 mg of QZ-7 on copper stuff by an X-ray detector of SEM-EDX (HITACHI-SU8220. Japan)(Rasulove *et al.* 2017), and was examined with Aztec: 22650005761133539 software. Which revealed the weight and atomic (%) of different elements present in the sample.

3.7.10 Thermogravimetric Analysis (TGA)

About 10 mg of purified QZ-7 was examined using a TGA analyzer (Perkin Elmer TGA 4000. USA) at temperature of 50-900˚C and at a heating degree of 10˚C/min in a constant nitrogen gas flow rate of 20 mL/min.

3.8 Factors Affecting the Flocculation Performance of Purified Bioflocculant QZ-7

The followings will describe the material and methods of the assays of the parameters tested for the determination of the effect on bioflocculant QZ-7 performance, such as QZ-7 concentration, pH, temperature, cation ions, salinity and initial kaolin on the flocculating efficiency of QZ-7 were investigated (Gomaa *et al.,* 2012).

3.8.1 Effect of Bioflocculant QZ-7 Concentration on the Flocculation Efficiency

Optimal concentration of QZ-7 was investigated. Jar-test was used for the amplification of kaolin clay suspension (4 g/L) at neutral pH 7. Different concentrations of pure bioflocculant were used ranging from 0.25 to 10 mg/mL (1.0 mg/L.) The samples of kaolin clay suspension mixture containing 100 mL (4 g/L, pH 7), 0.25 to 10 mg/mL of bioflocculant concentration and 3 mL of 1% $CaCl_2$ were stirred vigorously and kept standing for 2-5 min. Calcium ions were added to neutralize the negative repulsion

changes to enhance and facilitating flocculation process. Optical density (OD) of the clarified solution was measured at 550 nm. Experimental control was also organised, and bioflocculant QZ-7 solution was switched with distilled water. Flocculation activity was estimated according to the following Equation 3.3 (Gao *et al.*, 2009).

$$\text{Flocculation activity (\%)} = \frac{F1-F2}{F1} \times 100 \quad (3.3)$$

Where F_1 and F_2 are the control of the sample and optical densities (OD) at 550 nm, respectively.

3.8.2 Effect of pH on the Flocculating Efficiency of Purified Bioflocculant QZ-7

To evaluate the effect of pH on flocculating activities of QZ-7, pH of the kaolin suspension was adjusted to pH 2, 3, 4, 5, 6, 7, 9 and 11 with 1M HCl or 1M NaOH, the samples were kept standing at 4˚C for 1 day (He *et al.*, 2004). Bioflocculant QZ-7 a 2 mg/mL was dissolved in 10 mL of deionized water. The samples were mixed together and stirred for 2 min at 220 rpm, then another round of agitated for 10 min at 100 rpm and kept standing for 5 min. The purpose of using a wide range of pH is to determine the condition that enhances the flocculation process to occur with the aid of the bioflocculant and to obtain an optimal range as where it might perform. The measurement of flocculation activities was determined according using Equation 3.3 (Gao *et al.*, 2009).

3.8.3 Effect of Temperature on the Flocculation Efficiency of Bioflocculant QZ-7

Purified QZ-7 2 mg/mL was dissolved in 10 mL of distilled water to determine the optimum temperature for bioflocculant QZ-7 performance. The sample were tested at temperature of 25, 30, 35, 40, 45, 55, 65, 75, 85 and 100˚C for 60 min in a water bath (He *et al.*, 2004). The kaolin suspension (4g/L) was adjusted at pH 7, the mixed solution was

stirred 2 min at 220 rpm, and agitated again agitated at 100 rpm for 10 min, and kept to stand for 5 min. The flocculation efficiency was determined using above Equation 3.3.

3.8.4 Effect of Cations on the Flocculating Efficiency of Bioflocculant QZ-7

The influence of cations on the flocculation activity of the QZ-7 was studied. Calcium chloride ($CaCl_2$) solution formerly used as activated substance was replaced by different metal salts solution of 1% (v/v), of monovalent such as KCl, NaCl, LiCl, divalent as $MnCl_2$, $MgCl_2$, and trivalent $AlCl_3$, $FeCl_3$ (Manivasagan *et al.,* 2015). Flocculating efficiency were measured using Equation 3.3.

3.9 Applications of Bioflocculant QZ-7

The surface water and different industrial wastewaters such as sugars mill, palm oil, rubber effluent and hospital wastewater were selected for the experiments. The initial chemical oxygen demand (COD), total suspended solid (TSS) and turbidity for surface water (river water). Also, COD and biological oxygen demand (BOD) for the filtered raw wastewater were measured according to standard method for water and wastewater examination as shown in Table 3.2, (APHA. 2005).

Table 3.2: Analytical Methods for Major Parameters using Standard Methods.

Parameters	Method reference	Wave-length	Range	Unit
pH	APHA 4500	-	-	-
COD	APHA 5220 C	425nm	0-1500	mg/L
BOD	APHA 2210B			mg/L
NH3-N	APHA 4500	425nm	0-2.5	mg/L
Colour	APHA2120 B	455nm	0-500	*Pt-Co
Conductivity, EC	APHA2510 B			µS/cm
TSS			0-750	mg/L
TDS	APHA 2540 D			mg/L

*Platinum-Cobalt

3.9.1 Surface Water Treatment

Water samples were collected from Langat River, kajang, Selangor, Malaysia. A sets of 100 mL of raw water were prepared to examine different concentration of crude and pure bioflocculant, for example, 0.5, 1, 2, 4, 6, 8 and 10 mg/L, respectively. The Jar test experiment was carried out in accordance with description of Wang *et al.* (2010). Each water sample was mixed with 1% (v/v) $CaCl_2$ and with different dose of bioflocculant, and the mixture were vigorously agitated at 200 rpm for 2 min and slowly stirred at 50 rpm for 5 min. The samples were kept standing for 25 min, and then the supernatant was extracted for analysis. The measurement of turbidity, COD and TSS were determined according to the standard methods for examination of water and wastewater (APHA. 2005)

$$\text{Removal Efficiency (\%)} = \frac{Co - C}{Co} \times 100 \quad (3.4)$$

Where C_0 is the initial value and C is the value after the flocculation treatment.

3.9.2 Removal of COD from Wastewater with Bioflocculant QZ-7

Approximately 0.2 g of the prepared bioflocculant QZ-7 was added to 100 mL of filtered wastewater. The physio-chemical characteristics of the raw wastewater (Appendix D, Table 4). The system was agitated with a magnetic starrier at room temperature for 2 min at 200 rpm and for 10 min at 50 rpm. The samples were left to stand for 15 min and clarified through a 0.45 μm membrane filter paper. The final COD was determined by the closed reflux colorimetric method (APHA. 2005). The COD removal rate was calculated as follows:

$$\text{Removal rate} = (CODi - CODf)/CODi \times 100 \quad (3.5)$$

Where R is the removal rate in %, CODi is the COD concentration of the wastewater before treatment, and CODf is the COD concentration of the wastewater after treatment.

3.9.3 Removal of BOD from Wastewater with *B. salmalaya* strain 139SI

The removal of BOD from wastewater with *B. salmalaya* strain 139SI was carried out using two diluted samples. The initial dissolved oxygen (DOi) of the first sample was measured using a DO meter. The other sample was inoculated with 10% of bacterial suspension and incubated in a BOD incubator for 5 days. The final dissolved oxygen (DOf) was measured after 5 days. The BOD value was calculated using Equation 3.6 (APHA. 2005), and the BOD removal percentage was calculated using the following equation.

$$BOD\ (mg/L) = DOi - DOf \tag{3.6}$$

3.9.4 Heavy Metal Adsorption from Aqueous Solution

Removal of heavy metals using bioflocculant was measured according to the work Lin and Harichund (2011). The metal salts used comprised copper sulphate, lead acetate, sodium arsenate, zinc sulphate, cadmium chloride (Sigma Co). The effect of different initial concentrations of heavy metals (20, 40, 60, 80, and 100 mg/L), bioflocculant concentrations (20, 40, 60, 80 and 100 mg/L) and pH value (3, 4, 5, 6, 7, 9 and 11) on the metal adsorption were examined. Bioflocculant QZ-7 solutions (5mL) were added through a dialysis tube in flasks having accurate 100 mL of each metal-salts solution and kept for 2 h at room temperature under shaken at 100 rpm. Next, 2 mL of each the solution was filtered through an Amicon filter (Centrifree) and then acidified within 1% of nitric acid solution for residual metal determination. The metal amounts removed from the samples tested, that is the bounded polymers were measured previously, whereas, those that remained after 2 h were detected using Inductively Coupled Plasma Mass

Spectrometry (AAS; Model AA 6300, Shimadzu, Japan); removal percentage rate for each element was calculated (Gourdon *et al.*, 1990). Controls, 5 mL de-ionized water were prepared in the dialysis tube per different metal-salt solutions. Removal ratio (R) is expressed Equation 3.7.

$$\text{Removal ratio} = \frac{\text{Ci} - \text{Ce}}{\text{Ci}} \times 100 \quad (3.7)$$

Where Ci and Ce are original and equilibrium metal concentrations removed, respectively.

3.9.5 Removal of Heavy Metals from Wastewater using Bioflocculant QZ-7

Wastewater from rubber industry was selected for the experiments. Removal efficiency analysis was performed as stated by the standard methods for examination of water and wastewater (APHA, 2005). Filtered wastewater samples of 100 mL were poured into a dialysis tube, and different bioflocculant concentration 20, 40 and 60 mg/L, were added and stirred with a magnetic stirrer for 15 min at 100 rpm, slowly stirred for 5 min at 50 rpm and kept standing to settle for 15 min. The volume of supernatant was filtered through a filter paper and used for heavy metals analysis before and after bioflocculant treatment through inductively coupled plasma mass spectrometry (AAS; Model AA 6300, Shimadzu, Japan); metal removed percentage rate of each element was also calculated (Gourdon *et al.*,1990). Controls, 5mL deionized water were prepared in the dialysis tube per different metal-salt solutions. Removal ratio (R) is expressed in Equation 3.7.

3.9.6 Removal of Pharmaceuticals from Wastewater using Bioflocculant QZ-7

3.9.6.1 Preparation of Standard Solutions

The individual stock standard solution of pharmaceuticals (1000 µg/mL) was prepared by dissolving an appropriate amount of each analytical standard for pharmaceuticals in methanol. Each stock solution was then stored in a clean 15-mL amber glass vial with fluoropolymer-lined cap in a freezer at -15˚C. A mixed standard solution (1 µg /mL) was prepared from the stock solutions with a concentration of 1000 µg/mL by mixing 10 µL of each stock solution in a 10-mL volumetric flask with methanol. A series of calibration standard solutions was prepared by an appropriate dilution of the mixed standard solution in (2:1) water and methanol at pH 10. The water and methanol mixture was adjusted to pH 10 using ammonium hydroxide (2.0 M) and formic acid (2.0 M).

3.9.6.2 Sample Collection and Preparation

Influent and effluent wastewater was collected from a wastewater treatment plant at Sungi Boloh Hospital Malaysia using a homemade glass sampler. Then, the samples were kept in clean 1Liter amber glass bottles. The bottles were stored in an icebox at 0˚C while the samples were transported to the laboratory. Then the samples wereµ filtered using 1.2 µm glass fiber filter (Whatman, Maidstone, UK).The filtered samples were preserved by addition of 1g/L of sodium azide to prevent microbial degradation (APHA, 2005).

3.9.6.3 Solid-Phase Extraction (SPE)

Effluent and influent wastewater were collected from hospital water treatment plant around Kuala Lumpur. After water sampling, 1 g of sodium azide and 50 mg of ascorbic acid were added to the water samples to preserve the samples and prevent microbial degradation. All water samples were filtered through Whatman GF 6 filter, then the pH of the samples was adjusted to 7.0 with 2.0 M NaOH and 2.0 M HCl solutions. HLB SPE

Tube from Supelco (3 cc, 60 mg) (Bellefonte, USA) was used for the solid phase extraction (SPE). The extraction volumes were 100, 250 mL for sewage influent and effluent, respectively. The solid phase adsorbent was pre-conditioned with 5 mL of methanol and 3 mL of non-contaminated tap water (pH adjusted to 7.0). The samples were introduced to the cartridge using a manifold which holds multiple cartridges and combined with the large volume sampler. The sample flow rates in the system were 2, 5, and mL min^{-1} for sewage influent and sewage effluent water, respectively. After sample loading, the solid phase was washed with 2 mL of 5% methanol in ultra-pure water at pH 10. The cartridges were then dried for about 30 min and subsequently, the pharmaceuticals were eluted with 1×1 mL of METH and 2×1.5 mL of ACN-METH with 2% ammonium hydroxide. The extracts were evaporated to near dryness under the vacuum at 50 °C and then 0.5 mL of (2:1) water and methanol at pH 10 were added. The extracts were stored at -18°C until analysis (Kafeenah *et al.*, 2018).

3.9.6.4 UPLC-ESI-MS/MS Analysis

The LC analysis was performed using an UPLC Agilent 1290 Infinity system (Agilent Technologies, Germany) consisting of a binary pump, vacuum degasser, an autosampler, and a thermostatic column oven. The chromatographic separation was achieved with Accucore Polar Premium LC column (100 mm × 2.1 mm, particle size 2.6 µm, supplied by Thermo Scientific (Loughborough, UK). The injection volume was set at 1 µL with the column temperature 30 °C. The column was eluted for the new method with methanol (eluent B) and NH4Ac 5 mM/HAc in HPLC water (eluent A) at pH 4.6 at a flow rate of 0.1 - 0.2 mL/min. Where ammonium format and formic acid (NH4Fc/HFc) in HPLC water at pH 3.2 were used as (eluent A) in the positive ionisation mode method. Ammonium carbonate at pH 7 was used with the negative mode ionisation method (Kafeenah *et al.*, 2018).

3.9.6.5 Mass Spectrometry

For detection and quantification, the Agilent Technologies 6490 triple-quadrupole mass spectrometer (Agilent Technologies, Singapore) was connected to UPLC via an electrospray ionisation (ESI) interface. It is equipped with the Agilent Jet Stream system AJS ESI as an ionisation source. The capillary voltage was 3 kV while the nebuliser pressure was 45 psi for both modes. Nitrogen gas was used as desolvation and nebulising gas at a flow rate of 14 L/min and temperature 200°C. In addition, the mass hunter software was used for instrument control, peak detection, and integration. To increase sensitivity, selectivity, and data acquisition, a multiple reaction monitoring modes (MRM) were used both in the negative and positive mode simultaneously with a dwell time of 0.2 s (Kafeenah *et al.*, 2018).

3.10 Statistical Analysis

Statistical analyses for the data collected were analysed using SPSS statistical version 25 software. A minimum of the three replicates of treatment were made to detect variability and to avoid biases. Exploratory data analyses (EDA) with descriptive statistical as mean and standard deviation were determined for pattern trend and observation. Significant differences were analysed through analysis of variance ANOVA for the basis of making conclusion and predication (Zhu *et al.*, 2012). One-way ANOVA, A multiple comparison post-hoc tests was used for the determination of differences in each assay for cases of equality of variance assumed or not assumed, respectively. Post-hoc test results were used as the basis for determination of the highest flocculating activity achieved in the respective assays. Statistical significance was defined as a p-value < 0.05 mean value are shown for 3 individuals' ± SD.

3.11 Synthesis of Silver Nanoparticles (AgNPs) by using Bioflocculant

The synthesis of AgNPs using bioflocculant QZ-7, its characterisation and application was carried out in nutrient broth medium, using cell-free supernatant and purified bioflocculant.

3.11.1 In nutrient broth medium

The nutrient broth was supplemented with 1.0% of glucose since the bioflocculant producing bacteria need glucose as carbon source and 3 mM of $AgNO_3$, 2.0% of 24 h fresh culture broth of *B. salmalaya* 139SI strain was inoculated to the medium and the mixture was incubated in darkness at 35.5˚C at 140 rpm for 7 days (Zaki *et al.*, 2014).The extracellular synthesis of AgNPs was observed by visual check-up of the change in nedium colour from a clear yellow to brown. The control was prepared without addition of $AgNO_3$.

3.11.2 Using Cell-Free Supernatant

The basal bioflocculant producing medium containing (g/L): glucose, 20.0; yeast extract, 0.5g; ammonium sulphate, 0.2g and urea, 0.5g; K_2HPO_4 , 5.0g; KH_2PO_4 , 2.0g and $MgSO_4$, 0.2g, was inoculated with 2.0% of fresh culture broth of *B. salmalaya* 139SI, then incubated at 35.5˚C for 24 h. The broth was centrifuged at 4.000 rpm for15 min; about 10% of the cell-free supernatant was added to 100 mL of 3 mM $AgNO_3$ solution. The bottle was incubated in darkness on a shaker 120 rpm at 37˚C (Elkady *et al.*, 2011). The spectra were taken at every 24 h interval between 200 to 600 nm using UV-vis spectrophotometer. Subsequently, the peaks are achieved, the AgNPs having liquid was kept in oven at 60˚C for overnight until dried.

3.11.3 Using Purified Bioflocculant QZ-7

Prepared concentration of 3mM $AgNO_3$ was added to100 mL of 10% bioflocculant QZ-7 solution in 200 mL bottles. The bottles were incubated in darkness at 37˚C for 48 hrs at 140 rpm (Zaki *et al.*, 2014). The AgNPs synthesis was monitored as described above. The control was prepared without addition of $AgNO_3$.

3.12 Characterisation of Silver Nanoparticles (AgNPs)

3.12.1 UV-vis Spectrophotometry

The primary screening of AgNPs production was visual observation for a colour change from yellow to brownish, 5 mL of the sample was withdrawn and centrifuged at 5000 rpm for 10 min. The spectra of supernatant were conducted using a UV-Vis 1800 spectrophotometer in the range of 200-600 nm. Quartz cuvettes with optical path length of 10 mm were used in the measurement. 1mL of synthesized AgNPs was withdrawn and the absorbance was measured. The synthsized AgNPs was obtained by centrifugation at 10.000 rpm for 10 min. The pellet samples were dried at 60 ˚C in vacuum oven for overnight.

3.12.2 Fourier-Transform Infrared Spectroscopy (FT-IR)

The presence of functional groups in the synthesized AgNPs were determined using FT-IR spectra (Perkin Elmer spectrum 400. USA). The dried pellet of silver nanoparticles was subjected to FT-IR spectral characterisation in the frequency rang of 4000-400cm^{-1} and at resolution ratio of 1cm^{-1} (Rasulov *et al.,* 2016c; Rasulov *et al.*, 2017).

3.12.3 X-ray Diffractometry (XRD)

The dried powder of AgNPs was subjected to powder XRD using Empyrean. Cu-K radiation diffracted intensities were recorded from 10 and 80˚ in 2θ angles. XRD approves

the nature of powder material, which ever crystalline or amorphous. The AgNPs mean crystallite size was measured by the Debye-Scherre equation 3.8 (Monshi *et al.*, 2012)

$$D = K\lambda/\beta \cos\theta \quad (3.8)$$

K – Scherrer constant, related to crystalline shape, λ –Radiation wavelength, β – Full width at half maximum of the diffraction peak, and θ – Bragg's angle.

3.12.4 Field Emission Scanning Electron Microscopy (FE-SEM) and EDAX Analysis

The FE-SEM and EDAX were used to study the morphology, size, and elemental composition of the biosynthesized AgNPs. The surface imaging technique, detection of particles shape, size, surface morphology and size distribution of nanoparticles by using the instrument - FE-SEM (JEOL JEM-1230, Japan), and EDAX (INCApenta FETX3 OXFORD (Lawrence & Prakash, 2019). For this, AgNPs were suspended into water; a drop of it was placed on the copper grid and kept to dry at room temperature.

3.13 Antibacterial Activity

The antimicrobial activity was tested in both, solid and liquid media. As such liquid AgNPs were used in this study with slight modification in the method described by Wei *et al.* (2012). At present, 20 µL of AgNPs was added to tubes containing 10 mL of Muller-Hilton broth inoculated with 10µL of inoculum of pure isolates of *Escherichia coli* ATCC35401, *Salmonella enteritidis* ATCCBAA-711, *Pseudomonas aeruginosa* and *Staphylococcus aureus* ATCC2592 incubated at 37 ˚C at 120 rpm. The absorbance was measured at 600 nm at every 3 h interval up to 12 h and then 24 h of incubation to check the growth. On solid medium, well diffusion method was employed. Muller-Hilton agar plates were streaked with the test organisms with top agar, wells were made after solidification and different concentrations of AgNPs such as 20µL, 40µL, 60µL and 80µL

were pipetted into wells (i.e. 35.2, 70.4, 105.6 and 140.8 µg). The plates were incubated for 24 h at 37˚C and the zone of inhibition was measured.

CHAPTER 4: RESULTS

4.1 Introduction

In the present study, the production of the bioflocculant produced by *B. salmalaya* 139SI was investigated to determine the optimal culture medium composition and environmental condition required. Various factors influencing the production of bioflocculant, such as initial pH of culture medium, inoculum size, culture temperature, carbon and nitrogen sources, metal salts, shaking speed, static condition and time course were investigated. Chemical composition and properties of the bioflocculant were determined. Also, the application of bioflocculant in surface water and industrial wastewater treatment were investigated. Furthermore, the bioflocculant was tested to synthesized nanoparticles.

4.2 Selection of Bacterial Strain for Bioflocculant Production

4.2.1 Morphological Identification

Figure 4.1a, shows the *Bacillus salmalaya* strain 139SI colonies were large and white-opaque with a rough and irregular edge. The colonies were 2-3 mm in diameter and shows strong β-haemolytic activity after 18-24 h of incubation at 37˚C on 5% v/v sheep blood agar as showed in Figure 4.1b.

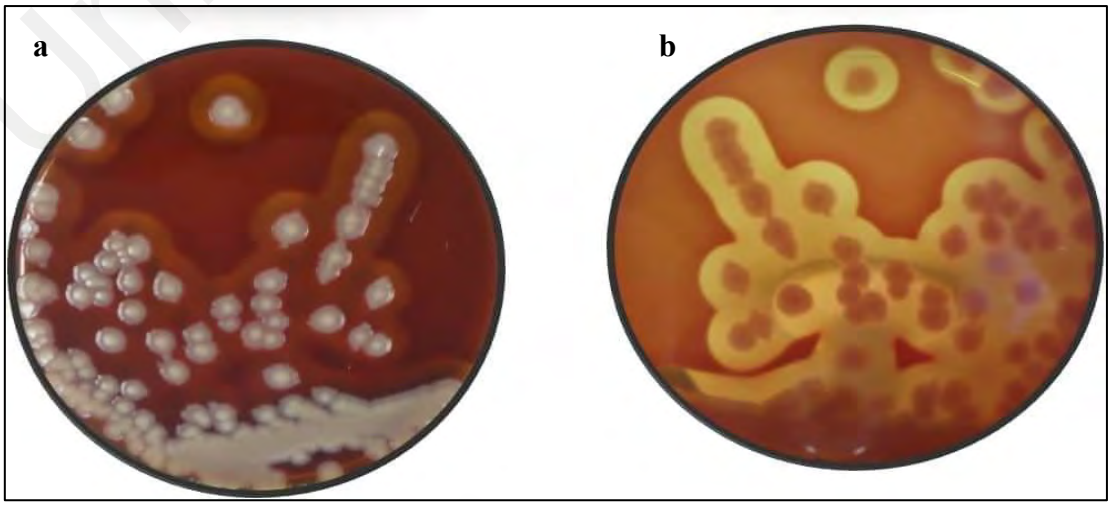


Figure 4.1: a. Front View of Pure Colony of *B. salmalaya* 139SI Strain; b. Back View of β-haemolytic *B. salmalaya* 139SI Activity.

4.2.2 Microscopic Examination

Figure 4.2, shows the *Bacillus salmalaya* strain 139SI under the microscope a gram positive, short rods in chains and the formation of endo-spore.

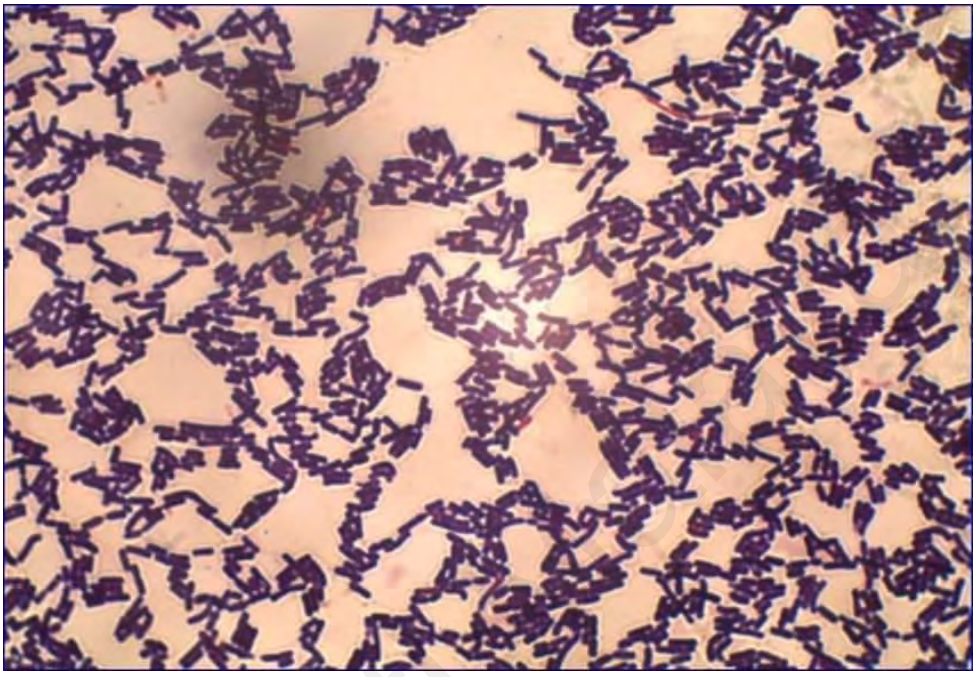


Figure 4.2: Gram Stain of *B. salmalaya* 139SI.

4.3 Screening Strain for Bioflocculant Production

The bacterial strain has been screened for bioflocculant production. From 15 strains appendix A-1, 5 strains have the highest flocculating activity as presented in Table 4.1.

Table 4.1: Flocculating Activity Values for the Selected Strains.

Strain code no	Flocculating activity (%)	Standard deviation
BS* 139SI-1	67.5	0.65
BS 139SI-5	54.2	0.77
BS 139SI-7	83.3	0.75
BS 139SI-8	72.2	1.95
BS 139SI-13	63.4	0.45

*BS means *Bacillus salmalaya* strain

4.4 Optimisation of Bioflocculant Production by *B. salmalaya* 139SI

4.4.1 Effect of pH on Bioflocculant Production

The inceptive pH of the fermentation media has a direct affected the bioflocculant production. Figure 4.3, and Table 4.2, shows the optimum pH value and the significant differences ($p<0.05$), between different pH value for the bioflocculant production.

Table 4.2: Analysis of Variances for the Effect of pH on Bioflocculant Production.

	Sum of Squares	Degrees of Freedom	Mean Square	F-ratio	P-value
Between Groups	2.011	7	0.287	1607.644	0.000
Within Groups	0.003	16	0.000		
Total	2.013	23			

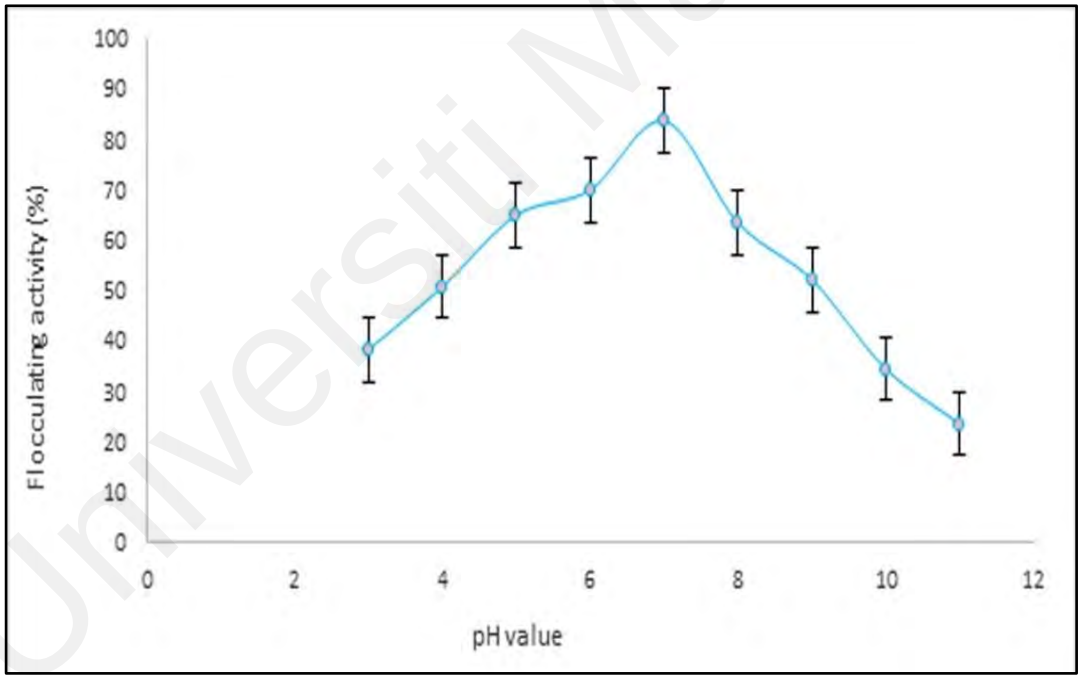


Figure 4.3: Effect of pH on Bioflocculant Production.

4.4.2 Effect of Inoculum Size on Bioflocculant Production

The influence of inoculum volume of bioflocculant production was determined according to zhang *et al.* (2008), using bacterial inoculum size ranging from 0.1 to 15 % (v/v). Figure 4.4, showed the differences effect in inoculum volume at certain change on

the cell mass and flocculation activity values. While, table 4.3, prevailed that there is a significant difference ($p<0.05$), of different inoculum size.

Table 4.3: Analysis of Variances for the Inoculum Size Effect on Bioflocculant Production.

	Sum of Squares	**Degrees of Freedom**	**Mean Square**	**F-ratio**	**P-value**
Between Groups	3042.443	6	507.074	547.177	0.000
Within Groups	12.974	14	0.927		
Total	3055.417	20			

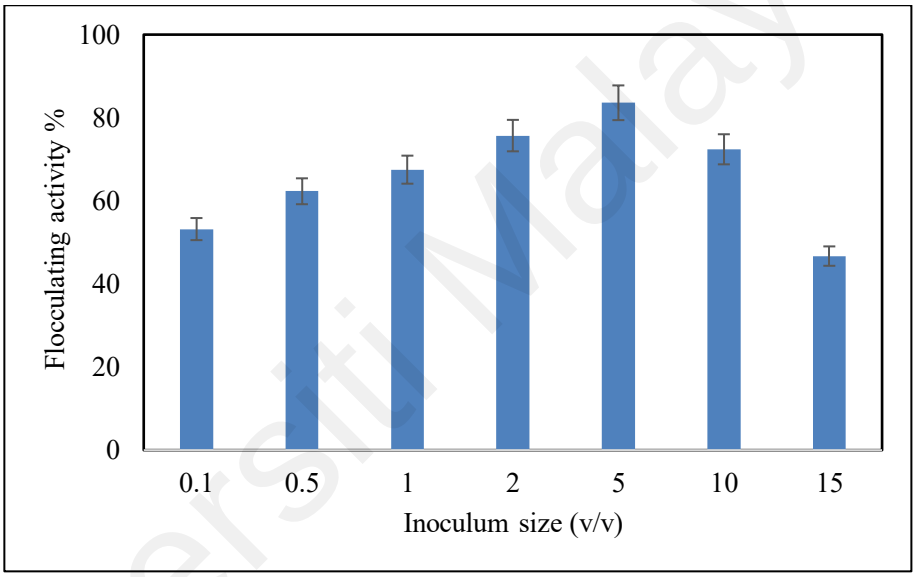


Figure 4.4: Inoculum Size Effect on Bioflocculant Production.

4.4.3 Effect of Temperature on Bioflocculant Production

The influence of temperature was used to investigate its effect on *B. salmalaya* 139SI for bioflocculant production. Figure 4.5, show that, the flocculating activity of bioflocculant was found to be 81.93%, when the culture temperature was at 35.5˚C, which was a best flocculating activity in the experiment.

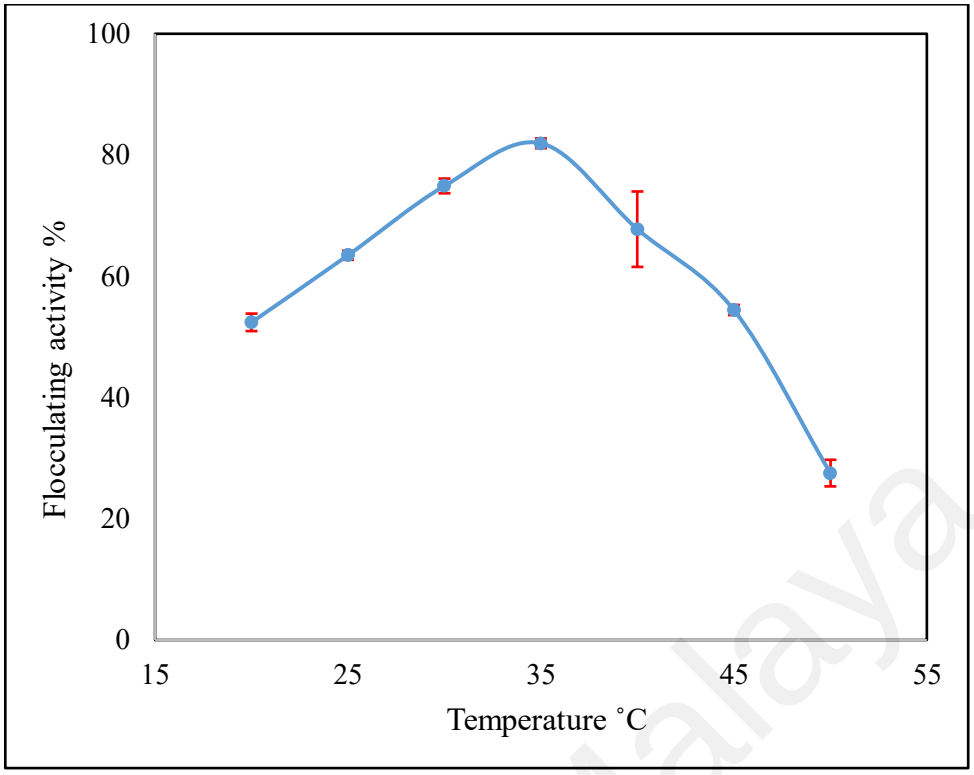


Figure 4.5: Effect of Cultivation Temperature on Bioflocculant Production.

Table 4.4, shows there is significant differences (P<0.05), for the effect of different temperature degrees on bioflocculant production.

Table 4.4: Analysis of Variances of Different Cultivation Temperature on Bioflocculant Production.

	Sum of Squares	Degrees of Freedom	Mean Square	F-ratio	P-value
Between Groups	5778.758	6	963.126	133.644	0.000
Within Groups	100.893	14	7.207		
Total	5879.651	20			

4.4.4 Effect of Shaking Speed on Bioflocculant Production

The effect of shaking speed on bioflocculant production is shown in Figure 4.6. The optimum shaking speed was found to be 160 rpm, and flocculating activity was 83.6

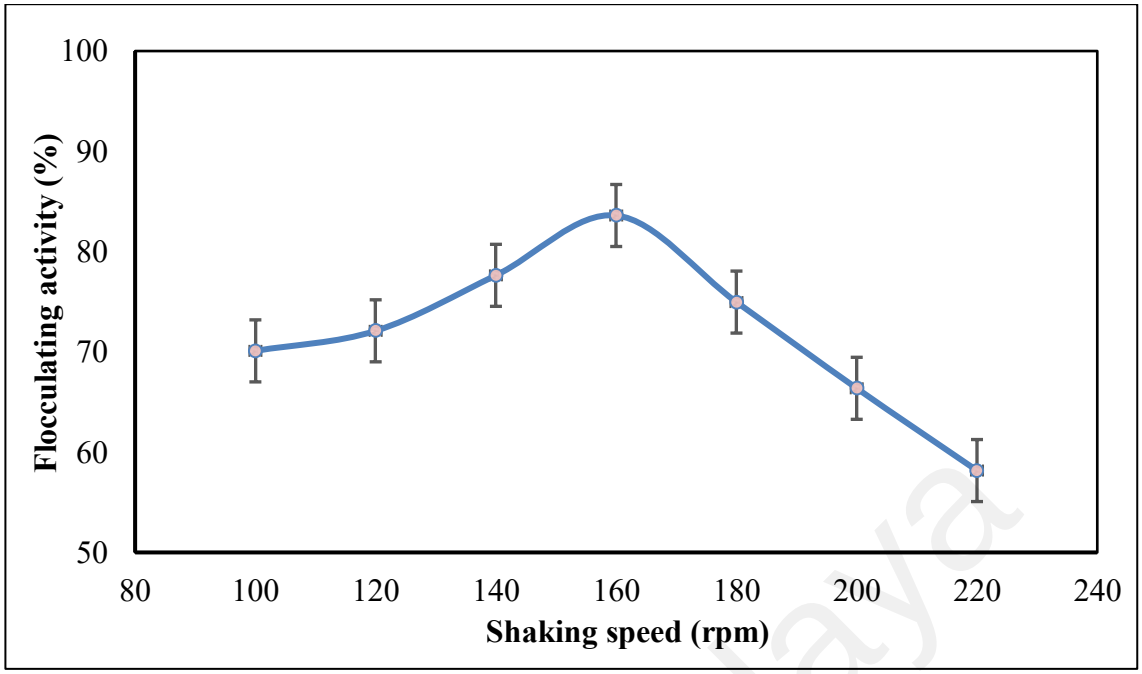


Figure 4.6: Effect of Shaking Speed on the Bioflocculant Production.

Table 4.5, showed that there is significant difference ($p<0.05$), between different shaking speed.

Table 4.5: Analysis of Variances for Effect of Shaking Speed on Bioflocculant Production.

	Sum of Squares	**Degrees of Freedom**	**Mean Square**	**F-ratio**	**P-value**
Between Groups	1203.763	6	200.627	601.881	0.000
Within Groups	4.667	14	0.333		
Total	1208.430	20			

4.4.5 Effect of Static Condition on Bioflocculant Production

When flocculating activity of the cultures under non-shaking condition it was found that the strain of *B. salmalaya* 139SI exhibited much less flocculating activity as shown in Figure 4.7.

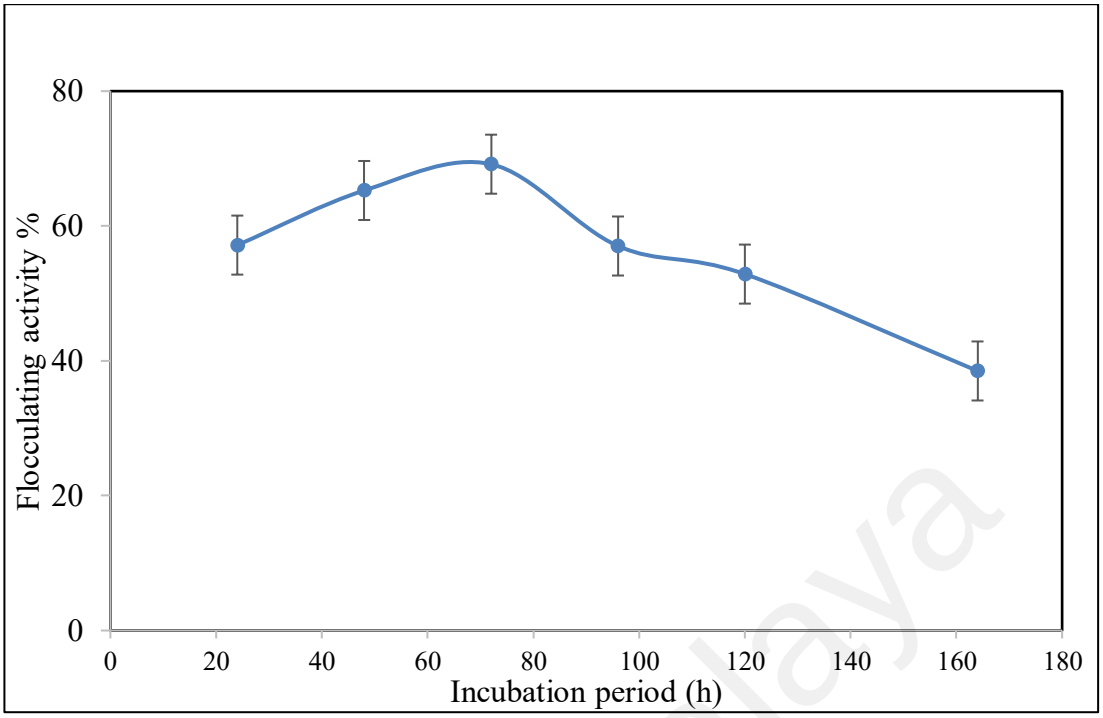


Figure 4.7: Effect of Static Condition on the Bioflocculant Production.

Table 4.6, presented that there is a significant difference ($p<0.05$), of the effect of static condition.

Table 4.6: Analysis of Variances for the Effect of Static Condition on Bioflocculant Production.

	Sum of Squares	**Degrees of Freedom**	**Mean Square**	**F-ratio**	**P-value**
Between Groups	1730.198	5	346.040	377.727	0.000
Within Groups	10.993	12	0.916		
Total	1741.191	17			

4.4.6 Effect of Carbon Source on Bioflocculant Production

The effect of carbon sources includes sucrose, glucose, lactose, maltose, fructose, and starch was evaluated for the production. The results of distinct carbon sources on the bioflocculant synthesis by *B. salmalaya* strain 139SI are shown Figure 4.8. The highest 90.1% and lowest 28.9% flocculating activities were obtained with a mixed carbohydrate (glucose and sucrose), source and starch with (1:1) mixing ratio, respectively.

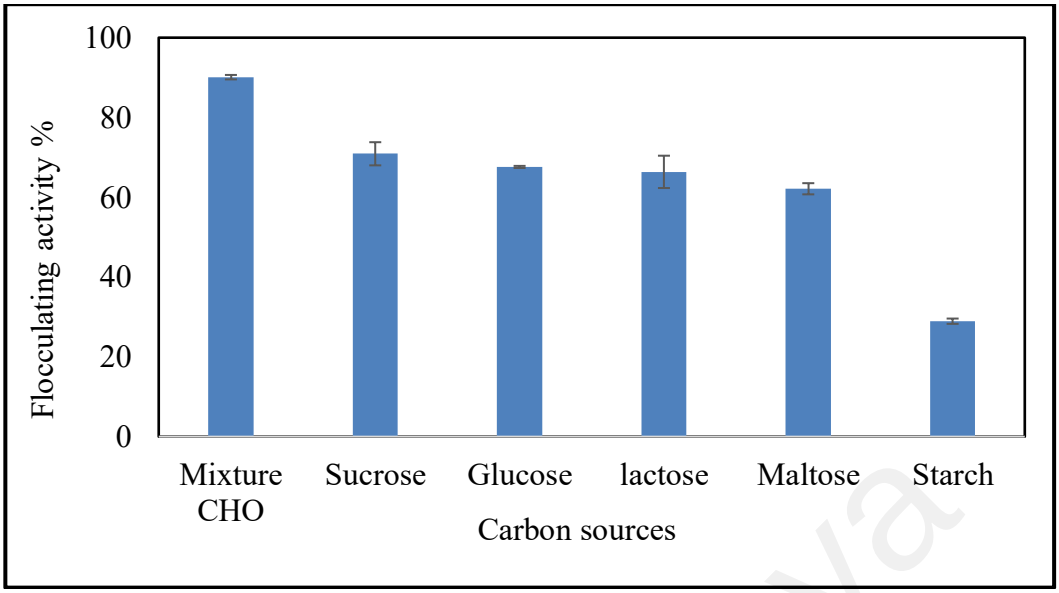


Figure 4.8: Carbon Sources Effect on Bioflocculant Production.

As shown in Table 4.7, there is a significant difference ($p<0.00$), between different carbon source.

Table 4.7: Analysis of Variance for the Effect of Carbon Source on Bioflocculant Production.

	Sum of Squares	**Degrees of Freedom**	**Mean Square**	**F-ratio**	**P-value**
Between Groups	2.229	5	0.446	241.474	0.000
Within Groups	.022	12	0.002		
Total	2.251	17			

4.4.7 Effect of Nitrogen Sources on Bioflocculant Production

Figure 4.9, showed the highest flocculating activity which obtained after 72 h of cultivation with mixture nitrogen such as yeast extract and urea 72.2%, whereas, as a single nitrogen source such as urea 67.7%, yeast extract 64.53%, while the lowest activity was obtained with peptone 42.5%. However, the inorganic nitrogen shows 58.86% of flocculating activity.

Table 4.8, revealed that there is a significant difference ($p<0.00$), between different nitrogen source.

Table 4.8: Analysis of Variance for the Effect of Nitrogen Sources on Bioflocculant Production.

	Sum of Squares	Degrees of Freedom	Mean Square	F-ratio	P-value
Between Groups	0.647	5	0.129	11.955	0.000
Within Groups	0.130	12	0.011		
Total	0.777	17			

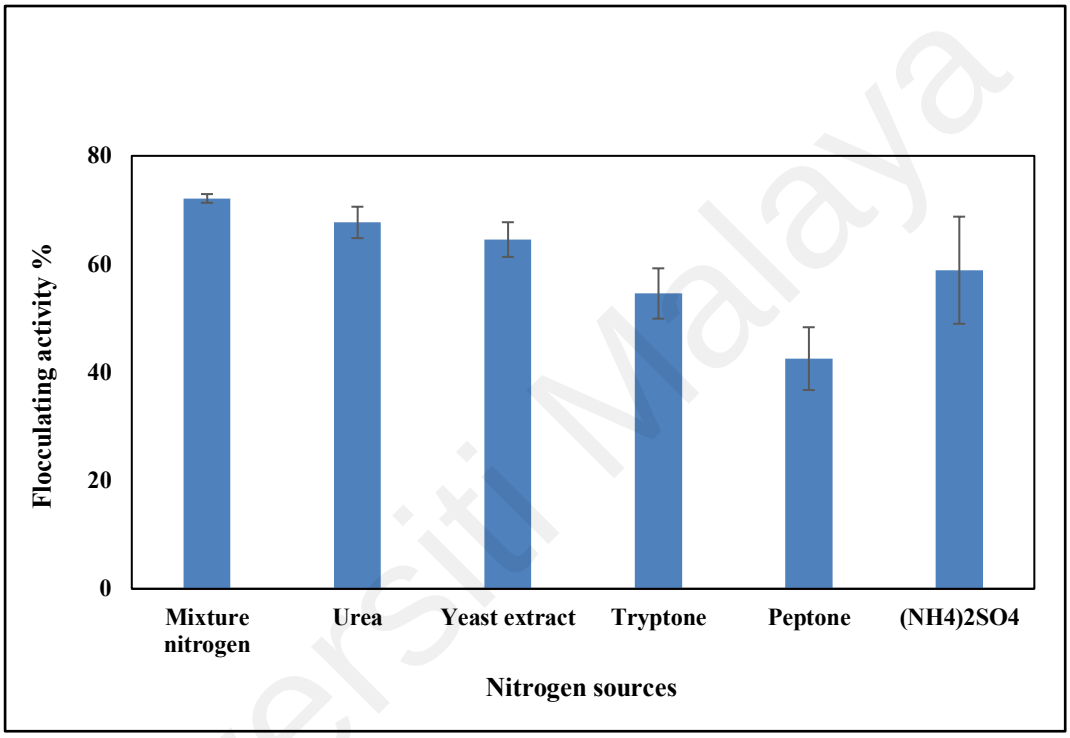


Figure 4.9: Effect of Nitrogen Sources on Bioflocculant Production.

4.4.8 Effect of Different Inorganic Salts

The effect of various salts on bioflocculant production were examined. Table 4.9. Show that the flocculating activity was nourished by several salt such as $CaCl_2$, KH_2P_4, K_2HPO_4, KCl, $FeCl_3$ and Na_2HPO_4, but were adversely affected by $Al_2(SO_4)_3$ and $CuSO_4$ Table 4.10, shows there is significant differences ($p< 0.00$), of the different of inorganic salts.

Table 4.9: Effect of Inorganic Salts on the Bioflocculant Production.

Salts	Flocculation activity (%)	Standard deviation
$CaCl_2$	76.8	1.7
KCl	73.0	1.6
$FeCl_3$	55.6	1.4
$CuSO_4$	40.2	1.2
$Al_2(SO_4)^3$	42.2	1.8
KH_2PO_4	75.7	1.7
K_2HPO_4	73.1	1.3
Na_2HPO_4	63.1	1.7
$MgSO_4$	67.0	1.5
0.1%K_2HPO_4+0.5% KH_2PO_4	91.8	1.5

Table 4.10: Analysis of Variance for the Effect of Inorganic Salts on the Bioflocculant Production.

	Sum of Squares	Degrees of Freedom	Mean Square	F-ratio	P-value
Between Groups	6957.467	9	773.052	280.430	0.000
Within Groups	55.133	20	2.757		
Total	7012.600	29			

4.5 Time Course of Bioflocculant Production by *B. salmalaya* 139SI

Optimal culture conditions were monitored for bioflocculant production by *B. salmalaya* 139SI. Figure 4.10, shows the time course assay of bioflocculant production. The production of bioflocculant at the early growth phase of *B. salmalaya* 139SI was reflected by the flocculating activity achieved through cultivation period. Results depicted in Table 4.11, prevailed that there is a significant difference ($p<0.00$), of the cultivation condition.

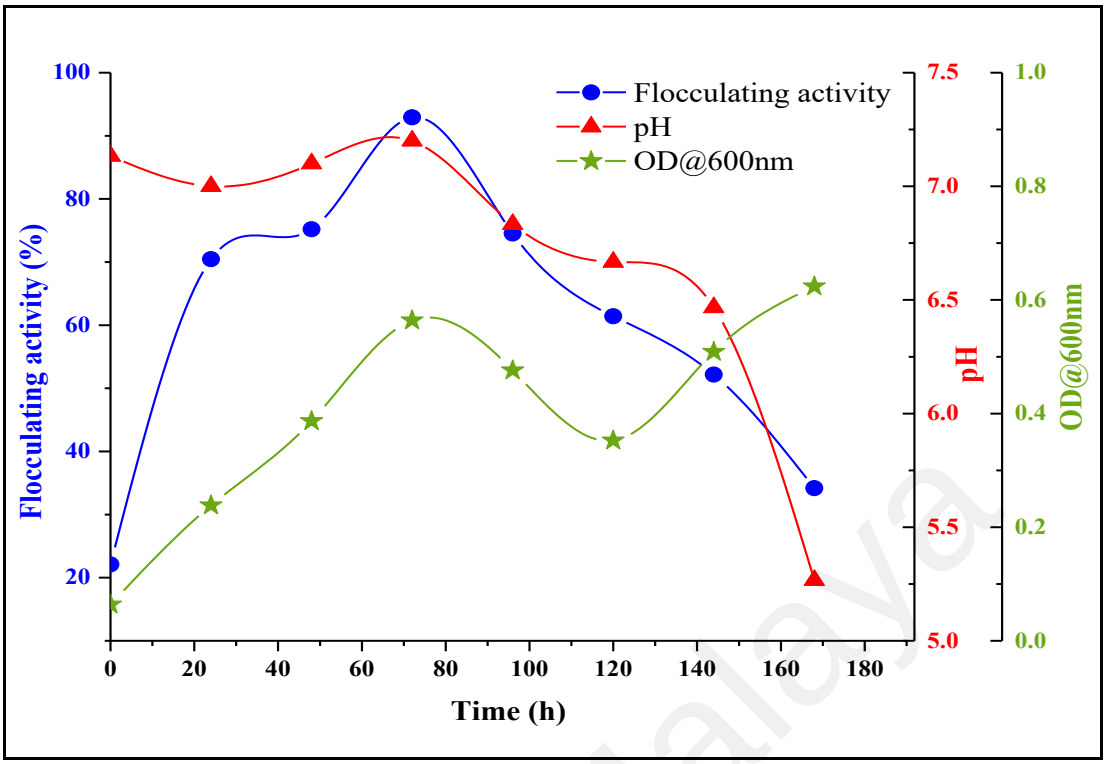


Figure 4.10: Time Course of Bioflocculant Production by *Bacillus salmalaya*139SI.

Table 4.11: Analysis of Variance for the Time Course of Bioflocculant Production.

	Sum of Squares	**Degrees of Freedom**	**Mean Square**	**F-ratio**	**P-value**
Between Groups	11248.807	7	1606.972	6536.837	0.000
Within Groups	3.933	16	0.246		
Total	11252.740	23			

4.6 Characterisations of Bioflocculant QZ-7

The composition of bacterial bioflocculant plays role in their flocculating activities (Wu & Ye, 2007).

4.6.1 Qualitative Chemical Analysis of the Bioflocculant QZ-7

Based on qualitative analysis of bioflocculant QZ-7, depicted in Table 4.12, presenting a clear indication that the bioflocculant QZ-7 composed of polysaccharides, protein and uronic acid was found.

Table 4.12: Qualitative Analysis of Bioflocculant QZ-7.

Reaction type	Analytical method	Occurrence
Polysaccharide	Anthrone test	+
	Phenol-sulphric acid test	+
	Molish test	+
Protein	Bradford test	+
	Folin-Lowry test	+
Uronic acid	Carbazole assay	+

4.6.2 Quantitative Chemical Analysis of the Bioflocculant QZ-7

On the basis of the quantitative analysis of the produced bioflocculant QZ-7. Figure 4.10, and Figure 4.11 showed the standard curve of the total carbohydrate and protein, were determined to be 79.08% and 15.4%, respectively.

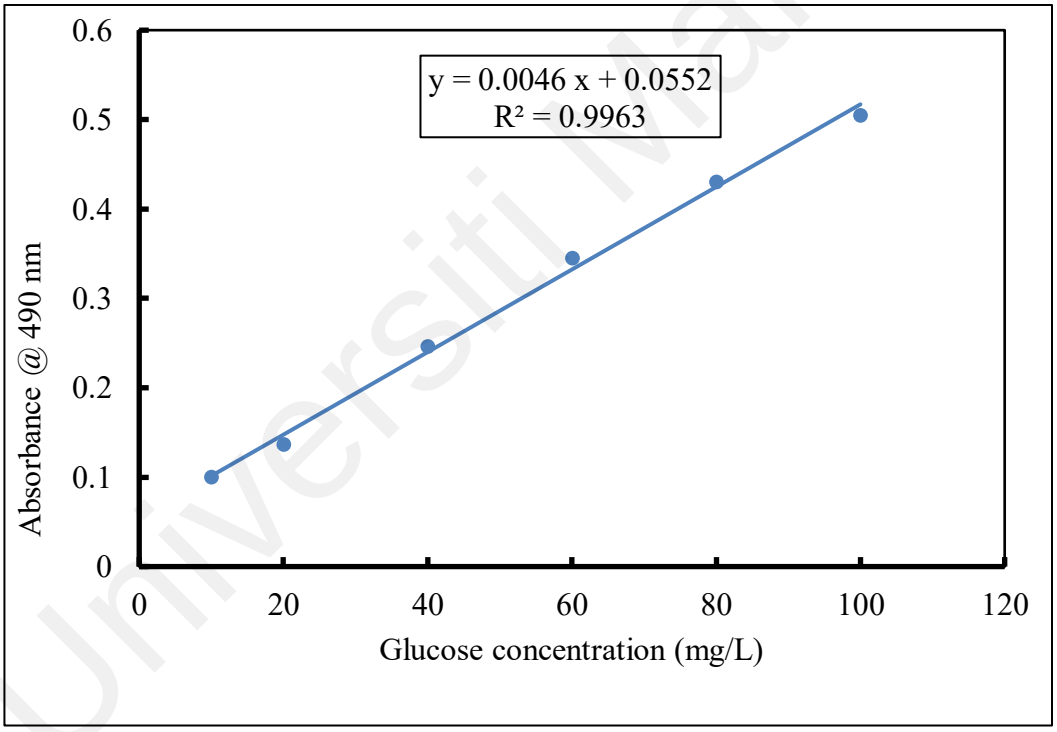


Figure 4.11: Determination of Carbohydrate Concentration.

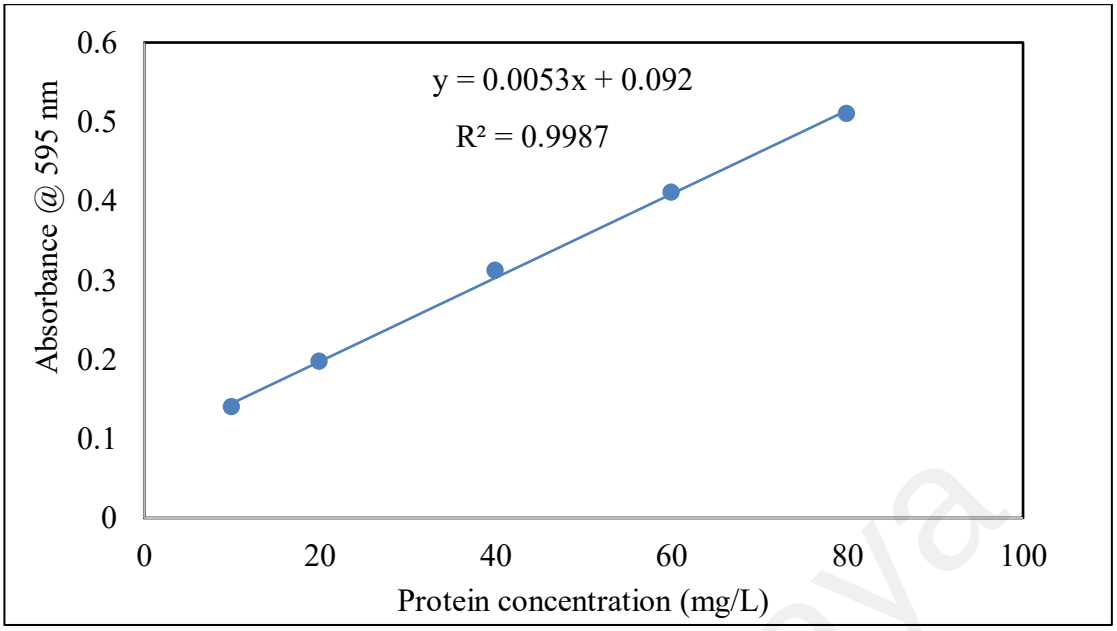


Figure 4.12: Determination of Protein Concentration.

4.6.3 Fourier-Transform Infrared Spectroscopy (FTIR)

Infrared spectrophotometry was used to analyze the purified bioflocculant QZ-7 as illustrated in Figure 4.13. Clear absorption peaks were revealed at spectrum of wave number 3420.56, 2929.82, 2437.35, 2176.08, 2073.19, 1658.90, 1432.97, 1187.49, 1109.66, 924.94, 618.50, 535.79, and 476.32 cm^{-1}.

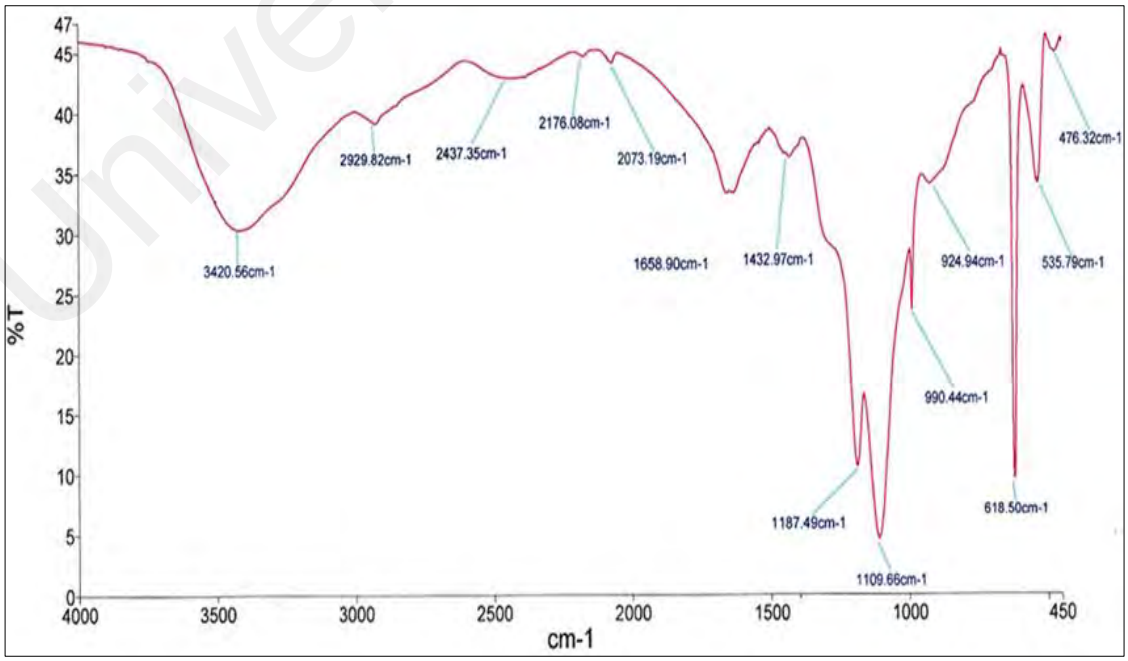


Figure 4.13: Fourier-Transform Infrared Spectroscopy Analysis of Bioflocculant QZ-7.

4.6.4 Molecular Weight Analysis

The HPGPC spectrum of the purified bioflocculant QZ-7 exhibited a symmetrical and sharp peak in the retention time of 6.53 and 9.95 (Figure 4.14). The molecular mass–retention time equation in accordance with the calibration curve was expressed as follows:

Log (molecular weight) = −0.1368T + 8.3496

The average weight of the bioflocculant was calculated to be 5.13× 10^5 Da, which was much higher than the weight of other bioflocculants (Yang *et al.*, 2015).

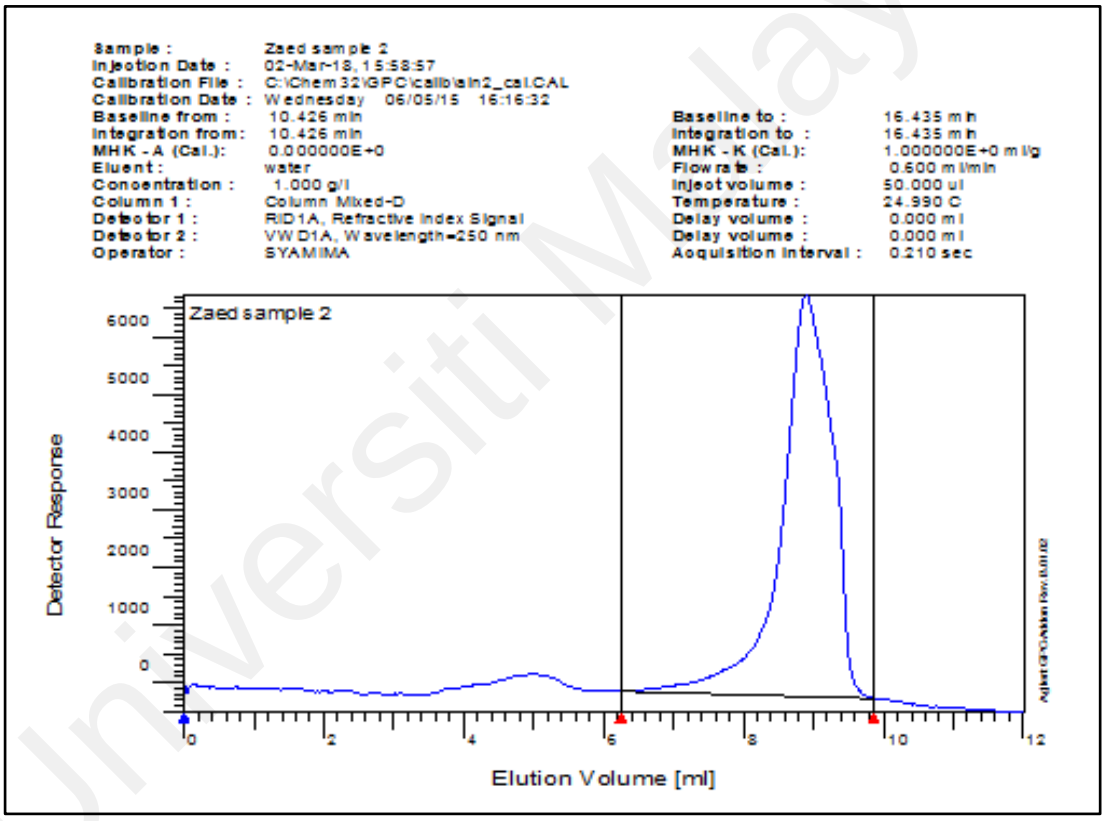


Figure 4.14: Gel Permeation Chromatography of the Purified Bioflocculant QZ-7.

4.6.5 H-NMR Spectrum

The spectral analysis of bioflocculant QZ-7 shown in Figure 4.15, 4.16 revealed the presence of hydroxyl, methyl, amino and carbonyl groups.

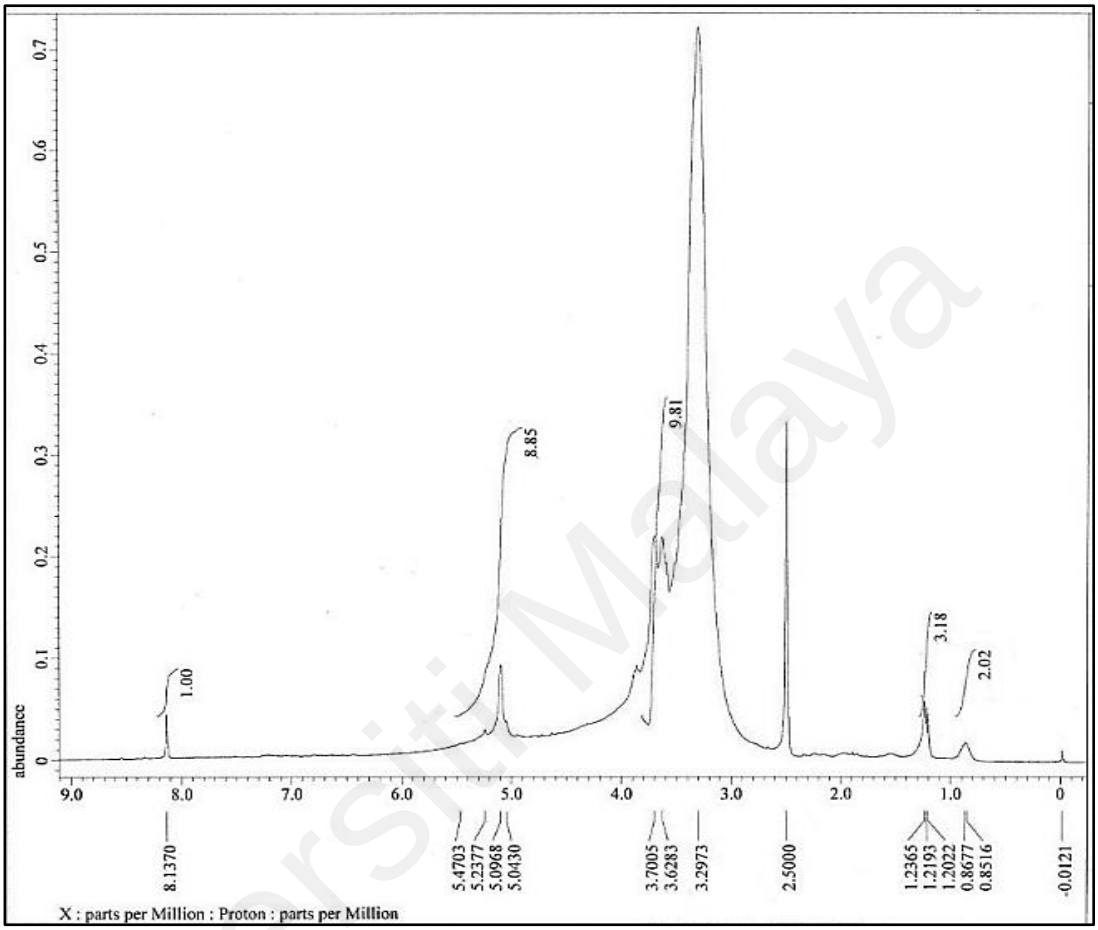


Figure 4.15: H^+ NMR Spectra of Purified Bioflocculant QZ-7.

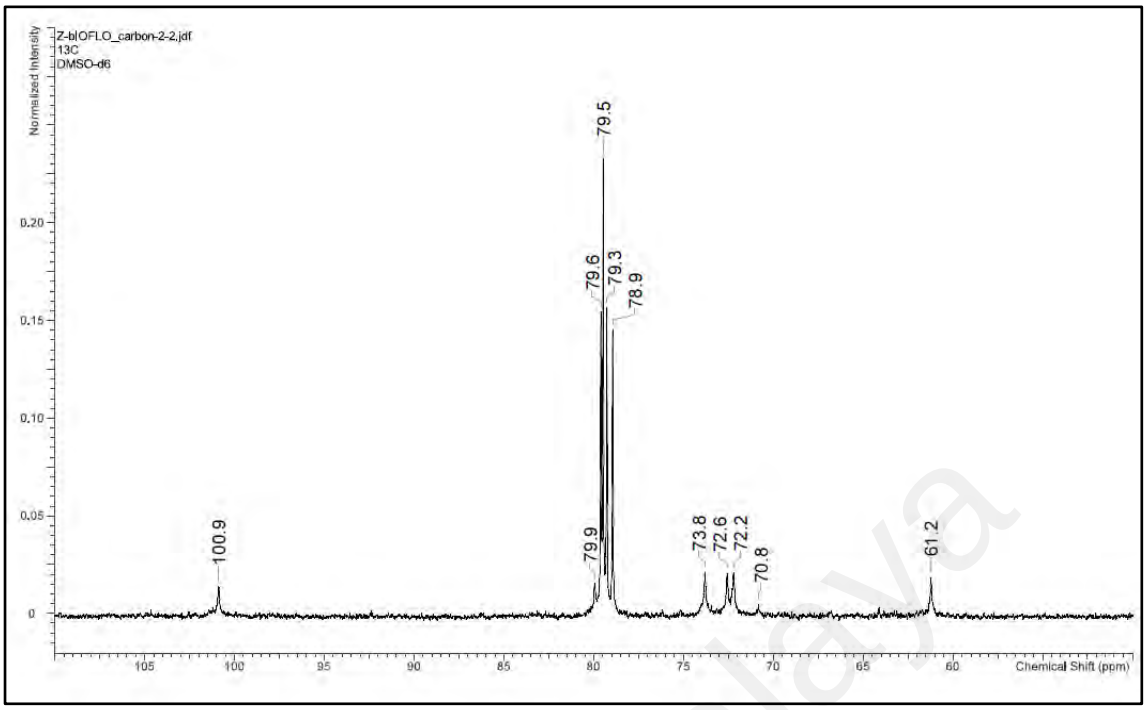
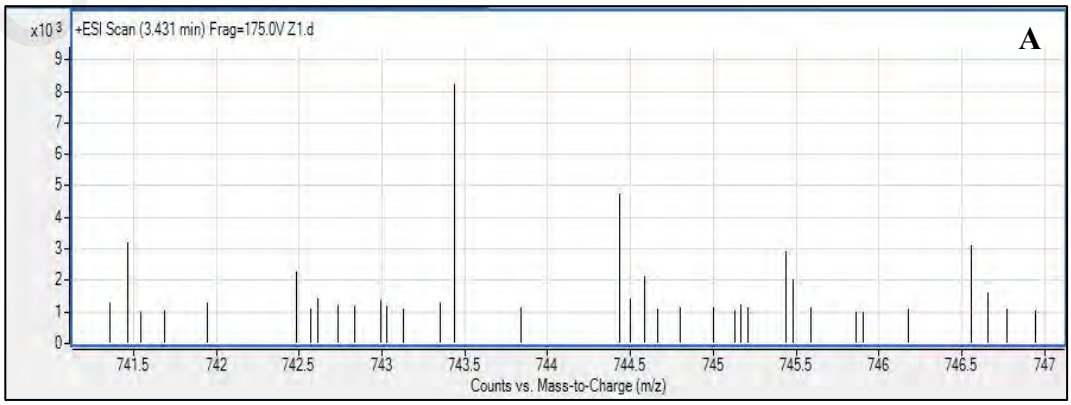


Figure 4.15: C^{13} NMR Spectra Of Purified Bioflocculant.

4.6.6 Liquid Chromatography Mass Spectrometry (LC-MS)

Through the LC/MS based analysis, adducts of Na^+, NH_4^+ and H^+ ions were identified for the sugar monomers depending on fragmentation and elution time in present case. Figure 4.17, shows the presence of carbohydrate such as glycoprotein (A) at 741 m/z-745 m/z at retention time 3.34. While, Na^+ adducts for glucose (B) appeared as (Glc+Na) + (182.96 m/z), glucuronic acid (C) at 212.85 m/z, at retention time 1.653, and rhamnose (D) as (Rha+Rha+Na) $^+$ (m/z 354.3) at retention time 1.576 and 1.529 min, respectively.



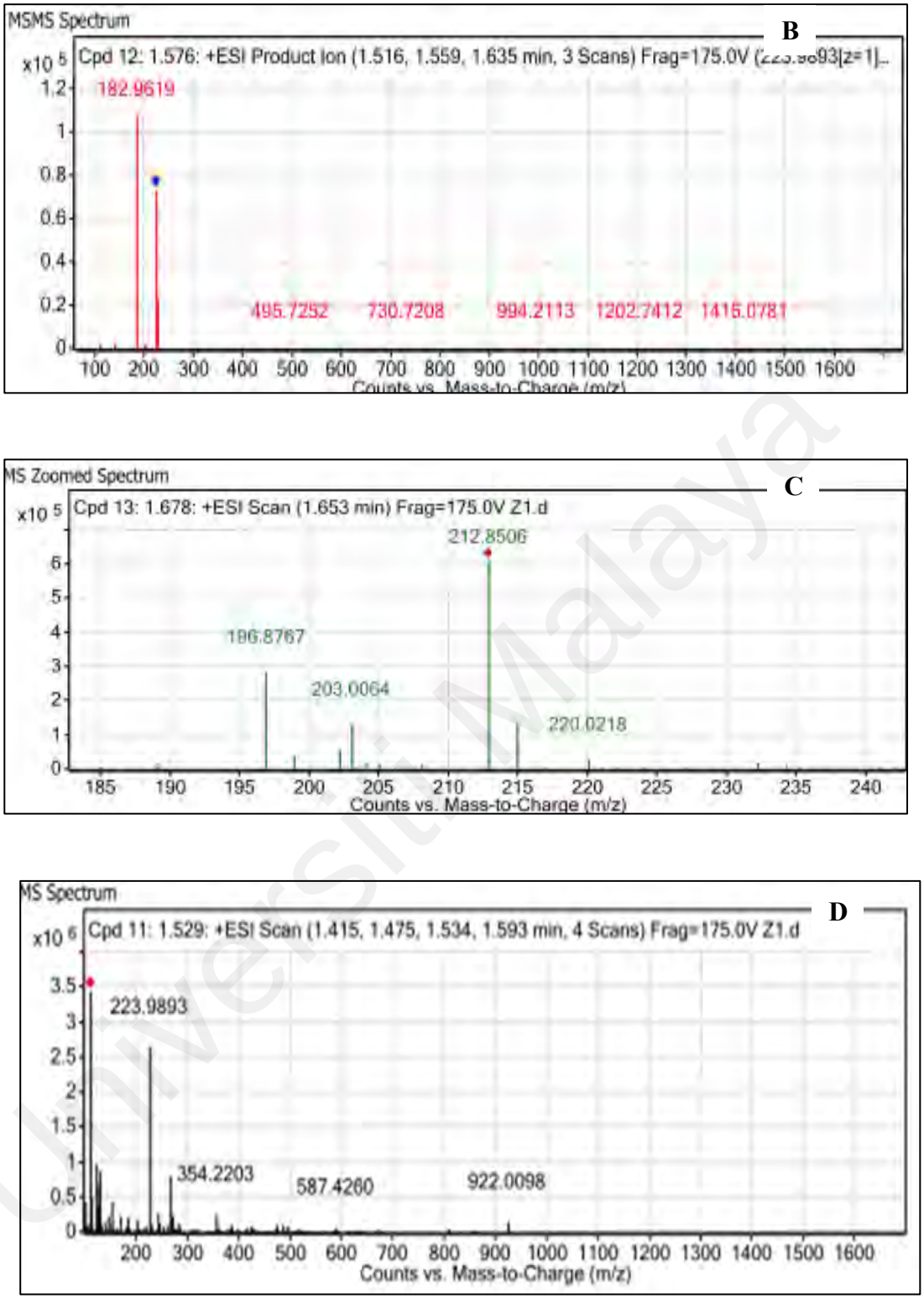


Figure 4.17: ESI Spectra of LC/MS Analyses of Bioflocculant QZ-7 Showing Glycoprotein (A), Na^+, H^+ and $NH4^+$ adducts of Glucose (B), Glucuronic Acid (C) and Rhamnose (D).

4.6.7 Scanning Electron Microscopy (SEM) Imaging

The morphological surface structure of QZ-7 were illuminated prior to and after flocculation process with kaolin clay particles. As shown in Figure 4.18A, the QZ-7 has

a greyish colour with a condensed crystalline brick shaped structure. Whereas, Figure 4.18B, showed the flocculation of the bioflocculant QZ-7 with kaolin clay.

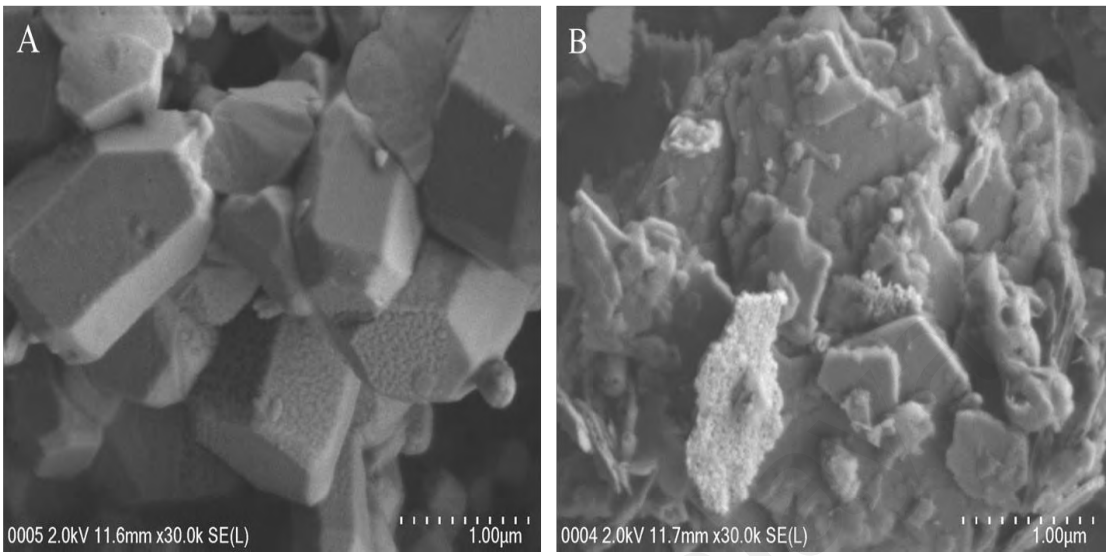


Figure 4.16: SEM Micrograph of Bioflocculant QZ-7 of *B. salmalaya strain* 139SI. A. Purified Bioflocculant QZ-7. B. Bioflocculant Aggregation with Kaolin Clay.

4.6.8 Energy Dispersive X-ray (SEM- EDX)

The elemental analysis of bioflocculant QZ-7 on the basis of SEM-EDX testing as shown in Figure 4.19, the existence of C, O, N, P and S in this macromolecule as 55.74%, 42.74%, 0.54%, 0.93% and 0.06%, respectively.

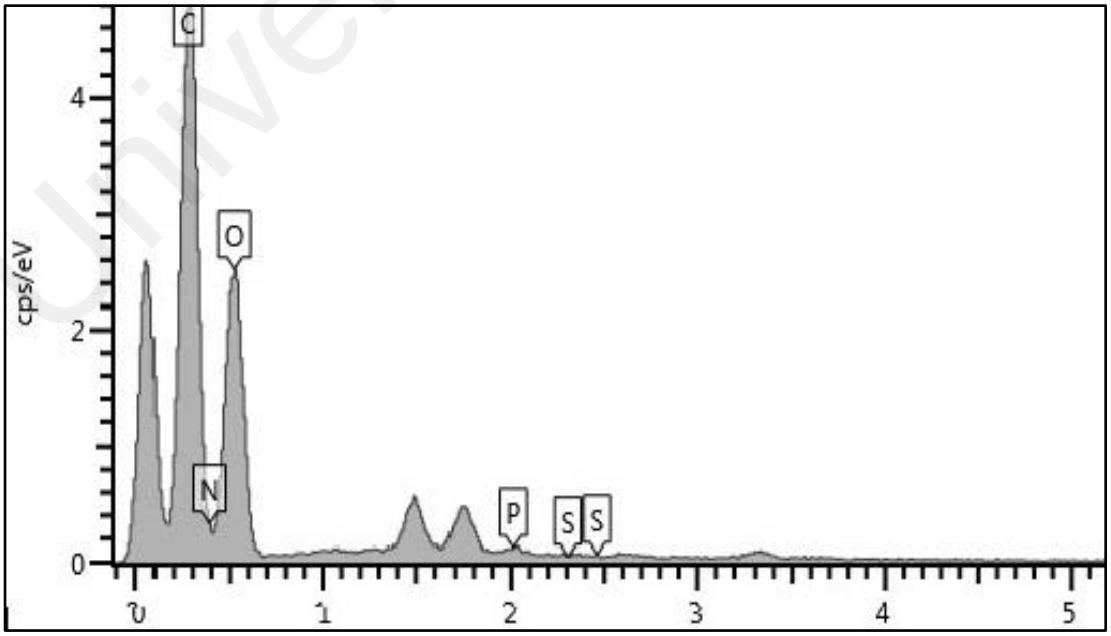


Figure 4.19: EDX Analysis of Bioflocculant QZ-7.

4.6.9 Thermogravimetric (TGA) Determination of the Bioflocculant QZ-7

Bioflocculant QZ-7 was tested to explain its property comportments while exposed to a different heat degree. For this reason, to assist us to recognise its pyrolysis property after exposed to a very elevated temperature. Results as depicted in Figure 4.20, approximately 12.14%, 23.76% in weight loss of QZ-7 at 100˚C, 200˚C, respectively, and a 45.28% weight loss of QZ-7 at 200-450˚C.

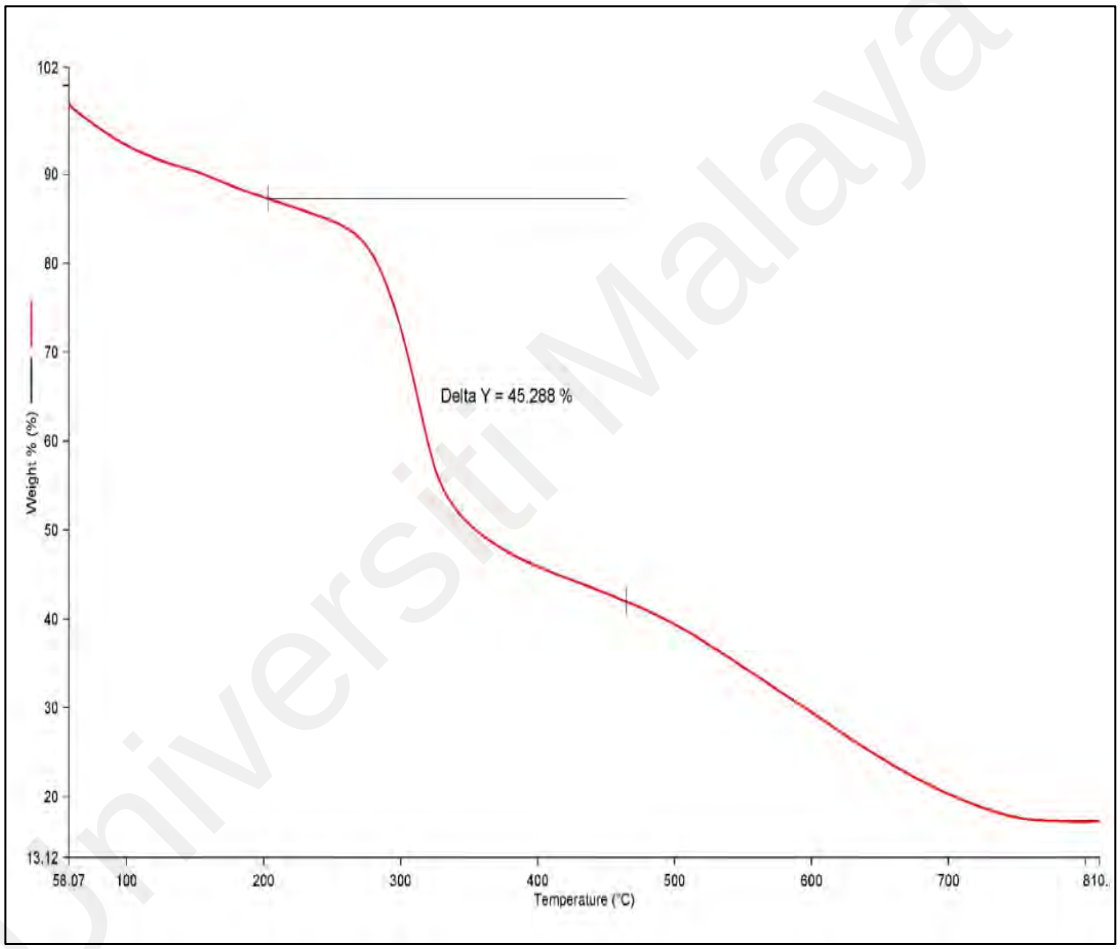


Figure 4.20: Thermogravimetric Analysis of the Purified Bioflocculant QZ-7.

4.7 Factors Affecting of Bioflocculant QZ-7 Performance

4.7.1 Effect of pH on the Flocculation Efficiency of QZ-7

As shown in Figure 4.21, bioflocculant QZ-7 was found to be a fairly steady at wider range of pH range (5-7), and above 70 % flocculating activity was accomplished at that pH series.

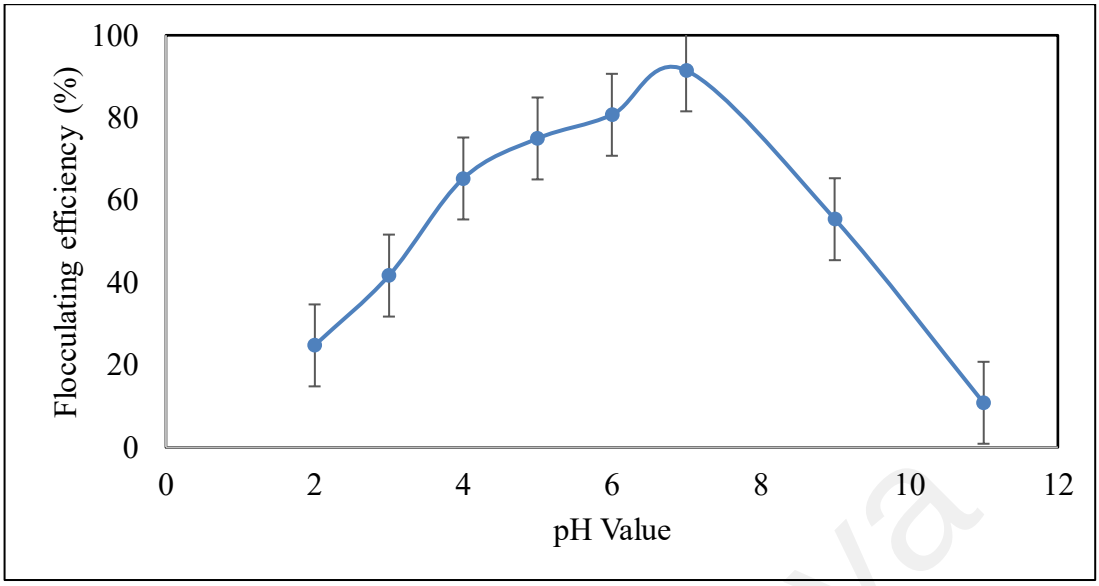


Figure 4.17: pH Stability of the Purified Bioflocculant QZ-7.

As depicted in Table 4.13, there is significant difference ($p<0.000$), of different pH value.

Table 4.13: Analysis of Variance for the Effect of pH on Flocculating Performance.

	Sum of Squares	**Degrees of Freedom**	**Mean Square**	**F-ratio**	**P-value**
Between Groups	16405.203	7	2343.600	2014.895	0.000
Within Groups	18.610	16	1.163		
Total	16423.813	23			

4.7.2 Effect of Temperature on the Flocculation Efficiency of QZ-7

The influence of temperature on the flocculation activity of the purified bioflocculant QZ-7 was investigated. Figure 4.22, showed that more than 80% of flocculating efficiency was sustained in the temperature series of 25- 45˚C, also the flocculating efficiency above 70% was found to be a constant at 55-65 ˚C. Moreover, above 60% of flocculating efficiency was obtained at 75-85 ˚C.

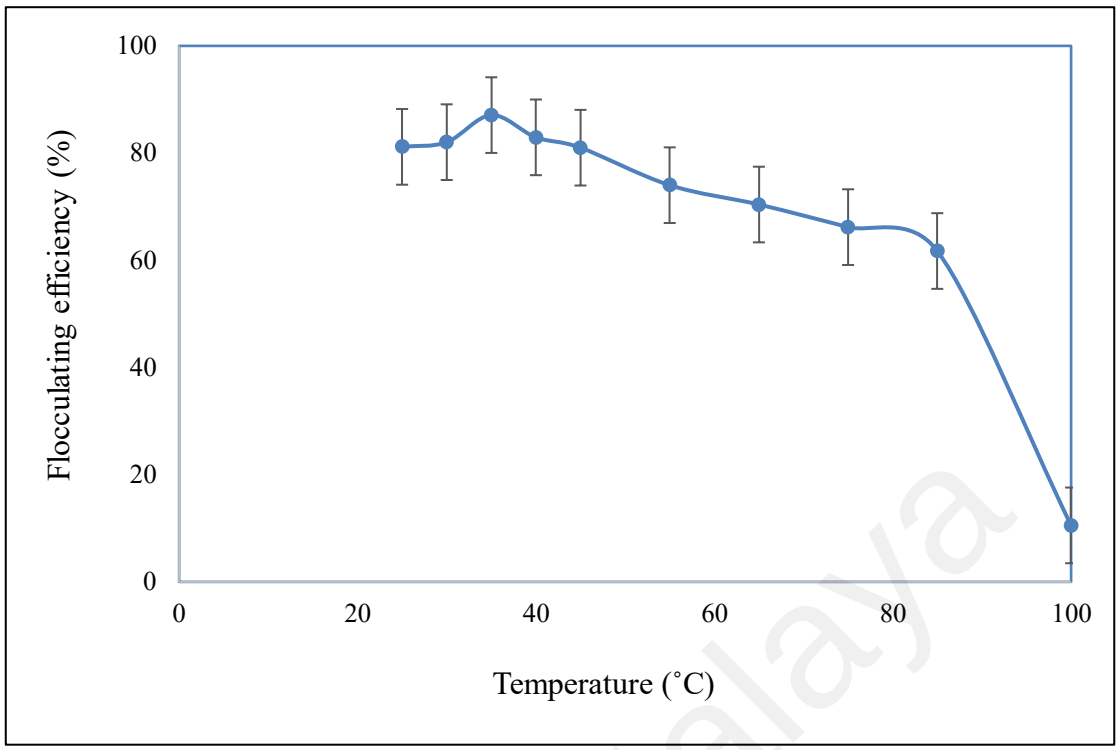


Figure 4.18: Thermal Stability of the Purified Bioflocculant QZ-7.

As shown in Table 4.14, the treatment analysis for the effect of different temperature degrees on flocculating activity revealed that there are significant differences ($p<0.00$).

Table 4.14: Analysis of Variance for the Effect of Temperature on Bioflocculant Performance.

	Sum of Squares	**Degrees of Freedom**	**Mean Square**	**F-ratio**	**P-value**
Between Groups	12932.466	8	1616.558	983.215	.000
Within Groups	29.595	18	1.644		
Total	12962.061	26			

4.7.3 Effect of Bioflocculant QZ-7 Concentration on the Flocculation Efficiency

Figure 4.23 showed the flocculating activity increased dramatically as the bioflocculant QZ-7 concentration increased from 0.25 to 2 mg/mL. The highest flocculating efficiency of 93.6% was obtained at 2 mg/mL of bioflocculant QZ-7. Also, QZ-7 show a good flocculating activity of 77.23% at 1mg/L, 73.2 % at 0.5mg/mL, 84.2 % at 4 mg/mL and over 64.93% at 6 mg/mL.

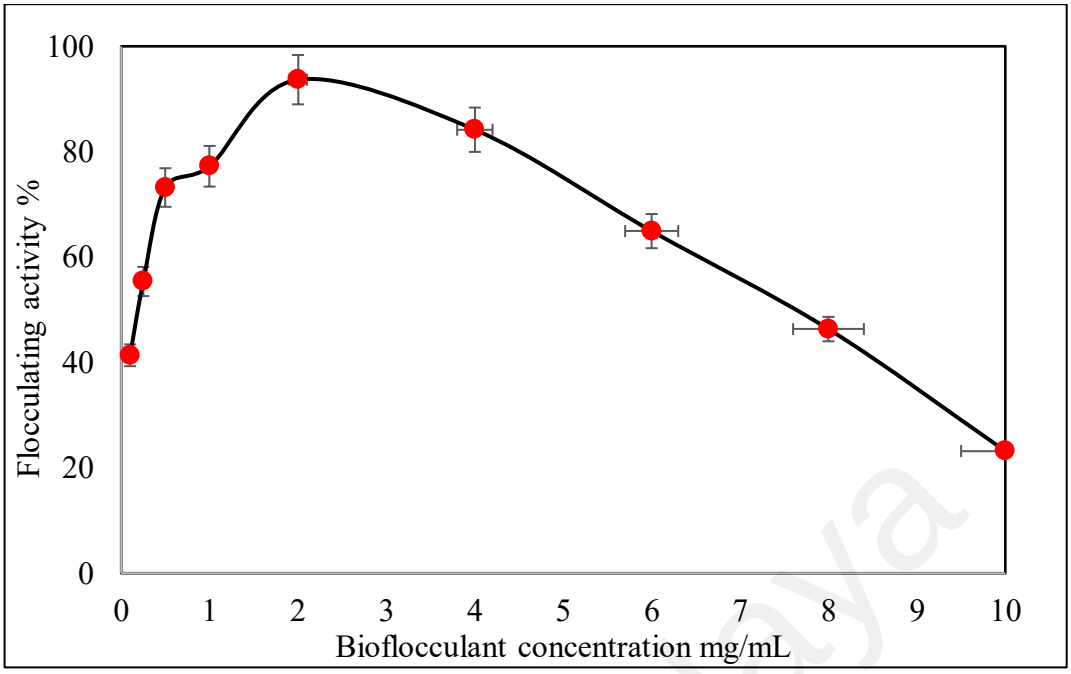


Figure 4.19: Bioflocculant QZ-7 Dosage Effect on Flocculating Activity.

Table 4.15, shows the result obtained is in accordance with the reported findings in the flocculation performance of different concentration of bioflocculant has significant differences ($p<0.00$).

Table 4.15: Analysis of Variance for the Effect of Bioflocculant QZ-7 Concentration.

	Sum of Squares	**Degrees of Freedom**	**Mean Square**	**F-ratio**	**P-value**
Between Groups	12241.856	8	1530.232	834.503	0.000
Within Groups	33.007	18	1.834		
Total	12274.863	26			

4.7.4 Effect of Cations on the Flocculation Efficiency of QZ-7

Figure 4.24 shown the monovalent and divalent cations, including Al^{+3} were facilitating the flocculating activity to a good point matched with the trivalent Fe^{+3} cations. The maximum flocculating activity 92.9 % was detected with Ca^{+2}, followed by Al^{+3} (83.3%), Mn^{+2}(75.6%), K^{+}(71.6%), Mg^{+2} (71.4%), Na^{+} (67.7%) and Li^{+} (64.0 %).

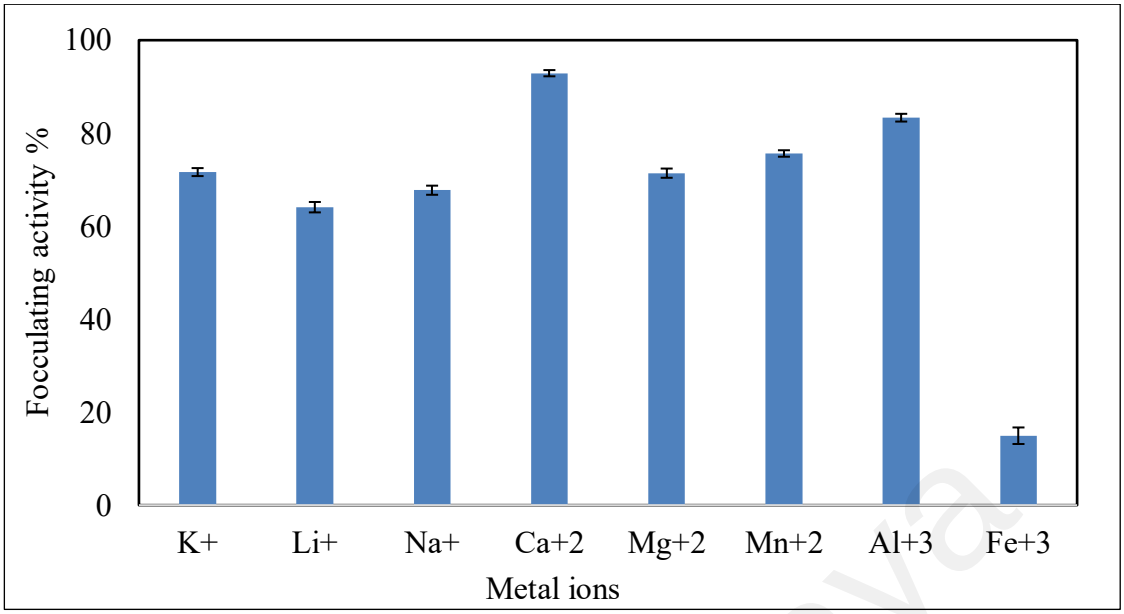


Figure 4.20: Effect of Metal Ions on Flocculating Activity of Purified Bioflocculant QZ-7.

The statistical analysis presented in Table 4.16, indicated that there is a significant difference ($p<0.000$) between different cations.

Table 4.16: Analysis of Variance for the Effect of Cations on Flocculating Activity.

	Sum of Squares	**Degrees of Freedom**	**Mean Square**	**F-ratio**	**P-value**
Between Groups	11327.947	7	1618.278	1016.387	0.000
Within Groups	25.475	16	1.592		
Total	11353.422	23			

4.7.5 Effect of Salinity on Flocculation Efficiency of QZ-7

Figure 4.25. Different concentrations of NaCl salt were tested to examine the effect of salinity on bioflocculant QZ-7 flocculation performance. As presented at 1% and 5% concentration of NaCl the bioflocculant QZ-7 maintained over 90 % of flocculating efficiency. While 10% (w/w) of NaCl in kaolin suspension has no effect on the flocculating efficiency of bioflocculant QZ-7.

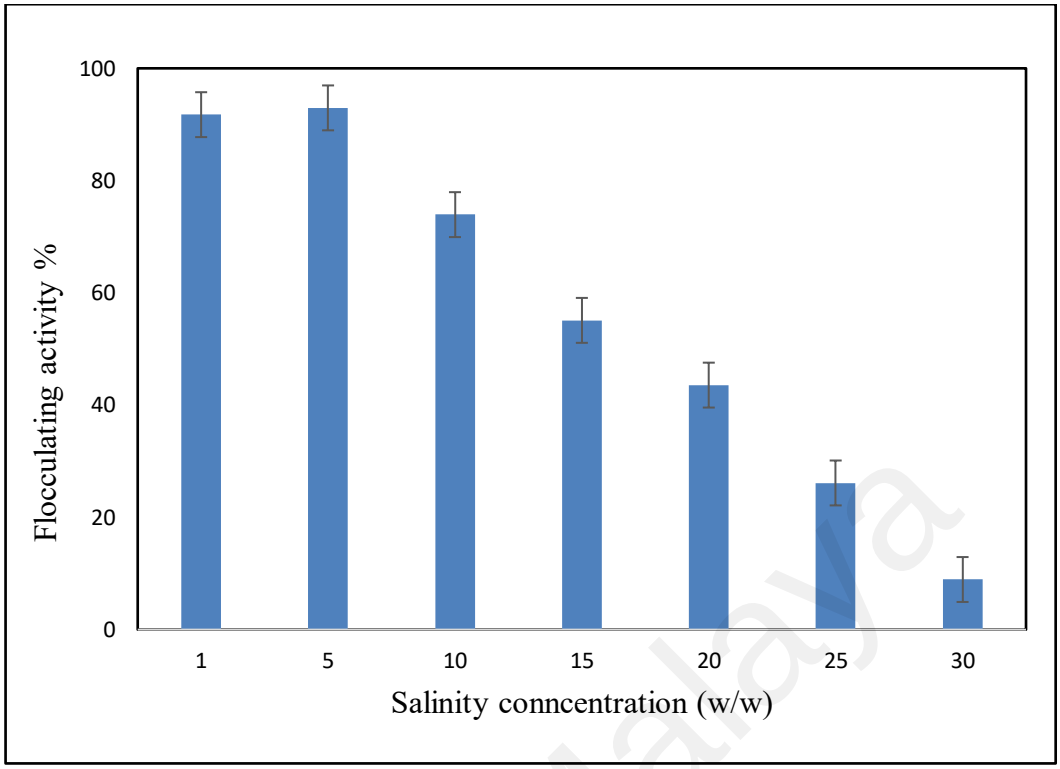


Figure 4.21: Effect of Salinity on Flocculation Performance of Bioflocculant QZ-7.

The statistical analysis of variance of the effect salinity concentration of flocculating activity is depicted in Table 4.17, revealed that there is significant difference between different concentration of salt 1-30% (w/w) found to be ($p<0.000$).

Table 4.17: Analysis of Variance for the Effect of Salinity on Flocculation Performance.

	Sum of Squares	**Degrees of Freedom**	**Mean Square**	**F-ratio**	**P-value**
Between Groups	18648.423	6	3108.071	1049.224	0.000
Within Groups	41.472	14	2.962		
Total	18689.895	20			

4.8 Application of Bioflocculant QZ-7

In this study the application of bioflocculant QZ-7 in water treatment and industrial wastewater were investigated by using surface water, sugar mill, rubber wastewater and hospital wastewater. Bioflocculant QZ-7 produced by *Bacillus salmalaya* 139SI shown different levels of COD, TSS, TDS, Turbidity and BOD removals, heavy metal adsorption. Differences in the attraction of metals for bioflocculants are due to attractive

interaction, charge density and polymers conformation natures with adsorbed ions (Morillo *et al.*, 2006). The metal biosorption mechanism and kinetics influenced by experimental conditions mainly, pH medium, bioflocculant concentrations and initial heavy metal concentrations (Converti *et al.*, 2006).

4.8.1 Surface Water Treatment by Bioflocculant QZ-7

The physical and chemical characteristic of Langat River are shown at Table 4.18**.**

Table 4.18: The Average Water Quality Characteristic of Langat River.

Parameter	**Values**
pH at 25˚C	6.72
Conductivity (ms/cm)	25.5
Chemical Oxygen Demand (mg/L)	67.4
Total suspended solid (mg/L)	194.6
Turbidity NTU	178.5

4.8.1.1 Removal of Turbidity

The flocculation performance of purified QZ-7 was shown in Figure 4.26. The turbidity removal of purified QZ-7 was higher than crude bioflocculant especially at low bioflocculant concentration. Purified QZ-7 concentration of 5 mg/L, reduced the turbidity of river water from 178.5 NTU to 5.2 NTU and the turbidity was 96.8%. The crude bioflocculant at 5 mg/L dosage produced water with final turbidity of 35.6 NTU with removal efficiency 83.3%.

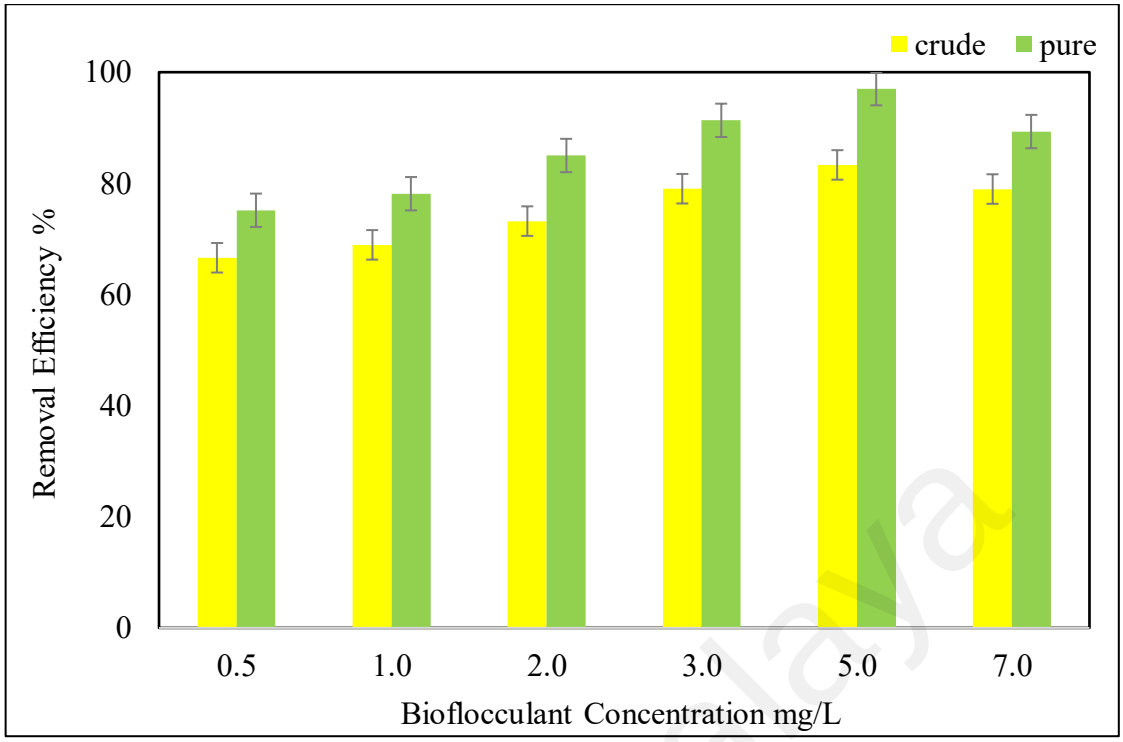


Figure 4.22: Effect of Bioflocculant QZ-7 Concentration on Turbidity Removal.

As depicted in Table 4.19 and Table 4.20 there is a significant difference ($p<0.000$), between different concentration of pure QZ-7.

Table 4.19: Analysis of Variance for the Effect of Pure Bioflocculant QZ-7 on Turbidity Removal.

	Sum of Squares	**Degrees of Freedom**	**Mean Square**	**F-ratio**	**P-value**
Between Groups	2813.491	5	562.698	677.950	0.000
Within Groups	9.960	12	.830		
Total	2823.451	17			

Table 4.20: Analysis of Variance for the Effect of Crude Bioflocculant QZ-7 on Turbidity Removal.

	Sum of Squares	**Degrees of Freedom**	**Mean Square**	**F-ratio**	**P-value**
Between Groups	1733.765	5	346.753	209.730	0.000
Within Groups	19.840	12	1.653		
Total	1753.605	17			

4.8.1.2 Total Suspended Solid Treatment

The following Figure 4.27, show the final TSS concentration removal of water treated by purified and crude bioflocculant QZ-7. In general, the TSS removal efficiency was gradually increased as the bioflocculant concentration increased. The TSS removal efficiency of purified bioflocculant QZ-7 was found a significant at concentration range of 0.5 to 7 mg/L. In addition, 5 mg/L of purified bioflocculant produced water with final TSS of 4.53 mg/L. (97.8%).

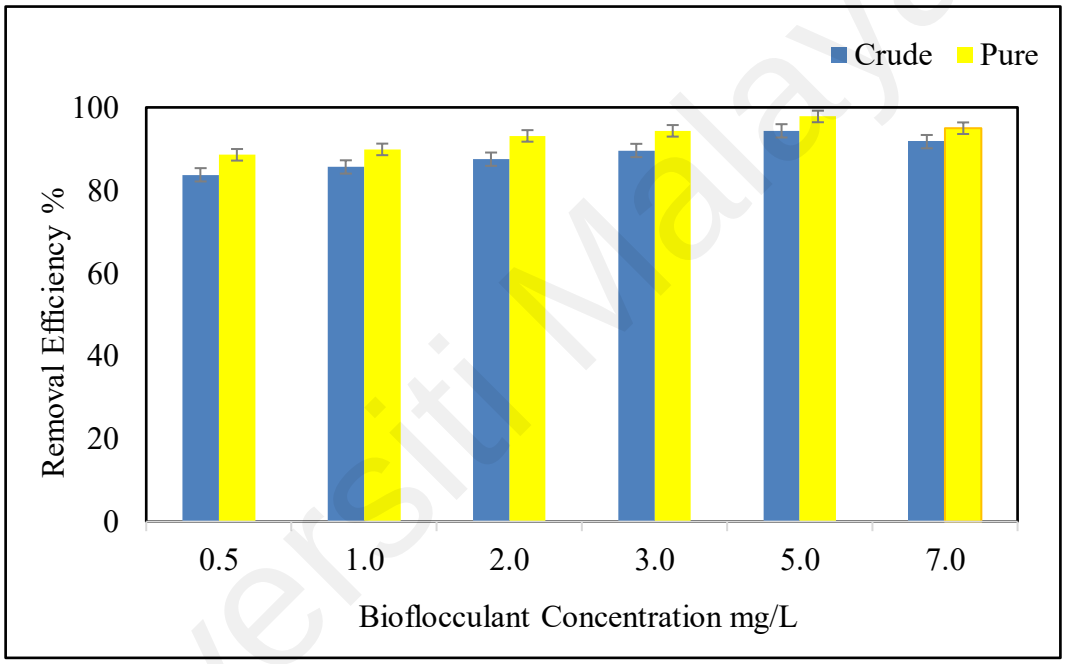


Figure 4.23: Effect of Bioflocculant QZ-7 Concentration on TSS Removal.

The result obtained by statistical analysis in Table 4.21 and Table 4.22 found that there is significant difference ($p<0.000$) between pure and crude bioflocculant on TSS removal.

Table 4.21: Analysis of Variance for the Effect of Pure Bioflocculant QZ-7 on TSS Removal.

	Sum of Squares	**Degrees of Freedom**	**Mean Square**	**F-ratio**	**P-value**
Between Groups	732.375	5	146.475	135.995	0.000
Within Groups	12.925	12	1.077		
Total	745.300	17			

Table 4.22: Analysis of Variance for the Effect of Crude Bioflocculant QZ-7 on TSS Removal.

	Sum of Squares	Degrees of Freedom	Mean Square	F-ratio	P-value
Between Groups	965.433	5	193.087	184.969	0.000
Within Groups	12.527	12	1.044		
Total	977.960	17			

4.8.1.3 Chemical Oxygen Demand (COD) Removal

Results showed in Figure 4.28, that the COD reduction was increased dramatically as the bioflocculant QZ-7 concentration increased from 0.5 to 5 mg/L. The highest reduction of COD was 91.2% of optimum bioflocculant concentration at 5 mg/L, then the COD reduction was dropped as the purified bioflocculant QZ-7 concentration increased from 5-7 mg/L.

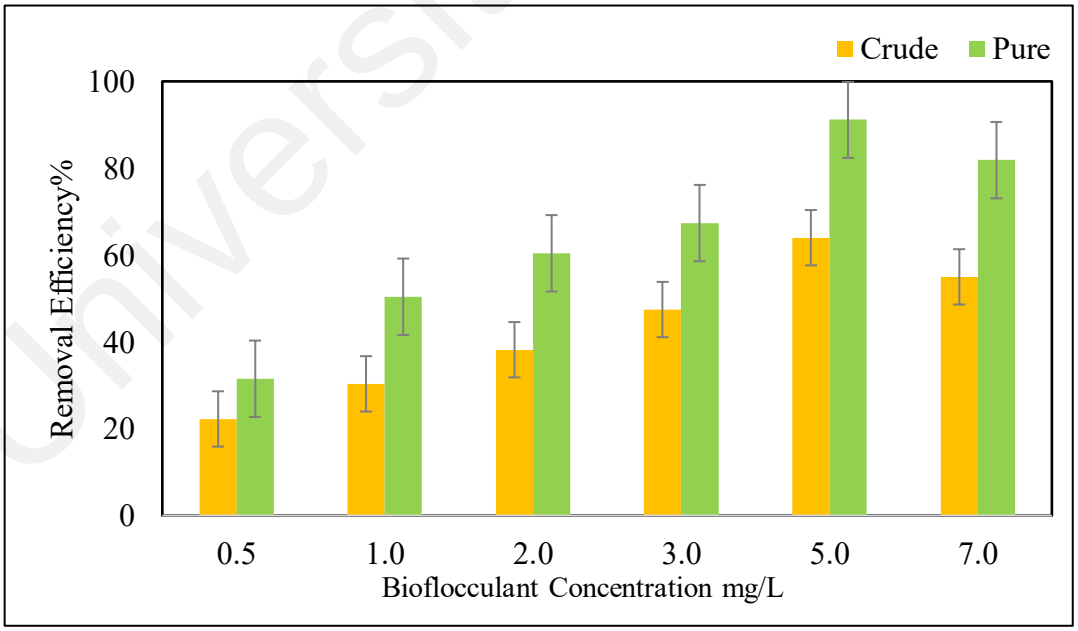


Figure 4.28: Effect of Bioflocculant QZ-7 Concentration on COD Reduction.

Based on the statistical analysis of the effect of pure and crude bioflocculant QZ-7 on COD removal are presented in Table 4.23 and Table 4.24, revealed that there is significant difference ($p<0.000$), between the two types of QZ-7.

Table 4.23: Analysis of Variance for the Effect of Pure Bioflocculant QZ-7 on COD Removal.

	Sum of Squares	Degrees of Freedom	Mean Square	F-ratio	P-value
Between Groups	3540.608	5	708.122	505.946	0.000
Within Groups	16.795	12	1.400		
Total	3557.403	17			

Table 4.24: Analysis of Variance for the Effect of Crude Bioflocculant QZ-7 on COD Removal.

	Sum of Squares	Degrees of Freedom	Mean Square	F-ratio	P-value
Between Groups	1905.352	5	381.070	330.090	0.000
Within Groups	13.853	12	1.154		
Total	1919.205	17			

4.8.2 Removal of Heavy Metals using Bioflocculant

4.8.2.1 Effect of pH on the Heavy Metal Adsorption.

Figure 4.29, showed that the highest adsorption 89.3% of As, 81.3% of Zn^{+2}, are reported at pH 7, whereas, the highest adsorption 76.1% of Cu^{+2}, 77.9% of Pb^{+2} and 68.7% of Cd^{+2} were reported at pH 9 and the highest adsorption 68.4% of Hg^{+2} was at pH 5.

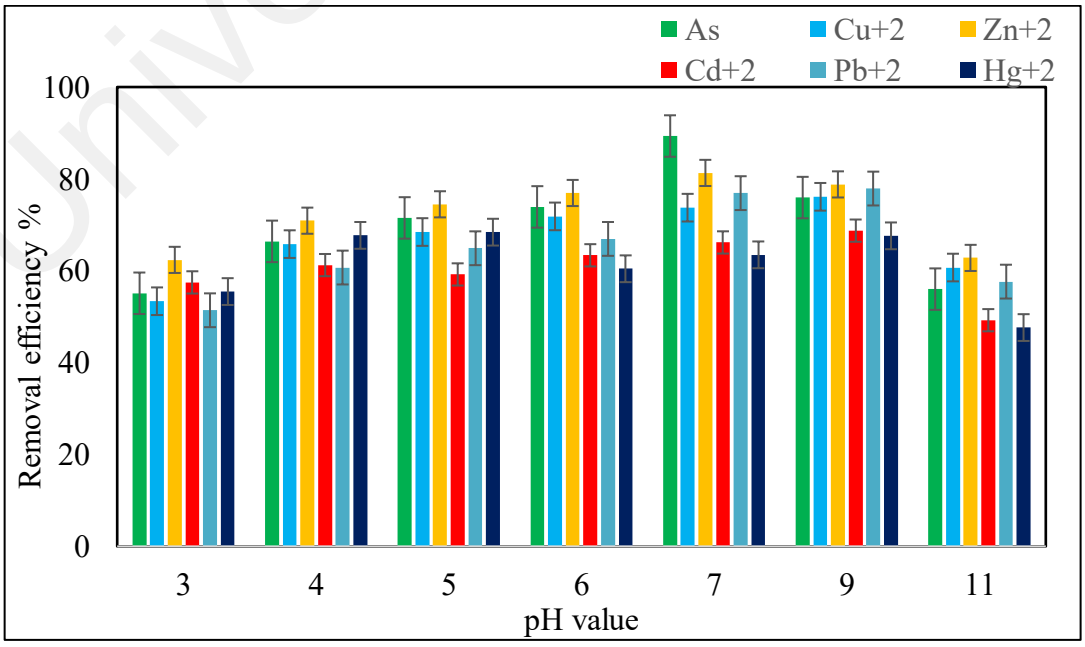


Figure 4.24: Effect of pH on Heavy Metal Adsorption using 20 mg/L of QZ-7.

Regarding to the analysis of variance of the effect of different pH value on arsenate removal efficiency is presented in Table 4.25. It is revealed that the different pH value has a significant difference ($p<0.00$) on as removal.

Table 4.25: Analysis of Variance for the Effect of pH on Arsenate Removal Efficiency.

	Sum of Squares	**Degrees of Freedom**	**Mean Square**	**F-ratio**	**P-value**
Between Groups	2555.898	6	425.983	274.912	0.000
Within Groups	21.693	14	1.550		
Total	2577.591	20			

As depicted in Table 4.26, there is a significant difference ($p<0.000$) between thedifferent pH value on cupper removal.

Table 4.26: Analysis of Variance for the Effect of pH on Cupper Removal Efficiency.

	Sum of Squares	**Degrees of Freedom**	**Mean Square**	**F-ratio**	**P-value**
Between Groups	1141.283	6	190.214	205.056	0.000
Within Groups	12.987	14	.928		
Total	1154.270	20			

Table 4.27, shows that there is a significant difference ($p<0.000$) between the different pH value on cadmium removal.

Table 4.27: Analysis of Variance for the Effect of pH on Cadmium Removal Efficiency.

	Sum of Squares	**Degrees of Freedom**	**Mean Square**	**F-ratio**	**P-value**
Between Groups	738.030	6	123.005	118.600	.000
Within Groups	14.520	14	1.037		
Total	752.550	20			

Table 4.28, presented the statistical treatment of the effect of different pH value on lead removal efficiency. It is revealed that there is a significant difference ($p<0.000$) between the groups.

Table 4.28: Analysis of Variance for the Effect of pH on Lead Removal Efficiency.

	Sum of Squares	**Degrees of Freedom**	**Mean Square**	**F-ratio**	**P-value**
Between Groups	1707.656	6	284.609	687.779	0.000
Within Groups	5.793	14	0.414		
Total	1713.450	20			

As depicted in Table 4.29, showed that there is a significant difference ($P<0.000$) between the different pH value on the zinc efficiency removal. However, multiple comparison analysis presented in appendix F23, found that no significant difference ($p>0.05$), have been detected between pH value such as pH 3 and 11, pH 6 and 9, and pH 7and 9.

Table 4.29: Analysis of Variance for the Effect of pH on Zinc Removal Efficiency.

	Sum of Squares	**Degrees of Freedom**	**Mean Square**	**F-ratio**	**P-value**
Between Groups	1040.038	6	173.340	131.603	.000
Within Groups	18.440	14	1.317		
Total	1058.478	20			

The result obtained by statistical analysis presented in Table 4.30, showed that there is a significant difference ($p<0.000$), between the different pH value of mercury removal efficiency. Moreover, the multiple comparison shown in appendix F24, revealed that there is no significant difference ($p>0.05$) between the following pH 3 and 6, pH 4 and 5, 7 and 9, and pH 6 and 7.

Table 4.30: Analysis of Variance for the Effect of pH on Mercury Removal Efficiency.

	Sum of Squares	**Degrees of Freedom**	**Mean Square**	**F-ratio**	**P-value**
Between Groups	1067.392	6	177.899	29.260	.000
Within Groups	85.120	14	6.080		
Total	1152.512	20			

4.8.2.2 Effect of the Bioflocculant QZ-7 Concentration on Heavy Metals Adsorption

The results obtained in Figure 4.30 showed that an exceed 50% of heavy metals were removed at 20 mg/L - 100 mg/L of bioflocculant QZ-7 concentration. Whereas, the highest metal removal was achieved at 60 mg/L of bioflocculant concentration, as 84% of As, 80.7% of Cu^{+2}, 78.5% of Zn^{+2} and 77.4% Cd^{+2} and Pb^{+2} 72.43% at 100 mg/L.

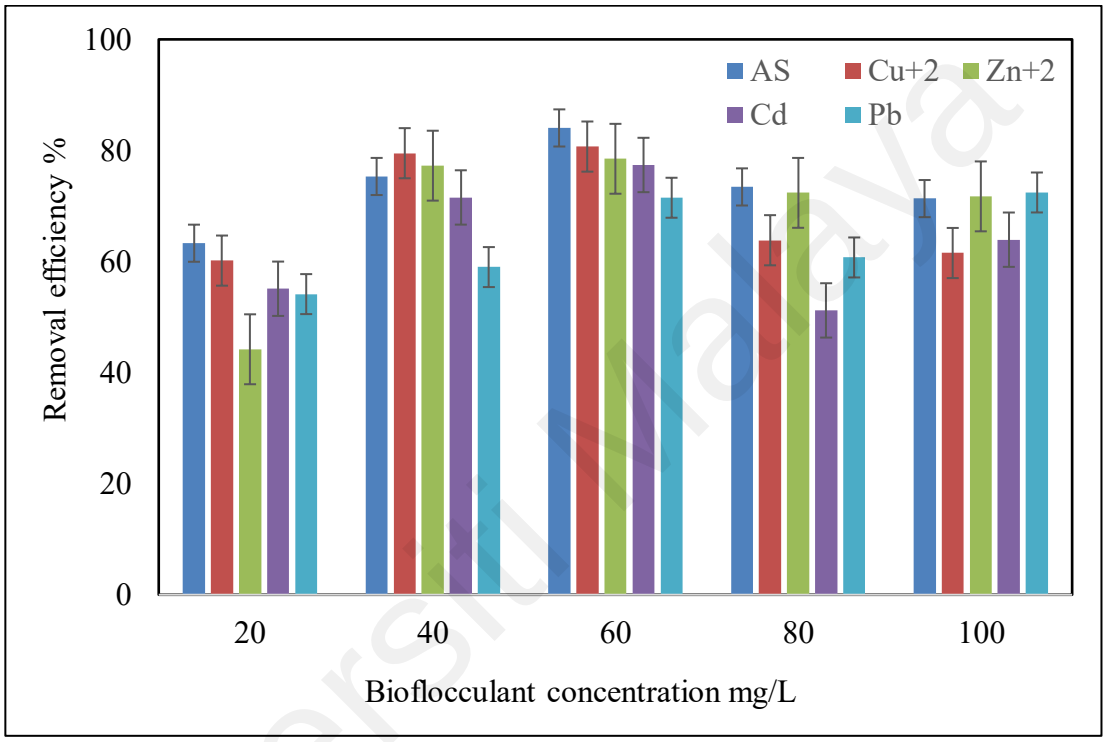


Figure 4.25: Effect of Bioflocculant QZ-7 Concentration on the Removal Efficiency of Heavy Metals.

As shown in Table 4.31, there is a significance difference ($p<0.000$) of different QZ-7 concentration. Besides, in accordance with the multiple analysis in appendix F26, revealed that there is no significant difference ($p>0.05$), between 40 mg/L and 80 mg/L and 100 mg/L.

Table 4.31: Analysis of Variance for the Effect of QZ-7 Concentration on Removal Efficiency of Arsenate.

	Sum of Squares	**Degrees of Freedom**	**Mean Square**	**F-ratio**	**P-value**
Between Groups	740.568	5	148.114	35.041	0.000
Within Groups	50.723	12	4.227		
Total	791.291	17			

Table 4.32, showed that there is a significant difference ($p<0.000$), between the different concentration of QZ-7 on cupper removal. However, as revealed in multiple comparison appendix F25. That there is no significant difference between 20 mg/L and 40 mg/L, also between 60 mg/L and 80 mg/L or 100 mg/L.

Table 4.32: Analysis of Variance for the Effect of QZ-7 Concentration on Removal Efficiency of Cupper.

	Sum of Squares	**Degrees of Freedom**	**Mean Square**	**F-ratio**	**P-value**
Between Groups	1959.031	5	391.806	263.448	0.000
Within Groups	17.847	12	1.487		
Total	1976.878	17			

Table 4.33, showed that there is a significant difference ($p<0.000$), between the different concentration on cadmium removal efficiency. Whereas, the multiple comparison appendix F27, showed that there is no significant difference ($p>0.05$), between 20 and 60 or 80 mg/L, or 40 and 60 mg/L and 80 and 100 mg/L.

Table 4.33: Analysis of Variance for the Effect of QZ-7 Concentration on Removal Efficiency of Cadmium.

	Sum of Squares	**Degrees of Freedom**	**Mean Square**	**F-ratio**	**P-value**
Between Groups	1728.051	5	345.610	187.483	0.000
Within Groups	22.121	12	1.843		
Total	1750.172	17			

Table 4.34, presented that there is a significant difference ($p<0.000$), of different concentration on Pb^{+2} removal efficiency. While, the multiple comparison in appendix F28, indicated that there is no significant difference between 40 mg/L and 80 mg/L or between 60 mg/L and 100 mg/L.

Table 4.34: Analysis of Variance for the Effect of QZ-7 Concentration on Removal Efficiency of Lead.

	Sum of Squares	**Degrees of Freedom**	**Mean Square**	**F-ratio**	**P-value**
Between Groups	983.998	5	196.800	95.405	0.000
Within Groups	24.753	12	2.063		
Total	1008.751	17			

Table 4.35, indicated that there is a significant difference ($p<0.000$) of different QZ-7 concentrations on the removal efficiency of zinc. Moreover, based on the multiple comparison analysis presented in appendix F29, revealed that there is no significant difference ($p>0.05$) between 20 mg/L and 40 mg/L, 60 mg/L and 100 mg/L of QZ-7 concentrated on the zinc removal. Also, between 40 mg/L and 80 mg/L or 100 mg/L.

Table 4.35: Analysis of Variance for the Effect of QZ-7 Concentration on Removal Efficiency of Zinc.

	Sum of Squares	**Degrees of Freedom**	**Mean Square**	**F-ratio**	**P-value**
Between Groups	3389.997	5	677.999	299.776	0.000
Within Groups	27.140	12	2.262		
Total	3417.138	17			

4.8.2.3 Effect of Heavy Metals Concentration on the Adsorption using of Bioflocculant QZ-7

Results presented in Figure 4.31 shows that the heavy metals adsorption exceeded 60%, with the increased of it's an initial concentration of 10 mg/L to 100 mg/L. For example, indicated the maximum removal achieved 94.3% of 60 mg/L As and the highest removal of Cu^{+2} 85.2%, Cd^{+2} (83.15%), Zn^{+2}(84.5) and Pb^{+2} (82.76%), of 100 mg/mL.

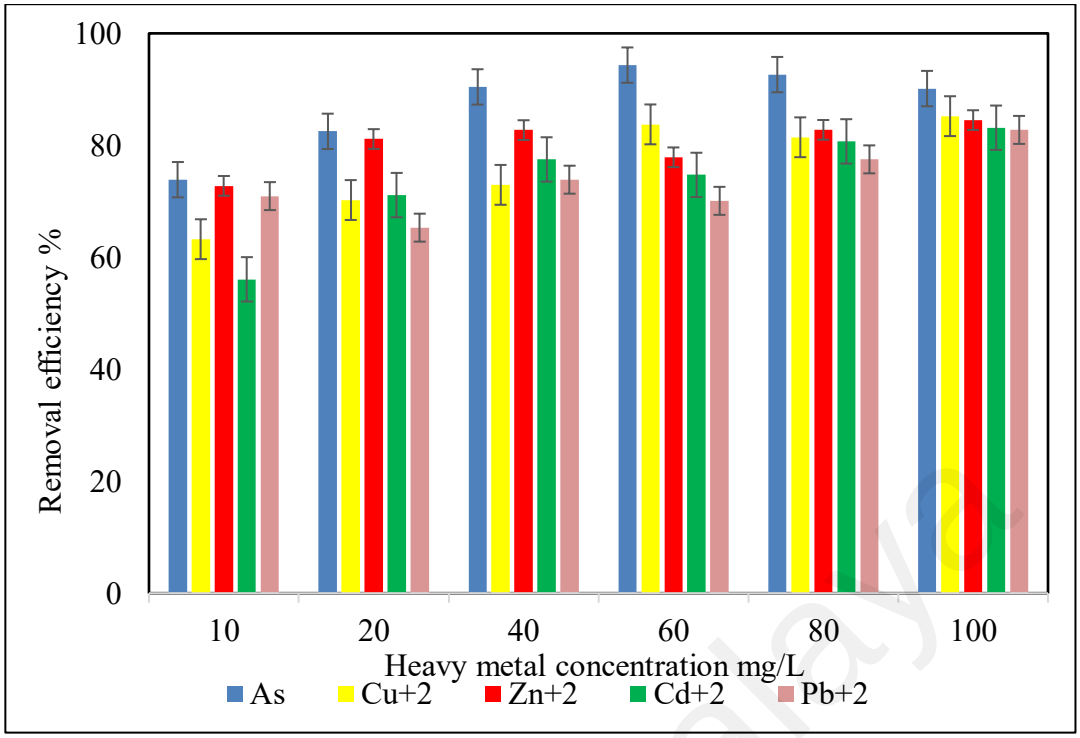


Figure 4.26: Effect of Metal Concentration on the Efficiency of Removal Heavy Metals using 60 mg/L of Bioflocculant QZ-7.

As presented in Table 4.36, there is a significant difference ($p<0.000$) of the different concentration of arsenate. Whereas, the multiple comparison analysis in appendix F30, showed that there is no significant difference ($p>0.05$), between 40 mg/L of arsenate and 80 mg/L or 100 mg/L, also 60 mg/L and 100 mg /L.

Table 4.36: Analysis of Variance for the Effect of Arsenate Concentration on QZ-7 Adsorption Efficiency.

	Sum of Squares	**Degrees of Freedom**	**Mean Square**	**F-ratio**	**P-value**
Between Groups	897.752	5	179.550	91.711	.000
Within Groups	23.493	12	1.958		
Total	921.245	17			

Table 4.37, showed that there is a significant difference ($p<0.000$), of the cupper concentration on the adsorption of QZ-7 performance. Furthermore, multiple comparison analysis in appendix F31, revealed that there is no significant difference ($p>0.05$), of 20 mg/L of cupper and 40 mg/L, or between 60 mg/L and 80 mg/L, 100 mg/L.

Table 4.37: Analysis of Variance for the Effect of Cupper Concentration on QZ-7 Adsorption Efficiency.

	Sum of Squares	**Degrees of Freedom**	**Mean Square**	**F-ratio**	**P-value**
Between Groups	1137.910	5	227.582	100.779	0.000
Within Groups	27.099	12	2.258		
Total	1165.009	17			

Table 4.38, showed that there is a significant difference ($p<0.000$), of cadmium concentration on the adsorption of QZ-7 performance. Despite the fact that, the results revealed in multiple comparison appendix F32, there is no significant difference ($p>0.05$) between 20 mg/L of cadmium and 60 mg/L, also between 40 mg/L and 60 mg/L or 80 mg/L.

Table 4.38: Analysis of Variance for the Effect of Cadmium Concentration on QZ-7 Adsorption Efficiency.

	Sum of Squares	**Degrees of Freedom**	**Mean Square**	**F-ratio**	**P-value**
Between Groups	1411.147	5	282.229	65.704	.000
Within Groups	51.545	12	4.295		
Total	1462.692	17			

Table 4.39, there is a significant difference ($p<0.000$), of lead concentration on the QZ-7 adsorption performance. Although, multiple comparison analysis in appendix F33, indicated that there is no significant difference ($p>0.05$), of 10 mg/L lead concentration and 40 mg/L or 60 mg/L, or between 20 mg/L, 60 mg/L, and 80 mg/L and 100 mg/L.

Table 4.39: Analysis of Variance for the Effect of Lead Concentration on QZ-7 Adsorption Efficiency.

	Sum of Squares	**Degrees of Freedom**	**Mean Square**	**F-ratio**	**P-value**
Between Groups	563.465	5	112.693	26.520	0.000
Within Groups	50.991	12	4.249		
Total	614.457	17			

Table 4.39, shows that there is a significant difference ($p<0.000$) of zinc on QZ-7 adsorption performance. Despite the fact that, the multiple comparison analysis in appendix F34, revealed that there is no significant difference ($p>0.05$), of 20 mg/L zinc and 40 mg/L, 60 and 80 mg/L, also between 40 mg/L and 80 mg/L and 100 mg/L.

Table 4.40: Analysis of Variance for the Effect of Zinc Concentration on QZ-7 Adsorption Efficiency.

	Sum of Squares	**Degrees of Freedom**	**Mean Square**	**F-ratio**	**P-value**
Between Groups	279.373	5	55.875	17.071	0.000
Within Groups	39.277	12	3.273		
Total	318.650	17			

4.8.3 Treatment of Real Wastewater

4.8.3.1 Removal of Heavy Metals from Industrial Wastewater by Bioflocculant QZ-7

Figure 4.32 showed the efficient removal of arsenate (As), from 71.9% to 89.85% with the increased of the amount of adsorbent concentration, as well as the Zn^{+2} removal was also increased from 63.85% to 77.3%. Whereas, the Cu^{+2} slightly removed from 53.2% to 58.4 %.

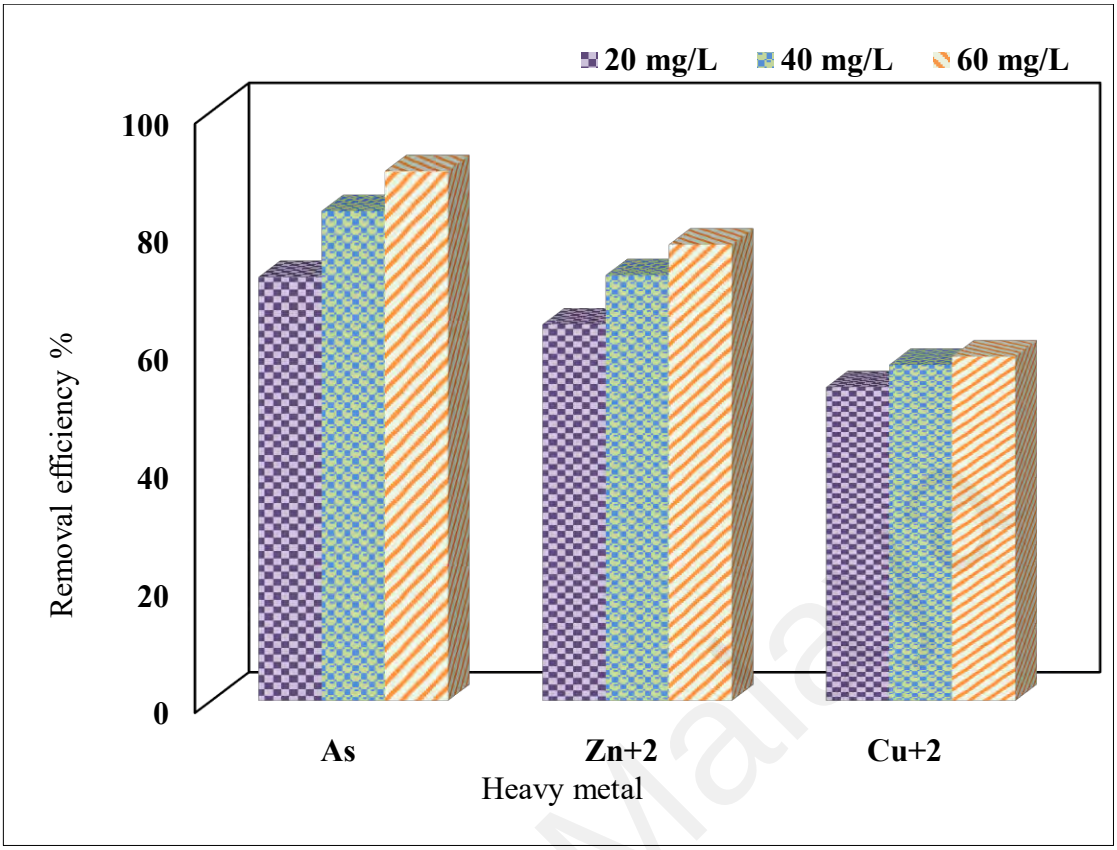


Figure 4.27: Removal Efficiency of Heavy Metals using Different Concentrations of Bioflocculant QZ-7.

4.8.3.2 Removal of BOD and COD from Industrial Wastewater

The conjugation of COD analysis test with BOD test are useful in sign of toxic circumstances and the incidence of biological resistance (Poddar & Sahu, 2017).

Table 4.41: Removal Efficiency of Bioflocculant QZ-7 in Industrial Wastewater Treatment.

Parameter	Removal Efficiency %		
	Sugar mill	Ethanol	Rubber
BOD mg/L	92.48	22.15	57.2
COD mg/L	93	25.8	61.1
Colour	39.4	0	62.2
Turbidity (NTU)	90.7	0	56.6
Total dissolved solid mg/L	88.2	52.1	61.2
Total suspended solid mg/L	89.69	null	61.5

4.8.3.3 Removal of Pharmaceutical Compounds using Bioflocculant QZ-7

The primary elimination of selected pharmaceuticals as shown in Table 4.42, from hospital wastewater was determined at the Laboratory.

Table 4.42: Efficiency of Bioflocculant QZ-7 in Removal of Pharmaceutical Compounds.

Pharmaceutical compounds	Removal efficiency %
Simvastatin	92.45939
Perindopril	25.49839
Nifedipine	0
Diclofenac	4.046219
Mefenamic acid	13.64833
Salbutamol	88.69775
Caffeine	66.52178
Acetaminophen	69.94135
Metformin	7.193886
Ibuprofen	8.773372

4.9 Synthesis of Nanoparticle

The biopolymers-based synthesis of AgNPs has been carried out since a decade and a variety of polymers viz., algal, bacterial, fungal and plant-based polymers were exploited greatly.

4.9.1 Nutrient Broth Medium

The nutrient broth supplemented with glucose showed synthesis of AgNPs which was primarily detected by visual colour change Figure 4.33 in a bottle on the 3rd day of inoculation with *B. salmalaya* 139SI.

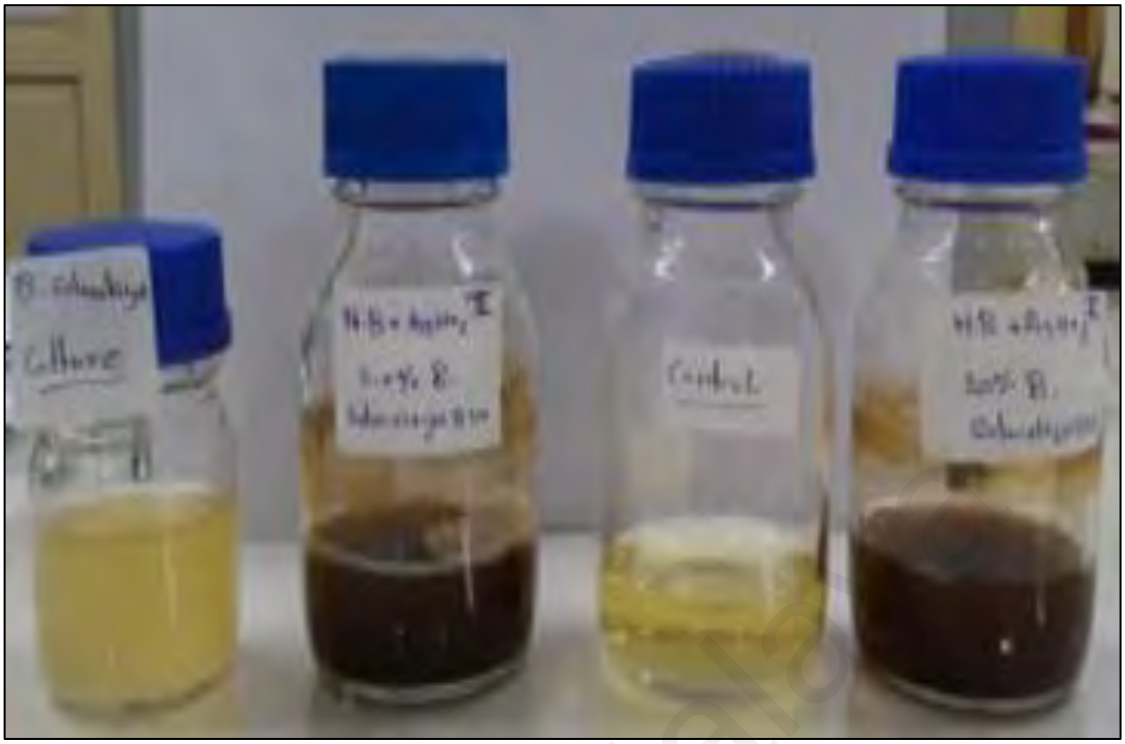


Figure 4.28: Synthesis of AgNPs in Nutrient Broth Inoculated with *B. salmalaya* 139 SI.

4.9.2 Using Cell- Free Supernatant

In the cell-free supernatant having bioflocculant QZ-7 the colour change was detected after 72 h of the incubation. The bioflocculant QZ-7 reduced $AgNO_3$ to produce AgNPs, Figure 4.34.

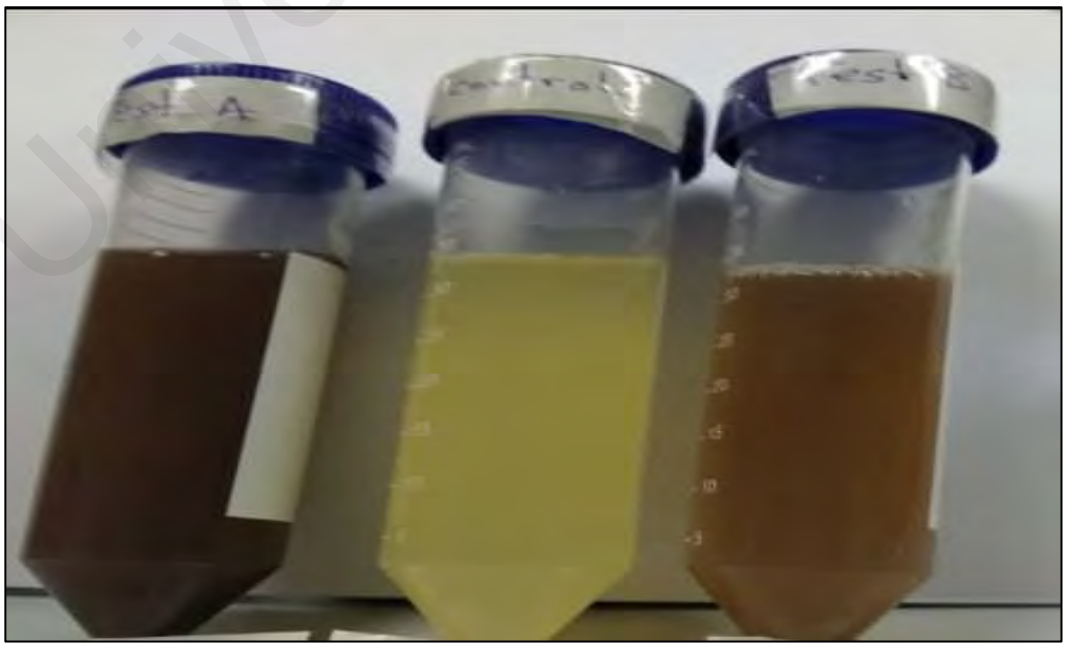


Figure 4.29: Synthesis of AgNPs using Cell-Free Supernatant.

4.9.3 Synthesis of AgNPs using Purified Bioflocculant QZ-7

Figure 4.35 showed the synthesis of AgNPs by using purified bioflocculant QZ-7 after 24h.

Figure 4.30: Synthesis of AgNPs by Purified QZ-7.

4.10 Characterisation of AgNPs.

4.10.1 Characterisation of Ag NPs by UV-Vis Spectroscopy

The primary synthesis of Ag NPs was recognized by UV–vis spectral analysis. In this particular absorption spectrum, a strong, broad peak located at approximately 400-450 nm, was observed for nanoparticles synthesized using the cell free supernatant of QZ-7 (Figure 36). This was revealing of the presence of nanoparticles and was reconfirmed by the manifestation of a similar strong, broad peak after a two-month period.

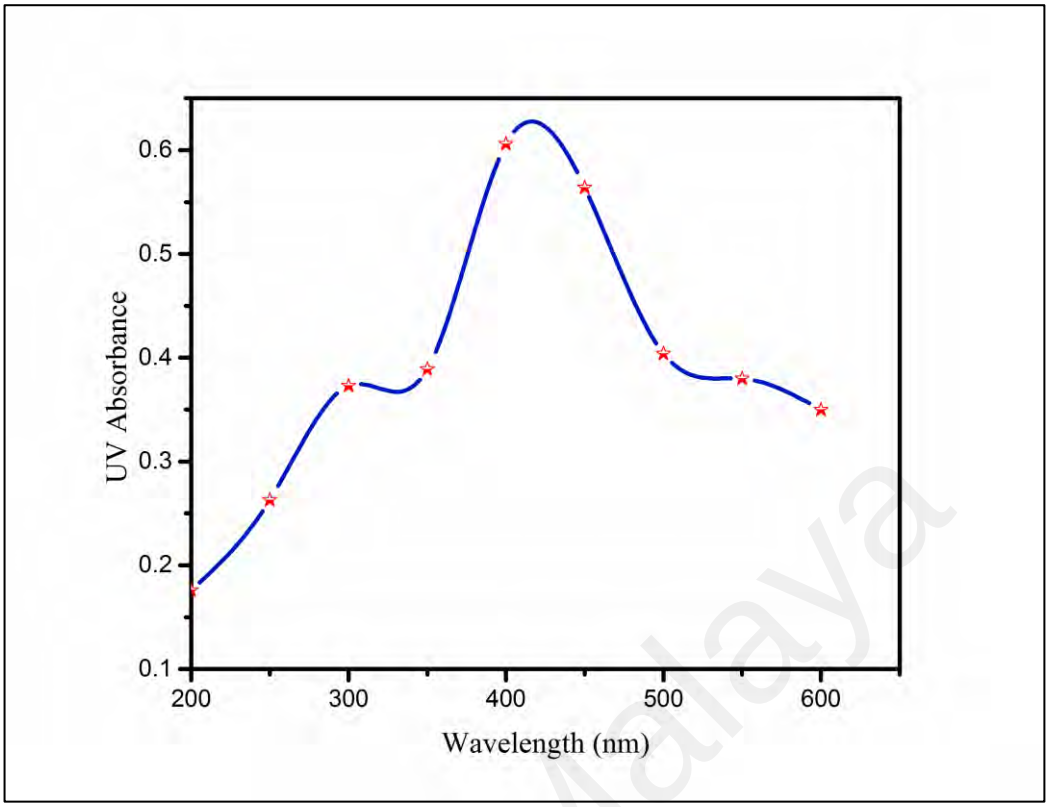


Figure 4.31: UV-vis Spectra of Synthesized AgNPs.

4.10.2 Characterisation of AgNPs by FTIR

FTIR spectroscopy was used to determine the interaction between glycoprotein and metallic particles. Figure 4.37, shown the reduction of silver ions and stabilisation of silver nanoparticles was reflected as intense absorption bands at 2921.48, 2851.09, 2321.94, 2163.83, 2120.46, 1578.26, 999.07, 478.31 and 463.30 cm^{-1}.

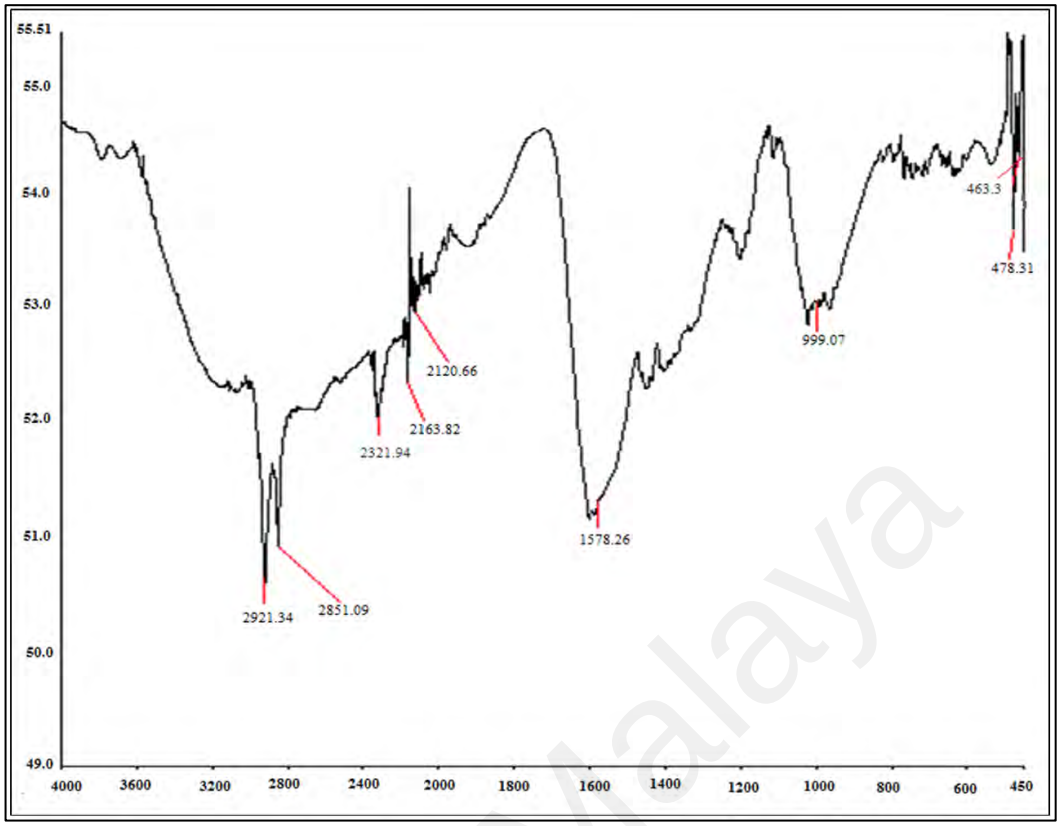


Figure 4.32: FTIR Spectrum of Synthesized AgNPs.

4.10.3 FE-SEM and EDAX Analysis

The FE-SEM and EDAX were carried to study the morphology and elemental composition of biosynthesized AgNPs. The FE-SEM clearly shows that the particles is spherical as shown in Figure 4.38a. The elemental composition of AgNPs is examined by EDAX spectroscopy and is a chemical analysis method combined with FE-SEM Figure 4.38b. (Lawrence & Prakash, 2019).

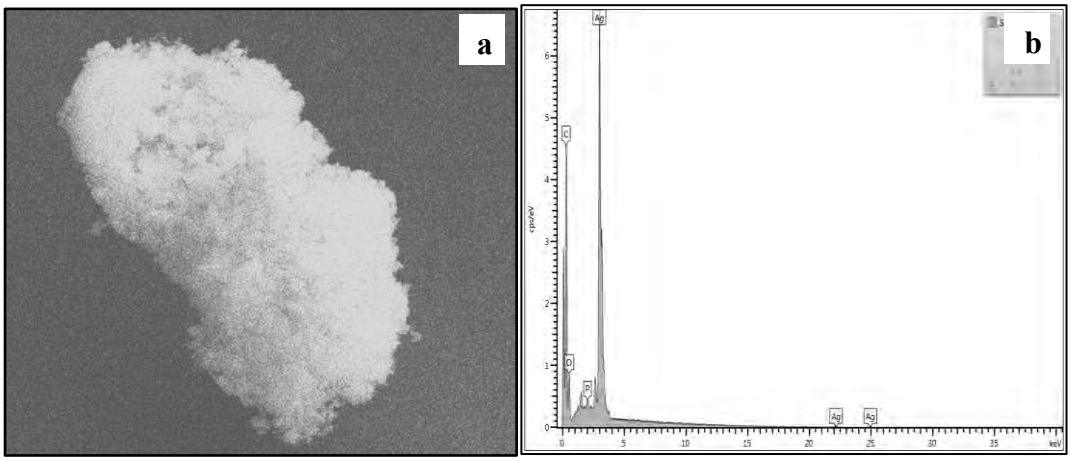


Figure 4.33: FE-SEM (a) and EDAX (b) Analysis of Synthesized AgNPs

4.10.4 Characterisation of AgNPs by XRD Analysis

As illustrated in Figure 4.39 the XRD spectrum of AgNPs showed 4 intense peaks in rang of 20-80˚ at 2θ value; particularly at 38.2667˚, 44.4964˚, 64.5751˚,77.5673˚ and 82.23˚with conforming degree of 111, 200, 220, 311 and 222, reflection of metallic silver. This data was found to be in agreement with database of the Joint Committeeon Powder Diffraction Standards (JCPDS File no. 04-0783).

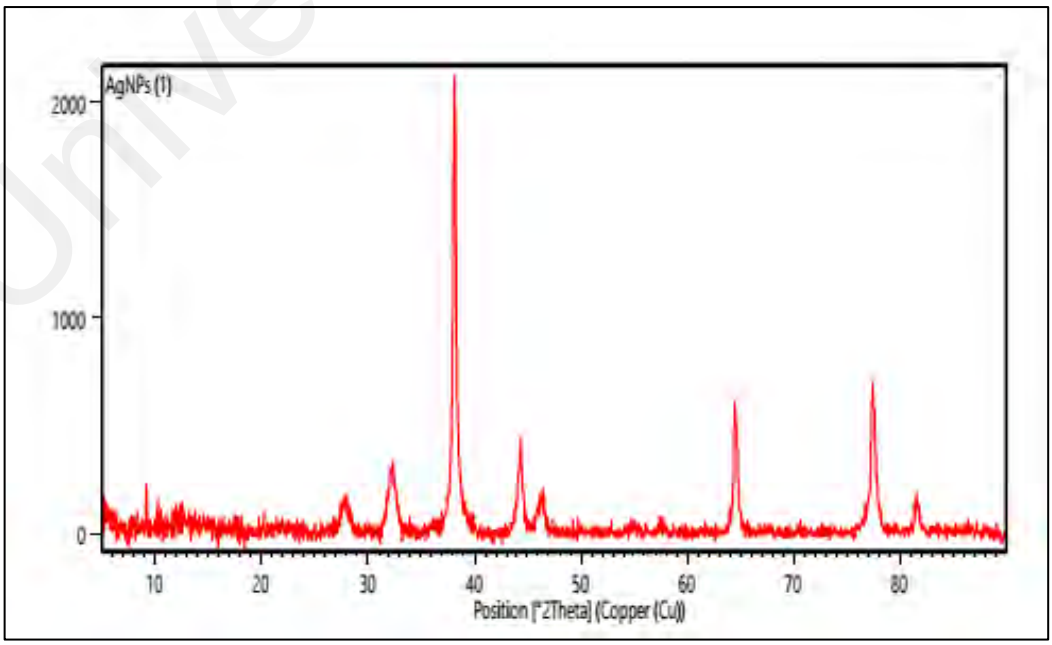


Figure 4.34: XRD Patterns for the AgNPs Synthesized by Pure Bioflocculant QZ-7.

4.11 Antibacterial Activity of AgNPs Synthesized by Bioflocculant QZ-7

The zone of inhibition was measured after 24 h and results were presented (Table 4.43 and Figure 4.40). *E. coli* ATCC35401, *Pseudomonase aeruginosa*, *Salmonella entertidis* ATCCBAA-711 and *staphylococcus aureus* ATCC2592 showed almost consistency in the zone of inhibition.

Table 4.43: Antimicrobial Activity of AgNPs in Solid Medium.

	Zone of inhibition at various concentration of AgNPs (mm)			
Microorganisms	**20 µL**	**40 µL**	**60 µL**	**80 µL**
E. coli ATCC35401	15	18	23	24.5
S. enteritidis ATCC BAA-711	16.5	21	24.5	26
P. aregonosa	16	19	22	22
*S.aureus*ATCC2592	15	16	18	17

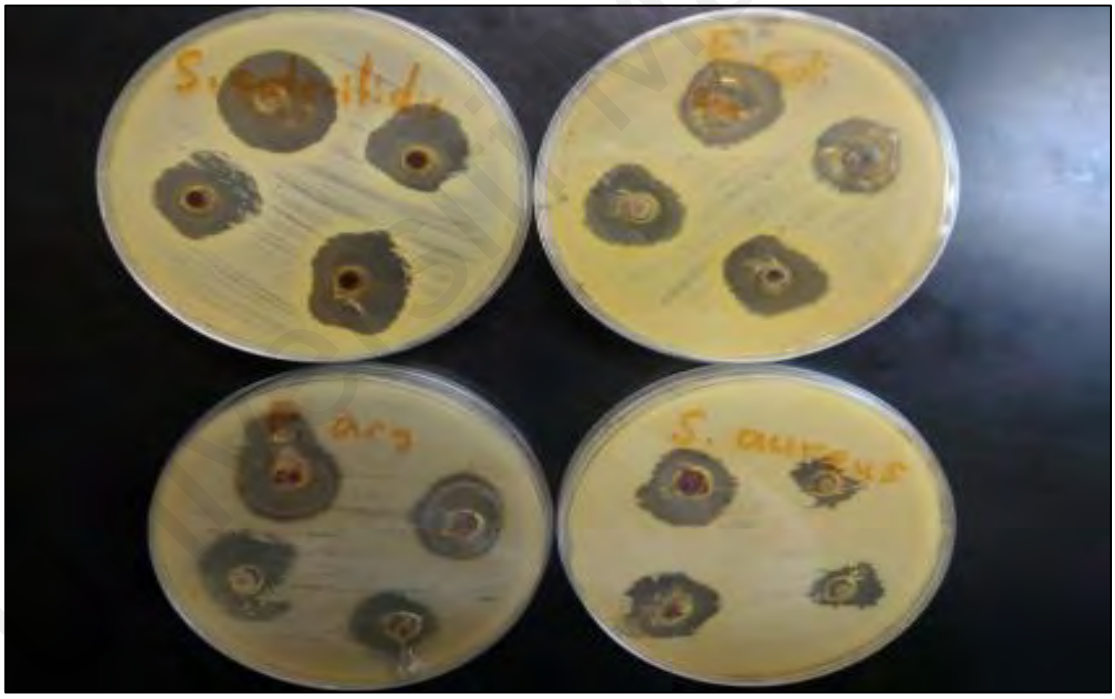


Figure 4.35: Antibacterial Activity of AgNPs against *E. coli* ATCC35401, *P. aeruginosa, S. entertidis* ATCC BAA-711 *and Staph. aureus* ATCC2592.

CHAPTER 5: DISCUSSION

5.1 Selection of Bacterial Strain for Bioflocculant Production

A total of 15 *Bacillus salmalaya* 139SI strains were screened for bioflocculant production. From 15 strains appendix A-1, five strains presented the highest flocculating activities when tested against kaolin suspension (4g/L) as shown in Table 4.1. The strain *B. salmalaya* 139SI (BS 139SI-7), that was selected for this study produced a bioflocculant that showed the highest flocculating activity of 83.3% for kaolin clay suspension. Moreover, it is the first time that *B. salmalaya* 139SI strain used for bioflocculant production.

Based on the morphological and physiological characteristics, the selected strain are shown Figure 4.1a shows that the *B. salmalaya* strain 139SI colonies were large and white-opaque with a rough and irregular edge. The colonies were 2 mm–3 mm in diameter and showed strong β- haemolytic activity after 18h–24 h of incubation at 37˚C on 5% v/v sheep blood agar, as shown in Figure 4.1b. Moreover. Figure 4.2, the *B. salmalaya* strain 139SI was observed under the microscope as gram-positive, short rods in chains and the formation of spore, which is characteristic of the *B. salmalaya* 139SI.

5.2 Optimisation of Bioflocculant Production by *B. salmalaya* 139SI

The inceptive pH of the fermentation media directly influenced the synthesis of the bioflocculant. The effect of initial pH of the culture broth on *B. salmalaya* 139SI growth and bioflocculant production are illustrated in Figure 4.3. At a glance it can be said that the growth of *B. salmalaya* 139SI and bioflocculant production was increased gradually by increasing of pH 3 to 7, while *B. salmalaya* 139SI growth and bioflocculant activity were decreased at pH higher than pH 7. The maximum flocculation activity in the fermentation broth culture was reported with 83.7% at pH 7. A similar result was obtained for bioflocculant production by *Halomonas* sp. and *Citrobacter* sp. TKF04 (Mabinaya *et*

al., 2011; Fujita *et al*., 2000), and the increases in bioflocculant yield differ in different strains at their optimal pH (Xia *et al.,* 2008). The nutrient absorption capability of the cells and the presence of high electric charge could influence the enzymatic reaction (Nakata & Kurane, 1999; Xia *et al*., 2008), thereby decreasing flocculating activity. At the neutral pH, the nutrient absorption capability of the cells was high due to the neutral electric charge, indicating that the highest flocculating activity was observed in the broth culture. Moreover, extreme pH not only inhibits microbial growth, but also bioflocculant (EPS) production, leading to changes in the morphological properties of cells and molecular weight of EPS compounds (Chug *et al*., 2016). The differences between the effect of pH values on bioflucculation activity was found to be significant ($p<0.000$) as reported in Table 4.2. Moreover, the multiple comparison analysis shows the differences between each pH values on bioflucculation activity as presented in appendix F1.

The influence of inoculum size on bioflocculant production was determined according to Zhang *et al.* (2013). The differences between inoculum sizes showed a certain effect on the flocculating production and cell mass values, as shown in Figure 4.4. The obtained optimal inoculum size was 5% (v/v). The outcome results were analogous to those reported by Wang *et al*. (2007), where an optimal inoculum volume at 5% was detected for *Klebsiella mobilis*. The maximum flocculation activity was not in agreement with the maximum cell growth, but the flocculation activity for *B. salmalaya* strain 139SI was maintained within a certain range of inoculum volume. This condition was due to the influence of inoculum volume on microbial growth. A small inoculum volume, extended the lag phase growth, while outsized inoculum made the niche of the strain overlap extremely. Subsequently inhibiting the bioflocculant production due to inadequate supply of nutrient (Zhang *et al*., 2014). While, the analysis of variance presented in Table 4.3, for the effect of the different inoculum size of *B. salmalaya* 139SI on flocculating activity was revealed a significant difference ($p<0.000$).

The influence of temperature on bioflocculant production by *B. salmalaya* 139SI was investigated. As shown in Figure 4.5, the highest bioflocculating activity 81.9% was obtained for the bioflocculant produced at 35.5˚C. However, the flocculating activity of bioflocculant start to decrease at higher temperatures than 35.5˚C, due to the metabolism of microorganisms is directly affected to a cultivation temperature degree (Xia *et al*., 2008; Zhang *et al*., 2007). Maximum enzymatic activation can only be obtained at an optimum temperature (Lixin *et al*., 2016). The optimal temperature for *B. salmalaya* 139SI was found to be 35.5˚C for the bioflocculant production. When the temperature was over 40˚C, the flocculating activity of bioflocculant gradually declined. This decrease could be due to denaturation of extracellular enzyme at the higher temperatures.(Zhong *et al*., 2018). Moreover, as showed in Table 4.4, the analysis of variance for the effect of different temperature on bioflocculant production, was revealed that there is a significant difference ($p < 0.001$).

The effect of shaking speed on bioflocculant production is shown in Figure 4.6. The flocculating activity start gradually to be increased under the effect of shaking speed between 100 to 160 rpm. While shaking speed higher than 160 rpm caused a decline in the flocculating activity. Agitation speed basically determines the concentration of dissolved oxygen, which can also affect microbial nutrient absorption and enzymatic reaction hence affecting both bacterial growth and bioflocculant production (Lixin *et al*., 2016). High-speed mixing have a tendency to break the flocs, causing the coagulated cells to be redispersed and presented again into the medium (Ahmed *et al*., 2011). Some scholars have recommended rapid mixing followed by slow mixing during the flocculation process. This phenomenon is caused by the restabilisation of the cells at high

mixing speed (Ahmed *et al*., 2005). Furthermore, the analysis of variance shown in Table 4.5, for the effect of shaken culture at 72 h of incubation on bioflocculant production was found that there is a significant difference of the flocculating activity of shaken speed ($p<0.000$). In the course of the early growth phase, the biomass and bioflocculant production were lower, resulting in lower viscosity of culture broth and oxygen demand, when the strain *B. salmalaya* 139SI proceed in the logarithmic and stationary phases, the biomass and the bioflocculant production increased, and the corresponding viscosity of the culture broth and the oxygen demand also increased.

When the broth cultures of *B. salmalaya* 139SI were cultivated in non-shaking condition, it was found that the flocculating activities was revealed very much decreased as shown in Figure 4.7. The subsequent measurement showed that the maximum bioflocculant production occurred between 24-72 h. Compared to the shaking speed there is significant decreased of 20 % flocculating activity can be seen at 72 h of incubation, due to the agitation of the culture medium (Salehizadeh *et al*., 2001). Furthermore, the concentration of dissolved oxygen can affect microbial nutrient absorption and enzymatic reaction, hence affecting both bacteria growth and bioflocculant production (Lixin *et al*., 2016). Therefore, the yield of bioflocculant produced by *B. salmalaya* 139SI is significantly reduced under static condition. Therefore, an experiment was conducted to check the effect of bioflocculant production and measurement in terms of flocculation activity under shaking and non-shaking condition. Table 4.6 showed the analysis of variance in bioflocculanting performance of a static culture condition at 24 -168 h of incubation it was revealed that there are significant differences between the flocculating activity of static condition ($p<0.000$) at different incubation period. Moreover, the

multiple comparison analysis designates there is no significant differences between the 24 h and 96 h ($p>0.05$), appendix F4.

The carbon source tends to be the precursor for the metabolic pathway. The effect of carbon sources including sucrose, glucose, lactose, maltose, and starch as single sources on bioflocculant synthesis by *B. salmalaya* 139SI was evaluated as shown in Figure 4.8. In addition, the bioflocculant synthesis was also tested under the effect of a mixture of carbohydrate (CHO) which includes sucrose and glucose. The highest 90.1% and lowest 28.9% flocculating activities were obtained with a mixed carbohydrate source and starch with (1:1) mixing ratio, respectively. Moreover, as single carbon sources such as sucrose, glucose, lactose, and maltose were markedly appropriate for bioflocculant production with flocculating activity exceeding 60% after 72 h of cultivation period. Aljuboori *et al.* (2013) found that sucrose is the most desirable to *Aspergillus flavus* for bioflocculant production. The optimal composition of the growth medium for alkaliphilic bacterium *Paenibacillus jamilae* was found to be sucrose 25 g/L, urea 5 g/L, and KH_2PO_4 5 g/L. (Zhong *et al.*, 2018). Moreover, based on analysis of variance revealed in Table 4.7, that the overall result analysed there are significant differences ($p<0.000$), between the bioflocculating activity of the different carbon source groups. Based on multiple comparison analysis presented in appendix F5, revealed that there is no significant difference between sucrose and glucose, lactose and maltose, respectively. To conclude, glucose and sucrose were the best carbon source to be utilized as they are relatively cheap. Glucose is favoured by all bacteria for the production of EPS e.g. *Cryseobacterium daequene* (Liu *et al.*, 2010), while, *Alkaligenus cupidus* KT201 preferred sucrose for maximum production (Toeda & Kurane, 1991).The novel bioflocculant REA-11 synthesised by *Corynebacteria glutamicum*, sucrose was utilised as a carbon source containing corn steep liquor (He *et al.*, 2004). It is well documented in the literature that various bioflocculant-producing microorganisms prefer organic carbon sources for

optimum bioflocculant production. On the other hand, the utilisation of inorganic carbon sources for bioflocculant production is quiet slight in literature (Makapela *et al*., 2016). The nitrogen source plays a crucial role in synthesis of bioflocculant (Cosa *et al*., 2011). The effect of nitrogen source on production was evaluated using different sources of organic origin, such as yeast extract, tryptone, peptone, urea and beef extract and ammonium sulphate was used as a source of inorganic nitrogen. In addition, the bioflocculant synthesis was also tested under the effect of mixture of nitrogen which include urea and yeast extract. Different microorganisms utilize nitrogen derived from both or either inorganic or organic sources for production (Okaiyeto *et al*., 2014). The influence of nitrogen sources, both organic and inorganic on the bioflocculant synthesis by *B. salmalaya* strain 139SI is illustrated in Figure 4.9. The highest flocculation activity reached at 72 h of cultivation was obtained with mixed nitrogen source 72.1%, while yeast extract gives 64.5% and urea 61.5%, whereas the lowest activity was detected with peptone 42.5%. A similar result was reported by You *et al*. (2008) that the *B. subtilis*, *B. fusiformis* and *B. flexus* preferred urea and yeast extract for the bioflocculant production. While the ammonium sulphate as inorganic nitrogen gives about 58.86% of flocculating activity. As reported by Ugbenyen *et al*. (2014), the flocculating activity was peaked with yeast extract followed by casein hydrolysate, tryptone, and beef extract. A similar finding indicated that organic nitrogen is more appropriate for bioflocculant production than inorganic nitrogen origin, because it is simply absorbed by the microbial cells (Cosa *et al*., 2012). Moreover, Ismail and Nampoothiri, (2010), indicated that yeast extract was a highly favourable nitrogen source that improved EPS production by *Lactobacillus planetarium* MTCC 9510. While, based on the analysis of variance shown in Table 4.8, indicated that there are significant differences of bioflocculant production of different nitrogen source ($p<0.000$). However, the multiple comparison analysis showed in

appendix F6 that there no significant differences between mixed nitrogen and urea or yeast extract, and between urea, yeast extract, tryptone and $(NH_4)_2 SO_4$ ($p>0.05$).

Bioflocculant production affected by the presence or absence of metal ions due to its responsible for activation of some of the biosynthetic pathways (Raza *et al.*, 2011).Table 4.9. Show that the flocculating activity was stimulated by several salt such as $CaCl_2$, KH_2P_4, K2HPO4, KCl, $MgSO_4$ and Na_2HPO_4, but were adversely affected by $CuSO_4$ and $Al_2 (SO_4)_3$. While combination of KH_2P_4, and K_2HPO_4 as inorganic salts in the production medium for *B. salmalaya* 139SI significantly enhance bioflocculant production. Moreover, the presence of KH_2P_4 and K_2HPO_4 in the production medium are considered a good buffer, it alleviates the effect of pH change during the cultivation period. While, the bioflocculant yield by *A. flavus* was enhanced by the addition of K^+, Na^+, Ca^{+2}, Mg^{+2} and Mn^{+2} into the fermentation medium (Aljuboori *et al.*, 2015). Table 4.10, indicate that the overall results of treatment analysis showed that there are significant differences ($p<0.00$) between the bioflocculant production of different inorganic salts. However, based on multiple comparison analysis as shown in appendix F7, that there are no significant differences between the following $CaCl_2$, KH_2P_4, K_2HPO_4 and KCl ($p>0.05$), and $MgSo_4$ and Na_2HPO_4 ($p>0.05$).

5.3 Time Course for Bioflocculant Production by *B. salmalaya* Strain 139SI

The bioflocculant production by an organism is associated to its stages of the growth phase, and bioflocculant can be produced during growth phase through, biosynthesis consuming available nutrients or during decline phase due to autolysis of cells. As shown in Figure 4.10, the production of bioflocculant at the early growth stage of *B. salmalaya* 139SI was reflected by the 70% flocculating activity achieved at 24 h of incubation. The subsequent measurements showed that the maximum bioflocculant production occurred between 24-96 h of incubation and the flocculating activities achieved above 70%.

Whereas, the highest bioflocculant activity 92.6% achieved at 72 h of incubation and pH 7.0 a logarithmic growth phase. The stationary growth phase was attained after 72 h of cultivation. Flocculating activity ran parallel to cell growth, thereby exhibiting a concomitant increase in bioflocculant production with cell growth. The optical density of the culture increased progressively during the period 24 h-72 h with an increase in flocculating activity produced, although, after the 72 h the OD decreased with decreased of flocculation activities produced. This observation indicated that the production of bioflocculant was a result of biosynthesis during bacterial growth and not via cell autolysis (Czemierska *et al.,* 2017). The decrease in flocculating activity detected after 72 h might be attributed to the existence of bioflocculant-degrading enzymes produced by the microorganisms (Li *et al*., 2009). A similar study was reported by Zheng *et al*. (2008), who found that the flocculating activity of the bioflocculant produced by *Serratia fiacre* and *Bacillus* sp. F19 reaches its maximum at the early stationary phase of 72 h. The initial pH of the production medium was adjusted to 7 and then monitored at regular intervals over the entire fermentation period. Therefore, factors affecting bioflocculant (EPS) production like growth phase, pH and temperature have been optimized for *A. beijreinckii* and *B. subtilis*. Both bacteria were found to produce maximum EPS at neutral pH (chung et al., 2016). Also, the flocculating activity of a novel bioflocculant from *Paenibacillus jamilae* increased significantly with time, achieving a peak rate of 87.5% at 72h and declining after 96h (Zhong *et al*., 2018). Moreover, the pH of the production medium governs the oxidation- reduction potential and the cells electrical charge thereby affecting enzymatic reaction and nutrient absorption (Zhang *et al*., 2013). Consequently, the pH of the fermentation medium decreased as cultivation period increased in time. The decrease in pH of the fermentation medium might be due to the production of organic acids as a result of glucose metabolism, because glucose was a constituent of the production medium or the presence of organic acids metabolically produced by bacteria

(Lu *et al*., 2005). The produced amount of bioflocculant was 2.72 g/L. While, based on the analysis of variance for the time cource of bioflocculant production was presented in Table 4.11, revealed that there is a significant difference ($p<0.00$).

5.4 Characteristics of Bioflocculant QZ-7

The composition of bacterial bioflocculants plays a role in their flocculating activities (Wu & Ye, 2007). Reports showed that many types of bioflocculants comprise proteins, polysaccharides, glycoproteins, and glycolipids (Salehizadeh & Shojaosadati, 2003). Table 4.12, presents a clear indication that the bioflocculant composed of polysaccharides, proteins and uronic acid was found. On the basis of the chemical analysis of the produced bioflocculant, shown in Figure 4.11and Figure 4.12, the total carbohydrate and protein compositions were found to be 79.08% and 15.4%, respectively. The bioflocculant mainly composed of polysaccharides and proteins. Further analysis revealed the presence of uronic acid in the bioflocculant. An adequate proportion of uronic acid molecules in the bioflocculant, carboxyl groups can be provided to the molecular chain. The carboxyl group present in the molecular sequence affords more active sites for constituent parts, so several molecules can bind to the elongated molecular chain (Aguilera *et al*., 2008).

Fourier transform infrared (FTIR) spectroscopy was used to analyse the purity of bioflocculant, as illustrated in Figure 4.13, FTIR spectroscopy of purified QZ-7 biopolymer indicted a broad stretching intense peak at 3425.56 cm^{-1} demonstrated the presence of hydroxyl and amino groups. The weak stretching band detected at 2929.82 cm^{-1} revealed the presence of aliphatic C—H stretching. This result is similar to the results obtained by Ayat *et al*. (2017). The peaks at 1658.90 and 1432.97 cm^{-1} were attributed to C=O and vibration COOH vibration, respectively, whereas, that at 1432.97 cm^{-1} was due to the C=O antisymmetric extension in the carboxylate (Sun *et al*., 2015a),

and thus the result showed the existence of carboxylate function groups in QZ-7. The carboxyl group may also serve as a functional moiety for the generation of modified or new polymers in different forms via different approaches, such as unique designed formulation by assembling such polymer to other synthetic polymers. Other bands observed at 1109.66 and 1187.49 cm^{-1} were identified to be classic characteristics of all compounds derived from sugar, including sugar derivatives like mannuronic acid, guluronic acid, and uronic acid (Suh *et al*., 1997). Other absorption bands at 924.94 and 990.44 cm^{-1} were related to the β-glycosidic bond linking the monomeric units present in sugars as recommended by the study Gupta *et al*., (1987). The corresponding small band absorption was observed at 446.32, 538.79, and 618.50 cm^{-1}, which indicated that the bioflocculant QZ-7 was a protein-bound polysaccharide (Yang *et al.*, 2013). Similar finding of Makapela *et al.* (2016); Gommaa. (2012) that the FTIR spectrometry analysis of purified bioflocculant from *Bacillus pumilus, Pseudomonas aeruginosa* ATCC-10145 respectively, showed the display of amino, hydroxyl and carboxyl groups. Furthermore, the FT-IR spectra of the EPS peroduced by *Leu. pseudomesenteroides* YB-2 was highly similar to those of *W. cibaria* JAG8 (Tingirikari *et al*., 2014) and *Leu. pseudomesenteroides* R2 (Paulo *et al*., 2012), indicating a similar structure. The α-(1→6) linkages were further confirmed by ^{1}H and ^{13}C NMR analysis.

The high-performance gel permeation chromatography (HPGPC) spectrum of the purified bioflocculant QZ-7 exhibited a symmetrical and sharp peak in the retention time of 6.53 and 9.95, as shown in Figure.4.14. The molecular mass–retention time equation in accordance with the calibration curve is expressed as following:

$$\text{Log (molecular weight)} = -0.1368T + 8.3496$$

The average weight of the bioflocculant QZ-7 was calculated to be 5.13×10^{5} Da, which was much higher than the weight of other bioflocculants (Yang *et al.*, 2015).

Bioflocculants with high molecular weight present stronger bridging, more adsorption points, and higher flocculating activities than those with low molecular weight (Salehizadeh & Shojaosadati, 2001). Besides, high molecular weight of EPSs were considered important for stimulating the formation of EPS-protein network structure and improving the consistency of fermented products (Du *et al*., 2018).

The ^{1}H-NMR spectra of bioflocculant QZ-7 produced by *B. salmalaya* strain 139SI significantly reflected the results obtained from FTIR analyses. In the ^{1}H NMR spectrum (Figure 4.15), the anomeric region (3.29–5.47ppm) signals often functioned to differentiate the anomeric protons of sugar residues in bioflocculant QZ-7.The ^{1}H NMR spectra of QZ-7 confirmed the resonance of hydrogen protons as corroboration of the glucosyl residue as the repetitive unit of the biopolymer (Ye *et al*., 2019). The spectral analysis of bioflocculant QZ-7 presented in Figure.4.15, revealed the presence of a signal at 2.5 ppm, indicating the presence of –NH group. The solvent peak showed at 3.29 ppm. Furthermore, signals at 5.04- 5.47, 3.6-3.7 and 1.2-1.43 ppm confirmed the presence of hydroxyl proton, non-anomeric protons and CH_2 linkage in the sample, respectively. Again, a peak at 2.0 ppm, indicated the presence of carbonyl linkage of glycoprotein molecule. The H NMR chemical shift (δ) value at 0.85ppm–0.86 ppm an indicator of the presence methylene (CH_3) groups, also at 1.20 ppm–1.33 ppm indicated the methyl (CH_2) groups consistence in sugar moiety. Therefore, the chemical shift was detected in the NMR spectra of many bioflocculants although a similar finding was stated by Wenliang *et al*. (2015). The value of CH-proton of carbon of deoxy sugar exhibited δ at 1.747 ppm (Bayoumi *et al*., 2010). The strong water signal (HOD) showed at 2.5 ppm, and clouds the β-anomeric proton signals at about 5.04 ppm. The δ value at 5.05-5.47 ppm indicated the proton on anomeric carbon of sugar moieties. The existence of NH_2 group of the protein in bioflocculant was detected in the range of δ value of 4.65 ppm–5.03 ppm. Likewise, the chemical shift of the bioflocculant QZ-7 produced by *B. salmalaya* 139SI

was found to be as significance and assignment of the bioflocculant produced by *leuconostoc lactis* KC117496 (Saravanan & Shetty, 2016). The ^{13}C NMR spectrum (Figure. 4.16) of bioflocculant QZ-7 included the anomeric carbon region (60–101 ppm) and ring carbon (60–79.9 ppm) region (Chen *et al*., 2016). The anomeric carbon signal at 79.9 ppm confirmed that the sugar residues were linked by α-glycosides (Ye *et al*., 2019). The peaks at 70.8, 72.2, 72.6, 73.8 and 78.9 ppm corresponded to C-2, C-3, C-4 and C-5, respectively (Seymour *et al*., 1979). The downfield shift of the C-6 carbon signal for the glucose unit was detected at 61.2 ppm (Chen *et al*., 2016). The result achieved from the NMR analysis showed the FTIR result and specified that the bioflocculant QZ-7 was a glycoprotein (Zaki *et al*., 2013).

Characterisation and determination of EPS monomers and even the associated proteins were been possible through HPLC, LCMS/MS, and LC/MS-ESI techniques (Jachlewski *et al*., 2015). As a result, obtained by the LC/MS based analysis, adducts of Na^+, NH_4^+ and H^+ ions were identified for the sugar monomers depended on fragmentation and elution time in present case. Figure 4.17, shows the presence of glycoprotein at 741 m/z-745 m/z at retention time 3.34. Meanwhile, Na^+ adducts for glucose appeared as $(Glc+Na)^+$ (182.96 m/z) and rhamnose as $(Rha^+Rha^+Na)^+$ (m/z 354.3) at retention time 1.576 and 1.529 min, respectively. The m/z ratio of 212.0 m/z represents glucuronic acid may form adduct as $(GlcA+NH4)^+$ at 1.678 min. The solvent peak appeared at 5.65 min. These results indicated that the bioflocculant consisted of four basic sugar monomers.

Scanning electron microscopy (SEM) imaging showed the morphological surface structure of QZ-7 were illuminated prior to and after flocculation process with kaolin clay particles. As shown in Figure.4.18a, QZ-7 was grey with a condensed crystalline brick-shaped structure. The structure served as an attachment site to which suspended particles and cations could bind (Salehizadeh & Shojaosadati, 2001). Figure 4.18b illustrates how

the bioflocculant aggregated the kaolin particles, which resulted in the formation of larger flocs that were easily sedimented. Therefore, SEM images of QZ-7 and flocculating kaolin particles indicated that bridging could be liable for the flocculation capability of QZ-7. In accordance with the observation, previous research also reported related incidences with some biofloccuants (He *et al*., 2010; Lian *et al*., 2008).

The elemental analysis of bioflocculant QZ-7 was on the basis of SEM-EDX testing. Figure 4.19 reveals that the bioflocculant QZ-7 from *B. salmalaya* 139SI had content of carbon (55.74%) oxygen (42.74%), nitrogen (0.54%), phosphate (0.93%), and sulphur (0.06%). Similar results were obtained by Salehizadah *et al*. (2014) where phosphorus and sulphur were present in low quantities.

Thermogravimetric (TGA) determination of purified bioflocculant QZ-7 was tested to elucidate its property compartments at different heat degrees. The results are depicted in Figure 4.20 to assist in recognising its pyrolysis property after exposure to significantly elevated temperatures. Figure 4.20 shows approximately 12.14%, 23.76% in weight loss at 100˚C, 200˚C respectively, and a 45.28% weight loss at 200 ˚C –450˚C. The pyrolytic process was almost completed at approximately 800 ˚C. TGA of bioflocculant QZ-7 exhibited thermo-labile and thermo-stable molecule contents, showing combination of sugar and protein substance, as indicated by examination. This stability was likely due to the polysaccharide composition of the main backbone of MBF-15 (Zhong *et al*., 2018). The first weight decline may be due to the loss of available moisture or the presence of carboxyl and hydroxyl groups in the protein portion related to glycoprotein-like molecules of QZ-7. Analogous observation was organised according to the work of Wang *et al*. (2011) with respect to the bioflocculant gained by mixed consortium of bacteria

5.5 Factors Affecting of Bioflocculant QZ-7 Performance

Some external factors are closely linked to the performance of ionic flocculants, in certain dosage and pH value (Liu *et al.*, 2017). In general, the inner factor of flocculants such as ionic groups exerts more significant impact on the flocculation performance (Yang *et al.*, 2013). As shown in Figure 4.21, bioflocculant QZ-7 was found to be a fairly steady at wider range of pH range 4-7, and over 70 % flocculating activity was observed at this pH value with significant different performance ($p<0.00$), as shown in Table 4.13. The highest flocculating activity was achieved at pH 7 with a mean difference of about 92.26 % from the lowest activity measured at pH 2-3 and 11 ($p<0.05$), as depicted in Table 4.13. Consequently, to the multiple comparison analysis revealed that pH 5 and 6 has the same effect on flocculating activity of QZ-7 appendix F12. Therefore, bioflocculant QZ-7 was considered useful in neutral and acidic conditions, whereas a pH greater than 9 decreased the degrees of flocculation activity. QZ-7 displays diverse electric statuses at varying pH range, and this could be due to the destabilisation of the kaolin particles which inhibited agglomeration and bridging by the bioflocculant (Natarajan, 2015). Similar finding was reported by Liu *et al*. (2010), for bioflocculant produced by *Chryseobacterium daequens* W6 which favoured condition at low acidic and low alkali with the same pH range 4-5-8.

The influence of temperature on the flocculation activity of the purified QZ-7 was investigated. As shown in Figure 4.22. The study found the flocculating activity of QZ-7 was quite sustained and stable at different temperatures of kaolin suspension series from 20- 60˚C, within this range, above 80% of flocculating efficiency was obtained. The thermal strength of this bioflocculant QZ-7 was attributed to the core backbone of QZ-7, which comprises polysaccharides (Lu *et al*., 2005). The bioflocculant with polysaccharides-based structure are generally thermo-stable, but those with protein are sensitive to temperature (Salehizadeh & Shojaosadati, 2001; Gong *et al*., 2008). A similar

study of MBF-15 indicated tremendous thermostability over a wide range of temperatures 1from 10 to100˚C. This stability was presumably due to the polysaccharide composition of the main backbone of MBF-15 (Luo *et al*., 2014). However, higher temperature may cause degradation of this polysaccharides chains and diminish flocculating activity (Zhang *et al.,* 2007). Also, the treatment analysis depicted in Table 4.14, for the effect of different temperature degrees on flocculating activity revealed that there are significant differences ($p<0.00$). While, the multiple comparison showed in appendix F13, that are no significant differences between 20 ˚C and 45˚C and between 55˚C and 65˚C ($p>0.05$).

Bioflocculant dosage concentration is one of the dynamic parameters deliberated, during investigating of optimum conditions for coagulant-flocculants production in the coagulation flocculation development (Hassan *et al*., 2009). Figure 4.23, shown the correlation concerning bioflocculant dosage and turbidity reduction in clay suspension. The performance of bioflocculant concentration applied through treatment can lead to a poor bridging phenomenon, thus resulting in low flocculating activities measured, while, excess concentration might induce re-stabilisation of the kaolin particles (Hassan *et al*. 2009; Gong *et al*., 2008). Consequently, bioflocculant optimal dosage should be set up to support reduction of cost and achieve better performance in treatment processes. Figure 4.23 showed the flocculating activity increased dramatically as the bioflocculant QZ-7 concentration increased from 0.25 to 2 mg/mL. The highest flocculating efficiency of 93.6% was obtained at 2 mg/mL of bioflocculant QZ-7. Also, QZ-7 show a good flocculating activity of 77.23% at 1 mg/L, 73.2 % at 0.5 mg/mL, 84.2 % at 4 mg/mL and over 64.93% at 6 mg/mL. However, at very low concentration of QZ-7 destabilisation and aggregation were not sufficiently to achieve high flocculating efficiency. Furthermore, the analysis of variance shown in Table 4.15, that the result obtained is in accordance with the reported findings in the flocculation performance of different concentration of bioflocculant has significant differences ($p<0.005$).

Cations improved the coagulation-flocculation process of neutralizing and destabilisation the negative charges residue of bioflocculant function groups through making linked that particle bonds to kaolin suspension (Zhang *et al*., 2013). For instance, Figure 4.24 shows that monovalent and divalent cations, including Al^{+3} stimulated the flocculating activity to a significant extent that matched that of trivalent Fe^{+3} cations. The maximum flocculating activity 92.7 % was detected with Ca^{+2}, because it is recognised that Ca^{+2} could neutralize and stabilize negative charges of the functional groups of bioflocculant which resulted in forming bridges between suspended particles (Yim *et al*., 2007). Also, followed by Al^{+3} (82.9%), Mn^{+2}(76.5%), K^{+} (70.6%), Mg^{+2} (72.7.4%), Na^{+} (67.7%) and Li^{+} (65.6%). These results are analogous to those stated by Manivasagan *et al*. (2015) described that in the presence of Ca^{+2} and Al^{+3} flocculating activity of bioflocculants, from *Bacillus licheniformis a*nd *Streptomyces* sp. was enhanced. Also, Okaiyeto *et al*. (2013). Wang *et al*. (2011), reported that multivalent of cations such as Ca^{+2}, Mn^{+2} and Al^{+3} increased the flocculation activity of bioflocculant produced by *Micrococcus* sp. and *Halomonas* sp., xn11 and xn7 and mixed culture of *Bacillus sphaeicus* F6, with *Rhizobium radiobacter* F2. A similar finding reported by Rasulov *et al*. (2017), that Ca^{+2} contributed to the enhanced flocculating activity of the bioflocculant, produced by *R. radiobacter* SZ4S7S14. Moreover, the bioflocculant produced by aquatic bacteria, *Oceanobacillus* sp. which was the most stimulated by cations such as calcium chloride and aluminium chloride was also detected (Cosa *et al*., 2013a). Moreover, bioflocculant produced by *Bacillus* sp. and *Virgibacillus* sp. were interactive, increasing the flocculating activity in the influence of Ca^{+2}, Mg^{+2} and Mn^{+2} (Nwodo *et al*., 2013). Calcium ions were more active and improved the assembly of larger flocs when matched with others. The statistical analysis presented in Table 4.16, indicated that there is a significant difference ($p<0.00$) between different cations. However, based on multiple

comparison analysis presented in appendix F9, showed that the potassium and magnesium have no significant difference between each other ($p>0.05$).

The effect of salt concentration on bioflocculation efficiency of bioflocculant QZ-7 is shown in Figure 4.25. Different concentrations of NaCl salt were tested to examine the effect of salinity on bioflocculant QZ-7 flocculation performance. As presented at 1% and 5% concentration of NaCl the bioflocculant QZ-7 maintained over 90 % of flocculating efficiency. While 10% and 15% (w/w), of NaCl in kaolin suspension has a 73.9%, 55.1% of the flocculating efficiency of bioflocculant QZ-7. In this experiment, the flocculation activity of QZ-7 was decreased as salt concentration increased. This might be due to the excessive concentration of Na^+ ion interfere between the kaolin particles and QZ-7, or the physical properties of QZ-7 have been changed in high concentration of Na^+ ion which affected the performance of bioflocculant QZ-7 activity. However, bioflocculant QZ-7 was found suitable for treating turbid water with salinity up to 15 % w/w. The statistical analysis of variance of the effect of salinity concentration on flocculating activity is depicted in Table 4.17, revealed that there is significant difference between different concentration of salt 1-30% (w/w) found to be ($p<0.000$). But, in accordance to multiple comparison appendix F11, showed that there is no significant difference between concentration 1% and 5% as ($p>0.05$).

5.6 Application of Bioflocculant QZ-7

In this study the application of bioflocculant QZ-7 in water treatment and industrial wastewater were investigated by using surface water, sugar mill, rubber wastewater and hospital wastewater. Bioflocculant QZ-7 produced by *Bacillus salmalaya* 139SI shown different levels of COD, TSS, TDS, Turbidity and BOD removals, heavy metal adsorption. Differences in the attraction of metals for bioflocculants are due to attractive interaction, charge density and polymers conformation natures with adsorbed ions

(Morillo *et al*., 2006). The metal biosorption mechanism and kinetics influenced by experimental conditions mainly, pH medium, bioflocculant concentrations and initial heavy metal concentrations (Converti *et al*., 2006).

5.6.1 Surface Water Treatment by Bioflocculant QZ-7

The main backbone of the bioflocculant QZ-7 was composed of several sugars and proteins as the side chains which provided numerous flocculant binding sites, while the long backbone ensured the formation of large flocs (Shi-Jie *et al*., 2011). The raw water sample collected from Langat River, in Kajang, Selangor, Malaysia. This river was the source of drinking water for more than one million people. The physical and chemical characteristics of Langat River are shown at Table 4.18. As indicated in Figure 4.26, the turbidity removal of purified QZ-7 and crude QZ-7 was increased gradually as the concentration increased. The turbidity removal of purified QZ-7 was found higher than crude bioflocculant, especially at low flocculant concentration. Purified QZ-7 concentration of 5 mg/L, reduced turbidity of river water from 178.5 NTU to 5.2 NTU and the turbidity was 96.8%. Gong *et al*., (2008), found that the bioflocculant produced by *Serratia ficaria* was used to remove some of the impurities from river water was able to reduce the initial turbidity by 84.2%. The crude bioflocculant at 5 mg/L dosage produced water with final turbidity of 35.6 NTU with removal efficiency of 83.3% because crude bioflocculant may contain some impurities which affect the flocculation performance and decreased the turbidity removal. This indicates that lower concentration of purified bioflocculant could be applied in water treatment. In present case occurrence of glucuronic acid in the bioflocculant QZ-7 from *B. salmalaya* 139SI this in confirmed through mass spectrometric analysis. Carboxyl groups present on the molecular chain of bioflocculants provide more sites for particle attachment and makes bridging between bioflocculant and discrete particles effective (Farooq *et al*., 2010). Based on the statistical analysis of the effect of pure and crude bioflocculant QZ-7 in Table 4.19 and Table 4.20,

it shows that there was a significant difference ($p<0.000$) on the different concentration of the two types of QZ-7.

In general, the TSS removal increased as the bioflocculant concentration increased as presented in Figure 4.27. The TSS removal efficiency by purified bioflocculant QZ-7 gradually increased at concentration range of 0.5 mg/L – 7 mg/L. In addition, 5 mg/L of purified bioflocculant produced water with final TSS of 4.53 mg/L (97.7%). However, beyond 3 mg/L of purified QZ-7 (optimum concentration), TSS removal was decreased. Because of excess purified QZ-7 concentration. Similar study by Lian *et al*. (2008), reported that the bioflocculant produced by *B. mucilaginosus* was able to reduce the TSS of wastewater by 93.3%. Also, Salehizadeh *et al*. (2001) mentioned that *Alc. Latus* bioflocculant was used to treat coke wastewater and final TSS of 80 mg/L could be obtained from the 370 mg/L (78% removal) in the presence of Ca^{+2}. The removal of crude bioflocculant was gradually increased as the bioflocculant concentration increased and the lowest final concentration of TSS 9.7 mg/L was obtained. The result obtained by statistical analysis in Table 4.21 and Table 4.22, found that there was a significant difference ($p<0.000$) between pure and crude bioflocculants on TSS removal.

Results in Figure 4.28 showed that the COD reduction was increased dramatically as the purified bioflocculant QZ-7 concentration increased from 0.5 mg/L to 5 mg/L. The highest reduction of COD was obtained at 91.2% of optimum bioflocculant concentration at 5 mg/L, while the COD reduction dropped as the purified bioflocculant QZ-7 concentration increased from 5 mg/L to 7 mg/L, which might be the excess concentration of the bioflocculant that raised the residual of COD. Gong *et al.* 2008; Lian *et al*. (2008), showed that the bioflocculant of *Serratia ficaria* reduced the COD of river water by 87.1% and the bioflocculant of *B. mucilaginosus* decreased the COD by 74.6%. The statistical analysis of the effect of pure and crude bioflocculant QZ-7 on COD removal is

presented in Table 4.23 and Table 4.24, revealed that there was a significant difference ($p<0.000$), between the two types of QZ-7.

5.6.2 Removal of Heavy Metals Aqueous Solution using Bioflocculant QZ-7

The most essential parameter as pH has been recognized that was very active on heavy metal sorption. It is directly linked through hydrogen ions interaction competency with metal ions on the biosorbent surface active sites (Loderio *et al.*, 2006). The spectroscopic analysis (FTIR), displayed that the pure bioflocculant QZ-7 had an assortment of functional groups, include carboxyl, hydroxyl and amino and these groups are virtually involved in binding mechanisms potentials. Furthermore, these functional groups contribute in metal ion binding are depend on the pH values of the aqueous solution (Dobrowolski *et al.*, 2017). Adsorption of heavy metals was high at neutral and alkaline pH values, this is described that at lower pH values, and a higher proton concentration competing on the same anionic sites of the polymer as the divalent cations (Figure 4.29). The proton mass leads to their preferred binding and so divalent cation binding is low (Sahoo *et al*., 1992). For instance, the increased of the pH towards optimal value, which is contrasted from metal ion to another, also the saturated superficial adsorbed by negative charges, lead to, to increase the efficiency of positive charges to bound and adsorb metal ions (Bayramoglu *et al.*, 2003). While at a higher pH than its optimal importance, hydroxide metals could be made and the adsorption sites on the surface did not bind the adsorbent (Kacar *et al.*, 2000). The stability of pH obtained in the present study could be advantageous in removing other heavy metals and pollutants too from various waste water streams by bioflocculants in required doses.(Pathak *et al*., 2017). Regarding to the analysis of variance of the effect of different pH value on heavy metal removal efficiency are presented in Table 4.25 to 4.30. It is revealed that the different pH value has a significant difference ($p<0.00$) on heavy metal removal. Whereas, the multiple

comparison analysis obtained in appendix F20 to F25, indicated that there is no significant difference between pH 3 and 11, pH 5 and 6, and pH 6 and 7or 7 and 9.

Biofloocculant QZ-7 displayed a good removal efficiency of heavy metals at low bioflocculant concentration as designated by other studies (Das & Santra, 2007). Figure 4.30 showed that an exceed 50% of metals removal were observed at 20 mg/L - 100 mg/L of bioflocculant QZ-7 concentration. Whereas, the highest metal removal was achieved at 60 mg/L of bioflocculant concentration, as 84% of As, 80.7% of Cu^{+2}, 78.5% of Zn^{+2} and 77.4 Cd^{+2} and Pb^{+2} 72.43% at 100 mg/L, besides, there is a significance difference ($p<0.000$) of different QZ-7 concentration as shown in Tables 4.31 to 4.35. Similarly, finding was reported by Gomma. (2012), the highest metal removal efficiency for the bioflocculant produced by *P. aeruginosa* was obtained at 60 mg/L of bioflocculant concentration i.e Zn^{+2} 80.5%, and As 72.96%, and Cd^{+2} 79.93% at 40 mg/L. The main backbone of the bioflocculant QZ-7 was poised of several sugars and proteins as the side chains which provided several flocculant binding sites, although the long backbone confirmed the formation of large flocs (Shi-Jie *et al*., 2011). Supporting the contribution of the functional groups of associated with bioflocculant, it was also manifest from studies on Zn^{+2} sorption on different bacterial strains which indicated presence of acidic, neutral and basic group on the cell surface essential for biosorption (Guinea *et al*., 2006). The interactions between the exopolysaccharide based bioflocculant, and Ni^{+2}, Zn^{+2} and other metal cations in aqueous solution leads to removal of the metal ions from water. The increased heavy metals removal efficiencies at low concentrations of bioflocculant develop more attractive in the industrial effluent wastewaters treatment. Besides, in accordance with the multiple analysis presented in appendix F26 to F30, revealed that there is no significant difference ($p>0.05$), between 10 mg/L and 20 mg/L, 20 mg/L and 100 mg/L, 40 mg/L and 80 mg/L and 100 mg/L.

Results of the effect of metals concentration on the removal efficiency presented in Figure 4.31 shows that the heavy metals adsorption exceeded 60%, with the increased of it's an initial concentration of 10 mg/L to 100 mg/L, also there is a significant difference ($p<0.00$) of the different concentration of heavy metals on bioflocculant QZ-7 efficiency adsorption was reaveled in Tables 4.36- 4.40. Whereas, the multiple comparison analysis in the appendix F30 –F35 showed that there is no significant difference ($p>0.05$), between 20 mg/L and 40 mg/L, 20 mg/L and 60 mg/L, 40 mg/L and 60 mg/L, 40 mg/L and 80 mg/L, also 80 mg/L and 100 mg/L. For example, indicated the maximum removal efficiency achieved of As 94.3% at 60 mg/L and the highest removal of Cu^{+2} (85.2%), Cd^{+2} (84.5%), Zn^{+2}(84.5) and Pb^{+2} (82.76%), at 100 mg/L. Moreover, the presence of carboxylate, hydroxyl, amino and phosphate groups in the bioflocculant displays involvement in metals binding capacity (Chug *et al*., 2016). In addition, the presence of Sulphur in bioflocculant QZ-7, enhances its binding capacity and makes it a perfect substrate for utilisation in heavy metals sequestration (Kumari *et al*., 2017). In addition, the improvement of metal adsorption might be due to an increase in electrostatic interactions, steadily lower affinity linking sites for metal ions (Puranik & Pakniker, 1999).

5.6.3 Removal of Heavy Metal from Industrial Wastewater

The treatment of industrial effluents with bioflocculant QZ-7 at different concentration of 20, 40 and 60 mg/L showed effective flocculation with a concomitant reduction in heavy metals. Likewise, the effect of the dosage of adsorbent on the adsorption of heavy metals by bioflocculant QZ-7 is depicted in Figure 4.32. For example, As removal increased from 72.6% to 89.2% with the increased of the amount of adsorbent concentration, while the Zn^{+2} removal also increased from 63.5% to 76.3%. Whereas, the Cu^{+2} removal slightly increased from 52.3% to 56.5 %. There is proportional of adsorbent concentration and removal efficiency of heavy metal due to bioflocculant QZ-7 has many

functional groups and sulphur ions which provides high affinity towards many heavy metals (Kumari *et al*., 2017). Similar results were obtained by Taha *et al*. (2011) that higher adsorption capacity of QZ-7 due to active functional groups such as hydroxyl and carboxylic group. In case of the removal of As, Zn^{+2}, Cd^{+2}, Cu^{+2} and Pb^{+2} using QZ-7 the ion exchange might be the dominant mechanism (Feng & Guo,. 2012). Furthermore, the presence of phosphate and carboxyl group on EPS produced by *B. subtilis* facilitates greater binding of Cd^{+2} in comparison to EPS derived from *Pseudomonas putida* (Wei *et al.,* 2011). Following the guidelines of MTCC, *B. subtilis* (MTCC 8363) is also capable to remove heavy metals.

5.6.4 Removal of BOD and COD from Industrial Wastewater

The conjugation of COD analysis test with BOD test is a useful toxic sign of circumstances and the incidence of biological resistance (Poddar & Sahu, 2017). The crude bioflocculant produced by *B. salmalaya* strain 139SI was used to treat an industrial wastewater with initial concentration showed in Appendix (D.4). After the treatment, results shown in Table 4.42, there was a significant removal rate of COD about 93%. Furthermore, crude QZ-7 showed exceptional turbidity removal of 90.7% for sugar mill effluent and 62.2% for rubber effluent. Besides, crude QZ-7 was capable to reduce TSS in sugar mill effluent and rubber effluentto 89.69%, 61.5%, respectively. While, QZ-7 revealed TDS removal by 88.2% of sugar mill effluent, 61.5% of rubber effluent and 52.1% of ethanol mill effluent. There were many studies reported on the reduction of COD and TSS by using bioflocculants. For example, bioflocculant from *Azotobacter indicus* was able to reduce BOD, COD and TSS in different wastewater samples in the range of 38%–80%, 37%–79% and 41%–68%, respectively, (Patil *et al*., 2011). Moreover, bioflocculant from *Klebsiella* sp. showed tremendous turbidity removal of 99% and COD of 68.4% at low concentration of 40 mg/L (Feng *et al*., 2009). The *B. salmalaya* strain 139SI used to treat an industrial wastewater of initial BOD concentration

of 4018 mg/L. After the treatment, the final BOD concentration was found to be 302 mg/L. Therefore, the percentage of BOD removal estimated to be 92.4%. Hence, *B. salmalaya* strain 139SI can be used for BOD removal from wastewater.

The removal of pharmaceuticals during wastewater treatment was estimated from concentration data in influent wastewater and effluent wastewater. Considering that pharmaceuticals have rather different physicochemical characteristics, their removal during treatment is expected to be diverse (Gracia-Lor *et al*., 2012) In the literature, the removal efficiency is generally computed as the percentage of reduction between the dissolved aqueous phase concentration of the contaminant in the influent and the dissolved aqueous phase concentration of the contaminant in the effluent. As far as hospital effluent wastewater was concerned, bioflocculant QZ-7 was effectively used to treat the hospital effluent sample, with removal of 88.5% of COD, 86.7% of BOD and 92.5% of TSS. Meanwhile, the primary removal of selected pharmaceuticals as shown in Table 4.42, from hospital wastewater was determined at the laboratory. The result obtained showed that the bioflocculant QZ-7 had a significant removal efficiency of simvastin 92.45 % and salbutmon 88.69 %, while acetaminophen and caffeine was found to be 69.94 %, 66.52 %, respectively. However, there was no significant removal of nifedipine, but for the perindopril, mefenamic acid diclofence was detected at 25.49 %, 13.64 %, 4.04 %, respectively.

5.7 Bioflocculant QZ-7 Mediated Synthesis of AgNPs, Characterisation, and Antibacterial Activity.

The biopolymers-based synthesis of AgNPs has been carried out for a decade and a variety of polymers viz., algal, bacterial, fungal and plant-based polymers were exploited greatly. The bacterial bioflocculant being conjugates of carbohydrates and proteins showed the best to bring out the reduction of $AgNO_3$ to particulate Ag-capped with

polymer, providing stability by preventing particles to avoid aggregation. The synthesis of metal nanoparticles has been extensively studied, among which AgNPs were intensively focused due to its application in the field of biomedical optical and solar. The antimicrobial activity, especially antibacterial effect was detected as the potent quality of silver for centuries.

5.7.1 Synthesis of AgNPs

The nutrient broth supplemented with glucose showed synthesis of AgNPs which was primarily distinguished by visual colour change (Figure 4.33) in a bottle on the 6th day of cultivation of *B. salmalaya* 139SI. While, the control bottle containing nutrient broth supplemented with and without $AgNO_3$ did not display change colour. The constant colour was changed from yellow to dark brown in testing bottle revealed the presence of Ag particles. The reduction of silver nitrate was carried out through bioflocculant QZ-7 and the mechanism is called excitation of surface plasmon response (Zaki *et al.*, 2014).

In the cell-free supernatant having bioflocculant QZ-7 the colour change was detected after 3rd day of the incubation. The bioflocculant QZ-7 reduced $AgNO_3$ to produce AgNPs (Figure 4.34). A sharp peak at 402 nm achieved was designated to AgNPs. Report of Zaki *et al.* (2014) showed that particular bioflocculant producing media provide a better effect on synthesis as the amount of extracellular substances increase in the particular medium. Therefore, the concentration of bioflocculant in free supernatant might be higher as compared to nutrient broth leading to the faster synthesis of AgNPs.

Ten mL of purified bioflocculant QZ-7 (1%) was added to 100 mL of 3 mM $AgNO_3$ and kept overnight on a shaker incubator at 37˚C, at 120 rpm under dark condition. The purified bioflocculant QZ-7 showed synthesis after 1st day as compared to cell-free supernatant which was acquired on 3rd day (Figure 4.35). Bioflocculant QZ-7 comprised of carbohydrate and protein, involve carboxyl and hydroxyl group supported in the

reduction and stabilisation of Ag^+ (Sharma *et al.*, 2009). Ag^+ ions oxidise the hydroxyl groups to carbonyl groups, reducing itself to elemental silver. The carboxylate and amino groups generated a capping that keeps the particles separated. The aggregation is a major problem of chemically synthesised AgNPs and requires a stabilising chemical. The bioflocculant not only synthesises nanoparticles but also provides stability to the particles (Sathiyanarayan *et al.*, 2013; Zaki *et al.*, 2014). Consequently QZ-7 performed as reducing and stabilising agent which is an advantage of biological synthesis of AgNPs over chemical synthesis. Sathiyanarayan *et al.* (2013) found that the bioflocculant produced by marine *B. subtilis* could produce AgNPs with 5% bioflocculant concentration with 48 h incubation time. Furthermore, bioflocculant has an advantage over other microbial techniques, whereby the separation of nanoparticles from the bacterial cell is difficult. The separation of bacterial cells from aqueous solution by using series processes; centrifugation accumulates Ag^+ particles together with bacterial cells, whereas some of the bacteria produce intracellular nanoparticles that need disruption of the cell (Singh *et al.*, 2015).

5.7.2 Characterisation of AgNPs.

Spectrophotometric analysis is the simplest, unique as well as the most sensitive technique to determine the presence of AgNPs and their size distribution as shown in Figure 4.36. The spectrum was determined every 24 h until the peak was detected at 412 nm and absorbance was 2.7 called excited surface plasmon response. This result indicated the presence of 10-40 nm sized AgNPs which was well described by Kumar *et al.* (2012). According to Shrivastava *et al.* (2007) defined that peak at 300 nm is labelled to $AgNO_3$ solution while peak around 400 nm is of AgNPs and range of peak from 400-450 nm exhibit particle size.

The reducing/capping protein responsible for the formation of AgNPs were recognised by FTIR spectrum. Therefore, the FTIR spectrum of silver nanoparticles between the wave numbers 400 cm^{-1} –4000 cm^{-1} proposed that the active bioflocculant QZ-7 was found to be responsible for the reduction of Ag+ ions into metallic silver nanoparticles, which were produced by bond stretching. The specific bond contribution in this phenomenon included O-H at 3421, C-H at 2921.48, =C-H 2851.09, C=C 2321.94, C=C at 2163.83, C-C at 1578.26, C-N at 999.07 and C-H at 478.31$cm^{-1}$and 463.3cm^{-1} as shown in (Figure 4.37). Moreover, the results of the present study clearly suggested that protein or amino acids were responsible for the stabilisation of the silver nanoparticles synthesised by using the purified bioflocculant QZ-7. The results achieved from the present study were in agreement with the findings described by Viswanathan *et al*. (2016).

The FE-SEM and EDAX were used to study the morphology, size and elemental composition of biosynthesized AgNPs. The elements present in the AgNPs were confirmed by EDAX spectrum spot profile made from the densely populated nanoparticles region of the slide surface. The synthesized nanoparticles at micro (10^{-6}) and nano (10^{-9}) can be identified by FE-SEM. The FE-SEM clearly shows that the particles is spherical and the size is found to be about 25 nm -87 nm for biosynthesised AgNPs as shown in Figure 4.38a. The elemental composition of AgNPs is examined by EDAX spectroscopy and is a chemical analysis method combined with FE-SEM Figure 4.38b. (Lawrence & Prakash, 2019). In EDAX, the optical absorption peak at 2kev is observed and strong signal for Ag was detected in which designates that the AgNPs were successfully made by the bioflocculant QZ-7. This shows that metallic silver nanocrystallites which are due to surface plasmon resonance. Sathiyanarayanan *et al.* (2013), reported 60 nm average sized particles synthesized by bioflocculant produced by *B. subtilis*. Recent literature also reports a similar synthesis procedure for AgNPs concerning the treatment of silver nitrate solution with free- supernatant from culture of

K. pneumoniae, in which the particles range in size from 28.2 to 122 nm and exhibited an average size of 52.5 nm (Viswanathan *et al.* 2016). In addition, the manifest of AgNPs synthesized by all bio-groups of *Morganella* spp. were found quasi-spherical in shape, and ranged between 10-50 nm in diameter (Parikh *et al.* 2011). Wei *et al* (2012) stated 4.8-23.7 nm sized AgNPs while Zaki *et al.* (2014), found 6-72 nm sized AgNPs synthesized by bioflocculant produced from *B. mojavensis*.

The XRD configuration shown metallic nature of AgNPs and in this technique, electromagnetic radiation (X-ray) with typical photon energy penetrates into the core of the material to distinguish the nature of bulk structure (Zaki *et al.*, 2014). As illustrated in Figure 4.39 the XRD spectrum of AgNPs showed 4 intense peaks in rang of 20-80˚ at 2θ value; particularly at 38.2667˚, 44.4964˚, 64.5751˚and 77.5673˚ with conforming degree of 111, 200, 220 and 311 respectively, and this result was found to be in agreement with database of the joint committee on Powder Diffraction Standards (JCPDS File no.04-0783). Which are particular to crystalline silver indexed to face centered cubic structure of silver. The AgNPs mean crystallite size was measured by the Debye-Scherrer formula $D = K\lambda/\beta \cos\theta$ (K – Scherrer constant, related to crystalline shape, λ –Radiation wavelength, β – Full width at half maximum of the diffraction peak, and θ – Bragg's angle). The average crystallite size of AgNPs was 40 nm (Monshi *et al*., 2012) Similar finding was reported by Manivasagan *et al.* (2015) that the bioflocculant synthesized by *Streptomyces* sp. is capable of producing AgNPs with similar XRD pattern. Likewise, diffraction patterns were gained with silver nanoparticles synthesized by cell free supernatant of *B. cereus* (Ganesh & Gunasekaran. 2009), *B. Bacillus casei* (Kalishwaral *et al*., 2010).

5.7.3 Antibacterial Activity of AgNPs Synthesised by Purified Bioflocculant QZ-7

Silver is known for its antimicrobial property since ancient times; hence, used widely in the biomedical field. Results are presented in Table 4.43 and Figure 4.40. *E. coli* ATCC35401. *Pseudomonase aeruginosa*, *Salmonella entertidis* ATCCBBA-711 and *staphylococcus aureus* ATCC 2592 showed almost consistent in the zone of inhibition. Meanwhile, *E. coli* ATCC35401 and *Salmonella enteritidis* ATCCBAA-711 showed a slight increase with an increase in the concentration of AgNPs. These values are compared with amoxicillin (standard). These results were supported by antimicrobial activity in liquid media. The bactericidal mechanism of AgNPs was not specific as antibiotics, but rather it has a multiplicity of mechanisms. Meanwhile, smaller particles that possess larger surface area accessible for interaction will contribute more bactericidal effects than larger particles. The possible mechanisms are cell disruption, protein denaturation and subsequent loss of enzyme activity, denaturation of nucleic acid and implication of oxidative stress by reactive oxygen species. Furthermore, the study showed that by using the scanning tunnelling electron microscopy (STEM), and the X-ray energy dispersive spectrometer (EDS) revealed AgNPs can penetrate inside the bacterial cell and cause damage by interacting with phosphorus and sulphur-containing compounds such as DNA (Panacek *et al.*, 2006).

CHAPTER 6: CONCLUSION AND RECOMMENDATIONS

6.1 Summary

In this thesis, the main experiments were completed and summarised as in Chapter 4, a novel bioflocculant named QZ-7 produced by *B. salmalaya* strain 139SI was investigated in this study. The flocculating activity of bioflocculant QZ-7 presented in the selected strain was found to be 83.3%. The effects of carbon source, nitrogen source, metal ions, initial pH, inoculum size, temperature, shaking speeds, static condition and time course on bioflocculant production were investigated. The maximum production was obtained when mixture of carbon source was sucrose and glucose and urea plus yeast extract as nitrogen source, the optimum initial pH 7 inoculum size 5% (v/v), temperature 35.5˚C and shaking speed 160 rpm. The maximum flocculating activity of the optimised strain *B. salmalaya* 139SI was found to be 92.6%.

Chemical analysis revealed that the pure bioflocculant QZ-7 consisted of 79.08% carbohydrates and 15.68% proteins. About 2.72 g of purified bioflocculant with average molecular weight of 5.13×10^5 Da was recovered from 1.0 L of fermentation broth. Fourier-transform infrared spectroscopy (FTIR) analysis showed the presence of carboxyl (COO-), hydroxyl (-OH), and amino ($-NH_3$) groups; which were typically of polysaccharides; and proteins. The NMR spectroscopy analysis confirmed the result of FTIR, through the presence of functional groups of the QZ-7. Scanning electron microscopy (SEM) analysis showed that QZ-7 exhibited a clear crystalline brick-shaped structure. LC-MS analysis confirmed that QZ-7 was a glycoprotein compound detected at 741m/z-745m/z. Moreover, the presence of glucose at 182.96 m/z, rhamnose at 354.3 m/z, and glucuronic acid at 212 m/z. The elemental analysis of purified QZ-7 revealed that the weight fraction of elements C, H, N, O and S were 55.75%, 0.54%, 42.74%, 0.93% and 0.06%, respectively. The TGA of bioflocculant was found to be heat-stable and its activity was only decreased by about 12.14% and 23.76% in weight at 100˚Cand

200˚C , respectively, with 45.28% loss of weight at 300˚C. Some physical properties of QZ-7 bioflocculant such as pH stability and thermo-stability were investigated as well. Results showed bioflocculant QZ-7 exhibited wide pH stability ranging from 4 – 7, with a flocculation activity above 70% at pH 7. Likewise, to study the thermo-stability of QZ-7, the QZ-7 was kept for 1h at different temperatures of 20 ˚C – 100˚C. Result revealed that QZ-7 was thermally stable and reserved more than 80% of its flocculating efficiency after being heated at 80 °C for 30 min.

In this study, the effect of different cation on flocculation efficiency was established by KCl, NaCl, LiCl (monovalent), $MnCl_2$, $MgCl_2$ (divalent), and $AlCl_3$, $FeCl_3$ (trivalent) and flocculating efficiency was measured. Over--all, most of the cations enhanced the flocculating efficiency of QZ-7, the highest flocculating activity (92.9%) was achieved for Ca^{+2} at pH 7, followed by Al^{+3} (83.3%), Mn^{+2} 75.6 K^{+2} 71.6, Mg^{+2} 71.4, 70%, Na^{+} 67.3 and Li^{+} 64%.

The treatment of river water was by 5 mg/L of bioflocculant QZ-7. Results showed that the final turbidity, total suspended solids and COD were found to be reduced by 96.8%, 97.7% and 91.2%, respectively. These results were the lowest in comparison with the water treated by crude QZ-7 at 5 mg/L.

Bioflocculant QZ-7 could successfully flocculate artificial and real wastewater, with a concomitant reduction in COD, TDS, turbidity, colour and heavy metals from wastewater of rubber, sugar mill, and ethanol industries. In addition, for the removal of heavy metals from wastewater, the results revealed that the bioflocculant QZ-7 was capable to remove heavy metals. For example, the maximum adsorption of As (89.3 %), and Zn^{+2} (81.3%) were detected at pH 7. Meanwhile, the removal of Pb^{+2} (77.9%), Cu^{+2} (76.1%) and Cd^{+2} (68.7%) was achieved at pH 9. Whereas, the optimal dosage of bioflocculant of 60 mg/Lfound that the removal efficiency of As, Cu^{+2}, Zn^{+2} and Cd^{+2} were detected at 84.4%,

80.7%, 78.5% and 77.4%, respectively. Furthermore, results for the removal of heavy metals from industrial wastewater revealed that the bioflocculant QZ-7 was capable of removing the heavy metals. For example, the maximum adsorption of As (89.8 %), and Zn^{+2} (77.4 %), and Cu^{+2} (58.4%).

The pharmaceuticals from hospital effluent wastewater were also treated with QZ-7 with a reduction in COD and suspended particles. Meanwhile, the removal efficiency of drug compounds, such as Simvastatin 92.45%, Salbutamol 88.69%, Acetaminophen 69.94, Caffeine 66.52% Perindopril 25.49%, and Mefenamic acid 13.65%.

Apart from flocculation, *B. salmalaya* 139SI, supernatant and pure bioflocculant QZ could synthesise AgNPs and were coated with biopolymer which created a repulsive force between the particles to keep them apart and escape aggregation. The antibacterial activity of AgNPs was obtained against different strain of bacteria as application of AgNPs.

The overall results analysed by the analysis of variance revealed that there are significant differences ($p<0.00$).

6.2 Recommendation for Future Research

This study creates a basal platform on fermentation technologies for the production of bio-products of environmental significance, particularly in the production of bioflocculant for the treatment of wastewater. Therefore, it is recommended for future research to further exploit the production through submerged fermentation (SmF) by scaling– up the process on a pilot-scale and industrial level to increase yield for possible mass production and potential commercialisation while maintaining the quality and performance. Moreover, the sugar processing industries wastewater must be studied as cheap substrate as the carbohydrates source for bioflocculant production by *B. salmalaya* 139SI.

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