EFFECT OF THE MORPHOLOGY OF *ASPERGILLUS SOJAE* ON PECTINASE ENZYME AND THE OPTIMIZATION OF FERMENTATION CONDITIONS

A Thesis Submitted to
the Graduate School of Engineering and Sciences of
İzmir Institute of Technology
in Partial Fulfilment of the Requirements for the Degree of

MASTER OF SCIENCE

in Food Engineering

By
Nihan GÖĞÜŞ

September 2006
İZMİR

We approve the thesis of **Nihan GÖĞÜŞ**

Date of Signature

..... **19.09.2006**

Asst. Prof. Dr. Canan TARI

Supervisor

Department of Food Engineering

İzmir Institute of Technology

..... **19.09.2006**

Asst. Prof. Dr. Sevcan ÜNLÜTÜRK

Department of Food Engineering

İzmir Institute of Technology

..... **19.09.2006**

Asst. Prof. Dr. Figen TOKATLI

Department of Food Engineering

İzmir Institute of Technology

..... **19.09.2006**

Asst. Prof. Dr. Duygu KIŞLA

Faculty of Fisheries and Fish Processing Technology Department of Fishing

Ege University

..... **19.09.2006**

Prof. Dr. Şebnem HARSA

Head of Department

İzmir Institute of Technology

.....

Assoc. Prof. Dr. Barış ÖZERDEM

Head of the Graduate School

ACKNOWLEDGEMENTS

Firstly, I would like to thank my advisor Assistant Prof. Canan Tarı, for her supervision, support, patience and confidence during my research. Infact, she has been more than an advisor to me.

I would also like to thank Associate Prof. Ahmet Yemenicioğlu, Assistant Prof. Figen Tokatlı and Assistant Prof. Sevcan Ünlütürk for their support, guidance and the valuable informations they provided.

I also want to express my thankfulness to my friends Fatih Yalçın Güneş Yener, Fatma Işık Üstok and Şelale Öncü for their all kind of support and help.

Finally, I offer sincere thanks to my family members for their support, patience and love.

ABSTRACT

EFFECT OF MORPHOLOGY OF *ASPERGILLUS SOJAE* ON THE PRODUCTION OF PECTINASE AND OPTIMIZATION OF FERMENTATION CONDITIONS

The control of the morphology of fungi needs great attention for the optimal potential production of the product. For this purpose *Aspergillus sojae* ATCC 20235, which has no available literature report on the pectinase production, is used as a model in the determination of the optimum regions for maximum polygalacturonase synthesis and biomass formation with desired pellet morphology by using low cost carbon and nitrogen sources.

Firstly, a full factorial statistical design, with the factors of, two taxonomically different strains, seven types of seed culture formulations (slants) and two types of fermentation media were used to investigate the effect of these parameters on the polygalacturonase (PG) production. According to statistical analysis, factors of strain types and fermentation media and the interaction between them were found significant on the enzyme activity. *Aspergillus sojae* in a complex media, inoculated with a seed culture prepared from molasses resulted in maximum PG activity (0.2 U/ml).

Then, a two step optimization procedure with four factors (concentrations of maltrin and corn steep liquor (CSL), agitation speed and inoculation ratio) was used to investigate the effect of these parameters on the PG activity, mycelia growth (biomass) and morphology (pellet size) of *Aspergillus sojae*. According to the results of response surface methodology (RSM), concentrations of maltrin, CSL and agitation speed were significant ($p<0.05$) on both PG synthesis and biomass formation. As a result, maximum PG activity (13.5 U/ml) was achievable at high maltrin (120 g/l), low CSL (0 g/l), high agitation speed (350 rpm) and high inoculation ratio ($2x10^7$ total spore). The diameter of pellets ranged between 0.05-0.63 cm. The second optimization step improved the PG activity by 74 % and the biomass by 40 %.

Furthermore characterization of the enzyme with respect to its optimum pH and temperature and the effect of these on the stability were considered. Determination of the thermal inactivation constant with its inactivation energy and the substrate specificity constant were estimated.

ÖZET

ASPERGILLUS SOJAE MORFOLOJİSİNİN PEKTİNAZ ENZİMİ ÜZERİNE OLAN ETKİSİ VE FERMANTASYON KOŞULLARININ OPTİMİZASYONU

Küf morfolojisinin kontrolü, potansiyel ürünün en uygun düzeyde üretimi açısından çok önemlidir. Bu amaçla bu çalışmada, pektinaz üretimi üzerine herhangi bir çalışma bulunmayan *Aspergillus sojae* ATCC 20235, maliyeti düşük carbon ve nitrogen kaynakları kullanarak istenilen pellet morfolojisi ile maksimum poligalakturonaz sentezi ve biyokütle oluşumu için optimum koşulların belirlenmesi amacıyla model olarak alınmıştır.

Öncelikle, taksonomik olarak farklı iki küf, yedi değişik aşılama ortamı ile iki farklı fermantasyon ortamının poligalakturonaz (PG) üretimi üzerine etkisi, bu parametrelerin faktör olarak alındığı tam faktöriyel istatistiksel tasarım ile planlanmıştır. Buna göre kullanılan küf ile fermantasyon ortamının ve bunların etkileşiminin PG aktivitesi üzerine etkili olduğu sonucuna varılmıştır. *Aspergillus sojae*'nin melas aşılama ortamında hazırlanan aşı kültürü ile aşılanan komplex besiyeri maksimum PG aktivitesi (0.2 U/ml) göstermiştir.

Daha sonra, maltrin ve mısır şırası şurubu (CSL) konsantrasyonları, çalkalama hızı ve aşılama oranı gibi parametrelerin *Aspergillus sojae* morfolojisi, biyokütle ve PG aktivitesi üzerine etkilerini incelemek amacıyla bu parametrelerin faktör olarak alındığı iki basamaklı optimizasyon prosedürü uygulanmıştır. Tepki Yüzey Yöntemi (RSM) sonuçlarına göre, maltrin ve CSL konsantrasyonları ve çalkalama hızının PG aktivitesi ve biyokütle üzerine etkili olduğu belirlenmiştir. Sonuç olarak, maksimum PG aktivitesine (13.5 U/ml) yüksek maltrin (120 g/l), düşük CSL (0 g/l), yüksek çalkalama hızı (350 rpm) ve yüksek aşılama oranı ($2x10^7$ toplam spor) koşullarında ulaşılmıştır. Pellet çapları ise 0.05–0.63 cm aralığında değişmiştir. İkinci optimizasyon basamağı ile PG aktivitesinde %74, biyokütlede ise %40 artış sağlanmıştır.

Enzim karakterizasyon çalışmalarında optimum pH, sıcaklık ve bunların stabiliteye etkileri belirlenmiş, ayrıca termal inaktivasyon sabiti ve enerjisi ile substrat spesifite sabiti hesaplanmıştır. Böylece üretilen poligalakturonaz enziminin karakteri hakkında bilgi sahibi olunmuştur.

TABLE OF CONTENTS

LIST OF FIGURES ix

LIST OF TABLES x

ABBREVIATIONS xi

CHAPTER 1. INTRODUCTION 1

CHAPTER 2. FUNGI 3

2.1. Fungal Nutrition 3

2.2. Fungal Taxonomy 4

2.3. Fungal Morphology 5

2.4. The Production of Biochemicals by Fungi 7

CHAPTER 3. PECTINOLYTIC ENZYMES 9

3.1. Classification of Pectic Enzymes 9

3.2. Application of Pectinases 13

3.2.1. Fruit Juice Extraction 13

3.2.2. Maceration of Plant Tissue 13

3.2.3. Liquefaction and Saccharification of Biomass 14

3.2.4. Retting and Degumming of Fiber Crops 14

3.2.5. Textile Processing and Bioscouring of Cotton Fibers 14

3.2.6. Treatment of Pectic Wastewater 14

3.2.7. Paper Making 15

3.2.8. Oil Extraction 15

3.2.9. Coffee and Tea Fermentation 15

3.2.10. Animal Feed 15

3.2.11. Purification of Plant Viruses 16

CHAPTER 4. MATERIALS AND METHODS 17
4.1. Materials 17
4.1.1. Microorganisms 17
4.1.2. Chemicals 17
4.2. Methods 18
4.2.1. Spore Production 18
4.2.2. Production Medium 19
4.2.2.1. Full Factorial Statistical Design 19
4.2.2.2. First and Second Step Optimization 19
4.2.3. Enzyme Assay 20
4.2.4. Protein Assay 20
4.2.5. Biomass Determination and Morphological Measurements 20
4.2.6. Experimental Design and Statistical Analysis 21
4.2.6.1. Full Factorial Statistical Analysis 21
4.2.6.2. First and Second Step Optimization 21
4.2.7. Effect of pH on Activity and Stability of Polygalacturonase 23
4.2.8. Effect of Temperature on Activity and Stability 23
4.2.9. Kinetics of the Thermal Inactivation and Estimation of the Inactivation Energy 23
4.2.10. Determination of Kinetic Constants 24

CHAPTER 5. PRELIMINARY STUDIES 25
5.1. *Rhizopus oryzae* Experiments 25
5.1.1. Effect of Media and Temperature on Sporulation 25
5.1.2. Shake Flask Experiments for *Rhizopus oryzae* 25
5.2. *Aspergillus sojae* Experiments 27
5.2.1. Effect of Media and Temperature on Sporulation 27
5.2.2. Effect of Slant Media on the Sporulation of *Aspergillus sojae* 28
5.2.3. Shake Flask Experiments for *Aspergillus sojae* 29

CHAPTER 6. RESULTS AND DISCUSSION 31
6.1. Full Factorial Statistical Design 31
6.1.1 Effect of Type of Strain 31
6.1.2 Effect of Seed Culture Composition 33

6.1.3. Effect of Production Media on the Pectinase Activity 35
6.2. First Optimization Step 38
6.3. Second Optimization Step 41
6.4. Validation of the Models 46
6.5. Effect of pH on Activity and Stability of Polygalacturonase 48
6.6. Effect of Temperature on Activity and Stability of Polygalacturonase 50
6.7. Kinetics of the Thermal Inactivation and Estimation of the Inactivation Energy 52
6.8. Calculation of Kinetic Constants 54

CHAPTER 7. CONCLUSIONS AND FUTURE PERSPECTIVES 56

REFERENCES 58

APPENDICES
APPENDIX A. SEED MEDIA FORMULATIONS 64
APPENDIX B. IMAGES TAKEN BY SCANNING ELECTRON MICROSCOPE 65
APPENDIX C. MORPHOLOGY OF PELLETS GROWN IN COMPLEX AND DEFINED MEDIUM 66
APPENDIX D. RESULTS OF FULL FACTORIAL DESIGN 67
APPENDIX E. RESULTS OF FIRST STEP OPTIMIZATION 69
APPENDIX F. RESULTS OF SECOND STEP OPTIMIZATION 71
APPENDIX G. STANDARD CALIBRATION CURVE FOR POLYGALACTURONASE ACTIVITY 73
APPENDIX H. STANDARD CALIBRATION CURVE FOR TOTAL PROTEIN 74

LIST OF FIGURES

Figure 2.1 Schematic representation of fungal morphologies in suspension culture 6
Figure 3.1 Mode of action of pectinases 11
Figure 6.1 Result of full factorial design: Effect of the interaction of fungal strain and seed culture media on the enzyme activity 32
Figure 6.2 Spore counts obtained using various seed media formulations by Aspergillus sojae and Rhizopus oryzae 34
Figure 6.3 Result of full factorial design: Effect of the interaction of fermentation media and seed culture media on the enzyme activity 35
Figure 6.4 First step optimization activity RSM plots 39
Figure 6.5 First step optimization biomass RSM plots 40
Figure 6.6 Second step optimization activity RSM plots 44
Figure 6.7 Second step optimization biomass RSM plots 45
Figure 6.8 Second step optimization pellet size RSM plots 46
Figure 6.9 Effect of pH on A.sojae polygalacturonase activity 49
Figure 6.10 Effect of pH on A.sojae polygalacturonase stability 50
Figure 6.11 Effect of temperature on A.sojae polygalacturonase activity. 51
Figure 6.12 Effect of temperature on the stability of A.sojae polygalacturonase 52
Figure 6.13 First order plots of the thermal denaturation of A.sojae polygalacturonase 53
Figure 6.14 Arrhenius plots for the determination of the inactivation energy of A.sojae polygalacturonase 54
Figure 6.15 Double reciprocal plots to determine the kinetic constants for polygalacturonic acid hydrolysis by A.sojae polygalacturonase 55
Figure B.1 Images taken by scanning electron microscope 65
Figure C.1 Morphology of pellets grown in complex and defined medium 66
Figure G.1 Standard calibration curve for Polygalacturonase activity 77
Figure H.1 Standard calibration curve for total protein 78

LIST OF TABLES

Table 2.1 Examples of industrial enzymes from fungi. 8
Table 3.1 An extensive classification of pectinolytic enzymes 12
Table.4.1 Chemicals used 17
Table 4.2 Two step optimization factors and their coded levels 22
Table 5.1 Fermentation media formulations used 25
Table 5.2 Results of shake flask experiments for Rhizopus oryzae 27
Table 5.3 Seed media formulations used 28
Table 5.4 Spore count results of Aspergillus sojae 29
Table 5.5 Results of shake flask experiments for Aspergillus sojae 30
Table 6.1 Morphological parameters of different fermentation media which showed pellet morphology 33
Table 6.2 Kinetic parameters for thermal inactivation of polygalacturonase from A.sojae 53
Table A.1 Seed media formulations used in the preparation of spore suspension 64
Table D.1 Effect of factors and interactions on the enzyme activity in full factorial design 67
Table D.2 The mean values and standard deviations of activity measurements in full factorial design 68
Table E.1 CCD results of first optimization step 69
Table E.2 First optimization: the analysis was done using coded units 70
Table F.1 CCD results of second step optimization 71
Table F.2 Experimental and predicted values for Polygalacturonase activity, biomass and pellet size at individual optimal conditions 72

ABBREVIATIONS

ANOVA	Analysis of Variance
ATCC	American Type of Culture Collection
AS	*Aspergillus sojae*
CCD	Central Composite Design
CM	Complex Medium
CSL	Corn Steep Liquor
DM	Defined Medium
DSB	Defatted Soybean
E	Activation energy ($kJmol^{-1}$)
k_0	Frequency factor (min^{-1})
k_d	First-order deactivation rate constant (min^{-1})
K_m	Michaelis-Menten constant (equilibrium constant)
PG	Polygalacturonase
R	Universal gas constant ($Jmol^{-1}K^{-1}$)
PGA	Polygalacturonic Acid
RSM	Response Surface Methodology
RO	*Rhizopus oryzae*
$t_{1/2}$	Half-life time of enzyme (min)
V_m	Maximum Forward Velocity of the Reaction
YME	Yeast Malt Extract
YMP	Yeast Malt Peptone

CHAPTER 1

INTRODUCTION

Enzymes that hydrolyze pectic substances, which contribute to the firmness and structure of plant cells, are known as pectinolytic enzymes or pectinases "(Kaur et al. 2004)". Pectinases are extensively used in the industrial clarification of wine and fruit juice, in tomato pulp and oil extraction, in chocolate and tea fermentation and in vegetable waste treatment. In the fruit juice extraction and clarification process, these are used together with amylases whereby a reduction of 50% in the filtration time is observed "(Jayani et al. 2005, Blanco et al. 1999)". Furthermore, in combination with other enzymes like cellulases, arabinases and xylanases they have shown to increase the pressing efficiency enormously "(Gailing et al. 2000)". Recent applications have emerged in the treatment and degumming of natural fibers used in paper and textile industry "(Almeida 2004, Kashyap 2001)". For example pectinases in conjunction with amylases, lipases, cellulases and hemicellulases have been used to remove the sizing agents from cotton, in a safe and eco-friendly manner by replacing toxic soda "(Hoondal et al. 2000)". Moreover, pectinases are used in animal feed production reducing the feed viscosity and increasing the absorption of nutrients "(Hoondal et al. 2000)". In the industrial market they contribute to almost 25 % of the global enzyme sales, where this contribution is estimated to increase further by the year 2009 "(Jayani et al. 2005)" Therefore in order to meet this high demand, it is highly important to produce pectinase enzyme in a cost effective and productive way.

It is well documented, that several organisms are able to produce pectin degrading enzymes including plants, filamentous fungi, bacteria and yeast "(Silva 2005, Vries 2001, Prade 1999, Behere 1993)". For industrial purposes moulds such as *Aspergillus niger, Coniotryrium diplodiela, Penicillum* and *Rhizopus* species are preferred because as much as 90% of the enzyme can be excreted into the culture medium "(Blandino 2001, Silva 2005, Schwan 2004, Souza 2003)". Pectinase production by filamenteous fungi varies according to the type of strain, cultivation conditions (pH, temperature, aeration, agitation and incubation time) and the growth medium composition (particularly carbon and nitrogen sources), "(Souza 2003, Fedurek 1989)". Therefore these have to be specified individually for each and every single

strain of interest. However, their filamentous growth characteristic creates a number of process engineering problems attributed to the morphological change accounted during the fermentation process in large scales. Therefore the relationship between morphology and the factors influencing these needs to be fully investigated.

In this study, *Aspergillus sojae* ATCC 20235 (from here on *Aspergillus sojae* only), which has been mostly used in the production of a well-known Japanese food (koji), by means of solid-state fermentation "(Bennet 2001)" is considered in the production of polygalacturonase enzyme (PG), which attracts the most attention among the family of pectionolytic enzymes due to its wide use. To best of our knowledge there is no literature report on the pectinase production by this organism in submerged or solid state fermentation. Therefore, this thesis will be one of the initial studies working in this field.

The objective of this study is the investigation of the effect of seed culture and production media compositions on morphology and product formation that is mostly disregarded in the industry and determination of the optimum regions for maximum polygalacturonase (PG) synthesis and biomass formation with a desired pellet morphology using low cost carbon and nitrogen sources. Furthermore characterization of the enzyme with respect to its optimum pH and temperature and the effect of these on the stability will be considered. Additionally thermal inactivation constant with its inactivation energy and the substrate specifity constant will be estimated. Therefore all these will provide information, which will benefit the food and enzyme industry, and also be a new reference point for the microbiology area by providing new knowledge regarding the growth requirement of *Aspergillus sojae*, which has been lacking so far in the literature.

CHAPTER 2

FUNGI

Fungi participate in human society as one of the most significant biotechnologically usefull living cells.

Novel developments are also being made on fungi in agricultural and environmental biotechnology, as well as in the industrial biotechnology beside the traditional fermentation industries.

In the traditional technologies, the manufacture and flavouring of foods and production of such biochemicals as citric acid and antibiotics (penicillin) are materialized by the use of fungi.

2.1. Fungal Nutrition

Fungi are strict obligate chemoheterotrophs, therefore energy-rich substrates are required for fungi to cover their energy and biomass requirements. They consume natural organic substrates such as cellulose, chitin, starch, sugars, hemicelluloses and lignin. Eventually, they fabricate a large number of extracellular enzymes, especially oxidases or hydrolases. Fungi usually use up charbohydrates as the main carbon source so as to get energy and synthesize the cellular material. In addition, alcohols, hydrocarbons, glycerol and starch can be utilized by fungi as carbon source.

Generally all fungi can use nitrate but they use nitrogen mainly in the form of ammonium. Urea, hydroxylamine, L-amino acids and peptides are the other main nitrate sources.

In the laboratory conditions defined media including sugars such as glucose and sucrose, or on polymers such as cellulose can be used to grow fungi. Moreover, complex media like potato-dextrose agar and vegetable based media can also be used for fungi growth. Inorganic nitrogen source is usually in the form of ammonium, nitrate, amides or amino acids and ammonia gas which is used in some industrial fermentation processes.

Other major mineral nutrients required by fungi for maximum yields are phosphorus, sulphur, potassium and magnesium. Such zinc, copper, molybdenum and

vitamins as minor nutrients are also necessary enzyme functionality under rapid growth conditions "(Wainwright 1992)".

2.2. Fungal Taxonomy

According to the production of their sexual spores, fungi have been classified into four classes. These are the;

Ascomycotina, Basidiomycotina, Deuteromycotina (or 'imperfect' fungi) and Phycomycetes, divided into the Mastigomycotina (zoosporic fungi) and the Zygomycotina (those forming zygospores).

The first three classes of fungi show a general increase in complexity, so that the Phycomycetes are known as the simplest of the true fungi, while fungal evolution reaches its top within the Basidiomycetes.

Phycomycetes contain water moulds like *Saprolegnia* and the pin moulds or *Mucor* species.

Ascomycotina is the largest class of fungi, containing the yeasts and a variety of fungi. This class contains the fungi such as *Claviceps purpurea* growing on grasses and as well as some edible morels.

Basidiomycotina are represented by the mushroom seen growing in fields and in mushroom farms.

Deuteromycotina include many industrially important filamentous fungi such as *Aspergillus niger, Penicillium notatum-chrysogenum* and *Trichoderma viride*. They were initially grouped together in this class because they were considered not having a sexual stage.

There are some 200 species of *Aspergillus*, found throughout the world growing on a vast array of substrates. Aspergilli are ubiquitous members of the air mycoflora and so frequently occur as contaminant of culture media. Aspergillus species produce numerous extracellular enzymes many of which are of great importance in biotechnology "(Wainwright 1992)".

2.3. Fungal Morphology

Fungal morphology is often considered as one of the key parameters in industrial production.

Most industrially important fungi are either single-celled yeasts or grow as filaments called hyphae (0.5 μm to 1.0 mm in diameter) and can aggregate to form a mycelium "(Wainwright 1992)".

Fungal spore may be thought as a sign of the onset and the end of the development of a fungus. In a fungal spore, when it is placed in a suitable environment, it is germinated and then the germinated cells often grow that will extend by a thread like cell, i.e., called hyphae. Usually these hyphae will be branched after a certain development. For a cellular growth and hyphal growth, many processes occur at the tip of the hyphae, i.e., the formation of youngest cell "(Panda et al. 2000)".

Fungal fermentation is extensively thought as a complex process with numerous problems. Different fungal morphologies is one of the reasons for this "(Cui et al. 1997)". Two major morphologies like pellet or mycelia are observed by fungal culture in submerged fermentations (Figure 2.1) and they are very much determined by several environmental and genetic factors "(Shukla et al. 2000, Panda et al. 2000)". These are;

- type of the strain,
- pH and composition of the media,
- inoculation ratio,
- type of the inoculum,
- agitation speed, aeration rate,
- feeding rate and
- genetic factors of the culture "(Reichl et al. 1991)".

In industrial applications the reason why pellet morphology is usually preferred in fermentations and in downstream processing is the non viscous (Newtonian) rheology of the broth "(Zhaou et al. 2000, Atkinson et al. 1976)". There are some advantages for the use of pellet form, first of all the mass transfer of oxygen and nutrients is significantly better, secondly, the subsequent separation of pellets from the medium is simpler "(Reichl et al. 1991)". Third, owing to the ease of agitation and aeration in this kind of system, the power input and the operating cost is lower. Unlike pellet

morphology, when the mycelia form is dominant, the broth is much more viscous (non Newtonian) although the cell growth and productivity might be higher. This viscous case causes heterogeneous and stagnant non-mixed zone formations which are harder and more expensive to operate "(Metz et al. 1977)". Hence, the balance between these parameters has to be established in an effective manner. As a conclusion, the control of the morphology is of great importance for productivity and rheological properties of the broth.

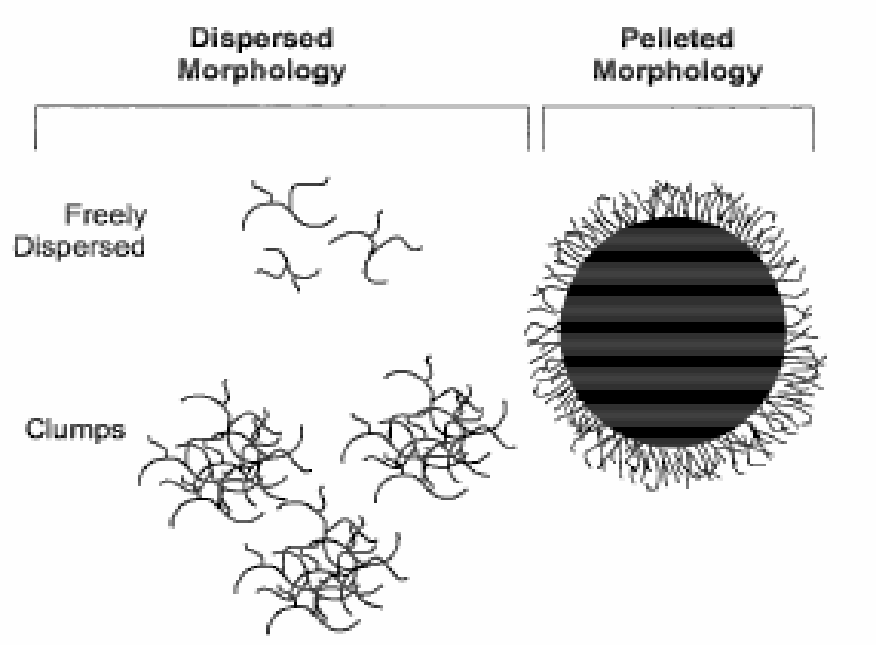


Figure 2.1**.** Schematic representation of fungal morphologies in suspension culture. (Source: Li et al. 2000)

In fungal fermentations different growth morphologies are needed for the purpose of optimum product yield.

The only process in which pellet growth is being widely used is the production of citric acid by Aspergillus niger, whereas pelleted plus filamentous growth have been suitable for penicillin production "(Metz et al. 1977 and Panda et al. 2000)".

Number and sizes of the pellets produced in submerged culture are affected by many factors. Problems related to nutrient limitation and product toxicity is proportionally more acute in larger pellets than smaller pellets. Fungal pellets also have potential applications as immobilized cell system "(Panda T. et al. 2000)".

Pellet formation is grouped into two types;

a) Coagulating type; the spores coagulate in the early stages of development, germinate gradually, and aggregate with other small agglomerates and ungerminated spores to form pellets.

b) Noncoagulating pellet formation; one spore grows out to form one pellet.

The type of pellet formation changes from strain to strain used. Pellets of *Aspergillus niger* were formed from agglomerates of up to 500 spores, whereas *Penicillium chrysogenum* has noncoagulative pellet formation.

Classification of pellets can be made in three groups;

a) Fluffy loose pellets; these pellets have a compact center and a much looser outer zone.

b) Compact smooth pellets; the whole pellet is compact and the outside of the pellet is smooth.

c) Hollow smooth pellets; the center of the pellet is hollow owing to autolysis and the outside is smooth.

The structural properties strongly depend on the culture conditions "(Metz et al. 1977 and Panda et al. 2000)".

2.4. The Production of Biochemicals by Fungi

Fungi produce a wide range of biochemicals, most of which are required by the fungus for growth and metabolism-the primary metabolites. On the other hand, there are also some compounds that are not essential for growth. These are termed as secondary metabolites, which are produced during the non-growing or stationary phase of the fungus.

One of the main biotechnological uses of fungi is the production with the aid of fermentation of a range of organic acids like citric, gallic, gluconic and itaconic acid.

Ethanol is by far the largest fungal fermentation product, in terms of both volume and value. Most of this production is devoted to wine and beer.

Two polysaccharides of fungal origin, scleroglucan and pullulan, are currently produced on an industrial scale.

Chitosan, which is the deacylated derivative of chitin, is found rarely in nature, but is potentially more beneficial in industry. In Japan, for example, crabshell chitin is processed into chitosan by fermentation and used as a flocculating agent for clarifying sewage.

Microorganisms that produce considerable amounts of oil are referred to as oleaginous. Oleaginous fungi produce a more diverse range of lipid types and fatty acids than yeasts. Certain fungi also contain unusual oils and so have been evaluated as a source of dietary essential fatty acids.

Gibberellins are fungal secondary metabolites. They are also formed as endogenous hormones in plants. They are of biotechnological interest because of their growth-promoting properties "(Wainwright 1992)".

Fungi produce a vast array of enzymes (Table 2.1). However, only a relative small number of these enzymes are produced on an industrial scale.

Table 2.1. Examples of industrial enzymes from fungi.
(Source: Wainwright 1992)

Enzyme	*ApplicationProduction species*	
Alpha-amylase	Bread making	Aspergillus niger, A.oryzae, A. awamori
Glucoamylase	Malting	A. niger, A. foetidus, Rhizopus foetidus
Lactase	Feed additive, digestive aid, whey processing	Aspergillus oryzae, A.niger, Kluyveromyces lactis, K. fragilis, Torula cremoris
Invertase	Sucrose conversion (confectionery industry)	Aspergillus oryzae, A.niger Saccharomyces cerevisiae
Pectinases	Fruit processing	Aspergillus niger, A.wentii, Rhizopus sp.
Lipases	Supplement for pancreatic lipase	Penicillium roqueforti, Rhizopus delmar
Penicillin acylases	Semisynthetic penicillin	Penicillium chrysogenum, Aspergillus ochraceus, Trichophyton mentagrophytes, others
Acid proteases	Bread making; chill-proofing bottled beer	Aspergillus saitoi, A. niger, A.oryzae, Trametes sanguineara, Mucor pusillus
Microbial rennin	Cheese making	Endothia parasitica, Mucor meihei, M. pusillus
Cellulases	Research	Trichoderma viride, Aspergillus niger

CHAPTER 3

PECTINOLYTIC ENZYMES

Pectinases or pectinolytic enzymes were one of the first enzymes that were used in homes. They were commercially used first in 1930 for the preparation of wines and fruit juices. Recently, pectinases are one of the promising enzymes of the commercial sector "(Kashyap et al. 2001)".

These enzymes degrade the long and complex molecules called pectin that serve as structural polysaccharides in the middle lamella and in the primary cell walls of plant cells "(Kashyap et al. 2001)". They are mostly found in bacteria, fungi and plants. In the industrial market they contribute to almost 25 % of the global enzyme sales, where this contribution is estimated to increase further by the year 2009 "(Jayani et al. 2005, Thakore 2004)".

Pectinase production by filamentous fungi varies according to the strain, the composition of the growth medium and the cultivation conditions (pH, temperature, aeration, agitation and incubation time) "(Sauza et al. 2003)".

3.1. Classification of Pectic Enzymes

Pectinases are classified under three main groups according to the following criteria:

- Whether pectin, pectic acid or oligo-D-galacturonate is the preferred substrate,
- whether pectinases act by trans- elimination or hydrolysis and
- whether the cleavage is random (endo-, liquefying of depolymerizing enzymes) or endwise (exo- or saccharifying enzymes)

An extensive classification of pectinolytic enzymes is given in table 3.1. The three major types of pectinases are as follows:

1. *Protopectinases:* degrade the insoluble protopectin and give rise to highly polymerized soluble pectin. Protopectinase catalyzes the following reaction:

$$\underset{\text{(insoluble)}}{\text{Protopectin}} + H_2O \xrightarrow{\text{PPase}} \underset{\text{(soluble)}}{\text{pectin}}$$

They are found in the culture filtrates of yeast, yeast-like fungi and *Bacillus* sp.

The enzymes catalyze the hydrolysis of polygalacturonic acid; they decrease the viscosity, slightly increasing the reducing value of the reaction medium containing polygalacturonic acid.

2. *Esterases:* catalyze the de-esterification of pectin by the removal of methoxy esters.

i. Pectin esterases (PE): catalyze the deesterification of methyl ester linkages of galacturonan backbone of pectic substances to release acidic pectins and methanol. The resulting pectin is then acted upon by polygalacturonases and lyases. Fungal PEs act by a multi-chain mechanism, removing the methyl groups at random. They are found in plants, plant pathogenic bacteria and fungi.

3. *Depolymerases:* catalyze the hydrolytic cleavage of the α-(1-4)-glycosidic bonds in the D-galacturonic acid moieties of the pectic substances.

Depolymerases subdivided into two categories. These are;

A. Hydrolases:

i. Polygalacturonase (PG): catalyzes the hydrolytic cleavage of the polygalacturonic acid chain with the introduction of water across the oxygen bridge. They are the most extensively studied ones among the family of pectinolytic enzymes. Endo-PGases are widely distributed among fungi, bacteria, many yeasts and higher plants. In contrast exo- PGases occur less frequently. Fungal exo-PGases produce monogalacturonic acid as the main end product; and the bacterial exo-PGases produce digalacturonic acid as the main end product.

ii. Polymethylgalacturonase (PMG): breakdowns pectin by the mechanism of hydrolysis.

B. Lyases(or transeliminases): perform non-hydrolytic breakdown of pectates or pectinates, characterized by a trans-eliminative split of the pectic polymer.

i. Polygalacturonate lyase (PGL): breakdowns pectate by β-elimination. They are produced by many bacteria and some pathogenic fungi with endo-PGLs being more abundant than exo-PGLs. They have been isolated from bacteria and fungi associated with food spoilage and soft rot.

ii. Polymethylgalacturonate lyase: breakdowns pectin by β-elimination.

Depending upon the pattern of action, these enzymes are termed as Endo or Exo enzymes “(Jayani et al. 2005)”.

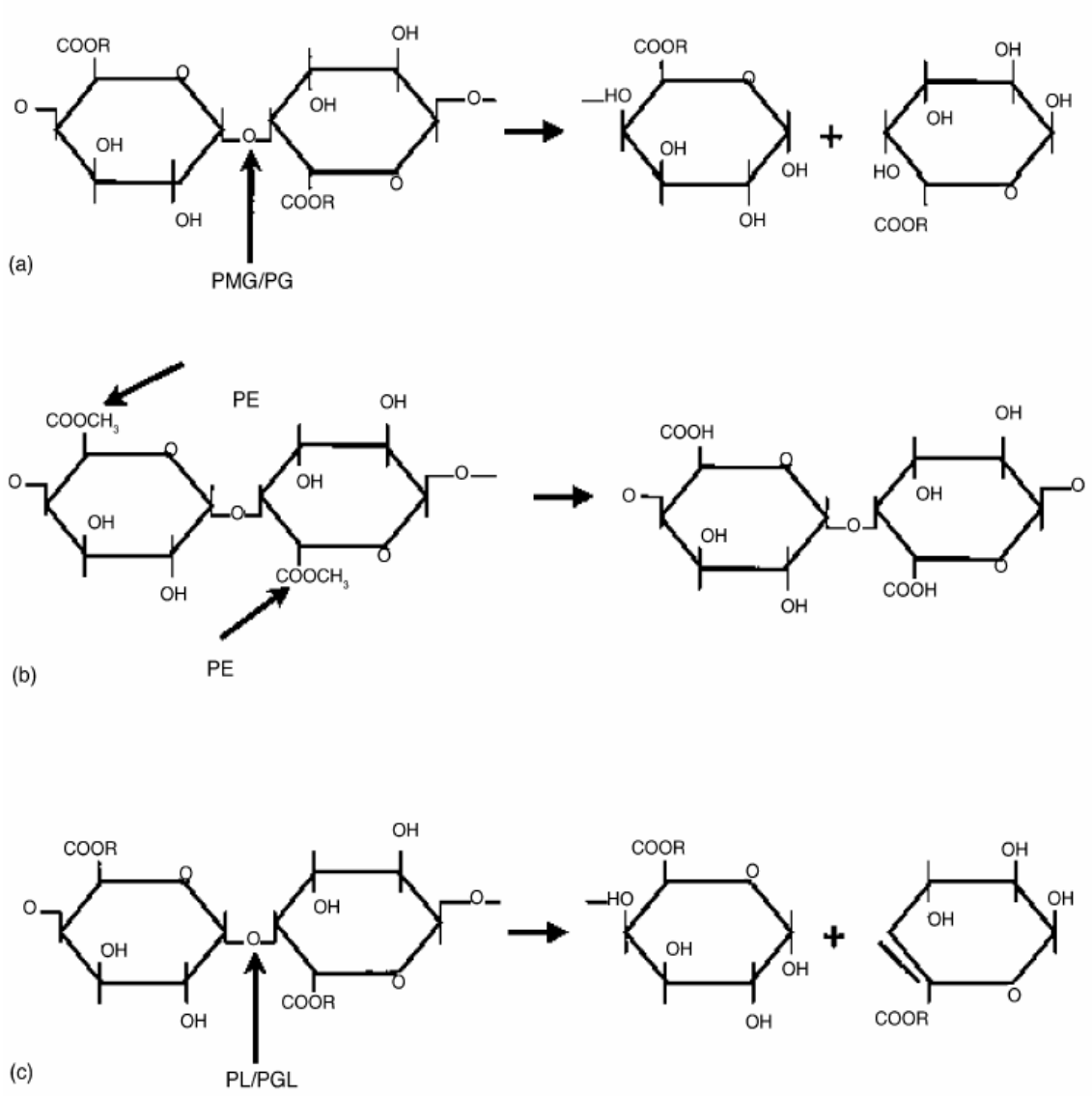


Figure 3.1. Mode of action of pectinases: (a) R = H for PG and CH_3 for PMG; (b) PE; and (c) R = H for PGL and CH_3 for PL. the arrow indicates the place where the pectinase reacts with the pectic substances. PMG, polymethylgalacturonases; PG, polygalacturonases; PE, pectinesterase; PL, pectin lyase (Source: Jayani et al. 2005).

Table 3.1. An extensive classification of pectinolytic enzymes

(Source: Jayani et al. 2005).

An extensive classification of pectinolytic enzymes

Enzyme	E.C. no.	Modified EC systematic name	Action mechanism	Action pattern	Primary substrate	Product
Esterase						
1. Pectin methyl esterase	3.1.1.11		Hydrolysis	Random	Pectin	Pectic acid + methanol
Depolymerizing enzymes						
a. Hydrolases						
1. Protopectinases			Hydrolysis	Random	Protopectin	Pectin
2. Endopolygalacturonase	3.2.1.15	Poly-(1-4)-α-D-galactosiduronate glycanohydrolase	Hydrolysis	Random	Pectic acid	Oligogalacturonates
3. Exopolygalacturonase	3.2.1.67	Poly-(1-4)-α-D-galactosiduronate glycanohydrolase	Hydrolysis	Terminal	Pectic acid	Monogalacturonates
4. Exopolygalacturonan-digalacturono hydrolase	3.2.1.82	Poly-(1-4)-α-D-galactosiduronate digalacturonohydrolase	Hydrolysis	Penultimate bonds	Pectic acid	Digalacturonates
5. Oligogalacturonate hydrolase			Hydrolysis	Terminal	Trigalacturonate	Monogalacturonates
6. Δ4:5 Unsaturated oligogalacturonate hydrolases			Hydrolysis	Terminal	Δ4:5(Galacturonate)$_n$	Unsaturated monogalacturonates & saturated (n-1)
7. Endopolymethyl-galacturonases			Hydrolysis	Random	Highly esterified pectin	Oligomethylgalacturonates
8. Endopolymethyl-galacturonases			Hydrolysis	Terminal	Highly esterified pectin	Oligogalacturonates
b. Lyases						
1. Endopolygalacturonase lyase	4.2.2.2	Poly-(1-4)-α-D-galactosiduronate lyase	Trans-elimination	Random	Pectic acid	Unsaturated oligogalacturonates
2. Exopolygalacturonase lyase	4.2.2.9	Poly-(1-4)-α-D-galactosiduronate exolyase	Trans-elimination	Penultimate bond	Pectic acid	Unsaturated digalacturonates
3. Oligo-D-galactosiduronate lyase	4.2.2.6	Oligo-D-galactosiduronate lyase	Trans-elimination	Terminal	Unsaturated digalacturonates	Unsaturated monogalacturonates
4. Endopolymethyl-D-galactosiduronate lyase	4.2.2.10	Poly(methyl galactosiduronate) lyase	Trans-elimination	Random	Unsaturated poly-(methyl-D-digalacturonates)	Unsaturated methyloligogalacturonates
5. Exopolymethyl-D-galactosiduronate lyase			Trans-elimination	Terminal	Unsaturated poly-(methyl-D-digalacturonates)	Unsaturated methylmonogalacturonates

3.2. Application of Pectinases

Acidic pectinases used in the fruit juice industries and wine making often come from fungal sources, especially from *Aspergillus niger*.

3.2.1. Fruit Juice Extraction

Pectins may cause problems in the food industry by giving rise to turbidity and viscosity during the extraction, filtration, and clarification of fruit juices "(Gonzales et al. 2004)".

In the case of sparkling clear juices (apple, pear and grape juices) enzymes are added in order to increase the juice yield during pressing. A combination of pectinases and cellulases gives a juice yield up to 100%.

Pectic enzymes from fungi, typically *Aspergillus niger*, *Penicillium notatum*, or *Botrytis cinerea* are useful in wine making "(Kashyap et al. 2001)".

A mixture of pectinases and amylases is used to clarify fruit juices. It decreases filtration time up to 50%.

Vacuum infusion of pectinases has a commercial application to soften the peel of citrus fruits for removal "(Jayani et al. 2005)".

Pectic enzymes containing high levels of polygalacturonase activity are added to fruit juices to stabilize the cloud of citrus juices, purees and nectars.

3.2.2. Maceration of Plant Tissue

Maceration is a process by which organized tissue is transformed into a suspension of intact cells, resulting in pulpy products used as base material for pulpy juices and nectar, as baby foods and an ingredient for dairy products such as puddings and yogurts.

The aim of enzymatic treatment is the transformation of tissue into a suspension of intact cells "(Kashyap et al. 2001)".

3.2.3 Liquefaction and Saccharification of Biomass

A large spectrum of enzymes (pectinases, cellulases, hemicellulases) is needed for the production of fermentable sugars from polysaccharides and for the disruption of the cell wall matrix, in order to liquefy the material and to release the intracellular carbohydrates.

Alkaline pectinases are mainly used in the degumming and retting of fiber crops and pre-treatment of pectic wastewater from fruit juice industries. These enzymes originate mostly from bacteria "(Kashyap et al. 2001)".

3.2.4. Retting and Degumming of Fiber Crops

Retting is defined as a fermentation process in which certain bacteria (e.g. *Clostridium, Bacillus*) and certain fungi (e.g. *Aspergillus, Penicillium*) decompose the bark and release fiber "(Kashyap et al. 2001)".

The fibers contain gum, which must be removed before its use for textile making. Biotechnological degumming using pectinases in combination with xylanases presents an ecofriendly and economic alternative to the chemical degumming treatment "(Jayani et al. 2005)".

3.2.5. Textile Processing and Bioscouring of Cotton Fibers

Pectinases have been used in conjunction with amylases, lipases, cellulases and hemicellulases to remove sizing agents from cotton in a safe and ecofriendly manner.

Pectinases have been used for bioscouring which is a process for removal of non-cellulosic impurities from the fiber with specific enzymes "(Jayani et al. 2005)".

3.2.6. Treatment of Pectic Wastewater

The wastewater from the vegetable food processing industries contains pectinaceous materials. Pre-treatment of these wastewaters with pectinolytic enzymes, facilitates removal of pectinaceous material and renders it suitable for decomposition by activated sludge treatment "(Jayani et al. 2005)".

3.2.7. Paper Making

Pulp and paper mills are beginning to use enzymes to solve problems in their manufacturing processes. Pectinase can depolymerise polymers of galacturonic acids, and subsequently lower the cationic demand of pectin solutions and the filtrate from peroxide bleaching "(Kashyap et al. 2001)".

3.2.8. Oil Extraction

Cell-wall-degrading enzymes, including pectinase, may be used to extract vegetable oil in an aqueous process by liquefying the structural cell wall components of the oil-containing crop.

Recently, the plant cell-wall-degrading enzyme preparation has begun to be used in olive oil preparation. The enzymes are added during grinding of the olives, thereby the oil is released easily in the subsequent separation techniques "(Kashyap et al. 2001)".

3.2.9. Coffee and Tea Fermentation

Fermentation of coffee using pectinolytic microorganisms is done to remove the mucilage coat from the coffee beans. Pectic enzymes are sometimes added to remove the pulpy layer of the bean, three-fourths of which consists of pectic substances. The fermentation stage of coffee processing is accelerated.

Fungal pectinases are also used in the manufacture of tea. Enzyme treatment accelerates tea fermentation and improves the foam-forming property of instant tea powders by destroying tea pectins "(Kashyap et al. 2001)".

3.2.10. Animal Feed

Pectinases used for the production of animal feeds, reduces the feed viscosity which increases adsorption of nutrients.

3.2.11. Purification of Plant Viruses

Alkaline pectinases and cellulases can be used to liberate the virus from the tissue to give very pure preparations of the virus “(Jayani et al. 2005)”.

Future studies on pectic enzymes should be devoted to the understanding of the mechanism of action of different pectinolytic enzymes of different microbial sources.

With this perspective, two strains of different taxonomies, such as *Aspergillus sojae* belonging to the subdivision of Deutoromycotina and *Rhizopus oryzae* belonging to the subdivision of Zycomycotina were used (Figure B1) as a model to unveil the problems associated with the morphology, and at the same time optimize the production of an industrially important enzyme (polygalacturonase) which has many applications in the food, textile, paper industries and waste treatment process.

CHAPTER 4

MATERIALS AND METHODS

4.1. Materials

4.1.1. Microorganisms

Aspergillus sojae ATCC 20235 and *Rhizopus oryzae* ATCC 4858 were purchased in the lyophilized form, from Procochem Inc., an international distributor of ATCC (American Type of Culture Collection) in Europe.

4.1.2. Chemicals Used

Table 4.1. Chemicals used

NO	CHEMICAL	CODE
1	Ammonium heptamolybdate-tetrahydrate	Merck 1.01182
2	Bacteriological Agar	Oxoid LP0011
3	Brain Heart Infusion Agar	Fluka 70138
4	Copper(II)sulphate-5-hydrate	Riedel-De Haën 12849
5	Corn steep liquor	Sigma C4648
6	D-Galacturonic acid	Fluka 48280
7	D-Glucose	AppliChem A3666
8	di-Sodium hydrogen arsenate heptahydrate	Fluka 71625
9	Folin-Ciocateu's phenol reagent	Merck 1.09001
10	Glycerol	Sigma G5516
11	Iron(II)sulphate heptahydrate	Riedel-De Haën 12354
12	K_2HPO_4	Riedel-De Haën 04243

Table 4.1. Chemicals used (cont.)

NO	CHEMICAL	CODE
13	KCl	Riedel-De Haën 31248
14	Malt Extract	BD 218630
15	Maltrin	Cargill Starch & Sweeteners
16	Manganese(II)sulphate monohydrate	Riedel-De Haën 13255
17	Molasses	Pakmaya Kemalpaşa Üretim Tesisi
18	$MgSO_4.7H_2O$	Merck 1.05886
19	NaCl	Riedel-De Haën 13423
20	NaOH	Merck 1.06462
21	Peptone	Acumedia 7182A
22	Polygalacturonic acid	Sigma P3850
23	Potassium sodium tartarate tetrahydrate	Sigma S6170
24	Protein standard, 2mg BSA	Sigma P5619-25VL
25	Sodium carbonate anhydrous	AppliChem A3900
26	Sodium dihydrogen phosphate monohydrate	Fluka 71507
27	Sodium phosphate di basic dihydrate	Riedel-De Haën 04272
28	Yeast Extract	Fluka 70161

4.2. Methods

4.2.1. Spore Production

The propagation of the cultures was done on YME agar slant medium containing, malt extract (10 g/l), yeast extract (4 g/l), glucose (4 g/l) and agar (20 g/l), incubated at 30 $^{\circ}$ C until well sporulation (1 week). Stock cultures of these strains were prepared with 20 % glycerol water and stored at -80 $^{\circ}$C.

The spore suspensions used as inoculum were obtained on molasses agar slants containing glycerol (45 g/l), peptone (18 g/l), molasses (45 g/l), NaCl (5 g/l),

$FeSO_4.7H_2O$ (15 mg/l), KH_2PO_4 (60 mg/l), $MgSO_4$ (50 mg/l), $CuSO_4.5H_2O$ (12 mg/l), $MnSO_4.H_2O$ (15 mg/l) and agar (20 g/l), after the pre-activation step performed on YME agar using the stock cultures. The incubation temperature and time for each of the steps were 30 °C and one week, respectively. The harvesting of the spores from the slants was done using 5 ml of Tween80-water (% 0.02). The spore suspension was collected in a sterile falcon tube and stored at 4 °C until the actual study. The initial spore counts and viability counts were recorded.

4.2.2. Production Medium

4.2.2.1. Full Factorial Statistical Design

Total of 56 shake flasks media (50 ml in 250 ml Erlenmeyer), including the duplicates, were prepared according to the full factorial design, with two different media formulations described as complex and defined media, with the compositions given in Table A1 without agar. These were inoculated with $5x10^5$ total spores, coming from the slants prepared in the previous section 4.2.1. and incubated for 96 hours at 250 rpm and 30 ° C. After this period, each flask was assayed for enzyme activity. The final pH of the flasks ranged between 3-3.5. Enzyme activity was determined on supernatant obtained after the centrifugation of the broth at 6000 rpm for 15 minutes.

4.2.2.2. First and Second Step Optimization

Total of 31 shake flasks media (50 ml in 250 ml Erlenmeyer), including the replicates, were prepared according to the CCD (Central Composite Design) (Table E1 and F1) for each optimization step and experiments were conducted at 30 °C for 96 hours. The basal medium consisted of glucose (25 g/l), peptone (2.5 g/l), disodium phosphate (3.2 g/l) and monosodium phosphate (3.3 g/l). In addition to this medium, maltrin and corn steep liquor were added according to the concentrations given in the design. After 96 hour of incubation, each flask was assayed for enzyme activity, fungal morphology and biomass. Enzyme activity was determined on supernatant obtained after the centrifugation of the broth at 6000 rpm for 15 minutes.

4.2.3. Enzyme Assay

PG (polygalacturonase) activity was assayed according to the procedure given by Panda et al. (1999) by using 2.4 g/l of polygalacturonic acid as substrate at pH 6.6 and 26 ^{o} C. The amount of substrate and enzymes used were 0.4 and 0.086 ml respectively. The absorbance was read on Varian Cary Bio 100 UV-Visible spectrophotometer at 500 nm. In this study, one unit of enzyme activity was defined as the amount of enzyme that catalyses the release of 1 µmol of galacturonic acid per unit volume of culture filtrate per unit time at standard assay conditions. Galacturonic acid was used as standard for the calibration curve of PG activity. Calibration curve was prepared using 50, 100, 200, 300, 400, 500 µl concentrations of the stock solution containing 500nmol galacturonic acid in 500 µl.

$$\text{Activity (U/ml)} = (\text{mg of galacturonic acid } /212.12) \times (1/20) \times (1/0.086) \qquad (4.1)$$

Where, 212.12 is the molecular weight of galacturonic acid (mg/mole), 20 is the reaction time (min.) and 0.086 is the amount of enzyme in the reaction mixture (ml).

4.2.4. Protein Assay

The total protein contents of the samples were determined according to the method described by Lowry. Protein standard calibration curve was prepared in the range of 0-300 µg/ml with the protein standard Bovine Serum Albumin. The absorbance was read at 750 nm using Varian Cary Bio 100 UV-Visible spectrophotometer.

4.2.5. Biomass Determination and Morphological Measurements

The biomass expressed as dry cell weight (g/l) was determined by means of gravimetric method. The fermentation broth was filtered through the pre weight Whatman No.1 filter paper, followed by drying to constant weight at 40 $^{\circ}$C for approximately 24 hours.

Pellet morphology was characterized by using image analysis (Cox and Thomas 1992). Pellet particles were analyzed for determination of the number of pellet per given volume and pellet size. Image analysis was performed with the software package Image-Pro Plus 4.5.1 (Media Cybernetics Inc., Silver Spring, MD, USA). The size of the pellet was quantified using the diameter corresponding to a circular area equivalent to the pellet projected area "(Lopez et al. 2005)".

4.2.6. Experimental Design and Statistical Analysis

4.2.6.1. Full Factorial Statistical Design

Full factorial design was used in order to identify important parameters in the screening analysis. The factors were the strain, media and slant types. As the strain types, *Aspergillus sojae ATCC 20235* and *Rhizopus oryzae ATCC 4858* were used. Two different fermentation media formulations were used as defined and complex media. As the slant types, seven different slant medium were used; YME, YMP, ATCC, molasses, DSB, CA and DA (Table A1). Total of 56 experiments were conducted with 2 replicates of each factorial combination. Analysis of data was done by Modde 7 statistical software.

4.2.6.2. First and Second Step Optimization

In this study, the effects of independent variables, concentrations of maltrin (X1) and corn steep liquor (X2), inoculation ratio (X3) and agitation speed (X4) were investigated on the responses of PG activity, biomass and morphology (pellet size) using RSM (Response Surface Methodology). A face centered CCD design with four factors were generated and conducted.

Analysis of data and generation of response surface graphics was done by Minitab statistical software (Release 13). After running the experiments and measuring the PG activity levels, biomass and pellet sizes, a second order model including interactions was fitted to the response data:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_i \sum_j \beta_j X_i X_j + \varepsilon \quad (4.2)$$

Where Y is the predicted response, k is the number of factor variables, $ß_o$ is the model constant, $ß_i$ is linear coefficient, X_i is the factor variable in its coded form, $ß_{ii}$, is the quadratic coefficient, $ß_{ij}$, is the interaction coefficient. The following equation is used for coding the actual experimental values of the factors in a range of [-1 +1]:

$$x = [\text{actual}–(\text{low level}+\text{high level})/2]/ (\text{high level}–\text{low level})/2 \quad (4.3)$$

The analysis of variance (ANOVA) tables were generated and the effect and regression coefficients of individual linear, quadratic and interaction terms were determined. The significances of all terms in the polynomial were judged statistically according to the p-value. P values were compared to the significance level of 5%. In the second optimization step 3 out 31 experiments resulted in clump type of morphology. The mean diameter of these was taken as 3.0 cm for the evaluation of the results.

Table 4.2. Two step optimization factors and their coded levels

Factors	First step optimization coded levels	Second step optimization coded levels
Maltrin conc.(g/l)	25(-1) and 75(+1)	50(-1) and 120(+1)
CSL conc. (g/l)	2.5(-1) and 15(+1)	0(-1) and 5(+1)
Agitation (rpm)	150(-1) and 300(+1)	150(-1) and 350(+1)
Inoculation rate (total spore)	$2.5x10^5$(-1) and $7.5x10^5$(+1)	$1.25x10^4$(-1) and $2x10^7$(+1)

4.2.7. Effect of pH on Activity and Stability of Polygalacturonase

The effect of pH on the activity of polygalacturonase was determined by assaying the enzyme activity at different pH values ranging from 3.0 to 7.0 using 0.1 M of the following buffer systems: citrate (pH 3.0), acetate (4.0, 5.0) and phosphate (pH 6.0, 7.0). The relative activities were based on the ratio of the activity obtained at certain pH to the maximum activity obtained at that range and expressed as percentage. The pH stability of *Aspergillus sojae* polygalacturonase was investigated in the pH range of 3.0-8.0 using 0.1 M of citrate (pH 3.0), acetate (4.0, 5.0) and phosphate (pH 6.0, 7.0, 8.0) buffer systems. Therefore, 2ml of the crude enzyme was mixed with 2ml of the buffer solutions mentioned above and incubated at 30ºC for 2 hours. Afterwards aliquots of the mixtures were taken to measure the residual polygalacturonase activity (%) with respect to control, under standard assay conditions.

4.2.8. Effect of Temperature on Activity and Stability

The effect of temperature on the activity of polygalacturonase was determined by performing the standard polygalacturonase assay procedure at different temperatures ranging from 25 to 75ºC (25, 37, 45, 55, 65, 75^{o}C). Before the addition of enzymes, the substrate (0.24% (w/v) polygalacturonic asit) was preincubated at the respective temperatures for 10 minutes. The relative activities (as percentages) were expressed as the ratio of the polygalacturonase activity obtained at certain temperature to the maximum activity obtained at the given temperature range. The thermostability of the crude polygalacturonase was investigated by measuring the residual activity after incubating the enzyme at various temperatures ranging from 25 to 55^{o}C (25, 37, 45 and 55^{o}C) for 15, 30 and 60 minutes.

4.2.9. Kinetics of Thermal Inactivation and Estimation of the Inactivation Energy

Thermal inactivation kinetics of polygalacuronase was studied by incubating the crude enzyme at different temperatures (65, 75 and 85^{o}C) in the absence of the substrate. Aliquotes were withdrawn at periodic intervals and cooled in an ice bath prior

to assay as described above. The residual activity was expressed as % of the initial activity. From a seminaturallogarithmic plot of residual activity versus time, the inactivation rate constants (k_d) were calculated (from slopes), and apparent half lives were estimated using the equation 4.4. The half-life ($t_{1/2}$) is known as the time where the residual activity reaches 50 %.

$$t_{1/2} = \frac{\ln 2}{k_d} \tag{4.4}$$

The temperature dependence of k_d was analyzed using the Arrhenius plot "(Shuler and Kargi 2002)". The inactivation energy was calculated from the Arrhenius equation as:

$$k_d = k_0 \exp\left(-\frac{E}{RT}\right) \tag{4.5}$$

or,

$$\ln (k_d) = \ln(k_0) - \left(\frac{E}{R}\right)\frac{1}{T} \tag{4.6}$$

The values of E and k_0 were estimated from the slope and intercept of the plot of ln (k_d) versus 1/T respectively. (4.6)

4.2.10. Determination of Kinetic Constant

The kinetic constants (V_{max} and K_m) were determined using Lineweaver –Burk double reciprocal (1/V versus 1/S) plot "(Shuler and Kargi 2002)" where different concentrations of polygalacturonic acid was used as substrate (0.125-1 mg/ml) at pH 6.6.

$$\frac{1}{v} = \frac{1}{V_m} + \frac{K_m}{V_m}\frac{1}{[S]} \tag{4.7}$$

With a slope of K_m/V_m and intercept of $1/V_m$ (equation 4.7) estimated K_m and V_m values were calculated as given in double-reciprocal plot (Figure 6.15).

CHAPTER 5

PRELIMINARY STUDIES

5.1. *Rhizopus oryzae* Experiments

5.1.1. Effect of Media and Temperature on Sporulation

In order to investigate the effect of temperature and media on the sporulation, *Rhizopus oryzae* ATCC 4858 was inoculated on malt extract agar, YMP agar, YME agar and ATCC media (corn meal agar) at three different temperatures (24^{o}C, 30^{o}C and 37^{o}C).

According to this study it was decided to incubate *Rhizopus oryzae* at 30^{o}C using malt extract agar where maximum sporulation was observed. This media served as slant media for further studies.

5.1.2. Shake Flask Experiments for *Rhizopus oryzae*

Shake flask experiment was done with seven different fermentation media formulations (Table 5.1) in order to observe the morphology of *Rhizopus oryzae* at the end of the fermentation.

Table 5.1. Fermentation media formulations used

Media Components	**Concentrations (g/l)**
Media I:	
Glucose	50
Yeast extract	2.5
Peptone	2.5
Disodyum phosphate	3.2
Monosodium phosphate	3.3
pH: 5.9-6.0	

Table 5.1. Fermentation media formulations used (cont.)

Media Components	Concentrations (g/l)
Media II:	
Glucose	25
Matrin 50	25
Yeast extract	2.5
Peptone	2.5
Disodyum phosphate	3.2
Monosodium phosphate	3.3
pH: 5.9-6.0	
Media III	
Glucose	50
Yeast extract	2.5
Peptone	2.5
Disodyum phosphate	3.2
Monosodium phosphate	3.3
$MgSO_4.7H_2O$	0.5
$ZnSO_4.7H_2O$	0.0176
$FeSO_4.7H_2O$	0.498 (mg)
pH: 5.9-6.0	
Media IV	
Glucose	50
Corn steep liquor	2
KH_2PO_4	0.6
$MgSO_4.7H_2O$	0.5
$ZnSO_4.7H_2O$	0.0176
$FeSO_4.7H_2O$	0.498 (mg)
pH: 5.9-6.0	
Media V	
Glucose	50
Yeast extract	2
KH_2PO_4	0.6
$MgSO_4.7H_2O$	0.5
$ZnSO_4.7H_2O$	0.0176
$FeSO_4.7H_2O$	0.498 (mg)
pH: 5.9-6.0	
Media VI	
Glucose	50
Peptone	2
KH_2PO_4	0.6
$MgSO_4.7H_2O$	0.5
$ZnSO_4.7H_2O$	0.0176
$FeSO_4.7H_2O$	0.498 (mg)
pH: 5.9-6.0	
Media VII	
Glucose	50
Yeast extract	1
Peptone	1
KH_2PO_4	0.6
$MgSO_4.7H_2O$	0.5
$ZnSO_4.7H_2O$	0.0176
$FeSO_4.7H_2O$	0.498 (mg)
pH: 5.9-6.0	

The inoculation ratio was $5x10^5$ total spores in the broth. The shaker speed was adjusted to 250 rpm and 30^oC throughout the experiment.

After 24 hours, morphology in the form of small long chain like pellets with clear liquid was observed in all media formulation exept for media 1. Media 1 was the only media that had white mycelia until the end of the experiment. After 144 hours fermentation was stopped. The final pH, measured in the total broth, and morphological observation are given in the following table.

Table 5.2. Results of Shake Flask Experiments for *Rhizopus oryzae*

Media	***Colour***	***Final PH (broth) at 23C***	***Morhology***
Media 1	Medium yellow	4.16	Loose Mycelia
2	Dark brown	7.32	Clump
3	Dark yellow	3.45	Clump
4	Light brown	2.82	Clump
5	Light yellow	2.7	Clump
6	Light yellow	2.5	Clump
7	Light yellow	2.52	Clump

As it is seen here except for media 2 the final pH at the end of the fermentation was very low and resulted into clump formation. One explanation is that this strain is forming only clump irrespective of the environmental conditions-because of its genetic make up. However there are couple of papers reporting pellet type of morphology. Therefore this feature has to be exploided in future studies.

5.2. *Aspergillus sojae* Experiments

5.2.1. Effect of Media and Temperature on Sporulation

In order to investigate the effect of temperature and media on the sporulation, *Aspergillus sojae* ATCC 20235 was inoculated on malt extract agar, YMP agar and ATCC media (Brooks Agar) at three different temperatures (24^{o}C, 30^{o}C and 37^{o}C).

The growth temperature at each level seemed not to matter, but 30°C seemed to be the best. YMP and ATCC media seemed to be good sporulated but for further investigations as discussed below several more slant media formulations were prepared.

5.2.2. Effect of slant media on the sporulation of *Aspergillus sojae*

Five different media slants (Table 5.3) were prepared and inoculated with a loop full culture of original ATCC culture. For each media 8 slants were used. All the slants after inoculation were incubated at 30°C. Humidity inside the incubator was controlled by a flask full of water placed inside.

Table 5.3. Seed media formulations used

Media	***Concentrations (g/l)***
YME (Yeast malt extract agar)	
Malt extract	10
Yeast extract	4
Glucose	4
Agar	20
YMP: (Yeast malt pepton agar)	
Malt extract	3
Yeast extract	3
Peptone	3
Glycerol	20
Agar	20
Molasses media:	
Glycerol	45
Peptone	18
Molasses	45
NaCl	5
$FeSO_4.7H_2O$	15 mg
KH_2PO_4 (1,2 %w/v)	5 ml of the stock
$MgSO_4$ (1 % w/v)	5 ml of the stock
$CuSO_4.5H_2O$ (0.24 % w/v)	5 ml of the stock
$MnSO_4.H_2O$ (0.3 % w/v)	5 ml of the stock
Agar	15
Defatted soybean media:	
Defatted soybean	5
Malt extract	20
KH_2PO_4	10
Agar	20
ATCC media: according the instructions	
Corn meal	5
Yeast extract	2
Casitone	1
Malt extract	1
$MgSO_4.7H_2O$	0.5
KH_2PO_4	1.5
Agar	20

After two weeks of incubation it was observed that best sporulation as given on Table 5.4 was observed on molasses media.

Table 5.4. Spore count results of *Aspergillus sojae* in 30 ml suspension.

Media	***Spore count (spore/ml)***
YMP	$1.16x10^7$
YME:	$0.76\ x10^7$
Molases media:	$\mathbf{4.24x10^7}$
ATCC media:	$0.0706x10^7$
Defatted soybean media:	$2.86x10^7$

5.2.3. Shake Flask Experiments for *Aspergillus sojae*

In order to determine the interaction of the seed media formulations with the two fermentation media (media 2 and 4) formulations, 10 shake flasks (65 ml) were inoculated with 5×10^5 total spores containing media 2 or media 4, (on Table 5.1) at initial pH of 5.9 and incubated at 250 rpm, 30^{o}C for 96 hours. The *Rhizopus oryzae* culture was used also in two shake flasks media 2 and 4 containing 0.13 % of tween 80 incubated under the same conditions. At 18–24 hours all *Aspergillus sojae* cultures resulted in pelleted growth with different sizes and densities, however both *Rhizopus oryzae* cultures resulted in clumps. The effect of seed media formulation might have had more effect on the size and densities of the pellets formed in both media (media 2 and 4) for *Aspergillus sojae*.

Table 5.5. Results of Shake Flask Experiments for *Aspergillus sojae*

Shake flask media media	Slant culture media of Aspergillus sojae	Final pH at 96 hours	Pellet morhology (dense , smooth, loose, dark or light centered or no center, mycelia, clump)
Media 2	YME	4.97	Light, no center, smooth pellet
Media 2	YMP	5.05	Light, no center, smooth pellet
Media 2	ATCC	4.58	Light, no center, different sized pellets
Media 2	Defatted	-	Light, no center, dense pellets
Media 2	Mollases	4.84	Light, no center small, dense pellets
Media 4	YME	3.24	Clump is grown, centered, small, dark, dense pellets
Media 4	YMP	3.25	One long clump and centered, too small dark pellets
Media 4	ATCC	3.21	One small clump, too small, dark, centered pellets
Media 4	Defatted	3.17	One big, dark clump
Media 4	Mollases	3.17	One dark clump
Media 2 with tween 80 Rhizopus		2.98	One clump
Media 4 with tween 80 Rhizopus		2.69	One dark clump

For *Aspergillus sojae* media 2 promoted more pellet type of growth with higher final pH. However media 4 resulted in a mixed type of morphology (clump with pellets) with a lower final pH. One conclusion would be that final lower pH was promoting more clump type of growth, which was very much pronounced for *Rhizopus oryzae* irrespective of the media formulation used.

CHAPTER 6

RESULTS AND DISCUSSION

6.1. Full Factorial Statistical Design

6.1.1. Effect of Type of Strain

There are number of reports showing that various fungal fermentations require different growth morphologies for optimum product yield "(Pamboukian et al. 1998)". For example pelleted growth is preferred for itaconic acid production by *Aspergillus terrreus*, whereas pelleted and filamentous growth has been used for penicillin production industrially. However, only filamentous growth is necessary for maximum pectic enzyme production by *Aspergillus niger* and for fumaric acid production by *Rhizopus arrhizus* "(Metz et al. 1997, Bryne et al. 1998 and Calam 1976)". Therefore, it is highly important to determine the right morphology required for the optimum desired product formation. With this regard, two strains of different taxonomies, such as *Aspergillus sojae* belonging to the subdivision of Deutoromycotina and *Rhizopus oryzae* belonging to the subdivision of Zycomycotina were used (Figure B1) in order to determine their effect on enzyme activity under study. Another objective was also to provide a comparison which of these two strains would be the right choice for pectinase production with the desired morphology.

Statistical analysis of full factorial design is presented in Table D1 and D2. Small p-values ($p < 0.01$) show that the parameter is significant on the response at the 1 % level of significance. Factors of strain types and fermentation media and the interaction between them were found significant on the enzyme activity, whereas the slant types were found insignificant. Interaction between strain types and slants, and interaction between fermentation media and slants were also found insignificant (p-value $>> 0.01$). *Aspergillus sojae* produced almost 4-times more pectinase enzyme than *Rhizopus oryzae* in defined medium (Figure 6.1.).

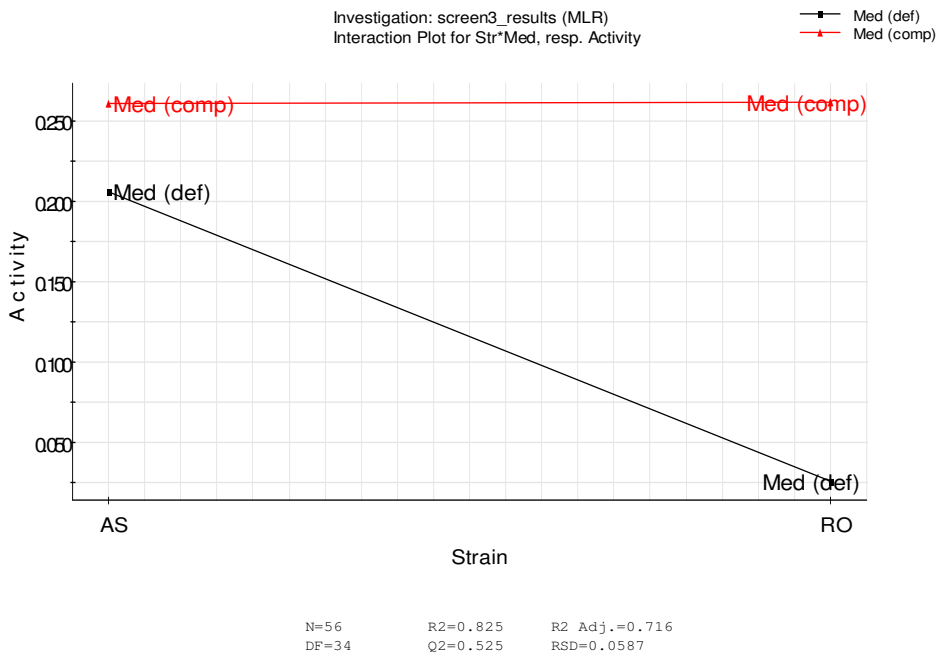


Figure 6.1. Result of full factorial design: Effect of the interaction of fungal strain and seed culture media on the enzyme activity (AS: *Aspergillus sojae*; RO: *Rhizopus oryzae;* def: defined; comp: complex)

However, in the complex medium this difference could not be detected. From here, one can get the impression that, *Rhizopus oryzae* has either more nutritional requirements that is not present in defined medium or there are some inhibitor(s) which might be present initially or formed later. On the other hand, *Aspergillus sojae* is demonstrating a more robust behavior with respect to the nutritional requirement.

Considering the morphological parameters, *Aspergillus sojae* resulted almost in all runs into pellet morphology (Table 6.1.) with higher enzyme activity, where Rhizopus *oryzae* on the contrary exhibited clump type morphology only. The enzyme synthesis of *Rhizopus oryzae* was not affected by the morphology. However, in the literature there are number of studies, "(Znidarsic et al. 2000 and Du et al. 2003)" reporting pellet morphology for *Rhizopus* spp. similarly there is no literature to best of our knowledge, on the morphology of *Aspergillus sojae* showing the present form in submerged fermentation. Hence, the current study will provide new perspective in this field.

Table 6.1. Morphological parameters of different fermentation media which showed pellet morphology.

Media	Bulk Pellet Density (kg/m^3)	Number of pellet (in 13 ml sample)	Pellet Volume Fraction (v/v)	Pellet % (w/v)	Enzyme activity (unit/ml)
ADYME	0.9576	36	0.0854	7.7725	0.045
ADYMP	0.9589	240	0.2000	19.0042	0.0093
AMATCC	0.7123	39	0.1026	6.9271	0.060
ADM	0.7568	160	0.2250	16.9570	0.052
ACM	**3.6846**	**103**	**0.0513**	**18.0470**	**0.2073**
ADDSB	0.8599	109	0.2000	14.6803	0.0568
ACDSB	0.9539	144	0.2308	21.1917	0.1848
ADCA	2.3532	49	0.1026	22.9508	0.1259

6.1.2. Effect of Seed Culture Composition

It is well documented that the fermentation media compositions with respect to its carbon, nitrogen sources and trace elements have significant influence on the optimum product formation and on the morphology of the culture "(Du et al. 2003, Jonsbu et al. 2002 and Taherzadeh et al. 2003)". However, there is no literature to best of our knowledge on the media compositions used for the preparations of the seed cultures and their effects. It is industrially very important to generate productive spore suspensions with high counts promoting the desired amount of product formation and type of morphology, which eases the down stream processing. These can only be obtained with the right media compositions and environmental conditions used in the preparation of the seed cultures, assuming that genetic factors of the culture is not a determining factor. Therefore, especially for industrial fermentations requiring spore type of inoculations, these become very critical issues requiring special attention.

With this perspective, 7 different compositions as outlined in Table A1 were considered in the preparation of the seed culture media. These compositions were based on literature and on our own experience "(Zhaou et al. 2000 and Difco and BBL Manual 2003)". The spore counts obtained from these spore suspensions are presented in Figure 6.2. As it is observed among all the compositions, the molasses and defatted soy bean media promoted more spore growth for *Aspergillus sojae.* However, the least favorable one was with composition suggested by ATCC. The same results were YMP and ATCC media for *Rhizopus oryzae,* respectively. It was very interesting to note that

for both cultures the media suggested by ATCC was not very favorable. The same problem was also encountered at the initial propagation step when they were obtained in lyophilized forms.

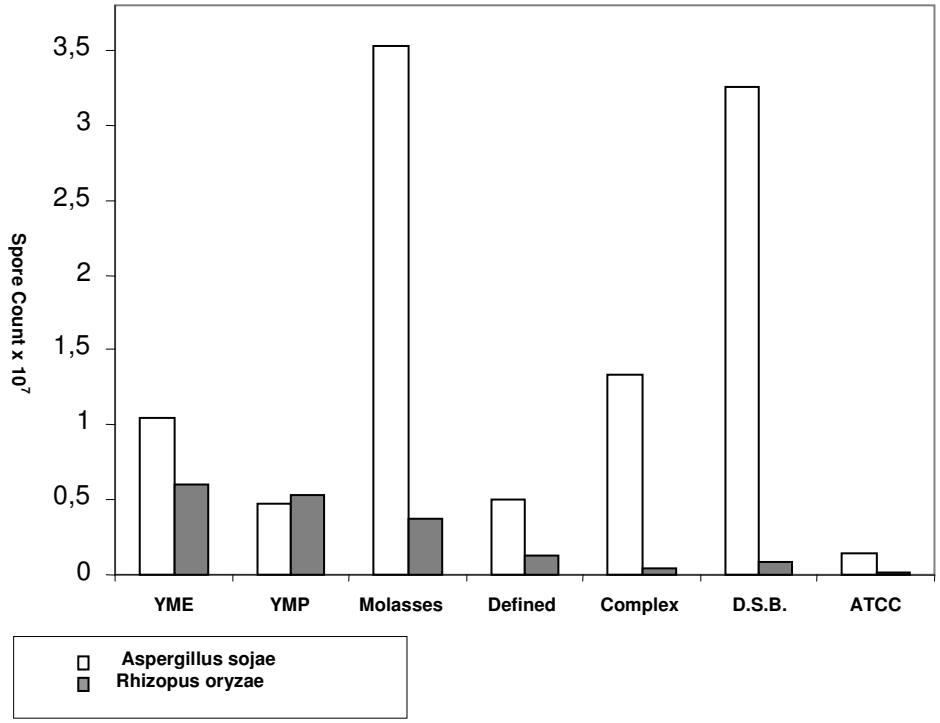


Figure 6.2. Spore counts obtained using various seed media formulations by *Aspergillus sojae* and *Rhizopus oryzae*. Standard deviation of spore counts of *Aspergillus sojae* varies in a range of 9.62e+06 and 1.98e+07. Standard deviation of spore counts of *Rhizopus oryzae* varies in a range of 9.48e+05 and 2.83e+07.

The spore counts of *Aspergillus sojae*, based on the favorable media formulation (molasses for *Aspergillus sojae* and YME for *Rhizopus oryzae)*, were almost 6 times higher than *Rhizopus oryzae* under the same incubation conditions (Figure 6.2). This result indicates that, different strains require different incubation period and conditions, requiring optimization. As a result, considering spore counts and economic feasibility, molasses or defatted soybean media would be the right choice for activating both cultures and promoting higher enzyme activity. Furthermore, the effect of the seed media composition was pronounced more in defined media (Figure 6.3).

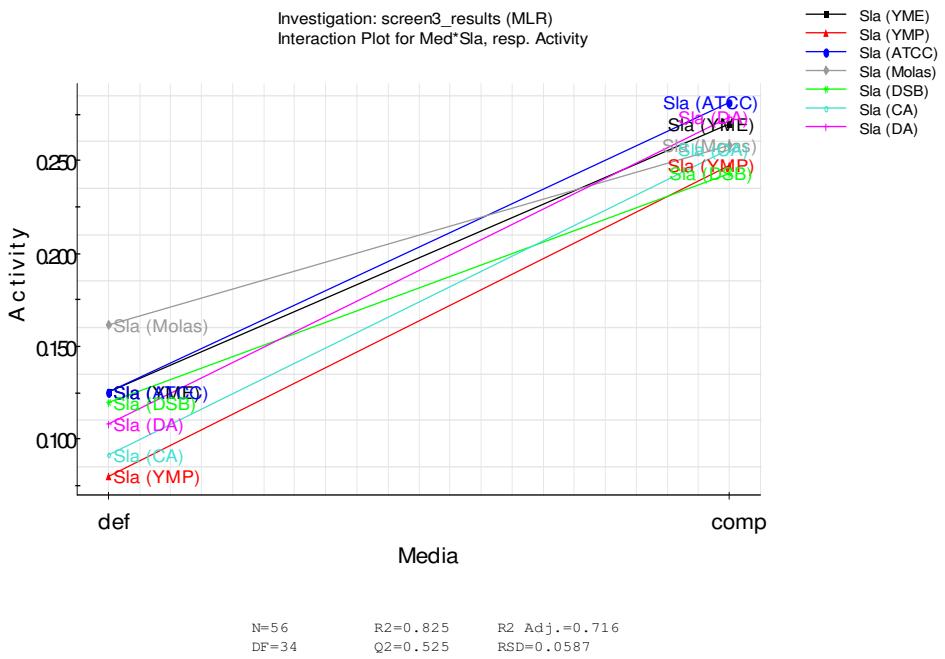


Figure 6.3. Result of full factorial design: Effect of the interaction of fermentation media and seed culture media (def: defined, comp: complex) on the enzyme activity.

Overall, if one wants to obtain high spore count, together with high enzyme production with pellet morphology, it would be a wise choice to grow the culture on molasses slant media for seed culture preparations. Although the seed culture composition did not affect the final morphology of *Rhizopus oryzae,* which just ended up as a clump of different sizes (just affected the size of the clump), it did cause a significant effect on the final morphology of *Aspergillus sojae* where different size and density pellets were observed (Table 6.1).

6.1.3. Effect of Production Media on the Pectinase Activity

The actual fermentation media composition has been always an important parameter worked on by many scientists and there is an enormous literature on the optimization of the formulations for different strains and products "(Puri et al. 2005, Elibol 2004 and Tellez et al. 2003)". There has also been a close correlation of defined media formulations for maximum secondary product formations in the literature "(Taherzadeh et al. 2003 and Tari et al. 1998)". Knowing the significance, the attempt was to determine which of the media composition (complex or defined) would favor the

maximum pectinase enzyme production with the desired morphology. Since there is no available literature on the interaction of seed media with the final fermentation media, it would greatly benefit to determine their effect on the enzyme activity and on the final morphology.

Based on these, two different media formulations (complex and defined) as presented in Table A1 were considered. Defined media compared to the complex one seemed to be more influenced by the slant media composition (Figure 6.3) and was not the right choice for *Rhizopus oryzae*. Complex media increased the enzyme activity 5 times more for *Rhizopus oryzae*, whereas this increase was only 1.25 times for *Aspergillus sojae* (Figure 6.1). Even though the increase was low for *Aspergillus sojae,* this was a positive effect. This showed that this organism would perform in spite of limited nutrients, baring the potential to be a good candidate for industrial use with possibly less expensive nutrient requirements.

There are few literatures on *Rhizopus oryzae* reporting its potential as pectinase producer. However since *Aspergillus sojae* so far was not considered for this purpose there is no available data "(Saito et al. 2004 and Saito et al. 2003)". Therefore, with this study it was proved that *Aspergillus sojae* could be even a better candidate with a desired morphology for pectinase production with less nutrient requirement. The final morphology of the fermentations differed, from different pellet sizes and densities for *Aspergillus sojae* to completely clump morphology for *Rhizopus oryzae*. The pellet morphology which is mostly preferred in industrial submerged fermentations due to its Newtonian nature is economically feasible with its lower operational cost and an easier downstream processing over mycelia type of fermentations, which are much more costly. Clump type morphology, even though the viscosity is of Newtonian type, causes difficulties during the fermentation by surrounding the impeller and agitator shaft, therefore increasing the power supply.

Factors like carbon and nitrogen sources and their concentrations have always been of great interest to the researchers in the industry for the low cost media design "(Parra et al. 2005, Tari et al. 2006)" especially when strains lacking information on their growth requirements, are under consideration such as the case here. It is also known that thirty to forty percent of the production cost of industrial enzymes is estimated to be the cost of growth medium "(Laxman 2005)". These sources together with factors like agitation speed and inoculation ratio, besides their effect on the product formation, have been determined to play significant role in the determination of the

final morphology of the culture "(Papagianni 2003)". Therefore, it is of great significance to optimize the conditions for cost-efficient enzyme production. With this perspective, a study was initiated with the goal of designing a low cost media using maltrin and cornsteep liquor as carbon and nitrogen sources, which would promote maximum PG synthesis and specifying the necessary agitation speed and inoculation ratio that would also result into pellet morphology preferred in large scale fermentations. As discussed above, another parameter that needs attention with respect to its effect on the fermentation run and downstream processing is the fungal morphology (pellet size). It is well known that a uniform small pellet size is preferred in much fungal fermentation due to its easier downstream processing and a better oxygen and nutrient mass transfer during the course of the fermentation "(Pamboukian et al. 1998)". Besides the PG enzyme synthesis, the fungal biomass was also considered due to its significance in applications of waste treatment and feed stocks. For such applications its maximization during the fermentation processes might be vital where it becomes the product of interest "(Field et al. 1992, Milstein et al. 1992, Zhang et al. 2000, Hoyos et al. 2002, Pascoal and Casio 2004)". For example in waste treatments, it is cheaper and more feasible to use the microorganism itself (biomass) as the source of the enzymes which are required to degrade the waste. Especially fungal organisms are very suitable for such treatments due to their capability of producing various enzymes. Based on this assumption, in order to utilize the biomass which is mostly taken as waste, the goal was also to provide the conditions for maximum biomass formation. All these three output variables were taken into consideration in the optimization procedure and their interactive relation was tried to be investigated.

Since it was not possible in a single step to obtain real optimum regions for maximum PG and biomass, a two step optimization procedure was applied using CCD. In the first step the four independent variables, concentrations of maltrin (X1) and corn steep liquor (X2), agitation speed (X3) and inoculation ratio (X4) were used. In the second step a CCD design with the same factors with new ranges based on the first step were applied. The first step served as a preliminary study for the determination of the initial levels, since there is no literature information available on this strain that could be used initially.

6.2. First Optimization Step

A face-centered central composite design with 31 experiments was performed. The respective low and high levels with the coded levels in parentheses for the factors were defined as 25 (-1) and 75 (+1) g/l for maltrin, 2.5 (-1) and 15 (+1) g/l for CSL, 150 (-1) and 300 (+1) rpm for agitation speed, and $2.5x10^5$ (-1) and $7.5x10^5$ (+1) total spore for inoculation ratio. The experimental runs with their response variables (PG activity, biomass) are presented in Table E1. After treatment combinations, the response data for maltrin and CSL, agitation speed, inoculation ratio and the interaction terms of CSL and agitation speed (X2xX3) and maltrin and CSL (X1xX2) yielded significant ($p<0.05$) terms with respect to PG activity. Similarly all the linear terms and the quadratic terms of maltrin (X1xX1) and agitation speed (X3xX3) were significant ($p<0.05$) with respect to biomass as the response variable. The coefficients of the models in coded units and their significance levels are presented in Table E2. The adjusted R^2 of the models were 83.2% for PG and 71.3 % for biomass with an insignificant lack of fit indicating a good fit between the models and the experimental data. According to the response surface graphic of X1, X2 and X3 (Figure 6.4) maximum PG activity (7.4 U/ml) could be achieved at higher maltrin and lower CSL concentrations and at higher agitation speeds.

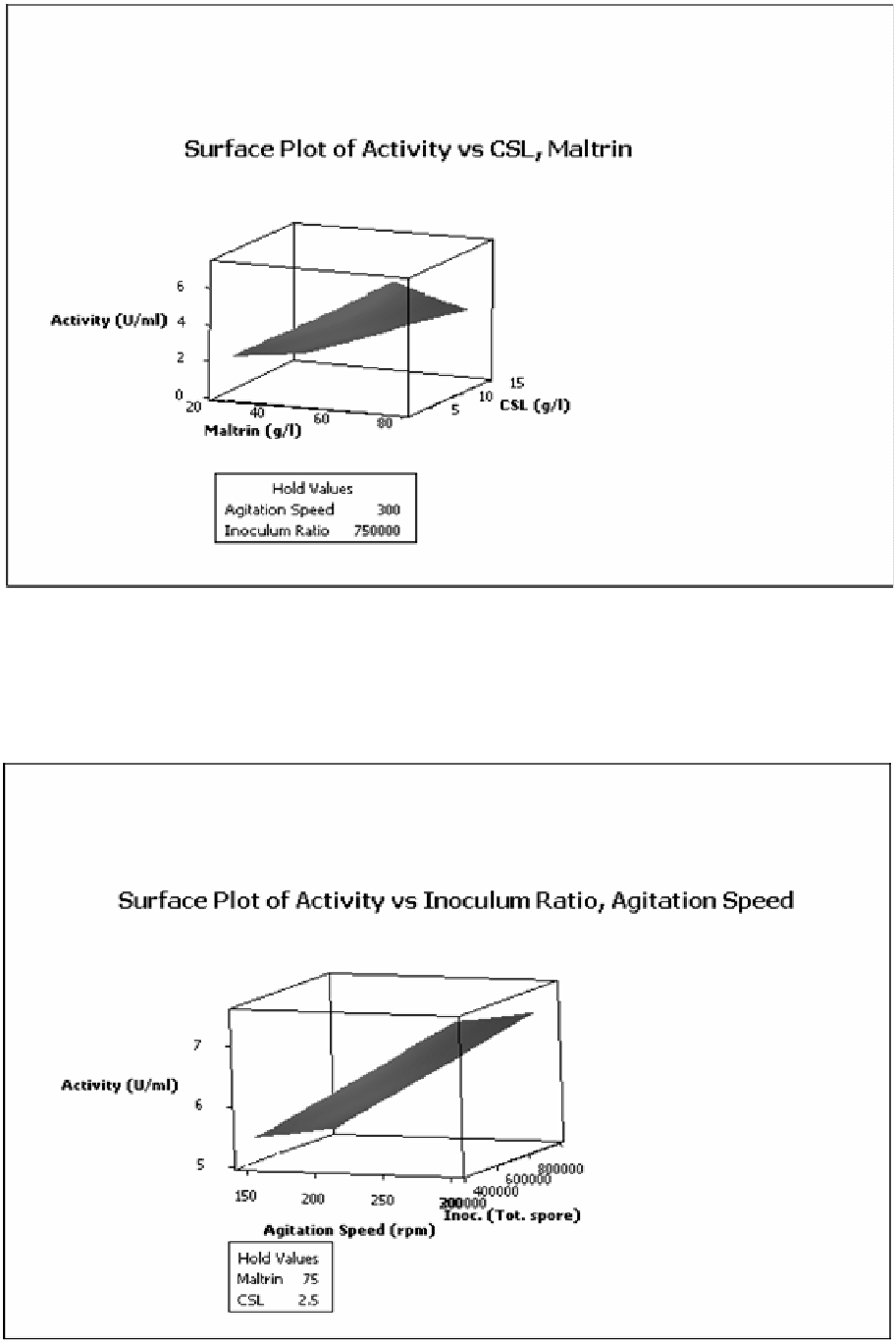
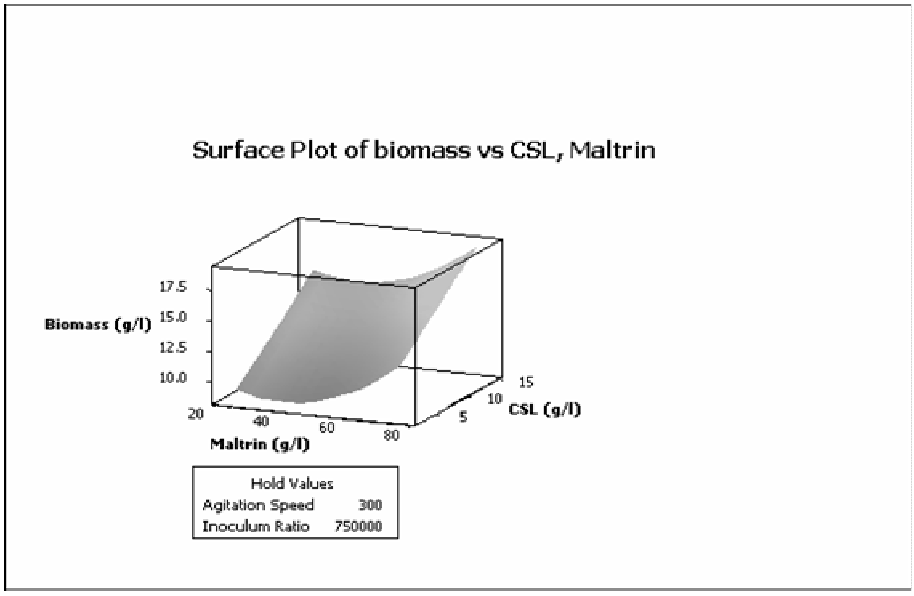


Figure 6.4. First step optimization activity RSM plots



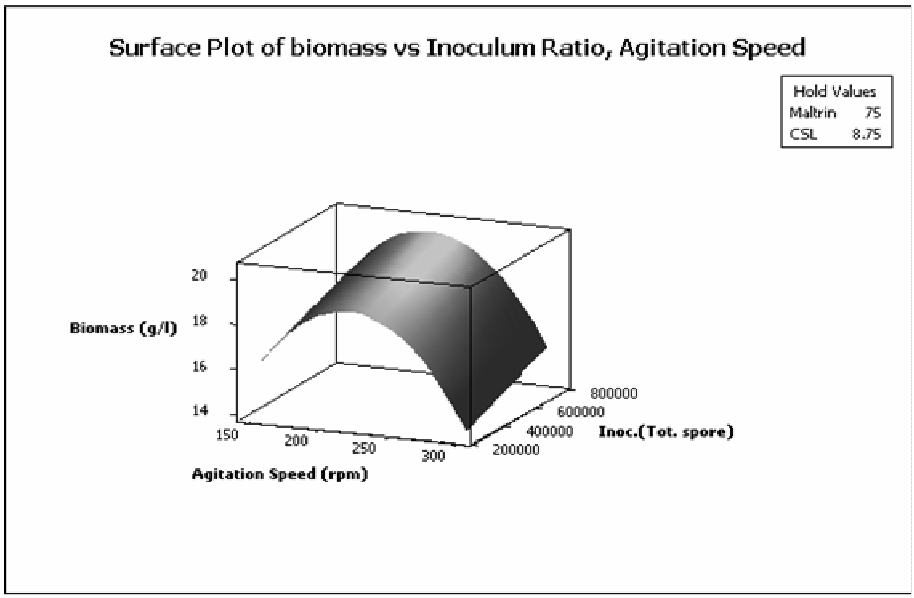


Figure 6.5. First step optimization biomass RSM plots

For maximum biomass formation, high maltrin and high CSL concentrations with an optimum agitation speed ranging between 200-250 rpm were required (Figure 6.5). The inoculation ratio was insignificant under ranges studied. However these values did not result into true optima yet. Therefore further optimization was suggested by decreasing the CSL level and increasing the Maltrin level and agitation speed further for maximization of the enzyme activity. Since the incubator shaker used was not suitable

for further increase in agitation speed, the maximum speed was leveled at 350 rpm. Although the inoculation ratio under the ranges studied did not affect the PG activity and biomass significantly, it did affect the morphology profoundly. Based on this, the ranges were widened further and inoculation ratio was also taken as an independent variable for the second optimization step.

6.3. Second Optimization Step

New factor values with higher maltrin (X1) and lower CSL (X2) concentrations and higher agitation speed (X3) and wider inoculation ratio (X4) range were proposed. An additional 31 experiment using 4-factor central composite design was performed. The experimental runs with their response variables (PG activity, biomass and morphology (pellet size) are presented in Table F1. The respective low and high levels with the coded levels in parentheses for the factors were defined as 50 (-1) and 120 (+1) g/l for maltrin, 0 (-1) and 5 (+1) g/l for CSL and 150 (-1) and 350 (+1) rpm for agitation speed and 1.25 $x10^4$ (-1) and $2x10^7$ (+1) total spore for inoculation ratio. After the treatment combinations all linear terms of the independent variables, quadratic term of CSL (X2), interaction terms of CSL with maltrin (X1xX2) and inoculum (X2xX4) and interaction terms of agitation and inoculation ratio (X3xX4) were included in the model for PG activity since these were significant ($p<0.05$). Even though variables X3 and X4 were not found statistically significant ($p > 0.05$), they were added to the model due to their significant interaction effects. Considering biomass as the response, all linear terms and quadratic term of CSL (X2xX2) together with the interaction terms of maltrin with inoculum (X1xX4) and interaction terms of agitation speed with inoculum (X3xX4) were significant and therefore included in the model. Similarly, taking pellet size as a morphological measurement, the linear terms of maltrin, CSL, agitation speed and inoculation ratio together with quadratic term of inoculation ratio (X4xX4) and interaction terms of agitation speed with Maltrin (X1xX3) and inoculation ratio (X3xX4) were included in the model equation. Data for the mean diameter of pellets were transformed to get the normally distributed data in the RSM by taking the inverse of the square root of pellet diameter. In all 31 experiments, diameter values varied between 0.05 – 0.63 cm except 3 experiments that ended with the clumps of 3 cm diameter. For all of the models the adjusted R^2 values were 92.5 %, 66.6 % and 66.5%

for PG activity, biomass and pellet size, respectively, with insignificant lack of fit values. The model equations for PG enzyme (Y_{PG}), biomass (Y_b) and 1/sqrt (pellet size) (Y_p) with the coefficients in coded units of factors are given below:

$$Y_{PG}=6.29+2.75X_1-2.657X_2+0.286X_3-0.147X_4+1.93X_2^2+1.107X_1X_2-0.518X_2X_4+0.577X_3X_4 \quad (6.1)$$

$$Y_b=17.86+2.436X_1+5.735X_2+1.508X_3+1.942X_4-4.469X_2^2-1.227X_1X_4+1.845X_3X_4 \quad (6.2)$$

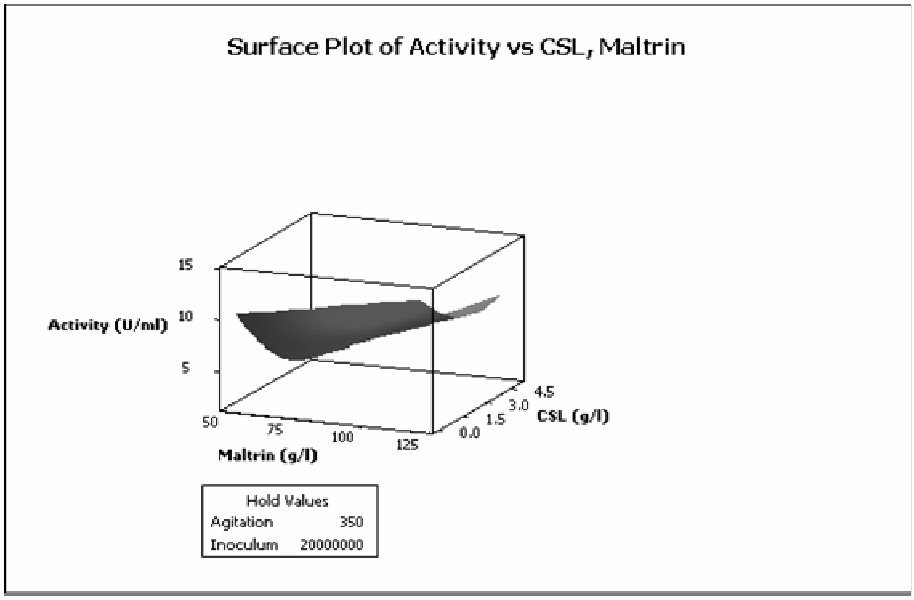
$$Y_p=2.97-0.089X_1-0.577X_2-0.356X_3+0.280X_4-0.788X_4^2+0.161X_1X_3-0.704X_3X_4 \quad (6.3)$$

From the response surface graphs (Figure 6.6) presenting the interaction of maltrin and CSL it is obvious that maximum PG activities can be obtained at high maltrin (120 g/l) and in the absence of CSL (0 g/l) concentrations. This confirms the result of the first optimization study. However, when the interaction of agitation speed and inoculation ratio is considered, the situation changes where two options become available (Figure 6.6). In this case maximum enzyme synthesis is achievable either at low agitation speed together with low inoculation ratio or high agitation speed together with high inoculation ratio. This is due to the fact that agitation speed influences the mass transfer of oxygen and nutrients. It is logical that high inoculum requires more oxygen and nutrient transfer which is supported through the higher agitation. Therefore, a lower inoculum of cells will require less oxygen and nutrient transfer. The same rule is valid for biomass formation which is depicted through the figures presented (Figure 6.7). In addition to these conditions maximum biomass formation required higher levels of CSL (Figure 6.7). This situation rises the following conclusion: If one wants to produce high enzyme, then the preference of CSL concentration has to be on the lower end, however if the primary goal is to produce biomass, the only difference would be to add more CSL. Comparing the two optimization steps, the second step improved the enzyme activity by 74 % and biomass by 40 %.

From the response surface graphs (Figure 6.8) showing the interaction of agitation speed with inoculation ratio on the response variable 1/sqrt (pellet size) at low CSL and low maltrin concentrations, it is observed that bigger pellets (see where low 1/sqrt (pellet size) is observed) are obtained either at very low inoculation ratio and low agitation speed or very high inoculation ratio and high agitation speed. This is due to the fact that higher agitation speed increases the mass transfer of nutrient and oxygen to the cells and also promotes aggregation of the mycelial biomass in the form of pellets and

clumps. This indicates indirectly the mechanism for pellet formation that can be related to aggregation of pellets at higher speeds "(Gibbs et al. 2000)". Therefore lower speed is not sufficient to bring the pellets together and to provide enough nutrient and oxygen mass transfer at high inoculation levels. Smaller pellet sizes (see where high 1/sqrt (pellet size) is observed) are obtainable, at low agitation speed, with an optimum inoculation ratio of $1.7x10^7$ total spore (in 50 ml of fermentation medium). Smaller pellets which can also be observed at low inoculation levels and at higher speeds could be due to the toxic effect of oxygen that might be in surplus.

Overall, in order to achieve a high PG activity, maltrin concentration should be 120 g/l, while CSL concentration should be as low as possible or none. Agitation speed and inoculum ratio should be 350 rpm and 2×10^7, respectively. For biomass on the other hand, CSL concentration should be kept higher (~ 4 g/l) to have a high biomass concentration. Other conditions match to the ones for high enzyme activity. For high enzyme activity, the mean diameter of pellets was observed as 0.32 cm, particles ranged between 0.05 – 0.76 cm in diameter.



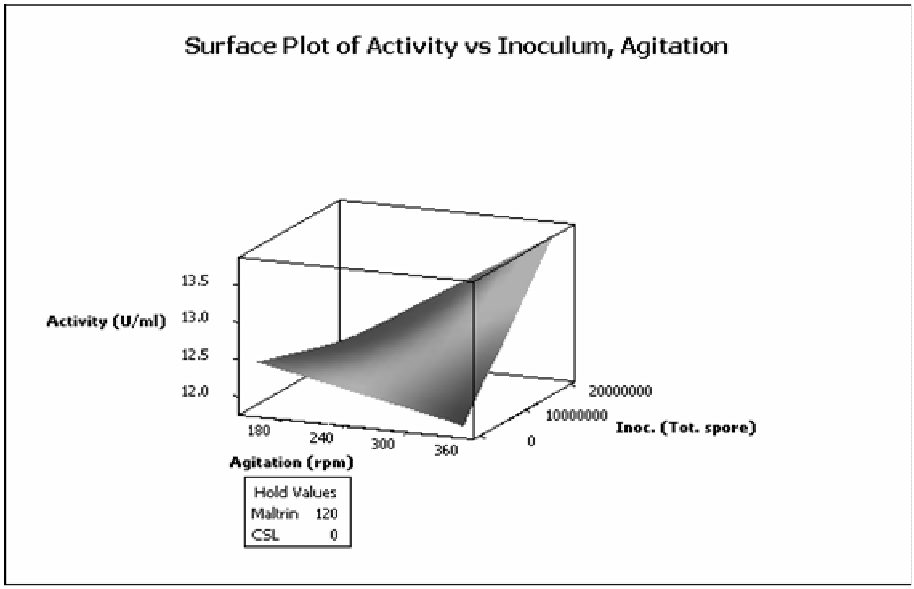
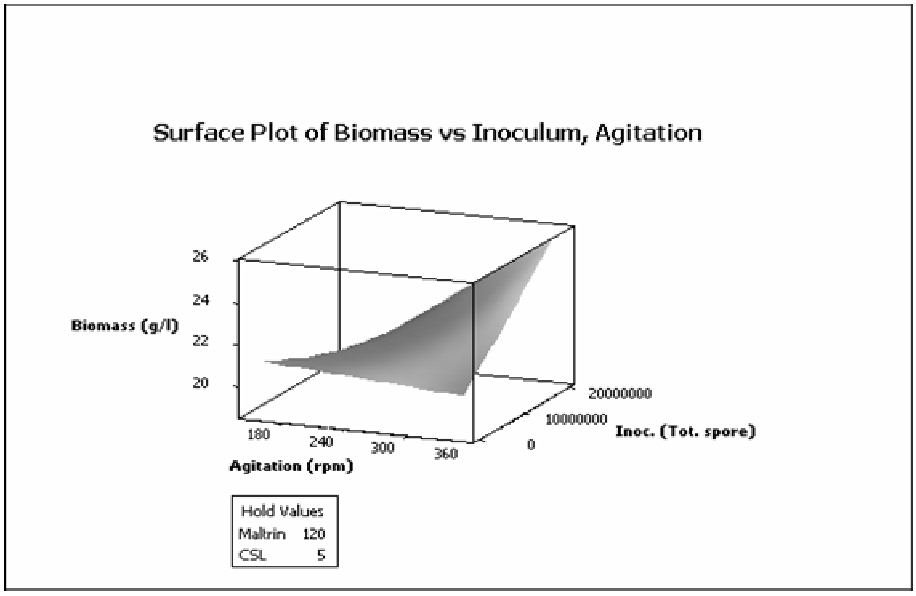


Figure 6.6. Second step optimization activity RSM plots.



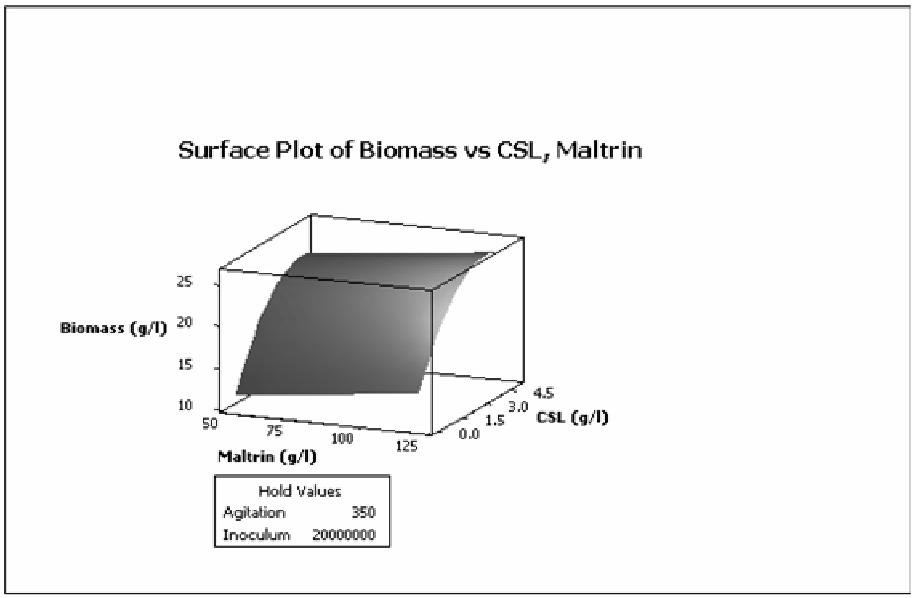
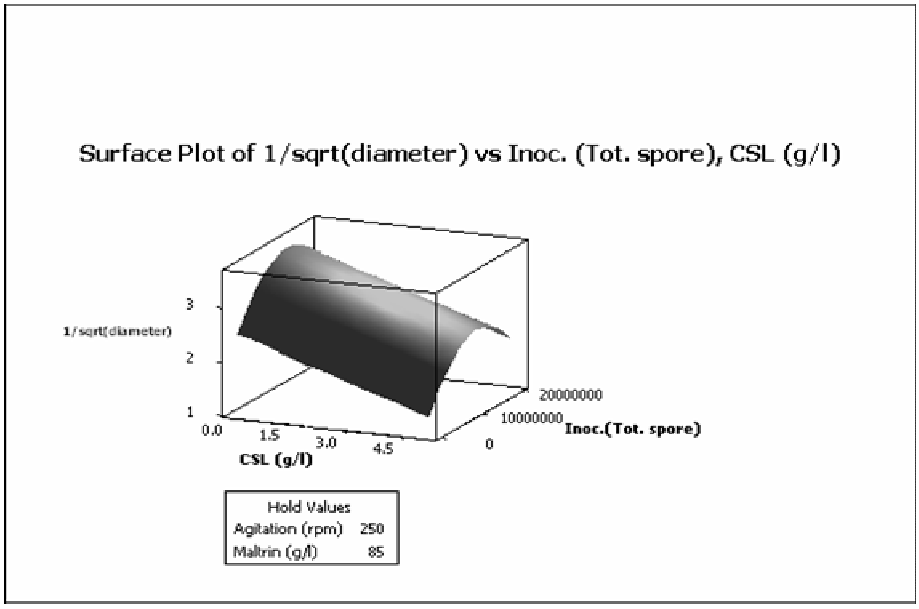


Figure 6.7. Second step optimization biomass RSM plots.



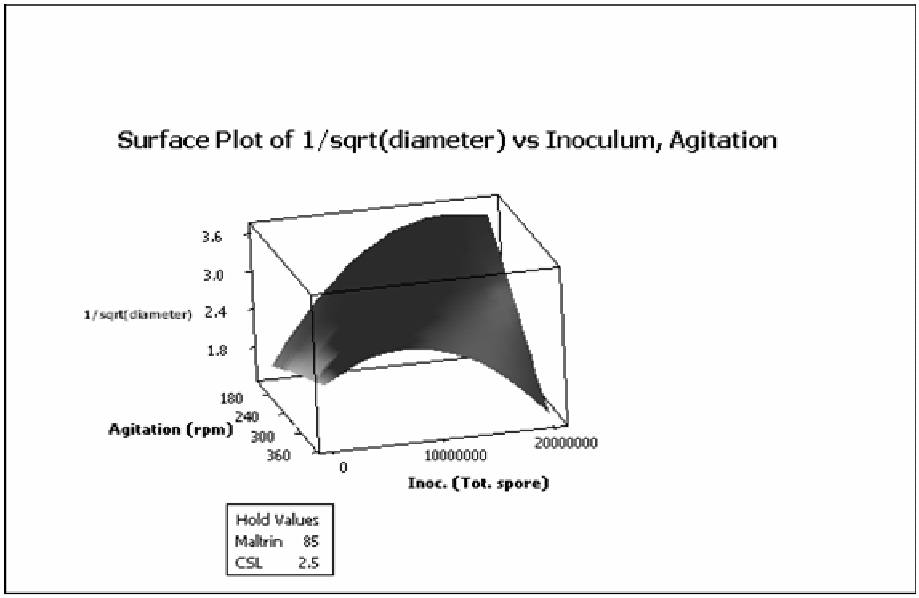


Figure 6.8 Second step optimization pellet size RSM plots.

6.4. Validation of the Models

In order to validate the adequacy of the model equations a total of 4 verification experiments that were repeated twice were carried out at the predicted optimum conditions. The mean values of the experimental data and the predicted responses are given in Table F2.

As it is seen from the table there was a good correlation between the experimental and predicted values of the PG activity indicating a good fit of the model. However, the experimental biomass values were observed higher than the predicted ones. Also increasing the maltrin concentration to 180 g/l increased the PG activity slightly. The pellets observed at the optimum conditions of the validation experiments revealed that large number of small size pellets (average of 0.078 cm in diameter) was obtained. The diameter of pellets ranged between 0.025 – 0.36 cm with an average of 0.078 cm. This was still in the range of 0.05 – 0.76 cm in diameter obtained in the optimization experiments. The clump morphology observed at maximum biomass formation was in agreement with clump formation in the validation experiment.

In the comparison of the current results with the literature studies conducted on pectinase production using different fungal organisms, reveals that the enzyme activity obtained in this study, is above the range of those results "(Kaur et al. 2004, Blandino et al. 2001 and Maldonado et al. 1998)". For example in a study conducted by Panda et al. in the multi response analysis of microbiological parameters affecting the production of pectolytic enzymes by *Aspergillus niger*, the maximum PG activity obtained was around 0.91U/ml which is lower than our results. Similarly in another study conducted by Blandino et al., using *Aspergillus awamori* in the polygalacturonase production, the maximum activity in defined medium was around 0.05 U/ml, which is below the results we obtained in this study. In the comparison of the biomass results with literature, our results are within the ranges of other researchers "(Blandino et al. 2001 and Maldonado et al. 1998)". The pellet sizes which are taken as a means of morphological measurements in this study are comparable to the results of various researchers who also relate these to the broth rheology during the fermentation "(Gibbs et al. 2000 and Metz et al. 1979)". With this perspective this study is indicative of providing informations on the conditions for biomass and certain pellet size formation that could be related to other rheological and model development studies for various fungal fermentations "(Papagianni 2004 and Olsvik et al. 993)". For example it is emphasized by a study conducted by Fatile and Olsvik, that the shape of mycelial aggregates (such as pellets) and the biomass concentration are correlated to the rheological properties of the broth. Broth rheology is important since it has profound effect on the mass transfer properties of nutrient and oxygen during the fermentation run and the downstream processing. In downstream processing problems due to the difficulties in establishing and maintaining fluid flow in pipe lines used to transport the broth to and from

fermentation vessels and biomass filtration for product recovery may arise from very viscous fermentation broth which are the result of the rheology of the broth "(Gibbs et al. 2000)".

This study clearly indicates that this strain with the optimized conditions can be considered as a potential pectinase producer for different industrial applications. Furthermore, with potential high biomass formation it can also be considered for waste treatment purposes with the possibility of producing other enzymes and metabolites that needs further investigation. These optimized conditions also provide a desired pellet size through the pellet morphology that exhibits throughout the course of the fermentation, which not only benefit the fermentation run but also the subsequent downstream processes by reducing the operational cost. Besides data on the pellet sizes and biomass will be useful in providing informations for the development of mathematical models describing fungal fermentations and be indicative in scale up procedures. In order to exploit new industrial potentials of polygalacturonase it is necessary to investigate new microbial strains and to understand the structure-stability relationship of this enzyme. The knowledge gained in this way would offer new possibilities in improving the potential and effective use of this enzyme in various fields and commercialization of its industrial production. Maintaining the desired level of enzyme activity over a long period of time and improving its stability are important parameters for the selection and design of pectinases "(Gummadi and Panda 2003)". Based on this, emphasis was given to the investigation of the effect of pH and temperature on activity and stability of polygalacturonase, together with the determinations of the deactivation and kinetic parameters.

6.5. Effect of pH on activity and stability of Polygalacturonase

As it is seen from figure 6.9., the enzyme was active over a broad pH range, displaying over 80% of its activity in the pH range of 5.0-7.0. Optimum polygalacturonase activity was observed at pH 5 (figure 6.9). Although optimum polygalacturonase activity was observed at pH 5.0, a further decrease from pH 5.0 to 3.0 decreased the polygalacturonase activity rapidly to 40% relative activity. However 80% activity was retained between the pH 6 to 7.0. These findings are in accordance with several earlier reports showing pH optima of 4.8 for polygalacturonase from

Aspergillus niger, pH optima of 5.0 for *Rhizopus stolonifer* "(Gummadi and Panda 2003)". The polygalactururonase of *Sporotrichum thermophile* Apinis was quite active over a wide pH range (3.0-9.0) "(Kaur et al. 2004)". Among the polygalacturonases obtained from different microbial sources, most have the optimal pH ranges of 3.5-5.5 "(Jayani et al. 2005)". The pectinase secreted by *Kluyveromyces wickerhamii* has optimum pH of 5.0 "(Moyo et al. 2003)". An optimum pH of 5.0 showed that, *Aspergillus sojae* produces an acidic polygalacturonase which can have wide applications in the fruit juice industries and wine making. It is well known that acidic pectinases mostly originate from fungal sources, especially from *Aspergillus niger* which has optimum pH of 4.5-6 "(Kashyap et al. 2001)".

The effect of pH on stability (Figure 6.10) showed that the polygalacturonase enzyme was very stable at the pH 5.0 and retained 65% and 70% of its activity at pH 4.0 and 6.0, respectively. The enzyme lost about 50-60% of its activity at pH 8.0. As shown in Figure 6.10., the enzyme was very stable in a broad pH range, maintaining over 60-70% of its original activity between pH 3.0 and 7.0. The enzyme was stable at both acidic and alkaline pH, but declined sharply to 40% of the initial activity at pH 8.0.

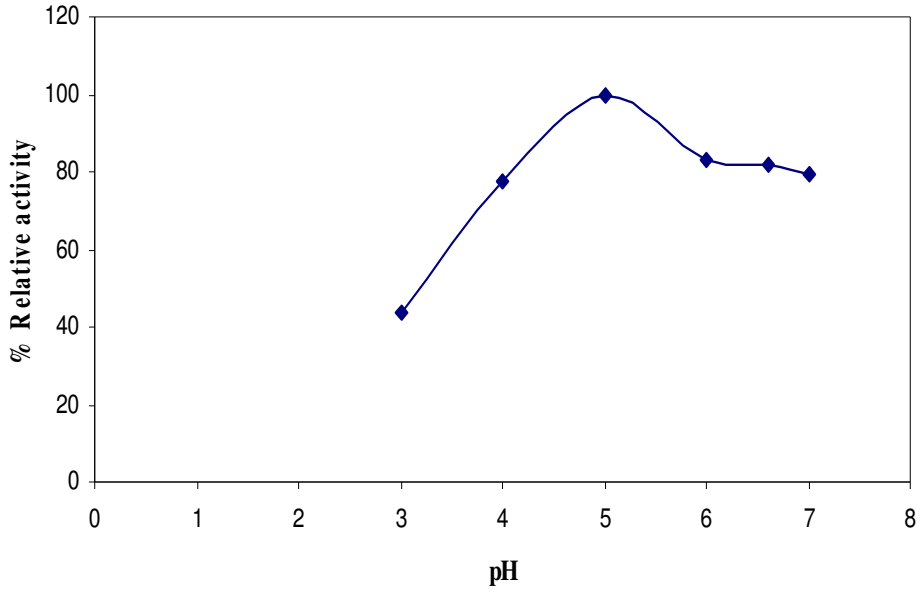


Figure 6.9. Effect of pH on *A.sojae* polygalacturonase activity

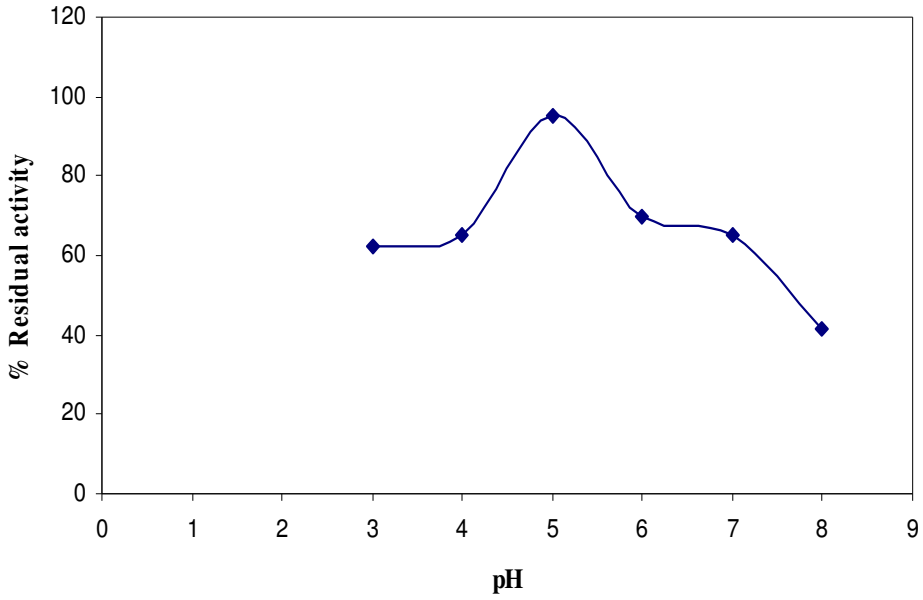


Figure 6.10. Effect of pH on *A.sojae* polygalacturonase stability

Therefore, polygalacturonase like the one characterized in this study could be a potential candidate for different applications in the industry requiring broader pH stability ranges.

6.6. Effect of Temperature on Activity and Stability of Polygalacturonase

Aspergillus sojae polygalacturonase was found to have an optimum temperature of 55ºC (Figure 6.11). The enzyme retained 75% of its activity at 65 and 75ºC. The polygalacturonase was active over a broad temperature range of 25-75ºC. This optimum temperature was in agreement with the studies conducted by several authors using different strains. For example Sakamoto et al. 2002 found the optimum temperature of an exo-polygalacturonase from *Aspergillus niger as* 60ºC. Similarly, maximum activity for commercial enzyme, Rapidase C80 was determined at 55ºC "(Ortega et al. 2004)". The partially purified polygalacturonase from *Sporotrichum thermophile Apinis* was optimally active at 55ºC, where this value was higher than the optimum temperature determined for *Aspergillus niger* polygalacturonase "(Jayani et al. 2005 and Kashyap et

al. 2001)". Another study performed with *Aspergillus niger* indicates the optimum temperature 60ºC which is close to our results "(Sakamoto et al. 2002)".

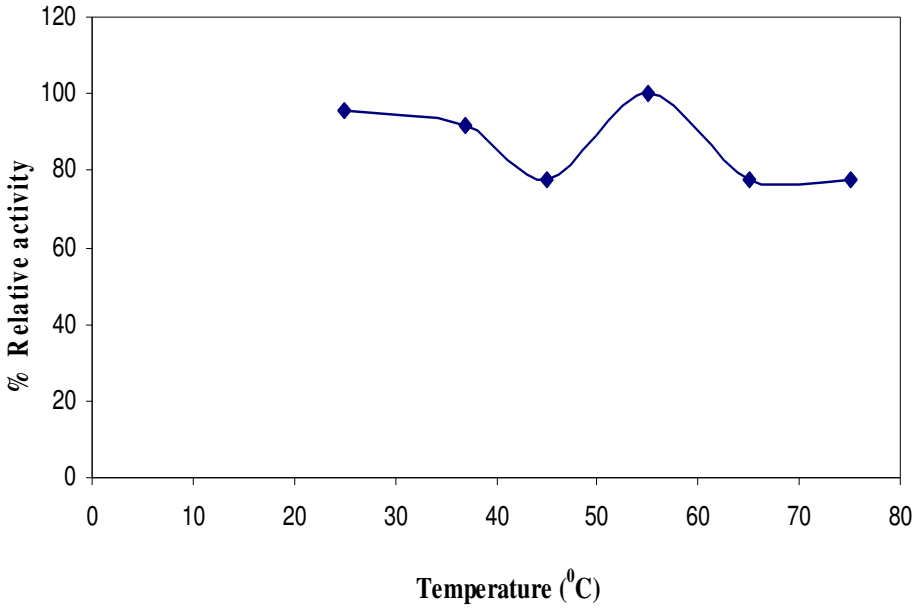


Figure 6.11. Effect of temperature on *A.sojae* polygalacturonase activity

Thermostability is the ability of enzyme to resist against thermal unfolding in the absence of substrates "(Bhatti et al. 2006)". The thermostability of the crude polygalacturonase was determined by measuring the residual activity of the enzyme after incubation at various temperatures ranging from 25 to 55ºC for 15, 30 and 60 minutes. As it is shown in Figure 6.12., after 15 and 30 minutes of incubation, the enzyme was stable at 25ºC but after 1 hour of incubation the enzyme lost 40% of its activity. The enzyme was stable also at 37, 45 and 55ºC for 15, 30 and 60 minutes with a residual activity ranging from 65% to 80%. The thermostability of pectinases is an important parameter in fruit juice extraction. Before the addition of pectinases, fruits are first cooked to release more juice. This releases most of the pectin into the juice giving a thick and a cloudy appearance. Apples, stone fruits and berries are normally processed at 30-50ºC for about 15 to 90 minutes "(Moyo et al. 2003)". As the polygalacturonase from *Aspergillus sojae* is thermostable up to 55ºC for an hour with an optimum temperature of 55ºC it has great potential to be used in the fruit juice industry. Since,

after the application the enzyme has to be inactivated, the estimation of thermal inactivation discussed in the next section has great importance.

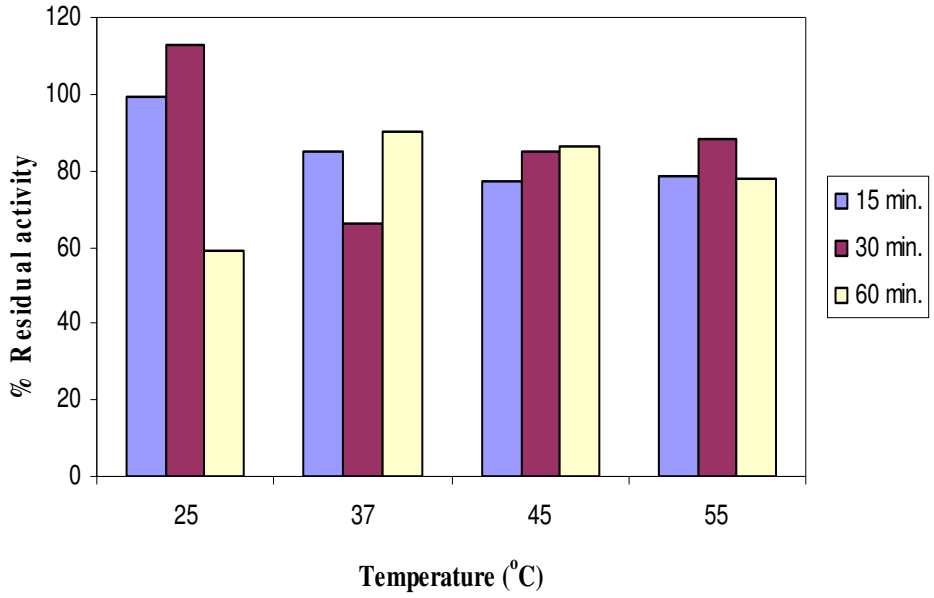


Figure 6.12. Effect of temperature on the stability of *A.sojae* polygalacturonase

6.7.Kinetics of Thermal Inactivation and Estimation of the Inactivation Energy

Inactivation is defined as a process where the secondary, tertiary or quaternary structure of a protein changes without breaking covalent bonds "(Naidu and Panda 2003)". Inactivation rate constants (k_d) of polygalacturonase which are presented in table 1 at 65, 75 and 85 ºC were calculated from the slope of seminaturallogarithmic plot of residual activity versus time (Figure 6.13). Similarly the half-life values estimated using these constants and equation 1, are presented in the same table 6.2. The half life of polygalacturonase at 65 ºC was 78.8 and 47 times higher than the half-life values at temperatures of 75 and 85ºC, respectively at pH 6.6. This issue once again reveals the thermal stability of this enzyme at 65 ºC and its easy inactivation at higher temperatures such as 75 and 85ºC.

Table 6.2. Kinetic parameters for thermal inactivation of polygalacturonase from *A.sojae*.

T (^{o}C)	k_d (min^{-1})	$t_{1/2}$ (min)	T (^{o}K)
65	0.0011	630.0909091	338
75	0.0878	7.894077449	348
85	0.0513	13.51072125	358

Inactivation energy of crude polygalacturonase was found to be E_d = 46.74 kcal mol^{-1}K^{-1}. This value is in lower side of the values (40-70 kcal mol^{-1}K^{-1}) estimated for many microbial enzymes "(Kargi and Shuler 2002)". The inactivation energy estimated for this enzyme is 1.35, 1.17 and 1.22 times higher than the commercial polygalacturonases like Rapidase C80, Pectinase CCM and Pectinex 3XL respectively, compared to a study conducted by Ortega et al. 2004.

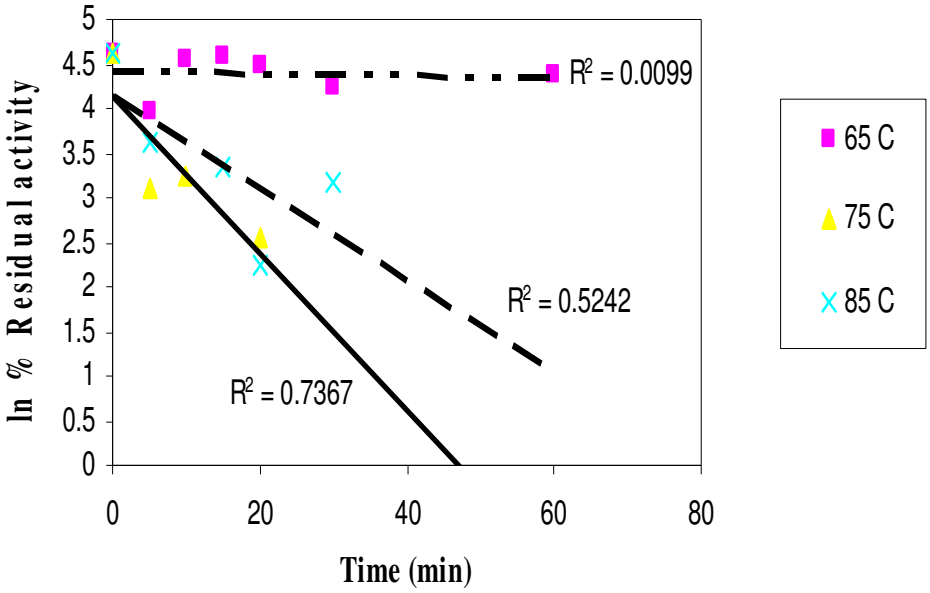


Figure 6.13. First order plots of the thermal denaturation of *A. sojae* polygalacturonase.

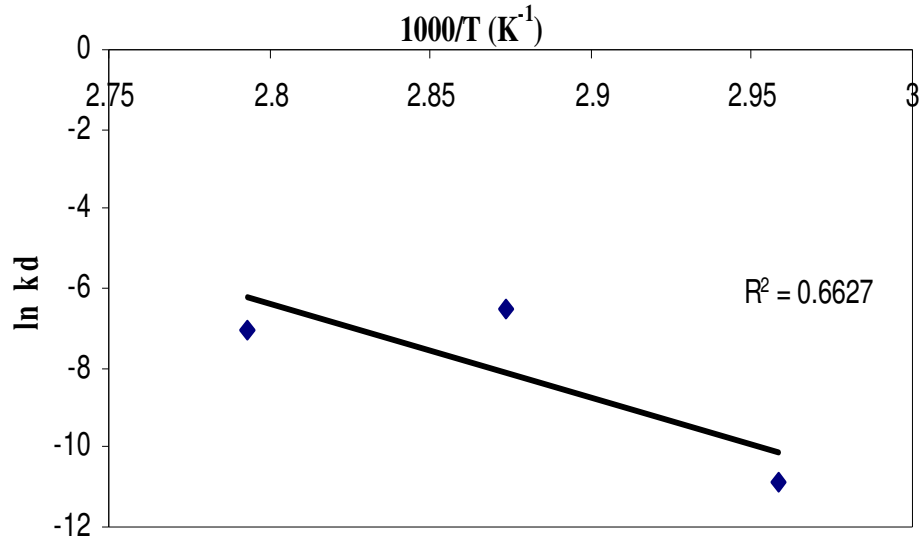


Figure 6.14. Arrhenius plots for the determination of the inactivation energy of *A. sojae* polygalacturonase.

6.8. Calculation of Kinetic Constants

The kinetic parameters of *Aspergillus sojae* polygalacturonase for hydrolysis toward polygalacturonic acitd (PGA) at pH 6.6 and 26 ºC were obtained by a typical double reciprocal Lineweaver Burk plot (Figure 6.15). The apparent K_m value and V_m value for hydrolyzing PGA were V_m = 80 µmole/min, K_m = 0.424 g/l respectively. The estimated Km value which indicates the affinity of the enzyme towards the substrate is 1.7 and 1.18 times lower than the *Aspergillus niger* and *Stereum purpureum* polygalacturonases, respectively using the same substrate. This indicates that the current polygalacturonase has a higher affinity for PGA compared to the other two "(Jayani et al 2005)". In a study which was done with *Sporotrichum thermophile* polygalacturonase, they found a similar K_m value (0.416 $mgml^{-1}$) with *Aspergillus sojae* polygalacturonase "(Kaur et al. 2004)". Another study about *Aspergillus niger* polygalacturonase indicates V_m value as 154 µmole/min which is in correlation with our results "(Singh and Rao 2002)".

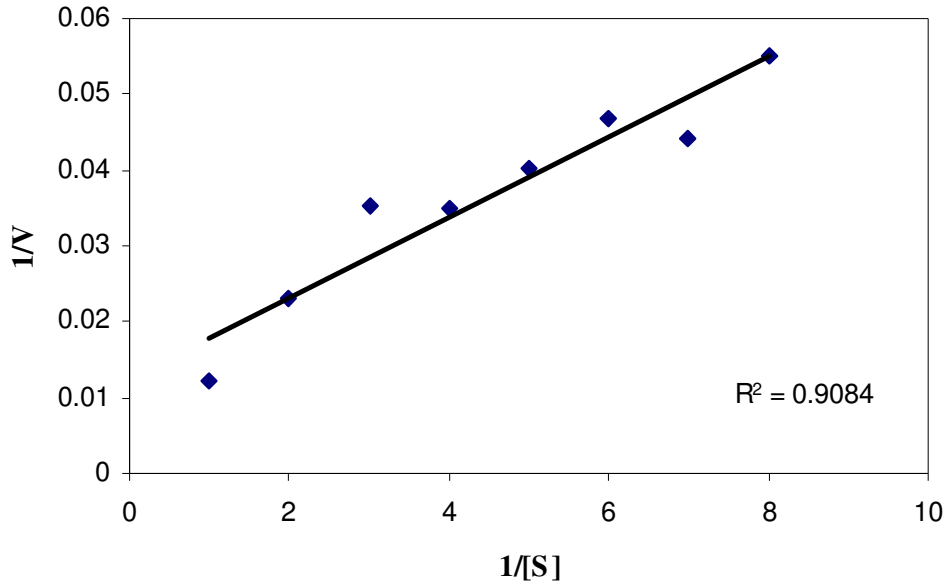


Figure 6.15. Double reciprocal plots to determine the kinetic constants for polygalacturonic acid hydrolysis by *A. sojae* polygalacturonase.

Overall the polygalacturonase characterized in this study, with its unique biochemical properties can be considered as a potential candidate for various applications in the food industry.

CHAPTER 7

CONCLUSIONS AND FUTURE PERSPECTIVE

A full factorial statistical analysis revealed that factors of strain types and fermentation media and the interaction between them was significant on the pectinase enzyme activity, whereas, the slant types were found insignificant at the significance level of 1%. Interaction between strain types and slants, and interaction between fermentation media and slants were also found insignificant (p-value >> 0.01). It was recommended, that, *Aspergillus sojae* grown on molasses and inoculated into complex media will result in pellet morphology, in the production of pectinase. The enzyme activities obtained in this study were comparable to the literature for various *Aspergillus* sp and *Rhizopus* sp. "(Panda et al. 1998 and Blandino et al. 2001)".

Furthermore, the investigation of the factors that would result in pellet morphology of *Rhizopus oryzae* will be another topic to investigate. With this study it was also shown that *Aspergillus sojae*, known mainly in solid state fermentation of the popular koji food, could be a good candidate in submerged fermentations, with the desired morphological characteristics, in the production of the pectinase as well as other potential enzymes. Even though, the type of slant media was insignificant on the enzyme activity, it had influence on the morphology of the final fermentation broth affecting the rheology and consequently the downstream processing. Besides, the type of seed media had great influence on the spore amounts obtained, which are very critical in the spore inoculum preparations of large scale productions.

To date, no reports are available in literature regarding the optimization of fermentation conditions for mycelial growth, pellet size and PG enzyme synthesis by *Aspergillus sojae*. Therefore, this study will serve as a base line of the initial studies in this field. The study does not only provide novel information on the growth requirement of this organism, but also serves as an example for the application of the statistical techniques to the fungal systems by giving the end user flexibility for choosing the optimized conditions depending on the responses of interest such as enzyme synthesis, biomass formation or the necessary pellet size for an easy downstream process. Through these optimization experiments, the optimal conditions for maximum PG enzyme activity were to use maltrin at 120 g/l, exclude CSL, with an agitation speed of

350 rpm and an inoculation ratio of $2x10^7$ total spore. Similarly if the primary goal is the maximization of biomass formation then the preferred conditions would be to include CSL in the concentration of 4 g/l to the given parameters above. The pellet sizes under these conditions would range from 0.05 – 0.76 cm in diameter for maximum PG synthesis and being mostly clump for maximum biomass formation.

Characterization experiments indicated that the optimum pH and temperature of A.sojae polygalacturonase were 5 and 55^{o}C, respectively. Furthermore the calculated inactivation energy (46.74 kcal) was in the range of the values estimated for many microbial enzymes. Besides the half-life of PG at 65^{o}C was higher than that of PG at 75 and 85^{o}C showing that the enzyme was more stable at 65^{o}C and easy to inactivate at 75 and 85^{o}C. Additionally kinetic constants (V_m=80 µmole/min and K_m=0.424 g/l) proved that *A.sojae* PG has high affinity for polygalacturonic acid as substrate.

REFERENCES

Almeida, C., Branyik, T., Ferreira, P.M., Teixeira, J. 2004. "Use of Two Different Carriers in a Packed Bed Reactor for Endopolygalacturonase Production by a Yeast Strain." *Process Biochem.* Vol. 40, No. 5, p.1937-1942.

Atkingson, B. and Daoud, L.S. 1976. "Microbial Flogs and Flocculation in Fermentation Process Engineering." *Adv. Biochem. Eng*. Vol. 4, p. 41-124.

Bai, Z.H., Zhang, H.X., Qi, H.Y., Peng, X.W., Li, B.J. 2004. "Pectinase Production by *Aspergillus niger* Using Wastewater in Solid-State Fermentation for Eliciting Plant Disease Resistance." *Bioresource Technol.* Vol. 95, p. 49-52.

Behere, A., Satyanarayan, V., Desai, R.P. 1993. "Separation and Limited Characterization of Three Polygalacturonases of *Aspergillus niger." Enzyme Microbial Technol*. Vol. 15, p. 158-161.

Benett, J.W. 2001. "*Aspergillus* and Koji: History, Practice and Molecular Biology." *Society of Industrial Enzymes News*. Vol. 51, p. 65-71.

Bhatti, H.N., Asgher, M., Abbas, A., Nawaz, R., Sheikh, M.A. 2006. "Studies on Kinetics and Thermostability of a Novel Acid Invertase from *Fusarium solani*." *J. Agric. Food Chem.* Vol. 54, p. 4617-4623.

Blanco, P., Sieiro, C., Villa, T.G. 1999. "Production of Pectic Enzymes in Yeasts." *FEMS Microbiology Letters.* Vol. 175, p. 1-9.

Blandino, A., Dravillas, K., Cantero, D., Pandiella, S.S., Webb, C. 2001. "Utilisation of Whole Wheat Flour for the Production of Extracellular Pectinases by Some Fungal Strains." *Process Biochem.* Vol. 37, p. 497-503.

Bryne, G.S. Ward, O.P. 1989. "Effect of Nutrition on Pellet Formation by *Rhizopus arrhizus." Biotechnol. Bioeng*. Vol. 33, p. 912-914.

Calam, C.T. 1976. "Starting Investigational and Production Cultures." *Process. Biochem.* Vol. 11, p. 1-7.

Carr, J.G., 1985. "Tea, coffee and cocoa." In: Wood BJB, editor. *Microbiology of Fermented Foods.* Vol. 2, p. 133-224.

Cox, P.W. and Thomas, C.R. 1992. "Classification and Measurement of Fungal Pellets by Automated Image Analysis." *Biotechnol. Bioeng.* Vol. 39, p. 945-952.

Cui, Y.Q., van der Lans, R.G.J.M., Luyben, K.C.A.M. 1997. "Effect of Agitation Intensities on Fungal Morphology of Submerged Fermentation." *Biotechnol. Bioeng*. Vol. 55, No. 5.

De Vries, R.P. and Viesser, J. 2001. "*Aspergillus* Enzymes Involved in Degradation of Plant Cell Wall Polysaccharides." *Microbiol. Mole. Biol. Rev.* Vol. 65, p. 497-522.

Du, L.X., Jia, S.J., Lu, F.P. 2003. "Morphological Changes of *Rhizopus chinesis* 12 in Submerged Culture and its Relationship with Antibiotic Production." *Process Biochem.* Vol. 38, p. 1643-1646.

Difco and BBL Manual. 2003. "Manual of Microbiological Culture Media" *Becton Dickonson and Company*. Sparks, Maryland.

Elibol, M. 2003. "Optimization of Medium Composition for Actinorhodin Production by *Streptomyces coelicolor* A3(2) with Response Surface Methodology." *Process Biochem*. Vol. 39, p. 1057-1062.

Esquivel, J.C.C. and Voget, C.E. 2004. "Purification and Partial Characterization of an Acidic Polygalacturonase from *Aspergillus kawachii*." *J. Biotechnol.* Vol. 110, p. 21-28.

Fatile, I.A. 1985. "Rheological Characteristics of Suspensions of *Aspergillus niger*: Correlation and Shape of the Mycelial Aggregate." *Biotechnol. Bioeng*. Vol. 21, p. 60-64.

Field, J.A., Jong, E., Feijo, C.G., deBont, J.A. 1992. "Biodegradation of Polycylic Aromatic Hydrocarbons by New Isolates of White Rot Fungi." *Appl. Environ. Microbiol.* Vol. 58, No. 7, p. 2219-2226.

Gadre, R.V., Driessche, G.V., Beeumen, J.V., Bhat, M.K. 2003. "Purification, Characterization and Mode of Action of an Endo-polygalacturonase from the Psychrophilic Fungus *Mucor flavus*." *Enzyme Microbiol. Technol*. Vol. 32, p. 321-330.

Gailing, M.F., Guibert, A., Combes, D. 2000. "Fractional Factorial Designs Applied to Enzymatic Sugar Beet Pulps Pressing Improvement." *Bioprocess Eng.* Vol. 22, p. 69-74.

Gibbs, P.A., Seviour, R.J., Schmid, F. 2000. "Growth of Filamentous Fungi in Submerged Culture: Problems and Possible Solutions." *Crit. Rev. Biotechnol*. Vol. 20, p. 17-48.

Godfrey, T., West, S. 1996. "Introduction to industrial enzymology." Industrial Enzymology second ed. Stocholm Press, New York. p. 1-7.

Gonzales, M.F., Ubeda, J.F., Vasudevan, T.G., Cordero Otero, R.R., Briones, A.I. 2004. "Evaluation of Polygalacturonase Activity in *Saccharomyces cerevisia* Wine Strains" *FEMS Microbiology letters*. Vol. 237, p. 261-266.

Gummadi, S.N. and Panda, T. 2003. "Purification and Biochemical Properties of Microbial Pectinases - a review." *Process Biochem.* Vol. 38, p. 987-996.

Hoondal, G.S., Tiwari, R.P., Tewari, R., Dahiya, N., Beg, Q.K. 2002. "Microbial Alkaline Pectinases and Their Applications: a review." *Appl. Microbiol. Biotechnol*. Vol. 59, p. 409-418.

Hoyos, S.E., Nieto, M.L., Rubio, F.C., Cormenzana, A.R. 2002. "Kinetics of Aerobic Treatment of Olive Mill Waste Water (OMW) with *Aspergillus terreus." Process Biochem*. Vol. 37, p. 1169-1176.

Jayani, R.S., Saxena, S., Gupta, R. 2005. "Microbial Pectinolytic Enzymes: A review." *Process Biochem.* Vol. 40, No. 9, p.2931-2944.

Jonsbu, E., McIntyre, M., Nielsen, J. 2002. "The Influence of Carbon Sources and Morphology on Nystatin Production by *Streptomyces nourse." J. Biotechnol*. Vol. 95, p. 133-144.

Kashyap, D.R., Vohra, P.K., Chopra, S., Tewari, R. 2001. "Applications of Pectinases in the Commercial Sector: a review." *Bioresource Technol*. Vol. 77, p. 215-227

Kaur, G., Kumar, S., Satyanarayana, T. 2004. "Production, Characterization and Application of a Thermostable Polygalacturonase of a Thermophilic Mould *Sporotrichum thermophile Apinis*." *Bioresource Technol*. Vol. 94, p. 239-243.

Laxman, R.S., Sonawane, A.P., More, S.V., Rao, B.S., Rele, M.V., Jogdand, V.V., Deshpande, V.V., Rao, M.B. 2005. "Optimization and Scale up of Production of Alkaline Protease from *Conidiobolus coronatus*." *Process Biochem*. Vol. 40, p. 9, p. 3152-3158.

Li, Z.J., Shukla, V., Fordyce, A.P., Pedersen, A.G., Wenger, K.S., Marten, M.R. 2000. "Fungal Morphology and Fragmentation Behavior in a Fed-Batch *Aspergillus oryzae* Fermentation at the Production Scale." *Biotechnol. Bioeng.* Vol. 70, p. 300-312.

Lopez, L.C., Perez, J.A.S., Sevilla, J.M.F., Porcel, E.M.J., Chisti, Y. 2005. "Pellet Morphology, Culture Rheology and Lovastatin Production in Cultures of *Aspergillus terreus."* J. *Biotechnol*. Vol. 116, p. 61-77.

Metz, B., Kossen, N.W.F., van Suijdam, J.C. 1979. "The Rheology of Mould Suspensions." *Adv. Biochem. Eng.* Vol. 11, p. 103-156.

Metz, B. and Kossen, N.W.F. 1977. "The Growth of Molds in the Form of Pellets, A literature review." *Biotechnol. Bioeng.* Vol. 19, p. 781-799.

Metzner, A.B. 1985. "Rheology of Suspensions in Polymeric Liquid." *Society Rheology.* Vol. 29, No. 6, p. 739-775.

Milstein, O., Gersonde, R., Hutterman, A., Chen, M.J., Meister, J.J. 1992. "Fungal Biodegradation of Lignopolystyren Graft Polymers." *Appl. Environ. Microbiol.* Vol. 52, No.10, p. 3225-3232.

Mitard, A. and Riba, J.P. 1987. "Morphology and Growth of *Aspergillus niger* ATCC 26036 Cultivated at Several Shear Rates." *Biotechnol. Bioeng.* Vol. 32, p. 835-840.

Mohamed, S.A., Farid, N.M., Hossiny, E.N., Bassuiny, R.I. 2006. "Biochemical Characterization of an Extracellular Polygalacturonase from *Trichoderma harzianum." J. Biotechnol.*

Moyo, S., Gashe, B.A., Collison, E.K., Mpuchane, S. 2003. "Optimising Growth Conditions for the Pectinolytic Activity of *Kluyveromyces wickerhamii* by Using Response Surface Methodology." *I. J. Food Microbiol.* Vol. 85, p. 87-100.

Nagai, M., Katsuragi, T., Terashita, T., Yoshikawa, K., Sakai, T. 2000. "Purification and Characterization of an Endo-Polygalacturonase from *Aspergillus awamori*." *Biosci. Biotechnol. Biochem.* Vol. 64, No. 8, p. 1729-1732.

Naidu, G.S.N and Panda, T. 1998. "Application of Response Surface Methodology to Evaluate Some Aspects on Stability of Pectolytic Enzymes from *Aspergillus nige*r." *Biochem. Eng*. p. 71-77.

Naidu, G.S.N and Panda, T. 2003. "Studies on pH and Thermal Deactivation of Pectolytic Enzymes from Aspergillus niger." *Biochem. Eng*. Vol. 16, p. 57-67.

Olsvik, E., Tucker, K.G., Thomas, C.R., Kristiansen, B. 1993. "Correlation of *Aspergillus niger* Broth Rheological Properties with Biomass Concentration and Shape of Mycelial Aggregates." *Biotechnol Bioeng.* Vol. 42, p. 1046-1052.

Ortega, N., de Diego, S., Perez-Mateos, M., Busto, M.D. 2004. "Kinetic Properties and Thermal Behaviour of Polygalacturonase Used in Fruit Juice Clarification." *Food Chem.* Vol. 88, p. 209-217.

Pamboukian, C.R.D., Facciotti, M.C.R., Schmidell, W. 1998. "Relationship between Morphology, Rheology and Glucoamylase Production by *Aspergillus awamori* in Submerged Cultures." *Braz. J. Chem*. Vol. 15, p. 1-8.

Panda, T., Naidu, G.S.N., Sinha, J. 1999. "Multiresponse Analysis of Microbiological Parameters Affecting the Production of Pectolytic Enzymes by *Aspergillus niger:* a statistical view." *Process Biochem*. Vol. 35, p.187-195.

Panda, T., Nair, S.R., Kumar, M.P. 2004. "Regulation of Synthesis of the Pectolytic Enzymes of *Aspergillus niger*." *Enzyme Microbiol. Technol*. Vol. 34, p. 466-473.

Papagianni, M. 2004. "Fungal Morphology and Metabolite Prodcution in Submerged Mycelial Processes." *Biotechnol. Adv*. Vol. 22, p. 189-259.

Parra, R., Aldred, D., Magan, N. 2005. "Medium Optimization for the Production of Secondary Metabolite Squalestatin S1 bty a Phoma sp. Combininig Orthogonal Design and Response Surface Methodology." *Enzyme Microbiol. Technol*. Vol. 37, No. 7, p. 704-711.

Pascoal, C. and Cassio, F. 2004. "Contribution of Fungi and Bacteria to Leaf Litter Decomposition in Polluted River. *Appl. Environ. Microbiol.* Vol. 70, No. 9, p. 5266-5273.

Pazauki, M. and Panda, T. 2000. "Understanding the Morphology of Fungi." *Bioprocess Eng.* Vol. 22, p. 127-143.

Prade, R.A., Zhan, D.F., Ayoubi, P., Mort, A.J. 1999. "Pectins, Pectinases and Plant-Microbe Interactions." *Biotechnol. Genetic Eng. Rev*. Vol. 16, p. 361-391.

Puri, M., Banerjee, A., Banerjee, U.C. 2005. "Optimization of Process Parameters for the Production of Naringinase by *Aspergillus niger* MTCC 1344." *Process Biochem.* Vol. 40, p. 195-201.

Reichl, U., King, R., Gilles, E.D. 1991. "Characterization of Pellet Morphology during Submerged Growth of *Streptomyces tendae* by Image Analysis." *Biotechnol. Bioeng.* Vol. 39, p. 164-170.

Riou, C., Freyssinet, G., Fevre, M. 1992. "Purification and Characterization of Extracellular Pectinolytic Enzymes Produced by *Sclerotinia sclerotiorum*." *Appl. Environ. Microbiol.* Vol. 58, No. 2, p. 578-583.

Ryoo, D. 1999. "Fungal Fractal Morphology of Pellet Formation in *Aspergillus niger*." *Biotechol. Techniques*. Vol. 13, p. 33-36.

Saito, K., Takakuwa, N., Oda, Y. 2004. "Purification of the Extracellular Pectinolytic Enzyme from the Fungus *Rhizopus oryzae* NBRC 4707." *Microbiol. Research*. Vol. 159, p. 83-86.

Saito, K., Kawamura, Y., Oda, Y. 2003. "Role of the Pectoloytic Enzyme in the Lactic Acid Fermentation of Potato Pulp by *Rhizopus oryzae*." *J. Ind. Microbiol. Biotechnol.* Vol. 30, p. 440-444.

Sakamoto, T., Bonnin, E., Quemener, B., Thibault, J.F. 2002. "Purification and Characterization of Two Exo-polygalacturonases from Aspergillus niger able to Degrade Xylogalacturonan and Acetylated Homogalacturonan."*Biochimica et Biophysica Acta.* Vol.1572, p. 10-18.

Schwan, R.F. and Wheals, A.E. 2004. "The Microbiology of Cocoa Fermentation and Its Role in Chocolate Quality." *Crital Rev. Food Sci. Nutr.* Vol. 44, p. 205-222.

Shuler, M.L. and Kargi, F. 2002. *Bioprocess Engineering Basic Concepts 2nd ed*., (Prentice Hall, Upper Saddle River), p. 57.

Silva, E.G., Borges, M.F., Medina, C., Piccoli, R.H., Schwan, R.F. 2005. "Pectinolytic Enzymes Secreted by Yeast from Tropical Fruits." *FEMS Yeast Research.* Vol. 5, p. 859-865.

Singh, S.A. and Rao, A.G.A. 2002. "A Simple Fractionation Protocol for, and a Comprehensive Study of the Molecular Properties of, Two Major Endopolygalacturonases from *Aspergillus niger*." *Biotechnol. Appl. Biochem.* Vol. 35, p. 115-123.

Souza, J.V.B., Silva, E.S., Maia, M.L.S., Teixeira, M.F.S. 2003. "Screening of Fungal Strains for Pectinolytic Activity: Endopolygalacturonase Production by *Peacilomyces clavisporus* 2A.UMIDA.1." *Process Biochem*. Vol. 39, p. 455-458.

Taherzadeh, M.J., Fox, M., Hjorth, H., Edebo, L. 2003. "Production of Mycelium Biomass and Ethanol from Paper Pulp Sulfite Liquor by *Rhizopus oryzae." Bioresource Technol*. Vol. 88, p. 167-177.

Tari, C., Genckal, H., Tokatli, F. 2006. "Optimization of a Growth Medium Using a Statistical Approach for the Production of an Alkaline Protease by a newly Isolated Bacillus *sp. L21." Process Biochem*. Vol. 41, p. 659-665.

Tari, C., Parulekar, S.J., Stark, B.C., Webster, A.D. 1998. "Synthesis and Excretion of alpha amylase in vgb+ and vgb- Recombinant *E.coli*: A Comparative Study." *Biotechnol Bioeng.* Vol. 59, p. 673-678.

Tellez, S.J., Moldes, A.B., Alonso, J.L., Vazquez, M. 2003. "Optimization of Lactic acid Production by *Lactobacillus delbuecki* through Response Surface Methodology." *J. Food Sci.* Vol. 68, p. 1454-1458.

Wainwright, M., 1992. *An Introduction to Fungal Biotechnology,* (John Wiley, New York), p. 8.

Zhang, H. and Qi, H.Y. 2000. "Pectinase Production by *Aspergillus niger* using Biomass Waste in Solid State Fermentation." *CN Patent 1271774A.*

Zhaou, Y., Du, J., Tsao, G.T. 2000. "Mycelial Pellet Formation by *Rhizopus oryzae* ATCC 20344." *Appl Biochem. Biotechnol.* 84-86, 779-789.

Znidarsic, P., Komel, R., Pavko, A. 2000. "Influence of Some Environmental Factors on *Rhizopus nigricans* Submerged Growth in the Form of Pellets." *World J. Microbiol. Biotechnol.* Vol.16, p. 589-593.

APPENDIX A

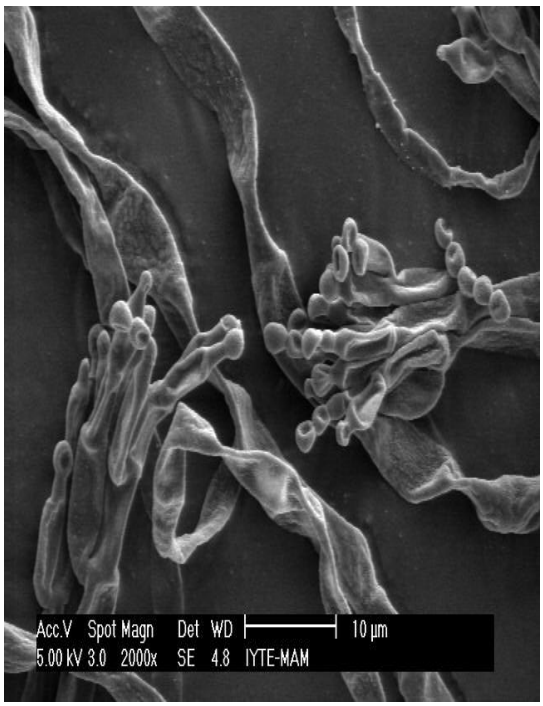
SEED MEDIA FORMULATIONS

Table A.1. Seed media formulations used in the preparation of spore suspensions

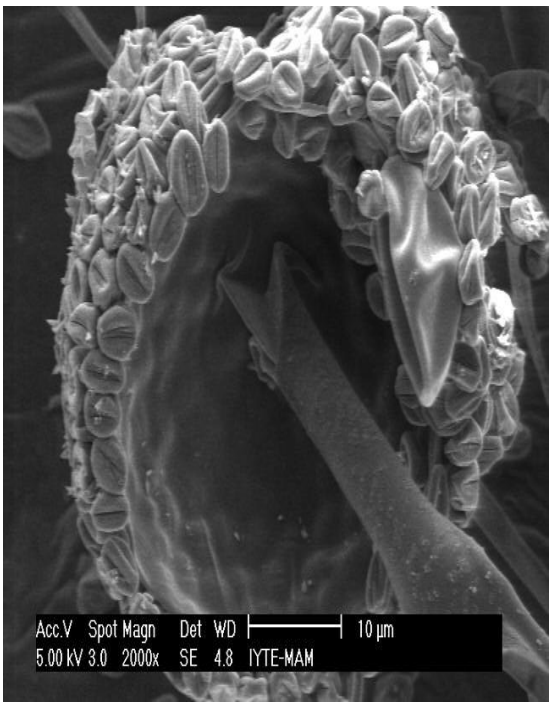
Media Code	Composition
Yeast Malt Extract Agar **(YME)**	Malt extract (10 g/l) , Yeast extract (4 g/l), Glucose (4 g/l), Agar (20 g/l)
Yeast Malt Pepton Agar **(YMP)**	Malt extract (3 g/l) , Yeast extract (3 g/l), Pepton (3 g/l), Glycerol (20 g/l), Agar (20 g/l)
Molasses Medium **(M)**	Glycerol (45 g/l), Pepton (18 g/l), Molasses (45 g/l) , NaCl (5 g/l), $FeSO_4.7H_2O$ (15 mg/l), KH_2PO_4 (60 mg/l), $MgSO_4$ (50 mg/l), $CuSO_4.5H_2O$ (12 mg/l), $MnSO_4.H_2O$ (15 mg/l), Agar (20 g/l)
***ATCC** media :* For Rhizopus this ise corn meal agar (purchased by Bacto) and for Aspergillus it is the one stated in next column	Corn meal (5 g/l), Yeast extract (2 g/l), Casitone (1 g/l), Malt extract (1 g/l), $MgSO_4.7H_2O$ (0.5 g/l), KH_2PO_4 (1.5 g/l), Agar (20 g/l)
Defatted Soy Bean **(DSM)**	Defatted soybean (5 g/l), Malt extract (20 g/l), KH_2PO_4 (10 g/l), Agar (20 g/l)
Complex Medium **(CM)**	Glucose (25 g/l), Matrin 50 (25 g/l), Corn steep liquor (2.5 g/l), Peptone (2.5 g/l), Disodyum phosphate (3.2 g/l), Monosodium phosphate (3.3 g/l), Agar (20 g/l)
Defined Medium **(DM)**	Glucose (50 g/l), Yeast extract (2 g/l), KH_2PO_4 (0.6 g/l), $MgSO_4.7H_2O$ (0.5 g/l), $ZnSO_4.7H_2O$ (0.0176 g/l), $FeSO_4.7H_2O$ (0.498 mg/l), Bacto agar (20 g/l)

APPENDIX B

IMAGES TAKEN BY SCANNING ELECTRON MICROSCOPE



Aspergillus sojae ATCC 20225



Rhizopus oryzae ATCC 4858

Figure B.1. Images taken by scanning electron microscope (Philips XL-30S FEG) of two taxonomically different strains *Aspergillus sojae 20235* and *Rhizopus oryzae 4858* (magnified 2000x)

APPENDIX C

MORPHOLOGY OF PELLETS GROWN IN COMPLEX AND DEFINED MEDIUM

Complex Medium

Defined Medium

Figure C.1. Morphology of pellets grown in complex and defined medium

APPENDIX D

RESULTS OF FULL FACTORIAL DESIGN

Table D.1. Effect of factors and interactions on the enzyme activity

Response: Activity	P-value
Factor: Strain	
AS	2.06376e-006
RO	2.06376e-006
Factor: Media	
def	8.20811e-011
comp	8.20811e-011
Factor: Seed culture	
YME, YMP, ATCC, Molasses, DSB, CA, DA	>>0.01 insignificant
Interaction: Strain*Media	
AS* def	1.70172e-006
AS* comp	1.70172e-006
RO* def	1.70172e-006
RO* comp	1.70172e-006
Interaction: Strain*Seed culture	>>0.01 insignificant
Interaction: Media*Seed culture	>>0.01 insignificant

Table D.2. The mean values and standard deviations of activity measurements in full factorial design.

Combination	Strain	Media	Slant	Activity_mean[a] (U/ml)	Activity_stdev[a] (U/ml)
1	AS	def	YME	0.237	0.023
2	RO	def	YME	0.014	0.020
3	AS	comp	YME	0.277	0.040
4	RO	comp	YME	0.262	0.001
5	AS	def	YMP	0.147	0.101
6	RO	def	YMP	0.013	0.019
7	AS	comp	YMP	0.246	0.070
8	RO	comp	YMP	0.248	0.003
9	AS	def	ATCC	0.184	0.052
10	RO	def	ATCC	0.066	0.024
11	AS	comp	ATCC	0.280	0.054
12	RO	comp	ATCC	0.282	0.095
13	AS	def	M	0.244	0.033
14	RO	def	M	0.079	0.112
15	AS	comp	M	0.258	0.042
16	RO	comp	M	0.257	0.058
17	AS	def	DSB	0.239	0.115
18	RO	def	DSB	0.000	0.000
19	AS	comp	DSB	0.232	0.023
20	RO	comp	DSB	0.254	0.081
21	AS	def	CA	0.175	0.100
22	RO	def	CA	0.009	0.013
23	AS	comp	CA	0.249	0.054
24	RO	comp	CA	0.263	0.080
25	AS	def	DA	0.216	0.069
26	RO	def	DA	0.000	0.000
27	AS	comp	DA	0.281	0.083
28	RO	comp	DA	0.265	0.085

Mean values and standard deviations are calculated based on two replications. AS: *A. sojae*; RO: *R. oryzae*; def: defined medium; comp: complex medium.

APPENDIX E

RESULTS OF FIRST STEP OPTIMIZATION

Table E.1. CCD results of first optimization step

Run Order	Maltrin (g/l)	CSL (g/l)	Agitation Speed (rpm)	Inoculum Ratio (total spore)	Activity (U/ml)	Biomass (g/l)
1	50	8.75	225	5.00E+05	2.212	17.481
2	25	2.5	150	7.50E+05	0.764	10.668
3	25	15	150	2.50E+05	1.568	15.390
4	25	15	300	2.50E+05	1.889	14.060
5	75	15	300	7.50E+05	4.813	22.115
6	50	8.75	300	5.00E+05	2.982	12.185
7	50	8.75	225	5.00E+05	3.548	18.588
8	25	15	150	7.50E+05	0.316	19.165
9	75	15	150	7.50E+05	4.188	19.954
10	75	2.5	150	7.50E+05	5.492	14.982
11	25	2.5	300	2.50E+05	1.717	7.814
12	50	8.75	225	5.00E+05	2.494	13.887
13	25	2.5	300	7.50E+05	2.717	9.929
14	75	2.5	300	7.50E+05	7.350	13.046
15	50	8.75	225	5.00E+05	3.129	18.878
16	75	15	150	2.50E+05	4.351	17.261
17	50	8.75	225	5.00E+05	1.697	13.939
18	50	8.75	150	5.00E+05	1.668	13.949
19	75	15	300	2.50E+05	3.359	15.685
20	50	8.75	225	7.50E+05	2.432	12.810
21	25	8.75	225	5.00E+05	0.906	17.148
22	75	2.5	300	2.50E+05	8.620	10.226
23	50	2.5	225	5.00E+05	4.353	10.344
24	25	2.5	150	2.50E+05	1.576	10.187
25	75	2.5	150	2.50E+05	4.569	13.138
26	25	15	300	7.50E+05	0.289	12.186
27	50	8.75	225	2.50E+05	3.629	16.602
28	50	8.75	225	5.00E+05	1.899	16.628
29	50	15	225	5.00E+05	2.153	20.693
30	75	8.75	225	5.00E+05	5.581	20.077
31	50	8.75	225	5.00E+05	2.521	14.614

Table E.2**.** First optimization: the analysis was done using coded units

Estimated Regression Coefficients for PG-activity

Term	Parameter estimates	p- value
Constant	3.0580	0.000
Maltrin	2.0322	0.000
CSL	-0.7906	0.000
Agitation Speed	0.5136	0.011
Inoculum Ratio	-0.1619	0.395
Maltrin*CSL	-0.4130	0.048
Agitation Speed*CSL	-0.5048	0.018

S = 0.7937 R-Sq = 86.6% R-Sq(adj) = 83.2%

Estimated Regression Coefficients for biomass

Term	Parameter estimates	p- value
Constant	15.995	0.000
Maltrin	1.6633	0.001
CSL	3.1207	0.000
Agitation Speed	-0.9693	0.045
Inoculation Ratio	0.8051	0.092
Maltrin*Maltrin	1.8781	0.085
Agitation Speed*Agitation Speed	-3.6679	0.002

S = 1.948 R-Sq = 77.0% R-Sq(adj) = 71.3

APPENDIX F

RESULTS OF SECOND STEP OPTIMIZATION

Table F.1. CCD results of second step optimization

Run Order	Maltrin (g/l)	CSL (g/l)	Agitation (rpm)	Inoculum (total spore)	Activity (U/ml)	Average Pellet size (cm)	Biomass (g/l)
1	50	0	350	2.00E+07	9.63	0.301	7.591
2	120	5	350	2.00E+07	8.73	3.0	31.488
3	85	2.5	250	1.00E+07	6.95	0.093	22.543
4	85	0	250	1.00E+07	10.46	0.146	10.096
5	85	2.5	250	1.00E+07	6.13	0.141	18.276
6	120	0	150	2.00E+07	11.29	0.055	11.025
7	120	0	350	1.25E+04	10.55	0.110	6.818
8	120	2.5	250	1.00E+07	7.87	0.164	23.018
9	50	5	150	1.25E+04	1.89	0.529	13.284
10	50	5	350	2.00E+07	2.37	3.0	20.498
11	85	2.5	250	2.00E+07	5.33	0.1312	18.499
12	50	5	150	2.00E+07	1.03	0.089	20.617
13	120	0	150	1.25E+04	13.50	0.155	7.561
14	85	2.5	150	1.00E+07	5.28	0.276	10.000
15	85	2.5	250	1.00E+07	6.36	0.76	13.128
16	50	2.5	250	1.00E+07	4.17	0.102	14.996
17	120	5	150	1.25E+04	10.47	3.0	26.630
18	85	2.5	250	1.00E+07	6.16	0.159	20.758
19	120	5	350	1.25E+04	11.02	0.399	18.462
20	50	0	350	1.25E+04	8.96	0.164	4.065
21	120	0	350	2.00E+07	15.43	0.321	12.043
22	85	2.5	250	1.00E+07	7.578	0.105	17.014
23	50	0	150	2.00E+07	9.32	0.049	7.840
24	85	5	250	1.00E+07	5.17	0.092	18.541
25	85	2.5	250	1.25E+04	7.37	0.302	13.503
26	85	2.5	250	1.00E+07	6.10	0.105	18.752
27	120	5	150	2.00E+07	8.13	0.160	10.087
28	50	0	150	1.25E+04	8.81	0.167	1.871
29	85	2.5	350	1.00E+07	6.86	0.063	22.558
30	50	5	350	1.25E+04	1.31	0.633	12.529
31	85	2.5	250	1.00E+07	5.69	0.116	19.136

Table F.2. Experimental and Predicted Values for PG Activity, Biomass and Pellet Size at Individual Optimal Conditions

Maltrin (g/l)	CSL (g/l)	Agitation rpm)	Inoculum (Total spore)	PG activity (U/ml)	Biomass (g/l)
120	0	350	2 x 10^7	13.162 (13.8)	5.08 (14.1)
150	0	350	2 x 10^7	13.818 (14.2)	6.266 (15.1)
180	0	350	2 x 10^7	16.645 (16.6)	9.823 (16.2)
120	4.10	350	2 x 10^7	5.817 (9.2)	40.366 (26.1)

* Predicted values of responses are in the paranthesis

APPENDIX G

STANDART CALIBRATION CURVE FOR ACTIVITY

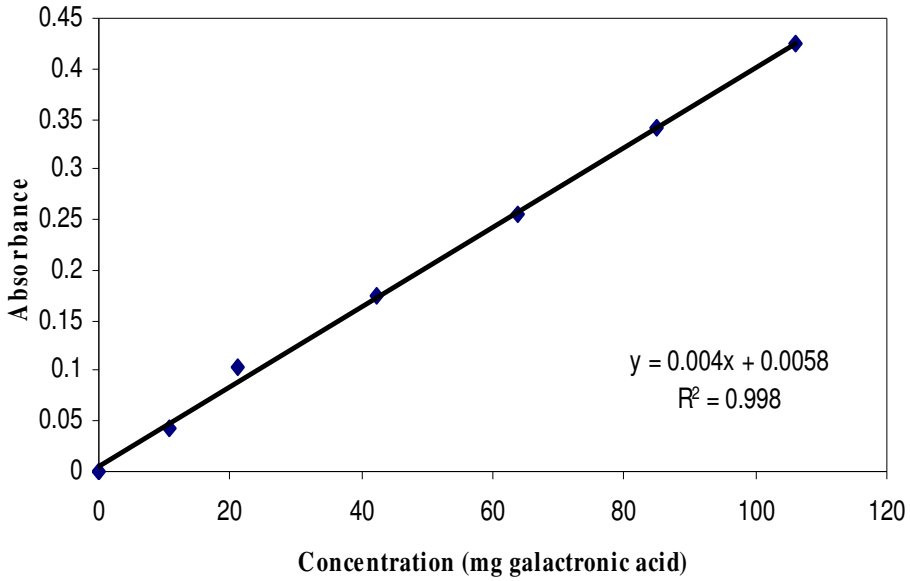


Figure G.1. Standard calibration curve for activity

APPENDIX H

STANDARD CALIBRATION CURVE FOR TOTAL PROTEIN

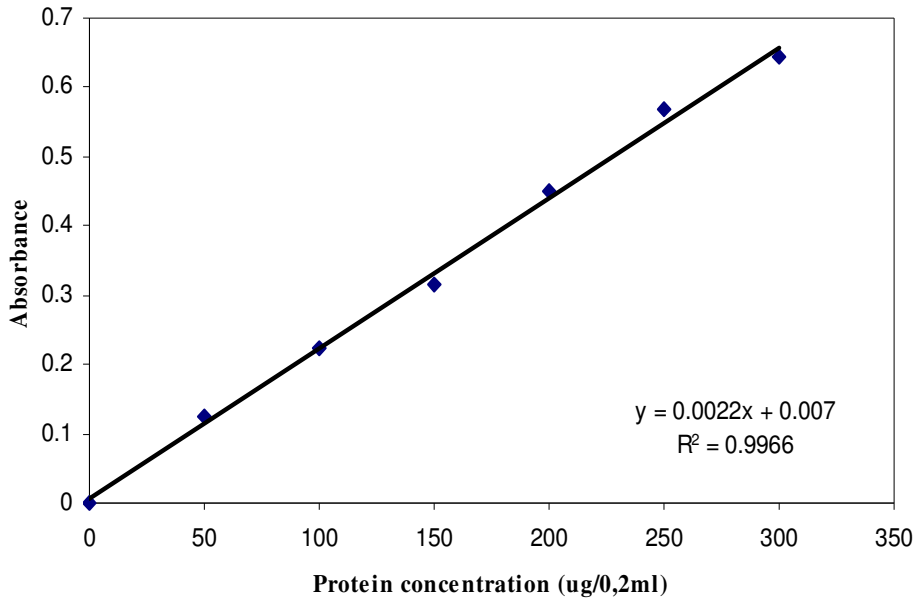


Figure H.1. Standard calibration curve for total protein