

PRODUCTION OF ENZYMES FOR FOOD INDUSTRY VIA SOLID-STATE
FERMENTATION AND OPTIMIZATION OF PROCESS CONDITIONS



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ABSTRACT

PRODUCTION OF ENZYMES FOR FOOD INDUSTRY VIA SOLID-STATE FERMENTATION AND OPTIMIZATION OF PROCESS CONDITIONS

Enzymes are biocatalysts, accelerating biochemical reactions *in vivo* or *in vitro*, with a wide range of applications in different industries like food, feed, detergent, paper, and pharmaceuticals. Aspartic protease, an acid protease, and Transglutaminase are important enzymes for the food industry, like milk, meat, brewery, and wine industries. Limited GRAS native strain and low extracellularly produced APase and TGase, makes the research for recombinant alternatives imperative. *Pichia pastoris* a well-known yeast host for heterologous protein production is a good alternative for recombinant expression of these enzymes. Once the production host is selected, the determination of fermentation steps is the key in enzyme production. Solid-state fermentation (SSF), which is performed in the near-absence of free water is considered to be a cheap and simple process with high protein titers. Despite being a promising process option, SSF presents several challenges, primarily because of its heterologous nature, e.g., maintaining fermentation temperature and pH constant, enhancing oxygen consumption, and homogeneous nutrient distribution. Addressing these challenges constitutes the main theme of this thesis.

This thesis covers molecular work, optimization of production via SSF of the two target enzymes, and application of these in various food processes. The molecular work covers the design, de novo synthesis and cloning, and construction of methanol inducible system with *P. pastoris* as host. Once the host is obtained, a series of submerged (SmF) and solid-state fermentation (SSF) were conducted to comparatively analyze growth and production yields as well as oxygen consumption and methanol evaporation rates. To find optimum conditions, SSF in a modified reactor with forced aeration and forced stirring were performed. The produced enzyme is tested in the different food products. SSF process is verified to be promising conditions for recombinant enzyme production, during which high cell density are reached. A higher oxygen uptake rate was proved by results compared with SmF. Both recombinant enzymes were successfully expressed when the complex medium was used. While, when the defined medium was used the production was in good titer only in the SSF process.

ÖZET

KATI FAZ FERMANTASYONU VE PROSES KOŞULLARININ OPTİMİZASYONU İLE YİYECEK ENDÜSTRİSİ İÇİN ENZİM ÜRETİMİ

Enzimler biyokimyasal reaksiyonları in vivo ya da in vitro hızlandıran, canlı hücreler tarafından üretilen biyokatalizörlerdir. Yiyecek, kağıt, deterjan ve ilaç gibi bir çok endüstriyel alanda kullanılırlar. Bir asit proteaz olan aspartik proteaz ve transglutaminaz, süt, et, bira ve şarap endüstrilerinde kullanımı açısından önemli enzimlerdir. Limitli GRAS doğal suşu olması ve düşük hücre dışı APaz ve TGaz üretimi bu alandaki rekombinant üretim çalışmaları için destek noktasıdır. İyi bilinen bir heterolog protein üreticisi olan *Pichia pastoris*, bu enzimlerin rekombinant ifadesi için iyi bir alternatiftir. Üreticinin önemi dışında, fermantasyon süreci de enzim üretiminin iyileştirilmesinde anahtar rol oynamaktadır. Neredeyse serbest su yokluğunda yapılan katı faz fermantasyonu yüksek protein üretim oranı sağlamasıyla basit ve ucuz bir yöntem olarak değerlendirilir. Ancak katı faz fermantasyonu uygulamasında, sistemin heterolog doğasından kaynaklanan sıcaklık ve pH'nın sabit tutulması, oksijen tüketiminin geliştirilmesi gibi zorluklar vardır.

Bu tezdeki çalışmalar, moleküler çalışmalar, biyoproses çalışmaları ve üretilen enzimlerin yiyecek sektörü ürünlerinde uygulanması şeklinde gruplanmıştır. Moleküler bölümünde, tasarlanan aspartik proteaz ve transglutaminaz genleri de novo sentezlenip klonlanarak metanol tarafından indüklenebilen gen ifade sistemi oluşturulmuştur. Koloni doğrulandıktan sonra, batık kültür ve katı faz fermantasyonları yapıp büyüme hızı, üretim verimi, oksijen tüketim ve methanol buharlaşma hızları açısından karşılaştırma yapılmıştır. Optimum koşulların bulunabilmesi için fermantasyonlar modifiye edilmiş reaktörlerde cebri havalandırma ve karıştırma uygulanmıştır. Üretilen enzim farklı gıda ürünlerinde test edilmiştir. Katı faz fermantasyonunun rekombinant enzim üretimi için gelecek vaat ettiği ve batık kültür fermantasyonuna göre oksijen alım hızının daha yüksek olduğu gösterilmiştir. Karmaşık besi yeri kullanıldığında iki rekombinant enzim de başarılı bir şekilde üretilmiştir ancak kimyasal bileşimi tanımlı besi yeri kullanıldığında ise sadece katı faz fermantasyonunda yüksek titrede elde edilebilmiştir.

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LIST OF SYMBOLS/ABBREVIATIONS

d	Day
g	Gram
x g	Relative centrifugal force
h	Hour
Da	Dalton
k	Kilo
kDa	Kilodalton
L	Liter
M	Molarity
m	Meter
mm	Millimeter
N	Normality
n	Nano
U	Unit
μ	Micro
w/v	Weight per volume
APase	Aspartic protease
BSA	Bovine serine albumin
CaCl ₂	Calcium chloride
DTT	Dithiothreitol
DNA	Deoxyribonucleic acid
DCW	Dry cell weight
dH ₂ O	Distilled water
ddH ₂ O	Double distilled water
EAI	Emulsifying activity index
EDTA	Ethylenediaminetetraacetic acid
Gal	Galactose
GAP	Glyceraldehyde 3-phosphate
gds	Gram dry substrate

<i>GTGase</i>	Guinea transglutaminase
HPLC	High pressure liquid chromatography
IDW	Initial dry weight
LB	Luria-bertani
MCU	Milk clotting units
<i>MTGase</i>	Microbial transglutaminase
$(\text{NH}_4)_2\text{SO}_4$	Amonyum sulfat
<i>PPAPase</i>	<i>Pichia pastoris</i> transformed with <i>APase</i> gene
<i>PPTGase</i>	<i>Pichia pastoris</i> transformed with <i>TGase</i> gene
RSM	Response surface methodology
SDS	Sodium dodecyl sulfate
SSF	Solid state fermentation
SmF	Submerged fermentation
SOC	Super optimal media with catabolite repression
TGase	Transglutaminase
YPD	Yeast extract, peptone and dextrose
Zeo	Zeocin

1. INTRODUCTION

1.1. MICROBIAL ENZYMES

Enzymes are functional proteins, accelerating metabolic, biochemical reactions [1]. Proteins are macromolecules, however, the region of the enzyme which is directly involved in the catalytic process called active sites consists of a few amino acids [2]. In the reaction, enzymes are required in a small concentration due to their ability to not being consumed during the reaction. For the first time, the term enzyme was used by Wilhelm Friedrich Kühne professor of physiology at the University of Heidelberg in 1877, when he was explaining the ability of yeast to produce alcohol from sugars [3]. However, usage of in fermentative process is mentioned in ancient era around 400 BC in Homer's Iliad.

Enzymes have an important role in several bio-products processing due to their unique properties, e.g., lowering the activation energy for biochemical reactions[1]. Based on the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology the Enzyme Commission number classifies enzymes into six classes, oxidoreductases, transferases, hydrolases, lyases, isomerases, ligases, and now there is a new class of enzyme translocases, which catalyze the movement of ions or molecules across membranes or their separation within membranes [2].

Enzymes are considered to be sustainable, environmentally benign, highly efficient, and easily biodegradable catalysts. Due to their ability for selective reactions, higher efficiency, and faster reaction demand for industrial enzymes is increasing day by day, the global enzymes market size value for 2019 was \$9.9 billion [4].

Enzymes are found in all living matter, human, plant, animal, and microorganisms. Microorganisms are major enzyme producers for the industry since they occupy small spaces and produce high quantities in a relatively short time, allow easier down-stream processing, and lower maintenance cost [5]. Resulting enzymes are, in turn, more attractive than animal or plant enzymes because they are more stable and active, with generally longer shelf-life

[6,7]. Many enzymes are produced via microorganisms for several industries such as detergent, pharmaceutical, food, feed, biofuel industry as well as for bioremediation [8]. Recently, several research has been initiated to isolate new microorganisms from extreme environments capable of producing enzymes that work in extreme conditions like temperature, pH, salinity, and heavy metals [9-12]. There are numerous enzymes from microbial sources, but, some of the most known produced in the industry are proteases, α -amylases, lipases, lactases, cellulases, xylanases, and catalases. Among those, proteases are the dominant group of enzymes with over 60 percentage share of the total microbial enzymes produced [4]. Moreover, aspartic protease is one of the main proteases used in the food industry, and it's going to be the main target of this thesis. The second target for production is transglutaminase, an enzyme with high demand in food industry that catalyzes the formation of isopeptide bond. From here on this thesis, we will talk about aspartic protease and transglutaminase production.

1.2. ASPARTIC PROTEASE

Proteases belong to the group of hydrolases, using water molecules to cleave the peptide bond releasing in the end as product peptides of amino acids. Based on the exact location of hydrolysis, proteases are divided into exopeptidases and endopeptidases. The first one, breaks down the terminal peptide bound resulting in a free amino acid or with a dipeptide. While the second group cleaves the internal peptide bond. Endopeptidases are further grouped such as serine, aspartic, cysteine, metallo, glutamine, and threonine peptidases based on the position of the active site (Figure 1.1) [4].

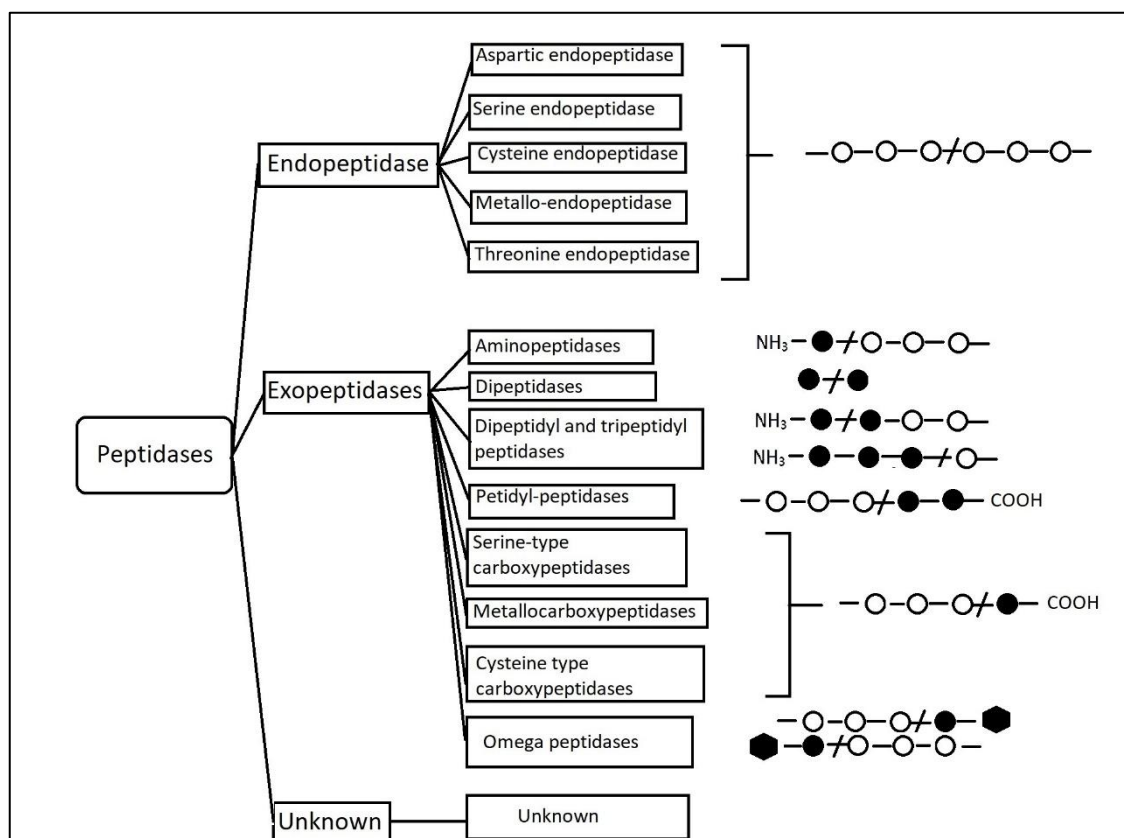


Figure 1.1. Protease classification

Aspartic proteases are mainly found in animal, plant, fungi, and yeast cells. Major producers of microbial aspartic proteases are filamentous fungi. The earliest work that I found for bacteria and fungi screening for aspartic protease production was Sardinas et al. 1968, where they isolated *Endothia parasitica* which yielded a protease capable of milk clotting [13]. Over time, the number of aspartic protease producing fungal strains increased, Ismail et al, 1984 isolated aspartic protease from *Rhizomucor pusillus* [14], Preetha et al, 1994 isolated from *Rhizomucor miehei* [15], Fernandez Lahore et al. 1999 isolated from *Mucor* species [16], yet most accepted aspartic proteases from dairy industry was still the enzymes produced using *Rhizomucor miehei* and *Rhizomucor pusillus*. Even though there is a considerable number of isolated producers of aspartic proteases, still the activity of the produced enzyme is low.

1.2.1. Aspartic Protease Characteristics and Structure

Based on the study of Tang et al., 1973 aspartic protease contains two active sites aspartic acid residues Asp-32 and Asp-215, near each other in three-dimensional structure [17], as further elucidated and visualized by Andreeva and Rumsh, where they analyzed the crystal structure of aspartic protease. The three-dimensional structure of aspartic protease contained two domains, in each an active site. The substrate-binding clefts are placed in each of these lobes and extend nearly the all width of the enzyme. An essential part of this junction of the domain plays a water molecule, which becomes deprotonated on substrate binding to initiate the base catalysis [18].

Fungal aspartic proteases consist of a single chain enzyme with a molecular weight between 35-39 kDa. They are mostly produced as zymogen inactive enzymes. In N-terminal Asp-32 propeptide of up to 50 amino acids long helps in the three-dimension folding of the enzyme, this region is cleaved upon activation. The zymogen is autocatalytically activated by peptide release at low pH [19]. Most of the aspartic proteases show the optimal activity between pH 3-4 with the isoelectric points in the pH range of 3-4.5 [20]. Sun et al, reported an optimal temperature 55 °C for recombinant aspartic protease from *Rhizomucor miehei* expressed in *Pichia pastoris* [21].

1.2.2. Mechanism of Action of Aspartic Protease

There has been a lot of discussion about the aspartic protease mechanism of the reaction, researchers proposed a mechanism of the intermediate covalent catalyzed reaction, which they failed to prove [22]. The lack of an intermediate covalent reaction was supported by experiments with isotope ^{18}O water. A water molecule is found equidistant from the carboxyls, Davies 1990 proposed a mechanism of action where Asp-215 has to be charged, while Asp-32 has to be protonated, in which case will enhance nucleophilicity in the attack on the carbon atom of the carbonyl group of the scissile peptide bond [18].

The catalytic mechanism involves residues of aspartic acid at the active site of the peptide chain, bound to a water molecule that acts as a nucleophile. There are no functional groups on these enzymes that provoke a nucleophilic attack against the carbonyl of the peptide bond that is to be cleaved, so there is no covalent intermediate between the enzyme and the substrate. Figure 1.2 shows how catalysis begins with the structure of the cycled active site (E), to which the substrate is bound (ES), and then, the electrons extend in a counterclockwise movement through the sensitive carbonyl (ES1) form to generate an intermediate bound to a deprotonated form of the enzyme (ES2). In the next step, the clockwise movement of the two electrons generates an intermediate zwitterion bound to the monoprotonated form of the enzyme (ES3). Finally, the zwitterion collapses, releasing the carboxyls from the enzyme (EP). This completes the chemical processing of the substrate but not that of the enzyme. The regeneration of the latter is completed when it is deprotonated and rehydrated (E') [23].

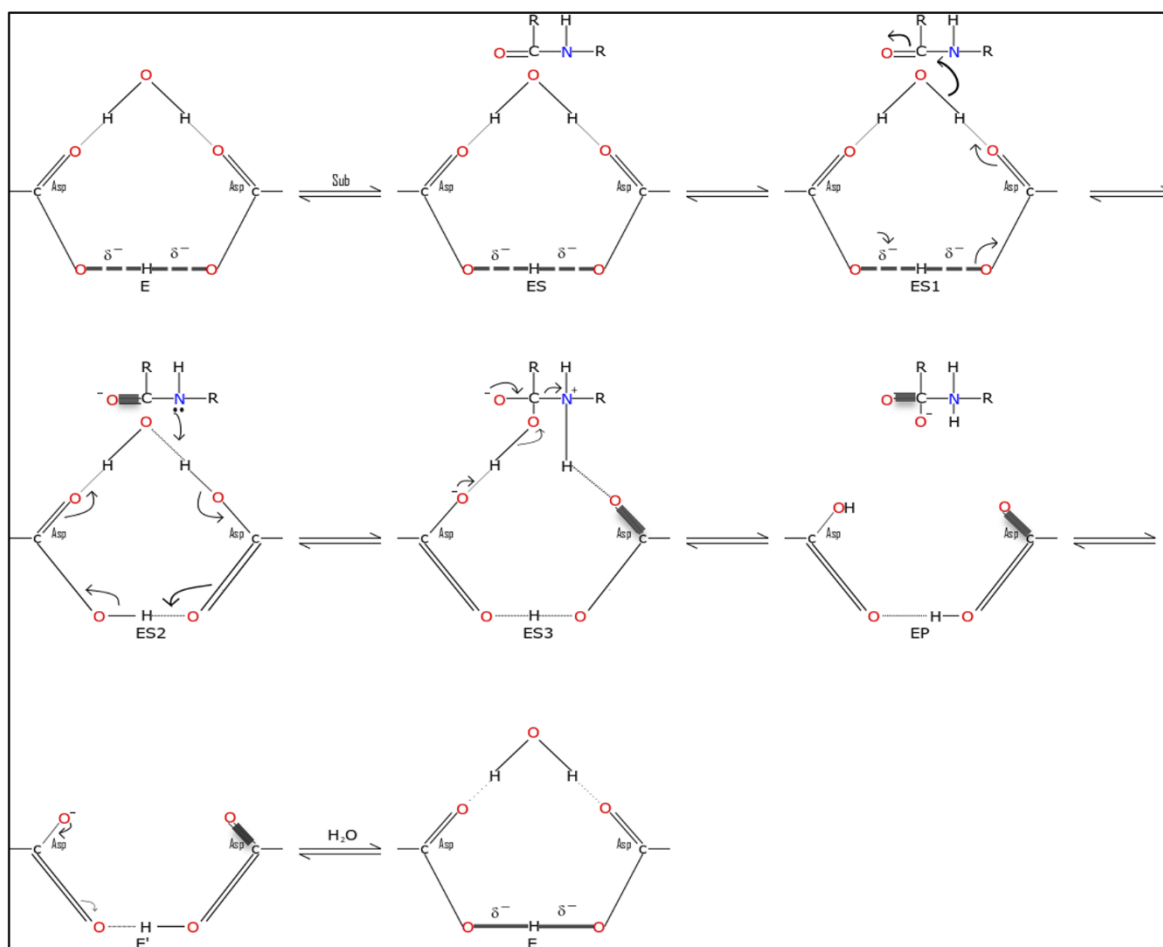


Figure 1.2. Mechanism of action of aspartic protease. E Enzyme, ES Enzyme substrate complex, EP Enzyme product complex

1.2.3. Recombinant Aspartic Protease Production

There have been isolated bacteria and fungi which are capable of aspartic protease production. There have been found also plants that can produce this enzyme. Even though high in the number, the activity of the enzyme from native producers was far from the desirable level for industrial production. In some cases, the native species also have a parasite background, which will make the produced enzyme not applicable in most of the industries. In this case, molecular biology was involved for recombinant expression of the isolated genes from this species to a more suitable industrial species.

In 1987 Dickinson *et al* isolated and expressed the gene encoding the aspartic protease from *Mucor miehei* in *Mucor circinelloides* by using a constructed vector named pMA67 which contained Ampicillin and Leucine auxotroph as a selection marker for *E. coli* and *M. circinelloides*, respectively. Gene was successfully expressed, isolated enzymes resulted with the same molecular weight as produced from the natural strain. The activity was measured in Christian Hansen units and the highest activity was obtained after 7 days of fermentation with 1.2 U/mL [24].

Aspartic protease SA76 from *Trichoderma harzianum* T88 is responsible for inhibition of the growth of phytopathogenic fungi. cDNA of this enzyme was expressed in *Saccharomyces cerevisiae* under control of the GAL1 promoter. The activity of the enzyme produced was 10.5 U/mL after 72 h induction with galactose. Produced enzyme effectively inhibited the mycelial growth of five phytopathogenic fungi [25].

Feijoo-Siota *et al.* isolated a novel aspartic protease from *Galim verum* plant which is used in cheese making. The isolated enzyme was used to generate cDNA, which was transformed to *Pichia pastoris* by using α -factor as a signal peptide. The produced enzyme by *P. pastoris* was capable of milk coagulation with 23 milk clotting units (MCU). The molecular weight was approximately 48 kDa [26].

Aspartic protease from *Mucor circinelloides* was expressed in *Pichia pastrois* by using constitutively promoter GAP. The expressed enzyme showed a molecular weight of around 37 kDa. The enzyme was analyzed for milk clotting activity and proteolytic activity. Obtained results were 137 U/ μ g milk clotting activity and 7.02 U/ μ g proteolytic activity, respectively. Showing a potential of the expressed enzyme for application in cheese making process [27].

Aspartic protease has found application in bioethanol production from raw materials. To do that, an aspartic protease gene from *Neurospora crassa* filamentous fungi was cloned in ethanol-producing yeast *Saccharomyces cerevisiae* by using PGK1 promoter. The highest activity obtained was 6.8 U/mL at pH 4.0 and 45 °C. In the fermentation process by recombinant strain, a quicker fermentation rate for about 22 h faster, rapid ethanol production, and fast sugar consumption rate were observed by the research. This yielded a concentration of ethanol between 50.2 to 50.5 g per 100g sugar. Calculated as 98 to 99 percentage of the theoretical maximum level of the ethanol [28].

Isolated marine yeast *Metschnikowia reukaufi* was used as a source of aspartic protease cDNA which was used to transform *E. coli* by using *pET-24a* plasmid and fused with 6-His Tag. The expressed aspartic protease was characterized with a high molar mass of 54 kDa with optimal activity at pH 3.5 and temperature 40 °C, this enzyme showed milk clotting activity too [29].

There are several other examples where aspartic proteases from different sources are expressed in different industrial hosts. This shows the importance of this enzyme application in numerous industries like food, feed, plant, and biofuels [21, 30, 31]. The results of the activity in the reported article show that there is still space for improvement.

1.2.4. Application of Aspartic Protease in Food Industry

Aspartic proteases or usually known as acid proteases are very important components of the food industry, they are used in a wide range of products to improve organoleptic characteristics of the food like taste, appearance, and texture. It also found application in product conservation by producing a whole new product from raw materials or increasing the nutrition value to the existing product by decreasing the processing expenses.

The earliest use of aspartic protease has been found in the dairy industry for milk coagulation in the cheese-making process. The first aspartic protease used in this industry was rennin-chymosin which was extracted from the fourth stomach of the young suckling ruminants like lamb and calf. Chymosin is a Ca^{++} -dependent enzyme in charge with cleavage of the Phe¹⁰⁵-Met¹⁰⁶ bond in the K-casein, generating para-casein and macromolecules [32]. The nonpolar side of K- casein is connected to the micelle structure while the negative polar side of K-casein is facing outside. Aspartic protease in this case rennin cut the negative polar side from K- casein allowing the micelles to bond together and coagulate the milk, shown in Figure 1.3.

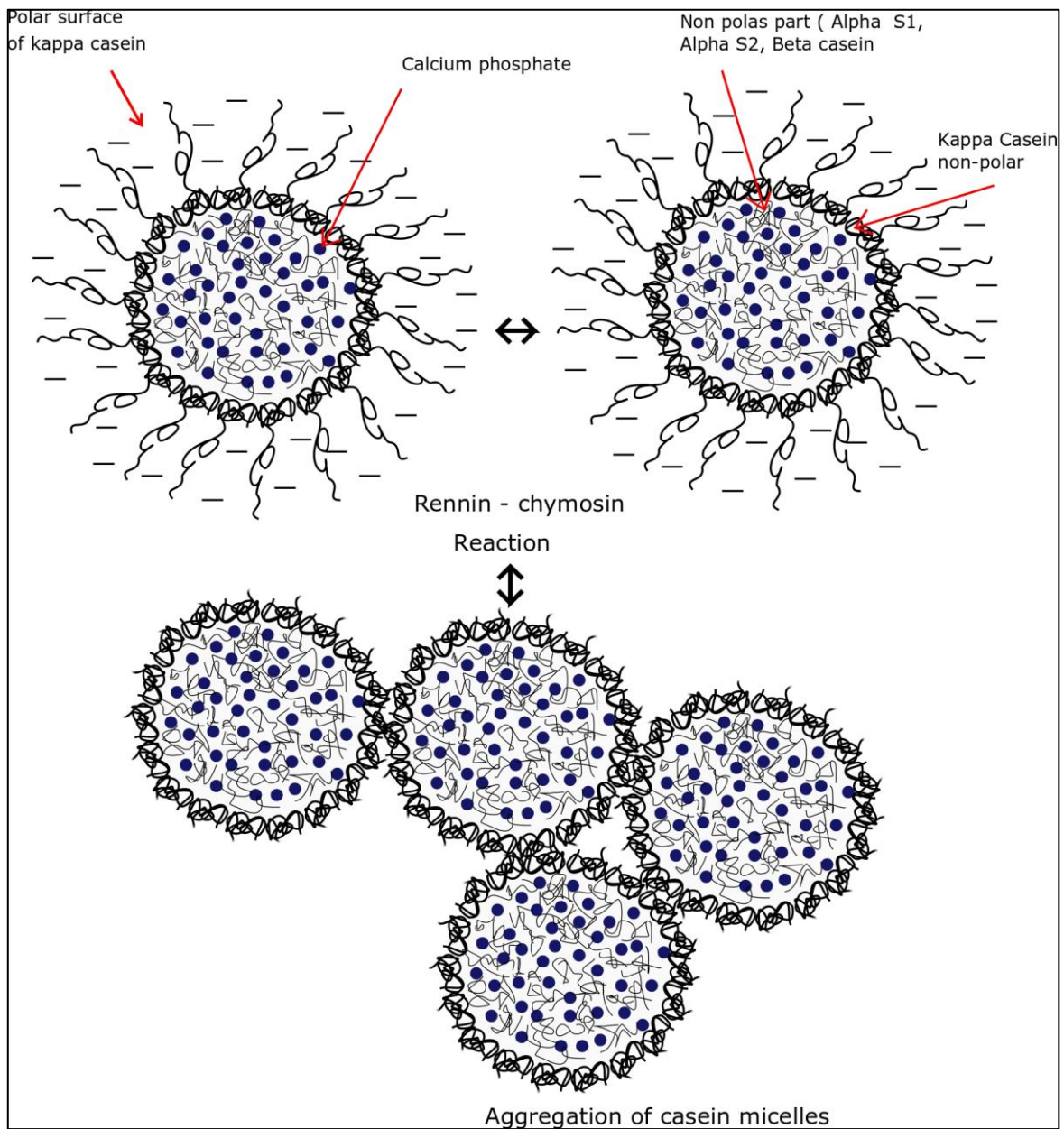


Figure 1.3. Aggregation of casein after the cleavage of bond between Phe¹⁰⁵-Met¹⁰⁶ of κ -casein

Earlier in 1919, the economic problems as a result of the war in Europe increased the lack of rennet. This problem forced many dairy industries to search for new enzyme alternatives. Merker studied the difference in cheese quality and the cheese making process by pepsin from pig stomach vs rennet from calf stomach. From their results, they concluded that there is no significant difference in the milk clotting process when pig pepsin or calf rennet are used. They disproved the earliest theory for the butter-fat loss when pepsin was used [33].

However, several years later Garg and Johri analyzed the feta cheese quality produced by pepsin and concluded that milk clotting takes place effectively while proteolysis is extended through the process making cheese less compact [34].

Based on the process of the cheese making, the effect of the rennet can be expanded until the last process of the cheese making which is the ripening of the cheese. Hesari *et al.* reported a high contribution to the proteolysis of rennet in the white ultra-filtered Iranian cheese during the storage time. There was a significant difference in free amino acid between cheese produced with and without rennet, by which they concluded that rennet is a necessary ingredient for coagulation as well as for the ripening process in cheese making [35].

Different sources of aspartic protease yielded cheese with different characteristics. It is reported that even though comparable white cheese produced by calf, camel, and rabbit rennet yielded cheese with different texture and odor [36].

The bakery industry is another sector where aspartic proteases find the application. Celiac disease is an immune disease with which people cannot eat food products containing gluten. Gluten is the main protein of wheat flour. Knowing that bread is one of the main components of the meal, authors M'hir *et al.* used the combination of enterococci and *Rhizopus oryzae* proteases in the dough making. Based on the results they reported threefold higher water-soluble peptides in enterococci and *Rhizopus oryzae* treated dough than in control which was treated with acidification. Based on the SDS page they reported full degradation of gliadins and partial degradation of gluten. Showing the opportunity of the aspartic protease in gluten-free food production [37]. The gluten from wheat flour treated with aspartic protease from *Aspergillus usamii* resulted in a radical increase in emulsifying activity index (EAI), water, and oil-holding capacity in the work of Deng *et al.* [38].

Rice is a gluten-free cereal crop in which flour is used for making gluten-free bread. Because of the lack of gluten structure formation during cooking, prepared bread with rice flour is always of a low quality. To increase the quality of the bread, Kawamura-Konishi *et al.* treated the dough of rice flour with different amounts of commercial protease from *Bacillus stearothermophilus*, produced bread showed higher quality like good crumb appearance, high volume, and soft texture [39].

Furthermore, aspartic protease is used in the beverage industry for nutritional value enhancement and haze treatment formed from protein. An example is the treatment of the

fresh juice from the pomegranate. In this case, pomegranate juice was treated with pectinase and protease. The treatment of the juice prevented the haze formation in about 6 NTU units of turbidity compared with untreated samples [40]. The beer industry is another sector in the beverage industry where aspartic protease finds application. Chill haze formation in beer production is a consequence of polyphenol reaction with proteins. Hence, Nelson and Young used aspartic protease during the fermentation process to control the chill-haze formation in beer during the storage. They reported no changes in the flavor of the beer while in the third group of fermentation where *Phellinus pini* protease was used as an addition, the fermentation progressed faster to lower specific gravity than the control fermentation. The final cell concentration was three times higher than the average amount of cell in the control fermentation, this might be due to an increase of the free amino nitrogen as a result of the proteolytic effect of protease [41].

Haze protein formation is also a problem for the wine industry. Until now, haze protein wine has been treated with filtration through bentonite which causes wine volume, aroma, and color loss. Marangon *et al.* developed a technique for haze wine treatment with acid protease. This technique included wine treatment with the temperature at 75 °C for 1 min to unfold the protein, making them more accessible for enzymes, and then the addition of the enzyme. Using this process, they succeeded to produce a wine highly heat stable and free from haze-forming proteins [42].

1.3. TRANSGLUTAMINASE

Transglutaminase (EC 2.3.2.13) (TGase) or known informally by the nickname “protein glue” [43] are an acyltransferase enzyme that catalyzes the reaction between γ -carboxyamides of peptide or protein-bound glutamyl residue (acyl donor) and primary amino group (acyl acceptor) [44] by generating covalent crosslinks between glutamine and lysine, without affecting bioavailability of essential amino acid [45], and subsequent release of ammonia. Transglutaminase is broadly disseminated in different living organisms, mammals, plants, and microorganisms [46]. For the first time transglutaminase was described by the work of Clarke *et al.* where they described *in vitro* incorporation of amines into proteins by using

Ca^{++} activated enzyme extracted from Guinea pig liver [47]. Until the 1980s the only source of transglutaminase was from Guinea pig liver or from mammalian blood, which did not find any application in the food industry for two main reasons: (a) It was Ca^{2+} dependent, and (b) extracted enzyme contained red pigmentation which makes the product not appropriate [48]. Several authors worked to find microbial sources for transglutaminase. After several attempts, in 1989 author Ando *et al.* [49], produced and purified the first microbial transglutaminase (MTGase) from *Streptoverticillium* S-8112, which was produced after 5000 strains isolated from soil were screened for transglutaminase activity by hydroxamate method. For more, *Streptoverticillium* sp. S-8112, an actinomycete strain was the only strain from 5000 which shows the transglutaminase activity. Purified produced enzyme showed 2.5 U/mL activity, that was the half of activity of guinea pig liver transglutaminase activity in that time. Moreover, purified MTGase showed 100 percentage activity in both cases, with or without calcium ions, proving that MTGase was calcium independent.

1.3.1. Microbial Transglutaminase, and its Mechanism of Action

Primary amino acid structure of MTGase produced by *Streptoverticillium* sp. S-8112 was unknown until 1993, in that year a group of scientists published a work where they characterized the structure by a combination of fast atom bombardment mass spectrometry and standard Edman degradation of peptide fragments. They reported that MTGase consisted of 331 amino acids in a single polypeptide chain with molecular weight 37863 Da, defined as monomeric simple protein, even though it has two potential glycosylation sites it was reported no similarities in the sequence of MTGase with Guinea Transglutaminase GTGase except to the Cys residue region. The active center of transglutaminase is composed of histidine, cysteine, and either asparagine or aspartate residues. This catalytic triad, based on the work of Kashiwagi *et al.* is structured by Cys⁶⁴ residue which exists at the bottom of the cleft, Asp²⁵⁵ residues at a position nearest to Cys⁶⁴ and is adjacent to His²⁷⁴ [50].

Animal transglutaminase is Ca^{2+} dependent, the presence of which is demonstrated to induce conformational changes in the enzyme molecule. Thus, its activity is promoted. In contrast,

microbial transglutaminase is calcium-independent, which puts MTGase in a better position for industrial usages [51].

From the hydropathy analysis it was reported that *Streptoverticillum* TGase is hydrophilic as a whole, but with some hydrophobic sequences. The isoelectric point of microbial transglutaminase was reported to be pI 9 [52,53]. Based on the literature the optimal pH for TGase activity is 7 [54] but is considered to have a stable activity between pH 5 and 9 as well [55]. Temperatures below 10 °C normally reduce the activity of MTGase, similarly, temperatures above 55 °C decrease the activity, while temperatures over 70 °C inactivate the enzyme in minutes.

The transglutaminase reaction occurs through a modified double-displacement process in which an intermediate acyl-enzyme is generated between the acyl portion of the glutamine substrate and the catalytically active cysteine residue sulfhydryl group on the enzyme. An acyl acceptor reacts with this intermediate, such as water or a primary amine, resulting in either deamidation or cross-linking reactions.

There are three reactions known catalyzed by transglutaminase. First, the reaction between the group of γ -carboxamides of glutamine residues as a donor and the first-order ϵ -amine groups of different compounds, proteins for instance as acceptors of acyl residue by forming an isopeptide bond (Figure 1.4 (a)). While the second group of reactions happens when lysine is the acceptor of the acyl group, a protein molecule is enriched with this amino acid. An example is the formation of cross-linking inter- or intramolecular cross-links ϵ -(γ -Glu) Lys, shown in Figure 1.4 (b). Third reaction type that transglutaminase catalysis is when water acts as an acyl acceptor, which realizes the deamination in the absence of amine groups (Figure 1.4 (c)). Reactions catalyzed by this enzyme result in significant changes in the physical and chemical properties of proteins, such as modifications in viscosity, thermal stability, elasticity, and resilience of proteins [56].

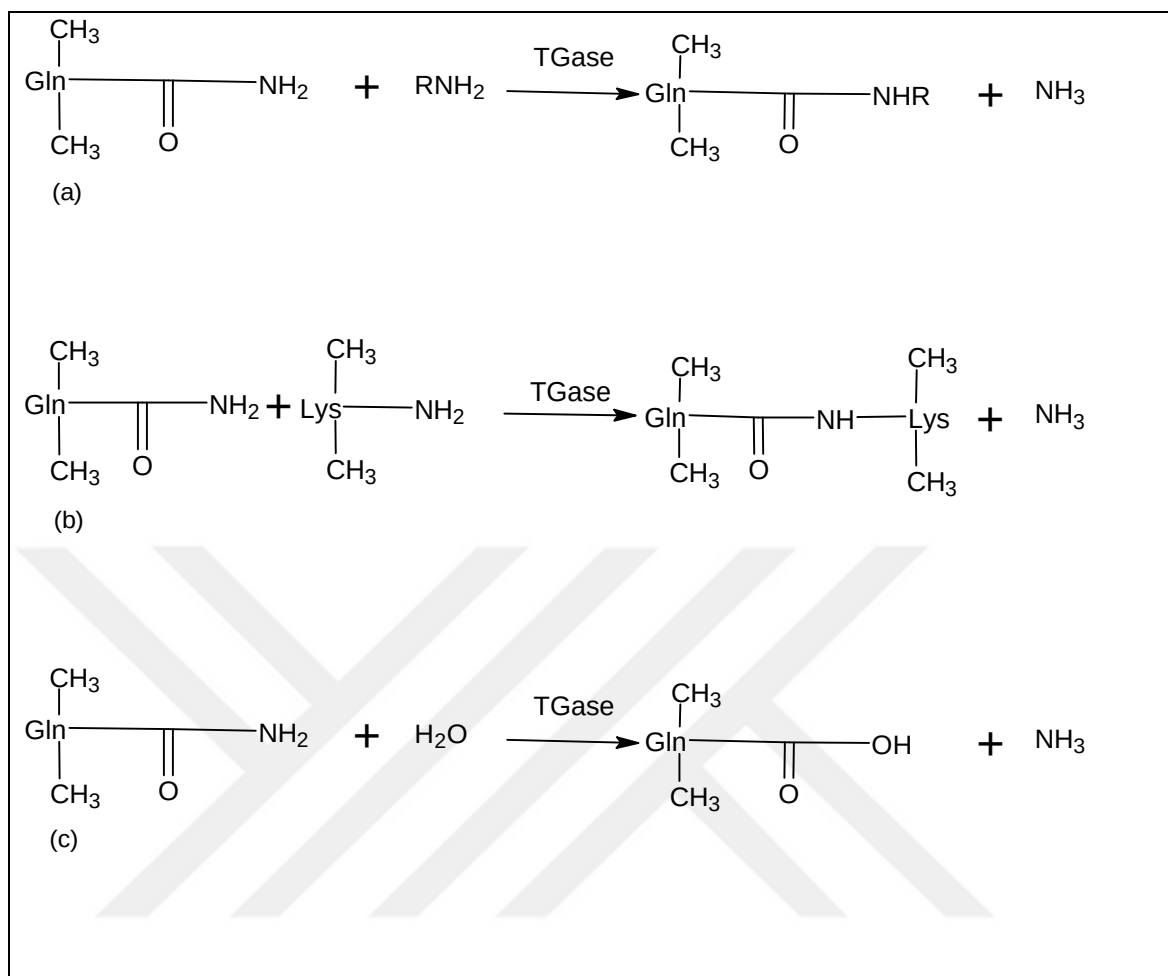


Figure 1.4. Reactions that are catalyzed by transglutaminase, (a) Acyl-transfer reaction, (b) Cross-linking between Gln and Lys of proteins, (c) Deamination

1.3.2. Transglutaminase in Food Industry

Transglutaminase catalysis crosslinking of several food proteins. These crosslinks are the reason for many rheological changes in food proteins. Moreover, the usage of transglutaminase incorporates amino acids or peptides in food proteins, hence the nutritive properties of food are enriched. When transglutaminase is used properties like water holding capacity, solubility, and thermal stability of food proteins is improved. Food proteins that are gelled by transglutaminase:

- Proteins that are not gelled by heating can be gelled;
- Gels that normally melt at an elevated temperature no longer melt after the MTGase gelation;
- Protein in oil-in-water emulsions, even in the presence of sugars or and/or sodium chloride, can be gelled;
- Gel firmness increases after heating;
- The gels can no longer be solubilized by detergents or denaturants [57].

Kuraishi *et al.* in their work assayed the production of reconstructed meat pork by using microbial transglutaminase. Fresh pork meat pieces were mixed with transglutaminase and stuffed in polyvinylidene. They incubated samples at 5 °C for 2 h for an enzyme reaction. The strength of the binding formed was analyzed by Rheometer. The strength of the binding meat was significantly improved when 0.1 percentage TGase and 3 percentage NaCl is used to treat the meat, with around 8 fold higher binding strength compared with a simple 0.1 percentage TGase treated piece of meat [58].

Caseinate, treated with MTGase, becomes viscous, which acts as a glue to hold different foodstuffs together. Meat pieces, including minced meat, can be bound together by MTGase without salt and phosphatase, resulting in healthier, fresh meat products. In the work of Kilic, microbial transglutaminase was used to analyze crosslink formation between chicken döner kebab meat with sodium caseinate. After MTGase treatment kebab meat was under survey for texture, sensory, pH, and color change. Moreover, SDS-PAGE and yield were investigated. SDS-PAGE results showed that crosslinks are formed between meat proteins with or without the addition of sodium caseinate. While texture analysis revealed that meat treated with MTGase and caseinate had higher hardness and chewiness values than that treated without caseinate. Sensory analysis showed that meat treated with MTGase increased juiciness and overall acceptability [59]. In general, meat products can be effectively treated with MTGase for meat reconstruction, and improvement of texture, sensory qualities, and nutrition values.

MTGase enables low-fat meat production, by using caseinate as a substitute for fat at sausages. A Bologna-type sausage was produced with transglutaminase when Na-caseinate was used as a fat substitute. From 29 percentage the fat content was reduced to 19.4

percentage, without any change in quality compared with the control one [60]. In the same trend, the substitution of the lipid was done in salami. MTGase concentration up to 15 U/g protein improved gelation of myofibrillar protein isolate [56]. While there were reports that the addition of 0.2 percentage of microbial TGase in sausages, increased their firmness and hardness resulting in a better protein network structure. Moreover, for preparing low-salt dry-cured hams the addition of 2 g of TGase per kg of raw meat muscle resulted in good binding properties without a negative effect on sensory parameters [61]. Surimi-beef was prepared with 3 percentage potato starch and with 0.5 percentage microbial TGase, showing the maximum strength [62].

MTGase was used in pasta and noodles. Dough density was reported to increase significantly when the MTGase was used in the work of Bellido and Hatcher [63]. In other studies, they investigated the inclusion of bran into spaghetti, by using MTGase as a protein crosslinking ingredient. They reported significant improving effects on spaghetti quality like firmness, stickiness, and bulkiness when they compared with the control where only bran and no MTGase was added [64]. Other examples where MTGase is applied in pasta and noodles are collected in a review paper by Gharibzahedi *et al.* in 2017 [45].

Furthermore, MTGase is used in yoghurt production. Yoghurt, which is produced by acidic fermentation with lactic bacteria. Serum separation is one of the problems that yoghurt suffers, which problem can be solved by treating MTGase by improving the water holding capacity of the gel formed. MTGase reaction makes it possible to produce dairy products like ice cream and cheese with low-fat contents or reduced content of non-fat solids. The transglutaminase reaction made it possible to produce yoghurt with a low content of fat and reduced content of non-fat solids, resulting in yoghurt that is perceived as a full-fat yoghurt. The shelf life of the yoghurt was analyzed by Tsevdou *et al.* [65] when the skim milk is treated firstly with TGase 2.2 U/g protein and incubated for 180 min at 42 °C. Then the transglutaminase activity was terminated by treating the milk at 80 °C for 1 min. After which milk was fermented with culture until the pH reached 4.75. Yoghurt produced with TGase treated milk resulted in shorter fermentation time, less whey separation and higher firmness. Low dry matter milk TGase treated was used to produce yoghurt [66], one unit of TGase was used for each gram of milk protein. Which resulted in low serum separation and gel firmness and a viscosity similar to yoghurt with higher dry matter content. Tyrosine levels in TGase treated milk were lower compared with untreated milk, which is explained by the

stability that milk proteins gained against proteolysis. 3 U per gram protein of milk plant TGase treatment resulted in a significant improvement in the texture of the yoghurt. While the microbial TGase brought significant improvement to all parameters of the yoghurt, when 1 U TGase per gram proteins was used [67]. Low-fat yoghurt with probiotics was produced with inactivated (pretreatment of milk with TGase than inactivate after 2 h) and not inactivated TGase (simultaneous addition of TGase with probiotics), in the concentration 0.02, 0.06, and 0.12 percentage with 100 U/g powder enzyme activity. The highest firmness, cohesiveness, viscosity, and consistency was shown in the yoghurt prepared with active TGase with 0.12 percentage concentration. In both cases fermentation time was shorter for 15-60 min than the control yoghurt. While the fermentation time was shorter in yoghurt made with active TGase for 20 min than in the one made with inactivated TGase [68]. Moreover, to enhance dairy products TGase is used in the process of cheese making. In the work of Oros [69], semi-hard cheese production was monitored after it was produced with TGase addition. The amount of TGase used was 0.01 percentage (w/v) with a known activity of 40 U. Cheese in their case was produced with two different fat concentrations in milk 3.5 and 5 percentage fat content. The parameters that were tested are cheese yield, yellowness, fat in dry matter, and protein of cheese. In each case of the cheese produced with TGase, the yield of cheese (1-2 percentage), yellowness, and protein of cheese concentration were higher than in the control one, while the fat in dry matter was lower in TGase treated milk than in untreated one. In the work of Ren et al. [70], they produced full and lower fat cheddar cheese by using TGase as an enzyme in the process 7 min after the rennet addition in the concentration 2 U/g protein. They researched the indication of TGase in recoveries of protein (protein retained in cheese $\times 100$ / total protein in milk), fat (fat retained in cheese $\times 100$ / full fat contained in milk), and yield (cheese weight from one vat $\times 100$ / milk used weight) of produced cheese from both full and lower fat content. TGase showed no effect based on their results in full fat cheese, while TGase showed significant effect in yield, protein and fat recoveries in the lower fat cheese by enhancing the water holding capacity based on more covalent bond formation. Edam cheese is a ripened cheese. TGase was used in the production of this cheese in concentration 132 U/kg skim milk. Standardized skim milk with 42 g/kg protein and 33 g/kg fat was pretreated with TGase for 2 h at 40 °C and then was pasteurized. Prepared cheese was ripened for 4 weeks at 12 °C, during this period parameters as yield, recovery of total solid and fat was measured. The yield was significantly increased, while there were no significant changes in recoveries of total solids and fat, however water

recoveries was higher than the control one. The authors comment that TGase treatment did not crosslink much of the whey protein but it did help to improve water holding capacity in cheese [71]. Improved yield of cheese was shown in the data of Özer et al. [72], where TGase was applied simultaneously with rennet. The activity of TGase was 100 U/g. Lower synergies and higher protein in dry matter were shown compared with the control one without TGase.

Transglutaminase 2 U/mL was used by Mahmoud and Savello [73], for edible packing film making with 100 mg of whey protein in a 1 mL reaction solution. Durability, water, and vapor transferability were affected by glycerol concentration in films. Membrane films produced with transglutaminase by milk protein is a useful material for packing material in the future, incorporating cells or enzymes immobilized bioreactor or medical application.

1.4. FERMENTATION

Fermentation is defined as a process of breakdown of organic substances by microorganisms which results in a release of the desired products and heat [74]. This is undoubtedly a crucial process in the food industry for several final products to the end-user, but also the production of several intermediate products or ingredients. Considering the free water content, there are two main groups, namely Submerged Fermentation (SmF) and Solid State Fermentation (SSF). Owing to the abundant water content, the continuous phase (filling the space between nutrients) is liquid in SmF, while in SSF the continuous phase consists of gas [75]. The solid material in SmF ranges between 1-7 percentage (up to 15 percentage for High Cell Density Fermentation), which mostly are the nutrients (carbon and nitrogen sources) typically homogeneously distributed in the liquid [76]. In contrast, SSF is characterized by low water content near the absence of the free water [77], or generally with the necessary amount of moisture to maintain the microbial growth [78,79]. However, according to Aidoo *et al.* solid-state fermentation is a process that takes place on solid or semisolid substrates or in inert solid support supplemented with nutritionally medium [80]. Hence, based on the nature of the solid phase, two types of SSF are known: a) SSF on a natural solid substrate which is also used as a carbon and nitrogen source referred by Bigelis [81] as solid-substrate

fermentation, and b) SSF on an inert material impregnated with a liquid as nutrition support referred by Bigelis as solid-state fermentation [81,82]. During this thesis material to both fermentations we are going to refer to as solid-state fermentation, however, the material used as solid particles will be stated. The typical appearance of SSF is dry or mash-like, and the content is rarely homogeneous, which is one of the challenges in solid-state fermentation. During fermentation, the substrate typically becomes contagious in texture.

Besides the water content, SSF is additionally characterized based on aeration and agitation applied, which also have an impact on the texture of the substrate. From the process point of view, this classification yields four different subgroups in SSF:

- unforced aeration, without mixing;
- forced aerated, without mixing;
- unforced aeration, forced mixing;
- forced aeration, forced mixing [83]

Solid-state fermentation recently has received great attention in scientific research and application in numerous industries, owing to its advantages compared to submerged fermentation e.g. simple process, concentrated fermented material translated as smaller equipment and space requirements, high product yield, easy and cheap downstream process, lack of foaming during fermentation [83]. Moreover, less water used in up-stream processing results in lesser wastewater generation in downstream processing, which reduces the costs of downstream processing. Additionally, SSF is conceived to be more natural for several microorganisms, in particular for fungi growing naturally in low moisture media, than SmF. SSF now is an approved process by the Food and Drug Administration even for the production of clinical drugs [82].

1.4.1. Advantages and Disadvantages of Solid-State Fermentation

The advantages and disadvantages of the solid-state fermentations are going to be shown in Table 1.1 below in the short outline. Metabolic overheating is described as one of the disadvantages of SSF when compared to submerged fermentation [76]. The increase in

temperature of the solid substrate during fermentation may damage the cells and decrease the production rate [84]. Ashley *et al.* reported using a mathematical model for packed bed bioreactor that periodic air reversal is not a good technique for preventing overheating, however, periodical stirring during a certain time was found to be useful to decrease the metabolic overheating temperature in a solid matter [84].

Another important factor is the water content since all living systems need water for growth. Compared to fungi, bacteria needs higher moisture content for optimal growth, which in turn, is used as a selection factor for fermentation, in favor of fungi [85]. Interestingly, high levels of water are also unfavorable for enzyme production using SSF, since it decreases the oxygen transfer, as reported by [85], where α -amylase is produced by *Bacillus licheniformis* M27 using wheat bran as a solid substrate. The authors concluded that optimal levels of water are needed for the production of enzymes with the highest activity. A list of advantages and disadvantages of solid-state fermentation from different research are shown in Table 1.1.

Table 1.1. Solid-state fermentation, advantages, and disadvantages

Factors	Advantages	Disadvantages	References
Water content	Water content in the medium is low	Evaporation water during the fermentation process	[86,87]
Concentrated product	Produced products are more concentrated, the production rate is high, high stability of the product	Hard to adjust the pH inside the solid matter	[88,89]
Downstream process	Easy and cheap downstream process, no need for complicated reactor	Hard to get rid of the solid material after the downstream process	[90,91]
Process	The simple fermentation process, no foam formation	Problems with scaling up agitation and aeration	[92,93]

Environmental concern	Environmentally friendly, low output waste	Realize of methane (which if it is used for energy can cause no harm)	[94,95]
Suitability for micro.	Suitable for work with filamentous fungi	Problem with bacteria as a reason of low water activity	[94,96]
Media and inoculum	Nutrients of the medium are more concentrated, Minimize catabolite repression	A high amount of inoculum more than 10% are necessary to homogenize the content	[90,97,98]
Structure	Can be performed in an open environment, Gas-phase as the continuous phase	Problems with substrate shrinkage, Sampling is more complicated than in submerged fermentation	[99,100]
Contamination, Indigenous culture	Easy bacterial contamination control, fermentation with indigenous culture, small workspace	Hard in controlling metabolite temperature realized during the process	[93]
Cost	The cheap agro-industrial, the material cheap overall process		[101,102]

1.4.2. Factors Affecting Solid-State Fermentation

Several factors strongly affect the growth of microorganisms and protein production during solid-state fermentation. These factors are temperature, water activity, pH, physiochemical nature of the solid material and its particle size, form, and void space. All these factors described above cross in a way or another with each other and affect the whole process of

fermentation. Due to the heterologous nature of SSF, gradient formation of these parameters through the solid substrate like nutrients, O₂, CO₂, and metabolic heat is common. Knowing them better and controlling them, improves the process of fermentation. Several researchers contributed with their work to define the effects of these factors in SSF.

1.4.2.1. Water Activity and Water Content

Water content is defended as the amount of the total water that is present in the solid material. Water content ranges between 30 to 80 percentage, depending also on the solid material characteristics [88,103,104]. For filamentous fungi, the moisture of the solid matrix could be between 20-70 percentage, however, in the case of bacteria, the moisture of the solid matrix should be over 70 percentage.

Metabolic heat, aeration, and absorption characteristics of material directly indicate the water content of the solid substrate. In their study, He *et al.* studied water supply strategies and usage of superabsorbent polymers in solid-state. From their result, they found that superabsorbent polymers can help with increasing the porosity of the solid substrate and made much easier the oxygen transfer and heat dissipation by decreasing the free water between the solid particles. While with the increase of the water content growth and production decreased because of biofilm formation which decreased oxygen transfer to the cells [105]. They stated that the inclusion of water-retention materials and application of negative-pressure auto-controlled irrigation during SSF increased the control for moisture content and temperature gradient through the process, as a consequence product yield was increased. This was stated also in the work of Gervais and Molin [106] that an insufficient amount of water does not allow a good diffusion of solutes and gas, causing a lack of nutrients or high concentration of them acting as inhibitors for the cell growth. Metabolic heat accumulation, evaporation as a result of aeration, and water retention capacity of the solid material are the reason for water loss during solid state fermentation. Shojaosadati *et al.* in their work stated that even water saturated air still causes evaporation in solid state fermentation. While using both saturated aeration and a jacket cooling system decreases the temperature gradient in the solid bed and decreases the water loss [107]. On the other hand,

increased concentration of water affects permeability and transport of nutrients through the cell membrane making the cell perturbed.

In general, water activity should be used as a parameter for the water requirement of microorganisms. Water activity, represent the free water that is free and available for microorganism growth. Below certain water activity depending on the microorganisms their growth might inhibit. Bacteria are considered generally to require higher 0.9 water activity content, while yeast and molds can grow also at lower content mostly not lower than 0.75. The water activity is dependent upon the water-binding properties of the solid substrate. By the time of the SSF process, the water activity might decrease, due to the evaporation process and moisture decrease. This may cause a decrease in specific growth rate, extend lag phase, and low biomass production. This implies the importance of the quantities of water to be added to the substrate for better support of microorganism growth [108].

1.4.2.2. Temperature

The most important physical variable affecting SSF performance is temperature. This variable affects the growth and the production of enzymes. In the case of yeast, this variable takes extra credits knowing the sensitivity of the yeast versus temperature changes. The heterogeneous nature of the solid-state substrate makes it more difficult for temperature control through the fermentation process. Because the continuous phase in SSF is gas, thus the system, in general, has poor thermal conductivity. As a result, temperature gradient through the system can be encountered. Adding the metabolic heat that is produced through the SSF process the problem is even larger. However, the evaporation process and aeration with water-saturated air may have a high impact on heat removal and prevent heat accumulation. This is shown in the work of Figueroa-Montero, where they concluded that heat removal from the tray reactor is improved with increasing the air circulation [109]. The authors Shojaosadati *et al.* concluded that a simple system of the cold water jacket is a suitable strategy for the reduction of the gradient temperature [107]. In general temperature control during SSF is reported to be a general problem.

1.4.2.3. Oxygen Transfer

Oxygen is an important substrate for yeast biomass growth. Limitation in oxygen transfer limits the biomass growth too. Aeration itself provides oxygen to the solid substrate, it removes out the produced CO₂ and other volatile compounds and metabolic heat produced during fermentation. However, this may also increase the evaporation rate, as a consequence decrease the moisture content of the solid substrate. Aeration rate is determined by growth requirements of the microorganism, production of byproducts like volatile metabolites, gaseous, and heat evaluation. Oxygen transfer on the other hand is affected by many parameters like air pressure and flow rate, the porosity of the solid material, the depth of the solid bed, moisture content of the solid substrate, reactor geometry, and impeller rotational and geometry [108]. Throughout the aerobic fermentation process, the oxygen is transferred from a rising gas bubble into a liquid phase and ultimately to the site of oxidative phosphorylation inside the cell [110].

1.4.3. Inert Material in Solid-State Fermentation

Solid-state fermentation performed with inert material as solid support is considered as a future of the SSF. Whether these support materials are natural substances or synthetic compounds, they are impregnated with a liquid medium suitable to support the growth of the microorganism. This fermentation decreases impurities in the fermentation product and creates a homogeneous aerated medium through the process. Moreover, it enables the control of the medium composition, it further facilitates the downstream process in SSF, and enables semi-continuous or continuous SSF process [111]. This system offers the possibility of using the medium with any desired composition and it has the potential for high-production commercial systems.

Sugarcane bagasse is a known inert support material used for the successful production of enzymes. An example is the work of Mazutti *et al.*, who used sugarcane bagasse

supplemented with corn steep liquor fermented by *Kluyveromyces marxianus* for production of inulinase. After the optimization process, they reached a concentration of 391.9 U/g [112]. Another example is the production of amylase by *Aspergillus niger* using sugarcane bagasse as solid support with 73 percentage moisture concentration while starch yeast extract and KH_2PO_4 were used as a supplement reaching an enzyme activity of 457.82 EU/g [113]. Another agriculture material, wheat straw is used as solid support for phytase production by *Aspergillus ficuum*. Different carbon and nitrogen sources were assayed as a supply for the wheat straw. The highest phytase activity achieved was 16.4 U/gds (gram dry substrate) when they supplied moisten wheat straw with 0.17 g/gds glucose and 0.068 g/gds ammonium sulfate [114].

Artificial (nonorganic) inert materials can be used also as a solid support material for solid-state fermentation. The process itself is convenient for laboratory scale for research but it has also potential on a commercial scale. In the research field, a support material with well-characterized properties and minimum interaction with the biological process is convenient for better evaluation of SSF kinetics and transfer mechanisms [115]. There are several solid artificial materials used like polyurethane foam, perlite, vermiculite, and glass fiber. Zhu *et al.* used polyurethane foam as inert solid support for nuclease production by using *Penicillium citrinum*. They reported comparable results with SSF on wheat bran [111]. Another inert solid support material used was ion-exchange resin by Richiard *et al.* the solid support material was impregnated with the chemical defined medium for the growth of *Aspergillus niger*. They successfully grow the biomass in solid support, and they measured the protein content, with the highest peak after 20 h of fermentation with a concentration around 7 mg/g IDW (initial dry weight) [115]. Lately, another material that attracted the attention of several scientists as an inert solid support, this material is high-density polyurethane foam. In several studies this inert solid support was used to cultivate fungi for protein production, an example is the work of Hu *et al.* where *Trichoderma veride* was used for the production of cellulase. In this study, they investigated the effect of PUF size and PUF weight percentage to solid substrate. They reported an enhancement of enzyme yields by 70 percentage when PUF material was used in the fermentation. During their study they attempt to establish a semi-continues SSF by reusing the PUF materials from the first fermentation to the second. The idea was based on the adsorption of the cells to the surface of the PUF. Their results showed a possibility for PUF material reuse with adsorbed biomass

[116]. Yeast also was cultivated in polyurethane foam, *Pichia pastoris* transformed with laccase gene from *Trametes versicolor* was cultivated in SSF and SmF. Different concentrations of glycerol were used to increase biomass production, while methanol was used as a product inducer and as a carbon source. Results showed that in low carbon source concentration no significant difference was observed between biomass produced by SmF. However, in high concentrations of carbon source more than 100 g/L, high cell density on solid-state fermentation was achieved, while the growth of biomass was inhibited on submerged fermentation due to lower oxygen transfer in a liquid medium. Contrary, product expression in SSF was lower than in SmF. However, when methanol was increased to 63 g/L they reported inhibition of *Pichia* growth in SmF, then laccase production was higher SSF [117]. In another study morphology and size of biofilm of *Pichia pastoris* were analyzed in SSF and compared with SmF, when three concentrations of glycerol were used. Maximal biomass growth was similar in both SmF and SSF when the concentration of initial glycerol where 20 g/L. Also, when the concentration of glycerol was increased by 100 g/L, they reported a significant difference in maximal biomass growth between SSF and SmF, with around 178 percentage higher in SSF than in SmF. They reported higher maintenance energy in SmF than in SSF by 33 percentage [118].

1.4.4. Protease Production in Solid-State Fermentation

Proteases (proteinases) are a group of enzymes capable of hydrolyzing the protein bonds resulting in the production of their constituents amino acids according to this, proteases are considered the most important industrial enzyme gaining around 60 percentage of the total enzymes in the market [119]. There are known three types of proteases based on the optimum pH: acidic, neutral, and alkaline proteases. Acidic proteases have an irreplaceable impact in industries like animal feed, food and cheese processing, etc. [120]. Neutral proteases are so important for the food industry for the reason of fewer bitterness byproducts realized after reaction due to intermediate rate reaction [120]. Meanwhile, alkaline proteases are widely used in industries such as detergent, leather, textile, photographic, food and feed, and chemical industry [119].

Protease production for dehairing in the leather industry was reported [119] by using solid-state fermentation. They used livestock waste as solid material supplemented with organic carbon and nitrogen sources and 140 percentage (v/w) moisture content using *Bacillus subtilis*. They reported a highly active enzyme and with good potential in the leather and detergent industry. As a follow-up, concomitant enzyme production of cellulase and protease using separately *Bacillus cereus* AT and *Bacillus subtilis* IND19 and reported significant improvement in protease production. Besides, for multi-enzyme production using cow dung, additional nutritional supplements are needed.

One of the most important advantages of SSF for enzyme production is the potentiality for using industrial residues at a low price. In this context, an article using feather residues from the poultry industry to produce protease was reported. Both SmF and SSF were used as fermentation processes to make a collation between their indications in protease production by *Streptomyces* sp. 594. The protease produced by SmF resulted in highly thermophilic and more active compared to protease produced in SSF. For the first time in this article, *Streptomyces* are used for protease production by SSF [121]. Moreover, residues like *Saccharomyces cerevisiae* dried cells from the fermentation process were used for alkaline protease production by using *Cellulosimicrobium cellulans*, this medium showed to highly support protease production even though salt enrichment were necessary [102].

The substrate components of a solid medium were screened to improve protease production by *Bervibacterium linens* DSM 20158, substrate components which were analyzed as organic carbon and nitrogen source like rice husk, wheat bran, rice bran, soybean meal, yeast extract, corn steep liquor, casein and beef extract were screened. Moreover, a series of salt solutions were used too, the best composition for protease production they found to be the substrate prepared with soybean meal, wheat bran, $(\text{NH}_4)_2\text{SO}_4$, and a certain size of the inoculum. They found that protease activity increased two-fold with screened composition than in random one, all the components used during their study were low price and easily found which means that cost production will be decreased [122].

Otherwise, to increase the production and to produce the specific proteases, the recombinant transformation was used as a technique. As an example the work of [123], where *Pichia pastoris* was transformed to produce extracellular protease, found in *Mucor circinelloides*. This protease was highly capable of milk coagulation, even though the good result of the transformation, the solid-state was not used as a substrate for recombinant cells. Moreover,

authors [124] compared the production of protease during SSF and SmF by using wild and mutant strains of *B. subtilis*, by treating the wild types of *B. subtilis* with ethyl methanesulfonate they prepared mutant types which they selected based on casein agar plates. Around ten agro-industrial byproducts were checked out for their indication in protease production, wheat bran with 2 percentage addition of soybean was found to give the best result for protease production in both wild and mutant strains, and furthermore protease activity was around 1.96 fold higher by mutant strains than the wild strain.

Inoculum preparation conditions were shown to have a significant indication in protease and amylase production during solid-state fermentation. During inoculum propagation, they used different carbon and nitrogen ratios when maltose and peptone were used as a source of them. A high ratio of carbon and nitrogen during inoculum preparation of *Aspergillus awamori* IOC-3914 brings a notable improvement in both amylase and protease production during solid-state fermentation in babassu cake with 70 percentage initiated controlled water content [125].

Interestingly, concomitant production of enzymes (when two or more enzymes are produced in the same conditions from the same microorganism at the same time) are successfully proceed by SSF. Protease and amylase are known as concomitant enzymes and was study point of the article [125], in their study they tried improvement of the production of this concomitant enzyme from *Bacillus megaterium* B69 by using different agro-industrial waste as a substrate statistical optimized by using designed screening method Placket-Burman. The best production of the concomitant enzyme was by using the mustard oilseed cake, they enriched the activity of both of the enzymes around two-fold by using response surface methodology (RSM) to screen the amount of the variables.

1.4.5. Scaling-up in Solid-State Fermentation

Considerable attention has been spent on either process optimization or selection of substrates in lab-scale for SSF, while studies on scale-up are comparatively untouched. We consider scale-up to be a significant problem in SSF, since optimum production processes

on a larger scale drastically change when compared with the lab-scale conditions. The most momentous parameters in large scale SSF are the temperature gradient through the solid content, evaporation and aeration control related to that, and local pH changes.

In 1997, a 50 L fed-batch reactor was prepared as a pilot plant for production of gibberellic acid. The amount of the wheat bran, in the beginning, was 6 kg plus for each kg of wheat bran, 250 g of soluble starch have been added. The humidity, temperature, and aeration flow temperature were monitored with probes inserted into the reactor. The reactor was fed with 500 g of cornstarch and 500 mL of distilled water, every 24 hours after the 72nd hour. The work aimed to minimize the temperature gradient (both vertically and horizontally) and control the dry matter percentage of the solid medium inside the reactor by using aeration, agitation, and the jacket from the outside of the reactor. They report that during the initial stages (up to 75-100 h), maintaining the outlet temperature is easy, while the inlet air temperature should be decreased considerably in later stages of the fermentation. The amount of gibberellic acid per dry matter produced was around 3 g per kg dry matter, and it is worth mentioning an aseptic auto-sampler is used to take samples every time during the fermentation process [126].

Oostra *et al.* reported a cooling system in SSF to produce *Coniothyrium minitans* spores which are used as biopesticides. For bioreactor selection and scale-up, they used lab experiments and mathematical simulation. Based on their results, conductive cooling through the wall in the packed-bed reactor (non-mixed) is unproductive but it gives a good effect in decreasing the temperature in a scraped drum reactor (mixed). Additionally, they reported forced aeration for temperature control inside the reactor, which is found to be effective but with a side effect as substrate shrinkage resulting from evaporation. Saturated air was found to be not effective. They conclude that mixed reactors with conductivity cooling seem the best option for better production in large scale reactors [127]. In contrast with previous work, by using Koji room 200 kg authors [128] suggested that increasing the humidity during the aeration step decreases the temperature (accumulated from the metabolic activity) and decreases the losses in water content. Based on their outcome authors [129] reported higher enzyme production from the scaled-up process than unscaled one, where they used soy fiber to produce alkaline protease in 500 mL Erlenmeyer with 100 g solid material for lab-scale and in a 4.5 L bioreactor with 1.25 kg of solid matter. They suggested that there is no negative effect on enzyme activity from scaling up the process.

This interestingly contradicts the report in [130] where they concluded that the production is 45 times lower in tray reactors than in lab scale, attributed to the metabolic heat accumulation in tray reactors. They concluded that the tray reactor was not appropriate for polygalacturonase production when cashew apple bagasse is used as substrate.

An alternative to tray reactors in SSF, fixed bed force aerated reactor was proposed in [131]. They used a 2.3 L capacity bioreactor aerated with humidified air. From their work, they suggested that babassu cake was well adapted for the production of hydrolase enzymes in fixed bed reactors. Moreover, inappropriate aeration in large scale reactors can cause additional problems in gas emission during fermentation, as reported in [132]. The most problematic contamination gas emitted during the fermentation process was CH₄ and ammonia, typically related to temperature accumulation.

1.5. HOST ORGANISM *PICHIA PASTORIS*

Pichia pastoris for the first time was identified and described by mycologist and cytologist Alexander Guilliermond in 1920 and named it *Zygosaccharomyces pastori*. While ecologist Herman Phaff in 1950 at the University of California isolated several related strains from oak trees and renamed them *Pichia pastoris*. In the 1970s, a company from Bartlesville called Phillips Petroleum developed media and medium to grow *Pichia* on methanol as a carbon source in high-cell densities >130 g/L, while during the 1980s contracted a biotechnological company to develop a system for heterologous gene expression. Vectors, strains, and molecular methods were developed. Petroleum Company sold its patent for the expression system to RCT and licensed Invitrogen to sell components of the system to researchers. While, in 1995 scientists Yamada *et al.* re-classified into the newly established genus *Komagataella*, split later into two species *K. species* and *K. paffi* [133]. *Pichia pastoris* is a single-celled yeast that is easy to manipulate and cultivate. Another advantage is growth to very high cell densities in a simple defined medium, strongly inducible promoters, high production titers, and numerous molecular manipulation tools are available. Moreover, *Pichia pastoris* cells are eukaryotic microorganisms meaning that are capable of many post-translational modification processes such as disulfide bond formation,

glycosylation, folding and proteolytic processing. These processes are so important for the expression of eukaryotic proteins, which often are produced by prokaryotic cells as inclusion bodies/ biologically inactive proteins. Additionally, *Pichia pastoris* secretes low levels of endogenous proteins, which simplifies the downstream processing consequently making the process cheaper [117]. The ability of these microorganisms to metabolize methanol as a sole carbon source defines them as methylotrophic yeast. Methanol oxidized is used for energy production, growth (biomass production), and product formation [133]. There are two main reasons for the success of the *P. pastoris* expression system. The first one is the strong and efficient methanol inducible promoter from alcohol oxidase I gene AOX1 which is frequently utilized to express foreign genes. Secondly, the preference of this microorganism for aerobic fermentation rather than anaerobic, thus avoiding the generation of ethanol and acetic acid during high-cell density [134]. *Pichia pastoris* is one of the four strains that possess methanol utilization pathways which are *Hansenula*, *Torulopsis*, and *Candida*. This capability is based on the highly expressed genes encoding enzymes for the methanol assimilation and dissimilation pathway. The methanol utilization pathway is shown in Figure 1.5, the utilization starts at the peroxisome where methanol is oxidized in formaldehyde by alcohol oxidase and hydrogen peroxide is released which further was processed by catalase into water and oxygen. Both these two steps occur in the peroxisome where the two enzymes are sequestered. There are two possible metabolic pathway routes for formaldehyde. A part of it is used to synthesize energy through oxidation to CO₂ and formate (dissimilation pathway). The other part of formaldehyde undergoes through assimilation pathway where condensation reaction with xylulose 5-monophosphate (Xu5P), the end products glyceraldehyde 3-phosphate (GAP) and dihydroxyacetone exit the peroxisome and undergoes through a cyclic pathway which convert them into raw material of cellular components, this reaction happens in cytosol [135].

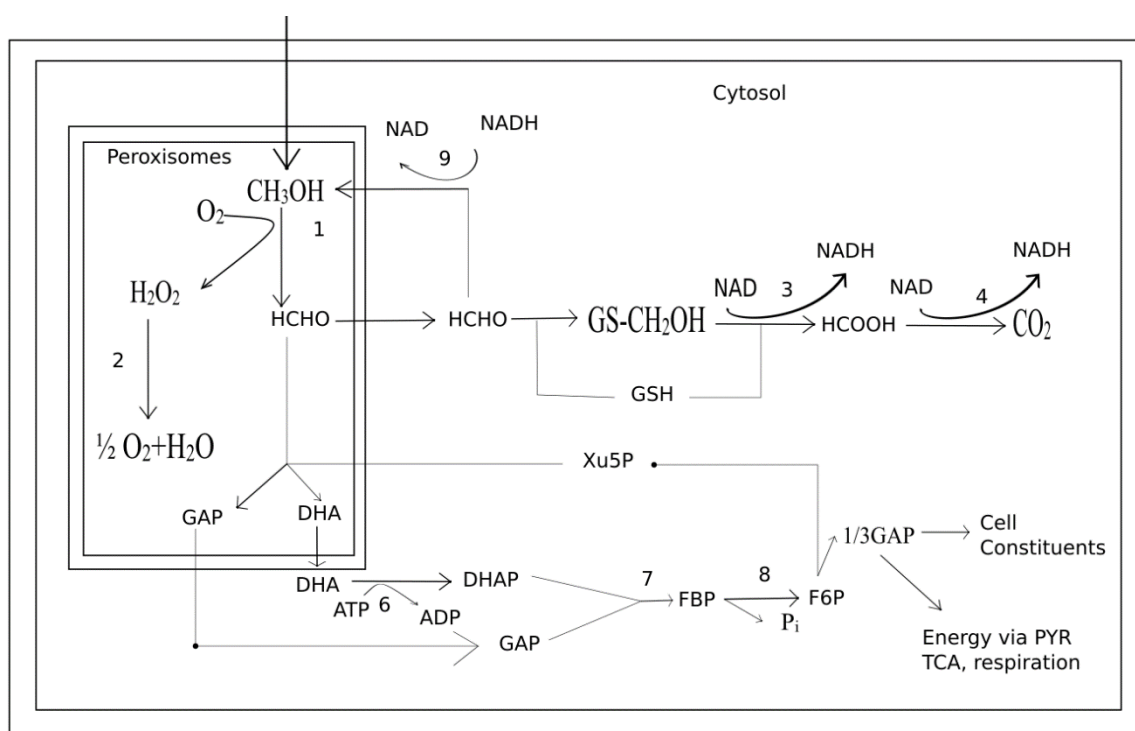


Figure 1.5. Methanol utilization pathways and their compartmentation in methylotrophic yeast. 1. Alcohol oxidase, 2. Catalase, 3. Formaldehyde dehydrogenase, 4. Formate dehydrogenase, 5. Dihydroxyacetone synthase, 6. Dihydroxyacetone kinase, 7. Fructose-1,6-bisphosphate aldolase, 8. Fructose-1,6-bisphosphatase, 9. Formaldehyde reductase [136]

There are two alcohol oxidase genes in the genome of *Pichia pastoris*, *AOX1* and *AOX2*. Regarding their presence in the genome of *Pichia pastoris* there are three phenotypes: 1) MUT^+ known as methanol utilization plus, which contains in its genome both *AOX* genes, 2) MUT^s methanol utilization slow phenotype which contains in its genome *AOX2* gene but lacks the *AOX1*, and 3) MUT^- methanol utilization minus phenotype, where both *AOX* genes are absent Figure 1.6 [137].

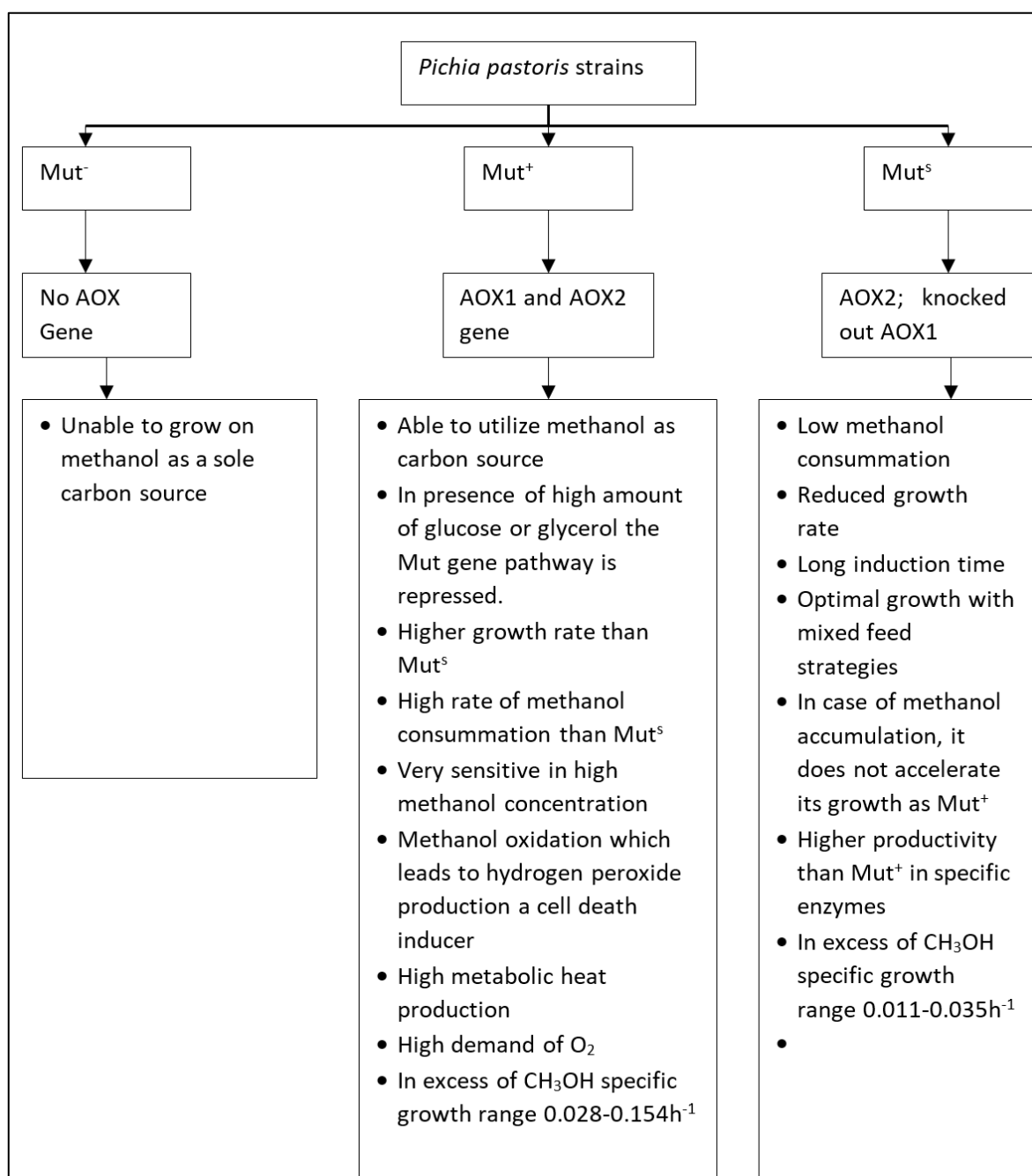


Figure 1.6. MUT strain characteristics of *Pichia pastoris*

These AOX genes enable the utilization of lower primary alcohols as a sole carbon source, methanol more exactly. Sequence analysis of each of them determined a similar protein of 663 amino acids encoded. Although they share 97 percentage identity in amino acid sequence, expression of AOX1 and AOX2 is controlled by different promoters (Cregg *et al.*, 1989). AOX1 constitutes the majority of AOX protein in the cell around 30 percentage and is the main contributor to methanol metabolism, which in some cases is considered a

drawback because of the significant amount of energy and amino acids spent for AOX1 synthesis [138].

AOX1 promoter is considered the most powerful and commonly used promoter for driving protein expression [139]. Being the most popular and strictly regulated methanol inducible promoter it was in the focus of numerous studies for enhancement by deletion or insertion, enriching its library with around 34 variants of AOX1 promoter-driven from native AOX1 promoter [140]. The activity of this promoter is strongly repressed by multiple carbon sources such as glucose and glycerol. However, in the work of Zhan *et al.*, they come up with a solution by deletion of the glycerol transporter GT1 gene in the genome of *Pichia pastoris*. GT1 gene was considered to play a role in the repression of the AOX1 promoter. Achieved mutant strain is reported to be the first one to express heterologous protein by AOX1 promoter induced by methanol in the presence of glycerol in the medium [138].

The presence of the AOX2 gene in the genome of *Pichia pastoris* was proved by the work of Cregg *et al.* in the work they used AOX1 mutant strains which grow slowly in methanol and have low AOX activity, this due to differences in the sequences located at 5' of the protein-coding portion of the AOX1 genes. From their laboratory examination they could not find any selective conditions for AOX2, but since *Pichia pastoris* has conserved the sequence for a second AOX gene (AOX2), argues that this gene provides a selective advantage in the native environment of *P. pastoris* [141].

Regarding metabolization of methanol there are three phenotypes of *Pichia pastoris*: 1) MUT⁺ known as methanol utilization plus, which contains in its genome both AOX genes, 2)MUT^s methanol utilization slow phenotype which contains in its genome AOX2 gene but lack the AOX1 gene, and 3) MUT⁻ methanol utilization minus phenotype where both AOX genes are absent(Figure 1.6) [137].

For secretion of heterologous proteins from *P. pastoris* the most commonly used secretion signal is the α -factor from *S. cerevisiae*. This signal consists of two parts: an N-terminal signal sequence that directs translocation into the endoplasmic reticulum (19-amino acid) and a pro-region that mediates receptor-dependent packaging into the endoplasmic reticulum derived COPII transport vesicles (66-amino acid) [117]. Several research are done in an attempt to optimize the secretion signal for recombinant *Pichia pastoris*, however, the α -factor secretion signal resulted in higher secretion. Such an example is the work of Li *et al.*

where four different secretion signals were used. α -mating signal, a native secretory signal of Human C-reactive protein, invertase signal, and a mutant of the α -mating signal were integrated into the pPICZ plasmid. *Pichia pastoris* cells transformed with a plasmid containing α -mating signal resulted in the highest Human C-reactive protein production. Another successful example is the secretion of immunoglobulin by *P. pastoris* which resulted in more resistance to aggregation than one produced by mammalian cells due to the amino acid residues left by α -factor [117]. Despite the successful secretion of heterologous proteins in *P. pastoris*, this α -factor has drawbacks. This secretion signal promotes posttranslational translocation into the endoplasmic reticulum (ER), so proteins that can fold in the cytosol may be inefficiently translocated and thus poorly secreted. Moreover, if a protein self-associated, the α -factor pro region can potentially cause aggregation, thereby hampering export from the ER [117]. In their research Barrero *et al.* used the Ost1 signal followed by a pro region of α -factor to express red fluorescent protein E2-Crimson. Higher titer was secreted from paired signals compared with only α -factor signals used.

1.6. TRANSFORMATION AND INTEGRATION

Stable integration of expression vector into the *Pichia pastoris* genome is generally based on homologous recombination between sequences shared by the vector and the host genome. These integrants are stable in the absence of the selective agent even when present as multiple copies. Promoters like AOX and GAP have unique restriction site which directs the integration in genome. An illustration of homologous recombination is given below in Figure 1.7.

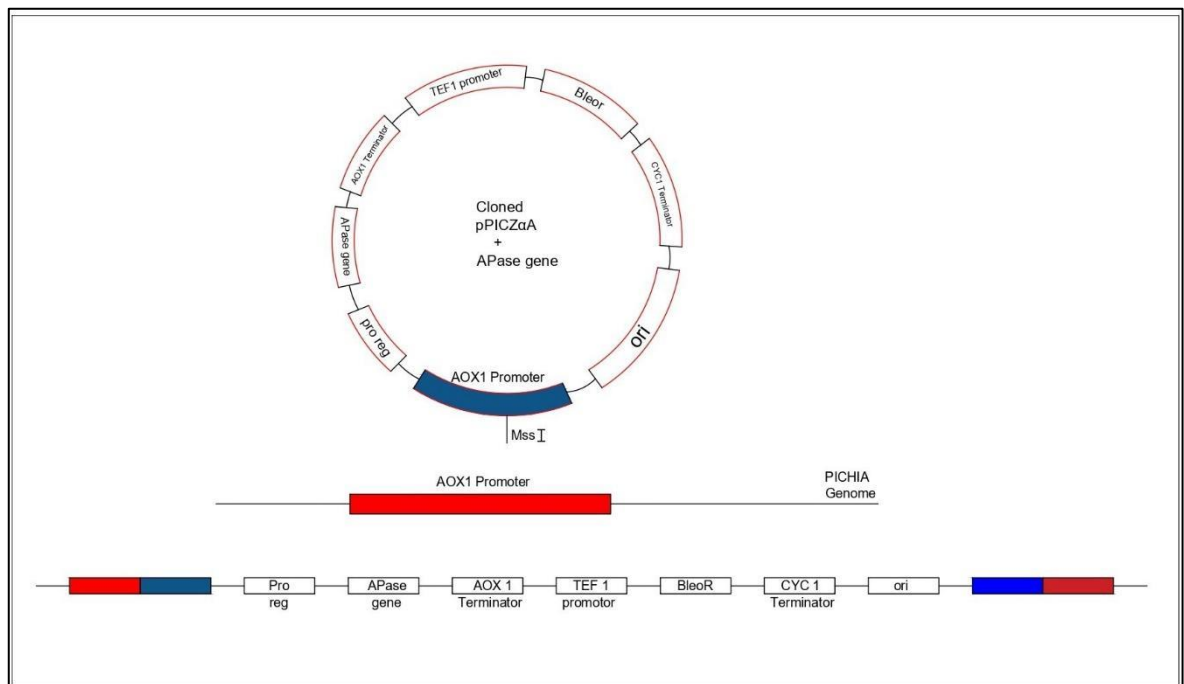


Figure 1.7. Integration of cloned pPICZαA+APase gene in *Pichia pastoris* genome by homologous recombination

However, that is not the only way of gene integration in the *Pichia* genome non-homologous end-joining Figure 1.8. Expression levels of the gene of interests may be influenced not only by the number of integrated genes but also by the integration locus which influences the transcript levels of the integrated genes.

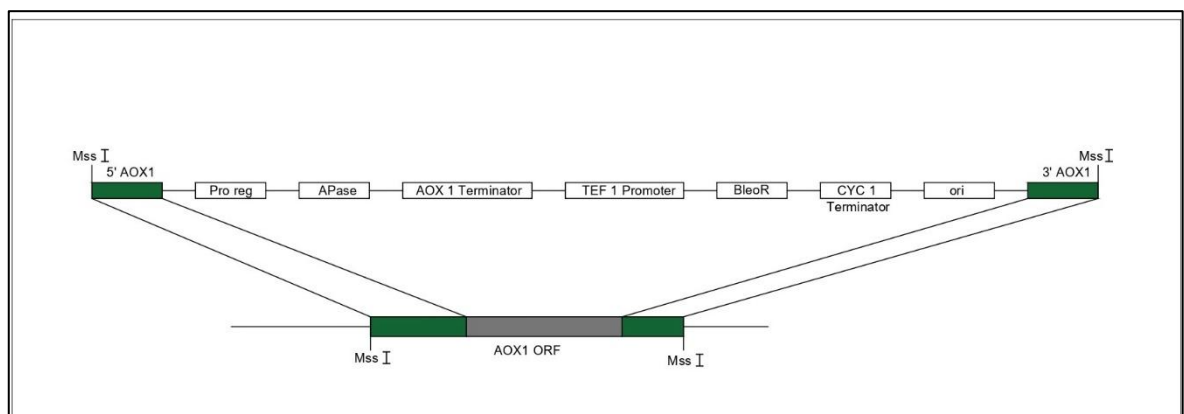


Figure 1.8. Non-homologous end-joining integration

1.7. AIM OF THE STUDY

The demand for specific enzymes for food processing is increasing, with the demand for healthier and eco-friendly food products. Two of these specific enzymes are aspartic protease and transglutaminase, which are used in different food industries like dairy, meat, brewery, and wine industry. These specific enzyme production from native sources are becoming insufficient with time. Hence, the recombinant production of these enzymes by model organisms with known genome sequences is a potential for the industry. *Pichia pastoris* is one of these eukaryotic microorganisms which can grow in a simple medium with high cell density, and easy genetical transformation tools. Recombinant enzyme production by this model microorganisms by using a solid-state fermentation process to our knowledge are scarce, so through this research, we aimed to produce both aspartic protease and transglutaminase recombinantly by transformed *Pichia pastoris* using pPICZ α vector containing AOX1 promoter induced by methanol in solid-state fermentation. Natural inert solid-substrate and artificial solid-substrate were optimized, for better enzyme production. Using inert solid-support moistened with a defined medium gives the advantage for a more controlled and monitoring SSF process. Oxygen transfer on the other hand is an important variable that indicates the biomass and protein produced. Monitoring and controlling it through the SSF is another challenge that we aimed to do by modifying a bioreactor for monitoring the oxygen uptake rate. By this process, we aimed to elucidate more about recombinant enzyme production in a more controlled, aerated SSF process by *Pichia pastoris* host.

2. MATERIALS

2.1. CHEMICALS

EcoRI, *XbaI*, *XhoI*, and *NotI* restriction enzymes were supplied from New England BioLabs. All buffer salts and media components were purchased as pure molecular biology grade chemicals from Sigma-Aldrich and Merck. Organic solvents (ethanol, methanol, isopropanol, acetonitrile) and ammonium acetate were all HPLC grade and purchased from Sigma-Aldrich. Zeocin antibiotic was purchased from Invitrogen.

Macherey-Nagel Nucleospin Plasmid Kit (Catalogue No. 740588.50) was used for plasmid isolation from *E. coli* transformants according to the manufacturer's instructions. Macherey-Nagel NucleoSpin Gel and PCR Clean-up Kit (Catalogue no. 740609.50) was used for gel purification of DNA samples according to manufacturer's instructions. Page ruler Ladder was purchased from Thermo Scientific (Catalogue No 26616). For electrophoresis as a marker, a GeneRuler DNA Ladder (Catalogue No. SM0331) was used.

For ligation process 4T DNA ligase (NEB with catalog No. M0202S). For polymerase chain reaction *Taq* DNA Polymerase from Thermo Scientific (Catalog No. 10342020) was used in the process. *Pichia pastoris* vectors pPICZ α A, B (Catalog No.V19520) were purchased from Thermo Scientific.

2.2. EQUIPMENT

For *Pichia pastoris* electroporation (Bio-Rad) GenePulser II was used, while for gene amplification by PCR T100™ Thermal Cycler by (Bio-Rad) equipment is used. As an incubator shaker (Innova 44) equipment was used. For cell growth with the controlled condition, a bioreactor produced by (Infors HT) named Minifors 2, connected with off-gas measurement (BlueInOne Cell), Air compressor, pH probe and DO probe from (Hamilton).

Centrifuges (Eppendorf 5424 and Gyrozen 1580R), Fume hood (GreenLab), HPLC system with Refractive Index detector (Shimadzu Nexera XR), Aminex HPX-87H Column (300x7.8 mm, Bio-Rad) and IC-Pak Ion Exclusion Column (78x300 mm, waters), Laminar Flow cabinet (GreenLab), Periplastic pump (Watson Marlow), pH meter (Mettler Toledo), water bath (Grant SUB aqua 12Puls), Spectrometer (Genesys 10SUV-VIS Thermo Scientific), Vacuum pump Vortex, Ultrapure water system (Milli-Q, Millipore), Ultra-low-temperature freezer (Thermo Scientific 88000).



3. METHODS

3.1. *ESCHERICHIA COLI* AND *PICHIA PASTORIS* STRAINS

In this study *Escherichia coli* DH5- α was used as an intermediate host, these are engineered to improve transformation efficiency. *Pichia pastoris* wild-type strain ATCC2848 is used as the final host for the expression of the gene of interest. This strain is MUT⁺ possessing both AOX1 and AOX2 genes. Both strains *E. coli* and *Pichia pastoris* were used from the Genetic and Bioengineering Department, Yeditepe University.

3.1.1. Plasmid

Pichia expression vector pPICZ α A from Invitrogen was used as a harboring plasmid for the aspartic protease gene. pPICZ α A is a methanol-inducible vector for the expression of the protein. This feature is based on a strong methanol AOX1 promoter. This plasmid was cut with the same restriction enzymes *Eco*RI (GAATTC) and *Xba*I (TCTAGA) as the gene of interest. While for *TGase* gene, pPICZ α B was used as a harboring plasmid. Restriction enzyme *Xho*I (CTCGAG) 5', *Not*I (GCGGCCGC) were used to cut the plasmid. The amplification of the plasmids were based on the transformed *E.coli* cells and isolation was done with LB-agar + zeocin 25 μ g/mL.

3.1.2. The sequence of *APase* and *TGase*

Codon optimized gene of interest has a positive impact on expression level, by increasing it for two-fold in the work of Mellitzer *et al.* [143], hence aspartic protease gene (JQ906105.1) of *Mucor circinelloides* [124] and *TGase* gene from *Streptomyces hygroscopicus*

(HM231108.1) are codon optimized for *Pichia pastoris*. Then, extra nucleotide and recognition sites for restriction enzymes *EcoRI* (GAATTC) 5', *XbaI* (TCTAGA) 3' and *XhoI* (CTCGAG) 5', *NotI* (GCGGCCGC) were added in the sequence of *APase* and *TGase*, respectively. Restriction enzymes were chosen based on the DNA sequence and multiple cloning sites of the pPICZα-A for *APase* and pPICZα-B for *TGase*. Designed sequences were *de novo* synthesis at Twist Bioscience as cloning fragments into the plasmid. This plasmid was equipped with an ampicillin resistance gene. Below in Figure 3.1 is shown the sequence of the optimized *APase* gene aligned with native *APase* gene. Where, nucleotides underline with asterisk shows similarities between sequences, while empty gaps represents the differences. Same was shown in Figure 3.2 for *TGase* gene.

Optimized	ATGAAATTCCTCCTAGTAAGTCTTGTGTCGCCCTTGTGGTTATGACTCTTGCTGTTGACGACGCCCATCTGGTTCCAAGAAGCTGAGTGTCCCTCTTGCTAAAAACGAAGACTACCAAG
Native	ATGAAATTCCTCCTAGTCTCTTCTTGTGTCGCTCTGGTTGTGATGACTCTGGCAGTCGATGCTGCTCCAGTGGTAGCAAGAAGCTTCCGTTCTCTTGGCTAAAAATGAAGACTACCAA

Optimized	CCTAATATCAAGCGTTCAATAGCTAAAGCTCGTGCCAAGTACATAAAGCACATAAATAACCCCTTAAGGGTGTACCAGCTGGTGTCTACTACTGACGCAACTGGTACTGTTCCAGTGACA
Native	CCCAACATCAAGCGCTCCATTGCTAAAGCCCGTGCAAGTATATCAAGCACATTATCAACCCCTCAAGGGTGTCCCGCCGGCGCTACTACTGATGCCACTGGTACTGTCCCGTTGCTACT

Optimized	GACTATGCCAACGATATAGAGTACTATGGCAGGTCAAAGTAGGTACGCCAGCTCAATCCCTTAAATTAATTTTACACAGGAAGTTGAGATTTTGGTTGTCATCTACGTTGTGCTCT
Native	GATTACGCAACGACATTGAATACTATGTTACTGTCAAGGTCGGTACTCCGCTCAATCCCTCAAGATCAACTTTGATACTGGTTCCTCTGATTCTGGTTTGGTTCACCTTTGTGCTCT

Optimized	ACTTGACAACACATACAAGGTATGACCAACGAAAAGTTCTACTTACGTGGCAGATGGAAGAGCCTGGAGTATCCAATACGGAGATGGTTCTACTGCTTCTGGTGTGCTAGCCAAGGAC
Native	ACTTGACCACTCACACTCGTTATGATCCCAAGTCCAGCACCTATGTTGCTGATGGTGTGCTGCTATCCAATACGGTATGGTTCACTGCTAGCGGTGCTTGGCCAAGGAT

Optimized	ACCGTGAACCTTGGCGGACTTACTATTAATCCCAACGATCAATCTTGCTAAAAAGGAATCATCTTCGCTTACAGCCCATAGATGGTTTAAATGGGATTGGGATTGACACCATT
Native	ACTGTCAACTTGGGTGGTCTACCATCAAGAGCCAACTATCAACTTGGCCAAGGAAGATCCAGCAGTTTGTAGTGATCCTATTGATGGTCTATGGGTCTCGGTTTGGACCACTC

Optimized	ACGACGGTTGACGGCATAAAGACACCAAGTGGATAAATTAATCAGTCAGGGTCTTATCAGTTACCCGCTATGGAAGTTTGGTTAAGTAAGGCCAAGAAGCGTGGTGGCGGTGAGTACTTA
Native	ACCAGTGTGCTGGTATCAAGACTCCTGTTGATAAATGATCAGCAAGGTTTGTCTCTTCTGTTACGGTGTCTGGCTCGGTAAAGCCAAAGATGGTGGTGGTGAATACTTG

Optimized	TTTGGCGGATTAATCCCAACCACTATACTGGAGCACTTACCACCGTCCAGTCGATAATTCTCAAGGATTCTGGGGTATTACTGTTGGTAGTTTGAAGGCAGGAACGACCTCCGTAACA
Native	TTTGGTGGTTCAACCCCAACCACTTACACTGGTGTGTTGACTACTGTTCTGTCGATAAATCTCAAGGCTTCTGGGGTATTACAGTTGGTAGTCTCAAGGCTGGTACTACCTCTGTCACT

Optimized	GGTAGTTTCTCTGGTATCCTGGATACGGGTACCAAGCTGCTGTTATTTCTCAATCAATAGCAAAATAAGTCGCGCTCAATATGGCGCCCGTGACAACGGAGACGGTACCTACACGATA
Native	GGTTCTTTTCAAGCGTATCCTGATACTGGTACCACTCTCTGCTCTTCCCAATCCATTGCCAACAAGGTTGCTGCTCAATACGGTGCCAGAGACAATGGTGACGGTACTTACACTATC

Optimized	TCTGCTCTACGGCAATTTAAACCTTTAACTTCACGATTAAATGGTGCTCAATTCCAGGTTCCAGTCGATTCACTGATCTTTGAACAAGAGGTTCAACGTGTTATGCAAGTTTCGGA
Native	AGCTGTAGCACCGCAACCTCAAGCCTCTTAACCTTACCATCAACGGTGCTCAATTCCAAGTTCTGTTGATTCCCTCATCTTTGAACAGGATGGTCCACTTGTATGCAAGCTTCGGT

Optimized	TATGCAGGTTTAGATTTCGCAATCCTTGGAGATGTTTCTTGA AAAACAATTATGTCATATTTAACGAAAGGTCGCCAAGTTCAGATTGCTAAGTCCGTTTAA
Native	TACGCTGGCCTTGACTTTGCTATTTGGGTGATGCTCTTGAAGAACACTATGTTATCTTTAACCAAAAGGTCCTCAAGTCCAATTGC AAAATCTGCTAA

Figure 3.1. Differences and similarities between codon optimized and native *APase* gene sequence. With gaps are labeled the differences, with asterisk are labeled matched nucleotides between sequences

Optimized	GGCGAGTGCCCTCTGCGAGGAGATCAGACCTGGCCAGAGAGGATAGGGTCATGGAGCGCTGCATACAGGAACCTTTCTTTGAGGAGATCATTGGCTTGCTCCCGGTCAAGGAAT
Native	GGCGGCGTCTCTTCTGTCGACGGCAGCGACCCAGGACAGCGCGCTGAGGTCACGGAGCGGCTGCTTACCGCAACCTCTCATTGCGTCGTTGCTCGCATGCCCGTTTCACGAAAT

Optimized	TCTACTACTAGAGAGAGTCTATTTTCATGTATAAGAGACGTAGTTTACTAGCTTTTGCTACTGTATCAGCCGCATTTTCACAGCCGAGTAATGCCTCCGCTCCCATGCAGCAAGT
Native	TCCACAACAAGGGAGTCACCGATTTTCATGTACAAGCGTCGGAGTTTACTCGCCTTCGCCACTGTGAGTGCGGCGATATTACCCTGGAGTCATGCCCTGGTCAGCCATGCCCGCAGC

Optimized	GGCGGAGACGGCGAAAGGGAAGGATCTATGCTGAGACCCACGGCCTTACCGCCGAGGATGTCAAAAATATTAAATGCCCTAAATAAGCGTGCTTTGACCGCTGGACAGCCAGGTAATTC
Native	GGCGGCGACGGGAAAGGAGGGGCTCTACGCCGAAACGACGCTGTGACGGCGGAGGACGTCAAGAACATCAACGCACTGAACAAAAGGGCCCTGACTCGGGTCAACCTGGCAATTCT

Optimized	TTGGCTGAATCTCTCCATCAGTATCAGCACTATTAGGGCCCTGATGAGCCGATGAGAGGGTCACCCCGCGAGAACCATTAAACAGAATGCCGATGCTTACAGAGCTTATGGT
Native	CTGGCGGAATTGCCCGCAGCGTCAGTGCGCTCTTCCGGGCCCGACGCTGCCGACGAGAGGGTGACCCCTCTGCGAGCCGCTCAACCGGATGCTGACGCTACCGGGCTACGGA

Optimized	GGCAGAGCCACTACAGTCGTTAATAATTACATAAGGAAATGGCAGCAGGTGTATAGTCATAGGGACGGAATTCAGCAGCAGATGACTGAAGAACAGCGTGAGAACTGAGTTACGGTTGT
Native	GGTAGGGCCACTACGGTCGTCAACAACATACATACGCAAGTGGCAGCAGGTCTACAGTCACCGCGACGGCATCCACAGCAAAATGACCGAAGAGCAGCGAGAAAGCTGTCTACGGCTGC

Optimized	GTGGGAGTTACTTGGGTCAACAGTGACCTTATCTACTAACAAGTTGGCCTTGGCTTCTTCGATGAGGACAAGTACAAGTCGACTTGGAAGACTCACGTCCTCGCTTCAACGAGACC
Native	GTCGGCGTCACTTGGGTCAATTCGGGCCCTACCCGACGAACAATTTGGCGTTTCGCTTCTTCGACGAGGACAAGTACAAGAGTGACCTGGAAAACAGCAGGCCACGCCCAATGAGACG

Optimized	CAAGCAGAATTCGAGGGCGTATTGTCAAAGATAGTTTCGATGAGGGTAAGGATTTAAGAGAGCTAGGGATGTGGCTTCAATAATGAATAAGCATTGGATAGTGCACATGACGAAGGA
Native	CAAGCCGAGTTTGAGGGGCGCATCGTCAAGGACAGTTTCGACGAGGGGAGGGGTTCAAGCGGGCGGTGATGTGGCTCATATGAACAGGCCCTGGATAGTGCACGACGAGGGG

Optimized	ACGTACATAGATAATCTAAAAAAGAGTTAGCCAATAAGAATGATGCATACGATACGAAGATTACGTTCCAATTTTATTACGCTTTGAGAATAACCCCTCCTTCAAAGAGCGTGAC
Native	ACTTACATCGACAACCTCAAGAAAGAGCTCGCAACAAAAATGACGCTCTGGCTACGAGGACAGTCGCTGAACTTTTACTCGGCGCTGAGGAATACGCCGCTCTTCAAGGAAAGGGAT

Optimized	GGAGGTAACATACGATCCATCAAAGATGAAAGCAGTAGTATTTCCAAGCATTCTTGGTCAGGTACAGGATCAGAGAGGATCTAGTGACAAGCGTAAGTATGGTGACCCCGACGATTACGG
Native	GGAGGCACTACGACCCATCCAAGATGAAGCGGTGGTCTACTCGAACAACCTTGGAGCGGGCAGGACCAAGCGGGCTCCTTGACAAGAGGAAGTACGGCAGCCCGGATGCCTTCGC

Optimized	CCCGATCAGGGAACCGGATTGGTAGATATGTCTAAGGATAGGAATATACCAAGATCTCCCGCAAGGCCGGAGAGTCATGGGTGAATTTTATTATGGATGGTTCCGGCAGACAGCCGAG
Native	CCCGACCAAGGGCAGAGCCTGGTCGACATGTGCAAGGACAGGAATATTCCGCGAGTCCCGCCGACCTGGCGAAAGTTGGGTGAATTTGACTACGGCTGGTTGGGGCTCAGACGGAA

Optimized	GCCGATGCCGATAAGACTATTGGACACATGCTAACCACTACCATGCTCCCAACGGCGGCTCGGACCAATGAATGTCTATGAGTCTAAATTCAGGAATTTGGTCCGGGATACGCCGAT
Native	GCGGATGCCGACAAAACCATATGGACCCAGCCAAACCATATCACGCGCCAAACGGCGGCTGGGGCCCATGAACGTATACGAGAGCAAGTTCCGGAACCTGGTCTGCCGGGTACGCGGAT

Optimized	TTTGATAGGGGCACTTACGTTATCACATTCTTCTAAGTCATGGAATACGGCCCCGAGAGGTGAAACAAGGTTGGTCCTAA
Native	TTGACCGCGGAACCTACGTCATCAGTTTCATACCAAGAGCTGGAACACCGCCCCGCGAGGTGAAGCAGGGCTGGTCGTAA

Figure 3.2. Differences and similarities between codon optimized and native *TGase* gene sequence. With gaps are labeled the differences, with asterisk are labeled matched nucleotides between nucleotides

3.2. CLONING

3.2.1. Preparation of Commercial Plasmid

Received plasmid with gene of interest was treated in accordance with the guideline of the company that synthesized them. Plasmids with the gene of interest were received in our laboratory in the 96 well plate as powder weight 1000 ng. In the well, 100 µl of the TE Buffer was added to dilute the plasmid. The content of the well was placed in a labeled sterile

Eppendorf tube, the final concentration of the plasmid was 10 ng/μl. Eppendorf tubes were stored at -20 °C.

3.2.2. *Escherichia coli* Competent Cell Preparation

E. coli DH5- α culture was streaked into fresh LB-agar and incubated at 37°C overnight. A fully grown single colony from the plate was transformed with a sterile loop into 5 mL LB-broth media in a 50 mL falcon tube and incubated at 37 °C, 150 rpm for 14 h. After 14 h of incubation, 2 mL of the culture is added into 200 mL LB-broth and incubated at 37 °C, 150 rpm for around 1.5-2 h until the OD 600 nm reaches between 0.35-0.45.

The content was transferred to 50 mL sterile falcon tubes which were placed on the ice for 5 min. Tubes were centrifuged at 2000 x g for 10 min at +4°C. Pellet was dissolved by using 20 mL of ice-cold improved buffer (15 percentage glycerol and 70 mmol CaCl₂), the content was incubated for 5 min in ice. After incubation tubes were centrifuged at 1000 x g for 6 min at +4°C. The supernatant was discarded and the pellet was resuspended with 1 mL of improved buffer in each tube. The content was collected in one tube and aliquoted into 1.5 mL Eppendorf tube as 200 μL culture. Cells were immediately stored at -80 °C [142].

3.2.3. Heat Shock Transformation

Tubes with prepared competent *E. coli* cells from stock were placed into ice, the same was done with a plasmid with a gene of interest. The water bath was set at 42 °C. Into 1.5 mL Eppendorf tubes 100 μL of competent cells are placed and 10 μL of the plasmid with the gene of interest. The content was mixed gently by pipetting. The content was kept in ice for 30 min then tubes were placed immediately in a water bath at 42 °C for 50 sec. Instantly, tubes were placed in the ice for extra 2 minutes. After the incubation on ice, the volume of the tubes was brought to 1 mL by pre-warmed SOC medium at 37 °C. Tubes were placed in a shaker at 37 °C, 150 rpm for 1 h and 45 min.

The process was followed by a centrifugation step at 3000 G for 4 min. 800 μ L of the supernatant was discarded the remaining 200 μ L was used to resolve the pellet. 100 μ L was spread to the pre-warmed LB agar plates with 100 μ g/mL ampicillin. Untransformed competent cells are used as blank. Plates are incubated at 37 °C overnight. The appearance of colonies means that we have positive transformation. No colonies should appear in the blank [142].

3.2.4. Plasmid Isolation

Nucleospin Plasmid Isolation Kit from Macherey-Nagel was used for plasmid isolation. The protocol was based on the guideline of the producer. A single transformed colony was used to prepare inoculum in 5 mL LB-Broth with 100 μ g/mL ampicillin and incubated at 37 °C at 150 rpm. After 18 h tubes were centrifuged at 4000 x g for 5 min. The supernatant was discarded and the pellet was dissolved by 250 μ L A1 buffer solution from the kit, content was transferred to 1.5 mL Eppendorf tubes. 250 μ L of A2 buffer was added too, tubes were inverted 8 times, and incubated at room temperature for 5 min. Then, 300 μ L of A3 buffer was added and content was mixed by inverting 10 times. Eppendorf tubes were centrifuged at 11000 x g for 5 min. Columns were placed in collection tubes 750 μ L of supernatant was poured into columns, then centrifuged for 1min at 11000 x g. Supernatant was discarded and columns were placed back to collection tubes. 600 μ L of the A4 solution was added, tubes were centrifuged for 2 min at 11000 x g, and columns were placed into Eppendorf tubes. To the columns, 50 μ L of the elution buffer was added and incubated at room temperature for 1min. Tubes with columns are centrifuged at 11000 x g. Columns were discarded, while the tubes with content are stored at -20 °C.

3.2.5. Measurement of Plasmid Concentration

The concentration of the plasmid and DNA was measured by a NanoDrop spectrophotometer. The machine was cleaned with dH₂O and laboratory papers. Then 1 μ L

of the elution buffer is placed in Nano-drop which was read as blank. After the blank measurement, we read the samples by taking from each sample 1 μL of sample and press the button read. Concentrations are read in $\text{ng}/\mu\text{L}$. While the purity of the DNA was provided by the ratio between absorbance A_{260}/A_{280} which should be between 1.8-1.9 [143].

3.2.6. Double Digestion Protocol

For the double digestion of the plasmid and gene of interest to form sticky ends in both of them, Thermo-scientific restriction enzymes and their protocol was used Table 3.1.

Table 3.1. Double digestion protocol from Thermo-scientific

<i>APase</i>		<i>TGase</i>	
Buffer O	2 μL	Buffer O	2 μL
<i>EcoRI</i>	1 μL	<i>XhoI</i>	1 μL
<i>XbaI</i>	4 μL	<i>NotI</i>	1 μL
DNA pPICZ α A/ <i>APase</i> gene	1 μg DNA	DNA pPICZ α B/ <i>TGase</i> gene	1 μg DNA
Nuclease free water	up to 20 μL	Nuclease free water	up to 20 μL

3.2.7. Gel Electrophoresis

1 percentage agarose gel was prepared with 1 X TAE buffer and 1.5 μL ethidium bromide. Prepared gel was carefully poured into the casting tray. To run the gel, 10 μL of the dye was mixed with 50 μL of DNA sample and loaded to the gel well. The gel was run at 100 V per 45 min [144].

3.2.8. Ligation

NEB enzymes and buffers are used for the ligation procedure between digested plasmid and gene of interest (Table 3.2.).

Table 3.2. Ligation procedure

TN4 Buffer	2 μ L
Ligase	1 μ L
Vector DNA	50ng
Gene of interest DNA	37.4ng
Nuclease free water	up to 20 μ L

Treated DNA was incubated at 16 °C overnight and was heat-inactivated at 65 °C for 10 min. Figure 3.3 is shown the ligation of the *APase* gene in the double digested plasmid of interest pPICZ α A. Both *APase* gene and pPICZ α A plasmid are cut in sticky ends by *Eco*RI and *Xba*I restriction enzymes.

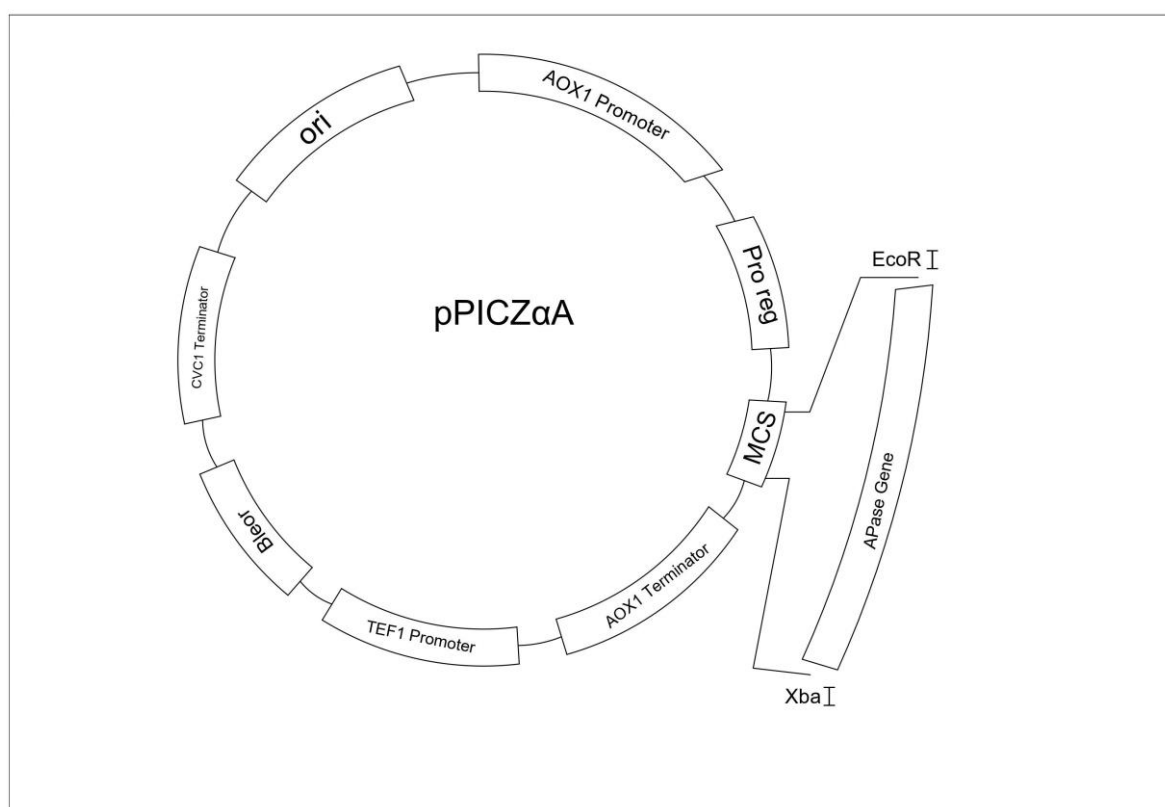


Figure 3.3. Cloned pPICZαA plasmid with *APase* gene

3.2.9. Linearization

Positive clones were linearized by using the *Pme*I unique restriction enzyme which is located in the AOX1 promoter site in the plasmid (Table 3.3). Linearized vector DNA can generate more stable transformants of *Pichia pastoris* via homologous recombination integration (Figure 1.7.) between the sequences shared by plasmid and host genome, single crossover [145].

Table 3.3. Linearization protocol

Buffer B	5 μ L
DNA of cloned plasmid with gene of interest	1 μ g
<i>Pme</i> I	2.5 μ L
Nuclease free water	Up to 50 μ L

The content was incubated at 37 °C for 1 h and 30 min. Electrophoresis is used to check if the linearization is finished. The reaction was terminated by heat treatment at 65 °C for 30 min.

3.2.10. Purification and Concentration of Linearized Clone (Phenol-Chloroform Treatment)

Phenol-Chloroform (5:1) was used to linearize DNA in ratio 1:1, the content was vortexed and centrifuged at 2000 rpm for 5 min. The top layer is pipette carefully to a new tube. 0.1 volume of the top layer of Sodium acetate 3 M pH 5.5 and 2.5 volume of the whole solution was added 100 percentage ice-cold alcohol was added to the top layer. Tubes were incubated at -20 °C for 12 h. Afterward, samples are centrifuged at 14000 rpm at +4 °C for 15 min. The supernatant was discarded, and 2 volumes of 80 percentage ethanol were added to the pellet, incubated for 5 min to room temperature. Then tubes are centrifuged for 5 min at 14000 rpm, +4 °C. Discard the ethanol, place the tubes at room temperature to evaporate the remaining ethanol for 15 to 20 min. Add 50 μ L nuclease-free water, measure the concentration in Nanodrop.

3.2.11. Preparation of *Pichia pastoris* Electrocompetent Cells

Pichia pastoris single cell was used to inoculate 10 mL YPD culture, culture is grown overnight in shaker 150 rpm at 30 °C. The overnight culture is used to inoculate 200 mL of YPD medium in 1 L Erlenmeyer with an OD600 0.1 abs.

The culture was grown until the OD600 reached between 1.3-1.5 abs. Content is divided into four 50 mL falcon tubes. The culture is harvested by centrifugation at 2000 x g, +4 °C for 10 min. Pellet in each tube was re-suspended with 10 mL medium composed of 80 percentage YPD broth and 20 percentage HEPES. Then, 0.250 mL of 1 M DTT was gently added to each tube, which was incubated at 30 °C for 15 min. Each falcon tube was filled with cold sterile water up to 50 mL. A centrifugation process was applied again at 2000 x g, +4°C for 10 min. Each tube was washed one more time by 25 mL of ice-cold sterile water. Followed by washing with 2 mL of 1 M sorbitol solution each, and the content was collected to one tube. After last centrifugation, the remaining pellet was re-suspended with 0.2 mL ice-cold 1 M sorbitol. 40 µL of prepared electrocompetent *Pichia pastoris* cells were aliquoted in a 1.5 mL Eppendorf tube and stored at -80 °C.

3.2.12. Electroporation

Electroporation cuvettes are placed in ice, tubes containing competent cells, and linearized DNA too. 100 ng of DNA were transferred to Eppendorf tubes containing 40 µL of competent cells. The content was mixed by pipetting and transferred to electroporation cuvettes 0.2 cm gap vial, cuvettes were incubated for 5 min in ice. After incubation, an electroporation pulse was applied at 1.5 kV, 25 µF, and 200 Ω. 1 mL of ice-cold 1 M sorbitol was used to dilute electrocompetent cells. Cells immediately were transferred to 15 mL falcon tubes. Tubes were incubated for 2 h in the incubator shaker at 30 °C, 150 rpm. Then 150 µL of culture is spread to YPDS agar with 100 or 200 ng/mL zeocin as a selection antibiotic. Single colonies appeared were streaked in the same agar two times more for a

better selection of the right clone. One colony was used to create the glycerol stock at -80 °C [147].

3.2.13. Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-Page is used to determine the molecular weight of protein-based on their migration through the SDS gel. The method is based on the protocol of Bio-rad. First, a Bio-rad casting system was set, and then resolving gel was prepared as shown in Table 3.4. Prepared resolving gel is poured into the SDS prepared plate, on top of it dH₂O is added. The content was let in rest for 30 min. After polymerization, stacking gel was prepared (Table 3.4.), from the SDS plate water was wiped with paper, and stacking gel was poured, immediately comb was placed to form the wells. The unknown protein sample was mixed with loading dye in ratio 1:1 and treated at 96 °C for 5 min. After treatment, 30 µL of samples were loaded to gel, which was run at 200 V for around 45 minutes until the dye front reaches the bottom of the gel [147].

Table 3.4. Resolving gel (A) and stocking gel (B) composition

Chemicals	A (mL)	B (mL)
H ₂ O	3.92	3.4
30% Acrylamide	4.8	0.83
1.5M Tris-HCl pH 8.8	3.04	-
1.0M Tris-HCl pH 6.8	-	0.63
10% sodium dodecyl sulfate	0.12	0.05
10% Ammonium persulfate	0.12	0.05
TEMED	0.0048	0.005

3.2.14. Polymerase Chain Reaction (PCR)

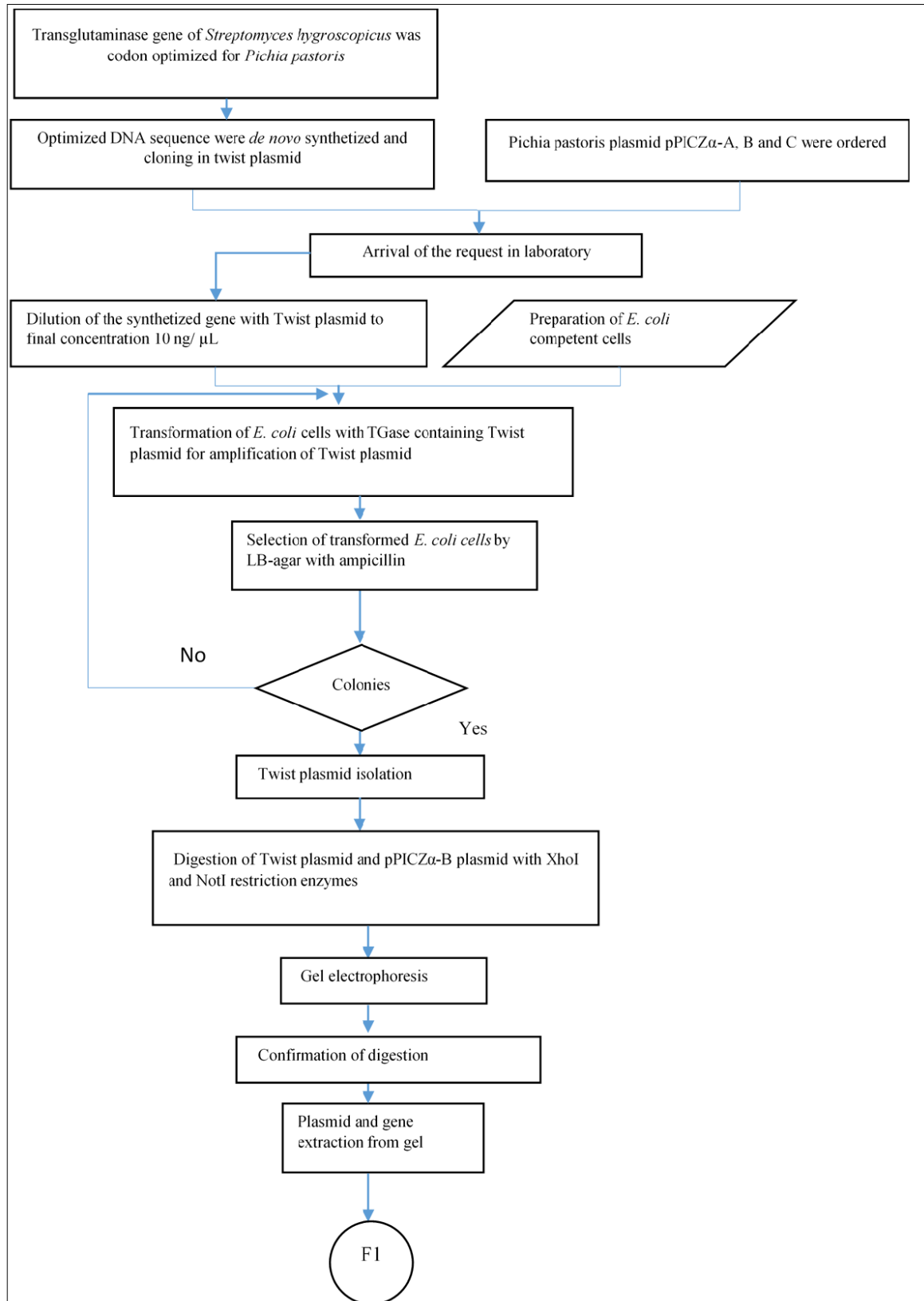
To make sure that integration of the gene of interest in the genomic DNA was done, PCR protocol with 5' specific (Table 3.5.) and 3' AOX1 primers was performed for each gene. In an attempt to optimize this protocol several changes in chemical amounts and in temperature and time of the protocol were tried. The protocol was based on the work of Liu *et al.* [149], with small changes. Initial denaturation was done at 95 °C for 4 min, followed by 40 cycles, (each at denaturation 94 °C for the 60 s, annealing at 55 °C for 60 s, extension at 72 °C for 150 s) and a final extension at 72°C for 10 min. The rest of the chemical and DNA template concentration used was: Template 300 ng; 5 µL of 10 X PCR buffer with 500 mM KCl, 8 µL of MgCl₂ 20 mM; 2 µL of 100 mM dNTP; 4 µL of both forward and reverse primers concentrated 10 mM; and 0.5 µL of Taq polymerase with activity 5 U/µL, and the rest was nuclease-free water up to 50 µL.

Table 3.5. Designed specific primers for aspartic protease gene and transglutaminase gene

Primers	Forward sequence	Reverse sequence
5'AP	TGTGTCGCCCTTGTGGTTATG	GCAAATGGCATTCTGACATCC
5'TG	GGAGTAATGCCTTCCGTCTCC	GCAAATGGCATTCTGACATCC

3.2.15. MUT Phenotype Assesment

MUT phenotype of the transformed *Pichia pastoris* cells was assesses by using Minimal Dextrose agar (2 percentage glucose), and Methanol agar (0.5 percentage methanol) plates. The other components were the same in both agar media, YNB 1.34 percentage, 4×10^{-5} percentage biotin, and 100 ng/mL zeocin as a selection of transformed colonies. In both agar plates, one colony was streaked at the same time and incubated at 30 °C. Plates were inspected each 12 h.



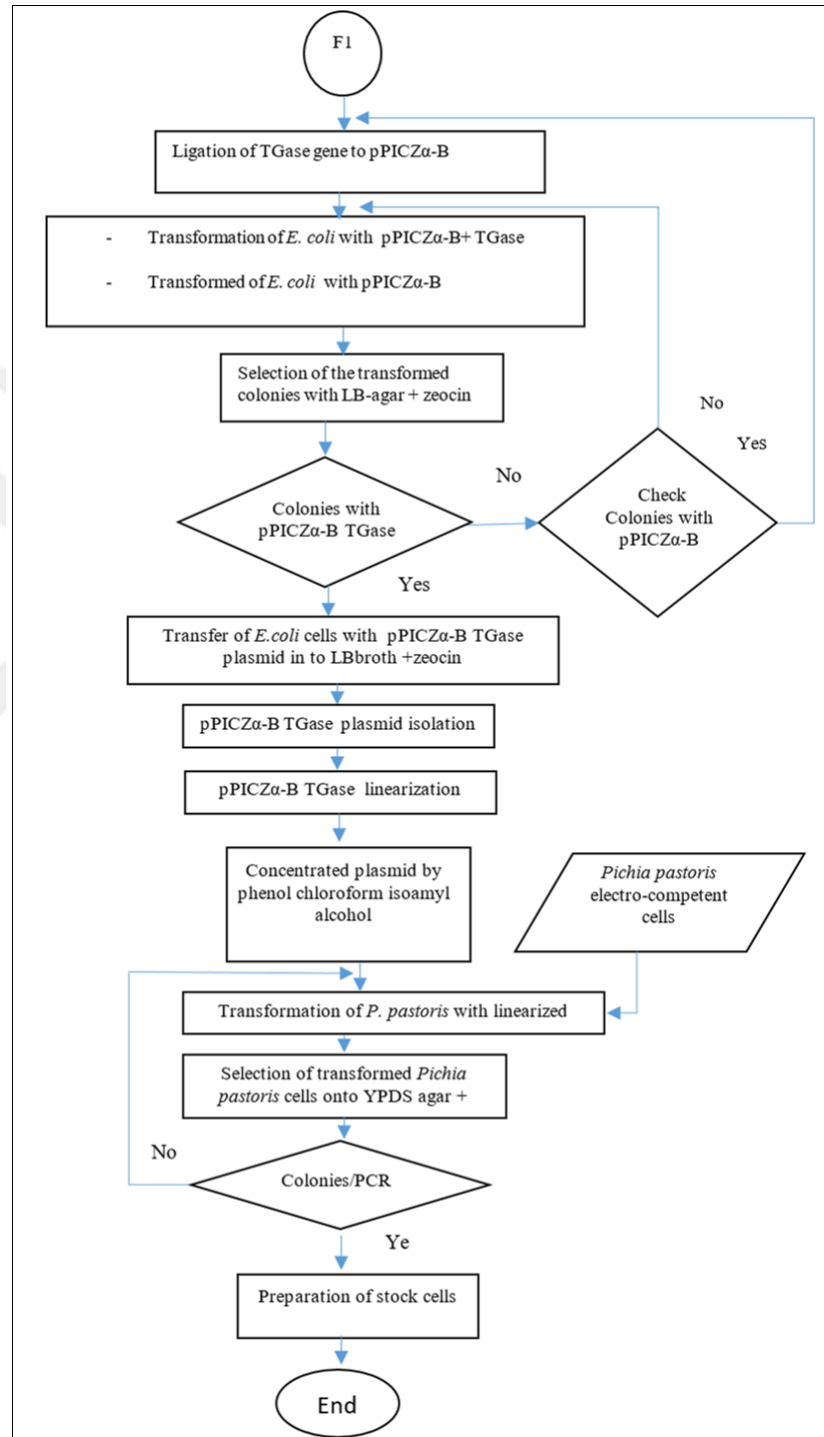


Figure 3.4. Flow chart of cloning process of *Pichia pastoris*

3.3. SUBMERGED FERMENTATION

3.3.1. Batch and Fed-Batch Submerged Fermentation

Cloned *Pichia pastoris* with the gene of interest -80 stuck was streaked in YPDS agar + zeocin 100 µg/mL. After 48 h at 30 °C, a single colony was used to prepare inoculum for the fermentation culture in 25 mL of BMGY medium in 250 mL Erlenmeyer incubated in a shaker at 30 °C and 150 rpm. Grown inoculum for 24 h, was used to prepare the culture in 1 L Erlenmeyer with 200 mL BMMY medium (0.65 percentage (v/v) methanol) incubated at 30 °C and 150 rpm. Samples were taken each 6 h, and growth, enzyme activity, protein content, and methanol utilization was assayed. After the first 24 h batch fermentation, fed-batch fermentation was started by the addition of 1 percentage methanol. All fermentation processes were performed in triplicate, while as a background control fermentation with untransformed *Pichia pastoris* was used.

3.3.2. Scale-up Fermentation in Bioreactor

Scale-up SmF process was performed in a controlled environment in a 6 L bioreactor with a 3 L working volume. Temperature, pH, percentage of dissolved oxygen, and stirring were used to control the fermentation process. The jacket cooling system kept the temperature of the medium at 30 °C, pH was maintained at 6 by 25 percentage ammonium hydroxide, 1 vvm airflow was used to maintain dissolved oxygen higher than 30 percentage. Methanol utilization was followed by HPLC. The inoculum was grown for 24 h in 500 mL Erlenmeyer with 50 mL working volume. Culture growth was initiated by inoculation with a 2 percentage inoculum. To achieve high cell density the first period of fermentation was performed in BMGY media prepared based on the work of Higgins *et al.* [149] with 10 g/L glycerol. After the glycerol depletion 0.4 g/L methanol pulse was sterile injected in the reactor to adopt cells

with a new carbon source. After the adaptation period when methanol was consumed 8 g/L methanol was injected into the culture. Methanol was monitored periodically and when the amount of methanol was lower than 0.1 g/L another pulse of 8 g/L methanol was injected.

3.4. SOLID-STATE FERMENTATION

3.4.1. Solid-State Fermentation with Natural Inert Solid-Support

Solid-state fermentation with natural inert solid support was performed by using 10 g of sieved (between 2-10 mm) wheat straw in each 1 L Erlenmeyer and autoclaved at 121 °C or 40 min. the concentration of solid support was adjusted to 5, 10, and 15 percentage by adding BMMY medium with 0.3 percentage methanol, which was used as moistening material. 10 percentage of 24 h grown inoculum of *Pichia pastoris* was used to inoculate the substrate. Erlenmeyer was incubated at an incubator shaker at 30 °C, 180 rpm. Every 24 h of fermentation 0.5 percentage v/v methanol was added, and three reactors are harvested by washing the solid support with sterile distilled water, 5 mL per 1 g solid support, for 15 min at 180 rpm. Then, a centrifugation step was applied by using 50 mL falcon tubes at 10000 rpm. The supernatant was divided as a crude enzyme and used further.

3.4.2. Solid-State Fermentation with Artificial Inert Solid-Support

Based on the nature of the solid support two different materials were possible, natural solid support and artificial inert solid support. Artificial inert solid support gives much more opportunities for controlled solid-state fermentation, and a much easier downstream process [150]. Hence, for SSF 4 g of 0.5 cm³ polyurethane foam particle with density 0.044 g/mL as solid support was placed in 1 L Erlenmeyer, while 100 mL Verduyn medium with 20 g/L glycerol was added as a moistening and as a nutrient source. 10 percentage of 24 h grown

inoculum in the Verduyn medium is used to start the fermentation process. Inoculated Erlenmeyer was incubated at 30 °C, 180 rpm.

After glycerol depletion, 4 g/L methanol was added in the beginning for an adaption period. Then, after the first pulse of MeOH is spent, 8 g/L methanol is added, this step is repeated after each pulse [151]. The supernatant was harvested by pressing the polyurethane foam in a 50 mL syringe, the collected substrate with biomass, and produced enzyme was centrifuged to separate biomass from the crude enzyme.

3.4.3. Solid-State Fermentation with Inert Solid-Support in Aerated Modified 3L Bioreactor

A solid-state fermentation process was performed in a modified 3 L bioreactor. 40 g of 0.5 cm³ polyurethane foam and 978 mL of Verduyn medium with 20 g/L glycerol were placed in the bioreactor vessel. The bioreactor was autoclaved and prepared for fermentation. Vitamins and minerals are added to the medium before starting the fermentation. 10 percentage v/v inoculum was added to start the fermentation. Aeration was adjusted in the beginning at 1.5 vvm for the first 36 h. After the methanol addition, cascade was activated with aeration and stirring between 1.5-2.5 vvm and 500-900 rpm, respectively. Dissolved oxygen was set to be maintained at over 30 percentage. Oxygen and CO₂ were measured at off-gas during the fermentation process. pH was maintained at 6 by 25 percentage hydroxide ammonium and 6 M phosphoric acid. Samples were taken each 6 h by syringe, the substrate in between the foam was used as a sample. After fermentation is finished, the substrate was collected by pressing the foam in the syringe, which was dried until constant weight. The difference in weight of PUF before and after fermentation was considered as biomass adsorbed in PUF.

As a control, the same conditions were used for SmF, in this case only polyurethane foam is not added.

3.5. ANALYZING THE FERMENTATION AND ITS PRODUCTS

3.5.1. Biomass Growth Estimation

In submerged fermentation biomass was inspected based on the optical density (OD) measurement at 600 nm. To measure the Dry cell weight (DCW) (g/L) produced, a correlation between OD and dry cell weight was calculated. The final fermented substrate was used to prepare 50 mL of 2, 4, 8 times diluted substrate in pre-weighed 50 mL falcon tubes. After the content was inverted well, OD 600 was measured. Samples were centrifuged at 7000 rpm for 10 min. Pellet was washed with 25 mL sterile distilled water two times. Then cell paste was dried at 100 °C for 24 h until the constant weight was reached. Before the last weight measurement tubes with the dry cells were cooled in desiccators. A linear regression equation was built and used for the other estimation of DCW [152].

3.5.2. Protease Activity Measurement

Quality APase activity was measured based on 5 percentage skim milk agar. 50 µL of supernatant from fermentation with transformed *Pichia* cells were placed well in the agar. The diameter of the clear halo formed around the well was to measure the APase activity expressed. As a control supernatant from fermentation with *Pichia pastoris* wild strain was used. Non-specific APase activity assay is based on the L-tyrosine reaction with Folin and Ciocalteu's Phenol reagent producing a blue-colored chromophore which can be measured as absorbance value in spectrophotometer. To perform the assay, 130 µL of 0.65 percentage w/v casein solution (pH 7.5 and 5.8, phosphate 50 mM and sodium acetate buffer 0.1 M, respectively) was used as substrate and placed in reaction with 25 µL sample for 10 min at 37°C. 130 µL of 10 percentage trichloroacetic acid was used to stop the reaction. After incubation for 20 min tubes were centrifuged at 10000 rpm for 5 min. 250 µL supernatant were placed in new tubes, 625 µL of 500 mM Sodium Carbonate, and 125 µL of Folin-

Ciocalteu were added to the supernatant. After incubation for 30 min at 37 °C, samples were read at 660 nm in 1 mL cuvettes. A standard curve was built with L-tyrosine, one unit of protease activity was defined as the amount of enzyme required to liberate 1 μmol of L-tyrosine/mL/minute [153].

$$\frac{U}{\text{mL}} = \frac{\mu\text{mol tyrosine} \times \text{Total volume of assay}}{\text{volume of enzyme} \times \text{time of reaction} \times \text{volume used in colorimetric determination}} \quad (3.1)$$

3.5.3. Quality Activity Assay for TGase

The quality test is based on analyzing the cross-links between the BSA proteins detected based on SDS PAGE. 5 μL of BSA (10 mg/mL in 0.1 M Tris-HCl, pH 8.0) are mixed with 10 μL of 0.1 M Tris-HCl with or without 1 mg/mL recombinant TGase. The total volume is made up to 40 μL by adding 2 mM Dithiothreitol DTT solution. The reaction solution is incubated for 2 h at 50 °C or 12 h at 4 °C, then 30 μL of content is run in 12 percentage SDS-PAGE and is stained with Coomassie Brilliant Blue R-250 [154].

3.5.4. Protein Measurement

Protein quantification by bicinchoninic acid (BCA) in complex medium fermentation often is interfered by media substances like glucose, glycerol, mercaptoethanol, and dithiothreitol [156]. The interference of these substances can be eliminated by protein precipitation. A precipitation assay was applied before the BCA method application. In 1.5 mL Eppendorf tubes, 1 mL of the sample was placed and 0.1 mL of sodium deoxycholate (DOC) 0.15 percentage w/v was added. The content was mixed and was incubated at room temperature for 10 min. Then, 0.1 mL of trichloroacetic acid (TCA) (72 percentage, w/v) was added. The content was vortexed and then centrifuged at 3000 x g for 15 min. The supernatant was discarded and the pellet was solubilized by vortexing with 0.05 mL of sodium dodecyl

sulfate (SDS) 5 percentagew/v prepared in 0.1 M NaOH. After this sample was ready for protein measurement.

The BCA method was applied based on the guideline of the producer of the kit (QuantiPro BCA Assay Kit, Sigma-Aldrich). The method was performed in 96 well plates where 25 μ L sample or standard was treated with 200 μ L of prepared WR reagent. The plate was incubated in a water bath at 37 °C for 30 min. It is cooled at room temperature and samples were read at 562 nm.

3.5.5. Glycerol Utilization Assay

Glycerol was measured based on HPLC method [155]. Supernatant from fermentation was siring filter in 0.2 μ m and then read in HPLC whereas a mobile phase H₂SO₄ 5 mM was used, the flow was 0.6 mL/min, while sample injection was 20 μ L. Detector that was used for sample reading was the Refractive index detector. Calibration was done by measuring standard points in the range of 1.25 to 10 g/L glycerol.

3.5.6. Methanol Utilization Assay

Methanol was measured based on the work of Krainer *et al.* with some changes. Supernatant from fermentation was siring filter in 0.2 μ m and then read in HPLC whereas a mobile phase H₂SO₄ 5 mM was used, the flow was 0.6 mL/min, while sample injection was 20 μ L. Detector that was used for sample reading was the Refractive index detector. Calibration was done by measuring standard points in the range of 0.2 to 4 percentage or 1-8 g/L.

3.6. APPLICATION OF PRODUCED ENZYME IN FOOD

3.6.1. Milk Clotting Assay

Milk clotting activity was determined according to the method of Preetha and Boopathy with some minor changes. This method is based on the visual evaluation of the appearance of the first clotting flakes and expressed in terms of Milk clotting units (MCU). One unit is defined as the amount of enzyme that clots 1 mL of the substrate in 40 min at 37 °C. To perform the assay, 0.1 mL of the sample (crude enzyme) was added to the Eppendorf tube containing 1 mL of reconstituted skim milk solution 10 percentage (w/v) pre-incubated at 37 °C for 10 min. The mixture was mixed well and the clotting time was measured with a chronometer. The clotting activity was calculated using the following formula:

$$MCU = \frac{2400 \times 1 \times D}{0.1 \times t} \quad (3.2)$$

Where, D is the dilution factor, and t is the clotting time in seconds [15].

3.6.2. Hazy Beer Treatment

The visual appearance of beverage products has an important impact on consumption. Parameters such as clarity, color, and foam are vital for consumers. Beer, a product of the brewing industry as a consequence of polyphenols and proteins reaction, faces the problem of haze beer. With digestion of the protein by protease, the polyphenols will not react anymore so the haze will be prevented from formation [8]. In the current work, 100 mL haze beer was degassed then placed in 250 mL Erlenmeyer and treated with 1 mL of cell-free supernatant for 18 h at 25 °C. Resulted product was compared with untreated haze beer by reading their absorbance at 360 nm.

3.6.3. Meat Myofibril Treatment with APase

Extracting buffer (100 mM KCl, 20 mM potassium phosphate (pH 7.0), 1 mM EDTA, and 1 mM sodium azide) was used to extract myofibril from fresh cow muscle tissue. All the procedure was performed at 4 °C based on the work of Olson *et al.* with some changes [157].

1.95 mL of myofibril extract with pH 5.5 (approximately the pH of the red meat) was aliquoted in a 15 mL tube and assayed with 50 µL crude enzyme extracts. The hydrolysis takes place in an orbital shaker 150 rpm at 37 °C for 12 h. The reaction was terminated by the addition of 2 mL of 15 percentage trichloroacetic acid. The mixture was cooled at room temperature for 15 minutes and centrifuged at 1000 x g for 15 min. Finally, the optical density of the supernatant was determined at 280 nm. The same experiment is repeated with a blank [12].

3.6.4. Meat Treatment with Transglutaminase

Minced fresh beef meat from the rump part was used for the test. 15 g of minced meat was placed in a sterile 50 mL falcon tube. 3 percentage w/w NaCl was added and the content was mixed for 30 sec, after this to the meat 2 mL of crude TGase was added. The meat inside the falcon tube was mixed for 1 min and was placed in plastic wrap. To the minced meat, rod shape was given with 15 mm diameter and 10 cm length. Covered with plastic wrap, treated minced meat was placed in incubation for 12 h at 4 °C. After incubation meat was placed in the freezer -20 °C for 16 h. To read the result, reconstructed meat was hung and placed under different weight pressures. The weight that was enough to part the reconstructed meat was registered [58].

4. RESULTS AND DISCUSSION

4.1. TRANSFORMATION OF PLASMID WITH THE GENE OF INTEREST IN *E. COLI* CELLS

Heat shock technique has been successfully implemented to transform the two genes of interest (codon optimized, *de novo* synthesized *APase*, and *TGase* in commercial (Twist) plasmid) into *E. coli* DH5- α cells and the transformants are grown on selective Luria Bertani (LB) agar + ampicillin at 37 °C with the negative control being untransformed *E. coli* cells. Successful, selective transformation is shown in Figure 4.1, where grown colonies appeared 24 h after transformation.

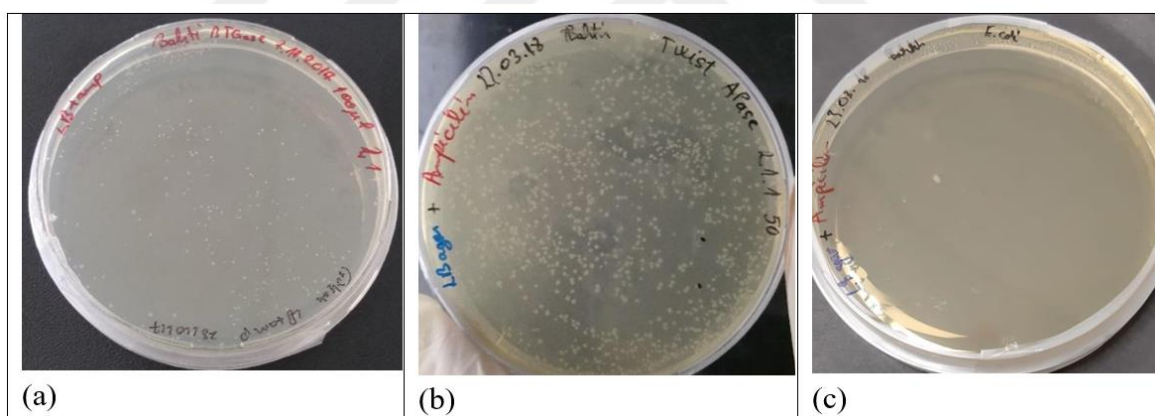


Figure 4.1. Colonies of transformed *E. coli* cells: (a) plasmid + *TGase*, (b) plasmid + *APase*, (c) Negative control, as untransformed *E. coli* cells

Single colonies were grown in selective LB for 18 h and used for plasmid isolation. The concentration and purity of isolated plasmids are shown in Table 4.1. The ratio OD_{260}/OD_{280} is used to assess the purity of isolated DNA. A ratio of around 1.8 is generally accepted as pure, while a lower ratio indicates contamination with protein or phenol [158], which is the case for both genes.

Table 4.1. Concentration and purity of isolated plasmid with genes of interest from transformed *E.coli* cells

	Concentration ng/ μ L	Purity OD ₂₆₀ /OD ₂₈₀
Plasmid+ <i>APase</i> 1	120	1.91
Plasmid+ <i>APase</i> 2	122.9	1.91
Plasmid+ <i>TGase</i> 1	32.5	1.73
Plasmid+ <i>TGase</i> 2	93.6	1.83

4.1.1. Double Digestion Results of Plasmid with Gene of Interest and pPICZ α A and B

The next step is to transfer the genes to the expression vector, pPICZ α A (for *APase*), pPICZ α B (for *TGase*). Amplified plasmids with *TGase* gene and expression plasmid pPICZ α B were separately and successfully double digested with *Xho*I and *Not*I restriction enzymes (Figure 4.2). *TGase* gene is 1404 bp long, and the multiple bands on Lanes 1-4 corresponds to *TGase* gene and what remains from the commercial vector.

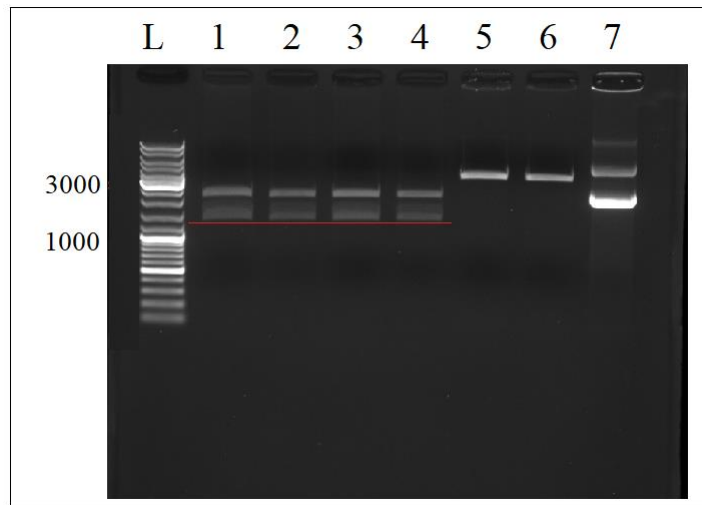


Figure 4.2. Electrophoresis slide of double digestion of the plasmid with *TGase* and pPICZαB plasmid. Column L is DNA ladder, 1-4 rows are digested plasmid with *TGase*, 5 and 6 rows digested pPICZαB, row 7 undigested pPICZαB

The same digestion process was performed for plasmid with *APase* (1185bp) gene and pPICZαA plasmid, only here *EcoRI* and *XbaI* restriction enzymes are used (Figure 4.3 (a)). Column three in this Figure 4.3.a shows the supercoiled band of undigested Plasmid with the *APase* gene.

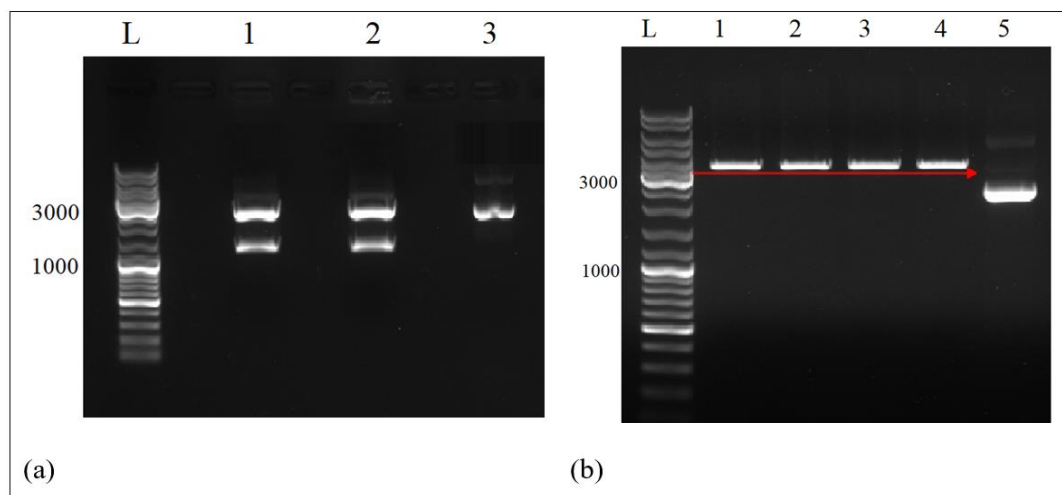


Figure 4.3. Double digestion of (a) columns 1 and 2 plasmid +*APase*, column 3 undigested plasmid +*APase*; (b) columns 1-4 digested pPICZαA plasmid, column 5 undigested pPICZαA plasmid; L is the DNA ladder

In both cases, genes of interest are extracted from the gel, and their concentrations and purities are shown in Table 4.2. Following the kit protocol, the expression vector and genes of interest are left overnight for ligation. Subsequently, the ligase was inactivated by heat treatment and the resulting expression vector is transferred to *E. coli DH5- α* cells. The transformed cells were grown on LB agar plates, selective with 25 $\mu\text{g/mL}$ zeocin (Figure 4.4).

Table 4.2. The concentration of digested genes of interest and their plasmids after gel extraction

Gene/ Plasmid	Concentration	Purity OD ₂₆₀ /OD ₂₈₀
<i>APase 1</i>	5.8 ng/ μL	1.84
<i>APase 2</i>	12.9 ng/ μL	1.88
<i>TGase 1</i>	10.1 ng/ μL	1.77
<i>TGase 2</i>	8.4 ng/ μL	1.73
pPICZ α A	21.6 ng/ μL	1.74
pPICZ α A	21.7 ng/ μL	1.85
pPICZ α B	19.6 ng/ μL	1.75
pPICZ α B	12.2 ng/ μL	1.84

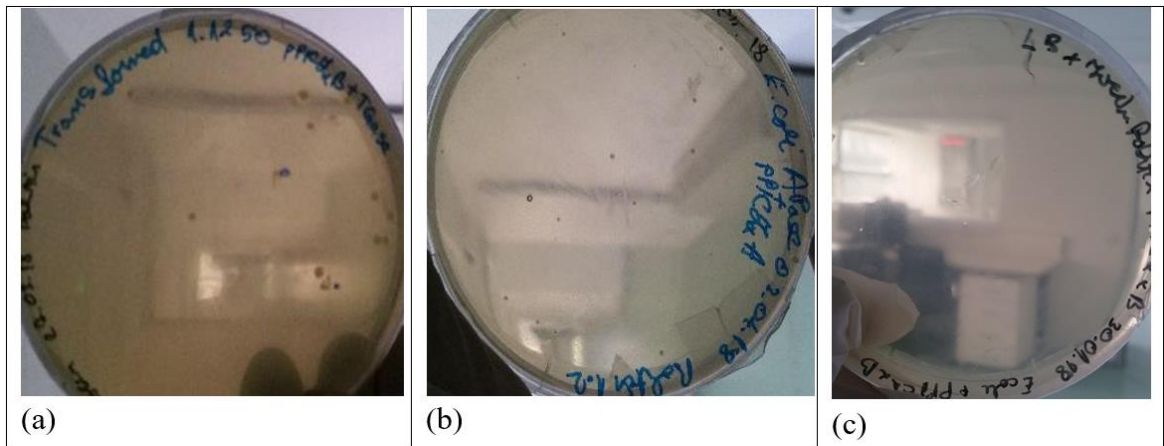


Figure 4.4. Transformed *E.coli* cells with (a) pPICZ α B+TGase, (b) pPICZ α A+APase; and negative control untransformed *E. coli*

4.1.2. Linearization and Purification Results

The expression vectors (pPICZ α A and pPICZ α B) ligated with genes of interest were then isolated, purified and finally linearized again by *Pme*I (Figure 4.5). The linearization is to increase the transformation efficiency as well as to allow its integration into the 5'AOX1 region of host genome.

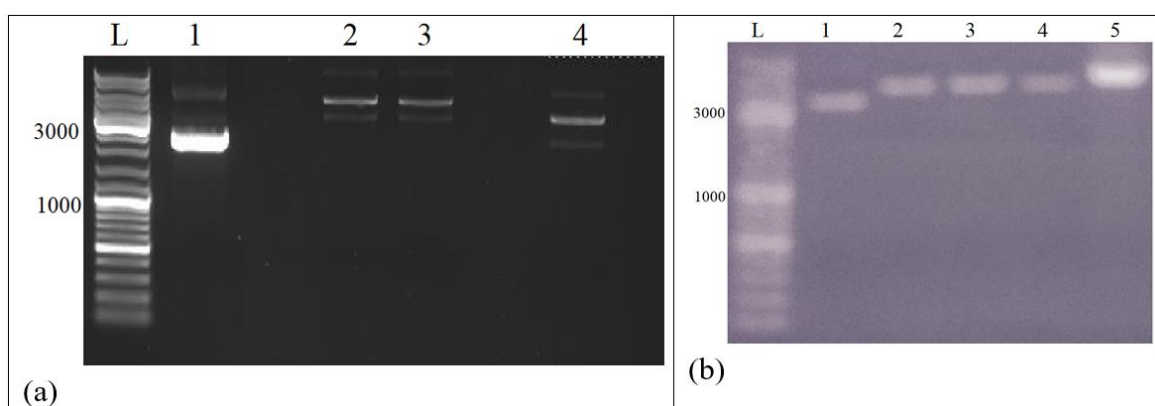


Figure 4.5. Linearization of cloned plasmids of interest with the genes of interest: (a) pPICZαB+*TGase* (L-ladder, 1- non-linearized pPICZαB, 2 and 3- linearized pPICZαB+*TGase*, 4-linearized pPICZαB), and (b) pPICZαA+*APase* (L-ladder, 1- linearized pPICZαA, 2-3- linearized pPICZαA+*APase*, 5-non-linearized pPICZαA)

Linearization was terminated by heat treating samples (65 °C, 30 min) and the linearized DNA was purified and concentrated by using the Phenol-Chloroform method (Table 4.3).

Table 4.3. Concentration and purity of linearized, cloned DNA of pPICZαB +*TGase* and pPICZαA +*APase*

Linearized DNA	Concentration	Purity 260/280
PCI Linearized pPICZαB + <i>TGase</i> 1	609ng/μL	1.22
PCI Linearized pPICZαB + <i>TGase</i> 2	423ng/ μL	1.75
PCI Linearized pPICZαA + <i>APase</i> 1	489.3ng/μL	1.89
PCI Linearized pPICZαA + <i>APase</i> 2	412.2ng/μL	1.81

As it is seen from the results in Table 4.3 concentration of linearized DNA and its purity were enough to perform transformation by electroporation to electrocompetent cells of *Pichia pastoris*. Once the sufficiently pure and concentrated DNA was obtained for both genes, these are transferred to *Pichia pastoris* via electroporation (1.5 kV, 25 μF, and 200 Ω). Upon electroporation the transformed cells were incubated on selective (100 ng/mL zeocin) YPDS agar (Figure 4.6). The selection procedure is repeated three times to make sure that we select for stable transformants. After the third passage on selective YPDS agar,

glycerol stocks were prepared for *Pichia pastoris* ATCC 28485 with *TGase* gene (*PPTGase*) and for *Pichia pastoris* ATCC 28485 with *APase* gene (*PPAPase*) and are kept at -80 °C.

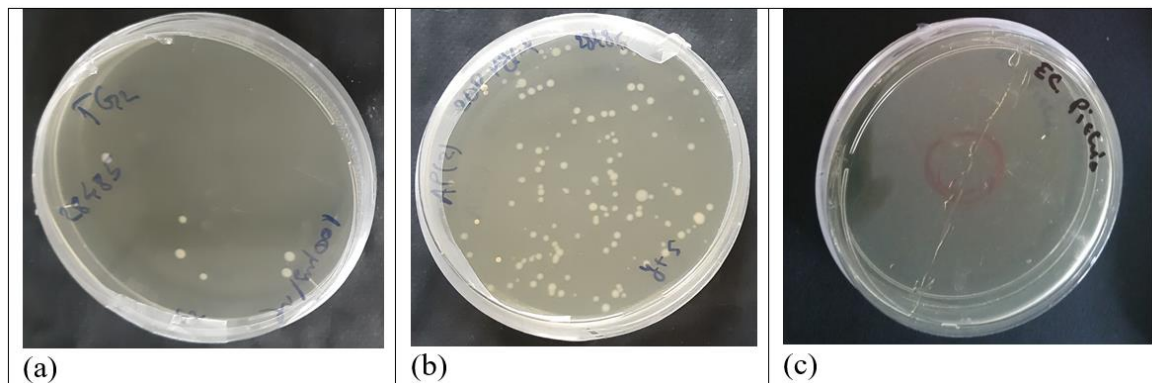


Figure 4.6. Analysis of transformation. (a) Transformed colonies of *Pichia pastoris* ATCC 28485 with *TGase* gene (*PPTGase*); (b) Transformed colonies of *Pichia pastoris* ATCC 28485 with *APase* gene (*PPAPase*); (c) Negative control, untransformed *Pichia pastoris* ATCC 28485 cells

4.2. CLONING CONFIRMATION

To further confirm that the transformants contain the genes of interest (and not only the linearized pPICZ α A/B vectors), further confirmations are performed (also related to the MUT phenotype explained in below section) and the results related to the confirmation are presented in this section.

4.2.1. MUT Phenotype

P. pastoris ATCC 28485 is a wild type, MUT⁺ phenotype strain. This strain contains both AOX1 and AOX2 genes which are responsible for Alcohol oxidase activity. During the homologous recombination, there are three possibilities of phenotype forming MUT⁺, MUT⁻, and MUT^s. Based on methanol utilization, a screening of the selected colonies on the

minimal agar medium with methanol or glucose was performed. Two selective agar plates were prepared (one with 2 percentage glucose and the other with 0.5 percentage methanol). Single colonies were streaked to both agars and cultivated at 30 °C (Figure 4.7). The only difference was the size of colonies, colonies grown in methanol were slightly smaller than colonies grown in glucose. The same results are gained with untransformed *P. pastoris* cells (no zeocin in agar plates). In this case, both transformed cells did not lose the AOX1 gene during the gene integration and they are both MUT⁺ phenotypes.

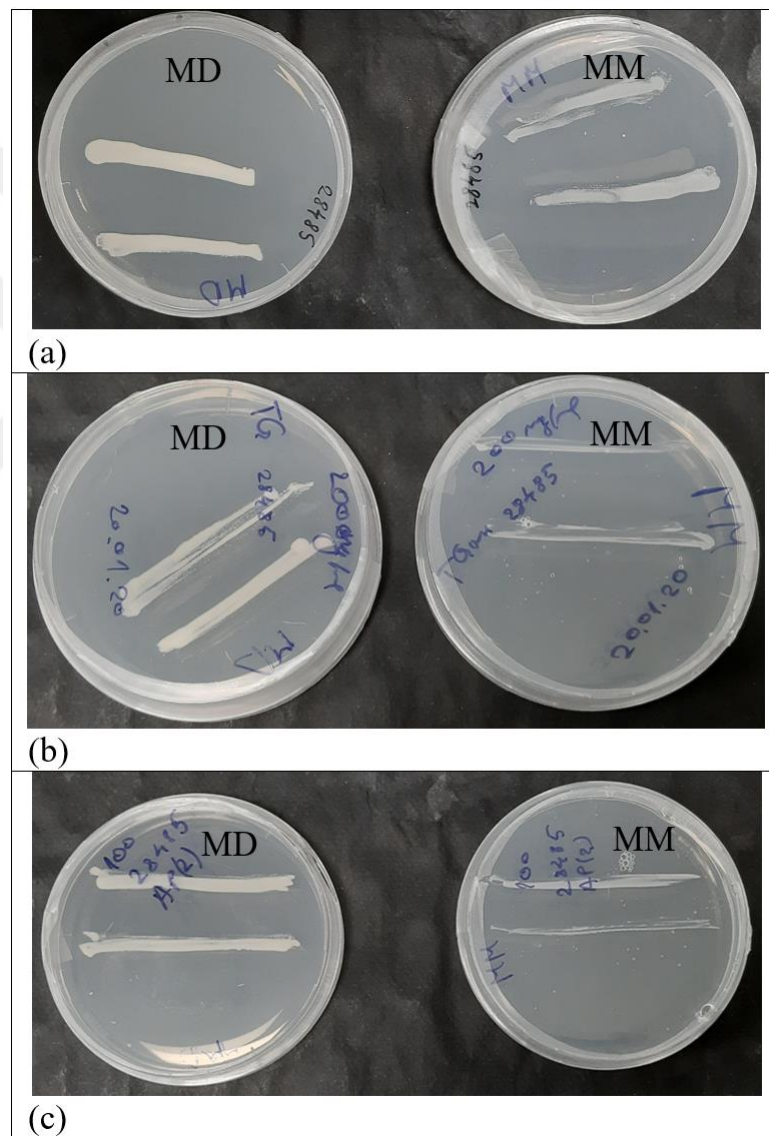


Figure 4.7. Screening results of transformed colonies in Minimal Methanol agar (MM) and/or glucose agar (MD). (a) Untransformed *P. pastoris* cells; (b) *PPTGase* cells; (c) *PPAPase* cells

4.2.2. Integration of the Gene of Interest in the Genomic DNA

To check the integration of the genes, PCR was used with specific primers for both *TGase* and *APase*. According to the manufacturer's guide for the expression vector, when forward specific primers are used for the gene of interest and the reverse AOX1 primer, additional 235 bp should be taken into account during the analysis of PCR products. The expected *APase* band is 1402 bp, 1167 bp being from the *APase* gene itself (amplified with specific primers), and 235 from the terminator region. Similarly for *TGase*, expected band is at 1440 bp (1205 + 235). Both of the bands for *APase* and *TGase* are around 1400 bp, shown in Figure 4.8 (Lanes 1 and 5) where additionally unsuccessful cases due to contamination, no integration etc. are presented.

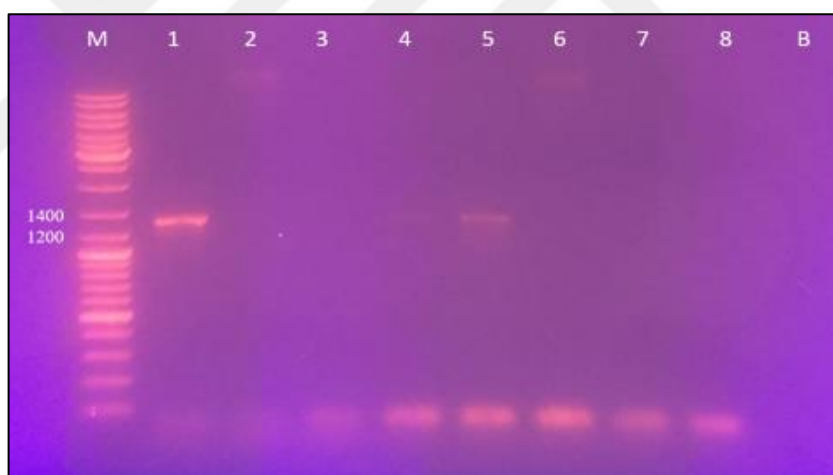


Figure 4.8. PCR product of genomic DNA from successfully and not successfully gene integration. M-marker; 1-*Pichia pastoris* genomic DNA with integrated *APase* gene. 2-*Pichia pastoris* genomic DNA not integrated *APase* gene. 3-Contaminated old stock. 4, 5-*Pichia pastoris* genomic DNA with integrated *TGase* gene. 6, 7-*Pichia pastoris* genomic DNA from not integrated *TGase* gene. 8-B-blank

4.2.3. Sequence Analysis

Upon amplification, the content was purified with a PCR-clean-up kit and sent for sequencing. Resulting chromatograms are aligned and merged by using the BioEdit tool, resulting in 88 percentage identity the gap being due to partial amplification of the DNA. The Stop codon was found in the 3' *APase* sequence flowing by 4 more bp which was the restriction enzyme site *XbaI*. The rest of the amplified 3' DNA was a perfect match with the sequence of the plasmid known as the c-myc epitope. Cutting the part of the c-myc epitope sequence from the amplified sequenced gene and the part after the 5' primer in the designed gene when we alignment these two sequences the percentage of identity is 98 percentage (APPENDIX A: Figure A.1).

The same workflow of aligning, merging and comparing to the designed sequence for *TGase*, 70 percentage identity was found. At the end of the 3' sequence stop codon and *NotI* restriction enzyme were found. When the c-myc epitope at the end of 3' sequence was taken out and the sequence of the designed *TGase* gene was taken into account only from the 5' specific primers 88 percentage identity was achieved (Figure B.1). Over the years, cloning was repeated in case there is a doubt for stock contamination and stock renewing. After each cloning and transformation process confirmation of clones was performed with PCR as explained above.

4.3. SUBMERGED FERMENTATION

In this section, the results related to the submerged fermentation for *APase* gene is presented, as the activity determination for *TGase* enzyme needs special attention. Figure 4.9 shows the growth curve (a), and protease activity (b), when transformed *PPAPase* is grown in BMMY medium with two different concentrations of methanol (0.3 percentage and 0.6 percentage) in the adaptation period (first 24 h) for production of the protein of interest.

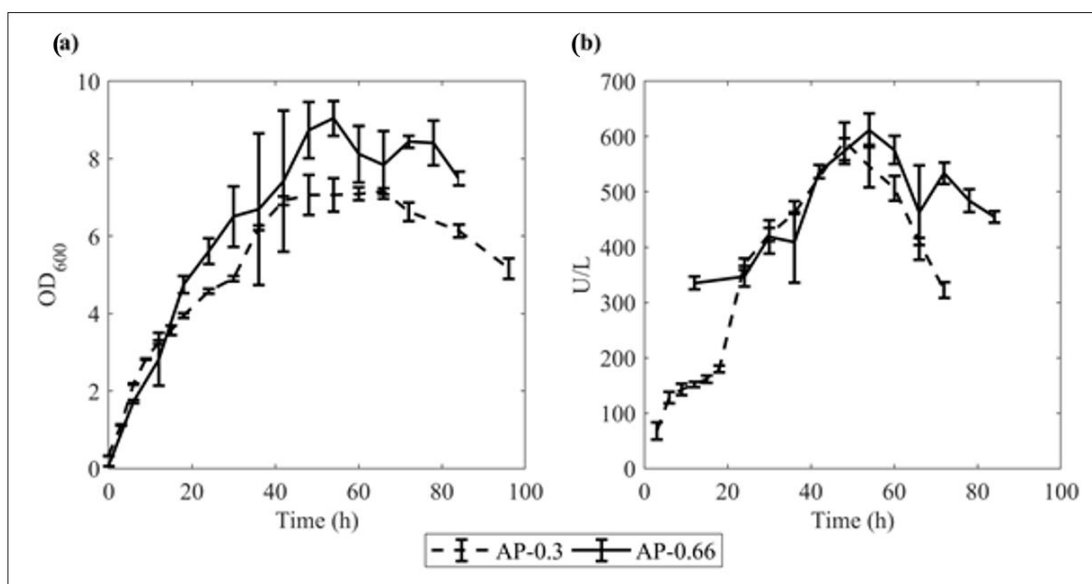


Figure 4.9. Fermentation in SmF. (a) Growth curve of *PPAPase* in 1L Erlenmeyer with 200 mL BMMY medium with methanol concentration in the adaptation period 0.6%, and 0.3%; (b) Protease activity expressed in U/L for 0.6%, and 0.3% methanol concentration

In Figure 4.9 (a) is shown that higher growth was achieved in *PPAPase* grown in BMMY medium with 0.6 percentage methanol at the beginning of the fermentation. The highest growth was achieved at 54 h of fermentation with OD_{600} 9 for 0.6 percentage methanol, at the same time highest protease activity was reached 610 U/L (Figure 4.9 (b)). While, with 0.3 percentage methanol concentration OD_{600} reached until 7, and protease activity 590 U/L obtained from culture supernatant. In the literature, the highest activity reported is 7 U/ μ g of aspartic protease, produced using engineered *Pichia*. However in that work, the recombinant protein is produced from high cell density culture and chromatographically purified [123]. Contrary, in my research unpurified culture samples are used to measure the activity.

In both concentration of Methanol, the correlation between growth and protease activity was analyzed resulting in a correlation factor of 0.857 and 0.912 for 0.6 percentage and 0.3 percentage methanol addition in the adaptation period, respectively.

4.4. SUBMERGED FERMENTATION IN 6 L BIOREACTOR WITH NEW CLONED *PICHIA PASTORIS* ATCC28485

After Erlenmeyer scale experiments, submerged fermentation was performed at benchtop scale, in a 6 L bioreactor with 3 L working volume, in feed-batch mode. Process was monitored by measuring OD, and Glycerol or methanol concentration at 3 h intervals, and manually controlled the process at the optimum substrate levels. Additionally, pH and dO₂ are automatically controlled (Figure 4.10).

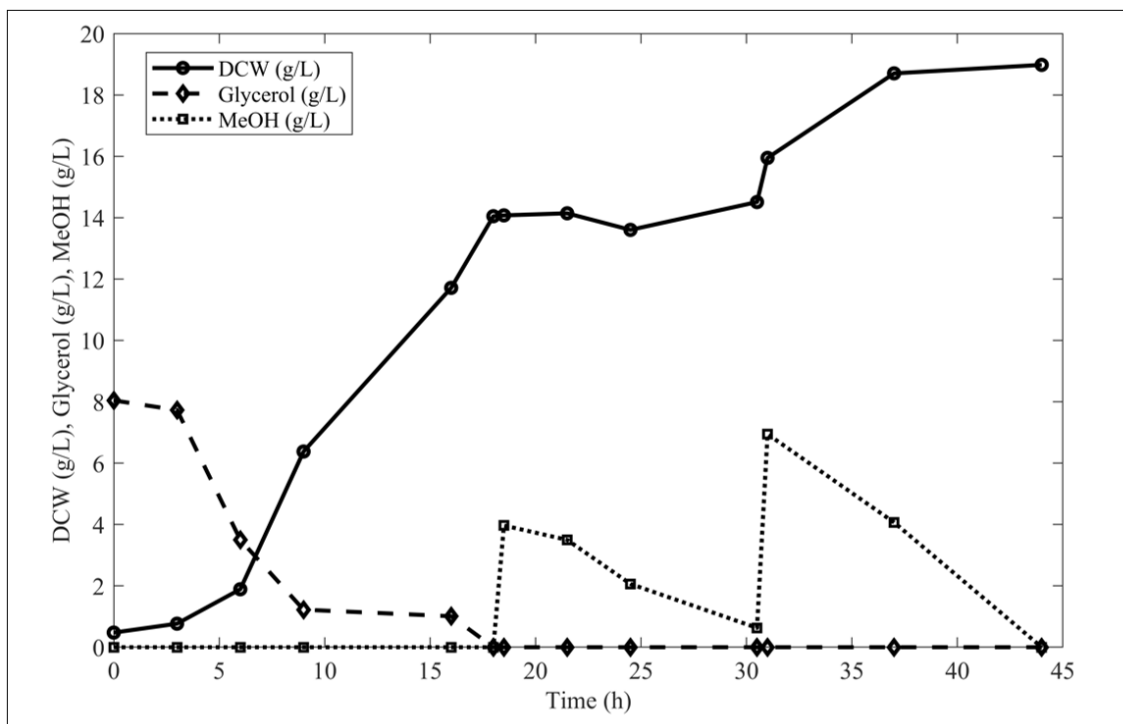


Figure 4.10. Dry cell weight (solid line with circles), Glycerol g/L (dash line with diamonds); Methanol g/L (dot line with rectangles) in 6 L Bioreactor fermentation with BMGY-BMMY medium

From Figure 4.10 is shown the growth of *Pichia* is significantly correlated with the type of carbon source consumption. After the glycerol depletion at 18 h, 4 g/L methanol was added. This period of fermentation is known as an adaptation period because of the drastic changes in carbon sources from glycerol to methanol. This adaptation period continued for a straight 7 h after that a slight increase in the growth was detected, methanol utilization started almost

immediately. The growth increased after the second 8 g/L methanol addition. Full methanol consumption was observed after the next 13 h of fermentation. The highest OD₆₀₀ achieved was 21, corresponding to 18.9 g/L based on the reconstructed linear relation ($DCW = 0.8996 \cdot OD_{600}$).

The specific glycerol consumption rate was -0.826 mmol/h/g DWC and specific methanol consumption rates are -0.616 for the adaptation phase and -0.933 for the production phase. However, biomass yield on glycerol was 1.68 g/g while biomass yield on methanol for adaption period was 0.13 g/g and 0.48 g/g for the production phase. Even though degree of reduction of methanol is higher than the one of glycerol (higher reduction degree, higher yield can be obtained), in all fermentation that were performed for this thesis project the yield of biomass on glycerol were higher than the one in methanol. An explanation is provided in the work of Geier *et al.*, based on the methanol metabolism pathway initial oxidation of methanol to formaldehyde in peroxisome does not generate any metabolic energy, which effect negatively the growth performance of *Pichia* in methanol. Moreover, diauxic growth was observed through all the growth curve when two carbon sources are used [158,159].

The aspartic protease activity of the broth supernatant after each period of fermentation was measured. Low activity resulted from the fermentation, but there is a significant difference in protease activity before and after methanol induction with the highest peak in activity after 6 h from the first induction with methanol, which is also a starting point of growth after the adaptation period. The activity in that peak reached 68 U/L (Figure 4.11.).

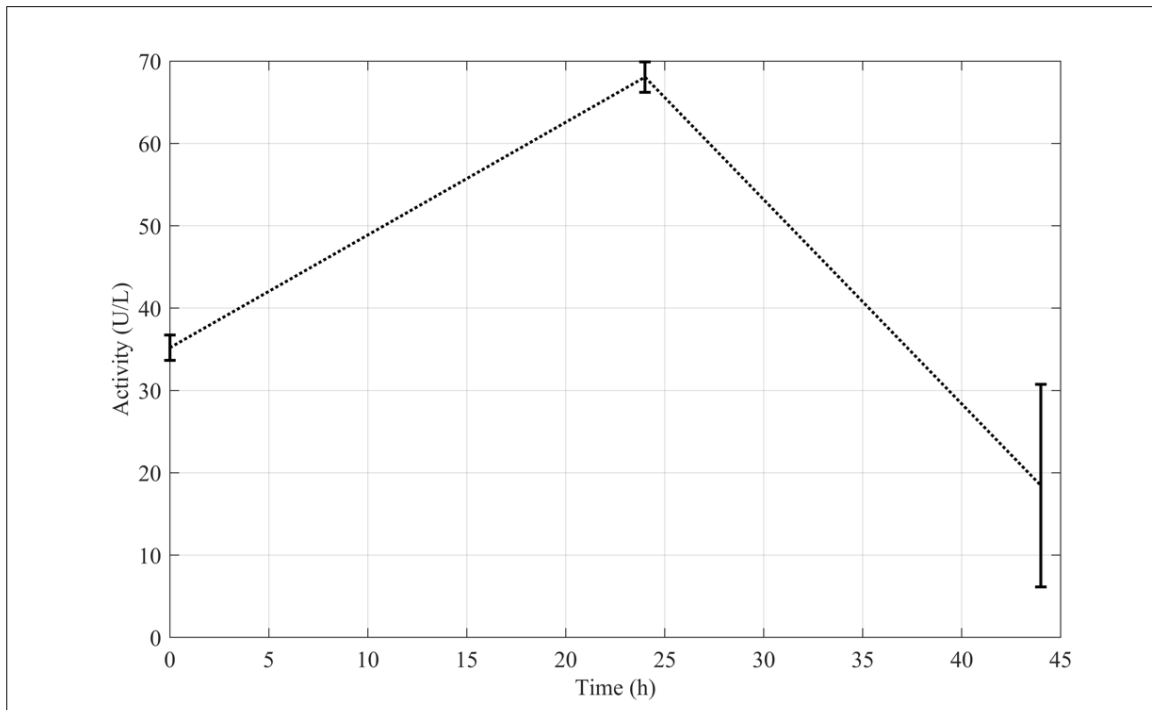


Figure 4.11. Aspartic protease activity from SMF fermentation in a 3L bioreactor with BMMY medium

During the fermentation, dissolved oxygen, pH, stirring rate, and airflow were monitored or controlled. Dissolved oxygen quickly increased when the carbon was depleting. Upon methanol feeding, dissolved oxygen started again to drop (Figure 4.12 (a)). In fed-batch fermentation, this could be a helpful variable to control the feeding time. As it is shown in Figure 4.12 (b), the oxygen inflow rate was stable during the fermentation.

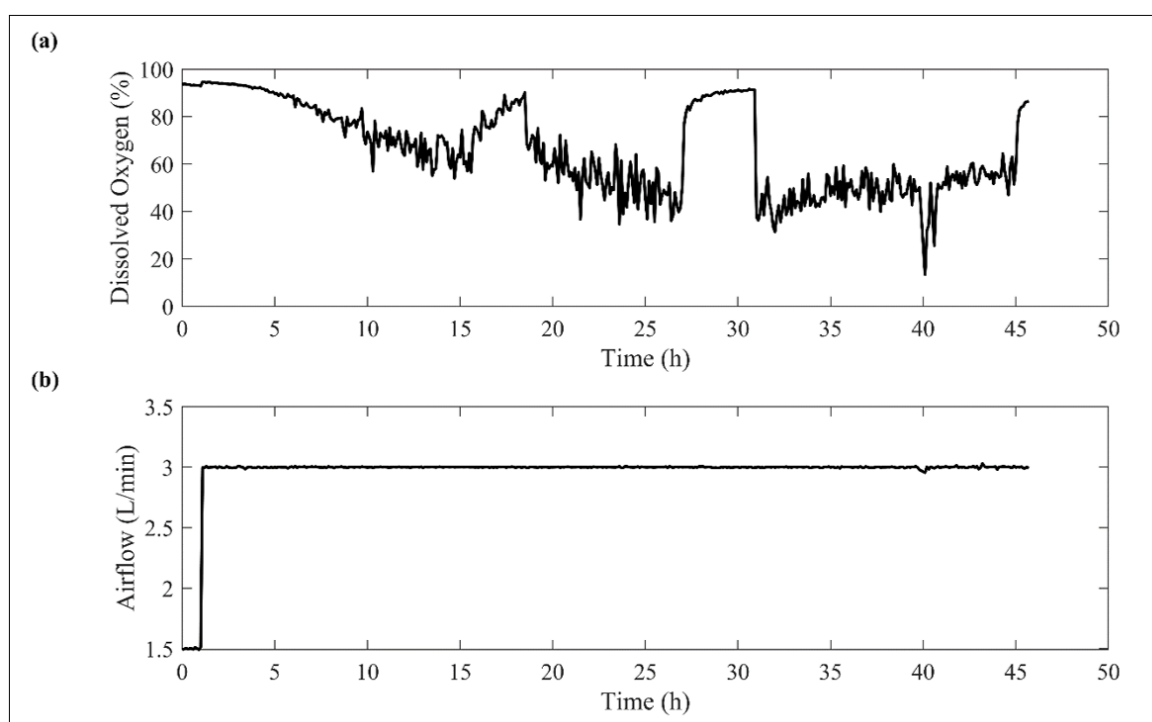


Figure 4.12. Aeration process. (a) Percentage of dissolved oxygen in the 3L reactor SMF, and (b) airflow supplied in the reactor. Methanol was added at $t = 18\text{h}$ and $t = 30.5$

4.5. SOLID-STATE FERMENTATION

Wild type *Pichia pastoris* transformed with aspartic protease gene were cultivated in a solid-state mode. In 1 L Erlenmeyer 20 g, wheat straw was placed. The content was sterilized at $121\text{ }^{\circ}\text{C}$ for 40 min. After cooling at room temperature, the solid medium was humidified with 100 mL of BMMY medium with 2.4 g/L methanol. The inoculum was prepared for 18 h in BMGY medium and was used as 15 percentage v/v in a solid substrate. For the extraction of the enzyme, for each 1g of the solid substrate, 10 mL of distilled water was used. Samples are taken each 24 h per 120 h. Samples were analyzed for protease activity by using the casein method at pH 5.8. Substrate utilization is determined by using HPLC, and protein content was determined using BCA method (Figure 4.13).

Results show that protease activity was increased by time until 72 h with the highest activity around 150 U/L. The methanol content is decreasing with the lowest point at 96 h at which

point another pulse of 2.4 g/L of methanol was added. The effect of addition is noticeable at the measurement of 120 h. For protein content, the highest point was achieved at 96 h and the protein content follows the activity profile.

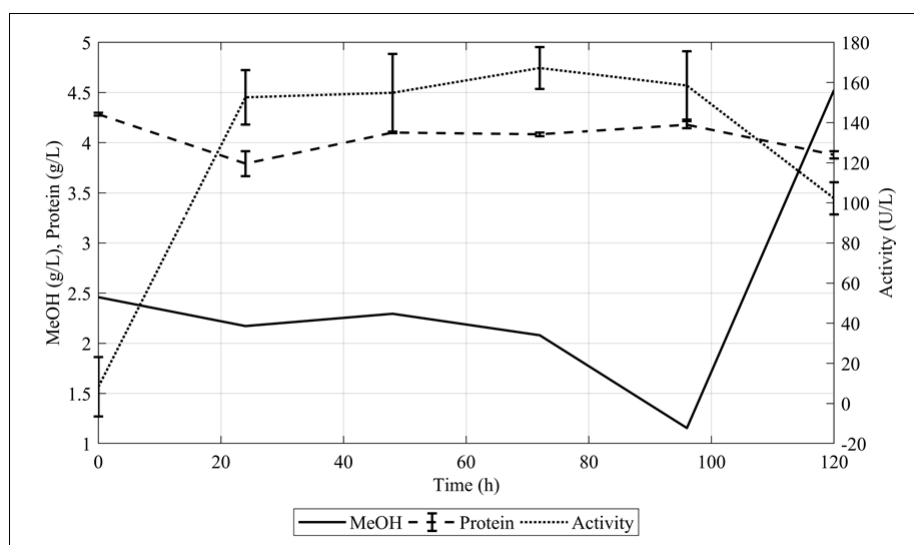


Figure 4.13. Results of SSF in 1L Erlenmeyer with wheat straw as solid support moistened with BMMY medium

4.6. EFFECT OF DIFFERENT CONCENTRATION OF NATURAL INERT SOLID-SUPPORT SUBSTRATE IN ENZYME PRODUCTION

Solid-state fermentation was performed by using wheat straw as the inert natural support for recombinant protein expression by *Pichia pastoris*. To the best of our knowledge, this is the first study of heterologous protein production by *Pichia pastoris* in SSF, by using wheat straw, despite previous reports on bioethanol production from *Saccharomyces cerevisiae* from wheat straw in co-fermentation with recombinant *Pichia pastoris* expressing xylanase [160].

Three different concentrations of solid materials are used 5, 10, and 15 percentage. In fermentation with 5 percentage solid material, the solid material was submerged in liquid (Figure 4.14 (a)). Even though solid-state fermentation is considered as the growth of microorganisms on moist substrates in the absence of free-flowing water [161], there is semi-

solid-state fermentation where free water is detected in medium e.g. Gupta and Jana [162] in their work they used 8 percentage of solid material for laccase production. In the fermentation where solid content was 10 and 15 percentage, no free water during the fermentation was shown Figure 4.14 (b) and Figure 4.14 (c), respectively.

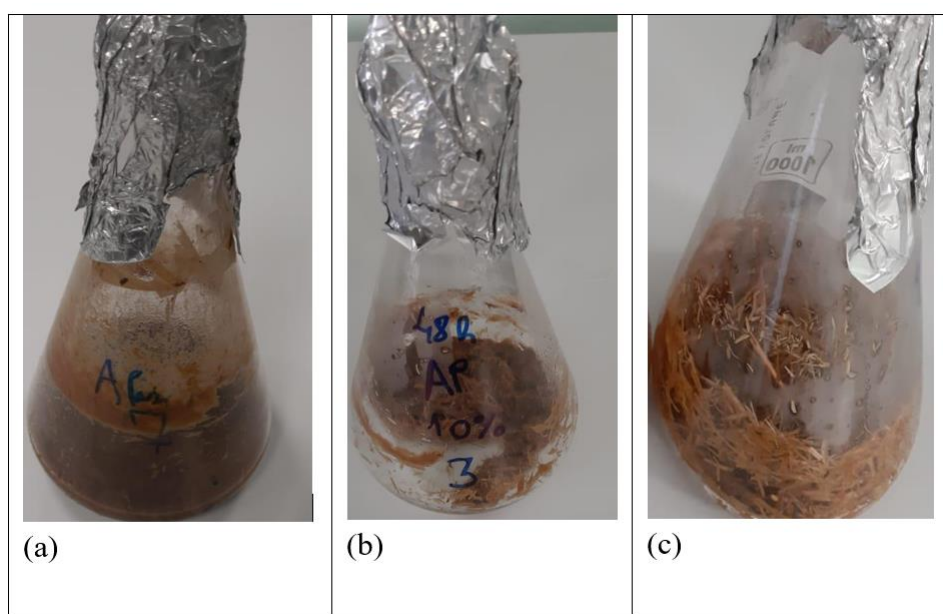


Figure 4.14. Solid-state fermentation in 1 L Erlenmeyer with different concentrations of wheat straw, (a) 5% wheat straw, (b) 10% wheat straw, and (c) 15% wheat straw

Fermentation was monitored each 24 h where one whole reactor (1 L Erlenmeyer) was harvested. For 5 percentage solid material fermentation liquid medium was just separated by solid material by centrifugation no washing step was used. However, for 10 and 15 percentage solid material fermentation 5 mL sterile dH₂O was used for 1 g solid medium. Washing process was followed by incubation in rotary shaker for 15 min at 180 rpm immediately after it, a 100 μ L sample was taken and used for CFU measurement. Extraction of the product then was followed by centrifugation. Samples were stored at +4 °C for further analysis. Samples were analyzed for protease activity at pH 5.8 and protein content. Results of protease activity and protein content are used for specific protease activity calculation. Each 24 h sample of the harvested reactor was spread on the YPD agar plate to analyze the host organism growth in solid-state fermentation, results are shown in Table 4.4.

Table 4.4. The number of colony forming units (cfu/mL) for SSF for each 24 h of fermentation. *PPAPase*- transformed *Pichia pastoris* with *APase* gene, Wild type- untransformed *Pichia pastoris* cells

Time	<i>PPAPase</i> 5% SSF	Wild type 5%SSF	<i>PPAPase</i> 10% SSF	Wild type 10%SSF	<i>PPAPase</i> 15% SSF	Wild type 15%
24	$10 \cdot 10^9$	$6 \cdot 10^9$	$12 \cdot 10^9$	$2 \cdot 10^9$	$40 \cdot 10^9$	$45 \cdot 10^7$
48	$20 \cdot 10^9$	$4 \cdot 10^9$	$7 \cdot 10^9$	$4 \cdot 10^9$	$4 \cdot 10^{11}$	$10 \cdot 10^9$
72	$23 \cdot 10^9$	0	$3 \cdot 10^9$	0	$10 \cdot 10^{11}$	$11 \cdot 10^{11}$
96	$10 \cdot 10^{11}$	0	$5 \cdot 10^7$	0	$18 \cdot 10^{11}$	$12 \cdot 10^{11}$

For protease activity measurement as a sample blank was used supernatant from 0 h harvested reactor, in which case we take into account the interference that comes from medium. To assay for the protease background from wild type *Pichia pastoris*, the same fermentation process was done with untransformed wild type. The highest activity was achieved from *PPAPase* culture in 10 percentage solid substrate after 24 h of fermentation. As well, protease activity of supernatant from 48 and 72 h of this culture fermentation was higher than their untransformed counterparts in all cultivation (Figure 4.15). A t-test was performed to assess 95 percentage significance of the difference in protease activity among varying solid/liquid ratio and separately upon transformation. The confidence interval was set as 95 percentage.

There was a significant difference 10 percentage SSF and 15 percentage SSF (*p-value*: 0.009) and transformation resulted in increased activity with 10 percentage SSF cultures (*p-value* of 0.012). A noticeable problem was the heterogeneity between the Erlenmeyer in the same fermentation process. In the fermentation process with wild type untransformed *Pichia pastoris* at 96 h with 10 percentage, solid substrate bacterial contamination was shown in YPD agar, this explains the high protease activity of that fermentation (Figure 4.15. (b) dot-line).

In general, a decrease in protease activity was observed after 24 h of fermentation, this might be due to the evaporation process of methanol in solid support and as a result, the methanol

level is decreased so the expression level too. Compare with the protein produced which was increased by time, by producing other secondary products that are not dependent on methanol induction.

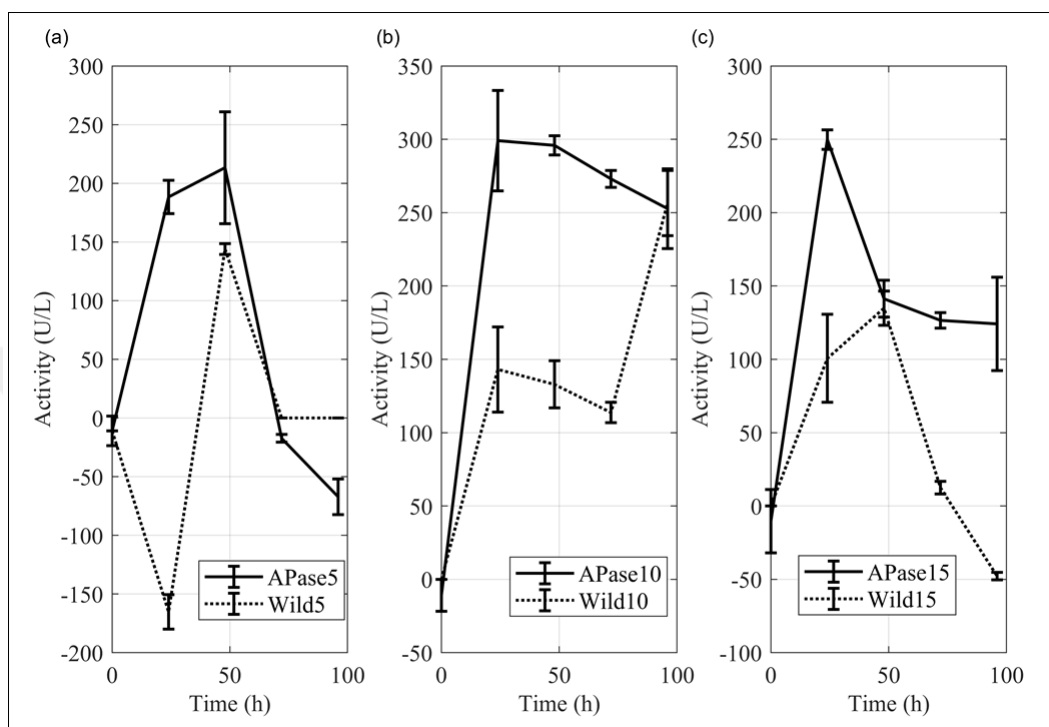


Figure 4.15. Aspartic protease activity at pH 5.8. Supernatants from different hours of fermentation with different percentages of solid material (wheat straw). APase stands for protease produced from transformed wild type *Pichia pastoris* with aspartic protease gene, while Wild is supernatant from untransformed wild type *Pichia pastoris*. 5, 10 and 15 % show the percentage of the solid substrate in the fermentation process

From the activity results, we can see the indication of the moisture content in the solid medium. In the work of Zimbardi *et al.* water content was considered the most important and critical parameter for SSF. At a low moisture content during SSF, lower substrate swelling occurs which reduces superficial area, solubility and diffusion of the nutrients, and stability of the extracellular proteins [163]. In this study, methanol is used as a carbon source and as an inducer for the expression. In this case diffusion in the solid substrate of this nutrient makes possible biomass production and enzyme expression. Moreover, an increased level for water content may cause aggregation of the substrate and limit the oxygen transfer by reducing the porosity of the solid substrate which in turn inhibits the growth and enzyme

production. This is shown in our result when enzyme activities were lower for both 5 percentage and 15 percentage solid substrate than in 10 percentage solid substrate which was shown to be the optimal concentration for the expression of the protein with over 300 U/L protease activity.

4.7. PACKED BED AERATED COLUMN BIOREACTOR

SSF was performed by using an in-house constructed stainless steel column reactor with volume 4 L. 75 g of wheat straw particles 2-10 mm were placed (5 percentage) in the reactor. 1425 mL of BMMY medium were used. The system is shown in Figure 4.16. and consisted of (a) Reactor, (b) Compressor with manometer, (c) Modified Humidifier with distilled water, and (d) Modified cooling jacket. Each 24 h fermentation was induced with 4 g/L of 100 percentage filter sterilized methanol to the total volume of fermentation medium. Sampling was the challenging part. 25 mL of supernatant was extracted each 24 h, the supernatant was analyzed for protease activity, and protein content.

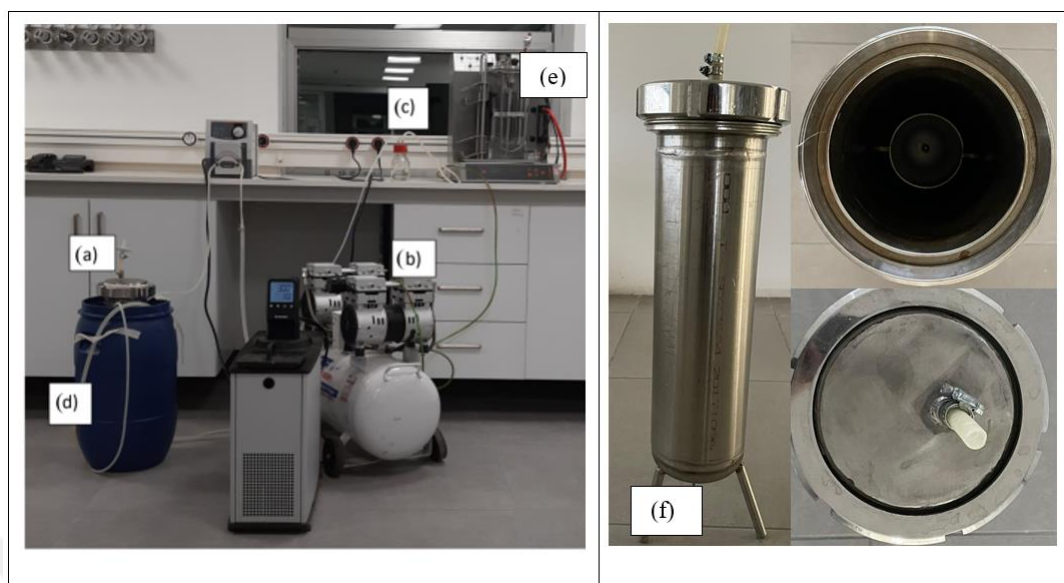


Figure 4.16. SSF system for packed bed column reactor with humidifying and aeration system. (a) Reactor, (b) Compressor with manometer, (c) Modified Humidifier with distilled water, (d) Modified cooling jacket, (e) Manometer, and (f) Reactor without the jacket

SSF model with Erlenmeyer is defined as the “Unforced aeration, forced mixing” type of fermentation. Withal, solid-state fermentation performed in packed bed aerated column reactors is defined in the group of forced aeration without mixing. In this fermentation, a continuous increase in the activity of the protease was monitored during the first 72 h where the highest peak of activity was achieved 149 U/L (Figure 4.17 dot-line) and continued with a slight decrease. This prolonged production phase might be a result of proper aeration and a decrease in the evaporation amount by using moisture saturated air by a humidifier. After 72 h an increase in protein production was monitored which were considered to be secondary products. The highest specific activity was 160 U/g also at 72 h of production (Figure 4.17 solid-line). Challenges during SSF fermentation in aerated column reactor were non-uniform fermentation through layers of the solid support in the reactor, agglomeration of the medium, and foam-forming which can be managed by periodically mixing, in the case of the mixed forced aerated reactor [164].

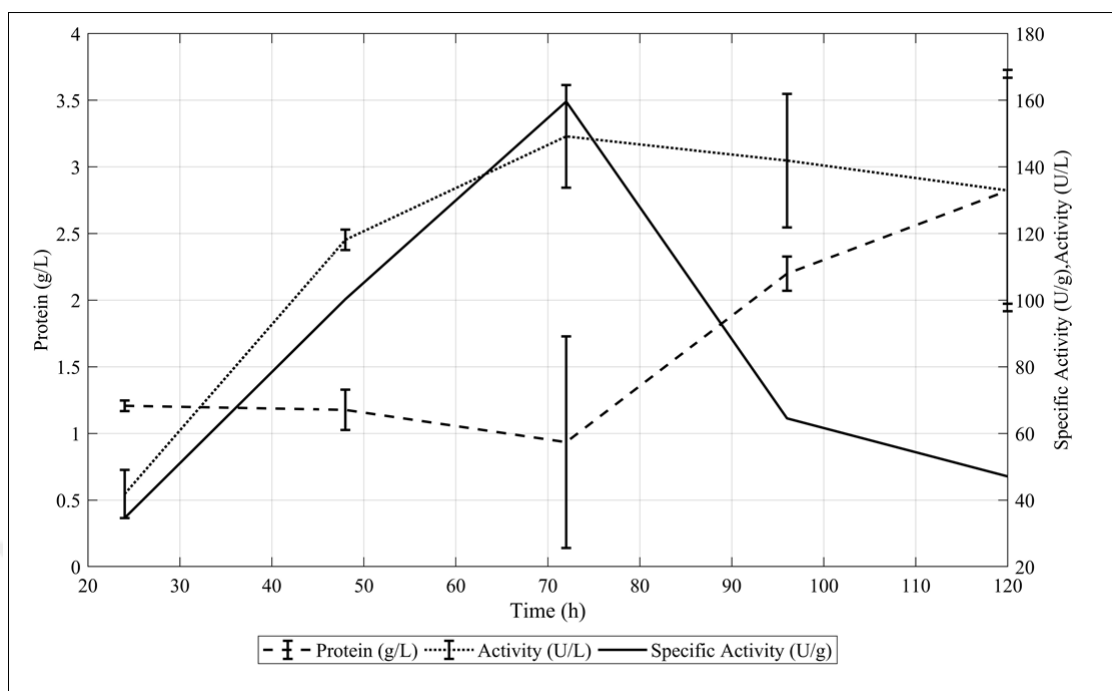


Figure 4.17. Results of SSF in packed bed aerated column reactor with *PPAPase*, using as inert solid support wheat straw

4.8. FERMENTATION RESULT WITH ARTIFICIAL SOLID-SUPPORT

Polyurethane foam (PUF), prepared in 0.5cm^3 (Figure 4.18 (a)) was used for fermentation with Verduyn medium. After fermentation, a syringe was used to harvest the fermented material and produced biomass from PUF, presented in Figure 4.18 (b). From the difference in the PUF weight before and after fermentation, and also visually was shown adsorption of the biomass in PUF material shown Figure 4.18 (c). The void spaces of the PUF material were filled by *Pichia pastoris* cells similar to concrete would be used build biofilms. Similar growth of biomass in the void of PUF material was reported in the work of Bastos *et al.*, where they cultivated the fungi *Drechslera monoceras* for allergenic protein production. Based on their results, they concluded that this fungus uses PUF material as a solid matrix for growth [165].

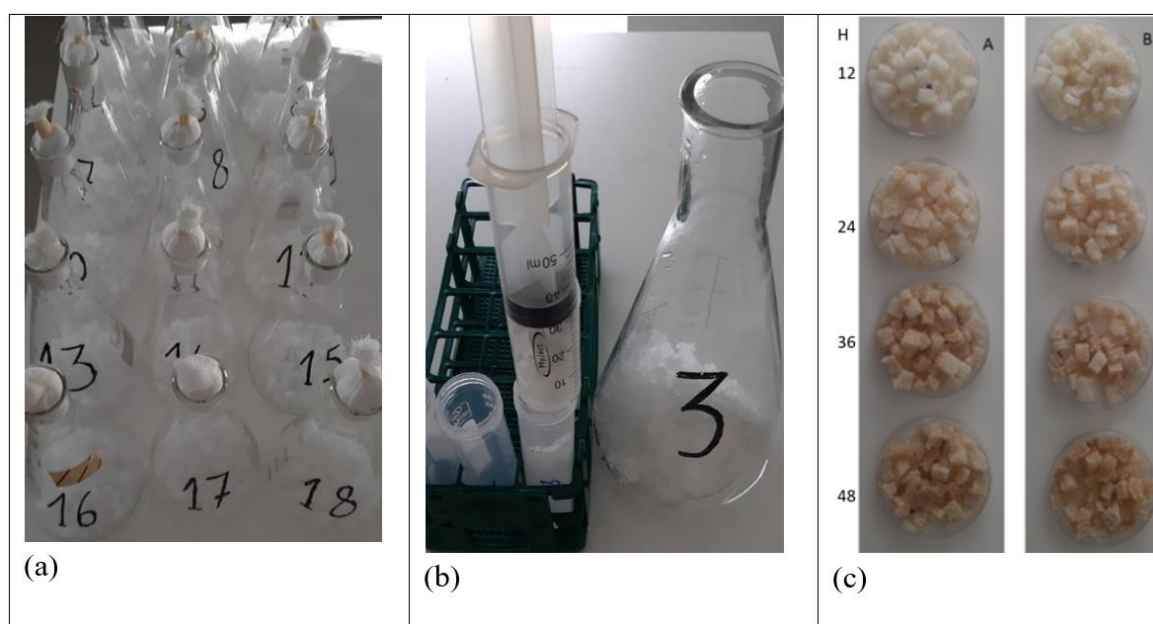


Figure 4.18. SSF with PUF as a solid support material. (a) Prepared 1 L Erlenmeyer with 4 g of PUF material, (b) Harvested culture from PUF material, (c) PUF material after the fermentation process with A *PPAPase*; B untransformed *P. pastoris*

Results of growth, methanol consumption, enzyme activity, and protein content from SSF in PUF material are shown in Figure 4.19. Results of growth are shown in g/L biomass, after the culture is harvested from PUF material, OD_{600} was measured and biomass level was calculated using the calibration line. The rest of the biomass that remained adsorbed in PUF material was estimated by the weight difference of PUF before and after fermentation. Biomass concentrations of relevant time from OD and PUF weight were added and the result is considered as Dry cell weight (DCW) g/L biomass.

High cell density fermentation was achieved during fed-batch fermentation. The highest concentration achieved was 29.12 g/L biomass shown in Figure 4.19 (a). After the first 24 h, 20 g/L glycerol was utilized, the result present in Figure 4.19 (a). The yield coefficient of biomass on glycerol was 0.66 g/g. However, the yield of biomass on methanol for the first 24 h was 0.07 g/g, which was increased to an average of around 0.31 g/g until 120h. Ren *et al.* reported an average biomass yield of *Pichia pastoris* on glycerol 0.45 g/g, and methanol 0.15 g/g [166]. Unrean also reported a higher biomass yield on glycerol than on methanol too [167]. This strain was proved to be MUT^+ , in the literature we found a reported yield of

biomass on methanol around 0.323 g/g for MUT^s, which is a slow methanol utilization phenotype strain [168].

There was also a difference in the specific growth rate when the carbon source was changed. During the batch phase with glycerol, the specific growth rate was 0.05 1/h, meanwhile, for methanol, the specific growth rate was around 0.002 1/h. The highest protein production achieved was 0.9 g/L shown in Figure 4.19 (b) solid line Secondary y-axis and protease activity 60 U/L (Figure 4.19 (b) dash-line). A correlation test between activity and protein content, activity and biomass concentration, and protein content and biomass were performed resulting in these correlating factors 0.6844, 0.821, and 0.807, respectively.

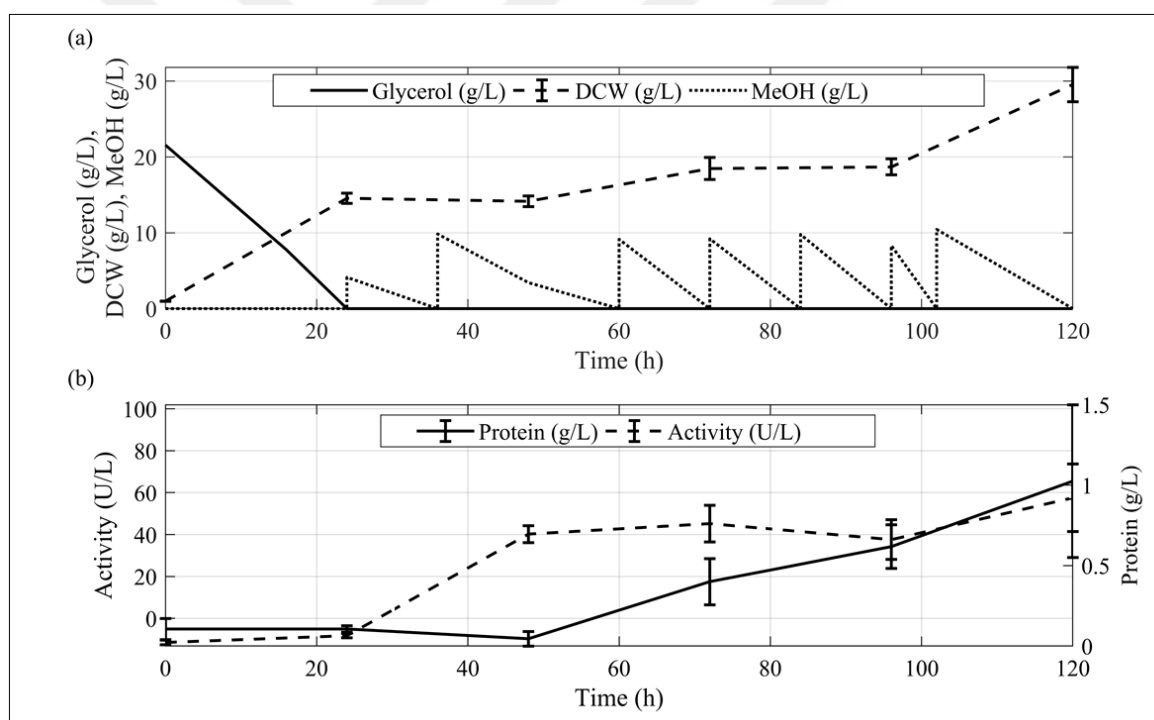


Figure 4.19. Fermentation results for (a) Glycerol utilization (solid line), dry cell weight (dash line), and methanol utilization (dot line), (b) Protease activity (dash line), protein content (solid line) from *PPAPase* in PUF material moistened with Verduyn medium

Pichia pastoris transformed with *TGase* gene was used for SSF with PUF and Verduyn medium. From the growth parameters in Figure 4.20., the highest biomass produced was around 16 g/L and was achieved at 48 h of fermentation (Figure 4.20). After 48 h of

fermentation, culture seems to be under a stationary phase which also explains the accumulation of methanol at 72 h of fermentation. The yield of biomass on glycerol was 0.41 g/g, while on methanol an average $Y_{x/s}$ was 0.18 g/g, which is almost the half of yield on methanol of its similar fermentation with wildtype and *APase* transformed *Pichia pastoris* (Table 4.5).

The highest protein content produced was 1.26 g/L at 48 h of fermentation after which followed a decreasing trend. In the work of Bourneow *et al.*, they reported growth and MTGase activity inhibition, due to nitrogen source crosslinking catalyzed by excreted MTGase [169]. This might be also the reason why in our case the growth of *Pichia pastoris* + *TGase* was inhibited after 48 h of fermentation, only 24 h after the first initiation with methanol, compared with *Pichia pastoris*+*APase* and untransformed *Pichia pastoris* which continue to grow exponentially even after 48 h of fermentation (Figure 4.19. and Figure 4.22.).

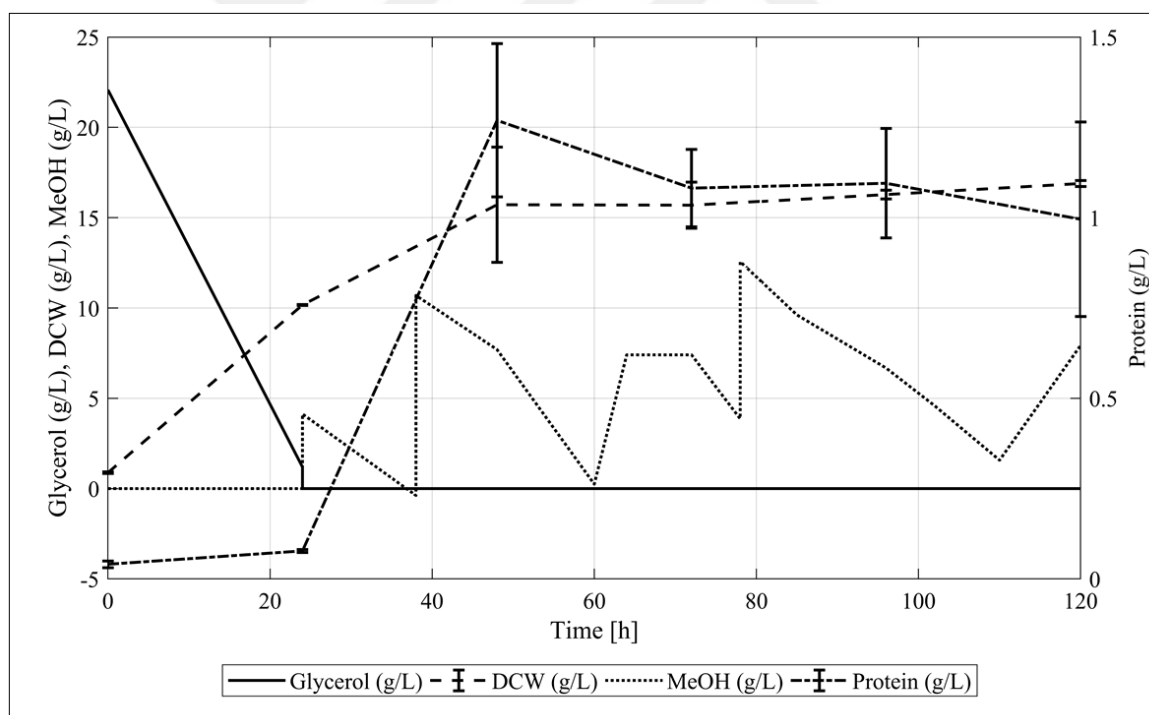


Figure 4.20. Fermentation results for Glycerol utilization (Solid line), Dry cell weight (dash line), MeOH utilization (dot line), and protein content (dash-dot line) from *PPTGase* in PUF material moistened with Verduyn medium

As for the TGase enzyme activity, a qualitative test was performed by SDS-Page and bovine serum albumin. This method is based on the reaction between the produced crude enzyme and bovine serum albumin (BSA) solution. If the TGase reacts, then bands of BSA fractions will fade or vanish. For this method, two different temperatures for incubation reactions were used, 50 and 4 °C.

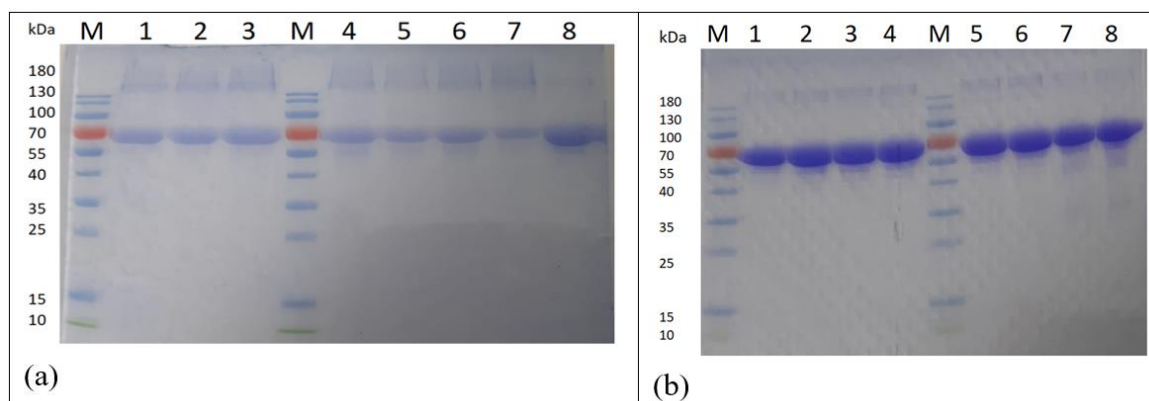


Figure 4.21. Results of the reaction of crud treated TGase with BSA. (a) At 50 °C, where columns, M-protein ruler, (1, 2, 3) 96 h treated supernatant with cleavage buffer, (4, 5, 6) 96 h untreated supernatant, (7) Blank treated in water bath same as samples, (8) Blank not treated in a water bath. (b) At 4 °C, where columns, M-protein ruler, (1, 2) 48 h supernatant, (3, 4) 72 h supernatant, (5, 6) 96 h supernatant, and (7, 8) BSA solution as blank

As seen from above in Figure 4.21, when we used high temperature at 50 °C, little to no TGase activity was observed (Figure 4.21. (a)). The blank band formed over 180 kDa was slightly thicker or equally with the samples treated with TGase. This might happen as a reason for BSA protein denaturation from temperature treatment. However, when the same supernatant was used to catalyze the BSA cross-linking at 4 °C Figure 4.21 (b), a difference between blank and TGase treated sample is observed. The band is over 180 kDa at columns 1, 2, 3, 4, 5, 6 even though faded seems to be thicker than the blank. Moreover, the fractions of BSA around 40- 55 kDa which appeared in blank are not seen in *TGase* treated samples due to the protein crosslinking.

To see the background activity from the host, a similar fermentation with untransformed *Pichia pastoris* was performed, where glycerol and MeOH utilization, cell density, protein production, and protease activity were also monitored. Unlike in *PPAPase* and *PPTGase*, glycerol was spent only after 36 h presented in Figure 4.22 (a) solid line. This was shown also by a specific glycerol consumption rate of -0.94 mmol/h/g DCW. The highest cell concentration was around 24 g/L (Figure 4.22 (a) dash line, while the highest protein produced is 0.9 g/L Figure 4.22 (b) solid line. During 120 h of fermentation, no protease activity was observed Figure 4.22 (b) dash line. This might be due to the lack of protein or peptide in the growing medium as stimulation for native protease production.

Methanol consumption rate was -1.08 mmol/h/g DCW. The yield of biomass on glycerol resulted in 0.51 g/g, meanwhile yield on methanol resulted in 0.31 g/g. The specific growth rate on glycerol was 0.031 1/h on methanol started with 0.0079 and continued to decrease until 0.002 1/h (Table 4. 5).

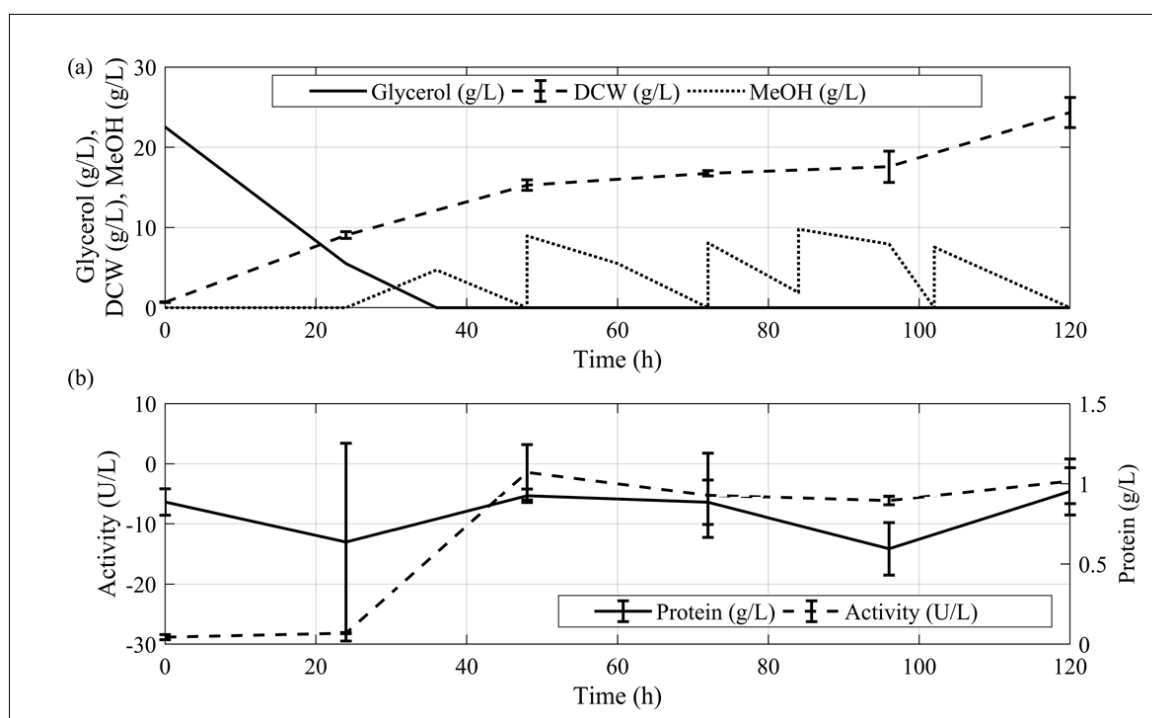


Figure 4.22. Fermentation results for (a) Glycerol utilization (solid line), dry cell weight (dash line), and methanol utilization (dot line); (b) protease activity (dash line), protein content (solid line) from untransformed *Pichia pastoris* in polyurethane foam moistened with Verdun medium

Same fermentation conditions were used for SmF with both transformed *Pichia* cells and the untransformed ones. Results for *PPAPase* are shown in Figure 4.23 with the highest dry cell weight 21 g/L, while the protein content did not exceed 0.45 g/L. The utilization of glycerol and methanol was lower than in SSF with a specific consumption rate -0.77 and -0.82 mmol/h/gDCW, respectively (Table 4.5). The yield of biomass on glycerol was 0.66, while on MeOH an average of 0.17 g/g resulted. In general, the biomass yield is observed to be lower on methanol, compared to glycerol.

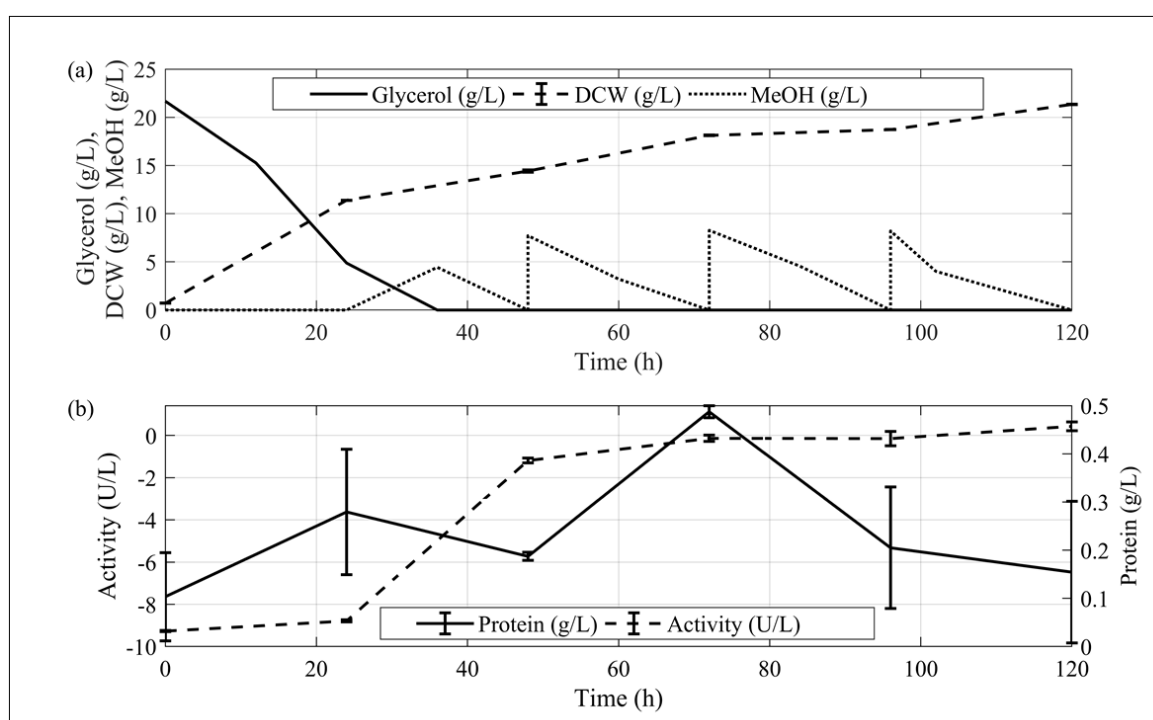


Figure 4.23. SmF results for (a) Glycerol utilization (solid line), dry cell weight (dash line), and methanol utilization (dot line); (b) protease activity (dash line), protein content (solid line) from *PPAPase* in Verduyn medium

Similar to *PPTGase* SSF, after 48 h of fermentation growth nearly stopped in SmF (Figure 4.24) around 20 g/L, while total protein had steady increase to 0.65 g/L, though 35 percentage less than produced in SSF. Specific glycerol consumption rate was around -0.84 mmol/h/gDCW, while methanol was only -0.55 mmol/h/g DCW. Biomass yield in glycerol was around double the value of the one in SSF, however, the yield of biomass on MeOH was slightly lower 0.16 g/g. The specific growth rate on glycerol as expected was 12 fold

higher than the one in methanol. Specific growth rates were similar for homologous fermentation in SSF and SmF.

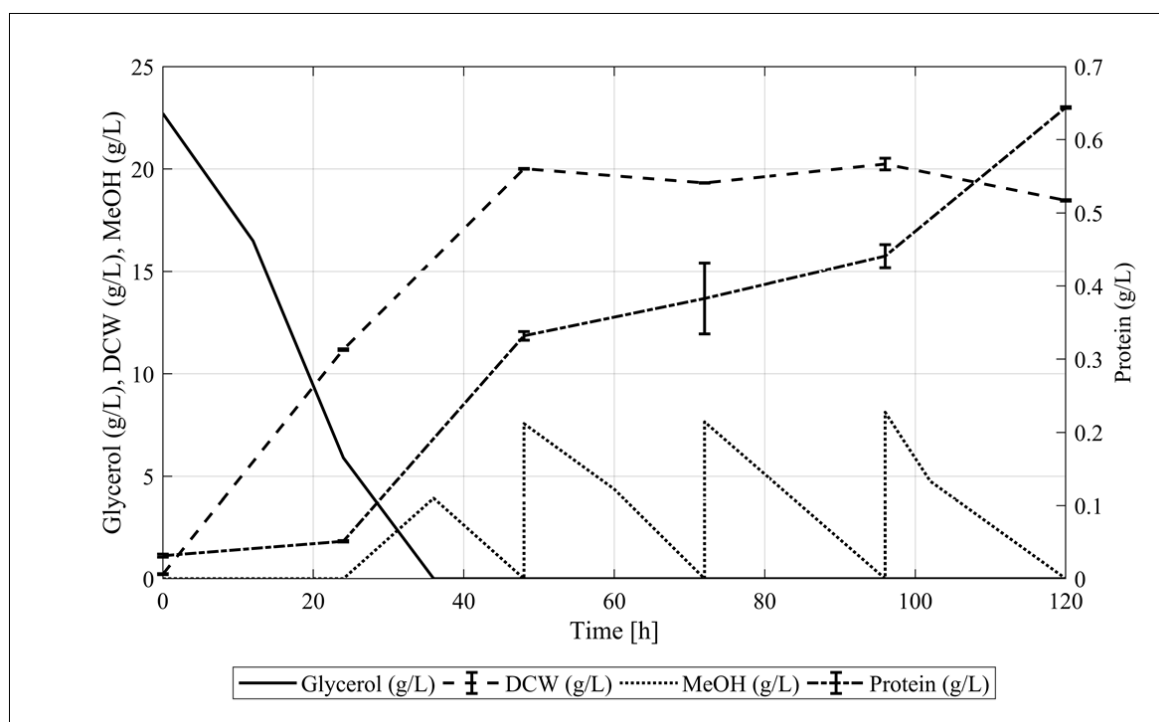


Figure 4.24. SmF results for Glycerol utilization (Solid line), Dry cell weight (dash line), MeOH utilization (dot line), and protein content (dash dot-line) from *PPTGase* in Verduyn medium

Untransformed *Pichia pastoris* cells were used to run a SmF fermentation too. This fermentation serves as a background fermentation for the growth and also for the protease activity. The highest biomass concentration reached was 19 g/L (Figure 4.25 (a) dash-line), while the protein produced was 0.55 g/L (Figure 4.25 (b) solid line). In general, during submerged fermentation biomass production and protein production were lower. The same findings were reported by Viniegra-Gonzalez *et al.* when they produced invertase, pectinase, and tannases by *Aspergillus niger*, with a higher titer of enzyme and biomass in SSF than in SmF. In their SSF process as solid support they used PUF material too, they reported that increasing the initial substrate increased the biomass production in both systems but significantly increased in SSF. Another factor might be the product stability, which is reported to be higher in SSF than in SmF. Moreover, in several research catabolite repression

on enzymes was proved to play a higher effect during submerged fermentation than in SSF [132,170]. These are the factors that might indicate that in our fermentation results, biomass, protein production, and enzyme activity to be higher in SSF than in SmF. Because we are working with yeast *Pichia pastoris*, which is a highly aerobic microorganism, and for biomass and enzyme production oxygen is necessary. In this case, overall oxygen transfer from the gas phase to the medium might be another important factor that makes the difference between SSF and SmF processes. This parameter will be analyzed on the fermentation in the modified reactor where aeration and off-gas measurements are possible.

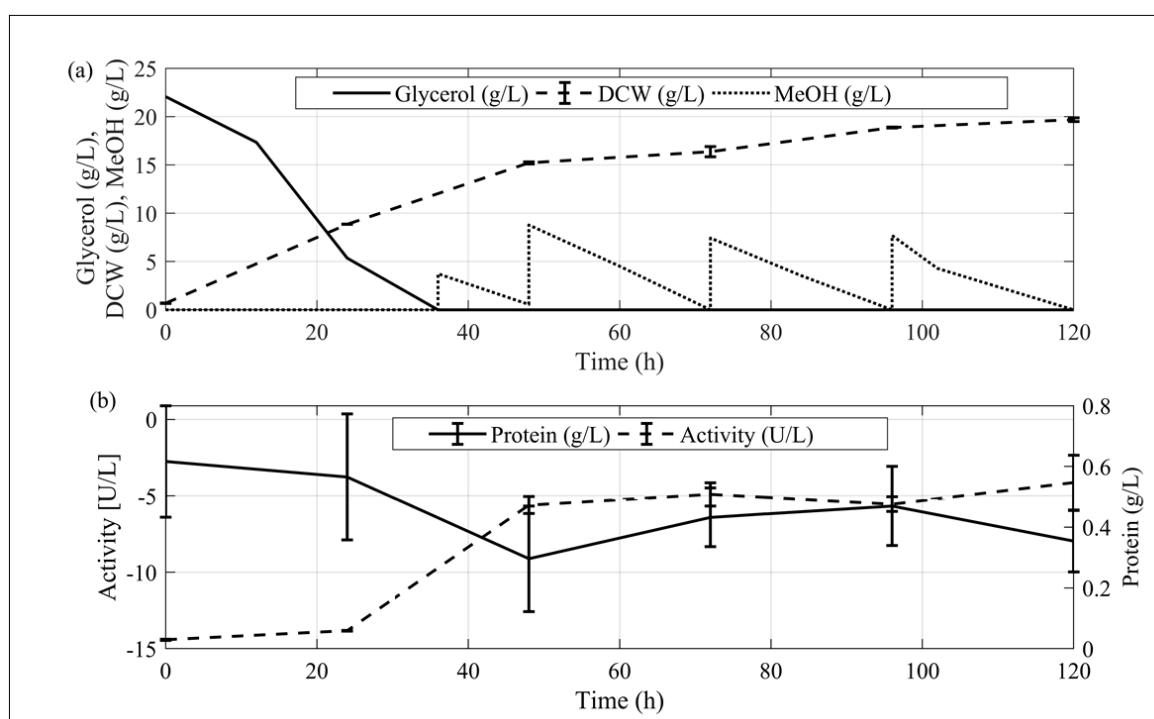


Figure 4.25. SmF results for (a) Glycerol utilization (solid line), dry cell weight (dash-line), and methanol utilization (dot line); (b) protease activity (dash line), protein content (solid line) from untransformed *Pichia pastoris* in Verduyn medium

Table 4.5. Calculated parameter for fermentation

	On Glycerol			On MeOH		
	q_s	$Y_{x/s}$	μ	q_s	$Y_{x/s}$	μ
<i>Pichia</i> + <i>APase</i> SSF Verduyn	-1.27	0.62	0.052	-1.07	0.31	0.0020
<i>Pichia</i> + <i>TGase</i> SSF Verduyn	-1.70	0.42	0.035	-0.76	0.18	0.0025
Untransformed <i>Pichia</i> SSF Verduyn	-0.94	0.51	0.030	-1.08	0.31	0.0030
<i>Pichia</i> + <i>APase</i> SmF Verduyn	-0.77	0.66	0.040	-0.82	0.17	0.0035
<i>Pichia</i> + <i>TGase</i> SmF Verduyn	-0.84	0.72	0.040	-0.55	0.16	0.0020
Untransformed <i>Pichia</i> SmF Verduyn	-1.01	0.57	0.031	-0.60	0.25	0.0020
<i>Pichia</i> + <i>APase</i> SSF Verduyn reactor SSF	-1.72	0.46	0.016	-1.17	0.31	0.0071
<i>Pichia</i> + <i>APase</i> SmF Verduyn reactor SmF	-0.88	0.83	0.080	-1.024	0.57	0.0220

To measure the methanol evaporation during the culture, a mock fermentation was prepared for both SSF and SmF by imitating all the fermentation conditions only instead of the medium dH₂O was used and no cell was added (also in triplicate). Erlenmeyers were sterilized in an autoclave, 8 g/L MeOH was added, and then Erlenmeyer were incubated in an incubator shaker. MeOH was measured every 3 hours, data were analyzed statistically with a t-test for any significant difference between MeOH evaporation in SSF and SmF fermentation. Based on the t-test a significant difference was observed in MeOH evaporation between SSF and SmF. Higher methanol evaporation during SSF resulted according to the test (Figure 4.26.). Higher carbon source evaporation and still higher biomass yield per carbon source during SSF than SmF is explained by higher maintenance energy during SmF, found also in the research of Lima-Perez *et al.* with 33 percentage higher maintenance energy in SmF than SSF [118]. In contrast, the yield on Glycerol was slightly higher in SmF than SSF. Glycerol in the fermentation is used only for biomass production, compared with methanol which is also used as an inducer for enzyme production.

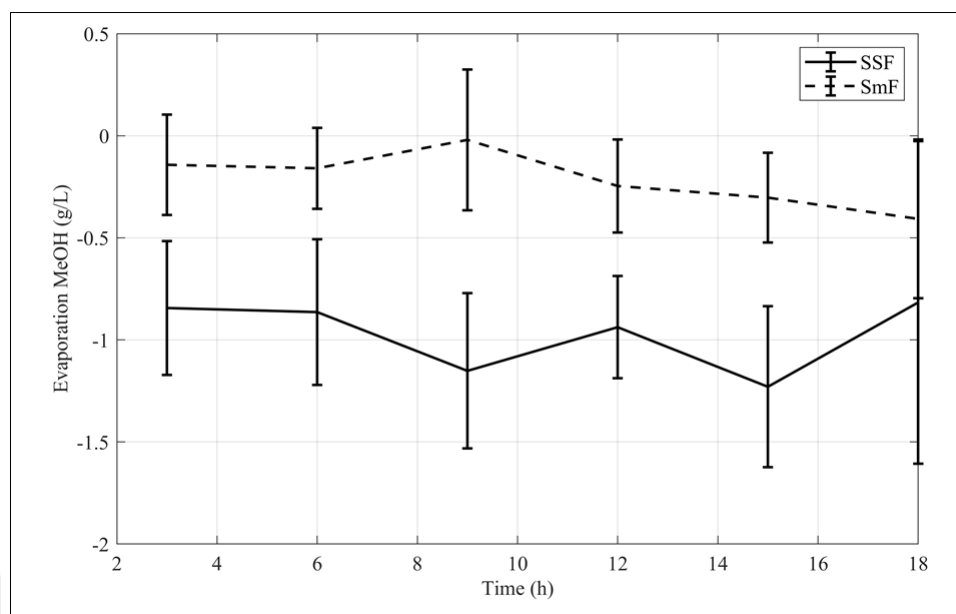


Figure 4.26. Methanol evaporation in solid-state fermentation and submerged fermentation

4.9. SOLID-STATE FERMENTATION IN BIOREACTOR

Solid-state fermentation on PUF support was performed in a modified aerated 3L bioreactor. Off-gas, growth, glycerol, methanol, protein, and enzyme activity were investigated. Parameters as specific substrate consumption, specific growth rate, the yield of biomass per substrate were calculated from the measured parameters and used to evaluate the fermentation. Results of growth, glycerol and methanol content and enzyme activity are presented in Figure 4.27 for SSF. In this fermentation cloned *APase Pichia pastoris* strain was used. Around 40 g/L dry cell weight was achieved, which is near the result of Lopez *et al.* [150] when they grow *Pichia pastoris* 55 g/L dry cell weight on PUF material for recombinant laccase production. In contrast with their results, higher biomass was achieved, but less recombinant protein is produced during SSF than SmF, in our case lower biomass but the higher recombinant protein was produced during SSF than SmF. In SmF around 100 g/L biomass was produced (Figure 4.28), while the enzyme activity was 100 U/L and 30 U/L in SSF and SmF, respectively (Figure 4.27). These results of fermentation in reactors with controlled aeration are in contrast with the results of fed-batch fermentation without aeration,

where biomass growth was higher in solid-state fermentation than SmF. Higher biomass production during SSF was explained with better oxygen mass transfer to the thin layers of the medium that are distributed throughout the polymer structure by author Lopez *et al.* Higher oxygen uptake rate per biomass in SSF than in SmF resulted during fermentation in the modified aerated reactor, where also the off-gas was monitored shown in Figure 4.29. The oxygen uptake rate (OUR) was increased drastically in SSF 24 h after methanol induction and continued through all fermentation, similar increased also noted in SmF but much more moderate. A 30 percentage increase in oxygen uptake rate, when methanol was used as a carbon source, was reported by Gao *et al.* [171]. A higher oxygen uptake rate might be explained by a higher oxygen transfer rate as a result of the PUF material. This increase in oxygen transfer rate might also help with the production of the protein of interest and explain the higher protease activity of the supernatant produced during SSF.

Table 4.6. Specific growth rate through time

Fermentation	μ on glyc	μ on MeOH			
	24 h	48 h	72 h	96 h	120 h
SmF reactor fermentation in Verduyn medium APase	0.082693	0.0268	0.02649	0.0129	0.021
SSF reactor fermentation in Verduyn medium APase	0.016	0.016	0.0031	0.0049	0.0041
SSF APase fermentation in Verduyn medium	0.052	- 0.0009	0.0035	0.000007	0.004
SSF TGase fermentation in Verduyn medium	0.035	0.007	-0.0002	0.0003	0.00018
SSF Wild fermentation in Verduyn medium	0.031	0.0079	0.00095	0.0004	0.0028
SmF APase fermentation in Verduyn medium	0.041	0.0039	0.0028	0.00033	0.0069
SmF TGase fermentation in Verduyn medium	0.042	0.0113	- 0.00053	0.000506	- 0.00076
SmF Wild fermentation in Verduyn medium	0.0315	0.0081	0.00089	0.00137	0.00035

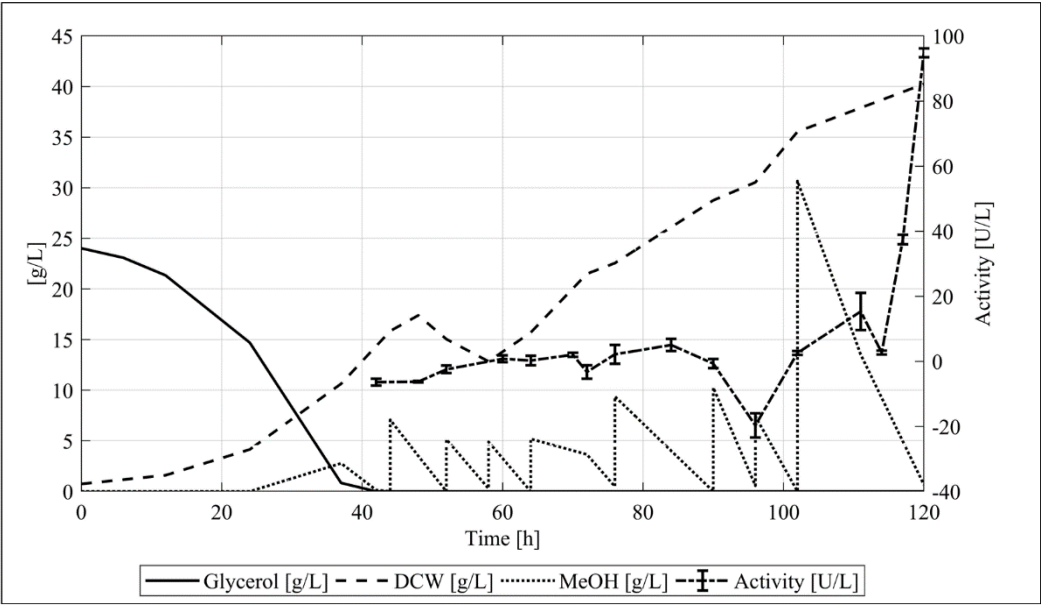


Figure 4.27. SSF with PUF solid-support fermentation by *PPAPase* in 3 L bioreactor

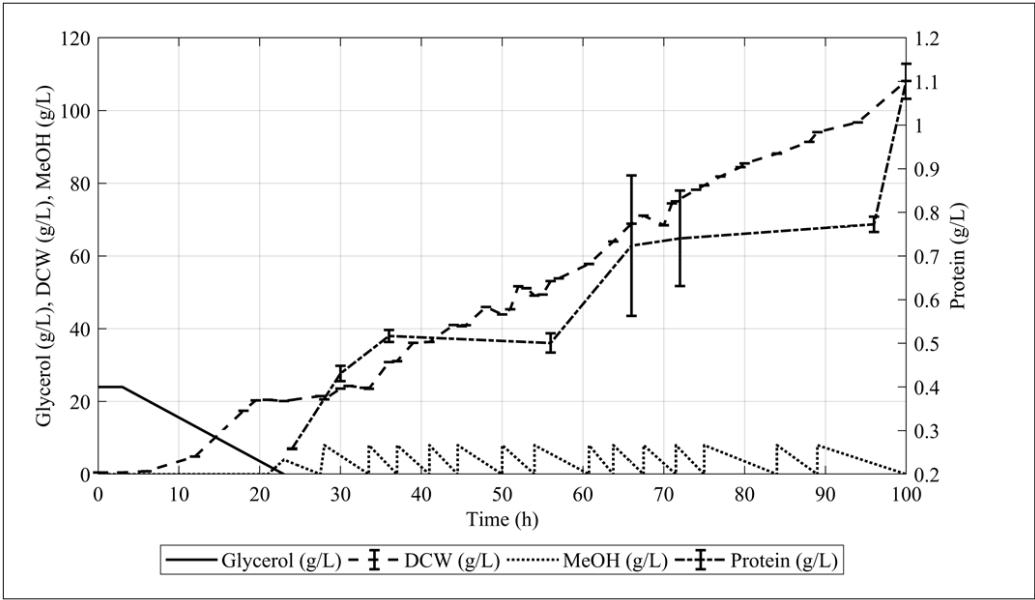


Figure 4.28. SmF fermentation with *PPAPase* in 3 L bioreactor in Verduyn medium

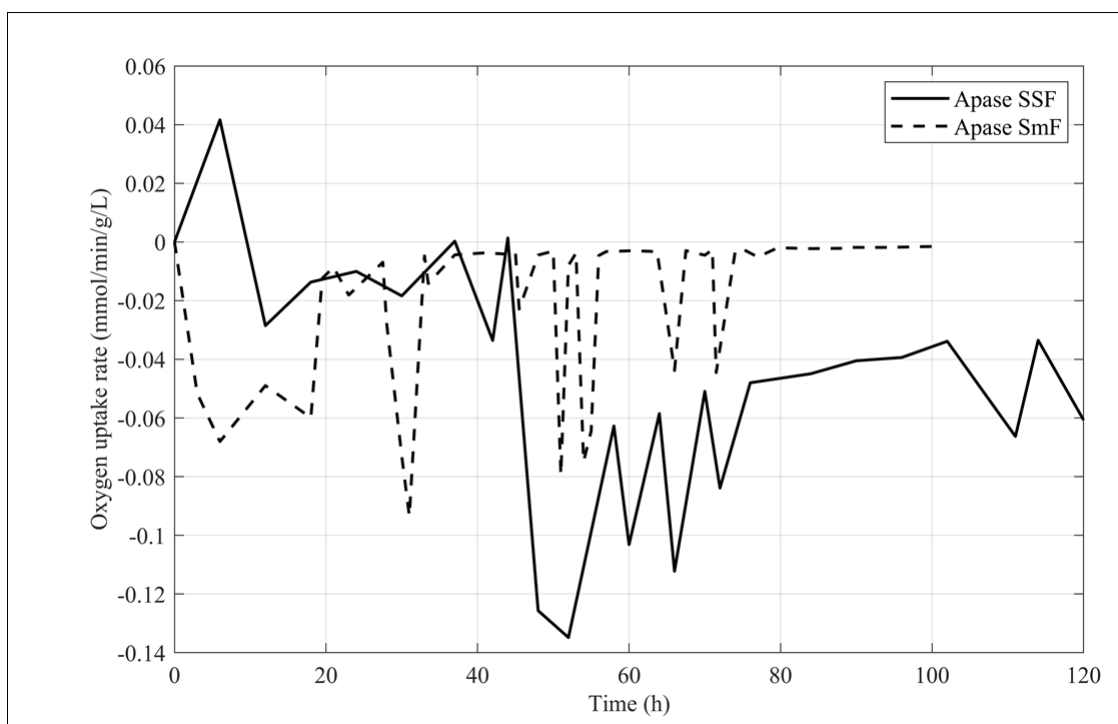


Figure 4.29. Oxygen uptake rate per g/L produced biomass in SSF and SmF

4.10. ADAPTION OF TRANSFORMED *PICHTIA PASTORIS* IN RAPID ADDITION OF METHANOL IN BOTH SSF AND SMF

To understand the effect of MeOH concentration and number of pulse feeds on the adaption of *Pichia pastoris* cells for growth, a set of experiments in 1 L Erlenmeyer was performed for both SSF (Figure 4.30 (a)) and SmF (Figure 4.30 (b)), where in one case 32 g/L is fed at the first feed (Rapid), while in the other case the MeOH concentration is increased from 4, to 8 then to 32, in three successive feedings (Gradual).

No difference in growth during SmF for both rapid and gradual addition of methanol was observed shown in Figure 4.30 (b). The same did not happen in SSF, as is shown in Figure 4.30 (a), samples, where methanol was gradually added, continue to grow, on the other hand, the one with rapid high methanol concentration addition undergoes a stationary phase. From the results, we can state that in SmF rapid addition results in similar yields, allowing shorter

fermentation time, while in SSF gradual addition results in higher biomass. Longer-term experiments are necessary for better conclusions.

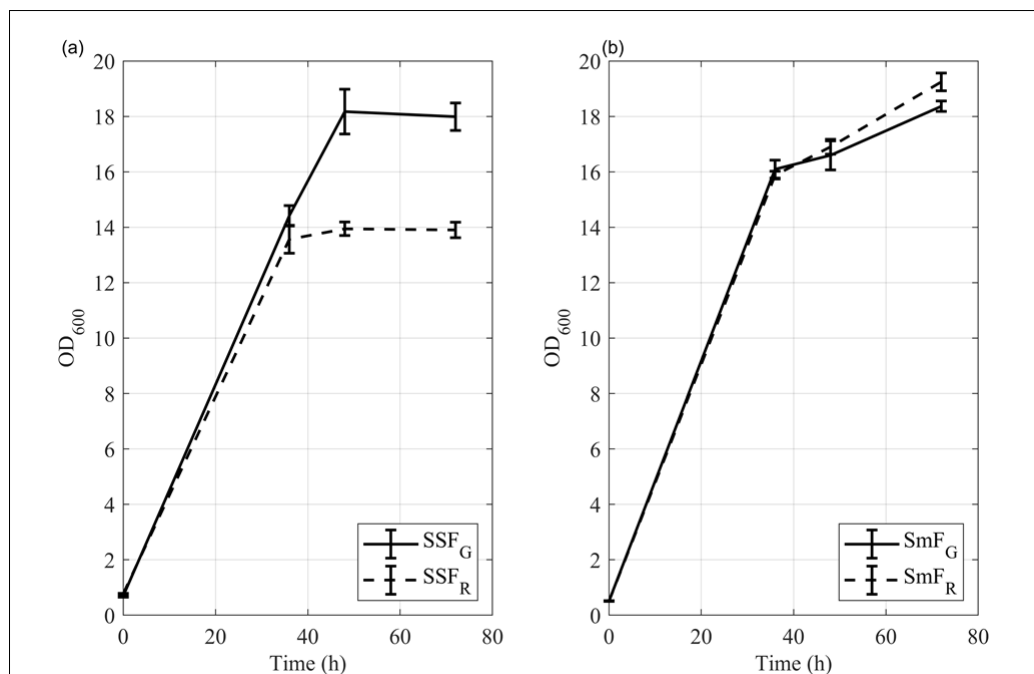


Figure 4.30. The growth of *PPAPase* in two types of fermentation (a) SSF, and (b) SmF with different types of methanol concentration addition

4.11. ENZYME APPLICATION IN FOOD

4.11.1. Protease

Supernatant samples from APase SSF culture, and untransformed *Pichia* SSF culture were collected and used to treat (i) milk for clotting, (ii) hazy beer for clarity, and (iii) meat myofibrils for ripening.

Results of the milk clotting are shown in the milk clotting unit and wet crud weight after coagulation. Reconstituted skim milk of 1 mL was treated with 100 μ L of supernatant and the tubes were visually checked for coagulation each 5 min for 90 min. Results are shown

as curd yield per milk in Figure 4.31 (a), where supernatant from APase SSF culture showed around three-fold higher crud yield per milk than untransformed cells. While visually (Figure 4.31 (b)) there was no coagulation observed in milk samples inoculated with supernatant from untransformed or medium used in SSF. Interestingly, milk coagulated only 15 min after inoculation with APase, which result in terms of milk clotting units is 26 MCU/mL. APase from SSF in PUF/Verduyn medium showed 6.5 MCU/mL while untransformed cells resulted with only 0.8 MCU/mL.

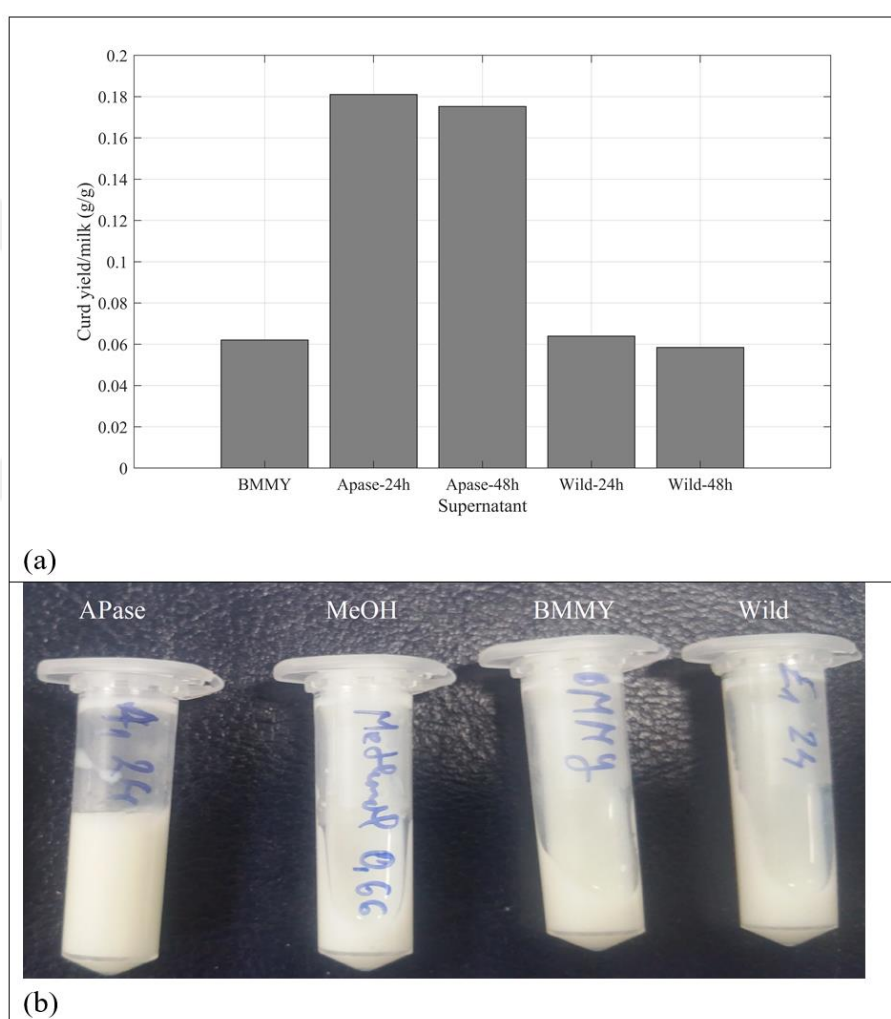


Figure 4.31. Milk clotting assay, (a) curd yield per milk and, (b) Milk treated with supernatant and with different blank

Haze beer is a common problem for the brewery industry and is formed as a reaction between beer protein and polyphenols. If proteins are digested the haze in beer will not be formed.

Another benefit of this process is that the nutrition value of the beer will not be filtered out of beer but will remain in beer in the form of peptide or amino acids

Result of hazy beer treatment with APase producing and WT *Pichia pastoris* are shown in Figure 4.32, where higher turbidity points to protein reaction with polyphenolics. When hazy beer was treated with *PPAPase* supernatant, OD₃₆₀ dropped down around -1.039 fold for 24h supernatant untransformed vs *PPAPase*. For 48h of fermentation it was -1.078 fold. The change was also visually inspected, in Figure 4.32 (b) the difference between a treated and untreated hazy beer can also be seen. The sample treated with APase was crystal clear with no foggy appearance and almost no precipitation.

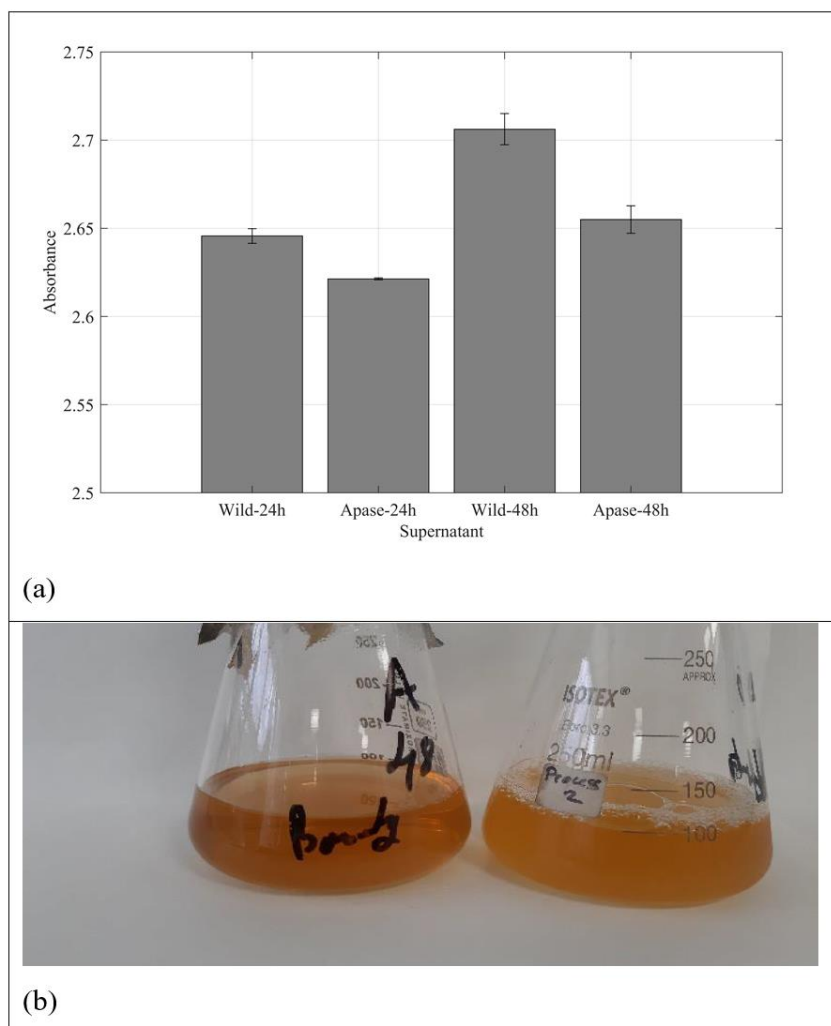


Figure 4.32. Beer treated with Supernatant from APase transformed and untransformed *Pichia pastoris* shown in (a) Abs results of treated beer and, (b) Treated beer with supernatant and blank

Meat tenderness is an important quality parameter in the meat industry. It is known that meat tenderness is improved with the fragmentation of the meat myofibrils and this process mostly happens during the postmortem stage. Natural tenderization of meat depends on the amount of connective tissue, age, species, breed, protein intake, and stress before and during the process of slaughtering, and the treatment of carcasses in the first 24 h of storage. Therefore, to improve the tenderness of the meat, where natural tenderization did not occur, a process called proteolytic tenderization is applied [172,173]. Hence, with aspartic protease produced during SSF and SmF by *APase* transformed *Pichia pastoris*, meat myofibril extraction was performed. Supernatant from untransformed *Pichia pastoris* was used too. As negative control meat myofibril extract was placed in reaction with a growth medium (Figure 4.33). Results are presented in Abs values, more myofibril digested by the enzyme higher Abs resulted. *APase* enzyme produced during SSF showed maximum activity even though the differences with blank were not significant. In this case, we can conclude that *APase* has limited activity in meat myofibrils. Regarding the fact that *APase* is in the group of endopeptidases, it might have self-limiting hydrolysis as explained also by Cronlund and Woychik, where they stated that microbial rennet hydrolyzed myofibrils to a 130 000-140 000 Da, and the rest was resistant to the hydrolysis [174]. This limited reaction is an advantage for the tenderization process compared with other proteolytic enzymes that cause excessive tenderization, in extreme cases until meats get mushy [175].

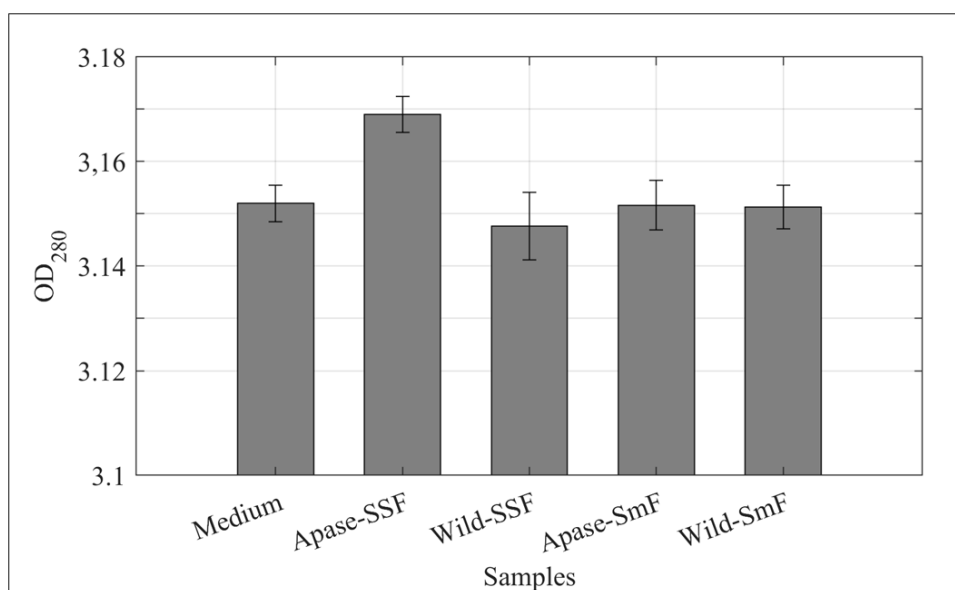


Figure 4.33. Spectrophotometric results of myofibril digestion

4.11.2. Result of Minced Meat Treated by Crude Transglutaminase

Raw minced meat samples 15 g were treated with 3 percentage NaCl, and 2 mL of crude Transglutaminase from culture. After the incubation process at 4 °C, then glued meat particles in a rod shape were subject to different weight pressure. The weight that was enough to separate the reconstructed meat was registered. Below are given the average result of triplicated data graphically for transglutaminase produced in different hours in SSF fermentation with PUF material as solid support (Figure 4.34). As a blank sample minced meat samples are treated with a medium instead of the enzyme.

Based on a two-tail t-test, a significant difference in weight necessary to separate reconstructed meat samples treated by *TGase* supernatant and blank is shown in the result in Figure 4.35. Around 3 fold higher weight was necessary for reconstructed meat samples treated with *TGase* to detach, compared with the sample treated with the medium. There was no significant difference between samples of meat treated with *TGase* supernatant produced 120 h and 96 h of fermentation. The ability of *TGase* to reconstruct minced meat can help the meat processing industry to produce more healthy meat products with less salt and fat addition. *TGase* forms a bond between glutamine and lysine between meat protein which enhances the texture, rigidity, and gel strength of meat. On the other hand, undesirable attributes such as excessive meat adhesiveness and stickiness do not appear according to Ahhmed *et al.* [176].

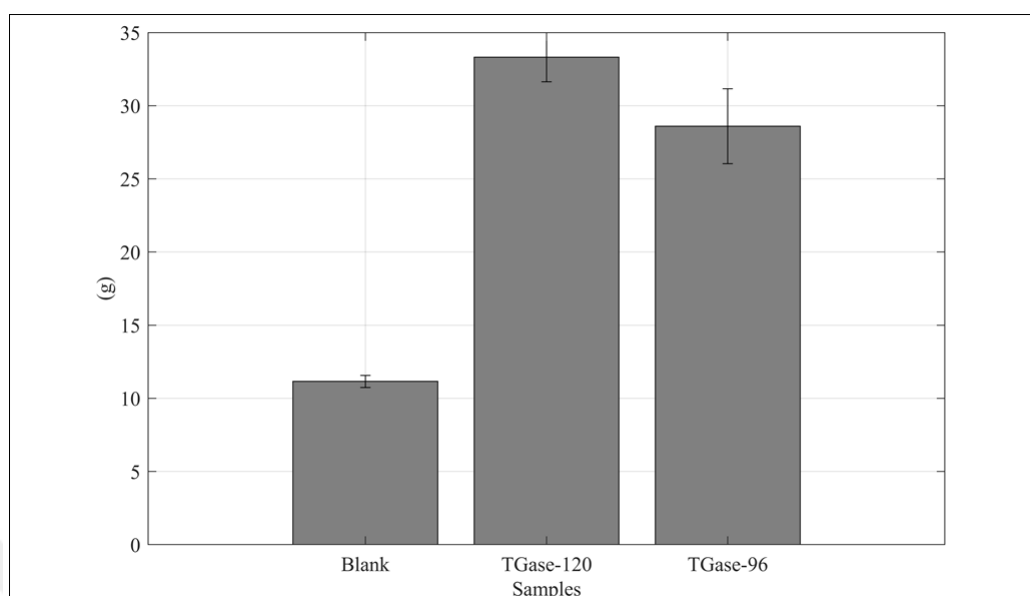


Figure 4.34. Minced meat samples treated with supernatant from *TGase* gene transformed *Pichia pastoris*

5. CONCLUSION

Enzymes are very important functional proteins for the food industry. For many industries, their food product quality depends on the enzyme. Aspartic protease and transglutaminase have shown an important role for several food industries. However their production is still challenging, hence industrially known host organisms are used for their production. One of them is *Pichia pastoris* which is a methylotrophic yeast with a well-studied genome and multiply optimized medium for growth. Growing it in a fermentation process such as solid-state fermentation has scarce research, which is an opportunity for further enhancement of recombinant protein production. Solid-state fermentation on the other hand is known for its advantage for a high concentration of enzyme production, fewer byproducts of materials, and easier downstream processes.

Within the content of this thesis, two genes of interest *de-novo* synthesized for aspartic protease enzyme and transglutaminase were successfully cloned to pPICZ α vector and transformed to *Pichia pastoris* cells. Methanol was used to induce the expression of the gene of interest. Submerged and solid-state fermentations were used to cultivate the microorganism to produce the recombinant enzymes. Two different solid support inert materials were used through the fermentation with two different moistening medium BMMY complex medium, and Verduyn a defined medium. A modified bioreactor was used to control the aeration, pH, temperature and monitor the off-gas. From the data, the oxygen uptake rate was calculated in an attempt to understand more the fermentation type and the oxygen transfer coefficient.

Wheat straw as cost-competitive agricultural byproduct resulted in a promising solid-support inert material to be used in SSF. The highest protease activity was achieved when 10 percentage of this solid support material was used in the fermentation process, where complex medium BMMY was used as a moistening material. Optimization of the moisture content of the SSF showed a significant indication in the production rate. Lower moisture content indicates the diffusion of nutrients in this case carbon source or the induction agent methanol which directly will affect the production rate. While higher moisture content lowered the porosity of the solid support, apropos lowered the oxygen transfer which indicated the production rate. We consider that moisture content should be the first to be

optimized in the SSF fermentation and it's a specific parameter that depends on the structure of the solid support. The medium leftover after the enzyme production in the wheat straw is an easily biodegradable and eco-friendly wheat straw cake.

Artificial inert solid-support like polyurethane foam has great consideration for usage in SSF. PUF rendered the SSF easier to control during the fermentation, the foam was easier to prepare and had a higher water holding capacity. Moreover, after fermentation with PUF, estimation of the biomass was possible, which in general in other SSF is impossible by the interference of the solid material. In all fermentation, oxygen uptake rate was higher in SSF than in submerged fermentation for which we suspect for higher oxygen transfer coefficient in SSF than SmF.

APase produced during SSF was successfully applied in milk, beer, and meat myofibril showing a potential usage in the food industry of the produced enzyme. However, the highest application potential was in the milk industry shortening the process of coagulation from 40 to 15 min. To make it applicable other quality parameters should be taken into consideration for the coagulated milk by the produced enzyme. TGase on the other hand was applied in minced meat where it showed the potential of application with a low amount of salt which in the future can be used by the industry to produce healthier meat products.

Based on the work presented in this thesis we give some recommendation for future work:

Further optimization of the solid state fermentation process parameters are necessary. One of the parameters with great indication is the particle size. In this research only one size of polyurethane foam was used. Further optimization of the particle size it may increase the contact of the biomass with carbon and nitrogen source and still maintain higher oxygen transfer rate than submerge fermentation.

Another recommendation is further optimization the modified reactor for solid state fermentations, specifically for higher homogenous substrate unique designed loops of agitator are necessary. Mixing should not only occur in the horizontal plane but also in the vertical one. Hence we recommend to design and construct a pilot reactor with better agitation system, which will support the specific needs of solid state fermentation.

Focus of future studies should also fall on designing the cloning process, especially for TGase. Designing a cloning process where this enzyme is produced as zymogene enzyme and it is activated by cleaving the pro part in vitro in a unique restriction site, which will

prevent the inhibition of growth and product production as the reason of activity of TGase during fermentation process.



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APPENDIX A: ALIGNED SEQUENCE OF AMPLIFIED DNA FROM GENOMIC DNA OF *PPAPASE* WITH DESIGNED GENE

* CGCCAT-----CGCA-----CATCTGCATTCA-GA-G	25
# TGTGTGCGCCCTTGTGGTTATGACTCTTGTGTTGACGACGCCCATCTGTTCCAAGAAG	60
* CTGAGTGTCCCTCTTGTCTAAAA-CGAAGACTACCAAGCCTAATATCAAGCGTTCAATAGCT	84
# CTGAGTGTCCCTCTTGTCTAAAAACGAAGACTACCAAGCCTAATATCAAGCGTTCAATAGCT	120
* AAAGCTCGTGCCCAAGTACATAAAGCACATAATTAACCCCTTAAGGGGTACCAAGCTGGT	144
# AAAGCTCGTGCCCAAGTACATAAAGCACATAATTAACCCCTTAAGGGGTACCAAGCTGGT	180
* GCTACTACTGACGCAACTGGTACTGTTCCAGTGACAGACTATGCCAACGATATAGAGTAC	204
# GCTACTACTGACGCAACTGGTACTGTTCCAGTGACAGACTATGCCAACGATATAGAGTAC	240
* TATGGCAGGTCAAAGTAGGTACGCCAGCTCAATCCCTTAAATTAATTTTGACACAGGA	264
# TATGGCAGGTCAAAGTAGGTACGCCAGCTCAATCCCTTAAATTAATTTTGACACAGGA	300
* AGTTCAGATTTTGGTTTGACATCTACGTTGTCTACTTGCACACACATACAAGGTAT	324
# AGTTCAGATTTTGGTTTGACATCTACGTTGTCTACTTGCACACACATACAAGGTAT	360
* GACCCAACGAAAAGTTCTACTTACGTGGCAGATGGAAGAGCCTGGAGTATCCAATACGGA	384
# GACCCAACGAAAAGTTCTACTTACGTGGCAGATGGAAGAGCCTGGAGTATCCAATACGGA	420
* GATGGTTCTACTGCTTCTGGTGTGTAGCCAAAGGACACCGTGAACCTTGGCGGACTTACT	444
# GATGGTTCTACTGCTTCTGGTGTGTAGCCAAAGGACACCGTGAACCTTGGCGGACTTACT	480
* ATTAATCCCAACGATCAATCTTGCTAAAAGGAATCATCCTCATTCGCTTCAGACCCC	504
# ATTAATCCCAACGATCAATCTTGCTAAAAGGAATCATCCTCATTCGCTTCAGACCCC	540
* ATAGATGGTTTAAATGGGATTGGGATTGACACCATTACGACGGTTGCAGGCATAAAGACA	564
# ATAGATGGTTTAAATGGGATTGGGATTGACACCATTACGACGGTTGCAGGCATAAAGACA	600
* CCAGTGGATAACTTAATCAGTCAGGGTCTTATCAGTTCACCCGCTATGGAGTTTGGTTA	624
# CCAGTGGATAACTTAATCAGTCAGGGTCTTATCAGTTCACCCGCTATGGAGTTTGGTTA	660
* TGGTAAGGCCAAGACGGTGGTGGCGGTGAGTACTTATTTGGCGGATCTAATCCCAACCA	684
# -GGTAAGGCCAAGACGGTGGTGGCGGTGAGTACTTATTTGGCGGATCTAATCCCAACCA	719
* CTATACTGGAGCACTTTACCACCGTTCCAGTCGATAATTCTCAAGGATTCTGGGGTATTA	744
# CTATACTGGAGCACTT-ACCACCGTTCCAGTCGATAATTCTCAAGGATTCTGGGGTATTA	778
* CTGTTGGTAGTTTGAAGGCAGGAACGACCTCCGTAACAGGTAGTTTCTCTGTTATCCTGG	804
# CTGTTGGTAGTTTGAAGGCAGGAACGACCTCCGTAACAGGTAGTTTCTCTGTTATCCTGG	838
* ATACGGGTACCAAGCTGCTGTTATTTCTCAATCAATAGCAAATAAAGTCGCCGCTCAAT	864
# ATACGGGTACCAAGCTGCTGTTATTTCTCAATCAATAGCAAATAAAGTCGCCGCTCAAT	898
* ATGGCGCCCGTGACAACGGAGACGGTACCTACAGGATATCCTGCTCTACGGCCAATTTAA	924
# ATGGCGCCCGTGACAACGGAGACGGTACCTACAGGATATCCTGCTCTACGGCCAATTTAA	958
* AACCTTTAACTTCACGATTAATGGTGTCTCAATCCAGGTTCCAGTCGATTCAGTGATCT	984
# AACCTTTAACTTCACGATTAATGGTGTCTCAATCCAGGTTCCAGTCGATTCAGTGATCT	1018
* TTGAACAAGACGGTTCAACGTGTTATGCAAGTTTCGGATATGCAGGTTTAGATTTCGCAA	1044
# TTGAACAAGACGGTTCAACGTGTTATGCAAGTTTCGGATATGCAGGTTTAGATTTCGCAA	1078
* TCCTTGGAGATGTTTTCTTGA AAAACAATTATGTCATATTTAACGAGAAGTCCCCCAAG	1104
# TCCTTGGAGATGTTTTCTTGA AAAACAATTATGTCATATTTAACGAGAAGTCCCCCAAG	1138
* TTCAGATTGCTAAGTCCGTTTAACTCTAGA	1133
# TTCAGATTGCTAAGTCCGTTTAACTCTAGA	1167

Figure A.1. Aligned sequence of amplified DNA from isolated genomic DNA from *PPAPase*, marked asterisk (*) with designed *APase* gene marked with Number sign (#).

APPENDIX B: ALIGNED SEQUENCE OF AMPLIFIED DNA FROM GENOMIC DNA OF *PPAPASE* WITH DESIGNED GENE

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* CG-----TTCA-TATTACATGC-----GTGCTGAGTAG-----ATCT 32
| | | | | | | | | | | | | | | | | | | | | | | | | | | |
# GGAGTAATGCTTCCTGCTCCCATGCGAGCAAGTGGCGGAGAGCGGCAAGGGAAGATCT 60

* TATGCTGAGACCCAGGCTTACCGCGAGGATGTCAAAATATAAAGCCCTAAATAAG 92
| | | | | | | | | | | | | | | | | | | | | | | | | | | |
# TATGCTGAGACCCAGGCTTACCGCGAGGATGTCAAAATATAAAGCCCTAAATAAG 120

* CGTGCTTTGACCGCTGAGACGCGAGTAATTCATTGGCTGAACCTCCTCATCAGTATCA 152
| | | | | | | | | | | | | | | | | | | | | | | | | | | |
# CGTGCTTTGACCGCTGAGACGCGAGTAATTCATTGGCTGAACCTCCTCATCAGTATCA 180

* GCACATTACAGGCGCCTGATGCGAGCGATGAGAGGCTCAACCCCGCAGAACCATTA 212
| | | | | | | | | | | | | | | | | | | | | | | | | | | |
# GCACATTACAGGCGCCTGATGCGAGCGATGAGAGGCTCAACCCCGCAGAACCATTA 240

* AACAGAATGCGCGCTTACAGAGCTTATGCTGCGAGCGCATACAGTGTAAATAAT 272
| | | | | | | | | | | | | | | | | | | | | | | | | | | |
# AACAGAATGCGCGCTTACAGAGCTTATGCTGCGAGCGCATACAGTGTAAATAAT 300

* TACATAAGGAATGCGAGCGCTGATAGTCATAGGAGCGAATTCAGCAGCAGTACT 332
| | | | | | | | | | | | | | | | | | | | | | | | | | | |
# TACATAAGGAATGCGAGCGCTGATAGTCATAGGAGCGAATTCAGCAGCAGTACT 360

* GAGAACAGCGCTGAGAACTGAGTTACGCTTGTGTGGAGTACTTGGGTCAACAGTGA 392
| | | | | | | | | | | | | | | | | | | | | | | | | | | |
# GAGAACAGCGCTGAGAACTGAGTTACGCTTGTGTGGAGTACTTGGGTCAACAGTGA 420

* CCTTATCTACTACAACTGGCGCTTTCCTTCTTGTGATGAGGACAGTACAGTCTGAC 452
| | | | | | | | | | | | | | | | | | | | | | | | | | | |
# CCTTATCTACTACAACTGGCGCTTTCCTTCTTGTGATGAGGACAGTACAGTCTGAC 480

* TTGGAGACTCAAGTCCCGCTTACAGAGCCCAAGCAGATTGAGGCGCTATTGTC 512
| | | | | | | | | | | | | | | | | | | | | | | | | | | |
# TTGGAGACTCAAGTCCCGCTTACAGAGCCCAAGCAGATTGAGGCGCTATTGTC 540

* AAAGTAGTTTGTGAGGCTAAAGGATTAAAGAGCTAGGAGTGTGGTCAATAATG 572
| | | | | | | | | | | | | | | | | | | | | | | | | | | |
# AAAGTAGTTTGTGAGGCTAAAGGATTAAAGAGCTAGGAGTGTGGTCAATAATG 600

* AATAAGCATTGGATGTCACATGAGGAGGAAGTACATAGATAATCTAAAAAGAG 632
| | | | | | | | | | | | | | | | | | | | | | | | | | | |
# AATAAGCATTGGATGTCACATGAGGAGGAAGTACATAGATAATCTAAAAAGAG 660

* TTAGCAATAAGATGATGGGAGGGTA--AA-----ACTTGGATT----- 674
| | | | | | | | | | | | | | | | | | | | | | | | | | | |
# TTAGCAATAAGATGATGGGAGGGTA--AA-----ACTTGGATT----- 720

* -----CCC--CTTCAAAA--GA-----ATGAAA----- 695
| | | | | | | | | | | | | | | | | | | | | | | | | | | |
# TTAGCAATAACACCTCTCAAGAGCGTGACGAGGTAACTACGATCATCAAGATG 780

* AAGCAGTTAGGTATATTTCCCAAGGCACTTTTGGGTCAGGGTCAGGATTCAG 755
| | | | | | | | | | | | | | | | | | | | | | | | | | | |
# AAGCAGTTAGGTATATTTCCCAAGGCACTTTTGGGTCAGGGTCAGGATTCAG 825

* -GAGGAGGATTTCTAGTGAACAAGGCGTAAGTTATGGTGAACCCCGAGCCATT 814
| | | | | | | | | | | | | | | | | | | | | | | | | | | |
# AGAGGA-----TCTAG-TGA-CAA--GCTAA-GT-ATGG-TGA---CCCCGAGC-ATT 869

* TCAGGCGCGGATTCAGGGGACCGGAGTATGGTATATGCTAAGGATAGGGAATAT 874
| | | | | | | | | | | | | | | | | | | | | | | | | | | |
# -CAGG-CGCG-AT-CAGGG-AACC-GGA-TTGGTATATGCTAAGG-ATAGG-AATAT 920

* AACCAGATCTCCCGCAGGCGCGGAGATCATGGGTGAATTTGATTATGGATGTTTC 934
| | | | | | | | | | | | | | | | | | | | | | | | | | | |
# AACCAGATCTCCCGCAGGCGCGGAGATCATGGGTGAATTTGATTATGGATGTTTC 978

* GGCGCAGACCGAGGCGGATGCGGATGAAGTATTGGACATGCTAACCACTACCA 994
| | | | | | | | | | | | | | | | | | | | | | | | | | | |
# GGCGCAGACCGAGGCGGATGCGGATGAAGTATTGGACATGCTAACCACTACCA 1037

* TGCTCCCAACGCGCGGCTCGGACCAATGATGCTATGAGTCTAAATCAGGAATTGTC 1054
| | | | | | | | | | | | | | | | | | | | | | | | | | | |
# TGCTCCCAACGCGCGGCTCGGACCAATGATGCTATGAGTCTAAATCAGGAATTGTC 1097

* CGCGGATAGCGGATTTGATAGGGGCACTTACGTTTACATTCATTC-TAAGTCATG 1113
| | | | | | | | | | | | | | | | | | | | | | | | | | | |
# CGCGGATAGCGGATTTGATAGGGGCACTTACGTTTACATTCATTC-TAAGTCATG 1157

* GAATACGGCGCGCAGAGGTGAACAGGT-GTCTTAAGCGCGCGC 1161
| | | | | | | | | | | | | | | | | | | | | | | | | | | |
# GAATACGGCGCGCAGAGGTGAACAGGTGCTCTTAAGCGCGCGC 1205

```

Figure B.1. Aligned sequence of amplified DNA from isolated genomic DNA from *PPTGase* marked as asterisk (*) with designed *TGase* gene marked with Number sign (#).