CLONING, HETEROLOGOUS EXPRESSION AND PURIFICATION OF VARIOUS WAX ESTER SYNTHASES IN *ESCHERICHIA COLI*

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Kamil OVACIK

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We approve the thesis of **Kamil OVACIK**

Examining Committee Members:

Assoc. Prof. Dr. Alper ARSLANOĞLU
Department of Molecular Biology and Genetics, İzmir Institute of Technology

Asst. Prof. Dr. Nur Başak SÜRMELİ
Department of Bioengineering, İzmir Institute of Technology

Assoc. Prof. Dr. Serap EVRAN
Department of Biochemistry, Ege University

26 July 2017

Assoc. Prof. Dr. Alper ARSLANOĞLU
Supervisor, Department of Molecular Biology and Genetics
İzmir Institute of Technology

Prof. Dr. Volkan SEYRANTEPE
Head of the Department of
Molecular Biology and Genetics

Prof. Dr. Aysun SOFUOĞLU
Dean of the Graduate School of
Engineering and Sciences

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ABSTRACT

CLONING, HETEROLOGOUS EXPRESSION AND PURIFICATION OF VARIOUS WAX ESTER SYNTHASES IN *ESCHERICHIA COLI*

Biodiesel, known all around the World, is a diesel fuel containing fatty acid methyl esters (FAMEs) and fatty acid ethyl esters (FAEEs) with different molecular weights. The recent studies which are about the development of FAEE focused on production of FAEEs in vivo syntheses. This synthesis is catalyzed by wax ester synthases (WS). Bifunctional wax ester synthase/acyl-coenzyme-A (acyl-CoA): diacylglycerol acyltransferase (WS/DGAT) synthesizes wax ester by processing a certain range of fatty alcohols and fatty acyl-CoAs. It is considered as the final enzyme in biosynthetic process of wax ester production.

Aim of the research is cloning, heterologous expression, purification and crystallization trial of was ester synthases from *M. aquaeolei* VT8 (MaWES) and *R. opacus* PD630 (RoWES). MaWES was cloned into pET expression vector and heterologous expression of MaWES was carried out in *E.coli* BL21 (DE3) strain. Three chromatography systems were used for purification of MaWES. After Immobilized Metal Affinity Chromatography (IMAC), buffer exchange and gel filtration chromatography, enzyme was purified with approximately 100 mg yield. This project can pave the way for structural studies WS/DGAT 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 FAEE based biodiesel.

ÖZET

ÇEŞİTLİ MUM ESTERİ SENTAZLARIN *ESCHERICHIA COLI* DE KLONLANMASI, HETEROLOG EKSPRESYONU VE SAFLAŞTIRILMASI

Son yüz yılda yakıt tüketiminin tüm dünya üzerinde artması ve fosil yakıtların çevreye verdikleri zarar ve yakın süreç içerisinde tükenecek olma kaygısı alternatif yakıt kaynakların oluşturulma çabasını arttırmıştır. Alternatif yakıt kaynaklarından olan biyodizel, sahip olduğu avantajlarla fosil yakıtlara alternatif olma özelliğine sahiptir. Biyodizel üretimi mevcut sistemlerde metanolün ve biyolojik yağların transesterifikasyon reaksiyonu sonucu gerçekleştirilmektedir. Son dönem de yapılan çalışmalar, biyodizel yakıtının üretiminde etanol kullanımının biyolojik organizmalar aracılığıyla gerçekleştirmesi üzerine odaklanmış bulunmaktadır. Mum esteri sentaz enzimi (WS/DGAT) yakın tarih içerisinde keşfedilmiş olup farklı mikroorganizmalar da yapılan çalışmalar ile mum esteri sentaz enzimi aracılığı ile pilot ölçekte yağ asidi etil ester (mikrodizel) üretimi gerçekleştirilmiştir. Fakat geniş substrat seçiciliği özelliüine sahip olan WS/DGAT enzimlerinin substrat olarak uzun zincirli yağlı alkolleri ve yağ asitlerini tercih etmesi etanol gibi kısa zincire sahip olan alkollerin büyük ölçüde dışarılanmasına sebep olmaktadır.

Bu çalışma da amaç, *M. aquaeolei* VT8 ve *R. opacus* PD630 kaynaklı farklı iki mum esteri sentaz enziminin klonlanması, heterolog ekspresyonu, saflaştırılması ve saflaştırılmış olan WS/DGAT'ler ile kristalizasyon denemesi amaçlanmaktadır. *M. aquaeolei* VT8 kaynaklı mum esteri sentaz enzimi pET22bTV vektörü içerisine klonlanmış ve *E.coli* BL21 (DE3) hücre hattı kullanılarak heterolog ekspresyon ile üretilmiştir. İmobilize metal afinite kromatagrafi ve jel filtrasyon kromatografi yöntemleri kullanılarak MaWES proteini saflaştırılmıştır. Bu çalışma; gelecek için önem arz eden biyodizel üretimi için kullanılacak olan WS/DGAT enziminin klonlanma ve saflaştırma aşamalarına ışık tutarak, gelecekte yapılması muhtemel protein mühendisliği çalışmalarına dolaylı olarak yardımcı olacaktır.

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CHAPTER 1

INTRODUCTION

1.1. Renewable Diesel

Reliable and sustainable energy resources are essential for sustainable economic and industrial development. As it is predicted that for energy resources the tendency will be shifted to raw materials, therefore different and rather new concepts will be required in R&D as well as production and economy (Naik et al., 2010). As the population increases, energy demand will surely increase as well. In this ever growing energy requirement mankind will not have a wide range of options for energy sources.

It is known that the world mostly relies on non-renewable energy sources such as petroleum based fuels and coal. Non-renewable energy sources are, no doubt, going to deplete and this depletion is going to bring about the energy crisis will be followed by an economic downfall sooner than expected, in only a few decades. CO_2 level is significantly increasing and using fossil fuels for various reasons just accelerates this process, thusly global warming is affected as well (Fortman et al., 2008; Lee, 2012; Mabee et al., 2005). That's why the issue of alternative energy sources and energy management must seriously be taken into account along with its positive effects on environment and climate (Röttig et al., 2010).

In principle, it is thought that the energy potential of renewable sources is extraordinary so much that it can meet energy demand a few times over (Herzog et al., 2001). Considering energy security and sustainability and also in order to solve environmental problems, it is obvious that there should be a sustainable, large scaled substitute for fossil fuels and petroleum based products (Goudriaan and Peferoen, 1990). Negative effects of greenhouse gas emissions have been understood and it led the scientists to find a sustainable and environment friendly energy source for both the industry and the people (Mabee et al., 2005). We can include biomass, wind, solar, hydropower and geothermal power as renewable sources (Herzog et al., 2001).

Biofuel production process includes biomass being turned into better intermediate

forms of energy. It is much more beneficial to convert solid biomass into liquid. This process may be a substitute for petroleum based fuels in transportation sector (Herzog et al., 2001). Biofuels are divided into two main groups, first generation and advanced biofuels.

First generation biofuels make up almost 90% of total biofuel market today. Bioethanol, fermented from corn, biogas and animal fat or vegetable oil esterified biodiesel are two of the most common types (Van Rensburg et al., 2014). First generation biofuels can be classified according to its ability to be added to petroleum based fuels and used in combustion engines with the current technology or its necessity to use a specific biodiesel system like FFVs (Flexible Fuel Vehicle). With its already 'established technology' large amounts of three different biofuels, biodiesel, ethanol and biogas, have been produced around the world (Van Rensburg et al., 2014)

Advanced biofuels, however, are produced from different raw materials such as wheat straw, forest waste and energy crops like switchgrass. These raw materials contain nonfood cellulosic biomass. Feedstocks mentioned above are easily cultivated; grow fast and relatively cheaper by products of agricultural practices. They contain high amounts of cellulosic biomass whereas do not compete with food and reduce water and fertilizer use. In practice, these advanced fuels should have high energy content, be stored and transported similarly and also have combustion properties good enough to be used in current gasoline, diesel and jet engines. Butanol, isopentanol, terpenes, fatty acid ethyl esters and alkanes can be included in these fuels (Balat, 2006).

Biomass-derived molecules which can be used in diesel engines are as follows; Alkanes/olefin mixtures, farnesane and fatty acid methyl esters (FAMEs; biodiesel) (Figure1.1.). However, it is also known that biodiesel group can sometimes contain fatty acid ethyl esters (FAEEs) as well (Steen et al., 2010). By applying hydrotreatment of triglycerides, alkanes/olefin mixtures can be produced; they are classified as 'renewable' diesel (epa rule). Farnesane, biomass-derived alkanes/olefins mixtures can also be covered under renewable diesel, where as we consider FAMEs and FAEEs as biodiesels. They are classified according to their fuel molecule, not production process. For example, Farnesane, a C15 isprenoid, is a single molecule diesel fuel while alkane/olefin fuels can be constructed by linear carbon chains possessing 8 to 22 carbons (Knothe, 2008; Kinast, 2003; Steen et al., 2010). Performance criteria of petroleum diesel can be met by renewable diesels as well but their performance can vary in different setups. (See Table 1.1.) For example in cetane and lubricity their application is rather useful, while in energy den-

sity and cloud point results are not as desired. We can also encounter instability due to oxygen presence and unsaturated bonds in the FAMEs (Westfall and Gardner, 2011).

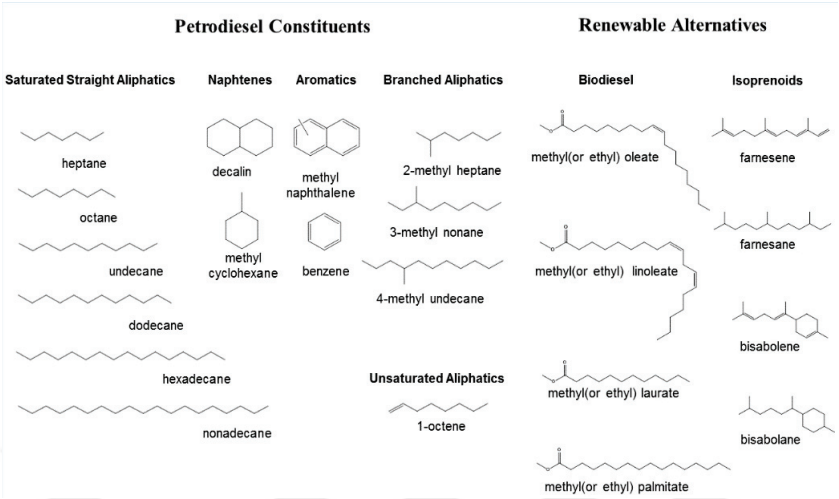


Figure 1.1. Petrodiesel and its renewable alternatives

Table 1.1. Diesel fuel properities

(Source: Westfall and Gardner, 2011)

Diesel Molecule	Synthesis method	Cetane	Cloud Point (C)	Energy density (MJ/kg)	Lubricity (mm)	Oxygen (%)	Stability
Farnesane	Fermentation	58	-40	43	400	0	Good
Alkanes via hydrotreatment or Fischer-Tropsch [15]	Fermentation or Chemical	70-90	-20 to 20	44	520	0	Good
FAMEs [1,3]	Fermentation or Chemical	47-59	0	38	250	11	Marginal
Alkanes/aromatics from Petrol [15]	Distillation	40	-5	43	<520*	0	Good

*Ultra-low sulfur diesel (<15 ppm sulfur) requires additives to achieve less than 510 mm lubricity.

1.2. Biodiesel

1.2.1. Overview of Biodiesel

Biodiesel which consists of methyl esters of fatty acids (FAMEs) and ethyl esters of fatty acids (FAEEs) is considered to be a substitute or an extender of diesel fuel can be produced by transesterifying from renewable feedstocks, as vegetable oil or animal fats with alcohol, mostly methanol. This process can be applied even to cooking greases. It is estimated that soybean will have the highest portion in biodiesel production; other vegetable oils are relatively more expensive as an input. Biodiesel and total biodiesel inputs should be waste fats and recycled oils. Although high amount of saturated fats in waste fats may cause undesired results such as low quality, they are much cheaper as a feedstock option. For example, yellow grease is one of the cheapest feedstocks but its supply is rather limited (Perlack et al., 2011). That's why different raw stocks have been put forward such as palm oil and jatropha especially in Asian countries. They are believed to be renewable sources with great potential (Mekhilef et al., 2011). Thanks to this process, fatty acid methyl esters or ethyl esters can be obtained and used as a fuel substitute or extender called biodiesel (Figure1.2.) (Krawczyk, 1996).

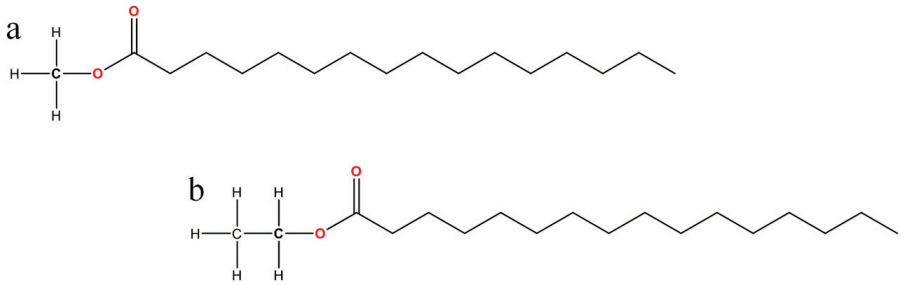


Figure 1.2. Chemical structures of fatty acid methyl esters (FAME) and fatty acid ethyl esters (FAEE). a methyl palmitate and b ethyl palmitate

As the fossil fuel resources are on the decline, biodiesel seems to become an even more important option (Meher et al., 2006). It is known that methanol and ethanol transesterification products biomass derived oils can be used as additives or even as neat fuel for

many diesel engines, which does not even require engine modifications. That's why many studies have been initiated in both European countries and in United States (Krawczyk, 1996). Biodiesel, a.k.a. nonpetroleum diesel is an important item in European export and import. This has also drawn the attention to other biofuels and biodiesel recently (Mekhilef et al., 2011; Krawczyk, 1996).

As we have mentioned before biodiesel mixtures contain FAMEs and FAEEs, these esters can vary from C8 to C22, however, the most common chain length is between C16 to C18 (Westfall and Gardner, 2011). Today, more and more countries join the biodiesel production trend as it is one of the most clean and sustainable fuel alternative (Westfall and Gardner, 2011; Fortman et al., 2008; Shi et al., 2011).

1.2.2. Advantages and Limitations of Biodiesel

It is an undeniable fact that the fossil fuels will not endure forever, which results in the search for sustainable, renewable and environmental friendly fuel sources. Biodiesel can be the answer as biodiesel production is a new industry sector, along with many positive factors such as reduced carbon emissions and costs, sustainable production and raw material flow, environment protection and energy output rival to that of fossil fuels. Biodiesel can be used in diesel engines and it does not require a serious modification to the engine itself. It can also free the world from fossil fuel dependence and reduce CO_2 emission which is a huge problem for environment. Biodiesel does not only reduce carbon emission by its sole use, it also helps this reduction by creating an alternative to fossil fuels thusly minimalize our dependence on them. It is suggested that biodiesel utilization can decrease pollution levels and possible carcinogens thanks to its properties such as clean combustion, biodegradability, low CO emission and toxicity (Mekhilef et al., 2011; Johansson and Burnham, 1993; Osamu and Carl, 1989; Lee, 2012).

Renewable diesels or biodiesel fuels have other important properties as well, such as narrow molecular size distribution, low aromatic content and almost zero sulfur. Additionally, methyl esters, part of this process, also have certain advantages. While their volatility and viscosity are considerably low, they have high flash point and penetration properties. They are not only biodegradable, also they have good water wetting features In this case for base engine oils, heat transfer and hydraulic fluids rapeseed oil for example can be a much better feedstock due to the fact that it contains relatively low polyunsatu-

rated fatty acid (Shahidi, 2005).

There were certain doubts of the scientists about the first generation biofuels. Its effect on the environment and carbon emission and balance were neither completely understood nor researched. For example, biodiesels have the lowest unburned hydrocarbon emission but they still emit NOx at a relatively higher level which can result in ozone formation (Knothe et al., 2006; Kinast, 2003). This created a setback in biodiesel production initially. Another negative aspect of biodiesel was the increased demand for oil. Biodiesel production requires oils and this brings the controversy of food or fuel. As biodiesel consumes more and more oil, it is inevitable to see an increasing trend in oil prices (Hill et al., 2006; LAURSEN, 2005).

FAME, which is another feedstock for biodiesel production, have some downsides as well, and the most apparent one is the cold-flow properties. In order to keep the fuel liquid, FAME may require special heated tanks. This requirement makes it harder to use and also sell in cold climate countries. In time FAME can degrade and form corrosive acid, especially when it contacts with air and water. Water also accelerates biological degradation of FAME (Council et al., 2012).

Due to the disadvantages of biodiesel, such as lower energy density, oxidative in stability and cold temperature viscosity, it cannot completely replace petroleum diesel considering current fuel distribution and diesel engine designs. That's exactly why both utilization and production can be limited. Feedstock price is another factor limiting the production (Kinast, 2003).

Although renewable biofuel research has skyrocketed due to concerns on fossil fuel scarcity and its harmful effects on the environment it is hard to come up with a fuel option such fuel must not only have good energy potential but also be economical. Besides it needs to be produced without causing food scarcity and environment friendly (Hill et al., 2006).

1.2.3. Chemical Production of Biodiesel

Transesterification, a.k.a. alcoholysis, can be defined as a chemical process where triglycerides from vegetable oils and animal fats are redacted into diglycerides first then into monoglycerides and then converted into methyl or ethyl esters with the help of alcohols. This reaction can be enhanced with a catalyst, also it is required to provide excess

of alcohols in order to acquire a desired reaction rate and yield. The reaction produces esters (biodiesel) and a byproduct (Figure1.3.). Although both biodiesel and glycerol are high value products, it is rather hard to find enough amount of lipids to be harvested for a cost effective, large scale production (Srivastava and Prasad, 2000) (Meher et al., 2006) (Lee, 2012).

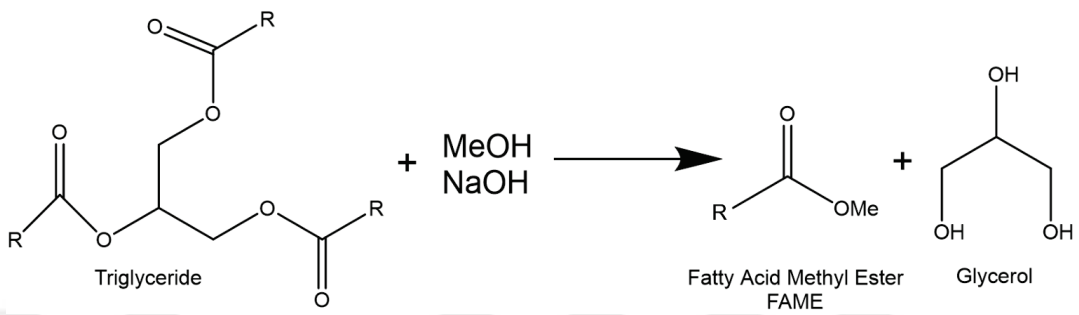


Figure 1.3. Production of biodiesel by transesterification

In transesterification alcohol within the ester is displaced by another. Although it is similar to hydrolysis, not water but alcohol is the basis. It is known to be used to decrease the viscosity of triglycerides. Transesterification can be reversed and also accelerated thanks to certain catalysts. For example, if the amount of free fatty acid and water is high, transesterification can be catalyzed with acid (Srivastava and Prasad, 2000; Meher et al., 2006; Freedman et al., 1986; Ma et al., 1998).

Biodiesel, also known as fatty acid alkyl esters, is named due to different reactants such as ethanol, methanol, propanol and butanol, which are used as starting alcohol in transesterification. Methanol and ethanol are quite prominent thanks to their common usage. Methanol FAMEs consists of methane generated from another finite mineral resource, natural gas. In certain applications, a different method is followed, fatty acid methyl ester, that is, methanol is utilized with alkali catalyzed method for the production of biodiesel. It costs a lot less. Ethanol has many advantages itself. It depends on agricultural resources, which means a more renewable and sustainable production process compared to the petroleum based methanol process. It is easier to handle ethanol thanks to its superior solvency properties. Moreover, triacylglycerides have a higher solubility in ethanol than methanol. In short ethanol is a better candidate for transesterification. However, its production cost is higher(Yusoff et al., 2014).

Transesterification process depends on many different variables such as reaction

time, temperature, molar ratio of alcohol to oil, type and amount of catalysts, reactant purity, free fatty acid and moisture. Reaction temperature plays an important part in the reaction rate. Although the reaction can be completed even at room temperature (this obviously takes a huge amount of time) preferred temperature is the boiling point of alcohol at atmospheric pressure (Meher et al., 2006; Çaylı and Küsefoğlu, 2008; Sharma et al., 2008; Srivastava and Prasad, 2000).

There are different types of catalysts used for transesterification. Alkali, acid, enzyme and heterogeneous catalysts are some of them. Sodium hydroxide, sodium methoxide, potassium hydroxide and potassium methoxide are considered to be more effective as it is already known that alkali catalyzed transesterification is faster than acid catalyzed reaction (Ma et al., 1998).

For enzymatic catalysts, such as lipases, it is not important if the system is aqueous or not. Such catalysts can be effective in both systems and overcome many problems. But when compared to alkaline catalysts, production cost of a lipase catalyst outweighs its benefits. Besides, alkaline catalysts are able to convert all the free fatty acids in waste oils and fats. During this reaction there can be byproducts as well. Glycerol is one of them and can easily be removed from the system (Al-Widyan and Al-Shyoukh, 2002; Meher et al., 2006).

1.2.4. Wax Ester Synthases for Microbial Production of Biodiesel

1.2.4.1. Definition of Wax Ester

Wax esters can be defined as high value neutral lipid compounds. They are also known as long chain fatty acids linked to long chain alcohols with an ester bond. They can be used for a wide range of purposes both in biological systems such as in some microorganisms protection against dehydration, UV light and pathogens, and industrial sectors such as biodiesel, cosmetics, medical formulations and food additives etc. (Jetter and Kunst, 2008; Samuels et al., 2008; Wältermann et al., 2005, 2007). They are widely found in organisms such as plants and animals, however, the same cannot be said for bacteria as only a small portion of bacteria can produce these esters as a potential carbon and en-

ergy storage. Studies shows that, polyhydroxyalkanoic acids, polyhydroxbutyrate, starch and glycogen are one of the most important forms of reduced carbon storage compounds whereas they have been some cases where bacteria also accumulate neutral lipids such as TAGs and wax ester (Ishige et al., 2003; Kalscheuer and Steinbüchel, 2003; Wältermann et al., 2005; Wältermann and Steinbüchel, 2005; Wältermann et al., 2007; Alvarez et al., 2008; Hernández et al., 2008; Stöveken and Steinbüchel, 2008; Manilla-Pérez et al., 2011; Barney et al., 2012). There are also known examples of wax esters serving a neutral lipid storage compounds such as jojoba or just like the triacylglyceride storages in oil crops (Jetter and Kunst, 2008; Metz et al., 2000; Voelker and Kinney, 2001). Additionally, wax esters can be found in the outer layer of the leaves acting as protection against water loss. Sperm whales also produce these wax esters especially in their spermaceti organ. They are believed to help the whale to stay afloat.

TAGs and WEs are mostly stored in the cytoplasm. Their shape and size can vary according to the lipid, strain and culture conditions (Kalscheuer et al., 2001; Wältermann and Steinbüchel, 2005). After whale hunting ban, the most widely used natural source for wax esters has been the jojoba oil. Liquid wax esters in jojoba plant contain carbon chain with the length of C38 to C44, which are mostly as follows; C20:1 fatty acid and C20:1 fatty alcohols (Miwa, 1971). As jojoba oil is quite expensive, its utilization is rather limited to medical and cosmetics only. Natural esters, owing to their unique features, can be used in cosmetics, lubricants, printing inks, candles and polishes. Wax esters with C20 carbons and C20 alcohols are essential in lubricant production. There is a certain variety of wax esters and they can be useful for different purposes. This depends on their chain length.

1.2.4.2. WAX ESTER SYNTHASES (WS)/DGAT

Although they are unrelated, it is known that three families of WS can be found in plants, mammals and certain bacteria. They can produce wax esters biosynthetically, through a process where activated fatty acids are reduced to fatty alcohols by reductases (Jetter and Kunst, 2008; Wältermann et al., 2005, 2007). It is also known as wax ester biosynthetic pathway (Hofvander et al., 2011; Lenneman et al., 2013; Reiser and Somerville, 1997; Wahlen et al., 2009; Willis et al., 2011). During the biosynthesis process of wax esters several enzymes take part. Wax ester synthase/acyl-coenzyme

A:diacylglycerol acyltransferase (WS/DGAT) (EC 2.3.1.75) is the bifunctional enzyme which catalyzes the esterification process of fatty acyl-coenzymes A (CoA) and fatty alcohols in biosynthetic pathway of bacterial wax ester. After the WS/DGAT acyltransferase reaction, as a second product free CoA is released (Fig. 1.4.). (Stöveken et al., 2005). WS/DGAT produces wax esters in bacteria from a certain range of fatty acyl-CoA pool, thanks to fatty acyl-CoA reductase and fatty aldehyde reductase (Stöveken et al., 2005; Reiser and Somerville, 1997; Wahlen et al., 2009; Willis et al., 2011; Manilla-Pérez et al., 2010; Barney et al., 2012).

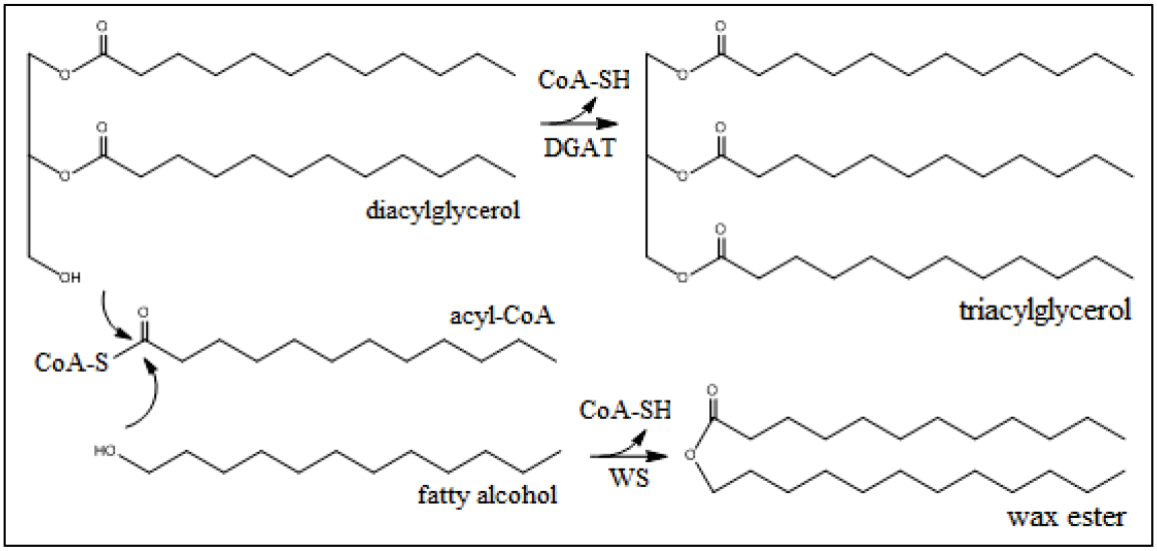


Figure 1.4. The wax ester synthase/acyl-coenzyme A:diacylglycerol acyltransferase enzyme (EC 2.3.1.75) is catalyst in fatty acid acyl-CoA and fatty alcohol esterification which yields free coenzyme A and wax ester. (Source: Barney et al., 2013)

WS/DGAT is the most essential enzyme in biosynthetic process of storage lipids in bacteria. It is the catalyst for the last steps in TAG and WE biosynthesis. It's described as an unspecific enzyme which catalyzes acyl-CoA-dependent acylation of diacylglycerols (DAGs) and fatty alcohols to triacylglycerol (TAGs) and wax ester (WEs). WS/DGAT shows no sequence homologies to any of the known acyltransferases taking part in biosynthesis of TAGs, WEs, steryl esters or phospholipids in eukaryote, that's why it is considered as a new class of acyl-CoA-dependent acyltransferase (Kalscheuer and Steinbüchel, 2003).

WS and DGAT enzymes are specifically membrane proteins. Unlike them, WS/DGAT is amphiphilic and an only electrostatic interaction holds them on the membrane (Stöveken et al., 2005; Lardizabal et al., 2000; Wältermann et al., 2005). Homologs specific to this protein have been found in certain bacteria, plants and mammals and these genes for WS/DGAT enzymes have been utilized by cloning and overexpressing for enzyme characterization. However, obvious regions of conservation can be seen in the primary amino acid sequence of WS/DGAT proteins. Despite these regions, the overall similarity between homologs of both different species and same species is rather low. This low similarity can bring up certain issues such as the differences occurring as a result of potentially affecting properties as substrate specificity (Barney et al., 2012).

It is stated that HHXXXDG motif can be found in many sequenced genomes of microbial strains which synthesize WEs and/or TAGS (Daniel et al., 2004; Stöveken et al., 2005; Holtzapple and Schmidt-Dannert, 2007) . Multiple sequence alignments have shown that highly conserved and condensed HHXXXDG domain is actively involved in catalytic center. This motif has also been located in almost all WS/DGAT homologues. It is estimated that this motif can activate the ester bond formation by catalyzing. But in most of the active acyltransferases it is either marginally modified or not modified at all. It is known that this motif has been located as the catalytic active site of nonribosomal peptide synthases as well (NRPS)(Stachelhaus et al., 1998; Bergendahl et al., 2002; Kalscheuer and Steinbüchel, 2003).

It is known that the first bacterial WS/DGAT was firstly found in *Acinetobacter calcoaceticus* ADP1 accumulating wax esters and TAGs as intracellular storage lipids. Furthermore, the first bacterial long chain acyltransferase (WS/DGAT) which is able to catalyze WE and TAG synthesis was found in 2003 in the gamma *proteobacterium Acinetobacter baylyi* ADP1 (used to be known as *A. calcoaceticus*) (Kalscheuer and Steinbüchel, 2003; Stöveken et al., 2005; Uthoff et al., 2005; Holtzapple and Schmidt-Dannert, 2007). In 2006, First experiment regarding biodiesel production in a living organism involves heterologous expression in *E.coli* by isolating pyruvate decarboxylase and alcohol dehydrogenase (ethanol generation pathway) from *Zymomonas mobilis* along with WS/DGAT (unspecific acyltransferase) from *Acinotobacter baylyi* strain ADP1FAEE concentration was around 1, 28 g l-1 and FAEE percentage was 26% of the dry mass of the cell thanks to fed batch fermentation with renewable carbon sources (Kalscheuer et al., 2006).

Although WS/DGAT from Acinobacter has been significantly focused, there are

other sources such as *Mycobacterium tuberculosis* 8798 (Lynn et al., 1972), *M. hydrocarbonoclasticus* DSM 8798 (Holtzapple and Schmidt-Dannert, 2007), *Rhodococcus opacus* PD630 (Alvarez et al., 2008), *Rhodococcus jostii* RHA1 (Hernández et al., 2008), *Marinobacter aquaeolei* VT8 (Wahlen et al., 2009), *Mus musculus* C57BL/6, *Psychrobacter arcticus* 273-4, *Psychrobacter cryohalolentis* K5 (Shi et al., 2012).

1.2.4.3. Substrate specificity of WS/DGAT

The hypothesis is that upstream of metabolic pathways producing fatty acyl-CoA and fatty alcohol substrates for the downstream WS/DGAT protein is the primary determinant of natural wax ester length and composition. This hypothesis can be supported by the different substrate specificities for fatty alcohol chain length in the different bacterium (Figure1.5.) (Barney et al., 2012).

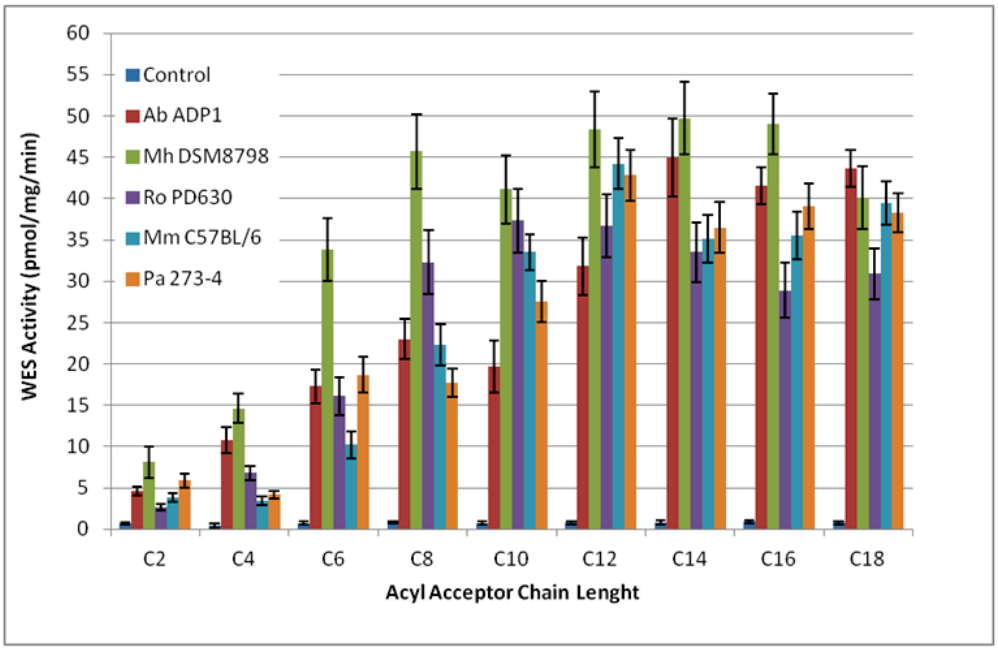


Figure 1.5. Substrate specificity profiles of WS/DGATs from various organisms towards linear alcohols with varying hydrocarbon chain lengths. (Source: Barney et al., 2012)

Specificity of WS/DGAT is one of the most noticeable feature which can be available for prodigiously wide range of substrates. It's been stated that, WSs are mostly prone

to interact with acyl-CoA groups possessing C14 to C18 carbon chains (Figure1.6.) and also with linear alcohols with carbon chains ranging between C12 to C18. But with short carbon chain alcohols the interaction activities are rather rare as seen Figure1.7. (Stöveken et al., 2005). It's been also found that WS/DGAT has a substantially broad substrate options and in some cases it even accepts monoacylglycerols (MAGs) as substrates (Uthoff et al., 2005).

WS/DGAT from *A.baylyi* ADP1 has many features, of which the most essential one is that it has a broad specificity towards acyl-CoAs of various chain lengths(Stöveken et al., 2005). Thanks to this feature, it is possible to use many different fatty acyl intermediates which can range from 2 to 20 carbon acyl chains with or without saturation for biodiesel synthesis (Figure 1.6.)(Stöveken et al., 2005). However, mid length chains are mostly preferred. It's also considered correct for the chain length of acyl acceptor alcohols which have a tendency towards alcohols with more than 2-carbon (Figure 1.7). This example can illustrate; It is 10 times less efficient for WS/DGAT from *A.baylyi* ADP1 to convert palmitoyl-CoA to ethyl palmitate (corresponding FAEE-based biodiesel) than to butyl palmitate (ester with 4- carbon alcohol) and 20 times less efficient to esters of alcohols with 14 or more carbons, which are nor suitable for biodiesel. Lately, studies have indicated that both WS/DGATs from *A.baylyi* ADP1 and other organisms such as bacteria and mammals (Figure 1. 6.)(Shi et al., 2012; Barney et al., 2012) disfavor ethanol as a co-substrate which is a huge disadvantage in terms of utilization in FAME- or FAEE-based biodiesel production process as short chain alcohols are preferred for more effective biosynthesis of biodiesel constituents.

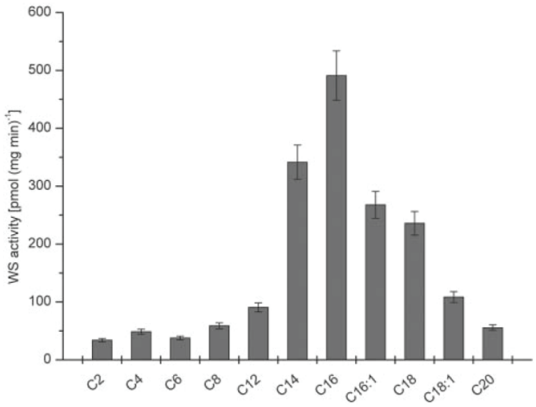


Figure 1.6. WS/DGAT specificity for different acyl-CoAs

(Source: Stöveken et al., 2005)

This broad substrate range for various acyl-CoA is an interesting feature but still WS/DGAT shows selectivity to a certain degree for specific substrates (Barney et al., 2012; Shi et al., 2012; Stöveken and Steinbüchel, 2008). It's also helped the popularity of WS/DGAT in esterification process including the production of an ethyl ester named "microdiesel" due to the fact that the fatty acid ethyl ester is directly produced by the microorganism (Kalscheuer et al., 2006). These bacterial wax esters are mostly solid at room temperature but as fuel, smaller esters are preferred thanks to their similarity to biodiesel (Kalscheuer et al., 2006; Rodriguez et al., 2014; Steen et al., 2010; Youngquist et al., 2013).

It is known that microdiesel production, if developed as desired, will be much more environmental friendly and the best alternative for methods based on transesterification. Considering the current methods require oil seeds, the biodiesel is a little area limited. However, using microorganisms will remove such restrictions and spread biodiesel availability worldwide. In other words, it is a sustainable and fulfilling method, should it be developed properly.

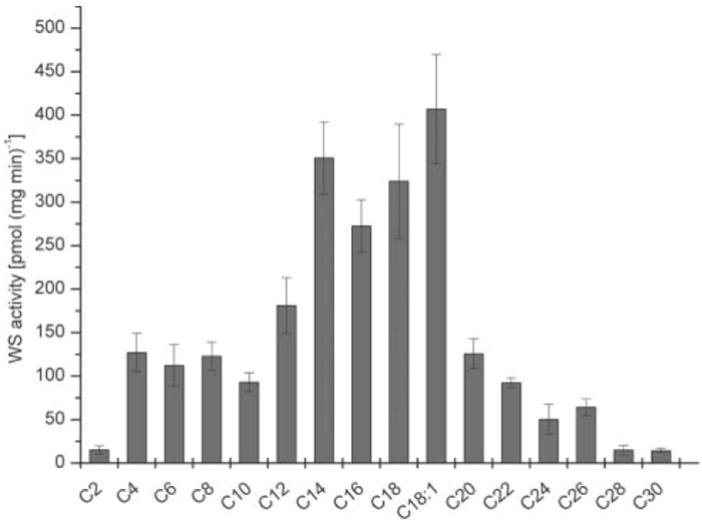


Figure 1.7. WS/DGAT Specifity for different linear alcohols

(Source: Stöveken et al., 2005)

1.2.4.4. Scope of This Study

This study focuses on wax ester synthases from *Marinobacter aquaeolei* VT8 and Rhodococcus opacus PD630. It is aimed to empower de novo biodiesel production by isolating essential enzymes catalyzing biodiesel formation. It is required for these isolation and purification processes to perform mechanistic and structural studies leading to elucidate the catalytic mechanism and eradicate the basic limitation for fatty acid ethyl ester (FAEE) production in microorganisms. Once the basic problem is solved, with the high substrate specificity of WS leaning towards ethanol, proper manipulations can take place in the active site therefore de novo biodiesel production can occur in larger amounts. This is much more advantageous than conventional biodiesel production. Besides, one can also be inspired to build a microbial cell factory thanks to using other organisms such as bacteria, yeast or algae. Considering their high photosynthesis efficiency and continuous oil reservations, algae can be the best candidate to meet biodiesel material demand. Should the microalgal systems be introduced into biodiesel or intermediate feedstocks in order to harvest solar energy, biosynthetic approaches have surpassed the desired amount to meet the global fuel needs without the necessity of large areas of cultivation of biodiesel crops.

CHAPTER 2

MATERIALS AND METHODS

2.1. Plasmid Constructs

E.coli XL1 Blue (Agilent Technologies) was used for all DNA amplification and construction. Plasmids pUC57Kan which contained the WS/DGAT template DNA from *Marinobacter aquaeolei* VT8 (MaWES) and *Rhodococcus opacus* PD630 (RoWES) with a C-terminal hexahistidine tag were synthesized by commercially (Genewiz Inc, USA). The DNAs were transformed to XL1 Blue and were streak out in the presence of 50μg/mL kanamycin at 37°C overnight for colony selection. Transformed *E.coli* cells were cultured in 25mL Luria-Bertani medium (LB) with 50μg/mL kanamycin at 37°C overnight. Afterwards, DNA was isolated with DNA isolation kit (MO BIO, USA). The genes were isolated from the commercial plasmid by NdeI and BamH1 restriction enzymes and controlled on the 1% agarose gel. Digested-inserts were ligated into pET 22bTV expression plasmid, a modified version of pET22b vector containing constitutively active T7 promoter by T4 DNA ligase (Thermo Scientific). Ligated DNAs were transformed to XL1 Blue and were streak out in the presence of 100μg/mL ampicillin at 37°C overnight for colony selection. Transformed *E.coli* cells were cultured in 50 mL LB with 100μg/mL ampicillin at 37°C overnight. DNA was isolated with DNA isolation kit (MO BIO) and sent to Izmir Institute of Technology, Biotechnology and Bioengineering Research and Application Center for sequence conformation that they contained no mistakes.

2.2. Gene Expression Tests

The WS/DGAT genes which encoding C-terminal His-tagged proteins in pET22bTV plasmid were then transformed to different type of *E.coli* cell lines which were used for heterologous expression test of WS/DGAT enzymes in Table 2. 1. In addition to that, culture medium (i.e., LB, TB), expression temperature (18°C, 20°C 25°C and 37°C) expres-

sion duration (5, 12 and 16 hours) and isopropyl-1-thio-ß-D-galactopyranoside (IPTG) (CarboSynth Inc., UK) concentration (0.25, 0.5, 1mM) were tested during expression test.

Table 2.1. List of strains used in this study and their genotypes

***E.coli* strains**	**Genotype**	**Source**
E.coli BL21 (DE3)	F^- *ompT hsdS*$_B$ (rB^-mB^-) *gal dcm* (DE3)	Invitrogen
E.coli BL21 (DE3) pLysS	F^- *ompT hsdS*$_B$ (rB^-mB^-) *gal dcm* (DE3) pLysS (Cam^R)	Invitrogen
One Shot® BL21 Star™ (DE3)	F^- *ompT hsdS*$_B$ (rB^-mB^-) *galdcmrne*131 (DE3)	Thermo Scientific
OverExpress™ C41(DE3)	F^- *ompT hsdS*$_B$ (rB^-mB^-) *gal dcm* (DE3)	Lucigen Corp.
Rosetta™ 2(DE3)pLysS	F^- *ompT hsdS*$_B$ (rB^-mB^-) *gal dcm* (DE3) pLysSRARE2 (Cam^R)	Merck Millipore Corp.
T7 Express *lysY/I*^{q} Competent *E. coli*	MiniF *lysY lacIq*(Cam^R) / *fhuA2 lacZ::T7 gene1 [lon] ompT gal sulA11 R(mcr-73::miniTn10--*Tet^S*)2 [dcm] R(zgb-210::Tn10--*Tet^S*) endA1 Δ(mcrC-mrr) 114::IS10*	New England Biolabs, Inc.
Lemo21(DE3) Competent *E. coli*	*fhuA2 [lon] ompT gal (λ* DE3*) [dcm] ΔhsdS/ pLemo(*Cam^R*) λ DE3 = λ sBamHIo ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5* pLemo = pACYC184-*PrhaBAD-lysY*	New England Biolabs, Inc

WS/DGAT transformed *E.coli* competent cells were streak out in the presence of 100μg/mL ampicillin at 37°C overnight for colony selection. Transformed *E.coli* cells were cultured in 5 mL LB with 100μg/mL ampicillin at 37°C overnight. During expression of WS/DGAT in the presence of GroEL/S chaperon, kanamycin antibiotic was used additionally. Following, 50 mL of selected media which contained same concentra-

tion of antibiotic was inoculated with 50μl of preculture at 37°C. At an optical density at 600nm of 1.1 in 1 over 5 dilutions, a protein expression was induced with 0.5mM isopropyl-1-thio-ß-D-galactopyranoside IPTG (CarboSynth Inc., UK) (after cooling on ice for 1min) .Before induction and 5, 12 and 16 hours after induction, 1 mL sample was taken from induced culture and harvested via centrifugation at 13,000 x g for 10 min in micro-centrifuge tube at 4°C. 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. Cell suspension was vortexed in cold room. After then, Lysozyme (0.25 mg/mL final concentration) (Bioshop Inc.) and DNase I (0.01 mg/mL final concentration) (Applichem) were added and rotated for 30 min in cold room. Cell disruption was carried out by bath sonication (Branson, USA) at 3 min in cold room. Before centrifugation, 50 μl disrupted sample (homogenate) were taken for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Cell debris was pelleted by centrifugation at 13000 g for 30min at 4°C. 50 μl of clear supernatant was taken a clean microcentifuge tube. 50 μl samples and 10 μl 6X Laemli's sample buffer were mixed and heated for 7 min at 95°C. Samples were loaded immediately after boiling to SDS-PA Gel. Coomassie Stain (Coomassie R250, 10% acetic acid, 40% methanol) was used for staining of gel. SDS-PA gel was destained via destaining solution (20% methanol, 10% acetic acid). Mentioned procedure used for all expression test conditions.

2.3. Heterologous Expression

Cultures (1L) of *E.coli* BL21 (DE3) which contains MaWES in pET22bTV and pET22bTV and pREP4-GroEL/S (Cole, 1996) plasmid were grown in Terrific- Broth (TB) and Luria-Bertani medium (LB) medium with supplemented with final concentration of ampicillin 100 μg/mL for GroEL/S expression additionally 50 μg/mL kanamycin added and 4 mL glycerol in 2L baffled flasks to at 37°C and continues shaking at 200 rpm. At an optical density at 600nm of 1.1 in 1 over 5 dilutions, a protein expression were induced with 0.5mM isopropyl-1-thio-ß-D-galactopyranoside IPTG (CarboSynth Inc.) at 18°C at 16 hours and continues shaking at 250 rpm. After expression all process were done at 4°C. Cells were then harvested via centrifugation (Beckman Coulter Avanti J-E) at 8,000 x g for 10 min in 450 mL bottles at 4°C. Per 1 L of culture roughly 15g cell

pellet were obtained. Thereafter, Frozen cell pellet was thawed on ice and cell pellet was suspended with Lysis Buffer (50 mM TRIS [pH8.0], 150 mM NaCI, 1mM DTT, 5% glycerol and 100 μM PMSF) two times the weight of the pellet at 4°C. Cell suspension was vortexed in cold room. After then, Lysozyme (0.25 mg/mL final concentration) (Bioshop Inc.) and DNase I (0.01 mg/mL final concentration) (Applichem) were added and rotated for 30 min in cold room. Cell disruption was carried out by sonication with Branson Ultrasonics Sonifier S-450 (Branson Inc.) using 5 cycles of 60 sec ON 60 sec OFF, 50% bursts, 60% power Cell debris was pelleted by ultracentrifugation at 40,000 x g for 1h at 4°C, twice.

2.4. Protein Purification

Attempts for heterologous expression of soluble protein was only successful for MaWES, hence the following purification steps were carried out only for MaWES. Fast protein liquid chromatography (FPLC) system was used for all purification process. After lysing cells as indicated above, the cleared lysate was filtered by 0.2-micron filter and was applied to 5 mL column (BioRad Inc, USA) packed with His60 Ni Superflow Resin (Clontech Laboratories, Inc.). Column was equilibrated with buffer E (50 mM TRIS [pH8.0], 150 mM NaCI, 5 mM ß-ME, 10% glycerol and 100 μM PMSF) at a flow rate of 1 mL/min using an ÄKTAprime plus FPLC system (GE Healthcare Bio-Sciences AB). After being washed with 70 mL of buffer E nonspecific proteins were removed with buffer E. Before elution of MaWES, column was washed by Elution buffer which contained 50 mM imidazole. The desired protein was eluted washing with elution buffer supplemented with 500mM imidazole (Buffer I) a linear gradient from 50 to 500mM at a flow rate of 1 mL min-1. Afterwards, fractions were selected, 50 μl fractions and 10 μl 6X Laemli's sample buffer were mixed and heated for 7 min at 95°C. Samples were loaded immediately after boiling to 12% SDS-PA Gel. Coomassie Stain (Coomassie R250, 10% acetic acid, 40% methanol) was used for staining of gel. SDS-PA gel was destained via destaining solution (20% methanol, 10% acetic acid). Mentioned procedure used for all collected fractions. The purified proteins were concentrated by using Vivaspin® 20 (Sarthorius Stedium Biotech.). WS/DGAT protein in Buffer I was exchanged with buffer see below Table 2.2 by using HiTrap desalting columns (GE Healthcare Bio-Sciences AB). After the buffer selection, purified enzyme was passed over a gel filtration/size exclusion chro-

matography column (HiLoad 16/600 Superdex 200 pg) equilibrated with buffer see below Table 2.2. at a flow rate 0.2 mL min-1. Fraction of desired protein were selected, combined, confirmed with 12% SDS-PAGE and concentrated to 20 mg mL-1. Nanodrop (Thermo Scientific) was used for determining the relative protein concentration of the combined fractions. After concentration, sample was flash frozen in liquid nitrogen and store -80°C for later analysis.

Table 2.2. Buffers of MaWES

Buffers	**Contents**
Buffer 1	50 mM PIPES [pH7.5], 150 mM NaCI, 1mM DTT, 20% glycerol, 100 µM PMSF
Buffer 2	50 mM TRIS [pH7.5], 150 mM NaCI, 1mM DTT, 20% glycerol, 100 µM PMSF
Buffer 3	50 mM HEPES [pH7.5], 150 mM NaCI, 1mM DTT, 10% glycerol, 100 µM PMSF

2.5. Crystallization trials for MaWES

Crystallization trials were carried out for MaWES protein using sitting drop vapor diffusion technique with 96-well plates (Hampton Research, USA) Following commercial crystallization screening kits were used for initial trials: Index Screen, Crystal Screen I-II, PEG/Ion Screen I-II, PEG/Ion Screen I-II, SaltRx Screen I-II (Hampton Research, USA), JBScreen JCSG++, JBScreen PACT++ 1- 4, JBScreen PACT++ 1- 4 (Jena Biosciences, Germany). For crystallization, 1 μL of protein solution of MaWES in buffer see below Table 2.2. was mixed with 1 μL of the mother liquor and the plate was sealed. Various buffer mixes and protein concentrations were tried during crystallization experiments as summarized in Table 2.3. Crystallization plates were checked for crystals under stereo light microscope just after setting up, after 24 hrs, 3 days, 1 week, 2 weeks, then monthly.

Table 2.3. Conditions of Crystallization Trials

Sample Name	Temperature	Screen Name	Chamber 1 (mg/mL)	Chamber 2 (mg/mL)	Chamber 3 (mg/mL)	Substrate	Buffer
2002/1	21°C	Hampton Index Screen	5	10	15		Buffer 1
2002/2	21°C	Hampton Crystal Screen I-II	4	8	10		Buffer 1
2002/3	21°C	Hampton PEGRx Screen I-II	5	10	15		Buffer 1
2002/4	21°C	Hampton PEG/Ion Screen I-II	5	10	15		Buffer 1
2002/5	21°C	Hampton SaltRx Screen I-II	5	10	15		Buffer 1
2002/6	21°C	JBScreen JCSG++	5	10	15		Buffer 1
2002/7	21°C	JBScreen Pentaerythritol 1-4	5	10	15		Buffer 1
2002/8	21°C	JBScreen PACT++ 1- 4	5	10	15		Buffer 1
2003/1	4°C	Hampton Index Screen	5	10	15		Buffer 1
2003/2	4°C	Hampton Crystal Screen I-II	5	10	15		Buffer 1
2003/3	4°C	Hampton PEGRx Screen I-II	5	7,5	15		Buffer 1
2003/4	4°C	Hampton SaltRx Screen I-II	5	7,5	15		Buffer 1
2003/5	4°C	Hampton PEG/Ion Screen I-II	5	7,5	15		Buffer 1
2003/6	4°C	JBScreen JCSG++	5	7,5	15		Buffer 1
2003/7	4°C	JBScreen Pentaerythritol 1-4	5	7,5	15		Buffer 1
2003/8	4°C	JBScreen PACT++ 1- 4	5	7,5	15		Buffer 1
2004/1	21°C	Hampton Index Screen	3,5	4,5	7		Buffer 1
2005/1	21°C	Hampton Index Screen	3,5	4,5	7	Palmitoyl Coenzyme A (2mM)	Buffer 1
2005/2	21°C	Hampton Index Screen	3,5	4,5	7	Palmitoyl Coenzyme A (2mM)	Buffer 1

(cont. on next page)

Table 2.3. (cont.)

Sample Name	Temperature	Screen Name	Chamber 1 (mg/mL)	Chamber 2 (mg/mL)	Chamber 3 (mg/mL)	Substrate	Buffer
2005/3	21°C	Hampton Index Screen	3,5	4,5	7	Palmitoyl Coenzyme A (2mM)	Buffer 1
2006/1	21°C	Hampton Index Screen	7,5	10	15	1-Hexanol + Lauroyl Coenzyme A	Buffer 2
2006/2	21°C	Hampton Index Screen	1,85	5,39	-	1-Hexanol + Lauroyl Coenzyme A	Buffer 1
2006-1	21°C	Hampton Index Screen	15	10	5		Buffer 2
2006-2	21°C	Hampton SaltRx Screen I-II	8	6	4		Buffer 2
2006/3	21°C	Hampton PEG/Ion Screen I-II	5	7,5	10	1-Hexanol + Lauroyl Coenzyme A	Buffer 2
2006/4	21°C	JBScreen JCSG++	4	6	8	1-Hexanol + Lauroyl Coenzyme A	Buffer 2
2006/5	21°C	Hampton Crystal Screen I-II	3	6	-	1-Hexanol + Lauroyl Coenzyme A	Buffer 2
2006/6	21°C	JBScreen Pentaerythritol 1-4	3	6	-	1-Hexanol + Lauroyl Coenzyme A	Buffer 2
2006/7	21°C	Hampton SaltRx Screen I-II	3	6	-	1-Hexanol + Lauroyl Coenzyme A	Buffer 2
2007/1	21°C	Hampton Index Screen	4	6	8		Buffer 1
2007/3	21°C	Hampton PEG/Ion Screen I-II	4	6	8		Buffer 1
2008/1	21°C	Hampton Index Screen	5 (1% benzyl alcohol)	7,5 (1% 1-Butanol)	10 (1% 2-Butanol)		Buffer 1
2008/2	21°C	Hampton SaltRx Screen I-II	5 (1% benzyl alcohol)	5 (1% 1-Butanol)	5 (1% 2-Butanol)		Buffer 1
2008/3	21°C	JBScreen JCSG++	4	6	8	2-Butanol	Buffer 1
2009/1	21°C	Hampton Index Screen	5	7,5	10	2-Butanol	Buffer 3
2009/2	21°C	Hampton SaltRx Screen I-II	5	7,5	10	2-Butanol	Buffer 3
2009/3	21°C	Hampton Crystal Screen I-II	5	7,5	10	2-Butanol	Buffer 3
2009/4	21°C	Hampton PEG/Ion Screen I-II	5	7,5	10	2-Butanol	Buffer 3

(cont. on next page)

Table 2.3. (cont.)

Sample Name	Temperature	Screen Name	Chamber 1 (mg/mL)	Chamber 2 (mg/mL)	Chamber 3 (mg/mL)	Substrate	Buffer
2009/5	21°C	Hampton PEGRx Screen I-II	5	7,5	10	2-Butanol	Buffer 3
2009/6	21°C	JBScreen JCSG++	5	7,5	10	2-Butanol	Buffer 3
2009/7	21°C	JBScreen Pentaerythritol 1-4	5	7,5	10	2-Butanol	Buffer 3
2009/8	21°C	JBScreen PACT++ 1- 4	5	7,5	10	2-Butanol	Buffer 3
2010/1	4°C	Hampton Index Screen	5	7,5	10	2-Butanol	Buffer 3
2010/2	4°C	Hampton SaltRx Screen I-II	5	7,5	10	2-Butanol	Buffer 3
2010/3	4°C	Hampton Crystal Screen I-II	5	7,5	10	2-Butanol	Buffer 3
2010/4	4°C	Hampton PEG/Ion Screen I-II	5	7,5	10	2-Butanol	Buffer 3
2010/5	4°C	Hampton PEGRx Screen I-II	5	7,5	10	2-Butanol	Buffer 3
2010/6	4°C	JBScreen JCSG++	5	7,5	10	2-Butanol	Buffer 3
2010/7	4°C	JBScreen Pentaerythritol 1-4	5	7,5	10	2-Butanol	Buffer 3
2010/8	4°C	JBScreen PACT++ 1- 4	5	7,5	10	2-Butanol	Buffer 3

CHAPTER 3

RESULTS

3.1. General Characteristics of MaWES and RoWES

The amino acid sequence of MaWES and RoWES genes is shown in Appendix A. According to the *E.coli* optimized sequence, some characteristics about MaWES and RoWES can be obtained from protein calculator tools. (http://protcalc.sourceforge.net/) Based on the amino acid sequence, MaWES had molecular weight of 52.44 kDa, estimated pI at 6.96 and extinction coefficient of 1.16 ((mg/mL)-1cm-1). Molecular weight of RoWES was 51.78 kDa. Estimated pI was nearly 8.76 and extinction coefficient was 1.03 ((mg/mL)-1cm-1).

3.2. Plasmid Construction

Two genes of WS/DGAT enzymes from two different bacteria were cloned with following procedure. The genes were obtained from the commercially synthesized pUC57 Kan plasmid by NdeI and BamHI restriction enzyme and the restricted vector band (2500 bp) and the desired insert band, MaWES (1374 bp) and RoWES (1395 bp) controlled on the 1% agarose gel and isolated (Figure 3.1.), then BamHI and NdeI restricted pET22bTV vector ligated with above mentioned restricted gene of WS/DGATs see Afterwards, MaW ES and RoWES genes firstly were confirmed with NdeI and BamH1 digestion (Figure 3.2.) and secondly send to sequence with T7 primers for conformation that it contained no mistakes.

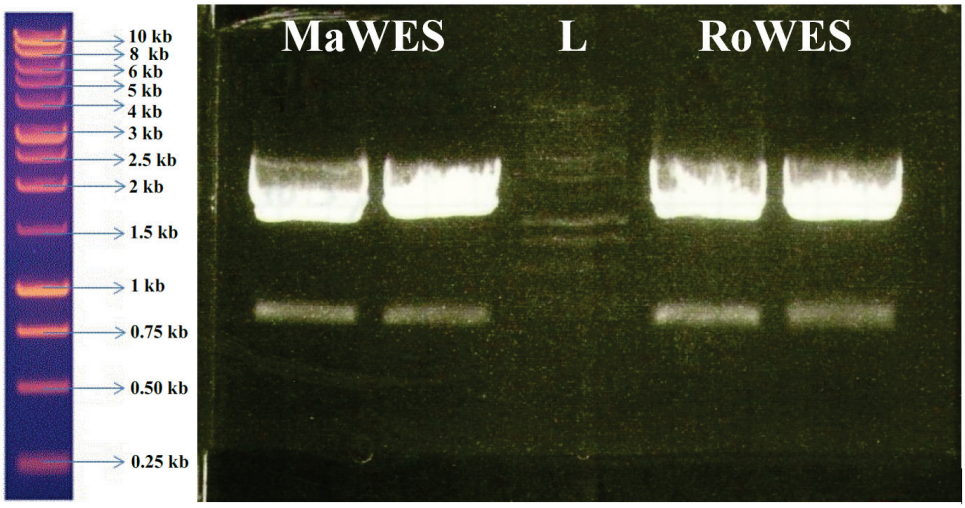


Figure 3.1. Agarose gel electrophoresis of pUC57Kan plasmid which contained MaWES and RoWES was restricted by using NdeI and BamHI. L is DNA ladder

3.3. Heterologous Expression

Heterologous expression of WS/DGAT from *M. aquaeolei* VT8 (MaWES) was obtained high level only in *E.coli* BL21 (DE3) among the tested cell lines (Figure3.9). Furthermore, MaWES was expressed with GroEL/S chaperon for increase protein folding and stability (Figure3.12.). Expression was done at an optical density at 600nm of 1.1 in 1 over 5 dilutions, a protein expression were induced with 0.5mM IPTG (CarboSynth Inc.) at 18 °C at 15 hours in TB medium and continues shaking at 200 rpm.

E.coli cell lines (BL21 DE3, pLysS, STAR, T7 Express LysI/Iq, Lemo 21) were used for heterologous expression of WS/DGAT from *R. opacus* PD630 (RoWES). RoWES was not obtained in a soluble form. Expressed protein became an inclusion body during expression and associated with cell debris during cell lysis and harvesting (Figure3.3., Figure3.4., Figure3.5., and Figure3.6.). Although GroEL/S chaperon was used for improving to solubility, RoWES protein was became an inclusion body in cell debris (Figure3.6. and Figure3.7.).

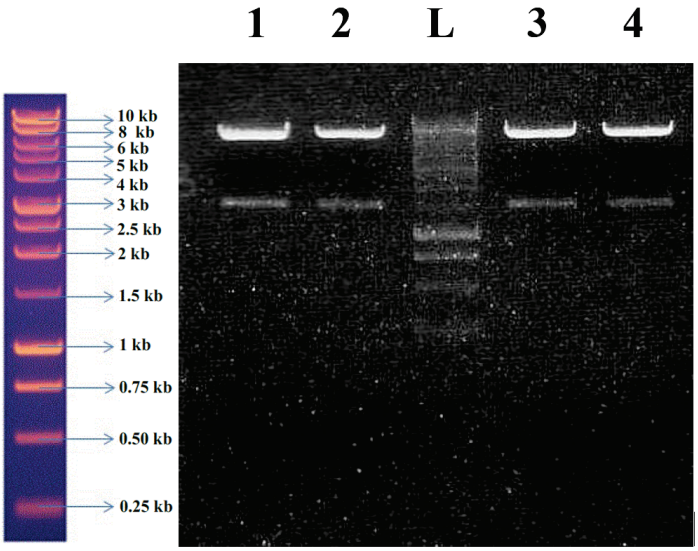


Figure 3.2. Agarose gel electrophoresis of ligation control of pET22bTV plasmid restricted with NdeI and BamHI. Lane 1 and 2 which are shown MaWES digestion and RoWES was represented by lane 3 and 4. L is DNA ladder.

3.4. Protein Purification

The MaWES was taken from the cleared lysate with a three-step purification which are IMAC, buffer exchange and size exclusion chromatography as described in detail in Materials and Methods. Afterwards, fractions from the different purification steps were selected, combined and confirmed with 12% SDS-PAGE. The results showed that apparent molecular mass of MaWES 50 kDa, which is the very near to theoretical molecular mass 52.44 kDa (Figure3.9. and Figure3.10.). A purification result of MaWES which was expressed with GroEL/S chaperon was showed in Figure3.11. and Figure3.12. After all purification steps, purified samples were concentrated via Vivaspin® 20 approximately 5 mL of 20mg/mL MaWES was obtained. Samples were flash frozen in liquid nitrogen and store -80°C for later analysis. That storage condition provides the stability of protein for many months stored in this manner.

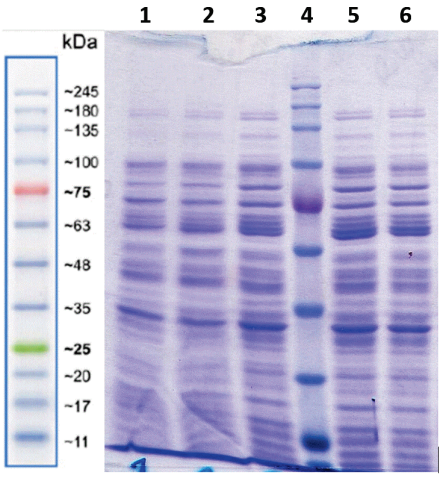


Figure 3.3. Expression of RoWES was performed using *E.coli* BL21 (DE3). Grown culture was induced by 1mM IPTG at 37°C. Lysate which is collected different hour was analyzed by SDS-PAGE. Lane 1; 0 hour, Lane 2; 1 hour, Lane 3; 3 hour, Lane 4; Protein standards, Lane 5; 4 hour, Lane 6; 5 hour.

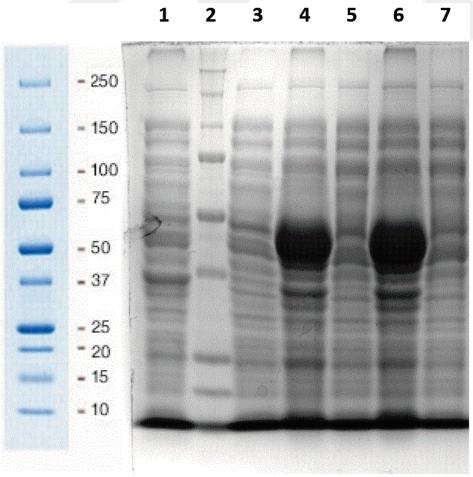


Figure 3.4. Expression of RoWES was performed using *E.coli* BL21 (DE3). Grown culture was induced by 1mM IPTG at 25°C. Lysates which is collected from different hour was analyzed by SDS-PAGE. Lane 1; 0 hour homogenate, Lane 2; protein standard, Lane 3; 0 hour lysate, Lane 4; 12 hour homogenate, Lane 5; 12 hour lysate, Lane 6; 16 hour homogenate, Lane 7; 16 hour lysate.

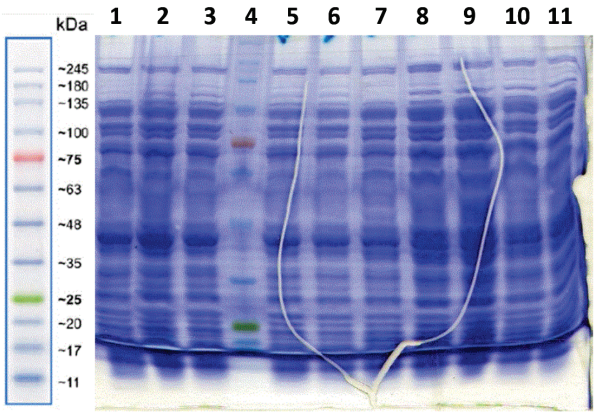


Figure 3.5. Expression of RoWES was performed using *E.coli* BL21 (DE3) and *E.coli* BL21 (DE3) pLysS. Grown culture was induced by 0.5 mM IPTG at 20°C. Lysates which is collected from different hour was analyzed by SDS-PAGE. Lane 1 to 6 was represented to lysate of expression test on *E.coli* BL21 (DE3) and Lane 7 to 11 was represented to lysate of expression test on *E.coli* BL21 (DE3) pLysS. Lane 1; 0 hour, Lane 2; 2 hour, Lane 3; 5 hour, Lane 4; protein standard, Lane 5; 10 hour, Lane 6; 16 hour, Lane 7; 0 hour, Lane 8; 2 hour, Lane 9; 5hour, Lane 10; 10 hour, Lane 11; 16 hour.

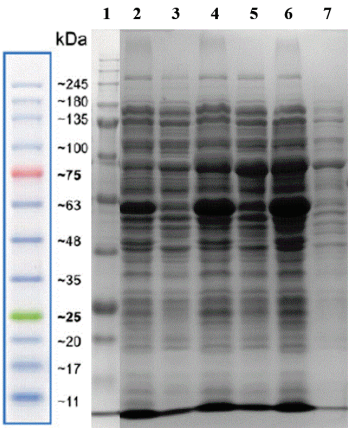


Figure 3.6. Expression of RoWES with GroEL/S was performed using *E.coli* BL21 (DE3). Grown culture was induced by 0.5 mM IPTG at 18°C. Homogenate and lysates which is collected from different hour was analyzed by SDS-PAGE. Lane 1; protein standard, Lane 2; 0 hour homogenate, Lane 3; 0 hour lysate, Lane 4; 10 hour homogenate, Lane 5; 10 hour lysate, Lane 6; 16 hour homogenate, Lane 7; 16 hour lysate.

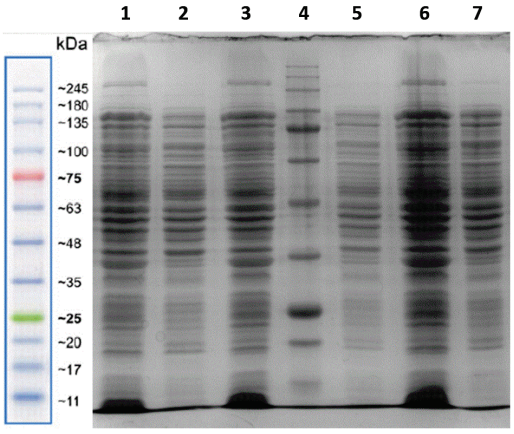


Figure 3.7. Expression of RoWES was performed using T7 express LysY/Iq. Grown culture was induced by 0.5 mM IPTG at 18°C. Lysates which is collected from different hour was analyzed by SDS-PAGE. Lane 1; 0 hour homogenate, Lane 2; 0 hour lysate, Lane 3; 12 hour homogenate, Lane 4; protein standard, Lane 5; 12 hour lysate, Lane 6; 16 hour homogenate, Lane 7; 16 hour lysate.

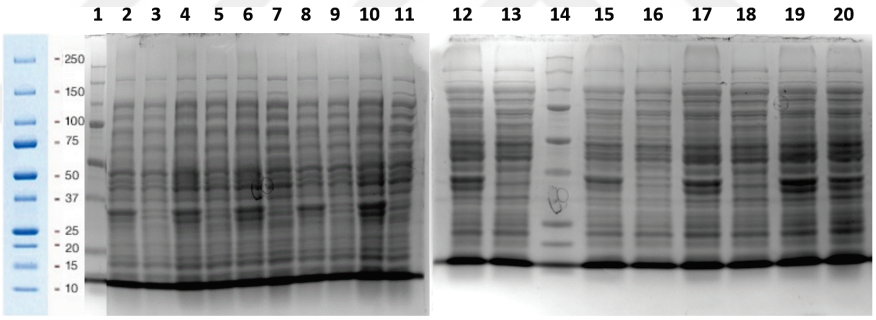
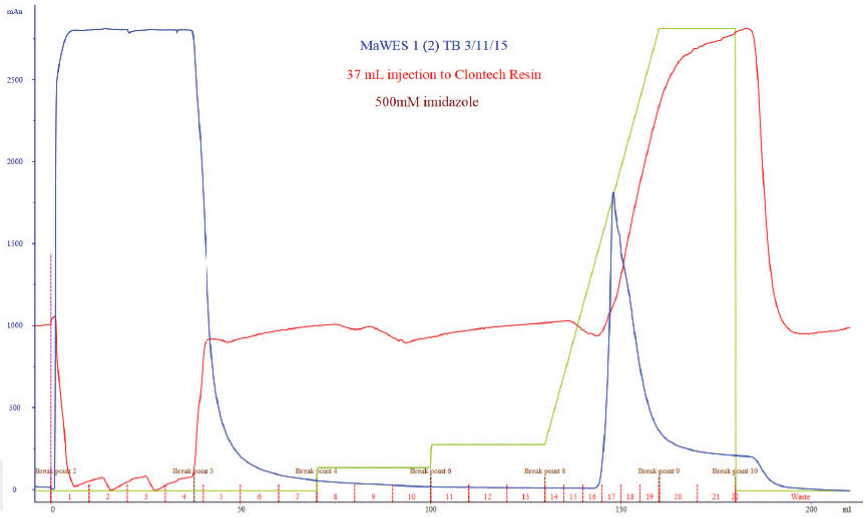


Figure 3.8. Expression of RoWES was performed using Lemo 21(DE3). Grown culture was induced by 0.5 mM IPTG and 500 μM, 750 μM and 1000 μM L-rhamnose at 25°C. Lysates which is collected from different hour was analyzed by SDS-PAGE. Lane 2 to 7 contain 500 μM L-rhamnose, 8 to 13 contain 750 μM L-rhamnose and 14 to 20 contain 1000 μM L-rhamnose. Lane 1; protein standard, Lane 2; 0 hour homogenate, Lane 3; 0 hour lysate, Lane 4; 12 hour homogenate, Lane 5; 12 hour lysate, Lane 6; 16 hour homogenate, Lane 7; 16 hour lysate, Lane 8; 0 hour homogenate, Lane 9; 0 hour lysate, Lane 10; 12 hour homogenate, Lane 11; 12 hour lysate, Lane 12; 16 hour homogenate, Lane 13; 16 hour lysate, Lane 14; protein standard, Lane 15; 0 hour homogenate, Lane 16; 0 hour lysate, Lane 17; 12 hour homogenate, Lane 18; 12 hour lysate, Lane 19; 16 hour homogenate and Lane 20; 16 hour lysate.



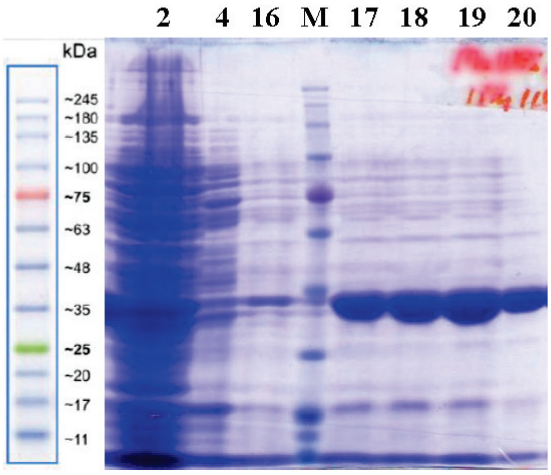


Figure 3.9. SDS-PAGE analysis of WS/DGAT from *M. aquaeolei* VT8 expressed in *E.coli* BL 21 (DE3) purification of Nickel Affinity Chromatography steps. Blue (–), red (–), green (–) lines depict absorbance at 280 nm, conductivity, and imidazole concentration, respectively. Lane 2,4,16,17,18,19 and 20 indicate fractions of chromatogram. M is molecular-weight protein marker (10 -245 kDa, AppliChem).

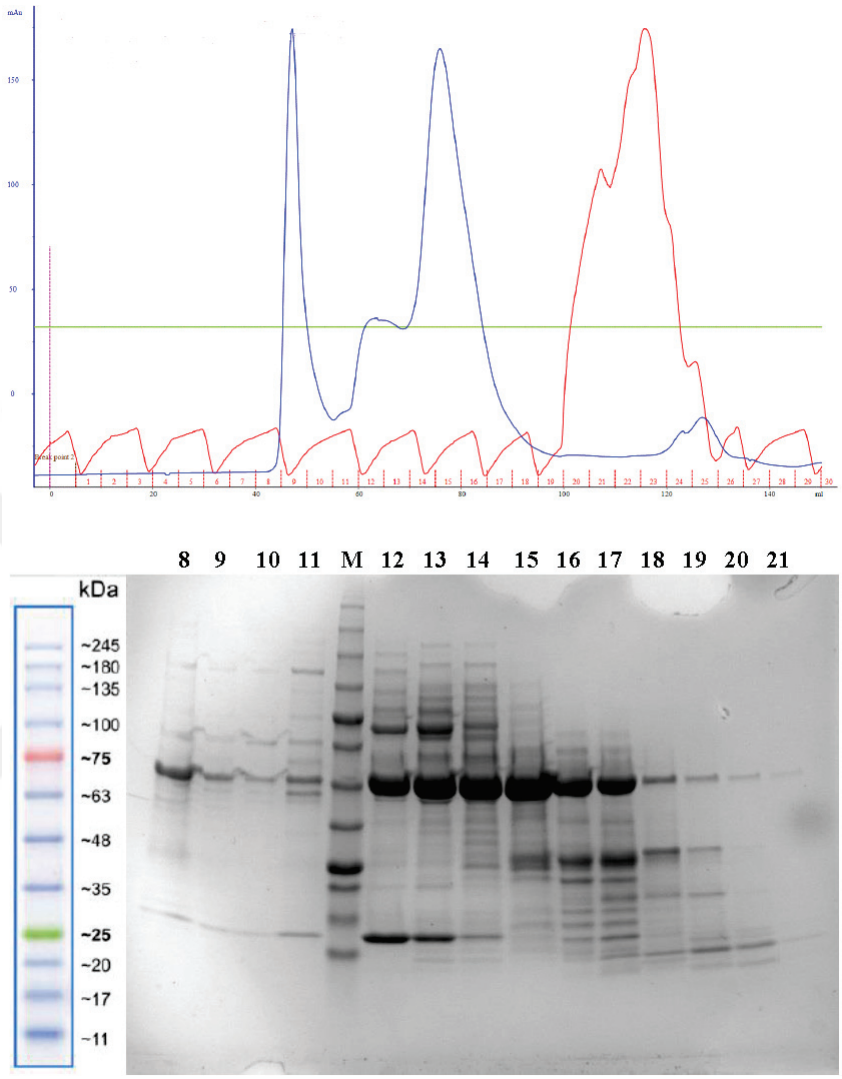


Figure 3.10. SDS-PAGE analysis of WS/DGAT from *M. aquaeolei* VT8 expressed in *E.coli* BL 21 (DE3) purification of Size Exclusion Chromatography steps. Blue (–), red (–), green (–) lines depict absorbance at 280 nm, conductivity, and salt concentration, respectively. Lane 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 and 21 indicate fractions of chromatogram. M is protein standards (10 -250 kDa, Bio-Rad All Blue).

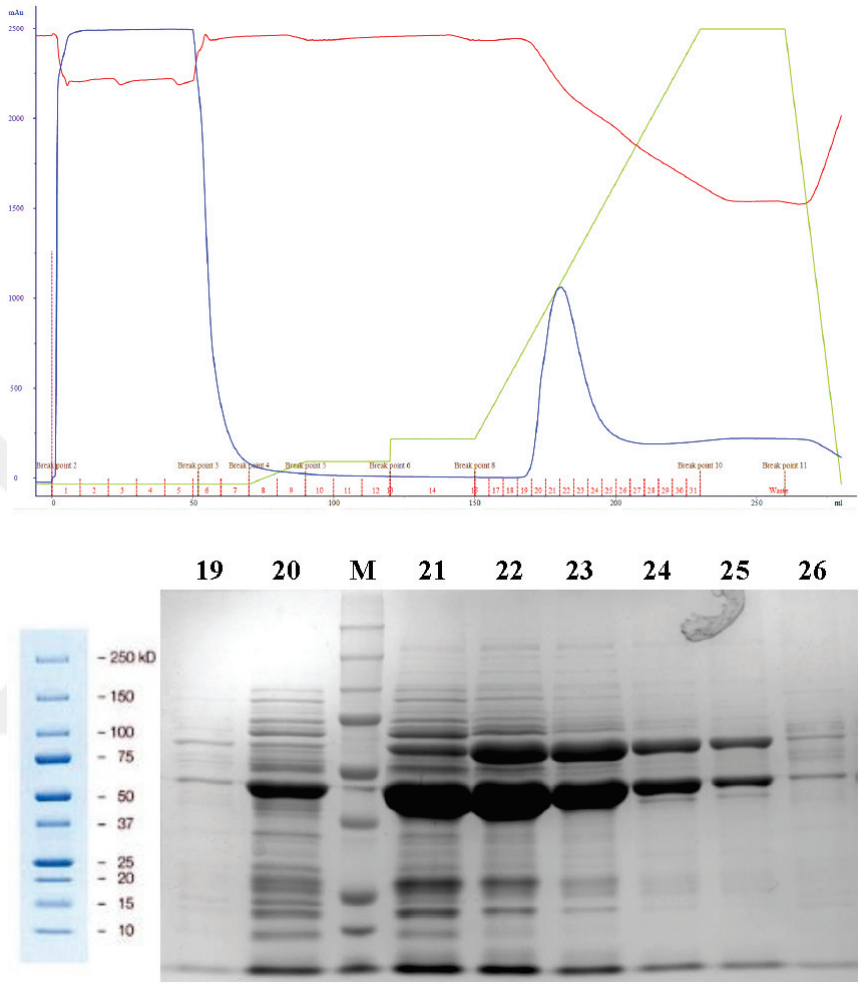


Figure 3.11. SDS-PAGE analysis of WS/DGAT from *M. aquaeolei* VT8 expressed with GroEL/S in *E.coli* BL 21 (DE3) was purified with Nickel Affinity Chromatography. Blue (–), red (–), green (–) lines depict absorbance at 280 nm, conductivity, and imidazole concentration, respectively. Lane 19, 20, 21, 22, 23, 24, 25 and 26 indicate fractions of chromatogram. M is protein standards (10 -250 kDa, Bio-Rad All Blue).

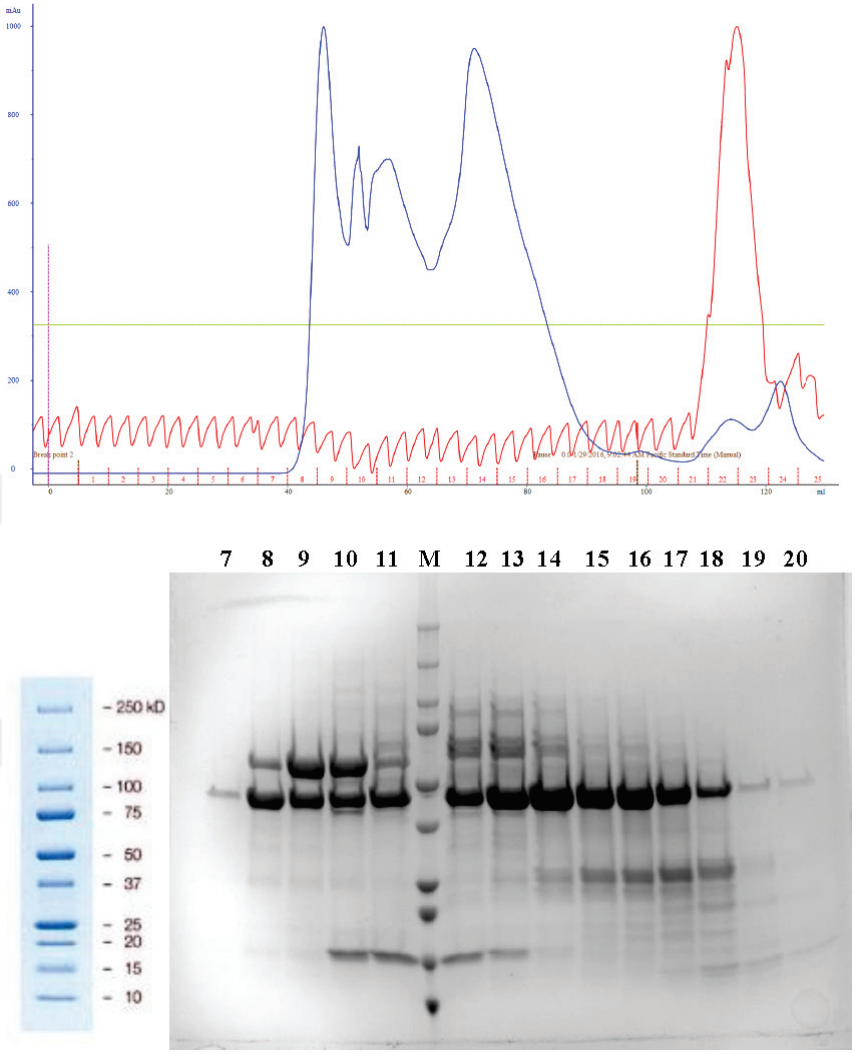


Figure 3.12. SDS-PAGE analysis of WS/DGAT from *M. aquaeolei* VT8 expressed with GroEL/S in *E.coli* BL 21 (DE3) was purified with gel filtration chromatography. Blue (–), red (–), green (–) lines depict absorbance at 280 nm, conductivity, and salt concentration, respectively. Lane 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 and 20 indicate fractions of chromatogram. M is protein standards (10 -250 kDa, Bio-Rad All Blue).

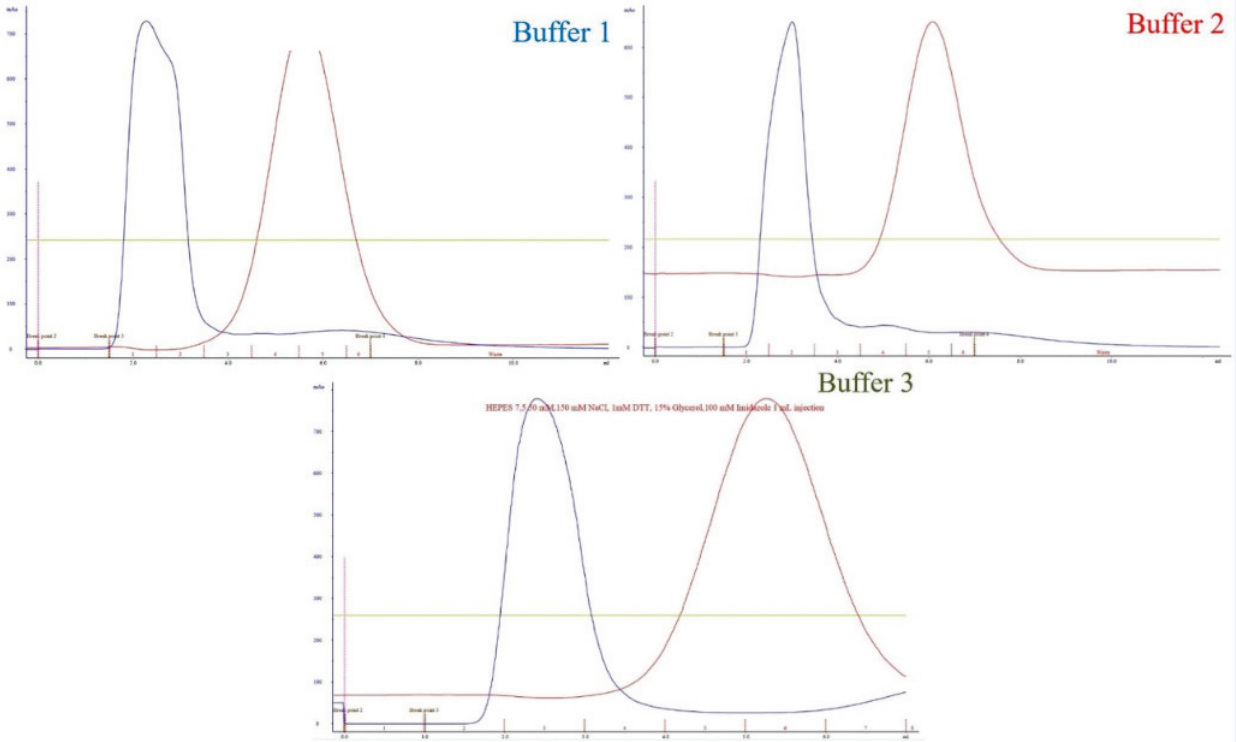


Figure 3.13. Buffer Exchange Chromatograms for MaWES buffer see bellow Table 2.2. Blue (–), red (–), green (–) lines depict absorbance at 280 nm, conductivity, and salt concentration, respectively.

3.5. Crystallization trials for MaWES

In crystallization trials, no crystals were detected in visual inspection of plates. Different types of protein precipitation were seen in crystallization plates (Figure14.).

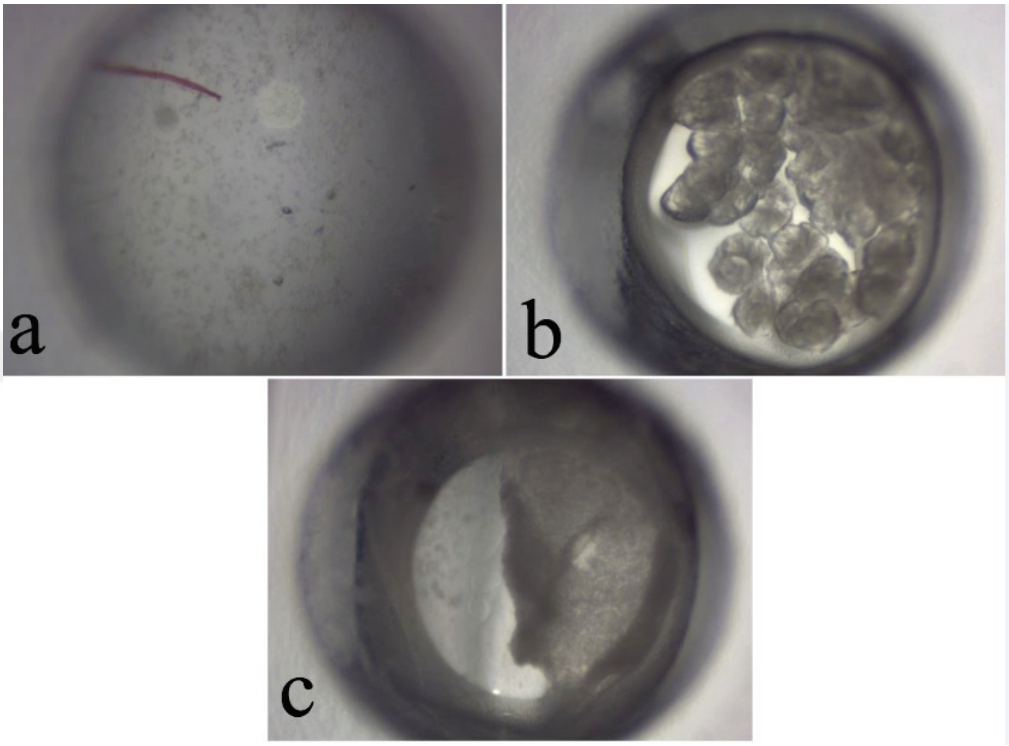


Figure 3.14. Protein precipitate in crystallization plates. a) Granular Precipitate, b) and c) Heavy (Amorphous) Precipitate

CHAPTER 4

CONCLUSION

In order to reduce our dependence on fossil fuels more and more attention has been channeled into renewable energy sources. Fatty acid (m)ethyl esters can be derived from plant oils and used in transportation sector as fuel, such as biodiesel. However, commercial production of biodiesel in a large scale is being disrupted due to limited availability of plant oils and high costs. Considering the crop yield it requires a large area to be allocated for such purposes. Thusly, researchers focus on different ways to overcome this obstacle. With the help of wax ester synthases (WS) introduced into the microbial hosts, such processes allowing ester production through microbial fermentation from traditional feed stock like sugar cane, wheat, corn or simply from biomass can be established.

In this project, two biosynthetic enzymes which have promising applications in the field of biodiesel 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.

Cloning the gene of was ester synthase from *M. aquaeolei* VT8 (MaWES) into pET22bTV expression plasmid was successfully completed. According to results from the expression test, heterologous expression of MaWES was carried out in *E.coli* BL21 (DE3) strain, at 18 °C with 0.5 mM IPTG concentration. Otherwise GroEL/S protein was expressed with MaWES in *E.coli* BL21 (DE3) strain, at 18°C with 0.5 mM IPTG to increase the solubility and folding ability. The desired protein was purified with yield 100 mg by using three chromatography systems; IMAC, buffer exchange and size exclusion chromatography. After size exclusion chromatography, the purified protein could not be obtained in the expected purity. The reason behind is thought that desired proteins during purification could also be bound to the column at the same level as the target protein. By using different chromatography systems such as ion exchange and hydrophobic interaction chromatography, it is aimed to increase the desired protein purity to a higher

level.

Cloning the gene of was ester synthase from *R. opacus* PD630 (RoWES) into pET22bTV expression plasmid was successfully carried out as well. Successful results were not obtained in the expression test studies for RoWES. The results of SDS-PAGE show that the protein has completed heterologous expression in *E.coli* strains. However, after lysis of cell the protein has formed an inclusion body in the cell debris. In order to overcome this problem, the expression with GroEL / S chaperone protein has been performed, yet the protein has formed an inclusion body. Recent studies have shown that protein solubility and purity percentage of proteins expressed in insoluble forms can be increased by using different protein tags.

This study will be the threshold for future studies focusing on structure guided protein engineering on WS/DGATs to manipulate their specificities towards acyl-CoAs or fatty alcohol substrates. Once structural studies are completed successfully, next step should be the mutational studies so that structure function relationships can be observed and evaluated and also WS/DGAT mutants catalyzing selective synthesis of desired esters can be obtained. The efficiency of biodiesel production through WS pathway process will be improved on the condition that short chain alcohols (i.e., methanol, ethanol) are used as co-substrates as they are favored by WS/DGATS. The efficiency of biodiesel production through WS pathway process will be improved on the condition that short chain alcohols (i.e., methanol, ethanol) are used as co-substrates as they are favored by WS/DGATS. This study will focus on different biochemical and biophysical features of WS/DGATs, namely; catalytic mechanism, stability, oligomerization state and surface features. This will allow a better adaptation of WS/DGATs in different industrial applications.

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APPENDIX A

AMINOACID SEQUENCES

The amino acid sequence of MaWES gene: MTPLNPTDQLFLWLEKRQQPMHVG GLQLFSFPEGAPDDYVAQLADQLRQKTEVTAPFNQRLSYRLGQPVWVEDEHLDLE HHFRF EALPTPGRIRELLSFVSAEHSHLMDRERPMWEVHLIEGLKDRQFALYTKVH HSLVDGVSAMRMATRMLSENPDEHGMPPIWDLPCLSRDRGESDGHSLWRSV THLL-GLSGRQLGTIPTVAKELLKTINQARKDPAYDSIFHAPRCMLNQKITG SRRFAAQSWCLK RI RAVCEAYGTTVNDVVTAMCAAALRTYLMNQDALPEKP LVAFVPVSLRRDDSSG-GNQVGVILASLHTDVQEAGERLLKIHHGMEEAKQR YRHMSPEEIVNYTALTLA-PAAFHLLTGLAPKWQTFNVVISNVPGPSRPLYW NGAKLEGMYPVSIDMDRLAL-NMTLTSYNDQVEFGLIGCRRTLPSLQRMLDY LEQGLAELELNAGLGSHHHHHH

The amino acid sequence of RoWES gene: MTQTDFMSWRMEEDPILRSTI-VAVALLDRRPDQSRFVDMMRRAVDLVPLFRRTAI EDPLGLAPPRWADDRDFDLSWHLR-RYTLA EPRTWDGVLDFARTAEMTAFDKRRPLWEFTILDGLNDGRSALVMKVHH-SLTDGVSG MQIAREIVDFTREGTPRPGRTDRATAVP HGGSSRPPSRLSWYRDTAAD-VTHRAANILGRNSVRLVRAPRATWREATALAGSTLRL TRPVVSTLSPVMTKRSTR-RHCAVIDVP VEALAQAAAAAAGSINDAFLAAVLLGMAKYHRLHGAEIRELRMTLPISL RTETDPL GGNRISLARFALPTDIDDPAELMRRVHA TVDAWRREPAIPFSPMIAGAVN-LLPASTLGNMLKHVDFVASNVAGSPVPLFIAGSEI LHYYAFSPTLGSAFNVTLMSYT-TQCCV GINADTDAVPDLATLTESLADGFRAVLGLCAKTTDTRVVVASGSHHHHHH

APPENDIX B

AMINOACID SEQUENCES

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

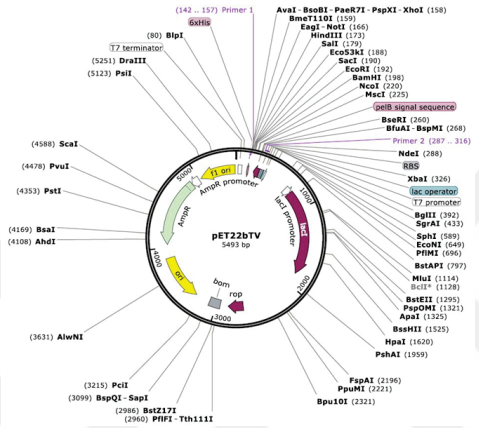

Figure B.1. pET22bTV vector map