PRODUCTION OF PECTINASE ENZYME FROM *Aspergillus sojae* IN BATCH AND FED-BATCH SYSTEMS

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ABSTRACT

PRODUCTION OF PECTINASE ENZYME FROM *ASPERGILLUS SOJAE* IN BATCH AND FED-BATCH SYSTEMS

Commercial preparations of pectinases derived from fungi are well known to have high biotechnological value in the industry. For this purpose, polymethylgalacturonase (PMG) and polygalacturonase (PG) were produced with high productivities by *Aspergillus sojae* ATCC 20235 by using low cost carbon (Maltrin) and nitrogen (Corn Steep Liquor, CSL) sources. There is no literature report to best of our knowledge on the fed-batch production, purification and characterization of polygalacturonase using this microorganism.

In this study batch fermentation was carried out in order to obtain the crude PG and to establish a baseline for the forth coming fed-batch experiments. The crude PG was partially purified using three-phase partitioning as an emerging bioseparation technique and characterized with respect to its biochemical and thermal properties. These studies showed that this enzyme holds a great potential to be a good candidate for various industrial applications.

To optimize fed-batch fermentation conditions, response surface methodology (RSM) was performed using face-centered central composite design. As a result, maximum PG activity (20.61 U/ml) and maximum biomass (34.23 g/l) were obtained at high maltrin (150 g/l) and high CSL (10 g/l) concentrations when the repeated feeding was done at 48th and 72nd hours. Maximum PMG activity (16.76 U/ml) was also achieved at higher maltrin and higher CSL concentrations at a feeding time of 72nd hours. Fed-batch fermentation has been successfully used to increase PG (33.74%) and PMG (23.96%) activities from *Aspergillus sojae*.

Finally, agar diffusion method was adapted as a rapid method for the selection of high pectinase producer in the strain improvement study.

ÖZET

KESİKLİ VE YARI-KESİKLİ SİSTEMLERDE *ASPERGILLUS SOJAE*'DEN PEKTİNAZ ENZİMİ ÜRETİMİ

Funguslardan elde edilen ticari pektinazlar endüstride yüksek biyoteknolojik değere sahip olmalarıyla bilinirler. Bu amaçla, *Aspergillus sojae* ATCC 20235 tarafından düşük maliyetli karbon (Maltrin) ve azot (Mısır Şırası Şurubu, MŞŞ) kaynaklarının kullanılması yoluyla yüksek verimlilikte polimetilgalakturonaz (PMG) ve poligalakturonaz (PG) üretilmiştir. Bu mikroorganizmanın kullanılmasıyla poligalakturonazın yarı-kesikli üretimi, saflaştırılması ve karakterizasyonu hakkında bilindiği kadarıyla rapor edilen hiçbir literatür bulunmamaktadır.

Bu çalışmada, ham PG elde etmek ve ilerideki yarı-kesikli deneylere bir temel oluşturmak için kesikli fermentasyon uygulanmıştır. Ham PG bir biyoayrılma tekniği olan üç faza ayırmanın kullanılmasıyla kısmen saflaştırılmış ve biyokimyasal ve termal özellikleri bakımından karakterize edilmiştir. Bu çalışmalar, bu enzimin çeşitli endüstriyel uygulamalar için iyi bir aday olarak büyük bir potansiyele sahip olduğunu göstermiştir.

Yarı-kesikli fermentasyon koşullarını optimize etmek için, yüzey-merkezli yüzeysel kompozit dizayn kullanılarak cevap yüzey yöntemi uygulanmıştır. Sonuç olarak, en yüksek PG aktivitesi (20.61 U/ml) ve en yüksek biyokütle (34.23 g/l) yüksek maltrin (150g/l) ve yüksek MŞŞ (10 g/l) konsantrasyonlarında 48. ve 72. saatlerde tekrarlı beslemenin yapılmasıyla elde edilmiştir. En yüksek PMG aktivitesine (16.76 U/ml) de yüksek maltrin ve yüksek MŞŞ konsantrasyonlarında 72. saatte beslemenin yapılmasıyla ulaşılmıştır. Yarı-kesikli fermentasyon *Aspergillus sojae*'den PG aktivitesini %33.74 ve PMG aktivitesini %23.96 arttırarak başarıyla kullanılmıştır.

Son olarak, suş geliştirme çalışmalarında yüksek pektinaz üreticisinin seçiminde için hızlı bir yöntem olan agar diffüzyon yöntemi uyarlanmıştır.

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CHAPTER 1

INTRODUCTION

In nature, microorganisms have been endowed with vast potentials. They produce an array of enzymes, which have been exploited commercially over the years. Pectinases of fungal origin are of great significance with tremendous potential to offer to industry. Among these applications are retting of flax and vegetable fibres, increasing the yield of fruit juice extraction, clarification of fruit juices, extraction of oils from vegetables and citrus peels, pre-treatment of pectic waste water, fermentation of coffee and tea, manufacturing of paper and pulp, production of poultry feed and purification of plant viruses (Saito, et al. 2004, Hoondal, et al. 2002). It is reported that pectinases hold a share of 25 % in the global sales of food enzymes where this value is expected to increase over time with the invention of new application areas (Jayani, et al. 2005). Novozymes (Denmark), Novartis (Switzerland), Roche (Germany) and Biocon (India) are some important commercial producers of pectinases (Gummadi and Panda 2003).

Pectolytic enzymes are classified mainly into two main groups of de-esterification and depolymerising enzymes based on their degradation mechanism (Saito, et al. 2004, Kashyap, et al. 2001, Gummadi and Panda 2003). The enzymes depolymerising polygalacturonic acid are further classified into polygalacturonase simply hydrolyzing the α-1,4- linkage and polygalacturonate lyase cleaving trans–eliminatively. Both include exo-type enzymes, which catalyze the release of single galacturonic acid residue from the non–reducing end and random fashion endo-type hydrolyzing enzymes which release oligosaccharidic chains of various lengths (Saito, et al. 2004).

Since applications of pectinases in various fields are broadening, it is important to discover new strains producing pectinases with novel properties and understand the nature and properties of these enzymes for their efficient and effective usage. With this perspective polymethylgalacturonase and polygalacturonase which attract the most attention due to their wide uses among pectinases were produced by *Aspergillus sojae ATCC 20235* (not considered for this purpose so far) in batch and fed-batch systems. In fed-batch studies pectolytic activity was enhanced by feeding carbon (maltrin) and

nitrogen sources (corn steep liquor) at different concentrations. Furthermore, optimization conditions for maximum polymethylgalacturonase and polygalacturonase were determined in fed-batch fermentations. Moreover, the crude polygalacturonase obtained from batch fermentation was purified by three phase partitioning (TPP) for a one-step purification based on a modified procedure reported by Sharma and Gupta, (Sharma and Gupta 2001). In this process (TTP) the protein extract is mixed with ammonium sulphate and t-butanol. Three phases are formed within short periods of time (about 1 hour): lower aqueous layer, interfacial protein precipitate and upper t-butanol layer (Kansal, et al. 2006). This technique is an emerging bio-separation technique, which employs collective operation of principles involved in numerous techniques such as conventional salting out, cosolvent, isoionic, osmolytic and kosmotropic precipitation of proteins (Roy and Gupta 2002).

The purified polygalacturonase was further characterized with respect to its activity and stability at various pH and temperature ranges. Determination of its K_m and V_{max} parameters together with its thermal inactivation kinetics was carried out. The effect of metal ions and certain compounds such as EDTA, glycerol, Tween 80, SDS and β-mercaptoethanol were also investigated. To date there is no literature report on the fed-batch production, purification and characterization of this enzyme using this organism. This study will be one of the initial studies in this field. In the previous studies (Ustok, et al. 2007, Tari, et al. 2007, Gogus, et al. 2006) it was reported that this organism holds a great potential for the batch production of this enzyme in submerged and solid state fermentation. Therefore in order to exploit new industrial potentials of polygalacturonase, it is necessary to investigate new microbial strains and understand the structure-stability relationship. In industrial processes separation and purification of proteins/enzymes, account a major fraction of the overall production cost. Hence using efficient bioseparation techniques reduces this cost significantly. Besides, 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, Roy and Gupta 2002). In view of this fact, knowledge gained in this way will offer new possibilities in improving the potential and effective use of pectinases in such diverse and broad areas.

CHAPTER 2

FUNGI

The universal phylogenetic tree (Figure 2.1) is the road map of life that describes the evolutionary history of the cells of all organisms showing three domains. Comparing the sequences of nucleotides in ribosomal RNA (rRNA), transfer RNA molecules, membrane lipid structure and sensitivity to antibiotics from different kind of cells demonstrates that there are three distinctly different cell groups: the eukaryotic domain is called the Eukarya and two different types of prokaryotes are the Bacteria and the Archaea (Tortora, et al. 2005). These terms explain the three domains of life, the domain being the highest of biological taxons (Madigan and Martinko 2006).

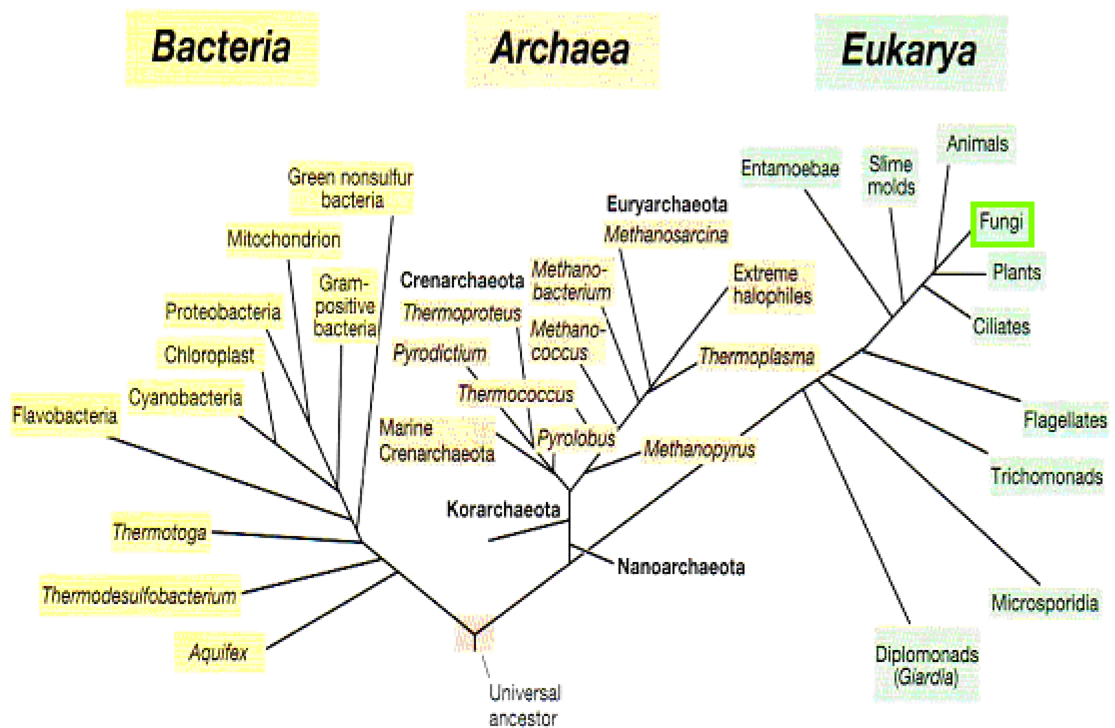


Figure 2.1. Universal phylogenetic tree as determined from comparative ribosomal RNA sequencing (Source: Madigan and Martinko 2006)

The Kingdom Fungi which is within the domain Eukarya (Figure 2.1) includes the unicellular yeasts, multicellular molds, and macroscopic species such as mushrooms. A fungus absorbs dissolved organic matter through its plasma membrane in order to obtain raw materials for vital functions. The cells of a multicellular fungus are commonly joined to take shape of thin tubes called hyphae that are usually divided into multinucleated units by cross-walls that have holes, so that cytoplasm can flow between the cell-like units. Fungi grow from spores or from fragments of hyphae (Tortora, et al. 2005).

2.1. Cell Walls, Metabolism and Nutrition

Fungal cell walls and plant cell walls resemble each other constructively, but not chemically. Most fungi consist of chitin, a polymer of the glucose derivative, N-acetylglucosamine in their cell walls. The chitin is laid down in microfibrillar bundles such as cellulose. Other polysaccharides like for example mannans, chitosans and galactosans replace chitin in some fungal cell walls. Fungal cell walls are typically 80-90% polysaccharide, with proteins, lipids, polyphosphates, and inorganic ions making up the wall-cementing matrix. An understanding of their chemistry is important due to the extensive biotechnological uses of fungi. In addition, the chemical nature of the fungal cell wall has been used in classifying fungi for research and industrial purposes (Madigan and Martinko 2006).

Fungi are aerobic or facultatively anaerobic; only a few anaerobic fungi are known. Many fungi can grow under environmental conditions of extremely high temperature (up to 62°C) or low pH. It makes these organisms common contaminants of food products, microbial culture media, and surfaces (Madigan and Martinko 2006).

All fungi are chemoheterotrophs, which use the electrons from hydrogen atoms in organic compounds as energy and carbon source (Tortora, et al. 2005). They consume natural organic substrates like cellulose, chitin, starch, sugars, hemicelluloses and lignin. As a result a large number of extracellular enzymes, particularly oxidases or hydrolases are produced. Carbohydrates as the main carbon source are utilized by fungi in order to get energy and to synthesize the cellular material. Furthermore, fungi can use up alcohols, hydrocarbons, glycerol and starch as carbon sources. All fungi generally can consume nitrate and utilize nitrogen in the form of ammonium. The other main

nitrate sources are urea, hydroxylamine, L-amino acids and peptides. In the laboratory conditions defined media containing sugars like glucose, sucrose and cellulose or complex media such as potato-dextrose agar and vegetable based media can be used to grow fungi. Inorganic nitrogen sources are generally in the form of ammonium, nitrate, amides or amino acids and ammonia gas which are used in some processes of industrial fermentations. Moreover, the major mineral nutrients required by fungi to obtain maximum yields are phosphorus, sulphur, potassium and magnesium. Minor nutrients like zinc, copper, molybdenum and vitamins are also essential for enzyme functionality under rapid growth conditions (Wainwright 1992).

2.2. Characteristics of Fungi

Since fungal colonies are composed of cells involved in catabolism and growth, they are defined as vegetative structures. The thallus (body) of a mold or fleshy fungus consists of long filaments of cells joined together; these filaments are called hyphae (singular: hypha) which can develop to enormous proportions. In most molds, the hyphae include cross-walls called septa (singular: septum), which divide them into distinct, uninucleate (one-nucleus) cell-like units. These hyphae are called septate hyphae as shown in Figure 2.2.a. In a small number of classes of fungi, the hyphae don't contain septa and appear as long, continuous cells with many nuclei. These are called coenocytic hyphae (Figure 2.2.b). Even in fungi with septate hyphae, there are generally openings in the septa that make the cytoplasm of adjacent "cells" continuous; these fungi are also actually coenocytic organisms. Hyphae grow by getting longer at the tips (Figure 2.2.c). Each part of a hypha is capable of growth, and when a fragment breaks off, it can elongate to form a new hypha. In the laboratory, fungi are usually grown from fragments obtained from a fungal thallus (Tortora, et al. 2005).

The part of a vegetative hypha that obtains nutrients is called the vegetative hypha; the part involved in reproduction is the reproductive or aerial hypha, so named due to the fact that it projects above the surface of the medium on which the fungus is growing. Aerial hyphae frequently bear reproductive spores. When environmental conditions are suitable, the hyphae grow to form a filamentous mass called a mycelium, which is visible to the unaided eye (Figure 2.3) (Tortora, et al. 2005).

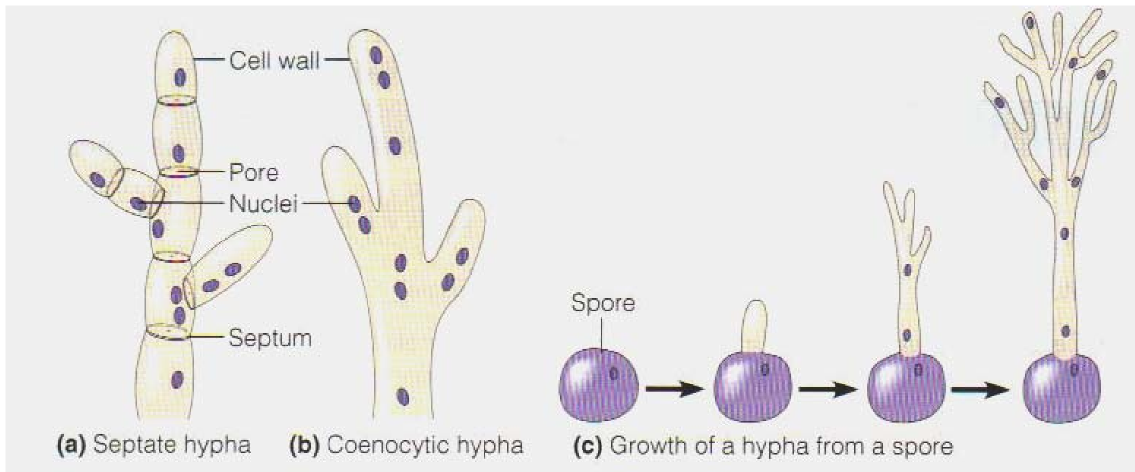


Figure 2.2. Characteristics of fungal hyphae (a) Septate hyphae have cross-walls, or septa, dividing the hyphae into cell-like units. (b) Coenocytic hyphae lack septa. (c) Hyphae grow by elongating at the tips (Source: Tortora, et al. 2005)

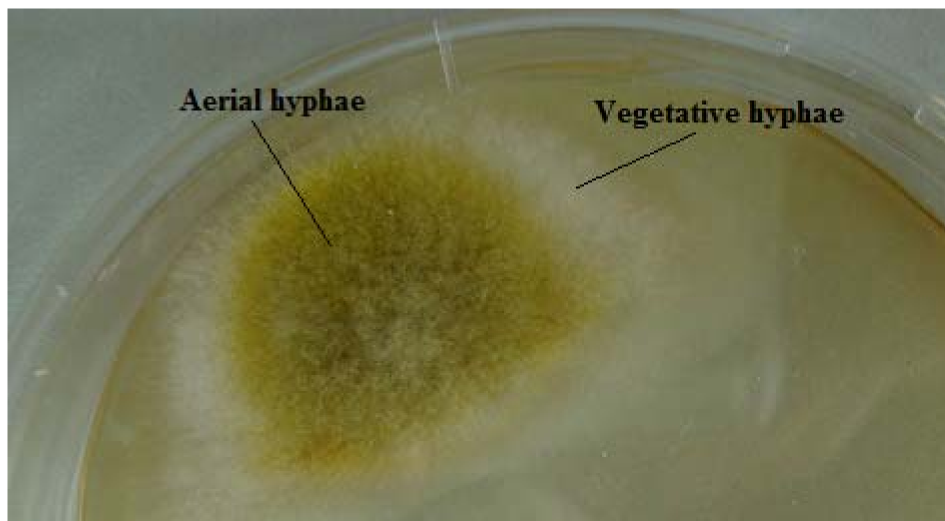


Figure 2.3. A colony of *Aspergillus sojae* grown on a YME agar plate, showing both vegetative and aerial hyphae

2.3. Life Cycle

Filamentous fungi are considered to go through a four stage life cycle: spore, germ, hypha and mature mycelium. They can reproduce asexually by fragmentation of

their hyphae (Figure 2.4) which can grow longer rapidly, at rates of up to several micrometers per minute. Furthermore, both sexual and asexual reproduction in fungi occurs by the formation of spores. Fungi can be identified depending on the type of spore they are formed from aerial hyphae. While asexual spores are formed by the hyphae of an organism, sexual spores result from the fusion of nuclei from two opposite mating strains of the same species of fungus. Fungi produce asexual spores more frequently than sexual spores (Tortora, et al. 2005).

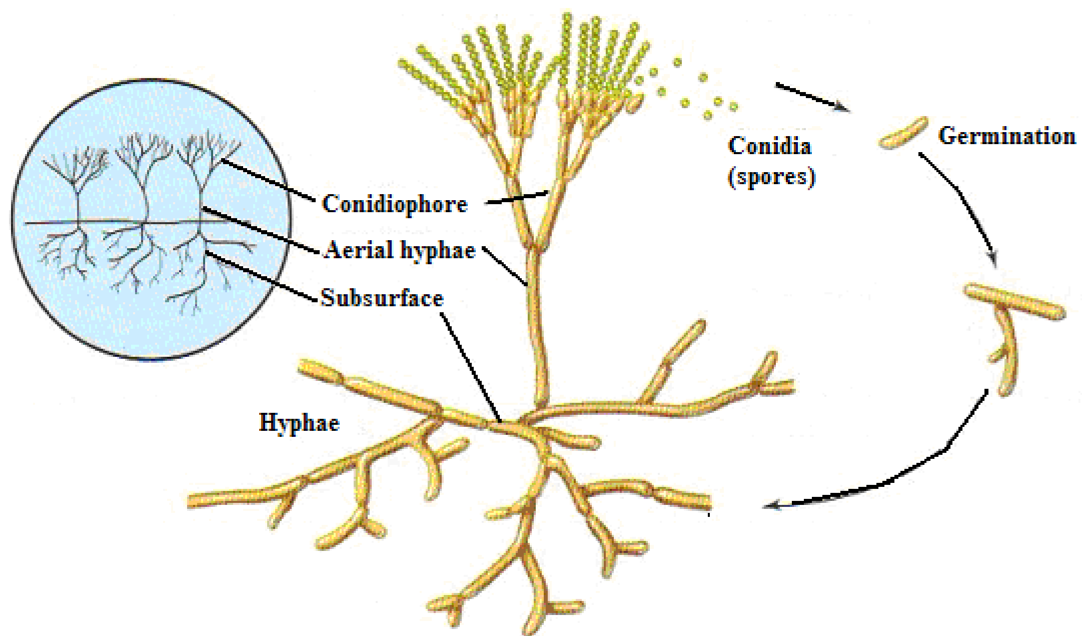


Figure 2.4. Diagram of a mold life cycle (Source: Madigan and Martinko 2006)

2.3.1. Asexual Spores

Fungi produce several types of asexual spores through mitosis and following cell division; there is no fusion of the nuclei of cells. One type of asexual spores is a conidiospore or conidium, a unicellular or multicellular spore that is not enclosed in a sac (Figure 2.5.a). *Aspergillus* produces conidia in a chain at the end of a conidiophore. One type of conidium, an arthrospore produced by *Coccidioides immitis*, is formed by the fragmentation of a septate hypha into single, slightly thickened cells (Figure 2.5.b). Another type of conidium, blastoconidia, consists of buds coming off the parent cell

(Figure 2.5.c). Such spores are found in some yeasts, such as *Candida albicans* and *Cryptococcus*.

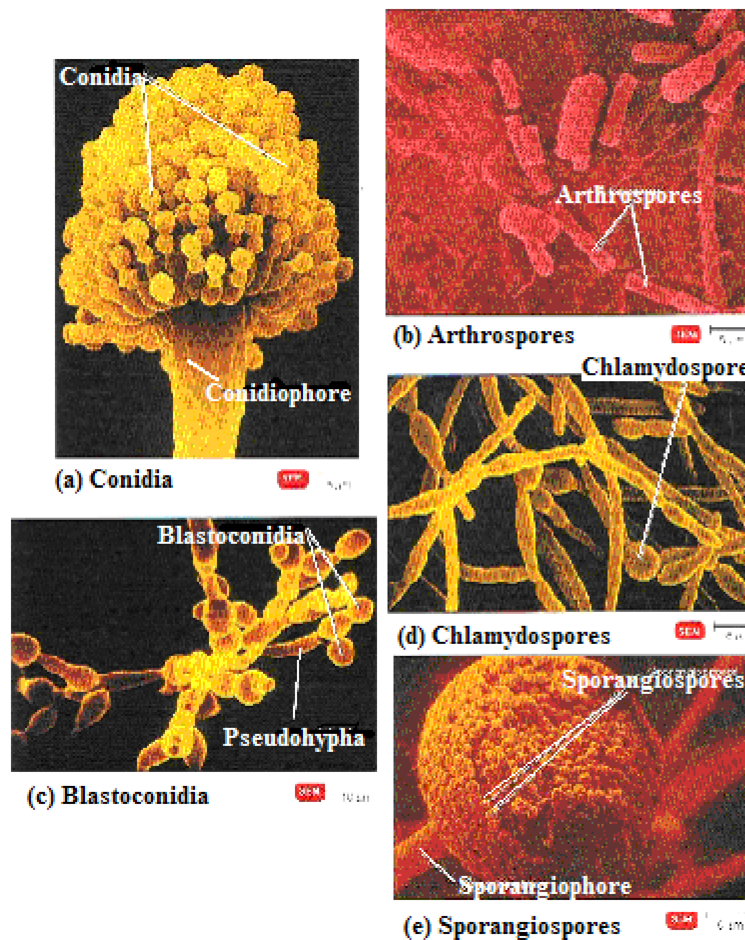


Figure 2.5. Representative asexual spores (a) Conidia are arranged in chains at the end of a conidiophore on this *Aspergillus flavus* (b) Fragmentation of hyphae results in the formation of arthroconidia in this *Coccidioides immitis* (c) Blastoconidia are formed from the buds of a parent cell of *Candida albicans* (d) Chlamydospores are thick-walled cells within hyphae of this *C. Albicans* (e) Sporangiospores are formed within a sporangium (spore sac) of this *Rhizopus* (Source: Tortora, et al. 2005)

A second type of spore is a chlamydospore, produced by the yeast *C. Albicans*, a thick-walled spore formed by rounding and enlargement within a hyphal segment (Figure 2.5.d). A third type of asexual spore is a sporangiospore, formed within a sporangium, or sac, at the end of an aerial hypha called sporangiophore. The sporangium can have

within hundreds of sporangiospores (Figure 2.5.e). This kind of spores is produced by *Rhizopus* (Tortora, et al. 2005).

2.3.2. Sexual Spores

A fungal sexual spore is the result of sexual reproduction, consisting of three phases; plasmogamy, a haploid nucleus of a donor cell (+) penetrates the cytoplasm of a recipient cell (-); karyogamy, the (+) and (-) nuclei fuse to form a diploid zygote nucleus; and meiosis, the diploid nucleus bring about haploid nuclei (sexual spores), some of which may be genetic recombinants.

In laboratory settings, most fungi produce only asexual spores. Historically, fungi whose sexual cycle had not been observed were put in a "holding category" called Deuteromycota. Nowadays, mycologists are using rRNA sequencing to classify these organisms. Most of these previously unclassified deuteromycetes are anamorph, which some Ascomycetes have lost the ability to reproduce sexually, phases of Ascomycota, and a few are basidiomycetes (Tortora, et al. 2005).

2.4. Fungal Taxonomy

The 100000 known species of fungi have been classified into four classes, based on how and where they specifically generate their sexual spores. These are Phycomycetes, Ascomycetes or Ascomycotina (sac fungi), Basidiomycetes or Basidiomycotina (club fungi) and Deuteromycetes or Deuteromycotina ('imperfect' fungi) (Waites, et al. 2001).

The first class is Phycomycetes that are the lower fungi, which are subdivided into Mastigomycotina (zoosporic, motile spores) and Zygomycotina (zygosporic). Industrially important members of the Zygomycotina including *Mucor*, *Rhizomucor* and *Rhizopus* species are used in some traditional food fermentations, whole cell and enzyme bioconversions. Secondly, Ascomycotina is the largest class of fungi including the yeasts used in many industrial fermentation processes. Other filamentous members that have industrial and commercial roles are *Neurospora* species, *Claviceps* species, and important edible fungi, including *Michaela* species (morels) and *Tuber* species (truffles). Thirdly, Basidiomycotina include the mushrooms, e.g. *Agaricus bisporus*

(button mushroom) and *Lycoperdon* species (puff balls). Other industrially important basidiomycetes are certain wood-rotting fungi involved in biodegradation and biodeterioration processes, e.g. *Phanerochaete chrysosporium* (white rot). Finally, Deuteromycotina contains a diverse group of around 25000 species, which have been grouped together simply because they lack a defined sexual (perfect) stage, or one has not been observed. It is thought that this group may represent the conidial stages of ascomycetes whose ascus stage has not yet been discovered or has been lost in the course of evolution. Some species has shown parasexuality, which has proved importance for genetic study and strain development. Many industrially important fungi are classified as deuteromycetes, including species of *Aspergillus*, *Cephalosporium*, *Fusarium*, *Penicillium* and *Trichoderma* (Waites, et al. 2001).

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

2.5. *Aspergillus sojae*

Filamentous fungi among microbial production organisms have a vital importance for the production of antimicrobial metabolites such as penicillins and cephalosporins and hydrolytic enzymes. Only a small quantity of fungal species is now used as protein production hosts. Among these fungi, *Aspergillus* species play a significant role. In fact they are very well represented among the strains used for traditional production processes. Moreover, several *Aspergillus*-derived food additive products had already retained a generally recognized as safe (GRAS) status from the regulatory authorities, thereby easing its recognition as a safe and reliable expression platform. As with other industrial environments, a wide mental property position was increased by a few of Biotech companies such as DSM, NOVOzymes, Genencor for particular *Aspergillus* species. As a consequence several parties start to identify alternative species and to assess the possibilities of employing the newly identified fungal expression hosts for heterologous gene expression. *Aspergillus sojae* was identified as a result of this search for new hosts (Gellissen 2005).

In addition, fungal taxonomy is a complex issue which is subject to changes. When deduced in particular from molecular data, the genus *Aspergillus* is classified into the *Ascomycetes*, now combining species with ascomycetous teleomorphs as well as species with no known teleomorphs which were previously classified into the *Deuteromycetes*. Furthermore, it was found that the genus *Aspergillus* consists of three different evolutionary lines by comparing 18S rDNA sequences. Based on monophyletic taxonomy, three subgenera were indicated that consist of 15 sections in total: *Aspergillus*, *Fumigati*, and *Nidulantes*. A phylogenetic tree of selected *Aspergillus* species shown in Figure 2.6 include species belonging to the section *Flavi*, which in turn can be divided into non-aflatoxigenic species like for example *Aspergillus oryzae* and *A. sojae*, and aflatoxigenic species such as *Aspergillus flavus* and *A. parasiticus*. The non-aflatoxigenic species have been extensively used in industry for food fermentation or enzyme production. Difference between the aflatoxigenic species and the non-aflatoxigenic species is a very important characteristic concerning their use in food applications, and represents a key criterion for their acceptance by regulatory authorities (Gellissen 2005).

A. sojae strains can be distinguished from taxonomically closely related species, such as *A. oryzae* and *A. parasiticus*. Traditionally, the identification of species of the section *Flavi* was based on several morphological characteristics. However, these species are morphologically very similar, which impedes a clear distinction between them. *A. flavus*, *A. oryzae*, *A. parasiticus*, and *A. sojae* which are varieties of a single species were based on the high degree of DNA complementarity. It was shown that the homology in total DNA hybridizations between *A. flavus* and *A. oryzae* is 100%, between *A. parasiticus* and *A. sojae* is 91%, and between these groups is 72%.

Furthermore, molecular genetic techniques have been used for the classification of the species in the section *Flavi* during the past decade. Reference is here made in particular to the PCR fragments derived from ver-1, aflR and rDNA sequences. For instance, it has been found that the very closely related *A. parasiticus* and *A. sojae* species can be differentiated from each other by random amplification of polymorphic DNA (RAPD) analysis or by the difference in resistance to bleomycin. In addition, it has been indicated that *Aspergillus oryzae* can be differentiated from *A. sojae* by comparison of the alpA sequences. It has also been found that the *A. sojae* genome harbors an XmnI restriction site at a specific location within the alpA gene, which is not present in *A. oryzae* (Gellissen 2005).

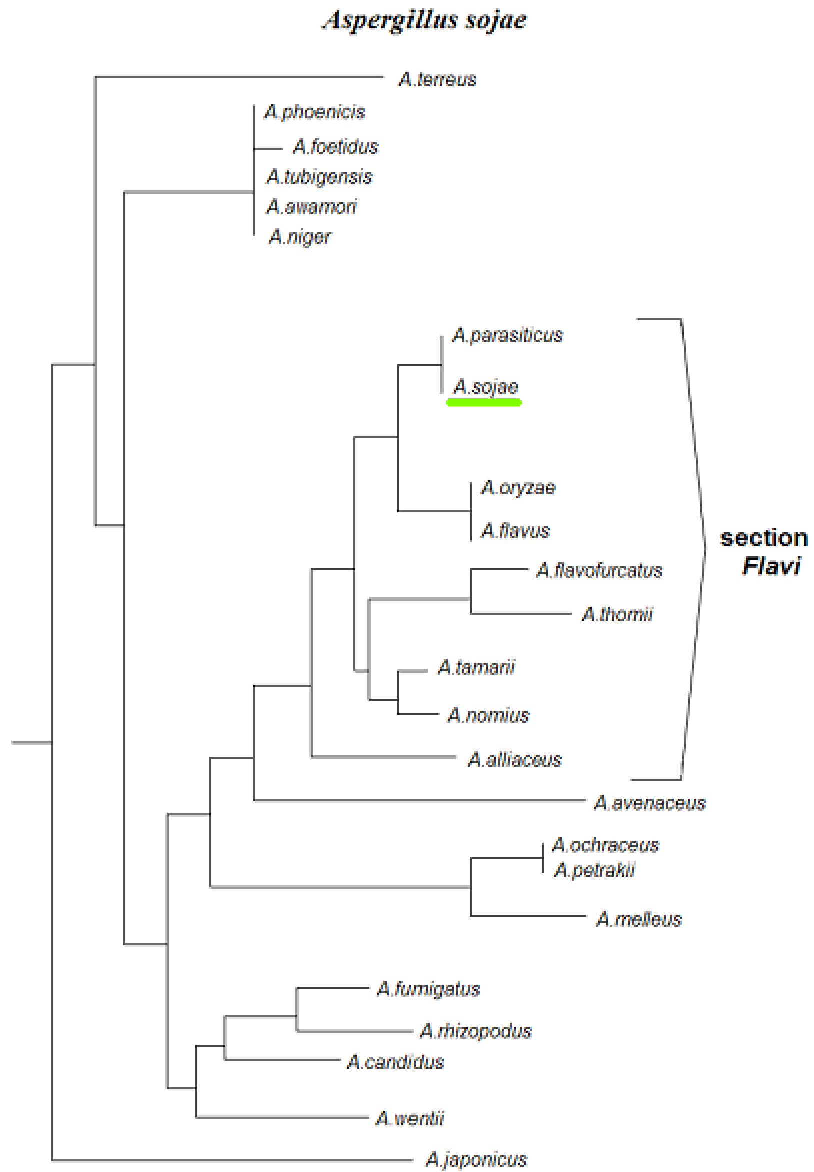


Figure 2.6. Molecular phylogeny of *Aspergillus* species, including species belonging to the section *Flavi*, based on ITS sequence data (Source: Gellissen 2005)

CHAPTER 3

PECTINOLYTIC ENZYMES

The biotechnological potential of microbial pectinolytic enzymes has drawn a great deal of attention world wide owing to various applications (Patil and Dayanand 2006). They were commercially used first in 1930 for wines and fruit juices industries. Recently, pectinases are one of the promising enzymes of the commercial sector. These enzymes degrade pectins, the long and complex molecules, which serve as structural polysaccharides in the middle lamella and in primary cell walls of plant cells (Kashyap, et al. 2001). Pectinases 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). Filamentous fungal pectinase production varies according to the strain, the composition of the growth medium and the cultivation conditions such as pH, temperature, aeration, agitation and incubation time (Sauza, et al. 2003).

3.1. Classification of Pectic Enzymes

Pectinases are classified under three headings according to 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 and also the important producers of pectinases as reported in the literature are given in Table 3.2. Moreover, different pectic substances and their mode of reaction are illustrated in Figure 3.1.

The three major types of pectinases are pectinesterases, depolymerizing enzymes and protopectinases.

Pectinesterases (pectinmethyl hydrolase, PE) catalyze deesterification of the methoxyl group of pectin forming pectic acid.

Depolymerizing enzymes are the enzymes hydrolyzing or cleaving glycosidic linkages.

Table 3.1. An extensive classification of pectinolytic enzymes
(Source: Jayani, et al. 2005)

Enzyme	E.C. no	Modified E.C. 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 digalactoronohydrolase	Hydrolysis	Penultimate bonds	Pectic acid	Digalacturonates
5. Oligogalacturonate hydrolase			Hydrolysis	Terminal	Trigalacturonate	Monogalacturonates

(cont. on next page)

Table 3.1. (cont.) An extensive classification of pectinolytic enzymes

(Source: Jayani, et al. 2005)

6. Δ4:5 Unsaturated oligogalacturonate hydrolases			Hydrolysis	Terminal	Δ4:5(Galacturonate)$_\eta$	Unsaturated monogalacturonates & saturated (η-1)
7. Endopolymethyl-galacturonases			Hydrolysis	Random	Highly esterified pectin	Oligomethylgalacturonates
8. Exopolymethyl-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

Table 3.2. Characterization of microbial pectinases
(Source: Kashyap, et al. 2001)

Producer	Type of pectinase	Optimum pH	Optimum temperature (°C)
Acidic pectinases			
Aspergillus niger CH4	Endo-pectinase, Exo-pectinase	4.5-6.0 3.5-5.0	Below 50
Penicillium frequentans	Endopolygalacturonase (Endo-PG)	4.5-4.7	50
Sclerotium rolfsii	Endo-PG	3.5	55
Rhizoctonia solani	Endo-PG	4.8	50
Mucor pusilus	PG	5.0	40
Clostridium thermosaccharolyticum	Polygalacturonate hydrolase	5.5-7.0	30-40
Alkaline pectinases			
Bacillus sp. RK9	PGL	10.0	-
Bacillus sp. NT-33	PG	10.5	75
Bacillus polymyxa	PG	8.4-9.4	45
Bacillus pumilis	PATE	8.0-8.5	60
Amucola sp.	Pectate lyase (PAL)	10.25	70
Xanthomonas compestris	PATE	9.5	25-30
Bacillus No. P-4-N	PG	10.0-10.5	65
Bacillus stearothermophilus	PATE	9.0	70
Penicillium italicum CECT 22941	Pectin lyase	8.0	50
Bacillus sp. DT 7	Pectin lyase	8.0	60
Bacillus subtilis	PAL	8.5	60-65
Pseudomonas syringae pv. Glycinea	PAL	8.0	30-40

Hydrolyzing glycosidic linkages include polymethylgalacturonase (PMG) and polygalacturonase (PG). PMG catalyzes the hydrolytic cleavage of α-1,4-glycosidic bonds and is classified as endo-PMG that causes random cleavage of α-1,4-glycosidic linkages of pectin, preferentially highly esterified pectin, and exo-PMG that causes sequential cleavage of α-1,4-glycosidic linkage of pectin from the non-reducing end of the pectin chain.

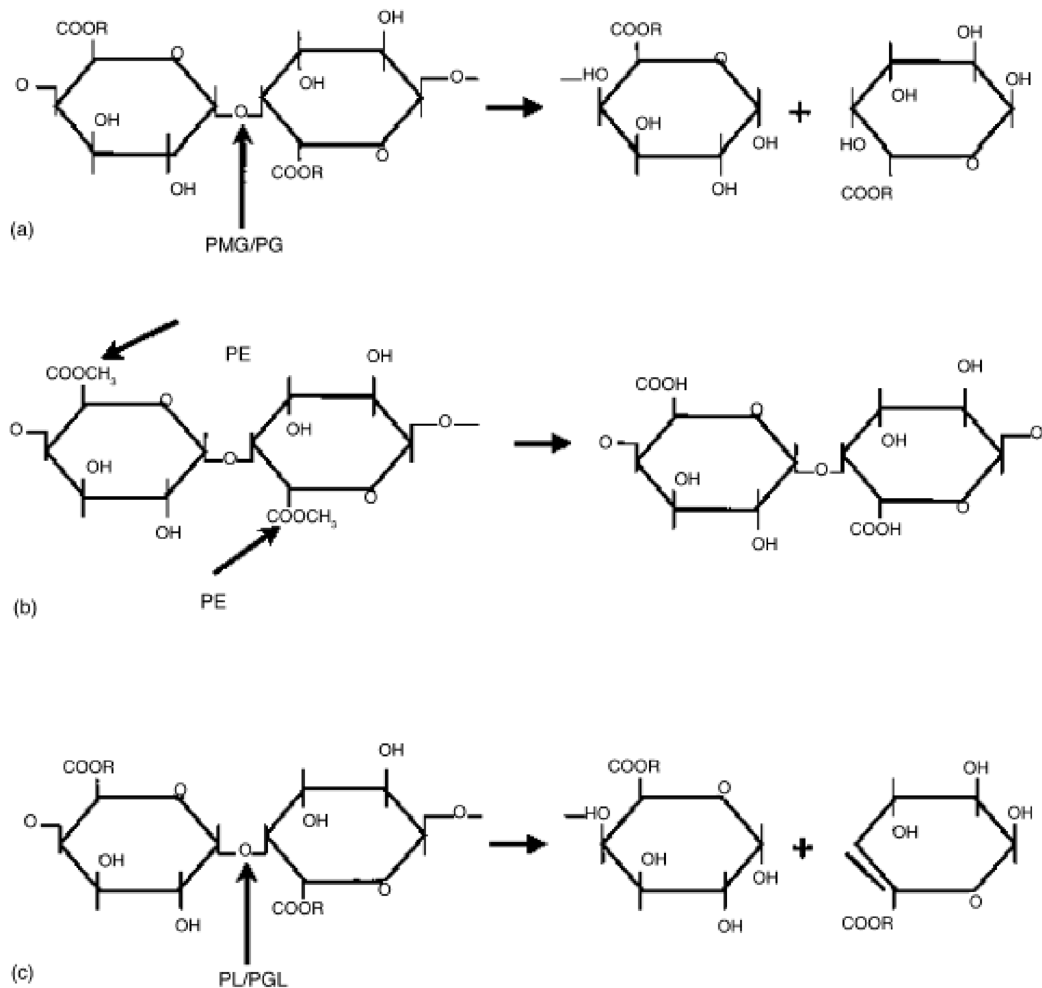


Figure 3.1. Mode of action of pectinases: (a) R = H for PG and CH3 for PMG; (b) PE; and (c) R = H for PGL and CH3 for PL (The arrow indicates the place where the pectinase reacts with the pectic substances. PMG, polymethylgalacturonases; PG, polygalacturonases (EC 3.2.1.15); PE, pectinesterase (EC 3.1.1.11); PL, pectin lyase (EC-4.2.2.10)) (Source: Gummadi and Panda 2003)

PG catalyzes hydrolysis of α-1,4-glycosidic linkages in pectic acid (polygalacturonic acid). This enzyme is also classified as endo-PG, known as poly (1,4-α-D-galacturonide) glycanohydrolase, which catalyzes random hydrolysis of α-1,4-glycosidic linkages in pectic acid and exo-PG known as poly (1,4-α-D-galacturonide) galacturonohydrolase, catalyzes hydrolysis in a sequential fashion of α-1,4-glycosidic linkages on pectic acid. Cleaving α-1,4-glycosidic linkages by trans-elimination, which results in galacturonide with an unsaturated bond between C4 and C5 at the non-

reducing end of the galacturonic acid is formed by polymethylgalacturonate lyases (PMGL) and polygalacturonate lyases (PGL). PMGL catalyzing the breakdown of pectin by trans-eliminative cleavage are classified as endo-PMGL, known as poly (methoxygalacturonide) lyase, which catalyzes random cleavage of α-1,4-glycosidic linkages in pectin, and exo-PMGL, which catalyzes stepwise breakdown of pectin by trans-eliminative cleavage. On the other hand PGL catalyzing the cleavage of α-1,4-glycosidic linkage in pectic acid by trans- elimination are also classified as endo-PGL, known as poly (1,4-α-D-galacturonide) endolyase, which catalyzes random cleavage of α-1,4-glycosidic linkages in pectic acid, and exo-PGL, known as poly (1,4-α-D-galacturonide) exolyase, which catalyzes sequential cleavage of α-1,4-glycosidic linkages in pectic acid.

Protopectinases solubilize protopectin forming highly polymerized soluble pectin (Kashyap, et al. 2001).

3.2. Applications of Pectinases

Pectinases have a significant importance in the current biotechnological area with their large applications in food, textile, waste water, pulp and paper industries. The largest industrial application of pectinases is in fruit juice extraction and clarification. A combination of pectinases and other enzymes which are cellulases, arabinases and xylanases, have been used to increase the pressing efficiency of the fruits for juice extraction. In addition, pectinases containing great levels of polygalacturonase activity are added to fruit juices to stabilize the cloud of citrus juices, purees and nectars. Furthermore, their addition to macerated fruits before the addition of wine yeast in the process of producing red wine results in improved visual characteristics such as colour and turbidity, as compared to the untreated wines. Maceration is a process by which organized tissue is transformed into a suspension of intact cells, causing pulpy products. These are used as base material for pulpy juices and nectar, as baby foods and an ingredient for dairy products such as puddings and yogurts. Pectinases from fungus are used in the manufacture of tea, where enzyme treatment increases the speed of tea fermentation and improves the foam-forming property of instant tea powders by destroying tea pectins (Kashyap, et al. 2001).

The use of pectinases in conjunction with amylases, lipases, cellulases and hemicellulases to remove sizing agents from cotton has decreased the use of harsh chemicals in the textile industry. This brings about a lower discharge of waste chemicals to the environment by improving both the safety of working conditions for textile workers and the quality of the fabric (Hoondal, et al. 2002). Pectinases have also been used for removal of non-cellulosic impurities from the fiber known as bioscouring (Jayani, et al. 2005). They have been used in retting of flax to split the fibers and completely getting rid of pectins. 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. Pectinolytic enzymes from *Actinomycetes* have shown good correlation between the pectate lyase activity and the degumming effects, causing good separation of the bast fiber (Kashyap, et al. 2001).

Pectin containing wastewaters as by-products are released in vegetable food processing industries. Pretreatment with pectinases remove pectinaceous material easily and render it suitable for decomposition by activated sludge treatment. Moreover, pulp and paper mills are beginning to use enzymes to solve problems in their manufacturing processes. During paper making, pectinases can depolymerise pectins and subsequently lower the cationic demand of pectin solutions and the filtrate from peroxide bleaching (Jayani, et al. 2005). Alkaline pectinase produced by *Bacillus sp.* and *Erwinia carotovora*, due to its strong macerating activity, has been used for retting of Mitsumata bast. These retted basts have been used for the preparation of Japanese paper (Kashyap, et al. 2001). Pectolytic enzymes which are used for the production of animal feeds, reduce the feed viscosity there by increasing the adsorption of nutrients (Jayani, et al. 2005).

CHAPTER 4

MICROBIAL FERMENTATIONS

Microbial fermentations in liquid media can be carried out as batch, fed-batch and continuous processes. Batch growth includes a closed system in which all nutrients are present at the start of the fermentation within a fixed volume. In fed-batch fermentations fresh medium or its components are fed continuously, intermittently or are added as a single addition and the volume of the batch increases with time. Continuous systems are open systems where fresh medium is continuously fed into the fermentation vessel, but the volume remains constant as spent medium and cells are removed at the same rate (Waites, et al. 2001).

4.1. Batch Growth

In batch fermentations a fermenter or Erlenmeyer flask is loaded, sterilized and inoculated in a closed system. The inoculated microorganisms are grown through a typical batch profile consisting of lag, acceleration, exponential growth, deceleration, stationary and death phases (Stanbury and Whitaker 1987). During batch fermentations certain environmental conditions continually change, particularly nutrient and product concentrations, as does the specific growth rate, because the cells must pass through the sequence of growth phases described above. Consequently, the system never achieves steady-state conditions (Waites, et al. 2001). Before restarting the system, the product is harvested and the fermenter or flask must be cleaned. This is a non-productive phase which is referred to as 'down-time'. Examples of this process involve the production of alcoholic beverages, most amino acids, enzymes, organic acids, etc. The advantages of batch systems are that initial capital expenditure is lower. In addition to this, if contaminant happens, it is very simple to end and begin again a new fermentation cycle. They are successfully used in the production of many traditional fermentation products; and for producing secondary metabolites, such as antibiotics. However, when producing biomass and primary (growth-associated) metabolic products, batch fermentations are less effective. Other disadvantages of batch fermentations are the batch-to-batch

variability of the product; plus increased non-productive down-time, involving cleaning, sterilizing, refilling and post-sterilization cooling. Additionally, the running costs are greater for fitting and maintaining stock cultures, and usually more personnel are required for operating batch processes (Waites, et al. 2001).

4.2. Fed-Batch Growth

In fed-batch culture, while effluent is removed discontinuously, nutrients are continuously or semicontinuously fed. Fed-batch culture which is also called the semicontinuous system or variable-volume continuous culture is generally used to overcome substrate inhibition or catabolite repression by sporadic feeding of the substrate. When the substrate is inhibitory, intermittent addition of the substrate improves the productivity of the fermentation by maintaining the substrate concentration low. An example of fed-batch culture is its use in some antibiotic fermentation. This method can be applied to other secondary metabolite fermentations such as lactic acid and other plant cell and mammalian cell fermentations, where the rate of product formation is maximal at low nutrient concentrations. (Shuler and Kargi 2002).

Fed-batch operations have been successfully used for producing baker's yeast and penicillin. For this, extra nutrients are added as the fermentation progresses, which increase the fermentation volume. Additions may be made continuously, intermittently or as a single addition, often when a batch culture moves towards the end of the rapid growth phase. Fed-batch system can extend the product formation phase and also may successfully solve problems associated with the use of repressive, rapidly metabolized, substrates. Besides, this method is useful where a substrate causes viscosity problems or is toxic at high concentrations. Fed-batch with recycle of cells (biomass) can also be used for specific purposes, e.g. some ethanol fermentations and waste-water treatment processes. Moreover, higher cell densities of up to $10x10^7$ cells mL^{-1} is feasible, and the productivity of the substrate is much higher (up to fivefold) in comparison to the batch fermentation mode (Gellissen 2005).

4.3. Continuous Growth

The continuous culture system is an open system in which fresh medium is continuously added and culture is simultaneously removed at the same rate, leading to a constant working volume. In continuous systems, cells grow exponentially for extended periods at a specified predetermined growth rate. Furthermore, the system has the property of reaching a steady state in which the concentration of limiting nutrient and the cell number do not vary with time. Consequently, these systems are more productive than batch systems. Continuous fermentations are particularly well suited for the production of biomass and growth-associated primary metabolites. Their reduced down-time and lower operating costs are also desirable attributes. However, they require higher initial capital expenditure and, to date, relatively few large-scale industrial examples have become established, other than for biomass, fuel/industrial ethanol and effluent treatment (Waites, et al. 2001).

Problems associated with continuous culture processes, other than waste-water treatment, include the fact that throughout their 20-50 days or longer operation, sterility must be maintained and a continuous supply of media of constant composition provided. However, these difficulties can be overcome by GMP and good microbiological practices. Nevertheless, the operating conditions place strong selection pressure on the organism. (Waites, et al. 2001)

CHAPTER 5

MATERIALS AND METHODS

5.1. Materials

5.1.1. Microorganism

Aspergillus sojae ATCC 20235 was purchased in the lyophilized form, from Promochem Inc., an international distributor of ATCC (American Type of Culture Collection) in Europe. The image taken by scanning electron microscope of strain *Aspergillus sojae 20235* (magnified 3500x) is shown in Figure A.1.

5.1.2. Chemicals

Chemicals listed in Table 5.1 were used in studies.

Table 5.1. Chemicals used

NO	CHEMICAL	CODE
1	Acetic acid (100%)	Riedel 27225
2	Ammonium heptamolybdate-tetrahydrate	Merck 1.01182
3	Ammonium sulfate	Riedel-De Haën 11225
4	Bacteriological agar	Oxoid LP0011
5	Boric acid	Merck 1.00165.1000
6	Brain heart infusion agar	Fluka 70138
7	Calcium chloride dehydrate	Riedel-De Haën 12022
8	Coomassie brilliant blue G-250	Fluka 27815
9	Copper (II) sulfate-5-hydrate	Riedel-De Haën 12849
10	Corn steep liquor	Sigma C4648
11	D-Galacturonic acid	Fluka 48280

(cont. on next page)

Table 5.1. (cont.) Chemicals used

12	D-Glucose	AppliChem A3666
13	Di-Sodium hydrogen arsenate heptahydrate	Fluka 71625
14	Di-Sodium hydrogen phosphate monobasic	Fluka 71662
15	Ethanol (95%)	Riedel-De Haën 32221
16	Glycerol	Sigma G5516
17	Hydrochloric acid (37%)	Riedel-De Haën 07102
18	Iron (II) sulfate heptahydrate	Riedel-De Haën 12354
19	Magnesium sulfate heptahydrate	Riedel-De Haën 13142
20	Malt extract	Bacto BD 218630
21	Maltrin (Nişkoz HM)	Pendik Nişasta 01521
22	Manganese (II) sulfate monohydrate	Riedel-De Haën 13255
23	Molasses	Pakmaya Kemalpaşa Üretim Tesisi
24	Molybdenum (VI) oxide	Merck 1.00401.0250
25	Pectin from citrus fruits	Sigma P9135
26	Pectinase from *Aspergillus niger*	Sigma P4716
27	Peptone	Acumedia 7182A
28	Phosphoric acid	Riedel-De Haën 04107
29	Polygalacturonic acid	Sigma P3850
30	Potassium chloride	Riedel-De Haën 31248
31	Potassium phosphate monobasic	Riedel-De Haën 04243
32	Potassium sodium tartrate tetrahydrate	Sigma S6170
33	Protein standard, 2mg BSA	Sigma P5619-25VL
34	Sodium acetate trihydrate	Merck K35710467 613
35	Sodium carbonate anhydrous	AppliChem A3900
36	Sodium chloride	Riedel-De Haën 13423
37	Sodium dihydrogen phosphate monohydrate	Fluka 71507
38	Sodium hydrogen carbonate	Sigma S8875
39	Sodium hydroxide	Merck B238062 315
40	Sodium phosphate dibasic dihydrate	Riedel-De Haën 04272
41	Sodium sulfate anhydrous	Carlo Erba 483007
42	Sulfuric acid (95-97%)	Riedel-De Haën 07208
43	Tert-Butanol	Merck 8.22264
44	Tween 80	Merck s4374087 529
45	Yeast Extract	Fluka 70161
46	Zinc sulfate heptahydrate	Merck 108883.0500

5.2. Methods

5.2.1. Spore Production

The microorganism was propagated on Yeast Malt Extract (YME) agar containing malt extract (10g/l), yeast extract (4 g/l), glucose (4g/l) and agar (20g/l). YME agar slant medium were incubated 30°C until well sporulation. Stock cultures of these strains were prepared with 20% glycerol water and stored at -80°C.

The spore suspension was used as inoculum in the growth media for the production of PMG and PG. Spores were transferred from YME agar slant medium to 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 (1.2%), $MgSO_4$ (1%)**,** $CuSO_4$.5H_2O (0.24%)**,** $MnSO_4$.H_2O (0.3%)**,** KCl (10%) and agar (20 g/l). Inoculated slants were grown in an incubator at 30°C for 1 week. Following this, 5 ml of 0.02% (v/v) Tween-80 water was added into each slant and spores were harvested in a sterile falcon tube. Then, the tween-80 water with spores was tested for sterility check by using Brain Heart Infusion (BHI) agar and spore count. The spore counts were done using Thoma bright line haemocytometer (Marienfield, Germany).

5.2.2. Polymethylgalacturonase and Polygalacturonase Assays

Polymethylgalacturonase (PMG) was assayed at 30°C and pH 5.3 by the procedure given by Panda et al. (Panda, et al. 1999), using pectin as substrate. PMG activity was calculated by Equation 5.1.

Activity (U/ml) = (mg of galacturonic acid/212.12) x (1/20) x (1/0.074) (5.1)

Polygalacturonase (PG) was determined according to the method modified by Dogan et al. (Dogan and Tari 2008), using polygalacturonic acid as substrate at pH 4.0 and 26°C. The activity for PG was calculated by Equation 5.2.

Activity (U/ml) = (mg of galacturonic acid/212.12) x (1/20) x (1/0.086) (5.2)

One unit of enzyme activity is described as the amount of PMG or PG that catalyzes the release of 1 μmol of galacturonic acid per unit volume of culture filtrate per unit time at standard assay conditions (pH 5.3 and 30°C for PMG and pH 4.0 and 26°C for PG).

5.2.3. Protein Determination

The total protein content of the samples was determined in three-phase partitioning experiments according to the method described by Lowry et al. (Lowry, et al. 1951); the protein standard used was bovine serum albumine (BSA).

The method of Bradford (Bradford 1976) with BSA as a standard was used to measure protein amount in fed-batch experiments instead of the method of Lowry.

5.2.4. Biomass Determination

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 37°C.

5.2.5. Batch Fermentation of Polygalacturonase in Shake Flasks

The cultivation was carried in a solution containing glucose (25 g/l), peptone (2.5 g/l), disodium phosphate (3.2 g/l), monosodium phosphate (3.3 g/l) and maltrin (120 g/l). The fermentation was conducted at 350 rpm at 30°C for 96 h. These were the pre-optimized conditions determined by the previous studies (Gogus et al., 2006). After 96 h of incubation, each flask was assayed for enzyme activity and protein determination. Crude enzyme activity was determined on supernatant obtained after the centrifugation of the broth at 6000 rpm for 15 min. Moreover, the supernatant was used in the three-phase partitioning which is a partially purification procedure.

5.2.6. Characterization of Three-Phase Partitioned Polygalacturonase

Even though PMG and PG were considered in this study, only PG was used in the characterization study due to the heavy experimental lot. Therefore all the characterization results are based on this enzyme only. In addition, each experiment was carried out three times in characterization studies.

5.2.6.1. Three-Phase Partitioning of the Polygalacturonase

Partially purification of the polygalacturonase enzyme was performed by three phase partitioning for a one-step purification step based on a modified procedure reported by Sharma and Gupta (Sharma and Gupta 2001). In order to adopt the mentioned procedure to our study, the crude enzyme solution (pH adjusted to 6.6) was brought to 25, 30 and 40 % (w/v) saturations using ammonium sulphate (Table 6.2). To this solution, *tert*-butanol was added in order to obtain 1:1 or 1:2 (v/v) ratio of crude enzyme to *tert*-butanol at the specified temperature (Table 6.2). After incubation for 1 h at 25 or 37°C, the mixture was centrifuged (2000 *x* g for 5 min) to facilitate separation of phases. The lower aqueous layer and the interfacial precipitate were collected; the latter was dissolved in 0.1 M sodium phosphate buffer, pH 6.6. The best conditions which resulted into maximum recovery were used as standard purification procedure. The purified enzyme obtained under these conditions was used for further characterization. The activity of the crude extract initially added was taken as 100%.

5.2.6.2. Effect of pH on Activity and Stability of the Purified Polygalacturonase

The effect of pH on the activity of the purified polygalacturonase was determined by assaying the enzyme activity at different pH values ranging from 3.0 to 12.0 using 0.1 M of the following buffer systems: acetate (3.0, 4.0, and 5.0), phosphate (pH 6.0 and 7.0), tris-HCl (pH 8.0, 9.0, and 10.0) and Na_2HPO_4-NaOH (pH 11.0 and 12.0) buffer systems (Atlas, et al. 1995). 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 *A. sojae* polygalacturonase was investigated in the pH range of 3.0-12.0. Therefore, 1 ml of the crude enzyme was mixed with 1 ml of the buffer solutions mentioned above and incubated at 30°C for 2 h. Afterwards aliquots of the mixtures were taken to measure the residual polygalacturonase activity (%) with respect to control, under standard assay conditions.

5.2.6.3. Effect of Temperature on Activity and Stability of the Purified Polygalacturonase

The effect of temperature on the activity of the purified polygalacturonase was determined by performing the standard assay procedure at different temperatures ranging from 25 to 75°C (25, 37, 45, 55, 65, and 75°C). Before the addition of enzymes, the substrate (0.24% (w/v) polygalacturonic acid) was preincubated at the respective temperature for 10 min. The relative activities (as percentages) were expressed as the ratio of the purified polygalacturonase activity obtained at certain temperature to the maximum activity obtained at the given temperature range.

The thermostability of the purified polygalacturonase was investigated by measuring the residual activity after incubating the enzyme at various temperatures ranging from 25 to 65°C (25, 37, 45, 55 and 65°C) for 10, 20, 30 and 60 min.

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

Thermal inactivation kinetics of the purified polygalacturonase was studied by incubating the enzyme at different temperatures (75, 80, 82.5 and 85°C) in the absence of the substrate. Aliquots 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 semilogarithmic 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 5.3. The half-life is known as the time where the residual activity reaches 50 %.

$$t_{1/2} = \frac{\ln 2}{k_d} \qquad (5.3)$$

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:

$$\ln(k_d) = \ln(k_0) - \left(\frac{E_d}{R}\right)\frac{1}{T} \qquad (5.4)$$

The values of E_d and k_0 were estimated from the slope and intercept of the plot of $\ln(k_d)$ versus 1/T, respectively. (R is the ideal gas constant =1.987 cal mol^{-1} K^{-1})

5.2.6.5. Estimation of Thermodynamic Parameters during Inactivation of Polygalacturonase

The enthalpy of inactivation (ΔH^*) for each temperature was calculated according to Equation 5.5.

$$\Delta H^* = E_d - RT \qquad (5.5)$$

The values for the Gibb's free energy (ΔG^*) of inactivation at different temperatures were calculated from the first-order constant of inactivation process by using Equation 5.6.

$$\Delta G^* = -RT \ln\left(\frac{k_d h}{\kappa T}\right) \qquad (5.6)$$

where h (=6.6262x10^{-34} Js) is the Plank constant, and κ (= 1.3806x10^{-23}) is the Boltzmann constant.

From Equations 5.5 and 5.6 the entropy of inactivation (ΔS^*) of polygalacturonase was calculated from Equation 5.7 (Ortega, et al. 2004).

$$\Delta S^* = \frac{\left(\Delta H^* - \Delta G^*\right)}{T} \qquad (5.7)$$

5.2.6.6. Determination of Kinetic Constants

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 were used as substrate (0.1-1 mg/ml) at pH 6.6 and 26°C. With a slope of K_m/V_m and intercept of $1/V_m$ (Equation 5.8) estimated K_m and V_m values were estimated.

$$\frac{1}{v} = \frac{1}{V_m} + \frac{K_m}{V_m}\frac{1}{[S]} \tag{5.8}$$

5.2.6.7. Effect of Metal Ions and Different Compounds on Activity

Metal ions (K^+, Na^+ , Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+} and Fe^{2+}) and various compounds (EDTA, glycerol, Tween 80, SDS and β-mercaptoethanol) were tested for their effect on purified polygalacturonase activity in Na-phosphate buffer, pH 6.6. The enzyme was preincubated for 30 min at 37°C with 1mM, 5mM and 10mM of listed ions and compounds (Table 6.6 and Table 6.7) as a final concentration prior to the substrate addition. Activity without added metal ions and compounds was taken as 100% activity. Each value represents the average of two experiments.

5.2.7. Fed-Batch Fermentation of Polymethylgalacturonase and Polygalacturonase in Shake Flasks

For fed-batch fermentation a total of 66 for optimizing shake flasks experiments were performed at 30°C and 350 rpm for 4 days in 250 ml Erlenmeyer flasks with a working volume of 50 ml and prepared according to the face-centered central composite design (CCD). The basal medium for fed-batch production of pectinases consisted of glucose (25 g/l), peptone (2.5 g/l), disodium phosphate (3.2 g/l) and monosodium phosphate (3.3 g/l). Fed-batch experiments were conducted by the addition of 5 ml of feed solutions, consisting of maltrin and corn steep liquor (CSL), according to the concentrations given in the face-centered CCD (Table D.1.) to the production medium

at specified time of growth. The pH was not controlled during the fermentation. After 4 days of incubation, each flask was assayed for determination of PMG and PG activities, biomass and final pH.

5.2.8. Experimental Design and Statistical Analysis (Face-Centered Central Composite Design) for Fed-Batch Fermentation

Based on previous batch fermentations, the most significant factors were considered as maltrin concentration (X_1), CSL concentration (X_2) and feeding time (X_3). Therefore, their effects were investigated on the responses of polymethylgalacturonase and polygalacturonase activities and biomass formation using Response Surface Methodology (RSM) in fed-batch fermentation. A total of 66 run in face-centered central composite design (FCCCD) with three factors and five center points were generated and employed (Table D.1.)

In all statistical studies, analysis of data and generation of graphics were done by the statistical software Design Expert 7.0.0 Trial Version, Stat-Ease Inc.

After running the experiments and determining PMG and PG activities and biomass, a second order polynomial model including interactions was fitted to the response data by multiple linear regression procedure (Montgomery 2001):

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i} \sum_{j} \beta_{ij} X_i X_j + \varepsilon \qquad (5.9)$$

where Y is the predicted response, k the number of factor variables, β_0 the model constant, β_i the linear coefficient, X_i the factor variable in its coded form, β_{ii} the quadratic coefficient, β_{ij} the interaction coefficient and ε is the error factor. The following Equation 5.10 is used for coding the actual experimental values of the factors in a range of [-1 +1]:

$$x = \frac{actual - ((lowlevel + highlevel)/2)}{(highlevel - lowlevel)/2} \qquad (5.10)$$

The respective low and high levels with the coded levels in parentheses for the factors were shown in Table 5.2. While X_1 and X_2 have low and high coded levels, X_3 has three coded levels due to being a categorical factor.

Table 5.2. Coding the actual experimental values of the factors using faced-centered central composite design

Factors	Coded levels
X_1 : Maltrin concentration (g/l)	50(-1) and 150(+1)
X_2 : CSL concentration (g/l)	0(-1) and 10(+1)
X_3: Feeding time (h) (categorical factor)	$48^{th}{}_{[1]}$ (+1), $48^{th}{}_{[2]}$ (0) $72^{nd}{}_{[1]}$ (0), $72^{nd}{}_{[2]}$ (+1) 48th&$72^{nd}{}_{[1]}$ (-1), 48th&$72^{nd}{}_{[2]}$ (-1)

The analysis of variance (ANOVA) tables were generated and the effects and regression coefficients of individual linear, quadratic and interaction terms in the polynomial were judged statistically according to their *p*-values. *P*-values were compared to the significance level of 0.05.

5.2.9. Agar Diffusion Method for Detection of Pectinase Production

The use of agar diffusion method allows the rapid determination of production and activities of pectinases from *Aspergillus sojae*. This method will lead the way of further studies on strain improvement. In future studies, *A. sojae* will be mutated in order to increase the productivity. After obtaining mutant strains, agar diffusion methods will have vital importance by providing the means of rapid detection of effective mutant strains with potentially high pectinase activity.

Based on this, the solid medium described by Hankin and Anagnostakis (Hankin and Anagnostakis 1975) was modified to detect the production of pectinases. This medium consisted of 500 ml of mineral salts solution, 1 g of yeast extract, 15 g of agar, 5 g of polygalacturonic acid (PGA) or pectin, and 500 ml of distilled water; the final pH of the mineral salt solution was adjusted to 5.3 for pectin and to pH 4.0 for PGA containing medium. The mineral salts solution used above contained per liter: $(NH_4)_2SO_4$, 2 g; KH_2PO_4, 4 g; Na_2HPO_4, 6 g; $FeSO4.7H_2O$, 0.2 g; $CaCl_2$, 1 mg; H_3BO_3,

10 μg; $MnSO_4$, 10 μg; $ZnSO_4$, 70 μg; $CuSO_4$, 50 μg; MoO_3, 10 μg. The medium at pH 4 and 5.3 was used to detect polygalacturonase and polymethylgalacturonase production, respectively. These media were inoculated with spore suspension of ($11.17x10^7$ spore/ml) *Aspergillus sojae*. After incubation at 30^oC for 2 days, plates were flooded with 5N of HCl to show the clear zone formation around a colony.

CHAPTER 6

RESULTS AND DISCUSSION

6.1. Batch Fermentation of Polygalacturonase in Shake Flasks

The batch fermentation was carried out in order to obtain the crude polygalacturonase enzyme which was intended to be subjected to purification and characterization procedure and to establish a baseline for the forthcoming fed-batch experiments. Therefore a solution containing maltrin, glucose, peptone, disodium phosphate and monosodium phosphate with the optimum concentrations and conditions given in the materials and method section 5.2.5 was used. In batch fermentation studies, profiling of polygalacturonase activity, total carbohydrate and protein amount, and pH values were determined (Table 6.1). Each experiment was carried out two times. As it can be observed Figure B.1.a, highest polygalacturonase activity was achieved at 43 hours. Moreover, total carbohydrate level was lower after 43 hours as seen from Figure B.1.b Protein amount was changeable (Figure B.1.c) because there were both consumption and production of proteins simultaneously in the living system and also a large portion of the variation came from the measurement of biological systems.

Table 6.1. Values of (a) polygalacturonase enzyme activity (U/ml) (b) total carbohydrate (g/l) (c) protein (mg/ml) and (d) pH profiles for *Aspergillus sojae* at optimum batch fermentation conditions for 96 h

Time (h)	PG Activity (U/ml)	Total Carbohydrate (g/l)	Protein (mg/ml)	pH
0	0 ± 0	129,57 ± 5.68	0,0182 ± 0.0053	6,40 ± 0
19	14,70 ± 2.15	138,74 ± 14.41	0,0215 ± 0.0032	6,37 ± 8.43E-08
26	13,45 ± 3.29	125,27 ± 2.17	0,0184 ± 0.0022	6,35 ± 1.19E-07
43	17,90 ± 1.64	114,99 ± 3.64	0,0163 ± 0.0035	6,32 ± 0.017
50	15,20 ± 1.00	121,50 ± 1.04	0,0164 ± 0.0015	6,30 ± 0.01
67	13,02 ± 0.84	123,24 ± 5.75	0,0263 ± 0.0114	6,31 ± 0.015
74	15,86 ± 1.06	121,20 ± 0.87	0,0240 ± 0.0099	6,30 ± 0.035
91	14,36 ± 2.19	121,34 ± 0.86	0,0301 ± 0.0144	6,31 ± 0.01
96	15,47 ± 2.73	120,58 ± 0.51	0,0349 ± 0.0186	6,31 ± 0.006

Besides, a slight decrease in pH values was observed until 50 hours, followed by a constant profile after this time (Figure B.1.d).

6.2. Characterization of Three-Phase Partitioned Polygalacturonase

In order to purify and characterize the PG enzyme, batch fermentations using two types of Erlenmeyer flasks (non-buffled and buffled) were carried out. In buffled Erlenmeyer flask, polygalacturonase activity was 15.45 (±7.35) % (U/ml) lower than PG activity produced in non-buffled Erlenmeyer flask. Furthermore, the type of Erlenmeyer flasks had a significant effect on the morphology of pellets, where small pellets in buffled Erlenmeyer flask were observed (Figure C.1). Besides, investigating the effect of the type of Erlenmeyer flasks on the activity and morphology the primary the goal of this study was to produce the crude enzyme and proceed with the partially purification and characterization study. Therefore three phase partitioning (TPP); a simple, fast and relatively recent technique was used for partially purification of PG.

6.2.1. Three-Phase Partitioning of the Polygalacturonase

In order to adapt the procedure developed by Sharma and Gupta (Sharma and Gupta 2001), to our study, various experiments at different percent saturations of ammonium sulphate, different crude extract to *tert*-butanol (v/v) ratio and different temperatures as given in Table 6.2 were performed. The effect of these parameters on the yield and on the degree of purification is presented in Table 6.2. The chosen parameters had major influence on the purification of the polygalacturonase. For example an increase of the saturation level of ammonium sulphate from 25 to 30 %, at 1:1 crude extract to *tert*-butanol (v/v) ratio and 25 ºC, increased the recovery by 2 and purification by 6.1 fold, however further increase to 40 % saturation decreased the same responses by 2.5 and 3.9, respectively. Similarly, an increase of the temperature from 25 to 37 ºC at 30 % saturation of ammonium sulphate and 1:1 crude extract to *tert*-butanol (v/v) ratio caused 2.89 and 9.57 fold of reduction in recovery yield and purification, respectively. The effect of the crude extract to *tert*-butanol (v/v) ratio was also very considerable where the change from 1:1 to 1:2 crude extract to *tert*-butanol (v/v) ratio, decreased the responses (recovery and degree of purification) by 3.26 and

4.47, respectively. Based on these observations the best results were obtained at 30% saturation with ammonium sulphate, 1:1 (v/v) ratio of crude extract to *tert*-butanol and 25°C where, 6.7-fold purification and 25.5% recovery was observed.

Table 6.2. Various conditions used for three phase partitioning (TPP) of the polygalacturonase enzyme

Condition	**Temperature (^{o}C)**	**Percent saturation with ammonium sulphate (w/v)**	**Enzyme: *tert*-butanol ratio (v/v)**	**Degree of purification**	**Activity recovery (%)**
1	25	25	1:1	1.1	12.7 ± 2.4
2	25	25	1:2	0.5	7.4 ± 5.9
3	25	30	1:1	6.7	25.5 ± 1.8
4	25	30	1:2	1.5	7.8 ± 3.2
5	25	40	1:1	1.7	10.3 ± 3.3
6	25	40	1:2	3.4	17.2 ± 1.3
7	37	25	1:1	0.1	1.0 ± 0.3
8	37	25	1:2	0.4	3.4 ± 0.8
9	37	30	1:1	0.7	8.8 ± 1.4
10	37	30	1:2	1.2	9.8 ± 1.4
11	37	40	1:1	2.8	21.6 ± 4.7
12	37	40	1:2	1.5	9.8 ± 1.5

TPP was carried out after the pH of the polygalacturonase enzyme preparation had been adjusted to 6.6. The starting volume of the enzyme was 2 ml under each condition.

The purified enzyme in the forthcoming characterization experiments was obtained by this adapted procedure. These conditions were in good correlation with various applications of TPP on different proteins, enzymes and soy bean oil. (Shah, et al. 2004, Dennison and Lovrien 1997, Sharma and Gupta 2001, Sharma, et al. 2003). The degree of purification of polygalacturonase obtained in this study was 1.68 , 3.52 and 5.73 times higher than the studies conducted by Keon and Waksman, Riou et al. and Sakamoto et al., respectively, where ammonium sulphate precipitation was used alone (Sakamoto, et al. 2002, Keon and Waksman 1990, Riou, et al. 1992). In those

studies ammonium sulphate precipitation serves as an intermediate step prior to other chromatographic steps used in the purification, however here a pure enzyme not requiring any other treatment is obtained in a single step. Therefore this treatment is economically more feasible and relatively simple. Besides when the degree of purification was compared to various studies where TPP was employed, the current results were relatively above those (Kansal, et al. 2006, Roy and Gupta 2002, Roy, et al. 2005, Roy and Gupta 2005). It was concluded that this modified procedure could be recommended as a single purification step of polygalacturonases of similar organisms and holds a potential to be applicable to industrial scales.

6.2.2. Effect of pH on Three-Phase Partitioned Polygalacturonase Activity and Stability

The effect of pH on the polygalacturonase activity is presented in Figure 6.1. The enzyme was active over a broad pH range, displaying over 71 % of its activity in the pH range of 4.0-9.0 with an optimum pH of 4. A slight increase to 78 % relative activity was observed at pH 9. A further distance from these pH values decreased the relative activity down to 17 %. These findings were in correlation with several earlier reports showing pH optima of 4.8 for polygalacturonase from *Aspergillus niger* and pH optima of 5.0 for *Rhizopus stolonifer* (Gummadi and Panda 2003). Similarly, the polygalacturonase of *Sporotrichum thermophile* Apinis was reported to be active over a wide pH range (3.0-9.0) as well (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 high relative activity in the alkali pH region in addition to the acidic pH optima, will open up new application windows for the current purified polygalacturonase unlike other polygalacturonases which are mostly known to have narrow pH ranges (Jayani, et al. 2005).

Investigations on pH stability by incubating the enzyme in suitable buffer systems for 2 h at 30 ºC (Figure 6.2) revealed, that the polygalacturonase enzyme was very stable at the broad pH range of pH 4 to 9, retaining more than 73 % of its activity. At its optimum pH of 4 the residual activity was 17.5 % higher than the control. However the stability was significantly reduced to 52% to 16 % between pH 10 to pH

12. It is reported that the inactivation process is found to be faster at high alkaline pHs due to disulfide exchange, which usually occurs at near neutral and alkaline conditions.

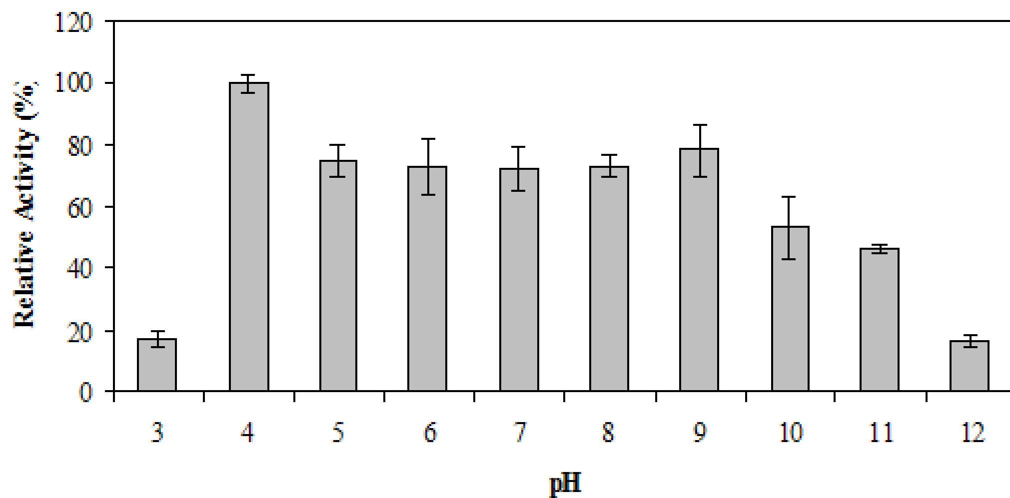


Figure 6.1. Effect of pH on the activity of three-phase partitioned polygalacturonase enzyme

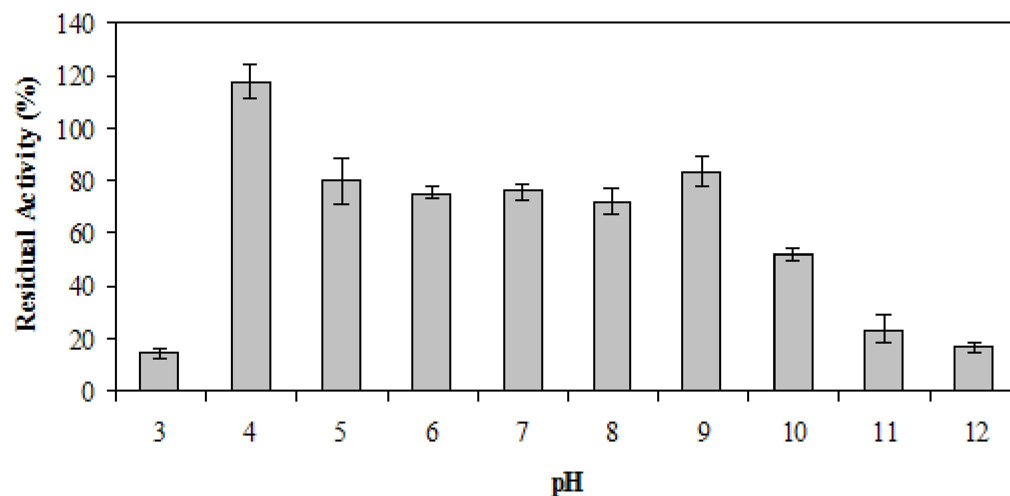


Figure 6.2. Effect of pH on the stability of three-phase partitioned polygalacturonase enzyme

Therefore, polygalacturonase like the one characterized in this study can be a potential candidate for different applications in the industry demanding either acidic or alkaline pH ranges. In addition, with the broad pH stability range, this enzyme can be considered suitable for applications requiring long exposure times. Hence, enhancing

the stability and maintaining the desired level of activity over long period of time are two significant points which are taken into account for the selection of enzymes in industrial applications.

6.2.3. Effect of Temperature on Three-Phase Partitioned Polygalacturonase Activity and Stability

The *Aspergillus sojae* polygalacturonase was active over a broad temperature range of 25-75ºC with an optimum temperature of 55ºC (Figure 6.3). It retained more than 66% of its activity at 65 and 75ºC. This optimum temperature was in agreement with the studies conducted by several authors using different strains. For example Sakamoto et al. (Sakamoto, et al. 2002) found the optimum temperature of a polygalacturonase from *Aspergillus niger* as 60ºC, similarly, maximum activity for commercial enzyme, Rapidase C80 was determined at 55ºC (Ortega, et al. 2004). Furthermore, the partially purified polygalacturonase from *Sporotrichum thermophile Apinis* was optimally active at 55ºC as well (Jayani, et al. 2005, Kashyap, et al. 2001).

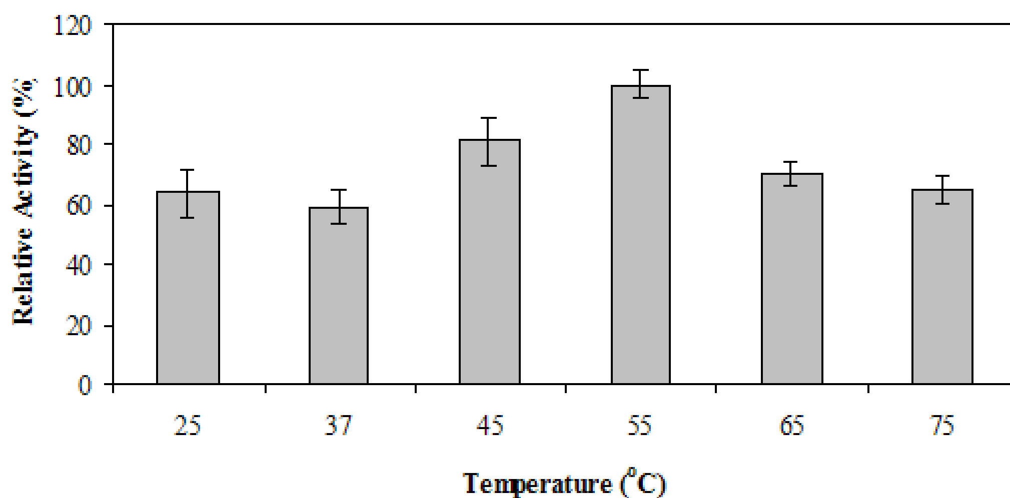


Figure 6.3. Effect of temperature on the activity of three-phase partitioned polygalacturonase enzyme

Thermostability is the ability of enzyme to resist against thermal unfolding in the absence of substrates (Bhatti, et al. 2006). The thermostability of the purified polygalacturonase was determined by measuring the residual activity of the enzyme after incubation at various temperatures ranging from 25 to 65ºC for 10, 20, 30 and 60

minutes. As it is shown in Figure 6.4, the increase in temperature and time caused an overall increase in the stability until 55 ºC and 20 minutes after which a decline was observed.

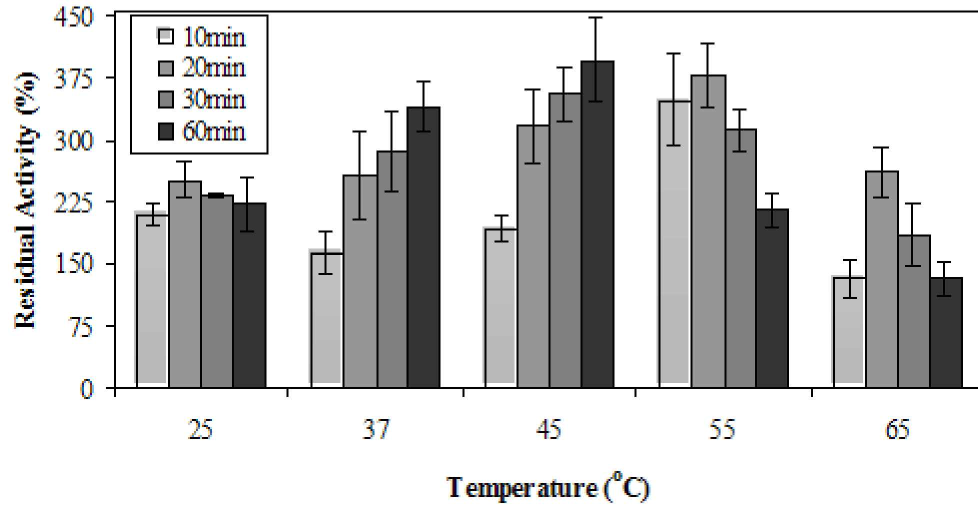


Figure 6.4. Effect of temperature on the stability of three-phase partitioned polygalacturonase enzyme

The maximum residual activity of 397 % was observed at 45 ºC and 60 minutes. The enzyme was very thermostable even at 65 ºC incubated for 60 minutes; it possessed 133 % of residual activity. This clearly demonstrated that the enzyme was induced with the temperature increase and especially at its optimum temperature of 55 ºC incubated for 20 minutes it was stable and exhibit 379 % of residual activity. This is an important finding pointing out that in various applications carried out at this temperature the total enzyme amount required will be reduced significantly which will be reflected eventually in the economics of the process. In order to increase productivity and reduce microbial contaminations, industrial processes may require reactions carried out at high temperatures. That's why thermostable enzymes have been the target of many studies involving the elucidation of thermal inactivation mechanism and development of strategies for stability enhancement (Ortega, et al. 2004). Therefore, the current polygalacturonase in this study will exhibit a new example for thermostable enzymes obtained from a new source (*Aspergillus sojae*) with unique properties. After the use of polygalacturonases in the industry it is important to remove or inactivate them, where the estimation of thermal inactivation comes in to the picture discussed below.

Following the studies on effects of pH and temperature on polygalacturonase activity, PG activity assay was modified and optimum conditions were determined as pH 4 and 26°C (Figure 6.5) to achieve higher PG activity.

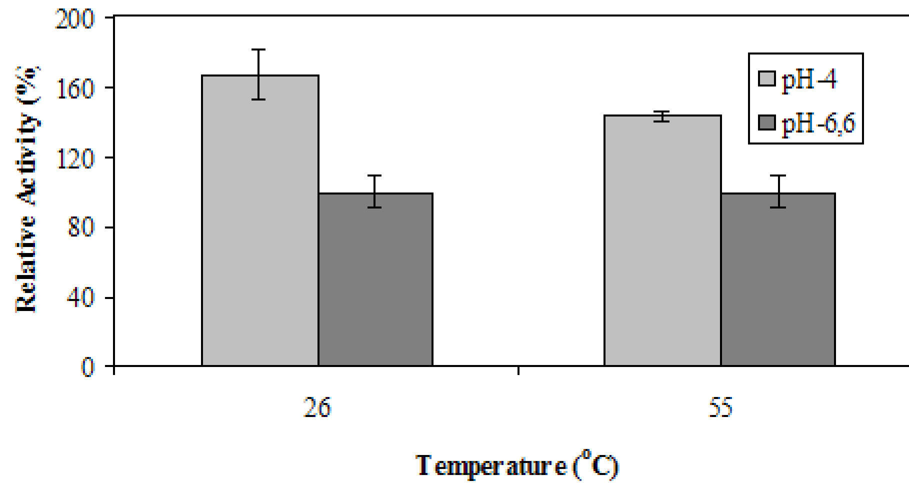


Figure 6.5. Modification to the standard PG assay

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

Enzyme inactivation is one of major constrains in biotechnological processes therefore it is important to understand the mechanism of inactivation. Inactivation is defined as a process where the secondary, tertiary or quaternary structure of a protein changes without breaking covalent bonds. The inactivation of pectolytic enzymes is assumed to follow first order kinetics (Naidu and Panda 2003). Inactivation rate constants (k_d) of polygalacturonase which are presented in Table 6.3 (at 75, 80, 82.5 and 85ºC) were calculated from the slope of semi logarithmic plot of residual activity versus time. Samples were incubated at 75°C (R^2=0.72), 80°C (R^2=0.95), 82.5°C (R^2=0.93) and 85°C (R^2= 0.98) (Figure 6.6). Similarly the half-life values estimated using these constants and Equation 5.3, are presented in the same table. The half life of polygalacturonase at 75ºC was 8.9, 10.96 and 15.36 times higher than the half-life values at temperatures of 80, 82.5 and 85ºC, respectively at pH 6.6. This issue once again reveals the thermal stability of this enzyme at 75 ºC and its easy inactivation at higher temperatures such as 80 and 85 ºC. From slope of the semilogaritmic plot of

residual activity versus time for each temperature, the inactivation rate constants (k_d) were calculated.

Table 6.3. Kinetic parameters for thermal inactivation of three-phase partitioned polygalacturonase enzyme

T (°K)	k_d (min^{-1})	$t_{1/2}$ (min)
348	0.0032	216.609
353	0.0285	24.321
355.5	0.0350	19.801
358	0.0492	14.088

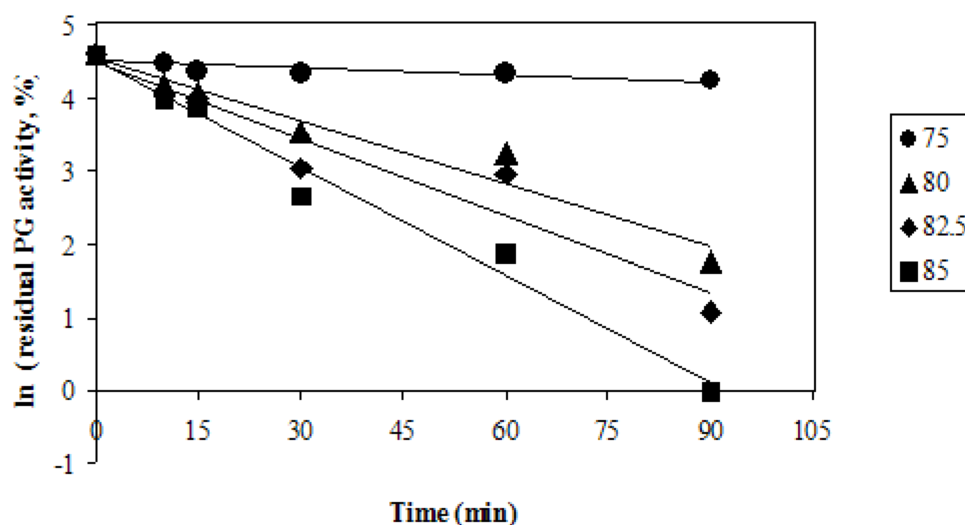


Figure 6.6. First-order plots of the effect of thermal denaturation of the purified polygalacturonase

Inactivation energy of purified polygalacturonase was determined as E_d = 68.41 kcal mol^{-1} from the slopes of the linear curve plotted by 1 /T versus ln (k_d) using Equation 5.4 (Figure 6.7). This value is in the range of the values (40-70 kcal mol^{-1}) estimated for many microbial enzymes (Shuler and Kargi 2002). The inactivation energy estimated for this enzyme was 1.96, 1.71 and 1.77 times higher than the commercial polygalacturonases like Rapidase C80, Pectinase CCM and Pectinex 3XL respectively, compared to a study conducted by Ortega et al (Ortega, et al. 2004). These close

approximations with the current commercial enzymes indicated that the purified enzyme could be a good candidate for various industrial applications.

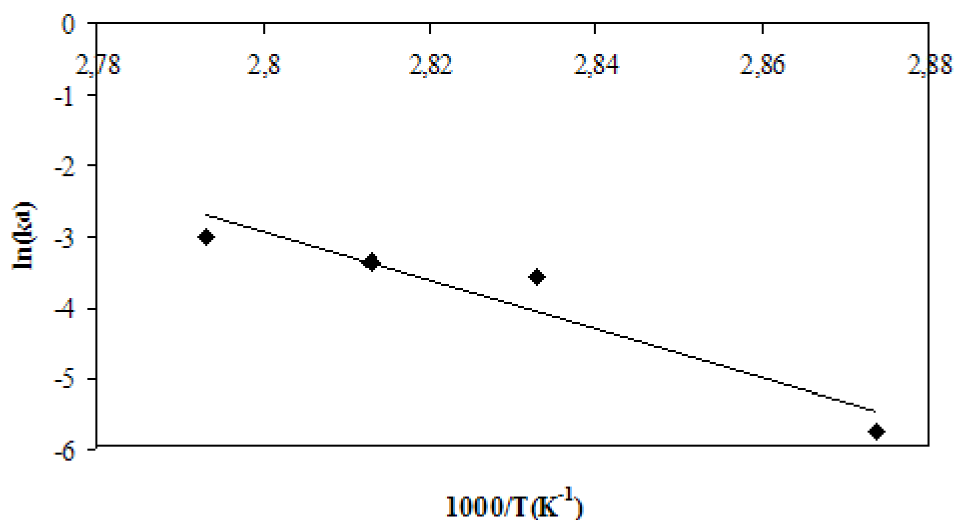


Figure 6.7. Arrhenius plot for the determination of energy of deactivation for thermal inactivation of the purified polygalacturonase (R^2=0.9066)

6.2.5. Estimation of Thermodynamic Parameters during Inactivation of Three-Phase Partitioned Polygalacturonase

The change in enthalpy (ΔH^*) and entropy (ΔS^*) for the thermal inactivation of polygalacturonase which have been calculated using transition state theory (Ortega, et al. 2004) according to Equations 5.5 and 5.7 are presented in Table 6.4. A positive ΔH^* and ΔS^* were determined at the temperature ranges studied. With the increase of temperature a slight decrease in ΔH^* whereas an increase in ΔS^* was observed suggesting the thermal denaturation of the enzyme possibly due to disruption of non-covalent linkages including hydrophobic interactions (Bhatti, et al. 2006, Georis, et al. 2000). The opening up of the enzyme structure is confirmed in fact by the increase in the entropy or disorder of inactivation (Bhatti, et al. 2006). The increase in ΔS^* also indicates an increase in number of protein molecules in transition activated stage, which results in lower values of ΔG^*. Also positive entropy values suggest that enzyme unfolding might be the rate determining step for the irreversible thermo inactivation of

polygalacturonase. Moreover solvent and structural effects are reported to be the two major factors influencing the numerical values of ΔH^* and ΔS^*. Especially, ΔS^* values are known to provide information regarding the degree of solvation and the degree of compactness of protein molecule (Tari, et al. 2008). Also the increase in the ΔH^* with respect to temperature increase reveals the fact that the confirmation of the enzyme was altered. The current results were in very close agreement with the results reported by Ortega et al. (Ortega, et al. 2004) using commercial pectinases such as Rapidase C80, Pectinase CCM and Pectinex 3XL. In fact this is a positive outcome since we demonstrated that the polygalacturonase in the current study is very close in biochemical as well as in thermal characteristic to commercial enzymes currently used.

Table 6.4. Thermodynamic parameters during inactivation of the purified polygalacturonase enzyme

T (^{o}K)	**ΔH^* (kJ mol^{-1})**	**ΔG^* (kJ mol^{-1})**	**ΔS^*(kJ $mol^{-1}K^{-1}$)**
348	283.34	102.30	0.5202
353	283.29	97.39	0.5266
355.5	283.27	97.49	0.5226
358	283.25	97.19	0.5197

6.2.6. Calculation of Kinetic Constants

The kinetic parameters of *Aspergillus sojae* polygalacturonase for hydrolysis toward polygalacturonic acid (PGA) at pH 6.6 and 26 ºC were obtained by a typical double reciprocal Lineweaver Burk plot (Equation 5.8, Figure 6.8). Values of substrate concentration and velocity were shown in Table 6.5. The K_m and V_m values for hydrolyzing PGA were 0.751 g/l and 1.139 µmole/min, respectively.

The estimated K_m value which indicates the affinity of the enzyme towards the substrate is 1.79 and 2.13 times lower than the *Aspergillus niger* and *Penicillium frequentans* polygalacturonases, respectively using the same substrate (Keon and Waksman 1990). This indicates that the current polygalacturonase has a higher affinity for PGA compared to the other two. Similarly, the current polygalacturonase had 1.38,

1.47 and 1.65 times more affinity towards PGA substrate than the commercial enzymes Rapidase C80, Pectinase CCM and Pectinex, respectively jugged from the K_m values (Ortega, et al. 2004). V_m which is an indication of the catalytic activity of an enzyme is usually desired to be as high as possible; therefore the current value obtained in this study was very comparable to many studies in the literature. For example, V_m values of polygalacturonases from *Rhizopus stolonifer* and *Mucor rouxii* were 1.3 and 0.045 μmole/min, respectively (Gummadi and Panda 2003, Saad, et al. 2007).

Table 6.5. Values of substrate concentration ([S], mg/ml) and velocity (V, U/ml) of the purified polygalacturonase enzyme

[S]	1/[S]	V	1/V
-0.751	-1.332	0	0
0	0	1.139	0.878
1	1	0.913	1.095
0.5	2	0.414	2.415
0.333	3	0.283	3.54
0.25	4	0.285	3.511
0.2	5	0.272	3.670
0.167	6	0.230	4.341
0.143	7	0.152	6.595
0.125	8	0.179	5.592
0.111	9	0.152	6.581
0.1	10	0.130	7.697

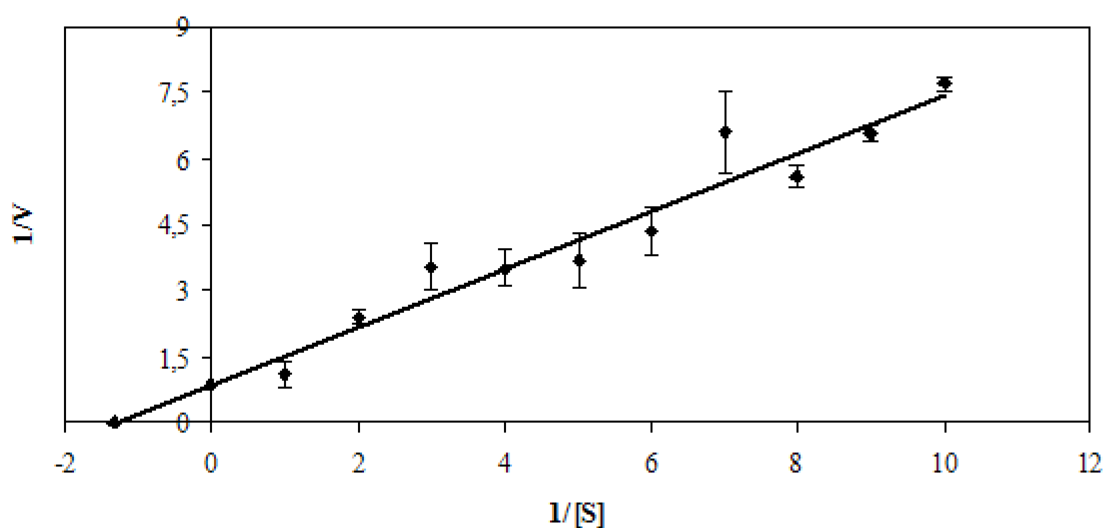


Figure 6.8. Double reciprocal plot to determine the kinetic constants for polygalacturonic acid hydrolysis by purified polygalacturonase enzyme (R^2=0.9266, y=0.6592x+0.8783)

6.2.7. Effect of Metal Ions and Different Compounds on Three-Phase Partitioned Polygalacturonase Activity

The effects of metal ions were examined by adding the chlorides of K^+, Na^+, Ca^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+} and the sulphates of Cu^{2+} and Fe^{2+} at different concentrations to the buffer solution. Activity without added metal ions was taken as 100% activity. As it can be observed from Table 6.6, the enzyme was induced in the presence of 1 mM of K^+, Na^+, Ca^{2+} ions, and completely inhibited in the presence of Mn^{2+} at both concentrations studied. Furthermore at 1 mM concentration, Cu^{2+} and Fe^{2+} reduced the enzyme activity where Zn^{2+} did not have any profound effect. The effect of these ions was concentration dependent.

Table 6.6. Effect of metal ions on the activity of the purified polygalacturonase enzyme

% Relative activity		
Control	**100**	
Metal ions	**1mM**	**10mM**
KCl	174.81 ± 3.95	94.35 ± 0.91
NaCl	124.57 ± 3.67	84.75 ± 3.19
$CaCl_2$	116.00 ± 2.58	101.25 ± 3.26
$MgCl_2$	93.51 ± 4.01	93.42 ± 3.93
$MnCl_2$	0 ± 0	0 ± 0
$ZnCl_2$	99.94 ± 1.12	112.79 ± 1.83
$CuSO_4$	55.47 ± 2.07	42.67 ± 3.17
$FeSO_4$	74.58 ± 2.20	97.66 ± 1.10

For example increasing the concentration of K^+, Na^+, Ca^{2+} from 1 mM to 10 mM, reduced the enzyme activity by 1.85, 1.47 and 1.15 fold, respectively. Similarly, increasing the concentration of Mg^{2+}, Zn^{2+} , Cu^{2+} and Fe^{2+} to 10 mM, resulted in no change in polygalacturonase activity for Mg^{2+}, a 1.3 fold reduction for Cu^{2+} and 1.12 and 1.31 fold of increase for Zn^{2+} and Fe^2 , respectively. In various studies reported in the literature different results were obtained depending on the ion used and the source of the polygalacturonase (Kapoor, et al. 2000, Riou, et al. 1992). For example in a study conducted by Kaur et al., (Kaur, et al. 2004), it was reported that the activity of pectinase was stimulated by Fe^{2+} and Mn^{2+} at both 1 mM and 5 mM and inhibited

strongly at 5mM by Mg^{2+}. These results were partly in agreement with our results. Similarly, the current results were comparable to a study conducted by Mohamed et al., (Mohamed, et al. 2006). Overall these results revealed that the polygalacturonase activity does not necessary depend on the metal ion except for Mn^{2+} and Cu^{2+}, which inhibit and significantly reduce the activity. The effect of metal ions which are concentration dependent could be important, where media formulations are prepared with raw materials of high salt content.

Various compounds such as listed in Table 6.7 in different concentrations were examined in order to determine their effect on the polygalacturonase activity. The activity without added compound was taken as 100% activity. EDTA as chealating agent had promoting effect proportional with concentration in the range of 1mM-10mM. This finding was reconfirming that polygalacturonase does not strongly depend on metal ions as discussed above, which otherwise would have been inhibited in the presence of EDTA. Similarly, glycerol which is mostly used as protective agent in preservation promoted polygalacturonase activity significantly with an increase in the concentration from 1mM to 10mM. Therefore, glycerol can be recommended for preservation of polygalacturonase enzyme during storage.

Table 6.7. Effect of different compounds on the activity of the purified polygalacturonase enzyme

Compounds	Concentration (mM)	% Relative activity
	1	132.98 ± 1.25
EDTA	5	165.03 ± 1.43
	10	195.26 ± 0.53
	1	111.66 ± 1.06
Glycerol	5	233.58 ± 1.89
	10	283.76 ± 1.68
Tween 80	(0.02%)	96.41 ± 1.97
SDS	(0.1%)	0 ± 0
β-mercaptoethanol	(0.1%)	363.16 ± 2.03

EDTA (Ethylenediaminetetraacetic acid); SDS (Sodium dodecyl sulphate).

Tween 80 known as surface active agent and very popular in the fermentation media formulations slightly inhibited the enzyme activity at a concentration of 0.02 %. SDS a protein denaturant, inhibited enzyme activity of the polygalacturonase completely, whereas β-mercarptoethanol increased the enzyme activity by 3.63 fold compared to control suggesting that disulphide linkages do not have a critical role in maintaining an appropriate conformation of the enzyme for its catalytic activity. This finding was partly in agreement with the study conducted by Kaur et al (Kaur, et al. 2004) who determined inhibitory effect of both SDS and β-mercarptoethanol on thermostable polygalacturonase from *Sporotrichum thermophile* Apinis.

6.3. Fed-Batch Fermentation of Polymethylgalacturonase and Polygalacturonase in Shake Flasks

Batch fermentations suffer from low cell mass and product recovery because of limited initial carbon source concentration, as high initial carbon source concentration may cause substrate inhibition. Fed-batch fermentations have been successfully used to increase the yields of pectinases from *Aspergillus* sp. and the production of ergosterol by *Saccharomyces albulus* (Gummadi and Kumar 2008). Therefore, the primary goal in this study was to observe the effect of fed-batch application on PG and PMG and to increase their yield. In this current study fed-batch operation was performed by addition of maltrin (carbon source), corn steep liquor (CSL, nitrogen source) and their combinations. The frequency of feeding time was determined by profiling of polygalacturonase activity and total carbohydrate (Figure B.1.a and B.1.b). Highest polygalacturonase activity was obtained at 43rd hours. Since total carbohydrate level was lower after 43 hours, the time of feeding was selected either as 48th h, 72th h or both of them.

As a result of batch and fed-batch fermentations experiments, it was observed that highest enzyme activity was obtained from fed-batch fermentation. Not only batch fermentations but also fed-batch fermentations were conducted three times. While PG activity was measured as 11.39 ± 1.40 U/ml in batch fermentation, it was 17.19 ± 0.96 U/ml for fed-batch fermentation. Similarly, PMG activity was 9.52 ± 0.90 U/ml in batch fermentation and 12.52 ± 2.03 U/ml for fed-batch fermentation as shown Figure 6.9.

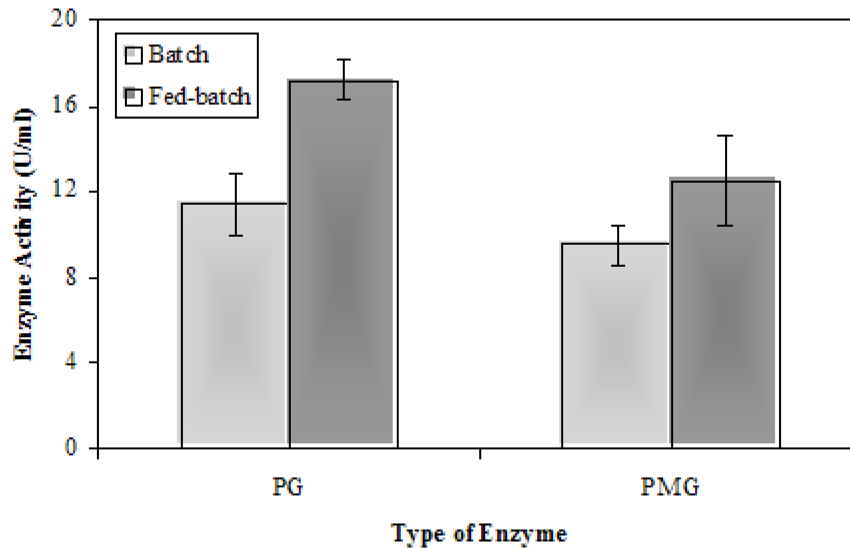


Figure 6.9. Comparison of PG and PMG activities in batch and fed-batch fermentations

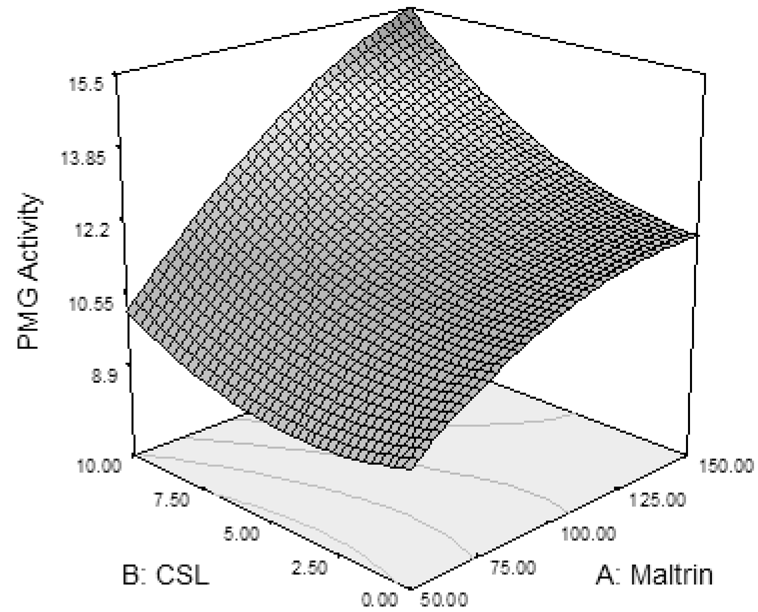
Consequently, the activity of pectinase obtained from fed-batch fermentation was higher than batch fermentation.

6.4. Experimental Design and Statistical Analysis for Fed-Batch Fermentation

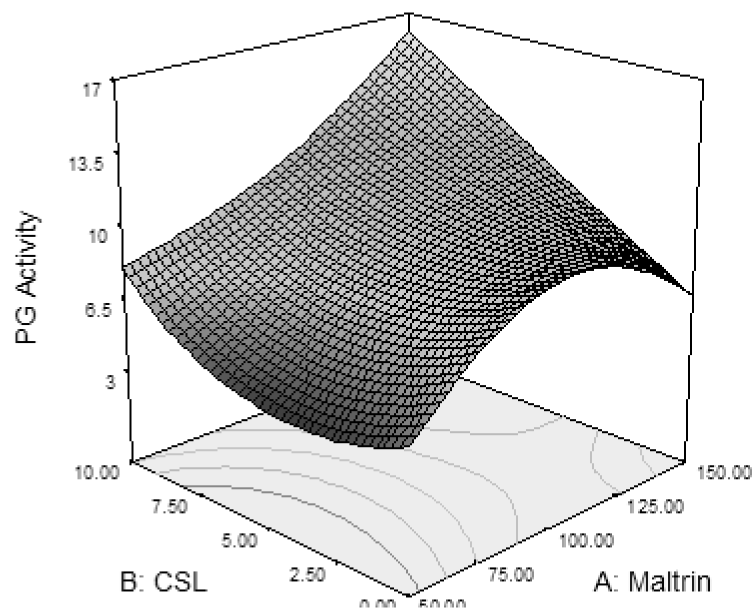
6.4.1. Face-Centered Central Composite Design

Based on the results of the previous studies (Tari, et al. 2007), factors such as maltrin concentration (50-150 g/l) (X_1), CSL concentration (0-10 g/l) (X_2) and feeding time (48th, 72th or both of them h) (X_3) were considered to have significant effect on the responses and therefore taken as factor variables in this study. Not only one feeding but also repeated feeding of this combination was carried out.

The experimental runs of faced-centered central composite design with PMG and PG activity measurements and biomass formation (response variables) are shown in Table D.1. Maximum PG activity (20.61 U/ml) and maximum biomass formation (34.23 g/l) were obtained from feedings of both 48th and 72th h at higher maltrin (150 g/l) and higher CSL (10 g/l) concentrations. However, when the feeding was done at only 72th h, maximum PMG activity (16.76 U/ml) was achieved at higher maltrin and higher CSL concentrations (Table D.1 and Figure 6.10).



(a) Surface plot of PMG Activity vs. Maltrin, CSL (Feeding time: 72nd h)



(b) Surface plot of PG Activity vs. Maltrin, CSL (Feeding time: 48th&72nd h)

Figure 6.10. Results of CCD in fed-batch fermentation: response surface plots showing the effect of maltrin (g/l) and CSL (g/l) on (a) PMG enzyme activity (U/ml) vs. Maltrin, CSL at Feeding time: 72nd h and (b) PG enzyme activity (U/ml) vs. Maltrin, CSL at Feeding time: 48th&72nd h

On the other hand maximum reduction in pH values was observed in the presence of corn steep liquor (5 g/l) and at higher maltrin concentration. Whereas, in the absence of CSL a slight decrease in pH values was recorded (Table D.1). The results of ANOVA tables (Table 6.8.a, 6.8.b, and 6.8.c) for all three responses (PMG and PG activities and biomass) revealed that the individual models were highly significant ($p<<0.005$). The lack of fit F-values of 1.12, 0.92 and 0.48 which corresponded to PMG, PG and biomass implied that the lack of fit was not significant relative to the pure error.

Table 6.8. Analysis of variance (ANOVA) tables: effect of parameters on (a) PMG activity, (b) PG activity and (c) biomass

(a) PMG Activity					
Source	**Sum of Squares**	**df**	**Mean Square**	**F-Value**	**P-value Prob>F**
Block	3.78	1	3.78		
Model	336.27	7	48.04	22.43	<0.0001 significant
Maltrin	140.38	1	140.38	65.55	<0.0001
CSL	43.63	1	43.63	20.37	<0.0001
Feeding Time	127.92	2	63.96	29.87	<0.0001
MaltrinxCSL	10.99	1	10.99	5.13	0.0273
MaltrinxMaltrin	6.89	1	6.89	3.22	0.0781
CSLxCSL	8.50	1	8.50	3.97	0.0512
Residual	122.06	57	2.14		
Lack of Fit	48.11	21	2.29	1.12	0.3769 not significant
Pure Error	73.96	36	2.05		
Cor Total	462.11	65			

(b) PG Activity (Transformed Squared Root)					
Source	**Sum of Squares**	**df**	**Mean Square**	**F-Value**	**P-value Prob>F**
Block	0.60	1	0.60		
Model	17.53	13	1.35	17.87	<0.0001 significant
Maltrin	5.14	1	5.14	68.04	<0.0001
CSL	4.55	1	4.55	60.34	<0.0001
Feeding Time	2.86	2	1.43	18.98	<0.0001
MaltrinxFeeding time	0.59	2	0.29	3.88	0.0270
MaltrinxMaltrin	1.15	1	1.15	15.19	0.0003
MaltrinxCSLxFeeding time	0.54	2	0.27	3.56	0.0356
MaltrinxMaltrinxCSL	1.46	1	1.46	19.37	<0.0001
MaltrinxCSLxCSL	0.74	1	0.74	9.85	0.0028
CSLxCSLxFeeding time	0.50	2	0.25	3.32	0.0442
Residual	3.85	51	0.075		
Lack of Fit	1.07	15	0.071	0.92	0.5480 not significant
Pure Error	2.78	36	0.077		
Cor Total	21.98	65			

(cont. on next page)

Table 6.8. (cont.) Analysis of variance (ANOVA) tables: effect of parameters on (a) PMG activity, (b) PG activity and (c) biomass.

(c) Biomass (Transformed Squared Root)					
Source	**Sum of Squares**	**df**	**Mean Square**	**F-Value**	**P-value Prob>F**
Block	1.57	1	1.57		
Model	70.98	3	23.66	282.41	<0.0001 significant
Maltrin	9.57	1	9.57	114.25	<0.0001
CSL	53.20	1	53.20	634.94	<0.0001
CSLxCSL	8.21	1	8.21	98.04	<0.0001
Residual	5.11	61	0.084		
Lack of Fit	1.28	25	0.051	0.48	0.9709 not significant
Pure Error	3.83	36	0.11		
Cor Total	77.66	65			

The second order polynomial equations for PMG enzyme (Y_{PMG}), PG enzyme (Y_{PG}) and biomass (Y_b) with the coefficients in coded units of factors are given in Equations (6.1), (6.2) and (6.3), respectively, where Y is the predicted response, X_1 is the coded value of maltrin concentration, X_2 is the coded value of CSL concentration and X_3 is the coded value of feeding time. Furthermore, estimated coefficients of PMG activity, PG activity and biomass were presented in Table D.2.a, Table D.2.b and Table D.2.c, respectively. The coefficient of determination (R^2) was 0.7337, 0.8200 and 0.9328 which corresponded to PMG, PG and biomass. This indicated that 73.37, 82.00 and 93.28% of total variation in the activities of PMG and PG and the biomass were explained by the fitted models (Equations 6.1-6.3).

$$Y_{PMG} = 9.89 + 1.97X_1 + 1.10X_2 - 0.10X_{3[1]} + 1.75X_{3[2]} + 0.68X_1X_2 - 0.71X_1^2 + 0.78X_2^2 \tag{6.1}$$

$$\begin{aligned} Y_{PG} = {} & 3.26 + 0.58X_1 + 0.071X_2 - 0.062X_{3[1]} + 0.35X_{3[2]} - 0.18X_1X_{3[1]} + 0.086X_1X_{3[2]} - 0.28X_1^2 \\ & - 0.057X_1X_2X_{3[1]} - 0.15X_1X_2X_{3[2]} + 0.43X_1^2X_2 - 0.30X_1X_2^2 - 0.14X_2^2X_{3[1]} - 0.10X_2^2X_{3[2]} \end{aligned} \tag{6.2}$$

$$Y_b = 4.31 + 0.52X_1 + 1.22X_2 - 0.76X_2^2 \tag{6.3}$$

The Predicted R-Squared of 0.6461, 0.6990 and 0.9215 was in reasonable agreement with the Adjusted R-Squared of 0.7010, 0.7741 and 0.9295 for PMG, PG and biomass, respectively, which showed that the model was good (for a good statistical model, the R^2 value should be in range of 0-1.0, and the nearer to 1.0 the value is, the more fit the model is deemed to be.) (Iyer and Singhal 2007, Reddy, et al. 2008). The adequate precision values were 17.685, 17.480 and 43.461 for responses PMG, PG and biomass respectively, suggesting that the model could be used to navigate the design space. The adequate precision value is an index of the signal-to-noise ratio, and values of higher than 4 are essential prerequisites for a model to be a good fit (Reddy, et al. 2008). The coefficient of variation "C.V." % of 14.73, 8.84 and 7.42 which corresponded to PMG, PG and biomass implied that there were no highly unexplained or residual variability in data as a percentage of the mean of all response variables.

6.4.2. Validation of the Model

Validation experiments were carried out to validate the adequacy of the model equations in shake flasks with two replicas under the predicted optimum conditions. The mean values of the experimental data and the predicted values of responses are given in Table 6.9. In the first experiment, 150 g/l of maltrin, 10 g/l of CSL were used at the feeding time of 72 nd h, whereas in the second and third experiments, 148.33 and 150 g/l of maltrin and 10 and 9.5 g/l of CSL were used at the feeding time of 72nd h, respectively. As it can be seen from Table 6.9 and Figure D.1, there was a very good compatibility of the models with experimental data implying the successful validation of the model.

Table 6.9.Validation experiments at optimum conditions

Experiment number	PMG activity[a] (U/ml)	PG activity[a] (U/ml)	Biomass[a] (g/l)
1	17.02 ± 0.64 (15.48)	18.09 ± 1.48 (15.78)	26.13 ± 1.66 (27.92)
2	15.88 ± 1.58 (15.44)	17.30 ± 0.88 (15.81)	28.18 ± 1.95 (27.98)
3	16.13 ± 0.27 (15.35)	18.31 ± 1.04 (15.86)	25.11 ± 1.26 (28.19)

[a] Predicted values of responses are in the parentheses.

6.4. Agar Diffusion Method for Detection of Pectinase Production

The studies on *A. sojae* strain improvement will be conducted in order to obtain higher activity for biotechnological applications and a rapid method is necessary to detect the potency of enzyme synthesis and activity from new mutant strain. Therefore, agar diffusion method was used and modified. It is a fast, cheap, easy and reliable technique which takes shorter time compared to analytical methods, in spite of the fact that it doesn't give exact activity results.

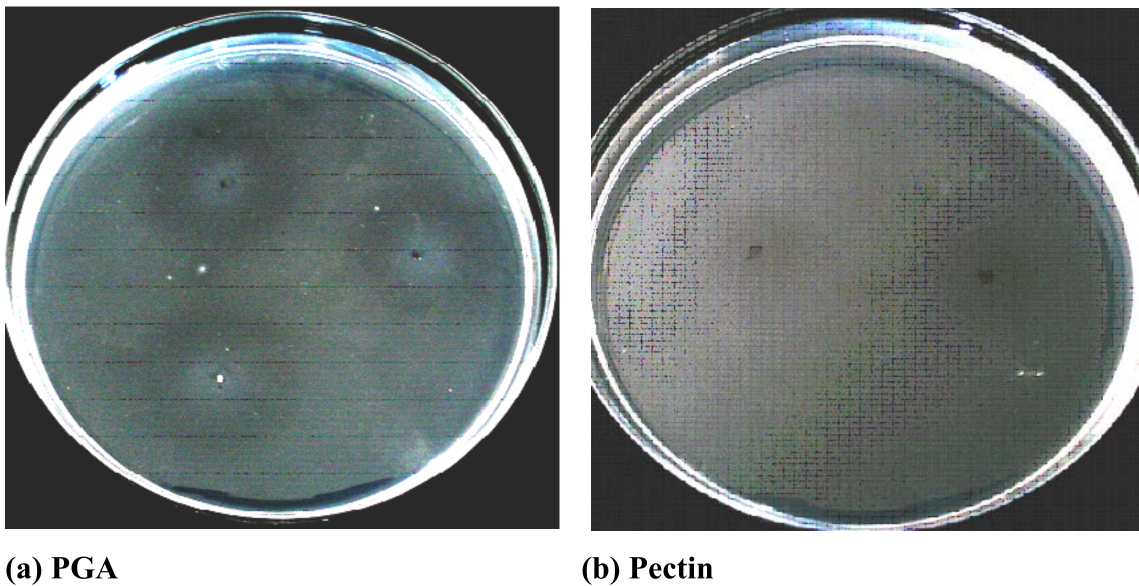
The term of pectinase production in the study is intended to mean the synthesis of the enzyme from *Aspergillus sojae*. The ability of this fungus to synthesize pectinases on solid media containing either polygalacturonic acid (PGA) at pH 4.0 or pectin at pH 5.3 as substrate is shown in Table 6.10 and is expressed in terms of average of radii of zones formed. Each experiment was carried out three times and all dilutions used were the same. The data showed that pectinases produced by *A. sojae* can easily be detected on solid media. The extracellular pectic enzymes polygalacturonase (PG) and polymethylgalacturonase (PMG) were active at pH 4.0 and 5.3, respectively, according to the optimum PG and PMG assay procedures. For all tests, plates were incubated at 30°C for 2 days and then flooded with 5N of HCl. It precipitates intact PGA or pectin in the medium and, forms a clear zone around a colony. It was observed that the medium containing PGA had bigger radii of zones than the medium with pectin (Table 6.10 and Figure 6.11).

Table 6.10 Pectinase production on solid media

	Pectinase Production (Radius of zone, cm)
PGA	1.367 ± 0.115
Pectin	0.333 ± 0.153

Moreover, with this technique viable colonies can be removed and purified by further isolation if the precipitant is not allowed to remain in contact with the cells for more than 5 min (Hankin and Anagnostakis 1975). This technique is very useful in

screening experiments when large number of isolates is under study. It is easy and fast when large number of isolates are considered, however it is not as accurate as analytic enzyme assays. Based on the substrate used it can provide a clear picture on the type of the enzyme produced. Especially strain improvement studies where lots of shake flask experiments are necessary, this technique can be a cheaper and less time consuming alternative. Since in the current study the ultimate aim is to improve the strain under study, it was thought to come up with a technique that is fast, cheap and reliable. Therefore initial experiments using this technique were applied to *Aspergillus sojae* in order to check its PG and PMG synthesis capability. This technique also provided an additional check to the analytical assays used. Finally, this technique demonstrated that it can be used in future strain improvement study and can be an alternative when large number of isolates is under consideration.



(a) PGA **(b) Pectin**

Figure 6.11. Solid media containing (a) polygalacturonic acid and (b) pectin for detection of enzyme production from spore solution

CHAPTER 7

CONCLUSION

This study demonstrated that repeated feeding of the combination of maltrin and CSL as low cost carbon and nitrogen sources had significant effect on polygalacturonase activity and biomass where the maximum PG activity (20.61 U/ml) and maximum biomass formation (34.23 g/l) were obtained from feedings at both 48th and 72th h at higher maltrin (150 g/l) and higher CSL (10 g/l) concentrations. However, when the feeding was done at only 72th h, maximum PMG activity (16.76 U/ml) was achieved at higher maltrin and higher CSL concentrations (Table D.1). Furthermore, the individual models in ANOVA table were highly significant and the lacks of fits were non-significant. It has been demonstrated that fed-batch cultures can be successfully used to increase the activities of both polymethylgalacturonase and polygalacturonase from *Aspergillus sojae*. Moreover, type of Erlenmeyer flasks had a considerable effect on the morphology, where small to medium sized pellets in non-buffled but very little pellets in buffled Erlenmeyer flasks were observed. In addition, rapid method for detection of pectinase production and activity of *A. sojae* has been modified to be used in further strain improvement studies.

After characterization of the polygalacturonase purified with an emerging bioseparation technique TTP, it was concluded that this enzyme could be a candidate with potential applications primary in food, waste treatment centers, paper and textile industries. As a polygalacturonase from a new strain (*Aspergillus sojae)*, which was not considered for this purpose so far, it will be a new reference point for the enzyme industry and microbiology area and will fill the gap in the literature. As it is known discovering new enzymes with novel properties is a tedious and difficult task to perform, therefore enzymes from new sources with unique properties such as the one here may have an enormous economic significance which should not be overlooked.

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APPENDIX A

IMAGE TAKEN by SCANNING ELECTRON MICROSCOPE

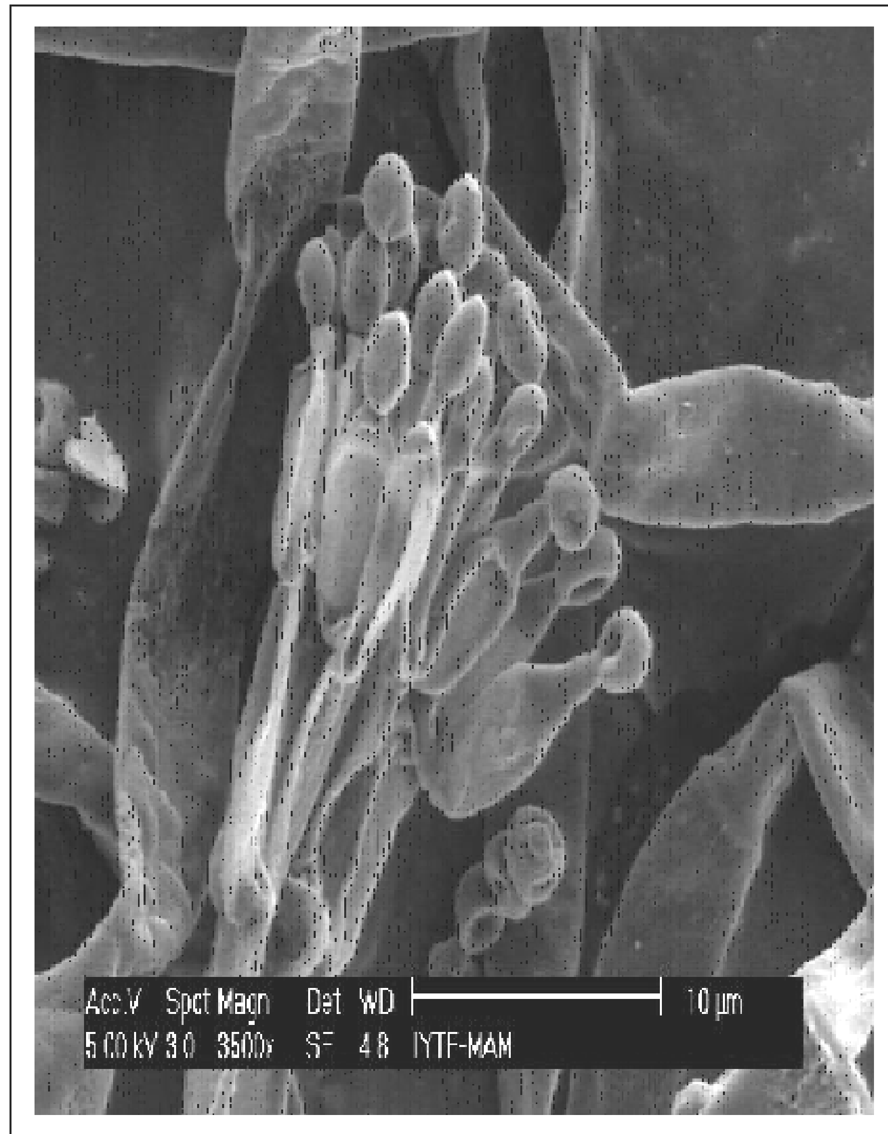

Figure A.1. Image taken by scanning electron microscope (Philips XL-30S FEG) of strain *Aspergillus sojae ATCC 20235* (magnified 3500x)

APPENDIX B

PROFILES of POLYGALACTURONASE ACTIVITY, CARBOHYDRATE, PROTEIN and pH

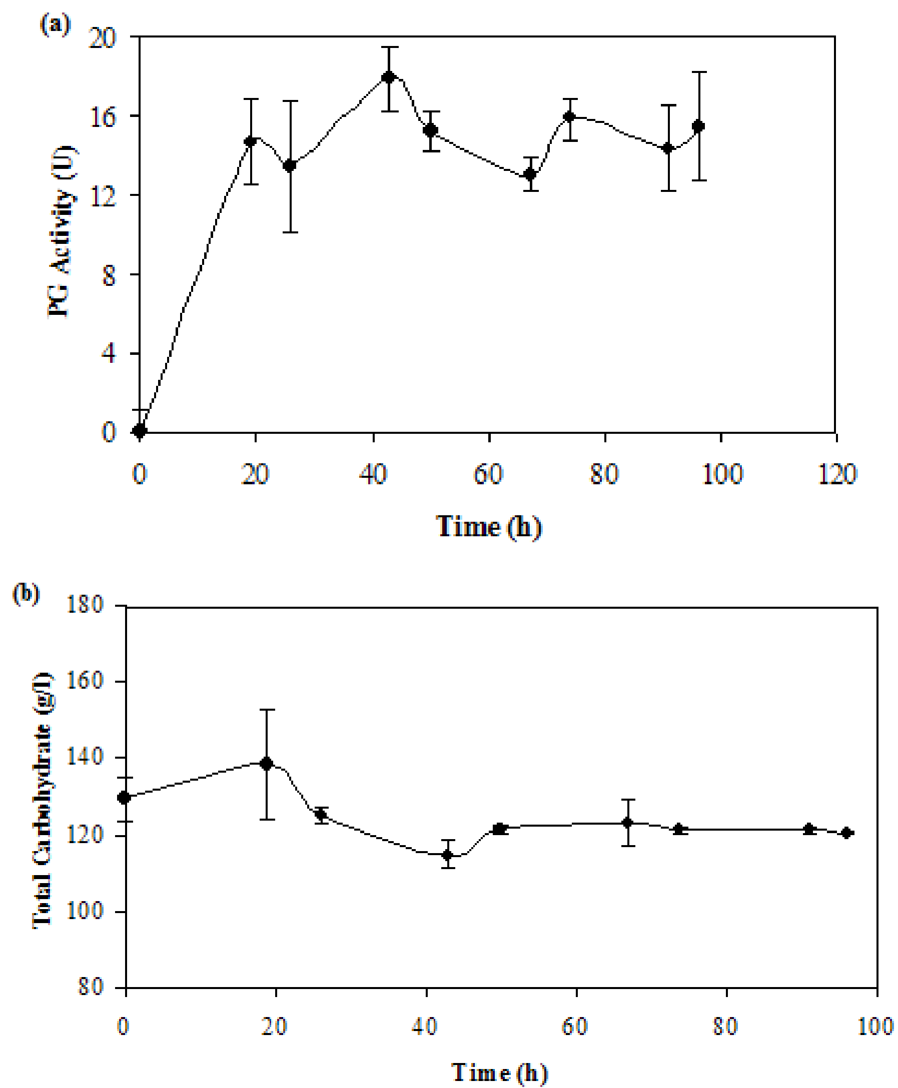

Figure B.1. Profiles of (a) polygalacturonase enzyme activity (U/ml) (b) total carbohydrate (g/l) (c) protein (mg/ml) and (d) pH for *Aspergillus sojae* at optimum batch fermentation conditions for 96 h

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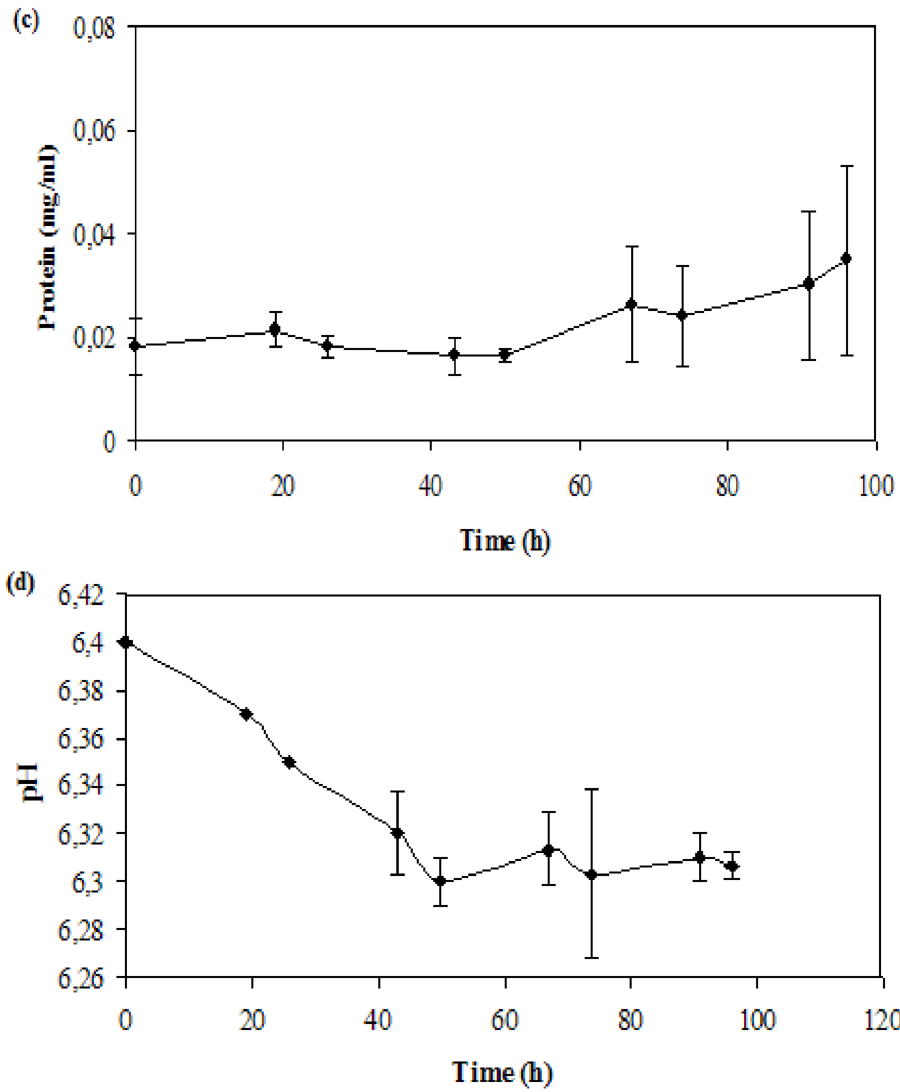
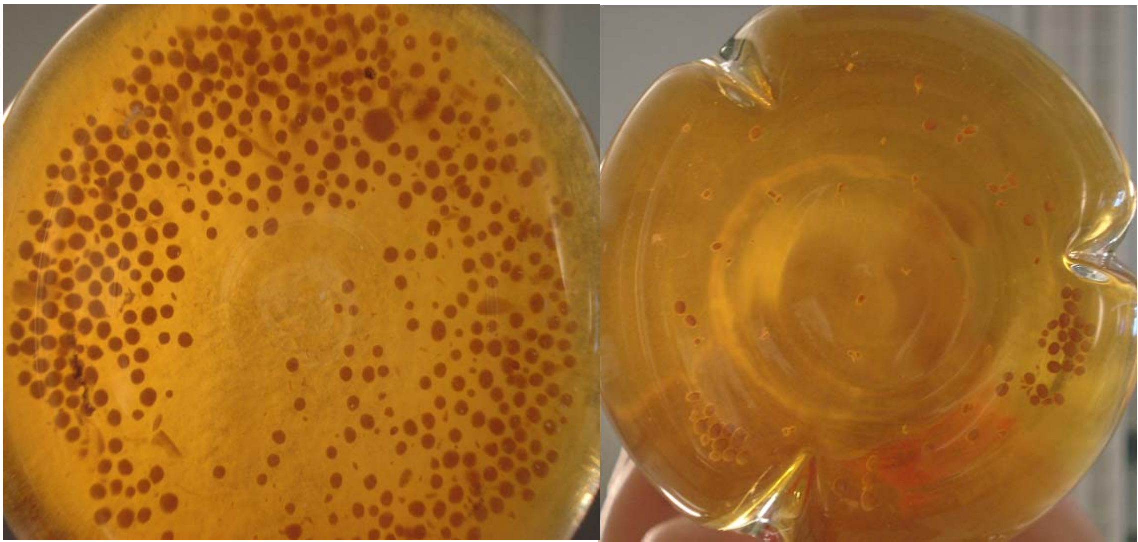


Figure B.1. (cont.) Profiles of (a) polygalacturonase enzyme activity (U/ml) (b) total carbohydrate (g/l) (c) protein (mg/ml) and (d) pH for *Aspergillus sojae* at optimum batch fermentation conditions for 96 h

APPENDIX C

MORPHOLOGY of PELLETS GROWN in NON-BUFFLED and BUFFLED ERLENMEYER FLASKS



(a) Non-buffled Erlenmeyer (b) Buffled Erlenmeyer

Figure C.1. Morphology of pellets grown in (a) non-buffled and (b) buffled Erlenmeyer flasks

APPENDIX D

CENTRAL COMPOSITE DESIGN RESULTS

Table D.1. Face-centered central composite design results of polymethylgalacturonase (PMG) activity, polygalacturonase (PG) activity, biomass and pH in fed-batch fermentation

Run order	X_1	X_2	X_3	Response 1 (PMG activity (U/ml))	Response 2 (PG activity (U/ml))	Response 3 (Biomass (g/l))	Response 4 (pH)
1	150	0	48th	8.836	5.870	7.652	6.16
2	50	0	48th	6.753	4.759	4.338	6.31
3	100	5	48th	12.402	14.064	20.226	3.84
4	150	0	72nd	12.127	9.701	10.186	6.2
5	100	5	72nd	11.570	10.492	15.950	3.87
6	150	0	48th	11.137	6.561	7.922	6.08
7	100	5	72nd	11.188	16.240	25.650	3.73
8	150	0	72nd	10.991	13.057	7.714	6.21
9	150	10	48th	15.857	13.424	30.646	4.06
10	150	0	48th&72nd	9.056	8.924	5.840	6.17
11	50	0	72nd	9.427	6.590	2.842	6.33
12	100	5	48th&72nd	10.001	7.640	16.970	3.9
13	50	10	72nd	10.311	10.593	13.546	4.25
14	100	5	48th&72nd	5.217	9.323	18.920	3.97
15	150	0	48th&72nd	7.565	6.302	7.678	6.16
16	100	5	48th	8.178	10.942	15.822	3.96
17	150	10	48th&72nd	10.036	17.9123	26.516	3.97
18	100	5	72nd	10.142	14.172	18.762	4.02
19	150	10	72nd	16.758	16.499	27.172	4.08
20	100	5	48th	10.788	12.895	16.350	3.93
21	150	10	48th	13.309	11.492	29.266	4.11
22	50	10	48th	6.353	11.902	15.528	4.18
23	50	0	48th&72nd	6.839	5.324	5.056	6.38

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Table D.1. (cont.) Face-centered central composite design results of polymethylgalacturonase (PMG) activity, polygalacturonase (PG) activity, biomass and pH in fed-batch fermentation

24	50	0	48th&72nd	5.695	6.075	2.710	6.35
25	150	10	48th&72nd	9.052	20.611	34.230	4.09
26	100	5	48th&72nd	6.101	9.744	20.208	3.81
27	50	10	48th	8.865	11.827	15.792	4.26
28	50	10	48th&72nd	6.406	7.950	16.382	4.01
29	50	0	48th	7.218	6.183	2.544	6.33
30	150	10	72nd	16.548	14.086	27.64	4.14
31	50	10	48th&72nd	6.745	10.791	16.438	3.77
32	50	10	72nd	10.657	13.154	23.972	4.39
33	50	0	72nd	7.799	5.489	2.858	6.32
34	150	5	72nd	14.832	15.010	21.158	3.71
35	100	0	72nd	11.867	13.848	6.434	6.3
36	100	5	72nd	13.659	10.063	24.502	4.2
37	50	5	48th&72nd	5.188	2.827	14.730	4.1
38	100	10	48th&72nd	10.218	10.597	27.680	4.2
39	100	0	48th	11.580	7.748	5.520	6.23
40	100	0	72nd	9.702	11.697	5.578	6.23
41	50	5	48th	6.246	6.614	15.664	4.35
42	100	0	48th&72nd	9.550	7.841	4.986	6.24
43	100	5	48th&72nd	8.274	9.500	16.422	3.63
44	150	5	48th	9.357	11.654	23.162	4.14
45	150	5	72nd	12.683	15.884	19.592	3.72
46	100	10	48th&72nd	11.496	12.669	26.802	3.81
47	50	5	48th	8.303	3.367	15.412	3.88
48	100	0	48th&72nd	6.804	8.618	5.456	6.22
49	100	5	48th	9.989	8.323	19.328	3.96
50	100	10	48th&72nd	12.901	9.884	21.494	4.3
51	50	5	72nd	9.864	8.129	17.012	3.71
52	100	10	48th	11.797	10.154	19.548	4.06
53	50	5	48th&72nd	7.144	5.446	14.504	3.83
54	100	5	48th	10.833	8.935	18.412	4.19
55	50	5	72nd	7.870	4.845	13.586	3.71
56	150	5	48th	10.177	8.295	23.982	3.98

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Table D.1. (cont.) Face-centered central composite design results of polymethylgalacturonase (PMG) activity, polygalacturonase (PG) activity, biomass and pH in fed-batch fermentation

57	100	0	48th	9.400	9.381	6.096	6.26
58	100	5	48th&72nd	9.513	8.856	17.864	3.72
59	100	5	72nd	13.823	14.190	14.332	4.03
60	100	10	48th&72nd	12.736	13.884	20.304	4.11
61	100	5	72nd	11.108	10.295	20.140	4.01
62	100	10	48th	11.933	7.295	22.310	4.26
63	150	5	48th&72nd	11.166	9.050	23.614	3.95
64	150	5	48th&72nd	9.287	10.780	22.068	3.6
65	100	5	48th	6.942	7.510	17.614	4.18
66	100	5	48th&72nd	9.478	5.730	22.134	3.85

Factors, $X_{1,}$ Maltrin Concentration (g/l); $X_{2,}$ CSL concentration (g/l); $X_{3,}$ Feeding time (h)

Table D.2. Estimated Coefficients for (a) PMG activity, (b) PG activity and (c) biomass

(a) PMG activity			
Term	**Coefficient Estimate**	**Standard Error**	**P-value Prob>F**
Constant	9.89	0.33	<0.0001
Block-1	-0.25		
Block-2	0.25		
Maltrin	1.97	0.24	<0.0001
CSL	1.10	0.24	<0.0001
Feeding Time[1]	-0.10	0.25	<0.0001
Feeding Time[2]	1.75	0.25	<0.0001
MaltrinxCSL	0.68	0.30	0.0273
MaltrinxMaltrin	-0.71	0.39	0.0781
CSLxCSL	0.78	0.39	0.0512
R-Sq=73.4%, R-Sq(adj)=70.1%, R-Sq(pred)=64.6%			

(b) PG activity			
Term	**Coefficient Estimate**	**Standard Error**	**P-value Prob>F**
Constant	3.26	0.052	<0.0001
Block-1	0.15		
Block-2	-0.15		
Maltrin	0.58	0.079	<0.0001
CSL	0.071	0.079	<0.0001
Feeding Time[1]	-0.062	0.071	<0.0001
Feeding Time[2]	0.35	0.071	<0.0001
MaltrinxFeeding time[1]	-0.18	0.065	0.0270
MaltrinxFeeding time[1]	0.086	0.065	0.0270
MaltrinxMaltrin	-0.28	0.073	0.0003
MaltrinxCSLxFeeding time[1]	-0.057	0.079	0.0356
MaltrinxCSLxFeeding time[2]	-0.15	0.079	0.0356
MaltrinxMaltrinxCSL	0.43	0.097	<0.0001
MaltrinxCSLxCSL	-0.30	0.097	0.0028
CSLxCSLxFeeding time[1]	-0.14	0.096	0.0442
CSLxCSLxFeeding time[2]	-0.10	0.096	0.0442
R-Sq=82.0%, R-Sq(adj)=77.4%, R-Sq(pred)=69.9%			

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Table D.2. (cont.) Estimated Coefficients for (a) PMG activity, (b) PG activity and (c) biomass

(c) Biomass			
Term	**Coefficient Estimate**	**Standard Error**	**P-value Prob>F**
Constant	4.31	0.055	<0.0001
Block-1	-0.016		
Block-2	0.016		
Maltrin	0.52	0.048	<0.0001
CSL	1.22	0.048	<0.0001
CSLxCSL	-0.76	0.077	<0.0001
R-Sq=93.3%, R-Sq(adj)=93.0%, R-Sq(pred)=92.2%			

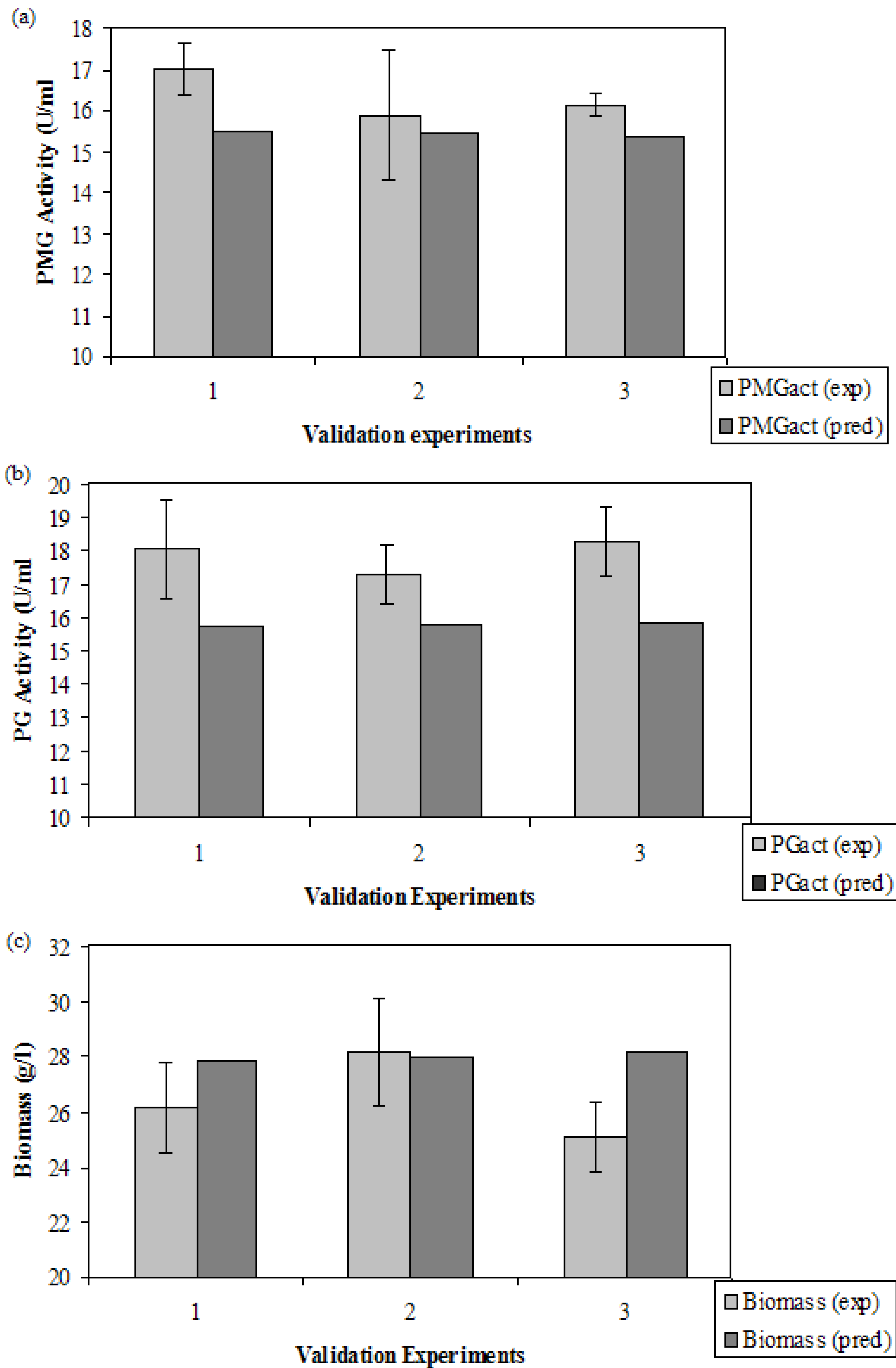

Figure D.1. Experimental and predicted values for (a) PMG activity (b) PG activity and (c) biomass formation in validation experiments

APPENDIX E

STANDART CALIBRATION CURVE for POLYGALACTURONASE ACTIVITY

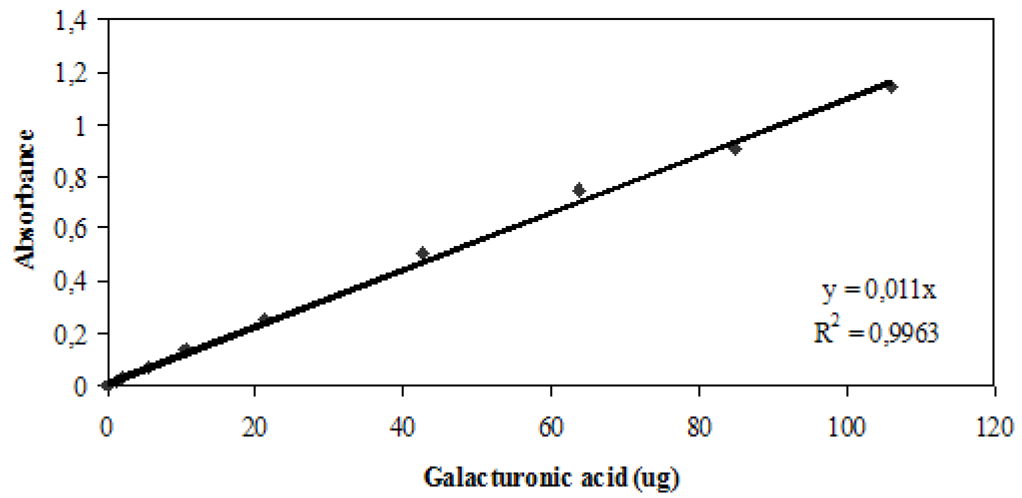

Figure E.1. Standard calibration curve for polygalacturonase activity

APPENDIX F

STANDART CALIBRATION CURVE for POLYMETHYLGALACTURONASE ACTIVITY

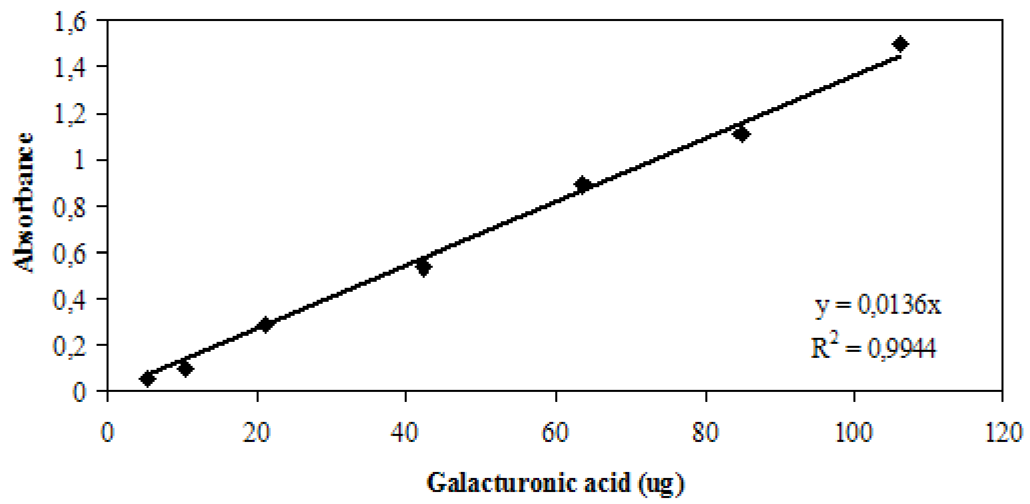

Figure F.1. Standard calibration curve for polymethylgalacturonase activity

APPENDIX G

STANDART CALIBRATION CURVE for TOTAL PROTEIN

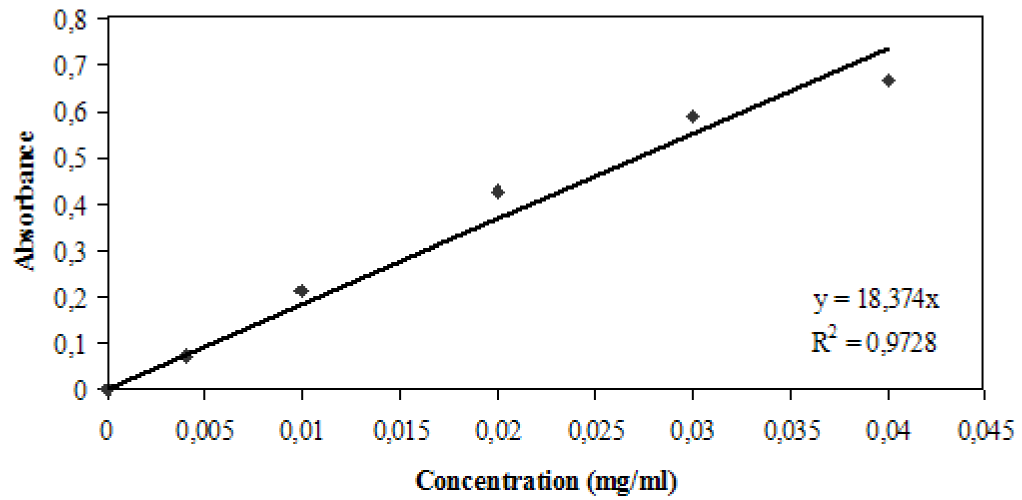

Figure G.1. Standard calibration curve for total protein

APPENDIX H

STANDART CALIBRATION CURVE for TOTAL CARBOHYDRATE

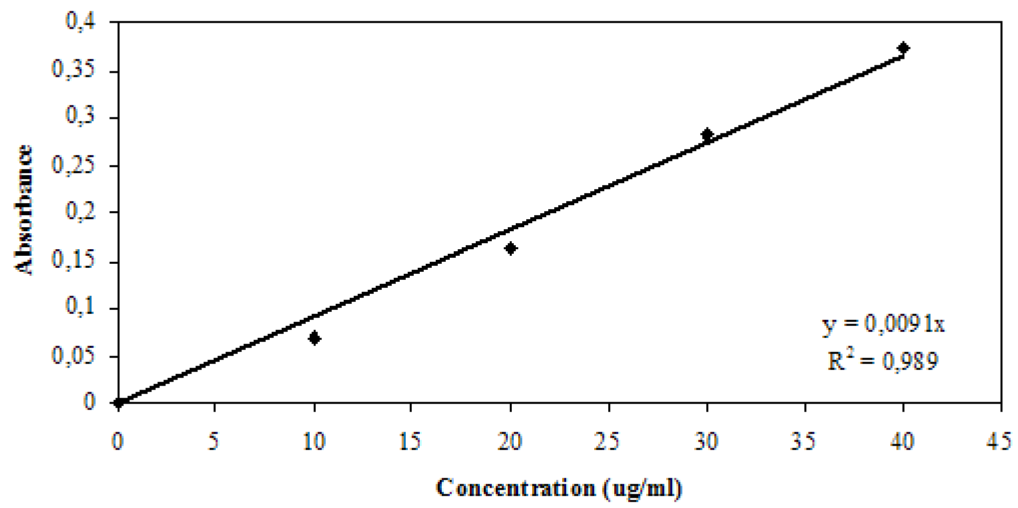

Figure H.1. Standard calibration curve for total carbohydrate