ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

IDENTIFICATION AND CHARACTERIZATION OF MEIOTHERMUS RUBER THIOLASE AND CLOSTRIDIUM ACETOBUTYLICUM THIOLASE

M.Sc. THESIS

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Department of Chemistry

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

CLOSTRIDIUM ACETOBUTYLICUM TİYOLAZ VE MEIOTHERMUS RUBER TİYOLAZ ENZİMLERİNİN TANIMLANMASI VE KARAKTERİZASYONU

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JUNE 2013

Meltem KUTLU, a M.Sc. student of ITU Graduate School of Science Engineering and Technology, student ID 509111024, successfully defended the thesis entitled "IDENTIFICATION AND CHARACTERIZATION OF MEIOTHERMUS RUBER THIOLASE AND CLOSTRIDIUM ACETOBUTYLICUM THIOLASE", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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Date of Submission : 3 May 2013
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To my family,

FOREWORD

I would like to acknowledge my advisor, Prof. Dr. Oya Atıcı for giving me courage to go abroud, also for supporting me. Moreover, I would like to thank my co advisor, Prof. Dr. Thomas Brück for giving me a chance to work in his laboratory and for helping me everytime.
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(Chemist)

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ABBREVIATIONS

APS **:** Amonium persulfate
CAB **:** Clostridium Acetobutylicum
CAPS **:** 3-(Cyclohexylamino)-1-propanesulfonic acid
DNAse **:** Deoxyribonuclease I from bovine pancreas
DTNB **:** (5,5'-Dithio-bis(2-nitrobenzoic acid)
HEPES **:** (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
IPTG **:** Isopropyl-β-D-1-thiogalactopyranoside
MOPS **:** 3-(N-morpholino)propanesulfonic acid
MR **:** Meiothermus Ruber
SDS-PAGE **:** Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
TEMED **:** N,N,N',N'-Tetramethylethylenediamine
Tris **:** Tris(hydroxymethyl)aminomethane

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IDENTIFICATION AND CHARACTERIZATION OF MEIOTHERMUS RUBER THIOLASE AND CLOSTRIDIUM ACETOBUTYLICUM THIOLASE

SUMMARY

Butanol is an important platform molecule for the chemical, textile, polymer and biofuels industry due to its favourable physical properties such as increased hydrophobicity and energy density. At present, biotechnological butanol production is based on anaerobic fermentation processes with *Clostridia* species, which convert sugars into the solvents butanol, ethanol and acetone (ABE fermentation).
Thiolase (EC 2.3.1.9) is a key enzyme in the biosynthetic conversion of glucose to butanol, where it is responsible for the formation of carbon-carbon bonds by catalysing a thioester dependent Claisen-condensation. Specifically, thiolase is responsible for the condensation of two Acetyl-CoA molecules, thereby forming Acetoacetyl CoA, the first dedicated intermediate in cell based butanol synthesis. The well characterised *Clostridia* thiolases show low resistance to oxygen and are neither solvent nor thermostable, which limits butanol yields. We have isolated and characterised a thermo- and solvent stable Thiolase derived from the bacterium *Meiothermus ruber*. The Mr thiolase gene encompasses 12 kb encoding a protein of 397 amino acids with a deduced molecular weight of 42.3 kDa. Steady-state kinetic properties, thermo- and solvent stability of Mr thiolase were analysed via the DTNB (5,5'-dithiobis-(2-nitrobenzoic acid)) assay. During this assay the thiol moiety of the released CoA molecule cleaves the disulfide bond of DTNB to form one equivalent of a yellow colored TNB^{2-} dianion. The kinetic constant of Mr thiolase in the condensation direction is: $K_M = 4{,}8 \pm 0{,}3$ mM. The enzyme exhibits an pH optimum of 10.0, while the highest activity was measured at 50 °C. Even in the presence of 4 % butanol, Mr thiolase showed no significant decrease in activity. Therefore, the new Mr thiolase enzyme system shows superior catalytic and process parameters to previous characterised thiolase enzymes. Moreover, we have isolated and characterized Thl derived from *Clostridium Acetobutylicum* to compare Mr-Thl.

CLOSTRIDIUM ACETOBUTYLICUM TİYOLAZ VE MEIOTHERMUS RUBER TİYOLAZ ENZİMLERİNİN TANIMLANMASI VE KARAKTERİZASYONU

ÖZET

Butanol gerek kimya gerek tekstil sektöründe büyük öneme sahiptir. Bunun yanısıra özellikle son zamanlarda biyoyakıt endüstrisinde hidrofobik özellikleri ve enerji yoğunluğu bakımında büyük öneme sahip olmuştur. Biyoteknolojik butanol üretimi, Clostridia türlerinin anaerobik fermentasyon prosesine dayanmaktadır. ABE fermentasyonu olarak bilinen bu proseste şekerler butanol, etanol ve asetona çevrilmektedir.

Tiyolaz enzimi de glikozun butanola biyosentetik yöntemle çevrilmesi sırasında kullanılan anahtar enzimdir. Tiyolaz enzimi Claisen kondenzasyonuna dayanarak karbon-karbon bağlarının tiyoesterin katalizlenerek oluşturulmasından sorumludur. Spesifik olarak, tiyolaz asetil-koa molekülünün kondenzasyonundan sorumludur. Bunun sonucunda asetoasetil-koa molekülü meydana gelmektedir.

Clostridia tiyolazlar oksijene karşı düşük direnç gösterirler ve ne çözelti ne de sıcaklık stabillikleri söz konusudur. Bu durumda butanol verimini düşürmektedirler. Bu çalışmamızda, hem çözelti hem de sıcaklık stabilliği gösteren bir enzim olan *Meiothermus Ruber*'dan tiyolaz enzimi izole edildi. Bu enzimin özellikleri daha önce üzerinde araştırmalar yapılmış *Clostridium Acetobutylicum* tiyolazı ile karşılaştırıldı.

İlk olarak ekspresyon sistemi ile hücrelerin proteini yüksek miktarda üretmesi sağlandı ve bir dizi aşamadan sonra enzim saflaştırıldı. Saflaştırma işlemi için ise adsorbsiyon temeline dayanan afinite kromatografisinin çeşitlerinden biri olan metal şelat afinite kromatografisini (IMAC) kullanıldı. Afinite kromatografisinin temeli kolon içerisinde yer alan sabit faza, ligandın kovalent olarak immobilize olmasına dayanır. Bu sayede saflaştırılmak istenen molekülü içeren karışım kolondan geçirildiğinde istenen molekül ligand tarafındna tutulur, safsızlıklar ise tampon çözelti ile kolondan ayrılır. Kolonda liganda bağlı olan molekül ise elüsyon çözeltisi ile kolondan ayrılır. Metal şelat afinite kromatografisinde ise Zn (II), Cu (II), Ni (II), Co (II) gibi geçiş metallerinin sistein, histidin ve triptofana karşı afinitesi önem taşımaktadır. Çünkü proteinlerin özellikle sistein, histidin ve triptofan gibi kısımları kolonun metal iyon koordinasyon alanlarına bağlanmasına yardımcı olur. Çalışmamızda ise bu temele dayanan, Ni afinite kromatografisi kullanıldı. Bağlayıcı tampon çözelti olarak 20 mM imidazol, %10 gliserin ve %0,1 tween20 içeren 50 mM HEPES kullanıldı. Elüsyon çözeltisi olarak ise 500 mM imidazol, %10 gliserin ve %0,1 tween20 içeren 50 mM HEPES kullanıldı. Fraksiyonlar SDS-PAGE kullanılarak analiz edildi. En yoğun enzim içeren fraksiyonlar toplanıp, osmatik membran içerisine konularak diyaliz işlemi uygulandı. Diyaliz işlemi, saflaştırma sırasında ve önceden meydana gelen küçük moleküllü safsızlıkların uzaklaştırılması için gerçekleştirildi.

Saflaştırma işleminin ardından tiyolaz aktivitesine bakıldı. 0,1 mM DTNB, 0,2 mM asetil-KoA, 50 mM HEPES (pH8.0/50°C) kullanılarak master mix hazırlandı ve bu karışım 50°C'de 10 dk boyunca inkübe edildi. Isıtılmış karışımdan 180 µl alınıp microplate'e konuldu ve üzerine 20 µl tiyolaz enzimi ilave edilerek reaksiyon 50°C'de başlatıldı. Bu işlemler ayrı ayrı hem Cab-tiyolaz için hem de Mr-tiyolaz için yapıldı. Reaksiyonda asetil-KoA'nın asetoasetil-KoA'ya dönüşümü sırasında açığa çıkan KoA molekülünün renksiz DTNB'nin disülfit bağlarının ayrılmasını ve sarı renkli TNB^{2-} dianyonunun ortaya çıkmasını sağladığı gözlemlendi. Reaksiyondaki bu değişim spektrofotometrik olarak kolaylıkla gözlemlendi. Elde edilen grafikler ve yapılan hesaplamalar sonucunda Mr-tiyolazın aktivitesi 0,6068 U/mg, Cab-tiyolazın aktivitesi ise 0,9239 U/mg olarak bulundu.

Enzim aktivitesi belirlendikten sonra enzim karakterizasyonu için denemeler yapıldı. pH 6.0-11.0 aralığında farklı tampon çözeltiler kullanılarak ölçümler alındı. Hem Cab- hem de Mr-tiyolazın optimum pH'ı 10.0 olarak bulundu. Tiyolaz enzimi üzerinde sıcaklığın etkisini görmek için 20°C'den 65°C'ye kadar farklı sıcaklıklarda ölçümler alındı ve Mr-tiyolaz için optimum sıcaklık 50°C ve Cab-tiyolaz için optimum sıcaklık 60°C olarak bulundu. Termostabilite testi için 40, 50 ve 60°C sıcaklıklarda 150 saate kadar ölçümler alındı ve hesaplamalar sonucunda her iki enzim için de 150 saat sonra bile enzim aktivitesi gözlendi.

Enzimin etanol ve butanol stabilitesi, tiyolaz enziminin etanol-butanol üretimlerinde görev almasından kaynaklı büyük önem taşımaktaydı, bu yüzden enzime %5, 10, 15 ve 20 oranlarında olmak üzere ethanol ilave edilerek 50°C'de ısıtılıp reaksiyon plate readerda okunup, ölçümler alındı. Hem Mr- hem de Cab- tiyolaz aktivitesinde azalma gözlemlendi ancak azalma oranının çok düşük olduğu görüldü. Ayrıca enzime %2, 4, 6, 8 ve 10 olmak üzere butanol ilave edilip, önceki koşullarla aynı ortamda reaksiyon gerçekleştirildi. Bu işlemden elde edilen sonuçlarda ise butanol ortamında Mr-tiyolaz aktivitesinde belirgin düşüş gözlenirken, Cab-tiyolazda o kadar büyük bir düşüş gözlenmedi.

Solvent stabilitesine bakmak için ise reaksiyon karışımına DMSO (%5, 10, 15) ilave edilerek analizler yapıldı. DMSO miktarı arttıkça Mr-tiyolazın aktivitesinin de arttığı diğer taraftan Cab-tiyolaz aktivitesinin de azaldığı görüldü.

Son olarak kinetik ve inhibisyon çalışmaları yapıldı. Değişen asetil-KoA konsantrasyonlarında tiyolaz aktivitesi ölçüldü. Elde edilen kinetik grafiğinin eğiminden ve denkleminden yararlanılarak kinetik sabitlere ulaşıldı. Mr-tiyolaz için kinetik sabit (K_M) 4,82 mM, Cab-tiyolaz içinse bu değer 3.07 mM olarak bulundu. Diğer taraftan V_{max} değerleri Mr-tiyolaz için 13,089 µmol min^{-1} mg^{-1} olarak Cab-tiyolaz için ise 11,806 µmol min^{-1} mg^{-1} olarak bulundu. NAD^+'ın inhibisyon etkisi görüldükten sonra değişen oranlarda NAD^+ kullanılarak da inhibisyon testleri yapıldı. Bu testler sonucunda elde edilen grafiklerden Mr-tiyolazın yarışmalı inhibisyon gösterdiği bulundu.

Sonuç olarak; Mr-tiyolazın aktivitesi Cab-tiyolazın aktivitesinden düşük olarak bulundu. Hem Mr- hem de Cab-tiyolaz için optimum pH'ın aynı olduğu gözlendi. Mr-tiyolazın optimum sıcaklığının Cab-tiyolazın optimum sıcaklığından düşük olduğu görüldü. Diğer taraftan Mr-tiyolazın termostabil olduğu kanıtlandı.

Etanol ve butanol varlığında, tiyolaz enziminin nasıl davranacağının taşıdığı önem bakımından yapılan deneylerde etanol varlığında Mr-tiyolazın Cab-tiyolaza göre daha stabil olduğu gözlenmesine rağmen butanol için tam tersi görüldü. Diğer bir açıdan, etanol için her iki enzimde de enzim aktivitesinde çok büyük düşüşler gözlenmedi.

Ancak butanolde özellikle Mr-tiyolazda %4 butanolden sonra enzim aktivitesinde yarıdan fazla düşüş gözlendi. Her şeye rağmen %4 butanol ilave edildiğinde enzim aktivitesinin hemen hemen sabit olması yararlı bulundu. Bu yüzden 120 saat boyunca belirli aralıklarla %4 butanol içeren karışımla yapılan ölçümlerde Mr-tiyolazın aktivitesinde ani düşüşler gözlenmedi. Bunun sonucunda da butanol üretimi sırasında ortamda %4 oranına kadar bulunan butanolün reaksiyon verimini çok fazla etkilemeyeceği görülmüş oldu.

DMSO varlığında Mr-tiyolazın aktivitesinin Cab-tiyolaza göre arttığı görüldü.

Cab-tiyolazın K_M ve V_{max} değerlerinin düşük olmasından dolayı Mr-tiyolazın substratları Cab-tiyolaza göre daha zayıf bağladığı bulundu. Son olarak Mr-tiyolazın Cab-tiyolaz gibi yarışmalı inhibisyon gösterdiği bulunmuş oldu.

Özetle, Mr-tiyolazın termostabil, solvent stabil olduğunu ayrıca Meiothermus Ruber'ın aerobic bir bakteri olmasından dolayı da Mr-tiyolazın oksijene dayanıklı olduğunu söyleyebiliriz. Ayrıca Cab-tiyolazın da daha önceki yapılan araştırmalardan farklı özellikler gösterdiği ve aktivitesinin, optimum pH'ının, termostabil özelliğinin daha iyi sonuçlar verdiği görüldü.

1. INTRODUCTION

1.1 Purpose of Thesis

In recent years, there has been an interest for the production of chemicals and fuels from renewable resources. Reasons for this trend includes growing concerns about global warming and climatic change. Depleting petroleum resources, fluctations in fuel prices, the harmful effects of the toxic and greenhouse gases generated by burning of petroleum have resulted in seeking an alternative fuel (Zheng et al., 2009; Garcia et al., 2011). One of them is butanol because energy density closer to gasoline, suitable for cars without modifying on a car, and reducing the emissions. Not only the butanol is used as fuel but also it is used as a solvent, in cosmetics, hydraulic fluids, detergent formulations, drugs, antibiotics, hormones and vitamins, as a chemical intermediate in the production of butyl acrylate and methacrylate.

Butanol which has a significant effect on industry is produced by oxo process, aldol process, ABE (acetone-butanol-ethanol) fermentation (Ezeji et al., 2007). Oxo process means reaction of propylene with carbon monoxide and hydrogen in the presence of an appropiate catalyst. Aldol process starts from acetaldehyde. In ABE fermentation, it is possible to convert plants to use cheaper cellulosic feedstocks (Green, 2011). On the other hand, in our research group, a new free cell pathway for butanol synthesis that is similar to solventogenic pathway have been worked, especially in my thesis, thiolase enzyme to convert two acetyl-CoA to acetoacetyl-CoA on butanol synthesis was studied (Figure 1.1). In early studies, especially thiolase derived from *Clostridium* species have been investigated. Particularly, *Clostridium acetobutylicum* which is anaerobic, mesophilic bacterium was worked for butanol synthesis. Therefore, thiolase from *Meiothermus Ruber* which is aerobic and thermophilic bacterium was researched in order to find better thiolase features such as, thermostable, solvent stable and high resistance to oxygen for used in butanol synthesis in my study. Also, both *Meiothermus ruber* thiolase and *Clostridium acetobutylicum* thiolase was characterized and they were compared.

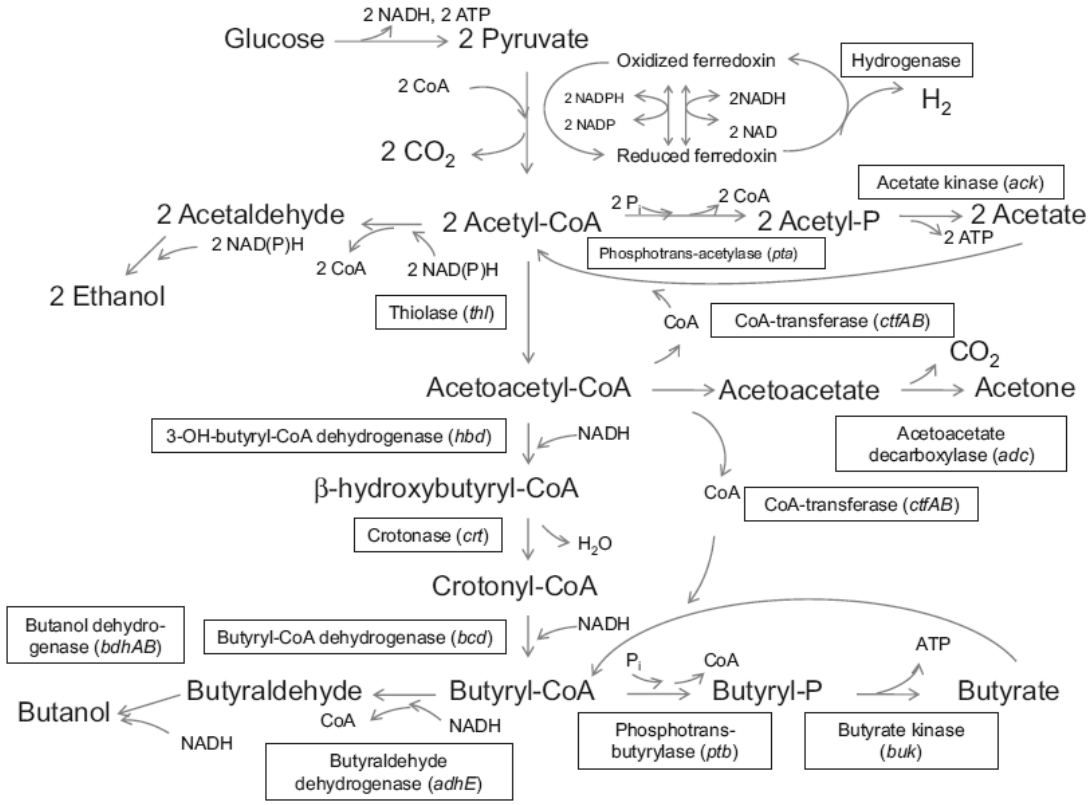

Figure 1.1: Acidogenic and solventogenic metabolic pathways in clostridia (Garcia et al., 2010).

1.2 Enzymes and Literature Review for Thiolase

Enzymes control the rate of biological reactions (Wilcox, 2010; Boyer, 2012). Most of the the biochemical reactions would not proceed at the required rate without enzymes. Enzymes have several factors, e.g. their extraordinary catalytic powers, and their selectivity.

In figure 1.2, reactants are converted to products. The activation energy of the reaction without catalyst is greater than the activation energy of the reaction with catalyst. This means catalyst (enzyme) lowers the activation energy required to initiate the reaction and makes the reaction easier. It must be known that enzyme does not change the energies of the original reactant and products, just changes the activation energy.

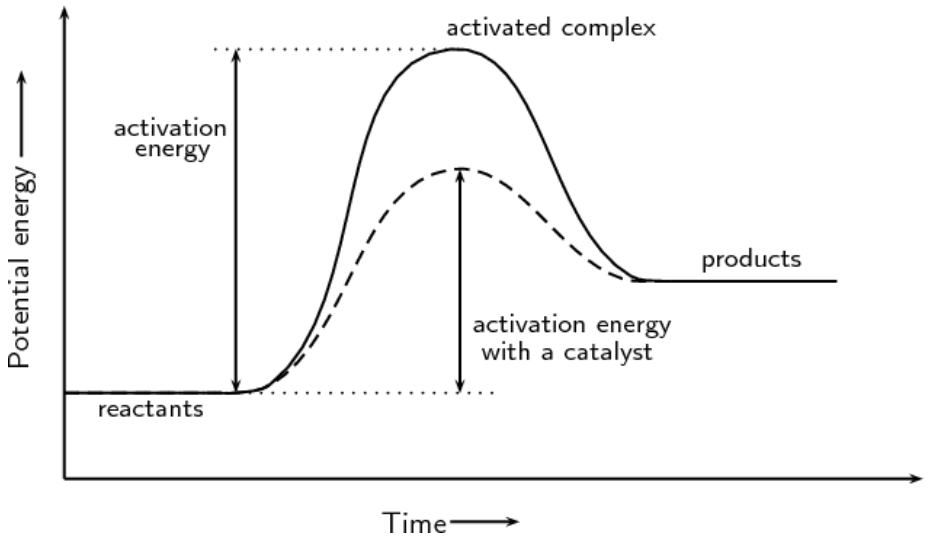

Figure 1.2: The effect of a catalyst on the activation energy of the chemical reaction (Url-1).

In enzyme catalyzed reactions, substrates bind to active sites of enzymes. There is a very significiant point in this step that the substrate must have a size, shape and polarity compatible with the active site of the enzyme. After that, product occurs, followed by the release of unmodified enzymes, as shown in equation (1.1).

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E + P \qquad (1.1)$$

E represents the enzyme, S the substrate, P the product and k_1, k_2, k_3, k_4 rate constants.

A lot of enzymes have been found out and isolated, confusion would influenced without some system for nomenclature and classification. In 1961, the first Enzyme Commission said that enzymes were separated into six main categories by means of the type of catalyzed reaction (Gerhartz, 1990; Grogan, 2009). They were described by using E.C. numbers including four elements (E.C. X.X.X.X). The first number indicates the number of the representative class of six which the enzyme belongs. The next two numbers indicate subclasses, e.g. in terms of the type of cofactor. The fourth is the number of the enzyme in its subclasses. The important six classes are oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases.

Oxidoreductases, encompasses all enzymes that catalyze oxidation and reduction reactions, for example, hydroxylation reactions, and the oxidation of amines. For

instance, lactate dehydrogenase (E.C. 1.1.1.27) catalyses the interconversion of lactic acid and pyruvic acid.

Transferases catalyzes specific group transfer reactions. For example, flavonol-3-*O*-glucosyltransferase (E.C. 2.4.1.91) transfers glucose from the activated sugar donor UDP-glucose to flavonoid acceptor substrate.

Hydrolases are enzymes that catalyse the hydrolotic cleavage of C-C, C-O, C-N, and some other bonds. For example, epoxide hydrolase enzymes (E.C.3.3.2.X) perform the ring opening of epoxides to form vicinal diols.

Lyases catalyze the addition of water or ammonia across double bonds. For example, phenylalanine ammonia lyase (E.C. 4.3.1.5) catalyzes the reversible addition of ammonia to a cinamic acid derived substrate.

Isomerases are enzymes that geometric or structural rearrangements within in a molecule, such as hexose pentose isomerisations. Mandelate racemase (E.C. 5.1.2.2) provide reacemisation of mandelic acid.

The last class is ligases that catalyze joining of two molecules, coupled with the hydrolisis of a pyrophosphate bond in ATP or another nucleoside triphosphate, such as aspartate-ammonia ligase (E.C. 6.3.1.1) catalyzes the ATP-dependent reaction of ammonia with L-aspartate to form L-asparagine.

The second class, transferases, have an importance on our study because this class includes thiolase enzyme. The information about thiolase was collected from several research.

In an early study, B-ketothiolase which was a key enzyme in poly-β-hydroxybutyrate metabolism was purified from fructose-grown cells of *Hydrogenomonas eutropha* H16 by purification method including cetyltrimethylammonium, DEAE-cellulose chromatography and exclusion chromatography on Sephadex G-200 (Oeding and Schlegel, 1972). The molecular weight was 147 000 – 150 000. The authors worked its kinetics and potential effectors for both condensation reaction and cleavage reaction (thiolysis). They found out that the specific activity of enzyme was 4.78 units/mg of protein after Sephadex G-200 also, the optimum pH for the cleavage reaction was 8.1 and for the condensation reaction 7.8, both reactions measured in Tris-HCl buffer. They measured the variation of the initial velocity as a function of the variation of the concentration of the first substrate and then of the second one, the

cleavage of acetoacetyl-CoA catalysed by β-ketothiolase is a two substrate reaction including acetoacetyl-CoA and CoASH. Moreover, they measured the reaction rate of condensation reaction of two molecules of acetyl-CoA to form acetoacetyl-CoA and free CoASH by a coupled optical test system. Finally, they found out that K_M value of condensation reaction was $3{,}9 \times 10^{-4}$M. Also, they indicated that free CoASH (0,1 mM) had a inhibitory effect physiologically on the enzyme catalysing the condensation reaction. That result had a significant effect on tricarboxylic acid cycle because the citrate synthase reaction generates free CoASH, this means that the high CoA concentrations resulted in an inhibition of the β-ketothiolase condensation reaction and poly-β-hydroxybutyrate synthesis.

In another study, thiolase in poly-β-hydroxybutyrate-synthesizing bacteria, the enzyme of *Clostridium Pasteurianum* was worked in the condensation direction (Berndt and Schlegel, 1975). *C. pasterianum* thiolase had optimal activity at pH 8.0, and its relative activity falls with decreasing pH. It was very sensitive to changes in pH that had a significant importance on acetone-butanol fermentation process.

A previous report showed that β-ketothiolase (E.C. 2.3.1.9) that its molecular weight was 190 000 determined by Sephadex G-200 was derived from *Zoogloea ramigera* I-16-M (Nishimura et al., 1978). For purification, they used DEAE-cellulose chromatography, phosphocellulose chromatography and Sephadex G-200 chromatography. The enzyme showed a pH optimum of 7.5 in the condensation reaction, and 8.5 in the thiolysis reaction. The K_M value for acetyl-CoA was $1{,}0 \times 10^{-5}$ and for CoA was $8{,}5 \times 10^{-6}$ in the thiolysis reaction. In the condensation reaction K_M value for acetyl-CoA was 0,33 mM. On the other hand, this group investigated the effect of sulfhydryl reagents, then they found out that 0.4 *p*-chloromercuribenzoate had strongly inhibitory effect. Furthermore, The condensation reaction was inhibited by CoA concentrations lower than 0.1 mM.

In the study , thiolase (E.C. 2.3.1.19) was derived from *Clostridium acetobutylicum* ATCC 824 which is one of the bacteria most commonly used to effect the acetone-butanol fermentation by purification with ammonium sulfate precipitation, DEAE-sephacel chromatography, blue sepharose CL-6B chromatography, and HPLC-anion exchange chromatography (Wiesenborn et al., 1988). In this study, they found out that K_m value for acetyl-CoA is 0,27 mM at 30°C, pH7.4. Thiolase had high activity over a broad range of pH values from at least pH 5.5 to greater than pH 7.0. On the

other hand, the condensation reaction is sensitive to competitive inhibition by CoASH. Also few studies described cloning and expression of the Thl-encoding gene and characterization of thiolase gene (Petersen and Bennett, 1991; Stim-Herndon et al., 1995).

Some authors showed that acetyl-CoA acetyltransferase (also known as acetoacetyl-CoA thiolase and 3-ketothiolase [E.C. 2.3.1.9]) was purified from *Rhizobium* sp. (*Cicer*) Strain CC 1192 (Kim and Copeland, 1997). The optimum pH was found out as 7.8 for 3-ketothiolase in thiolysis reaction. Furthermore, they found out that K_m values for acetoacetyl-CoA and CoA was 42 and 56 μM, also, V_{max} was 296 μmol of product min^{-1} mg of $protein^{-1}$. For condensation reaction, they investigated that K_m values for acetyl-CoA was 1,06 mM and V_{max} was 35 μmol of product min^{-1} mg of $protein^{-1}$. Also, in condensation reaction CoA showed competitive inhibition, giving an estimatte of 11 μM for the K_i.

In an another research, acetoacetyl-CoA thiolase was purified from isolated rat liver peroxisomes by DEAE-fast flow sepharose column, phospho-cellulose column, and blue-sepharose CL-6B column (Antonenkov et al., 2000). This thiolase enzyme was different from others because the peroxisomal acetoacetyl-CoA thiolase probably catalyzed the first reaction in peroxisomal cholesterol and dolichol synthesis.

Some scientists purified an acetoacetyl-CoA thiolase from *Halobacterium* sp ZP-6 with methods including DEAE-cellulose chromatography, benzyl sepharose chromatography, and hydroxylapatite treatment (Liu et al., 2002). For this enzyme, the optimum pH was 7.9 for acetoacetyl-CoA thiolysis, also when the pH was higher than 8.6, there was no activity. The presence of Mg^{+2} caused inhibitory effect, in contrast KCl and NaCl stimulated the thiolytic activity.

A study showed that β-ketoacyl-CoA thiolase (E.C. 2.3.1.9), which is the first enzyme of most common biosynthetic pathway for polyhydroxyalkanoates (PHAs), was purified from Thermus thermophilus (Pantazaki et al., 2005). The methods that were DEAE-52 cellulose chromatography, phenyl-sepharose, octyl-agarose chromatography,and hydroxylapatite chromatography were used for the purification of β-ketoacyl-CoA thiolase. The optimum pH was 8.0 and optimum temperature was 65°C both direction of the reaction (thiolysis and condensation). In condensation reaction, K_m value was 0,25 mM for acetyl-CoA. In thiolysis reaction, K_m values

were 11 μM, and 25 μM respectively for, CoA, and acetoacetyl-CoA. Also, thermostability test was done, at higher temperatures than 70°C, the activity started to decrease.

A previous report said that a thiolase gene from *Clostridium pasteurianum* was cloned (Liu and Meng, 2006). The thiolase was purified by a nickel nitrilotriacetic acid-agarose (Ni-NTA) affinity chromatography. The optimum pH was 8.2 in Tris/HCl buffer. The K_m value for acetoacetyl-CoA was 0,13 mM and V_{max} value was 46 μmol min^{-1} mg^{-1}.

According to Gehring and Lynen, the thiolase family members were divided into two categories which are degradative (E.C. 2.3.1.16) and biosynthetic (E.C. 2.3.1.9) thiolases (Modis and Wierenga, 1999). The thiolytic cleavage of 3-ketoacyl-CoA molecules to yield acetyl-CoA and a shortened acyl-CoA species was catalyzed by degradative thiolases which had importance on β-oxidation pathway of fatty acids. On the other hand, Walsh indicated that the biological Claisen condensation of two acetyl-CoA molecules to form acetoacetyl-CoA that is both the endergonic condensation and exergonic thiolytic cleavage and the reverse degradation reaction were catalyzed by biosynthetic thiolases (Modis and Wierenga, 1999; Mann and Lütke-Eversloh, 2013).

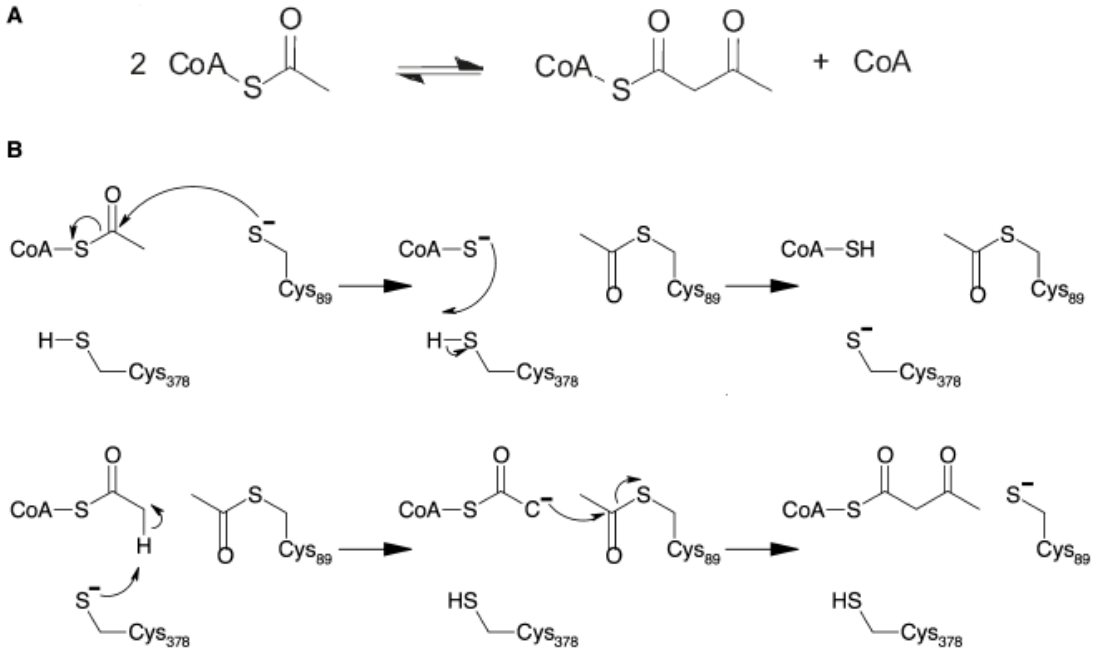

Figure 1.3: The thiolase reaction and mechanism (Merilainen et al., 2008; 2009).

The importance of the sulfur atoms of the substrate CoA and the catalytic cysteine on thiolase reaction mechanism was studied by a group (Merilainen et al., 2008). In the biosynthetic direction, the overall reaction starts with two molecules of Ac-CoA and generate AcAc-CoA and CoA (Figure 1.3a). The figure 1.3b shows that His348 attacked nucleophilically to activate Cys89 and the nucleophile was generated by proton abstraction from the C2 of Ac-CoA.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Reagents

Monosodium phosphate	: NaH_2PO_4, Sigma
Disodium phosphate	: Na_2HPO_4, Roth
(5,5'-Dithio-bis(2-nitrobenzoic acid)	: DTNB, AppliChem
Acetyl-CoenzymeA trilithium salt	: AppliChem, Endotherm
(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)	: HEPES, Roth
Glycerin	: Roth
Sodium chloride	: NaCl, Roth
Trypton (Pepton from Casein, pancreatic, granulated)	: Roth
Hefeextract (Yeast)	: AppliChem
Monopotassium phosphate	: KH_2PO_4, Roth
Dipotassium phosphate	: K_2HPO_4, Roth
Kanamycin sulfate	: Roth
Isopropyl-β-D-1-thiogalactopyranoside	: IPTG, Roth
Imidazole	: Roth
Tween 20	: Roth
Deoxyribonuclease I from bovine pancreas	: DNAse, Serva
Dodecylsulfate sodium salt for electrophoresis	: SDS, Serva
Ammonium persulfate	: APS, Serva
N,N,N',N'-Tetramethylethylenediamine for electrophoresis	: TEMED, Roth
Acrylamid; N,N-Methylen-	: Roth

Bisacrylamid-Solution 40% (w/v)	
Unstained Protein Ladder (Page Ruler)	: ThermoScientific
Ethanol	: Roth
3-(Cyclohexylamino)-1-propanesulfonic acid	: CAPS, AppliChem
Butanol	: Vwr
Dimethylsulfoxide	: Vwr
Magnesium chloride hexahydrate	: $MgCl_2.6H_2O$, Sigma
Potassium chloride	: KCl, Roth
Coenzyme A	: AppliChem
Tris(hydroxymethyl)aminomethane	: Tris, Serva
Hydrochloric acid	: HCl, Vwr

2.1.2 Buffers and solutions

4x Lower gel buffer: 1L 1,5M Tris was prepared (Don't fill flask until 1L). 0,8% (w/v) SDS-sodium salt was added to Tris. The solution was adjusted to pH8,8 with HCl. H_2O was added until volume of solution.

4x Upper gel buffer: 1L 0,5M Tris was prepared (Don't fill flask until 1L). 0,8% (w/v) SDS-sodium salt was added to Tris. The solution was adjusted to pH6,8 with HCl. H_2O was added until volume of solution. Spatula point/L was added.

10% APS solution: 10% (w/v) was prepared in water.

SDS-PAGE gels (12% concentration in gel): First, running gel was prepared and then stacker gel was prepared. First of all, spacer and short plates were bought together, locked into BioRad clips. To prepare two running gel, 3,75 mL Acrylamid-bis, 3,54 mL distilled water, 2,5 mL Lower buffer (4x), 100 µL 10% APS and 10 µL TEMED were mixed in a falcon tube. The mixture was added to between plates as quickly as possible. A small layer of bidistilled water was added in order to produce straight, clean top of the gel. After the mixture in the falcon was polymerized, the water was poured from the glass. Stacker gel mixed with 0,65 mL Acrylamide-bis, 3 mL distilled water, 1,25 mL Upper buffer (4x), 50 µL 10% APS and 5L TEMED was prepared. As quickly as possible it was added on top of the running gel. The comb was put on top slowly to prevent air bubbles. After polymerization, the comb was removed. Gels were stored at 4°C wrapped in SDS buffer solution.

5x SDS loading buffer: 12,5 mL 1,25M Tris-HCl (pH6.8), 25% (w/v) Glycerol, 12,5 mL 0,5M EDTA (pH8.0), 12,5% (v/v) β-mercaptoethanol, 5% (w/v) SDS-sodium salt were mixed. Then, 50 mg bromphehol blue was added. H_2O was added until 100 mL volume.

5x SDS running buffer: 0,125M Tris-Base, 0,96M Glycin, 0,5% (w/v) SDS-sodium salt were mixed in 2,5L distilled water. The solution pH was adjusted to pH8,3, then the bottle was filled its volume (3L).

Staining solution: 0,2% (w/v) Coomassie Brilliant Blue G250 and R250, 50% (v/v) Ethanol, 10% (v/v) Acetic acid (100%) were mixed in a bottle (1L). Then it was filtrated and the bottle was wrapped aluminium foil.

1M IPTG solution: 11,915 g IPTG was weighed. It was put in 50 mL falcon and distilled water was added until level of 50 mL. IPTG was stored at -20°C.

30 mg/mL Kanamycin solution: 1,5 gram Kanamycin-sulphate was weighed and put in 50 mL falcon. Distilled water was added until level of 50 mL. This solution was stored at -20°C.

50 mM HEPES with 10% glycerol buffer (pH7.5/RT): 11,9g HEPES was dissolved in 800 mL distilled water and 100 mL Glycerol. The pH was adjusted to 7,5 and the water was added to level of 1L.

100 mM Phosphate buffer (pH7.2): 0,4363 g monosodium phosphate monohydrate and 1,8326 gdisodium phosphate heptahydrate were added to 100 ml water. This buffer was used to solve DTNB. Also, pH6.07 (pH6.00/50°C) and pH6.57 (pH6.50/50°C) solutions of phosphate buffer were prepared by adding NaOH at room temperature.

50 mM CAPS buffer: 2,2132 g CAPS was dissolved in 200 ml of water. pH of this buffer were arranged to pH9.73 (pH9.50/50°C), pH10.23 (pH10/50°C), pH10.73 (pH10.50/50°C), pH11.23 (pH11/50°C) by adding NaOH at room temperature.

50 mM HEPES buffer: 2,38 g HEPES was dissolved in 200 ml of water. The pH of this buffer was adjusted to pH7.35 (pH7/50°C), pH7.85 (pH7.50/50°C), pH8.21 (pH8/40°C), pH8.35 (pH8/50°C), pH8.49 (pH8/60°C), and pH8.85 (pH8.50/50°C) by adding NaOH at room temperature.

Binding buffer: 50 mM HEPES, 20 mM imidazol, 10% glycerin and 0.1% tween20 were mixed in bidistilled water. pH was adjusted to 8 at 25°C. This mixture was filtrated.

Elution buffer: 50 mM HEPES, 500 mM imidazol, 10% glycerin and 0.1% tween20 were mixed in bidistilled water. pH was adjusted to 8 at 25°C. This mixture was filtrated.

Stripping buffer: 50 mM sodiumphosphote, 300 mM NaCl, 100 mM EDTA were mixed in water and the pH was adjusted to pH8 at room temperature by adding NaOH. Then, the volume was completed to 500 ml by H_2O.

TB media: 15 g trypton, 24 g hefeextract/yeast and 4 ml glycerin were mixed in the bottle, and it was completed with bidistilled water until volume of 900 ml. After this solution was autoclaved, 100 ml phosphate buffer x10 was added in clean bench.

5mM DTNB solution: 0,0040 g DTNB was dissolved in 2 ml 100 mM phosphate buffer (pH7.2/RT) and stored in the freezer.

10 mM Acetyl-CoA solution: 0,0175 g Acetyl-CoA was dissolved in 2 ml H_2O and stored in the freezer.

10 mM CoA solution: 0,0082 g CoA was dissolved in 1 ml H_2O and stored in the freezer.

8M Hainstoff: 24 g dry urea was dissolved in 25 ml of water.

2.1.3 Equipments

Electrophoresis gel system	: Bio-Rad Mini Protean® Electrophoresis System
Incubator	: Innova®44 Incubator Shaker Series
Heater	: Eppendorf® ThermoStat Plus Interchangeable Block
Macrocentrifuge	: Beckman Coulter, Avanti® J-20 XP
Microcentrifuge	: Harmony, Mini
Water bath	: Memmert
Vortex	: Scientific Industries, Vortex-Genie 2
Microplatereader	: Perkin Elmer, EnSpire® 2

pH Meter	: Mettler Toledo, SevenGo
Weighing device	: Ohaus Scout™ Pro and Ohaus Pioneer
High pressure homogenizer (pump)	: Avestin Emulsiflex-B15
Molecular Imager	: Gel Doc™ XR + System with Image Lab™ Software
UV photometer	: Hewlett Packard
Magnetic Stirrer	: Hanna instruments HI 200M

2.1.4 Bacterial strains

Clostridium Acetobutylicum and Meiothermus Ruber.

2.2 Methods

2.2.1 Expression and purification of thiolase

Enzymes always obtained from various sources, especially microorganisms. (Gerhartz, 1990). These microorganisms plays important role on the gene transferring. The genomic changes on the microorganisms make it easy to isolation and purification.

In our study, thiolase enzyme was derived from microorganisms which are bacteria, called *Clostridium acetobutylicum* and *Meiothermus ruber*.

Preparation of overnight culture: This process was made for augmentation of growth of the organism (Grogan, 2009). It was needed to be enough biomass of biocatalyst for isolated enzyme experiments.

In clean bench, 10 mL TB media including phosphate buffer and 20 µl Kanamycin (30 mg ml-1) were mixed in 100 ml autoclaved erlenmeyer flask. A single colony of M. Ruber was picked and added to the erlenmeyer flask. This flask is put on incubator at 37°C and 120 rpm as overnight culture.

Expression: The OD600 of the overnight culture was measured, according to this measurement, calculated amount of overnight culture was added to 250 ml TB media to reach an OD600 of 0.1. 1 µl Kanamycin (30 mg ml-1) per ml was added to the culture and this was incubated at 37°C and 120 rpm. After reaching the OD600 of between 0.5-0.8, 1 µl IPTG (1M) per ml was added to the culture. Expression was induced at 120 rpm and 37°C for 4 h. 5

Harvesting: The cells were centrifuged at 4500 rpm for 10 min at 4°C to harvest, supernatant discarded, pellet was stored at -20°C until further use.

Resuspension: The pellet was weighed and dissolved in 5 times weight of pellet binding buffer (50 mM HEPES, 20 mM Imidazol, 10% Glycerin, 0.1% Tween20, pH8.0) and 1 µl DNAse per ml by vortex to resuspention.

Disruption: Different biological buffers (e.g. phosphate, Tris-HCl, MOPS, and HEPES) are used to disrupt cells (Grogan, 2009). pHs of buffers are very significant connected to the nature of the enzyme. Moreover, in order to improve the overall solubility of the enzyme by removing this from the membrane fracion of the cell debris, some chemicals are added, such as detergant, glycerol.
To disruption of our cells, HEPES buffer (pH 8) was used, also, imidazole glycerin and tween were added to the buffer.

A few methods are both, ultrasonication and high pressure homogenization are used in the microorganisms for cell disruption (Gerhartz, 1990).

In our study, high pressure homogenizer was used for disruption. Basically, the cell suspension is pressed through a valve and hits an impact ring (e.g Manton-Gaulin homogenizer). Shearing forces and simultaneous decompressionare used the cells to make shatter.

Separation of solid matter: **S**eperation of enzymes from cells or cellular fragments is next step that is difficult (Gerhartz, 1990). Because there is a little difference between density of the cell and size of bacterial cells are small. continuous filtration, centrifuges,extraction, flocculation and flotation method are used for this process. In our study, centrifugation method was used to separate materials.

The disrupted cells were centrifuged at 20000 rcf at 4°C for 30 min. Supernatant and pellet were separated.

Purification: Crystallization, electrophoresis and chromatography are used for enzyme purification (Gerhartz, 1990).

Enzyme can be crystallized from ammonium sulfate solution. For this method, very pure solutions are used, because of that generally crystallization is not commonly used to purify enzymes.

One of the pure enzyme building methods is electrophoresis (zone electrophoresis, isotachophoresis, or porpsity gradients). This method has problems including association with a scale up because of the heat generated in electrophoresis and the interference caused by convection.

Molecules are seperated according their physical properties, chemical properties, or biological properties by chromatography of fundamental importance (Table2.1).

Table 2.1: Chromatographic methods (Gerhatz, 1990).

Type of chromatography	Principle	Separation according to
Adsorption	Surface binding	Surface affinity
Distribution	Distribution equilibrium	Polarity
Ion exchange	Ion binding	Charge
Gel filtration	Pore diffusion	Molecular size, molecular shape
Affinity	Specific adsorption	Molecular structure
Hydrophobic	Hydrophobic chelation	Molecular structure
Covalent	Covalent binding	Polarity
Metal chelate	Complex formation	Molecular structure

In the study, affinity column chromotography was used to purification.

One of the chromatograpy types is that affinity chromatography is characterized by using of the highly specific molecular recognition of certain biomolecules and has highest specifity and selectivity (Switzer and Garrity, 1999; Manz et al., 2004). Stationary phase material attached a specific ligand like an antigen adsorbs the matching antibody specificially and reversibly. Molecular recognition does not only occur between antigens and antibodies but also other bonding partners exist, including enzyme and co-enzyme, receptor protein and hormone.

For the purification and isolation of biomolecules even at low concentration, affinity chromatography can be selected. The process has four step: sample introduction, adsorption, washing and desorption. Agarose or cellulose beads as a stationary phase on which ligand molecules have been covalently attached are found in the chromatographic column. On the beads they are absorbed in a affinity for the ligand with molecules and is retained with the stationary phase, and this happens after addition of crude sample (Figure 2.1). The substances with no affinity fort he ligand

must be eluted from the column. To elute the adsorbed substances is the nex step. Disruption the non-covalent interaction between the biomolecules is necessary. a pH decrease, an increase in ionic strength, addition of a denaturing agent, such as urea or the addition of an organic solvent are found in all of these methods.

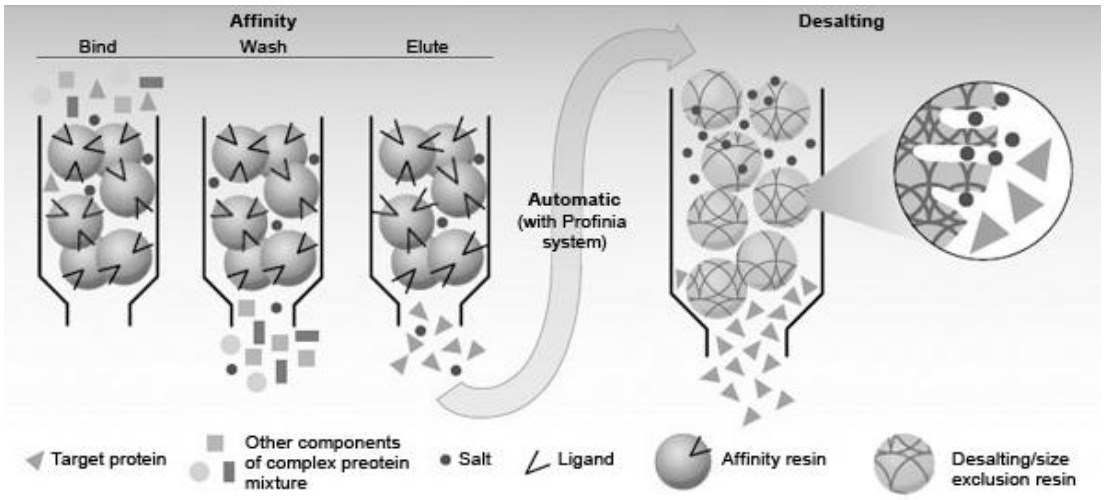

Figure 2.1: Affinity-tag and desalting purification (Url-2).

Although these type of desorption are non-specificon the stationary phase, Specific desorption can be achieved by introducing a species that binds to the analyte more strongly than the ligand.

On the solid phase, the free ligand competes with the bound ligand and this competition is for the protein binding sites. The protein is eluted from the column bound to the free ligand. For recurrent using, the seperation matrix can be reproduced.

Porath and coworkers suggested the idea of immobilized metal affinity chromatography (IMAC) in 1975. The affinity of transition metal ions like Zn(II), Cu(II), Ni (II), and Co(II) ions toward cysteine, histidine, and tryptophan in aqueous solutions are the principles of IMAC (Cheung et al., 2012). Iminodiacetic acid (IDA) was used to immobilize the metal ions to agarose by Porath et al. (1975). Certain amino acid residues like cysteine, histidine, and tryptophan help to the proteins to bind specifically to the metal ion coordination sites. Purification of histidine-tagged fusion proteins is the best known application.

The metal ions and produce metal chelates can coordinated by electron-donor atoms (N, S, and O) in the chelating compounds that are attached to the chromatographic support. To according to the number of occupied coordination bonds, the chelating

compounds are called as bidentate or pentadentate. The metal coordination sites that stay usually contain water or buffer molecules and can suffer exchanges with electrondonor groups from the protein surface. Some residues including Glu, Asp, Arg, Lys, Tyr, His, Cys, and Met, can participate in the binding process but the actual protein binding in IMAC determined by the availability of histidine residues.

In our study, in IMAC, after equilibration of column, the supernatant was loaded to the column, flowthrough was collected and column was washed with five column volumes of binding buffer. Finally, thiolase was eluted with 1 mL elution buffer (50 mM HEPES, 500 mM Imidazol, 10% Glycerin, 0.1% Tween20, pH 8.0) from the column.

20 µl of each fraction was mixed with 5xSDS loading buffer and this mixture was incubated at 95°C for 10 min. After quick centrifugation, 5 µl mix of each fraction was added to gel and under 25 mA the process was continued for 60 min. After detection, fractions including much more thiolase were collected and dialysed using osmatic membrane in 50 mM HEPES (10% Glycerin, pH7.5).

Dialysis: According to the dimensions of the dissolved molecules is called the allocation method of dialysis (Grogan, 2009; Boyer, 2012). A semi-permeable membrane is required for dialysis and this membrane pores are too small to allow diffusion of macromolecules of molecular weight greater than about 10,000 and that permits the migration of small molecules (e.g. salt, ammonium sulfate). The principle of dialysis is equilibration of the solutions between within and outside, the bag within the dialysis buffer (Figure 2.2). So, the concentration of ammonium sulfate in the protein sample decreases.

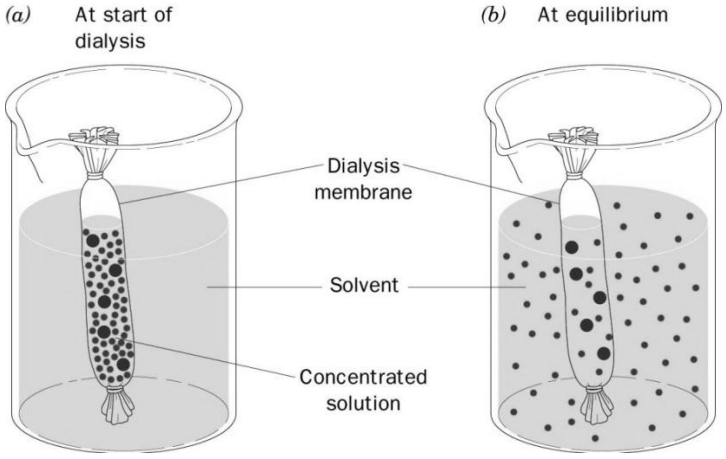

Figure 2.2: Dialysis technique (Url-3).

In our study, 50 mM HEPES (including 10% Glycerol) was used as dialysis buffer. The fractions collected from IMAC was put in osmatic membrane and closed with clips. This membrane was put in prepared dialysis buffer, fish was added, and the beaker was placed into the fridge. This solution stayed in the fridge during one day. After that, the membrane was taken from the old dilaysis buffer and it was put in new dilaysis buffer. After 1 hour, the enzyme was collected from the membrane and stored in the freezer.

2.2.2 Thiolase activity assays

Figure 2.3 shows the reaction between thiolase, acetyl-CoA and DTNB. DTNB is very important in this reaction because the activity of thiolase was determined by means of DTNB convertion to TNB^{-2}.

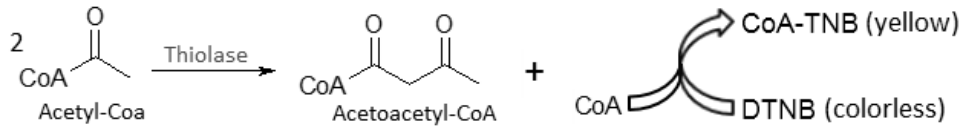

Figure 2.3: Thiolase reaction in butanol synthesis.

Figure 2.4 showed convertion of colorless DTNB, known as Ellman's reagent, to the yellow 5-mercapto-2-nitrobenzoic acid in the presence of thiol compounds (Riddles et al., 1983).

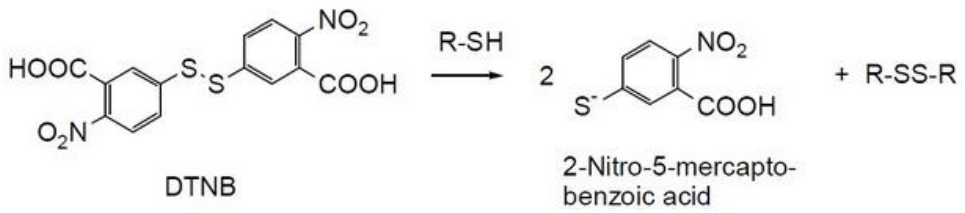

Figure 2.4: The reaction of DTNB in the presence of thiol compounds (Riddles et al., 1983).

As 5-mercapto-2-nitrobenzoic acid has an absorption maximum at 412 nm, the absorption spectrum of DTNB does not interfere with thiol detection (Figure 2.5). Therefore, it is easy to change in reaction spectrophotometrically.

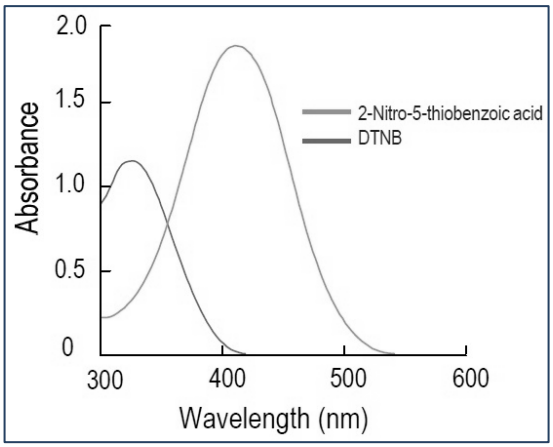

Figure 2.5: Absorption spectra of DTNB and reduced DTNB (Url-4).

Spectrophotometrically, thiolase activity was measured at 412 nm for per 60s. In this reaction, Acetyl-Coa was used as a substrate and decrease in absorption of Acetoacetyl-CoA was determined to find activity.

Firstly, to find out activity of thiolases, master mix (HEPES, DTNB, Acetyl-CoA) was prepared, incubated at 50°C for 10 min. In reaction, 50 mM HEPES, 0.1 mM DTNB and 0.2 mM Acetyl-CoA were used. The reaction was started by addition of 0.1 mM thiolase in reaction. In each assay measurement, standard assays was also measured without thiolase.

Phosphate buffer (pH6-6.5), HEPES buffer (pH7-8.5) and CAPS buffer (pH9.5-11) were used to collect information about optiumum pH. From 20°C to 65°C, assays were measured to find optimum temperature, and also HEPES buffer (pH8/20-50°C) for different temperatures arranged pHs was used. For thermostability experiments, the condition was HEPES buffer (pH8/40-50°C), 30 min, 0,1 mM DTNB, 0,2 mM Acetyl-CoA. For ethanol, butanol and DMSO stabilities, the conditions were same (HEPES buffer (pH8/50°C), 10 min, 0,1 mM DTNB, 0,2 mM Acetyl-CoA), just different percentages of ethanol, butanol and DMSO was added to thiolase enzyme. Inhibition were found out with NAD^+. Kinetic works were made according to changing acetyl-CoA concentrations.

3. RESULTS AND DISCUSSION

3.1 Purification of Thiolase

Pools of fractions collected from IMAC produced the protein profile on 12% SDS-PAGE as shown in figure 3.1 (Pellet (P), supernatant (S), flow through (FT), wash step (W), eluted enzyme (E) and marker (M)). Thiolase enzymes derived from both M. Ruber and C. Acetobutylicum were determined by SDS-PAGE with the molecular mass of about 42.3 kDa. After this results, the fractions including much more enzyme were collected to dialyse. After dialyses, enzymes were characterized.

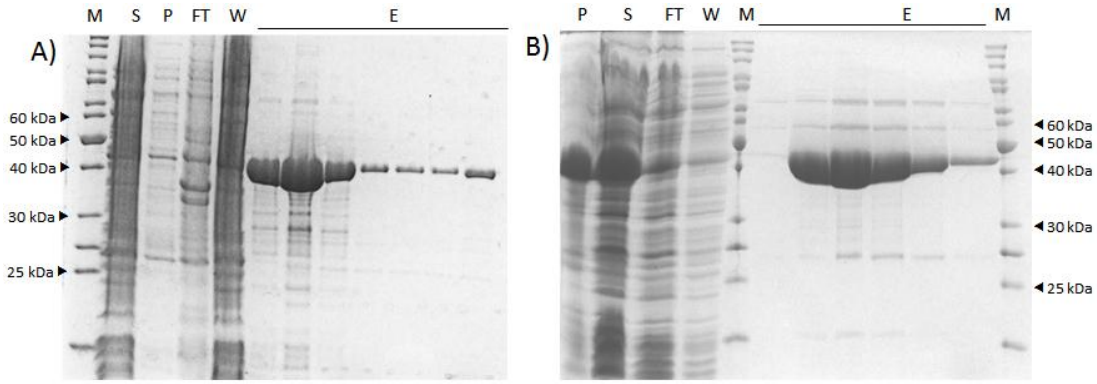

Figure 3.1: Purification of C-terminal His-Tagged Thiolase a) from *Meiothermus ruber* and b) from *Clostridium Acetobutylicum.*

3.2 Thiolase Activity

The extinction of MR-thiolase versus time was measured by DTNB, Acetyl-CoA and HEPES buffer. The equation (3.1) where ΔE is called measured optical density change, V called volume of the solution in the plate, dF called dilution factor, ε called extinction coefficient of the light absorbing substance, d called light path of the plate, V_E called volume of the enzyme taken for the determination, and c_E called concentration of the enzyme was used in order to calculate activity of Mr-thiolase and Cab-thiolase.

$$A_E = \frac{\Delta E/min \,.\, V \,.\, dF}{\varepsilon \,.\, d \,.\, V_E \,.\, c_E} \qquad (3.1)$$

The activity of Mr-thiolase was expressed in terms of ΔAbs 412 nm/30min. The values which are 0,2 ml for *V*, 86 for *dF*, 14,15 $M^{-1}cm^{-1}$ for ε, 0,59 cm for *d*, 0,02 ml for V_E, and 4,3 mg/ml for c_E were used to find activity of Mr-thiolase. The slope of reaction curve (Figure 3.2) which is 0.025 was calculated and the value of slope was put on equation (3.1). Finally, the activity of Mr-thiolase was found out as 0,6068 U/ml.

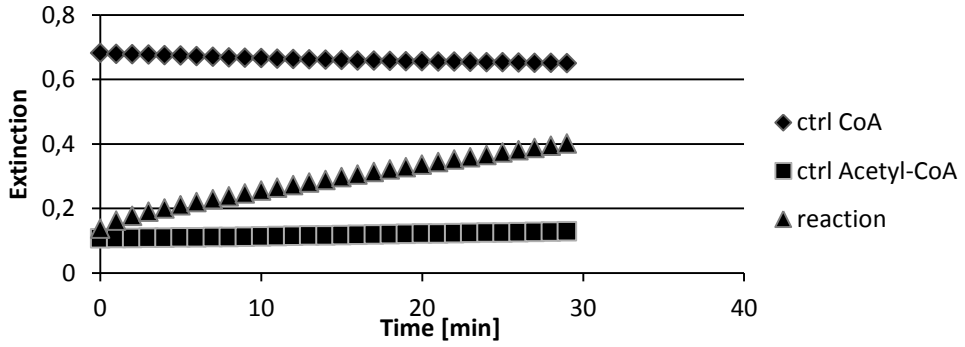

Figure 3.2: Extinction values of reaction of Mr-thiolase and controls for 30 min.

In same way, the activity of Cab-thiolase was expressed in terms of ΔAbs 412 nm/30min. The values which are 0,2 ml for *V*, 52 for *dF*, 14,15 $M^{-1}cm^{-1}$ for ε, 0,59 cm for *d*, 0,02 ml for V_E, and 2 mg/ml for c_E were used to find activity of Cab-thiolase. The slope of reaction curve (Figure 3.3) which is 0.030 was calculated and the value of slope was put on equation (3.1). Finally, the activity of Cab-thiolase was found out as 0,9239 U/ml. It was found out that the activity of Cab-thiolase is higher than Mr-thiolase.

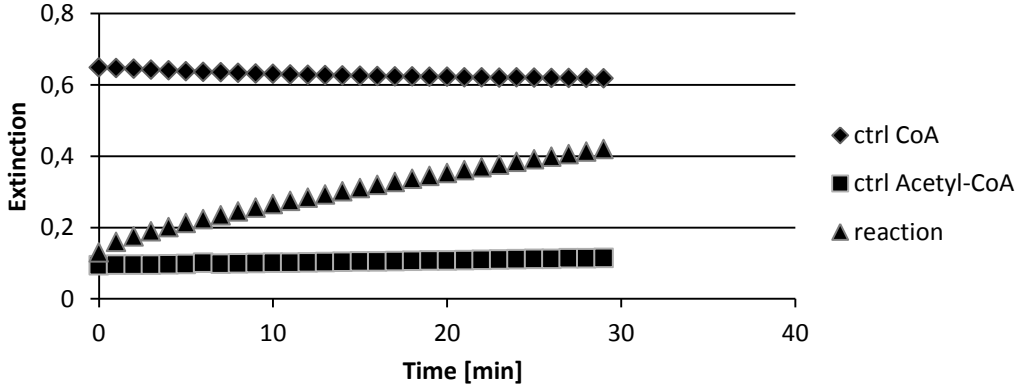

Figure 3.3: Extinction values of reaction of Cab-thiolase and controls for 30 min.

3.3 Effect of pH on Thiolase Activity

pH is an important parameter that affects the activity of enzymes. The enzyme is most active at pH is called an optimal pH (Cornish-Bowden, 2011). Extremely high or low pH values generally result in complete loss of activity for most enzymes. So,for each enzyme there is also a region of pH optimal stability.

To find out which pH conditions is better for thiolases, activities were measured in different buffers including different pH values. Thiolase activities of *Meiothermus ruber* is shown in Table 3.1. Based on these results, the highest activity value was 2,0682 U/ml. Relative activity was calculated with reference of the activity of the enzyme showing maximum value at pH10.

Table 3.1: Activities and relative activities of Mr- and Cab-thiolases in different pH values.

	Mr-thiolase	Cab-thiolase
pH	Specific Activity U/ml	Specific Activity U/ml
6	0,1198	0,1437
6,5	0,2555	0,3034
7	0,3913	0,3833
7,5	0,6548	0,6388
8	0,7187	0,8664
8,5	0,9702	1,0062
9,5	1,3056	1,4933
10	2,0682	2,4356
10,5	1,3096	1,2697
11	0,5191	0,3993

Figure 3.4 shows us the maximum point of Mr thiolase activity. Also, this graph presents the effect of pH. Thiolase was found to be active over a broad range of pH values between 6.0 and 10.0 at 50°C. After pH10, the increasing pH values gave us decreasing activity. As a result, optimum pH for Mr- thiolase was pH10.

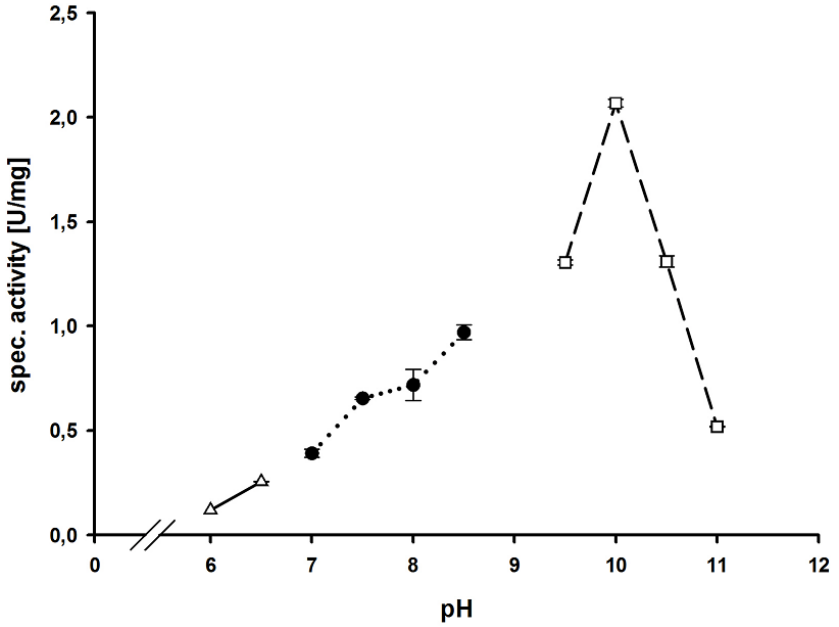

Figure 3.4: Effect of pH on Mr thiolase activity.

Thiolase activities of *Clostridium acetobutylicum* is shown in Table 3.1. The highest activity of Cab-thiolase was 2.4356 U/ml at pH10. This value is nearly similar to Mr-thiolase result. Also, in Figure 3.5, as you can see here, behaviour of Cab-thiolase is similar to Mr-thiolase.

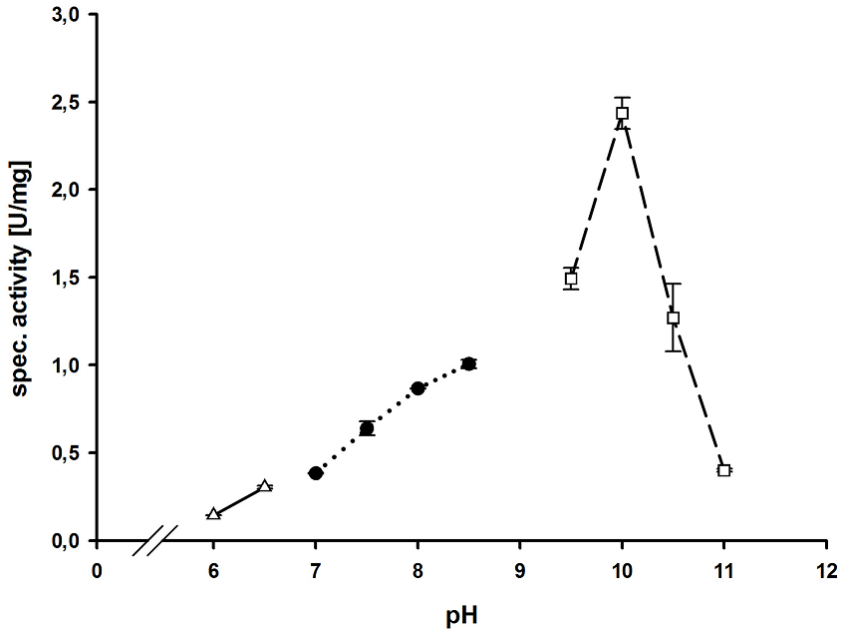

Figure 3.5: Effect of pH on Cab-thiolase activity.

Most studies indicated that the optimum pH for condensation reaction of thiolase was range from 7.5 to 8.0. Our findings differ from previously recorded data because both Mr-thiolase and Cab-thiolase showed optimum pH on 10.0.

3.4 Effect of Temperature on Thiolase Activity

Temperature is a factor affecting the rate of an enzyme-catalyzed reaction (Cornish-Bowden, 2011). Each 10-degree increase in the tempertaure will increase the activity of most enzymes by 50 to 100%. Even small changes in temperature may 10 to 20% affect the results of the reaction. At the high temperatures many enzymes are adversely affected. Because of most animal enzymes rapidly become denatured at temperatures above 40°C, the reaction rate increases with temperature to a maximum level, then abruptly declines. In some cases, enzymes will be deactivated at even moderate temperatures. So, storage of enzymes at 5°C or below is generally the most suitable. Some enzymes lose their activity when it is frozen.

3.4.1 Optimum temperature

The thiolase activity of purified enzyme was measured at temperatures ranging from 20°C to 65°C. Optimum activity of Mr-thiolase was exhibited at 50°C in figure 3.6. The optimum temperature studies indicated that thiolase was active over a fairly wide range of temperature values from 20°C to 65°C.

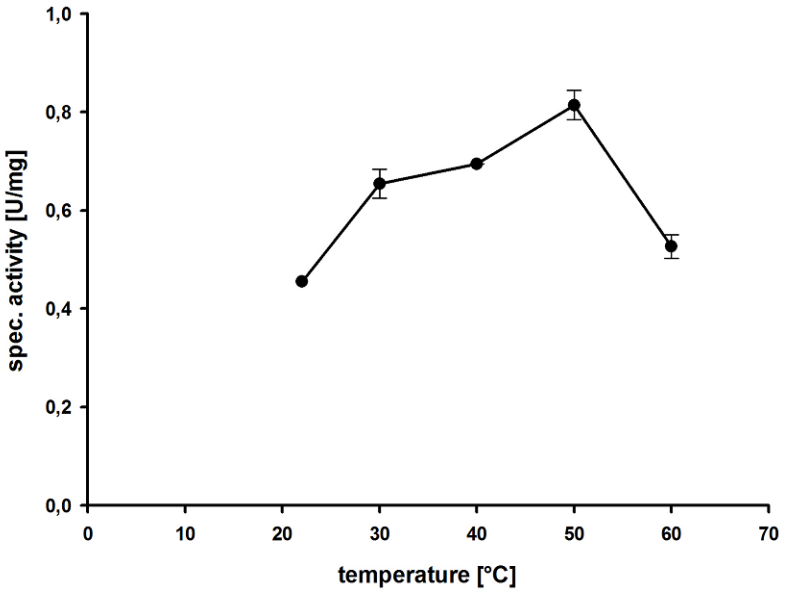

Figure 3.6: Optimum temperature graph of Mr-thiolase.

On the other hand, the activity of Cab-thiolase was the highest on 60°C in figure 3.7. Also, in similar way, Cab-thiolase was active over a wide range of temperatures. In similar way, Pantazaki et al. indicated that optimum temperature of that β-ketoacyl-CoA thiolase (E.C. 2.3.1.9) was 65°C both direction of the reaction (2004).

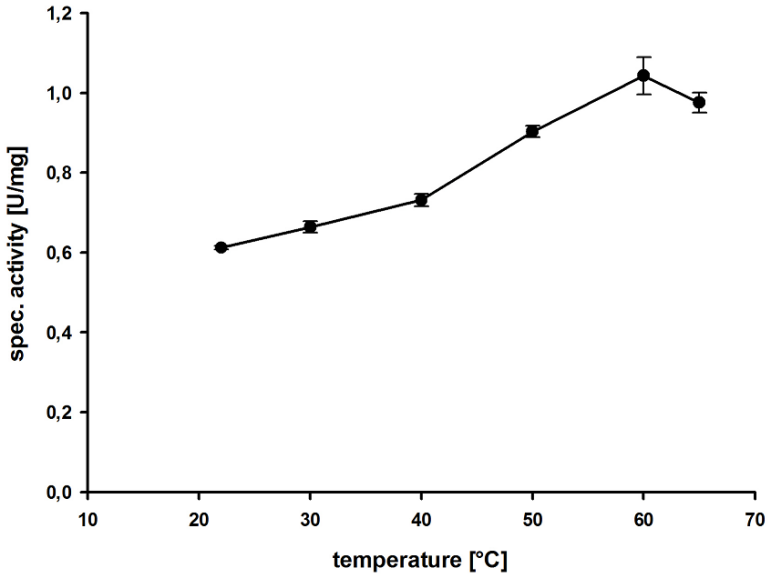

Figure 3.7: Optimum temperature graph of Cab-thiolase.

3.4.2 Thermostability

The termostability study of thiolase was carried out at temperature ranging from 40°C to 60°C and it lasted 150 hours. As you can see in Figure 3.8, the activity of Mr-thiolase decreased periodically after 30 hours. Especially, at 60°C the activity decreased much more.

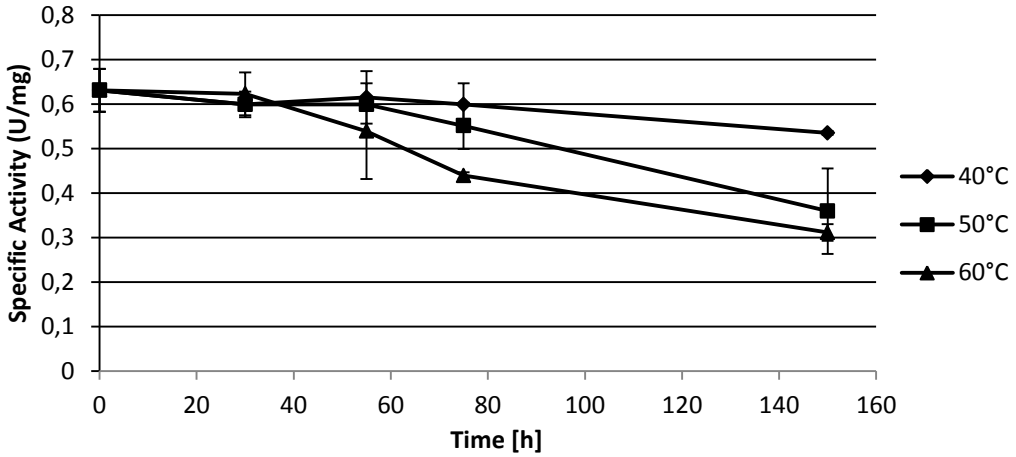

Figure 3.8: Thermostability graph of Mr-thiolase.

Figure 3.9 shows that the activity of Cab-thiolase decrased after 30 hours like Mr-thiolase.

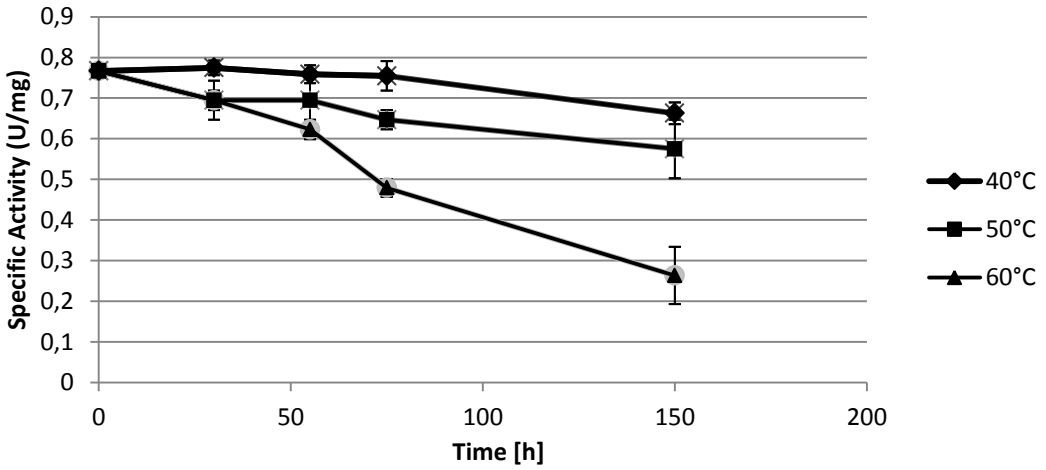

Figure 3.9: Thermostability grapf of Cab-thiolase.

3.5 Ethanol and Butanol Stability of Thiolase

It is very important to know about behaviour of enzymes, especially for thiolase in ethanol and butanol solutions. Due to the fact that thiolase enzyme is used in ABE fermentation, in that process ethanol and buthanol were produced. It is need to know how the enzyme behaves presence ot these solutions. Therefore, ethanol and butanol stabilities were investigated in our study.

Figure 3.10 showed that the activity of Mr-thiolase stayed between 0% and 10% ethanol. It showed a dramatic decrease on 15% ethanol. On the other hand, the activity of Mr-thiolase continued to be stable on 20% ethanol. Also, the Mr-thiolase activity was 0,651 U/mg at the begining, after addition of 20% ethanol in enzyme solution, the activity was 0,519 U/mg. In general, there is no big difference between activities of Mr-thiolase in different percentage of ethanol solutions.

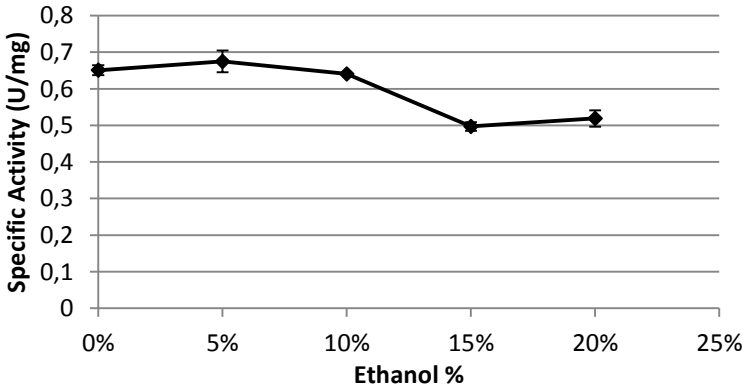

Figure 3.10: Ethanol stability of Mr-thiolase.

On the other hand, figure 3.11 showed that Cab-thiolase activity changed in every addition of ethanol. In every step, the activity of Cab-thiolase decreased. At the beginning, activity was 0,783 U/mg, in addition of 20% ethanol, activity was 0,455 U/mg. The result proved that Cab-thiolase was not totally stable in ethanol solution, but it was not exactly true that Cab-thiolase was not stable, because the thiolase was still active in the presence of ethanol, in addition, there was no rapid decrease on activity.

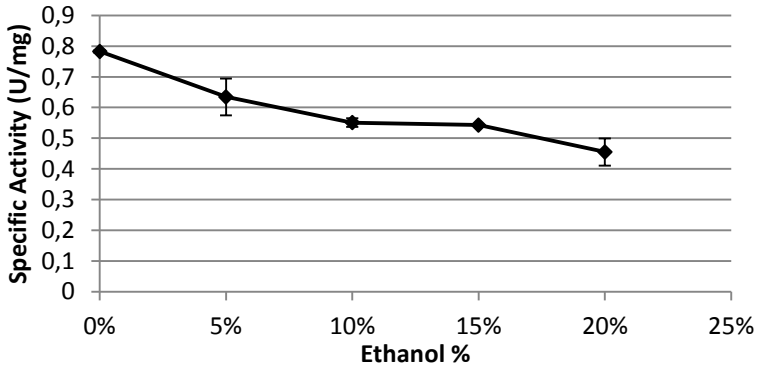

Figure 3.11: Ethanol stability of Cab-thiolase.

In conclusion, it could be said that both Mr- and Cab-thiolase was stable in the presence of ethanol solution. However, in comparison Mr-thiolase gave better results than Cab-thiolase, because the activity of Mr-thiolase in the presence of 20% ethanol was much more close the beginning activity than Cab-thiolase.

Thiolase enzyme is very significant in production of butanol. As it is shown in figure 3.12, firstly, the activity of Mr-thiolase was 0,623 U/mg. After addition of 5% butanol, the activity changed marginally until addition of 6% butanol. When 6% of butanol was added to enzyme, the activity of Mr-thiolase fell down dramatically, in addition, the decline of activity which is 0,047 U/mg continued depending on addition of butanol.

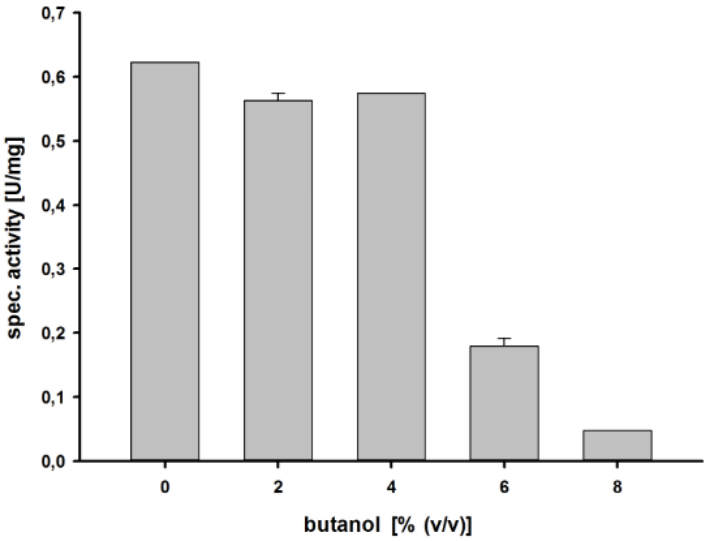

Figure 3.12: Butanol stability of Mr-thiolase.

Cab-thiolase activity did not show rapidly decrease depending on butanol addition (Figure 3.13). At the beginning, the activity of Cab-thiolase was 0,783 U/mg, while the activity was 0,467 U/mg when 8% butanol was added.

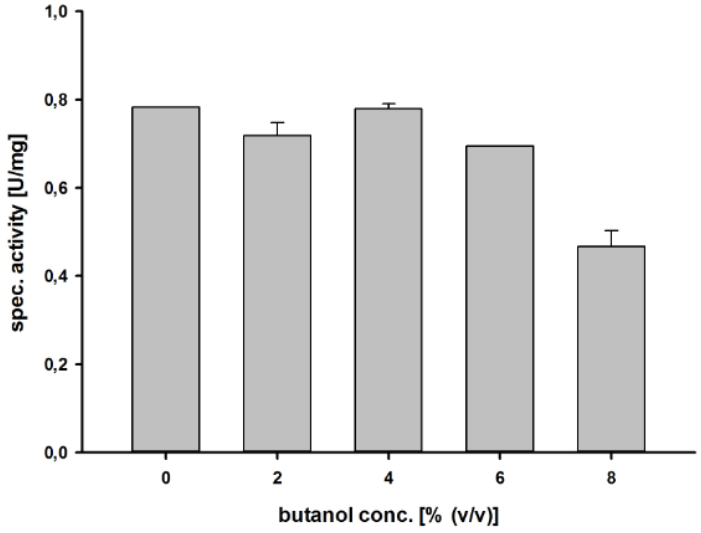

Figure 3.13: Butanol stability of Cab-thiolase.

It is obvious that Cab-thiolase gave better results than Mr-thiolase. Cab-thiolase had better butanol stability than Mr-thiolase.

Moreover, how thiolase behaves long hours in butanol solution was needed to find. That is the why the activity of both Mr- and Cab-thiolase was researched in 4% butanol solution during 120 hours. Mr-thiolase showed slightly decrase in activity during hours, the activity of Mr- thiolase decreased from 0,599 U/mg to 0,491 U/mg (Figure 3.14).

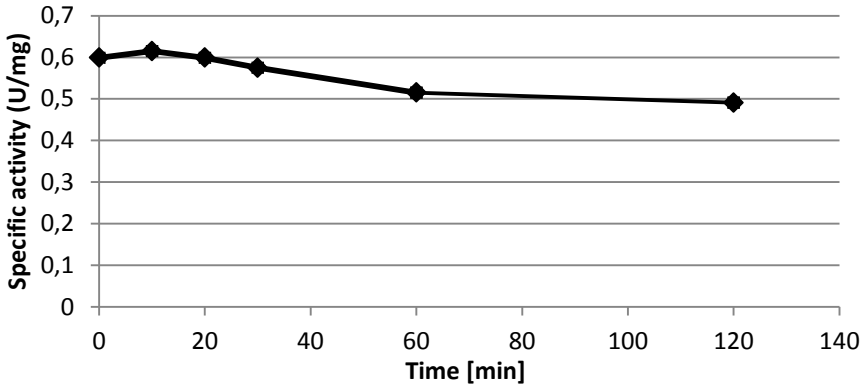

Figure 3.14: 4% butanol stability of Mr-thiolase.

In similar way, Cab-thiolase did not show dramatic decrease in activity during hours. The activity of Cab-thiolase fell down marginally from 0,850 U/mg to 0,791 U/mg. The activity of Cab-thiolase decreased less than the activity of Mr-thiolase.

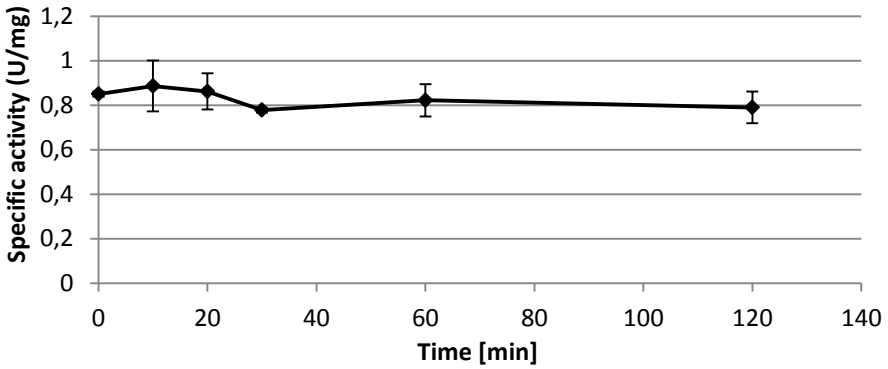

Figure 3.15: 4% butanol stability of Cab-thiolase.

These findings advocated that Cab-thiolase was more resistant to butanol than Mr-thiolase; hence, it can be said that Mr-thiolase was stable in butanol.

3.6 DMSO Stability of Thiolase

It is very important to know about behaviour of enzymes in organic solvents is caused by the usage of solvent in production process which uses enzymes. In our study, DMSO stability was worked in order to find how thiolase reacts.

Figure 3.16 showed the behaviour of Mr-thiolase in DMSO. As it shown in figure, the activity of Mr-thiolase increased from 0,645 U/mg to 0,759 U/mg depending on the addition of DMSO.

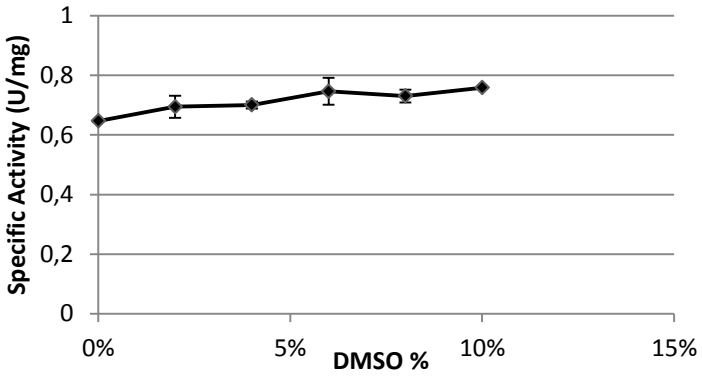

Figure 3.16: DMSO stability of Mr-thiolase.

On the other hand, the activity of Cab-thiolase decreased slightly from 0,783 U/mg to 0,759 U/mg. These results proved that Mr-thiolase showed better behaviour to DMSO than Cab-thiolase; hence, the changes in activities of Cab- and Mr- was not so much. Therefore, it can be said that not only Mr-thiolase but olsa Cab-thiolase was stable to DMSO.

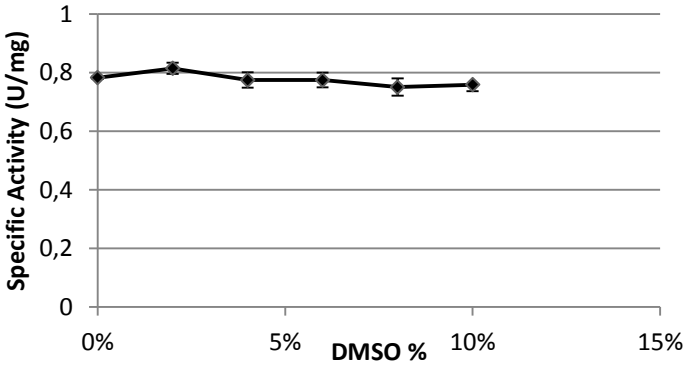

Figure 3.17: DMSO stability of Cab-thiolase.

3.7 Thiolase Kinetics

As it is shown in figure 3.18, the initial reaction velocity (v_0) of an enzyme catalyzed reaction alters with the substrate concentration ([*S*]) (Boyer, 2012). To clarify the kinetic properties of enzymes, the Michaelis-Menten equation has been derived. Commonly, the equation is,

$$\boldsymbol{v_0 = \frac{V_{max}[S]}{K_M + [S]}} \qquad \textbf{(3.2)}$$

Where v_0 is initial reaction velocity, V_{max} is maximal reaction velocity, means when all enzyme active sites are filled with substrate molecules, [S] is substrate concentration, K_M is Michaelis constant.

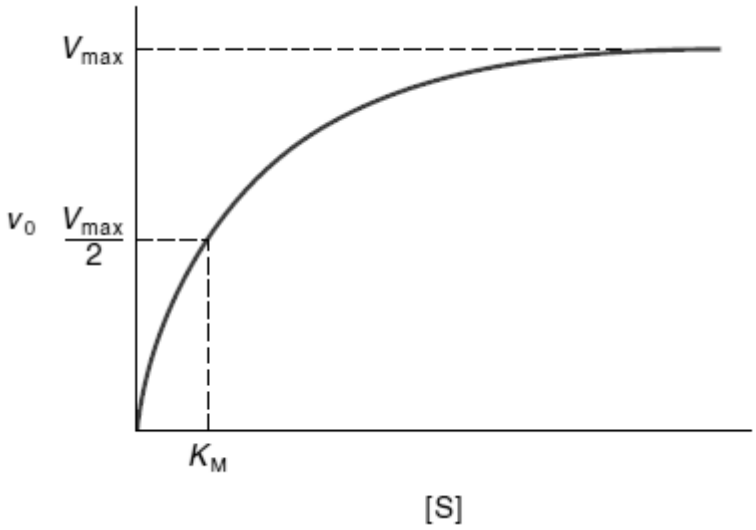

Figure 3.18: Michaelis-Menten plot for an enzyme catalyzed reaction (Hardin and Knopp, 2013).

From the graph (Figure 3.18), V_{max} and K_M, which are significant kinetic constants, can be determined. Equation 3.2 and figure 3.18 have all of the disadvantages of nonlinear kinetic analysis. The asymptotic nature of the line causes estimation of V_{max}. The value of K_M, the substrate concentration that results in a reaction velocity of $V_{max}/2$, depends on V_{max}, therefore both are just an estimation. The Michaelis-Menten equation is converted into the Lineweaver-Burk relationship by taking the reciprocal of both sides of the equation,

$$\frac{1}{v_0} = \frac{K_M}{V_{max}} \times \frac{1}{[S]} + \frac{1}{V_{max}} \qquad (3.3)$$

This equation is in the form $y = mx + b$, also, if $1/v_0$ is plotted versus $1/[S]$, the equation gives a straight line (Figure 3.19). The intercept on the $1/v_0$ axis is $1/Vmax$, and the intercept on the $1/[S]$ axis is $-1/K_M$. On the other hand, Lineweaver-Burk plot has a disadvantage, too. The data points are compressed in the high substrate concentration region.

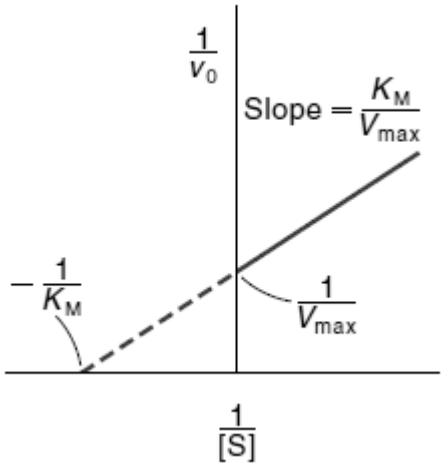

Figure 3.19: Lineweaver–Burk plot for an enzyme-catalyzed reaction obeying Michaelis–Menten kinetics (Hardin and Knopp, 2013).

The Michaelis constant (K_M) is very significant for an enzyme substrate interaction. One of importance of K_M is the substrate concentration that results in the filling of one-half of the enzyme active sites. The other importance is the equation,

$$K_M = \frac{k_2 + k_3}{k_1} \tag{3.4}$$

This equation is very important because if $k_2 \gg k_3$, so $K_M = k_2/k_1$, this means that K_M is equivalent to the dissociation constant of the ES complex. Moreover, large K_M values mean weak binding between E and S. In contrast, small K_M means strong interaction between E and S.

V_{max} is important because of finding k_3, another kinetic constant, turnover number. The turnover number means the number of substrate molecules converted to product by one enzyme with one active site per molecule per unit time. A measure of the efficiency of an enzyme is the turnover number.

In the light of these, in our study, after preparation of graph of activity versus concentration of Acetyl-CoA, 1/activity versus 1/concentration diagram was drawn. From that diagram, the constants (a and b) of equation (y=ax+b) of line was found. The equation 3.2 is used to find V_{max} and K_M values.

$$V_{max} = 1/b$$
$$K_M = V_{max}.a \tag{3.5}$$

By usage of Lineweaver-Burk plot, V_{max} and K_M values of both Mr- and Cab-thiolase was found out. Also, kinetic diagrams of Mr- and Cab-thiolase were obtained (Figure 3.20 and figure 3.21).

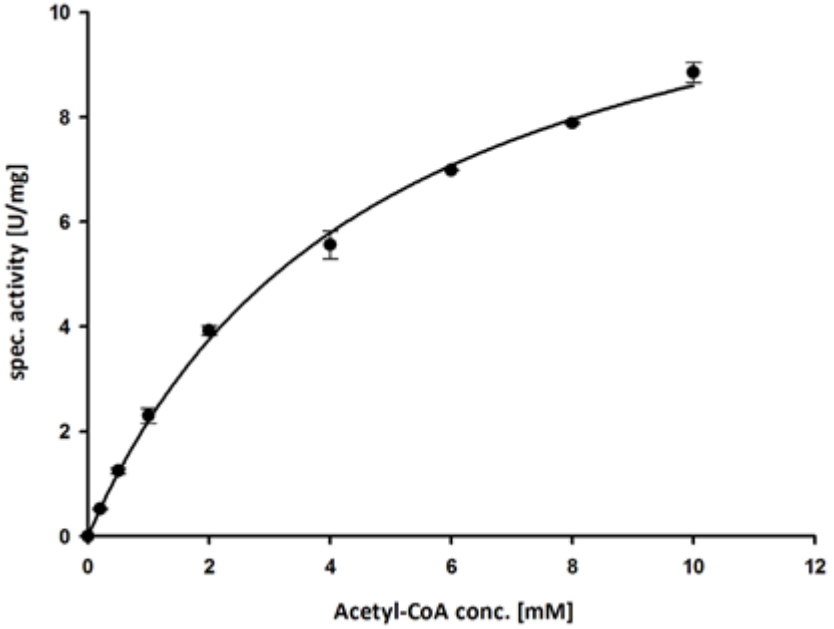

Figure 3.20: Kinetic diagram of Mr-thiolase.

In early studies, different K_M and V_{max} values were found out in condensation reactions as indicated below;

- The K_M value of β-ketothiolase from *Hydrogenomonas eutropha* H16 was 0,39 mM (Oeding and Schlegel, 1972).
- The K_M value of β-ketothiolase (E.C. 2.3.1.9) from *Zoogloea ramigera* I-16-M for acetyl-CoA was 0,33 mM (Nishimura et al., 1978).
- The K_M value of thiolase (E.C. 2.3.1.19) derived from *Clostridium acetobutylicum* ATCC 824 was 0,27 mM for acetyl-CoA (Wiesenborn et al., 1988).
- The K_M value of acetyl-CoA acetyltransferase (E.C. 2.3.1.9) purified from *Rhizobium* sp. (*Cicer*) Strain CC 1192 was 1,06 mM for acetyl-CoA and V_{max} was 35 μmol min^{-1} mg^{-1} (Kim and Copeland, 1997).
- The K_M value of β-ketoacyl-CoA thiolase (E.C. 2.3.1.9) purified from *Thermus thermophilus* was 0,25 mM for acetyl-CoA (Pantazaki et al., 2004).

- The K_M value of a thiolase gene from *Clostridium pasteurianum* was 0,13 mM and V_{max} value was 46 μmol min^{-1} mg^{-1} (Berndt and Schlegel, 1975).

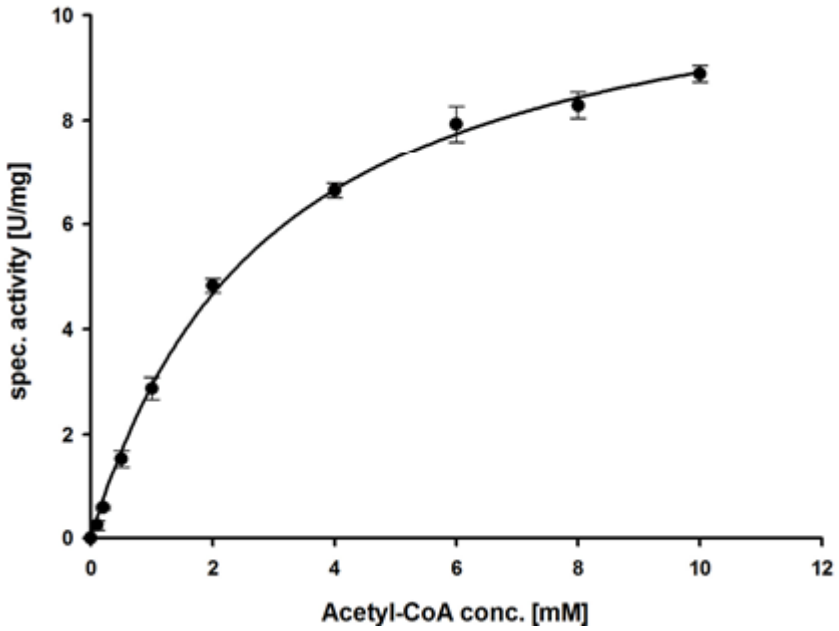

Figure 3.21: Kinetic diagram of Cab-thiolase.

In contrast to earlier findings, the K_M value and V_{max} value of Mr-thiolase were observed respectively as 4,82 mM and 13,089 μmol min^{-1} mg^{-1}. For Cab-thiolase, the K_M value and V_{max} value was respectively 3.07 mM and 11,806 μmol min^{-1} mg^{-1}.

K_M values of our enzymes were found higher than earlier enzymes. This means that Mr-thiolase and Cab-thiolase binded substrates weakly.

3.8 Inhibition of Thiolase

Inhibitors reduce the rate of an enzyme-catalyzed reactions and interferes with the action of an enzyme (Campbell and Farrell, 2012). The action of an inhibitor divided into two categories: reversible and irreversible. A reversible inhibitor can bind to the enzyme and subsequently be released, leaving the enzyme in its original condition. In reversible inhibition means reacting with the enzyme to produce a protein that is not enzymatically active and from which the original enzyme cannot be regenerated. An irreversible inhibitor frequently forms a stable compound with the enzyme by covalent bonding with an aminoacid residue at the active site (Gerhartz, 1990).

There are three important types of reversible inhibition: competitive inhibition, noncompetitive inhibition, and uncompetitive inhibition.

Competitive inhibitors compete with the substrate for the active site of enzyme (Wilcox, 2010).

A noncompetitive inhibitor binds to the enzyme at a site that is distinct from the substrate binding site (active site). So, both the free enzyme and the enzyme–substrate complex can be filled with enzyme.

An uncompetitive inhibitor does not bind to the free enzyme; instead, it binds reversibly to the enzyme-substrate complex to provide an inactive ESI complex.

In our study, to find inhibition type, NAD^+ showed in figure was used.

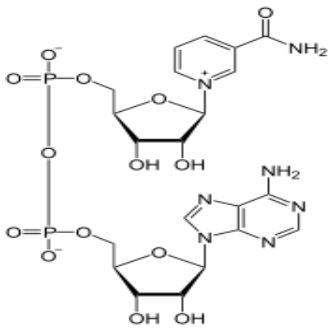

Figure 3.22: The chemical structure of NAD^+.

In this study, the activities of Mr-thiolase were measured in changing concentration of NAD^+. First, 0,5 mM NAD^+ was added to master mix, and the activities were measured in changing concentration of acetyl-CoA. Finally, figure 3.22 was obtained.

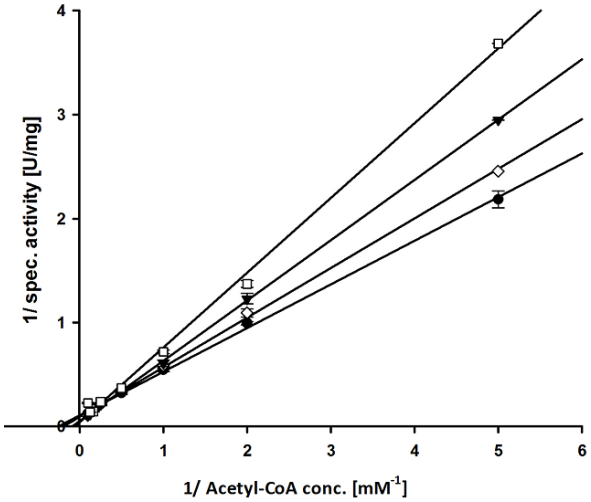

Figure 3.23: Inhibition diagram of MR-thiolase.

This diagram (figure 3.22) was compared to different types of inhibition diagrams in figure 3.23. It is clear that the inhibiton diagram of Mr-thiolase was similar to competitive inhibiton diagram. In conclusion, it is found out that Mr-thiolase with NAD^+ showed a competitive character (Figure 3.23). Similar results that acetyl-CoA acetyltransferase derived from *Rhizobium* sp. (*Cicer*) Strain CC 1192 showed competitive inhibition have been demonstrated by Kim and Copeland (1997).

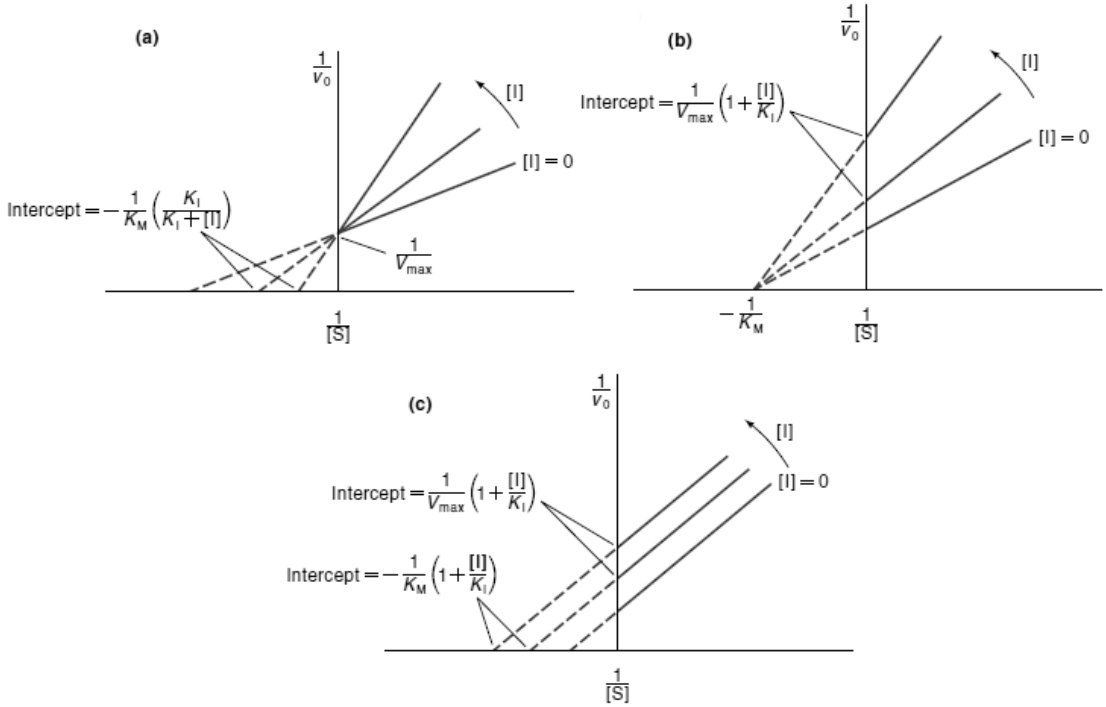

Figure 3.24: Lineweaver–Burk plots: (a) competitive inhibition, (b) noncompetitive inhibition, and (c) uncompetitive inhibition (Url-5).

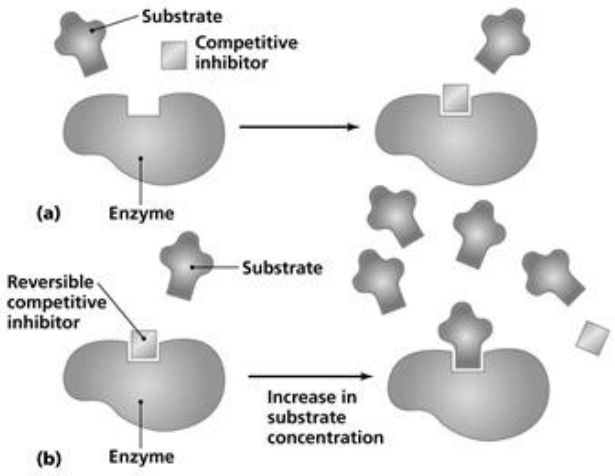

Figure 3.25: Competitive inhibition mechanism (Url-6).

4. CONCLUSIONS AND RECOMMENDATIONS

We compared two enzymes, thiolase derived from *Meiothermus Ruber* and *Clostridium Acetobutylicum*. As a result, we found out;

- The activity of Mr-thiolase was found out as 0,6068 U/ml and the activity of Cab-thiolase was found as 0,9239 U/ml.
- Both Mr-thiolase and Cab-thiolase had same optimum pH, which is 10.00. This result was better than the earlier studies.
- The optimum temperature of Mr-thiolase was 50°C and the optimum temperature of Cab-thiolase was 60°C. This result was similar to earlier studies.
- The thermostability of either Mr-thiolase or Cab-thiolase had a same effect. After 30 hours, the activity started to decrease. Especially, at 60°C, the decrase was very clear; hence, after hours both thiolases were active. It can be said that Mr- and Cab-thiolase were thermostable.
- Mr- and Cab- thiolase were stable in the presence of ethanol solution. Even in the presence of 20% ethanol, both thiolases did not show dramatic decrease in activity. Furthermore, Mr-thiolase was much more stable than Cab-thiolase.
- Mr- and Cab- thiolase were stable in the presence of butanol solution. This test gave better results for Cab-thiolase. But, in the presence of 4%butanol , the activity of Mr- and Cab- thiolase did not show rapid decrease.
- DMSO stability results showed that it enhanced the activity of Mr-thiolase, on the other hand, slightly it decreased the activity of Cab-thiolase.
- The K_M and V_{max} values for Mr -thiolase were found respectively as 4,82 mM and 13.089 μmol min^{-1} $mg^{-}1$. The K_M and V_{max} values for Cab-thiolase were respectively 3,07 mM and 11,806 μmol min^{-1} mg^{-1}. Several studies showed that K_M and V_{max} values were exactly different from our results.

- NAD^+ had an inhibition effect on Mr-thiolase. Finally, the competitive inhibition character of Mr-thiolase was found.

As a consequence of our findings, we recommend that salt effects of enzyme should be done in order to reach information about which salts increase activity of enzyme. Moreover, our study was carried out in conditions including, pH8 and 50°C. For better results, the process can be conducted in pH 10.0 and 60°C.

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