Optimization for Biopolymer Production by *Enterobacter cloacae* WD7

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Thesis Title	Optimization for Biopolymer Production by *Enterobacter cloacae* WD7
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ชื่อวิทยานิพนธ์	สภาวะที่เหมาะสมต่อการผลิตพอลิเมอร์ชีวภาพโดยเชื้อ *Enterobacter cloacae* WD7
ผู้เขียน	นายสันทัด วิเชียรโชติ
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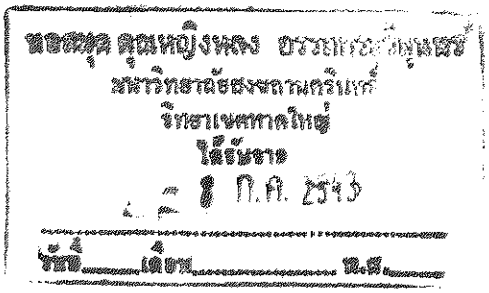
บทคัดย่อ

จากการศึกษาปัจจัยที่มีผลต่อการผลิตพอลิเมอร์จากเชื้อ *Enterobacter cloacae* WD7 เมื่อเลี้ยงในอาหารสูตรพื้นฐาน (พีเอช 7.0) โดยใช้ 1 เปอร์เซ็นต์ กลูโคสเป็นแหล่งคาร์บอนเป็นเวลา 5 วัน ที่อุณหภูมิ 30 องศาเซลเซียส พบว่าเชื้อผลิตพอลิเมอร์ได้สูงสุด 2.14 กรัมต่อลิตรในวันที่ 3 เมื่อเปรียบเทียบผลจากการใช้แหล่งคาร์บอนชนิดต่างๆ พบว่า กาแลคโตสและซูโครสให้พอลิเมอร์ (2.50 และ 2.45 กรัมต่อลิตร ตามลำดับ) สูงกว่าใช้ มอลโตส ฟรุกโตสและกลูโคส เลือกซูโครสเป็นแหล่งคาร์บอนเนื่องจากมีราคาถูกกว่า ความเข้มข้นที่เหมาะสมของซูโครสคือ 3 เปอร์เซ็นต์ ได้พอลิเมอร์ 2.63 กรัมต่อลิตร การเติมแหล่งไนโตรเจนไม่ว่าอนินทรีย์ไนโตรเจน (แอมโมเนียมซัลเฟต แอมโมเนียมคลอไรด์ และแอมโมเนียมไนเตรต) และอินทรีย์ไนโตรเจน (โพลีเปปโตน) ไม่ทำให้ผลผลิตพอลิเมอร์สูงขึ้น ความเข้มข้นของยีสต์สกัดที่เหมาะสมคือ 0.05 เปอร์เซ็นต์ ซึ่งเท่ากับที่มีในอาหารสูตรพื้นฐาน พีเอชเริ่มต้นที่เหมาะสมคือ 7.0 ที่อุณหภูมิ 30 องศาเซลเซียส ผลการเปรียบเทียบค่าจลนพลศาสตร์การเลี้ยงเชื้อในอาหารสูตรพื้นฐานและสูตรเหมาะสมที่พีเอช 7.0 และ 30 องศาเซลเซียส พบว่าอัตราการเจริญจำเพาะ (μ) เท่ากับ 0.14 และ 0.15 ต่อชั่วโมง ตามลำดับและกำลังการผลิตสูงสุด (R_m) เท่ากับ 0.03 และ 0.04 กรัมพอลิเมอร์ต่อลิตร-ชั่วโมง เวลาที่ใช้เพิ่มเซลล์เป็น 2 เท่า (g) เท่ากับ 4.95 และ 4.62 ชั่วโมง การควบคุมพีเอชเป็น 7.0 ระหว่างการเลี้ยงเชื้อให้พอลิเมอร์สูงกว่าการไม่ควบคุมพีเอช การเพิ่มอัตราการให้อากาศจาก 0.5 เป็น 2.0 ปริมาตรอากาศต่อปริมาตรอาหารต่อนาที ที่อัตราการกวน 200 รอบต่อนาที สามารถเพิ่มความเข้มข้นของพอลิเมอร์เป็น 4.80 กรัมต่อลิตร แต่การเพิ่มอัตราการกวนทำให้เชื้อผลิตพอลิเมอร์ลดลง การใช้น้ำนึ่งปลาทูน่าแทนการใช้ยีสต์สกัด (0.05 เปอร์เซ็นต์) ในปริมาณไนโตรเจนที่เท่ากัน เชื้อให้ผลผลิตพอลิเมอร์สูงกว่าการใช้น้ำนึ่งปลาทูน่าเข้มข้น 10 เปอร์เซ็นต์เป็นอาหารเลี้ยงเชื้อ (4.66 และ 3.24 กรัมต่อลิตร ตามลำดับ) ส่วนการใช้ซูโครสทางการค้าแทนซูโครสเกรดวิเคราะห์พบว่าได้พอลิเมอร์ต่ำกว่า (4.18 และ 4.90 กรัมต่อลิตร ตามลำดับ) พอลิเมอร์ที่ผลิตได้มีคุณสมบัติเป็นสารดูดซับน้ำโดยมีค่าการดูดซับน้ำของพอลิเมอร์ 80.3 กรัมต่อกรัมพอลิเมอร์แห้ง การเปรียบเทียบค่าจลนพลศาสตร์การเลี้ยงเชื้อในฟลาก์สและถังหมักแบบกะโดยใช้อาหารและสภาวะเลี้ยงเชื้อที่เหมาะสม

ได้ค่าดังนี้ อัตราการเจริญจำเพาะเท่ากับ 0.15 และ 0.29 ต่อชั่วโมง เวลาที่ใช้เพิ่มเซลล์เป็น 2 เท่า เท่ากับ 4.62 และ 2.39 ชั่วโมง สัมประสิทธิ์ผลผลิตของเซลล์ ($Y_{X/S}$) เท่ากับ 0.03 และ 0.04 กรัมเซลล์ต่อกรัมซูโครส สัมประสิทธิ์การเปลี่ยนสารอาหารเป็นผลิตภัณฑ์ ($Y_{P/S}$) เท่ากับ 0.25 และ 0.52 กรัมพอลิเมอร์ต่อกรัมซูโครส อัตราผลิตผลิตภัณฑ์จำเพาะ (q_p) เท่ากับ 0.60 และ 0.56 ต่อชั่วโมง อัตราการใช้สารอาหารจำเพาะ (q_s) เท่ากับ 5.0 และ 7.25 ต่อชั่วโมง กำลังการผลิตผลิตภัณฑ์สูงสุด (R_m) 0.04 และ 0.07 กรัมพอลิเมอร์ต่อลิตร-ชั่วโมง และค่าคงที่อิ่มตัว (K_s) เท่ากับ 1.30×10^{-5} และ 2.60×10^{-5} กรัมซูโครสต่อลิตร ตามลำดับ การเลี้ยงเชื้อแบบกึ่งกะโดยเติมสารละลายน้ำตาลซูโครส (10 เปอร์เซ็นต์) เพื่อควบคุมให้ได้ความเข้มข้นในถังหมักเป็น 3 เปอร์เซ็นต์ ทุกๆ 3 วัน ได้พอลิเมอร์สูงสุดเท่ากับ 6.19 กรัมต่อลิตร ในวันที่ 5 ของการเลี้ยงเชื้อ อัตราเจือจาง (D) ของการเลี้ยงเชื้อแบบต่อเนื่องที่ 0.05 ต่อชั่วโมง ได้พอลิเมอร์สูงสุดเป็น 7.28 กรัมต่อลิตร ค่าจลนพลศาสตร์ของการเลี้ยงเชื้อแบบกึ่งกะมีค่าดังนี้คือ สัมประสิทธิ์ผลผลิตของเซลล์ เท่ากับ 0.05 กรัมเซลล์ต่อกรัมซูโครส กำลังการผลิตผลิตภัณฑ์สูงสุด เท่ากับ 0.05 กรัมพอลิเมอร์ต่อลิตร-ชั่วโมง และสำหรับการเลี้ยงเชื้อแบบต่อเนื่อง (อัตราเจือจางเท่ากับ 0.05 ต่อชั่วโมง) มีค่าดังนี้ อัตราเจือจางวิกฤต (D_C) เท่ากับ 0.49 ต่อชั่วโมง อัตราเจือจางสูงสุด (D_m) เท่ากับ 0.485 ต่อชั่วโมง สัมประสิทธิ์ผลผลิตของเซลล์ เท่ากับ 0.03 กรัมเซลล์ต่อกรัมซูโครส และกำลังการผลิตผลิตภัณฑ์สูงสุด เท่ากับ 0.06 กรัมพอลิเมอร์ต่อลิตร-ชั่วโมง



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Abstract

Factors affecting the production of exopolysaccharide (EPS) from *Enterobacter cloacae* WD7 cultivated in basal medium (pH 7.0) using 1% glucose as carbon source for 5 days at 30 °C were investigated. The maximum EPS yield of 2.14 g/l was obtained after 3 days cultivation. Among the carbon sources (1%) tested, galactose and sucrose gave higher EPS yields (2.50 and 2.45 g/l, respectively) compared to maltose, fructose and glucose. Sucrose was selected due to its lower cost. The optimum concentration of sucrose was found to be 3% giving the EPS yield of 2.63 g/l. The addition of either inorganic nitrogen [$(NH_4)_2SO_4$, NH_4Cl and NH_4NO_3] or organic nitrogen (polypeptone) sources had no effect on the EPS yield. The optimum concentration of yeast extract was found to be 0.05% which was the same concentration as in the basal medium. The optimum initial pH was 7.0 at 30 °C. A comparison on growth and EPS production in the basal and optimized media with the initial pH of 7.0 at 30 °C revealed that their specific growth rates (μ) were 0.14 and 0.15 h^{-1} with a maximal productivity (R_m) of 0.03 and 0.04 g EPS/l.h, respectively and generation time (g) of 4.95 and 4.62 h. Control of pH at 7.0 during batch cultivation gave higher EPS yield than under uncontrolled pH condition. An increase in the aeration rate from 0.5 to 2.0 vvm at agitation speed of 200 rpm could elevate the EPS yield to 4.80 g/l while further increase the agitation speed reduced the production of EPS. In addition, 0.05% of yeast extract could be replaced by tuna condensate based on equal nitrogen concentration which gave higher EPS yield than using 10% tuna

condensate medium (4.66 and 3.24 g/l, respectively) as culture medium. In addition, replacement of analytical sucrose by the commercial sucrose gave lower EPS yield (4.90 and 4.18 g/l, respectively). The EPS produced was found to be a water absorbent with the water absorption capacity of 80.3 g/g dried EPS. Comparison on the kinetic parameters of the shake-flask and batch fermentor culture gave the following values; μ = 0.15 and 0.29 h^{-1}, g = 4.62 and 2.39 h, the cellular yield coefficient ($Y_{x/s}$) = 0.03 and 0.04 g cell/g sucrose, the conversion yield ($Y_{p/s}$) = 0.25 and 0.52 g EPS/g sucrose, the specific rate of product formation (q_p) = 0.60 and 0.56 h^{-1}, the specific rate of substrate utilization (q_s) = 5.0 and 7.25 h^{-1}, R_m = 0.04 and 0.07 g EPS/l.h, and saturation constant (K_s) = 1.30×10^{-5} and 2.60×10^{-5} g sucrose/l. For fed-batch culture, addition of 10% sucrose solution to maintain the final concentration in the fermentor at 3% sucrose every 3 days gave the highest EPS yield of 6.19 g/l at 5 days cultivation. Optimal dilution rate (D) of continuous culture was found to be 0.05 h^{-1} which gave the highest EPS yield of 7.28 g/l. The kinetic parameters of fed-batch culture were as follow; $Y_{x/s}$ = 0.05 g cell/g sucrose and R_m = 0.05 g crude EPS/l.h. The kinetic parameters of continuous culture (D = 0.05 h^{-1}) were as follow; the critical dilution rate (D_c) = 0.49 h^{-1}, the maximum dilution rate (D_m) = 0.485 h^{-1}, $Y_{x/s}$ = 0.03 g cell/g sucrose and R_m = 0.06 g crude EPS/l.h.

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Santad Wichienchot

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List of Abbreviations

°C	=	degree Celsius
CFU/ml	=	colony forming unit per millilitre
conc	=	concentration
Da	=	Dalton
g	=	gram
× *g*	=	gravitational force
h	=	hour
L	=	litre
mg/l	=	milligram per litre
min	=	minute
ml	=	millilitre
mM	=	millimolar
nm	=	nanometre
OD	=	optical density
rev/min	=	revolutions per minute
rpm	=	rounds per minute
s	=	second
w/v	=	weight by volume
w/w	=	weight by weight
%	=	percent
g/mol	=	gram per molar
DO	=	dissolved oxygen
pO_2	=	partial pressured oxygen
h^{-1}	=	per hour
vvm	=	volume of air per volume of medium per minute
cP	=	centi poise
mPa.s	=	milli Pascal per second

Chapter 1

Introduction

Under the proper culture condition, the majority of bacterial species secret mucoid substances of high molecular weight (Margaritis and Pace, 1985). When these viscous materials remain associated with the cell, they are called various capsules, sheaths, or lime layers. Whereas capsules and sheaths are well-defined layers external to the cell wall, slime accumulates in large quantities outside the cell wall and diffuses into the medium. Most of the extracellular polymers produced by bacteria are polysaccharides, although a few bacteria produce capsules made up of polypeptides of amino acid residues. The molecular weight and even the composition of these extracellular polysaccharides may vary depending on the culture conditions (Glazer, 1995; Sutherland, 1990). Nowadays industrial polysaccharides are produced from microorganisms substituting those of plant and alga sources (Lee, 1996). Several microbial polysaccharides are now produced commercially by many species of bacteria, as well as some alga and fungi (Lee, 1996) and displaced marine algae and plant gum. The most important microbial polysaccharide is xanthan. Other important microbial polysaccharides include dextran, alginate and pullulan (Blanch and Clark, 1996). The exopolysaccharides produced by microorganisms are widely used in the food, pharmaceutical and chemical industries. The novel microbial polysaccharides are used in various fields such as bioflocculant, bioabsorbent, and heavy metal removal, etc (Bender *et al.*, 1994). Microbial flocculants have been suggested to replace organic high-polymer flocculants such as polyacrylamide which are inexpensive and highly effective, some of them are not easily degraded and some of the monomers can be harmful to humans due to their biodegradability and the harmlessness of their degradation intermediates to the environment. Among the

microbial flocculants reported previously, protein polysaccharide, glycoprotein, polyglutamic acid and xanthan have been investigated (Yokoi *et al.*, 1997). Recently, exopolysaccharide produced by *Enterobacter cloacae* WD7 was reported to have high flocculating activity and could be used as a bioflocculant. However, *E. cloacae* WD7 could produced only 2.27 g/l of crude exopolysaccharide with the flocculating activity of 151.78. *E. cloacae* WD7 was gram negative, facultative anaerobe, short rod, motile, acid production from various sugars such as glucose, galactose, fructose, maltose, sucrose and lactose (Dermlim, 1999).

In this study, optimization for exopolysaccharide production by this strain to increase its yield by optimization of both medium compositions and culture conditions in batch, fed-batch and continuous cultivation were investigated.

Literature Review

1. Types and functional properties of microbial polysaccharide

1.1 Types of microbial polysaccharide

Microbial polysaccharide formation results from the condensation of monosaccharide units by eliminating water between the C1 hydroxyl group of one unit and an available hydroxyl group of another monosaccharide (Margaritis and Pace, 1985). Microbial polysaccharides, which serve different functions in a microbial cell, may be distinguished into three types (Margaritis and Pace, 1985; Shibaev, 1986); intracellular polysaccharides, structural polysaccharides and extracellular polysaccharides or exopolysaccharides

1.2 Mechanism of synthesis or chemical compositions

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

1.2.1 Homopolysaccharides

Homopolysaccharides contain only one type of sugar moiety such as dextran and pullulan, their synthesis involving a single or simple enzyme system. Others are curdlan and scleroglucan (Gibbs and Seviour, 1996; Glazer, 1995), levan, and some alginates (Han, 1990). The synthesis of alginate from fluorescent *Pseudomonas* sp differs from that of other microbial polysaccharides in that the final epimerization step takes place after the export of the polymer (Leigh and Coplin, 1992).

1.2.2 Heteropolysaccharides

Heteropolysaccharides are common microbial exopolysaccharides, which consist of linear arrangements of repeating units containing neutral sugars and uronic acid as well as non-carbohydrate substituents. Some may contain amino

sugars in place of uronic acids or along with them. In addition, they may contain acetyl groups or, more rarely, other acyl groups such as formate and succinate. Microorganisms from any utilizable carbon source usually produce heteropolysaccharides such as xanthan, alginate, and gellan (Glazer, 1995). The biosynthesis of these polysaccharides involves four stages:

(i) Synthesis of nucleotide sugar diphosphate intermediates

(ii) Stepwise assembly of the repeating oligosaccharide subunit of the polymer by transfer of monosaccharides from the corresponding nucleotide to the carrier lipid (C-55 undecapenyl phosphate) located in the cell membrane.

(iii) Addition of decorating, such as pyruvate, acetate, succinate, and sulfate.

(iv) Transfer of the growing polysaccharide chain from its carrier lipid to the new subunit, which is followed by the polysaccharide being transported to the cell surface through Bayer adhesion zones between the outer and inner membranes (Leigh and Coplin, 1992).

1.3 The electrical charge property

Polysaccharides may be classified as naturally neutral, anionic (acidic) or cationic (basic) based on their charge properties (Margaritis and Pace, 1985). The partial purified EPS of *E. cloacae* WD7 was classified according to the electric charge revealing that it contained acidic groups in its structure due to the interaction with quarternary ammonium of the cetylpyridinium chloride (CPC). It could thereafter be concluded that this polymer was an acidic polysaccharide, its component can be the acidic groups of pyruvate, succinate, uronic acid, acetate, or sulphate (Dermlim, 1999). However, uronic acids are the principal charged component of the anionic polysaccharide (Pace, 1981). On the other hand, the cationic polysaccharide is composed of basic groups such as the amino groups in the structure of chitosan (polyglucosamine) (Crestini *et al.*, 1996). Examples of neutral polysaccharides are levan, pullulan, dextran, scleroglucan which is

composed of only glucose repeating units. The example of an anionic polysaccharide is xanthan, which consists of glucose, mannose, glucuronic acid, acetate and pyruvate. The example for a cationic polysaccharide is chitosan which is composed of glucosamine.

2. Factors affecting polysaccharide production

2.1 Medium compositions

The techniques used to produce the microbial polysaccharides may be altered, with the aim of both improving the product and lowering the cost through improved yields or reduced energy input. Both improved technology and genetic improvement of strains appear be worthy of consideration by the producer of industrial polysaccharides. At each stage in polysaccharide synthesis, control mechanisms can affect the production of polymer. The initial level of control concerns the various mechanisms of substrate uptake which consist of simple diffusion, facilitated diffusion, active transport and/or group translocation mechanisms. During or after uptake, the substrate is phosphorylated prior to conversion to sugar nucleotides. Control is also exerted at this stage through the sugar nucleotide pyrophosphorylases. After the formation of oligosaccharide subunits of the extracellular polymer, attached to the isoprenoid lipid, they may either be excreted directly or transferred to some acceptor molecule at or near the cell surface (Sutherland and Ellwood, 1979).

Fermentation processes usually employ two media, one for inoculum development and the other for growth and production of the product. Medium compositions are also affecting the quality of the polysaccharide such as the molecular size of homopolysaccharide which was varied by substrate changes. However, some microorganisms produced extracellular heteropolysaccharide which are identical in composition and constant molecular weight (McNeely, 1967).

Fialho *et al.* (1999) found that altering the growth medium can markedly affect the polysaccharide yield, acyl substitution level, polymer rheological properties, and susceptibility to degradation. Although the production yields, compositions, structures, and properties of bacterial EPS are generally determined, it is possible to influence these factors by modifying culture conditions, such as temperature, dissolved oxygen tension, and growth medium composition (i.e., the concentration of cations and the carbon source used). Gellan polysaccharide produced by the industrial strain *S. paucimobilis* ATCC 31461 in a laboratory-defined production base medium containing lactose (2%, w/v) gave yields of around 70% of those from the other two gellan samples (glucose and galactose as carbon source). These results indicated that the same primary carbohydrate structure contained different levels of acetate and glycerate. Gellan modification may be strictly regulated and may depend not only on the enzyme activities that catalyzed the corresponding biosynthesis steps but also on the intracellular concentrations of the acyl activated precursors, which may vary depending on cell metabolism in the different growth medium. The results on the chemically modified gellans suggested that the rheology and conformation depend on both the level of acetate and glycerate substitution. In addition, it was found that enzymes degrade deacylated gellan due to extracellular eliminase types of enzymes (lyase), which cleave the sequence -β-D-glucosyl-(1,4)-β-D-glucuronosyl- in the tetrasaccharide repeat unit but exhibit negligible activity against the native acylated gellan polysaccharides.

Ananthalakshmy and Gunasekaran (1999) found that the increased rates of levan from *Zymomonas mobilis* mutated by nitrosoguanidine (NTG) are attributed to their higher levansucrase activity.

2.1.1 Carbon sources

Generally, the structure of polysaccharide produced by a microbial species is independent of the carbon substrate used. However, in some plant

pathogenic *Pseudomonas* species the polysaccharide synthesized depends on the substrate used. If this is sucrose, the product is a levan whereas on glucose, alginates are formed (Sutherland, 1990).

For some microorganisms, the carbon source determines both the quantity of the polysaccharide formed and its quality (Lawson and Sutherland, 1978; Sutherland, 1990). For instance, *B. polymyxa* synthesized a heteropolysaccharide consisting of glucose, mannose and fructose if grown on a medium containing sucrose as a carbon source, whereas, when grown on a medium containing a monosaccharide such as fructose, glucose, arabinose or galactose, the polysaccharide was made up of glucose, mannose and uronic acid (Clarke *et al.*, 1991). However, a strain of *B. polymyxa* produced a heteropolysaccharide when the liquid medium contained glucose as carbon source but a homopolysaccharide containing only fructose when sucrose was used as the carbon source (Cox and Steer, 1981). *B. polymyxa* NRRL B-18475 produced a large quantity of fructan or levan when grown on 4-16% sucrose solution (Clarke *et al.*, 1991). The organism converted the fructose moiety of sucrose to fructan; glucose is the most often used carbon source for microbial growth. No fructan was produced when the organism was grown on glucose or fructose. When various sources of sucrose from beet juice, beet molasses and cane molasses were used, the yields were 28, 22 and 57% levan, respectively.

In contrast, the extracellular heteropolysaccharides synthesized by *X. campestris* did not change significantly in composition or molecular weight as a result of changes in the nature of the substrate (Lawson and Sutherland, 1978). Sucrose was also used for the production of dextran production. because sucrose induced the enzyme dextransucrase which converted sucrose to dextran (Jeanes, 1977). Lactose and hydrolyzed starch were used as carbon source preferably to glucose, sucrose, and maltose for Zanflo, trade name of a heteropolysaccharide produced by *Erwinia tahitica* (Lawson and Sutherland, 1978).

The carbon sources used for xanthan were glucose, sucrose, starch syrup and whey; dextran is produced from sucrose; pullulan is produced from glucose, sucrose and maltose; alginate is produced from sucrose and glucose while curdlan uses glucose as carbon source. Besides sugars, hydrocarbon and low-molecular weight petrochemical substrate such as glycerol, methane, methanol are also used (Tam and Finn, 1977; Slodki and Cadmus, 1978).

Investigations into the effect of carbon sources on succinoglucan production by *Agrobacterium tumefaciens* ID95-748 found that various mono- and disaccharides tested (glucose, galactose, sucrose, maltose, mannitol and sorbitol) yielded conversion rates into the EPS ranging from 36 to 45% with sucrose proving the most efficiently converted carbon substrate. Conversely, polysaccharides such as starch and dextrins were not metabolized, suggesting a lack of the specific enzymes for their degradation. Alditols like sorbitol and mannitol were also suitable for succinoglucan production, whereas ethanol and citrate supported good growth, which was not associated with significant EPS synthesis. Other tested substrates (oil, acetate and isopropanol) were not metabolized.

The effect of carbon sources on EPS production was suggested to be dependent on the sugar uptake system, metabolic route and the energetic balance in terms of ATP generated and consumed in the oxidation/reduction reactions occurring during carbon substrate metabolism and EPS synthesis (Stredansky *et al.*, 1999). The effect of initial sugar concentration on the kinetic aspects of molasses fermentation by *A. pullulans* for pullulan production was studied. The polysaccharide concentration increased significantly with the increase of initial sugar concentration from 30 to 50 g/l and increased slightly thereafter with the highest concentration of pullulan (17.0 g/l) at 70 g/l after 120 h of incubation. The biomass dry weight followed a pattern similar to polysaccharide concentration with a maximum concentration of 16 g/l (Roukas, 1998).

2.1.2 Nitrogen Sources

While a nitrogen source is necessary for cell growth and the enzyme synthesis associated with the polysaccharide formation, an excess of nitrogen generally reduces the amount of polysaccharide produced by reduction of the conversion of the carbohydrate substrate to the conversion to the heteropolysaccharide (Lawson and Sutherland, 1978). It appears from the nitrogen-limited growth data that polymer production is non-growth associated (Tam and Finn, 1977).

Studies on the effect of varying the nitrogen source on succinoglucan yield and on the viscosity of fermentation broth in *Rhizobium hedisari* HCNT1 cultures resulted in sodium glutamate, lysine, sodium nitrate and ammonium sulfate giving yields of 8.8, 8.7, 5.9 and 7.5 g/l respectively. More viscous fluid was obtained when lysine was used as the nitrogen source, which was very likely to be due to a higher polymerization degree of the EPS produced in the presence of lysine (Stredansky and Conti, 1999).

For the production of succinoglucan by *A. tumefaciens* ID95-748 using varying nitrogen sources, those with buffering capacity (sodium nitrate and sodium glutamate) had a beneficial effect on both bacterial growth and metabolism, preventing a marked acidification of the fermentation broth. However, the highest yield was obtained with lysine as the sole nitrogen source in the presence of calcium carbonate to buffer the medium acidification (Stredansky and Conti, 1999).

The production of xanthan is promoted by a high ratio of carbon to nitrogen in the substrate. In ammonium-deficient media, a high xanthan production was observed (Becker *et al.,* 1998). The addition of ammonium nitrate and dipotassium hydrogen phosphate (both 0.2%, w/v) to the hydrolyzed starch waste improved the yields of biomass and EPS remarkably (160% and 139%) more than unsupplemented hydrolyzed starch waste. Furthermore, adding the salts at 0.1% (w/v) to olive oil waste, molasses and the mixture of olive oil waste plus

molasses increased the biomass but had less effect on the yield of EPS (Israilides *et al.*, 1998).

Urea and potassium nitrate were effective inorganic nitrogen sources, while ammonium chloride, ammonium sulfate and ammonium nitrate were not, in the case of polysaccharide (bioabsobent) production from *Alcaligenes latus* B-16 (Nohata and Kurane, 1994). Furthermore, the addition of methionine, glutamic acid, arginine and glycine increased, the culture broth viscosity increased but vitamins had no effect on the level of bioabsorbent production (Nohata and Kurane, 1997).

The effect of nitrogen sources on EPS recovery by *Rahnella aquatilis* revealed that urea and ammonium chloride gave similar higher yields (1.5 and 1.7 g/l) than using potassium nitrate, corn steep liquor and peptone (Matsuyama, 1999). Wachenheim and Patterson (1992) evaluated the effect of ammonium sulfate, urea and vitamin-free casien hydrolysate (VFCH) as nitrogen sources for EP production by *Butyrivibrio fibrisolvens* nyx. The effects of ammonium sulfate in the range of 0 to 1.92 g/l were linear for growth but not for EP production. Urea was tested at 0 to 8.64 g/l and the effects were significant but not linear for growth and increasing urea above 0.86 g/l did not affect EP production. VFCH was tested to provide an array of amino acids while avoiding vitamin effects. VFCH at 0 to 16 g/l affected growth and EP production in linear fashion. In enriched whey broth, peptone concentrations greater than 0.14% (w/v) significantly increased the broth viscosity of *Klebsiella oxytoca* (Dlamini and Peiris, 1997).

2.1.3 Yeast extract

The use of complex nitrogen sources like yeast extract and various peptones were detrimental to succinoglycan production by *A. tumefaciens* ID95-748 and, in the case of peptones, also to cell growth, which might indicate restricted proteolytic activity of the strain used (Stredansky and Conti, 1999). Guillouet *et al.* (1999) studied the effects of yeast extract on the production and

the quality of zooglan produced by *Zoogloea ramigera* 115SLR. It was found that one or more component(s) of yeast extract affected the production, composition, molecular weight and molecular weight distribution of the polysaccharide. It was found that zooglan produced in yeast extract medium had a higher molecular mass (1×10^7 compared to 6×10^6 g/mol) and lower polydispersity index than deficient yeast extract medium. It could have an effect on polymerase or depolymerase activity or synthesis. Although it has been reported that the degree of polymerization of polysaccharide during a culture changed with the oxygenation conditions and with the genetic background of strains (Guillouet *et al.*, 1999).

Israilides *et al.* (1998) found that supplementing the grape skin pulp extract (GSPE) with yeast extract (0.2% w/v) had very little effect on pullulan produced from *A. pullulans* over the 7-day period of fermentation, though hydrolyzed starch waste (SW) with added yeast extract gave increased biomass (41%) and slightly increased EPS (15%).

Nohata and Kurane (1994) showed that yeast extract at 0.5% was the most favorable organic nitrogen source for bioabsorbent production from *Alcaligenes latus* B-16 compared to beef extract and malt extract. Lo *et al.* (1997) reported that the effect of yeast extract on xanthan production was opposite to the effect of glucose. At the same concentration, a higher yeast extract concentration gave a higher cell yield and specific growth rate, but a lower specific xanthan production rate and xanthan yield. Yeast extract was important for the culture to reach a high xanthan production rate, but did not increase the amount of EPS produced by *Methanobacterium formicicum* T1N (Veiga *et al.*, 1997).

2.1.4 Phosphate

The concentration of phosphate in the rage of 0.3-0.6% was required for the bioabsorbent formation by *Alcaligenes latus* B-16. Keith *et al.* (1991) found that polymer yields from a number of different phosphates were similar, while Veiga *et al.* (1997) reported that the reduction in the concentrations of phosphate

(5 mM) resulted in the significantly increase of EPS from *Methanobacterium formicicum* T1N.

Phosphate salts; 0.2% potassium dihydrogen phosphate, 0.3% dipotassium hydrogen phosphate, 0.4% disodium hydrogen phosphate/sodium dihydrogen phosphate, 0.4% disodium hydrogen phosphate and 0.4% sodium dihydrogen phosphate gave 25.7, 24.0, 14.3, 20.3 and 11.8% substrate conversion yields from *E. herbicola* (Keith *et al.*, 1991).

Alginate production by *Azotobacter vinelandii* was found to be enhanced by decreasing the phosphate level in the medium. Alginate yield from biomass ($Y_{p/x}$) reached its highest value of 0.66 g/g at the lowest phosphate level (100 mg/l), compared to 0.40 g/g and 0.25 g/g at higher phosphate levels (200 mg/l and 400 mg/l, respectively). In contrast, biomass formation behaved differently and the growth yield ($Y_{x/s}$) decreased with decreasing phosphate concentrations. Moreover, the respiratory quotient (RQ) of the culture was depending on the initial phosphate concentration, especially in the phosphate-limited phase of growth. As the initial phosphate level decreased from 400 mg/l to 100 mg/l, the average RQ value of the culture declined from 1.46 to 0.89. In addition, the molecular mass and composition of alginate were also found to be affected by both phosphate and oxygen concentrations (Sabra *et al.*, 1999).

2.1.5 Trace elements

The highest viscosity of polysaccharide was obtained from *Alcaligenes latus* B-16 by addition of ferrous or ferric ion, among the trace minerals. Ferric (or ferrous) ion has been reported to be a cofactor of oxygenase and respiratory chain proteins such as cytochroms, which mediate the production of high-energy phosphorous compounds such as ATP and UTP. These high-energy phosphorous compounds participate in polysaccharide biosynthesis. Therefore the effect of ferric (or ferrous) ion on production of the bioabsorbent might stimulate

production of high-energy phosphorous compounds which would contribute to the biosynthesis of the polysaccharide bioabsorbent (Nohata and Kurane, 1997).

West and Strohfus (1997) reported that manganese supplementation resulted in higher polysaccharide levels by more than 8 or 17-fold after cultivation for 7-d of the fungus *Aureobasidium pullulans* ATCC 4203 on sucrose or corn syrup, respectively, as a carbon source. The effect of adding calcium ion to cultures of *A. pullulans*, which were initially in the yeast like form, was examined in shake- flasks and a stirred-tank reactor. In shake-flasks, addition of calcium ion led to a decrease in the proportion of the culture in mycelium form and a marked increase in biopolymer synthesis. In the stirred-tank reactor, the effects of calcium ion on morphology were less pronounced, although an effect on the molecular mass of pullulan was apparent (Madi *et al*, 1997).

2.2 Environmental parameters

Control of the composition of the fermentation medium, its addition and other environmental parameters are critical in achieving the desired rates of synthesis and yields of microbial polysaccharides, and the complex utilization of the full heat and mass transfer capabilities, or energy input, of existing fermentors. These parameters are also prime determinants of the purity of the product, its chemical composition and molecular weight, which in turn determine its performance. The growth and production of exopolysaccharides by microorganisms are determined by a wide range of environmental parameters, in addition to the effects of the culture medium described in the previous section. However, the influences on the fermentation of variables such as temperature, pH, dissolved oxygen, agitation speed, aeration rate, are described as follows (Margaritis and Pace, 1985).

2.2.1 pH

The pH of *B. polymyxa* culture fell from 7.0 to 4.7 indicating acid production. In reports of other production, maintaining pH above 5.5 was important because the optimum pH for fructansucrase is between 5.5-7.0 and fructan may be hydrolyzed at a lower pH (Clarke *et al.*, 1991). The pH control also affected the molecular weight of dextran produced from *Leuconostoc mesenteroides* and pullulan produced from *A. pullulans* (Jeanes, 1977). The optimum pH for the synthesis of bacterial polysaccharide is 6.0-7.5. For fungi, the optimum is 4.0-5.5 (Lawson and Sutherland, 1978). The pH cultured decreased during fermentation of xanthan by *X. campestris* because anionic groups were formed as part of the polysaccharide structure so that pH maintained at 6.0-7.5 was necessary (Baird and Pettitt, 1991).

The pH of the medium is important in polysaccharide production by *A. pullulans* and affects the morphology of the organism, which influences pullulan production. The polysaccharide concentration remained practically constant with increasing initial pH up to 5.5 and then increased. Maximum polysaccharide concentration (32.0 g/l) was obtained in cultures grown at pH 6.5-7.5. The biomass dry weight increased with the increase of pH from 3.5 to 6.5 and then decreased. Maximum biomass concentration (33.8 g/l) was achieved at pH 6.5. The polysaccharide yield and the sugar utilization remained constant between pH 6.5-7.5 and 4.5-7.5 respectively (Roukas, 1998).

The pH-controlled batch fermentation of *Lactobacillus* strain LB 121 and LB180 produced approximately the same amount of EPS (less than 10 g/l) at pH 4.8 and pH 5.8 (t=30h), although at pH 4.8 less biomass was produced and less sucrose was consumed while without pH control resulted in abundant EPS production (over 10 g/l) by both strains (Geel-Schutten *et al.*, 1998).

It is clear that both batch and continuous culture, pH significantly affect the morphological form of *A. pullulans*; low pH favors filamentous, while high pH favors yeast cell-type forms. The optimum pH for both biomass and pullulan

production was pH 4.5 (McNeil *et al.*, 1989). Gassem *et al.* (1997) showed periodic adjustment of pH to 6.2 during fermentation, for exoopolysaccharide production from whey lactose by *Lactobacillus delbrueckii* ssp. *bulgaricus*, resulted in greater viscosity in all media than when there was no pH adjustment.

Maintenance of higher pH might result in increased exopolysaccharide production by increasing the time the culture was in log phase. Higher pH resulted in a longer stationary phase, which would decrease peptidoglycan and teichoic acid syntheses and could result in an increased exopolysaccharide production. There was little change in polymer yield at neutral pH, while at the pH extremes of 5 and 8, the yields dropped off to zero. A mid point of pH 7.2 was chosen for levan production by *Erwinia herbicola* (Keith *et al.*, 1991).

2.2.2 Temperature

Optimum temperature is often a critical factor in polysaccharide synthesis, and is in general the same for growth and product formation. All commercial polysaccharide-producing microorganisms are mesophiles (Lawson and Sutherland, 1978). Temperature is one of the most important parameters affecting xanthan gum fermentation, higher temperatures leading to xanthan biosynthesis while lower temperatures favor cell growth (Shu and Yang, 1991). Polysaccharide production by *L. delbrueckii* spp. *bulgaricus*, viscosity was higher at 32 °C than at 37 °C and 44 °C and was lowest at 44 °C.

Other studies have shown that incubating lactic acid bacteria at below optimum growth temperatures (25 °C) resulted in greater production of exopolysaccharide (Cerning *et al.*, 1992). Lower incubation temperatures (25 °C) resulted in slower growth rate, which promoted longer logarithmic and stationary phases. The least lactose was utilized at 32 °C, confirming slower growth and biomass production than at 37 °C or 44 °C (Gassem *et al.*, 1997).

Shu and Yang (1991) studied the effect of temperatures between 22 °C and 33 °C on growth and xanthan formation. Growth rate constants k increased to

a maximum (0.16 to 0.29 h^{-1}) at 31 °C and then decreased to zero at 35 °C. The optimal temperature for EPS production from *Methanobacterium formicicum* T1N was 30 °C, the yield decreased at higher temperatures (37 °C and 42 °C) while optimal temperature for growth was 40 °C (Veiga *et al.*, 1997).

2.2.3 Dissolved oxygen

Most polysaccharide-produced microorganisms are either aerobes or facultative anaerobes. In the latter, exopolysaccharide synthesis generally occurs only when the microorganism is grown aerobically. Thus aeration of culture medium is an important requirement for polymer production (Sutherland, 1990).

Controlling dissolved oxygen (DO) was achieved with two control variables: air flow rate and agitation speed. The optimal DO for the formation of EPS can be different from that of biomass production. Controlling DO concentration seems not easy to manipulate because the fermentation conditions are unpredictably changing. Another problem in DO concentration control is sensor reliability. The most popular DO electrode dynamics is better than PID controller and adaptive algorithms without the DO electrode dynamics (Lee *et al.*, 1991).

Commercial heteropolysaccharides are expected to be produced by an aerobic process. Anaerobic fermentation of carbohydrates is too inefficient as a pathway for energy production to be utilized (McNeely, 1967). As the viscosity of the medium increases with polysaccharide formation, oxygen transfer to the cells becomes increasingly more difficult. To investigate the possible influence of oxygen limitation, increasing medium volumes (20 to 100 ml) were used in shake-flasks (250 ml) so as to vary the oxygen availability in the solution. They found that the EPS produced by *A. tumefaciens* ID95-748 was strongly inhibited by oxygen limitation, with polymer yield decreasing from 9.40 to 2.06 g/l as the cultivation volume increased. To overcome the problem of oxygen limitation in shake-flask culture, laboratory-scale fermentators were used in which the soluble

oxygen level was maintained at 50±10% of saturation. Under these conditions, a maximum yield of 9.56 g/l succinoglucan was achieved. Under non-limited oxygen conditions a polymer with higher molecular weight was produced. A similar effect was also described for xanthan production (Stredansky and Conti, 1999).

The dissolved oxygen level under no limitation of oxygen and the oxygen transfer rate under oxygen-limited conditions were reported to affect the average molecular weight of xanthan significantly and the differences observed in the homogeneity of the polysaccharides can not be related to oxygen or micro-mixing problems (Guillouet *et al.*, 1999). Kouda *et al.* (1997a) showed that the bacterial cellulose production rate was dependent on the oxygen transfer rate which declined as the broth viscosity increased, accompanied by bacterial cellulose accumulation. Increasing the partial pressure of oxygen by sparging with oxygen-enriched air and/or raising the operating pressure improved the oxygen supply.

Gibbs and Seviour (1996) showed that controlling oxygen at 7% saturation after 32 h incubation resulted in no stimulation of exopolysaccharide production compared to when no oxygen control was exercised. However, when the oxygen was maintained at 7% for the entire or at first after 8 h cultivation, polysaccharide yield increased (8.17 g/l). However a further increase in oxygen to 30% and 50% saturation for the first 16 h cultivation gave no stimulation in polysaccharide production compared to when no oxygen control was exercised at 1000 rpm.

Xanthan productivity was inefficient when the organism was grown under oxygen-limited conditions. To overcome oxygen transfer limitation, fermentation was carried out at 1000 rev/min in which the minimum dissolved oxygen was 50% of saturation. Due to better oxygen transfer and mixing behavior in the fermentation broth, xanthan concentration reached 27 g/l and the yield of xanthan also increased from 0.49 (at 600 rev/min) to 0.59 (at 1000 rev/min) (Amanullah *et al.*, 1998b).

Xanthan synthesis requires energy (ATP) which being available from the oxidation of $NADH+H^+$ and $NADPH+H^+$ produced through the TCA cycle. These reduced cofactors have to be oxidized through oxidative phosphorylation to produce ATP, NAD and NADP. The net energy yield of the cell was therefore depending on the oxygen available (Jana and Ghosh, 1997). The provision of sufficient oxygen could be important to maintain cell availability throughout the stationary phase in the whole of the vessel. Xanthan could continue to be produced at a level governed by a specific oxygen uptake rate, unless there were biological limitations preventing the formation of the product. Sufficient oxygen transfer rates were dependent on the extent of bulk mixing which was dictated by impeller design and operational parameters such as power input and fluid properties (Amanullah *et al.*, 1998a).

Sabra *et al.* (1999) concluded that alginate production by *Azotobacter vinelandii* preferred 2-3 % pO_2 in batch culture and 2-5 % pO_2 in continuous culture. These data also suggested that the synthesis and/or expression of an alginase could be controlled by the dissolved oxygen tension of the culture. An increase in the molecular mass of the capsular alginate with the increase in pO_2 was of advantage for the survival of the extremely oxygen-sensitive cells. The density and/or the size of this alginate capsule may in fact be a self-regulating mechanism for controlling the intracellular O_2 concentration.

2.2.4 Agitation speed

It has been hypothesized that exopolysaccharide produced by bacteria may form a layer on the cell surface and acts as a diffusion barrier. Thus, in addition to affecting bubble size and distribution, the shear rate distribution and mixing throughout the fermentor may also be important indirectly affecting the rate of polymer production by determining the effective size of such a diffusion barrier. Air agitated vessels appear to be unsuited for exopolysaccharide production as air sparging alone is insufficient to promote the liquid turbulence

necessary for small bubble generation and in preference mechanically agitated, aerated vessels are widely used (Pace, 1981).

Efficient heat and mass transfer are achieved when the fermentor contents are fully mixed, with the effectiveness of a given impeller being determined by the fluid rheology. The proper selection of an agitation system for a fermentor producing microbial exopolysaccharides requires a full appreciation of the complex rheological behavior of the culture fluid and its impact on various transport phenomena throughout the fermentation. For example, impellers such as disc turbines, having high velocity heads, give good small bubble formation but poor mixing in highly viscous systems (Pace, 1981).

To improve the bacterial cellulose productivity in aerated and agitated culture, the agitator configuration was improved by using *Acetobacter xylinum* subsp. *sucrofermentans* BPR3001A. Firstly, impellers such as Maxblend and Gate with turbine were suitable for bacterial cellulose because they mixed the culture broth well and had large K_La, and secondly, production rate and yield of bacterial cellulose were dependent on K_La and the oxygen consumption rate and thirdly, the static gassing-out method to measure K_La was useful for characterization of the agitation conditions and the agitator configuration (Kouda *et al.*, 1997b).

Bacterial cellulose production was decreased when cultured in aeration and agitation conditions. One of the reasons for the decrease was the genetic instability of *Acetobacter xylinum* subsp. *scrofermentans* BPR3001A. However some producers could produce a large quantity of the cellulose (Yoshinaga *et al.*, 1997). The suitable impeller for gellan production was obtained with a helical ribbon impeller and led to a gellan exhibiting a molecular weight twice as high as that obtained with Ruston turbines. The composition of the polymer itself was also affected since the degree of esterification of gellan could vary with the fermentation conditions (Dreveton *et al.*, 1996).

The influence of agitation rate on exopolysaccharide production by *A. pullulans* was studied with a total of six different agitation rates (Gibbs and

Seviour, 1996), ranging from 125 rpm to 1250 rpm. High agitation rates (750 rpm and above) clearly resulted in substantially diminished exopolysaccharide yields compared to those seen at low speeds, while biomass yield was slightly increased. Culture growth at agitation rates of 125 rpm and 250 rpm gave similar high final exopolysaccharide yields of approximately 11 g/l, with almost 40% of the glucose initially provided converted into exopolysaccharide. Increasing the agitation rate to 500 rpm led to an increase in biomass yield and a clear reduction in exopolysaccharide production to about 7.5 g/l and therefore a substantially lower exopolysaccharide/biomass ratio (i.e $Y_{p/x}$=0.64 compared with more than 1.2 at 125 rpm to 250 rpm). The proportion of glucose converted into polysaccharide was also reduced. Additional increases in the agitation rate to 750, 1000 and 1250 rpm led to further marked and reproducible reductions in polysaccharide yields, to levels approximately half of those achieved at 125 rpm and 250 rpm.

Amanullah *et al.* (1998b) reported that agitation speed affected both the rheological property and the rate of oxygen transfer to culture broth of xanthan fermentation. The combination of these two effects causes the dissolved oxygen concentration and its special uniformity to change with agitator speed. Separating these complex interactions was achieved in the following ways.

First, under controlled dissolved oxygen conditions, the results demonstrated that biological performance of the culture was independent of agitation speed as long as broth homogeneity could be ensured. With the increasing rheological complexity leading to stagnant regions, the superior bulk mixing was achieved at 1000 rpm which leading to an increased proportion of the cells in the fermentor to be metabolically active and hence higher microbial oxygen uptake rates.

Second, the effects of varying dissolved oxygen were compared with a control in each case with an agitator speed of 1000 rpm to ensure full motion, but with a fixed, non-limiting dissolved oxygen of 20% air saturation. The specific oxygen uptake rate of the culture in the exponential phase was found to be

independent of dissolved oxygen above 6% air saturation, whereas the specific growth rate of the culture was not influenced by dissolved oxygen. In the production phase, the critical oxygen level was determined to be 6% to 10% so that both specific xanthan production rate as well as specific oxygen uptake rate decreased significantly.

3. Culture types

3.1 Batch culture

The process of Biopolymer PS87 production using *Bacillus polymyxa* in batch fermentation did not require the addition of alkali to control the pH of the culture medium. It was observed that the pH usually dropped to about 5 after 10 to 20 h, and then increased to about 6 to 7 and generally maintained at this level for the remainder of the incubation period. In order to obtain rapid and efficient production of the heteropolysaccharide, it is essential to have a sufficient quantity of oxygen available for growth of *Bacillus polymyxa* NCIB 11429.

Wachenhiem and Patterson (1992) compared the specific growth rate of *Butyrivibrio fibrisolvens* nyx with that of *X. campestris* in batch culture (μ=0.15 and 0.19 h^{-1}, respectively). *Alteromonas macleodii* HYD-1545 was cultivated in a 2-liter fermentor containing the 2216E medium supplemented with glucose (30 g/l), which allowed an optimization of EPS production. The production started at the end of the exponential phase of growth and continued during the stationary phase. All the substrate was utilized after 4 days of cultivation, and the $Y_{p/s}$ was 37%. The pH of the culture decreased to 6.5 as glucose was consumed and then increased to 8.3 with the utilization of residual peptone (Vincent *et al.*, 1994).

Leathers (1998) reported that alternan produced by *Leuconostoc mesenteroides* NRRL B-1355 in conventional medium (20 g/l sucrose as carbon source) reached stationary growth phase within about 12 h, although polysaccharide levels continued to increase up to approximately 24 h. Cultures grown in a medium containing 1.5% CCDS (corn condensed distiller's soluble)

exhibited higher growth rates and yield than those in conventional medium and polysaccharide also accumulated more rapidly and to greater yields. Polysaccharide continued to accumulate during this cell death phase. These results explain the apparent contradiction between low viable counts and high polysaccharide yields observed at 48 h in 0.25% CCDS medium and suggested that stationary phase cultures required nutrients or growth factors for continued viability.

Batch xanthan gum fermentation generally can be distinguished by two stages: (i) the trophophase in which there is propagation of cells and (ii) the idiophase in which xanthan gum production occurs. A two-stage fermentation process, one to optimize cell growth and one to optimize xanthan production, has thus been proposed to improve xanthan fermentation (Lo *et al.*, 1997).

In comparing the cell yields, specific growth rates, xanthan yields and specific xanthan production rates from two-stage and fed-batch fermentation with those from batch fermentation, it was found that both cell yield and specific growth rate from the two-stage and fed-batch processes were similar to those from batch fermentation. Therefore, the two-stage batch fermentation, which provides two optimal conditions, was preferred to the conventional single-stage batch fermentation. It seems that for microorganisms, which produced EPS during exponential phase, such as xanthan, two-stage batch fermentation is necessary (Lo *et al.*, 1997).

A summary of selected recent publications on batch xanthan fermentations was included in Table 1. The typical values of maximum xanthan gum concentrations, xanthan yields on glucose and productivity obtained were between 15-30 g/l, 0.27-0.86 and 0.12-0.43 g/l/h respectively. Very few studies have reported concentrations of xanthan gum in excess of 30 g/l.

Cultivation of *Sphingomonas paucimobilis* GS1 in 2.0-liter bioreactor gave 6.5 g of EPS/l, almost 75% of it being produced during exponential growth

(up to 48 h); the remainder was accumulated in the stationary phase (Ashtaputre and Shah, 1995).

Acidic exopolysaccharide produced by *Enterobacter* sp. was performed in shake-flask. The crude polysaccharide yield increased with increasing bacterial growth, decreasing in the stationary growth phase. When the maximum crude yield of polysaccharide was 5.8 g/l on the third day of cultivation, the conversion ratio of sucrose to the polysaccharide was 13.5% w/w. Over 90% of the sucrose were consumed in the logarithmic growth phase. pH was stable in the range of 7 to 8 throughout the cultivation(Shimada *et al.*, 1997).

Mulchandani and Luong (1988) proposed a modified form of equitable logistic for quantifying the batch kinetics of microbial growth during the biosynthesis of extra- and intracellular polymers; i.e. for *Aureobasidium pullulans*, for representing the reported data on pullulan and xanthan. In comparison to the logistic and Monod kinetics, this model fitted the data better and more accurately described the overall fermentation.

Pestalotiopsis sp. KCTC 8637P producing a flocculant was cultivated in a jar fermentor with the aeration of 2-3 vvm, agitation speed 100-300 rpm, at 25 °C for 5 days. The flocculating activity and cell growth were highest at 108 h (3000 and 11 g/l, respectively), whereas pestan production and viscosity of cultural supernatant were highest at 120 h cultivation (13 g/l and 800 cP, respectively) (Kwon *et al.*, 1996).

Table 1 Summary of selected recent publication on batch xanthan fermentations

Glucose added (g/l)	Maximum xanthan (g/l)	Xanthan yield	Global xanthan production (g/l/h)	Researchers
50	16	0.34	0.32	Krebser *et al.* (1988) (STR)
50	25	0.54	0.33	Krebser *et al.* (1988) (Loop reactor)
50	18	0.36	0.20	Peters *et al.* (1989)
50	26	0.52	0.29	Peters *et al.* (1992)
45	20	0.48	0.20	Zaidi *et al.* (1991)
50	25	0.50	0.19	Suh *et al.* (1992) (Bubble column)
50	15	0.27	0.12	Suh *et al.* (1992) (Airlift reactor)
50	24	0.50	0.48	Kessler *et al.* (1993)
45	28	0.62	0.51	Flores *et al.* (1994)
40	27	0.67	0.39	Garcia-Ochoa *et al.* (1995)

Source: adapted from Amanullah *et al.* (1998a)

In shake-flask culture for exopolysaccharide production by *Rahnella aquatilis*, the pH decreased rapidly (from 6 to 4) up to 4-d during cultivation, the concentration of sucrose decreased (10.5 to 3.0 g/l) more rapidly and the concentration of EPS increased (2.5 g/l). Insoluble EPS were produced at the beginning of cultivation, but thereafter their concentration decreased. On the other

hand, the production of soluble EPS continued to increase despite the concentration of sucrose becoming low (Matsuyama *et al.*, 1999).

During Zooglan production by *Zooglan ramigera* 115SLR, the exponential cell growth rate was 0.18 h^{-1} and the bacteria consumed two-thirds of the glucose and produced 4.4 g/l exopolysaccharide with an overall specific productivity of 0.13 g EPS/g cell dry weight/h (Guillouet *et al.*, 1999). The glucose was completely exhausted after 60 h of fermentation and 1.5 g/l biomass and 5.2 g/l EPS were produced. During the stationary phase, the growth and EPS production (0.6 g/l) were reduced. When the glucose to EPS conversion yield remained at 30%, the specific productivity was dramatically decreased (0.012 g EPS/g biomass/h). The maximum $Y_{p/s}$ was 35% and the maximum overall specific productivity was 0.18 g EPS/g cell dry weight/h.

During batch cultivation of levan producing *Zymonas mobilis* for 18 h at 30 °C it was found that during the initial fermentation (0.8 h), the rate of sucrose hydrolysis was high (about 13 g/l/h) while the rate of levan production was low in both mutans ZML1 and ZML2 (0.58 and 0.40 g/l/h, respectively), and in the parent strain (0.18 g/l/h). In contrast, the rates of sucrose hydrolysis were low 2.38 g/l/h, during 8 to 16 h while the levan production rates of mutants ZML1 and ZML2 (1.72 and 1.68 g/l/h, respectively) and the parent strain (0.69 g/l/h) were high as shown in Table 2. These results were in agreement with the correlation between the rates of sucrose hydrolysis and levan formation proposed by Viikari and Gisler (Ananthalakshmy and Gunasekaran, 1999).

Table 2 Fermentation kinetic parameters of *Z. mobilis* B4286 and mutants ZML1 and ZML2 on sucrose medium

Parameters	*Z. mobilis* strains		
	B4286	ZML1	ZML2
Levan (g/l)	16.0	21.0	20.0
Biomass (g/l)	2.0	1.8	1.8
Residual sucrose (g/l)	3.75	1.06	1.55
Rate of levan formation (g/l/h)	0.69	1.72	1.68
Rate of sucrose hydrolysis (g/l/h)	2.38	2.38	2.38
Specific growth rate (μ, h^{-1})	0.10	0.068	0.068

Source: adapted from Ananthanlakshmy and Gunasekaran (1999)

Naritomi *et al.* (1998) showed bacterial cellulose (BC) production by *Acetobacter xylinum* subsp. *sucrofermentans* in batch cultures when using CSL-Fru medium. The highest BC production rate and BC yield, 0.40 g/l and 28%, respectively, were obtained using CSL-Fru medium with 70 g/l initial fructose. Sanchez *et al.* (1997) compared cultivation of *Xanthomonas* strains grown in baffled shake-flask and batch fermentor (Table 3). The results clearly showed that the shake-flask productivity test was a quick and simple, but preliminary, method of screening. It is, however, necessary to characterize the isolates under more controlled conditions (in fermentors) in order to make a fair and complete comparison among isolates. Productivity in fermentors was approximately twice that obtained in buffled shake-flasks because shake-flasks culture is limited since the pH cannot be controlled, and this is disadvantageous for xanthan production. Moreover, xanthan quality (pyruvic acid content) and the proportion of high molecular weight polymers were influenced by the dissolved oxygen tension during culture and this was not readily controlled in shake-flask either.

Table 3 Comparison between batch fermentor and shake-flask

	Batch-fermentation				Shake-flask			
	10	11	16	E2	10	11	16	E2
Fermentation times (h)	54.0	49.0	48.8	48.0	48.0	48.0	48.0	48.0
Specific growth rate (h^{-1})	0.09	0.11	0.14	0.12	-	-	-	-
Final cell concentration (g/l)	4.80	4.31	2.78	4.84	-	-	-	-
Final xanthan concentration (g/l)	19.4	20.4	18.3	19.1	9.0	11.0	9.0	10.0
Final apparent viscosity (mPa.s)	3200	4400	3800	2200	900	900	930	1140
Productivity (g/l/h)	0.36	0.43	0.37	0.39	0.16	0.22	0.18	0.20

Source: Sanchez *et al.* (1997)

EPS production by *Bacillus thermoarcticus* was conducted in a 3-liter fermentor and in a 1-liter batch culture (static culture) (Manca *et al.*, 1996). The EPS production in fermentors reached 400 mg/l in the presence of mannose as carbon source; this level was 2.4-fold higher than that obtained in batch culture (Table 4). It is noteworthy that the highest production of EPS in fermentors was obtained when the principal physiologic parameters (O_2, stirring, and pH control) were also at the optima for biomass production.

Table 4 Production of EPS in batch and fermentor culture

Carbon source	Batch culture		Fermentor culture	
	EPS (mg/l)	OD_{540}	EPS (mg/l)	OD_{540}
Mannose	165	0.750	400	1.600
Glucose	65	0.620	150	1.300
Sucrose	100	0.690	250	1.450

Sources: Manca *et al.* (1996)

3.2 Fed-batch

In a batch culture, the transition from exponential growth to stationary phase may occur for a variety of reasons, including the depletion of an essential nutrient substrate or the building up of a toxic metabolite product. When the transition results from nutrient depletion, growth will continue if fresh medium is added. The medium addition rate and the culture volume must be increased exponentially to maintain a constant rate of exponential growth. This technique for exponential growth is usually called fed-batch culture.

Kurtanjek (1991) reported that fed-batch operation enabled superior control as it provided different avenues in the substrate addition for control through distinct phases of biomass growth and metabolite production. By programming substrate feeding, one can control important phenomena such as substrate-inhibited growth and glucose effect

The fed-batch mode will allow substantial improvements in cell mass or product productivity over an ordinary batch operation. This technique can also be used to supply large quantities of a potentially toxic substrate while maintaining a low concentration of the substrate in the medium (Gerhardt and Drew, 1994). Lo *et al.* (1997) produced xanthan using fed-batch conditions, the additional glucose (2.5%) was added in five equal parts (75 g glucose) at 5 h intervals during the

stationary phase, xanthan production was poor throughout the entire period and reached only about 18 g/l even though cells grew well (1.25 g/l) in the growth phase.

For xanthan production from *X. campestris*, 50 g/l of glucose was initially added in a fed-batch mode in order to obtained high gum concentrations (40 g/l) followed by an addition of 10 g/l once the cells entered the stationary phase. The biomass concentration increased exponentially almost immediately after inoculation, reaching maximum concentration of 3.7 (STR, standard Ruston turbine), 3.3 (LRT, large Ruston turbine), 4.2 (PMD, Prochem Maxflo T), and 3.0 g/l (SRGT, scaba 6SRGT) at the point when nitrogen became limiting. In all cases, the maximum specific growth rate in the exponential phase was similar (0.09-0.11 h^{-1}), after which the cell concentration remained essentially constant, about 29.0 to 34.2 g/l, throughout the stationary phase and xanthan yields were 0.58 to 0.68 g xanthan/g glucose/h (Amanullah *et al.*, 1998a).

The methylan production by *Methylobacterium organophilum* in the bioreactor was carried out by the DO-stat fed-batch mode of methanol. When the total added content of the ammonium ion was 0.75 g/l, methylan accumulated to 17.6 g/l and cells grew to 23.6 g/l. The production of cells and methylan in a bioreactor were 35 and 40%, respectively. The DO level was always over 20% throughout the fermentation process of the bioreactor. The methylan yield was 60% (Oh *et al.*, 1997).

The biosynthesis of xanthan by *X. campestris* was found to be affected by the addition of citric acid in fed-batch mode. Citric acid (0-2.60 g/l) was added after 24 h, then added every 8 h until the end of fermentation. Under oxygen-limiting condition, up to 2.6 g citric acid per liter improved cell viability as well as increasing xanthan yield by up to 80%. Citric acid feeding increased specific productivity even at low agitation (600 rev/min). Specific xanthan productivities in citric acid fed-batch and normal batch processes were the same at 1000 rev/min.

Moreover, citric acid addition after 48 h did not have any positive effect on xanthan synthesis (Jana and Ghosh, 1997).

3.3 Continuous culture

Chemostat cultivation is always preceded by transient batch cultivation during which time the cell mass accumulates at the expense of the substrate. Continuous culture was started when the batch cultivation is in the exponential phase of growth; this will minimize oscillations due to nutritional step-up and avoid inadvertent washout due to physiological lag. Start the dilution at a rate less than the desired operational dilution rate, and then increase to the operational rate within one residence time, the average time that a cell remains in the vessel. This will minimize oscillations from toxic substrate, offer high productivity and uniform of production rate (Gerhardt and Drew, 1994; Glazer, 1995; Sutherland, 1990).

The conventional method of exopolysaccharide production from microorganisms is batch cultivation with relatively long incubation times to ensure maximum production of polysaccharide and maximum utilization of substrate. It would clearly be advantageous to use the continuous culture mode in terms of production from a given size of fermentor but, until recently, no commercial size continuous culture to microbial polysaccharide formation was available (Sutherland and Ellwood, 1979; Amanullah *et al.*, 1998b).

Lawford (1981) patented the continuous process for the production of gelable exopolysaccharide, a two-stage continuous process for the production of curdlan. In the first stage, a curdlan-producing strain of microorganism was grown aerobically in an aerated, agitated culture medium containing assimilable carbon, nutrients and organic salts. The amount of nitrogen in the first stage was so limited that the affluence contains substantially no organic nitrogen. The effluence was introduced into a second stage in a constant volume fermentor wherein it was mixed with a nitrogen-free carbohydrate. The resultant mixture was aerated and

mixed at pH 5.5 to 6.5 at a temperature of 25 °C to 35°C, the volume and dilution rate in the reactor being selected. The product was subsequently isolated. The production of biopolymer PS 87 in continuous fermentation in a 3 liter stirred tank fermentor was used, pH and dilution rate were controlled. The concentration of polysaccharide in the broth was found to increase with decreasing dilution rate (D in the range of 0.03-0.10 h^{-1}) i.e. from about 0.4% at D=0.1 to about 0.8% (D=0.03) (Cox and Steer, 1981).

Continuous production of xanthan has been investigated but not commercially implemented. The alginate production in continuous culture has also been studied, and the yield from sucrose could increase to 50%, compared to 25% in batch culture (Lee, 1996). The existing methods of continuous cultivation can be subdivided into two groups of mechanisms providing the stability of growth. In the first group, represented mostly by chemostat, the stability is maintained by the culture itself. The second group method (turbidostat, pH-auxostat) is based on the use of special automatic device that controls different growth-linked parameters. Chemostat operates successfully within the region of growth limitation by any substrate. Contrary to it, methods of the second group are operable on the plateau of specific growth rate, or at the excess substrate inhibition (Minkevich *et al.*, 1989).

L. delbrueckii subsp. *bulgaricus* grown in continuous culture in defined medium (D=0.075h^{-1}) on 167 mM fructose produced 24 mg/l EPS (Grobben *et al.*, 1998). At 45 h, the cell growth and EPS yields were 2.3 (OD_{600}) and 25 mg/l, respectively. After 45 h, the carbohydrate source was switched to 83 mM fructose + 83 mM glucose and after 220 h to 167 mM glucose . At 220 h, the cell growth of 2.4 and EPS of 110 mg/l were observed and had no further effect on the growth, EPS production or sugar composition of the EPS.

BC was produced by *Acetobacter xylinum* subsp. *sucrofermentans* in continuous culture experiment in which CSL-Fru medium containing 30 g/l fructose was fed at a dilution rate of 0.07 h^{-1}. The BC production rate (0.62 g/l/h)

and BC yield (36%) were higher than those obtained in the batch cultures using CLS-Fruc medium. The BC production rate and yield increased with increase in the dilution rate, but wash-out occurred when the dilution rate was 0.12 h^{-1}. A BC production rate of 0.74 g/l/h and a BC yield of 35% were obtained at dilution rate of 0.10 h^{-1} (Naritomi *et al.*, 1998).

When various fructose concentrations were fed at a fixed dilution rate of 0.07 h^{-1}, the BC production rate increased with increases in the fructose concentration in the feed medium, and reached 1.0 g/l/h at 100 g/l fructose. However, both the BC yield and total yield of BC declined when the fructose concentration was higher then 45 g/l. To determine whether supplementing lactate to the feed medium also improved the fructose consumption and BC production at a fixed dilution rate of 0.07 h^{-1}, the following experiments were carried out. The optimum lactate concentration for BC production was 12.5 g/l, at which the production rate and BC yield of BC were 0.80 g/l/h and 38%, respectively. Then the BC production rate was examined at various dilution rates using feed medium supplemented with 12.5 g/l lactate. A BC production rate of 0.90 g/l/h and a BC yield of 36% were obtained at a dilution rate of 0.10 h^{-1}, but wash-out occurred when the dilution rate exceeded 0.12 h^{-1} (Naritomi *et al.*, 1998).

Polysaccharide production from *P. aeruginosa* in continuous culture, under nitrogen-limited conditions at dilution rates of 0.05-0.10 h^{-1} was studied. Results indicated a conversion rate of 56-64% polymer from substrate. The specific rate of polysaccharide production increased with increasing dilution rate. At a dilution rate of 0.026 h^{-1}, the yield of xanthan based on the glucose consumed was 68% and the production rate for the xanthan was a function of the pH and dilution rate (Sutherland and Ellwood, 1979).

Lawson and Sutherland (1978) reported the influence of dilution rate on the production of polysaccharide from different microorganisms. At a dilution rate of 0.05 h^{-1}, *Pseudomonas aeruginosa* gave 8.9 g/l of polysaccharide and 1.3 g/l of biomass while *X. campestris* produced 7.0 g/l EPS and 1.6 g/l biomass. However

at a dilution rate of 0.15 h^{-1}, *Azotobacter vinelandii* produced polysaccharide of 2.0 g/l and biomass was 1.5 g/l.

Xanthan production in continuous culture was only investigated on laboratory scale at a dilution rate of 0.05 h^{-1} for 20 h cultivation, because continuous processes would have a high risk of contamination. The processes used in the production of microbial polysaccharides of commercial importance are shown in Table 5.

Pace (1980) reported that the specific growth rate of alginate synthesis from *A. vinelandii* was independent of specific growth rates over a range of dilution rates from 0.05-0.25 h^{-1} which was similar to *Pseudomonas* sp. while *Xanthomonas* was virtually unchanged over the growth rate range of 0.05-0.20 h^{-1}. The quantity of polysaccharide produced per unit of cell mass thus increased with decreasing growth rate when the lower dilution rates were used. In contrast, *Pseudomonas aeruginosa* produced the same quantity of alginic acid per unit of cell mass over the growth rate range 0.05-0.10 h^{-1}; at all growth rates the ratio of polysaccharide to cell mass was about 5.

Alginate production by *A. vinelandii* under microaerobic condition at four different dilution rates (D=0.08, 0.15, 0.22 and 0.26 h^{-1}) found that a dilution rate of 0.15 h^{-1} gave highest biomass and alginate yields (1.0 g/l and 1.5 g/l, respectively) (Sabra *et al.*, 1999).

Table 5 Processes used in the production of microbial polysaccharides

Polysaccharide	Microorganism	Process	Reference
Pullulan	*Aureobasidium pullulans*	Batch, fed-batch and continuous ($D=0.075\ h^{-1}$)	Youssef *et al.* (1999) and McNeil *et al.* (1989)
Gellan	*Sphingomonas paucimobilis*	Batch	Fialho *et al.* (1999)
Dextran	*Leuconostoc mesenteroides* NRRL B-21138	Batch	Leathers (1998)
Microbial alginate	*Azotobacter vinelandii*	Continuous ($D=0.08\text{-}0.26\ h^{-1}$)	Sabra *et al.* (1999)
Bacterial cellulose	*Acetobacter xylinum* subsp. *sucrofermentans*	Batch and continuous ($D=0.02\text{-}0.12\ h^{-1}$)	Naritomi *et al.* (1998)
Xanthan	*Xanthomonas campestris* NRRL B-1459	Fed-batch	Amanullah *et al.* (1998a)

Objectives

1. To investigate the effect of medium composition on polymer yields in shake-flask culture
2. To study the effect of environmental conditions for polymer production in batch, fed-batch and continuous cultures
3. To determine the water absorption capacity of the polymer compare to a commercial synthetic water absorbent

Chapter 2

Materials and Methods

Materials

1. Media

The basal medium contained (g/l): glucose, 10, $(NH_4)_2SO_4$, 0.5, polypeptone, 2, yeast extract, 0.5, K_2HPO_4, 5, KH_2PO_4, 2, $MgSO_4.7H_2O$, 0.5 and NaCl, 0.1. Glucose was sterilized separately and the pH was adjusted to 7.0 (Yokoi *et al.*, 1995).

Modified optimal medium contained (g/l): sucrose, 30, K_2HPO_4, 5.0, KH_2PO_4, 2.0, $MgSO_4.7H_2O$, 0.5 and tuna condensate 45.3 ml/l. The quantity of tuna condensate added was calculated based on the equal nitrogen concentration to that of yeast extract (0.5 g/l). The total nitrogen concentrations of yeast extract and tuna condensate were 65% and 0.76%, respectively. NaCl was not added in this medium as it was present in the tuna condensate. Sucrose was sterilized separately and the pH was adjusted to 7.0.

The tuna condensate medium contained 10% tuna condensate with the addition of optimal carbon source and concentration. The tuna condensate was diluted for 10% due to the initial NaCl content in tuna condensate was 7% and after dilution, its concentration was 0.7% NaCl which was less than the maximum NaCl concentration (1.0%) for good growth of this strain (result from preliminary experiment).

2. Microorganism

Enterobacter cloacae WD7 isolated from activated sludge of a seafood processing plant by Dermlim (1999) was used in this study.

3. Chemicals

Chemicals used in the analysis were analytical grade: acetonitrile (J.T. Baker), ethanol (J.T. Baker), methanol (May & Baker) and sucrose (Carlo Erba). In medium optimization studies, commercial sucrose (purified cane sugar) bought from local market was used while tuna condensate was kindly provided by a seafood processing plant, Royal Canning Co., Ltd. Commercial synthetic water absorbent is trade mark of Uniseeds Co., Ltd.

4. Fermentor Operation

A 3-l fermentor containing 1.8-l of culture medium was autoclaved at 121 °C for 15 min. After it was cooled, pH, DO and anti-foam probes were connected to the controller. Controlling parameters such as agitation speed, aeration rate, pH-set point were selected to give desired culture conditions.

5. Instruments

Instruments	Model	Company
Double-beam Spectrophotometer	U-2000	Hitachi, Ltd.
Fermentor and	MDL300	B.E. Marubishi
Controller	MDL-6C	B.E. Marubishi
Hot air oven	ULM. 500	Memmert
Incubator	-	K.S.L. Engineering Co., Ltd.
Incubator-shaker	3525-1C	Lab-Line Instrument, Inc.
Liquid chromatograph and	LC-10AD	Shimadzu Corporation
refractive index detector	RID-10A	Shimadzu Corporation
Peristaltic pump	EP-1	Bio Rad
pH meter	D-12	Denver Instrument
Refrigerated centrifuge	SCR 20B	Hitachi Koki

Analytical Methods

1. Cell growth measurement

Culture broth was collected and diluted with distilled water to obtain optimum dilution. After mixing, the absorbance was measured at 660 nm (Kurane *et al.*, 1994).

2. Cell dry weight determination

The diluted viscous culture broth (50 ml) was centrifuged at 13,000 rpm (12,846*xg*) for 15 min at 5 °C to remove the cells. The cells precipitates were resuspended in distilled water (50 ml) and centrifuged again to wash the cells. The washed cells were dried for 24 h at 105 °C in a hot air oven and then weighed to constant weight after cooling in a desiccator (Dermlim, 1999).

3. Crude exopolysaccharide (EPS) yield

The supernatant (after cell removal) was diluted and precipitated with 4 volumes of cold 95% ethanol, left for 2 h in a freezer (-20 °C) and centrifuged at 10,000 rpm (7,600*xg*) for 15 min at 5 °C. The obtained precipitate was dried for 24 h in a hot air oven at 105 °C and weighed to constant weight after cooling in a desiccator (Adapted from Dermlim, 1999).

4. Residual sugar determination

The supernatant (after cell removal) was diluted and determined for sugar content by HPLC consisting of a Shim-pack CLC-NH_2 column and a refractive index detector. Mobile phase flow rate was 1.5 ml/min and acetonitrile/water (74:26) was used as the mobile phase and the standard sugar solutions of 0.05 and 1.0% sucrose were prepared and used as a control (Adapted from Liu and Steinbuchel, 1997).

5. Water absorption capacity

The water absorption capacity of the biopolymer was determined (Adapted from Kurane and Nohata, 1994) by soaking the weighed dried sample (0.10 g) in distilled water for 30 min, 24 h and 36 h then removed and left for 1 h to drain off the excess water using cheese cloth. This soaked sample was then put into a known weight tube and weighed. The water absorption capacity (g) per gram of dried sample was then calculated from the equation

Water absorption capacity

$$= \frac{\text{Sample weight after absorption (g)- Sample weight before absorption (g)}}{\text{Dried sample weight (g)}}$$

Methods

1. Optimization for polymer production in batch culture

Starter preparation

One loop of 24 h culture of *Enterobacter cloacae* WD7 was inoculated into 200 ml nutrient broth in a 500 ml flask. Cultivation was performed on a rotary shaker (200 rpm) at 30 °C for 12 h. The culture was diluted with sterile distilled water to obtain the viable cell counts of 10^8 CFU/ml, determined from the calibration curve as given in the appendix 1. This was used as the starter culture.

1.1 Time course of exopolysaccharide production in shake-flask cultivation

The starter culture (5%) was transferred into the basal medium (200 ml) and cultivated on a rotary shaker (200 rpm) at 30 °C for 5 days. Samples were taken at first 6 h and every 12 h to measure pH and cell dry weight. The crude polymer yield was measured at the end of cultivation.

1.2 Effect of nutrients on exopolysaccharide production

The cultivation of *E. cloacae* WD7 in the basal medium was performed as described above (section 1.1). The influence of the following nutrients was investigated. The samples were taken up to 3 days cultivation.

1.2.1 Type of carbon source

The effect of different carbon sources; monosaccharide such as glucose, fructose, galactose, and disaccharide such as sucrose and maltose at 1% (w/v) concentration, was studied.

1.2.2 Carbon source concentration

Cultivation was performed in basal medium containing a suitable carbon source (from section 1.2.1) with concentrations at 0, 1.0, 2.0, 3.0 and 4.0% (w/v).

1.2.3 Nitrogen source

Cultivation was carried out in basal medium containing the optimum carbon source and concentration (from section 1.2.2), the nitrogen source was varied as following.

1.2.3.1 Inorganic nitrogen

The inorganic nitrogen such as $(NH_4)_2SO_4$, NH_4NO_3 and NH_4Cl at 0, 0.05, 0.10 and 0.15% (w/v) were tested.

1.2.3.2 Organic nitrogen

The concentration of organic nitrogen (polypeptone from enzymatic digest of protein) was studied at 0, 0.1, 0.2 and 0.3% (w/v).

1.2.4 Yeast extract concentration

E. cloacae WD7 was cultivated in the basal medium containing optimum carbon and nitrogen sources (from 1.2.3) and the concentrations of yeast extract were tested at 0, 0.05, 0.10 and 0.20% (w/v).

1.2.5 Initial pH

The initial pH of the optimal medium (from 1.2.4) was adjusted to 6.0, 6.5, 7.0, 7.5 and 8.0 using 6 N HCl or 3 N NaOH.

1.2.6 Incubation temperature

The cultivation was performed on a rotary shaker (200 rpm) at 30, 35 and 40 °C for 3 days.

1.2.7 Time course on exopolysaccharide production in optimal medium

E. cloacae WD7 was cultivated in the optimal medium (from section 1.2.6). Samples were taken at first 6 h and every 12 h to measure for pH, cell dry weight, crude EPS yield and residual sugar and kinetic parameters were determined. The kinetic parameter, K_s, was evaluated by cultivation in the optimal medium with various sucrose concentrations (0-4%) and cell dry weight were measured.

1.3 Effect of environmental condition on exopolysaccharide production

The 10^8 CFU/ml inoculum (5%) was transferred into a 3-l fermentor containing 1.8-l of optimal medium and the temperature was controlled at optimal value (from section 1.2.6). Samples were taken and all parameters were analyzed as mentioned in the shake-flask cultivation. The effects of the following conditions were investigated.

1.3.1 Effect of controlling pH

Cultivation under uncontrolled and controlled pH (from section 1.2.5) were compared. The agitation speed of the fermentor was 200 rpm with the aeration rate of 0.5 vvm.

1.3.2 Aeration rate

The aeration (compressed air) rates were varied from 0, 0.5, 1.0 and 2.0 vvm at the agitation speed of 200 rpm.

1.3.3 Agitation speed

The cultivation was performed at the agitation speeds of 200, 400, 600 and 800 rpm at the optimal aeration rate (from section 1.3.2).

1.3.4 Time course on exopolysaccharide production under optimal condition

E. cloacae WD7 was cultivated under optimal cultivation condition (from section 1.3.3) for 72 h. The kinetic parameters were determined and compared to those from shake-flask culture.

1.4 Effect of carbon and nitrogen sources substitution

1.4.1 Effect of using commercial sucrose as carbon source

The cultivation was carried out as described in section 1.3.4 but the carbon source was replaced by the commercial sucrose (purified cane sugar) at concentrations of 1% and 3% (w/v) and the EPS yields were compared.

1.4.2 Effect of using tuna condensate as nitrogen source

The cultivation was carried out in two media; the modified optimal medium and the 10% tuna condensate medium.

The EPS yields achieved from these media were compared to those from the optimal media.

2. Water absorption capacity of the biopolymer

The crude EPS and the commercial synthetic water absorbent were examined for water absorption capacity in which the soaking periods of these polymers in distilled water were compared at 30 min, 24 h and 36 h.

3. Fed-batch culture

The starter and the three batch cultivations were carried out as described previously (section 1). The following conditions were carried out.

3.1 Effect of initial batch cultivation period

A 10% sterile sugar solution (to obtain concentration of 3%) was added after 1, 2, 3 and 4 days cultivation and the incubation were continued for 7 days in each condition.

3.2 Effect of initial sugar concentration

Batch fermentation was performed for 3 days with the initial sugar concentrations at 1% and 3%. Then the batch culture was started by adding 10% sugar solution (to obtain concentration of 1% and 3%, respectively) every 3 days. Time course of EPS production was determined with the optimal sugar concentration and feeding period. Samples were taken every 24 h to determine for EPS yield, cell dry weight and residual sugar whereas pH and DO values were recorded by a recorder.

4. Continuous culture

4.1 Effect of dilution rate

The batch cultivation was carried out under the optimal medium and conditions for 3 days. Then, the optimal medium was fed at the dilution rates of 0.01, 0.05 and 0.10 h^{-1}.

4.2 Effect of batch cultivation period

The batch culture was performed in the optimal medium and conditions for 1, 2 and 3 days, after that the optimal medium was fed at the dilution rate of 0.05 h^{-1}. Time course of continuous culture was examined and the cultivation time was extended to 7 days. Samples were taken every 24 h to measure for EPS yield, cell dry weight and residual sugar whereas pH and DO values were recorded by a recorder.

Under the optimal conditions for each type of cultivation, the kinetic parameters were calculated by the following equations:

Kinetic parameters for batch and fed-batch cultures were as following (Prasertsan, 1993; Doelle, 1997):

1. Specific growth rate (μ) = $(\ln X - \ln X_0)/\Delta t$ (h^{-1})

when X = final biomass concentration (g/l)

X_0= initial biomass concentration (g/l)

Δt= elapsed time (h)

2. Maximum specific growth rate (μ_m) was calculated by plotting $1/\mu$ vs. $1/S$ obtained a linear slope that intercept value on ordinate was $1/\mu_m$

or $(\mu_m) = \mu (K_s+S)/S$

when μ = specific growth rate (h^{-1})

K_s= saturation constant (g/l)

S = carbon source concentration (g/l)

3. Cellular yield coefficient ($Y_{x/s}$) = $(X-X_0)/(S_0-S)$ (g cell/g substrate)

when S_0= initial substrate concentration (g/l)

S= final substrate concentration (g/l)

4. Conversion yield of substrate to product ($Y_{p/s}$) = $(P-P_0)/(S_0-S)$ (g product/ g substrate)

when P= final dried weight of crude EPS (g/l)

P_0= initial dried weight of crude EPS (g/l)

5. Specific rate of product formation (q_p) = $\mu/(Y_{p/s})$ (h^{-1})

or $q_p = (1/X)(\Delta P/\Delta t)$ (h^{-1})

when ΔP = elapsed of dried weight of crude EPS (g/l)

6. Specific rate of substrate utilization (q_s) =$\mu/(Y_{x/s})$ (h^{-1})

or $q_s = (1/S)(\Delta S/\Delta t)$ (h^{-1})

when ΔS= elapsed of substrate concentration (g/l)

7. Maximum productivity (R_m) = P_m/t (g crude EPS/l.h)

when P_m= maximum product concentration (g/l)

8. Generation time (g) = t/n (h)

or $g = (\ln 2)/\mu$ (h)

when t = divided time (h)

ln = natural logarithm

9. Saturation constant (K_s) calculated by plotting $1/\mu$ vs. $1/S$ obtained a linear slope that had the value of K_s/μ_m and the intercept value on abscissa was $-1/K_s$ while intercept value on ordinate was $1/\mu_m$

or $K_s = (1/\mu-1/\mu_m)\ \mu_m S$ (g/l)

Kinetic parameters for continuous culture were as following:

1. Dilution rate (D) = F/V (h^{-1})

   when F = medium flow rate (l/h)

   V = culture volume (l)

2. Critical dilution rate $(D_c) = (\mu_m S_0)/(K_s+S_0)$ (h^{-1})

3. Maximum dilution rate $(D_m) = D_c\{1-(K_s/K_s+S_0)^{1/2}\}$ (h^{-1})

4. Cell yield coefficient $(Y_{x/s}) = X/\{S_0-DK_s/ \mu_m-D)\}$ (g cell/g substrate)

5. Maximum productivity $(R_m) = P_m/t$ (g crude EPS/l.h)

Chapter 3

Results and Discussions

1. Optimization for polymer production in batch culture

1.1 Time course of exopolysaccharide production in shake flask cultivation

Time course on growth and exopolysaccharide (EPS) production by *E. cloacae* WD7 in basal medium (pH 7.0) using 1% glucose as carbon source for 5 days at 30 °C is shown in Fig. 1. The bacterium grew rapidly within the first 6 h. This was correlated to the rapid decline of pH which due to the sugar was metabolized by cells and formed acidic metabolites and EPS (Lawson and Sutherland, 1978, Clarke, 1991). Furthermore, the EPS produced from *E. cloacae* WD7 are acidic heteropolysaccharide consisting of neutral sugar (29.4%) and uronic acids (14.18%) (Dermlim, 1999). After 6 h, pH increased rapidly and kept constant after 24 h which may cause by the depletion of carbon source and the generation of ammonia from nitrogen source consumption. The maximum EPS yield (2.22 g/l) was obtained after 3 days cultivation which was in the stationary phase of growth. This indicated that *E. cloacae* WD7 produced EPS not correlated with cell growth or non-growth associated.

E. cloacae WD7 produced EPS during the stationary phase and excreted it into the culture broth leading to the viscous characteristic that is typically exhibited as pseudoplastic behavior (Pace, 1980). Because the EPS produced by *E cloacae* WD7 contains hydroxyl groups so that it could be hydrated, swell and give viscous solutions (Dermlim, 1999). The biopolymer could be biosynthesized either through a block mechanism or a monomeric mechanism (Shibaev, 1986). *E. cloacae* WD7 is anticipated to synthesize the EPS via the second mechanism as almost all of the Enterobacteriaceae do (Tonn and Gander, 1979). After it was synthesized, the polysaccharide would be released into the culture broth (Sutherland and Ellwood, 1979).

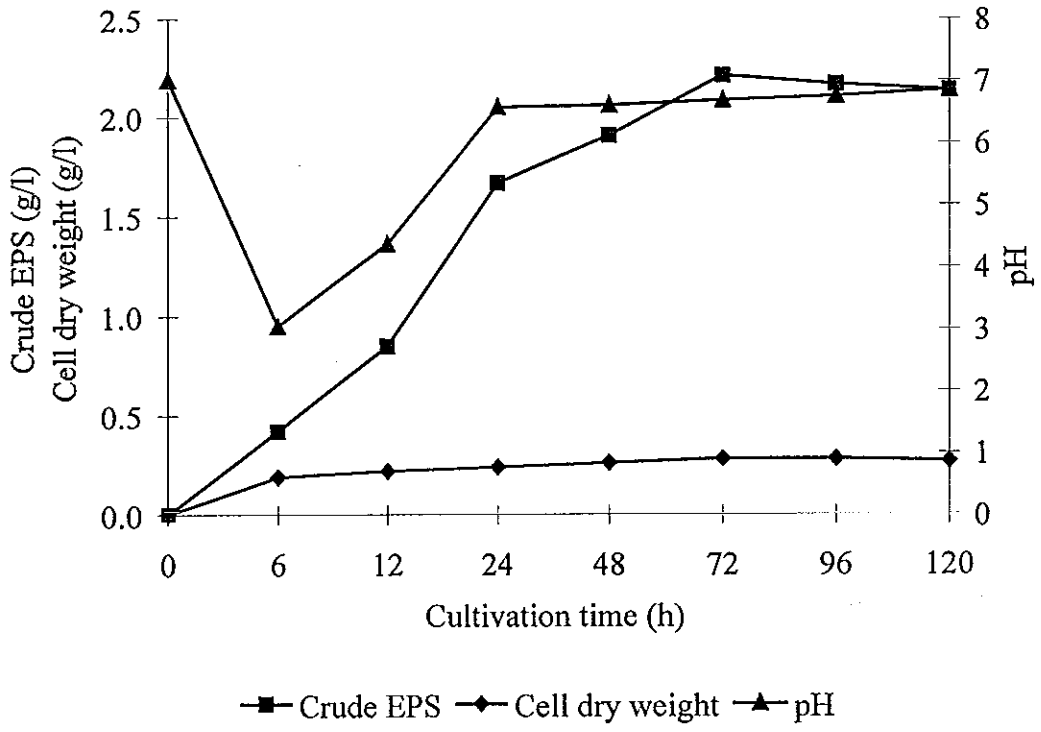


Fig. 1 Time course on EPS production and growth of *E. cloacae* WD7 in basal medium under shake-flask culture

The highly viscous culture broth reduced the oxygen transfer rate, hence, slowing down growth in the stationary phase (after 2 days cultivation). *E. cloacae* WD7 gave the highest EPS yield of 2.14 g/l after 3 days cultivation. The EPS produced from *E. cloacae* WD7 is therefore a non-growth associated product and similar to almost of microbial exopolysaccharides which have generally been considered as secondary metabolites (not growth related) (Pace, 1980). However, some microorganisms produced growth associated exopolysaccharides such as *Butyrivibrio fibrisolvens* nyx (Wachenheim and Patterson, 1992) and *Klebsiella. oxytoca* (Dlamini and Peiris, 1997).

The kinetic parameters were as follow; specific growth rate (μ) was 0.14 h^{-1}, the maximum productivity (R_m) was 0.03 g crude EPS/l.h and the generation time (g) was 4.95 h. These results indicated that *E. cloacae* WD7 had similar growth rate but lower EPS yield when compared with growth and exopolysaccharide production from *Butyrivibrio fibrisolvens* nyx (μ= 0.15 h^{-1}, R_m= 0.14 g EPS/l.h) and *X. campestris* (μ=0.19 h^{-1}, R_m= 0.34 g EPS/l.h) (Wachenhiem and Patterson, 1992).

1.2 Effect of nutrients on exopolysaccharide production

1.2.1 Carbon sources

The effect of various carbon sources (1%) on growth and EPS production from *E. cloacae* WD7 are given in Fig. 2 and Fig. 3, respectively. The microorganism could grow on all of the carbon sources tested (mono- and di-saccharides) with no significant difference ($p<0.05$) (Fig. 2(a)). Rapid growth occurred in the first 12 h of cultivation and increased with slower rate thereafter. The maximum growth (OD_{660}=3.83) occurred when sucrose was used as carbon source. For EPS production by *B. thermoantarcticus,* the maximum growth obtained from using mannose (OD_{540}=1.60) was higher than those from sucrose (OD_{540}=1.45) and glucose (OD_{540}=1.30) (Manca *et al*, 1996). The pH changes of culture broth in various carbon sources (Fig. 2(b)) were correlated to cell growth,

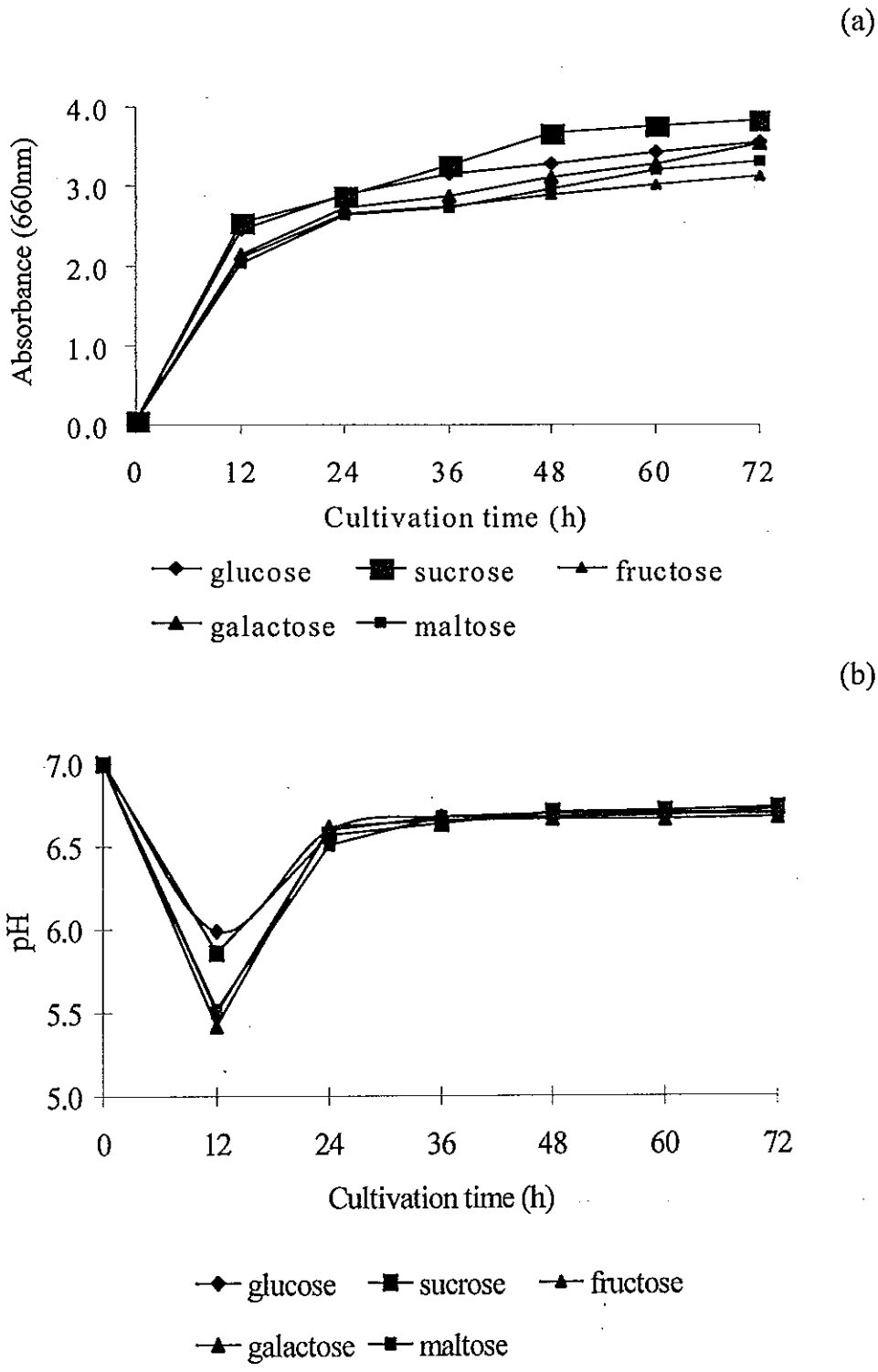


Fig. 2 Growth (a) and pH changes (b) during cultivation of *E. cloacae* WD7 in the basal medium with various carbon sources (1%) on a shaker (200 rpm) at 30 °C

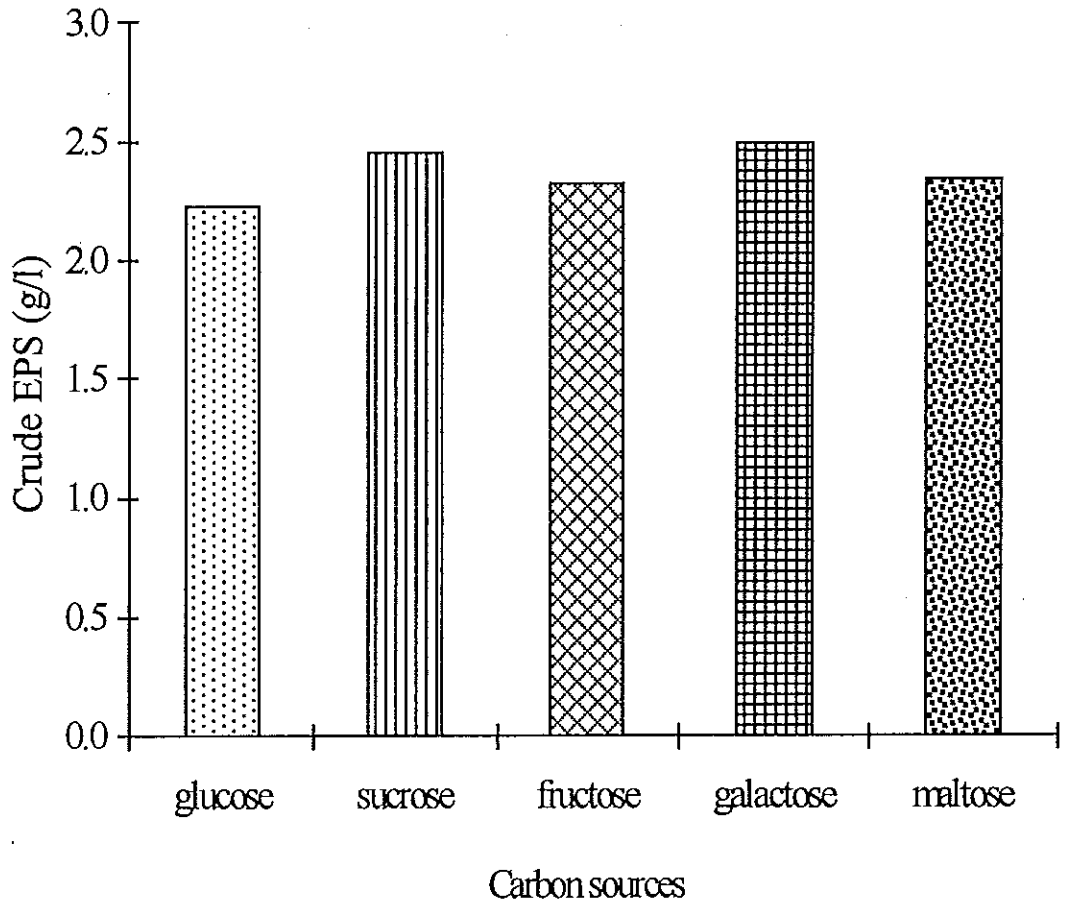


Fig. 3 Effect of carbon sources (1%) on the EPS production from *E. cloacae* WD7 in shake-flask culture after 3 days cultivation

as it decreased rapidly from pH 7.0 to about 5.5 in the first 12 h. This is in agreement with previous reports (Cerning *et al.*, 1994). When glucose and sucrose were used as carbon sources, the final pH were similar (5.9 and 6.0). This is also the case for the pH changes for galactose, maltose and fructose (5.42, 5.52 and 5.50, respectively). However, after 24 h the pH of cultures returned to nearly neutral pH (6.70) and remained constant. The difference in pH changes of culture medium when using different carbon sources were also found in previous reports (Roukas, 1998; Clarke *et al.*, 1995; Shin *et al.*, 1989).

The effect of various carbon sources on EPS production (Fig. 3) revealed that fructose and maltose gave similar yields (2.32 and 2.34 g/l) ($p<0.05$) while glucose, sucrose and galactose gave different EPS yields (2.23, 2.45 and 2.50 g/l, respectively) ($p<0.05$). These were correlated to cell growth. Cell growth and EPS production was reported to depend on carbon sources which affected quality, sugar component and/or molecular weight of EPS (Wachenheim and Patterson, 1992). On the other hand, the molecular size of an extracellular heteropolysaccharide produced by a particular microbial species appears to be identical in composition and of constant molecular weight no matter what changes are made in the substrate but not for homopolysaccharide (McNeely, 1967).

Although the maximum EPS yield was obtained from galactose but sucrose is a cheaper substrate. So sucrose was chosen as optimal carbon source for EPS production from *E. cloacae* WD7. Sucrose was also the best carbon source for levan production by *Erwinia herbicola* because the energy from the hydrolysis of the disaccharide glycosidic bond was required for polymerization while fructose alone yielded little polymer (Keith *et al.*, 1991). *E. cloacae* WD7 gave similar results. The production of EPS from *L. casei* CG11 gave low EPS yield when glucose was used as carbon source due to the PEP-fructose phosphotransferase and 1-phosphofructokinase repression by glucose (Cerning *et al.*, 1994). So the low yield from glucose in this experiment may be caused by the suppression of any enzymes involved in the polymer biosynthesis. Carbon sources

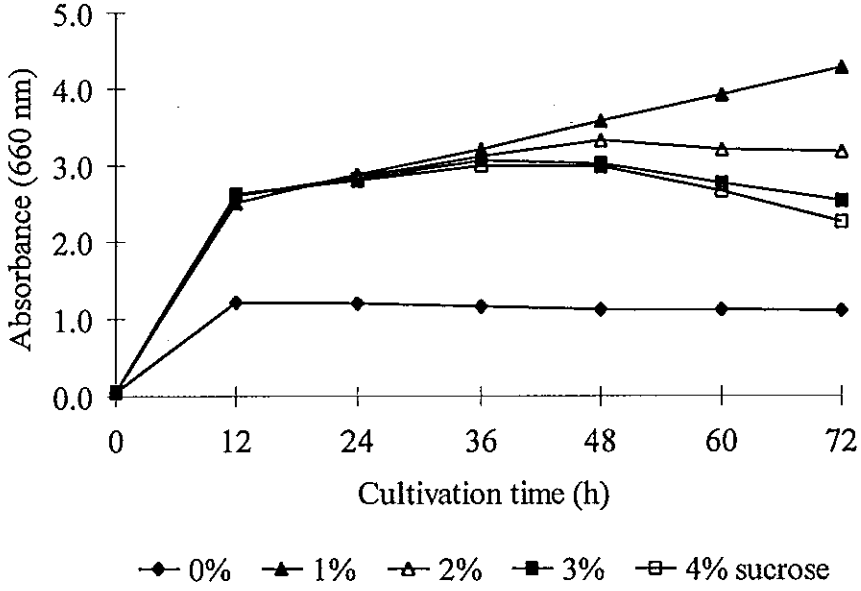
also generate ATP used in polymerization, the content of oxidized groups and the energy metabolism of the cell (Margaritis and Pace, 1985). Typically, changes in the carbon source cause the development of capsules on the cells then extracellular polysaccharide was produced (McNeely, 1967). It could be concluded that the carbon source affected both cell growth and EPS yield and sucrose was selected as a suitable carbon source for *E. cloacae* WD7.

1.2.2 Carbon source concentrations

The effect of sucrose concentration for growth and EPS production from *E. cloacae* WD7 was examined (Fig. 4 and Fig. 5, respectively). Without sucrose, slight growth was observed in the first 12 h and decreased thereafter (Fig. 4(a)). This result was similar to the result using 0% glucose (maximum OD_{660}=0.50) for cultivation of *Alteromonas maeleodii* HYD-1545 (Vincent *et al.*, 1994) while previous reports showed no growth when 0% glucose was used for the production of EPS by *Butyrivibrio fibrisolvens* nyx (Wachenheim and Patterson, 1992). The sucrose concentrations of 1, 2, 3 and 4% gave rapid growth rates (maximum OD_{660}=4.28, 3.18, 2.54 and 2.27, respectively) ($p<0.05$) within the first 12 h and decreased thereafter. Therefore, only 1% sucrose seemed to be sufficient for cell growth. Trace amounts of sucrose was required for cell growth although dextran produced by *Leuconostoc mesenteroides* used mainly glucose as repeating units of its polymer and generated ATP for cell metabolism (Margaritis and Pace, 1985).

The changes of pH during the cultivation of *E. cloacae* WD7 in various sucrose concentrations were different (Fig. 4(b)). Without sucrose (0%), the pH increased from 7.0 to about 7.45 at the end of cultivation (72 h). At 1 and 2% sucrose concentrations, the pH declined sharply within 12 h, then increased and kept constant (pH about 6.90 at 72 h). This indicated that in the presence of sucrose, acidic metabolites were produced which correlated to cell growth. However the results at 3% and 4% sucrose were different as pH decreased within the first 36 h, then increased slowly from pH 4.88 and 4.26 to pH 6.43 and 5.09,

(a)



(b)

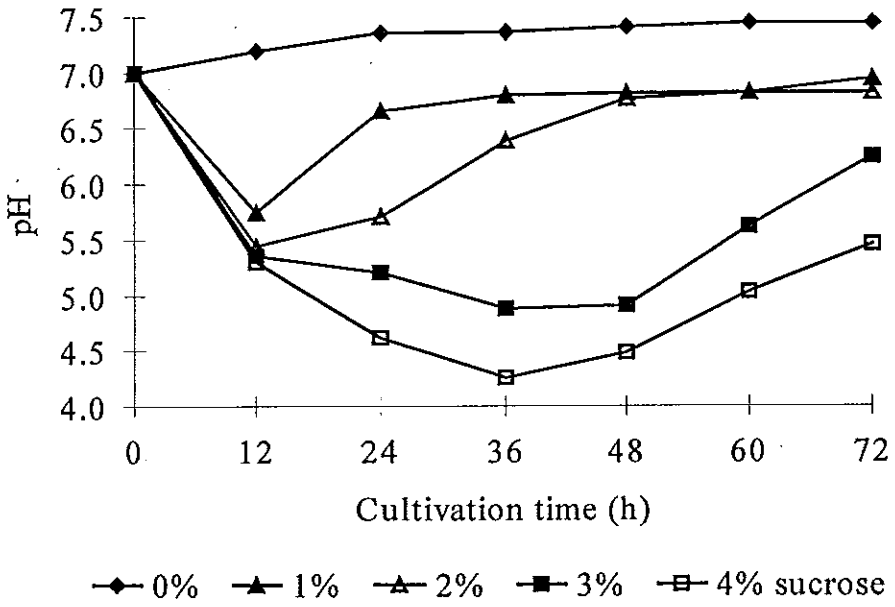


Fig. 4 Growth (a) and pH changes (b) during cultivation of *E. cloacae* WD7 in the basal medium with various sucrose concentrations on a shaker (200 rpm), at 30 °C

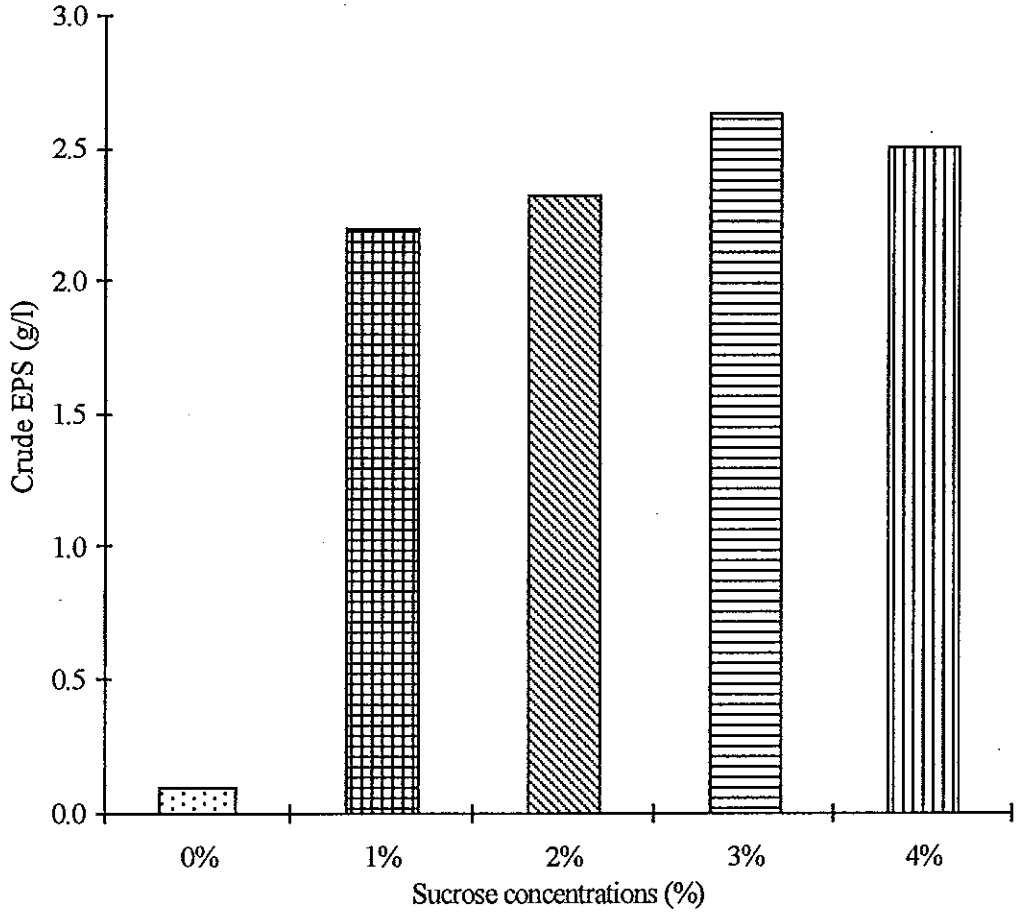


Fig. 5 Effect of carbon source concentrations on the EPS production from *E. cloacae* WD7 in shake-flask culture after 3 days cultivation

respectively. Therefore, higher sucrose concentrations gave higher concentrations of acidic metabolites with much lower pH values.

The EPS from *E. cloacae* WD7 increased significantly ($p<0.05$) as the sucrose concentrations increased up to 3% with the values of 2.23, 2.24 and 2.72 g/l at 1, 2 and 3% sucrose respectively and 2.51 g/l at 4% sucrose (Fig. 5). Therefore, the optimal sucrose concentration for *E. cloacae* WD7 was 3% while it was 2.5% for *Agrobacterium tumefaciens* (Stredansky and Conti, 1999), 10% for *Lactobacillus* strain LB 80 (Geel-Schutten *et al.*, 1998) and 2% for *Lactobacillus casei* CG11 (Cerning *et al.*, 1994), giving the maximum EPS concentrations of 23.5, 21.0 and 0.05 g/l, respectively.

The carbon source concentration affected the efficiency of substrate conversion to polysaccharides (Lawson and Sutherland, 1978). The high carbon source concentrations, resulting in high polymer concentration, may lead to oxygen limitations or heterogeneity due to increased viscosity within the broth before exhaustion of the carbon source, and this might indirectly affect yield as well as the overall production rate (Magaritis and Pace, 1985). For instance, increasing glucose concentration can decrease the conversion efficiency of glucose to polysaccharide by *X. campestris* while the sugar concentration had a marked effect on EPS yield of *Lactobacillus casei* CG11 (Nohata and Kurane, 1994).

1.2.3 Nitrogen sources

1.2.3.1 Inorganic nitrogen sources

The effect of various inorganic nitrogen sources on growth and EPS production from *E. cloacae* WD7 when 3% sucrose used as carbon source, are presented in Fig. 6 and Fig. 7, respectively. Ammonium chloride (NH_4Cl) gave the highest cell growth (OD_{660}=3.78) while ammonium nitrate (NH_4NO_3) and ammonium sulfate ($(NH_4)_2SO_4$) gave similar low growth values (OD_{660}=3.41 and 3.31, respectively) ($p<0.05$) (Fig. 6(a)). Without the addition of any inorganic

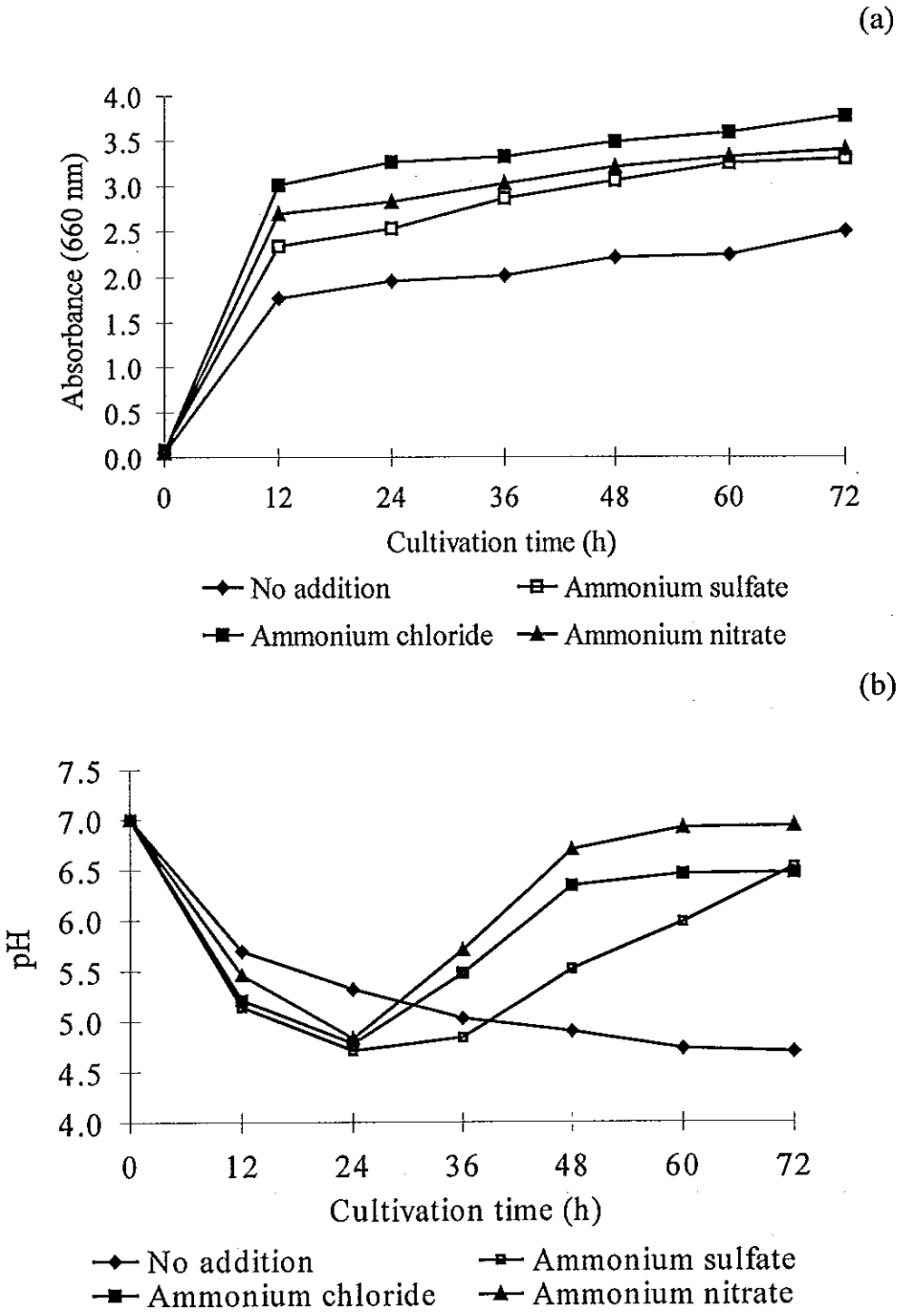


Fig. 6 Growth (a) and pH changes (b) during cultivation of *E. cloacae* WD7 in the basal medium containing 3% sucrose as carbon source with various inorganic nitrogen sources (0.05%) on shake-flask culture (200 rpm) at 30 °C

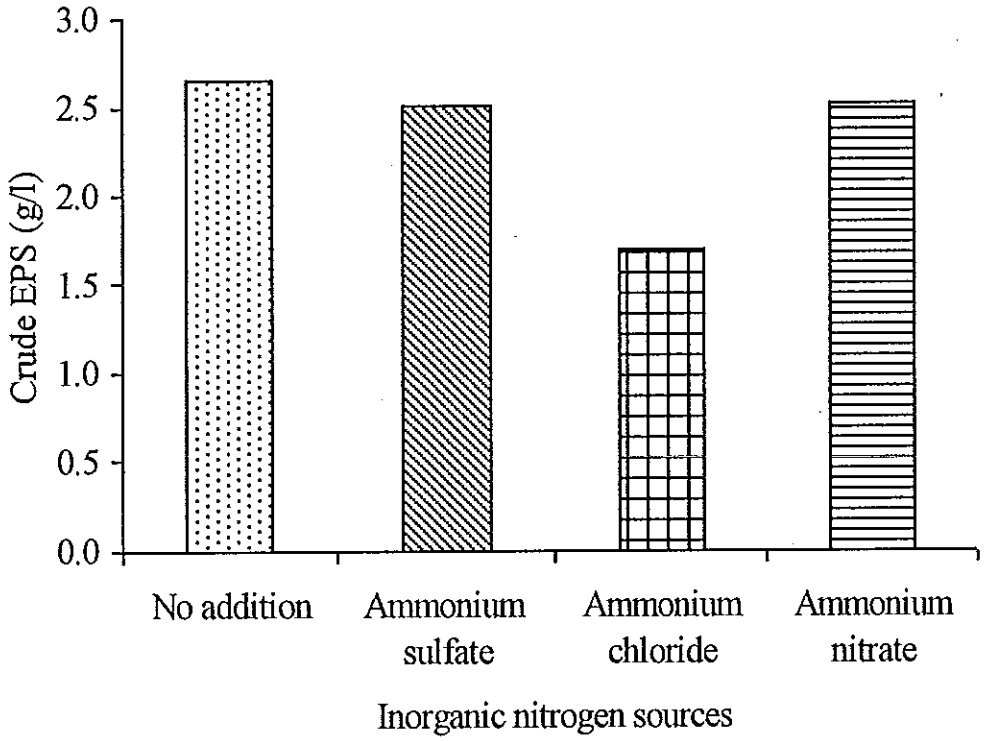


Fig. 7 Effect of inorganic nitrogen sources (0.05%) on the EPS yield from *E. cloacae* WD7 in shake-flask culture after 3 days cultivation

nitrogen source, slight growth was observed which may be due to the presence of organic nitrogen sources (yeast extract and polypeptone) in the basal medium.

The pH declined sharply within the first 24 h cultivation in all cases and increased to the values of pH 6.5-7.0 at 72 h, except in the case of no inorganic nitrogen addition (Fig. 6(b)). The latter case may be due to the lower buffering capacity of the medium (Stredansky *et al.*, 1998).

The crude EPS yields of 2.53, 2.51 and 1.69 g/l were obtained when NH_4NO_3, $(NH_4)_2SO_4$ and NH_4Cl were used as nitrogen sources (Fig. 7). However, the highest EPS achieved (2.73 g/l) was obtained without any inorganic nitrogen added which correlated to the decline of pH. In general, optimum medium for EPS from microorganisms prefer a high C:N ratio (>10) (Lawson and Sutherland, 1978). The C:N ratio of this experiment was found to be 10 (3% sucrose as carbon source and 0.3% nitrogen source from $(NH_4)_2SO_4$, polypeptone and yeast extract). Although nitrogen source is necessary for cell growth and the synthesis of the enzymes necessary for polysaccharide formation, an excess of nitrogen generally reduces the conversion of the carbohydrate substrate to the heteropolysaccharide (Lawson and Sutherland, 1978). Furthermore, the apparent encouragement of heteropolysaccharide production by low nitrogen levels may be a survival effect (McNeely, 1967). *E. cloacae* WD7 might not need inorganic nitrogen for EPS formation as there was enough nitrogen from organic nitrogen in the basal medium. For the production of exopolysaccharide (ZanflO) from *Erwinia herbicola*, inorganic nitrogen (0.2% NH_4NO_3) was necessary (Lawson and Sutherland, 1978). Ammonium sulfate (0.096%) gave a correlated linear to cell growth of *B. fibrisolvens* nyx but not for EPS production while urea at 0.86 g/l increased cell growth significantly (Wachenheim and Patterson, 1992). Urea and potassium nitrate were the effective inorganic nitrogen sources for the production of bioabsorbent by *Alcaligenes latus* B-16 while ammonium chloride, ammonium sulfate, and ammonium nitrate were not (Nohata and Kurane, 1994). Initial

ammonium sulfate (0.06%) is presumed to contribute to the decrease of pH in culture broth (Shin *et al.*, 1989).

1.2.3.2 Effect of organic nitrogen (polypeptone) concentrations

The effect of organic nitrogen concentrations on growth and EPS production from *E. cloacae* WD7 are shown in Fig. 8 and Fig. 9, respectively. The maximum cell growth with the OD_{660} values of 2.67, 2.00, 1.51 and 1.08 were achieved in the presence of polypeptone at 0.3, 0.2, 0.1 and 0%, respectively (Fig. 8(a)). The pH of the culture broth decreased more rapidly as the concentrations of polypeptone increased (Fig. 8(b)). Without polypeptone added the data showed very low decline of pH and kept nearly constant value (pH 6.67).

The EPS concentrations of 2.70, 2.01, 2.00 and 1.85 g/l were obtained with the addition of polypeptone at 0, 0.1, 0.2 and 0.3%, respectively (Fig. 9). This clearly indicated that polypeptone was unnecessary for production of EPS but affecting cell growth. The amount of nitrogen in minimal medium did not influence the amount of EPS produced except when the concentration became limiting; cell density decreased but EPS increased (Anton *et al.*, 1988). Biopolymer produced from *K. oxytoca* was enhanced when nitrogen supply was limited and carbon sources were in excess (Dlamini and Peiris, 1997). The high C:N ratio gave higher EPS yield with lower cell growth because the pathway for growth was blocked then changed to EPS biosynthesis pathway, nevertheless, nitrogen source should be available at beginning of the stationary phase (Lo *et al.*,1997). Xanthan production was poor if the nitrogen concentration in the medium was too high (Lo *et al.*, 1997).

1.2.4 Effect of yeast extract concentrations

Studied on growth and EPS production from *E. cloacae* WD7 in various yeast extract concentrations in the optimized medium (pH 7.0) containing 3% sucrose without the addition of inorganic nitrogen and polypeptone cultivated at 30 °C were carried out. Cell growth was higher at higher concentration of yeast

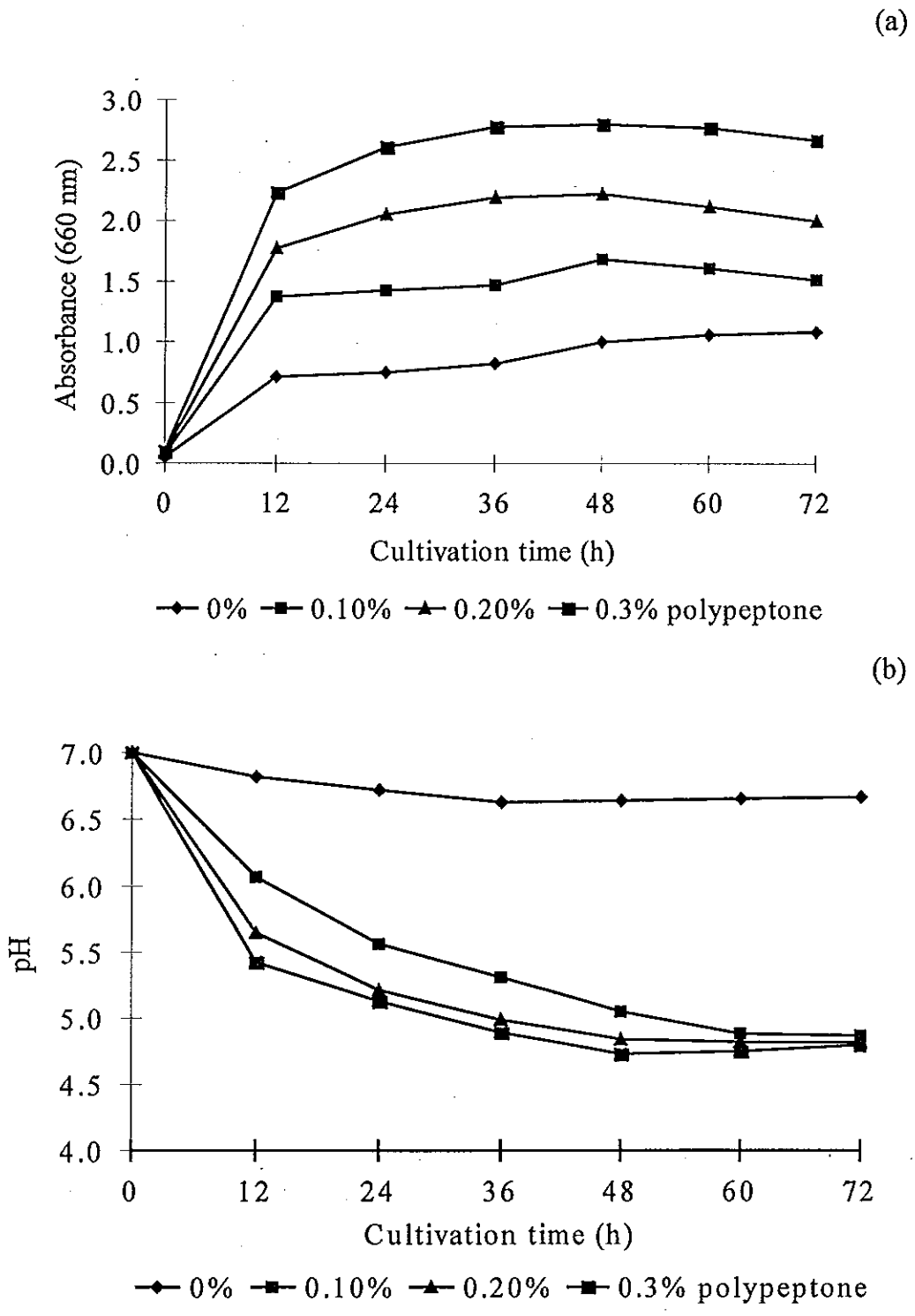


Fig. 8 Growth (a) and pH changes (b) during cultivation of *E. cloacae* WD7 in the basal medium containing 3% sucrose as carbon source with various polypeptone concentrations on shake-flask culture (200 rpm) at 30 °C

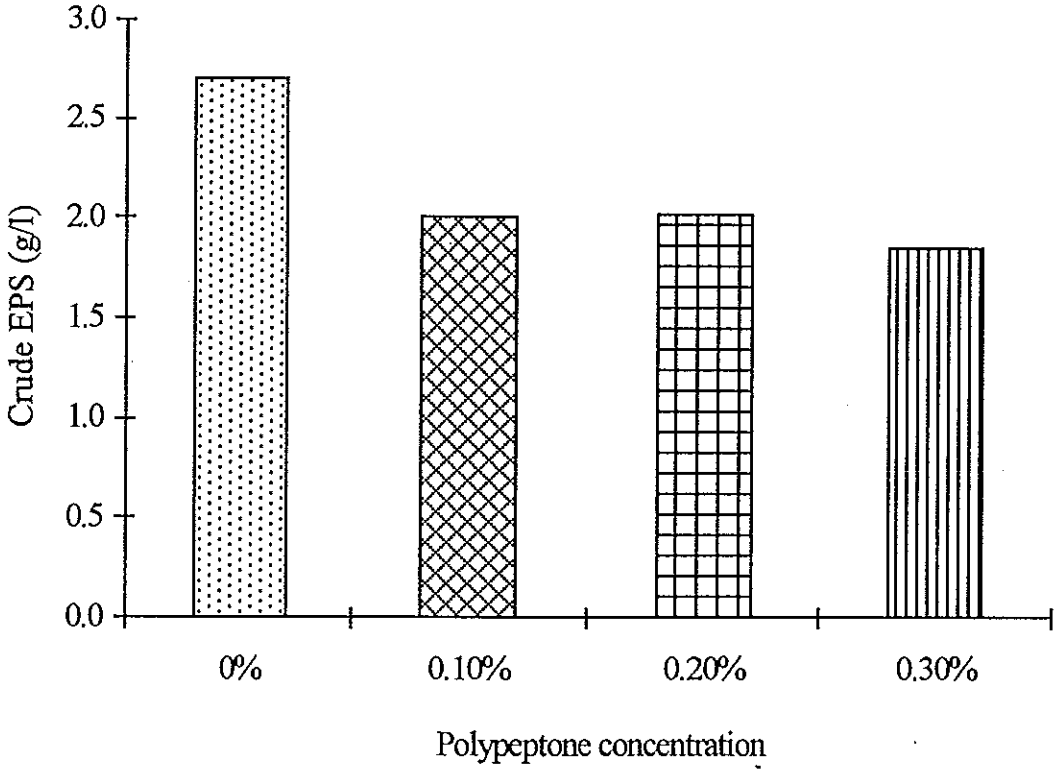


Fig. 9 Effect of polypeptone with various concentrations on the EPS production from *E. cloacae* WD7 in shake-flask culture after 3 days cultivation

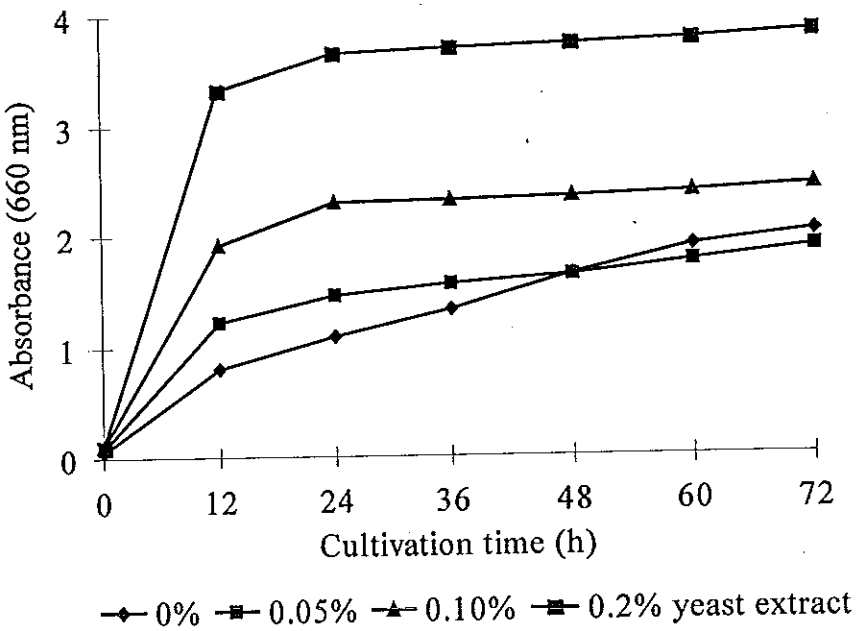
extract ($p<0.05$) (Fig. 10(a)). Yeast extract may be considered as the sole nitrogen source in the medium. Its effect on pH changes during cultivation (Fig. 10(b)) indicated that at low amount of yeast extract (0 and 0.05%) pH declined slightly, giving values of pH 6.64 and 6.54 respectively while at higher concentrations (0.10 and 0.20%) the culture broth became more acidic (pH 5.17 and 4.65, respectively). This is contrary to the report that yeast extract mainly contributed to the cell growth without significant decrease of pH of the culture broth (Shin *et al*, 1989).

The EPS yields decreased as yeast extract concentrations increased (Fig. 11). Without yeast extract added, EPS is still produced (1.97 g/l) and the maximum yield (2.71 g/l) was achieved from 0.05% yeast extract. At 0.10% and 0.20%, the EPS yields decreased to 2.68 and 1.75 g/l, respectively. Therefore, *E. cloacae* WD7 required at least 0.05% yeast extract to maintain cell growth and the highest EPS. This concentration was 10 times lower than the optimal yeast extract concentration (0.5%) for bioabsorbent production from *Alcaligenes latus* B-16 (Nohata and Kurane, 1994). Increasing yeast extract concentrations also gave higher pullulan yield from a mixed culture of *Aureobasidium pullulans* and *Kluyveromyces fragilis* (0.1% as sole nitrogen source gave 11.0 g/l EPS) (Shin *et al.,* 1989). One or more component(s) of yeast extract affected the polydispersity of the zooglan produced by *Zoogloea ramigera* 115SLR and on polymerase or depolymerase activity or synthesis (Guillouet *et al.*, 1999).

1.2.5 Effect of initial pH

E. cloacae WD7 was cultivated in the optimal medium consisting of 3% sucrose, 0.05% yeast extract, 0.2% KH_2PO_4, 0.05% $MgSO_4.7H_2O$ and 0.01% NaCl at 30 °C. The initial pH was adjusted in the range of 6.0 to 8.0. The results indicated that at initial pH of 6.0, the growth increased within 12 h and decreased thereafter. At initial pH of 6.5, results were similar to those at pH 7.5 but gave

(a)



(b)

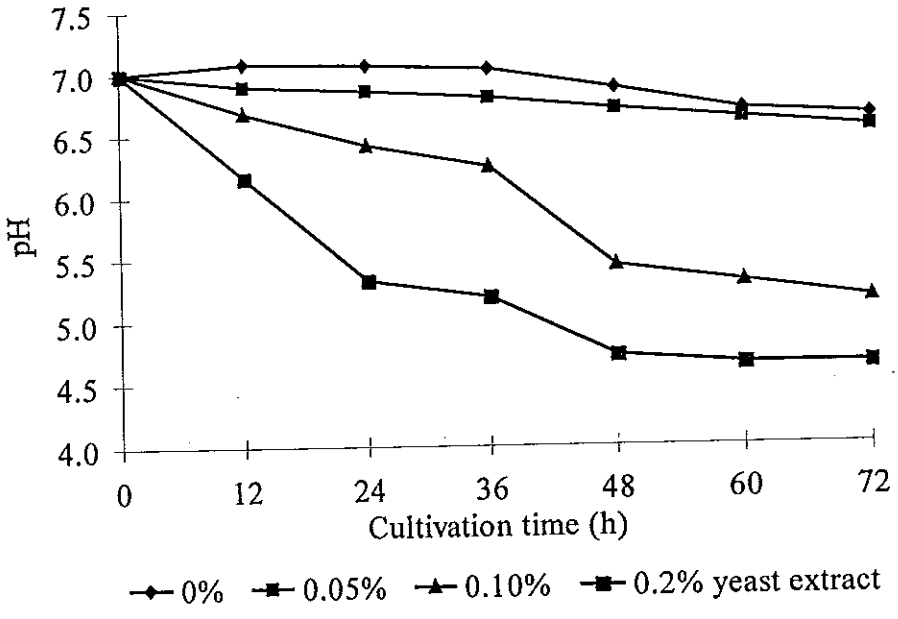


Fig. 10 Growth (a) and pH changes (b) during cultivation of *E. cloacae* WD7 in the basal medium containing 3% sucrose as carbon source with various yeast extract concentrations on shake-flask culture (200 rpm) at 30 °C

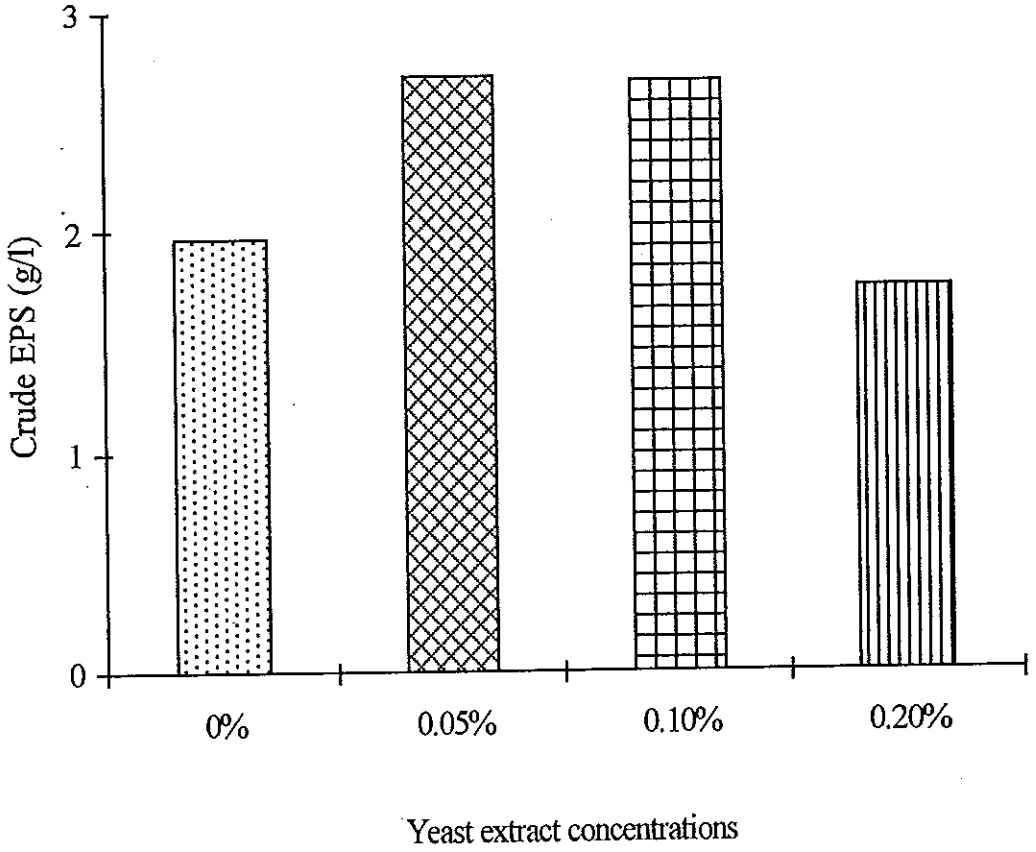


Fig. 11 Effect of yeast extract with various concentrations on the EPS production from *E. cloacae* WD7 in shake-flask culture after 3 days cultivation

lower growth. The most suitable initial pH of medium for cell growth was found to be 7.0 (Fig. 12(a)).

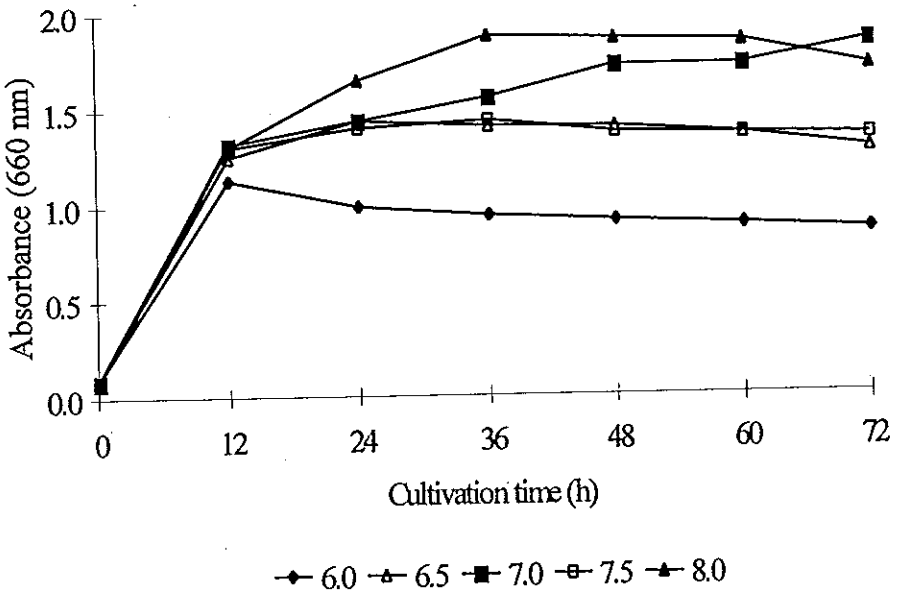
The pattern of pH changes was the same in cultures with the initial pH of 6.5, 7.5 and 8.0 while at initial pH of 7.0, the pH declined throughout the cultivation period (Fig. 12(b)). Almost all microorganisms prefer neutral pH conditions, but optimum for growth and for EPS production might not be the same value (Lawson and Sutherland, 1978). Nevertheless, this is not the case for *E. cloacae* WD7.

The crude EPS yields obtained at initial pH of 6.0, 6.5, 7.0, 7.5 and 8.0 were 1.32, 1.97, 2.72, 2.36 and 2.07 g/l, respectively (Fig. 13). These results therefore indicated that the optimum pH for cell growth and EPS production from *E. cloacae* WD7 was 7.0. This optimum pH for *E. cloacae* WD7 agreed with the report that the optimum pH for the synthesis of bacterial polysaccharides were 6.0-7.5, for fungi were 4-5.5 (Lawson and Sutherland, 1978). For xanthan production by *X. campestris*, the pH must not be lower than 5.0. The initial pH also affected the molecular weight of the product (Jeans, 1977), for example, the initial pH of 5-7 and 7-8 gave high ($1.5\text{-}4\times10^6$) and low ($5\text{-}10\times 10^4$) molecular weight pullulan respectively from *A. pullulans*. (Margaritis and Pace, 1985). The optimum initial pH for levan production from *Erwinia herbicola* was found to be 7.2 (Keit *et al.*, 1991). Acidic condition gave lower yield because the polymer (fructan) could be hydrolyzed at low pH (Clarke, 1991). The pH had more influence on polysaccharide production than on cell growth (Pace, 1980).

1.2.6 Effect of incubation temperatures

Results on the effect of incubation temperature on growth and EPS production are given in Fig. 14 and Fig. 15, respectively. The optimal temperature at 30 °C was the same as that of *Enterobacter* sp. (Grimont and Gromont, 1992). *E. cloacae* WD7 could grow at all temperatures tested (Fig. 14(a)). The incubation

(a)



(b)

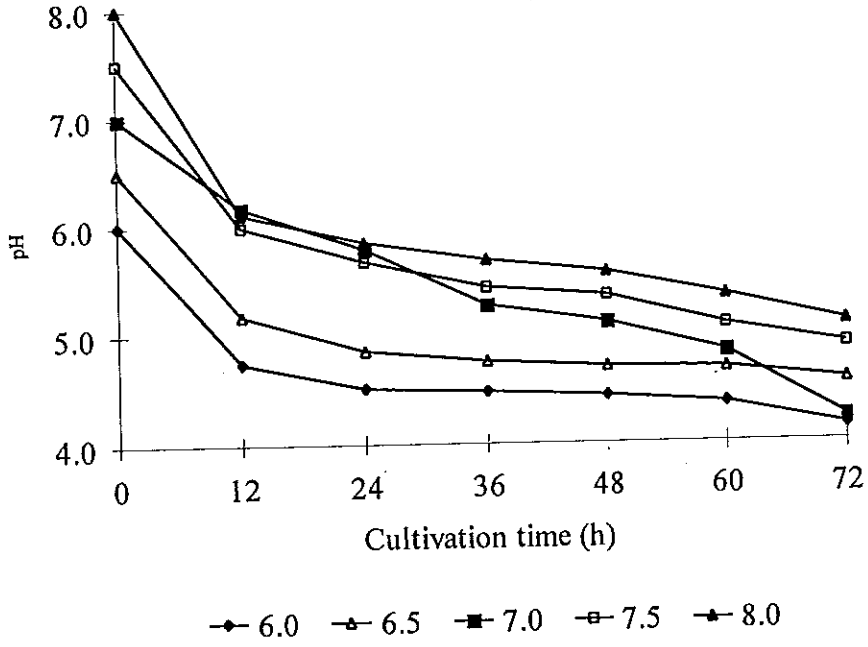


Fig. 12 Growth (a) and pH changes (b) during cultivation of *E. cloacae* WD7 in the optimal medium containing 3% sucrose as carbon source, 0.05% yeast extract with various initial pH on shake-flask culture (200 rpm) at 30 °C

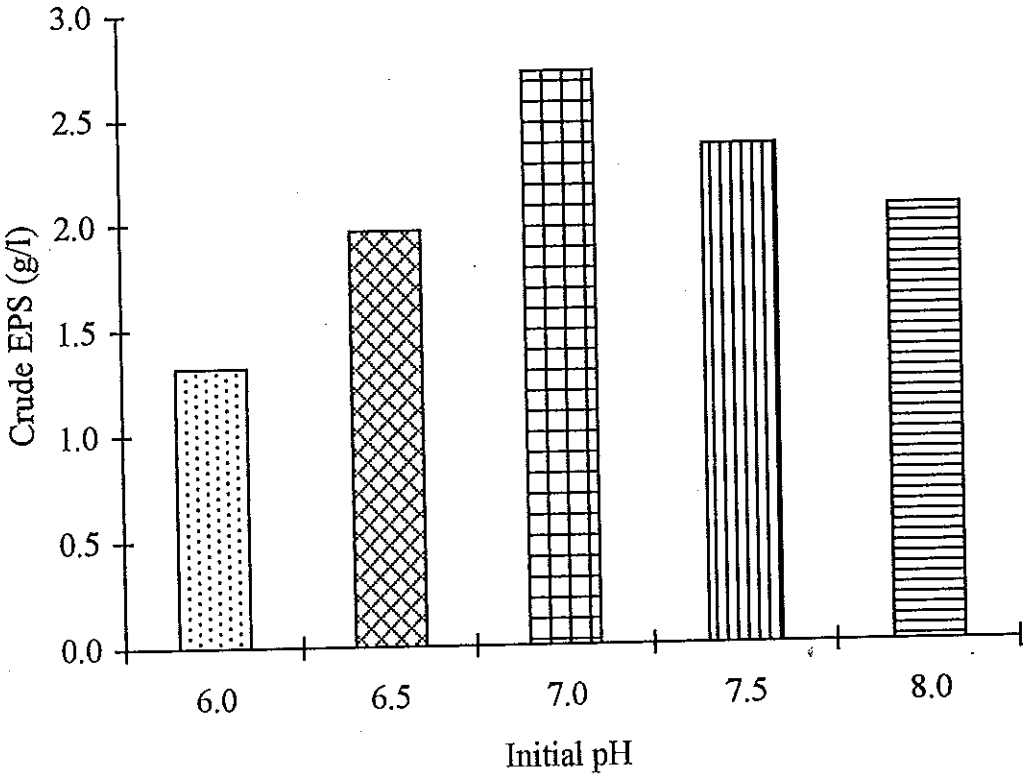


Fig. 13 Effect of various initial pH of optimal medium on the EPS production from *E. cloacae* WD7 in shake-flask culture after 3 days cultivation

temperature did not highly influence pH changes (Fig. 14(b)) ($p<0.05$). The crude EPS yield at 30, 35 and 40 °C were 2.71, 1.20, 0.66 g/l, respectively (Fig. 15).

Incubation temperature is often a critical factor in polysaccharide synthesis. All commercial polysaccharide-producing microorganisms are mesophiles (Lawson and Sutherland, 1978). The optimum temperature for polysaccharide production depends on the type of microorganism, *E. herbicola* prefers 30 °C and *Alteromonas* HYD-1545 prefers 25-29 °C while *X. campestris* prefers 28 °C (Vincent *et al.*, 1994). The optimal temperature for growth during xanthan production was 30 °C and no growth occurred at 35 °C (Shu and Yang, 1991). The optimum temperature for polysaccharide production by lactic acid bacteria must be lower than growth temperature. At lower temperature slow growth was caused by promoting to longer logarithmic and stationary phase (Gassem *et al.*, 1997).

1.2.7 Time course of exopolysaccharide production in optimal medium

Cultivation of *E. cloacae* WD7 in the optimal medium (initial pH 7.0) at 30 °C on a shaker is given in Fig. 16 and the kinetic parameters are shown in Table. 6. The specific growth rate (μ) was 0.15 h^{-1} while it was 0.14 h^{-1} when grown on basal medium (Fig. 1). This showed that the optimized medium is more suitable than basal medium for growth of *E. cloacae* WD7. The maximum productivity increased from 0.03 to 0.04 g EPS/l.h while the higher yield increased from 2.22 g/l on basal medium to 2.71 g/l in optimized medium, with the increase of 0.22 fold.

The association of low cell mass with greater polysaccharide production has been explained by Sutherland (1990). When cells grew slowly, synthesis of cell wall polymer was also slower, making more isoprenoid phosphate available for exopolymer synthesis. In the optimized medium used, the conversion of substrate to product ($Y_{p/s}$) was 25%, which is higher than those from *Enterobacter* sp. (13.5%) and over 90% of the sucrose was consumed during the logarithmic

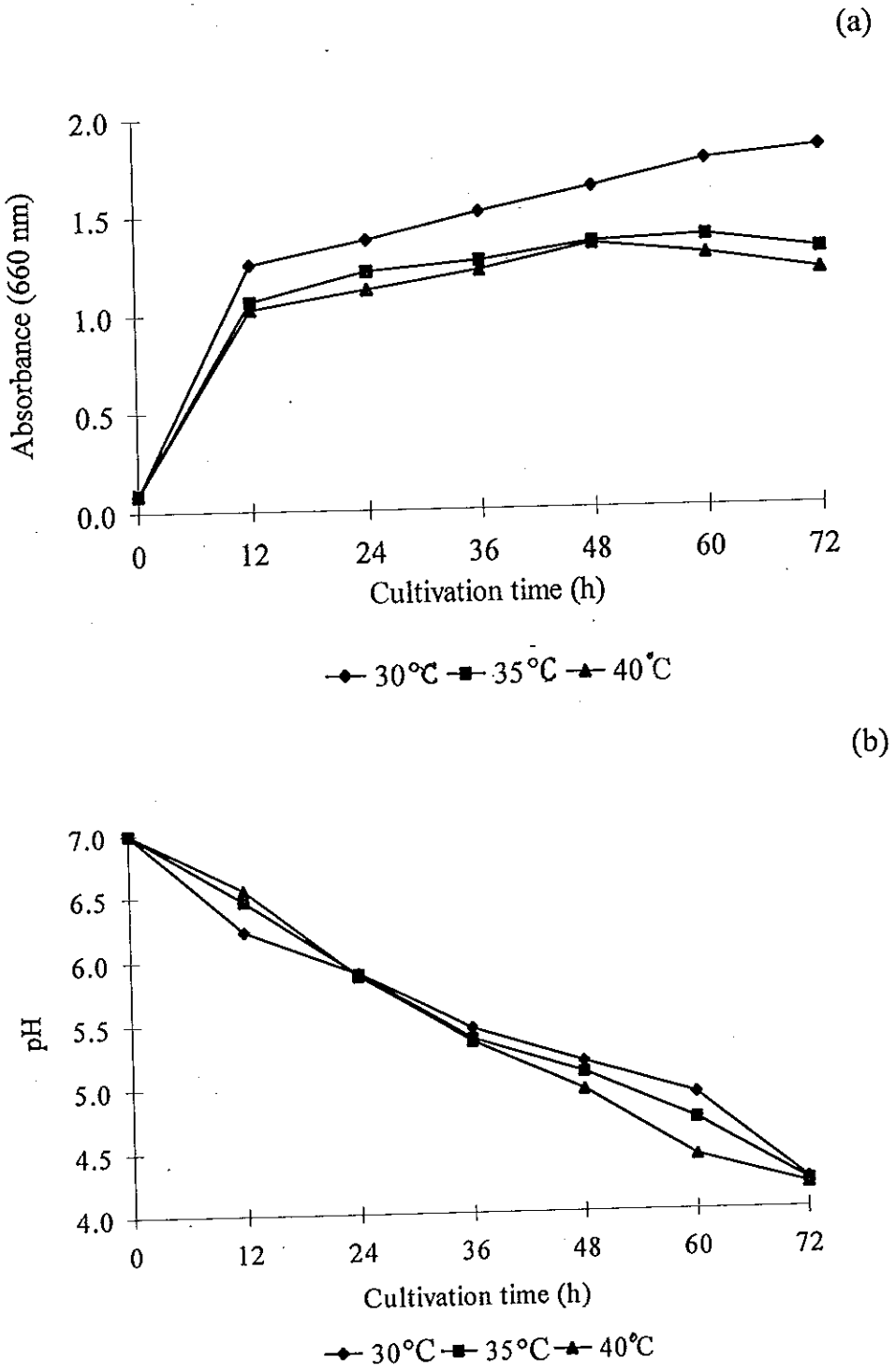


Fig. 14 Growth (a) and pH changes (b) during cultivation of *E. cloacae* WD7 in the optimal medium (pH 7.0) containing 3% sucrose as carbon source, 0.05% yeast extract with various temperatures on shake-flask culture (200 rpm)

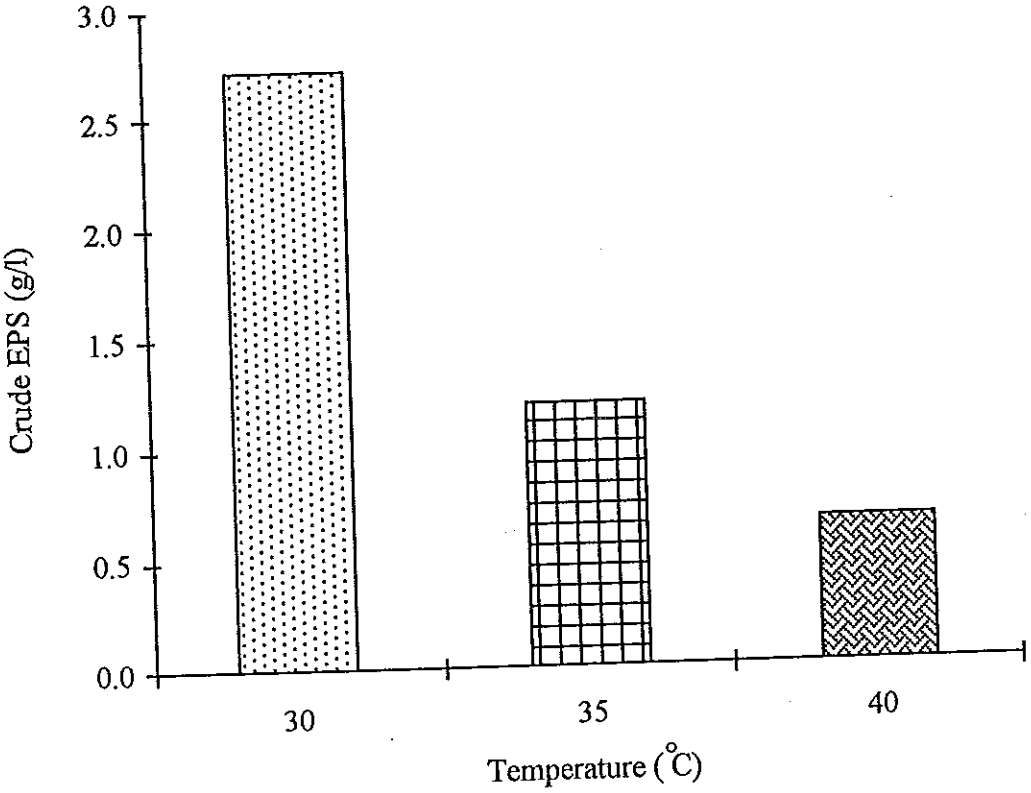


Fig. 15 Effect of various incubation temperatures of optimal medium (pH 7.0) on the EPS production of *E. cloacae* WD7 in shake-flask culture after 3 days cultivation

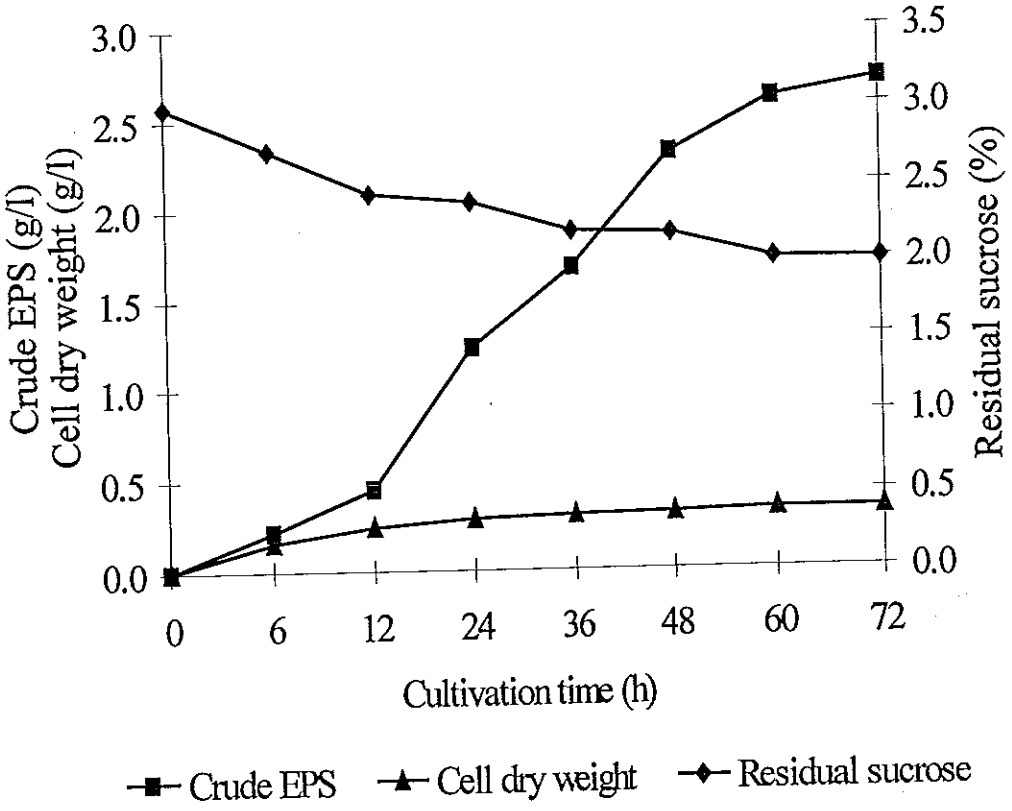


Fig. 16 Changes of exopolysaccharide production, cell dry weight and residual sucrose during fermentation of *E. cloacae* WD7 in optimal medium (pH 7.0) on shake-flask at 30 °C

growth phase (Shimada *et al.*, 1997). The conversion efficiency, kinetics and composition of exopolysaccharide could be affected by environmental conditions and growth rate (Pace, 1981). Almost 75% of polymer from *S. paucimobilis* GS1 was produced during exponential growth and the remainder was accumulated in the stationary phase (Ashtaputre and Shah, 1995).

The cultivation was performed in shake-flask culture by varying sucrose concentrations (0-4%) to yield K_s value of *E. cloacae* WD7 which was found to be 1.30×10^{-5} g/l (Fig. 17). The specific growth rate (μ) in shake-flask culture was 0.15 h^{-1} when 3% sucrose was used as carbon source. The maximum specific growth rate (μ_m) in shake-flask culture was found to be 0.25 h^{-1}. It indicated that *E. cloacae* WD7 had similar cell growth with other microbial polysaccharide producing microorganisms.

Table 6. Kinetic parameters from shake-flask culture of *E. cloacae* WD7 in optimal medium (3% sucrose as carbon source), pH 7.0 at 30 °C for 3 days

Kinetic parameters	Units	Values
μ	h^{-1}	0.15
μ_m	h^{-1}	0.25
$Y_{x/s}$	g cell/g sucrose	0.03
$Y_{p/s}$	g crude EPS/g sucrose	0.25
q_p	h^{-1}	0.60
q_s	h^{-1}	5.0
R_m	g crude EPS/l.h	0.04
g	h	4.62
K_s	g sucrose/l	1.30×10^{-5}

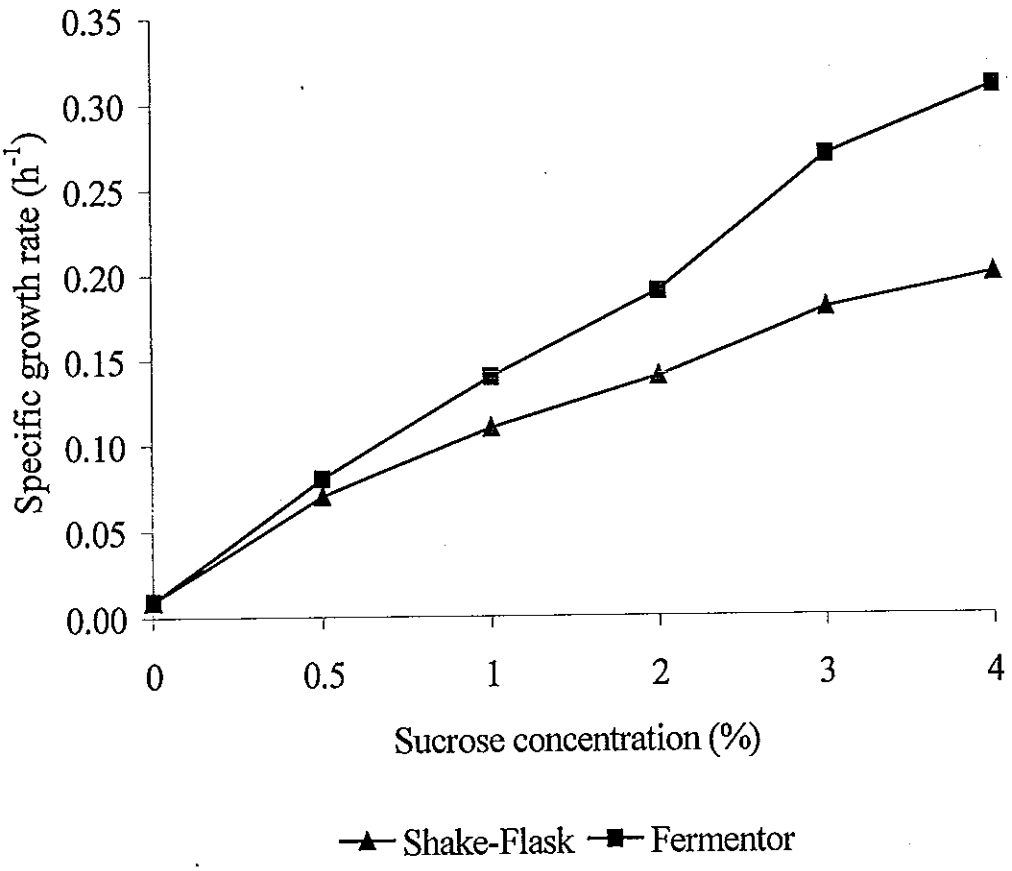


Fig. 17 Specific growth rate of *E. cloacae* WD7 cultivated on optimal medium (pH 7.0) on shaker (200 rpm, 30 °C) and batch fermentor (200 rpm, aeration rate of 0.5 vvm, 30 °C)

1.3 Effect of environmental condition on exopolysaccharide production

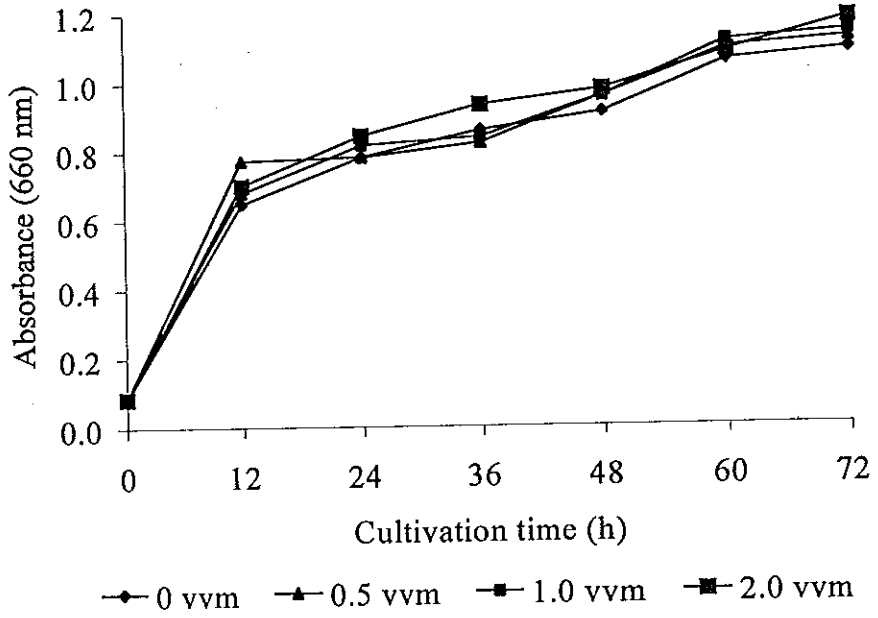
1.3.1 Effect of uncontrol and control of pH during cultivation

The cultivation was performed in two 3-l fermentors containing 1.8-l optimal medium with the aeration rate of 0.5 vvm, agitation speed of 200 rpm and incubation temperature of 30 °C, with and without control of pH (7.0). The results showed that *E. cloacae* WD7 grow well both under uncontrolled and controlled pH in the first 2 days cultivation and the growth continue to increase in the case of controlled pH (Fig. 18(a)).

E. cloacae WD7 gave higher crude EPS yield under controlled pH condition than uncontrolled pH condition (2.91 and 2.11 g/l, respectively) (Fig. 18 (b)). Under uncontrolled pH condition, the pH declined rapidly within 24 h and gradually increased (Fig. 19(a)) while uncontrolled pH gave slightly constant. It was reported that pH also affected directly to enzymes synthesis repressible for EPS production, which need a specific pH (Lawson and Sutherland, 1978). Gassem *et al.* (1997) reported that the pH was adjusted to 6.2 during fermentation for production of exopolysaccharide by *L. delbrueckii* ssp. *bulgaricus* which resulted in greater viscosity than unadjusted pH treatments. Growth in pH adjusted medium resulted in greater lactose utilization (5%) compared with unadjusted media (3.5%) and greater viscosity obtained (450 cP from 20 cP) by controlling environment condition such as pH (Dlamini and Peiris, 1997). Control of pH is likely to be needed for most production process as the polysaccharides of commercial interest are generally acidic (Paces, 1980).

During cultivation, the dissolved oxygen (DO) declined rapidly within 24 h (Fig. 19(b)) as the culture broth became very viscous due to the production of EPS so that oxygen could not penetrate into culture broth. In the case of uncontrolled pH, dissolved oxygen decreased rapidly within 24 h then increased. This showed that large amount of EPS was produced in early cultivation period and slow production rate occurred after that which may be caused by oxygen limitation. Under controlled pH condition, the culture broth was less viscous in the

(a)



(b)

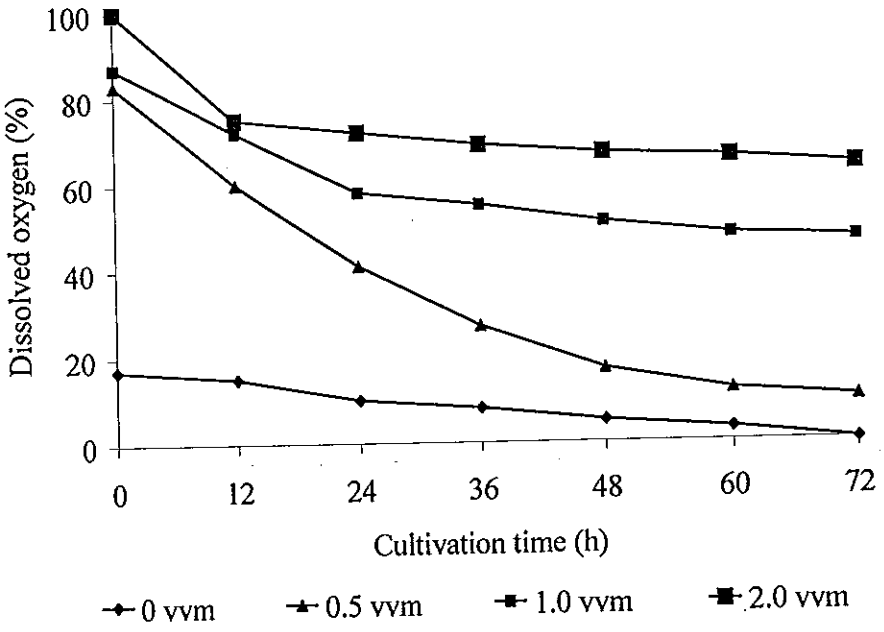


Fig. 20 Growth (a) and dissolved oxygen (b) during cultivation of *E. cloacae* WD7 in optimal medium at various aeration rates and control of pH at 7.0

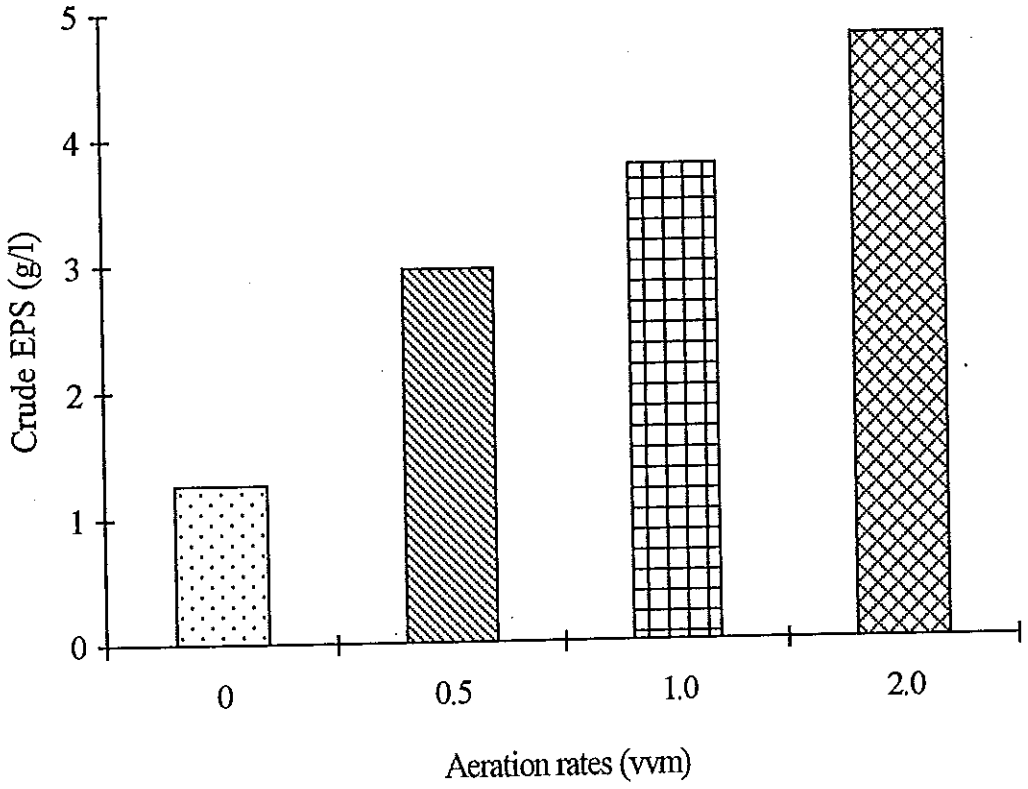
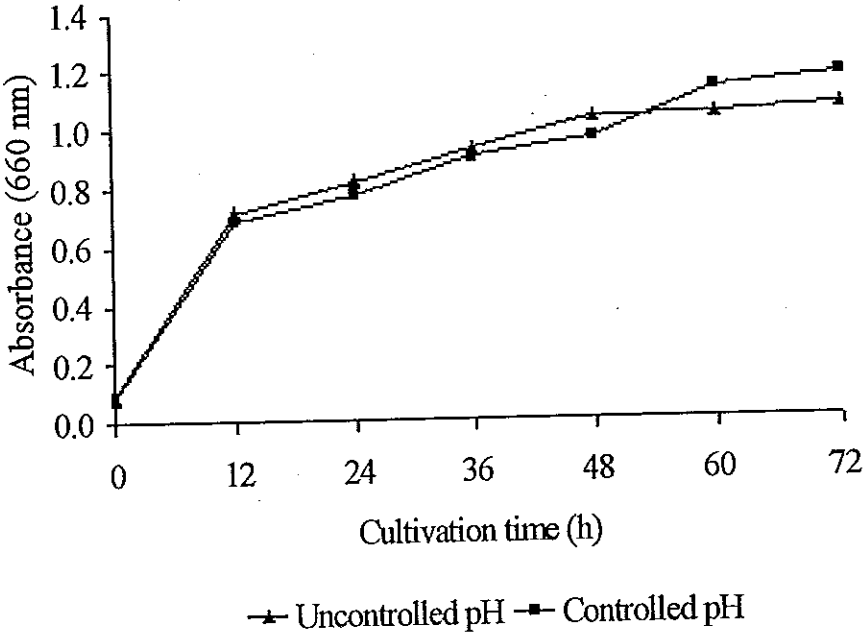


Fig. 21 Crude EPS from *E. cloacae* WD7 in optimal medium at various aeration rates and control of pH at 7.0

(a)



(b)

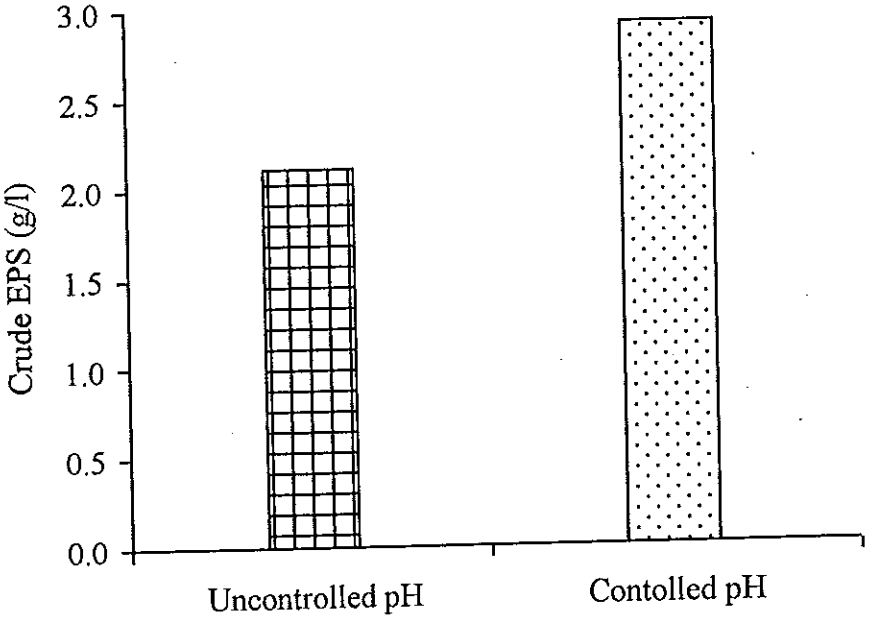
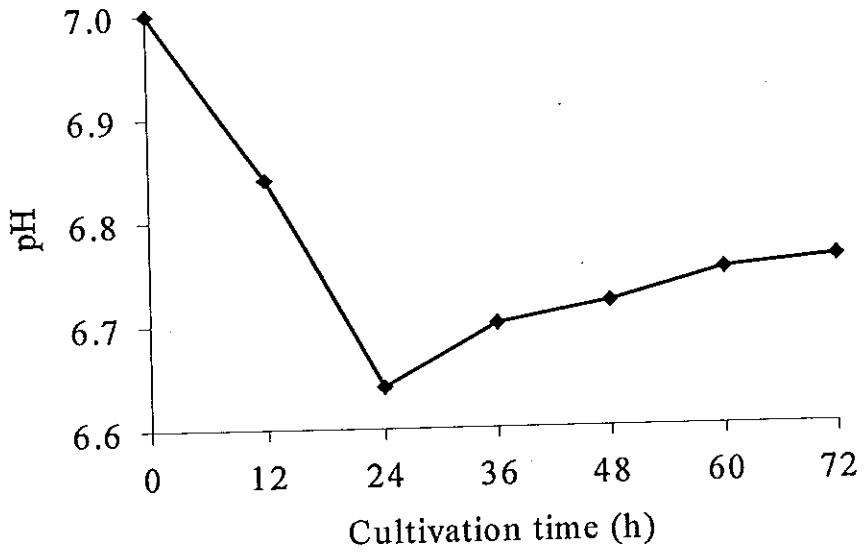


Fig. 18 Growth (a) and crude EPS (b) from *E. cloacae* WD7 cultivated under uncontrolled and controlled pH (7.0) after 3 days cultivation

(a)



(b)

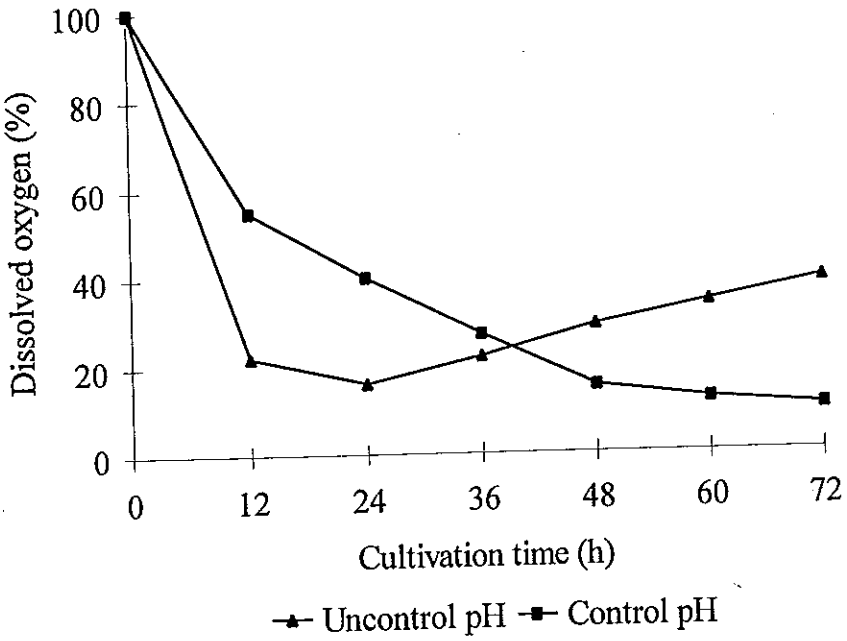


Fig. 19 pH (a) and dissolved oxygen (DO) changes (b) in culture broth of *E. cloacae* WD7 cultivated under uncontrolled and controlled pH (7.0) conditions

first 2 days than that of uncontrolled pH condition, the results under controlled pH gave much better results.

1.3.2 Effect of aeration rates

Higher aeration rates gave slightly higher cell growth (Fig. 20(a)) and higher dissolved oxygen (Fig. 20(b)). The growth under no aeration condition may due to the fact that *E. cloacae* WD7 was facultative anaerobic bacteria (Dermlim, 1999) and the initial dissolved oxygen was found to be 17% without aeration (0 vvm), dissolved oxygen decreased and depleted at the end of cultivation (3 days). The value of 17% DO, on the other hand, may due to the effect of agitation speed (200 rpm). At aeration rate of 0.5 vvm, the initial dissolved oxygen was 83% and was consumed during cultivation with 10% remained after 72 h cultivation. For 1.0 and 2.0 vvm aeration rates, the initial dissolved oxygen values were 87% and 100%, respectively, giving the residual DO of 58% and 60% at the end of cultivation. The crude EPS yields increased as the aeration rates increased giving 1.26, 2.96, 3.79 and 4.80 g/l of EPS at the aeration rates of 0, 0.5, 1.0 and 2.0 vvm, respectively (Fig. 21). The higher EPS yields were correlated with cell growth (Fig. 20) and dissolved oxygen consumption (Fig. 21). As a result, the optimal aeration rate for both cell growth and EPS production by *E. cloacae* WD7 was 2.0 vvm. In order to increase oxygen transfer rate, usually air is sparged through the medium with high degree of aeration (Lawson and Sutherland, 1978). Oxygen is usually required for both the synthesis of polymer components (e.q. sugar acid) or indirectly in the oxidation of reduced pyridine nucleotides generated (Margaritis and Pace, 1985).

An increase in viscosity could cause oxygen transfer limitation resulting in the accumulation of reduced cofactors ($NADH+H^+$ and $NADPH+H^+$) in the cells of *X. campestris* (Jana and Ghosh, 1997). The oxygen availability by control aeration rate and agitation speed can maintain the dissolved oxygen not less than 30% which is suitable for polymer production (McNeely, 1967). Bacteria utilized

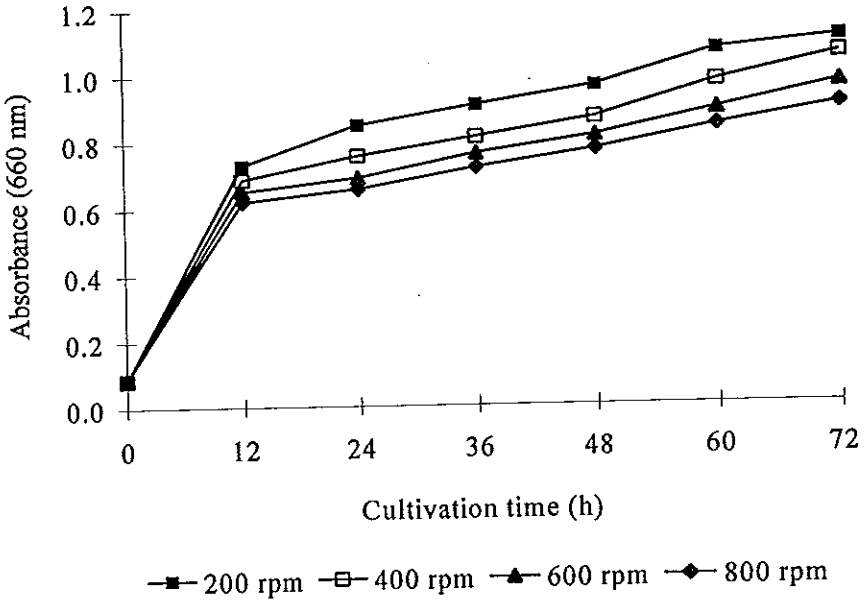
only dissolved oxygen consequent the rate of dissolved oxygen supply must at least equal the rate of oxygen demand (Gerhardt and Drew, 1994). For alginate production by *Azotobacter vinelandii*, the microaerobic conditions (3-5% pO_2) enhanced the polymer production (Sabra *et al.*, 1999).

1.3.3 Effect of agitation speeds

The cultivation carried out at the aeration rate of 2.0 vvm, controlled pH at 7.0 and maintained incubation temperature at 30 °C. Cell growth decreased as the agitation speeds increased (Fig. 22(a)) which might due to the cell destruction Therefore, the minimum agitation rate at 200 rpm was most preferred. Dissolved oxygen curves showed that higher agitation speeds gave higher dissolved oxygen ($p<0.05$) (Fig. 22(b)). This indicated that agitation speed could elevate dissolved oxygen levels as agitation seems to play a role in addition to that of maintaining oxygen tension, possibly by improving mass transfer between the medium and cells (Slodki and Cadmus, 1978). So that 200, 400, 600 and 800 rpm of agitation speeds could maintain dissolved oxygen over 60%, 70%, 80% and 90%, respectively.

The crude EPS yield was lower at higher agitation speeds (Fig. 23). Higher agitation speeds might cause reduction both yield and cell growth because high agitation speed could destroy cell and/or EPS production. Moreover high agitation speed gave excess dissolved oxygen which in turn affected to cells growth and/or EPS production. The high agitation rates (above 750 rpm) clearly resulted in substantially diminished exopolysaccharide yields by *A. pullulans* compared to those at lower speeds (125 to 500 rpm) while biomass yield increased, if only slightly dissolved oxygen decreasing to minimum of around 70% saturation (Gibbs and Seviour, 1996). For xanthan production, dissolved oxygen at 60% and the critical oxygen level was found to be between 6% was required and 10%, and below 6% both specific xanthan production rate as well as specific oxygen uptake rate decreased significantly (Amanullah *et al.*, 1998b).

(a)



(b)

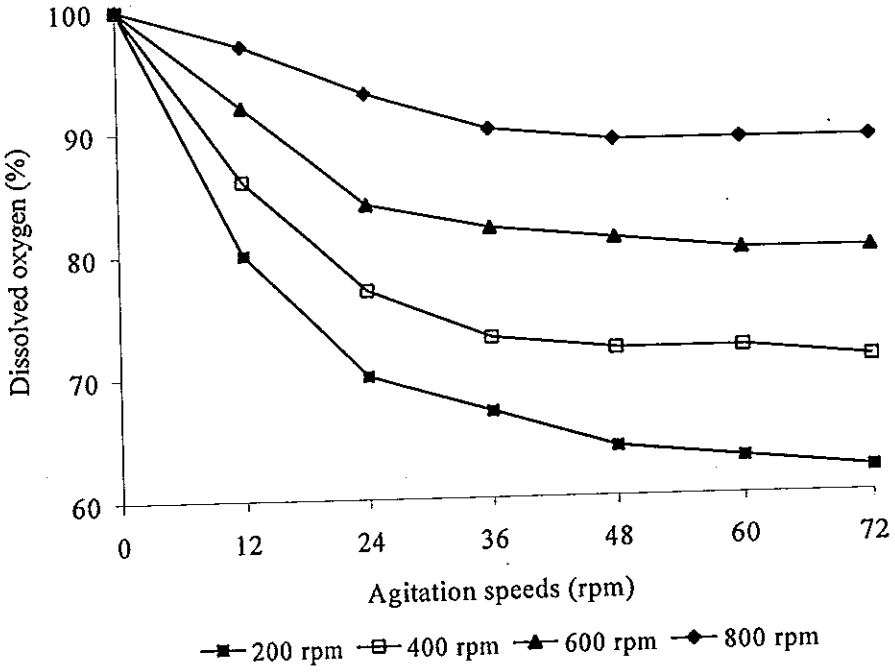


Fig. 22 Growth (a) and dissolved oxygen (b) during cultivation of *E. cloacae* WD7 in optimal medium at various agitation speeds with the aeration rate of 2.0 vvm and pH control at 7.0

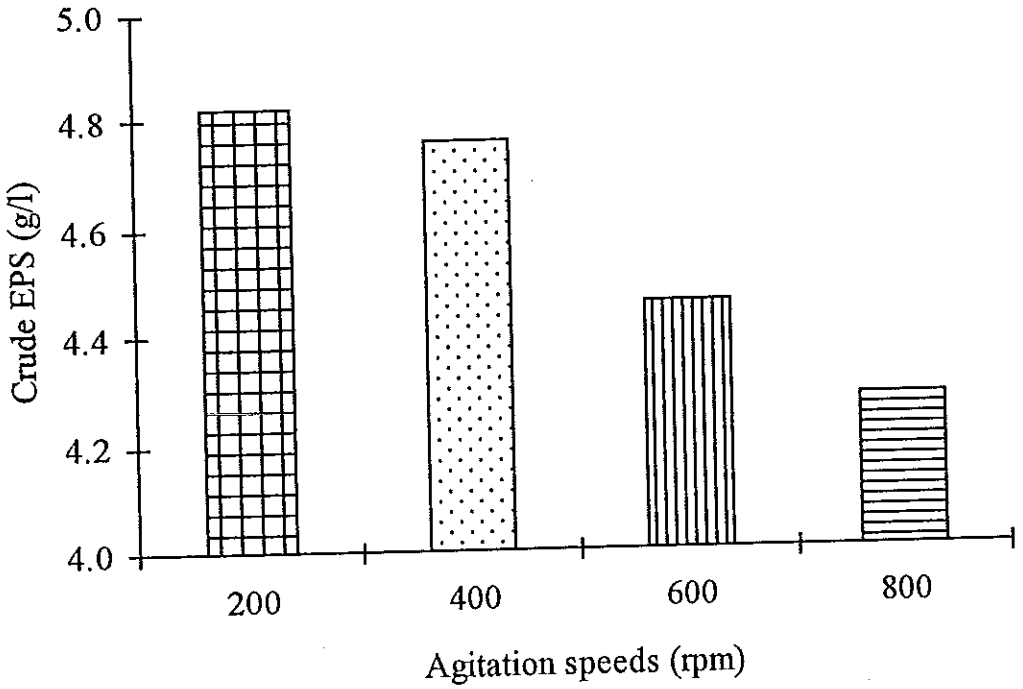


Fig. 23 Crude EPS from *E. cloacae* WD7 in optimal medium at various agitation speeds with the aeration rate of 2.0 and pH control at 7.0

1.3.4 Time course on exopolysaccharide production under optimal condition

E. cloacae WD7 was cultivated in the optimal medium, pH controlled at 7.0 and incubation at 30 °C for 3 days. The cultivation was performed in a 3-l fermentor with aeration rate of 2.0 vvm and agitation speed of 200 rpm. The results were given in Fig. 24 and kinetic parameters were calculated (Table 7). The K_s value was determined by cultivation in the fermentor with various concentrations of sucrose (0-4%). These values were about two times higher than those from batch shake-flask culture. The specific cell growth (μ) was 0.29 h^{-1}, the maximum specific cell growth (μ_m) was 0.49 h^{-1}, the cellular yield coefficient ($Y_{x/s}$) was 0.04 cells/g sucrose, the conversion yield of substrate to product ($Y_{p/s}$) was 0.52 g crude EPS/g sucrose, the specific rate of product formations (q_p) was 0.56 h^{-1}, the specific rate of substrate utilization (q_s) was 7.25 h^{-1}, the maximum productivity (R_m) was 0.07 g crude EPS/l.h and the saturation constant (K_s) was 2.60×10^{-5} g sucrose/l. The generation time decreased from 4.62 h to 2.39 h.

Table 7 Kinetic parameters of cell growth and EPS production from *E cloacae* WD7 cultivating in optimal medium and environmental condition

Kinetic parameters	Units	Values
μ	h^{-1}	0.29
μ_m	h^{-1}	0.49
$Y_{x/s}$	g cell/g sucrose	0.04
$Y_{p/s}$	g crude EPS/g sucrose	0.52
q_p	h^{-1}	0.56
q_s	h^{-1}	7.25
R_m	g crude EPS/l.h	0.07
g	h	2.39
K_s	g sucrose/l	2.60×10^{-5}

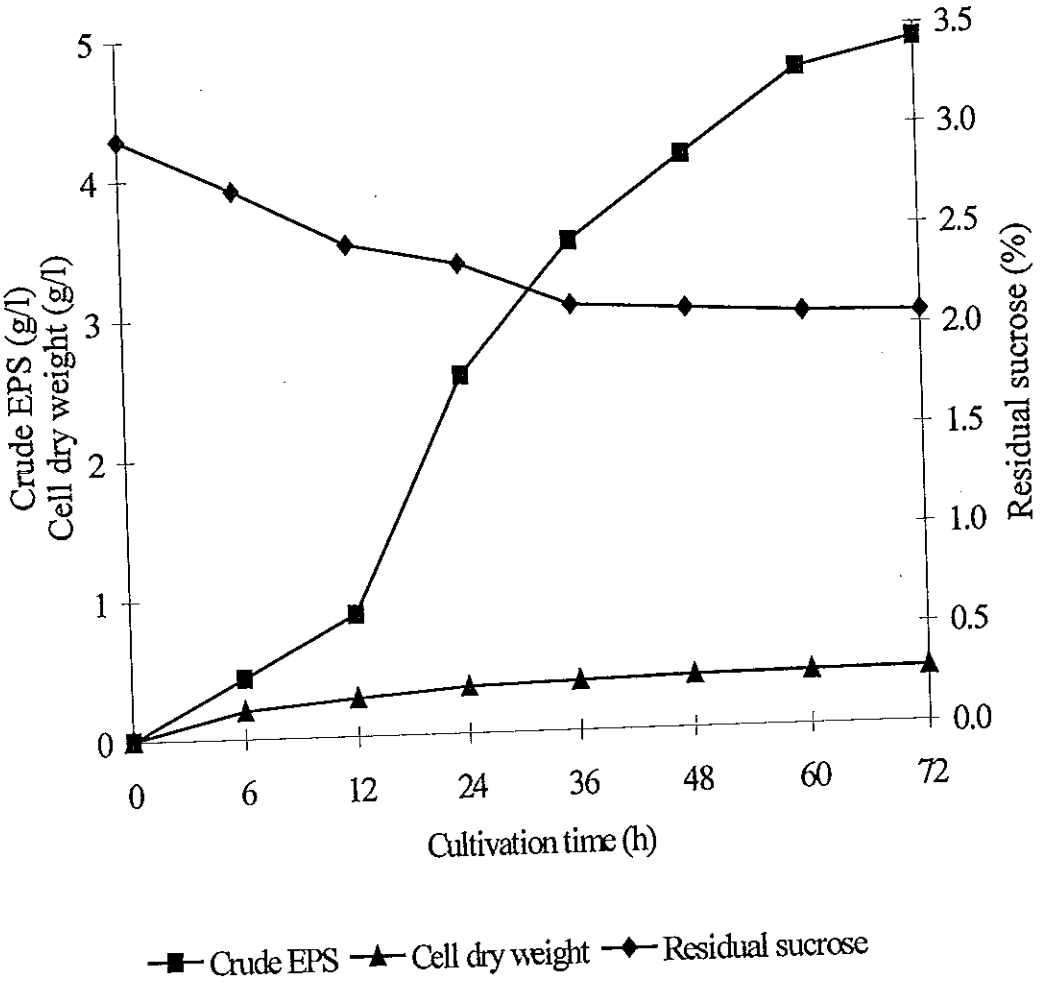


Fig. 24 Growth and exopolysaccharide production from *E. cloacae* WD7 cultivating in a 3-l fermentor at 30 °C, with aeration rate of 2 vvm and agitation speed of 200 rpm and pH control at 7.0

Conversion of 70-80% of utilized glucose into crude polymer is commonly found in high yielding polysaccharide fermentation (Magaritis and Pace, 1985). The microorganism had quite high conversion yield of substrate to product (52%) when compared to that from *Alteromonas* HYD-1545 using glucose as carbon source (37%) (Vincent *et al.*, 1994). The yields of biomass and pullulan (based on glucose utilization) were 0.18 and 0.52, respectively (Mulchandani and Luong, 1988). The specific growth rate from a mixed culture of *Aureobasidium pullulans* and *Kluyveromyces fragilis* was 0.13 h^{-1} (5% sucrose as carbon source) (Shin *et al.*, 1989) and it was 0.09 h^{-1} for pullulan production from *A. pullulans* (0.5% peat hydrolysate as carbons source) (Boa and LeDuy, 1987). The methylan production gave specific growth rate of 0.23 h^{-1}, the conversion yield of substrate to product was 0.66 g/l, the specific rate of product formation was 0.52 g/l (Oh *et al.*, 1997) which were quite similar to those values from *E. cloacae* WD7. Cultivation of *E. cloacae* WD7 under optimal condition in the 3-l fermentor resulted in the increase of EPS yields and cell growth twice higher than those under shake-flask condition. *E. cloacae* WD7 had similar growth rate to other exopolysaccharide producing microorganisms while crude EPS yield was in moderate range.

1.4 Effect of carbon and nitrogen sources substitution

1.4.1 Effect of using commercial sucrose as carbon source

EPS production from *E. cloacae* WD7 using commercial sucrose was compared to that using analytical grade of sucrose at the concentration of 1% and 3% (Fig. 25). Using analytical sucrose as substrate gave higher performance than those from using commercial sucrose (purified cane sugar) at both levels ($p<0.05$). The maximum yields of EPS obtained were 4.90 and 4.18 g/l using analytical and commercial sucrose (3%), respectively. Higher EPS yield from using analytical sucrose was due to its higher purity than the commercial sucrose, hence higher sugar content was transformed to higher EPS yield (Stredansky *et al.*, 1998).

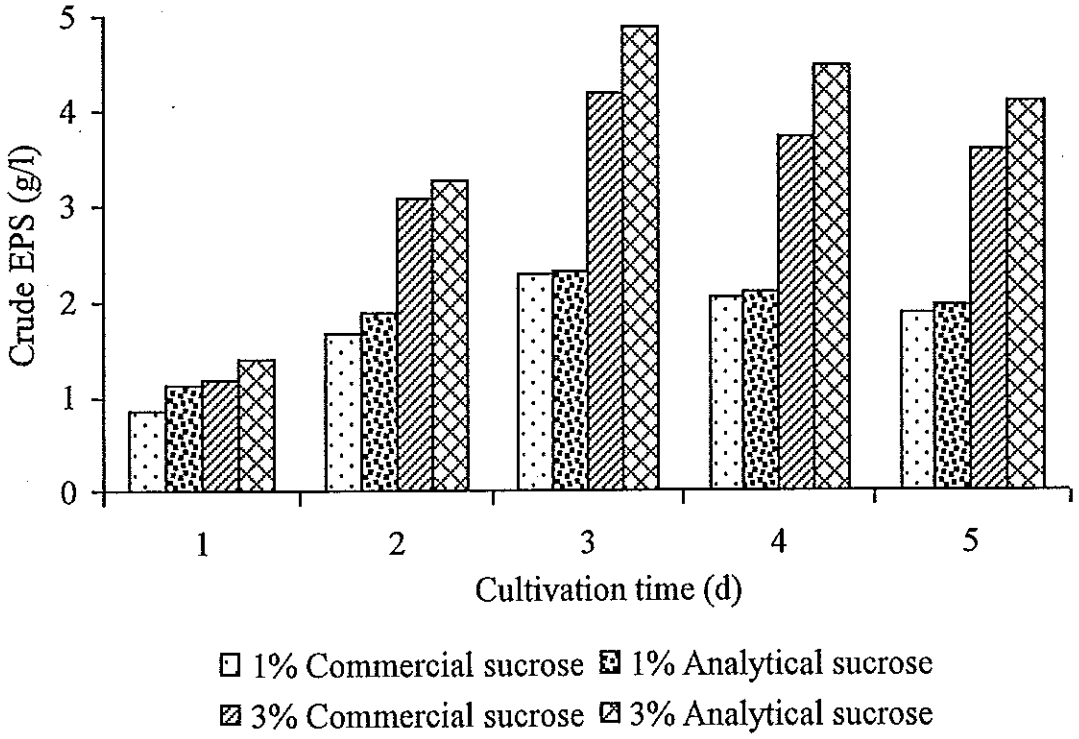


Fig. 25 The EPS production from *E. cloacae* WD7 cultivated in optimal medium containing commercial and analytical sucrose under optimal condition

Moreover, some impurities in commercial sucrose may suppress or inhibit enzymes involved in polymer production. Nevertheless, commercial carbon sources are always used for commercial scale because of its lower cost.

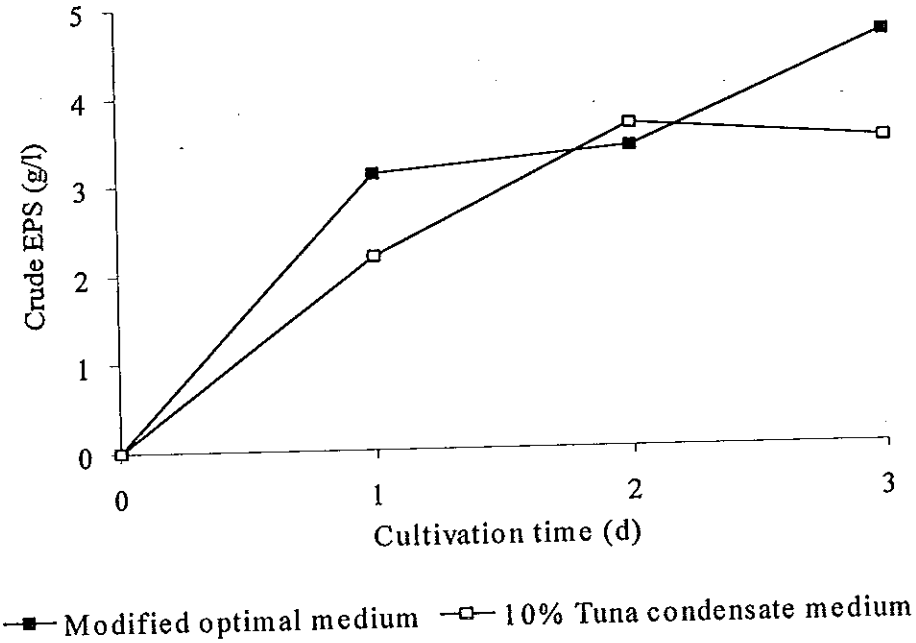
Some reports revealed that commercial carbon sources such as molasses, fruit juice and various agro-industrial wastes gave higher polymer yield than those from analytical sources (Clarke *et al.*, 1991; Lawson and Sutherland, 1978; Roukas, 1998). However, these agro-industrial wastes should be supplemented with other components such as nitrogen and phosphorus sources in order to obtain the maximum pullulan reached (30.8 g/l) (Israilides *et al.*, 1998).

1.4.2 Effect of using tuna condensate as nitrogen source

Tuna condensate from a seafood processing plant was used to replace yeast extract (0.05%) based on equal nitrogen concentration (0.72%) by using tuna condensate 4.5% (v/v) replaced yeast extract in optimal medium. It was found that using 10% tuna condensate medium gave lower EPS yield than modified optimal medium significantly (4.66 g/l and 4.90 g/l, respectively) (Fig. 26(a)). In large scale, tuna condensate could replace yeast extract in optimal medium because tuna condensate is a low cost by-product. The growth of *E. cloacae* WD7 in 10% tuna condensate medium gave higher than modified optimal medium (Fig. 26(b)) due to 10% tuna condensate medium contained higher nitrogen source than those from modified optimal medium. However, a little undesirable odor occurred during fermentation may come from amino or amine derivative and the odor was stronger as cultivation time prolonged.

The 10% tuna condensate medium could not replace the optimum medium as it gave the EPS yield of 3.44 g/l and caused strong bad odor. Moreover, the culture broth became brown color also resulted in the brownish color of the derived EPS. The lower yield may due to the very high nitrogen content (7.6 g/l) of the tuna condensate which may unsuitable for polymer production (Prasertsan *et al.*, 1993).

(a)



(b)

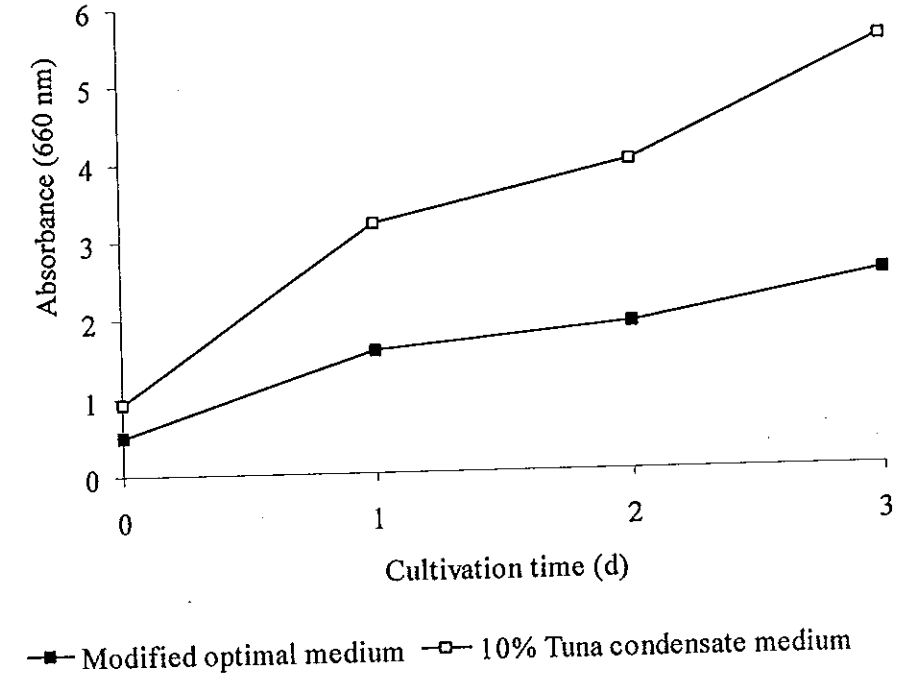


Fig. 26 The EPS production (a) and cell growth (b) of *E. cloacae* WD7 cultured in modified optimal medium compared to 10% tuna condensate medium

2. Water absorption capacity

The EPS produced was tested for the water absorption capacity. The results (Fig. 27) showed that the EPS had lower water absorption capacity than the commercial synthetic water absorbent ($p<0.05$) and both polymers revealed the maximum absorption after 24 h. In addition, the EPS possessed the water absorption capacity 3 times lower than the commercial synthetic polymer (80.3 compared to 292.9 g/g dried EPS).

Furthermore, the water absorption capacity of the EPS of this study also lower than those of the polysaccharide produced by *Alcaligenes latus* (about 1300 g/g EPS), high grade synthetic high-polymer absorbent: co-polymer of acrylate and vinyl alcohol (249.4) and anionic synthetic high-polymer absorbent: polyacrylamide derivative (363.6). However, the value was higher than those of other materials (g/g dried polymer) such as pulp (3.8), silica gel (1.4), ion-exchange resin (2.5), polyacrylate (4.6) (Kurane and Nohata, 1994). Moreover, study on the effect of NaCl concentrations (1-10%) in optimal medium on water absorption capacity of EPS revealed that the highest water absorption capacity of EPS was achieved at 1.0% NaCl with the value of 87.1 g/g dried EPS.

3. Fed-batch culture

3.1 Effect of initial batch cultivation periods

The cultivation was performed in four batches in which 10% sucrose solution was added to reach the final concentration of 3% after batch cultivation for 1, 2, 3 and 4 days. The maximum EPS yields obtained were 2.20, 2.50, 6.19 and 5.76 g/l, respectively (Fig. 28(a)) and the residual sucrose for each condition is shown in Fig. 28(b).

3.2 Effect of initial sugar concentrations

The fed-batch culture was performed after 3 days batch cultivation in optimal medium (3% sucrose as carbon source) and under optimal conditions and

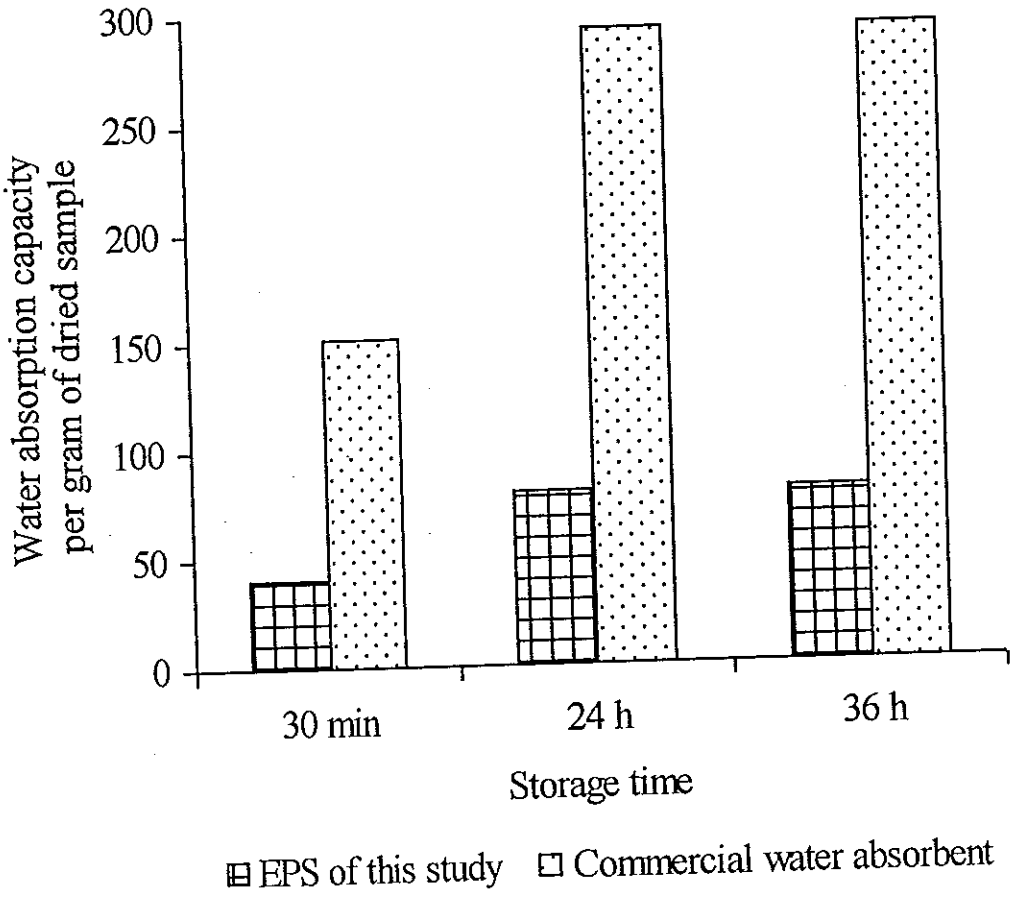
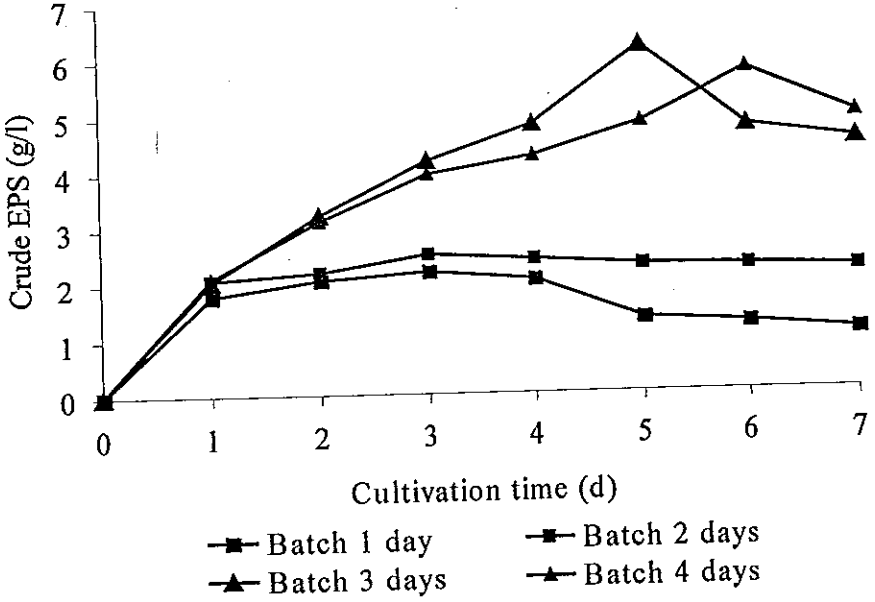


Fig. 27 Water absorption capacity of the EPS from *E. cloacae* WD7 and commercial water absorbent after storage at 30 min, 24 h and 36 h

(a)



(b)

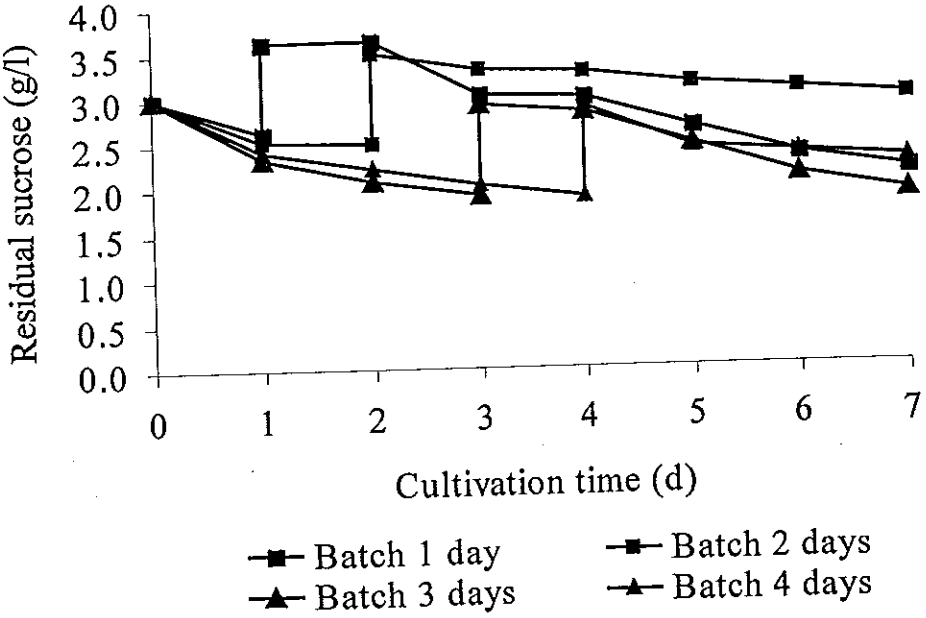


Fig. 28 Profiles of EPS (a) and residual sucrose (b) during fed-batch culture of *E. cloacae* WD7 after batch cultivation for 1, 2, 3 and 4 days with the addition of 10% sucrose solution

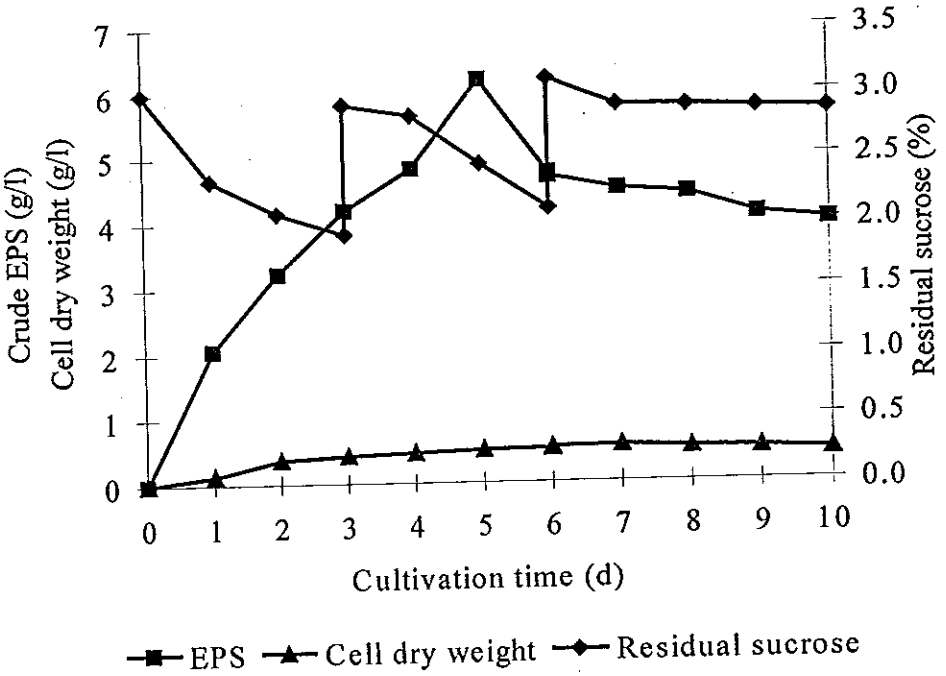
10% sucrose solution was added every 3 days to reach the final concentration of 3%. It was found that the maximum EPS yield of 6.19 g/l was obtained after 5 days cultivation. However, further addition of 10% sucrose solution on the 6th day of cultivation resulted in a gradually decrease of the EPS yield (Fig. 29 (a)). Using 1% sucrose as carbon source in the optimal medium and conditions in batch culture gave the maximum EPS yield of 2.55 g/l after 4 days of cultivation. Further addition of 10% sucrose solution on the 6th day did not further increase the EPS yield (Fig. 29 (b)).

The fed-batch culture therefore gave a higher EPS yield (6.19 g/l) than the batch culture (4.80 g/l) (Fig. 24). However, the productivity of fed-batch was slightly lower value (1.24 g/l/d) compared to that from the batch culture (1.60 g/l/d). When the sucrose solution (10%) was fed at day 4, the cell growth was similar but gave lower EPS yield (5.76 g/l) after 6 days of cultivation. The most suitable period for addition of 10% sucrose solution was on the 3rd day of cultivation and the maximum EPS yield was found to be 6.19 g/l on the 5th day of cultivation. Addition of sucrose solution on the 3rd and 4th day cultivation gave lower values of EPS yields after 5 days cultivation. This may be due to the degradation of polymer by some enzymes (such as pullulanase and alginate lyase) as reported previously (Stoll *et al.*, 1999; Nankai *et al.*, 1999).

Batch culture was proven to be a better fermentation system for the production of pullulan than the fed-batch culture system although fed-batch culture gave a higher yield but required a longer cultivation time. However, fed-batch culture of *A. pullulans* produced a pullulan which was often better if one considered the molecular size of the polymer produced but it still depend on cultivation time. Moreover, the molecular size usually decreased as fermentation progressed for both batch and fed-batch culture (Youssef *et al.*, 1999).

The kinetic parameters for fed-batch culture were given as following: the cellular yield coefficient ($Y_{x/s}$) = 0.05 g cell/g sucrose and the maximum productivity (R_m) = 0.05 g crude EPS/l.h.

(a)



(b)

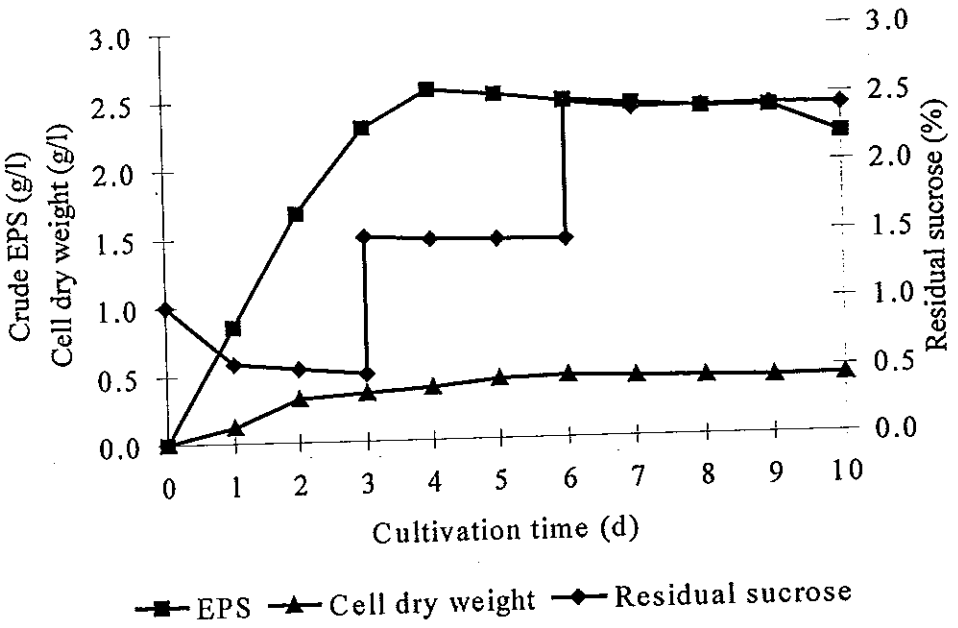


Fig. 29 Fed-batch culture of *E. cloacae* WD7 in optimal medium with the initial sucrose concentrations of 3% sucrose (a) and 1% sucrose (b) and addition of 10% sucrose solution every 3 days during cultivation

4. Continuous culture

4.1 Effect of dilution rates

The continuous culture was performed in the optimal medium (1.8-l) and optimal conditions at dilution rates of 0.01, 0.05 and 0.10 h^{-1} (Fig. 30) after 3 days batch cultivation. The optimal dilution rate was found to be 0.05 h^{-1} which gave the highest both cell growth (OD_{660}=1.24) and EPS production (7.28 g/l).

4.2 Effect of batch cultivation periods

Batch cultures were performed for 1, 2 and 3 days before starting continuous culture at the dilution rate of 0.05 h^{-1}. The suitable time for batch culture was found to be 3 days (Fig. 31). Fresh medium was fed continuously at the rate of 1.25 ml/min. The maximum EPS yields were 2.75, 5.56 and 7.28 g/l, for 1, 2 and 3 days of batch culture, respectively ($p<0.05$) (Fig. 31). Therefore, continuous culture should start after 3 days of batch culture. The maximum EPS yield (7.28 g/l) was obtained after 5 days cultivation (Fig. 32).

Typically, continuous culture started while the batch cultivation is in the exponential phase of growth to minimize oscillations due to nutritional step-up and avoid washout due to physiological lag (Gerhardt and Drew, 1994; Glazer, 1995). However, the maximum EPS yield from *E. cloacae* WD7 was obtained when the continuous culture started within stationary phase.

This study showed that the optimal dilution rate was 0.05 h^{-1} and higher dilution rate encountered with cell washout. However, the production rate and yield of cellulose production from *Acetobacter xylinum* subsp. *sucrofermentans* increased with the increase of dilution rate and cell washout occurred when the dilution rate was 0.12 h^{-1} (Naritomi *et al.*, 1998). Studies on alginate production by *A. vinelandii* at four dilution rates (D=0.08-0.26 h^{-1}) revealed that dilution rate of 0.15 h^{-1} gave the highest yield (Sabra *et al.*, 1999).

Polysaccharide produced by *P. aeruginosa* in continuous culture at dilution rates of 0.05-0.10 h^{-1} showed that at each dilution rate indicated a

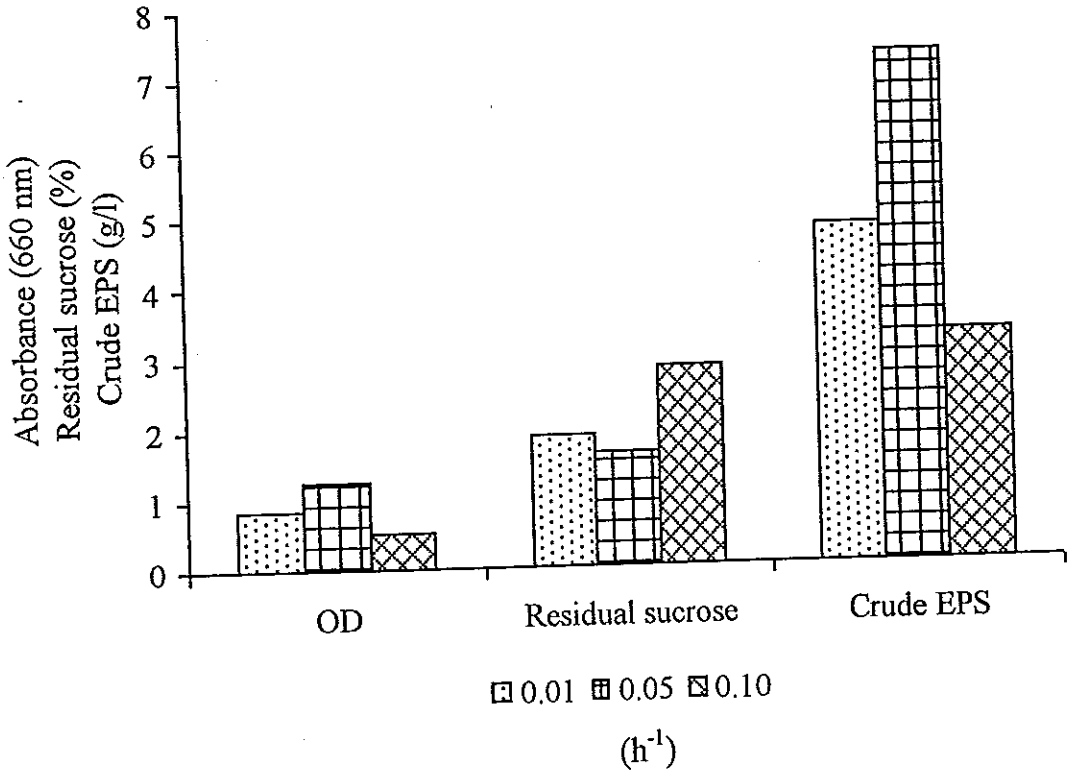


Fig. 30 Effect of dilution rates (D) on EPS production from *E. cloacae* WD7 in continuous culture, feeding with the optimal medium containing 3% sucrose

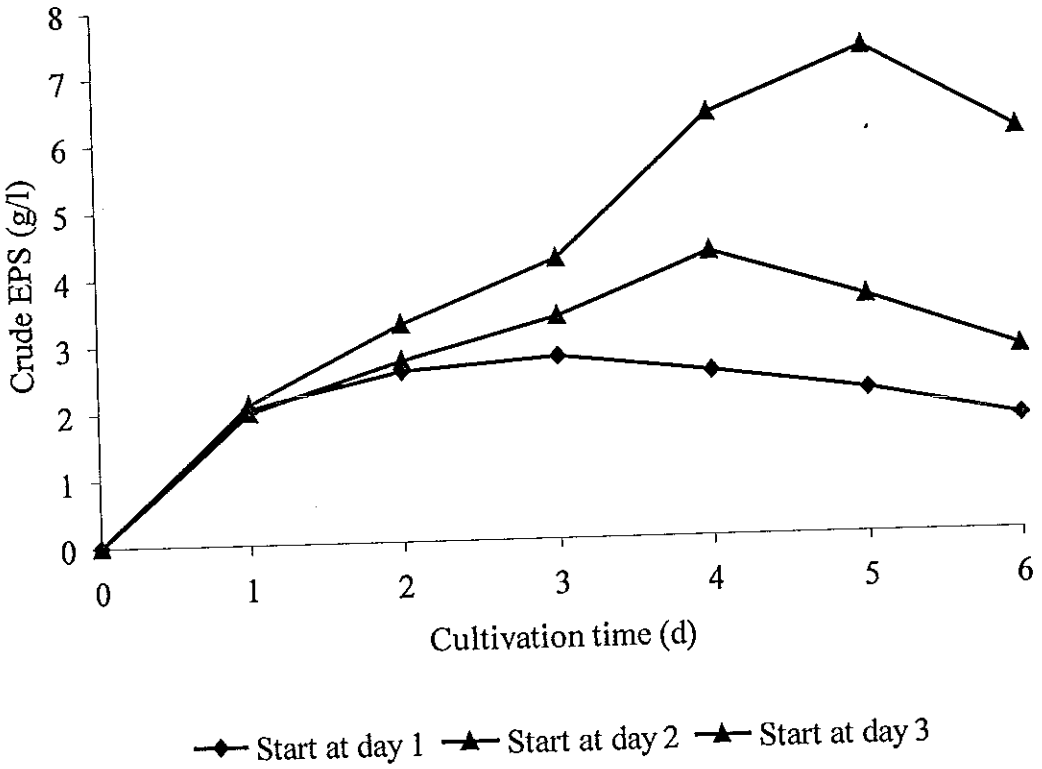


Fig. 31 Effect of batch culture periods on EPS production from *E. cloacae* WD7 in continuous culture (D=0.05 h^{-1}), feeding with the optimal medium containing 3% sucrose

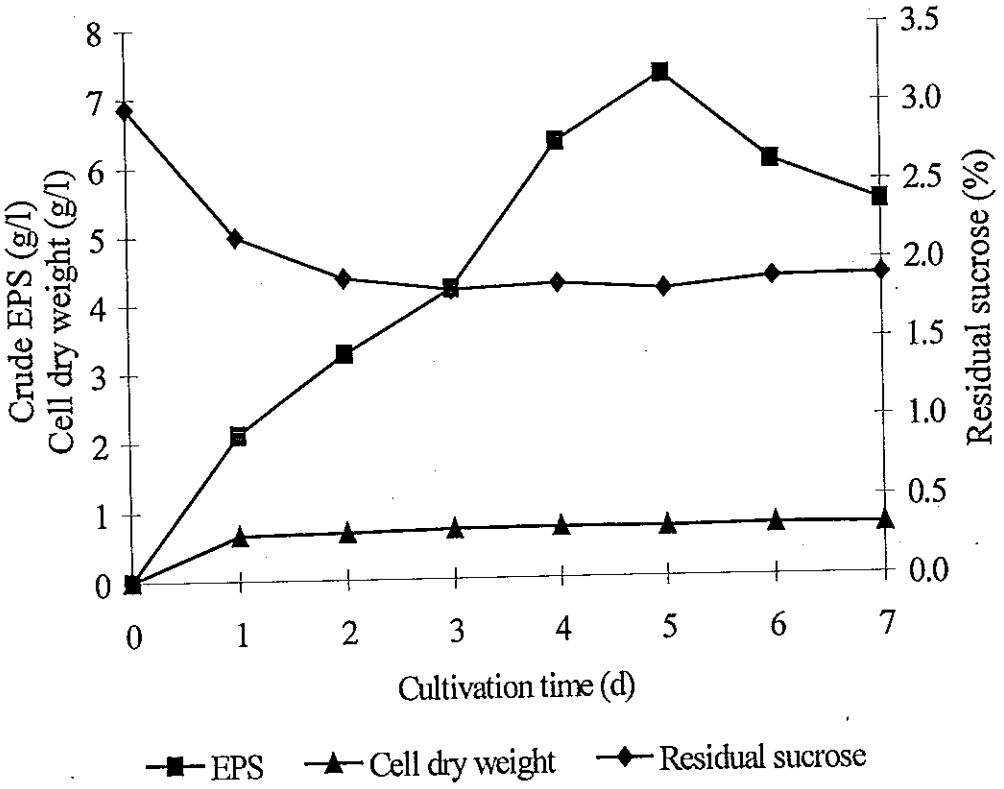


Fig. 32 Profile of continuous culture of *E. cloacae* WD7 feeding with the optimal medium containing 3% sucrose at dilution rate of 0.05 h^{-1} after 3 days of batch cultivation

conversion rate of 56-64% polymer from substrate (Lawson and Sutherland, 1978) which also similar to this study. Alginate production from *A. vinelandii* in continuous culture using sucrose as carbon source could increase to 50%, compare to 25% in batch culture (Lee, 1996). Continuous culture for bacterial cellulose gave higher yield than batch culture (Naritomi *et al.*, 1998; Lee, 1996). Because continuous culture need longer cultivation time which may affect the quality of polymer produced and is not easy to operate, continuous culture for industrial production of microbial polysaccharide has not been made (Amanullah *et al.*, 1998b).

The kinetic parameters from continuous culture were summarized in Table 8. It was found that the continuous culture had higher EPS yield (7.28 g/l) than fed-batch (6.19 g/l) and batch culture (4.90 g/l). If considered the maximum productivity per day, batch culture had more advantage than continuous and fed-batch cultures, the values of maximum productivity per day were 1.63, 1.46 and 1.24 g EPS/day, respectively.

Table 8 Kinetic parameters of cell growth and EPS production from *E. cloacae* WD7 in continuous culture using the optimal medium (containing 3% (w/v) sucrose) and conditions after 3 days of batch culture

Kinetic parameters	Units	Values
D	h^{-1}	0.05
D_c	h^{-1}	0.49
D_m	h^{-1}	0.485
$Y_{x/s}$	g cell/ g sucrose	0.03
R_m	g crude EPS/l.h	0.06

Chapter 4

Conclusion

Enterobacter cloacae WD7 was grown on basal medium containing various carbon sources (1%) with the initial pH of 7.0 at 30 °C for 72 h. Sucrose supported strongly cell growth, and exhibited, like galactose higher EPS yields (2.50 and 2.45 g/l, respectively) compared to maltose, fructose and glucose. Sucrose was selected due to its lower cost. The optimal concentration of sucrose was found to be 3% giving an EPS yield of 2.63 g/l. Inorganic (0-0.15%) of $(NH_4)_2SO_4$, NH_4NO_3 and NH_4Cl and organic (polypeptone) nitrogen sources had no effect on EPS yield, but influence on growth. The optimal concentration of yeast extract on EPS production was 0.05%. The optimized medium consisting of 3% sucrose, 0.05% yeast extract, 0.5% K_2HPO_4, 0.2% KH_2PO_4, 0.05% $MgSO_4.7H_2O$ and 0.01% NaCl gave EPS of 0.22 fold higher than those from basal medium.

The optimal environmental conditions for EPS production were as following; initial pH of 7.0, temperature of 30 °C. Control of pH at 7.0 during cultivation was necessary for both cell growth and exopolysaccharide production. The optimal aeration rate was 2.0 vvm while the minimum agitation speed (200 rpm) was the most preferred one. The highest yield obtained was (4.80 g/l).

The performance in the batch fermentor resulted in about 2-fold higher yield compared to that from shake-flask culture. Among all parameters involved; aeration rate had the most influence on EPS yield. Tuna condensate, a by-product of seafood processing could be used to substitute yeast extract (0.05%) in the medium giving the EPS yield of 4.66 g/l. In 10% tuna condensate with the addition of 3% sucrose gave the EPS yield of 3.64 g/l. In addition, commercial sucrose could also replace analytical grade sucrose although slightly lower yield

was obtained (4.18 g/l compared to 4.90 g/l at 3% sucrose). The maximum EPS yields for each condition in shake-flask and batch fermentor are given in Table 9.

Table 9 Influence of medium and culture conditions on exopolysaccharide (EPS) production from *E. cloacae* WD7 in shake-flask and batch fermentor

Medium composition and culture condition	Maximum EPS yield (g/l)
Carbon sources (1%) (glucose, sucrose, galactose, maltose)	2.50 (galactose)
Sucrose concentrations (0-4%)	2.63 (3%)
Inorganic nitrogen sources (0.05%) [$(NH_4)_2SO_4$, NH_4NO_3, NH_4Cl]	2.65 (No addition)
Organic nitrogen concentrations (polypeptone) (0-0.3%)	2.70 (0%)
Yeast extract concentrations (0-0.2%)	2.71 (0.05%)
Initial pH (6-8)	2.72 (pH 7.0)
Incubation temperature (30-40 °C)	2.71 (30 °C)
Control of pH	2.91
Aeration rates (0-2.0 vvm)	4.80 (2.0 vvm)
Agitation speed (200-800 rpm)	4.82 (200 rpm)
Effect of tuna condensate (substituted 0.05% yeast extract, 10% tuna condensate as culture medium)	4.66 (yeast extract substituted by tuna condensate)
Sources of sucrose (1% and 3%) (Analytical and commercial grade)	4.90 (3% analytical grade)

EPS from *E. cloacae* WD7 was found to be an absorbent. The water absorption capacity of the EPS was compared to that of the commercial synthetic water absorbent at the soaking time of 30 min, 24 h and 36 h. Both polymers could absorb water at saturation level after 24 h soaking time with the water absorption capacity of 80.3 and 292.9, respectively.

In fed-batch culture, the suitable time for addition of 10% sucrose solution every 3 days to maintain at 3% sucrose was 3 days after batch culture which resulted in an EPS yield of 6.19 g/l at 5 days of cultivation.

For continuous culture, the optimal dilution rate was 0.05 h^{-1}. The suitable time for starting continuous cultivation was 3 days after batch cultivation. The maximum EPS yield was obtained after 5 days of cultivation (7.28 g/l).

The kinetic parameters of batch (shake-flask and fermentor), fed-batch and continuous culture are given in Table 10. It could be concluded that continuous culture had higher EPS yield than fed-batch and batch culture with 7.28, 6.19 and 4.90 g/l, respectively. But if the production was calculated in EPS yield per day, batch culture had higher advantage than continuous and fed-batch with the productivity of 1.63, 1.46 and 1.24 g EPS/l.day, respectively.

Table 10 Kinetic parameters and EPS production from *E. cloacae* WD7 under optimal medium and conditions of shake-flask, batch fermentor, fed-batch and continuous (D=0.05 h^{-1})

Kinetic parameters	Units	Shake-Flask	Batch Fermentor	Fed-Batch	Continuous
μ	h^{-1}	0.15	0.29	-	-
μ_m	h^{-1}	0.25	0.49	-	-
D	h^{-1}		-	-	0.05
D_c	h^{-1}		-	-	0.49
D_m	h^{-1}		-	-	0.485
$Y_{x/s}$	g cell/g sucrose	0.03	0.04	0.05	0.03
$Y_{p/s}$	g crude EPS/ g sucrose	0.25	0.52	-	-
q_p	h^{-1}	0.60	0.56	-	-
q_s	h^{-1}	5.0	7.25	-	-
R_m	g crude EPS/l.h	0.04	0.07	0.05	0.06
g	h	4.62	2.39	-	-
K_s	g sucrose/l	1.30×10^{-5}	2.60×10^{-5}	-	-
EPS conc	g/l	2.71	4.80	6.19	7.28

Suggestions

The results of this work lead to the following suggestions:

1. *E. cloacae* WD7 should be identified whether it is a pathogenic bacteria or not.
2. Investigation on the compositions and structure of the EPS produced from *E. cloacae* WD7.
3. Large scale production of EPS using low cost by-product as culture medium such as commercial sucrose as carbon source and tuna condensate as nitrogen source.
4. Minimising recovery costs using lower volume of ethanol and reuse.
5. Study on the polymer synthesis of *E. cloacae* WD7 and biodegradability of the EPS with various microorganisms and the optimal conditions.
6. Applications of the polymer such as flocculant for cell precipitation for industry use, thickening agent, blend with synthetic polymers, oil recovery agent etc.
7. Strain improvement for higher polymer yield by mutation and genetic engineering techniques.

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Appendices

Appendix 1

Standard Curve of OD_{660} and Viable Cells Count of *E. cloacae* WD7

Microorganism

Enterobacter cloacae WD7

Method

Growth profile of *E. cloacae* WD7

One loop of 24 h culture of *Enterobacter cloacae* WD7 was inoculated into 200 ml nutrient broth in a 500 ml flask. Cultivation was performed on a rotary shaker (200 rpm) at 30 °C for 24 h. Every 4 h, the culture broth was taken to measure OD_{660} (5 time dilution) and viable cells count.

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

The result of growth profile of *E. cloacae* WD7 (from triplicate samples) in nutrient broth was illustrated in Fig. 1.1 and Fig. 1.2. OD_{660} values were related to the viable cells count (log CFU/ml). With the suitable time and viable cells count, therefore the cultivation time for 12 h was chosen to determine the standard curve of OD_{660} (5 time dilution) and viable cells count for *E. cloacae* WD7.

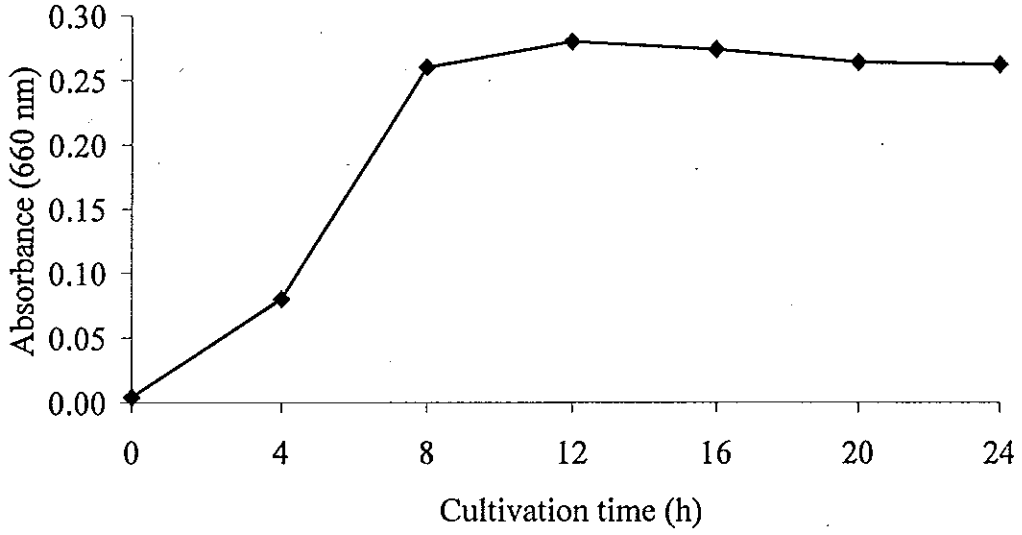


Fig. 1.1 Changes of OD_{660} (5 times dilution) of *E. cloacae* WD7 in nutrient broth for 24 h cultivation on shaker of 200 rpm at 30 °C

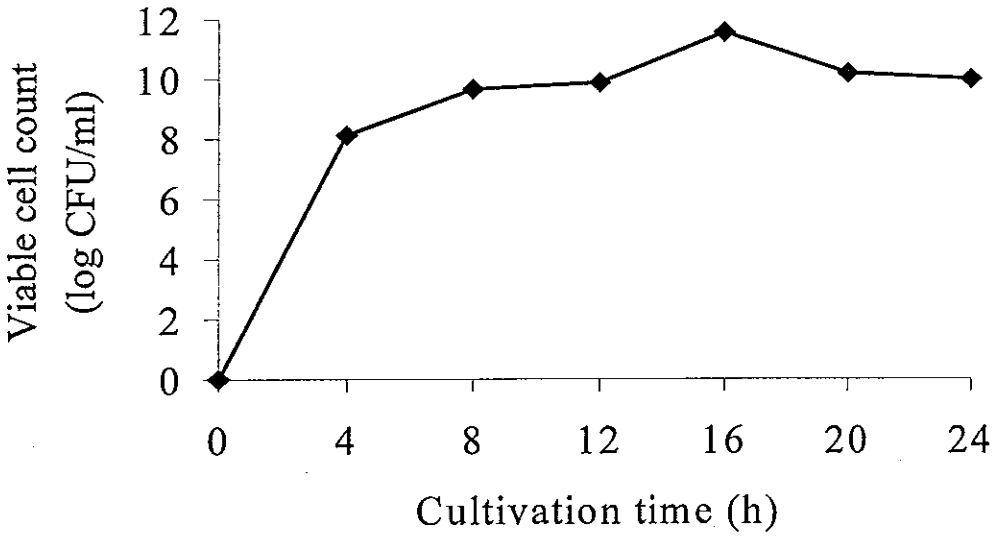


Fig. 1.2 Changes of viable cell count of *E. cloacae* WD7 in nutrient broth for 24 h cultivation on shaker of 200 rpm at 30 °C

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

Cultivation of *E. cloacae* WD7 for 12 h was carried out as above. The 12 h culture broth of *E. cloacae* WD7 was diluted with 0.1% peptone water for 0, 2, 5 and 10 fold, diluted culture broth was taken to measure OD_{660} (5 time dilution) and viable cells count as described above. The result (from triplicate samples) was shown in Fig. 1.3.

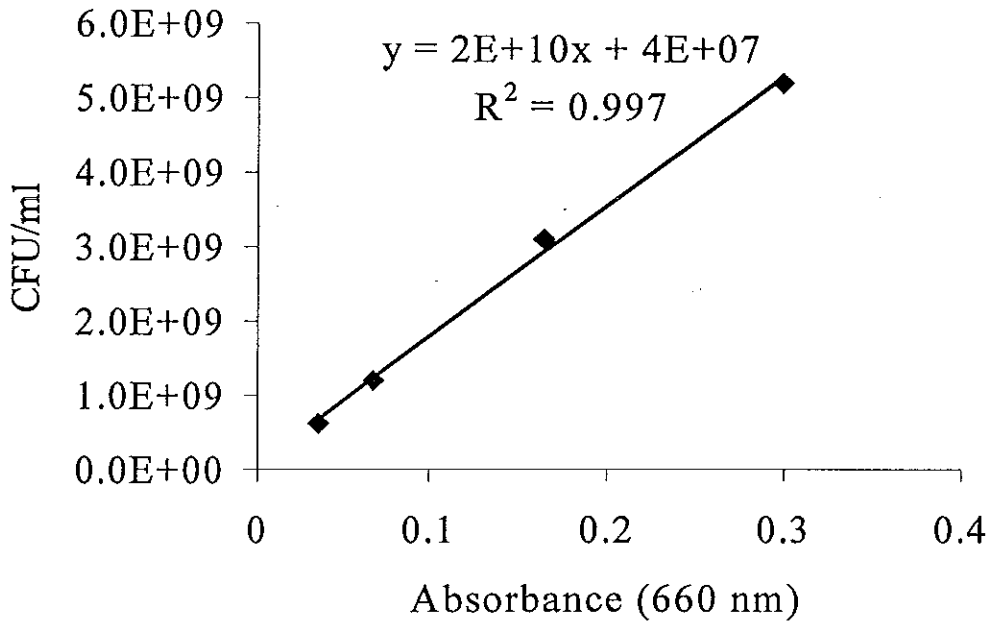


Fig. 1.3 Standard curve of OD_{660} (5 time dilution) and viable cells count of *E. cloacae* WD7 in nutrient broth

Appendix 2

Summary of Pictures of Thesis

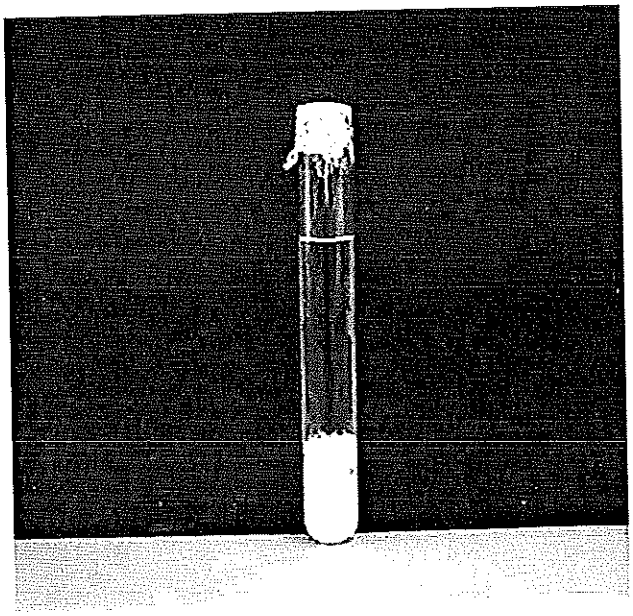


Fig.2.1 Exopolysaccharide precipitated with cold 95% ethanol

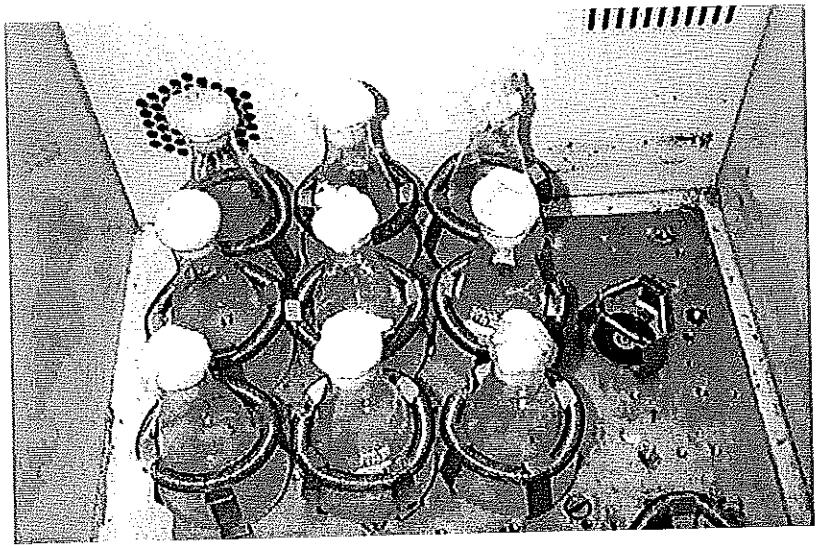


Fig.2.2 Shake-flask culture performed on incubated rotary shaker

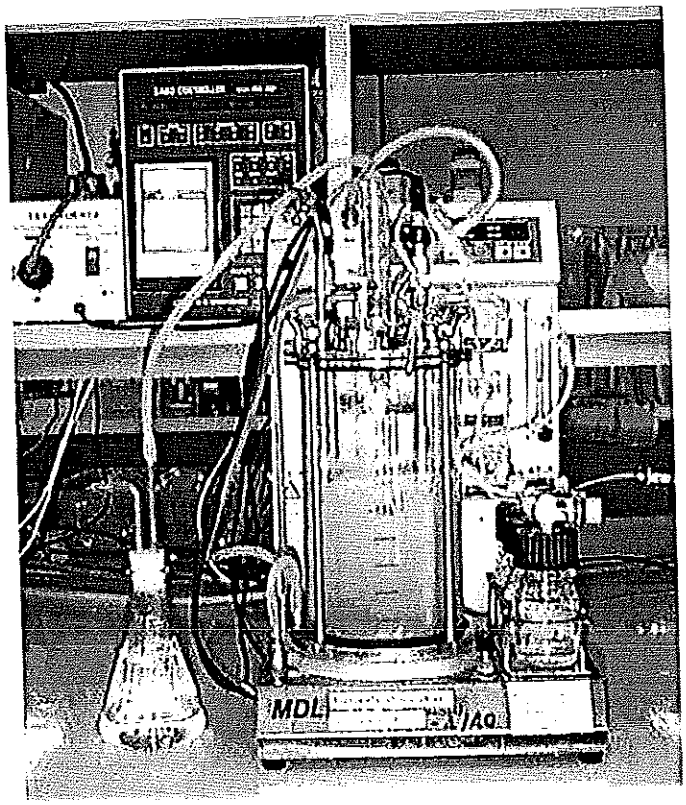


Fig. 2.3 Batch fermentor culture

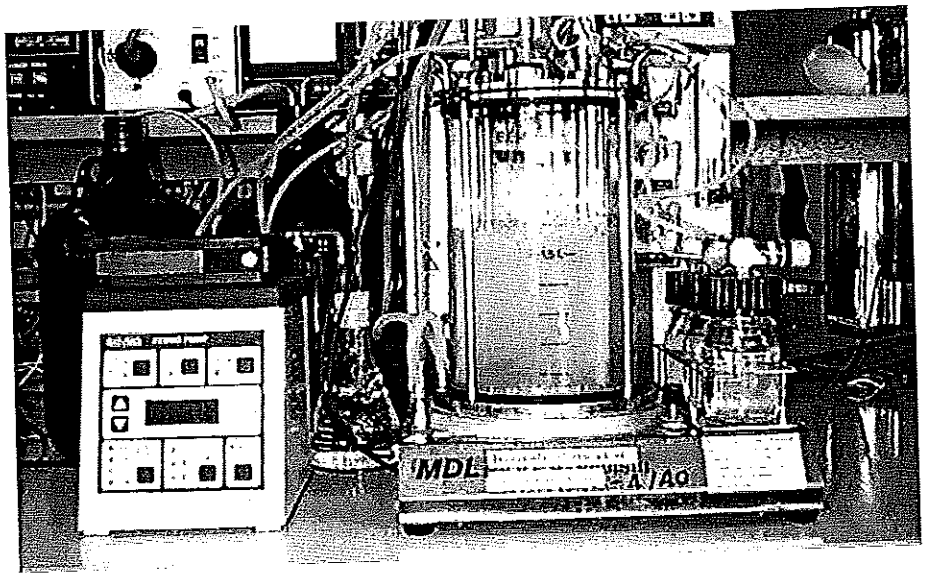
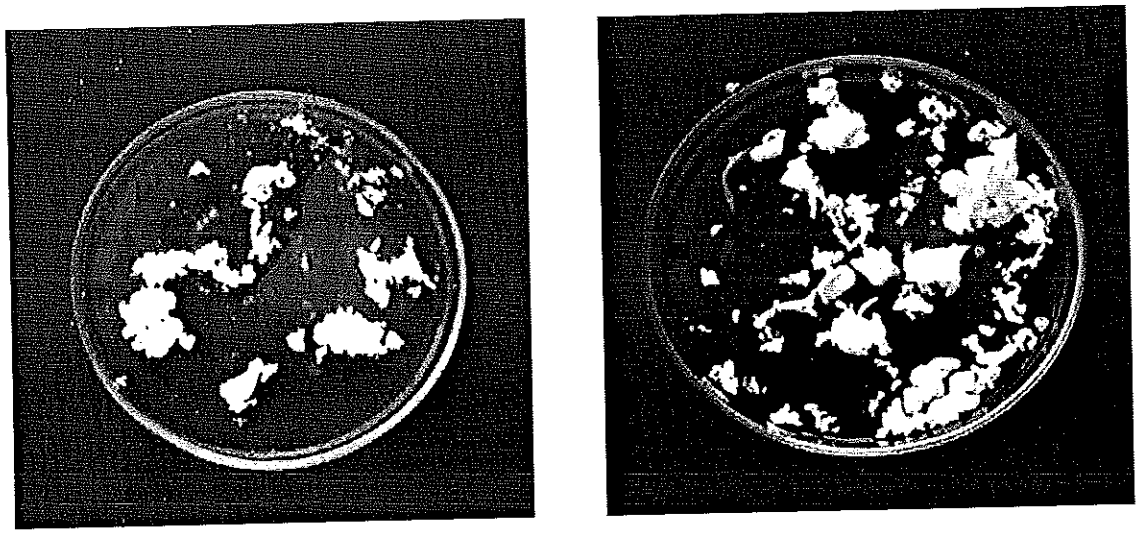
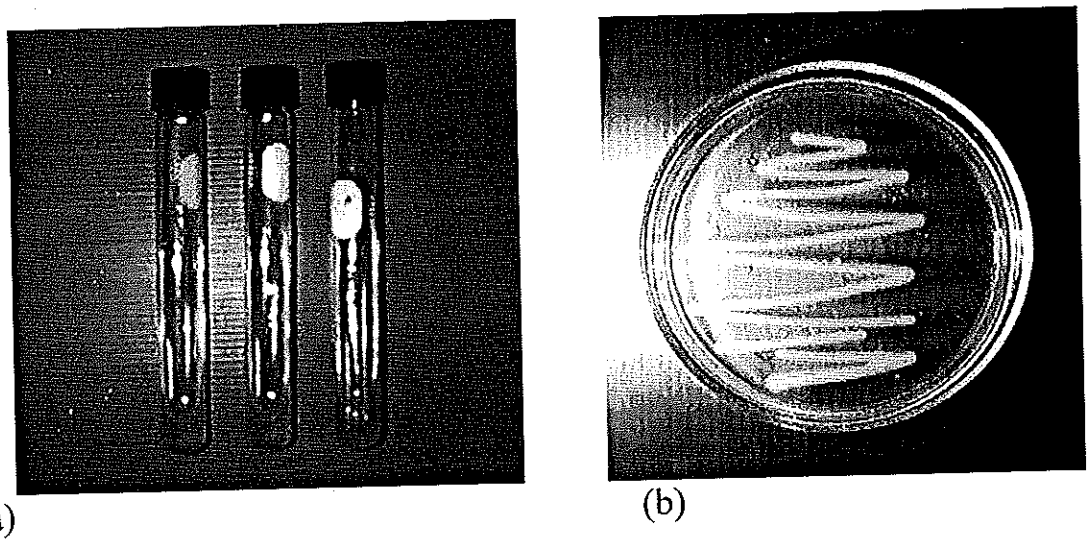


Fig. 2.4 Continuous culture



(a) (b)

Fig. 2.5 Dried crude EPS (a) and partially purified EPS (b)



(a) (b)

Fig. 2.6 Growth of *E. cloacae* WD7 on slant (a) and plate (b)

Appendix 3

Table 3.1 Time course of EPS production and growth of *E. cloacae* WD7 in basal medium under shake-flask culture

Time (h)	Cell (g/l)	pH	EPS (g/l)
0	0.001	7.00^{a}	0^{g}
6	0.19	4.05^{b}	0.42^{f}
12	0.22^{a}	4.36^{b}	0.85^{f}
24	0.24^{a}	6.57^{c}	1.67^{e}
48	0.26^{a}	6.60^{c}	1.91^{d}
72	0.28^{a}	6.68^{c}	2.22^{a}
96	0.28^{a}	6.75^{c}	2.17^{b}
120	0.27^{a}	6.85^{c}	2.14^{c}

Table 3.2 Growth, final pH and EPS production of *E. cloacae* WD7 at 3 days cultivation in the basal medium with various carbon sources (1%) on a shaker (200 rpm) at 30 °C

Type of carbon source	OD_{660}	pH	EPS (g/l)
glucose	3.56^{a}	6.73^{a}	2.23^{d}
sucrose	3.83^{a}	6.74^{a}	2.45^{b}
fructose	3.14^{a}	6.70^{a}	2.32^{c}
galactose	3.54^{a}	6.68^{a}	2.50^{a}
maltose	3.31^{a}	6.71^{a}	2.34^{c}

Table 3.3 Growth, final pH and EPS production of *E. cloacae* WD7 at 3 days cultivation in the basal medium with various sucrose concentrations on a shaker (200 rpm) at 30 °C

Sucrose concentration (%)	OD_{660}	pH	EPS (g/l)
0	1.10^{e}	7.45^{a}	0.09^{e}
1	4.28^{a}	6.95^{b}	2.20^{d}
2	3.18^{b}	6.83^{c}	2.32^{c}
3	2.54^{c}	6.25^{d}	2.63^{a}
4	2.27^{d}	5.46^{e}	2.51^{b}

Table 3.4 Growth, final pH and EPS production of *E. cloacae* WD7 at 3 days cultivation in the basal medium with various inorganic nitrogen sources on a shaker (200 rpm) at 30 °C

Type of inorganic nitrogen	OD_{660}	pH	EPS (g/l)
No addition	2.51^{d}	4.70^{c}	2.65^{a}
$(NH_4)_2SO_4$	3.31^{c}	6.53^{b}	2.51^{b}
NH_4Cl	3.78^{a}	6.47^{b}	1.69^{c}
NH_4NO_3	3.41^{b}	6.94^{a}	2.53^{b}

Table 3.5 Growth, final pH and EPS production of *E. cloacae* WD7 at 3 days cultivation in the basal medium with various organic nitrogen (polypeptone) concentrations on a shaker (200 rpm) at 30 °C

Polypeptone concentration (%)	OD_{660}	pH	EPS (g/l)
0	1.08^{d}	6.67^{a}	2.70^{a}
0.1	1.51^{c}	4.87^{b}	2.00^{b}
0.2	2.00^{b}	4.82^{c}	2.01^{b}
0.3	2.67^{a}	4.80^{c}	1.85^{c}

Table 3.6 Growth, final pH and EPS production of *E. cloacae* WD7 at 3 days cultivation in the basal medium with various yeast extract concentrations on a shaker (200 rpm) at 30 °C

Yeast extract concentration (%)	OD_{660}	pH	EPS (g/l)
0	2.02^{c}	6.64^{a}	1.97^{b}
0.05	1.88^{d}	6.54^{b}	2.71^{a}
0.10	2.44^{b}	5.17^{c}	2.68^{a}
0.20	3.84^{a}	4.65^{d}	1.75^{c}

Table 3.7 Growth, final pH and EPS production of *E. cloacae* WD7 at 3 days cultivation in the optimal medium with various initial pH on a shaker (200 rpm) at 30 °C

Initial pH	OD_{660}	pH	EPS (g/l)
6.0	0.86^{e}	4.14^{e}	1.32^{c}
6.5	1.28^{d}	4.56^{c}	1.97^{b}
7.0	1.84^{a}	4.22^{d}	2.72^{a}
7.5	1.35^{c}	4.89^{b}	2.36^{a}
8.0	1.71^{b}	5.09^{a}	2.07^{b}

Table 3.8 Growth, final pH and EPS production of *E. cloacae* WD7 at 3 days cultivation in the optimal medium with various initial pH on a shaker (200 rpm) at 30 °C

Incubation temperature (°C)	OD_{660}	pH	EPS (g/l)
30	1.82^{a}	4.23^{a}	2.71^{a}
35	1.30^{b}	4.22^{a}	1.20^{b}
40	1.19^{b}	4.19^{a}	0.66^{c}

Table 3.9 Time course of EPS production during cultivation of *E. cloacae* WD7 in the optimal medium (pH 7.0) in shake-flask culture (200 rpm) at 30 °C

Time (h)	Cell (g/l)	Residual sucrose (%)	EPS (g/l)
0	0.0013^{d}	3.00^{a}	0^{h}
6	0.14^{c}	2.72^{b}	0.22^{g}
12	0.24^{b}	2.44^{c}	0.45^{f}
24	0.28^{a}	2.38^{c}	1.23^{e}
36	0.30^{a}	2.18^{d}	1.67^{d}
48	0.31^{a}	2.16^{d}	2.31^{c}
60	0.33^{a}	2.00^{d}	2.61^{b}
72	0.33^{a}	1.99^{d}	2.71^{a}

Table 3.10 Growth, final dissolved oxygen (DO) and EPS production of *E. cloacae* WD7 at 3 days cultivation in the optimal medium under uncontrolled and controlled pH (7.0) on 3-l fermentor, agitation speed of 200 rpm, aeration rate of 0.5 vvm at 30 °C

Condition	OD_{660}	DO (%)	EPS (g/l)
Uncontrolled pH	1.07^{a}	39^{a}	2.13^{b}
Controlled pH (7.0)	1.17^{a}	10^{b}	2.91^{a}

Table 3.11 Growth, final DO and EPS production of *E. cloacae* WD7 at 3 days cultivation in the optimal medium under controlled pH (7.0) on 3-l fermentor, agitation speed of 200 rpm at 30 °C

Aeration rate (vvm)	OD_{660}	DO (%)	EPS (g/l)
0	1.09^{c}	0^{d}	1.26^{d}
0.5	1.12^{b}	10^{c}	2.96^{c}
1.0	1.14^{b}	47^{b}	3.79^{b}
2.0	1.18^{a}	64^{a}	4.80^{a}

Table 3.12 Growth, final DO and EPS production of *E. cloacae* WD7 at 3 days cultivation in the optimal medium under controlled pH (7.0) on 3-l fermentor, aeration rate of 2 vvm at 30 °C

Agitation speed (rpm)	OD_{660}	DO (%)	EPS (g/l)
200	1.10^{a}	62^{d}	1.26^{d}
400	1.05^{a}	71^{c}	2.96^{c}
600	0.97^{b}	80^{b}	3.79^{b}
800	0.90^{b}	89^{a}	4.80^{a}

Table 3.13 Time course of EPS production during cultivation of *E. cloacae* WD7 in the optimal medium and conditions on 3-l fermentor

Time (h)	Cell (g/l)	Residual sucrose (%)	EPS (g/l)
0	0.0014^{d}	3.00^{a}	0^{h}
6	0.20^{c}	2.74^{a}	0.43^{g}
12	0.27^{b}	2.46^{a}	0.87^{f}
24	0.33^{a}	2.35^{a}	2.56^{e}
36	0.35^{a}	2.13^{b}	3.50^{d}
48	0.37^{a}	2.10^{b}	4.10^{c}
60	0.38^{a}	2.07^{b}	4.70^{b}
72	0.39^{a}	2.06^{b}	4.90^{a}

Table 3.14 EPS production from *E. cloacae* WD7 at 3 days cultivation on optimal medium and conditions on batch fermentor with 1 and 3% analytical and commercial sucrose as carbon source

Type and concentration of sucrose (%)	EPS (g/l)
1% analytical sucrose	2.33^{c}
1% commercial sucrose	2.29^{d}
3% analytical sucrose	4.90^{a}
3% commercial sucrose	4.18^{b}

Table 3.15 Growth and EPS production from *E. cloacae* WD7 at 3 days cultivation on tuna condensate medium and 10% tuna condensate on batch fermentor

Medium	OD_{660}	EPS (g/l)
Modified optimal medium	2.50^{b}	4.66^{a}
10% tuna condensate medium	5.52^{a}	3.44^{b}

Table 3.16 Water absorption capacity of crude EPS from *E. cloacae* WD7 and commercial synthetic absorbent after soaking in distilled water for 24 h

Type of absorbent	Water absorption capacity (g/g dried polymer)
Crude EPS (this studies)	80.3^{b}
Commercial absorbent	292.9^{a}

Table 3.17 Fed-batch culture on optimal medium and conditions with 10 % sucrose solution was added at several times

Time (d)	Residual sucrose (%)	Maximum EPS (g/l)
1	2.15^{c}	2.06^{d}
2	2.96^{a}	2.50^{c}
3	1.90^{d}	6.19^{a}
4	2.27^{b}	5.76^{b}

Table 3.18 Fed-batch culture on optimal medium and conditions with 1 and 3% sucrose (final conc) were added every 3 days cultivation

Sucrose concentration (%)	Cell (g/l)	Residual sucrose (%)	Maximum EPS (g/l)
1%	0.43^{a}	2.41^{b}	2.56^{b}
3%	0.46^{a}	2.85^{a}	6.19^{a}

Table 3.19 Time course of fed-batch culture with 1% sucrose (final conc) addition every 3 days cultivation

Time (d)	Cell (g/l)	Residual sucrose (%)	EPS (g/l)
0	0.0016^{e}	3.00^{a}	0^{f}
1	0.12^{d}	2.33^{c}	2.04^{e}
2	0.36^{c}	2.07^{d}	3.22^{d}
3 (before added)	0.43^{b}	1.91^{d}	4.18^{c}
3 (after added)	0.43^{b}	2.91^{a}	4.18^{c}
4	0.46^{b}	2.82^{b}	4.82^{b}
5	0.49^{a}	2.45^{c}	6.19^{a}
6 (before added)	0.52^{a}	2.10^{d}	4.75^{b}
6 (after added)	0.53^{a}	3.10^{a}	4.70^{b}
7	0.56^{a}	2.90^{a}	4.50^{c}
8	0.52^{a}	2.89^{a}	4.43^{c}
9	0.50^{a}	2.87^{a}	4.10^{c}
10	0.46^{b}	2.85^{b}	3.98^{c}

Table 3.20 Continuous culture in optimal medium and conditions with various dilution rates (D) after batch culture done for 3 days

Dilution rate (h^{-1})	OD_{600}	Residual sucrose (%)	EPS (g/l)
0.01	0.83^{b}	1.87^{b}	4.82^{b}
0.05	1.24^{a}	1.62^{c}	7.28^{a}
0.10	0.52^{c}	2.84^{a}	3.27^{c}

Table 3.21 Continuous culture in optimal medium and conditions, continuous culture (D=0.05 h^{-1}) was started at various times

Starting time (d)	Maximum EPS (g/l)
1	2.75^{c}
2	4.27^{b}
3	7.28^{a}

Table 3.22 Time course of continuous culture using optimal medium containing 3% sucrose was fed at dilution rate of 0.05 h^{-1} at 3 days of batch cultivation

Time (d)	Cell (g/l)	Residual sucrose (%)	EPS (g/l)
0	0.001^{c}	3.00^{a}	0^{h}
1	0.65^{b}	2.18^{b}	2.10^{g}
2	0.68^{b}	1.91^{c}	3.26^{f}
3	0.72^{a}	1.83^{d}	4.20^{e}
4	0.73^{a}	1.86^{d}	6.32^{b}
5	0.72^{a}	1.82^{d}	7.28^{a}
6	0.74^{a}	1.89^{c}	6.02^{c}
7	0.73^{a}	1.96^{c}	5.42^{d}

Note: The same superscript-letter means not significantly different ($p<0.05$)

These values are the average of triplicate for shake-flask cultures and water absorption capacity of the polymers and duplicate for fermentor cultures

Publications

Wichienchot, S., Prasertsan, P. and Doelle, H.W. 1999. Optimization for biopolymer production by *Enterobacter cloacae* WD7. The 5th Asia-Pacific Biochemical Engineering Conference 1999 and The 11th Annual Meeting of the Thai Society for Biotechnology. November 15-18, 1999, Phuket, Thailand.

Vitae

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