Screening for Polymer-Producing Bacteria from Seafood Activated Sludge and Bioflocculant Characterization

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ชื่อวิทยานิพนธ์ การคัดเลือกแบคทีเรียที่ผลิตโพลิเมอร์จากระบบแอคติเวตเต็ตสลัดจ์ของโรงงานแปรรูปอาหารทะเลและการศึกษาสมบัติของสารตกตะกอน

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บทคัดย่อ

การแยกเชื้อแบคทีเรียที่สามารถผลิตโพลิเมอร์จากสลัดจ์หมุนเวียนของระบบแอคติเวตเต็ตสลัดจ์ของโรงงานแปรรูปอาหารทะเลในพื้นที่จังหวัดสงขลา โดยใช้อาหารแยกเชื้อที่มีกลูโคสหรือซูโครส 1% เป็นแหล่งคาร์บอน พบว่าได้แบคทีเรียจำนวน 188 สายพันธุ์ เมื่อเลี้ยงแบคทีเรียเหล่านี้ในอาหารเหลวภายใต้สภาวะการเลี้ยงเชื้อแบบเขย่า พบว่าแบคทีเรีย 3 สายพันธุ์คือ WD 7, WD22 และ WD50 ให้น้ำหมักที่มีความหนืดและมีค่ากิจกรรมการตกตะกอนสารแขวนลอย kaolin ที่สูงในสภาวะที่มี $CaCl_2$ ทั้ง 3 สายพันธุ์เป็นแบคทีเรียแกรมลบ รูปท่อน และจำแนกเป็น *Enterobacter cloacae, Pseudomonas alcaligenes* และ *Enterobacter agglomerans* ตามลำดับ การศึกษาการผลิตโพลิเมอร์ภายใต้สภาวะการเลี้ยงเชื้อแบบเขย่าที่ความเร็วรอบ 200 รอบต่อนาที ณ อุณหภูมิห้อง เป็นเวลา 5 วัน โดยใช้อาหารเหลวซึ่งมีแหล่งคาร์บอนคือกลูโคส สำหรับ *E. cloacae* WD7, *P. alcaligenes* WD22 และซูโครสสำหรับ *E. agglomerans* WD50 พบว่า *E. cloacae* WD7 ให้น้ำหมักลักษณะหนืดที่มีค่ากิจกรรมการตกตะกอนสูงสุด (10.28) และให้ผลผลิตของโพลิเมอร์ 2.27 กรัม/ลิตร ที่ระยะเวลา 3 วัน การทำให้บริสุทธิ์บางส่วนของโพลิเมอร์ที่ผลิตจาก *E. cloacae* WD7 กระทำโดยการตกตะกอนด้วยเอทานอล 95% ไดอะไลซิส และทำแห้งแบบ freeze dry โพลิเมอร์ที่บริสุทธิ์บางส่วนมีสมบัติเป็นเฮทเทอโรโพลิแซคคาไรด์ชนิดเป็นกรด ประกอบด้วยน้ำตาลชนิดเป็นกลาง (29.4%) กรดยูโรนิค (14.18%) และน้ำตาลอะมิโน (amino sugar) (0.93 %) การวิเคราะห์หมู่ฟังก์ชันโดย FT-IR สเปกโตรสโกปี บ่งบอกถึงการมีหมู่ไฮดรอกซิล คาร์บอกซิล คาร์บอนิล และเมททอกซิล เป็นองค์ประกอบ การศึกษาสมบัติด้านความร้อน (thermal analysis) โดย DSC พบว่าโพลิแซคคาไรด์มีการเปลี่ยนแปลงโครงสร้างผลึกและ

อุณหภูมิหลอมตัวของผลึก (crystalline melting point ; T_m) เท่ากับ 300 องศาเซลเซียส โพลิแซคคาไรด์นี้ละลายในน้ำและไม่ละลายในตัวทำละลายอินทรีย์ที่ใช้ทดสอบ สารละลายโพลิแซคคาไรด์นี้มีสมบัติการเกิดเจลได้ภายใต้สภาวะที่เป็นด่าง เมื่อมีเกลือชนิดประจุ 2+ เช่น $CaCl_2$, $MgSO_4$ หรือ $CuSO_4$ โดย $CuSO_4$ ให้การเกิดเจลที่ดีที่สุด จากการศึกษาคุณสมบัติการตกตะกอนพบว่าโพลิแซคคาไรด์ชนิดนี้มีความเสถียรที่อุณหภูมิช่วง 4 - 60 องศาเซลเซียส และช่วงพีเอช 5 - 7 ความเข้มข้นที่เหมาะสมสำหรับกิจกรรมการตกตะกอน คือ โพลิแซคคาไรด์เข้มข้น 2 มิลลิกรัมต่อลิตร $CaCl_2$ เข้มข้น 40 มิลลิโมลาร์ นอกจากนี้โพลิแซคคาไรด์ยังสามารถตกตะกอนสารแขวนลอย kaolin ได้ในช่วงกว้างของค่าพีเอช (พีเอช 2 - 8) และอุณหภูมิ (4 -50 องศาเซลเซียส) ในสภาวะที่มี $CaCl_2$

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Abstract

A total of 188 polymer-producing bacterial strains were isolated from recycled sludge of five seafood processing plants in the Songkhla region using a screening medium containing 1% glucose or sucrose as carbon sources. Three (3) isolates designated as WD7, WD22 and WD50 were selected from shake-flask cultures based on their viscosity and high flocculating activity against kaolin suspension in the presence of $CaCl_2$. All three strains were rod shaped, Gram-negative bateria and identified as *Enterobacter cloacae*, *Pseudomonas alcaligenes* and *Enterobacter agglomerans*, respectively. The time course for polymer production using shake-flask cultures (200 rpm) at room temperature for 5 days using glucose for *E. cloacae* WD7 and *P. alcaligenes* WD22 and sucrose for *E. agglomerans* WD50 as carbon sources in basal medium revealed that *E. cloacae* WD7 possessed a viscous culture broth exhibiting the highest flocculating activity (10.28) and a crude polymer yield of 2.27 g/l after 3 days cultivation. Partial purification of the polymer produced from *E. cloacae* WD7 was performed by precipitation with 95% ethanol, dialysis and freeze-drying. This partially purified polymer was characterized as an acidic heteropolysaccharide, composed of neutral sugars (29.4%), uronic acids (14.18%) and amino sugars (0.93%). The functional group analysis by FT-IR spectroscopy showed the presence of hydroxyl, carboxyl, carbonyl and methoxyl groups. Thermal analysis by DSC showed the crystalline transition

and the crystalline melting point (T_m) at 300 °C. This polysaccharide was soluble in water and insoluble in any organic solvents tested. Gelation of this polysaccharide occurred under alkaline conditions in the presence of divalent cations such as $CaCl_2$, $MgSO_4$ or $CuSO_4$. The best gelation was obtained in the presence of $CuSO_4$. Studies on the flocculation property revealed that this polysaccharide was stable in the temperature range of 4-60 °C and pH 5-7. The optimal concentrations for the flocculating activity were 2 mg/l polysaccharide and 40 mM $CaCl_2$. Moreover, this polysaccharide could flocculate the kaolin suspension over a wide pH range (pH 2-8) and temperature (4-50 °C) tested in the presence of $CaCl_2$.

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List of Abbreviations

°C	=	degree Celsius
CFU/ml	=	colony forming unit per millilitre
Da	=	Dalton
g	=	gram
x *g*	=	gravitational force
h	=	hour
lb/in^2	=	pound per square inch
L	=	litre
mg/l	=	milligram per litre
min	=	minute
ml	=	millilitre
M	=	molar
MWCO	=	molecular weight cut off
nm	=	nanometre
OD	=	optical density
rpm	=	revolutions per minute
s	=	second
T_m	=	crystalline melting point
w/v	=	weight by volume
%	=	percent
µl	=	microlitre

Chapter 1

Introduction

Flocculants are useful in the aggregation of colloids, cells, cell debris etc. Various flocculants are widely used in industrial fields such as wastewater treatment, water treatment, dredging and downstream processes in fermentation industries. Flocculants are generally divided into three major groups ; (1) inorganic flocculants such as aluminium sulfate and polyaluminium chloride, (2)organic synthetic high polymers such as polyacrylic acid and polyacrylamide derivatives, (3) bioflocculants such as chitosan, guagum, sodium alginate, gelatin and microbial polymers (Kurane and Matsuyama, 1994). Although the organic and inorganic synthetic flocculants are cost-effective and strong agents, they are not easily degraded in nature. Some of them, especially polyacrylamides, contain acrylamide monomers which are both neurotoxic and strong human carcinogens (Yokoi, *et al.*, 1995). Moreover, the aluminium, a major component of polyaluminium chloride, also induces Alzheimer disease (Kurane and Matsuyama, 1994). On the other hand, the natural occurring flocculants which are mostly used in food industries are safe and biodegradable but they show weak flocculating activities (Takagi and Kadowaki, 1985 ; Kwon, *et al.*, 1996). Therefore, bioproducts produced by microorganisms are expected to be useful flocculating substances because they are safe for the environment and also they can be produced uniformly and reliably by fermentation. Studies on flocculating substances from microorganisms have been examined from various viewpoints, such as coagulation of kaolin clay suspension and removal of microorganisms in the fermentation industries (Kurane and Matsuyama, 1994 ; Kurane, *et al.*, 1986 ; Toeda and Kurane, 1991). Most microbial polymeric flocculants are polysaccharide flocculants

which are mostly produced by bacteria such as *Alcaligenes cupidus* KT-201 (Toeda and Kurane, 1991), *A. latus* B-16 (Kurane and Nohata, 1991), *Bacillus* sp. DP-152 (Suh, *et al.*, 1997) and *Enterobacter* sp. BY-29 (Yokoi, *et al.*, 1997). A few strains of actinomycetes, such as *Nocardia amarae* YK-1 (Takeda, *et al.*, 1992) and *Rhodococcus erythropolis* S-1 (Kurane, *et al.*, 1986 ; Takeda, *et al.*, 1991), which produced protein flocculants. The polyglutamate flocculants are predominantly produced by bacteria in the genus *Bacillus* (Yokoi, *et al.*, 1995, 1996 ; Tanaka, *et al.*, 1993).

In this paper, the screening and characteristics of a biopolymeric flocculant produced by a microbial strain isolated from activated sludge are reported.

Literature Review

1. Microbial polymers

1.1 Definition

Microbial polymers represent a group of polymers formed by microorganisms such as bacteria, actinomycetes and fungi (Table 1) by the polymerization reaction (condensation or dehydration) of repeating subunits (monomers). Depending on their biosynthesis, microbial polymers are classified as (Takagi and Kadowaki, 1985) :

a) Intracellular polymers, which are synthesized inside the cell and require cell extraction for their isolation and purification. Examples of these polymers are DNA, proteins, enzymes and celluloses ;

b) Extracellular polymers or exopolymers, which are synthesized inside the cells and excreted outside the cell wall in 2 different forms, either be attached to the cell wall (capsules) or released into the surrounding environment (soluble slime). Examples of these polymers are proteins, enzymes, glycoproteins, polyglutamates, polysaccharides and glycolipids.

1.2 Functional properties of microbial polymers

Microbial polymers are differently used according to their different functional properties. Microbial exopolymers have greater potential for industrial development than the intracellular polymers because most of them are water-soluble which can be recovered in large quantities from the culture broth. Microbial exopolymers, especially polysaccharides, are widely used as stabilizer, suspending agent, dispersant, thickener, film-forming agent, water-retention agent, lubricant or friction reducer, etc. in many industries such as detergent and laundry products, textiles, adhesives, paper, paint, food, pharmaceutical and cosmetic and others (Sandford, 1979). Although they are widely applied as several above functions, the function as "flocculant" was

Table 1 Polymeric flocculants produced by microorganisms

Microorganism	Polymer	Reference
Alcaligenes cupidus KT201	anionic polysaccharide	Toeda and Kurane (1991)
Alcaligenes latus B-16	anionic polysaccharide	Kurane and Nohata (1991)
Arcuadendron sp. TS-49	glycoprotein	Lee, *et al.* (1995)
Aspergillus sojae	glycoprotein	Nakamura, *et al.* (1976a,b)
Bacillus anthracis, *B. natto* , *B. licheniformis*, *B. subtilis*	polyglutamate	Tanaka, *et al.* (1993)
B. subtilis PY-90	polyglutamate	Yokoi, *et al.* (1995)
B. subtilis IFO 3335	polyglutamate	Yokoi, *et al.* (1996)
Bacillus sp.DP-152	anionic polysaccharide	Suh, *et al.* (1997)
Corynebacterium sp.	?	Zagic and Knettig (1971)*
Dematium sp.	?	Shinohara (1983)*
Enterobacter sp.BY-29	anionic polysaccharide	Yokoi, *et al.* (1997)
Nocardia amarae YK-1	protein	Takeda, *et al.* (1992)
Paecilomyces sp. I-1	cationic polysaccharide	Takagi and Kadowaki (1985)
Pestalotiopsis sp. KCTC 8637P	anionic polysaccharide	Kwon, *et al.* (1996)
Rhodococcus erythropolis S-1	protein	Kurane, *et al.* (1986), Takeda, *et al.* (1991)
R. erythropolis S-1	glycolipid	Kurane, , *et al.*, (1994)
mixed culture of *Oerskovia*, *Acinetobacter*, *Agrobacterium* and *Enterobacter*	anionic polysaccharide	Kurane and Matsuyama (1994)

Remark : ? not identified

* cited by Kurane and Matsuyama (1994)

also interesting and should be developed for wastewater treatment, water treatment, dredging, and downstream processes in fermentation industries (Kurane and Matsuyama, 1994) to replace the generally used chemically synthetic polymers which are not friendly to the environments.

1.3 Types and properties of microbial polymers

Microbial exopolymers have been widely studied and developed as flocculants. Types of these microbial polymers can be divided into 4 types according to the different compositions as follows :

1.3.1 Protein polymer

Protein polymer produced by *R. erythropolis* S-1 was insoluble in water, butanol, or acetone and it can be digested by Pronase E (protease). Elemental analysis showed the content of nitrogen was 11%. In an infrared spectrum, two significant absorption bands (1500-1550 and 1650-1700 cm^{-1}) were considered to correspond to mono-substituted amide.When this polymer was analyzed by filter paper electrophoresis, a single protein band was detected. However, many bands were detected on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Takeda, *et al.*, 1991). Moreover, this polymer solution was unstable to heat and the flocculating activity was reduced by about 50% after treatment at 100 °C for 15 min (Kurane, *et al.*, 1986).

Arcuadendron sp. TS-49 produced a glycoprotein polymer due to hexosamine, uronic acid, neutral sugar and a large portion of protein were contained in the flocculant. This polymer was stable to wide range of pH (pH 2-12) and maintained its stability up to 100 °C and thus seemed to be relative thermostable (Lee, *et al.*, 1995).

1.3.2 Polyglutamate polymer

Poly (γ-glutamic acid) or polyglutamate (PGA) is a poly(amino acid), a macromolecular peptide or naturally occurring polyamide (Cromwick, *et al.*, 1996), which is not protein and does not have a specific sequence. It was polymerized from a single amino acid with a molecular mass as widely polydispersed as that of a polysaccharide. The molecular structure of PGA is different from that of proteins. PGA contains glutamic acid residues connected between the α-amino group and the γ-carboxyl group (Kunioka, 1997) in which the γ-glutamyl bond functions in the linear elongation of glutamic acid repeating units (Tanaka,*et al.*,1993). PGA is water-soluble and biodegradable with a high relative molecular mass (Mr ; 100,000-1,000,000).

PGA has been isolated from many kinds of organisms (bacteria, cyanobacteria, and plant seeds) and natto (a traditional Japanese fermented food) (Yokoi, *et al.*, 1995). Several bacterial strains produced PGA outside the cells as a capsular material or an extracellular viscous material (Murao, 1969 ; Troy, 1975; Cheng, *et al.*, 1989 cited by Kunioka and Goto, 1994 ; Kunioka, 1995). Strains of *B. antracis* and *B. licheniformis* are encapsulated with PGA, which consists of the D-isomer of glutamic acid alone, but various strains of *B. subtilis* and *B. natto* are known to produce PGA as an extracellular viscous material which consists of both glutamic acid isomers in varying quantities such as PGA from *B. subtilis* F-2-01 consists of D- and L-glutamic acid in the ratio of 62:38 (Tanaka, *et al.*, 1993).

The biopolymer, produced by *B. subtilis* PY-90 in the liquid medium containing 5% L-glutamic acid as nitrogen source, was a homopolymer composed of glutamic acid residues and was presumed to be poly (γ-glutamic acid). It was soluble in water and gave high viscous solution. However, the polyglutamate solution was unstable to heat at 100 °C (more than 40 min) either under an air or argon atmosphere since no residual activity was detected

(Yokoi, *et al.*, 1995). The PGA produced by *B. subtilis* IFO 3335 was similar to that of strain PY-90 as it was composed of glutamic acid residues and its molecular weight was about 1.5×10^6 (Yokoi, *et al.*, 1996).

1.3.3 Glycolipid polymer

The polymer produced by *Rhodococcus erytropolis* S-1 was found to exist as huge assemblies in aqueous solution, the molecular mass (over one million daltons) composed of various polypeptides and lipids. The culture broth was isolated and purified by ultracentrifugation, extracting with 90% acetone, and two successive silica gel chromatographies. ^{1}H-NMR and HPLC studies showed that it was a kind of glycolipid that contained 2 fractions, non-polar fraction (long methylene chain) and polar fraction (glucose moiety). So it can be dissolved in non-polar organic solvent such as chloroform and also dissolved in water (Kurane, *et al.*, 1994).

1.3.4 Polysaccharide polymer

Polysaccharides are high-molecular weight carbohydrates in which monosaccharides (or their derivatives such as the uronic acids or amino sugars) have been glycosidically linked (Whistler, 1969).

Industrial usage of polysaccharides (gums) still relies extensively on materials obtained from plants such as starch or from marine algae such as alginate, etc. Although these substances are valuable commercial products, they have some disadvantages such as lack of an assured supply and variations in quality (Sutherland, 1996). Unlike traditional gums, microbial polysaccharides can be produced uniformly and reliably by fermentation. Thus, microbial polysaccharides are not subject to crop failure, climatic conditions, or to marine pollution, although it does require inexpensive substrates, high technology equipment, well trained staff and adequate power and water

supplies. The products are less subject to variability and output can be carefully controlled and accurately costed. At present, microbial polysaccharides are more interesting and favorable due to varying in unique properties which are applicable requirement for industries (Harada, 1980).

Microbial polysaccharides can be divided into several types according to the different criterias as follows:

1.3.4.1 Relation to cell structure or function in the cells

Microorganisms produce polysaccharides of three distinct types (McNeely and Kang, 1973 ; Margaritis and Pace, 1985) :

1.3.4.1.1 Intracellular (storage) polysaccharides

Intracellular polysaccharides may provide mechanisms for storing carbon or energy for the cell such as glycogen in *Enterobacteriaceae* and starch in *Clostridium*.

1.3.4.1.2 Structural polysaccharides

Structural polysaccharides are components of cell structures such as lipopolysaccharides and teichoic acids present as integral components of cell walls.

1.3.4.1.3 Extracellular polysaccharides

Extracellular polysaccharides or exopolysaccharides - found outside the microbial cell wall and membrane are common products of microbial cells. Exopolysaccharides are found in two different forms : (a) capsules as discrete physical structures, that are integral with the cell wall or attached to the microbial cells which synthesize them and (b) slimes that accumulate outside of the cell wall and diffuse constantly into the culture medium.

These extracellular polysaccharides contribute a gummy texture to bacterial colonies on a solid medium and an increased viscosity during growth in a liquid medium. The capsular components can be separated from the

amorphous loose slime by centrifugation. The slime formers may produce slime in large quantities and, in some cases, the viscosity becomes so great a liquid culture remains in place when the culture flask is inverted. The polysaccharides produced by slime formers have the greatest potential for industrial development because the gums can be recovered in large quantities from the culture fluids.

1.3.4.2. Mechanism of synthesis or chemical compositions

Microbial exopolysaccharides can be divided into two groups according to their chemical components (Sutherland, 1996 ; Sutherland and Ellwood, 1979 ; Margaritis and Pace, 1985).

1.3.4.2.1 Homopolysaccharides

Homopolysaccharides contain only one type of sugar moiety such as dextran and pullulan which are composed of glucose units only in their structures. Homopolysaccharides synthesis involves a single or simple enzyme system.

1.3.4.2.2 Heteropolysaccharides

Heteropolysaccharides are common microbial exopolysaccharides which composed of two or more sugar moieties such as neutral sugars and, commonly, uronic acids. Some may contain amino sugars in place of uronic acids or along with them. In addition, many contain acetyl groups or, more rarely, other acyl groups such as formate and succinate. Heteropolysaccharides are usually produced by microorganisms from any utilizable carbon source and complex enzyme systems are usually involved.

1.3.4.3 The electrical charge property

Polysaccharides may be classified as naturally neutral, anionic or cationic based on their charge properties (Margaritis and Pace, 1985).

1.3.4.3.1 Neutral polysaccharides

Neutral polysaccharides are composed of only neutral sugars and without any uronic acids or other ionizable groups. Examples of these are levan, pullulan, dextran, scleroglucan which composed of only glucose repeating units.

1.3.4.3.2 Anionic polysaccharides

Anionic polysaccharides, sometimes called acidic polysaccharides, contain neutral sugars and acidic groups such as carboxyl, phosphate or sulfate in the structures of polysaccharides. The example is xanthan which composed of glucose, mannose, glucuronic acid, acetate and pyruvate.

1.3.4.3.3 Cationic polysaccharides

Cationic polysaccharides, sometimes called basic polysaccharides, are rarely found in nature such as chitosan (polyglucosamine), which are most abundant in crustaceans, insects and fungi. Commercial quantities are present in the shells of lobster, crabs and shrimp (Whistler, 1969). It was the component of fungal cell wall in the order Mucorales such as *Mucor rouxii, Absidia coerulea.* The cationic charge property come from free amino group ($-NH_2$) in its structure (Sandford, 1979).

Application of polysaccharides as flocculants is relatively small, about 7% (Sandford, 1979). However, nowadays the environmental problems have been widespread and are more interesting. So microbial polysaccharides have been studied and developed to apply as flocculant for waste and wastewater treatment. Several microbial strains could produce the polysaccharides with capable of flocculation suspended solid, the chemical components of these polysaccharides were shown in Table 2.

Table 2 The chemical components of some microbial polysaccharide flocculants

Microorganism	Component	Reference
Alcaligenes cupidus KT201	glucose, galactose, glucuronic acid, acetic acid	Toeda and Kurane (1991)
Bacillus sp.DP-152	glucose, mannose, galactose, fucose, acetic acid, pyruvic acid, uronic acid	Suh, *et al.* (1997)
Enterobacter sp.BY-29	glucose, galactose, xylose, galacturonic acid	Yokoi, *et al.* (1997)
Paecilomyces sp. I-1	galactosamine, acetyl, formyl	Takagi and Kadowaki (1985)
Pestalotiopsis sp. KCTC 8637P	glucose, glucosamine, glucuronic acid, rhamnose	Kwon, *et al.* (1996)
mixed culture of *Oerskovia*, *Acinetobacter*, *Agrobacterium* and *Enterobacter*	glucose, galactose, succinic acid, pyruvic acid	Kurane and Matsuyama (1994)

Toeda and Kurane (1991) cultivated *Alcaligenes cupidus* KT 201 in liquid medium containing 2% sucrose and 0.01% $(NH_4)_2SO_4$ as sole carbon and nitrogen sources, respectively. The polymer (Al-201) was found to aggregate a kaolin suspension without cations, although its flocculating activity was significantly enhanced by the addition of bivalent/trivalent cations such as Ca^{2+} and Fe^{3+}. Al-201 was purified by precipitation with ethanol and cetylpyridinium chloride, and had a gel filtration chromatography molecular weight of over $2x10^6$ Da. This polymeric flocculant was an acidic

polysaccharide which composed of glucose, galactose, and glucuronic acid (molar ratio of 6.34 : 5.55 : 1), with this polymer occurring in 10.3% acetic acid as *n*-acetyl ester group without pyruvic or succinic acid. The total sugars was 72.25% and uronic acid was 21.90%. The elements of this polymer were carbon (36.94%), hydrogen (5.42%) and trace amount of nitrogen, phosphorous and sulfur. The infrared (IR) spectrum of this polymer appeared the absorption peaks at 1720 cm^{-1} and 1605 cm^{-1} indicating the presence of a carboxy ester and carboxylic acid, respectively.

Kurane and Matsuyama (1994) cultivated the R-3 mixed strains which were comprised of four strains belonging to the genera *Oerskovia*, *Acinetobacter*, *Agrobacterium* and *Enterobacter*. These mixed strains efficiently produced a bioflocculant (APR-3) in liquid culture of production medium, especially that containing starch and glucose (1:1) as carbon sources. This polymer was purified electrophoretically to homogeneity by ethanol and cetylpyridinium chloride precipitation. Its molecular mass is at least $2x10^6$ Da. APR-3 is an acidic polysaccharide made of glucose, galactose, succinic acid, and pyruvic acid (molar ratio of 5.6 : 1: 0.6 : 2.5). The total sugar was 64.7%, the proportion of carbon and hydrogen of polymer were 38-42% and 5-7%, respectively. In addition, APR-3 also contained traces of nitrogen, sulfur and phosphorous. The IR-spectrum of APR-3 showed the absorption at 2700-3700 cm^{-1} represents a hydroxyl group, that at 1730 and 1160 cm^{-1} represents carboxyester bonds, and that at 1600 and 1400 cm^{-1} represents ionized carboxyl groups. APR-3 was soluble in water, highly soluble in alkali and insoluble in methanol and acetone. Moreover, it demonstrated pseudoplastic flow property in the same manner as xanthan gum and it had higher viscosity than xanthan gum.

2. Process development for the production of microbial polymers

2.1 Screening for polymeric flocculant-producing microorganisms

Several researchers reported the methods for screening the polymeric flocculant-producing microorganisms from several sources such as soil, activated sludge, wastewater and the culture collection. Mostly, based on the capability of culture broth which contained the exopolymers for flocculation of kaolin clay suspension and removal of microorganisms in the fermentation industry as follows :

Takagi and Kadowaki (1985) screened for flocculant-producing fungi from 25 strains of fungi from culture collection and 50 strains of fungi isolated from soil. All strains were cultivated in glucose yeast extract (GYe) medium containing 2% glucose and 0.3% yeast extract. The culture broths were tested for the ability to flocculate *E. coli* cells suspension. The strain I-1 was selected and identified as *Paecilomyces* sp.

Kurane, *et al.* (1986) screened for flocculant-producing bacteria by cultivation of thirty strains of phthalate ester assimilating bacteria (isolated from Japanese soils) in nutrient broth and complete defined medium containing phthalate ester as a sole carbon source for 7 days at 30 °C. The culture broths were tested for the ability to flocculate kaolin suspension. Flocculation was found in *Rhodococcus erythropolis, Nocardia resticta*, *Nocardia calcarea*, *Nocardia rhodnii*, and *Corynebacterium* sp. The microorganisms which had the strongest flocculating activity among those tested were *Rhodococcus erythropolis* S-1 and 260-2. Moreover, the nutrient broth appeared more favorable than the complete defined medium such as phathalate medium.

Kurane and Nohata (1991) screened for flocculant-producing bacteria from about one hundred cultures in the laboratory using basal medium (pH 6) containing 1% fructose, 0.05% urea, 0.05% yeast extract, 0.04% K_2HPO_4, 0.44% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$, and 0.01% NaCl. The culture broth of

strain B-16 possessed the highest flocculating activity against kaolin suspension. This strain was identified as *Alcaligenes latus*.

Toeda and Kurane (1991) isolated the flocculant-producing microorganisms from soil by using screening medium which consisted of 1% starch and 0.1% $(NH_4)_2SO_4$ as carbon and nitrogen sources. The mucoid colonies from agar plates were then suspended in water. The cell-free mucous solution was tested to flocculate the kaolin and cerite suspensions. Fifty six strains isolated from 53 soil samples have the ability to flocculate kaolin but either no or weak ability to flocculate cerite. The strain KT201 possessed the highest flocculating activity for kaolin only and it was identified as *Alcaligenes cupidus*.

Kurane and Matsuyama (1994) screened the flocculant-producing microorganisms from activated sludge by agar plates containing phthalic acid as sole carbon source. The slime forming colonies among phthalic acid assimilating microbes were cultivated in the production medium containing 0.5% starch and 0.5% glucose as carbon sources. Among thirty-one colonies, a group of mixed microbes (R-3 mixed strains) had the highest flocculating activity. R-3 mixed strains were composed of 4 strains belonging to the genera *Oerskovia*, *Acinetobacter*, *Agrobacterium* and *Enterobacter*.

Lee, *et al.* (1995) isolated the flocculant-producing microorganisms from the soil samples collected from many places in Korea by using a kaolin suspension as a flocculation test. One fungal isolate among several strains which had the highest flocculating activity was identified as *Arcuadendron* sp. TS-49.

Yokoi, *et al.* (1995) isolated the flocculant-producing microorganisms from soil using a basal medium (pH 7) consisting of 2% glucose, 1% polypeptone, 0.05% yeast extract, 0.2% K_2HPO_4 and 0.05% $MgSO_4 \cdot 7H_2O$. The mucoid colonies were cultivated in liquid basal medium, strain PY-90

possessed high flocculating activity on kaolin suspension and considered to belong to *Bacillus subtilis*.

Kwon, *et al.* (1996) isolated the flocculant-producing microorganisms from soil or rotted leaves samples by agar plates containing potato dextrose agar supplemented with 2% glucose. One isolate among several selected fungal strains had the highest flocculating activity for kaolin suspension. This strain was identified as *Pestalotiopsis* sp. KCTC 8637P.

Suh, *et al.* (1997) isolated flocculant-producing bacteria from many kinds of soil samples using screening medium (pH 7) containing 4% glucose and 0.1% NH_4NO_3 as carbon and nitrogen sources. More than 200 bacterial strains which excreted mucous material on plates were selected. The culture broth of each isolate was tested for its ability to flocculate kaolin clay, activated carbon and industrial wastewaters. Strain DP-152 showed the highest flocculating activity and considered to belong to *Bacillus* sp.

2.2 Optimization for the production of microbial polymers

Processes for the production of most microbial polymers are characterized by the extreme rheology of the fermentation broth, the low product concentration at which this occurs and the diversity of subtle structure and conformational changes which can occur throughout the entire process. The successful design of the fermentation of general microbial product including microbial polymer relies on the environmental control and equipment design and operation as shown in Fig. 1 (Margaritis and Pace, 1985).

Most microbial polymers which have been reported, were exopolymers excreted into the liquid medium. The properties and yields of these exopolymers are affected by the above environmental control such as fermentation medium and other environmental parameters- pH, temperature, etc.; and the equipment design and operation as following :

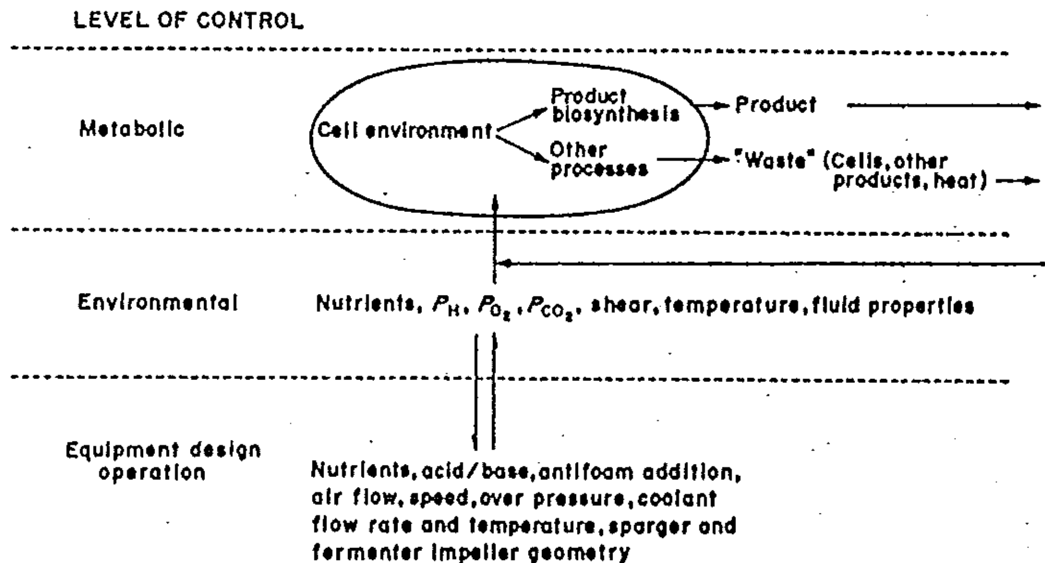

Fig. 1 Level of control governing microbial processes

Source : Margaritis and Pace (1985)

2.2.1 Fermentation medium

The growth media which are suited to the production of different microbial polymers by microorganisms vary widely (Table 3) and this probably reflects different role of each exopolymer in nature. Although it is difficult to draw general conclusions, it is instructive to consider the medium components affecting to polymer biosynthesis rates, yields and compositions.

The composition of the growth medium can also indirectly affect polymer yield, for example by governing the pH changes which can occur during fermentation without pH control. In addition, high media concentrations resulting in high polymer concentrations, may lead to oxygen limitations or heterogeneity due to increased viscosity within the fermentor before exhaustion of the carbon source, and this might indirectly affect yield as well as the overall

Table 3 The medium components and cultural conditions for microbial flocculants production

Microorganism	Medium component / cultural condition	Reference
Anionic polysaccharide		
Alcaligenes cupidus KT201	2%sucrose, 0.02% yeast extract, 0.01% $(NH_4)_2SO_4$, 0.16% KH_2PO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.01% NaCl, 0.002% $CaCl_2 \cdot 2H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$ / 30 °C, 6-8 days, 1 vvm, 300 rpm	Toeda and Kurane (1991)
Bacillus sp. DP-152	4% glucose, 0.1% NH_4NO_3, 0.01% yeast extract, 0.01% soytone, 0.01% tryptone, 0.03% K_2HPO_4, 0.03% KH_2PO_4, 0.01% $MgSO_4 \cdot 7H_2O$, 0.01% $MnSO_4 \cdot 4H_2O$, 0.005% NaCl, 0.04% $CaCO_3$ / 30 °C	Suh, *et al.* (1997)
Enterobacter sp. BY-29	2% glucose, 1% polypeptone, 0.05% yeast extract, 0.2% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$ / 30 °C, 2 days	Yokoi, *et al.* (1997)
Pestalotiopsis sp. KCTC 8637	potato dextrose broth (PDB) containing 2% glucose / 25 °C, 5 days, 2-3 vvm, 100-300 rpm	Kwon, *et al.* (1996)
mixed culture of *Oerskovia*, *Acinetobacter*, *Agrobacterium* and *Enterobacter*	1% starch, 0.05% yeast extract, 0.05% $(NH_4)_2SO_4$, 0.5% K_2HPO_4, 0.2% KH_2PO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.01% NaCl / 30 °C, 7 days, 1 vvm, 200-400 rpm	Kurane and Matsuyama (1994)

Table 3 (continue)

Microorganism	Medium component / cultural condition	Reference
Cationic polysaccharide		
Paecilomyces sp. I-1	2% starch, 0.3% polypeptone, 0.5% $CaCl_2$ / 25 °C, 5 days	Takagi and Kadowaki (1985)
Protein		
Nocardia amarae YK-1	1% glucose, 0.4% yeast extract, 0.5% $(NH_4)_2SO_4$, 0.6% Na_2HPO_4 / 30 °C, 7-14 days	Takeda, *et al.* (1992)
R. erythropolis S-1	1% glucose, 0.05% urea, 0.05% yeast extract, 0.5% K_2HPO_4, 0.2% KH_2PO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.01% NaCl (pH 9.5) / 30 °C, 0.01-0.5 vvm, 100-200 rpm	Kurane, *et al.* (1986)
Glycoprotein		
Arcuadendron sp. TS-49	3% glucose, 0.2%yeast extract, 0.1% NH_4Cl, 0.01% $MgSO_4 \cdot 7H_2O$, 0.05% $MnSO_4 \cdot 4H_2O$ / 30 °C, 4-5 days	Lee, *et al.* (1995)
Aspergillus sojae	2% casein, 0.1% KH_2PO_4, 0.1% $MgSO_4 \cdot 7H_2O$ (pH 6) / 30 °C, 3 days, 0.25 vvm, 300 rpm	Nakamura, *et al.* (1976b)
Glycolipid		
R. erythropolis S-1	1% glucose, 0.05% urea, 0.05% yeast extract, 0.5% K_2HPO_4, 0.2% KH_2PO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.01% NaCl (pH 9.5) / 30 °C, 0.01-0.5 vvm, 100-200 rpm	Kurane, *et al.* (1986)
Polyglutamate		
Bacillus subtilis PY-90	2% glucose, 0.05% yeast extract, 5% L-glutamic acid, 0.2% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$ (pH 7) / 30 °C, 2 days	Yokoi, *et al.* (1995)

production rate. Moreover, changing the composition of growth medium is possible to change the degree of substitution of the repeating unit by various groups and the degree of polymerization (molecular weight).

2.2.1.1 Carbon source

The amount of carbon (substrate) converted to the polymer by the cells depends on the composition of the growth medium and, under certain conditions, the product may not be made at all. Generally, media containing a high carbon to limiting nutrient ratio, often nitrogen, are favoured component to induce growth limitation and stimulate exopolysaccharide formation (Sutherland, 1977 ; 1996). These have now been shown to favour polymer production in the Enterobacteriaceae, in *Xanthomonas campestris*, *Pseudomonas* sp. and many other bacteria as well as in many fungi.

Ordinarily, the conversion efficiency of a carbon source into polysaccharide is inversely related to the carbon substrate concentration. For example, it has been reported that the conversion efficiency of D-glucose to polymer by *Xanthomonas campestris* decreased markedly with an increase in D-glucose concentration (McNeely and Kang, 1973).

Various carbon sources were used for production of microbial polymer such as carbohydrates, vegetable oils, alcohols and hydrocarbons which differently affect growth and polymer yield. In fermentation processes, glucose is the most frequently used monosaccharides; the most often used oligosaccharides are sucrose and lactose, polysaccharide with wide application is starch (Sikyta, 1983). Glucose at concentrations of 2-5% (w/v) is usually the preferred carbon substrate as it is utilised by a vary wide range of microbial species (Sutherland, 1996).

Kurane, *et al.* (1986) studied the effect of various carbon sources on protein flocculant production by *Rhodococcus erythropolis* S-1. Glucose,

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fructose and sorbitol provided higher cell growth and polymer production than those of mannose, galactose, arabinose, xylose, lactose, maltose, cellobiose and sucrose. Moreover the water-insoluble carbon sources such as olive oil would provide the cell growth and polymer production better than those of glucose and fructose but gave the very low flocculating activity, and *n*-hexadecane would exhibit the cell growth less than fructose and olive oil but gave the flocculating activity higher than that of olive oil.

Takeda, *et al.* (1991) cultivated *R. erythropolis* S-1 in liquid medium containing 1% *n*-pentadecane, the cells would assemble as fibrous floc floated at the top layer of the culture broth, however the produced-polymer was attached to the cell surface. The cell dry weight and polymer yield were 0.38-0.44 g and 5 mg, respectively in 100 ml of culture broth. When 1% glucose was used as carbon source, the cell and polymer would disperse in the culture broth. The cell dry weight and polymer were 0.31-0.37 g and 20-50 mg, respectively in 100 ml culture broth.

Kurane, *et al.* (1994) produced protein flocculant from *R. erythropolis* S-1 in liquid medium (pH 8) with various carbon sources (1%) such as sugars, sugar alcohols, starch, organic acids and alcohols. Consequently, sorbitol, mannitol and ethanol were effective carbon sources for cell growth and polymer production as well as glucose and fructose. Although sucrose was the most effective carbon source for cell growth, the flocculating activity did not increase. Organic acids such as acetic acid and citric acid were not good for polymer production. Among several types of alcohol such as methanol, propanol, ethylene glycol and glycerol ; ethanol had the highest potential for polymer production.

Toeda and Kurane (1991) cultivated *Alcaligenes cupidus* KT201 in liquid medium containing 1% starch and 0.1% $(NH_4)_2SO_4$ as carbon and nitrogen sources. Under this condition low polymer was produced and this

would be reversed by decreasing the concentration of $(NH_4)_2SO_4$ to be 0.01%. Among various carbon sources (2%); glucose, galactose and sucrose resulted in better flocculating activity than either starch or maltose for polymer production. Therefore, the favourable production medium was 0.01% $(NH_4)_2SO_4$ and 2% sucrose as carbon and nitrogen sources.

Nohata and Kurane (1994) studied the production of polysaccharide flocculant from *Alcaligenes latus* B-16. The suitable carbon source (1%) for polymer production would be glucose and sucrose. Moreover, xylose, fructose, rhamnose and cellobiose also exhibited the high viscous culture broth, this indicated that mono- and di- saccharide were suitable for polysaccharide production.

In large-scale fermentation, the carbon substrates will almost certainly be a complex product, most probably obtained as by-products from the processing of agricultural or other plant materials (Sutherland, 1996).

2.2.1.2 Nitrogen source

Inorganic and organic substances containing nitrogen in various forms may serve as source of nitrogen for microbial growth. Inorganic nitrogen such as $(NH_4)_2SO_4$ and NH_4Cl and organic nitrogen such as yeast extract, peptone or amino acids are most often used in growth medium. Although many microorganisms assimilate inorganic nitrogen, they often grow faster in the presence of an organic nitrogen source (Sikyta, 1983). Although a nitrogen source is necessary for both cell growth and product formation, an excess of nitrogen, reduces conversion of the carbohydrate substrate to extracellular polysaccharide (McNeely and Kang, 1973).

Kurane, *et al.*(1986) studied the effect of addition of organic nitrogen (0.05%) and inorganic nitrogen (0.05%) on protein flocculant produced by *R. erythropolis* S-1. Urea and yeast extract were most suitable inorganic and

organic nitrogen, respectively. However, NH_4Cl and NH_4NO_3 could stimulate the cell growth, and gave the culture broth which possessed the flocculating activity about 60-70% of that from urea.

Toeda and Kurane (1991) reported that 0.01% $(NH_4)_2SO_4$ and 0.02% yeast extract were suitable nitrogen sources for polysaccharide production by *Alcaligenes cupidus* KT201. Cell growth and polysaccharide production increased rapidly within 3 days and the growth still increased continuously, consequently, the viscosity of culture broth increased but the flocculating activity was constant.

Nohata and Kurane (1994) studied the effect of nitrogen sources on polysaccharide production by *Alcaligenes latus* B-16. Among tested organic nitrogen such as yeast extract, meat extract and malt extract, yeast extract would be most effective and most suitable concentration was 0.5% from the range of 0.005%-1%. Amongst inorganic nitrogen ; urea, potassium nitrate were effective whereas NH_4Cl, $NH_4(SO_4)_2$ and NH_4NO_3 were unsuitable.

Kubota, *et al.* (1993) studied the effect of addition of amino acids on polyglutamic acid (PGA) production by *Bacillus subtilis* F-2-01 in liquid medium containing 2% veal infusion broth and 0.1% glucose. L-glutamic acid (1%) gave the PGA yield of 6.3 g/l which was higher than that of other amino acids (in L-form at concentration of 7%) such as aspartic acid, alanine, leucine, phenylalanine and histidine. Addition of L-glutamic acid at various concentrations of 1, 3, 5, 7, 10 and 15%, it revealed that at 7% would give the highest PGA yield of 48 g/l which was higher than that of D-glutamic acid (7%) which was 15.9 g/l.

Yokoi, *et al.,* (1995) produced polyglutamate from *B. subtilis* PY-90. The liquid medium containing L-glutamic acid was more favourable than polypeptone and L-glutamic acid (2-5%) would result in high viscous culture broth.

2.2.1.3 Phosphate and trace elements

Phosphate containing in liquid medium was inorganic phosphate dissociated from K_2HPO_4 or KH_2PO_4.The optimal concentrations of phosphate were in the range of 0.3-0.6% for polysaccharide production from *Alcaligenes latus* B-16. The excess amount of phosphate would be residued in dried polysaccharide, consequently decrease its water adsorption (Nohata and Kurane, 1994). Moreover, the high concentrations of phosphate would suppress production of polysaccharide, this may be due to the increase of buffering capacity would prevent change to favourable pH for microbial polysaccharide synthesis (Badr-Eldin, *et al.*, 1994). And the concentration of phosphate in the polymer would be expected to markedly affect its rhelogical properties, through polyelectrolytic effects on the polymer's conformation (Margaritis and Pace, 1985).

Microorganisms contain calcium, potassium, sulfur and sodium, and these elements should therefore be added into the medium (Sikyta, 1983). The trace elements added in the liquid medium were favourable in inorganic forms such as NaCl and $MgSO_4$. Sometimes, the elements added may suppress the formation of products (McNeely and Kang, 1973). So the requirement of elements for biopolymer production was varied according to each species such as 0.02% $MgSO_4$ and 0.01% NaCl for *Alcaligenes latus* B-16 (Kurane and Nohata, 1991) ; 0.02% $MgSO_4$, 0.002% $CaCl_2$, 0.01% NaCl and 0.001% $FeSO_4$ for *A. cupidus* KT201 (Toeda and Kurane, 1991).

2.2.2. Environmental parameters

Although the culture medium described in the previous section affect growth and production of exopolymer by microorganisms, the influence of the fermentation variables such as temperature, pH, dissolved oxygen, carbon dioxide, shear, etc. appear to have significant roles (Margaritis and Pace, 1985).

2.2.2.1 Temperature

Temperature is particularly important parameter affecting the conversion efficiency of substrate into cell mass where the substrate is the carbon/energy source. The optimal temperature for growth may not be that best suited to product formation, especially where the product is predominantly non-growth associated as in the case of many secondary metabolites (Forage, *et al.*, 1985). Production of most polysaccharides are performed at or near 30 °C, although cultivation at suboptimal temperatures frequently favours polysaccharide production (Sutherland, 1996). In a steady state of continuous culture under nitrogen limited condition for exopolysaccharide production by *Pseudomonas* sp. NCIB 11264 showed that the cell concentration remained constant between 20 to 37.5 °C, whereas the conversion efficiency of glucose to polymer and culture broth viscosity varied sharply with temperature showing a maximum at 30 °C. Interestingly, the polymer composition appeared unaffected by the fermentation temperature (Williams and Wimpenny, 1978 cited by Margaritis and Pace, 1985).

Kurane, *et al.* (1986) produced protein flocculant by *R. erythropolis* S-1, the temperature at 30 °C was suitable for growth and polymer production. The rate of polymer production at 30 °C was 2 folds higher than those at 25 and 37 °C.

2.2.2.2 pH

pH control is likely to be needed for most production processes as the polysaccharide of commercial interest are generally acidic (Pace and Righelato, 1980). Lack of pH control and poor medium buffering results in a rapid fall in the polysaccharide formation with a cessation of growth and production of polymer. An optimal pH of 7 was reported for the production of an acidic exopolysaccharide by *Xanthomonas campestris*, *Pseudomonas* sp. NCIB 11264

and other microorganisms (Margaritis and Pace, 1985). Alteration of the medium pH (pH 5.5-8.25) for production of polyglutamate by *Bacillus licheniformis* had little or no significant effects on the product quality as measured by stereochemical composition and molecular weight (Cromwick, *et al.*, 1996).

2.2.2.3 Dissolved oxygen

Oxygen is usually required in both the polymer synthesis or indirectly in the oxidation of reduced pyridine nucleotides generated (Margaritis and Pace, 1985). So during polysaccharide fermentation by microorganisms, a major problem is a rapidly increasing media viscosity caused by an increasing heteropolysaccharide concentration. The increasing culture broth viscosity in turn caused a markedly decreased oxygen transfer rate between the air bubbles and the culture fluid (McNeely and Kang, 1973). A similar phenomenon was reported for polyglutamate, where it was shown that its volumetric oxygen mass transfer could be improved by increasing the available oxygen through increased agitation rate and /or oxygen partial pressure in the air pumped through the fermentation vessel (Cromwick, *et al.*, 1996).

2.3 Recovery and purification of microbial polymers

2.3.1 Polysaccharide polymer

Production of microbial polysaccharide resulted in the increase of viscosity during fermentation. Centrifugation was performed to remove the cells from the culture broth. Then the polysaccharide was precipitated from cell-free supernatant by organic solvents such as ethanol, acetone, methanol or isopropyl alcohol. Sometimes, the viscous culture broth may be pasteurized at the temperature near the boiling point of water to kill the viable cells. Then the

polysaccharide was precipitated afterwards, the obtained polysaccharide was dried and milled (Baird and Pettitt, 1991 ; Smith and Pace, 1982).

The viscous culture broth of *Alcaligenes cupidus* KT201 was diluted with water followed by cell removal by centrifugation. Finally, the polysaccharide was precipitated from supernatant by ethanol and cetylpyridinium chloride (Toeda and Kurane, 1991). In contrast, polysaccharide produced by *Alcaligenes latus* B-16 was excreted and coated as thin layer on cell surface and was insoluble in the culture broth, resulting in high viscosity of culture broth. It was difficult to isolate the polysaccharide by normal techniques such as centrifugation or filtration. So the combination of diluting the culture broth with 0.02% NaOH and heating at 121 °C for 10 min (at 1 atm) was applied to dissolve the polysaccharide from cell surface. Then the cells were removed and polysaccharide was precipitated with ethanol (Nohata and Kurane, 1994). Recovery by this method needed to take the larger volume of ethanol and longer time, so the applicable, easy and low cost method by fractional precipitation with 2-propanol at concentration of 33% and 66% was used to dissolve polysaccharide from cell surface and precipitation of polysaccharide, respectively. Finally, the repeated precipitation for desalting was done to obtain the purified polysaccharide (Kurane and Mita, 1996).

2.3.2 Polyglutamate polymer

After cultivation of *B. subtilis* PY-90 in liquid medium containing L-glutamic acid as nitrogen source, the viscous culture broth was diluted with water. After cells removal, the supernatant was then precipitated by cold ethanol to get the crude polyglutamate. The polyglutamate precipitate was redissolved in distilled water and repeated precipitation with ethanol was done. Finally, dialysis and freeze-drying were performed to obtain partially purified polyglutamate (Yokoi, *et al.*, 1995).

2.3.3 Glycolipid polymer

Biopolymer produced by *R. erythropolis* S-1 exists as huge assemblies in aqueous solution and composed of various polypeptides and lipids which can be removed by ultrafiltration or ultracentrifugation. Afterthat, the lipid component was isolated by extracting with acetone (90%), followed by centrifugation at 12000 x *g* for 15 min. The acetone-soluble component was passed through silica gel chromatography 2 times to obtain the purified glycolipid (Kurane, *et al.*, 1994).

2.3.4 Protein polymer

R. erythropolis S-1 produced the polymer which composed of protein and formed micelles in culture broth. Cell growth and polymer production were different when glucose and *n*-pentadecane were used. When S-1 was cultivated in medium containing *n*-pentadecane, cells formed fibrous flocs that floated at the surface and the polymer was attached to cell surface. In contrast, when glucose was used, the cells and polymer were dispersed into culture broth. Consequently, the method for recovery the polymer were different as follows:

2.3.4.1 Glucose as carbon source

Ammonium sulfate and butanol were added into culture broth and mixed vigorously. The mixture was centrifuged and a thin biopolymer layer was formed at the water and butanol interface. This biopolymer polymer was separated and washed with acetone, followed by resuspension in distilled water and dialysis to obtain the dialysate containing polymer (Takeda, *et al.*, 1991).

2.3.4.2 *n*-Pentadecane as carbon source

The cells were separated from culture broth by centrifugation, then washed with the pyridine solution (50% v/v) to break the cells completely. The

washed solution was added with acetic acid to neutralize, followed by the addition of butanol and saturated $(NH_4)_2SO_4$. After centrifugation the mixture, the thin layer of polymer at the boundary phase was present. This layer was separated and performed as described above (2.3.4.1) (Takeda, *et al.*, 1991).

3. Flocculation of colloidal suspension

Coagulation and flocculation are essential stages in many solid-liquid separation processes. In water and wastewater treatment, coagulation and flocculation are important physicochemical process for removing suspended solids, mainly colloidal particles.

3.1 The phenomena of coagulation and flocculation

Although the two words coagulation and flocculation are frequently used as being synonymous, it is important to notice that in fact they describe different phenomena which may be distinguished in terms of mechanisms, physical form of the agglomerated material, and industrial areas in which they are normally applied. The situation is perhaps sometimes confused by the fact that both phenomena may occur either simultaneously or consecutively (Purchas, 1971 ; 1981).

3.1.1 Coagulation

Coagulation describes the phenomenon whereby the colloidal particles in the suspension are destabilized by the addition of chemical agents resulting in a decrease of the negative charges on the surface particles and thus neutralization occurs. As a result, the destabilized particles approach each other and lead to the aggregation.

3.1.2 Flocculation

Flocculation describes the phenomenon whereby the destabilized particles are induced to come together by the long chain polymer acting as

bridges between the particles, make contact and thereby form the larger agglomerate or three dimensional network of flocs.

3.2 Mechanisms of flocculation

Coagulation and flocculation are processes which are able to accomplish destabilization of colloidal particles or dissolved materials. The principal underlying destabilization of a colloid is the interaction that occurs between colloidal particles, their counter ions, and various metal ions/or polymers added as coagulating agents (Green and Kramer, 1979). By both coagulation and flocculation, the different coagulants (flocculants) can bring about the destabilization of the suspension in different ways. The possible proposed mechanisms are : (1) double layer compression, (2) charge neutralization, (3) sweep flocculation, (4) interparticle bridging, and (5) electrostatic patch (Faust and Aly, 1983).

3.2.1 Double layer compression

The addition of a simple salt (indifferent electrolyte) causes no specific interaction between it and the particle, i.e. the electrolytes are not adsorbed on the surface. These counter ions enter into the double layer. Under this condition, the particle's charge remains constant but the double layer thickness will decrease and the Nernst and zeta potential will decrease. The repulsive interaction between similar colloidal particles decreases, thus the particles approach each other and aggregate.

The efficacy of an indifferent electrolyte is strongly dependent on the valency of the counter ions according to the Schulze-Hardy rule which states that coagulation effectiveness of these ions increases with charge. That is, a trivalent ion will require a lesser concentration than a divalent and monovalent ion such that the concentration of mono-, di-, and trivalent required for

destabilization would be in the ratio of 800:12:1 respectively. For a given suspension, as the ionic strength is incrementally increased due to the increasing concentration of electrolyte, the transition from stability to destabilization occurs over a narrow range of electrolyte concentration. Furthermore, if the indifferent electrolyte is increased to excess, there is no effect on destabilization that means restabilization occur. In addition, the electrolyte concentration required for destabilization is independent of particle concentration.

3.2.2 Charge neutralization (charge reduction)

Charge neutralization is most frequently brought about by inorganic salt (metal ions) or cationic polymers (Green and Kramer, 1979). Adsorption of these coagulants on the surfaces of the colloidal particle will cause reduction and eventual reversal of the particle's negative charge. In the case of iron or aluminium salts used as coagulants, these metals form hydrides and then insoluble hydroxides upon hydrolysis (see sweep flocculation below). However, positively charged hydrolysis products can also react to help reduce the colloidal particle charge.

When the colloidal particle's electrostatic charge is significantly reduced the colloid is destabilized, attractive forces are effective, and a coagulant-colloid complex is formed which settles out. Overdosing the colloidal particle with excess cationic ions will reverse the original particle charge and forms a positively charged coagulant-colloid complex, resulting in the particles being restabilized in suspension again.

3.2.3 Sweep flocculation (Enmeshment in a precipitate)

Multivalent metal ions such as Al^{3+}or Fe^{3+}do not exist as free species in solution, but attract dipole H_2O to form hydrated ions of $Al(H_2O)_6^{3+}$ and

$Fe(H_2O)_6^{3+}$, respectively. As the concentration of these hydrides of metal ions increases, there is a tendency for hydrolysis to occur. This leads to rapid precipitation of insoluble neutral hydroxide such as $Al(OH)_{3(s)}$ or $Fe(OH)_{3(s)}$ which enmesh with each other and neighboring colloidal particles to form a gelatinous precipitate (Amirtharajah and O'Melia, 1990 ; Green and Kramer, 1979)

3.2.4 Interparticle bridging

An electrostatic mechanism is not the only means of destabilization. A "bridging" theory was proposed by La Mer and Healy (1963 ; cited by Faust and Aly, 1983) to account for the destabilization of colloidal systems by high molecular weight polymer. Adsorption of the polymer on specific sites of the colloid plays an important role in the "bridging" theory. A "particle-polymer-particle" complex is formed in which the polymer serve as a "bridge".

Akers (1975) and Gregory (1987) presents several stages in the bridging mechanism :

(A) Dispersion of polymer in the suspension

(B) Adsorption of polymer at the solid-liquid interface

(C) Compression of the adsorbed polymer

(D) Bridge formation

Each of these stages, schematically represented in Fig.2, is discussed in detail below :

(A) *Dispersion of polymer in the suspension*

Because of its high molecular weight, the polymer in the solution exhibits high viscosity and low diffusion rate. So it is essential to disperse the polymer throughout the suspension in the shortest practicable time, to permit adsorption on to the surface of particles. Dispersion can be done by mechanical

agitation and vigorous mixing should be avoided due to rupture the long chain polymer.

(B) *Adsorption of polymer at the solid-liquid interface*

After step (A), where polymers have diffused to the solid-liquid interface, adsorption of initially one functional group will occur while the rest of the chain is free and extends into the solution. As time proceeds, due to continuous Brownian movement, the chain becomes successively attached at more points, with different configuration such as loops, trains and tails as illustrated in Fig. 3, along its length until eventually there are no dangling ends extending into the solution. The final polymer configuration is of loops extending from the surfaces. The size of the loops depending on many physical properties of the system polymer/solid/solution.

The mechanism of adsorption of polymer segments to a solid surface depends on both the chemical characteristics of the polymer and the adsorbent surface. For example, adsorption could be due to electrostatic linkages, hydrogen bonding or ionic bonding for anionic polymer adsorption to clays.

The polymer adsorption is effectively irreversible and a monolayer as described by the Langmuir adsorption isotherm as illustrated in Fig. 4. In the case of polymer and particles carrying charges of like sign, adsorption may strongly depend on ionic strength. Ionic strength may influence the adsorption by two effects :

(i) reduce repulsion between similarly charged particles surfaces and polymer segments, thus permitting adsorption to occur and

(ii) reduce the size of the polymer coil thus permitting more polymer chains to be accommodated on the particles surface.

(C) *Compression of adsorbed polymer at the interface*

From the onset of adsorption, progressively greater numbers of polymer segments will become adsorbed on the particles surface and, consequently, the polymer chain will become compressed to the surface. By the way, the adsorbed polymer chains would be re-arranged (or re-conformed) to give an equilibrium configuration. The configuration of the adsorbed polymer chain depends upon the size of the polymer (molecular weight), its structure, flexibility, charge density and so on. Under optimum flocculation conditions, the adsorbed polymer is at fairly low surface coverage and re-conformation may be much more rapid than the particle collision rate. If slow, then particles will collide before an equilibrium configuration has been achieved, with the adsorbed polymer in a more extended state. This process is indicated by the dash line in Fig. 2. And should lead to an enhanced bridging opportunity and improve flocculation.

(D) *Bridge formation*

After adsorption has taken place as described in the preceeding sections, polymer loops extending into solution from the particle surfaces will further become adsorbed onto adjacent particles thus forming a number of bridges and thereby increasingly larger flocs. The strength of the flocs thus formed depends on the number of bridges formed, which in turn is dependent on the number of loops available. Such availability depends on the concentration of polymer added. If an excess of polymer is added, the particles may be re-stabilized by the adsorption of excess polymer, as shown in Fig. 2. Under these conditions the particle surface is almost saturated by adsorbed polymer and there is little opportunity for attachment of polymer chains adsorbed on other particles.

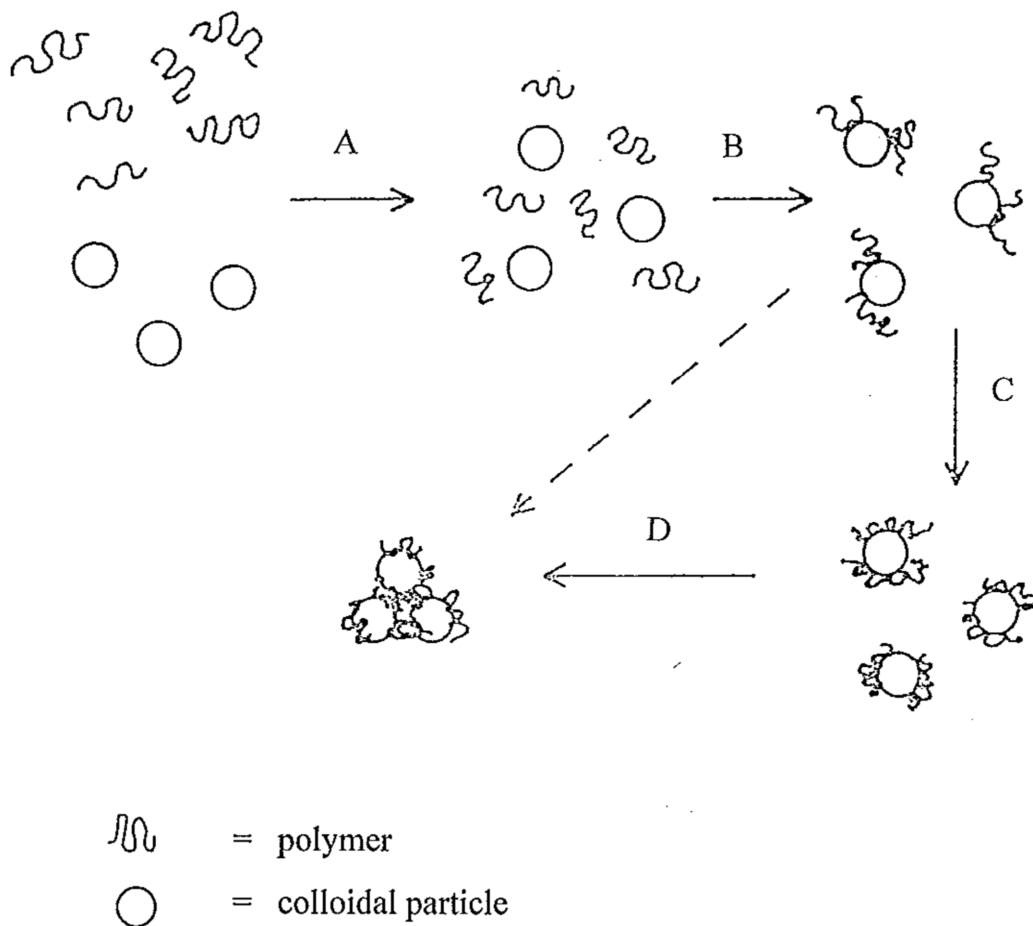


Fig. 2 Stages in the bridging mechanisms of destabilization with polymeric flocculants

(A) Dispersion of polymer in the suspension

(B) Adsorption of polymer at the solid-liquid interface

(C) Compression of the adsorbed polymer

(D) Bridge formation

Source : Akers (1975)

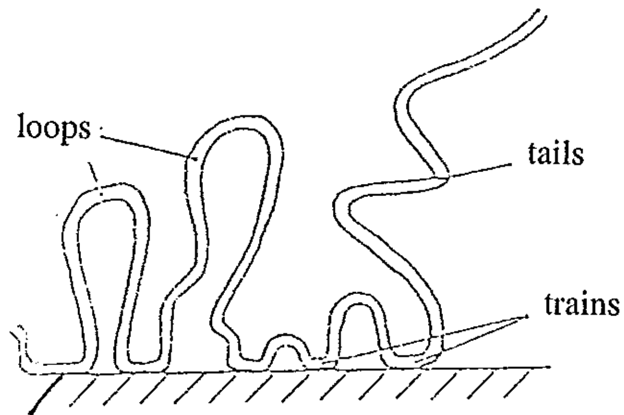


Fig. 3 Adsorbed polymer configuration with loops, trains and tails
Source : Amirtharajah and O'Melia (1990)

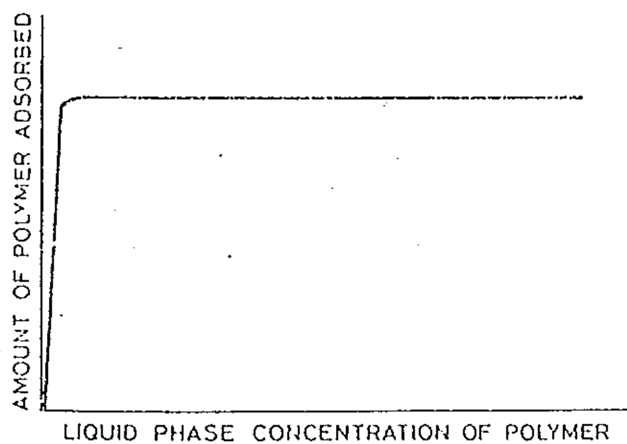


Fig. 4 Adsorption isotherm of polymer at solid/liquid interface
Source : Akers (1975)

3.2.5 The electrostatic patch mechanism

For the case of non-ionic and anionic polymeric flocculants applied to a negatively charged colloidal suspension, a destabilization mechanism described by the bridging model is adequate. However, for the case of charged polymer applied to dispersion with particles carrying surface charges of opposite sign, the bridging model is often inadequate. Such systems include cationic polymer applied to a negative colloidal dispersion and could include anionic polymer applied to dispersions destabilized with metal coagulants i.e. as flocculant aids to particle-metal hydrolysis product aggregates, which may be positively charged.

The electrostatic patch model is resemble to the electrical double layer model of colloid destabilization whereby ionic polymer, bearing a charge of opposite sign to the suspension, are adsorbed and thereby reduce the potential energy of repulsion between adjacent colloids.

3.3 Flocculating and coagulating agents

In order to flocculate a suspension, the particles must be adequately destabilized and this often requires the use of chemical additives. These may act simply to reduce the repulsion between particles or may play a more positive role in the flocculation process, such as improving the stronger aggregates. In all cases the additives may be referred to as " flocculating agent" and " coagulating agent". Sometimes the terms "flocculant" and "coagulant" are employed.

3.3.1 Simple salts

Simple salts such as sodium chloride are sometimes called indifferent electrolytes. This means that the ions produced in solution (Na^+ and Cl^- in this case) act as point charges and have no chemical characteristics such as

hydrolysis and adsorption in the reaction. So the simple salts cause the colloidal particles to be destabilized by double-layer compression mechanism. According to the Schulze-Hardy rule, the destabilization by an indifferent electrolyte is brought about by ions of opposite charge to the colloid (counter ions) and that the coagulation effectiveness of the ions increases markedly with ion charge (Amirtharajah and O'Melia, 1990).

However, simple salts are of no practical interest as coagulating agents, because of the large amount of such additives that would be required to produce adequate destabilization. Nevertheless, such salts are always present in natural waters and they may considerably influence the flocculation behaviour when other additives are used (Gregory, 1986).

3.3.2 Metal salts (metal ion coagulants)

The most common inorganic coagulating agents are aluminium and iron salts. The aluminium coagulants include aluminium sulphate, aluminium chloride, polyaluminium chloride and sodium aluminate. The iron coagulants include ferric sulphate, ferrous sulphate, chlorinated copperas and ferric chloride. Other chemicals used as coagulants include hydrated lime [$Ca(OH)_2$] and magnesium carbonate [$MgCO_3$]. The popularity of aluminium and iron coagulants arises not only from their effectiveness as coagulants but also from their ready availability and relatively low cost. These metal salts are the typical agents used for water and sewage treatment. Destabilization of the colloidal suspension by these metal salts are brought about by charge neutralization or sweep flocculation mechanisms (Bratby, 1980).

3.3.3 Polymeric flocculants

Polymeric flocculants are water soluble macromolecular compounds which have the ability to destabilize or enhance flocculation of the colloidal

suspension. The polymeric flocculants possess the characteristics of both polymers and electrolytes and are therefore, called polyelectrolytes. They are medium to high molecular weight compounds containing functional groups located at intervals along the chain.

The polyelectrolytes become charged either positively or negatively, depending on the specific functional groups present, and are thus referred to as cationic or anionic polyelectrolytes, respectively. Polyelectrolytes which possess both positively and negatively charged sites are referred to as ampholytic, whereas those that possess no ionizable functional groups are termed nonionic polyelectrolytes. These polymeric flocculants can destabilize the colloidal suspension by two mechanisms - bridging mechanism and the electrostatic patch mechanism. Polymeric flocculants can be classified as chemically synthetic polymers and bio polymers (Bratby, 1980).

3.3.3.1 Synthetic polymers

The use of synthetic polymers has gradually become more widespread, although natural polymers have the advantage of being virtually toxic free. In general, they are more effective as flocculants principally due to the possibility of controlling properties such as the number and type of charged units, molecular weight, etc. (Bratby, 1980). Although they are considerably more expensive than primary coagulants, the dose rate is much lower, typically 0.1-0.15 mg/l of substrate to be treated. The types of synthetic polymer which are considered useful are summarized in Fig. 5 (Hughes, 1990). Obviously the anionic/cationic character can be altered by co-polymerization of the various monomers and any product is then characterized by its average molecular weight and the charge density distribution within the polymer chain. Molecular weight can be classified to high ($\sim 2\times10^7$), medium (1×10^7), low (5×10^6) and very low ($<1\times10^6$).

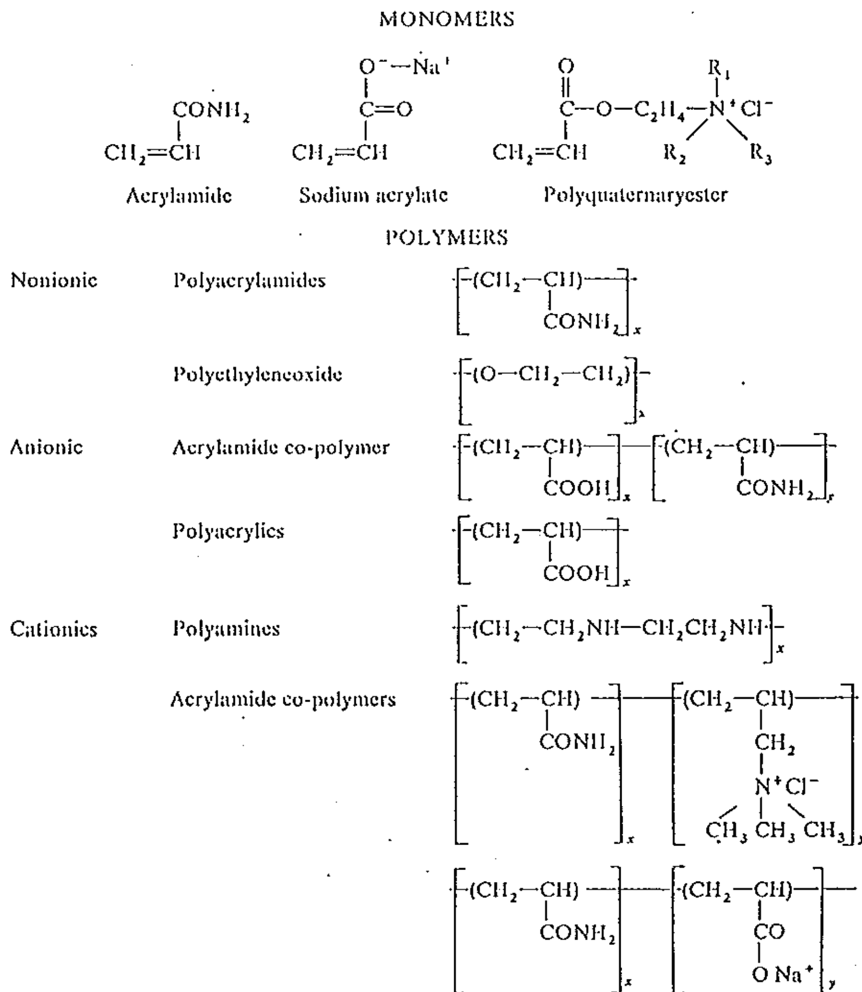


Fig. 5 Chemically synthetic polymers and their monomers

Source : Hughes (1990)

3.3.3.2 Biopolymers

There are several such naturally or microbiological derived substances used as polyelectrolytes, most of them being based on a polysaccharide skeleton with charged property due to the presence of functional groups. The natural polymers have recently been explored with particular application to the food industry such as the clarification of beer and wine (Gregory, 1986). Solids recovered by natural flocculants could be used as animal feeds. The examples of natural flocculants are gums, glue and gelatin, starches, sodium alginate, tannins, chitosan, fish skin and skale etc. In general, the natural polymeric flocculants have to be used at a considerably higher dose than synthetic polymers, and may give rise to biochemical oxygen demand (BOD) problems in discharged effluents. The high dose required are offset by their low cost, but in general natural flocculants are being replaced by synthetic polymers that are used at very low dose rates (Akers, 1975).

Such natural polymers derived from plants or algae have low potential for flocculation. Consequently, microbial polymers are of greater interest and more widespread in use at present due to its high polymer yield, variety of unique properties, biodegradability and friendliness to the environment.

The polymeric flocculants can be applied as primary coagulants, flocculant aids or sludge conditioners. The application as primary coagulants in wastewater treatment represents predominantly turbidity removal, organic colour removal, removal of microorganisms and various industrial applications. In case where replacement of metal coagulants by polymeric flocculants is possible, the potential advantages are as follows :

(1) soluble metal ion species carry-over from sedimentation is prevented

(2) the need for extensive pH adjustment is eliminated

(3) carry-over of light flocs is minimized

(4) sludge volume is reduced

(5) the amount of soluble anions are reduced.

3.4 Parameters affecting flocculation by microbial polymer

The important property for application of polymer as flocculant in water and wastewater treatment was the ability to flocculate the suspended or colloidal particles in water. The flocculation can be estimated from the flocculating activity in the reaction containing the polymer, cationic salt and colloidal particles such as kaolin suspension. So the efficiency of biopolymer for flocculation also depended on several factors as follows:

3.4.1 Biopolymer flocculant concentration

The relationship between flocculant concentrationn and flocculating activity of several microbial flocculants were similar, i.e. the flocculating activity initially increased but then decreased at the excess flocculant concentration due to the adsorption of excess flocculant restabilized the particles. This relationship can be seen in a polyglutamate flocculant produced by *B.subtilis* PY-90 (Yokoi, *et al.*, 1995) in Fig. 6A, protein flocculant produced by *R. erythropolis* S-1 (Takeda, *et al.*, 1991) in Fig. 6B and polysaccharide flocculants produced by *Pestalotiopsis* sp. KCTC 8637P (Kwon, *et al.*, 1996), *Bacillus* sp. DP-152 (Suh, *et al.* 1997) and *Enterobacter* sp. (Yokoi, *et al.*, 1997). However, higher concentration of glycolipid flocculant produced by *R. erythropolis* S-1 possessed higher flocculating activity in reaction with the presence of Ca^{2+} (Kurane, *et al.*, 1994).

3.4.2 Types of suspended solids

Various microbial polymers had different ability to flocculate different types of suspended solids. The polymer produced by *Paecilomyces* sp. I-1 could flocculate several species of microorganisms such as *Bacillus*

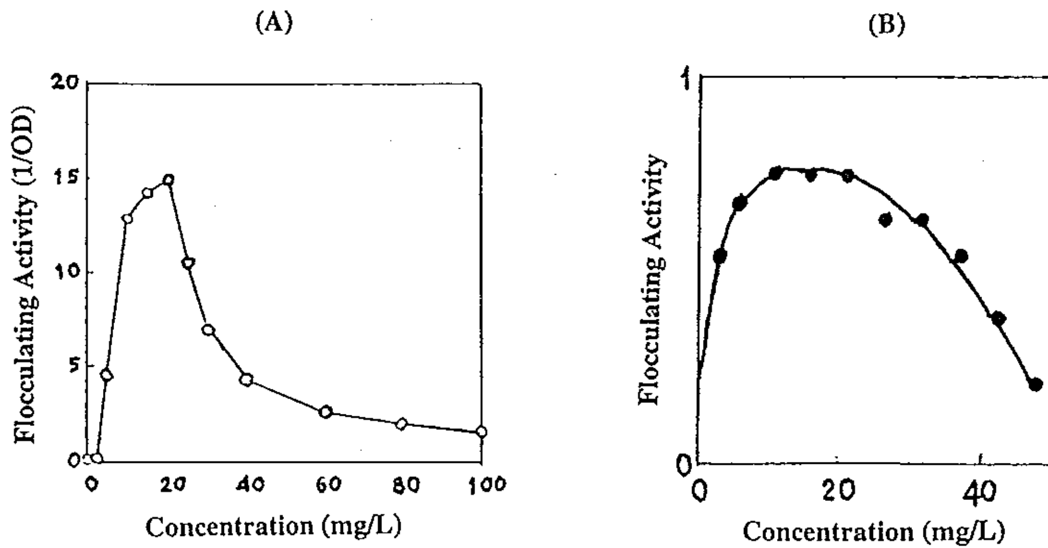


Fig. 6 Effect of bioflocculant concentration on the flocculating activity

(A) polyglutamate flocculant (with addition of Ca^{2+} 4.5 mM)

(B) protein flocculant (with addition of Al^{3+} 1.2 mM)

Source : Yokoi, *et al.* (1995) and Takeda, *et al.* (1991)

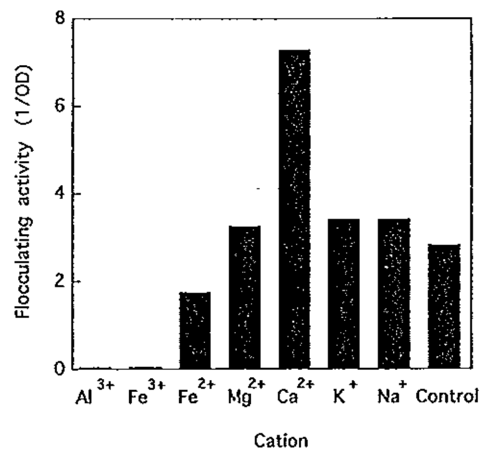


Fig. 7 Effect of various cations (4.5 mM) on the flocculating activity of polyglutamate flocculant (20 mg/l)

Source : Yokoi, *et al.* (1995)

subtilis, *B. amylosacchariticus*, *B. natto*, *Escherichia coli*, *Candida utilis* and *Saccharomyces cerevisiae* in which the flocculating activity differs in each species. Moreover, this polymer could flocculate several suspended solids such as blood cell, cellulose powder, agar powder, activated charcoal, aluminium oxide and silica gel (Takagi and Kadowaki, 1985).

3.4.3 Cations

The flocculating activity of several microbial flocculant such as *R. erythropolis* S-1 protein flocculant, *Alcaligenes cupidus* polysaccharide flocculant, *Nocardia amarae* protein flocculant (Takeda, *et al.* 1992) increased upon addition of several cations (e.g., Na^{+}, Ca^{2+}, Al^{3+} or Fe^{3+}). For *B. subtilis* PY-90 polyglutamate flocculant, the flocculating activity would increased markedly in the presence of Ca^{2+} and decreased with addition of Al^{3+} and Fe^{3+} as illustrated in Fig. 7. The optimal Ca^{2+} concentration for the highest flocculating activity was 2 - 8 mM. In contrast, when Al^{3+} or Fe^{3+} concentration was increased, the flocculating activity would decrease rapidly and flocculation could not be observed at concentrations of 0.2 and 0.5 mM, respectively (Yokoi, *et al.*, 1995).

The flocculating activity of glycolipid flocculant produced *by R. erythropolis* S-1 reached the highest value at the concentration of Ca^{2+} above 5 mM. Although the flocculation was not observed without Ca^{2+}, only Ca^{2+} 1 mM was effective to flocculate the suspension to half of the maximum level. Divalent cations, Mg^{2+} and Mn^{2+} were as effective as Ca^{2+} but monovalent cation of Na^{+} was less effective (Kurane, *et al.*, 1994).

For polysaccharide flocculant produced by the mixed culture of *Oerskovia*, *Acinetobacter*, *Agrobacterium* and *Enterobacter*, the monovalent cations had no effect on flocculating activity, while synergistic effect appeared resulting from addition of bivalent and trivalent cations consisting of Ca^{2+},

Al^{3+} and Fe^{3+}. Synergistic effect resulting from the addition of trivalent cations was stronger than in the case of addition of bivalent actions (Kurane and Matsuyama, 1994).

3.4.4 pH of reaction

The flocculating activity of *B. subtilis* PY-90 polyglutamate flocculant on kaolin suspension was high in an acidic pH range of 3 to 5, and optimum pH was 4 (Yokoi, *et al.*, 1995). The effect of pH on the ability to flocculate *E. coli* cells suspension revealed that the flocculating activity was not affected in the pH range of 4 to 8 (Takagi and Kadowaki, 1985). The maximum flocculating activity of *Enterobacter* sp. polysaccharide flocculant in kaolin and cellulose suspension containing 0.2 mM Fe^{2+} was observed at pH 3.0 and the activity decreased with increasing pH (Yokoi, *et al.*, 1997).

3.4.5 Temperature of reaction

Temperature in the range of 0-100 °C never affected the flocculating activity for flocculation of *E. coli* cells suspension by *Paecilomyces* sp. I-1 polysaccharide flocculant (Takagi and Kadowaki, 1985).

Objectives

1. To isolate, select and identify the polymer-producing bacteria from activated sludge
2. To study time course of polymer production by the selected strains
3. To determine the physical and chemical properties of the produced polymer

Chapter 2

Materials and Methods

Materials

1. Activated sludge samples and preparation

Recycled sludge samples from sedimentation tanks were taken from the following five seafood processing plants in Songkhla regions :

- Chotiwat Manufacturing Co.,Ltd.
- King Fisher Holding Co.,Ltd.
- Kiang Huat Sea Gull Trading Frozen Food Public Co.,Ltd.
- Narong Seafood Co.,Ltd.
- Songkhla Canning Public Co.,Ltd.

All collected samples were kept in ice-containers and immediately used for isolation. The samples were centrifuged at 7,000 rpm (6,442 x *g*), 5 °C for 5 min. After washing with 0.85% NaCl solution, the precipitate was weighed and 10 g was mixed with 90 ml of 0.85% NaCl solution. The mixture was homogenized at 2,500 rpm, 5 °C for 10 min and used for isolation (modified from Shimizu and Odawara, 1985).

2. Media

Screening medium and basal medium (pH 7.0) contained 1% glucose (or sucrose), 0.05% $(NH_4)_2SO_4$, 0.5% K_2HPO_4, 0.2% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$ and 0.01% NaCl. Glucose (or sucrose) was sterilized separately.

3. Dialysis membrane

Dialysis membrane was regenerated tubular membrane (Cellu·Sep® T2, nominal MWCO 6000-8000, Membrane Filtration Products, Inc.).

4. Chemicals

Chemicals used were analytical grade. The major chemicals were Anthrone, Carbazole, Cetylpyridinium chloride, D(+)-Galactosamine hydrochloride, D(+)-Glucosamine hydrochloride, D-glucuronic acid, Ninhydrin, *p*-dimethylaminobenzaldehyde (Sigma) and Acetyl acetone (Merck).

5. Instruments

Instruments	Model	Company
Differential scanning calorimeter	DSC 7	Perkin-Elmer
Double-beam spectrophotometer	U-2000	Hitachi,Ltd.
Fourier transform spectrophotometer	series 1600 FT-IR	Perkin-Elmer
Freeze-dryer	Dura-Dry^{TM} μp	FTS System
Incubator	-	K.S.L. Engineering Co.,Ltd.
Incubator-shaker	3525-1C	Lab-Line Instrument,Inc.
Light microscope	CHS	Olympus Optical Co.,Ltd.
Microcentrifuge	centrifuge 5415C	Eppendorf
Refrigerated centrifuge	RC-5B plus	Sorvall
Scanning electron microscope	JSM-5800 LV	JEOL
Shell freezer	Just-A-Tilt^{TM}	FTS System

Analytical Methods

1. Cell dry weight determination

Strain WD7 : The viscous culture broth (4 ml) was diluted 5 times with distilled water, 0.2 ml of 1% formaldehyde was added and mixed. The mixture was heated at 60 °C for 5 min and centrifuged at 13,000 rpm (12,846 x *g*) for 15 min at 5 °C. The cells were washed in distilled water and centrifuged again in a microcentrifuge, dried for 24 h at 105 °C and weighed.

Strain WD22 : The non-diluted culture broth (20 ml) was used for the determination of cell dry weight using the same procedure as for strain WD7.

Strain WD50 : The viscous culture broth (4 ml) was diluted 5 times with distilled water, 0.2 ml of 1% formaldehyde was added and mixed.The mixture was heated at 100 °C for 5 min followed by centrifugating at 16,000 rpm (19,459 x *g*) for 20 min at 5°C. The cells were washed in distilled water and centrifuged again in a microcentrifuge, dried for 24 h at 105 °C and weighed.

2. Crude polymer yield determination

After cells removal, the supernatant was precipitated with 4 volumes of cold 95% ethanol, left overnight and centrifuged at 10,000 rpm (7,600 x *g*) for 15 min at 5 °C. The precipitate was dried for 24 h at 105 °C and weighed.

3. Flocculating activity assay

Flocculating activity of culture broth or biopolymer solution was measured from the turbidity of a kaolin clay suspension after aggregation. The procedures were modified from the method as described by Kurane, *et al.* (1986) and Suh, *et al.* (1997).

In a 50 ml cylinder (or beaker), 4.5 ml of $CaCl_2$ solution (1% w/v) was added to the 45 ml kaolin suspension (5,000 mg/l) and stirred with a glass rod (or magnetic bar) for 20 s. After stirring, 0.5 ml of culture broth or polymer solution was added. The pH of the mixture was adjusted to 7 with 0.5 M or 6 M of HCl or NaOH solution and stirred again. After the mixture was kept standing for 5 min at room temperature, the sample was taken at the middle of the container to measure the absorbance at 550 nm using a spectrophotometer. The control was prepared using the same method but replacing the sample with deionized water. The flocculating activity (or flocculation rate) was calculated according to the equations ;

$$\text{Flocculating activity} = [1/(OD_{550})_s] - [1/(OD_{550})_c]$$

$$\text{Flocculation rate (\%)} = \frac{(OD_{550})_c - (OD_{550})_s}{(OD_{550})_c} \times 100$$

$(OD_{550})_s$ = absorbance of the sample

$(OD_{550})_c$ = absorbance of control

4. Determination of biopolymer components

A. Qualitative analysis

Determination of α-amino acids (by ninhydrin reaction) and aromatic amino acids (by xanthoproteic reaction) were carried out following the procedures of Plummer (1978). (Appendix 1)

B. Quantitative analysis

The procedures for the determination of neutral sugars by anthrone reaction (Trelyan and Harrison, 1952), total sugars by phenol-sulfuric acid reaction (Dubois, *et al.*, 1956), amino sugars (Elson and Morgan, 1933), uronic acid by carbazole-sulfate reaction (Bitter and Muir, 1962) were followed as described by Chaplin and Kennedy (1986). (Appendix 1)

5. Determination of the electric charge of the biopolymer

The electric charge of the biopolymer was determined by precipitation with cetylpyridinium chloride (Scott, 1965). To 10 ml of a partially purified biopolymer solution (0.1% w/v) was added 2 ml of 1% (w/v) cetylpyridinium chloride (CPC) (polysaccharide : CPC was 1:2 by weight), and 1 ml of 0.13 M Na_2SO_4 (final concentration was 0.01 M) to increase the rate of aggregation of the precipitate. The mixture was mixed and incubated overnight at 37 °C.

Methods

1. Isolation of polymer-producing microorganisms from activated sludge

1.1 Characteristics of activated sludge samples

The activated sludge samples were recorded for colour, pH and settling characteristic by SV_{30} [the sludge volume after 30 min of settling (King and Forster, 1990 cited by Urbain, *et al.*, 1993)].

1.2 Isolation procedures

One ml of each homogenized sludge sample was mixed with 9 ml of sterile 0.85% NaCl solution using a vortex mixer. Serial dilutions were prepared and 0.1 ml of each dilution was dropped onto screening medium and spread over the agar surface. After incubation at 37 °C for 3 days, the mucoid colonies or slime-forming colonies were selected and streaked onto new agar plates. The procedure was repeated until pure cultures were obtained. The isolates were kept on nutrient agar slants and, after incubation at 37 °C for 2 days, were stored at 4 °C until used (modified from Yokoi, *et al.*, 1995).

2. Selection and identification of polymer-producing microbes with the highest flocculating activity

2.1 Selection

One loop of each isolated strain was inoculated into 50 ml of basal medium. Cultivation was carried out on a rotary shaker (200 rpm) at room temperature (31± 2 °C) for 3 days. At the end of cultivation, the culture broth was taken to measure for OD_{660}, flocculating activity and pH. The culture broth viscosity was observed visually. The strains with the culture broth possessing the highest flocculating activity were selected for further studies.

2.2 Identification

The selected isolates were identified according to Bergey's Manual of Determinative Bacteriology (Holt, *et al.*, 1994) and biochemical tests followed the methods in " Biochemical Tests for Identification of Medical Bacteria" (Mac Faddin, 1980).

3. Time course of polymer production by the selected strains

3.1 Starter preparation

One loop from each of the selected strains (24 h) was inoculated into 50 ml nutrient broth in 250 ml flasks and cultivated on a rotary shaker (200 rpm) at room temperature for 12 h. The culture broth was then diluted with sterile sugar-free basal medium to adjust the OD_{660} to achieve the viable cell counts equivalent to 10^8 CFU/ml obtained from the standard curve (Appendix 2).

3.2 Cultivation

Starter with a viable cell count of 10^8CFU/ml was used as inoculum. A 5% (2.5 ml) inoculum was transferred into 50 ml of basal medium and cultivations were carried out on a rotary shaker at room temperature for 5 days. Samples were taken every 24 h to measure pH, cell dry weight, crude polymer yield and flocculating activity.

4. Physical and chemical properties of the partially purified biopolymer from the selected strain

Preparation of partially purified biopolymer

The selected strain (strain WD7) was cultivated for an optimal period (result from 3), its viscous culture broth (1.2 litre) was diluted 5 times with distilled water, 1% formaldehyde was added, heated at 60 °C for 5 min and centrifuged at 13,000 rpm (12,846 x *g*). The supernatant (6 litre) was concentrated in a vacuum dryer at 50 °C, 20 lb/in^2 for 7 h. The concentrated supernatant (3 litre) was mixed with 4 volumes of cold 95% ethanol and left overnight. After centrifugation at 10,000 rpm (7,600 x *g*), 5 °C for 15 min, the precipitate polymer was redissolve in 1 L distilled water and concentrated again. The concentrate (150 ml) was dialysed against distilled water at 4 °C for 24 h and the dialysate was freeze-dried to obtain the partially purified biopolymer.

4.1 Analysis of the components of the partially purified biopolymer by the colorimetric methods

The partially purified biopolymer was classified as polysaccharide, protein, glycolipid, or polyglutamate according to its components using qualitative and quantitative analysis. The biopolymer would be classified as a polysaccharide if it contained higher amounts of total sugars compared to other components (Kurane and Matsuyama, 1994).

4.2 Electric charge property of the partially purified biopolymer

The appearance of the precipitate would indicate that the biopolymer was an anionic or an acidic polysaccharide. If no precipitation occurred, 95% ethanol would be added and the appearance of the precipitate would indicate that the biopolymer was a neutral polysaccharide.

4.3 Functional groups analysis of the partially purified biopolymer by Fourier-transform infrared (FT-IR) spectroscopy

Prior to analysis, the biopolymer sample was prepared according to the potassium bromide (KBr) disk method as follows : the biopolymer was ground and mixed with KBr powder and uniformly pressed to get a disk. Infrared absorption spectra were measured on KBr disk with a FT-IR spectrophotometer (Perkin-Elmer series 1600 FT-IR). Infrared spectra were given in term of % transmittance.

4.4 Thermal analysis of the partially purified biopolymer by differential scanning calorimetry (DSC) method

Thermal property of the biopolymer was investigated using the thermal analysis technique based on energy change-differential scanning calorimetry (DSC). The investigation was carried out using a Perkin-Elmer model DSC-7 (mode power-compensation DSC). Two empty pans in the sample and reference chamber were heated and the temperature was programmed as follows ; (1) holding at 200 °C for 1 min, (2) heating from 200 °C to 420 °C at 10 °C/min increment, to obtain the steady baseline. The transition of the biopolymer was determined by heating the sample chamber (a pan containing 1.020 mg polymer) and reference chamber (empty pan) with the same programmed temperature as above. The temperatures of the sample and reference are maintained at the same temperature throughout the controlled temperature program. The net energy difference in the independent power supplies to sample and reference is recorded versus the programmed temperature.

4.5 Solubility of the partially purified biopolymer

One milligram of the biopolymer was tested for its solubility in several solvents (1 ml) such as distilled water, acetone, carbon tetrachloride, ethanol, isopropanol, hexane, methanol and nitrobenzene. The biopolymer-solvent mixtures were allowed to stand for 24 h, and the solubility was observed (Collins, *et al.*, 1973).

4.6 Gelation property of the partially purified biopolymer with metal salts

Monovalent cations (NaCl and KCl) and divalent cations ($CaCl_2 \cdot 2H_2O$, $MgSO_4 \cdot 7H_2O$ and $CuSO_4 \cdot 5H_2O$) were used for the investigation of gelation of the biopolymer. Two milligrams of each cationic salt and 0.2 ml of 2M NaOH were added into 1 ml of the biopolymer solution (0.5% w/v) (Shimada, *et al.*, 1997). After mixing, the phase transition from solution to semi-solid phase of gel was visually observed, thus indicating the gelation.

4.7 Flocculation property of the partially purified biopolymer

The partially purified biopolymer was measured for the flocculating activity against kaolin suspension according to the analytical method described above. The properties studied are as following :

4.7.1 Temperature (thermal) stability

The temperature (thermal) stability of the biopolymer was determined by measuring the residual activity after 30 min incubation (Kwon, *et al.*, 1996 ; Lee, *et al.*, 1995) at various temperatures ranging from 4 to 120 °C and compared with that of 30 °C (room temperature). The final concentration of biopolymer in the reaction mixture was 2 mg/l.

4.7.2 pH stability

The pH stability of the biopolymer was determined by measuring the residual activity after 24 h (Lee, *et al.*, 1995) of preincubation at various pH values ranging from 2-12 and compared with that of normal polymer solution at pH 6.3. The final concentration of biopolymer in the reaction mixture was 2 mg/l.

4.7.3 Effect of polymer concentration on the flocculating activity

The flocculating activity was measured from the reaction mixture containing the different final concentrations of biopolymer ranging from 0 to 7 mg/l.

4.7.4 Effect of cation types and concentrations on the flocculating activity

The flocculating activity was measured from the reaction mixture containing the optimum biopolymer concentration (from 4.7.3) and various types of salts (NaCl, KCl, $CaCl_2 \cdot 2H_2O$, $MgSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$ and $FeCl_3 \cdot 6H_2O$) as the source of cations with final concentrations at 0, 0.01, 0.1, 1, 10 and 100 mM.

4.7.5 Effect of pH of reaction on the flocculating activity

The flocculating activity was measured from the reaction mixture containing the optimum type and concentration of cationic salt (from 4.7.4), the pHs of the reactions were adjusted from 2 to 12 (using HCl or NaOH) prior flocculation.

4.7.6 Effect of temperature of reaction on the flocculating activity

The flocculating activity was measured from the reaction mixture performed at the optimal pH reaction (from 4.7.5) at different temperatures ; 4, 30, 40 and 50 °C.

Chapter 3

Results and Discussions

1. Isolation of polymer-producing bacteria from activated sludge

Recycled activated sludge samples from five seafood processing plants had different characteristics as shown in Table 4. Most samples had brown colour and neutral pH range (pH 6.97-7.32) except that of Narong Seafood Company, Ltd. (pH 5.39). The settleability characteristic from SV_{30} indicated that these samples may have different structure, density and strength of the flocs, therefore, affected on its flocculation in activated sludge systems (Magara and Nambu, 1976).

Table 4 Characteristics of recycled sludge from five seafood processing plants

Plant	Type of processing	Characteristics		
		pH	Colour	SV_{30} (ml)
Chotiwat Manufacturing Company Limited	Canning	7.14	dark brown	400
Kiang Huat Sea Gull Trading Frozen Food Public Company Limited	Frozen	6.98	brown	840
King Fisher Holding Company Limited	Frozen	6.97	light brown	975
Narong Seafood Company Limited	Frozen	5.39	greenish brown	500
Songkhla Canning Public Company Limited	Canning	7.32	dark brown	1000

Activated sludge was chosen for the isolation of polymer-producing bacteria due to the high probability to be present, since activated sludge primarily consists of flocs which are bound together by means of exopolymer component produced by the bacterial cells due to metabolism and cell autolysis and compounds in the wastewater (Dugan, 1987 ; Urbain, *et al.*, 1993 ; Nielsen, *et al.*, 1996). Moreover, activated sludge may contain new and good polymer-producing strains due to the presence of a complicated mixed cultures of viruses, bacteria, protozoa and other organisms. The predominant and most active organisms are heterotrophic bacteria (Ganczarczyk, 1983). The presence of floc-forming bacteria causes the secretion of an exocellular binding agent during the early declining or stationary phase of growth which was reported to occurr in sedimentation tanks (Ganczarczyk, 1983). With these reasons, it was of interest to isolate the microbes from activated sludge to produce polymer for their application as bioflocculant and to replace the synthetic flocculant in wastewater treatment.

Besides the activated sludge, several carbohydrate-rich environments such as sugar refineries, paper and pulp mill, potato crisp or chip processing plants, brewery or distillery effluents and corn or vegetable processing plants also were interesting sources for the isolation of polysaccharide-producing microbes. Obtaining microbial species from such environments is that they should be capable of converting the waste carbohydrate to an extracellular polysaccharide which hopefully will be of commercial value (Lawson and Sutherland, 1978).

A total of 188 slime-forming or mucoid bacteria anticipated to produce an extracellular polymer were isolated from activated sludge samples using the spread plate technique (Table 5). The screening medium (pH 7) contained either 1% glucose or sucrose and 0.05% $(NH_4)_2SO_4$, therefore, its carbon to nitrogen ratio was high as nitrogen is usually the favored component to induce

Table 5 Number of isolated mucoid bacterial strains from each source of activated sludge

Source*	Number of isolates		
	Glucose screening medium	Sucrose screening medium	Total
CC	22	36	58
KF	8	11	19
KS	17	23	40
NS	5	4	9
SC	37	25	62
Total	89	99	188

* CC = Chotiwat Manufacturing Co.,Ltd.
KF = King Fisher Holding,Co.,Ltd.
KS = Kiang Huat Sea Gull Trading Frozen Food Public Co., Ltd.
NS = Narong Seafood Co.,Ltd.
SC = Songkhla Canning Public Co., Ltd.

growth limitation and stimulate exopolymer formation (Sutherland, 1996). All bacterial isolates appeared on the agar plates within 2 to 3 days at 37 °C incubation. They were convex, rounded, entire edge with cream or light brown colour colonies. Some were irregular shape or less convex due to their loosen slime on the medium and some were highly ropy colonies.

The number of isolated bacteria on both screening media (Table 5) indicated that most isolates could assimilate both glucose and sucrose and convert both to extracellular polymer. Moreover, the number of isolates from the canning seafood factories was higher than those from frozen seafood factories as illustrated by a larger number of isolates from the two canning seafood factories (SC and CC). This may be due to the higher organic loading wastewater generated from the canning factories compared to the frozen factories, resulting in more abundance of bacteria. Moreover, the microbial community of the activated sludge flocs may vary significantly, depending on the conditions at which an aeration tank is operated (Li and Ganczarczyk,

1990). On the contrary, the number of isolates from Narong Seafood Co.,Ltd. was the least as a result of the lower pH condition (pH 5) of the samples, thus most bacteria can not grow well and consequently affected the flocs quality. Therefore, pH was also an essential parameter influencing on the abundance and types of bacteria in activated sludge. Also, the recycle of activated sludge flocs controls the viable/non-viable cell ratio in the floc for any given organic substrate concentration. Low recycling results in a very active floc with a small non-viable fraction (McKinney, 1956 cited by Atkinson and Daoud, 1976).

Although many isolates grew on the screening media, some may be identical strains due to their similar morphologies. However, some isolates could not grow on both solid and liquid media. On the other hand, some bacterial isolates grew well and produced a polymer on solid media but either grew poorly or not at all in liquid media. Some may need a surface to allow them to grow as a biofilm. It should be noted, however, that despite the perfectly satisfactory medium for isolation, the screening medium may not be able to isolate all biopolymer-producing bacteria. Some bacterial isolates would need growth factors, normally yeast extract (0.05-0.1% w/v), which was added to the screening medium (Sutherland, 1996).

2. Selection and identification of polymer-producing bacteria with the highest flocculating activity

All 188 bacterial isolates were cultivated in basal medium for 3 days to select the isolates possessing high flocculating activities. Most isolates can grow well according to growth measurement (OD_{660}) and low pH. Only 30 isolates showed high flocculating activities against kaolin suspension in the presence of 1% $CaCl_2$ (Table 6), and only a few isolates possessed a viscous culture broth which was obviously different from the others. The viscous culture broth (0.5 ml) of these 3 strains gave a low flocculating activity due to

high amount of polymers in the culture broth causing a restabilization of the kaolin suspension. So the performance to achieve the flocculation by the viscous culture broth were carried out. The results (Table 7) revealed the following :

a) $CaCl_2$ (1 % w/v ; 68 mM) for 4.5 ml, with a final concentration of 6.12 mM in the reaction mixture, can flocculate kaolin suspension (flocculation rate, 65.37%).

b) The culture broth (500 μl) of strain WD7, WD22 and WD50 showed very low flocculating activity and thus a low flocculation rate. This indicated that the viscous culture broth can not achieve flocculation of kaolin suspension.

c) The viscous culture broth of all 3 strains showed different flocculating activities and flocculation rates in the presence of 1% $CaCl_2$. At the optimum volume of culture broth, the maximum flocculating activity or flocculation rate was achieved. Lower or higher volumes than the optimum ranges resulted in a low flocculating activity. Culture broth of strain WD7 (50 μl) exhibited the highest flocculating activity or flocculation rate. Strain WD22 and WD50 also showed high flocculating activities with 2 times of culture broth (100 μl).

d) Flocculation of the kaolin suspension with the culture broths of the strain WD7, WD22 and WD50 in the presence of 1% $CaCl_2$ gave the flocculation rate of 87.43%, 78.22% and 86.89% respectively which were higher than that of using only $CaCl_2$ without the culture broth (65.37%). However, $CaCl_2$ plays the important role for flocculation of kaolin suspension.

Table 6 Final pH, flocculating activity, OD_{660} and broth viscosity of 30 isolated strains after cultivation in basal medium for 3 days

Isolate	Code[a]	Final pH	Flocculating activity	OD_{660}	Broth viscosity[b]
WD3	CC-G3	4.87	0	1.065	-
WD7	CC-G7	5.30	0.26	1.030	++
WD22	CC-G22	6.64	0.95	0.430	+
WD41	CC-S19	4.89	1.50	1.460	-
WD47	CC-S25	4.59	-0.04	1.405	-
WD48	CC-S26	4.98	0.78	1.155	-
WD50	CC-S28	4.54	0.10	1.410	+
WD51	CC-S29	4.55	1.70	1.570	-
WD52	CC-S30	4.47	0.81	1.675	-
WD54	CC-S32	4.22	-0.48	1.960	-
WD57	CC-S35	4.78	1.92	2.340	-
WD58	CC-S36	4.51	0.18	1.680	-
WD68	KF-S2	4.99	0.45	1.225	-
WD79	KS-G2	4.61	0.44	3.140	-
WD85	KS-G8	4.64	0.92	1.355	-
WD86	KS-G9	4.58	1.46	1.530	-
WD89	KS-G12	4.64	0.97	1.555	-
WD90	KS-G13	3.70	3.73	1.790	-
WD93	KS-G16	4.53	1.22	1.070	-
WD99	KS-S5	4.67	1.13	1.445	-
WD110	KS-S16	4.55	0.69	1.625	-
WD118	NS-G1	3.58	2.26	1.370	-
WD121	NS-G4	3.74	3.19	1.370	-
WD124	NS-S2	4.64	1.91	1.545	-
WD139	SC-G13	3.60	0.24	1.830	-
WD140	SC-G14	4.60	0.74	2.410	-
WD141	SC-G15	3.31	1.52	1.340	-
WD150	SC-G24	4.54	1.54	4.460	-
WD161	SC-G35	3.56	1.34	1.375	-
WD162	SC-G36	4.17	1.50	1.375	-

a : code indicated source of the isolates

CC = Chotiwat Manufacturing Co.,Ltd.
KF = King Fisher Holding,Co.,Ltd.
KS = Kiang Huat Sea Gull Trading Frozen Food Public Co., Ltd.
NS = Narong Seafood Co.,Ltd.
SC = Songkhla Canning Public Co., Ltd.

G = glucose basal medium, S = sucrose basal medium

for example : **CC-G3** = the third isolatefrom Chotiwat Manufacturing Company Limited (CC) with glucose basal medium as screening medium

KF-S2 = the second isolate from King Fisher Holding, Ltd. (KF) with sucrose basal medium as screening medium

b : broth viscosity by visual observation

- = not viscous culture broth
+ = little viscous culture broth
++ = medium viscous culture broth

Table 7 Flocculating activity of the viscous culture broth (3 days cultivation) of three selected strains

Treatment	Strain WD7		Strain WD22		Strain WD50	
	Flocculating Activity	Flocculation rate (%)	Flocculating Activity	Flocculation rate (%)	Flocculating Activity	Flocculation rate (%)
Kaolin + $CaCl_2$	0.707	65.37	0.707	65.37	0.707	65.37
Kaolin + culture broth (500 µl)	0.005	1.20	0.009	2.41	0.006	1.66
Kaolin + $CaCl_2$ + culture broth						
25 µl	2.24	67.39	1.37	55.80	0.40	27.19
50 µl	**7.53**	**87.43**	2.46	69.45	1.33	55.04
100 µl	1.88	63.49	**3.89**	**78.22**	**7.18**	**86.89**
200 µl	0.95	46.80	2.17	66.74	2.63	70.86
300 µl	0.53	32.83	1.87	63.27	1.55	58.94
400 µl	0.38	25.78	1.27	53.95	0.43	28.49
500 µl	0.26	19.18	0.95	46.70	0.10	8.56

Among them, three strains; WD7,WD22 and WD50 showed very high flocculating activities on their respective carbon source. They were Gram-negative bacteria, rod shaped with different size (Fig.8). According to their morphological and biochemical characteristics (Table 8), the three selected isolates were identified to be *Enterobacter cloacae*, *Pseudomonas alcaligenes* and *Enterobacter agglomerans*, respectively.

3. Time course of polymer production by the three selected strains

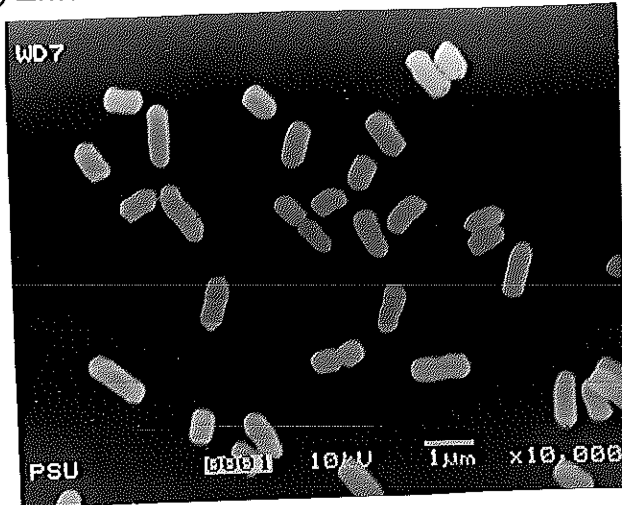
The time courses on growth and polymer production of the three selected strains in basal medium using glucose (strain WD7 and WD22) or sucrose (strain WD50) as carbon source for 5 days are given in Fig. 9-11. All 3 strains exhibited a similar pattern of growth ; they grew rapidly within 1 day as indicated by the increase of cell dry weight. Their fast growth correlated with a rapid decline in pH due to the metabolism of the sugar (as carbon source) and ammonium sulfate. During the growth, they produced the polymer and excreted it into the culture broth which led to the viscous characteristic of the broth. Consequently, the oxygen transferred into the culture broth would be reduced resulting in a slow growth at stationary phase (after 2 days cultivation). It was observed that the increase of cell growth of *Enterobacter cloacae* WD7 and *Pseudomonas alcaligenes* WD22 were less than that of *Enterobacter agglomerans* WD50 after 2 days cultivation. The highest polymer yields were obtained during stationary phase with values of 3.41, 2.27 and 0.83 g/l for *P. alcaligenes* WD22, *E. cloacae* WD7 and *E. agglomerans* WD50, respectively. These polymers were white but were different from each other by visual observation after ethanol precipitation. The polymer from *E. cloacae* WD7 in particular had fibrous characteristics which could be removed easily by low speed centrifugation or filtration.

Table 8 Taxonomical and biochemical characteristics of the three selected strains

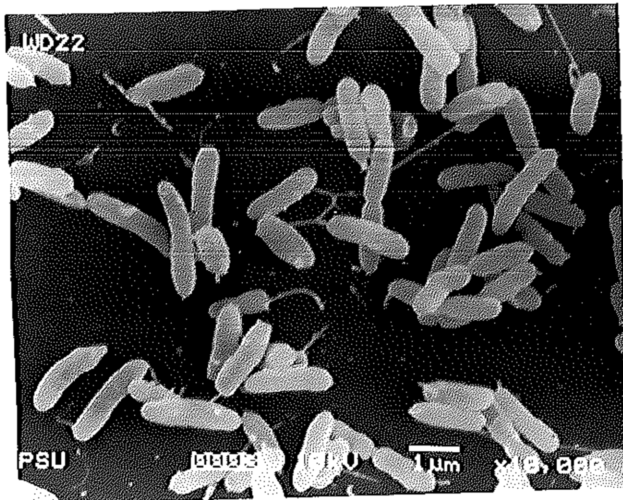
Test	WD7	WD22	WD50
Gram stain (24h)	negative	negative	negative
O_2 requirement	facultative anaerobe	strictly aerobe	facultative anaerobe
Cell morphology	short rod	rod	short rod
Cell size (µm)	0.45x1.02	0.54x1.94	0.58x0.87
Motility	+	+	+
Oxidase	-	+	-
Catalase	+	+	+
Indole production	-	-	-
Methyl red	+	-	+
Voges-Proskauer	+	-	-
Citrate (Simmons)	+	+	+
TSI reaction	A/A,G	NC/NC	A/A,G
H_2S production (TSI)	-	-	-
Esculin hydrolysis	+	-	+
Urea hydrolysis	-	-	+
Gelatin hydrolysis	-	+	-
Starch hydrolysis	-	-	-
Lipid hydrolysis	-	+	-
Casein hydrolysis	-	-	-
Nitrate reduction	+	-	+
Lysine decarboxylase	-	NC	-
Arginine dihydrolase	+	NC	-
Ornithine decarboxylase	+	NC	-
KCN growth	+	-	+
Oxidation-Fermentation (O-F)	F	non oxidizer	F
Litmus milk	acid,clot	peptonization	acid, clot
Acid production from :			
glucose	+	-	+
galactose	+	-	+
fructose	+	-	+
ribose	+	-	+
arabinose	+	-	+
xylose	+	-	+
rhamnose	+	-	+
mannitol	+	-	+
glycerol	-	-	+
dulcitol	-	-	-
sorbitol	+	-	+
inositol	-	-	+
maltose	+	-	+
sucrose	+	-	+
lactose	+	-	+
cellobiose	+	-	+
trehalose	+	-	+
raffinose	+	-	+
inulin	-	-	-
Identified as	*Enterobacter cloacae*	*Pseudomonas alcaligenes*	*Enterobacter agglomerans*

N.B. : + = positive result , - = negative result , A = acid , G = gas , NC = no change

A) *Enterobacter cloacae* WD7



B) *Pseudomonas alcaligenes* WD22



C) *Enterobacter agglomerans* WD50

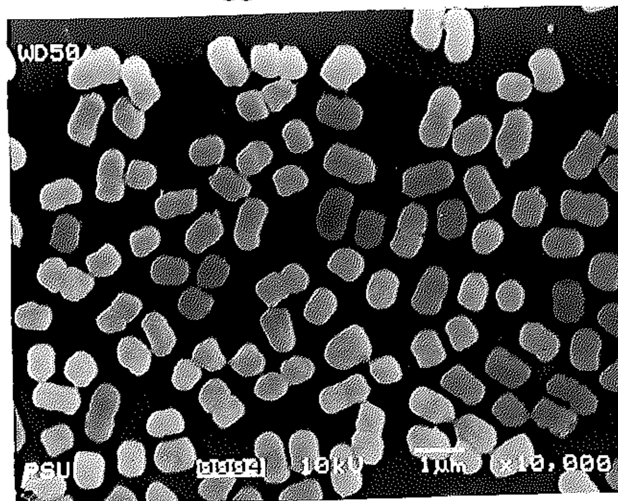


Fig. 8 Scanning electron micrograph of three selected strains

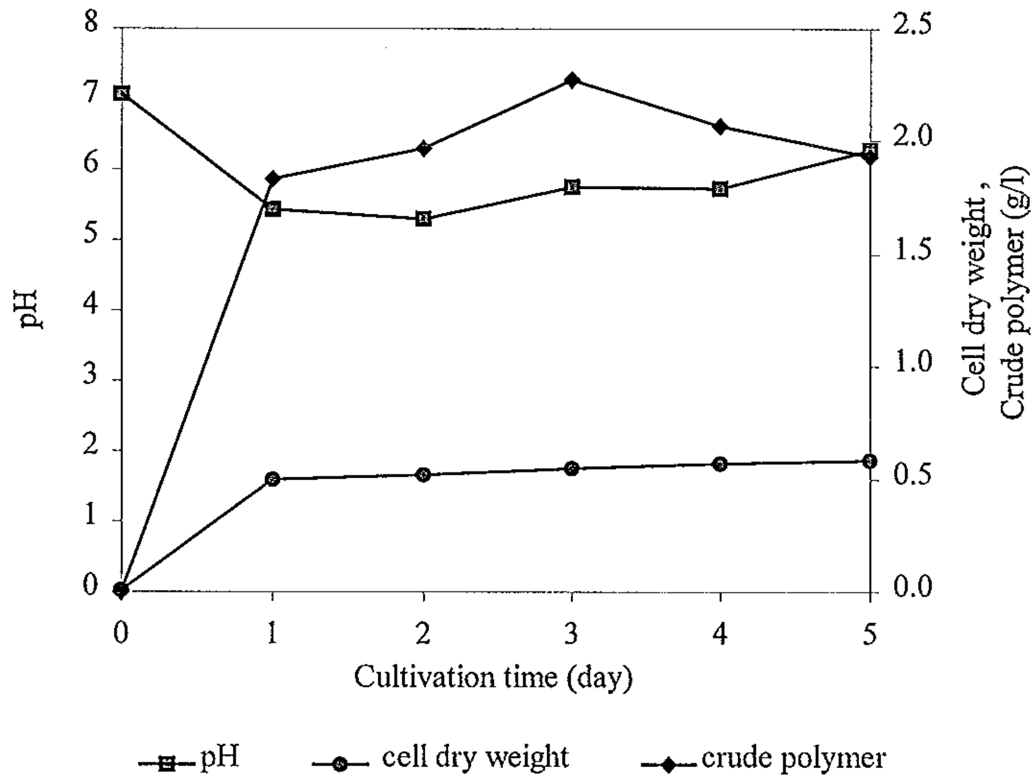


Fig. 9 Time course of polymer production by *Enterobacter cloacae* WD7 in basal medium (1% glucose as carbon source)

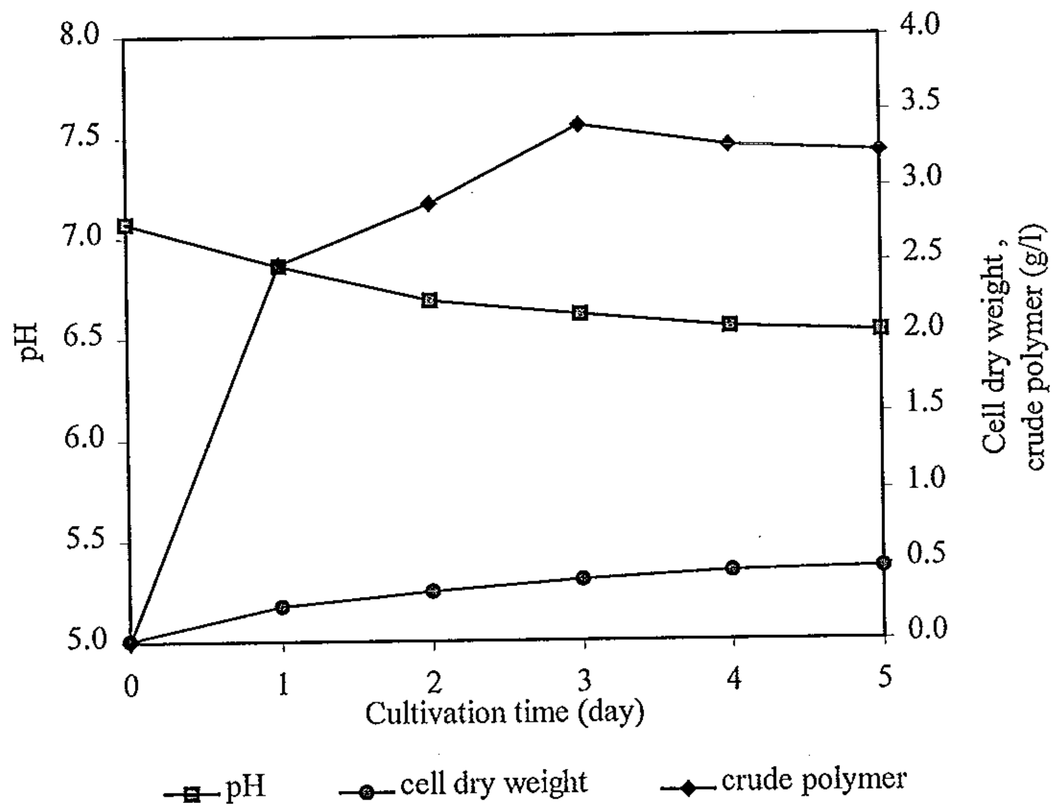


Fig. 10 Time course of polymer production by *Pseudomonas alcaligenes* WD22 in basal medium (1% glucose as carbon source)

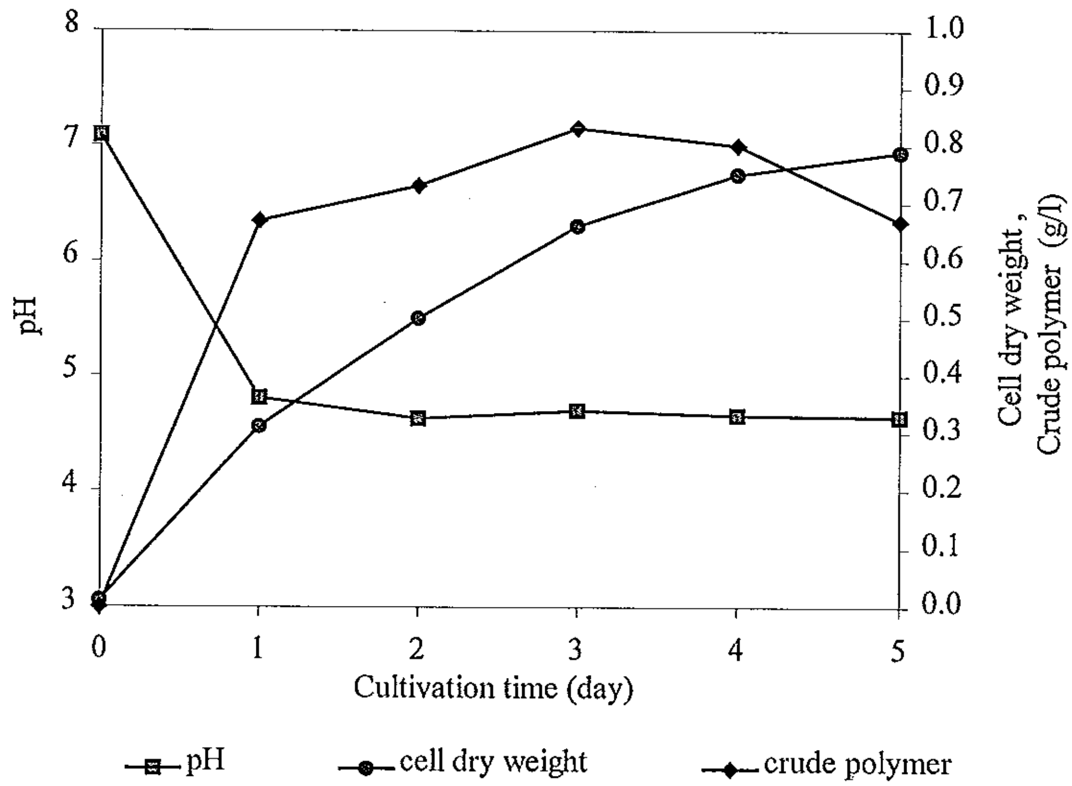
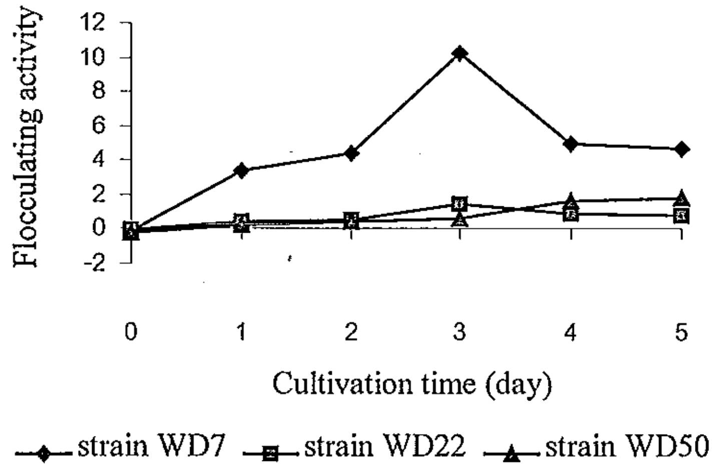


Fig. 11 Time course of polymer production by *Enterobacter agglomerans* WD50 in basal medium (1% sucrose as carbon source)

Culture broth at different stages of growth was tested for its flocculating activity on kaolin suspension. As shown in Fig.12A, the flocculating activity of *E. cloacae* WD7 and *P. alcaligenes* WD22 increased and reached their maximum (10.28 and 1.43 respectively) after 3 days cultivation and decreased thereafter, which may indicate that both strains possessed the polymer-degrading enzyme (Kurane and Nohata, 1991). The flocculating activity of *E. agglomerans* WD50, however, increased during 5 days cultivation. Since the flocculating activity was assayed using the whole culture broth, it was probable that the flocculation resulted from both the extracellular biopolymer and the cells. Although the majority of the produced biopolymer dispersed in the culture broth, some remained on the surface of the cells and appeared as capsule (india ink staining). The flocculation caused by the cells is anticipated to be the result of kaolin particles binding by the capsule.

The appearance of floc after the flocculation was also different within these strains ; *E. cloacae* WD7 showed the large, coarse and more compact floc than those of *E. agglomerans* WD50 and *P. alcaligenes* WD22, respectively. In comparing the flocculation rate, it was found that *E. cloacae* WD7 and *P. alcaligenes* WD22 possessed a flocculation rate of 91% and 55%, respectively after 3 days whereas *E. agglomerans* WD50 gave a flocculation rate of 60% after 5 days (Fig. 12B). These results indicated that *E. cloacae* WD7 was the most suitable strain for polymer production due to its highest flocculating activity (10.28) and the highest flocculation rate (91%) although its polymer yield (2.27 g/l) was lower than that of *P. alcaligenes* WD22 (3.41 g/l).

(A)



(B)

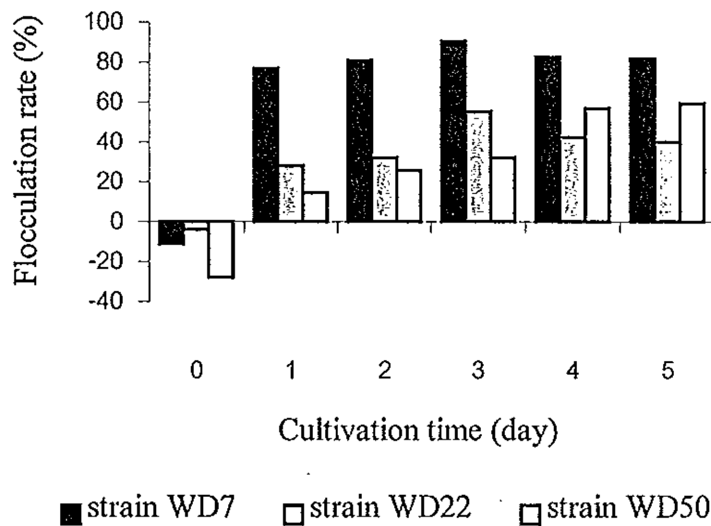


Fig. 12 Flocculating activity (A) and flocculation rate (B) change during cultivation of three selected strains in basal medium (glucose basal medium for *E. cloacae* WD7 and *P. alcaligenes* WD22, sucrose basal medium for *E. agglomerans* WD50) for 5 days

E. cloacae WD7 (50 μl of culture broth)

P. alcaligenes WD22 (100 μl of culture broth)

E. agglomerans WD50 (100 μl of culture broth)

4. Physical and chemical properties of the partially purified biopolymer from the selected strain

Enterobacter cloacae WD7 was selected and cultivated in basal medium for 3 days, then the polymer was recovered, partially purified, and freeze-dried as described above. The crude biopolymer obtained from precipitation the supernatant with 95% ethanol was about 4.32 g/l (wet weight) and the yield of freeze-dried partially purified biopolymer was about 0.54 g/l. The freeze-dried partially purified biopolymer was white.

4.1 Analysis of the components of the partially purified biopolymer by the colorimetric methods

The components of the freeze-dried, partially purified biopolymer were determined by various colorimetric methods. Results showed that this polymer was composed of neutral sugar and uronic acids as the major and minor components, respectively, with a little amount of amino sugar (Table 9). Neither alpha amino acids nor aromatic amino acids were detected indicating that it contained no amino acids or protein in its molecule. Therefore, the polymer produced by *Enterobacter cloacae* WD7 was a polysaccharide. The uronic acids contained in its molecule might be glucuronic acid or galacturonic acid as often found in most acidic polysaccharides (Margaritis and Pace, 1985 ; Pace and Righelato, 1980).

Table 9 Components of the partially purified biopolymer

Method	Analyzed Item	% (w/w)
Qualitative test		
1. Ninhydrin reaction	α-amino acids	-
2. Xanthoproteic reaction	aromatic amino acids	-
Quantitative test		
1. Anthrone reaction	neutral sugar	29.40
2. Phenol-sulfuric acid reaction	total sugar	41.15
3. Carbazole-sulfate reaction	uronic acids	14.18
4. Elson-Morgan reaction	amino sugar	0.93

\- = not detected

4.2 Electric charge property of the partially purified biopolymer

The partially purified biopolymer of *Enterobacter cloacae* WD7 was further classified according to the electric charge. The precipitation occurred after the addition of cetylpyridinium chloride (CPC) into the polysaccharide solution indicating that it contained acidic groups in its structure due to the interaction with the quarternary ammonium (QN^+) of the CPC, resulting in the formation of a polysaccharide-CPC complex (Scott, 1965). It could therefore be concluded that this polymer was an acidic polysaccharide. Normally the polysaccharide structure is composed of neutral sugars and other derivatives of various chemical groups. In the case of an acidic polysaccharide, its component can be the acidic groups of pyruvate, succinate, uronic acid, acetate or sulphate (Pace, 1980 ; Sutherland, 1977). These acidic groups may play an important role causing the anionic (or acidic) charge of the polysaccharide (Margaritis and Pace, 1985). However, uronic acids are the principal charged component of the anionic polysaccharide (Pace and Righelato, 1980). On the other hand, the cationic (or basic) polysaccharide is composed of basic groups such as amino groups in the structure of chitosan (polyglucosamine) (Sandford, 1979).

4.3 Functional group analysis of the partially purified biopolymer by Fourier-transform infrared (FT-IR) spectroscopy

From the infrared spectroscopy of the biopolymer with a KBr disk, the functional groups were analyzed (Fig. 13). The broad absorption band at 3455 cm^{-1} was characteristic of O-H stretching from the bound hydroxyl groups. The occurrence of hydrogen bonding in general causes a broadening of the absorption band (Sawyer, *et al.*, 1984). The peaks in the range from 2900 to 2800 cm^{-1} were an indication of aliphatic C-H stretching. The peak at 1716 cm^{-1} representing C=O stretching indicated the presence of a carbonyl group. The spectra also displayed an asymmetrical stretching band at 1608 cm^{-1} and a

weak symmetrical stretching band near 1400 cm^{-1} which were consistent with the presence of carboxylate ions and thus indicating the presence of uronate in this polysaccharide. These two absorption peaks of carboxylate ions were the characteristic pattern for uronate of the polysaccharides produced by *Butyvibrio fibrisolvens* (Ha, *et al.*, 1991) and *Bacillus* sp. (Pfiffner, *et al.*, 1986). Moreover, the band stretching of the carboxylate ion at around 1608 cm^{-1} was the characteristic of uronate in alginate and pectate (Churms, 1995). The other absorption peaks were S=O stretching / bending at 1250 cm^{-1} which indicated the presence of sulphate, C-O antisymmetrical stretching at 1136 cm^{-1} and 1075 cm^{-1}, indicating the presence of methoxyl groups ($O\text{-}CH_3$). The strong absorption peaks observed in the range from 1000-1200 cm^{-1} are generally known to be typical characteristics of all sugar derivatives (Suh, *et al.*, 1997)

Peaks below 1400 cm^{-1}, resulting from skeletal vibration of polysaccharides, are parts of fingerprint region. Many of the bands in this region are dependent on the overall molecular structure rather than individual functional group. The pattern in this region is therefore very specific for a molecule and the comparison with a reference spectrum can positively identify a compound (Brauer and Kline, 1995).

The infrared spectra of this polysaccharide thus showed the presence of carbonyl, hydroxyl, carboxyl and methoxyl groups. This polysaccharide may be a methylated polysaccharide containing uronic acid due to the presence of O-H broad at 3700-3000 cm^{-1} and the intensity of absorption due to $O\text{-}CH_3$, at 1150-1050 cm^{-1} (Churms, 1995). The functional groups are responsible for its physical and chemical properties, for example, carboxylate groups can serve as binding sites for divalent metal ions. In addition, the carboxylate groups of the polysaccharide might be used as functional groups to link the polysaccharide to starch or man-made polymers to form new polymers with unique properties (Ha, *et al.*, 1991).

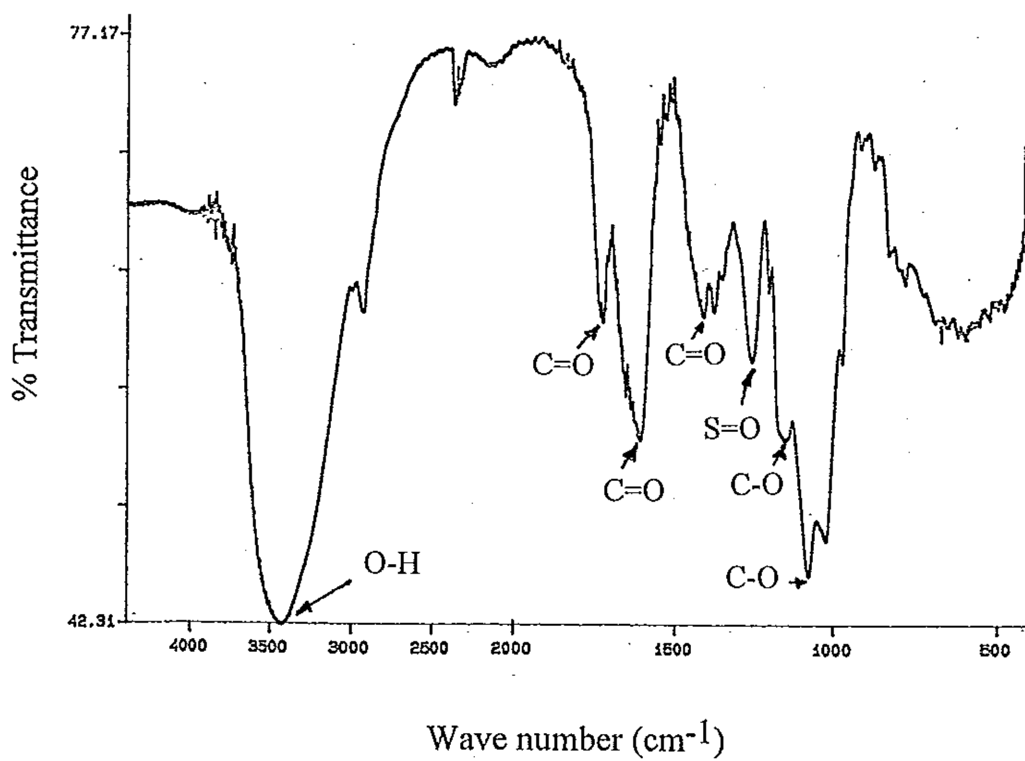


Fig. 13 FT-IR spectra of the partially purified biopolymer from *Enterobacter cloacae* WD7

4.4 Thermal analysis of the partially purified biopolymer by differential scanning calorimetry (DSC)

Thermal property of this polysaccharide determined by DSC was illustrated as the heat flow-temperature curve (Fig.14). Initially, there was no occurrence of the glass transition in this polysaccharide since there was no indication of an initial baseline shift (Hill, 1995 ; Brown, 1988). As the temperature slowly increased up to 272 °C, the solid polysaccharide may recrystallize resulting in the sharp drop in the specific heat curve (exotherm) as exhibited in Fig.14. As the temperature increased to 282-390 °C the crystalline polymer melted with a corresponding rise in the specific heat curve (endotherm) resulting in a peak at 300 °C, thus indicating the crystalline melting point (T_m) of this polysaccharide. According to the crystalline transition, the physical nature of this solid polysaccharide may be the partial crystalline polymer (customarily called crystalline polymer) which consist of crystalline and amorphous regions (Tadokoro, 1979). These crystalline and amorphous regions indicated a high, little or no degree of ordering of polymer chain interaction, respectively (Kroshwitz, 1990). Since crystalline polymers are strong, tough, stiff and generally more resistant to solvents and chemicals than their non-crystalline counterparts, it is possible to improve the desirable properties of this polysaccharide for material applications.

4.5 Solubility of the partially purified biopolymer

Results from solubility tests showed that this polysaccharide was only soluble in water but insoluble in all tested organic solvents (Table 10). This confirms that *Enterobacter cloacae* WD7 secreted the water-soluble polysaccharide into the culture broth, leading to the high viscosity.

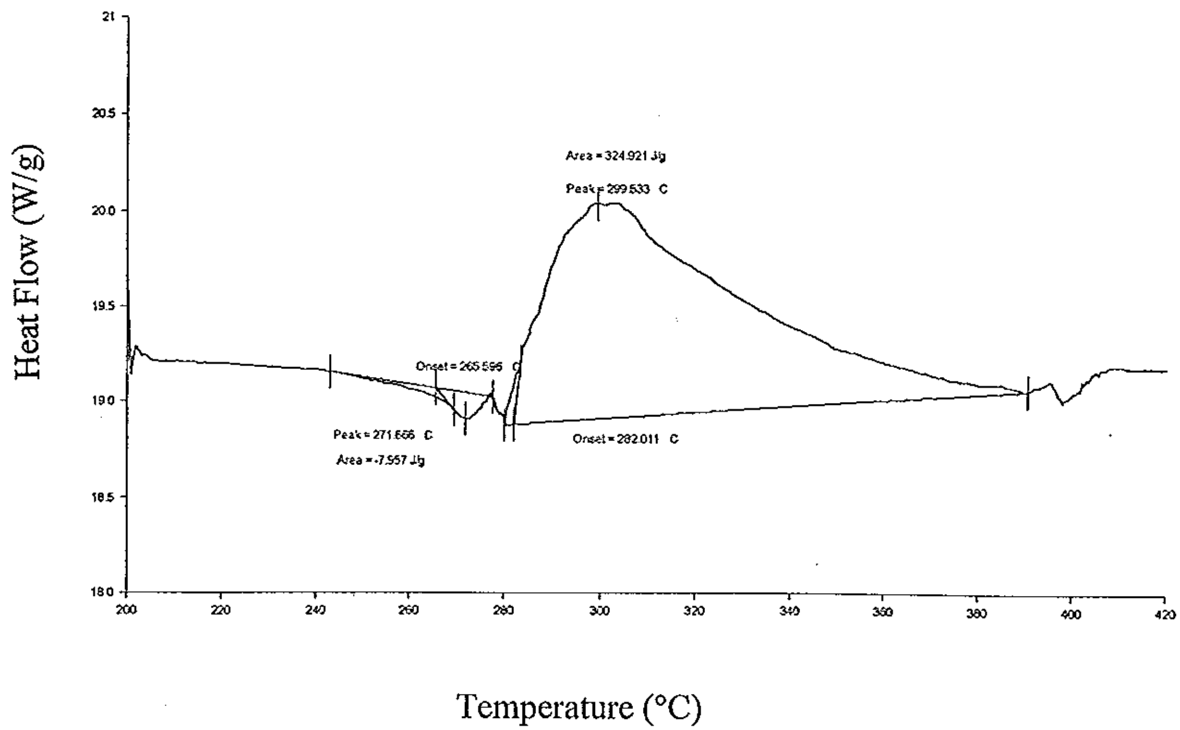


Fig. 14 Differential scanning calorimetry (DSC) curve of the partially purified biopolymer from *Enterobacter cloacae* WD7

Table 10 Solubility test of the partially purified biopolymer in the solvents

Solvent	Solubility
Water	+
Acetone	-
Carbon tetrachloride	-
Ethanol	-
Isopropanol	-
Hexane	-
Methanol	-
Nitrobenzene	-

+ = soluble , - = insoluble

Consequently, it can be recovered from the cell-free supernatant by precipitation with organic solvents such as ethanol.

Since this polysaccharide was only water-soluble, thus solubility of the polysaccharide should be based on the principle "like dissolves like"; i.e., a solute will dissolve in a solvent having similar properties (James, 1986). The concept is frequently expressed in the form of rule "polar solutes dissolve in polar solvents or non-polar solutes dissolve in non-polar solvents."

Since the polysaccharide contains hydroxyl groups, it has the possibility of hydrogen bonding to one or more water molecules. Also the ring oxygen atom and the glycosidic oxygen atom connecting one sugar ring to another can form hydrogen bonds with water. Therefore, polysaccharides have a high affinity for water and readily hydrate when water is available. In an aqueous system, polysaccharide particles can take up water, swell and usually undergo partial or complete dissolution (BeMiller and Whistler, 1996 ; Billmeyer, 1971). The abundance of hydroxyl groups build up strong forces of attraction between polysaccharide molecules, resulting in relative hard crystalline solids. These forces are too great to be broken by organic solvents, so the polysaccharide was insoluble in organic solvents (James, 1986).

4.6 Gelation property of the partially purified biopolymer with metal salts

All metal salts can not form a gel on this polysaccharide without the addition of NaOH indicating that gelation would occur in alkaline condition. Moreover, only divalent metal salts can form a gel in alkaline aqueous solution of this polysaccharide (Table 11). This may be due to the molecular chains of polysaccharide in alkaline solution spread owing to repulsion between dissociated acidic groups. In contrast, the reverse change occurs at high ionic strength or in the presence of salt (Clark, 1991 ; Shimada, *et al.*, 1997). This indicating that the polysaccharide gel may result from intermolecular associations which give rise to three dimensional network (Morris, 1986 ; Chinachoti, 1996). The different types of divalent cations also gave the different appearance of gels with the same amount (2 mg) of each divalent metal salts used with combination of 0.5% polysaccharide solution (1 ml) and NaOH (0.2 ml), thus the ratio of polysaccharide to metal salt was 2.5 : 1 by weight. $CuSO_4$ (final concentration of 6.68 mM) gave a stronger gel than $CaCl_2$ (final concentration of 11.3 mM) and $MgSO_4$ (final concentration of 6.76 mM) (Table 11). Gelation with metal salts of this polyasccharide was similar to that of an acidic polysaccharide from *Enterobacter* sp. as reported by Shimada, *et al.* (1997).

Many natural acidic polysaccharides in addition to bacterial exopolysaccharides undergo gelation in the presence of divalent cations. For example, cappa-carrageenan, alginate and gellan gum undergo gelation in the presence of Ca^{2+} (Ogino, *et al.*, 1993 cited by Shimada, *et al.*, 1997). Gelation in the presence of metal ions is useful in various applications such as alginate, which undergoes gelation in the presence of Ca^{2+},was used as an eliminator for Ca^{2+} from solution or as an immobilization agent (Kokufuta, *et al.*, 1988 cited by Shimada, *et al.*, 1997). The presented partially purified polysaccharide has

Table 11 Gelation of the partially purified biopolymer with metal salts

Treatment*	Gelation**
Polysaccharide + NaOH	-
Polysaccharide + NaCl	-
Polysaccharide + NaOH + NaCl	-
Polysaccharide + KCl	-
Polysaccharide + NaOH + KCl	-
Polysaccharide + $CaCl_2 \cdot 2H_2O$	-
Polysaccharide + NaOH + $CaCl_2 \cdot 2H_2O$	+
Polysaccharide + $MgSO_4 \cdot 7H_2O$	-
Polysaccharide + NaOH + $MgSO_4 \cdot 7H_2O$	+
Polysaccharide + $CuSO_4 \cdot 5H_2O$	-
Polysaccharide + NaOH + $CuSO_4 \cdot 5H_2O$	++

* Each treatment contained 2 mg of the appropiate metal salt / 0.2 ml NaOH/ 1 ml of 0.5% (w/v) polysaccharide solution

** gelation was visually observed, the phase transition from solution to the semi-solid phase of gel

- = No gelation, + = weak gelation, ++ = medium-strong gelation

also a high affinity for calcium and copper ions (Table 11). It therefore can act as an eliminator of both divalent cations in wastewater. It is assumed that the biopolymer acts as immobilizing agent. So the application of this biopolymer for wastewater treatment would be effective due to the acidic groups in its molecule which can bind the metal ions and may be simultaneously enhance forming inter-particle bridging between biopolymer chains and particles in wastewater to larger agglomerate (flocs) and settled down. Further experiments were carried out on the flocculation property of this biopolymer.

4.7 Flocculation property of the partially purified biopolymer

4.7.1 Temperature (thermal) stability

The temperature range of 4-60 °C did not affect the flocculating activity of the polysaccharide (Fig. 15). However, at 60 °C the flocculating activity is highest due to the increase in entropy (Lee, *et al.*, 1995) or this temperature may be the optimum temperature for enhancing the polysaccharide chain to have highly effective flocculation. In contrast, the temperatures above 60 °C affected the flocculating activity of the polysaccharide because the polysaccharide chain may be broken down to be shorter and lead to the low potential to form bridges with the kaolin particles. The comparison of temperature stability of polymer produced by different microorganisms are shown in Table 12.

4.7.2 pH stability

The pH range of 5-7 did not affect the stability of the polysaccharide (Fig.16) since it could maintain more than 80% of its activity. But in highly acidic solution at pH 2-3, the flocculating activity decreased to about 10%. This indicated that the polysaccharide did not stabilize in such condition due to the glycosidic bonds in the polysaccharide chain being degraded or hydrolyzed. At highly basic solutions in the pH range of 8-12, the flocculating activity decreased as the pH increased. This is due to the alkaline degradation of polysaccharide, which causes several changes such as molecular rearrangement of its residue or fragmentation of the polysaccharide chain (Aspinall, 1982). Moreover, the polysaccharide in highly basic solution possessed higher flocculating activity than those of highly acidic solution due to its higher stability in such basic solution.

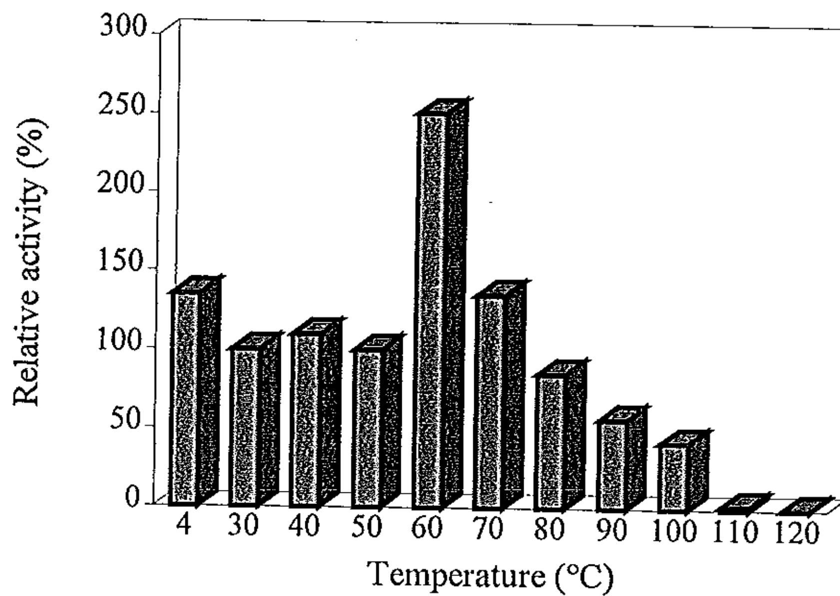


Fig. 15 Temperature stability of *Enterobacter cloacae* WD7 polysaccharide

Table 12 Comparison of temperature stability of various polymers from different microorganisms

Microorganism	Polymer	Temperaure stability	Reference
Arcuadendron sp.	glycoprotein	up to 100 °C	Lee, *et al.* (1995)
Pestalotiopsis sp.	acidic polysaccharide	up to 70 °C	Kwon, *et al.* (1996)
E. cloacae WD7	acidic polysaccharide	up to 60 °C	This study

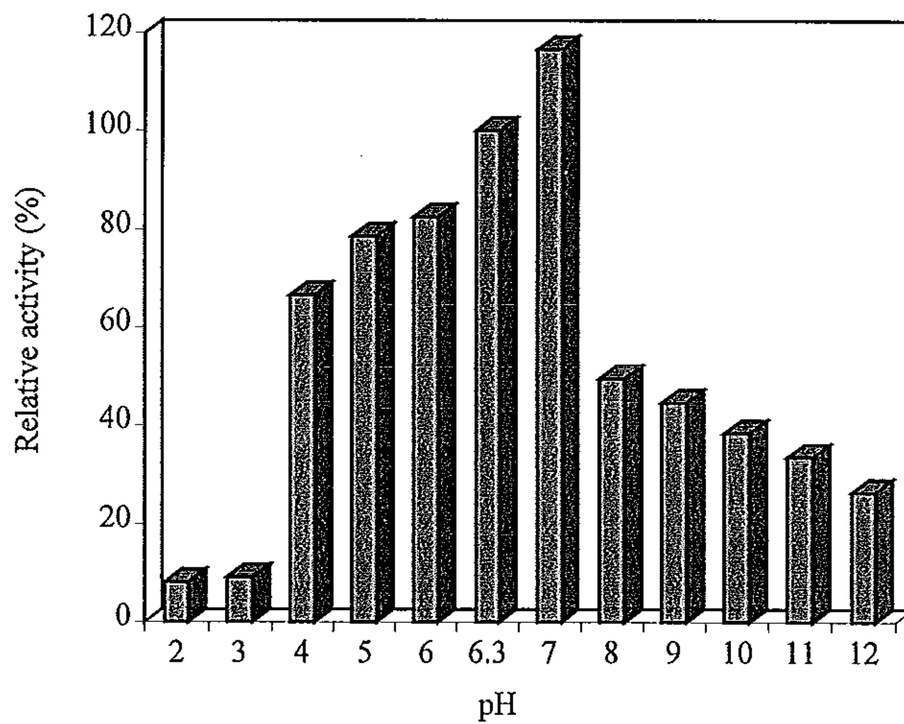


Fig. 16 pH stability of *Enterobacter cloacae* WD7 polysaccharide

4.7.3 Effect of polysaccharide concentration on the flocculating activity

The optimum concentration of polysaccharide for the flocculating activity was found to be 2 mg/l (Fig. 17). The polysaccharide dosage and the size of floc were related to each other at the increasing polysaccharide up to 2 mg/l and lead to the highest flocculating activity. The activity initially increased with increasing polysaccharide dosage, but then decreased as the adsorption of excess polysaccharide restabilized the kaolin particles. The results could be explained as follows :

1) The incomplete dispersion of excess polysaccharide, only the kaolin particles around the polysaccharides participated in the flocculation reaction. Therefore, other kaolin particles did not participate in the reaction (Yokoi, *et al.*, 1997).

2) The excess polysaccharide was over saturated on many binding sites of the surface of kaolin particles, thus the attractive force of the other particles was reduced and the flocculating activity decreased (Kwon, *et al.*, 1996).

This polysaccharide showed much higher flocculating activity than those of other flocculants previously reported (Table 13). So it is possible that this polysaccharide could be substitued for a commercial polymer in respect to flocculation.

Although the polysaccharide concentration affected directly the flocculating activity, the flocculating activity was also effectively increased only at an adequate concentration of kaolin clay. Moreover, the rate of flocculation was influenced by the size of floc and also by the density of both polysaccharide and kaolin clay. Thus, either the deficiency or excess amount of polysaccharide and kaolin clay decreased or even prevented the flocculating activity (Lee, *et al.*, 1995).

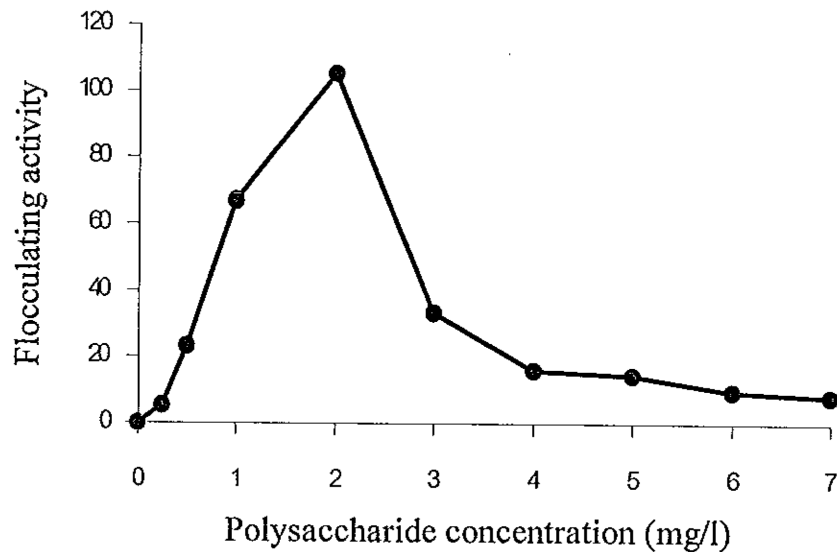


Fig. 17 Effect of *Enterobacter cloacae* WD7 polysaccharide concentration on the flocculating activity

Table 13 Comparison on the flocculating activity at the optimum dosage of various flocculants

Microorganism	Polymer	Dosage (mg/l)	Flocculating activity	Reference
Bacillus sp.	acidic PS*	1	43	Suh, *et al.* (1997)
Bacillus subtilis	polyglutamate	20	15	Yokoi, *et al.* (1995)
B. subtilis	polyglutamate	20	20	Yokoi, *et al.* (1996)
Rhodococcus erythropolis	protein	20	33	Takeda, et al. (1991)
Enterobacter sp.	acidic PS	20	125	Yokoi, *et al.* (1997)
Arcuadendron sp.	glycoprotein	2	17	Lee, *et al.* (1995)
Pestalotiopsis sp.	acidic PS	1	50	Kwon, *et al.* (1996)
Zoogloea ramigera	acidic PS	3	15	Suh, *et al.* (1997)
Enterobacter cloacae	acidic PS	2	105	This study

* = acidic polysaccharide

Flocculation depended greatly upon the concentration ratio of polymer to colloidal surface area, but of equal importance was the qualitative properties of the polymer, in particular the configuration and chain length necessary for optimal colloidal bridging. The primary function of the ionic functional groups was to maintain polymer elongation necessary for bridge formation, and that other polymer sites were involved in the specific sorptive reactions with the solid surface. Moreover, the elongation of the polymer chain would increase the probability of bridging (Harris and Mitchell, 1973).

4.7.4 Effect of cation types and concentrations on the flocculating activity

The results, as shown in Fig. 18, revealed the following :

1) The flocculation of kaolin clay suspension was observed only in the presence of a cation and not in their absence, even if the polysaccharide was added. So the addition of a cation to the reaction mixture was necessary to induce the effective flocculation. This suggests that the polysaccharide could not bind to kaolin clay directly without a cation. Consequently, the flocculation mechanism may be explained by forming complexes of the polysaccharide and kaolin clay mediated by a cation (Kurane, *et al.*, 1994).

2) All cations at low concentration (0.01 and 0.1 mM) had less effectiveness for enhancing the flocculation. However, increasing the concentration of most cations resulted in an increase of the flocculating activity. The optimum concentration of each cation was summarized in Table 14. It was found that $CaCl_2$ in the range of 10-100 mM and 1 mM $FeCl_3$ were more effective than other cations in kaolin flocculation. Although $FeCl_3$ possessed a slightly higher flocculating activity compared to $CaCl_2$ at 10 mM, both of them gave the different appearance of flocs. The Ca^{2+} enhanced the

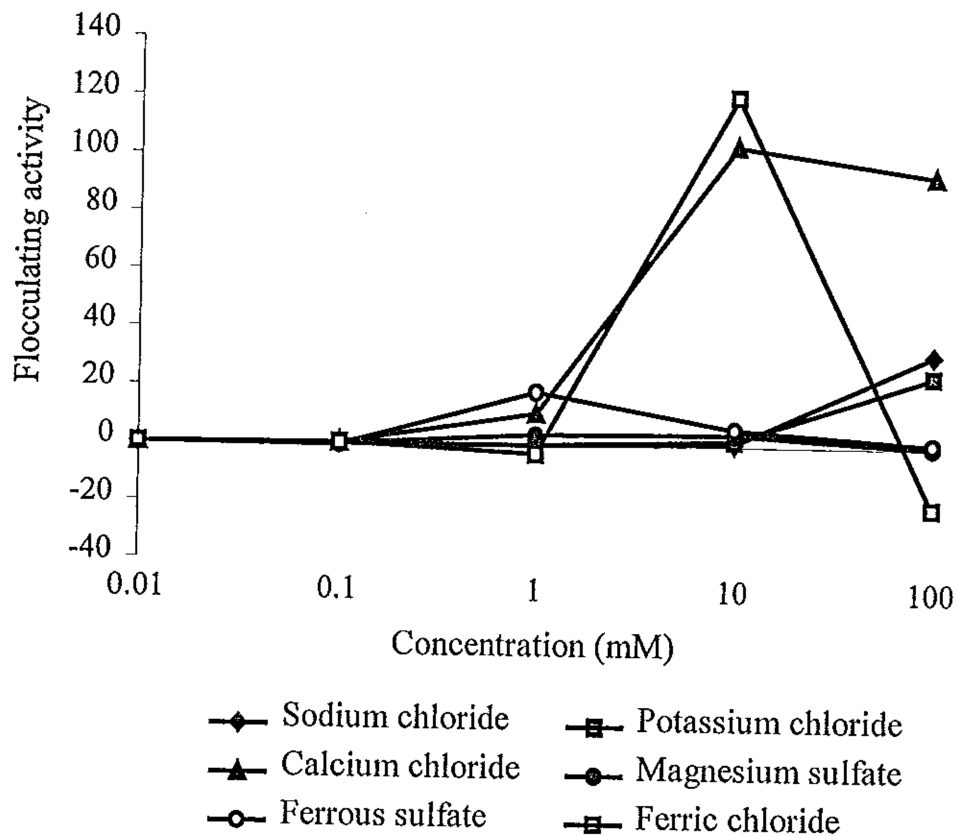


Fig. 18 Effect of cationic salt types and concentration on the flocculating activity of *Enterobacter cloacae* WD7 polysaccharide

Table 14 The optimum concentration of each cationic salt for flocculation of kaolin suspension

Cationic salt	Optimum dosage* (mM)	Flocculating activity
NaCl	100	32
KCl	100	25
$CaCl_2$	10 - 100	94 - 104
$MgSO_4$	1 - 10	3.5 - 4
$FeSO_4$	1	18
$FeCl_3$	10	120

* Note : Concentration range tested was 0.01 - 100 mM

formation of the large and compact floc whereas Fe^{3+} formed the gelatinous precipitates which were more difficult to remove.

Therefore in practical application, $CaCl_2$ was more interesting and effective than other cationic salts. Since $CaCl_2$ was the cation selected for enhancing the flocculation, further investigation on its optimum concentration for flocculation of kaolin clay was found to be 40 mM (Fig.19). The flocculating activity increased with increasing $CaCl_2$ concentration and reached its highest value at 40 mM then decreased rapidly at higher concentrations. The excess dose of $CaCl_2$ may cause the restabilization of the kaolin suspension and lead to the low flocculating activity.

The divalent calcium ion may affect anionic polysaccharide-clay systems in the following ways (Faust and Aly, 1983) :

(a) By compressing the thickness of the double layer of the clay particles, thereby reducing interparticle repulsive forces.

(b) By reducing the repulsive forces between the anionic polysaccharide and clay particles.

(c) By reducing interaction between polysaccharide molecule adsorbed on clay particle surfaces.

3) Mechanisms involved in flocculation by these cations and polysaccharide

Normally these cations can act as chemical coagulants to bring about the destabilization of the suspension, however, in flocculation reaction which also contains polysaccharide, the polysaccharide acts as high molecular weight polymer to destabilize colloidal dispersions. As mentioned above, polysaccharide itself could not flocculate the kaolin particles so the cation

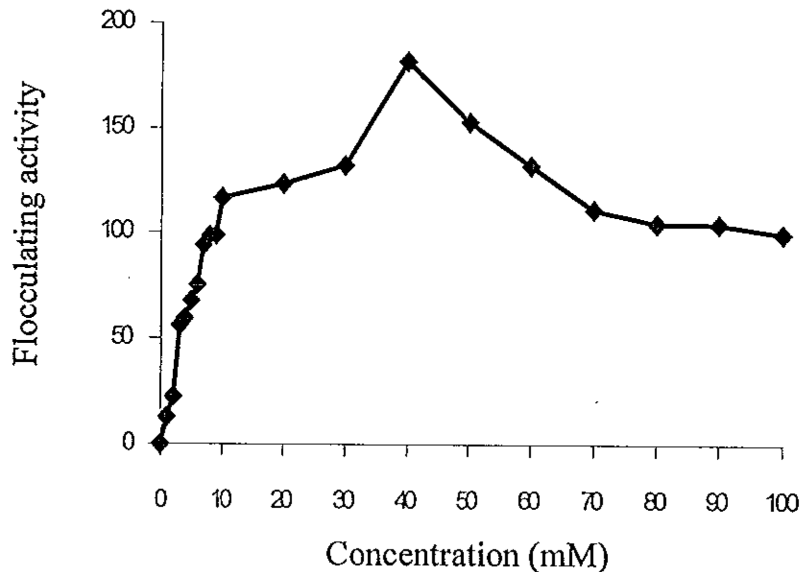


Fig. 19 Effect of $CaCl_2$ concentration on the flocculating activity of *Enterobacter cloacae* WD7 polysaccharide

plays the synergistic effect on kaolin flocculation. The surface charge of kaolin particles in aqueous solution was negative, by adding the cation, the electrically repulsive forces exist between the polysaccharide and kaolin particles and/or among kaolin particles decreased as the binding distance was shortened (Kwon, *et al.*, 1996).

Moreover, the cation could stimulate the flocculation by neutralization and destabilization of residual negative charges of carboxyl groups of uronic acid in an acidic polysaccharide, forming bridges which bind kaolin particles to each other (Yokoi, *et al.*, 1997). This was related to the explanation of Faust and Aly (1983) that flocculation, principally with the Ca^{2+}, occurs by adsorption of polysaccharides on the colloidal surface and from bridging of polysaccharide chains between solid particles if an appropriate content of an electrolyte is present. Complex formation occurs in the vicinity of the colloid's surface between the counterion and functional groups of the polyelectrolyte.

This mechanism apparently plays a major role in the attachment of anionic polyelectrolytes to negative hydrophobic colloids.

4.7.5 Effect of pH on the flocculation reaction

The results (Fig. 20) revealed that the flocculating activity was not affected by the pH of reaction mixture at high acidic to neutral pH in the range of pH 2-8, indicating a wide range pH for flocculation of this polysaccharide. However, the highest flocculating activity was observed at pH 6. At higher pH of 9-12, the flocculating activity decreased gradually. This suggested that the hydroxide ion (OH^-) may interfere with the complex formation of the polysaccharide and kaolin particles mediated by Ca^{2+}, consequently the kaolin particles were suspended in the mixture. The pH range for flocculation reaction of the polymer from *E. cloacae* WD7 was wider than those of the polyglutamate from *Bacillus subtilis* PY-90, with the pH of reaction mixture in the range of 3-5 (Yokoi, *et al.*, 1995), and the cationic polysaccharide from *Paecilomyces* sp.I-1 in the pH range of 4-8 for the flocculating activity against *E. coli* cells suspension (Takagi and Kadowaki, 1985).

Normally, changes in pH would tend to reduce the repulsive potential between particles and thus increased the particles contact. Such reductions in surface potential were subsidiary to bridge formation by the polysaccharide (Harris and Mitchell, 1973). The fluctuation in pH also altered the attraction and repulsion of the functional groups within the polysaccharide, led to the decreased elongation at constant molecular weight and lowered the probability of polysaccharide bridging.

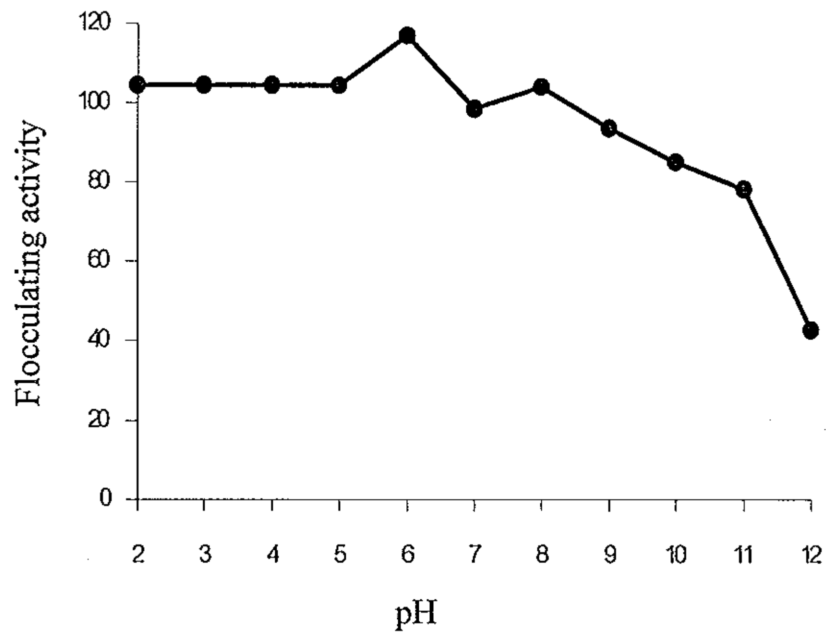


Fig. 20 Effect of pH on the flocculation reaction of *Enterobacter cloacae* WD7 polysaccharide

4.7.6 Effect of temperature on the flocculation reaction

The flocculating activity was influence by the temperature during flocculation reaction as it increased with increasing reaction temperature (Fig. 21). This was due to the increase in entropy and also indicated the wide range of temperature for flocculation of this polysaccharide. The acceleration by high temperature (50 °C) of this polymer better than the acidic polysaccharide produced by *Enterobacter* sp. BY-29 in which the optimum flocculation reaction temperature against kaolin suspension was 25 °C (Yokoi, *et al.*, 1997). However, it was previously reported that the reaction temperature in the range of 0-100 °C did not influence the flocculation against cell suspension of *E. coli* by cationic polysaccharide produced by *Paecilomyces* sp. I-1 (Takagi and Kadowaki, 1985).Furthermore, optimum temperature for flocculation of polyglutamate from *Bacillus subtilis* IFO 3335 was approximately 70 °C in kaolin suspension and the flocculating activity decreased markedly at temperatures higher than 80°C (Yokoi, *et al.*, 1996). The influxes of temperature did not greatly affect the rate of flocculation, and the rate of colloid particle collisions (aggregation) were more affected by the rate of fluid motion (mixing) than by temperature (Mohtadi and Rao, 1973 cited by Green and Kramer, 1979).

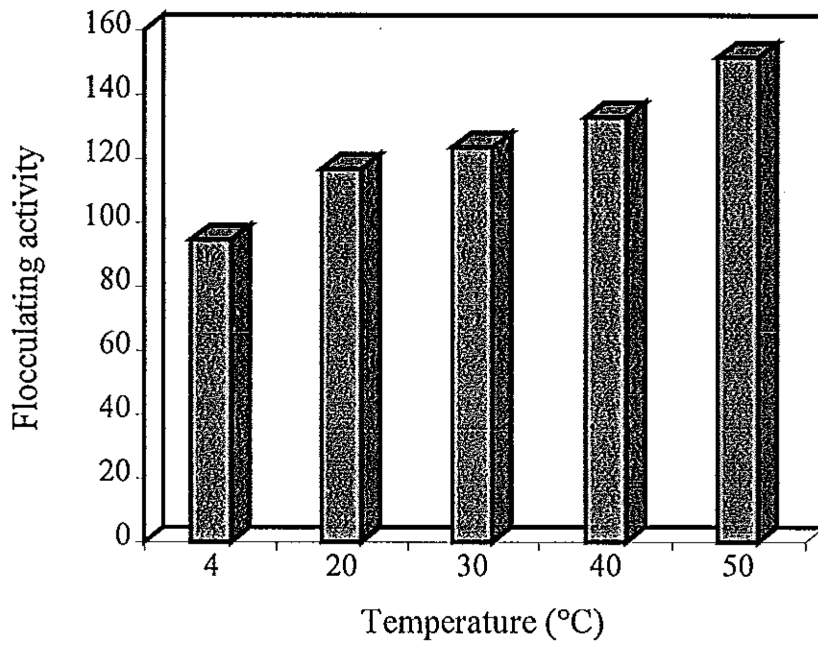


Fig. 21 Effect of temperature on the flocculation reaction of *Enterobacter cloacae* WD7 polysaccharide

Chapter 4

Conclusions

Seafood activated sludge was chosen as a source for the isolation of polymeric flocculant-producing bacteria. This sludge is in general a type of bioflocculation system primarily consisting of flocs which are bound together by means of exocellular polymers produced mainly by bacteria and chemical compounds in the wastewater. A total of 188 strains of polymer-producing bacteria were isolated during these studies, using a screening medium with 1% glucose or sucrose as carbon source. The isolation of high potential strains was affected by the formulation of media and environmental parameters such as pH, temperature and oxygen.

In comparing the ability of the produced polymer to flocculate a kaolin suspension, strain WD7 was selected on the observation of possessing the highest flocculating activity (10.28) after 3 days cultivation in basal medium (glucose 1% as carbon source). This strain was identified as *Enterobacter cloacae.*

The partially purified biopolymer from *E. cloacae* WD7 was characterized as an acidic heteropolysaccharide containing neutral sugar (29.4%) and sugar derivatives of uronic acids (14.18%) and amino sugar (0.93%), in which uronic acids are the principal charged components. The hydroxyl, carboxyl, carbonyl and methoxyl groups present in this polysaccharide are responsible for its physical and chemical properties. The major hydroxyl groups possess a strong affinity for water, so the polysaccharide can be hydrated, swelled and usually underwent partial or complete dissolution, exhibiting the viscous solution. This polysaccharide can therefore be applied as a thickening agent in foods and also to modify/control the flow properties and textures of liquid food and beverage products. Moreover, these hydroxyl groups

build up strong forces of attraction between polysaccharide molecules, resulting in a relative hard crystalline structure which was correlated to its thermal property by DSC indicating the amorphous and crystalline regions and exhibiting the crystalline melting point (T_m) at 300 °C. The degree of high T_m resulted in strong, tough, stiff and more resistancy to solvents and chemicals. Consequently, it is possible to develop a desirable and effective polymeric material by blending or mixing with this polysaccharide. Besides the hydroxyl groups, the carboxyl groups occurring in uronic acids also play important roles for this polysaccharide such as causing the acidic charge of the polysaccharide and also serving as binding sites for cations. This polysaccharide can therefore also act as a chelating agent to bind some metal ions. Moreover, its carboxyl groups might be used to link this polysaccharide to starch or man-made polymers to form polymers having different properties. Although a high concentration of this polysaccharide solution can become "gel-like", its diluted solution (low concentration) can form true gels, "the ionic gels", with divalent ions such as Ca^{2+} and Cu^{2+} under alkaline condition. Thus, this polysaccharide can act as an eliminator or remover for Ca^{2+} and Cu^{2+} in wastewater or as an immobilization agent.

Flocculation property of the biopolymer is shown below :

Item	Range
• Temperature stability	up to 60 °C
• pH stability	pH 5-7
• Optimum biopolymer concentration	2 mg/l
• Optimum cation type and concentration	$CaCl_2$ 40 mM
• pH of reaction	pH 2-8
• Temperature of reaction	4 - 50 °C

The thermal stability (up to 60 °C) and pH stability (pH 5-7) of this polysaccharide are useful for any aqueous systems which use low level heating or weak acidic to neutral conditions. For the application of this polysaccharide as a flocculant, it was more interesting and favorable than using metal coagulants such as aluminium and iron salts and synthetic polymers (polyelectrolytes) such as polyacylamide and its derivatives, because it is a biopolymer which can be easily degraded in nature. Due to its polyelectrolyte behavior (high molecular weight polymer with acidic charged property), a replacement of the chemical synthetic polymers with this biopolymer is possible and of advantage.

It is furthermore of considerable interest to apply this polysaccharide for solid-liquid separation as flocculant not only for wastewater treatment (primary and secondary stages, sludge thickening and sludge dewatering) but also for fermentation industries especially in downstream processess. With several functional properties, this polysaccharide can be widely used in many fields such as food and non-food applications. However, its properties can be improved by either chemical or enzymic modification to obtain more potential applications.

Suggestions

The results of the research work presented lead to the following suggestions :

1. **Improving the knowledge on the biopolymer characteristics**
    a) further elucidation of the structure of the biopolymer using modern analytical procedures such as HPLC, GC, NMR etc.
    b) the behaviour of a polymer mixture, when the biopolymer is being mixed with a synthetic polymer at various ratios.
2. **Scale-up of the biopolymer production** from *Enterobacter cloacae* WD7. This would require further investigations into
    a) minimising the production costs in using cheap carbon (e.g. molasses etc.) and nitrogen sources (e.g. tuna condensate etc.)
    b) minimising recovery costs using simple methods such as fractional precipitation with organic solvents such as ethanol, methanol etc.
3. **Market research for the application of the biopolymer**, which would require further investigations into the use of
    a) as a flocculant in wastewater to remove suspended solids
    b) as a flocculant in fermentation industries to remove microbial cells
    c) as a thickening agent in the food industry, e.g. ice cream, gelatin, etc.
    d) as an agent for the more economic production of oil from oil wells.
4. **Biodegradability** of the biopolymer through exposing the polymer to various microorganisms and studying the biodegradability.
5. **Lipase production**

Pseudomonas alcaligenes WD22 has been shown to possess a strong lipid hydrolysing activity and thus should be explored for the possibility of lipase production.

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Appendices

Appendix 1

Qualitative Analysis of Biopolymer

Ninhydrin reaction (Plummer, 1978)

Objective : To detect alpha-amino acids

Reagents

-biopolymer solution, 1 mg/ml

-standard amino acid of L-leucine, 1 mg/ml

-ninhydrin reagent : dissolve 0.04 g of ninhydrin in 20 ml of absolute ethanol (kept avoid from light)

Method

1. Two ml of amino acid solution/biopolymer solution/water was added into test tube.
2. Ninhydrin solution 0.4 ml was added and mixed together.
3. Boil in water for 2 min.
4. Observe the change of colour of solution, if there is amino acid moiety, the solution would be violet/blue.

Interpretation :

ninhydrin positive : violet colour after heating

Xanthoproteic reaction (Plummer, 1978)

Objective : To detect aromatic amino acid

Reagents

-biopolymer solution, 1 mg/ml

-standard amino acid of tryptophan, 1 mg/ml

-conc. nitric acid (HNO_3)

-3 M NaOH

Method

1. One ml of standard amino acid solution/biopolymer solution/water was added into test tube.
2. Conc.HNO_3 3 ml was added, then warmed over boiling water.
3. Cool and observe the colour changes of solution in acid condition.
4. NaOH solution was added and observe change of colour of solution in alkali condition.

Interpretation :

Xanthoproteic positive : yellow colour in acid condition

: orange colour in alkali condition

Quantitative Analysis of Biopolymer

Phenol sulfuric acid reaction (Dubois, *et al.*, 1956 cited by Chaplin and Kennedy, 1986)

Objective : To determine amount of total carbohydrate

Reagents

-phenol solution : dissolve phenol in water (5% w/v)

-conc. H_2SO_4

-biopolymer solution, 0.2 mg/ml

-standard solution of glucose, 0-100 μg/ml

Method

1. Biopolymer solution/standard/water each 600 μl was mixed with 600 μl of phenol reagent in test tube.
2. Add 3 ml of conc. H_2SO_4 rapidly and directly to the solution surface without allowing it to touch the sides of the tube
3. Leave the solution undisturbed for 10 min before vigorous shaking.
4. Measure the absorbance at 490 nm after a further 30 min.

Anthrone reaction (Trelyan and Harrison, 1952 cited by Chaplin and Kennedy, 1986)

Objective : To determine amount of neutral sugar

Reagents

-Anthrone reagent : add 50 ml conc. H_2SO_4 in 20 ml distilled water, then dissolve 0.392 g of anthrone (must prepare freshly).

-biopolymer solution, 0.2 mg/ml

-standard solution of glucose, 0-100 μg/ml

Method

1. Add 5 ml anthrone reagent into screw cap test tube (prevention of evaporation) and cool in ice bath.
2. Add 1 ml biopolymer solution/standard/water on surface of anthrone reagent.
3. Leave for 10-15 min to get the complete diffusion of mixture.
4. Mix the solution by vortex mixing, then heating at 100 °C for 10 min.
5. Cooling in ice bath and determine the absorbance of greenish-blue solution at 620 nm.

Carbazole-sulfate reaction (Bitter and Muir, 1962 cited by Chaplin and Kennedy, 1986)

Objective : To detect amount of uronic acids

Reagents

-Reagent A : dissolve sodium tetraborate decahydrate (borax) 0.95 g in 2 ml of hot water, then add 98 ml of conc.H_2SO_4 (prepare early 1 day before use)

-Reagent B : dissolve carbazole 0.125 g in 100 ml of ethanol (keep in dark, closed container and cool)

-biopolymer solution, 0.2 mg/ml

-standard solution of glucuronolactone, 0-100 μg/ml

Method

1. Add 750 μl biopolymer solution/standard/water in screw cap test tube and cool in ice bath.
2. Add 4.5 ml cold reagent A, mix with vortex mixer and cool in ice bath.
3. Heat the mixture at 100 °C for 15 min.
4. Cool rapidly in ice bath.
5. Add 150 μl of reagent B and mix well.
6. Reheat the mixture at 100 °C for 15 min.
7. Cool rapidly to room temperature and measure the absorbance at 525 nm.

Elson-Morgan reaction (Elson and Morgan, 1933 cited by Chaplin and Kennedy, 1986)

Objective : To determine the amount of amino sugar such as hexosamine (glucosamine and galactosamine)

Reagents

-Reagent A : dissolve 1 ml acetylacetone in 50 ml of 0.5M Na_2CO_3

-Reagent B : dissolve 0.4 g 4(N,N-dimethylamino)benzaldehyde in 15 ml absolute ethanol, then mix with 15 ml of conc. HCl

-standard mixed solution of glucosamine and galactosamine, 0-100 μ g/ml

-absolute ethanol

-0.2 and 4 M HCl

-0.5 and 4 M NaOH

-phenolphthalein solution (dissolve 1 mg of phenolphthalein in 5 ml of ethanol then mixed with 45 ml of water)

Method

1. Preparation of biopolymer hydrolysate

   1.1 dissolve 1 mg of biopolymer in 1 ml of 4 M HCl, then hydrolysed at 95 °C for 4 h.

   1.2 adjust pH of hydrolysate to pH 10 as following : add phenolphthalein 8 drops in 2 ml of hydrolysate , then add 4 M NaOH until the solution became to pink-red colour and add 4M HCl until solution was clear (red colour disappeared).

   1.3 find the total volume of hydrolysate after adjusting pH.

2. Mix 750 μl hydrolysate solution/standard solution/water with 750 μl reagent A in test tube, then adjust final volume of solution to be 1800 μl with distilled water.
3. Stopper the test tube, heat at 100 °C for 20 min and cool the solution rapidly to room temperature.
4. Add 3 ml absolute ethanol carefully to wash liquid from condensation (at the sides of tube).
5. Add 750 μl reagent B and dilute with absolute ethanol to adjust the final volume of solution to be 7.2 ml.
6. Heat at 65 °C for 10 min.
7. Cool in ice bath and measure the absorbance at 530 nm.

Appendix 2

Standard Curve of OD_{660} and Viable Cells Count of Strain WD7, WD22 and WD50

Microorganisms

Enterobacter cloacae WD7, *Pseudomonas alcaligenes* WD22 and *Enterobacter agglomerans* WD50

Method

Growth profile of selected strains in nutrient broth

One loopful of each microorganism, grown on nutrient agar plates for 24 h, was inoculated into 50 ml nutrient broth in 250 ml flask. Cultivations were carried out on a rotary shaker at 200 rpm at room temperature for 24 h. Every 4 h, the culture broth was taken to measure OD_{660} (5 times dilution) and viable cells count.

Enumeration of viable cell count was carried out by spread plate method as follows : One ml of culture broth was serially diluted with 0.1% peptone water. Each serial dilution for 0.1 ml was dropped on standard plate count agar (PCA) which consisted 0.5% tryptone, 0.1% glucose, 0.25% yeast extract and 1.5% agar (pH 7.0). Then it was spread over the surface of agar. All plates were incubated at 37 °C for 24 h, the amount of microbe were reported as CFU/ml.

The result of growth profiles of the 3 strains in nutrient broth were illustrated in Fig.1 and Fig.2. OD_{660} values were related to the viable cells counts (log CFU/ml). With the suitable time and viable cells count, therefore the cultivation time for 12 h was chosen to determine the standard curve of OD_{660} (5 times dilution) and viable cells count for these strains.

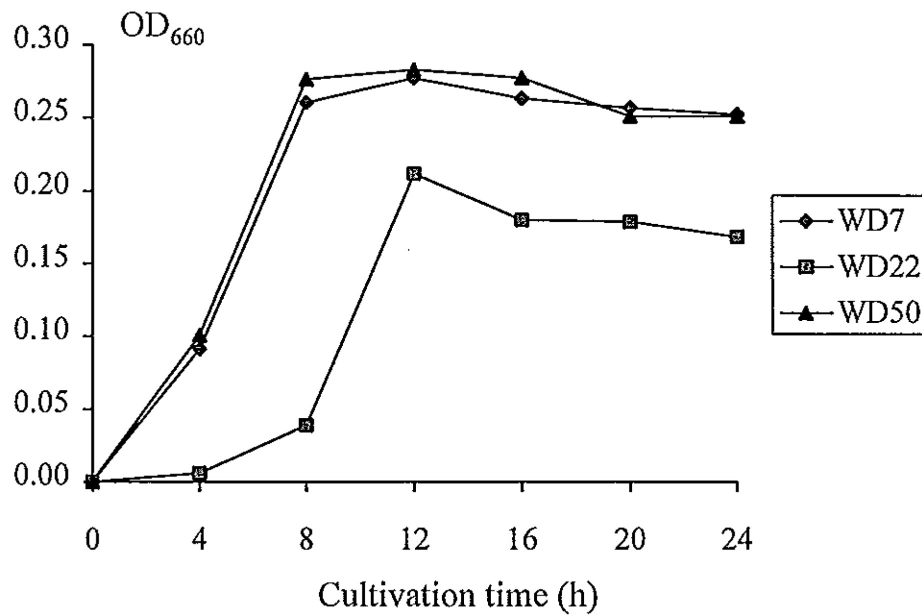

Fig. 1 Changes of OD_{660} (5 times dilution) of 3 selected strains in nutrient broth for 24 h cultivation under shaken condition at 200 rpm , room temperature

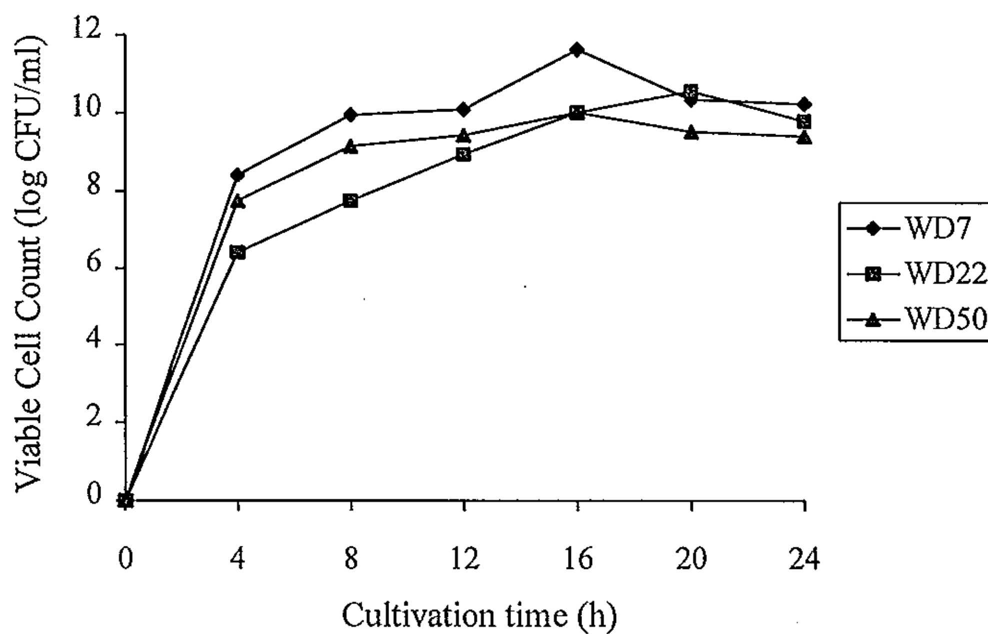

Fig. 2 Changes of viable cell count of 3 selected strains in nutrient broth for 24 h cultivation under shaken condition at 200 rpm, room temperature

Standard curve of OD_{660} and viable cells count at 12 h cultivation

Cultivation of each strain for 12 h was carried out as above. The 12 h culture broth of each strain was diluted with 0.1% peptone water for 0, 2, 5 and 10 fold, diluted culture broth was taken to measure OD_{660} (5 times dilution) and viable cells count as above. The results were shown in Fig. 3(a), (b) and (c).

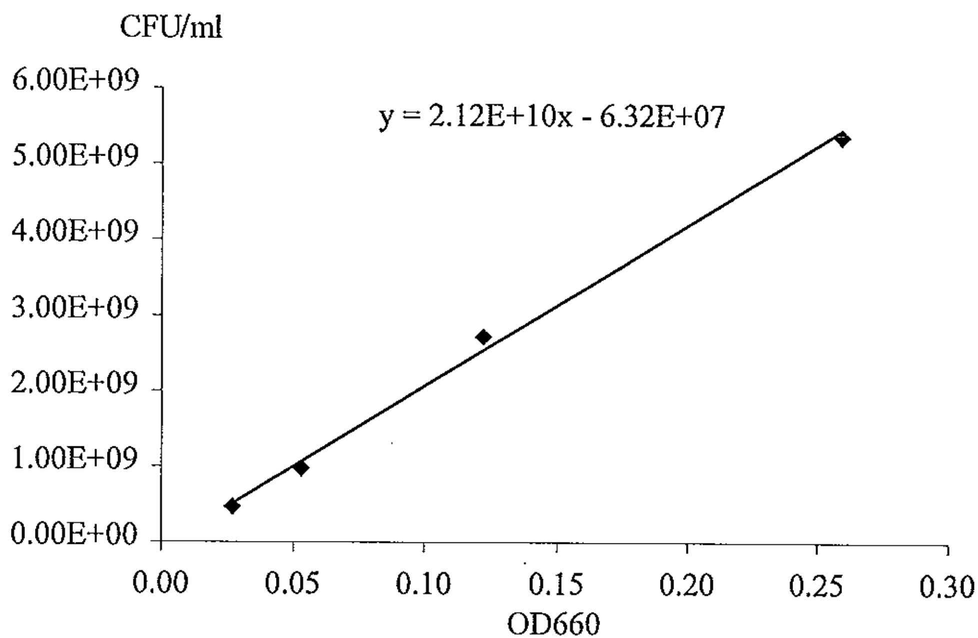

Fig. 3 (a) Standard curve of OD_{660} (5 times dilution) and viable cells count of *Enterobacter cloacae* WD7 in nutrient broth

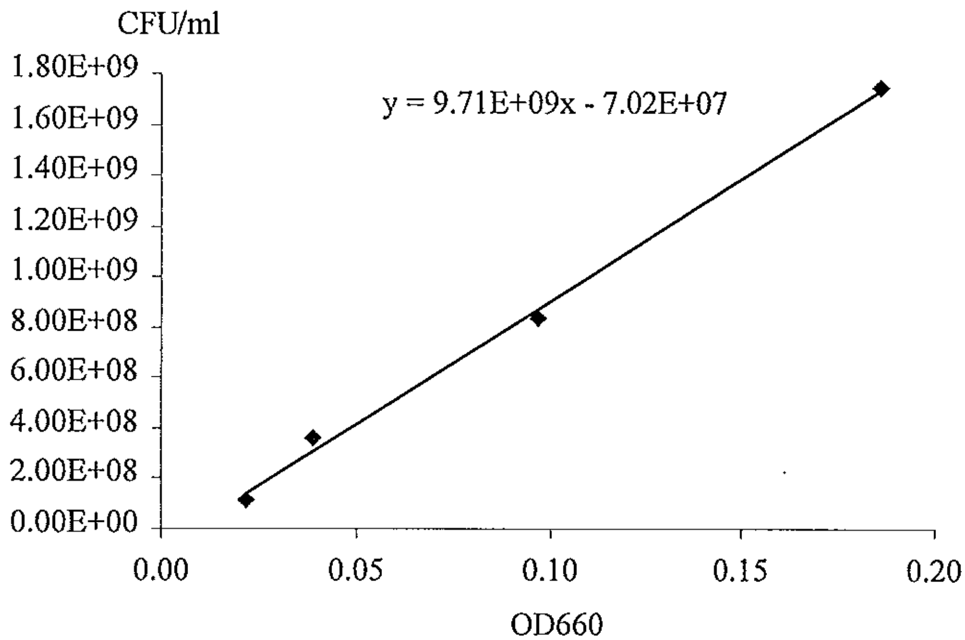

Fig. 3 (b) Standard curve of OD_{660} (5 times dilution) and viable cells count of *Pseudomonas alcaligenes WD22* in nutrient broth

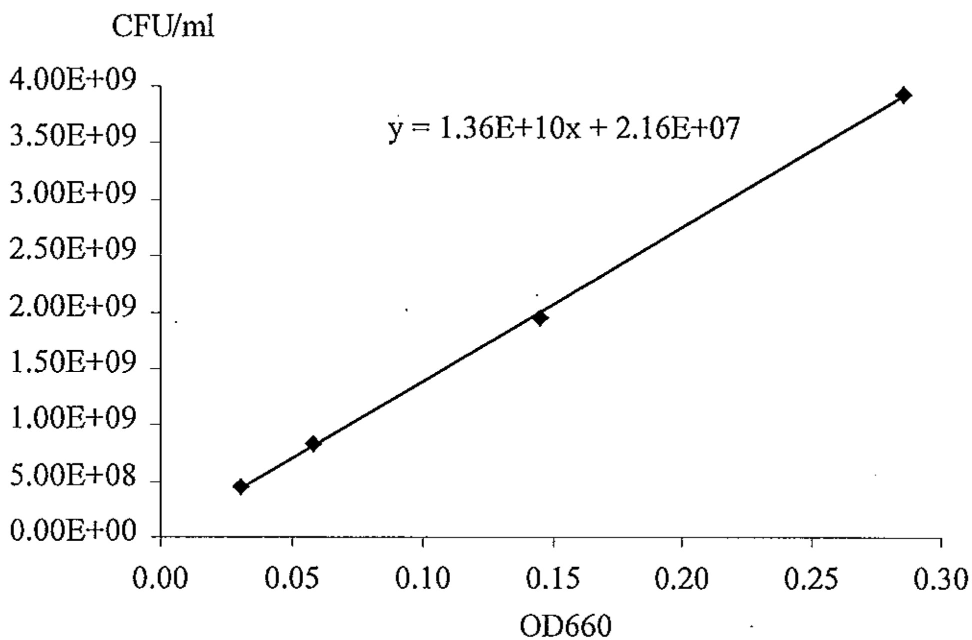

Fig. 3 (c) Standard curve of OD_{660} (5 times dilution) and viable cells count of *Enterobacter agglomerans* WD50 in nutrient broth

Appendix 3

Table 1 Time course of polymer production by *Enterobacter cloacae* WD7 in basal medium (1% glucose as carbon source)

Parameter	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
pH	7.07	5.43	5.29	5.75	5.73	6.27
Cell dry weight (g/l)	0.01	0.50	0.52	0.55	0.57	0.58
Crude polymer (g/l)	0	1.83	1.97	2.27	2.07	1.93
Flocculating activity	-0.10	3.42	4.37	10.28	4.95	4.72
Flocculation rate (%)	-10.82	77.00	80.77	90.74	82.40	81.68

Table 2 Time course of polymer production by *Pseudomonas alcaligenes* WD22 in basal medium (1% glucose as carbon source)

Parameter	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
pH	7.07	6.86	6.68	6.61	6.55	6.52
Cell dry weight (g/l)	0.01	0.23	0.33	0.40	0.45	0.48
Crude polymer (g/l)	0	2.49	2.89	3.41	3.27	3.23
Flocculating activity	-0.04	0.44	0.54	1.43	0.85	0.78
Flocculation rate (%)	-4.30	27.62	32.14	55.35	42.21	40.35

Table 3 Time course of polymer production by *Enterobacter agglomerans* WD50 in basal medium (1% sucrose as carbon source)

Parameter	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
pH	7.08	4.80	4.62	4.70	4.65	4.64
Cell dry weight (g/l)	0.01	0.31	0.50	0.66	0.75	0.79
Crude polymer (g/l)	0	0.67	0.73	0.83	0.80	0.67
Flocculating activity	-0.26	0.20	0.40	0.56	1.58	1.82
Flocculation rate (%)	-28.20	14.30	25.24	31.84	56.64	59.60

Table 4 Temperature stability of *E. cloace* WD7 polysaccharide

Temperature (°C)	Flocculating activity	Relative activity (%)
4	223.75	135.26
30	165.42	100.00
40	182.08	110.08
50	165.42	100.00
60	415.42	251.13
70	223.75	135.26
80	141.61	85.61
90	94.20	56.95
100	70.18	42.42
110	3.28	1.98
120	0.84	0.50

reference temperature = 30 °C

Table 5 pH stability of *E. cloacae* WD7 polysaccharide

pH	Flocculating activity	Relative activity (%)
2	11.48	8.11
3	13.01	9.18
4	94.17	66.52
5	111.22	78.56
6	116.77	82.48
6.28	141.57	100.00
7	165.38	116.82
8	70.14	49.55
9	63.30	44.71
10	54.44	38.46
11	47.53	33.57
12	37.41	26.42

reference pH = 6.28

Table 6 Effect of *E. cloacae* WD7 polysaccharide concentration on the flocculating activity

Concentration (mg/l)	Flocculating activity	Flocculation rate (%)
0	0.01	0.00
0.2	5.25	81.10
0.5	23.23	94.29
1	67.05	97.83
2	105.27	98.83
3	33.36	96.50
4	15.84	92.76
5	14.38	92.16
6	9.60	88.78
7	8.16	87.09

Table 7 Effect of cationic salt types and concentrations on the flocculating activity

Salt	Concentration (mM)	Flocculating activity	Flocculation rate (%)
NaCl	0.01	-0.013	-3.27
	0.10	0.004	0.94
	1.00	0.009	2.08
	10.00	0.94	68.61
	100.00	32.20	98.20
KCl	0.01	-0.004	-0.92
	0.10	0.013	3.08
	1.00	0.262	38.27
	10.00	2.116	82.17
	100.00	24.61	97.18
$CaCl_2{\cdot}2H_2O$	0.01	-0.007	-1.66
	0.10	0.307	42.21
	1.00	10.87	93.82
	10.00	103.93	98.45
	100.00	94.14	98.62
$MgSO_4{\cdot}7H_2O$	0.01	-0.003	-0.83
	0.10	0.045	9.58
	1.00	3.506	88.43
	10.00	4.016	77.72
	100.00	0.134	13.28
$FeSO_4{\cdot}7H_2O$	0.01	-0.006	-1.53
	0.10	-0.264	-66.49
	1.00	18.24	58.79
	10.00	5.96	51.67
	100.00	1.30	41.98
$FeCl_3{\cdot}6H_2O$	0.01	0	-0.12
	0.10	0.440	43.00
	1.00	-2.928	-25.36
	10.00	120.86	78.00
	100.00	-20.83	-20.00

Table 8 Effect of $CaCl_2$ concentrations on the flocculating activity

Concentration (mM)	Flocculating activity	Flocculation rate (%)
0	0.006	1.37
1	12.88	95.30
2	22.58	94.34
3	55.92	97.78
4	59.30	97.74
5	67.64	97.96
6	75.12	97.66
7	93.94	98.42
8	98.41	98.41
9	98.71	98.71
10	116.37	98.57
20	123.36	98.68
30	132.06	98.60
40	181.80	99.16
50	153.00	98.86
60	132.00	98.56
70	110.97	98.62
80	104.18	98.69
90	104.02	98.54
100	99.47	98.46

Table 9 Effect of pH of the reaction on the flocculating activity

pH	Flocculating activity	Flocculation rate (%)
2	104.48	98.98
3	104.46	98.96
4	104.44	98.95
5	104.42	98.92
6	116.86	98.96
7	98.55	98.55
8	104.00	98.52
9	93.52	97.98
10	84.94	97.67
11	78.24	97.10
12	42.73	93.99

Table 10 Effect of temperature of reaction on the flocculating activity

Temperature (°C)	Flocculating activity	Flocculation rate (%)
4	94.64	99.14
20	116.84	98.96
30	123.56	98.85
40	133.00	98.60
50	151.78	98.05

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- Dermlim, W., Prasertsan, P. and Doelle, H.W. 1998. Screening and characterization of bioflocculant produced by isolated *Klebsiella* sp. Paper presented at UNESCO International Training Course on Current Trends in Microbial Technology for a Sustainable Environment : Exploring Microbial Biodiversity for Novel Processes. October 12-24, 1998, Kuala Lumpur, Malaysia.

- Dermlim, W., Prasertsan, P. and Doelle, H.W. 1998. Production of microbial flocculant. The 10th Annual Meeting of the Thai Society for Biotechnology and The 1998 Annual Meeting of the National Center for Genetic Engineering and Biotechnology on Biotechnology for a Self-Sufficient Economy. November 25-27, 1998, Bangkok, Thailand.

Vitae

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