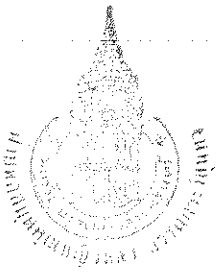

Scale-up for Production and Characterization of Biopolymer from *Enterobacter cloacae* WD7

Chaiwat Bandaiphet

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ชื่อวิทยานิพนธ์ การขยายขนาดสำหรับการผลิตและลักษณะของพอลิเมอร์จาก *Enterobacter cloacae* WD7

ผู้เขียน นายชัยวัฒน์ บรรใดเพ็ชร

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บทคัดย่อ

จากการศึกษาการขยายขนาดการผลิตพอลิเมอร์ของ *Enterobacter cloacae* WD7 ในถังหมักขนาดใหญ่ (72 ลิตร) เปรียบเทียบกับผลที่ได้จากการหมักในถังหมักขนาดเล็ก (5 ลิตร)ภายใต้การหมักแบบใช้อากาศโดยการคงค่าสัมประสิทธิการส่งผ่านออกซิเจน (k_La) พบว่าผลผลิตพอลิเมอร์จากการหมักในถังหมักขนาดเล็กเพิ่มขึ้นเมื่อเพิ่มอัตราการให้อากาศ แต่ลดลงเมื่อเพิ่มอัตราการกวน ในถังหมักขนาดใหญ่การเจริญและผลผลิตพอลิเมอร์เพิ่มขึ้นตามการเพิ่มขึ้นของทั้งอัตราการให้อากาศและการกวน อัตราการส่งผ่านออกซิเจน(OTR)สูงกว่าอัตราการใช้ออกซิเจน(OUR) ทั้งช่วง exponential (ชั่วโมงที่ 18) จนถึง stationary phases (ชั่วโมงที่ 36) ของการหมัก ค่า k_La ของการหมักทั้งสองระดับเพิ่มขึ้นเมื่อเพิ่มการให้อากาศและการกวน เมื่อเปรียบเทียบภายใต้สภาวะที่เหมาะสม ค่า k_La ในถังหมักขนาดเล็กในช่วง exponential และ stationary phases มีค่าเท่ากับ 9.97 และ 9.72 h^{-1} ตามลำดับ (ที่ 1.25 vvm และ 200 rpm) ขณะที่ในถังหมักขนาดใหญ่มีค่าเท่ากับ 9.68 และ 9.50 h^{-1} ตามลำดับ (ที่ 2.00 vvm และ 600 rpm) การขยายขนาดการหมักโดยการคงค่า k_La ในช่วง stationary phase ได้ค่า k_La ใกล้เคียงกันที่สุดเท่ากับ 9.72 และ 9.50 h^{-1} ในถังหมักขนาดเล็กและขนาดใหญ่ตามลำดับ คิดเป็นอัตราการขยายขนาดของ k_La เท่ากับ 0.98 (9.50/9.72) ซึ่งให้ผลผลิตพอลิเมอร์เท่ากับ 3.20 และ 3.07 กรัมต่อกรัมเซลล์ จากถังหมักขนาดใหญ่และขนาดเล็ก ตามลำดับ

การไหลของสารละลายพอลิเมอร์ที่ได้จาก *E. cloacae* WD7 เป็นแบบ pseudo-plastic behavior ในช่วงความเข้มข้นที่ 0.25 - 1.00 % (w/v) และเจลที่ความเข้มข้นช่วง 2-10% (w/v) พอลิเมอร์ชนิดนี้ไม่ทนต่อสภาวะที่มีค่าพีเอชที่สูงและต่ำมากรวมถึงที่อุณหภูมิสูงเนื่องจากความหนืดลดลง อุณหภูมิและพีเอชที่เหมาะสมคือ ที่ 50°ซ และพีเอชช่วง 6-7 การใช้ความร้อนสูงมากกว่า 100°ซ ทำให้พอลิเมอร์เปลี่ยนรูปและความหนืดลดลงอย่างมาก แต่การใช้อุณหภูมิที่ 65°ซ นาน 120 นาที มีผลต่อการเปลี่ยนแปลงความหนืดน้อยมาก นอกจากนี้สารละลายและเจลของพอลิเมอร์สามารถเก็บรักษาที่อุณหภูมิ 4°ซ และ -20°ซ ได้เป็นเวลาอย่าง

น้อย 2 เดือน ยิ่งกว่านั้นยังสามารถแช่แข็งและละลายน้ำแข็งสลับกันโดยไม่พบการเปลี่ยนแปลงพฤติกรรมการไหลและความหนืด สารที่มีประจุบวกหนึ่งบางตัว (Li^+ และ Na^+) และสารที่มีประจุบวกสองบางตัว (Mn^{2+} และ Mg^{2+}) สามารถเพิ่มการเกิดเจลของสารละลายพอลิเมอร์นี้

พอลิเมอร์ที่ผลิตจาก *E. cloacae* WD7 สามารถเตรียมให้อยู่ในรูปผง ฟิล์มและฟองน้ำ สภาวะที่เหมาะสมสำหรับการขึ้นรูปฟิล์มโดยเทคนิคของการระเหยน้ำออกจากสารละลายพอลิเมอร์ (2-10% w/v) คือที่อุณหภูมิที่ 50°ซ และความชื้นสัมพัทธ์ที่ 50% ได้ฟิล์มที่เปราะและไม่ยืดหยุ่น การปรับปรุงลักษณะและคุณสมบัติของฟิล์มของพอลิเมอร์นี้ทำโดยการเติม plasticizers (กลีเซอรอล, ซูโครส, พอลิเอทธิลีนไกลคอล และซอร์บิทอล) ที่อัตราส่วนที่ 1.0:0.5 พบว่า ความยืดหยุ่นของฟิล์ม (elongation at break) เพิ่มขึ้นจากเดิม 1.38% เป็น 43.55, 31.80, 30.60 และ 25.78 % ตามลำดับ ขณะที่ค่าความแข็งแรง(tensile strength) ของฟิล์ม ลดลงจาก 1.358 ปาสคาล เป็น 0.477, 0.217, 0.473 และ 0.247 ปาสคาล ตามลำดับ กลีเซอรอลเป็น plasticizer ที่ดีที่สุดที่ให้ค่าความยืดหยุ่นของฟิล์มมากกว่า 40 เท่าของฟิล์มปกติอย่างไรก็ตามทั้งฟิล์มปกติและฟิล์มดัดแปรสามารถละลายได้ในน้ำ สารละลายบัฟเฟอร์, สารละลายกรด (1M HCl) และด่าง (1M NaOH) และให้ค่าการส่งผ่านไอน้ำ (water vapor permeability) ได้ต่ำมาก ส่วนฟองน้ำของพอลิเมอร์จาก *E. cloacae* WD7 ทำโดยการขึ้นรูปในแม่แบบพลาสติกด้วยวิธีการทำแห้งแบบเยือกแข็งที่อุณหภูมิต่ำกว่า -80°ซ ฟองน้ำที่ได้มีความนุ่ม ดัดให้โค้งงอได้และมีความหนาเพิ่มขึ้นตามความเข้มข้นของพอลิเมอร์ เพื่อปรับปรุงคุณสมบัติของฟองน้ำ, พอลิเมอร์ WD7 ถูกผสมด้วยอัลจีเนท พบว่าฟองน้ำที่ผสมอัลจีเนทที่อัตราส่วน 1:1 มีข้อดีคือความนุ่มและความยืดหยุ่นที่ได้จากคุณสมบัติของพอลิเมอร์ WD7 และแข็งแรงขึ้นจากคุณสมบัติที่ดีของอัลจีเนท

จากการวิเคราะห์ด้วยเทคนิคทางโครมาโตรกราฟฟี (TLC และ HPLC) พอลิเมอร์จาก *E. cloacae* WD7 เป็น acidic heteropolysaccharide ที่ประกอบด้วยน้ำตาลโมเลกุลเดี่ยว แรมโนส กลูโคส กาแลคโตสและกลูคูโรนิคแอซิด เป็นองค์ประกอบหลัก พอลิเมอร์นี้มีขนาดน้ำหนักโมเลกุล 50.7-56.6 กิโลดาลตันจากการวิเคราะห์ด้วย GPC ขณะที่สเปคตรัม ^{13}C NMR ของสารละลายพอลิเมอร์ชนิดนี้อยู่ในช่วง 97.514 -101.898 และ 139.831 ppm แสดงให้เห็นว่าพอลิเมอร์นี้เป็นพอลิแซคคาไรด์ นอกจากนี้ พอลิแซคคาไรด์จาก *E. cloacae* WD7 ไม่มีกิจกรรมการต้านแบคทีเรีย เชื้อรา ไวรัส และมาเลเรียบางชนิดที่ความเข้มข้นสูงสุดที่ใช้ในการทดสอบ (> 50 ไมโครกรัมต่อมิลลิลิตร) และไม่มีคุณสมบัติเป็นสารปฏิชีวนะ แต่มีคุณสมบัติเข้ากันได้กับเม็ดเลือดแดงของมนุษย์ โดยให้ค่าการปลดปล่อยสารในเม็ดเลือดแดงออกมาน้อยกว่า

50% ที่ความเข้มข้นของพอลิแซคคาไรด์ต่ำกว่า 3 มิลลิกรัมต่อมิลลิลิตร ไม่มีความเป็นพิษต่อเซลล์จากการทดสอบแบบ *in vitro* และไม่มีกิจกรรมที่ป้องกันการเจริญของเซลล์มะเร็งของมนุษย์ (เซลล์มะเร็งเยื่อบุโพรงจมูก, เซลล์มะเร็งเต้านม และเซลล์มะเร็งปอด) ด้วยค่า IC_{50} มากกว่า 50 ไมโครกรัมต่อมิลลิลิตร จากการทดสอบแบบ *in vitro* พบว่าพอลิเมอร์ชีวภาพนี้ไม่มีกิจกรรมยับยั้งการสังเคราะห์สาร prostaglandin ที่เป็นสาเหตุของอาการอักเสบได้ ดังนั้นพอลิแซคคาไรด์จาก *E. cloacae* WD7 จึงไม่มีสารที่มีฤทธิ์ทางชีวภาพในการบำบัด ป้องกันหรือรักษาโรคได้

Thesis Title	Scale-up for Production and Characterization of Biopolymer from *Enterobacter cloacae* WD7
Author	Mr. Chaiwat Bandaiphet
Major Program	Biotechnology
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ABSTRACT

Scale-up for biopolymer production from *Enterobacter cloacae* WD7 at pilot plant scale (72L) compared with bench scale (5L) was studied under aerobic fermentation base on the same oxygen transfer coefficient (k_La). The biopolymer yield at bench scale increased with increasing aeration rate but decreased with increasing agitation rate. Cell growth and biopolymer production at pilot scale increased with increasing of both aeration and agitation rates. Oxygen transfer rate (OTR) was higher than oxygen uptake rate (OUR) in both exponential phases (18^{th} h) and stationary phase (36^{th} h). The k_La values at both scales increased with increasing aeration and agitation rates. Comparison under the optimum condition, the k_La of bench scale in exponential and stationary phases were 9.97 and 9.72 h^{-1}, respectively (at 1.25 vvm and 200 rpm), while those of pilot scale were 9.68 and 9.50 h^{-1}, respectively (at 2.00 vvm and 600 rpm). Scale-up by fixing k_La at stationary phase gave the closest k_La of 9.72 and 9.50 h^{-1} at bench and pilot scales, respectively and carried out with ratio of 0.98 (9.50/9.72), giving the biopolymer yields of 3.20 and 3.07 g/g cells at pilot and bench scales, respectively.

Flow curve of biopolymer solution from *E. cloacae* WD7 revealed the pseudoplastic behavior at the concentration range of 0.25 - 1.00% (w/v) and gel at the concentration of 2-10%(w/v). WD7 biopolymer was not stable at strong acidic and alkaline condition and at high temperature because of the decrease of viscosity. The optimum temperature and pH were found at 50°C and pH 6-7, respectively. High heat (over 100°C) caused dramatically decreasing of viscosity but heating at 65°C for 120 min had little effect on polymer viscosity. This biopolymer solution and gel can be stored at 4°C and -20°C for at least 2 months. In addition, the polymer can be alternatively freeze-thawed without affecting on flow rheological behavior and viscosity. Some mono-

(Li^+ and Na^+) and divalent cations (Mn^{2+} and Mg^{2+}) could increase the gelation of WD7 biopolymer.

Biopolymer of *E. cloacae* WD7 could be prepared as powder, film and sponge. Optimal condition for film casting by evaporation technique from biopolymer solution (2-10% w/v) was at 50°C and 50%RH. The films were brittle and not flexible. Improving of film appearance and property by adding plasticizers (glycerol, sucrose, polyethyleneglycol and sorbitol) at the ratio of 1:0.5 increased the flexibility expressed as the elongation at break values from 1.38% to 43.55, 31.80, 30.60 and 25.78%, respectively. However, the tensile strengths lowered from 1.358 Pa to 0.477, 0.217, 0.473 and 0.247 Pa, respectively. Glycerol was the best plasticizer, giving about 40 times higher values of elongation at break compared to the native film. Both native and modified films dissolved in water, buffer solutions, acid (1M HCl) and base (1M NaOH) solution and showed low water vapor permeability. Sponge of *E. cloacae* WD7 prepared by freeze drying method at temperature below -80°C was very soft and flexible. Thickness of sponges increased with the increase of WD7 polymer concentration. To improve the property of sponge, WD7 biopolymer was blended with alginate and it was found that WD7 EPS-alginate sponge at mixing ratio of 1:1 was soft and flexible as the properties of WD7 biopolymer sponge and stronger as the property of alginate sponge.

With chromatographic techniques (TLC and HPLC), the biopolymer of *E. cloacae* WD7 was characterized as an acidic heteropolysaccharide that composted of rhamnose, glucose, galactose and glucuronic as major components. The average molecular weights (M_w) of this biopolymer were 50.7-56.6 kDa by GPC technique. The signals of ^{13}C NMR spectrum at 97.514 -101.898 and 139.831 ppm confirmed that this biopolymer was a polysaccharide. In addition, the polysaccharide of *E. cloacae* WD7 had no antibacterial, antifungal, antiviral and antimalarial activities at the maximum concentration tested (>50 µg/ml) and no antibiotic activity. It was heamocompatibility with less lysis of human red blood cell, <50% heamoglobin release at lower 3 mg EPS/ml. The biopolymer showed non-cytotoxicity via *in vitro* testing, and no antitumor activity (against human epidermoid carcinoma of cavity, breast cancer cell line and small cell lung cancer) at IC_{50} >50 µg/ml. *In vitro* testing revealed that this polymer did not inhibit the synthesis of prostaglandin which caused the inflammation. Thus, this EPS had no bioactive compound for healing, protection or cure.

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Chaiwat Bandaiphet

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CHAPTER 1
INTRODUCTION

Enterobacter cloacae WD7 is the one of 188 strains isolated and selected from the sedimentation tank of recycled sludge of five seafood processing plants in Songkhla region, Thailand (Dermlim, 1998). This strain produced biopolymer in 3-L fermenters with batch cultivation giving the highest yield of polymer (4.99 g/l) (Wichenchot, 2000). The massive of WD7 biopolymer need to be prepared to be used for analysis and application studies. Thus the next step of this reserch would be the scaling-up for WD7 production in pilot scale bioreactor. Since *E. cloacae* WD7 can grow and produce the biopolymer under optimal aerated and agitated condition. Thus, the simplest scale-up in order to consider the highest biopolymer production as an important aim in a pilot plant fermenter with different geometry from a small scale may be possibly considered by fixing of k_La. Effects of OTR and OUR on cell growth and product formation in viscous broth and its fluid flow behavior were investigated in this work. Scale-up of the biopolymer production by fermentation of *E. cloacae* WD7 in pilot scale (72-L) stirred tank fermenter was studied.

Most of precise structure and features/diversity of microbial biopolymer are often unknown. Compositional and structural characterization is therefore required in order to satisfy regulatory authorities. Biopolymer of *E. cloacae* WD7 was partially purified and preliminary characterized its chemical structure and physical properties by Dermlim (1999). It is an acidic heteropolysaccharide composted the hydroxyl, carboxyl, carbonyl and methoxyl groups as functional groups. This polysaccharide can become gel with a divalent cation such as $CuSO_4$. It can solute in water and has high water absorption capacity and is stable in a broad range of temperature from 4 to 60^{o}C and pH 5 to 7. It can be applied as flocculating agent as it possesses high flocculation activity. In addition, it was expected to use in other areas especially in phamaceatical and medical applications that can increase the added value of this product. For this study, the WD7 biopolymer was investigated to elucidate its chemical structure by repeating some testings and carried out a deep analysis with chromategraphy (GPC, TLC and HPLC) and NMR techniques. Since its solution and gelation properties, the rheological property of this biopolymer should be investigated to show some basic properties before being prepared into other forms and be applied to many application areas.

Some acidic polysaccharides such as alginate, hyaluronate and others were used in pharmacological and medical areas as the wound managing aids or dressing (Lloyd *et al.*, 1998). In this study, the physical and biological properties of this biopolymer were studied to be used for evaluation the performance and possibility of this WD7 biopolymer to be applied wound managing device. The biological activities of polymer were also investigated for wound healing property. Some physical properties were investigated to enhance its structural properties of this polysaccharide to give information and understanding in its structure. Finally, making a variety form of product such as powder, fiber, film, gel, hydrogel, sponge, etc. is important for consideration the potential form and use for applications. Thus, this thesis was focused in four main topics as present in the "OBJECTIVES" part.

OBJECTIVES

1. To study the scale-up for production of biopolymer from *Enterobacter cloacae* WD7 in a large bioreactor by fixing k_La.
2. To characterize the chemical composition of WD7 biopolymer.
3. To investigate some physical and biological properties of this biopolymer.
4. To prepare some potential forms and study their properties.

CHAPTER 2

LITERATURE REVIEW

2.1 Microbial Exopolysaccharides

Polysaccharide is the one of main biopolymer groups produced by bacteria, fungi or microalgae (Sutherland, 1990). Most commercial polysaccharides derived from microorganisms are of the exopolysaccharides (EPS) that are found external to the structural outer surface of microbial cells. Number of microbial EPS has been gradually increasing and extensively used and applied for commercial importance in many areas today with three main reasons. Firstly, due to their nature, microbial polysaccharides exhibit polydispersity, have water binding capacity, and have high solution viscosity at low concentrations (Johns and Noor, 1991). Secondly, they can be produced by fermentation and scaled up for industrial production (Roller and Dea, 1992). Finally, production is more rapid and can be manipulated by genetic engineering, resulting in uniformity of product (Morris, 1996).

2.2 Chemical Structure of Polysaccharide

Polysaccharides are branched or linear natural polymers of sugars and are polyacetyls which have a high degree of polymerization with molecular mass of above 10,000 kD, and upon hydrolysis, they yield many monomeric sugars (El Khadem, 1988). They can be produced by the condensation of many monosaccharide subunits to form polymer with high molecular weight, forming long, often fibrous molecules. Monosaccharide residue linked together by glycosidic linkages between the dehydrated anomeric carbon atom hydroxyl group (C_1) of one monosaccharide residue and any of the other hydroxyl groups on the adjacent monosaccharide residue (C_2, C_3, C_4 or C_5). The linkages are defined as alpha (α) or beta (β), depending on the orientation of the anomeric carbon hydroxyl group forming the linkage (α is axial and β is equatorial for D-aldohexoses), and according to the carbon atoms on adjacent monosaccharide residues that are linked, e.g. α-D-Glc*p*-(1$\longrightarrow$4)-D-Glc (maltose) is formed by glycosidic linkage between the anomeric carbon hydroxyl group of α-D-glucopyranose and the C_4 hydroxyl group of another D-glucose molecule.

Most EPS are polymers of hexose sugars such as D-glucose, D-galactose and D-mannose. A few numbers of bacterial polysaccharides contain 6-deoxy-sugars, and ketoses (Graber *et al.*, 1988). Some EPS may contain uronic acids such as D-glucuronic

acid or galacturonic acid, to make such polysaccharide polyanionic (Sutherland, 1990). Some may also contain organic substituents such as pyruvate, found in *E. coli* and *Z. ramigera* polysaccharides. Therefore, they may also contain inorganic substituents such as sulphates and phosphates (Sutherland, 1990).

2.3 Types of Polysaccharides

Polysaccharide may be a neutral, basic, acidic polymer or indeed have the potential to have mixed / variable charge according to characterization of monosaccharide residues (which can be neutral, basic acidic or combinations). They are divided into two groups: **Homopolysaccharide**; this type is composted of the same type of monosaccharide residues. They may be homopolymers composted of a single type monosaccharide residue (Rolle and Dea, 1992). **Heteropolysaccharide**; this type contains the different types of monosaccharide residues. There are many potential combinations of different monosaccharide residues and linkages which results in a wide variety of potential repeating units. Most EPS are heteropolysaccharides formed by two or three different sugars and various acyl constituents. Majority of heteropolysaccharides are polyanionic and their repeat units vary in size from disaccharides to octasaccharide. Each repeat unit frequently consists of 1 mole of uronic acid. While some contain short side chains, relatively few have linear structure (Sutherland, 1990). Some chemical structures of heteropolysaccharide are shown in Table 1.

Alginate is a good example of a polyanionic heteropolysaccharide produced by either *Azobacter vinelandii* or *Pseudomonas aeruginosa*. It is composed of D-manuronic acid and L-guluronic acid, and carries acetyl groups on the C2 and C3 position (Roller and Dea, 1992). *Enterobacer* NCIB 11870 and Klebsiella aerogenes produced some bacterial heteropolysaccharides, XM6 and K54, respectively (Morris, 1988 and Sutherland, 1996a). The K54 carries O-acetyl groups on every L-fucose residue of alternate repeat unit but XM6 is a gelling polysaccharide in the presence of monovalent and divalent cations, where else K54 displays neither a sharp defraction pattern no anygelation. After the removal of acetyl groups K54 show properties similar to XM6.

2.4 Polysaccharides from Gram-negative Bacteria

Large numbers of Gram-negative bacterial EPS are potentially available, but bacteria may be pathogenic, production costs may be very high, product quality may be difficult to maintain and to guarantee, or the product may not achieve regulatory acceptability, and

Table 1 Chemical structure of some heteropolysaccharides

Exopolysaccharides	Structure (repeating sequence)
Gellan	→3)-β-D-Glc-(1→4)-β-D-GlcA-(1→4)-β-D-Glc-(1→4)-α-L-Rha-(1→
Welan	→3)-β-D-Glc-(1→4)-β-D-GlcA-(1→4)-β-D-Glc-(1→4)- α-L-Rha-(1→ ↑3 1 α-L-Rha
Xanthan	→4)-β-D-Glc-(1→4)-β-D- Glc-(1 ↑3 1 β-D-Man-(1→4) -β-D-GlcA-(1→2)-α-D- Man-6-OAc 4 X 6 HO_2C CH_3
Scleroglucan	β-D-Glc-(1→3)-β-D- Glc-(1→3)-β-D-Glc ↑6 1 β -D-Glc
Curdland	β-D-Glc-(1→3)-β-D- Glc-(1→3)-β-D-Glc
Hyaluronic acid	→3)-β-D-GlcNAc-(1→4)-β-D-GlcA-(1→
K54 from *Klebsiella aerogenes*	→4)-α-D-GlcA-(1→3)-α-L-Fuc-(1→3)-β-D-Gluc-(1→ 2 or 4 ↑ ↑4 Q-Acetyl 1 β -D-Glc
XM-6 from *Enterobacer* sp. NCIB 11870	→3)-β-D-Glc-(1→4)-α-D- GlcA-(1→3)-α-L-Fuc-(1→ ↑4 1 β -D-Glc

Source: Sutherland (1996a)

very commonly there is no market niche. Despite these problems, several products from Gram-negative bacteria are accepted products of modern biotechnology; several more may be developed in the next few years, especially as renewable resources for alternatives to products of chemical industry.

Structure of many Gram-negative bacterial polysaccharides is relatively simple, comprising either homo- (usually polymers composed of D-glucose) or heteropolysaccharides that are normally composed of regular repeating units ranging in size from disaccharides to octasaccharides with 2-4 types of monosaccharide and many contain acyl group as additional adornments (Table 2). The commonest acyl substituents are acetate esters and pyruvate ketals; succinyl half esters are also a feature of some EPS. The presence of the ketals or of uronic acids, results in linear polyaionic macromolecules.

Table 2 Non-carbohydrate substituents of exopolysaccharides from Gram-negative bacteria

Substituent	Linkage	Charge conferred	Occurrence
Organic acids			
Acetate	Ester	None	Very common-e.g. *Klebsiella* spp.
Glycerate	Ester	Nagative	*Sphingomonas paucimobilis*
Hydroxybutanoate	Ester	None	*Rhizobium trifolii*; *R. leguminosarum*, etc.
Propionate	Ester	None	Rare-some *Escherichia coli*
Pyruvate	Ketal	Negative	Very common-e.g. *Klebsiella spp.*
Succinate	Half ester	Negative	*Rhizobium spp*; *Agrobacterium spp.*
Inorganic acids			
Phosphate		Negative	Found in some genera
Sulphate		Negative	Cyanobacteria; *Haloferax mediterranea*

Source: Sutherland (2001)

Many Gram-negative bacteria can produce more than one type of EPS (Sutherland, 2001). Within a number of *Enterobacterial* sp., the ability to produce the EPS colonic acid is wild spread Sutherland (2001). EPS from *Enterobacter sp* BY-29 contained glucose, galactose, xylose and galacturonic acid (Yokoi et al., 1997). EPS from *Acetobacter xylinum* share a tetrasaccharide structure of their repeating unit with xanthan and are synthesized as alternatives to the bacterial cellulose. *Azotobacter chroococcum* can produce different EPS depending on the substrate provided, when growth on glucose yields a bacterial alginate, while a levan homopolysaccharide is formed on sucrose. Species, which differ taxonomically, may produce the same or an almost identical polysaccharide. Alginates are one example of this, being synthesized by *Pseudomnas* and *Azotobacter spp.* In contrast, *Agrobacterium* and some *Rhizobium spp* appear to show simultaneous production of two polysaccharides (succinoglycan and curdland) (Sutherland, 1996b). Non-identical microbial polysaccharides including the galactoglycans produced by Gram-negative bacteria were given in Table 3.

2.5 *E. cloacae* WD7 and Its Biopolymer

E. cloacae WD7 is Gram negative facultative anaerobic short rod bacteria with acid production and oxidation-fermentation and can produce the slime biopolymer that cause of

Table 3 Galactoglucans of Gram-negative bacteria

Gram (-) bacteria	Repeating sequence of Polysaccharides
Rhizobium meliloti	→[-4-α-D-Glc-(1→3)-β-D-Gal-1-]→ ↑6 ▲ $CH_3CO.O.$ Pyr
Rhizobium meliloti Strains YE-2(S1), RM1021	→[3-β-D-Glc-(1→3)-α-D-Gal-1-]→ ↑6 ▲ $CH_3CO.O.$ Pyr
Agrobacterium radiobacter Strain II; *Burkholderia cepacia*	→[3-β-D-Glc-(1→3)-α-D-Gal-1-]→ ▲ Pyr
Pseudomonas marginalis HTO41B	Succ ▼ →[3-β-D-Glc-(1→3)-α-D-Gal-1-] ▲ Pyr
Escherichia coli K37	→[3-β-D-Glc-(1→3)- α -D-Gal-1-] 4 ↑ 1 pyr ▶ α -D-Gal
Klebsiella pneumoniae SK1	→[3-β-D-Gal-(1→3)-β-D-Gal-(1→3)-α-D-Glc-1-]→ ↑4 ↑2 1 1 β-D-GlcA- α -D-Gal
Enterobacter sp. Strain XM-6	→3)-β-D-Glc-(1→4)-α-D-GlcA-(1→3)-α-L-Fuc-(1→ ↑4 1 β-D-Glc

Source: Sutherland (2001)

viscous broth. Dermlim (1999) showed the time course on growth and biopolymer production in batch shake flask of *E. cloacae* WD7 in basal medium using glucose as carbon source for 5 days. Production medium was optimized and contained of 3%sucrose, 0.05%yeast extract, 0.5%K_2HPO_4, 0.2%KH_2PO_4, 0.05%$MgSO_4 \cdot 7H_2O$ and 0.01% NaCl and optimal conditions in batch cultivation were at pH 7.0, 30^{o}C, 2.0 vvm and 200 rpm

(Wichienchot, 2000). This biopolymer was characterized as an acidic heteropoly-saccharide with components of neutral sugar (29.4%), uronic acid (14.18%) and amino sugar (0.93%) with no amino acids or protein in its molecule (Dermlim, 1999). The expected neutral sugar fractions might be glucose, mannose, fucose and rhamnose. Hydroxyl, carboxyl, carbonyl and methoxyl function groups were found in structure when detected by FT-IR spectroscopy.

2.6 Scale-up

Scale-up is the study of the problem associated with transferring data obtained in laboratory and pilot-plant equipment to industrial production (Aiba *et al.*, 1973). Main idea of scaling-up is to produce the large amount yield of interested product with enduring the results obtained in small scale or improvement something on accepted principle basis. It will be good in operation with less possible step from the beginning of shaken flask to laboratory fermentation, pilot-scale fermenter and industrial or production fermenter, respectively. The values of parameter in larger scale must be set and stand as same or close as the variable of operation in smaller scale to expectably obtain the same results or product yield. Then, the results from each step are compared together to prove the problems or errors of operation.

Scale-up should be carried out by using the same geometrically larger fermenter if possible. Working volume of each larger scale is should be 10 time of smaller scale, for example 30, 300, 3,000, 30,000 l etc., in order to easy to operation, control and prove of problems and errors. Operation in large scale about the large volume is deal with transferring of momentum, mass and energy that affects on processes and high volume, such as pressure and force of massive liquid and gasses, heat exchange, thermal capacity, controlling of mixing and foam formation, etc. Consequently, the performance of equipment and instrument should be appropriated and readily.

2.7 Scale-up of Biopolymer Production

Microbial biopolymer was normally produced by aerobic process under non-limited oxygen condition (Sutherland, 1990). The viscosity of broth increased with the increase biopolymer which form a layer on the cell surface and acts as a diffusion barrier for oxygen transfer to the cells and usually is the limiting operation in industrial bioprocesses, scale-up, and economy of aerobic biosynthesis systems (Al-Masry, 1999; Elibol and Ozer, 2000; Garcia-Ochoa *et al.*, 2000 and Merritt *et al.*, 2000). High oxygen demand

(fast growing microorganisms, high biomass) or when the rheological properties of broth (in production of biopolymer or exopolysaccharides) offer a high resistance to the mass transfer, for example in xanthan gum production (Casas *et al.*, 2000; Lo *et al.* 2001).

Merritt *et al.* (2000) scaled the production of capsular polysaccharide from bacteria *Heamophilus influenzae* type b, which is important for the production of effective conjugate vaccines for Pneumonia, Cellulitis, Septic arthritis and Bacteremia. It was produced in liquid culture by fed-batch fermentation at controlling pH 7.3 by 5N NaOH, 36.5^{o}C, 400 rpm and 0.6-0.8 vvm for maintain dissolved oxygen about 50%. This process was successfully scaled from 1.5L development scale to the 500l manufacturing scale using 327L of basal medium with 3L of mid-log phase inoculum and approximately 40L of feed medium. No criteria and stoichiometric equation was considered in this case.

Characterization and behavior of fluid broth in the large bioreactor may be affected on the microorganism and their activities from adaptation of process controlling of scale-up (Aiba *et al.*, 1973). There were many factors affect on oxygen transfer such as aeration rate, agitation rate, mixing, air pressure, air flow rate, fluid properties, flow pattern of broth, rheology and chemical reagent (such as protein in medium composition or antifoam etc.), are main problem and usually used as the criteria of scale-up in aerated fermentation processes. Rheology of fermented fluid can offer a high resistance to the mass transfer of oxygen in scale-up process of extracellular polysaccharides production. To reduce the complication of various variables and factors, the traditional method for scaling-up a fermentation system is usually based on various empirical criteria. Scaling-up technique using criteria may be considered that are proper to the owing process and operation and an optimal criterion is used as retained value for investigation the other variable affect on process (Hsu *et al.*, 2002).

2.8 Scale-up by k_La Criterion

The aeration efficiency in operation of aeration and mixing-sparging bioreactor depends on the oxygen mass transfer rate (OTR), oxygen solubilization and diffusion rate, oxygen consumption of cell (OUR) and bioreactor capacity to satisfy the oxygen demand of microbial population (Wernersson and Trägårdh, 1998; Thiry and Cingolani, 2002). Dissolved oxygen in the broth is limited by OTR and OUR and to maintain the growth of microorganism, the OTR should be control at higher than OUR. But OTR were affected by several factors, such as the geometrical and operational characteristics of vessels, liquid properties (viscosity, superficial tension, etc.), biocatalysts properties, concentration and

morphology of microorganisms, and the dissipated energy in the fluid, which depends on the air flow rate, the stirrer speed, etc Galaction *et al.* (2000). While, the OUR was limited by the increase of viscosity resulting from polymeric property (Eickenbusch *et al.*, 1995; Çalik *et al.*, 2000 and Kwon, *et al.*, 1996). To reduce the complication of various variables and factors based on the principles of similarity, scaling-up for biopolymer production should be studied by consideration the oxygen transfer parameters.

The k_La, the constant of oxygen transfer coefficient, is the most commonly used criteria in aerobic fermentations (Nüske *et al.*, 2002; Yuh-Lih and Wen-Teng, 2002). The rationale of the criterion of constant k_La is to ensure a certain mass transfer capability that can cope with the oxygen demand of the culture. However, it must be realized that what really matters is not the transfer capability (k_La), but is the OTR, which is the product of k_La and the mass transfer potential (C^*-C_L). Diaz and Acevedo (1999) studied the results of k_La on scale-up with rheological behaviour of many important industrial fermentation processes. They summarised that a method for scale-up of fermentations with non-Newtonian broths should be based on geometric similarity. It is a fact that the operation at dissolved oxygen concentrations below the critical value is clearly inconvenient. Many factors, especially the dissolved oxygen concentration and the rheology of broth had effect on calculation k_La and discussion.

Four methods are available to estimate the overall k_La value: static method, dynamic method, gaseous oxygen balance and carbon dioxide balance (Pouliot *et al.*, 2000). The static method with sulphile oxidation was used to estimate the k_La value for scale-up of cellulose yield from *Acetobacter xylinum* (Kouda *et al.*, 1997). This method has the advantages for establishing the effect of media components on oxygen mass transfer and does not involve chemical reactions that could affect the measurement precision. However, this method is adequate for non-respiring systems only.

Dynamic method is well-known and used to determine the k_La and qO_2 in scale-up for pigment fermentation of *Monascus* cells in viscous broth (Hyun *et al.*, 2002), xanthan production controlled by oxygen consumption of cell and transferring of oxygen (Garci'a-Ochoa *et al.*, 2000), pullulan production (Gibbs and Seviour, 1996) and for scale-up of L-lactic acid production (Miura *et al.*, 2003). It is correlated to stirrer speed and superficial gas velocity, and broth viscosity in the case of the non-Newtonian fluid (Reyes *et al.*, 2003).

Dynamic gassing-out technique is more complicate than the static gassing-out, but it has more advantage with giving the OUR value which presents to respiration rate and

activity of tested cells during bacterial cellulose cultivation (Kouda *et al.*, 1997). The major limitation of k_La measurement by dynamic gassing-out technique is the allowance of oxygen concentration to drop below its critical level during the degassing period giving specific oxygen uptake rate. This oxygen limited condition influenced the cells change, metabolism and biopolymer production although the oxygen level during fermentation was controlled not lower than 10% saturation for cells survival. The occurrence of deoxygenation step gave the stress to cells, while the k_La value was measured in reoxygination step without oxygen stress (Ni *et al.*, 1995). This method has effected directly on physical property (OTR, k_La, viscosity, biopolymer character), and has exactly negative effect on biological phenomenon (cells growth, OUR and metabolism, biopolymer production).

The k_La by gas balance technique was about two-fold higher than k_La by both gassing-out techniques due to some oxygen fraction from sparged bubbles was transferred to both of fluid and the remaining small bubbles, thus dc_O/dt was smaller than that measured in the absence of small bubbles (Tuffile and Pinho, 1970). The k_La by static gassing-out technique was able to be calculated proportional to k_La by gas balance method due to it was not concerned with microorganism. Heijnen *et al.* (1980) explained that in homogeneous phase the oxygen solubility of the "liquid-small bubble dispersion" was high because the oxygen concentration in air is about 32 times that in water, thus it was considered the reason why k_La by gassing-out technique was lower than k_La by gas balance.

2.9 Some Properties of WD7 Biopolymer

Some intrinsic properties of this WD7 biopolymer were reported by Dermlim (1999). This biopolymer is only soluble in water, leading to the high viscosity but insoluble in the tested organic solvents such as acetone, carbon tetrachloride, ethanol, isopropanol, hexane, methanol and nitrobenzene due to having charge (polar). This polymer is an anionic (or acidic) charged polysaccharide due to the acidic group in its structure and formation of the precipitated polysaccharide-Cetylpyridinium Chloride (CPC) complex from reaction between the acid groups in the polysaccharide structure and quaternary ammonium (QN^+) of (CPC).

In an aqueous system, it can take up water, swell and usually undergo partial or complete dissolution (Dermlim, 1999). Consequently, it can be recovered from broth by precipitation with these organic solvents. Therefore, this WD7 EPS could be formed the

true gel (ionic gel) only in alkaline aqueous solution and the divalent metal salts such as $CaCl_2 \cdot 2H_2O$, $MgSO_4 \cdot 7H_2O$, especially $CuSO_4 \cdot 5H_2O$. Different types of divalent cations and the ratio of polysaccharide to metal salt gave the different appearance of gels. Copper sulfate ($CuSO_4 \cdot 5H_2O$) gave a stronger gel than $CaCl_2 \cdot 2H_2O$ and $MgSO_4 \cdot 7H_2O$. It was found to possess the water adsorption capacity (80.3 g/g dried EPS) with 3 times lower than commercial synthetic water absorbent (292.9 g/g dried EPS). The maximum absorption of this polymer occurred after 24 h. The highest water absorption capacity of EPS was achieved at 1.0% NaCl with the value of 87.1 g/g dried EPS (Wichienchot, 2000).

Dermlim (1999) found that the temperature range of 4-60^{o}C and pH 5-7 did not affect the flocculating activity. But the temperature above 60^{o}C, this polysaccharide chain may be broken down to be the shorter chain and lead to the low potential to form bridges with the kaolin particle and low flocculating activity. This polymer is not stable in the strong acidic and basic condition due to the glycosidic bonds in this polysaccharide chain can be easily hydrolyzed in nature. From DSC results, the partially purified WD7 polysaccharide composted of crystalline and amorphous fraction. Crystalline polymer melted with increasing of temperature increased from 282^{o}C to 390^{o}C with the melting point (T_m) of 300^{o}C.

2.10 Flow Rhelogical Properties of Polysaccharide Solution and Gel

Rheology can be defined as "the study of flow and deformation of matter". A stress is applied to a material and the resulting motion is monitored. The stress may be extensional, compresional, torsional, flexural, shear, etc. It can be resolved into extensional and shear components. Fluid rheology is used to describe the consistency of products, normally by the two components viscosity and elasticity. Viscosity is usually described as resistance to flow or thickness, and elasticity as stickiness or structure. The viscosity of a material is defined as the shear stress divided by the shear rate. The greater a material's resistance to flow, therefore the greater its viscosity was. Since the units of shear stress are Pascals (Pa), and those of shear stress are inverse seconds (s^{-1}), viscosity has the units of Pascal second (Pa.s).

Polymer solution should be grouped as Non-Newtonian that the viscosity is not independent of the rate of shear and therefore to characterize the flow properties a flow curve must be plotted according to the related equation that may be expressed as: $F/A = \eta(dV/dX)^n$ where F/A is the shearing stress (force/area), dV/dX is the rate of strain

(rate of shear or velocity gradient), η is the coefficient of dynamic viscosity (Pa.s) which is dependent on temperature but is independent of the rate of shear and n is flow index. The flow describes the relationship between the stress (related to the force applied) and the shear rate [the speed (velocity gradient caused by the stress) with which the fluid flows of a sample. Flow curve is a plot of shear stress versus shear rate.

For a Newtonian fluid, the flow curve will be a straight line passing through the origin; the slop is equal to the viscosity. Most fluids are non-Newtonian and their viscosity remains independent of the shear rate. Idealized flow profile (Figure 1) shows two Newtonian plateaus which represent the zero shear and infinite shear viscosities, separated by a power law region where the sample structure is being destroyed. At the zero shear stresses, predictions about the appearance of the sample can be made, as well as the suspension stability as sedimentation, leveling and other similar processes occur under very small gravitational forces. The higher the value, the higher the resistance to particles falling through the medium, so the more likely that the sample is not going to suffer from sedimentation or sagging, but too high and ease of handling may become a concern. The point at which the sample start to shear thin (enters the power law region) dictates the energy needed to make the sample flow easier. This can affect the pump ability and type of packaging used.

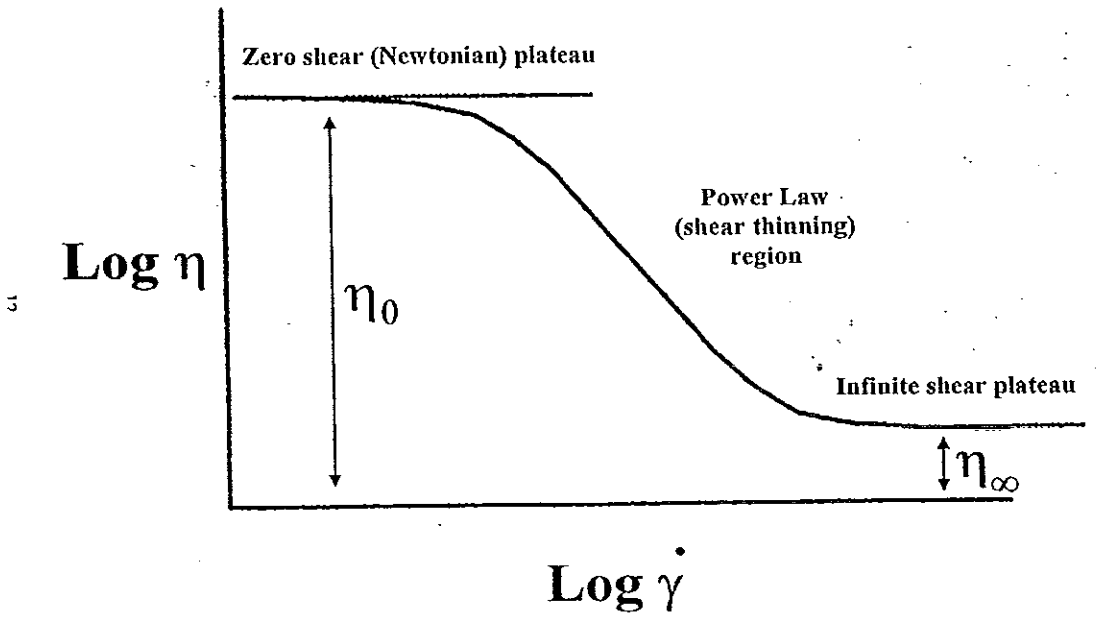


Figure 1 Idealized rheological flow profile between Log of shear rate and Log of viscosity
Source: Latif (2001)

Zero shear viscosity: There are Zero shear (Newtonian plateau), Power law (shear thinning) region and Infinite shear plateau on the three profile lines, they mean having little or no effect on the observed viscosities of the material (initially obeys the Newtonian's law of liquid flow). The magnitude of zero shear viscosity (η_0) is directly related to the molecular weight of system, thus the higher the zero shear viscosity the greater the molecular weight (for materials of similar concentration). While the length of the plateau can indicate the molecular weight distribution, the larger the distribution the smaller the plateau. Length of the zero shear viscosity plateau is inversely proportional to the molecular weight distribution, i.e., the shorter the plateau, the more polydisperse the material. The higher the length of this plateau, the greater the molecular weight was found.

Infinite shear viscosity: This usually represents the final application viscosity. At this infinite shear viscosity, the easiest state for pumping, rubbing, etc. Too high a value and the sample may be difficult to apply, too low and the final finish is more likely to be saturated or messy. **Shear thinning**: the point at which a sample starts to shear thin dictates the energy needed to make the sample flow easier. The shear thinning region is where the sample structure is being destroyed and shows a decrease in viscosity, at the shear rate is increased. It is known as the Power Law region and can be described mathematically by the Power Law model: $\eta = K \gamma^{(n-1)}$, where n is the shear rate index (n), η = apparent viscosity and K is the consistency index. This is where the material exhibits an increase in viscosity as the shear rate increases. It is less common than shear thinning ($n<1$) while the shear thickening can also be described with $n>1$.

Materials having a constant viscosity over the range measured are considered to be Newtonian in performance, since they obey Newton's law of liquid flow. Those for which the viscosity decreases with increasing shear stress (or shear rate) are said to be **shear thinning** (also be called pseudoplastic) such as paint, shampoo, slurries, fruit juice concentrates and ketchup. Those for which the viscosity increases are **shear thickening** (or dilatant) such as wet sand and concentrated starch suspensions. In addtition, some fluid must be applied with the certain shear stress before flow occurs; it's called **Plastic** fluid behaviour, such as tomato paste, tooth paste, hand cream and grease. Many industrial materials exhibit different forms of flow behavior at different shear stresses.

2.11 Gelation of Polysaccharides

Gelation of this EPS may be owing to repulsion between dissociated acidic groups in polysaccharide and due to intermolecular associations which give rise to three dimensional networks (Chinachoti, 1996). Most of several microbial EPS were used as gelling agent in food industries, pharmaceutical, medical field and healthcare (Sutherland, 1998) such as gel of xanthan, alginate and dextran and in medical of hyaluronan (glycosaminoglycan) gel (Lloyd *et al.*, 1997). Gels are extensively used as thickening or gelling agents, texture enhances, stabilizers in food industry or in pharmaceutical/cosmetic industries, in lotions, creams, toothpaste, etc.

Gel-forming polysaccharide must have a structure that allows partial but not overall association, since the later could cause precipitation or insolubility (Silva *et al.*, 2003). The mechanism for gelation of ionic polysaccharide could involve formation and subsequent aggregation of double helices, as in gellan or carageenan, or ionic interchain association mediated by divalent cations, described as the 'egg-box model', as in alginate and low-methoxy pectin (Grant *et al.*, 1973). Even so, more than one mechanism could also be involved as in gel formation in high-methoxy pectin, which is governed by both hydrogen bonds and hydrophobic interactions. In neutral polysaccharides, for example dextran, gels are formed by interactions of the individual polymer chain that are mediated by specific interactions between the hydroxyl groups and/or the bound water molecules (Naji *et al.*, 2003). In schizophyllan, another neutral polysaccharide, triples helices are formed.

Some EPS gels were achieved by combinations of polymer and metal cations, to form a variety of gel textures generating the potential for a wide range of uses as gelling agent to biological and physiological importance and its use as a thickening, suspending and gelling agent (Stephan *et al.*, 1995, Sanderson, 1990, Olivera *et al.*, 2001). Mono- and multivalent cations may establish different kinds of interactions with anionic polysaccharide and promote interaction between chains and an increase in viscosity (Oliveira *et al.*, 2001). Silva *et al.*, (2003) found that the gel strength of Na-*Sterculia. striata* polysaccharide in the presence of divalent salt follows the order: Mg > Ca > Sr > Ba, i.e. inversely proportional to the cation radius.

Biopolymer of *E. cloacae* WD7 is an acidic heteropolysaccharide which can form the true gel (ionic gel) by addition of some divalent metal compounds to obtain the stronger gel (Dermlim, 1999). The addition of counterions to "neutralize" polymers resulted in viscosity data which were more consistent with those of uncharged polymers

such as gellan (Tang *et al.*, 1996), λ-carrageenan, alginate (Draget *et al.*, 1998, Zheng *et al.*, 1997) and xanthan (Iseki *et al.*, 2001). There are many factors had effect on biopolymer properties added with various ions such as conditions of diluted and concentrated polymer, temperature, pH, ion intensity etc. The decrease in ionic strength as a polymer is diluted causes polymer chain reorientation, ultimately affecting viscosity.

Proposed gelation model presented by Silva *et al.* (2003), shows the interaction of different structural parts of polyanion and cations as shown in Figure 2. Gelation could be due to the formation of intermolecular junction zones, involving ionic interaction between homogalacturonic segments of different chains and calcium ions giving an 'egg-box model' (zone A), and hydrogen bonding between rhamnose segments in the hydrophobic areas (zone B). Ionic interactions could also occur between two or more galacturonic acid residues on different main chains or glucuronic acid in branched or non-regular chain segments (zone C).

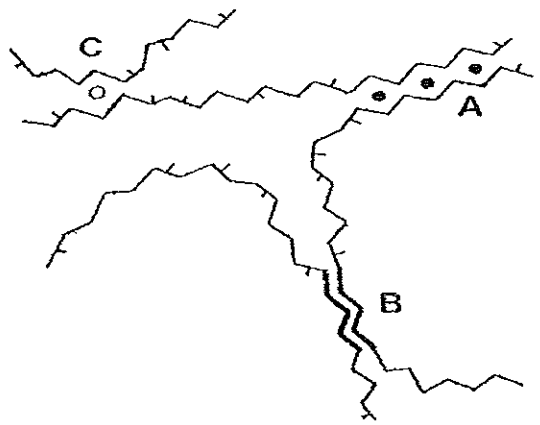


Figure 2 Proposed model for gelation: showing ordered ionic interaction between galacturonic acid residues and calcium ions (●) (zone A); hydrogen bonding between rhamnose segments (Zone B) and single ionic interaction involving galacturonic acid and counter ions (calcium or orther metal ion)(○) (zone C)

Source: Silva *et al.* (2003)

The formation of intermolecular junction zones in the proposed model includes ionic interaction and hydrogen bonding. I onic interactions between two galacturonic acid residues of different principal chains and counter ions or two glucuronic acid residues of branching are predominantly of individual character. The ionic interaction between

homogalacturonic segments of different chains and calcium ions, such as 'egg-box model', could also be possible, but to a smaller extent. Hydrogen bonding may occur between rhamnose segments. This model can illustrate the gelification and gelation enhancement of other EPS interacted with cations.

Silva *et al.*, 2003 reported the effects of monovalent salt on gelation of some polysaccharides could be:

1) decreasing gel strength by overshadowing the effect of the original counter ion b screening the charge of the polymer and decreasing the cross-links between the polysaccharide chains (high salt concentration and multivalent counter ion);
2) increasing gel strength by the promotion of a non-specific shielding of the long range electrostatic repulsions between the highly negative charged polysaccharide chains, thus facilitating multivalent counter ion interchain cross-linking (low salt concentration an multivalent counter ion);
3) increasing gel strength by augmenting the shielding of electrostatic repulsion, promoting the effective participation of the monovalent cation in cross-link through in polyanion/water/M^{+}/water/M^{+}/polyanion interaction (high salt concentration and monovalent counter ion).

Whist the effects of divalent salt on gelation of somepolysaccharide could be (Silva *et al.*, 2003):

1) increasing gel strength due to the substitution of monovalent counter ion by divalent cation and increasing cross-links (low salt concentration and monovalent counter ion).
2) decreasing gel strength due to the repulsive forces between positive net charges on the polymer chains, originating from the excess of the divalent cations and formation of $M^{2+}(COO^{-})$ bonds, instead of the $M^{2+}(COO^{-})_2$, which characterize the cros-links (any salt concentration and divalent counter ion or high salt concentration and monovalent counter ion).

2.12 Applications of Microbial Polysaccharides

The application of microbial polysaccharides is found in food, industrial, environmental and medical (pharmaceutical). Medical application of microbial polysaccharides also involves the exploitation of their biological properties; biocompatibility, hydrophilicity, biodegradability and non toxicity effects (Yalpani and Sandford, 1987). Some EPS was reported for using as antiviral agents, application as

vaccines and antitumour agents (Bell and Torrigiani, 1987). Some may be beneficial in a wound management aid or indeed may participate in the wound healing process such as bacterial alginate in form of calcium alginate fibers and Celllose (Sutherland; 1994). Hyaluronic acid is a mucopolysaccharide from microbial fermentation (such as *Streptococcus* sp, *Pseudomonas aeruginosa* and *Streptococcus zooepidemicus* that is used in the areas of ophthalmology, orthopaedic surgery, wound healing and treatment of swollen joints (Amstrong and Johns, 1996). It has also therapeutic application for tumor treatment, and has pharmaceutical uses such as in lotions for skin rejuvenation and wound healing (Sutherland, 1998 and Mahlabacher *et al.*, 1992).

2.13 Use of Polysaccharide as Wound Management

Polysaccharides used as wound managing can be grouped by into neutral, basic and acidic polysaccharides that included their derivative products after structural modification in case of their primary structure were not suitable. Some possess potentially useful biological properties that may make the suitable wound healing. A wound would be healed faster when a 'moist' dressing was applied compared with traditional 'dry' dressing found in natural skin (recognized as ideal wound dressing) with its 85% water content and inherent permeability (Lloyd, *et al.*, 1998)

Lloyd, *et al.* (1998) reported that the properties of wound dressing material should:

(1) be capable of maintaining high humidity at the wound-dressing interface whilst removing through adsorption excess wound exudate and associated toxic compounds ;

(2) permit the exchange of gasses whilst maintaining an impermeable layer to microorganisms so preventing secondary infections ;

(3) provide thermal insulation ;

(4) all components of the dressing, including the adhesives, must be biocompatible and not provoke any allergic reaction through their prolonged contact with tissue ;

(5) there must be minimal adhesion to the surface of the wound so that the dressing can, when required, be removed without trauma ;

(6) the dressing must be physically strong even when wet;

(7) be produced in a sterile form and

(8) easy to dispose of when removed at the end of use.

Polysaccharide can be prepared to be potential physical forms (powder, suspension, fibre, film, gel, hydrogel, hydrocolloid) and some actual polysaccharide or polysaccharide derivative participate activity as potential wound management aids (Lloyd *et al.*, 1998).

2.14 Preparation Polysacchride Forms

Polysacchararides can be produced and invented by chemical and physical method to obtain many different forms as described following:

2.14.1 Powder

There are many methods for powder formation of macromolecules; conventional methods for powder production, including freeze-drying, spray-drying and precipitation, have considerable disadvantages, for these applications. Freeze-drying produced particles of a broad-size distribution which may require subsequent processing by milling and sieving. Spray-drying can produce particles of high temperatures during the process, which can result in significant lodd of biological activity. Precipitation often uses a precipitation agent that is not wanted in the final formation. It also requires a secondary drying step to remove residual solvent.

Tservistas *et al.* (2001) offers the possibility to produce suitable dry powder formulations of novel drugs on biological macromolecules by supercritical fluid technology. This method facilitated controlled partical formation at near-ambient temperature and integrates particle formation and solvent removal into a single step. Most commonly applied fluid is carbon dioxide which does not leave toxic residues associated with the product. Supercritical CO2 has been used as medium in extraction and enzymatic reactions. The more recently developed technique of solution enhanced disperse by supercritical fluids is able to produce dry fluids particles from an aqueous solution, widening the range of potential applications for the formation of bioactive macromolecults.

2.14.2 Gel

One of the most important properties of several of the industrially useful microbial polysaccharides is their ability to form gels. Some of these polysaccharides require the presence of ions for gel formation (e.g. alginates or gellan) while others form gels without the involvement of ions (e.g. curdland). Gels owe their charecterstics to the formation of networks of polymer chains which are cross linked. The actual mechanism of gelation varies; in some systems hydrogen bonding is involved, whereas in others there is covalent linking through multivalent cations. The site-binding of cations is important in a number of polysaccharides in maintaining the stability of the ordered conformation.

Counter-ion selectivity appears to be explained by the sandwiching of arrays of cations, either between two folded buckled ribbons as in alginate or pectin, or between double helices as in carrageenan.

Hydrogel is a three-dimensional network of hydrophilic polymer chains in which at least 20% by weight is retained water (if greater than 95% called a superabsorbent). Hydrogels swell and shrink in the presence/absence of water, respectively and if the water is completely removed they collapse to form a xerogel. The shrinking/swelling process is reversible in the presence of water so the xerogel will swell to give the hydrogel structure. In order to maintain the three-dimensional structure of hydrogel, the hydrophilic polymer chains were cross-linked either by covalent bonds or non-covalently by electrostatic, hydrophobic or Van der Waals interactions (Lloyd *et al.*, 1998). Hydrogels/xerogels are used as wound management aids as dressing materials. They can be manufactured so that they are flexible, durable, non-antigenic and permeable to water vapour and metabolites whilst also securely covering the wound so preventing infection by bacteria.

Microbial exopolysaccharide gel: Primary structure of a microbial exopolysacharide has a very great effect on its capacity to form gels. This can be seen in a number of polysaccharides which posses closely related structures. Thus, curdland forms gels, whereas the structurally similar scleroglucan, differing only in the presence of glucosyl side-chains, does not. Similarly, gellan is capable of gel-formation, wheras none of the other five polymers that are structurally related to it possess the ability to form gels although all are capable offorming highly viscous solutions.

Mechanism of polysaccharide gel formation: Alginates form gels by a very different mechanisms by selective cooperative binding of divalent caions. From Figure 3, X-ray diffraction studies revealed a three-fold ribbon-like conformation for polymannuronic acid and a buckle two-fold structure for polyguluronic acid (Atkin *et al.*, 1973). Both types of structure bind calcium ions, but homopolymeric polyguluronate sequences of 18-20 residues or more show a sigmoidal increase in binding, which promotes the formation of junction zones. Mixed sequences and polymannuronic acid segments may have only minor roles. This coorperative binding process has been confirmed by competitive ion binding studies and by the large change in circular dichroism observed on addition of Ca^{2+} to alginate solutions or to polyguluronic acid preparations (Sutherland, 1998). The specific site binding is seen in circular dichroism studies: the negative trough derived from the acid monomer sequences is converted to a positive value

on controlled addition of Ca^{2+}. The magnitude of the observed difference is correlated in alginates with the proportion of polyguluronic acid sequence.

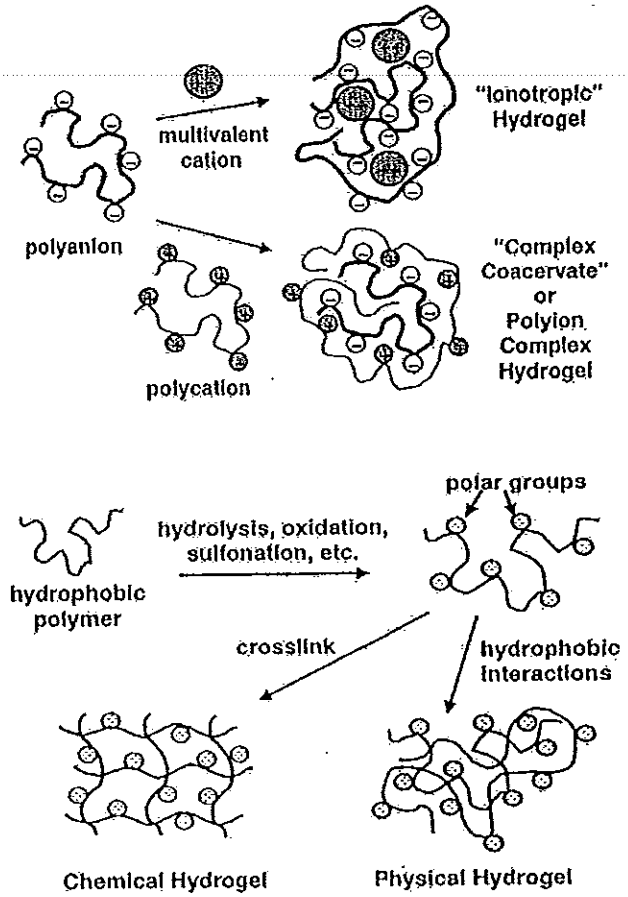


Figure 3 Mechanical of gel formation

Source: Sutherland (1990)

Morris *et al.* (1988) proposed that cross-linking of alginate gels involves the cooperative binding of Ca^{2+} in the interstices of aligned ribbons of polyguluronate sequence, giving a close ion-pair association with the carboxylate anion. This yields a so-called "egg-box" model, which is relatively undisturbed by the removal of water. The junction zones are terminated when the polyguluronate sequence gives way to polymannuronic acid or mixed sequences, which have a lesser role in gelation. Like curdland gels, the alginate gels are thermostable and only melt at temperatures in excess of $120^{o}C$. The physical properties of alginate gels greatly depend on the ratio of D-mannuronic acid to L-guluronic acid. Brittle gels are obtained from alginate with a high

guluronic acid content; weaker, more flexible gels result if the D-mannuronic acid content is high (Sutherland, 1990).

Gellan form gels in the presence of either monovalent or divalent cations. The setting temperature and the strength of this gel depend on the nature and concentration of the cation as well as on the polysaccharide concentration. This gel contains polysaccharide chins organized in parallel to form an intertwined doulble helix. In the gel formed with K^+, the cations are cooridated to the carboxylate group which is involved in interchain hydrogen bonding to yield a stable duplex. Gelation may even occur in two steps: chain ordering followed by chain association. The melting temperature of this gel is $60^{\circ}C$ and the setting temperature is $45^{\circ}C$. In the absence of salt, the transition of gellan shows no detectable thermal hysteresis. There is a simple conformational transition from a double helix at low temperature to a single-coil structure at high temperature. Gels formd with K^+ set at lower temperature than those with Ca^{2+}. Althrough gels of the same strength can be formed with K^+ or Ca^{2+}, the concentration of divalent cation needed is much lower. This is similar to the gelation of polysaccharide XM6 with Na^+ and Ca^{2+}, in which higher concentrations of monovalent ions are also required; the gel formed shows a very sharp transition at $30^{\circ}C$, which is independent of the ion used.

Polysaccharide from *E. clocae* WD7 could be formed the true gel (ionic gel) only in alkaline aqueous solution and the divalent metal salts such as $CaCl_2 \cdot 2H_2O$, $MgSO_4 \cdot 7H_2O$, especially $CuSO_4 \cdot 5H_2O$ (Dermlim *et al.*, 1999). Ratio of polysaccharide to metal salt about 2.5:1 by weight (1 ml of 0.5% w/v polysaccharide with combination of 2 mg of each divalent metal salts in 0.2 ml of 2M NaOH) was used. The different types of divalent cations and the ratio of polysaccharide to metal salt gave the different appearance of gels. $CuSO_4 \cdot 5H_2O$ gave a stronger gel than $CaCl_2 \cdot 2H_2O$ and $MgSO_4 \cdot 7H_2O$. However, if calcium ions are exchanged for monovalent ions such as sodium, the cross-linking is lost ans the gel losses its rigidity and stability (Lloyd *et al.*, 1999).

2.14.3 Bead

Bead, microsphere and encapsution were the different names applied to use in various objectives or areas. Polysaccharide gel beads offer the advantage of being usable for microbial cell immobilization (Guiseley, 1989). Numerous techniques were reported to use in polysaccharide bead preparation (encapsulation) such as dripping, emulsification or coacervation, atomization, rotating disc atomization, electrostatic dripping, mechanical cutting and the vibrating nozzle technique, all of which suffer from certain limitations. No technique allows for the simultaneous production of beads with a

narrow size distribution, a high production rate, a satisfactory level of material utilization under mild and nontoxic conditions, under completely sterile conditions and with the ability to scale-up.

The simplest well known technique of polysaccharide bead preparation is dripping method that is to lay down a saponification process. It is the basic method for bead formation of polysaccharide that has been growing interest due to basic technique being in the field of cell immobilization that has led to the development of immobilized cell technology and encapsulation. An aqueous suspension of polysaccharide was extruded from a syringe into a dripping solution that presence of suitable ions, for example; solution of calcium chloride (0.3M) for bead of sugar beet pectin (2.5-3% w/v) (Harel *et al.*, 1998) and solution of calcium sulphate for alginate bead etc. Then, the gel particles were stirred in the precipitation bath and washed with distilled water. The results were namely citrus pectin and calcium alginate, respectively.

Most method of bead preparation from polysaccharide still be the droping polysaccharide gel or viscous solution into solution offer the bead making, or in contrast, being droping of some chemical solution into polysaccharide gel or solution to produce bead as encpsulation. Polysaccharide can be formed into bead or gel bead by own properties or using some chemical as linker, filler, supporter or nuclease of bead. Condition for bead preparation is the important control point to influence to shape, surface and properties of beads, in addition to preparation conditions for polysaccharide and bead making solution.

The fillers and other ingredients influenced on external and internal shape and texture. Presence of filler in the bead increased the number of mid-area 'craters' and reduced the number of the smaller 'craters' formed on the bead's dried surface. Smaller-sized fillers (such as kaolin) gave a smoother surface; a rougher surface was achieved with bigger-diameter filler, such as bentonite. These parameters are very important since the surface of the bead is the first part to come into contact with its fluid, solid or gaseous environment and together with its internal structure, will influence, if not determine, its suitability to a predetermined task. Starch can be incorporated into freeze-dried alginate beads, serving as a carbon source and filler.

Process of alginate bead was successful example. It can be divided into three steps; liquid distribution, droplet formation and bead formation by droplet solidification. Senuma *et al.* (2000) prepared the alginate beads ,30 μm diameter, were formed by droplet solidification upon entering the $CaCl_2$ bath the sodium alginate droplets solidify

due to the exchange of the monovalent Na^{+} for the bivalent Ca^{++}, providing a physical ionic crosslinking between carboxylate ($-COO^{-}$) groups on the alginate main chain. Using this ionic crosslinking, solid particles can be formed directly. As the alginate drop enters the bath, a self-arrangement of the di-block copolymer occurs at the interface with the hydrophilic block pointing toward the outside and the hydrophobic one toward the inside, because the alginate molecular chain contains hydrophobic segments. This forces the hydrophilic crosslinking solution to cover the entire drop and results in immediate and complete wetting and the formation of spherical beads. Therefore, floating drops may collide with subsequent drops, thus producing twins or other undesired shapes. To avoid this problem, a di-block copolymer, polyethylene glycol/polypropylene glycol (Pluronic L68) surfactant, is dissolved in the $CaCl_2$ bath.

However, in adequate viscosity, the drops undergo deformation and produce pear-like particles. Solidified particles indeed exhibit tails, indicative of high deformation upon landing in the bath and gelation prior to regaining a spherical shape. In contrast, viscous drop (with higher alginate concentrations) withstand deformation and essentially form spherical beads. It is likely that a small deformation of the droplet might occur upon landing, but that it is regained prior to gelation, resulting in nearly perfectly spherical beads. This is particularly true for large particles, whereas small particles tend to show a tail more easily. Goosen *et al.* (1989) observed that the capsule sphericity depends on sodium alginate viscosity, but also on the sodium alginate solution purity. Crude alginate solutions resulted in capsules exhibiting tails and striations, and having a granular texture with surface irregularities. In general, dried hydrocolloid beads are stronger than non-dried gel beads, their porosity can be controlled such that produced gases can be liberated from their matrix without interfering with its structure, and they are important as carriers for food and biotechnological operations (Nussinovitch, 2001).

2.14.4 Fiber

Fibres have been extensively used in wound dressing applications because of their unique/advantageous properties, such as high surface area, softness, absorbency and ease of fabrication into many product forms. Fibres made from natural sources, especially polysaccharides, have been considered the most promising due to their excellent biocompatibility, non-toxicity, and potential bioactivity at the wound surface and beyond. Many commercial wound dressing products (woven and non-woven dressings) are made from such natural polymers and their derivatives, the simplest being retention bandages, support and compression bandages, absorbents, gauzes, tulle dressings, and wound

dressing pads produced from woven cellulose fibres (cotton and viscose) (Lloyd *et al.*, 1998, Kennedy *et al.*, 2000). Polysaccharide fibers have been considered the most promising due to their excellent biocompatibility, non-toxicity, and potential bioactivity at the wound surface and beyond. Polysaccharide-based dressings have increasingly become viable alternatives to somewhat incompatible and often problematic cotton or viscose gauzes traditionally used for wound dressings. Abundant availability of alginates and their relative ease of reversible solubility in particular have been instrumental in their development into fibers and lately their application as vehicles for delivering drugs (Miraftab *et al.*, 2003).

The fiber formation process is simple in operation and is essentially based on exchange of ions, as same as bead preparation. In preparation of insoluble fiber of calcium alginate from seaweed alginate, sodium ions (Na^{+}) present in soluble sodium alginate 'dope' and calcium ions (Ca^{2+}) present in the coagulating calcium chloride bath. Alginate fibers are generally prepared by injecting a solution of water-soluble alginate (usually sodium alginate) into a bath containing an acidic solution and/or calcium salt solution to produce the corresponding alginic acid and/or calcium alginate fibers, respectively, which can be used to produce yarns and fabrics for medical applications (Miraftab *et al.*, 2003; Qin *et al.*, 1997; Chen *et al.*, 2001).

Among the various fibrous and hydrogel products, alginate-based products are currently the most popular ones used in wound management, since they offer many advantages over traditional cotton and viscose gauzes (Horncastle, 1995; Qin and Gilding, 1996). They are biocompatible and form a gel on absorption of wound exudate. Alginate fibres are generally prepared by injecting a solution of water-soluble alginate (usually sodium alginate) into a bath containing an acidic solution and/or calcium salt solution to produce the corresponding alginic acid and/or calcium alginate fibres, respectively, which can be used to produce yarns and fabrics for medical applications (Chen *et al.*, 2000; Miraftab *et al.*, 2003).

Although chitosan can be produced in fibre and fabric forms by gel-spining (Notin, 2006), products made from pure chitosan fibres have not been commercially viable due to the high processing costs involved (deproteination, demineralisation and deacetylation processes are required to produce chitosan materials of adequate purity). Therefore, the availability of such purified material is still insufficient for large industrial scale fibre production. Chitosan has been used to coat calcium alginate filaments (utilising the cationic interaction of the chitosan with the anionic nature of the alginate to

produce a tight interaction). However, due to its high molecular weight the chitosan must be used in very low concentrations, as precipitation occurs in the presence of calcium ions, resulting in very low levels of chitosan incorporation into the fibres (<0.2% w/w). Problems in the direct production of chitosan/alginate fibres have been overcome using a variety of different approaches. Alginate and chitosan fibres have been separately produced and subsequently blended, and chitosan has been utilised as the insolubilising cation for production of an alginate fibre.

2.14.5 Sponge

Preparation and characteristics of novel gelatin-alginate sponge for use as artificial skin (Choi *et al.*, 1999). An absorbable sponge was prepared by new crosslinking method that improved the efficiency of crosslinking. The crosslinking degree was characterized by trinitrobenzenesulfonic acid (TNBS) assay. As the alginate content in the sponge increased, the porosity increased, resulting in the enhanced water uptake ability. For water uptake ability test, sponges were loaded with silver sulfadiazine or gentamicin sulfate slowly released drugs for up to four days. Crosslinked sponge resisted *in vitro* collagenase digestion for up to three days. An *in vivo* animal test using witar rat showed rather good wound healing effect of this sponge containing AgSD than vasseline gauze in our full-thickness skin defect model.

Drug-impregnated polyelectrolyte complex (PEC) of chitosan and sodium alginate sponge with silver sulfadiazine (AgSD) was studied for wound dressing application Kim *et al.* (1999). Morphological structure of this wound dressing was observed to be composed of a dense skin outer layer and a porous cross-section layer by SEM. The release of AgSD from AgSD-impregnated PEC in PBS buffer (pH 7.4) was dependent on the number of repeated *in situ* complex formations. Antibacterial capacity of AgSD-impregnated sponge was examined in agar plate against *Pseudomonas aeruginosa* and *Staphylococcus aureus*. PEC wound dressing containing antimicrobial agents could protect the wound surfaces from bacterial invasion and effectively suppress bacterial proliferation. In cytotoxicity test, cellular damage was reduced by the controlled release of AgSD from sponge matrix of AgSD-medicated wound dressing. *In vivo* tests showed that granulation tissue formation and wound contraction for the AgSD plus dihydroepiandrosterone (DHEA) impregnated PEC wound dressing were faster than any other groups.

2.14.6 Film

Films are defined as thin layer of material which can provide some main properties needed on applications such as being a barrier to moisture, gas and solute movement. Films may be devided to two groups, non edible and edible films. Non-edible films can be used in food area, not as food, but as packaging or protection materials which are proved for safe and no-reaction to give some toxic and/or contamination into food. E dible films are films can be eaten as food and non-toxic material against consumers and usually have some important properties as being a barrier to moisture, oxygen and solute movement (Guilbert, 1986). They can be prepared from some raw materials used as food being such as well-known film from protein, lipid and especially carbohydrate. Edible films can be formed as food and coatings and free-standing films and have potential to be used with food as gas aroma barrier (Kester and Fennema, 1986). Production of edible films causes less waste and pollution, however, their permeability and mechanical properties are generally poorer than synthetic films.

The advantages of edible films over other traditional non edible polymeric packaging materials are summarized by Gennadios *et al.* (1994) as followings:

1. They could be consued with the package products. This was obviously of critical importance since it represents the environmentally ideal package.
2. There was no package to dispose of even if the films were not consumed they could still contributed to the reduction of environmental pollution.
3. The films were produced exclusively from renewable, edible ingredients and therefore were anticipated to degrade more readily than polymeric materials.
4. The films could be enhanced for the organoleptic properties of packaged foods provided that various components (flavorings, colorings sweeteners).
5. The films could be supplemented for the nutrition vale of the foods.
6. The films could be used for individual packaging of small portion of food, particularly products that currently wee not individually packaged for praical reasons such as pears, beans, nut and strawberries.
7. The films could be applied inside heterogeneous foods at the interfaces between different layers of components. They could be tailored to prevent deteriorative intercomponent moisture and solute migration in foods such as pizzas, pies and candies.

8. The films could be functioned as carriers for antimicrobial and antioxidant agents. In a similar application they also could be used at the surface of food to control the diffusion rate of preservative substances from the surface to the interior of the food.
9. The films could be very conveniently used for microencapsulation of foodflavoring and lesavening agents to efficiently control their additional and released into the interior or foods.
10. Another possible application for edible films could be their uses in multilayer food packaging materials together with nonedible films. In this case, the edible films would be the internal layers in direct contact with food materials.

2.15 Preparation of Polysaccharide Film

Polysaccharides used for edible films include cellulose and derivatives, starch and derivatives pectin, seaweed extracts (alginate), exudates gums, microbial fermentation gums and chitosan (Krochta and Mulder-Johnson, 1997). Polysaccharides are generally very hydrophilic resulting in poor water vapor and gas barrier properties. Although coating by polysaccharide polymers may not provide a good water vapor barrier, these coatings can act as sacrificing agent retarding moisture loss from food products (Kester and Fennema, 1986).

Edible film can be produced from material with film forming ability. During manufacturing, film matrials must be dispersed and dissolved in the solvent such as water, alcohol or mixture of water and alcohol or mixture of other solvents. Plasticizer, antimicrobial agent, colors or flavor can be added in this process. Adjusting pH and / or heating the solutions may be done for the specific polymer to facilitate the dispersion. Film solution is then casted and dried at desired temperature and relative humidity to obtain a free-standing film (Donhowe and Fennema, 1993). Composite films can be prepared from heterogeneous natural film blend of polysaccharides, protein and / or lipids). In the food application, film solutions could be applied to food by several methods such as dipping, spraying, brushing and panning followed by a drying step. There are many examples of polysaccharide film found nowadays.

A plasticizer may be defined as a compound, when added to another material and under given conditions, modifies certain physical and mechanical properties of the material, less likely to break and more flexible and stronger. This was due to reduction of the intermolecular bonds between the polymer chains, and thus the overall cohesion, facilitated elongation of the films and reduced its glass transition temperature. This is

manifested by a reduction in the barrier properties to gasses, vapors, and film solutes (Banker, 1966; Kumins, 1965).

Cellulose films: Carboxy methyl cellulose (CMC), methyl cellulose (MC), hydroxyl propyl cellulose (HPC) and hydroxyl propyl methyl cellulose (HPMC) films possess good film-forming charecteristis. These films were generally odorless and tasteless, flexible and were of moderate strength, transparent, resistance to oil and fats, water-soluble, moderate to moisture and oxygen transmission. They have been used to retard oil absorption in deep frying food product (Kester and Fennema, 1986; Williams and Mittal, 1999). MC was the most resistant to water and could be applied as coating on confectioner products as berrier to lipid migration. Cellulose could also be chemically modified to ether, ethyl cellulose (EC), which is biodegradable but not edible. EC films could either be cast from non-aqueous solutions or extruded. Like the other cellulose ethers, EC films were poor moisture barrier, but they have been reported to be good oil and fat barriers (Hanlon, 1992).

Pectin films: Pectin was a complex group of structural polysaccharides found in the middle lamella of plant cells which composted of (1,4) α-D-galactopyranosyluronic acid units with varying degrees of esterification (DE). Chemical de-esterification yielded low-methoxyl pectins which when dissolved in aqueous media was capable of forming gels in the presence of calcium ions. The function of the ionic calcium is to bridge free carboxyl group on adjacent polymer molecules. Pectin with DE >50% was classified as a high-methoxyl and as a low methoxyl pectin at DE <50%. Pectin might form film alone or blended with other polymers (Coffin and Fishman, 1993). These films or coating gave a glossy, non sticky surface, high water vapor permeability. Although pectinate coating was poor in moisture barrier, they could reduce the moisture loss from food product by sacrificing (Kester and Fennema, 1986).

Chitin and chitosan films: Chitin is the second most abundant naturally occurring biopolymer (after cellulose) and it is found in the exoskeleton of crustaceans, in fungal cellwalls and other biological materials. Chitosan could form semi-permeable coatings, which could modify the internal atmosphere, thereby delaying ripening and decreasing transpiration rates in fruits and vegetables. Films from aqueous chitosan were clear, tough, flexible and good oxygen barriers. Carbon dioxide permeability could be improved by methylation of polymer. Butler *et al.* (1996) observed that films from chitosan were ratcher stable and mechanical and barrier properties changes only slightly during storage. Chitosan coatings were usually used on fruit and vegetable products such as strawberries,

cucumbers, and bell peppers as antimicrobial cotings and on apples, pears, peaches and plums as gas barrier

Starch films: Strach consists of amylose an amylopectin, the ration of amylase and amylopectin depends on thetype and variety of raw material. Combined effect of plasticizers and surfactants on the physical properties of starch based edible film was studied by Rodríguez (2006). High amylose starch as conn starch is a good source for films formation. Free-standing films could be produced from aqueous solution of gelatinized amylase and drying. Normal corn starch consisted of approximately 25% amylase and 75% amylopectin. Mutant varieties of corn were produced which contained starch with up to 85% amylose.

Starch hydrolysates (dextrin) of low dectrose rquivalent (DE) have been suggested for use a protective coating. Althrough hydrophilic in nature, starch hydrolysates provide a limited resistance to transport of water vapor. It was evaluated the relative barrier properties of edible film materials by coating them onto a cellulose acetate upport. Starch films displayed minimal resistance to water transport, while films of low-DE dextrin and corn syrup were approximately 2- and 3-fold more resistant, espectively. Murray *et al.* (1973) coated almond nutmeats with a 50% solution of a 10-DE starch hydrolysate. Sensory evaluation indicated that the coated nuts maintained a more desirable texture than uncoated controls during storage. Presumably, this was attributable to a reduction in the rate of moisture absorption by coated almonds. Films of starch hydrolysates may exhibit some resistance to oxygen transmission. Dipping of fresh sliced apples in a 40% solution of a 15-DE hydrolysate prior to dehydration prevented browing of tissue, probably by retarding the enhancement of oxygen (Murray *et al.*, 1973).

Film of seaweed and gum polymer: Alginate, carragenan and agar are seaweed products and have good film formimg characteristics. Alginate is the salt of alginic acid, a linear (1->4) linked polyuronic acid extracted from brown seaweed. Film formations, which may or may not involve gelation, can be achieved by evaporation, electrolyte crosslinking, or injection of a water-miscible nonsolvent for alginate (Kelco, 1976). Alginate coating possessed good oxygen and lipid barrier but poor water vapor barrierproperties (Cottrell and Kovacs, 1980; Conca and Yang, 1993). Additionally, coating with alginate could improve flavor, texture and batter adhesion.

Carragenan is an estract from red seaweed which consists of a family of sulfated polysaccharides of D-galactose and 3, 6-anhydro-D-galactose. Upon cooling, a warm aqoueus solution of the polymer, gelation occurs presumably by the formation of a

double-helix structure to yield a three-dimensional polymer network. Coating with carrageenan has been used in food to incorporate antimicrobial agents and reduce moistur loss, oxidation or disintegration (Krocha and Mulder-Johnson, 1997).

2.15.1 Formation of Polysaccharide Films

Polysaccharide films could be formed with different methods of evaporation of water or solvent (surface film formation and deposition method), precipitation (extrusion method) and radiation. Films by these methods can be converted chemically into different composition and properties without change of form. It is often convenient to convert the film to the original raw material (status) as an intermediate step. Evaporation: is the simpliest method by removing of water from a solution leaving a film of soluble polysaccharide tested such as alginate film preparation (McDowell, 1974).

Surface film formation: Films were obtained by prolong heating of film solutions and films were periodically harvested from the surface, drained and dried. During heating, the high molecular weight polysaccharide was broken down into lower molecular weight moiety. Interfacial forces might initiate the formation of a polysaccharide matrix capable of trapping oil droplets and water released from the surface and facilitating the formation of polysaccharide matrixes (Fanum *et al.*, 1976).

Deposition method: Films obtained from this method generally are made by casting and drying film-forming solution on a non-stick surface (such as on Teflon coated baking pan). Films from this technique were more uniform compared to the surface forming method. Film thickness could be controlled by the amount of total solids in the film solutions which was not the case when the surface formation method was employed. Most researchers have been using the deposition technique in recent years to produce edible films. However, casting material and casting temperature might vary depending upon the state and ype of substrates. This method has been used to make polysachharide film.

Extrusion: Film can be formed by precipitation the solution of soluble polysaccharide sample into a precipitating bath to give an insoluble film (e.g. calsium alginate film by extrusion into a solution of a calcium salt). This method is also applicable to the formation of filament.

Radiation: Gamma irradiation is an alternative method for the manufacture of sterilized packaging with increased storage stability and microbiological safety. PHB (poly 3-hydroxybutyrate) films are typically brittle. Many scientists have attempted to reduce this brittleness by blending PHB with PEG (polyethyleneglycol) and casting film by

irradiation at a dose rate of 5.72 kGy/h with a ^{60}Co. Water vapour transmission of films decreased with increasing irradiation dose, indicating that the films performance as water vapour barrier had improved. The vapour barrier property of PHB/PEG blend was enhanced at low irradiation doses, probably owing to the cross-linking effect that reduced the pore size within the blend structure, thus avoiding the water vapour transmission observed in pure PHB films.

2.15.2 Factors Affecting on Polysaccharide Films

Many factors have affected on formation and properties of polysaccharide films.

Types of material: Polysaccharide films have weak water vapour barrier properties because of their hydrophilic characteristics; their water vapour permeability is related to their thickness. Some carbohydrates such as alginate, carrageenan, pectin, starch, cellulose and cellulose derivatives provided a strong matrix free standing films, but these films had poor water barrier properties because hydrophilic nature of raw materials used (Kester and Fennema, 1986).

Polymer chemistry: The regular structure molecule of polysaccharide is more diffusible than irregular stereochemical structure whereas branched molecule may provide a greater cohesive strength than non-branch molecule. Lower molecular weight fraction shows a greater cohesion and a greater change in cohesion with temperature change. In highly polar polymer such as cellulose, a regid ring structure chain back bone, self-adhesion by diffusion is not significant due to the minimal flexibility and fixed order of the macromolecule caused by the internal molecular forces holding the polymer chains (Banker, 1966). The desire molecular characteristic of polysaccharides for film formation as 1) high soluble molecules promoted rapid diffusion; 2) the large molecules allowed more interactions at the interface resulting in strong film; 3) amphiphatic molecules provided unbalanced distribution of charged and apolar residuals for improved interfacial interaction; 4) flexible domains facilitated phase behavior and unfolding at interface; 5) dispersion of charged groups affected protein-protein interaction in the films and charge repulsion between neighboring bubbles; 6) polar residue could provide hydratable or charged residues to keep bubbles apart, binding and retaining water; 7) retention of structure could enhance overlap and segmental interactions in film; 8) intractive regions could affect depositions of different functional segments and facilitate secondary interactions in the sir and aqueous phases.

pH: The pH plays an important role in polysaccharide films made from water-soluble materials since the cohesive forces between the solute molecules are neutralized by unions with the solvent molecules during the dissolution (Banker, 1966). The functionality of the polymer was related to solution properties which further influenced film charecteristics. The charged groups repelled each other and produced a stretching of the polymer chain when the functional groups on a linear polymer became ionized during dissolution. The greater the degree of dissolution and more extensively the chain was charged, the greater was uncoiling of chain. The interection between the charged polymer molecules and the molecules of the polar solvent increased with increasing charge on the chain.

Casting teperature: Temperature has influence on the interaction forces in polysaccharide structure. Hydrophilic interaction increase, hydrogen bonds and electrostatic interaction decrease when temperature increase (Kinsella and Phillips, 1989) resulting in facilitation of adhesion between polymer films and substrate (Banker, 1966). High temperature (70-100^{o}C) affected the forming or rigid structure in polysaccharide solutions due to breaking of glycosidic bond. The excessive heat or excessive solvent evaporation rate during process might produce non-cohesive films (Guilbert, 1986). Water soluble polysaccharide needed a medium temperature and time for films formation storter than film from alcohol-soluble polysaccharide. The higher drying temperature of water-soluble based films might limit films use. However, low relative humidity could also be employed for film formation at low temperature as reported.

Casting time: In thin films, the short drying time made the molecules too stretched annd could give a tensile strength, lower than for thicker films (Jansson and Thuvander, 2004). The films of 2.5 mm had enough time for the molecules to relax and orient themselves. It was only in the films of 1 mm that the molecules had induced crystallization caused by some stretch of the molecules. Therefore, the drying of the films is difficult parameter to deal with. It seems like 1 mm thick films drying fast enough to stretch the molecules yet slow enough to prevent molecular relaxation, resulting in molecular orientation. Thin films dry to fast in 23^{o}C which seems to result in molecular orientation and thick films dry to slow the molecules seems to have time to relax.

Concentration: Concentration of film solutions affected the self adhesion of high polymers and rate of matrix forming in film preparation. At low concentration of high concentration, self-diffusion was promoted. At optimum concentration of film solutions, an intermediate viscosity could be obtained which resulted in the highest cohesive strength (Banker, 1966; Guibert, 1986).

Film addifives: Plasticizer was a major component of edible films. A plasticizer may be defined as a compound, when added to another material and under given conditions, modifies certain physical and mechanical properties of the material. The addition of a plasticizer to films produced films, which were less likely to break and more flexible and stronger. The reduction of intermolecular bonds between the polymer chains, and thus the overall cohesion, faciliatated elongation of films and reduced its glass transition temperature. This is manifested by a reduction in the barrier properties to gasses, vapors, and film solutes (Mali *et al.*, 2004). However, it was important to remember that the formation of the whole film system (polymer, solvent, plasticizer, and other additives) had a direct effect on the nature and characteristics of the films produced. Polymer and the plasticizer must not only be compatible, but also have similar solubility in the solvent used. A soluble plasticizer will be generally be sought for the development of soluble coating and an insoluble plasticizer (or dispersible) for an insoluble coatings or for a slow solubilization (Guilbert, 1986).

2.15.3 Effect of Plasticizer on Properties of Polysaccharide Films

Plasticizers are the basic material added to the film forming polymers. They reduce intermolecular forces, increase the mobility of the biopolymer chains and thereby improve the mechanical properties of films. In addition, a plasticizer is necessary in order to overcome the film brittleness, improve the barrier properties of film and prevent it from cracking during packing and transportation. On the other hand, plasticizers generally increase the transferal of gases, water vapour and the soluble solid materials through the films. Edible films can improve the appearance of food and protect its properties during their storage and handling. Hydrophilic edible films contain oxygen and carbondioxide barrier properties at low relative humidity (Aydinli and Tutas, 2000).

Generally two types of plasticizers, internal and external plasticizers, were distinguished. Internal plasticization was a result of modifications to the chemical structure of the polymer, for example, by copolymerization or selected hydrogenation or transesterification in the case of edible fats or similar. External plastification is obtained by adding an agent which modifies the structure and energy within the three-dimensional arrangement of the film polymer (Banker, 1966). It was the second method which, on the basis of the type of material and the technology, was mainly used for edible packaging and coatings.

The permanence of plasticizer was also of prime importance since this influence the physical and mechanical stability of the films. The plasticizer should not be

volatile (or only very slightly volatile) and its degree or retention by the films should be high. Other properties, such as its chemical stability, hygroscopicity, color and flavour andso on, were also more or less important depending on the type of films uner consideration. In addition, the concentration of plasticizer necessarity varied from 10-60% (dry base) according to the nature and type of films and the method of application (Guilbert and Biquet, 1996).

Most plasticizers often used in the field of edible films are (a) mono-, di- and oligosaccharide (generally glucose syrups or glucose-fructose honey) (b) polyols (principally glycerol and its derivatives) (c) lipids and its derivatives (fatty acids, monoglycerides and their ester, phospholipids (d) other emulsifiers and (e) protein.

Glycerol and polyethylene glycol were found to be the most effective plasticizers for some polysaccharide. Park *et al.* (1993) studied the effect of three plasticizer comprising polyethylene glycol (PEG), propylene glycol (PG), glycerine (G) at 4 level concentrations on cellulose based films. Decrease in tensile strength and increase in elongation were found when plasticizer content increase. PEG was found to be most effective to improve flexibility among glycerin and PG. However, PEG did not affect film's permeability properties. Glycerine did not have effect on oxygen permeability of cellulose films but affected water vapor permeability that increased with increase of glycerin but decreased with decrease of cellulose. Water vapor and oxygen permeability of cellulose films increased with an increase of PG.

In pectin films casting, Coffin and Fishman (1993) found that glycerin performed better than urea and PEG. The mechanical properties (elongation and tensile strength) improved with increasing glycerin (9-19% w/w). Guo (1994) investigated the effect of PEG-600 on sucrose permeability of cellulose acetate films and reported that permeability of sucrose showed decrease with increasing plasticizer concentration; however when the plasticizer concentration increased above 30% dramatic increase in sucrose permeability was found. Butler *et al.* (1996) investigated the effect of glycerin on properties of chitosan films. As they expected, the barrier properties and elongation at brake increased but tensile strength decreased with increasing glycerin.

PEG was the effective plasticizers to improve flexibility of some polysaccharide films from the decrease in tensile strength (TS) and increase in elongation (E) when plasticizer content increase. However, PEG did not affect film's permeability properties. Guo (1994) investigated the effect of PEG600 on sucrose permeability of cellulose acetate films and reported that permeability of sucrose showed decrease with

increasing plasticizer concentration; however when the concentration of plasticizer increased above 30% dramatic increase in sucrose permeability was resulted.

Aydinli and Tutas (2000) found that water sorption of edible films containing Locast Bean Gum (LBG) and PEG200 as plasticizer increase sharply above aw 0.65. Permeability of edible LBG films were found to increase with increasing amounts of plasticizer, PEG (200, 400, 600), which are liquid at room temperature. Jansson and Thuvander (2004) reported that glycerol was the common plasticizer for hydroxyl propylated native potato starch films preparation with 30 parts by weight of glycerol above 20% glycerol, phase separation was suggested to occur. Some reports presented that the mechanical properties of amylose and amylopectin films added with glycerol exceeded 20% were changed dramatically, while their efficient oxygen barrier properties were lost above 20% water. Both stiffness and strength showed a strong dependence on film thickness, stronger than expected from linear fracture mechanics.

Gaudin *et al.* (1999) found that wheat starch was plasicised with sorbitol below 27% (dry basis) as plasticizer and the films were brittle while a plastic behaviour was observed at higher concentrations. Ma *et al.* (2004) studied the using of glycerol, urea and formamide as a mixed plasticizer for thermoplastic wheat flour (TPF) by a single screw extruder. TPS plasticized with glycerol, a conventional TPS, was thought to tend to retrogadation after being stored for a period of time and this retrogadation embrittled TPS. Urea was proven to prevent retrogradation. It is, however, a solid with little internal flexibility and hence urea-plasticized TPS becomes regid and brittle. Formamide could availably suppress the retrogradation of thermoplastic corn starch and make thermoplastic starch more flexible, but the tensile failure stress was weak. Since formamide is a good solvent for urea, a combination of urea and formamide could effectively restrain TPS retrogradation and improve mechanical properties of TPS.

Butler *et al.* (1996) investigated the effect of glycerin on properties of chitosan films. As they expected, the barrier properties and elongation at brake increased but tensile strength decreased with increasing glycerin. In order to decrease water vapor permeability on whey protein isolate (WPI) films by using glycerol or sorbitol as plasticizer. Sodium dodecyl sufate (SDS) could not be used as plasticizer by itself, however, when used as co-plasticizer with sorbitol at mass ratio of SDS to WPI of1:2, films improved in flexibility and solubility without water vapor permeability change. When SDS was used with glycerol at the same ratio of SDS to WPI, a less flexibility and solubility with slight increase in water vapor permeability were shown.

2.15.4 Some Application of Polysaccharide Films

The use of natural hydrophilic polymer as drug carriers has received considerable attention in the last few years, especially from the viewpoint of cost, environmental pollution and safety (Remuñán-López and Bodmeier, 1997). In particular, polysaccharides such as alginates and chitosan and their derivatives are suitable candidates for sustained or controlled drug-release systems.

Chitosan had the importance resides in their antimicrobial properties in conjution with their cationicity and their film-forming properties. It has been extensively used over a wide range of applications, such as a biomaterial in medicine either on its own or as a blend component, a membrane filter for water treatment, a biodegradable, edible coating or film in food packaging, a dietary fibre, and a medicine against hypertension because of its scavenging action for chloride ions. It was expected to use as food coating and casing applications such as sausage casings, poultry coatings and meat industry (Arvanitoyannis *et al.* 1998).

The gel forming ability of alginates with $CaCl_2$ has been used in immobilizing living cells or in the controlled release of drugs, many applications can be found in pharmaceutical technology. Natrajan and Sheldon (2000) examine the use of 0.5% calcium alginate based films containing bacteriocin (nisin) formulations for inhibiting *Salmonellae* on fresh broiler drumstick skin (coated with the film). Varying concentrations of nisin (0-500 microg/ml) plus 3% citric acid, 5.0 mM EDTA, and 0.5% Tween 80 were incorporated into films at a 20% level (wt/wt) and then tested on *Salmonella typhimurium* skin samples (log_{10} 5.0) at a 1:2 weight ratio (film: skin). Contaminate population reductions ranged from 1.98 to 3.01 log cycles after a 72-h exposure at 4°C. *S. typhimurium* NAr population was reduced ranging between 1.8 to 4.6 log cycles after 72 to 96 h of exposure at 4°C. The level of kill was affected by film type and gel concentration (i.e., gel network formation), exposure time, and nisin concentration.

Yan *et al.* (2001) prepared the chitosan-alginate polyelectrolyte complex (PEC) film for potential applications in packaging, controlled release systems and wound dressings. Coacervation between chitosan and alginate was rapid, but the rate may be controlled with the addition of water miscible organic solvents. Suspensions of fine, uniformly dispersed coacervates were produced by a dropwise addition of 0.25% w/v chitosan solution (solvent: 1:1 v/v of 2% acetic acid and acetone) into 0.25% w/v sodium alginate solution in water under rapid agitation. The PEC films were transparent

and flexible. They exhibited high permeability to water vapor, but resisted complete dissolution in 0.1M HCI, distilled water and pH 7.4 phosphate buffer solution. Microscopic heterogeneity in the films could be reduced by immersion in aqueous media, but this was accompanied by modifications in the thickness, permeability and mechanical property of films.

Some polysaccharide films were produced to reduce fat absortion of food product after frying. Gellan gum, Methyl cellulose and Hydroxy-propyl cellulose (HPC) films reduced the fat absorption by 50% to 91% during frying, but MC film reduced the final fat content in the food more than the other films (Williums and Mittal, 1999).

2.16 Biological Properties and Activity

Biological properties of some polysaccharide biopolymers from microorganisms were usually caused from having some bioactive compound or compartments in structure of those polysaccharides or being substituted with pyruvate, acetate, formate, sulfate, phosphate and other groups (Sutherland, 1990). This also included a wild variety of sugars and, in some cases, several non-carbohydrate side groups such as fatty acids, proteins, hydrocarbons and other polymers of various sizes, as found in polymers produced from prokaryote cells.

Polysaccharides from different sources have various biological properties that can be responsible for the effects associated with food, pharmaceutical and medical application, especially from plants. Although the large numbers of bacterial EPS are potentially available, but bacteria may be pathogenic, production costs may be very high, product quality may be difficult to maintain and to guarantee, or the product may not achieve regulatory acceptability. However, very commonly there is no market niche. Despite these problems, several products from Gram-negative bacteria are accepted products of modern biotechnology. Several more may be developed in the next few years, especially as renewable resources for alternatives to products of the chemical industry.

In general, most of biological activities basically tested are antimicrobial activities such as antibacteria, antivirus, antimalaria, and cytotoxicity against normal or cancen cells that are testing of infection prevention. Mode of action is an important topic for consideration the appropriate and potential application of product. In present, the bioactivity testing can be done in a rapid and easily, reproducible and sensitive fashion and be reliable by using many developing biotechnological techniques. For example, the sulforhodamine B (SRB) protein staining assay for the *in vitro* measurement of cellular

protein content of adherent and suspension cultures was established by Skehan *et al.*, (1990). The dye binds to basic amino acids of cellular proteins and colorimetric evaluation provides an estimate of total protein mass which is related to cell number which is available and well known used as international standard method. Cytotoxicity tests against various cancer cell line and pathogen from various human organs can be investigated by using chemosensitivity testing in microplates that has been widely used for in vitro anti-cancer drug screening such as MTT colorimetric tetrazolium assay and other tetrazolium-based assays (XTT, MTS, WST-1) were developed. Using these assays a large number of tests can be carried out in a rapid, reproducible and sensitive fashion.

Currently, some polysaccharides were studied and showed that be interesting for medical and pharmaceutical application with having or substituted and derivatised with some bioactive compound or constituents (Sutherland, 1998). Some was considered and possible to be produced as high adding valued products in commercial. Some polysaccharide such as chitosan, have been widely investigated for applications as alternative biomaterials that accelerate the wound healing process by inducing production of different types of cytokines including to Interleukine-8 (IL-8), a cytokine that recruits neutrophils to the wounded site as well as stimulates vascularization. Consequenctly, WD7 EPS should to be tested for its potential biological activity and evaluation in case of safty for using and usage.

Alginate was edible polysaccharide due to it has no toxic properties in the human and animal digestive system. Commercially available refined alginates are practically free form any taste or odour. In the British "Emulsifiers and Stabilisers in Food Regulations", alginic acid and sodium and calcium alginates are classified as natural products which are such well established constituents of a number of foods that no legislation is required to limit their use. Propylene glycol alginate is used as the permitted stabilizers with no restriction to its general use that can be digested to some extent and can form part of the carbohydrate of the diet. Normally, however, when an alginate is eaten the grater part remains undigested and if taken in sufficient quantity acts as a bulk laxative (McDowell, 1974). Alginate applied in the form of fibres or powder that has a marked haemostatid action. Furthermore, they are gradually absorbed by vascular tissues without ill effect and are therefore used as surgical dressings which can be left in the body. Therefore, a low molecular weight alginate solution is used by injection as an antigen, followed by a calcium salt to give a gelled deposit, greatly increases the response of the organism to the antigen.

In common with other natural organic substances, the alinates are attacked by various microorganisms, but highly purified alginates do not support the groth of organisms. However, its solutions used in practice contain sufficient nitrogen and salts to allow growth to take place and was depolmerisation by bacteria and mould. Furthermore, alginate solutions are conveniently protected from microbiological attack by using of some preservatives, formaldehyde, chlorinated phenol, ester of p-hydroxy benzoic acid, phenyl mercuric acetate and other bactericides (McDowell, 1974).

Most are *in vitro* testing of toxicity, biocompatibility and healing properties including to anti-bacterial, fungal, viral activity test, anti-TB test, anti-tumor or cancer activities. For human use, some biological properties of this WD7 EPS especially on skin tests were also tested such as heamo-compatibility test (human red blood cell lyses testing) and inflammatory test (Samuelsen, 2000 ; Diallo *et al.*, 2001).

CHAPTER 3
MATERIALS AND METHODS

3.1 Materials

3.1.1 Microorganisms

Enterobacter cloacae WD7 isolated from the recycle sludge in the sedimentation tank of the seafood processing plant in Songkhla region by Dermlim (1999) was used in this study. It was kept in ampoule containing nutrient broth and glycerol (50% v/v) at -80°C, and subcultured on nutrient agar slants kept at 4°C.

Starter of *E. cloacae* WD7 was prepared by inoculating one loop of 18h culture into 100 ml optimized medium (in section 3.1.2) in a 250 ml flask and cultivated on a rotary shaker (200 rpm) at 30°C for 18 h. The culture in log phase (10^6 CFU/ml) was added as starter (5%) in shake flask and fermentor.

Microorganisms used for biological activity assay of the biopolymer (used in section 3.6) were obtained from lyophilized cultures purchased from Microbiological Resources Centre (MIRCEN) of Thailand Institute of Scientific and Technological Research (TISTR). They were Gram positive bacteria (*Staphylococcus aureus*, *Bacillus cereus*), Gram negative bacteria (*Escherichia coli* and *Salmonella typhi*), some fungi and yeast; *Aspergillus flavas* and *Trichoderma viride*, *Saccharomyces cerevisiae* and *Candida albicans* ATCC 90028. Some microorganisms such as virus (Herpes simplex virus type 1, HSV-1) strain ATCC VR260), malaria parasite (*Plasmodium falciparum*, K1, multi drug resistant strain) and bacteria (*Mycobacterium tuberculosis* $H_{37}Ra$) were prepared by National Center for Genetic Engineering and Biotechnology.

Human cells (vero cell line ATCC CCL-81 and normal dermal human fibroblasts), animal cells (immortalized mouse PGH-1 and PGH-2 null cells) and cancer cells (cell lines of human colon cancer, HT 29, human epidermoid carcinoma of cavity ATCC CCL-17, KB), breast cancer cell line, BC and human, small cell lung cancer, NCI-H187) were also prepared by BIOTEC.

3.1.2 Media

The optimized medium contained 3% sucrose (or 5% glucose), 0.05% yeast extract, 0.5% K_2HPO_4, 0.2% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$ and 0.01% NaCl with pH adjusted to 7.0 (Wichienchot, 2000). Commercial sucrose was used as carbon source for production of biopolymer.

Potato dextrose agar (PDA, Himedia) and nutrient broth (NB, Labscan) were prepared (Skehan *et al.*,1990) and autoclaved at 121°C, 15 min. Some instant medium for biological properties tests such as RPMI 1640 and Dulbecco's modified Eagle's medium (Gibco) were used by BIOTEC.

3.1.3 Human Red Blood Cells

Human red blood cells used for heamolysis test (RBCs release test or heamocompatibility test) was obtained from human non-group identified blood without contamination and disease free. They were obtained from blood bank of Songklanakarin Hospital, Thailand.

3.1.4 Chemicals and Reagents

All chemicals and reagents employed were of analytical or reagent grade and commercial grade. Their lists and sources were shown in Table 20 of Appendix 7.1.1.

3.1.5 Instruments

Many instruments used in this works were in Faculty of Agro-Industry, Prince of Songkla University, Thailand. Instruments used in this thesis were supported by Faculty of Pharmacy (Prince of Songkla University) such as some instruments in biological testing, Faculty of Engineering and Industrial Technology (Silpakorn University) such as Texture analyzer, National Metal and Materials Technology Center (MTEC, Thailand Science Park) such as NMR, Thailand and Chembiotech Laboratories (University of Birmingham Research Park, Birmingham, UK). All list and source of instruments were given in Table 4.

3.1.6 Equipments

3.1.6.1 Fermentors

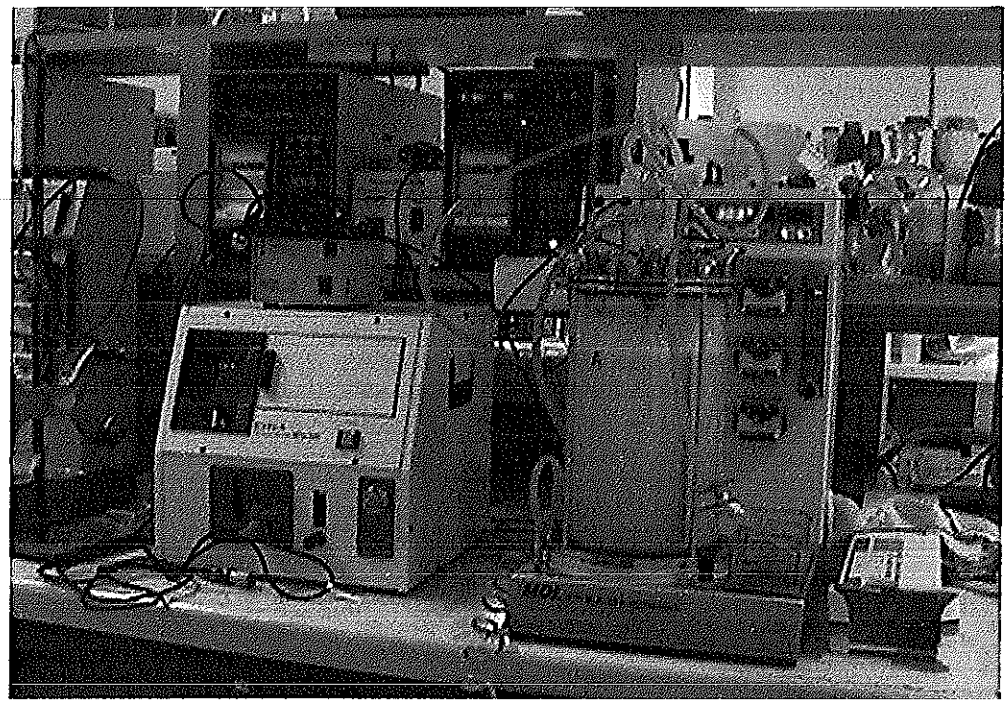
Two continuous stirrer tank reactors; bench scale (5 l) and pilot plant (72 l) fermentors, used in this study and their technical information were shown in Table 5 and Figure 4.

Bench scale fermentation was performed in a 5-L pyrex glass fermentor equipped with DO electrode (DY-220, TOA Electrodics Co. Ltd., Japan) and a controller (Labo Controller MDL-4CR). Agitation was a magnetic coupling system with two of six flat-blade impellers. Aeration system was composed of air inlet through ring sparger, air-flow meter, sterilized plastic filter with cellulose membrane and DO probe. Oxygen content was measured as percentage of dissolved oxygen tension (%DOT).

Table 4 List of some instruments

Instrument name	Model	Source
Autoclave	-	Tommy, USA
^{13}C NMR	DPX-300	Bruker, Germany
CHNS-O Analyzer	Flash 1112 EA	CE Instrument, UK
Colorimeter	MiniScan XE	HunterLab, USA.
Dial thickness gauge	H (0.01-1.0 mm)	Peacock Ozaki Meg, Japan
Differential scanning calorimeter	DSC 7	Perkin-Elmer, USA
Double-beam spectrophotometer	U-2000	Hitachi, Japan
Electronic scale	-	Sartorious, Germany
Fermentor (Bench scale)	MDL 300	B.E. Marubishi, Japan
Fermentor (Pilot plant scale)	Biostat-M	B. Braun, Germany
Freeze-dryer	Dura-Dry™ μp	FTS system, USA
FT-IR- spectrometer	EQUINOX55 series 1600	Perkin-Elmer, USA
Gas chromatography	GC model 5890	Hewlett-Packard, USA
Gel permeation chromatograph	Dionex 500system	UK
Hot air oven	ULM500	Memmert, USA
Incubator shaker	3525-1C	Lab-Line Instrument, USA
Incubate water bath	4-100°C	K.S.L. Engineering, Thailand
Incubator with air circulation	Model 2000	Rumed, Germany
Mass spectrometer	MS model 5971	Hewlett-Packard, USA
Moisture analyzer	HR 73 Halogen	Mettler Toledo, USA
NMR spectrometer	DPX-300	Bruker, Germany
pH meter	D-12	Denver Instrument, USA
Refrigerated centrifuge	SCR20B	Hitachi Koki, Japan
Rheometer	AR1000	TA 'Rheolyst', Instruments, UK
Rotary Evaporator	SB 651	Rikakai Co.LTD., Tokyo
Scanning electron microscope	JSM-5800 LV	JEOL, USA
Spectrophotometer	Series 1600	Perkin-Elmer, USA
Tensile testing instrument	Model 1122	Instron, USA
Texture analyzer	TA-XT2I	Stable Micro Systems, UK
UV-VIS spectroscopy	Ultrospec 4000	Pharmacia Biotech, UK

(a)



(b)

Figure 4 The 5-L (a) and 72-L (b) continuous stirred tank reactors (CSTRs) used for bench scale and pilot scale experiments in this work

Table 5 Technical information of two-sized fermentors used for scale-up study

Properties	Bench scale	Pilot scale	Unit
Total volume	5.00	72.00	litre
Working volume	4.0	50.0	litre
Height of fermentor; H_t	0.25	1.00	m
Height of liquid; H_L	0.23	0.70	m
Diameter of fermentor; D_f	0.15	0.40	m
Diameter of impeller; D_i	0.08	0.10	m
No. of impeller	2	3	-
No. of baffles	3	4	-

Pilot scale fermentation was operated in a 72-L stainless steel reactor equipped with temperature probe, pH electrode, antifoam probe and a dissolve oxygen electrode (InPro 6000 Series, membrane type T-96 Ingold, Mettler Toledo, Switzerland). It was non-geometrically similar to small fermentor (5-L). Agitator consisted of three of six flat-blade impellers which was equipped with electric motor and controlled through a controller. Aeration was done through spider sparger, air filter, air flow meter (DK800N, Krohne Dulsburg, Germany) and shown as %DOT. Steam and cool water circulation was used for controlling temperature and sterilization within fermentor.

3.1.6.2 Rheometer

Rheological flow measurements were performed using a controlled stress rheometer, which utilizes a drag cup motor to apply a known torque to a drive shaft and measuring geometry mounted on an almost friction free air bearing. The displacement of the sample in response to the applied stress is monitored by a very sensitive optical encoder. Temperature control is by means of a standard Peltier plate which has an operating range of -10 to 99.9°C (a maximum ramp rate of ~20°C/ min). The AR1000 was controlled using TA Instruments 'Rheology Solutions' software package which is split into two modules. The 'Run' module enables the operator to set up the experimental procedure and performs the actual experiments, whilst the 'Data' module is utilized to manipulate and present the collected data.

TA 'rheolyst' rheometer was set for measuring the specific flow behaviour, parameters and the conditions for testing were as followed:

Geometry of measurement: 4 cm steel flat plate, gap 50 μm,

Calibrate inertia: 22.16 μ.Nm.s^2,

Instrument inertia: 14.38 μ.Nm.s^2,

Geometry inertia: 7.781 μ.Nm.s^2.

Pre-experiment step: Initial temperature; 20 °C, Shear stress (Applied value); 1.768 Pa, Preshear duration: 10 s, Equilibration duration; 10 s.

Flow step: Step type; Stepped ramp; Temperature 20°C; Controlled variable; Shear stress (0.5-500 Pa), Start value; 0.5 Pa, End value; 500 Pa.

Biopolymer solution sample was loaded on the plate, tested at above conditions. The resulting profiles were analyzed by TA Instrument programs.

3.1.6.3 Brookfield Viscometer

Apparent viscosity (cp or mPa.s) of broth was measured using a Brookfield viscometer at various shear rates (γ = 1.32 x N sec^{-1}, where N = rpm) with spindle SC4-18/13R at room temperature for samples with apparent viscosities 0-200 cP. The relationships among apparent viscosity, shear rate and shear stress were given in the Brookfield viscometer manual as followed:

Apparent viscosity (cP) = (shear stress x 100) /shear rate

Shear stress (dyne/cm^2) = (apparent viscosity x shear rate) / 100

3.1.6.4 Texture Analyzer

A texture analyzer was TA Instruments serial no. 980646AR1000 (Rheolyst) with TA instrumental CPU, 'Techne' temperature controlled water bath and test head (Geometry 4 cm flat steel plate). It was used for measurement the maximum adhesion load. Raw data form individual test comprised of the monitored load measured as a function of time (probe displacement). The point at which the load-time carve crosses the X axis (time) at the start of phase 2 of data displayed and recorded graphically. Maximum load value attained in this phase, and the time at which the load subsequently first returns to zero were the key data utilized in the calculation and assessment of sample behavior. This instrument was supported by Chembiotech Laboratories, University of Birmingham Research Park, Esbaston, UK.

3.2 Analytical Methods

3.2.1 General Analysis

Determination of Dry Cell Weight: Cell growth was determined as dry cell weight (DCW) by centrifugation the broth at 13,000 rpm (12,846 x g) for 15 min at 4

°C and the washed cell sample was dried at 105°C overnight and then weighed (Dermlim, 1999).

Measurement of Biopolymer Content: The supernatant was precipitated with 4 volumes of 95% cold ethanol kept in a freezer (-20°C) and the biopolymer was centrifuged at 10,000 rpm (7,600 x g) for 15 min, dried at 50°C and weighed (Dermlim, 1999).

Apparent Viscosity Measurement: Apparent viscosity (cp or mPa.s) of the broth was measured using a Brookfield viscometer.

3.2.2 Chromatographic Methods

Gel Permeation Chromatograph (GPC): GPC was used for measurement and estimated the various types of molecular weights of WD7 biopolymer samples. The equipment and operation of GPC was described in section 7.1.4 of Appendix.

Thin Layer Chromatography (TLC): Monosaccharide identification was tested on TLC plate (Silica gel 60 F254, plastic roll, Merck). Hydrolysate of WD7 biopolymer was spotted into this plate comparison with various standard monosaccharides (1 mg/ml) such as glucose, arabinose, fructose galactose, rhamnose, ribose, sorbitol, xylose. The TLC plate was placed into the glass chamber which put the solution as developing (mobile) phase that consisted of a) isopropyl alcohol + acetone + 0.1 M lactic acid (2:2:1) and b) acetonitrile + water (9:1). The treatment was left until developing phase diffusing to the top of plate and it was dried in an oven 105°C for 5 min. Then, the dried TLC plate was sprayed with 0.5% Naphol solution (compose of Naphol 1g, absolute ethanol 190 ml and H_2SO_4 conc. 10 ml) and dried again in hot air oven for 20 min. The diffusion distance (Rf value) of color from each spot on TLC plate was measured. The color of spots also used to indicate sugar types (Chaplin and Kennedy, 1987).

High Performance Liquid Chromatography (HPLC): Monosaccharide types and contents in hydrolysate samples of WD7 biopolymer were identified by HPLC as followed:

HPLC 1 (for identify monosaccharides from WD7 hydrolysate after TFA hydrolysis)

Equipment: HPLC (Shimaszu, Model CR 6A Chromatipac)

Column: Hibar pre packed column, RT 250-4, Lichrosorb NH_2 (5 μm), Cica Merck.

Detector: RI model LC-10AD (liquid chromatograph), Shimaszu

Column temperature: 25°C

Eluent: Acetonitrile and water (90:10)

Flow rate: 1 ml/min

Injection: 10 microlitres

HPLC 2 (for identify monosaccharides from WD7 hydrolysate after MSA hydrolysis)

Equipment: HPLC (Shimaszu, Model CR 6A Chromatipac)

Column: Anion Exchange HPLC equipped with CarboPac PA10 analytical column, (250 x 4.0 mm ID, Dionex company) and CarboPac PA10 guard column, (50 x 4.0 mm ID), Waters 625 LC non-metallic system and Waters Whips 712 Autoinjector SSI Model 350 self-flushing pump.

Detector: Waters 464 Pulsed Amperometric Detector (PAD)

Column temperature: 25°C

Eluent: Ultra High Quality (UHQ) water

Flow rate: 1 ml/min

Injection: 20 microlitres with injection interval of 1.40 h (100 min total analysis time and data collection at 90 min)

3.2.3 FT-IR Spectroscopy

FT-IR spectroscopy was used for the qualitative identification of functional group of WD7 biopolymer samples with FT-IR spectrophotometer. Absorption bands were interpreted against the data in computer software of LEHMANN & VOSS manufacturer. Prior to analysis, a sample was prepared followed the potassium bromide (KBr) disk method. Sample was ground and mixed with KBr powder and uniformly pressed to get a disk. Infrared adsorption spectra were measured on KBr disk with a FT-IR spectrophotometer. Infrared spectra were given in term of % transmittance.

3.2.4 Nuclear Magnetic Resonance Spectrometer (NMR) Analysis

NMR spectrometer was used for configuration analysis and monosaccharide type in WD7 biopolymer structure. Dry sample (50 mg) was dissolved in Deuterium oxide, D_2O (2 ml) and Sodium Deuteroxide (NaOD, 40% wt, 50 µl) used as an internal reference. The ^{13}C-NMR spectra were recorded.

3.2.5 CHNS-O Analysis

CHNS-O Analyzer equipped with dynamic flash combustion was analyzed for carbon, hydrogen, nitrogen, sulphur and oxygen. Analytical condition for CHNS was

set at furnace temperature of 900°C, oven temperature at 65°C, carrier flow of 130 ml/min, oxygen flow of 250 ml/min, reference flow 100 ml/min. Analytical condition for oxygen was set at furnace temperature of 1,060°C, oven temperature 65°C, reference flow 100 ml/min.

3.2.6 Physical Property Analysis

Morphology of Biopolymer: Morphology of biopolymer and its products was studied by Scanning Electron microscopy (SEM). Coated samples with gold-palladium under an argon atmosphere to achieve the 20 nm thickness were observed with a SEM.

Rheological Flow Analysis: The WD7 biopolymer dispersed in ultra high quality water was investigated by controlled stress rheometer. Profiles were analyzed by fit curve models; Power Law, Cross, Carreau, Williamson and Sisko models with equations shown in Table 6. Flow data in log forms of shear stress (τ, Pa) vs shear rate (γ, sec^{-1}) were plotted as Y and X-axis. Zero shear viscosity (η_0), the infinite shear viscosity (η_∞), consistency flow index (K), or rate index value (n) were calculated to identify the characteristics. The n value from Power Law model can identify the flow characteristic of sample as shear thinning ($n<1$), or shear thickening ($n>1$).

Table 6 Equation types of fit curve models

Model Types	Equation Types	X-axis	Y-axis
Power Law	Stress = b X $Rate^C$ or $\tau = K(\gamma)^n$	Log of shear rate (1/s)	Log of shear stress
Cross	[Viscosity-b]/[a-b] = $1/[1+(c \times Rate)^d]$	Log of shear rate (1/s)	Log of viscosity (Pa.s)
Carreau	[Viscosity-b]/[a-b]=$1/[(1+(c \times Rate)^2)^{(d/2)}]$	Log of shear rate (1/s)	Log of viscosity (Pa.s)
Williamson	Viscosity = $a / [1 + (b \times Rate)^c]$	Log of shear rate (1/s)	Log of viscosity (Pa.s)
Sisko	Viscosity = $a + b \times Rate^{(c-1)}$	Log of shear rate (1/s)	Log of viscosity (Pa.s)

Source: Latif *et al.* (1978)

Thermal Analysis: Thermal analyses of WD7 biopolymer samples were performed using differential scanning calorimetry (DSC). Thermograms were recorded as a function of temperature. The apparatus was flushed with nitrogen. The samples (10

mg) were placed in aluminium pans, and sealed with aluminium covers. Then, they were heated from room temperature to 600°C with a heating rate of 10°C/min. Two pans, the sample pan and the empty pan, were put in the sample and reference chamber. They were heated and the temperature was programmed as follows; (i) holding at 200°C for 1 min, (ii) heating at increasing 10 °C/min from 200 °C to 420°C, to obtain the steady baseline. Transition of the biopolymer was determined by heating the sample chamber (a pan containing 1.0 mg polymer) and reference chamber (empty pan) with the same programmed temperature as above. Temperature of the sample and reference was maintained at the same temperature throughout the controlled temperature program. The net energy difference in the independent power supplies to sample and reference was recorded versus the programmed temperature.

3.3 Methods

3.3.1 Scale-Up for WD7 Biopolymer Production

To produce large quantity of WD7 biopolymer and compare the predicted yield, scaling-up from small scale fermentor (5L) to pilot scale bioreactor (72L) with different geometry was studied under controlled aeration and agitation condition. Effect of the oxygen supply and consumption on biopolymer production had been previously reported to be the important parameters (Wichienchot, 2000) and was investigated in both scales in this work by fixing oxygen transfer coefficient (k_La) (Garci'a-Ochoa *et al.*, 2000).

Bench fermentor was performed as prototype scale with 4.0 liters of working volume. Medium (3.8 liters) was prepared and sterilized at 121°C for 15 min and left to room temperature and then 5% inoculum (200 ml from shake flask cultivation) was added into a bench fermentor. For pilot scale operation, the medium (47.5 liters) was prepared and inoculum (2.5 liters) was added for operation in 72-L fermentor (with 50 liters of working volume). The pH value was controlled at 7.0 by addition of 0.1 N and 0.5 N NaOH for bench and pilot scale fermentor, respectively, with proper feeding rate. Antifoam probe was set for foam detection by using the sterilized tween80 solution as antifoam agent.

3.3.1.1 Effect of Aeration and Agitation Rates on Biopolymer Production

Batch fermentation in a 5L fermentor with 4L working volume as bench scale was carried out at 30°C and controlled pH at 7.0 with 0.1N NaOH for 3 days. Effect of aeration rate was studied by varying aeration rate at 0.5, 0.75, 1.00 and

1.25 vvm and agitation rate at 200, 400, 600 and 800 rpm. Samples were taken to determine for the dry cell weight (DCW), biopolymer concentration, viscosity and measurement of % dissolve oxygen tension (DOT) were recorded every 6h of cultivation.

3.3.1.2 Determination of OUR, OTR and k_La Values

During cultivation, the OUR, OTR and k_La were determined by dynamic gassing out technique according to the method of Garci'a-Ochoa *et al.* (2000). From mass balance equation, dissolve oxygen changes during fermentation time (dCo/dt) could be determined from the equation:

$$dCo/dt = OTR - OUR = k_La\,(C^* - C) - (Qo_2 \cdot Cx) \quad ...(1)$$

where C^*= saturated oxygen concentration, C = experimental oxygen concentration, Qo_2= oxygen consumption, Cx = DCW and k_La (C^*-C) and ($Qo_2 \cdot Cx$) were the volumetric OTR and OUR, respectively.

For OUR determination, the inlet of air flow was interrupted and the DO decreased due to cellular respiration. According to Eq. (2), OUR = slope of graph (%DO vs time);

$$dCo/dt = OUR = -(Qo_2 \cdot Cx) \quad ...(2)$$

For OTR determination, the air inlet was restarted and caused the increase of % DOT according to Eq. (1), OTR = slope of graph of the change %DO vs time. For k_La determination, Eq. (1) could be integrated when the aeration began ($t = t_1$, so $Co_2 = C_1$) and another time ($t = t_2$, so $Co_2 = C_2$), yielding the Eq. (3):

$$(Qo_2 \cdot C_x)(t_2 - t_1) + (C_2 - C_1) = k_La \cdot \int (C^* - C).dt \quad ...(3)$$

k_La value was calculated by solving Eq. (3) from graph (DOT vs time) by mean of the integration algorithm taking. The OUR, OTR and k_La, value in exponential and stationary phase (at 12 and 36 h cultivation) were investigated at various aeration rates and agitation rates. The k_La value was used for scale-up study.

3.3.1.3 Scale-up Based on k_La Criterion for Biopolymer Production

Batch cultivation in pilot plant scale was carried out in a 72L fermenter with 50 l working volume at 30°C, pH 7.0 for 3 days of cultivation using the aeration and agitation rates that gave the equal or very similar of k_La values obtained from bench scale. A k_La value (which usually be value at giving the maximum biopolymer yield) at bench scale is fixed. Then the fermentation at pilot plant scale was performed at a condition gave a k_La value as similar to that of bench scale. Time course, the OTR, OUR and k_La values and their effects from pilot plant fermentation were investigated and

the EPS yield of both scales were compared.

3.3.2 Preparation and Characterization of Crude and Partially Purified WD7 Biopolymer Samples

Viscous broth from fermentation was centrifuged at 13,000 rpm (12,846 x g). The supernatant was concentrated in a vacuum dryer at 50°C (20 lb/in^2) until haft of water was evaporated. Concentrated supernatant was precipitated with 4 volumes of cold 95% ethanol at -20°C overnight. The biopolymer was easily separated from ethanol solution, dried in an oven at 50°C and finally, ground in mortal. The crude biopolymer (CB) was re-dissolved in distilled water, evaporated at 50°C until half volume of water was evaporated then dialyzed in dialysis membrane (Cellu.Sep® T2, nominal MWCO 10,000, Membrane Filtration Products, Inc.) against distilled water at 4°C overnight. Dialysate was freeze-dried to obtain the partially purified biopolymer (PB). This method was described and modified from Dermlim (1999).

This polymer solution was precipitated with Cetylpyridinium Chloride (CPC), cold ethanol and dialysis for 2-3 times to prepared the purified biopolymer samples for structural (chemical analysis) analysis. The WD7 biopolymer was purified from reaction between the acid groups in the polysaccharide structure and quaternary ammonium (QN^+) of CPC (Dermlim, 1999).

3.3.3 Characterizations of WD7 Biopolymer

3.3.3.1 Determination of Molecular Weight

Crude and partially purified WD7 biopolymers from cultivation of *E. cloacae* WD7 in glucose and sucrose containing media were dissolved in PBS at 4°C overnight (1 mg/ml). Then the biopolymer solution was filtered (using 0.2 mm Titan® Nylon filters) before placing in GPC vials. The average molecular weight (M_w) of WD7 biopolymer was determined by GPC analysis using pullulan as the standard M_w material. Pullulan standards (M_w from 5.8 to 853 kDa) were dissolved overnight in PBS at 4°C with 1 mg/ml concentration and 100 µl injection.

3.3.3.2 Chemical Characterizations of WD7 Biopolymer

Some chemical characteristics of WD7 biopolymer were investigated. Using the colorimetric reactions, the polysaccharide samples were tested for the presence of neutral sugars by anthrone reaction, total sugar by phenol-sulfuric acid, ketose (phenol-boric acid-sulphuric acid assay), total carbohydrate (hexose and pentose

contents by L-cysteine hydrochloride assay) (Chaplin and Kennedy, 1987). Amino sugars (by Morgan-Elson techniques) were determined using glucosamine as standard. Protein was estimated by Comassy blue assay using bovine serum albumin as standards (Chaplin and Kennedy, 1987). Moisture contents of biopolymer solution samples (0.5 g) were determined using an HR73 halogen moisture analyzer with drying at 80°C. The C, H, O, N and S composition of crude and partially purified WD7 biopolymer samples were analyzed by the C, H, O, N, S analyzer.

In addition, the qualitative analysis of α-amino acids (by ninhydrin reaction) and aromatic amino acids (by xanthotropic reaction) in the WD7 biopolymer samples were carried out following the procedures of Plummer (1978). Therefore, some acid constituents in WD7 biopolymer such as the uronic acid were determined by Carbazole-sulfate assay, described by Chaplin and Kennedy (1987). The details of all analysis were described in Appendix 7.3.

3.3.3.3 Functional Groups Analysis

FT-IR spectroscopy was used for the qualitative identification of functional group of WD7 biopolymer samples in term of % transmittance with FT-IR spectrophotometer. WD7 biopolymer was ground and mixed with potassium bromide (KBr) powder and uniformly pressed to get a disk. Infrared adsorption spectra were measured with a FT-IR spectrophotometer and given in term of % transmittance in the range of wave number 4000-370 cm^{-1}.

3.3.3.4 Monosaccharide Identification

The contents of neutral sugars and monosaccharide in biopolymer after acid hydrolysis were determined by using chromatographic techniques; TLC and HPLC. Three acids (Sulphuric acid, Methane sulphonic acid (MSA) and Trifluoro acetic acid, TFA) were used for hydrolysis of WD7 biopolymer in this study.

Hydrolysis with sulphuric acid: The biopolymer samples were heated in 1M H_2SO_4 at 40°C for 2 h and neutralized with $BaCO_3$. The reaction mixture was then centrifuged at 5000 g for 10 min at 4°C to remove the precipitate. Supernatant was filtered through a membrane filter with 0.45 μm pores, and the filtrate was concentrated on rotary evaporator at 45°C to obtain the hydrolysate and then determined the monosaccharide compositions in samples with TLC and HPLC.

Hydrolysis with methane sulphonic acid: MSA (4M, 5 ml) was added to weighted samples (50 mg) in vial with caps. Mixture was then hydrolyzed in a thermostatically controlled water bath at 80°C for 0.5, 1.0, 1.5, to 8.0 h (30 min

interval) and 28 h. The sample vials were picked up and stopped the reaction by putting into cold water. Then the samples were adjusted to pH 5.0 by adding NaOH solution (4M, 5 ml) in the same vial, and were diluted to 100 ml with ultra high quality water in a volumetric flask. Aliquots of the diluted sample solutions were filtered (0.2 μm Titan® Nylon filters) before testing on TLC or being subjected to HPLC analysis.

Hydrolysis with Trifluoro acetic acid: The weighed WD7 biopolymer samples (50 mg) in the glass vial with cap were added with TFA (100%, 2 ml), mixed and left for 1-8 h at 100°C in water bath. The hydrolysate was neutralized with 2M NaOH and made up volume to 5 ml. Hydrolysates were filtrated (0.2 μm Titan® Nylon filters) and then determined the monosaccharide compositions in samples with chromatographic analysis (TLC and HPLC).

Determination of monosaccharide by TLC and HPLC: The hydrolysate and standard monosaccharide solutions were spotted on the TLC (Silica gel 60 F254, plastic roll, Merck). The developing reagent (mobile phase) systems were the solution of acetonitrile in water (9:1). TLC plate was dried and sprayed with 0.5% Naphol solution (Naphol 1g, absolute ethanol 190 ml and H_2SO_4 conc., 10 ml) (Petry, 1988). The color and Rf values of monosaccharide bands were considered to estimate the monosaccharide types and constituents of this biopolymer samples. Hydrolysate samples and monosaccharide standard solution (50 mg/ml) were filtered through filter (0.2 μm Titan® Nylon filters) and applied to HPLC systems.

3.3.3.5 ^{13}C NMR Analysis

Configuration of monosaccharides was determined by ^{13}C NMR techniques of WD7 biopolymer. Structure of the WD7 polysaccharide repeating unit was determined by ^{13}C NMR technique. Dry sample (50 mg) was dissolved in Deuterium oxide, D_2O (2 ml) and sodium deuteroxide (NaOD, 40% wt, 50 μl) used as an internal reference.

3.3.4 Some Factors Affecting on Apparent Viscosity of WD7 Biopolymer Solution

Dried powder of WD7 biopolymer was dissolved at low and high concentrations to form solution. Crude and partially purified dried powders of WD7 biopolymer were dissolved in ultra high quality water at low concentrations. Effect of some parameters on apparent viscosity of WD7 biopolymer solution was studied. Apparent viscosity (η, mPa.s or cp) was expressed as a function of shear rate (γ, rps) and

shear stress (τ, mPa) as shown in equation; $\eta = \tau/\dot{\gamma}$ and as relation to shear stress by equation of Power Law model ($\eta = K\dot{\gamma}^n$) as described by Audet *et al.* (1998) where K and n were consistency constant and flow index.

Effect of (low) concentration (0-0.5 mg/100 mL or 0-0.5 x 10^{-4} g/dL) on the apparent viscosity and flow rheological behavior of WD7 biopolymer dispersion was measured by Brookfield viscometer and rheometer, respectively. Therefore, the effects of temperature (4, 20, 30, 40, 60 and 80°c) and pH (2.0-12.0) were studied by Brookfield viscometer at 30°C.

3.3.5 Factors Affecting Flow Rheological Behaviour of WD7 Biopolymer Gel

Gel is a basic form of biopolymer for preparing to other forms (film, sponge, bead etc). Flow behavior and apparent viscosity of WD7 gel was tested at various effects:

3.3.5.1 Effect of Biopolymer Concentrations

Flow rheology and the apparent viscosity of crude and freeze-dried WD7 biopolymer samples at concentrations in ranges of 0-10% (w/v) were determined.

3.3.5.2 Effect of Temperature

Apparent viscosity and flow rheology of WD7 biopolymer was a function of shear rate and temperature that were investigated at various temperatures. Crude WD7 gels (2% w/v) were investigated at 20, 30, 40, 50, and 60 °C, pH 5.0 by a Brookfield viscometer and a 'Rheolyst' rheometer. In addition, the flow rheology of crude and partially purified (freeze dried) WD7 biopolymer gel (2% w/v) was investigated at 37°C for 48-72 h in an incubator. Therefore, the crude WD7 gels (2.0%w/v) were pasteurized (65°C, 30 min), boiled (105°C for 0-2 h) and sterilized (121°C, 20 min for 1-2 times as single- and double-autoclave).

Therefore, the effects of various temperatures and times on the stability of WD7 biopolymers were investigated to study the appropriate condition for storage, removal some microbial contaminants and human application. To consider the stability and storage temperature (freezing, refrigeration or chilling and room temperatures), rheological flow and the apparent viscosity of crude WD7 gel (2% w/v) was investigated at -20°C, 4°C, and 30°C for 60 days and checked every 15 days.

3.3.6 Effect of Cation Variation on Enhancement of WD7 Gelation

Effect of many cations on gel enhancement was tested by measuring viscosity and flow behavior.

3.3.6.1 Effect of Monovalent Cations on Flow Rheological Behavior of WD7 Biopolymer Gel

Crude (2% w/v) and partially purified (0.25% w/v) of WD7 biopolymer were dispersed (with ratio 1:1) in cation solution of metal chloride salts [NaCl, KCl, LiCl, NH_4Cl,]. Mixtures were mixed completely overnight before performing flow rheology assessments.

Therefore, flow rheology of partially purified WD7 biopolymer gel (0.5-2.0% w/v) in various concentrations of NaCl and $MgCl_2 \cdot 6H_2O$, (0-5 M) were determined.

3.3.6.2 Effect of Divalent Cations on Flow Rheological Behavior of WD7 Biopolymer Gel

Effect of divalent metal sulphate salts; $(NH_4)_2SO_4$, $CuSO_4 \cdot 5H_2O$, Na_2SO_4, $MgSO_4 \cdot H_2O$, $MnCl_2 \cdot 4H_2O$, $CaCl_2$ and $CaCl_2 \cdot 2H_2O$ were studied with crude (2% w/v) and partially purified (0.25% w/v) of WD7 biopolymer dispersed (with ratio 1:1) in UHQ water. Mixtures were mixed completely overnight before performing flow rheology assessments.

3.3.6.3 Effect of Cations on Adhesive force of WD7 Biopolymer Gel

The adhesive forces of this biopolymer with various mono and divalent cations were also tested. Adhesive force of WD7 biopolymer solutions and gels at 0-10%(w/v). The WD7 solution (0.25% w/v) treated with cation solution of various metal chloride salts and divalent metal sulfate salts (with ratio 1:1) in 1 M NaOH solution and NaCl (0-5 M) were investigated.

3.3.7 Some Factors Affecting on Film Preparation and Properties of WD7 Film

The method for film preparation of WD7 biopolymer was adapted from the preparation of calcium alginate film (Aslani, et al., 1996) and thin film of sodium alginate (Sartori *et al.*, 1997). Concentration, temperature and relative humidity were important parameters for film casting by evaporation method.

3.3.7.1 Effect of Biopolymer Concentration on Casting and Properties of WD7 Film

The WD7 biopolymer was dissolved in distilled water to prepare film solutions at various concentrations (1, 2, 4, 6, 8 and 10% w/v) at pH 6.5. WD7 biopolymer solution (25 ml) was poured onto leveled nonstick trays (29.1 x 29.3 cm) with initial thickness of 0.3 cm. Any bubbles formed were removed with gentle suction

by pipette. Film ofWD7 biopolymer was cast in incubation oven with humidity control without disturb until completely dried. After setting, the trays were leaved to room temperature in desiccators before peeling the films off. Films were stored in plastic bags and held in desiccators for further testing (Sartori *et al.*, 1997 and Aslani *et al.*, 1996). Test of film properties were described in 3.3.7.5.

3.3.7.2 Effect of Heating Temperature on Casting and Properties of WD7 Film

The WD7 biopolymer solution (at 2% and 4% w/v) was cast in the incubation oven at 40, 60 and 80°C by controlling humidity at 60% until dry at various given times (1-5 days depending on controlled condition). Test of film properties were described in 3.3.7.5.

3.3.7.3 Effect of Relative Humidity on Casting and Properties of WD7 Film

The WD7 biopolymer solution (at 2% and 4% w/v) was cast in the incubation oven controlling the relative humidity at 20, 40 and 60% at optimal temperature until dry film was obtained. Test of film properties were described in 3.3.7.5.

3.3.7.4 Effect of Plasticizers on Characteristic and Properties of WD7 Film

The WD7 biopolymer solution (at 2% w/v) was added with plasticizer [sorbitol, sucrose, glycerol, PEG (polyethylene glycol; MW. 400) and PPG (polypropylene glycol; MW. 400) on a biopolymer to plasticizer with ratio of 1:0, 1:0.25, 1:0.5, 1:0.75 and 1:1 (w/w) at optimal condition. The blends were poured into a Teflon pan and dried overnight in an oven at 50°C to produce a film. Then, an appropriate plasticizer was chosen and tested again by varying of temperature and humidity to find the optimal condition for film casting of biopolymer mixture. Test of film properties were described in 3.3.7.5.

3.3.7.5 Tests of Film Properties

Before testing of film properties, the WD7 films were conditioned prior to subjecting them to permeability and mechanical tests. Films used for testing water vapor permeability (WVP), tensile strength (TS) and elongation at break (E) were conditioned at 50% relative humidity (RH) and 23±2°C by placing them in a desiccator over a saturated solution of $Mg(NO_3)_2 \cdot 6H_2O$ for 48 h or more. For other tests, film samples were transferred to plastic bags after peeling and placed in desiccators. Films

sample was tested according to described and adapted from Remunan-Lopez and Bodmeier (1993), Sartori *et al.* (1997). and Aslani *et al.* (1996) as below:

Appearance of WD7 Film: Appearance of film and all modified films were studied by observing the visual appearance and under microscope (Sartori *et al.*, 1997). Surface, pore size and density of tested films were reported including the texture characteristics.

Film Thickness Measurement: Thickness of the film and all modified films with plasticizer addition was measured with a dial thickness Gauge (0.01-1.0 mm) at five random locations on the film (Sartori *et al.*, 1997). Mean thickness values for each sample were calculated and used in WVP and TS calculations.

Color Measurement of WD7 Film: A portable colorimeter was used to determine the L*, a* and b* color value of film and all modified films with plasticizer addition (where L* = 0 (black) to 100 (white); a* = -60 (green) to +60 (red); and b* = -60 (blue) to +60 (yellow). Yellow standard plate (calibration plate CR-A47, L* = 85.45, a* = -0.15 and b* = 54.55) was used as a standard. Color (means of five measurements at different locations on each specimen) was measured on 10 cm x 10 cm segment of film. Film specimens were placed on a black plate when measurements were performed. Total color difference (ΔE^*_{ab}), hue angle (H) and chroma (C) were calculated using the following equations:

$$\Delta L^* = L^*_{sample} - L^*_{standard}, \qquad \Delta a^* = a^*_{sample} - a^*_{standard}, \qquad \Delta b^* = b^*_{sample} - b^*_{standard}$$

$$\Delta E^*_{ab} = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{0.5} \qquad C = [(a^*)^2 + (b^*)^2]^{0.5}$$

where ΔL^*, Δa^* and Δb^* are the differentials between the color parameter of the samples and the color parameter of the white standard (L* = 94.8, a* = -0.78, b* = 1.43) used as the film background (Aslani *et al.*, 1996).

Water Vapor Permeability (WVP): WVP of WD7 native film and all modified films with plasticizer addition was tested by the gravimetric modified cup method of Remunan-Lopez and Bodmeier (1993). The test cups were filled with 20 g of desiccant (bead, Siligar gel) to produce a 0% RH below the film. The WD7 film samples were held on the cup with O-ring. The air gap was set at approximately 0.6 cm between films surface and desiccant. The cup was weighed placed into an incubator at 50% RH and 25^{o}C. The water vapor transmission rates (WVTR) of films were measured. Weight gain measurements were taken by weighing the test cup to the nearest 0.001g with an

electronic scale every 2 h for 16 h. A plot of weight gained versus time was used to determine the WVTR. Slope of the linear portion of this plot represents the steady state amount of water vapor diffusing through the film per unit time (g/h). WVTR was expressed in unit of grams per meter square per day, and then WVP of WD7 films was calculated.

Tensile Strength and Elongation at Break (TS and E): The WD7 native film and all modified films with plasticizer addition were tested for TS and E values to indicate their strength and flexibility, respectively. TS were performed using an Instron universal testing instrument (Model 1122, Instron Corp., Canton, MA). Three samples (2 cm x 8 cm each) were cut from each film. Initial gap separation and cross-head speed were set at 50 mm and 50 mm/min, respectively. TS were calculated by dividing the maximum force at break by initial specimen cross-sectional area, and percent elongation at break was calculated from $E = (d_{after} - d_{before}) / d_{before}$ where d was the distance between grips holding the specimen before or after the break of the specimen (Sartori *et al.*, 1997).

3.3.8 Some Factors Affecting on Preparation and Properties of WD7 Sponge

Sponges of WD7 biopolymer was prepared by method adapted from the preparation of calcium alginate sponge by evaporation method (Aslani, et al., 1996) and thin film of sodium alginate (Sartori *et al.*, 1997). Various parameters for sponge of biopolymer were investigated as described below:

3.3.8.1 Effect of Biopolymer Concentrations on Preparation and Properties of WD7 Sponge

The sponge preparation protocol was adapted from the method of Lai *et al.* (2003). The WD7 biopolymer solutions were poured into four plastic cups (~25 ml each) with 3 cm diameters, frozen overnight at -18°C and then freeze dried overnight in a freeze dryer. Foaming agents were not used in this study to prevent any possible effect and sponge may incur on the morphological structure or mechanical strength of the sponges. The WD7 biopolymer was prepared into sponge form at various concentrations (1, 2, 4, 6, 8 and 10 % w/v). Appearance of sponge; thickness, texture (softness and fluffiness) and color, was considered.

3.3.8.2 Effect of Mixing Ratios on Preparation and Properties of WD7-Alginate Composite Sponge

WD7 biopolymer solution was mixed with sodium alginate solution (1% w/v) at varying weight ratios (3:1, 1:1 and 1:3). These solutions were

formed into sponges at room temperature. Their visual appearance of WD7 native and composite sponges was investigated.

3.3.8.3 Testing of Sponge Properties

Microscopic structure (including porosity and wall thickness) of WD7 native and composite sponges were investigated. The WD7 sponges (10 x 10 nm) were investigated. The pore length, width and all thickness (i.e. the average distance between neighboring pores) and the effective size (d) of the pores was reported from microscope determination.

3.3.9 Study on Preparation of Other Forms of WD7 Biopolymer

3.3.9.1 Bead Preparation of WD7 Biopolymer

In this study, the WD7 biopolymer solution and gel in water at various concentration of 1, 2, 4 and 6% (w/v) were extruded from a syringe into the KCl, NaCl, LiCl, MnCl, $CaCl_2$, $CuCl_2$, $MgCl_2$, Ca_2SO_4 and KH_2SO_4) at tested concentration of 0.1, 0.2, 0.5 and 1.0 M. Dripping method was used for bead preparation of this WD7 biopolymer (Knill *et al.*, 2004).

3.3.9.2 Preparation of WD7 Biopolymer Fiber

Soluble WD7 biopolymer (4 and 6% w/v) was injected from syringe and drawn as the line into all cation (KCl, NaCl, LiCl, MnCl, $CaCl_2$, $CuCl_2$, $MgCl_2$, Ca_2SO_4 and KH_2SO_4) and some inorganic solutions (ethanol, methanol and hexane) as coagulants to form the fiber. Formation of WD7 biopolymer fiber from the line drawn was studied after left in cold at freezing temperature ($-20^{o}C$) and after drying at room temperature.

3.3.10 Investigation of Biological Properties of WD7 Biopolymer

For application in food and pharmaceutical areas, some biological activities of WD7 biopolymer were tested without consideration of the active compound. Most are *in vitro* testing of toxicity, biocompatibility and healing properties including anti-bacterial, anti-fungal, anti-viral activity tests, anti-tuberculosis (TB) test, anti-biotic, anti-tumor or anti-cancer assays. For human use, some biological properties of this WD7 biopolymer especially on skin tests were also tested such as heamocompatibility test (human red blood cell lyses testing) or heamolysis test and anti-inflammatory assay (Samuelsen, 2000; Diallo *et al.*, 2001). The procedures for these biological properties testing of WD7 biopolymers were given as following sections.

3.3.10.1 Testing of Antimicrobial Activities

Crude and purified WD7 biopolymer powder was pasteurized at 60°C for 1 h before *in vitro* testing of antibacterial, antifungal, antiviral (anti-herpes simplex activity), antiplasmodial, anti-tuberculosis (TB) activities against target test microorganism with methods as described in Appendix 7.2.1-7.2.5. These testings were analyzed by staff at Bioassay Laboratory, National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand Science Park. The results of testing were reported as positive or negative activities.

3.3.10.2 Antibiotic Activity Testing

Test Microorganisms: The test bacteria were Gram positive (*Staphylococcus aureus*, *Bacillus cereus*) and Gram negative (*Escherichia coli* and *Salmonella typhi*). These were incubated at 37°C and all were activated by incubating for a period of 24 h in a nutrient broth, NB (Oxoid). Some fungi and yeast were *Aspergillus flavas* and *Trichoderma viride*, *Saccharomyces cerevisiae* and *Candida albicans* were incubated at 30°C for 24 h in PDA plates. They were used for antibiotic testing of this WD7 biopolymer solution.

Testing of Antimicrobial Activity by Disc Methods: The inhibitory effect on test microorganism of WD7 crude and partially purified biopolymer were carried out based on clear zone determination by disc method. All bacteria were spread on NA medium in sterile petri dish (100 mm diameter) and incubated at the appropriate temperature for 24 h. Sterilized paper disc of 6 mm diameter was dip into WD7 biopolymer solution (5 mg/ml) and some standard antibiotic solutions, left until dry, and then put the dried disc on each agar plates inoculated with target strains. The inoculated plates were incubated for 24 h at their optimum growth temperatures, and the diameter of the inhibition zone was measured as mm. Antimicrobial activity was compared with standard antibiotic discs used for control as follows: erythromycin (E; 15 µg, Oxoid), vancomycin (VA; 30 µg, Difco), penicillin-G (P; 10U, Oxoid), chloramphenicol (C; 30 µg, Difco) at 0.1 mg/ml.

3.3.10.3 Cytotoxicity Test

Cytotoxicity test of crude and purified biopolymer samples were carried out by sulforrhodamide B colorimetric assay adapted from Skehan *et al.* (1990). This method was described in Appendix 7.3.6.

3.3.10.4 Anticancer Assays

KB (Human epidermoid carcinoma of cavity, ATCC CCL-17), BC (Breast cancer cell line) and NCI-H187 (human, small cell lung cancer) were determined by colorimetric cytotoxicity assay that measured cell growth from cellular protein content (Plumb *et al.*, 1989 and Skehan *et al.*, 1990). Elliptine and doxorubicin were used as positive control. DMSO was used as negative control. This method was described in Appendix 7.3.7.

3.3.10.5 Heamolysis Test (Hemocompatibility)

This heamolysis test (heamocompatibility) test was human red blood cell lysis (or RBCs release test) that was adapted from Carreño-Gómez and Duncan (1997). The RBCs were separated from serum by centrifugation at 1,000 rpm for 5 min and pipetted into the fresh phosphate buffer pH 7.4 with 0.9% (w/v) NaCl at 4°C. Tritron-X100 solution (1% v/v, Sigma) and chitosan (1% v/v) were used as positive and negative control of RBCs lysis tests, respectively. All equipment, materials and methods in these tests have to be operated under sterilization and aseptic technique condition.

Erythrocytes were collected by centrifuging the human blood three times in chilled phosphate buffered saline (PBS at 4°C) at 1000 x g for 10 min. The final pellet was resuspended in PBS to give a 2% w/v solution. Using a spectrophotometer assay, 100 µl of the erythrocyte solution was added to soluble WD7 biopolymer (0-0.5 mg/ml). Samples were then incubated for 1 h or 24 h, the appendoff tubes were centrifuged then at 1000 x g for 10 min and the supernatants transferred into new ones. Heamoglobin release was determined with spectrophotometer at 550 nm. The detergent Triton X-100 (1.0% v/v) was used to produce 100% haemoglobin release as positive control. Results were expressed as the amount haemoglobin release induced by the polymer as percentage of the total. Erythrocyte morphology was also visualized by using the binocular microscope with camera after exposure to soluble biopolymer comparison with control agents.

3.3.10.6 Healing Properties (Anti-Inflammation Assay)

Crude and partially purified WD7 biopolymer was tested for inhibitory activity of PEG that showed anti-inflammation in mouse by method described in Appendix 7.3.8.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Scale-up for WD7 Biopolymer Production

Since the role of oxygen on microbial growth and metabolism is important, the oxygen uptake rate (OUR) and oxygen transfer rate (OTR) into a system during fermentation need to be investigated. The fermentation of *E. cloacae* WD7 was carried out at bench scale by changing of aeration rate (0.5, 0.75, 1.0 and 1.25 vvm) and agitation rate (200, 400, 600 and 800 rpm) for k_La value determination, especially in exponential phase and stationary phase. Effects of aeration rate and agitation rate on fermentation of *E. cloacae* WD7 at bench scale are illustrated in Figure 5 and 6, respectively. Results on % dissolved oxygen tension (DOT), DCW, biopolymer production and viscosity are discussed in section 4.1.1 to 4.1.3, respectively.

4.1.1 Effect of Aeration and Agitation Rates on DOT at Bench Scale

From Figure 5a and 6a, all DOT values of both aeration and agitation rates decreased dramatically to a minimum level within 24h cultivation, and then kept constant afterwards at 10-30%. The remaining DOT levels in stationary phase (after 24 h fermentation) increased with increase of aeration rates tested, but did not depend on agitation rates. The viscosity of the culture broth increased corresponding to the cell growth with high oxygen consumption. However, the DOT values in all runs were never below 10%, indicating that oxygen supply was enough for consumption of cells.

However, agitation, as is well-known, affects both air bubble distribution and the mixing of the system. Mechanically agitated aerated vessels are widely used rather than only aerated vessels which can be inadequate to promote the liquid turbulence necessary for small air bubble generation. Although the agitation could maintain available dissolved oxygen in the fermenter, but the inappropriate speed of agitation results was poor oxygen transfer especially in high viscous broths. The fully mixed fermenter with the effective impeller is efficient on heat and mass transfer under fluid rheological behavior of biopolymer production.

4.1.2 Effect of Aeration and Agitation Rates on DCW at Bench Scale

In the exponential phase (12 h cultivation), the DCW values did not vary in the tested ranges of aeration rate (0.75, 1.00, 0.75 and 0.98 g/l at 0.5, 0.75, 1.00, and 1.25 vvm, respectively) (Figure 5b), but decreased when the agitation rate increased (0.98,

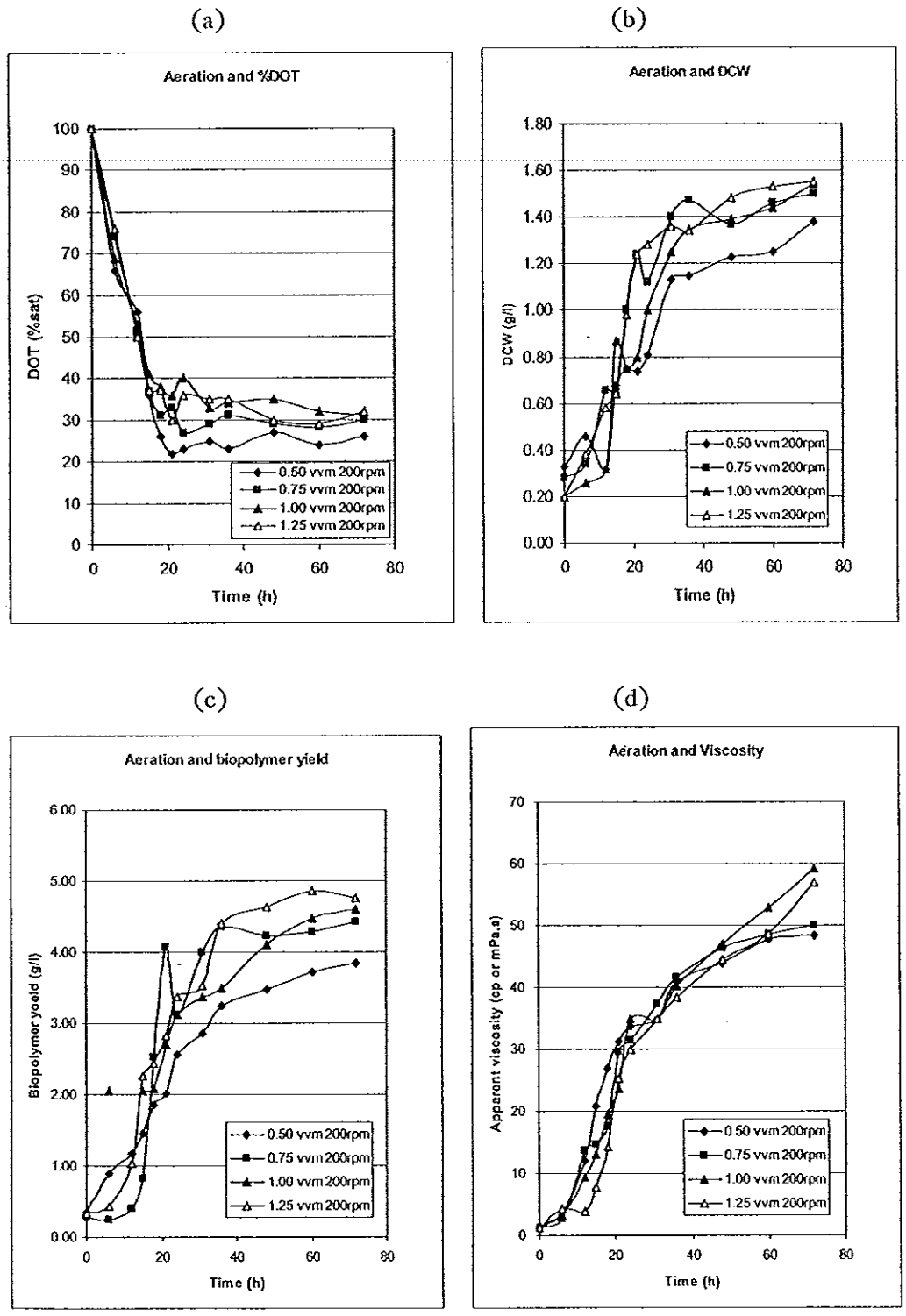


Figure 5 Effect of aeration rate (0.50-1.25 vvm) on DOT (a) dry cell weight (b), biopolymer yield (c) and apparent viscosity (d) during cultivation of *E. cloacae* WD7 in a 5L fermenter containing the optimal medium at 30°C, pH 7.0

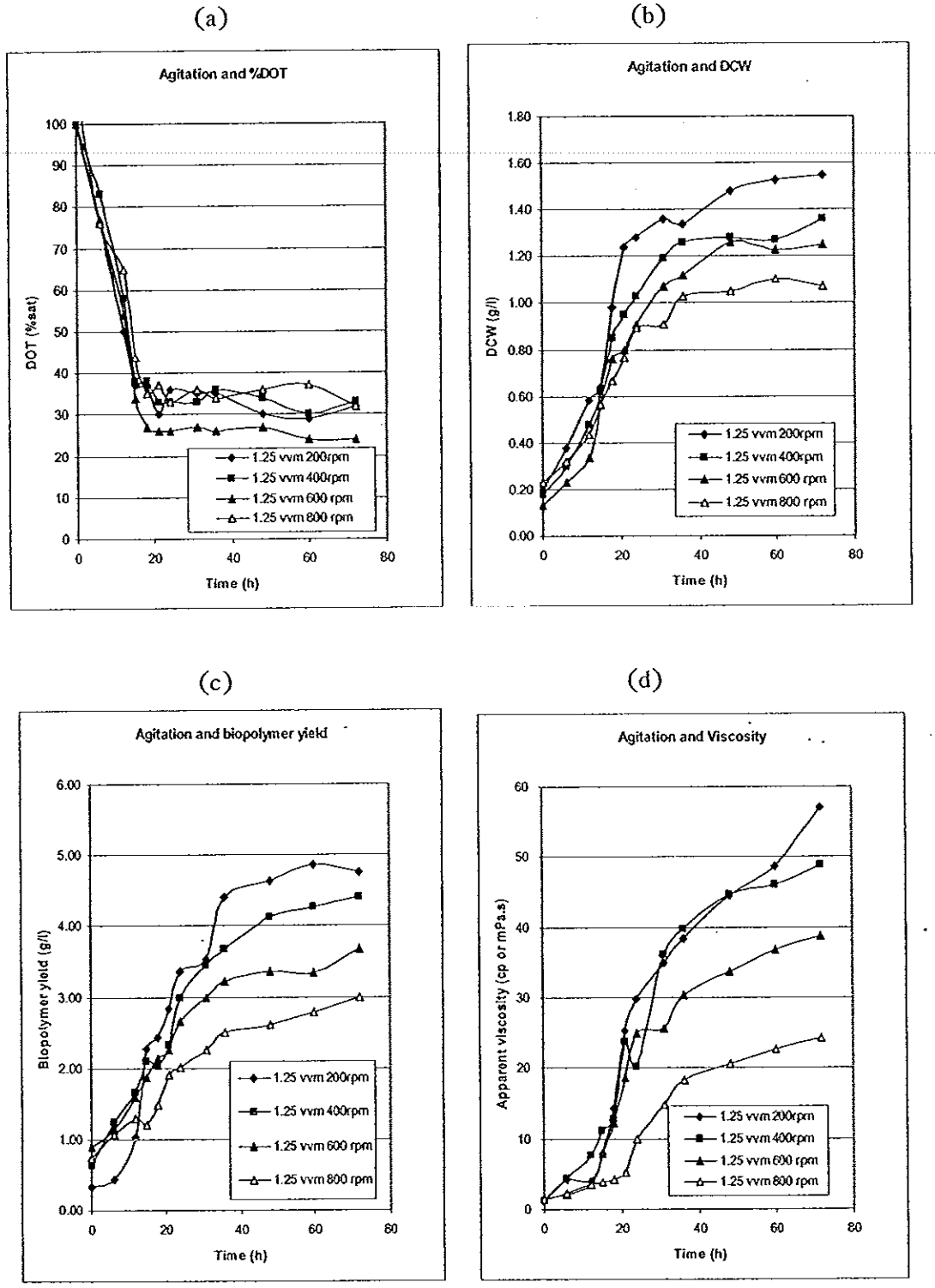


Figure 6 Effect of agitation rate (200 - 800 rpm) on DOT (a) dry cell weight (b), biopolymer yield (c) and apparent viscosity (d) during cultivation of *E. cloacae* WD7 in a 5L fermenter containing the optimal medium at 30°C, pH 7.0

0.85, 0.76 and 0.67 g/l at 200, 400, 600 and 800 rpm, respectively) (Figure 6b). In stationary phase, the DCW increased with the increase of aeration rates (0.81, 1.12, 1.00 and 1.28 g/l at 0.5, 0.75, 1.00, and 1.25 vvm, respectively) (Figure 5b), but became decreased at high agitation rates (1.28, 1.03, 0.91 and 0.89 g/l at 200, 400, 600, and 800 rpm, respectively) (Figure 6b). Thus, the higher in aeration rates, the higher the DCW, but it decreased when agitation rates increased.

From Figure 6b, the DCW value was highest at 200 rpm agitation rate, but decreased at higher speed (400-800 rpm). This was due to shear force and mixing effects. In the exponential phase, some growing cells were separated from medium and became stuck to the glass vessel walls over the broth at too high agitation rate. This caused heterogeneous (poor) mixing. However this problem did not happen in stationary phase since cells were suspended in high viscosity of biopolymer. But DCW decreased at high shear speed giving high shear stress that had influences on both of oxygen transfers and cell activities in the system (Gibbs and Seviour, 1996).

Shear rate is one of indispensable parameters used in the design of aerobic fermenter for viscous non-Newtonian systems. Vigorous mixing at high agitation speed always decreases the oxygen transfer that caused the cell growth and activities inhibition (Al-Masry, 1997). High shear fields resulting from the fluid physical properties and hydrodynamics cause damage to fragile microorganism and reduced product formation (Al-Masry, 1997). Inappropriate agitation rates can inhibit cells growth because of heterogeneous mixing and shear stress effect. Hewitt *et al.*(2000) reported that small-scale well-mixed fermentation gave the highest biomass yield, but surprisingly, the lowest cell viability for *E. coli.*

Oxygen supply into the broths constitutes one of the decisive factors for microorganism growth and can play an important role in scale-up of aerobic biosynthesis systems. At constant agitation speed (200 rpm), as the aeration rate increased, higher DCW values were found as shown in Figure 5b. The highest DCW yield (1.55 g/l) was obtained at the maximum rate of aeration (1.25 vvm). Oxygen content in the cultivation should be enough to supply on demand of the tested microorganism but may be supplied at higher content if it has no effect on the microorganisms or microorganisms are not sensitive on oxygen toxic (Galaction *et al.*, 2004). Thus, not surprisingly, the growth and biopolymer production of *E. cloacae* WD7 were found at 1.25 vvm (maximum rate in this work), while they were found at 2 vvm (maximum rate in 3L fermenter with 1.8 l working volume with the same conditions, medium and control) by Wichienchot (2000). The aeration

efficiency depends on oxygen solubility and diffusion rate into the broths on the bioreactor capacity to satisfy the oxygen demand of microbial population (Galaction *et al.*, 2004).

Cell growth and product formation should be related to both aeration rate and agitation at different rates, especially under viscous condition. Galaction *et al.* (2004) reported that aeration with optimal turbulence agitation may counteract the increase of blocking effect and biomass coalescence in the high viscosity broth with non-Newtonian behavior. Too high biomass may influence growth and oxygen transfer, both by reducing the oxygen solubility in media - especially surface aeration, and by blocking the aeration and agitation system. The contribution of surface aeration to the total mass transfer of oxygen for some bacterial fermentation was about 15-20 times lower than for viscous broth without biomass.

4.1.3 Effect of Aeration and Agitation Rates on Biopolymer Production at Bench Scale

In this work, the biopolymer formation at bench scale of all experiments increased when aeration rate increased (3.85, 4.43, 4.60 and 4.76 g/l at 0.5, 0.75, 1.00 and 1.25 vvm, respectively) (Figure 5c). On the other hand, they decreased when agitation speed increased (4.76, 3.80, 3.67 and 3.00 g/l at 200, 400, 600 and 800 rpm, respectively) (Figure 6c). Increase of aeration rate and decrease of agitation rate elevated the biopolymer yield. This was a typical relationship showing the greater the increase in cell growth, the greater the increase in the product formation. Maximum biopolymer yield (4.76 g/l or 3.07 g/g DCW) was found at 1.25 vvm and 200 rpm (maximum aeration rate and minimum agitation rates in the tested ranges, respectively). This was similar to previous report by Wichienchot (2000), the highest biopolymer yield (4.80 g/l) of *E. cloacae* WD7 cultivation was obtained at 2.0 vvm and 200 rpm (maximum aeration rate and minimum agitation rate). This showed the results obtained were different from those of Wicheinchot (2000) who studied range of aeration rate in 3L fermentor compared with 5L and 72L fermentors in this work. This is due to the different of sizes of vessel and working volumes of fermentation with different ranges of aeration rate. Thus, with the same aeration and agitation system, the maximum aeration rate was studied by control at maximum rate of 1.25 vvm for the 5L fermenter used in this study, while it was 2 vvm for the 3L fermenter used by Wichenchot (2000).

Oxygen transfer into microbial cells in aerobic fermentation processes strongly affects product formation by influencing metabolic pathways and changing metabolic fluxes

(Çalik *et al.*, 2000). Therefore, oxygen transfer in a fermenter depends on microorganism physiology and bioreactor efficiency. Most laboratory-scale stirred-tank fermentation systems are fitted with high speeds which impart a high shear stress on the medium, and this is known to reduce metabolite yields, such as in *Penicillium chrysogenum*, *Aspergillus* species, especially, pullulan of *A. pullulans* (Gibb and Seviour, 1996). High biopolymer yields were obtained under the combined conditions of low shear stress and optimal dissolved oxygen concentration. The biosynthesis of bacterial cellulose was mainly dependent on the energy compounds derived from respiration, i.e. processing (by oxidation in glycolysis or TCA cycle or other metabolisms) of macromolecular compounds (carbohydrate, protein, lipid) in the nutrient that can give the energy compounds such as AMP, ADP, ATP, etc. which are used in metabolism, activity or product formation of cells, and correlated with OUR (Kouda *et al.*, 1997). Thus, at too high a speed of agitation, the biopolymer yields and cell growth decreases if OUR value is unsuitable, although the efficiency and ability of oxygen transfer increases.

A reduction in metabolite production at high agitation rate has also been blamed on the cell deformation and damage suffered as a result of exposure to high shear rates. Therefore, the extreme flow and stress condition may inhibit the growth and activity of microorganisms by blocking the nutrient transfer and consumption of cells during growth. Therefore, the excessive oxygen supply caused the decrease in bacterial cellulose productivity because of a loss of substrate by direct oxidation. In addition, too high an agitation rate resulted in no mixing or slow movement of highly viscous broth outside of the stir zone, ultimately causing product decrease. However, the actual oxygen transfer to the cells would be reduced at low agitation rate that caused the large air-bubble size and poor bubble break-up, leading also to some viscous, non-stirred zones formation (Gibbs and Seviour, 1996). Consequently, the OTR related to aeration rate and bioreactor performance and OUR related to agitation rate. Consequently, the OTR related to aeration rate and bioreactor performance but the OUR related to agitation rate, mixing and shear stress, while both rates had effects on growth and activities of cells.

4.1.4 Effect of Aeration and Agitation Rates on Viscosity at Bench Scale

Viscosity values increased if aeration rate increased (48.40, 50.70, 57.00 and 59.20 mPa.s at 0.5, 0.75, 1.00 and 1.25 vvm, respectively) as presented in Figure 5d, but decreased if agitation rate increased (59.20, 48.80, 43.80 and 24.30 mPa.s at 200, 400, 600 and 800 rpm, respectively) as presented in Figure 6d. Increase of broth viscosity from fast growing microorganisms, high biomass and biopolymer lead to oxygen limitation

or broth heterogeneity and acts as a diffusion barrier. Therefore, if oxygen is a limiting nutrient in the processes and the transfer of oxygen from liquid phase to cells also is difficult, the product yield and the growth of microorganism will be affected directly. The rate of dissolved oxygen supply should be at least equal to the rate of oxygen demand. Hence the OTR and OUR, were studied to obtain maximum biopolymer yield.

Agitation rate has a direct effect on mixing which is an important factor that reduces the effect of high viscosity and increases the oxygen solubility and transfer, including enhancing the cells assimilation of dissolved oxygen and product formation at optimal agitation rate. In some CSTR, since the distance between some parts of the culture broth volume and the impellers was large, the homogeneous mixing was difficult to achieve in the high viscous culture broth furthest from the impeller (Kouda *et al.*, 1997). Kouda et al. (1997) found that the highest shear rates were encountered in the bottom zone of the vessel; the shear rate was high in the impeller zone and low in the zone furthest from the impeller. The sluggish flow of highly viscous broth was found at agitation rates higher than 600 rpm. Stagnant zones have also been known to be developed in many biopolymer cultivations such as in fermentation of bacterial cellulose (Kouda *et al.*, 1997), xanthan and hydroxypropyl guar gum (Eickenbusch *et al.*, 1995) and *Monascus* sp. J101 biopolymer (Kim *et al.*, 2002).

Shear rate is an important factor affecting the system viscosity, consequently vigorous mixing at high agitation speed is always used in viscous cultivation to increase the oxygen transfer and cause the inhibition of cells growth and activities with shear effect (Al-Masry, 1998). Therefore, too high a rate of aeration and agitation causes a flooding phenomenon (a decrease in viscosity with increasing shear) around the impellers and destroys the pseudoplastic product. High viscosity induces bubble coalescence and the mass-transfer characteristics deteriorate, while the oxygen mass transfer may become rate-controlling in aerobic cultivations (Eickenbusch *et al.*, 1995; Kim *et al.*, 2002). Consequently, the aeration and agitation had more effect on oxygen transfer in WD7 biopolymer fermentation due to having high viscosity and pseudoplastic characteristic.

4.1.5 Effect of Aeration and Agitation Rates on OTR and OUR at Bench Scale

An example of an experimental graph obtained from dynamic gassing-out technique during the exponential phase of fermentation at 1.25 vvm, 200 rpm of *E. cloacae* WD7 cultivation in the 5L fermenter, is shown in Figure 7. The decline of initial curve showed the decrease of dissolved oxygen content in the fermenter (due to oxygen consumption of cell, OUR value) when air supply was stopped. The later part of the curve

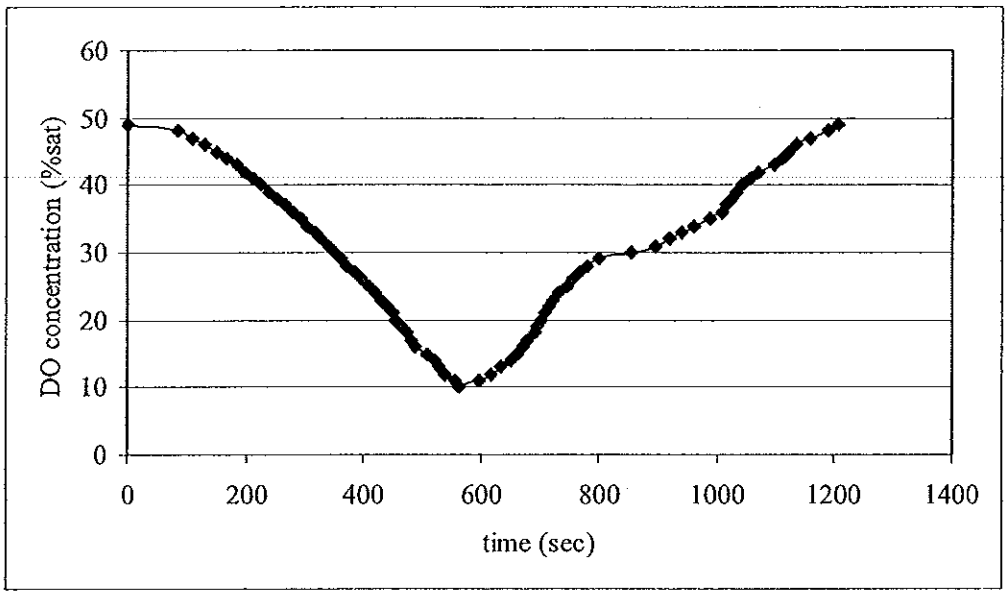


Figure 7 Experimental graph obtained during dynamic gassing-out technique for the OUR and OTR determination during exponential phase of *E. cloacae* WD7 fermentation at 1.25 vvm, 200 rpm in 5L fermenter

showed increase of dissolved oxygen content in the fermenter due to the air supply being re-started while the oxygen still was consumed by cells, indicating the change of oxygen content in the fermenter (dCo/dt value). Decrease of dissolved oxygen in the fermenter during oxygen consumption of cells took a shorter time than the oxygen diffusion after restarting the oxygen supply and both values at exponential phases took shorter times than those in stationary phase. These indicated that oxygen transfer was difficult in viscous broth. The effectiveness of oxygen transfer (OTR value) can be considered from OUR and dCo/dt values determined by equation 3 (section 3.3.1.2). These parameters are important for determination of the efficiency of oxygen transfer and are widely used as the aeration and agitation controls in aerobic processes.

The OUR and OTR values in exponential and stationary phases at various aeration and agitation rates were summarized in Figure 8. At various aeration rates tested (0.5-1.25 vvm), the OUR values were 6.47 - 6.90 and 4.67 - 5.07 (x 10^{-5} mol/l.h), whereas the OTR values were 9.70 - 10.70 and 6.17 - 6.98 (x 10^{-5} mol/l.h) in exponential and stationary phase, respectively. The OTR of both phases were higher than OUR indicating the oxygen supply was enough for cultivation. The OUR and OTR values during exponential phase were slightly higher than stationary phase because the supplied oxygen was used for both cells growth and product formation and it was not interrupted by

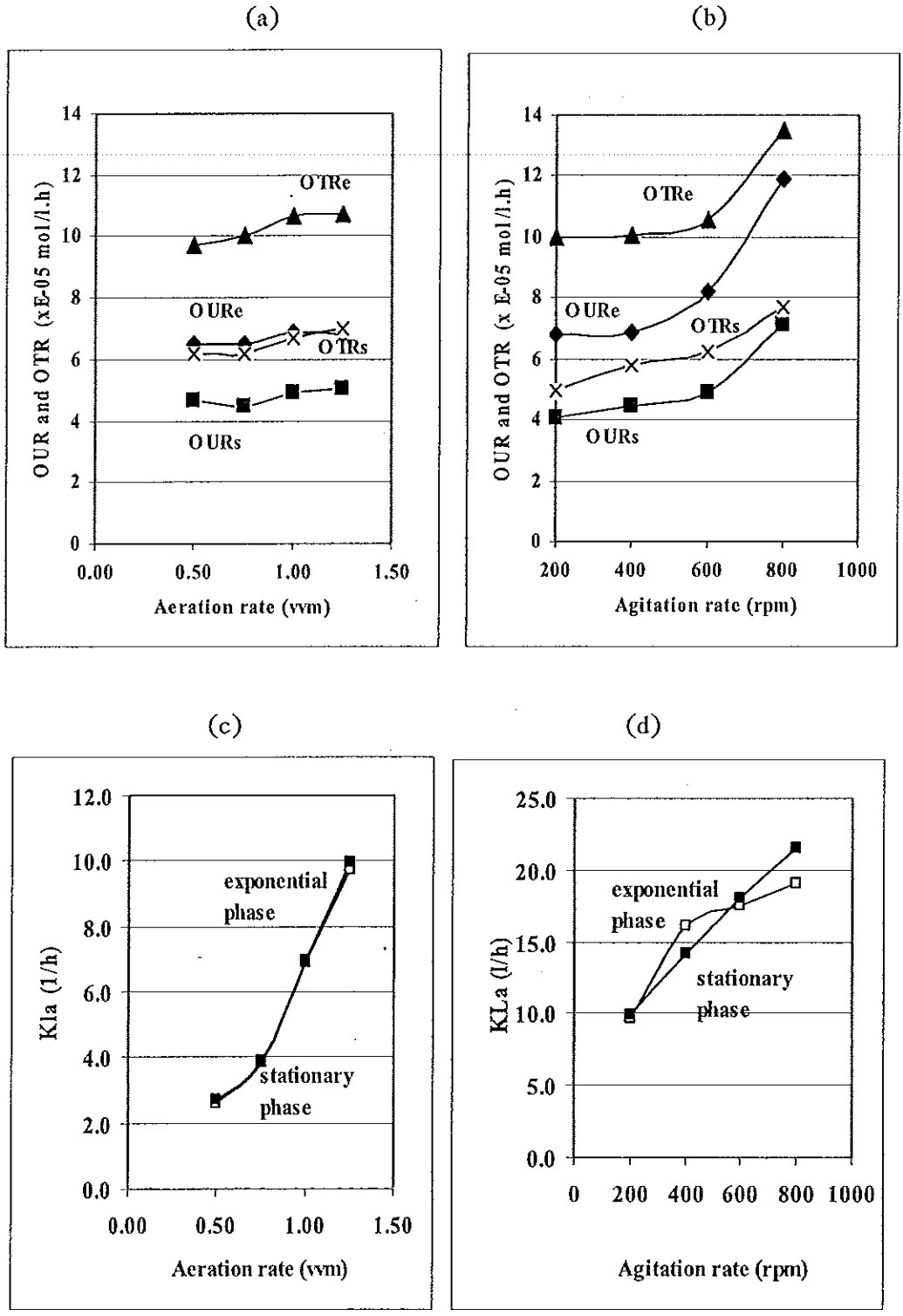


Figure 8 Correlations of aeration and agitation rate on OUR, OTR (a, b) and k_La (e, f) during *E. cloacae* WD7 fermentation at bench scale (5L fermenter) comparison between exponential (e,□) and stationary (s ,■) phases

viscosity as found in stationary phase. The OUR and OTR values increased with increasing aeration rate. The highest values of 6.90 and 5.07 ($x10^{-5}$ mol/l.h) for OUR and 10.69 and 6.9 ($x\ 10^{-5}$ mol/l.h) for OTR in exponential and stationary phase, respectively were found at the maximum aeration rate (1.25 vvm).

From Figure 8, at various agitation rates tested (200-800 rpm), the OUR values in exponential and stationary phase were 6.79 - 11.90 and 4.07 - 7.14 ($x10^{-5}$ mol/l.h), respectively and the OTR values were 10.02 - 13.51 and 4.98 - 7.69 ($x\ 10^{-5}$ mol/l.h), respectively. The OUR and OTR values increased with the increase of agitation rate. The highest OUR and OTR values were found at the maximum agitation speed (800 rpm) in both exponential and stationary phases. This showed that increasing of agitation rate not only increased the OTR, but also increased the OUR value. It indicated that if the oxygen was more transferred to the system; it was more taken-up by cells. Consequently, the OTR also had an effect on OUR.

In this study, most OTR values were higher than OUR values in all runs resulting from enough of oxygen supply for cultivation since the DOT did not drop lower than 10%. Rapid decrease of DOT in exponential phase was due to a high oxygen uptake rate by the cells. The OUR and OTR in all operations slightly increased when the aeration and agitation rates increased, although the biopolymer yield in the stationary phase reduced with the increase of agitation rate. These indicated that *E. cloacae* WD7 can survive under oxygen-limiting condition with polymer viscosity. In contrast, the exopolysaccharide (EPS) produced by *Agrobacterium tumefaciens* ID95-748 was strongly inhibited by oxygen limitation, with the decrease of polymer yield from 9.40 to 2.06 g/l, and under non-limited oxygen condition a polymer with higher molecular weight was produced(Sutherland, 1990).

Both OUR and OTR in the exponential phase were higher than in the stationary phase, with increasing aeration rate and agitation rate (Figure 8a and b). Although OUR and OTR were still high at 800 rpm, the cell growth, biopolymer yield and broth viscosity were all less than those at a lower agitation rate. Increasing shear rate enhanced the OTR value, but it caused the diminution of cells growth and biopolymer yield by mechanical damage. In aerobic cultivation of *Aureobasidium pullulans*, the OUR values increased for cells survival despite the cells concentration decreased (Gibbs and Seviour, 1996). However, the OUR in the viscous broth of *Monascus sp.* fermentation decreased at high agitation rate (>500 rpm) while the DOT level was maintained as high as 40%, despite the fact that the results for OTR were opposite to the OUR of cells. This suggests the presence of mechanical cell damage resulting from strong shear stress at high speed of

agitation (Kim *et al.*, 2002).

4.1.6 Effect of Aeration and Agitation Rates on k_La values at Bench Scale

The k_La values increased with the increase of aeration and agitation rate (Figure 8c and 8d). At various aeration rates (0.5-2.0 vvm), the k_La values were about 2.63-9.72 h^{-1} and 2.71 -9.97 h^{-1} in the exponential and stationary phases, respectively. At various agitation rates, the k_La values were 9.72-19.10 h^{-1} and 9.97-21.54 h^{-1} in the exponential and stationary phases, respectively. Increase of k_La values with agitation rate were higher than the increase of aeration rate. Increase of agitation rate (500-1200 rpm) gave an increase of k_La value (approximately from 10 to 90 h^{-1}), by using a static gassing-out technique, during cellulose production by *Acetobacter xylinum* (Kouda *et al.*, 1997). During fermentation, the k_La values of *Monascus* sp. increased gradually from 0.003 to 0.029s^{-1} as the rotational speed increased from 200 to 700 rpm (Kim *et al.*, 2002).

The k_La values at optimal condition (1.25 vvm 200 rpm) were 9.72 and 9.97 h^{-1} in the exponential and stationary phases, respectively. The k_La values increased with the increase of aeration rate in both phases (Figure 8c). The k_La values in the exponential phase at high agitation rate seem to be decreased compared to the results at stationary phase; this was due to the increase of OUR (Figure 8d). These results indicated that agitation had more effect on k_La value than aeration rate especially during the exponential phase. In addition to aeration and agitation rates, many factors influenced the k_La values during fermentation, such as the mixing, broth viscosity, product formation, biomass content, etc. There are many factors influencing the OTR, OUR and k_La values that were not considered in this study (i.e. distribution and size of air bubble, mixing delay time, shear force and stress) as reported by others (Galaction *et al.*, 2004) and they should be further studied subsequently.

4.1.7 Correlation of k_La on Biopolymer Productivity at Bench Scale

In this work, the k_La values at bench scale increased with increasing aeration and agitation rates, while cell growth and biopolymer yield decreased with increasing agitation rate. Thus, the correlation of k_La values on biopolymer productivity with aeration effect was positive, but negative for correlation of k_La values on biopolymer productivity is found with agitation effect. These correlations were typical in many studies of fermentation for production of biopolymers. Garćia-Ochoa *et al.* (2000) reported that the xanthan gum concentration increased when k_La value decreased at high agitation rate, but low productivity was found although the oxygen transfer increased. High shear stress (high agitation rate)

affected the structure and property of biopolymer (i.e. viscosity) and depressed cell growth and activity. Consequently, the DCW and biopolymer yield were low due to too high agitation rate and heterogeneous mixing, resulting in low viscosity. However, the EPS produced by *Aureobasidium pullulans* was reduced with increasing agitation rate but no shear forces occurred (Gibb and Seviour, 1996).

Basically, the biopolymer productivity of most aerobic processes increased with the increasing aeration rate and agitation speed and the negative effect of agitation speed was stronger than aeration rate. This was anticipated in a mechanically agitated vessel since the agitation affected the oxygen transfer rate stronger than aeration (Elibol and Ozer, 2000). DO concentration directly correlated with the OTR and k_La values. The data for k_La under various aeration rates and agitation rates indicated a stronger dependence of OTR on agitation than on aeration; therefore, the productivity with increasing k_La was a satisfactory correlation.

Mixing was another important factor for oxygen transfer and cell activity in the bioprocess and its scale-up. Therefore, mixing had a significant impact on the rate of biomass production and on product polysaccharide physical properties (Audet *et al.*, 1998). The mixing intensification may lead to a reducing of k_La and this effect was more pronounced at lower aeration rate and lower biomass concentration, being the results of finest dispersion of air and, consequently, of easier adsorption of cells on to the bubble surfaces (Galaction *et al.*, 2004). The increasing of k_La value was the result of bubble surfaces being blocked by cells adsorption, phenomena that can be described by means of the ratio between k_La value for viscous suspensions with and without biomass. The magnitude of blocking effect on k_La values also depends on microorganism type. Therefore, the k_La around the impellers was high since the mixing is appropriate while k_La was low where mixing was heterogeneous (Kouda *et al.*, 1997). Heterogeneous mixing was also found during WD7 biopolymer production at too high agitation rate (>600 rpm) which affected the k_La value.

4.1.8 Effect of Aeration and Agitation Rate on k_La at Pilot Plant Scale

When the scale of operation increased, the broth became more and more heterogeneous and oxygen can be depleted in some areas of the reactor (Thiry and Cingolani, 2002). The supply of oxygen to the growing cells was usually the limiting operation in scale-up. In this study, the DOT values in all runs at pilot scale decreased in the exponential phase, then increased and kept constant in the stationary phase; i.e. the same as found at bench scale. The residue DOT levels were high at high rate of aeration and agitation, with higher than 10% of DOT in all runs where there was sufficient oxygen

support in the system. The effect of aeration (0.5, 1.0, 1.5 and 2.0 vvm) and agitation rates (200, 400 and 600 rpm) on OTR, OUR and k_La values were also studied at pilot scale. By the dynamic gassing out technique, the OTR and OUR values increased with increasing aeration and agitation rate. Agitation rate had still more effect on these parameters than aeration rate, and the k_La values at various rates of aeration and agitation are shown in Figure 9.

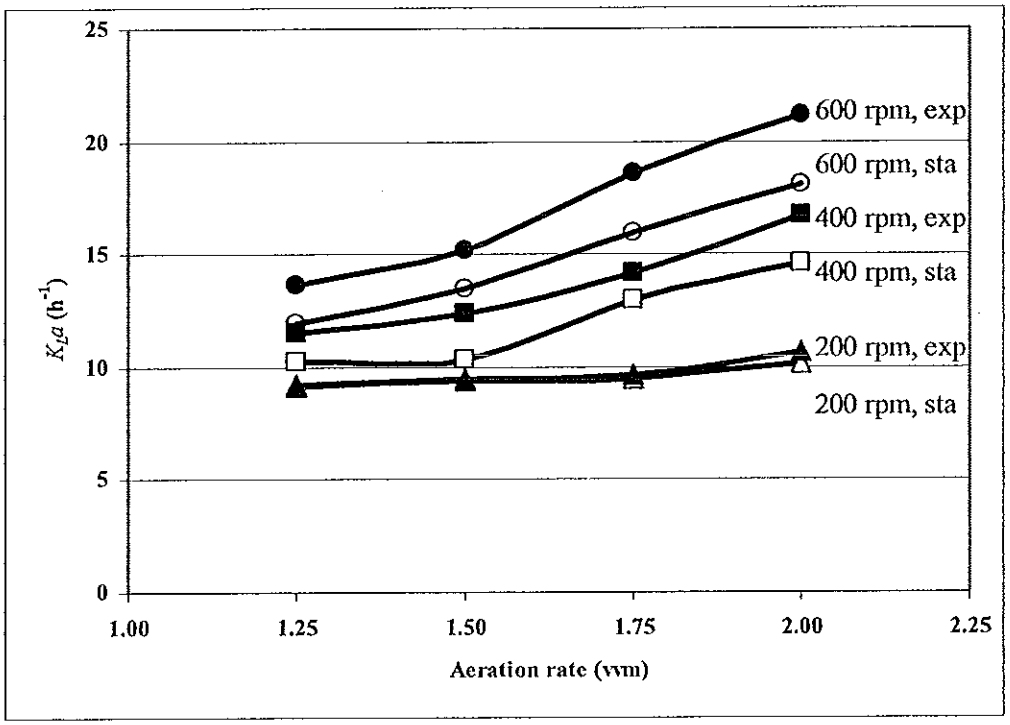


Figure 9 Correlations of aeration and agitation rate on k_La during fermentation of *E. cloacae* WD7 on pilot scale (72L fermenter) at exponential (exp) and stationary (sta) phases

The k_La values increased when agitation and aeration rates increased in both phases. With various aeration rates (1.25 -2.00 vvm), the k_La ranges at 200 rpm were 9.25 -10.67 and 9.09-10.18 h^{-1}, 11.51-16.75 and 10.21-14.57 h^{-1} at 400 rpm, while the maximum k_La values (21.16 and 18.11 h^{-1}) were found at 2.00 vvm and 600 rpm in the exponential and stationary phase, respectively. The maximum k_La value was still found at maximum rates of aeration and agitation and still higher in the exponential phase rather than stationary phase because of high OUR under high viscosity. By comparison, under the same conditions (1.25 vvm and 200 rpm as the optimal condition at bench scale, the k_La values at pilot sale (9.25 and 9.09 h^{-1}) were lower than those at bench scale (9.97

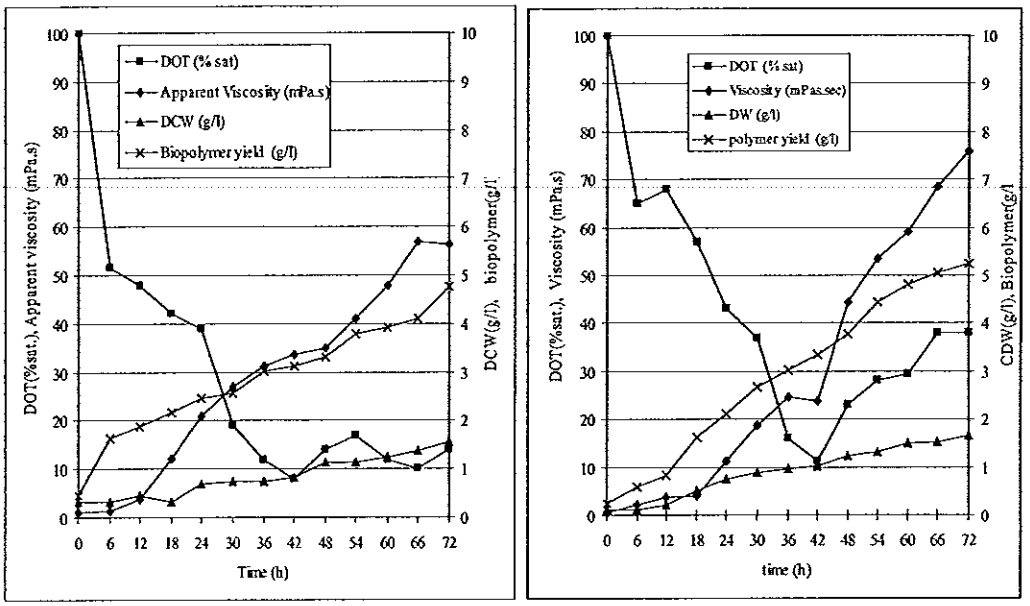
and 9.72 h^{-1}) in exponential and stationary phases, respectively. The k_La values at pilot scale (9.68 and 9.50 h^{-1}) found at 1.75 vvm, 200 rpm, were very close to the k_La value at optimal condition at bench scale (9.97 and 9.72 h^{-1}), therefore these were chosen for scale-up of WD7 biopolymer production.

4.1.9 Scale-up by Fixing k_La

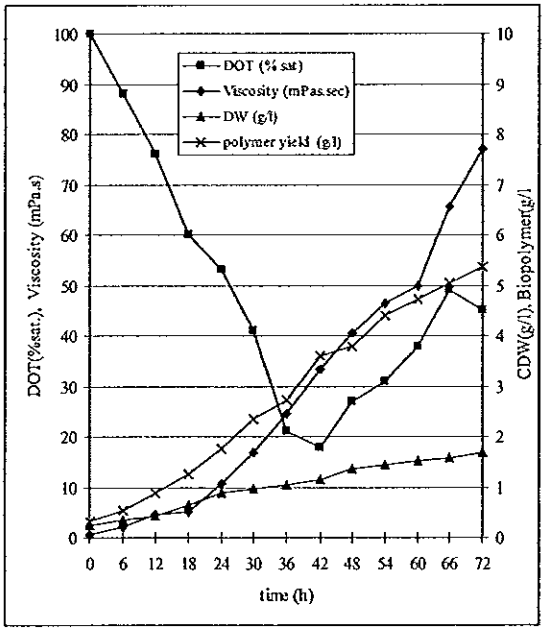
Fermentations at pilot scale were carried out for the massive production of WD7 biopolymer. Three batch cultivations at various conditions in the pilot fermenter were chosen as follows: a) 1.25 vvm and 200 rpm, an optimal condition from bench scale, b) 2.00 vvm and 200 rpm, the aeration rate increased while keeping the agitation rate constant and c) 2.00 vvm and 600 rpm, the maximum rate of aeration and agitation at pilot scale. Time courses and some data of fermentations at pilot scale under these different conditions are shown in Figure 10 (a, b and c). All time courses showed similar characteristics to those of the bench scale, the DCW, biopolymer yield and viscosity concentration still increased corresponding to cultivation time.

Therefore, when aeration and agitation rates increased, at operating conditions of a), b), and c), the DCW values were 1.58, 1.65 and 1.67 g/l, the biopolymer yields were 3.07, 3.18 and 3.20 g/g respectively with corresponding viscosities of 66.8, 76.0, and 77.2 cp (mPa.s), respectively (Table 7). The best condition to achieve the highest biopolymer production at pilot plant scale was 2.00 vvm and 600 rpm, which differed from those at bench scale (at 1.25 vvm and 200 rpm). The maximum biopolymer yield was found at the maximum aeration rate at both scales. Effect of shear stress on biopolymer production (as found at bench scale) was not found at pilot scale because of differences in geometry characteristics, impellers size and number and diameter ratio of impeller to fermenter. Thus, no effect of shear stress on cell growth and biopolymer production occurred at pilot plant scale in aeration and agitation ranges tested.

Since the k_La values at bench scale were different from those at pilot scale and both fermenters were different in geometric characteristics and agitation systems, the scale up in this study was operated by fixing equal k_La at bench and pilot scales. By comparison, the k_La values at pilot scale (ps) were 9.50 and 9.68 h^{-1} at 1.75 vvm and 200 rpm, which were very similar to those of bench scale (9.97 and 9.72 h^{-1}) at 1.25 vvm and 200 rpm at



(a) (b)



(c)

Figure 10 Time courses of biopolymer production of *E. cloacae* WD7 in 72 L fermenter [containing optimal medium at 30°C, pH 7.0 and (a) 1.25 vvm and 200 rpm, the optimal condition from bench scale (b) 2.00 vvm and 200 rpm and (c) 2.0 vvm and 600 rpm (▲ = cell concentration, x = biopolymer concentration, ■ = dissolve oxygen tension and ● = viscosity)]

Table 7 Effect of various conditions on biopolymer fermentation of *E. cloacae* WD7 (at 30°C, pH 7 after 3 days cultivation in 72L fermenter when comparison with results of cultivation at 5L fermenter for scale-up consideration)

Fermenters	72L			5L
Parameters	1.25 vvm, 200 rpm	2.00 vvm, 200 rpm	2.00 vvm, 600 rpm	1.25 vvm, 200 rpm
Final cell concentration (g/l)	1.58	1.65	1.67	1.55
Biopolymer concentration (g/l)	4.86	5.25	5.35	4.76
Biopolymer Yield based on biomass, Y p/x (g/g)	3.07	3.18	3.20	3.07
Apparent viscosity (mPa.s)	68.8	76.0	77.2	59.2
k_La at exponential phase (h^{-1})	9.09	9.50	18.11	9.72
k_La at stationary phase (h^{-1})	9.25	9.68	21.16	9.97

both exponential and stationary phases, respectively. These gave k_La ratios of 1.03 ($k_La_{(ps)}$ / $k_La_{(bs)}$ = 9.97 / 9.68) in exponential phase and 0.98 ($k_La_{(ps)}$ / $k_La_{(bs)}$ = 9.50/9.72) in stationary phase. In the successful scale-up for WD7 biopolymer production, biopolymer yield (3.20 g/g) was slightly higher than at bench scale (3.07 g/g) (Table 7). This is normally found in other scale-ups of biopolymer production due to different geometric characteristics with aeration and agitation systems (Flores *et al.*, 1997; Galaction *et al.*, 2004; Garćia-Ochoa *et al.*, 2000).

In this work, although heterogeneity is usually found in viscous culture, no such problem was found in the tested ranges of variables that could not be solved easily by using appropriate agitation rate control. Using the appropriate geometry, stirrer and impeller types one can increase the oxygen supply and reduce the highest level of flow. At bench scale with only two impellers, the flow caused serious stress conditions for microorganisms which were higher than for the pilot reactor with three impellers with six flat blades type. Oxygen was highly transferred into the pilot plant system by the control of agitation with suitable mixing and without shear stress. Cell concentration and biopolymer yield at pilot scale were high, with high values of OTR, OUR and k_La as well.

In addition to the effect of agitation and aeration, there are also many factors affecting on aerobic process and scale-up such as shear stress, mixing, growth of

microorganism, product concentration and viscosity, which are important and directly affecting on oxygen transfer rate in different scales and on k_La determination (Hsu and Wu, 2002). If the fermentation system tested in a small-scale fermenter is insensitive to a wide range of these environmental variables, scale-up is not a problem. Therefore, the knowledge of how a cell reacts is needed so one can accurately predict the, process parameters, influence of environmental factors and product yield in scale-up (Hewitt *et al.*, 2000).

Cell growth and biopolymer yield of *E. cloacae* WD7 under aerobic fermentation increased with increasing aeration and agitation rates at pilot scale (72L), but decreased when agitation increased at bench scale (5L), because of shear thinning. The highest biopolymer yield at bench scale (3.07 g /g) was found at optimum condition (1.25 vvm and 200 rpm), but the increase of biopolymer yield (3.20 g/g) was found at 2 vvm and 600 rpm in pilot scale cultivation. The OTR was higher than OUR in both phases indicating adequate oxygen supply in both systems. The OUR and OTR values in exponential phase were lower than those in the stationary phase due to having high concentration of cell and biopolymer with high viscosity. The k_La values at both scales increased with increasing aeration and agitation rate. At 1.75 vvm and 200 rpm, the k_La values in exponential and stationary phases at bench scale were 9.97 and 9.72 h^{-1}, which were very close to those of pilot plant scales (9.68 and 9.50 h^{-1}), respectively. Under these conditions, scale-up differences in geometry and stirrer types were successfully overcome by fixing k_La (with ratio of fixing k_La of 1.03 and 0.98 in exponential and stationary phases, respectively) giving biopolymer yields of 3.07 and 3.20 g/g per gram of DCW at bench and pilot scales, respectively, without system failure.

4.2 Preparation and Characteristics of Crude and Partially Purified WD7 Biopolymer Samples

After fermentation, the viscous broth was centrifuged to remove some bacterial cells, the supernatant was concentrated, and then precipitated with cold absolute alcohol (about 1:4). The fibrous white biopolymer (Figure 11a) was dried at 50°C to obtain dry polymer (Figure 11b) which was prepared to be as powder after grinding in a ceramic mortar (Figure 11c). When the powder biopolymer was dissolved in water, and concentrated in a vacuum rotary evaporator, then dialyzed in a dialysis bag and freeze dried, the partially purified biopolymer like soft sponge was obtained (Figure 11d).

Thus, the WD7 biopolymer powder was produced in two types; crude dried biopolymer and partially purified freeze dried biopolymer that could be kept in sealed plastic bag at room

temperature for a long time until used. Drying at 50°C was used to evaporate ethanol used for precipitation and recovery of crude biopolymer. Freeze drying, on the other hand, was a complicate and energy intensive method to remove the solvent (or water) at low temperature and appropriate for high valuable biopolymer product. However, the freeze dried WD7 biopolymer obtained still had high water content and unable to be ground into small pieces or powder as found in dried sample.

Dried and freeze dried biopolymers were re-dissolved in water and became biopolymer solution with high viscosity. Partially purified freeze dried biopolymer lost its viscosity since it was probably degraded by heat at high temperature during concentration step in the rotary evaporator. A lthough the temperature in water bath was set at 50°C but the actual temperature in rotary glass bottle of evaporator was too high because of high vacuum condition. Thus, the freeze dried WD7 biopolymer had lower viscosity when compared to the dried crude sample at the same concentration.

Both dried and freeze dried WD7 biopolymer could be prepared into solution and gel at low and high concentration of biopolymer, respectively. The WD7 biopolymer appeared as solution at the concentrations of 2 and 4% (w/v) while formed gel at the concentrations of 6, 8 and 10% (w/v) of WD7 biopolymer (Figure 12). WD7 solution at low concentration had white color while its gel had yellow color. In addition, the properties of WD7 biopolymer solution and gel were very different, such as their viscosity and rheology. Rheological properties of the broth culture offered a high resistance to the mass transfer, the supply of oxygen to the growing cells usually being the limiting operation in scale-up. When scale is increased, the broth will become more and more heterogeneous and oxygen can be depleted in some areas of the reactor (Thiry and Cingolani, 2002). Characteristics and flow behavior of the viscous broth were studied in order to describe some of the phenomena found in biopolymer fermentation.

4.3 Characterization of WD7 Biopolymer

4.3.1 Chemical Characterization

Some preliminary chemical characteristics of partially purified WD7 biopolymer were investigated by colorimetric reactions (Table 8). Total sugar and neutral sugars of WD7 biopolymer were 54.36 and 34.65% (w/w), respectively. Monosaccharide of WD7 biopolymer was pentose (31.2%) and hexose (20.4%). The ketose (fructose) type content

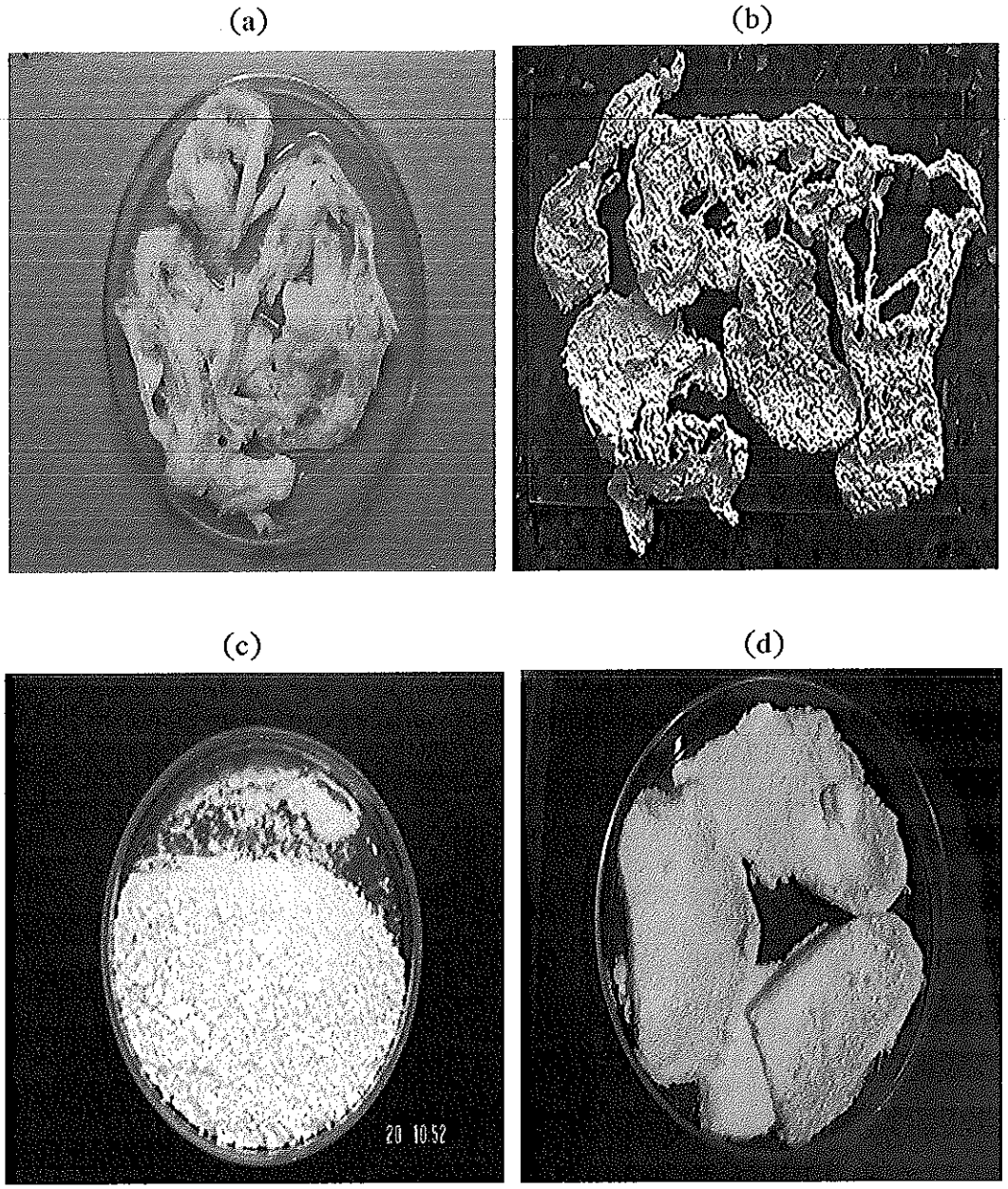


Figure 11 Biopolymer forms of *Enterobacter cloacae* WD7 [after precipitation with cold absolute ethanol (a), drying in oven at 50°C (b), grinding in mortar (c) and partially purification and freeze drying (d)]

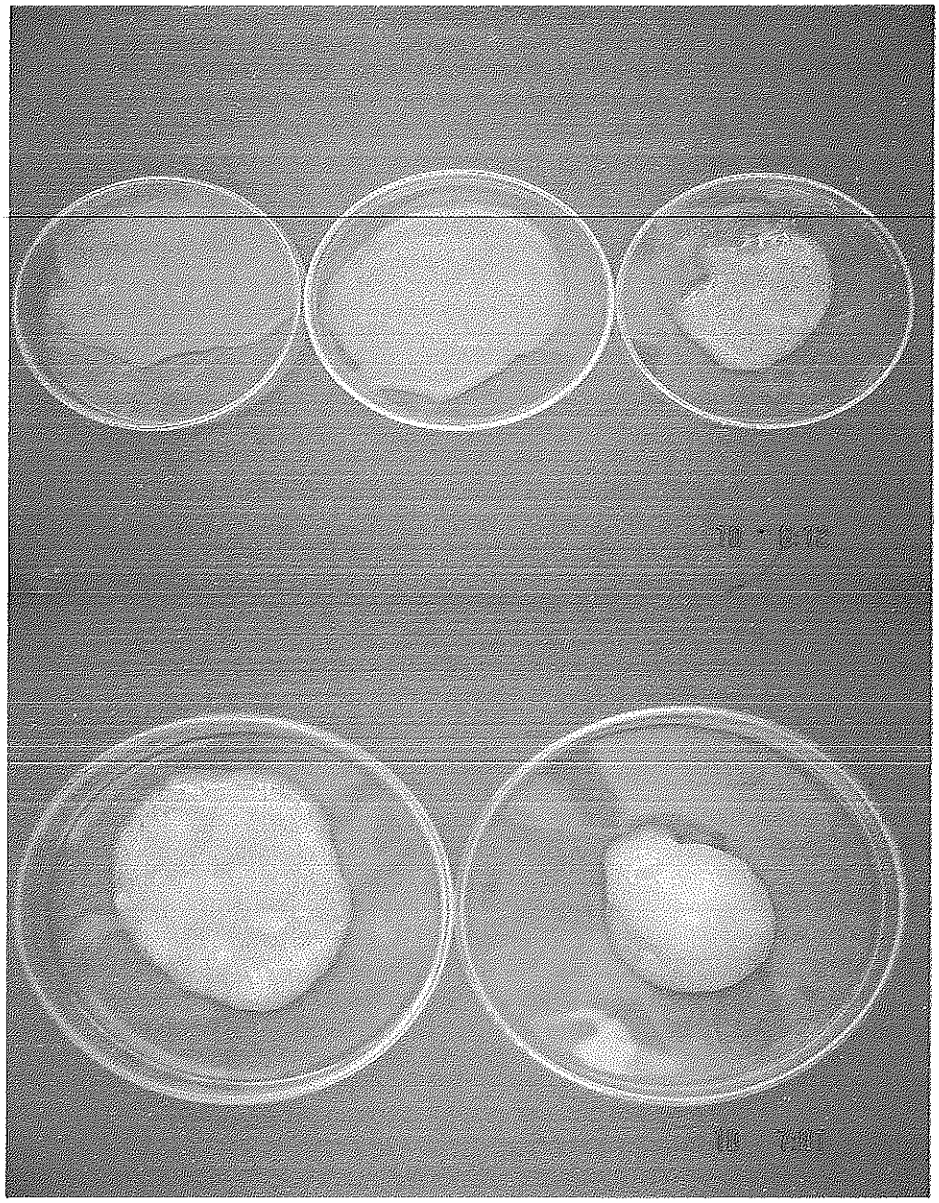


Figure 12 WD7 biopolymer solution and gel prepared at concentrations of 2, 4, 6, 8 and 10% (w/v) (from left to right)

of partially purified freeze dried WD7 biopolymer was very low about 1.95% (w/w). Fructose might be a contaminant from culture medium.

Constituents of carbon, hydrogen, oxygen were 6.01, 2.23 and 32.32% RSD, for purified WD7 biopolymer. Clearly, this WD7 biopolymer was carbohydrate (exo-polysaccharide) with trace amount of nitrogen and sulphur compositions (< 0.01% RSD).

Moisture content of WD7 EPS samples were about 4.40 and 4.80 % (w/w) for crude and partially purified freeze dried samples, respectively. The protein and amino sugar content is very low about 1.05 and 0.78 % (w/w), respectively and no α-amino acids or aromatic amino acids, these were the same as previously reported by Dermlim (1999). In addition, the uronic acid content in this WD7 EPS was 13.83 %. This might be sugar substitute in WD7 EPS structure.

Table 8 Components of the partially purified WD7 biopolymer

Methods	Analyzed item	Partially purified WD7 biopolymer %(w/w)
Qualitative tests		
1. Ninhydrin reaction	α-amino acids	negative
2. Xanthoproteic reaction	aromatic amino acids	negative
Quantitative tests		
1. Anthrone reaction	neutral sugar	34.65
2. Phenol sulphuric acid reaction	total sugar	54.36
3. L-cysteine hydrochloride assay	total sugar	51.60
	hexose contents	20.40
	pentose contents	31.20
4. Phenol boric acid-sulphuric acid assay	ketose content	1.95
5. Comassy blue assay	protein content	1.05
6. Elson-Morgan reaction	amino sugar	0.78
7. Carbazole-sulphate reaction	uronic acids	13.83
8. HR 73 Halogen Moisture Analyser	Moisture contents	4.40
Structural components		
C,H,O,N and S analyzer	Carbon (% RSD)	16.01
	Hydrogen (% RSD)	2.23
	Oxygen (% RSD)	32.32
	Nitrogen (% RSD)	<0.01
	Sulphur (% RSD)	<0.01

4.3.2 Molecular Weight Determination of WD7 Biopolymer

By GPC analysis, the average molecular weights (M_w) were 59.4 and 56.6 kDa for crude and partially purified biopolymer from fermentation by using glucose as carbon source in the modified medium (CG and PG, respectively) and 48.9 and 50.7 kDa for crude and partially purified biopolymer from fermentation by using sucrose as carbon source in the modified medium (CS and PS, respectively) with various molecular weights by mathematical analysis of WD7 biopolymer as shown in Table 9.

Table 9 Mathematical analysis of GPC chromatograms of crude (C) and partially purified biopolymer (P) of *E. cloacae* WD7 [cultivated in modified media with glucose (G) and sucrose (S) as carbon sources]

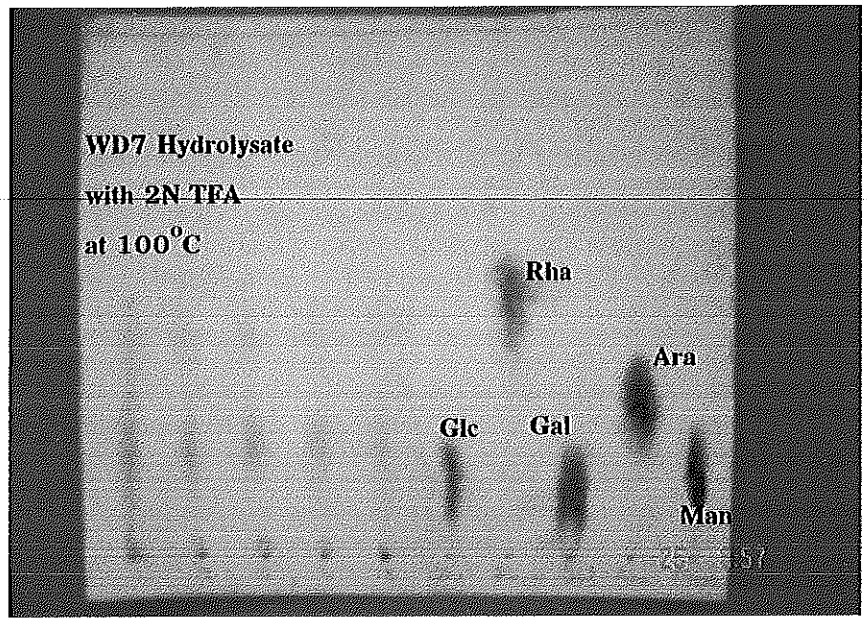
WD7 biopolymer Sample	M_p (kDa)	M_n (kDa)	M_w (kDa)	M_z (kDa)	M_{z+1} (kDa)	M_v (kDa)	d
CG	9.8	15.0	59.4	281.5	483.0	44.3	4.0
PG	11.6	16.7	56.6	252.5	472.6	43.6	3.4
CS	16.5	17.7	48.9	199.6	456.7	39.7	2.8
PS	10.0	14.2	50.7	266.5	506.4	37.9	3.6

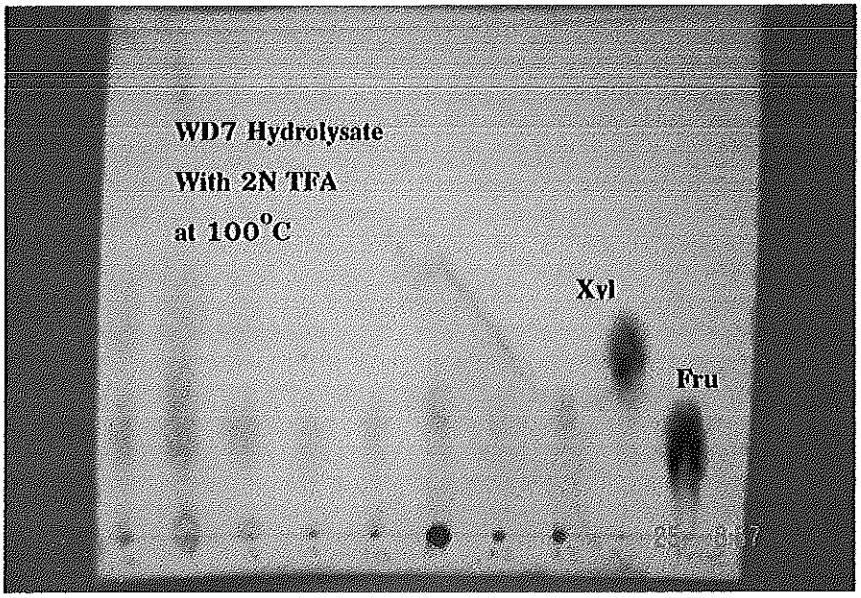
4.3.3 Investigation of Monosaccharides of WD7 EPS

4.3.3.1 Monosaccharides Identification by TLC

TLC chromatograms for identification of WD7 biopolymer by hydrolysis with 2N trifluoroacetic acid (TFA) at 100°C for 8 h, compared with standard monosaccharides were shown in Figure 13. Rf values of each standard monosaccharides; glucose, arabinose, fructose, galactose, rhamnose, mannose and xylose, were 0.248, 0.347, 0.296, 0.238, 0.428, 0.220 and 0.358 with violet, blue, violet, purple, orange, purple and brown color, respectively. After TLC run, TFA hydrolysates at various times (1-8h) clearly showed the rhamnose, glucose and galactose, with Rf values of 0.355, 0.250 and 0.240 with orange, violet and purple colour, respectively.

(a)





(b)

Figure 13 TLC of WD7 hydrolysate after TFA hydrolysis for 1-8 h. [for 1-5h (a) and 1-8h (b), respectively. [Monosaccharide standards; glucose (Glc), rhamnose (Rha), galactose (Gal), arabinose (Ara), mannose (Man), xylose (Xyl) and fructose (Fru) were used and the developing phase was 90% acetonitrite].

4.3.3.2 Monosaccharide Composition Analyzed by HPLC

Figure 14 showed the HPLC chromatogram of hydrolysate after methane sulponic acid (MSA) hydrolysis (8h) of mixed neutral sugars standards (A) and WD7 biopolymer hydrolysate (B). Retention times of mixed standard monosaccharides corresponded to: 12.067 = rhamnose; 13.650 = arabinose; 16.050 = galactose; 18.017 = glucose; 21.808 = mannose; 24.150 = fructose; 42.200 = glucuronic acid. While the retention times of monosaccharides in WD7 biopolymer hydrolysate corresponded to three monosaccharides (16.726 = galactose; 18.725 = glucose; 42.283 = glucuronic acid). The WD7 EPS revealed the presence of galactose and glucose with MSA hydrolysis by detection of this HPLC-1.

After strong hydrolysis with 2N Sulphuric acid for 2 h at 80°C, the HPLC chromatogram (Figure 15) showed five peaks in WD7 hydrolysate indicating of galactose, glucose, rhamnose and fructose at retention times of 8.771, 9.945, 10.983 and 12.049 min, respectively. Mixed standard monosaccharide was injected separately to HPLC showed the retention time (min) of galactose, glucose, rhamnose and fructose at 8.082, 9.052, 10.242 and 12.582 min, respectively. The WD7 EPS revealed the presence of rhamnose and glucose by sulphuric acid hydrolysis, while galactose was not found by detection of this HPLC-2. Fructose content was detected by H_2SO_4 may be contaminant since it was found by TLC.

There were three peaks of HPLC chromatogram of hydrolysate after hydrolysis with 2N trifluoroacetic acid (TFA) method for 6h, indicating they were rhamnose, glucose and galactose detected by HPLC-2. After calculation, the rhamnose (16.6%) was the dominant monosaccharide, while glucose and galactose was present 7.7 and 5.77%, respectively. Further work is required to elucidate the structures of these WD7 polysaccharides. Considering these results, it was concluded that the major monosaccharide for this WD7 biopolymer are rhamnose, glucose and galactose with ratio of 2.88:1.33:1.00.

4.3.4 Functional Groups Analysis by FTIR

FT-IR spectroscopy is used for the qualitative identification of polymers and its infrared spectra are given in term of % transmittance (Sartori *et al.*, 1997; Dermlim, 1998). Results of FT-IR spectroscopy analysis of freeze dried WD7 biopolymer samples from fermentation using sucrose and glucose as carbon source were shown in Figure 16. Both had similar pattern of broad absorption bands and can be identified by matching against the

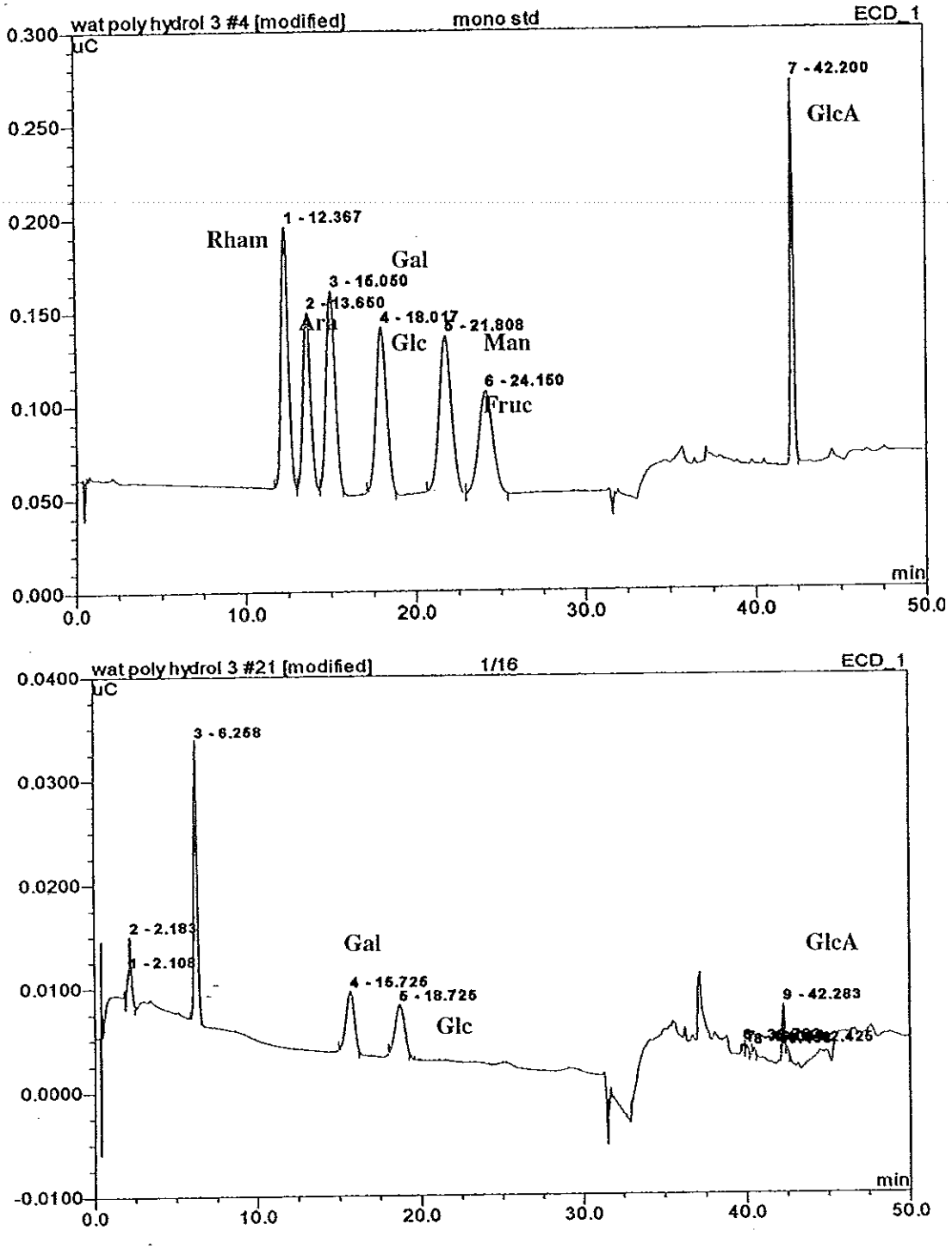


Figure 14 HPLC chromatogram of WD7 hydrolysate after methane sulphonic acid (MSA) hydrolysis (8h) (lower) comparison to which of the mixed neutral sugars standards (upper)

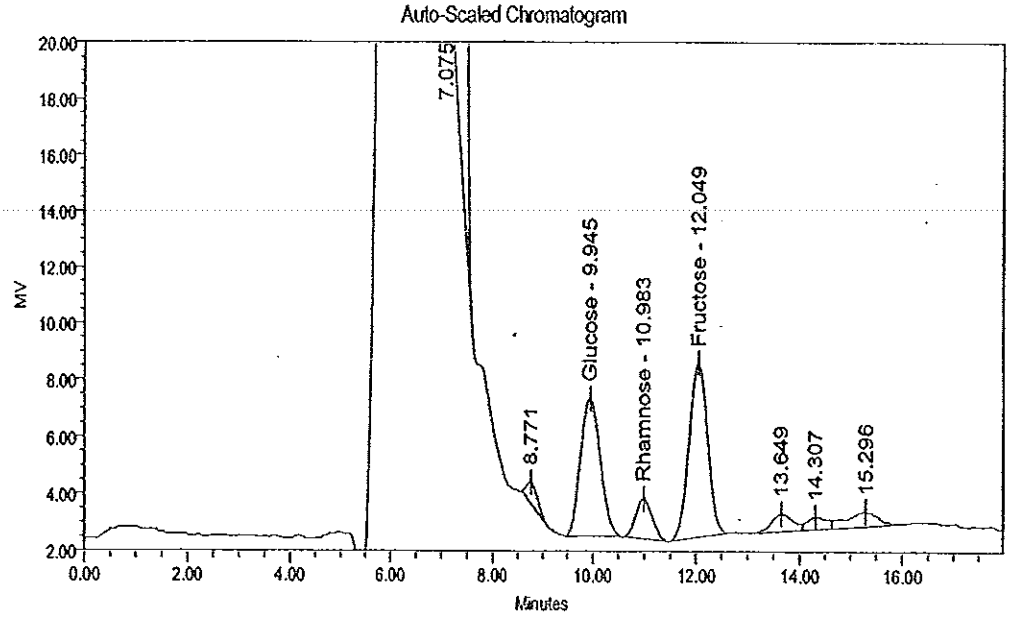


Figure 15 HPLC chromatogram of WD7 hydrolysate after H_2SO_4 hydrolysis for 2h

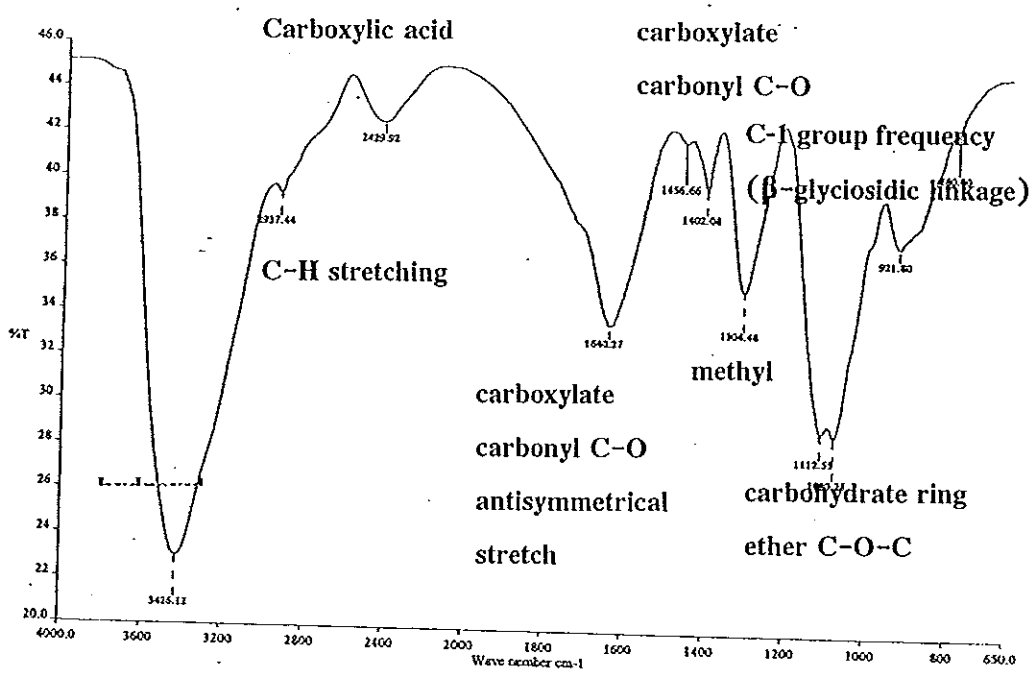


Figure 16 FT-IR chromatograms of WD7 polysaccharides

data library in software disc. Interpreting the absorption bands against the data library in computerize software can show the chemical structures and functional groups and atomic bonding of tested biopolymer sample.

Infrared spectra of WD7 biopolymer demonstrated the presence of carbonyl, hydroxyl, carboxyl and methoxyl groups. This biopolymer was a polysaccharides with C-H stretching with C-1 (β-glycosidic linkage), containing some acids due to the presence of O-H stretching and C=O of carboxylic acid. The functional groups are responsible for its physical and chemical properties, for example carboxylate groups can serve as binding sites for divalent metal ions. The interpretation results of the WD7 biopolymer samples were compared with the previous descriptive characteristics of biopolymer from glucose fermentation by Dermlim (1999).

The IR spectrum exhibited a broad O-H stretching absorption band centered at frequencies of 3421-3455 cm^{-1}. A minor C-H stretching band at 2429.92 (2922) cm^{-1} and several C-O absorption bands in 1400-900 cm^{-1} region were found including a set strong C-O stretching bands at 1042 and 1072 cm^{-1}. The spectrum also displayed adsorption bands of carboxylate groups from 1670 to 1663 cm^{-1}. However, the spectrum did not display absorption bands for sulfate groups (i.e., bands at 1250 and 820 cm^{-1}, attributable to S=O and C-O-S stretching vibrations), which is in agreement with the absence of sulfur in the elemental analysis.

4.3.5 ^{13}C-NMR Spectroscopy Analysis

The ^{13}C-NMR spectroscopic analysis was performed to investigate the anomeric linkage configuration of the WD7 EPS. This allows elucidation of the polymer backbone and can also be employed to evaluate the type of side-chains branching along the backbone. The ^{13}C-NMR spectrum of WD7 polysaccharide in solution of Deuterium oxide, (D_2O) and Sodium Deuteroxide (NaOD) is illustrated in Figure 17. An average repeating structure unit of WD7 polysaccharide, elucidated from NMR spectroscopy and characterization of oligosaccharide. Assignments of ^{13}C NMR spectrum were expected. From the signals of ^{13}C NMR spectrum at δ 97.514 -101.898, it was confirmed that this WD7 EPS was polysaccharide.

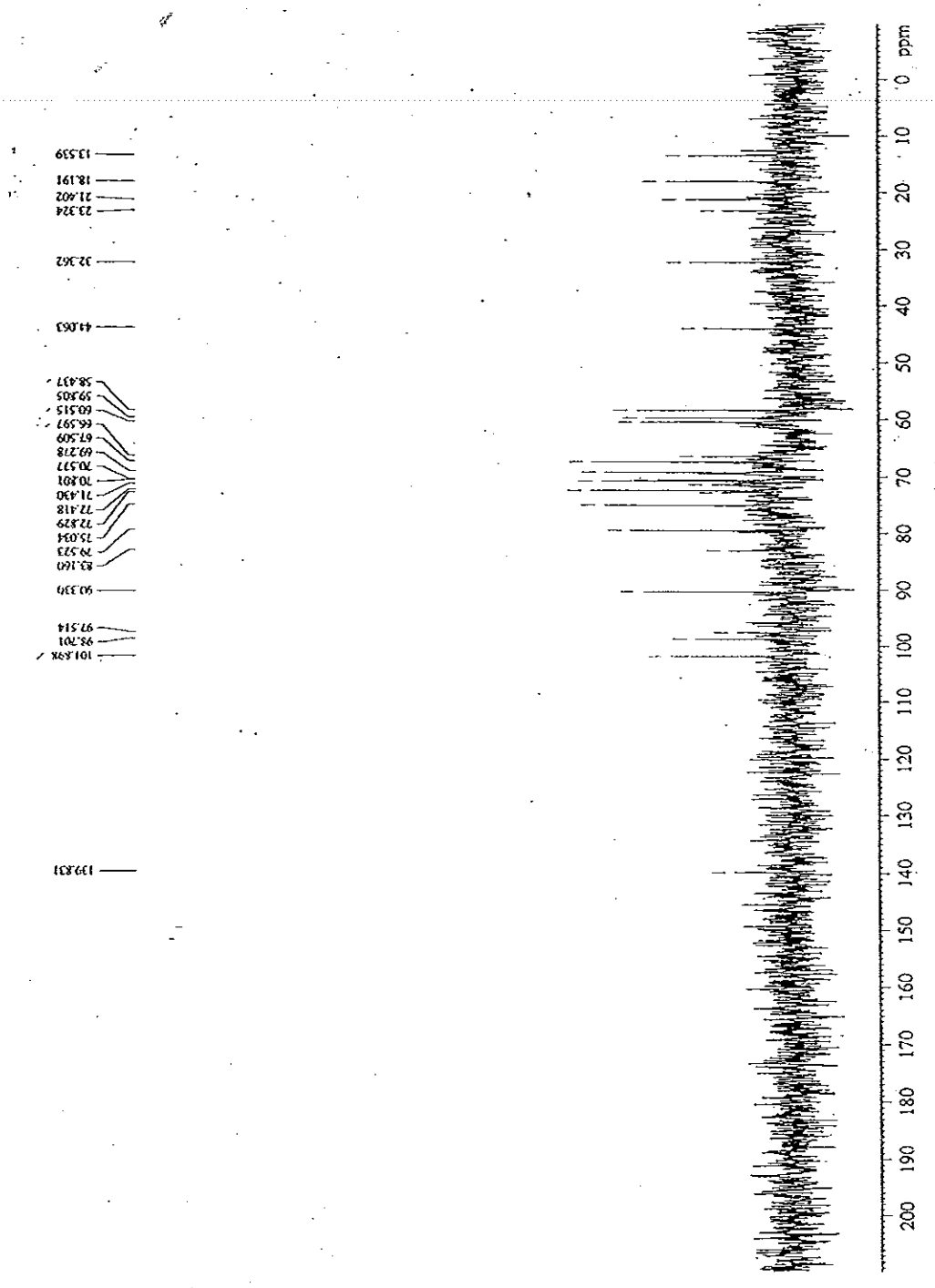


Figure 17 ^{13}C-NMR chemical shift data of WD7 EPS in solution of Deuterium oxide, (D_2O) and Sodium Deuteroxide (NaOD) at 40^{o}C

4.4 Some Factors Affecting on Apparent Viscosity of WD7 Biopolymer Solution

Apparent viscosities of dilute solution of WD7 biopolymer were measured by using Brookfield viscometer due to its measuring capacity at low viscosity ranges. The relationship of apparent viscosity and shear rate at various concentrations (0.1-0.5% w/v) of WD7 biopolymer are shown in Figure 18. It was found that the higher biopolymer concentration, the higher its viscosity, while indicated the decrease of viscosity when shear rates increased. Table 10 presents the values of viscosity (3.18, 4.90, 5.34, 7.35 and 12.80 mPa.s) at different shear stress (419.76, 646.80, 704.88, 970.20 and 1,689.60 mPa), the K values were about 6.92, 20.41, 26.30, 38.02 and 83.18 x 10^{-3} Pa.s^{c} and n values about 0.85, 0.74, 0.68, 0.66 and 0.63, respectively. These results confirmed that theWD7 biopolymer possessed pseudoplastic characteristics due to n<1.

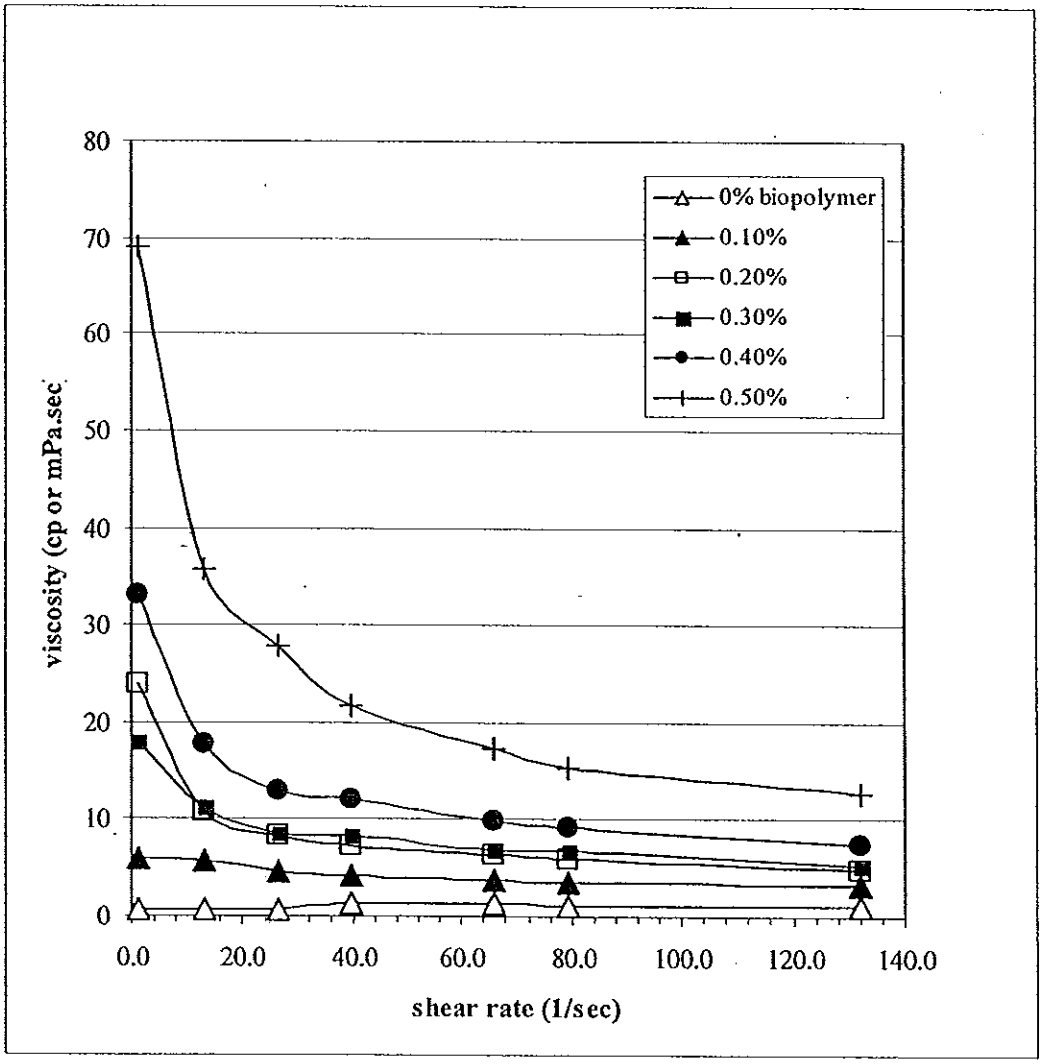


Figure 18 Relationship between shear rate (s^{-1}) and viscosity (mPa.s) of WD7 biopolymer at various concentration measured by Brookfield viscometer

Table 10 The K and n values (consistency index and flow index) of WD7 biopolymer at various concentrations obtained from curves of log shear rate vs log shear stress

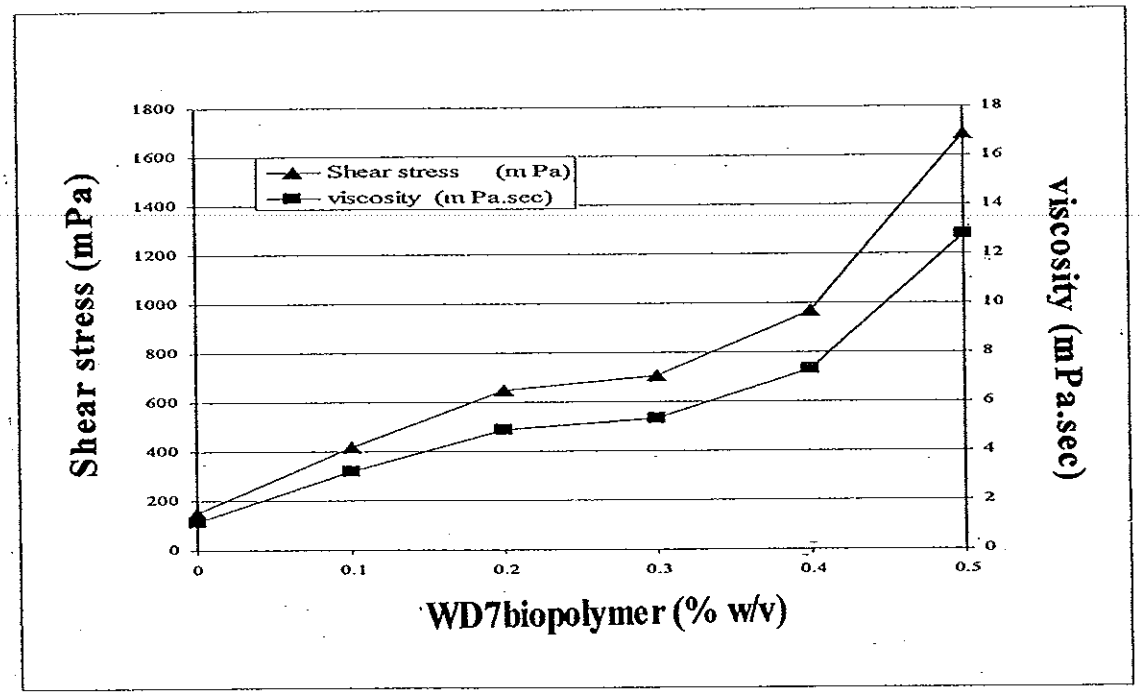
Biopolymer concentration (%)	Viscosity (x 10^{-3} Pa.s)	K values (x 10^{-3}Pa.s^{c})	Flow index (n)
0	1.14	4.9	1.19
0.1	3.18	6.92	0.85
0.2	4.90	20.41	0.74
0.3	5.34	26.30	0.68
0.4	7.35	38.02	0.66
0.5	12.8	83.18	0.63

This behavior was also found in many polymer fluids such as the most well-known biopolymer; xanthan and pestan, alginate gum and carboxymethyl cellulose (CMC), as well as in many polymer products, such as paint, shampoo, slurries, fruit juice concentrate and ketchup (Sutherland, 1998). Normally, the substances in the pseudoplastic class are long molecules, randomly oriented and with no connected structure. These fluids need an efficient rate of agitation for over all mixing to homogeneity. The application of a shearing stress tends to align the molecules so that the viscous resistance between the adjacent layers diminishes, resulting in a progressive fall in viscosity as the shear rate increases. Therefore, the shear rate causes the stress that inhibits cell growth and biopolymer production (Audet *et al.*, 1998) as was also found in this study.

4.4.1 Effect of Biopolymer Concentration

Effect of concentrations on apparent viscosity of WD7 biopolymer solution was studied at 30°C by using Brookfield viscometer. Combined relationship of apparent viscosity and shear stress to shear rate at various concentrations (0-0.5% w/v) of crude biopolymer is shown in Figure 19. Shear stress and apparent viscosity of biopolymer increased with increasing concentration in the tested ranges (Figure 19a). Therefore, shear stress (mPa) at all biopolymer concentration declined at high shear rate (1/sec) (Figure 19b). For example, the apparent viscosity of biopolymer solution at 0.5% (w/v) was gradually decreased from 69-18 mPa.s at shear rate ranges of 1.3-132 sec^{-1}. Logarithm of shear stress was linearly regressed on logarithm of shear rate at each biopolymer concentration and the K and n values were calculated by Power-Law relationship. The K values increased (6.92, 20.41, 38.02, 26.30 and 83.18 mPa.s^{c}), while n values

(a)



(b)

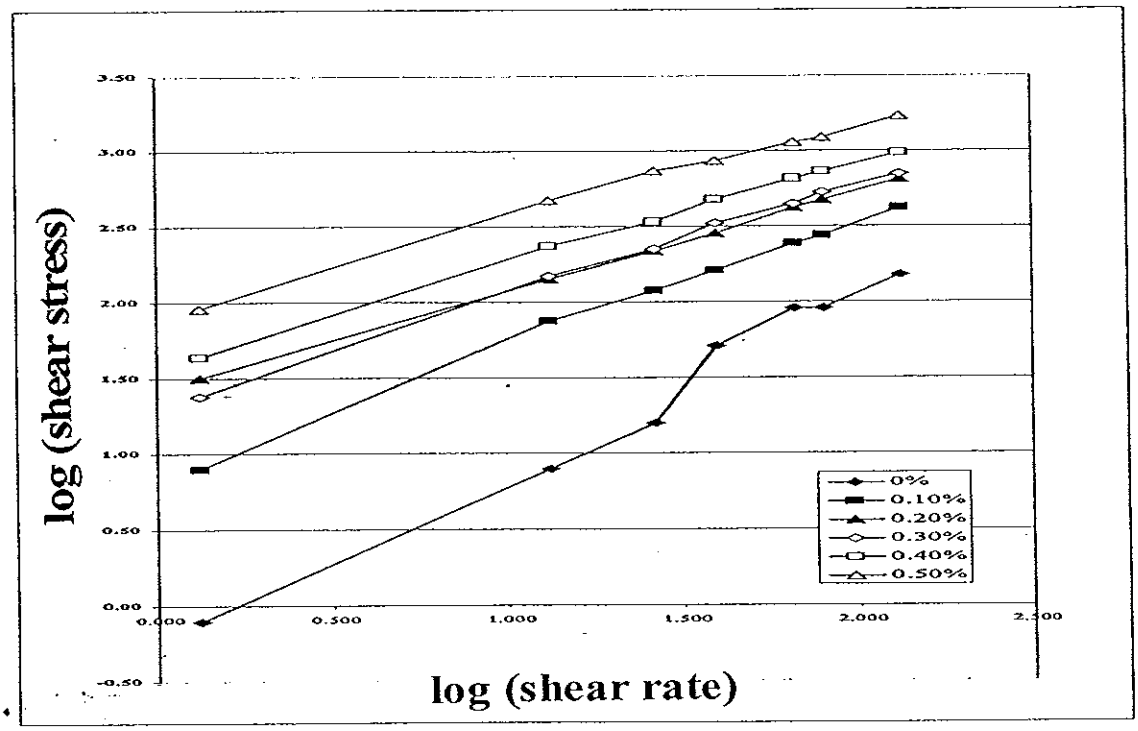


Figure 19 Relationship between shear stress and apparent viscosity (a), and logarithmic shear rate and shear stress used for calculation of K and n values (b) at various concentrations of WD7 biopolymer and 30 ^{o}C by using Brookfield viscometer

decreased (0.85, 0.74, 0.68, 0.66 and 0.63) with increasing biopolymer concentration (a range of 0.1-0.5% w/v, respectively).

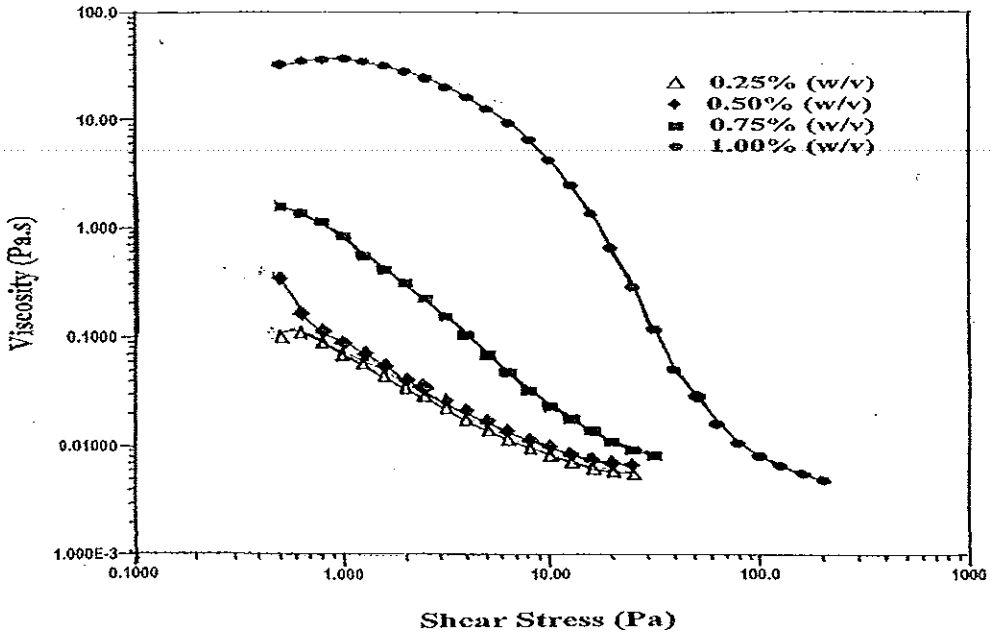
Rheometer was an effective instrument for accurate viscosity measurements across a wide range of shear and expressed as the flow rheological behavior. Importance of flow behavior is that from one experiment a large amount of information can be gained that simulates a wide range of application processes and performance properties. In this study, the effect of WD7 biopolymer concentrations on rheological flow was studied. The results of dilute concentration of WD7 biopolymer solution on flow rheological are shown in Figure 20. All curves had two Newtonian plateaus which represent the zero shear viscosity (η_0) and infinity shear viscosity (η_∞), separated by a Power Law region. In the initial regions (at low shear stress), the increasing shear stress/shear rate had no effect on the observed viscosity (obeys Newton's law of liquid flow). They displayed higher η_0 at higher biopolymer concentrations. First Newtonian plateau was longer at higher biopolymer concentrations and showed high resistance to shear force as considered to be gel condition, which was found at shear stress higher than 100 Pa at 10% (w/v) of this WD7 biopolymer.

Flow profiles of diluted WD7 biopolymer solutions at various concentrations (0.25-1.0% w/v) were given in Figure 21a. First Newtonian plateaus were not found at low concentrations (<1.0% w/v), which showed the low resistance to shear stress. Power Law region, as linear slope, was clearly observed as generally revealed in non-Newtonian fluid, which presents the η_0 decrease with increasing of shear stress/shear rate (obeys Newton's law of liquid flow). All flow profiles still displayed the η_0 which was very low as biopolymer solution and having higher η_0 at higher biopolymer concentrations. The sol/gel transition was observed in the concentration range of 0.75-1.0% w/v.

4.4.2 Effect of Temperature and pH

Effect of temperature and pH on apparent viscosity of WD7 biopolymer solution was investigated. Apparent viscosity of dilute solution (1.0% w/v) of the biopolymer was measured at steady shear rate (100 rpm or 132 sec^{-1}) at different temperatures (10, 20, 30, 40, 60 and 80^{o}c) and pH (2-12) at 30^{o}C by using Brookfield viscometer (Figure 21). The apparent viscosities of this biopolymer solution at strong acidic pH (pH 2 and 4), were lower than those at pH range of 5-7. The apparent viscosity of WD7 biopolymer was declined at alkaline condition (pH 8-12). The highest value of

(a)



(b)

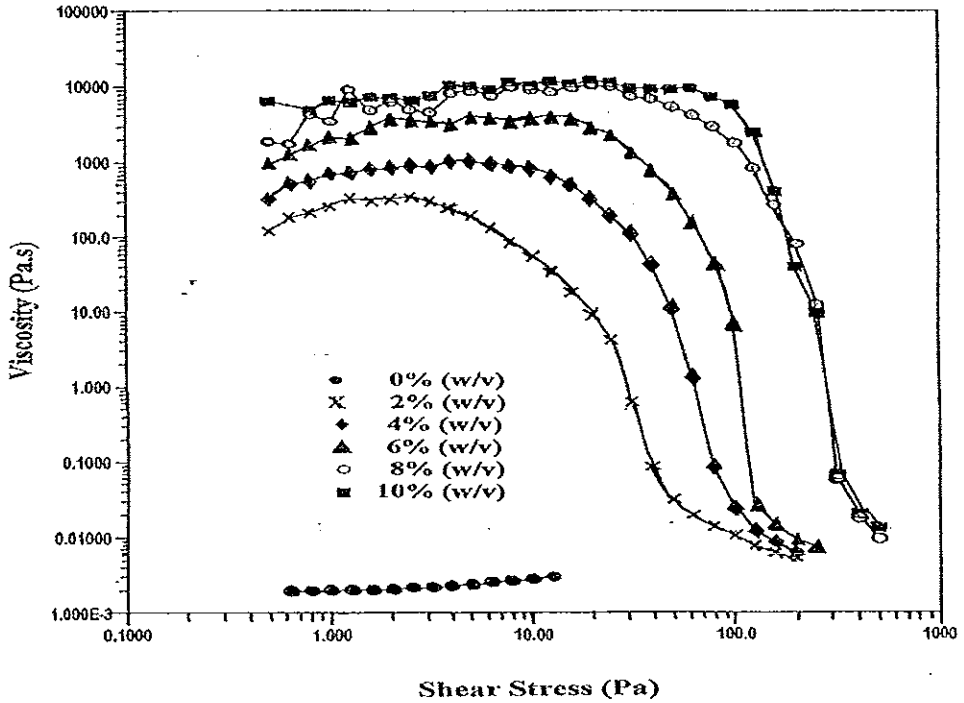


Figure 20 Effect of concentration on flow rheograms of WD7 biopolymer solution at 4 ^{o}C of (a) solution at 0.5 -1.0% (w/v) and (b) gel at 2-10% (w/v)

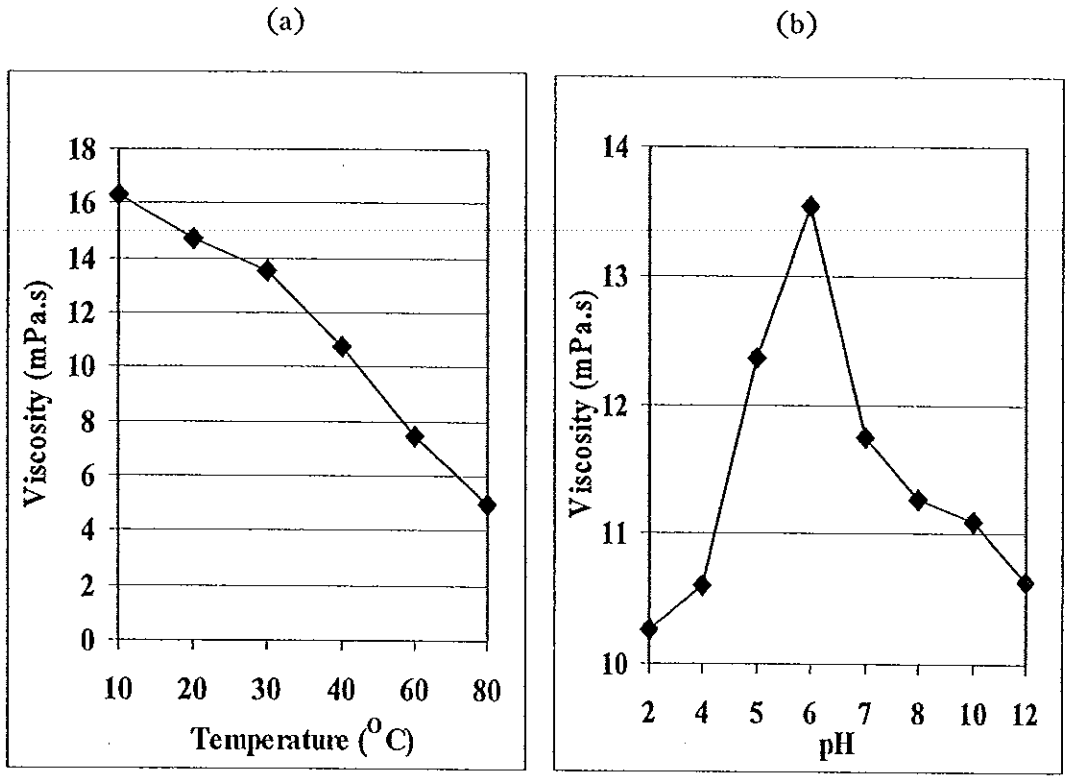


Figure 21 Effect of various temperature (a) and pH (b) on apparent viscosity of WD7 biopolymer solution (1% w/v)

apparent viscosity (13.5 mPa) was obtained at pH 6.0. The viscosity of this biopolymer solution decreased at strong acidic and alkali solution, which agreed with the previous results (Dermlim, 1999). This biopolymer was most stable at pH 6, which was similar to the optimal pH for xanthan (Iseki *et al.*, 2001) and alginate (McDowell, 1974).

The viscosity of WD7 biopolymer solution decreased with increasing temperatures. Relationship between temperature and viscosity may be explained by Arrhenius-Frenkel-Eyring equation: $\eta = A^{(Ea/RT)}$ or $\ln(\eta) \sim 1/T$, where temperature, T (^{o}K), A = frequency factor and Ea = activation energy (kJ/mol) (Jampen *et al.*, 2000). The linear curve obtained from a plot of ln (η) and 1/T, and slope of the line which was the apparent Ea value (Jampen *et al.*, 2000). Low Ea value indicated few inter- and intra-interactions between polysaccharide chains, thus, so much change of viscosity indicated the degradation or conformational transitions occurrence during heating. The Ea values (low or high) indicated the weak or strong bonds of branch or linear chains in sodium carboxymethyl cellulose and arabic gum. Apparent viscosity of WD7 biopolymer decreased when heated.

4.4.3 Factors Affecting Flow Rheological Behaviour of WD7 Biopolymer Gel

In this study, flow profiles were studied under various conditions and factors to consider the flow characters of this biopolymer.

4.4.3.1 Effect of Biopolymer Concentration

Flow profiles of WD7 biopolymer gel at concentration higher than 2.0% (w/v) were shown in Figure 21b. From Table 11, the rheological parameters and η_0 values analyzed by three models, Cross, Carreau and Williumson models. All models were compared and given as similar values. The η_0 values increased (287.3, 792.6, 2,969, 6,160 and 8,405 Pa.s by Cross Model analysis) when the WD7 biopolymer concentration increased (a range of 2 to 10% w/v, respectively) indicated the development of a stronger structure. The n values did not follow any definite trend, which no conformation occurred. In second Newtonian plateau, the η_∞ values of all profiles were very low at high shear stresses (up to 100 Pa.s) and depended on biopolymer concentration. Fluid behavior of crude WD7 biopolymer solution was shear thinning behavior, it became more pronounced if concentration increased.

At high concentration ranges, the high resistance to shear (strength) of this biopolymer were obtained and the inter-particulate attraction of biopolymer rise to non-Newtonian materials of increasing complexity as pseudoplastic character (a decrease in viscosity with increase of shear stress). Therefore, the differences in the η_0 magnitudes and point of shear thinning can be related to the molecular weight and distribution, and could be related to any stabilizing effect the gels could impart to another system. Alginate and xanthan gum solution (4% w/v) exhibited the shear thinning properties, promising in regard to applications as a stabilizer in the food industry (Moreno *et al.*, 1999).

In Power Law region, the viscosities of all profiles dramatically collapsed at higher shear stresses, this exhibited a region of shear thinning (Power Law region). Therefore, the decrease of viscosity as a function of increasing shear stress (Figure 22) confirmed the shear thinning characteristics of this biopolymer with all n values< 0.4. Different n values were obtained from the influence of biopolymer concentrations on viscosity which was depend on the shear rate. It is well-known that the increase of viscosity with biopolymer concentration was less pronounced with the increase of shear stress. For practical applications of biopolymer, the difference in viscosity is expected at different operative shear rates associated with their performance as stabilizer, or with operations such as pouring, mixing and pumping. Pseudoplastic property is important in providing good

sensory qualities, such as mouth feeling, flavor releasing, and suspending properties of food products (Charalambous and Doxastakis, 1989).

Table 11 Rheological parameters analyzed by using various models; Cross, Carreau, Williumson and Power law for dilute and concentrated biopolymer solution of *E. cloacae* WD7

WD7 solutions (% w/v)	η_0 (Pa.s) Cross	η_0 (Pa.s) Carreau	η_0 (Pa.s) Williumson	K Pa(s^C) Power Law	n Power Law
0.25	0.164	0.134	0.106	0.118	0.6439
0.50	0.366	0.219	0.154	0.129	0.6333
0.75	2.019	1.910	1.752	1.564	0.4815
1.00	44.18	34.21	40.95	2.120	0.4191
2.00	287.3	244.7	316.4	2.775	0.3990
4.00	792.6	729.3	816.5	24.20	0.1680
6.00	2,969	2,811	3,027	43.51	0.1544
8.00	6,160	5,908	6,221	101.80	0.1469
10.00	8,405	8,231	8,544	133.40	0.2204

Rheograms of WD7 biopolymer (2% w/v) in each step of purification process were determined. By Cross Model analysis, the η_0 value of crude biopolymer sample (287.3 Pa.s) was higher than those of purified ones (19.69 and 10.13 Pa.s in steps of dialysis and freeze drying, respectively). After purification process, the η_0 decreased more than 14 folds. The loss of viscosity of purified biopolymer was due to re-arrange of biopolymer chain or deformation of biopolymer structure.

4.3.4.2 Effect of Temperature

Effects of temperatures (20-80^{o}C) on flow rheology of crude WD7 biopolymer (2% w/v) at pH 6.0 were determined and their flow rheograms are presented in Figure 22. The viscosity decreased when incubation temperatures increased

with decrease of η_0 values from all analysis models. Flow profiles patterns at high temperature (60-80 ^{o}C) were different from those at low temperatures (20-40 ^{o}C). It demonstrated that non-Newtonian behavior was more pronounced at lower temperatures due to the reduction of η_0 values from 84.80, 68.30, 68.89, 15.41 to 7.00 Pa.s over a temperature range of 20^{o}C to 80^{o}C, respectively) although rate index (n values) still indicated the shear-thinning. Thus, this WD7 biopolymer can be simply degraded by high temperature, thus, the appropriate temperature was <60^{o}C. Temperature is related to the viscosity of biopolymer following Arrhenius relationship; the viscosity generally decreased with increasing temperature (Jampen *et al.*, 2000).

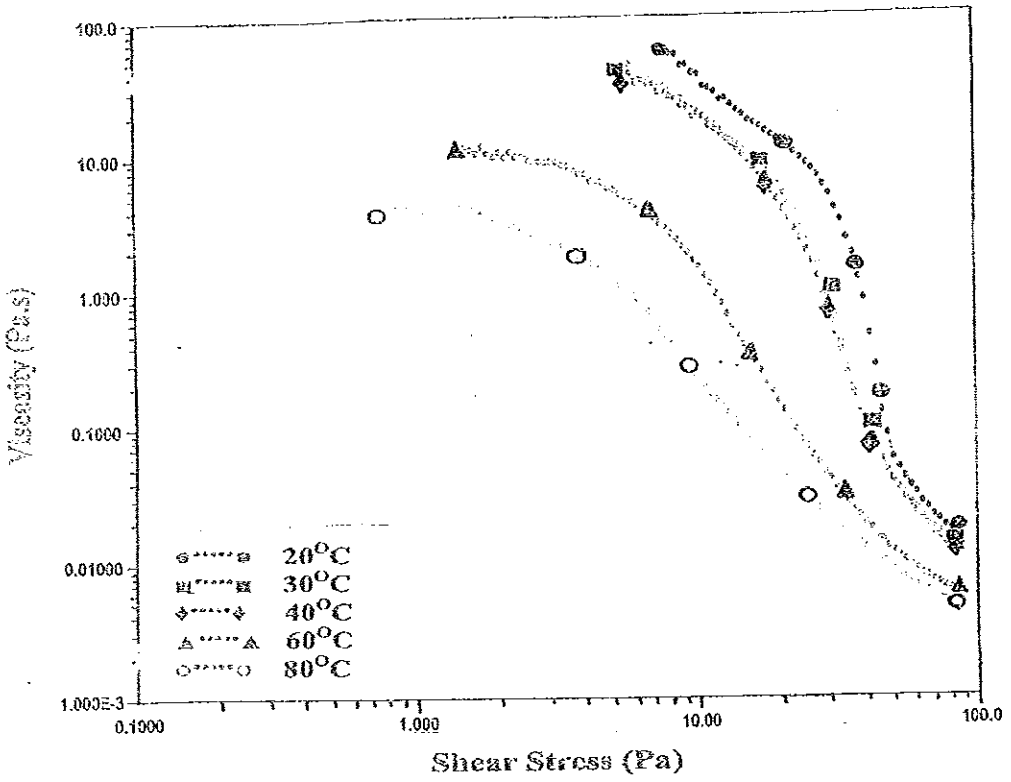


Figure 22 Effect of temperature on flow rheogram of WD7 biopolymer (2% w/v)

Effect of steam sterilization (121^{o}C, 20 min) and pasteurization (at 65^{o}C for 120 min) and boiling (105^{o}C for 120 min) temperatures/times on WD7 biopolymer solution (2.0% w/v) was studied. Viscosity of the sterilization sample decreased dramatically. The η_0 values decreased 15-20 folds from 52.6 to 5.46 Pa.s. This biopolymer was destroyed and simply degraded by high heat. Thus, the steam sterilization

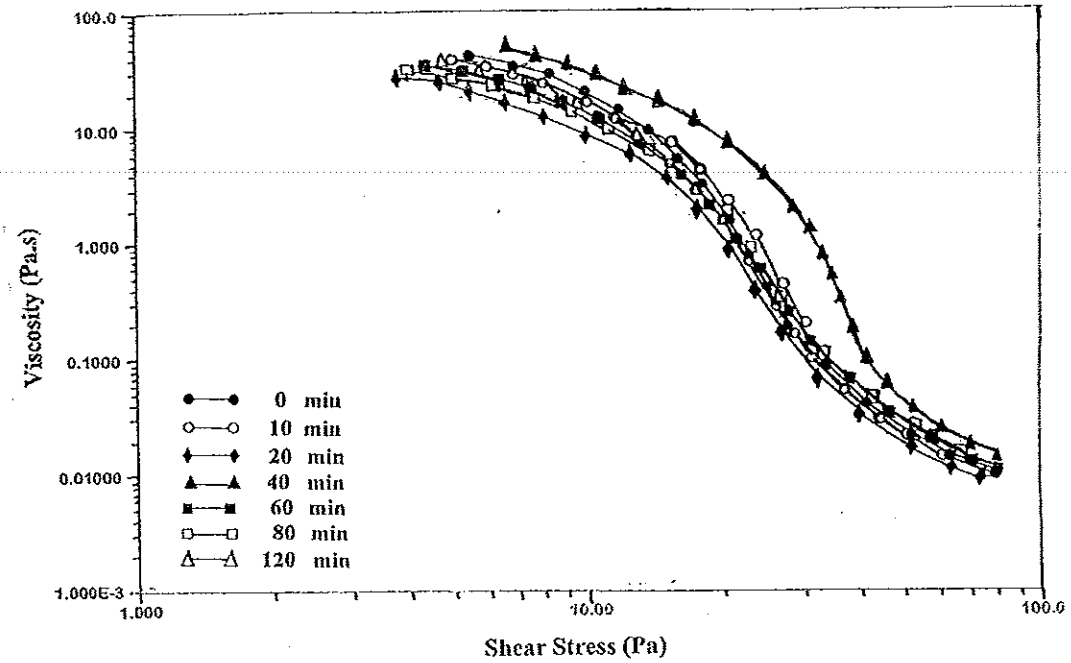
for 20 min was not the suitable sterilization of the biopolymer. In addition, the biopolymer powder was burnt and turned to brown liquid after autoclave.

Stability tests of biopolymer solution were carried out at 65°C in water bath and heating at 105°C for 120 min. Their rheograms and parameters were shown in Figure 23. At pasteurization temperature (Figure 23a), the η_0 values were not different during 120 min of testing. Flow profiles at 105°C showed much difference in viscosity which reduced dramatically from 71.51 to 0.05 Pa.s during 120 min test (Figure 23b). These indicated that this biopolymer solution was not stable at boiling temperature. The pasteurization was the suitable method to remove microbial contaminant of WD7 biopolymer.

Effects of various storage temperatures and times (stability test during storage) on equilibrium flow of WD7 biopolymer were analyzed. At the beginning, the effects of freezing (-20°C) and refrigeration (4°C) temperature/time on flow rheology of WD7 biopolymer solutions (2% w/v) were determined for 60 days (Figure 24). Flow profiles during storage at freezing (-20°C) from the first day to the day 60 did not show difference at various shear stresses (Figure 24a). The η_0 value of 77.6 Pa.s at the first day increased to 95.7 Pa.s at the last day by analysis with Cross model. The viscosity of samples increased due to the water evaporation during freeze-thaw before testing. Therefore, the entanglement and interaction of polymer chains might be reversible, consequently, the increase of viscosity during storage was found. The η_0 values had also the same trends by analyzing with Cross, Carreau and Williumson Models.

In addition, the n values less than 0.4 showed shear thinning behavior during 60 days storage. Flow rheograms of biopolymer solutions (2% w/v) incubated in refrigerator (at 4°C) for 60 days were shown in Figure 24b. The η_0 values increased slightly from 77.6 Pa.s (at the first day) to 99.9 Pa.s (at day 60). Flow profiles were still the similar patterns by analyzing with models. Shear thinning behavior of this biopolymer was found during 60 days storage. Thus, this biopolymer can be stored in freezing and refrigeration temperature at least 60 days without viscosity loss and no flow property changes.

(a)



(b)

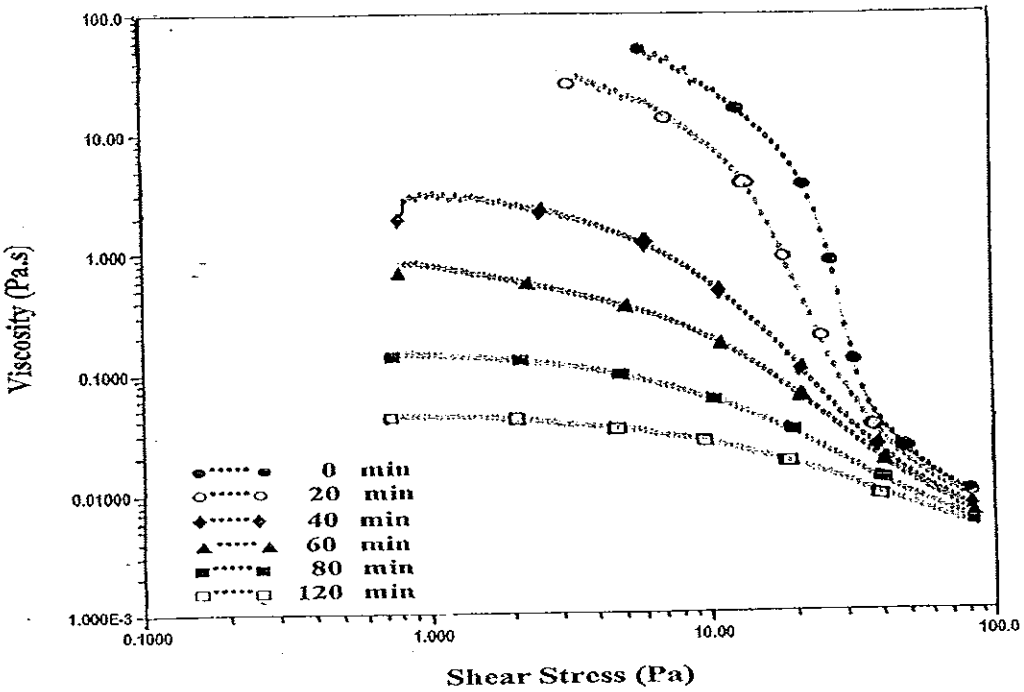
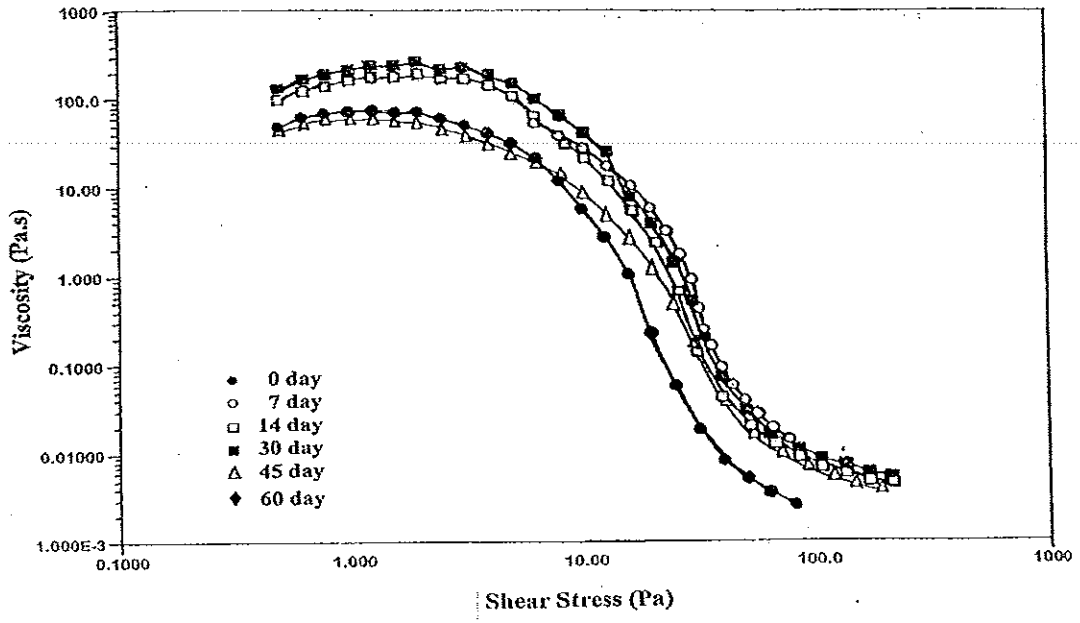


Figure 23 Effect of pasteurization temperature (65°C, a) and boiling temperature (105°C, b) on stability of WD7 biopolymer solution (2% w/v)

(a)



(b)

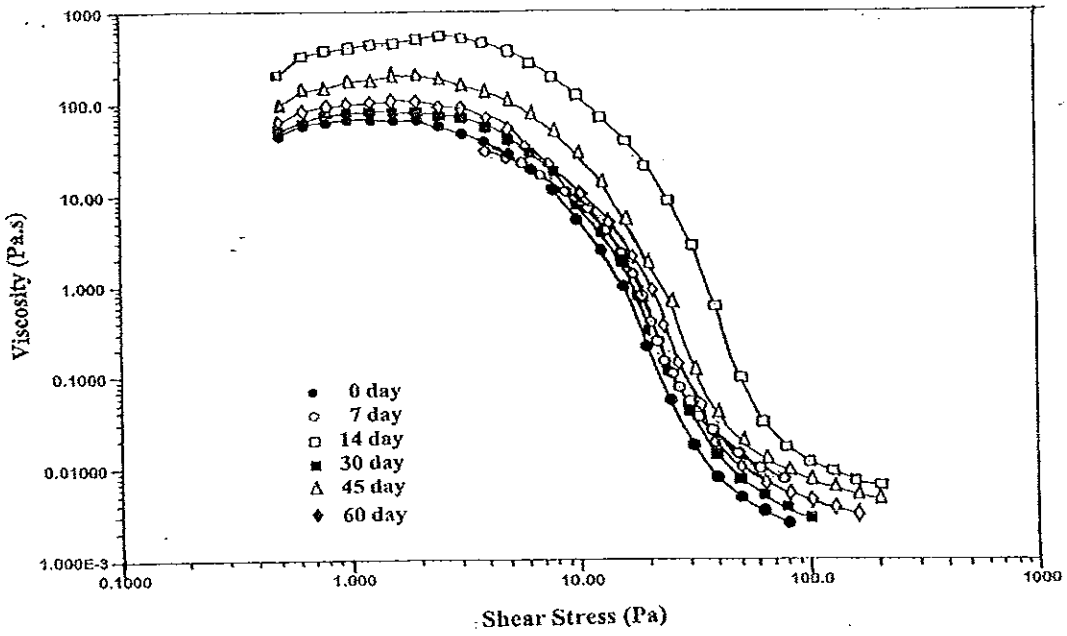


Figure 24 Stability tests of WD7 biopolymer (2% w/v) during storage in a freezer ($-20\,^{o}C$, a) and in refrigerator ($4\,^{o}C$, b) for 60 days

Stability test of biopolymer solutions (2% w/v) at room temperature (30°C) was carried out for 48 h in an incubator. Flow profiles of all testing (Figure 25) had no difference and their viscosity values decreased with the increased shear stress. Power law region was not different and shear thinning behaviors were still found in all profiles. The η_0 value was about 97.6 Pa.s at the beginning and decreased to 90.05 at the end of incubation time (48 h) with having bad smell by microbial degradation. Thus, this biopolymer solution can be tested at room temperature (30°C) without viscosity lost and no change of flow profiles within 18h and should not leave this biopolymer at room temperature (30°C) more than 18 h (Figure 25a).

To study the possible application to human and animal, effect of temperature on the properties of this 2% (w/v) biopolymer was tested at 37°C in an incubator for 48 h. From Figure 25b, all flow profiles with shear thinning behavior by Power Law model showed no difference. Thus, this biopolymer can be applied at body temperature (37°C) within 24h without loss of viscosity. However, further increase in the shear stress resulted in higher decrease in the viscosity. The η_0 values of biopolymer for complete swelling increased from 97.60 to 102.8 Pa.s during the first 24 h incubation, but afterward they decreased to 46.79 Pa.s at 48 h test.

4.4.4 Effect of Cation Variation on Enhancement of WD7 Biopolymer Gelation

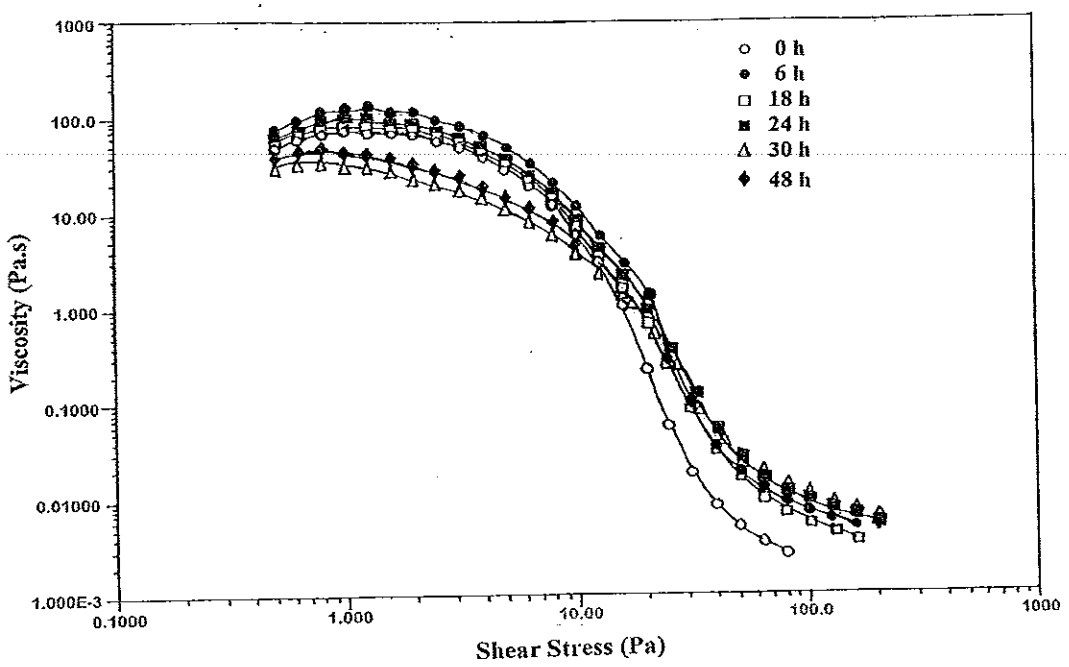
In this study, the effect of some monovalent and divalent cations on enhancement of WD7 biopolymer gelation (expressed as flow rheological behavior) was assessed.

4.4.4.1 Effect of Monovalent Cations on WD7 Biopolymer Gel

Effect of monovalent cations on flow property of WD7 biopolymer was studied in order to improve gelation. The partially purified biopolymer solution was mixed with monovalent and divalent metal salts in the ratio of 1:1 by weight. Their rheograms were presented in Figure 26.

Flow rheograms of biopolymer (0.5, 1.0, 1.5 and 2.0% w/v) in the presence of sodium chloride at low (0.10-0.50 M NaCl) and high (1.0-4.0 M NaCl) concentrations were shown in Figure 26a, b, c and d, respectively. The first Newtonian plateau of mixtures at low biopolymer concentrations w ere shorter than those at high concentrations and the η_0 values were lower with lower biopolymer concentrations because of lower viscosity. However, the shear thinning characters were found at all tested biopolymer

(a)



(b)

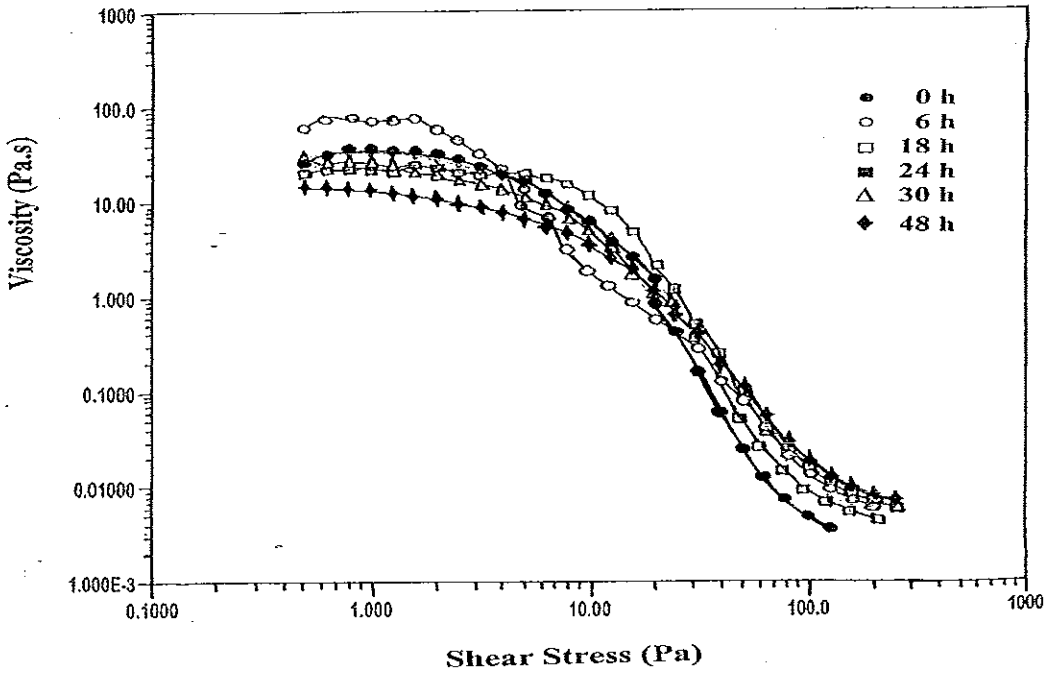
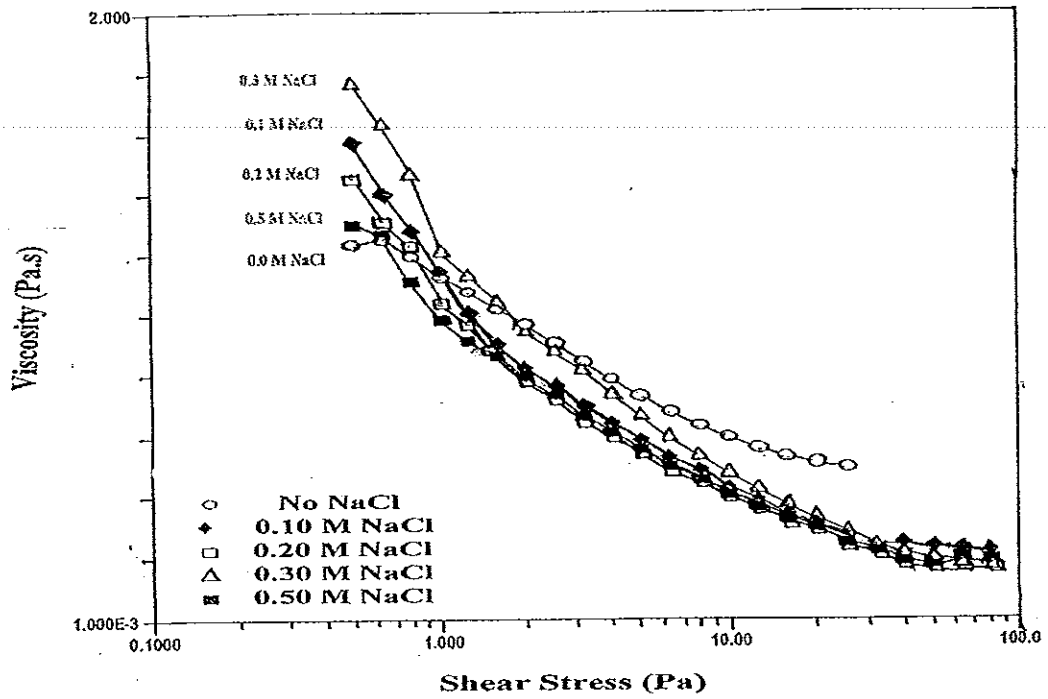


Figure 25 Stability tests of WD7 biopolymer (2% w/v) at room temperature (30°C, a) and body temperature (37°C, b) for 48 h

(a)



(b)

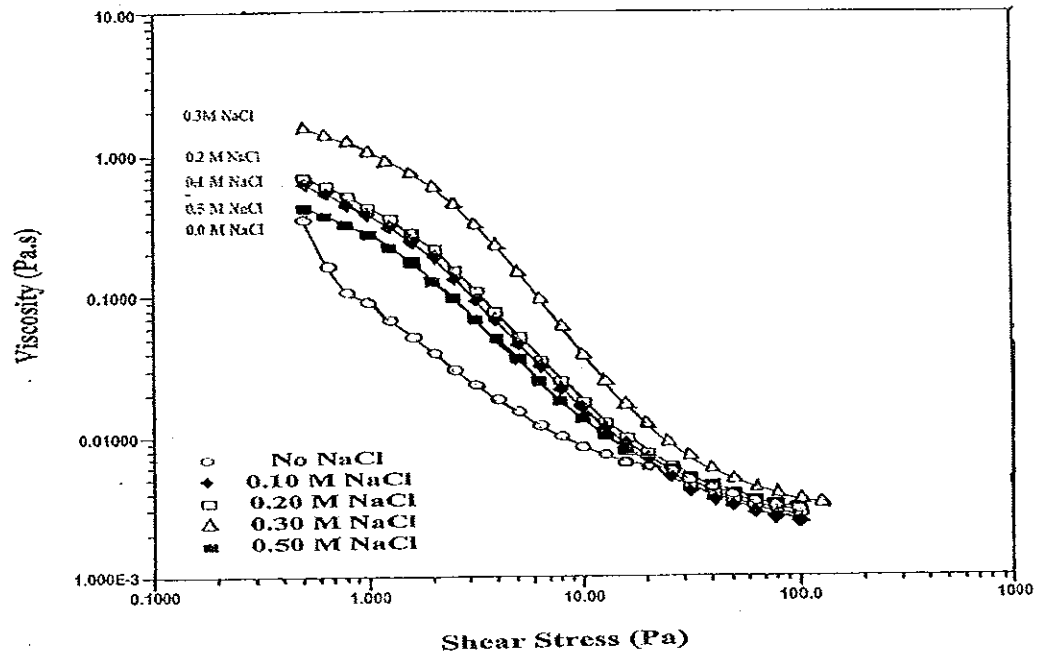
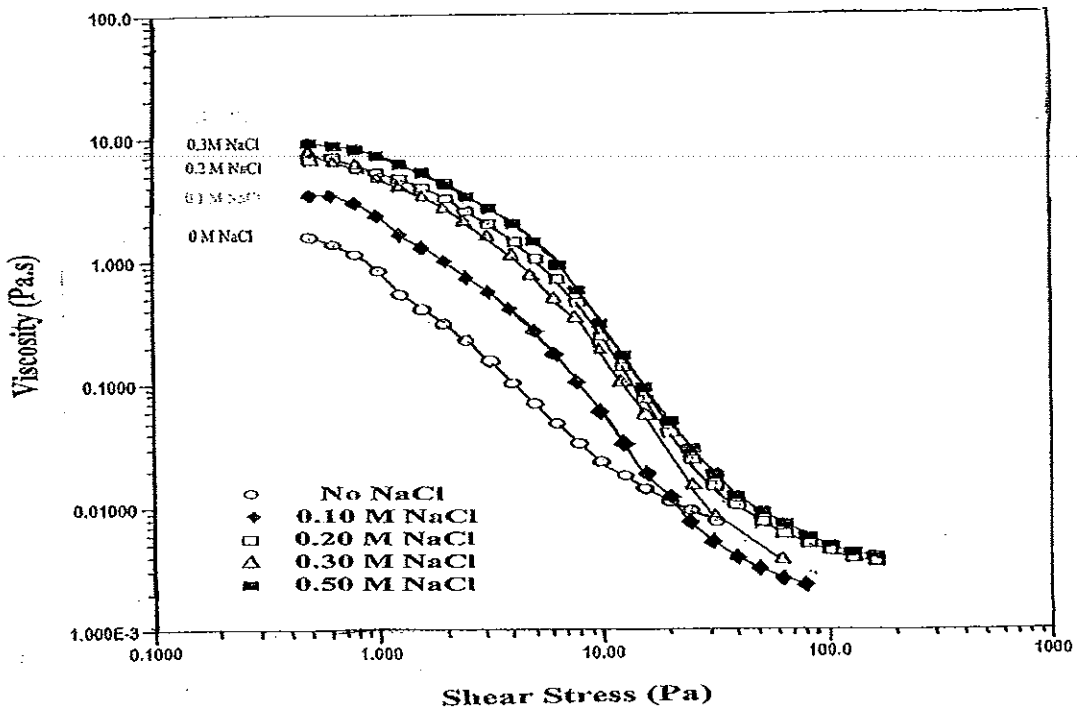


Figure 26 Flow rheograms of WD7 biopolymer at 0.10, 0.25, 1.0 and 2.0% (w/v) (a-d, respectively) in NaCl solutions at low concentrations (0.10 - 0.5M)

(c)



(d)

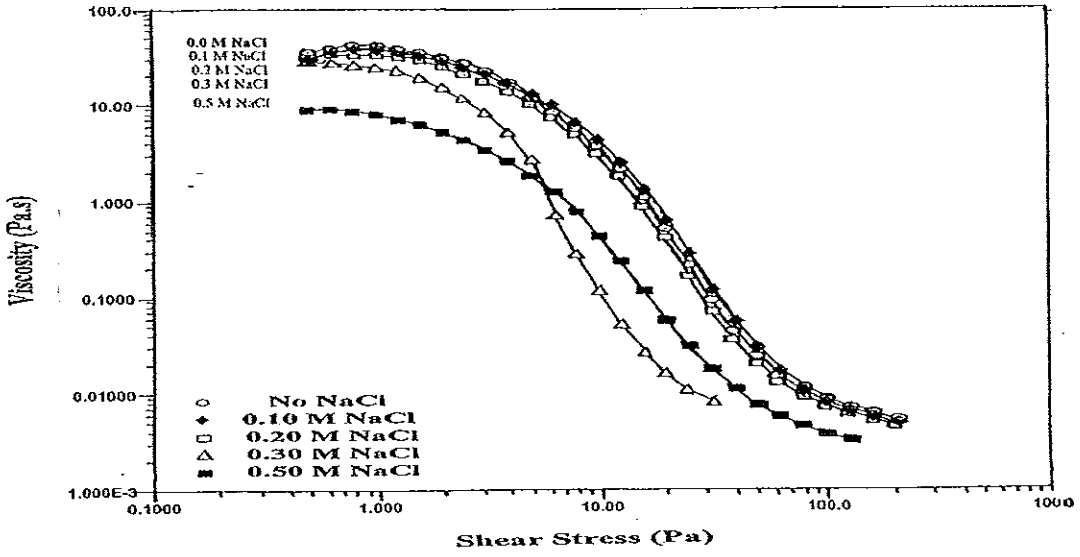


Figure 26 (cont.) Flow rheograms of WD7 biopolymer at 0.10, 0.25, 1.0 and 2.0% (w/v) (a-d, respectively) in NaCl solutions at low concentrations (0.10 - 0.50 M)

concentrations. At low NaCl concentration, the gel strength (viscosity) increased at 0.5-1.5% (w/v) biopolymer (Figure 26 a, b and c) and decreased at 2.0% (w/v) since the NaCl contents might be not enough to react with high contents of biopolymer.

The salt concentrations (0.1, 0.3 and 0.5M NaCl) gave the highest η_0 values for biopolymer concentration of 0.1, 0.25 and 1.0% (w/v), respectively. Nevertheless, their η_0 values of all treatments were higher than the samples without cation addition (control). These results showed that there was an optimal NaCl concentration that enhance or conserve the gel strength at optimal biopolymer concentration. The viscosity decreased at high biopolymer concentration (2% w/v) and high NaCl concentration comparison with sample without salt addition (as control, native biopolymer). Increase in Na^+ concentration resulted in the decrease of viscosity (Figure 26d).

Effect of monovalent salt (Na^+) on gelation could be conducted in two ways by increasing and decreasing gel strength of biopolymer. For karaya gum, (polyanion polysaccharide) (Silva *et al.*, 2003); at low Na^+ salt concentration, the increase of gel strength occurred by promotion of a non-specific shielding of the long range electrostatic repulsions between the highly negative charged polysaccharide chains, thus facilitating multivalent counter ion inter chain cross-linking. At higher salt concentration, the increasing of gel strength was due to augmenting the shielding of electrostatic repulsion, promoting the effective participation of monovalent cations in cross-link through polyanion/water/cation/water/cation/polyanion interaction (Silva *et at.*, 2003).. In contrast, the decrease of gel strength after salt (Na^+) treatment was presented by overshadowing effect of the original counter ion by screening the charge of the polymer and decreasing the cross-links between the polysaccharide chains with high salt concentration (Silva *et at.*, 2003). Thus, the increasing of gel strength of WD7 biopolymer at low concentration (<2% w/v) by the influence of Na^+ salt may be due to the diffusion of monovalent cation (Na^+) caused gelation.

At high salt concentrations (1-4 M NaCl) tested with the WD7 biopolymer at 2% (w/v), their rheograms were presented in Figure 27 (the flow profiles at 0.25M and 0.5M NaCl were also included for comparison). Most flow profiles clearly still had the shear thinning behavior. The viscosity decreased with addition of salt solutions. However, the increase to higher concentration of Na^+ caused higher decrease of gel strength. At 2%(w/v), the WD7 biopolymer appeared as gel, thus, no further increase of gel strength after Na^+ addition. The cause of sodium salt on the decrease of viscosity was due to the diffusing cations which could shield the electrostatic repulsion of biopolymer molecules (due

to interaction of nearby carboxylate groups) thereby disrupting the ordered network of biopolymer solution adopts. This would allow the chains to slide over each other more easily (i.e. flow), leading to a decrease in viscosity (Kobayashi *et al.*, 1994).

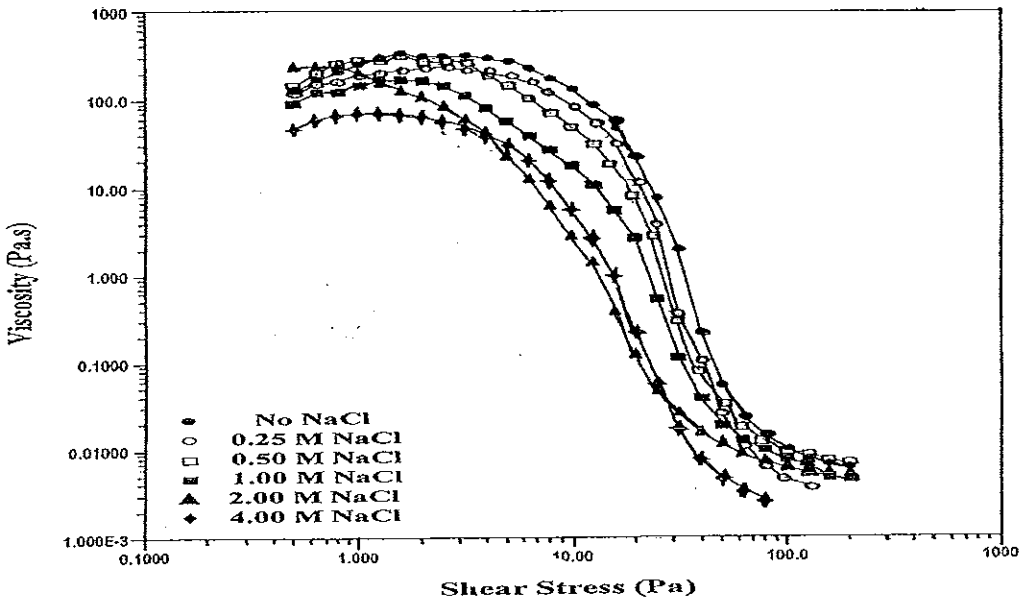


Figure 27 Flow rheogram of WD7 biopolymer (2 % w/v) tested in NaCl solutions at high concentrations (1.0, 2.0 and 4.0 M) (including to 0.25 M and 0.50 M for comparison)

In addition of Na^+, the effect of other monovalent cations on gelation was tested at low WD7 biopolymer concentration (0.25% w/v) in the ratio 1:1 by weight of mixtures. Flow rheogram (Figure 28) of most samples were plotted between the viscosity and shear rate, they showed shear thinning characters in all treatments. The η_0 values of WD7 biopolymer solution in water without cation (0.1698 Pa.s) as control sample was higher than the samples in chloride salt solutions with various monovalent cations of LiCl, NaCl, KCl and NH_4Cl (0.2423, 0.2146, 0.0186 and 0.0108 Pa.s, respectively). LiCl and NaCl ion caused the gelation while KCl had no effect on gelation and NH_4Cl caused the decrease of gel strength when compared to control sample. Li^+ and Na^+ gave 2 times higher viscosity than that of the control, while NH_4^+ decreased the viscosity about 6 and 10 times, respectively.

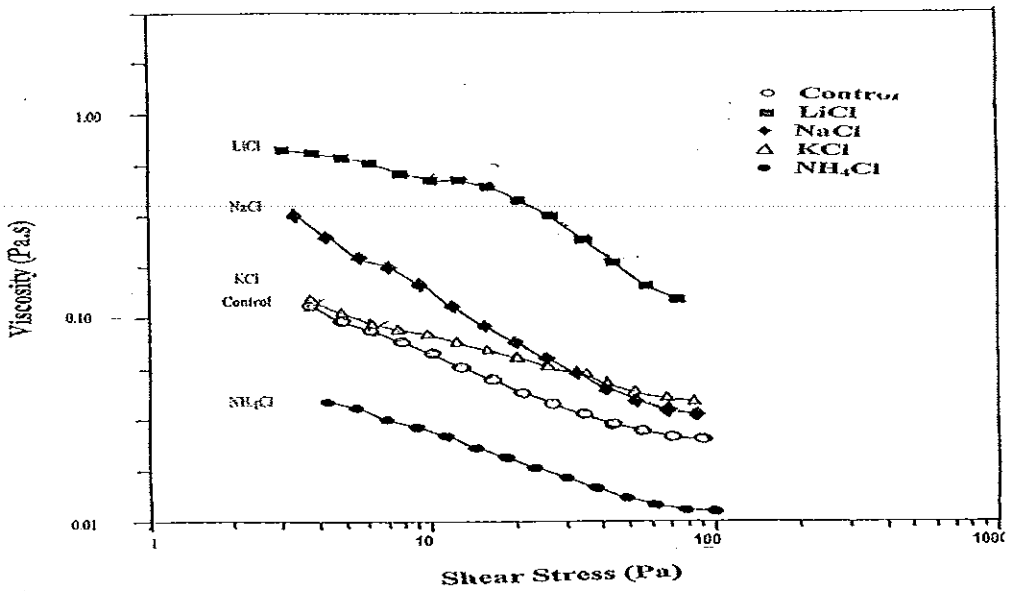


Figure 28 Flow rheogram of partially purified WD7 biopolymer (0.25 % w/v) with various monovalent cations at ratio 1:1 (w/w)

Two effects of monovalent salts on gellan gum, increase or decrease of viscosity were found by Oliveira *et al.*, (2001). Two forms of lateral aggregation of gellan helices in the presence of metal ions were reported. The addition of monovalent salts to native *Sterculia striata* polysaccharide increased the gel strength with more responses of KCl > NaCl >LiCl, respectively (Silva *et al.*, 2003). First type forms junction zones that lead to network formation and gelation, while the second type leads to the formation of isolated fibers of aggregated helices and inhibition of gelation. Some polyanion biopolymer carrying acidic groups interact with monovalent cation but more dissociation of the chain structure occurred, resulting in a decrease in viscosity. Monovalent cation can dissociate the chain-to-chain interaction in structure of polymer. The more cations present, the less chain-to-chain interaction occurs between the chain.

Affinity of polysaccharide for metal ions depended on the charge and ionic radius and ratio (Oliveira *et al.*, 2001). Addition of screened charge counter-ion attracted towards the poly-ion domain, made possible the contraction of the macromolecule (de Paula and Rodrigues, 1995). Therefore, the viscosity of polyelectrolyte solutions decreased in the presence of electrolytes. A strong ionic selectivity existed in the polysaccharide gel phase corresponding to $NH_4^+ > K^+ > Na^+ > Li^+$. Thus, the presence of higher charges of K^+ and NH_4^+ might induce the aggregation of double helices in polymer structure. This would allow the chains to slide over each other more easily (i.e. flow),

leading to a decrease in η_0 value. Furthermore, the structure of native biopolymer can support or protect the interactions of polysaccharide side chain and the strange ions.

The WD7 biopolymer is acid polysaccharides (polyanion) with uronic acid which may contain metal ions as neutralized cations. The nature and content of these cation constituents depend on the composition of culture medium, in this study, which composted of K_2HPO_4, KH_2PO_4, $MgSO_4 \cdot 7H_2O$ and NaCl salts. Major cation in medium was possible K^+ (92% of these ions). From X-ray diffraction chromatogram in (Figure 29), this crystalline of both crude and partially purified WD7 biopolymer powder contained the peaks of KH_2SO_4 and KH_2PO_4 that were the constituents of cultivation medium. Thus, the WD7 biopolymer may be assumed to be predominantly in a potassium salt. Although high charge of Na^+ possessed stronger affinity for chain binding sites of this biopolymer but its content was very low in medium composition. Thus, the addition of Li^+ and Na^+ with high charge gave the increase gelation, while addition KCl had no effect on gelation since having equal charge when compared to K^+ (COO^-) which had no net charge on polymer chain. In addition, the excess of added ion also induces the occurrence of repulsive forces that decreased the gel strength instead (Silva *et al.*, 2003).

4.4.4.2 Effect of Divalent Cation on WD7 Biopolymer Gel

Effect of various divalent metal salts on gelation enhancement of WD7 biopolymer was studied. Flow rheogram for WD7 biopolymer in presence of divalent metal ions were illustrated in Figure 30.

The η_0 values of biopolymer without cation (0.1698 Pa.s) and with $MnCl_2 \cdot 4H_2O$, $MgSO_4 \cdot H_2O$, $CaCl_2$, $CuSO_4 \cdot 5H_2O$, Na_2SO_4, and $(NH_4)_2SO_4$ were 0.234, 0.1805, 0.0440, 0.0337, 0.01763, 0.0169 and 0.01302 Pa.s, respectively. In this work, some divalent ions (Mn^{2+} and Mg^{2+}) enhanced the viscosity of WD7 biopolymer. But some divalent cations ($Ca^{2+}$and Cu^{2+}) decreased the viscosity due to degradation of biopolymer. The effect of $2(Na)^+$ and $2(NH_4)^+$ions were negative on this biopolymer gelation as was previously found in the monovalent ion test due to the effect of metal ions as $2(Na)^+$ and $(NH_4)^{2+}$ dissociated the chain interaction within biopolymer structure as found and described above.

Increasing gel strength by Mn^{2+} and Mg^{2+} resulted from increasing of cross-links due to the substitution of monovalent counter ion of biopolymer by divalent cation or the interaction of anionic polysaccharide with divalent cations may be direct

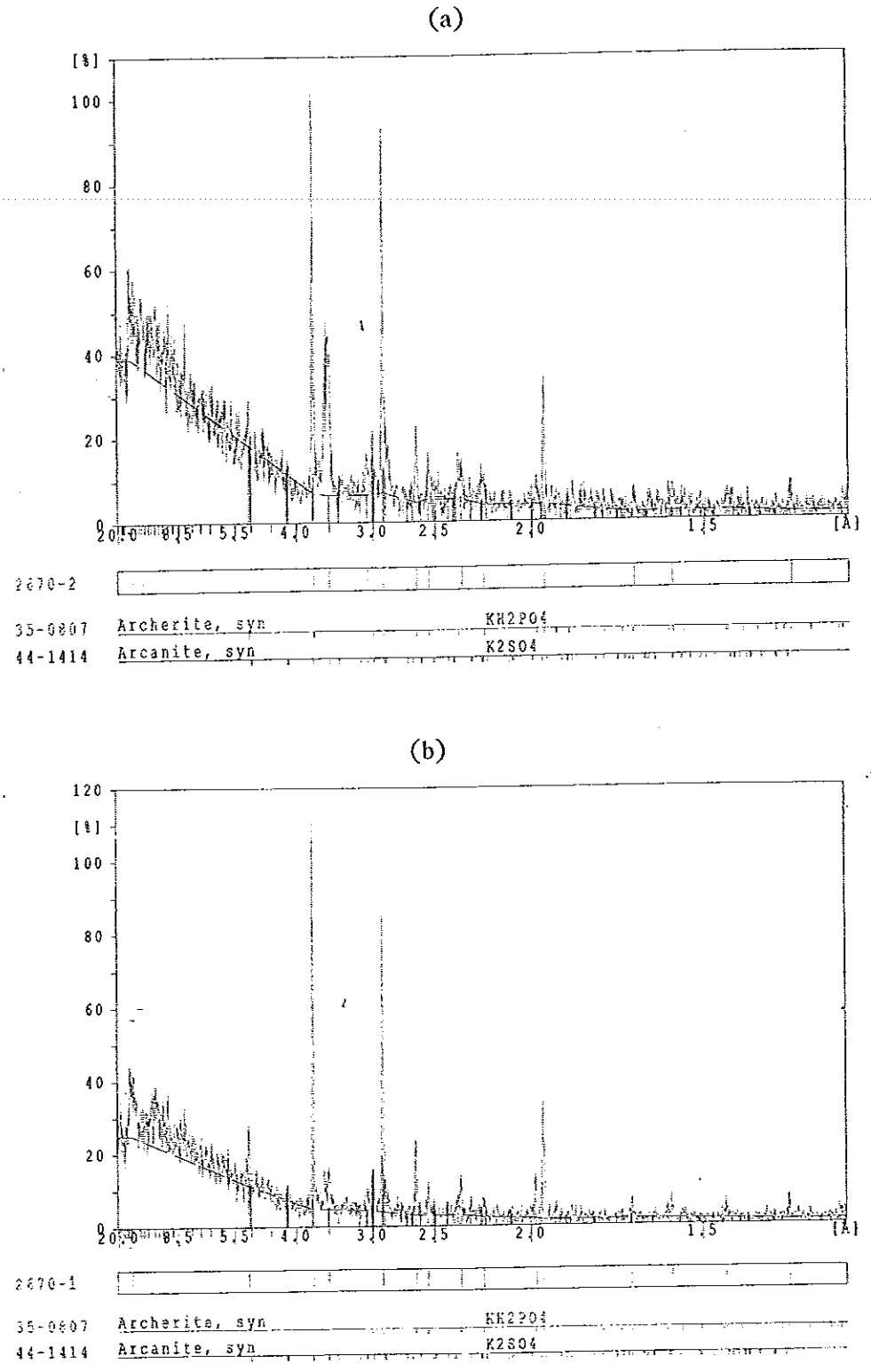


Figure 29 Chromatograms of X-ray diffraction of crude (a) and partially purified WD7 biopolymer (b)

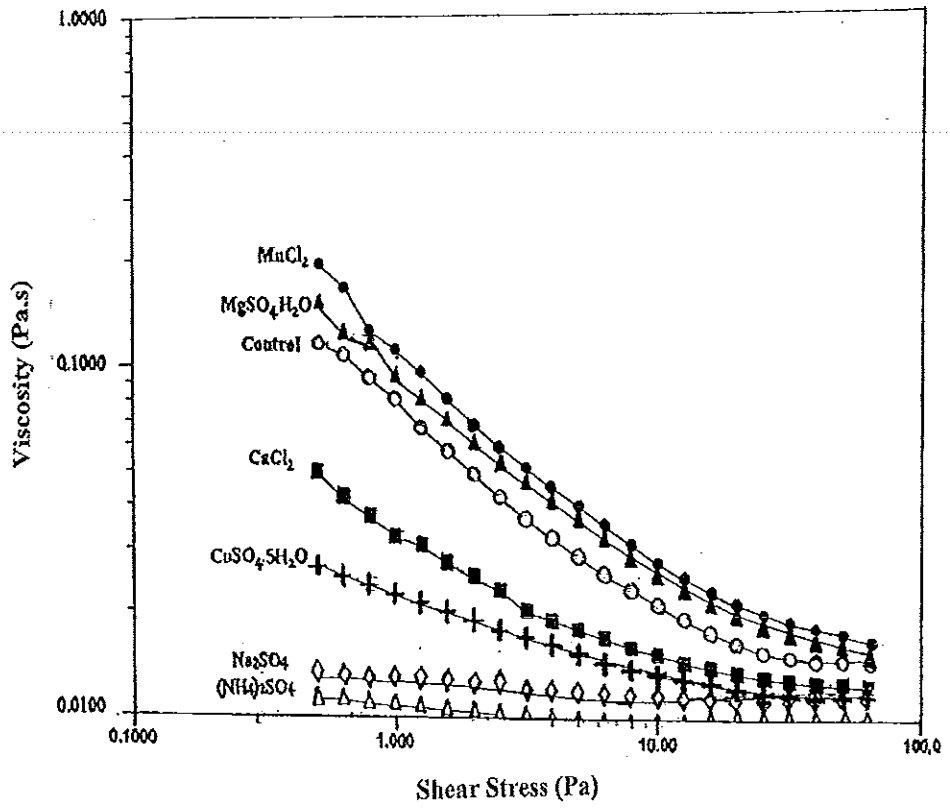


Figure 30 Flow rheogram of partially purified WD7 biopolymer (0.25 % w/v) with various divalent cations (ratio 1:1 w/w)

(polyanion- cation-polyanion). This involved the ionic bonds between the metal ions and carbohydrate groups belonging to adjacent chains (interchain crosslinks) (Silva *et al.*, 2003). A small number of these interactions could form a junction or a junction zone. In contrast, the decrease of gel strength by Ca^{2+}, Cu^{2+}, $2(Na)^{+}$ and $2(NH_4)^{+}$ ions may be due to the repulsive forces between positive net charges on the polymer chains, originating from the excess of divalent cations and formation of $M^{2+}(COO^{-})$ bonds, instead of the $M^{2+}(COO^{-})_2$, which characterize the cross-links (Silva *et al.*, 2003). The effects of divalent salts on increase and decrease of gelation were reported by many investigators (Silva *et al.*, 2003; Oliveira *et al.*, 2001 and de Paula *et al.*, 1995).

Affinity of WD7 biopolymer for Mn^{2+} and Mg^{2+} was found in this test. Polysaccharides can interact with different divalent salts by their own affinity (Oliveira *et al.*, 2001). Silva *et al.*, (2003) found that the gel strength of Na-*Sterculia striata* polysaccharide in the presence of divalent salt follows the order: $Mg^{2+} > Ca^{2+} > Sr^{2+} > Ba^{2+}$,

i.e. inversely proportional to the cation radius. The affinity of gum exudates of *Enterolobium conortisilliquum* for cations depends on the [cation]/[carboxylate] ratio. The limit ratio is 0.5, which corresponds to the stoichiometric requirement of the carboxylate groups in gels by divalent cations. T his gum exudates with Ca^{2+} are stronger and weaker than the corresponding gels with Mg^{2+} and Sr^{2+}, at a ratio lower and higher than 0.5, respectively (Oliveira *et al.*, 2001).

4.4.4.3 Effect of Metal Cations on Adhesive force of WD7 Biopolymer Gel

Effect of cation variations (both of mono- and disaccharide cations) on improvement of biopolymer gel properties were confirmed by adhesion tests (Figure 31). Most of monovalent metal ion had an effect on adhesive force response of this biopolymer mixture the same as the divalent metal ions. It can be concluded that addition of the cations had no effect on biopolymer viscosity, but may give a significant effect on the adhesive characters of this biopolymer.

The adhesive force of this biopolymer (0.25% w/v) containing variation of sodium chloride (0-4 M NaCl) were found to be about 116.0, 110.1, 105.9, 105.6 98.1 and 98.5 g at the test ranges from 0 to 4.0 M NaCl, respectively (Figure 32). The results showed that the adhesive forces decreased when NaCl concentration increased. Although the Na^{+} (of NaCl solution) decreased the adhesives force, the NaCl solution can be used for dissolution of this WD7 biopolymer (especially at low NaCl concentration) without flow rheological changed and viscosity lost that were benefit for application in medical or pharmaceutical areas.

Although two divalent salts, $MnCl_2$ and $MgCl_2$ increased the viscosity of WD7 biopolymer, $MgCl_2$ was used as additive solution in food, medical or pharmaceutical field and without toxicity more than using $MnCl_2$. Thus, the adhesive force of WD7 biopolymer (0.25% w/v) dissolved in $MgCl_2$ solution at various concentration of 0 - 4 M was measured. It was found that the adhesive force of mixture were about 115.8, 118.1, 120.5, 127.9, 131.0, and 134.0 g at the test ranged from 0 to 4.0 M $MgCl_2$, respectively (Figure 33). The results showed that the adhesive forces increased when $MgCl_2$ concentration increased. $MgCl_2$ solution was another interesting saline solution that increased the viscosity and adhesion of WD7 biopolymer that could be beneficial benefit for application in food, medical or pharmaceutical areas.

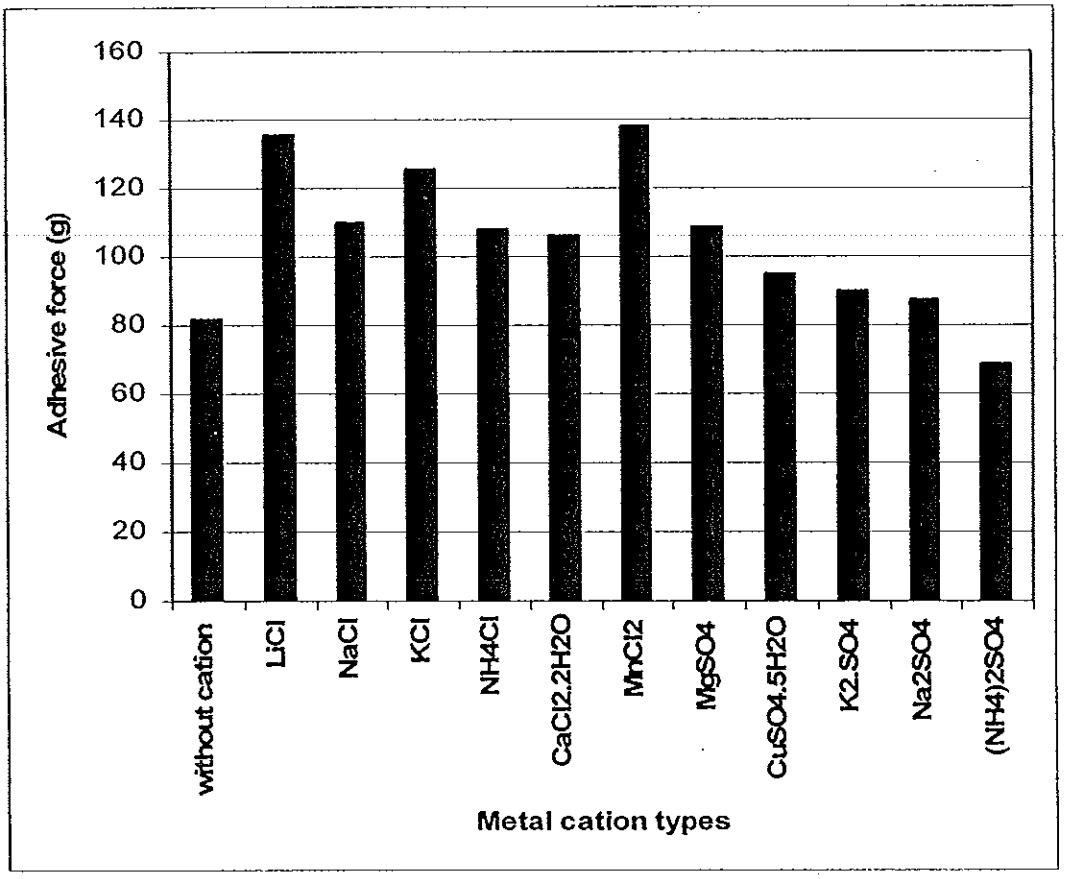


Figure 31 Adhesive forces of WD7 biopolymer (0.25 % w/v) with various cations

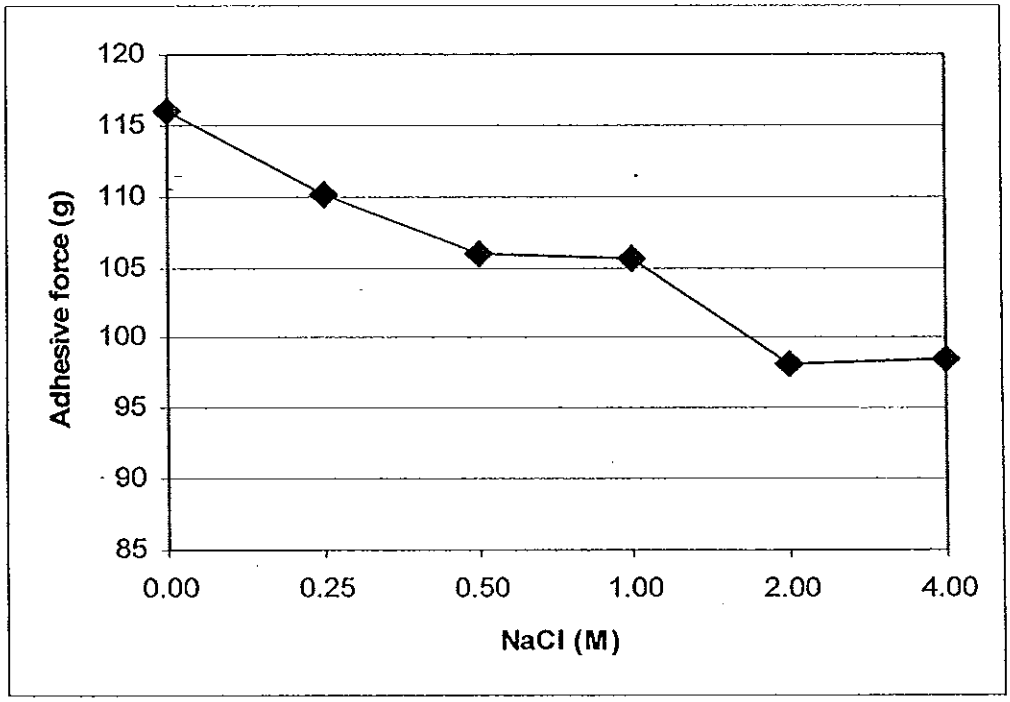


Figure 32 Adhesive forces of WD7 biopolymer (0.25% w/v) at various concentrations of NaCl

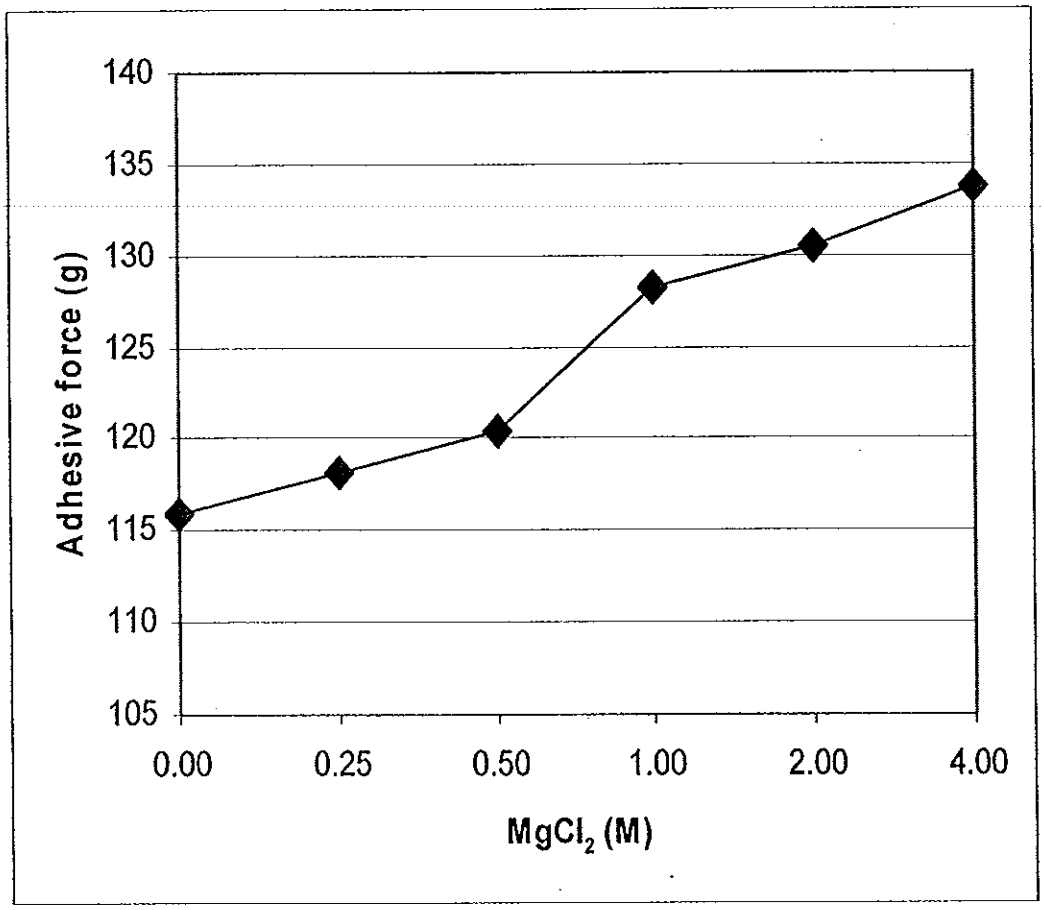


Figure 33 Adhesive forces of WD7 biopolymer (0.25% w/v) at various concentrations of $MgCl_2$

4.5 Some factors Affecting on Preparation of WD7 Film

In general, the evaporation method is the efficient film preparation due to it is convenience to peel the film from the mould and give the good film with a required aspect and properties. In this study, the films was formed from WD7 solution by this method and the effects of some parameters; biopolymer concentration, temperature of evaporation and relative humidity on WD7 biopolymer film appearance and the general properties such as color, thickness, softness, strength and elasticity (tensile strength and elongation at break) were evaluated as the preliminary study. In addition to film properties, such as the barrier ability to water permeability, water absorption, solubility and film swelling were also studied. The modification of film was studied by simple method with addition of some plasticizers for appropriate changes of property and appearance. The properties of natural and modified films were compared.

4.5.1 Effect of WD7 Biopolymer Concentration on Properties of WD7 Film

All films prepared from WD7 biopolymer (2-10% w/v, pH 6.5) in the incubator with air circulation at 50°C and 60%RH were thin. All films were not homogeneous with white to yellow colors. Thickness of WD7 biopolymer films after casting at 50°C and 60% RH increased (0.116, 0.345, 0.415, 0.517, 0.623 mm) when concentration increased (at 2, 4, 6, 8 and 10% w/v, respectively) as shown in Figure 34. Thin film was found at concentration of 2, 4 and 6% (w/v) and the films were easy to be torn and shrink after drying, especially at 2% (w/v). WD7 films at higher

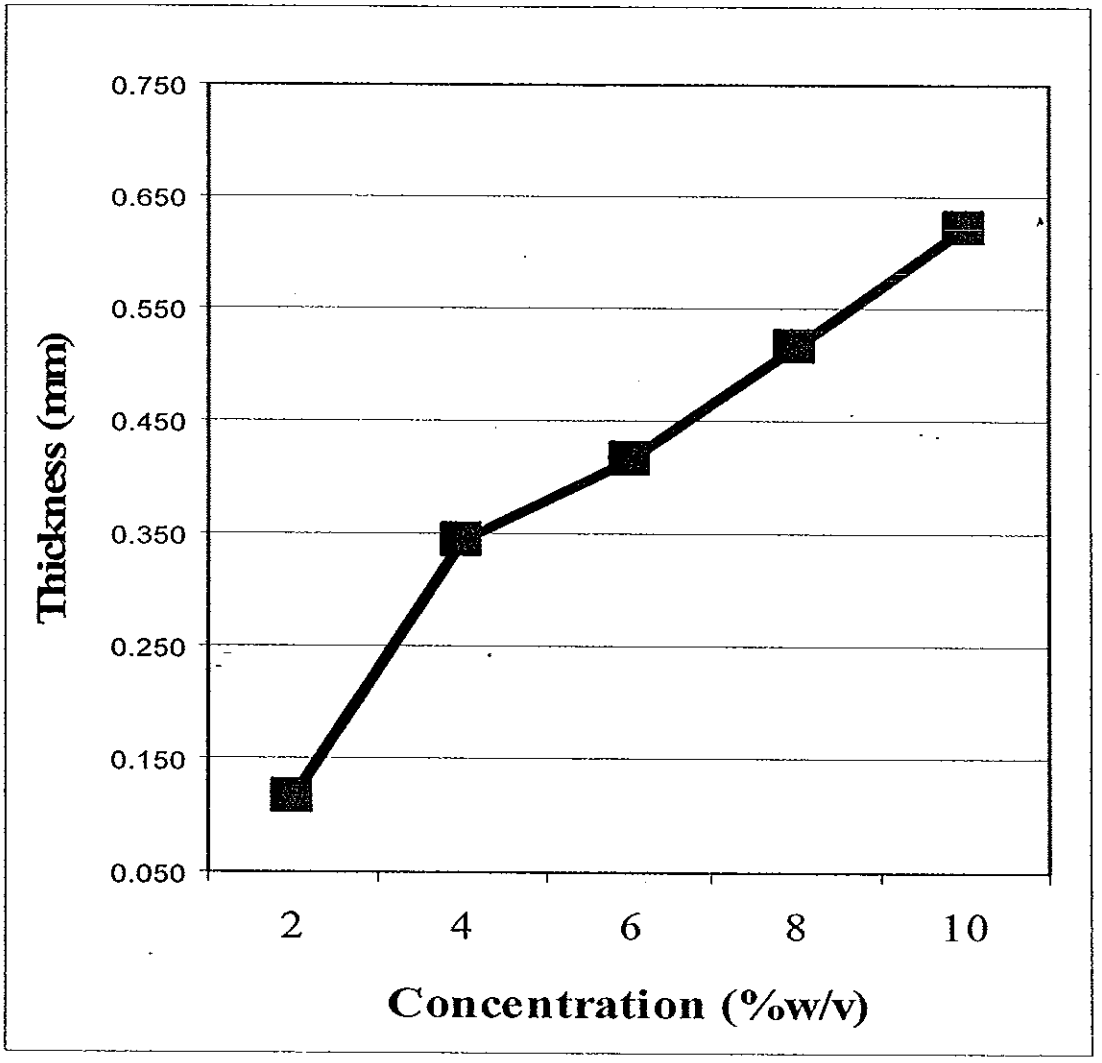


Figure 34 Effect of WD7 biopolymer concentrations (2-10% w/v) on film thickness at casting condition of 50°C and 60%RH

concentration of biopolymer (8 and 10% w/v) were thick and they were full sheets but had very rough surface. However, all tests were performed without drying time control due to the film forming time (evaporation time) of WD7 biopolymer solutions at high concentrations were longer than those at low concentrations. All WD7 biopolymer solutions in this test were dried and left until the highest concentration of polymer at 10% (w/v) were completely dried at day 3 of drying time while the drying time for the lowest concentration of polymer at 2% (w/v) was shortest about 18 h.

From this test, the WD7 biopolymer film at 2% (w/v) was chosen for further investigation to improve its appearance by optimization the casting condition at various temperatures and relative humidity. Effect of relative humidity (ranges over 30-60% RH at 50°C) and temperature (ranges over 30-80°C at 50%RH) on film appearance and properties was studied.

4.5.2 Effect of Relative Humidity and Temperature on Properties of WD7 Film

Effect of relative humidity (ranges over 30-60% RH) on film thickness prepared from WD7 biopolymer (2% w/v) at 50°C was shown in Figure 35a. Thickness of WD7 films did not change (0.095, 0.102, 0.103, and 0.103 mm) when the increase of relative humidity (at 30, 40, 50 and 60% RH), respectively. The incubation (casting or evaporating) times were 74, 72, 65 and 53 h at range over 30-60% RH, respectively.

At 2% (w/v) of WD7 biopolymer solution, the film should be cast at 50% RH within shorter time when compared with those at higher concentrations. Effect of temperature (30-70°C) on film thickness prepared from WD7 biopolymer (2% w/v), at 50%RH was shown in Figure 35b. Thickness of WD7 biopolymer films were 0.056, 0.086, 0.116, 0.106 and 0.062 mm at testing temperature of 30, 40, 50, 60 and 70°C, respectively. Film thickness was highest at 50°C and reduced at high temperature (>60°C). Thus, the condition for film formation of 2% (w/v) of WD7 biopolymer solution in the incubator should be controlled at 50°C and 50%RH until dry to achieve the required thickness about 0.11 mm.

4.5.3 Effect of Plasticizers on Characteristic and Properties of WD7 Film

In this study, WD7 biopolymer solution (at 2% w/v) was added with plasticizer [sorbitol, sucrose, glycerol, polyethyleneglycol400 (PEG400), polypropylene-glycol400 (PPG400), tween 80 and glycerin] on various ratios of biopolymer to plasticizers

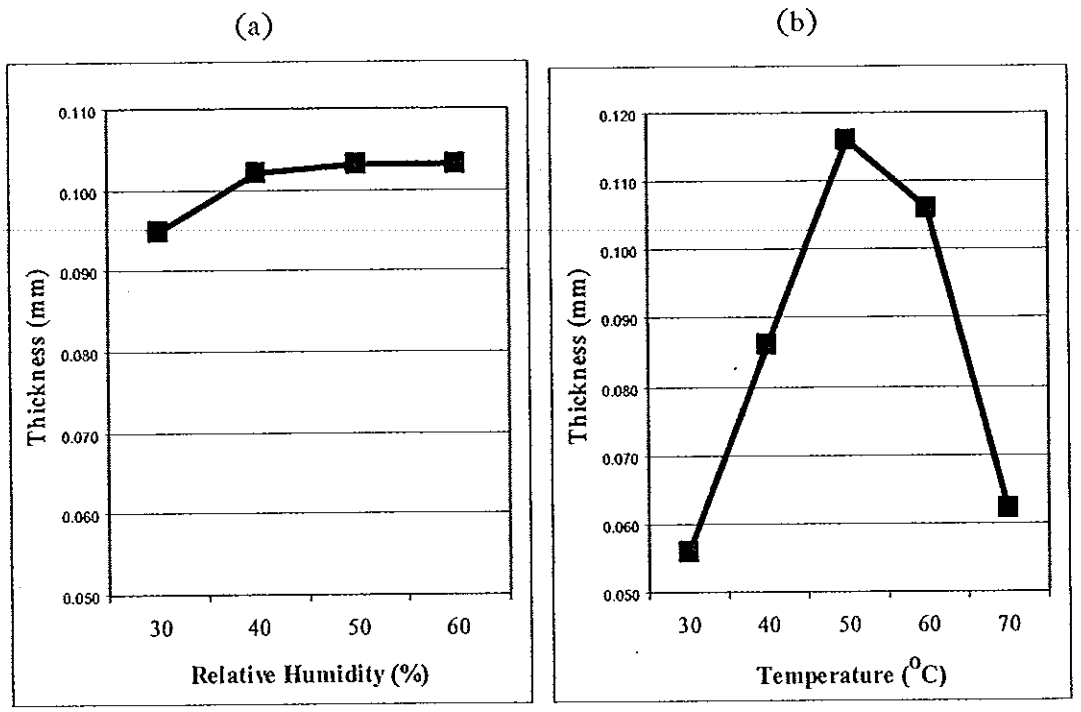


Figure 35 Effect of (a) relative humidity (30-60%RH) and (b) temperature (30-80 °C) on film thickness prepared from WD7 biopolymer (2% w/v) at casting condition of 50°C and 50%RH

(1:0.25, 1:0.50 and 1:0.75 or 25, 50 and 75% by weight, respectively) at optimal condition for casting of natural biopolymer. By nature, all plasticizers used in this work could not form the films by themselves. The WD7 biopolymer blended with PPG400, tween 80 and glycerin could not form the film, which may be due to their high molecular weights and/or unsuitable concentration ranges.

After drying at 50°C and 50% RH, the soft films were formed. Sorbitol, glycerol, gucrose and PEG400 were the effective plasticizers for forming the soft films found at the ratios (1:0.25 and 1:0.5 or 25 and 50% w/w). No films were formed at higher concentration of plasticizers (1:0.75 or 75% w/w). Only soft film was obtained with PEG at various concentrations that were softer than using other plasticizers. WD7 films obtained were yellow to brown color but was pale color at low concentrations of WD7 biopolymer. The difference of ΔL^*, Δa^* and Δb^* values as color parameters of the native film samples prepared from WD7 biopolymer solution (at 2-10% w/v) were shown in Table 12. The film color was more yellowish (or brown) when biopolymer concentration increased with high minus values of ΔL^*, increasing of a^*value, total color difference (ΔE^*_{ab}), and chroma (C^*). In addition, the WD7 film was more rough (not glossy) at

higher biopolymer concentrations due to the reduction of color disperse (decrease of hue angle, h*). The concentration of WD7 biopolymer solutions affected on film color possibly because the relation of dried solids by surface of films was maintained constant after drying.

Table 12 Comparison of color parameters of WD7 films (at 2% w/v) and its modified films with some plasticizers prepared at 50%RH and 50°C

Film samples	Parameters of film color					
	ΔL*	Δa*	Δb*	ΔE*ab	C*	h*
2% WD7 Biopolymer	-6.56	6.37	26.97	811.04	26.00	84.70
4% WD7 Biopolymer	-13.03	5.01	26.97	922.37	30.62	78.87
6% WD7 Biopolymer	-8.71	10.12	26.97	905.62	30.35	81.90
8% WD7 Biopolymer	-11.88	16.18	26.97	1130.40	35.17	76.28
10% WD7 Biopolymer	-30.53	14.89	26.97	1881.42	39.48	66.92
2% WD7 Biopolymer + Glycerol (1:0.25)	-12.35	2.46	26.97	885.83	37.71	80.50
2% WD7 Biopolymer + Glycerol (1:0.50)	-9.36	1.16	26.97	816.24	35.73	85.33
2% WD7 Biopolymer + Sucrose (1:0.25)	-12.62	1.16	26.97	888.09	40.13	79.92
2% WD7 Biopolymer + Sucrose (1:0.50)	-5.90	8.31	26.97	831.19	31.06	88.13
2% WD7 Biopolymer + Sorbitol (1:0.25)	-9.94	2.75	26.97	833.79	31.45	83.34
2% WD7 Biopolymer + Sorbitol (1:0.50)	-8.80	4.66	26.97	826.57	30.07	83.79
2% WD7 Biopolymer + PEG (1:0.25)	1.43	1.06	26.97	730.56	17.18	91.96
2% WD7 Biopolymer + PEG (1:0.50)	-8.08	1.16	26.97	794.01	29.66	83.07
2% WD7 Biopolymer + PEG (1:0.75)	-45.10	7.59	26.97	2819.00	14.69	83.32

All WD7 modified films with glycerol, sorbitol and sucrose were yellow, while it was white color when blended with PEG (Table 12). All coating films were not uniform. All blended films were softer as the concentration of plasticizer increased in

comparison to original film. But the surface texture of original film was more rough and heterogeneity than the blended films. The color testing of modified films comparison with native film was shown in Table 12. At the same tested concentration of WD7 biopolymer (2% w/v), all blend soft films was thicker and also had the same color with no difference in brightness (ΔL^*), total color (ΔE^*ab), color intensity (C^*) and light reflect (h^*) values when compared to the original one. While all coating films were more opacity with lower value of color intensity (C^*) but higher value of light reflect (h^*) than the soft film. Especially, WD7 film blended with PEG showed more pale color than the origin film with low values of brightness (ΔL^*), total color(ΔE^*ab), color intensity (C^*) but high value of light reflect (h^*). Addition of PEG gave soft and clear film, but different surface texture on both sides (glossy and rough) of film with very oily. The thickness of WD7 film (at 2% w/v as control) and its modified films that were plasticized at ratio of 1:0.5 with sucrose, glycerol, sorbitol and PEG was 0.105, 0.134, 0.147, 0.118 and 0.158 mm, respectively as shown in Figure 36. Most modified films were higher than the control.

Effect of concentrations, casting conditions (relative humidity and temperature) on the film strength (as tensile strength value) and elasticity or stretch capability (as elongation at brake value) of WD7 film (at 2% w/v) was reported in Table 13. In addition, only the WD7 soft films with some plasticizers (sorbitol, sucrose, glycerol and PEG at 1:0.25 and 1:0.5) were chosen for testing of these mechanical properties compared to the native film. Characteristics of WD7 biopolymer (at 2% w/v) with all plasticizers were softer than the native one. The tensile strength values (0.238, 0.772, 1.358, 1.419 and 1.526 Pa) and the elongation at break (4.636, 4.685, 1.380, 1.062 and 0.541 %) were obtained with the increased concentrations of WD7 biopolymer solutions (at 2, 4, 6, 8 and 10% w/v, respectively). Thus the highest values of tensile strength (1.526 Pa) and elongation at brake (4.685%) were found at 10% and 4% native WD7 film, respectively, by formation at optimal condition; 50^{o}C and 50%RH.

All modified films with addition of glycerol, sucrose, sorbitol and PEG400 (at the ratio of 1:0.5) gave the higher elongation at break (43.55, 31.80, 25.78 and 30.60 %, respectively) than the native WD7 films at 2% w/v (4.636%). Modified films had higher tensile strengths (0.477, 0.247, and 0.473 Pa for glycerol, sorbitol and PEG400 respectively), while sucrose did not increase the tensile strengths (0.217 Pa). All plasticizers increased the elasticity of film with highest value of elongation at break by glycerol addition. Glycerol and PEG400 can improve the tensile strength while only glycerol gave the highest elongation at break value.

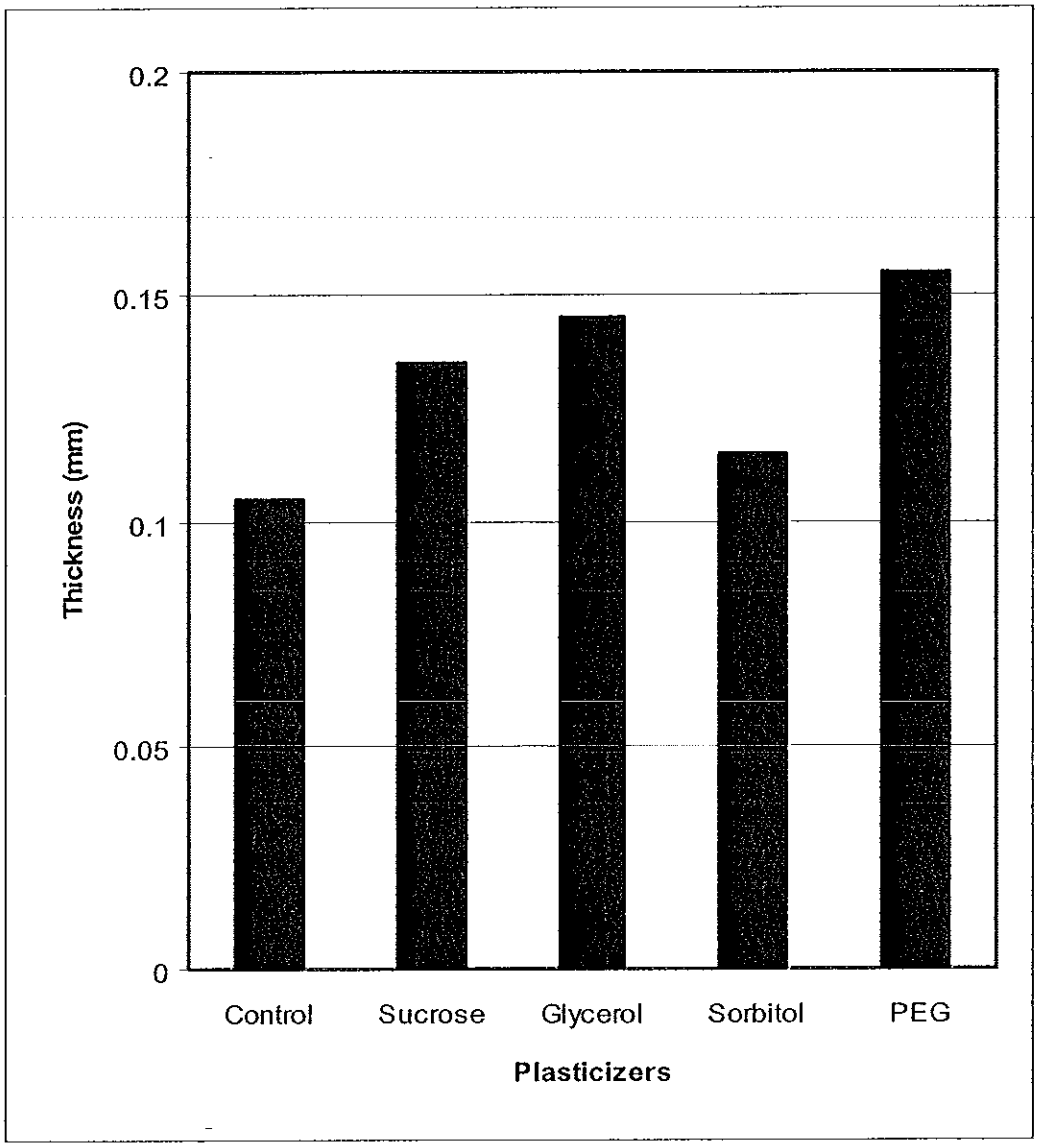


Figure 36 Effect of various plasticizers on thickness of modified WD7 films (at 2% w/v) with various plasticizers at ratio of 1.0:0.5 and at optimal casting condition, 50°C and 50% RH

A plasticizing agent must be compatible with the film-forming polymer and be permanently presented within the solvent-polymer system under the conditions used. Formation of the whole film system (polymer, solvent, plasticizer, and other additives) had a direct effect on the nature and characteristics of the films produced. As a result, the polymer and the plasticizer must not only be compatible, but must also have similar solubility in the solvent used. A soluble plasticizer will be generally be sought for the development of

soluble coating and an insoluble plasticizer (or dispersible) for an insoluble coatings or for a slow solubilization (Guilbert, 1986).

Table 13 Tensile strength and elongation at break of native WD7 films prepared from WD7 biopolymer solution at various concentrations prepared at 50°C and 50% RH

Concentration of WD7 biopolymer solution	Tensile strength (kN/m or Pa)	Elongation at break (%)
2%	0.238	4.636
4%	0.772	4.685
6%	1.358	1.380
8%	1.419	1.062
10%	1.526	0.541
glycerol	0.477	43.550
sucrose	0.217	31.800
sorbitol	0.247	25.780
PEG400	0.473	30.600

Plasticizer was also of prime importance since this had the influence on the physical and mechanical stability of the films. The plasticizer should not be volatile (or only very slightly volatile) and its degree or retention by the films should be high. Other properties, such as its chemical stability, hygroscopicity, color and flavour and so on, were also more or less important depending on the type of films under consideration. In addition, the concentration of plasticizer necessarily varied from 10-60% (dry basis) according to the nature and type of films and the method of application. The efficiency, stability, compatibility and permanence of a plasticizing agent could be evaluated by various semi-empirical tests (Guilbert and Biquet, 1996).

The native WD7 film and all modified films were hydrophilic and could dissolve immediately in water, even in acidic (1M HCl) and basic solutions (1M NaOH solution) at tested pH ranges of 2, 4, 6, 8 and 10. They were also dissolved immediately after soaking in Tris-buffer and phosphate buffer solution at pH 6 and 6.5, respectively and also in 70% and 95% ethanol. Thus, this WD7 films were water soluble.

In this study, the water vapor permeability (WVP) of the native WD7 films and modified films with sucrose, glycerol, sorbitol and PEG400 were 5.53, 5.67, 4.56, 4.23 and 5.13

kPa, respectively at ratio of 1:0.5 (Table 14). The WVP of WD7 biopolymer with sucrose was highest, while the native WD7 biopolymer was higher than the WD7 biopolymer with PEG400, glycerol and sorbitol, respectively.

Table 14 Average values of water vapor permeability of WD7 films and modified films with plasticizers tested at 30°C and 60%RH

Samples	Water vapor permeability ($g\ mm/m^2\ d\ kPa$)
2% WD7 biopolymer	5.53
2% WD7 biopolymer with 1% sucrose	5.67
2% WD7 biopolymer with 1% glycerol	4.56
2% WD7 biopolymer with 1% sorbitol	4.23
2% WD7 biopolymer with 1% PEG400	5.13

4.6 Some Factors Affecting on Characteristic and Properties of WD7 Sponge

WD7 sponges were prepared from the WD7 biopolymer solution at the concentration ranges of 1 - 10% (w/v). All WD7 sponges had flexible texture with considerable bending possible without fracture. Most WD7 sponges had brown color due to the color of crude biopolymer material. They were very soft and can be flexible. However, their surface was not smooth and some parts looked like fiber.

Appearance of WD7 sponges was not fluffy because of three main reasons. First, the treatments were tested at too low concentration ranges (1-10% w/v). Secondly, the volume of WD7 biopolymer solution added into the mould was inappropriate (too low) (25 ml/mould) of WD7 biopolymer solution. Finally, the plastic mould was too shallow that could put the WD7 biopolymer solution with limiting volume and initial thickness before freeze drying. These caused the thickness of sponges to be too low (1 cm) after forming process that made the square of WD7 sponges. However, the sponges prepared by this method were still used throughout this study on basis of economic and save the process times. As well known the microbial biopolymer is very high valuable and cost because it was difficult to produce and recover at very low mass although scale-up for production was successful. Thus it should be most benefit if its application or testing was done at lowest concentration as possible. Consequently, the sponge preparation in this study was carried out at low volume and mass production. In addition, sponge was prepared within a short time in

freeze-dry for 24-36 h depending on concentration ranges used. However, increase of higher concentration of WD7 biopolymer solution resulted in the higher increase of the thickness of WD7 sponge, while the softness and pliability were still found.

4.6.1 Effect of Biopolymer Concentration on Characteristic and Properties of WD7 Sponge

Concentration had influence on the appearance and property of native WD7 sponges, as found in other polysaccharides such as sponges of arabinogalactan (Shapiro and Cohen, 1997), arabinogalactan (Ehrenfreund-Kleinman, 2002), chitosan and complex polysaccharides (Lai *et al.*, 2003). Table 15 showed the effect of various WD7 biopolymer concentrations on the appearance and mechanical properties of WD7 sponges. All WD7 sponges were soft in texture and looked like the fiber. Size of the WD7 sponges depended on the concentration but not much different between each treatment. However, the size of all sponges was smaller than size of mould (55 x 55 mm). At low concentration (1, 2 and 4 % w/v), the WD7 sponges were not in full size since the biopolymer mass (concentration) was not appropriate and caused the sponge shrink after freeze drying. While it was full sized if sponged was prepared at high concentration (6, 8 and 10 % w/v).

Table 15 Effect of various WD7 biopolymer concentrations on appearance and some mechanical properties of WD7 sponges prepared by freeze drying method

Concentration of WD7 biopolymer (%w/v)	Sponge size (mm x mm)	Thickness (mm)	Average pore size (μm)
1	30.0 x 30.0	5.6	55
2	37.0 x 38.0	18.0	25
4	37.5 x 37.0	54.0	20
6	38.0 x 40.0	63.0	15
8	40.0 x 40.0	83.5	10
10	42.0 x 44.0	93.0	5

In addition, the concentration of WD7 biopolymer solution had also influence on thickness and strength of WD7 sponge. The thickness of WD7 sponges were 0.56, 1.80, 5.40, 6.30 8.35 and 9.30 mm at WD7 biopolymer concentration of 1, 2, 4, 6, 8 and 10% (w/v), respectively. However, they were lower than the initial thickness of solution before freeze dry. Microstructure of WD7 sponge was presented in terms of pore size, the

number of pores and their distribution, the distance between the pores (such as wall thickness), was highly dependent on the concentration of WD7 biopolymer solution used in the preparation of the sponges. The average pore sizes were 55, 25, 20, 15, 10 and 5 μm at concentration of 1, 2, 4, 6, 8 and 10% (w/v), respectively. This indicated that the smaller pore sizes of WD7 sponge samples were obtained at higher WD7 biopolymer concentration.

Figure 37 showed microstructure of sponges for example prepared from 10% w/v (a and b) and 2% w/v WD7 biopolymer solution (c and d) with magnitudes of 400 times and 3,000 times, respectively. They had the high porosity with good interconnected pore structure. It appeared that sponge looked like fiber character was obtained at 10% (w/v), while it looked like the honeycomb with uniform pore size and distribution at 2% (w/v) of WD7 sponges.

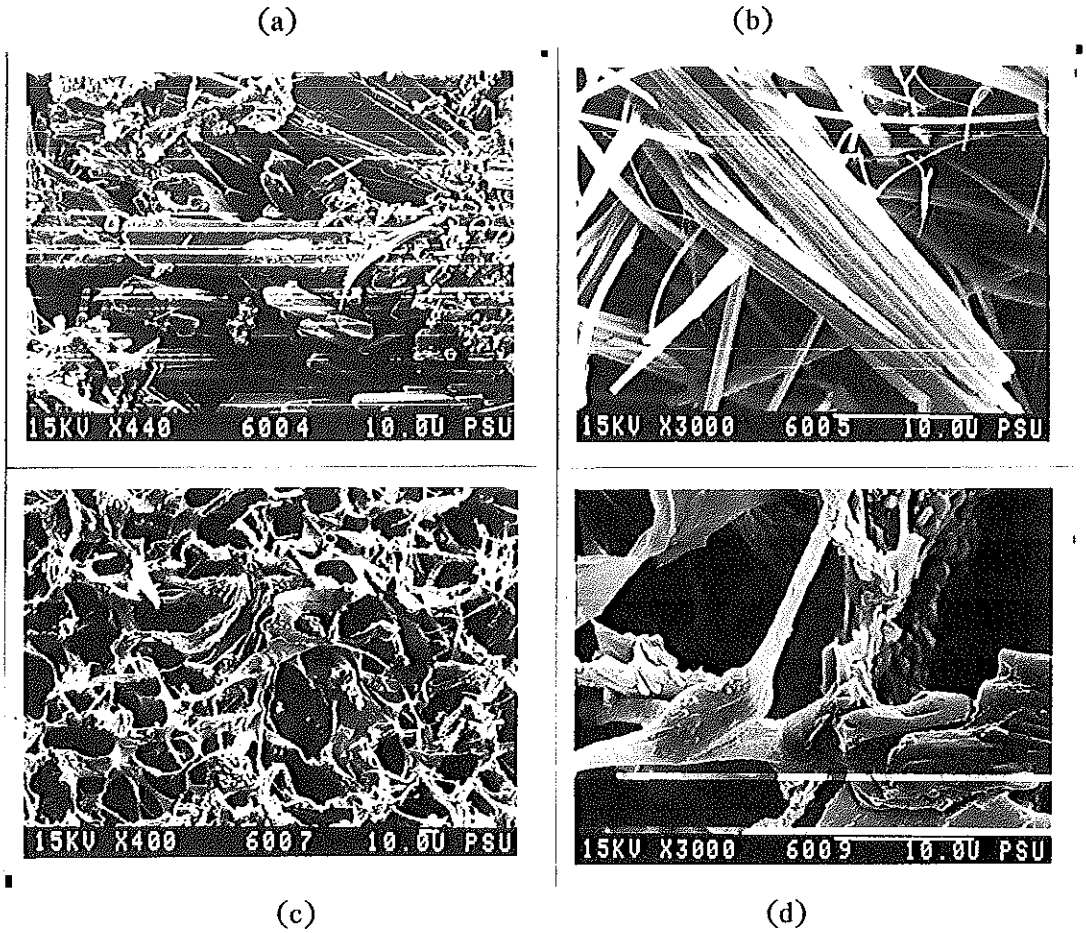


Figure 37 Microstructures of WD7 sponges prepared from WD7 biopolymer concentrations of 10% (a and b) and 2% (w/v) (c and d)

Alginate sponges prepared at the concentration ranges of 1-10% (w/v). Most alginate sponges had white color while the appearance of WD7 sponges was fluffier, but harder than WD7 sponges when compared at the same concentration. However, the surface character of all alginate sponges was smooth at low concentration (<4% w/v) and showed heterogeneous structured fiber at the surface at high concentration (6-10% w/v). At low concentration (2 and 4% w/v), they had a homogeneous structure which was expressed in term of pore distribution and size. They displayed a highly porous, good interconnected pore structure. However, the WD7 sponge was pliability comparison with the alginate sponge at the same concentration tested (Figure 38).

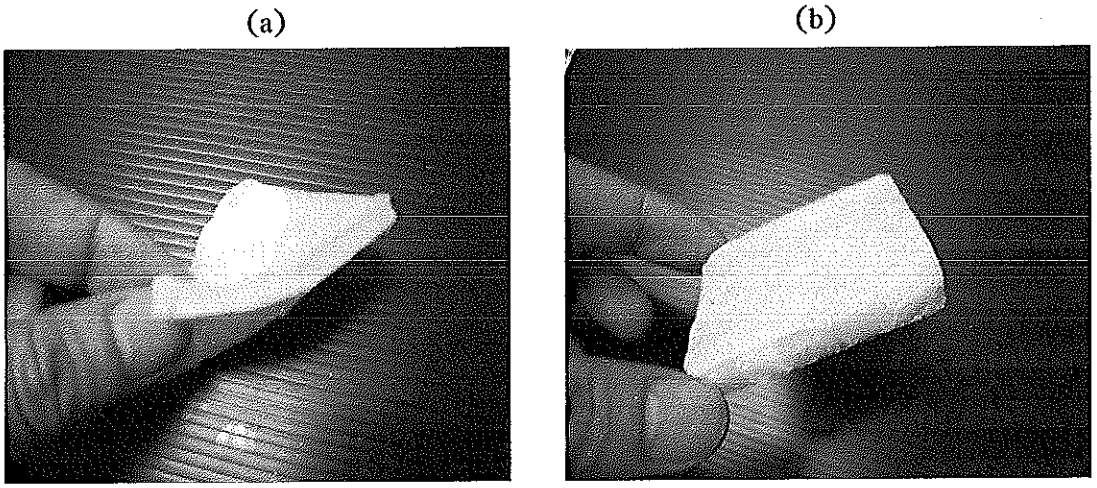


Figure 38 Photograph of WD7 sponge (a) compared with alginate sponge (b) at the same concentration (2%w/v)

Table 16 showed some appearance and mechanical properties of alginate sponges at various concentrations. All alginate sponges were not soft in texture and looked like fiber at high concentration. Size of the alginate sponges depended on the concentration and not much difference between each concentration. Size of all alginate sponges was almost equal to the size of mould with full size at high concentration at 10% (w/v) after freeze drying. As well as the WD7 sponge, the alginate concentration had an effect on thickness, pore size and strength of WD7 sponge. Alginate sponges were thicker than WD7 sponges when compared at the same concentration and the higher the concentration, the higher the thickness of alginate sponges. Thickness of alginate sponges were 6.10, 7.00, 7.15, 7.30, 7.70 and 7.90 mm at the concentrations of 1, 2, 4, 6, 8 and 10% (w/v), respectively.

Table 16 Effect of alginate concentrations on appearance and some mechanical properties of alginate sponge prepared by freeze drying method

Conc. of alginate solution (%w/v)	Sponge size (mm x mm)	Thickness (mm)	Average pore Size (μm)
1	40.0 x 45.0	6.10	15
2	48.0 x 49.0	7.00	12
4	49.0 x 49.0	7.15	10
6	50.0 x 50.0	7.30	8
8	52.0 x 52.5	7.70	5
10	55.0 x 54.5	7.90	5

However, all alginate sponges were harder and stronger than WD7 sponge compared at the same concentration. The number of pores and their distribution in alginate sponges were higher than those of WD7 sponge. The wall thickness of the sponges was highly dependent on the concentration of WD7 biopolymer solution used. The average pore sizes were 15, 12, 10, 8, 5 and 5 μm at the concentrations of 1, 2, 4, 6, 8, and 10% (w/v), respectively, indicating that the smaller pore size was obtained at higher WD7 biopolymer concentration. Shapiro and Cohen (1997) reported that the alginate solution concentration significantly affected the sponge microstructure with an increasing pore size and decreasing wall thickness at decreasing polymer concentration. However, some other parameters also had an effect on pore size such as type and constituent of biopolymer. Grant *et al.* (1973) found that alginate gel had egg-box model structure with the bivalent cation bridge the negative charged guluronic acid (G) and mannuronic (M) residues which play only a subordinate role in the gel framework. Thus, increasing the G content generally produces a matrix with an increased pore size.

4.6.2 Effect of Mixing Ratios on Preparation and Properties of WD7-Alginate Composite Sponge

All alginate sponges had strong and thick texture, small pore size and good distribution of pore with honeycomb character, but they were not soft and fluffy with no pliability when bending. On the other hand, the WD7 sponges were very soft and had fluffy texture with high flexibility and pliability when compared with alginate sponges. To obtain the sponge with good characters and properties showing the advantages of both polymers, the WD7-alginate sponge was made by blending the solution of WD7 biopolymer

and alginate at various concentrations and ratios before preparation of sponge by evaporation method. Complex sponges were prepared by blending both polymers using two solution concentrations (1% and 2% w/v) at three mixing ratios (3:1, 1:1 and 1:3) before sponge preparation. Then their appearance and property of WD7-alginate sponges were investigated.

All WD7-alginate blending sponge was flexible, not too hard or too soft (medium fluffy) and pliable when bended. This indicated the first advantage of blending of these polymers. All complex sponges had white color and all had the strength with harder texture than those of native WD7 sponges, but less than those of native alginate sponge. Their surface characters were glossy as found in alginate sponge. Thus, the characters of complex sponge might be in between one of the native WD7 and alginate sponges. No difference in texture was found between the two groups (1% and 2% w/v). Therefore, the surface texture of complex sponges at high mass of WD7 biopolymer was rougher than those at low mass. At low mass of WD7 biopolymer (ratio of 1:3), they presented a high porous, better interconnected pore structure than those found at high mass (1:1 and 3:1, respectively). Therefore, the WD7-alginate sponge at ratio of 3:1 was pliability compared with the complex sponge at ratios of 1:1 and 1:3, respectively at the same concentration tested.

Some characteristics of complex sponges were given in Table 17. Thickness of complex sponges was dependent on material concentration and mass ratio. The thicknesses of native WD7 sponge and alginate sponge at 2% (w/v) were 25 and 12 mm, respectively, while the complex sponges [prepared from 2% (w/v) WD7 biopolymer + 2% (w/v) alginate at ratios of 1:3, 1:1 and 3:1] were 52, 55 and 50 mm, respectively. Consequently, at the same concentration, the complex sponges had increasingly thickness when blended alginate mass increased. However, blending of two polymers gave the complex sponge with higher thickness when compared with the native sponges. Therefore, the complex sponge thickness increased. The thickness of a WD7-alginate sponge (2% WD7 biopolymer + 2% alginate at ratio of 1:1) was comparable to that native WD7 sponge at 4% (w/v) of WD7 biopolymer solution. This indicated that the complex sponges possessed higher fluffiness.

Complex sponge (prepared from 2% w/v of alginate and 2% w/v WD7 biopolymer solution) had small dense average pore size with 8, 10 and 15 μm at blending ratios of 1:3, 1:1 and 3:1, respectively, while the average pore size of the native WD7 biopolymer sponge and alginate sponge were 25 and 12 μm. This indicated that the smaller pore size was found at higher alginate concentration blended. In addition, the pores number

and distribution of complex sponges were higher than those of native sponge but lower than native alginate sponge.

Table 17 Effect of sponge composition on size, thickness and porosity of WD7 sponge and its modified sponges (ND = Not determined)

Sponge Samples	Mixing ratio	Sponge size (mm x mm)	Thickness (mm)	Average pore size (μm)
2%(w/v) WD7 biopolymer	-	37 x 38	18	25
2%(w/v) alginate	-	48 x 49	7	12
2%(w/v) WD7 biopolymer	1 : 3	48 x 45	52	8
+	1 : 1	50 x 52	55	10
2% (w/v) alginate	3 : 1	49 x 48	50	15
1%(w/v) WD7 biopolymer	1 : 3	45 x 47	38	
+	1 : 1	50 x 50	20	ND
1% (w/v) alginate	3 : 1	50 x 48	28	

The mechanical properties were assessed quantitatively using texture analysis. Figure 39a showed the maximum force ("hardness") for the sponges under compression as a function of composition. The alginate sponge was clearly more resistant to compression than the WD7 sponge or mixed systems, consideration from force (N) values compressed on of alginate sponges (2%), mixing sponges (of WD7 biopolymer and alginate) at mixed ratios of 1:3, 1:1 and 3:1 and WD7 sponge (2%) with 0.065, 0.225, 0.235, 0.274, 0.350 N, repectively. Similarly the WD7 alone gave the weakest sponge, while the mixed system at the same concentration (2% (w/v) WD7 biopolymer + 2% (w/v) alginate) showed intermediate "hardness" values. Clearly, therefore, the expected interaction between the two materials has resulted in a more robust structure, but softer of the mixed sponge. It is also interesting to note that the compression profiles were almost identical between the first and second cycles, indicating that in all cases recovery was effectively complete.

The tensile force profile of the sponges (Figure 39b) showed a different rank order with respect to sponge composition in that the alginate and WD7 biopolymer alone showed greater and less breaking strength than the mixed systems. The elongation values were similar in all cases (no statistically significant differences), with only very limited extension taking place prior to breakage. The lack of correlation between the hardness and

tensile force is of interest as it indicated that while the alginate sponges showed favorable rigidity (hardness) and resistance to breakage (tensile force), the WD7 sponges appeared to be more pliable while still having a relatively high strength. Clearly the use of either of the polysaccharides alone or the mixed systems affords the operator a range of options with regard to mechanical characteristics.

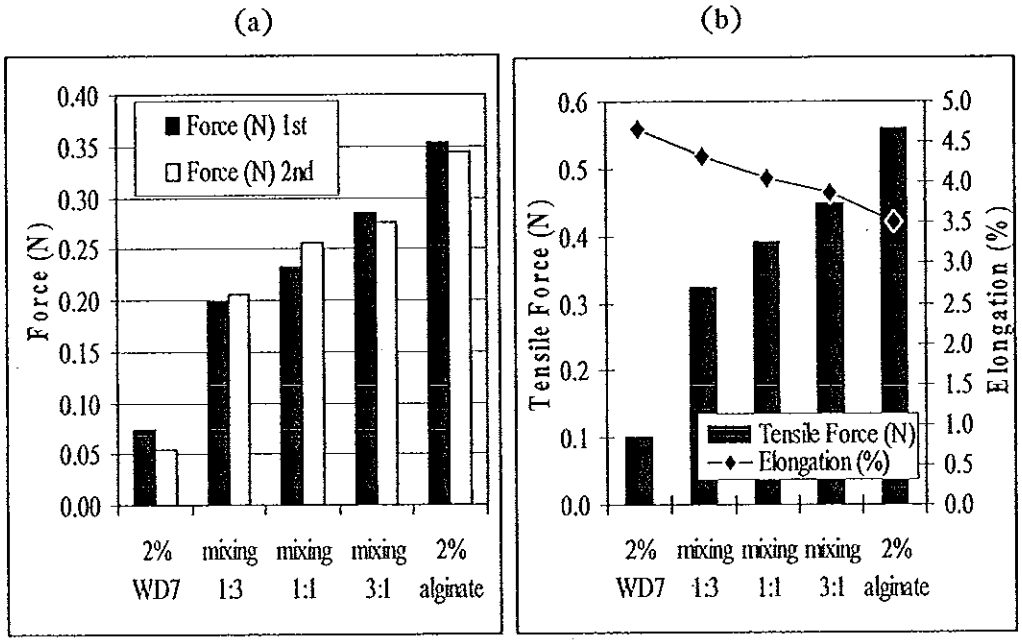


Figure 39 Texture analysis data showing (a) the compression force on first and second compression (b) the tensile force and elongation for mixed and single component of WD7 biopolymer and alginate sponges with 2% WD7 biopolymer+ 2% alginate at ratio of 1:3, 1:1 and 3:1

Alginate sponge was stronger and harder than WD7 sponges which had more pliability. To obtain the sponge with good characters and properties showing the advantages of both polymers alone, the sponge was the first attention made by blending the alginate solution with WD7 biopolymer. All WD7-alginate sponge had flexible texture with considerable bending with no fracture. They were not either too hard or too soft (medium fluffy) and pliable after being bended showing the first advantage of blending of two polymers. Therefore, they had more strength with hard texture than those of native WD7 sponges, but less than those of native alginate sponge. This has indicated that the mechanical and morphological properties of WD7-alginate sponges are highly dependent on composition. The resistance to compression was greatest for the alginate alone and was

markedly lower for the mixed systems and WD7 biopolymer alone while the resistance to breakage was greater for the single component systems. The microscopic studies indicated a more regular structure for the single component systems, possibly due to a pre-freezing interaction between the two polysaccharides.

4.7 Study on Preparation of Other Forms of WD7 Biopolymer

4.7.1 Bead Preparation of WD7 Biopolymer

The viscosity and rhelogical flow profiles of WD7 solution and gel increased by both monovalent and divalent cations, thus, this WD7 biopolymer was expected to be prepared as gel bead. In this study, the WD7 biopolymer solution and gel in water at various concentration of 1, 2, 4 and 6% (w/v) were extruded from a syringe into the KCl, NaCl, LiCl, MnCl, $CaCl_2$, $CuCl_2$, $MgCl_2$, Ca_2SO_4 and KH_2SO_4 at tested concentration of 0.1, 0.2, 0.5 and 1.0 M. Dripping method was used for bead preparation of this WD7 biopolymer. The results showed that no WD7 biopolymer bead was formed in these cation solutions neither at room temperature ($30^{\circ}C$) nor freezing temperature ($-20^{\circ}C$). Soluble WD7 biopolymer drops presented at the bottom of coagulating bath and they were dissolved immediately when stirred.

Some polysaccharides are polyelectrolyte substances, so their bead formation process is essentially based on exchange of ions. Many polyelectrolytes with nontoxic for cells are reported to rapidly form gelation by cross-linkage through chelate formation (egg box model) involving the divalent cations and carboxyl (and hydroxyl) groups of the polysaccharide chain. In alginate bead preparation, sodium ions (Na^+) present in soluble sodium alginate were replaced by calcium ions (Ca^{2+}) present in the coagulating calcium chloride bath. A solution of water-soluble alginate (usually sodium alginate) was injected into a bath containing an acidic solution and/or calcium salt solution to produce the corresponding calcium alginate bead (Knill *et al.*, 2004). Although some polyelectrolytes were hence suitable for gelation, but some are readily destabilized in the presence of Ca^{2+} complexants or monovalent cations, thus impeding further application (Serp *et al.*, 2000).

Since WD7 biopolymer is precipitated in some inorganic solvents (Dermlim, 1999), then it was tested to form the bead by these solutions. After testing, no bead were formed using the WD7 biopolymer of 2, 4 and 6% (w/v) formed in various concentrate solvents of dichrolomethane, chloroform, hexane, ethanol and methanol, in comparison to water as a control, at room temperature ($30^{\circ}C$) and freezing temperature ($-20^{\circ}C$).

Dripping method as the simplest method was used for bead preparation of this WD7 biopolymer for preliminary testing. WD7 biopolymer was mixed with some monovalent (Li^+, Mn^+, Na^+) and divalent cations (Ca^{2+}, K^{2+} and Mg^{2+}) mixed before dropping into these tested solvents. No bead formation was observed and mixed WD7 biopolymer dissolved immediately in strong acid and base solution, thus, it was not tested in acid or basic solutions. From these experiments, it could be concluded that the WD7 biopolymer cannot be prepared in bead form in all tested cations solution and inorganic solvents as mentioned above.

4.7.2 Preparation of WD7 Biopolymer Fiber

In this study, WD7 biopolymer could not be formed into fiber in coagulating bath with all tested cations solution after leaving for an hour. In inorganic solvents, WD7 biopolymer fiber was formed when the polymer was extruded into very cold ($-20^{\circ}C$) ethanol, methanol and hexane. WD7 biopolymer formed a short fiber (2-3 cm) due to the limitation of needle length and high concentration of polymer (4 and 6% w/v). After leaving at room temperature until dry, all obtained fibers were hard but brittle and easy to break in all tested concentrations (2, 4 and 6% w/v). They were not fibrous and neither flexible, stretched nor elongated.

4.8 Testing of Some Biological Activities of WD7 Biopolymer

Crude and partially purified WD7 biopolymer was tested for different types of biological activities and biocompatibility. Results of some antimicrobial activities of WD7 biopolymer were tested at various concentrations against various strains of tested microorganism as given in Table 18.

4.8.1 Antibacterial Activity

From Table 18, both crude and partially purified WD7 biopolymer samples had no antibacterial activity on *Staphylococcus aureus* at the maximum tested concentration of 50 µg/ml. Orange color developed in each well was detected indicating growth of *S. aureus*, hence, no inhibitory effects of WD7 biopolymer. Amphotericin B, used as a positive control, demonstrated the antibacterial activity with no color change.

4.8.2 Antifungal Activity

Both crude and purified WD7 biopolymer had no antifungal activity on *Candida albicans* at the maximum tested concentration of 50 µg/ml (Table 18). The inhibitory effects of WD7 biopolymers were inactive indicating by changing the medium orange color. Amphotericin B, used as a positive control, gave the antifungal activity with no change of color.

Table 18 Biological activities of WD7 biopolymer from *E. cloacae* WD7

Biological activity testings	Results of cytotoxicity testing		
	IC_{50} (µg/ml)	Activity	Positive control / IC_{50} (µg/ml)
Antibacterial: (*Strephylococcus aureus*)	>50	inactive	Amphotericin B, (0.35 ± 0.12)
Antifungal: (*Candida albicans* ATCC 90028)	>50	inactive	Amphotericin B, (0.35 ± 0.12)
Antiviral***: (Herpes simplex virus type 1 ; HSV-1 strain ATCCVR 260)	>50	inactive	Ellipticine (0.6 ± 0.2)
Antimalaria: (*Plasmodium falciparum* ; K1 multi drug resistant strain)	>10	inactive	Dihydroartemisinine (4.2 nM)
Anti-TB: (*M. tuberculosis* H37Ra)	>10	inactive	Dihydroartemisinine
Cytotoxicity assays against human cancer cell lines of:			
- epidermoid carcinoma of cavity (KB)	>20	inactive	Ellipticine (0.32 ± 0.14) Doxorubicine (0.11 ± 0.03)
- breast (BC)	>20	inactive	Ellipticine (0.32 ± 0.19) Doxorubicine (0.20 ± 0.09)
-lung (NCI-H187)	>20	inactive	Ellipticine (0.32 ± 0.17) Doxorubicine (0.03 ± 0.01)

*** Resistant activity <25% when Acryclovir was used as positive control with IC_{50} = 1.6 ± 0.4 µg/ml

4.8.3 Antiviral Activity

In vitro cytotoxicity test against vero cell line (ATCC CCL-81) and determination of anti-herpes simplex virus type 1 (HSV-1 strain ATCC VR 260) activity were performed on crude and purified WD7 biopolymer samples. Crude and purified WD7 biopolymer samples were non-cytotoxic and weakly active cytotoxic, respectively against vero cell at tested maximum concentration of 50 µg/ml when compared with cytotoxicity results of ellipticine as positive control with the concentration that inhibited viral activity by 50% (IC_{50}) = 0.6 ± 0.2 µg/ml. Both samples did not inhibit virus more than 50%; then the IC_{50} were not determined further.

The WD7 biopolymer samples had no activity for antiviral test against Herpes simplex virus at the tested ranges less than 50 µg/ml. For anti-HSV-1 test, acyclovir was used as positive control with IC_{50} of 1.6 ± 0.4 µg/ml. Consequently, this WD7 biopolymer was non cytotoxicity against vero cell line ATCC CCL-81 and had no antiviral activity against HSV-1 strain ATCC VR 260.

4.8.4 Antiplasmodial Activity

The ability of crude and purified WD7 biopolymer (at concentration >50 µg/ml in DMSO) to inhibit the malaria parasites was performed *in vitro* antiplasmodial activity against *Plasmodium falciparum* (K1, multi drug resistant strain) and dihydroartemisinine (DHA) was used as a positive control. After 24 h incubation, levels of incorporated radioactively labeled hypoxanthine indicating parasite growth in both samples, hence, no inhibition activity occurred. Results showed that both crude and partially purified samples had no antiplasmodial activity against *Plasmodium falciparum* at the tested ranges of 0-50 µg/ml. The test of inhibition concentration (IC_{50}) was not further performed in this parasite growth, while the cytotoxicity results of DHA had the IC_{50} value of 4.2 nM.

4.8.5 Anti-Tuberculosis Activity

Anti-tuberculosis (TB) (Alamar Blue Susceptibility Test; MABA) testing was performed in 96-well microplates with visual consideration of color change of MABA. No change of color from blue to pink was found after 24 h observation. All tested wells consisted of bacteria only (B) and medium only (M) that remained blue, were tested daily until a color change occurred, at this time MABA reagents were added to all remaining wells. After incubation at 37°C, no change of color was observed at >50 µg/ml DMSO of both crude and purified WD7 biopolymer samples after 24 h post-reagent addition.

Consequently, the activity to inhibit the TB test was performed *in vitro* against *M. tuberculosis* $H_{37}Ra$ and found to be negative in both crude and partially purified samples.

4.8.6 Antibiotic Activity Investigation

Disc containing crude and purified WD7 biopolymer solution were applied to bacterial cultures that have traditionally been used to treat ailments of a bacterial or fungal origin. The complementary assays were carried out against the human pathogenic bacteria (*Staphylococcus aureus, Bacillus cereus, Escherichia coli* and *Salmonella typhimurium*), fungi (*Saccharomyces cerevisiae* and *Candida albican*) on agar plates. The selected test microorganisms are common indicators in food and medical fields. Diameter of inhibition zone (mm) exhibited against test bacteria and fungi of standard antibiotics and crude and purified samples (0.1 mg/ml) are given in Table 19.

In this study, penicillin G and chloramphenicol showed higher inhibitory effect than antibiotic vancomycin. Most tested bacteria were resistant to vancomycin, but most were sensitive to chloramphenicol at higher level of antimicrobial activity than the antibiotic penicillin G. On the other hand, *E.coli* was sensitive to vancomycin and resistant to penicillin G. It appeared that both fungi were resistant towards vancomycin and penicillin G, but were sensitive to Chloramphenicol.

Table 19 Antibiotic activities expressed as inhibition zone (mm) of crude and partially purified biopolymer solution samples of *Enterobacter cloacae* WD7 (CB and PB, respectively) against tested microorganisms by disc method tested with 0.1 mg/ml of both samples and standard antibiotic

Test Microorganisms	Diameter of clear zone (mm) observant at 24 h of incubation				
Bacteria	**vancomycin**	**penicillin G**	**chloramphenicol**	**CB**	**PB**
S. aureus	4^R	8^S	7^S	NC	NC
B. cereus	5^R	20^S	14^S	NC	NC
E. coli	13^S	4^R	12^S	NC	NC
S. typhi	6^R	15^S	11^S	NC	NC
Fungi					
S. cerevisiae	R	4^R	6^S	NC	NC
C. albicans	R	6^R	8^S	NC	NC

Note: CB and PB = crude and purified samples, respectively
R= resistant, S=sensitive, NC=no clear zone

No inhibition zone was found in all tested plates of both samples (the maximum concentration of 0.5 mg/ml) after incubation for 24 h. Thus, this WD7 biopolymer had no activity for inhibiting target microorganisms tested of both Gram negative (*E. coli* and *Salmonella typhimurium*) and Gram positive bacteria (*Staphylococcus aureus* and *B. cereus*) and fungi (no antimycotic activity). Then, higher concentration of WD7 biopolymer samples was tested to confirm the bioactivities. However, the WD7 biopolymer samples in both forms (at 0.5 mg/ml) were not sensitive against all isolates tested. Consequently, this WD7 biopolymer had no bioactive compounds or no antibiotic activity. This result was consistent with the previous results of antimicrobial bioactivities by *in vitro* colorimetric assay in 96 well plates.

4.8.7 Human Cytotoxicities and Anticancer Activities

4.8.7.1 Cytotoxicity Testing on HT 29 Human Colon Cancer

Cytotoxicity test on the viability of HT 29 cell lines of WD7 biopolymer solution was evaluated *in vitro* with chitosan (AR grade, as negative control). The amount of surviving cells after incubation was determined by SRB assay and cell viability was converted to toxicity expressed in percent. Figure 40 showed that toxicity on

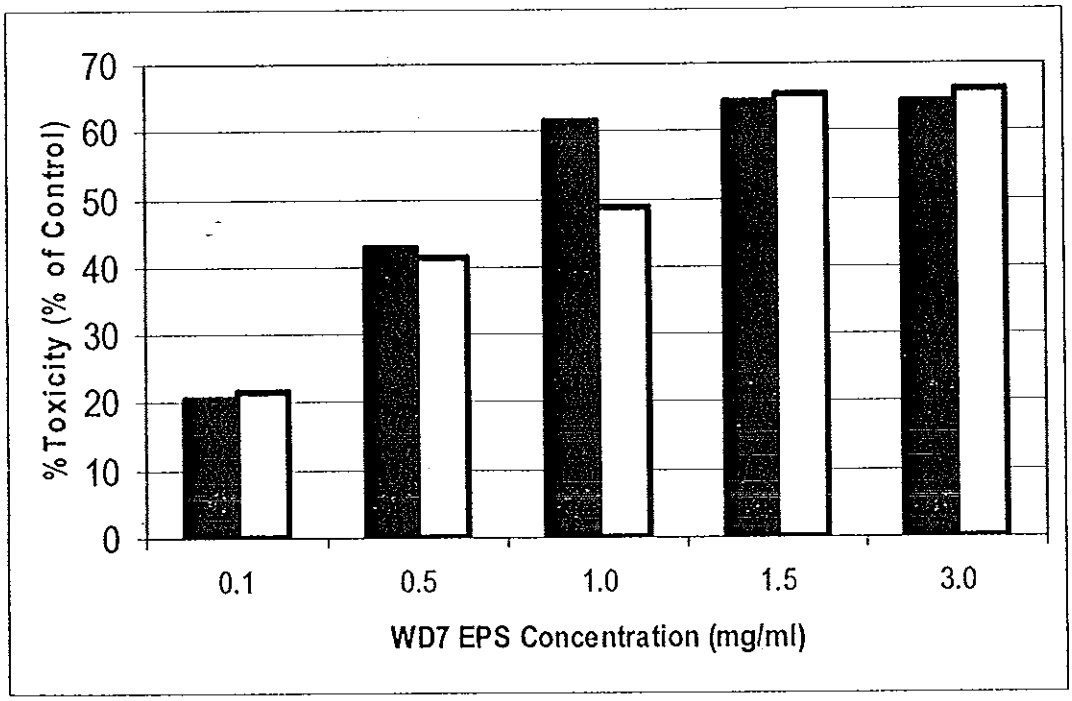


Figure 40 Cytotoxicity test against on HT 29 human colon cancer cell lines of crude (■) and partially purified WD7 biopolymer samples (□)at various concentrations

tested HT 29 cell depended on concentration of WD7 biopolymer after 92 h incubation, giving 20.36, 42.85, 64.26, 64.48 and 61.58% for crude samples and 21.59, 41.52, 48.63, 65.24 and 66.19% for partially purified samples, at increasing concentrations of 0.1, 0.5, 1.0, 1.5 and 3.0 mg/ml, respectively. Both crude and purified WD7 samples had the same effect on toxicity against HT 29 cell lines and they were almost non-toxicity against HT 29 cell lines at low concentration with %survival of cells > 80% at 0.1 mg/ml and ~ 40% at 1.0% w/v of WD7 biopolymer.

4.8.7.2 Anticancer Assay (KB, BC and NCI-H187)

Anticancer activities for human mouth carcinoma (KB) and breast cancer (BC) were tested at various concentrations of crude and partially purified WD7 biopolymer. The results showed that this WD7 biopolymer was inactive at tested maximum concentration of 20 μg/ml. For anticancer BC test, Ellipticine and Doxorubicine were used as positive controls with IC_{50} of 0.32 ± 0.14 and 0.11 ± 0.03 μg/ml, respectively. For anticancer KB test, Ellipticine and Doxorubicine were used as positive controls with IC_{50} of 0.32 ± 0.19 and 0.20 ± 0.09 μg/ml, respectively. The WD7 biopolymer were also inactive for anticancer activities against NCI-H187 at tested maximum concentration of 20 μg/ml. Ellipticine and Doxorubicine were used as positive controls with IC_{50} of 0.32 ± 0.17 and 0.03 ± 0.01 μg/ml, respectively. In conclusion, WD7 biopolymer was inactive against three tested human cancer cells; KB, BC and NCI-H187.

4.8.7.3 Heamocompatibility Testing

The WD7 biopolymer was investigated for human heamo-compatibility for using in pharmaceutical and medical purposes. Crude and partially purified WD7 biopolymer samples were tested for toxicity against human red blood cell. Erythrocyte morphology after 1 h and 24 h exposure to soluble WD7 biopolymer was visualized by using the microscope with camera in comparison to control agents; phosphate buffer solution (PBS) and triton X surfactant as positive and negative control, respectively. The result of various concentrations of crude and partially purified WD7 biopolymer against human red blood cells lysis was investigated after 1 h and 24 h exposure.

Crude and partially purified WD7 biopolymer solution caused the relative lysis depended on concentration. Toxicity on RBCs was of WD7 biopolymer when increasing concentration to 5.0 mg/ml (Figure 41). After 1 h incubation, RBCs lysis was approximately 1.43, 2.18, 3.53, 13.16 and 14.29% for crude samples and 0, 0, 1.43, 2.18 and 7.07% for partially purified samples, at increasing concentrations of 1.0, 2.0, 3.0, 4.0, and 5.0 mg/ml, respectively. After 24 h incubation, the RBCs lysis was approximately

33.74, 44.18, 62.47, 74.07 and 88.18% for crude samples and 18.47, 32.40, 39.80, 43.73 and 42.84% for purified samples, at increasing concentrations of 1.0, 2.0, 3.0, 4.0 and 5.0 mg/ml, respectively.

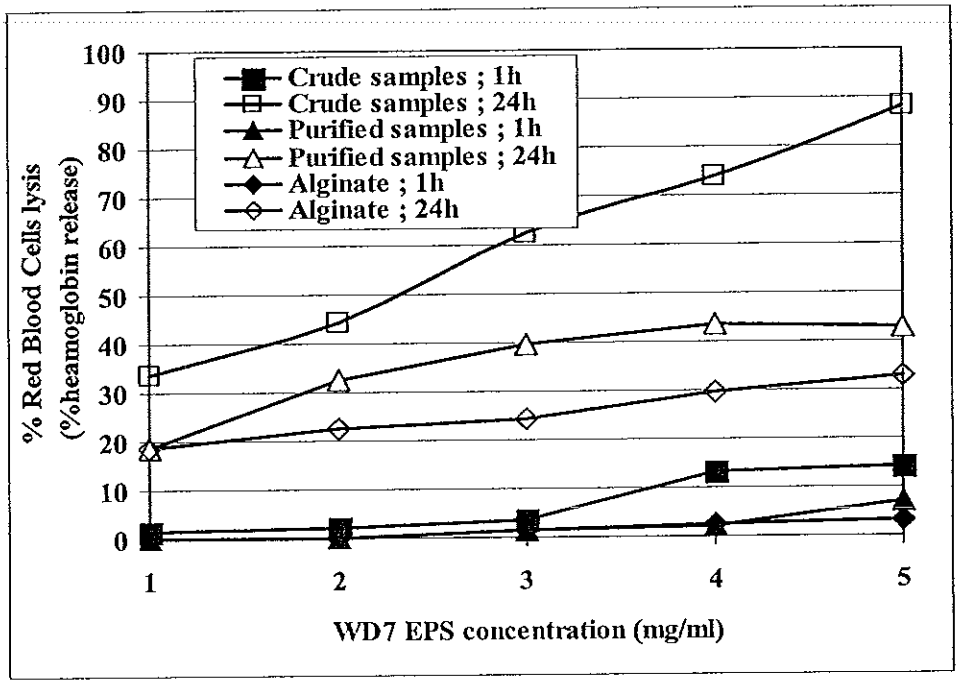


Figure 41 Red blood cells lysis after 1h and 24h exposure of crude and purified WD7 biopolymer

The RBCs lysis was very low after exposed with WD7 biopolymer samples while it reached maximum value in Triton X treatment. Crude sample had more effect on hemoglobin release and abnormal shape of RBCs than partially purified samples at all concentrations tested (0-5 mg/ml). The extent of hemoglobin release depended on polymer concentration after exposure for 1 and 24 h. The WD7 biopolymer may be possibly used as curing material for human if it is purified at least by dialysis and at low concentrations with RBCs lysis lower than 20%.

The RBCs exposed to WD7 biopolymer samples and also PBS treatments were aggregated and most of them were swollen when examined under microscope (Figure 42). No RBCs lysis was found after incubation in PBS buffer (Figure 42 a1 and a2), but the whole RBCs were completely lysed with Tritron X-100 which was used as negative control (Figure 42 b1 and b2) at 1h and 24h incubation, respectively. The RBCs were swollen and some were lysed when exposure in WD7 biopolymer and

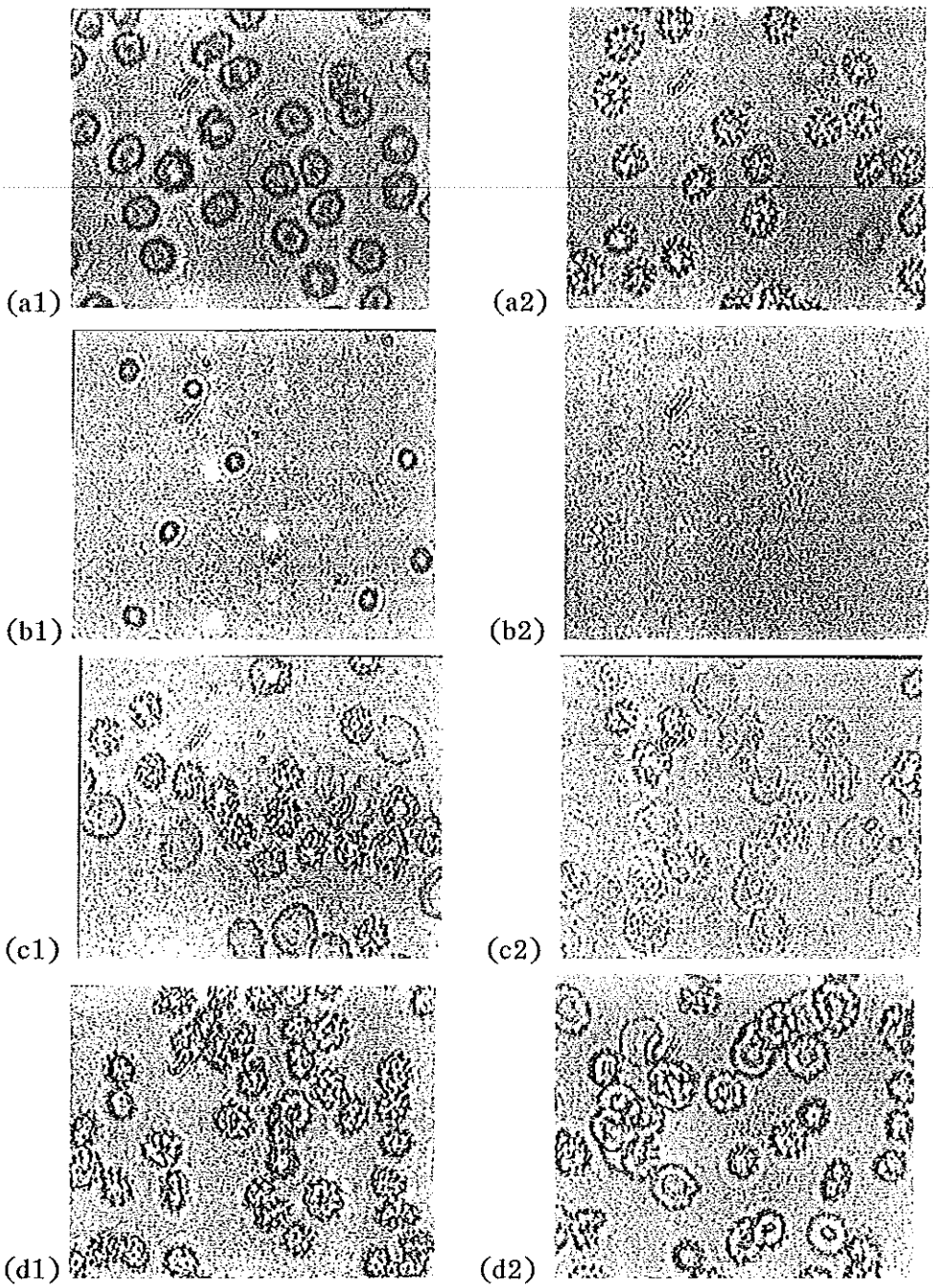


Figure 42 Microscopic illustration of human RBCs after exposure to:

a) PBS buffer solution as negative control at 1 h (a1) and 24 h (a2)

b) Tritron X100 solution as positive control for 1 h (b1) and 24 h (b2)

c) Crude WD7 biopolymer at 5 mg/ml for 1 h (c1) and 24 h (c2)

d) Purified WD7 biopolymer at 5 mg/ml for 1 h (d1) and 24 h (d2)

increased as the incubation times increased. The swelling and haemoglobin released after 1 h and 24 h incubation were shown in Figure 42c and 42d. After 1-h incubation, both crude and partially purified WD7 biopolymer samples caused the swelling of RBCs with the increase of absorbance higher than PBS control treatment. After 24h incubation, the hemoglobin release occurred in crude biopolymer exposure more extensive than the purified one.

Characteristic of RBCs were destroyed due to immediate lysis when exposured to Triton X surfactant as positive control. The results indicated that RBCs lysis depended on the biopolymer concentrations. Increasing biopolymer concentrations leaded to complete lysis of RBCs. Increasing of biopolymer concentration leaded to complete lysis of RBCs. Different biopolymer charactristics or derivative types gave different heamolysis efficiency. Carreño-Gómez and Duncan (1997) reported that hydroglutamate chitosan and glycol chitosan caused the 75% RBCs lysis and little lysis, respectively at the same tested concentrations (3 mg/ml).

Many reports presented that heamolytic activity and RBCs lysis depended on various parameters and conditions in tested such as pH, biopolymer molecular weight, osmotic mechanism, and oxidation reaction, suspending media, type of charge functionality and biopolymer characteristics and properties (Carreño-Gómez and Duncan, 1997; Tosteson *et al.*, 1985). Heamolytic activity was found when explored to glycoprotein and some anionic polymer as found in hyaluronan (sulphate hyaluronic acid) (Abatangelo *et al.*, 1997).

Charge of polymer has an effect on RBCs and caused haemolysis. Chitosan had the protonated amino group that could bind to the anionic glycoproteins of RBCs easily, thus, it induced membrane ruptures and heamoglobin release. Anionic carboxylated methacrylic acid (MAA) copolymer type C and D, giving 100% lysis from both types at 3 mg/ml (Yessine *et al.*, 2003). MAA copolymer could absorb to lipid bilayer of RBC membrane and caused the sufficient binding and membrane lysis, in addition to increasing of hydrophobic condition during testing. The RBC lysis was different after exposure with various molecular weights of a polymer. Anionic carboxylated MAA copolymer types B, C and D (at 2 mg/ml in PBS pH 7.4) with molecular weight of 26,240, 31510 and 23,030 gave 20, 65 and 80% blood lysis, respectively (Yessine *et al.*, 2003).

Therefore, the effect of ionic strength and osmolarity on heamolysis was studied especially in most surfactant polymer. Shalel *et al.* (2002) reported that the

cationic dodecyl trimethylmonium bromide (DTAB) with the presence of glucose caused the heamolysis faster than DTAB in the presence of NaCl at constant osmolarity. Increase of ionic strength decreased the heamolysis of biopolymer as found in DTAB with sucrose when compared with the presence of glucose. This also was found in other surfactants such as SDS and Triton X-100 which showed that heamolysis rate occured slowly when reduced the ionic strength.

4.8.8 Anti-inflammatory Activities

Anti-inflammatory compound screening assay was the test on the ability of compounds to inhibit cyclooxygenase (COX)-1 and COX-2 enzyme activities via the measurement of prostaglandin produced from mouse COX-1 and COX-2 null cell lines. Prostaglandins are a group of arachidonic acid metabolites that play a beneficial role in the regulation of normal cell and organ function, as well as mediate the inflammatory process, a cause of pain, fever and swelling. Prostaglandin biosynthesis is largely controlled by cyclooxygenases (COXs), enzymes that convert arachidonic acid into prostaglandin H2, a prostaglandin precursor. There exist two isoforms of COX: COX-1, which is believed to be the so-called 'good' COX and COX-2, which is thought to be the cause of inflammation. Due to the high incidence of adverse effects caused by the use of non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin and indomethacin which exert their effects by inhibiting both COXs, there is a growing demand for COX-2 selective drugs. However, there are a number of studies that show interchangeable roles of COX-1 and COX-2 and studies that indicate the compensation between the two COX isoforms, when one of the isoforms is missing or being suppressed. Based on these data, this test was currently investigating the regulatory mechanism that control the expression and compensation between COX isoenzymes, using mouse lung fibroblast cell lines derived from normal mice and mice lacking either COX-1 or COX-2 gene as our model system. In this study, the aqueous WD7 biopolymer had no activities for anti-inflammatory related to inhibition of prostaglandin synthesis *in vitro* test at IC_{50} > 50 mg/ml.

CHAPTER 5
CONCLUSIONS

1. Scale up for WD7 biopolymer production

Growth of E. cloacae WD7 and biopolymer yield under aerobic fermentation increased with increasing aeration and agitation rates at pilot plant scale (72L), but decreased when agitation increased at bench scale (5L) because of shear thinning. The optimum condition for bench scale was 1.25 vvm and 200 rpm, but it was at 2 vvm and 600 rpm in pilot plant cultivation. By fixing k_La with ratio of 1.03 and 0.98 in exponential and stationary phases, respectively, the highest biopolymer yields of 3.07 and 3.20 g/g were achieved at bench and pilot plant scales, respectively, without system failure.

2. Characterization of WD7 Biopolymer

Biopolymer from *E. cloacae* WD7 was an acidic heteropolysaccharide that composed of glucose, galactose and rhamnose with glucuronic acid, without protein or other component, hence, it was a polysaccharide with C-H stretching with C-1 (β-glycosidic linkage) and contained some acids due to the presence of O-H stretching and C=O of carboxylic acid. WD7 biopolymer was confirmed that it was polysaccharide from analysis the obtained signals of ^{13}C NMR spectrum at δ 97.514 -101.898. Average molecular weights (Mw) of this WD7 biopolymer was 50.7-56.6 kDa.

3. Rheology Behavior of WD7 Biopolymer

Rheological behavior is a property of polymeric solution and gel. Flow curve of this WD7 biopolymer solution revealed the pseudoplastic behavior. This biopolymer was not stable at strong acidic and alkaline condition. The appropriate temperature and pH were found at <50^{o}C and pH 6-7, respectively. This biopolymer solution and gel can be stored at 4^{o}C and -20^{o}C for at least 2 months and can be freeze-thawed for many times without flow rheological behavior changing and viscosity loss. Some mono- (Li^{+} and Na^{+}) and some divalent cations (Mn^{2+} and Mg^{2+}) can enhance the gelation of WD7 biopolymer. The addition of excess cation (or high salt) caused the decrease of gelation (viscosity), such as found in Na^{+}. The decrease of gelation by some divalent cations (Ca^{2+} and Cu^{2+}) was found in the WD7 biopolymer.

4. Preparation of WD7 Biopolymer Film

Optimal condition for film casting from biopolymer solution (2-10% w/v) of *E. cloacae* WD7 was at 50°C and 50%RH. Biopolymer concentrations influenced on film thickness but not response to tensile strengths and elongation at break. To improve the film appearance or property, the WD7 biopolymer solution (2% w/v) was added with the selected plasticizer, glycerol at ratios of 1:0.5 before casting and gave about 40 times higher values of elongation at break compared to the native film. Both nature and all modified films dissolved immediately in water, buffer solutions (Tris and acetate buffer at pH 6.5), acid (1M HCl) and base (1M NaOH) solution and showed low water vapor permeability.

5. Preparation of WD7 Biopolymer Sponge

To obtain the sponge with good characters and properties, the WD7 biopolymer (2% w/v) blended with alginate (2% w/v) at ratio of 1:1 and evaporated at 50°C until dried (at least 48 h) gave the flexible texture.

6. Biological Activity of WD7 Biopolymer

This WD7 EPS had no antibacterial, antifungal, antiviral and antimalarial activities at 50 µg/ml and no antibiotic activity. It was heamocompatibility with less lysis of human red blood cell (<50% heamoglobin release at lower 3 mg EPS /ml), but increased with EPS concentration. Therefore, it was non-cytotoxicity via *in vitro* testing, and no antitumor activity against human epidermoid carcinoma of cavity, breast cancer cell line and small cell lung cancer (at IC_{50} >50 µg/ml). Thus, this EPS had no bioactive compound affected on human pathogen and antitumor activities. It also had no healing activities from *in vitro* testing of anti-inflammation assay (inhibition of prostaglandin synthesis).

Further Works

This WD7 biopolymer can be scaled-up for massive production with high yield, thus from this study, it will be benefit for industrial development. However, the recovery process of this biopolymer is still a problem as bottle-neck for production since some solvent for precipitation the biopolymer are expensive and very toxic that will be as environmental pollutant. For further work, WD7 biopolymer should be

1. elucidated for complete chemical structure with investigation on glycosidic linkage and configuration of monosaccharide in polymeric chain.

2. synthesis of the biopolymer derivative and structural analysis using HPLC, GC and NMR etc.
3. studied on the structure of biopolymer modified by chemical, physical and/or enzymatic methods to obtain the required properties such as a stronger biopolymer gel, and prepare the modified WD7 biopolymer forms (such as gel, fiber, sponge, bead etc.) suitable for the application.
4. investigated on application in various fields.

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APPENDICES

APPENDICES

7.1 Reagent and Chemical Analysis

7.1.1 List of Chemicals

All chemicals and their source in this study were given in Table 20.

Table 20 List of some chemicals

Lists	Sources
Acids;	
Acetic acid (CH_3COOH; F.W. 60.05), ACS reagent, ≥ 99.7%	Labscan
Arachidonic acid ($C_{20}H_{31}O_2Na$; F.W. 326.45), ~99%	Sigma
Ascorbic acid ($C_6H_8O_6$; F.W. 176.12, puriss. p.a., ≥99.0%	Fluka
Boric acid (H_3BO_3; F.W. 61.83), puriss. p.a., ACS reagent, ≥99.5%	BDH
N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) ($C_8H_{18}N_2O_4S$; F.W. 238.30), meets USP testing specifications	Merck
Glacial acetic acid (CH_3CO_2H; F.W. 60.05)	Labscan
Hydrochloric acid (HCl; F.W. 36.46)	Labscan
Lactic acid ($CH_3CH(OH)COOH$; F.W. 90.08) puriss., analytical specification, 88-92% as total acid	Riedel-de Haen
Methane sulphonic acid (MSA)	Sigma
3-(N-morpholino) propanesulfonic acid (MOP; $C_7H_{15}NO_4S$; F.W. 209.26, ≥ 99.5% (titration)	Sigma
Pyruvatic acid ($CH_3COCOOH$; F.W. 88.06), purum, ≥98.0%	Fluka
Sulphuric acid (H_2SO_4; F.W. 98.08), puriss. p.a. plus, ≥95%	Labscan
Trifluoro acetic acid (TFA, CF_3COOH; F.W. 114.02, reagent grade, ≥ 98% (titration)	Aldrich

Table 20 (cont.) List of some chemicals

Lists	Sources
Antibiotic;	
Acyclovir ($C_8H_{11}N_5O_3$; F.W. 225.20), ≥95%	Sigma
Amphotericin B ($C_{47}H_{73}NO_{17}$; F.W. 924.08), cell culture tested	Difco
Chloramphenicol ($Cl_2CHCONHCH(CH_2OH)CH(OH)C_6H_4NO_2$; F.W. 323.13, ampule of 1000 mg, 98%	Aldrich
Doxorubicin elliptine	Oxoid
Erythromycin ($C_{37}H_{67}NO_{13}$, F.W. 733.93) potency: ≥ 850 μg/mg	Sigma
Hygromycin B ($C_{20}H_{37}N_3O_{13}$; F.W. 527.52), from *Streptomyces hygroscopicus* powder, ≥60% (HPAE), cell culture tested, insect cell culture tested	Sigma
Penicillin-G (sodium salt, $C_{16}H_{17}N_2NaO_4S$, F.W. 356.37), powder, cell culture tested	Sigma
Streptomycin sulfate salt ($C_{21}H_{39}N_7O_{12}\cdot 1.5H_2O_4S$; F.W. 728.69), powder, ≥98%	Difco
Vancomycin ($C_{66}H_{75}Cl_2N_9O_{24}\cdot HCl$; F.W.: 1485.71), from *Streptomyces orientalis*, ≥ 900 μg per mg	Carlo Erba
Detergents;	
Triton X-100 [4-(C_8H_{17}) $C_6H_4(OCH_2CH_2)_nOH$, n~10]	Aldrich
Polymers;	
Dextran ($[C_6H_{10}O_5]_n$, for GPC, standard-Set M_p 1,000-400,000)	Merck
Polyethylene glycol (PEG, $H(OCH_2CH_2)_nOH$; average M_w 400)	Merck
Polypropylene glycol(PPG, $H[OCH(CH_3)CH_2]_nOH$; average M_w400)	Merck
Pullulan (for GPC, Mp 342-710,000)	Riedel de Haen
Sodium alginate (viscosity 500 cP)	BP

Table 20 (cont.) List of some chemicals

Lists	Sources
Salts;	
Ammonium chloride (NH_4Cl; M.W. 74.56), 99.5%, A.R. grade	Fisons
Ammonium sulphate [$(NH_4)_2SO_4$;M.W. 132.14], ≥99.5%,A.R. grade	Fisons
Calcium chloride ($CaCl_2$; F.W. 110.98), granule size 1-2 mm	Fisher
Calcium chloride dihydrate($CaCl_2.2H_2O$; F.W. 147.02), 98%, A.C.S.	Aldrich
Copper sulphate ($CuSO_4$; M.W. 159.68), 98.5%	Univar
Cupric sulphate ($CuSO_4.5H_2O$; M.W. 249.68), ≥ 98.5%, S.L.R. grade	Fisons
Potassium Bromide (KBr, M.W. 119.01), ≥ 99.5%, A.R. grade	Fisons
Potassium chloride(KCl; M.W.74.56), ≥ 99%	Fisons
Potassium sulfate (K_2SO_4; F.W.174.3), 99.5%, A.C.S. reagent,	Sigma
Lithium chloride (LiCl; M.W.42.40), ≥ 98.5%	BDH
Magnesium chloride ($MgCl_2 .6H_2O$; F.W.203.3), ≥ 99.0%	Sigma
Magnesium sulphate ($MgSO_4.H_2O$; M.W. 120.48), 62-70%, G.P.R.	BDH
Magnesium sulphate heptahydrate($MgSO_4.7H_2O$; MW.246.48),99.5%	H&W
Manganeasechloride ($MnCl_2.4H_2O$; M.W.197.91), ≥ 97%, G.P.R.	BDH
Sodium chloride (NaCl; M.W. 58.43, ≥ 99.5%), C.A.S. grade	Acros
Sodium sulfate anhydrous(Na_2SO_4; M.W.142.04), > 99%	Fisons
Serums;	
Fetal calf serum (FCS) frozen liquid	Gibco
Fetal bovine serum (FBS) Dialyzed by ultrafiltration against 0.15M NaCl, USA origin, sterile-filtered, cell culture tested	Gibco

Table 20 (cont.) List of some chemicals

Lists	Sources
Substances;	
Barium carbonate ($BaCO_3$)	Fluka
2,3-bis(2-methoxy-4-nitro-5-sulfonylphenyl)-5-[(phenylamino)	-
carbonyl]-2H-tetrazolium hydroxide	-
Bovine serum albumin	Sigma
Casitone (7H9GC)	-
Coomassie Brilliant blue G, ($C_{47}H_{48}N_3O_7S_2Na$)	Sigma
Deuterium oxide (D_2O)	Aldrich
Dihydroartemisinine (DHA)	-
Disodium hydrogen phosphate (Na_2HPO_4)	BDH
EDTA	Aldrich
[^{3}H] hypoxanthine	-
L-cysteine hydrochloride, ($CH_2(SH).CH(COOH).NH_2HCl$	BDH
L-glutamine	-
Magnesium nitrate hexahydrate [Mg $(NO_3)_2 \cdot 6H_2O$]	-
Methyl imidazole	Aldrich
N-methylphenazolium methosulfate (XTT tetrazolium/PMS)	-
Naphol	Riedel-de Haen
Parraffin	Unilab
Phenol	Carlo Erba
Potassium bromide (KBr)	Merck
Potassium dihydrogen orthophosphate (KH_2PO_4)	Fluka
Potassium hydrogen phosphate (K_2HPO_4)	Fluka
Potassium sorbate	Fluka

Table 20 (cont.) List of some chemicals

List	Sources
Sodium chloride (NaCl; M.W. 58.43), ≥ 99.5%,C.A.S. grade	Aldrich
Sodium chlorite ($NaClO_2$)	Aldrich
Sodium borohydride ($NaBH_4$)	Riedel-de Haen
Sodium deuteroxide (NaOD)	Sigma
Sodium dihydrogenphosphate monohydrate ($NaH_2PO_4.H_2O$; F.G. 137.99), ≥ 98%, A.C.S.reagent	Aldrich
Sodium phosphate dibasic (Na_2HPO_4; F.G. 141.96), ≥ 99%, A.C.S. reagent	Aldrich
Sodium hydrogen carbohydrate ($NaHCO_3$)	Fluka
Sodium Hydroxide (NaOH; F.W.40.00), ≥ 98%, pellets	Fisons
Sulforhodamine B (SRB)	–
Tetramethylsilane (TMS)	Gibco
Tween 80	Univar
Yeast extract	Merck
Solvents;	
Acetic anhydride [$(CH_3CO)_2O$; F.W. 102.09], ≥ 98.0%, GC	Fluka
Acetone (CH_3COCH_3; F.W. 58.08) puriss. p.a., absolute, ACS reagent, ≥ 99.5%	Labscan
Acetonitrile (CH_3CN, F.W. 41.05) for HPLC, ≥ 99.9%	Labscan
Chloroform ($CHCl_3$; F.W. 119.38)puriss, for GC, ≥ 99.9%	Labscan
Dichloromethane (CH_2Cl_2; F.W. 84.93), puriss, ≥ 99.9%	Unilab
Dimethyl sulfoxide [DMSO, $(CH_3)_2SO$; F.W. 78.13], ≥99.0% (GC)	Univar
Ethanol (EtOH, CH_3CH_2OH; F.W. 46.07), ≥ 99.5%, for HPLC	Merck
Glycerol [$HOCH_2CH(OH)CH_2OH$; F.W. 92.09], puriss. p.a., ACS reagent, anhydrous, dist., ≥ 99.5% (GC)	Labscan
Hexane [$CH_3(CH_2)_4CH_3$; F.W. 86.18], ≥99.0%, (GC)	Merck
Isopropyl alcohol [$(CH_3)_2CHOH$; F.W. 60.10,] , ≥ 99.7%	Aldrich

Table 20 (cont.) List of some chemicals

List	Sources
Sugar and monosaccharide;	
L-(+)-Arabinose ($C_5H_{10}O_5$; F.W. 150.13), ≥ 99%	Sigma
D-(−)-Fructose ($C_6H_{12}O_6$; F.W. 180.16), for HPLC, ≥ 98.0%	Fluka
D(+)-Galactose ($C_6H_{12}O_6$; F.W. 180.16), ≥99%	Sigma
D(+)-Galactosamine hydrochloride($C_6H_{13}NO_5$·HCl; F.W. 215.63), ≥99%	Sigma
D(+)-Glucose ($C_6H_{12}O_6$; F.W. 180.16), SigmaUltra, 99.5% (GC)	Sigma
D-Glucuronic acid (GlcA, $C_6H_{10}O_7$; F.W. 194.14), purum, ≥97.0%	Sigma
D-Glucosamine hydrochloride($C_6H_{13}NO_5$·HCl; F.W.215.63) HPLC, ≥99%	BDH
D-(+)-Glucuronic acid γ-lactone ($C_6H_8O_6$; F.W.176.12), ≥99%	Sigma
D-Mannose ($C_6H_{12}O_6$; F.W. 180.16), ≥ 99.5% (HPLC)	Fluka
Myo-inositol ($C_6H_{12}O_6$; F.W. 180.16), purum, ≥98.0% (HPLC)	Fluka
α-L-Rhamnose ($C_6H_{12}O_5$·H_2O; F.W. 182.17), (HPLC), ≥99.0%	Fluka
D-(−)-Ribose ($C_5H_{10}O_5$; F.W. 150.13), ≥99.0% (HPLC)	Fluka
Sorbitol ($C_6H_{14}O_6$; F.W. 182.17), crystallized, ≥99.0% (HPLC)	–
Sucrose (commercial grade)	Mitphol Co.Ltd.
D(+)-xylose ($C_5H_{10}O_5$; F.W. 150.13), ≥99.0% (HPLC)	BDH

7.1.2 Quantitative Analysis

7.1.2.1 Total Carbohydrate

Total carbohydrate contents of the WD7 biopolymer samples were determined using Phenol-sulphuric acid assay or L-Cysteine sulphuric acid assay.

Phenol-sulpuric acid assay : Total carbohydrate contents of the WD7 biopolymer samples were determined using Phenol sulphuric acid assay (Chaplin and Kennedy, 1994).

Reagents :

Reagent A: Phenol dissolved in water (5% w/v). This solution is stable indefinitely.

Reagent B: Concentrated sulphuric acid

Standard : Prepare the stock solution of D-glucose (0.10 mg/ml) in UHQ water and dilute into various concentration, 0-30 μg/ml.

Samples : WD7 biopolymer (10 mg) in UHQ water of 10 ml (1,000 μg/ml)

Procedure : Mix samples, standards and control solutions (200 µl containing up to 100 µg carbohydrate) with 200 µl of reagent A. 1.0 ml of reagent B was added rapidly and directly to the solution surface without touching the sides of the tube. Leave the solutions undisturbs for 10 min before shaking vigorously. Determine the absorbance at 490 nm after a further 30 min. The carbohydrate content was plotted against the corresponding absorbance resulting in the calibration curve (Figure 43) which was used to determine the total of carbohydrate content of WD7 samples. Other aldoses, ketose and alduronic acids respond to different degrees.

Reducing Sugar : Reducing sugar (hexose as glucose) contents of the WD7 biopolymer samples were determined using Dinitrosalicylic acid (DNS) assay (Chaplin and Kennedy, 1994).

Reagent A : Dissolve 0.25 g of 3,5-dinitrosalicylic acid and 75 g sodium potassium tartrate (Rochelle salt) in 50 ml 2M sodium hydroxide (made by dissolving 4 g NaOH in 50 ml water) and dilute to 250 ml with water. This is stable for several weeks.

Standard : glucose 0.5-50 µg/µl

Samples : WD7 biopolymer (10 mg) in UHQ water 10 ml (1,000 µg/ml).

Procedure: Mixed well of the samples, standards and controls (100 µl) with 1.0 ml of reagent. Heat the mixtures at 100^{o}C for 10 min. After rapid cooling to room temperature, determine the absorbance at 570 nm. The weight of glucose was plotted against the corresponding absorbance resulting in the calibration curve (Figure 44) which was used to determine the fructose contents of WD7 samples. Dissolved molecular oxygen interferes with this assay. This may be overcome either by purging the assay solutions with nitrogen or helium prior to the assay or by the addition of a fixed known small amount of glucose (~20 µg) to all samples in order to raise the total reducing sugars concentration above a critically low value. Non-carbohydrate reducing agents also interfere with this assay. Some metal ions [e.g. manganous, cobalt (II), and calcium] may increase the assay response.

L-Cysteine Sulphuric Acid Assay : Total carbohydrate as reducing sugar, xylose (as pentose) and glucose (as hexose) contents were determined using L-Cysteine sulphuric acid assay (Chaplin and Kennedy, 1994).

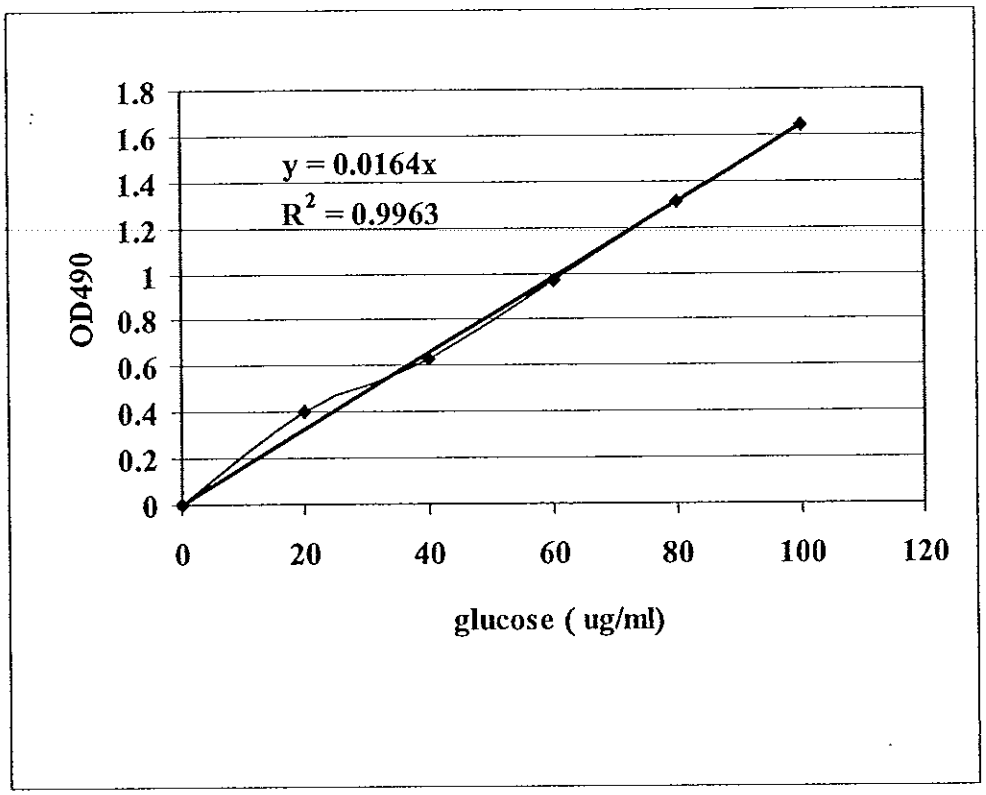


Figure 43 Calibration curve for total sugar content (as glucose) at OD_{490} determined by phenol sulphuric acid assay

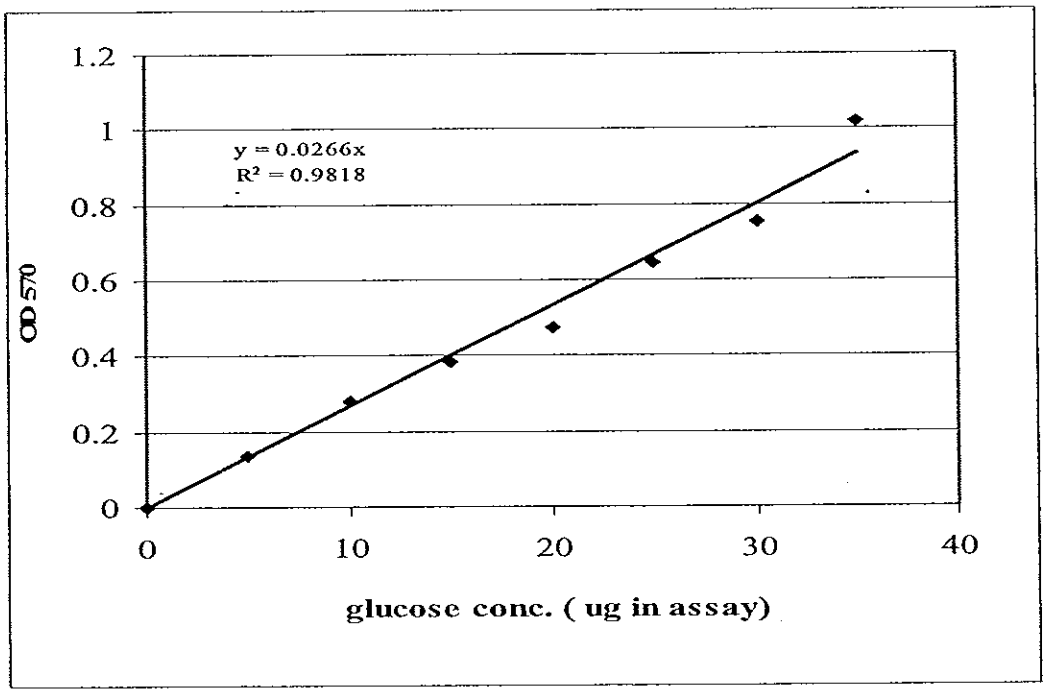


Figure 44 Calibration curve of glucose at OD_{570} for determination of reducing sugar (hexose) content by DNS method

Reagents :

Reagent A: Ice-cold 86% (v/v) sulphuric acid, prepared by the very careful addition of 860 ml high quality concentrated sulphuric acid to 140 ml of water. The reagent is stable if kept dust-free in a stoppered container.

Reagent B: L-cysteine hydrochloride (700 mg/l) in reagent A was prepared no earlier than the day before use.

Standard : Prepare the stock solution of D-xylose (0.2 mg/ml) and D-glucose (0.1 mg/ml) in UHQ water and dilute into various concentration, 0-40 μg/ml for D-xylose and 0-30 μg/ml for D-glucose.

Samples : WD7 biopolymer (10 mg) in UHQ water of 10 ml (1,000 μg/ml)

Procedure: Reagent B (1ml, precooled to 4°C) was added to precooled standard solutions (containing 0-35 μg xylose and 0-25 μg glucose) and samples (200 μl) with immediate thorough mixing in an ice bath. The reactants were heated at 100°C for 3 min in glass stoppered test tube and rapidly cooled to ambient temperature. Absorbances were determined at wavelengths of 415 nm and used for preparation the calibration curves (Figure 45) of D-glucose obtained from this assay.

7.1.2.2 Ketose Content

The ketose sugar (as fructose) contents of the WD7 biopolymer samples were determined using Phenol-boric acid-sulphuric acid assay (Chaplin and Kennedy, 1994).

Reagents :

Reagent A: Phenol solution (recrystallized from methanol and ethanol, 2.5 g in 50 ml in water) was added with acetone droppwise with constant stirring over a period of 10 min and stir the mixture for a further 10 min at room temperature. The boric acid (2 g) was dissolved in this mixture. The reagent is table for at least 2 weeks at 4°C.

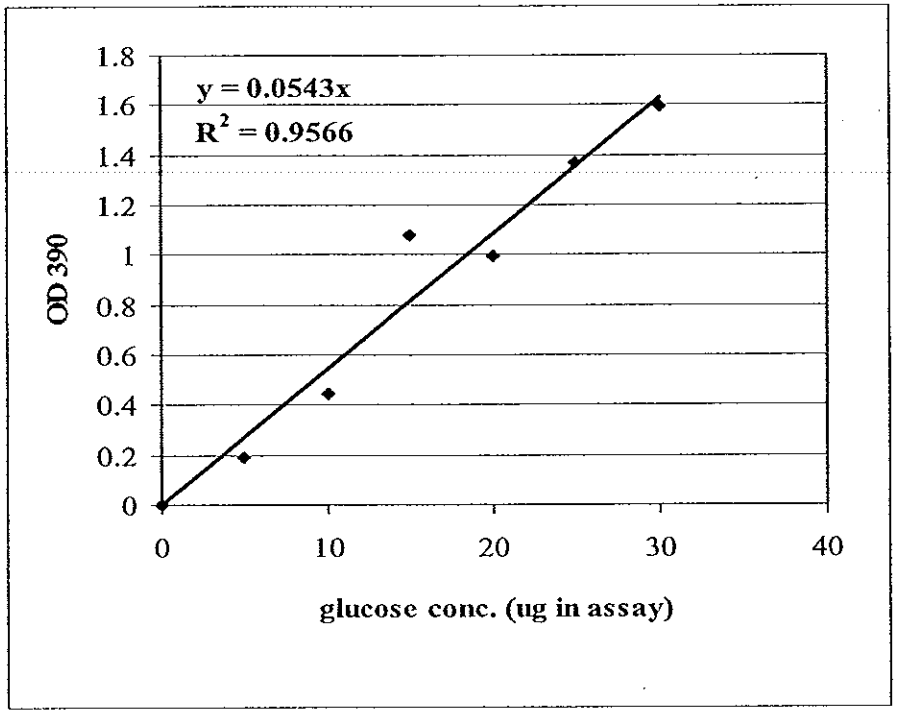
Reagent B : Concentrated sulphuric acid

Standard : Fructose 0.5-50 μg/μl

Samples : WD7 biopolymer (10 mg) in UHQ water 10 ml (1,000 μg/ml)

Procedure : Mixed well of the samples, standards and control solutions (100 μl) with 0.5 ml of reagent A .and then the reagent B (1.4 ml) was added rapidly to the surface, avoiding the sides of the tubes. After thorough mixing, the solutions was left for 5 min at room

(a)



(b)

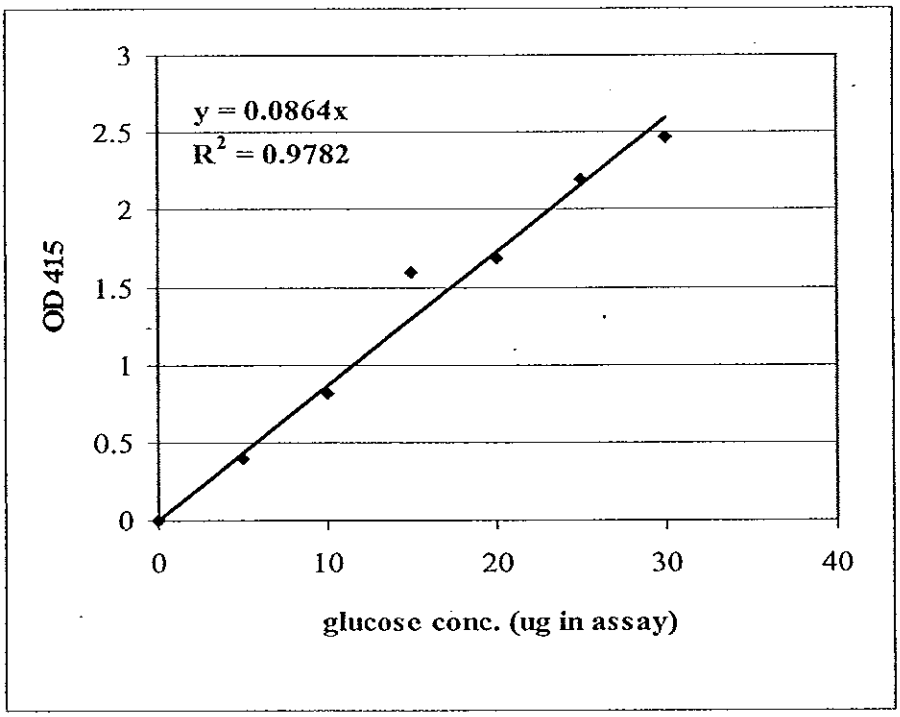


Figure 45 Calibration curve of xylose (pentose, a) and glucose (hexose, b) for determination of total carbohydrate by L-cystein assay at OD_{390} and OD_{415}, respectively

temperature and incubated at 37°C for 1 h. The absorbance was determined at 568 nm.

Standard calibration curve of fructose at 568 nm for determination of ketose content was given in Figure 46. Different ketoses give differing adsorbances in this assay. Interferences from non-ketose carbohydrate is slight (<1%) to non-existent. The reproducibility of this assay is strongly dependent on the manner of the addition of the sulphuric acid.

7.1.2.3 Pentose Contents

The pentose contents of the WD7 biopolymer samples were determined using Ferric-orcinol assay (Chaplin and Kennedy, 1994).

Reagents :

Reagent A: Trichloroacetic acid solution in water (10% w/v). This is stable indefinitely.

Reagent B: Freshly prepared solution of ferric ammonium sulphate (1.15% w/v) and orcinol (0.2% w/v) in 9.6 M hydrochloric acid (made by diluting five parts concentrated HCl with one part water).

Standard : glucose 0.5-50 μg/μl

Samples : WD7 biopolymer (10 mg) in UHQ water 10 ml (1,000 μg/ml).

Procedure: Mixed well of the samples, standards and control solutions (200 μl) containing up to 40 μg pentose with 200 μl of reagent A. Heat the mixtures at 100°C for 15 min. Cool the solution rapidly to room temperature. Add reagent B (1.2 ml) and mix well. Reheat the solution at 100°C for a further 20 min. Then cool the solution to room temperature and determine the absorbance at 660 nm. The pentose content was plotted against the corresponding absorbance resulting in the calibration curve (Figure 47) which was used to determine the total of pentose content of WD7 samples.

Hexose interfere in this assay but can be accounted for by additionally determining the absorbance at 660 nm at which wavelength they have a strong absorbance. It is recommended that both hexose and pentose standard are used where the hexose content of the samples might be considerable.

7.1.2.4 Protein Content

Protein contents of the WD7 biopolymer samples were determined using Comassy Blue assay (Chaplin and Kennedy, 1994).

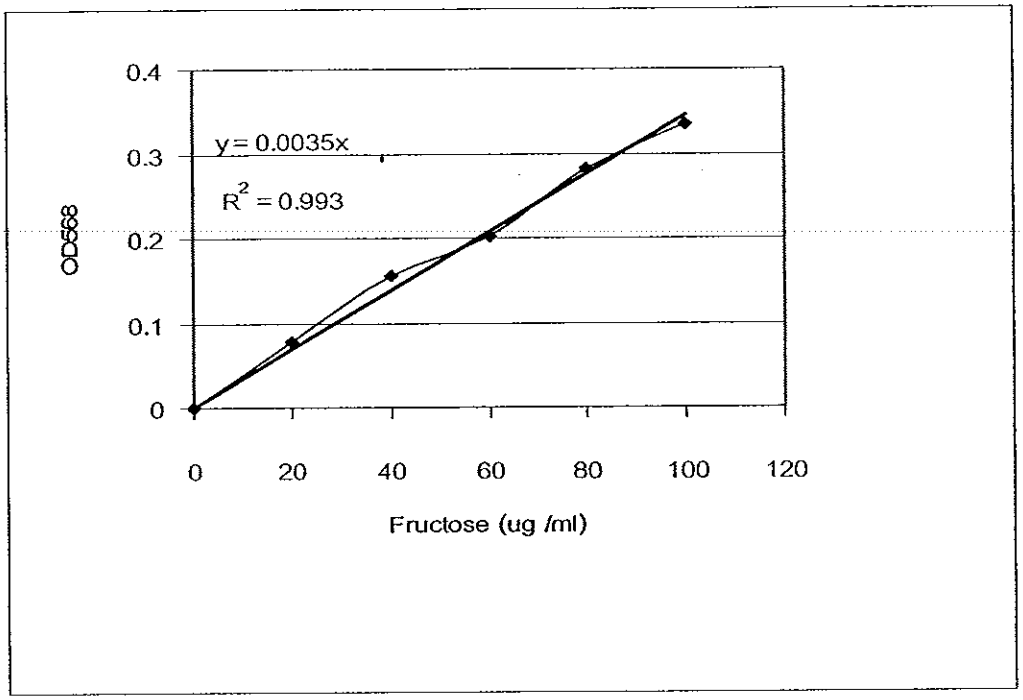


Figure 46 Standard calibration curve of fructose at OD_{568} for determination of ketose content by phenol-boric acid-sulphuric acid assay

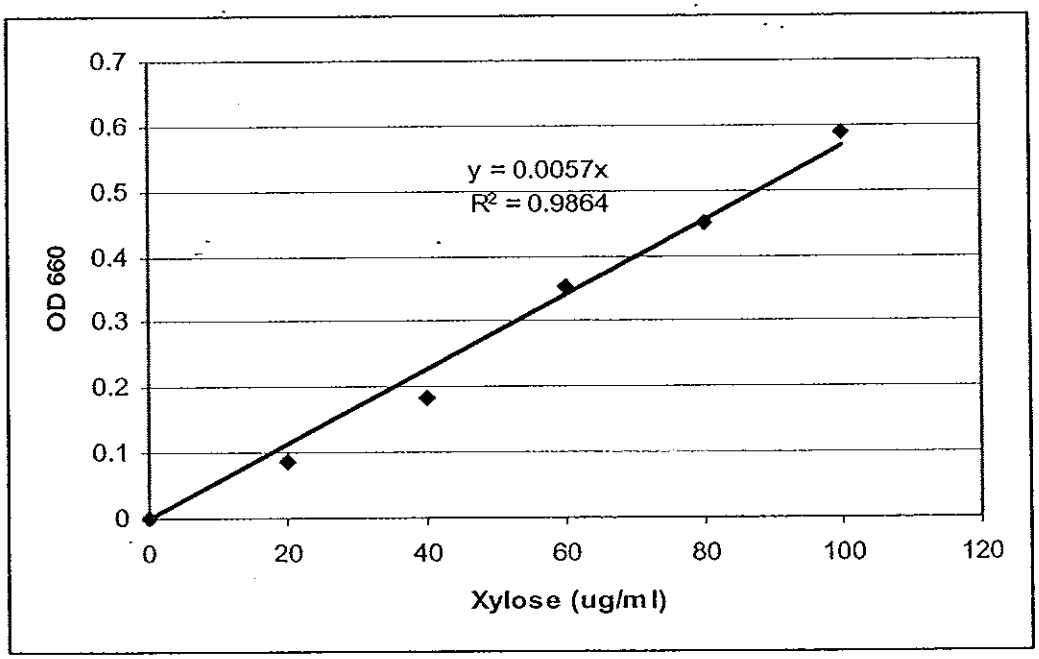


Figure 47 Standard calibration curve of xylose at OD_{560} by Ferric-orcinol assay for determination of pentose content

Reagents :

A) Dye reagent: Comassie Brilliant Blue G (10 mg) was dissolved in the mixture of 85% phosphoric acid (10 ml) and 95% ethanol (5ml). After the dye had completely dissolved, the volume of the dye reagent was made up volume to 100 ml with cold Ultra-high quality (UHQ) water.

B) 1M NaOH: Weight 4.670 g of NaOH and dissolve into 100 ml of UHQ water.

C) Standard: Stock solution of BSA 0.25 mg/ml concentration and dilute with UHQ water into the 0-25 μg protein were prepared:

D) Samples: WD7 samples (1.0 mg/ml or 1,000 μg/ml in UHQ water)

Procedure : Pipette all standard solutions of BSA and sample solutions, 20 μl into the test tubes, add 1M NaOH (50 μl) mix well and then dye reagent (A), 1 ml. The contents were mixed by vortexing and incubate at room temperature for 5 min. Then the mixtures in glass cuvettes were measured by spectrophotometer at the absorbances at 590 nm. The weight of protein was plotted against the corresponding absorbance resulting in the calibration curve (Figure 48) which was used to determine the protent contents of samples.

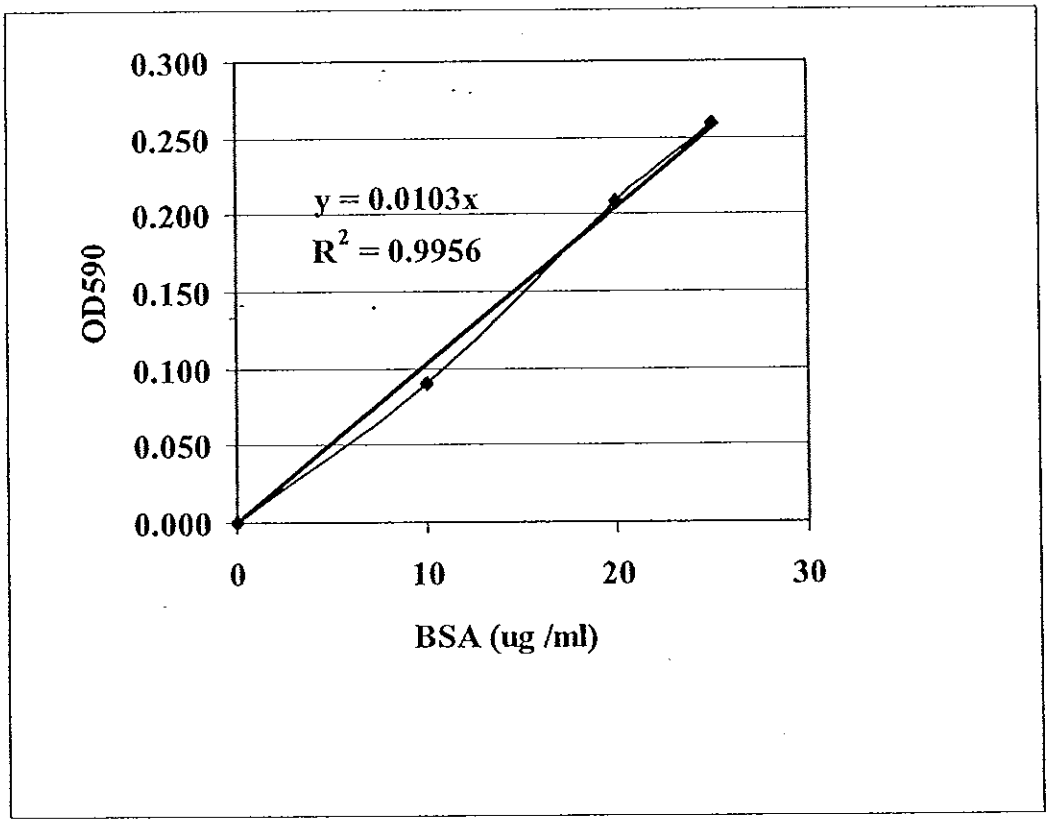


Figure 48 Standard calibration curve of protein (Bovine serum albumin, BSA) at OD_{590} by Comassy Blue assay

7.1.2.5 Uronic acid contents

The uronic acid contents of WD7 biopolymer samples were determined using Carbazole assay (Chaplin and Kennedy, 1994).

Reagents :

A) Ice-cold reagent: Dissolve 0.95 g of sodium tetraborate decahydrate (borax) in 0.025 M of ice-cold concentrated sulphuric acid carefully with stirring and leave dissolved overnight. This regent is stable indefinitely if refrigerated.

B) Carbazole reagent: Dissolve 125 mg of Carbazole (recrystallized from ethanol) in 100 ml of absolute ethanol to give a stable reagent.

Standard : D-Glucuronolactone (25 mg) dissolve in 25 ml UHQ water [(1.0 mg/ml), 1,000 µg/ ml] and dilute with UHQ water into the 0, 50, 100, 150, 200, 250 µg/ml.

Samples : WD7 biopolymer (10 mg in 10 ml of UHQ water).

Procedure: Pipetted the samples, standards and controls (250 µl) and put into the tubes, (the used samples should contain 0-20 µg of hexuronic acid). Added 1.5 ml of dye reagent (A) ($4^{o}C$), mixed well. The contents were mixed by vortexing and incubated at $100^{o}C$ in water bath for 10 min, then cooled rapidly in the ice-bath,added 50 µl of reagent B and mixed well. Reheated at $100^{o}C$ for 15 min, cooled rapidly to room temperatur and determined the absorbance scanning, 400-700 nm (or 525nm). The weight of uronic acid was plotted against the corresponding absorbance resulting in the calibration curve (Figure 49) which was used to determine the uronic acid contents of samples.

7.1.3 Monosaccharide Identification by Thin-layer chromatography (TLC)

The purified dried polysaccharide (5mg) is heated and hydrolyzed in glass vial with cap by hydrolysis reagent (10 ml) of

a) 2N- Trifluoro acetic acid at $100^{o}C$ for 4 h (Petry, 1988)

b) Concentrated H_2SO_4 at $105^{o}C$ for 2 h and over night (Laity, 1995)

and then neutralized with 1M NaOH. The reaction mixture is left to precipitate. The supernatant is removed and evaporated in oven to concentrate for 30 min. The concentrated acid hydrolysate is spotted into the silica plate comparison with many monosaccharides sugar (1 mg/ml) such as glucose, arabinose, fructose galactose, rhamnose , rhibose, sorbitol, xylose as standard reagent for TLC method. This TLC plate

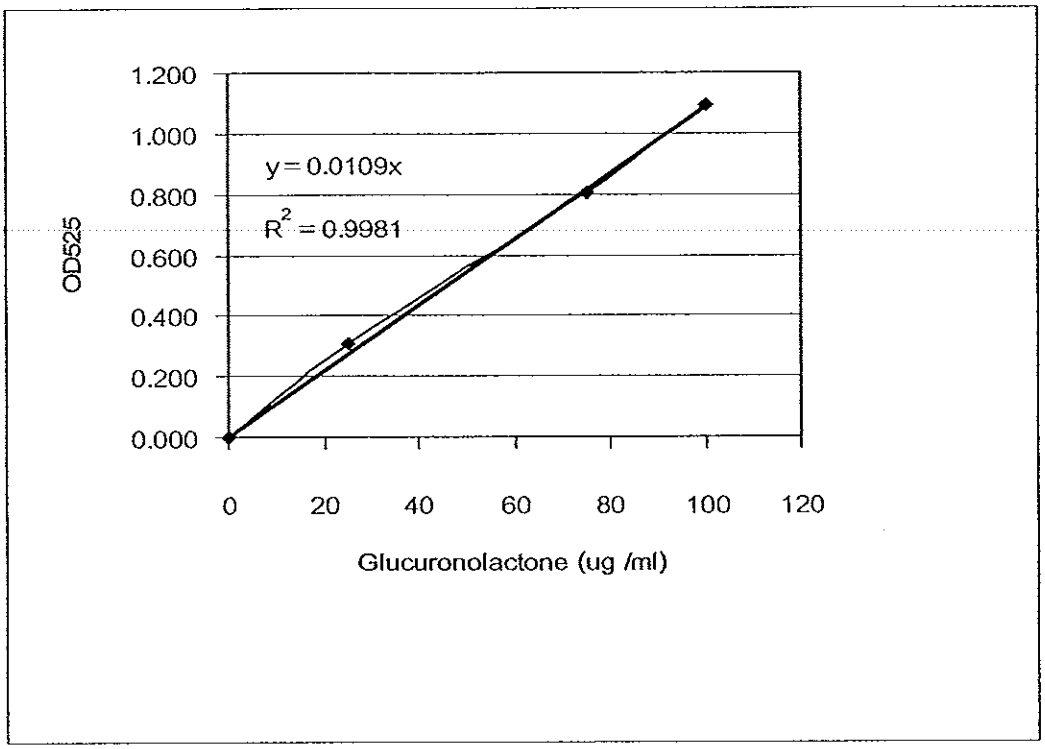


Figure 49 Calibration curve of D-glucuronolactone at OD_{525} for determination of uronic acid by Carbasol assay

will be placed into the glass chamber which put the solution as developing (mobile) phase, here are isopropyl alcohol + acetone + 0.1 M lactic acid (2:2:1) for a) and acetonitrile +water (9:1) for b). Leave it until developing phase diffuse to the top of TLC plate by request, take it out, put into the aluminum tray and then dry it in the oven 105°C for 5 min. After that, spray it with 0.5% Naphol solution (compost of Naphol 1 g, absolute ethanol 190 ml and H_2SO_4 conc. 10 ml) in fume removing cupboard to protect poison smell and dry it again in hot air oven for 20 min. The color spots of samples will appear on TLC plate, measure the distance of diffusion (cm) of each spot divided with the distance of developing phase, that is called Rf value. The color of spots also indicate to sugar types. The color and Rf value of the hydrolyzed WD7 biopolymer sample spot will be compared with each standard sugar spots for indication and identification of the sugar and monosaccharide constituents of this WD7 biopolymer.

7.1.4 Gel Permeation Chromatograh (GPC)

GPC was used in this thesis was operated under setting of parameters as following:

Equipment: GPC (Dionex 500 system) comprising: Dionex GP50 gradient pump; Dionex AS50 autosampler; Dionex UI20 Universal Interface (for DRI-PC Link)

Columns: Progel™-TSK PW_{XL}guard, Progel™-TSK G6000 PW_{XL}, Progel™-TSK G4000 PW_{XL}, Progel™-TSK G2500 PW_{XL}, linked in series

Detector: Knauer differential refractive index (DRI) detector

Eluent: Helium degassed phosphate buffered saline (PBS) solution, final pH 7.0 containing of sodium dihydrogen phosphate ($NaH_2PO_4 \cdot H_2O$, 0.0003M), 0.05M disodium hydrogen phosphate (Na_2HPO_4, 0.0016M) and sodium chloride (0.145M NaCl).

Flow rate: 0.5 mL/min

Calibrants: Pullulan polysaccharides (M_W 853, 380, 186, 100, 48, 23.7, 12.2 and 5.8 kDa) dissolved overnight in phosphate buffer solution (PBS) at 4°C (1 mg/mL, 100 μL injection).

Software: Data was collected from the DRI detector via the UI20 interface using Dionex Chromeleon software and analysed using Polymer Laboratories PL Caliber Reanalysis software.

7.2 Analysis of Physical Properties

7.2.1 Rheological Properties

The rheological flow behavior (power-law parameters, K and n) were investigated by using a TA Instruments AR1000 'Rheolyst' controlled stress rheometer with geometry 4 cm flat steel plate as test head, TA Instrument serial no. 980646. Temperature control is by means of a standard Peltier plate which has an operating at 30°C (a maximum ramp rate of ~ 20 °C/min). The consistency (K), flow index or rate index value (n) determinations were performed by non-linear regression with the data provided by the viscometer, i.e. the apparent viscosity (η) as a function of shear rate (γ) (Audet *et al.*, 1998) according to the equation ; $\eta = K\gamma^n$.

Profiles were analyzed by TA Instrument programs with the 'Rheology Solutions' software package and fit curve models; Power Law , Cross model, Carreau model, Williamson, and Sisko models with their equations shown following below. It allows the zero shear viscosity (η_0), the infinite shear viscosity (η_∞), K and n values and Thixotropy (T). The n value was calculated to identified how shear thinning (n > 1), or shear thickening (n <

1) the WD7 biopolymer solutions are. These model focuses on the shear thinning region of the grap where the zero shear viscosity ($\eta_o >> \eta >> \eta_\infty$).

7.2.2 Texture Analysis for Adhesion and Gel Strength

The maximum adhesion load measurements were performed using a Stable Micro Systems TA-XT2I Texture Analyzer. The texture analyzer utilizes a load cell to measure the loads associated with the forces generated within a test sample as a function of time and/or distance, depending on the sample probe and test methodology employed. Probe movement is controlled via a linear speed motor, an integral part of the analyzer head also incorporating the load cell. The load cell used for these experiments (5 kg) was calibrated each day with a 2 kg brass calibration weight prior to the commencement of measurements. Instrument operation is PC-controlled: the software package provides individual graphics, spreadsheet, macro and chart windows for maximum flexibility in data manipulation.

Maximum adhesion load measurements; These were carried out using a 45 mm diameter cylindrical probe, the sample being situated on the center of the base platen platform. The sample was centered using 'target" rings engraved on the upper face of the platen: the area covered by the sample was circular, its diameter being 40 mm. Prior to the start of each individual measurement the probe height was calibrated (to 40.0 mm in this case) : this was essential since the probe and base platen were removed for cleaning and drying between each test measurement. The gel was spread within the 40 mm target area to a uniform height of ca. 2-3 mm. This ensured that there was sufficient sample to fill completely the 45 mm x 0.5 mm high cylindrical space between the probe face and base platen when the probe was at its lowest point, without excessively large amounts of gel being squeezed out. The test procedure consisted of two different phases.

i) The probe was lowered from a height of 40.0 mm above the base platen towards and into the sample at a velocity of 2.0 mm/s, with the load cell monitoring the increasing load, until the distance between the probe and platen was 0.5 mm. At this point, the second phase was triggered.

ii) The direction of probe travel was then reversed, its velocity being increased to 10.0 mm/s. The load cell monitored changing load as a function of time as long as physical contact was maintained between platen, gel and probe.

The raw data for each individual test comprise the monitored load measured as a function of time (probe displacement). The data are displayed and recorded graphically. The point at which the load-time carve crosses the X (time) axis at the start of Phase 2, the

maximum load value attained in this phase, and the time at which the load subsequently first returns to zero are the key data utilized in the calculation and assessment of sample behavior.

7.3 Aanalysis of Biological Activities

Biological activities and biocompatibility of were studied in this topics as below. Crude samples in powder form and partially purified samples after dialysis and prepared into freeze dried forms were prepared as solution and used for this testings. The WD7 biopolymer samples at optimal concentrations (5 - 50 μg / ml) were used for this testing.

7.3.1 Antibacterial Test

Strephylococcus aureus was grown on potato dextrose agar (PDA) plate at 30°C for 3 days. Three to five single colonies were then suspended in RPMI 1640 and cultured in a shaking flask until cell density reaches 2x 10^6 CFU/ml. The 100 μl of the culture was added to each well of 96 well plate containing 100 μl of biopolymer solution sample and incubated at 37°C for 4 hrs. 50 μl of XTT/PMS mixure solution (mix 1 mg/ml of XTT in RPMI 1640 with 1.53 mg/ml of PMS in PBS at the ratio of 1:6 was added to each well and incubated at 37°C for an additional 4hrs. Subsequently absorbance at 450 nm was determined using the multilabel counter Victor3V. Orange color developed in each well indicates growth of *S. aureus* while inhibitory effects of the sample result in no change of color. Amphotericin B and DMSO were used as a positive and a negative control, respectively.

Interpretation of testing: if sample have bioactivity on tested microorganism in the range of low concentration which show the moderate and strong activity, serial dilution of samples for IC_{50} test should be investigated.

7.3.2 Antifungal Test

Candida albican ATCC 90028 was grown on potato dextrose agar (PDA) plate at 30°C for 3 days. Three to five single colonies were then suspended in RPMI1640 and cultured in a shaking flask until cell density reaches 2 x 10^6CFU/ml. The 100 μl of culture was added to each well of 96 well plate containing 100 μl of biopolymer solution sample and incubated at 37°C for 4 hrs. The 50 μl of XTT/PMS mixure solution (mix 1 mg/ml of XTT in RPMI 1640 with 1.53 mg/ml of PMS in PBS at the ratio of 1:6 was added to each well and incubated at 37°C for an additional 4 hrs. Subsequently absorbance

at 450 nm was determined using the multilabel counter Victor3V. Orange color developed in each well indicates growth of *C. albicans* while inhibitory effects of the sample result in no change of color. Amphotericin B and DMSO were used as a positive and a negative control, respectively.

Interpretation of testing : if sample have bioactivity on tested microorganism (*Candida albicans*) in the range of low concentration which show the moderate and strong activity, serial dilution of samples for IC_{50} test should be investigated.

7.3.3 Antiviral test

Anti-herpes simplex virus type HSV-1 activity of WD7 biopolymer was tested against HSV-1 strain ATCC VR 260, using colorimetric microtiter plate assay that determines host cell growth by measuring cellular protein content, as described by Skehan *et al.*(1990). The growth of host cells, vero cell line ATCC CCL-81, infected with virus and treated with extract was compared with control cells, infected with virus only. Acyclovir and DMSO were used as positive and negative control, respectively. The biopolymer samples were tested at non-cytotoxic concentrations inhibit cell growth (<50%). Extracts that inhibit virus more than 50% were tested further to determine the concentrations that inhibit viral activity by 50% (IC_{50}). Ellipticine and Acyclovir agent were used as positive controls for cytotoxicity test and anti-HSV-1 test, respectively.

Interpretation of testing : if sample have cytotoxicity, results in the test range of high concentration which show the strong activity, IC_{50} on virus test should be investigated.

7.3.4 Antiplasmodial activity

Plasmodium falciparum (K1, multi drug resistant strain) was cultivated *in vitro* according to Trager & Jensen (1976) in RPMI 1640 medium containing 20 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), 32 mM $NaHCO_3$ and 10% heat activated human serum with 3% erythrocytes and incubated at 37°C in an incubator with 3% CO_2. Cultures were diluted with fresh medium and erythrocytes every day according to cell growth. Quantitative assessment of antimalarial activity *in vitro* was determined by microculture radioisotope techniques based upon the methods described by Desjardins *et al.* (1979).

A mixture of 200 µl of 1.5% erythrocytes with 1% parasitemia at the early ring stage was pre-exposed to 25 µl of the medium containing a test sample dissolved in 1% DMSO (0.1% final concentration) for 24 hr employing the incubation conditions described

above. Subsequently, 25 µl of [^{3}H] hypoxanthine (Amersham, USA) in culture medium (10 µCi) was added to each well and plates were incubated for an additional 24 hr. Levels of incorporated radioactively labeled hypoxanthine indicating parasite growth were determined using the TopCount microplate scintillation counter (Packard, USA). Inhibition concentration (IC_{50}) represents the concentration which indicates 50% reduction in parasite growth. The standard sample was Dihydroartemisinine (DHA).

7.3.5 Anti-TB test (Alamar blue susceptibility test; MABA)

Alamar blue susceptibility test (MABA) ; Preparation of bacterial seed lots ; *Mycobacterium tuberculosis* $H_{37}Ra$ was grown in 100 ml of 7H9GC containing 0.005% Tween 80. Culture was incubated in 500 ml plastic flask on a rotary shaker at 200 rpm and 37°C until they reached an optical density of 0.4-0.5 at 550 nm. Bacteria were washed and suspended in 20 ml of phosphate-buffered saline and passed through an 8-µm-pore-size filter to eliminate clumps. The filtrates were aliquoted, stored at −80°C.

Antimicrobial susceptibility testing was performed in 96-well microplates. Outer perimeter wells were filled with sterile water to prevent dehydration in experimental wells. Initial screened-sample dilutions were prepared in either dimethyl sulfoxide or distilled deionized water. The dissolved-screened samples were then diluted by Middlebrook 7H9 media containing 0.2% v/v glycerol and 1.0 gm/L casitone (7H9GC), and subsequent twofold dilutions were performed in 0.1 ml of 7H9GC in the microplates. Frozen inocula were diluted 1: 100 in 7H9GC. Addition of 0.1 ml to the well resulted in final bacteral titers of about $5x10^4$ CFU/ml. Wells containing sample only were used to determine whether the tested-samples themselves can reduce the dye or not. Additional control wells consisted of bacteria only (B) and medium only (M). Plates were incubated at 37°C. Starting at day 6 of incubation, 20 µl of Alamar Blue solution and 12.5 µl of 20% Tween 80 were added to one B well and one M well, and plates were reincubated at 37°C. Wells were observed at 24h for a color change from blue to pink. If the B wells became pink by 24h, reagent was added to the entire plate. If the well remained blue, additional M and B wells were tested daily until a color change occurred, at which time reagents were added to all remaining wells. Plates were then incubated at 37°C, and results were recorded at 24h post-reagent addition. Visual MICs were defined as the lowest concentration of sample that prevented a color change (Collins and Franzblau, 1997).

7.3.6 Cytotoxicity test on HT 29 human colon cancer (Sulforrhodamide B colorimetric assay)

The cell lines of HT 29 (human colon cancer) were grown as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) at 37°C in an atmosphere containing 5% CO_2 in air and 100% relative humidity. Medium was supplemented with 10% fetal bovine serum, Antibiotic-Antimycotic [penicillin G sodium, Streptomycin sulfate and amphotericin B as Fungizone in 0.85% saline]. Adherent cells at a logarithmic growth phase (18-24 h) were plated (100 µl per well) in 96 well flat-bottom microplates at densities of 3,000 cells per well. The biopolymer were pasteurized add to the plates at various concentrations (0.1-3 mg/ml) in a volume 100 µl/well (five replicate wells per cell density). Microplates were lift for 92 h at 37 °C so that cells were able to attach to the bottom of the wells before the fixation protocol was carried out. In fixation protocol, cold (4°C) 50% Trichloroacetic acid (TCA) was added in each well to avoid fluid shearing forces which could result in cell detachment and loss. Microplates were left for 1 h and subsequently washed 5 times with distilled water and then left to dry at room temperature (Skehan *et al.*, 1990).

The SRB assay (0.4% w/v sulforhodamine B in 1% acetic acid solution) was added to each well and left at room temperature for 30 min and then washed 5 times with distilled water to remove SRB before air drying. Bound SRB was solubilized with unbuffered Tris-base solution (Sigma) and left on a shaker for at least 10 min. Absorbance was read in a 96 well plate reader at 492 nm. The test optical density (OD) value of WD7 biopolymer was defined as the absorbance in each individual well, minus the blank value. Dose-response curves were plotted % toxicity and % survival comparison to positive control, chitosan.

7.3.7 Anticancer assay (KB, BC and NCl-H187)

The KB (Human epidermoid carcinoma of cavity, ATCC CCL-17, BC (Breast cancer cell line) and NCl-H187 (human, small cell lung cancer) were determined by colorimetric cytotoxicity assay that measured cell growth from cellular protein content. Elliptine and doxorubicin were used as positive control. DMSO was used as negative control. Briefly, Cells at a logarithimic growth phase were harvested and diluted to 105 cells/ml with fresh medium and gently mixed. Test compounds were diluted in distill water and put into microtiter plates in total volume 200µl. Plates were incubated at 37°, 5% CO_2 for 72h.

After incubation period, cell were fixed by 50% trichloroacetic acid. The plates were incubated at 4°C for 30min, washed plates with tap water and air-dried at room temperature. The plates were stained with 0.05% sulforhodamine B dissolved in 1% acetic for 30 min. After staining period, SRB was removed with 1% acetic acid. Plates were air-dried before bound dye was solubilized with 10 mM Tris base for 5min on shaker. OD was read in microtiter plate reader at wavelength of 510 nm. Ellipticine and Doxorubicine was used as positive controls for both cancer cells (Plumb *et al.*, 1989 and Skehan *et al.*, 1990).

Interpretation of testing: if sample have cytotoxicity, results in the test range of low concentration which show the strong activity, IC_{50} on anticancer test should be carried out.

7.3.8 Anti-inflammation assay

Cell culture and treatment : Immortalized mouse PGH-1 and PGH-2 null cells at the concentration of $1x10^5$ cells/ml incomplete Dubelcco' s Modified Eagle Meduim (DMEM) high glucose supplemented with hygromycin B (200 µg/ml), non essential amino acid (0.1 mM), L-glutamine (50 mg/L), ascorbic acid (0.05 mg/ml) and 10% fetal calf serum (FCS) were seeded into 96-well flat bottom tissue culture plates(83 µl/well). Cells were incubated 37°C in humidified incubator with 5%CO_2 for 72 h, then washed with DMEM medium without FCS and preincubated for 30 min with 83 µl of serum-free DMEM medium containing vehicle or drugs. Following the preincubation period, the medium was removed and cells were immediately treated with serum-free medium containing vehicle or drugs and 20 µM arachidonic acid (AA) or 2 µM A23187 for 30 min. Culture supernatants were then collected from wells and analyzed for PGE_2 concentrations by radioimmunoassay (RIA).

PGE_2 Measurement: The RIA method used for measuring PGE_2 concentrations in the culture supernatant is based on the competition between PGE_2 in the samples and ^3H labeled PGE_2 for anti-PGE_2 antibody binding sites. The assay was performed on ice as following. To 1.5 ml microcentrifuge tubes 50 µl of culture supernatant, diluted 1:10 in DMEM or, for blank and zero % binding tube, 50 µl of DMEM were added. Then, 50 µl of anti-PGE_2 antibody in RIA buffer (0.1 mM phosphate buffer, pH 7.4, containing 0.9% sodium chloride, 0.1% sodium azide and 0.1% gelatin) was added to every tube accept for the blanks, in which 50 µl RIA buffer was added. Subsequently, 50 µl of ^3H-PGE_2 (1.12 µci/ml) , was added to each tube, vortexed briefly and incubated overnight at 4°C. Then,

100 µl at 2% charcoal-dextran suspension in RIA buffer was added to each tube. After 15 min incubation on ice, the tube were centrifuged at 3800 rpm (1500 g) at 4°C for 10 min. Supernatants were then transfered to new 1.5 ml microcentrifuge tubes containing liquid scintillation cocktail, vortexed and counted for radioactivity. The resulting radioactive counts were used to calculate % binding of ^{3}H-PGE_2, which were then used for the estimation of PGE_2 concentrations from standard curves.

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List of Publications and Proceedings

Publication

Bandaiphet, C., and Prasertsan, P. 2006. Effect of aeration and agitation rates and scale-up on oxygen transfer coefficient, $k_L a$ in exopolysaccharide production from *Enterobacter cloacae* WD7. Carbohydrate Polymer. 66:216-228.

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