

Production and Isolation of Fungal Chitosan by Submerged Fermentation

**By
Seda ALPER**

**A Dissertation Submitted to the
Graduate School in Partial Fulfillment of the
Requirements For the Degree of**

MASTER OF SCIENCE

**Department: Biotechnology and Bioengineering
Major: Biotechnology**

**İzmir Institute of Technology
İzmir, Turkey**

September, 2003

We approve the thesis of **Seda ALPER**

Date of Signature

.....


10.09.2003

Prof.Dr. Şebnem HARSA

Supervisor

Department of Food Engineering

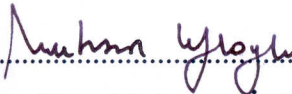
.....


10.09.2003

Assist.Prof.Dr. Ali Fazıl YENİDÜNYA

Co-Supervisor

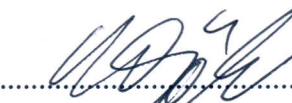
Department of Biology

.....


10.09.2003

Prof.Dr. Muhsin ÇİFTÇİOĞLU

Department of Chemical Engineering

.....


10.09.2003

Assist.Prof.Dr. Yekta GÖKSUNGUR

Department of Food Engineering

Ege University

.....


10.09.2003

Prof.Dr. Şebnem HARSA

Head of Interdisciplinary

Biotechnology and Bioengineering Program

ACKNOWLEDGEMENTS

I gratefully acknowledge the guidance and support of my thesis advisor, Prof. Dr. Şebnem HARSA, who showed tremendous enthusiasm for this topic and encouraged me at all times. I would also like to acknowledge Assist. Prof. Dr. Ali Fazıl YENİDÜNYA, Assist. Prof. Dr. Yekta GÖKSUNGUR and Dr. İhsan YAŞA who showed me an interest in my research efforts, provided critical feedback, and offered encouragement.

This work has benefited significantly from comments and suggestions received from various academics. A special thank you goes to all my colleagues and friends, especially Berna ÜZELYALÇIN, Rukiye ÇİFTÇİOĞLU, Naz GÜLTEKİN, Ali Emrah ÇETİN, Hande GENÇKAL, Aslı UÇAN, Berna ŞENGÜL and Elif TOKUÇ who provided me with valuable information. And also a special thank to Zelal POLAT for sharing me her experiences in the laboratory.

Finally, this thesis could not have been undertaken without the support, love, and patience of all my family.

I dedicate this thesis to the lovely memory of my mother.

ABSTRACT

Chitosan is the N-deacetylated derivative of chitin which is the supporting material of crustaceans and insects. Chitosan together with chitin are recommended as suitable functional materials because of their excellent biocompatibility, biodegradability, non-toxicity and adsorption properties and can be used in agriculture, biotechnology and food industry. Although chitosan is produced by chemical deacetylation of chitin molecule, it is also a natural component of cell walls of fungi belonging to Zygomycetes and can be produced by extraction from fungus cell walls. Fungi are thus the promising alternative sources of chitosan. Fungi can be manipulated to give chitosan of more consistent and desired physico-chemical properties compared to chitosan obtained from crustacean sources.

In this study, *Absidia spp*, *Aspergillus niger*, *Rhizopus arrhizus*, *Cunninghamella elegans*, and *Mucor rouxii* were examined for biomass growth. At first, all five species were grown on synthetic medium at 28°C, 180 rpm in shake-flask incubator. *Mucor rouxii* which gave the maximum biomass concentration was also grown on molasses. The maximum biomass concentration of *Mucor rouxii* was found to be higher than that of synthetic medium. The best growth conditions obtained were 4% sucrose, 0.2% yeast extract, 1% peptone and 10^6 spores in 40 ml. The mycelia harvested at late exponential phase was treated with alkali to remove proteins and chitosan was extracted from cell wall by using acetic acid. The yield of extractable chitosan obtained from cell wall of *Mucor rouxii* was 2500 mg / l and it is almost 20 % of biomass and approximately 35 % of alkali insoluble fraction.

ÖZ

Kitosan, deniz kabukluları ve böceklerin destek materyali olan kitinin N-deasetilasyonu ile elde edilir. Kitin ve kitosan, biyouyumluluk, biyobozunurluk, toksik olmama ve adsorpsiyon gibi önemli özelliklere sahiptirler. Bu nedenle, ziraat, biyoteknoloji ve gıda endüstrisi gibi birçok alanda geniş kullanım olanakları bulunmaktadır. Kitosan, kitin molekülünün kimyasal deasetilasyonu ile elde edilir. Aynı zamanda, Zygomycetes sınıfına ait küflerin hücre duvarlarında da doğal olarak bulunmaktadır ve kitosan üretiminde alternatif bir kaynak olan bu küflerin hücre duvarından ekstrakte edilebilirler. Deniz kabuklularından elde edilen kitosanla karşılaştırıldığında, küflerden daha kararlı ve istenilen fizikokimyasal özelliklere sahip kitosan üretilebilir.

Bu çalışmada, kitosan üretimini gerçekleştirmek üzere *Absidia* spp., *Aspergillus niger*, *Rhizopus arrhizus*, *Cunninghamella elegans* ve *Mucor rouxii* tarandı. İlk olarak, beş küf türü sentetik ortamda, 28 °C, 180 rpm'de, çalkalamalı inkübatörde geliştirildi. Daha sonra, en yüksek biyomas konsantrasyonuna sahip *Mucor rouxii*, melas içeren ortamda geliştirilerek; elde edilen biyomas miktarı, sentetik ortamla karşılaştırıldı. Melaslı ortamdan elde edilen biyomas miktarının, sentetik ortamdan daha yüksek miktarda olduğu gözlemlendi. En yüksek biyomas konsantrasyonu %4 sukroz, %0.2 yeast extract, %1 peptone ve 10^6 spor konsantrasyonu / 40 ml ortamda elde edildi. Geç büyüme fazında, miseller fermantasyon sıvısından alınarak, proteinleri uzaklaştırmak için önce alkali ile, daha sonra da kitosan ekstraksiyonu için asetik asit ile muamele edildi. *Mucor rouxii*'den elde edilen kitosan miktarının 2500 mg / l olduğu belirlendi. Elde edilen kitosan, biyomasın % 20'si ve alkalide çözünmeyen kısmın yaklaşık olarak % 35'idir.

TABLE OF CONTENTS

LIST OF FIGURES.....	VIII
LIST OF TABLES.....	XI
Chapter 1. INTRODUCTION.....	1
Chapter 2. CHITIN AND CHITOSAN	3
2.1. History	6
2.2. Chitin and Chitosan Biosynthesis.....	7
2.3. Properties of Chitosan	10
2.4. Applications of Chitosan	13
2.5. Production of Chitosan	14
2.5.1. Chemical Synthesis	15
2.5.2. Microbial Fermentation.....	17
2.5.2.1. Microorganisms.....	18
2.5.2.2. Raw Materials.....	19
2.5.2.3. Fermentation Process.....	21
2.5.2.4. Extraction of Chitosan	23
2.6. Characterization of Chitosan	24
2.6.1. Degree of Deacetylation	24
2.6.2. Molecular Weight.....	25
Chapter 3. MATERIALS AND METHODS	26
3.1. Materials	26
3.2. Methods	27
3.2.1. Culture Conditions.....	27
3.2.2. Growth of Fungal Biomass in Shake Flask.....	28
3.2.2.1. Effect of Mineral Salts.....	30
3.2.2.2. Effect of Spore Inoculum Concentration.....	30
3.2.2.3. Effect of Production Medium Volume	30
3.2.2.4. Effect of Initial Sucrose Concentration	30
3.2.3. Chitosan Fermentation in Fermenter	30
3.2.4. Chitosan Extraction	31
3.2.5. Analyses	33
3.2.5.1. Dry Cell Weight Measurement.....	33

3.2.5.2. Sucrose and Lactose Analyses.....	33
3.2.5.3. Metal Ions Determination in Molasses.....	34
3.2.5.4. Enzyme (protease) Assay	35
Chapter 4. RESULTS AND DISCUSSION.....	36
4.1. Growth Kinetics of Different Fungal Strains Produced on Complex Medium 1.....	36
4.2. Effect of Medium Composition and Mineral Salts on Growth of <i>Mucor rouxii</i>	38
4.3. Biomass Yields of <i>Mucor rouxii</i> on Different Food Industry By- products	40
4.4. Effect of Spore Inoculum Concentration on Growth of <i>Mucor rouxii</i>	42
4.5. Effect of Production Medium Volume on Growth of <i>Mucor rouxii</i>	43
4.6. Effect of Initial Sucrose Concentration on Growth of <i>Mucor rouxii</i>	44
4.7. Growth of <i>Mucor rouxii</i> in Shake-Flask and Stirred Tank Bioreactor	45
4.8. Chitosan Extraction and Yield Determination	47
Chapter 5. CONCLUSIONS AND RECOMMENDATIONS.....	49
REFERENCES	50
APPENDIX	53
A. Growth curves of five fungal strains	53
B. Effect of mineral salts on growth of <i>Mucor rouxii</i>	56
C. Effect of different food industry by-products on growth of <i>Mucor</i> <i>rouxii</i>	59
D. Effect of initial spore inoculum concentration, production medium volume and sucrose concentration on growth of <i>Mucor rouxii</i>	63
E. Calibration Curves	72
F. Definitions of Fermentation Evaluation Terms.....	74

Figure A.1. Growth

Figure A.2. Growth

Figure A.3. Growth

Figure A.4. Growth

Figure A.5. Growth

Figure B.1. Growth

LIST OF FIGURES

Figure 2.1. Structures of cellulose, chitin and chitosan	5
Figure 2.2. Pathway for the synthesis of chitin in crustaceans	8
Figure 2.3. Pathway for the biosynthesis of chitin in fungi	9
Figure 2.4. Pathway of chitosan synthesis	10
Figure 2.5. Deacetylation of chitin	15
Figure 2.6. Chitin and chitosan manufacturing process.....	16
Figure 2.7. Production of chitin and chitosan in Asian countries.....	17
Figure 3.1. Flowsheet for the extraction of chitosan	32
Figure 4.1. Growth kinetics of different fungal strains.....	37
Figure 4.2. Growth of <i>Mucor rouxii</i> on complex medium 1 vs. YPG.....	38
Figure 4.3. Effect of mineral salts on growth	39
Figure 4.4. Effect of mineral salts on growth at stationary phase	39
Figure 4.5. Growth curves of <i>Mucor rouxii</i> on different food industry by- products	40
Figure 4.6. Effect of different food industry by- products on growth at stationary phase	41
Figure 4.7. Effect of spore inoculum concentration on growth	42
Figure 4.8. Effect of production medium volume on growth	43
Figure 4.9. Effect of initial sucrose concentration on growth.....	44
Figure 4.10. Effects of initial sucrose concentration, spore concentration and medium volume for growth of <i>Mucor rouxii</i> on YPM medium	45
Figure 4.11. Growth of <i>Mucor rouxii</i> and sucrose consumption in shake-flask in the best condition for fermentation.....	46
Figure 4.12. Changes of biomass, AIM and chitosan concentration during fermentation of <i>Mucor rouxii</i>	47
Figure A.1. Growth of <i>Mucor rouxii</i> on complex medium 1	53
Figure A.2. Growth of <i>Rhizopus arrhizus</i> on complex medium 1	53
Figure A.3. Growth of <i>Absidia</i> on complex medium 1	54
Figure A.4. Growth of <i>Aspergillus niger</i> on complex medium 1	54
Figure A.5. Growth of <i>Cunninghamella elegans</i> on complex medium 1	55
Figure B.1. Growth of <i>Mucor rouxii</i> on Complex Medium 1 without MgSO ₄	56

Figure B.2. Growth of <i>Mucor rouxii</i> on Complex Medium 1 without CaCl ₂	56
Figure B.3. Growth of <i>Mucor rouxii</i> on Complex Medium 1 without (NH ₄) ₂ SO ₄	57
Figure B.4. Growth of <i>Mucor rouxii</i> on Complex Medium 1 without K ₂ HPO ₄	57
Figure B.5. Growth of <i>Mucor rouxii</i> on Complex Medium 1 without NaCl	58
Figure C.1. Growth of <i>Mucor rouxii</i> on molasses.....	59
Figure C.2. Growth of <i>Mucor rouxii</i> on whey.....	59
Figure C.3. Growth of <i>Mucor rouxii</i> on YPM.....	60
Figure C.4. Growth of <i>Mucor rouxii</i> on YPW	60
Figure C.5. Growth of <i>Mucor rouxii</i> on YM.....	61
Figure C.6. Growth of <i>Mucor rouxii</i> on PM.....	61
Figure C.7. Growth of <i>Mucor rouxii</i> on Complex Medium 2	62
Figure C.8. Growth of <i>Mucor rouxii</i> on Complex Medium 3	62
Figure D.1. Growth of <i>Mucor rouxii</i> in YPM medium inoculated 0.025x10 ¹ spore inoculum	63
Figure D.2. Growth of <i>Mucor rouxii</i> in YPM medium inoculated 0.025x10 ² spore inoculum	63
Figure D.3. Growth of <i>Mucor rouxii</i> in YPM medium inoculated 0.025x10 ³ spore inoculum	64
Figure D.4. Growth of <i>Mucor rouxii</i> in YPM medium inoculated 0.025x10 ⁴ spore inoculum	64
Figure D.5. Growth of <i>Mucor rouxii</i> in YPM medium inoculated 0.025x10 ⁵ spore inoculum	65
Figure D.6. Growth of <i>Mucor rouxii</i> in YPM medium inoculated 0.025x10 ⁶ spore inoculum	65
Figure D.7. Growth of <i>Mucor rouxii</i> in YPM medium inoculated 0.025x10 ⁷ spore inoculum	66
Figure D.8. Growth of <i>Mucor rouxii</i> in YPM medium inoculated 0.025x10 ⁸ spore inoculum	66
Figure D.9. Growth of <i>Mucor rouxii</i> in 10 ml of YPM medium.....	67
Figure D.10. Growth of <i>Mucor rouxii</i> in 20 ml of YPM medium.....	67
Figure D.11. Growth of <i>Mucor rouxii</i> in 30 ml of YPM medium.....	68
Figure D.12. Growth of <i>Mucor rouxii</i> in 40 ml of YPM medium.....	68
Figure D.13. Growth of <i>Mucor rouxii</i> in 50 ml of YPM medium.....	69

Figure D.14. Growth of <i>Mucor rouxii</i> in YPM medium containing 1 % sucrose	69
Figure D.15. Growth of <i>Mucor rouxii</i> in YPM medium containing 2 % sucrose	70
Figure D.16. Growth of <i>Mucor rouxii</i> in YPM medium containing 3 % sucrose	70
Figure D.17. Growth of <i>Mucor rouxii</i> in YPM medium containing 4 % sucrose	71
Figure D.18. Growth of <i>Mucor rouxii</i> in YPM medium containing 5 % sucrose	71
Figure E.1. Calibration curve for sucrose standard (HPLC)	72
Figure E.2. Calibration curve for sucrose standard (UV-VIS. Spect.)	72
Figure E.3. Calibration curve for lactose standard (UV-VIS. Spect.)	73

Table 3.7

Table 3.1

Table 3.2

Table 3.3

Table 3.4

Table 3.5

Table 3.6

Table 3.8

LIST OF TABLES

Table 2.1.	Chitin content of selected Crustacean, Insects, Molluscan Organs, and Fungi	4
Table 2.2.	Properties of chitosan	12
Table 2.3.	Applications of Chitosan	13
Table 2.4.	The composition of Turkish beet molasses.....	20
Table 2.5.	Gross composition of liquid and dried whey.....	20
Table 2.6.	Amino acid, vitamin and mineral content of whey powder	21
Table 2.7.	Some of the chitosan fermentation studies.....	22
Table 3.1.	Trace element composition of molasses.....	26
Table 3.2.	Chemicals and their producers	27
Table 3.3.	Media used in the shake flask fermentations.....	28
Table 3.4.	The properties of the HPLC column and analysis conditions	34
Table 3.5.	The wavelenghts used for elemental analyses by ICP.....	35
Table 3.6.	ICP-AES (Axial Liberty) operating conditions.....	35
Table 4.1.	Concentrations of mycelia, AIM, chitosan, sugar and yields of extractable chitosan of <i>Mucor rouxii</i>	48

CHAPTER 1

INTRODUCTION

Polysaccharides are widely distributed in nature. Their molecular structures and hence their properties vary over a broad range. They have been regarded primarily as structural materials and as suppliers of water and energy. These structures are considered less significant than other natural polymers such as proteins and nucleic acids in view of their biological functions. They are, however, attracting increased attention as their inherent biological activities and physicochemical properties are being better understood. Polysaccharides will therefore become increasingly important in various fields since they possess unique structures and characteristics that are quite different from those of typical synthetic polymers (Kurita, 2001).

Chitin is a naturally abundant polysaccharide in nature, in addition to cellulose and starch. It is known to consist of 2- acetamido- 2- deoxy- β – D - glucose through β - (1-4) linkage. Chitin can be found in crustaceans, such as crabs, lobsters, and shrimp. It can also be found in insects, worms and fungi.

Chitin can be processed into many derivatives, which are chitin oligomers, chitosan and chitosan oligomers. Chitosan, the most readily available, is N-deacetylated derivative of chitin. However, this N- deacetylation is almost never complete.

Chitosan has a number of important industrial uses because of its high amine content and polycationic nature. These uses are related to its molecular size and unusually high charge density. Chitosan has been used to produce biodegradable films and fibres, to flocculate proteinaceous solids and chelate metal ions in waste water treatment, to produce contact lenses and blood anticoagulants. Chitosan has also been used as a feed additive, a cell immobilization and permeabilization substrate, and has been shown to possess antimicrobial properties.

Commercially, chitosan is derived by the chemical deacetylation of chitin from waste crustacean exoskeletons with strong alkali. This harsh conversion process, as well as variability in source material, leads to inconsistent physicochemical characteristics. Production and purification of chitosan from the cell walls of fungi grown under controlled conditions offer greater potential for a more consistent product. The cell walls of Zygomycetes are characterized by the joint occurrence of chitosan and chitin, which have protective and supportive functions. Physicochemical properties of chitosan

isolated directly from a fungus may be manipulated by control of fermentation and processing parameters (Arcidiacono *et al.*, 1992).

The objective of this study is the production and extraction of chitosan from cell walls of fungi grown by submerged fermentation. Within this context, five fungal strains such as *Absidia* spp, *Aspergillus niger*, *Rhizopus arrhizus*, *Cunninghamella elegans*, and *Mucor rouxii* were used to obtain the highest biomass concentration. Moreover, additional purpose was to find the best condition for biomass production of *Mucor rouxii*.

CHAPTER 2

CHITIN AND CHITOSAN

Of the many kinds of polysaccharides, cellulose, chitin and chitosan are the most important biomass resources; cellulose is synthesized mainly in plants, chitin is synthesized mainly in lower animals. In fact, they are the most abundant organic compounds on earth. Although cellulose has been studied extensively, only limited attention has been paid to chitin and chitosan, principally to its biological properties. Despite its huge annual production and easy accessibility, chitin and chitosan still remain as unutilized biomass resources (Kurita, 2001).

Chitin is very widely distributed, especially in animals, and exists also in less evolved taxonomic groups, such as Protozoa. In plants, chitinous cell walls are only found for supporting structures such as fungi and molds, which, like animals, find considerable nitrogen in their food. On the contrary, photosynthetic plants utilize nitrogen-free sugars almost exclusively for their supporting structures; chitin is however believed to constitute the cell membrane of some lowergreen plants like Chlorophyceae. Chitin is associated with other polysaccharides in the fungal cell walls, while in animal forms chitin is associated with proteins. In hard structures, the proteins are tanned by phenolic derivatives (Muzzarelli, 1977).

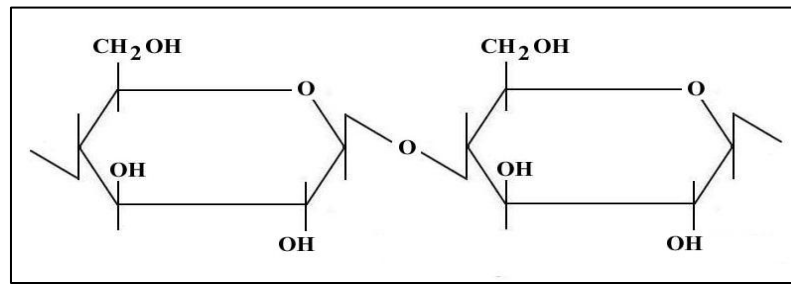
The occurrence of chitin in various organisms is given in Table 2.1.

Table 2.1. Chitin content of selected Crustacean, Insects, Molluscan Organs, and Fungi (Tharanathan *et al.*, 2003).

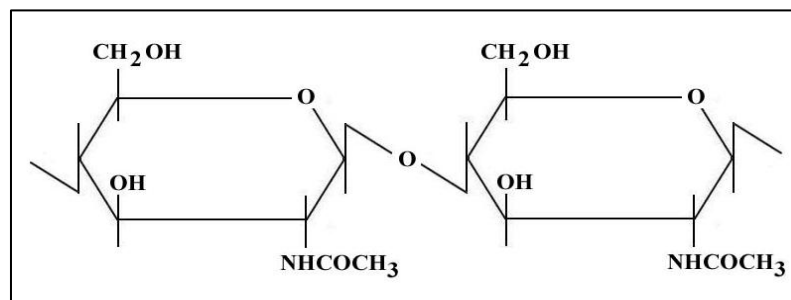
Type	Chitin Content (%)	Type	Chitin Content(%)
<u>Crustacean</u>		<u>Insects</u>	
Cancer (crab)	72.1 ^c	Periplaneta (cockroach)	2.0 ^d
Carcinus (crab)	64.2 ^b	Blatella (cockroach)	18.4 ^c
Paralithodes (king crab)	35.0 ^b	Colcoptera (beetle)	27-35 ^c
Callinectes (blue crab)	14.0 ^a	Diptera (truefly)	54.8 ^c
Crangon (shrimp)	69.1 ^c	Pieris (sulfur butterfly)	64.0 ^c
Alasakan shrimp	28.0 ^d	Bombyx (silk worm)	44.2 ^c
Nephrops (lobster)	69.8 ^c	Calleria (wax worm)	33.7 ^c
Homarus (lobster)	60-75 ^c		
Lepas (barnacles)	58.3 ^c	<u>Fungi</u>	
<u>Molluscan Organs</u>		<i>Aspergillus niger</i>	42.0 ^e
Clamshell	6.1	<i>Penicillum notatum</i>	18.5 ^e
Oyster shell	3.6	<i>Penicillum chrysogenum</i>	20.1 ^e
Squid, Skeletal pen	41.0	<i>Saccharomyces cereviseae</i>	2.9 ^e
Krill, deproteinized shell	40.2	<i>Mucor rouxii</i>	44.5
		<i>Lactarius vellereus</i>	19.0
		(mushroom)	

- a. Wet body weight, b. Dry body weight, c. Organic weight of cuticle,
d. Total dry weight of cuticle, e. Dry weight of the cell wall

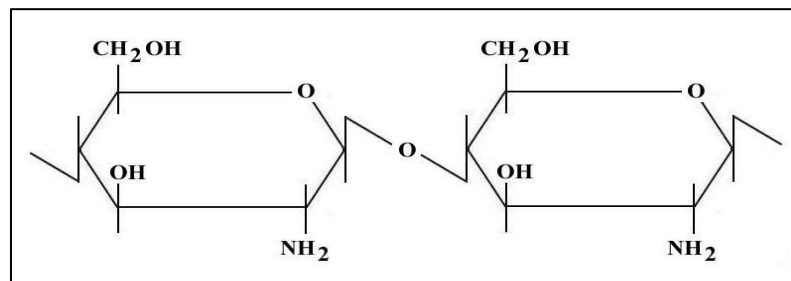
Chitin is a polysaccharide constituted of β - (1 \rightarrow 4) 2- acetamido- 2- deoxy- D- glucose units, some of them being deacetylated. This natural polymer that can be called poly-N-acetyl-D-glucosamine, can be formally considered a derivative of cellulose where the C-2 hydroxyl groups have been completely replaced by acetamido groups (Muzzarelli, 1973). Chitosan is the N-deacetylated derivative of chitin. In chitin, the amino group is acetylated, thus chitin is an amide of acetic acid; in chitosan the amino group is free and therefore chitosan is a primary amine. However, chitin can not be sharply distinguished from chitosan, because fully acetylated and fully deacetylated chitins do not normally occur in nature and are difficult to prepare. Experimentally, chitosan can be distinguished from chitin because of differences in solubility in acids (Muzzarelli, 1977). The structures of cellulose, chitin and chitosan are shown in Figure 2.1.



(a)



(b)



(c)

Figure 2.1. Structures of cellulose (a), chitin (b) and chitosan (c) (Kumar, 2000).

Chitin is a white, hard, inelastic, nitrogenous polysaccharide and the major source of surface pollution in coastal areas. The immunogenicity of chitin is exceptionally low, in spite of the presence of nitrogen. It is a highly insoluble material resembling cellulose in its solubility and low chemical reactivity. Like cellulose, it functions naturally as a structural polysaccharide, but differs from cellulose in its properties. Chitin is highly hydrophobic and is insoluble in water and most organic solvents. It is soluble in hexafluoroisopropanol, hexafluoroacetone, chloroalcohols in

conjugation with aqueous solutions of mineral acids and dimethylacetamide containing 5% lithium chloride. Chitosan, the deacetylated product of chitin, is soluble in dilute acids such as acetic acid, formic acid, etc. Chitin and chitosan are of commercial interest due to their high percentage of nitrogen (6.89 %) compared to synthetically substituted cellulose (1.25 %) (Kumar, 2000).

Ideally, the monomer of chitin is 2-acetamido-2-deoxy- β -D-glucose, while the monomer of chitosan is 2-amino-2-deoxy- β -D-glucose; for practical purposes, however, the extent of deacetylation in both the natural and the modified polymers has to be measured. The official name of chitin (a fully acetylated product) is, (1 \rightarrow 4)- 2-acetamido-2-deoxy- β -D-glucan and the official name of chitosan (a completely deacetylated product) is, (1 \rightarrow 4)-2-amino-2-deoxy- β -D-glucan (Muzzarelli, 1977).

Chitosan, a cationic β - (1-4) linked 2- amino- 2- deoxy- D- glucosamine, is rarely found in nature. Cell walls of some fungi, particularly Zygomycetes, contain both chitin and chitosan (Arcidiacono *et al.*, 1989).

2.1. History

Chitin was described for the first time by Bracannot (1811) who was professor of Natural History, director of the Botanical Garden and member of the Academy of Sciences, Nancy, France. In the pursuit of his research on mushrooms, he treated *Agaricus volvaccus* and other mushrooms with dilute warm alkali and isolated chitin, possibly slightly contaminated with proteins. From the dry distillation of this product, called fungine, he obtained a liquid that, upon distillation with potassium hydroxide, yielded ammonia. He described the lack of reaction of chitin with dilute alkali solutions, the compound formed with tannin, the degradation produced by sulfuric acid, the release of acetic acid and several other reactions of chitin. He stated that ‘fungine seems to contain more nitrogen than wood’ and concluded that it is ‘a quite distinct substance among those identified in plants’. The first observation about the occurrence of chitosan in plant cell walls and, more broadly, in living systems was made by Kreger (1954) who reported its occurrence in the mycelia and sporangiophores of *Phycomyces blakesleeanus*. Chitosan was later demonstrated to be the most abundant component of the cell walls of filamentous and yeast-like forms of *Mucor rouxii* grown respectively under air and under carbon dioxide (Muzzarelli, 1977).

2.2. Chitin and Chitosan Biosynthesis

Chitin biosynthesis in crustaceans differs significantly from that of fungi. However, they do share common biosynthetic steps, and the enzymatic machinery exhibit more or less similar catalytic regulation.

Figure 2.2. shows the pathway for the synthesis of chitin in crustaceans. The non-reducing disaccharide, trehalose is the most common sugar in insects. It is hydrolyzed by trehalase, and the glucose units are converted to uridine diphospho- N-acetylglucosamine (UDP- GlcNAc). The terminal polymerization step has been the contentious issue in chitin biosynthesis in insects for which several explanations and speculation were given. However, based on the available evidence, it is now proposed that the epidermis in insects using UDP- GlcNAc as the precursor synthesizes soluble oligosaccharides that are linked to polyprenol lipids such as dolichol phosphate, transported outside the cell and attached to specific residues, possibly asparagine, on a receptor protein. The resultant primer (attached to receptor protein) is extended by the sequential addition of GlcNAc residues via chitin synthetase (Tharanathan *et al.*, 2003).

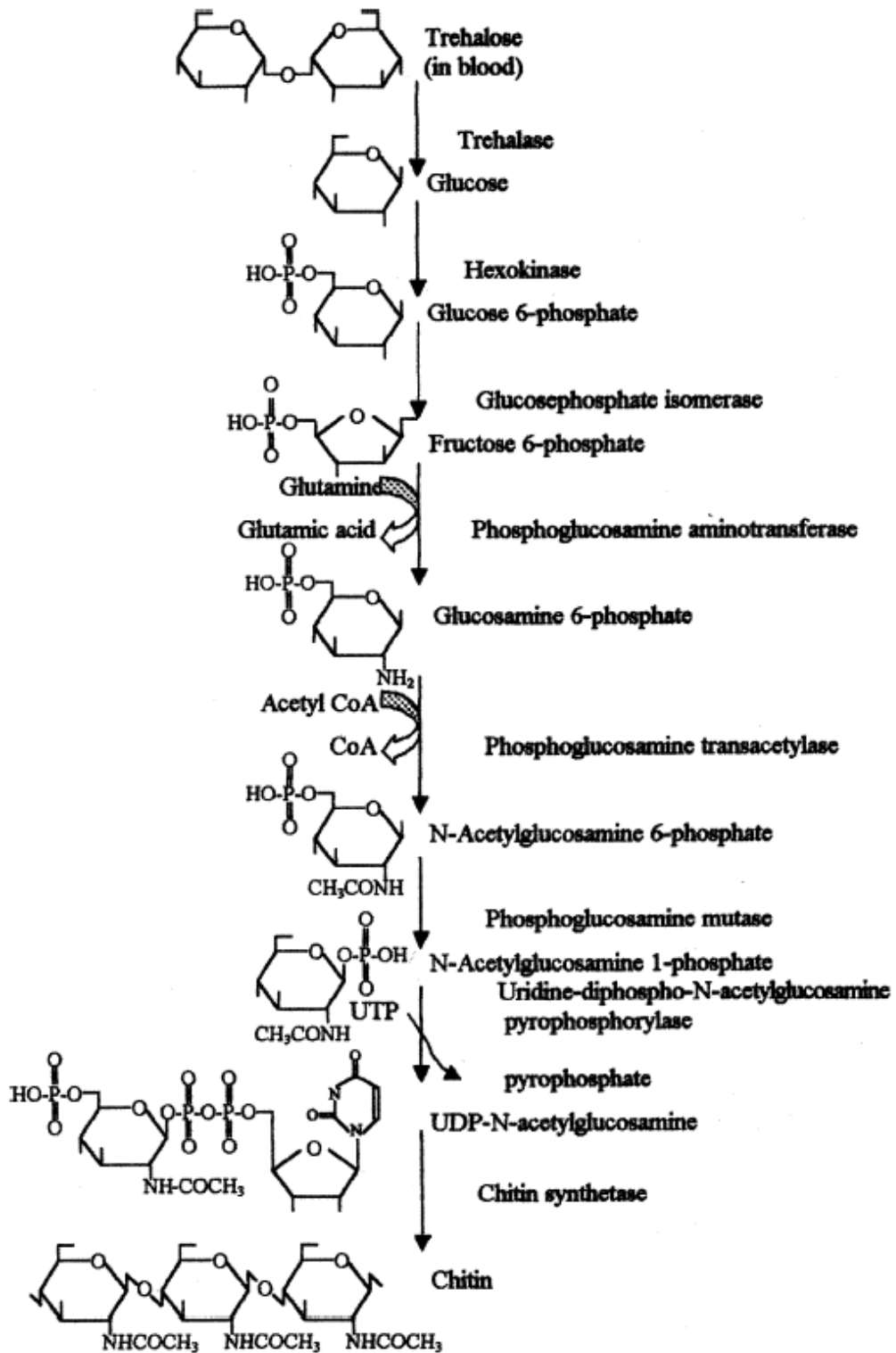


Figure 2.2. Pathway for the synthesis of chitin in crustaceans (Tharanathan *et al.*, 2003).

In fungi, chitin synthesis is highly compartmentalized. The key enzyme chitin synthetase, like its counterpart in crustaceans, occurs in zymogen form from where it is distributed to specific regions on the cell surface in specialized vesicles called chitosomes. The macromolecular assembly begins outside the cytoplasm wherein the protease (activator) on the cell surface activates the zymogen. Once the energy- rich uridine diphospho- N- acetylglucosamine is produced (from glucose), chitin synthetase successfully catalyzes the transfer of GlcNAc to primers forming chitin (Tharanathan *et al.*, 2003).

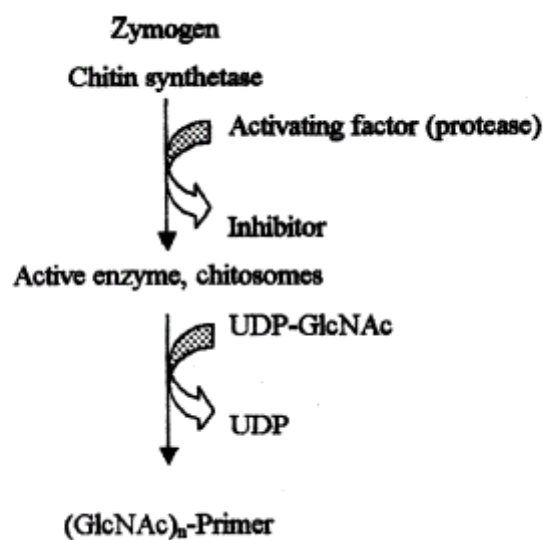


Figure 2.3. Pathway for the biosynthesis of chitin in fungi (Tharanathan *et al.*, 2003).

The formation of chitosan in fungi cell walls is a result of the complex action of two enzymes which are chitin synthase and chitin deacetylase. In the first step, chitin synthase builds a chain of chitin using the chitin precursor, uridino- di phospho N- acetylglucosamine (UDP- GlcNAc). Next, chitin deacetylase hydrolyzes acetic groups from N- acetylglucosamine (GlcNAc), transforms it into glucosamine (GlcN) and finally chitosan is formed.

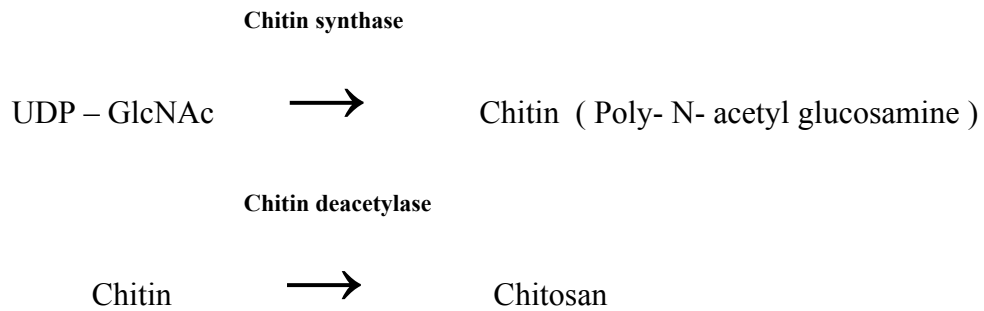


Figure 2.4. Pathway of chitosan synthesis (Arcidiacono *et al.*, 1989).

The activity of chitin synthase influences the length of the chitin chain (that is, the molecular weight of the biopolymer), and the activity of chitin deacetylase influences the content of GlcNAc groups in the chain of chitosan (that is, the degree of acetylation). The properties of nascent chitosan (degree of acetylation and molecular weight) depend on the activity of these two enzymes (Jaworska, 2001).

2.3. Properties of Chitosan

Chitosan is a natural, non-toxic, biodegradable, high molecular weight polymer. Key properties of chitosan are its ability to act as a cationic flocculent, humectant, viscosifier, and selective chelator of metal ions. Properties of chitosan can be categorized into four groups that are cationic, biological, chemical and solution properties.

Most of the naturally occurring polysaccharides, e.g. cellulose, dextran, pectin, alginic acid, agar, agarose and carragenans, are neutral or acidic in nature, whereas chitin and chitosan are examples of highly basic polysaccharides. Chitosan is linear polyelectrolyte at acidic pH's. It has a high charge density, one charge per glucosamine unit. Since many materials carry negative charges (*e.g.* proteins, anionic polysaccharides, nucleic acids, etc.), the positive charge of chitosan interacts strongly with negative surfaces to give an electric neutrality. Chitosan is an excellent flocculent due to its vast number of $-NH_3^+$ groups that can interact with negatively charged colloids. Chitosan adheres readily to natural polymers such as hair and skin, which are composed of negatively charged mucopolysaccharides and proteins. Chitosan forms complexes with metal ions. Thus, it is useful in chelating iron, copper, and magnesium

and can also be used to remove toxic heavy metal ions such as silver, cadmium, mercury, lead, nickel and chromium. (Sandford, 1989 and Kumar, 2000).

Many of biomedical applications of chitosan rely on its non- toxic and biodegradable properties. Chitosan has been shown to facilitate wound healing, reduce serum cholesterol levels and stimulate the immune system. Chitosan, when coated on seeds, results in increased crop yields, apparently due to chitosan inducing a protective response by the germinating plant (Sandford, 1989).

Chitosan, being a high molecular weight polymer, is a linear polyamine whose amino groups are readily available for chemical reactions and salt formation with acids. Since chitosan can be viewed as a cellulose derivative, the primary (C- 6) and secondary (C- 3) hydroxyl groups can be used to make derivatives. The scientific literature contains many interesting chemical derivatives of chitosan with commercial potential (Sandford, 1989 and Kumar, 2000).

Chitosan has rather specific solutions properties. First, when in the amine form, chitosan is not soluble in water at neutral pH. At acidic pH, the free amino groups (-NH₂) become protonated to form cationic amine groups (-NH₃) (Sandford, 1989).

Table 2.2. summarizes the properties of chitosan.

Table 2.2. Properties of chitosan (Sandford, 1989).

Chitosan	
Cationic properties	<ul style="list-style-type: none"> • Linear polyelectrolyte • High charge density • Excellent flocculent • Substantive to hair, skin • Chelates metal ions <ul style="list-style-type: none"> -Iron (Fe), Copper (Cu) -Toxic metals (Cd, Hg, Pb, Cr, Ni) -Radionucleides (Pu, U)
Biological properties	<ul style="list-style-type: none"> • Biocompatible <ul style="list-style-type: none"> -non-toxic -biodegradable -natural polymer • Bioactivity <ul style="list-style-type: none"> -wound healing accelerator -reduce blood cholesterol levels -immune system stimulant
Chemical properties	<ul style="list-style-type: none"> • Linear polyamine (poly-D- gluc.) • Reactive amino groups • Reactive hydroxyl groups (C3-OH, C6-OH)
Solution properties	<ul style="list-style-type: none"> • Free amine (-NH₂) <ul style="list-style-type: none"> -soluble in acidic solutions -insoluble at pH > 6.5 -insoluble in H₂SO₄ -limited solubility in H₃PO₄ -insoluble in most organic solvents • Cationic amine (-NH₃⁺) <ul style="list-style-type: none"> -soluble at pH < 6.5 -forms viscous solutions -solutions shear thinning -forms gels with polyanion -will remain soluble in some alcohol - water mixtures

2.4. Applications of Chitosan

Chitosan has some unique properties such as non-toxicity, biodegradability, biocompatibility, chelating ability with metal ions and etc. It is produced commercially in some countries such as United States of America and Japan as a solution, flaked and fine powder, bead, fiber, film, gel, spray forms. Chitosan's availability in a variety of useful forms and its unique chemical and biological properties make it a very attractive biomaterial.

In Table 2.3. The applications of chitosan are summarized.

Table 2.3. Applications of Chitosan (Sandford, 1989).

Chitosan Applications	Key Uses
Clarification and Purification Non-Food Related	<ul style="list-style-type: none"> • Sewage effluents • Sand and gravel wash • Metal finishing electroplating wastes • Paper mills • Radioactive wastes
Clarification and Purification Food Related	<ul style="list-style-type: none"> • Purify drinking water • Recover protein for animal feed • Fruit juices • Recover microalgae
Pharmaceutical	<ul style="list-style-type: none"> • Wound healing • Drug delivery • Bioengineering material • Cholesterol reducing agents
Wound Healing	<ul style="list-style-type: none"> • Bandages • Sutures • Synthetic skin • Eye bandage
Cosmetics and Personal Care	<ul style="list-style-type: none"> • Natural, non-toxic, cationic polymer • Hair treatment <ul style="list-style-type: none"> Clear solutions from clear films Substantive to hair Obtain gels in water: alcohol mixtures Viscosity can be varied; low to high to suit application • Skin care <ul style="list-style-type: none"> Moisturizer Substantive to skin Excellent tactile properties

Agriculture	<ul style="list-style-type: none"> • Coat seeds (wheat, rice, peas) Fungistatic, protect plants Non-toxic, biodegradable, biocompatible Increase crop yields EPA approved • Animal feed Approved flocculent for recovering proteinaceous wastes
Biotechnology	<ul style="list-style-type: none"> • Immobilization of enzyme and cells Entrapment and absorption Crosslinked with dialdehydes Charge complexes with anionic polymers • Chitosan gels Stable to phosphate buffers Easily prepared at room temperature Non-toxic High cell loads/activities • Purify/Recover Biologicals

Chitin and chitosan have a wide range of applications. They may be employed, for example, to solve numerous problems in environmental and biomedical engineering. Chitin derivatives including partially deacetylated chitosan can be easily molded to various forms and their derivatives are digested in vivo by lysosomal enzymes. Thus, it appears that this material can be a most interesting candidate for use as a carrier of a variety of drugs for controlled release applications. Lately, the transdermal absorption promoting characteristics of chitosan have been exploited, especially for nasal and oral delivery of polar drugs to include peptides and proteins and for vaccine delivery. These properties, together with the very safe toxicity profile, make chitosan an exciting and promising excipient for the pharmaceutical industry for present and future applications.

2.5. Production of Chitosan

Chitosan can be produced by chemical synthesis or by microbial fermentation. However, chitosan is produced on an industrial scale by chemical deacetylation of chitin with concentrated hydroxides at high temperatures.

2.5.1. Chemical Synthesis

In chitosan manufacturing process, firstly, minerals such as calcium carbonate and calcium phosphate are extracted with hydrochloric acid. Proteins are removed by treating with sodium hydroxide to obtain chitin. After that, chitin is first treated with very strong sodium hydroxide to hydrolyze the N- acetyl linkage, than chitosan is produced. Also it is possible to prepare chitosan oligosaccharide, chitosan salt and chitosan succinamide as seen in Figure 2.5 and Figure 2.6 (Sandford, 1989).

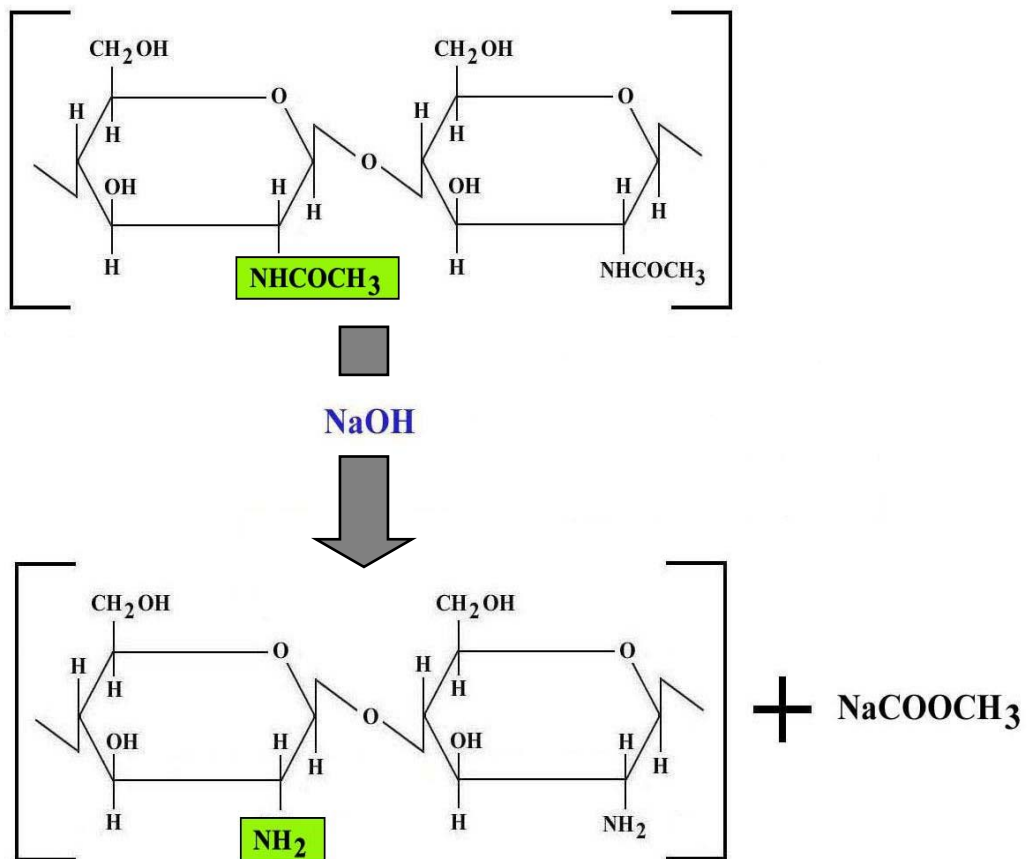


Figure 2.5. Deacetylation of chitin.

CHITIN & CHITOSAN MANUFACTURING PROCESS

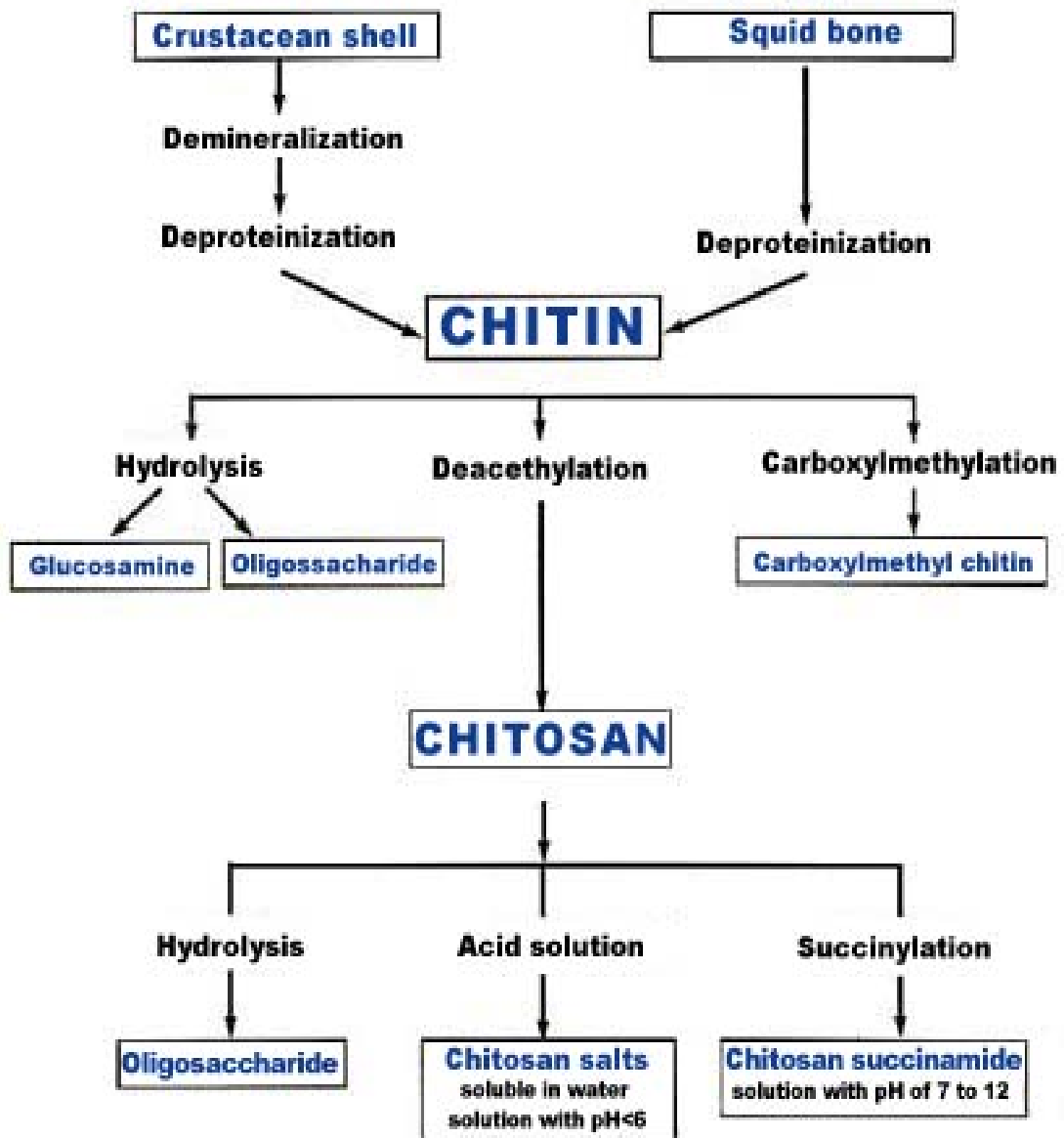


Figure 2.6. Chitin and chitosan manufacturing process.

Total world production of chitin was estimated roughly to be 20,000 million tons in 1997. Production of chitin and chitosan in Asian countries was estimated to be around 3,000 million tons (Fig. 2.7.) (Chen, 2000).

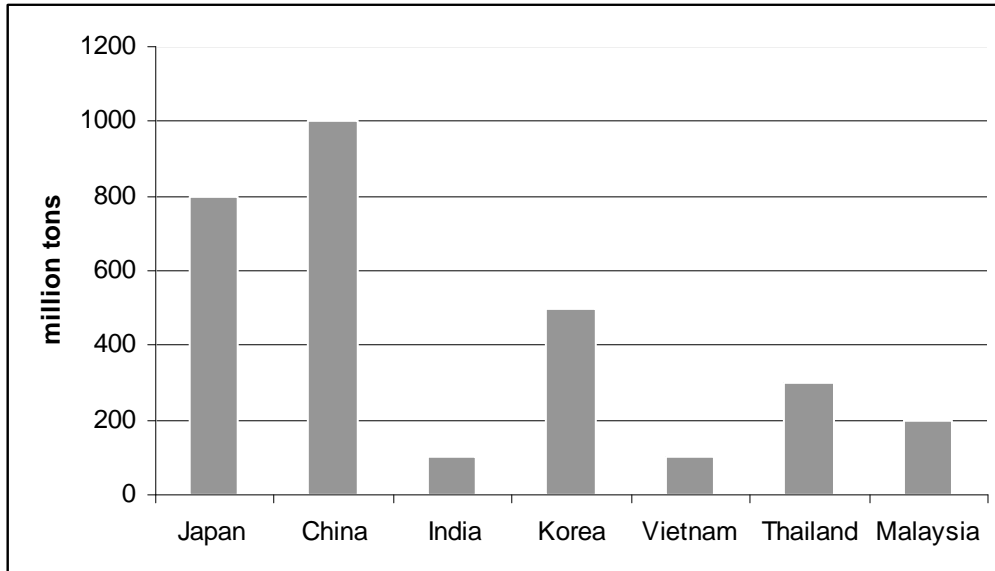


Figure 2.7. Production of chitin and chitosan in Asian countries (Chen, 2000).

2.5.2. Microbial Fermentation

The traditional source of chitin and chitosan is shellfish waste from shrimp, Antarctic krill, crab and lobster- processing. However, the industrial isolation of this polymer is reduced by the problems of seasonal and limited supply in some countries and environmental pollution while collecting large amounts of shell waste. Moreover, the conversion of chitin to chitosan, using a strong base solution at high temperature, causes variability of the product properties, decreases the chitosan quality and increases the processing costs. This also produces waste liquid containing base, proteins and protein degradation products (Synowiecki *et al.*, 1997).

Chitosan is also a natural component of cell walls of fungi belonging to Zygomycetes (e.g. *Absidia*, *Mucor*, *Rhizopus*, *Gongronella*) and can be produced by extraction from fungus cell walls. Therefore, the zygomycetes, has been considered as an alternative source of chitosan. This method is not yet used on an industrial scale, although it can provide an alternative to chemical deacetylation, independent of chitin production at the fishery. Also, there are several advantages of using these fungi to

produce chitosan. The most important is that the cell wall of zygomycetous fungi contains a large quantity of chitosan and the physicochemical properties of this chitosan can be manipulated and standardized by controlling the parameters of fermentation. For instance, chitosans of different molecular weights (MW) are produced by these fungi when they are grown on media comprising different pH and composition. Fungi are also easily cultured using relatively simple nutrients. As a result, chitosan can be produced in a controlled environment all year round and be independent of the seasonal shellfish industry (Tan *et al.*, 1996 and Jaworska *et al.*, 2001).

In recent years, chitin obtained by extraction from fungal mycelia is gaining importance. Fungal mycelia can be cultivated throughout the year by fermentation that is rapid, synchronized and can be organized in a closed or semi- closed technological circuit to comply with modern ecological requirement. In addition, fungal mycelia are relatively consistent in composition and are not associated with inorganic materials; therefore no demineralization treatment is required to recover fungal chitin (Teng *et al.*, 2001).

2.5.2.1. Microorganisms

Several yeast and filamentous fungi, for example, *Schizosaccharomyces pombe*, *Candidia albicans*, *Saccharomyces cerevisiae*, *Mucor rouxii*, *Phycomyces blakesleeanus*, *Colletotricum lindemuthianum*, *Absidia coerulea*, *Coprinus cinereus*, *Neurospora crassa*, *Trichoderma reesei*, *Rhizopus* spp., *Absidia* spp., *Mucor* spp., *Mortierella isabelina* and *Lentinus edodes* have been reported containing chitin and chitosan in their cell wall and septa so they are alternative sources of chitin and chitosan. And they can also be readily cultured in simple nutrients and used as an alternative source of chitosan (Synowiecki *et al.*, 1997 and Suntornsuk *et al.*, 2002).

Especially best chitosan producing fungi that are, *Absidia* spp., *Gongronella* spp., *Mucor* spp., *Rhizopus* spp., *Lentinus edodes*, *Cunninghamella echinulata*, *Aspergillus niger*, *Zygorhynchus moelleri*, *Candida albicans*, *Zygosaccharomyces rouxii* were used in research (Synowiecki *et al.*, 1997).

2.5.2.2. Raw Materials

The cells are readily cultured in a medium containing a carbon source such as glucose, dextrose, a high concentration of an ammonium salt, trace quantities of mineral salts, yeast extract and by maintaining a pH of about 4.5 (McGahren *et al.*, 1984).

Generally, synthetic medium containing glucose (20g), peptone (10g), yeast extract (1g), $(\text{NH}_4)_2\text{SO}_4$ (5g), K_2HPO_4 (1g), NaCl (1g), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.5g), $\text{Ca}_2\text{Cl}_2 \cdot 2\text{H}_2\text{O}$ (0.1g) in 1L deionized water or yeast extract (0.2%), peptone (1%), glucose (2%) or yeast extract (1%), peptone (3%), dextrose (0.4%) and BASF pluronic L61 antifoam (0.01%) was used for chitosan production (White *et al.*, 1979, Shimara *et al.*, 1989, Tan *et al.*, 1996, Synowiecki *et al.*, 1997, Jaworska *et al.*, 2001).

Also some researchers used natural products or food industry by-products such as soybean and mungbean residues, ground corn and rice, shochu distillery waste water and sweet potato enriched with mineral salts because of their cheap costs (Yokoi *et al.*, 1998, Hang, 1990, Nwe *et al.*, 2001, Suntornsuk *et al.*, 2002). After preparing all media, pHs of media were adjusted to 4.4, 4.5 and 6.3 using HCl, H_2SO_4 .

Some industrial by- products such as molasses or whey can be used as an inexpensive carbon source to grow fungi. This may also help to alleviate some environmental pollution concerns (Tan *et al.*, 1996).

Molasses is the by-product of sugar industry. It contains a high concentration of sucrose, it is cheap and abundant. It is used as a substrate in the production of baker's yeast, citric acid, lactic acid and acetone. The composition of beet molasses is tabulated in Table 2.4.

Table 2.4. The composition of Turkish beet molasses (Gökşungur, 1998 and Üzelyalçın, 2002).

Brix	85.50
Dry weight, %	84.31
Humidity, %	15.69
Total sugar, %	54.89
Inverted sugar, %	0.40
Ash, %	5.18
Nitrogen, %	2.14
Protein (N x 6.25), %	13.38
Protein (Lowry), %	7.81
Formol nitrogen, %	0.42
pH	7.45
Ca (mg / kg molasses)	3480.5
Cu (mg / kg molasses)	2.84
Mg (mg / kg molasses)	129.8
Zn (mg / kg molasses)	5.96

Whey is the liquid by-product of cheese manufacturing. Approximately 1-2 kg of cheese is produced from 10 kg of milk and the residual liquid is the whey. The composition of whey varies with cheese manufacture procedures. The compositions of liquid and dried whey are given in Table 2.5. and 2.6.

Table 2.5. Gross composition of liquid and dried whey (Nutting 1970, Irvine and Hill 1985, Atkinson and Mavituna 1991).

Component	Fluid Whey	Dried Whey
Total solids, %	6.35-7	96.3-96.5
Protein, %	0.8-0.9	13.0-13.1
Lactose, %	4.85-5.1	68.0-75.0
Fat, %	0.3	0.8-1.0
Lactic Acid, %	0.05	0.2
Ash, %	0.5-0.6	7.3-9.6

Table 2.6. Amino acid, vitamin and mineral content of whey powder (Atkinson and Mavituna 1991, Irvine and Hill 1985).

Amino acids		Vitamins	Minerals
L-Arganine	0.4 %	Biotin 0.4 mg kg ⁻¹	P 0.66%
L-Cystine	0.4 %	Choline 2420 mg kg ⁻¹	Ca 0.77%
L-Glycine	0.7 %	Nicotinic acid 11.0 mg kg ⁻¹	K 2.54%
L-Histidine	0.2 %	Pantothenic acid 48.5 mg kg ⁻¹	Mg 0.12%
L-Isoleucine	0.7 %	Pyridoxine 2.9 mg kg ⁻¹	Na 0.83%
L-Leucine	1.2 %	Riboflavin 19.8 mg kg ⁻¹	
L-Lycine	1.0 %	Thiamin 4.0 mg kg ⁻¹	
L-Methionine	0.4 %		
L-Phenylalanine	0.5 %		
L-Threonine	0.6 %		
L-Tryptophan	0.2 %		
L-Tyrosine	0.5 %		
L-Valine	0.6 %		

2.5.2.3. Fermentation Process

Fungal chitosan is produced from fungi cultivated in either submerged or solid-state cultures; the first method is more common (Jaworska, *et al.*, 2000).

The yield of chitosan from a fungal mass, or from a unit of culture medium, depends on several factors including: the strain of fungi used, cultivation method (shaking culture, batch culture, continuous culture, solid-state culture), and cultivation parameters (pH, temperature, mixing rate, length of cultivation). An increase in the chitosan yield can be obtained either by increased biomass yield or by an increase in the cell wall content of chitosan (Jaworska *et al.*, 2001).

Table 2.7. Some of the chitosan fermentation studies.

Microorganisms	Fermentation Type	Fermentation Medium	Fermentations Parameters	Reference
<i>Absidia coerulea</i>	SMF	Synthetic medium		McGahren <i>et al.</i> , 1984
<i>Mucor rouxii</i>	SMF	Synthetic medium	25 °C, 125 rpm	Arcidiacono <i>et al.</i> , 1992
<i>Mucor rouxii</i>	SMF	YPG	28 °C	Synowiecki <i>et al.</i> , 1997
<i>Gongronella butleri</i>	SSF SMF	Sweet potato medium	28 °C 28 °C, 200 rpm	Nwe <i>et al.</i> , 2001
<i>Mucor rouxii</i>	SMF	Synthetic medium	28 °C, 400 rpm	White <i>et al.</i> , 1979
<i>Absidia butleri</i>	SMF	Synthetic medium	24 °C, 150 rpm	Shimahara <i>et al.</i> , 1988
<i>A. niger</i> <i>R. oryzae</i> <i>Z. rouxii</i> <i>C. albicans</i>	SSF	Soybean and Mungbean residues	30 °C	Suntornsuk <i>et al.</i> , 2002
<i>Lentinus edodes</i>	SSF SMF	Wheat straw Synthetic medium	28 °C 28 °C, 400 rpm	Crestini <i>et al.</i> , 1996
<i>R. oryzae</i> <i>R. arrhizus</i> <i>R. microsporus</i> <i>R. stolonifer</i> <i>R. oligosporus</i> <i>Absidia glauca</i> <i>Mucor sp</i> <i>M. hiemellis</i> <i>Zygorhynchus moelleri</i> <i>Cunninghamella echinulata</i> <i>Gongronella butleri</i>	SMF	Synthetic medium	25 °C, 200 rpm	Tan <i>et al.</i> , 1996
<i>Rizopus oryzae</i>	SMF	Corn and rice medium	30 °C 240 rpm	Hang, 1990

Suntornsuk *et al.* (2002), used four fungal strains on both soybean and mungbean residue for chitosan by solid state fermentation. And 10^7 cells/ml suspension of *Rhizopus oryzae* TISTR 3189 produced the highest yield of chitosan when inoculated on soybean residue after 3 days of cultivation (4.3 g chitosan / kg substrate).

Shimahara *et al.* (1989), demonstrated that chitosan yields from 43 strains of *Mucor* species ranged from 65 to 215 mg / l in a complex medium and also, the amounts of extractable chitosan from 2 days old and 4 days old cultures of *Absidia butleri* HUT 1001 were found to be 0.8 to 0.9 and 0.9 to 1 g / l, respectively. They were about 14 and 11 % on dry cell weight bases. Jar-fermentor cultivation of *Mucor rouxii* ATCC 24905 reported by White *et al.* (1979) produced from 9 to 14 g / l of dry cells, of which 4 to 8 % was extractable chitosan.

In another study, Crestini *et al.* (1996) used *Lentinus edodes* to produce chitosan by solid state and submerged fermentation. *Lentinus edodes* was grown in a 25 l stirred tank bioreactor at 28° C with a synthetic medium without air flow. The maximum dry cell biomass reached 3 g / l at 9 days and maximum chitosan amount was 120 mg / l at 12 days. McGahren *et al.* (1984) found that *Absidia coerulea* mycelia harvested at the end of the active growth phase gave a lower yield because chitosan was more difficult to solubilize. Nwe *et al.* (2001) reported that, *Gongronella butleri* USB 0201 gave maximum biomass 56.3 g / kg sweet potato after 3.5 day incubation using 1.8×10^9 spores / kg by submerged fermentation and chitosan yield 9.2 % of biomass. Hang (1990) used 2×10^7 viable spores of *Rhizopus oryzae* on corn and rice media for producing chitosan. The maximum chitosan concentration was obtained at 72nd hour, that were 460 and 700 mg / l, respectively. Tan *et al.* (1996) demonstrated that, biomass concentration of thirteen strains of Zygomycetes, inoculum concentration of 1.8×10^6 spores / ml, were found range from 4.4 to 8.15 g / l.

2.5.2.4. Extraction of Chitosan

The extraction process in obtaining chitosan from fungal cell wall is simpler and milder than the chemical process used to obtain chitosan from crustacean shells, and produces less waste materials. Most studies on the screening of the fungal chitosan content typically harvest the mycelia at a fixed incubation period. This is because chitosan is more difficult to extract from fungi after their active growth phase. However, this disregards the differences in the growth rate of different fungi.

Furthermore, there are brief reports that allude to the late exponential phase as the most likely phase to give the highest yield of chitosan (Tan *et al.*, 1996).

2.6. Characterization of Chitosan

The chitosans separated from the fungal biomass were characterized by two parameters: DD (degree of deacetylation) and MW (molecular weight) (Jaworska *et al.*, 2001). Physicochemical properties of chitosan isolated directly from a fungus may be manipulated by control of fermentation and processing parameters. Additional control through genetic manipulation of the fungal system may also be possible in the future. The list of applications for chitosan is extensive, including adhesives, food processing, paper and textile additives, wound healing accelerants, and waste water treatment. For these and other potential applications, control of physicochemical characteristics (*e.g.*, weight-averaged molecular weight and degree of deacetylation) is often critical due to effects on mechanical and chemical properties when chitosan is processed into films, powders, and fibers (Arcidiacono *et al.*, 1992).

2.6.1. Degree of Deacetylation

The chitin conversion process results in the production of chitosans with low degrees of deacetylation. Recent advances in fermentation technology suggest that many of these problems can be overcome by culturing chitosan-producing fungi (Crestini *et al.*, 1996).

The properties of chitin as well as chitosan depend greatly on the extent of deacetylation, which is one of the most important parameters to examine closely is the degree of N- acetylation in chitin, i.e. the ratio of 2- acetamido- 2- deoxy- D- glucopyranose to 2- amino- 2- deoxy- D- glucopyranose structural units for specifying chitin and chitosan. This ratio has a striking effect on chitin solubility and solution properties. Chitosan is the universally accepted non-toxic N-deacetylated derivative of chitin, where chitin is N- deacetylated to such extent that it becomes soluble in dilute aqueous acetic and formic acids. In chitin, the acetylated units prevail (degree of acetylation typically 0.90). Chitosan is the fully or partially N- deacetylated derivative of chitin with a typical degree of acetylation of less than 0.35.

It is therefore critical to determine the degree of deacetylation as accurately and simply as possible. Many methods have been reported for assessing the degree of deacetylation including elemental analysis, hydrolysis of acetamide groups, titration of free amine groups, dye adsorption, spectroscopies such as IR, UV, CD, and NMR, enzymatic degradation, and pyrolysis. Compared to other methods reported, IR spectroscopy is a convenient way for determining the degree of deacetylation (Kumar, 2000, and Kurita, 2001).

2.6.2. Molecular Weight

Chitosan molecular weight distributions and the weight- average molecular weight (M_w) of chitin and chitosan can be determined by using HPLC and light scattering. Viscometry is a simple and rapid method for determination of molecular weight. Converting chitin into chitosan lowers the molecular weight, changes the degree of deacetylation, and thereby alters the charge distribution. The weight – average molecular weight of chitin is between 1.03×10^6 and 2.5×10^6 Da, but the N-deacetylation reaction reduces this to 1×10^5 to 5×10^5 Da (Kumar, 2000).

CHAPTER 3

MATERIALS AND METHODS

3.1. Materials

Molasses samples were supplied from Pakmaya Yeast Factory (İzmir, Turkey) and stored at +4° C. Sucrose concentration of molasses was 45%. Different concentration of molasses were added to the fermentation broth in order to obtain the desired initial sucrose concentration. The elemental composition of molasses used as a medium in this study is given in Table 3.1.

Table 3.1. Trace element composition of molasses

Trace Elements	Concentrations (mg / kg molasses)
Na	3300,3
K	11000
Ca	3160
Mg	81,6
Al	48,9
Fe	32,6
Mn	10,2
Ni	2,2
Pb	1,7
P	1,3
Zn	6,35
Cu	1,33

Whey powder samples were supplied from Pınar Dairy Products, Inc. (İzmir, Turkey) and kept at room temperature. Lactose concentration of whey powder was approximately 60%. Amount of whey powder added to the fermentation broth was adjusted to obtain the desired initial lactose concentration of the medium.

Microorganisms used in this work, *Absidia spp* TEM, *Aspergillus niger* TEM, *Cunninghamella elegans* CECT 2123, *Rhizopus arrhizus* NRRL 2286, *Mucor rouxii* CECT 2655, were obtained from CECT (Colección Española de Cultivos Tipo),

NRRL (United States Department of Agriculture, National Center for Agricultural Utilization Research) and Ege University, Basic Industrial Microbiology Department. The fungi were supplied in lyophilized form and activated in the propagation medium.

Deionized and bidistilled water were used in all preparations.

The chemicals and their producers are listed in Table 3.2.

Table 3.2. Chemicals and their producers.

Chemicals	Producer	Product number
Yeast extract	Acumedia	7184 A
Peptone	Applichem	A 2210, 0500
Sodium hydroxide	Sigma	S 5881
Glucose	Sigma	G 7528
Sulfuric acid	Fluka	84716
Magnesium sulphate heptahydrate	Fluka	63138
Sodium chloride	Sigma	S 7653
Calcium chloride	Sigma	C 5426
Ammonium sulphate	Sigma	A 2939
Potassium hydrogen bisulphate	Sigma	P 5035
Sucrose	Acumedia	7276
Lactose	Sigma	L 3625
Potato Dextrose Agar	Merck	1.10130

3.2. Methods

3.2.1. Culture Conditions

The lyophilized fungal strains were activated on Potato Dextrose Agar (PDA) medium. All cultures were maintained at 4 °C on PDA plates. Whenever required, all cultures were subcultured on PDA tubes, incubated at 30 °C and exposed to blacklight to stimulate sporulation. The cultures were allowed to grow for 7 days for spore formation. The spore stocks were prepared by washing 7-day-old tubes with 0.5 % Sodium-laurile sulphate solution and spores were counted by using hemocytometer. Then spore suspension was inoculated to the propagation medium.

3.2.2. Growth of Fungal Biomass in Shake Flasks

The complex medium 1 was used to screen the best biomass producing fungi. Then, several different media were examined to increase biomass amount of *Mucor rouxii*. The media used in the experiments are shown in Table 3.3. After preparing all media components, pH of the broth was adjusted to 4.5 with H₂SO₄. Erlenmeyer flasks of 100 ml containing 40 ml of production medium were used for shake flask fermentations. The fermentation medium was sterilized at 121 °C for 15 minutes. The spore suspensions prepared from a 7-day-old slant were inoculated 10⁶ spores per flasks (2.5 x 10⁴ spores / ml) into sterilized media. The tops of the flasks were covered with aluminium foil. Fermentations were carried out in a temperature controlled flask shaker (Gerhardt) operated at 180 rpm, 28 °C for 7 days (Synowiecki *et al.*, 1997). Unless otherwise stated, the experiments were performed by using two flasks to be removed at each day of fermentation and flask components were analysed to determine the biomass, sucrose and protease concentrations. The details of analyses are given in Section 3.2.5.

Table 3.3. Media used in the shake flask fermentations.

Medium	Components
Complex Synthetic Medium 1	2 % glucose 1 % peptone 0.1 % yeast extract 0.5 % (NH ₄) ₂ SO ₄ 0.1 % K ₂ HPO ₄ 0.1 % NaCl 0.05 % MgSO ₄ .7H ₂ O 0.01 % CaCl ₂ .2H ₂ O
YPG (Yeast extract, peptone, glucose)	2 % glucose 0.2 % yeast extract 1 % peptone
Molasses (M)	2 % sucrose

Whey (W)	2 % lactose
Molasses, Yeast extract, Peptone (YPM)	2 % sucrose 0.2 % yeast extract 1 % peptone
Whey, Yeast extract, Peptone (YPW)	2 % sucrose 0.2 % yeast extract 1 % peptone
Complex Medium 2	2 % sucrose 1 % peptone 0.1 % yeast extract 0.5 % (NH ₄) ₂ SO ₄ 0.1 % K ₂ HPO ₄ 0.1 % NaCl 0.05 % MgSO ₄ .7H ₂ O 0.01 % CaCl ₂ .2H ₂ O
Complex Synthetic Medium 3	2 % lactose 1 % peptone 0.1 % yeast extract 0.5 % (NH ₄) ₂ SO ₄ 0.1 % K ₂ HPO ₄ 0.1 % NaCl 0.05 % MgSO ₄ .7H ₂ O 0.01 % CaCl ₂ .2H ₂ O
Molasses, Yeast extract (YM)	2 % sucrose 0.2 % yeast extract
Molasses, Peptone (PM)	2 % sucrose 1 % peptone

3.2.2.1. Effect of Mineral Salts

In order to determine the effect of each salt, one of the salts was discarded from the formulation and fermentations were carried out for each salts at 28 °C, 180 rpm.

3.2.2.2. Effect of Inoculum Spore Concentration

In order to determine the amount of spore inoculum concentration on biomass production, shake flasks containing 0.2 % yeast extract, 1.0 % peptone, 2 % sucrose from molasses were used. Inoculum concentrations used were 0.025×10^1 , 10^2 , 10^3 , 10^4 , 10^5 , 10^6 , 10^7 , 10^8 spores / ml at 28 °C, 180 rpm.

3.2.2.3. Effect of Production Medium Volume

In order to determine the effect of medium volume, the shake flasks containing 2 % sucrose, 0.2 % yeast extract, 1% peptone medium were used. The medium volume used were 10, 20, 30, 40 and 50 ml and spore inoculum concentration was 2.5×10^4 spores / ml for the fermentation studies performed at 28 °C, 180 rpm.

3.2.2.4. Effect of Initial Sucrose Concentration

Fermentations were carried out at 28 °C, 180 rpm in the shake flasks containing 1, 2, 3, 4, 5 % sucrose, 0.2 % yeast extract, 1% peptone.

3.2.3. Chitosan Fermentation in Fermenter

Mucor rouxii which gave the maximum biomass was used in fermenter experiments. Continuous stirred tank bioreactor (Bioengineering, type ALF) with a working volume of 3 L was used during the experiments. The heating jacket around the fermentation tank provided the temperature control. Agitation was provided by a magnetic stirrer. The system was equipped with temperature, pH, agitation speed controllers, aeration and a computer controlled by software, FERM. The medium components were sterilized together with the vessel at 121°C, 15 minutes. The fermentation conditions and aeration rate were 28 °C, 200 rpm, 2.5×10^4 spores / ml and

2 vvm (vol/ vol/ min). The medium used for the growth of *Mucor rouxii* in the fermenter was YP molasses medium (4% sucrose, 0.2% yeast extract, 1% peptone).

3.2.4. Chitosan Extraction

The chitosan from mycelia was isolated according to the flowsheet given in Figure 3.1. In this process, cells were harvested from submerged fermentation broth by vacuum filtration and the washed mycelia was homogenized in the blender at high speed rate. Then, mycelia were autoclaved at 121 °C, in 1M NaOH (30:1, v/w) solution for 15 minutes to remove proteins. Alkali insoluble fraction (AIF) was recovered after centrifugation, alternative washing with water and ethanol. The residue was separated from the precipitate and extracted in 2% acetic acid (40:1, v/w) at room temperature for 30 minutes with frequent shaking and the residue was washed once with deionized water. All supernatants were combined and the mixture was adjusted to pH 8.5 with NaOH and then centrifuged. The residue was washed and dried in oven and this fraction was identified as chitosan (Shimahara *et al*, 1989, Crestini *et al.*, 1996, and Synowiecki *et al.*, 1997).

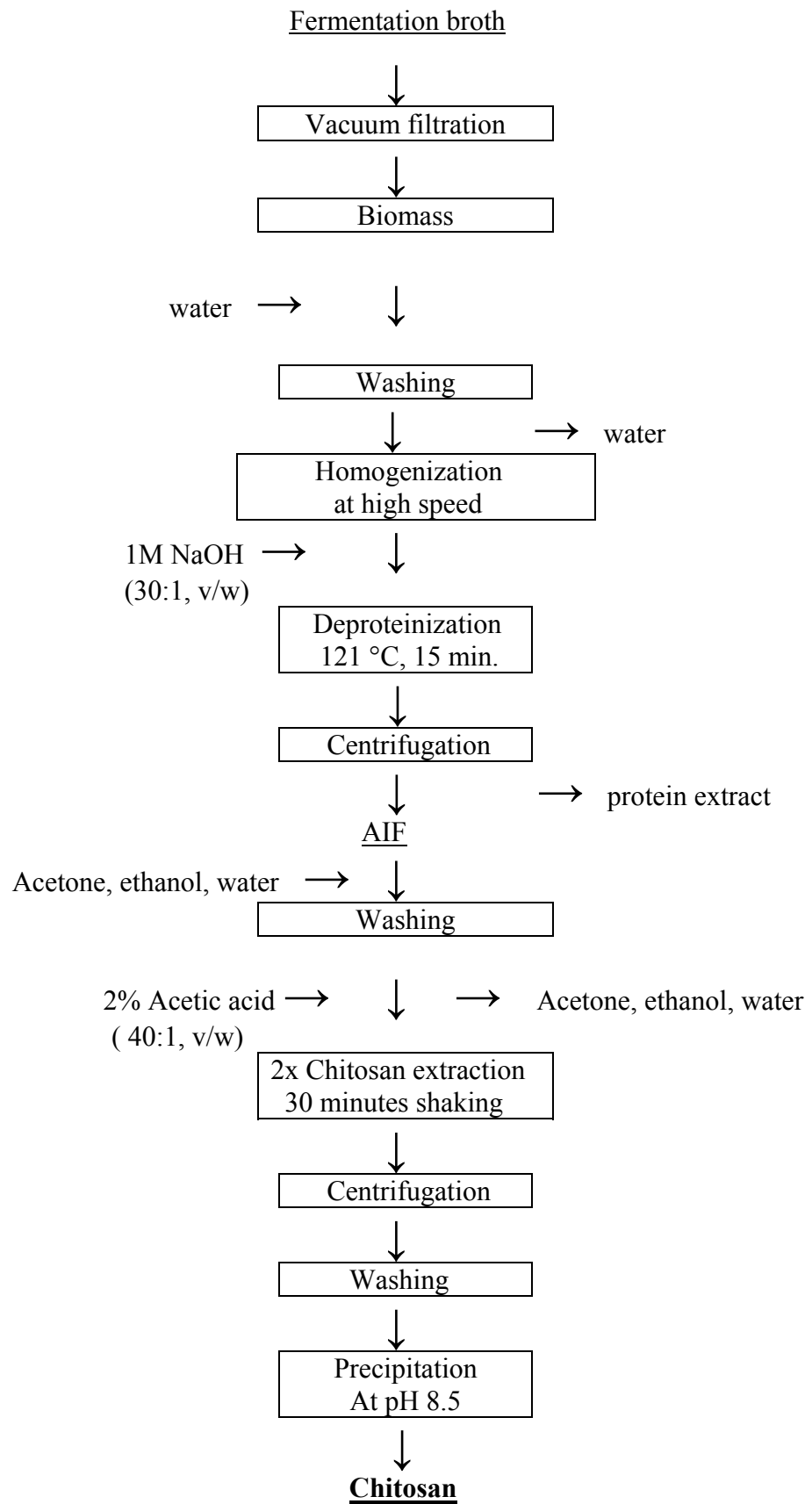


Figure 3.1. Flowsheet for the extraction of chitosan.

3.2.5. Analyses

3.2.5.1. Dry Cell Weight Measurement

Fungal biomass was collected by vacuum filtration using coarse filter paper and twice washed with distilled water. The solid fraction which contains cell biomass was dried overnight at 60 °C until constant weight. Biomass concentrations were expressed as dry weight (g / l) in all experimental procedure and results.

3.2.5.2. Sucrose and Lactose Analyses

The sucrose and lactose concentrations in molasses and whey were measured by using Shimadzu 2450 UV-VIS spectrophotometer. Carbohydrate content of the samples were determined using their calibration curves. For calibration curves, different concentration of pure sucrose and lactose were used and absorbances were measured at 490nm. Calibration curves for sucrose and lactose are shown in Figure E.2 and E.3 in Appendix E.

Sucrose content of supernatant was determined by HPLC. The HPLC system was composed of Perkin Elmer Series 200 pump, Series 200 refractive index detector, Series 900 interface and a computer. The system was controlled by the software, Turbochrom Navigator.

The HPLC analyses were done under isocratic conditions using an Aminex HPX- 87H cation exchange column (Bio – Rad Laboratories). The column temperature was maintained at 45 °C with a MetaTherm column oven. The column was eluted with 5 mM H₂SO₄ at a flow rate of 0.6 ml min⁻¹ for 15 minutes. Under these conditions retention times for sucrose 7.5 minutes. The properties of the HPLC and the analysis conditions and properties are given in Table 3.4.

Table 3.4. The properties of the HPLC column and analysis conditions.

Property	Values or Attributes
Column	Aminex HPX 87H ion exclusion column
Column length	300 mm
Column diameter	7.8 mm
Particle size	9 μm
Guard cartridge	Micro-Guard cation-H cartridge (30x4.6mm)
Column cleaning solvent	5 % CH_3CN in 5mM H_2SO_4 , 30 % CH_3CN in 5mM H_2SO_4
Mobile phase	5 mM H_2SO_4
Flow rate	0.6 ml min^{-1}
Temperature	45 $^\circ\text{C}$
Detector	Refractive index

Calibration standard was prepared from analytical grade chemicals. Calibration curve is shown in Figure E.1. in Appendix E. The R- squared value for sucrose calibration curve was greater than 0.999.

3.2.5.3. Metal Ions Determination in Molasses

The concentration of metal ions in molasses that was used for fermentation medium was determined by Inductively Coupled Plasma – Atomic Emission Spectrometry (Varian, ICP-AES, Axial Liberty). 0.5 gram of homogenized molasses sample was placed into test tube. Next, 1ml HNO_3 about 65 % in concentrated form and 1ml deionized water were added into test tube. It was placed in a water bath and heated at 80 $^\circ\text{C}$ overnight. The following day digests were treated with 30 % H_2O_2 , added dropwise and heated at 100 $^\circ\text{C}$ for several hours, repeating the H_2O_2 treatment until sample digests were clear. The digests were heated again overnight at 80 $^\circ\text{C}$. Then 1ml of 37 % HCl was added and the digests were heated for 3-4 hours. The digests were filtered through ashless 7 cm (D) Whatman No: 41 filter paper and they were diluted to a final volume of 50 ml (Miller, 1996). The wavelengths used for ICP measurements are tabulated in Table 3.5. and the instrument operating conditions of ICP-AES are described in Table 3.6.

Table 3.5. The wavelengths used for elemental analyses by ICP.

Element	Wavelength (nm)
Ca	317.933
Cu	324.754
Fe	259.940
K	766.490
Mg	279.079
Mn	257.610
Na	588.995
Ni	231.604
Zn	213.856

Table 3.6. ICP-AES (Axial Liberty) operating conditions.

Argon gas flow	15 l / min
Argon auxiliary flow	1.50 l / min
PMT voltage	650 V
Sample uptake	30 sec
Rinse time	10 sec
Spray chamber	Glass cyclonic
Nebulizer	Glass nebulizer

3.2.5.4. Enzyme (protease) Assay

Protease activity was measured using casein as substrate. To 1 ml of 2 % casein in 50 mM glycine – NaOH buffer, pH 10, 1 ml diluted enzyme was added and incubated at 40 °C. After 20 min the reaction was terminated by adding 2 ml of 10 % trichloroacetic acid solution and centrifuging at 5000g for 10 min. To 0.5 ml of supernatant 2.5 ml of 0.5 M sodium carbonate was added, followed by 0.5 ml 1N Folin – Ciocalteu’s phenol reagent. After 30 min incubation at room temperature absorbance was measured at 660 nm against an appropriate reagent blank. One unit of protease activity was defined as the amount of enzyme which released 1 µg of amino acid equivalent to tyrosine per min under the above assay conditions (Gessesse, 1997).

CHAPTER 4

RESULTS AND DISCUSSION

In this chapter, experimental results on batch fermentation of fungi, selection of the best chitosan producing fungus were presented together with the emphasis on several effects on biomass growth. Both the shake flask experiments and fermentor studies were presented. Experimental results included selection of the best biomass producing fungus, production medium, initial spore inoculum concentration, production medium volume and sucrose concentration to extract maximum amount of chitosan.

All the experiments were done in duplicates at least and mean values were reported. Fermentations were evaluated according to their biomass concentration. In every section the biomass concentrations were shown in charts. Generally, the optimum values determined in experiments or other type of results were evaluated and used for the subsequent experiments.

4.1. Growth Kinetics of Different Fungal Strains Produced on Complex Medium 1

The growth kinetics of different fungal strains were investigated on complex medium 1 by submerged fermentation at 28 °C, 180 rpm. The inoculum spore concentrations were 2.5×10^4 spores / ml.

Dry cell biomass concentrations of different fungal strains are shown in Figure 4.1. All strains grew well on synthetic medium and growth reached to a maximum after 96 hours of cultivation and then remained nearly constant and declined. As seen from the figure, *Mucor rouxii* adopted the medium easily and reached maximal value for each day among them. The maximum biomass concentrations obtained were 7.6, 5.8, 8.1, 8.5 and 9.52 g / l for *Absidia*, *Rhizopus arrhizus*, *Cunninghamella elegans*, *Aspergillus niger* and *Mucor rouxii*, respectively.

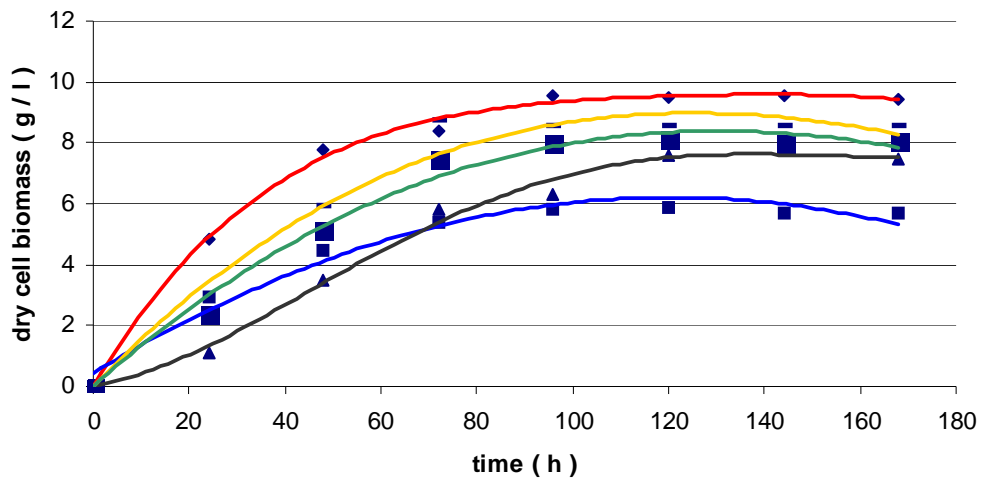


Figure 4.1. Growth kinetics of different fungal strains.

(◆ *Mucor rouxii*; ■ *R. arrhizus*; ▲ *Absidia*; - *A. niger*; ■ *C. elegans*)

The individual growth curves for each fungus are presented in Appendix A. In this study, the highest dry cell biomass was obtained by *Mucor rouxii* (9.52 g / l) and it seemed to be the most suitable fungus for chitosan production. There have been several studies in the literature for chitosan fermentation. The values obtained for dry cell biomass were found to be in the range of approximately 3 to 6 g / l for different fungus strains. For example, Synowiecki *et al.* (1997) reported that weight of dry mycelia was about 4 g / l. Although the growth parameters were similar (28 °C, 170 rpm) with this study, the growth medium they used was different (YPG).

A few studies could be found in the literature related with the chitosan production from *Mucor rouxii* biomass. Tan *et al.* (1996) found that, biomass concentration of *Rhizopus arrhizus* and *Mucor* spp. were 4.85, 5.85 g / l, respectively. In another study, *Lentinus edodes* was grown under submerged cultivation and after 9 days of cultivation the concentration of dry cell biomass reached maximal value of 3 g / l (Crestini *et al.*, 1996). The biomass concentration obtained for *Mucor rouxii* in this study was found to be the highest among the other research.

For chitosan manufacture by fermentation, strain producing large amount of biomass is preferable. Since chitosan is a cell wall material and the amount of chitosan is related with amount of biomass. Therefore, *Mucor rouxii* having the highest biomass is used for further fermentation studies yielding to maximum chitosan concentrations.

4.2. Effect of Medium Composition and Mineral Salts on Growth of *Mucor rouxii*

The cultivation profiles of *Mucor rouxii* on Complex Medium 1 and YPG medium are shown in Figure 4.2. The growth curves showed different trend when the media composition was changed.

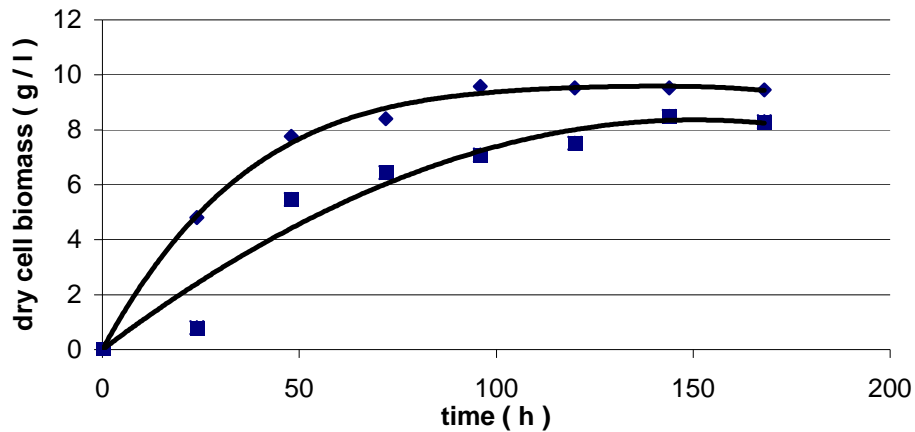


Figure 4.2. Growth of *Mucor rouxii* on Complex Media 1 vs YPG.

(◆ Growth on complex medium 1, ■ Growth on YPG)

As seen from the Figure 4.2. biomass concentrations increased up to almost 96 and 144 hours and they were 9.5 and 8 g / l for Complex Medium 1 and YPG Medium, respectively. Similar to the results of Arcidiacono *et al.* (1989), the biomass yield of *Mucor rouxii* increased rapidly up to 48 hours of growth. However, the final density of the culture reached during this time was about 4 g / l dry mycelia. According to the study of White *et al.* (1979), dry cell weight of *Mucor rouxii* reached 8 to 14 g / l after 12 and 50 h of incubation in the fermenter, perhaps due to the existing differences in aeration of the samples. These growth times having maximum dry cell weights were significantly shorter than our results.

The maximum biomass concentration was obtained when Complex Medium 1 was used as the fermentation medium. The growth was also faster than that of YPG medium. This also may be due to the Complex Medium 1 composition; as it contained mineral salts for the growth requirements of *Mucor rouxii*. To investigate the most effective salt in the medium; experiments were conducted with Complex Medium 1 and at each run, one of the salts were discarded from medium to be able to understand which

one of the salt or salts are the most effective on the biomass growth. The individual growth curves for each media are presented in Appendix B. The growth kinetics of *Mucor rouxii* on Complex Medium 1 without $(\text{NH}_4)_2\text{SO}_4$ and K_2HPO_4 salts are shown in Figure 4.3. as an example; the effect of all salts are summarized in Figure 4.4.

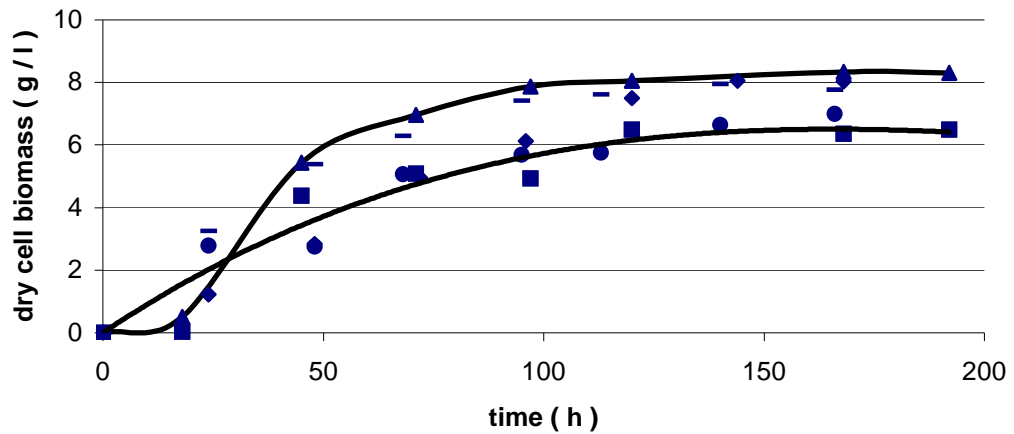


Figure 4.3. Effect of mineral salts on growth.

(♦ Complex medium 1 without NaCl , ▲ Complex medium 1 without $(\text{NH}_4)_2\text{SO}_4$
 ■ Complex medium 1 without K_2HPO_4 , ● Complex medium 1 without MgSO_4 ,
 - Complex medium 1 without CaCl_2)

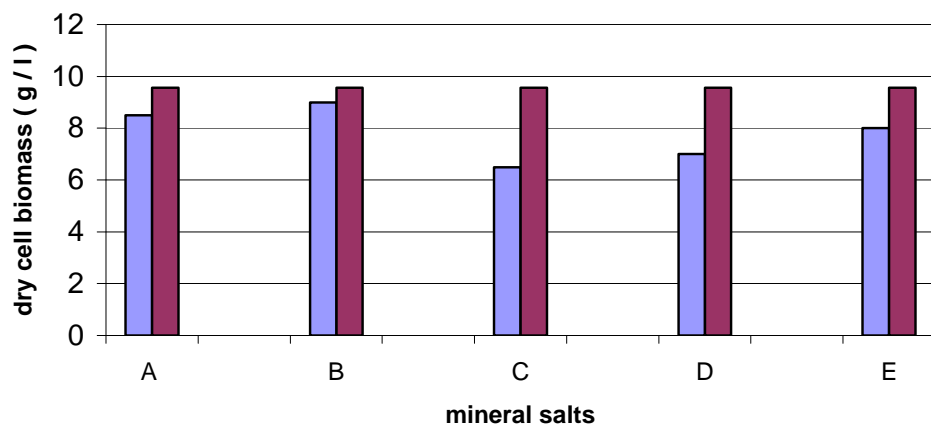


Figure 4.4. Effect of mineral salts on growth at stationary phase.

(A ; Complex Medium 1 without NaCl + Complex Medium 1
 B ; Complex Medium 1 without $(\text{NH}_4)_2\text{SO}_4$ + Complex Medium 1
 C ; Complex Medium 1 without K_2HPO_4 + Complex Medium 1
 D ; Complex Medium 1 without MgSO_4 + Complex Medium 1
 E ; Complex Medium 1 without CaCl_2 + Complex Medium 1)

The growth rate of *Mucor rouxii* was slower without the mineral salts of K_2HPO_4 and $MgSO_4$, and fermentation was prolonged up to 200 hours. Fermentation was completed within 96 hours when all the salts are present in the medium. Without $(NH_4)_2SO_4$, NaCl and $CaCl_2$ the stationary phase was obtained after 100 hours; but the maximum biomass concentrations were 8-9 g / l still less than the findings when the original medium was used (9.8 g / l). It can be summarized that all mineral salts are effective on growth but K_2HPO_4 and $MgSO_4$ are found to be the most effective ones. *Mucor rouxii* needed K^+ and Mg^{2+} ions for its metabolism.

4.3. Biomass Yields of *Mucor rouxii* on Different Food Industry By-products

Since the mineral salts are found to be effective on the growth of *Mucor rouxii*; different food industry wastes containing these salts were used for the fermentation medium.

Figure 4.5. and 4.6. show the comparison of biomass concentrations for *Mucor rouxii* using by-products and synthetic media. The cell dry biomass obtained for all media, ranged in between 1.5 to 10 g / l in molasses, whey, YPM, YPW, YM, PM, CM 2 and CM 3 media. The individual growth curves for each media are presented in Appendix C.

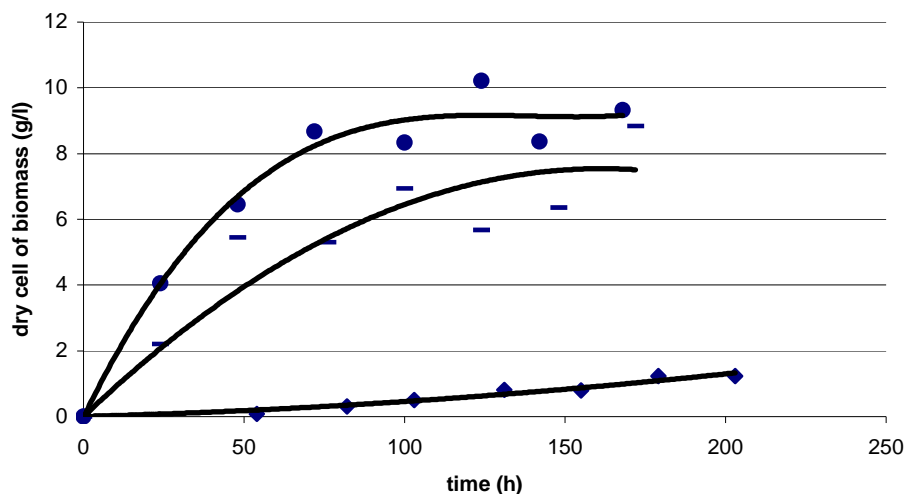


Figure 4.5. Growth curves of *Mucor rouxii* on different food industry by-products.

(♦ molasses , - yeast extract + molasses, ● yeast extract + peptone + molasses)

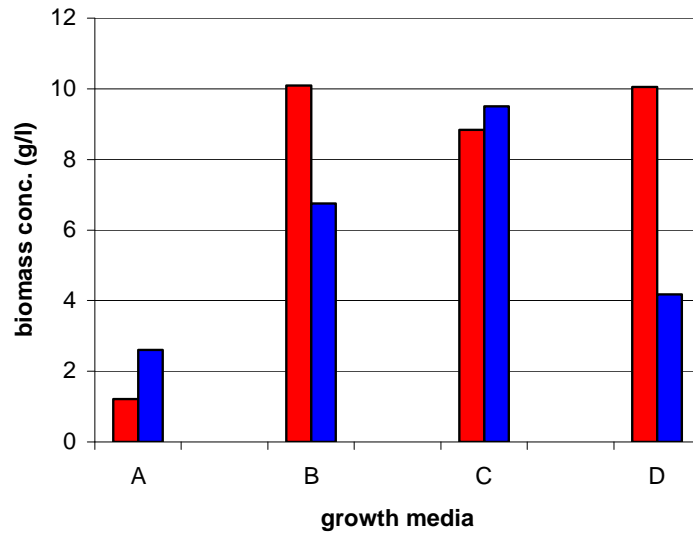


Figure 4.6. Effects of different food industry by- products on growth at stationary phase.

(A; molasses (M) + whey (W)

B; YPM + YPW

C; YM + PM

D; Complex Medium 2 + Complex Medium 3)

As seen from the figures, whey and molasses medium yielded to 1.0 and 2.5 g / l biomass concentrations, respectively. The highest growth was obtained when the molasses was added into YP medium and the stationary phase was observed in shorter periods such as 96 hours. In this case, the maximum biomass concentration obtained was approximately 10 g / l which was the highest among all media. When YP molasses medium was used, the similar trend obtained with those of the values of Complex Medium 1 (10 g / l biomass concentration). Complex Medium 1 contains salts and these salts are available in molasses, adding the mineral salts to the medium, the salts required can be supplied from molasses. This may have economical implications in case of industrial production of chitosan.

The differences in biomass concentrations for different medium compositions can be more easily seen in Figure 4.6. Since molasses and whey does not contain nitrogen sources other than minerals; the biomass concentrations were very low. When yeast extract and peptone (N source) was added to molasses and whey medium, the increase in biomass concentration is clearly seen. Therefore, for chitosan production from cell walls of *Mucor rouxii*, the carbon source, nitrogen source and mineral salts are

very effective. YP molasses is thought to be as the best medium for chitosan production since maximum biomass concentration can be obtained with it.

4.4. Effect of Spore Inoculum Concentration on Growth of *Mucor rouxii*

To determine the best spore inoculum concentration obtaining highest *Mucor rouxii*, 1 to 8 (0.025×10^x) spores / ml were inoculated to YPM medium at the same fermentation conditions. Figure 4.7. shows the effect of inoculum concentration on the growth of *Mucor rouxii*. The individual growth kinetic curves for each spore inoculum concentrations are presented in Appendix D.

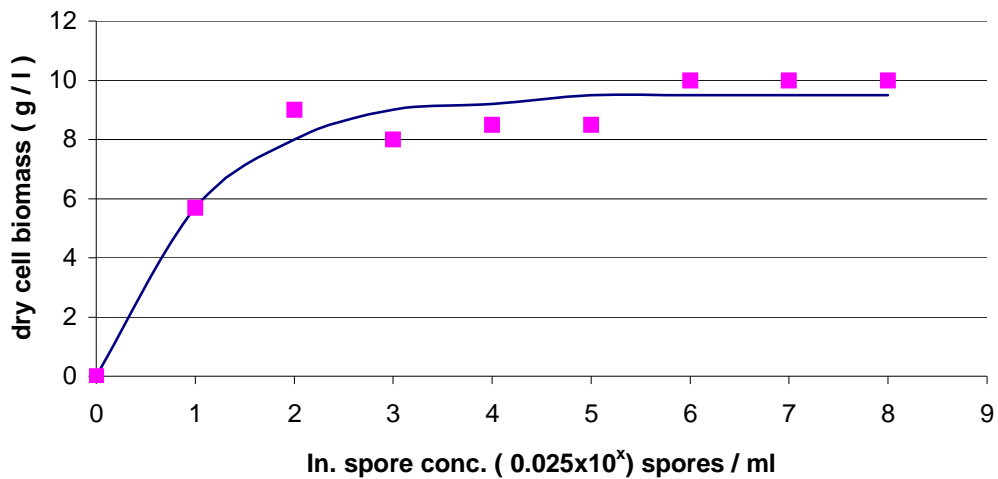


Figure 4.7. Effect of spore inoculum concentration on growth.

As seen from Figure 4.7., 2.5×10^4 spores / ml inoculum concentration gave the maximum dry cell biomass, approximately 9 g / l. Inoculating the medium with higher spore concentrations, yielded to similar biomass concentration in the study conducted by to Synowiecki *et al.* (1997), the amount of *Mucor rouxii* inoculum of 5×10^7 cells per 200 ml flask reached about 4 g / l dry mycelia at 48th hour. Their medium was YPG and found lower values.

In study of White *et al.* (1979), biomass concentration of *Mucor rouxii* in 14 liter fermenter reached almost 10 g / l by inoculating 5 % inoculum (v / v).

4.5. Effect of Production Medium Volume on Growth of *Mucor rouxii*

Production medium volume of 10, 20, 30, 40 and 50 ml were examined to obtain maximum biomass concentration and results are shown in Figure 4.8. The individual growth curves for each medium volume are presented in Appendix D.

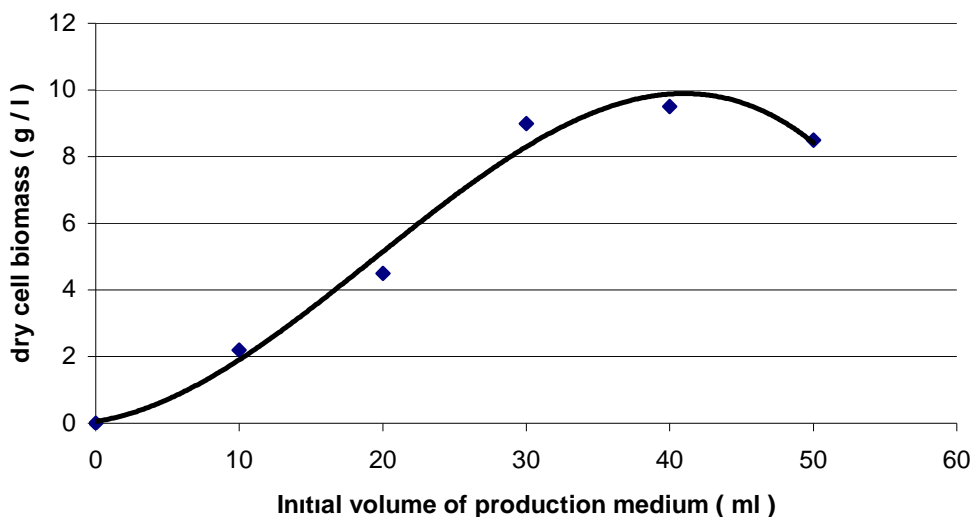


Figure 4.8. Effect of production medium volume on growth.

As seen from Figure 4.8., 40 ml production medium volume gave the maximum biomass concentration approximately 10 g / l because of the differences in oxygen transfer and substrate limitation. And similar results were obtained in a study conducted by Shimahara *et al.* (1989). They used 200 ml complex synthetic medium in 500 ml Erlenmeyer flask to obtain the highest biomass concentration.

Some researchers have used different medium volumes to obtain maximum biomass yields and hence chitosan concentrations. Arcidiacono *et al.* (1991) used, 750 ml YPG and BG medium to produce chitosan which gave 51.5 and 78 % biomass yield at 72nd hours. Synowiecki *et al.* (1997) reported that, 200 ml of liquid medium for fermentation of *Mucor rouxii* reached maximum biomass concentration about 4 g / l biomass concentration at 48th hour.

4.6. Effect of Initial Sucrose Concentration on Growth of *Mucor rouxii*

Effect of initial sucrose concentration on cell growth of *Mucor rouxii* was also investigated and results are presented in Figure 4.9. Growth kinetic curves belong to different initial sucrose concentrations can be seen in Appendix D.

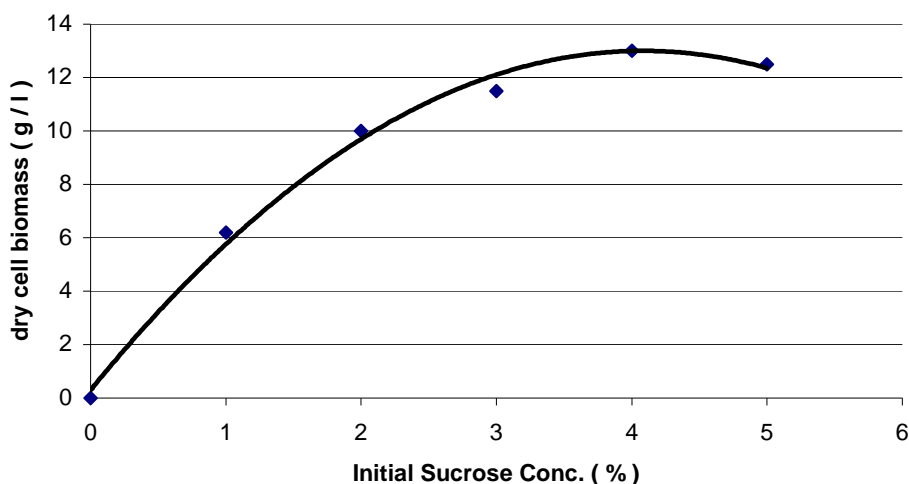


Figure 4.9. Effect of initial sucrose concentration on growth.

As seen from Figure 4.9., maximum biomass concentration of 13 g / l was obtained when 4 % sucrose was present into the YPM medium.

A linear approximation can be made for the sucrose concentrations below 2%. At higher carbohydrate concentrations, biomass growth reaches to a plateau slowly and reaches at its maximum value at 4% sucrose concentration. A small decrease can be seen at sucrose concentrations higher than 4%. This may be due to the substrate inhibition; excessive concentration of carbohydrate can inhibit or even poison cell growth. Therefore, it is common to observe this type of hyperbolic trends since metabolic end products may disrupt further cell growth at the critical substrate concentrations.

Fermentation studies showed that YPM medium can be used to obtain the maximum biomass concentration. Other findings on the effect of initial substrate concentration, spore inoculum concentration, medium volume when YPM medium was used are summarized in Figure 4.10.

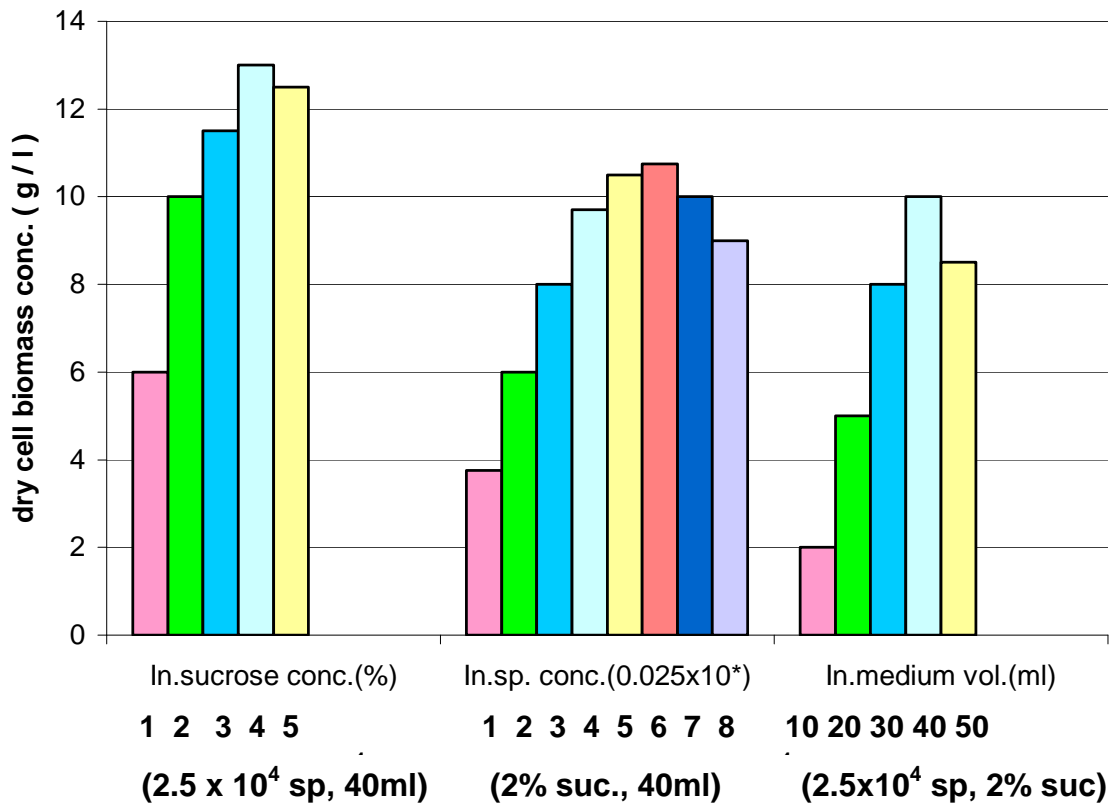


Figure 4.10. Effects of initial sucrose concentration, spore concentration and medium volume for growth of *Mucor rouxii* on YPM medium.

As it can be seen from Figure 4.10., YPM medium contained 4% sucrose, 10^6 spores / 40 ml yielded to maximum *Mucor rouxii* cell growth at 28 °C, 180 rpm. Therefore fermenter and shake flask studies were conducted under the best conditions described above, and given in the following section.

4.7. Growth of *Mucor rouxii* in Shake-Flask and Stirred Tank Bioreactor

Submerged fermentations in both shake flask and fermenter were performed under the best growth conditions for *Mucor rouxii*. The comparison of results obtained from the fermenter and shake flask could not be made because of differences e.g. the working volumes, the geometric shapes, the agitation types and aeration in these system. The shake flask fermentation medium had a greater surface area due to the shape of the flask and shaking.

The growth of *Mucor rouxii* biomass and consumption of sucrose versus time in shake flasks are given in Figure 4.11.

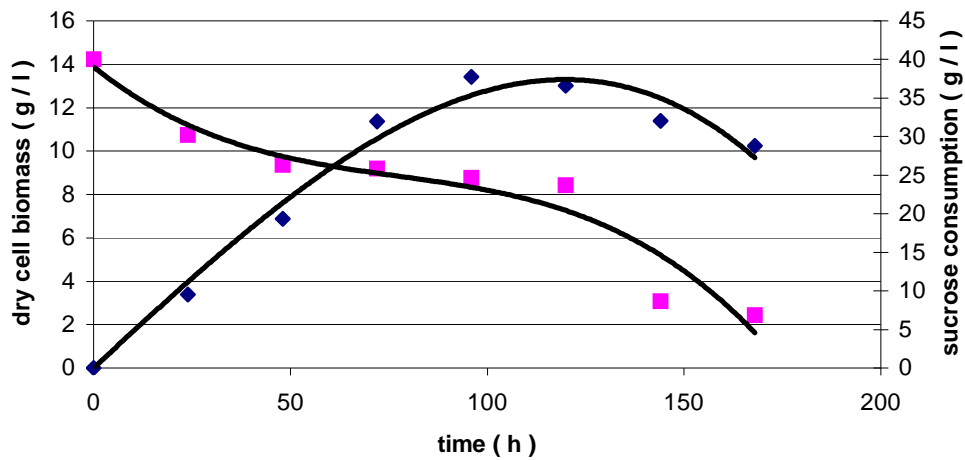


Figure 4.11. Growth of *Mucor rouxii* and sucrose consumption in shake-flask in the best condition for fermentation

(♦ biomass concentration, ■ sucrose concentration)

The growth kinetics of *Mucor rouxii* could not be investigated in the fermenter, it was difficult to remove samples during the fermentation because of the fungus morphology. In the fermenter, the fungus grew as large agglomerates or pulp causing difficulties with pumping out the broth with the biomass from the bioreactor and also they grew on the inside surface of the fermenter, electrodes and impeller. Because of these reasons, it could not be possible to take sample every day from fermentor, homogeneously. Only two samples were obtained for 4 and 7 days.

As seen from the graph, biomass concentration was increased linearly up to 100 hours. The stationary phase was obtained in between 100-120 hours of fermentation with the productivity value of 0.11 g / l / h. The biomass concentration and hence the productivity value decreased to 10 g / l and 0.06 g / l / h, respectively at the end of fermentation. And also total sucrose consumption was approximately 35 g / l during this fermentation.

In shake flask fermentation, the pH of the medium increased from starting value of 4.5 to 8. In fermenter, the pH of the medium increased from 4.5 to 6 until 100 hours and then decreased to 5.5 during growth of *M.rouxii*. Aeration in the fermenter can be the reason of low pH changes and lower biomass concentration.

The extracellular fluid was screened for protease enzyme activity. However, protease activity could not be detected in the supernatant.

4.8. Chitosan Extraction and Yield Determination

The main components of the mycelia were water, proteins and an AIF (alkali insoluble fraction) containing chitin, chitosan and acidic polysaccharides (Synowiecki *et al.*, 1997). Chitosan was extracted from the cell walls of *Mucor rouxii* grown under the best fermentation conditions. The extraction procedure was given in Section 3.2.4. according to this procedure, firstly AIF was obtained from the biomass with NaOH, then chitin and chitosan were separated from each other. Since chitin is not soluble in acetic acid; chitosan was dissolved in it, the end product obtained as chitosan. AIF and chitosan extracted from AIF followed during fermentation are presented in Figure 4.12. together with biomass concentrations.

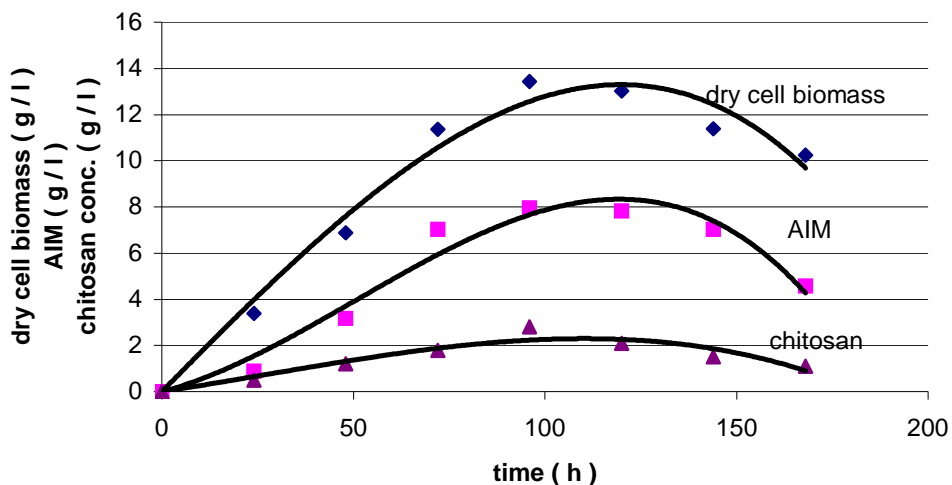


Figure 4.12. Changes of biomass, AIM and chitosan concentration during fermentation of *Mucor rouxii*

The dry weight of biomass, AIM and extractable chitosan of *Mucor rouxii* increased over a period of time. The fungal biomass increased rapidly during the first 72 hours of incubation and continued to increase until 96 hours. Beyond this point, the growth slowed down and the fungus appeared to enter stationary phase. The amount of AIM also increased steadily with incubation time to 72 hours, although its maximum was reached only at about 96 hours. In the case of chitosan, the absolute amount of

extractable chitosan increased slowly with incubation time, peaking at the 96th hour, followed by a slow decline when the cultures were incubated beyond this point.

The decline of the extractable chitosan seen in growth kinetics curve might be due to physiological changes in the fungal cell wall (McGahren *et al.*, 1984). Chitosan is produced in the fungal cell wall by deacetylating its precursor, nascent chitin. During the exponential phase, the ratio of free chitosan molecules is relatively high, due to the active growth. Once the culture enters the stationary growth phase, more of the chitosan is anchored to the cell wall of the Zygomycetes by binding to chitin and other polysaccharides and extraction becomes more difficult. Therefore, although the fungal biomass was highest during the stationary growth phase, less chitosan is obtained (Crestini *et al.*, 1996). This case was also observed in our study.

Table 4.1. summarizes the biomass, alkali insoluble material and chitosan concentrations together with sugar consumption. The extractable chitosan yields are calculated based on the biomass produced, AIM extracted and sugar consumed throughout fermentation period.

Table 4.1. Concentrations of mycelia, AIM, chitosan, sugar and yields of extractable chitosan of *Mucor rouxii*

Days	A:Biomass (g/l)	B:AIM (g/l)	C:Chitosan (g/l)	D:Sugar (g/l)	C/A (%)	C/B (%)	C/D ((%)
1	4	1.5	0.5	25	12.5	33.3	2
2	7.5	3.8	1.2	27.5	16	31.5	4.3
3	11	6	2	25	18.1	33.3	8
4	12.5	7.8	2.5	23	20	32	10.8
5	13.5	8.5	2.4	20	17.7	28.2	12
6	12.5	7	1.8	15	14.4	25.7	12
7	10	4.5	1	5	10	22.2	20

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

The objective of this study was to obtain chitosan from the cell walls of fungi having the maximum biomass concentration. Therefore, five fungal strains were screened for their biomass growth. Among the fungus species of *Absidia spp*, *Aspergillus niger*, *Rhizopus arrhizus*, *Cunninghamella elegans* and *Mucor rouxii*, *Mucor rouxii* was found as the producer of the highest amount of biomass.

Effect of different medium components (YPG, M, W, YPM, YPW, YM, PM, Complex Medium 1, Complex Medium 2, Complex Medium 3), initial sucrose concentrations (1, 2, 3, 4, 5 %), initial production medium volume (10, 20, 30, 40, 50 ml) and initial spore inoculum concentrations (1 to 8, 0.025×10^x) were investigated on the growth of *Mucor rouxii*. The maximum biomass concentration of *Mucor rouxii* was obtained at its late exponential phase on molasses YP medium containing 4% sucrose, 10^6 spores in 40 ml at 28 °C, 180 rpm by submerged fermentation in shake flasks.

The effect of salts added into the fermentation medium was also examined and K_2HPO_4 and $MgSO_4$ were found to be the most effective salts on the growth of *Mucor rouxii*.

This study showed that molasses can be used as a convenient substrate for the production of chitosan. Molasses which is a by product of food industry contains sucrose and mineral salts which constitutes a good formulation needed for the growth of fungal biomass.

The yield of extractable chitosan obtained from cell wall of *Mucor rouxii* was 2.5 g / l and it is almost 20 % of biomass and approximately 35 % of alkali insoluble fraction.

Since the studies in the literature focusing specifically on chitosan production by fungi are rare, the results of this study may contribute to the following research. Due to the time limitations of the present work, some aspects could not be studied. For future studies characterization of chitosan (determination of molecular weight, degree of deacetylation,...), biocompatibility and antimicrobial activities, production of films, membranes, beads for different purpose could be worth to investigate.

REFERENCES

1. Arcidiacono, S. and Kaplan, D.,L., 'Mw Distrubition of chitosan isolated from *Mucor rouxii* under different culture and processing conditions', *Biotechnology and Bioengineering*, 39, 3, 1992, 281-286.
2. Arcidiacono, S., Lombardi, S.J., Kaplan, D.L., 'Fermentation, processing and enzyme characterization for chitosan biosynthesis by *Mucor rouxii*', In *Chitin and Chitosan: Sources, chemistry, biochemistry, physical properties and applications*, G. Skjak-Braek, T. Anthonsen, P. Sandford, eds., Elsevier, N.Y., 1989, 319-332.
3. Atkinson, B., Mavituna, F., 'Biochemical engineering and biotechnology handbook', McMillan Press, New York, 1991.
4. Chen, R.H., 'Progress and perspective of chitin and chitosan science and industry in Asia', In *chitin and chitosan in life science*, T. Uragami, K. Kurita, T. Fukamizo, eds., Kodansha, 2000, 487-496.
5. Crestini, C., Kovac, B., Giovannozzi-Sermanni, G., 'Production and isolation of chitosan by submerged and solid-state fermentation from *Lentinus edodes*' *Biotechnology Bioengineering*, 50, 1996, 207-210.
6. Gessesse, A., 'The use of nug meal as a low – cost substrate for the production of alkaline protease by the alkaliphilic *Bacillus* sp. AR-009 and some properties of the enzyme', *Bioresource Technology*, 62, 1997, 59-61.
7. Göksungur, Y., 'Application of different production techniques on the production of lactic acid from beet molasses and optimization of process parameters', Ph.D. Thesis study, 1998.
8. Hang, Y.D., 'Chitosan production from *Rhizopus oryzea* mycelia', *Biotechnology Letters*, 12, 1990, 911-912.
9. Irvine, D.M., Hill, A.R., 'Cheese technology', *Comprehensive Biotechnology*, 3, edited by M.Moo-Young, Pergamon Press,1985, 523-526.
10. Jaworska, M.M., and Konieczana, E., 'The influence of supplemental components in nutrient medium on chitosan formation by the fungus *Absidia orchidis*', *Original Paper*, Springer- Verlag, 2001.
11. Jaworska, M.M., Szewczyk, K.W., 'The continuous cultivation of *Absidia orchidis* fungi', In *chitin and chitosan in life science*, T. Uragami, K. Kurita, T. Fukamizo, eds., Kodansha, 2000, 519-523.

12. Kumar, M.N.V.R., 'A Review of chitin and chitosan applications', *Reactive and Functional Polymers*, 40, 2000, 1-27.
13. Kurita, K., 'Controlled functionalization of the polysaccharide chitin', *Progress in Polymer Science*, 26, 2001, 1921-1971.
14. McGarhen, W.J., Perkinson, G.A., Growich, J.A., Leese, R.A., Ellestad, G.A., 'Chitosan by fermentation', *Process Biochemistry*, 19, 1984, 88-90.
15. Miller-Ihli, N.J., 'Trace element determinations in food and biological samples using inductively coupled plasma atomic emission spectrometry and flame atomic absorption spectrometry', *Journal of Agricultural Food Chemistry*, 44, 1996, 2675-2679.
16. Muzzarelli, R.A.A., 'Chitin', Oxford: Pergamon Press, 1977.
17. Muzzarelli, R.A.A., 'Natural Chelating Polymers', Oxford: Pergamon Press, 1973.
18. Nutting, G.C., 'The by-products of milk' In *By-products from milk*, edited by H.B. Webb, E.O. Whittier, The Avi Publishing Company, Inc, Connecticut, 1970.
19. Nwe, N., Chandkrachang, S., Stevens, W.F., Maw, T., Tan, T.K., Khor, E., Wong, S.M., 'Production of fungal chitosan by solid state and submerged fermentation', *Carbohydrate Polymers*, 2001, Article in press.
20. Sandford, P.A., 'Chitosan: commercial uses and potential applications', In *Chitin and Chitosan: Sources, chemistry, biochemistry, physical properties and applications*, G. Skjak-Braek, T. Anthonsen, P. Sandford, eds., Elsevier, N.Y., 1989, 51-69.
21. Shimara, K., Takiguchi, Y., Kobayashi, T., Uda, K., Sannan, T., 'Screening of Mucoracea strains suitable for chitosan production', In *Chitin and Chitosan: Sources, chemistry, biochemistry, physical properties and applications*, G. Skjak-Braek, T. Anthonsen, P. Sandford, eds., Elsevier, N.Y., 1989, 171-178.
22. Suntornsuk, W., Pochanavanich, P., Suntornsuk, L., 'Fungal chitosan production on food processing by-products', *Process Biochemistry*, 37, 2002, 727-729.
23. Synowiecki, J., and Al-Khateeb, N.A.A.Q., 'Mycelia of *Mucor rouxii* as a source of chitin and chitosan', *Food Chemistry*, 60, 4, 1997, 605-610.
24. Tan, S.C., Tan, T.K., Wong, S.K., Khor E., 'The chitosan yield of zygomycetes at their optimum harvesting time', *Carbohydrate Polymers*, 30, 1996, 239-242.
25. Teng, W.L., Khor, E., Tan, T.K., Lim, L.Y., Tan, S.C., 'Concurrent production of chitin from shrimp shells and fungi', *Carbohydrate Research*, 332, 2001, 305-316.
26. Tharanathan, R.N., and Kittur, F.S., 'Chitin – The undisputed biomolecule of great potential', *Critical Reviews in Food Science and Nutrition*, 43 (1), 2003, 61 – 87.

27. Üzelyalçın, B., 'Effects of trace elements on the production of Baker's yeast (*Saccharomyces cerevisiae*)', MSc. Thesis study, 2002.
28. White, S.A., Farina, P.R., Fulton, I., 'Production and isolation of chitosan from *Mucor rouxii*', Applied Environmental Microbiology, 38, 1979, 323-328.
29. Yokoi, H., Aratake, T., Nishio, S., Hirose, J., Hayashi, S., Takasaki, Y., 'Chitosan production from Shochu Distillery wastewater by fungus', Journal of Fermentation and Bioengineering, 85, 2, 1998, 246-249.

APPENDIX A

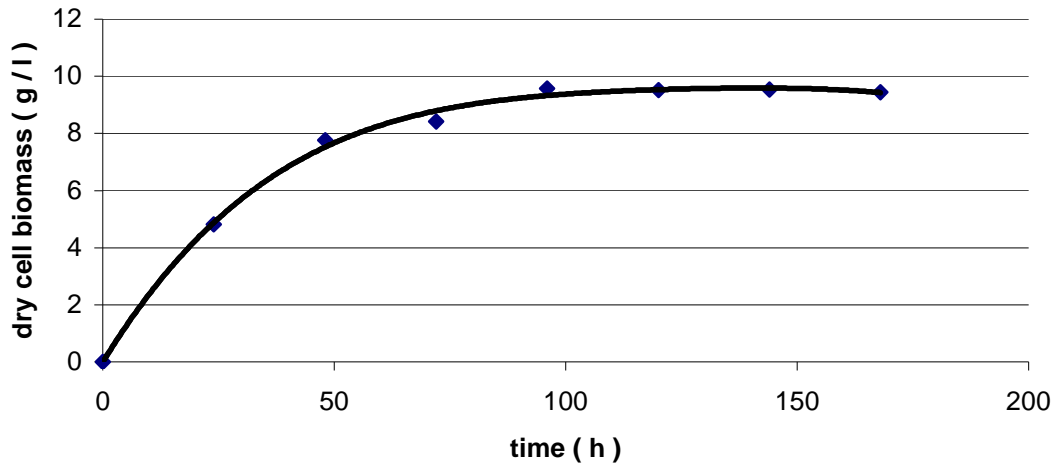


Figure A.1. Growth of *Mucor rouxii* on complex medium 1

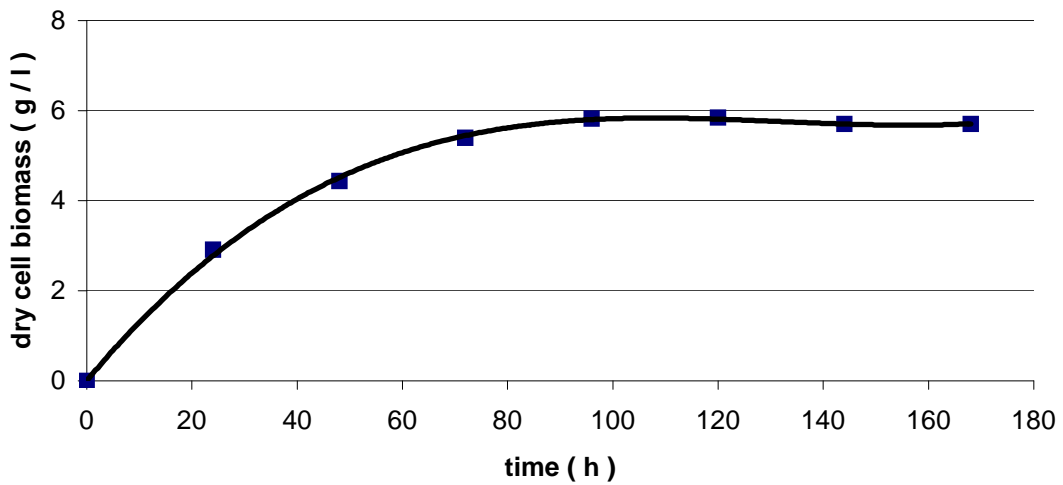


Figure A.2. Growth of *Rhizopus arrhizus* on complex medium 1

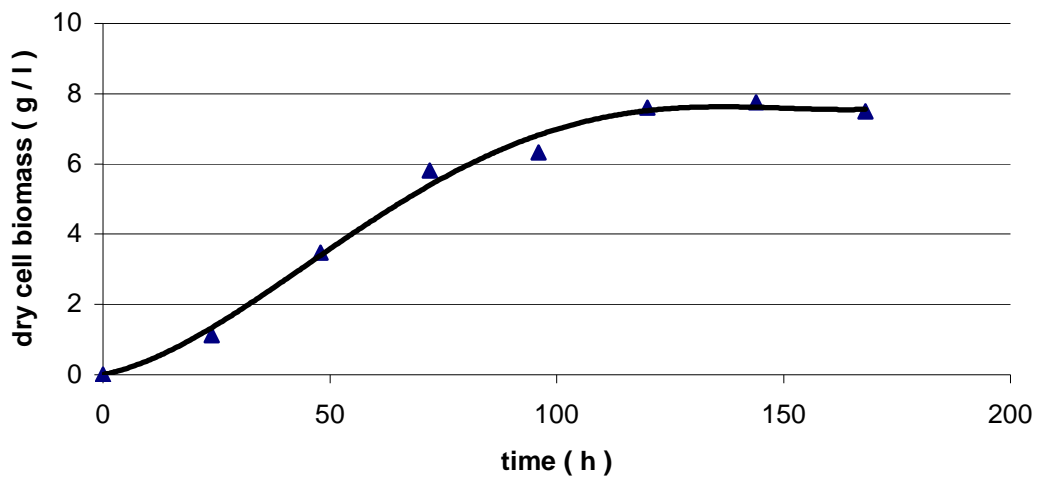


Figure A.3. Growth of *Absidia* on complex medium 1

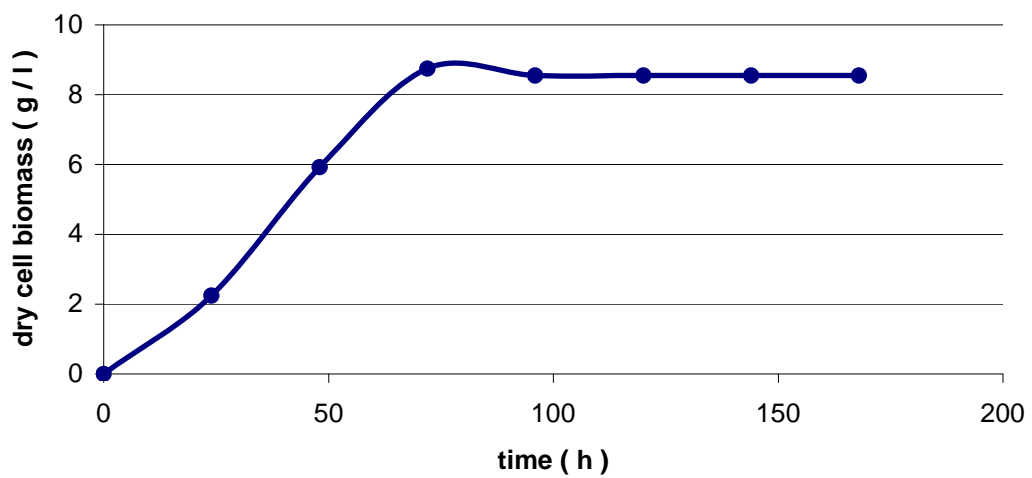


Figure A.4. Growth of *Aspergillus niger* on complex medium 1

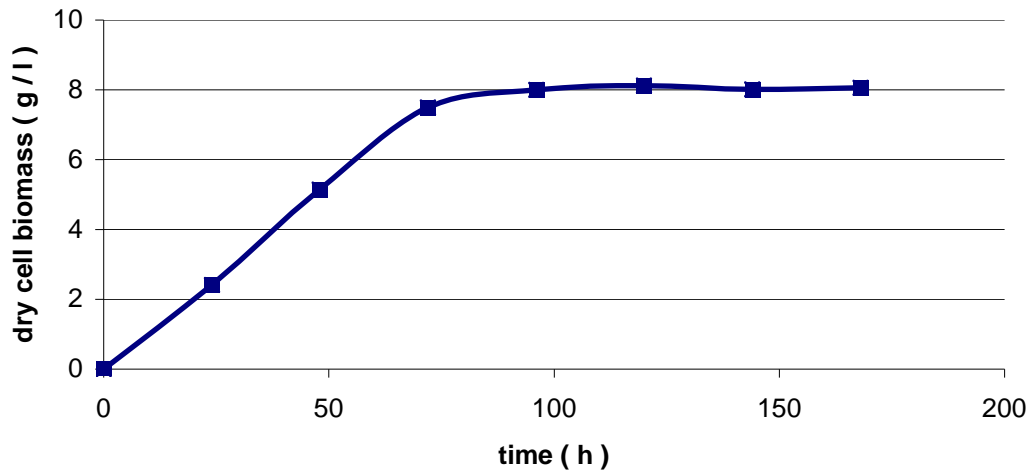


Figure A.5. Growth of *Cunninghamella elegans* on complex medium 1

APPENDIX B

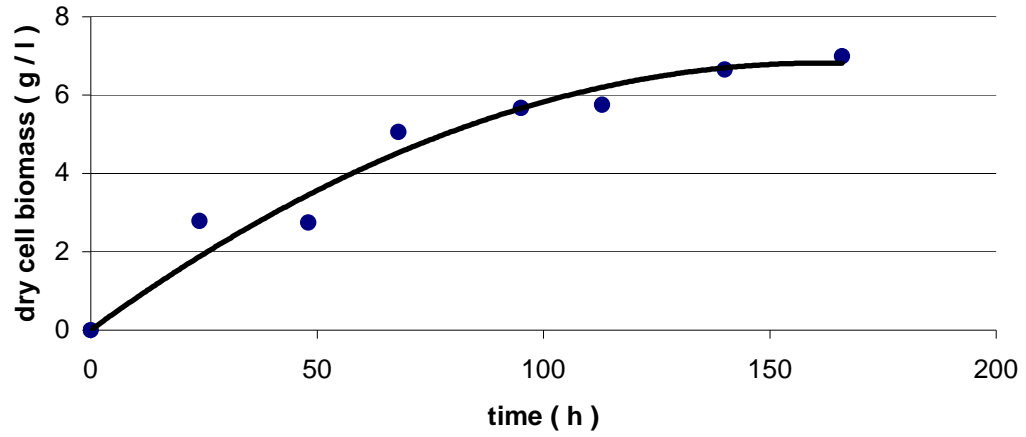


Figure B.1. Growth of *Mucor rouxii* on Complex Medium 1 without $MgSO_4$

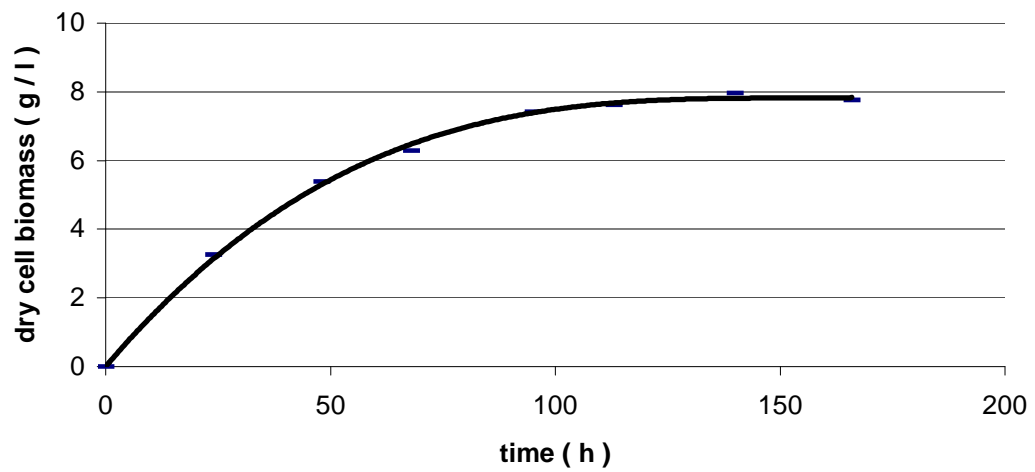


Figure B.2. Growth of *Mucor rouxii* on Complex Medium 1 without $CaCl_2$

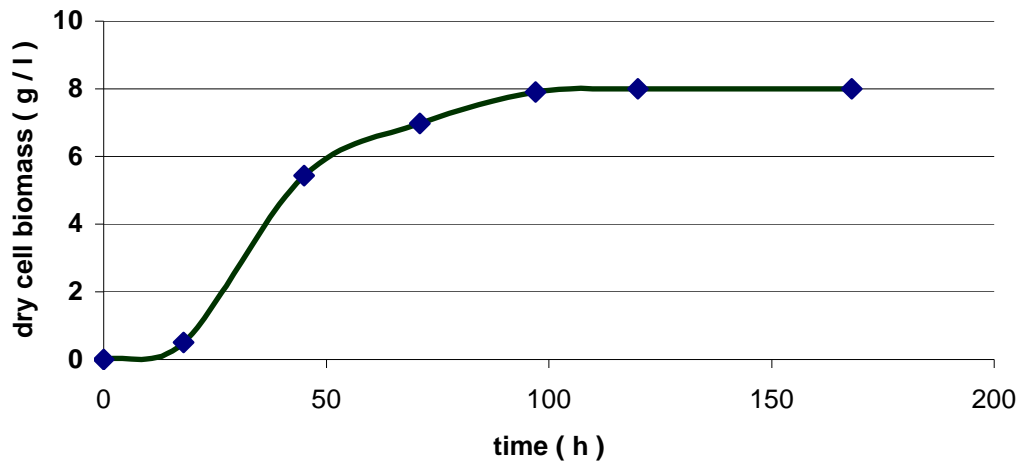


Figure B.3. Growth of *Mucor rouxii* on Complex Medium 1 without $(\text{NH}_4)_2\text{SO}_4$

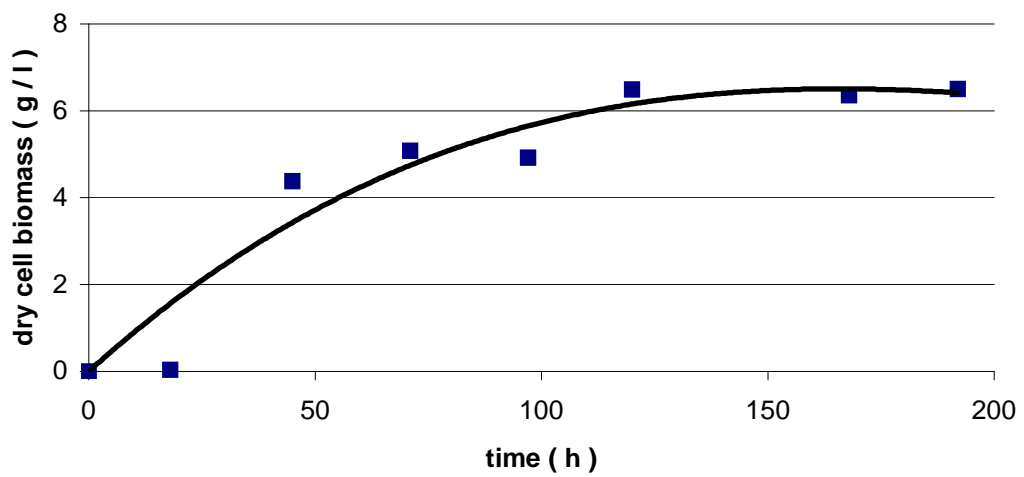


Figure B.4. Growth of *Mucor rouxii* on Complex Medium 1 without K_2HPO_4

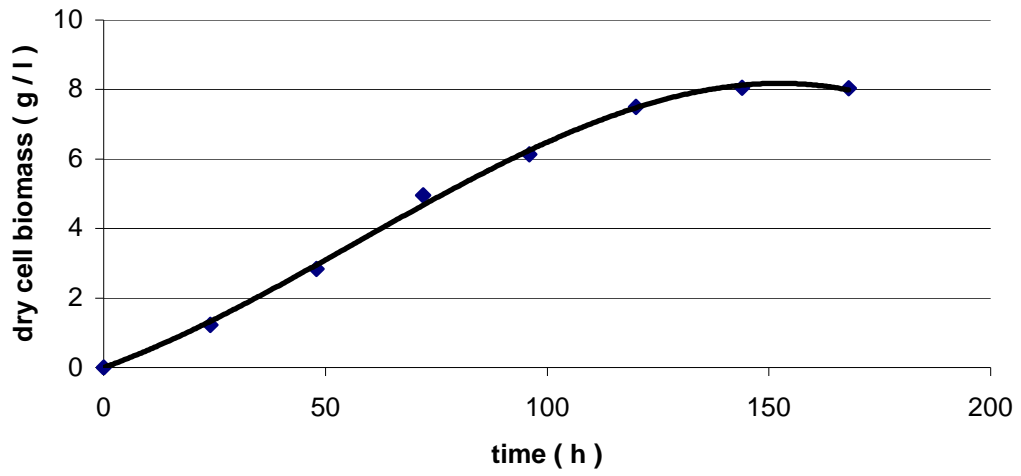


Figure B.5. Growth of *Mucor rouxii* on Complex Medium 1 without NaCl

APPENDIX C

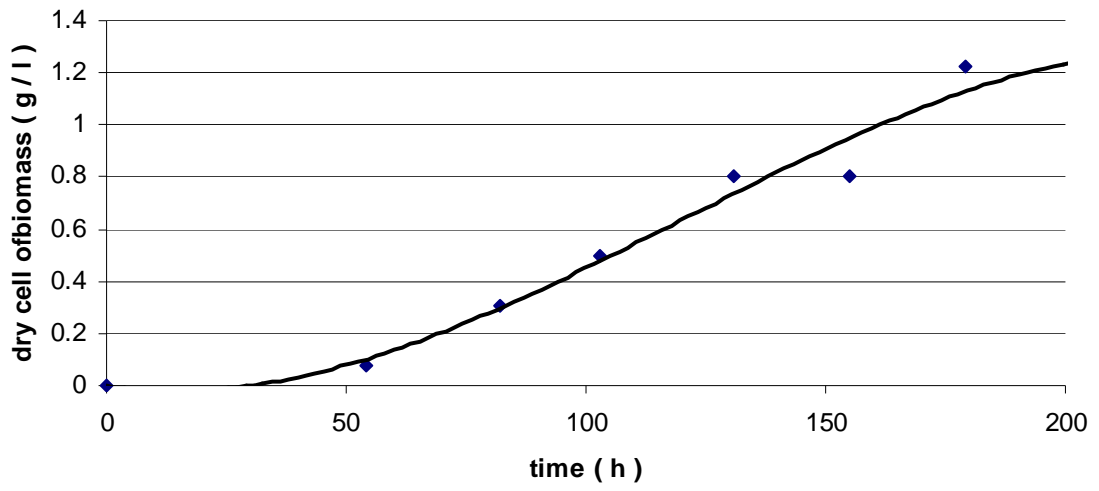


Figure C.1. Growth of *Mucor rouxii* on molasses

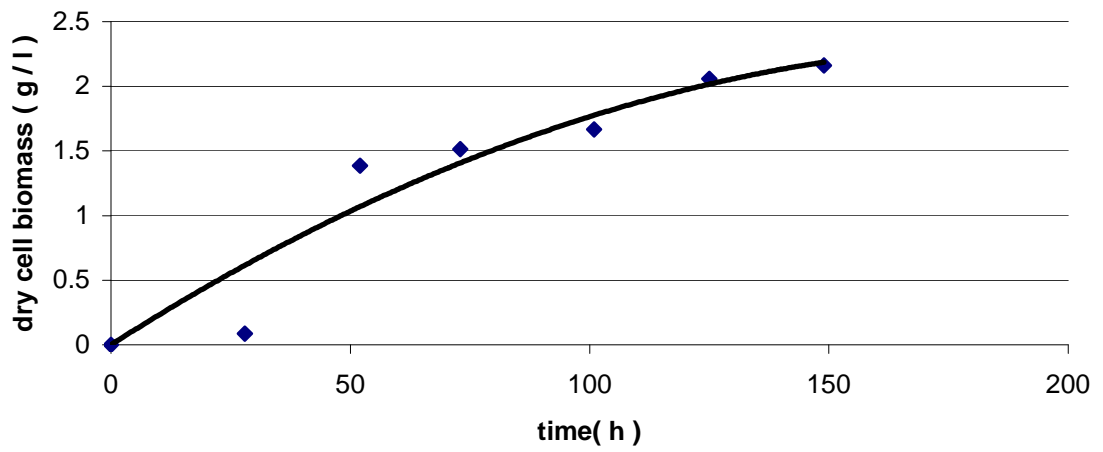


Figure C.2. Growth of *Mucor rouxii* on whey

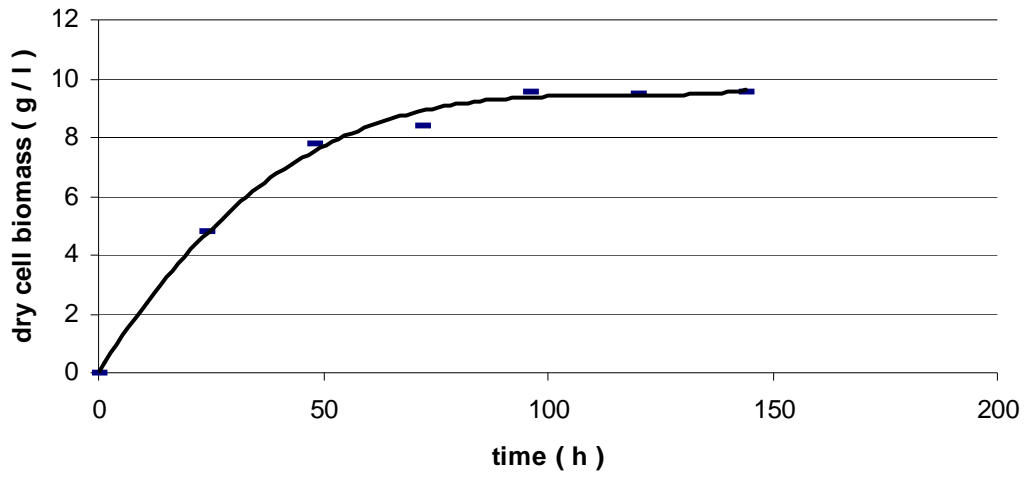


Figure C.3. Growth of *Mucor rouxii* on YPM

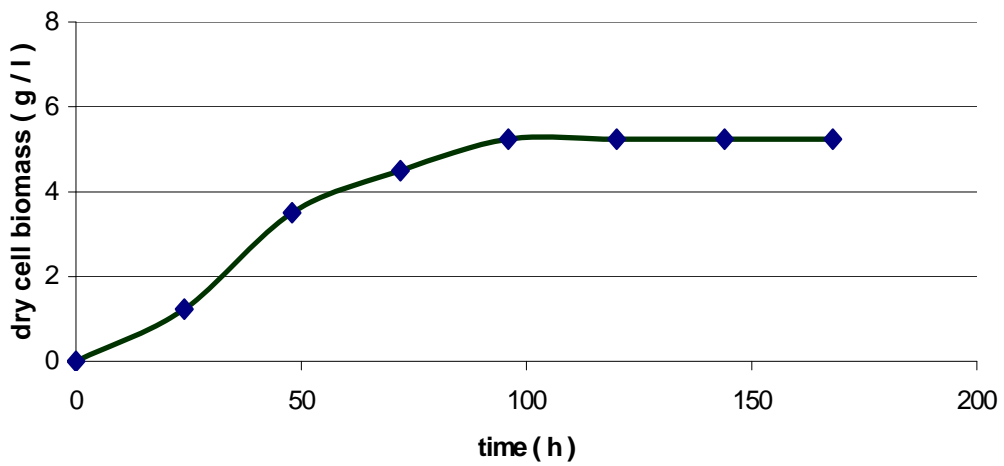


Figure C.4. Growth of *Mucor rouxii* on YPW

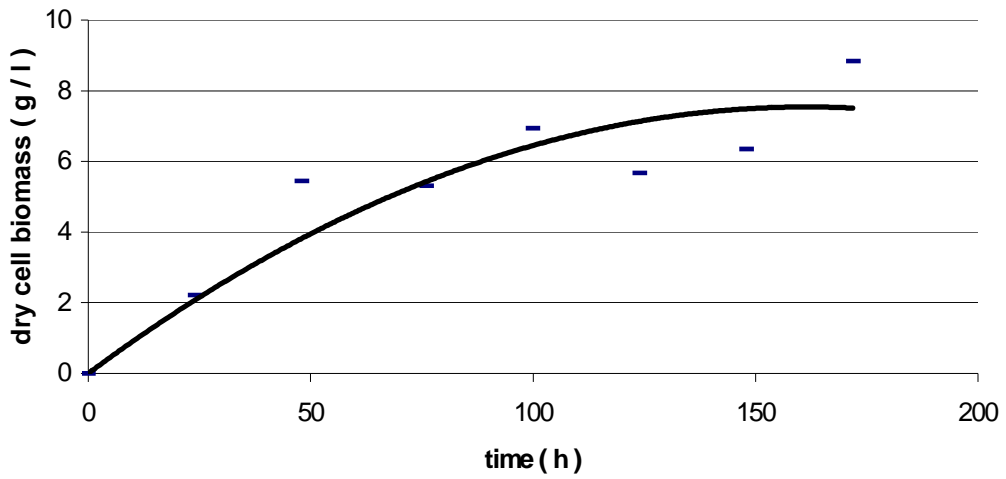


Figure C.5. Growth of *Mucor rouxii* on YM

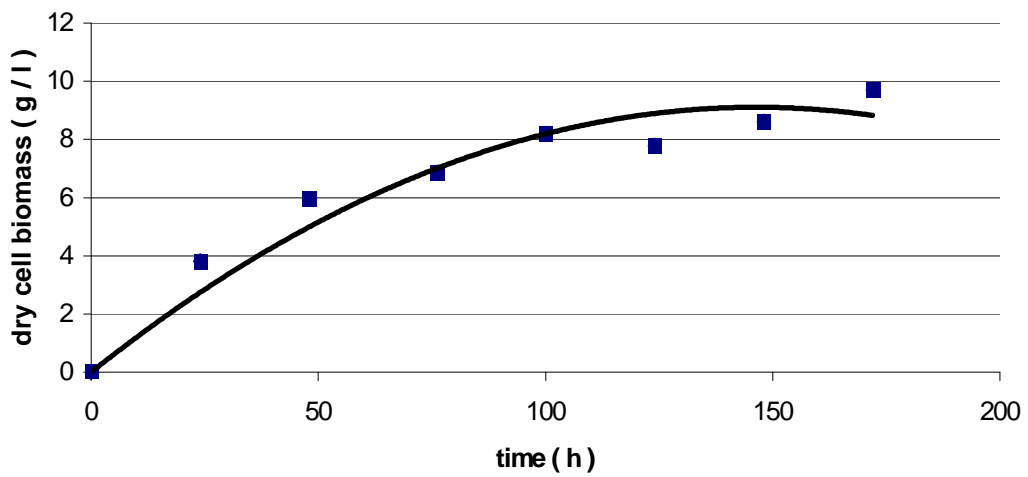


Figure C.6. Growth of *Mucor rouxii* on PM

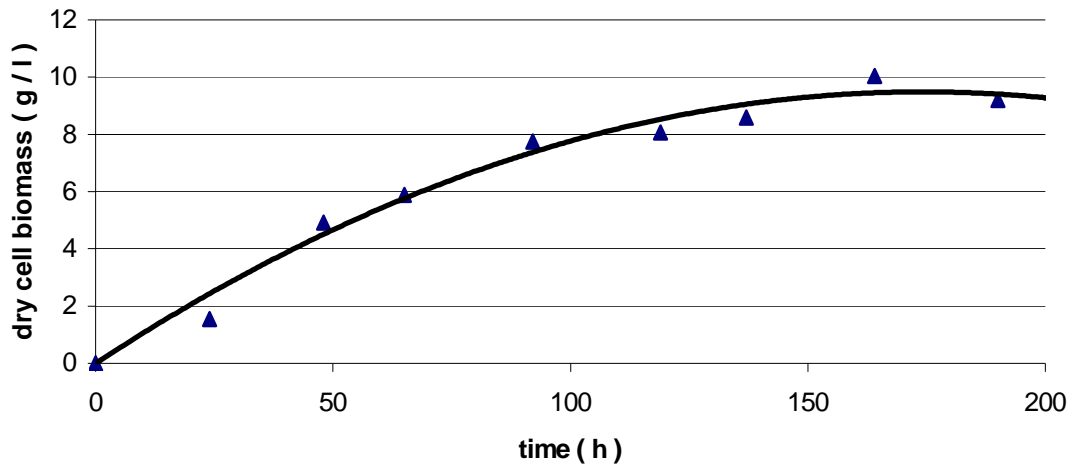


Figure C.7. Growth of *Mucor rouxii* on Complex Medium 2

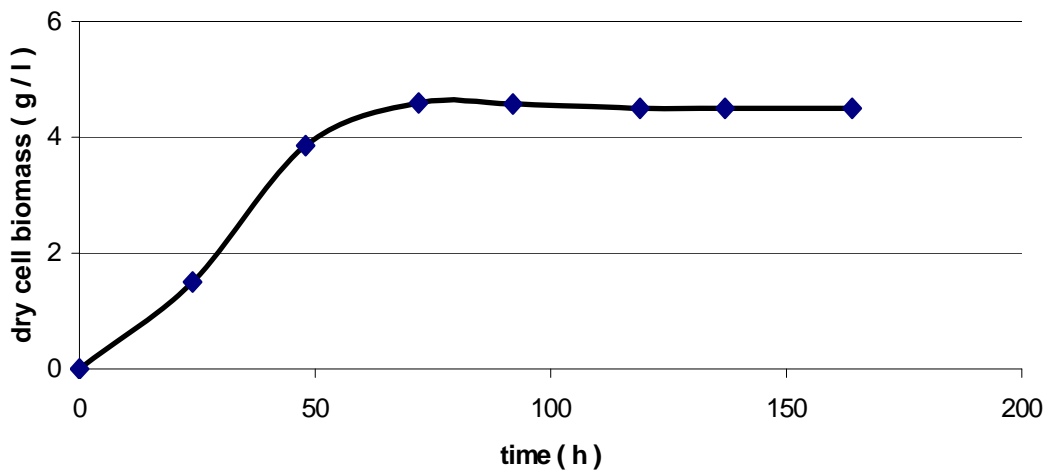


Figure C.8. Growth of *Mucor rouxii* on Complex Medium 3

APPENDIX D

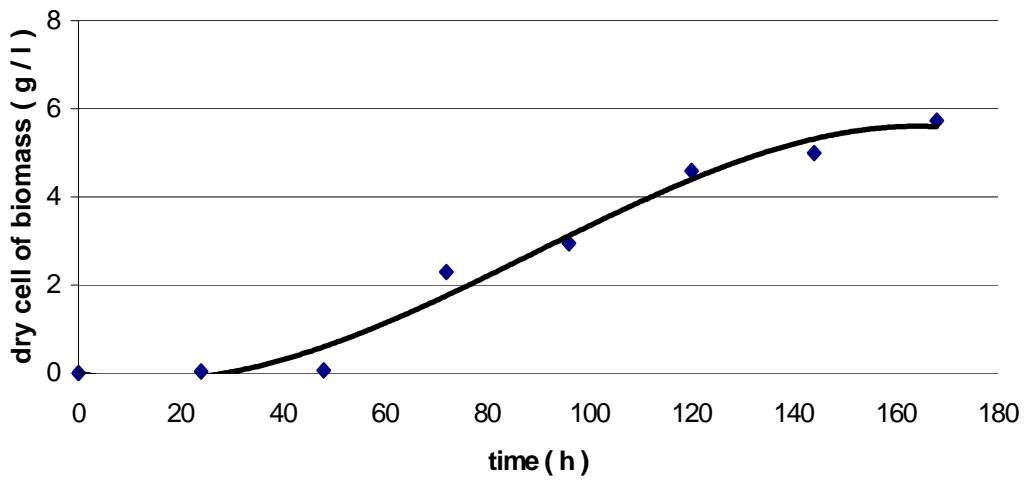


Figure D.1. Growth of *Mucor rouxii* in YPM medium inoculated 0.025×10^1 spores

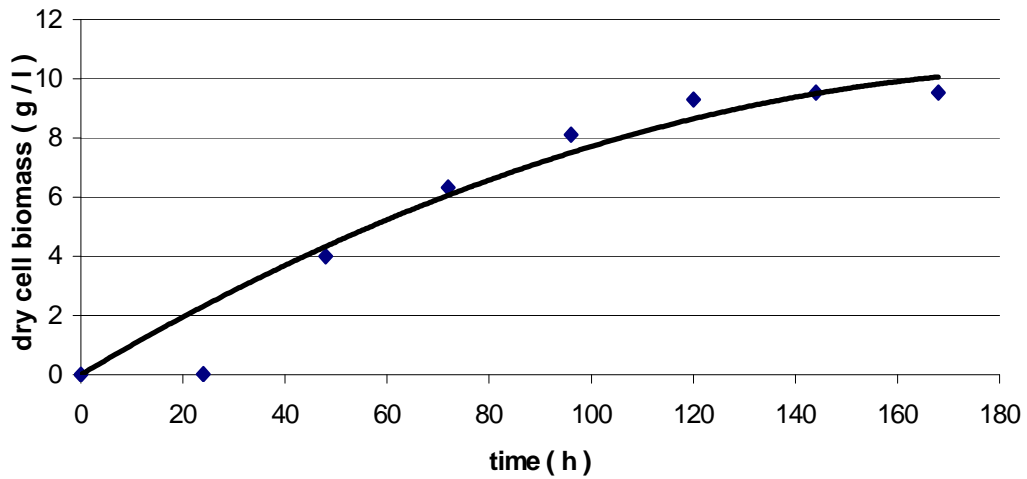


Figure D.2. Growth of *Mucor rouxii* in YPM medium inoculated 0.025×10^2 spores

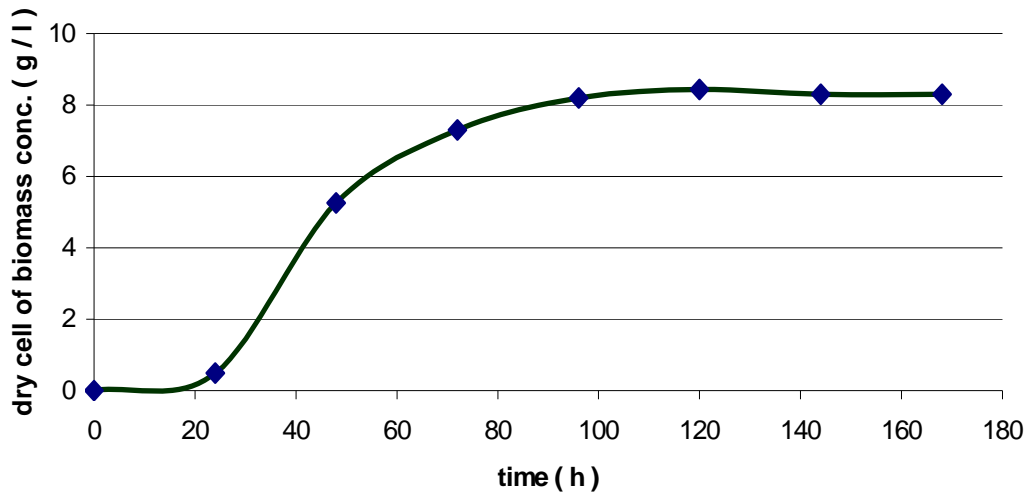


Figure D.3. Growth of *Mucor rouxii* in YPM medium inoculated 0.025×10^3 spores

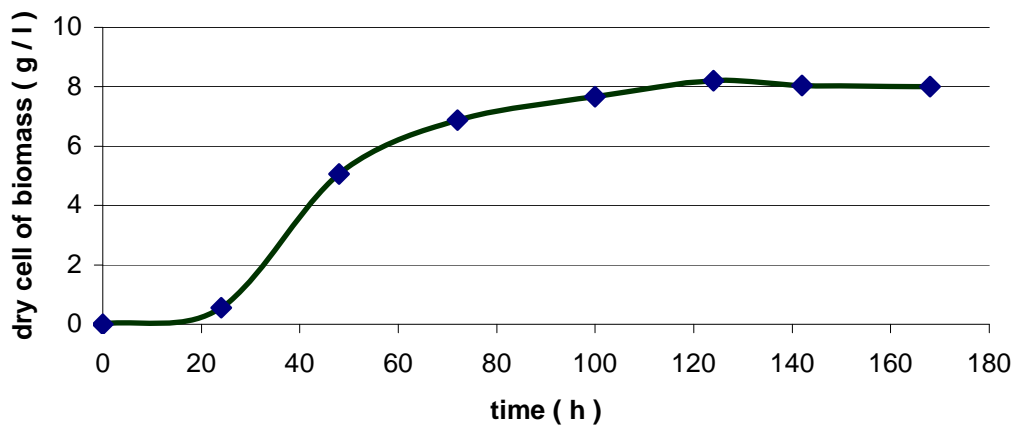


Figure D.4. Growth of *Mucor rouxii* in YPM medium inoculated 0.025×10^4 spores

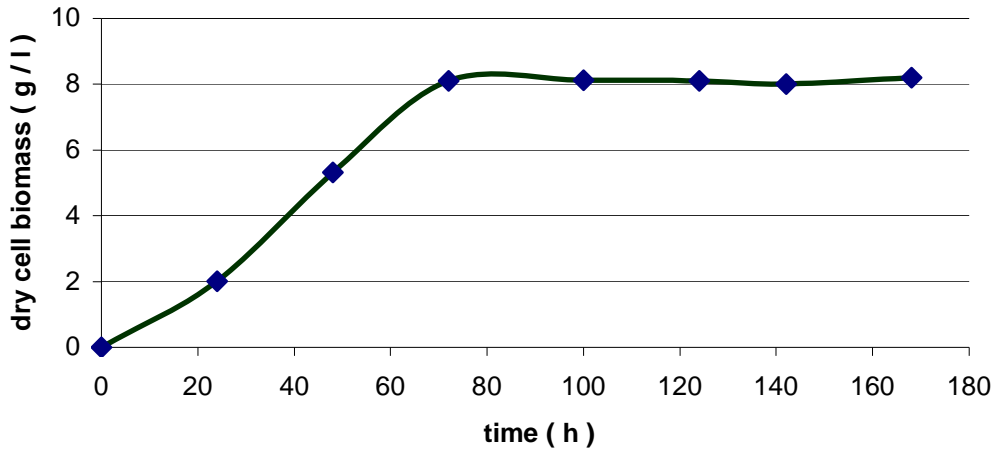


Figure D.5. Growth of *Mucor rouxii* in YPM medium inoculated 0.025×10^5 spores

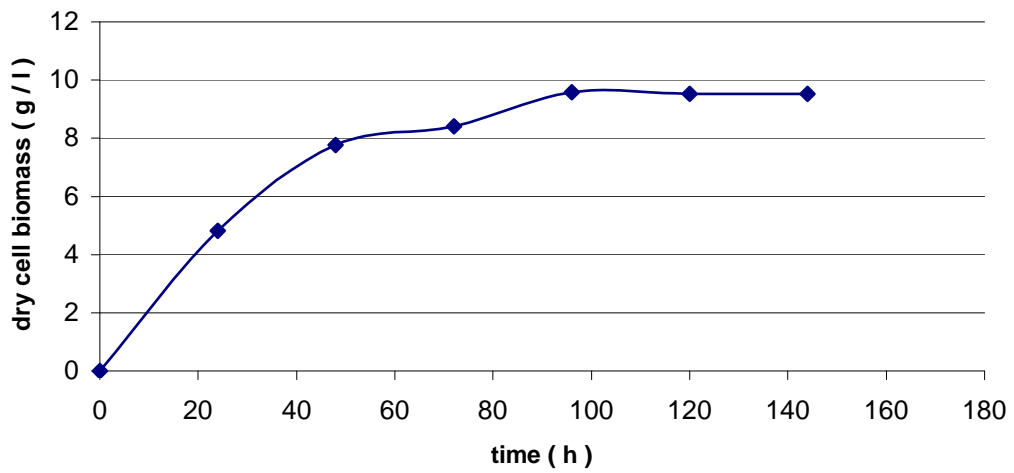


Figure D.6. Growth of *Mucor rouxii* in YPM medium inoculated 0.025×10^6 spores

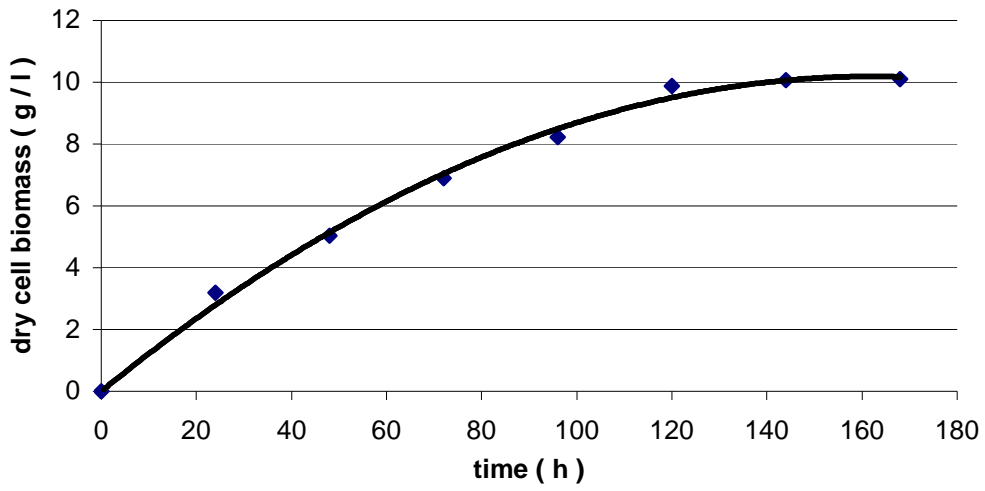


Figure D.7. Growth of *Mucor rouxii* in YPM medium inoculated 0.025×10^7 spores

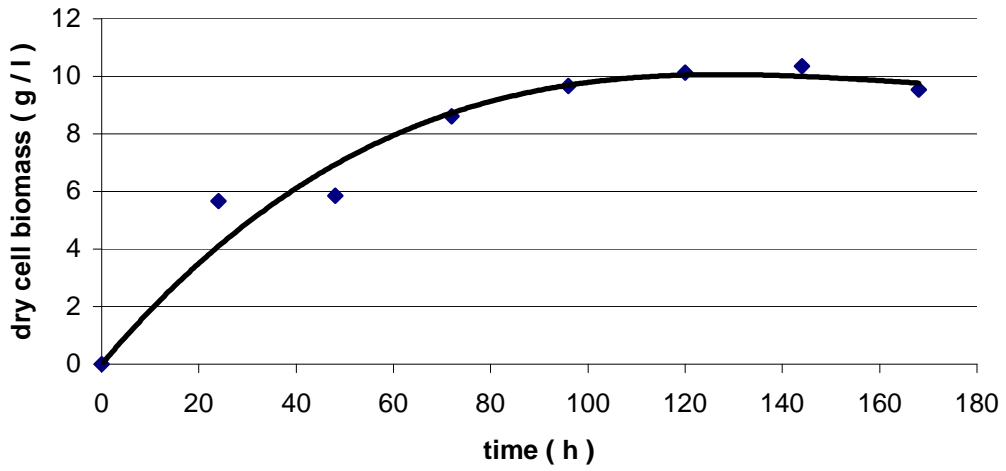


Figure D.8. Growth of *Mucor rouxii* in YPM medium inoculated 0.025×10^8 spores

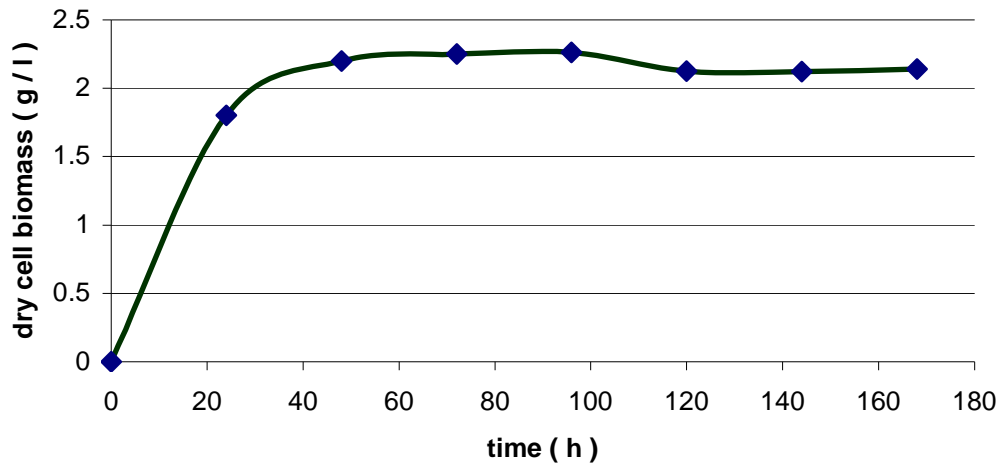


Figure D.9. Growth of *Mucor rouxii* in 10 ml of YPM medium

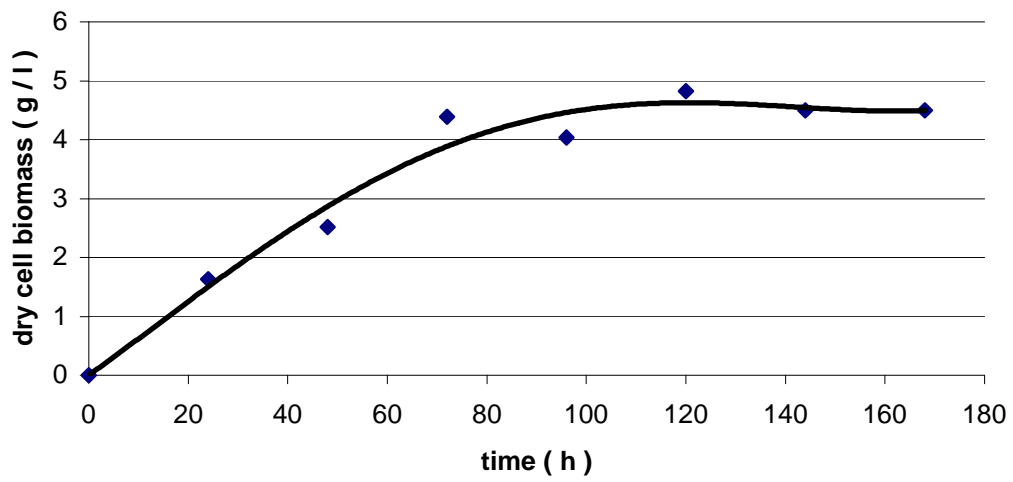


Figure D.10. Growth of *Mucor rouxii* in 20 ml of YPM medium

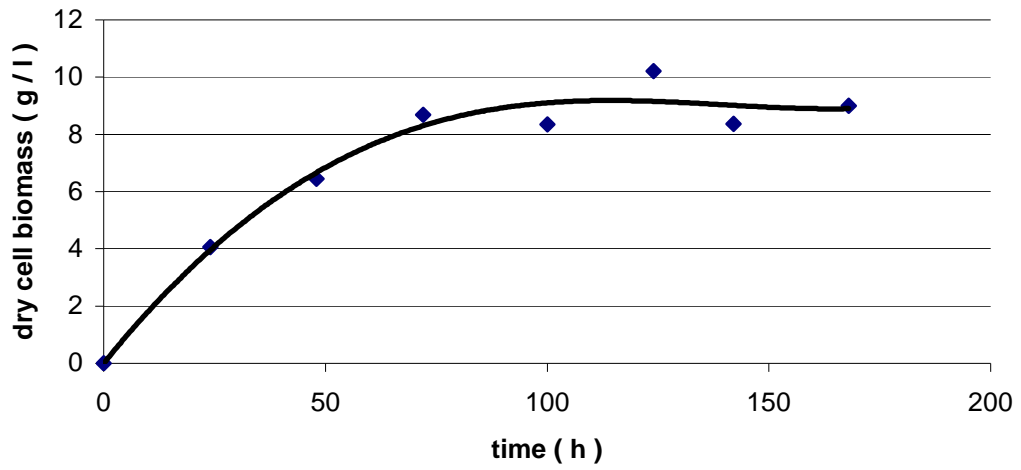


Figure D.11. Growth of *Mucor rouxii* in 30 ml of YPM medium

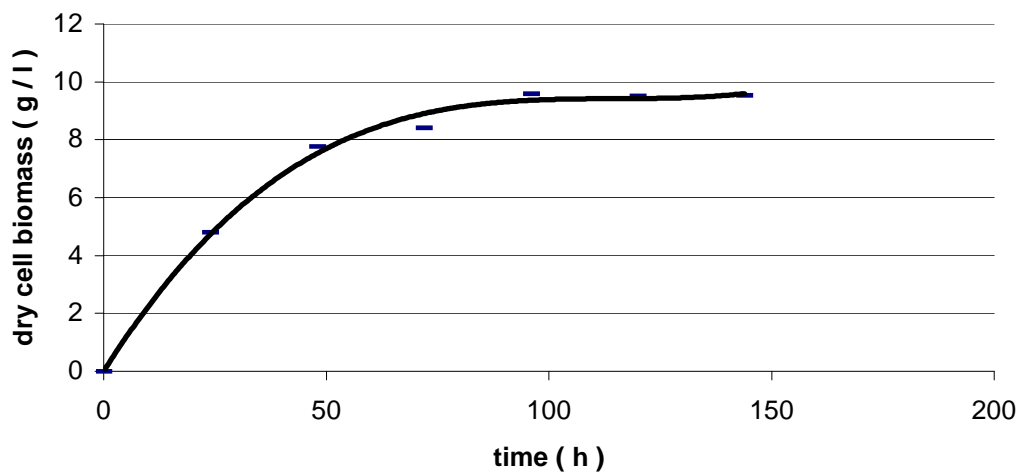


Figure D.12. Growth of *Mucor rouxii* in 40 ml of YPM medium

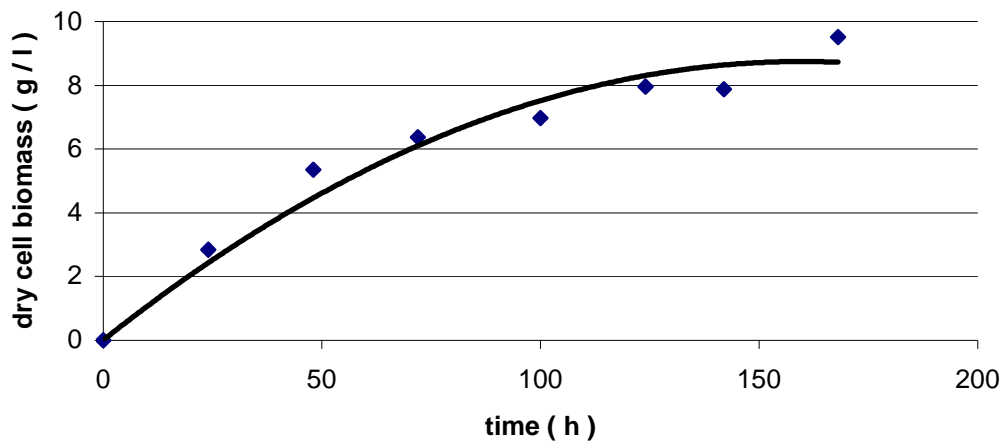


Figure D.13. Growth of *Mucor rouxii* in 50 ml of YPM medium

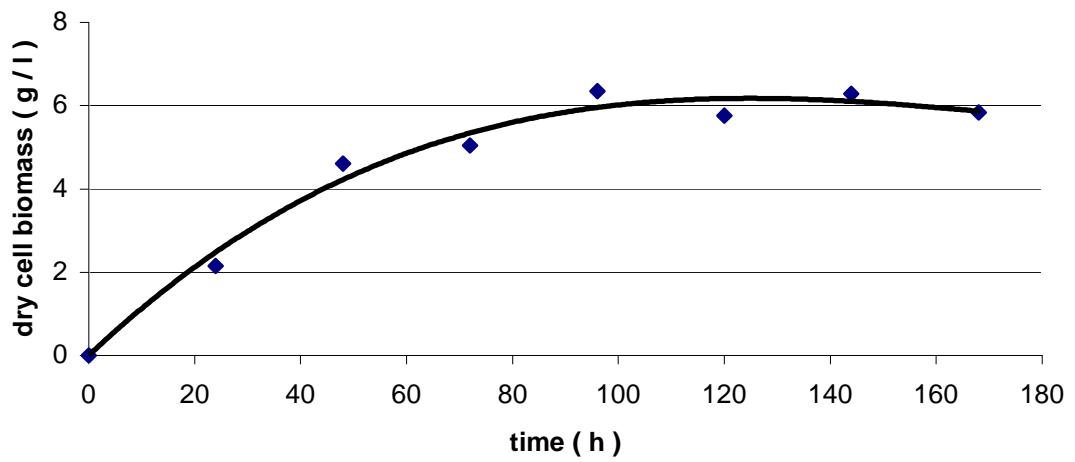


Figure D.14. Growth of *Mucor rouxii* in YPM medium containing 1 % sucrose

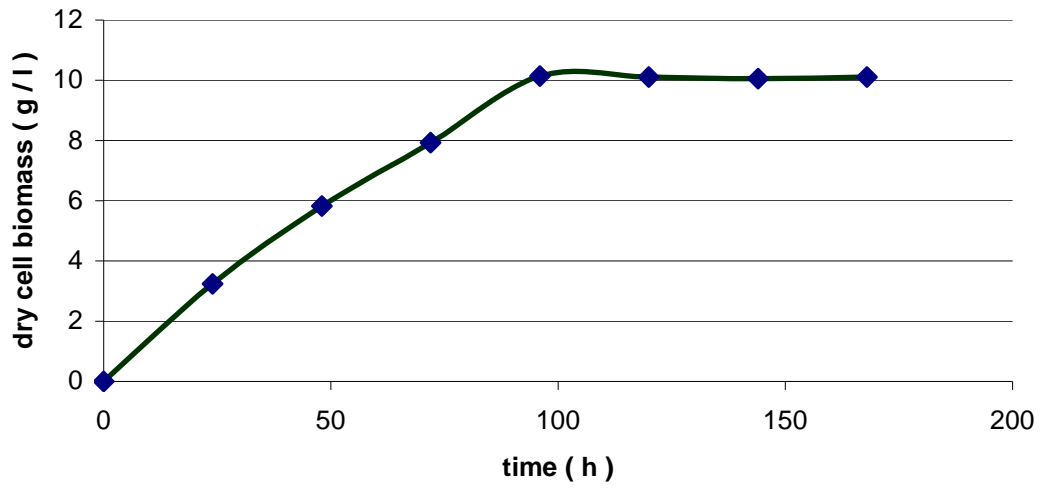


Figure D.15. Growth of *Mucor rouxii* in YPM medium containing 2 % sucrose

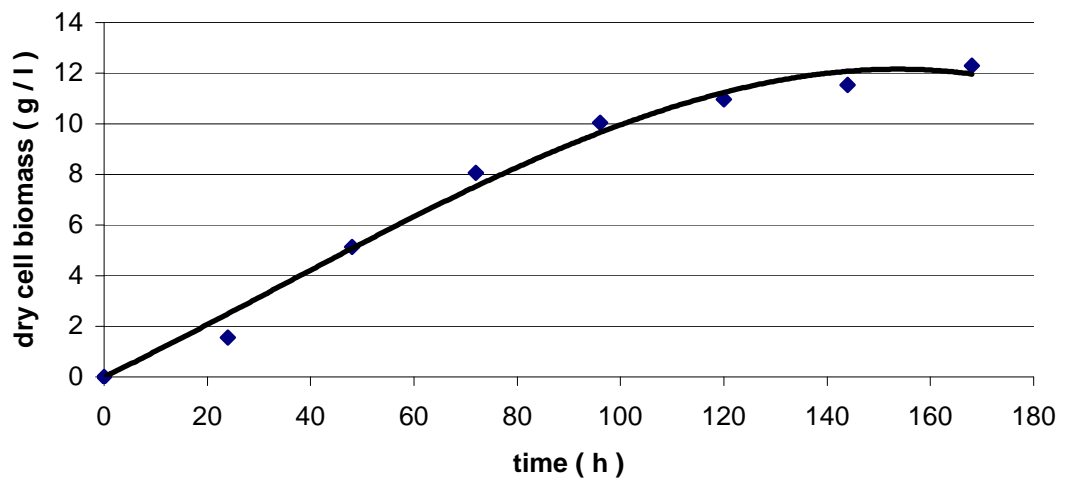


Figure D.16. Growth of *Mucor rouxii* in YPM medium containing 3 % sucrose

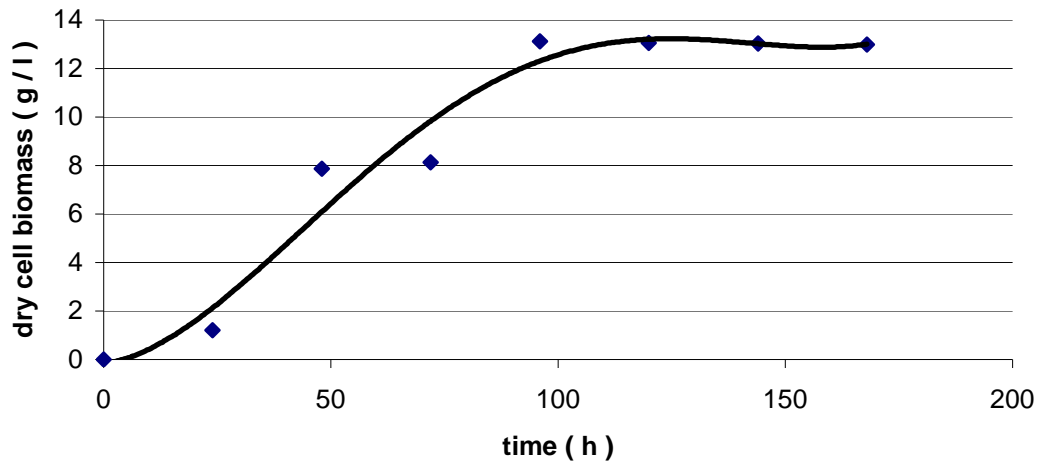


Figure D.17. Growth of *Mucor rouxii* in YPM medium containing 4 % sucrose

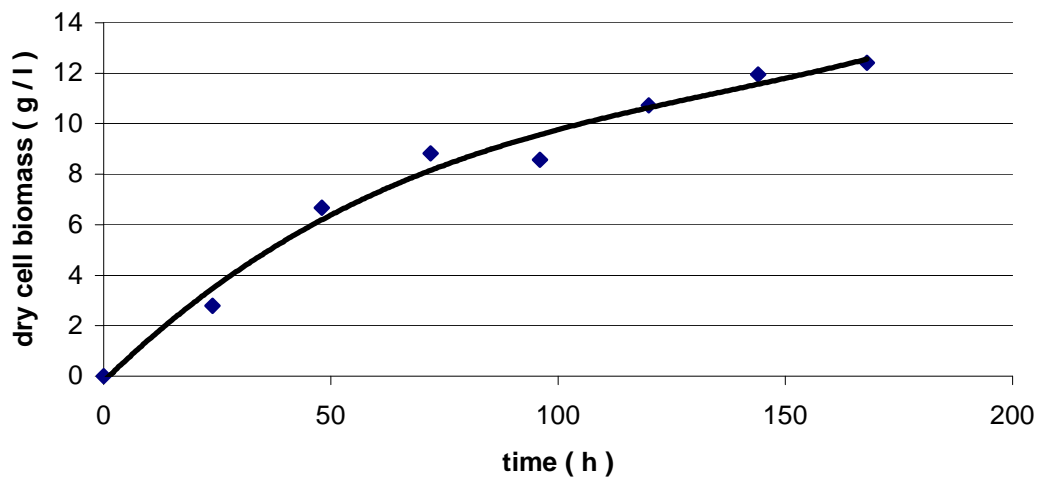


Figure D.18. Growth of *Mucor rouxii* in YPM medium containing 5 % sucrose

APPENDIX E

Calibration Curves

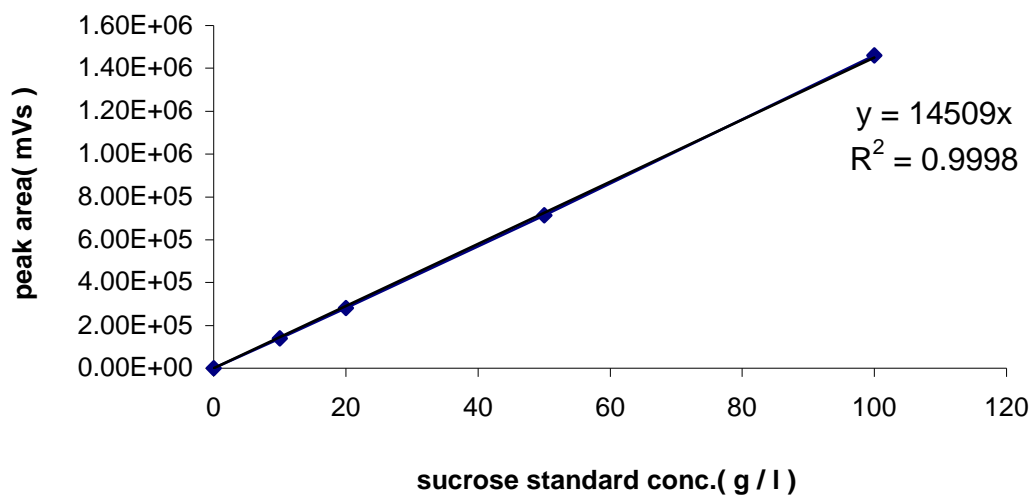


Figure E.1. Calibration curve for sucrose standard (HPLC)

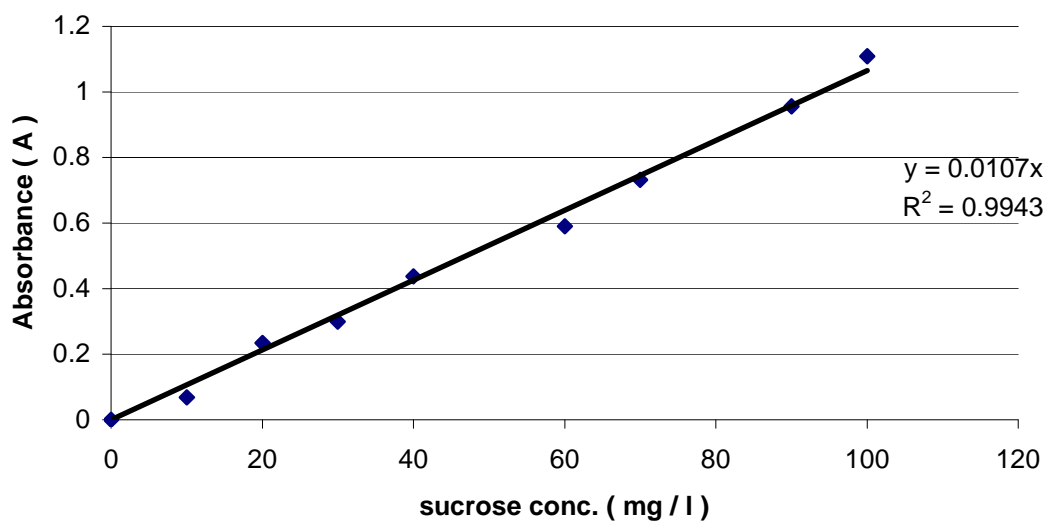


Figure E.2. Calibration curve for sucrose standard (UV-VIS.Spectrophotometry)

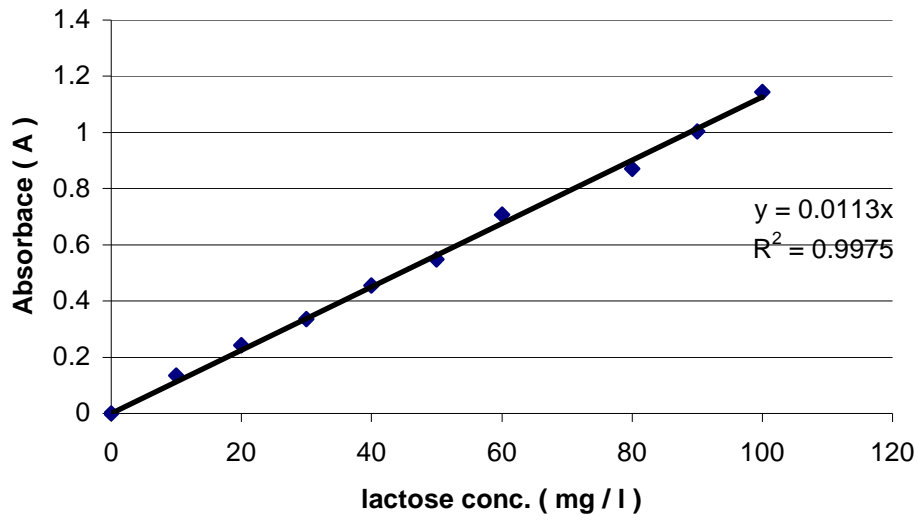


Figure E.3. Calibration curve for lactose standard (UV-VIS. Spectrophotometry)

APPENDIX F

Definitions of Fermentation Evaluation Terms

Productivity : Productivity is the amount of product produced per volume and per time.

$$\text{Productivity, (g / l / h)} = \frac{P_f - P_i}{t_f - t_i}$$

P = product (chitosan) concentration (g.L⁻¹)

t = time (h)

i = initial

f = final