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FACULTY OF GRADUATE AND POSDOCTORAL STUDIES

Nan Wu

AUTEUR DE LA THÈSE / AUTHOR OF THESIS

M.A.Sc. (Chemical Engineering)

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TITRE DE LA THÈSE / TITLE OF THESIS

C. Lan

DIRECTEUR (DIRECTRICE) DE LA THÈSE / THESIS SUPERVISOR

CO-DIRECTEUR (CO-DIRECTRICE) DE LA THÈSE / THESIS CO-SUPERVISOR

A. Macchi

E. Baronova

Gary W. Slater

Le Doyen de la Faculté des études supérieures et postdoctorales / Dean of the Faculty of Graduate and Postdoctoral Studies

Production and Rheological Studies of Microalgal Biopolymer from Lactose Using a Green Alga Strain

By

Nan Wu

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ABSTRACT

It was discovered that a green alga strain had the capacity of producing up to 5 g/l high viscosity biopolymers from lactose under mixotrophic cultivation conditions. The molecular weight of the polymer was determined to be around 505,000 daltons. The polymers were shown to have similar rheological characteristics as xanthan gum, a commercial bacterial polysaccharides, which has found numerous applications in a large variety of different fields. The ability of this strain to utilize lactose for biopolymer production promises the potential of valuable biopolymer production from cheese whey, a waste liquor stream of the cheese industry that contains approximately 50 g/l lactose. Using Plackett-Burnman experiment design and statistical analysis, it was determined that the major parameters affecting biopolymer production were the concentrations of sodium nitrate and lactose and temperature as well. Further studies on polymer characterization, production optimization, and applications are under way.

RÉSUMÉ

Il a été découvert qu'une souche d'algue verte aant la capacité de produire jusqu'à 5 g/l de bio-polymères à haute viscosité à partir de lactose dans des conditions de culture mixotrophes. La masse moléculaire du polymère trouvé était environ de 505,000 daltons. Les polymères ont montré des caractéristiques similaires à ceux de la gomme xanthane, un polysaccharide bactériologique commercial, qui a de nombreuses applications dans plusieurs domaines. La capacité de cette souche à utiliser le lactose pour produire du bio-polymère promet le potentiel d'une production de bio-polymère de valeur à partir de fromage de lactosérum, une liqueur résiduaire de l'industrie fromagère contenant approximativement 50 g/l de lactose. À l'aide du plan d'expérience de Plackett-Burnman et d'une analyse statistique, les principaux paramètres affectant la production de bio-polymères ont été identifiés comme étant le nitrate de sodium, le lactose et la température. Des études plus poussées sur la caractérisation du polymère, l'optimisation de la production et les applications sont en cours.

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NOMENCLATURE

ACV	Acyclovir
ADP	adenosine diphosphate
AIDS	acquired immunodeficiency syndrome
APTT	activated partial thromboplastin time
Ara	Arabinose
ATP	adensine triphosphate
CMP-NeuAc	cytidine monophosphate -N- acetyleuraminic acid
Fuc	Fucose
G1P	glucose-1-phosphate
G6P	glucose-6-phosphate
Gal	Galactose
galE	uridine diphosphate-glucose 4'-epimerase
galU	uridine diphosphate-glucose pyrophosphorylase
Glc	Glucose
HIV	human immunodeficiency virus
HK	phosphotrasnferase system (Hexokinase)
HSV-1	herpes simplex virus type 1
HSV-2	herpes simplex virus type 2
Man	Mannose
PEP	Phosphoenolpyruvate
pgi	phosphoglucose isomerise
pgm	Phosphoglucomutase
Pyr	Pyruvate
Rha	Rhamnose
Rib	Ribose
RPS	Exopolysaccharide
SAGD	steam assisted gravity drainage
TT	thrombin time

UDP	uridine diphosphate
UDP--GA	uridine diphosphate -glucuronic acid
UDP-Gal	uridine diphosphogalactose
UDP--GalNAc	uridine diphosphate -N-acerylgalactosamine
UDP-Glc	uridine diphosphoglucose
UTP	uridine triphosphate
VAPEX	vapour extraction
Xyl	Xylose

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CHAPTER 1

INTRODUCTION

1.1 Background

Algae play an important role on earth. As primary producers of the food chain, algae are original sources for a large array of valuable products. Algae cultivations were studied and developed over a century and the earliest algal culture techniques were described by Moore (Moore 1903). Algal culture techniques are also described in detail in several earlier books and articles (Kufferath 1928; Bold 1950; Pringsheim 1951). Algae have much faster growth-rates than terrestrial crops. Studies have shown the photosynthetic efficiency of microalge could well be in the range of 10–20% or higher (Richmond 2000) (Huntley and Redalje 2007). It is estimated that the biomass productivity of microalgae could be 50 times more than that of switchgrass, which is the fastest growing terrestrial plant (Nakamura 2006) (Demirbaş 2006). For the last 50 years, tremendous efforts have been made to exploit microalgae as a source of food, feed, lipids, vitamins, pigments, fertilizers, pharmaceuticals and other speciality chemicals. During the past two decades, subsequent studies illustrate that research developments are enabling the commercial potential of microalgae to shift from aquaculture and health food to fuel productions (de la Noue and de Pauw 1988). Microalgae including microalgal oil, have emerged as one of the most promising feedstock for biofuel production. The benefits of biofuels are not only as a greenhouse gas (GHG) neutral energy source, but also as an opportunity to reduce reliance on fossil fuel. However, to make the

microalgal biofuel production and CO_2 sequestration economical viable, enormous efforts are required to lower the cost of microalgal production and increase the value of end products. The employment of a high value co-product strategy through the integrated biorefinery approach is expected to significantly enhance the overall cost effectiveness of microalgal biofuel production (Li, Horsman et al. 2008). Polysaccharides (carbohydrate polymers) are one of these high-value products that have a vast market in a diversity of potential fields of application.

1.2 Project Objectives

It was accidentally discovered in our studies on the mixotrophic growth of different microalgal strains that a green alga strain could produce large quantities of biopolymers using lactose as the sole carbon source, resulting a gel-like broth at the end of cultivation. The current investigation focuses on microalgal biopolymer production from different carbon sources and different cultivation conditions and its rheological properties.

The main objectives of this research are summarized as follow:

1. Microalgae growth and polymer production under photoautotrophic, heterotrophic and mixotrophic conditions.
2. Production of microalgal polymer from lactose, and statistic experiment design for optimization.
3. Rheological properties of microalgal polymers

1.3 Structure of the Thesis

The main body of this thesis consists of three chapters, Chapters 2, 3 and 4, in a paper

format. All these papers will be submitted for publication shortly after the thesis defence.

Chapter 2 presents a literature survey on the works carried out in microalgal cultivation, microalgal polymer production and applications of microalgal biopolymers.

Chapter 3 presents the results of studies on the effects of macroelements and microelemenets on cell growth of the alga strain under photoautotrophic condition. The statistic experiment design for optimization of the microalgal polymer production from lactose under mixotrophic condition is also presented.

Chapter 4 presents the rheological properties of the microalgal biopolymers, which were produced under mixotrophic cultivation condition. The rheological properties of microalgal polymers show that they have potentials in applications such as enhanced oil recovery.

Finally, Chapter 5 presents the conclusions and recommendations for future work.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

During the past decades, much interest has been focused on the biotechnological potential of microalgae. The enormous biodiversity and consequent variability in the biochemical composition of the biomass obtained from the microalgal cultures have allowed this group of organisms to be one of the most promising sources of valuable products.

Consequently, extensive researches on different fundamental and applied aspects of phycology have been carried out that demonstrated that algal biomass can be used for various other applications such as human food, animal feed, and bio-fertilizer. In addition, the developments in genetic engineering and the establishment of technology in massive production have allowed various species to be commercially cultivated. In this sense, microalgae cultivations have been developed aiming at the biomass production not only for use in food and feed for ecological applications but also for the obtainment of natural compounds with high value in the world market.

According to many years of scientists' significant efforts, Algae, due to their structural simplicity and functional complexity, have become the vehicles for important discoveries and experiments. In this connection, the algae cultivation constructs a bridge between the study areas and application fields. More and more study areas in microalgal biotechnology

have been explored, such as taxonomy, morphology, physiology, biochemistry, genetic engineering, pharmacology, aquaculture, and environmental engineering, which are summarized in Figure 2.1.

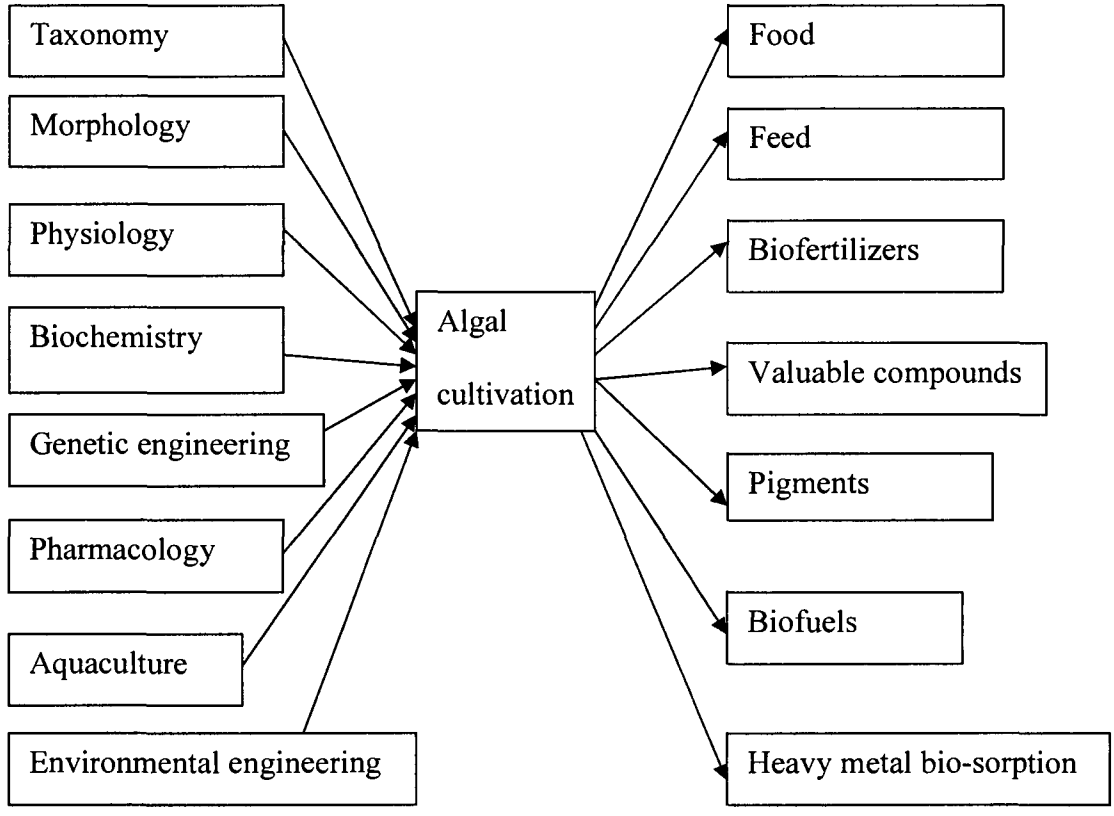

Figure 2.1. Algae study fields and applications.

What is more, latest developments have established the potential application fields from their various productions such as polysaccharides, lipids, proteins, carotenoids, pigments, vitamins, sterols, enzymes, antibiotics, pharmaceuticals, and biofuels (methane, alcohol, diesel, and hydrogen). These potential applications are summarized in table 2.1.

Table 2.1 Potential applications of microalgal biomass.

Application fields	Products /Applications	Reference
Food	Algal biomass or extracts are used as ingredient and supplement in health food recipes and products which include high protein content, high concentrations of minerals, trace elements, and vitamins.	(Yalcin, Hicsasmaz et al. 1994; Plaza, Cifuentes et al. 2008) (Derner, Ohse et al. 2006) (Pulz and Gross 2004; Richmond 2004) (Liang, Liu et al. 2004) (Gudin 1983; Kay 1991)
Feed	Algal biomass or extracts are used as feeds for farm animals (poultry and livestock), pets, fish, bivalves and mussels.	(Barnhart 2006; Spolaore, Joannis-Cassan et al. 2006) (Pulz and Gross 2004; Richmond 2004)
Biofertilizers	Algae are used as soil fertilizer in coastal regions all over the world which can increase water-binding capacity and mineral composition of the soil.	(Peña 2008) (Critchley 1998)
Pigments	β-Carotene as food colorant and food supplement (provitamin A); Phycocyanin as food colorant, in diagnostics, cosmetics and analytical reagents; Astaxanthin for coloring muscles in fish; Lutein, zeaxantin and canthaxantin for chicken skin coloration, or for pharmaceutical purposes.	(Spolaore, Joannis-Cassan et al. 2006) (Furuki, Maeda et al. 2003) (Olaizola 2003) (Pulz and Gross 2004)

Application fields	Products /Applications	Reference
Valuable compounds	Polyunsaturated Fatty acids (PUFAs) such as Eicosapentaenoic acid (EPA), Aocosahexaenoic acid (DHA) and Arachidonic acid (AA) ; Biotin (vitamin H), ascorbic acid (vitamin C) and α-tocopherol (vitamin E). Glycerol used in foods, beverages, cosmetics, pharmaceuticals. Polysaccharides and amino acids.	(Cardozo, Guaratini et al. 2007) (Valencia, Ansorena et al. 2007) (Bigogno, Khozin-Goldberg et al. 2002); (Baker, McLaughlin et al. 1981) (Survase, Bajaj et al. 2006) (Bremus, Herrmann et al. 2006) (Running, Severson et al. 2002); (Rinaudo 2008) (Brown 1991; De Philippis, Sili et al. 2001)
Biofuels	Biodiesel, bioethanol, and biosyngas (i.e. hydrogen and methane)	(Li, Horsman et al. 2008) (Cardozo, Guaratini et al. 2007) (Li, Horsman et al. 2008) (Wang, Li et al. 2008)
Heavy metal biosorption	The removal of heavy metal ions such as Cd^{2+}, Cu^{2+}, Zn^{2+}, Pb^{2+}, Cr^{3+}, and Hg^{2+}, or heavy metals such as K, Mg, Ca, Fe, Sr, Co, Cu, Mn, Ni, V, Zn, As, Cd, Mo, Pb, Se, and Al.	(Davis, Volesky et al. 2003) (L. Brinza 2007)

2.2 Polysaccharide Producers and Production

Beginning in the 1830s, algae were classified into major groups based on their colours (e.g., red, brown, and green), which are a reflection of different chloroplast pigments, such as chlorophylls, carotenoids, and phycobilins.

Algae are defined in this thesis as a group of photosynthetic microorganism including both prokaryotic (nucleus-lacking) blue-green algae (cyanobacteria) and eukaryotic algae. Some scientists suggested in the 1970s that the study of the blue algae should be incorporated into the study of bacteria because of certain shared cellular features between these two groups. However, other scientists consider the oxygen-producing photosynthetic capability of blue-green to be as significant as cell structure. Therefore, these organisms continue to be classified as a group of microalgae.

During the past decades, significant progress has been made in discovering and developing polysaccharides that possess novel and highly functional properties. Hundreds of alga strains including cyanobacterial (*Anabaena, Nostoc, Cyanospira, Cyanothece, Oscillatoria* and *Spirulina*), thirteen species of green algae (*Caulerpa, Cladophora, Bryopsis, Boodlea, Chaetomorpha, Ulva, Enteromorpha, Valoniopsis, Monostroma, Codium, Oedogonium, Penium,* and *Botryococcus*), seventeen species of red algae (*Pachymeniopsis, Champia, Acrotylus, Amphiplexia, Antrocentrum, Hennedya, Ranavalona, Corynocystis, Tichocarpus, Porphyridium, Grateloupi, Hypnea, Gracilaria, Botryocladia, Gracilariopsis, Gelidium, Phaeocystis*), nine species of brown algae (*Sargassum, Macrocystis, Fucus, Sargassum, Ascophyllum, Ecklonia, Spatoglossum, Laminaria,* and *Macrocystis*) and ten species of diatoms (*Amphora, Cylindrotheca, Thalassiosira, Skeletonema, Chaetoceros, Skeletonema,*

Cylindrotheca, Navicula, Nitzschia and *Chaetoceros*) have been investigated with regard to the production polysaccharides. However, very few products with real potential have been identified or developed. Table 2.2 presents the current developments in microalgal biopolymer production.

Table 2.2 Polymer production from algae.

Algae	Species(Scientific name)	Characteristics/ Monosaccharide composition	Applications	References
Cyanobacteria (blue-green algae)	*Nostoc flagelliforme*	Glc, Gal, Xyl and Man	Antiviral Activity	(Kanekiyo, Hayashi et al. 2008)
	Cyanospira capsulata		Heavy metal sorption	(De Philippis, Paperi et al. 2007)
	Nostoc PCC7936			
	Nostoc flagelliforme		Anti-herpes simplex virus	(Kanekiyo, Hayashi et al. 2007)
	Cyanothece sp.	Man, Glu, Xyl and Rib		(Parikh and Madamwar 2006)
	Oscillatoria sp.			
	Nostoc sp.			
	Nostoc carneum			
	Cyanothece CE4		Thickening agents, stabilization of emulsions or as bioflocculants. capability to bind water molecules for cosmetic industry	(De Philippis, Sili et al. 2001)
	Cyanospira capsulata			
	Nostoc commune	Glu, Xyl, and Gal		(Huang, Liu et al. 1998)
	Nostoc flagelliforme			
	Nostoc sphaeroides			

Algae	Species(Scientific name)	Characteristics/ Monosaccharide composition	Applications	References
	Spirulina platensis	Mainly composed of rhamnose	Inhibition of tumor invasion	(Mishima, Murata et al. 1998)
	Anabaena sp. ATCC 33047		Used in the food, textile, painting, cosmetic, paper and pharmaceutical industries as emulsifiers, stabilizers or thickening agents	(Moreno, Vargas et al. 1998)
	Cyanospira capsulate		Used in food, cosmetics, pharmaceutical and oil industries as thickening, emulsifying and viscosifying agents	(De Philippis, Sili et al. 1996)
	Cyanothece sp.	Gal, Glu, Man, Xyl and Fuc		(De Philippis, Margheri et al. 1993)
Green algae	*Caulerpa lentillifera* *C. sertularioides*	1,4-α-D-glucans Glc, Gal, Man, and Xyl		(Shevchenko, Burtseva et al. 2009)

Algae	Species(Scientific name)	Characteristics/ Monosaccharide composition	Applications	References
	Ulva rigida		Antioxidant activity	(Godard, Décordé et al. 2009)
	Monostroma nitidum	Rha, Xyl and Glu.	Anticoagulant activity	(Mao, Fang et al. 2008; Mao, Li et al. 2009)
	Codium isthmocladum	Gal, Ara and Man		(Farias, Pomin et al. 2008)
	Oedogonium bharuchae f. minor.			(Estevez, Leonardi et al. 2008)
	Codium yezoense	D-galactose, Ara and Xyl		(Bilan, Vinogradova et al. 2007)
	Codium fragile		Anticoagulant activity	(Athukorala, Lee et al. 2007)
	Ulva conglobata	Mainly consisted of rhamnose with variable contents of glucose and fucose, trace amounts of xylose and glactose	Anticoagulant activity	(Mao, Zang et al. 2006)

Algae	Species(Scientific name)	Characteristics/ Monosaccharide composition	Applications	References
	Penium margaritaceum	Xyl and Fuc		(Domozych, Kort et al. 2005)
	Ulva armoricana,		It could be used as a filling or reinforcement material in the manufacture of composite materials.	(Carrasco and Pagès 2004)
	Ulva lactuca,			
	Ulva rotundata			
	Ulva rigida.			
	Codium dwarkense and *C. tomentosum*	α-L-arabinofuranose	Anticoagulant activity	(Siddhanta, Shanmugam et al. 1999; Shanmugam, Mody et al. 2001; Shanmugam, Mody et al. 2001)
	Codium cylindricum		Anticoagulant activity	(Matsubara, Matsuura et al. 2001)
	Codium pugniformis	Mainly consisted of glucose with minor amounts of arabinose and galactose.	Anticoagulant activity	(Matsubara, Matsuura et al. 2000)

Algae	Species(Scientific name)	Characteristics/ Monosaccharide composition	Applications	References
	Botryococcus braunii UC 58	Gal and Fuc		(Lupi, Fernandes et al. 1994)
Red algae	*Pachymeniopsis elliptica*		Anticoagulant activity	(Ekanayake, Nikapitiya et al. 2008)
	Champia feldmannii		Exploited as antithrombotic and anticoagulant agents and suggested to be immunostimulants	(Assreuy, Gomes et al. 2008)
	Acrotylus australis *Amphiplexia hymenocladioides* *Antrocentrum nigrescens* *Hennedya cris* *Ranavalona duckerae* *Corynocystis prostrata*	kappa (κ)-/beta (β)-carrageenans		(Chiovitti, Kraft et al. 2008)
	Tichocarpus crinitus	Gal	Anticoagulant activity	(Barabanova, Shashkov et al. 2008)
	Porphyridium sp		Biolubricant	(Arad, Rapoport et al. 2006; Canter 2007)

Algae	Species(Scientific name)	Characteristics/ Monosaccharide composition	Applications	References
	Grateloupia longifolia *Grateloupia filicina*		Anti-HIV-1 activity	(Wang, Bligh et al. 2007)
	Hypnea musciformis	k- carrageenan		(Bi, Mahmoodul ul et al. 2007)
	Gracilaria *Gracilariopsis* *Polycavernosa*	Agar		(Oliveira, Alveal et al. 2000)
	Botryocladia occidentalis		Anticoagulant activity	(Farias, Valente et al. 2000)
	Porphyridium sp.(UTEX637)	Xylose, glucose, and galactose	Use in cosmetics as anti-herpes drugs, as growth promoters in agriculture, and in the health food market	(Li, Shabtai et al. 2000)
	Gelidium sesquipedale	Agar		(Carmona, Vergara et al. 1998)
	Phaeocystis globosa	Ara, Rha, Xyl, Man, Gal and Glu		(Van Rijssel, Janse et al. 2000)

Algae	Species(Scientific name)	Characteristics/ Monosaccharide composition	Applications	References
Brown algae	*Sargassum horneri*		Anticoagulant activity	(Athukorala, Lee et al. 2007)
	F. vesiculosus	Fucoidans	Anticoagulant, hemorrhagic activities and platelet aggregation	(Carvalho G. de Azevedo, Bezerra et al. 2009)
	Macrocystis pyrifera	Alginate	It can be used as a stabilizer of suspensions and as thickener in food industries	(Gomez, Pérez Lambrecht et al. 2009)
	Fucus evanescens	Fucoidan		(Anastyuk, Shevchenko et al. 2009)
	S. scroederi	Fucan	Inhibition of reverse transcriptase activity of HIV	(Queiroz, Medeiros et al. 2008)
	D. mertensii	Fucan		
	F. vesiculosus	Fucoidan		
	L. variegata	Galactofucan		
	Sargassum terrarium	Sodium Alginate		(Bi, Mahmood et al. 2007)

Algae	Species(Scientific name)	Characteristics/ Monosaccharide composition	Applications	References
	Sargassum patens		Inhibit the in vitro replication of both the acyclovir (ACV)-sensitive and -resistant strains of Herpes simplex virus type 1 (HSV-1)	(Zhu, Chiu et al. 2006)
	Ascophyllum nodosum	Fucoidan	Exhibit some heparin/heparan sulphate properties, are able in vitro to stimulate dermal fibroblast proliferation and extracellular matrix deposition. involved in connective tissue breakdown	(Senni, Gueniche et al. 2006)
	Ecklonia cava		Ant proliferative and antiradical activities	(Athukorala, Kim et al. 2006)
	Spatoglossum schr oederi	Xyl and Gal	Hemostatic Activities	(Rocha, Moraes et al. 2005)
	Sargassum fusiforme	Alginate		(Mao, Li et al. 2004)

Algae	Species(Scientific name)	Characteristics/ Monosaccharide composition	Applications	References
	Sargassum patens	Fuc, Xyl, Man, Glu and Gal	Anti-herpes activity against herpes simplex viruses which are the cause of cold sores (HSV-1) and genital herpes (HSV-2)	(Zhu, Ooi et al. 2003)
	Sargassum horneri	Fucan	Inhibitory activity against replication of herpes simplex virus type 1	(Preeprame, Hayashi et al. 2001)
	Fucus vesiculosus	Fucan	Anticoagulant Activity	(Pereira, Mulloy et al. 1999)
	Fucus vesiculosus	Alginate	Anti-HIV activity	(Béress, Wassermann et al. 1993)
Diatom	*Amphora sp.*	Fuc, Rha, Ara, Gal, Glu, Xyl, and Man		(Zhang, Xu et al. 2008)
	Chaetoceros mülleri	β-D-(1→3)-glucan		(Størseth, Hansen et al. 2004)
	Navicula salinarum / *Cylindrotheca closterium*	Glu and Xyl		(Staats, De Winder et al. 1999)

2.3 Polysaccharide Biosynthesis

Polysaccharides are composed of monosaccharide subunits. Synthesis of polysaccharides from monosaccharides generally follows a common pathway: the sugar is transferred from the nucleotide sugar to the lipid to form a glycosyl-containing lipid-linked sugar intermediates, and then the glycosyl residues are transferred either to an oligosaccharide-lipid intermediate or a growing saccharide chain. Not all glycosyltransferases transfer the sugar to a lipid; some transfer directly from the nucleotide to the growing polymer (Mayer and Ginsburg 1964).

2.3.1 Carbon requirement

Polysaccharides synthesis requires sugar nucleotides as precursors. These sugar nucleotides are also major components in cell wall biosynthesis. Thus production of these carbohydrates is carbon intensive and competes with the cell's metabolism for available carbon sources. Production of the sugar nucleotide precursors required for polysaccharide synthesis interface with the cell primary metabolism. . Figure 2.2 presents the metabolic pathway for production of two sugar nucleotides, UDP-glucose and UDP-galactose.

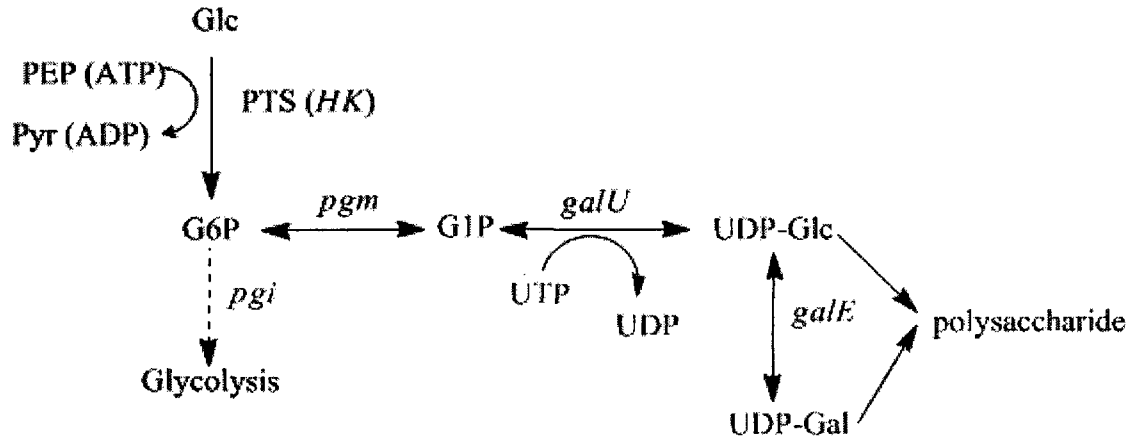

Figure 2.2. UDP-glucose and UDP-galactose biosynthesis pathway (Rehm January 2009)

Abbreviations: ADP: adenosine diphosphate, ATP: adenosine triphosphate, G1P: glucose-1-phosphate, G6P: glucose-6-phosphate, *galE*: uridine diphosphate-glucose 4'-epimerase, *galU*: uridine diphosphate-glucose pyrophosphorylase, Glc: glucose, PTS (*HK*): phosphotrasnferase system (Hexokinase), Pyr: pyruvate, UDP: uridine diphosphate, UDP-Gal: Uridine diphosphogalactose, UDP-Glc: uridine diphosphoglucose, UTP; uridine triphosphate, PEP: phosphoenolpyruvate, pgi: phosphoglucose isomerise, pgm: phosphoglucomutase.

2.3.2 Energy requirement

Polysaccharide synthesis is not only carbon-intensive; it is also an energy-intensive process. As shown in the Figure 2.3 two high energy compounds are consumed for biosynthesis of each sugar nucleotide. One high energy compound, either ATP or phosphoenolpyruvate depending upon the sugar transport system, is used to activate by the sugar component. This step is illustrated by the conversion of glucose to glucose-6-phosphate in Figure 2.2. A

second high energy compound, UTP or another nucleoside triphosphate, is required for synthesis of the sugar nucleotide precursor. Taking into account both the requirement of sugar nucleotide biosynthesis and the potential energy loss from an unutilized carbon source, a cell will lose up to 40 high energy compounds for each sugar nucleotide synthesized in polysaccharide production.

2.3.3 Sugar Nucleotides precursors

In nucleotide sugar metabolism a group of biochemicals known as nucleotide sugars act as donors for sugar residues in the glycosylation reactions that produce polysaccharides. (Ginsburg 1978) Most glycosylation takes place in the endoplasmic reticulum and golgi apparatus, there are a large family of nucleotide sugar transporters that allow nucleotide sugars to move from the cytoplasm, where they are produced, into the organelles where they are consumed.(Gerardy-Schahn, Oelmann et al. 2001) (Handford, Rodriguez-Furlán et al. 2006)

The synthesis of most polysaccharides begins with the formation of a nucleoside diphosphate glycosyl derivative from a nucleotide triphosphate and a glycosyl phosphate ester. This process requires nucleoside triphosphates (such as UTP or GTP) and a glycosyl-1-P (monosaccharide with a phosphate at the anomeric carbon). Several variations are used, but regardless of the monosaccharide, all must be either activated by a kinase (reaction 1) or generated from a previously synthesized activated nucleotide sugar (reactions 2):

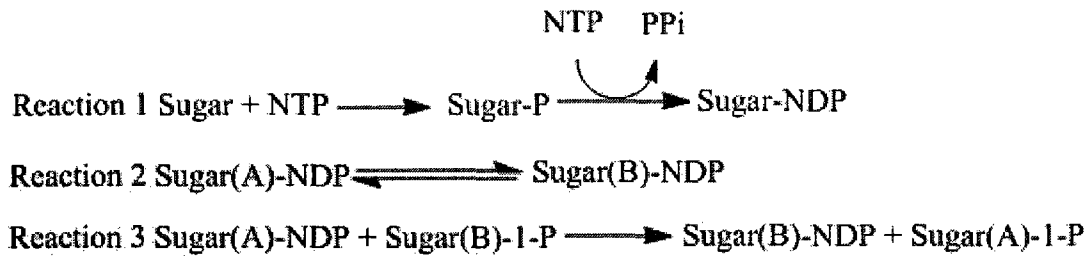

Figure 2.4. Formation of nucleoside diphosphate glycosyl, N could be A, T, G, C or U (Varki 2009)

In some instances, one nucleotide sugar can be formed from another by a nucleotide exchange reaction (reaction 3 above). For example, UDP-Gal is made from UDP-Glc by exchange of Gal-1-P for Glc-1-P.

Glucose is the central monosaccharide in carbohydrate metabolism, and it can be converted into all other sugars. UDP-glucose serves as a platform for synthesis of other sugar nucleotides: UDP-galactose, UDP-glucuronic acid (UDP--GA), UDP-N-acerylgalactosamine (UDP--GalNAc), GDP-fucose, and CMP-N- acetyleuraminic acid (CMP-NeuAc).

2.3.4 Enzymes catalyzing glycosidic linkage

Enzymes that are capable of synthesizing glycosidic linkage belong to two categories: glycosidases and glycosyltransferases. Glycosidases typically hydrolyse glycosidic bonds while glycosyltransferases naturally catalyse oligosaccharide formation within a cell and thus have regio and stereo selectivity as well as high yields. Approximately sixty nucleoside diphosphate sugars are known at the present time. Some of these are formed directly from an alpha-D-glycosyl-1-phosphate and a nucleoside triphosphate, but many are products of

oxidation, reduction, and epimerization reactions that are catalyzed by specific soluble enzymes. The enzymes that catalyze the transfer of glycosyl residues from nucleoside diphosphate sugars to the oxygen residues of other saccharide moieties in general show absolute specificity with respect to the linkage position and anomeric configuration. Since, as far as is known, all nucleoside diphosphate sugars have the same absolute configuration (=α-D) at the phosphate-glycosyl linkage some transfer reactions clearly take place with retention of this configuration at the glycosyl position while other transfer reactions lead to inversion of configuration. In either case the energy of the system is preserved, presumably by way of covalent intermediates; thus, it is possible that transfer reactions that take place with retention of the glycosyl carbon atom configuration are actually double inversion reactions. According to this concept, the enzyme that will catalyze transfer with retention of configuration reacts with the nucleoside diphosphate sugar with displacement of the nucleoside diphosphate and the formation of an enzyme-sugar covalent intermediate. This intermediate has the inverted glycosyl configuration and reaction with the final acceptor in a second displacement reaction leads to restoration of the original glycosidic configuration. Reactions that involve over-all inversion, on the other hand, would be visualized as simple displacement reactions on the nucleoside diphosphate sugar glycosyl linkage by the acceptor group oxygen moiety. While these simple schemes would account adequately for the occurrence of both types of glycosidic linkages in polysaccharides, little serious work has yet been carried out on the mechanisms of transfer reactions (Robbins, Wright et al. 1966).

2.3.5 Noncarbonhydrate Substituents

Structural studies show that a variety of different noncarbohydrate substituents could be attached to polysaccharides, leading to the production of different polysaccharide molecules. Because all of these reactions occur in the Golgi body, clearly there must be carriers or transporters that deliver and orient activated donors for efficient synthesis. As additional modifications of sugar chains made in the endoplasmic reticulum (ER)-Golgi pathway are uncovered, they will likely require specific transporters to carry the activated donors into the lumen of these compartments. Only a few reports describe the reactions for the incorporation of noncarbohydrate constituents into polysaccharides. These include methyl (Gray and Ballou 1971), glycerol (Ivatt and Gilvarg 1979), fatty acid (Hungerer, Fleck et al. 1969), pyruvate (Melton, Mindt et al. 1976), ethanolamine and choline (Mosser and Tomasz 1970) residues.

2.4 Effect of Nutrient and Cultivation Condition

Growth medium must provide sufficient nutrients for microalgal growth. Carbon, nitrogen, phosphorus, and sulfur are the most important elements constituting algal cells. Other essential elements include calcium, iron, magnesium and trace elements.

2.4.1 Nitrogen

Nitrogen, besides carbon, is quantitatively the most important element in algal nutrition. Generally, algae are able to utilize nitrate, ammonia or organic sources of nitrogen such as urea (Lourenço, Barbarino et al. 1998). Nitrogen is not only important for cell growth but also commonly used as a nutritional factor that could be controlled to trigger the biosynthesis of polysaccharide in microalgae. For example, during the polysaccharide production by the

Cylindrotheca closterium, when nitrogen was added to the culture in the stationary phase, growth was resumed and the accumulation of polysaccharides was delayed (Staats, Stal et al. 2000). This example indicated that nitrogen depletion caused cessation of growth, and stimulated polysaccharide accumulation.

2.4.2 Phosphorus

Phosphorus is an anothermajor nutrient required for normal growth of algae cells. It is essential for almost all cellular processes, i.e. biosynthesis of nucleic acids, energy transfer, etc. The major form which algae acquire phosphorus is as inorganic phosphate, either as $H_2PO_4^-$ or $H_2PO_4^{2-}$ (Gauthier and Turpin 1997; Becker 1994). It has been reported that, similar to the effects observed with algae grown under nitrogen starvation, phosphate-deficient algae cultures tend to accumulate large amount of polysaccharides (Staats, Stal et al. 2000) (Urbani, Magaletti et al. 2005), while the content of protein, chlorophyll and nucleic acids decreases.

2.4.3 Sulfur

The nutrient sulfur plays an important part in the structure and function of proteins. It is a constituent of some essential amino acids (methionine, cysteine, cystine), vitamins, sulfolipids, sulfate esters, sulfate polysaccharide and a variety of other compounds. It is generally provided as inorganic sulfate in the culture medium (Barsanti and Gualtieri 2006).

2.4.4 Trace elements

Algae grow in culture not only depends on essential macroelements, but also on a number of Trace metals normally refer to zinc, selenium, iron, manganese, nickel, cobalt, copper,

chromium and molybdenum. These elements are required in very small amount of micro-, nano- or even pictograms per liter. Trace elements play critical roles in a variety of metabolic pathway. Iron is needed for the growth of all phytoplankton. It serves essential metabolic functions in photosynthetic electron transport, respiratory electron transport. A major use for zinc is in carbonic anhydrase, an enzyme critical to CO_2 transport and fixation. Cooper serves its function in cytochrome oxidase, and essential protein in the respiratory electron transport chain. It also serves in plastocyanin in photosynthesis, which can substitute for the iron protein cytochrome in some algal species. Cobalt is required by all algae capable of synthesizing vitamin B12 since it is one of its constituents (Krauss 1955). Molybdenum and nickel, like iron, play important roles in nitrogen assimilation. Molybdenum occurs with iron in the enzymes nitrate reductase and nitrogenase. Nickel is present in the enzyme urease and thus is required for phytoplankton grown on urea as a nitrogen source. Selenium is also essential element which occurs in glutathione peroxidase, an antioxidant enzyme that degrades hydrogen peroxide and organic peroxides.

2.4.5 Light intensity

Light is the source of energy for the photosynthesis of microalgae and in this regard intensity, spectral quality, and photoperiod are all important parameter to be considered. Light intensity plays an important role and the requirement varies with the culture depth and the density of the algal culture. The light intensity must be increased to penetrate through the culture at higher depths and cell concentrations. However, the higher light intensity may lead to a higher rate of photorespiration and photoinhibition (Lebeau, Gaudin et al. 2002). Most commonly employed light intensities range from 100 to 200 $\mu E\ sec^{-1}m^{-2}$, which corresponds to about 5-10% of daylight ($2000\mu E\ sec^{-1}m^{-2}$). Many microalgae species do not grow well

under constant illumination, and hence a light/ dark (LD) cycle is used (maximum 16:8 LD, usually 14:10 0r 12:12) (Redalje and Laws 1983).

2.4.6 Temperature

Most commonly cultured species of microalgae tolerate temperatures between 16 and 27 °C, although this may vary. 18-20 °C is most often employed. Temperatures lower than 16°C and higher than 35 °C will slow down growth; even will inhabit a number of species.

2.4.7 Agitation

Agitation is necessary to prevent sedimentation of the algae, to ensure that all cells of the population are equally exposed to the light and nutrient, and to improve gas exchange between the culture medium and the air.

2.5 Applications

2.5.1 Food

Microalgae have been consumed by human being for centuries. For instance, some *Nostoc* species are regionally being used as food which has been consumed by Chinese dates back 2000 years (Spolaore, Joannis-Cassan et al. 2006). As another example, *Spirulina* (*Arthrospira*) has a history of human consumption, primarily in Mexico and Africa. To date, more than 150 species of algae have become commercially important food sources, and over $2 billion of seaweed is consumed each year by humans (Pulz and Gross 2004). Algae are considered nutritious because of their high protein content and high concentrations of minerals, trace elements, and vitamins, even the high iodine content of many edible algae.

Currently, most microalgal products launched to serve the health food market are supplied as tablets capsules, pastilles, liquids and powders (Spolaore, Joannis-Cassan et al. 2006). *Chlorella* and *Spirulina* dominate the microalgal health foods market. During the past decades, microalgal biomass was predominately utilized in the health food market, accounts with more than 75% of the annual microalgal biomass production and it is believed that health foods will continue to be a stable market in the future. The human consumption of microalgal biomass is restricted by food safety regulations.

2.5.2 Medicinal and pharmaceutical uses

Numerous algal polysaccharides have demonstrated to be with biological and pharmacological activity and have great potentials as therapeutics.

2.5.2.1 Anticoagulant activities

Some sulfated polysaccharides (containing sulfate ester groups) were compared with that of heparin by assays of activated partial thromboplastin time (APTT), prothrombin time (PT) and thrombin time (TT) are exploited as in vivo and in vitro antithrombotic and anticoagulant agents. They are usually mediated by blood coagulation inhibitors such as heparin cofactor II (HC II) or antithrombin III (AT III) (Mao, Fang et al. 2008) (Matou, Helley et al. 2002). Such anticoagulant compound could be extracted from green algae (e.g., *Ulva rigida, Monostroma nitidum, Codium fragile, Ulva conglobata, Codium cylindricum)* (Godard, Décordé et al. 2009) (Mao, Fang et al. 2008; Mao, Li et al. 2009) (Athukorala, Lee et al. 2007) (Mao, Zang et al. 2006) (Matsubara, Matsuura et al. 2001), red algae (e.g., *Pachymeniopsis elliptica, Tichocarpus crinitus, and Botryocladia occidentalis*) (Ekanayake, Nikapitiya et al. 2008) (Barabanova, Shashkov et al. 2008) (Farias, Valente et al. 2000) and

brown algae (e.g., *Sargassum horneri*) (Athukorala, Lee et al. 2007).

Some sulphated polysaccharides exhibited highest activity, which is comparable to or better than heparin. The anticoagulant activity usually increases with the amount of sulphate branch. The anticoagulant activity also is related to high molecular weight or a complex form with carbohydrate and protein (proteoglycan) (Athukorala, Lee et al. 2007). It is reported that sulphated polysaccharides have several advantages over traditional anticoagulant agents. They show concentration dependent inhibition of thrombin generation from platelets; exhibit concentration dependent inhibition of thrombin induced platelet aggregation; lack the hypotensive effect found in thrombin; reduce the sticking of polymorphonuclear leukocytes to rabbit aorta; and show a dose dependent inhibition of thrombin induced thrombosis. (Trento, Cattaneo et al. 2001). These features make them promising candidates in pharmaceutical and biomedical applications.

2.5.2.2 Anti-HIV activity

A number of algal extracts were found to be remarkably active in protecting human lymphoblastoid T-cell from the cytopathic effects of HIV (human immunodeficiency virus) infection, which is implicated as a causative agent of AIDS (acquired immune deficiency disease syndrome) (Gustafson KR 1989). It was also demonstrated that some compounds extracted from algae have in vitro or in vivo antiviral activity or inhibition of reverse transcriptase (RT) activity (Wang, Bligh et al. 2007) (Queiroz, Medeiros et al. 2008). Compounds isolated from algae that have been tested against HIV include steroids, sulfoglycolipids, and sulfated polysaccharides. Sulfated polysaccharides have been demonstrated to be able to block HIV replication in cell culture at concentrations as low as

0.1 to 0.01 μg/ml without toxicity to the host cells at concentrations up to 2.5 μg/ml (Witvrouw and De Clercq 1997).

Compounds present in algae can affect HIV infectivity by various mechanisms. Numerous studies have examined the mode of action of sulfated polyanions (SP). Sulfated polysaccharides and other SP bind to lymphocyte CD4 and inhibit binding of monoclonal antibodies to the first two domains of CD4 which is an important target for chemotherapeutic agents against AIDS (Schols 1989). The presence of the sulfate group is necessary for anti-HIV activity, and it capacity increases with the degree of sulfation (Schaeffer and Krylov 2000).

Several papers have reported on synergism between azidothymidine (abbreviate AZT, inhibits replication of some retroviruses) and sulfated polysaccharides, e.g., fucoidan (Sugawara, Itoh et al. 1989), pentosan polysulfate (Anand, Nayyar et al. 1990), cyclodextrin polysulfate (Anand, Nayyar et al. 1990), and dextran sulfate (Ueno and Kuno 1987). The use of combinations of low-toxicity, synergistically acting antiviral agents that target different sites in the HIV replicative cycle could prevent the emergence of drug-resistant HIV mutants (Anand, Nayyar et al. 1990; Witvrouw and De Clercq 1997).

2.5.3 Industrial applications

2.5.3.1 Thickening agents

The common industrial use of algal polysaccharides is related to the capability of these biopolymers to alter the rheological behaviour of water, acting as thickening agents. In this connection, the rheological characteristics of algal polysaccharides have been investigated to

use in the food and cosmetic areas. The polysaccharide from Chlorella sp. was found to be a potential thickening agent. Its thickening and secondary emulsifying effects were found to be compatible with most of the food-grade salts when these salts are used in the recommended range (1 to 30 g/L) for foods (Yalcin, Hicsasmaz et al. 1994). Sutherland studied their capability to bind water molecules which can be exploited for the cosmetic industry (Sutherland 1994).

Among the algal polysacchsrides studied for their rheological behaviour, the polymers produced by *Anabaena* ATCC 33047 (Moreno, Vargas et al. 2000), *Cyanospira capsulate* (De Philippis, Sili et al. 1996), and *Cyanothece* (De Philippis, Sili et al. 2001) strains seem to be the most interesting, showing values of viscosity comparable to, or even better than, those of aqueous solutions of xanthan gum at comparable concentrations. In addition, the polysaccharide produced by *Nostoc* PCC 7423 shows appreciable stability of the viscosity of its aqueous solutions over a wide range of pH, temperatures and NaCl concentrations (De Philippis, Ena et al. 2000).

2.5.3.2 Enhanced oil recovery

The marked differences in monosaccharidic composition so far observed in cyanobacterial polysaccharides (Vincenzini M 1990) suggest the possibility of finding new strains that produce polymers possessing chemical or theological properties as to make them suitable for new applications. Cells of red microalgae encapsulated within sulphated polysaccharides, are thought to have a wide range of potential industrial applications (Geresh and Arad Malis 1991). The biopolymer of *P. aerugineum* has been applied as thickening agent for aqueous driving fluids to enhance recovery of oil trapped in the porous space of reservoir rocks

(Ramus and Kenney 1989). Thickening agents can be used to boost oil production, which is known as polymer flooding or chemical flooding. Chemical flooding is of increasing interest and importance due to high oil prices and the need to increase oil production, particularly for application in mature water flooding.

Viscosity is the property of a liquid that resists flowing, coursed by internal friction. It is measured in centipoises. Crude oil, for instance, has a viscosity of around 90 centipoises, while the viscosity of water in reservoir conditions is only about half a centipoise. As a result, water flows in effective about 200 times more easily than oil through the reservoir rock. This means, when water flooding is used as a recovery technique, the water will tend to overtake the oil and flow out past it, rather than pushing the oil out. Adding polysaccharide to the water is designed to make it thicker and more viscous, bringing the ratio of mobility much closer to the crude oil and so making for a more effective sweep. Another advantage of polymer flooding is that it can be used in thin heavy oil formations with low viscosity where steam assisted gravity drainage (SAGD) and vapour extraction (VAPEX) are not suitable.

In recent years, significant progress has been made in developing enhanced oil recovery (EOR) by chemical flooding. At lab scale, many researchers use micromodels to simulate the reservoir (Nasr, Soudi et al. 2007; Soudmand-asli, Ayatollahi et al. 2007). The micromodels were made by etching the mirror image pattern of pore networks onto a glass plate using hydrofluoric acid. The etched plat has an inlet and an outlet port drilled at either end. A second glass plate was then placed over the etched side and fused together in a programmable furnace. To manufacture uniform-depth glass micromodels, the quality of the etching processes was controlled by adjusting the sequence of the processing operations, time of etching and the concentration of the acid. The pore volume was determined by

weighting the etched plates before and after the etching process. The pore void area was analyzed using an image analyzer software. Image analysis software (or matlab) was developed to measure the total area occupied by dyed oil. The relatively uniform depth of the models allowed us to determine oil saturation by measuring the area occupied by the dyed oil.

In large scale, ARC (Alberta Research Council) was among the first to research polymer flooding for heavy oil reservoirs. Over the last 15 years, they have taken the technology from an idea to a viable technology that is increasing the recovery of heavy oil by 10 per cent. Three field pilots are underway in Alberta, and more companies are looking to use polymer flooding to increase recovery.

2.5.3.3 Stabilization of emulsions or as bioflocculants

Another promising application of algal polysaccharides, related with the presence of both hydrophilic and hydrophobic groups in the macromolecles, is the use of these polymers for the stabilization of emulsions or as bioflocculants. In this connection, the released polysaccharides (RPSs) produced by some algal strains (e.g.: Aphanocapsa, Cy*anothece, Nostoc, Phormidiumnd Synechocystis* strains) seem to be quite promising, wing to large amounts of acetyl groups, deoxysugars and/or peptidic moieties.

2.5.3.4 Heavy metal biosorption

For several years it has been repeatedly suggested by many researchers that heavy metals from polluted aqueous systems may be removed by phytoplankton. Algae are of special interest as novel biosorbents, which have proven to be the most effective and promising

substrates among many low cost sorbents. Algae displays high sorption capacity metal uptake and selectivity for removal of toxic heavy metal ions, such as Cd^{2+}, Cu^{2+}, Zn^{2+}, Pb^{2+}, Cr^{3+}, and Hg^{2+}, according to the review by (Davis, Volesky et al. 2003). Brinza (L. Brinza 2007) reviewed some marine micro and macro algal species as biosorbents for heavy metals. These algae were reported to be able to adsorb one or more heavy metals, including K, Mg, Ca, Fe, Sr, Co, Cu, Mn, Ni, V, Zn, As, Cd, Mo, Pb, Se, Al, with good metal uptake capacity. It was concluded that this method, including the separation of the metal saturated algae from the medium, is an economic method for removing heavy metals from waste waters, resulting in high quality reusable effluent water and valuable algal biomass, which could be used for different purposes (production of biogas, fertilizer, fodder, etc)

The efficiency of using algae for heavy metal removal are determined principally by the following five parameters: 1) growth rate of the algae; 2) metal concentration factor of the algae; 3) concentration of heavy metals in the medium; 4) desired percentage of metal removal from the medium; 5) metal recovery in relation to capital and operating costs.

2.5.3.5 Other applications

Funori is a polysaccharide from the red alga *Gloiopeltis furcata* and has become well-known to conservators as an exceptionally suitable product to consolidate matte paint (Geiger and Michel 2002). It recently reported a naturally derived polysaccharide obtained from red microalgae could be an alternative biolubricant that may surpass the superior characteristic yet deteriorating performance of hyaluronic acid. (Canter 2007).

2.6 Conclusion

The characteristics of some of the algal polysaccharide described above indicate that algae can be considered an exciting and promising source of polysaccharides of tremendous potentials in many fields including biomedical, food, and industrial applications. Many features make polysaccharide materials a natural fit for sustainable development, to deal with polysaccharides as fascinating polymers with a bright future.

However, further investigations are imperative. The relations among structure and properties of most algal polysaccharides still have to be elucidated, in order to determine the proper application of polysaccharides. In this connection, it has to be stressed that the development of any algal polysaccharide into a biotechnological product requires a multidisciplinary approach which combine many different expertises (i.e. microbiology, biochemistry, chemistry, genetics, engineering etc.), may fill the existing gap between the current knowledge and the exploitation of algae for the production of useful polymers.

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CHAPTER 3

Production of Microalgal Biopolymer from Lactose

Abstract

It was discovered for the first time that a green alga strain could produce large quantities of biopolymer utilizing lactose as the sole carbon source under mixotrophic cultivation conditions. Potentially, this strain can use cheese whey as carbon source to reduce the cost in the future industry application. Using Plackett-Burnman experiment design and statistical analysis, we identified the main effect factors affecting biopolymer production, which were $NaNO_3$, lactose, and temperature, among the seven parameters tested.

Keywords: Green algae, biopolymer, mixotrophic cultivation, Plackett-Burnman experiment design.

3.1 Introduction

Algae play an important role on earth as primary producers of the food chain. They are original source for many valuable products. Algae cultivations were studied and developed over a century and the earliest algal culture techniques were described by Moore (Moore 1903). Algal culture techniques are also described in detail in several earlier books and articles (Kufferath 1928; Bold 1950; Pringsheim 1951). During the past two decades, microalgae have emerged as one of the most promising feedstock for biofuels production which is considered renewable and environmentally friendly alternative energy sources. However, the high costs of algal cultivation processes demands the implementation of a high-value co-product strategy, which inspired the search and development of a large array of novel bioproducts produced from algae. Polysaccharides are one of the novel bioproducts that can be produced by microalgae to enhance the overall cost effectiveness of microalgal cultivation since they have tremendous potentials for a large array of different applications.

Polysaccharides have a unique combination of functional properties and biodegradable features. They are generally recognized as safe for human consumption and have been used for the production of foods such as baking icings, jelly candies, salad dressing, and food stabilizers (Renn 1997; Spolaore, Joannis-Cassan et al. 2006). Agarose, which is extracted from red algae, is an industrially important high value material and is extensively used in biotechnology and molecular biology applications (Renn 1984). Agarose is good material for electrophoresis in special applications such as DNA and protein analysis and separation. On a related note, agar is a popular solidification agent used in the preparation of solid media for cell culture. In pharmaceuticals industry, some algal polysaccharides have been demonstrated

to have superb anticoagulant activity (Mao, Fang et al. 2008; Mao, Li et al. 2009), anti-HIV activities (Queiroz, Medeiros et al. 2008; Wang, Bligh et al. 2007) and anti-HSV activity (Zhu, Chiu et al. 2006; Zhu, Ooi et al. 2003). In the oil industry, polysaccharides alter the rheological behaviour of water, acting as thickening agents and to stabilise the flow properties of their aqueous solutions under drastic changes of temperature, ionic strength and pH (Sutherland 1998; De Vuyst and Degeest 1999). Such properties can be used to boost oil production, which is known as polymer flooding or chemical flooding. Another promising application of algal polysaccharides, related with the presence of both hydrophilic and hydrophobic groups in the macromolecles, is the use of these polymers for the stabilization of emulsions or as bioflocculants.

In recent years, significant progress has been made in discovering and developing new algal polymers that possess novel and highly functional properties. A few red and brown algae have been known as a potential source for polysaccharide (Assreuy, Gomes et al. 2008; Arad, Rapoport et al. 2006; Canter 2007; Wang, Bligh et al. 2007; Carvalho G. de Azevedo, Bezerra et al. 2009; Gomez, Pérez Lambrecht et al. 2009; Zhu, Chiu et al. 2006; Queiroz, Medeiros et al. 2008). However, studies in this area have so far been concentrated in polysaccharide production of intracellular and exocellular polysaccharides via photoautotrophic cultivation of microalgae, which is characterized by low productivity and low polysaccharide concentration, ranging from 174 milligrams per liter to 557 milligrams per liter in previous literature research (Moore and Tischer 1964).

We discovered a green alga strain that could produce large quantity of high-viscosity polysaccharides using lactose as the primary carbon source under illuminated conditions. Preliminary optimization studies have resulted in the production of polysaccharides ranging

from 3.6 to 5 gram per liter, remarkably higher than any microalgal polysaccharide production reported in the literature.

3.2 Materials and Methods

3.2.1 Microalgal strain

A unicellular green algae purchased from the algae culture collection at the University of Texas in Austin was found to be able to produce large quantities of algal polymer from lactose under mixotrophic conditions. This microalgal strain was used throughout this study.

3.2.2 Media

Medium for photoautotrophic cultivation: Modified soil extract (SE) medium was used as basic medium for photoautotrophic microalga cultivation. It includes the following components (per liter): 0.15 g $K_2HPO_4 \cdot 3H2O$, 0.15 g $MgSO_4 \cdot 7H_2O$, 0.05 g $CaCl_2 \cdot 2H_2O$, 0.35 g KH_2PO_4, 0.05 g NaCl. Chu Micronutrient Solution (0.5 ml per litre of medium) and P-IV metal solution (6 ml per litre of medium) were used to provide microelements for cell growth. The soil extract solution in the original SE medium was eliminated due to it unpredictable effects on microalga growth. Medium pH was adjusted to 6.5 before autoclave. Chu micronutrient solution including components as follow (per liter): 0.02 g $CuSO_4 \cdot 5H_2O$, 0.044 g $ZnSO_4 \cdot 7H_2O$, 0.02 g $CoCl_2 \cdot 6H_2O$, 0.012 g $MnCl_2 \cdot 4H_2O$, 0.012 g $Na_2MoO_4 \cdot 2H_2O$, 0.62 g H_3BO_3, 0.05 g $Na_2EDTA \cdot 2H_2O$. P-IV metal solution including components as follow (per liter): 0.75 g $Na_2EDTA \cdot 2H_2O$, 0.097 g $FeCl_3 \cdot 6H_2O$, 0.041 g $MnCl_2 \cdot 4H_2O$, 0.005 g $ZnCl_2$, 0.002 g $CoCl_2 \cdot 6H_2O$, 0.004 g $Na_2MoO_4 \cdot 2H_2O$.

Media for heterotrophic and mixotrophic cultivation of the alga strain: The media used for

heterotrophic and mixotrophic cultivation were the same modified soil exact medium except that glucose or lactose was added as organic carbon/energy source. The concentration of sugar is indicated in the text.

3.2.3 Photoautotrophic cultivation conditions

The microalga strain was grown in a light box with continuous illumination at 8000 lux (approximate 109 μE $sec^{-1}m^{-2}$). An enriched air stream containing 5% CO_2 was first saturated with water by passing through a water bottle and then filtered using a 0.45 μ m microfiltration cartridge before being bubbled into the cultivation bottle from the bottom at a flow rate of 0.5 vvm. Temperature inside the box was controlled around 28±1℃ by forced circulation of ambient air using two fans located on two sides of the box. Agitation was achieved by combined bubbling and magnetic stirrer. A detailed description of the cultivation system can be found in our previous work (Li, Horsman et al. 2008).

3.2.4 Heterotrophic cultivation conditions

Under the heterotrophic cultivation conditions, glucose or lactose was used as carbon/energy source. The microalga strain was grown in 500 ml flasks on a shaker without light at 28 ℃ and 200 rpm.

3.2.5 Mixotrophic cultivation conditions

For mixotrophic cultivation, glucose or lactose was used as carbon/energy source in the medium. The microalgae strain was grown in 500 ml flask containing 100 ml medium at 8000 lux (approximate 109 μE $sec^{-1}m^{-2}$), 28±1℃, and 200 rpm. Cultivations were carried out using a lightened shaker with refrigeration (DHZ-032LR, Chemostar, Shanghai, China).

3.2.6 Polymer separation and purification

At the end of cultivation, gel-like broth was heated to approximately 80℃ and centrifuged at 12000 rpm for 10 min to remove algal cells under reduced broth viscosity. Cell-free broth was then concentrated under reduced pressure by rotational evaporator, dialyzed in cellulose membrane tubing (molecular weight cut off 8000D) against de-ionized distilled water, which was changed twice a day for three successive days. The retained fraction was recovered, concentrated under reduced pressure, and precipitated by addition of 4 volumes of 99% (v/v) ethanol. Precipitate was then washed twice using 99% ethanol, followed by drying at 40℃ to obtain a crude polysaccharide (Mao, Fang et al. 2008).

3.2.7 Plackett-Burman statistical experiment design for biopolymer production

Plackett-Burman design, a 2-level fractional factorial design, was used to identify the most significant parameters affecting biopolymer production. Seven factors at two levels were investigated using 12 experiments. These factors and their levels are shown in Table 3.2 and the design and results of the 12 experiments are shown in Table 3.3. Experimental data were analysed using Minilab 15 statistical software (Minilab Software, Canada).

3.2.8 Analytical methods

Biomass concentration was measured by monitoring the optical density at 600 nm (OD_{600}) using a spectrophotometer. Samples were diluted by appropriate ratios to ensure that the measured OD_{600} values were in the range of 0.2–0.6. Biomass concentration was then calculated by multiplying OD_{600} values with 0.4, a predetermined conversion factor converting the OD_{600} value to dry cell weight (DCW). The conversion factor was established

by plotting OD_{600} versus DCW of a series of samples of different biomass concentrations. DCW of a sample was determined gravimetrically after drying the algal cells collected from samples with centrifugation (3,000×g, 10 min) and wash.

Molecular weight was determined by high performance gel permeation chromatography (HPGPC) using an HPLC system (Agilent Technologies, California, USA) loaded with a shodex sugar KS-805 column (8.0mmID*300mm) at 50°C, using deionized distilled water as eluent. Molecular weight was also measured using a Zetasizer (Zetasizer Nano S, Mulvin Instruments, US). The molecular weight is calculated by the Rayleigh equation:

$$\frac{KC}{R_\theta} = \left(\frac{1}{M} + 2A_2C\right) \qquad Eq\ 3.1$$

Where K is an optical constant, R_θ is the Rayleigh ratio of the scattered to incident light intensity, M is the weight-average molecular weight, A_2 is the second virial coefficient and C is the polymer concentration. Therefore, a plot of KC/R_θ versus C is expected to be linear with an intercept equivalent to 1/M and a slope equal to the second virial coefficient A_2. Algal polymer was prepared in the water, and a dñ/dC (differential refractive index increment) value of 0.140ml/g was used for the measurements.

The pH of samples was measured using a Fisher Scientific Accumet pH Meter (Acumetab15). Light intensity was measured using a Dual-Range Light Meter (Fisher brand) was used to read light intensity. Total sugar content was measured by phenol-sulfuric acid assay using lactose as the standard (Dubois, Gilles et al. 1956; Izydorczyk, Cui et al. 2005). Viscosity of algal broth was measured using a rotational dial reading viscometer (Brookfield LV Model).

3.3 Results

3.3.1 Effects of microelements and macroelements on microalgal cell growth under autotrophic conditions

Effect of microelements (i.e., Zn^{2+}, Mn^{4+}, Co^{2+}, Cu^{2+}, Mn^{6+}, BO_3^{3-}) and effect of macroelements (i.e., $NaNO_3$, K_2HPO_4, KH_2PO_4, $CaCl_2{\cdot}2H2O$, $MgSO_4{\cdot}7H_2O$, NaCl, $FeCl_3{\cdot}6H_2O$) on the cell growth of the microalgal strain were studied separately. As shown in Figure 3.1, there were no remarkable differences among the growth profiles obtained with media containing different folds of microelements. Thus, we can conclude that the concentration of microelements is not important factors influencing the growth microalgae under the tested conditions. Figure 3.2 shows the effects of the concentration of macroelements on the cell growth of the microalgal strain. It is obvious that the maximum growth rate was observed when 5 folds of macroelements as that used in the original SE medium presented under the tested conditions.

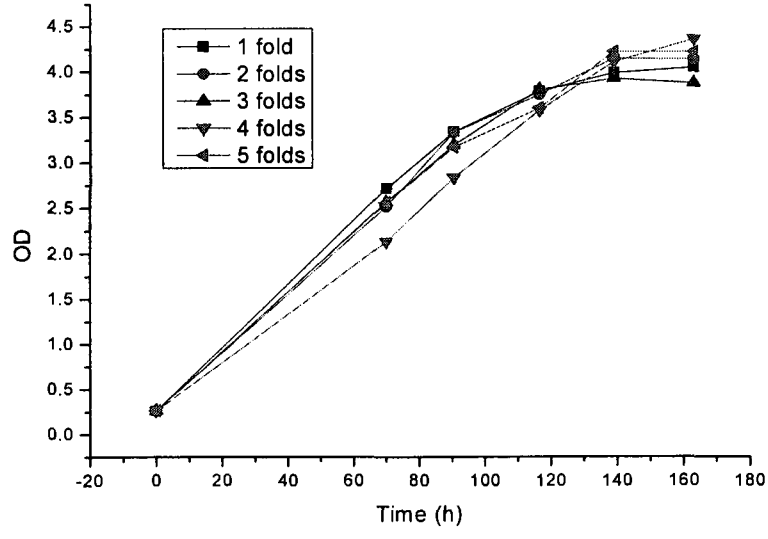

Figure 3.1. Microalgae cultivated with various concentrations of microelements.

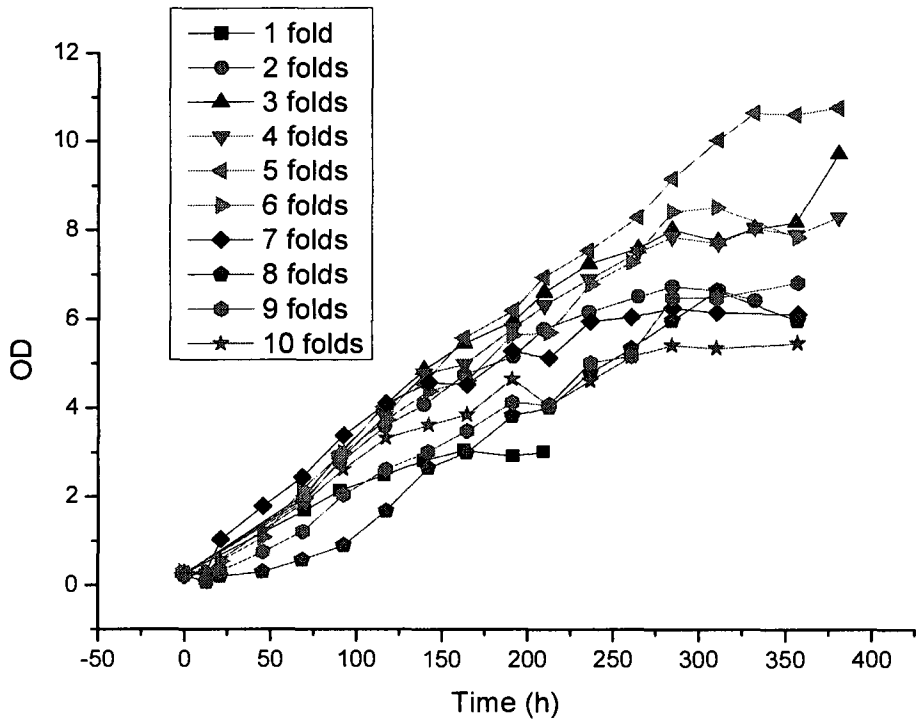

Figure 3.2. The green alga cultivated with various concentrations of macroelements.

3.3.2 Mixotrophic cultivation with lactose as carbon source

This green alga has the capability of utilizing lactose for mixotrophic cultivation. Lactose concentration was selected in the range from 5g/L to 20g/L as carbon source. Figure 3, presents the high lactose concentration is benefit for algae growth.

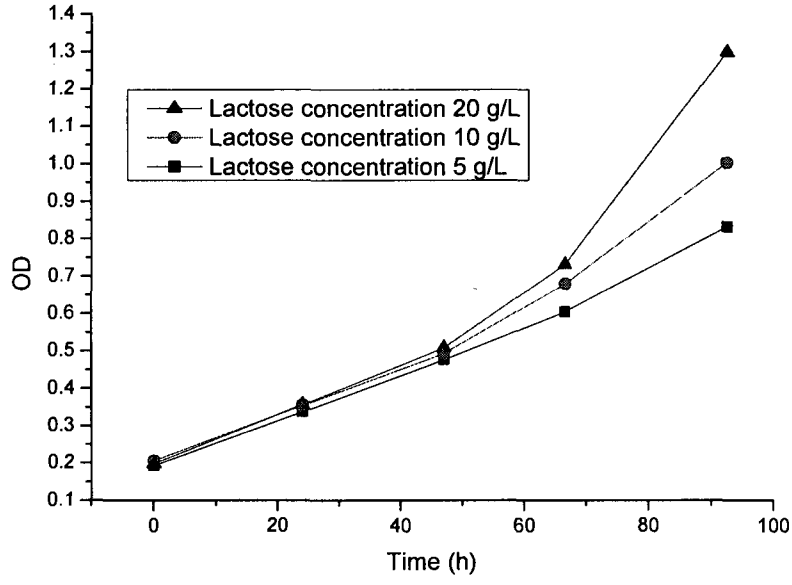

Figure 3.3. Microalgae cultivated with lactose at various concentrations

3.3.3 Biopolymer production by the green alga strain under different conditions

Table 3.1 shows the cell growth and biopolymer production of the algal strain under photoautotrophic, heterotrophic and mixotrophic cultivation conditions using CO_2, glucose, or lactose as carbon source. As shown in the table, cell growth was the best under photoautotrophic cultivation condition. However, no biopolymer was produced under this condition. Glucose is better than lactose when it was used as carbon source for both heterotrophic and mixotrophic cultivation. When the same carbon source (i.e., glucose or lactose) was used, biopolymer production was significantly higher under the mixotrophic cultivation condition than that of under heterotrophic cultivation condition. In fact, the only scenario in which large quantities of biopolymers were produced was mixotrophic cultivation using lactose as carbon source.

Under photoautotrophic cultivation conditions, there is no detectable algal polymer production. Under heterotrophic cultivation, glucose and lactose were used as carbon source in the medium for algae growth. At the end of cultivation, we found algae grow faster and more in the medium with glucose than the lactose medium.

In the mixotrophic cultivation studies, it was observed that the green alga strain has the capability of utilizing lactose for mixotrophic growth and producing large quantities of algal polymer. As a result, the broth become very viscous at the end of cultivation and a highest polymer concentration of 5 g/L was achievable with 10 g/L lactose in the medium.

Table 3.1 Biopolymer production by the green alga strain under different conditions.

Experimental No*		1	2	3	4	5
Carbon Source	Glucose	-	+	-	-	+
	Lactose	-	-	+	+	-
	CO_2	+	-	-	-	-
Illumination	Light	+	-	-	+	+
	Dark	-	+	+	-	-
Cultivation Mode		P	H	H	M	M
Cell Growth		++++	++	+	++	+++
Polymer Production		-	+	++	++++	+

* P represents photoautotrophic cultivation, H represents heterotrophic cultivation and M represents mixotrophic cutivation

3.3.4 Plackett Burman experiment results

To identify the parameters that affect the polymer production, we tested seven different factors using Plackett-Burman experiment design. Seven parameters were chosen from medium and cultivation conditions. The factors and their levels are listed in Table 3.2.

Table 3.2 Experimental range and levels of independent variables in the Plackett-Burman experiment.

Variable	Level	
	-1	1
$NaNO_3$ (*X1*)	6 mM	15 mM
$MgSO_4$ (*X2*)	0.6 mM	1.5 mM
K_2HPO_4:KH_2PO_4 (*X3*)	0.4:1.3	2:6.5
Microelements (*X4*)	2 folds	5 folds
Lactose (*X5*)	5 g/L	20 g/L
Inoculum size (*X6*)	10%	20%
Temperature (*X7*)	28°C	32°C

In this work, viscosity was chosen as response value, statistical analysis was carried out using Minilab software. Figure 3.4 presents the viscosity profile of algae cultivation broth measured under room temperature, ranging from 24.0 to 25.5 °C. The results of Plackett Burman experiments design were shown on Table 3.3, the maximum viscosity, which was measured at room temperature with a spindle rotation speed of 30 rpm, was 2252.0 mPa·s (Experiment No. 10).

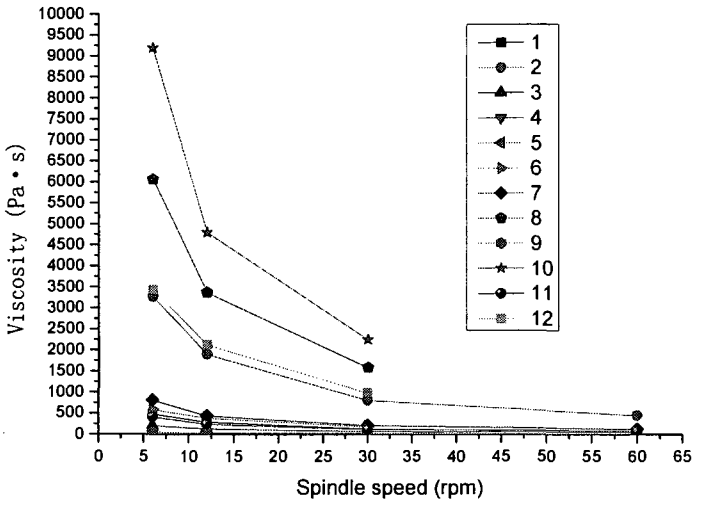

Figure 3.4. Viscosity profile of algae cultivation broth

Table 3.3 Result of Plackett Burman experiments design

	Pattern	*X*1	*X*2	*X*3	*X*4	*X*5	*X*6	*X*7	Biomass (g/l)	Viscosity (mPa·s)
1	--+++-+	-1	-1	1	1	1	-1	1	0.87	138.8
2	-+++-++	-1	1	1	1	-1	1	1	1.27	813.0
3	--++-+--	-1	1	1	-1	1	-1	-1	1.50	76.2
4	---+++-	-1	-1	-1	1	1	1	-1	0.98	5.0
5	-------	-1	-1	-1	-1	-1	-1	-1	0.86	3.2
6	++-+---	1	1	-1	1	-1	-1	-1	1.94	191.0
7	+--++-+-	1	-1	1	1	-1	1	-1	0.92	281.5
8	+---+++	1	-1	-1	-1	1	1	1	0.95	1590.0
9	+-+---+	1	-1	1	-1	-1	-1	1	0.85	27.3
10	++-++-+	1	1	-1	1	1	-1	1	2.66	2252.0
11	-+---++	-1	1	-1	-1	-1	1	1	1.48	125.5
12	+++-++-	1	1	1	-1	1	1	-1	2.24	987.0

*The viscosity values were selected at spindle rotation speed 30 rpm

The main effect of each variable was determined according to the following equation (Plackett and Burman 1943):

$$E_{xi} = \frac{(\sum M_{1+} - \sum M_{i-})}{N} \qquad Eq\ 3.2$$

Where Exi is the variable main effect, Mi+ and Mi- are the response percentage in trials, in which the independent variable (xi) was present in high and low concentrations, respectively, and N is the half number of trials.

Table 3.4 Main effects of the seven factors affecting biopolymer production

	*X*1	*X*2	*X*3	*X*4	*X*5	*X*6	*X*7
M1+	5266	4444.7	2260.8	3618.3	5049	3739	4946.6
M1-	1162	1982.8	4166.7	2809.2	1378.5	2688.5	1480.9
Exi	684	410.31	-317.65	134.85	611.75	175.08	577.62

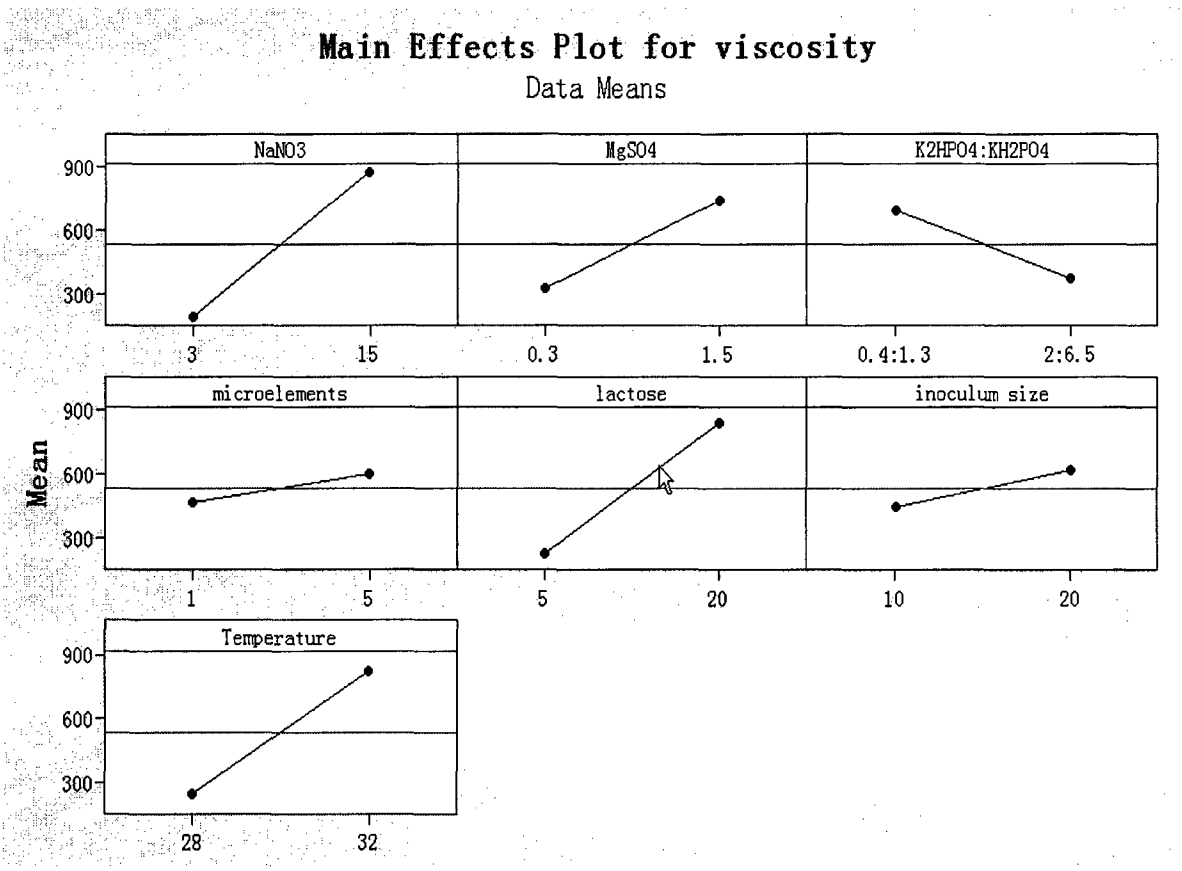

Figure 3.5. Main effects plot of the seven factors affecting biopolymer production.

As can be seen from Table 3.4 and Figure 3.5, $NaNO_3$(*X1*) concentration, lactose concentration (*X5*), and cultivation temperature (*X7*) were the three factors that showed remarkable effects on the viscosity of the algal broth at the end of cultivation.

3.3.5 Molecular weight of algal polymer

Figure 3.6 shows Debye Plot for a series of concentrations range from 0.25 to 1 g/l of algal polymer samples prepared in water and measured on a Zetasizer Nano S.

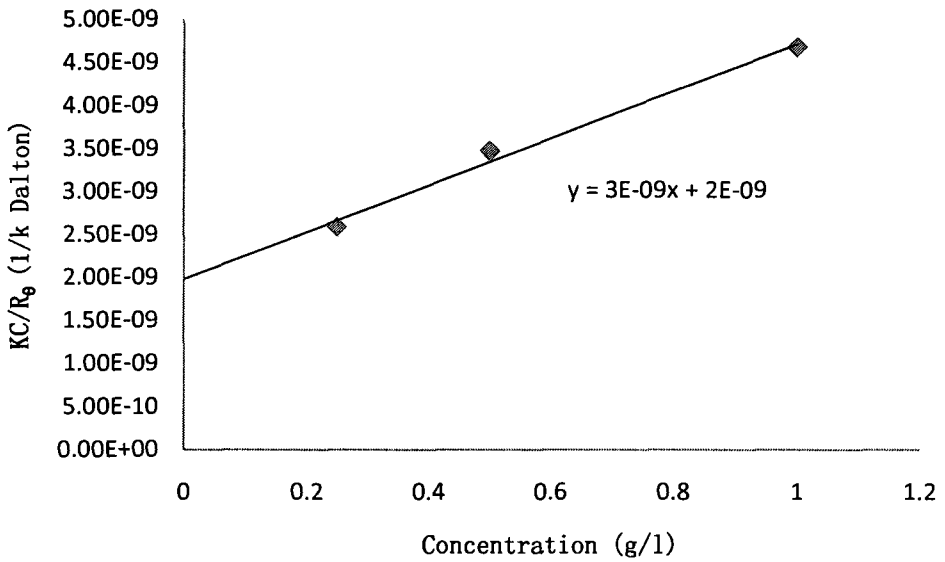

Figure 3.6. Debye plot for algal polymer in water.

Zetasizer Nano software measures the intensities of the scattered light of the algal polymer and automatically calculates second virial coefficient (A_2) from the data using a Debye plot. The calculated molecular weight is based on. The intercept of Debye plot is equivalent to 1/M of Rayleigh equation. From above figure, we get the value of intercept is 2×10^{-9} (1/k Dalton). Therefore the calculated molecular weight of algal polymer is 505 kDalton. The values are shown on the Table 3.5.

Table 3.5. Parameter values of Rayleigh equation

C (g/l)	KC/R_θ (1/k Dalton)	Intensities (kcps)	Second virial coefficient A_2 (ml mol/g^2)
0.25	2.58E-09	206.9	
0.5	3.47E-09	299.7	0.00136
1	4.67E-09	437.7	

The second virial coefficient A_2 was calculated to be a positive number, 0.00136 ml mol/g^2,

indicating that the aqueous polymer solution was stable under the condition.

3.4 Discussions

It was discovered that a green alga strain could produce large quantities of exopolysaccharides from lactose under mixotrophic cultivation conditions. It was observed that neither heterotrophic cultivation using lactose as carbon source nor mixotrophic cultivation using glucose as carbon source led to the production of significant quantities of biopolymers although the algal strain grew well in both conditions. These results suggest that both light and lactose were required for the production of the biopolymer of this algal strain. A possible explanation is that lactose or galactose might be the monomer for the polysaccharides and one or more light-dependant enzyme is involved in the biosynthesis of the polymer. This hypothesis needs to be verified by studies such as the analysis of the single sugar composition of the polysaccharide and elucidation of the biosynthetic pathways involved in the polymer production.

Algal polymer synthesis requires sugar nucleotides as precursors. The production of these carbohydrates is carbon intensive and competes with the cell's metabolism for available carbon sources.

Lactose is a disaccharide that consists of galactose and glucose, which presents the metabolic pathway for production of two sugar nucleotides, UDP-glucose and UDP-galactose. Based on the phenol-sulphuric acid assay, we conclude the configuration of the 3,6-anhydrogalactose present in our algal polymer. The is a major constituent of the agarose

fraction of agar, of kappa carrageenan, and of some other polysaccharides from algae (Yaphe 1963). As sugar 3,6-anhydrogalactose can be generated quantitatively from galactose (Navarro and Stortz 2003), lactose is considered as the best carbon source for algal polymer production among the tested sugars.

Light is another factor for algal polymer production. Light plays an important role for the photosynthesis of microalgae. Through photosynthesis reaction, light energy gathered by chlorophylls is stored in the form of adenosine triphosphate (ATP). Algal polymer synthesis is not only carbon-intensive; it is also an energy-intensive process. Energy is required for synthesis of the sugar nucleotide precursor. In the cell, either ATP or phosphoenolpyruvate depending upon the sugar transport system is used to activate by the sugar component. We also hypothesize there is a kind of enzyme for catalyzing algal polymer production need light to active it.

In this context, it not difficult to understand there are more algal polymer production under mixotrophic cultivation condition, and less algal polymer production under heterotrophic condition.

Based on the statistical analysis of Plackett-Brunman experiment results, $NaNO_3$, lactose, and temperature are the major factors affecting biopolymer production of the microalgal strain under the investigated conditions. Further optimization, purification and characterization of algal polymer are undergoing.

Lactose is the main component of the cheese whey, which is the waste liquor from cheese manufacturing that typically containing 50 g/l lactose and is discharged into the environment at large quantities worldwide. Potentially, this strain can use cheese whey, the waste liquor of

cheese manufacturing, as carbon source for cost effective production of valuable biopolymers for applications in a large diversity of different fields including the oil industry, the food industry, the cosmetic industry, the chemicals industry.

3.5 Acknowledgement

Financial supports from the Natural Science and Engineering Research Council (NSERC) of Canada and Canadian Foundation of Innovation (CFI) are gratefully acknowledged.

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CHAPTER 4

Rheological Studies on a Microalgal polymer

Abstract

Polysaccharides are natural polymers with a variety of important applications. Emphasis on rheological properties investigation was undertaken since it will ultimately dictate many significant commercial applications of biopolymers. For instance, chemical enhanced oil recovery by polymer flooding is a field-proven method that improves overall oil recovery. High oil prices and dropping reserves replacement has stimulated vast interests in these technologies, particularly for application in mature water flooding. Rheological tests were carried out for microalgal polymer produced by a green alga strain. In the temperature range of 15-75°C, simulating the temperature into the reservoirs was studied. Viscosity of polymer solution is sensitive to temperature, decreasing rapidly with the temperature in this range. In the brine solution, the viscosity of microalgal polymer significantly increases at the present of NaCl. It was concluded that the rheological properties of the microalgal biopolymer was comparable to that of xanthan gum, the most commonly used commercial biopolymer in polymer in enhanced oil recovery.

Keywords: Microalgal polymer; rheological properties; viscosity; temperature.

4.1 Introduction

It was demonstrated in the previous chapter that a green alga strain has the capacity to utilize lactose as carbon source for heterotrophic and mixotrophic growth and to produce large quantities (3.6-5 g/L) of high molecular weight biopolymer under mixotrophic cultivation conditions. Lactose is the major component of cheese whey, the waste liquid from cheese main factory after separation of curds during casein or cheese making it typically contains 4 to 5 % (w/v) lactose, 0.8 to 1% (w/v) proteins and trace amount of minerals and vitamins (González Siso 1996; Horton 1995). It is expected that this algal strain would be able to produce valuable biopolymers costeffectivly from cheese whey. From another perspective, whey has a very strong polluting capacity, with a biological oxygen demand (BOD) of 40,000 to 45,000 mg/L (Kemp and Quickenden 1989). Although several uses of whey have been reported (Jelen 1979; Teuber 1981; González Siso 1996; Smithers, Ballard et al. 1996; Gill, Duggal et al. 1999; Tripathy, Vijayalakshmi et al. 2003; Qureshi and Maddox 2005; Kapdan and Kargi 2006; Horton 1995; Marwaha and Kennedy 1988; Mawson 1994), there is evidence that the disposal of whey still present serious problems (Horton 1995) (Marwaha and Kennedy 1988; Mawson 1994). As a result, production of high value biopolymer using cheese whey promises a way to convert an environment harzard to high value products. Only a few microbial species are found to have this capacity (Fu and Tseng 1990; Dlamini and Peiris 1997; Ashraf, Soudi et al. 2008) (Silva, Fornari et al. 2009).

Biopolymers are of great interests to many industries including the food industry, the pharmaceutical industry, the scientific reagent industry, and the oil industry. The applications of biopolymers these industries are generally related to their rheological properties. For

instance, the ability of these biopolymers to alter the rheological behaviour of water, acting as thickening agents (Sutherland 1998; De Vuyst and Degeest 1999) are widely used to enhance oil recovery from oil fields, which is known as polymer flooding or chemical flooding.

Currently on average only about 30 - 35 per cent of the oil in fields is brought to the surface, leaving on average 65 - 70% of total oil behind (Vossoughi 2000). It is therefore not surprising for Dr. Willem Schulte, Shell's Chief Scientist Reservoir Engineering, to say that enhanced oil recovery (EOR) is one of the most powerful methods and that "in a little over twenty years time, EOR could account for up to 20 million barrels of oil production per day." (Schulte 2008).

During the past four decades, a large number of enhanced oil recovery (abbreviated as EOR below) methods have been developed and applied to mature and mostly depleted oil reservoirs to obtain the maximum amount of recoverable oil. EOR is also called improved oil recovery or tertiary recovery (as opposed to primary and secondary recovery). These methods improve the efficiency of oil production compared with primary (pressure depletion) and secondary (water flooding) oil recovery methods (Stevens and Donnell 1999). As summarized in Figure 4.1, these methods can be categorized into the following groups: 1) miscible displacement (hydrocarbon gas, CO2, and nitrogen); 2) thermal recovery (steam flooding, hot water drive, in situ combustion); 3) chemical flooding (polymer, surfactant, emulsion) (Mothe, Correia et al. 2006; Satter 2008); 4) microbial enhanced oil recovery (MEOR), which represents the use of microorganisms to extract the remaining oil from reservoirs (Moses 1987; Almeida, Moreira et al. 2004).

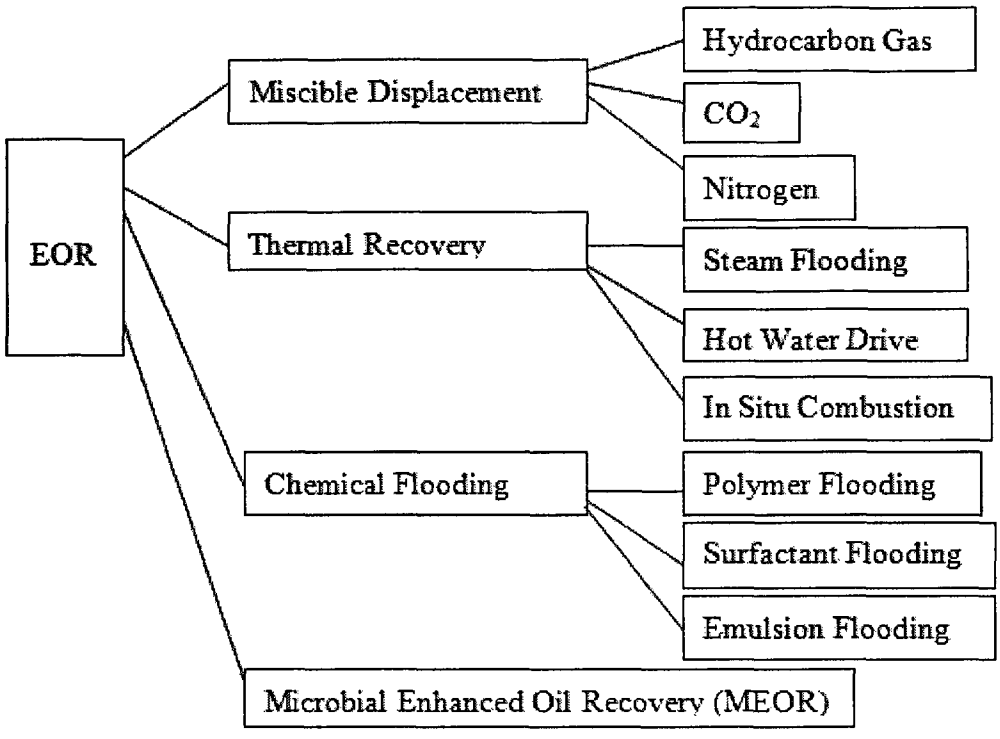

Figure 4.1. Classification of EOR methods

In essence, EOR is based on one or both of the following principles: 1) to reduce the in situ viscosity of oil to ease its flow (e.g., in situ combustion); and/or 2) to literally push oil out of the pores of rocks by lowering the mobility of flooding water by means of adding chemicals such as polymers and/or surfactants to the water (i.e., chemical flooding).

In situ combustion is a thermal method in which oil is ignited underground, creating a combustion front that is propagated through the reservoir by continuous air injection. In this process, a small portion of the oil in place is burned, producing heat that reduces the viscosity of the oil to increase its mobility (Kök 1999; Bagci and Kok 2001).

Another strategy that involves mobility change used in EOR is to use chemical flooding. Chemical flooding is of increasing interest and importance due to high oil prices and the need to increase oil production, particularly for application in mature water flooding

(achieved by secondary oil recovery water flooding). The most commonly used chemicals in chemical flooding include polymers, surfactants and the combination of the two. If polymer is involved, chemical flooding is also called polymer flooding. In this case, a water soluble polymer is added to the flood water, water mobility is reduced due to an increase in its viscosity. Water-soluble polymers are employed in EOR as viscosity agents of aqueous solutions used on oil recovery projects (Flores Candia and Deckwer 2002). The main role of the use of the polymers is to reduce the fluid displacement mobility. A secondary effect is a decrease in the relative water permeability in the reservoirs as a consequence of polymer adsorption. Flooding oil reservoir with high-viscosity polymer solution may be regarded as the most economic tertiary chemical oil recovery method (Littmann 1997) because it largely permits the use of existing oil field facilities. Another advantage of using polymers is that they are non-toxic and do not cause serious environmental problems (Kok and Alikaya 2003).

This objective of this paper is to investigate the rheological behavours of the microalgal biopolymer produced in previous studies at different conditions such as shear stress, temperature, concentration, and salinity to estimate the potential of the polymers in applications such as EOR.

4.2 Materials and Methods

4.2.1 Microalgal polymer preparation

Microalgal cultivation broth prepared as described in Chapter 3 was heated at temperature 80 °C, and then removes algae cells by centrifugation 12000rpm for 10min. The supernatants were concentrated under reduced pressure by rotational evaporator, dialyzed in cellulose membrane tubing (molecular weight cut off 8000 Dalton, fisher brand) against de-ironed distilled water changing twice a day for three successive days. The retained fraction was recovered, concentrated under reduced pressure, and precipitated by addition of 4-fold

volume of 99% (v/v) ethanol and washed twice, followed by drying at 40°C to obtain a crude polysaccharide (Mao, Fang et al. 2008).

4.2.2 Rheological tests

Rheological tests were carried out by using an Anton Paar rheometer. The measuring geometry was a cone/plate sensor (DG42-SN14302) and dynamic measurements were realized at shear rates between 0.1 to 1000 s^{-1}. In the 15-75℃ temperature range the effect of higher temperature, simulating the temperature into the reservoirs was studied. The temperature control was performed using a RTE-4 Refrigerated Bath /Circulators. The samples analyzed were in from 1000 ppm to 10000 ppm solution. The algal polymer dispersed in deioned distilled water or NaCl brine (0.3mol and 0.6mol) agitated by magnetic stir. The solution remained at rest for 24h in refrigerator before the rheological measurements.

The study of solutions in brine is justified by the use of polymers in offshore operations, since oil fields in the offshore environment, especially in the deepwater, represent a significant resource available to meet the expected increases in oil demand over the next decades (Bondor, Hite et al. 2005) (Mothe, Correia et al. 2006).

4.2.3 The rheological model based on the Cross Equation

The rheological model based on the Cross Equation is one of the most popular in use today. We consider one equation that describes the whole curve, which is Cross model, named after Malcolm Cross.

The equation is described by the following form,

$$\eta = \eta_{\infty} + \frac{\eta_0 - \eta_{\infty}}{1 + (K\dot{\gamma})^m} \qquad Eq\ 4.1$$

Where, η0 is the Zero Shear Viscosity, η∞ is the Infinite Shear Viscosity, K is known as the Consistency has the dimensions of time, and m is known as the (Cross) Rate Constant which is dimensionless. The experimental data of 0.5% (w/v) algal polymer were selected to fit cross model. The RHEOPLUS analysis software which companies with Anton Paar rheometer is employed for data analysis.

4.3 Results

4.3.1 Effects of temperature, shear rate and polymer concentration

Figures 4.2 to 4.4 show the viscosity curves of different concentration of microalgal polymer ranging from 0.1% to 1% (w/v) in de-ionized distilled water respectively. The polymer solutions showed a pseudoplastic behavior, since the viscosity decreased as shear rate increases in all temperatures and polymer concentrations. It is also evident that the rheological behaviors of the microalgal polymer were very sensitive to temperature and the viscosity decreased remarkable when temperature increased from 15 to 75 °C. It is interesting to notice that, when the temperature was above 45 °C, the viscosity of the polymer solutions become less sensitive to the increase of shear rate. Indeed, the viscosity profiles became flat at temperatures 65 °C and 75 °C in all polymer concentrations.

As shown in Figure 4.5, microalgal polymer concentration from 0.1% (w/v) to 1% (w/v), the viscosity of microalgal polymer increase dramatically. The maximum change of viscosity is over 80 times at 0.1 s^{-1}. However, the viscosity of biopolymer solution at 1% was very

sensitive to sheer stress and decrease to a level similar to that of 0.1% solution of high shear rate above 100 s^{-1}.

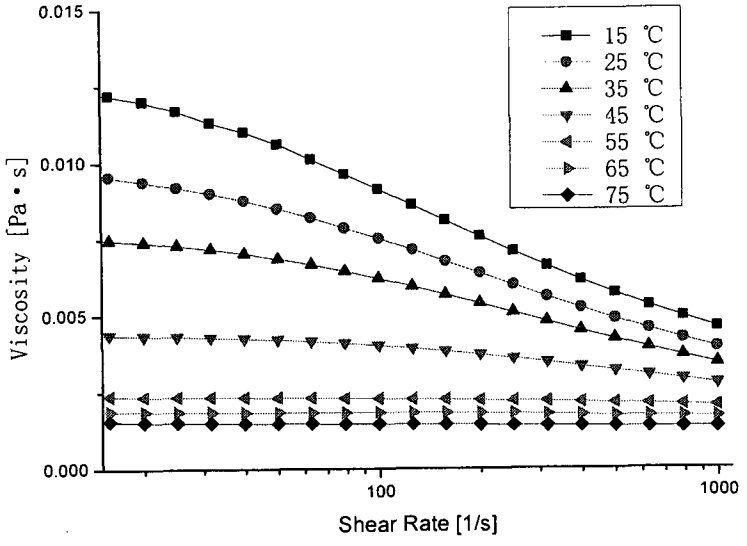

Figure 4.2. 0.1% Algal polymer viscosity profile

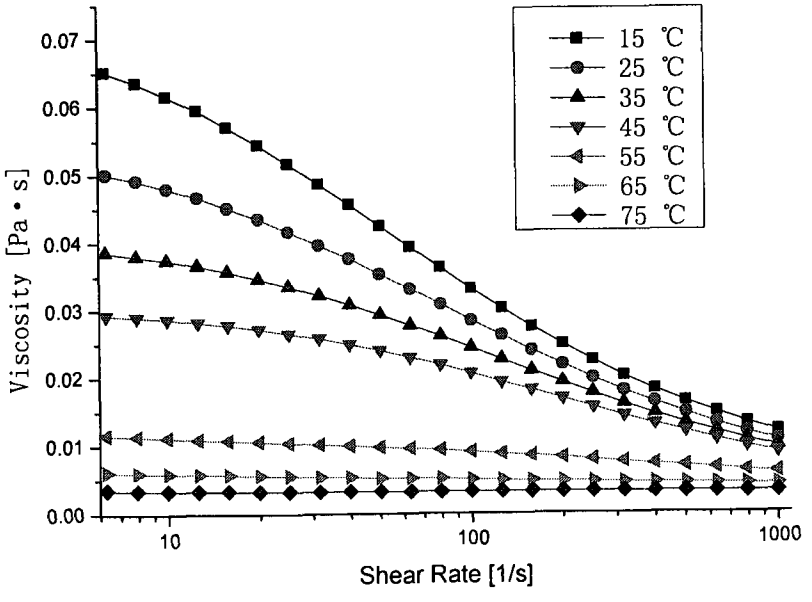

Figure 4.3 0.5% Algal polymer viscosity profile

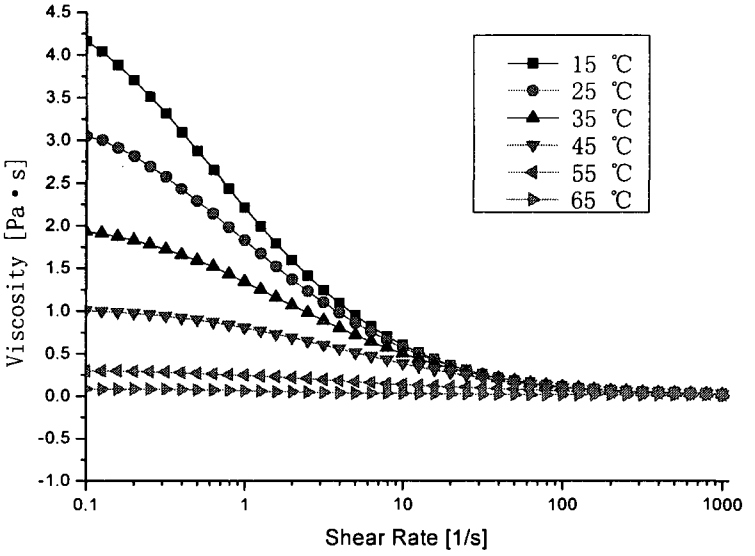

Figure 4.4. 1% Algal polymer viscosity profile

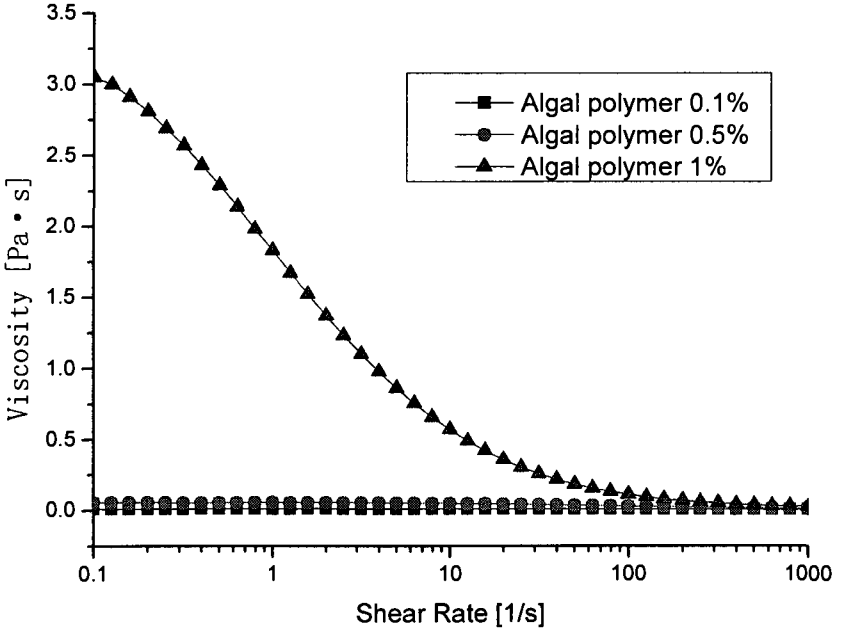

Figure 4.5. Different concentration of Algal polymer viscosity profile at 25℃

4.3.2 Effects of salt concentration

Viscosity curves of microalgal polymer plus NaCl are exhibited in Figs 4.6 and 4.7, which present the viscosity curves of microalgal polymer in brine solution with the NaCl concentration 0.3 mol and 0.6 mol respectively. From Figure 4.8, we found microalgal polymer was also sensitive to salt concentration because the viscosity of its solutions presented a large increase when it was compared with that in the absent of salt solution. In the range of 0 to 0.6 mol of NaCl, the viscocity increases with salt concentration. However, at the concentration of NaCl 0.3mol and 0.6mol, there is not much difference between viscosity curves was observed through the temperature profiles.

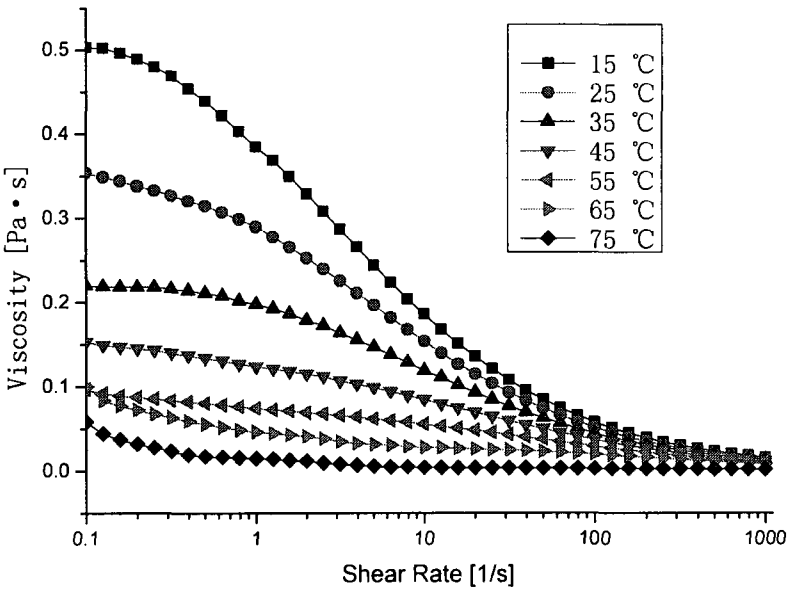

Figure 4.6. 0.5% Algal polymer + 0.3 mol NaCl viscosity profile

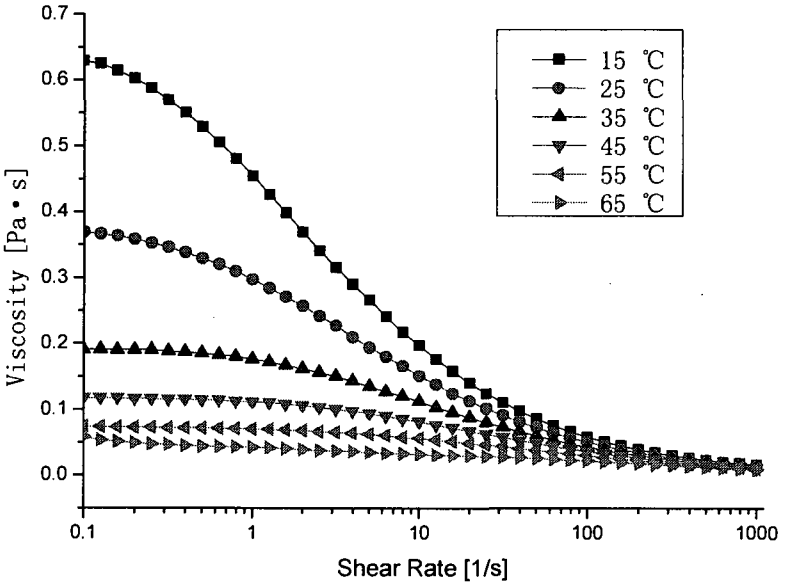

Figure 4.7. 0.5% Algal polymer + 0.6 mol NaCl viscosity profile

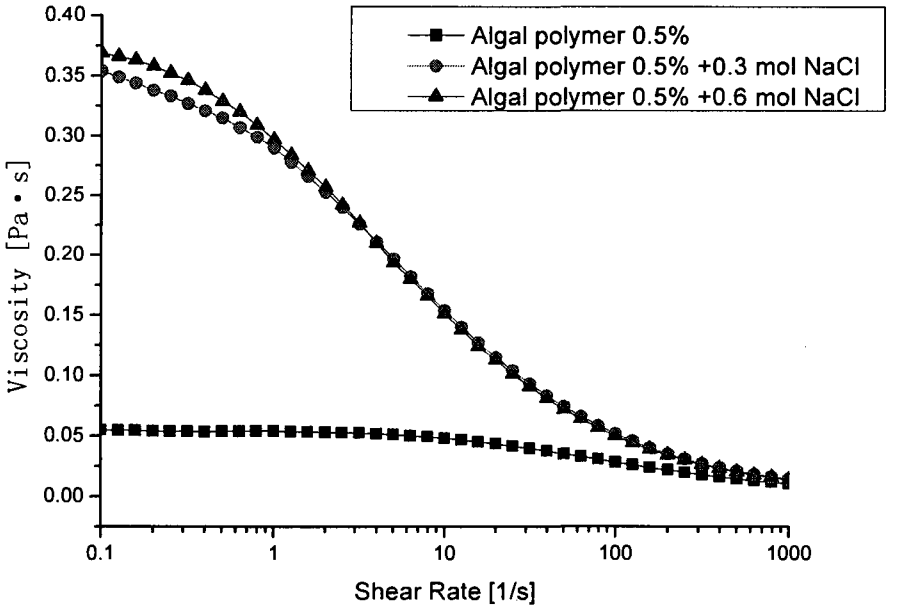

Figure 4.8. 0.5% Algal polymer + different concentration of NaCl viscosity profile at 25℃

4.3.3 Fit the experimental data to Cross model

Most of Non-newtonian fluids such as polymer solutions have mathematically described the flow of pseudoplastic fluids by power law. However, the power law only well describe the shear thinning region, is not sufficient to describe the whole viscosity curve. The Cross model describes the viscosity curve of a material with a zero shear viscosity, a constant viscosity at high shear rate values and a shear thinning region in between. This cross equation is considered fits well to biopolymers. Indeed, this model held the key to the successful application of rheology to polymer characterization. When this model is used to describe non-Newtonian liquids, the degree of shear thinning is dictated by the value of m, with m tending to zero describes more Newtonian liquids, while the most shear-thinning liquids have a value of m tending to unity. Table 4.1 lists the Cross equation parameters of the 0.5% polymer solution at different temperatures in the range of 15 - 65 ℃. It is clearly from the data that the values of η_0 and η_{inf} decreased with temperature, which is reasonable. The data also indicate that the value of the dimensionless exponent (m) was almost constant in the investigated temperature range, fluctuating slightly in the range of 0.81 to 0.86, indicating that the polymer solution was a pseudoplastic (shear-thinning) fluid in the tested temperature range.

Table 4.1 Parameters of the cross model calculated by RHEOPLUS analysis software for 0.5% (w/v) algal polymer

T (°C)	eta_0 (Pa · s)	eta_inf (Pa · s)	m	K
15	0.073111	0.00552	0.82053	0.015400
25	0.054919	0.004391	0.81092	0.011064
45	0.054351	0.004503	0.81946	0.010806
55	0.04106	0.00384	0.82647	0.007639
65	0.03047	0.00363	0.85897	0.005204

As it is shown on Figure 4.9, the cross model well describes the experimental data for 0.5% (w/v) algal polymer range from15 to 65 ℃ respectively.

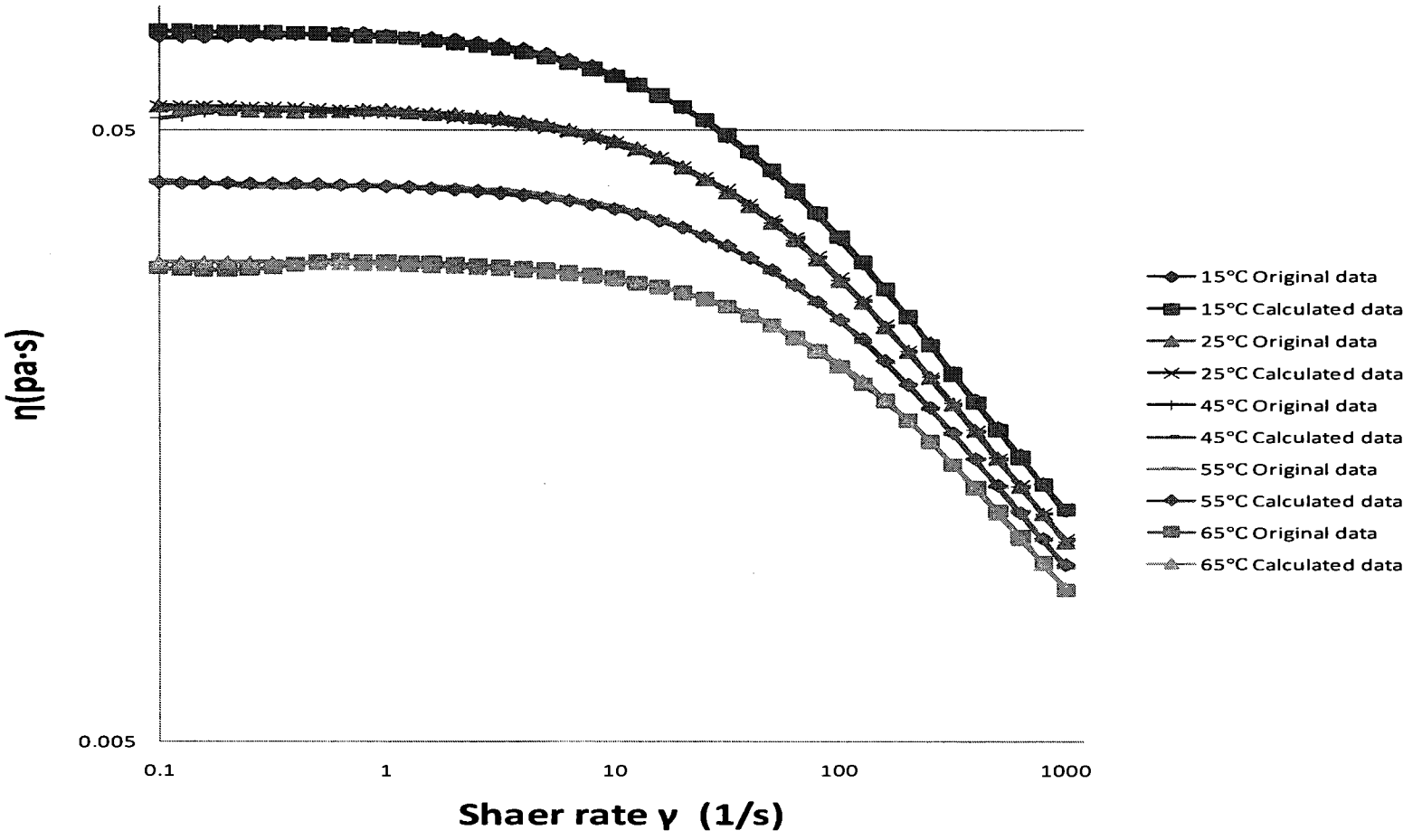

Figure 4.9. Fit the experimental date to cross model. The experimental data were obtain with 0.5% polymer solution.

4.4 Discussions

The concentration of polymer is a major factor affecting the viscosity profiles, with 1% (w/v) polymer aqueous solution showing magnitude higher viscosity than that of 0.5% (w/v) polymer solution. At the low shear rate from 0.1 to 10 s^{-1}, there are 5 to 80 times different in the same share rate. This observation may have significant implications in the choice of optimal polymer concentration for a particular practical application such as polymer flooding EOR.

It was interested to notice that the viscosity of polymer solution was much higher when prepared with 0.3M and 0.6 M of NaCl solution then distilled water. This result is not surprising because stronger intermolecular associations of microalgal polymer molecules in the presence of salts. In some cases, manufacturers add salts to the polymer material to minimize the variations in solution viscosity (Rochefort and Middleman 1987). The fact that the polymer solution has a high viscosity at salinity close to that of seawater is of significant practical relevance because this implies the applicability of the polymer in EOR in offshore oil fields. Besides, since there was not much difference between the viscosity profiles of the polymer solution with 0.3 M and 0.6 M NaCl, the polymers should provide consistent performance in environment with different groundwater salinities.

Viscosity of polymer solution is sensitive to temperature, viscosity decrease with the temperature incensement. Once the molecule is in the ordered state, it is relatively insensitive to further increases in temperature. There was a tendency change of viscosity curve in viscosity with temperature beginning at temperature 45°C and in brine solution the viscosity curve changed was delayed until temperature 55°C, indicating that the ordered structure was

stabilized by the addition of salt.

In comparison to the rheological properties of commercial Xanthan gum reported in literature (Nasr, Soudi et al. 2007; Ashraf, Soudi et al. 2008), the microalgal biopolymers exhibited comparable or superb viscosities at different temperature, sheer rate, salinity and polymer concentration.

In summary, this study shows that the rheological properties of the microalgal polymer produced by the microalgal strain from lactose have potentials in enhanced oil recovery.

4.5 Acknowledgement

Financial supports from the Natural Science and Engineering Research Council (NSERC) of Canada and Canadian Foundation of Innovation (CFI) are gratefully acknowledged.

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CHAPTER 5

SUMMARY AND CONCLUSIONS

Experiments were performed with the green alga growth under various experimental conditions in order to study the polysaccharide production conditions and study its rheological properties. The conclusions that were reached in this project concerning the production of polysaccharide are listed below.

In photoautotrophic cultivation experiments, it is worth noting that no detectable algal polymers were produced of this particular green alga strain.

In heterotrophic cultivation condition, glucose, lactose and sucrose were used as carbon source relatively for this green alga strain. Glucose is the best carbon source for growth, however, it hardly to observe any polysaccharide produced.

In our mixotrophic cultivation studies, it was observed that a green alga strain has the capability of utilizing lactose for mixotrophic growth and producing large quantities of algal polymer. As a result, the broth become very viscous at the end of cultivation and a highest polymer concentration of 5 g/L was achievable with 10 g/L lactose in the medium (data not shown). Lactose is main component of the cheese whey which is considered relatively economic second product. Potentially, this strain can use cheese whey as carbon source reduce the cost in the future industry application.

A Plackett-Brunman statistical experiment design was employed to study the optimization of

polysaccharide production. We draw the results that $NaNO_3$, lactose, and temperature are the primary factors affecting biopolymer production of the microalgal strain under the investigated conditions.

Our biopolymer is comparable to the other biopolymers in term of viscosity. Such as the xanthan gum production presenting by literature (Nasr, Soudi et al. 2007) (Ashraf, Soudi et al. 2008). The concentration of polymer is major effect of viscosity profile in study range from 0.1% to 1%. Viscosity of polymer solution is sensitive to temperature, viscosity decrease with the temperature incensement. Studies on the rheological properties of microalgal polymer show that it has potentials in enhanced oil recovery.

Recommendations

The following studies are recommended:

1) Microalgal polymer production utilizing cheese whey as the feedstock. This study will include feasibility studies and optimization studies;

2) Characterization of the microalgal biopolymers; more systematic studies are recommended to measure the molecular weight and molecular structure of the biopolymer (e.g., monosaccharide composition and specialized branch, functional groups and bonds);

3) Application studies such as the utilization of the polymer for EOR using desk-top devices micromodels system;

4) Potential applications of the biopolymers as anticoagulant agents by measuring the activated partial thromboplastin time (APTT), prothrombin time (PT) and thrombin time (TT);

5) Scale up of the biopolymer production process to pilot scale.

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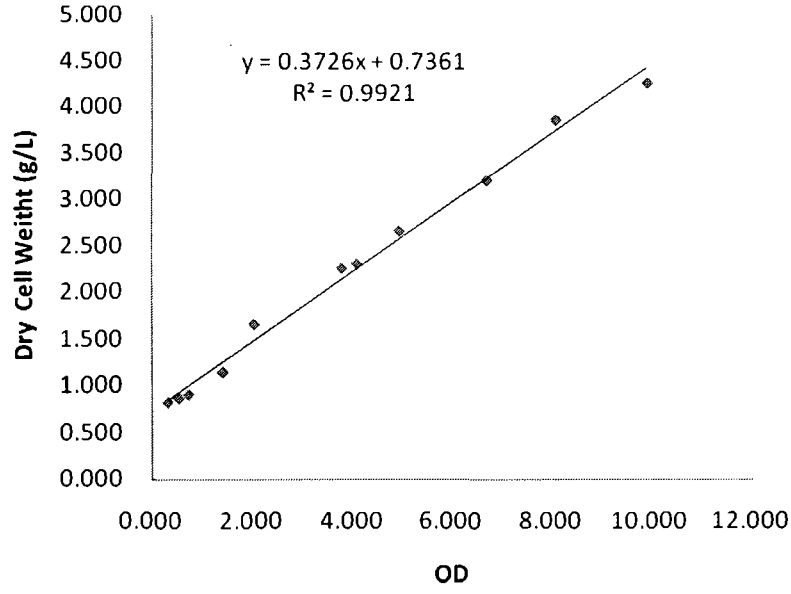
APPENDICES

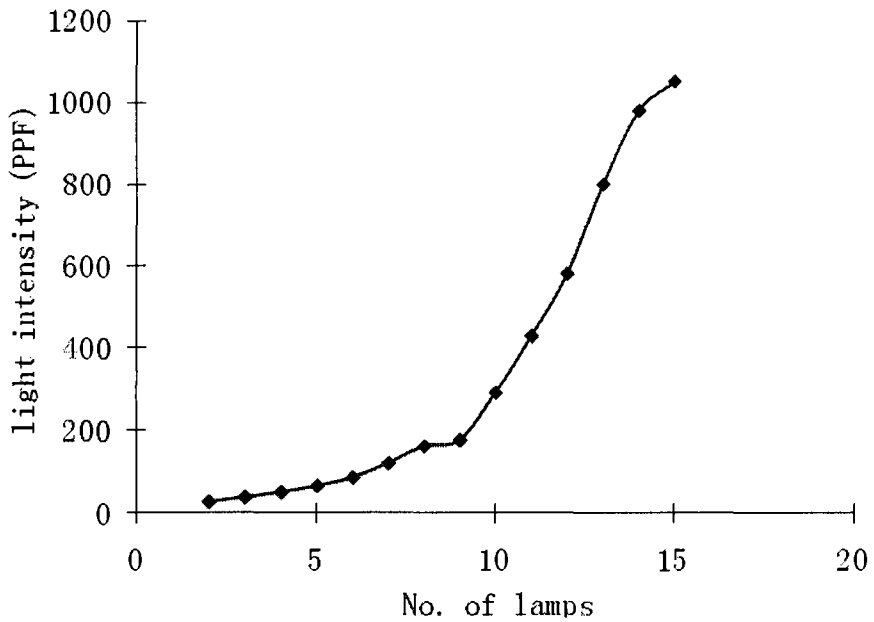
Appendix A. Calibration curve between the cell mass concentration and the optical density at a wavelength of 600 nm measured using a spectrophotometer.

Optical density (OD) VS Dry cell weight (DCL)

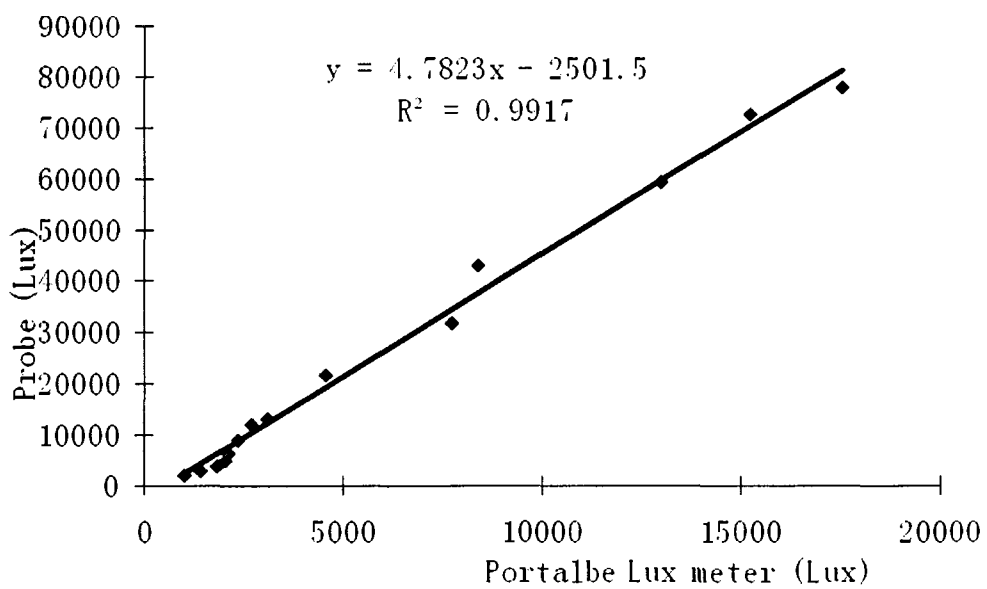


Appendix B. Light intensity measurements

The relationship between the number of lamps and light intensity



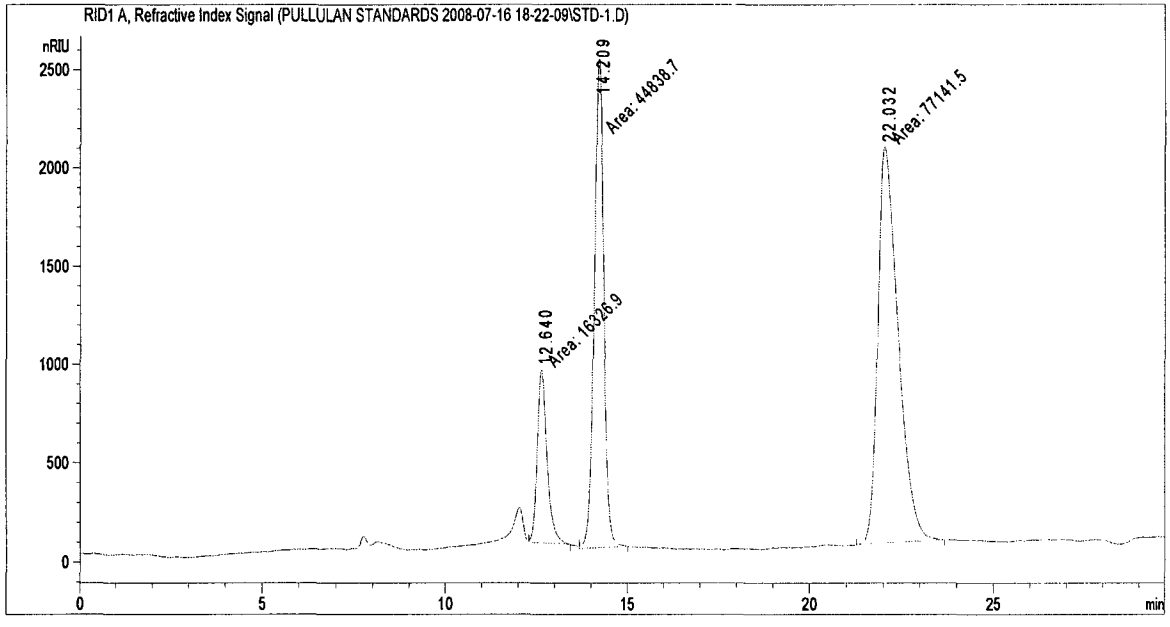
Comparison of light intensity measurements between probe and portable lux meter



Appendix C. Chromatograms of Pullulan standards and algal polysaccharide (A) Pullulan Standard curves (B) algal polysaccharide.

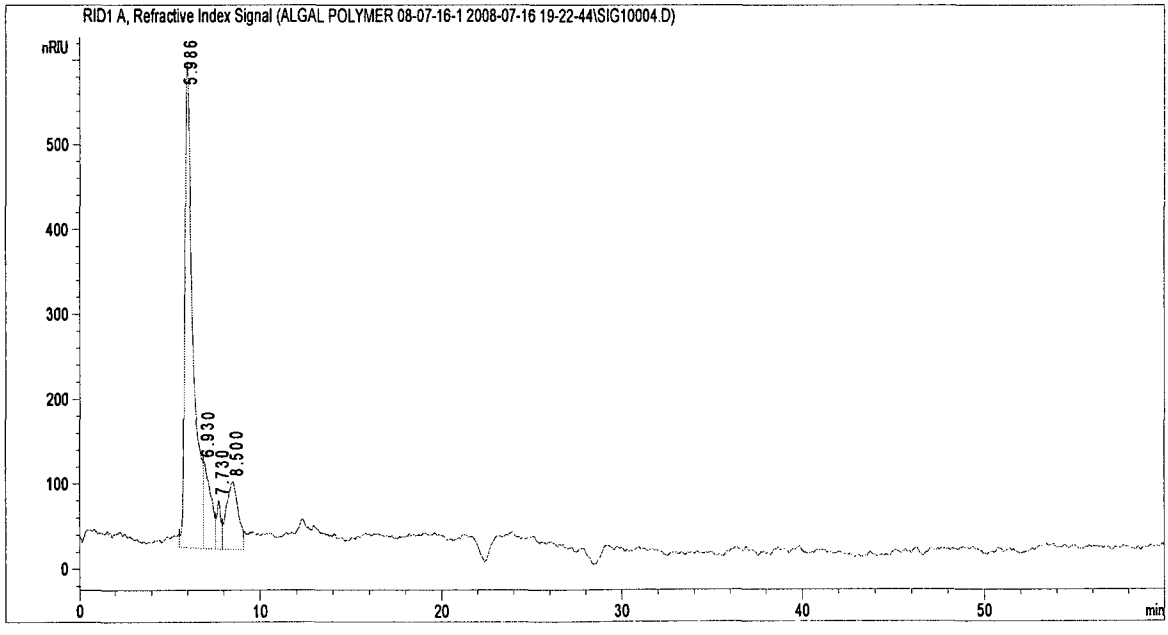
A

Pullulan Standards (70 k, 270 k, 850 k)



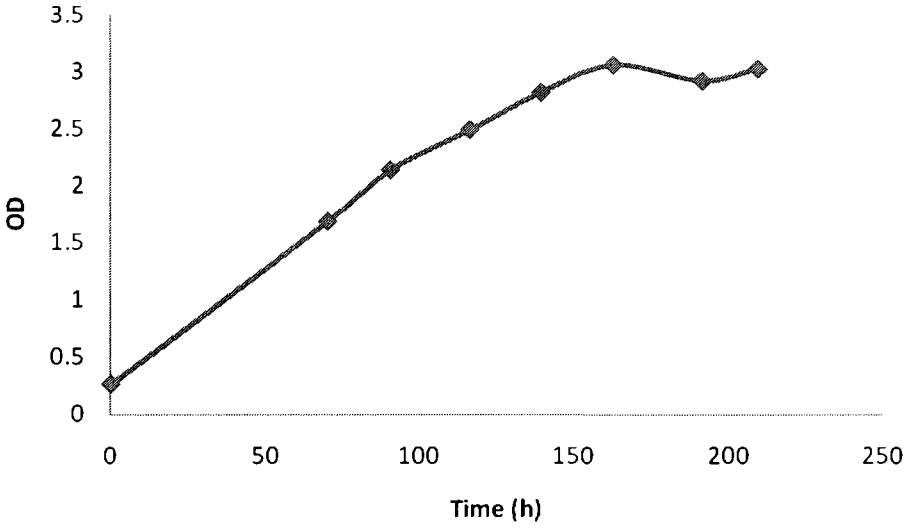
B

Algal polysaccharide

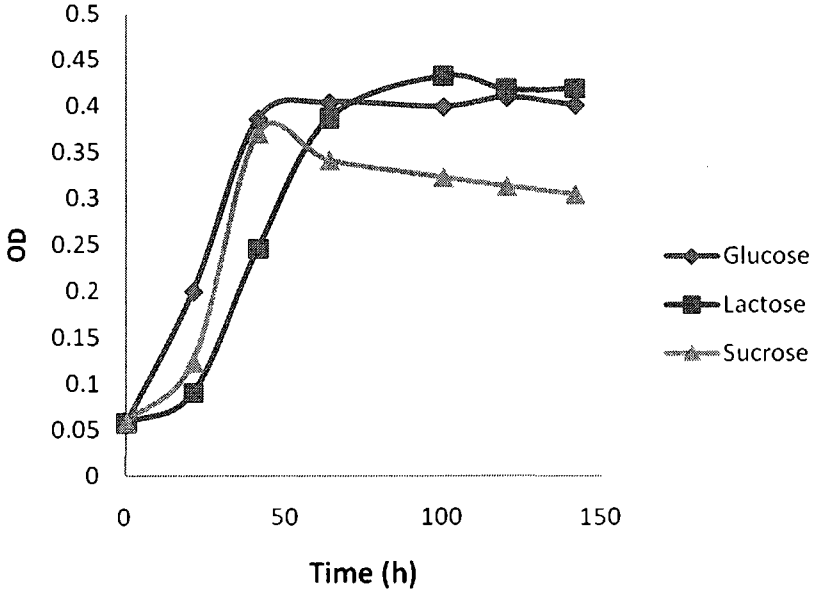


Appendix D. Growth curves of the *green alga* strain when cultivated in different cultivation conditions: (A) Photoautotrophic cultivation (B) Heterotrophic cultivation (C) Mixotrophic cultivation

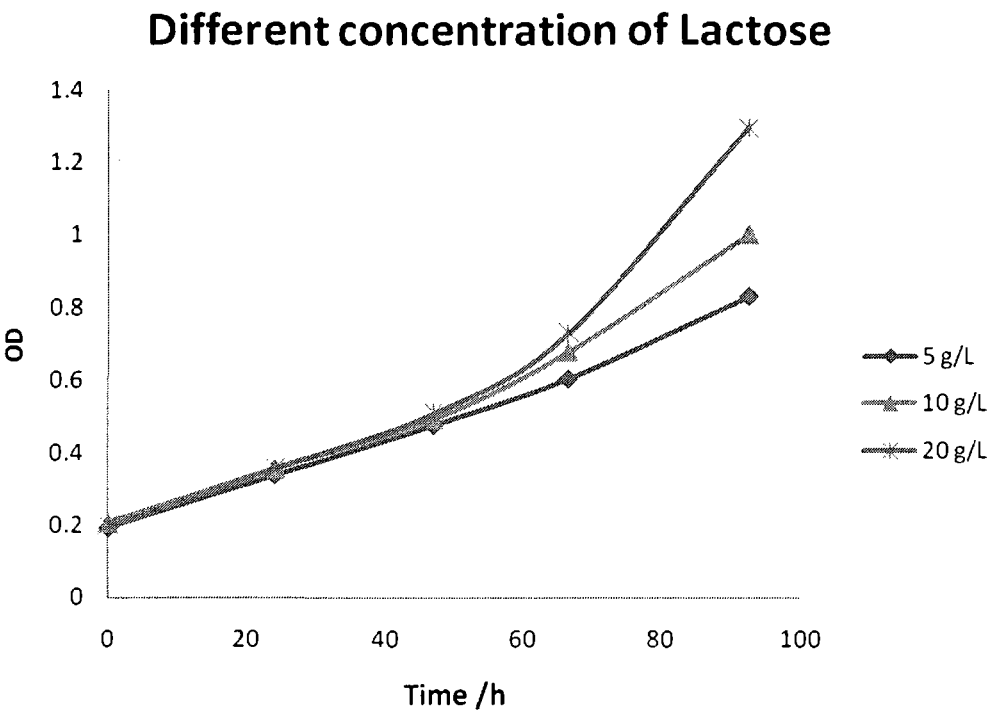
A



B



C


Different concentration of Lactose
1.4
1.2
1
0.8
0.6
0.4
0.2
0
OD
0
20
40
60
80
100
Time /h
5 g/L
10 g/L
20 g/L