RECOMBINANT PRODUCTION OF PECTINOLYTIC ENZYMES IN *PICHIA PASTORIS* AND PROCESS OPTIMIZATION FOR FOOD INDUSTRY

by
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ABSTRACT

RECOMBINANT PRODUCTION OF PECTINOLYTIC ENZYMES IN *PICHIA PASTORIS* AND PROCESS OPTIMIZATION FOR FOOD INDUSTRY

Pectinolytic and ligninolytic enzymes degrade pectin and lignin respectively, main constituents of plant-based biomass, used in several industries ranging from food, feed and energy, wastewater treatment, textile, oil extraction, retting of plant fibers and paper making industries. Pectinolytic enzymes dominate 10% of total enzyme market and 25% of the worldwide food enzyme market. A key bottleneck in these domestic industries is the supply of affordable enzymes to local markets. Heterologous enzyme production is a key technology that uses the powerful tools of molecular biology, allowing to manufacture tailor-made products for specific applications, e.g. desired enzyme blends.

Pichia pastoris is a methylotrophic yeast and a promising host for industrial biotechnology, as it can be used for high level of protein production. Additionally, suitability for high cell density cultivation, capacity to secrete produced proteins, rendering in turn downstream processes cheaper, availability of genome sequence, ease in genetic manipulations and capability of post translational modifications makes *P. pastoris* an interesting host for heterologous protein production.

In this thesis, five pectinolytic and ligninolytic enzymes namely, polygalacturonase (EC 3.2.1.15), pectin methyl esterase (EC 3.1.1.11), pectin lyase (EC 4.2.2.10), laccase (EC 1.10.3.2) and lignin peroxidase (EC 1.11.1.14) were heterologously produced in *P. pastoris*. The recombinant enzymes are produced in lab scale (both in Erlenmeyer flasks as well as in controlled bioreactor conditions), under the methanol-inducible AOX1 promoter and the activities of enzymes from the crude extracts (analyzed separately by SDS-PAGE) are measured based on released products. Subsequently, five different *Pichia pastoris* strains are engineered via recombinant DNA technology to produce target enzymes.

ÖZET

GIDA ENDÜSTRİSİNDE KULLANILAN PEKTİNAZ ENZİMİNİN *PİCHİA PASTORİS*'TE REKOMBİNANT ÜRETİMİ VE SÜREÇ OPTİMİZASYONU

Pektinolitik ve ligninolitik enzimler sırasıyla bitki bazlı biyokütle bileşenleri olan pektini ve lignini parçalarlar.Bu enzimlerin atık-su muamelesi, tekstil, yağ ekstraksiyonu, bitki fiberlerinin havuzlaması ve kağıt gibi birçok farklı endüstride ticari uygulamaları vardır. Pektinolitik enzimler, enzim gıda pazarının yaklaşık %10'unu, gıda enzim pazarının ise %25'ini oluştururlar. Uygun fiyatlı yerel enzimlerin üretimi bu endüstri alanlarının darboğazıdır. Heterolog enzim üretimi moleküler biyoloji araçlarının kullanıldığı önemli bir teknolojidir. Böylelikle üretime yönelik özgül uygulamalarda (ör:istenilen enzim harmanları) kullanılabilecek ürünler oluşturulabilir.

Pichia pastoris metanolü kullanabilen ve yüksek seviyede protein üretimi için endüstriyel biyoteknolojide kullanılan önemli bir konaçkıdır. Ek olarak, yüksek hücre yoğunlu kültürlemelerine uygun olması, üretilen proteini yüksek miktarlarda salgılayabilme kapasitesi, genom bilgisinin ulaşılabilir olması, daha uygun maliyetli saflaştırma imkanları sağlaması, kolay genetik manipülasyonu ve translasyon sonrası modifikasyon yapması *P. pastoris*'i heterolog protein üretimi açısından ilginç kılar.

Bu tezde beş tane pektinolitik ve ligninolitik enzimler; poligalakturonaz (EK 3.2.1.15), pektin metil esteraz (EK 3.1.1.11), pektin liyaz (EK 4.2.2.10), lakkaz (EK 1.10.3.2) ve lignin peroksidaz (EK 1.11.1.14) heterolog olarak *P. pastoris*'te üretilmiştir. Rekombinant enzimler metanolle uyarılabilen AOX1 promotörü altında laboratuvar ölçeğinde (erlen ve kontrollü biyoreaktör koşullarında) üretilip, aktiviteleri kaba özütten (SDS-PAGE ile analiz edilmiş) salınan ürünler aracılığıyla ölçülmüştür. Sonuç olarak, hedef enzimleri üretebilen beş farklı *P.pastoris* suşu rekombinant DNA teknolojisi kullanılarak tasarlanmıştır.

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LIST OF SYMBOLS/ABBREVIATIONS

d	Day
g	Gram
h	Hour
kDa	Kilodalton
L	Liter
M	Molarity
α	Greek letter ‘alpha’
β	Greek letter ‘beta’
μ	Greek letter ‘mu’
ρ	Greek letter ‘rho’
Amp	Ampicillin
APS	Ammonium per sulfate
BMGY	Buffered glycerol complex medium
BMMY	Buffered methanol complex medium
BSA	Bovine serine albumin
$CaCl_2$	Calcium chloride
Da	Dalton
DNA	Deoxyribonucleic acid
DNS	3,5-Dinitrosalicyclic acid
DTT	Dithiothreitol
dH_2O	Distilleted water
ddH_2O	Double distilleted water
EDTA	Ethylenediaminetetraacetic acid
NaOH	Sodium hydroxide
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
SDS	Sodium dodecyl sulfate
SSF	Solid state fermentation

SmF	Submerged fermentation
SOC	Super optimal media with catabolite repression
Lac	Laccase
LB	Luria-bertani
LP	Lignin peroxidase
MW	Molecular weight
MWCO	Molecular weight cut off
pelC	Pectin lyase
PGase	Polygalacturonase
PME	Pectin methyl esterase
RT	Room temperature
TEMED	Tetrametiletilendiamin
YPD	Yeast extract, peptone and dextrose
YE	Yeast extract
YPDS	Yeast extract, peptone, dextrose and sorbitol
Zeo	Zeocin

1. INTRODUCTION

1.1. PECTIN

Pectin is a polysaccharide that found in primary cell wall and middle lamella of plants [1]. Pectin was discovered by Louis Nicolas Vavquelin in 1970. However the term pectin that comes from Greek word, pektikos, was presented by Henri Braconnot [2]. Pectin acts as cementing agent with using cellulose fibrils and also intracellular pectin is used as channel to passage of water and nutrients [1]. Also, pectin provides cell growth and cell extension in plant cell wall. Backbone of pectin consists of (1→4)-linked a α-D-galacturonic acid unit that is also known as galactosyluronic acid [2]. Structure of pectin is shown in Figure 1.1.

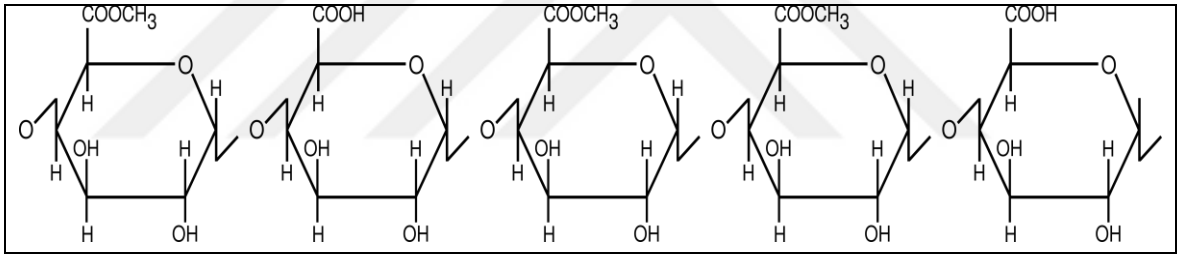


Figure 1.1. Pectin structure [3]

Amount of pectin can vary according to plant type. Pectin is most abundant in citrus fruits, which are oranges, lemons and grapefruits, and then in apple. According to fruit type, quantity of galacturonic acid can change. Galacturonic acid composition is 25.2 % for apple pomace while it is 26% for citrus peels [4]. There are two different regions of pectin, which are hairy and smooth regions. In smooth region, there are only carboxyl groups of galacturonic acid while there are also sugars such as galactose, arabinose and xylose in hairy regions [5]. Likewise, there are three different pectic polysaccharides that are homogalacturonan, rhamogalacturonan I and rhamogalacturonan II [4]. Homogalacturonan consists of (1→4)-linked α-D-galacturonic acid residues [1].

While the rhamnogalacturonan I composed of linear repeating structure of [→2)-α-L-Rhap-(1→4)-α-D- GalpA-(1→]. Also, rhamnogalactuonan II states a homogalacturonan with four side chains and consists of rare sugars as aceric acid, D-apiose, L-fucose [1].

Carboxyl groups of pectin are methylated with different degrees and according to the degree of methylation, the pectin is called as highly methoxylated (>%50 of pectin is methylated) or low methoxylated (<%50 of pectin is methylated) [6]. While low methoxylated gel is occured with using divalent cations (e.g. calcium ions), high methoxylated gel is appeared with acids or sugars [4].

There are different pectin extraction methods but most commonly hot acidified water treatment method is used. For this purpose, high temperature (60-100 °C), low pH (1,5 - 3) and long treatment times (up to 6 hours) are utilized. After the extraction of pectin, there are some methods to remove other parts of plant and among the different methods, washing with ethanol is most frequently used [4].

The alcohol treatment step has advantage and disadvantage. As an advantage, the end product can be modified with acid treatment to produce high methoxylated pectins or ammonia is used to get low methoxy pectin. However, alcohol can cause enhancing of hydrogen bonding between cell wall constituents or it can affect extraction process. Consequently, the steps of extraction method are determined according to property of end product [4].

There are many industrial application areas of pectin especially in food industry. Pectin is used as gelling, thickening agent and as a stabilizer in different areas as milk drinks, jam, jellies and fruit juice [1, 6]. Because of these properties pectin is used as food additives (E440) [1]. However, there are some disadvantages of pectin especially in food industry. Pectin decreases quality of end product (e.g. disturbing clearness of fruit juice) and causes bottlenecks (e.g. filtration process and increasing process time) in the process. Therefore, degradation of pectin is essential for the initial phases of bioprocess.

1.2. PECTINOLYTIC ENZYMES

Pectinolytic enzymes, also known as pectinases, were first isolated and described in 1825 by Henri Braconnot and pectinase was started to be used commercially in 1930 [7]. There are different types of pectinases and they are classified according to their reaction, substrate and cleavage types [8]. Polygalacturonase, pectin methyl esterase and pectin lyase are more essential ones. They provide to degrade pectin according to their action modes through de-polymerization and de-esterification reactions (Figure 1.2) [5, 9].

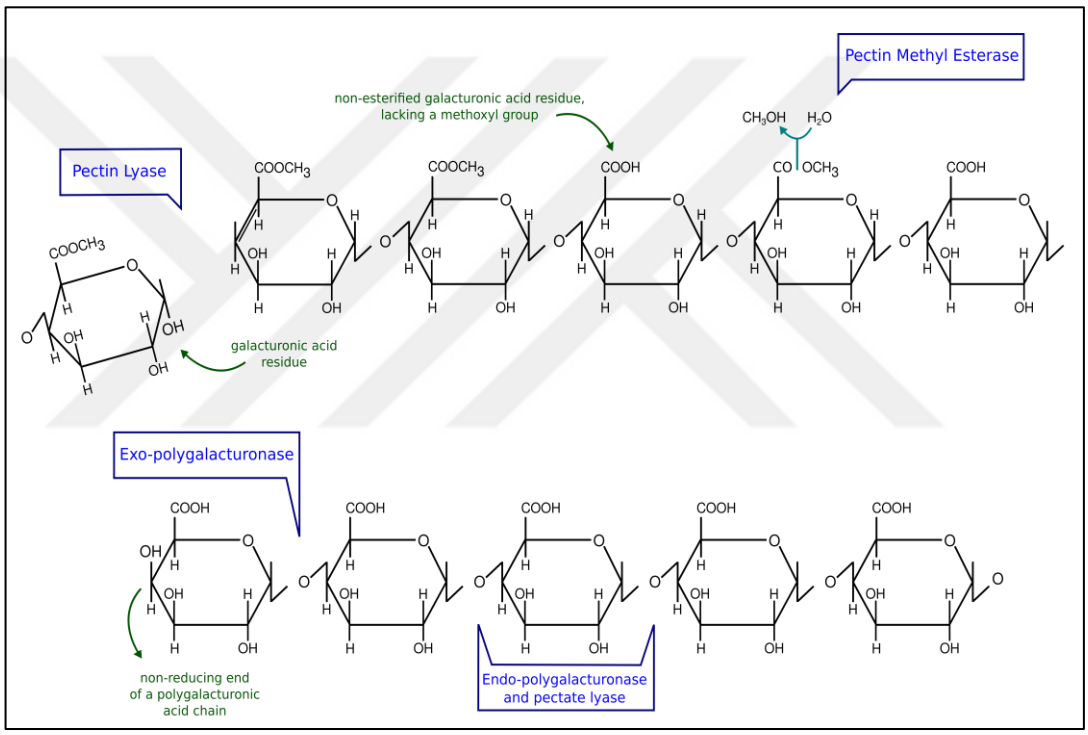


Figure 1.2. Action modes of pectinolytic enzymes on pectin

Pectinolytic enzymes are produced by fungi, bacteria and yeasts. *Bacillus*, *Erwinia*, *Kluyveromyces*, *Aspergillus*, *Trichoderma and Penicillium* are some of the microorganisms for pectinolytic enzymes production [10]. Besides, recombinant pectinolytic enzymes production is essential for the design of tailor-made process to have enzyme blends with different activities or with different kinds of enzymes. In this purpose, *Penicillium griseoroseum*, *Penicillium expansum*, *Bacillus halodurans*, *Aspergillus oryzae* and *Pichia pastoris* are used for recombinant pectinolytic enzyme studies [11-14]. There are different production parameters (temperature, pH, incubation time) for production of pectinolytic enzymes and Table 1.1 shows some of these parameters according to literature studies.

Table 1.1. Parameters for pectinolytic enzymes production

Microorganism	Substrate (g/L)	Process Parameters	Activity	Reference
Erwinia carotovora	Pectin (2.5)	30°C, 48 h	-	[15]
Kluyveromyces marxianus	Pectin (10)	28°C, 48 h	1 U/mL	[16]
Trichoderma harzianum	Citrus peel	28°C, 72 h, 200 rpm	16.2 U/mL/min	[17]
Penicillium griseoroseum	Pectin (3)	25°C, 150 rpm	15 065 ± 1280 U/mL	[11]
Bacillus subtilis	Polygalacturonic acid	pH 9, 37°C, 200 rpm	160 U/mL	[18]
Aspergillus oryzae	Lemon peel	30°C, 120 h	290 ± 4.5 U/mL	[19]
Pichia pastoris	Methanol	30°C, pH 6, 96 h	503 ± 3 U/mL	[14]
Aspergillus niger	Orange peel	30°C, pH 5.5, 180 rpm, 120 h	117 ± 3.4 μm/mL/min	[20]

1.2.1. Polygalacturonase

Polygalacturonases (3.2.1.15) provide hydrolysis of polygalacturonic acid chain in the presence of water. Also, it contributes to convert insoluble protopectin to soluble form. According to literature, polygalacturonase is one of the most studied pectinolytic enzymes [21]. There are two types of polygalacturonases as endo-polygalacturonase (3.2.1.15) and

exo-polygalacturonase (3.2.1.67). Endo-polygalacturonase catalyzes hydrolysis of alpha 1,4-glycosidic linkages in pectic acid randomly while exo-polygalacturonase catalyze in a sequential fashion [8].

1.2.2. Pectin Methyl Esterase

Pectin methyl esterase (EC 3.1.1.11) enzyme is attacking methoxyl groups of pectin via de-esterification reaction and provides to forming pectic acid and methanol. Pectin is degraded firstly by pectin methyl esterase because of methyl ester groups, after that polygalacturonase and pectate lyases can act to non-esterified substrates [5].

1.2.3. Pectin Lyase

Pectin lyase (4.2.2.10) provides to cleave α-1,4 glycosidic linkage by transelimination. As a result of the reaction, galacturonide and unsaturated bond between C_4 and C_5 at the non-reducing end of galacturonic acid are formed. There are different types of pectin lyase enzyme as exo-pectin lyase (4.2.2.9), endo-pectin lyase (4.2.2.2), endo-polymethylgalacturonate (4.2.2.10) and exo-polymethylgalacturonate [22, 23].

1.2.4. Pectinolytic Enzyme Activities

Polygalacturonase activity can be defined through 3,5-dinitrosalicyclic acid (DNS) method [24]. Galacturonic acid which is a reducing sugar is the end product of the degradation of pectin by polygalacturonase. Since, DNS is used to determine reducing sugars, activity of the enzyme can be determined [25].

Pectic acid and methanol are products from degredation of pectin through pectin methyl esterase [5]. Therefore, the enzyme activity can be determined either pectic acid or methanol measurement. Pectin methyl esterase activity can be determined by titration method in case of pectic acid measurement [26].

Gas chromotography is used to measure methanol concentration [27]. Also, amount of methanol can be determined through formaldehyde (the product that is from oxidation of

methanoly) or alcohol oxidase enzyme [28]. Besides, the enzyme activity can be measurd through galacturonic acid [26, 29].

Furthermore, galacturonic acid measurement determine pectin lyase activity. Also, thiobarbituric acid (TBA) method can be used for this purpose [30]. The increasement in absorbance (235 nm) will provide to calculate enzyme activity [31].

1.2.5. Applications of Pectinolytic Enzymes

Pectinolytic enzymes dominate about 10% of total enzyme market and 25% of the worldwide food enzyme market [5, 32]. Commercial applications of pectinolytic enzymes have range spectrum such as wastewater treatment, textile, oil extraction, retting of plant fibers and paper making industries [33].

In wastewater treatment, pectinolytic enzymes can degrade pectic substances while they are used to remove sizing agents from cotton in textile industry [34]. Also, they remove emulsifying properties of pectin and so preventing aggregation of oils from citrus peels. In addition to this, pectinolytic enzymes are essentially used in food industries for animal feed, wine production and fruit juice extraction [33, 35].

Main commercial uses of pectinases are in fruit extraction and clarification since the first time they were used to clarify apple juice in 1930 [33, 35]. Pectinolytic enzymes, especially acidic pectinases, are used in fruit juices because pectin cause viscosity and turbidity which increases the filtration time [5, 33]. Therefore, they improve product yield, remove gel structure, reduce viscosity, stabilize juice and cloudiness of juice [5, 34, 35]. However the main advantages of using pectinolytic enzymes are to speed up process and get more purified product [5]. According to literature, usage of pectinolytic enzymes causes decrease in viscosity up to 35% in apple juice [22].

In commercial applications of the enzymes, acidic (pH range is between 2.5 and 6) pectinases are used in fruit juice applications while alkaline pectinases are used in wastewater treatment, oil extraction and textile industry [35, 36]. Also, concentration of the enzymes can differ from 0,06% to 0,135 % in fruit juice industry [35].

Yield of fruit juice extractions is increased more than 90% with enzymatic method in comparision with conventional methods. Besides, pectinolytic enzymes strengthen flavour and colour of the wine and fruit juices [5, 35]. Also, concentration of alcohol is increased while pectin methyl esterase is used because methanol is released. However, high concentration of methanol can cause toxic effect, so enzyme concentration must be optimized. In animal feed, the enzymes are used for production of ruminant feed to decrease feed viscosity and raising nutrient absorption [35].

Commercial pectinolytic enzymes are produced by different companies in world. Some of the companies and product trade names are given in Table 1.2.

Table 1.2. Commercial pectinolytic enzymes and their manufacturers [35]

Product Trade Name	**Company**
Pectinase	Biocon Pvt Ltd, India
Pectolase	Grinsteelvaeket, Denmark
Pectinase Mash	Novozyme, Denmark
Ultrazyme	Ciba-Geigy A.G., Switzerland
Klerzyme	Clarizyme, Wallerstein, Co., USA
MaxLiq	Danisco, Denmark
Sclase	Kikkoman Shoyu, Co., Japan
Pectinex	Schweizerische Ferment, A.G., Switzerland
Pectinex Ultra SP-L and Pectinex CLEAR	Novo Nordisk Ferment Ltd., Switzerland
Pectinol	Rohm, GmbH, West Germany
Ly Peclyve PR	Lyven, France

Panzym	C.H. Boehringer Sohn, West Germany
Rohapect MA Plus	AB Enzymes, Finland
Rapidase	Societe Rapidase, S.A., France

1.3. LIGNIN

Lignin is an aromatic polymer on plant cell wall and it is attached to cellulose and hemi-cellulose [37, 38]. Lignin is the most ample natural biopolymer in the world and it provides to growth and improvement of plant cells [37, 39]. There are three structural units of lignin as syringyl (S), guaiacyl (G) and ρ-hydroxyphenyl (H). They are classified according to their methoxyl levels. In fact, syringly is dimethoxylated, guaiacyl is monomethoxylated while ρ-hydroxyphenyl is non-methoxylated. Lignin composition of a plant can differentiate according to plant type. Lignin content is highest in softwoods (G units) than hardwoods (S and G units) [40].

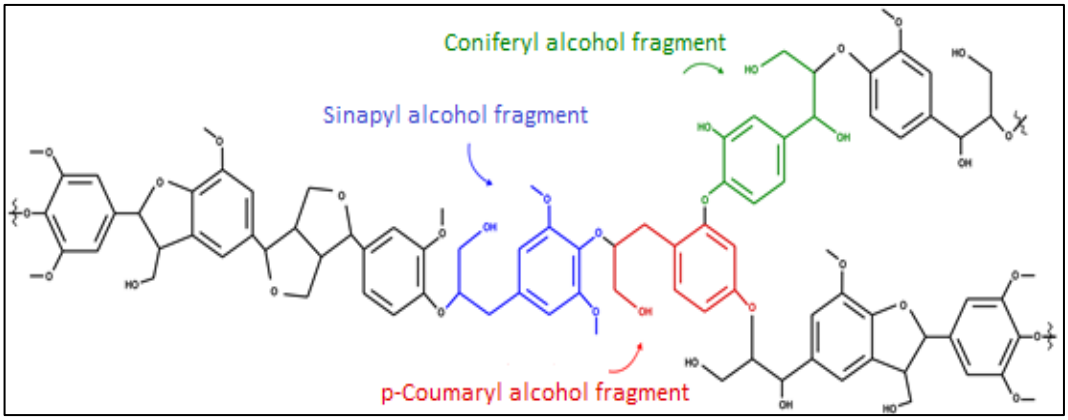


Figure 1.3. Lignin structure [41]

Besides, lignin acts as barrier to solutions and enzymes and so degredation of lignin is arduous [39]. There are mainly two ways of degrading lignin, chemical and enzymatic treatments. Strong acids/bases, organic solvents and ammonia can be used for chemical treatment. Hydrochloric acid (HCl), sulfuric acid (H_2SO_4) and nitric acid (HNO_3) are widely used strong acid for delignification process. Sodium hydroxide (NaOH), potassium

hydroxide (KOH) and calcium hydroxide $Ca(OH)_2$ are strong bases. Benzene, ether, ethanol and methanol are examples for organic solvents. Acid pretreatment is more efficient for degradation of hemi-cellulose while alkaline pretreatment is favorable for lignin removal [42, 43]. Nowadays, enzymatic pretreatment is an outstanding approach for delignifaction process because it is eco-friendly, more efficient and decreases process [42]. Ligninolytic enzymes (laccase, lignin peroxidase, manganese peroxidase and versatile peroxidase) are used as enzymatic pretreatment [38, 39].

Table 1.3. Natural fibers compositions (%)

Fiber	Cellulose	Lignin	Hemicellulose	Pectin	Wax	Moisture	Ash	Reference
Hemp	57-77	3.7-13	14-22.4	0.9	0.8	10	0.8	[44-46]
Cotton	82.7	-	5.7	-	0.6	-	-	[45, 47]
Kenaf	31-57	15-19	21.5-23	-	-	-	2-5	[44-46]
Ramie	68.6-91	0.6-0.7	5-16.7	1.9	0.3	8	-	[44, 45]
Coir	36-43	41-45	10-20	3-4	-	8	-	[45, 48]
Fiber flax	71	2.2	18.6-20.6	2.3	1.7	10	-	[44, 45]
Seed flax	43-47	21-23	24-26	-	-	-	5	[44]
Areca	-	13-24.8	35-64.8	-	-	-	4.4	[48]

1.4. LIGNINOLYTIC ENZYMES

Ligninolytic enzymes are produced by different microorganisms especially by basidiomyceteous white-rot fungi [38, 42]. Degradation of lignin is achieved via aerobic process slowly while aromatic rings cannot break down through anaerobic process [38]. *Trametes versicolor*, *Heterobasidium annosum*, *Phanerochaete chrysosporium* and *Irpex lacteus* are some of examples for white-rot fungi [49-51].

Table 1.4. Parameters for ligninolytic enzymes

Microorganism	Substrate (g/L)	Process Parameters	Activity	Reference
Trametes versicolor	Wheat bran	30°C, 168 h	-	**[52]**
Phanerochaete chrysosporium	Glycerol	pH 5, 37°C, 192 h	-	**[53]**
Phanerochaete chrysosporium	Wheat bran (1.25)	39°C, 120 h	2600 U/L	**[54]**
Marasmiellus palmivorus LA1	Galactose (8)	pH 5, 28°C	667.4 ±13 IU/mL	**[55]**
Arthrospira maxima (SAE-25780)	Sucrose	pH 6, 30°C, 96 h	60.914 U/mL	**[56]**
Pichia pastoris X-33	Methanol	pH 6, 30°C, 250 rpm	15 U/L	**[57]**

1.4.1. Laccase

One of the important ligninolytic enzymes is laccase (EC 1.10.3.2) that belongs to multicopper oxidase family. Laccase provides to oxidize phenolic compounds and it has four different coppers [58]. According to litearature, there are different enzymes that are described and laccase is found in this group [59]. Fungi secrete laccase enzyme initially in case of ligninolytic enzymes production and phenoxy radicals (mediators) are formed after oxidation reaction [60].

1.4.2. Lignin peroxidase

Lignin peroxidase (EC 1.11.1.14) is classified in oxidoreductases family. The enzyme can degrade lignin through H_2O_2 dependent oxidative depolymerization [38]. Lignin peroxidases (LiPs) have high redox capacity and broad substrate utilization. By means of this, LiPs can be used in different industrial areas. Compared to laccase, mediators are not

essential to catalyze high redox potential compounds for LiPs. However, H_2O_2 is necessary for initation of reaction [60].

1.4.3. Ligninolytic Enzyme Activities

Laccase activity is determined through different substrates as ABTS (2,2'-azino-bis(3-ethylbenzenthiazoline-6-sulfonic acid), syringaldazine, guaiacol [61]. Besides, ABTS is the most susceptible substrate for laccase activity [62]. It is oxidized by laccase enzyme and cation radicals will appear as product [63]. These radicals can be measured through spectrophotometer and enzyme activity can be determined.

Lignin peroxidase activity can be measured by spectrophotometrically through diverse substrates as veratryl alcohol, azure B, methylene blue [64-66]. In case of usage veratryl alcohol, veratraldehyde which has absorbance value at 310 nm will be product after oxidation of substrate by lignin peroxidase. Besides, capacity of dye oxidation will determine enzyme activity while dye-based substrates (azure B, methylene blue) are used [66].

1.4.4. Applications of Ligninolytic Enzymes

Ligninolytic enzymes are used in several industrial areas as food, pulp and paper, textile, bioremediation, organic synthesis, cosmetics and so on [60].

In food industry, laccase is used to clearify fruit juice and wine. Also, it provides to improve colour of food and dispose of phenolic compounds [67]. Furthermore, aromatic flavours (vanillin, β-carotene) are produced through others ligninolytic enzymes (LiPs and MnPs) [68, 69].

Delignification capacity of ligninolytic enzymes is their outstanding feature. Therefore, the property is used in industrial areas especially in pulp and paper industry. In that respect, laccase enzyme is frequently used in bleanching process for kraft pulp [70]. Also, LiPs and MnPs are used to remove color of kraft pulps [71, 72]. Due to color removal property, these enzymes are used in textile and dye industries [73, 74].

1.5. RECOMBINANT DNA TECHNOLOGY

Recombinant DNA (rDNA) technology involves changing genetic material (DNA) of an organism through insertion or deletion of gene(s) [75]. There are three main parts as cutting both gene sequence and related vector with restriction enzymes, ligation of DNA fragments through T4 DNA ligase enzyme and transformation of recombinant vector to host via different methods as heat-shock or electroporation [76, 77].

Recombinant DNA technology was started with bacterial hosts but eukaryotic hosts are used frequently right now [78]. There are different bacterial and eukaryotic hosts for rDNA technology. *Escherichia coli, Bacillus subtilis, Pichia pastoris, Saccharomyces cerevisiae* and *Aspergillus niger* are used more frequently [76, 79]. Eukaryotic expression systems are more favored than bacterial ones especially in case of heterologous and extracellular protein expression owing to advantages [80].

Pichia pastoris is an essential host for recombinant protein production. It is more advantages than prokaryotic and other eukaryotic hosts such as reaching high cell density, having high level protein production and secretion capacity, averting bacteriophage contamination, ease of genetic manipulations and providing various post translational modifications such as glycosylation, methylation and acetylation [81].

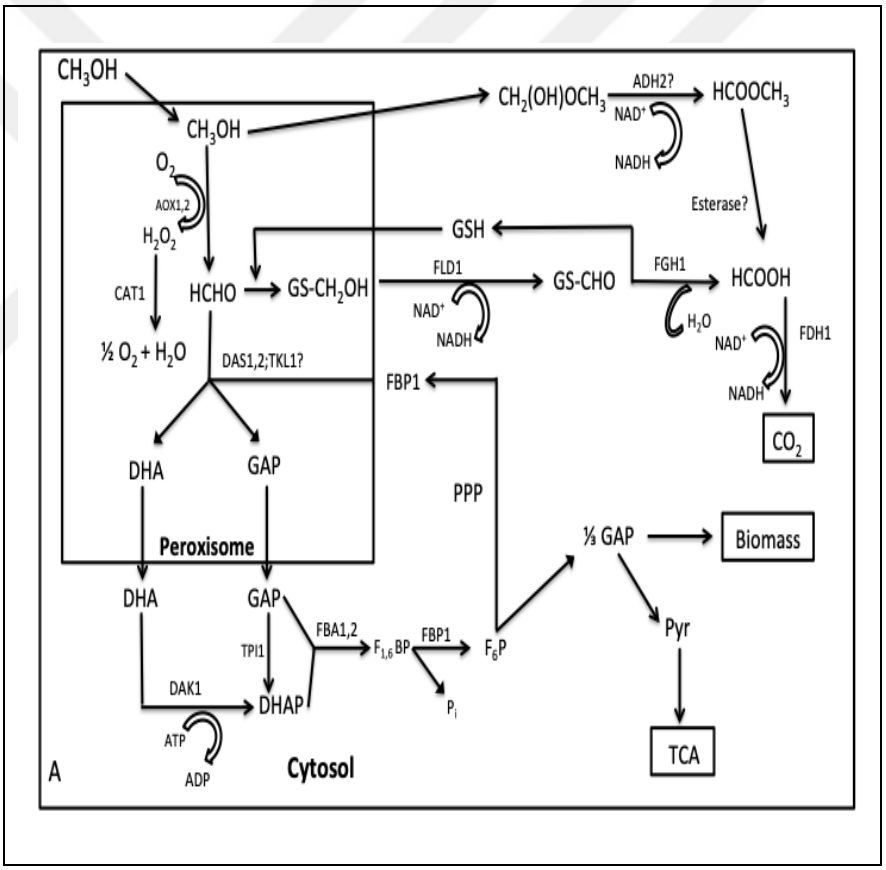
1.6. *PICHIA PASTORIS* EXPRESSION SYSTEM

Methylotrophic yeasts can utilize methanol for carbon and energy source when there is not any repressing carbon source [79, 82]. *Hansenula, Candida, Torulopsis* and *Pichia* are examples for methylotrophic yeast [82]. In 1919, *Pichia pastoris* was isolated from chestnut tree but ability of methanol utilization was discovered after half a century [83].

Pichia pastoris is generally recognized as safe (GRAS) by Food and Drug Administration (FDA) [84]. In 1995, *Pichia pastoris* was moved to new genus known as *Komagataella sp*. because of phylogenetic analyses [83]. *Komagataella pastoris* (ATCC 28485-wild type strain) has 4 chromosome and genome size is 9.6 Mb. Also, there are 5241 coding genes and GC content is 41.5 %. [85].

There are three phenotypes of *Pichia pastoris* according to methanol utilization capacity owing to alcohol oxidase genes (AOX1 and AOX2). Wild type (Mut^+) strain represent that they can grow on methanol. However, Mut^- strains cannot grow on methanol while Mut^S can grow slowly because of deletion in one or both of AOX1 and AOX2 genes [81].

Methanol utilization (MUT) pathway starts with introducing of methanol to peroxisome (Figure 1.4). AOX1 and AOX2 genes provide methanol oxidation. Formaldehyde and hydrogen peroxide are released after the oxidation of methanol. After that, formaldehyde is oxidized to CO_2 or it is altered to dihydroxyacetone and glyceraldehyde-3-phosphate through xylulose-5-phosphate [83, 86].



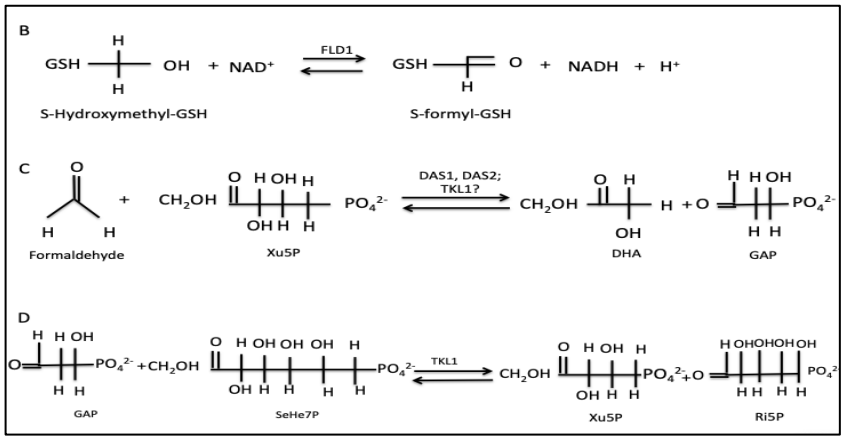


Figure 1.4. Metanol utilization pathway [86]

Wild type strain of *Pichia pastoris* is frequently used owing to yield high production capacity. However choosing the correct strain depends on process and the strains can provide high yield [81].

There are different promoters for *Pichia pastoris* such as AOX1, AOX2, GAP, FLD and PEX8 [81, 87]. Nevertheless, AOX1 promoter is one of the strongest and closely regulated eukaryotic promoters [79]. AOX1 promoter system provide some advantages.

Firstly, methanol is used as an inducer for transcription and transcription level can be regulated. Also, foreign product can be expressed in high concentrations without considering toxic effect. Besides, initial carbon source provide to prevent transcription and so high biomass will be obtained. In addition to these, high concentration of methanol utilization can cause a toxic effect and fire. Also, monitoring of methanol can be difficult due to unreliability of on-line probes [87].

There are different selectable marker genes for *Pichia pastoris* expression strains derived from NRRL-Y11 430 (Northern Regional Research Laboratories, IL, USA). Some of them are biosynthetic as *HIS4*, *ARG4* (argininosuccinate lyase), *ADE1*(PR-amidoimidazole succinocarboxamide synthase) and *URA3* (orotidine 5'-phosphate decarboxylase) while others are drug-resistances markers as kan^R and *Sh ble* genes. *Streptoalloteichus hindustanus* is encoding *Sh ble* gene that has resistance to bleomycin-related drug zeocin [81]. Zeocin is light sensitive and it has optimal selection in low salt medium [88, 89].

Protease enzymes, especially vacuolar proteases, can cause degradation of secreted foreign proteins in *Pichia pastoris* culture [81, 87]. Some features of *Pichia pastoris* can be used to

overcome the problem as growth capacity even low pH (3-7). Besides, decreasing of temperature can prevent protease activity arising from dead cells. There are studies with higher enzyme activity at different temperatures values (30 to 20°C). Optimal pH and temperature values of *Pichia pastoris* fermentations can change according to product [87].

Also, extra substrates (e.g. casamino acid, peptone) can be added to fermentation medium and these substrates can repress protease expression because of nitrogen limitation [87]. Furthermore, protease-deficient strains can be used to prevent protease degradation (Table 1.5) [81].

Table 1.5. Protease deficient strains of *Pichia pastoris* [81, 83]

Strain	**Genotype**	**Phenotype**
SMD1163	his4 pep4 prb1	$Mut^{+}His^{-}Prot^{-}$ (A^{-}, B^{-}, $CarbY^{-}$)
SMD1165	his4 prb1	$Mut^{+}His^{-}Prot^{-}$(B^{-})
SMD1168	his4, ura3, pep4::URA3	(A^{-}, B^{S}, $CarbY^{-}$)

Two genes (pep4 and prb1) are deleted to overcome protease activity [81, 83]. Proteinase A is a vacuolar aspartyl protease and it is encoded by pep4. Proteinase A is essential for activation of carboxypeptidase Y and proteinase B that are also vacuolar protease. Proteinase A provides to activate about half of proteinase B that is encoded by prb1 gene, so deletion of pep4 gene will affect two types of proteases [81, 87].

An important feature of *Pichia pastoris* is the ability of integration of expression cassette into the genome through homologous recombination. Alcohol oxidase 1 locus is the most attractive target for chromosomal integration since high expression levels and tight regulation through AOX1 promoter can be achieved via methanol induction [90].

1.7. AIM OF THE STUDY

The aim of this thesis is to produce commercially relevant pectinolytic and ligninolytic enzymes through eukaryotic host, *Pichia pastoris* using recombinant DNA technology with high production yield. In this context, upstream workflow for industrial biotechnology from -*de novo* gene synthesis to production and characterization of enzymes in laboratory scale- is set-up and completed.

2. MATERIALS

2.1. STRAINS

E. coli DH5-α

Pichia pastoris SMD1168H

2.2. KITS

TaKaRa BCA Protein Assay Kit (Catalog Number: T9300A)

Macherey-Nagel Nucleospin Plasmid (Catalog Number: 1809/002)

Macherey-Nagel Nucleospin Gel and PCR Clean-Up (Catalog Number: 1705/005)

2.3. CHEMICALS

6X DNA loading dye, acetic acid with glacial, agarose, ammonium persulfate, ammonium sulfate, ampicillin sodium salt, biotin, calcium chloride, citrus pectin, coomassie blue, D-(+)-glucose, D-galacturonic acid, dithiothreitol (DTT), EcoRI (100,000 units/mL), ethanol, ethidium bromide, generuler™ DNA ladder mix, glycerol, HEPES, hydrochloric acid, laemmi buffer, liquid nitrogen, luria bertani agar, luria bertani broth, magnessium sulfate, methanol, nucleasefree water, page ruler prestained protein ladder, peptone (bacteriological), phenol:chloroform (25:24), PmeI (10,000 units/mL), potassium phosphate dibasic, potassium phosphate monobasic, pPICz-α A, SOC medium (15544034), sodium acetate, sodium chloride, sodium dodecyl sulfate, sodium hydroxide, sodium nitrate, sorbitol, T4 DNA ligase, TEMED, tris acetate-edta buffer, tris base, twist plasmid, XbaI (100,000 units/mL), yeast extract, yeast nitrogen base without amino acids, zeocin.

2.4. LABORATORY EQUIPMENTS AND DEVICES

2.4.1. Equipments

96 well plate, bottles (50 mL, 100 mL, 250 mL, 500 mL, 1000 mL), cryo tubes, electrophoresis cuvettes, electrophoresis tanks, erlenmeyer flasks (100 mL, 250 mL, 500 mL, 1000 mL), eppendorf tubes (1.5 mL, 2 mL), falcon tubes (50 mL, 250 mL), graduated cylinders (25 mL, 50 mL, 100 mL, 500 mL, 1000 mL), inoculation loops, micropipettes (1-10 µL, 10-100 µL, 20-200 µL, 100-1000 µL), micropipette tips (10 µL, 200 µL, 1000 µL), petri dishes, serological pipettes (5 mL, 10 mL, 25 mL), spreader, suction bulbs.

2.4.2. Devices

Autoclave, balance (Shimadzu), Biorad genepulser II, centrifuge (Beckman Coulter, Eppendorf™ 5424, Gyrozen 1580R), fridge (Arçelik), incubator (Binder), incubator shaker (Sartorius stedim), laminar flow, magnetic stirrer (Benchmark), microwave, Minifors bioreactor (3L), spectrophotometer, pH-meter (Mettler Toledo), transilluminator, vortex (Scilogex MX-S), water bath (Grant SUB Aqua 12 Plus), water source (Sartorius).

3. PROCEDURE

There are two main parts of this study as (i) molecular biology work including cloning and transformation and (ii) fermentation at erlenmeyer and bioreactor scale. Figure 3.1. shows the diagram for the workflow followed in this thesis. According to diagram, there are essential steps for recombinant production (strain design, gene synthesis, transformation, selection, cloning and fermentation) and characterization (enzyme activity and SDS-PAGE) of pectinolytic and ligninolytic enzymes.

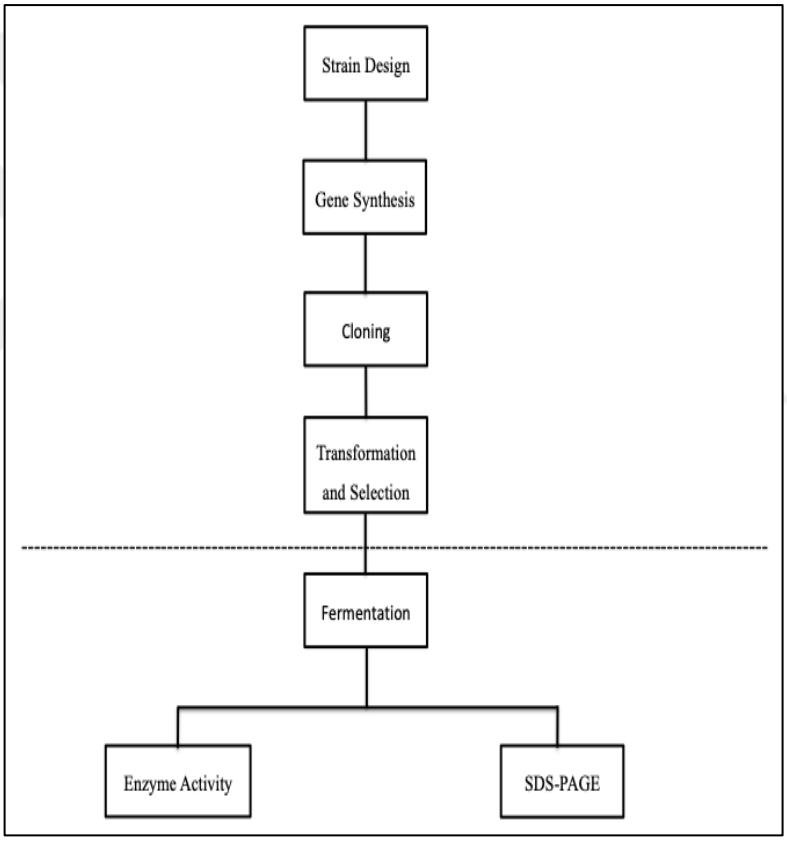


Figure 3.1. The workflow followed in this thesis. The dashed line separates the two main parts of this work, namely molecular biology and fermentation.

3.1. CLONING OF POLYGALACTURONASE, PECTIN METHYL ESTERASE, PECTIN LYASE, LACCASE AND LIGNIN PEROXIDASE GENES TO *PICHIA PASTORIS*

3.1.1. Gene Synthesis

The sequences of the pectinolyitc and ligninolytic enzymes are obtained from NCBI database. The list is provided in Table 3.1.

Table 3.1. Properties of genes

Enzymes	Donor organism	Length (bp)	GC Content (%)	Accession Nr
PGase	*Enterobacter*	1326	54	AGN88055.1
PME	*Bacillus pumilis*	981	43	ABV62479.1
pelC	*Bacillus subtilis*	666	44	936594
Lac	*Trametes versicolor*	1560	63	KR492189.1
LP	*Phanerochaete chrysosporium*	1119	62	AAA53109.1

The above genes are *de novo* synthesized with adding EcoRI (GAATTC) and XbaI (TCTAGA) restriction enzyme recognition sites as flanking sequences. Synthesized genes are delivered on company-plasmids at 1000 ng/µL and need further dilution to 10 ng/µL using 100 µL TE buffer.

3.1.2. Competent Cell Preparations for Bacterial Transformation

E.coli cells were streaked onto LB agar plates and incubated at 37°C for overnight. A single colony from the plate is aseptically transferred as inoculum to 5 mL LB broth in 15 mL falcon and further incubated at 37°C and 150 rpm for overnight. From this culture, 2 mL is mixed with 200 mL fresh LB broth and incubated until the OD_{600} reached 0.35-0.45. Cells were then transferred to sterile 50 mL falcons and incubated on ice for 5 minutes. Cells were centrifuged at 2000 g and 4°C for 10 minutes. Pellet was then resuspended in

20 mL ice cold improved buffer (15% glycerol and 70 mmol/L $CaCl_2$) and incubated on ice for 5 min.

The culture is then centrifuged twice at 1000 g and 4°C for 6 min. Supernatants were discarded and 4 mL ice cold improved buffer was used to resuspend pellets. Finally, the cells were aliquoted as 200 µL frosted through liquid nitrogen and stored at -80°C.

3.1.3. Bacterial Transformation for Twist Plasmids

Twist plasmids are multipled by transforming those into *E.coli* using heat shock by combining 50 µL competent cells with 5 µL plasmids on ice and the workflow on Table 3.2.

Table 3.2. Heat shock method for transformation

Temperature	**Time**
On ice	30 minutes
42 °C	50 seconds
On ice	2 minutes

Finally, the volumes is completed to 1 mL with SOC medium and cells were incubated at 37°C and 150 rpm for 1 h 45 minutes. Cells were harvested at 3000 g for 5 minutes by centrifugation. 800 µL of supernatant was discarded and pellets were dissolved with remaining 200 µL reactions. 100 µL cells were poured to each LB plates+amp (100 µg/mL) and the plates were incubated at 37°C overnight.

3.1.4. Plasmid Isolation

Twist plasmids were isolated from *E.coli* cells by using NucleoSpin Plasmid kit of Macherey Nagel following the kits procedure. Isolated plasmids were stored at -20°C.

3.1.5. Double Digestion Protocol

Double digestion reaction was applied to Twist and pPICz-α A plasmids according to the manufacturers recommendations. The reaction consists of 1 µg DNA, 1X NEB buffer, 10 units/µL of XbaI and EcoRI. Volume of the reaction was completed to 50 µL with nuclease free water. The tube containing reaction mixture was incubated at 37°C for 30 minutes, followed by heat inactivation at 65°C for 10 minutes.

3.1.6. Gel Electrophoresis and Gel Extraction

1 g agarose gel was combined with 100 mL TAE buffer and boiled in microwave. Ethidium bromide was added to the solution to final concentration of 1.5%. The solution solidifies on the electrophoresis cassette at room temperature. Products of double digestion reaction (50 µL) and 6X loading dye (10 µL) were combined and loaded to the gel with a marker. Electrophoresis was run at 100 V for 30 minutes. The desired bands are then extracted from the gel according to NucleoSpin Gel and PCR Clean-up kit of Macherey-Nagel.

3.1.7. Ligation Protocol

Digested genes and pPICz-α A plasmid were ligated according to NEB ligation protocol (Table 3.3). Reaction tube was incubated at 16°C overnight and followed by heat inactivation at 65°C for 10 minutes.

Table 3.3. NEB Ligation protocol [91]

Samples	**Concentrations**
T4 DNA ligase buffer	2 µL
Vector DNA	50 ng
Insert DNA	37.5 ng

T4 DNA ligase	1 µL
Nuclease free water	up to 20 µL

3.1.8. Bacterial Transformation and Plasmid Isolation for Ligation Products

Ligation reaction mixture (5 µL) is transformed into *E.coli* cells (50 µL) using heat shock. As positive control, uncut pPICz-α A vector (1.7 µL) are transformed into competent cells (100 µL). The cells were spreaded onto LB agar + zeocin (25 µg/mL) covered with aluminum folio and incubated at 37°C overnight.

Ligation plasmids were isolated from *E.coli* cells by using NucleoSpin Plasmid kit of Macherey Nagel. Isolated plasmids were stored at -20°C.

3.1.9. Linearization of Ligation Plasmids

pPICz-α A plasmids that contain genes were linearized with using PmeI restriction enzyme after the isolation of plasmids. The reaction was incubated at 37 °C for 1 hour 30 minutes. Heat inactivation was applied for 20 minutes at 65°C. 5 µL of the reaction was used for gel electrophoresis to check linearization.

Table 3.4. Linearization protocol

Samples	**Concentrations**
10X Buffer B	5 µL
DNA	0.5-1 µg/µL
PmeI	2,5 µL
Nuclease free water	up to 50 µL

3.1.10. Phenol Chloroform Extraction and Ethanol Precipitation

1:1 phenol:chloroform (25:24) pH:8 was added to linearization tubes and vortexed. Upper phase of solutions were removed carefully and put into the new tubes after they were centrifuged at 2000 rpm for 5 minutes. 3 M sodium acetate buffer (0.1 fold) and 100% ethanol (2.5 fold) were added to tubes. The tubes were incubated at -20°C overnight and then centrifuged at 10.000 rpm and 4°C for 10 minutes. Supernatants were discarded and 80% cold ethanol (20 µL) was added. Ethanol was evaporated and nuclease free water was added to tubes. Tubes were stored at -20°C after their concentrations were measured via spectrophotometer.

3.1.11. Preparation of Electrocompetent *P. pastoris* Cells

Pichia pastoris cells were streaked to YPD plates and incubated at 30°C overnight. A single colony from the plate is transferred as inoculum to 10 mL YPD broth in 50 mL falcon and further incubated at 30°C and 150 rpm for overnight. Main culture is started YPD broth (200 mL) as beginning OD_{600} is around zero and incubated at 30°C and 150 rpm for overnight. When OD_{600} of culture was around 1.3-1.5 and cells were harvested at 2000 g for 10 minutes at 4°C and supernatants were discarded. YPD medium/HEPES (40 mL) and 1 M DTT (1 mL) were used to resuspend the pellets and tubes were incubated at 30°C for 15 minutes. Volume was brought to 200 mL with ice cold sterile water and centrifugation was done at 2000 g and 4°C for 10 minutes. Pellets were resuspended with ice cold water (100 mL) and centrifugation step was repeated. After the supernatants were discarded, ice cold 1 M sorbitol (8 mL) was used to dissolve pellets. Centrifugation step was repeated for one more time and pellets were dissolved with 1 M sorbitol (0.2 mL). The tubes were incubated on ice until use.

3.1.12. Yeast Transformation Protocol

Electroporation method is used to transfer the plasmids to *Pichia pastoris*. Linearized plasmids (100 ng) and electrocompetent *Pichia pastoris* cells (40 µL) are combined in tubes on ice. Samples were transferred to electroporation cuvettes (0.2 cm gap) and

cuvettes are incubated on ice for 5 minutes. Parameters of the protocol is 1.5 kV, 200 Ω and 25 μF. After electroporation, 1 M ice cold sorbitol (1 mL) was added to cuvettes immediately and cells were transferred to 15 mL sterile falcons. Falcons were incubated at 30°C and 150 rpm for 2 hours. Following the incubation, the samples were poured to each YPDS + zeo plates (100 μg/mL). Plates were incubated at 30°C until colonies appeared. A single colony was streaked to new YPDS + zeo plates and incubated at 30°C until get single colonies.

3.1.13. Glycerol Stock Preparations

BMGY medium (25 mL) is incubated with a single colony of *Pichia pastoris* strains with linearized plasmids in erlenmeyer flask (250 mL) at 30°C for overnight until the OD_{600} reached between 2 and 6. When the OD_{600} was between 2 and 6, 800 μL cells and 200 μL 30% glycerol were combined in sterile cryo tubes. Tubes were stored at -80°C.

3.1.14. SDS-PAGE

Resolving gel (12%) and stacking gel (5%) are prepared with 30% acrylamide bis acrylamide, 10% SDS, 10% APS, 1.5 M Tris-HCl pH 8.8, 1 M Tris-HCl pH 6.8, TEMED and double distillated water. 2X laemmli buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue and 0.125 M Tris HCl, pH 6.8) and supernatants (1:1) are combined and boiled at 95°C for 7 minutes to denature proteins.

The gel is run at 200 V for 10 minutes (stacking gel) and 300 V for 45 minutes (resolving gel). Gels were incubated with coomassie blue (0.1% commassie blue dye, 50% methanol, 10% glacial acetic acid and 40% dH_2O) at RT and 40 rpm for 40 minutes. Gel is washed with distilleted water and incubated with destaining solutions for overnight. They are visualised with ChemiDoc instrument on the following day.

3.2. FERMENTATION AND ANALYTICAL METHODS

3.2.1. Growth Curve for *Pichia pastoris*

BMGY (25 mL) medium were inoculated with a single colony of *Pichia pastoris* with plasmids and incubated at 30°C for overnight until the OD_{600} reached to between 2 and 6. Cells were harvested by centrifugation and supernatants were discarded. The pellets were washed with BMMY (25 mL) medium and culture was centrifuged. Supernatant is discarded and pellet is solved with BMMY (25 mL) medium. Main culture is started using BMMY (100 mL) with 2% inoculum in flask (500 mL). Initial OD_{600} of main culture is around 0.02. OD_{600} is measured every three hours and promoter is induced with 1% pure methanol every 24 hours during 2 days.

3.2.2. Gene Expression

Main culture is started with an initial OD_{600} of 1 in BMMY (150-200 mL) medium in 1 L flasks. Pure methanol (1%) is provided every 24 hours and samples are taken every 12 hours during 4 days.

3.2.3. Bench Top Fermentor (3L) Cultivation

Inoculum (3%) is started in BMGY (100 mL) medium and incubated at 30°C overnight. Main culture is started with 3% inoculum (50 mL) in 3 L bioreactor with 1.5 L working volume that has sterilized 1450 mL BMMY medium and 1 mL antifoam after washing step. The pH of the medium is controlled at 6 with 3 M HCl and 30% ammonium hydroxide. Aeration rate is 1 vvm/L and temperature is arranged to 28°C. Pure methanol is used to induce promoter.

3.2.4. Total Protein Determination

Total protein amount is determined with TaKaRa BCA kit. Standard curve is plotted using gradually decreasing concentrations of 2 μg/mL bovine serum albumin (BSA). Supernatants (25 μL) and working solutions (200 μL, provided with kit) are mixed and

incubated at 37°C for 30 minutes. Optical densities are measured at 562 nm with spectrophotometer in 20 minutes.

3.2.5. Ammonium Sulfate Precipitation and Dialysis

Supernatants are mixed to dissolve ammonium sulfate (80% w/v) at 4°C overnight. Samples are centrifuged at 6000 rpm and 4°C for 30 minutes. Pellets are dissolved with 0.1 M sodium acetate buffer pH 5 and samples are poured into membranes for dialysis against 0.1 M sodium acetate buffer and incubated at 4°C overnight.

3.2.6. Ultrafiltration

Proteins of main culture are seperated by passing the supernatant through Amicon Ultra centrifugal filter unit at 10.000 rpm for 10 minutes. Serial filtration of the samples are carried out with filter units of different MWCO values (100, 30 and 3 kDa). All samples are stored at 4°C for further studies.

3.2.7. Enzyme Activity Assays for Pectinolytic Enzymes

3.2.7.1. Pectin Lyase Activity

Pectin lyase activity is determined via galacturonic acid measurement. Pectin (0.5 %) is solved in 0.05 M Tris-HCl buffer (pH 8.0). Supernatant (0.5 mL) and pectin (1 mL) are combined and incubated at 30°C for 1 hour then 0.5 M HCl (3.5 mL) is added to tubes immeaditely. Measurements are taken at 235 nm through spectrophotometer. Blank tubes has 0.5 M HCl at the beginning of the reaction.

3.2.7.2. Polygalacturonase Activity

Polygalacturonase activity is determined via 3,5-Dinitrosalicylic acid (DNS). Supernatant (0.5 mL), 0.5 % pectin (1 mL) and 0.1 M sodium acetate buffer pH 4.2 (0.5 mL) are incubated at 30°C for 10 min. DNS (2 mL) are added to reaction and incubated at 95 °C for

15 minutes. Measurements are taken at 545 nm through spectrophotometer after tubes are cooled to RT.

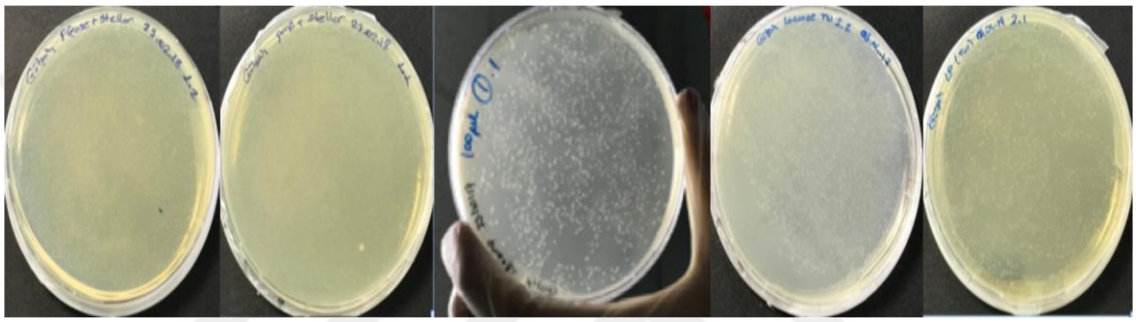
3.3. APPLICATION OF ENZYMES FOR FOOD INDUSTRY

Citrus peels (3 g) were incubated with two enzyme blends as pectinolytic enzymes, pectinolytic and ligninolytic enzymes at different temperatures (30 °C, 37 °C and 50 °C). Distilled water was used as control.

4. RESULTS

4.1. BACTERIAL TRANSFORMATION FOR TWIST PLASMIDS

The plasmids containing target enzymes were transferred to *E. coli*. After 24 h, colonies were observed in LB+amp plates (Figure 4.1).



a b c d e

Figure 4.1. Transformed *E. coli* cells containing Twist plasmids with a) PGase, b) PME, c) pelC, d) Lac and e) LP

4.2. PLASMID ISOLATION

The *E. coli* cells with the corresponding plasmids were cultivated at 5mL and the plasmids are isolated using Plasmid isolation kit. Concentrations of the plasmids were measured by spectrophotometer against elution buffer as blank (Table 4.1).

Table 4.1. Concentrations of isolated Twist plasmids and pPICzα-A

Tube Numbers	Concentration (ng/ µL)	Purity (A260/280)
Twist + PGase 1	160.7	1.88
Twist + PGase 2	180.7	1.90
Twist + PGase 3	168.3	1.88
Twist + PGase 4	127.0	1.87

Twist + PME 1	68.5	1.85
Twist + PME 2	78.8	1.84
Twist + PME 3	93.1	1.83
Twist + PME 4	75.5	1.80
Twist + pelC 1	244.4	1.81
Twist + pelC 2	211.2	1.82
Twist + pelC 3	257.0	1.83
Twist + pelC 4	190.3	1.82
Twist + LP 1	136.4	1.88
Twist + LP 2	128.4	1.88
Twist + LP 3	144.7	1.88
Twist + LP 4	150.2	1.86
Twist + LAC 1	139.4	1.87
Twist + LAC 2	157.8	1.87
Twist + LAC 3	116.4	1.85
Twist + LAC 4	157.2	1.89
pPICzα-A 1	83.1	1.88
pPICzα-A 2	89.4	1.89
pPICzα-A 3	81.2	1.88
pPICzα-A 4	89.3	1.82

Polygalacturonase (tube number 1 and 2), pectin methyl esterase (tube number 2 and 3), pectin lyase (tube number 1 and 3), lignin peroxidase (tube number 3 and 4), laccase (tube number 2 and 4) and pPICzα-A (tube number 2 and 4**)** were chosen for further experiments.

4.3. DOUBLE DIGESTION

Isolated Twist plasmids and pPICz-α A vector are double digested with EcoRI and XbaI. Resulting products and the marker were loaded to 1% agarose gel (Figure 4.2).

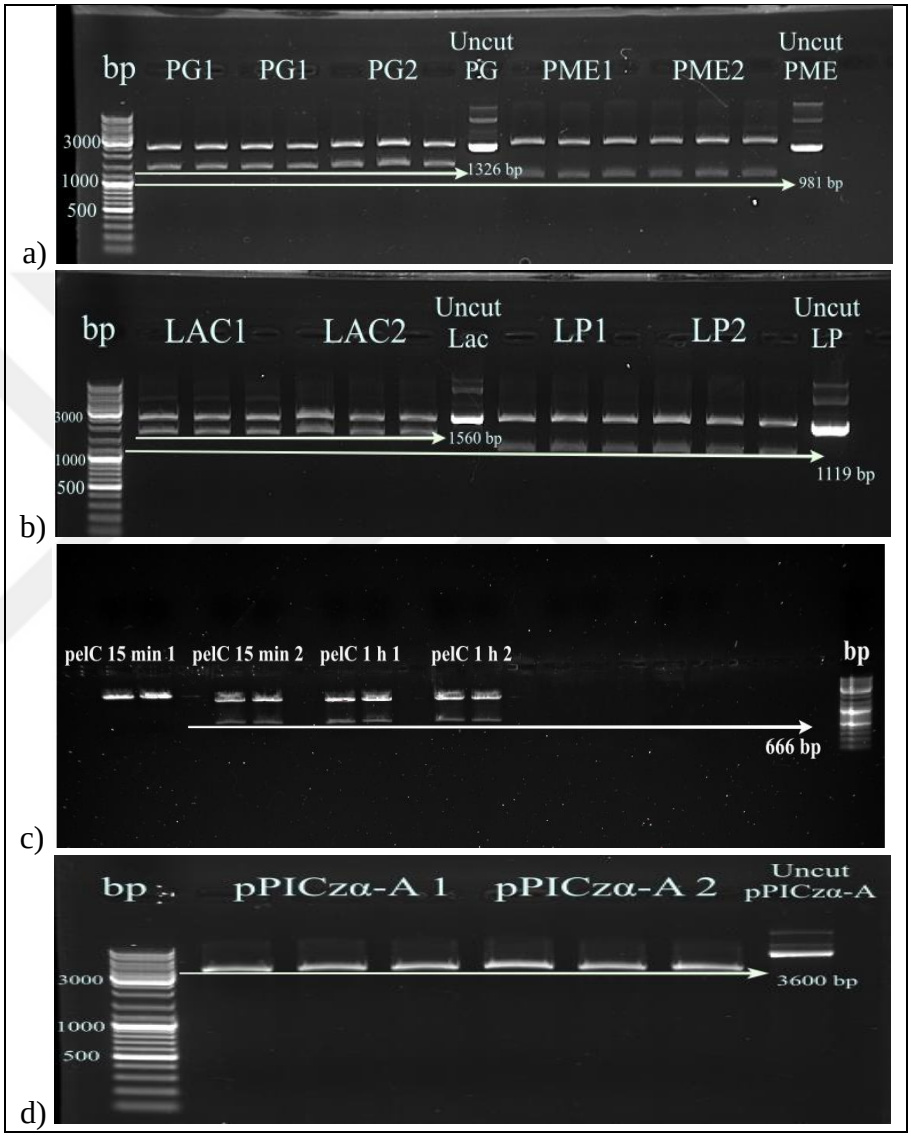


Figure 4.2. Gel electrophoresis results for double digestion protocol of Twist plasmids with a) PGase, PME and uncut plasmids b) Lac, LP and uncut plasmids c) pelC d) pPICzα-A and uncut plasmid

4.4. GEL EXTRACTION

After the electrophoresis, bands with target genes or pPICzα-A were extracted from gel and their concentrations are determined spectrophotometrically against elution buffer as blank (Table 4.2)

Table 4.2. Concentrations of DNA after gel extraction

Tube Number	Concentration (ng/µL)	Purity (A260/280)
PGase 1	10.1	1.68
PGase 2	9.8	1.72
PME 1	9.3	1.87
PME 2	6.7	1.63
pelC 1	12.0	1.86
pelC 2	9.1	1.66
LAC 1	6.7	1.64
LAC 2	11.1	1.65
LP 1	6.7	1.65
LP 2	8.1	2.08
pPICzα-A 1	11.2	1.91
pPICzα-A 2	9.3	1.91

Polygalacturonase (tube number 1), pectin methyl esterase (tube number 1), pectin lyase (tube number 1), lignin peroxidase (tube number 2), laccase (tube number 2) and pPICzα A (tube number 1) were chosen for further experiments.

4.5. LIGATION

After ligation protocol, plasmids are transferred into *E. coli* through heat shock method and they are grown on LB+zeo agars (Figure 4.3).

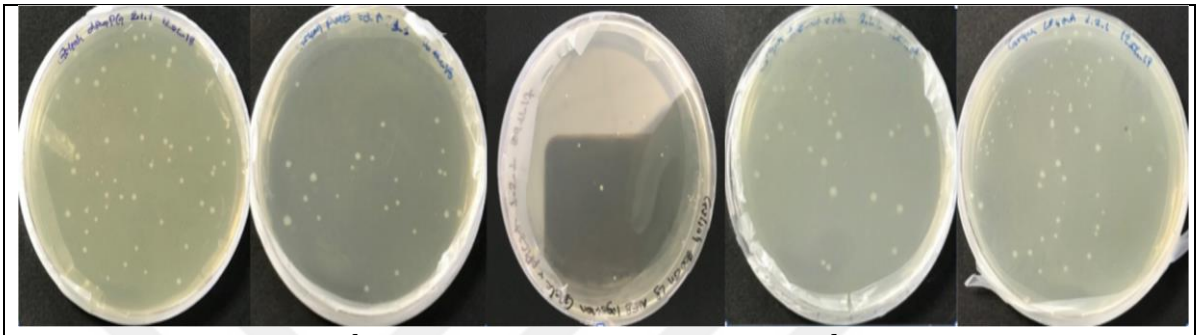


Figure 4.3. Transformed *E. coli* cells containing pPICzα A with a)PGase, b)PME, c) pelC, d) Lac and e) LP

Upon cultivation, the plasmids are isolated and their concentrations are determined (Table 4.3).

Table 4.3. Concentration of ligation plasmids

Tube Numbers	Concentration (ng/ µL)	Purity (A260/280)
pPICzα A+PGase 1	58.5	1.81
pPICzα A+PGase 1	54.6	1.80
pPICzα A+PGase 4	53.6	1.84
pPICzα A+PME 1	72.2	1.79
pPICzα A+PME 2	60.2	1.80
pPICzα A+PME 3	46.7	1.78
pPICzα A+pelC 1	27.8	1.72
pPICzα A+pelC 2	21.2	1. 76
pPICzα A+LAC 1	66.8	1.82
pPICzα A+LAC 2	48.3	1.80

pPICzα A+LAC 3	68.3	1.83
pPICzα A+LP 1	71.7	1.84
pPICzα A+LP 2	88.8	1.83
pPICzα A+LP 3	80.1	1.83
pPICzα-A 1	91.0	1.83
pPICzα-A 2	58.7	1.84

Polygalacturonase (tube number 1 and 2), pectin methyl esterase (tube number 1 and 2), pectin lyase (tube number 1), lignin peroxidase (tube number 2 and 3) laccase (tube number 1 and 3) and pPICzα-A (tube number 1) were chosen for further experiments.

4.6. LINEARIZATION

Plasmids are linearized PmeI restriction enzyme to be further transformed to *Pichia* (Figure 4.4).

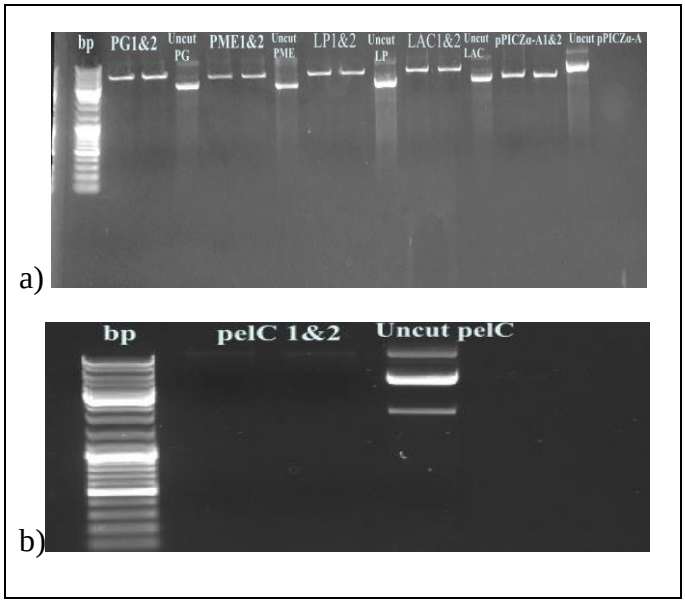


Figure 4.4. Gel electrophoresis result after linearization of a) pPICzα-A plasmids with PGase, PME, Lac, LP, and non linearized plasmids b) pPICzα-A plasmid with pelC and non linearized plasmid

Phenol chloroform extraction and ethanol precipitation methods were applied to linearized plasmids and their concentrations are determined (Table 4.4)

Table 4.4. Concentration of linearized plasmids

Tube Numbers	Concentration (ng/µL)	Purity (A260/280)
pPICzα A+PGase 1	53.0	1.52
pPICzα A+PGase 2	45.5	1.51
pPICzα A+PME 1	25.8	1.49
pPICzα A+PME 2	92.6	1.56
pPICzα A+pelC 1	458.0	1.78
pPICzα A+pelC 2	592.4	1.71
pPICzα A+LP 1	445.7	1.93
pPICzα A+LP 2	50.9	1.51
pPICzα A+LAC 1	36.1	1.53
pPICzα A+LAC 2	172.1	1.65
pPICzα-A 1	83.6	1.56
pPICzα-A 2	65.5	1.54

Polygalacturonase (tube number 1), pectin methyl esterase (tube number 2), pectin lyase (tube number 1), lignin peroxidase (tube number 1) laccase (tube number 2) and pPICzα-A (tube number 1) were chosen for further experiments.

4.7. ELECTROPORATION

Linearized plasmids were transformed to *Pichia* by electroporation. The transformants are cultivated on YPDS+zeo (100 µg/mL) agars at 30°C until colonies appeared (up to 3-4 days). Single colonies are selected from plates (Figure 4.5) then streaked to new YPDS+zeo (100 µg/mL) agars and incubated at 30°C until single colonies appeared (around 2-3 days).

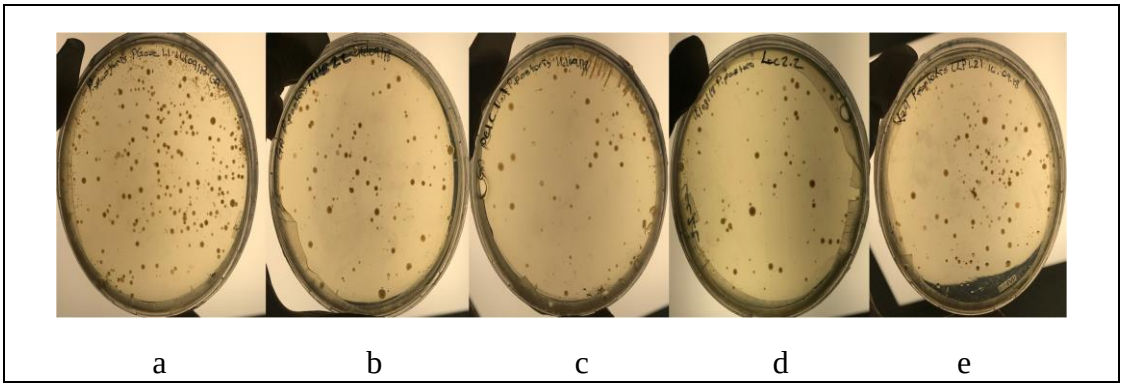


Figure 4.5. Colonies of *Pichia pastoris* with pPICzα-A plasmid and inserts a)PGase, b) PME, c) pelC, d) Lac, e) LP

4.8. GROWTH CURVES FOR *PICHIA PASTORIS*

Growth curves for *Pichia pastoris* SMD1168H strain was constructed using data for 48 hours for all constructs (Figure 4.6), using culture media (BMMY) as blank. Expectedly, the growth profiles for all enzymes are similar.

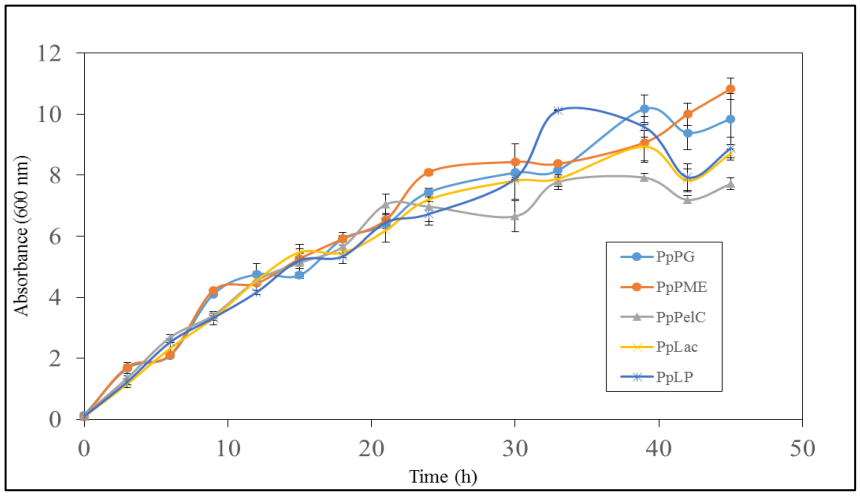


Figure 4.6. Growth curves with inserts *Pichia pastoris* SMD 1168H

4.9. DETERMINATION OF TOTAL PROTEIN

Protein concentrations were decreased from beginning to end of the fermentation according to Figure 4.7.

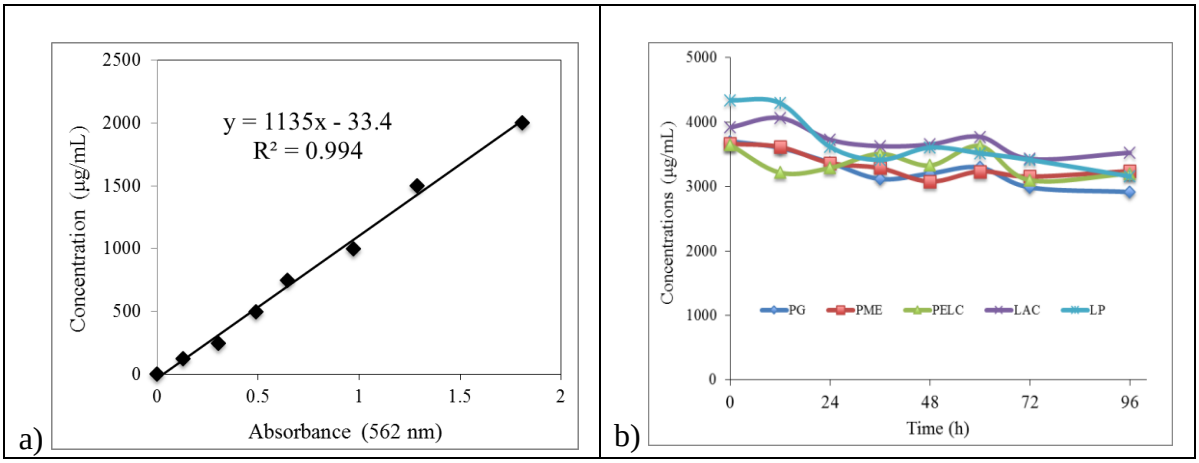


Figure 4.7. a) BSA standard curve and b) protein concentrations

4.10. SDS-PAGE

Theoretical molecular weight of polygalacturonase gene is 47 kDa, pectin methyl esterase is 36 kDa, pectin lyase is 24 kDa, laccase is 55 kDa and lignin peroxidase is 39 kDa. The results were as expected (Figure 4.8).

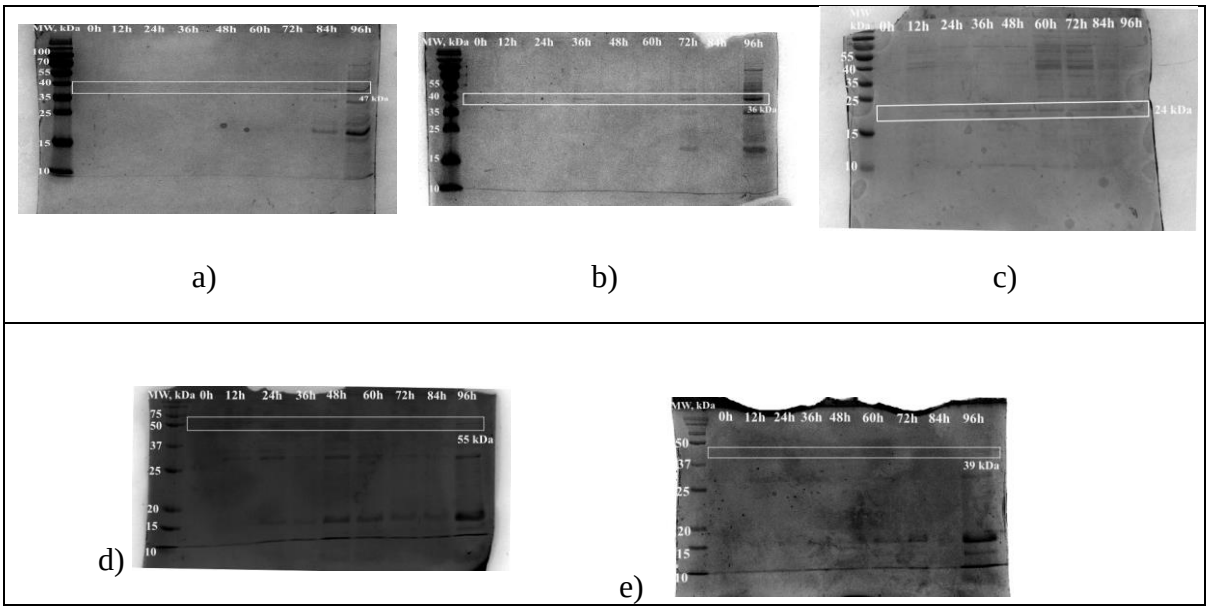


Figure 4.8. SDS-PAGE results for a)PGase, b)PME, c)pelC, d)Lac and e)LP

4.11. ENZYME ACTIVITY

4.11.1. Pectin Lyase Activity Assay

Different galacturonic acid concentrations were used plot standard curve for calculation of pectin lyase enzyme activity. According to pectin lyase activity calculation, highest enzyme activity is at 48th h (Figure 4.9).

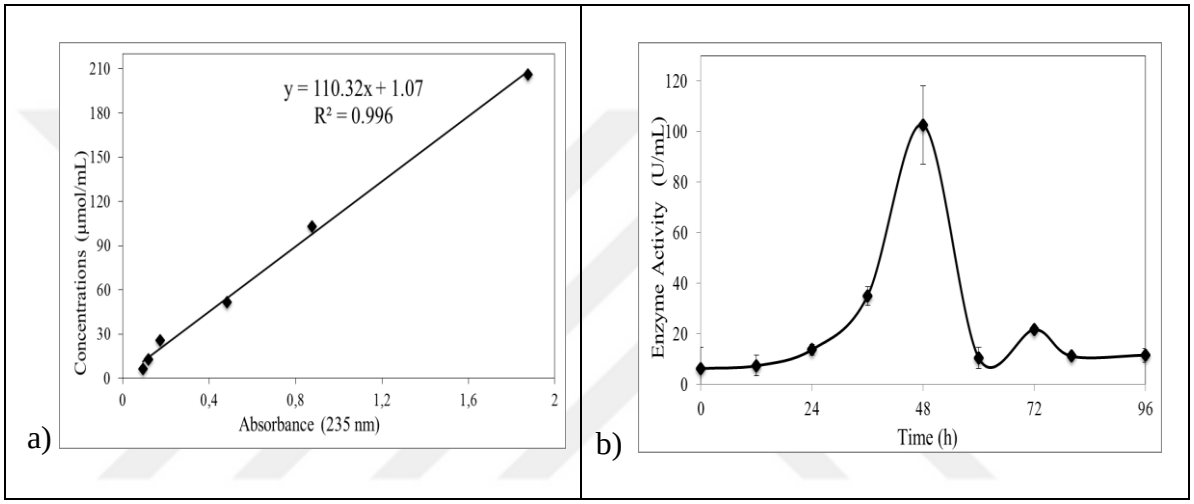


Figure 4.9. a) Galacturonic acid standard curve and b) pectin lyase enzyme activity

4.11.2. Polygalacturonase Activity Assay

Polygalacturonase activity was found through DNS method (Figure 4.10). Also, ultrafiltration method was used to find purified polygalacturonase activity (Figure 4.10). MW of PGase is 47 kDa and the enzyme activity was increased 2.5 fold through ultrafiltration (Figure 4.10).

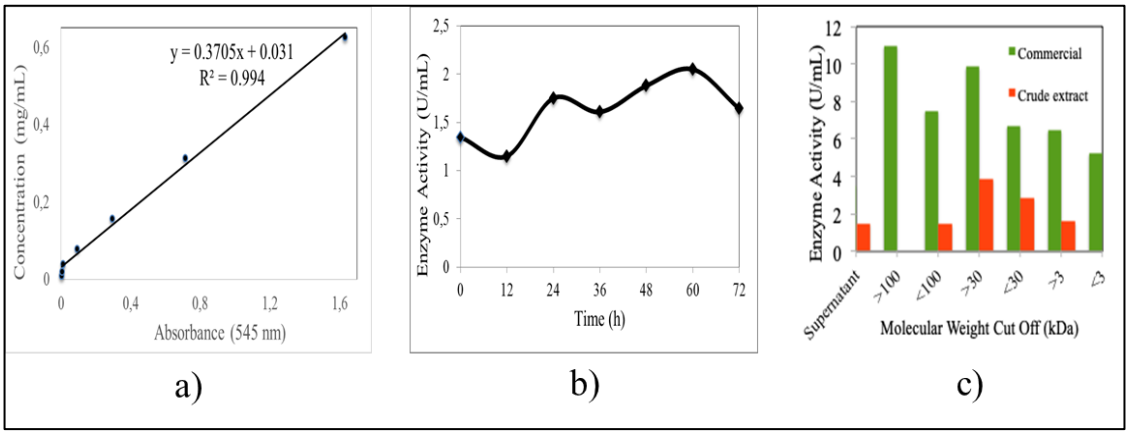


Figure 4.10. a) Standard curve for DNS protocol, b) polygalacturonase activity c) polygalacturonase activity after concentrated

4.12. BENCH TOP FERMENTOR (3L)

Fermentation was occured with *Pichia pastoris* (pectin lyase insert) in 3L bench top fermentor during 5 days. OD_{600} value was increasing after 1% methanol induction and highest OD_{600} value was found 21 (Figure 4.11).

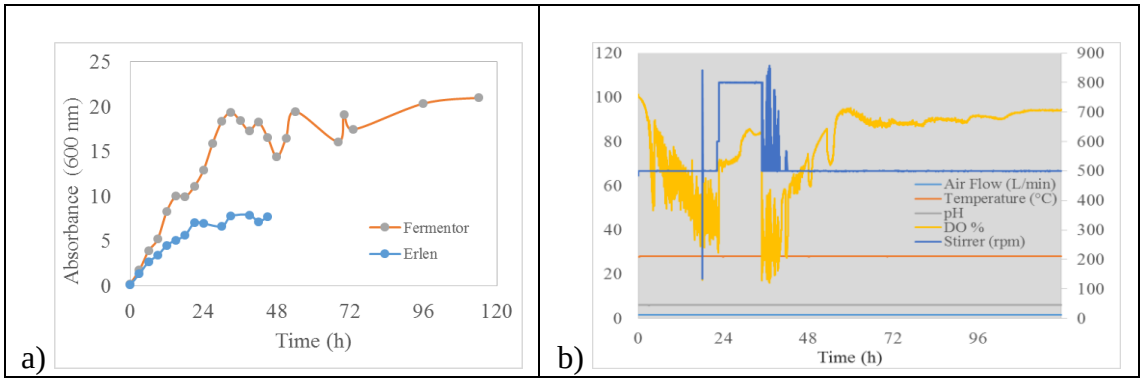


Figure 4.11. Bench top fermentor results for *Pichia pastoris* (SMD 1168H) with pelC gene a) growth curve, b) fermentor parameters

4.13. APPLICATION OF ENZYMES

Enzyme blends were used to see enzymes activities on citrus peels. Upper parts of figure show before incubation while bottom part is after incubation at different temperatures. The order is control, pectinolytic enzymes and combination of pectinolytic and ligninolytic enzymes. Also, OD545 measurements were shown in Figure 4.12.

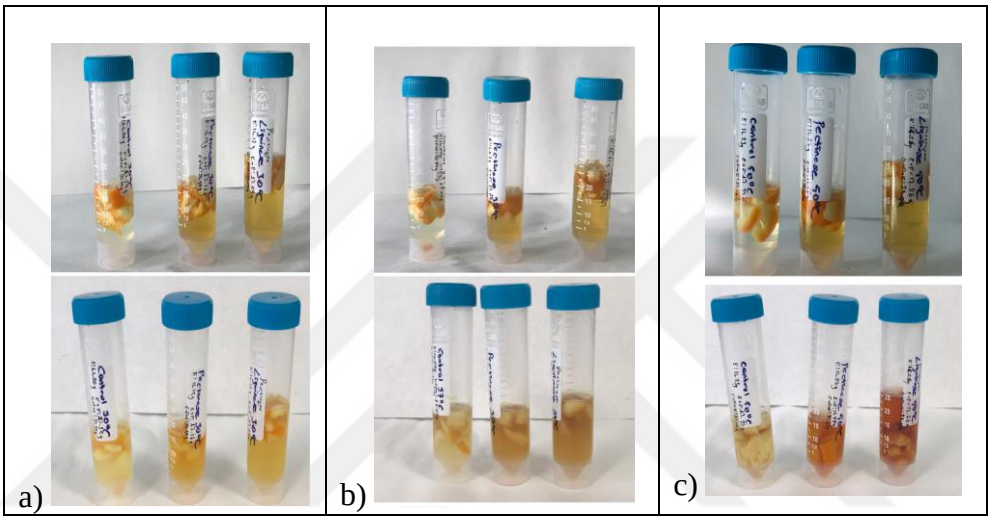


Figure 4.12. Application of enzymes on citrus peel at; a) 30°C, b) 37°C and c) 50°C

5. DISCUSSION

There are mainly two parts of the study as cloning and production. At the beginning, sequence of the genes must be obtained, either by amplifying these from a donor genome by PCR or by *de novo* synthesis. In this thesis, *de novo* synthesis was used, since it allows bypassing codon bias as well as practical problems during PCR [92], decreasing thereby untimately time and cost of process if run routinely.

EcoRI and XbaI sites were added to gene sequences before synthesizing for genetic manipulations as digestion and ligation. There are some important points for choosing correct restriction enzymes. Firstly, the restriction enzymes must not cut gene sequence otherwise related product will not be expressed. Also, the enzymes must be found in multiple cloning site of the plasmid that will transformed to host (*Pichia pastoris*) for occuring flanking regions to ligation protocol.

Synthesized genes, indivually cloned on Twist plasmids, were transformed to competent *E. coli* (DH5α). There is an incubation time to adapt resistance gene and grow cells after transformation. Thereafter, cells were harvested through centrifugation and they were resuspended with less volume of SOC medium to increase concentration. Twist plasmids has ampicillin resistance gene and so they were pourred to plates that has LB agar+amp. The plates were incubated overnight at 37°C which is the optimum temperature for growth of *E. coli*.

Single colony was grown on LB broth+amp to isolate plasmids. The reason of using single colony is to ensure that all cells have same properties. In determination of the plasmid DNA concentration, high concentration is important but purities of plasmids are equally crucial. Accordingly, A260/280 ratio should be ~1.8 for pure DNA [93]. Therefore, the tubes were chosen with high concentration and purity for further steps.

Double digestion upon plasmid multiplication was completed without any noticeable hiccup. Linearization is needed to integrate plasmids with corresponding inserts to the chromosome. Upon linearization with PmeI, the product is extracted with phenol:chloroform and precipitated with ethanol to increase pPICz-α A plasmid

concentration. Upper part (DNA) must be taken carefully without touching the bottom part (phenol, chloroform) to prevent any contamination risk.

An other important point is to prepare cells for electroporation to increase transformation efficiency [94]. For this purpose, electrocompetent cells were prepared with HEPES and DTT. They render the cell wall more permeable to DNA [94]. Also, sorbitol and DTT were used for osmatic stabilization [94]. These steps were successful compared to the prelimiary experiments (data not shown), since transformation efficiency was considerably high with several colonies in selective petris (Figure 4.5).

Growth curves for *Pichia pastoris* (SMD 1168H) with different inserts indicates that there are no significant difference of growth profile among *Pichia* with different inserts (Figure 4.6).

Protein concentrations were determined through different BSA concentrations. Interestingly highest protein concentration was at the beginning of fermentations for all inserts (Figure 4.7), attributed to the composition of BMMY medium with yeast extract and peptone. Protein concentrations were decreased till 48^{th} h and then there were an increase till 72 h for all inserts. Highest enzyme concentrations should be in this time-frame. Chemical media need to be used to overcome this problem in future studies. Highest activity is indeed obtained during 2^{nd} or 3^{rd} days of the fermentation, (48^{th} h for pectin lyase, 60^{th} hour for polygalacturonase)

SDS-PAGE was performed to show existence and molecular weights of proteins. Theoretical molecular weights of proteins are 47 kDa for PGase, 36 kDa for PME, 24 kDa for pelC, 54 kDa for Lac and 37 kDa for LP. The bands were found at expected places, yet some of them were not clear. Purification and/or concentration methods need to be applied to see darker bands.

Care must be taken for environmental parameters during activity measurements. Polygalacturonase activity was measured at pH 4.2, while pectin lyase activity needs to be determined at alkali conditions. This further supports the underlying aim of the thesis, i.e. individual production and tailor-made manufacture of these enzymes, since each enzyme would have optimum production and activity conditions.

Also, ultrafiltration method was used to purify enzyme. In this case, serial filtration was performed with filter units of different MWCO values (100, 30 and 3 kDa). Molecular weight of polygalacturonase enzyme was 47 kDa. Enzyme activity increased approximately 2.5 fold through ultrafiltration (Figure 4.10). This method could be further used to concentrate the enzymes in the crude extract.

Pichia pastoris (+pelC insert) was cultivated at 3 L bench top fermentor for 5 days. Growth curve was similar to the growth curves from erlenmeyer flask experiments (Figure 4.11), yet higher biomass levels are reached owing mainly to controlled aeration, therefore oxygen supply. Interestingly, condierably higher amount of HCl is used during the fermentation. The reason of this can be alkali property of pectin lyase enzyme.

Different enzyme blends were used to effect of enzymes on pectin in citrus peel. For this purpose, three different temperatures were used as 30°C, 37°C and 50°C. According to color changes, 50°C is optimum temperature. Besides, combination of pectinolytic and ligninolytic enzymes has the most significant color change hinting highest activity.

6. CONCLUSION

This thesis presents a successful application of recombinant DNA techonology for the production of five pectinolytic and ligninolytic enzymes. Despite successful upstream applications, final enzyme titers are low and so improvement of downstream process is essential for further applications. In line with this, additional upstream manipulations (e.g. using stronger promoter, multiple copies, higher cell density) need to be considered to increase titers and productivites.

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