การปรับปรุงการผลิตบีตากลูแคนใน *Saccharomyces cerevisiae* โดยสารเติมแต่งบางชนิด

นางสาว นฤมล มงคลธนวัฒน์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาเทคโนโลยีทางอาหาร ภาควิชาเทคโนโลยีทางอาหาร
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2553

IMPROVEMENT OF β-GLUCAN PRODUCTION IN *Saccharomyces cerevisiae* USING SOME ADDITIVES

Miss Naruemon Mongkontanawat

A Dissertation Submitted in Partial Fulfillment of the Requirements

for the Degree of Doctor of Philosophy Program in Food Technology

Department of Food Technology

Faculty of Science

Chulalongkorn University

Academic year 2010

Thesis Title	IMPROVEMENT OF β-GLUCAN PRODUCTION IN *Saccharomyces cerevisiae* USING SOME ADDITIVES
By	Miss Naruemon Mongkontanawat
Field of Study	Food Technology
Thesis Advisor	Assistant Professer Romanee Sanguandeekul, Ph.D.
Thesis Co-Advisor	Assistant Professer Cheunjit Prakitchaiwattana, Ph.D.
Thesis Co-Advisor	Associate Professer Lynne A. McLandsborough, Ph.D.
Thesis Co-Advisor	Pawadee Methacanon, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirement for the Doctoral Degree

……..Dean of the Faculty of Science
(Professor Supot Hannongbua, Dr.rer.nat)

THESIS COMMITTEE

……Chairman
(Assistant Professor Pasawadee Pradipasena, Sc.D.)

……...Thesis Advisor
(Assistant Professor Romanee Sanguandeekul, Ph.D.)

……Thesis Co-Advisor
(Assistant Professor Cheunjit Prakitchaiwattana, Ph.D.)

……Thesis Co-Advisor
(Associate Professor Lynne A. McLandsborough, Ph.D.)

……Thesis Co-Advisor
(Pawadee Methacanon, Ph.D.)

……Examiner
(Kanitha Tananuwong, Ph.D.)

……External Examiner
(Thunyarat Pongtharangkul, Ph.D.)

นฤมล มงคลธนวัฒน์ : การปรับปรุงการผลิตบีตากลูแคนใน *Saccharomyces cerevisiae* โดยสารเติมแต่งบางชนิด.(IMPROVEMENT OF β-GLUCAN PRODUCTION IN *Saccharomyces cerevisiae* USING SOME ADDITIVES) อ. ที่ปรึกษาวิทยานิพนธ์หลัก:ผศ.ดร.รมณี สงวนดีกุล, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : ผศ.ดร. ชื่นจิต ประกิตชัยวัฒนา, Assoc. Prof. Dr. Lynne A. McLandsborough, ดร. ภาวดี เมธะคานนท์, 153 หน้า.

จากการคัดเลือก *S. cerevisiae* 3 สายพันธุ์ *S. cerevisiae* Angel® เป็นสายพันธุ์ที่เหมาะสมเพื่อใช้ในการศึกษาผลของสารเติมแต่ง (EDTA, SDS, NaCl) ต่อปริมาณและคุณภาพของของบีตากลูแคน เนื่องจากมีปริมาณบีตากลูแคน เปอร์เซ็นต์น้ำหนักเซลล์แห้ง และมีปริมาณบีตากลูแคนสุทธิสูงสุด จากการเติมสารเติมแต่ง (EDTA, SDS, NaCl) ที่ระดับความเข้มข้นต่างๆ ในอาหารเลี้ยงเชื้อ YPD เลี้ยงภายใต้เครื่องเขย่าที่ความเร็วรอบ 200 รอบ/นาที พีเอช 4 อุณหภูมิ 30 องศาเซลเซียส พบว่าการเจริญของยีสต์เป็น sigmoid curve สามารถอธิบายโดยใช้สมการ non-linear regression ในสมการของ shifted logistic equation ระดับการเจริญของยีสต์สูงกว่ากลุ่มควบคุม เมื่อเติม SDS 100 และ 200 พีพีเอ็ม ในทางกลับกันระดับการเจริญลดลงเมื่อเติม EDTA 100 200 พีพีเอ็ม NaCl 30,000 และ 60,000 พีพีเอ็ม นอกจากนี้เมื่อเลี้ยงยีสต์ด้วยสารเติมแต่งทำให้เวลาที่ยีสต์ใช้ตั้งแต่เริ่มต้นจนถึงมีอัตราการเจริญสูงสุดเพิ่มขึ้น อย่างไรก็ตามไม่พบการเจริญและเซลล์ตายเมื่อเลี้ยงยีสต์ด้วยสารเติมแต่งที่ความเข้มข้นสูง และสารเติมแต่งผสม จากผลการทดลองที่ได้พบว่าสภาวะที่เหมาะสมของสารเติมแต่งชนิดเดียวคือ EDTA 50 พีพีเอ็ม SDS 100 พีพีเอ็ม และ NaCl 30,000 พีพีเอ็ม จากนั้นประมาณความเข้มข้นของสารเติมแต่งผสมที่เหมาะสมรวม 4 ชุด โดยใช้โปรแกรมคอมพิวเตอร์ พบว่า (1) ผลรวมของ EDTA 5 พีพีเอ็ม SDS 10 พีพีเอ็ม (2) ผลรวมของ EDTA 5 พีพีเอ็ม NaCl 3,000 พีพีเอ็ม (3) ผลรวมของ SDS 20 พีพีเอ็ม NaCl 3,000 พีพีเอ็ม และ (4) ผลรวมของ EDTA 5 พีพีเอ็ม, SDS 10 พีพีเอ็ม และ NaCl 3,000 พีพีเอ็ม จากนั้นศึกษาผลของสารเติมแต่งชนิดเดียวและสารเติมแต่งผสมรวม 8 ชุด ต่อรูปร่างเซลล์ พื้นผิวเซลล์ การผลิตบีตากลูแคน และองค์ประกอบของเซลล์ พบว่าเซลล์ค่อนข้างกลม (ความแตกต่างระหว่างความยาวและความกว้างเพียง 0.84 ไมโครเมตร) มีจำนวนรอยแผลของการแตกหน่อ และปริมาณบีตากลูแคนมากที่สุด ในขณะที่กลุ่มที่ 2 เซลล์รูปไข่ ยาว และยาวมากที่สุดในหน่วยทดลองที่เติม EDTA 5 พีพีเอ็ม และ NaCl 3,000 พีพีเอ็ม (ความแตกต่างระหว่างความยาวและความกว้าง 2.00 นาโนเมตร) จาก FTIR สเปกตรัม หน่วยทดลองที่เติมสารเติมแต่งจะมีปริมาณของโพลีแซคคาไรด์สูงกว่ากลุ่มควบคุม ยกเว้นเมื่อเลี้ยงในส่วนผสมของ EDTA 5 พีพีเอ็ม และ NaCl 3,000 พีพีเอ็ม จากผลการทดลองนี้สรุปว่าสารเติมแต่งกระตุ้นการสร้างบีตากลูแคนและการสังเคราะห์ผนังเซลล์ นอกจากนี้ปริมาณของโปรตีนต่ำที่สุดเมื่อเลี้ยงยีสต์ในอาหารเลี้ยงเชื้อที่มี SDS 100 พีพีเอ็ม และสูงที่สุดเมื่อเลี้ยงในส่วนผสมของสารเติมแต่ง 3 ชนิด อัตราส่วน β-(1,6) : β-(1,3) สูงขึ้นเมื่อเพาะเลี้ยงยีสต์ด้วยสารเติมแต่งยกเว้น EDTA 50 พีพีเอ็ม อย่างไรก็ตามหลังจากสกัดบีตากลูแคน พบว่าอัตราส่วน β-(1,6): β-(1,3) ในหน่วยทดลองที่เติมสารเติมแต่งต่ำกว่ากลุ่มควบคุม จากการศึกษาน้ำหนักโมเลกุลพบว่าบีตากลูแคนจากยีสต์ที่เลี้ยงด้วย SDS 20 พีพีเอ็ม, NaCl 3,000 พีพีเอ็ม และ ผลรวมของ EDTA, SDS และ NaCl พบว่ามีน้ำหนักโมเลกุลเฉลี่ยมากกว่ากลุ่มควบคุมและหน่วยทดลองอื่น สำหรับการศึกษาสมบัติของบีตากลูแคนในการกระตุ้นภูมิคุ้มกันจะเปลี่ยนบีตากลูแคนเป็น CM-glucan ก่อนการทดสอบ พบว่าความเข้มข้นของ CM-glucan และปริมาณคาร์บอกซีเมททิลกรุ๊ปเป็นปัจจัยสำคัญที่มีผลต่อการกระตุ้นเม็ดเลือดขาวและความเป็นพิษต่อเซลล์ โดยปัจจัยดังกล่าวมีแนวโน้มแปรผันตรงกับสมบัติทางชีวภาพทั้งสอง

ภาควิชา<u>เทคโนโลยีทางอาหาร</u> ลายมือชื่อนิสิต.....

สาขาวิชา<u>เทคโนโลยีทางอาหาร</u> ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ปีการศึกษา <u>2553</u> ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

4973866123 : MAJOR FOOD TECHNOLOGY
KEYWORDS : β-GLUCAN/ ADDITIVES/ *Saccharomyces cerevisiae*/ CELL MORPHOLOGY/ CM-glucan

NARUEMON MONGKONTANAWAT : IMPROVEMENT OF β-GLUCAN PRODUCTION IN *Saccharomyces cerevisiae* USING SOME ADDITIVES. THESIS ADVISOR : ASSIT. PROF. ROMANEE SANGUANDEEKUL, Ph.D., THESIS CO-ADVISOR : ASSIT. PROF. CHEUNJIT PRAKITCHAIWATTANA, ASSOC. PROF. LYNNE A. MCLANSBOROUGH, PAWADEE METHACANON, Ph. D., 153 pp.

After screening of *S. cerevisiae* strains, *S. cerevisiae* Angel® was selected as the test culture to study the effect of additives on quantity and quality of yeast β-glucan on the basis of high net β-glucan yield. EDTA, SDS and NaCl were supplemented into the medium at various concentrations. Cells were cultivated at pH 4, 30 °C with a reciprocal shaking at 200 rpm for 48 h. Sigmoid curve of yeast growth was analyzed using non-linear regression and presented as a function of shifted logistic equation. Treatments with SDS at 100 ppm and 200 ppm gave higher growth level than control. On the other hand, 50 and 100 ppm EDTA resulted in reduction of growth level. Similarly, the growth level of yeast cell was reduced in 30,000 ppm and 60,000 ppm NaCl. In addition, the time to reach the highest growth rate was longer in the presence of additives. Conditions with no growth and cell death were found in treatments with high concentrations and combination of additives. Based on this observation, the optimum concentration of each additive supplemented solely was 50 ppm EDTA, 100 ppm SDS and 30,000 ppm NaCl. Using computer software for estimation, 4 suitable combination of additives were EDTA 5 ppm and SDS 10 ppm; EDTA 5 ppm and NaCl 3,000 ppm; SDS 20 ppm and NaCl 3,000 ppm; and the combination of EDTA 5 ppm, SDS 10 ppm and NaCl 3,000 ppm. For further investigation, eight treatments (four single additives and four combination of additives) were set to study the effect of additives on cell shape, wall surface, β-glucan production and composition of cell in *S. cerevisiae* Angel®. Yeast cultured in medium supplemented with 100 ppm SDS exhibited rounder cell shape (the difference between major axis and minor axis length is 0.84 μm) with highest number of bud scars and highest β-glucan content (8.15 %w/w). Whilst, the second group was long oval cell shape and the longest cell was exhibited in treatment with combination of 5 ppm EDTA and 3,000 ppm NaCl (the difference between major axis and minor axis length is 2.00 μm). From FTIR spectra of yeast cell, treatments with additives showed slightly higher amount of polysaccharide region compared to control, with the exception in the presence of combination of 5 ppm EDTA and 3,000 ppm NaCl. Interestingly, these results implied that additives could activate β-glucan formation and cell wall synthesis. The ratio of protein region when yeast was cultivated with 100 ppm SDS was lower than control and other treatments. The highest protein was found when yeast was cultured with three combination of additives. For the β-(1,6) : β-(1,3) in yeast cell, calculation showed that the ratios were higher than control when yeast was cultivated with additives, with the exception of treatment with 50 ppm EDTA. However, after β-glucan extraction, the β-(1,6) : β-(1,3) was lower in treatments with additives. The higher weight average molecular weight than control were found when cultivated yeast with combination of 20 ppm EDTA, 3,000 ppm NaCl and three combination of EDTA, SDS and NaCl. For immunomudulatory properties, the β-glucan was derivatized to carboxymethylglucan (CM-glucan) before test. It was found that degree of substitution (DS) and concentration of CM-glucan seemed to affect significantly stimulation activity of macrophage cell as well as their cytotoxicity. The higher DS and concentration, the higher activity tended to be.

Department : Food Technology

Field of Study : Food Technology

Academic Year : 2010

Student's Signature .....

Advisor's Signature .....

Co-Advisor's Signature .....

Co-Advisor's Signature .....

Co-Advisor's Signature .....

ACKNOWLEDGMENTS

I would like to express my respect and appreciation to all the members on my advisory committee: Asst. Prof. Dr. Romanee Sanguandeekul, Asst. Prof. Dr. Cheunjit Prakitchaiwattana, of Department of Food Technology, Faculty of Science, Chulalongkorn University, Bangkok, Thailand, Dr. Pawadee Methacanon, National Metal and Materials Technology Center (MTEC), Thailand Science Park, Pathumthani, Thailand, Asst. Prof. Dr. Hang Xiao and Assoc. Prof. Dr. Lynne A. McLandsborough Department of Food Science, University of Massachusetts, Amherst, USA, for their guidance and support. Special thanks to my examining committees, Asst. Prof. Dr. Pasawadee Pradipasena**,** Dr. Kanitha Tananuwong and Dr. Thunyarat Pongtharangkul, for their valuable suggestions.

I am extremely thankful to Asst. Prof. Dr**.** Cheunjit Prakitchaiwattana, for her supporting, sharing her time, useful discussions and her assistance during the preparation of manuscript. I am extremely thankful to Asst. Prof. Dr. Hang Xiao and Assoc. Prof. Lynne A. McLandsborough for graciously providing me the equipments used in this research during the time I did my experiment at Department of Food Science, University of Massachusetts, Amherst and giving me constructive suggestions throughout the research.

I admire Asst. Prof. Dr. Romanee Sanguandeekul's intellectual vitality and consider myself very fortunate that she accepted me as her graduate student. Dr. Romanee Sanguandeekul 's guidance has roused my interest enormously in Food Science and Technology.

I would like to thanks Asst. Prof. Dr. Pasawadee Pradipasena for kindly help in determination weight average molecular weight of β-glucan by using MALLS (Multi Angle Laser Light Scattering) and Dr. Sanya Hogputsa, Research and Development Institute, Government Pharmaceutical Organization, Bangkok, Thailand for providing the equipment.

I would like to thanks Dr. Pawadee Methacanon for help in structure analysis of β-glucan by using Fourier Transform Infrared Spectroscopy (FTIR).

I would like to thank Professor Micha Peleg and Mr. Mark Normand of the Department of Food Science; University of Massachusetts, Amherst, USA., for their kind help in performing non-linear regression analysis.

I would like to thank Asst. Prof. Dr. Hang Xiao and Miss Shanshan Gua for kindly help on NO (Nitric Oxide assay) and MTT assay analysis.

I am grateful to Rajamangala University of Technology Tawan-ok for challenging me with the scholarship to pursue my doctoral studies at Department of Food Technology, Faculty of Science, Chulalongkorn University, Bangkok, Thailand.

I also would like to thank my dear friends for their affection and care.

I would like to express my overwhelmingly gratitude to my dearest mom and dad including to my brothers and sister, for their unconditional and moral support.

CONTENTS

PAGE

CONTENTS (cont.)

CHAPTER PAGE

CONTENTS (cont.)

CHAPTER PAGE

CONTENTS (cont.)

PAGE

LIST OF TABLES

TABLE **PAGE**

LIST OF TABLES(cont.)

TABLE **PAGE**

LIST OF FIGURES

FIGURE **PAGE**

LIST OF FIGURES (cont.)

FIGURE **PAGE**

LIST OF FIGURES (cont.)

FIGURE **PAGE**

LIST OF FIGURES (cont.)

FIGURE **PAGE**

LIST OF FIGURES (cont.)

FIGURE **PAGE**

CHAPTER I
INTRODUCTION

β-Glucans are a group of polysaccharides which compose of glucose units linked with beta-glycosidic bonds. They have been used in many industries: pharmaceutical industry, food and feed industries and cosmetics industry. The functional properties of food products such as thickening property, water holding capacity, emulsifying property can be improved by β-glucans. The medicinal properties such as antitumor, antiviral, antimicrobial, antioxidant, mycotoxins absorption, immune response stimulation in animals, reduction of cholesterol and glucose in blood also exhibited. They have been found in many natural sources such as bacteria, yeast, fungi and plants with differences in structure and functional properties. Interestingly, β-glucan produced by *S. cerevisiae* has shown to be the most effective immune enhancing source. The structure of β-glucan is composed of a long chain β-(1,3)-glucan and branch β-(1,6)-glucan. β-Glucan synthesis takes place in plasma membrane by uridine diphosphoglucose pyrophosphorylase (UGPase) and β-glucan synthetase group.

There are many reports about the effect of the environmental stress factors on stimulating β-glucan formation in fermentor and agar culture such as component of media, weak acid, SDS, hygromycin B and K1 killer toxin. In addition, the previous study revealed that EDTA activated β-(1,3)-glucan synthase in cell free extracts of *S. cerevisiae*. SDS has been shown to activate actin cytoskeleton, FKS1 and PIM1 gene. Sodium chloride stimulated UDP-glucose production, which is the substrate of β-glucan synthesis and actin synthesis and increased cell wall thickness. Consequently, they stimulate β-glucan formation. From the many previously reports, the additives could be activate β-glucan synthesis. Surprisingly, there is no report on the combination effect of additives on improvement of β-glucan formation in *S. cerevisiae*. Therefore, this research aimed to investigate the effect of additives on growth, β-glucan production, structure and molecular weight of β-glucan. Finally, the immunomodulatory properties of β-glucan from *S. cerevisiae* were assessed.

1.1 Objectives

The specific objectives of this study were to investigate influences of additives namely EDTA, SDS and NaCl on β-glucan of *S. cerevisiae*, structure and properties of β-glucan produced .

CHAPTER II
LITERATURE REVIEW

2.1 β-Glucans

β-Glucans are a group of polysaccharides which are composed of glucose units linked with beta-glycosidic bonds (Klis *et al*., 2002). They can not be dissolved in water at room temperature but they show high water-holding capacity and thickening properties which allow them to be used as a fat replacer and a stabilizer (Reed and Nagodawithana, 1991; The National Innovation Agency(NIA), 2005). Consequently, they have been used in many industries: the pharmaceutical industry, food and feed industries and cosmetics industry (Reed and Nagodawithana, 1991; Zülli and Saceker, 1994; Zülli *et al.*,1996; Suphantharika *et al*., 2003; Kogan and Kocher, 2007; Satrapai and Suphantharika, 2007; Kunlayavattanakul and Lourith, 2008). β-Glucans also exhibit medicinal properties such as antitumor, antiviral, antimicrobial, antioxidant, mycotoxins absorption, immune response stimulation in animals such as shrimp, weaned pigs and mice; and the reduction of cholesterol and glucose in blood (Ostroff, 1997; Nicolosi *et al*.,1999; Ross *et al*., 1999; Hayen and Pollmann, 2001; Ortuno *et al*., 2002; Chen and Seviour, 2007).

β-Glucans have been found in many natural sources such as bacteria, yeast, fungi, algae and higher plants with differences in structure and functional properties (Chen and Seviour, 2007). For examples, bacteria have been shown to contain only β-(1,3)-glucosidic linkages with linear chains (Fig. 2.1), fungi have been found to have β-(1,3)-glucan linear chains and the short branch of β-(1,6)-glucan (Fig. 2.2), in plants have been shown to include linear chains and be composed of a mixture of β-(1,3) and β-(1,4) linkages (Fig. 2.3). β-Glucan structure different from sources are summarized in Fig 2.4. The macromolecular structure of β-glucans depends on both the source and method of isolation, varying in the distribution and length of side chains, which provide for complex tertiary structures stabilized by inter-chain hydrogen bonds. In *S. cerevisiae*, cell

walls are composed of β-glucan about 55-65 %, which consists of long chains β-(1,3)-glucan representing about 85 %. This class of glucan has an average of 15,000 glucose residues per molecule and short chains of β-(1,6)- glucan which predominantly harbors β-(1,6)-linkage, but also contains some β-(1,3)-linked branch points and exhibits a degree of polymerization of approximately 140 glucose residues, represent about 15 % (Fig. 2.5) (Klis *et al*., 2002; Ha *et al.*, 2006).

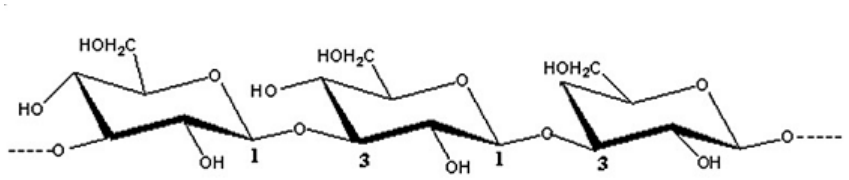


Fig 2.1 The structure of β-glucan from bacteria; curdlan from *Alcaligenes faecalis*

Source: Chen and Seviour (2007)

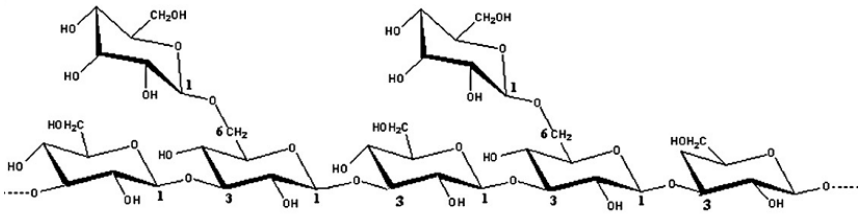


Fig 2.2 The structure of β-glucan from fungi; lentinan from *Lentinula edodes*

Source: Chen and Seviour (2007)

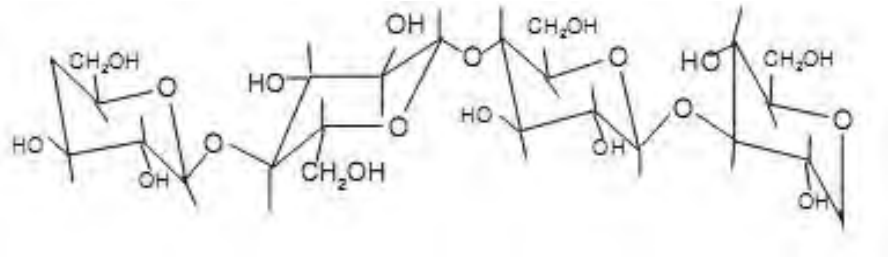


Fig. 2.3 Structure β-glucan from plant : oat

Source: Kim *et al.* (2006)

β-Glucan type	Structure	Description
Bacterial		Linear β1,3 glucan (i.e. Curdlan)
Fungal		Short β1,6 branched, β1,3 glucan (i.e. Schizophyllan)
Yeast		Long β1,6 branched, β1,3-glucan (i.e. WGP β-glucan, Betafectin™)
Cereal		Linear β1,3/β1,4-glucan (i.e oat, barley, rye)

Fig 2.4 The structure of β-glucan from different sources

Source: Volman, Ramakers and Plat (2007)

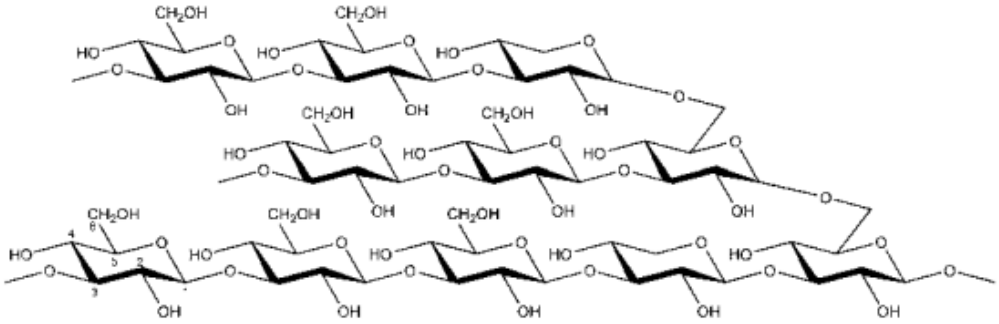


Fig 2.5 β-Glucan from *S. cerevisiae*

Source: Barsanti *et al.*(2011)

2.1.1 Structure of β-glucan from *S. cerevisiae*

In *S. cerevisiae*, β-glucan are composed of the linear long chains β-(1,3)-glucan and the branching β-(1,6)-polymer, which is amorphous in structure, and acts as flexible glue by forming covalent cross-link to β-(1,3)-glucan, chitin and cell wall mannoprotein (Lesage and Bussey, 2006). Krainer *et al.* (1994) studied the structure of β-glucan from *S. cerevisiae*. The spectra indicate that the β-glucan in the cell wall assumes a helical conformation (Fig 2.6). β-(1,3)-Glucan can show three different conformations; triple helical, single helical and random coil. Triple helical conformation is stable at neutral pH and ambient temperature. An increase in pH will change the single helical conformation to random coil structure (Leung *et al.*, 2006). The intramolecular force which maintains the single helical conformation and intermolecular force which maintains the triple helical conformation are hydrogen bonds (Reed and Nagodawithana, 1991;

Zekovic *et al*., 2005). An increase in temperature, hydroxide ion concentration or chaotropic agent concentration; dimethyl sulfoxide (DMSO), will disrupt hydrogen bonds and causes to change from triple helix to single helix, and single helix to random coil (Fig. 2.7) (Leung *et al.*, 2006)**.**

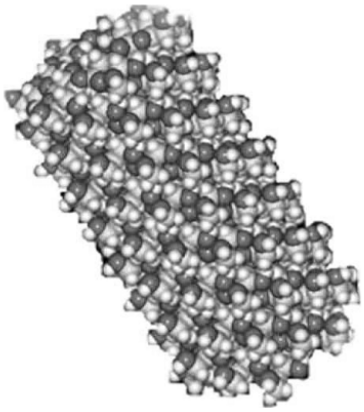


Fig 2.6 Triple helix structural conformation of β-(1,3)-D-glucan forming the backbone of the yeast cell wall
Source**:** Zekovic *et al*.(2005**)**

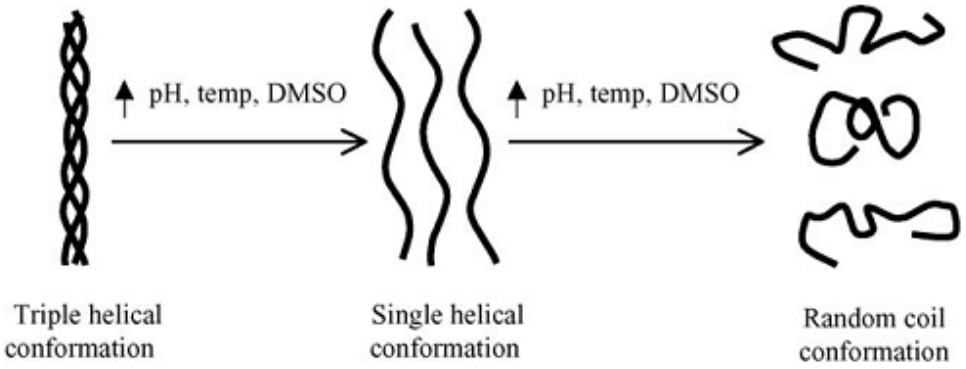


Fig 2.7 The transition of β-glucan triple helical conformation to single helical conformation and random coil conformation in response to physical and chemical treatment
Source**:** Leung *et al*.(2006)

Yeast cell wall consists of alkali–insoluble β-glucan in the inner layer and alkali–soluble β-glucan in the middle layer. The alkali-soluble glucan constitutes a minor component (15-20 % w/w of total glucan) of the cell wall, and has little structural importance. The major glucan component in the cell

wall is the alkali-insoluble, which composed of about 35 % of the wall (Walker, 1999). This glucan consists of a β-(1,3)-linked backbone with a high molecular weight (240,000), which consists of 3 % β-(1,6)-interaction linkages, and is responsible for both the structure and intergrity of the cell wall (Ha *et al.*, 2006).

The solubility of β-glucan associates with the degree of polymerization (DP). β-Glucans are completely insoluble in water when DP ›100. Solubility increases when DP decreases. β-Glucans can be classified according to their solubility properties as below:

a. alkali-insoluble, acetic acid insoluble β-(1,3)-glucan

b. alkali-soluble β-(1,3)-glucan

c. highly branched β- (1,6)-glucan (Zekovic *et al.*, 2005)

2.1.2 Properties of β-glucan from *S. cerevisiae*

The functional, nutritional and medicinal properties of β-glucan from *S. cerevisiae* have been shown as follow:

2.1.2.1 Functional properties

Satrapai and Suphantharika (2007) studied β-glucan as fat replacer in mayonnaise by partially substitution at level 25, 50 and 75 %, which indicated that high level of β-glucan could reduce fat particle size and caloric value of the products, and increased water-holding capacity and water activity of the products. The replacement of β-glucan (BG) in 6 % rice starch (RS) with the mixing ratio of RS /BG at level 6.0/0.0, 5.7/0.3, 5.4/0.6, and 5.0/1.0 (w/w) were investigated. It was found that β-glucan could increase viscosity of the RS/BG mixed suspensions during pasting and also retard the retrogradation, syneresis and hardness of the mixed gels during refrigerated storage.

2.1.2.2 Nutritional properties

β-Glucans are not degraded by human enzymes, thus provide them with nutritional fiber properties as a dietary fiber (Reed and Nagodawithana, 1991; The National Innovation Agency (NIA), 2005; Zekovic *et al*., 2005). The greastest interest in these fibers is due to the fact that they demonstrated protective hypocholesterolemic effect and reducing risk of chronic diseases (Mantovani *et al*., 2008). It is also known that β-glucan can reduce blood cholesterol levels. The ingestion of β-glucan increased intestinal viscosity and reduced choresterol absorption, thereby promoting its excretion (Kim *et al.*, 2006).

2.1.2.3 Medicinal properties

β-Glucan from *S. cerevisiae* has been found as an immunodulator in human and animals, antitumor, absorption mycotoxin, antimicrobial agent and antioxidant as follow:

2.1.2.3.1 Immunodulator in human and animals

Many studies have been shown that β-glucan from *S. cerevisiae* exhibits immunomodulating activity. The efficiency of the activity depends on the effect of binding to specific sites (receptors) on monocytes, macrophages and granulocytes. β-Glucan receptors are CR3 (complement receptor 3), lactosylceramind (Zimmerman *et al.*, 1998; Akramienė *et al*., 2007; Goodridge, Wolf and Underhill, 2009), scavenger receptors and dectin-1 (Kogan and Kocher, 2007; Goodridge, Wolf and Underhill, 2009). Adams *et al.* (2008) showed that dectin-1 is highly specific for glucans with a pure (1→3)-β-linked backbone structure. Structure analysis demonstrated that glucan backbone chain length and (1→6)-β-side chain branching strongly influenced dectin-1 binding affinity (Tsoni and Brown, 2008). Dectin-1 is a C-type lectin–like pattern recognition receptor that binds glucans and induces innate immune responses to fungal pathogens (Chen and Seviour, 2007). The stimulating activity leads to the production of monocytes, granulocytes, increased

antibody titres, boosted cytokine release (including IL-1, IL-2, IL-6 and TNF-α) and prostaglandin E_2 production (Chen and Seviour, 2007; Volman, Ramakers and Plat, 2007; Novak and Vetvicka, 2008). Ross *et al.* (1999) found that soluble yeast β-glucan iC3b-recepters opsonized tumor cells, phagocyte and NK cells, then secretion of the cytokines TNFa, IFNg and IL-6 were produced. In addition Ostroff (1997) determined that poly-β-(1,6)-glucotriosyl-β-(1,3)-glucopyranose glucan (PGG-glucan) increased the formation of neutrophil and monocyte in rats. Moreover, Wakshull (1999) showed that PGG-glucan is an immunomodulator which enchances leukocyte anti-infective activity and enhances myeloid and megakaryocyte progenitor proliferation. The effect of immunoadjuvants depends on primary structure, degree of branching (DB), solubility, molecular weight, the charge of polymers and structure in aqueous media, are involved in the biological activity (Okazaki *et al.*, 1995; Ross *et al.*, 1999; Zekovic *et al.*, 2005; Chen and Seviour, 2007; Volman, Ramakers and Plat, 2007). Generally, β-glucan from *S.cerevisiae* has been exhibited DB as 0.2-0.3 (Novak and Vetvicka, 2008). β-Glucan with high molecular weight and lower branching ratios induced TNF-α released by macrophages (Okazaki *et al.*, 1995). β-Glucans with $0.2 \leq DB \geq 0.3$, $100 \leq MW \geq 200$ KDa, and a triple-helix structure are more effective biologically activity (Zekovic *et al.*, 2005; Mantovani *et al*., 2008). Many researchers showed that β-glucan from *S. cerevisiae* stimulated immunity in animals. For instance, Hayen and Pollmann (2001) found that *S. cerevisiae* β-glucan stimulated the growth of weaned pigs. In addition, Suphantharika *et al.* (2003) found that brewer's yeast β-glucan (BYG) has immuno stimulating properties. *In vitro*, this glucan significantly enhanced phenoloxidase (PO) activity of black tiger shrimp hemolymph as compared to controls without added glucan. Also *in vivo*, an oral administration of 0.2 % (w/w) in diets for 3 days significantly increased the PO-activity of the shrimp. Similarly, spent brewer's yeast β-glucan has been shown to induce release of prophenoloxidase from intact black tiger shrimp haemocytes, when fed to shrimp at 0.2 % (w/w of the feed) for 3 days (Thanardkit *et al.*, 2002). In addition, *S. cerevisiae* β-glucan 0.1 % in feed has been found that it enhanced MPO (Myeloperoxidase) and lysozyme levels, superoxide production,

haemagglutination titre and level of protection against *A. hydrophila in* Asian catfish, *Clarias batrachus* (Kumari and Sahoo, 2006). In deed, Brattgjerd, Evensen and Lauve (1994) studied the immunostimulatory effect of M-glucan from *S. cerevisiae* on Atlantic salmon. The results indicated that M-glucan elevated the activity of the non-specific part of the immune system. Moreover, Cain *et al.* (2003) showed that *S. cerevisiae* glucan exhibited enhanced protection from bacterial challenge (*Vibrio anguillarum*, *V. salmonicida* and *Yersinia ruckeri* infections) in Catfish (*Ictalurus punctatus*, Racnesque). Furthermore, β-glucan from *S. cerevisiae* has exhibited the ability to increase plasma interleukin-6, plasma interleukin-10 and tumer necrosis facter-α in weaned piglets (Li *et al.*, 2005; Li *et al*., 2006).

There are many methods for immunomodulatory assay, Nitric Oxide(NO) assay is one of the methods. Nitric Oxide (NO) is produced from active macrophage cell by nitric oxide synthase (Fig. 2.8). Macrophage cells; RAW 264.7 used in this study were presented in Fig. 2.9. Then NO is oxidized to NO^-_2 (nitrite), NO^-_3 (nitrate) and determined as a colored azo-dry product of Griess reaction, which composed of N-1-napthylethylenediamine dihydrochloride (NED) and phosphoric acid that absorbed visible light at 540 nm (Fig. 2.10 and Fig. 2.11) (Marletta, 1993; Wink and Mitchell, 1998; Promega Corporation, 2010).

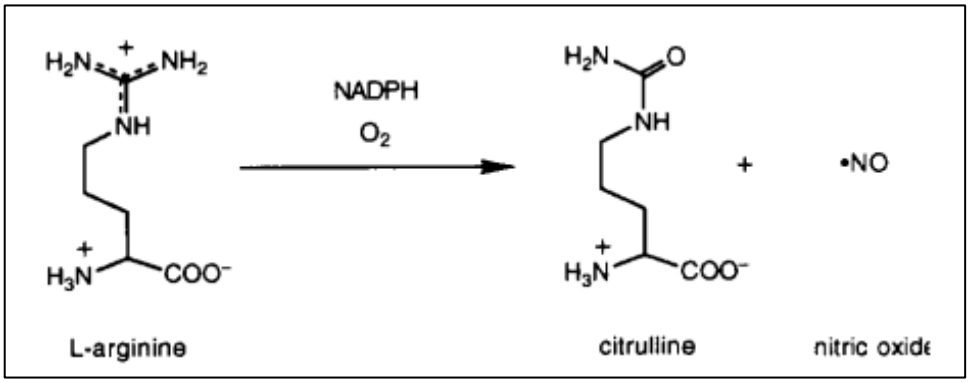


Fig. 2.8 Reaction catalyzed by nitric oxide synthase

Source: Marletta (1993**)**

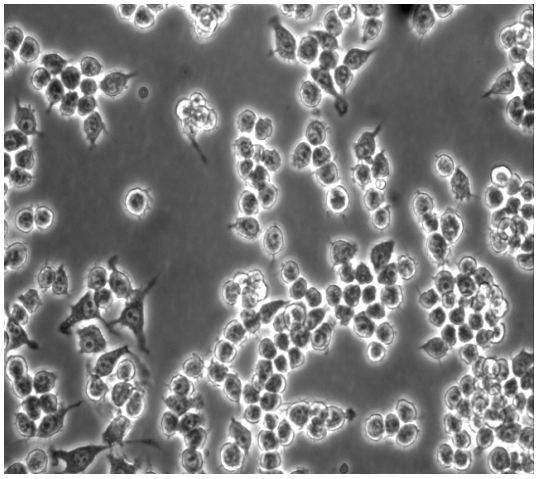


Fig. 2.9 Macrophage cells (RAW 264.7) cultured in DMEM medium for 24 h. The image was taken by using light microscope at magnification 400X

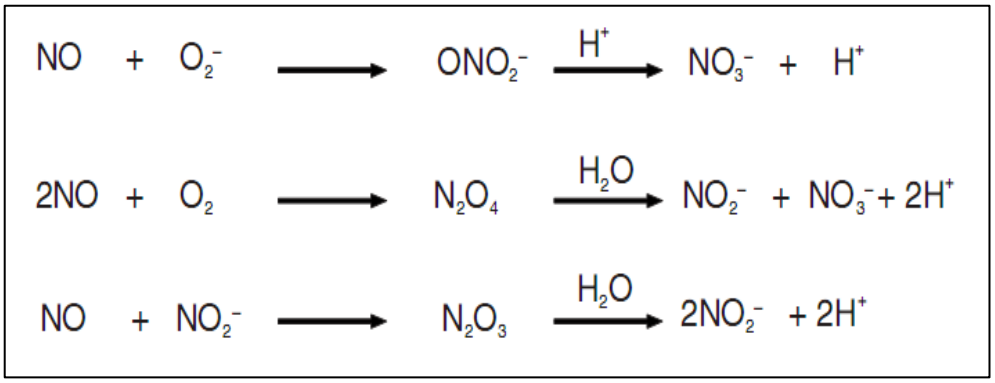


Fig. 2.10 The conversion of Nitric Oxide (NO) into nitrate (NO^-_3) and nitrite (NO^-_2) by reaction in system

Source: Promega Corporation (2010)

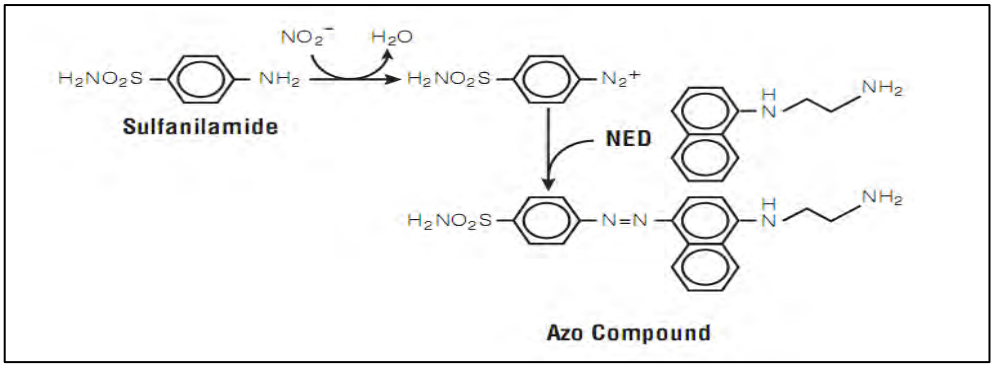


Fig. 2.11 Chemical reactions involved in the measurement of nitrite (NO^-_2) using the Griess Reagent System

Source: Promega Corporation (2010)

The method for test the viability of macrophage cell is MTT assay. This method is based on the cleavage of the yellow tetrazolium salt (MTT; 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to purple formazan crystals by mitochondrial reductase in metabolic active cell (Bopp and Lettieri, 2008). In the reaction, the MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble colour (dark purple) formazan product. The cell are then solubilised with organic solvent (eg. DMSO, isopropanol) and the solubilised formazan reagent is measured by using spectrophotometer at 570 nm (Fig 2.12) (Ebada *et al*., 2008).

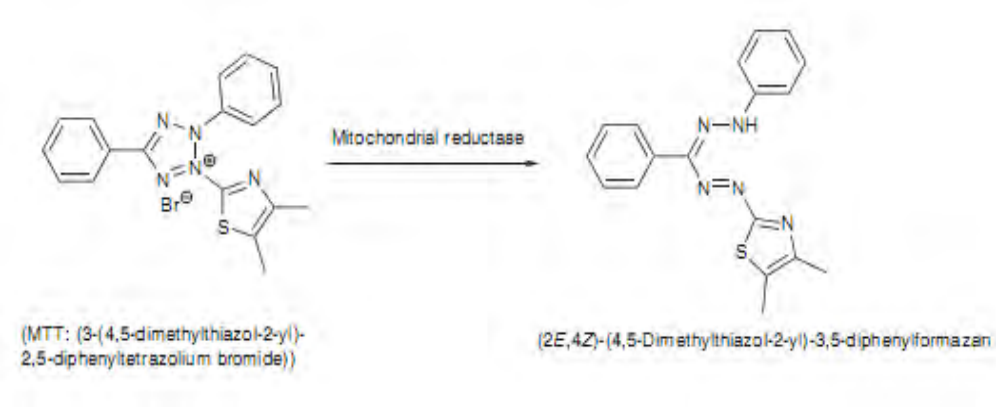


Fig. 2.12 Principle of cytotoxicity MTT assay
Source: Ebada *et al*. (2008)

2.1.2.3.2 Antitumor

Akinobu (2003) measured the immunological activity, antitumor effects, and radioprotective effects of β-(1,3)-D-glucan (Macroglucan) extracted from *S. cerevisiae*. Macroglucan 0, 200, 400, and 800 mg/kg were administered intraperitoneally to C3H/HeJ mice. The results showed that the levels of cellular cytotoxicity, NK(Natural Killer) and LAK(Lymphokine-activated killer) cell activity in the group administered Macroglucan were significantly higher than those in the control group. Furthermore, β-glucan from *S. cerevisiae* (20 or 100 μg/ml of culture) exhibited the activation of interferon-γ (IFN-γ) production *in vitro* and *in vivo* (Pelizon *et al.*, 2003). Moreover, Khan

(2004) found that zymozan can stimulate the activity of PMNs (polymorphonuclear leukocytes) both in blood and fluid PMNs in Wistar male rats.

2.1.2.3.3 Mycotoxins absorption

S. cerevisiae β-glucan has been shown to absorb various mycotoxins; such as zearalenone, aflatoxin β1, deoxynivalenol, ochratoxin A and patulin. Its admixture in the animal feed has been reduced the toxic effect of mycotoxin substances (Kogan and Kocher, 2007). The absorption properties were expected from hydrogen bonds and van der walls force in β-glucan structure (Novak and Vetvicka, 2008). Moreover, Yiannikouris *et al*. (2004) found that alkali-insoluble β-glucan fraction had greater affinity to absorb zearalenone (up to 50 %) than the alkali-soluble fraction (ca. 16 %).

2.1.2.3.4 Antimicrobial agent

Lowry *et al*. (2005) reported that β-glucan decreased the incidence of *Salmonella enterica* serovar Enteritidis(SE) organ invasion in immature chickens. In addition, Hetland and Sandven (2002) found that β-glucan from baker's yeast inhibited the growth of *Mycobacterium tuberculosis in vitro* and significantly stimulated the concentration of interferon-r and nitric oxide in bronchoalveolar lavage fluid from pigs (Jung *et al.*, 2004). Moreover, *Artemia* supplemented with β-glucan has been shown to resist to the effects of pathogens: *Vibrio campbellii* and *V. proteolyticus* (Marques *et al.*, 2006). Furthermore, Jung *et al.* (2004) investigated the potential antiviral effect of *S. cerevisiae* β-glucan on the pneumonia induced by swine influenza virus (SIV). The result was shown that *S. cerevisiae* β-glucan reduced the pulmonary lesion score and viral replication rate in SIV-infected pigs. In addition, poly-β-(1,6)-glucotriosyl-β-(1,3)-glucopyranose (PGG) glucan significantly reduced *Escherichia coli* and *Staphylococcus aureus* in animal (Onderdonk *et al.*, 1992). Other authors showed that PGG-Glucan (Betafectin®); a highly purified of

soluble β-(1,6)-branched-β-(1,3)-link glucan isolated from *S. cerevisiae* enhanced clearance of a multiple an antibiotic resistant *Staphylococcus aureus* and an increase in monocytes and neutrophils (Liang *et al.*, 1998).

2.1.2.3.5 Antioxidant property

Jaehrig (2007) studied the antioxidative activity of a derivative of alkali-insoluble β-(1,3)-glucan in water-soluble by carboxymethylation compared with water-soluble D-mannitol. β-(1,3)-glucan derivative has the scavenging activity much stronger than D-mannitol. Similarly, carboxymethyl β-(1,3)-glucan (CMG) from *S. cerevisiae* showed high radical scavenging activity by using the technique of electron paramagnetic resonance (Kogan *et al.*, 2005).

2.1.2.3.6 Reduction of blood cholesterol

When β-glucan was supplemented in diet 15 g/day in obesity human for 14 days, the result indicated that the total cholesterol concentration was significantly reduced while the HDL-cholesterol concentration was increased (Nicolosi *et al.***,** 1999). Moreover, Reed and Nagodawithana (1991) confirmed that yeast β-glucan is an excellent dietary fiber for in human for formulation and can reduce incident of coronary heart disease. In addition, Betamune; insoluble yeast derived glucan and whole glucan particle (WGP) have been found to reduce blood cholesterol level in mice (Vetvicka and Vetvickova, 2009).

2.2 Applications of β-glucans in industry

Nowsaday, β-glucan can be produced from by-product in brewing and baking industries. Moreover, *S. cerevisiae* can be grown easily and rapidly in many culture mediums at a low production cost and its whole genome is already

known. Yeast β-glucan has various properties which are better than that found in other sources (Nguyen, Fleet and Rogers, 1998). In addition, the U.S. Food and Drug Administration (FDA) has given the β-glucan derived from baker's yeast extract a GRAS (Generally Recognized as Safe) rating (The National Innovation Agency (NIA), 2005; Zekovic *et al.,* 2005).

There are many researches showing that β-glucan from *S. cerevisiae* has more benefit than other sources. In general, *in vitro* studies have suggested that large molecular weight or particular β-glucans (such as zymosan) can directly activate leukocytes, stimulate their phagocytic, cytotoxic, and antimicrobial activities, including the production of reactive oxygen and nitrogen intermediates (Chen and Seviour, 2007; Volman, Ramakers and Plat, 2008). Moreover, intermediate or low molecular weight β-glucans (such as glucan phosphate) have shown biological activity *in vivo* (Akramienė *et al.* 2007). Moreover, the long branched side chain structure of β-glucan from *S. cerevisiae* has been shown to be the most effective immune enhancing source and it could be used to improve the functional properties of the food products (Reed and Nagodawithana, 1991; The National Innovation Agency (NIA), 2005; Satrapri and Suphantharika, 2007). *S. cerevisiae* β-glucans exhibited immunomodulatory activities by enhancing the host immune function and activating macrophages, neutrophils and NK (Natural Killer) cells. In deed, yeast β-glucan showed ability to bind specifically with β-glucan receptor in macrophages and to stimulate the production of cytokines such as dectin-1, TNF (tumor necrosis factor)-alpha and AL(interlukin)-1β. Furthermore, β-glucans showed potential to be used as anti-infective, anti-tumor, radioprotective, wound-healing agents (Chen and Seviour, 2007).

However, β-glucan isolated from cell wall of baker's yeast; *S. cerevisiae* is a water insoluble particulate polymer which is not suitable for pharmaceuticals application (Zülli *et al.,* 1996; Zülli *et al.,* 1998; Wang *et al.,* 2005). Thus, development of β-glucan structure for improving immunomudulatory properties factors for the production of cytokines from macrophage is important. For examples, Zülli *et al.* (1996) and Zülli *et al.* (1998) modified it to carboxymethylglucan (CM-glucan), a water soluble product (Fig. 2.13) for use as cosmetic ingredient. In addition, the combination of sonication and spray-drying

has been shown to receive a homogenous preparation of 1-2 μm diameter β-glucan enhanced phagocytosis of mouse peritoneal macrophages significantly (Hunter Jr, Gault and Berner, 2002). Moreover, Hromádková *et al*. (2003) recovered the aqueous medium as water-insoluble particles of *S. cerevisiae* β-glucan by different drying methods: solvent exchange(GE), lyophilization(GL), and spray drying (GS). The drying method has been shown to affect the microstructure of the glucan particle leading to differences in their physical properties (particle size and shape, swelling capacity, interparticle hydrogen bonding) as well as in the flow and viscoelastic properties. Also, its immunomodulatory activity was two-fold higher than the GL and GE samples. Furthermore, Williams *et al.* (1991) studied the method to change the water insoluble glucan to water soluble glucan (glucan phosphate); it has the ability to prepare an immunologically active, non toxic glucan and greatly to use in clinical purpose.

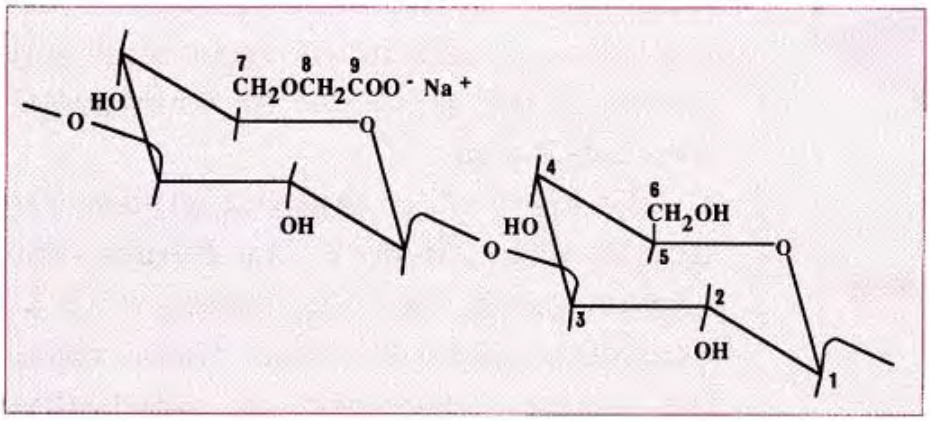


Fig 2.13 Structure of CM-Glucan
Source: Zülli and Saecker (1994)

2.2.1 Pharmaceutical industry

The pharmaceutical industry is exploring the therapeutic potential of yeast glucan as a biomedical agent to prevent infections and cancer. The National Innovation Agency (NIA)(2005) reported that Innovacan™; β-glucan from *S. cerevisiae* is a supplement for improving the serum lipid profile in human, lowering risk of heart disease and improving cardiovascular health in human. In

addition, zymosan from *S. cerevisiae* activated the immune response in humans administered the human immunodeficiency virus (HIV) vaccine by stimulating Th cell-mediated immunity through activation of the complement system and interferon gamma (Ara *et al*., 2001).

2.2.2 Food and Feed industries

In the food industry, yeast β-glucan is employed as thickening agent to improve the texture of food as stabilizer, fat replacer, used as a thickener, water-holding agent or emulsifying stabilizer in food products such as soups, sauces, desserts, sausage, meat-based product, ice cream and salad dressing (Reed and Nagodawithana, 1991; The National Innovation Agency (NIA), 2005). For examples; Innovacan™; β-glucans from *S. cerevisiae*; is employed for improving food texture. Moreover, Immunose™; β-glucans and mannan oligosaccharide from *S. cerevisiae* have increased the growth rate, the number of granulocytes and survival rate of shrimp (The National Innovation Agency(NIA), 2005). Bio-Mos® and MTB 100®; specific commercials yeast cell wall polysaccharide by Alltech Inc. (Nicholasville, KY) have been found to block fimbriae of pathogenic bacteria; such as *E. coli*, *Salmonella* sp. and *Clostridium* sp.; and exhibited to absorb a wide rang of mycotoxins, respectively. Taken together, they have been used as feed supplement to enhance weanling piglets protection from bacteria infections and increase weight gain (Kogan and Kocher, 2007).

2.2.3 Cosmetic industry

Many researchers found that carboxymethylglucan from *S. cerevisiae* was an efficient ingredient in cosmetic products. For instance, Zülli *et al*.(1996) showed that carboxymethylated β-(1,3)-glucan 0.04 %, 0.4 % in o/w emulsion increased in skin humidity after tested on human skin at forearm skin of volunteers over a 14 days period and decrease of transepidermal water loss. Furthermore, they exhibited the increase in rate of SC (Stratum Corneum) cell renewal. Moreover, 0.04 %, 0.2 % of CM-glucan in o/w emulsion showed the

inhibition of squalene peroxidation after UVA exposure. In deed, 0.005 % and 0.01 % of CM-glucan in o/w emulsion exhibited human keratinocytes protection from the depletion of antioxidant molecules (Zülli *et al.*, 1998). Moreover, CM-glucan 100 μg/ml has been shown to protect fibroblast and keratinocyte from the antioxidant molecule under oxidative stress caused by UV-A radiation (Zülli *et al.*, 1995). From that result, CM-glucan from baker's yeast is a new potent biological agent for cosmetic and dermatological applications (Zülli and Saecker, 1994; Zülli *et al.*, 1996; Zülli *et al.*, 1998; Kanlayavattanakul and Lourith, 2008).

2.3 *S. cerevisiae*

S. cerevisiae is a species of budding yeast in the Saccharomycetaceae family, Saccharomycetales order, Saccharomycetes class, Saccharomycotina subphylum and Ascomycota phylum (Fig. 2.14). It is an eukaryotic unicellular fungi, which plays important role in baking and brewing (Alexopoulos, Mims and Blackwell, 1996).

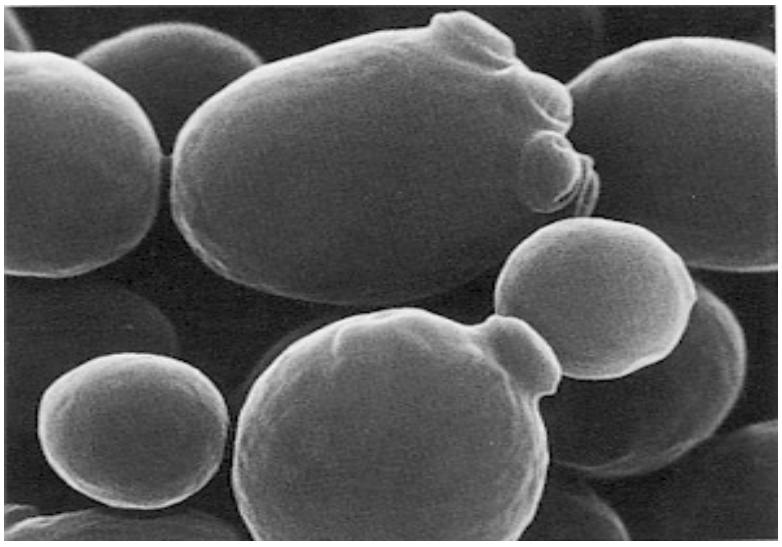


Fig 2.14 Scanning electron micrograph of *S. cerevisiae*
Source: Franklin (2009)

2.3.1 Cell wall of *S. cerevisiae*

Yeast cell walls of *S. cerevisiae* have been considered as an interesting source of β-glucan production. In a cell, approximately 30 % of cell dry weight is the cell wall, which was ca. 70-100 nm thick (Fig. 2.15) (Klis *et al.*, 2002) and comprises of 15 % protein and 85 % polysaccharides (Reed and Nagodawithana, 1991). The cell wall is an external envelope that surrounds yeast cells. This structure is essential for maintaining cell morphology and to protect cells from the external environment by preserving their osmotic integrity (Popolo, Guatieri and Ragni, 2001; Klis, Boorsma and Groot, 2006). The yeast cell wall comprises of layers of polysaccharides and mannoprotein. β-Glucans and chitin are responsible for rigidity of the cell wall affecting its morphology and shape. Mannoprotein and its carbohydrate portion (α-mannan) are responsible for cell–cell recognition and interactions with the environment. They also determine immunological specificity of the yeast (Klis *et al*., 2002; Liu, 2008) (Fig 2.16, Table 2.1). The study of *S. cerevisiae* cell wall demonstrated the composition varies from 1.4 to 7.9 % of chitin, 28 to 67 % of mannan, 28 to 46 % of β-(1,3)-glucan and 5 to 11 % of β-(1,6)-glucan which depends on environmental condition (Aguilar- Uscanga and Francois, 2003).

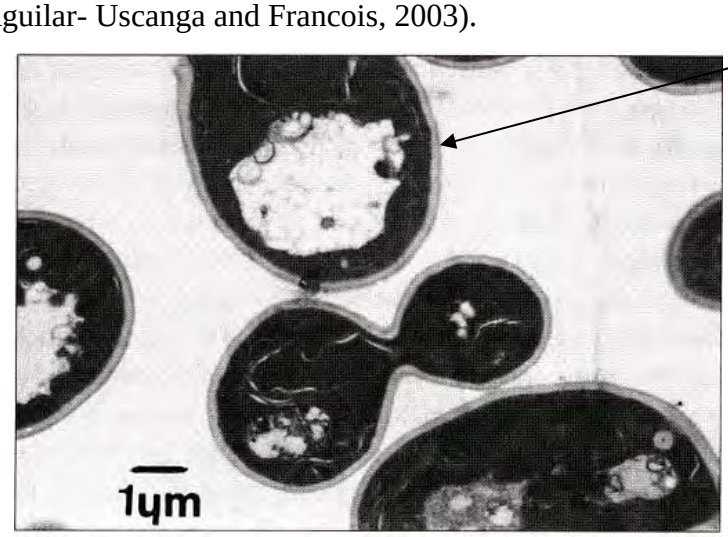


Fig 2.15 The cell wall of *S. cerevisiae*

Source : Zülli and Saecker (1994)

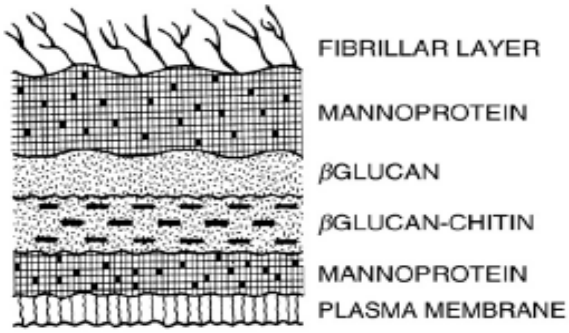


Fig 2.16 Diagram *S. cerevisiae* cell wall showing the major polysaccharide components

Source : Kogan and Kocher (2007**)**

Table 2.1 Macromolecules of the cell wall of *S. cerevisiae*

Macromolecule	% of wall mass	Mean M_r(DP) (kDa)
Mannoprotein	30 - 50	Highly variable
β-(1,6)-glucan	5 - 10	24(150)
β-(1,3)-glucan	30 - 45	240(1500)
Chitin	1.5 - 6	25(120)

Source : Klis, Boorsma and Groot (2006**)**

The cell wall of *S.cerevisiae* is composed of β-(1,3)-glucan, β-(1,6)-glucan, chitin, and mannoprotein. These components are linked to each other as macromolecular complexes in which β-(1,6)-glucan acts as a cross-linker, being attached to β-(1,3)-glucan and mannoproteins, and occasionally to chitin. β-(1,3)-Glucan is covalently linked to chitin to make up the inner cell wall, which is the main element responsible for the mechanical strength of this structure. Mannoprotein form the outer cell wall layer. Two main classes of proteins are coupled covalently to cell wall polysaccharides: GPI-dependent cell wall proteins (GPI-CWPs), which are generally linked to β-(1,3)-glucan through a β-(1,6)-glucan chain, and PIR proteins (PIR-CWPs), which are directly linked to β-(1,3)-glucan (Fig. 2.17) (Klis *et al.*, 2002; Klis, Boorsma and Groot, 2006). β-(1,3)-Glucan is responsible for the mechanical strength of the cell wall (Klis, 1994).

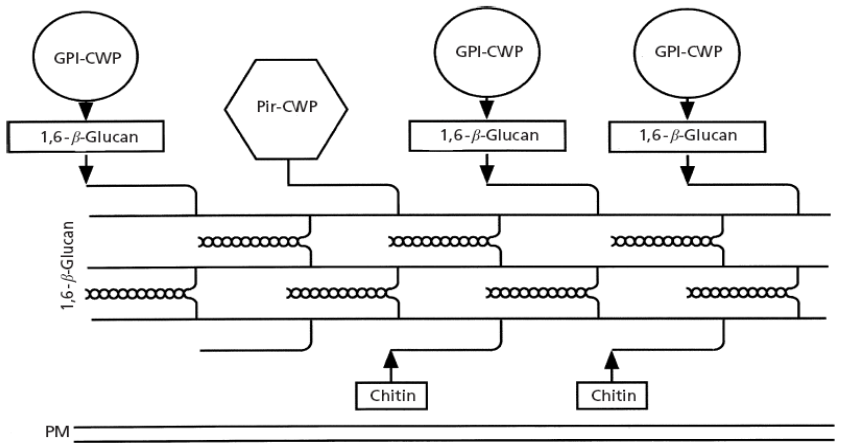


Fig 2.17 Model for the molecular architecture of the *S. cerevisiae* cell wall
Source: Smits, Ende and Klis (2001)

2.3.2 Cell wall biosynthesis in *S. cerevisiae*

Cell wall synthesis regulates in the cell cycle during budding process (Cabib, Drgonov´ and Drgon, 1998). In initiation of a new cell cycle, a chitin ring forms at bud site. Bud growth occurs at the tip (apical growth)(cell wall synthesis is switch on) until the bud reaches a size of approximately two thirds of the mother (cell wall synthesis is switch off). During cytokinesis and cell separation, cell wall synthesis takes place in the mother-bud neck. After degradation of the chitinous primary septum, the two individual moves over to the surface (Fig. 2.18) (Cabib *et al*., 1993; Cabib *et al.*, 2001; Smits, Ende and Klis, 2001). Rho1 is essential regulator of the synthesis (Cabib, Drgonov´ and Drgon, 1998; Inoue *et al.*, 1999; Smits, Ende and Klis, 2001). The shift of Rho1 between the active (Rho1p-GTP) and inactive state (Rho1p-GDP) provide a switching on cell wall synthesis and β-(1,3)-glucan (Cabib *et al.*, 2001). Moreover, fraction B is a protein for cell wall synthesis (Fig. 2.19) (Cabib, Drgonov´ and Drgon, 1998). PCK1 MAP (Mitogen Activated Protein) kinase pathway is the major regulator of cell wall biosynthesis and maintenance of cell wall integrity in *S. cerevisiae* (Igual, Johnson and Johnston, 1996; Martín *et al*., 2000; Wojda *et al.*, 2003).

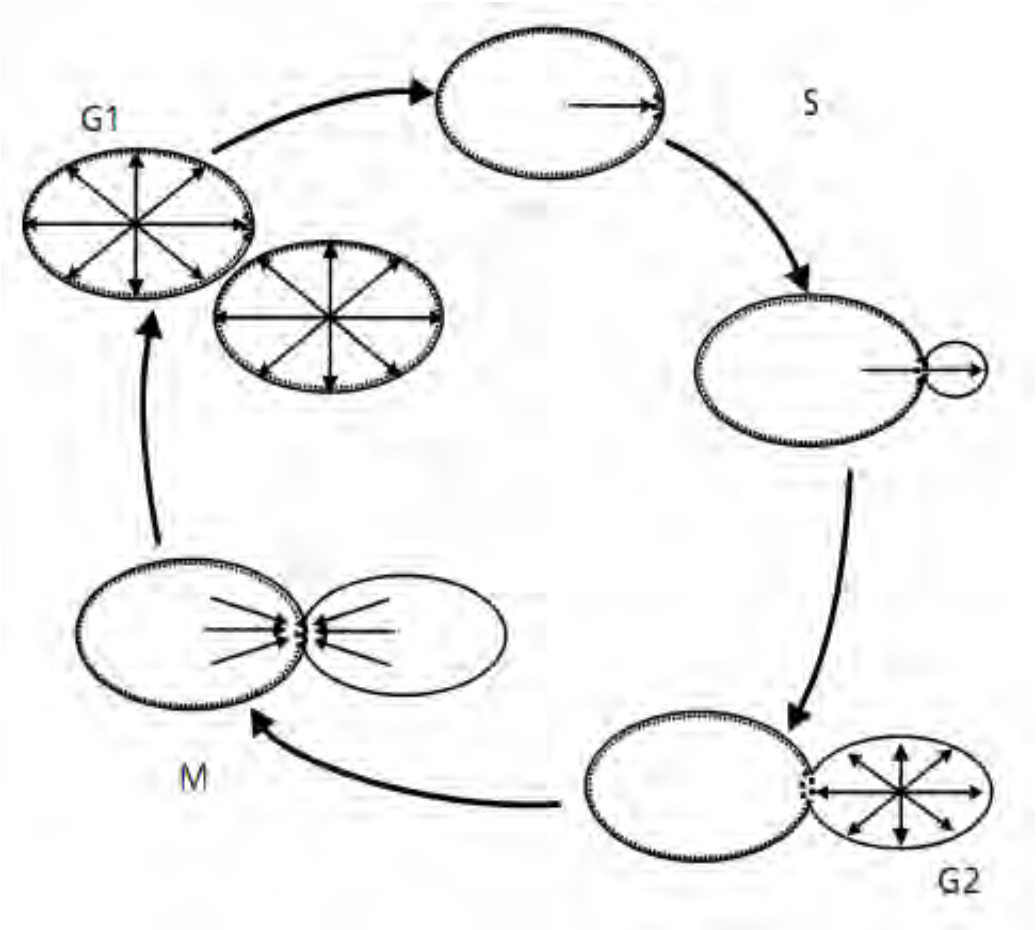


Fig. 2.18 Cell wall biosynthesis occurs during the cell cycle. Dashed lines indicate the presence of chitin, which is generally synthesized only after cytokinesis. Arrows indicate the polarization of the actin cytoskeleton in the stage of the division cycle
Source: Smits, Ende and Klis (2001)

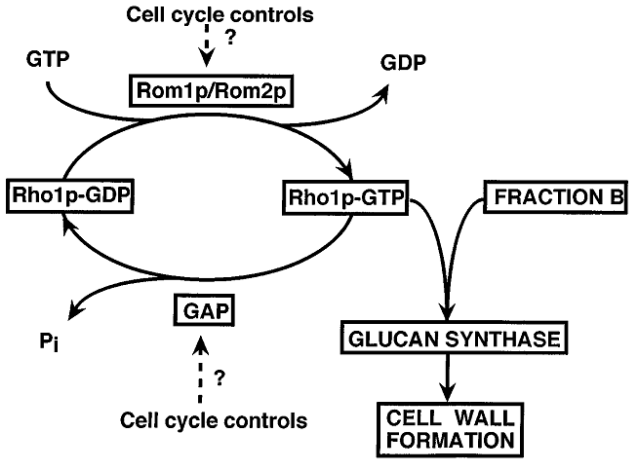


Fig. 2.19 The regulation of Rho1 activity and β-(1,3)-glucan synthesis
Source: Cabib, Drgonov´ and Drgon (1998)

2.3.3 β-Glucan synthesis in *S. cerevisiae*

In *S. cerevisiae,* the β-(1,3)-glucan synthase (EC 2.4.1.34) is a plasma membrane localized enzyme that transfers glucose to producing polymer of β-(1,3)-glucan. Fks1p and Fks2p, are intergral plasma membrane subunits proteins and the activity of the β-(1,3)-glucan synthase is regulated by the intracellular GTP-binding protein Rho1p, which detected at the bud site (Fig. 2.20, Table 2.2) (Cabib *et al*., 1993; Mazur and Baginsky, 1996; Cabib, Drgonov´ and Drgon, 1998; Smits, Ende and Klis, 2001; Klis *et al*., 2002; Klis, Boorsma and Groot, 2006). It has been reported that β-glucan synthesis takes place in the plasma membrane during the budding process of the cell growing under proper conditions (Frost *et al.,* 1994). β-Glucan synthase level is the highest in early exponential phase and declined in stationary phase (Frost *et al*., 1994). GTP (Guanosine triphosphate) is an important activator (Frost, Drakef and Wasserman, 1992; Cabib *et al*., 1993; Frost *et al*., 1994). Cabib, Drgonov´ and Drgon (1998) explained that β-glucan synthesis composed of Rho1-GTP, GDP-glucose and β-glucan synthase complex, consequently actin filament has been happening around plasma membrane (Fig. 2.21). For β-(1,3)-glucan synthesis and β-(1,6)-glucan synthesis, many of the identified gene products such as FKS1, GSC2, RHO1,

FKS3, KRE1 and KRE5 were localized throughout the secretory pathway (Lesage and Bussey, 2006). This led to the hypothesis that the synthesis of β-(1,6)-glucan begins in the endoplasmic reticulum, the product is extended in the golgi, and final processing steps take place at the cell surface and plasma membrane (Montijn *et al.*, 1999; Cabib *et al*., 2001; Vink *et al*., 2004; Klis, Boorsma and Groot, 2006). The pathway of β-glucan synthesis comprises of 2 steps. Step 1, a formation of sugar nucleotides (UDP-glucose) is catalysed by uridine diphosphoglucose pyrophosphorylase (UGPase) as shown in the following equation:

$$\text{Glucose 1-phosphate} + \text{UTP} \rightarrow \text{UDP-glucose} + \text{PPi} \qquad (2.1)$$

Step 2, the formation of β-glucan is catalysed by the β-glucan synthetase group. The β-glucan synthetase group comprises of β-(1,3)-glucan synthase, β-(1,6)-glucan synthase, β-(1,3)-glucan elongase, exo-β-(1,3)-glucanase, exo-β-(1,6)-glucanase and endo-β-(1,3)- glucanase (Walker, 1999; Lesage and Bussey, 2006).

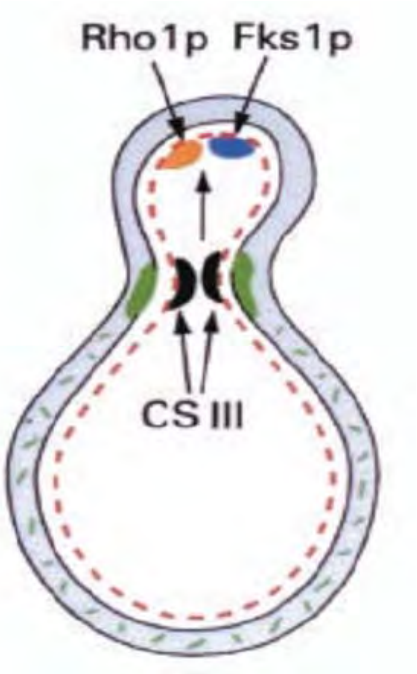


Fig 2.20 The position of Rho1 and Fks1p in budding site of *S. cerevisiae*
Source: Cabib *et al*.(2001)

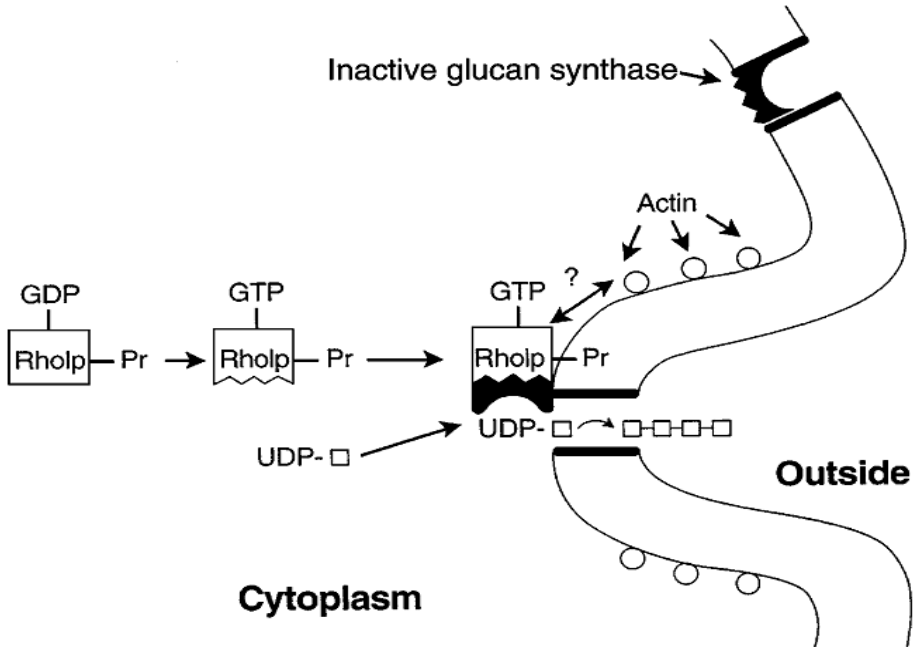


Fig. 2.21 Model for the regulation of β-(1,3)-glucan synthesis at the site of bud emergence. Pr, prenyl group
Source: Cabib, Drgonov´ and Drgon (1998)

2.4 Effect of stress condition on growth and β-glucan production in *S. cerevisiae*

There are several factors which influence the components and morphology of the cell wall during the growth process. Hohmann (2002; 2003) mentioned that when environment condition change, the cell must rapidly adjust itself for growth at the new condition. Moreover, polysaccharide composition, structure and thickness of the cell wall are varied, depending on environment conditions (Waker, 1999; Aguilar-Uscanga and Francois, 2003; Klis, Boorsma and Groot, 2006). For example, the factors that were reported included the type of culture medium and carbon source, pH, temperature, aeration rate and culturing conditions. The study of β-glucan production in a batch fermentor demonstrated

that the most suitable culture medium and type of carbon source, optimum pH, temperature and aeration condition were Yeast Peptone Dextrose (YPD), galactose as carbon source, pH 4, 37 °C and well-aerated conditions ($_pO_2$ > 50% saturation in the medium). However, in a shake flask cultivation, the best carbon source of β-glucan production was sucrose, and the β-glucan yield was higher than that in batch fermentor cultivation (Aguilar-Uscanga and Francois, 2003). It was reported that chemical substances such as 0.5 % of Congo red could directly inhibit the β-glucan synthesis in the protoplast of a cell (Kopeckfi and Gabriel, 1992). On the other hand, the factors affecting β-(1,3)-glucan synthase activity produced by *S. cerevisiae* in cell free extracts has been found as the stimulating agents such as EDTA, fluoride and GTP. The mechanism has been recently proposed for the activation of yeast hexokinase by chelators. In this case it could liberate GTP, from endogenous GTP-cation complexs. GTP (or other nucleotide triphosphate) has been shown to activate β-(1,3)-glucan synthase only in the present of EDTA (Leal *et al.*, 1984). In addition, the best stimulation of β-(1,3)-glucan synthase activity was EDTA in combination with GTP (Guillen *et al.*, 1985).

There are many works reported that salt stimulated phosphoglucomutase (PGM2) and uridine diphosphoglucose pyrophosphorylase (UGPase) which are enzymes of UDP-glucose synthesis. It also increased protein synthesis involving the ACT1 gene actin synthesis (Blomberg, 1995; 2000) but stimulated trehalose turnovered (Parrou, Teste and Francois, 1997). Moreover, exposure of *S. cerevisiae* to hyperosmotic solution of NaCl resulted in cell shrinkage, loss of viability cell, reduction of cell volumn and cell size decreased. Nevertheless, on return to isotonic solution the wall were reversible. In addition, the cell wall and structure have been increased in thickness and change (Morris *et al.*, 1983; Attfield, 1997). HOG (High Osmolarity Glycerol) MAP kinase pathway has been used to response in hyperosmotic condition, due to reorganization of actin cytoskeleton and cell wall; respectively (Slaninová *et al.*, 2000; Wojda *et al.*, 2003). Furthermore, Maneesri *et al.* (2005) found that the β-(1,6)-glucan and total β-glucan were increased when added SDS 0.003 %, hygromycin B 3 ug/ml and the K1 killer toxin (the polypeptide is secreted from *Hansenular mrakii* and

kills a strain of yeast by interfering with the synthesis of DNA, RNA, protein and lipid (Kasahara *et al*., 1994)) 3 ug/ml in YPD agar plate. Kasahara *et al.* (1994) showed that HM-1 killer toxin inhibited the growth of *S. cerevisiae* cell by interfering with β-(1,3)-glucan synthesis. Addition of HM-1 killer toxin with several kinds of oligosaccharides has been blocked either β-(1,3)-glucan or β-(1,6)-glucan whereas α-(1,4)-glucan and chitin did not. Moreover, Delley and Hall (1999) found that SDS (an ionic detergent) destabilised the plasma membrane and cell wall at very low concentrations (Cosano *et al*., 2001; Popolo, Gualtieri and Ragni, 2001). SDS (0.02 %) stimulated the actin cytoskeleton and FKS1 gene. Furthermore, PIM1 gene; which encodes the MAPK (Mitogen Activated Protein Kinase) and the key enzyme in the signal transduction pathway has been activated with 0.03 % SDS (Cosano *et al.,* 2001). Indeed, SDS also activated GDP/GTP exchange activity to RHO1 gene (Bickle *et al.*, 1998). Moreover, cell wall intergrity pathway is activated in hypoosmotic conditions (SDS) due to reorganization of cell wall. The pathway may affected the synthesis of structure of cell wall (Smits, Ende and Klis, 2001; Levin, 2005; Klis, Boorsma and Groot, 2006). In addition, polyamine has been found to affect abnormalities of cell, to increase mannan and decrease β-glucan level in *S. cerevisiae* I 79-5; an ornitine decarboxylase mutant (spc-1) (Miret *et al*., 1992). Furthermore, Coen *et al*. (1994) found that cilofungin and papulaeandin B, two inhibitors of glucan synthase, inhibited galactose incorporation into glucan. Gracía *et al.* (2009) demonstrated that High Osmolarity Glycerol (HOG) and cell wall integrity pathways invole in adaptation in yeast survival to cell wall stress mediated by zymolyase, which hydrolyzes the β-(1,3)-glucan network. Inoue *et al.* (1999) reported that when yeast cells were cultured in YPD medium with 50 mM or 100 mM $CaCl_2$, it was found that the higher Ca^{2+} concentration caused the critical damage to the plasma membrane and glucan synthase. Detergents Brij-35 (β-lactoglobulin) 0.5 % have stimulated β-(1,3)-glucan synthase in *S. cerevisiae* (Frost, Drakef and Wasserman, 1992). Moreover, this enzyme significantly stabilized more than 12 days (4 °C) compared with one without this detergent

only 5 days. However, glucolipid; papulacandin B and lipopeptides; echinocandin B and cilofungin have been shown to inhibit glucan synthesis (Frost *et al*., 1994).

In stress conditions (temperature, pH, hypoosmotic stress), sensors (the protein Wsc1p, Wsc2p, Wsc3p and Mid2p) received signals and transmitted to Rho1p (Inoue *et al.*, 1999). Consequently, mitogen activated protein kinase cascade and MAPK (Mitogen Activated Protein Kinase) pathway, which regulated cell wall integrity and repolarization of actin cytoskeleton were responded (Delley and Hall, 1999; Popolo, Gualtieri and Ragni, 2001; Cabib *et al*., 2001). This is responsible for depolarize glucan synthase FKS1 for repairing general cell wall damage (Delley and Hall, 1999). In addition, FKS2/GSC2 is encoded for alternative subunits of the β-(1,3)-glucan synthase complex in stress condition (Fig. 2.22) (Klis *et al.*, 2002).

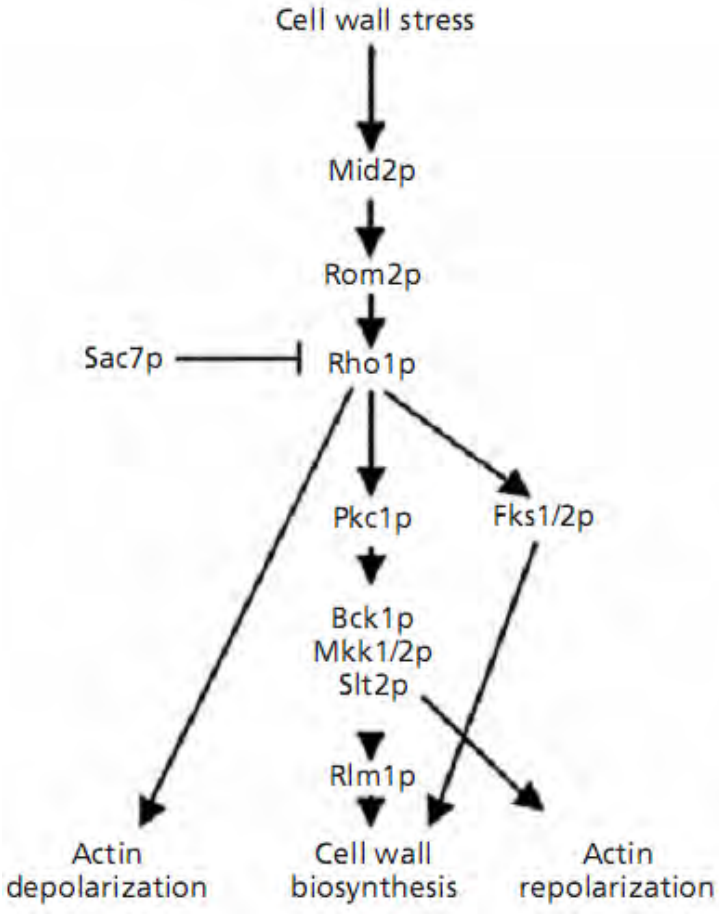


Fig. 2.22 The stress responses in *S. cerevisiae*

Source: Smits, Ende and Klis (2001)

CHAPTER III
MATERIALS AND METHODS

3.1 Materials

3.1.1 Microorganisms

In this study, two commercial baker's yeasts were used including *Saccharomyces cerevisiae* Fermipan® and *S. cerevisiae* Angel®. *S. cerevisiae* TISTR 5059 isolated from pineapple was obtained from Microbiological resources center, Thailand Institute of Scientific and Technological Research. The microorganisms were maintained on YPD agar, incubated at 30 °C for 24 h and then stored at 4 °C prior to use as the starter. Subculture was performed every 30 d. Starter culture was prepared in YPD broth, pH 7 at 30 °C for 24 h.

3.1.2 Apparatuses and Chemical Reagents

3.1.2.1 Apparatuses

Name	Model	Country
Autoclave	TOMY SS-325, Hiclave HVA-85	Japan
Centrifuge	Hettich Universal 32 R	Germany
Incubator shaker	NB-205	Korea
Laminar flow	Nuaire	USA
Magnetic stirrer	Gorning PC-400	USA
pH meter	Qis M360	Germany
Electronic balanced (3 points)	Satorius BP 221S	Germany
Electronic balanced (4 points)	Mettler Toledo AB204	USA
Fourier Transform Infrared Spectrophotometer (FTIR spectroscopy)	IR Prestige 21	Japan

Confocal microscope	Nikon 80i microscope	Japan
Multi Angle Laser Light Scattering (MALLS)	DAWN EOS and Optilab DSP	USA
Microtiter plate reader	Biotek	USA
Freeze dryer	Free Zone® 6,12, and 18 liter	USA
Sonicator	Sonic Dimortrator Gorning PC-4100	USA

3.1.2.2 Chemical Reagents

Name	**Company**	**Country**
Yeast extract	Himedia	India
Peptone	Himedia	India
D-glucose	Himedia	India
Ethylene diamine tetracetic acid (EDTA)	Fisher Sciencetific	USA
Sodium dodecyl sulfate (SDS)	Fluka Biochemica	USA
Sodium chloride (NaCl)	Fisher Sciencetific	USA
Yeast β-glucan Assay Kit	Megazyme	Ireland
Savinase 16L type EX	Sigma	USA
Sodium hydroxide	Fisher Sciencetific	USA
Hydrochloric acid	Fisher Sciencetific	USA
Potassium hydroxide	Fisher Sciencetific	USA
Hydrochloric acid	Fisher Sciencetific	USA
Methanol	Merck	Germany
Acetone	Merck	Germany
Acetic acid	Merck	Germany
Mono-chloroacetic acid	Acros, Organics	USA
Iso-propanol	Fisher Sciencetific	USA
Laminarin	Sigma-Aldrich	USA
Dulbecco's Modified Eagle Medium (DMEM medium)	Invitrogen	USA
Sulfanilamide	Acros, Organics	USA

Phosphoric acid (H_3PO_4)	Acros, Organics	USA
Naphthylethylenediamide	Acros, Organics	USA
Thiazolyl Blue Tetrazolium Bromide (MTT powder)	Acros, Organics	USA
Dimethyl sulfoxide (DMSO)	Fisher Sciencetific	USA
Bicinchoninic acid (BCA) assay kit	Thermo Sciencetific	USA
Bovine serum albumin (BSA)	Thermo Sciencetific	USA
Fetal bovine serum	Thermo Sciencetific	USA
Dextran	Fluka	USA
Lithium bromide (LiBr)	Sigma-Aldrich	USA
Lipopolysaccharides(LPS)	Invitrogen	USA
Toluene	Merck	USA
Polystyrene	Pressure Chemical	USA

3.2 Methods

3.2.1 Selection of *S. cerevisiae* strains and optimum conditions in yeast cultivation

3.2.1.1 Yeast strains selection

Three yeast strains; *S. cerevisiae* Fermipan®, *S. cerevisiae* Angel® and *S. cerevisiae* TISTR 5059 were cultured in a YPD medium (1 % w/v yeast extract, 2 % w/v peptone and 2 % w/v D-glucose; Appendix A), pH 4 (Aguilar-Uscanga and Francois, 2003)) under reciprocal incubator shaking (200 rpm) at 30 °C for 48 h. The cultured yeast was collected by using centrifugation at 8000 x g for 10 min., freeze-dried and analyzed for β-glucan content using a Yeast Beta-Glucan Assay Kit (Appendix A) as described below and % yield of cell dry weight was determined (Arnold, 1972; Jaehrig *et al*., 2008).

β-Glucan content was calculated from total glucan minus α-glucan. The protocol for determining total glucan and α-glucan contents were mentioned as below:

For total glucan content, 10 mg of yeast cell were placed in eppendorf tube then 0.15 ml of hydrochloric acid 37 % was added and the solution was mixed and incubated at 30 °C for 45 min (vortexed every 15 min). Then, 1 ml of distilled water was added, mixed and incubated at 100 °C for 2 h before the addition of 0.5 ml of 4 M KOH. After that, 200 μl solution was taken. The volume was adjusted to 1 ml with sodium acetate buffer pH5 (800 μl) and mixed. The mixtures were centrifuged at 13,000 x g for 5 min. Samples (20 μl) were taken and placed in the well of 96-well plates (in duplicates). A mixture of exo-β-(1,3)-glucanase (EC. 3.2.1.58) plus β-glucosidase (EC. 3.2.1.21) (10 μl) was added and then incubated at 37 °C for 90 min. Finally, 200 μl of glucose oxidase/peroxidase (EC. 1.1.3.4 and EC. 1.11.1.7) were added and incubated at 37 °C for 30 min. The absorbance was measured at 490 nm with microtiter plate reader. The concentration of glucose in the sample was calculated from D-glucose standard curve (Appendix B).

For α-glucan content, 100 mg of milled yeast were placed in test tubes. Then 2 M KOH (2 ml) was added and the pellets were suspended by stirring in ice bath with a magnetic stirrer for 20 min. Next, 8 ml of 1.2 M sodium acetate buffer (pH 3.8) was added to the mixture. Then, 1 ml of sample was taken in eppendorf tube. Amyloglucosidase (EC. 3.2.1.3) plus invertase (3.2.1.26) (20 μl) were added and incubated at 40 °C for 30 min. After that, the mixture was centrifuged at 13,000 x g for 5 min. Samples (20 μl) were taken to microtiter plate. Glucose oxidase / peroxidase (200 μl) were added to each well and incubated at 37 °C for 30 min. The absorbance was measured at 490 nm with microtiter plate reader. The concentration of glucose in the sample was calculated from the standard curve of D-glucose (Appendix B).

3.2.1.2 Effect of concentration of additives on yeast growth

The yeast strain used in this part was the best strain obtained from 3.2.1.1. Various combination of additives (EDTA, SDS and NaCl) and concentrations (0, 50, 100; 0, 100, 200 and 0, 30,000, 60,000 ppm for EDTA,

SDS and NaCl, respectively) were added to the growth medium as described in Table 3.1. The properties of three additives were described in Appendix C. Starter culture (1 ml) was inoculated into flask containing 100 ml YPD medium. Cells were incubated at 30 °C, pH 4 in reciprocal shaking 200 rpm for 48 h. The yeast cultured was collected every 8 h and determined the cell viability using spread plate technique on YPD plate (Imai and Ohno, 1995).

Growth rate and growth parameter model were estimated by using non-linear regression. Sigmoid curves of growth can be explained with a shifted logistic equation (Peleg, 2006; Corradini and Peleg, 2006; Dai *et al.*, 2010) as the following equation 3.1:

$$\Delta y_{(t)} = \Delta y_{asym}\left\{\frac{1}{1+\exp[k(t_c - t)]} - \frac{1}{1+\exp[kt_c]}\right\} \quad (3.1)$$

where; $\Delta y_{(t)}$ represents adjusted ratio of log $N_{(t)}/N_O$ at time. $N_{(t)}$ is the momentary number of cells at time t and N_O is the initial numbers of cells. Δy_{asym} represents adjusted ratio of log $N_{(t)}/N_O$, which roughly representing the growth level at the stationary phase; t_c representing the time to reach the highest growth rate that is, the inflection point's location, and k is its slope at this point, which representing the growth rate.

Central Composite Design, 3 factors and 3 levels, was used as statistical analysis for 3 replications at the centre point (Majumder, Singh and Goyal, 2009). The experimental design was also shown in Table 3.1. Selection of levels for independent variables was based on the result from preliminary observation. Each value was represented as the mean of three replications. The obtained results were used to create quadratic equations in order to determine the correlation of data by SPSS program version 17 and to determine response surface curves ($p \leq 0.05$) and a contour plot by computer program (Fox *et al.*, 2004)

Table 3.1 Experimental design for selected parameters for optimum conditions prediction

Treatments	Concentrations of EDTA (ppm)	Concentrations of SDS (ppm)	Concentrations of NaCl (ppm)
1	0(-1)	0(-1)	0(-1)
2	0(-1)	100(0)	0(-1)
3	0(-1)	200(1)	0(-1)
4	50(0)	0(-1)	0(-1)
5	50(0)	100(0)	0(-1)
6	50(0)	200(1)	0(-1)
7	100(1)	0(-1)	0(-1)
8	100(1)	100(0)	0(-1)
9	100(1)	200(1)	0(-1)
10	0(-1)	0(-1)	30,000(0)
11	0(-1)	100(0)	30,000(0)
12	0(-1)	200(1)	30,000(0)
13	50(0)	0(-1)	30,000(0)
14	50(0)	100(0)	30,000(0)
15	50(0)	200(1)	30,000(0)
16	100(1)	0(-1)	30,000(0)
17	100(1)	100(0)	30,000(0)
18	100(1)	200(1)	30,000(0)
19	0(-1)	0(-1)	60,000(1)
20	0(-1)	100(0)	60,000(1)
21	0(-1)	200(1)	60,000(1)
22	50(0)	0(-1)	60,000(1)
23	50(0)	100(0)	60,000(1)
24	50(0)	200(1)	60,000(1)
25	100(1)	0(-1)	60,000(1)
26	100(1)	100(0)	60,000(1)
27	100(1)	200(1)	60,000(1)

3.2.2. Effect of additives on yeast cell and the quantity of β-glucan from *S. cerevisiae*

The yeast strain and treatments used in this part were the best strain and optimum conditions obtained from section 3.2.1.1 and 3.2.1.2, respectively.

3.2.2.1 Determination of cell shape

Starter culture (1 ml) was inoculated into flask containing 100 ml YPD medium. Cells were incubated at 30 °C, pH 4 in reciprocal shaking 200 rpm for 24 h. The cells were sampling. The cell shape was determined by using Confocal microscope with DIC (Differential Interference Contrast) mode at magnification 400X (Nikon C1 Digital Eclipse, Japan). The images were analyzed with software ImageJ. Average size, distance of major axis length, distance of minor length and elongation (distance of major axis length - distance of minor axis length) of every single cells (at least 18 cells per treatment) were investigated (Coelho *et al.*, 2004). For confirmation of cell shape, the yeast cells were collected and filtered with 3 μm membrane filter. Then, the specimens were fixed with 2.5 % glutaraldehyde in 0.1 M phosphate buffer pH 7.2 at 4 °C. After that, the specimens were rinsed twice in phosphate buffer for 10 min and one in distilled water for 10 min. They were dehydrated with a series of ethanol (30 %, 50 %, 70 % for 10 min per each) and absolute ethanol for 10 min 3 times. They were put in Critical point dryer (Critical point dryer, Balzers model CPD O2O) and coated with gold (Sputter coater, Salzers model SCD 040). The specimens were photographed under a Scanning Electron Microscope (SEM) (JEOL, model JSM-5410LV, Japan) at magnification of 5,000X.

3.2.2.2 Determination of wall surface

Starter culture (1 ml) was inoculated into flask containing 100 ml YPD medium. Cells were incubated at 30 °C, pH 4 in reciprocal shaking 200 rpm for 24 h. The cells were prepared as described in 3.2.2.1. The wall surface of cells were observed under a Scanning Electron Microscope (SEM) (JEOL, model JSM-5410LV, Japan) at magnification of 15,000X.

3.2.2.3 Determination of β-glucan content

Starter culture (5 ml) was inoculated into flask containing 500 ml YPD medium. Cells were incubated at 30 °C, pH 4 in reciprocal shaking 200 rpm for 24 h. The yeast cells were collected by using centrifugation at 8,000 x g for 10 min, freeze-dried and analyzed for β-glucan yield by using a Yeast Beta-Glucan Assay Kit as described in 3.2.1.1.

3.2.3 Effect of additives on composition of cell and quality of β-glucan

3.2.3.1 Determination of polysaccharide, protein and lipid ratio and β-(1,6) : β-(1,3) ratio of yeast cell

Starter culture (5 ml) was inoculated into flask containing 500 ml YPD medium. Cells were incubated at 30 °C, pH 4 in reciprocal shaking 200 rpm for 24 h. The yeast cells were collected by using centrifugation at 8,000 x g for 10 min, freeze-dried and subjected to polysaccharide, protein and lipid ratio determination by using FTIR. Spectra were recorded from 600-4,500 cm^{-1}. The spectral resolution was 4 cm^{-1}, 64 scans per spectrum in absorption mode. The data were analyzed by curve fitting using computer software (Šandula *et al.*, 1999; Galichet *et al*., 2001; Ha *et al*., 2002; Adt *et al.*, 2006; Weeraya, 2008).

3.2.3.2 Determination of the quality of β-glucan

Starter culture (5 ml) was inoculated into flask containing 500 ml YPD medium. Cells were incubated at 30 °C, pH 4 in reciprocal shaking 200 rpm for 24 h. The cultured yeasts were collected by using centrifugation at 8,000 x g for 10 min, β-glucan was extracted, and protein was eliminated as described below.

3.2.3.2.1 β-Glucan extraction

NaCl (3 % w/v) was added to 30 % w/w cell suspension and incubated at 55 °C, pH 5 for 24 h with mild agitation (120 rpm). Then, the mixture was heated to 85 °C, maintained at that temperature for 15 min and cooled down to 25 °C. The mixture was centrifuged at 4,500 x g for 10 min. Next, the cell debris was adjusted to 30 % w/v with sodium phosphate buffer, 0.02 M, pH 7.5, with glass spheres (0.4 mm). The suspension was autoclaved at 121 °C for 4 h. The insoluble cell wall residue was separated, washed three times, and centrifuged at 4,500 x g for 7 min at room temperature. After that, the residue (10 %w/v) in distilled water was sonicated at 20 KHz, 150 W in an ice batch for 6 min and centrifuged at 4,500 x g for 15 min at 10 °C. Lipid was extracted with acetone by using cell wall residue : acetone = 1:1 (w/v) for 2 h and centrifuged at 4,500 x g for 7 min. Cell wall residue without lipid was washed with distilled water for 4 times and separated by centrifugation. Finally, 0.3 % v/v of the cell wall residue was treated with 0.3 % (w/v) of Savinase 16L type Ex (EC. 3.4.21.62) at 55 °C pH 10 for 4 h to remove protein (Freimund *et al.*, 2003; Varunya, 2006; Liu *et al*., 2008; Magnani *et al*., 2009). Crude β-glucan was obtained (β-glucan I). To reduce the protein content to below 2 % w/w, the suspension of 0.5 %w/v β-glucan I in distilled water was adjusted pH to 10 with NaOH 0.1 M. Savinase 16L type EX (3 % v/v) was added and incubated at 55 °C, 150 rpm for 4 h for repeat protein elimination. More purified β-glucan (β-glucan

II) was obtained. Then, β-glucan II was freeze-dried, protein content was analysed, weight average molecular weight and the immunomodulatory properties were determined.

3.2.3.2.2 Determination of protein content of β-glucan

Protein content was determined by using BCA (Bicinchoninic acid) assay kit (Waker, 2002). The β-glucan samples were dissolved in DMSO before used and 5 μl of solution was added to each well. Then, the 200 μl of BCA reagent (BCA reagent was prepared by mixing solution A : solution B = 50 : 1 before the assay) and added to each well before incubated at room temperature for 2 h. The optical density was measured at 570 nm. The concentration of protein was calculated from the standard curve of known concentration of BSA (Bovine serum albumin) dissolved in DMSO (Appendix B).

3.2.3.2.3 Determination of β-(1,6) : β-(1,3) ratio of β- glucan

Starter culture (5 ml) was inoculated into flask containing 500 ml YPD medium. Cells were incubated at 30 °C, pH 4 in reciprocal shaking 200 rpm for 24 h. The yeast cultures were collected by using centrifuged at 8,000 x g for 10 min. β-Glucan was extracted as described in 3.2.3.2.1, freeze-dried and determined β-(1,6) : β-(1,3) using FTIR. Spectra (both β-glucan I and β-glucan II) were recorded from 600 – 4,500 cm^{-1}. The spectral resolution was 4 cm^{-1}, 64 scans per spectrum in absorption mode. The data were analyzed by curve fitting using computer software (Šandula *et al.*, 1999; Galichet *et al*., 2001; Ha *et al*., 2002; Adt *et al.*, 2006).

3.2.3.2.4 Determination of weight average molecular weight of β-glucan

After the protein was eliminated as described in 3.2.3.2.1, the obtained β-glucan II was freeze-dried and the weight average molecular weight ($\overline{M}w$) was determined by Multi Angle Laser Light Scattering (MALLS) using flow cell in batch mode with a flow rate of 0.4 ml/min. MALLS was calibrated with HPLC grade toluene and validated with standard polystyrene having number average molecular weight ($\overline{M}n$) of 30 and 200 K Daltons in toluene. Normalization was done using standard dextran of $\overline{M}n$ of 25 K Dalton in 50 mM LiBr in dimethyl sulfoxide (DMSO) (Fisher Sciencetific). Three concentrations of β-glucan (treatment 5) in the range of 0.3 - 3 mg/ml were prepared and filtered through 0.45 μm nylon syringe filters for determination of differential refractive index increment of the solvent-solute solution (*dn/dc*) using Optilab DSP (Wyatt Technology), and the second virial coefficient (A_2) was analyzed through Zimm's plot by Astra V software (Version 5.1, Wyatt Technologies). *dn/dc* and A_2 were determined to be 0.160 ml/g and 6.30 x 10^{-5} mol ml/g^2, respectively. Samples (0.2 mg/ml) were dissolved in 50 mM LiBr in DMSO. The weight average molecular weight of β-glucan for other treatments was analyzed using the first-order Debye method (Astra V software, Version 5.1, Wyatt Technologies) (Debye, 1947; Wyatt Technology Corporation, 2004) as described in equation 3.2.

$$\frac{R_\theta}{K^* c} = \overline{M}w - 2A_2 c \overline{M}w^2 \qquad (3.2)$$

Where; R_θ represents the Rayleigh ratio (cm^{-1}). $K^* = 4\pi^2 \eta_0^2 (dn/dc)^2 \lambda_0^{-4} N_A^{-1}$ is an optical constant where η_0 is the refractive index of the solvent, equal to 1.478 (Park, Ibanez and Shoemaker, 2007), λ_0 is the wavelength, equal to 690 nm, N_A is Avogadro' number, equal to 6.022 x 10^{23} mol^{-1}. c is the concentrations of the solute molecules in the solvent (g/ml). $\overline{M}w$ is the weight average molecular weight (daltons).

3.2.3.2.5 Determination of immunomodulatory properties of β-glucan

Nitric Oxide (NO) Assay and MTT assay were used to determine the immunomodulatory properties of β-glucan. β-Glucan II was derivatized to carboxymethylglucan (CM-glucan) by slightly modified methods of previous works (Šandula *et al.*, 1999; Magnani *et al.*, 2009). β-glucan (0.5 g) was mixed with isopropanol (14 ml) and agitated with 140 rpm for 30 min at room temperature. Next, 30 % (w/v) NaOH (1.50 ml) was added and agitated with 140 rpm for 60 min and then agitation rate was increased to 280 rpm for 90 min at room temperature. After that, monochloroacetic acid (0.65 g) was added and agitated with 140 rpm at 65℃ for 5 h. The solution was filtered through filter paper (Whatman^{TM} No. 1) and washed with methanol: acid acetic (7:3 v/v), methanol: water (4:1 v/v); methanol and acetone; respectively. The precipitate was suspended in distilled water and dialyzed using membrane with molecular weight cut off 3,500 at 4 °C for 48 h. The CM-glucan was collected, centrifuged and separated into water soluble CM-glucan and water insoluble CM-glucan and lyophilized both parts before test. Then degree of substitution (DS) of CM-glucan of the selected treatments was determined by using vibrational spectroscopic methods. The absorbance intensity of carboxymethyl group area (1605 cm^{-1}) and internal standard β-glycosidic linkage area (890 cm^{-1}) were analyzed by curve-fitting using computer software. The band area ratio of carboxymethyl group to internal standard β-glycosidic linkage were calculated for determination DS by using xanthan gum standard equation (3.3) as report in Yuen *et al.*, 2009

$$y = 49.6\ x + 8.31 \qquad (3.3)$$

Where; y represents ratio of carboxymethyl group (1605 cm^{-1}) to internal standard β-glycosidic linkage (890 cm^{-1}). x is degree of substitution (DS)

Nitric Oxide(NO) Assay (Ha *et al.*, 2006; Aldridge *et al.*, 2008) was performed. Macrophage cells (RAW 264.7)

were cultured in 200 μl DMEM (Dulbecco's Modified Eagle Medium, Invitrogen) medium supplemented with 10 % (v/v) fetal bovine serum and 1 % (v/v) penicillin in a 96 well plate (2.5×10^4 cell per well) and incubated at 37℃ in 5 % (v/v) CO_2 incubator for 24 h. After 24 h incubation, the medium was decanted. Serial dilution of CM-glucan samples (100 μl) in DMEM medium were added to each well, and then 100 μl of 1 μg/ml LPS (Lipopolysacharides) in DMED medium was added to each well immediately. The plate was incubated for 24 h. Sample (100 μl) was taken out from the culture supernatant of each well and mixed with an equal amount of freshly prepared Griess reagent (Griess reagent was prepared by mixing an equal amount of solution A containing 2 (%w/v) sulfanilamide, 4 (%w/v) H_3PO_4 and solution B containing 0.2 (%w/v) naphthylethylenediamide before the assay). The mixture was incubated for 10 min and then the optical density was measured at 542 nm. The concentration of nitrite occurred in the supernatant was calculated from the standard curve of known concentration of sodium nitrite salt dissolved in DMEM medium.

MTT assay protocol (Ebada *et al.*, 2008): the cell viability was measured by MTT assay. After taking a 100 μl sample from the cell culture supernatant for NO assay, the residual medium was decanted. 0.1 %(w/v) MTT solution in DMEM medium (100 μl) was added in each well and incubated at 37℃ for 2 h. The solution was decanted. DMSO (100 μl) was added to each well and incubated for 10 min. Then the optical density was measured at 570 nm. The cell viability was calculated compared with control.

3.2.4 Statistical Analysis

The data were collected from triplicates. Exception in the protein determination and % Yield of β-glucan were done in duplicated. Analysis was performed by SPSS software version 17, $p \leq 0.05$ (two-tailed) considered as statistically significant. The data were analyzed with Analysis of Variance (ANOVA) and multiple comparision with Duncan's Multiple range test (Steel and Lorrie, 1980). The details were shown in appendix D.

CHAPTER IV
RESULTS AND DISCUSSION

4.1 Selection of *S. cerevisiae* strains and optimum conditions in yeast cultivation

4.1.1 Yeast strains selection

Screening of *S. cerevisiae* strains on β-glucan production were presented in Table 4.1. The β-glucan content from *S. cerevisiae* Fermipan®, *S. cerevisiae* Angel® and *S. cerevisiae* TISTR 5059 were 7.10, 8.95 and 8.54 (%w/w), respectively, which were not significantly different ($p \leq 0.05$).

Table 4.1 β-Glucan content (as % (w/w dry weight) of the cell) and the % yield of cell dry weight of *S. cerevisiae* Fermipan®, *S. cerevisiae* Angel® and *S. cerevisiae* TISTR 5059 isolates

Strains	β-Glucan content (% w/w of cell)^{ns}	Yield (% of cell dry weight)	Net β-glucan yield (%)
S. cerevisiae Fermipan®	7.10 ± 1.55	0.43 ± 0.13^{a}	3.05 ± 1.53^{a}
S. cerevisiae Angel®	8.95 ± 0.66	0.35 ± 0.05^{a}	3.13 ± 0.62 ^{a}
S. cerevisiae TISTR 5059	8.54 ± 1.15	0.14 ± 0.03^{b}	1.21 ± 0.32^{b}

Data are shown as the mean ± one SD and are derived from three replicates. ^{ns} not significantly differences ($p \leq 0.05$). Means within a column followed by different letter are significantly different ($p < 0.05$).

However, when three yeast strains were cultivated under the same condition; it was found that *S. cerevisiae* Fermipan® exhibited the highest cell dry weight as 0.43 % (w/v) which was not significantly different that from *S. cerevisiae* Angel® ($p \leq 0.05$); 0.35(w/v). While, the lowest cell dry weight, 0.14 % (w/v), was found in *S. cerevisiae* TISTR 5059. According to the results of β-glucan yield, % yield of cell dry weight and net β-glucan yield, *S. cerevisiae* Angel® was therefore selected for further studies such as the effect of additives on cell shape, wall surface and β-glucan production in *S. cerevisiae*, since it could grow well and produced the highest net amount of β-glucan compared with other two strains.

4.1.2 Effect of concentration of additives on yeast growth

Growth curves from 27 treatments were exhibited in Fig. 4.1, 4.2 and 4.3, based on growth characteristics as cell growth (Group I), cell death (Group II) and no growth (Group III), respectively.

According to Fig 4.1, sigmoid curve of the yeast growth, the parameters of growth model were estimated by non-linear regression in the function of shifted logistic equation (3.1). The growth rate, the growth level at the stationary phase and time to reach the highest growth rate were explained with k value, y_{asym} and t_c, respectively. Concentrations of SDS at 100 and 200 ppm (treatment 2, 3) resulted in higher growth level (3.67 and 3.77) compared to control (3.63) while the time to reach the highest growth rate was longer (12.13, 12.73 and 10.87 h). On the other hand, 50 and 100 ppm EDTA (treatment 4, 7) resulted in reduction of growth level to 3.50 and 3.24, respectively.

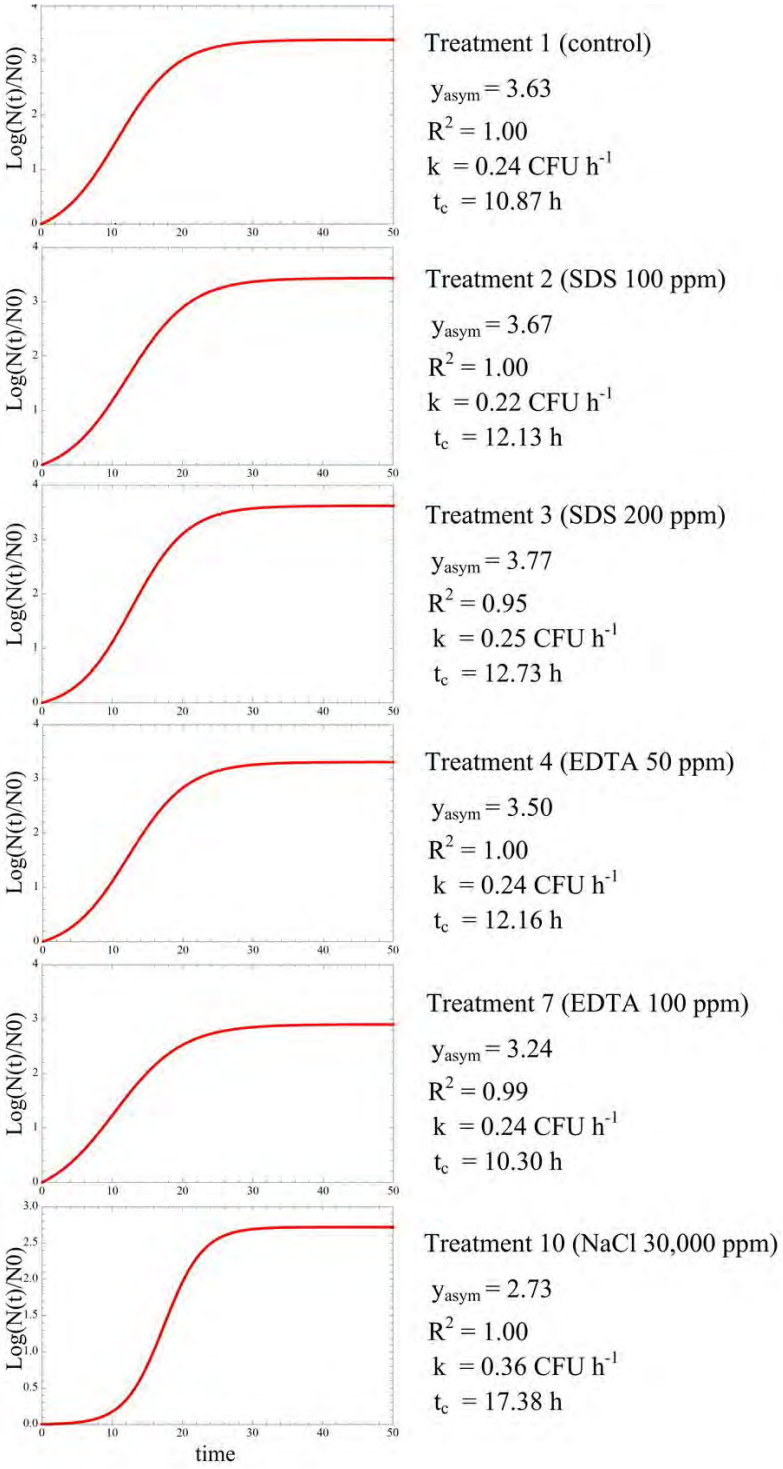


Fig 4.1 Growth characteristics of *S. cerevisiae* Angel® when yeast was cultured in YPD medium with different concentrations of addtives; EDTA, SDS and NaCl at different concentrations for 48 h, group I : yeast growth

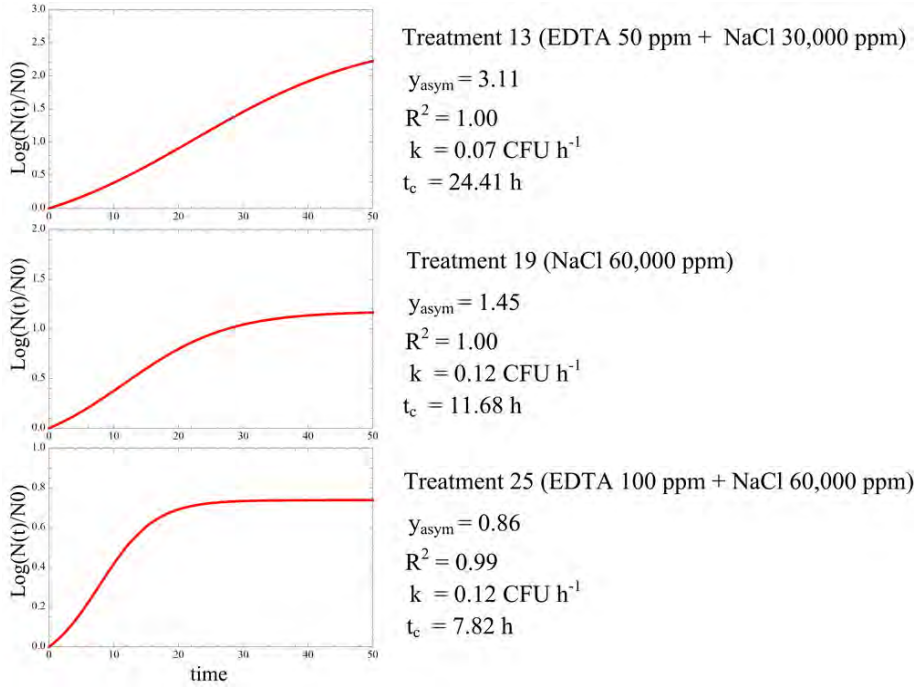


Fig 4.1 Growth characteristics of *S. cerevisiae* Angel® when yeast was cultured in YPD medium with different concentrations of addtives; EDTA, SDS and NaCl at different concentrations for 48 h, group I : yeast growth (cont.)

Similarly, the growth level of yeast cell was reduced to 2.73 in 30,000 ppm NaCl (treatment 10) and led to 1.45 when the concentration was increased to 60,000 ppm (treatment 19). Moreover, combination of additives were found to reduce the growth level of yeast cells until cell can not tolerant that stress and finally resulted in cell death in other treatments (Fig. 4.2 and 4.3). SDS at 200 ppm (treatment 3) and 30,000 ppm NaCl (treatment 10) stimulated growth rate to 0.25 and 0.36 CFU h^{-1}, respectively compared to control (0.24 CFU h^{-1}). The growth rate was found to be lower than that of control in other treatments except at 50 ppm EDTA (treatment 4) which showed the same value as in control. *S. cerevisiae* Angel® grew slower than control when supplemented with 100, 200 ppm SDS (treatment 2, 3); 50 ppm EDTA (treatment 4); 30,000 ppm NaCl (treatment 10); combination of 50 ppm EDTA and 30,000 ppm NaCl (treatment 13) and 60,000 ppm NaCl (treatment 19), according to t_c which showed longer (12.13, 12.73, 12.16, 17.38, 24.41 and 11.68 h, respectively) than that of the

control (10.87 h). In contrast, 100 ppm EDTA (treatment 7) and combination of 100 ppm EDTA and 60,000 ppm NaCl (treatment 25) showed shorter t_c (lower to 10.30 and 7.82 h; respectively) than control. However, the growth level at stationary phase was lower than control. This stress might be due to abnormal growth.

From Fig 4.2 and Fig 4.3, cell death and no growth were found when yeast was cultivated with high concentrations and combination of additives. The growth of yeast could not be explained by using the equation (3.1) in this case. High concentrations of anionic detergent (SDS), chelating agent (EDTA) and hyperosmotic pressure (NaCl) affected the diffusion permeability of cell membrane and disturbed the medium osmosis to yeast cell and might destroy cell wall (Hohmann, 2003). Moreover, EDTA can form chelation with divalent metals such as Mg^{2+} and Ca^{2+}. Thus, the activity of enzyme inside cell was blocked and yeast growth was reduced (Kubo, Lee and Ha, 2005). In addition, in hyperosmotic conditions, it was found that water diffused out of the cell and caused the change in cell wall properties and cell shrinkage. Consequently, cell viability was decreased (Morris *et al.*, 1983). From these results, it showed that yeast cell can not torelate high concentration of additives. So, it can not adapt itseft to toxic stress conditions (Hohmann, 2003). Similarly, high concentration of additives can damage cell membrane and the activity of enzyme is blocked, result in cell death (Smits and Brul, 2005). In the case of low concentration of additives, it was found that yeast could grow but the the time to reach the highest growth rate was longer than that in control, decrease of growth rate and the growth level were observed.

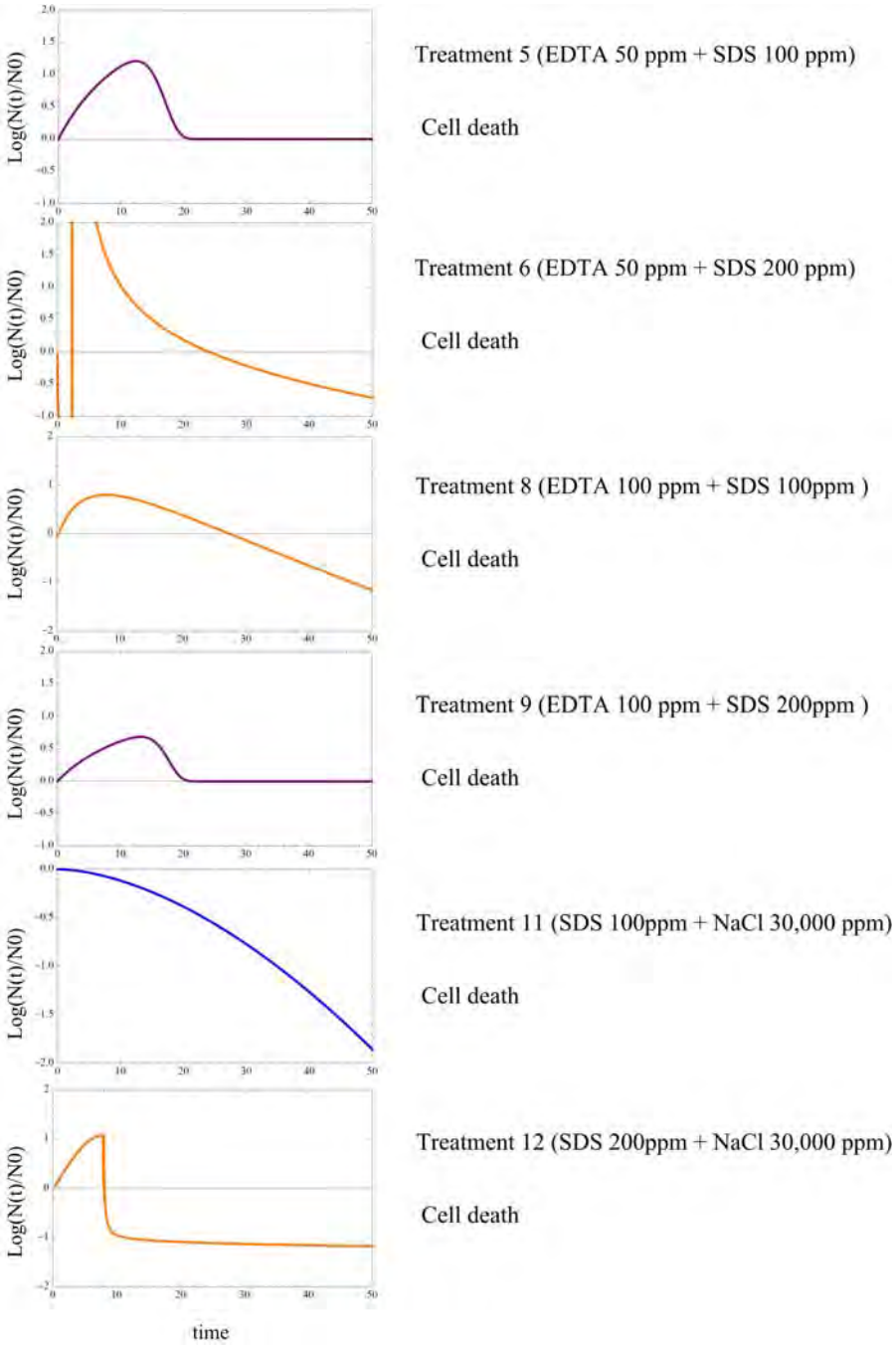


Fig 4.2 Growth characteristics of *S. cerevisiae* Angel® when yeast was cultured in YPD medium with different concentrations of addtives; EDTA, SDS and NaCl at different concentrations for 48 h, group II : cell death

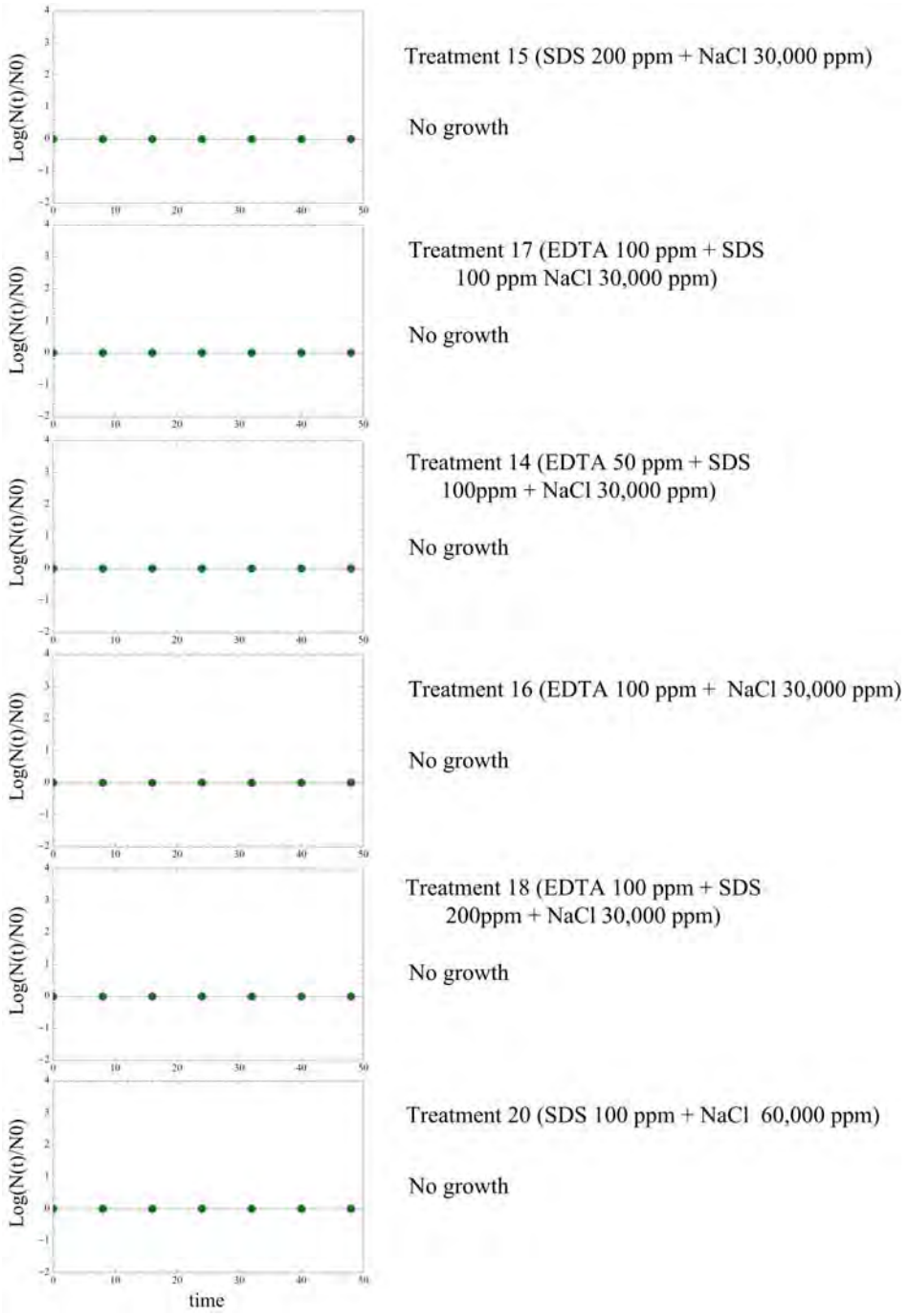


Fig 4.3 Growth characteristics of *S. cerevisiae* Angel® when yeast was cultured in YPD medium with different concentrations of addtives; EDTA, SDS and NaCl at different concentrations for 48 h, group III : no growth

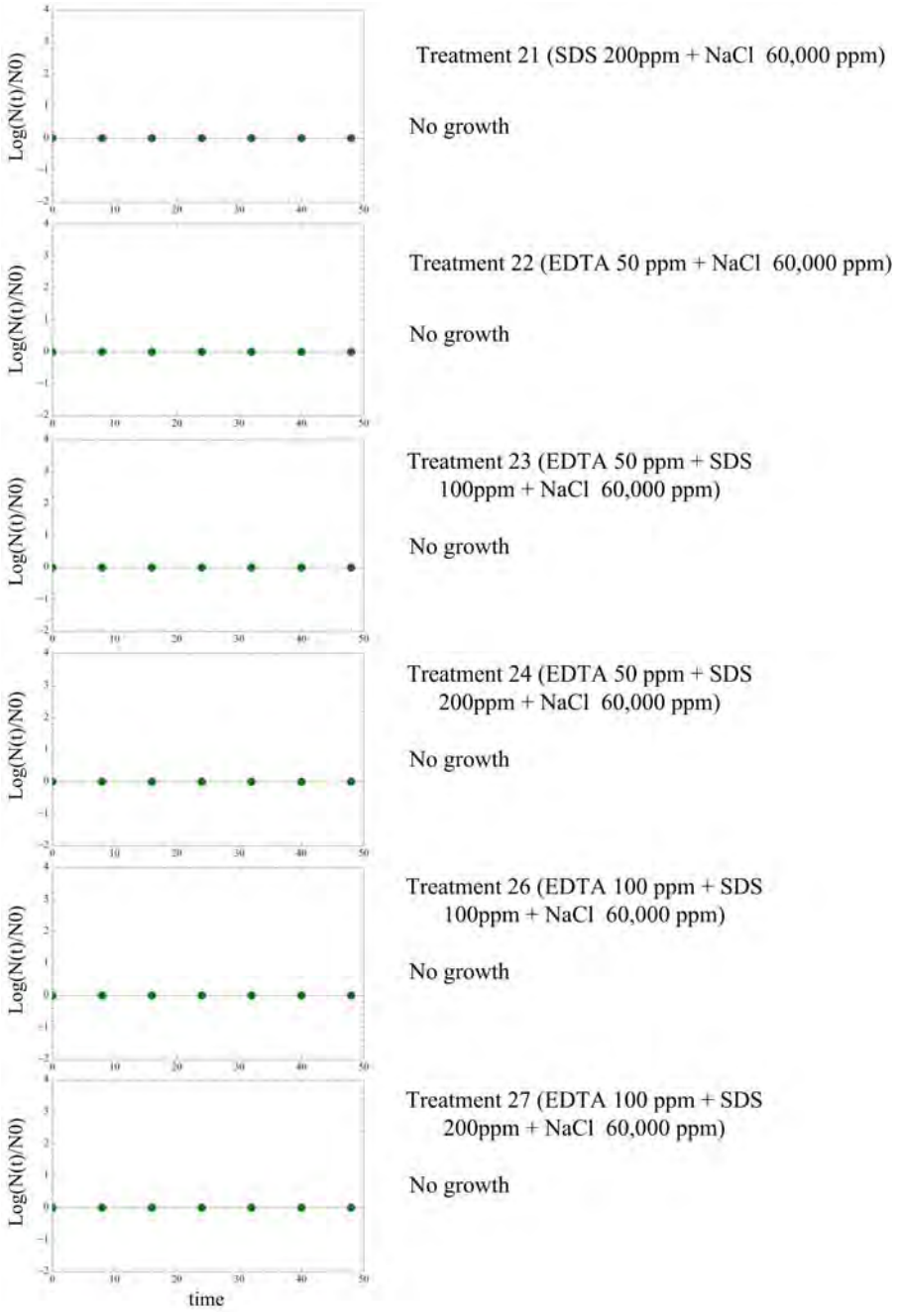


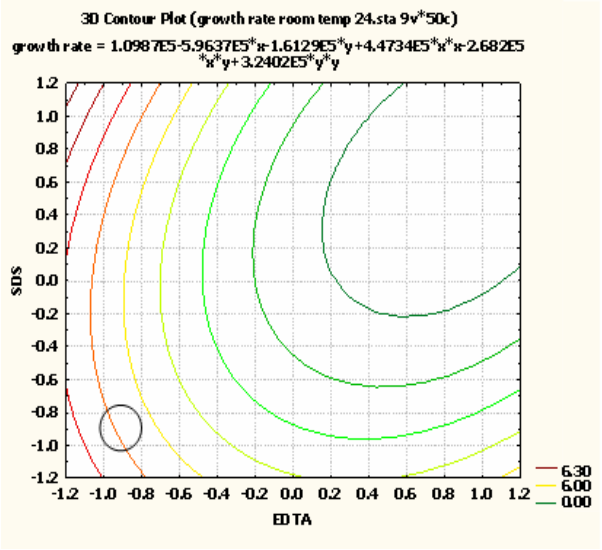
Fig 4.3 Growth characteristics of *S. cerevisiae* Angel® when yeast was cultured in YPD medium with different concentrations of addtives; EDTA, SDS and NaCl at different concentrations for 48 h, group III : no growth (cont.)

This effect was supported by the increase of t_c, decrease of k value and y_{asym}, respectively (Fig. 4.1). Previous researchers showed the toxic effect of high concentrations of EDTA at 400 μg/ml (400 ppm) (Kubo, Lee and Ha, 2005),

SDS over 0.014 % (140 ppm) (Sirisattha *et al*., 2004) and NaCl 1.5 M (87,500 ppm) (Almagro *et al.*, 2000) on *S. cerevisiae* growth rate. In this research, the yeast could tolerate to these additives at low concentration of EDTA 100 ppm, SDS 200 ppm and NaCl 60,000 ppm. The tolerant ability depends on yeast strains and culture condition used (Hohmann, 2002). The t_c or the time to reach the highest growth rate was long when cells were cultured with additives. This might be because cells tried to adapt themselves in new environmental conditions by using actin depolymerization mechanism, consequently the cell cycle was delayed (Smits, Ende and Klis, 2001; Kikuchi *et al.*, 2007). Some previous researches demonstrated that lag phase was extended when *S. cerevisiae* was cultured with NaCl in the range of 14,610–87,660 ppm. In addition, yeast used more energy for maintaining electrochemical gradient of sodium ions, therefore the growth level was lower than control (Watson, 1970). With the exception of yeast grew under 100 and 200 ppm SDS, the growth levels were higher than that of control. This could be due to the influence of certain concentration of SDS which stimulated FKS1 and PIM1, the gene controlling cell wall synthesis and actin cytoskeleton in order to increase budding process of *S. cerevisiae* cell. Therefore, cell wall components could be increasingly generated, growth rate and the growth level at stationary phase could be increased (Delley and Hall, 1999). Based on growth parameters as evaluated in term of $y_{asym,}$ k and t_c value, treatments with SDS (100 ppm), EDTA (50 ppm) and NaCl (30,000 ppm) were selected for further evaluation. Since under SDS at 100 ppm, growth model of the yeast exhibited low t_c, 12.13 h, high y_{asym} and k value, this indicated that in this condition *S. cerevisiae* Angel® exhibited growth parameters model close to other concentration (200 ppm). Moreover, the lowest amount of additives was used in this condition. For the treatment with 50 ppm EDTA, the model exhibited the value of y_{asym}, 3.50 and k, 0.24 CFU h^{-1} and low t_c, 12.16 h. While NaCl 30,000 ppm led to y_{asym}, 2.73, k, 0.36 CFU h^{-1} and t_c, 17.38 h, which indicated that *S. cerevisiae* Angel® could still grow well under these two conditions.

Based on parameters of growth model for each condition as evaluated, the optimum concentration of single additives were 50 ppm EDTA, 100 ppm SDS and 30,000 ppm NaCl. Therefore, the data of growth rate from Fig. 4.1 were

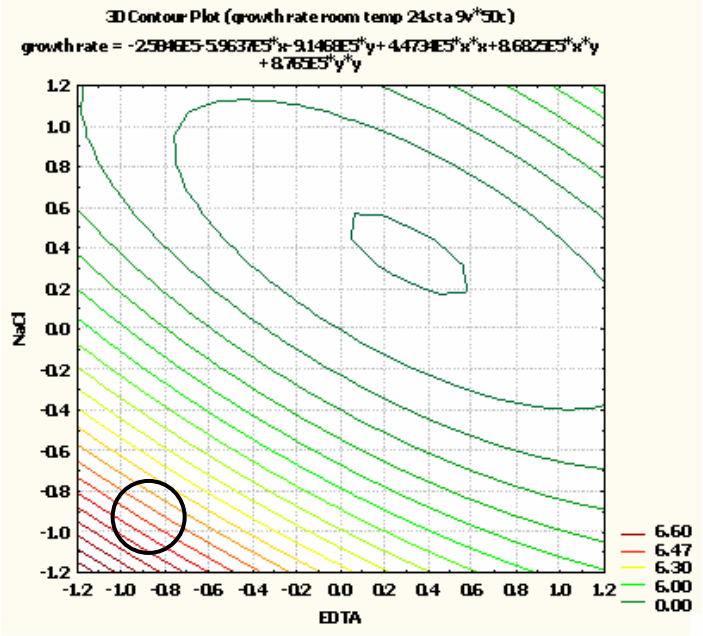
further calculated to predict additional optimum condition for combination of additives by using computer software. Consequently, four additional conditions were obtained and presented in Fig 4.4- Fig 4.7.



$R^2 = 0.78$

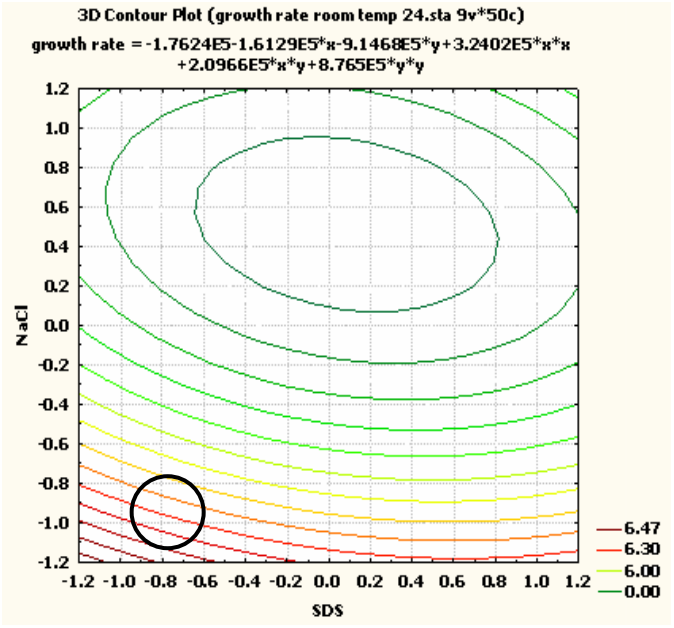
Fig 4.4 Contour plots for optimization of growth in *S. cerevisiae* Angel® between combination of EDTA and SDS

Fig. 4.4 presents the relationship between the effect of concentration of EDTA and SDS on growth rate of yeast. Based on the growth region, it was found that high concentration of additives (100 ppm EDTA and 200 ppm SDS; code 1) decreased the growth rate. The growth rate increased when yeast was cultured with low concentration of additives (0 ppm; code -1). Consequently, the optimum concentration of EDTA and SDS were 5 and 10 ppm, respectively. The estimation of growth rate was about 6.00 CFU h^{-1}. The relationship between the effect of concentration of EDTA and NaCl, SDS and NaCl and three combination of additives on growth rate of yeast were presented in Fig. 4.5 – 4.7, respectively.



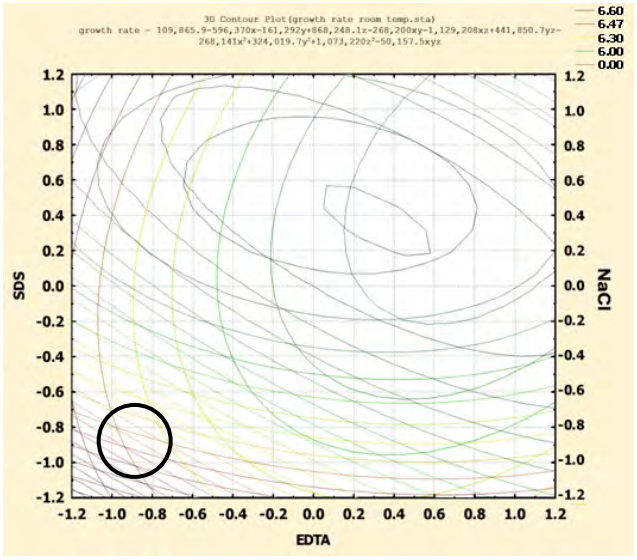
$R^2 = 0.78$

Fig 4.5 Contour plots for optimization of growth in *S. cerevisiae* Angel® between combination of EDTA and NaCl



$R^2 = 0.78$

Fig 4.6 Contour plots for optimization of growth in *S. cerevisiae* Angel® between combination of SDS and NaCl



$R^2 = 0.78$

Fig 4.7 Contour plots for optimization of growth in *S. cerevisiae* Angel® between combinations of EDTA, SDS and NaCl

From Fig 4.5-Fig 4.7, similar trends were found, high concentration of additives reduced the growth rate. Four additional combination conditions of additives were obtained, which were combination of EDTA and SDS (5 and 10 ppm); combination of EDTA and NaCl (5 and 3,000 ppm); combination of SDS and NaCl (20 and 3,000 ppm); and combination of EDTA, SDS and NaCl (5, 10 and 3,000 ppm). For further investigation, eight growth conditions for the optimum condition of single and combination of additives were used for study the effect of additives on cell shape, wall surface, β-glucan production and composition of cell in *S. cerevisiae* Angel®.

4.2 Effect of additives on yeast cell and quantity of β-glucan from *S. cerevisiae*

From previous results**,** the *S. cerevisiae* Angel® was cultured under reciprocal incubator shaking 200 rpm, pH 4 at 30 °C for 24 h. Then, the cells were collected for determination of the effect of additives on cell shape, wall

surface, β-glucan production, composition of cell and β-(1,6) : β-(1,3) ratio in cell.

4.2.1 Determination of cell shape

From previously researches, the high content of β-glucan and β-1,6-glucan were found when yeast was cultivated in the presence of stress conditions (Maneesri *et. al.*, 2005). In addition, many researches reported on the effect of additives on genetic mechanisms, with associated in β-glucan synthesis (Leal *et al.*, 1984; Guillen *et al.*, 1985; Bickle *et al.*, 1998; Delley and Hall, 1999; Blomberg, 2000). However, no research showing the correlation of cell shape and β-glucan synthesis was found. Since the cell shape might be affected by the β-glucan content, so the relationship between cell shape and β-glucan content was determined in this study. The cultured yeast was collected and photographed with Confocal microscope in Differential Interference Contrast (DIC) mode at magnification 400X (Nikon C1 Digital Eclipse, Japan). The images were analyzed with software ImageJ (NIH Image, USA.). Average size, distance of major axis length, distance of minor axis length and elongation (distance of major axis length - distance of minor axis length) of every single cell were investigated (Coelho *et al.*, 2004). The images after program processing were shown in Fig 4.8. It was found that no significant differences of single cell size were observed among treatments (Table 4.2). However, based on the length of two axeses (elongation), cell shape could be divided into three groups. The first group was rounder shape (major axis length longer than minor axis length only 0.84 um). This group exhibited when yeast was cultured with 100 ppm SDS and interestingly the elongation of single cell was significantly different from other treatments ($p \leq 0.05$) (treatment1) (Fig.4.8b, Fig. 4.9). While, the second group, long or oval shape was found under the condition of combination of 5 ppm EDTA and 3,000 ppm NaCl (treatment 5) (longest elongation of single cell; 2.00 um) (Fig. 4.8f, Fig. 4.9). The last group, oval shape which was similar to control was found (treatment 2, 3, 4, 6, 7) (Fig. 4.8c, d, e, g, h, Fig. 4.9).

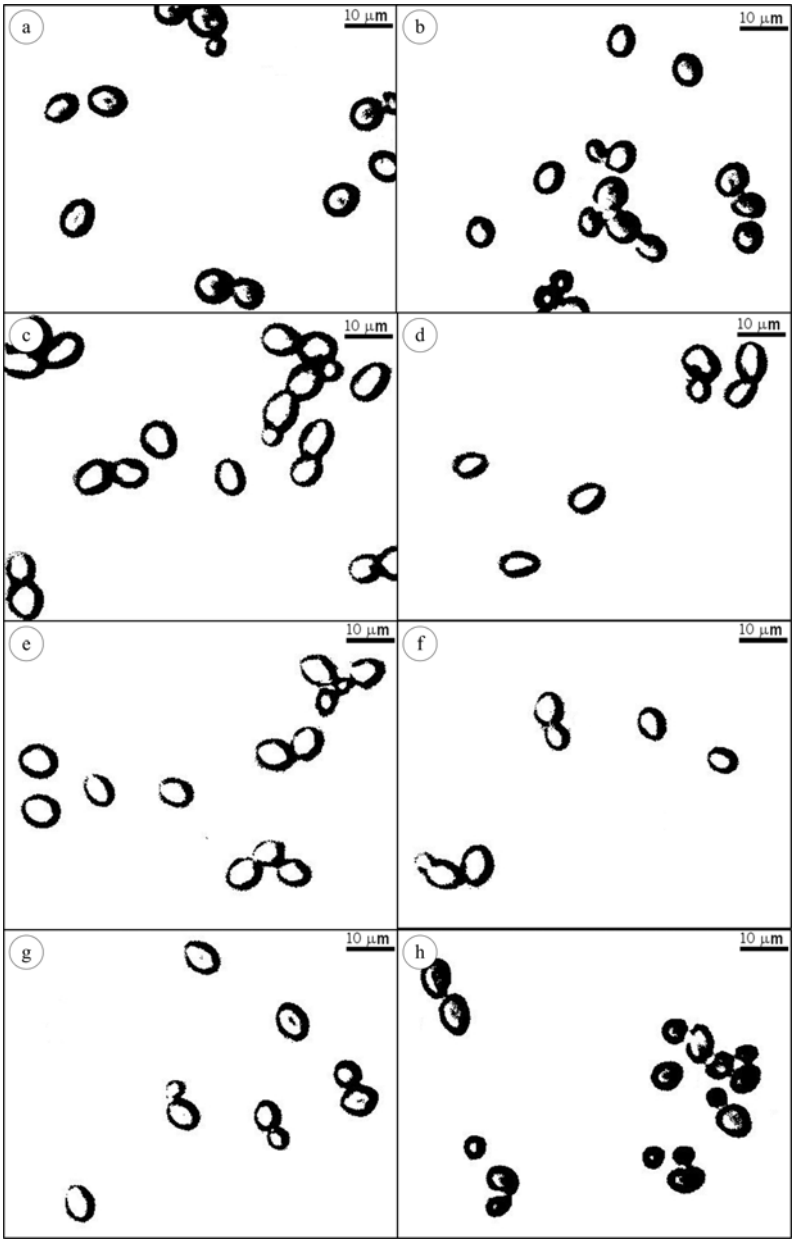


Fig. 4.8 Cell shape of *S.cerevisiae* Angel® when yeast was cultured with some additives in YPD medium for 24 h as followed; a = control ; b = 100 ppm SDS ; c = 50 ppm EDTA; d = 30,000 ppm NaCl; e = combination of 5 ppm EDTA and 10 ppm SDS; f = combination of 5 ppm EDTA and 3,000 ppm NaCl, g = combination of 20 ppm SDS and 3,000 ppm NaCl, h = combination of 5 ppm EDTA, 10 ppm SDS and 3,000 ppm NaCl

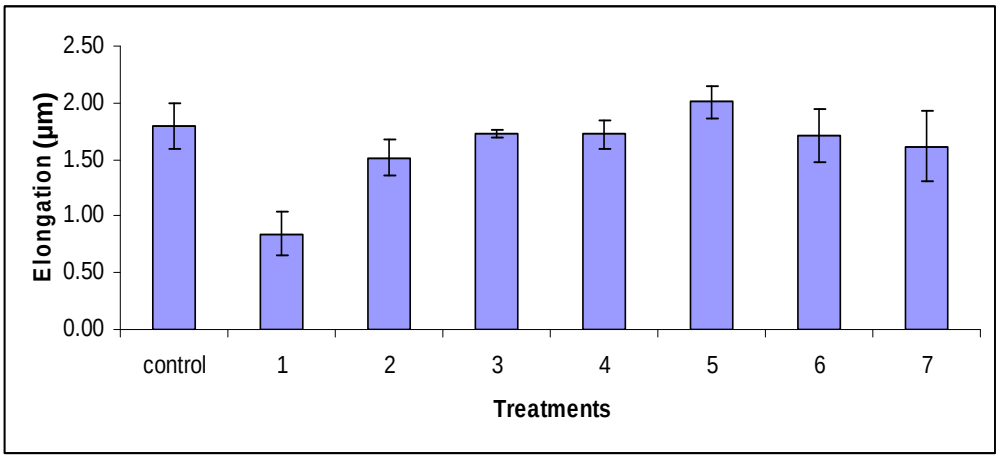


Fig. 4.9 Elongation of cells from *S.cerevisiae* Angel® when yeast was cultured with some additives as followed; 1 = 100 ppm SDS; 2 = 50 ppm EDTA; 3 = 30,000 ppm NaCl; 4 = combination of 5 ppm EDTA and 10 ppm SDS; 5 = combination of 5 ppm EDTA and 3,000 ppm NaCl, 6 = combination of 20 ppm SDS and 3,000 ppm NaCl, 7 = combination of 5 ppm EDTA, 10 ppm SDS and 3,000 ppm NaCl

The cell shape was confirmed by using Scanning Electron Microscope (SEM) at magnification 5,000X as presented in Fig. 4.10. This figure showed that rounder cell was also found when yeast was cultivated with 100 ppm SDS (treatment 1) and the longest cell was exhibited when yeast was cultured with 5 ppm EDTA and 3,000 ppm NaCl (treatment 5). These results were corresponded well with the cell shape from Confocal microscope (Fig. 4.8).

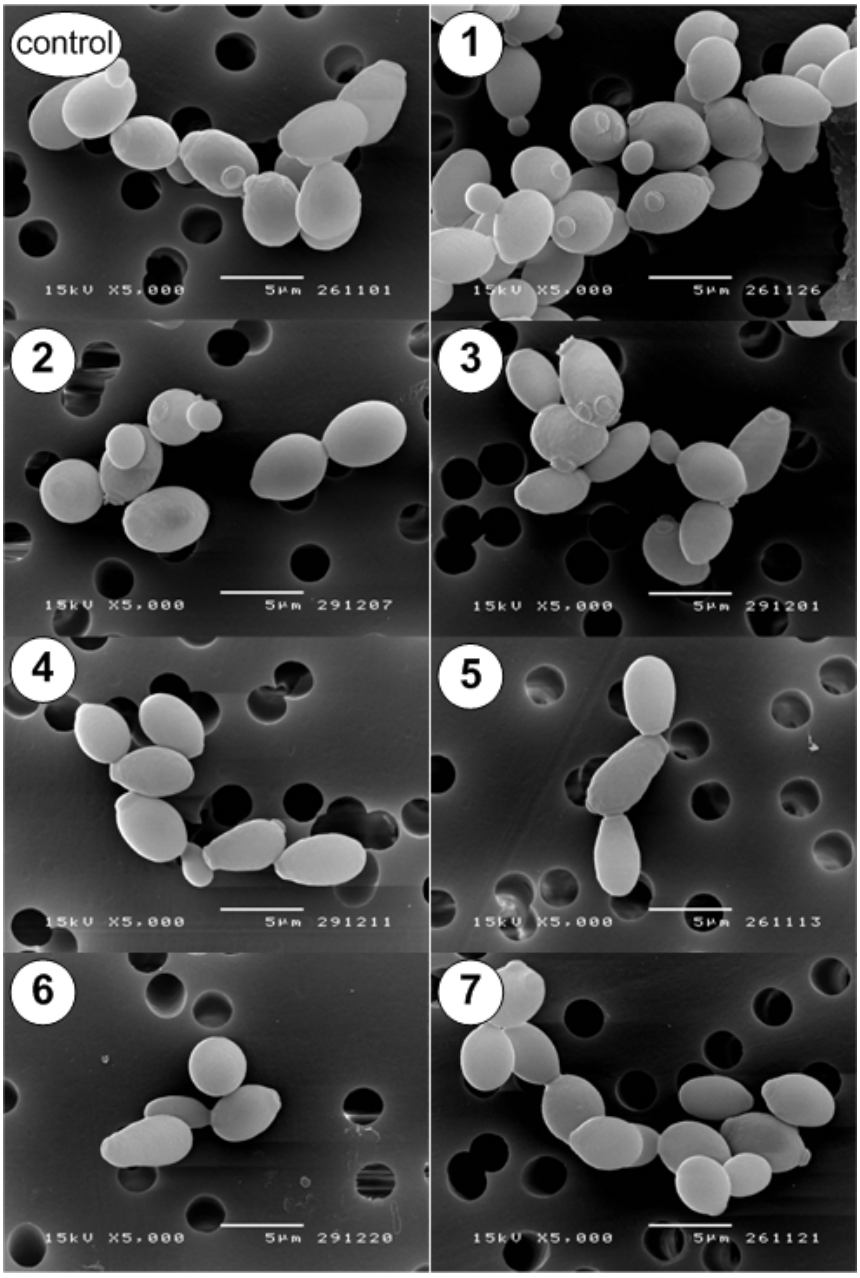


Fig. 4.10 Cell shape of *S.cerevisiae* Angel® when yeast was cultured with some additives in YPD medium for 24 h as followed; 1 = 100 ppm SDS ; 2 = 50 ppm EDTA; 3 = 30,000 ppm NaCl; 4 = combination of 5 ppm EDTA and 10 ppm SDS; 5 = combination of 5 ppm EDTA and 3,000 ppm NaCl, 6 = combination of 20 ppm SDS and 3,000 ppm NaCl, 7 = combination of 5 ppm EDTA, 10 ppm SDS and 3,000 ppm. The images were taken by using SEM at magnification 5,000X

4.2.2 Determination of wall surface

The yeast cultures were cultivated in the same condition, collected and photographed with Scanning Electron Microscope (SEM) at the magnification of 15,000X. The images were shown in Fig. 4.11, rounder shape and the highest number of bud scars was exhibited when yeast was cultivated with 100 ppm SDS (treatment 1). The longest oval shape was shown when cells were cultured with combination of 5 ppm EDTA and 3,000 ppm NaCl (treatment 5). In addition, the long oval cell shape and cell shrinkage were exhibited when yeast was cultured with 30,000 ppm NaCl (treatment 3; Fig. 4.11). From this observation, NaCl could affect reduction of the cell volume, morphological abnormalities and the cell became longer. Based on these results, it could be concluded that the rounder cell was observed in both Confocal microscope and SEM at magnification 5,000 X. The lowest elongation (rounder shape) was found in the case of 100 ppm SDS (treatment 1). This effect could be explained that the certain concentrations of SDS could activated FKS1 and PIM1 which are the genes that activate actin cytoskeleton and control cell size, respectively (Delley and Hall, 1999; Casano *et al*., 2001). During this process, actin patches and actin cables synthesis are randomly distributed before budding process commence (Motizuki *et al.*, 2008). Thus, the rounder cells are increased due to actin cytoskeleton. Moreover, the distance of major axis length could be shorter than that of control. Rounder morphology and multiple bud scars were therefore observed due to rapid synthesis of actin patches and actin cables (Delley and Hall, 1999; Kikuchi *et al.*, 2007).

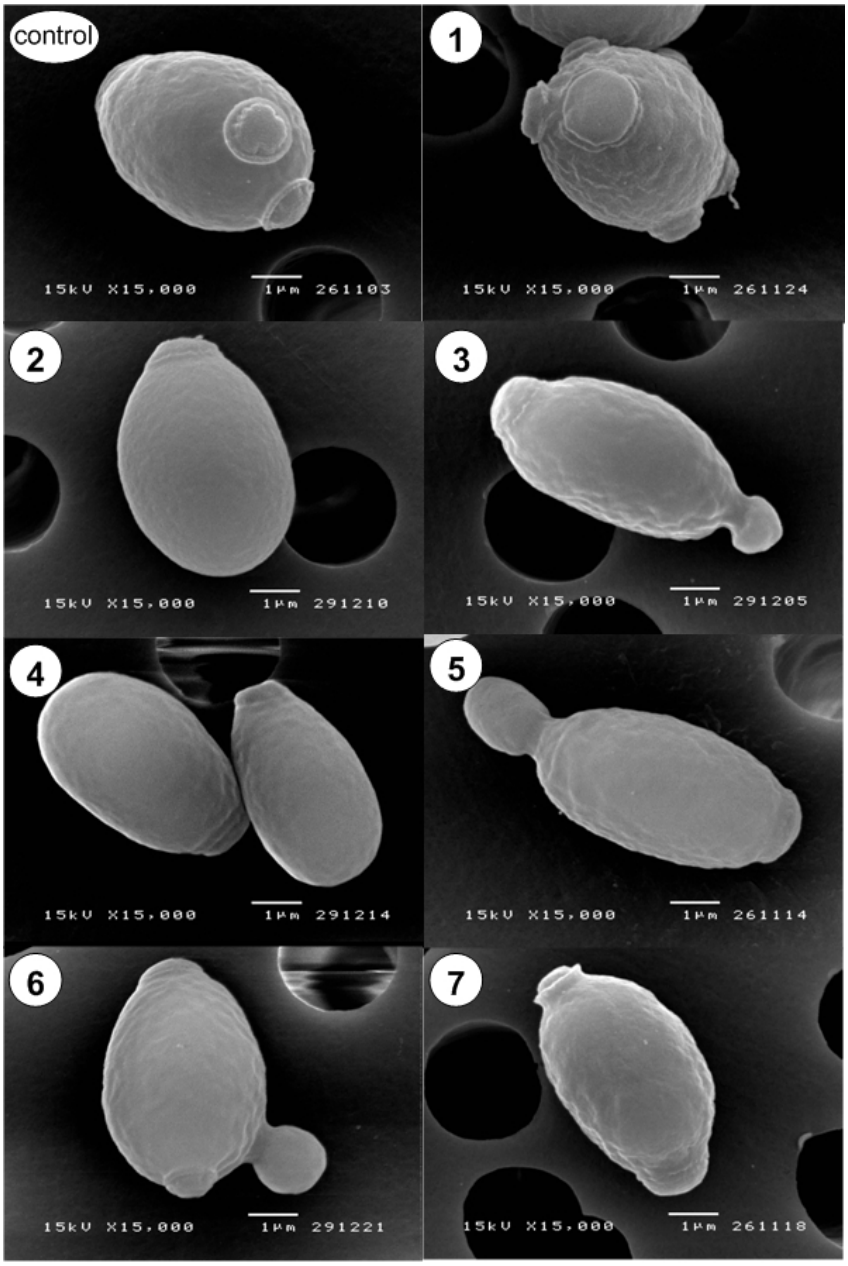


Fig. 4.11 Wall surface of *S. cerevisiae* Angel® when yeast was cultured with some additives in YPD medium for 24 h as follow; 1 = 100 ppm SDS, 2 = 50 ppm EDTA, 3 = 30,000 ppm NaCl, 4 = combination of 5 ppm EDTA, 10 ppm SDS, 5 = combination of 5 ppm EDTA and 3,000 ppm NaCl and 6 = combination of 20 ppm SDS and 3,000 ppm NaCl. and 7 = combination of 5 ppm EDTA, 10 ppm SDS and 3,000 ppm NaCl. The images were taken by using SEM at magnification 15,000X

In addition, the destabilization of cell wall by SDS (Popolo, Gualtieri and Ragni, 2001) may lead to the protective formation of a rigid rounder cell. The longer cell was exhibited when the culture medium was supplemented with other additives. The observed yeast cells were mainly oval shape and had lower number of bud scars which were similar to morphology of cell from control condition. Little information about the effect of EDTA on cell morphology of *S. cerevisiae* is available. However, some reports about the effect of NaCl on cell have been explained. In the case of high NaCl concentration (58,440 – 146,100 ppm), hyperosmotic condition resulted in rapid water efflux from cell, therefore cell volume was reduced, causing cell shrinkage, increase in internal solute concentration (Morris, 1983; Attfield, 1997) and also reflecting in the organization of cytoskeleton element (Hohmann, 2002; 2003). This process could result in asymmetric shape of the cell. This result corresponded to the work of Chowdhury, Smite and Gustin (1992) who demonstrated that the cell cultured in the medium having high osmolarity would exhibit loss or short actin cytoskeleton and morphological abnormalities. Moreover, osmotic stress was responsible for decrease in cell size (Morris *et al.*, 1983; Hohmann, 2003). Other researchers reported that HOG (High Osmolarity Glycerol) MAP kinase pathway has been used to response in hyperosmotic condition, due to reorganization of actin cytoskeleton and cell wall synthesis, respectively (Slaninová *et al*., 2000; Wojda *et al.*, 2003).

4.2.3 Determination of β-glucan content

The yeast cells were cultivated in the same condition, centrifuged and freeze-dried. β-Glucan content was determined by using β-glucan assay kit. The total-glucan, α-glucan, β-glucan contents (the difference between total-glucan and α-glucan) and % yield of cell dry weight were investigated. The results were shown in Fig. 4.12, Table 4.2 and Fig. 4.13, respectively.

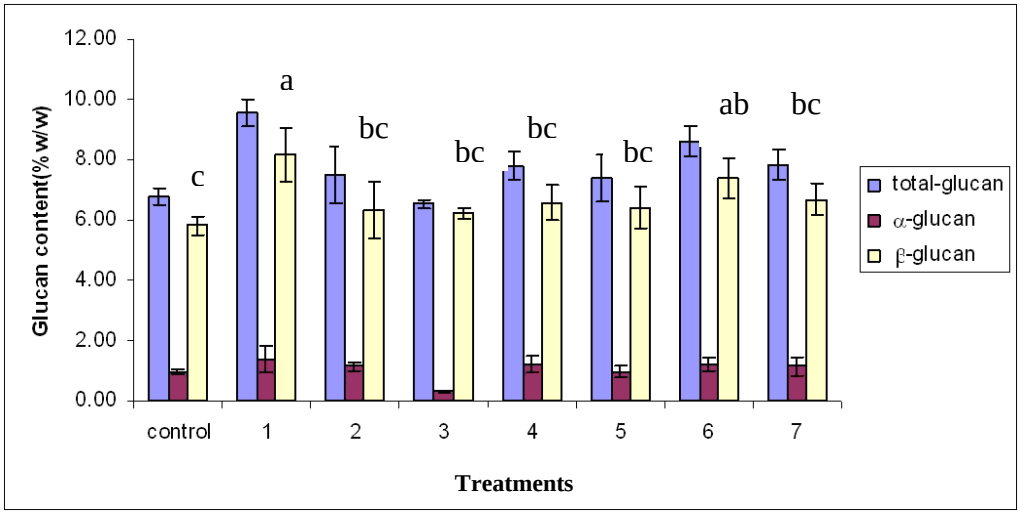


Fig. 4.12 Total-glucan, α-glucan and β-glucan contents of *S.cerevisiae* Angel® when yeast was cultured in YPD medium with some additives as followed; 1 = 100 ppm SDS; 2 = 50 ppm EDTA; 3 = 30,000 ppm NaCl; 4 = combination of 5 ppm EDTA and 10 ppm SDS; 5 = combination of 5 ppm EDTA and 3,000 ppm NaCl, 6 = combination of 20 ppm SDS and 3,000 ppm NaCl, 7 = combination of 5 ppm EDTA, 10 ppm SDS and 3,000 ppm NaCl. The different letters mean significantly differences of β-glucan ($p \leq 0.05$)

It was found that β-glucan yield was higher than control. The total-glucan, α-glucan and β-glucan contents exhibited similar trend. In addition, the highest total-glucan, α-glucan and β-glucan yields were found when yeast was cultured with 100 ppm SDS (treatment 1). The lowest α-glucan content was found when yeast was cultured with 30,000 ppm NaCl (treatment 3). This result might be because NaCl stimulated trehalose turnovered (Parrou, Teste and Francois, 1997) and in this stress, yeast used more energy for maintaining electrochemical gradient of sodium ions, so the storage polysaccharide was lower (Watson, 1970; Walker, 1999). Moreover, the lowest β-glucan yield was found in control. From these results, additives could activate β-glucan formation process and yeast cell wall synthesis. The β-glucan content, average single cell size, cell shape and % yield of cell dry weight were summarized in Table 4.2.

Table 4.2 Elongation of single cell, β-glucan content and % yield of cell dry weight in *S. cerevisiae* Angel® when yeast was cultured in YPD medium supplemented with additives at different concentrations for 24 h

Treatments	Elongation of single cell (μm) ±SD	Shape	Average single cell size(μm^2)^{ns}	β-Glucan content(%w /w ±SD)	Yield (% of cell dry weight)
control	1.79±0.19^{ab}	oval	0.034	5.82±0.30^{c}	0.39±0.15^{ab}
1	0.84±0.18^{c}	rounder	0.034	8.15±0.87^{a}	0.33±0.06^{ab}
2	1.51±0.15^{b}	oval	0.034	6.33±0.92^{bc}	0.42±0.15^{a}
3	1.72±0.03^{ab}	oval	0.034	6.22±0.16^{bc}	0.30±0.18^{b}
4	1.72±0.12^{ab}	oval	0.034	6.56±0.57^{bc}	0.40±0.01^{ab}
5	2.00±0.14^{a}	long oval	0.034	6.41±0.71^{bc}	0.39±0.15^{ab}
6	1.71±0.23^{ab}	oval	0.034	7.39±0.65^{ab}	0.32±0.14^{ab}
7	1.61±0.30^{b}	oval	0.034	6.69±0.51^{bc}	0.35±0.18^{ab}

Values were expressed as mean and standard deviation of triplicate determinations. ^{ns} not significantly differences ($p \leq 0.05$). Mean within a column followed by a different letter are significantly different ($p \leq 0.05$). Treatment 1 = 100 ppm SDS; 2 = 50 ppm EDTA; 3 = 30,000 ppm NaCl; 4 = combination of 5 ppm EDTA and 10 ppm SDS; 5 = combination of 5 ppm EDTA and 3,000 ppm NaCl, 6 = combination of 20 ppm SDS and 3,000 ppm NaCl, 7 = combination of 5 ppm EDTA, 10 ppm SDS and 3,000 ppm NaCl.

Two groups of β-glucan yield were exhibited as shown in Table 4.2. The first one, the highest β-glucan yield (8.15 and 7.39 %w/w) was found when cells were cultured with 100 ppm SDS (treatment 1), combination of 20 ppm SDS and 3,000 ppm NaCl (treatment 6). This group had significantly higher β-glucan yield than control ($p \leq 0.05$). The second one, slightly higher in β-glucan content than control (6.22-6.69 %w/w) were observed when yeast was cultured in other treatments. However, the β-glucan content in this group was not significantly different from control ($p \leq 0.05$). For % yield of cell dry weight, highest % yield

of cell dry weight was exhibited when yeast cultivated with 50 ppm EDTA (treatment 2) wherase the lowest % yield of cell dry weight was shown in the presence of NaCl 30,000 ppm (treatment 3). Nevertheless, the % yield of cell dry weight in all treatments was not significantly different ($p \leq 0.05$). While, the comparision of β-glucan content with control was shown in Fig 4.13

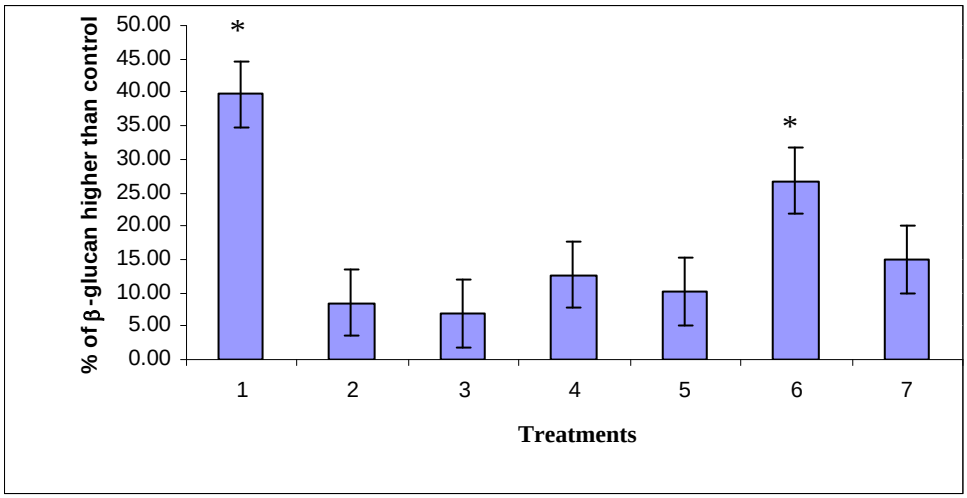


Fig. 4.13 Comparision of β-glucan content with control of *S.cerevisiae* Angel® when cultured yeast with some additives as followed; 1 = 100 ppm SDS; 2 = 50 ppm EDTA; 3 = 30,000 ppm NaCl; 4 = combination of 5 ppm EDTA and 10 ppm SDS; 5 = combination of 5 ppm EDTA and 3,000 ppm NaCl, 6 = combination of 20 ppm SDS and 3,000 ppm NaCl, 7 = combination of 5 ppm EDTA, 10 ppm SDS and 3,000 ppm NaCl, respectively**.** ∗Significant difference ($p \leq 0.05$) compared to the control group

When cells were cultured in these seven treatments of additives, the β-glucan content in all treatments were higher than control as 39.96 % in 100 ppm SDS (treatment1), 26.86 % in combination 20 ppm SDS and 3,000 ppm NaCl (treatment 6), 14.84 % in combination of additions of 5 ppm EDTA , 10 ppm SDS and 3,000 ppm NaCl (treatment 7), 12.70 % in combination of 5 ppm EDTA and 10 ppm SDS (treatment 4), 10.01 % in combination of 5 ppm EDTA and

3,000 ppm NaCl (treatment 5), 8.69 % in 50 ppm EDTA (treatment 2) and 6.78 % in 30,000 ppm NaCl (treatment 3).

Based on these observations, rounder shape cell and the highest numbers of bud scars which showed the highest β-glucan content was observed when cultured yeast with 100 ppm SDS (treatment 1), whereas oval shape exhibited lower β-glucan yield. However, there are no significant differences ($p \leq 0.05$) in elongation compare with other treatments and no obvious correlation in β-glucan yield was observed in the rest of the treatments tested.

The results showed that higher β-glucan content compared to control was observed from yeast supplemented with additives. This could be explained that yeast cultured under this conditions tried to produce larger amount of cell wall component to protect cell from stress causing by additives. Therefore, β-glucan synthase is redistributed in response to cell wall stress to repair general cell wall damage (Delley and Hall, 1999; Smits, Ende and Klis, 2001, Klis *et al.*, 2002; Hohmann, 2003). The highest β-glucan content was generated in *S. cerevisiae* Angel® when it was cultured with 100 ppm SDS. As previously described, a certain level of SDS stimulated depolarizing of β-glucan synthase gene (FKS1) and activated PIM1 the encode gene in MAPK (Mitogen Activated Protein Kinase pathway) in yeast and actin cytoskeleton; activated actin patch and cables (Delley and Hall, 1999; Casano *et al.*, 2001; Lesage and Bussey, 2006). Consequently, increase of the budding process, cell wall synthesis and the number of cell were observed and β-glucan content was then increased respectively as found in this study. Cell wall is biosynthesized mostly during budding process (Frost *et al.*, 1994; Cabib, 2001). SDS, a detergent could act on the plasma membrane; which could increase β-(1,3)-glucan synthase activity, and affect on wall stress component (Frost, Drakef and Wasserman, 1992; Frost *et al.*, 1994). Therefore, under SDS condition, the yeast cells try to reorganize cell wall through MAPK pathway by increasing of actin depolymerization biosynthesis (Smits, Ende and Klis, 2001; Klis *et al.*, 2002). Through these genetic pathway, cell wall synthesis increased therefore the wall component, in particular β-glucan synthesis, could be increased as observed in this study. In the case of EDTA, the

β-glucan content was slightly higher than control. This effect could be explained by the findings of Leal *et al*. (1984) and Guillen *et al.* (1985) who found that EDTA could stimulate β-(1,3)-glucan synthase activity. Thus, β-glucan content could be increased. In the case of NaCl, this additive could activate β-glucan production higher than control for approximately 7 %. This observation could be supported by the work of Blomberg (2000) which explained that NaCl could activate enzymes associated with UDP-glucose synthesis, resulted in the increase of β-glucan synthesis. Moreover, salt could also stimulate ACT1, the actin production control gene (Blomberg, 1995), which involves in the increase of wall component production in *S. cerevisiae* under the hyperosmotic condition (Morris *et al.*, 1983).

4.3 Effect of additives on composition of cell and quality of β-glucan

4.3.1 Determination of polysaccharide, protein and lipid ratio and β-(1,6) : β- (1,3) ratio of yeast cell

S. cerevisiae Angel® was cultivated with various additives (treatment 1-7) under reciprocal incubator shaking at 200 rpm, pH 4, 30 °C for 24 h. The cells were collected and freeze-dried. Polysaccharide: protein: lipid ratio was determined and β-(1,6)-glucan to β-(1,3)-glucan ratio was also estimated by using Fourier Transform Infrared Spectroscopy (FTIR). The example spectra of yeast cells were recorded from 600 cm^{-1} – 4,000 cm^{-1} from triplicate as shown in Fig. 4.14.

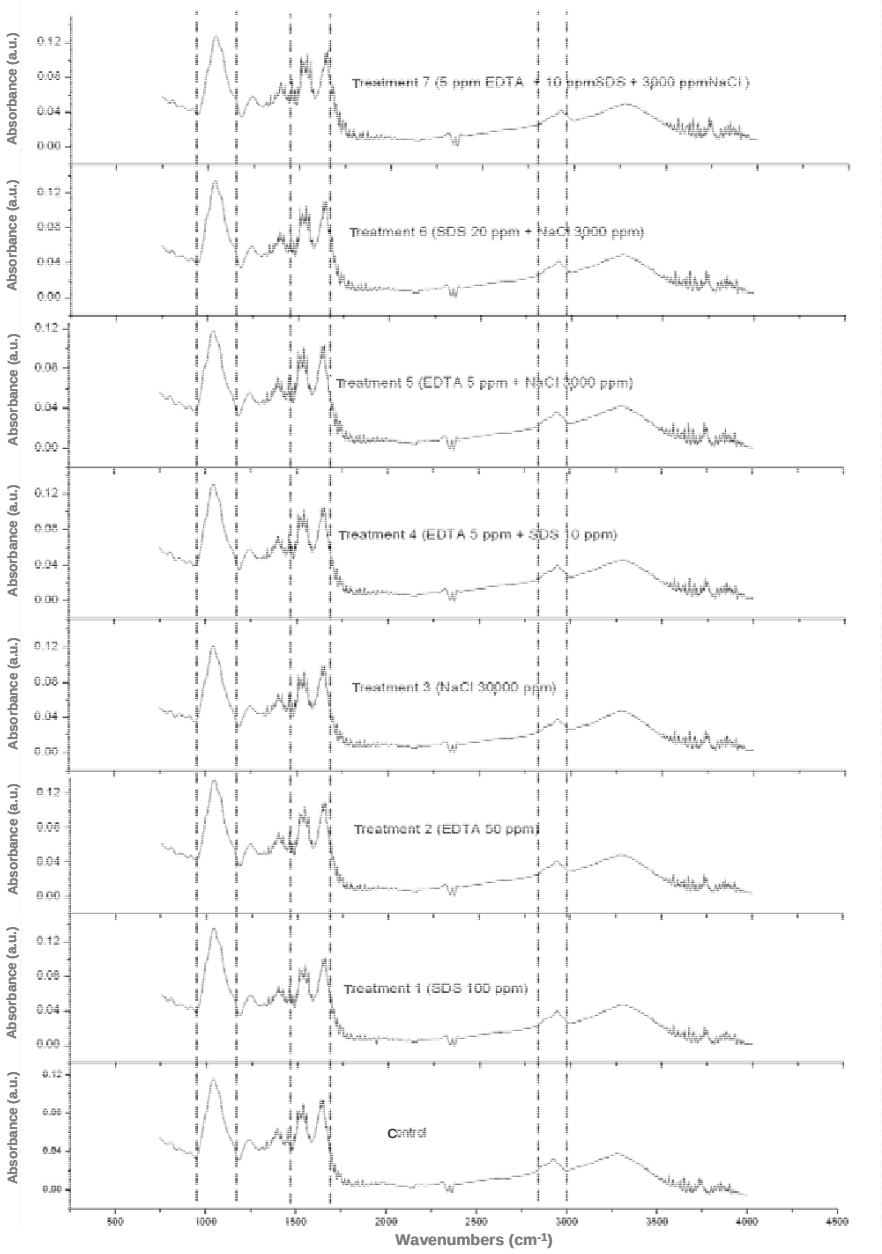


Fig. 4.14 Spectra of yeast cell of *S. cerevisiae* Angel® when yeast was cultured with some additives in different concentrations for 24 h

From Fig. 4.14, the spectra of yeast cell exhibited in 3 regions that is polysaccharide (950 – 1,185 cm^{-1}), protein (1,480 – 1,700 cm^{-1}) and lipid (2,840 – 3,000 cm^{-1}). The spectra of yeast cell in all treatments displayed similar spectral pattern but the absorbance intensities were different. This indicated similar chemical compositions in all treatments. The highest absorbance intensity was found in polysaccharide region followed by protein and lipid regions in all treatments. Curve-fitting was used to analyse structural modifications in three spectral region absorptions: polysaccharide (950 – 1,185 cm^{-1}), protein (1,480 – 1,700 cm^{-1}), and lipid (2,840 – 3,000 cm^{-1}) (Šandula *et al*., 1999; Galichet *et al*., 2001; Adet *et al*., 2006; Karreman *et al*., 2007; Burattini *et al*., 2008; Weeraya, 2008). In this study, spectra of cell with curve-fitting were shown in appendix E. The curve-fitting procedure is based on a least-square method using Gaussian bands. The accuracy of the fit is given by the chi-square value (χ^2). The lower the value of χ^2, the better is the fit (Galichet *et al*., 2001, Adet *et al*., 2006). After curve-fitting, the wavenumbers of spectra and the spectral assignments were summarized from polysaccharide, protein and lipid regions were represented in Table 4.3, 4.4 and 4.5, respectively.

From Table 4.3, six bands were found from curve-fitting the data of polysaccharide regions (950-1,185 cm^{-1}). The band identified at ca. 990 cm^{-1} is assigned to β-(1,6)-glucan (Šandula *et al*., 1999; Galichet *et al*., 2001; Adet *et al*., 2006). The two bands indentified at ca. 1,077, 1,097 cm^{-1} are assigned to β-(1,3)-glucan (Šandula *et al*., 1999; Galichet *et al*., 2001; Karreman *et al*., 2007). Mannan is indentified at the band that is 969 cm^{-1}(Adet *et al*., 2006; Burattini *et al*., 2008).

Table 4.3 Wavenumbers of spectra of cells of *S. cerevisiae* Angel® when yeast was cultured in YPD medium supplemented with additives at different concentrations in polysaccharide absorption (950 – 1,185 cm^{-1})

Wavenumbers									
Treatments									Assignment
laminarin	control	1	2	3	4	5	6	7	
none	969	969	969	968	969	969	969	969	Mannan
996	991	990	990	989	990	990	990	990	β-1,6-glucan
1033	1037	1035	1036	1039	1036	1037	1035	1035	β-1,4-glucan
1072	1078	1078	1078	1076	1077	1077	1077	1077	β-1,3-glucan
1103	1100	1097	1101	1104	1099	1100	1098	1097	β-1,3-glucan
1157	1149	1150	1149	1149	1148	1148	1149	1148	ether

χ^2 values for curve-fitting spectra were in the range 10^{-7}-10^{-8}**.** Treatment 1 = 100 ppm SDS; 2 = 50 ppm EDTA; 3 = 30,000 ppm NaCl; 4 = combination of 5 ppm EDTA and 10 ppm SDS; 5 = combination of 5 ppm EDTA and 3,000 ppm NaCl, 6 = combination of 20 ppm SDS 3,000 ppm NaCl, 7 = combination of 5 ppm EDTA, 10 ppm SDS and 3,000 ppm NaCl**.** Assignment from Šandula *et al*., 1999; Galichet *et al*., 2001; Adet *et al*., 2006; Karreman *et al*., 2007; Burattini *et al*., 2008

Table 4.4 Wavenumbers of spectra of cells of *S. cerevisiae* Angel® when yeast was cultured in YPD medium supplemented with additives at different concentrations in protein absorption (1,480 – 1,700 cm^{-1})

Wavenumbers								Assignment
Treatments								
control	1	2	3	4	5	6	7	
1488	none	1489	none	none	none	none	1488	none
1497	1494	none	1495	1495	1495	1495	1497	none
1506	1506	1501	1506	1506	1506	1506	1506	none
1519	1520	1524	1519	1519	1519	1519	1520	"tyrosine band"
1540	1541	1540	1540	1540	1540	1540	1540	Amide II, protein
1557	1556	1556	1557	1557	1557	1557	1557	Amide II, chitin
1568, 1575	1570	1570	1570	1569	1569	1570	1568, 1575	Amide II, chitin,acetamide
1597	1597	1606	1597	1597	1597	1597	1598	Chitin, acetamide Amide I
1631,1635	1630,1635	1628, 1635	1629, 1635	1635	1629, 1635	1629, 1635	1628, 1635	Amide I
1650	1650	1650	1650	1650	1650	1650	1650	Amide I
1668	1668	1669	1669	1668	1668	1669	1669	Amide I
1683	1683	1683	1683	1683	1683	1683	1683	none

χ^2 values for curve-fitting spectra were in 10^{-6}. Treatment 1 = 100 ppm SDS; 2 = 50 ppm EDTA; 3 = 30,000 ppm NaCl; 4 = combination of 5 ppm EDTA and 10 ppm SDS; 5 = combination of 5 ppm EDTA and 3,000 ppm NaCl, 6 = combination of 20 ppm SDS and 3,000 ppm NaCl, 7 = combination of 5 ppm EDTA, 10 ppm SDS and 3,000 ppm NaCl**.** Assignment from Byler and Susi, 1988; Šandula *et al*., 1999; Maquelin *et al*., 2002; Bahmed *et al.*, 2003; Adet *et al*., 2006; Karreman *et al.*, 2007

From Table 4.4, twelve bands were found from curve-fitting the data of protein regions (1,480-1,700 cm^{-1}). The band identified at ca. 1,519 – 1,575 cm^{-1} are assigned to amide II and chitin (Šandula *et al*., 1999; Maquelin *et al*. 2002; Adet *et al*., 2006). Amide I and chitin are indentified at the bands of ca. 1,598-1,669 cm^{-1} (Bahmed *et al.*, 2003; Adet *et al*., 2006; Karreman *et al.*, 2007).

Table 4.5 Wavenumbers of spectra of cells of *S. cerevisiae* Angel® when yeast was cultured in YPD medium supplemented with additives at different concentrations in lipid absorption (2,840-3,000 cm^{-1})

Wavenumbers								Assignment
Treatments								
control	1	2	3	4	5	6	7	
2852	2852	2852	2852	2852	2852	2852	2852	ν_{sym} CH_2 lipids
2869	2868	2869	2868	2869	2869	2869	2868	ν_{sym} CH_3 lipids
2894	2896	2894	2896	2900	2900	2899	2897	CH deformation of CH_3, lipids, protein and peptides
2925	2925	2926	2926	2926	2926	2926	2926	ν_{sym} CH_2 lipids
2957	2955	2957	2956	2955	2955	2955	2955	ν_{sym} CH_3 lipids

χ^2 values for curve-fitting spectra were in 10^{-9}. Treatment 1 = 100 ppm SDS; 2 = 50 ppm EDTA; 3 = 30,000 ppm NaCl; 4 = combination of 5 ppm EDTA and 10 ppm SDS; 5 = combination of 5 ppm EDTA and 3,000 ppm NaCl, 6 = combination of 20 ppm SDS and 3,000 ppm NaCl, 7 = combination of 5 ppm EDTA, 10 ppm SDS and 3,000 ppm NaCl**.** Assignment from Šandula *et al*., 1999; Maquelin *et al*. 2002; Adet *et al*., 2006; Burattini *et al*., 2008. ν_{sym} is stretching of symmetric carbon (Maquelin *et al*., 2002; Karreman *et. al.*, 2007)

From Table 4.5, five bands were found from curve-fitting the data of lipid regions (2,840-3,000 cm^{-1}). The bands identified at ca. 2,852 cm^{-1}, 2,926 cm^{-1} are assigned to ν_{sym} CH_2 lipids (Šandula *et al*., 1999; Maquelin *et al*., 2002; Adet *et al*., 2006). ν_{sym} CH_3 lipids are indentified at two bands of ca. 2,868 cm^{-1} and 2,955 cm^{-1}(Maquelin *et al*., 2002; Adet *et al*., 2006; Burattini *et al*., 2008). Then the normalized

total area from curve-fitting of polysaccharide, protein and lipid regions were determined. The results were exhibited in Table 4.6.

Table 4.6 Polysaccharide: protein: lipid ratio of cells of *S. cerevisiae* Angel® when cultured yeast in YPD medium supplemented with additives at different concentrations for 24 h

Treatments	Normalized total area of different wavenumber regions			Polysaccharide: protein: lipid ratio
	Polysaccharide (950-1,185 cm^{-1})	Protein(1,480 -1,700 cm^{-1})	Lipid(2,840-3,000 cm^{-1})	
Control	0.37	0.19	0.04	10.8:5.5:1.0
1	0.38	0.18	0.03	11.0:5.0:1.0
2	0.38	0.19	0.03	11.7:6.0:1.0
3	0.35	0.19	0.03	11.3:6.0:1.0
4	0.37	0.19	0.03	11.1:5.7:1.0
5	0.36	0.20	0.03	10.5:5.9:1.0
6	0.39	0.20	0.04	11.1:5.8:1.0
7	0.37	0.20	0.03	11.4:6.1:1.0

Treatment 1 = 100 ppm SDS; 2 = 50 ppm EDTA; 3 = 30,000 ppm NaCl; 4 = combination of 5 ppm EDTA and 10 ppm SDS; 5 = combination of 5 ppm EDTA and 3,000 ppm NaCl, 6 = combination of 20 ppm SDS and 3,000 ppm NaCl, 7 = combination of 5 ppm EDTA, 10 ppm SDS and 3,000 ppm NaCl

These results are related to absorbance intensity of the spectra. The highest absorbance intensity was found in polysaccharide region, followed by protein region and lipid region. That is lowest total area of lipid was observed, followed by protein area and polysaccharide area. Based on the same amount of lipid, the polysaccharide area was approximately 2 folds higher than protein region in all treatments. This result was slightly similar to Varunya (2006) report which showed that polysaccharide was 1.5 fold higher than protein in *S.cerevisiae* SC 90. However, the chemical components in yeast cell depend on the environment culture conditions (Smits *et al.*,

1999; Klis *et al.*, 2002). According to Aguilar-Uscanga and Francois (2003) work, the culture conditions at pH 4, 37 °C enhanced β-glucan production. In this research the culture condition at pH 4, 30 °C was used, so high polysaccharide was found as expected. In addition, Kapteyn *et al.* (2001) reported that low chitin was found when yeast was cultured at pH 3.5-4.0, so low protein should exhibited when compared with normal condition. The ratio of protein region showed lower than control and other treatments when yeast was cultivated with 100 ppm SDS (treatment 1) and slightly higher amount of polysaccharide compared with control because some proteins in cell wall can be extracted with SDS, so the lowest protein was obserbed (Montijn *et al.*, 1994; Vaart *et al.*, 1995). Moreover, yeasts released some protein compounds (purines, pyrimidines, mononucleotides) when treated with SDS (Asami, Hanai and Koizumi, 1977). Overall, the amount of polysaccharide in all treatments was slightly higher than control, with exception in treatment with combination of 5 ppm EDTA and 3,000 ppm NaCl (treatment 5). In this treatment, long oval shape was found (Fig. 4.10, Table 4.2), consequently thinner cell wall possibly caused the lowest polysaccharide content. The highest polysaccharide part was shown when yeast was cultured with 50 ppm EDTA (treatment 2), which can be explained that EDTA stimulated β-(1,3)-glucan synthase (Leal *et.al.*, 1984). Therefore, the absorbance intensity of polysaccharide region increased because β-(1,3)-glucan is one of the constituents of the polysaccharide in cell wall.

The % area of two bands identified for β-(1,6)-glucan and β-(1,3)-glucan were calculated. Then the β-(1,6)-glucan to β-(1,3)-glucan ratio in cell was estimated as shown in Table 4.7. The two bands identified at ca. 991 cm^{-1} and ca.1,078 cm^{-1}, are assigned to β-(1,6)-glucan and β-(1,3)-glucan, respectively. These assignments were based on spectra of laminarin standard (Šandula *et al.*, 1999; Galichet *et al.*, 2001; Adet *et al.*, 2006; Karreman *et al.*, 2007; Burattini *et al.*, 2008). The calculations showed that when yeast was cultivated with additives the β-(1,6)-glucan to β-(1,3)-glucan ratios were higher than control. Moreover, the highest β-(1,6)-glucan to β-(1,3)-glucan ratio (2.57) was exhibited when yeast was cultured with 30,000 ppm NaCl (treatment 3).

Table 4.7 β-(1,6) : β-(1,3) ratio of cells of *S. cerevisiae* Angel® when yeast was cultured in YPD medium supplemented with additives at different concentrations for 24 h

	1,6-Glucan		1,3-Glucan		β-(1,6) : β-(1,3)
Treatments	Wavenumbers (cm^{-1})	Band area (A1)(%)	Wavenumbers (cm^{-1})	Band area (A2)(%)	(A1/A2)
control	991	3.07	1078	1.88	1.63
1	990	4.08	1078	1.95	2.08
2	990	3.03	1078	1.90	1.59
3	989	5.44	1076	2.11	2.57
4	990	3.75	1077	2.03	1.84
5	990	4.00	1077	1.95	2.05
6	990	4.63	1077	2.06	2.24
7	990	3.93	1077	1.92	2.04

Treatment 1 = 100 ppm SDS; 2 = 50 ppm EDTA; 3 = 30,000 ppm NaCl; 4 = combination of 5 ppm EDTA and 10 ppm SDS; 5 = combination of 5 ppm EDTA and 3,000 ppm NaCl, 6 = combination of 20 ppm SDS and 3,000 ppm NaCl, 7 = combination of 5 ppm EDTA, 10 ppm SDS and 3,000 ppm NaCl. Assignment from Šandula *et al*., 1999; Galichet *et al*., 2001; Adet *et al*., 2006; Karreman *et al.*, 2007; Burattini *et al*., 2008

When yeast was cultivated with 50 ppm EDTA (treatment 2), the β-(1,6)-glucan to β-(1,3)-glucan ratio (1.59) was slightly lower than control. This result can be explained that EDTA stimulates β-(1,3)-glucan synthase, so the β-(1,3)-glucan is higher therefore the lowest ratio of β-(1,6)-glucan to β-(1,3)-glucan was observed (Leal *et al*., 1984; Guillen *et al*., 1985). For other treatments, higher β-(1,6)-glucan than control was found. In the stress condition, cells try to reorganize cell wall by MAPK (Mitogen Activated Protein Kinase pathway) in order to protect themself, so β-(1,6)-glucan increases because a higher β-(1,6)-glucan component is expected to increase the rigidity of the glucan matrix and stronger cell wall stability (Jamas, Rha and Sinskey, 1986; Smits and Brul, 2005). Moreover, previous research reported that

the degree of branching of β-glucan depended on the environmental growth conditions (Klis *et al.*, 2002). In addition, Kapteyn *et al*. (2001) reported that highest β-(1,6)-glucan content was found when yeast was cultivated at pH 3.5-4.5.

4.3.2 Determination of the quality of β-glucan

4.3.2.1 Determination of β-(1,6) : β-(1,3) ratio of β-glucan

S. cerevisiae Angel® was cultivated under eight cultural conditions for 24 h. The cells were collected, β-glucan was extracted and freeze-dried. The β-(1,6)-glucan to β-(1,3)-glucan ratio was determined by using Fourier Transform Infrared Spectroscopy (FTIR). The spectra of β-glucan I (one cycle of Savinase treatment) were recorded from 600 cm^{-1} – 4,000 cm^{-1} as shown in Fig. 4.15. Curve-fitting was used to analyze polysaccharide absorption region: 950–1,185 cm^{-1}(Šandula *et al*., 1999; Galichet *et al*., 2001; Adet *et al*., 2006; Karreman *et al*., 2007; Burattini *et al*., 2008). β-Glucan I spectra with curve-fitting were exhibited in appendix F. The wavenumbers of spectra from β-glucan I were represented in Table 4.8.

The spectra of β-glucan I also exhibited in 3 regions which are polysaccharide, protein and lipid (Fig. 4.15). However, the absorbance intensity of β-glucan was higher than that in yeast cell (Fig. 4.14). A similar spectral pattern was shown in all treatments but the absorbance intensity was different. The lowest absorbance intensity of protein region and the highest absorbance intensity of polysaccharide regions were found when yeast was cultured with 100 ppm SDS (treatment 1). This spectrum was corresponded well with the polysaccharide: protein: lipid ratio shown in Table 4.6. The lowest protein part in cell was found when yeast was cultivated with 100 ppm SDS (treatment1). Consequently, it was easy to remove protein from cell wall in extraction process. Thus, the lowest absorbance intensity in protein region was observed in this treatment. The highest protein region was shown in β-glucan I from yeast cultured with combination of 5

ppm EDTA, 10 ppm SDS and 3,000 ppm NaCl (treatment 7). This result could be explained that this stress resulted in more complexcity of mannoprotein portion, thus it is difficult to remove protein from cell.

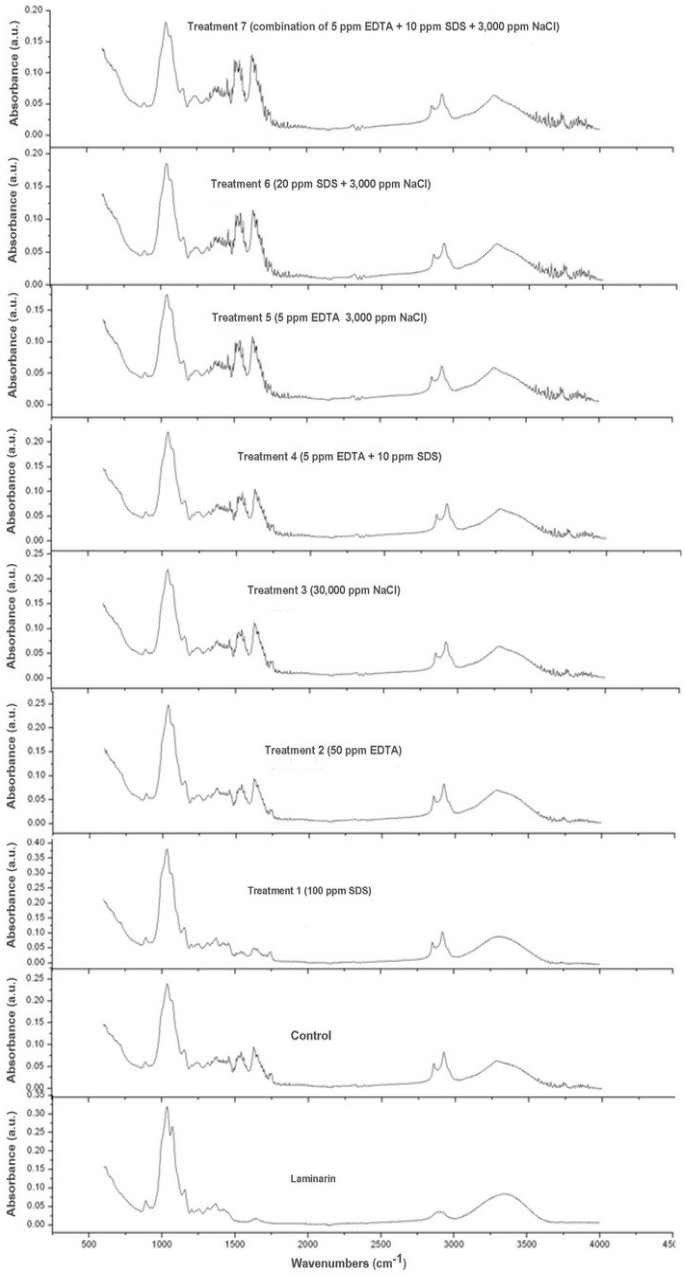


Fig. 4.15 Spectra of β-glucan I (one cycle of Savinase treatment) of *S. cerevisiae* Angel® cultured with some additives at different concentrations

Table 4.8 Wavenumbers of spectra of β-glucan I (one cycle of Savinase treatment) of *S. cerevisiae* Angel® when yeast was cultured with some additives at different concentrations

Wavenumbers									Assignment
Treatments									
laminarin	control	1	2	3	4	5	6	7	
none	966	966	966	none	none	none	none	none	Mannan
996	995	994	996	1000	999	1000	999	1001	β-1,6-glucan
1033	1036	1033	1036	1039	1038	1038	1038	1039	mannan, glycogen
1072	1074	1074	1074	1074	1074	1074	1074	1074	β-1,3-glucan
1103	1103	1104	1103	1104	1104	1104	1104	1104	β-1,3-glucan
1157	1155	1155	1155	1155	1154	1155	1154	1155	ether

χ^2 values for curve-fitting spectra were in 10^{-7}. Treatment 1 = 100 ppm SDS; 2 = 50 ppm EDTA; 3 = 30,000 ppm NaCl; 4 = combination of 5 ppm EDTA and 10 ppm SDS; 5 = combination of 5 ppm EDTA and 3,000 ppm NaCl, 6 = combination of 20 ppm SDS and 3,000 ppm NaCl, 7 = three combination of 5 ppm EDTA, 10 ppm SDS and 3,000 ppm NaCl

From Table 4.8, six bands were found from curve-fitting for the data of polysaccharide regions (950-1,185 cm^{-1}). The band identified at ca. 999 cm^{-1} is assigned to β-(1,6)-glucan (Šandula *et al*., 1999; Galichet *et al*., 2001; Adet *et al*., 2006). Two bands indentified at ca. 1,074, 1,104 are assigned to β-(1,3)-glucan (Šandula *et al*., 1999; Galichet *et al*., 2001; Karreman *et al*., 2007). Mannan or glycogen are indentified at two bands of ca. 966, 1,038 cm^{-1}, respectively (Adet *et al*., 2006; Burattini *et al*., 2008). The bands indentified at ca. 1,150 cm^{-1} is assigned to ether (glycosidic bond) (Adet *et al*., 2006).

From Table 4.9, two bands identified at ca. 999 cm^{-1} and ca. 1,074 cm^{-1}, was assigned to β-(1,6)-glucan and β-(1,3)-glucan, respectively (Šandula *et al*., 1999; Galichet *et al*., 2001; Adet *et al*., 2006; Karreman *et al*., 2007; Burattini *et al*., 2008). The calculations showed that the lower β-(1,6)-glucan to β-(1,3)-glucan ratio in β-glucan I than in yeast cells (Table 4.7) was observed. This reason might be because during extraction process, β-(1,6)-glucan can be broken down easier than β-(1,3)-glucan linear chain.

Table 4.9 β-(1,6) : β-(1,3) ratio in β-glucan I (one cycle of Savinase treatment) of *S. cerevisiae* Angel® when yeast was cultured with some additives at different concentrations

Treatments	1,6-Glucan		1,3-Glucan		β-(1,6) : β-(1,3) (A1/A2)
	Wavenumbers(cm^{-1})	Band area(A1)(%)	Wavenumbers(cm^{-1})	Band area(A2)(%)	
control	995	19.63	1074	17.33	1.13
1	994	21.09	1074	17.88	1.17
2	996	20.61	1074	18.37	1.12
3	1000	28.54	1074	21.73	1.31
4	999	27.04	1074	22.17	1.21
5	1000	27.39	1074	21.68	1.26
6	999	26.14	1074	23.07	1.13
7	1001	27.45	1074	23.93	1.14

Treatment 1 = 100 ppm SDS; 2 = 50 ppm EDTA; 3 = 30,000 ppm NaCl; 4 = combination of 5 ppm EDTA and 10 ppm SDS; 5 = combination of 5 ppm EDTA and 3,000 ppm NaCl, 6 = combination of 20 ppm SDS and 3,000 ppm NaCl, 7 = combination of 5 ppm EDTA, 10 ppm SDS and 3,000 ppm NaCl

When focusing on β-glucan I, slightly higher β-(1,6)-glucan to β-(1,3)-glucan ratio (1.31) was found when yeast was cultured with 30,000 ppm NaCl (treatment 3). This result could be explained that NaCl stress caused the reduction in cell volume (cell shrinkage), increase in cell wall thickness, and high β-(1,6)-glucan and β-(1,3)-glucan ratio in cell as previous mentioned. With thick cell wall, it might be more difficult to break cell and to remove β-(1,6)-glucan in extraction of β-glucan I from whole cell. Therefore, higher β-(1,6)-glucan to β-(1,3)-glucan ratio was observed when yeast was cultured with NaCl.

High impurity of protein content in the range of 0.12 – 3.58 % w/w were shown in β-glucan I (Fig. 4.16). The lowest protein content was found in treatment 1. This result correlated with the FTIR spectrum, which the lowest absorbance intensity of protein region (ca.1,480 – 1,720 cm^{-1}) was exhibited (Fig. 4.15). However, high

protein content was found in the other treatments. Since high protein content retards biological activity of β-glucan (Southeastern Pharmaceutical, 2010) and Xiao *et al*. (2004) reported that β-glucan used for activating immunity had protein content about 2 % w/w, so, the protein should be extracted out from β-glucan I. The protein content of β-glucans II after repeated protein extraction was shown in Figure 4.17.

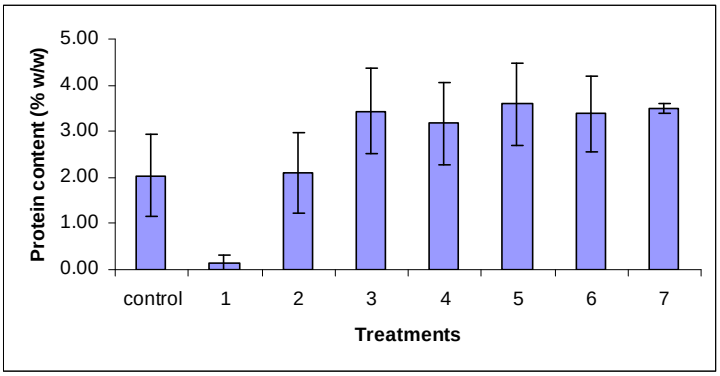


Fig. 4.16 Protein content of β-glucan I in different treatments. Treatment 1 = 100 ppm SDS; 2 = 50 ppm EDTA; 3 = 30,000 ppm NaCl; 4 = combination of 5 ppm EDTA and 10 ppm SDS; 5 = combination of 5 ppm EDTA and 3,000 ppm NaCl, 6 = combination of 20 ppm SDS and 3,000 ppm NaCl, 7 = combination of 5 ppm EDTA, 10 ppm SDS and 3,000 ppm NaCl

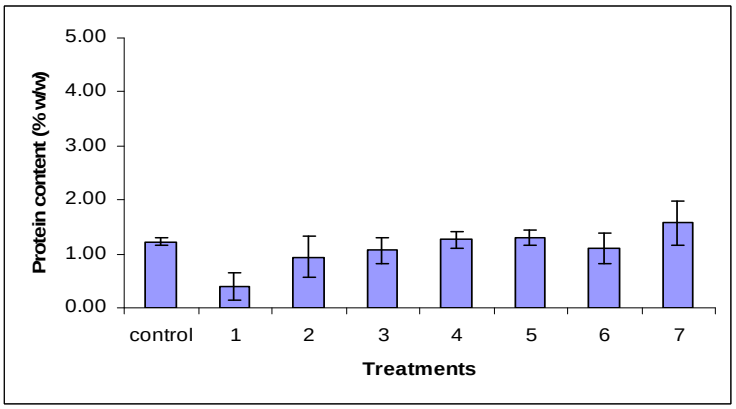


Fig. 4.17 Protein content of β-glucan II in different treatments. Treatment 1 = 100 ppm SDS; 2 = 50 ppm EDTA; 3 = 30,000 ppm NaCl; 4 = combination of 5 ppm EDTA and 10 ppm SDS; 5 = combination of 5 ppm EDTA and 3,000 ppm NaCl, 6 = combination of 20 ppm SDS and 3,000 ppm NaCl, 7 = combination of 5 ppm EDTA, 10 ppm SDS and 3,000 ppm NaCl

From Figure 4.16 and 4.17, the protein content of β-glucan II after repeated extraction with Savinase 16L type EX was lower than that of the corresponding β-glucan I. The protein content after repeated protein extraction process accounted for 0.30 – 1.5 % w/w which was 39 – 69 % reduction, with the exception of β-glucan II from yeast when cultured with 100 ppm SDS (treatment 1) that the protein content was slightly higher. Yield of β-glucan I and β-glucan II were exhibited in Fig 4.18. High yield of β-glucan I was found when yeast was cultivated with 50 ppm EDTA (treatment 2) and combination of 5 ppm EDTA and 3,000 ppm NaCl (treatment 5). The lowest yield was found in treatment with 100 ppm SDS (treatment 1). Then β-glucan I was repeated protein elimination. The lower yield of β-glucan II were exhibited when compare with β-glucan I, the β-glucan I yield in the decreased in the range of 7.38 – 38.34 % (w/w) due to protein removal.

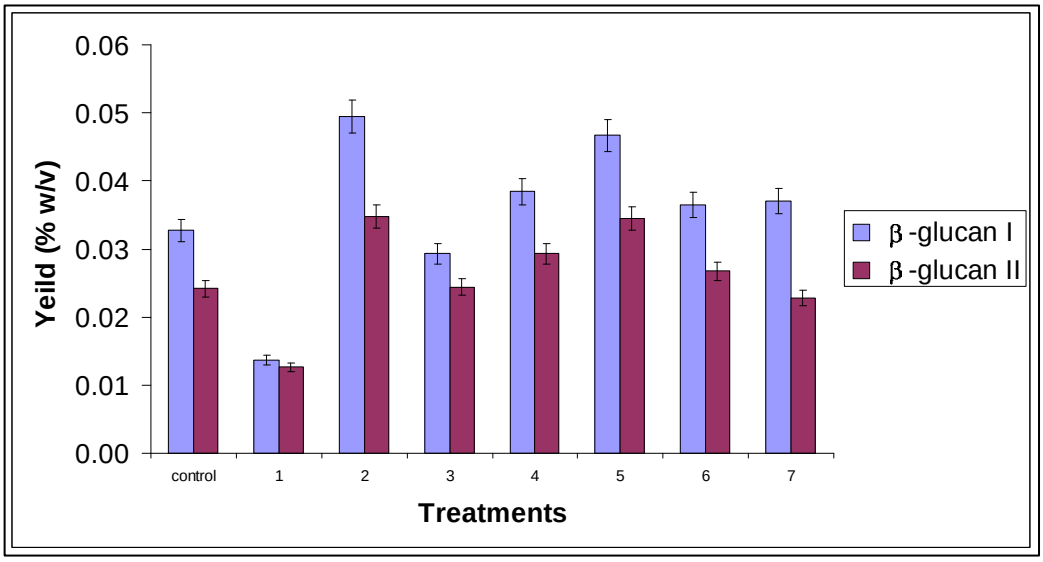


Fig. 4.18 Yeild of β-glucan I and β-glucan II. Treatment 1 = 100 ppm SDS; 2 = 50 ppm EDTA; 3 = 30,000 ppm NaCl; 4 = combination of 5 ppm EDTA and 10 ppm SDS; 5 = combination of 5 ppm EDTA and 3,000 ppm NaCl, 6 = combination of 20 ppm SDS and 3,000 ppm NaCl, 7 = combination of 5 ppm EDTA, 10 ppm SDS and 3,000 ppm NaCl

The spectra of β-glucan II after repeated protein extraction were shown in Fig. 4.19. These spectra showed the lower protein region intensity absorbance of spectra

of β-glucan II and higher polysaccharide region absorbance intensity than β-glucan I. The spectra of β-glucan exhibited in 3 regions that are polysaccharide, protein and lipid. Moreover, the absorbance intensity was higher than in cell and before repeated protein extraction (Fig. 4.14, Fig. 4.15). The similar spectra pattern was obtained in all treatments but the absorbance intensities were different. The lowest absorbance intensity of protein regions (ca. 1,480 – 1,720 cm-1) and the highest absorbance intensity of polysaccharide regions were also found when yeast was cultured with 100 ppm SDS (treatment1) (Fig. 4.15, Fig. 4.16). Curve-fitting was used to analyze structural modifications in polysaccharide spectral regions: 950–1,185 cm^{-1} for the calculation of β-(1,6)-glucan to β-(1,3)-glucan ratio in β-glucan (Šandula *et al*., 1999; Galichet *et al*., 2001; Adet *et al*., 2006; Karreman *et al*., 2007; Burattini *et al*., 2008). The wavenumbers and the spectral assignments of polysaccharide region of β-glucan II were summarized in Table 4.10.

From Table 4.10, five bands were found from curve-fitting for the data of polysaccharide absorption regions (950-1,185 cm^{-1}). The band identified at ca. 999 cm^{-1} was assigned to β-(1,6)-glucan (Šandula *et al*., 1999; Galichet *et al*., 2001; Adet *et al*., 2006). The two bands at ca. 1,074, 1,108 cm^{-1} were assigned to β-(1,3)-glucan (Šandula *et al*., 1999; Galichet *et al*., 2001; Karreman *et al*., 2007). Mannan was indentified at band of ca. 1,034 cm^{-1}, respectively (Adet *et al*., 2006; Burattini *et al*., 2008). The bands at ca. 1,153 cm^{-1} was assigned to ether (Adet *et al*., 2006).

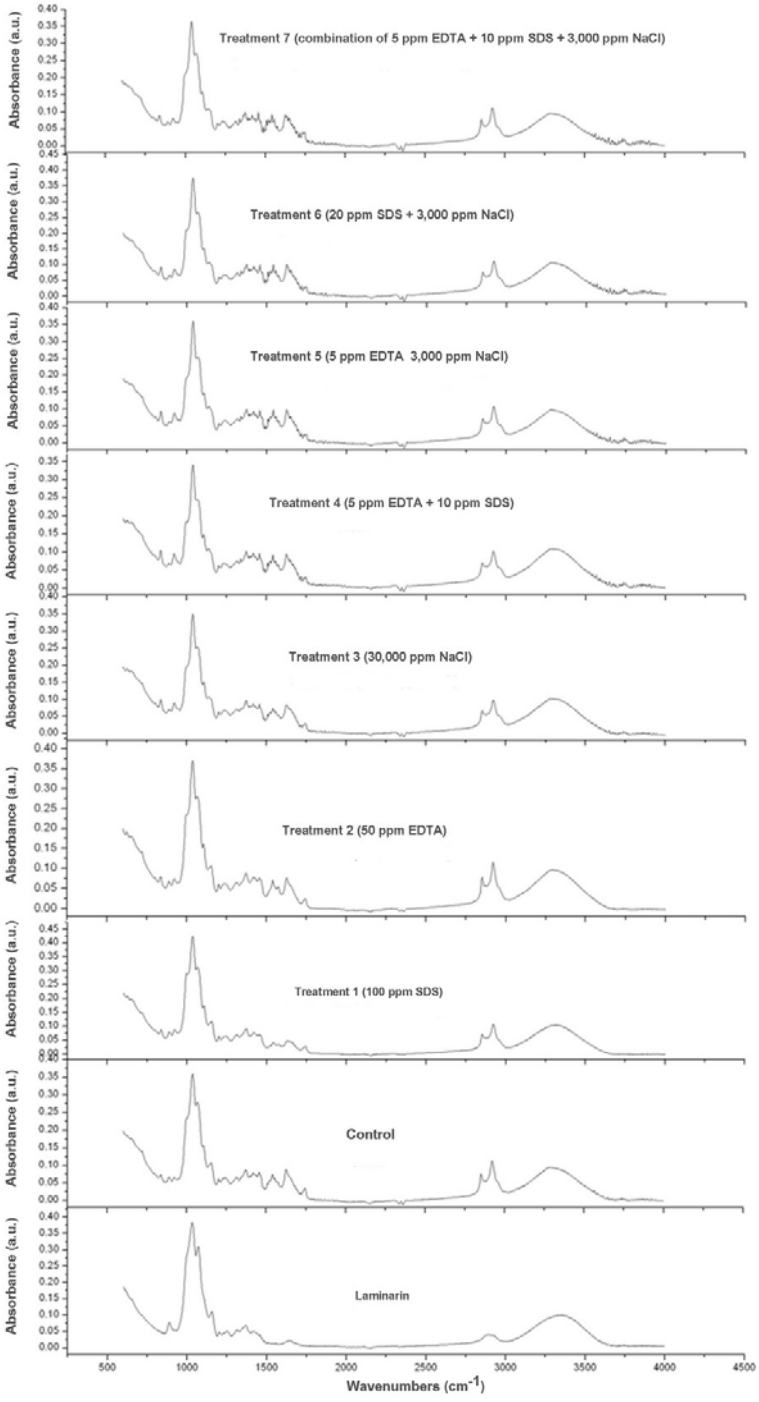


Fig 4.19 Spectra of β-glucan II (two cycles of Savinase treatment) of *S.cerevisiae* Angel® when yeast was cultured with some additives at different concentrations

Table 4.10 Wavenumbers of spectra of β-glucan II (two cycles of Savinase treatment) of *S. cerevisiae* Angel® when yeast was cultured with some additives at different concentrations

Wavenumbers									Assignment
Treatments									
laminarin	control	1	2	3	4	5	6	7	
996	999	997	998	999	999	999	998	1000	β-1,6-glucan
1033	1036	1034	1035	1037	1037	1037	1036	1037	mannan, glycoge
1072	1073	1072	1072	1073	1074	1074	1073	1072	β-1,3-glucan
1103	1108	1108	1108	1108	1108	1108	1108	1108	β-1,3-glucan
1157	1154	1154	1154	1150	1149	1152	1151	1153	ether

χ^2 values for curve-fitting spectra were in 10^{-5}- 10^{-6}. Treatment 1 = 100 ppm SDS; 2 = 50 ppm EDTA; 3 = 30,000 ppm NaCl; 4 = combination of 5 ppm EDTA and 10 ppm SDS; 5 = combination of 5 ppm EDTA and 3,000 ppm NaCl, 6 = combination of 20 ppm SDS and 3,000 ppm NaCl, 7 = combination of 5 ppm EDTA, 10 ppm SDS and 3,000 ppm NaCl

From Table 4.11, the two bands identified at ca. 999 cm^{-1} and ca.1,073 cm^{-1}, were assigned to β-(1,6)-glucan and β-(1,3)-glucan, respectively (Šandula *et al.*, 1999; Galichet *et al.*, 2001; Adet *et al.*, 2006; Karreman *et al.*, 2007; Burattini *et al.*, 2008). From calculations showed that the β-(1,6)-glucan to β-(1,3)-glucan ratio was slightly lower than control when yeast was cultured with additives. The lowest β-(1,6)-glucan to β-(1,3)-glucan ratio was shown when yeast was cultivated with 5 ppm EDTA and 10 ppm SDS (treatment 4). This result is similar with the work of Freimund *et al.* (2003), they showed that the loss of β-glucan after treated with Savinase was predominantly β-(1,6)-glucan. Since, β-(1,6)-glucan was probably broken easier than β-(1,3)-glucan in repeated protein extraction process, so the lower β-(1,6)-glucan to β-(1,3)-glucan ratio was observed when repeated protein extraction was done.

Table 4. 11 β-(1,6) : β-(1,3) ratio in β-glucan II (two cycles of Savinase treatment) of *S. cerevisiae* Angel® when yeast was cultured with additives at different concentrations

	1,6-Glucan		1,3-Glucan		β-(1,6) : β-(1,3)
Treatments	Wavenumbers (cm-1)	Band area(A1)(%)	Wavenumbers (cm-1)	Band area(A2)(%)	(A1/A2)
control	999	23.94	1073	28.31	0.84
1	997	22.48	1072	26.92	0.83
2	998	22.67	1072	28.53	0.79
3	999	18.86	1073	27.91	0.77
4	999	18.86	1074	27.20	0.69
5	999	19.29	1074	26.89	0.71
6	998	21.20	1073	27.12	0.78
7	1000	23.09	1072	29.40	0.78

Treatment 1 = 100 ppm SDS; 2 = 50 ppm EDTA; 3 = 30,000 ppm NaCl; 4 = combination of 5 ppm EDTA and 10 ppm SDS; 5 = combination of 5 ppm EDTA and 3,000 ppm NaCl, 6 = combination of 20 ppm SDS and 3,000 ppm NaCl, 7 = combination of 5 ppm EDTA, 10 ppm SDS and 3,000 ppm NaCl

4.3.2.2 Determination of weight average molecular weight of β-glucan

Fig 4.20 shows the effect of additives used in culturing yeast on weight average molecular weight of β-glucan. Molecular weight obtained from treatment 6 and 7 was one order of magnitude higher than that of other treatments including control. They were 2.36×10^5 and 2.80×10^5 daltons, respectively.

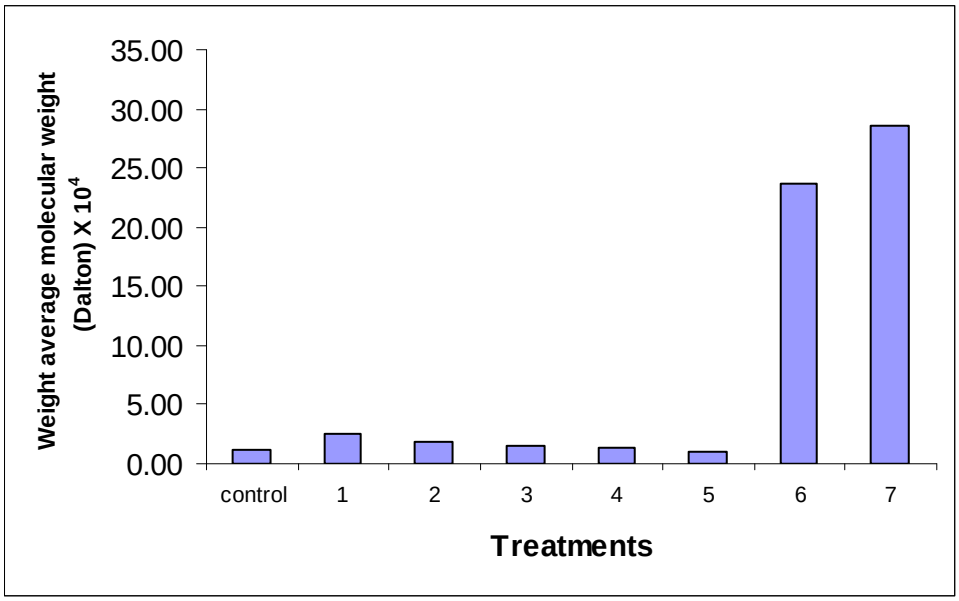


Fig. 4.20 Weight average molecular weight of β-glucan from *S. cerevisiae* Angel® when yeast was cultured with additives at different concentrations. Treatment 1 = 100 ppm SDS; 2 = 50 ppm EDTA; 3 = 30,000 ppm NaCl; 4 = combination of 5 ppm EDTA and 10 ppm SDS; 5 = combination of 5 ppm EDTA and 3,000 ppm NaCl, 6 = combination of 20 ppm SDS and 3,000 ppm NaCl, 7 = combination of 5 ppm EDTA, 10 ppm SDS and 3,000 ppm NaCl

Low molecular weight in the range of 1.07 – 2.50 X 10^4 daltons was found in other treatments including control. This result was similar with the work of Manners *et al*.(1973) and Williams *et al*. (1991) which was reported that β-glucan and β-glucan phosphate from *S. cerevisiae* exhibited the molecular weight of 2.40 X 10^5 daltons and 1.10 X 10^5 daltons, respectively. Both researches used alkali-acid method in β-glucan extraction process, so the molecular weight should probably be slightly different. From this obtained result, it is of interest to further investigate why SDS and NaCl affect on weight average molecular weight of β-glucan.

4.3.2.3 Determination of immunomodulatory properties of β-glucan

Due to a similar value of the β-1,6-glucan to β-1,3-glucan ratio of β-glucan II in all treatments (section 4.3.2.1), β-Glucan II in control and treatment with 100 ppm SDS (treatment 1) were selected for the determination of immunomudulatory properties. After they were derivatized to carboxymethylglucan (CM-glucan), two different parts of CM-glucan were obtained. They were water soluble CM-glucan and water insoluble CM-glucan. The spectra of CM-glucan from control and selected treatments were presented in Fig. 4.21- 4.22.

From Fig. 4.21, when spectra of CM-glucans from *S. cerevisiae* Angel® were compared to β-glucan (control), higher absorbance intensities at ca. 1305, 1426, 1605 cm^{-1} were found in CM-glucan (both in water insoluble part and water soluble part). In addition, the same trend was also shown in treatment 1 (Fig. 4.22) (Pushpamalar *et al*., 2006; Yuen *et al*., 2009). Degree of Substitution (DS) was assessed. DS is defined as the number average degree of carboxymethyl group substituted per anhydroglucose unit. So, the position of C-2, C-4 and C-6 in the glucose molecule are the point of carboxymethylation of hydroxyl group with mono-choloroacetic acid (Eyler, Kluy and Diephuis, 1947; Conner and Eyler, 1950). From preliminary determination of DS, curve-fitting of the absorbance intensity spectra between marker band of carboxymethyl group (1,300 – 1,700 cm^{-1}) and internal standard of β-glycosidic linkage (850 – 950 cm^{-1}) were performed.

Then, the DS was calculated by using standard curve that reported in Yuen *et al.* (2009), who studied the determination of DS of polysaccharide by vibrational spectroscopic methods. The results showed that DS of CM-glucan of control in water insoluble, water soluble parts, CM-glucan of the selected treatment in water insoluble and water soluble parts were shown in Table 4.12 and 4.13. Šandula, Machová and Hribalová (1995) reported that the solubility of CM-glucans in water depends on their DS. Samples with DS of 0-0.5 were insoluble or partially soluble, samples with DS 0.56, 0.75 and 0.89 gave opalescent solutions, and those with DS 1.08 or 1.15 gave clear solutions at a concentration of 2 mg/ml.

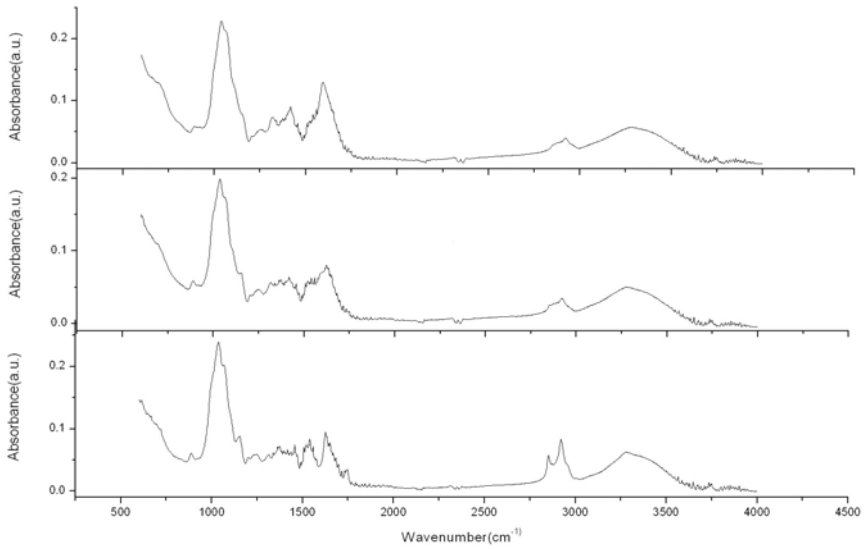


Fig. 4.21 FTIR Spectra of β-glucan and CM-glucan from *S. cerevisiae* Angel® when yeast was cultivated in YPD medium without additives (control). lower curve: β-glucan spectrum ; middle curve: water insoluble CM-glucan spectrum and upper curve: water soluble CM-glucan spectrum

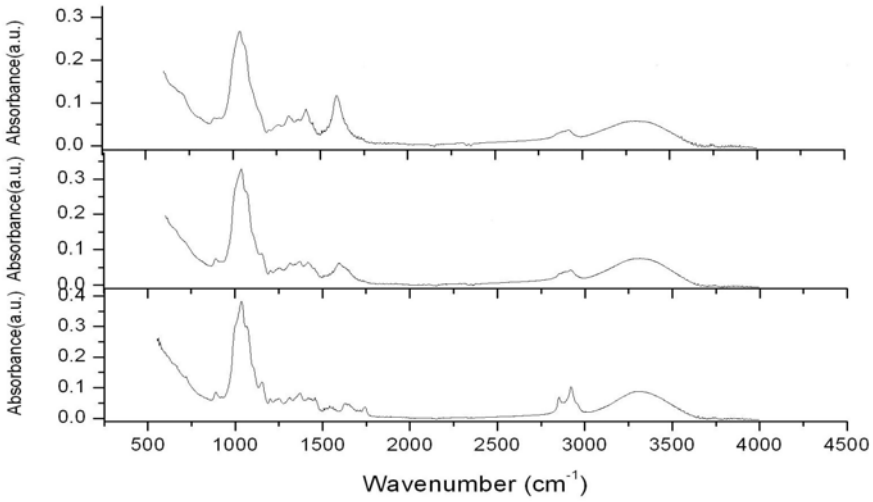


Fig. 4.22 FTIR Spectra of β-glucan and CM-glucan from *S. cerevisiae* Angel® when yeast was cultivated in YPD medium with 100 ppm SDS (treatment 1). lower curve: β-glucan spectrum ; middle curve: water insoluble CM-glucan spectrum and upper curve: water soluble CM-glucan spectrum

Nevertheless, the DS in this study was higher than that previously reported. Since Yuen *et al.* (2009) used xanthan gum for the calculation of standard curve after

curve-fitting process, so this is probably not suitable for β-glucan. However, the CM-glucan in water soluble part had higher DS than that in water insoluble one, resulting in higher water solubility. This result was similar with the work reported by Šandula *et al.* (1999). After that the water insoluble and water soluble CM-glucan from control and the selected treatment with different DS were tested to macrophage cell (RAW. 264.7) by using Nitric Oxide (NO) assay and MTT assay. The results were shown in Table 4.12 and 4.13, respectively.

Table 4.12 $NaNO_2$ concentrations (μM) from RAW 264.7 when cells were treated with CM-glucan from *S. cerevisiae* Angel® in control and treatment with 100 ppm SDS(treatment 1).

Treatments	DS (degree of substitution)	$NaNO_2$ concentrations (μM)			
		Concentrations (μg/ml)			
		500	1,000	2,000	4,000
control -water insoluble	0.67	0.06±0.03	0.16±0.06	0.60±0.13	1.98±0.93
control -water soluble	1.35	0.27±0.09	1.06±0.55	2.04±0.21	13.92±1.55
treatment 1 -water insoluble	0.48	1.83±0.21	2.72±0.19	2.22±0.12	1.48±0.26
treatment1 -water soluble	0.74	0.26±0.18	0.57±0.19	1.15±0.47	1.87±0.48

Value were expressed as mean and standard deviation of eight replications

As shown in Table 4.12, the macrophages can be compelled to release nitric oxide when incubated with CM-glucan from *S. cerevisiae* Angel® in control and treatment with 100 ppm SDS with differences DS. In the case of DS 0.67, 0.74 and 1.35, high concentrations of CM-glucan were found to highly induce of nitric oxide generation of macrophage cell. With the exception of low DS (0.48), the highest stimulation activity was exhibited at the concentration of 1,000 μg/ml. These results could be explained that high concentration of CM-glucan might be toxic to macrophage cell, so cells became weak and low releasing activity of nitric oxide was observed. The highest stimulation

activity of water insoluble CM-glucan in control was found. Since the β-(1,6)-glucan to β-(1,3)-glucan ratio of control were highest, high activation of macrophage activity should be exhibited. Previous researchs showed that high ratio of β-1,6-glucan had high activity to stimulate macrophage cell (Jamas *et al*., 1996; Cleary, Kelly and Husband, 1999; Kubala *et al*., 2003; Pang *et al.*, 2005). The stimulation requires specific β-glucan receptor cross-linking (Cleary, Kelly and Husband, 1999; Chen and Seviour, 2007), so branching has good interaction than linear chain. Thus, low stimulation activity was observed in this study. Nevertheless, the immunomodulatory properties of CM-glucan depend on DS, polymer charge, the degree of branching (β-(1,6) : β-(1,3) ratio), molecular weight (MW), β-glucan conformation, the water solubility and the properties of macrophage cell (Yadomae, 2000; Mantovani *et al.*, 2008). Othor authors reported that CM-glucan with DS = 0.75, $0.2 \leq DB \geq 0.3$, $100 \leq MW \geq 200$ KDa and triple-helix structure showed the highest activity for stimulating macrophage cell (Šandula, Machová and Hribalová, 1995; Zekovic *et al*., 2005). The weight average molecular weight of β-glucan from this research exhibited lower 100 KDa and higher than 200 KDa (Fig. 4.20). The positive immunity should be found, but not in this study. This might be because there are many factors affecting the biological activity as described above.

Table 4.13 % cell death in RAW 264.7 when cells were treated with CM-glucan from *S. cerevisiae* Angel® in control and treatment with 100 ppm SDS (treatment 1).

Treatments	DS (degree of substitution)	Cell death (%)			
		Concentrations (μg/ml)			
		500	1,000	2,000	4,000
control -water insoluble	0.67	23.72±1.91	25.34±2.64	30.51±2.13	38.36±3.03
control -water soluble	1.35	34.72±1.73	37.58±2.08	38.42±1.63	41.25±1.71
treatment1 -water soluble	0.74	28.33±2.39	34.02±2.60	40.75±1.33	48.63±2.49

Value were expressed as mean and standard deviation of eight replications

Based on the minimal toxic concentration (TC_{50}) that killing 50 % of macrophage cells (Kostoryz *et al*., 1999; Tempone *et al*., 2005), the CM-glucan samples were non-toxic (Table 4.13). However, it is noticeable that high concentration of CM-glucan (more than 4,000 μg/ml) seemed to be toxic. This results can be explained that high concentration of CM-glucan had high negative charge (Rice *et al*., 2002). The carboxyl groups in the derivative might react with macrophage cell membrane and show toxicity (Seljelid *et al.*, 1984).

In summary, from the polysaccharide: protein: lipid ratio in the cell and base on the same amount of lipid, the polysaccharide region in yeast cell was increased when yeast was cultured with additives. The highest polysaccharide region was found when yeast was cultured with 50 ppm EDTA (treatment 2). While, the lowest protein region was observed when cultured yeast with 100 ppm SDS (treatment 1). Furthermore, the absorbance intensity of spectrum in this treatment exhibited the lowest in wavenumber 1,480–1,700 cm^{-1} (protein region) too. Considering β-(1,6) : β-(1,3) ratio in cell, the ratios were higher when cultured yeast with additives, with exception of the treatment with 50 ppm EDTA (treatment 2). The highest ratio was shown when yeast was cultured with 30,000 ppm NaCl (treatment 3). Then, comparing β-glucan I with control, slightly lower β-(1,6)-glucan to β-(1,3)-glucan ratios were found. The β-glucan in control and selected treatment (treatment 1) were derivatized to water soluble derivative (CM-glucan) in order to increase the solubility of β-glucan in DMEM medium for *in vitro* immunomudulatory properties testing. This result provided that the high activity for releasing nitric oxide of CM-glucan depends on the concentration in the high DS group. With exception in low DS group, the high stimulation activity was exhibited at 1,000 μg/ml. Moreover, the high inhibitory activity of high concentration of CM-glucan were found in all treatments. The biological activity from this result was still not clearly demonstrated, however there was a tendency that the DS and concentration of CM-glucan seemed to affect stimulation activity of macrophage cell as well as their cytotoxicity. These results provide a basic information about the effect of additives on quantity, quality of β-glucan production from *S. cerevisiae* and modification β-glucan derivative for using in food industry, cosmetic industry in the future.

CHAPTER V

CONCLUSION

After screening of *S. cerevisiae* strain, *S. cerevisiae* Angel® was selected for further study, on the basis of high β-glucan yield, % yield of cell dry weight and net β-glucan yield. Then the effect of type and concentration of additives on yeast growth was investigated. No growth and cell death were found when cultured yeast with high concentration and combination of additives. In the case of yeast growth, it was shown that sigmoid curve of yeast growth can be explained by using non-linear regression in the function of shifted logistic equation. SDS at 100 ppm and 200 ppm gave higher growth level than control. Thus, high budding process was found and the number of cells was higher. On the other hand, 50 and 100 ppm EDTA resulted in reduction of growth level. Similarly, the growth level of yeast was reduced in 30,000 ppm NaCl and 60,000 ppm. In addition, the time to reach the highest growth rate was longer when yeast was cultured with additives. Based on these observations, the optimum concentrations of single additives (four treatments) on cell shape, wall surface, β-glucan production, composition of cell and β-(1,6)-glucan to β-(1,3)-glucan ratio in yeast cell were 50 ppm EDTA, 100 ppm SDS and 30,000 ppm NaCl under reciprocal shaking incubator at 200 rpm, pH 4 at 30 °C for 24 h. Then, the suitable combination of additives were estimated by using computer software. Additional combination conditions of additives (four treatments) were EDTA 5 ppm and SDS 10 ppm; EDTA 5 ppm and NaCl 3,000 ppm ; SDS 20 ppm and NaCl 3,000 ppm; and combination of EDTA 5 ppm, SDS 10 ppm and NaCl 3,000 ppm. For further investigation, eight treatments were used to study the effect of additives on cell shape, wall surface, β-glucan production, composition of cell and β-(1,6)-glucan to β-(1,3)-glucan ratio in *S. cerevisiae* Angel®. The results demonstrated that type and concentration of additives used did not significantly affect an average single cell size but played role in cell shape during growth. Rounder shape (major axis length longer than minor axis length

only 0.84 um;), and the highest β-glucan content (8.15 %w/w) were exhibited when cultured yeast with 100 ppm SDS (treatment 1) and this group was significantly different from other treatments ($p \leq 0.05$). Moreover, β-glucan content was higher than control approximately 40 %. Whilst, the second group with long and oval shape was found in combination of 5 ppm EDTA and 3,000 ppm NaCl (longest elongation of single cell; 2.00 um; treatment 5) and the last one, oval shape which was similar to control was found in the rest treatments. Moreover, the cell shape from Confocal microscope was related to those from SEM at magnification 5,000X. Wall surface representatives were determined by SEM at magnification 15,000X. The cells treated with 100 ppm SDS (treatment 1) having rounder shape, multiple bud scars on the wall surface were found more than control and other treatments. Based on these results, cell shape seemed to correlate with β-glucan production. From this study, it was observed that the additive SDS at certain concentration (100 ppm) could stimulate *S. cerevisiae* growth by increasing the budding process. The cells with oval or long shape observed in other conditions had significantly lower β-glucan content than rounder shape. The β-glucan content among these conditions were not significantly different. Moreover, the cell became shrink and longer when cultured yeast with NaCl. Highest % yield of cell dry weight was found when yeast was cultivated with 50 ppm EDTA (treatment 2). From FTIR spectra in yeast cell, similar patterns of absorbance intensity were shown among treatments. The absorbance intensity of polysaccharide region (950-1,185 cm^{-1}) was the highest, followed by protein region (1,480-1,700 cm^{-1}) and the lowest in lipid (2,840-3,000 cm^{-1}). Based on the same amount of lipid, higher amount of polysaccharide region was shown when yeast was cultured with additives. Thus, the cell wall could be increased when yeast was cultured with additives. Furthermore, the highest polysaccharide region was exhibited when yeast was cultured with 50 ppm EDTA (treatment 2). The protein region was lower than control and other treatments when yeast was cultivated with 100 ppm SDS (treatment 1). For the β-(1,6)-glucan to β-(1,3)-glucan ratio in cell, it was shown that the ratios were higher when yeast was cultivated with additives, with exception of the yeast cultured in 50 ppm EDTA. The highest β-(1,6)-glucan to

β-(1,3)-glucan ratio was exhibited when yeast was cultured with 30,000 ppm NaCl (treatment 3). The β-(1,6)-glucan to β-(1,3)-glucan ratio was lower than control in β-glucan from yeast cultivated with some additives after β-glucan extraction. For immunomodulatory properties, the β-glucan was derivatized to CM-glucan before test. It was found that degree of substitution (DS) and concentration of CM-glucan seemed to affect significantly the stimulation activity of macrophage cell as well as their cytotoxicity. The higher DS and concentration, the higher activity tended to be. However, the biological activity on activation of macrophage cell of CM-glucan depended on many factors and needs further investigation.

Overall, from results obtained, the additives affect to cell shape, wall surface, the chemical compositions and β-glucan production of yeast cell. Based on the similar amount of protein, β-glucan from yeast was cultivated with 50 ppm EDTA (treatment 2) and combination of 5 ppm EDTA and 3,000 ppm NaCl (treatment 5) were recommended to use, because high % yield of β-glucan I and β-glucan II were found. However, in pharmaceutical or cosmetic industries, β-glucan I from yeast cultured with 100 ppm SDS (treatment 1) was suggested to use, because the lowest protein was exhibited.

In conclusion, the β-glucan extraction and modifier derivatization process were suggested to optimize for increasing the immunomudulatory properties before future application.

REFERENCES

Adams, E. L. et al. 2008. Differential high-affinity interaction of Dectin-1 with natural or synthetic glucans is dependent upon primary structure and is influenced by polymer chain length and side-chain branching. The Journal of Pharmacology and Experimental Therapeutics 325(1) : 115-123.

Adt, I., Toubas, D, Pinon, J.M., Manfait, M., and Sockalingum, G.D. 2006. FTIR spectroscopy as a potential tool to analyse structural modifications during morphogenesis of *Candida albicans*. Archives Microbiology 85 : 277–285.

Akinobu, Y. 2003. Antitumor and radiation protection effects of β-1,3-D-glucan extracted from yeast (*Saccharomyces cerevisiae*). Radioisotopes 52(12) : 687-691.

Akramienė, D., Kondrotas, A., Didžiapetriene, J., and Kėvelaitis, E. 2007. Effects of β-glucan on the immuno system. Medicina(Kaunas) 43(8) : 597-604.

Aldridge, B.A., Razzak, C.A., Babcock, T.A., Helton, W.S., and Espat, N. J. 2008. Lipopolysaccharide-stimulated RAW 264.7 macrophage inducible nitric oxide synthase and nitric oxide production is decreased by and omega-3 fatty acid lipid emulsion. Journal of Surgical Research 149 : 296–302.

Alexopoulos, C.J., Mims, C.W., and Blackwell, M. 1996. Introductory mycology. Fourth edition. John Wiley & Sons, Inc. United States of America.

Almagro, A. et al. 2000. Effects of salts on *Debaryomyces hansenii* and *S. cerevisiae* under stress conditions. International Journal of Food Microbiology 56 : 191–197.

Aguilar-Uscanga, B., and Francois, J.M. 2003. A study of the yeast cell wall composition and structure in response to growth conditions and mode of cultivation. Letters in Applied Microbiology 37 : 268–274.

Ara, Y. et al. 2001. Zymosan enhances the immune response to DNA vaccine for human immunodeciency virus type-1 through the activation of complement system. Immunology 103 : 98-105.

Arnold, W.N. 1972. The structure of the yeast cell wall; solubilization of a maker enzyme, β-fructofuranosidase, by the autolytic enzyme system. Journal of Biological Chemistry 247 : 1161-ll69.

Asami, K., Hanai, T., and Koizumi, N. 1977. Dielectric properties of yeast cells: effect of some ionic detergent on the plasma membrances. Journal Membrance Biology 34 : 145-156.

Attfield, P.V. 1997. Stress tolerance: the key to effective strains of industrial baker's yeast. Nature Biotechnology 15 : 1351-1357.

Bahmed, K., Quilès, F., Bonaly, R., and Coulon, J. 2003. Fluorescence and infrared spectrometric study of cell walls from *Candida*, *Kluyveromyces*, *Rhodotorula* and *Schizosaccharomyces* yeasts in relation with their chemical composition. Biomacromolecules 4 : 1763-1772.

Bickle, M., Delley, P.A., Schmidt, A., and Hall, M.N. 1998. Cell wall integrity modulates RHO1 activity via the exchange factor ROM2. The EMBO Journal 17(8) : 2235-2245.

Blomberg, A. 1995. Global changes in protein synthesis during adaptation of the yeast *Saccharomyces cerevisiae* to 0.7 M NaCl. Journal of Bacteriology 177 : 3563 – 3572.

Blomberg, A. 2000. Metabolic surprises in *Saccharomyces cerevisiae* during adaptation to saline conditions: questions, some answers and a model. FEMS Microbiology Letters 182 : 1-8.

Bopp, S., and Lettieri, T. 2008. Comparison of four different colorimetric and fluorometric cytotoxicity assays in a zebrafish liver cell line. BMC Pharmacology 8(8) : 1-11.

Brattgjerd, S., Evensen, 0., and Lauve, A. 1994. Effect of injected yeast glucan on the activity of macrophages in Atlantic salmon, *Salmo salar* L., as evaluated by *in vitro* hydrogen peroxide production and phagocytic capacity. Immunology 83 : 288-294.

Burattini, E. et al. 2008. A FTIR microspectroscopy study of autolysis in cells of the wine yeast *Saccharomyces cerevisiae*. Vibrational Spectroscopy 47 : 139–147.

Byler, D.M., and Susi, H. 1988. Application of computerized infrared and raman spectroscopy to the conformation studies of casein and other food proteins. Journal of Industrial Microbiology 3 : 73-88.

Cabib, E., Mol, P.C., Shaw, J.A., and Choi, W.J. 1993. Biosynthesis of cell wall and septum during yeast growth. Archives of Medical Research 24(3) : 301 – 303.

Cabib, E., Drgonov´, J., and Drgon, T. 1998. Role of small G proteins in yeast cell polarization and cell biosynthesis. Annual Review of Biochemistry 67 : 307-333.

Cabib, E., Roh, D.H., Schmidt, M., Crotti, L.B., and Varma, A. 2001. The yeast cell wall and septum as paradigms of cell growth and morphogenesis. The Journal of Biological Chemistry 276(23) : 19679–19682.

Cain, K.D., Grabowski, L., Reilly, J., and Lytwyn, M. 2003. Immunomodulatory effects of a bacterial-derived β-1,3-glucan administered to tilapia (*Oreochromis nilotocus* L.) in a Spirulina-based diet. Aquaculture Research 34 : 1241-1244.

Chen, J., and Seviour, R. 2007. Medicinal importance of fungal β-(1/3),(1/6)-glucans. Mycological Research 3 : 635-652.

Chowdhury, S., Smite, K.W., and Gustin, M.C. 1992. Osmotic stress and the yeast cytoskeleton: phenotype specific suppression of an actin mutation. Journal of Cell Biology 118 : 561-571.

Cleary, A., Kelly, G., and Husband, A. J. 1999. The effect of molecular weight and β-1,6-linkages on priming of macrophage function in mice by (1,3)-β-D-glucan. Immunology and Cell Biology 77 : 395 - 403.

Coelho, M.A.Z. et al. 2004. Effect of hyperbaric stress on yeast morphology: study by automated image analysis. Applied Microbiology Biotechnology 66 : 318 - 324.

Coen, M.L., Lerner, C.G., Capobianco, J.O., and Goldman., R.C. 1994. Synthesis of yeast cell wall glucan and evidence for glucan metabolism in a *Saccharomyces cerevisiae* whole cell system. Microbiology 140 : 2229-2237.

Conner, A.Z., and Eyler, R.W. 1950. Analysis of sodium carboxymethylcellulose: copper salt precipitation method for determining assay: value and degree of substitution. Analytical Chemistry 22(9) : 1129 – 1132.

Corradini, M.G., and Peleg, M. 2006. Prediction of vitamins loss during non-isothermal heat processes and storage with non-linear kinetic models. Trends Food Sci & Technology 17 : 24–34.

Cosano, I.C., Martiń, H., Flandez, M., Nombela, C., and Molina, M. 2001. Pim1, a MAP kinase involved in cell wall intergrity in *Pichia pastoris*. Molecular Genetics Genomics 265 : 604- 614.

Dai, Y., McLandsborough, L.A., Weiss, J., and Peleg, M. 2010. Concentration and application order effects of sodium benzoate and eugenol mixtures on the growth inhibition of *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii*. Journal of Food Science 75(5) : 482-488.

Debye, P. 1947. Molecular weight determination by light scattering. Journal of Physical Colloid Chemistry 51: 18-32.

Delley, P.A., and Hall, M.N. 1999. Cell wall stress depolarizes cell growth via hyperactivation of RHO1. Journal Cell Biology 147 : 163–174.

Ebada, S., Edrada, R.A., Lin, W., and Proksch, P. 2008. Methods for isolation, purification and structural elucidation of bioactive secondary metabolites from marine invertebrates. Nature Protocols 3 (12) : 1820-1831.

Eyler, R.W., Kluy, E.D., and Diephuis, F. 1947. Determination of degree of substitution of sodium carboxymethycellulose. Analytical Chemistry 19(1) : 24 – 27.

Fox, T. E. et al. 2004. Bioavailability of selenium from fish, yeast and selenate: a comparative study in humans using stable isotopes. European Journal of Clinical Nutrition 58 : 343–349

Franklin, B. 2009. *Saccharomyces cerevisiae*[online]. Available from: http://harrisonmacsam.pbworks.com/w/page/4785850/FrontPage[2009, June 2].

Freimund, S., Sauter, M., Kappeli, O., and Dutler, H. 2003. A new non-degrading isolation process for 1,3-β-D-glucan of high purity from baker's yeast *Saccharomyces cerevisiae*. Carbohydrate Polymers 54 : 159 –171.

Frost, D.J., Drakef, R.R., and Wasserman, B.P. 1992. 1,3-β-Glucan synthase from *Saccharomyces cerevisiae*: *In vitro* activation by β-lactoglobulin or Brij-35, and photoaffinity labeling of enriched microsomal fractions with 5-azido-UDP-Glc and 8-Azido-GTP. Current Microbiology 24 : 295-300.

Frost, D.J., Brandt, K., Capobianco, J., and Goldman, R. 1994. Characterization of 1,3-β-glucan synthase in *Candida albicans*: microsomal assay from the yeast or mycelial morphological forms and a permeabilized whole-cell assay. Microbiology 140 : 2239-2246.

Galichet, A., Sockalingum, G.D., Belarbi, A., and Manfait, M. 2001. FTIR spectroscopic analysis of *Saccharomyces cerevisiae* cell walls study of an anomalous strain exhibiting a pink-colored cell phenotype. FEMS Microbiology Letters 197 : 179-186.

García, R., Rodríguez-Peña, J.M., Bermejo, C., Nombela, C., and Arroyo, J. 2009. The high osmotic response and cell wall integrity pathways cooperate to regulate transcriptional responses to zymolyase-induced cell wall stress in *Saccharomyces cerevisiae*. The Journal of Biological Chemistry 284(16) : 10901–10911.

Goodridge, H., Wolf, A.J., and Underhill, D. 2009. β-Glucan recognition by the innate immune system. Immunological Reviews 230 : 38-50.

Guillien, A., Leal, F., Andaluz, E., and Larriba, G. 1985. Endogenous factors that modulate yeast glucan synthetase in cell-free extracts. Biochimica et Biophysica Acta 842 : 151-161.

Ha, C.H., Lim, K.H., Kim, Y.T., Lim, S.T., Kim, C.W., and Chang, H.I. 2002. Analysis of alkali-soluble glucan produced by *Saccharomyces cerevisiae* wild-type and mutants. Applied Microbiology Biotechnology 58 : 370–377.

Ha, C.H. et al. 2006. Immune-enhancing alkali-soluble glucans produced by wild-type and mutant *Saccharomyces cerevisiae*. Journal Microbiology Biotechnology 16 : 576-583.

Hayen, G.D., and Pollmann, D.S. 2001. Animal feeds comprising yeast glucan. US Patent 6214337.

Hetland, G., and Sandven, P. 2002. β-1,3-Glucan reduces growth of *Mycobacterium tuberculosis* in macrophage cultures. FEMS Immunology and Medical Microbiology 33 : 41-45.

Hohmann, S. 2002. Osmotic stress signaling and osmoadaptation in yeasts. Microbiology and Molecular Biology Review 66 : 300-372.

Hohmann, S. 2003. Yeast Stress Response. First edition, Springer-Verlag Berlin Heideberg. Germany.

Hromádková, Z., Ebringerová, A., Sasinková, V., Šandula, J., Hríbalová, V., and Omelková, J. 2003. Influence of the drying method on the physical properties and immunomodulatory activity of the particulate β-(1,3)-D-glucan from *Saccharomyces cerevisiae*. Carbohydrate Polymers 51 : 9–15.

Hunter Jr, K.W., Gault, R.A., and Berner, M.D. 2002. Preparation of microparticulate β-glucan from *Saccharomyces cerevisiae* for use in immune potentiation. Letters in Applied Microbiology 35 : 267–271.

Igual, J.C., Johnson, A.L., and Johnston, L.H. 1996. Coordinated regulation of gene expression by the cell cycle transcription factor SWI4 and the protein kinase C MAP kinase pathway for yeast cell integrity. The EMBO Journal 15(18) : 5001-5013.

Imai, T., and Ohno, T. 1995. The relationship between viability and intracellular pH in the yeast *Saccharomyces cerevisiae*. Applied Environmental Microbiology : 3604–3608.

Inoue, S.B., Qadota, H., Arisawa, M., Watanabe, T., and Ohya, Y. 1999. Phenylation of Rho1p is required for activation of yeast 1,3-β-glucan synthase. The Journal of Biological Chemistry 274(53) : 38119- 38124.

Jaehrig, S.C., Rohn, S., Kroh, L.W., Fleischer, L.G., and Kurz, T. 2007. *In vitro* potential antioxidant activity of (1-3),(1-6)-β-D-glucan and protein fractions from *Saccharomyces cerevisiae* cell walls. Journal of Agricultural and Food Chemistry 55 : 4710−4716.

Jaehrig, S.C. et al. 2008. Antioxidative activity of (1-3), (1-6)-β-D-glucan from *Saccharomyces cerevisiae* grown on different media. LWT 41 : 868–877.

Jamas, S., Rha, C.K., and Sinskey, A. 1986. Morphology of yeast cell wall as affected by genetic manipulation of β-(1,6)-glycosidic linkage. Biotechnology and Bioengineering 28 : 769-784.

Jamas, S., Davidson, D., Easson, Jr., and Ostroff, G.R. 1996. Method for immune system activation by administration of a β-(1,3)-glucan which is produced by *Saccharomyces cerevisiae* strains R4. US Patent 5504079.

Jung, K. et al. 2004. Antiviral effect of *Saccharomyces cerevisiae* β-glucan to swine influenza virus by increased production of interferon-r and nitric oxide. Journal of Veterinary Medicine 5 : 72-76.

Karreman, R.J., Dague, E., Gaboriaud, F., Quilès, F., Duval, J.F.L., and Lindsey, G.G. 2007. The stress response protein Hsp12p increases the flexibility of the yeast *Saccharomyces cerevisiae* cell wall. Biochimica et Biophysica Acta 1774 : 131–137.

Kasahara, S. et al. 1994. Involvement of cell wall β-glucan in the action of HM-1 killer toxin. FEBS Letters 348 : 27-32.

Khan, H.A. 2004. Zymosan-induced luminol dependent chemiluminescence response of circulating and extravasated leukocytes in experimental sepsis. Mediators of Inflammation 13 : 123-125.

Kikuchi, Y., Mizuuchi, E., Nogami, S., Morishita, S., and Ohya, Y. 2007. Involvement of Rho-type GTPase in control of cell size in *Saccharomyces cerevisiae*. FEMS Yeast Research 7 : 569–578.

Kim, S.Y., Song. H.J., Lee, Y.Y., Cho, K.H., and Rho, Y.K. 2006. Biomedical issues of dietary fiber β-glucan. Journal Korean Medical Science 21 : 781-789.

Klis, F. 1994. Cell wall assembly in yeast. Yeast 10 : 851-869.

Klis, F., Mol, P., Hellingwerf, K., and Brul, S. 2002. Dynamic of cell wall structure in *Saccharomyces cerevisiae*. FEMS Microbiology Reviews 26 : 239–256.

Klis, F.M., Boorsma, A., and Groot, P.W.J.D. 2006. Cell wall construction in *Saccharomyces cerevisiae*. Yeast 23 : 185-202.

Kogan, G. et al. 2005. Antioxidant properties of yeast β-(1,3)-D-glucan studied by electron paramagnetic resonance spectroscopy and its activity in the adjuvant arthritis. Carbohydrate Polymers 61 : 18–28.

Kogan, G., and Kocher, A. 2007. Role of yeast cell wall polysaccharides in pig nutrition and health protection. Livestock Science 109 : 161-165.

Kopeckfi, M., and Gabriel, M. 1992. The influence of congo red on the cell wall and (1,3)-β-D-glucan microfibril biogenesis in *Saccharomyces cerevisiae*. Archives of Microbiology 158 : 115-126.

Kostoryz, E.L., Tong, P.Y., Chappelow, C.C., Eick, J.D., Glaros, A.G., and Yourtee, D.M. 1999. *In vitro* cytotoxicity of solid epoxy-based dental resins and their components. Dental Materials 15 : 363-373.

Krainer, E., Stark, R., Naider, F., Alagramam, K., and Becker, J. 1994. Direct observation of cell wall glucans in whole cells of *Saccharomyces cerevisiae* by Magic-Angel Spinning ^{13}C-NHR. Biopolymers 34 : 1627-1635.

Kubala, L., Ruzickova, J., Nickova, K., Šandula, J., Ciz, M., and Lojeka, A. 2003. The effect of (1,3)-β-D-glucans, carboxymethylglucan and schizophyllan on human leukocytes *in vitro*. Carbohydrate Research 338 : 2835 - 2840.

Kubo, I., Lee, S.H., and Ha, T.J. 2005. Effect of EDTA and combination with polygodial on the growth of *Saccharomyces cerevisiae*. Journal of Agricultural and Food Chemistry 53 : 1818-1822.

Kumari, J., and Sahoo, P.K. 2006. Dietary ß-1,3-glucan potentiates innate immunity disease resistance of Asian catfish, *Clarias batrachus* (L.). Journal of Fish Diseases 29 : 95–101.

Kunlayavattanakul, M., and Lourith, N. 2008. Carboxymethyglucan in cosmetics. Thai Pharmaceutical and Health Science Journal 3(3) : 378-382.

Leal, F., Ruiz-Herrera, J., Villanueva, J.R., and Larriba, G. 1984. An examination of factors affecting the instability of *Saccharomyces cerevisiae* glucan synthetase in cell free extracts. Archives of Microbiology 137 : 209 - 214.

Lesage, G., and Bussey, H. 2006. Cell wall assembly in *Saccharomyces cerevisiae*. Microbiology and Molecular Biology Reviews 70 : 317–343.

Leung, M.Y.K., Liu, C., Koon, J.C.M., and Fung, K.P. 2006. Polysaccharide biological response modifiers. Immunology Letter 105 : 101-114.

Levin, D.E. 2005. Cell Wall Integrity Signaling in *Saccharomyces cerevisiae*. Microbiogy and Molecular Biology Reviews : 262–291.

Li, J. et al. 2005. Effects of ß-glucan extracted from *Saccharomyces cerevisiae* on humoral and cellular immunity in weaned piglets. Archives of Animal Nutrition 59(5) : 303 – 312.

Li, J., Li, D.F. , Xing, J.J., Cheng, Z.B., and Lai, C.H. 2006. Effects of ß-glucan extracted from *Saccharomyces cerevisiae* on growth performence, and immunological and somatotropic responses of pigs challenged with *Escherichia coli* lipopolysaccharide. Journal of Animal Science 84 : 2374-2381.

Liang, J. et al. 1998. Enhanced clearance of a multiple antibiotic resistant *Staphylococcus aureus* in rats treated with PGG-glucan is associated with increased leukocyte counts and increased neutrophil oxidative burst activity. International Journal of Immunopharmacology 20 : 595-614.

Liu, X.Y., Wang, Q., Cui, S.W., and Liu, H.Z. 2008. A new isolation method of β-D-glucans from spent yeast *Saccharomyces cerevisiae*. Food Hydrocolloids 22 : 239–247.

Lowry, V.K., Farnell, M.B., Ferro, P.J., Swaggerty, C.L., Bahl, A., and Kogut, M. H. 2005. Purified β-glucan as an abiotic feed additive up-regulates the innate immune response in immature chickens against *Salmonella enterica* serovar Enteritidis. International Journal of Food Microbiology 98 : 309–318.

Magnani, M., Calliari, C.M., Macedo Jr., F.C., Mori, M.P., Cólus, I.M.S., and Castro-Gomez, R.J.H. 2009. Optimized methodology for extraction of (1,3)(1,6)-β-D-glucan from *Saccharomyces cerevisiae* and *in vitro* evaluation of the cytotoxicity and genotoxicity of the corresponding carboxymethyl derivative. Carbohydrate Polymers 78(4) : 658-665.

Majumder, A., Singh, A., and Goyal, A. 2009. Application of response surface methodology for glucan production from *Leuconostoc dextranicum* and its structural characterization. Carbohydrate Polymers 75 : 150–156.

Maneesri, J. et al. 2005. Deletion of MCD4 involved in glycosylphosphatidy-linositol (GPI) anchor synthesis leads to an increase in β-1,6-glucan level and a decrease in GPI-anchored protein and mannan levels in the cell wall of *Saccharomyces cerevisiae.* Journal of Bioscience and Bioengineering 99 : 354–360.

Manners, D. J., Masson, A. J., and Patterson, J. C. 1973. The structure of a β-(1-3)-D-glucan from yeast cell walls. Biochemistry Journal 135 : 19-30.

Mantovani, M.S., Bellini, M.F., Angeli, J.P.F., Oliveira, R.J., Silva, A.F., and Ribeiro, L.R. 2008. β-Glucans in promoting health: Prevention against mutation and cancer. Mutation Research 658 : 154–161.

Maquelin, K. et al. 2002. Identification of medically relevant microorganisms by vibrational spectroscopy. Journal of Microbiological Methods 51 : 255 – 271.

Marletta, M.A. 1993. Nitric oxide synthase : function and mechanism. Chemistry and Biology of Pteridines and Folates 281-283.

Marques, A., Dhont, J., Sorgeloos, P., and Bossier, P. 2006. Immunostimulatory nature of β-glucan and baker's yeast in gnotobiotic *Artemia* challenge tests. Fish & Shellfish Immunology 20 : 682-692.

Martín, H., Rodríguez-Pachón, J.M., Ruiz, C., Nombela, C., and Molina, M. 2000. Regulatory mechanisms for modulation of signaling through the cell integrity Slt2-mediated pathway in *Saccharomyces cerevisiae*. The Journal of Biological Chemistry 275(2) : 1511–1519.

Mazur, P., and Baginsky, W. 1996. *In vitro* activity of 1,3-β-D-glucan synthase requires the GTP-binding protein Rho1. The Journal of Biological Chemistry 271(24) : 14604–14609.

Megazyme International Ireland Limited. 2008. Mushroom and Yeast Beta-glucan [online]. Avilable from: http://www.megazyme.com/downloads/en/data/K-YBGL.pdf. [2008, March 31]

Miret, J.J., Solari, A.J., Barderi, P.A., and Goldemberg, S.H. 1992. Polyamines and cell wall organization in *Saccharomyces cerevisiae*. Yeast 8 : 1033-1041.

Montijn, R.C. et al. 1999. Localization of synthesis of β-1,6-glucan in *Saccharomyces cerevisiae*. Journal of Bacteriology 181(24) : 7414–7420.

Morris, G.J., Winters, L., Coulson, G.E., and Clarke, K.J. 1983. Effect of osmotic stress on the ultrastructure and viability of yeast *Saccharomyces cerevisiae*. Journal of General Microbiology 129 : 2023-2034.

Motizuki, M., Yokota, S., and Tsurugi, K. 2008. Effect of low pH on organization of the actin cytoskeleton in *Saccharomyces cerevisiae*. Biochimica et Biophysica Acta 1780 : 179-184.

Nguyen, T.H., Fleet, G.H., and Rogers, P.L. 1998. Composition of the cell wall of several yeast species. Applied Microbiology Biotechnology 50 : 206-212.

Nicolosi, R., Bell, J.S., Bistrian, R.B., Greenberg, I., Forse, R.A., and Blackburn, L.G. 1999. Plasma lipid changes after supplementation with β-glucan fiber. American Journal of Clinical Nutrition 70 : 208-212.

Novak, M., and Vetvicka, V. 2008. β-Glucan, History and present: immunomodulatoty aspect and mechanisms of action. Journal of Immunotoxicology 5 : 47-57.

Okazaki, M., Adachi, Y., Ohno, N., and Yadomae, T. 1995. Structure activity relationship of β-(1,3)-D-glucans in the induction of cytokine production from macrophage *in vitro*. Biological Pharmaceutical Bulletin 18(10) : 1320-1327.

Onderdonk, A.B., Cisneros, R.L., Hinkson, P., and Ostroff, G. 1992. Anti-infective effect of poly-1,3-ß-glucotriosyl-1,6-ß-glucopyranose glucan *in vivo*. Infection and Immunity 60(4) : 1642-1647.

Ortuno, J., Cuesta, A., Rodriguez, A., Esteban, M.A., and Meseguer, J. 2002. Oral administration of yeast *Saccharomyces cerevisiae* enhances the cellular innate immune response of gilthead seabream (*Sparus aurata* L.). Veterinary Immunology and Immunopathology 85 : 41-50.

Ostroff, G.R. 1997. Inhibition of infection-stimulated oral tissue destruction by β-(1,3)-glucan. US Patent 5622940.

Pang, Z. et al. 2005. Structure of β-glucan oligomer from laminarin and its effect on human monocytes inhibit the proliferation of U937 cells. Bioscience Biotechnology Biochemistry 69 (3) : 553- 558.

Park, I.M., Ibáñez, A.M., and Shoemaker, C.F. 2007. Rice starch molecular weight size and its relationship with amylose content. Starch/Stärke 59 : 69-77.

Parrou, J.L., Teste, M.A., and Francois, J. 1997. Effect of various types of stress on the metabolism of reserve carbohydrate in *Saccharomyces cerevisiae*: genetic evidence for a stress-induced recycling of glycogen and trehalose. Microbiology 143 : 1891-1900.

Peleg, M. 2006. Advanced Quantitative Microbiology for Foods and Biosystems; Models for Predicting Growth and Inactivation. CRC Press, New York. United States of America.

Pelizon, A.C., Kaneno, R., Soares, A.M.V.C., Meira, D.A., and Sartori., A. 2003. Down modulation of lymphoproliferation and interferon-γ production by β-glucan derived from *Saccharomyces cerevisiae*. Mem Inst Oswaldo Cruz, Rio de Janeiro 98(8) : 1083-1087.

Perry, D.L., and Phillips, S.L. 1995. Handbook of Inorganic compounds. First edition. CRC Press, INC. United States of America.

Popolo, J., Gualtieri, T., and Ragni, E. 2001. The yeast cell wall salvage pathway. Medical Mycology 39 : 111-121.

Promega Corporation. 2010. Griess Reagent System [online]. Available from: www.promega.com [2010, July 9].

Pushpamalar, V., Langford, S.J., Ahmad, M., and Lim, Y.Y. 2006. Optimization of reaction conditions for preparing carboxymethylcellulose from sago waste. Carbohydrate Polymers 64 : 312–318.

Reed, G., and Nagodawithana, T.W. 1991. Yeast-derived products and food and feed yeast. In: Yeast Technology. New York: Van Nostrand Reinhold: United States of America.

Rice, P. J., et al. 2002. Human monocyte scavenger receptors are pattern recognition receptors for (1,3)-β-glucans. Journal of Leukocyte Biology 72 (7): 140-146.

Ross, G.D., Vetvicka, V., Yan, J., Xia, Y., and Vetvickova, J. 1999. Therapeutic intervention with complement and β-glucan in cancer. Immunopharmacology 42 : 61-74.

Šandula, J., Machová, E., and Hribalová, V. 1995. Mitogenic activity of particulate yeast β-(1,3)-D-glucan and its water-soluble derivatives. International Journal of Biological Macromolecules 17(6) : 323-326.

Šandula, J., Kogan, G., Kacurakora, M., and Machová, E. 1999. Microbial (1,3)-β-D-glucan, their preparation, physico-chemical, characterization and immunomodulatory activity. Carbohydrate Polymers 38 : 247-253.

Satrapai, S., and Suphantharika, M. 2007. Influence of spent brewer's yeast β-glucan on gelatinization and retrogradation of rice starch. Carbohydrate Polymers 67 : 500–510.

Seljelid, R., Bogwald, J., Hoffman, J., and Larm, O. 1984. A soluble β-1,3-D-glucan derivative potentiates the cytostatic and cytolytic capacity of mouse peritoneal macrophages *in vitro*. Immunopharmacology 7 : 69-73.

Slaninová, I., Šesták, S., Svoboda, A., and Farkaš, V. 2000. Cell wall and cytoskeleton reorganization as the response to hyperosmotic shock in *Saccharomyces cerevisiae*. Archives Microbiology 173 : 245–252.

Sirisattha, S., Momose, Y., Kitagawa, E., and Iwahashi, H. 2004. Toxicity of anionic detergents determined by *Saccharomyces cerevisiae* microarray analysis. Water Research 38 : 61–70.

Smits, G.J., Kapteyn, J.C., Ende, H., and Klis, F.M. 1999. Cell wall dynamics in yeast. Current Opinion in Microbiology 2 : 348-352.

Smits, G.J., Ende, H., and Klis, F.M. 2001. Differential regulation of cell wall biogenesis during growth and development in yeast. Microbiology 147 : 781-794.

Smits, G.J., and Brul, S. 2005. Stress tolerance in fungi - to kill a spoilage yeast. Current Opinion in Biotechnology 16 : 225–230.

Southeasterm Pharmaceutical. 2010. Beta-glucan # 300 [online]. Available from :http:// www.beta-glucan .com/type_of_beta_glucan.htm [2011, March, 31].

Steel, R.G.D., and Lorrie, J.H. 1980. Principles and Procedures of Statistics. McGraw Hill, New York, United States of America.

Suphantharika, M., Khunrae, P., Thanardkit, P., and Verduyn, C. 2003. Preparation of spent brewer's yeast β-glucans with a potential application as an immunostimulant for black tiger shrimp, *Penaeus monodon*. Bioresource Technology 88 : 55–60.

Tempone, A.G. et al. 2005. Antiprotozoal activity of Brazilian plant extracts from isoquinoline alkaloid-producing families. Phytomedicine 12 : 382–390.

Thanardkit, P., Khunrae, P., Suphantharika, M., and Verduyn, C. 2002. Glucan from spent brewer's yeast: preparation, analysis and use as a potential immunostimulant in shrimp feed. World Journal of Microbiology & Biotechnology 18 : 527–539.

The National Innovation Agency (NIA). 2005. Yeast product; high quality of biotechnology. First edition. Ministry of science and technology. Thailand.

Tsoni, S.V., and Brown, G.D. 2008. β-Glucans and Dectin1. Annals of the New York Academy of Sciences 1143 : 45-60.

Vaart, J.M.V.D., Caro, L.H.P.,Chapman, J.W., Klis, F.M., and Verrips, C. T. 1995. Identification of three mannoproteins in the cell wall of *Saccharomyces cerevisiae*. Journal of Bacteriology 7 : 3104-3110.

Varunya Pornchalearn. 2006. Production of glucan from spent brewer's yeast and its functional properties. Master's Thesis, Department of Biotechnology, Faculty of Science, Chulalongkorn University.

Vetvicka, V., and Vetvickova, J. 2009. Effect of yeast-derived β-glucans on blood cholesterol and macrophage functionality. Journal of Immunotoxicology 6(1) : 30-35.

Vink, E. et al. 2004. An *in vitro* assay for β-(1,6)-D-glucan synthesis in *Saccharomyces cerevisiae*. Yeast 21 : 1121–1131.

Volman, J.J., Ramakers, J.D., and Plat, J. 2007. Dietary modulation of immune function by β-glucans. Physiology & Behavior 94 : 276-284.

Walker, G.M. 1999. Yeast Physiology and Biotechnology. First edition. John Wiley & Sons Ltd. United States of America.

Walker, J.M. 2002. The bicinchoninic (BCA) assay for protein quantitation, The Protein Protocols Handbook, second Edition. J.M. Walker © Human Press Inc., Totowa, NJ. United States of America.

Wakshull, E. 1999. PGG-Glucan, a soluble β-(1,3)-glucan, enhances the oxidative burst respone, microbicidal activity, and activates an NF-Kb-like factor in human PMN: evidence for a glycophingolipid β-(1,3)-glucan receptor. Immunopharmacology 41 : 89-107.

Wang, Y.J., Yao, S.J., Guana, Y.X., Wu, T.X., and Kennedy, J.F. 2005. A novel process for preparation of β-(1,3)-D-glucan sulphate by a heterogeneous reaction and its structural elucidation. Carbohydrate Polymers 59 : 93–99.

Watson, T.G. 1970. Effects of sodium chloride on steady-state growth and metabolism of *Sacharomyces cerevisiae*. Journal of General Microbiology 64 : 91-99.

Weeraya Janpaisaeng. 2008. Structure and functional properties of B-glucan extracted from different species and strains of Saccharomyces**.** Master's Thesis, Department of Food Technology, Faculty of Science, Chulalongkorn University.

Wink, D.A., and Mitchell, J.B. 1998. Chemical biology of nitric oxide insights into regulators, cytotoxic, and cytoprotective mechanism of nitric oxide. Free Radical Biology and Medicine 25(4) : 434-456.

Wojda, I., Alonso-Monge, R., Bebelman, J.P., Mager, W.H., and Siderius, M. 2003. Response to high osmotic conditions and elevated temperature in *Saccharomyces cerevisiae* is controlled by intracellular glycerol and involves coordinated activity of MAP kinase pathways. Microbiology 149 : 1193-1204.

Worrasinchai, S., Suphantharika, M., Pinjai, S., and Jamnong, P. 2006. β-Glucan prepared from spent brewer's yeast as a fat replacer in mayonnaise. Food Hydrocolloids 20 : 68-78.

Williams, D. et al. 1991. A method for the solubilization of a 1,3-β-D-glucan isolated from *S. cerevisiae*. Carbohydrate Research 219 : 203- 213.

Wyatt Technology Corporation. 2004. Astra V user's guide. First edition. 6300 Hollister Avenue Santa Barbara, CA , United States of America.

Xiao, Z., Trincado, C.A., and Murtaugh, M. P. 2004. β-Glucan enhancement of T cell IFNγ response in swine. Veterinary Immunology and Immunopathology 102 : 315–320.

Yadomae, T. 2000. Structure and biological activities of fungal β-1,3-glucans. Yakugaku Zasshi 120(5) : 413 – 431.

Yiannikouris, A. et al. 2004. Alkali extraction of β-D-glucan from *Saccharomyces cerevisiae* cell wall and study of their adsorptive properties toward zearalenone. Journal of Agricultural and Food Chemistry 52 : 3666-3673.

Yuen, S.N., Choi, S.M., Phillips, D.L., and Ma, C.Y. 2009. Raman and FTIR spectroscopic study of carboxymethylated non-starch polysaccharides. Food Chemistry 114 : 1091–1098.

Zekovic, D.B., Kwiatkowski, S., Vrvić, M.M., Jakovljević, D., and Moran, C.A. 2005. Natural and modified β-(1,3)-D-glucans in health Promotion and Disease Alleviation. Critical Review in Biotechnology 25 : 205–230.

Zimmerman, J.W., Lindermuth, J., Fish, P.A., Palace, G.P., Stevenson, T.T., and DeMong, D.E. 1998. A novel carbohydrate-glycosphingolipid interaction between a β-(1,3)-glucan immunomodulator, PGG-glucan, and lactosylceramide of human leukocytes. Journal of Biological Chemistry 273 : 22014–22020.

Zülli, F., and Saecker. 1994. CM-Glucan a new yeast polysaccharide for cosmetic use. Cosmetic and Toiletries Manufacture Worldwide 131-136.

Zülli, F., Applegate, L.A., Frenk, E., and Suter, F. 1995. Photoprotective effect of CM-glucan an cultured human skin cell. Euro Cosmetrics 11 : 46-50.

Zülli, F., Suter, F., Biltz, H., Nissen, H.P., and Birman, M. 1996. Carboxymethylated β-(1,3)-glucan, A beta-glucan from baker's yeast helps protect skin. Cosmetics & Toiletries 11 : 91-98.

Zülli, F., Suter, F., Biltz, H., and Nissen, H.P. 1998. Improving skin function with CM-glucan, a biological response modifier from yeast. International Journal of Cosmetic Science 20 : 79–86.

APPENDICES

APPENDIX A

Reagent, Medium and buffer preparation

Yeast β-glucan assay kit

The kit are composed of:

Bottle 1: exo-1,3-β-glucanase (100 U/mL) plus β-glucosidase (20 U/mL) suspension, 2.0 mL. Stable for > 4 years at 4°C.

Bottle 2: Amyloglucosidase (1630 U/mL) plus invertase (500 U/mL) solution in 50 % v/v glycerol, 20 mL. Stable for ~ 2 years at 4°C or > 4 years at -20°C.

Bottle 3: Glucose reagent buffer (concentrate; 50 mL). Stable for ~ 2 years at 4°C or for > 4 years at -20°C.

Bottle 4: Glucose determination reagent. Reagent concentrations after dissolution in buffer: Glucose oxidase > 12,000 U/litre. Peroxidase > 650 U/litre. 4-Aminoantipyrine 0.4 mM. Stable for > 4 years at -20°C.

Bottle 5: D-Glucose standard solution (5 mL, 1.00 mg/mL) in 0.2 % w/v benzoic acid. Stable for > 4 years at room temperature.

Bottle 6: Control yeast β-glucan preparation (~ 2 g, β-glucan content stated on the bottle label). Stable for > 5 years at room temperature.

Preparation of reagent solutions

1. Add 8 mL of 200 mM sodium acetate buffer (pH 5.0) to bottle 1 (i.e. dilute the contents of the vial to 10 mL). Divide into appropriately sized aliquots and store in polypropylene tubes at -20°C between use and on ice during use. Once diluted, the reagent is stable for > 2 years at -20 °C.

2. Use the contents of bottle 2 as supplied. Stable for ~ 2 years at 4 °C or > 4 years at -20 °C.

3. Dilute the contents of bottle 3 to 1.0 L with distilled or deionised water. Stable for > 2 years at 4 °C.

4. Dissolve the contents of bottle 4 in the diluted contents of bottle 3. Divide this reagent mixture (GOPOD reagent) into aliquots of desired volume for storage. Stable for 2-3 months at 4 °C in a dark bottle, or for > 12 months at -20 °C.

5. Use the contents of bottle 5 as supplied. Stable for > 4 years at room temperature.
6. Use the contents of bottle 6 as supplied. Stable for > 5 years at room temperature (Megazyme International Ireland Limited, 2008)

Reagents

1. Sodium acetate buffer (200 mM, pH 5.0).
Glacial acetic acid (1.05 g/mL) 11.6 mL was added to 900 mL of distilled water and pH 5.0 was adjusted by using 4 M (16 g/100 mL) sodium hydroxide solution. The volume was adjusted to 1 litre. Stable for ~ 1 year at 4 °C.
2. Sodium acetate buffer (1.2 M, pH 3.8).
Glacial acetic acid (1.05 g/mL) 69.6 mL was added to 800 mL of distilled water and pH 3.8 was adjusted using 4 M sodium hydroxide. The volume was adjusted to 1 litre with distilled water.
3. Potassium Hydroxide (2 M).
KOH 112 g was added to 800 mL of distilled water and the solution was dissolved by stirring. The volume was adjusted to 1 litre.
4. Hydrochloric acid (37 % v/v; ~10 M). (Megazyme International Ireland Limited, 2008)

YPD (Yeast Extract Peptone Dextrose) Broth

Yeast extract 10 g
Peptone 20 g
Dextrose 20 g
Distilled water 1,000 ml
pH 4 ± 0.1

YPD (Yeast Extract Peptone Dextrose) Agar

Yeast extract 10 g
Peptone 20 g
Dextrose 20 g
Distilled water 1,000 ml
Agar powder 15 g
pH 7 ± 0.1

Sodium Phosphate buffer 0.02 M pH 7.5

Solution A 0.02M of sodium phosphate buffer monobasic ($Na_2H_2PO_4$) = 2.39 g/l
Solution B 0.02M of sodium phosphate buffer dibasic (Na_2HPO_4 $7H_2O$) = 5.31 g/l
16 ml of solution A mixed with 84 ml of solution B in preparation 100 ml

APPENDIX B

Standard curve in β-glucan and protein determination

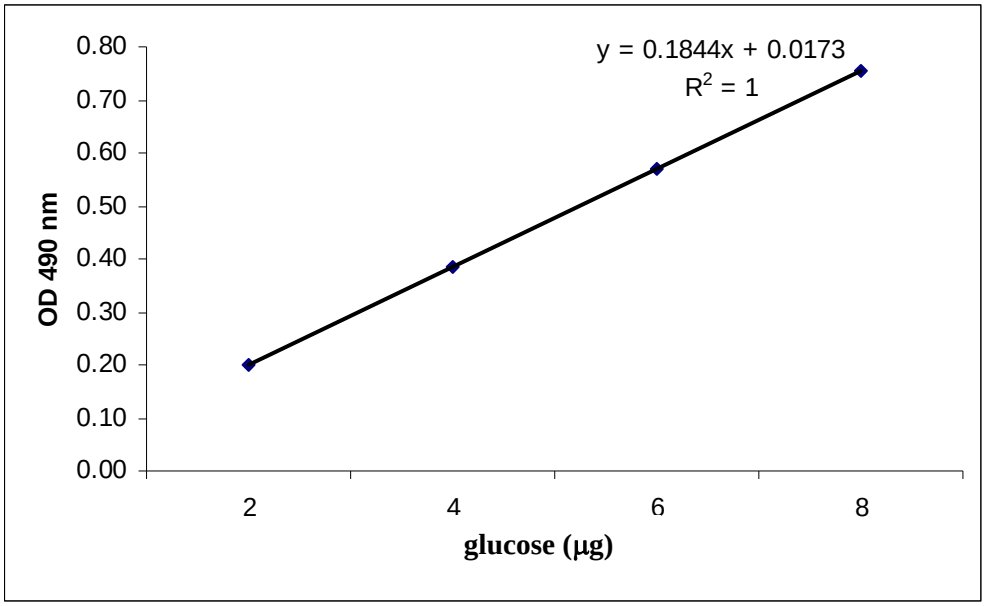


Fig B-1 Standard curve of D-glucose for total-glucan determination

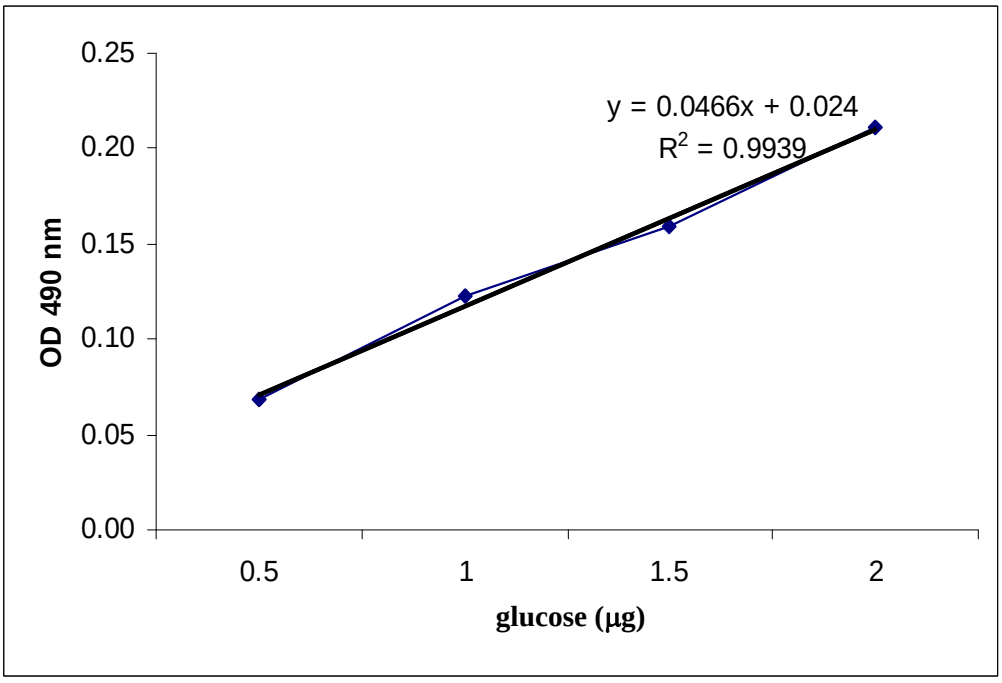


Fig B-2 Standard curve of D-glucose for α-glucan determination

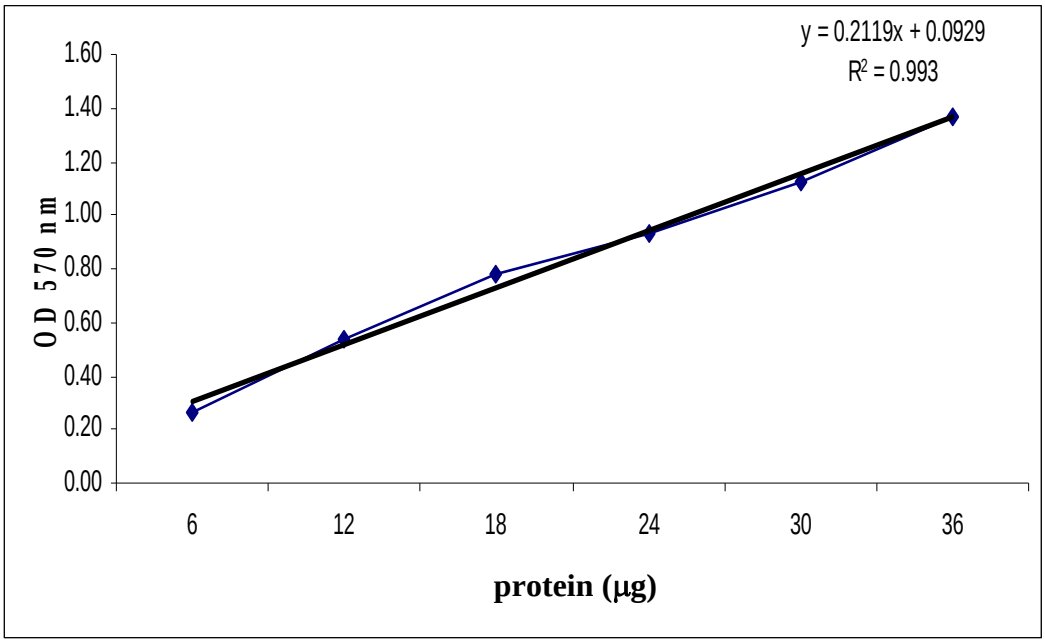


Fig B-3 Standard curve of bovine serum albumin for protein determination

APPENDIX C

Properties of additives

EDTA

Chemical name : EDTA

Orther names : H_4EDTA, Diaminoethanetetraacetic acid, Edetic acid, Edetate, Ethylenedinitrilotetraacetic acid,Versene, Ethylene diamine tetracetic acid

Chemical formular : $C_{10}H_{16}N_2O_8$

Molecular mass : 292.25 g/mol

Solubility : 0.5 g/L H_2O (25 °C)

Melting point : 220 °C

Structure

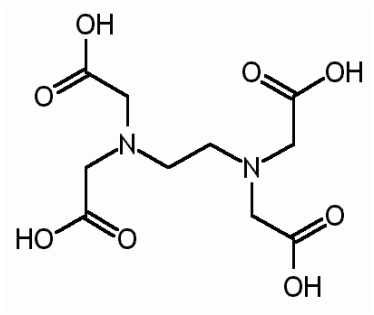


Fig C-1 Structure of EDTA

Properties

EDTA is a widely-used acronym for the chemical compound ethylenediamine tetraacetic acid. EDTA refers to the chelating agent with the formula $(HO_2CCH_2)_2NCH_2CH_2N(CH_2CO_2H)_2$. This amino acid is widely used to sequester di- and trivalent metal ions. EDTA binds to metals via four carboxylate and two amine groups. EDTA forms specially strong complexes with Mn(II), Cu(II), Fe(III), and Co(III) (Perry and Phillips, 1995).

SDS (sodium dodecyl sulfate)

Chemical name : SDS or NaDS (sodium dodecyl sulfate)

Orther names : Sodium lauryl sulfate (SLS)

Chemical formular : $C_{12}H_{25}SO_4Na$

Other name: Sodium monododecyl sulfate; Sodium monolauryl sulfate; Sodium dodecanesulfate; dodecyl alcohol, hydrogen sulfate, sodium salt; n-dodecyl sulfate sodium; Sulfuric acid monododecyl ester sodium salt;

Molecular mass : 288.38 g /mol

Density: 1.01 g/cm³

Melthing point: 206 °C

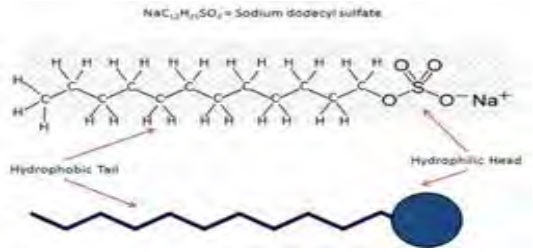


Fig C-2 structure of SDS

Sodium dodecyl sulfate is an anionic surfactant used in many cleaning products. The molecule has a tail of 12 carbon atoms, attached to a sulfate group, giving the molecule the amphiphilic properties required of a detergent. SDS is a highly effective surfactant used in the removal of oily stains and residues. This compound is found in high concentrations in industrial products including engine degreasers, floor cleaners, and car wash soaps. In household products, SDS is used in lower concentrations with toothpastes, shampoos and shaving foams. The product can also be used to aid in lysing cells during DNA extraction.

Synthesis

Sodium dodecyl sulfate (SDS) is synthesized by reacting lauryl alcohol with sulphuric acid to produce hydrogen lauryl sulfate, then neutralized through the addition of sodium carbonate. SDS can be converted by ethoxylation to sodium laureth sulfate (sodium lauryl ether sulfate; SLES) (Perry and Phillips, 1995).

Sodium chloride

Molecular formular: NaCl
Molecular mass: 58.443 g/mol
Appearance : Colorless/white crystalline solid
Oder : Oderless
Density: 2.165 g/cm3
Melting point: 801 °C (1074 K)
Boiling point: 1465 °C (1738 K)
Solubility in water : 35.6 g/100 mL (0 °C), 35.9 g/100 mL (25 °C), 39.1 g/100 mL (100 °C)
Solubility : soluble in glycerol, ethylene glycol, formic acid , insoluble in HCl
Solubility in methanol: 1.49 g/100 mL
Solubility in ammonia: 2.15 g/100 mL
Refractive index: 1.5442 (589 nm)

Sodium chloride, also known as common salt or table salt, is an ionic compound with the formula NaCl. As the major ingredient in edible salt, it is commonly used as a condiment and food preservative (Perry and Phillips, 1995).

APPENDIX D

STATISTICAL ANALYSIS

Table D-1 Table of statistical analysis of β-glucan yield between *S. cerevisiae* Fermipan®, *S. cerevisiae* Angel® and *S. cerevisiae* TISTR 5059 (CRD)

ANOVA of β-glucan yield

	df	Sum of Squares	Mean Square	F	Sig.
Between Groups	2	15.577	7.788	2.022	0.213
Within Groups	6	23.115	3.852		
Total	8	38.692			

Duncan[a]

1, Fermipan®; 2, Angel®; 3,TISTR5059	N	Subset for alpha = 0.05
		1
1	3	7.103
3	3	8.544
2	3	8.952
Sig.		0.114

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table D-2 Table of statistical analysis of % of cell dry weight between *S. cerevisiae* Fermipan®, *S. cerevisiae* Angel® and *S. cerevisiae* TISTR 5059 (CRD)

ANOVA of % of cell dry weight

	df	Sum of Squares	Mean Square	F	Sig.
Between Groups	2	0.129	6.456E-02	8.704	0.017
Within Groups	6	4.451E-02	7.418E-03		
Total	8	0.174			

Duncan[a]

1, Fermipan®; 2, Angel®; 3,TISTR5059	N	Subset for alpha = 0.05	
		1	2
3	3	0.144	
2	3		0.347
1	3		0.428
Sig.		1.000	0.291

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table D-3 Table of statistical analysis of effect of additive (EDTA, SDS and NaCl) on elongation of cells of *S. cerevisiae* Angel® (CRD)

ANOVA of elongation

	df	Sum of Squares	Mean Square	F	Sig.
Between Groups	7	2.452	0.350	9.857	0.000
Within Groups	16	0.569	3.554E-02		
Total	23	3.021			

Duncan[a]

Treatments	N	Subset for alpha = 0.05		
		1	2	3
1	3	0.8477		
2	3		1.517	
7	3		1.615	
6	3		1.715	1.715
4	3		1.723	1.723
3	3		1.726	1.726
Control	3		1.795	1.795
5	3			2.006
Sig.		1.000	0.127	0.106

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table D-4 Table of statistical analysis of effect of additive (EDTA, SDS and NaCl) on β-glucan content from *S. cerevisiae* Angel® (CRD)

ANOVA of β-glucan content

	df	Sum of Squares	Mean Square	F	Sig.
Between Groups	7	11.473	1.639	4.022	0.010
Within Groups	16	6.520	0.408		
Total	23	17.993			

Duncan[a]

Treatments	N	Subset for alpha = 0.05		
		1	2	3
control	3	5.828		
3	3	6.223	6.223	
2	3	6.334	6.334	
5	3	6.411	6.411	
4	3	6.568	6.568	
7	3	6.693	6.693	
6	3		7.393	7.393
1	3			8.156
Sig.		0.157	0.061	0.162

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table D-5 Table of statistical analysis of effect of additive (EDTA, SDS and NaCl) on % yield of cell dry weight from *S. cerevisiae* Angel® (CRD)

ANOVA of % yield of cell dry weight

	df	Sum of Squares	Mean Square	F	Sig.
Between Groups	7	3.997E-02	5.710E-03	1.798	0.157
Within Groups	16	5.080E-02	3.175E-03		
Total	23	9.077E-02			

Duncan[a]

Treatments	N	Subset for alpha = 0.05	
		1	2
3	3	0.297	
6	3	0.324	0.324
1	3	0.328	0.328
7	3	0.346	0.346
control	3	0.391	0.391
5	3	0.391	0.391
4	3	0.399	0.399
2	3		0.419
Sig.		0.067	0.087

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

APPENDIX E

Spectra of yeast cell by using Fourier Transform Infrared Spectroscopy (FTIR)

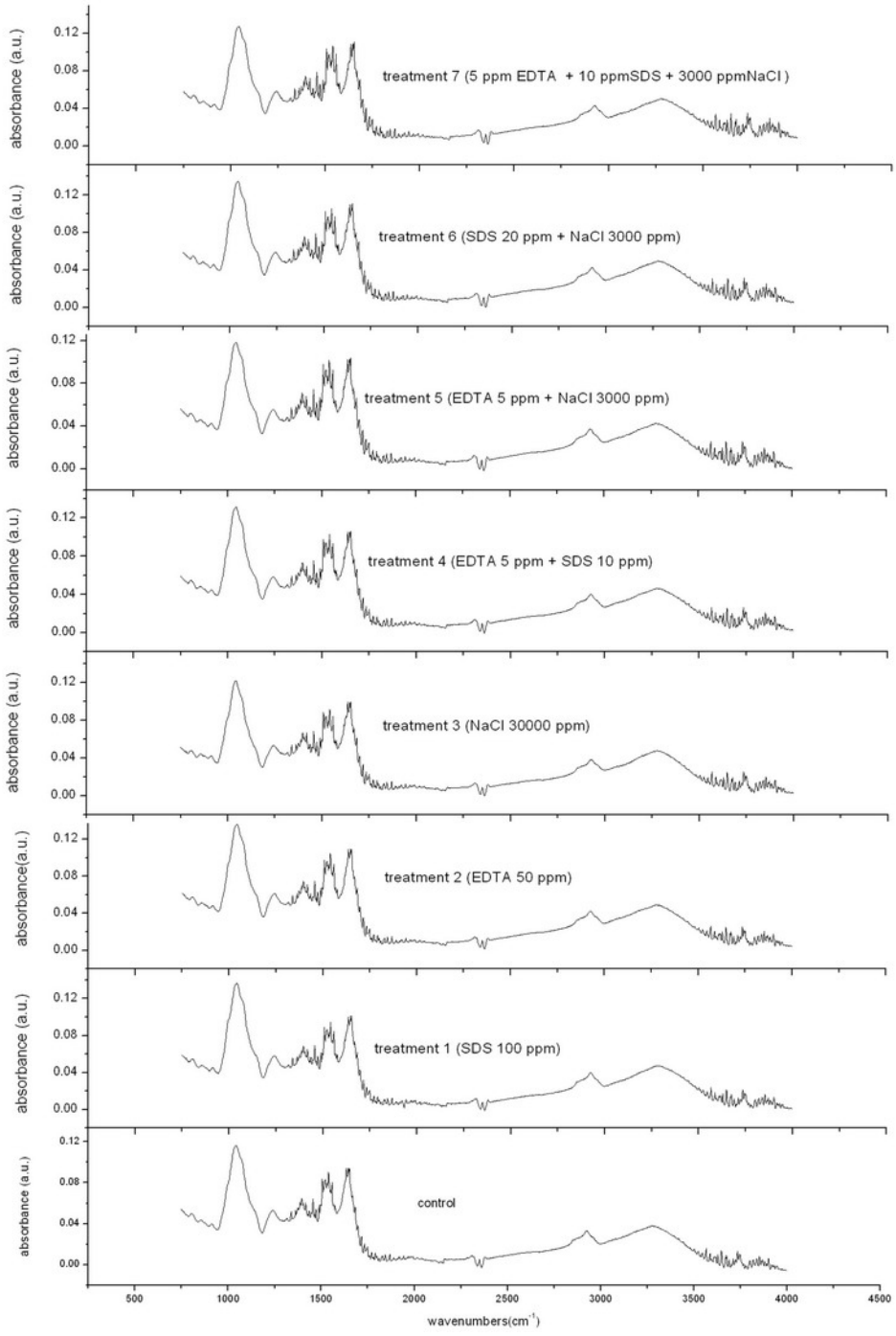


Fig E-1 spectra of yeast cell of *S. cerevisiae* Angel® when yeast was cultivated with some additives in different concentrations for 24 h in replication II

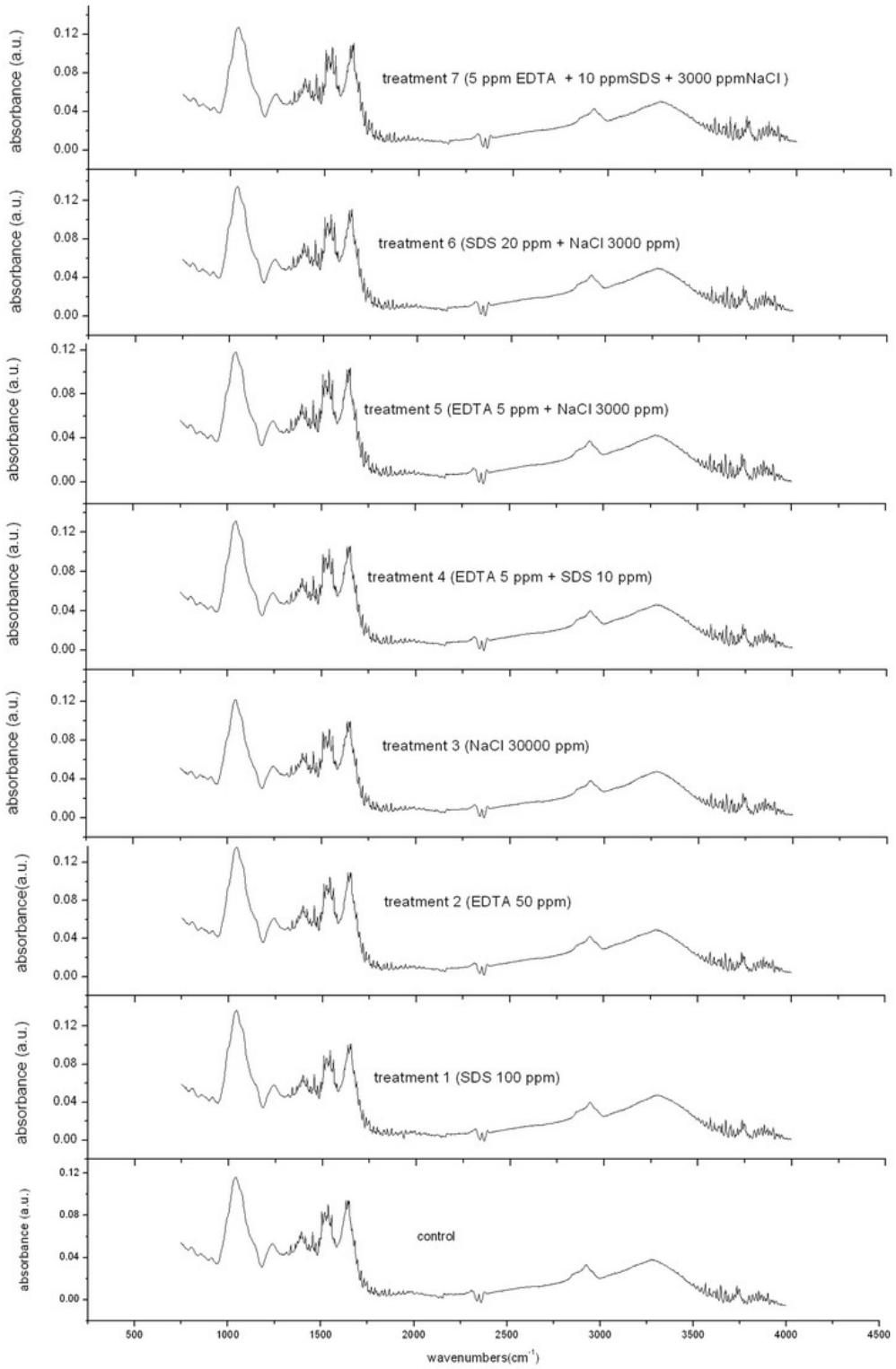


Fig E-2 spectra of yeast cell of *S. cerevisiae* Angel® when yeast was cultivated with some additives in different concentrations for 24 h in replication III

Spectra of polysaccharide region by using FTIR

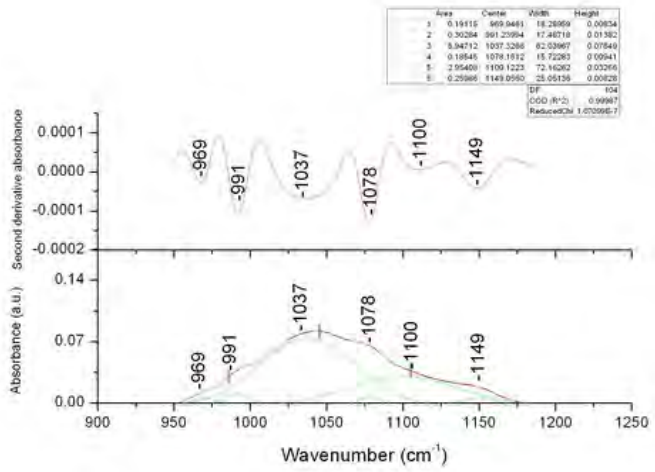


Fig E-3 FTIR spectra of polysaccharide from *S. cerevisiae* Angel® when cultured in YPD medium without additives (control) in wavenumber 950- 1,185 cm^{-1} from curve- fitting (lower) and second derivative plot (upper)

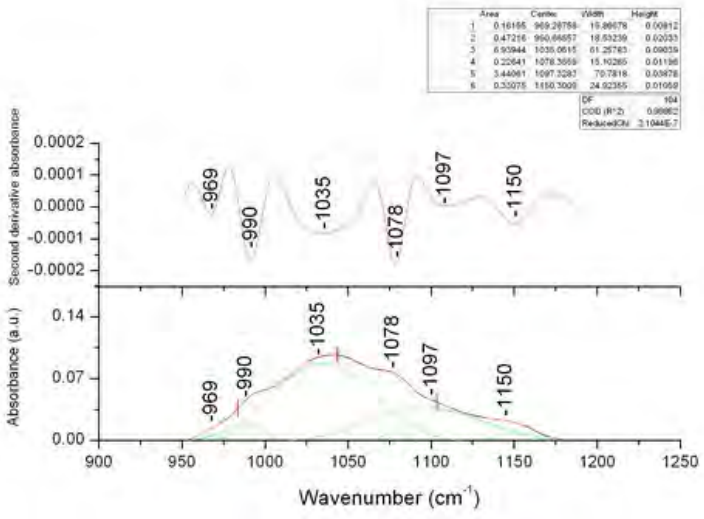


Fig E-4 FTIR spectra of polysaccharide from *S. cerevisiae* Angel® when cultured in YPD medium with SDS 100 ppm (treatment 1) in wavenumber 950- 1,185 cm^{-1} from curve- fitting (lower) and second derivative plot (upper)

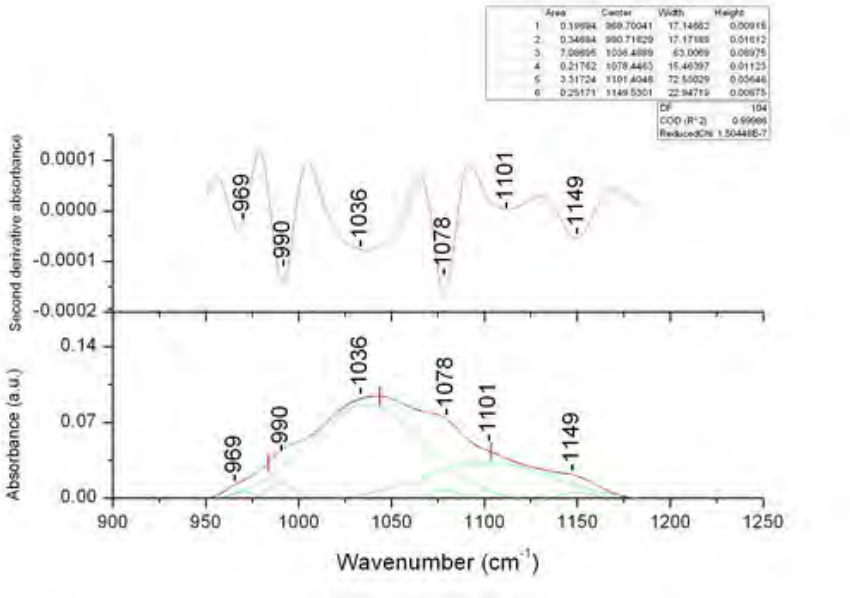


Fig E-5 FTIR spectra of polysaccharide from *S. cerevisiae* Angel® when cultured in YPD medium with EDTA 50 ppm (treatment 2) in wavenumber 950- 1,185 cm^{-1} from curve- fitting (lower) and second derivative plot (upper)

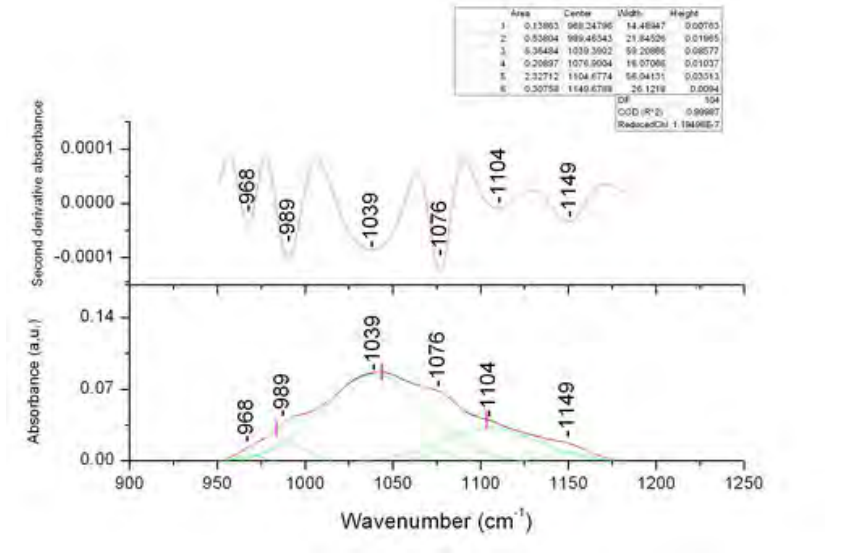


Fig E-6 FTIR spectra of polysaccharide from *S. cerevisiae* Angel® when cultured in YPD medium with NaCl 30,000 ppm (treatment 3) in wavenumber 950- 1,185 cm^{-1} from curve- fitting (lower) and second derivative plot (upper)

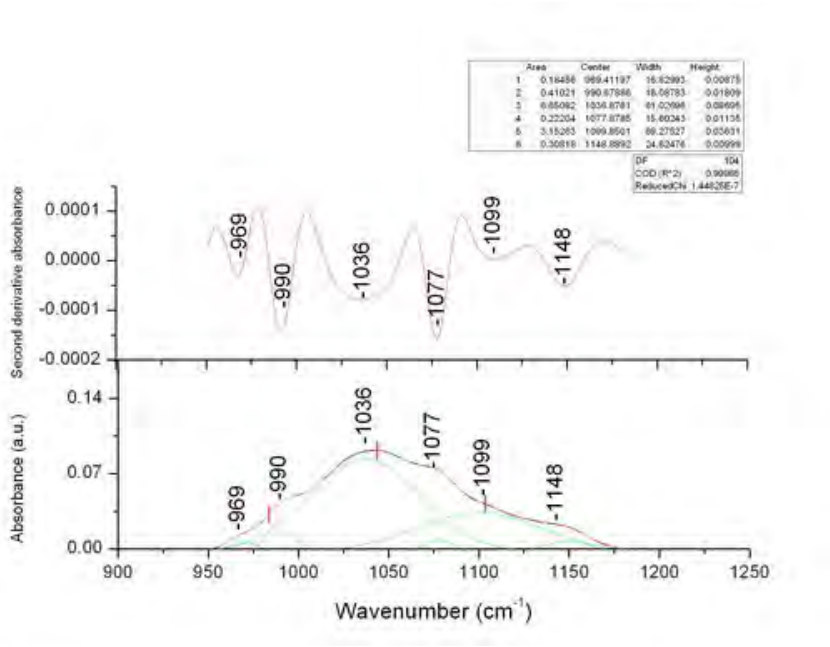


Fig E-7 FTIR spectra of polysaccharide from *S. cerevisiae* Angel® when cultured in YPD medium with combination of EDTA 5 ppm and 10 ppm EDTA (treatment 4) in wavenumber 950- 1,185 cm^{-1} from curve-fitting (lower) and second derivative plot (upper)

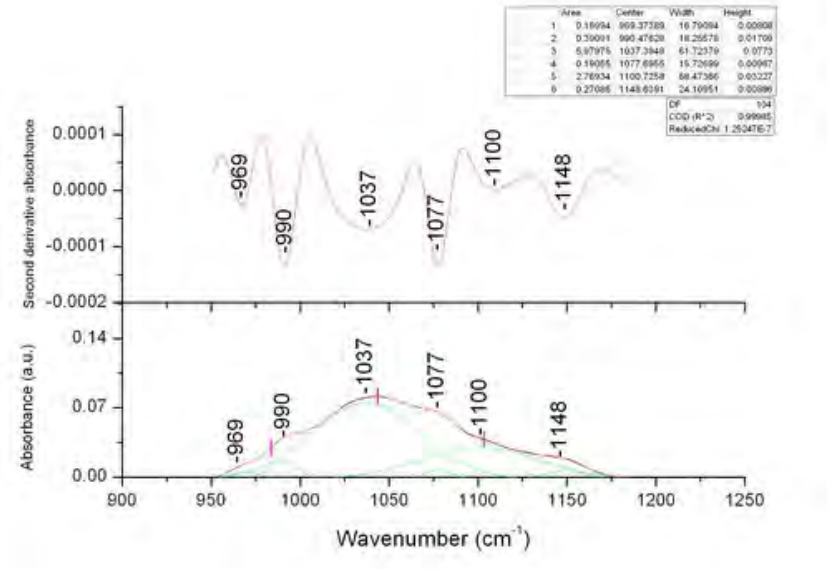


Fig E-8 FTIR spectra of polysaccharide from *S. cerevisiae* Angel® when cultured in YPD medium with combination of EDTA 5 ppm and 3,000 ppm NaCl (treatment 5) in wavenumber 950- 1,185 cm^{-1} from curve- fitting (lower) and second derivative plot (upper)

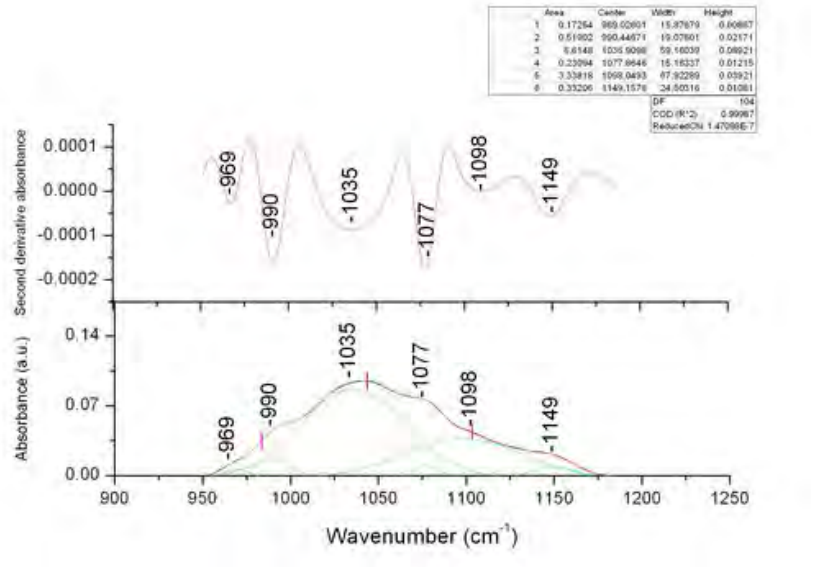


Fig E-9 FTIR spectra of polysaccharide from *S. cerevisiae* Angel® when cultured in YPD medium with combination of SDS 20 ppm and 3,000 ppm NaCl (treatment 6) in wavenumber 950- 1,185 cm^{-1} from curve-fitting (lower) and second derivative plot (upper)

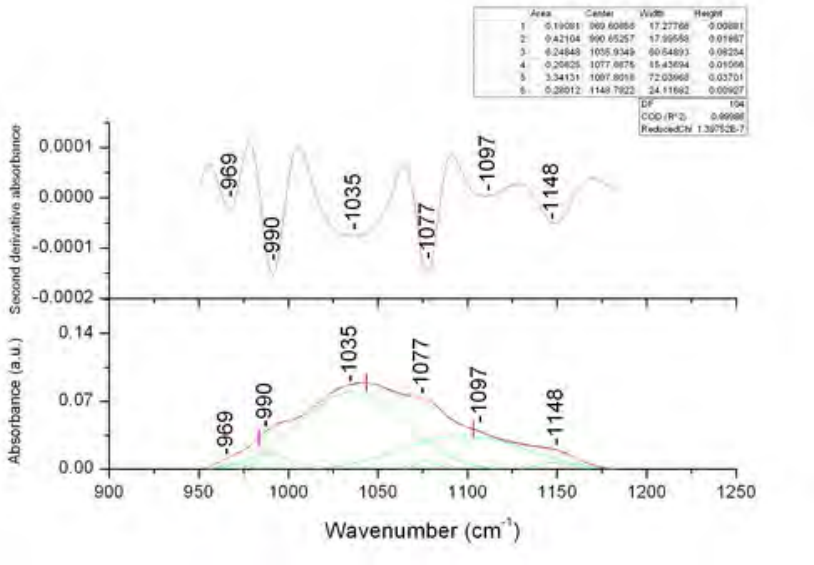


Fig E-10 FTIR spectra of polysaccharide from *S. cerevisiae* Angel® when cultured in YPD medium with combination of EDTA 5 ppm, SDS 10 ppm and 3,000 ppm NaCl (treatment 7) in wavenumber 950- 1,185 cm^{-1} from curve- fitting (lower) and second derivative plot (upper)

Spectra of protein region by using FTIR

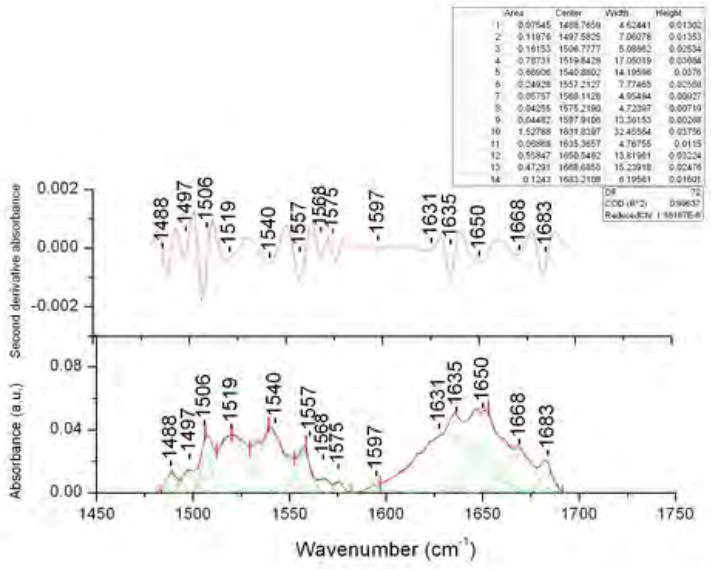


Fig E-11 FTIR spectra of protein from *S. cerevisiae* Angel® when cultured in YPD medium without additives (control) in wavenumber 1,480 – 1,700 cm^{-1} from curve- fitting (lower) and second derivative plot (upper)

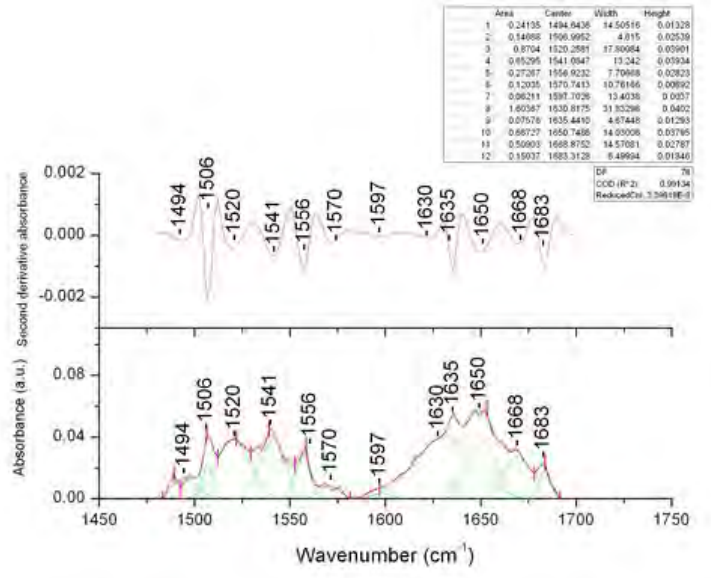


Fig E-12 FTIR spectra of protein from *S. cerevisiae* Angel® when cultured in YPD medium with SDS 100 ppm (treatment 1) in wavenumber 1,480 – 1,700 cm^{-1} from curve- fitting (lower) and second derivative plot (upper)

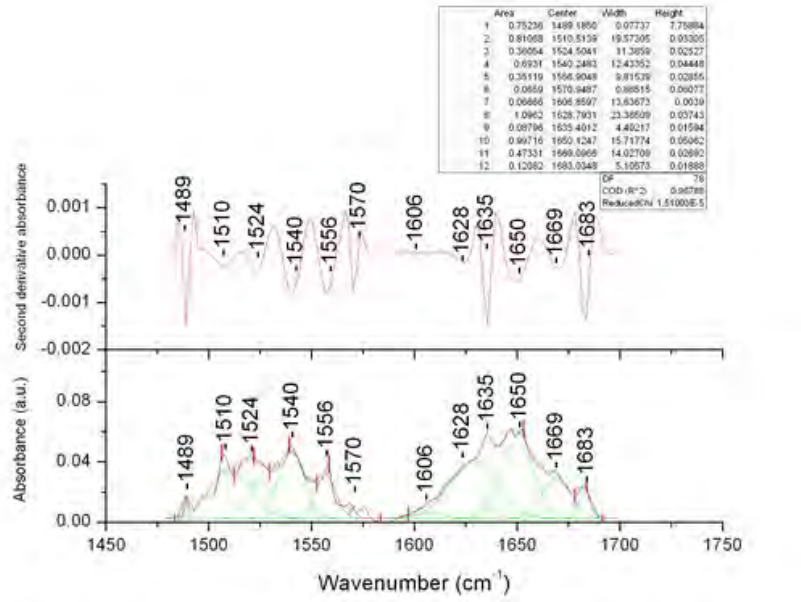


Fig E-13 FTIR spectra of protein from *S. cerevisiae* Angel® when cultured in YPD medium with EDTA 50 ppm (treatment 2) in wavenumber 1,480 – 1,700 cm^{-1} from curve- fitting (lower) and second derivative plot (upper)

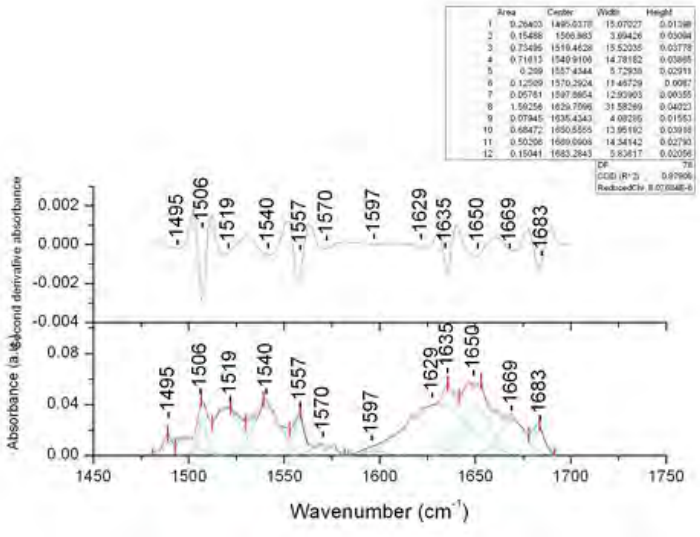


Fig E-14 FTIR spectra of protein from *S. cerevisiae* Angel® when cultured in YPD medium with NaCl 30,000 ppm (treatment 3) in wavenumber 1,480 – 1,700 cm^{-1} from curve- fitting (lower) and second derivative plot (upper)

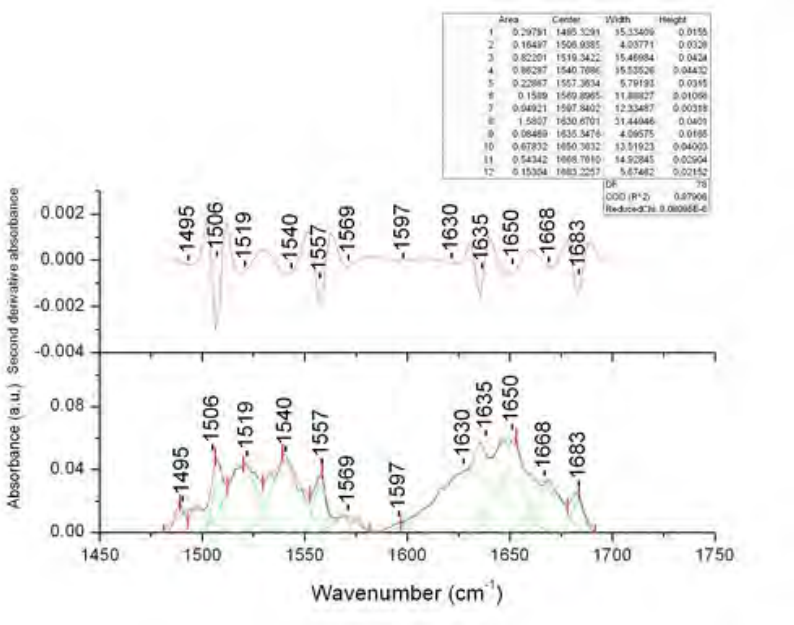


Fig E-15 FTIR spectra of protein from *S. cerevisiae* Angel® when cultured in YPD medium with combination of EDTA 5 ppm , SDS 10 ppm (treatment 4) in wavenumber 1,480 – 1,700 cm^{-1} from curve- fitting (lower) and second derivative plot (upper)

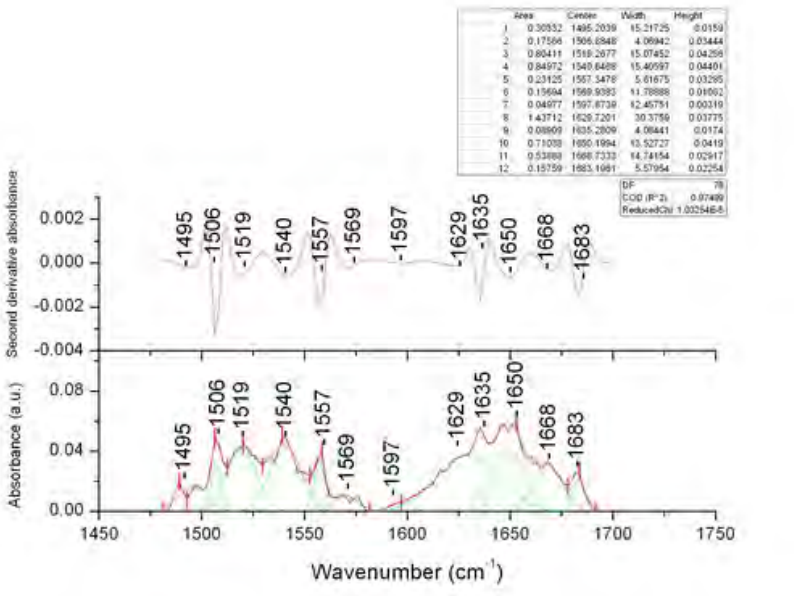


Fig E-16 FTIR spectra of protein from *S. cerevisiae* Angel® when cultured in YPD medium with combination of EDTA 5 ppm and NaCl 3,000 ppm (treatment 5) in wavenumber 1,480 – 1,700 cm^{-1} from curve-fitting (lower) and second derivative plot (upper)

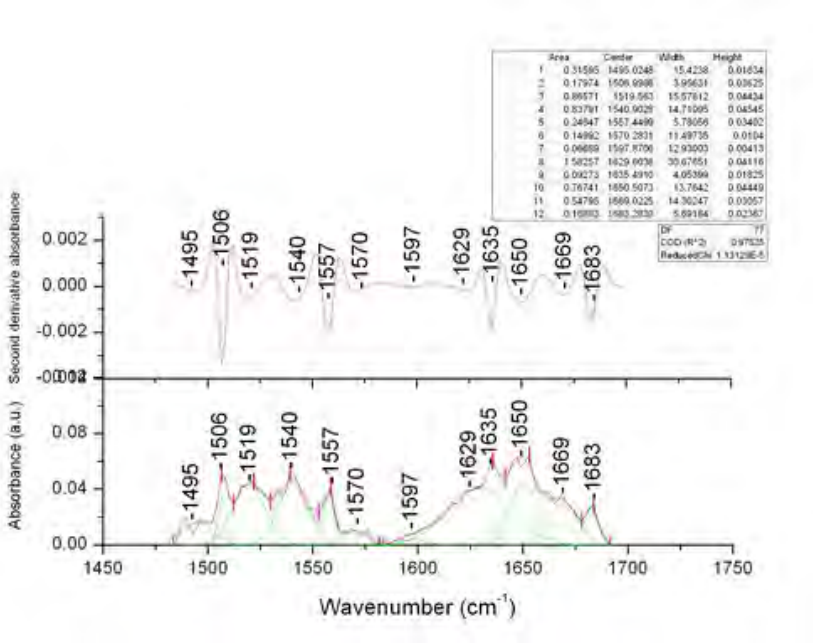


Fig E-17 FTIR spectra of protein from *S. cerevisiae* Angel® when cultured in YPD medium with combination of SDS 20 ppm and NaCl 3,000 ppm (treatment 6) in wavenumber 1,480 – 1,700 cm^{-1} from curve-fitting (lower) and second derivative plot (upper)

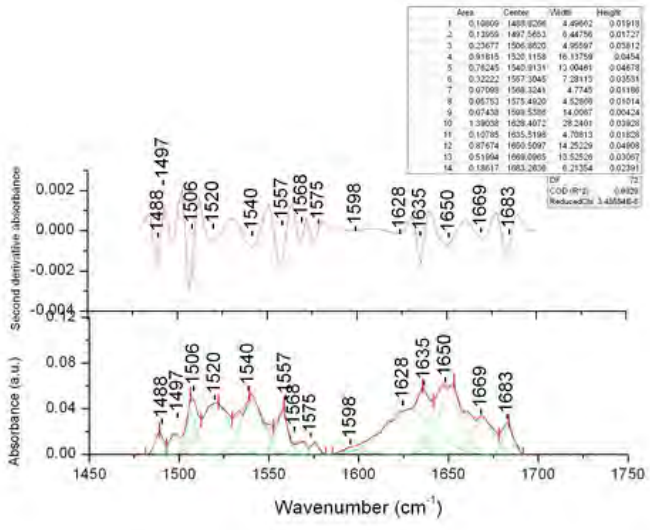


Fig E-18 FTIR spectra of protein from *S. cerevisiae* Angel® when cultured in YPD medium with combination of EDTA 5 ppm , SDS 10 ppm and NaCl 3,000 ppm (treatment 7) in wavenumber 1,480 – 1,700 cm^{-1} from curve- fitting (lower) and second derivative plot (upper)

Spectra of lipid region by using FTIR

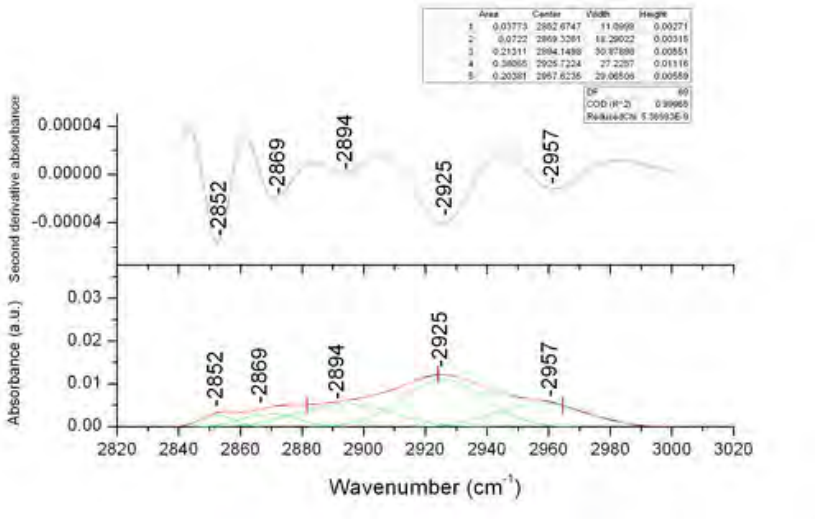


Fig E-19 FTIR spectra of lipid from *S. cerevisiae* Angel® when cultured in YPD medium without additives(control) in wavenumber 2,840 - 3,000 cm^{-1} from curve- fitting (lower) and second derivative plot (upper)

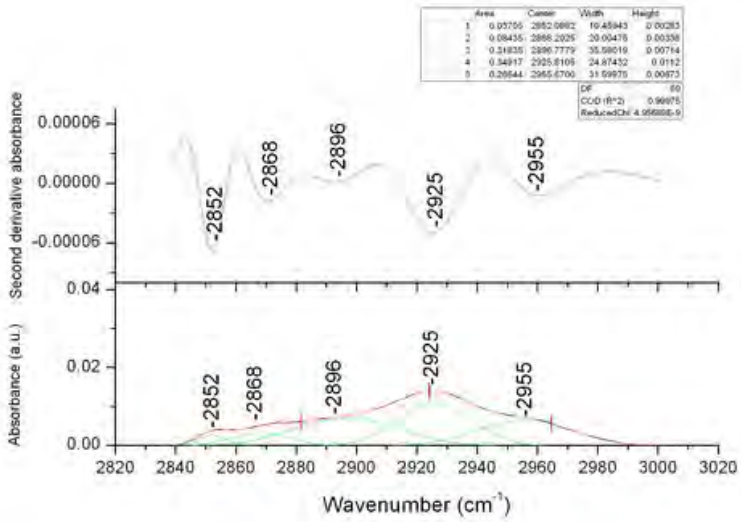


Fig E-20 FTIR spectra of lipid from *S. cerevisiae* Angel® when cultured in YPD medium with SDS 100 ppm (treatment 1) in wavenumber 2,840 – 3,000 cm^{-1} from curve- fitting (lower) and second derivative plot (upper)

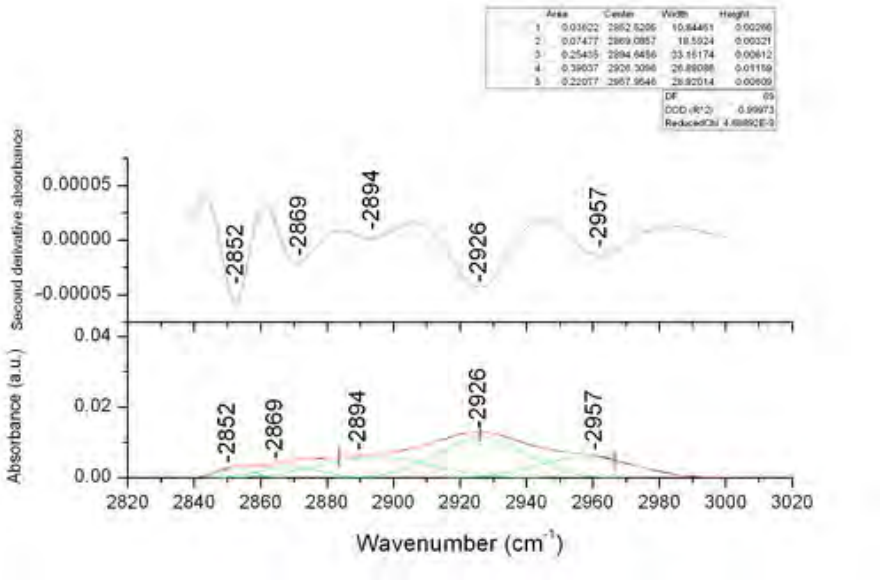


Fig E-21 FTIR spectra of lipid from *S. cerevisiae* Angel® when cultured in YPD medium with EDTA 50 ppm (treatment 2) in wavenumber 2,840 – 3,000 cm^{-1} from curve- fitting (lower) and second derivative plot (upper)

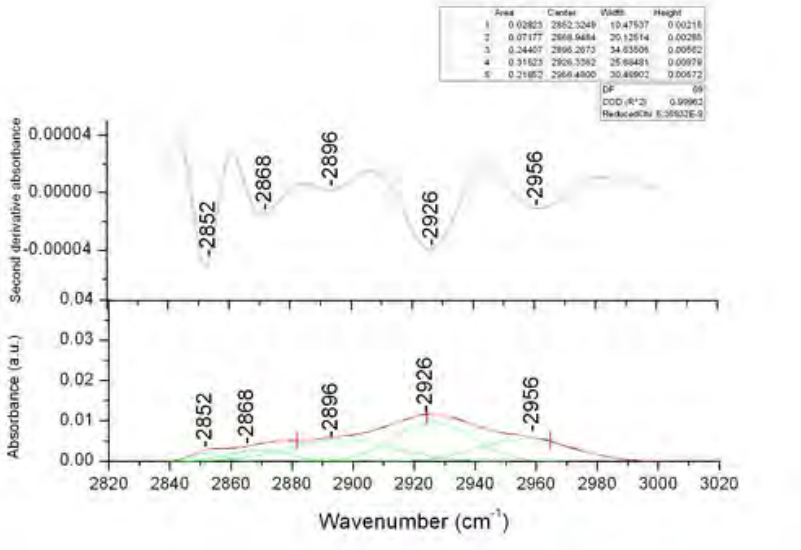


Fig E-22 FTIR spectra of lipid from *S. cerevisiae* Angel® when cultured in YPD medium with NaCl 30,000 ppm (treatment 3) in wavenumber 2,840 – 3,000 cm^{-1} from curve- fitting (lower) and second derivative plot (upper)

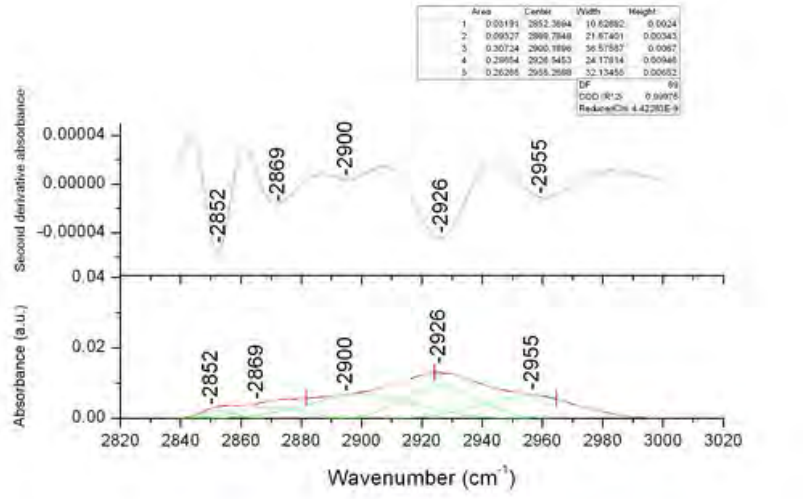


Fig E-23 FTIR spectra of lipid from *S. cerevisiae* Angel® when cultured in YPD medium with combination of 5 ppm EDTA and 10 ppm SDS (treatment 4) in wavenumber 2,840 – 3,000 cm^{-1} from curve- fitting (lower) and second derivative plot (upper)

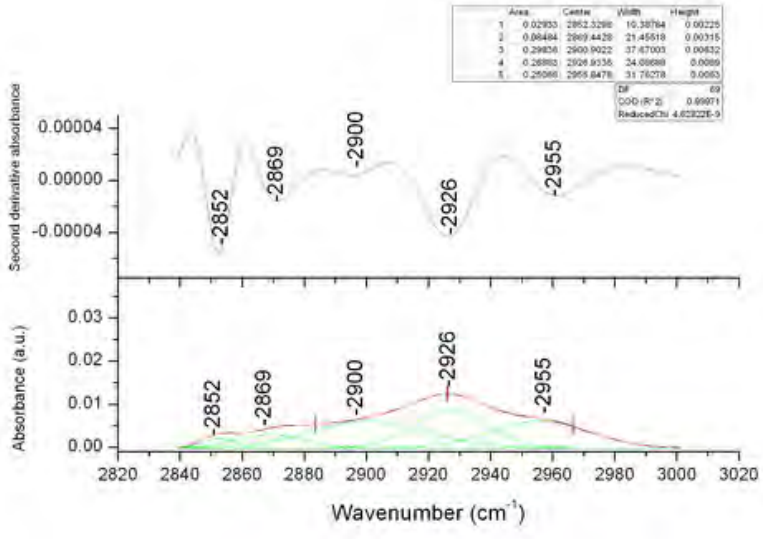


Fig E-24 FTIR spectra of lipid from *S. cerevisiae* Angel® when cultured in YPD medium with combination of 5 ppm EDTA and 3,000 ppm NaCl (treatment 5) in wavenumber 2,840 – 3,000 cm^{-1} from curve-fitting (lower) and second derivative plot (upper)

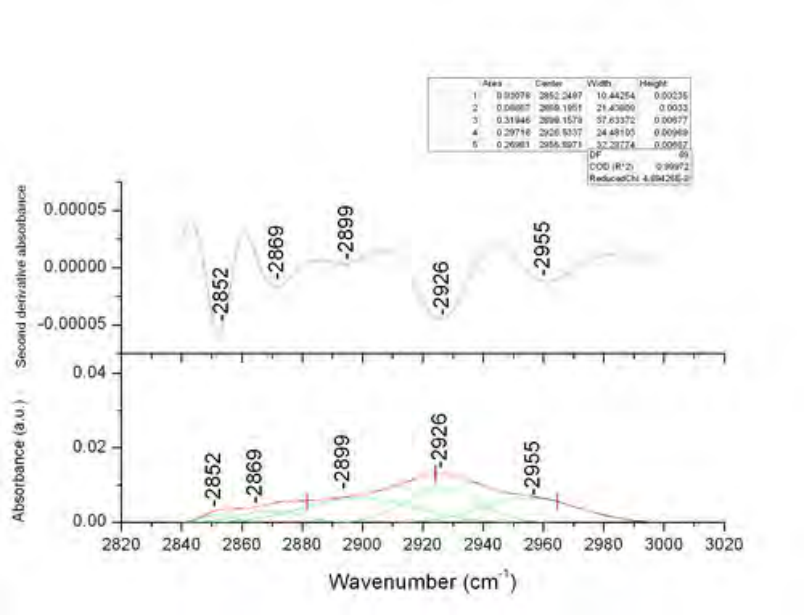


Fig E-25 FTIR spectra of lipid from *S. cerevisiae* Angel® when cultured in YPD medium with combination of 20 ppm SDS and 3,000 ppm NaCl (treatment 6) in wavenumber 2,840 – 3,000 cm^{-1} from curve-fitting (lower) and second derivative plot (upper)

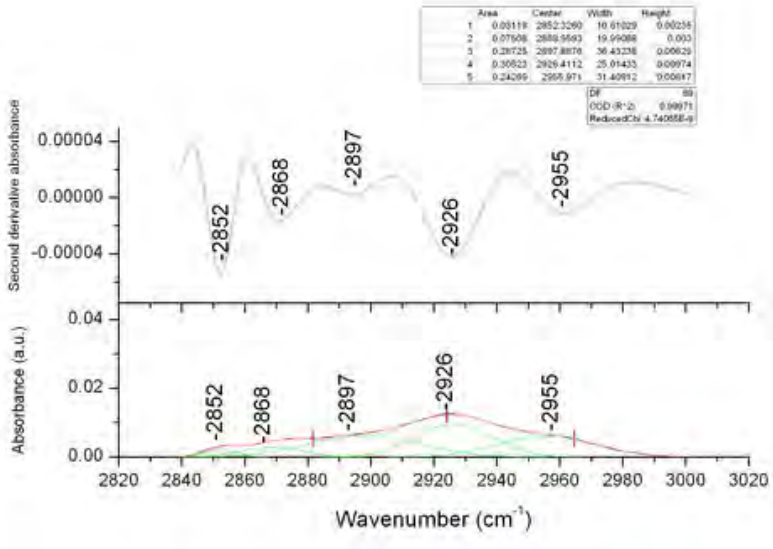


Fig E-26 FTIR spectra of lipid from *S. cerevisiae* Angel® when cultured in YPD medium with combination of 5 ppm EDTA, 10 ppm SDS and 3,000 ppm NaCl (treatment 7) in wavenumber 2,840 – 3,000 cm^{-1} from curve- fitting (lower) and second derivative plot (upper)

APPENDIX F

Spectra of β-glucan I by using Fourier Transform Infrared Spectroscopy (FTIR)

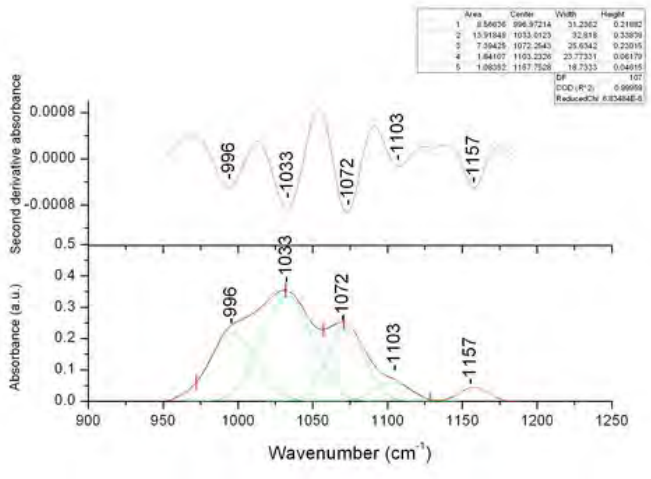


Fig F-1 FTIR spectra of β-glucan I of laminarin from *Laminaria digidata* in wavenumber 950- 1185 cm^{-1} from curve- fitting (lower) and second derivative plot (upper)

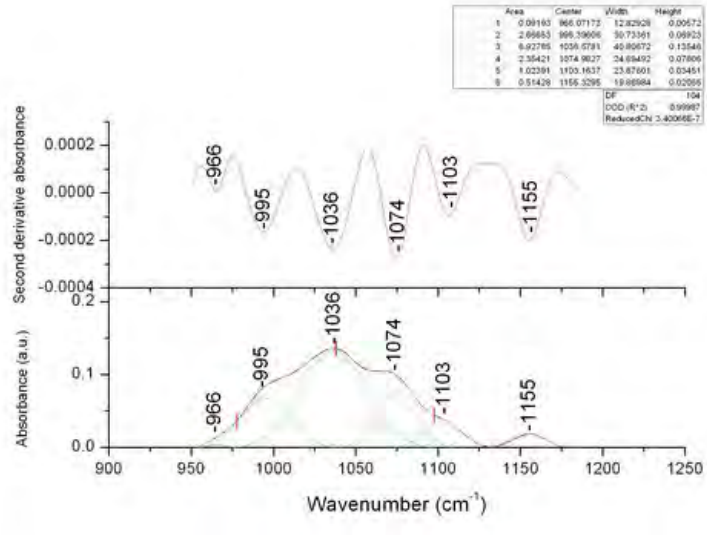


Fig F-2 FTIR spectra of β-glucan I from *S. cerevisiae* Angel® when cultured in YPD medium without additives (control) in wavenumber 950- 1,185 cm^{-1} from curve- fitting (lower) and second derivative plot (upper)

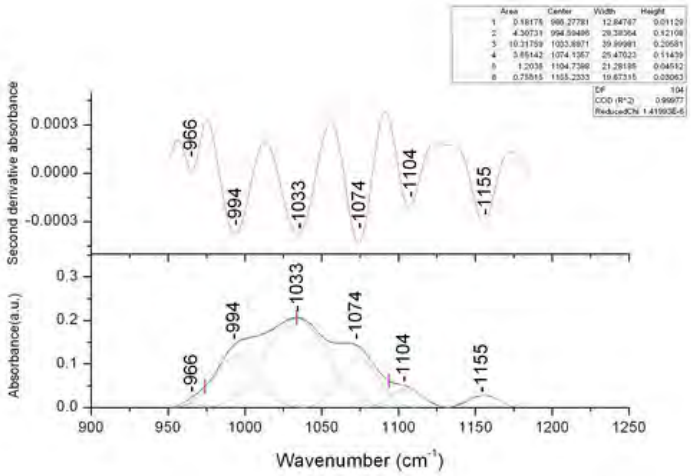


Fig F-3 FTIR spectra of β-glucan I from *S. cerevisiae* Angel® when cultured in YPD medium with SDS 100 ppm (treatment 1) in wavenumber 950-1,185 cm^{-1} from curve- fitting (lower) and second derivative plot (upper)

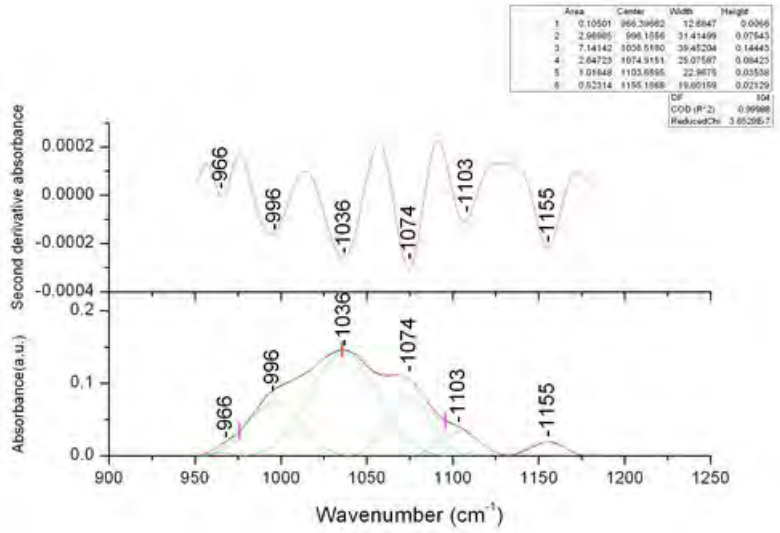


Fig F-4 FTIR spectra of β-glucan I from *S. cerevisiae* Angel® when cultured in YPD medium with EDTA 50 ppm (treatment 2) in wavenumber 950- 1,185 cm^{-1} from curve- fitting (lower) and second derivative plot (upper)

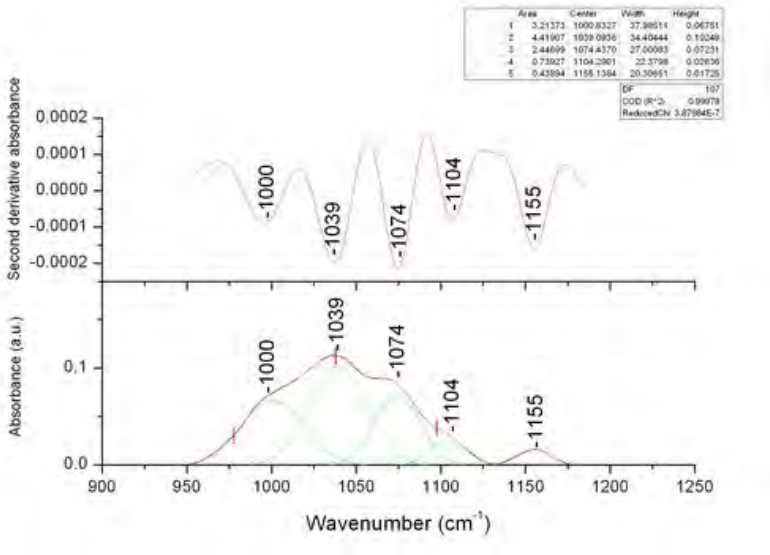


Fig F-5 FTIR spectra of β-glucan I from *S. cerevisiae* Angel® when cultured in YPD medium with NaCl 30000 ppm (treatment 3) in wavenumber 950- 1185 cm^{-1} from curve- fitting (lower) and second derivative plot (upper)

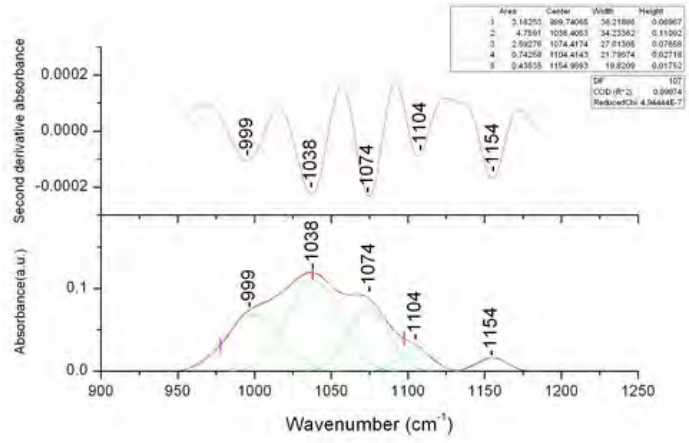


Fig F-6 FTIR spectra of β-glucan I from *S. cerevisiae* Angel® when cultured in YPD medium with EDTA 5 ppm and SDS 10 ppm (treatment 4) in wavenumber 950- 1,185 cm^{-1} from curve- fitting (lower) and second derivative plot (upper)

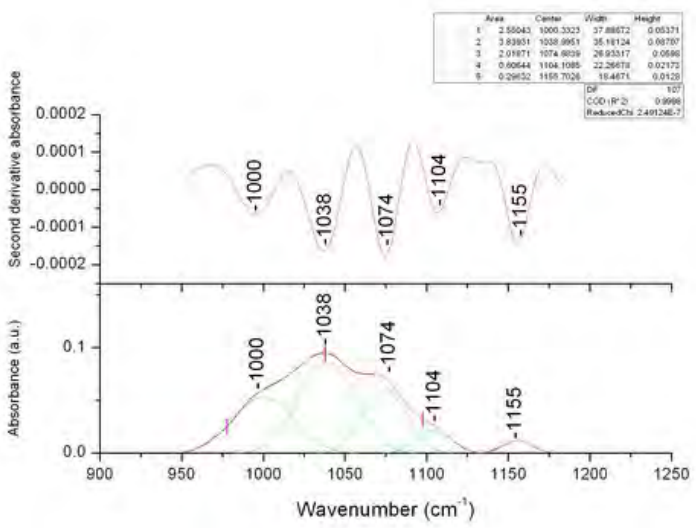


Fig F-7 FTIR spectra of β-glucan I from *S. cerevisiae* Angel® when cultured in YPD medium with EDTA 5 ppm and NaCl 3000 ppm (treatment 5) in wavenumber 950- 1,185 cm^{-1} from curve- fitting (lower) and second derivative plot (upper)

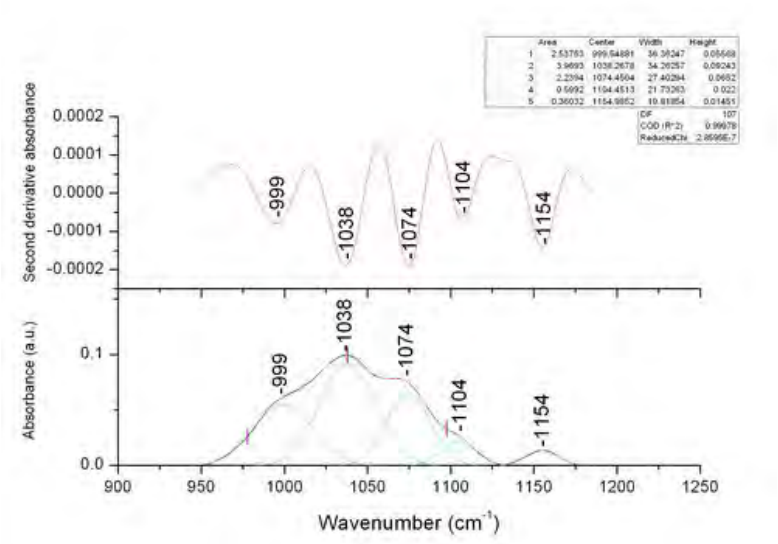


Fig F-8 FTIR spectra of β-glucan I from *S. cerevisiae* Angel® when cultured in YPD medium with SDS 20 ppm and NaCl 3,000 ppm (treatment 6) in wavenumber 950- 1,185 cm^{-1} from curve- fitting (lower) and second derivative plot (upper)

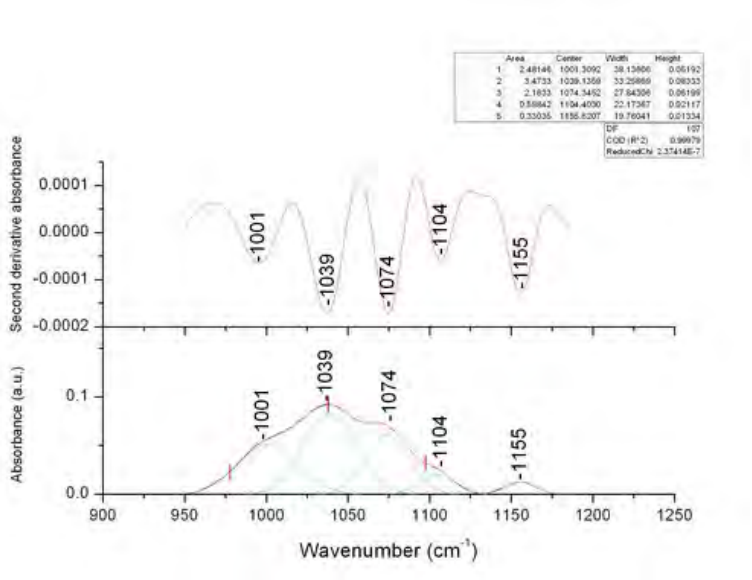


Fig F-9 FTIR spectra of β-glucan I from *S. cerevisiae* Angel® when cultured in YPD medium with EDTA 5 ppm, SDS 10 ppm and NaCl 3,000 ppm (treatment 7) in wavenumber 950- 1,185 cm^{-1} from curve- fitting (lower) and second derivative plot (upper)

APPENDIX G

Spectra of β-glucan II by using Fourier Transform Infrared Spectroscopy (FTIR)

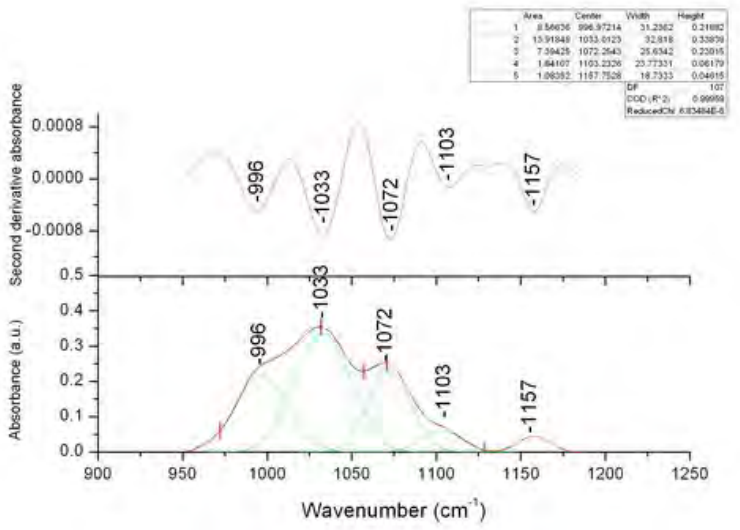


Fig G-1 FTIR spectra of laminarin from *Laminaria digidata* in wavenumber 950- 1185 cm^{-1} from curve- fitting (lower) and second derivative plot (upper)

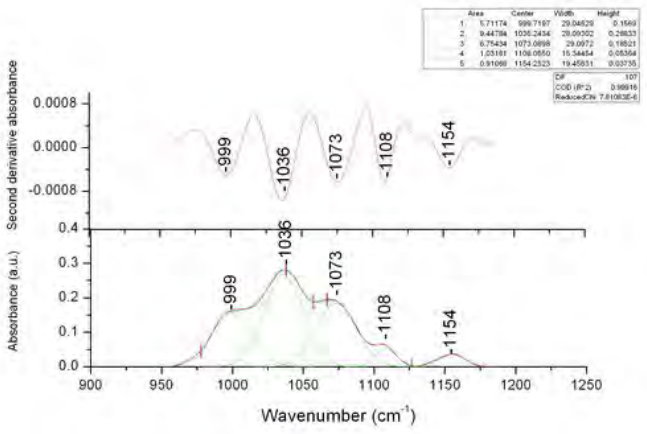


Fig G-2 FTIR spectra of β-glucan II from *S. cerevisiae* Angel® when cultured in YPD medium without additives (control) in wavenumber 950- 1,185 cm^{-1} from curve- fitting (lower) and second derivative plot (upper)

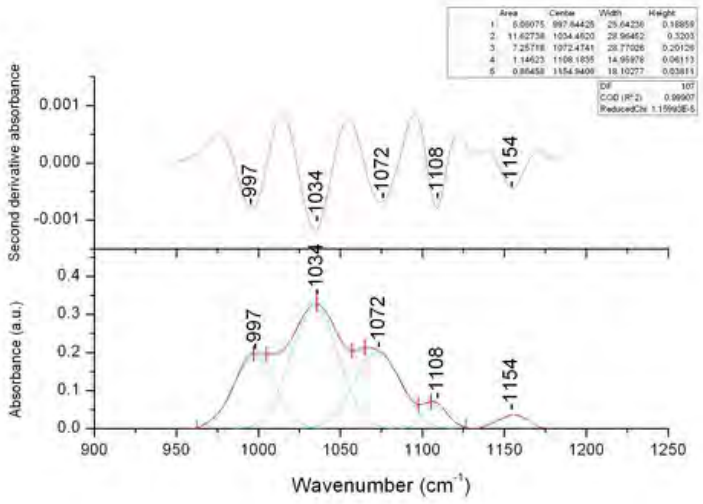


Fig G-3 FTIR spectra of β-glucan II from *S. cerevisiae* Angel® when cultured in YPD medium with SDS 100 ppm (treatment 1) in wavenumber 950- 1,185 cm^{-1} from curve- fitting (lower) and second derivative plot (upper)

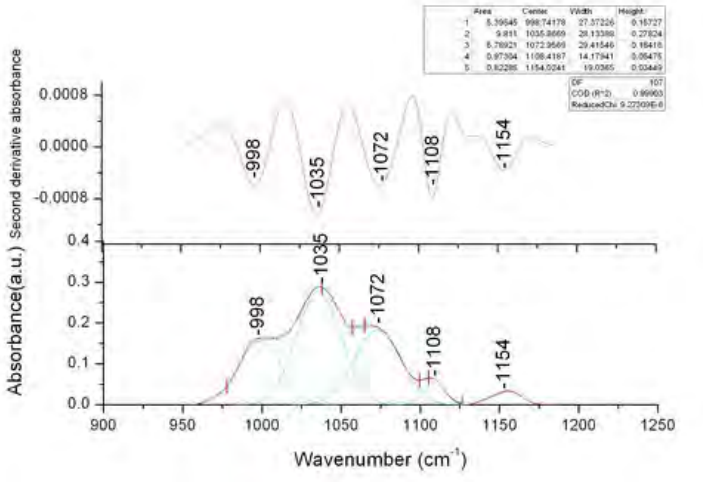


Fig G-4 FTIR spectra of β-glucan II from *S. cerevisiae* Angel® when cultured in YPD medium with EDTA 50 ppm (treatment 2) in wavenumber 950-1,185 cm^{-1} from curve- fitting (lower) and second derivative plot (upper)

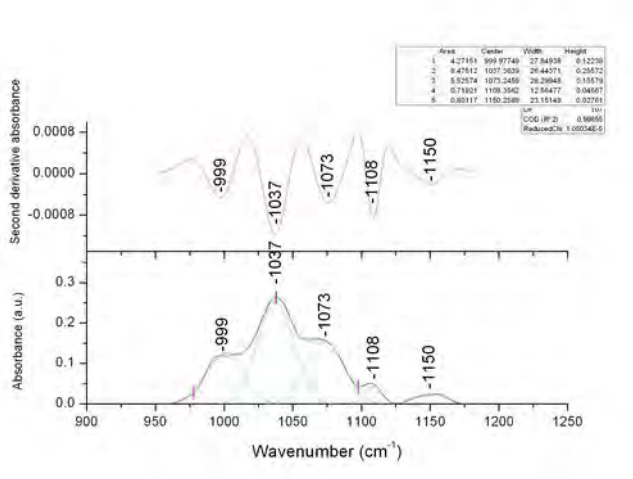


Fig G-5 FTIR spectra of β-glucan II from *S. cerevisiae* Angel® when cultured in YPD medium with NaCl 30000 ppm (treatment 3) in wavenumber 950- 1185 cm^{-1} from curve- fitting (lower) and second derivative plot (upper)

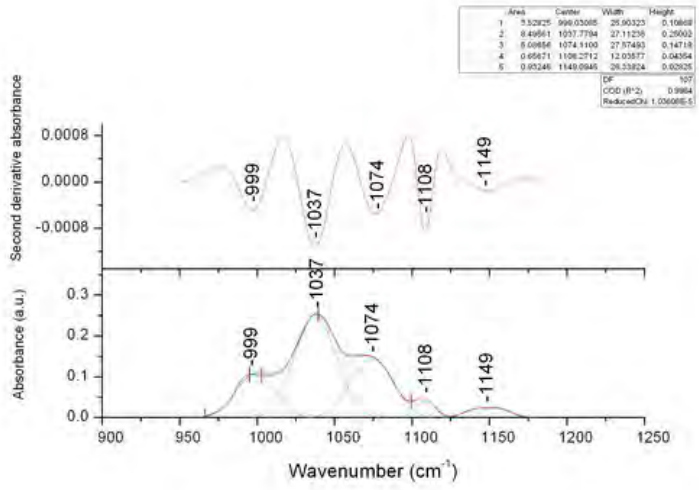


Fig G-6 FTIR spectra of β-glucan II from *S. cerevisiae* Angel® when cultured in YPD medium with EDTA 5 ppm and SDS 10 ppm (treatment 4) in wavenumber 950- 1,185 cm^{-1} from curve- fitting (lower) and second derivative plot (upper)

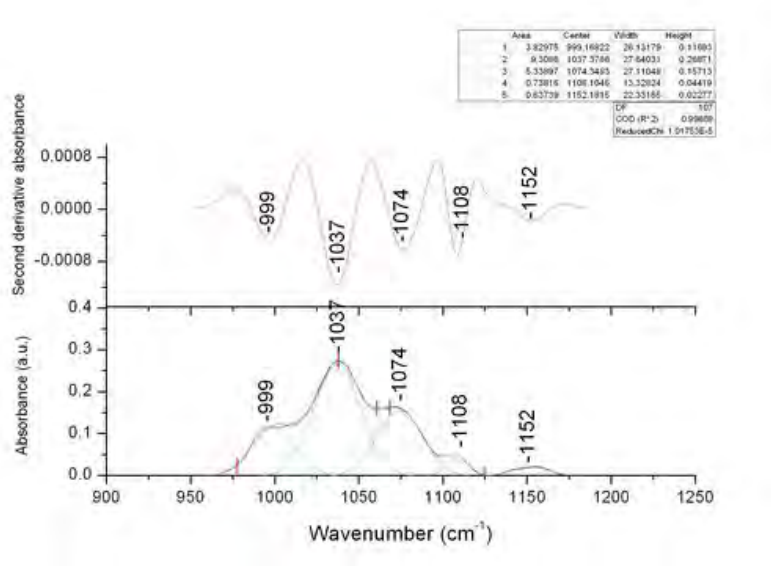


Fig G-7 FTIR spectra of β-glucan II from *S. cerevisiae* Angel® when cultured in YPD medium with EDTA 5 ppm and NaCl 3000 ppm (treatment 5) in wavenumber 950- 1,185 cm^{-1} from curve- fitting (lower) and second derivative plot (upper)

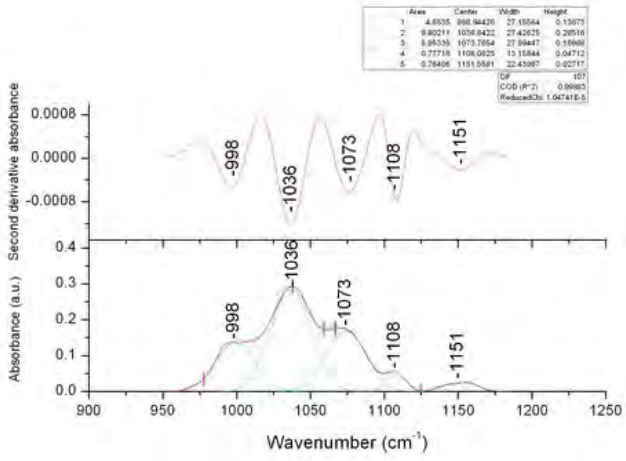


Fig G-8 FTIR spectra of β-glucan II from *S. cerevisiae* Angel® when cultured in YPD medium with SDS 20 ppm and NaCl 3,000 ppm (treatment 6) in wavenumber 950- 1,185 cm^{-1} from curve- fitting (lower) and second derivative plot (upper)

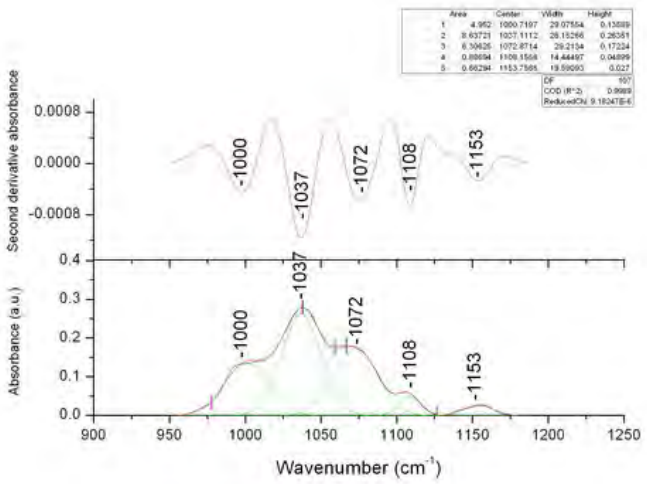


Fig G-9 FTIR spectra of β-glucan II from *S. cerevisiae* Angel® when cultured in YPD medium with EDTA 5 ppm, SDS 10 ppm and NaCl 3,000 ppm (treatment 7) in wavenumber 950- 1,185 cm^{-1} from curve- fitting (lower) and second derivative plot (upper)

VITAE

Miss Naruemon Mongkontanawat was born on March 31, 1975 in Chonburi, Thailand. She obtained a B.Sc. degree and a M. Sc. in Biotechnology from King Mongkut's Institute of Technology Ladkrabang, Bangkok in 1998 and 2002, respectively. She started her first job as a lecturer at Panusniklom college, Chonburi, Thailand during 1998-1999. During 2003-2006, she worked for the Department of Agro-Industrial, Faculty of Agro-Industry, Rajamangala University of Technology Tawan-ok, Chantaburi Campus. At the age of 30 she received a full scholarship from Rajamangala University of Technology Tawan-ok to pursue her Ph.D. studies at the Department of Food Technology, Chulalongkorn University. After she graduates, she will join the Department of Agro-Industrial, Faculty of Agro-Industry, Rajamangala University of Technology Tawan-ok, Chantaburi Campus.

Permanent mailing address: nmongkontanawut@hotmail.com
Department of Agro-Industrial,
Faculty of Agro-Industry,
Rajamangala University of Technology Tawan-ok, Chantaburi Campus.
Koukitchakut, Chantaburi, 22210
Thailand