PRODUCTION OF CRUDE BIOPOLYMER FROM BACTERIA IN DRAINAGE SYSTEM

by

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TABLE OF CONTENTS

LIST OF PLATES

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS

PAM	polyacrylamide
ALUM	aluminum sulfate
PGA	polyglutamic acid
16rRNA	partial nucleotide sequence analysis
FTIR	fourier transform infrared spectrometry
SEM	scanning electron microscope
EPS	exopolysaccharides/extracellular polymericsubstances
PAC	polyaluminum chloride
PAA	polyacrylamide
$MgSO_4.7H_2O$	magnesium sulphate heptahydrate
$Al_2Si_2O_5(OH)_4$	kaolin clay
HDPE	high density polyethylene
GPC	gas permeation chromatography
THF	tetrahydrofuran
NCBI	National Center for Biotechnology Information
O-H	hydroxyl group
NH_2	amino group
C-O	carboxyl group
BSIA	*bacillus subtilis* industrial area
BMRA	*bacillus megaterium* residential area

BMIR	*bacillus megaterium* industrial residential
CWIA	crude water industrial area
CWRA	crude water residential area
CWIR	crude water industrial residential

PENGHASILAN BIOPOLIMER MENTAH OLEH BAKTERIA DARIPADA SISTEM PERPARITAN

ABSTRAK

Kerja ini bertumpu kepada pengekstrakan biopolimer yang dihasilkan oleh bakteria untuk rawatan air sisa secara khusus dalam proses pembukuan. Tiga daripada strain bakteria terpencil (WIR, WRA, WIA) mampu untuk mengeluarkan biopolimer sebagai bahan pembukuan telah diasingkan daripada sistem perparitan industri dan perparitan daripada kawasan kediaman. Kesemua bakteria telah dikenal pasti sebagai *Bacillus megaterium* BMRA, *Bacillus megaterium* BMIR dan *Bacillus subtilis* BSIA melalui ciri fisiologi (mucoid, berlendir dan kental) dan turutan separa 16SrRNA mereka. CWIR biopolimer mentah (perindustrian + kediaman) dan biopolimer CWRA (kediaman) telah dihasilkan oleh *Bacillus megaterium* BMIR dan BMRA manakala biopolimer CWIA (industri) dihasilkan daripada *Bacillus subtilis* BSIA . Bakteria ini menghasilkan biopolimer mereka pada 40 ºC selama 2 hari tempoh pengeraman dengan pH 7.0 ± 0.03 masing-masing. Biopolimer mentah boleh didapati daripada supernatan hasil daripada pemendakan etanol bersama kaldu bakteria dan dikeringkan dalam ketuhar vakum. CWIR, CWRA dan CWIA mengandungi 4.01 %, 5.20 % dan 5.45 % protien masing-masing. Jumlah kandungan gula dalam setiap biopolimer adalah 1.48 % untuk CWIR, 1.65 % untuk CWRA dan 1.79 % untuk CWIA. Analisis elemen menunjukkan bahawa CWIR mempunyai 31.60% C, 4.81 % H dan 6.32 % N manakala CWRA mengandungi 31.37 % C, 5.02% H dan 6.12% N. Untuk CWIA ia menunjukkan 31.25% C, 4.88% H dan 5.69% N. Berat molekul bagi setiap biopolimer mentah CWIA , CWIR dan CWRA adalah pada 31,788 kDa ,

55,344 dan 38,791 kDa masing-masing. Analisis mikroskop stereo menunjukkan struktur gula setiap biopolimer mentah. Pengimbasan analisis mikroskop elektron (SEM) menunjukkan bahawa biopolimer mempunyai struktur berbentuk kristal linear. Hasil analisis spektroskopi inframerah (FT-IR) untuk CWIR, CWRA dan WIA menunjukkan kehadiran kumpulan karboksil, asid amino dan hidroksil serta sekumpulan gula. Aktiviti pembukuan (%) bagi *Bacillus subtilis* BSIA telah dicapai pada 90.13%, *Bacillus megaterium* BMIR adalah 89.58 % dan *Bacillus megaterium* BMRA adalah 90.50 % masing-masing.

PRODUCTION OF CRUDE BIOPOLYMER FROM BACTERIA IN DRAINAGE SYSTEM

ABSTRACT

This work focuses on the extraction of biopolymer from bacteria in relation to wastewater treatment, specifically in flocculation process. Three isolates of bacterial strain (WIR, WRA, WIA) which are able to produce biopolymers as flocculants were identified from industrial and residential drainage systems. These bacteria were identified as *Bacillus megaterium* BMRA, *Bacillus megaterium* BMIR and *Bacillus subtilis* BSIA through physiological characteristics (mucoid, slimy and ropy) and the partial sequences of their 16SrRNA. Crude biopolymers CWIR (industrial + residential) and CWRA (residential) were produced by *Bacillus megaterium* BMIR and BMRA while CWIA (industrial) was extracted from *Bacillus subtilis* BSIA. These bacteria produced their respective biopolymers at 40ºC given a two-day incubation period, with pH 7.0 ± 0.03. The crude biopolymers were recovered from the supernatant of the culture broth by ethanol precipitation and vacuum-dried in vacuum oven. CWIR, CWRA and CWIA contained 4.01%, 5.20% and 5.45% of protein respectively. Total sugar content in each biopolymer was 1.48% for CWIR, 1.65% for CWRA and 1.79% for CWIA. Elemental analyses showed that the CWIR obtained had 31.60% of C, 4.81% of H and 6.32% of N, while WRA contained 31.37% of C, 5.02% of H and 6.12% of N. WIA showed a composition comprising 31.25% of C, 4.88% of H and 5.69% of N. The molecular weight for each of the biopolymers CWIA, CWIR and CWRA was measured at 31.788 kDa, 55.344 kDa and 38.791 kDa respectively. Stereo microscopic analysis showed the structure of each

biopolymer similar to sugar. Scanning electron microscopic analysis (SEM) revealed that the polymers had crystal linear structures. Fourier-transform infrared (FT-IR) spectroscopic analysis for CWIR, CWRA and CWIA showed the presence of carboxyl, hydroxyl, amino acid and sugar derivative groups. Flocculating activity (%) for the broth culture *Bacillus subtilis* BSIA stood at 90.13%, 89.58% for the *Bacillus megaterium* broth culture, and 90.50% for the *Bacillus megaterium* broth culture.

CHAPTER 1.0

INTRODUCTION

1.1 Background of the study

The amount of wastewater, increasing at a rapid pace in recent years, has become among the major concerns as far as environmental problems go, given its dumping into the ecosystem which poses a serious problem for the survival of mankind. Most pollutants from industrial and residential sources affect the quality of air, water and soil, which remain essential components to the environment. Water pollution has triggered the public to raise their awareness in this area; as such, wastewater needs to be treated before its disposal or reuse in ensuring that favorable water qualities are maintained.

Wastewater treatment is a multistage process consisting of screening, sedimentation, coagulation and flocculation. Flocculation is the process of separating treated water and suspended solids by chemical flocculants. However, the process involves neurotoxic and carcinogenic acrylamide monomers that are harmful to humans and the environment, considering that their usage may cause Alzheimer's disease, which restricts their usage (Vanhoric and Moens, 1983, Dearfield and Ambermathy, 1988; Yim et al., 2007; Yu et al., 2009; Bhunia et al., 2012). For example, in synthetic high polymer flocculants, some of their monomers have neurotoxicity or carcinogens, while some inorganic flocculants compounds include Aluminium (Al). So if these flocculants are let out to the environment without treatment of the affected water,

environmental pollution may ensue. However, the recent years have seen the utilization of microbial flocculants in resolving these environmental issues, anticipated due to their biodegradability and the harmlessness of their degradative intermediates (Salehizadeh et al., 2000; Yu et al., 2009).

1.2 Problem statement

The use of bacteria as a flocculant in flocculation processes to replace chemical flocculants in wastewater treatment applications has recently garnered great interest. However, the limitations for utilizing bacteria include the insufficiency of substrates, the composition of the wastewater (physiochemical characteristics of wastewater) and the competition between the introduced species and the indigenous biomass. Therefore, the development of biodegradable biopolymers is an essential alternative in replacing the bacteria itself. Biopolymers that have flocculating activity are generally biodegraded readily and are harmless to the environment and to humans, indicating their potential in taking over existing chemical flocculants (Jang et al., 2001).

A biopolymer is a polymer secreted by bacteria and originates from their extracellular compound. A biopolymer is a type of flocculant which has great potential compared to commercial inorganic flocculants in the area of wastewater treatment. Theoretically, a biopolymer is biodegradable because it is produced by microorganisms. A biopolymer is a kind of biodegradable macromolecular flocculant secreted by microorganisms, with high flocculating activity (Gao et al., 2006). Its degradation process is harmless to humans and the environment (Kurane and Matsuyama, 1994).

Biopolymers with high flocculating activity was suggested as being the best for industrial applications, including for wastewater treatment, industrial processes and separation of cells from fermentation broths (Kurane et al., 1986; Nwodo and Okoh, 2013).

Due to their eco-friendly characteristics, biopolymers can be a potential replacement to conventional synthetic flocculants in downstream processing when concerning food and medicinal industries, as well as for wastewater treatment (Bhunia et al., 2012). The mechanism of flocculation activity by biopolymers depends on the physical characteristic of the biopolymer concerned, such as the number and type of functional groups, molecular weight, solubility, chemical composition and physical appearance., All these characteristics need to be studied to determine the effective component in bioflocculation processes prior to the purification of a biopolymer

There is a lot of work from different sources employing the same method to isolate bacteria in producing biopolymers, but their works focused on one species from a single source (Suryani et al., 2011; Kumar et al., 2004; Zhang et al., 2002; Jang et al., 2001). There are many possible sources of bacteria that could influence flocculating activity; examples include soil (He et al., 2001; Deng et al., 2003; Mahmoud, 2012), oil fields (Illias et al., 2001; Albahry et al., 2013) and freshwater (Nwodo et al., 2013). However, studies on same or different bacterial strains isolated from different sources, studied concurrently, is either lagging or missing altogether.

1.3 Objective

This work includes isolation and screening of bacteria, extraction of extracellular cells and the characterization of the biopolymer in order to produce biopolymers from bacteria for the flocculation process. Following are activities to achieve the objective:

a) Isolate, screen and identify biopolymer producing bacteria from different sources.

b) Characterize extracted crude biopolymer from different strains.

c) Evaluate flocculating activity (%) of selected biopolymer producing bacteria using kaolin suspension.

CHAPTER 2.0

LITERATURE REVIEW

2.1 Flocculant in flocculation process at wastewater treatment system

Flocculation is the process of aggregation of smaller particles to form larger sized clusters via physical, chemical and biological processes (Droppo et al., 2005). Flocculants have been used in wastewater treatment plants and play an important role in settling suspended solids from various wastewaters. Flocculants are used for separating treated water and suspended solids in wastewater treatment plants, pulp industries, food industries, tap water production, dredging, downstream processing and fermentation (Salehizadeh et al., 2000). Flocculants conventionally used for wastewater treatment and drinking water processes can be classified into three groups (Fujita, 2000) (Figure 2.1) : inorganic flocculants, such as aluminum sulphate, ferrite flocculants or polyaluminium chloride (PAC); synthetic organic flocculants, such as polyacrylamide (PAA) derivatives or polyethylene imine; and natural flocculants or bioflocculants (biopolymer) such as sodium alginates, chitosan or microbial flocculants (Bank et al., 2006; Polizzi et al., 2002; He et al., 2002; Salehizadeh and Shojaosadati, 2001; Salehizadeh et al., 2000; Yim et al., 2006).

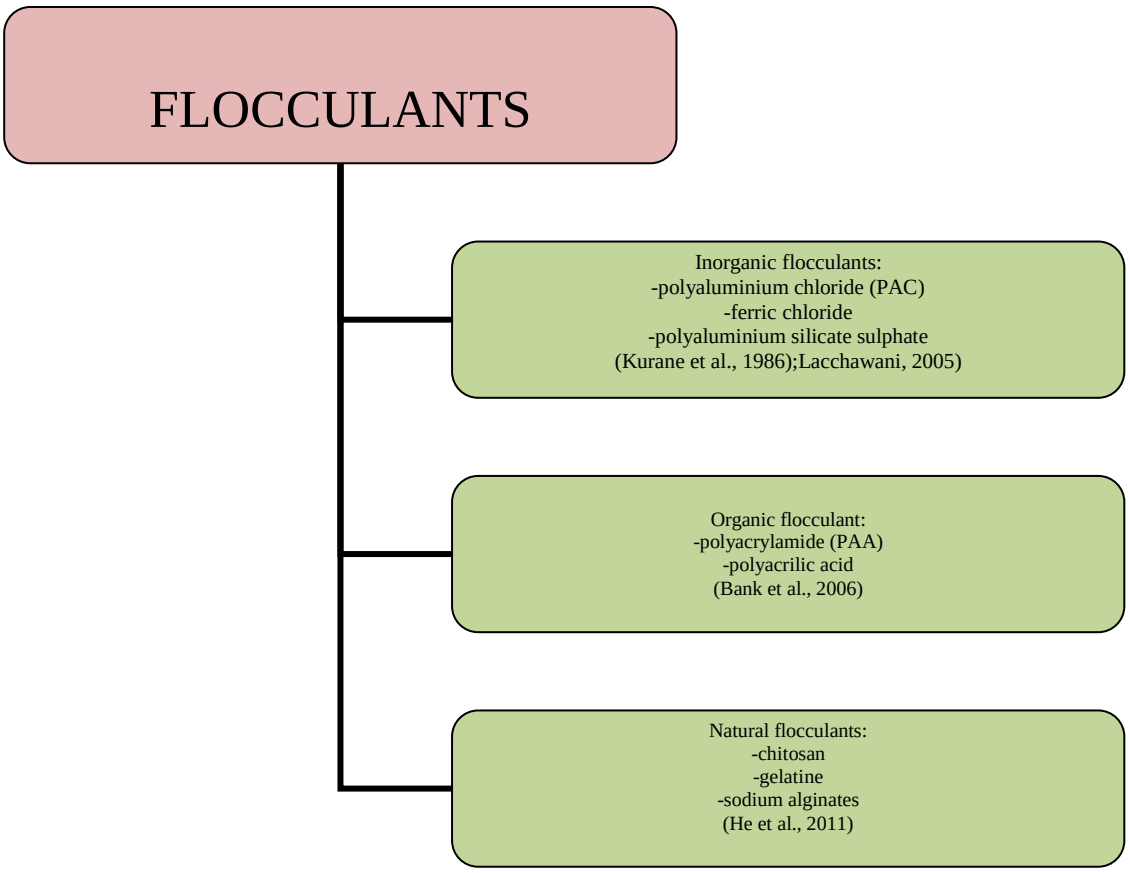

Figure 2.1 Different types of commercial flocculant (Kurane et al., 1986; Lachhwani, 2005; Bank et al., 2006; He et al., 2011)

Among the flocculants, the synthetic chemical polymeric flocculants are commonly used because of their effectiveness and low cost. Although synthetic organic flocculants are the most frequently used due to their cost-effectiveness, they are not biodegradable and show strong human carcinogenic and neurotoxic potential (Dearfield and Ambermathy, 1988). For example, ferrite flocculants can be costly, and produce iron in excess, potentially causing unpleasant metallic taste, odor, color, corrosion, foaming or staining. Organic flocculants such as polyacrylamide (PAA) are not easily degraded in nature, while some of the monomers derived from synthetic polymers are harmful to the human body (Vanhorick and Moens, 1983; Yokoi *et al*., 1995). Besides

that, poly-aluminum chloride (PAC) also contributes to health problems, but has been used for water and wastewater treatment. Health problems such as Alzheimer's disease occur due to the aluminum salts from PAC (Kowall et al., 1989). Synthetic/organic flocculating polymer agents pose environmental problems, in that some of them are not readily biodegradable, while intermediate products in the course of their degradation are harmful to humans. Synthetic flocculants are widely use in industrial wastewater treatment plants due to their cost effectiveness and high flocculating activity. However, these kinds of flocculants can cause health and environmental problems due to their carcinogenic and neurotoxic monomers (Suh et al., 1997). Natural flocculants such as chitosan, gelatin and microbial polysaccharides are safe, cheap and non-toxic to the environment (Sharma et al., 2006). Previous researchers solved these problems by investigating natural biodegradable biopolymer flocculants produced by microorganisms.

2.2 Applications and advantage of microorganisms as natural bioflocculants

Many technological applications are currently researched for the scientific application of bacteria to improve human life. Prokaryote microorganisms which secrete a biopolymer known as exopolysaccharides (EPS) have contributed as important resources to biotechnology and biopharmaceutical industries (Maugeri et al., 2002, Nicolaus et al., 2004, Novas et al., 2004). Microorganisms are expected to be useful in secreting bioproducts such as biopolymers (Kumar et al., 2007). These biopolymers, present in nature, are suitable for biotechnological applications. Biopolymers are widely

used in wastewater treatment, drinking water purification and fermentation processes in industries (Shih et al., 2001).

A lot of microbial polysaccharides have been developed previously such as xanthan, curdlan, dextran, gellan, and pullulan (Sutherland 1998, 2001). Some of these microbial polysaccharides were obtained from bacteria. These polysaccharides are normally used as microbial flocculants. Recently, utilization of microbial flocculants (biopolymer) has been anticipated to solve environmental problems due to their biodegradability and harmlessness to environment. Microbial polymers, especially polysaccharides, were used as suspending agents, stabilizers, thickeners, water retention agents, dispersants, etc. in many industries (Sanford, 1979).

Microorganism secreted biopolymers have received more attention of late given environmental concerns and claims that there are no adverse effects to human health (Kurane and Matsuyama, 1994). Over the past decades, some microorganisms, including algae,actinomyces, fungi and bacteria, have been reported to produce bioflocculants/biopolymers (Takagi and Kadowaki, 1985; Zhang et al., 1999; Huang et al., 2005). Biopolymers are released to environment by microorganisms either for protection from ionic, biotic and abiotic stress factors like osmotic, heat, water, desiccation, invasion by other organisms or nutrient and virulence against other organisms (Cowan et al., 2000; Chavant et al., 2002; Ullrich,2009; Ashraf et al., 2013). Because of the limitation in some flocculants or polymers, bioflocculants, or formerly known as biopolymers, which are produced by microorganisms through the synthesis of

extracellular polymers by living cells, have been investigated as alternative flocculants (Li et al., 2008).

Extracellular polymers found outside the cell wall are a common feature in eukaryotic and prokaryotic microorganisms (algae, fungi). These polymers have been defined as substances of biological origin that participate in the formation of microbial aggregates. They including organic macromolecules such as polysaccharides, proteins, nucleic acids and lipids have received much attention recently due to the awareness in environmental problems, which inevitably affects the health of human beings; notwithstanding, they show weak flocculating activities (Kaewchai and Prasertsan, 2002).

Table 2.1 shows previous studies on bioflocculant/biopolymer producing microorganisms by different researchers.

Table 2.1 Previous studies on bioflocculant-producing microorganisms

Microorganisms	References
Acidithiobacillus ferrooxidans	Natarajan and Das, 2003
Acidithiobacillus thiooxidans	Natarajan and Das, 2003
Aspergillus paraciticus	Deng *et al*., 2005
Bacillus sp. DP-152	Suh *et al*., 1997
Bacillus sp. I-450	Kumar *et al*., 2004
. *Bacillus sp.* AS-101	Salehizadeh *et al*., 2000
Bacillus firmus MS-102	Salehizadeh and Shojaosadati, 2002
Bacillus subtilis SM-29	Kaewchai and Prasertsan, 2002
Bacillus subtilis WD-90	Kaewchai and prasertsan, 2002
Bacillus subtilis DYU1	Wu and Ye, 2007
Bacillus polymyxa	Santhiya *et al*., 2002

Bacillus licheniformis CCRC 12826	Shih *et al*., 2001
Chryseomonas luteola TEM05	Ozdemir *et al*., 2005
Chromobacterium violaceum	Suryani *et al.,* 2011
Citrobacter koseri	Suryani et al., 2011
Citrobacter sp. TKF04	Fujita *et al*., 2000
Corynebacterium glutamicum	He et *al*., 2002
Enterobacter aerogenes WF-1	Lu *et al*., 2005
Enterobacter sp. BY-29	Yokoi *et al*., 1997
Enterobacter agglomerans SM-38	Kaewchai and Pasertsan, 2002
Erwinia chrysanthemi spp	Ding *et al*., 2003
Gyrodinium impudicum KG03	Yim *et al,* 2007
Klebsiella sp.	Sheng *et al*., 2006
Paenibacillus polymyxa	Sharma *et al*., 2001
Proteus mirabilis TJ-1	Xia *et al*., 2007
Rhodovulum sp. PS-88	Watanabe *et al*., 1999
Serratia ficaria	Gong *et al*., 2007
Vogococcus sp. W31	Gao *et al*., 2006

Biopolymers secreted from bacteria have high potential to replace chemical flocculants that greatly contribute to environmental pollution. Biopolymers extracted from bacteria are isolated from soil, wastewater and sludge (Salehizadeh and Shojaosadati, 2001). These bacteria produced biopolymers during their growth (Xia et al., 2008). Significantly, many reported bacteria which could secrete biopolymer flocculants belong to *Bacillus* sp that are rod shaped (Shih et al., 2001; Salehizadeh and Shojaosadati, 2002; Suh et al., 2002; Deng et al., 2003; Ganesh Kumar et al., 2004). Different bacteria have different shapes and arrangements. Plate 2.1 illustrates the shapes and arrangement of bacteria cells that occur as rods, spheres, chains and spirals.

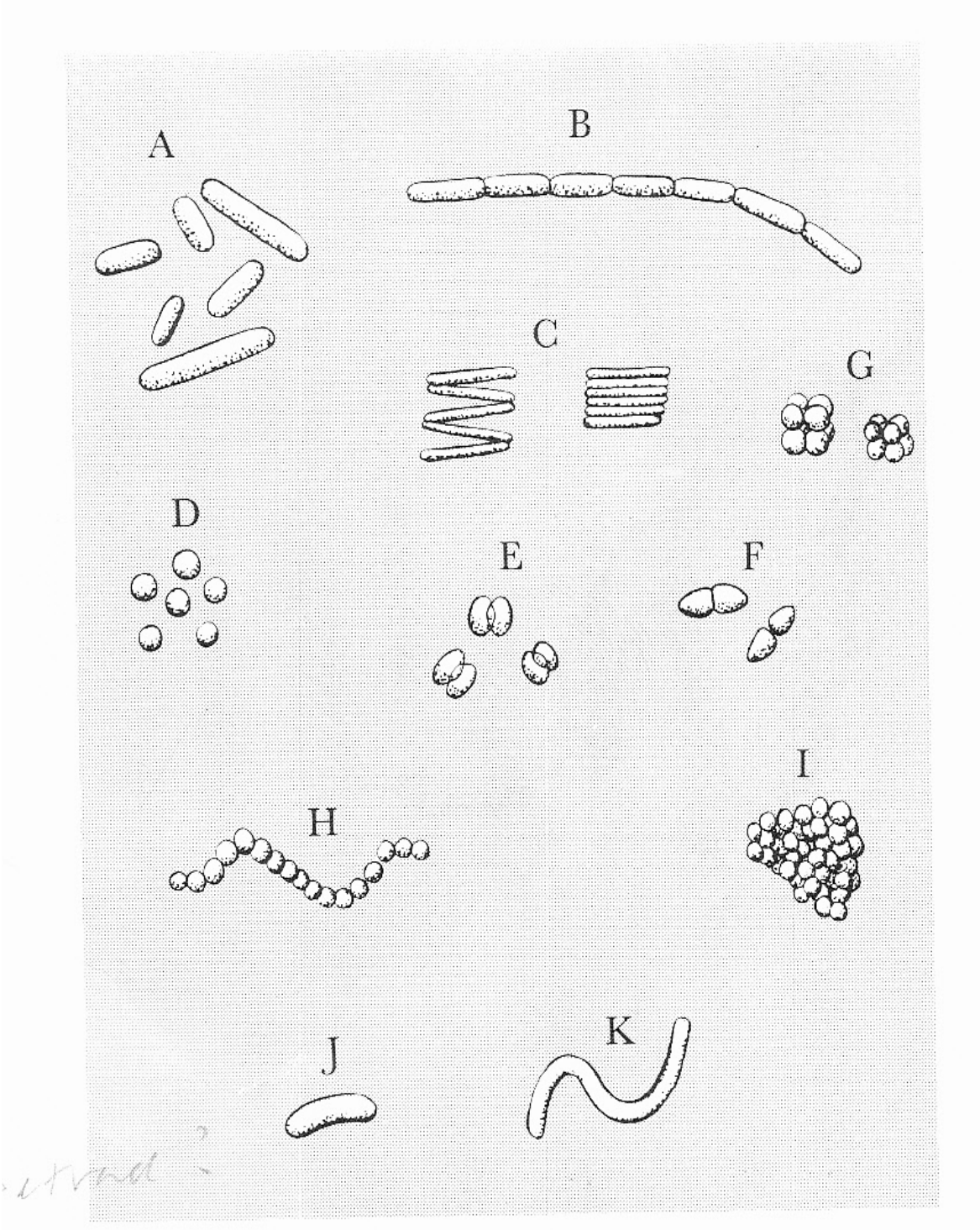

Plate 2.1 Shapes and arrangements of bacteria. A:short and long rods occurring singly; B:rods in chain: C:palisade arrangement of rods; D:single cocci(spheres); E:paired flattened cocci; F:paired elongate cocci; G:cubical packets of cocci(sarcina); H:a chain of cocci (streptococcus); I:an irregular cluster of cocci (staphylococcus); J:comma-shaped or bent rod; K:spiral rod (Carpenter, 1972).

2.3 Characteristic of biopolymer producing Bacteria

Characteristics of biopolymer producing bacteria were observed through solid media. Plate 2.2 illustrates colonial characteristics of bacteria on the solid media (Pollack et al., 2002).

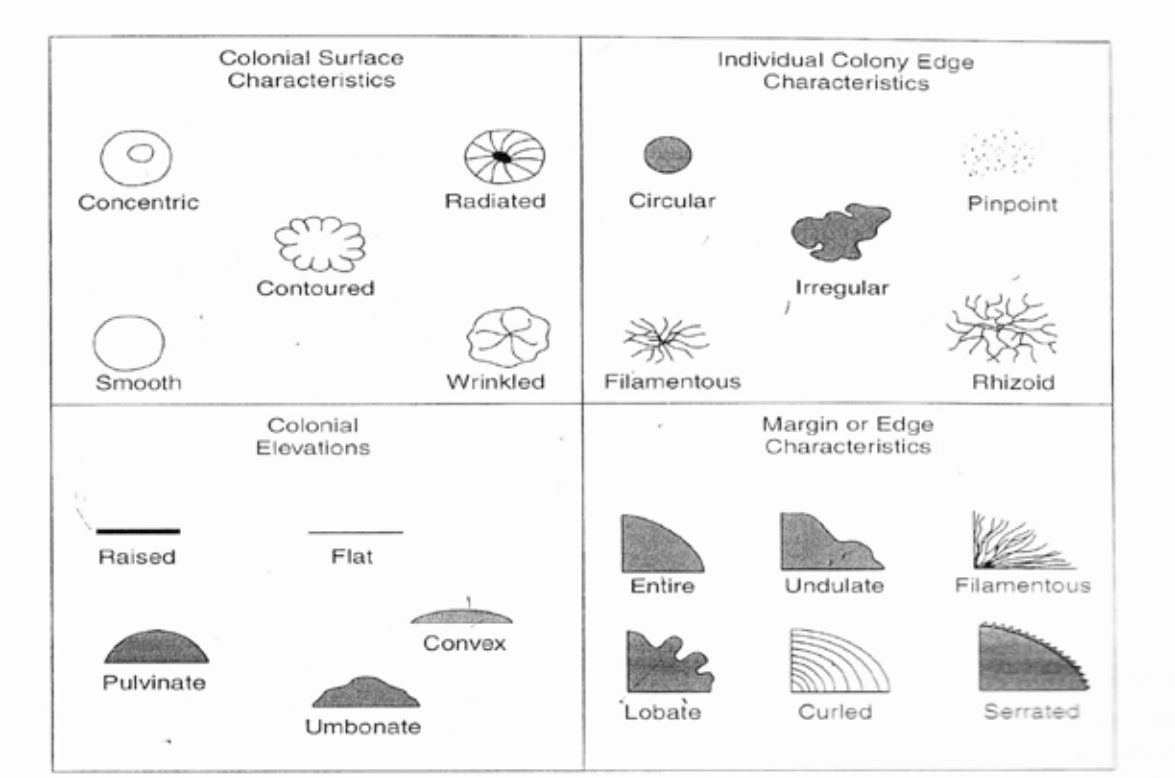

Plate 2.2 Colonial characteristics of bacteria (Pollack et al ., 2002).

Bacteria cell structures are divided into two types which are Gram positive and Gram negative. Plate 2.3 depicts the cells of gram-positive and gram-negative bacteria. The outer layer of bacteria is a capsule, microcapsule or loose slime. Slime and capsule layers consist polysaccharide polymers that prevent the bacterial cells from drying, or protect it from harmful agents (Ruas-Madiedo and Reyes-Gavilan, 2005). Besides,

capsules and slime from gram positive bacteria normally contains amino acids and sugar. Gram positive bacteria with rod shape and motile appearance also secreted EPS with high flocculating activity (Desouky et al., 2008)

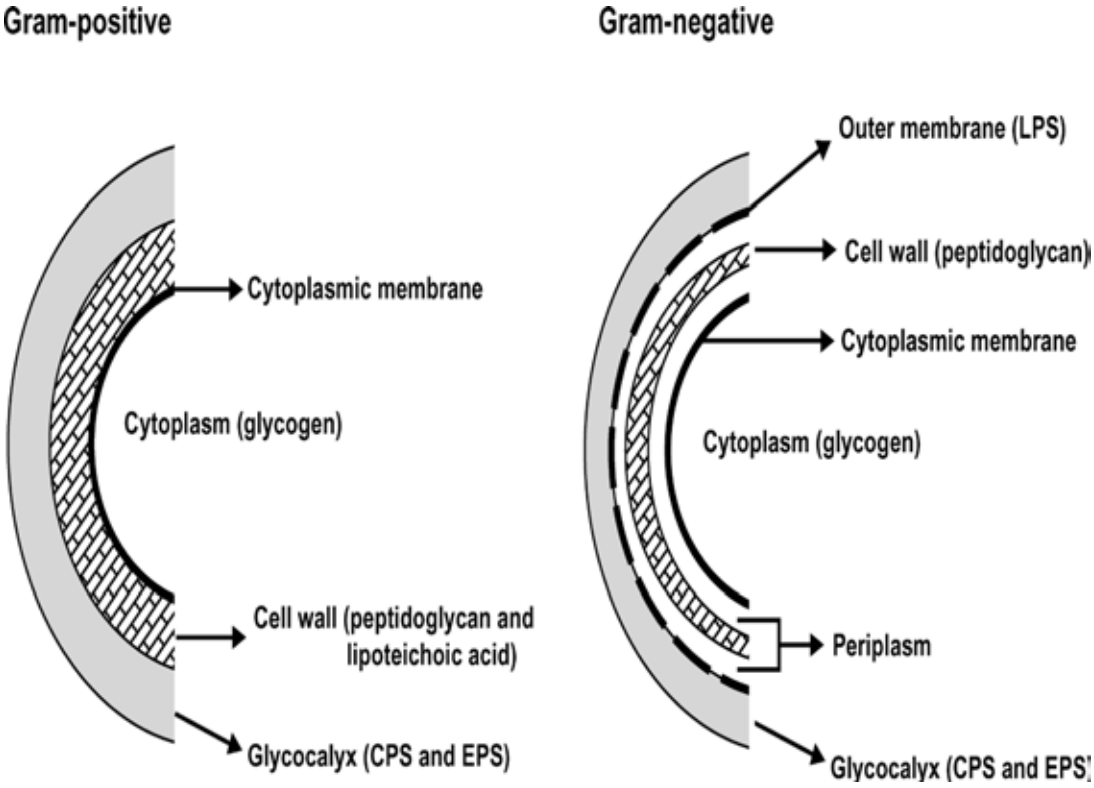

Plate 2.3 Cell of gram-positive and gram-negative bacteria. CPS = capsular polysaccharides (capsule), EPS = exopolysaccharides (slime layer) (Ruas-Madiedo and Reyes-Gavilan, 2005)

Colony morphological studies of bacteria with mucoid, slimy and ropy appearance proved the production of biopolymers (Takagi and Kadowaki, 1985; Zaki et al., 2013). Colonies of bacillus are famous in producing biopolymers. *Bacillus* sp. I-450 produces extracellular polysaccharides with mucoid and a ropy morphology (Kumar et al., 2004). *Bacillus subtilis* IFO 3335 grown on IFO medium showed the ability in

production of poly (glutamic acid) (PGA), known as a biodegradable biopolymer from fermentation processes (Richard and Margaritis, 2003).

Capsular bacteria highly contribute to the production of biopolymers as well. The capsule consists of polysaccharides, which is a major component in biopolymers (Todar, 2004). Plate 2.4 shows the morphology of a biopolymer producing bacteria on solid agar with mucoid and a slimy appearance. The mucoid colonies have a glistening and slimy appearance on agar plates but are not able to produce strands when extended with an inoculation loop (Ruas-Madiedo and Reyes-Gavilan, 2005).

Plate 2.4 Colonies of *Bacillus anthracis* with slimy and mucoid appearance evidence of capsule production (Todar, 2004).

Ropy strands formed (Plate 2.5) off extracellular bacteria always screened as a strain that has high potential in flocculating activity (Kumar et al., 2004, Gao et al., 2006).

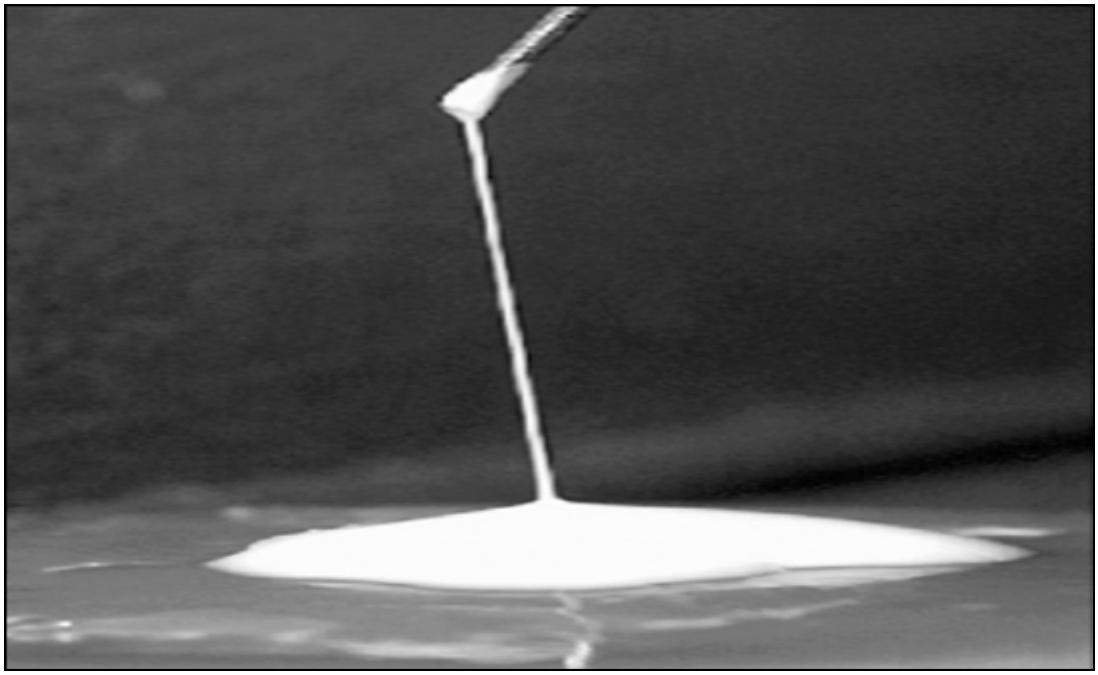

Plate 2.5 Macroscopic appearance of the 'ropy' strand formed by the cellular mass of a commercial EPS-producing LAB strain growing on the surface of de Man, Ragosa, and Sharpe (MRS) agar plates (Ruas-Madiedo and Reyes-Gavilan, 2005)

Biopolymers are essentially produced by bacteria during growth with their flocculating activities being dependent on the characteristics of the polymers (Xia et al., 2008). Important characteristics of biopolymers that play an important role in flocculation processes include their molecular weight, functional groups, solubility, surface structures and chemical compositions (Deng et al., 2005; Kurane et al., 1994; Salehizadeh and Shaojadasadati, 2001; Zhang et al., 2010).

2.4 Extraction of crude biopolymer

The extracted crude biopolymer was obtained through an ethanol precipitation process (Kumar et al., 2004a; Lu et al., 2005). A similar procedure was implemented by Falk et al. (1996) on the biopolymer extracted from *Sphingomonas paucimobilis* strain I-886. The crude biopolymer was extracted from the supernatant of a culture broth by ethanol precipitation, stored at 4ºC for 24 hours. This process can enhance the precipitation of biopolymers (Kumar *et al*., 2004). According to Yim et al. (2007), polysaccharide p-KG03 was separated from a supernatant by addition of ethanol and stored for 24 hours at 4ºC.

2.5 Physico-chemical characteristics of biopolymer secreted from bacteria

The physical method used for characterization and visualization of biopolymers divided into solubilization analysis, fourier transform infrared spectroscopy analysis (FT-IR), molecular weight analysis using gas permeation chromatography (GPC), and scanning electron microscopy (SEM)(Al-Bahry et al., 2013; Suryani et al., 2011; Takagi et al., 1985).

Solubility of biopolymers in aqueous solutions was due to the hydroxyl groups present in the biopolymer which presents the possibility of hydrogen bonding with water molecules. A polymer with a high number of hydroxyl groups exhibits high solubility in aqueous solutions (BeMiller and Whistler, 1996). The more the number of hydroxyl groups present in a given polymer, the greater the decrease in the insolubility of polysaccharides in organic solvents (James, 1986). The functional groups in the molecular chains of polymers play an important role in flocculating activity.

FT-IR analysis for biopolymers produced by *Bacillus velezensis* showed the presence of carboxyl, hydroxyl and amino groups (Zaki et al., 2013). Hydroxyl, carboxyl, amino and sugar derivative groups are the effective groups for flocculation processes (Zajic and Knetting, 1971; Kurane and Matsuyama, 1994; Xiuhang et al., 2013). Biopolymer flocculants extracted from haloalkalophilic *Bacillus sp.* indicated the presence of carboxyl and hydroxyl groups, based on its IR spectrum (Figure 2.2) (Kumar et al., 2004). MBF-5 secreted from *Klebsiella pneumonia,* consisting of polysaccharide and protein, showed the presence of carboxyl and hydroxyl groups (Zhao et al., 2013).

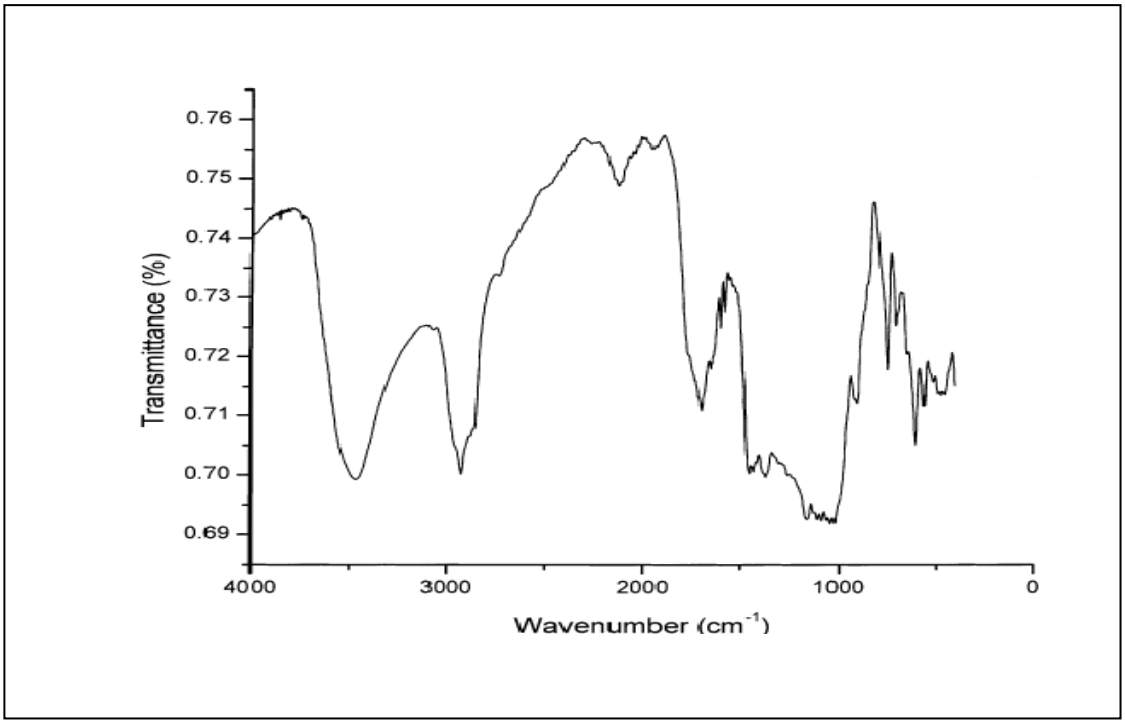

Figure 2.2 Infrared spectra of the purified biopolymer EPS471 from *Bacillus sp*. I-471(Kumar et al., 2004).

Biopolymers with ropy appearance have a high ability to become flocculants in wastewater treatment. The strong ropy character of biopolymers extracted from bacteria is one of the main characteristics of biopolymers (Plate 2.5). Biopolymers with ropy appearances will have high molecular weights. Biopolymers with high molecular weights have many functional groups which are important factors in flocculating activity (Kurane and Matsuyama, 1994). High molecular weights contribute to the effective bridging between the biopolymers and particles (Deng et al., 2003). Molecular weight is an important factor in flocculation performance. Polysaccharide bioflocculants have high molecular weights and many functional groups (Sharma et al., 2001). The biopolymer produced by *Proteus mirabilis* TJ-1 is a heteroglycan that contains protein and acid polysaccharides as major components with a molecular weight of 120000 Da (Xia et al., 2007); a high molecular weight can ensure absorbability and bridging ability (Zhang et al., 2010).

Plate 2.6 and 2.7 depicts the surface structure of purified biopolymers. A larger area in surface structure will bind more particles. Biopolymers with a linear structure under scanning electron microscopy analyses can assure more adsorption points to be functional and bridge more particles to form flocs (Salehizadeh and Shojaosadati, 2001). There is indication that amorphous structures in biopolymers contribute to high flocculating activity (Wang et al., 2011) (Plate 2.8).

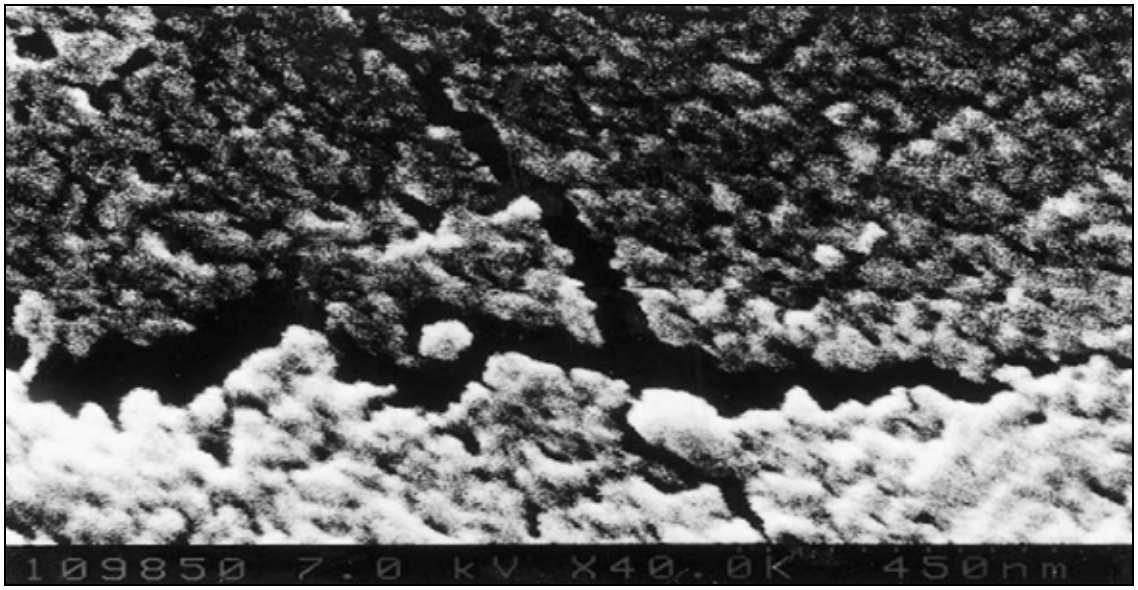

Plate 2.6 SEM microphotograph of the purified biopolymer EPS450 from *Bacillus* sp. I-450 showing the surface morphology. In the micrograph, a crack is also seen within the polymer surface, which was formed during scanning due to the high power of the electron beam(Kumar et al., 2004).

Plate 2.7 SEM micrograph image of the purified bioflocculant by *Proteus mirabilis* TJ-1 having a molecule linear in structure that allowed more particles bind to this bioflocculant (Xia et al., 2007).

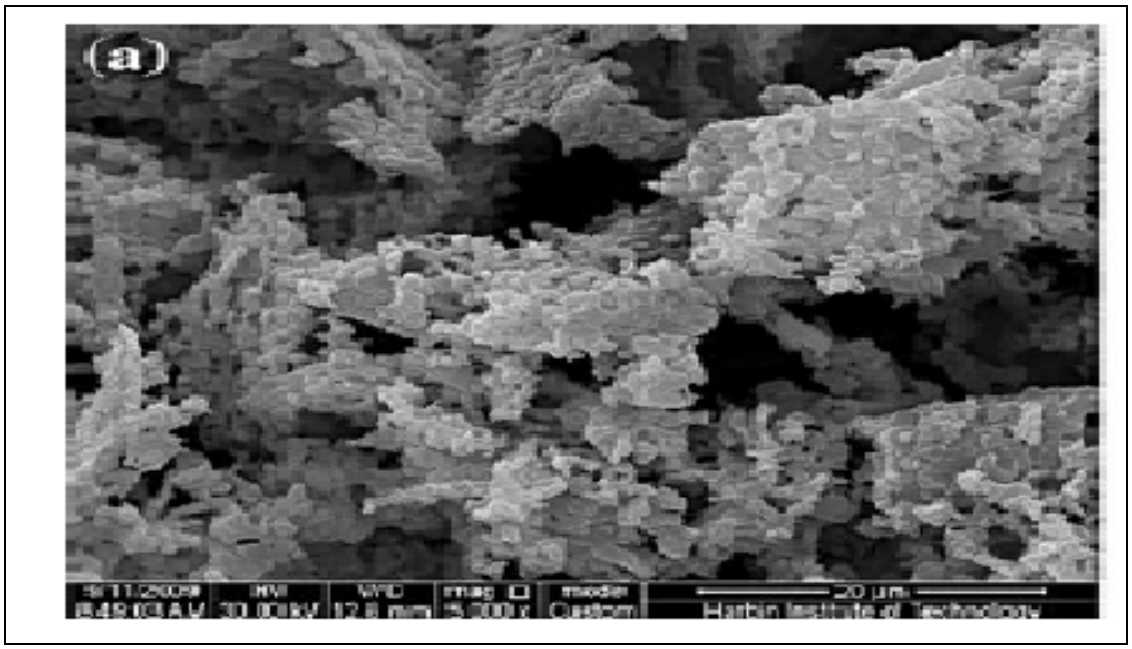

Plate 2.8 SEM micrograph image of purified CBF-F26 of mixed culture of *Rhizobium radiobacter* F2 and *Bacillus sphaeicus* F6 having amorphous structure was revealed high flocculating activity (Wang et., 2011).

Components and structures of biopolymers are complex, with different biopolymers produced by different bacteria having different properties (Deng et al., 2003). The structure and properties of biopolymers depend on the culture medium composition and growth conditions (Margaritis and Pace, 1985). Biopolymer producing bacteria have components that include peptidoglycan, lipopolysaccharides, capsules and exopolysaccharides, that prevent the bacterium from surviving harsh environments (Ullrich, 2009). If the major component of a biopolymer is a glycoprotein, the biopolymer is usually not thermally stable, as protein can be destroyed during heating processes; for example, the protein content in the NOC-1 bioflocculant, produced by *R.erythropolis,* decreased 50% after 30 minutes of heating at 100^{o}C (Takeda et al., 1991). The flocculating activity decreases after heating if the main backbone of a

biopolymer is a polysaccharide or protein (Lu et al., 2005, Salehizadeh and Shojaosadati, 2001) Salehizadeh and Shojaosadati, 2001). SF-1 showed a 15% decrease in flocculating activity after being heated to 100 ^{o}C (Gong et al., 2007).

Bioflocculants are essentially polymers produced by microorganisms during growth, with their flocculating activities being dependent on the characteristics of the flocculants. They are also known as extracellular polymeric substances (EPS), composed of organic substances such as carbohydrates, humic substances, proteins, amino acids, uronic acids, lipid compounds and deoxyribonucleic acids (Forster and Lewin, 1972; Liu and Fang, 2003; Pere et al., 1993; Urbain et al., 1993; Wawrznczyk et al., 2007).

The compositions of most bioflocculants are reported to be polysaccharides and protein (Deng *et al.*, 2005). *Gyrodinim impudicum* KG03, a bioflocculant secreted by marine dinoflagellate, is an acidic heteropolysachharide that contains galactose and uronic acid as main components (Yim *et al*., 2007). Molecular weight and functional groups in molecular chains are the important factors in flocculating activity of bioflocculants. Kurane *et al.* (1994*)* reported that for protein bioflocculants, the amino and carboxyl groups are the effective groups for flocculation. A polymer based on a C-C backbone tends to resist degradation (Zhang et al., 1993).

2.6 Application of biopolymer in wastewater treatment

Bioflocculation is a dynamic process resulting in the formation of stable aggregates or flocs due to the secretion of extracellular polymers by living cells, known as biopolymers (Gutcho, 1977; Dugan, 1987). Biopolymers synonymous to exopolysaccharides, extracellular polymers, exocellular polymers and exopolymers are expected to be useful in flocculation processes as a bioflocculants because they are safer than chemical flocculants, biodegradable, non-toxic and lack secondary pollution agents (Kumar et al., 2004; Desouky et al., 2008). Because of their biodegradability, harmlessness and lack of secondary pollution agents, biopolymers have gained much wider attention and research as of 1999 (Li, 1999). So they may potentially be applied in drinking and wastewater treatment, downstream processing and fermentation processes and industrial fields (Desouky et al., 2008). Bioproducts produced by bacteria are expected to be useful flocculating substances. Studies on flocculating substances from bacteria involve examination from various viewpoints, such as the coagulation of kaolin clay and the removal of microorganisms in the fermentation industry (Nakamura *et al*., 1976). Results from research on *Bacillus sp.* QUST2, *Bacillus sp.* QUST6 and *Bacillus sp.* QUST9 showed high flocculating activity in kaolin clay suspensions (Desouky et al., 2008).

CHAPTER 3.0

MATERIAL AND METHOD

3.1 Sampling of biopolymer producing bacteria

Three locations of wastewater sources were selected for bacteria isolation, namely, a drainage system located in the industrial Free Track Zone aree (FTZ), a drainage system located in Kampung Jawa, Bayan Baru, which comprises a mix of industrial and residential areas, and a drainage system at the Sungai Dua residential area, near USM. Wastewater samples were collected from each location using High Density Polyethylene (HDPE) (1L) sampling bottles, while sediment was collected using HDPE sampling bags. All samples were labeled and sent to the laboratory and immediately stored at 4ºC (APHA, 2000). A flowchart for the determination of biopolymer producing bacteria is shown in Figure 3.1.

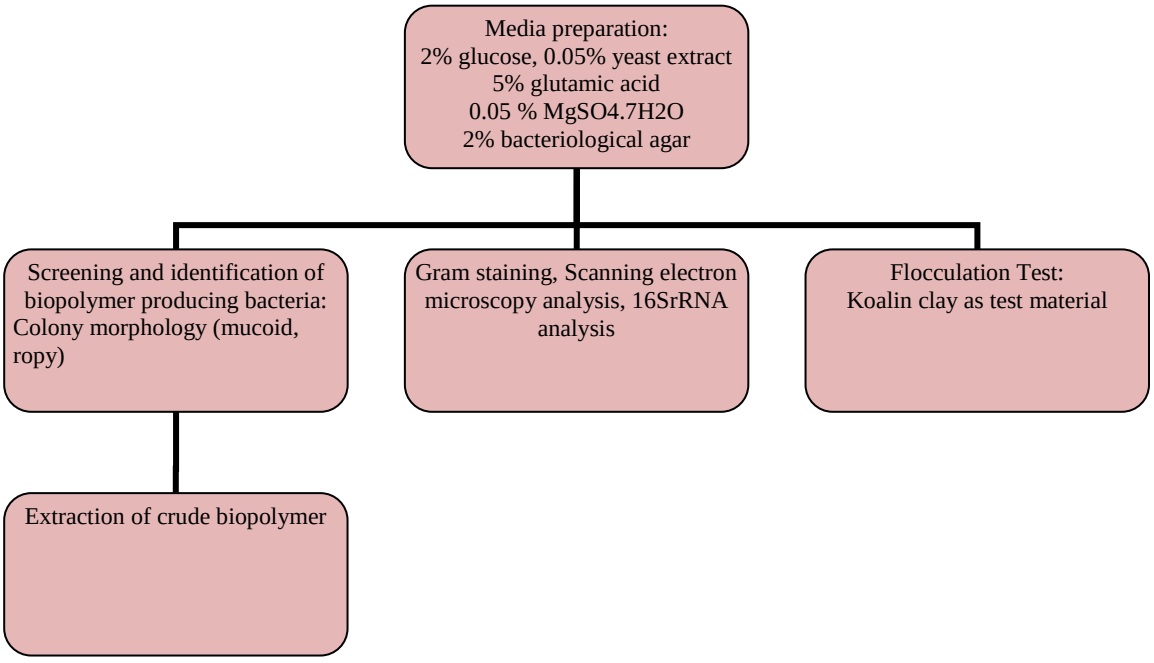

Figure 3.1 Flowchart of isolation, screening and identification of biopolymer producing bacteria (Yokoi et al., 1995; Lu et al., 2005).

3.2 Cultivation of bacteria on polyglutamic acid (PGA) media

The medium used to grow biopolymer producing bacteria was Polyglutamic Acid (PGA). The composition of the medium for screening these biopolymer producing bacteria is as follows: 20 g/L glucose, 0.5 g/L yeast extract, 50 g/L L-glutamic acid and 0.5 g/L magnesium sulphate heptahydrate ($MgSO_4.7H_2O$); the medium was adjusted to a pH of 7.0, measured using an HACH pH meter, while 15 g of bacteriological agar powder was added in 1.0 L deionized water to solidify the growth medium (Yokoi et al., 1995). After the culture broth reached the boiling point, 50 mL of boiled medium was poured into a 100 mL conical flask. The conical flask with the PGA medium was then covered with cotton and aluminum foil on the top open end before being sterilized using an autoclave machine. Unused media was kept in a refrigerated incubator at 4 ºC to avoid contamination.

Biopolymer producing bacteria were isolated from wastewater using specifically a solid PGA media. 1 mL of the wastewater sample was added to 9 mL of sterile distilled water and stirred vigorously with the appearance of a vortex for5 minutes. The suspension obtained was considered as a 1:9 dilution sample. The isolation was performed by using a ‘pour plate’ technique. After being cultured on PGA agar, the culture plates were incubated in an incubator oven at 40 ºC for a 2 day incubation period. After the incubation period, a mucoid and slimy colony appeared on the heterogeneous culture. A similar procedure as described above was performed in order to get a single colony of bacteria.