CLONING AND PURIFICATION OF BIOSYNTHETIC ENZYMES

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by

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İZMİR

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ABSTRACT

CLONING AND PURIFICATION OF BIOSYNTHETIC ENZYMES

Bioproducts have become prominent with their sustainable, eco-friendly and renewable features. In recent years, research and development studies focused on production of biodiesel and pharmaceuticals. Biodiesel can be synthesized in the form of fatty acid ethyl ester (FAEE) by *in vivo* activity. This synthesis is catalyzed by wax ester synthases (WS). This study aims cloning and purification of wax ester synthases from *Psychrobacter arcticus* 273-4 (PaWES) and *Mus musculus* C57BL/6 (MmWES). PaWES was cloned and expressed by *Escherichia coli* BL21(DE3) strain, at the proper conditions with using pET expression system. It was purified with approximately 1 mg yield. Cloning of the second wax ester synthase (MmWES) was achieved to *Saccharomyces cerevisiae* and it was purified with lower than 1 mg yield. The other aim of this study is related to taxadien-5α-ol-O-acetyltransferase from *Taxus cuspidata* (TcT5AT). This enzyme belongs to the biosynthesis pathway of Taxol, which is the most commonly used chemotherapy drug. Cloning and purification studies of this enzyme were successfully performed. It was expressed by *Escherichia coli* BL21(DE3) Star strain and purified with the yield of 23 mg. Immobilized Metal Affinity Chromatography (IMAC) is used for all three enzymes as a purification strategy. This project can pave the way for structural studies of all biosynthetic enzymes mentioned above. In summary, the findings of this study will circuitously help for solving the relationship between function and structure of these enzymes. It may lead to increased generation of Taxol and FAEE based biodiesel.

ÖZET

BİYOSENTETİK ENZİMLERİN KLONLANMASI VE SAFLAŞTIRILMASI

Biyoürünler, petrol kaynaklı ürünlere göre sahip oldukları avantajlarla ve yenilenebilir kaynaklara dayalı, çevre dostu bir üretime sahip olmalarıyla her geçen gün daha da önem kazanmaktadırlar. Biyoürün sınıflarına baktığımızda, araştırma ve geliştirme çalışmalarının en yoğun olduğu alanlar ilaç ve biyoyakıt üretimidir. Biyoyakıt olarak en büyük potansiyele sahip olan biyodizel, mikroorganizmalar kullanılarak da üretilebilmektedir. Bu sentezdeki kilit role sahip olan mum esteri sentaz enzimlerinin, önceki çalışmalarda farklı organizmalardan izole edildiği belirtilmiştir. Bu çalışmada, *Psychrobacter arcticus* 273-4 (bakteri) ve *Mus musculus* C57BL/6 (fare) kaynaklı iki mum esteri sentaz enziminin klonlanması ve saflaştırılması amaçlanmıştır. *P. arcticus* 273-4 kaynaklı mum esteri sentaz enzimi pET22bTV vektörüne klonlanmış, *Escherichia coli* bakterisinin BL21(DE3) hücre hattı kullanılarak üretilmiş ve yaklaşık 1 mg verimle saflaştırılmıştır. *Mus musculus* C57BL/6 (fare) kaynaklı diğer mum esteri sentaz enzimi de *Saccharomyces cerevisiae* maya türüne klonlanmış ve 1 mg'dan daha düşük bir verimle saflaştırılmıştır. Çalışılan son enzim *Taxus cuspidata* kaynaklı taxadien-5α-ol-O-asetil-transferaz, en yaygın kullanılan kemoterapi ilacı Taxol'ün biyosentezinde önemli bir role sahiptir. Bu enzim, gen ifade vektörü olan pET22bTV sistemine klonlanmıştır. Daha sonra enzimin üretimi, *Escherichia coli* bakterisi ile BL21 (DE3) Star hücre hattı kullanılarak gerçekleştirilmiştir. Saflaştırma işlemleri sonucunda yaklaşık 23 mg protein elde edilmiştir. Temel yaklaşım olarak, üç enzimin de saflaştırma çalışmaları immobilize metal afinite kromatografi metodu ile gerçekleştirilmiştir. Bu çalışma; gelecek vaat eden ürünlerin biyosentezinde görev alan üç önemli enzimin klonlanması ve saflaştırılması koşullarını açığa çıkararak, gelecekteki yapısal protein mühendisliği çalışmalarına dolaylı olarak yardımcı olmuştur.

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CHAPTER 1

INTRODUCTION

1.1. Bioproducts

1.1.1. Overview of Bioproducts

Biotechnological applications, which include life sciences and engineering methods, were noticed in the late 19th century. After the energy crisis in the 1970s, especially in manufacturing and industry, these applications had an increasing trend of using biomass. (Dunfold, 2012)

Biomass can be used with the aim of forming renewable chemicals and materials. According to European Committee for Standardization (EN16575), products that are based on whole or parts of plants, trees, algae, marine organisms, microorganisms, animals and industrial or agricultural waste etc. are called bioproducts.

A single cell or a system of cells in an organism can be a source for a bioproduct. It can be either a structural substance from a cell or a component which is generated from a various structural parts of an organism. Additionally, bioproducts can be created from a material that is formed by transformations into a different host organism. (Kudra, Strumillo, 1998) As a basis, the production goes on three different strategies; improvement of several ways for commercial production of natural entities, applying various genetic manipulations which are aimed to generate components naturally, or creation of completely new components. (Barakat, 2012) All of these combinations cause diversity of bioproducts.

Mainly, bioproducts are divided into four types; biochemicals, biomaterials, biofuels and bioenergy. (Agricultural Government of Canada Statistics, 2015) Bioplastics, biofibres and biorubbers are couple of examples for biomaterials. Beside this, pharmaceuticals, antibodies, biocosmetics, food ingredients and solvents are shown as biochemicals. Bioethanol, biodiesel, biogas and biohydrogen are at the forefront as biofuels. Moreover, the utilization of biomass for heat and power can be denoted as bioenergy. (Thimmanagari, 2010)

This project aims to isolate and purify the enzymes involved in synthesis of biofuels and important pharmaceutical products.

1.1.2. Benefits & Risks of Bioproducts

Bioproducts have been a remarkable concept due to their low environmental impact in the production and consumption.

British Petroleum announced that, according to their calculation, the amount of remainder petroleum reserves is nearly 171.1 thousand million tons. By taking into account of the daily consumption with 11.6 million tons of petroleum reserves all over the world, it can be easily realized that the global economy and energy demand will require a new resource. Furthermore, as a result of the crude oil based combustion, greenhouse gas emissions (GHGs) led to an irremediable climate change. (Pinzi et al., 2013) The solution to displace these problems is an alternative and renewable resource; like biomass based products. (Röttig et al., 2010) Some advantages promised by bioproducts are having environmental friendly production process, less greenhouse gas emissions, decreased need of petroleum resources, low toxicity, comparable features with petroleum-based products, reduced total energy usage, inexpensive recycle procedures, development of rural economy, employment opportunities, diminished contamination and wastes at manufacturing plants. (Biddy et al., 2016; Sparling et al., 2006) Also high productivity from biomass resources can enlarge the accessibility of energy all over the world.

In contrast, there are some concerns about bioproducts according to the researchers and community. Decreasing biodiversity, lacking area for food stocks, usage of genetically modified crops and microorganisms for efficient production, the probability of slaying the wild life habitat, and high costs requirement for research and commercialization are the disadvantages of bioproducts. (BIOCAP Canada, 2004; Dale, 2016) To prevent these disadvantages before being a problem for the industry, laws and policies can regulate the usage and production. Likewise, promotions, grants and assistances can guide the innovations for increasing the bioproducts market potential. (Staffa et al., 2013; Guo et al., 2007)

1.1.3. Market Analysis of Bioproducts

Producing alternative biobased products has increased the interest about research and development of bioproducts concept. These innovations have provided a sustainable investment area and economy, which is called bioeconomy. (Staffa et al., 2013) According to the Organisation for Economic Co-Operation and Development (OECD), the idea behind bioeconomy comprises of biotechnological knowledge, renewable biomass and combination of them into applications. The growing capacity of developments in production and effective resources which are based on biological processes are highlighted by the OECD. (OECD Report, 2009)

Biorenewable global market potential value was reported as approximately $2.4 billion in 2010. (Jong et al., 2012) According to the current analysis, the share of market total sales about biochemicals is $19.7 billion. (Biddy et al., 2016) If the revenue from biofuel and bioenergy counts in that calculation, the annual earning of bioproducts reaches to $57 billion, anounced by European Commission in 2016. Another study performed by U.S. Department of Agriculture detailed that 300 million gallons of crude oil per year is replaced with bioproducts. This amount is equal to removing 200.000 cars from the road. (Biddy et al., 2016)

Biomass resources provides nearly 10% of the industrial energy, however it is estimated that this share will be increase with higher demand for bioresources. (Kutnar, Muthu, 2016) 30% usage of petroleum resources in present can be displaced with biobased fuels by 2030 with the obtained resources. (Dang et al., 2016)

The tendency to being a part of bioeconomy can be seen easily in the statistics of firms. 65% of them has participated in research and development studies about alternative bioproducts. (Kutnar, Muthu, 2016) As a result, biochemicals-especially in pharmaceutical area-and biodiesel production play huge role in industry due to their promising features.

1.2. Biodiesel

1.2.1. Overview of Biodiesel

The first emergence of biofuels can actually be seen in 1900 with the invention of a diesel engine, which used peanut oil as combustible agent, by Rudolf Diesel in Paris World Exhibition. However, this engine started to be used with petroleum-based diesel because of the high accessibility and low costs of crude oil. Until the energy crisis and diminishing petroleum, biofuels have lost their remarkability to being a basis of energy. Biodiesel is the most suitable alternative with its comparable features with petroleum-diesel among bioethanol, biomethanol, biogas and biohydrogen. (Kamarudin, Yaakub, 2011)

According to European Committee for Standardization (EN14214) and US Standard Specification for Biodiesel (ASTM D6751), a fuel comprised of monoalkyl esters of long chain fatty acids derived from vegetable oils or animal fats.

Detailed features of biodiesel are presented in Table 1.1. Biodiesel has nearly same viscosity with petrol-based diesel. Non-flammability, biodegradability, nonexplosiveness with 423K flash point and non-toxicity features are some significant differences from conventional diesel. (Demirbas, 2009)

There is no need to modify diesel engines to use biodiesel in pure form or blended with petrodiesel. 100% pure biodiesel is coded as B100 by the published standards sheet (ASTM D6751). In this BXX code, XX changes with the ratio of blended biodiesel like B20 blend composed of 20% biodiesel and 80% petroleum based diesel. It is the most common blend and it has been used commercially in Germany, Italy and Malaysia. (Kamarudin, Yaakub, 2011)

Table 1.1. Detailed feature of biodiesel
(Source: Demirbas, 2009)

Common Name	Biodiesel
Common chemical name	fatty acid (m)ethyl ester
Chemical formula range	C_{14}-C_{24} methyl esters or $C_{15\text{-}25}H_{28\text{-}48}O_2$
Kinematic viscosity range (mm^2/s, at 313 K)	3.3-5.2
Density range (kg/m^3, at 288 K)	860-894
Boiling point range (K)	>475
Flash point range (K)	420-450
Distillation range (K)	470-600
Vapor pressure (mm Hg, at 295 K)	<5
Solubility in water	insoluble in water
Physical appearance	light to dark yellow, clear liquid
Odor	light musty, soapy odor
Biodegradability	more biodegradable than petroleum diesel
Reactivity	stable but avoid strong oxidizing agents

1.2.2. Advantages & Disadvantages of Biodiesel

Biodiesel has taken attention with its important advantages in the energy sector. Due to its production based on renewable source as oilseed crops, the handling, transporting and storing are safer than the conventional diesel. It also has environmental friendly process, less sulfur and aromatic substances which is decreased 20-50 times, and lower exhaust emissions. It can be degraded four times faster than petrodiesel because of its oxygen content. Higher oxygen content also led to higher combustion efficiency. Increased cetane number and lubricity are some other advantages that improves engine performance. Less friction wear in engine parts are supplied with high lubricity. The rise of lubricity with 30% can be observed even the content of biodiesel is below 1%.

High price, decreased engine speed and power, less energy content are some of disadvantages of biodiesel. Additionally, it is not suitable to long-term storage due to the probability of autoxidation. Also cold start problem and increased copper strip corrosion properties of biodiesel are the other problems for a functional engine. (Demirbas, 2009; Moser, 2009)

1.2.3. Production of Biodiesel

The conventional biodiesel production process is based on a chemical reaction called transesterification, in which triglyceride and alcohol act as reactants, fatty acid alkyl esters is the product, glycerol is formed as a byproduct, in the presence of a catalyst. (Figure 1.1.) This process composes of three sequential reversible reactions. In the first step, triglycerides from vegetable oils and animal fats are transformed to diglycerides and later on, they are converted to monoglycerides. As a result of third reaction, glycerol is produced from monoglycerides. In each conversion, one mole of ester molecule is produced. Consequently as seen in Figure 1.1., three moles of esters and one mole of glycerol are obtained from one triglycerides molecule.

Transesterification reaction is performed with different methods, such as:

- acid catalysis
- alkali catalysis
- enzyme catalysis
- noncatalytic

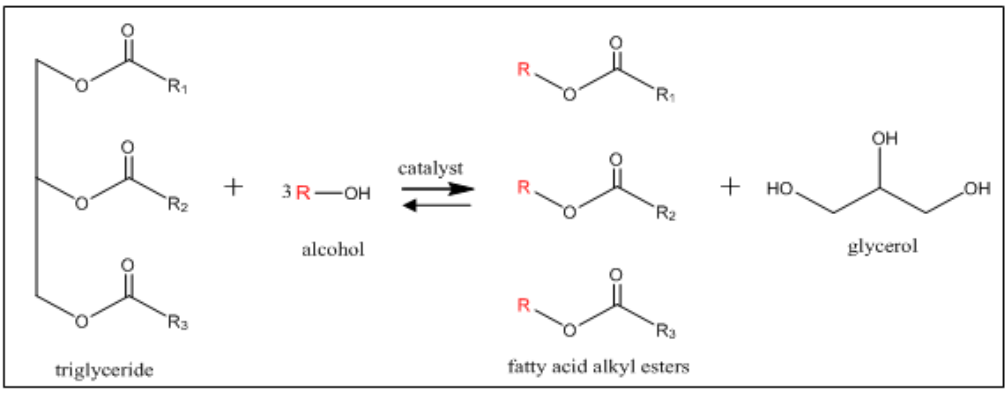

Figure 1.1. Fatty acid alkyl ester production

A catalyst is needed to provide better reaction rate and efficiency. The catalyst should be selected as acid or alkali according to the free fatty acids (FFA) in the oil which is used in reaction. Enzymes can also be used as catalyst for this reaction. The most preferred acid catalysts are sulfuric acid, hydrochloric acid and sulfonic acid. They catalyze the conversions very efficiently however the rate of reactions are slow and the usage of acid catalyst is hazardous. Sodium hydroxide and potassium hydroxide are the most common alkali catalysts. They are mixed with alcohol which is preferably metha-

nol when pumped into the reaction. Despite of higher activity of potassium methoxide in reaction, sodium methoxide is widely used at industrial applications, due to the hazardous properties of handling metallic potassium. The rate of alkali catalyzed reaction is higher than acid-catalyzed reaction. It has been reported that transesterification takes 2-5 minutes with sodium methoxide at room temperature, effectively. However, this method has some disadvantages like the difficulty of product separation and removing of the catalyst, increased energy and cost requirements. In both alkali and acid catalyzed reactions, alcohol used in excess amount in reaction to shift the equilibrium to products. The last type of catalyst is lipase which is providing an enzymatic reaction. Although it has a simpler separation process as an advantage, high costs of that catalyst make it unsuitable to industry. (Kamarudin, Yaakub, 2011; Adamczak, Bornscheuer, 2009) Furthermore, there are other production methods via supercritical alcohol transesterification and BIOX processas noncatalytic types. Supercriticial reaction results in purer product, more eco-friendly process, decreased need of energy and time. On the other hand, this reaction proceeds with the conditions of 525-675 K temperature, 35-60 MPa pressure. Triglycerides will be degraded under the following conditions. According to this, undesired byproducts and low quality biodiesel are inevitable to observe. The other noncatalyzed method is BIOX process which contains two production steps. The conversion of free fatty acids is the main aim of the first step. After that, triglycerides will be transesterified with alcohol. In both steps the usage of a co-solvent, most commonly tetrahydrofuran, is the essential difference of BIOX process. Free fatty acids and water lead to soap production and the soap affects the production yield of biodiesel. This problem is solved by using BIOX process. However, toxic feature of the co-solvent requires the additional removing step, which means extra costs for separation and purification. Biodiesel cannot be fitted for the international standards without the separation of co-solvent. (Demirbas, 2009; Badday et al., 2012)

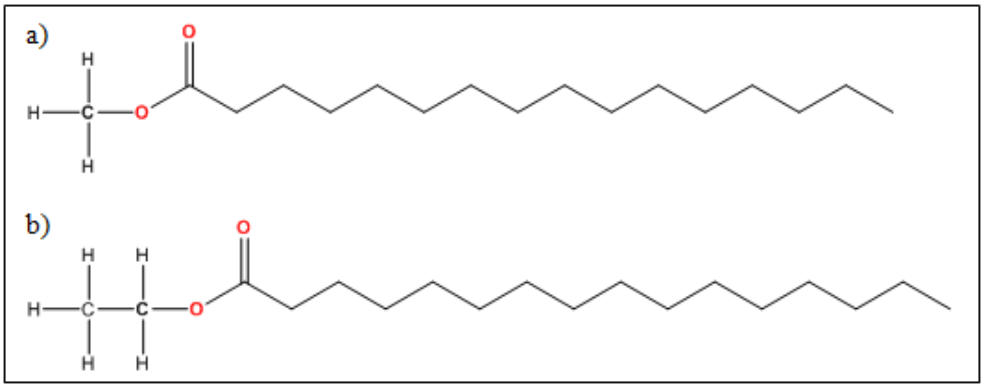

Figure 1.2. a) Fatty acid methyl esters (FAME), b) Fatty acid ethyl esters (FAEE)

Biodiesel which is called as fatty acid alkyl esters is named corresponding to the various types of reactants. Ethanol, methanol, propanol and butanol are suitable for performing as starting alcohol in transesterification. Methanol and ethanol become prominent with their common usage. In industrial applications, methanol is used with alkali catalyzed method for the production of biodiesel, which is called fatty acid methyl ester. (Figure 1.2.a.) The primary reason of that usage is its low cost. However, ethanol has many other advantages as a reactant. In terms of green chemistry, ethanol generation is based on agricultural sources, which make the production process more renewable and sustainable than petroleum based methanol process. According to their toxicity, the handling of methanol is more difficult than ethanol, which has superior solvency properties. In addition, triacylglycerides in ethanol show higher solubility than methanol. Taken together, ethanol is the most suitable candidate for transesterification. When ethanol is used, the product will be named as fatty acid ethyl ester. The structures of two esters can be seen in Figure 1.2. The biggest disadvantage of ethanol is high costs of its production. (Firdaus et al., 2014)

Lastly, the main criteria which control the production of biodiesel are; temperature of reaction, type of catalyst and oil, purity of product, content of water, mixing rate, quality of reactants and products, molar ratio of alcohol:vegetable oil, performance of the product. (Adamczak, Bornscheuer, 2009) For commercialization of sustainable biodiesel production, process costs are also leading criteria for the industry.

1.2.4. Market Analysis of Biodiesel

In the biodiesel production process, the basic cost is feedstock purchases, as seen in Figure 1.3. Thus, the production is limited by the availability of oilseed with the transesterification method.

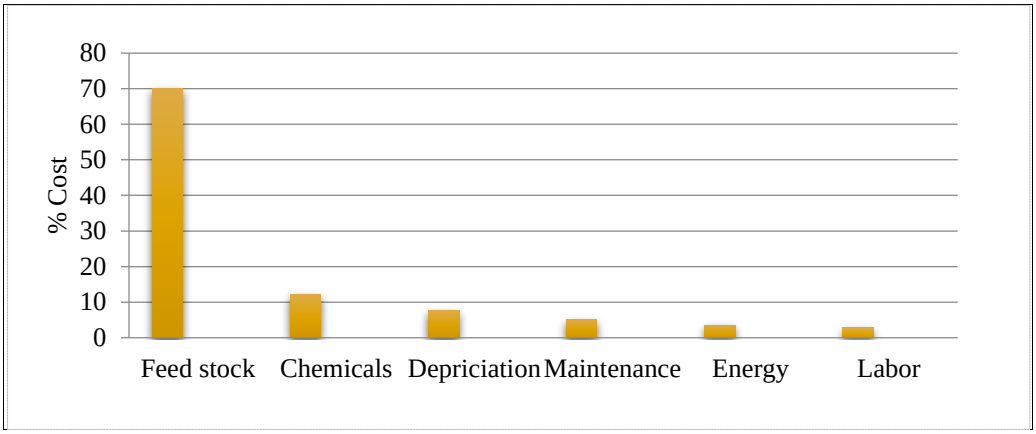

Figure 1.3. Cost of biodiesel process

(Source: Badday et al., 2012)

The biodiesel production around the world was raised up to approximately 15 billion liters between 2000 and 2009. The biggest producer is European Union with the %55 of sales revenue. Especially, the policies about fuel support this high market share. (Sanchez, Adlercreutz, 2015) This capacity has been increasing for every year with developments in the industry. According to U.S. Energy Information Administration, 529.9 million liters biodiesel is produced during July 2016, in the United States. The production is based on 94 oil crops with the potential of nearly 8 billion liters for each year. However, analysis shows that the amount of requested need of transport fuels is much more than the obtained output from oilseed and animal fats. Researchers have some concerns about the decreased utilization from arable lands, which are used for seeding of biofuel plants instead of food supply. (Schenk et al., 2008) The European Parliament voted for recently; at least 10% of energy from biofuels is targeted in transport and 7% limit on the usage of oil crops in biofuel production by 2020. An alternative method is required for the production of greener biodiesel which is based fatty acid ethyl esters to replace the conventional transesterification that over costing, energy and time consuming.

The goal of this project is isolation and purification of the key enzymes which lead to more renewable and advantageous biodiesel production.

1.2.5. Microbial Production of Biodiesel: Wax Ester Synthases

Biodiesel as indicated fatty acid ethyl esters (FAEE) can be produced as a result of an *in vivo* activity, which occurs by an enzyme called wax ester synthase (WS). Triacylglycerols (TAGs) and wax esters (WEs) are the accumulated neutral lipid compounds. Because of their unique features, they have a great variety of usage areas which are food additives, dietetics, cosmetics, candles, polishes, lubricants, printing inks and pharmaceutical applications in industry. (Kalscheuer, Steinbüchel, 2003; Shi et al., 2012) Accumulation of TAGs and WEs is ranked as second after polyhydroxyalkanoic acids in prokaryotes for the need of energy and carbon in the cellular actions. This storage is observed more frequently in eukaryotes. (Stöveken et al., 2005) Biosynthesis of TAG has important roles in animals such as; providing coordinated membrane fluidity, accumulation in adipocytes and formation of milk. Seed oils production is the basic result of TAG storage in plants. WEs also carry a key role in some processes like epicuticular waxes generation in plants for coating, tend to be a guard from ultraviolet light, dehydration, pathogens and acting as energy storage compounds in some seeds. (Kalscheuer, Steinbüchel, 2003)

TAGs and WEs based natural lipids are synthesized by a series of reactions with long chain fatty acids and long chain alcohols with various enzyme classes in eukaryotes. A new class of acyltransferases has been observed in bacteria with an ability to catalyze TAG or WE formation at the last step of reaction from a substrate of acyl-CoA. This enzyme is called wax ester synthase/acyl coenzyme A: diacylglycerol acyltransferase (WS/DGAT). (Stöveken et al., 2005) It is a bifunctional enzyme that performs two different processes. One of those is acylation of fatty alcohols from its hydroxyl group with the aim of wax ester formation which is termed as acyl-CoA:fatty alcohol acyltransferase (wax ester synthase: WS) function. The other one is adding acyl group to diacylglycerol (DAG) in which the targeted area is hydroxyl group, for the production of TAGs called acyl:CoA diacylglycerol acyltransferase (DGAT) process. Acetyl coenzyme A is an acylating agent in both generation which is described below in Figure 1.4. (Kalscheuer, Steinbüchel, 2003)

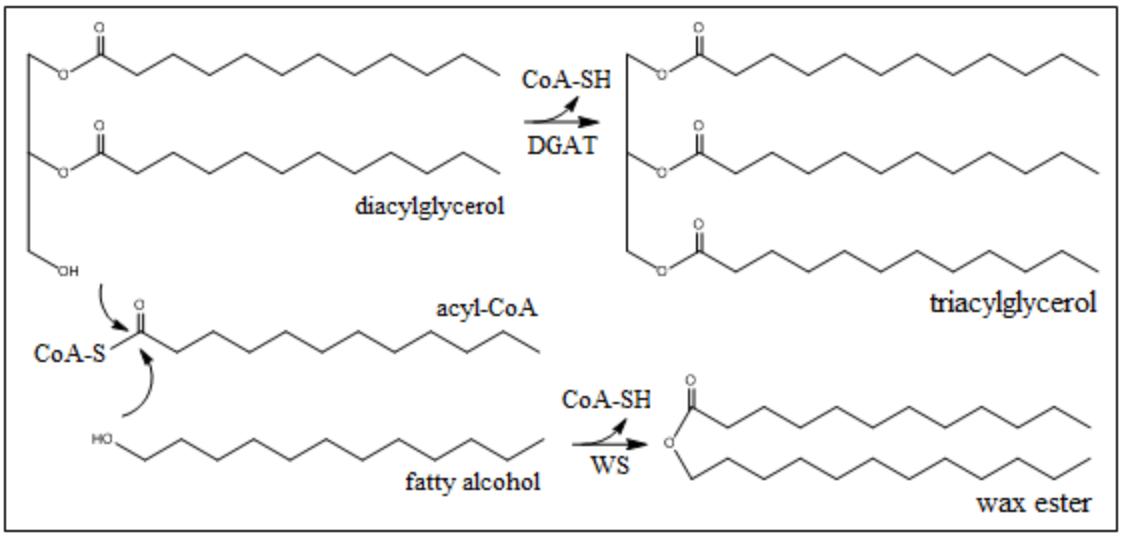

Figure 1.4. WS/DGAT catalyzed reactions

The first WS/DGAT was reported in 2003 with a study, involved *Acinetobacter baylyi* sp. ADP1. It is characterized as amphiphilic and through the electrostatic interactions it can attach to membrane weakly. (Kalscheuer, Steinbüchel, 2003) Hence, biosynthesis of FAEE in microorganisms which is indicated as "Microdiesel" has become an applied research instead of remaining a theory. In 2006, Kalscheuer et al. announced that they reached FAEE production with 26% of the cellular dry mass by using metabolically engineered *Escherichia coli* as the result of combined pathways about WS/DGAT synthesis from *Acinetobacter baylyi* strain ADP1 and ethanol generation pathway from *Zymomonas mobilis.* This study proves that WS/DGAT has an ability to catalyze the formation of FAEE from ethanol and fatty acyl-CoA. However, some limitations, in which substrate specificity is the main one, to form high yielded FAEE were observed about WS/DGAT. (Kalscheuer, Stölting, Steinbüchel, 2006)

Specificity of WS/DGAT is one of the most noticeable feature which can be available for prodigiously wide range of substrates. About alcohol specificity, from C_2 to C_{30} are accepted linear alcohols by WS/DGAT as seen in Figure 1.5. Medium chain alcohols which are C_{14}-C_{18} result in observed maximum activity. Same outcome can be seen about acyl-CoA specificity of WS/DGAT. According to Figure 1.6., it tends to give higher activity with middle length acyl-CoAs especially towards palmitoyl-CoA (C_{16}). (Stöveken et al., 2005; Stöveken, Steinbüchel, 2008)

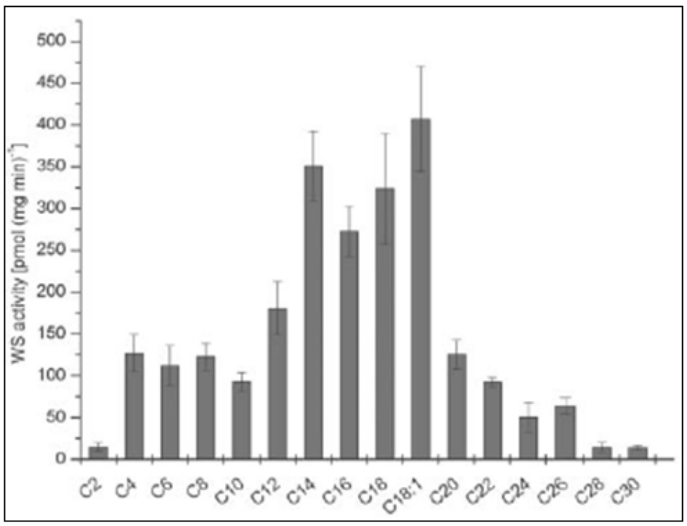

Figure 1.5. WS/DGAT Specifity for different linear alcohols
(Source: Stöveken et al., 2005)

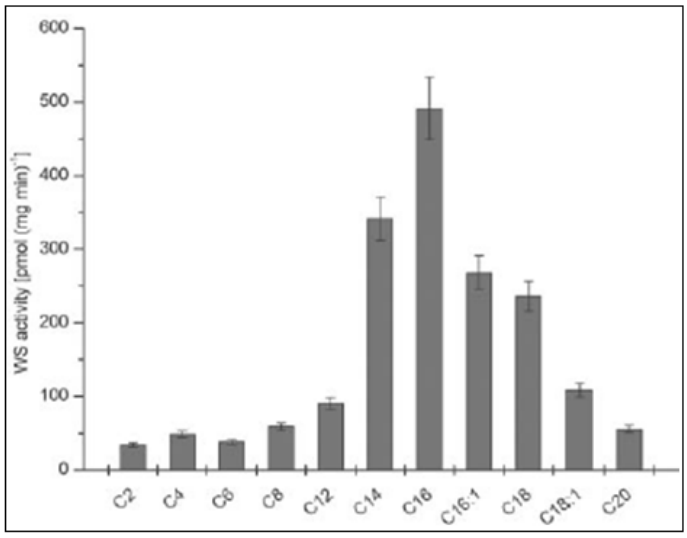

Figure 1.6. WS/DGAT specificity for different acyl-CoAs
(Source: Stöveken et al., 2005)

After determination of WS/DGAT from *Acinetobacter baylyi*, new members of this acyltransferase enzyme class have been announced in various types of bacteria. WSs from *Mycobacterium* species, marine species of *Marinobacter*, *Alcanivorax*, *Psychrobacter*, oleaginous species of *Rhodococcus*, *Streptomyces* and with several studies; mammals like *Mus musculus* C57BL/6 can be exemplified. (Röttig et al., 2013) A study was reported in 2012, for an effective comparison of WSs from different microorganisms, which are *Acinetobacter baylyi* ADP1, *Marinobacter hydrocarbonclasticus* DSM8798, *Rhodococcus opacus* PD630, *Mus musculus* C57BL/6 *Psychrobacter arcticus* 273-4 with heterologous expression in *Saccharomyces cerevisiae* H1246. The

increased trend was observed in all studied wax esters to accept C_{12}-C_{18} alcohols as substrates also indicating in Figure 1.7., below. (Shi et al., 2012)

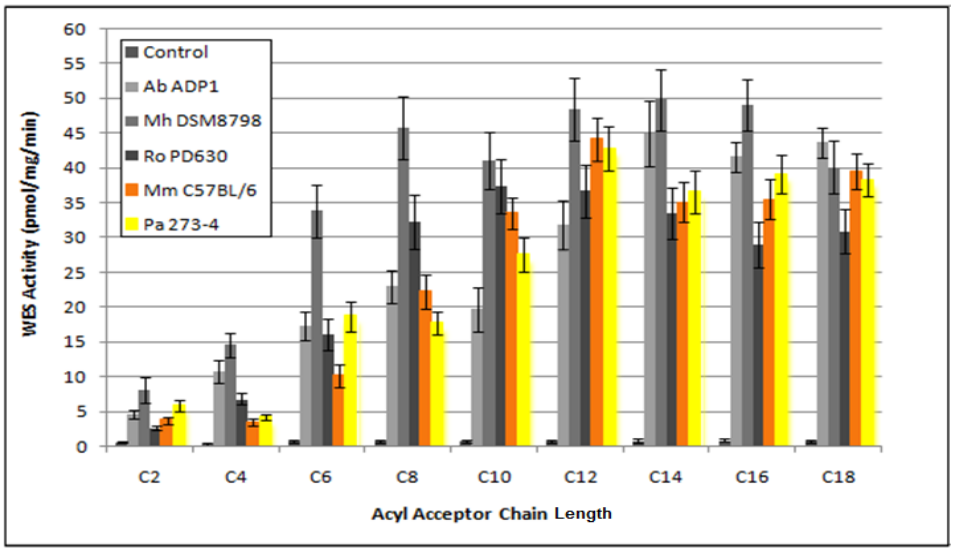

Figure 1.7. Substrate specificity of different WSs
(Source: Shi et al., 2012)

According to the main purpose of efficient FAEE formation, high WS activity is required with a selection of ethanol as substrate. Besides, if microdiesel production will be developed properly, it will be the best alternative for displacing the current methods which are based on transesterification. Microbial production of biodiesel is more environmental friendly and lower costed process rather than conventional generation. Moreover, usage of microorganisms removes the restriction about biodiesel accessibility which is only available in the definite oil seed production regions with the current methods. In short, it is more sustainable production way with the features that fulfilled the requirements.

1.2.6. Scope of This Study

Here, wax ester synthases from *Psychrobacter arcticus* 273-4 and *Mus musculus* C57BL/6 are studied. This project aims to aid *de novo* biodiesel production by isolation of critical enzymes that can catalyze biodiesel formation. Isolation and purification of wax ester synthases are required to perform mechanistic and structural studies that will lead to elucidate the catalytic mechanism, and eliminate the basic limitation for fatty acid ethyl ester (FAEE) production in microorganisms. If the basic problem is overcome

and high substrate specificity of WS towards to ethanol is provided with the proper manipulations in active site, *de novo* biodiesel production can be obtained in larger amounts as a result of *in vivo* reactions which are much more advantageous than conventional diesel production. Furthermore, it would be inspired to develop a microbial cell factory with the usage of other microorganisms such as bacteria, yeast or algae. Especially, due to its high photosynthesis efficiency and continuous oil reservations, algae will be the best candidate to meet the increasing demand of biodiesel. Foremost among them, successful cloning, optimized heterologous expression and efficient purification steps are essential preparation steps to define high resolution structures of these enzymes.

1.3. Pharmaceuticals

1.3.1. Terpenoid Natural Products

Usage of plants or herbal extracts, bandaging a wound with leaves and similar cases have been shown as some proofs for the existence of natural products term as a treatment of illness and injuries since the ancient times. (Ji et al., 2009) Basically, natural products are defined as small molecules which are derived from biological sources. This derivation refers various ways like isolation from nature, *in vitro* synthesis or production with metabolically engineered organism. (Krause, Tobin, 2013) It is obvious that natural products are obtained as a result of primary and secondary metabolism pathways. Primary metabolites are substances from essential metabolic reactions for growth, development or reproduction. Proteins, lipids, nucleic acids and carbohydrates can be shown as examples for primary metabolites. Substances from adaptation and survival reactions which are not essential like primary metabolites termed as secondary metabolites. Especially acetyl CoA, shikimic acid, mevalonic acid and 1-deoxyxylulose-5-phosphate are the most common secondary metabolites, which are taking main role in various pathways and mechanisms. Secondary metabolites are generally known as natural products. (Dias et al., 2012) Natural products have different categories related to their sources. There are plant, animal, microorganism and marine source based natural products used in especially drug development. (Siddiqui et al., 2014) This concept has been progressed with the improvements in biochemistry,

chemistry, structural and molecular biology. According to the understandings in the field, these natural products can be produced with chemical synthesis. As total or semi synthesis, there are many natural products used in the industry such as pharmaceuticals, additives in cosmetics, foods and dietary products. (Krause, Tobin, 2013) Alkaloids, terpenoids, steroids, antibiotics, nucleic acid components, pyrrole pigments, carbohydrates, lipids, peptides, tannins, phenolids are some types of natural products.

The largest class of natural products is terpenoids with over 60.000 substances, mostly based on plant sources. (Köksal et al., 2011) They have an ability to be involved in many different biological activities which result as huge diversity of terpenoids. This ability is also observed in the usage areas. Flavors, fragrances, food supplements as sweeteners, vitamins, pesticides, pharmaceuticals, feed stocks are the most commonly known areas for terpenoid utilization. This diversity supplies also an advantage for some specific plant terpenoids. Advantage of obtaining the same product with different pathways has a high potential for effective production in the industry. In addition, researches shows the alternative production ways, which are led by natural or synthetic terpenoids. (Bohlmann, Keeling, 2008; Moses et al., 2013) According to this concept, diterpenoid anticancer drug Taxol is the leading example for high value generation with biological and semisynthetic methods. (Chen et al., 2011)

This project aims to isolate a critical enzyme in Taxol biosynthesis. Isolation and purification of this enzyme will lead to structural studies and as a result, increased Taxol biosynthesis can be obtained.

1.3.2. Paclitaxel (Taxol)

Paclitaxel is a commonly used chemotherapy drug to provide the treatment of ovarian, metastatic breast, pancreatic and lung cancers. (Wheeler et al., 2001) It was announced as Taxol with a trademark of Bristol-Myers Squibb. The effect of Taxol can be indicated with providing a control mechanism for tubulin polymerization. It binds β-tubulin to stabilize microtubule assembly and results with mitosis inhibition. (Croteau et al., 2006) Due to its promising effects on treatment, its annual market value has reached several billion dollars. (Bohlmann, Keeling, 2008) Although it was described at 1971, because of the lack amount for commercialization, it became an approved chemotherapeutic drug in 1990s according to the U.S. Food and Drug Administration. The

bark of the Pacific yew *Taxus brevifolia* is the first source for the production of Taxol. It is isolated from that barks of the tree, which grows slowly and can be found only in specific areas around Pacific Northwest and North America. (Croteau et al., 2006; Guerra-Bubb et al., 2012) The production can be supplied from different Taxus species with a maximum yield of 0.069%. Besides, it cannot be termed as a sustainable method with becoming a menace against existence of Taxus species. Because of this low yielding, high costed and complicated purification features of Taxus based production, alternative ways have been researched in response to increasing demand of Taxol. Development of new production methods depends on the understandings about Taxol biosynthesis pathway. It includes initial generation of most common diterpenoid precursor geranylgeranyl diphosphate (GGDP) and 19 enzymatic steps with over 400 taxoid metabolites. Getting hundreds of similar metabolites can be produced as a result of the same biosynthesis pathway in Figure 1.8. There are still some unknown steps in this metabolic pathway. In contrast, many enzymes which are related to the specific genes involved in biosynthesis were defined. According to this pathway, steps are reported for diterpenoid product Taxol; GGDP formation as starting compound by geranylgeranyl diphosphate synthase, then reaching to taxa-4(5), 11(12)-diene in consequence of cyclization of GGDP by taxadiene synthase, following with the hydroxylation by cytochrome P450 taxadiene 5α-hydroxylase and the acetylation by taxa-4(20),11(12)-dien-5α-ol-O-acetyltransferase of taxadien-5α-ol, then the hydroxylation to taxadien-5α-yl acetate, the benzoylation to 2-O-debenzoyl taxane, the acetylation of 10-deacetyl baccatin III forming baccatin III, finally the addition of side chain from C_{13} position. (Walker, Croteau, 2001; Guo et al., 2006)

Currently, semi synthesis of Taxol is used as commercial production. In this method, needles, leaves and stems are used with plant cell and tissue culture techniques. *T. baccata*, *T. cuspidata*, *T. brevifolia*, *T. chinensis* and hybrids species were reported for Taxol extraction. Optimized large scale production is also required growth regulators, sugar additives and elicitors. This method has advantages like increased sustainability, easier separation and faster production. The maximum yield of Taxol is achieved with nearly 0.5% of dry mass with using cell cultures in the presence of methyl jasmonate as an elicitor. However, high cost and unsteady yield of production are the disadvantages of this method.

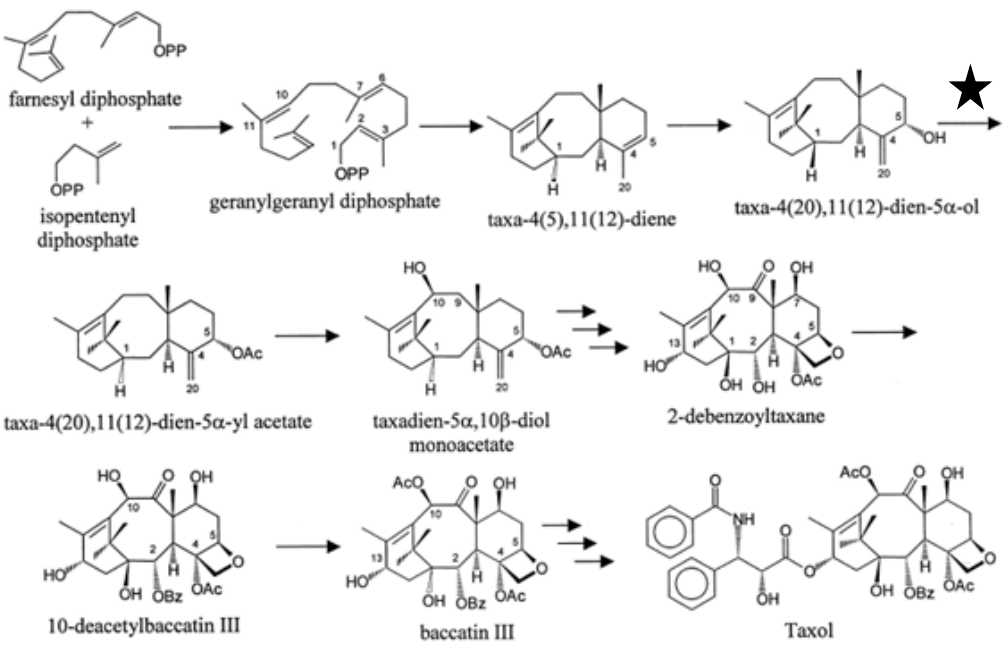

Figure 1.8. Biosynthetic pathway of Taxol. Multiple arrows used as undefined steps. Star shows the studied enzyme that is defined in scope of this study section. (Source: Walker, Croteau, 2001)

Endophtyic fungi can be shown as another alternative source of Taxol. These fungi are found in plant without any harmful effect. They promote to generate a fungicide with the aim of competing the others and getting self-survivability. Taxol can be also termed as fungicide. Minimum 18 various fungal species are reported as potential producers. Although increased growth rate is very promising feature of endophytes, fewer amount of products and instability problems make this method unsuitable.

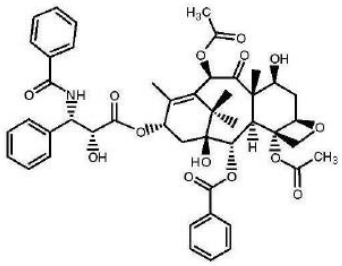

Figure 1.9. Chemical structure of Taxol
(Source: Badi et al., 2015)

The structure of Taxol, as seen in Figure 1.9. consists of four different rings which are a cyclohexene, a cyclooctane, a cyclohexane, a oxetane with N-benzoyphenylisoserine side chain and a benzoate group added in the proper positions. Chemical

synthesis of this structure is another way for production. It was reported that in 1994, the total chemical formation was performed with 2.7% production yield. However, this synthesis comprises over twenty steps, in which some have very low efficiency. In short, chemical synthesis provides more complicated and overpriced process for commercialization.

Developments in genetic and metabolic engineering are the leading factors of the high efficient Taxol production. Solving of every step in the pathway can give a chance to modify some limiting steps, find more efficient precursors, and eliminate undesired compounds. As a result of this improvements, bioengineered microorganisms may allow the renewable production of Taxol in large quantities like 100-fold higher with low costs. (Badi et al., 2015; Guo et al., 2006; Haas, 2010)

1.3.3. Acetyltransferases in Taxol Biosynthesis

Acetyltransferases are the enzyme class that transfer an acetyl group from acetyl coenzyme A. They take very important roles in Taxol biosynthesis pathway. They have basic functions in some critical catalysis steps. It has been reported that acetylated taxoid substrates are more efficient and they yield highly functionalized products in Taxol biosynthesis pathway. Besides, the huge diversity of other taxoids which are obtained from yew trees and affect Taxol production adversely, is supplied by acetyltransferases. These enzymes have different taxoid substrate and acyl-CoA specificities and even their regio selectivity feature can influence the activity in pathway. Moreover, defining of their functions provides the direct flux control to Taxol production.

Taxadien-5α-ol-O-acetyltransferase, 10-deacetyl-baccatin III-10β-O-acetyltransferase and taxane 2α-O-benzoyl-transferase are the responsible enzymes for catalysis of some steps in Taxol biosynthetic pathway. Isolation, expression and characterization studies have been performed about these enzymes. It is expected to be identified and characterized at least nine more acetyltransferases related to Taxol biosynthesis pathway. (Walker, Croteau 2001; Chau et al., 2004; Croteau et al., 2006)

1.3.4. Scope of this Study

Taxadien-5α-ol-O-acetyltransferase is the basic subject of this study. This enzyme takes role in acetylation of taxadien-5α-ol. The reaction step is showed with a star in Taxol biosynthesis pathway, in Figure 1.8. Isolation and purification of this enzyme will pave the way for mechanistic and structural studies. Thus, it will give chance to promote the direct and increased generation of Taxol with the manipulation of the substrate selectivity. Additionally, this selective manipulations on the enzyme towards the substrate provide the elimination of side-routes during biosynthesis. The requirement processes as preliminary studies for determination of the structure are successful cloning and overexpression. According to this aim, experiments were performed to express and purify taxadien-5α-ol-O-acetyltransferase from *Taxus cuspidata*.

CHAPTER 2

MATERIALS AND METHODS

A. Wax Ester Synthase from *Psychrobacterarcticus* 273-4 (PaWES)

2.1. Cloning, Bacterial Transformation and Sequence Confirmation

Wax ester synthase gene sequence from *Psychrobacter arcticus* 273-4 was codon optimized for expression in *Escherichia coli*. Codon optimized *Psychrobacter arcticus* 273-4 sequence with a C-terminal hexahistidine tag was obtained from GENEWIZ (South Plainfield, USA). The sequence of interest which is under control of T7 promoter was transformed into *E.coli* XL1-Blue (Agilent) for propagation with heat shock bacterial transformation protocol. (Invitrogen) It was isolated by plasmid isolation kit (MOBIO). Double digestion with *Bam*HI and *Nde*I was performed. Then, it was ligated with the 1:3 ratio of Vector:Insert into expression vector pET22bTV (Köksal et al., 2011) This plasmid was transformed into XL1-Blue cells, isolated via plasmid isolation kit (MOBIO) and sent to Izmir Institute of Technology, Biotechnology and Bioengineering Research and Application Center for sequence analysis. The sequence was confirmed with the NCBI based Basic Local Alignment Search Tool. The next transformations were done with different cell strains according to their unique expression features.

Furthermore, to increase the solubility, folding ability of the expressed proteins, the GroEL/ES chaperone system, which is 57 kDa, was employed. This chaperon prevents the occurrence of insoluble aggregates and promotes the proper folding process by its conformational change. Additionally, it was reported recently that, these chaperons can take some roles in various post translational modifications. (Ellis R.J., 2013) Some expression conditions were tried with the addition of GroEL/ES. A plasmid containing GroEL/ES genes (pGroE) was transformed into several expression strains and then, competent cells of these strains were prepared. Lastly, the plasmid containing the sequence of interest, which is PaWES in this case, was transformed to this new competent cells that included pGroE.

2.2. Growth Curve Preparation

To find the proper optical density at 600 nm (OD_{600}) value observed in late exponential phase, which was required for the optimum time of induction, transformants were cultured in a 100 ml flask containing Luria-Bertani (LB) (Fischer Scientific) medium with 100 mg/ml ampicillin at 37˚C. The OD_{600} was measured with spectro-photometer (Thermo Scientific Multiskan Go) in every 20 minutes until the stationary phase. According to the values obtained, a graph was formed.

2.3. Gene Expression Test

Gene expression test was applied to find optimum conditions for overexpression. Host strains, media selection, glucose content, temperature, length of expression, inducing agent concentration are the critical factors that can affect the expression. (Novagen, 2003) At first, performed protocol from Invitrogen Manual aimed to find optimum expression level in compliance with induction time. The combinations of affecting factors, as mentioned above, were tried later individually.

Transformant of appropriate *E.coli* expression cell containing PaWES was streaked out on the proper selective antibiotic containing plate (100 mg/ ml ampicillin). After overnight growth at 37˚C, a single colony was inoculated to 5 ml proper media with the proper antibiotic as a starter culture. Approximately 16 hours later, 1 ml was taken from the starter culture, added into 50 ml refresh media with antibiotic. The midi culture was grown at 37˚C with 200 rpm of shaking speed. According to the growth curve, when the appropriate OD_{600} was obtained, the flask was put into ice bath for 30 seconds to stop the growth. In the meantime, the shaker was adjusted with the suitable expression temperature. After the ice bath step, adding of isopropyl β-D-1-thio-galactopyranoside (IPTG) as inducing agent with the proper concentration was the beginning point of protein expression. 1 ml samples were taken from the culture before the induction as 0^{th} time and periodically during expression (at 0^{th} hour, continued nearly 5^{th} hours, 10^{th} hours, and 15^{th} hours). These samples were centrifuged at 4000xg for 10 minutes. Cell pellets were stored at -80˚C. For fast determination of gene expression samples, the appropriate procedure from European Molecular Biology Laboratory was performed. All cell pellets were thawed, which should be happen

always on the ice, and resuspended with 230 µl extraction buffer. This extraction buffer contained 20 mM Tris-HCl, ph=8.0, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mg/ml lysozyme and 0.05% Triton X-100. They were incubated for 30 minutes at 4˚C with the extraction buffer, and later treated with ultrasonic bath for 3 minutes. 10 µl samples from homogenate were taken to be analyzed via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The rest of the samples was centrifuged at 13000 rpm for 10 minutes. Again, 10 µl samples were taken from supernatant and analyzed with SDS-PAGE to check for protein solubility. Another expression test protocol was belonged to Invitrogen manual. The steps were nearly similar with the protocol above. Few differences can be shown like the amount of taken samples which are 50 ml in the beginning to store and buffer ingredients. It is detailed in Section 2.12. Both protocols were performed for gene expression tests of PaWES.

2.4. SDS-PAGE Analysis

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was applied for analyzing proteins according to their molecular weight during all this thesis study. In this method, lysates and protein samples were warmed up to approximately 95˚C with Laemmli Sample Buffer (60 mM Tris-Cl pH=6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue) and loaded to the polyacrylamide gel. SDS denatured the protein, and with heat effect, it provided similar charge on the linear protein molecules. β-mercaptoethanol also broke the disulphide bonds to ensure unfolding of tertiary or quaternary structures of proteins. The protein samples, which were negatively charged by SDS, were separated according to the migration based on their molecular weights with the supply of electrical field through the gel. The movement of high molecular weighted proteins was slower than the lower ones. Under the guidance of protein ladder or protein molecular weight marker in the same gel, the protein sample can be easily identified by its size. (JoVE, 2006) Coomassie dye was used for gel staining.

2.5. Cultivation and Expression

The conditions obtained from SDS-PAGE analysis of overexpression in preliminary gene expression tests were applied for huge volume expression. The 1 liter culture was started with nearly 3500 µl culture from the overnight culture which was 50 ml. This culture grew at 37ºC, 200 rpm of shaking speed until the appropriate OD_{600} value was reached. At this growth point, protein expression was induced with IPTG and the culture started to grow at proper expression temperature.

2.6. Extraction

The cells were harvested according to the thickest band time in SDS-PAGE from preliminary gene expression tests, which was most suitable. The cells were pelletized with centrifugation at 6000xg for 15 minutes. Then, the pellets were weighed and resuspended with the lysis buffer, which should be at least twofold of the pellet weight. Equilibration buffer (E-Buffer) contained 50 mM K_2HPO_4 pH=8.0, 10% glycerol, 5 mM β-mercaptoethanol, 300 mM NaCl, 100 µM PMSF, and Imidazole buffer (I-Buffer) comprises of the same ingredients with E-Buffer together with 250 mM Imidazole. Lysis buffer was formed with 96% E-Buffer and 4% I-Buffer. In addition, lysozyme (0.25 mg/ml) and DNaseI (0.01 mg/ml) were added to both buffers. Attention should be paid to the temperature, which was 4ºC for all steps. The mixture was rotated at least for 30 minutes. Then, they were sonicated on ice with 5 cycles of the appropriate program. (ON for 60 seconds, OFF for 60 seconds, 50% bursts, 60% power) The sonicated cell pellets were centrifuged at 20000xg for 1 hour. After that, the supernatant part was taken and the centrifuge step was repeated for another 1 hour. The clean part was obtained as lysate of the cells according to the procedure (Köksal et al., 2012). It was tested with SDS-PAGE for confirmation.

2.7. Purification

For initial purification method to obtain quick result from the lysate, Nickel loaded Nitrilotriaceticacid (Ni-NTA) Spin Column Purification procedure was used. This was done with HisPur NiNTA Resin (Thermo Fisher Scientific), which was used

to separate His-tagged fusion proteins based on their metal affinity. Based on the manufacturer protocol, low amounts were used. 200 µl resin was mixed with the same amount of E-Buffer. They were centrifuged at 700xg for 2 minutes. Then, E-Buffer was added to the resin twice as much for equilibration. The centrifugation step was repeated. Filtered cell lysate was added to EconoSpin column (Epoch Life Science) and the mixture was rotated at 4ºC for 30 minutes. After the centrifugation step, the collected sample was termed as flow-through (FT). The washing buffer was formed with 75% E-Buffer and 25% I-Buffer. The column was washed with the washing buffer, which was twofold amount of resin. As a result of the centrifugation step, the obtained fraction was called as wash (W). This step was repeated two more times. Lastly, the column was treated with elution buffer, which contained 100% I-Buffer, and centrifuged. It was repeated twice and elution fraction was obtained as a concluded fraction (E). According to this protocol, if obtained fraction (E) has the expected band in SDS-PAGE, then the purification can be done with larger volumes via system of AKTÄ Prime Plus Fast Protein Liquid Chromatography (FPLC).

Immobilized metal ion affinity chromatography (IMAC) method was performed for purification of 1-5 liter expression cultures to obtain PaWES. The lysate was filtered via 0.2 µm syringe filter and loaded to FPLC. Before the lysate entered to C Column (GE Healthcare Bio-Sciences) which was packed with 5 ml HisPur NiNTA resin, the equilibration step was applied with 50-80 ml E-Buffer. After that, the sample injected column was washed with 50 ml E-Buffer at 1 ml/min flow rate. The second washing step contained 30 ml E-Buffer which included step wise addition of imidazole from 0 to 50 mM. Then, the last step was resulted with elution fraction via 50 ml E and I-Buffer mixture with the gradient change of 50 to 250 mM imidazole, at 2 ml/min flow rate.

In short, Histidine-tagged proteins bind strongly to Nickel charged resin when the lysate passed through the column. After elimination of other components with washing step, imidazole was used as elution agent for disassembling of the bound His-tagged proteins which are the desired ones. (Strategies for Protein Purification, GE Healthcare)

2.8. Measurement of PaWES Protein Concentration

Protein concentration of PaWES in elution fraction was calculated with using the absorption value at 280 nm (A280) from Nanodrop measurement and Beer's Con-

centration Law. According to the Beer's Law, absorbance (A) is related to concentration (C), extinction coefficient (Ɛ) of that protein and the path length (b). (Thermo Scientific Tech Tip #6) For reaching the concentration value in mg/ml, absorbance is divided by extinction coefficient with the unit of $(mg/ml)^{-1}.cm^{-1}$ and path length of 1 cm, as seen in the equation below.

$$C = {}^{A}/_{\varepsilon . b}$$

2.9. Dialysis

Separation of protein from undesired small compounds and giving time to protein for folding was accomplished by dialysis method. This was done to overcome the observed column binding problem about PaWES. The denaturating agent Guanidium hydrocloride (GuHCl) was added with the concentration of 6M to the sample from flow through fraction of the IMAC run. Peptide backbone tends to interact with guanidium moiety. Therefore, the ordered structure of protein was destabilized and became randomly coiled at high concentrations of GuHCl. Filtered FT sample including GuHCl was injected to the FPLC. It was eluted with GuHCl containing E-Buffer and I-Buffer with 500 ml volumes for each. The elution fractions were ready for dialysis, which was preferred because of its simplicity. First, the sample was loaded inside a semi-permeable membrane (Spectra/Por 6 Dialysis Membrane). Then, this membrane was put into proper buffer through the dialysis tubing closures. The volume of the buffer was 200 fold higher than the sample. The applied step-wise dialysis was consisted of four steps. Buffer of the dialysis system was changed at intervals of 5 hours. The concentration of GuHCl in the buffers was decreased at each steps and the amount of glycerol was increased, comparatively. (Tsumoto et al., 2003; Thermo Fisher Scientific Dialysis Procedure) Same process was repeated with using another denaturizing agent 8M Urea instead of 6M GuHCl. The samples which were taken from supernatant and aggregate, which were separated by centrifugation, were analyzed with SDS-PAGE.

B. Wax Ester Synthase from *Mus musculus* C57BL/6 (MmWES)

2.10. Cloning, Transformation and Sequence Confirmation

Two different organisms, primarily *E. coli* and *S. cerevisiae* were used to obtain wax ester synthase from *Mus musculus* C57BL/6 (MmWES).

The codon optimized sequence for *E.coli* was synthesized by GENEWIZ (South Plainfield, USA). It included fusion tag with six Histidine (His) residues at C terminal. Propagation with XL-1Blue, restriction with *Nde*I and *Bam*HI, then ligation with pET22bTV vector were performed according to the Section 2.1. The obtained clone was sent to sequencing for confirmation step. Transformation to expression cell strains and adding GroEL/ES to some of the expression tests were also the applied in steps to obtain MmWES.

2.10.1. Gateway Cloning Technology

Gateway cloning technology was used in expression of MmWES in *S. cerevisiae.* Instead of restriction enzyme cloning, the transfer of MmWES sequence to yeast strains was performed with Gateway Cloning Technology, which provided easy and rapid shuttle between multiple vector systems. This cloning method had two different reactions to obtain desired gene in preferred vector with using site-specific recombination (Figure 2.1.). One of those was BP reaction, which provided the formation of entry clone by using donor vector, the other one was LR reaction that generated expression clone in destination vector. pDONR221 was used as the donor vector in BP reaction, and the destination vector in LR was pAG423GAL-ccdb with Histidine (His) selectable marker. This destination vector has GAL1 promoter, which led to galactose inducible protein expression. The Gateway Cloning Technology procedure was applied according to the Invitrogen Manual.

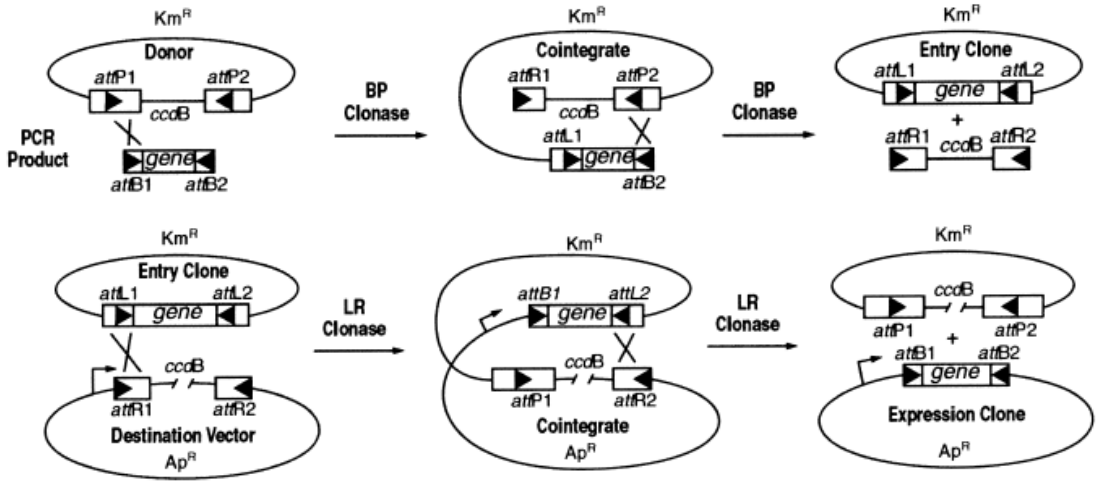

Figure 2.1. Gateway Cloning Technology
Km ^{R}: kanamycin resistant, Ap ^{R}: ampicillin resistant
(Source: Hartley et al., 2000)

For the utilization of Gateway Cloning Technology, it was required to have specific *att*B-flanked DNA fragment on the sequence of interest. The primers contained *att*B sites which are shown in Table 2.1. were designed and synthesized by Microsynth (Balgach, Switzerland). The sequence was amplified via PCR. The reaction conditions for amplification were explained in Table 2.2. All cloned DNA samples were confirmed initially by the restriction with *Bsr*GI (Fermentas).

Table 2.1. Designed primers with *att*B sites

Name of primer	Sequence of primer
MmWES_*att*B_ Forward	[5'-3'] GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAACC ATGGCCCATATGTTCTGGCCGACCAAAAAAG
pET22bTV_*att*B _Reverse	[5'-3'] GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAGTGGTGGTGGTGGT GG

Table 2.2. PCR reaction conditions

Step	Temperature	Time
Initial Denaturation	98˚C	30 seconds
30 cycles	98˚C	10 seconds
	59˚C -59.7˚C -62.1˚C -64.2˚C -65.7˚C -67˚C	30 seconds
	72˚C	50 seconds
Final Extension	72˚C	2 minutes
Hold	4˚C	

2.10.2. Yeast Transformation and Sequence Confirmation

DNA samples which resulted from BP-LR reactions were transformed to *E. coli* JM109 competent cells with standard bacterial transformation protocol. The cloned plasmids were isolated via plasmid isolation kit (MOBIO) and sent to sequencing for confirmation. According to the sequence analysis, the cloned gene was transformed to BY4741 yeast strain by LiAc method. (Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual)

2.11. Growth Curve Preparation

The preparation strategy of growth curves was the same for both *E. coli* and *S. cerevisiae*. For bacterial growth curve, the applied strategy was mentioned in Section 2.2. The main differences between bacterial and yeast growth curve preparation steps were the measuring time, growth temperature and the types of the media. Due to its longer doubling time, the OD_{600} was measured for each 90 minutes for *S. cerevisiae*. In addition, for *S. cerevisiae*, the type of medium used as starter culture was YPD (Difco), which contained 2% peptone, 1% yeast extract, 2% dextrose, and the expression culture was done with SD medium (Difco) that included 0.7% select yeast nitrogen base without amino acids, 2% glucose, 0.3% amino acids without Histidine. The starter culture was used as an initial step for 100 ml culture with SD medium. *S. cerevisiae* cultures were grown at 30˚C until the determination of the optimum OD_{600} value before the stationary phase. Then, the proper graphs were obtained with using these data.

2.12. Gene Expression Test

For bacterial expression, procedure from European Molecular Biology Laboratory was applied. In addition, gene expression test was repeated via another protocol about obtaining non-denaturing and denaturing lysates from Invitrogen Manual for determination of protein solubility. The steps were proceed in the same route until the addition of induction agent. In this protocol, 50 ml samples were taken at the mentioned time points in Section 2.3. Then, all cell pellets were thawed and weighed. They were resuspended in ND-Buffer (50 mM Tris-HCl pH=7.5, 100 mM NaCl, 1 mM DTT, 5% Glycerol) with 2 volumes of pellet. After adding 1 mg/ml lysozyme, they were shaken at 4˚C for 2 hours. Followed by the sonication step, which was done for 2 times with the appropriate program; 30 sec on 30 sec off by using microtip. Then, the samples were centrifuged at 18000xg for 1 hour. Supernatant was taken in another clean tube and labeled as non-denaturating lysate. This lysate was ready for SDS-PAGE analysis. The pellet part of the sample was resuspended in 500 µl D-Buffer which was similar to ND-Buffer with the exception of removal of glycerol, and addition of 6M GuHCl. The last centrifugation step was repeated with the samples including D-Buffer. As a result, these supernatants were called as denaturating lysates. These denaturating lysates were treated according to Trichloroacetic acid (TCA) Precipitation Protocol. 100 µl samples were mixed with the same amount of 10% TCA. After 20 minutes, the centrifugation step was performed for 15 minutes with the speed of 18000xg. Then, the pellet were washed with 100 µl ice-cold ethanol and resuspended in 20 µl Laemmli sample buffer. The SDS-PAGE analysis was applied for these samples.

2.13. Cultivation and Expression

For cultivation and expression in *S. cerevisiae*, transformed yeast was inoculated from glucose containing plate to 10-100 ml YNB-His culture. This starter culture medium contained 2% raffinose and 0.1% glucose as carbon source. The amount of glucose should be very low, because glucose had an ability to repress the expression of interested gene under the GAL1 promoter. (Park et al., 1999) Raffinose was a trisaccharide which can activate the expression with the addition of galactose, rapidly. (Berman H.M., 1970) Raffinose had no ability to express the gene, however it can be

used as adapter medium. After overnight growth at 30˚C, the yeast cells were added into 100-1000 ml appropriate media and started to grow approximately for 4 hours at 30˚C, until the OD_{600} reached to mid-log phase. The culture was centrifuged and then, pellet was washed three times with pre-chilled autoclaved dH_2O. All the samples were kept on ice. Fresh YNB-His with 2% final galactose was added and the culture was allowed to grow for 5-6 hours more. The culture was harvested by centrifugation and then, washing step was repeated with ddH_2O. (Keogh Lab Protocols, Albert Einstein College of Medicine) Obtained pellet was ready for further steps.

2.14. Cell Lysates Derivation from Yeast

Cell lysate was obtained with using glass beads vortexing according to the proce-dure form European Molecular Biology Laboratory. At the beginning, glass beads were prepared with HCl and distilled water treatments. They were cooled in 4˚C before the extraction. The pellet was resuspended with cold lysis buffer (50 mM Tris-HCl pH=8.0, 1% DMSO, 300 mM NaCl, %5 Glycerol, 1 mM TCEP-HCl, 0.1 mM EDTA, 1 mM PMSF, 1 µg/ml leupeptin, 1 µg/ml pepstatin A). PMSF and then 1-3 g cold glass beads were added to the suspension for each gram of cell wet weight. It was mixed 3-5 times for 1 minute and the glass beads were removed from the mixture. The centrif-ugation step at 45000 rpm for 30 minutes at cold room was the next step. The existed supernatant was the lysate from the yeast.

2.15. Purification

MmWES could only be expressed in *S. cerevisiae,* efficiently. NiNTA Spin Column Purification method (European Molecular Biology Laboratory) was used for separation of this protein from lysate. It is detailed in Section 2.7. Due to the low amount of obtained lysate, the quick spin column purification step was sufficient for protein purification from yeast.

2.16. Measurement of MmWES Protein Concentration

Protein concentration of MmWES from spin column purification method was calculated with using the A280 value and Beer's Concentration Law. The equation is detailed in Section 2.8.

C. Taxadien-5-alpha-ol-O-acetyltransferase from *Taxus cuspidata* (TcT5AT)

2.17. Cloning, Transformation and Sequence Confirmation

Taxadien-5-alpha-ol-O-acetyltransferase from *Taxus cuspidata* (TcT5AT) was synthesized from GENEWIZ (South Plainfield, USA) in codon optimized form for *E.coli.* It has poly-His tag at C terminal. Cloning with double restriction by *Nde*I and *Bam*HI, ligation with pET22bTV, bacterial transformation to XL1-Blue for propagation and other strains for expression were performed for TcT5AT. After ligation step, the sequence confirmation were done with the same procedure at Section 2.1.

2.18. Growth Curve Preparation

Growth curves of TcT5AT with different strains were prepared from the OD_{600} values which are obtained from 100 ml volume LB cultures. The steps were performed as mentioned in Section 2.2.

2.19. Gene Expression Test

The optimum expression conditions were tried to be reached with the methods from European Molecular Biology Laboratory and Invitrogen, which are detailed in Section 2.3 and 2.12, respectively. Different bacterial strains, LB medium with appropriate antibiotics, various temperatures, IPTG concentrations and duration times were the attempted conditions.

2.20. Cultivation

According to the cultivation procedure on Section 2.5., 1-10 liters LB medium were grown at 37˚C, 200 rpm of shaking speed for 15 hours.

2.21. Purification

Initial purification with NiNTA Spin Column and then, huge purification of 1-10 liters expressed culture with FPLC were applied for TcT5AT. It was purified with Tris IMAC Buffers. The ingredients of Tris E-Buffer were 50 mM Tris, 250 mM NaCl, 5 mM β-mercaptoethanol, 5% glycerol, 100 µM PMSF. Tris I-Buffer contained the same components and 500 mM imidazole, additionally. Both of buffers had pH 8.0. The FPLC program run with the similar instructions in Section 2.7. 5 ml HisPur NiNTA packed Glass-Econo Column (Bio-Rad) was used for IMAC. After equilibration step, 70 ml wash with E-Buffer and 80 ml additional wash with gradiently 0 to 50 mM imidazole content of E-Buffer were performed, respectively. The final step was elution step which includes gradient washing with 50 to 500 mM imidazole change in E-Buffer. In addition, the Talon resin (Clontech) was also used to elute TcT5AT with Spin Column Purification method. (Thermo Fisher Scientific) Talon resin had Cobalt ions instead of Nickel, which had higher binding specificity towards His-tagged proteins.

2.22. Buffer Optimization

Various buffers were attempted to obtain the highest concentration of purified protein without aggregation. These are MOPSO Buffer with pH=7.5 (50 mM MOPSO, 5 mM $MgCl_2$, 1 mM DTT, 5% glycerol), TrisHCl Buffer with pH=8.3 (10 mM TrisHCl, 100 mM NaCl, 100 mM $MgCl_2$), Tris-DTT Buffer with pH=8.0 (100 mM TrisHCl, 1 mM $MgCl_2$, 1 mM DTT, 0.1 mM NaCl, 10% glycerol), HEPES Buffer with pH=7.4 (30 mM HEPES, 200 mM NaCl, 3 mM DTT). Buffer exchange method was applied with FPLC for this purpose. It aimed for the removal of the salts and exchanging of the used buffer salts with the target buffer salts. (Thermo Fisher Scientific Manual) In the beginning, HiTrap Desalting Column (GE Healthcare Bio-Sciences) was washed with 15 ml target buffer. Then, 500-1000 µl sample, which was initially concentrated with

concentration tubes (Sarthorius Stedium Biotech.), was added to the column and the sample buffer was exchanged with the target one. This was followed by measurements of sample concentrations from different buffers, periodically. (Strategies for Protein Purification, GE Healthcare)

2.22.1. Measurements for TcT5AT Protein Concentration

According to Beer's Concentration Law and A280 values from Nanodrop measurements, protein concentrations of TcT5AT were calculated. Section 2.8. explains the detailed calculations about protein concentration.

2.23. Size Exclusion Chromatography

Size exclusion chromatography method was based on a separation of the components, such as salts or contaminants, in purified sample from IMAC according to their size. The sample was used with the buffer, which showed the highest concentration steadily from buffer optimization step. This separation can be provided by the pores of the column packing gel, which includes spherical beads. Larger molecules did not stumble into these pores, therefore they flew faster. In contrast, the small molecules flew slower and get retained for longer in the column. This method was performed to enhance the purity of the desired protein by separation protein mixture. HiLoad 16/600 Superdex 200 pg column (GE Healthcare Bio-Sciences) was used during the gel filtration via FPLC.

CHAPTER 3

RESULTS AND DISCUSSION

A. Wax Ester Synthase from *Psychrobacter arcticus* 273-4 (PaWES)

3.1. General Characteristics of PaWES

The amino acid sequence of PaWES gene is shown in Appendix A. According to the *E.coli* optimized sequence, some characteristics about PaWES can be obtained from protein calculator tools. (http://protcalc.sourceforge.net/) Based on the amino acid sequence, PaWES had molecular weight of 55.36 kDa, estimated pI at 8.78 and extinction coefficient of 0.77 $((mg/mL)^{-1}cm^{-1})$.

3.2. Cloning of PaWES

Cloning of PaWES was achieved by restriction enzyme cloning method. The vector (pUC57), which includes the synthesized gene, was cut with restriction enzymes *Nde*I and *Bam*HI. After this reaction, the restricted vector band (2500 bp) and the desired insert band (1434 bp) containing PaWES gene were separated by agarose gel electrophoresis as seen in Figure 3.1. The expression vector, which is pET22bTV, was cut with the same enzymes and the insert was ligated into restricted pET22bTV. The ratio of Vector:Insert in the ligation reaction was 1:3. After ligation, transformation and plasmid isolation were performed. Lastly, the cloning was confirmed by sequence analysis using T7 primers.

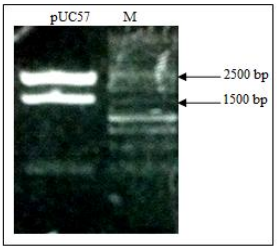

Figure 3.1. Restriction band of the gene. (pUC57; 2459 bp, PaWES; 1434 bp).
M indicates 1kb DNA marker

3.3. Growth Curve of PaWES

After transformation of the sequence of interest to the proper expression strains, 100 ml cultures was started. The optical density at 600 nm of these cultures was measured at every 20 minutes. According to the obtained values, growth curve was formed for all approved strains. The expected result of expression was accomplished by *E.coli* BL21(DE3) strain. The growth curve of transformed BL21(DE3) is shown in Figure 3.2., below. Based on this growth curve, starting point of induction was decided to be around the optical density value of 1.5.

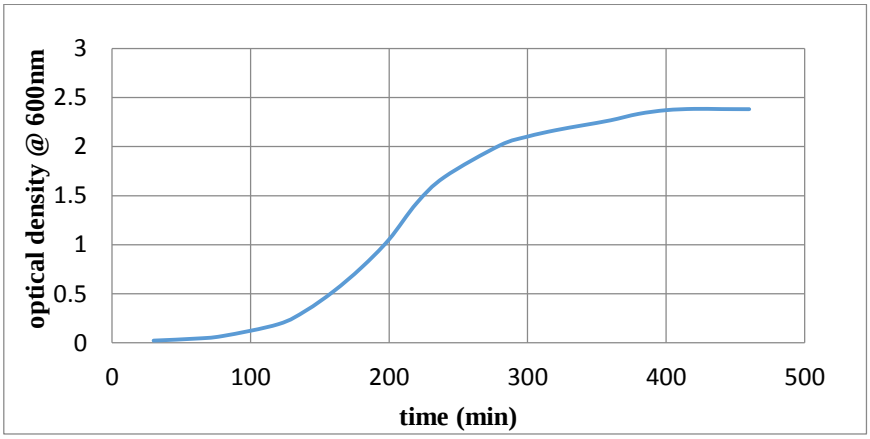

Figure 3.2. Growth curve of BL21(DE3) transformed with PaWES plasmid

3.4. Gene Expression Tests of PaWES

Gene expression tests for obtaining PaWES were carried out with different strains, IPTG concentrations, induction temperatures, additives and chaperons. They are listed in Table 3.1. Based on this expression tests, highest PaWES was observed in BL21(DE3) cells. Highest level of expression of PaWES was achieved in the soluble form in LB medium with the conditions of 20˚C induction temperature, 0.5mM IPTG concentration in the absence of additional glucose. (labeled * in Table 3.1.) According to SDS-PAGE analysis of expression test, which was performed under the conditions mentioned in the Materials and Methods Section, the maximum amount of protein was obtained at 15th hour, as seen in Figure 3.3.

Table 3.1. Attempted Conditions for PaWES Expression. * shows the condition under which highest level of soluble PaWES was obtained.

Strains	Antibiotic	IPTG Conc.	Induction Temp.
BL21(DE3)*	Ampicillin*	0.25 mM 0.5 mM* 1 mM	15˚C 20˚C* 25˚C - 37˚C
BL21(DE3) 2% glucose	Ampicillin	0.25 mM 0.5 mM 1 mM	18˚C 37˚C
BL21(DE3) 1% glucose	Ampicillin	0.5 mM 1 mM	18˚C 37˚C
BL21(DE3) Star	Ampicillin	0.5 mM- 1 mM	18˚C- 25˚C
BL21(DE3) Star 2% glucose	Ampicillin	0.25 mM- 0.5 mM- 1 mM	18˚C 25˚C
BL21(DE3) Star 1% glucose	Ampicillin	0.5 mM 1 mM	18˚C 25˚C
BL21(DE3) GroEL/ES	Ampicillin Kanamycin	0.5 mM 1 mM	18˚C 25˚C
BL21(DE3) GroEL/ES	Ampicillin Chloramphenicol	0.5 mM 1 mM	18˚C 25˚C
BL21(DE3)Star GroEL/ES	Ampicillin Kanamycin	0.5 mM 1 mM	18˚C 25˚C
BL21(DE3)Star GroEL/ES	Ampicillin Chloramphenicol	0.5 mM 1 mM	18˚C 25˚C
C41(DE3)	Ampicillin	0.25 mM- 0.5 mM- 1 mM	18˚C- 25˚C
C41(DE3) GroEL/ES	Ampicillin Kanamycin	0.5 mM 1 mM	18˚C 25˚C
BL21(DE3) pLysS	Ampicillin Chloramphenicol	0.25 mM-0.5 mM- 1 mM	18˚C 25˚C
Lemo T7 Express	Ampicillin Chloramphenicol	0.5 mM 1 mM	18˚C 25˚C
T7 Express LysY	Ampicillin	0.5 mM	18˚C
T7 Express LysY/Iq	Ampicillin	0.5 mM	18˚C

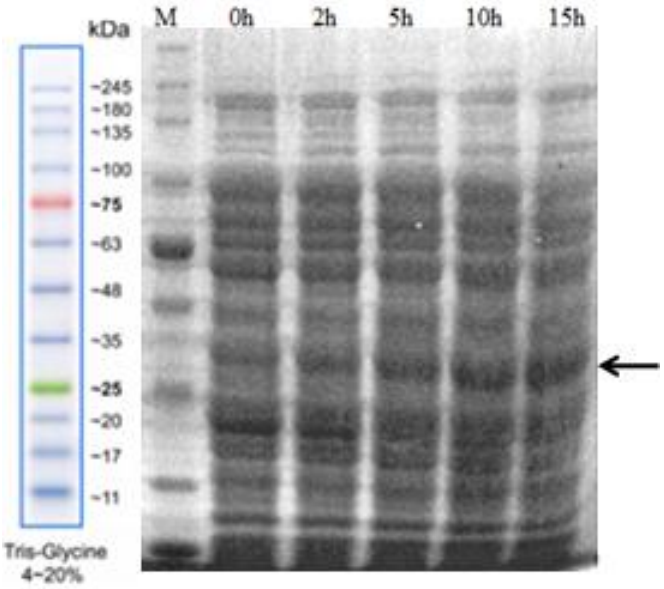

Figure 3.3. SDS-PAGE Analysis of Expressed BL21(DE3) Samples from 0-15th hours. M indicates protein marker, arrow shows the interested protein.

3.5. Purification of PaWES

The conditions that resulted in the highest level of soluble protein, as described above, were applied for large scale expression. Figure 3.4. shows the SDS-PAGE of purified samples via spin column method.

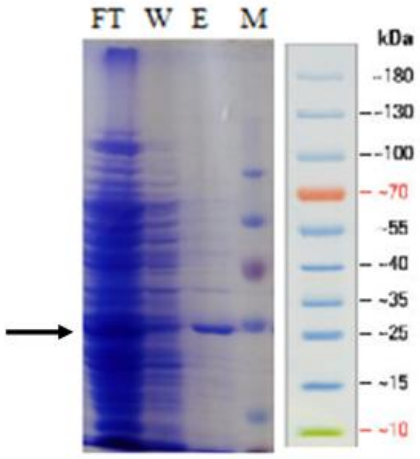

Figure 3.4. SDS-PAGE of spin column purified samples. Arrow indicates the desired protein band from different fractions. FT: flow through, W: wash, E: elution, M: marker

Purification method for large scale lysate which was approximately 35 ml was performed with IMAC. Fractions of flow through, wash and elution are shown in the

chromatogram which is in Figure 3.5. Samples from these fractions were analyzed by SDS-PAGE. It can be easily realized in Figure 3.6. that flow through fractions also contained the desired protein band. Because of that, one of the fractions from flow through (FT1) was taken and loaded to FPLC, again. However, the protein cannot be eluted as a result of this purification. It means this protein has a binding problem to the column. This binding problem may account for misfolding or the location of His-tag. Changing the His-tag location from C terminus to N-terminus may be the solution of this binding problem. The results of PaWES are not reproducible.

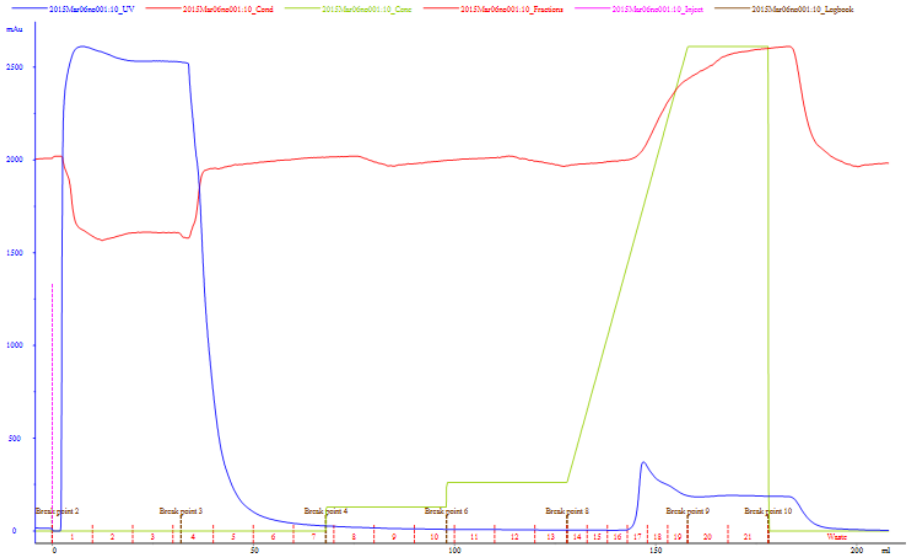

Figure 3.5. Purification chromatogram of PaWES. Blue (--), red (--), green (--) lines depict absorbance at 280 nm, conductivity and I-buffer concentration, respectively.

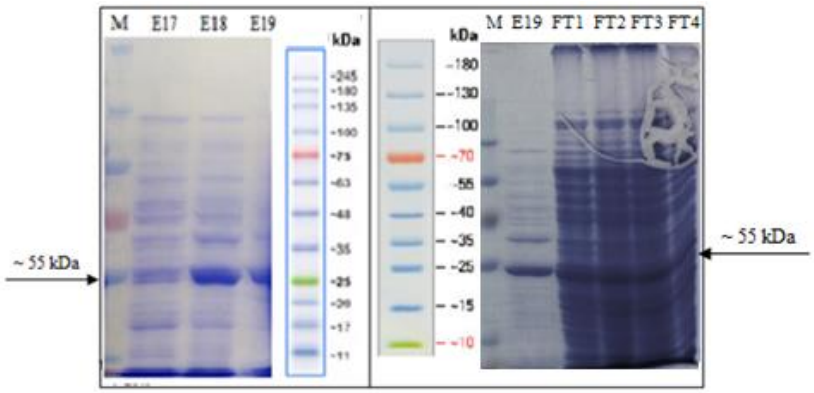

Figure 3.6. SDS-PAGE of purified samples from fractions of flow through (FT1-FT2-FT3-FT4) and elution (E17-E18-E19; fractions 17-19 in Figure 3.4.) that were taken from FPLC. M indicates the protein marker. Arrows show the estimated location of PaWES band.

3.6. Dialysis

Dialysis method was attempted stepwise for the refolding of the desired protein which has a binding problem to the column during IMAC. The samples were taken from W and E fractions of the FPLC that was run with 6M GuHCl denaturated sample at first. In the other trial, 8M Urea was used as denaturizing agent instead of GuHCl. Both of them gave the same result. Protein aggregation was observed as shown in Figure 3.7. Aggregation and supernatant samples were taken from the dialysis membrane, centrifuged for total separation and loaded in SDS-PAGE. The gel results are given in the Figure 3.8.

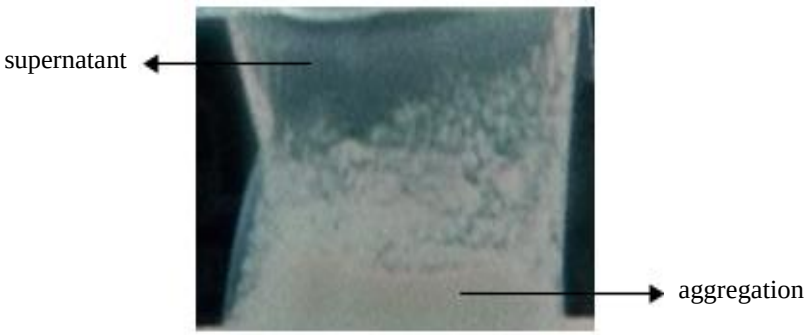

Figure 3.7. Visual of dialysis membrane

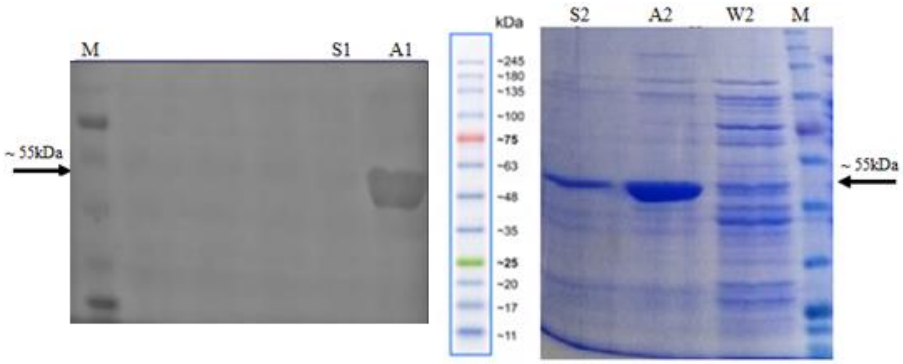

Figure 3.8. SDS-PAGE of aggregation and cleared part from dialysis membrane. Arrows show the expected location of the protein band, M: protein marker. GuHCl used dialysis; S1: supernatant part, A1: aggregation part. Urea used dialysis; S2: supernatant, A2: aggregate, W2: wash

Briefly, nearly 1 mg PaWES was successfully obtained from 1 liter LB culture, with BL21(DE3) cells, at 20˚C induction temperature and 0.5 mM IPTG concentration. However, the amount of protein was lower than expected. In addition, the productivity

value was also below 1 mg/hr. During IMAC, protein was lost in flow through fraction because of the binding problem of PaWES to HisPur NiNTA column.

B. Wax Ester Synthase from *Mus musculus* C57BL/6 (MmWES)

3.7. General Characteristics of MmWES

The amino acid sequence of MmWES gene is shown in Appendix A. Some estimated features about MmWES were obtained from protein calculator tools (http://protcalc.sourceforge.net/) with using the sequence. Molecular weight of MmWES was approximately 39 kDa. Estimated pI was nearly 9.42 and extinction coefficient was 1.95 $((mg/mL)^{-1}cm^{-1})$.

3.8. Cloning of MmWES

MmWES cloning strategy contained the target of bacterial strains and yeast strain. For bacterial studies, double restriction with *Nde*I and *Bam*HI and following ligation were applied strategy to achieve cloning of MmWES to pET22bTV. Figure 3.9. presents the SDS-PAGE of restricted gene with 1008 bp and pUC57 with nearly 2500 bp. After ligation step with the ratio Vector:Insert of 1:3, colonies were obtained from transfor-mation and sent to sequencing. Sequencing analysis also showed the cloning was succeeded.

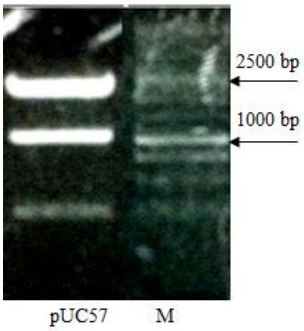

Figure 3.9. SDS-PAGE of restricted insert. M indicates 1 kb DNA marker.

3.8.1. Gateway Cloning Technology

Gateway Technology aimed to shuttle of the interested gene into multiple vectors. This *att*B site based recombination technology contained two different reactions. In the beginning of this technology, the 1008 base paired gene was amplified with designed *att*B primers via PCR. Figure 3.10. indicates the amplification products.

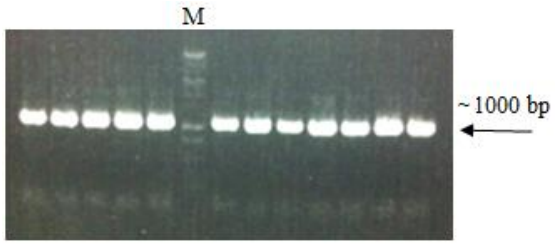

Figure 3.10. PCR products of MmWES. M; 1kb DNA marker

The second step was BP reaction, which targeted the transfer of the gene to pDONR221 as donor vector. BP reaction was confirmed with *Bsr*GI restriction. If empty pDONR221 was restricted with *Bsr*GI, the bands will be approximately at 800 bp, 1500bp and 2500 bp. Because of the BP reaction with PCR product, gene was expected to be integrated into pDONR221 and when restriction was applied, the bands will be estimated around 1008 bp and 2500 bp. According to the Figure 3.11., some of BP reaction trials with different PCR products (BP2, BP6) were achieved the formation of entry clone.

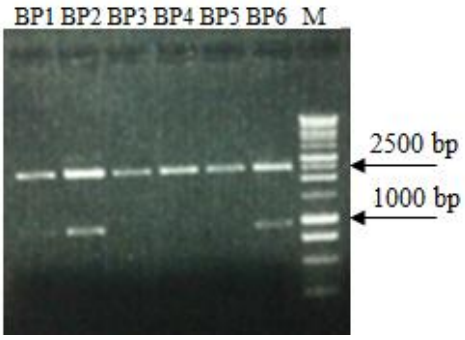

Figure 3.11. Restriction results of six BP reaction trials. M shows 1 kb DNA marker.

Then, LR reaction was performed with the aim of destination vector modification which will include MmWES gene sequence as a result. Confirmation of LR reaction was also done with *Bsr*GI enzyme. pAG423GAL-ccdb was used for

destination vector. Restriction of empty pAG423GAL-ccdb with *Bsr*GI resulted the band of 6280bp, 1286 bp, 402 bp and 229 bp. If MmWES gene was inserted to the vector, the bands will be obtained at 6280 bp and 1008 bp. Figure 3.12. showed the restriction result of LR reacted pAG423GAL-ccdb. LR1 and LR2 were the selected samples for further studies. Additionally, sequence analysis also supported the LR reaction results with the desired gene shuttle.

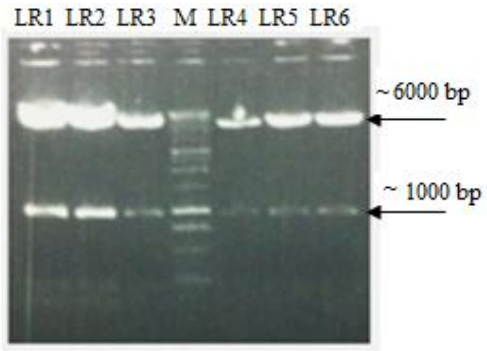

Figure 3.12. The *Bsr*GI restriction results of LR reaction products.
M: 1 kb DNA marker

3.9. Growth Curve of MmWES

The OD_{600} values from bacterial strains and yeast strain were used for the growth curve preparation. Figure 3.13. indicates the growth curve of *S. cerevisiae* BY4741 strain by which protein showed highest level of expression.

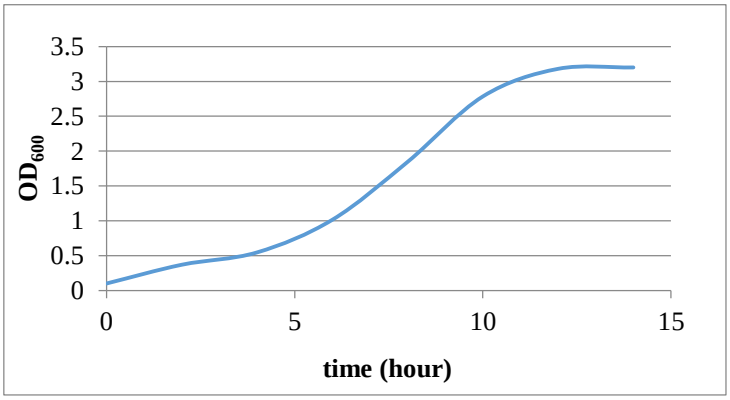

Figure 3.13. Growth curve of *S. cerevisiae* BY4741 strain

3.10. Gene Expression Tests of MmWES

The tested strains for bacterial expression of MmWES are shown in Table 3.2. Due to its eukaryotic protein features, it may need additional chaperones and foldases or some of post-translational modifications which are limited in bacterial strains, such as phosphorylation and glycosylation. The protein folding may not be achieved by bacteria because of the non-native disulphide formation. Additionally, stability of mRNA also affects to eukaryotic protein expression in bacteria. (Khow O., Suntrarachum S., 2012) Although nearly all troubleshooting methods, which were utilization of different *E.coli* strains, lower expression temperature, media additives, chaperone co-expression like GroEL/ES, were tried to resolve the expression problem of MmWES, the positive result cannot be obtained via bacterial expression. Under the conditions tested in Table 3.2., MmWES cannot be obtained with bacterial expression.

The eukaryotic characteristics of *S. cerevisiae*, like its short generation time and easily cultured ability, made it the best candidate for initial attempt of protein expression in yeast. MmWES was obtained with *S. cerevisiae* BY4741 included pAG423GAL-ccdb at the conditions of 30˚C temperature, 2% galactose as an inducing agent and 6 hours induction progressing time.

Table 3.2. The conditions tried for MmWES bacterial expression test

Strains	Antibiotic	IPTG concentrations	Induction Temperature
BL21(DE3)	Ampicillin	0.25 mM- 0.5 mM- 1 mM	15˚C- 20˚C- 37˚C
BL21(DE3) 2% glucose	Ampicillin	0.25 mM- 0.5 mM- 1 mM	18˚C 37˚C
BL21(DE3) 1% glucose	Ampicillin	0.5 mM 1 mM	18˚C 37˚C
BL21(DE3) Star	Ampicillin	0.25 mM- 0.5 mM- 1 mM	15˚C- 18˚C- 25˚C
BL21(DE3) GroEL/ES	Ampicillin Kanamycin	0.5 mM 1 mM	18˚C 25˚C
BL21(DE3) GroEL/ES	Ampicillin Chloramphenicol	0.5 mM 1 mM	18˚C 25˚C
BL21(DE3)Star GroEL/ES	Ampicillin Kanamycin	0.5 mM	18˚C 25˚C
BL21(DE3)Star GroEL/ES	Ampicillin Chloramphenicol	0.5 mM	18˚C
C41(DE3)	Ampicillin	0.25 mM- 0.5 mM- 1 mM	15˚C- 18˚C- 25˚C
C41(DE3) GroEL/ES	Ampicillin Kanamycin	0.5 mM	18˚C- 25˚C
BL21(DE3) pLysS	Ampicillin Chloramphenicol	0.25 mM- 0.5 mM- 1 mM	15˚C- 18˚C- 25˚C
T7 Express Lemo	Ampicillin Chloramphenicol	0.5 mM	18˚C- 25˚C
T7 Express LysY	Ampicillin	0.5 mM	18˚C
T7 Express LysY/Iq	Ampicillin	0.5 mM	18˚C

3.11. Purification of MmWES

The amount of lysate was 750 µl, which was extracted from 1500 ml culture. Due to the significantly low amount of lysate, spin column purification method was preferred. Figure 3.14. shows the SDS-PAGE of purified samples. According to the gel, the location of the band was seen as an expected, however the amount of protein obtained was low as seen in the thickness of the band. The reason for low level expression may be the ethanol accumulation as a result of fermentation process in *S. cerevisiae*. This accumulation can hinder the cell growth. Therefore the low density of cells are related to low amount protein production. Another reason of low level

expression may be the codon preference in *S. cerevisiae* during the translation. If the sequence of interest was codon optimized for *S. cerevisiae*, then the amount of expressed proteins may be increased.

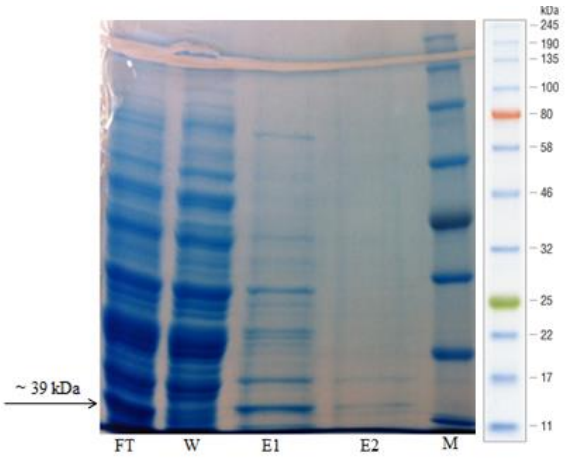

Figure 3.14. SDS-PAGE of MmWES purified samples. Arrow shows the expected band. FT: flow through, W: wash, E1: initial elution, E2: elution, M: protein marker

In short, MmWES was purified from *S. cerevisiae* culture with BY4741 strain, at 30˚C, with the volume of 2% galactose. The obtained amount of MmWES was lower than 1 mg. Also, the productivity value was below 1 mg/hr. This production can be increased with codon optimized sequence or selection of different yeast strain which has high density cell growth.

C. Taxadien-5-alpha-ol-O-acetyltransferase from *Taxus cuspidata* (TcT5AT)

3.12. General Characteristics of TcT5AT

The amino acid sequence of TcT5AT gene is shown in Appendix A. The estimated values of molecular weight, pI and extinction coefficient can be reached with the sequence via protein calculator tools. (http://protcalc.sourceforge.net/) Based on the amino acid sequence of TcT5AT was expected to have molecular weight of 50.2 kDa, pI of 6.3 and extinction coefficient at 280 nm of 0.99 $((mg/mL)^{-1}.cm^{-1})$.

3.13. Cloning of TcT5AT

Cloning of TcT5AT was performed as a combination of restriction and ligation reactions. TcT5AT gene insert and pET22bTV vector were restricted by *Nde*I and *Bam*HI, then insert was integrated into pET vector to being prepared for the further expression studies. The cloning result was also verified by sequencing.

3.14. Growth Curve of TcT5AT

The OD_{600} values of *E.coli* BL21(DE3) Star strain were taken every 20 minutes. This data is shown in TcT5AT growth curve, Figure 3.15. The value, which is starting point for induction, was approximately OD_{600} at 1.43.

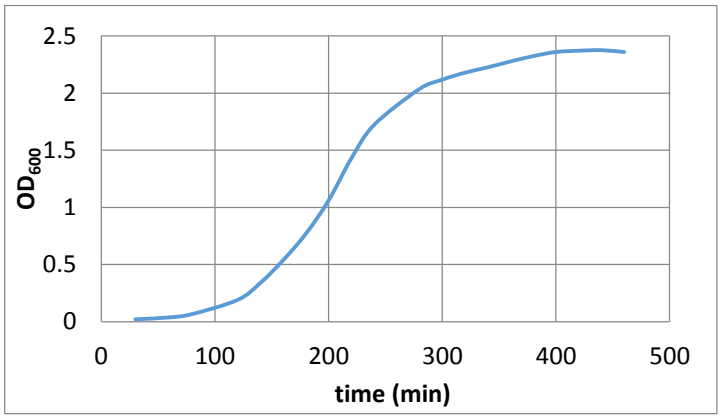

Figure 3.15. Growth curve of the protein obtained strain BL21(DE3) Star

3.15. Gene Expression Tests of TcT5AT

TcT5AT expression was tested with different strains and IPTG concentrations under various conditions. Table 3.3. shows the tried conditions to obtain soluble protein.

Table 3.3. Tested overexpression conditions for TcT5AT. * denotes condition that showed the highest expression level.

Strain	Antibiotic	IPTG concentration	Induction Temp.
BL21(DE3)	Ampicillin	0.5 mM- 1 mM	18˚C- 25˚C
BL21(DE3) GroEL/ES	Ampicillin Chloramphenicol	0.5 mM 1 mM	18˚C 25˚C
BL21(DE3) Star*	Ampicillin*	0.5 mM* 1 mM	18˚C* 25˚C
BL21(DE3)Star GroEL/ES	Ampicillin Chloramphenicol	0.5 mM- 1 mM	18˚C 25˚C
BL21(DE3) pLysS	Ampicillin Chloramphenicol	0.25 mM 0.5 mM- 1 mM	15˚C- 18˚C- 25˚C

Best condition that observed TcT5AT was BL21(DE3) Star strain in LB medium, 18˚C induction temperature with 0.5 mM IPTG at 15th hour. Figure 3.16. shows the SDS-PAGE of the samples from expression test, in which the band of TcT5AT quickly distinguishable.

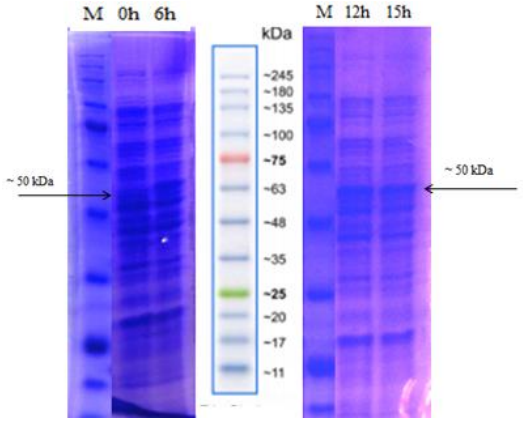

Figure 3.16. SDS-PAGE of samples from 0-15th hour at expression test.
M: protein marker

3.16. Purification of TcT5AT

TcT5AT was purified initially via spin column method. HisPur NiNTA resin and Talon resin were used for purification. Their results can be seen from SDS-PAGE in Figure 3.17.

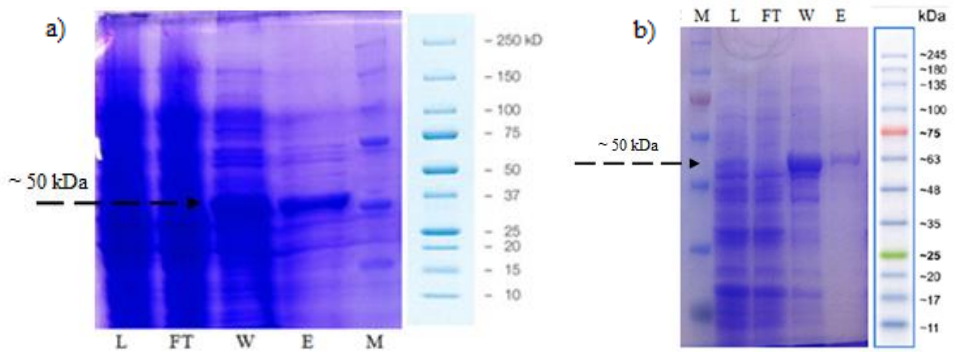

Figure 3.17. a) SDS-PAGE of Purified TcT5AT via Spin Column with HisPur NiNTA resin, b) with Talon resin. Arrows show the estimated location of TcT5AT. L: lysate, FT: flow through, W: wash, E: elution, M: protein marker

For the first FPLC run of TcT5AT, 3 liters expressed culture was harvested with two fold lysis buffer according to Invitrogen manual. However as a result of this trial, protein sample was obtained in a viscous solution. In addition, at the end of the IMAC, there was a high level of impurity observed in elution fractions and desired protein band was also observed in flow through fractions; at a low but detectable level. Therefore, the amount of lysis buffer was increased to five fold of pellet volume in extraction step for the next run of FPLC. Then, 8 liters of expressed culture, in which 60 ml lysate was obtained and was purified by IMAC with HisPur NiNTA resin. Samples from the fractions that have peaks in the chromatogram, indicated in Figure 3.18., were loaded to SDS-PAGE for verification of the protein existence. This gel is shown in Figure 3.19., below.

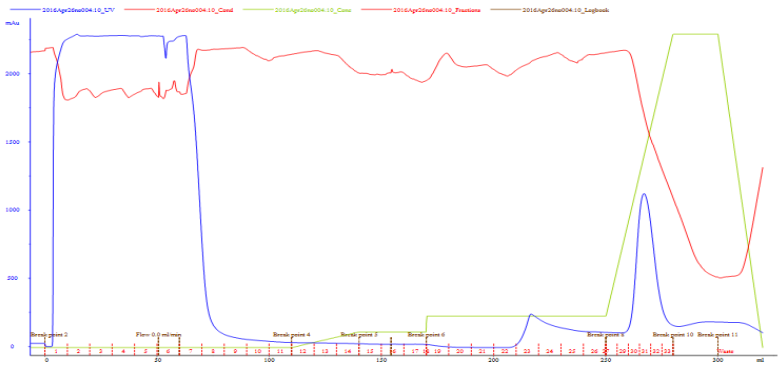

Figure 3.18. Chromatogram of purified TcT5AT from 60 ml lysate. Blue (--), red (--), green (--) lines depict absorbance at 280 nm, conductivity, and I-buffer concentration, respectively.

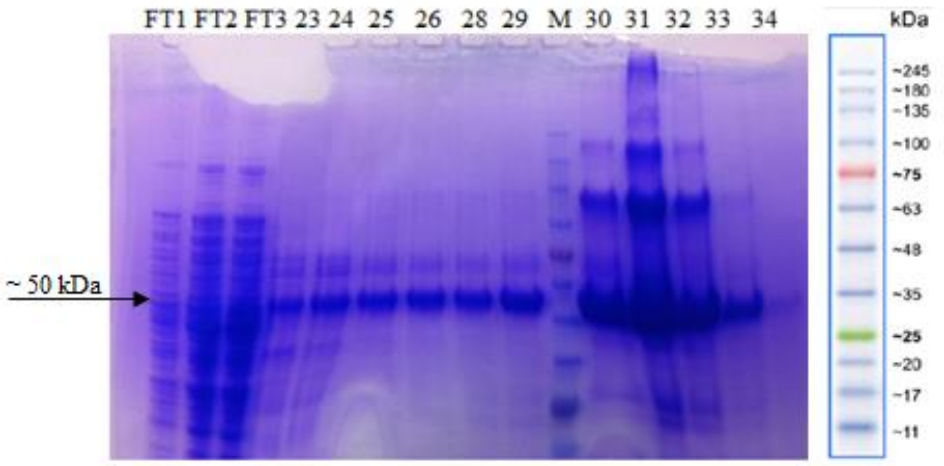

Figure 3.19. SDS-PAGE of numbered fractions from IMAC.
Arrow shows the TcT5AT band. FT: flow through, M: protein marker

3.17. Measurements of TcT5AT Concentration

Protein concentration was measured via Nanodrop with the samples belonged to IMAC of TcT5AT. According to the Beer's law for concentration with A280 value, protein concentration was calculated. Macrosep Advance Concentration tubes (Pall Corporation) were used to concentrate the eluted sample until the protein concentration reaches to ~20mg/ml in Elution Buffer, which was Tris-IMAC. After buffer exchange steps for four different buffers, the protein concentrations were measured again. The main purpose of this experiment was finding the most suitable buffer, where TcT5AT protein can remain stable for a long period of time. This was accomplished by determination of protein concentration after incubation of the protein in an extended period of time.

3.18. Buffer Optimization of TcT5AT

Four different buffers with various pH values were tried with the aim of preventing the protein aggregation. 500 µl concentrated samples from IMAC and HiTrap Desalting Columns were used for buffer exchange steps. 2500 µl samples were obtained as a result of buffer exchange programs via FPLC. Chromatograms belonged to these experiments can be seen in Figure 3.20. and Figure 3.21. Protein concentrations in these buffers were observed periodically. The values are shown in Table 3.4. The protein concentration resulted in a range from 5 mg/ml to 0.5 mg/ml with replacing the

buffers. According to this measurements, optimum buffer for TcT5AT was its original elution buffer in FPLC. Size Exclusion Chromatography (Gel Filtration) was applied with that Tris-IMAC Buffer. Regarding to the high stability effect of the reducing agent DTT to TcT5AT protein solubility, 1 mM DTT was added to Tris-IMAC Buffer while using in Gel Filtration. The oxidation of protein and the formation of unwanted di-sulfide bonds can be prevented by DTT at low amounts. Higher than 1 mM of DTT is not recommended, it makes the protein in unfolded form because of its reducing feature.

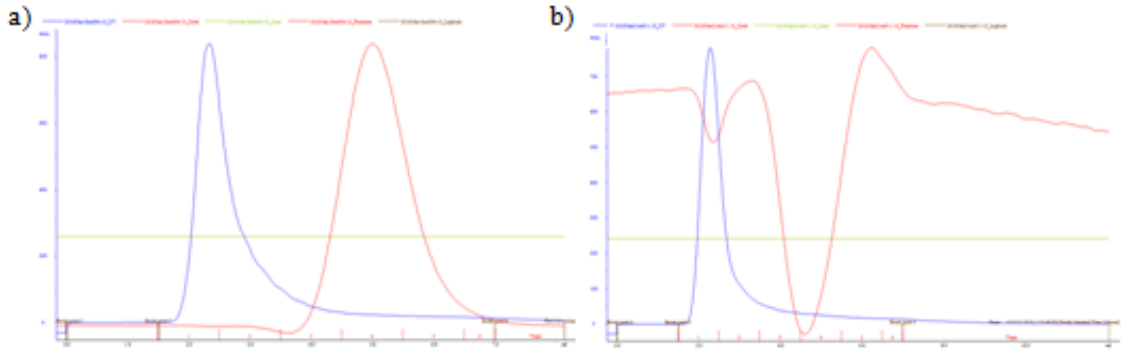

Figure 3.20. Buffer Exchange Chromatograms for a) MOPSO Buffer, b) HEPES Buffer. Blue (-), red (-), green (-) lines depict absorbance at 280 nm, conductivity, and buffer concentration, respectively.

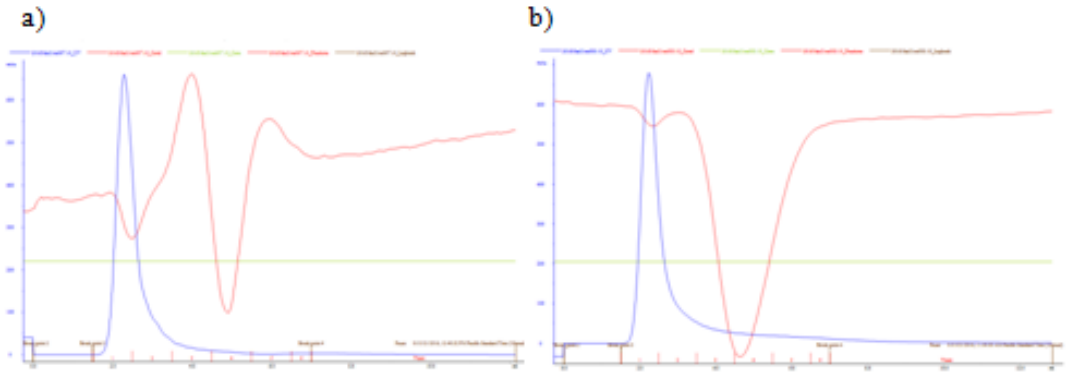

Figure 3.21. Buffer Exchange Chromatograms for a) Tris-DTT Buffer, b) Tris-HCl Buffer. Blue (--), red (--), green (--) lines depict absorbance at 280 nm, conductivity, and buffer concentration, respectively.

Table 3.4. Protein concentrations of buffer exchanged samples

Buffer	1st day	3rd day	7th day
Elution Buffer	4.2 mg/ml	3 mg/ml	3 mg/ml
MOPSO	5 mg/ml	4 mg/ml	1.4 mg/ml
HEPES	1.9 mg/ml	1.6 mg/ml	1.4 mg/ml
Tris-DTT	0.85 mg/ml	0.7 mg/ml	0.7 mg/ml
TrisHCl	1.4 mg/ml	1.0 mg/ml	0.5 mg/ml

3.19. Size Exclusion Chromatography for TcT5AT

Size Exclusion Chromatography was applied for the separation the substances by their size via gel filtration column. The chromatogram belonged this program was demonstrated, below in Figure 3.22. Samples from peak observed fractions were analyzed with SDS-PAGE. According to the gel in Figure 3.23., the purity of obtained TcT5AT from gel filtration was not very high. In contrast, based on the thickness of the band, the concentration of TcT5AT was not highly lost as a result of size exclusion chromatography. After size exclusion chromatography, the samples were concentrated and the protein concentration was reached to approximately 23 mg.

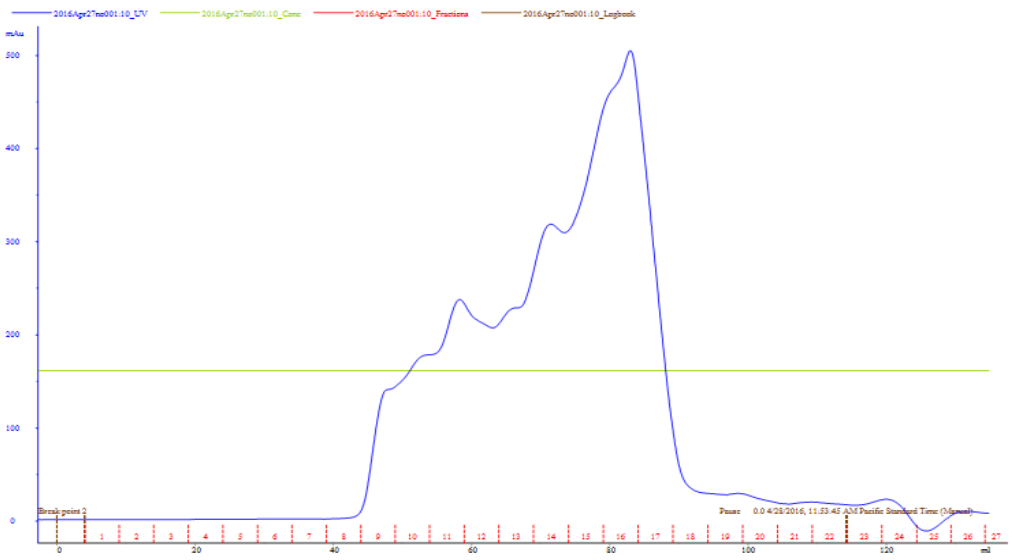

Figure 3.22. Size Exclusion Chromatogram of TcT5AT. Blue (--) and green (--) lines depict absorbance at 280 nm and buffer concentration, respectively.

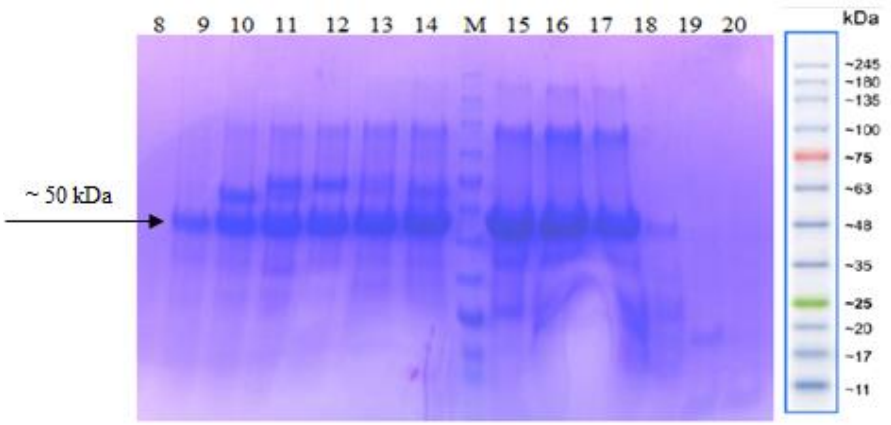

Figure 3.23. SDS-PAGE of Fractions (8-14, 15-20) from Size Exclusion Chromatography. Arrow shows the estimated location of TcT5AT, M indicates protein marker.

In brief, approximately 23 mg TcT5AT was obtained efficiently, from 8 liter LB cultures of *E. coli* BL21(DE3) Star cells, at induction temperature of 18˚C, with 0.5 mM IPTG. In addition, the productivity value was reached to ~1.6 mg/hr.

CHAPTER 4

CONCLUSION

In this project, three biosynthetic enzymes which have promising applications in the field of biodiesel and pharmaceutical production are studied.

Biosynthesis of fatty acid ethyl esters in microorganisms is more advantageous and sustainable compared to production by transesterification based conventional diesel method. In this biosynthesis, the key role belongs to an enzyme called wax ester synthase. Cloning and purification studies of wax ester synthases are the main subject of this study. *Psychrobacter arcticus* 273-4 based wax ester synthase (PaWES) was successfully cloned into pET22bTV expression vector system. As a result of the expression tests, PaWES was obtained by *E.coli* BL21(DE3) strain, at 20˚C, with 0.5mM IPTG. It was purified via IMAC with the yield ~1 mg. As also the His-tagged PaWES protein had a binding problem to the IMAC column, changing the location of His-tag may increase the efficiency of purification step.

Wax ester synthase from *Mus musculus* C57BL/6 (MmWES) is the other investigated enzyme of this study. Cloning of MmWES was achieved for two different organisms, which are pET system in bacteria and GAL system in yeast. Especially, solution methods for the key problems in expression of an eukaryotic enzyme with bacteria were investigated. Expression of MmWES was accomplished by *S. cerevisiae* BY4741 strain, at 30˚C, with 2% galactose and it was purified with lower than 1 mg yield. The expression level was lower than the expected, the reason of that may be due to the fact that the used sequence was not codon optimized for *S. cerevisiae*. The expression and purification yield can also be increased with using different yeast species as host organism. As providing a model for future studies, cloning and purification of these enzymes is essential for renewable production of biodiesel. Especially with the structural studies of this enzymes, the catalytic mechanism can be elucidated and the limitations about FAEE production can be solved. Additionally, high amount *de novo* biodiesel production in microorganisms can be inspired by this studies.

The third studied enzyme belonged to acetyltransferases class in the biosynthesis of Taxol, which is a drug predominantly used in chemotherapy. The acetyltransferases have important functions in the critical steps of the Taxol biosynthesis pathway.

Cloning and purification of taxadien-5α-ol-O-acetyltransferase (TcT5AT) from *Taxus cuspidata* is one of the main purposes of this study. Following the previous studies, TcT5AT was cloned into pET22bTV vector and expressed with the yield of 23 mg, by *E. coli* BL21(DE3) Star strain, at 18˚C, 0.5 mM IPTG, successfully. According to the performed protein purification methods with immobilized metal affinity chromatography and size exclusion chromatography, it was obtained with estimated 80% purity. The purification studies of TcT5AT are in progress. The purity of protein can be increased with different chromatographic methods, such as ion exchange chromatography, in protein purification applications. In the future, this purified protein can be used in mechanistic and structural analysis of TcT5AT and influence the development of new precursors coupled with increased biosynthesis of Taxol for therapeutic applications.

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APPENDIX A

AMINOACID SEQUENCES

The amino acid sequence of PaWES gene:

MRLLTAVDQLFLLLESRKHPMHVGGLFLFELPENADISFVHQLVKQMQ
DSDVPPTFPFNQVLEHMMFWKEDKNFDVEHHLHHVALPKPARVRELLMYVSR
EHGRLLDRAMPLWECHVIEGIQPETEGSPERFALYFKIHHSLVDGIAAMRLVKK
SLSQSPNEPVTLPIWSLMAHHRNQIDAIFPKERSALRILKEQVSTIKPVFTELLNN
FKNYNDDSYVSTFDAPRSILNRRISASRRIAAQSYDIKRFNDIAERINISKNDVVL
AVCSGAIRRYLISMDALPSKPLIAFVPMSLRTDDSIAGNQLSFVLANLGTHLDDP
LSRIKLIHRSMNNSKRRFRRMNQAQVINYSIVSYAWEGINLATDLFPKKQAFNLI
ISNVPGSEKPLYWNGARLESLYPASIVFNGQAMNITLASYLDKMEFGITACSKA
LPHVQDMLMLIEEELQLLESVSKELEFNGITVKDKSEKKLKKLAPGSHHHHHH

The amino acid sequence of MmWES gene:

MFWPTKKDLKTAMEVFALFQWALSALVIVTTVIIVNLYLVVFTSYWPV
TVLMLTWLAFDWKTPERGGRRFTCVRKWRLWKHYSDYFPLKMVKTKDISPD
RNYILVCHPHGLMAHSCFGHFATDTTGFSKTFPGITPYMLTLGAFFWVPFLRDY
VMSTGSCSVSRSSMDFLLTQKGTGNMLVVVVGGLAECRYSTPGSTTLFLKKRQ
GFVRTALKHGVSLIPAYAFGETDLYDQHIFTPGGFVNRFQKWFQKMVHIYPCA
FYGRGLTKNSWGLLPYSQPVTTVVGEPLPLPKIENPSEEIVAKYHTLYIDALRKL
FDQHKTKFGISETQELVIVGSHHHHHH

The amino acid sequence of TcT5AT gene:

MEHAVWKDLNVKSFDPVMVKPSIPLPKTVLHLSTVDNLPVLRGNLFNS
LIVYKASDKISADPVKVIREALSKVLVYYFPFAGRLRYKENGDLEVDCNGEGA
AFVEAMVDCNLSVLGDLDDLNPSYEDLLFALPQNTDIVDLHLLVVQVTRFACG
GFVVGVSFHHSICDGRGAGQFLQSLAEIARGEDKLSCEPIWNRELLKPEDPIHLQ
FYHLYSLPPSGPTFEEWVHASLVINPATIKHMKQSIMEECNEVCSSFEIVAALTW
RARTKALQIPQTQNVKLLFAVDMKKSFNPPFPKGYYGNAIGFACAMDNAHDLI
NGSLLHAVNIIRKAKAYLFEQCSKSSVAVNPSALDANTGQESVVALTDWRRLG
FNEVDFGWGDAVNVCPVQRMTNGLVMPNYFLFLRPSEDMPDGIKILMCMASS
MVKSFKFEVEDMINKYVPAVGSHHHHHH

APPENDIX B

USED VECTOR MAPS

Differently from pET-22b(+) vector, pET22bTV includes the following forward and reverse primers: GCAGGATCCCACCACCACCACCACC and GCACATATGTA-TATCTCCTTCTTAAAGTTAAAC. (Köksal et al., 2011)

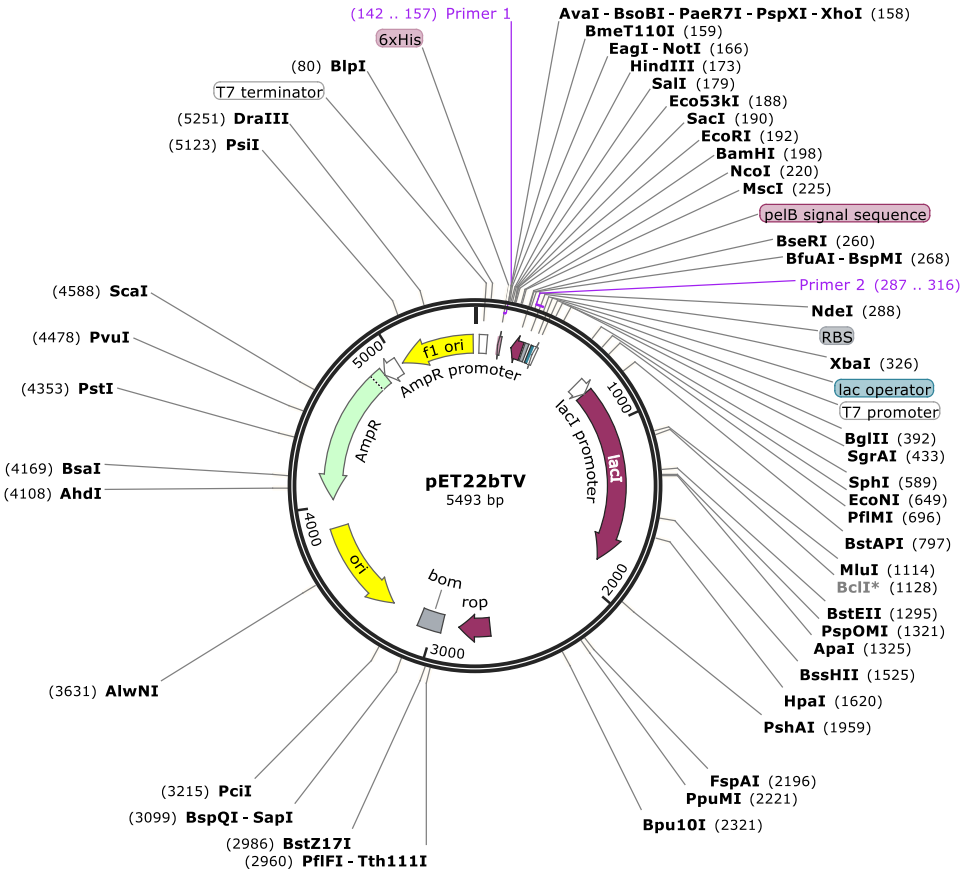

Figure B.1. pET22bTV vector map

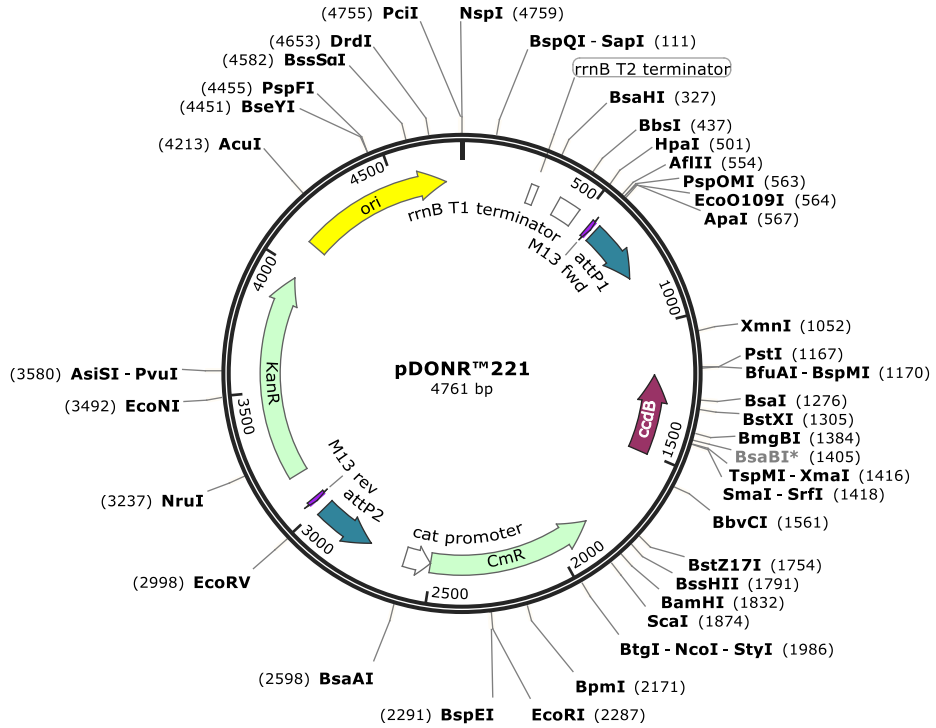

Figure B.2. pDONR221 vector map

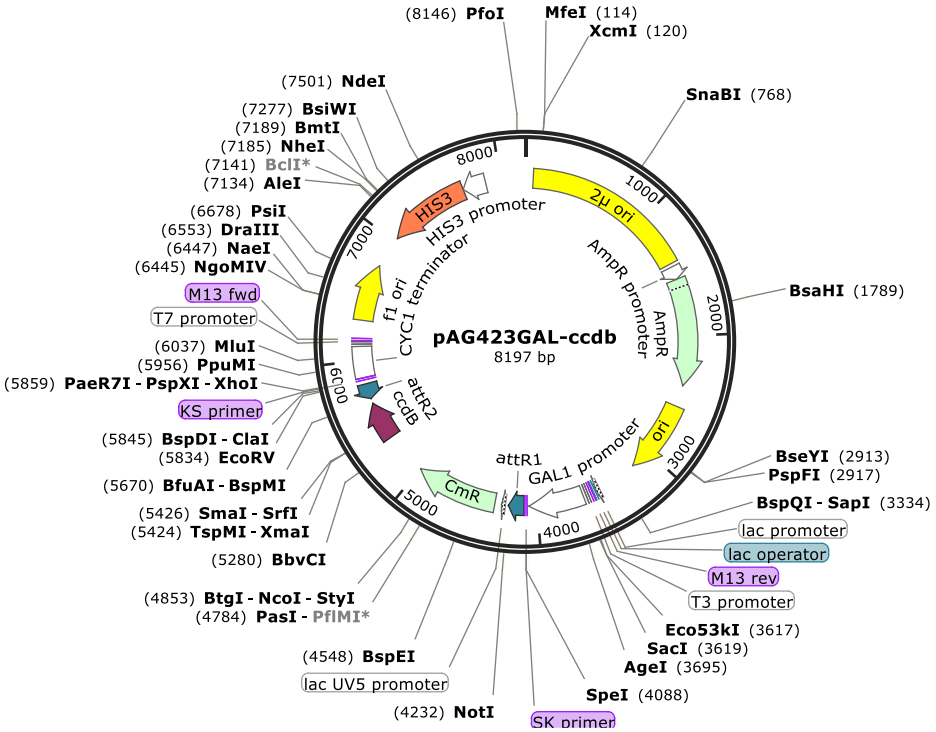

Figure B.3. pAG423GAL-ccdb vector map