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Biocatalytic Esterification of Lactones in Buffer

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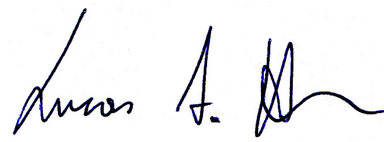

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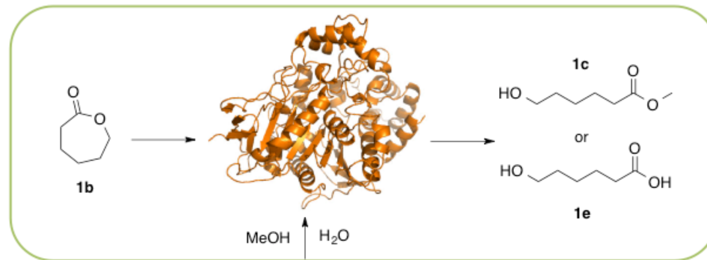
Zusammenfassung

Biokatalytische Veresterung von Lactonen in Buffer



Die Veresterung von ε-Caprolacton (**1b**) zu Methyl-6-hydroxyhexanoat (**1c**) ist ein Schlüsselschritt für die mögliche biokatalytische Herstellung von Nylon, einem Kunststoff mit weitem Anwendungsbereich (z.B.: Bekleidung, Sportartikel etc.). Manche Hydrolasen - wie die Pferdeleberesterase (HLE) und die Schweineleberesterase (PLE) - katalysieren die gewünschte Umsetzung dieser Lactone in der Gegenwart von 10% v/v MeOH oder EtOH.

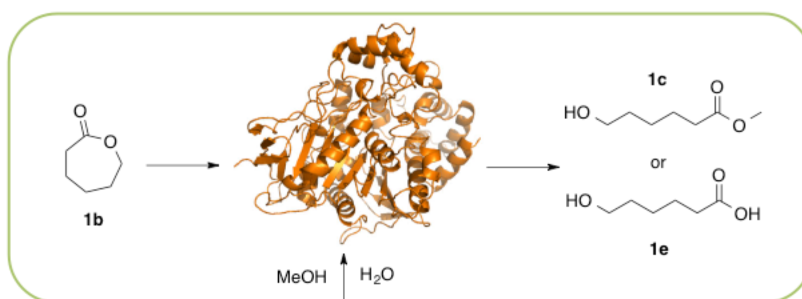
Drei Varianten der HLE (Sequenzidentität 92.57% - 96.28%) und eine Hydrolase aus *Bacillus subtilis* (pdb code 2R11) wurden erfolgreich kloniert, in *E. coli* – kostengünstig und effizient – exprimiert und aufgereinigt. Die optimalen Reaktionsbedingungen dieser Enzyme wurden für die Veresterung von ε-Caprolacton (**1b**) bestimmt. Variante 1_1 der HLE zeigte hauptsächlich Hydrolyse von Lacton **1b** zur 6-Hydroxyhexansäure (**1e**), während Varianten 1_2 und 1_3 bevorzugt das gewünschte Methyl-6-hydroxyhexanoat (**1c**) produzierten. Der gebildete Ester wurde erst danach langsam zur Säure **1e** hydrolysiert. Die Hydrolase aus *Bacillus subtilis* veresterte und hydrolisierte das Lacton **1b** zu gleichen Teilen. Der gebildete Ester wurde jedoch – im Gegensatz zu den HLE Varianten 1_2 und 1_3 nicht mehr von der Hydrolase zur Säure umgesetzt.

Zusätzlich wurde die Erkennung von chiralen Zentren für die Enzyme getestet. Dafür wurde ein racemisches Substrat (6-Methyl-ε-caprolacton) verwendet. Für diese Umsetzung wurde nur eine geringe Erkennung des chiralen Zentrums bestimmt, was für eine mögliche industrielle Anwendung im Polymersektor von Vorteil sein könnte.

Interessanterweise bevorzugte Variante 1 der HLE eine andere Reaktion als Variante 2, obwohl die Sequenzidentität (96.28%) sehr hoch ist (525 von 546 Aminosäuren sind identisch). Kristallstrukturen von den beiden Varianten könnten zu wichtigen Informationen über potentielle Tunnel führen, welche die Bevorzugung von Alkoholen gegenüber Wasser als Nukleophil und *vice versa* steuern. Die Ergebnisse aus dieser Masterarbeit sowie die Erkenntnisse aus der möglichen Strukturaufklärung der Biokatalysatoren wären von enormer Bedeutung für rationales Proteindesign und würde das Verständnis über die Funktion und Wirkung dieser Katalysatoren aus der Natur signifikant erweitern.

Abstract:

Biocatalytic Esterification of Lactones in Buffer



Esterification of ε-caprolactone (**1b**) to methyl-6-hydroxyhexanoate (**1c**) is a key step for the possible biocatalytic access to the nylon-6 monomer. Some hydrolases such as horse liver esterase (HLE) and pig liver esterase (PLE) possess esterification activity towards lactones in the presence of 10% v/v MeOH or EtOH, followed by subsequent hydrolysis of the produced ester to the corresponding carboxylic acid.

Three HLE isoenzymes with high sequence identity (92.57% - 96.28%) and a hydrolase of *Bacillus subtilis* (pdb code: 2R11) were successfully cloned and partly expressed as soluble protein in *E. coli* followed by purification *via* HisTrap Ni^{2+} affinity chromatography. Temperature and pH optimum of the heterologously expressed enzymes were determined for the esterification of ε-caprolactone (**1b**). Lyophilized cell free extracts (CFE) of the corresponding isoenzymes were used during the optimization studies. HLE isonenzyme 1 favored hydrolysis of ε-caprolactone (**1b**) to 6-hydroxyhexanoic acid (**1e**), while isoenzymes 2 and 3 preferably produced methyl-6-hydroxyhexanoate (**1c**) followed by hydrolysis to 6-hydroxyhexanoic acid (**1c**). Hydrolase from *Bacillus subtilis* (pdb code 2R11) esterified and hydrolyzed lactone **1b** simultaneously. However, 2R11 was – in contrast to isoenzymes 1_2 and 1_3 – not capable of hydrolyzing ester **1c**.

Additionally the chiral recognition of the enzymes was tested employing racemic 6-methyl-ε-caprolactone as substrate. However a low E value 6 was obtained which indicates that the enzymes are not selective for the examined application.

It is very interesting that HLE isoenzyme 1_1 favors a different reaction than isoenzyme 1_2, even though they share a sequence identity of 96.28% (525 of 546 amino acids are identical). Obtaining the crystal structures of these enzymes could lead to more information about potential tunnels that might explain the preference of alcohols over water as a nucleophile and *vice versa*. This knowledge would be very useful for rational protein design and would extend the understanding of protein mechanistic in general.

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1 Introduction

1.1 Biocatalysis[1]

Biocatalysis uses enzymes for the transformation of non-natural substrates in organic synthesis. Enzymes as catalysts from nature can be found in every living system. A vast variety of enzymes have been discovered so far. They are organized in 6 groups depending on the reaction type they catalyze (**Table 1.1-1**).

Table 1.1-1: Summary of enzyme classes and the type of reaction they catalyze.

Enzyme class	Reaction type
1. Oxidoreductases	Oxidation-reduction
2. Transferases	Transfer of groups
3. Hydrolases	Hydrolysis, formation of esters, amides, nitriles etc.
4. Lyases	Addition of small molecules on C=O, C=N, C=C
5. Isomerases	Isomerization reactions
6. Ligases	Formation or cleavage of bonds with concomitant triphosphate cleavage

For the classification of enzymes a system of 4 numbers was developed. The general form is [EC A.B.C.D.]. [EC stands for ‘Enzyme Commission; A - the main type of reaction (see **Table 1.1-1**); B – subtype, indicating substrate; C – indicates the nature of co-substrate; D – individual enzyme number].

Nowadays enzymes can be heterologously expressed in different organisms such as *E. coli* (prokaryotic), *Sacchermoyces cervisiae,* (eukaryotic), *Picchia pastoris* (eukaryotic) and more. Heterologous expression gives a fast and cost efficient access to biocatalysts.

1.1.1 Advantages and disadvantages of enzymes

Enzymes are proteins and therefore they are biodegradable. They are able to work in aqueous systems. Not necessarily water is needed as the reaction medium, but highest reaction rates are obtained in water. Most enzymes tend to act under mild conditions (pH 6-8; 30 °C), which minimize problems of side reactions. On the other hand enzymes require narrow reaction parameters (pH, T), because they can denature when exposed to harsh reaction conditions. Water can act as a ‘lubricant’ for enzymes, thus the addition of co-solvents may

decrease the rigidity of the enzyme, lowering down the reaction rate, but due to sterical effects increase the selectivity.[2]

Except of proteases, many enzymes are compatible with each other. That means that enzymes, which work under similar reaction conditions can be employed in cascades, also in a one-pot system.[3] Enzymes are not limited to their natural substrates, but in most cases to their natural co-factors. Most reactions carried out by enzymes are reversible, therefor the equilibrium needs to be pushed or pulled in the direction of the desired product. Since all enzymes are naturally made from L-amino acids they are chiral catalysts. Hence they are able to recognize chiral centers in substrates and some enzymes show high chemo-, regio- and/or enantioselectivity.

A disadvantage of using enzymes is that they might cause allergic reactions if not handled with care. Also inhibition phenomena that lower reaction rates (substrate, product inhibition) may occur.[4]

In nature enzymes undergo evolution, which means that they are adapted to their environment *in vivo*. In the last decades also the directed evolution of enzymes *in vitro* became a very important tool to optimize enzyme functions.[5]

1.1.2 Isolated enzymes vs. whole cell systems

Biotransformations can be performed employing isolated enzymes or whole cell systems. Depending on the type of reaction, cofactor recycling and the scale of the biotransformation, it can be decided to use purified enzymes, isolated enzymes (cell free extract), or whole cell microorganisms. Also the assessment of using free or immobilized enzymes is important.

The advantages of using isolated enzymes over whole cell catalysts are high enzyme activities, simple workup and better productivity. In immobilized form the recovery and the reuse of enzymes can be easy. Isolated enzymes show less stability than enzymes protected by a 'microorganism shell'. Enzyme purification and cofactor recycling can be difficult and cost intensive and during immobilization of enzymes a loss of activity is possible.

Employing whole cell systems for biotransformation no purification steps and no cofactor recycling is necessary, but workup is laborious because of large volumes of cell cultures. Microorganisms show a low tolerance of organic solvents and side reactions are common due to uncontrolled metabolism. By heterologous expression of the introduced enzyme, the catalytic protein becomes the dominant part of the cells proteome and side reactions become subsidiary. Competing enzymes in the host organism can also be knocked out if they are not

of vital importance for the cells. In immobilized form whole cell catalysts may be reused, but they also show a decrease in activity.

1.1.3 Biotransformations in organic solvents

As mentioned before biocatalysts show high activity in water as their natural solvent. Since water is a poor solvent for most reactions in organic chemistry – due to its polarity, side reactions and removal can be tedious because of its high boiling point – the addition of organic solvents can be necessary and advantageous.[6] Some of these advantages are: increased solubility of lipophilic substrates, elimination of side reactions involving water, decreased inhibitory effects and in the case of lipases controlling reaction equilibrium in the direction of synthesis instead of hydrolysis.

Some enzymes show activity in organic solvents miscible with water, at the cost of catalytic efficiency, but on the other hand selectivity may be increased.[7]

Solvent systems for biotransformations containing organic solvents can be classified in three types:

- 1.) Monophasic aqueous-organic solution.
- 2.) Biphasic aqueous-organic solution.
- 3.) Monophasic organic solution.

ad 1.) Enzymes and substrates are dissolved in monophasic solution consisting of water and a water miscible organic solvent (MeOH, DMSO, THF, acetone etc.). Mostly the concentration of organic solvent is 10% v/v but in some cases it may be increased to 50% v/v (e.g. lipases). In some rare exceptions enzymes remain active in a monophasic aqueous-organic solvent system with extremely low water content.[8]

ad 2.) In a two-phase system the enzyme is present in the aqueous phase and mostly substrate and product are present in the organic phase. Thus inhibitory effects (caused by substrate or product) on the enzyme are decreased. An efficient mass transfer between the phases and shaking/stirring are crucial parameters for biotransformations in biphasic systems.[9]

ad 3.) In a monophasic organic system the enzyme is suspended in neat organic solvents. It is crucial that the structural bound water is retained.[10] Special parameters have to be considered to perform biotransformations in monophasic organic solvents.

pH-Memory: The enzyme should by lyophilized in a buffer at the enzyme's pH optimum. Thus the optimal pH is 'saved' in the structural water.[11] Also the physical state in which the enzyme is used should be considered (lyophilized, precipitated, crystalline, immobilized).

Another possibility for water free solvents are ionic liquids. They consist of salts, which do not crystallize at room temperature (pyridinium-, ammonium-, phosphonium-kations and citrate-, triflate-, other heaviliy fluorinated anions). Disadvantages of ionic liquids are: high costs, toxicity and they are not biodegradable.[12]
Nowadays enzymes can also be engineered to suit harsh process conditions by mutagenesis methods.[13]

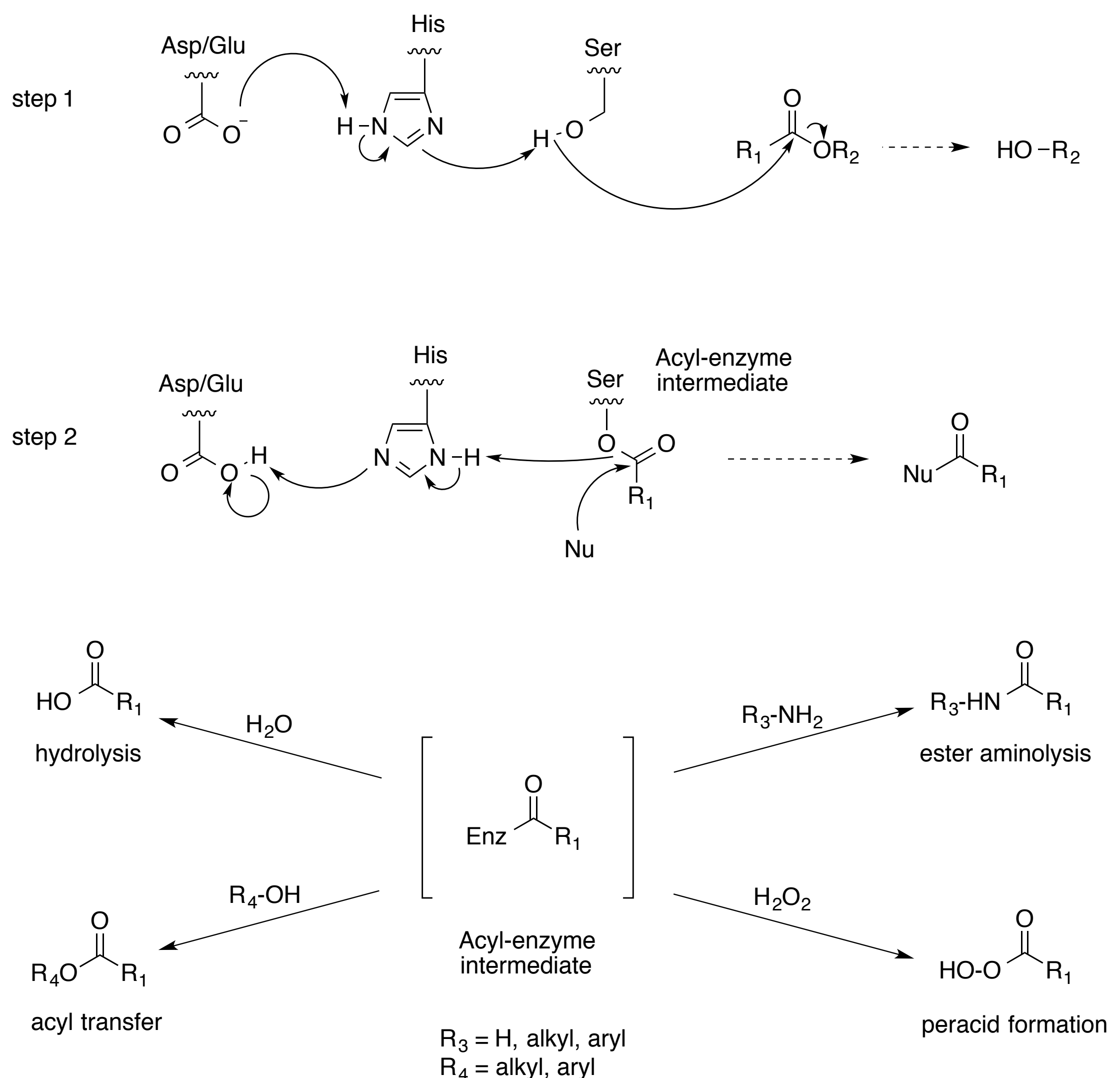
1.1.4 Hydrolases

More than 60% of enzymes explored in the field of biocatalysis are hydrolases.[14] Hydrolytic reactions are easy to perform using biocatalysts because they don't require cofactors and they show relaxed substrate specificities. Many enzymes such as esterases, lipases and proteases are used for hydrolytic reactions.
The mechanism of these hydrolyzing enzymes is similar to the mechanism observed for the base catalyzed hydrolysis. The mechanism for serine hydrolases is described in detail (**Scheme 1.1-1**).[15]
The catalytic triad consists of aspartic acid (Asp), histidine (His) and a serine (Ser) residue, which provides a hydroxy group as actual chemical operator of the catalyzed reaction. The aspartic acid and histidine act as acid and base to reduce the pKa of the neighboured serine hydroxy group. This enables the serine residue to perform a nucleophilic attack on the carbonyl group of the substrate (**Scheme 1.1-1**, step 1). R_2-OH of the substrate act as a leaving group and an acyl-enzyme intermediate is formed. The acyl-enzyme intermediate is attacked by another nucleophile (Nu) to regenerate the enzyme and in case of water as Nu, a carboxylic acid as product is formed (step 2).
In a water poor environment, other nucleophiles can compete with water for the nucleophilic attack leading to different useful products (**Scheme 1.1-1**).

- Interesterification (or so called acyl transfer) may occur by attack of an alcohol leading to esters. [16,17]
- Substituted amines or ammonia as nucleophiles give N-substituted amides (R-NH_2 as nucleophile) or carboxamides (NH_3 as nucleophile) as products. Employing ammonia

as nucleophile the reaction is called aminolysis[18,19] or in case of amines as nucleophiles ester aminolysis.[20,21]

- If hydrogen peroxide is used as nucleophile peracids may be formed.[22]

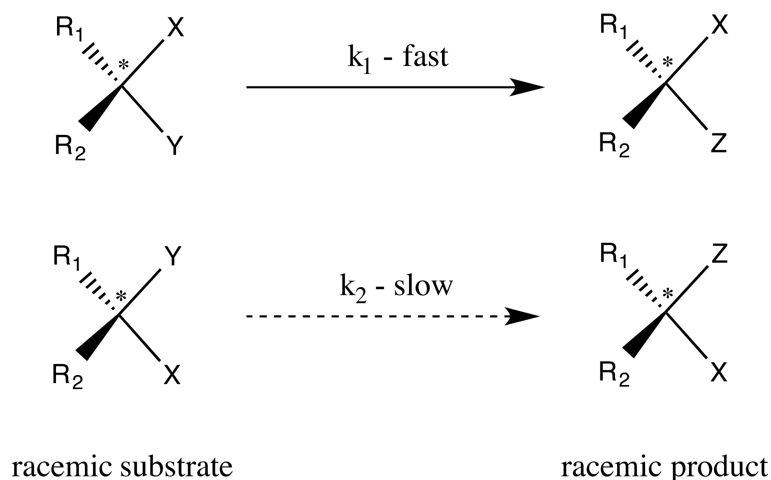


Scheme 1.1-1: Serine hydrolase mechanism. Adapted from Kurt Faber: Biotransformations in organic chemistry.[23]

Aspartic acid (Asp) can be replaced by glutamic acid (Glu), which has the same chemical function for this reaction (deprotonate the histidine residue). This exchange in amino acids has been reported in acetylcholine esterase from *Electrophorus electricus*[24] and a lipase from *Geotrichum candidum.*[25]

1.1.5 Kinetic and dynamic kinetic resolution of racemates by hydrolases

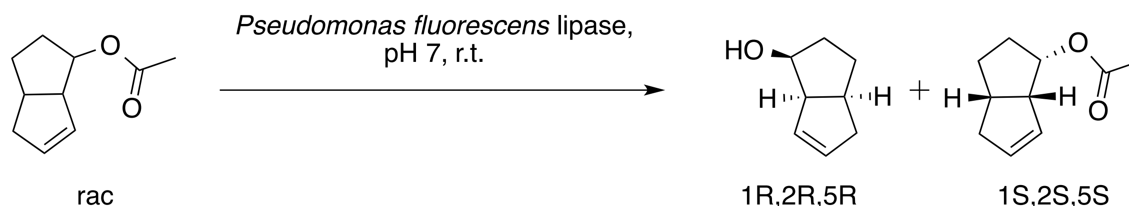
As mentioned before, enzymes are able to recognize chiral centers of molecules, since they harbor chiral active sites. Thus when using a racemic substrate, one enantiomer may fit better in the active site than the other and is therefore preferably converted. This results in a kinetic resolution of the racemic substrate. Giving the enzyme enough time to convert the substrate, it will end up producing both product enantiomers (50% each) (**Scheme 1.1-2**).



Scheme 1.1-2: Enantiomer differentiation *via* kinetic resolution.

After the preferred enantiomer of the substrate is fully converted ideally only the other enantiomer is left and the enzyme converts it until a racemic product is obtained. The enantiomeric ratio is defined as $E = \frac{k1}{k2}$, and the enantiomeric excess (*e.e.*) becomes a function of the conversion (**Scheme 1.1-2**).

In some ideal cases enzymes prefer one enantiomer significantly over the other, that it will end up producing 50% of the desired product enantiomer and leave 50% of substrate unconverted (**Scheme 1.1-3**).[26]

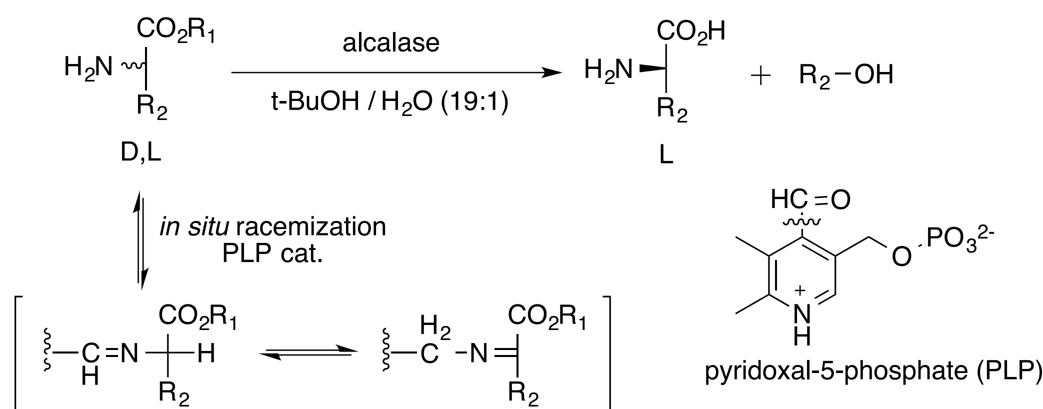


Scheme 1.1-3: Example for kinetic resolution employing lipase from *Pseudomonas fluorescens* as biocatalyst.[26]

A further approach is dynamic kinetic resolution.[27,28,29] This combines a classical kinetic resolution, while the substrate constantly racemizes during the biotransformation. Since several reactions occur simultaneously their reaction rates determine the stereochemical outcome of the process. A suitable dynamic kinetic resolution should fulfill the following criteria:[30,31]

- No spontaneous hydrolysis
- No racemization of the product
- The enzyme should provide a high preference towards the conversion of one enantiomer of the substrate
- Racemization of the substrate should occur at a higher or at least at the same rate as the biotransformation to ensure that always a sufficient amount of the favored enantiomer is present.

For dynamic kinetic resolution the *e.e.* is not a function of the biocatalytic conversion. A big advantage of the dynamic resolution approach is that 100% of the desired enantiomer may be produced and not a maximum of 50% as for the kinetic resolution approach. An example for a dynamic resolution is given in **Scheme 1.1-4**.



Scheme 1.1-4: Dynamic resolution of amino acid esters employing a protease from *Bacillus licheniformis*.[32]

Pyridoxal-5-phosphate (vitamin B_6) forms a Schiff base with the amino acid ester, thereby racemization occurs (**Scheme 1.1-4**).[33]

1.1.6 Ester substrates for hydrolysis catalyzed by esterases and proteases

Amide bonds are stronger than ester bonds. Hence it is reasonable that proteases are capable of cleaving ester bonds, but esterases are in general not capable of amide hydrolysis. Ester substrates show two possibilities of carrying a chiral center and may therefor be grouped into two substrate types (**Figure 1.1-1**).

R_1,R_2 = alkyl, aryl; R_3 = Me, Et

Figure 1.1-1 Types of substrates for esterases and proteases.

The chiral centre may be at the 'acid moiety' (Type 1) or at the 'alcohol moietyt' (Type 2, alcohol is released after hydrolysis reaction).

For the selection of substrates for proteases and esterases some rules may be applied:

- H is required at the chiral center, otherwise the substrate becomes too bulky (some exceptions are reported in literature).[34,35,36]
- For selective conversions the chiral center should be located as close to the carbonyl group as possible.
- Substituents R_1 and R_2 should differ in size to obtain high selectivity of the enzymes. At these positions charged or highly polar functional groups (–OH, -COOH, - NH_2, etc.) should be avoided. If those highly polar groups are required, a lipophilic group may protect them.
- R_3 of Type 1 substrates should be as short as possible. The same is applicable for Type 2 substrates, while electron withdrawing-substituents (e.g. halogens) may increase the reaction rate.[37]

1.1.7 Pig liver esterase (PLE)

Compared to other liver esterases (chicken, hamster, rats, etc.) PLE shows an exceptional versatility and has therefor an important role in industrial processes.[38] The natural function of PLE is the hydrolysis of esters present in the diet of the mammalians, which explains the wide substrate tolerance. For industrial applications a crude acetone powder is used, which can easily be prepared from pig liver offal.[39] PLE consists of several isoenzymes, which show differences in isoelectric point, molecular weight and substrate specificity.[40]

To overcome the undesirable presence of several isoenzymes and the interference of other enzymes present in the crude preparation, 5 isoenzymes were successfully expressed heterologously in *E. coli*. For this purpose a signal-sequence (18 amino acids) was omitted.[41] The expressed isoenzymes showed different characteristics regarding sensitivity towards inhibitors and differences in the specific activity towards chiral esters. Further studies with the isoenzymes showed different enantiopreferences in desymmetrization reactions of acetates.[41]

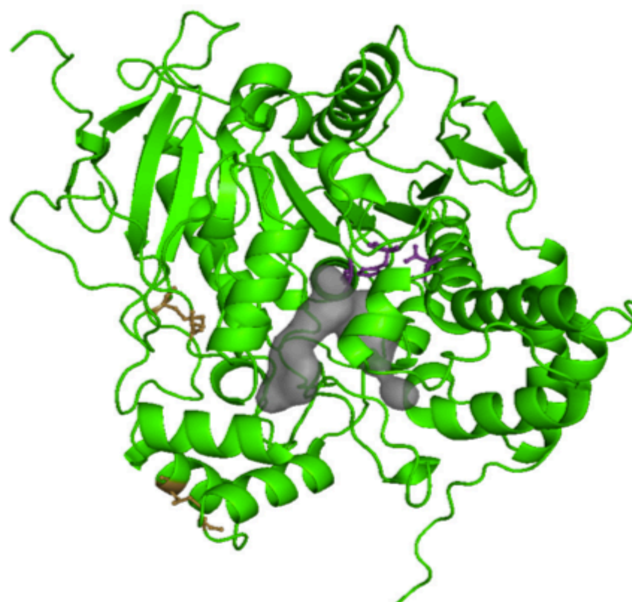


Figure 1.1-2: Model of a PLE monomer. The active site is shown in grey. Model was calculated on the base of the crystal structure of human carboxylesterase (pdb code: 1MX1).

PLE contains 5 cysteine residues, which may form 2 disulfide bridges (70 - 99, and 256 – 267).[42] Thus for expression in *E. coli*, helper proteins (chaperones such as DsbC) lead to better soluble enzyme.[43]

1.1.8 Horse liver esterase (HLE)

In some cases horse liver esterase (HLE) showed to be a useful substitute for PLE.[44,45] The natural function of HLE is also the hydrolysis of esters present in the food of horses. Crude preparation of HLE contains 6 isoenzymes.[46] The isoenzymes carry 4-5 cysteine residues, which might form 2 possible disulfide bonds.

Heterologous expression of the isoenzymes was not reported yet and is part of this thesis. Since HLE shares a lot of characteristics of PLE and disulfide bridges might be essential for active protein, similar expression conditions might lead to successful expression in *E. coli*.

HLE and PLE are suitable enzymes for enzymatic resolution chiral lactones with high to moderate *e.e.* of the produced hydroxy acids and the supplied lactones.[47]

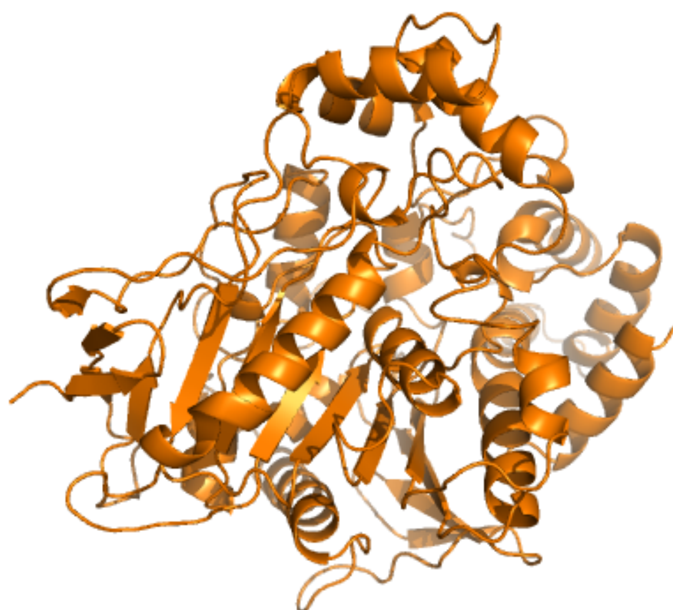


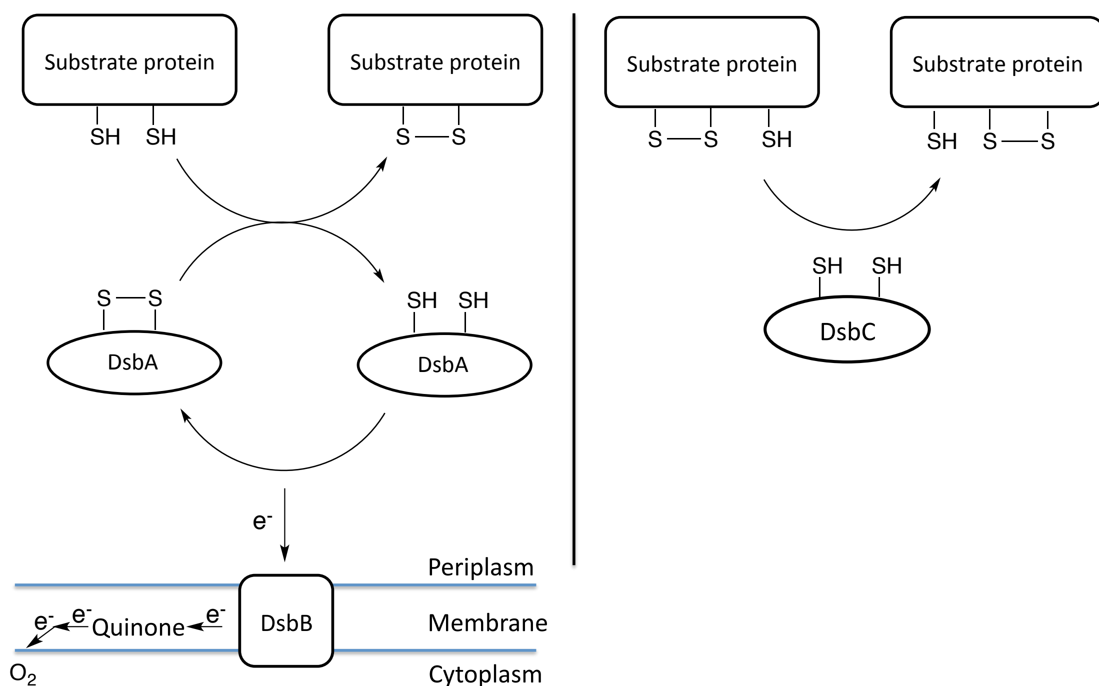
Figure 1.1-3: Model of a HLE monomer. The model was calculated on the base of the crystal structure of human carboxylesterase (pdb code: 1MX1).

1.2 Disulfide bridge formation in *E. coli*

Disulfide bonds are post-translationally formed protein modifications between thiol groups of cysteines. They may be essential for correct protein folding and increase the stability of proteins. Additionally they may have catalytic (oxidoreductases) and signaling roles (stress

response). In prokaryotic cells, they are usually formed in the periplasm and they mainly occur in proteins secreted to the extracellular environment.[48]

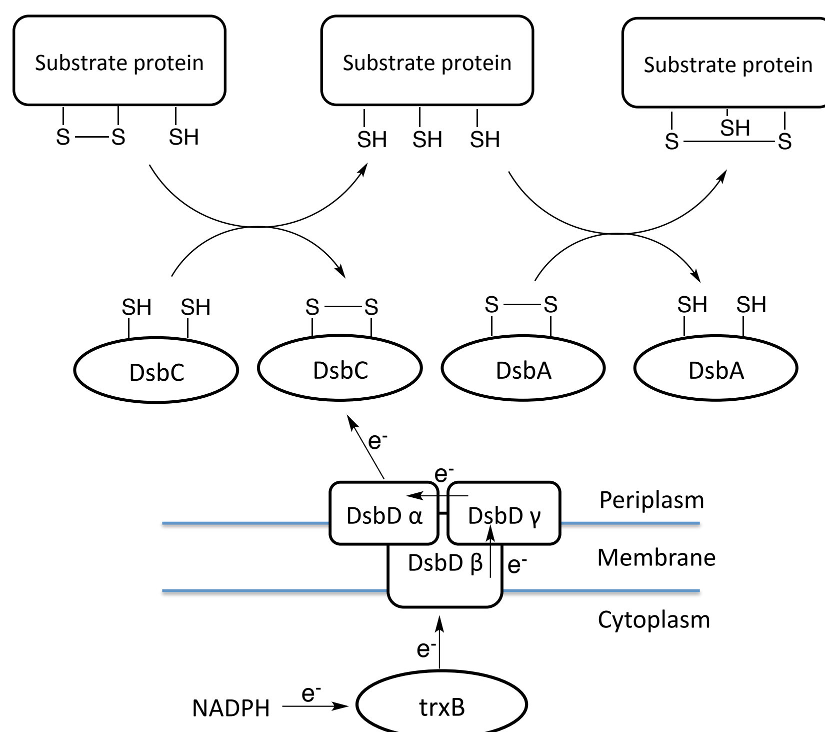
Gluthatione reductase (gor) and thioredoxin reductase (trxB) are two enzymes, involved in reducing mechanism of disulfide bonds in the cytoplasm of *E. coli* cells. Disulfide bond formation may be supported by chaperones such as the enzymy DsbA in the periplasm. DsbB is a membrane bound protein able to reoxidize the reduced form of DsbA. Consequently DsbB passes the electrons to quinones and then they are passed further to molecular oxygen (**Scheme 1.2-1**).[49]



Scheme 1.2-1: Disulfide bond formation by DsbA and isomerization by DsbC.

If two or more disulfide bonds are present in a protein, mispairing may occur, which may cause misfolding. The enzyme DsbC is capable of breaking disulfide bonds and form new ones. Thus it acts as disulfide bond isomerase and it is thereby released as reduced form (**Scheme 1.2-1**).

DsbC is also capable of exchanging disulfide bonds of a misfolded protein releasing DsbC in an oxidized form and the substrate protein in a reduced form. DsbA then reoxidizes the substrate protein and the membrane bound DsbD regenerates DsbC. DsbD is kept reduced by trxB, present in the cytoplasm utilizing NADPH as electron donor (**Scheme 1.2-2**).[49,50]

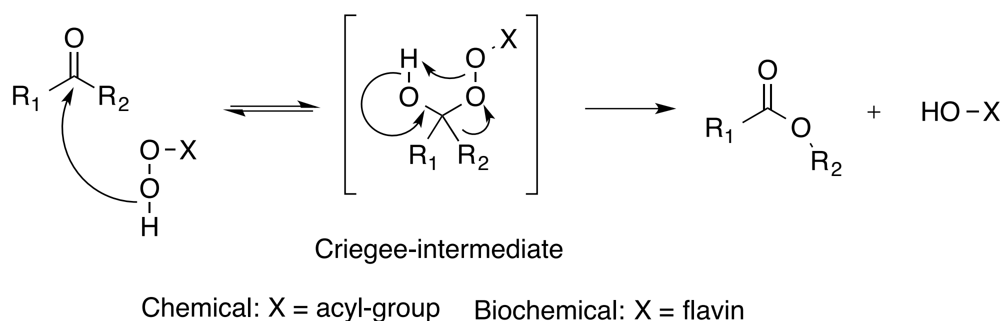


Scheme 1.2-2: DsbC reduces the misfolded disulfide bond. Afterwards DsbA introduces a disulfide bond in the substrate protein. DsbD regenerates DsbC. Within DsbD electrons flow from β domain to the γ and α domains subsequently.

1.3 Baeyer-Villiger oxidation

For preparation of lactones or esters the Bayer-Villiger oxidation is a suitable method.[51,52] Chemical Bayer-Villiger oxidation is carried out employing peroxy acids (peroxybenzoic acid, peracetic acid, *meta*-chloroperoxybenzoic acid, etc.). For biocatalytic Bayer-Villiger oxidation monooxygenases are used, which require an oxidized flavin species as cofactor to perform the nucleophilic attack.

The mechanism of chemical and biocatalytic Bayer-Villiger oxidation are closely related (**Scheme 1.3-1**).[53,54,55]



Scheme 1.3-1: Proposed mechanism for chemical and enzymatic Bayer-Villiger oxidation.

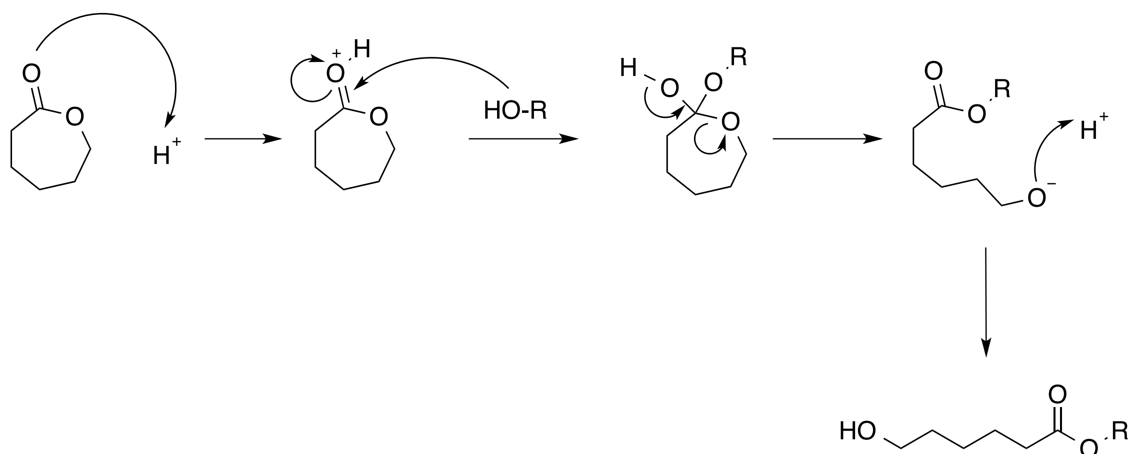
The carbonyl group of the ketone is attacked by the peracid to form the Criegee intermediate.[56] The carboxylate ion is expulsed and a C-C bond is migrated, which lead to the formation of lactones or esters. Employing asymmetric ketones, the position of the oxygen insertion can usually be predicted. The carbon atom, which has the best ability to support a positive charge, will migrate if not sterically hindered.[57]

General migration tendency: *tert*-alykl > *sec*-alkyl > *prim*-alkyl.

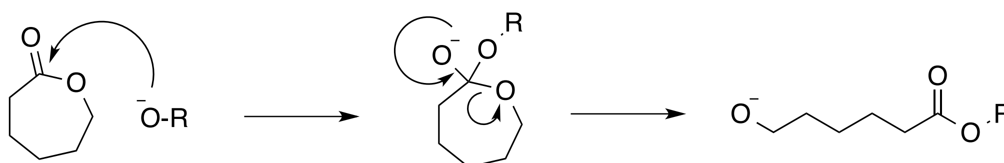
The strengths of Bayer-Villiger monooxygenases are chiral recognition and symmetric cyclic ketones may be oxidized to asymmetrical lactones.[58] These enzymes are also capable of kinetic resolutions using racemic substrates.[59]

1.4 Chemical esterification of lactones

Chemical esterification of lactones may occur acid or base catalyzed and gives hydroxy esters as product.[60,61] Proposed mechanisms for both reactions are shown in **Scheme 1.4-1** and **Scheme 1.4-2**. In aqueous media the reaction is followed by subsequent hydrolysis of the produced hydroxy esters.



Scheme 1.4-1: Proposed mechanism for acid catalyzed esterification of ε-caprolactone.



Scheme 1.4-2: Proposed mechanism for base catalyzed esterification of ε-caprolactone.

Hydroxy esters are intermediates for a vast variety of reactions[60] and can also be used for polymerization reactions leading in general to polycaprolactone (PCL).

1.4.1 Polycaprolactone (PCL)

Polycaprolactone (PCL) is an important thermoplast because of its mechanical properties, its miscibility with other polymers and its biodegradability.[62] PCL has applications in different fields such as adhesives,[63] scaffolds in tissue engineering,[64] microelectronics,[65] packaging material and as contraceptives.[66]

In general there are 2 possibilities to obtain PCL: polycondensation of 6-hydroxyhexanoic acid and ring opening polymerization of ε-caprolactone (ROP).[67]

Synthesis of PCL *via* polycondensation of 6-hydroxyhexanoic acid is performed under vacuum. The water is removed to push the equilibrium towards the polymerization. No catalyst needs to be added to the reaction and the temperature is gradually increased from 80 °C – 150 °C.[68]

Also biocatalytic methods for polycondensation of 6-hydroxyhexanoic acid to PCL were established employing lipase from *Candida antarctica*.[69]

Another method to produce PCL is described by polymerization of ethyl 6-hydroxyhexanoate employing a lipase from *Pseudomona* sp.[70]

Also a cascade starting from cyclohexanol to give acces of PCL-oligomers was reported in literature.[71]

Compared to polycondensation, ROP gives a polymer with higher molecular weight and a lower polydispersity.[67]

1.5 Biocatalytic access to nylon-6 monomer

Two co-factor self-sufficient biocatalytic cascade modules were combined to give the successful transformation of cyclohexanol into nylon-6 monomer (6-aminohexanoic acid) only at the expense of oxygen and ammonia (**Scheme 1.5-1**). It was reported that the opening of intermediate ε-caprolactone to 6-hydroxyhexanoic acid led to an dead end of the cascade. Therefor an *in-situ* capping strategy of the carboxylic functionality by opening ε-caprolactone to methyl 6-hydroxyhexanoate was introduced employing horse liver esterase (HLE). The precursor ε-caprolactone was converted to methyl 6-hydroxyhexanoate in aqueous buffer in the presence of 10% v/v methanol. This was the first time it was shown that horse liver esterase is able to perform the selective ring opening of ε-caprolactone with preference for methanol over water as nucleophile.[72]

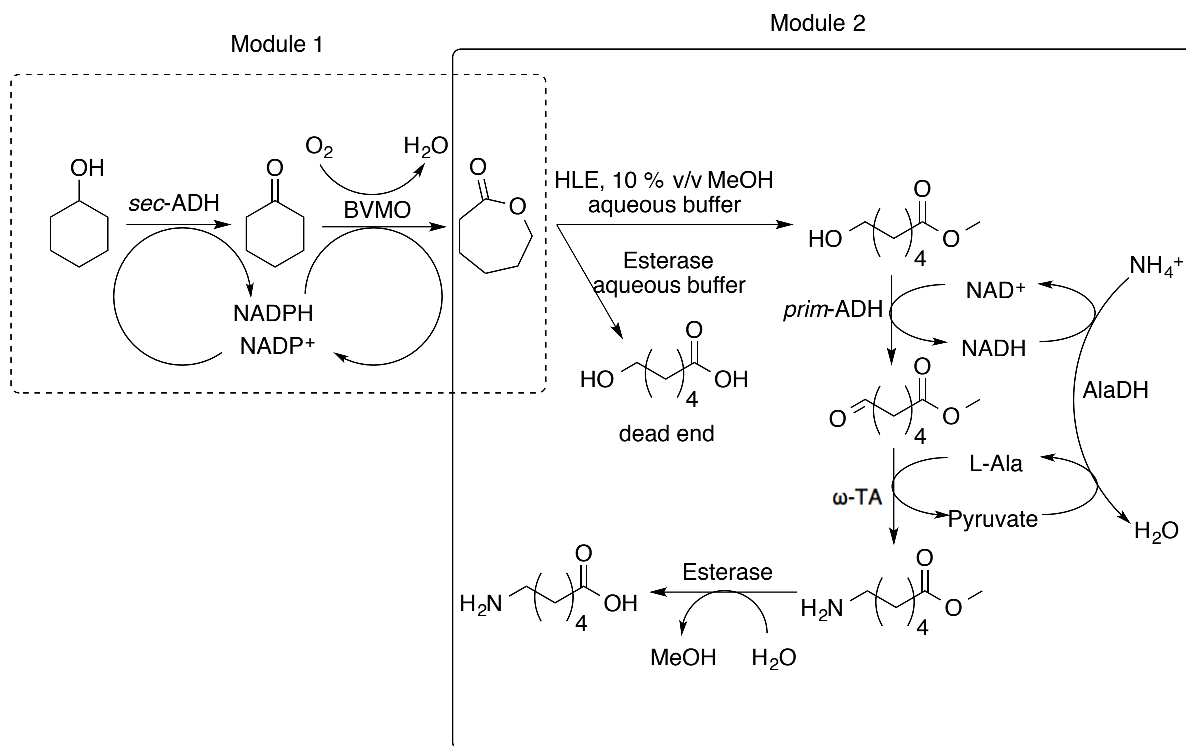


Figure 1.5-1: Biocatalytic cascade for production of nylon 6-monomer.[72]

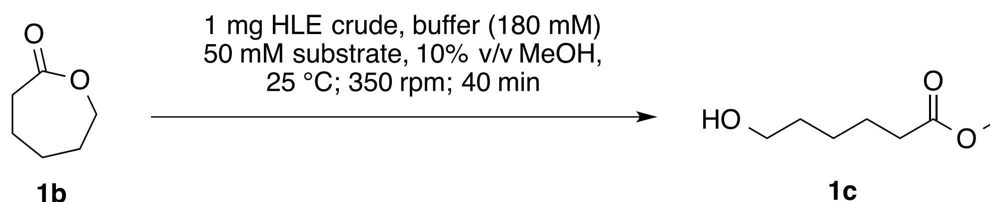
2 Results & Discussion

2.1 HLE crude preparation studies

Studies with a commercially available crude preparation of horse liver esterase (HLE) were performed. First of all optimal reaction conditions such as temperature and pH were investigated. Afterwards the biocatalytic conversion of ε-caprolactone (**1b**) to methyl-6-hydroxyheptanoate (**1c**) (MeOH was used as nucleophile) and 6-hydroxyhexanoic acid (**1e**) was followed over time. In addition to that EtOH was tested as a second nucleophile using the same reaction conditions as for MeOH.

2.1.1 HLE crude preparation buffer studies

Different buffers were prepared to show stability of HLE at different pH values. A list of the prepared buffers is shown in **Table 2.1-1**. Reaction conditions are shown in **Scheme 2.1-1** and results are summarized in **Figure 2.1-1.**



Scheme 2.1-1: Reaction conditions for buffer optimization study.

Table 2.1-1: List of prepared buffers for pH study.

pH	Buffer	Concentration [mM]
5	Citric Acid - Na_2HPO_4	200/100
6	Phosphate	200
7	Phosphate	200
7.5	Phosphate	200
8	Phosphate	200
9	TRIS-HCl	200
10	CAPS	200
11	CAPS	200

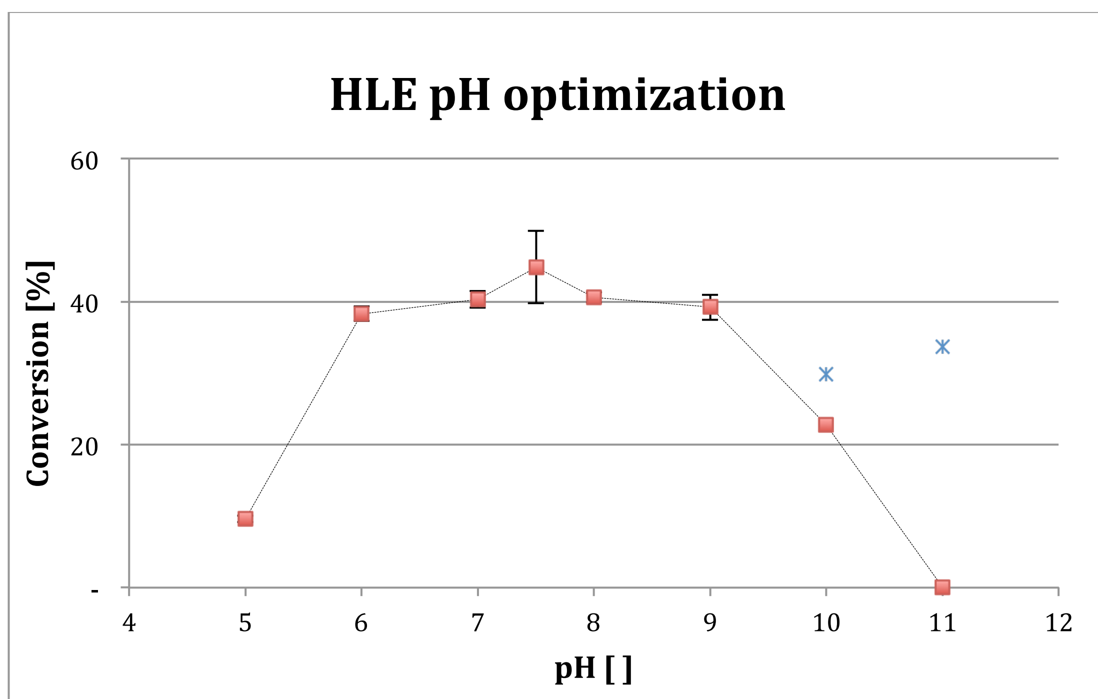
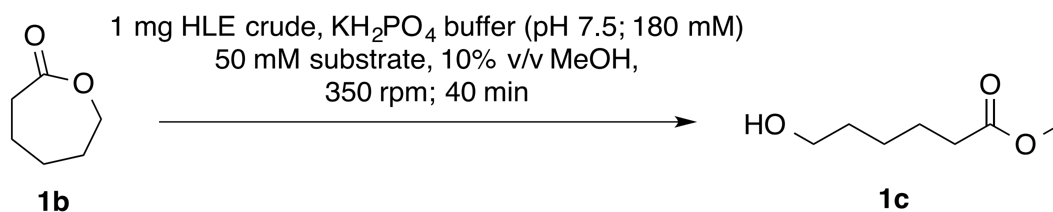


Figure 2.1-1: pH optimization study results. Formation of methyl-6-hydroxyhexanoate (**1c**) was followed (squares). Error bars represent standard deviation of triplicate measurements. Stars show the results of the background reactions (reaction performed without enzyme). For pH 10 and 11, blank reaction results were subtracted from biotransformation results (squares).

As shown in **Figure 2.1-1** the highest yield of methyl-6-hydroxyhexanoat (**1c**) was obtained using phosphate buffer at pH 7.5. At pH 10 and pH 11, ester formation was detected in the blank reactions (without enzyme). HLE shows best activity between pH 6 and pH 9.

2.1.2 HLE crude preparation temperature studies

Since the temperature is crucial for biocatalytic applications, a temperature optimization study was carried out at constant pH but varied reaction temperatures. The reaction conditions are given in **Scheme 2.1-2** and the results in **Figure 2.1-2.**



Scheme 2.1-2: Reaction conditions for temperature optimization study. Enzyme was dissolved in buffer and pre-incubated for 2 h at the varied temperature. Afterwards substrate and MeOH were added.

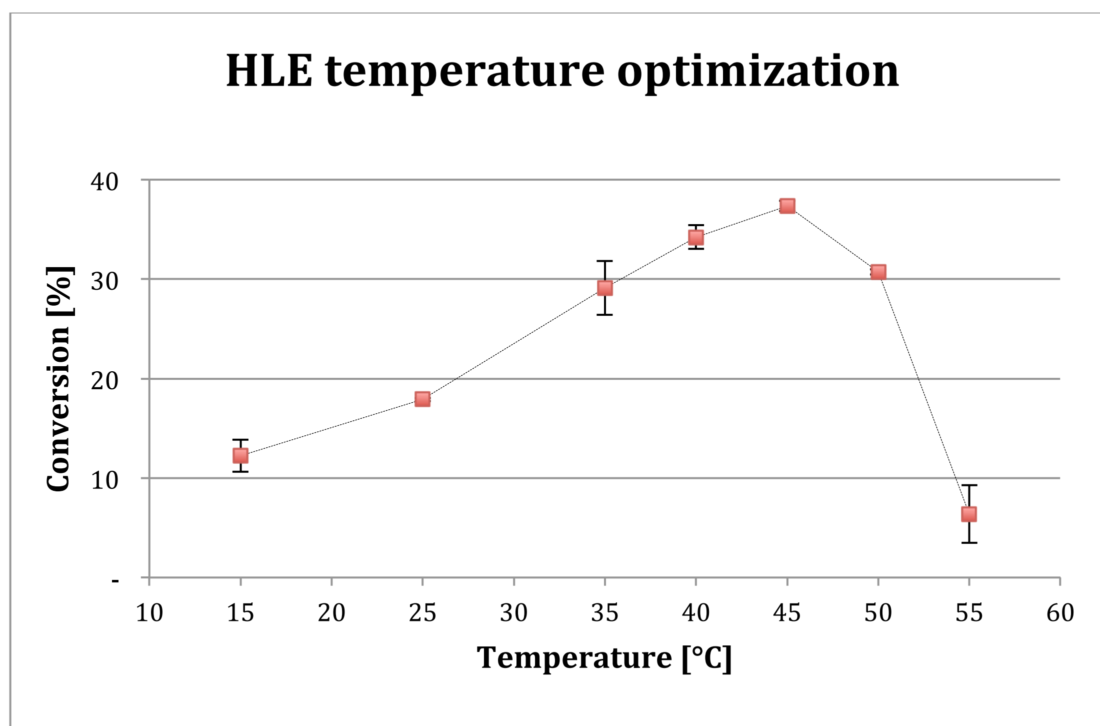
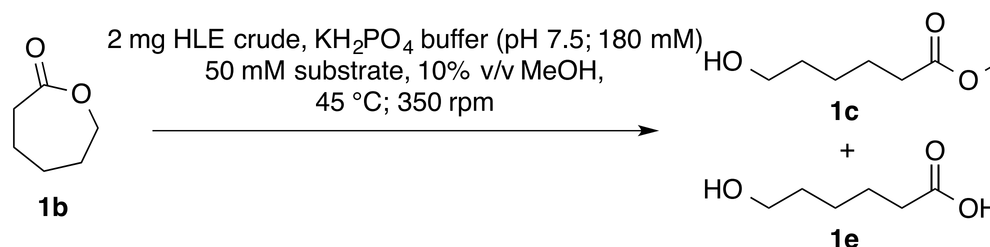


Figure 2.1-2: Temperature optimization study results. Formation of methyl-6-hydroxyhexanoate (**1c**) was followed. Error bars represent standard deviation of triplicate measurements.

Highest conversion to methyl-6-hydroxyhexanoate (**1c**) was obtained at 45 °C (**Figure 2.1-2**). At 55 °C the activity of HLE decreased significantly which indicates denaturation. 45 °C was chosen as the optimal reaction temperature for the conversion of ε-caprolactone (**1b**) to methyl-6-hydroxyhexanoate (**1c**) employing HLE crude preparation.

2.1.3 HLE crude preparation time studies

Since the natural function of esterases is to cleave esters to form the corresponding acid it is crucial to follow the reaction over time to see whether ester formation and hydrolysis are taking place subsequently or in parallel. Hence time studies were performed. As nucleophiles MeOH and EtOH were used separately. Reactions conditions are shown in **Scheme 2.1-3** and **Scheme 2.1-4**.



Scheme 2.1-3: Reaction conditions for time study employing MeOH as nucleophile. To equilibrate the temperature the reaction mixtures were pre-incubated without enzyme for 1 h.

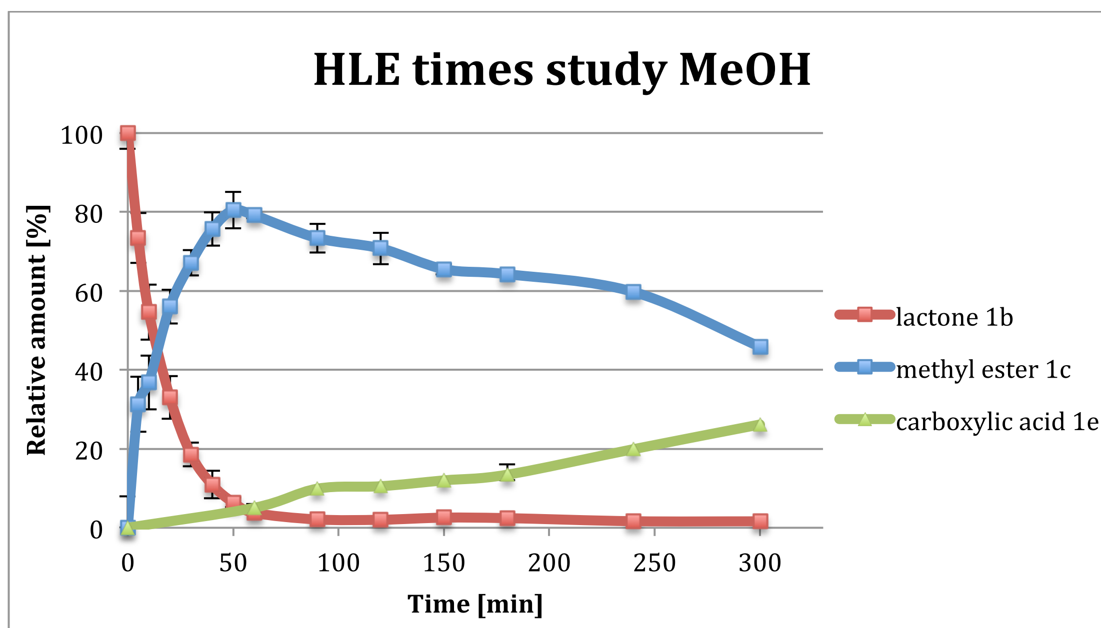
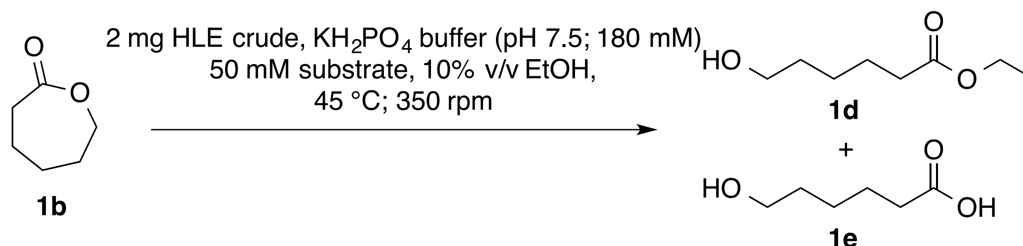


Figure 2.1-3: Time study results employing MeOH as nucleophile. Error bars represent standard deviation of triplicate measurements.

Figure 2.1-3 shows that HLE prefers to convert lactone **1b** to the corresponding hydroxy ester **1c** over hydrolysing the lactone to the corresponding hydroxy acid **1d**. The highest yield of ester **1c** obtained was around 80% and was reached after 1 hour. Lactone **1b** was fully

consumed after 1 hour and the enzyme started to hydrolyze ester **1c**. From that time on, the hydrolytic part of the reaction took over and already after 24 hours no significant amount of ester **1c** was detected anymore.



Scheme 2.1-4: Reaction conditions for time study employing EtOH as nucleophile. To equilibrate the temperature the reaction mixtures were pre-incubated without enzyme for 1 h.

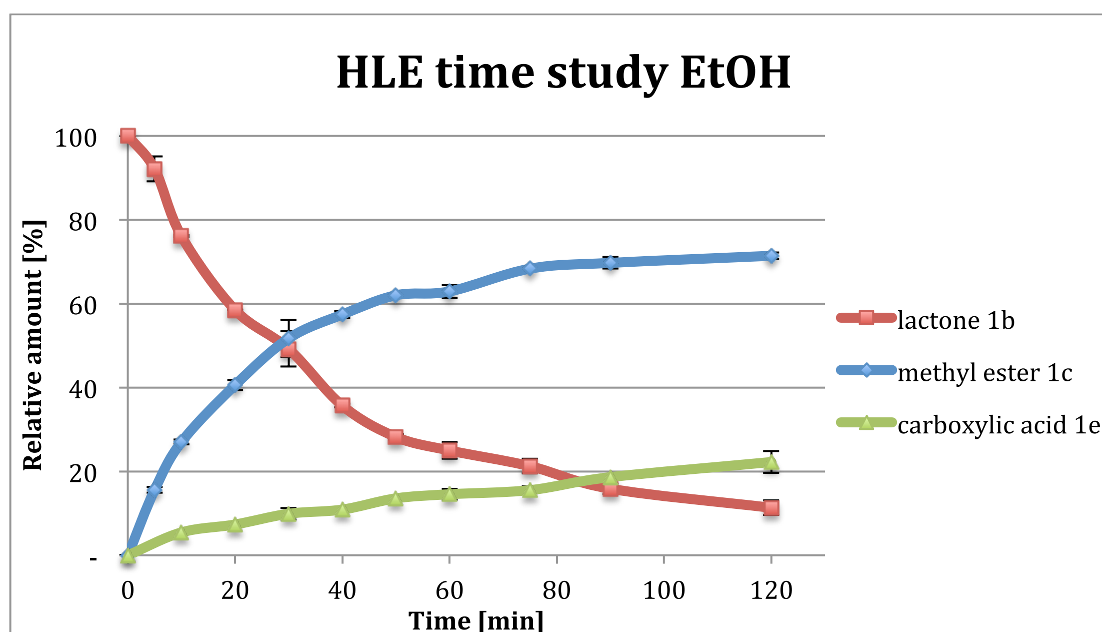


Figure 2.1-4: Time study results employing EtOH as nucleophile. Error bars represent standard deviation of triplicate measurements.

Figure 2.1-4 shows that employing EtOH as nucleophile leads to a decrease of the reaction rate compared to MeOH. The reaction was monitored for 120 minutes. At this time around 10% of lactone **1b** was still detectable, while already 20% of acid **1e** was formed. This indicates that acid formation occurs faster than with MeOH as nucleophile (**Figure 2.1-3**).

2.1.4 HLE crude preparation nucleophile studies

Different alcohols and methylamine were tested as nucleophiles for esterification and amidation of lactone **1b**. However, when employing alcohols with 3 or more carbon atoms (1-propanol, isopropanol, 1-butanol, 1-pentanol) as nucleophiles additional signals in the GC chromatograms appeared, but the products could not be identified *via* GC-MS. To give more information on the acceptance of the different longer chained nucleophiles and the potential amidation of lactone **1b** further investigation need to be done.

2.2 Gene cloning and expression

Genetic codes of 6 HLE isoenzymes were reported in literature.[73] To identify similar enzymes with the potential of esterification of lactone **1b** a BLAST search was performed, using isoenzyme 1_1 as template. A HLE carboxyl esterase (CES5) and an enzyme from *Equus przewalskii* with high sequence identity were found.

Genes coding for the 6 isoenzymes and the two related enzymes were adapted for expression in *E. coli.* Additionally a hydrolase from *Vibrio cholerae* (pdb code: 1R3D) harboring a hydrophobic active site pocket was previously cloned in IBA7Plus and expressed in *E. coli*. Since the enzyme didn`t show any activity (at 25 °C, pH 7.5) towards the esterification of lactone **1b**, no further studies were performed with it.

2.2.1 Protein structure prediction and signal sequence prediction

Based on an esterase of the human body (pdb code: 1MX1), models of the HLE enzymes were calculated and compared. Except of CES5, all of the found enzymes contained the active site residues (Ser, His, Glu) as shown in **Figure 2.2-1**. CES5 didn't contain glutamic acid (Glu) in the area of the active site and was not active. Therefore, there were no further studies performed with CES5.

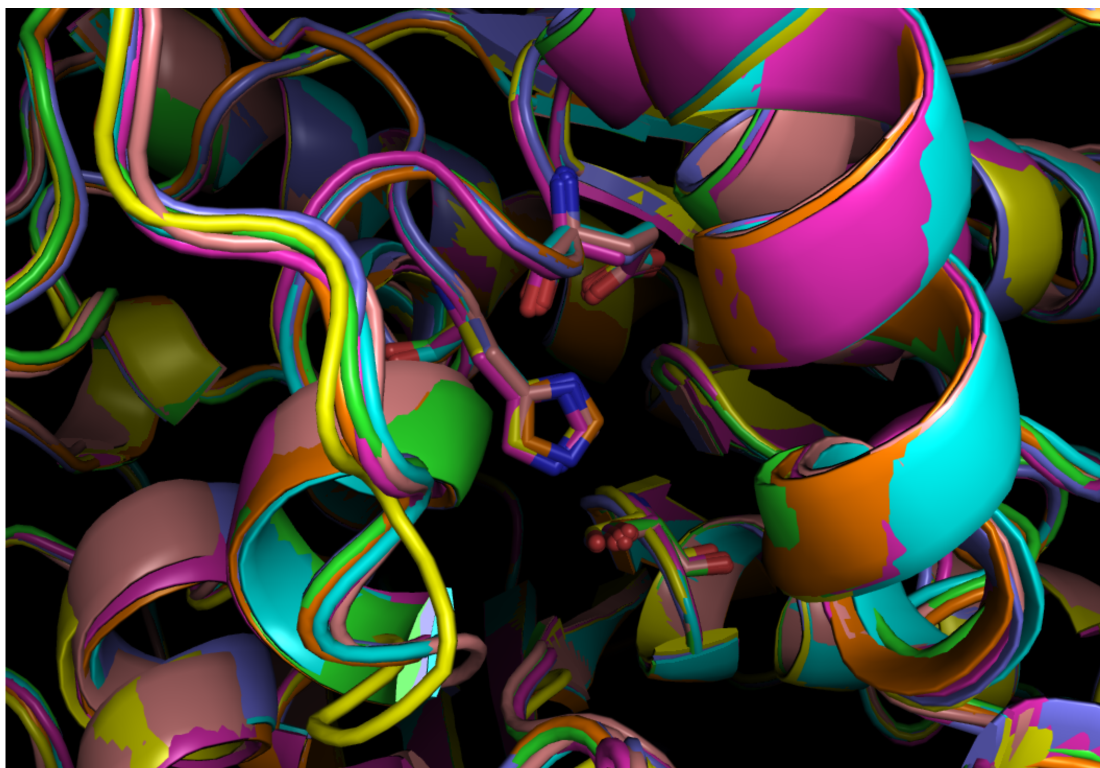


Figure 2.2-1 Overlay of all 6 HLE isoenzymes models and the model of *przewalskii* enzyme. Screenshot of the active site was taken in PyMOL.[74] The residues of the active site are shown in sticks. (Glu) top; (His) middle; (Ser) bottom. Models were prepared using Yasara and are based on an esterase of the human body (pdb code: 1MX1)

The found proteins derived from a higher eukaryotic organism (*horse*), which have complex pathways, often involving signal sequences for secretion. Since the aim was to express the enzymes in *E. coli* (prokaryotic organism), the proteins were checked for signal sequences using the SignalP- 4.1 tool.[75] A signal sequence, which ends between position 18 (Gly) and 19 (His) was found for all enzymes (**Figure 2.2-2**). This predicted signal sequences were deleted and genes were further processed for expression in *E. coli*.

The identity and the differences in amino acid sequence were compared by using CLC Workbench and are listed in **Figure 2.2-3**.

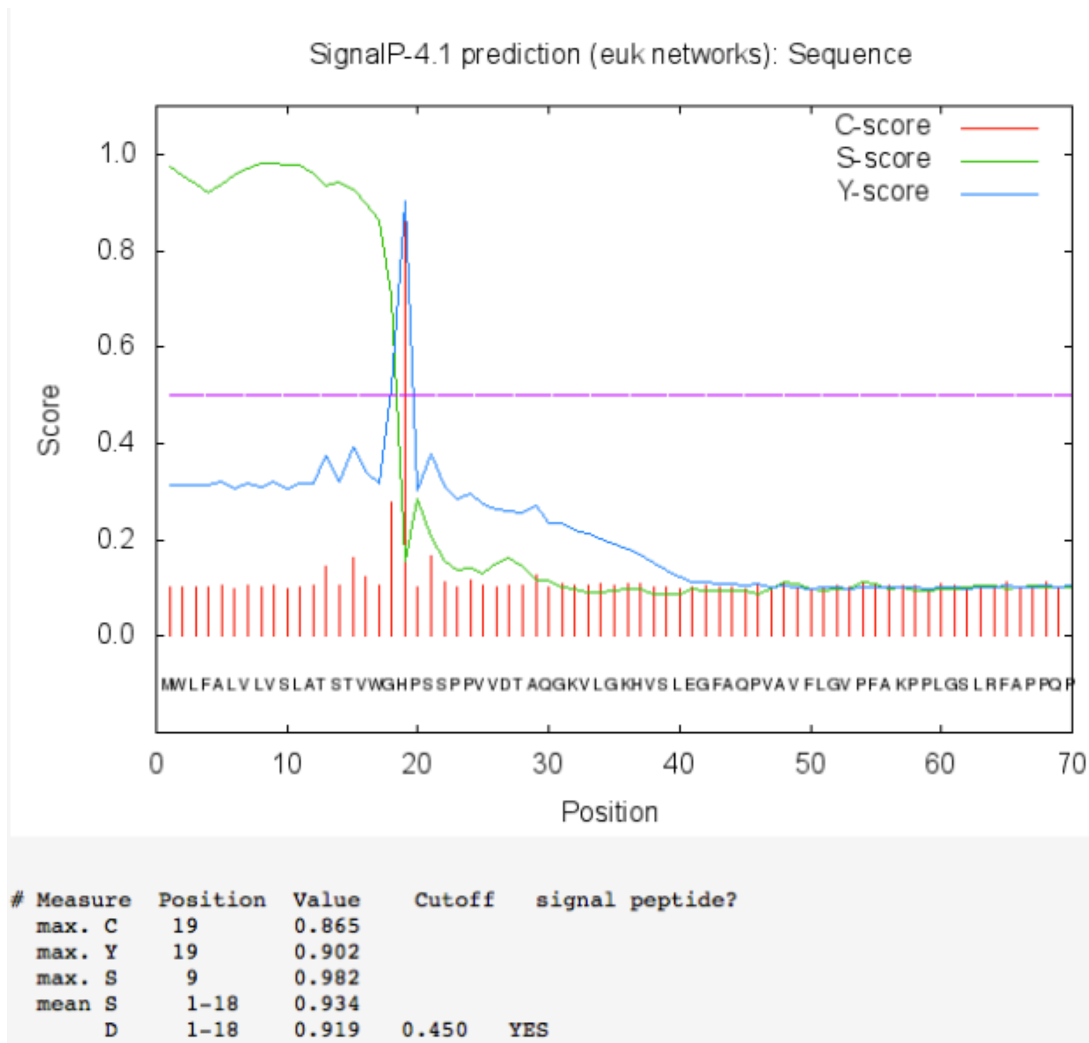


Figure 2.2-2: Example for SignalP results. Isoenzyme 1_1 was checked for signal sequences. Positive hits were found between position 18 (G) and 19 (H).[75]

		1	2	3	4	5	6	7
HLE_CES1.1	1		21	42	85	86	71	38
HLE_CES1.2	2	96.28		36	83	84	70	32
HLE_CES1.3	3	92.57	93.63		80	81	63	9
HLE_CES1.4	4	84.96	85.31	85.84		1	79	81
HLE_CES1.5	5	84.81	85.16	85.69	99.82		78	82
HLE_CES1.6	6	87.46	87.63	88.87	86.04	86.22		63
przewalskii	7	93.27	94.34	98.41	85.66	85.51	88.87	

Figure 2.2-3: Comparison of the 6 HLE isoenzymes and the esterease origination from *Equus przewalskii*. Identity is given in %. Integers show number of differing amino acids (HLE_CES – Horse liver carboxyl esterase, przewalskii – esterase from *Equus przewalskii*).

Lowest identities were found for isoenzyme 1_5 compared with 1_1 (84.81%). High identities were found for 1_4 and 1_5, where only one amino acid was different (insertion of Gln at position 361) (99.82%). 1_2 shows high identity with 1_1 (96.28%), enzyme from *Equus przewalskii* (93.27%) and 1_3 (92.57%). 1_3 show high identity with enzyme from *Equus przewalskii* (98.41%) and 1_1 (92.57%). The enzyme from *Equus przewalskii* also shows high identity with isoenzyme 1_1 (93.27%).

2.2.2 Gene design for cloning in pET-28a(+) and transformation into *E. coli*

Genes were ordered from LGC genomics as strings. To compare the importance of the signal sequence, construct 1_1 was ordered with and without the region coding for signal sequence. For all other constructs the coding region for the signal sequence was deleted. The pET-28a(+) vector harboring N-terminal His_6-tag was chosen for expression. NdeI and XhoI were used as restriction sites. Stuffer DNA was added at both ends of the genes to ensure digestion by the restriction enzymes. Vector map of pET-28a(+) is shown in **Figure 2.2-4.** Codons were optimized for expression in *E. coli* by LGC genomics. Final nucleotide sequences of the ordered genes are stored on enclosed CD "ordered genes" and are listed in the appendix section.

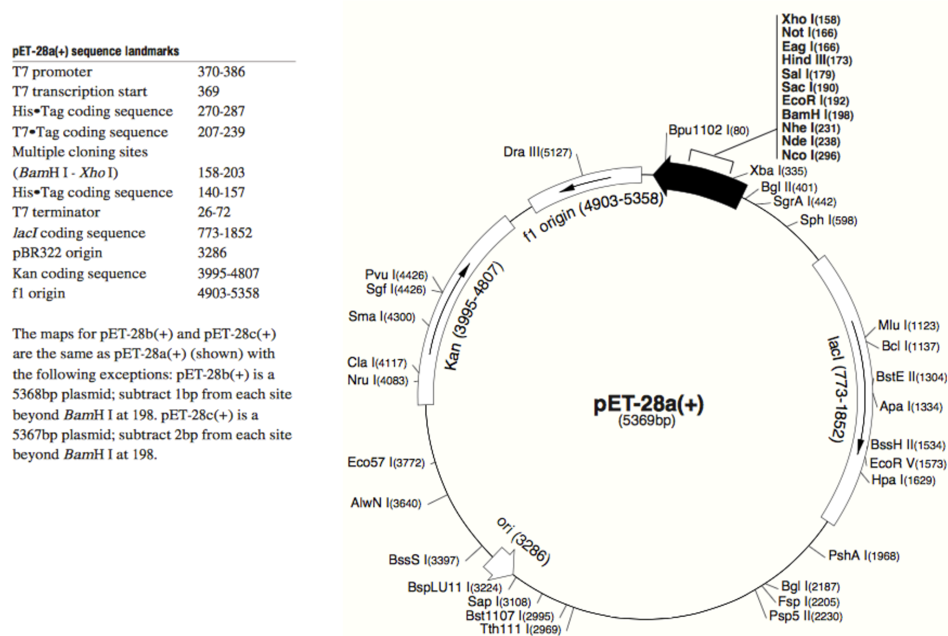


Figure 2.2-4: Vector map and features of pET-28a(+).[76] NdeI and XhoI were chosen as restriction sites for cloning. Thereby a N-terminal His_6-tag was provided.

The ordered genes were digested with the restriction enzymes NdeI and XhoI. The experimental setup for the restriction is given in **Table 4.9-1**. After digestion, the samples were analyzed on a 1% agarose gel (**Figure 2.2-5**).

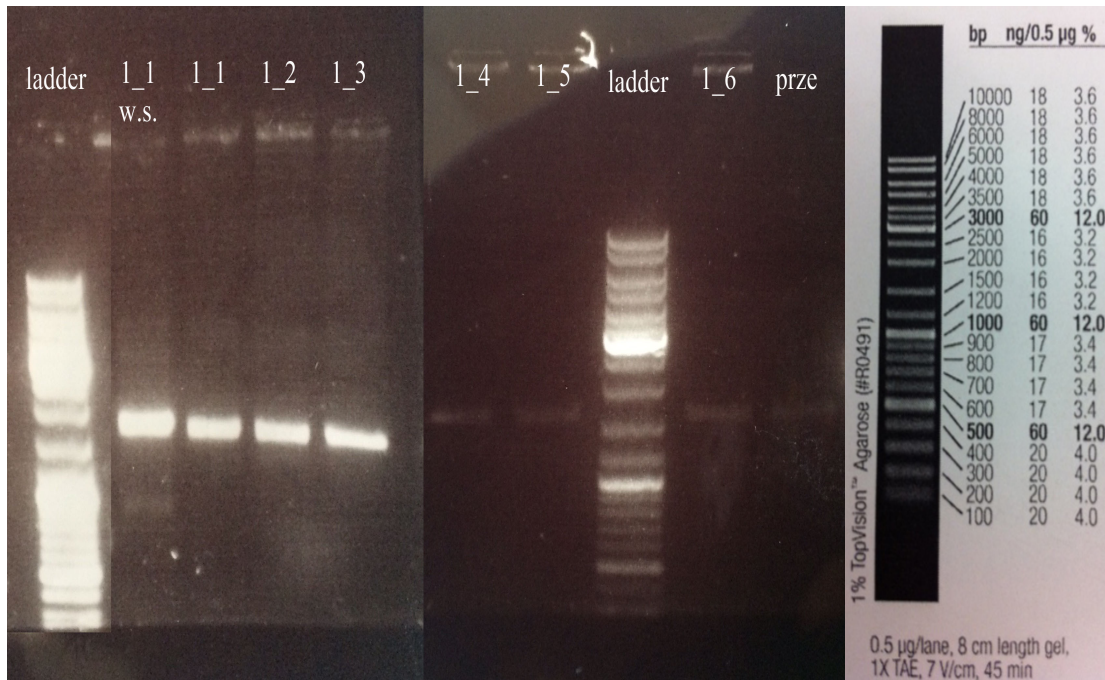


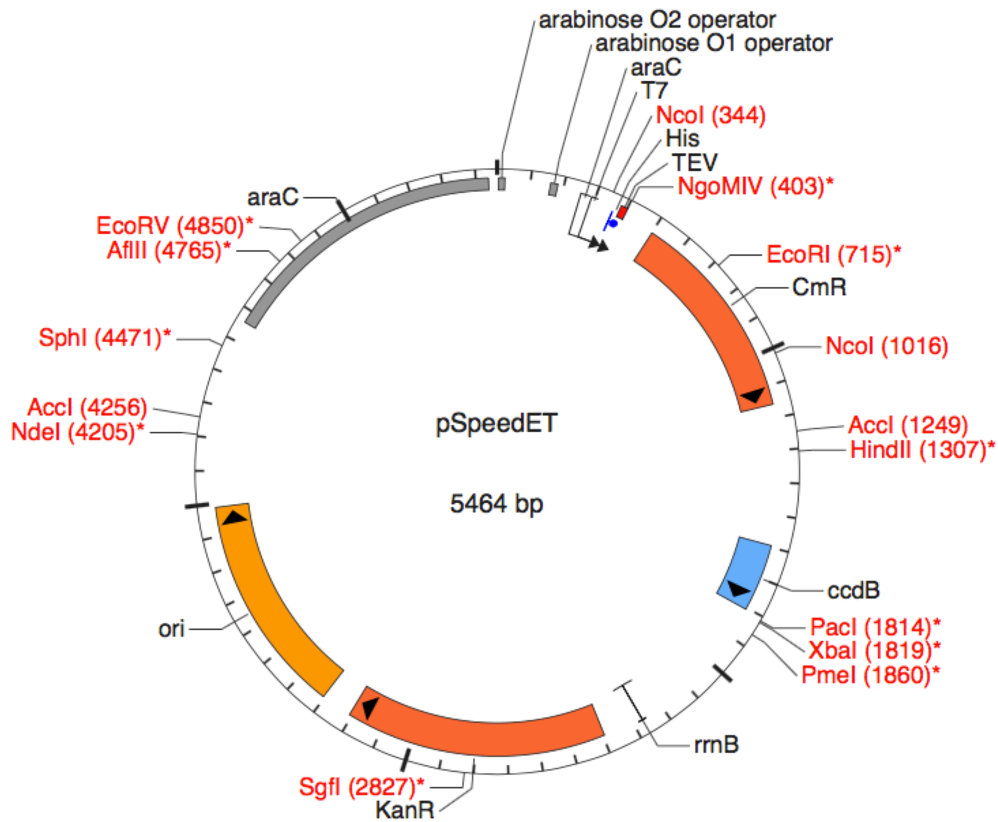
Figure 2.2-5: Agarose gel after digestion of the ordered genes with NdeI and XhoI. All samples showed the expected size (around 1700 bp). (Numbers indicate the number of isoenzyme; prze – enzyme from *Equus przewalskii*; 1_1 w.s – isoenzyme 1_1 with signal sequence).

The digested genes were purified *via* agarose gel followed by the isolation using a gel extraction kit (QIAGEN) and afterwards ligated into pET-28a(+) vector, which was digested before with the same restriction enzymes. After ligation, the recombinant plasmids were transformed into *E. coli* NEB5*α* cells in order to amplify the containing plasmid. The genes were cloned in a way, that the expressed proteins were equipped with a N-terminal His_6-tag. Transformants were plated on LB agar plates (KAN) and plasmids were isolated by QIAprep Spin Miniprep Kit. The mini-prep samples were sent for sequencing. After sequencing (all positive, see attached CD “sequencing”) the isolated plasmids were transformed into *E. coli* Shuffle® T7 cells.

2.2.3 Heterologous protein expression in *E. coli* Shuffle® T7

Cells were grown in LB Medium, protein expression was induced with IPTG (0.3 mM) and cells were harvested after 24 hours. The cells were resuspended and washed in phosphate buffer (200 mM, pH 7.5). After the cells were disrupted by ultrasonication (see Materials & Methods section), SDS PAGE was performed to check for overexpression (**Figure 2.2.7**). Supernatants and pellets were lyophilized afterwards.

Additionally recombinant *E. coli* Shuffle® T7 strain was grown carrying a *Bacillus subtilis* hydrolase (pdb code: 2R11 cloned into a pSpeedET vector; N-terminal His_6-tag, for vector features see **Figure 2.2.6**). Also a variant (Lys93Asn) of the *Bacillus subtilis* hydrolase was expressed successfully. Since it didn`t show enhanced activity compared to the wild type enzyme, the variant was not further investigated.



Part	Description	From	To	Type
ori	ColE1-type origin of replication	3306	3988	Bacterial Origin
araC	araC coding sequence	5438	4563	Other
ccdB	ccdB death cassette gene (lost in with-insert form)	1596	1805	CDS
araC	araC promoter	256	285	Promoter
T7	T7 promoter	290	310	Promoter
TEV	TEV protease cleavage site	382	402	Construction Suffix
CmR	chloramphenicol resistance gene (CmR) (lost in with-insert form)	502	1158	Antibiotic Resistance
KanR	kanamycin resistance gene	2396	3208	Antibiotic Resistance
His	N-terminal 6xHis tag	364	381	Signalling Peptide
arabinose O2 operator	arabinose O2 operator	5	23	Other
arabinose O1 operator	arabinose O1 operator	161	182	Other
rrnB	rrnB T2 transcription terminator	2258	2284	Terminator

Figure 2.2-6: pSpeedET vector map and features.[77] The *Bacillus subtilis* hydrolase gene was cloned into the vector allowing the expression with a N-terminal His$_6$-tag.

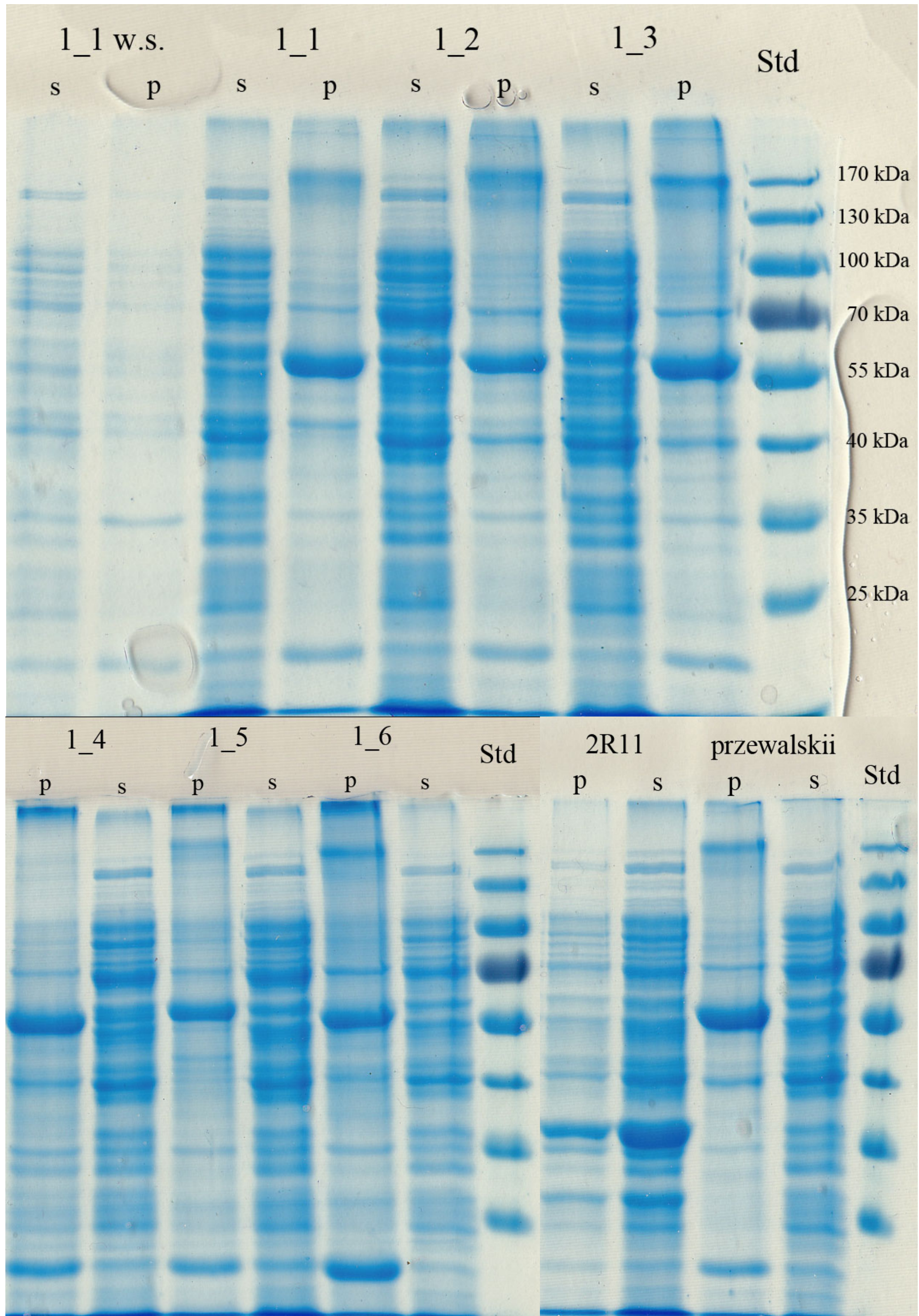


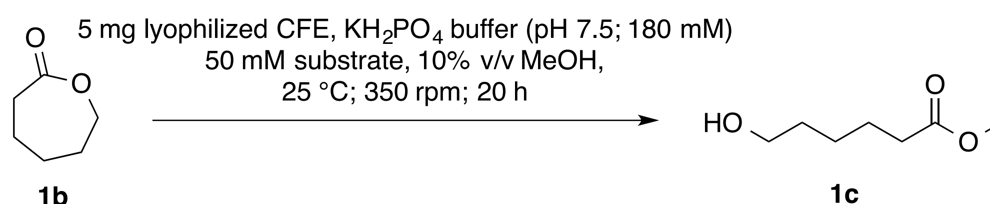
Figure 2.2-7: SDS PAGE results after expression trial in *E. coli* Shuffle® T7. For the HLE isoenzymes and esterase from *Equus przewalskii*, most of the enzyme is located in the pellet fraction (p - pellet fraction; s - supernatant fraction; std – standard; numbers indicate the number of isoenzyme; 1_1 w.s – isoenzyme 1 with signal sequence).

The SDS PAGE gels (**Figure 2.2-7**) showed some thick bands at around 60 kDa for the HLE enzymes and for the esterase from *Equus przewalskii* in the pellet fraction. There were also bands visible in the supernatant fraction. Overexpression of the *Bacillus subtilis* hydrolase (pdb code: 2R11; around 35 kDa) could be obtained as soluble protein in the supernatant and pellet fraction.

For isoenzyme 1_1 containing the signal sequence predicted by SignalP (**Figure 2.2-2**) no significant expression of the enzyme was detected. Already during the cultivation the expression host showed a reduced growth rate.

2.3 HLE isoenzymes and *Bacillus subtilis* hydrolase studies

First activity studies were performed employing lyophilized pellet and supernatant samples, obtained after cell disruption. Reaction conditions are given in **Scheme 2.3-1**. Results are shown in **Figure 2.3-1**.



Scheme 2.3-1: Reaction conditions for heterologous expressed enzyme screening. Lyophilized pellet and lyophilized supernatant samples were used and incubated at 25 °C. This reaction temperature was chosen since HLE crude preparation also showed highest activity at 25 °C. Consumption of lactone **1b** and formation of ester **1c** was followed by GC. Formation of carboxylic acid was calculated from the amount of lactone and ester (100% – lactone % – ester %).

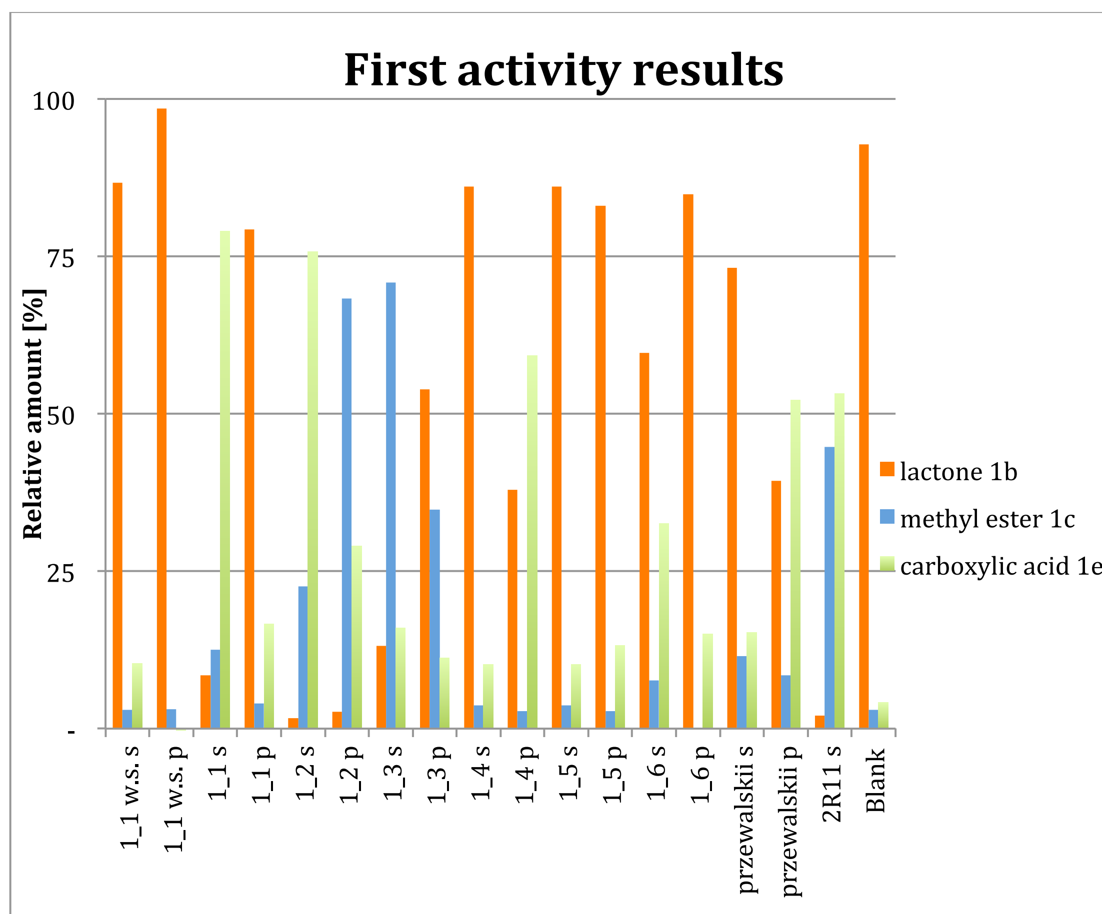


Figure 2.3-1: Activity results of heterologously expressed enzymes. (p – pellet; s – supernatant; w.s. - with signalsequence, 2R11 – hydrolase from *Bacillus subtilis*; numbers indicate the number of isoenzyme; Blank – no enzyme was added**).** Reactions were performed in duplicates. The sum of all components is 100%.

These initial activity tests (**Figure 2.3-1**) showed ester formation for the samples 1_2 (supernatant and pellet), 1_3 (supernatant and pellet), hydrolase from *Bacillus subtilis* (pdb code: 2R11, only supernatant was tested) and minimal for esterase from *Equus przewalskii* (supernatant and pellet).

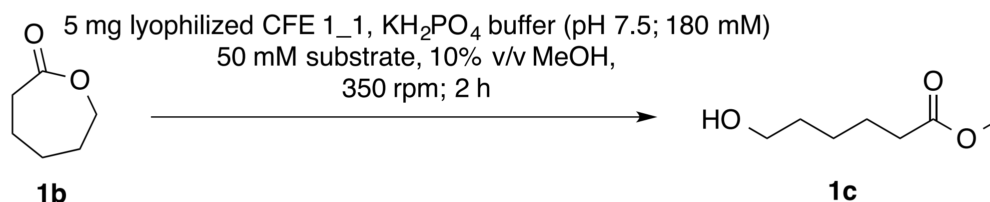
Carboxylic acid **1e** formation was observed in the samples 1_1 (supernatant and minor formation detected in pellet), 1_2 (supernatant and pellet), 1_3 (supernatant and pellet), 1_4 (pellet) 1_6 (minor formation detected in supernatant), esterase from *Equus przewalskii* (minimal for supernatant, 50% for pellet) and by hydrolase from *Bacillus subtilis* (only supernatant was tested).

The blank (no enzyme added) didn't show any conversion of lactone **1b**. Also 1_1 w.s. (containing the signal sequence), 1_4 (supernatant), 1_5 (supernatant and pellet) and 1_6 (pellet) showed no activity.

Since the best results were obtained with isonenzymes 1_1, 1_2, 1_3 and by the hydrolase from *Bacillus subtilis* (pdb code: 2R11), these enzymes were used for more detailed studies.

2.3.1 HLE Isoenzymes and *Bacillus subtilis* hydrolase temperature studies

The optimal reaction temperatures of the active enzymes were determined. Reaction conditions are shown in **Scheme 2.3-2**. All reactions were pre-incubated without enzyme for 2 hours. Consumption of lactone **1b** and formation of ester **1c** were followed by GC. Formation of carboxylic acid was calculated from the amount of lactone and ester (100% – lactone % – ester %).



Scheme 2.3-2: Reaction conditions for temperature optimization of isoenzyme 1_1. 5 mg of lyophilized cell free extract (CFE) was used.

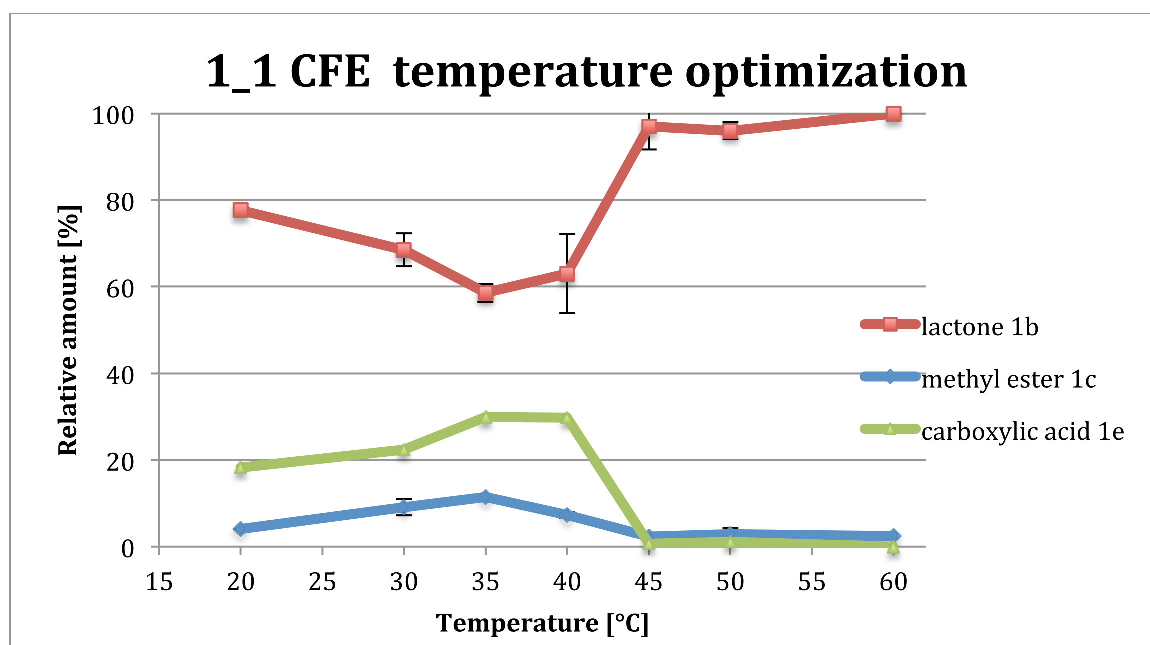
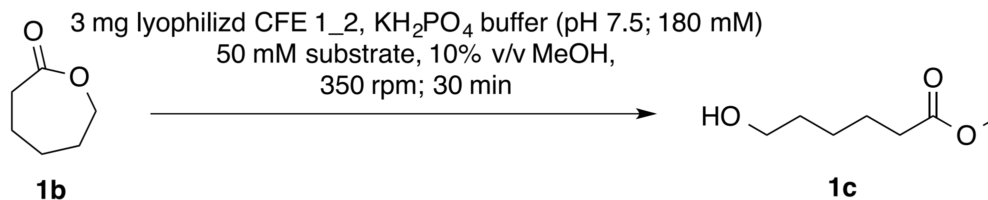


Figure 2.3-2: Results of temperature optimization study for isoenzyme 1_1. Error bars represent standard deviation of triplicate measurements.

Highest yield of ester **1c** and lowest amount of lactone **1b** was obtained at 35 °C. Carboxylic acid formation **1e** correlated with ester formation At 45 °C no ester formation and over 90% of lactone **1b** were detected for isoenzyme 1_1 which indicates denaturation (**Figure 2.3-2**).

35 °C was chosen as optimal reaction temperature for further studies employing isoenzyme 1_1.



Scheme 2.3-3: Reaction conditions for temperature optimization of isoenzyme 1_2. 3 mg of lyophilized cell free extract (CFE) was used.

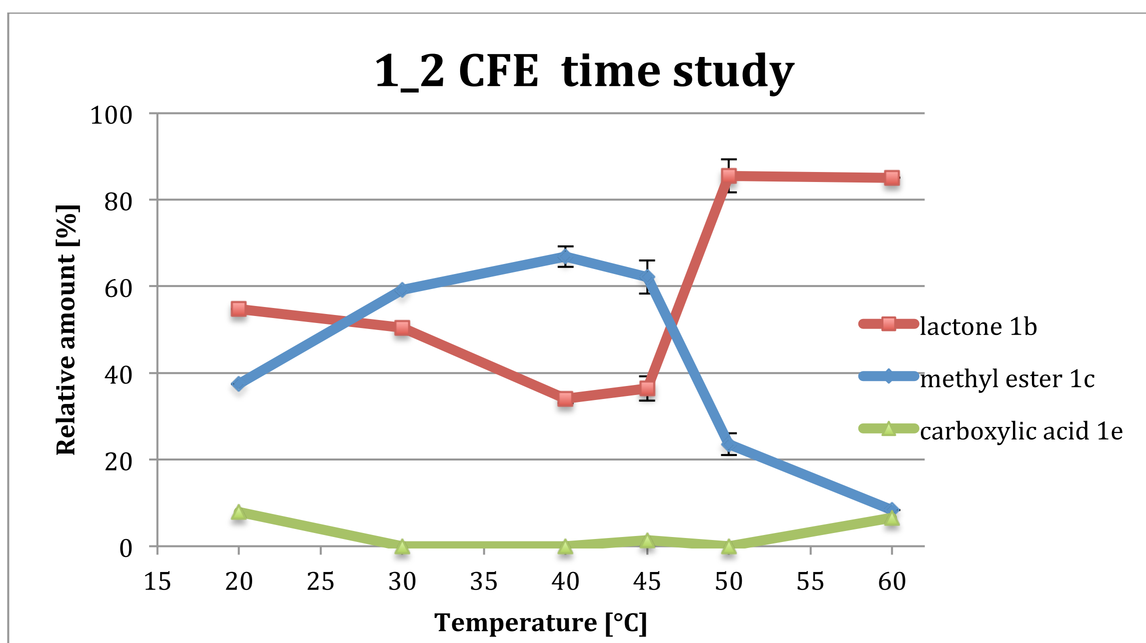
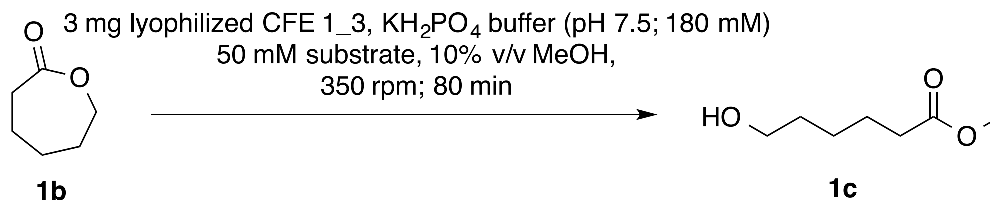


Figure 2.3-3: Results of temperature optimization study for isoenzyme 1_2. Error bars represent standard deviation of triplicate measurements.

Highest yield of ester **1c** and lowest amount of lactone **1b** was obtained at 40 °C employing isoenzyme 1_2. Carboxylic acid formation was not observed at that temperature. At 50 °C ester formation decreased significantly which indicates denaturation (**Figure 2.3-3**). 40 °C was chosen as optimal reaction temperature for further studies employing isoenzyme 1_2.



Scheme 2.3-4: Reaction conditions for temperature optimization of isoenzyme 1_3. 3 mg of lyophilized cell free extract (CFE) was used.

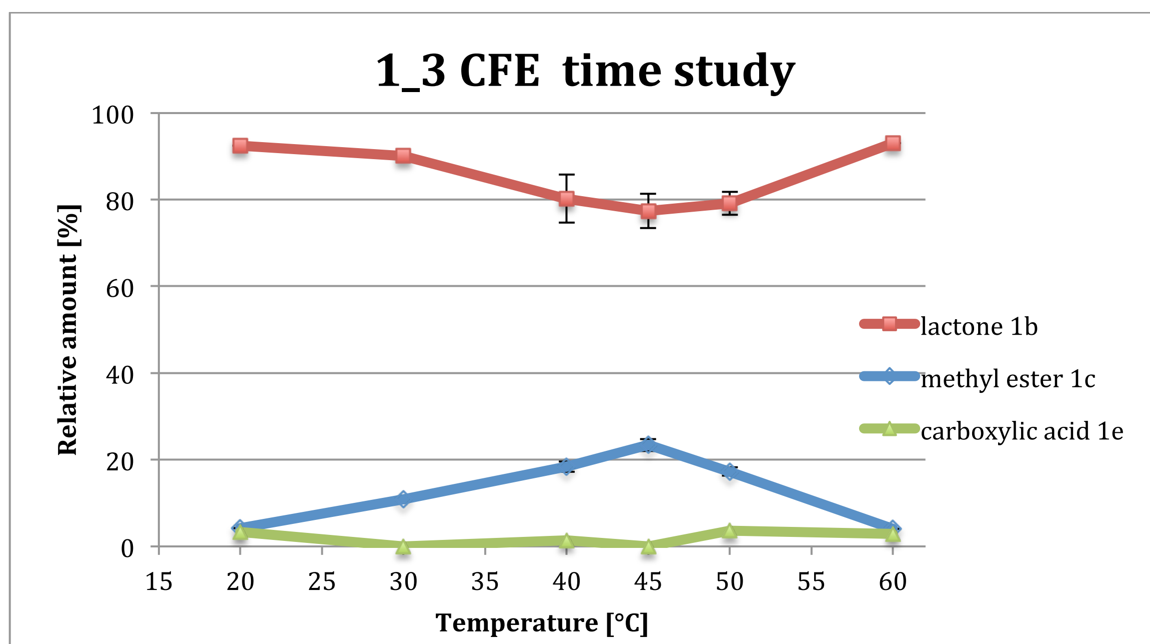
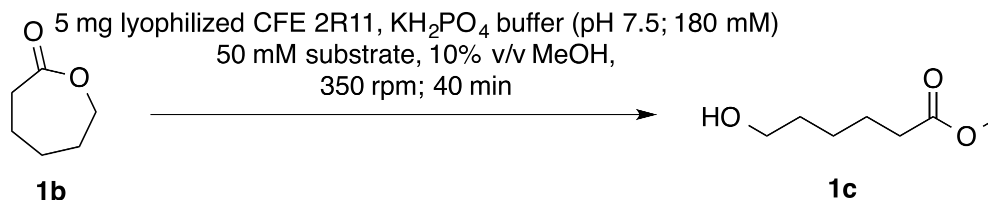


Figure 2.3-4: Results of temperature optimization study for isoenzyme 1_3. Error bars represent standard deviation of triplicate measurements.

Employing isoenzyme 1_3, highest yield of ester **1c** and lowest amount of lactone **1b** was obtained at 45 °C. At this temperature no carboxylic acid formation was observed. At 60 °C ester formation decreased significantly and over 90% of lactone was detected which indicates denaturation (**Figure 2.3-4**). 45 °C was chosen as optimal reaction temperature for further studies employing isoenzyme 1_3. This is the same temperature optimum identifiet for HLE crude preparation.



Scheme 2.3-5: Reaction conditions for temperature optimization of *Bacillus subtilis* hydrolase (pdi code: 2R11). 5 mg of lyophilized cell free extract (CFE) was used.

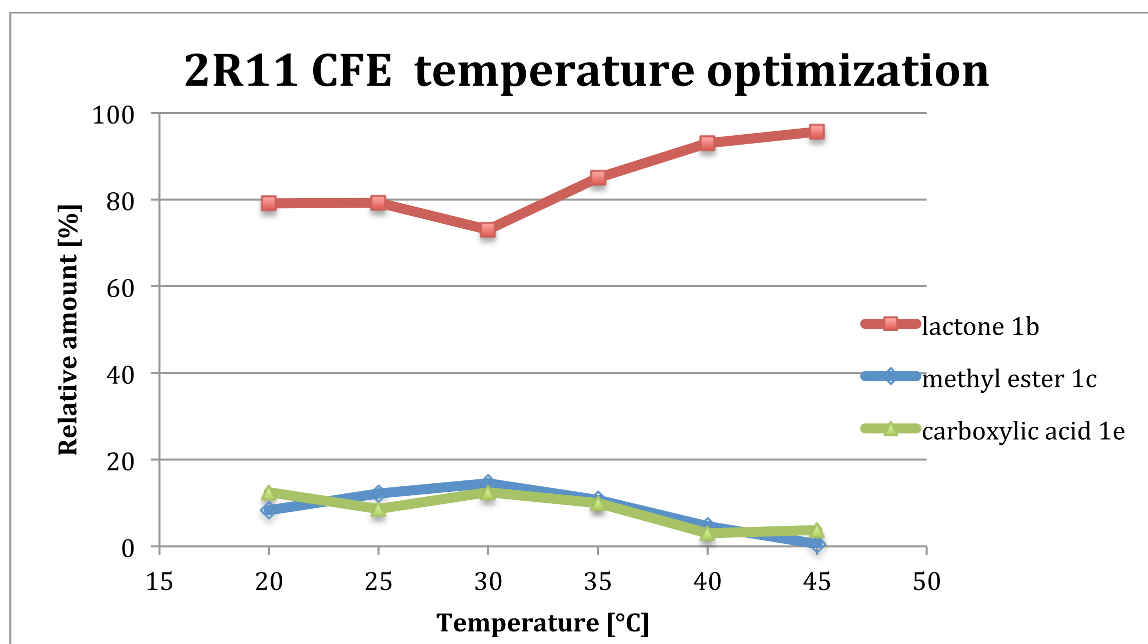


Figure 2.3-5: Results of temperature optimization study results for *Bacillus subtilis* hydrolase (pdi code: 2R11). Reactions were performed in duplicate.

Highest yield of ester **1c** and lowest amount of lactone **1b** was obtained at 30 °C employing hydrolase from *Bacillus subtilis* (pdi code: 2R11). Carboxylic acid formation correlated with ester formation at each temperature. At 40 °C ester formation decreased significantly. At 45 °C however there still seems to be conversion from lactone **1b** to the corresponding carboxylic acid taking place (**Figure 2.3-5**). 30 °C was chosen as optimal reaction temperature for further studies on ester formation employing hydrolase from *Bacillus* subtilis.

2.3.2 Determination of specific activity

To compare the different enzymes it was decided to determine specific activity to balance the units for further studies. In this study units were defined as the amount of lyophilized cell free extract (CFE) which is necessary to convert 1 µmol substrate in 1 minute at the given temperature.

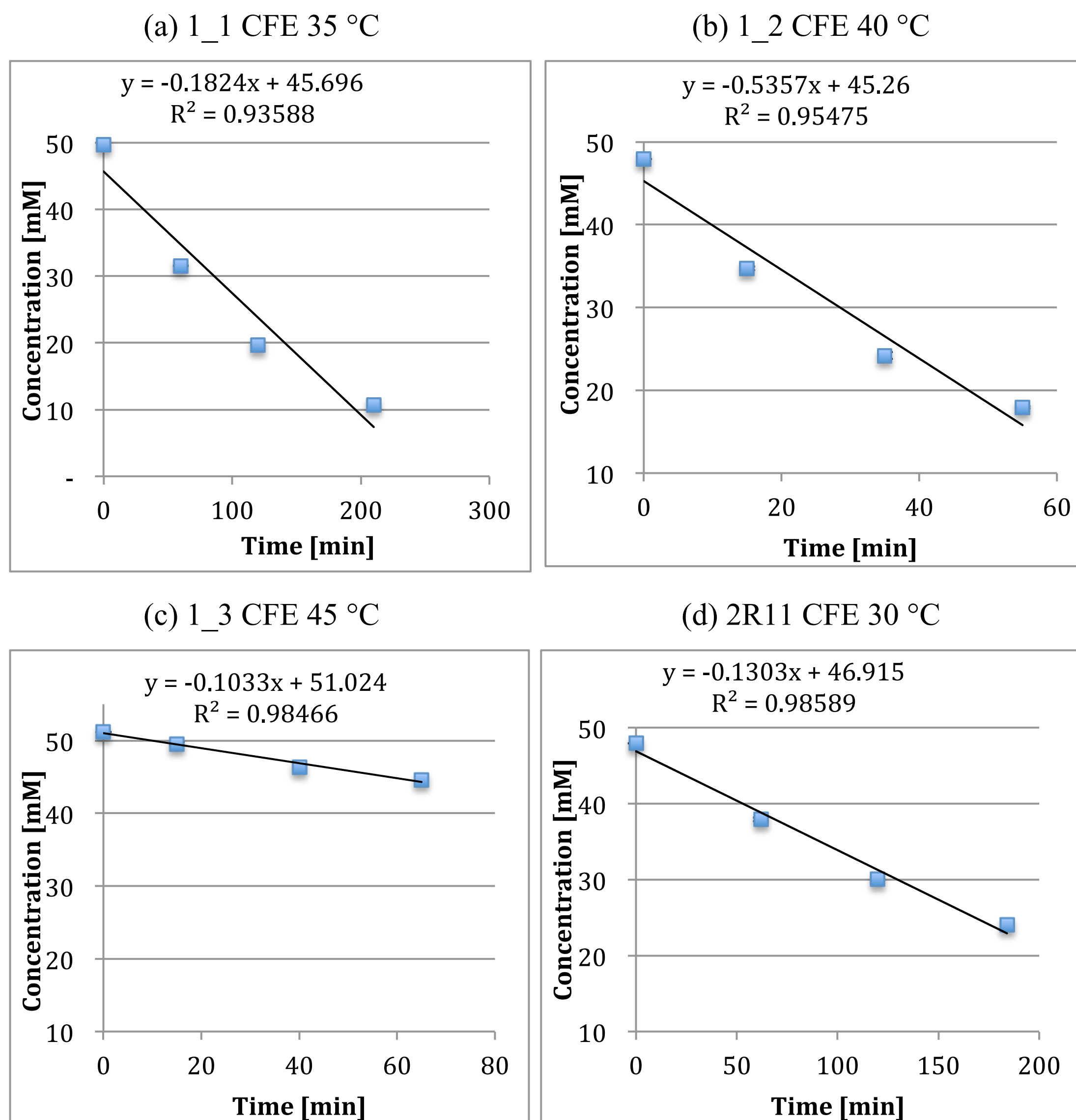


Figure 2.3-6: Specific activity study results. The decrease of the amount of lactone **1c** was detected over time. (a) 5 mg of 1_1 CFE was used and reaction was carried out at 35 °C. (b) 1 mg of 1_2 CFE was used and reaction was carried out at 35 °C. (c) 1 mg of 1_3 CFE was used and reaction was carried out at 45 °C. (d) 2 mg of hydrolase from *Bacillus subtilis* (2R11) CFE was used and reaction was carried out at 45 °C.

Table 2.3-1: Specific activity study results summary.

Enzyme lyophilized CFE	Slope [mM/min]	CFE [mg]	Temperature [°C]	U/mg (CFE)
1_1	0.1824	5	35	0.036
1_2	0.5357	1	40	0.536
1_3	0.1033	1	45	0.103
2R11	0.1303	2	30	0.065

In **Table 2.3-1** the results of the specific activity study are summarized. 1_2 CFE shows highest activity of the tested enzymes with 0.536 U/mg (CFE).

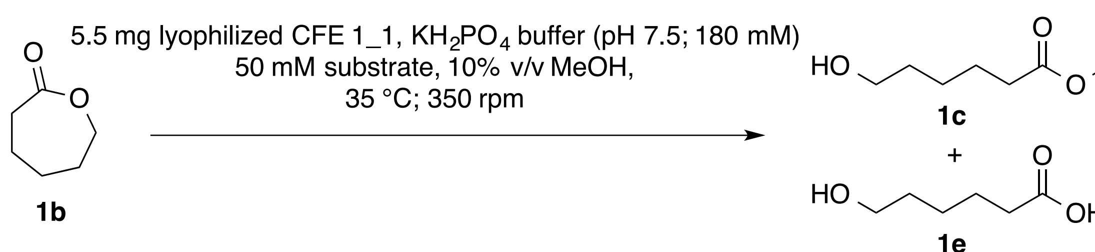
2.3.3 HLE isoenzymes and *Bacillus subtilis* hydrolase time studies

Time studies were performed to follow the reaction progress. To balance the activities, the amount of CFE were chosen to use an activity of 0.2 units (**Table 2.3-2**). For all experiments consumption of lactone **1b**, formation of ester **1c** and formation of carboxylic acid **1d** were followed over time using GC.

Table 2.3-2: Amount of CFE used to reach an activity of 0.2 units.

Enzyme	Units	CFE [mg]	Temperature optimum [°C]
1_1	0.2	5.5	35
1_2	0.2	0.4	40
1_3	0.2	1.9	45
2R11	0.2	3.1	30

In **Table 2.3-2** the amount of CFE used to reach an activity of 0.2 units per minute, at the optimized reaction temperature is summarized for each enzyme. The reaction conditions are given in **Schemes 2.3-6** to **2.3-9**.



Scheme 2.3-6: Reaction conditions for time study employing 1_1 CFE.

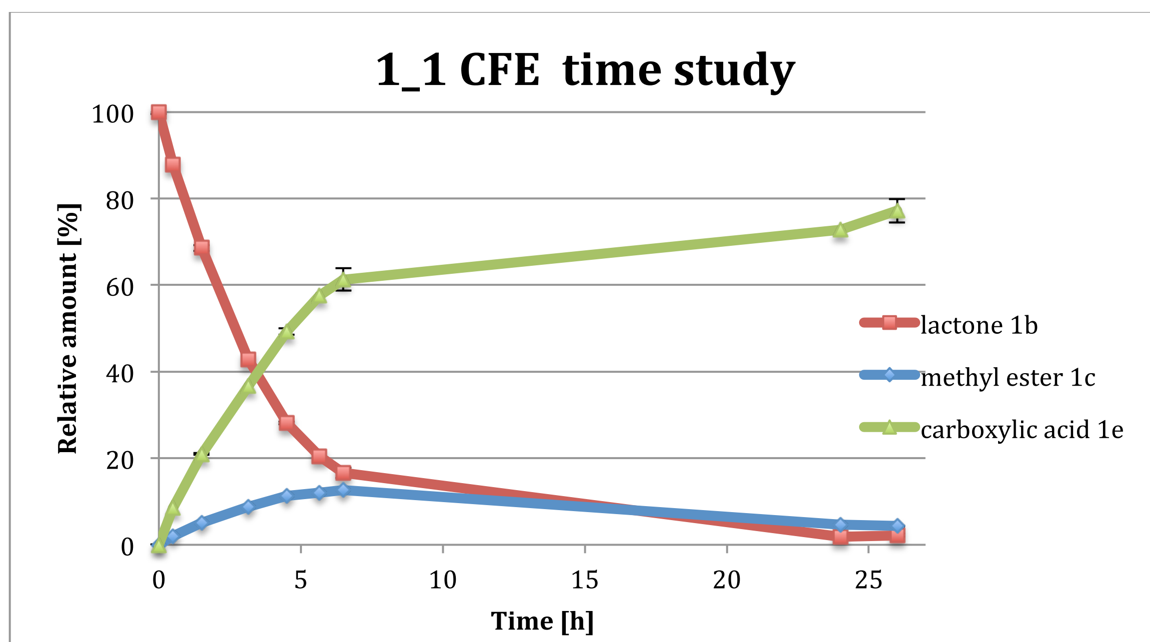
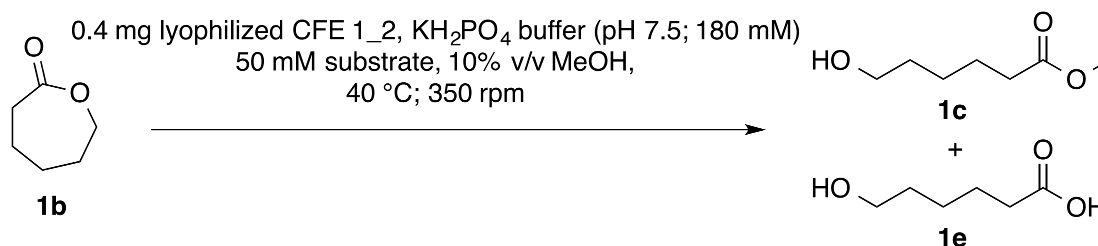


Figure 2.3-7: Time study results employing 1_1 CFE. Error bars represent standard deviation of triplicate measurements.

Figure 2.3-7 shows that isoenzyme 1_1 prefers hydrolysis of lactone **1b** to the corresponding carboxylic acid **1e** over esterification to **1d**. The highest amount of ester was detectable after 6 h 30 min (up to 15%). At this time already 60% of carboxylic acid was detected. Hydrolysis of the ester **1c** occurred at slower rate than hydrolysis of lactone **1b**. After 26 hours no significant amount of lactone **1b** was detected anymore while there was up to 4.5% of ester **1c** left.



Scheme 2.3-7: Reaction conditions for time study employing 1_2 CFE.

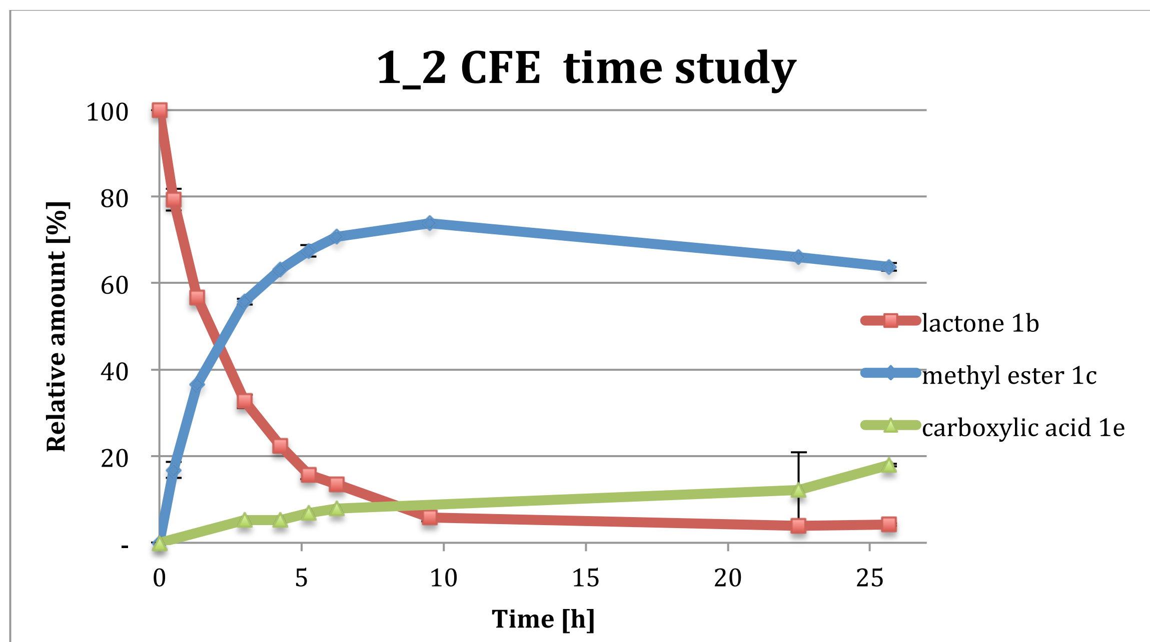
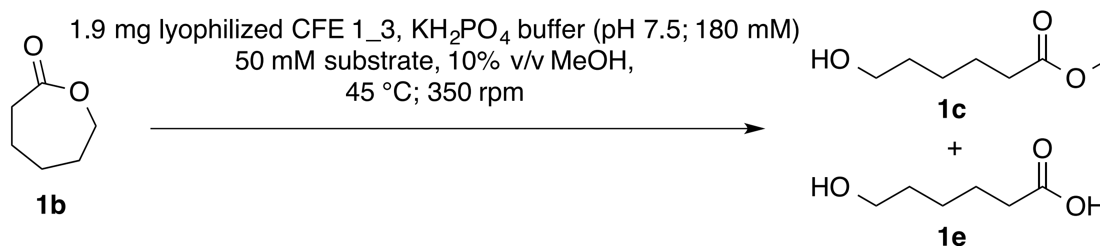


Figure 2.3-8: Time study results employing 1_2 CFE. Error bars represent standard deviation of triplicate measurements.

Figure 2.3-8 shows the preference of isoenzyme 1_2 for esterification of lactone **1b** over hydrolysis to the corresponding carboxylic acid **1e**. The highest amount of ester was detectable after 9 h 30 min (up to 73%). At that time most of lactone **1c** was consumed. Hydrolysis of ester **1c** occurred at slower rate than esterification of lactone **1b**. After 9 h 30 min no significant amount of lactone **1b** was detected anymore while after 25 h 40 min hydrolysis of ester **1c** to carboxylic acid **1e** was still observed.



Scheme 2.3-8: Reaction conditions for time study employing 1_3 CFE.

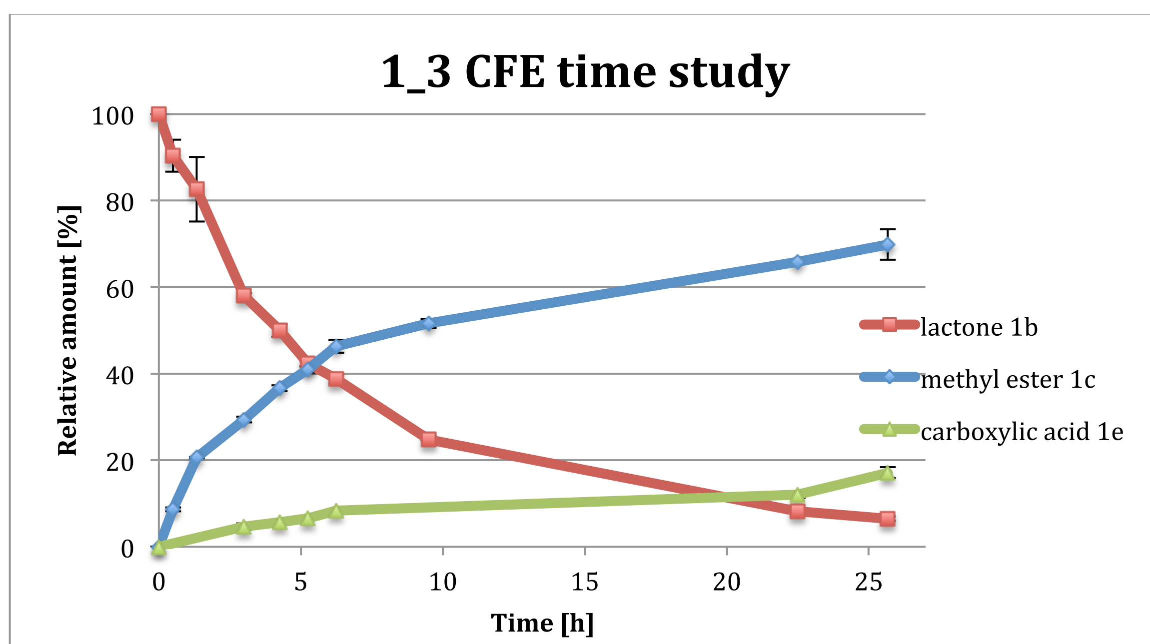
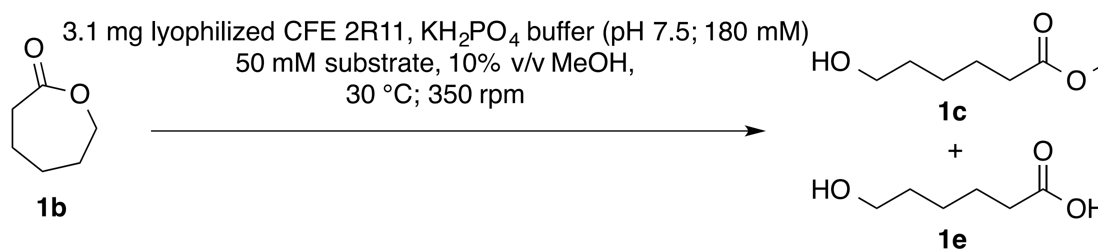


Figure 2.3-9: Time study results employing 1_3 CFE. Error bars represent standard deviation of triplicate measurements.

Figure 2.3-9 shows the preference of isoenzyme 1_3 for esterification of lactone **1b** over hydrolysis to carboxylic acid **1e**. After 24 h 40 min esterification of lactone **1c** was still detected. At that time only 6.5% of lactone **1b** was left and the concentration of carboxylic acid **1e** still increased. After 3 h already some formation of carboxylic acid **1e** was observed.



Scheme 2.3-9: Reaction conditions for time study employing hydrolase from *Bacillus subtilis* (pdb code: 2R11) CFE.

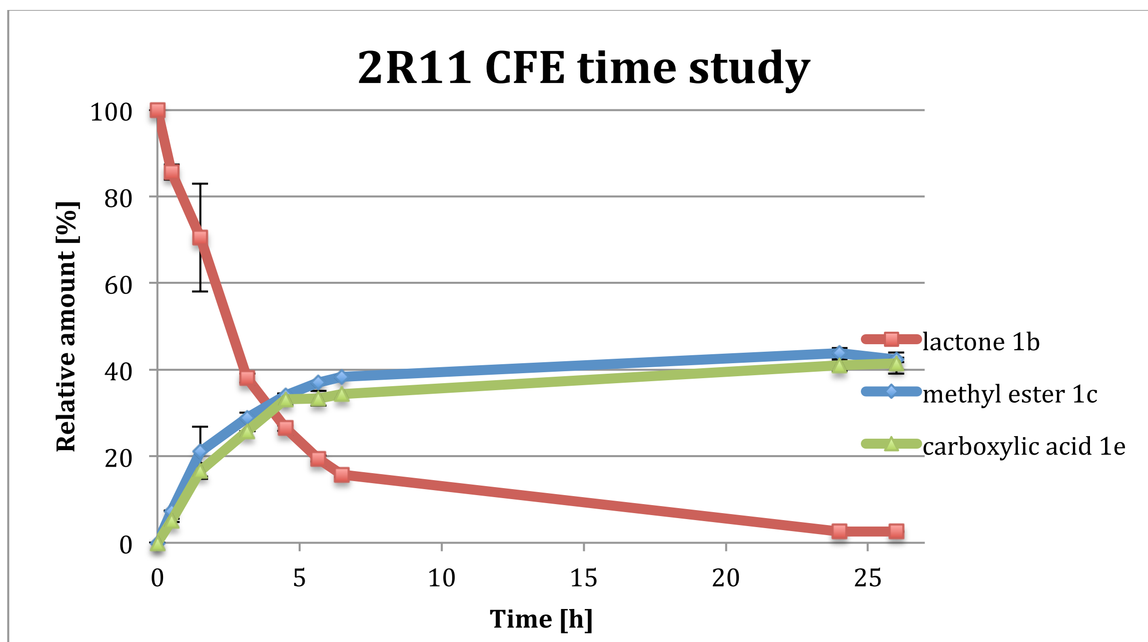


Figure 2.3-10: Time study results employing hydrolase from *Bacillus subtilis* (2R11) CFE . Error bars represent standard deviation of triplicate measurements.

Bacillus subtilis hydrolase (pdb code: 2R11) showed similar preference for esterification or hydrolysis of lactone **1b**. Ester formation and carboxylic acid formation was detected simultaneously at nearly the same rate (**Figure 2.3-10**). After 24 h no significant amount of lactone **1b** could be detected and the amount of ester **1c** and carboxylic acid **1e** (each around 42%) didn't change. Hydrolase from *Bacillus subtilis* (pdb code: 2R11) seems to be able to hydrolyze and esterify lactone **1b** but not to hydrolyze ester **1c**.

2.4 Protein purification

Enzymes were purified from cell free extracts (CFE) produced by recombinant *E. coli* Shuffle® T7 over a HisTrap^{TM} column (5 ml) and concentrated. SDS PAGE (**Figure 2.4-1)** showed prominent bands at the expected size (~62 kDa).

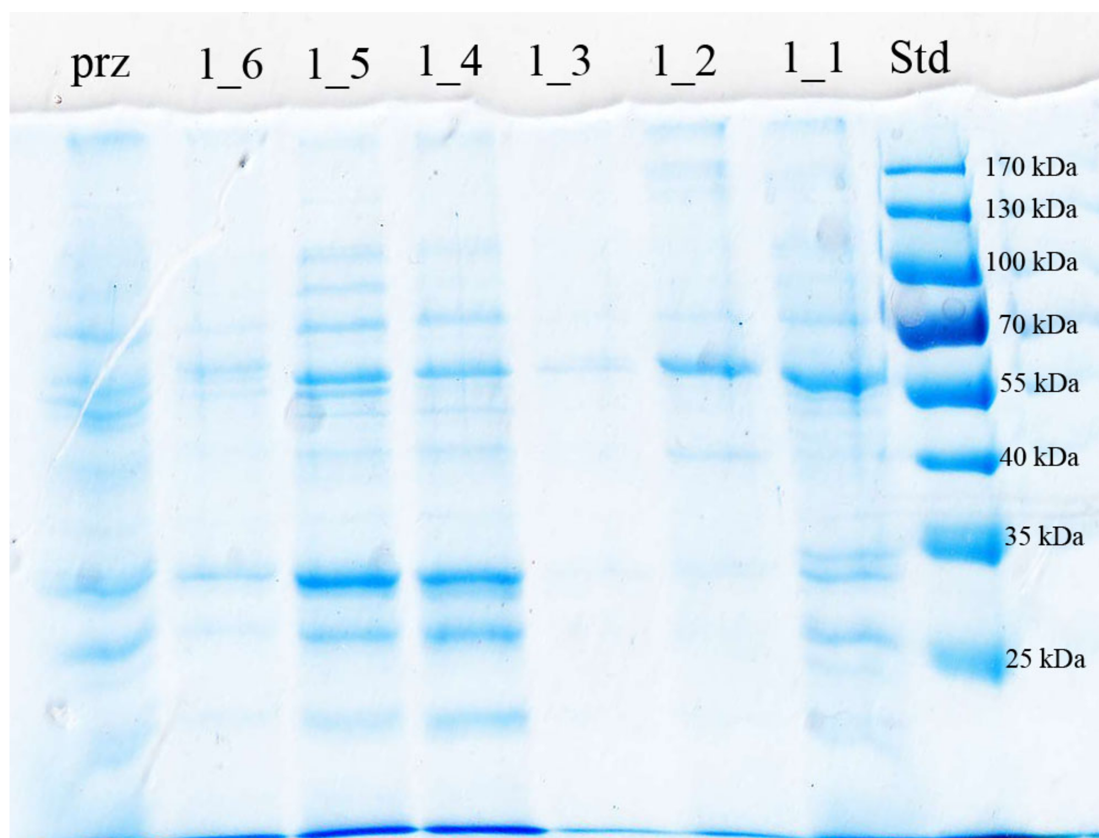


Figure 2.4-1: SDS PAGE results after His-tag purification. (Numbers indicate the corresponding isoenzyme; prz – esterase from *Equus przewalskii*; Std – standard).

A band at the expected size was detected for all samples. Since isoenzymes 1_4; 1_5; 1_6 and the esterase from *Equus przewalskii* didn't show activity in the first screening (**Figure 2.4-1**), but after purification bands at the expected size (~62 kDa) were obtained, it can be concluded that the produced proteins are either inactive or do not catalyze the tested reaction.

Isoenzyme 1_2 and 1_3 were purified again. Also the hydrolase from *Bacillus subtilis* (pdb code: 2R11) was purified and all samples were desalted to remove the remaining imidazole. Afterwards SDS PAGE analysis was performed (**Figure 2.4-2**).

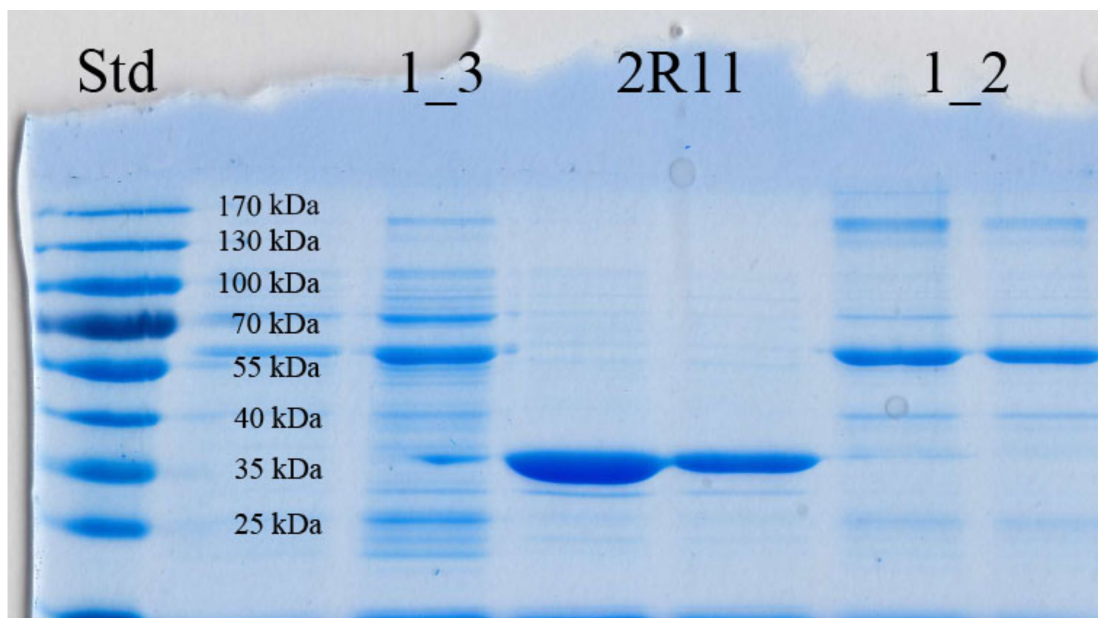


Figure 2.4-2: SDS PAGE after His-tag purification, concentration and desalting. Hydrolase from *Bacillus subtilis* (pdi code: 2R11) and isoenzyme 1_2 were loaded twice. (Numbers indicate the number of isoenzyme; 2R11 – *Bacillus subtilis* hydrolase; Std – standard).

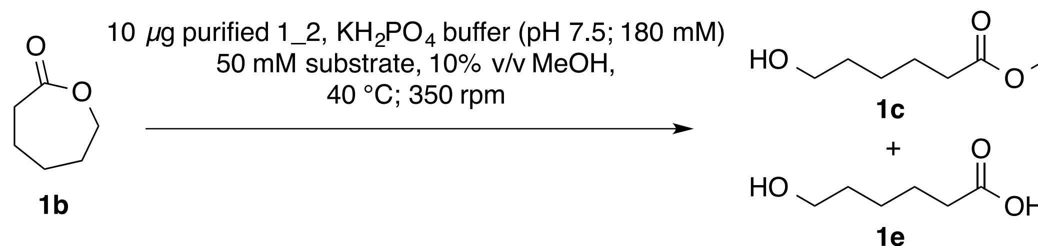
Figure 2.4-2 shows a prominent band for all samples at the expected size [1_3 and 1_2 at ~62 kDa; hydrolase from *Bacillus subtilis* (pdi code: 2R11) at ~35 kDa]. Due to higher expression levels, hydrolase from *Bacillus subtilis* (pdi code: 2R11) was purified significantly better than the HLE isoenzymes. For 1_2 some other protein bands were also observed and for 1_3 many other proteins could be detected after purification. However the most prominent band was always at the expected size.

2.5 Purified enzyme studies

The purified enzymes were used for further investigation. Time studies were performed employing 10 µg of purified enzyme dissolved in deionized H_2O. Protein concentration and volume used are listed in **Table 2.5-1.**

Table 2.5-1: Protein concentration and used volume of purified enzymes for time studies.

Sample	**Concentration [mg/ml]**	**Volume [µl]** corresponding to 10 µg
1_2	1.57	6.4
1_3	0.17	58.8
2R11	0.76	13.2



Scheme 2.5-1: Reaction conditions for time study employing purified 1_2.

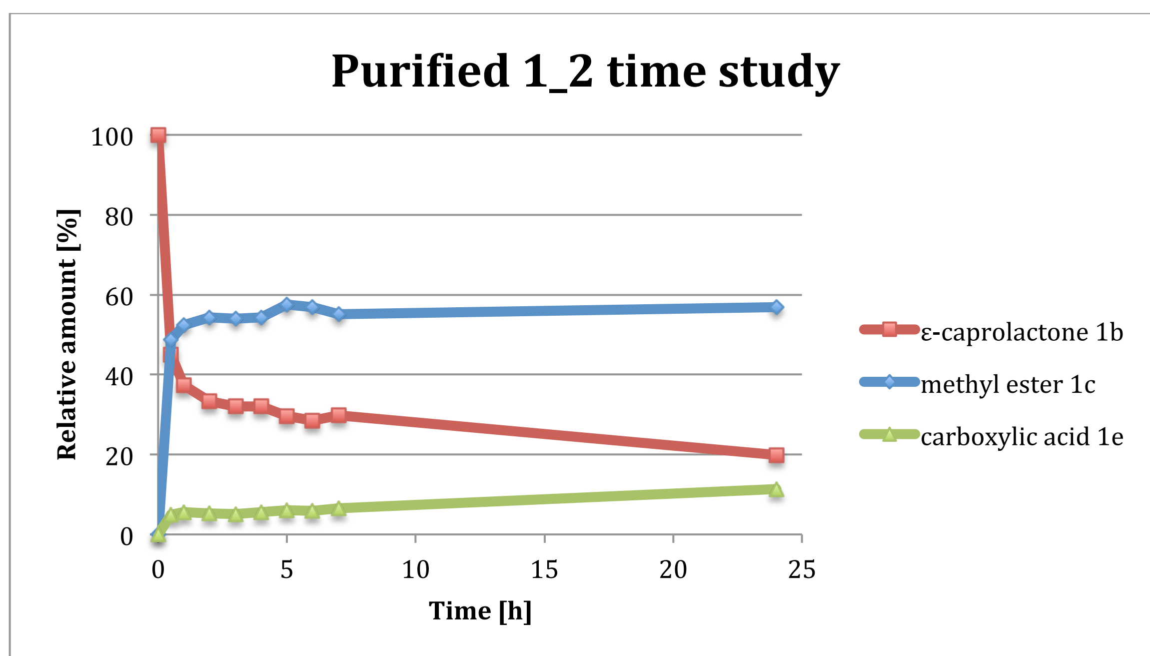
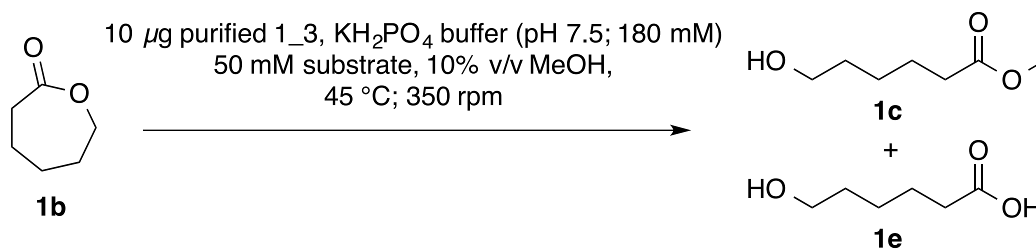


Figure 2.5-1: Time study results employing purified 1_2 solution. Reactions were performed in duplicates.

Figure 2.5-1 shows rapid production of ester **1c** during the first 30 min. Somehow the reaction rate decreases tremendously afterwards, but there was still some decrease of lactone **1b** and increase of carboxylic acid **1e** detectable after 24 h. As shown in **Figure 2.3-8** (employing 1_2 CFE) also employing pure 1_2 enzyme, ester formation is favored over hydrolysis of lactone **1b**. To give information on enzyme instability or inhibitory effects, further studies need to be done.



Scheme 2.5-2: Reaction conditions for time study employing purified 1_3.

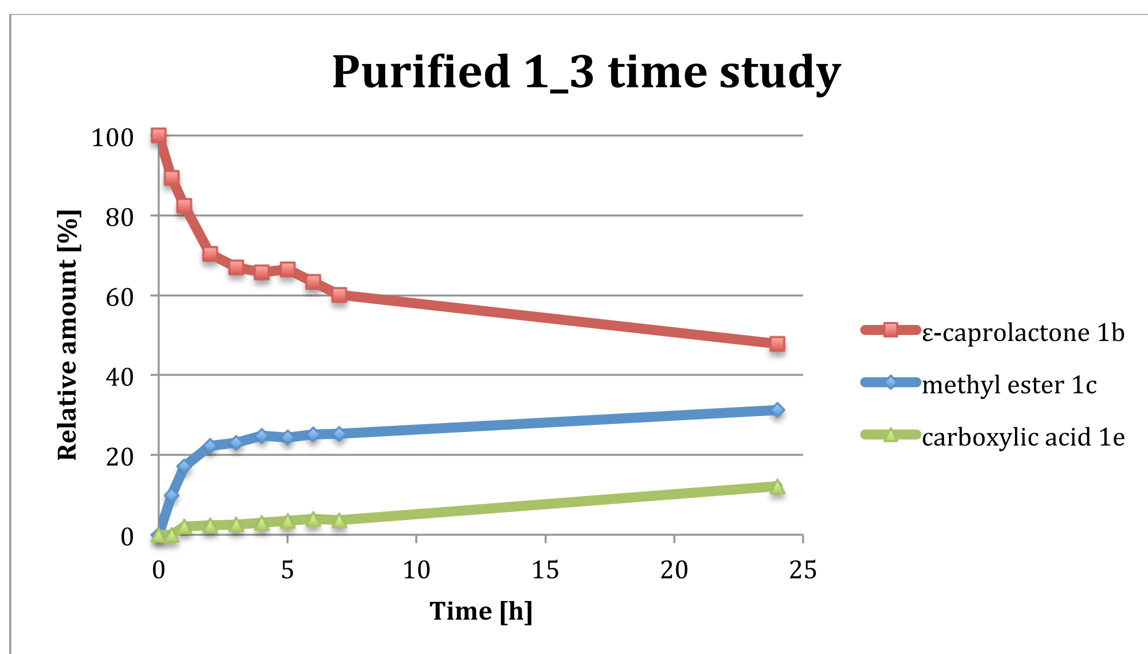
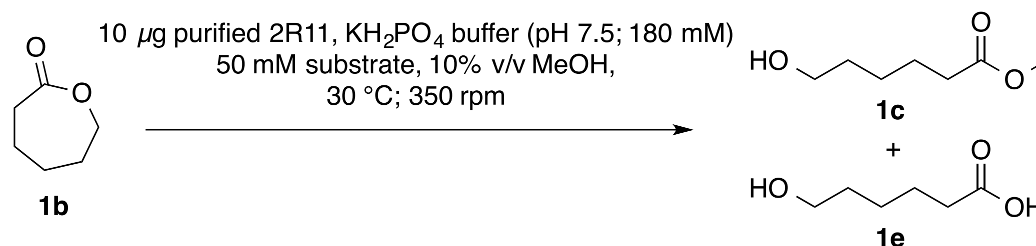


Figure 2.5-2: Time study results employing purified 1_3 solution. Reactions were performed in duplicates.

Figure 2.5-2 indicates formation of ester **1c** during the first 3 h. Afterwards the reaction rate decreases drastically, but reaction still occurs after 24 h. As expected also with pure 1_3 enzyme ester formation is favored over hydrolysis of lactone **1b**.



Scheme 2.5-3: Reaction conditions for time study employing purified 2R11.

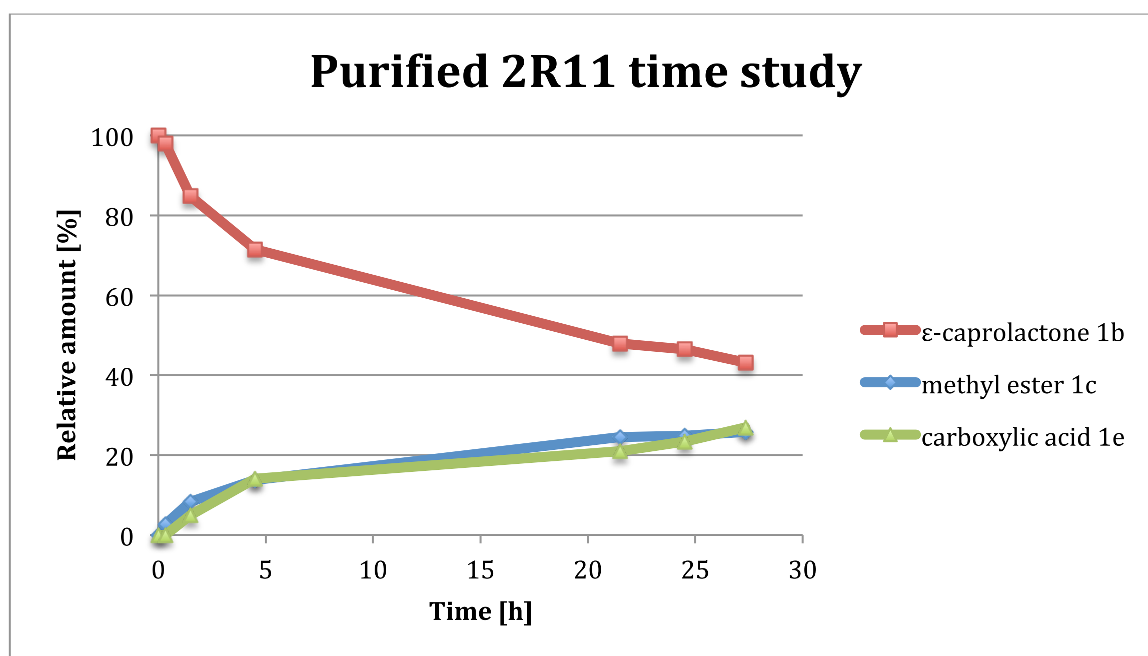


Figure 2.5-3: Time study results employing purified hydrolase from *Bacillus subtilis* (pdb code: 2R11) solution. Reactions were performed in duplicates.

Figure 2.5-3 indicates simultaneous formation of ester **1c** and carboxylic acid **1e** at nearly the same reaction rate. Even when employing 20 µg of the purified hydrolase from *Bacillus subtilis* (pdi code: 2R11) enzyme after 27 h the reaction was not complete.

2.5.1 Different concentration of purified 1_2 and 1_3 isoenzymes

Since the studies performed with 10 µg of purified 1_2 and 1_3 enzyme solutions showed rapid formation of ester **1c** and then a drastic decrease of reaction rate, further tests with 20 µg and 30 µg of enzyme solution were carried out. The results listed in **Table 2.5-2** were obtained after 30 min reaction time.

Table 2.5-2: Used purified protein and yields of lactone **1b** ester **1c** after 30 min reaction time. Reactions employing 10 µg of enzyme were performed 1 week before reactions employing 20 µg and 30 µg. Reactions were carried out in duplicates.

Sample	**Enzyme amount [µg]**	**Yield [%]** lactone **1b**	**Yield [%]** methyl ester **1c**
1_2	10.0	45.00	48.66
	19.9	49.74	41.83
	34.5	32.39	56.54
1_3	10.0	89.35	9.97
	21.7	75.31	21.87
	30.1	62.61	30.17

The formation of ester directly correlates with the amount of purified 1_3 enzyme used. Even after keeping the enzyme solution in the fridge for 1 week no significant loss of activity was detected.

For purified 1_2 enzyme solution a loss of activity during one week of storage in the fridge (4 °C) was detected. Even after doubling the amount of enzyme only 42% of ester **1c** was detected. One week before, 49% of ester **1c** was obtained when using 10 µg of the purified enzyme. When employing 35 µg of purified enzyme 1_2, 57% of ester **1c** was obtained. In order to get more information on the limiting factors of the reaction, further studies with higher amount of enzyme or tests on the long-term stability of the enzyme preparation need to be done.

2.6 Isoenzyme 1_2 harmonization

To enhance soluble expression of isoenzyme 1_2, codon harmonization was performed. For this purpose the occurrence of each codon triplet in the original organism (*horse*) was adjusted to *E. coli* manually. To establish the comparison between *horse* and *E. coli,* graphical codon usage analyzer was used.[78] The harmonized genetic code is given below:

Harmonized 1_2 (NdeI and Xho1 restriction sites are marked in red)

```
GTGCCGCGCGGCAGCCATATGTCGTCACCCCCAGTGGTGGATACCGCTCAGGGCAAAGTTCTGGGTAAACATGTTAGCCTGGA
GGGGTTCGCACAGCCGGTGGCGGTCTTTCTGGGTGTTCCATTCGCGAAGCCGCCGCTGGGGTCGCTGCGCTTCGCCCCCCCCC
AGCCAGCAGATCCTTGGCCGTTTGTGAAAAATGCGACCTCGTATCCACCGATGTGCTCGCAGGATACGGTGGCAGGTCAGATG
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ACGACGGTCTGGCGTTGAGTGCGCACGAGAATGTGGTGGTGGTGACCATCCAGTATCGGTTGGGCATTTGGGGGTTTTTGTCT
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TGGCGAAAAATTTGTTTCATAGAGCGATTAGTGAATCTGGCGTGACCTATACTGCCGGCCTGGTTCAGAAAGATTCCAAAGCG
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AGACGAATTGCTGGAAACATCACTGAAAATGAAGTTCTTGTCTTTAGATCTATTCGGGGAGCCGCGTGAAAGCCACCCGTTTC
TGCCGACTGTGGTCGACGGGGTGCTGCTGCCTCGCATGCCAGAAGAAATCCTGGCCGAGAAAACATTTAATACTGTCCCGTAT
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CTGCCGCATTGGCCTGTGTATGATAGAAAAGAGGGUTATCTCCAGATCGGAGTTACCACTCAGGCAGCTCAGAAACTGAAGGA
TAAAGAGGTGGCCTTTTGGACTGAATTGCTGGCGAAAGAAGCAGCAGAGAAACAGCAGCAGACGGAGCACGTCGAGTTATAAC
TCGAGCCACTGAGATCCGGC
```

```
Amino acid sequence: (signal sequence was deleted)
MSSPPVVDTAQGKVLGKHVSLEGFAQPVAVFLGVPFAKPPLGSLRFAPPQPADPWPFVKNATSYPPMCSQDTVAGQMLSDLFT
NRKENIPVQISEDCLYLNIYTPADLTKKSRLPVMVWIHGGGLMVGGASTYDGLALSAHENVVVVTIQYRLGIWGFLSTGDEHS
PGNWGHLDQVAALRWVQENIANFGGDPGSVTIFGESAGGESVSVLVLSPLAKNLFHRAISESGVTYTAGLVQKDSKAAAQQIA
VFAGCKTTTSAVIVHCLRQKTEDELLETSLKMKFLSLDLFGEPRESHPFLPTVVDGVLLPRMPEEILAEKTFNTVPYIVGINK
QEFGWIIPTMMGYPFSEGKMDQRTATSLLQNSSTLLHIPEELTPVAIEKYLGGTDDPVKKKDLFLDLMGDVMFGVPSVTVARL
HRDAGASTFMYEFQYRPSFSSAMRPKTVIGDHGDEIFSVFGAPFLKEGASEEEIKLSKMVMKFWANFARTGNPNGEGLPHWPV
YDRKEGYLQIGVTTQAAQKLKDKEVAFWTELLAKEAAEKQQQTEHVEL
```

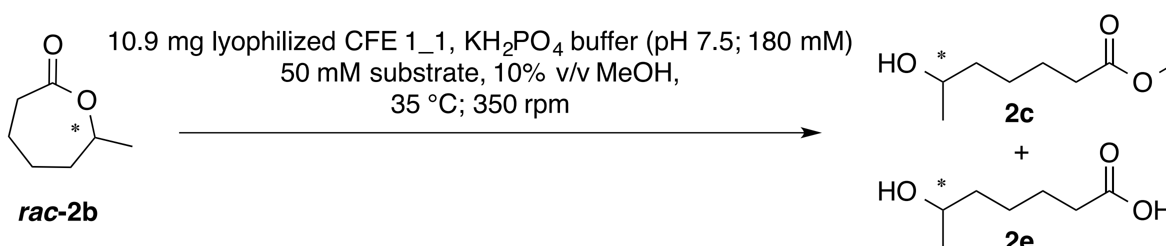
The SDS PAGE analysis didn't show any improvement in soluble expression of the protein, but first activity tests indicated that the enzyme was active.

2.7 6-Methyl-ε-caprolactone (2b) substrate studies

In order to check for chiral recognition of the enzymes time studies were performed employing 6-methyl-ε-caprolactone (**2b**) as substrate. **Table 2.7-1** summarizes the used amount of lyophilized cell free extracts (CFE) to reach an activity of 0.6 units [measured employing ε-caprolactone (**1b**) as substrate]. For isoenzyme 1_1, an activity of 0.4 units was chosen, because otherwise too much CFE had to be used for a reaction volume of 1 ml (over 11 mg). Additionally to the conversion, the enantiomeric excess of lactone **2b** and ester **2c** were monitored over time. To determine the absolute configuration the substances were synthesized before and analyzed on GC. The retention times were compared.

Table 2.7-1: Amount of CFE used for time studies of 6-methyl-ε-caprolactone **(2b).**

Enzyme	Units	CFE [mg]
1_1	0.4	10.9
1_2	0.6	1.1
1_3	0.6	5.8
2R11	0.6	9.2



Scheme 2.7-1: Reaction conditions for time study employing 1_1 CFE and racemic compound **2b** as substrate.

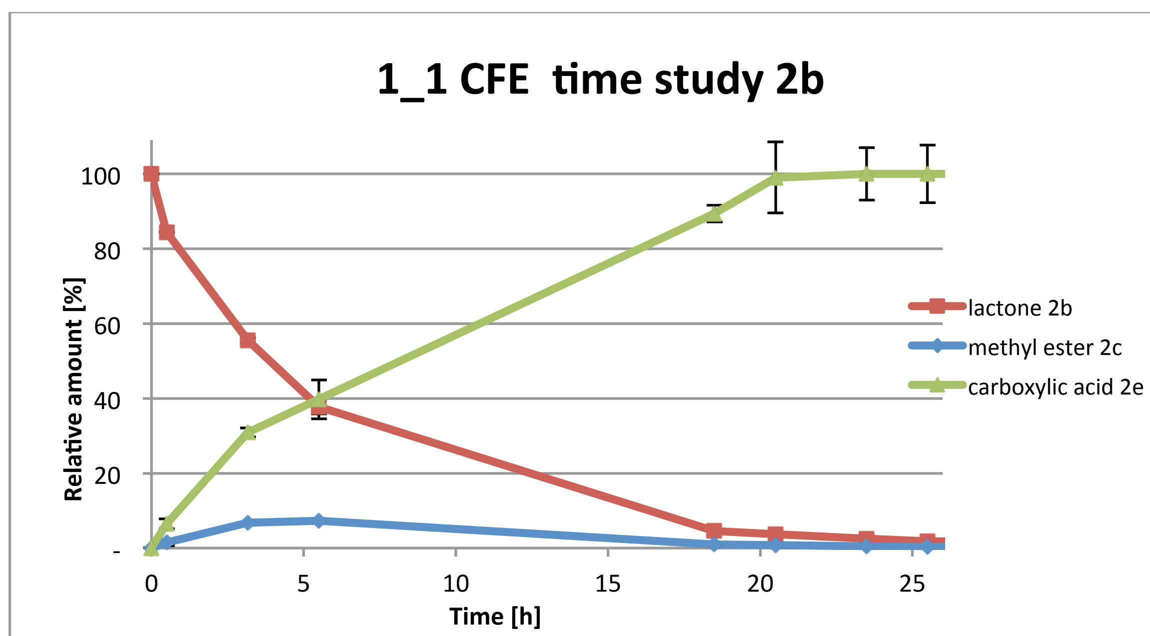


Figure 2.7-1: Time study fot the transformation of *rac*-**2b** employing 1_1 CFE. Error bars represent standard deviation of triplicate measurements.

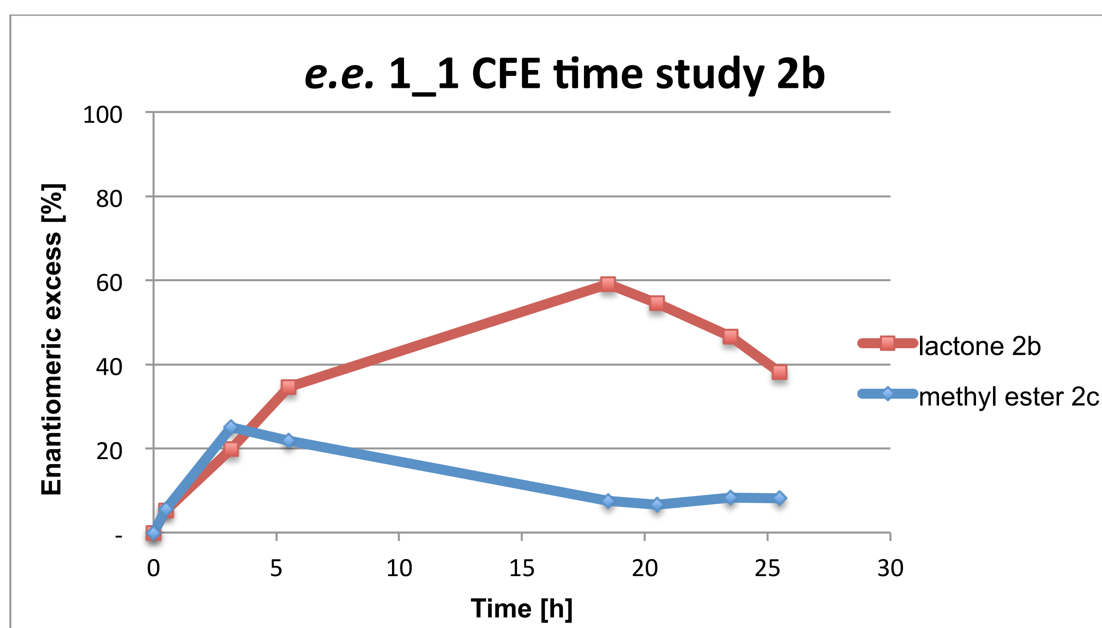
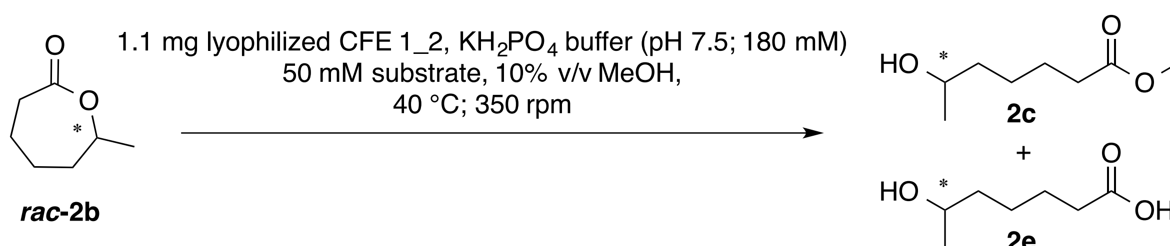


Figure 2.7-2: Enantiomeric excess monitored over time employing isoenzyme 1_1 and racemic compound **2b** as substrate. For the lactone **2b** the (*R*)-enantiomer is in excess and for the methyl ester **2c** the (*S*)-enantiomer.

Employing lactone **2b** as substrate gave similar results as lactone **1b**. Isoenzyme 1_1 prefers hydrolysis of lactone **2b** to the corresponding hydroxy acid **2e** over esterification to **2c** (**Figure 2.7-1**). The highest amount of ester was detected after 5 h 30 min (up to 7.5%).

After 5 h 30 min already 40% of carboxylic acid **2e** was detected. Hydrolysis of ester **2c** occurred at slower rate than hydrolysis of lactone **2b**. After 20 h 30 min no significant amount of lactone **2b** and ester **2c** could be detected while 100% of carboxylic acid **2e** was detected.

However a low enantiomeric excess (*e.e.*) was obtained for ester **2c** and lactone **2b** (**Figure 2.7-2**). Since the yield of the ester product **2c** did not exceed 30% no E-value was calculated.



Scheme 2.7-2: Reaction conditions for time study employing isoenzyme 1_2 CFE and racemic compound **2b** as substrate.

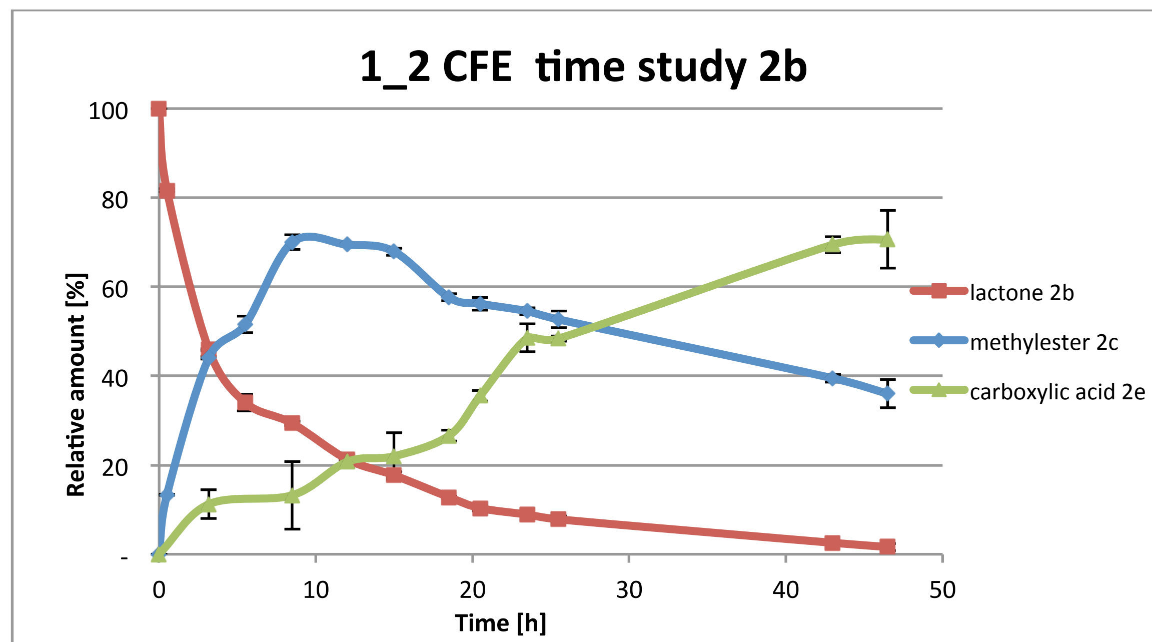


Figure 2.7-3: Time study fot the transformation of *rac*-**2b** employing 1_2 CFE. Error bars represent standard deviation of triplicate measurements.

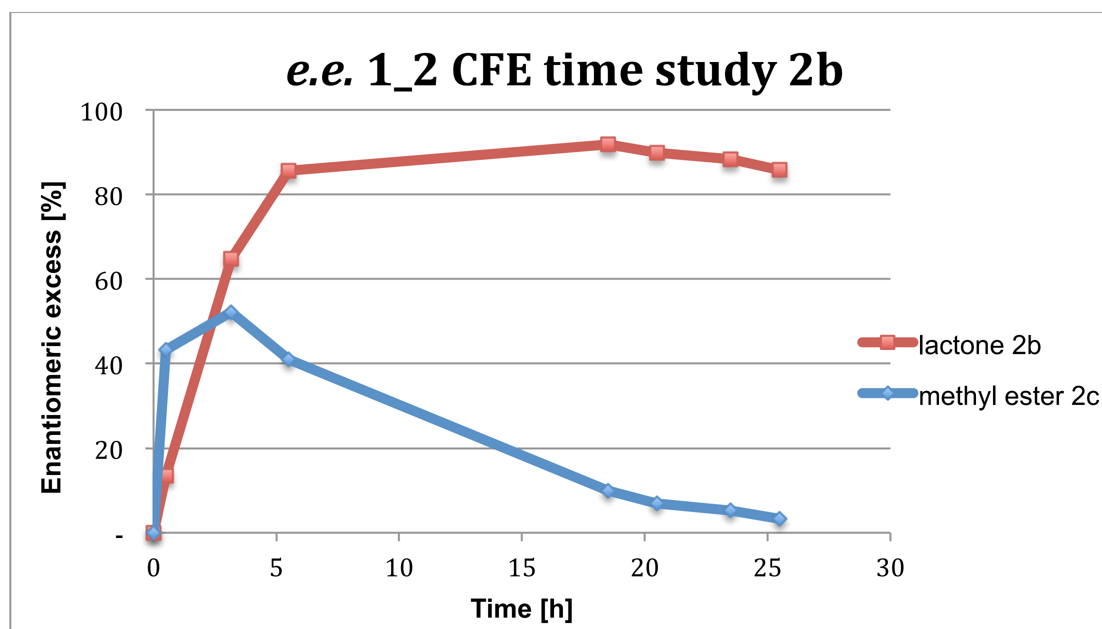
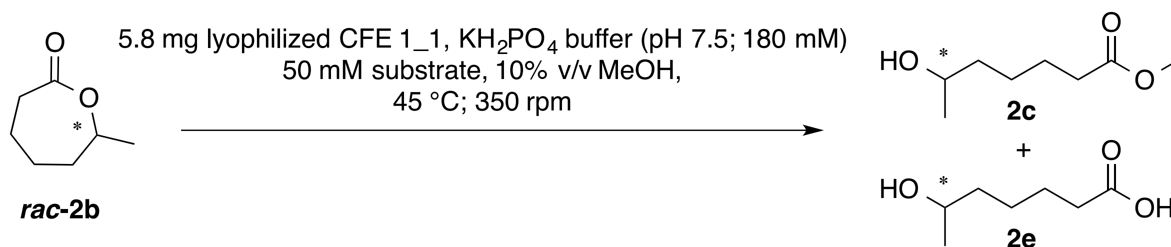


Figure 2.7-4: Enantiomeric excess monitored over time employing isoenzyme 1_2 and racemic compound **2b** as substrate. For the lactone **2b** the (*R*)-enantiomer is in excess and for the methyl ester **2c** the (*S*)-enantiomer.

Figure 2.7-3 shows the preference of isonenzyme 1_2 for esterification of lactone **2b** over hydrolysis to the carboxylic acid **2e**. Ester formation and acid formation were detected right from the beginning. After 8 h 30 min already 70% of ester **2c** and 13% of carboxylic acid **2e** were formed. Employing 0.6 units of isoenzyme 1_2 showed that after 43 h, most of the lactone was consumed. Hydrolysis of ester **2c** was still detected after 46 h 30 min. The conversion of lactone **2b** to ester **2c** occurred at a slower rate than conversion of lactone **1b** to ester **1c** (**Figure 2.3-8**).

Highest *e.e.* for ester **2c** was obtained after 3.2 h reaction time (53%) and for lactone **2b** after 18 h 30 min (92%). At that time 13% of lactone **2b** was detected, which indicates that isoenzyme 1_2 shows a slight preference towards the conversion of the (*S*)-enantiomer of lactone **2b**. Since the *e.e.* of the produced hydroxy ester decreases after 18 h 30 min it is cocluded that the preferably produced (*S*)-enantiomer is also favored for hydrolysis (**Figure 2.7-4**). An E-value of 6 was calculated for 3.2 h reaction time, which indicates poor selectivity. For calculation of the E-value the online software Enzyme Kinetics Tool Set was used.[79]



Scheme 2.7-3: Reaction conditions for time study employing isoenzyme 1_3 CFE and racemic compound **2b** as substrate.

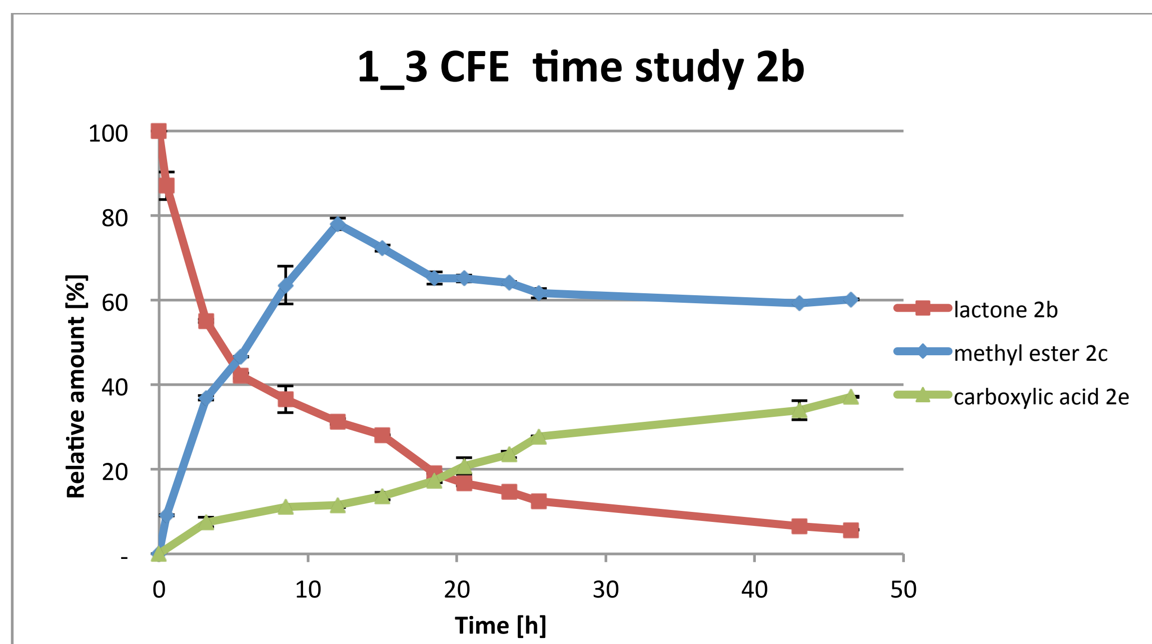


Figure 2.7-5: Time study fot the transformation of *rac*-**2b** employing 1_3 CFE. Error bars represent standard deviation of triplicate measurements.

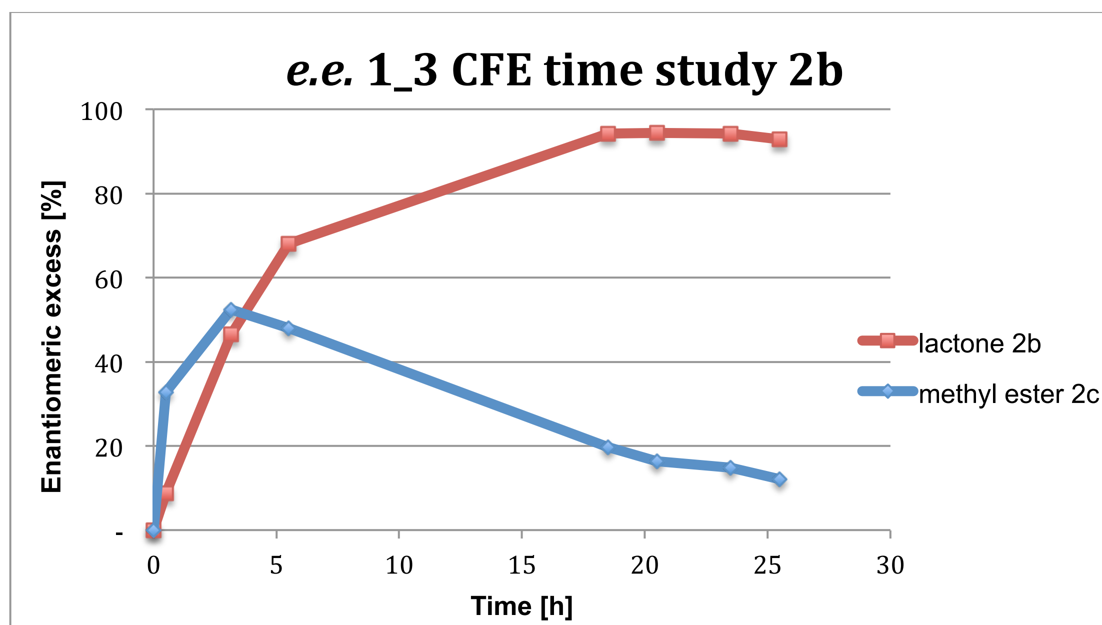


Figure 2.7-6: Enantiomeric excess monitored over time employing isoenzyme 1_3 and racemic compound **2b** as substrate. For the lactone **2b** the (*R*)-enantiomer is in excess and for the methyl ester **2c** the (*S*)-enantiomer.

Figure 2.7-5 shows the preference of isonenzyme 1_3 towards the esterification of the lactone **2b** over hydrolysis forming the carboxylic acid **2e**. Ester formation and carboxylic ester formation occured simultaneously. The highest amount of ester **2c** (78%) was detected after 12h reaction time. At that time already 12% of carboxylic acid **2e** was obtained. After 46 h 30 min most of lactone **2b** was consumed and hydrolysis of ester **2c** was still detected.
Highest *e.e.* for ester **2c** was obtained after 3 h 10 min reaction time (52%) and for lactone **2b** after 18 h 30 min (94%). At that time 13% of lactone **2b** was detected, which indicates that isoenzyme 1_3 shows slightly preference to convert the (*S*)-enantiomer of lactone **2b** (**Figure 2.7-6**). The decrease of the *e.e.* of the hydroxy ester indicates that the preferably produced (*S*)-enantiomer is also favored for hydrolysis. An E-value of 5 was calculated for 3 h 10 min reaction time, which indicates poor selectivity. For E-value calculation the online software Enzyme Kinetics Tool Set was used.[79]

2.7.1 7-Phenyloxepan-2-one (3b) substrate studies

As an additional chiral substrate 7-phenyloxepan-2-one (**3b**) was synthesized. Due to the lipophilic characteristics of this lactone it could not be solubilized and was therefor not accessible for the biotransformation. Even the addition of 5% v/v heptane and doubling the volume of MeOH to 20% v/v didn’t show any increase in solubility of substrate **3b**.

3 Summary & Outlook

Methyl-6-hydroxyhexanoat (**1c**) was successfully obtained employing HLE crude preparation using ε-caprolactone (**1b**) as substrate [in a buffer – MeOH (10%) solution]. After lactone **1b** was consumed, the hydrolysis of methyl-6-hydroxyhexanoat (**1c)** to 6-hydroxyhexanoic acid (**1e**) was catalyzed. The optimal temperature (45 °C) (**Table 3-1**) and the optimal pH (7.5) were identified for the esterification reaction. Additionally different nucleophiles were tested. Employing short chain alcohols such as MeOH and EtOH (decrease of reaction rate) gave positive results. Longer chained alcohols and amines showed some reaction, but to give trustworthy results, further studies need to be performed.

It was reported in literature that HLE crude preparation is able to perform the esterification of ε-caprolactone (**1b**).[72] The genetic code of 6 HLE isoenzymes was also reported[73] and to find similar enzymes capable to perform the investigated reaction, BLAST search was performed using HLE isoenzyme 1_1 as template. An enzyme with high identity from *Equus przewalskii* was found. The genes coding for the 6 isoenzymes and the esterase from *Equus przewalskii* were successfully cloned into pET-28a(+) vector and expressed (signal sequence was removed) partly solubly in *E. coli* Shuffle® T7. Additionally a hydrolase from *Bacillus subtilis* (pdb code: 2R11) was soluble expressed in *E. coli* Shuffle® T7 (cloned in pSpeedET vector). First activity tests at 25 °C showed that 3 out of the 6 isoenzymes (1_1; 1_2; 1_3) and the hydrolase from *Bacillus subtilis* (pdb code: 2R11) were able to convert substrate **1b**. Temperature optima for these enzymes were investigated in additional studies (**Table 3-1**) using lyophilized cell free extracts (CFE).

Table 2.7-1: Temperature optima of investigated enzymes.

Enzyme	Temperature optimum [°C]
HLE crude	45
1_1	35
1_2	40
1_3	45
2R11	30

Following the reaction progress of these enzymes employing ε-caprolactone (**1b**) as substrate showed that the enzymes are capable of catalyzing two different reactions. HLE isoenzyme 1_1 favored hydrolysis of ε-caprolactone (**1b**) to 6-hydroxyhexanoic acid (**1e**), while

isoenzymes 1_2 and 1_3 preferably produced methyl-6-hydroxyhexanoate (**1c**) followed by hydrolysis to 6-hydroxyhexanoic acid (**1e).** The hydrolase from *Bacillus subtilis* (pdb code: 2R11) esterified and hydrolyzed lactone **1b** simultaneously. However, this enzyme was – in contrast to isoenzymes 1_2 and 1_3 – not capable of hydrolyzing ester **1c**.
The same results were obtained employing racemic 6-methyl-ε-caprolactone (**2b**) as substrate [hydrolase from *Bacillus subtilis* (pdi code: 2R11) was not tested]. The chiral recognition of the investigated enzymes was very low with E-values of 5-6, which could be positive for industrial applications (since 100% of the substrate may be transformed quickly). Employing longer chained alcohols, such as EtOH, could improve the *e.e.* because longer chained alcohols occupy more space in the active site of the enzyme than shorter chained alcohols. This steric effect might improve the chiral recognition of the enzymes. Also the increase of cosolvent concentration might improve the *e.e.* for the reaction.
Polymerization reactions have to be considered though. In literature hydrolysis of racemic 6-methyl-ε-caprolactone (**2b**) to the corresponding carboxylic acid **2e** employing HLE was reported with an *e.e.* of 47% [(*S*)-enantiomer of the carboxylic acid was produced preferably]. It was also reported that the enzymatic resolution of 9-methyloxonan-2-one and 10-methyloxecan-2-one by HLE gave *e.e.* of >95% for the corresponding optical active lactones and *e.e.* of >95% for the corresponding optical active hydroxy acid.[93] These large-ring lactones are also promising substrates for the enantiopure esterification.

The investigated enzymes were purified using a HisTrap™ column. SDS PAGE showed that after purification some other proteins were still present in the collected fractions. Thus the samples could not be used for crystallization experiments. Adding additional purification steps such as size exclusion chromatography could provide samples pure enough for crystallization experiments.
Obtaining crystal structures of the investigated enzymes could lead to more information about potential protein tunnels that might explain the preference of alcohols over water as a nucleophile and *vice versa*. This knowledge would be very useful for rational protein design and would extend the understanding of protein mechanistic in general.

Following the reaction over time using the purified enzymes showed that the reaction rate slowed down drastically after 30 min to 1 h. To give further information about enzyme instability and/or inhibitory effects, further studies need to be performed.

Codon harmonization[80] of isoenzyme 1_2 did not show any enhancement in soluble expression of the enzyme. The harmonization was carried out manually, which incorporated some subjective decisions about the codon usage. Since there are many different approaches of codon harmonization, there is still a chance of enhancing the soluble expression by a different approach of codon harmonization.

The synthesis of substrates **2b** and **3b**, as well as the synthesis of reference materials **1c**, **1d, 1e, 2c** and **2e** was successfully completed. Also the synthesis of smaller chained hydroxy esters was tried, but they showed tendency for spontaneous cyclization back to the corresponding lactones (γ-lactones and δ-lactones). These esters and carboxylic acids need to be analyzed directly after purification (cyclization in GC possible) and/or should be derivative to obtain stable products.

Rational protein design could also decrease the hydrolysis ability of the enzymes by incorporating mutations. For example, random mutagenesis gave positive results in suppressing the hydrolysis activity of phosphatases in literature,[81] which could also be practicable for the HLE isoenzymes.

4 Experimental Part

Compounds, materials and methods used in this thesis are listed in this section.

4.1 Compounds used for buffer preparation

Compound	Purity [%]	Supplier	Lot No.	Unit size
KH_2PO_4 anhydrous	99.5	Sigma-Aldrich	SZBE0870V	1 kg
K_2HPO_4 anhydrous	n.a.	Merck	AMP0430204445	1 kg
Na_2HPO_4 x 7 H_2O	≥98	Sigma-Aldrich	SLBB5378V	500 g
TRIS PUFFERAN®	≥99.9	Roth	172154673	1 kg
CAPS (*N*-cyclohexyl-3-aminopropanesulfonic acid)	≥98	Sigma-Aldrich	SLBC1535V	25 g
Imidazole	≥99.5	Sigma-Aldrich	SZBE0500V	1 kg

4.2 Compounds used for cell cultivation

Compound	Purity [%]	Supplier	Lot No.	Unit size
Tryptone	n.a.	Oxoid	n.a.	500 g
Yeast extract	n.a.	Oxoid	n.a.	500 g
Agar bacteriological	n.a.	Oxoid	n.a.	500 g
NaCl	≥99.8	Roth	9265.1	1 kg
Glycerol	≥99.5	Sigma-Aldrich	BCP0365V	1 L
Isopropyl-β-D-1-thiogalactoside (IPTG)	n.a.	Peqlab	13411043	25 g
Kanamycin sulfate	n.a.	Sigma-Aldrich	SLBB0945V	25 g

4.3 Materials used for DNA isolation, restriction, ligation and desalting

Material	Supplier	Lot No.	Unit size
QIAquick Gel Extraction Kit (250)	QIAGEN	148017448	n.a.
QIAquick PCR Purification Kit (250)	QIAGEN	145036739	n.a.
QIAprep Spin Miniprep Kit (250)	QIAGEN	148047164	n.a.
SYBR safe DNA gel stain	Invitrogen	1621150	400 µl
Fast Digest XhoI endonuclease	Thermo Scientific	00240103	400 µl
Fast Digest NdeI endonuclease	Thermo Scientific	00173608	300 µl
Fast Digest buffer 10x	Fermentas	n.a.	1 ml
10x T4 DNA ligase buffer	Thermo Scientific	00136629	1.5 ml
T4 DNA lgase	Thermo Scientific	00157494	n.a.
GeneRuler^{TM} DNA ladder mix	Fermentas	00099230	50 µg
UltraPure^{TM} 10x TAE buffer	Invitrogen	15558-026	4 L
Uvette ® 220-1600 nm cuvettes	Eppendorf	D158000O	n.a.
BioPhotometer Plus	Eppendorf	n.a.	n.a.
MINI-SUB® CELL GT agarose gel station	BIO-RAD	n.a.	n.a.
Thermomixer comfort	Eppendorf	n.a.	n.a.
PD-10 desalting column	Ge Healthcare	9611730	n.a.

4.4 Materials used activity assay, biotransformation and synthesis

Compound	Purity [%]	Supplier	Lot No.	Unit size
Horse liver esterase (HLE crude preparation)	n.a.	Sigma-Aldrich	n.a.	100 mg
ε-Caprolactone	97	Sigma-Aldrich	MKBG027 5V	100 g
2-Methylcyclohexanone	98	Alfa Aesar	FAO26227	100 ml
2-Phenylcyclohexanone	98	Sigma-Aldrich	n.a.	5 g
Methanol	>99	Roth	n.a.	1 L
Ethanol	96	VWR CHEMICALS	n.a.	5 L
Isopropanol	>99.9	Sigma-Aldrich	n.a.	1 L
Dodecane	≥99	Sigma-Aldrich	08022BI-101	100 ml
H_2SO_4	95-98	Sigma-Aldrich	00411DH	100 ml
NaOH pellets	n.a.	Merck	0656540	1 kg
DMAP (4-dimethylaminopyridine)	≥98	Fluka	1345047 40508085	50 g
Acetic anhydride	98	Riedel-de Haen	32990	1 L
Urea powder	n.a.	Sigma-Aldrich	BCBJ5121 V	1 kg
Toluene	99.8	Sigma-Aldrich	STBD5155 V	1 L

4.5 Buffer preparation for biotransformation

All buffers were stored at room temperature.

100 mM citric acid – 200 mM Na_2HPO_4 buffer pH 5: 0.93 g citric acid and 1.46 g Na_2HPO_4 were dissolved in 100 ml deionized H_2O.

200 mM phosphate buffer pH 6: 5.29 g KH_2PO_4 and 1.66 g Na_2HPO_4 x 7 H_2O were dissolved in 250 ml deionized H_2O.

200 mM phosphate buffer pH 7: 2.34 g KH_2PO_4 and 8.18 g Na_2HPO_4 x 7 H_2O were dissolved in 250 ml deionized H_2O.

200 mM phosphate buffer pH 7.5: 3.84 g KH_2PO_4 and 45.07 g Na_2HPO_4 x 7 H_2O were dissolved in 1 L deionized H_2O.

200 mM phosphate buffer pH 8: 0.32 g KH_2PO_4 and 12.7 g Na_2HPO_4 x 7 H_2O were dissolved in 250 ml deionized H_2O. pH was adjusted with HCl.

200 mM TRIS-HCl buffer pH 9: 4.85 g TRIS was dissolved in 200 ml deionized H_2O. pH was adjusted with HCl.

200 mM CAPS buffer pH 10: 4.43 g CAPS was dissolved in 100 ml deionized H_2O. pH was adjusted with NaOH.

200 mM CAPS buffer pH 11: 4.43 g CAPS was dissolved in 100 ml deionized H_2O. pH was adjusted with NaOH.

4.6 Agarose gel electrophoresis

For the preparation of 1% agarose gels, agarose (400 mg) was dissolved in TAE-buffer (40 ml) by heating in a microwave. After cooling down, SYBRsafe-dye (4 µl) was added and the mixture was poured into the gel cast to solidify.

DNA samples were mixed with MassRuler 6x loading dye (8 µl) and loaded onto the well (20 µl). As standard, GeneRuler^{TM} DNA Ladder Mix (5 µl) was used.

The gels were run at 100 V for 50 minutes and then a picture was taken under UV-light.

4.7 SDS PAGE

The SDS PAGE consisted of the separating gel and the stacking gel. For the preparation of the separating gel 30% acrylamide solution (5 ml) was mixed with H_2O (4.093 ml), 1 M Tris-HCl buffer (pH 8.8, 5.625 ml), 10% SDS solution (150 µl), 10% APS solution (120 µl) and temed (12 µl). The mixture was added between the glass plates, overlaid with isopropanol and allowed to polymerize for 30 minutes.

The stacking gel was prepared by mixing 30% acrylamide solution (0.833 ml), 1 M Tris-HCl buffer (pH 6.8, 0.625 ml), water (3.462 ml), 10% SDS solution (50 µl), 10% APS solution (25 µl) and temed (5 µl). After removing the isopropanol, the stacking gel solution was poured onto the separating gel, the combs were placed and the mixture was allowed to polymerize.

Protein concentration of the samples was determined using Bradford assay (BIORAD reagents). The supernatant samples (volume corresponding to 15 ng) were mixed with same volume Laemmli loading buffer 2x. The pellet samples were mixed with urea (700 µl, 6 M) and the same volume as for the supernatant (15 ng) was mixed with Laemmli loading buffer. Then the samples were heated (at 95 °C for 5 minutes) in a thermo shaker and loaded (whole volume) in each slot. As reference, Page Ruler™ Prestained Protein Ladder (5 µl) was loaded. The gels were run for 30 minutes at 80 V and later increased to 120 V. After that, the gels were stained with ColloidalCoomassie “Blue Silver” staining solution (58.82 ml 85% *ortho*-phosphoric acid, 50 g Ammonium sulphate, 0.6 g Coomassie G-250R, 100 ml EtOH, 500 ml deionized H_2O) for 1 hour. The gels were destained overnight in deionized H_2O and documented by scanning.

4.8 Determination of protein concentration based on Bradford assay

In the method by Bradford Coomassie Blue is used as dye, which binds to the proteins present in the solution leading to a change of absorption (measured at 595 nm).

A dye stock solution was prepared by mixing 1 part of Bradford solution with 4 parts of water. 20 µl of the protein sample were mixed with 980 µl of the dye solution and then incubated at room temperature for 5 minutes.

To determine the protein concentration samples were measured undiluted, 1:10 and 1:20 diluted on Eppendorf BioPhotometer *plus* which provided concentration in mg/ml.

4.9 Digestion, Extraction and ligation of the genes

Digestion mixtures for the gene fragments are summarized in **Table 4.9-1**. All components were mixed in sterile Eppendorf tubes in the given order and incubated at 37 °C for 1 hour 30 minutes without mixing.

For digestion of vectors restriction mixtures are listed in **Table 4.9-2**. All components were mixed in sterile Eppendorf tubes in the given order and incubated at 37 °C for 30 minutes without mixing.

Table 4.9-1: Reaction mixture for restriction of gene fragments.

Component	Volume [µl]
H_2O (nuclease free)	x
Fast DigestR Green Buffer 10x	2
Fast DigestR XhoI	1
Fast DigestR NdeI	1
DNA solution (200 ng)	y
Total	20

Table 4.9-2: Reaction mixture for restriction of vectors

Component	Volume [µl]
H_2O (nuclease free)	x
Fast DigestR Green Buffer 10x	2
Fast DigestR XhoI	1
Fast DigestR NdeI	1
DNA solution (500 ng)	y
Total	20

For extraction the DNA-fragments were cut out of the agarose gel and isolated using the QIAquick® Gel Extraction Kit. The DNA was eluted with deionized H_2O (50 µl) and the DNA concentration was measured with a spectrophotometer (OD 260). To calculate the amount of substances needed for the ligation **equation 1** was used.

$$V\,insert = \frac{V\,plasmid * B\,insert * f * c\,plasmid}{B\,plasmid * c\,insert}$$

equation 1

V…...volume [µl]

B…...seize [kBp]

f……dilution factor (5) [ratio of gene and vector 1:5]

c……concentration [ng/µl]

Components for ligation mixtures are listed in **Table 4.9-3** and were put into a thermo shaker (at 22 °C without shaking, for 3 hours). Then it was heated (to 70 °C for 5 minutes) to deactivate the ligase. After this step the ligated vector was transformed to *E. coli NEB 5α* (competent cells).

Table 4.9-3: Reaction mixture used for ligation

Component	Volume [µl]
H_2O (nuclease free)	x
T4 DNA ligase Buffer 10x (Fermentas)	2
Vector DNA solution (31 ng)	y
Insert DNA solution	z
T4 DNA ligase (Fermentas)	1
Total	20

4.10 Transformation of vectors into *E. coli* cell lines and plasmid isolation

The vials containing the ligation mixture were centrifuged (10 sec), and placed on ice. For each transformation 100 µl of chemical competent cells were thawed on ice. Ligation mixture (3 µl) was pipetted directly into the vial of competent cells and incubated on ice (30 min). Afterwards the vials were incubated in a thermo shaker without shaking (30 sec, 42 °C). The vials were placed on ice and 900 µl LB medium was added. Vials were incubated in a thermo shaker sideways (1 h, 300 rpm, 37 °C). Afterwards the mixtures were pelleted and the pellets were resuspended in 100 µl LB medium followed by spreading on LB agar plates. The plates were incubated overnight (30 °C) and the grown colonies were analyzed by plasmid isolation and sequencing.

4.11 Expression trial of the enzymes in *E. coli* cell lines and growth medium preparation

Overnight cultures (ONCs) for the transformants were made in 50 ml tubes (10 ml LB Medium + 30 µg/ml Kanamycin). The tubes were incubated at 30 °C and shaken (at 120 rpm) overnight. ONCs were used for glycerol stocks (500 µl culture + 500 µl 60% glycerol stock) and stored in the freezer at -20 °C. Plasmid mini-prep of the samples was made according to the protocol of the QIAGEN plasmid Mini-prep kit.

LB agar plates and LB medium were used for bacteria growth. Ingredients are listed in **Table 4.11-1** (medium was made with dest. H_2O). Baffled shaking flasks (volume 1 L) were filled with medium (330 ml) and autoclaved. After cooling to room temperature, kanamycin (30 µg/ml) was added. The prepared medium was inoculated with the ONC (2 ml) and shaken at 28 °C and 120 rpm, [for hydrolase from *Bacillus subtilis* (pdb code 2R11) 120 rpm at 37 °C]. When the cultures reached an OD_{600} between 0.6 - 0.8, the cultures were cooled to room temperature and induced with IPTG (0.3 mM). After induction the flasks were shaken overnight at 20 °C and 120 rpm. The next day the cells were harvested by centrifugation (8000 rpm, 20 min, 4 °C). The cell pellet was washed with phosphate buffer (pH 7.5, 200 mM) After washing, the pellet was resuspended in the same buffer. To disrupt the cells the suspension was ultrasonicated on ice (10% amplitude, 2 sec pulse on, 2 sec pulse off for 4 min). The mixture was centrifuged (10 000 rpm, for 20 minutes, at 4 °C) and the supernatant (soluble fraction) was used for further experiments. The pellet was resuspended in phosphate buffer (per g pellet 10 ml buffer was used, 200 mM, pH 7.5) and a sample was taken (insoluble fraction).

The remaining suspension was frozen (round bottom flasks) in liquid nitrogen and lyophilized. The lyophilized cells were stored at -20 °C

Table 4.11-1: Ingredients of medias used for cell growth

LB Medium	LB Agar plates
10 g/L Tryptone	10 g/L Tryptone
5 g/L Yeast Extract	5 g/L Yeast Extract
5 g/L NaCl	5 g/L NaCl
	15g/L Agar

4.12 General procedure for activity determination

Reactions were carried out in Eppendorf tubes (2 ml). ε-caprolactone **1b** (5.7 mg, 50 μmol) [for 6-methyl-ε-caprolactone **2b** (6.4 mg, 50 μmol)] was suspended in phosphate buffer (694 μl, 200 mM Na_2HPO_4/KH_2PO_4, pH 7.5), MeOH (100 μl) was added and the reaction mixture was pre-incubated at the given temperature. After 10 minutes, enzyme {dissolved in 200 ml phosphate buffer [HLE crude preparation 51 U/mg (1 U corresponds to the amount of enzyme which hydrolyses 1 μmol ethyl butyrate per minute at pH 8.0 and 25 °C)[82]]} was added. After shaking (350 rpm, temperature and time varied for each enzyme) the reaction was saturated with NaCl, standard [dodecane (2.25 μg, 13 μmol) dissolved in 20 μl toluene] was added and the reaction was stopped by extraction with EtOAc (3 x 400 μl). The combined organic layers were dried over Na_2SO_4 and analyzed by GC.

DB 1701 capillary column (30 m, 0.25 mm, 0.25 μm film) was used to analyze lactones and corresponding esters. Temperature program: 100 °C hold 1 min, ramp 1: 20 °C/min – 170 °C – hold 4 min, ramp 2: 30 °C/min – 250 °C hold 1 min postrun 280 °C.

To extract the corresponding carboxylic acid the aqueous layers were acidified with HCl (35%, 50 μl), standard [dodecane (2.25 μg, 13 μmol) dissolved in 20 μl toluene] was added and the reaction was extracted with 2-PrOH (3 x 400 μl). The combined organic layers were dried over Na_2SO_4 and analyzed by GC.

Agilent HP-5 capillary column (30 m, 0.32 mm, 0.25 μm film) was used to analyze carboxylic acids. Temperature program: 80 °C hold 1 min, ramp 1: 20 °C/min – 140 °C – hold 4 min, ramp 2: 40 °C/min – 300°C hold 1 min, postrun 300 °C.

4.13 Derivatisation of hydroxy ester 2c for chiral analytic

Acetic anhydride (100 μl) and DMAP (4-dimethylaminopyridine, cat.) were added to the dried organic phase containing the product hydroxy ster **2c**. The reaction mixture was shaken at 30 °C, 500 rpm for 18 hours. The reaction was quenched by the addition of deionized H_2O (300 μl) and the organic phase was dried over anhydrous Na_2SO_4. The samples were analyzed on a Varian Chirasil Dex CB column (25 m x 0.32 mm x 0.25 μm film). Temperature program: 100 °C hold 1 min, ramp: 10 °C/min – 180 °C – hold 1 min, postrun 180 °C.[83]

4.14 His-tag purification

To purify the His-tagged enzymes a Ni-NTA column (QIAGEN) was used. After harvesting the cells, the pellet (centrifuged at 4 °C for 20 minutes at 8000 rpm) was washed with phosphate buffer (200 mM, pH 7.5) and resuspended in lysis buffer (for 10 g cell pellet 100 ml lysis buffer were used). The suspension was ultrasonicated (10% amplitude, 2 sec pulse on, 2 sec pulse off for 4 min) and centrifuged again (at 4 °C for 20 minutes at 8000 rpm). The column (5 ml HisTrap) was washed with water (50 ml, degased) and lysis buffer (50 ml). Afterwards the sample (dissolved in lysis buffer) (100 ml) was loaded onto the column and eluted with a linear gradient of lysis buffer and elution buffer [(0% / 100% → 100% / 0% 100 ml)]. Fractions (5 ml) were collected and every step was done at 10 °C with a flowrate of 5 ml/min. After elution, the column was washed with cleaning buffer (50 ml), water (degased) and stored in aqueous 20% EtOH (degased) at 4 °C. Fractions were concentrated by centrifuging in VIVASPIN tubes (20 ml; Membrane: 10 000 MWCO PES; at 4 °C at 3500 rpm). After concentration, samples were desalted (PD-10 Desalting Columns, GE Healthcare) and stored in the fridge at 4 °C. Components of the used buffers are listed in **Table 4.14-1**.

Table 4.14-1: His-tag chromatography buffers used *(K Pi* – K_2HPO_4 2.14 g/L / KH_2PO_4 1.04 g/L). pH was adjusted with HCL or NaOH.

Lysis buffer	Elution buffer	Cleaning buffer
300 mM NaCl	300 mM NaCl	300 mM NaCl
20 mM K Pi	20 mM K Pi	20 mM K Pi
20 mM Imidazol	300 mM Imidazol	500 mM Imidazol
pH 7.5	pH 7.5	pH 7.5
(degased)	(degased)	(degased)

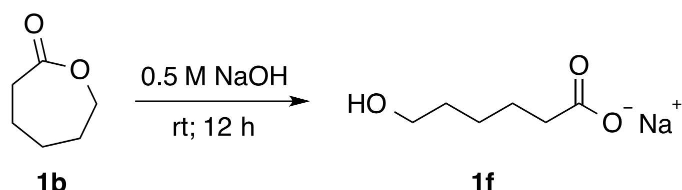
Table 4.14-2: Constructs used in this thesis.

Construct	Enzyme	Vector	Resistance	Expression Host
pEG A	HLE 1_1	pET28a(+)	Kanamycin	*E. coli* Shuffle® T7
pEG B	HLE 1_2	pET28a(+)	Kanamycin	*E. coli* Shuffle® T7
pEG C	HLE 1_3	pET28a(+)	Kanamycin	*E. coli* Shuffle® T7
pEG D	HLE 1_4	pET28a(+)	Kanamycin	*E. coli* Shuffle® T7
pEG E	HLE 1_5	pET28a(+)	Kanamycin	*E. coli* Shuffle® T7
pEG F	HLE 1_6	pET28a(+)	Kanamycin	*E. coli* Shuffle® T7
pEG G	Esterase from *Equus przewalskii*	pET28a(+)	Kanamycin	*E. coli* Shuffle® T7
pEG H	Hydrolase from *Bacillus subtilis*	pSPeedET	Kanamycin	*E. coli* Shuffle® T7

4.15 Synthesis

In this section the synthesis of substrates (lactones) and product references (carboxylic acids, esters) are described.

4.15.1 Synthesis of sodium 6-hydroxyhexanoate (1f)



A solution of ε-caprolactone (**1b**) (1.08 g, 9.46 mmol) in 0.5 M NaOH (20 ml) was stirred at room temperature for 12 h. The solution was cooled on ice and neutralized (pH 7) with Amberlite IR-120 (H^+). The aqueous phase was extracted with diethyl ether (3 x 20 ml). The aeqous layer was lyophilized to give sodium 6-hydroxyhexanoate (**1f**) as white solid (1.46 g, 100% yield).

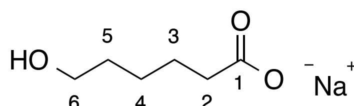
Sodium 6-hydroxyhexanoate (**1f**): white solid; m. p. 203 °C, R_f= 0.36 (CH_2Cl_2/EtOAc/AcOH 20:1:0.5);

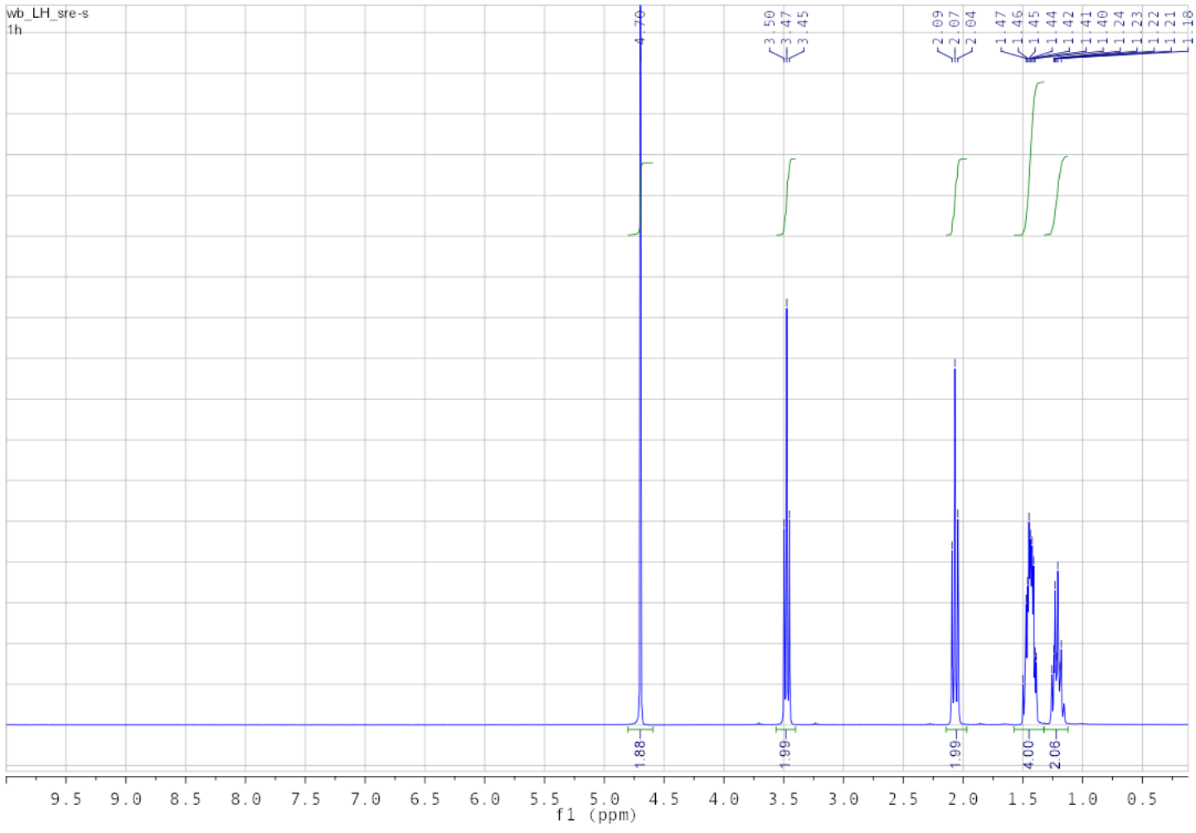
^{1}H NMR (300 MHz, D_2O): δ_H [ppm]: 1.18-1.26, (2H, m), 1.39-1.50 (4H, m), 2.07 (2H, t), 3.47 (2H, t).

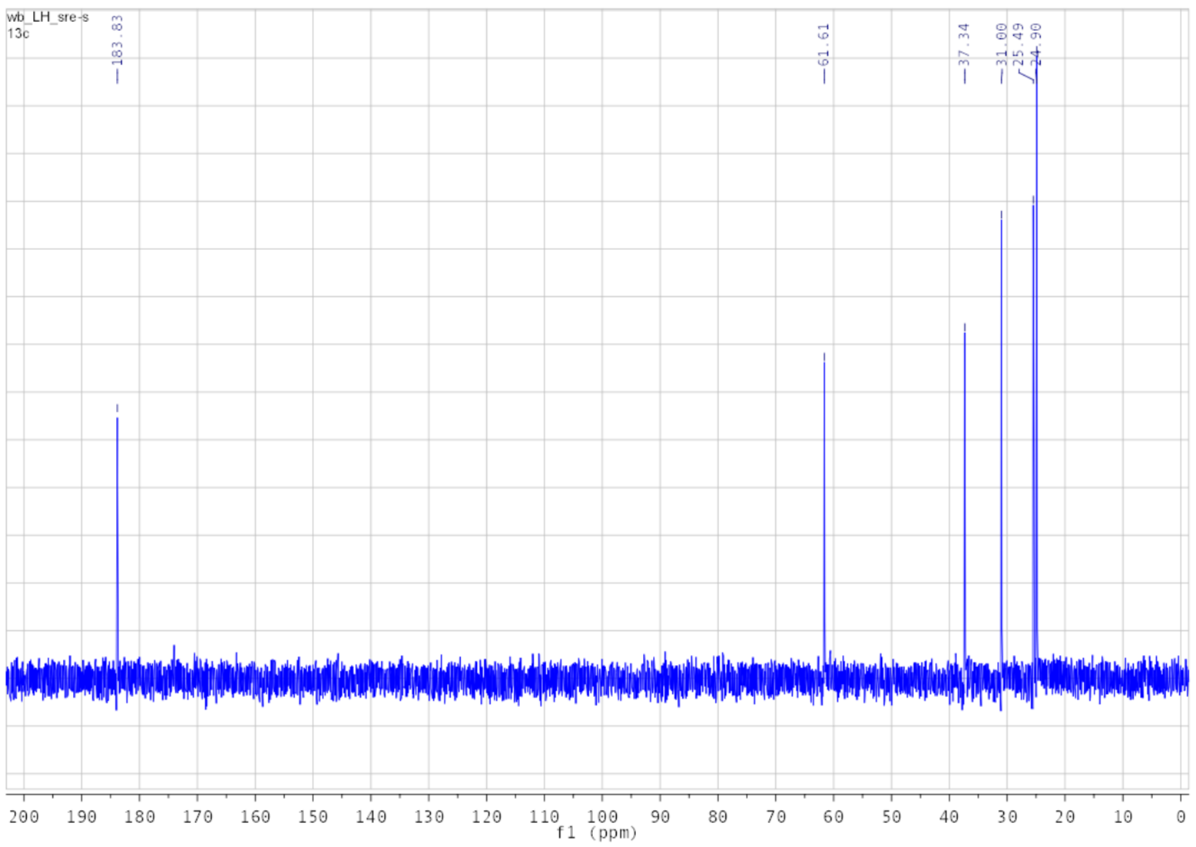
^{13}C NMR (75 MHz, D_2O): δ_C [ppm]: 24.9, 25.5, 31.0, 37.3, 61.6, 183.8.

IR (FT, cm^{-1}): 3307 br, 2938, 2859, 1557, 1443, 1415, 1072, 1050, 1030, 992, 958, 924, 840, 786, 725, 695,

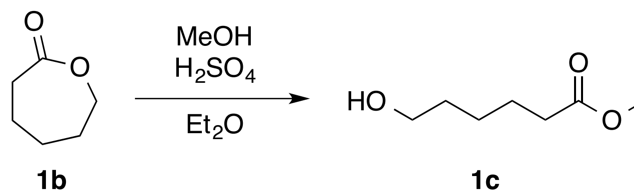
NMR data in accordance with literature.[84]




wb_LH_sre-s
1h
4.70
3.50
3.47
3.45
2.09
2.07
2.04
1.47
1.46
1.45
1.44
1.42
1.41
1.40
1.24
1.23
1.22
1.21
1.18
1.88
1.99
1.99
4.00
2.06
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5
f1 (ppm)


wb_LH_sre-s
13c
183.83
61.61
37.34
31.00
25.49
24.90
200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)

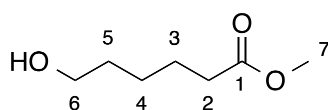
4.15.2 Synthesis of methyl 6-hydroxyhexanoate (1c)

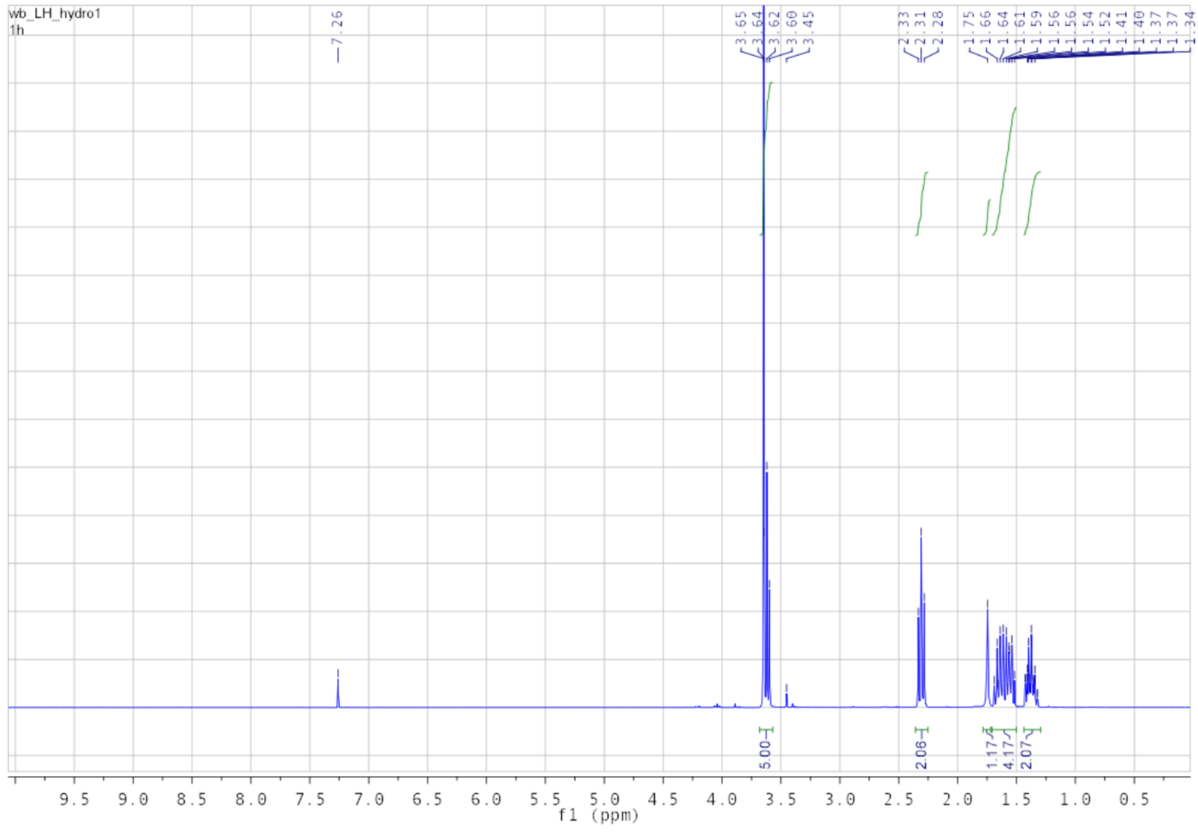


One drop of concentrated aqueous H_2SO_4 was added to a solution of ε-caprolactone (**1b**) (1.08 g, 1.0 ml, 9.4 mmol) in MeOH (9 ml) and stirred for 20 min at room temperature.
The mixture was diluted with diethyl ether (5 ml) and washed with distilled water (5 ml). The aqueous layer was extracted with diethyl ether (3 x 10 ml). Combined organic layers were dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (petrol ether/EtOAc 5:1) to give methyl 6-hydroxyhexanoate (**1c**) (1.09 g, 78.8% yield).[85]

Methyl 6-hydroxyhexanoate (**1c**): colorless oil; R_f = 0.16 (petrol ether/EtOAc 5:1);
^{1}H NMR (300 MHz, $CDCl_3$): δ_H [ppm]: 1.34-1.43, (2H, m), 1.52-1.69 (4H, m), 2.31 (2H, t), 3.60-3.65 (5H, m).
^{13}C NMR (75 MHz, $CDCl_3$): δ_C [ppm]: 24.7, 25.4, 32.4, 34.1, 51.6, 62.7, 174.3.

NMR data in accordance with literature.[86]

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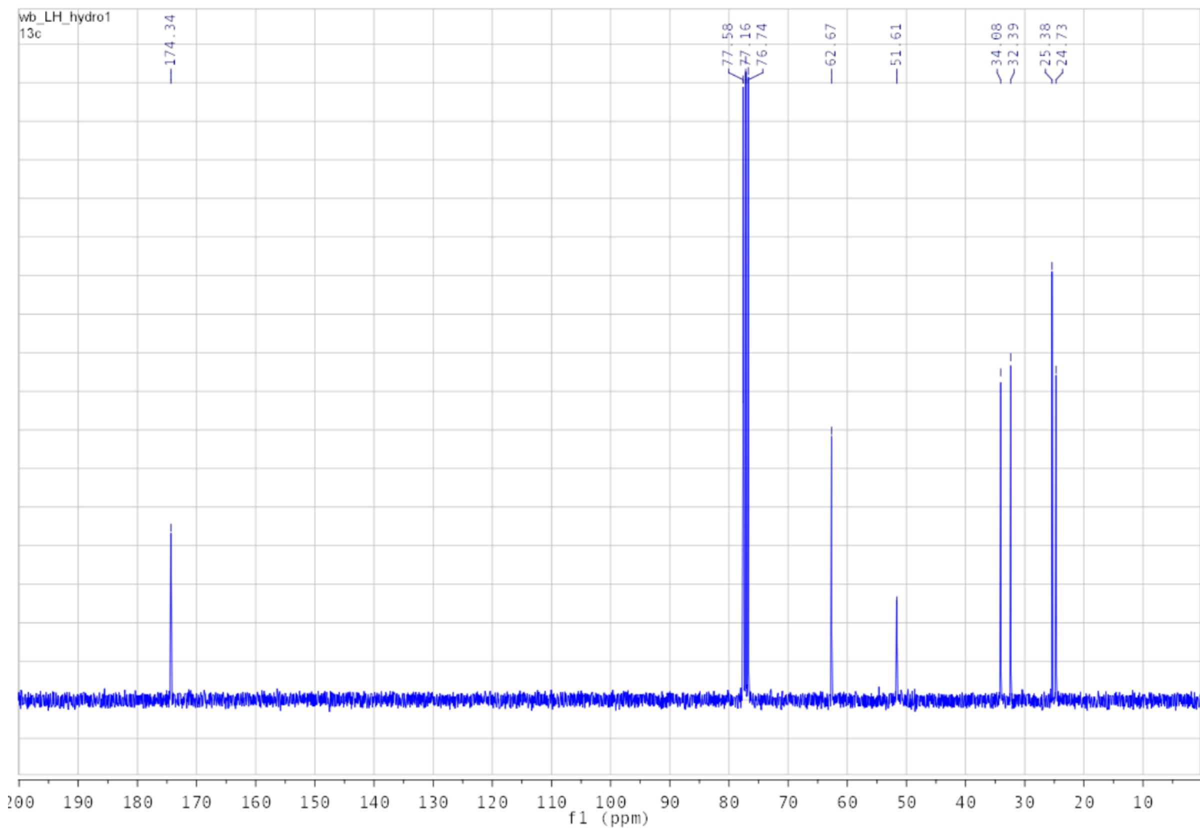
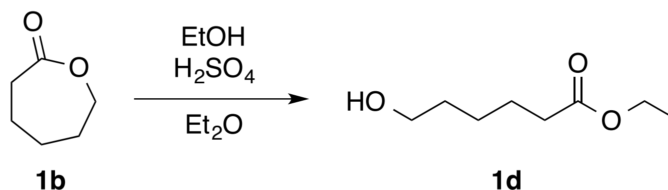
1h
7.26
3.65
3.64
3.62
3.60
3.45
2.33
2.31
2.28
1.75
1.66
1.64
1.61
1.59
1.56
1.56
1.54
1.52
1.41
1.40
1.37
1.37
1.34
5.00
2.08
1.17
4.17
2.07
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5
f1 (ppm)

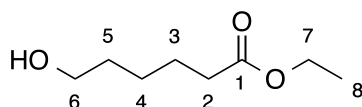

wb_LH_hydro1
13c
174.34
77.58
77.16
76.74
62.67
51.61
34.08
32.39
25.38
24.73
200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10
f1 (ppm)

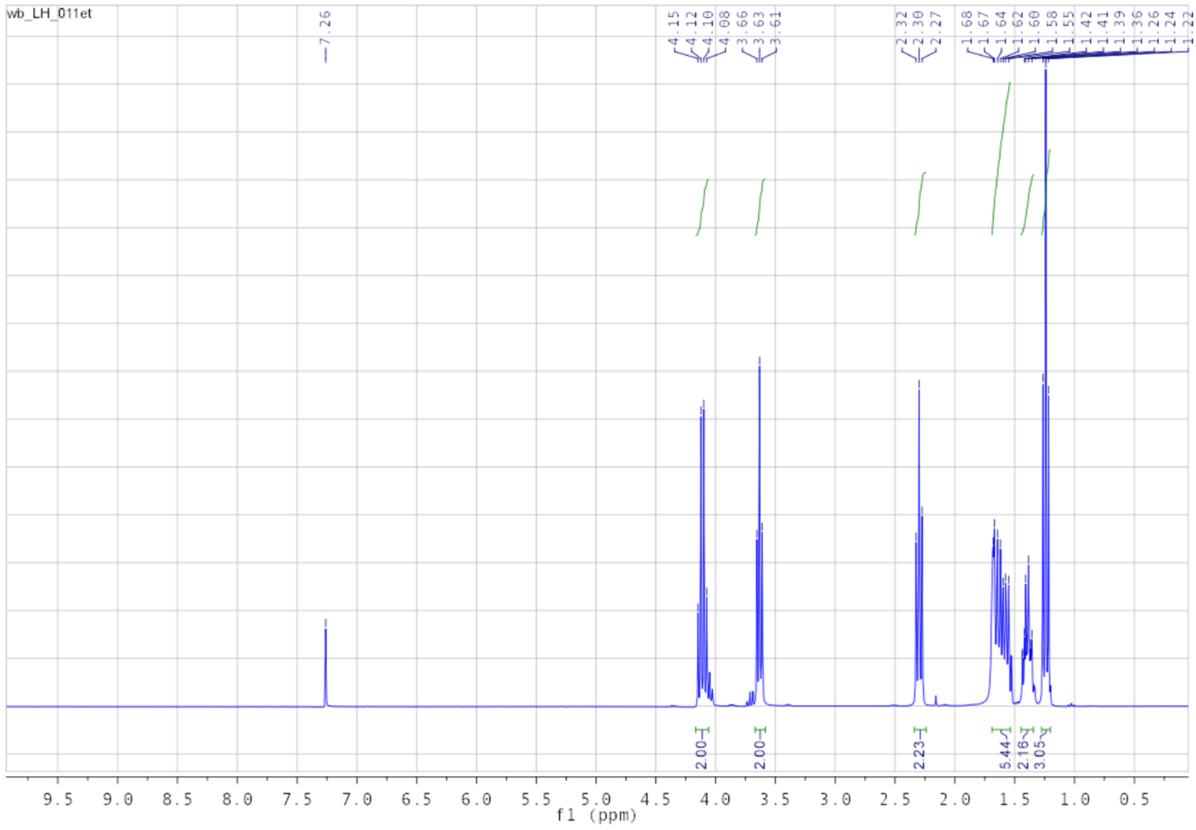
4.15.3 Synthesis of ethyl-6-hydroxyhexanoate (1d)

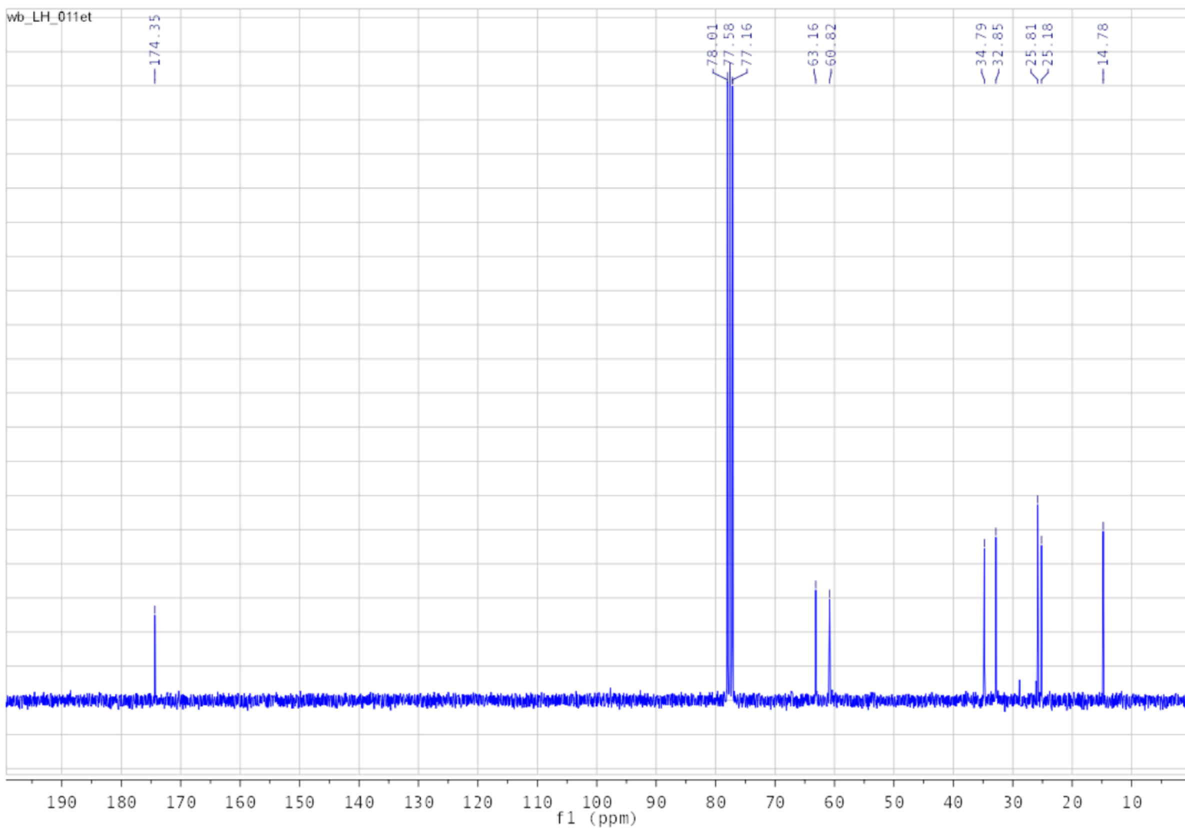


One drop of concentrated aqueous H_2SO_4 was added to a solution of ε-caprolactone (**1b**) (1.08 g, 1.0 ml, 9.4 mmol) in EtOH (9 ml) and stirred for 30 min at room temperature. The mixture was diluted with diethyl ether (5 ml) and washed with distilled water (5 ml). The aqueous layer was extracted with diethyl ether (3 x 10 ml). Combined organic layers were dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (CH_2Cl_2/EtOAc 7:3) to give ethyl-6-hydroxyhexanoate (**1d**) (1.104 g, 72.9% yield).

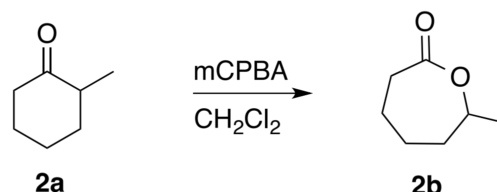
Ethyl-6-hydroxyhexanoate (**1d**): colorless liquid; $R_f = 0.44$ (CH_2Cl_2/EtOAc 7:3);
^{1}H NMR (300 MHz, $CDCl_3$): δ_H [ppm]: 1.24, (3H, t), 1.36-1.42 (2H, m), 1.55-1.68 (4H, m), 2.30 (2H, t), 3.63 (2H, t), 4.51 (2H, q).
^{13}C NMR (75 MHz, $CDCl_3$): δ_C [ppm]: 14.4, 24.8, 25.4, 32.4, 34.4, 60.4, 62.7, 173.9.
Data in accordance with literature.[87,88]




wb_LH_011et
7.26
4.15
4.12
4.10
4.08
3.66
3.63
3.61
2.32
2.30
2.27
1.68
1.67
1.64
1.62
1.60
1.58
1.55
1.42
1.41
1.39
1.36
1.26
1.24
1.22
2.00
2.00
2.23
5.44
2.16
3.05
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5
f1 (ppm)


wb_LH_011et
174.35
78.01
77.58
77.16
63.16
60.82
34.79
32.85
25.81
25.18
14.78
190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10
f1 (ppm)

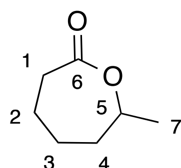
4.15.4 Synthesis of 6-methyl-ε-caprolactone (2b)

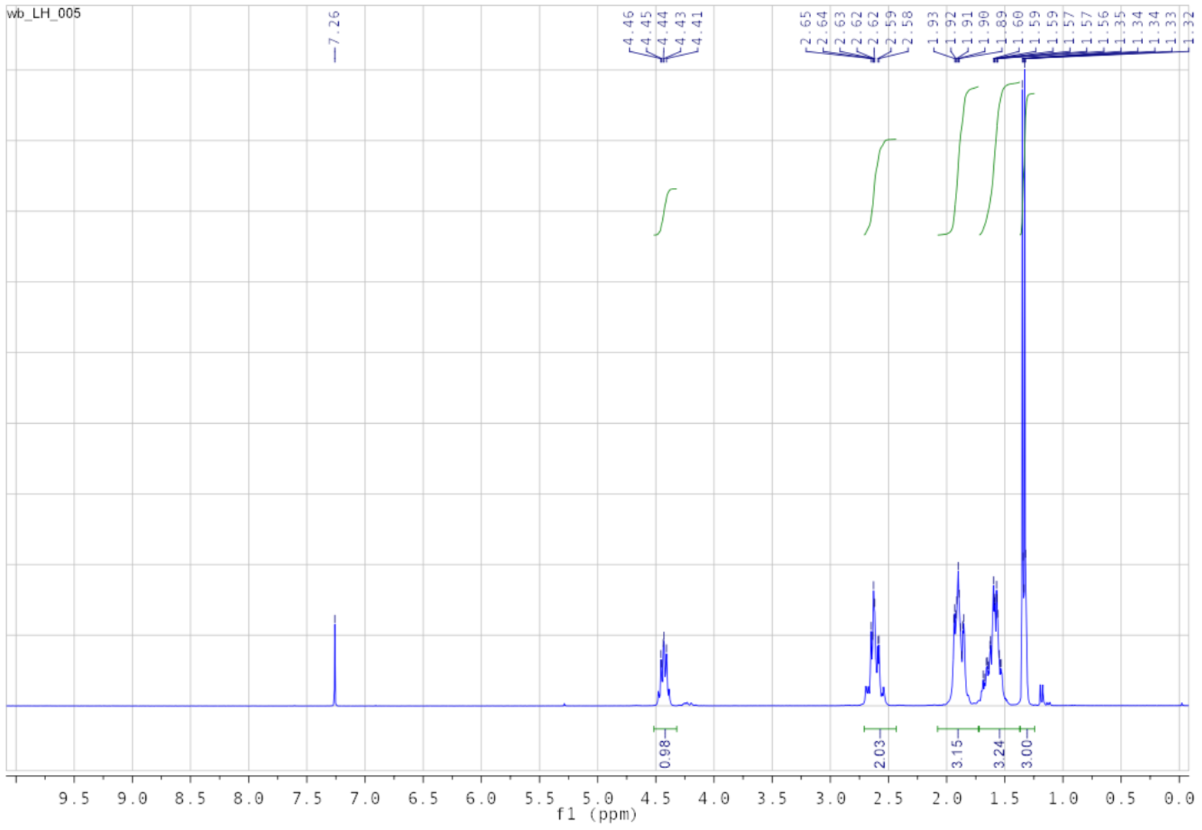


*m*CPBA (2,65 eq, 76%, 26.0 mmol, 5.9 g) was added to a solution of 2-methylcyclohexanone (**2a**) (1.00 eq, 9.8 mmol, 1.2 ml, 0.6 M in CH_2Cl_2) was added 76% *m*CPBA (2,65 eq, 26.0 mmol, 5.9 g) at 0 °C. After stirring overnight at room temperature, the reaction mixture was quenched by the addition of aqueous 10% K_2CO_3 and a saturated aqueous solution of $Na_2S_2O_3$. The aqueous layer was separated and extracted with CH_2Cl_2 (3 x 25 ml). The combined organic layers were dried with Na_2SO_4, filtered and concentrated under reduced pressure. The resulting product was purified by silica gel column chromatography (petrol ether/EtOAc, gradient from 0% to 30%) to give 6-methyl-ε-caprolactone (**2b**) as colorless oil (1.03 g, 82.2% yield).[89]

6-Methyl-ε-caprolactone (**2b**): colorless oil; R_f = 0.31 (petrol ether/EtOAc 7:3); ^{1}H NMR (300 MHz, $CDCl_3$): δ_H [ppm]: 1.33 (3H, d), 1.56-1.60 (3H, m), 1.89-1.93 (3H, m), 2.58-2.65 (2H, m), 4.41-4.46 (1H, m).
^{13}C NMR (75 MHz, $CDCl_3$): δ_C [ppm]: 22.7, 23.0, 28.4, 35.1, 36.2, 77.0, 175.8.

Data in accordance with literature.[89]




wb_LH_005
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4.46
4.45
4.44
4.43
4.41
2.65
2.64
2.63
2.62
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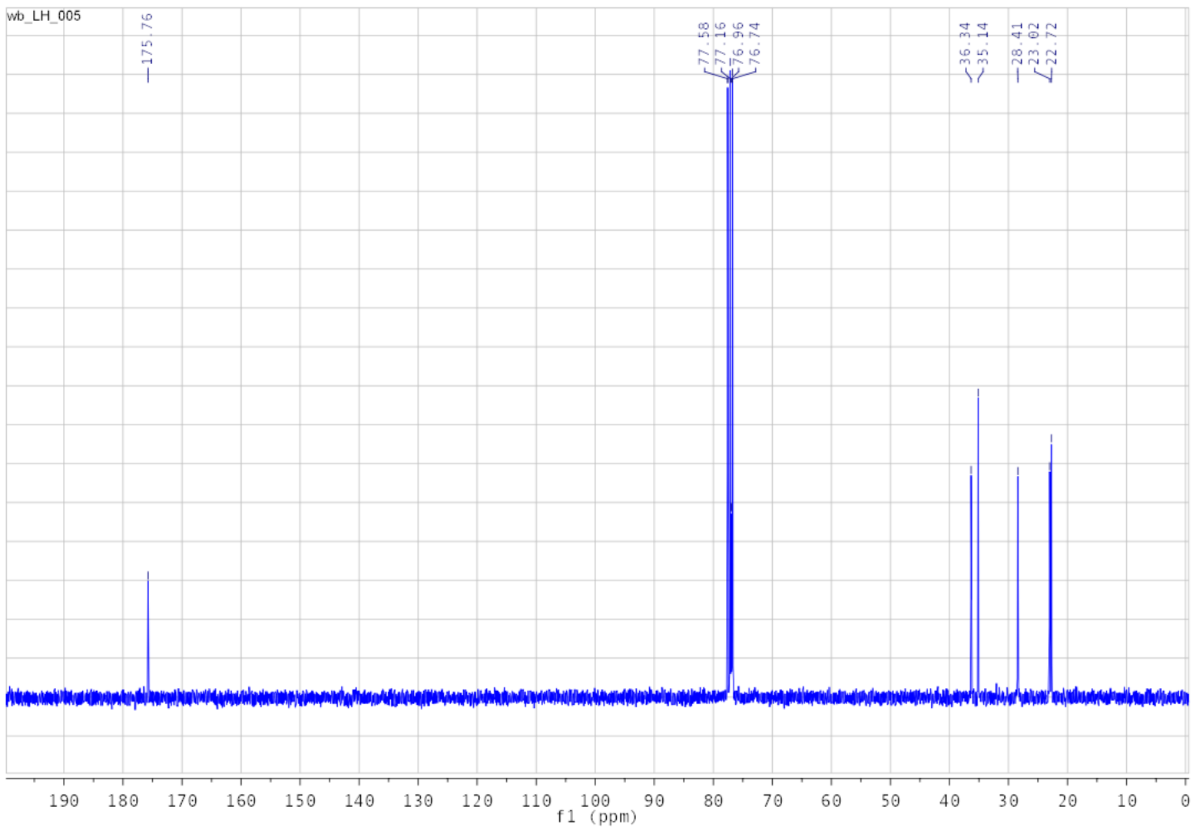
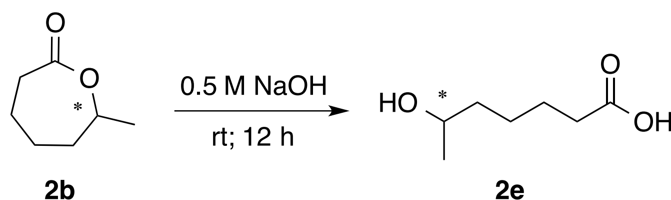
2.59
2.58
1.93
1.92
1.91
1.90
1.89
1.60
1.59
1.59
1.59
1.57
1.56
1.35
1.34
1.34
1.33
1.32
0.98
2.03
3.15
3.24
3.00
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
f1 (ppm)


wb_LH_005
175.76
77.58
77.16
76.96
76.74
36.34
35.14
28.41
23.02
22.72
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

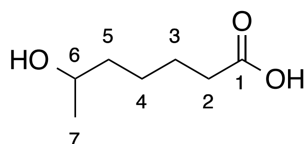
4.15.5 Synthesis of 6-hydroxyheptanoic acid (2e)

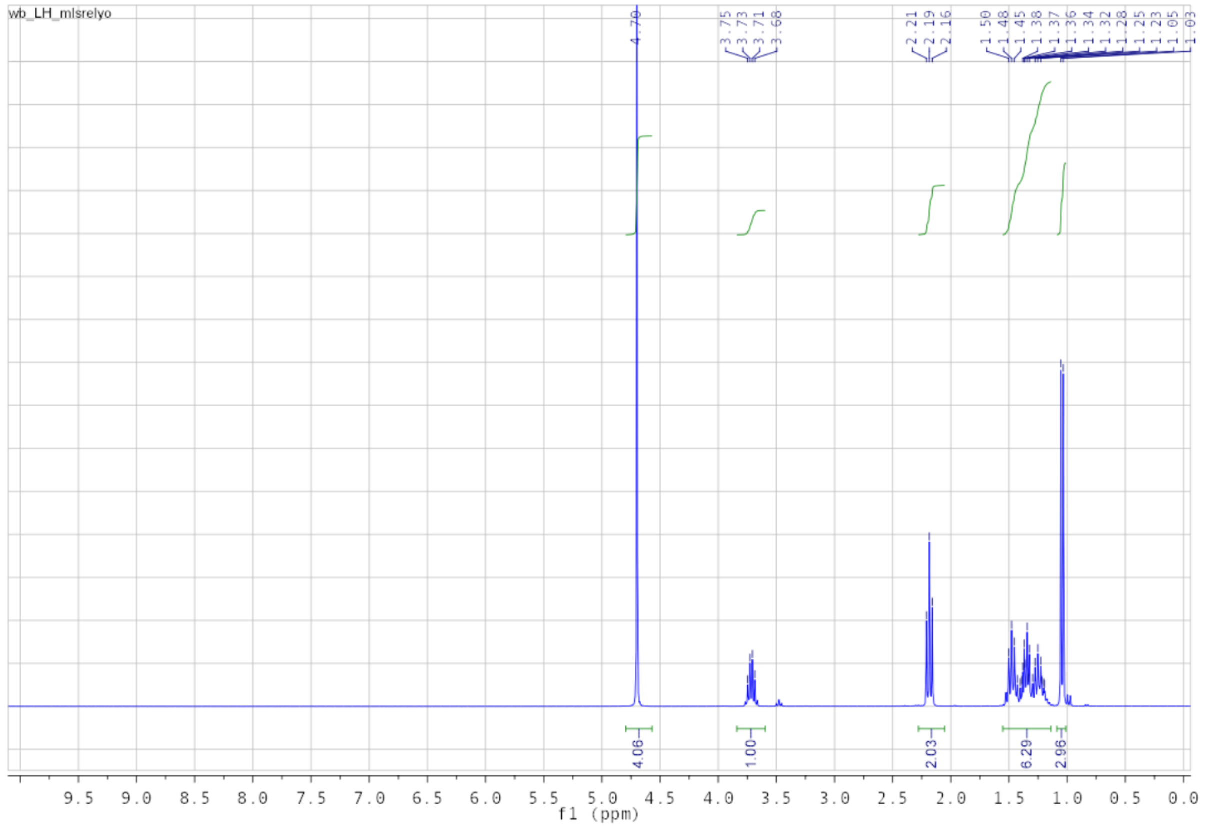


A solution of 6-methyl-ε-caprolactone (**2b**) (102 mg, 0.80 mmol) in 0.5 M NaOH (5 ml) was stirred at room temperature for 12 h. The solution was cooled on ice and neutralized (pH 7) with Amberlite IR-120 (H^+). The aqueous phase was extracted with diethyl ether (3 x 10 ml) and afterwards lyophilized. The resulting product was purified by silica gel column chromatography (CH_2Cl_2/EtOAc/AcOH 14:6:0.1) to give 6-hydroxyheptanoic acid (**2e)** as colorless oil (113 mg, 97.2%).

6-Hydroxyheptanoic acid (**2e**): colorless oil; $R_f = 0.24$ (CH_2Cl_2/EtOAc/AcOH 14:6:0.1);
^{1}H NMR (300 MHz, D_2O): δ_H [ppm]: 1.04, (3H, d), 1.23-1.50 (6H, m), 2.19 (2H, t), 3.72 (1H, q).
^{13}C NMR (75 MHz, D_2O): δ_C [ppm]: 21.7, 24.5, 24.9, 35.4, 37.5, 67.7, 181.4.

^{13}C NMR data in accordance with literature.[90] Literature data was obtained using $CDCl_3$ as solvent

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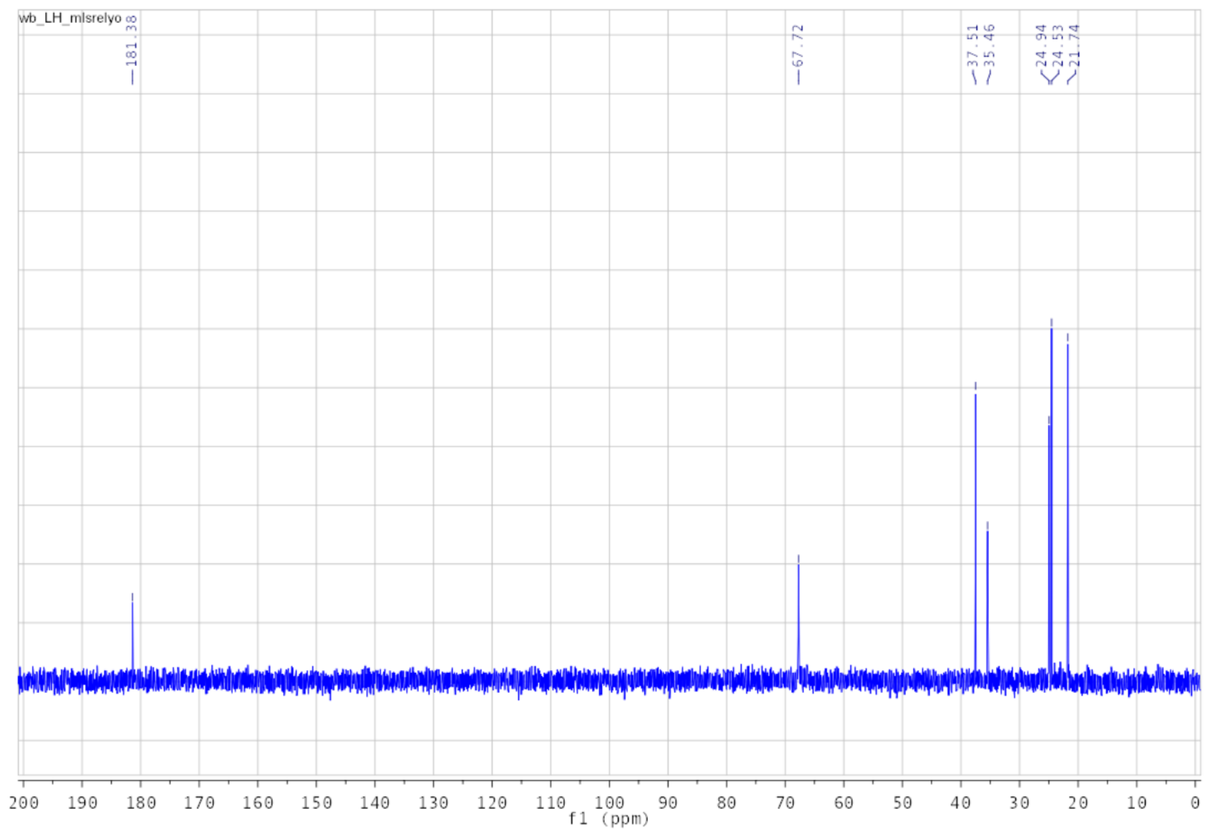
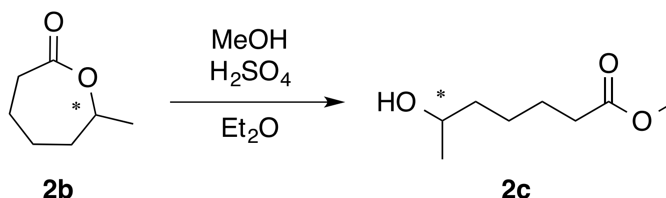
4.70
3.75
3.73
3.71
3.68
2.21
2.19
2.16
1.50
1.48
1.45
1.38
1.37
1.36
1.34
1.32
1.28
1.25
1.23
1.05
1.03
4.08
1.00
2.03
6.29
2.98
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)


wb_LH_mlsrelyo
181.38
67.72
37.51
35.46
24.94
24.53
21.74
200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)

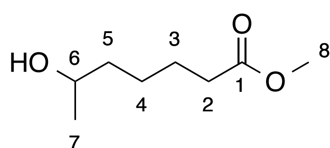
4.15.6 Synthesis of methyl 6-hydroxyheptanoate (2c)

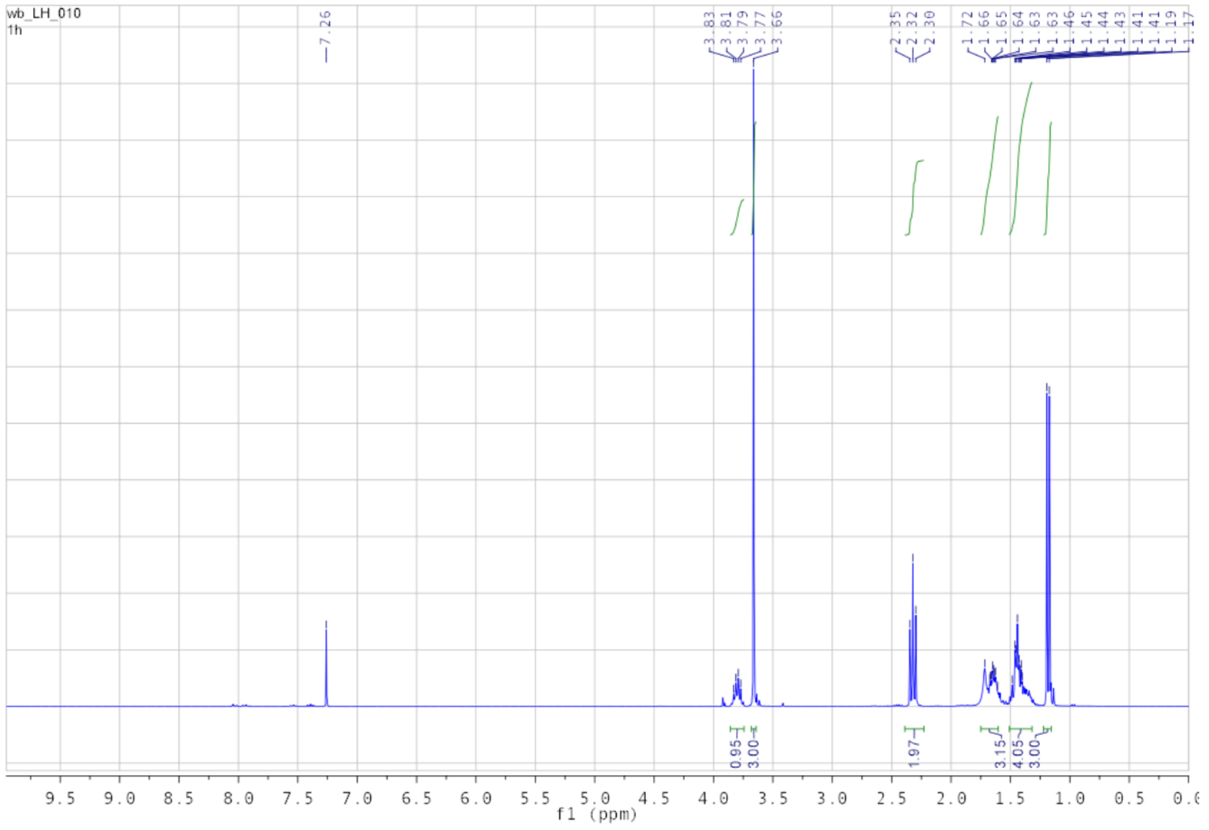


One drop of concentrated aqueous H_2SO_4 was added to a solution of 6-methyl-ε-caprolactone (**2b**) (0.26 g, 0.27 ml, 2.0 mmol) in MeOH (3 ml) and stirred for 30 min at room temperature. The mixture was diluted with diethyl ether (3 ml) and washed with distilled water (3 ml). The aqueous layer was extracted with diethyl ether (3 x 3 ml). Combined organic layers were dried over anhydrous Na_2SO_4, filtered and concentrated under reducd pressure. The residue was purified by silica gel column chromatography (petrol ether/EtOAc 5:1) to give methyl 6-hydroxyheptanoate (**2c**) (colorless oil, 0.18 g, 55,4% yield).

Methyl 6-hydroxyheptanoate (**2c**): colorless oil; R_f = 0.49 (petrol ether/EtOAc 1:1); ^{1}H NMR (300 MHz, $CDCl_3$): δ_H [ppm]: 1.18, (3H, d), 1.40-1.49 (4H, m), 1.61-1.72 (2H, m), 2.32 (2H, t), 3.66 (3H, s) 3.80 (1H, q).
^{13}C NMR (75 MHz, $CDCl_3$): δ_C [ppm]: 23.7, 25.0, 25.4, 34.1, 39.0, 51.7, 68.0, 174.3.

NMR data in accordance with literature.[91]

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1h
7.26
3.83
3.81
3.79
3.77
3.66
2.35
2.32
2.30
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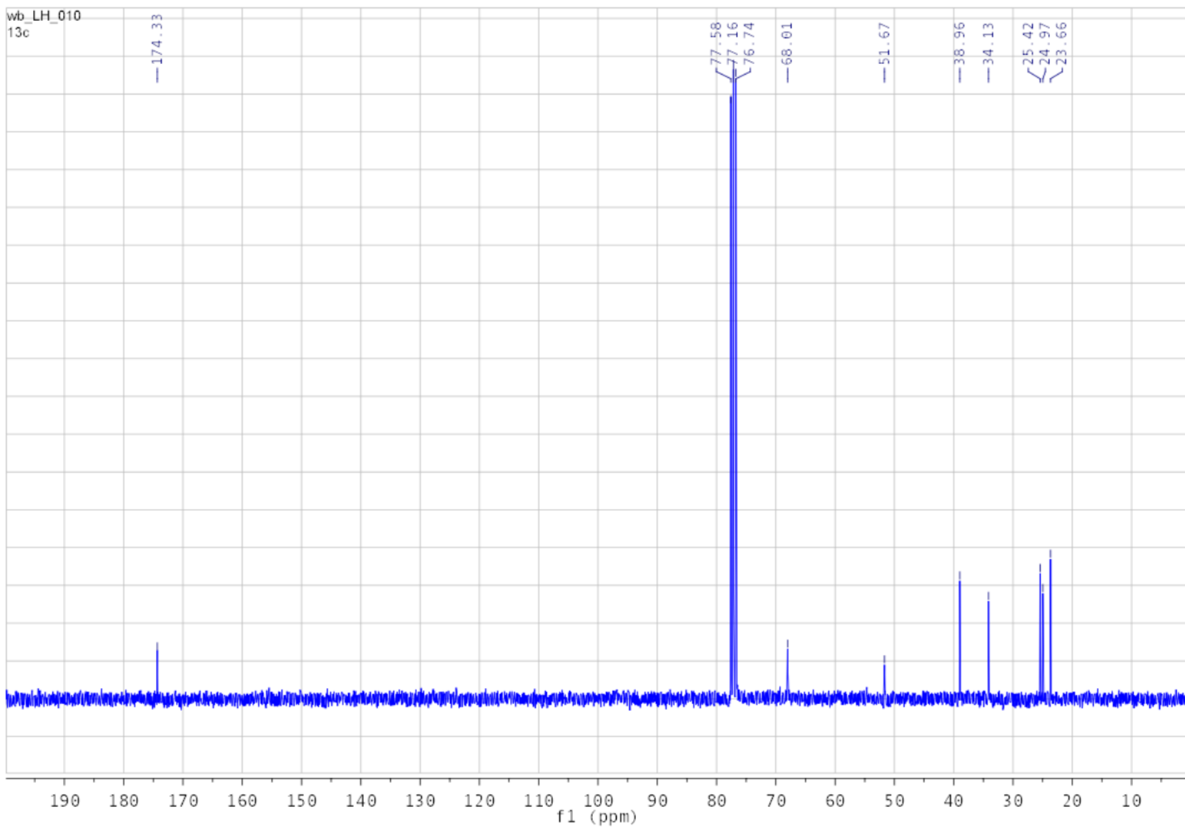
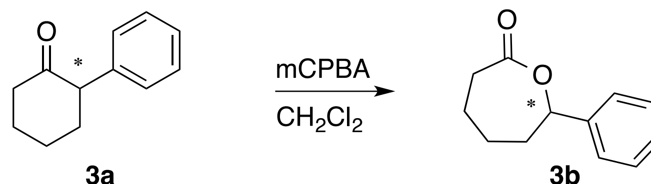
1.66
1.65
1.64
1.63
1.63
1.46
1.45
1.44
1.43
1.41
1.41
1.19
1.17
0.95
3.00
1.97
3.15
4.05
3.00
f1 (ppm)


wb_LH_010
13c
174.33
77.58
77.16
76.74
68.01
51.67
38.96
34.13
25.42
24.97
23.66
f1 (ppm)

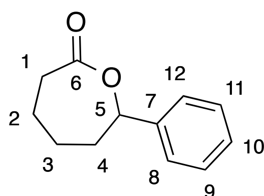
4.15.7 Synthesis of 7-phenyloxepan-2-one (3b)

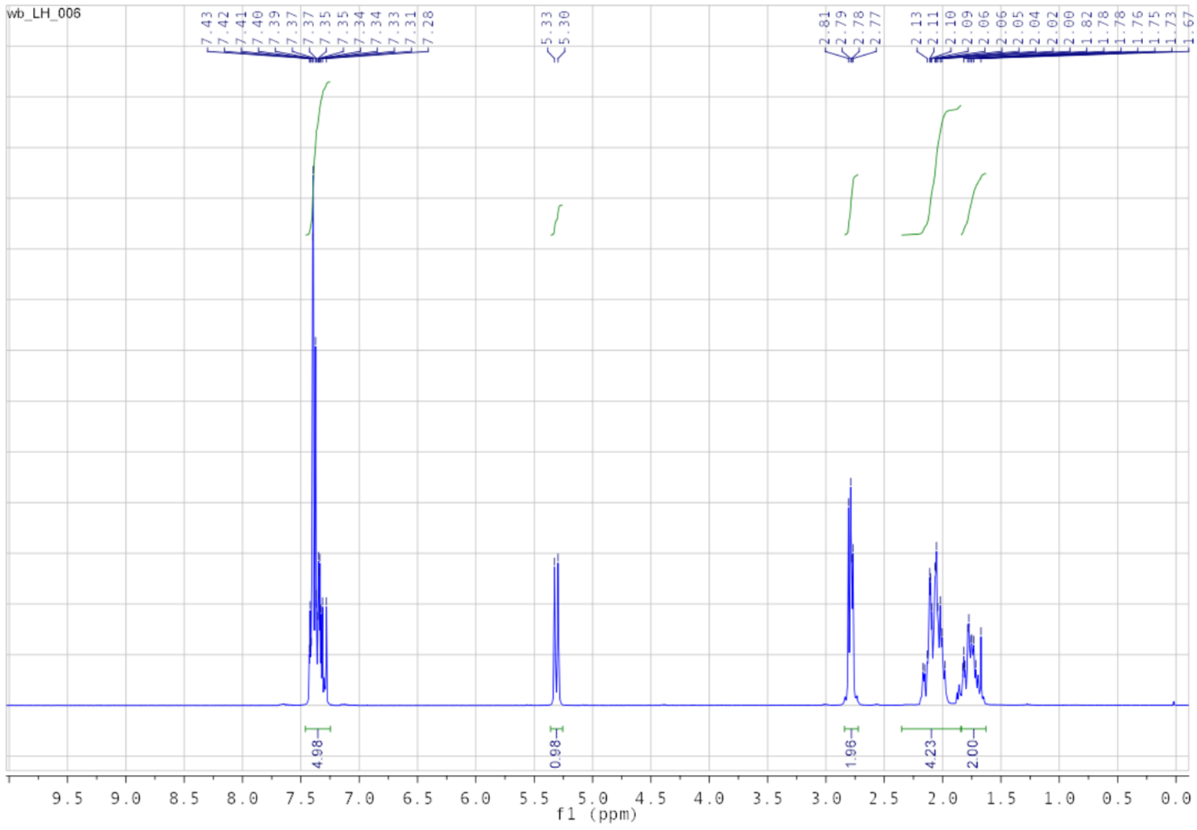


To a solution of 2-phenylcyclohexanone (**3a**) (1.00 eq, 2.85 mmol, 500 mg, 0.2 M in CH_2Cl_2)) was added 76% *m*CPBA (2.44 eqiv. 7.0 mmol, 1.2 g) at 0 °C. After stirring overnight at room temperature, the reaction mixture was quenched by the addition of 10% K_2CO_3 and a saturated aqueous solution of $Na_2S_2O_3$. The aqueous layer was separated and extracted with CH_2Cl_2 (3 x 200 ml). The combined organic layers were dried with Na_2SO_4, filtered and concentrated under reduced pressure. The resulting product was purified by silica gel column chromatography (petrol ether/EtOAc, gradient from 10% to 30%) to give 7-phenyloxepan-2-one (**3b**) as white solid (504 mg, 92.3% yield).

7-Phenyloxepan-2-one (**3b**): white solid; m. p. 70 °C, R_f = 0.48 (petrol ether/EtOAc 7:3);
^{1}H NMR (300 MHz, $CDCl_3$): δ_H [ppm]: 1.67-1.82 (2H, m), 2.00-2.13 (4H, m), 2.77-2.81 (2H, m), 5.32 (1H, d, J = 9.2 Hz), 7.28-7.43 (5H, m).
^{13}C NMR (75 MHz, $CDCl_3$): δ_C [ppm]: 22.9, 28.6, 35.0, 37.5, 82.1, 125.9, 128.1, 128.6, 140.8, 174.9.
IR (FT, cm^{-1}): 3029, 2928, 2868, 1715, 1495, 1443, 1327, 1256, 1227, 1164, 1138, 1086, 1038, 1009, 895, 853, 753, 698, 586;

NMR data in accordance with literature.[92]

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7.39
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7.31
7.28
5.33
5.30
2.81
2.79
2.78
2.77
2.13
2.11
2.10
2.09
2.06
2.06
2.05
2.04
2.02
2.00
1.82
1.78
1.78
1.76
1.75
1.73
1.67
4.98
0.98
1.96
4.23
2.00
9.5
9.0
8.5
8.0
7.5
7.0
6.5
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5.5
5.0
4.5
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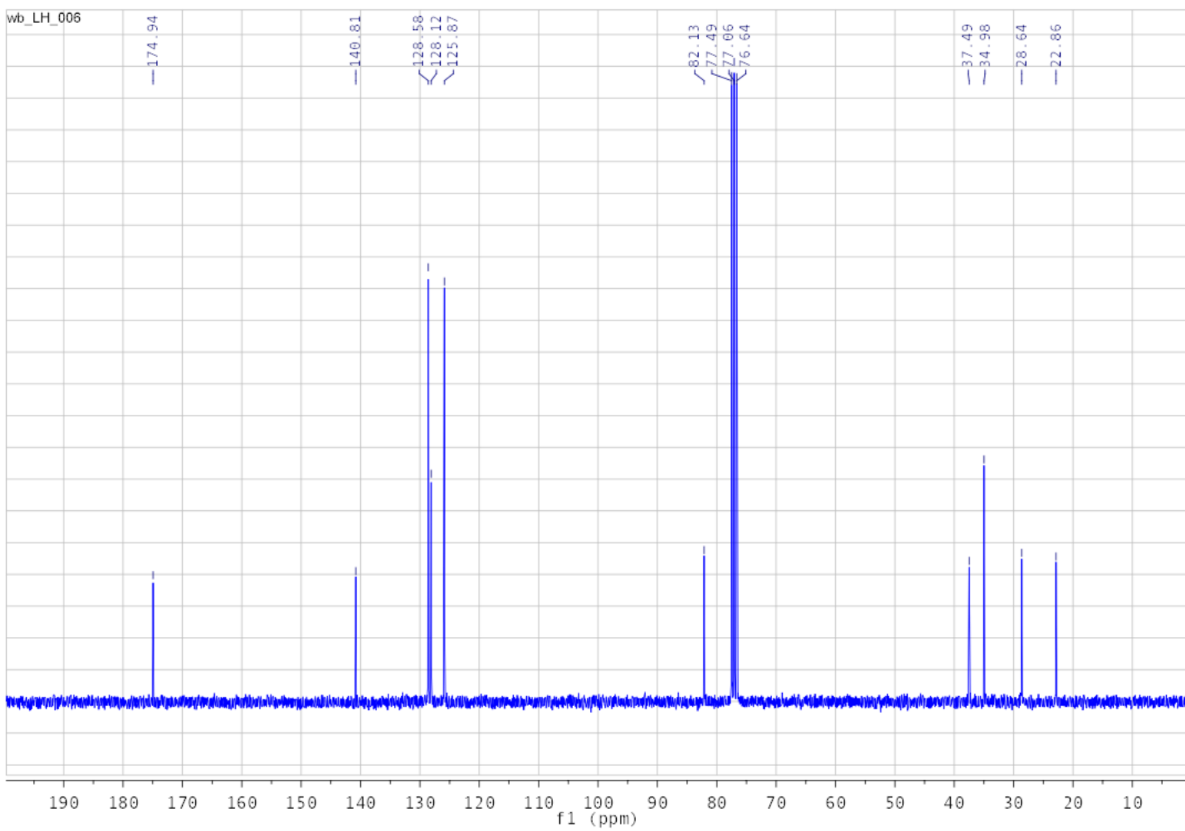
0.0
f1 (ppm)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77.49
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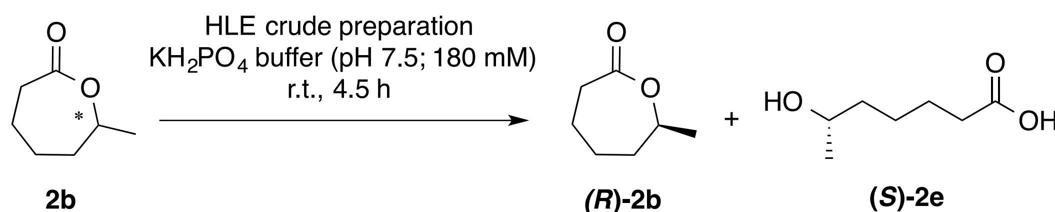
28.64
22.86
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

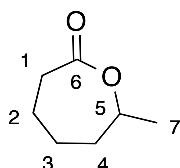
4.15.8 Enzymatic resolution of 6-methyl-ε-caprolactone (2b) to (*R*)-6-methyl-ε-caprolactone [*(R)*-2b] and (*S*)-6-hydroxyheptanoic acid [(*S*)-2e]

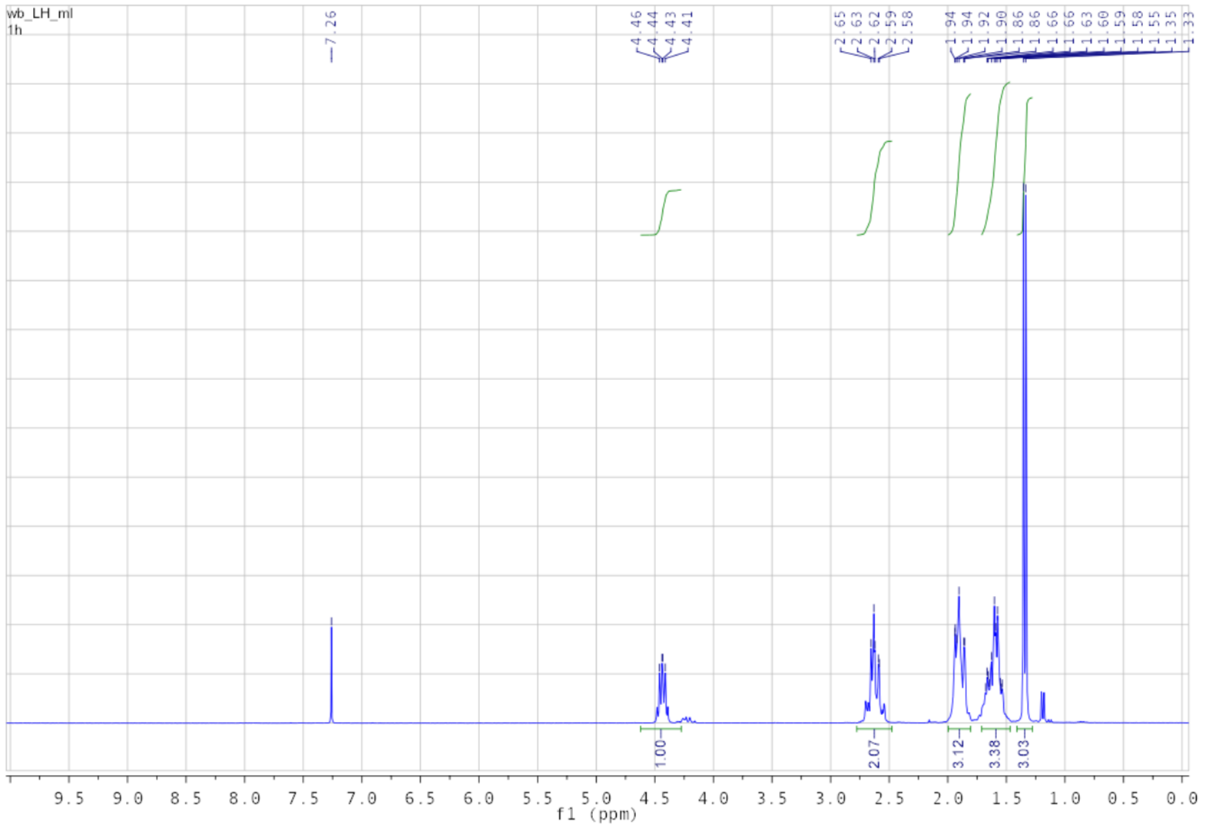


Phosphate buffer (5 ml, 200 mM, pH 7.5) was mixed with racemic 6-methyl-ε-caprolactone (**2b**) (419 mg, 3.3 mmol) and stirred at room temperature. 10 minutes later HLE crude preparation was added (177 mg, 0.51 U/mg). After 4 hours 30 minutes stirring at room temperature, ice (1 g) and Celite (0.5 g) were added. After stirring for 5 minutes the mixture was filtered through Celite and the cake was washed with Et_2O (2 x 2.5 ml). The aqueous phase was extracted with Et_2O (3 x 4 ml). The organic phase was washed with aqueous 10% $NaHCO_3$ (3 ml), dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to give (*R*)-6-methyl-ε-caprolactone [***(R)*-2b** (278 mg, 58.0% yield, *e.e.* = 52.4%)].[93]
The combined aqueous layers were heated for 5 min at 70 °C. After cooling Celite (0.5 g) and 2 M HCl (1 ml) were added. The mixture was filtered and the filtrate was extracted wit Et_2O (3 x 4 ml). The combined organic phases were dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to give (*S*)-6-hydroxyheptanoic acid (**2e**) as colorless oil [(67.6 mg, 30% yield) no *e.e.* was determined].[93]

6-Methyl-ε-caprolactone (**2b**): colorless oil; R_f= 0.65 (petrol ether/EtOAc 1:1);
^{1}H NMR (300 MHz, $CDCl_3$): δ_H [ppm]: 1.34, (3H, d), 1.55-1.66 (3H, m), 1.86-1.94 (3H, m), 2.58-2.65 (2H, m) 4.41-4.46 (1H, m).
^{13}C NMR (75 MHz, $CDCl_3$): δ_C [ppm]: 22.7, 23.0, 28.4, 35.2, 36.4, 77.0, 175.8.

NMR data in accordance with literature.[89]

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1.92
1.90
1.86
1.86
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1.66
1.63
1.60
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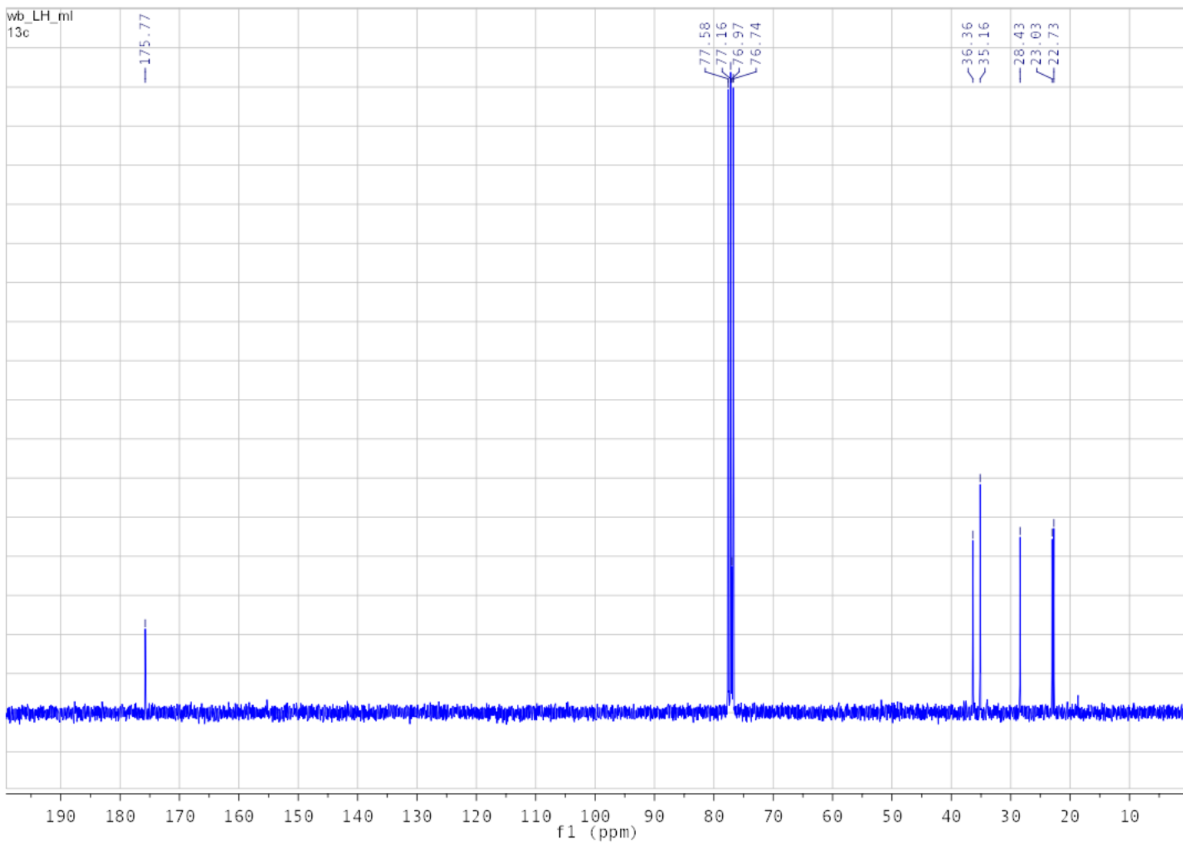
1.58
1.55
1.35
1.33
1.00
2.07
3.12
3.38
3.03
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)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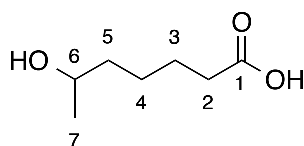
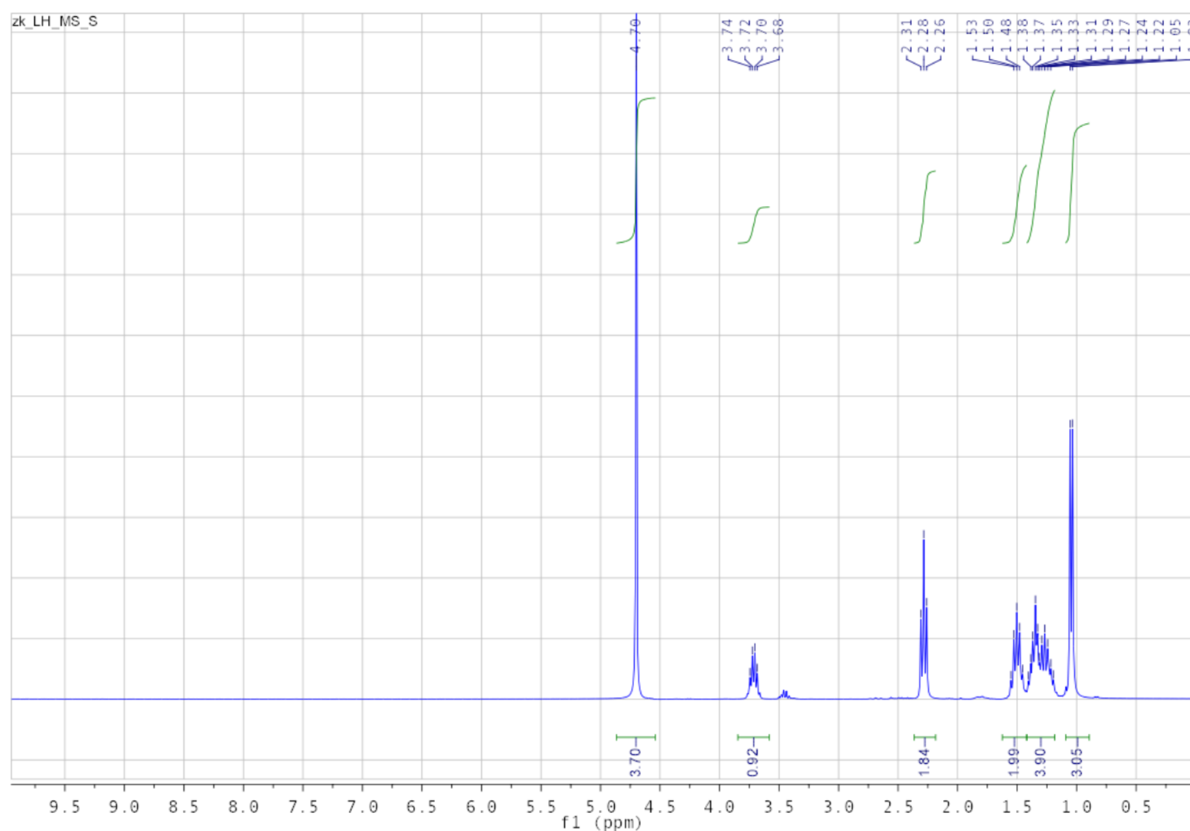
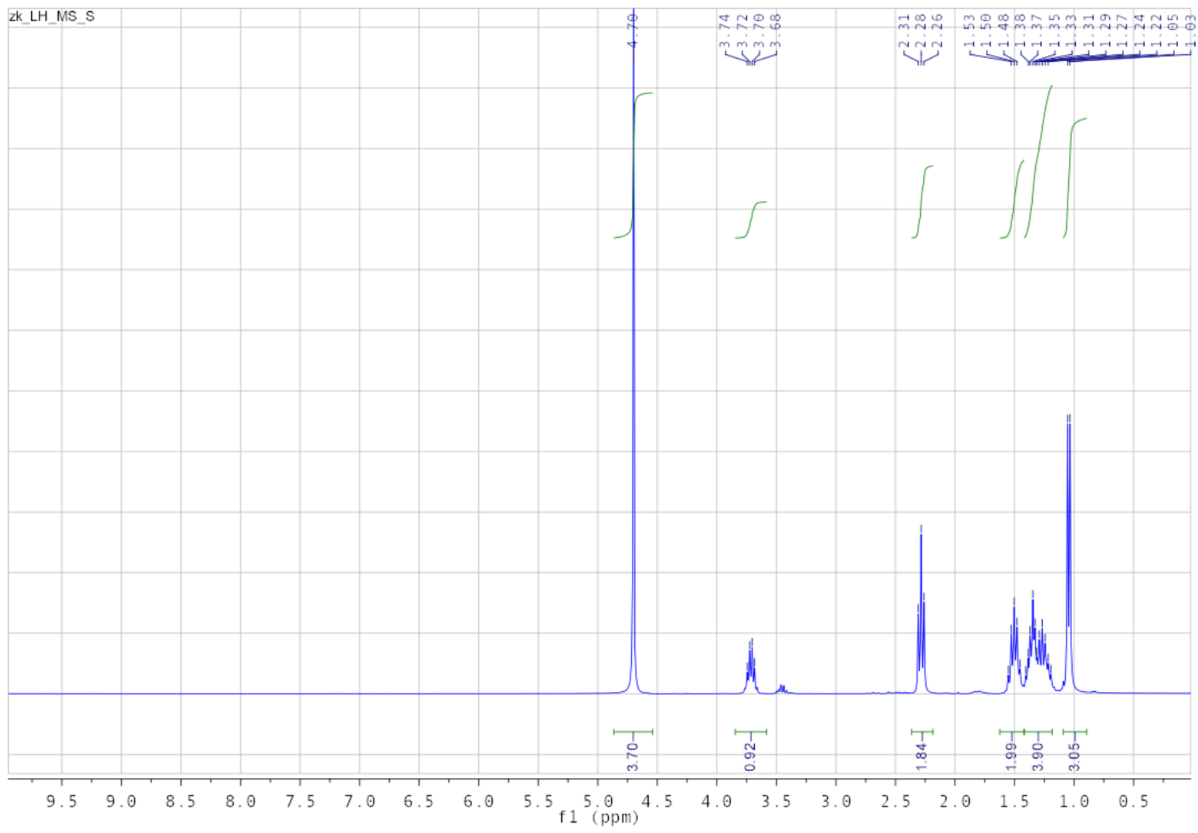
77.16
76.97
76.74
36.36
35.16
28.43
23.03
22.73
190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10
f1 (ppm)

6-Hydroxyheptanoic acid (**2e**): colorless oil; R_f = 0.24 (CH_2Cl_2/EtOAc/AcOH 14:6:0.1);
^{1}H NMR (300 MHz, D_2O): δ_H [ppm]: 1.04, (3H, d), 1.28-1.40 (4H, m), 1.45-1.55 (2H, m), 2.28 (2H, t), 3.71 (1H, q).
^{13}C NMR (75 MHz, D_2O): δ_C [ppm]: 21.8, 24.2, 24.3, 33.7, 37.4, 67.7, 179.1.

^{13}C NMR data in accordance with literature.[90] Literature data was obtained using $CDCl_3$ as solvent



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1.33
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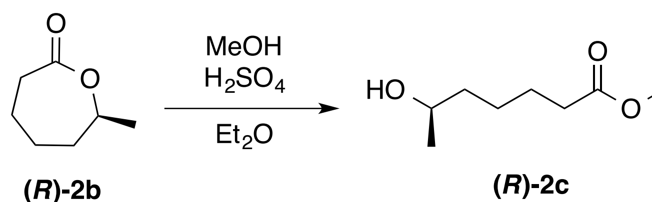
1.29
1.27
1.24
1.22
1.05
1.03
3.70
0.92
1.84
1.99
3.90
3.05
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5
f1 (ppm)

4.15.9 Synthesis of (*R*)-methyl 6-hydroxyheptanoate [(*R*)-2c)



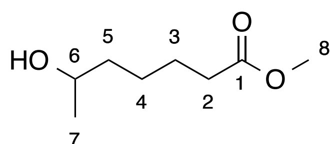
One drop of concentrated aqueous H_2SO_4 was added to a solution of (*R*)-6-methyl-ε-caprolactone [(***R***)-**2b**, *e.e.* 52.4%] 76 mg, 73 µl, 0.6 mmol) in MeOH (3 ml) and stirred for 30 min at room temperature.

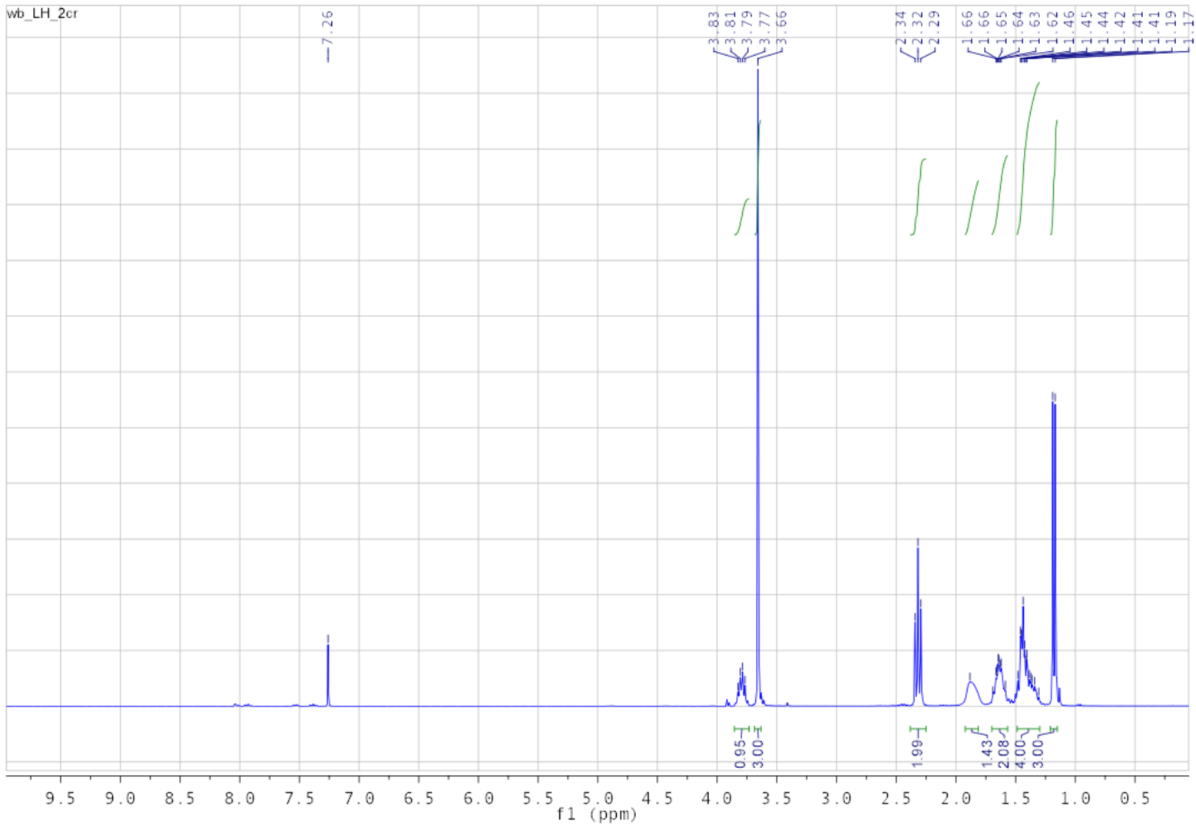
The mixture was diluted with with diethyl ether (3 ml) and washed with distilled water (3 ml). The aqueous layer was extracted with diethyl ether (3 x 3 ml). Combined organic layers were dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (petrol ether/EtOAc 5:1) to give (*R*)-methyl-6-hydroxyheptanoate [(***R***)-**2b**] (57 mg, 59.3% yield, *e.e.* 52.3%).

Methyl-6-hydroxyheptanoate (**2c**): colorless oil; R_f= 0.49 (petrolether/EtOAc 1:1); ^{1}H NMR (300 MHz, $CDCl_3$): δ_H [ppm]: 1.18, (3H, d), 1.31-1.48 (4H, m), 1.59-1.69 (2H, m), 2.32 (2H, t), 3.66 (3H, s) 3.80 (1H, q);

^{13}C NMR (75 MHz, $CDCl_3$): δ_C [ppm]: 23.6, 25.0, 25.4, 34.1, 38.9, 51.7, 68.0, 174.4.

NMR data in accordance with literature.[91]

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1.64
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1.62
1.46
1.45
1.44
1.42
1.41
1.41
1.19
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0.95
3.00
1.99
1.43
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3.00
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5
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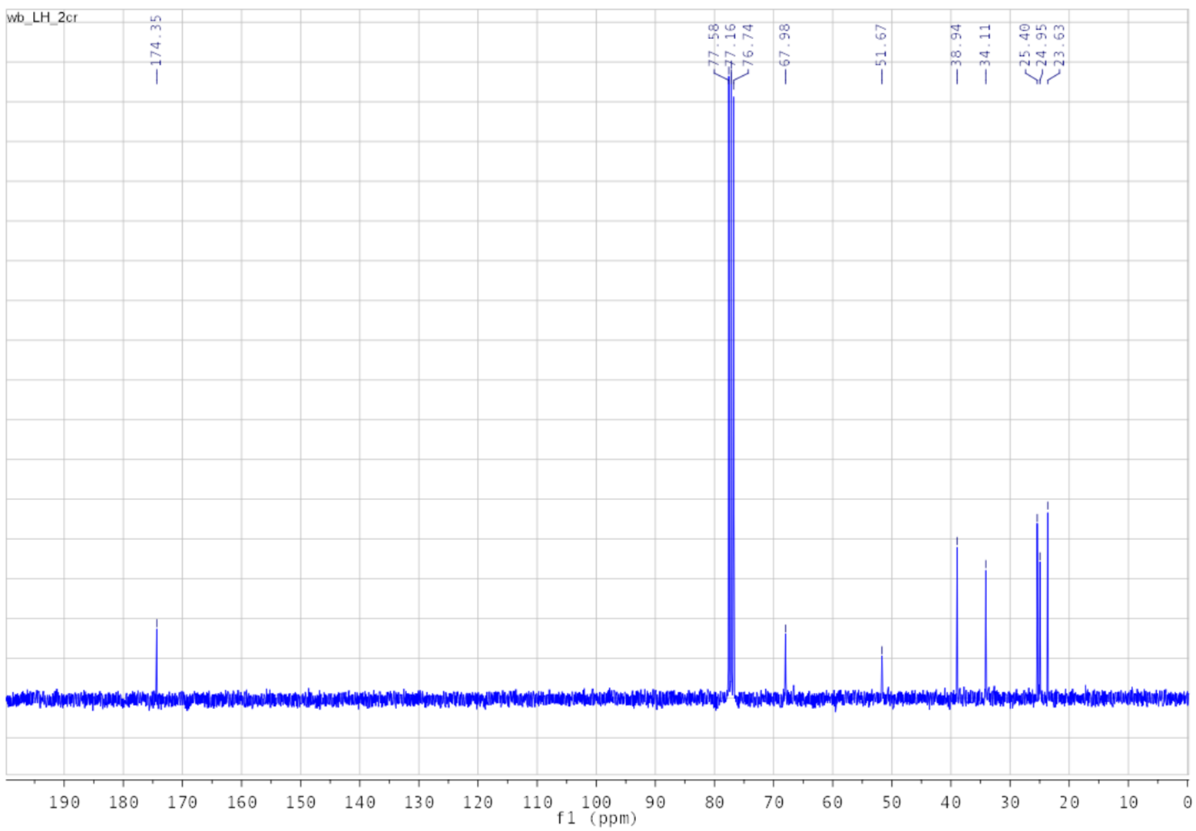
wb_LH_2cr
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77.16
76.74
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51.67
38.94
34.11
25.40
24.95
23.63
190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)

5 Appendix

5.1 Calibration of ε-caprolactone (1b), methyl 6-hydroxyhexanoat (1c), 6-hydroxyhexanoate (1e) and ethyl 6-hydroxyhexanoat (1d)

ε-Caprolactone (**1b**), methyl-6-hydroxyhexanoat **(1c)** and sodium 6-hydroxyhexanoate (**1f**) were weighted and dissolved in phosphate buffer (894 µl, 200 mM Na_2HPO_4/KH_2PO_4, pH 7.5) and MeOH (100 µl) was added. The mixture was saturated with NaCl, standard [dodecane (2.25 µg, 13 µmol) dissolved in 20 µl toluene] was added and extracted with EtOAc (3 x 400 µl). The combined organic layers were dried over Na_2SO_4 and analyzed by GC.

DB 1701 capillary column (30 m, 0.25 mm, 0.25 µm film) was used to analyze lactones and corresponding esters. Temperature program: 100 °C hold 1 min, ramp 1: 20 °C/min – 170 – hold 4 min – 30 °C/min – 250 °C hold 1 min postrun 280 °C.

To extract the corresponding carboxylic acid aqueous layer was acidified with HCl (35%, 50 µl), standard [dodecane (2.25 µg, 13 µmol) dissolved in 20 µl toluene] was added and the acid was extracted with 2-PrOH (3 x 400 µl). The combined organic layers were dried over Na_2SO_4 and analyzed by GC.

Agilent HP-5 capillary column (30 m, 0.32 mm, 0.25 µm film) was used to analyze carboxylic acid. Temperature program: 80 °C hold 1 min, ramp 1: 20 °C/min – 140 °C hold 4 min, ramp 2: 40 °C/min – 300 °C hold 1 min, postrun 300 °C.

Calibration of ethyl-6-hydroxyhexanoat **(1d)** was performed equivalent to methyl 6-hydroxyhexanoat **(1c).**

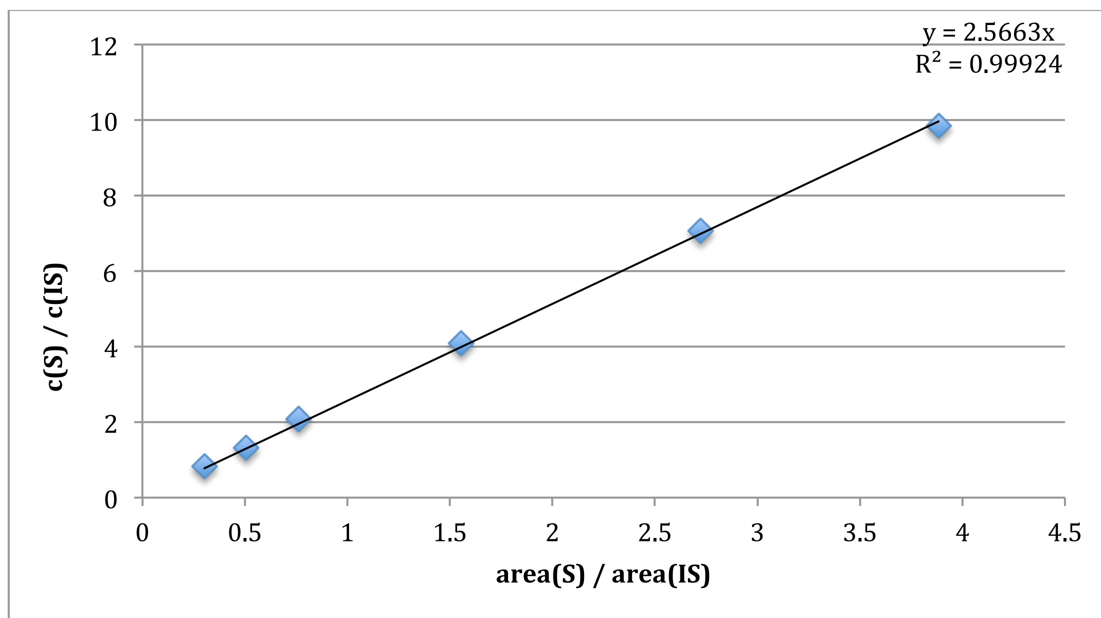

Figure 5.1-1: Calibration of ε-caprolactone (**1b**).

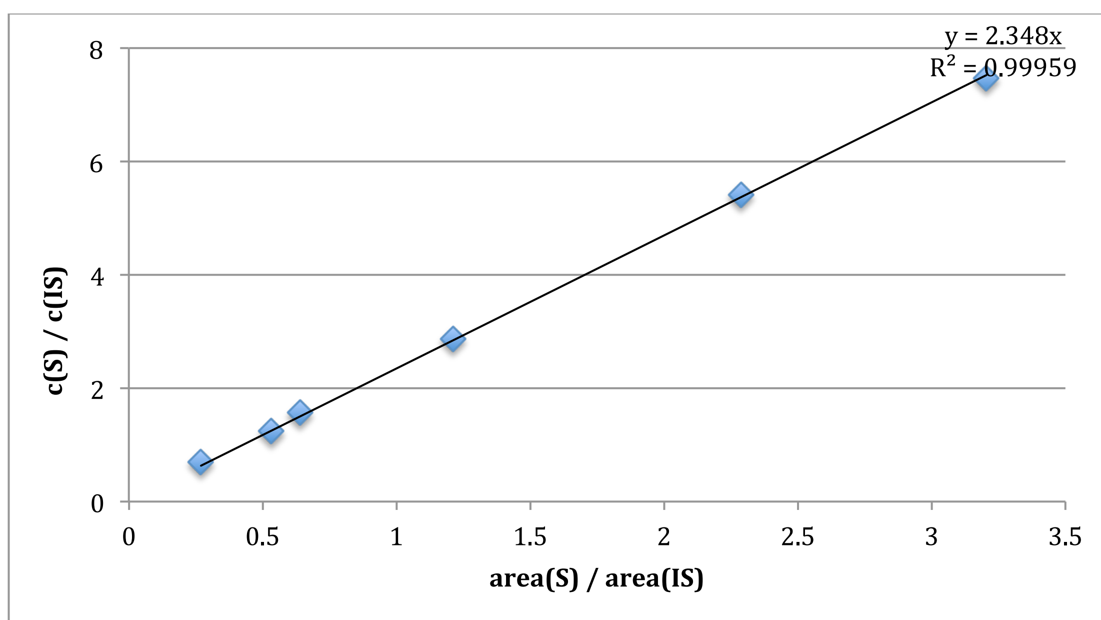

Figure 5.1-2: Calibration of methyl 6-hydroxyhexanoate (**1c**).

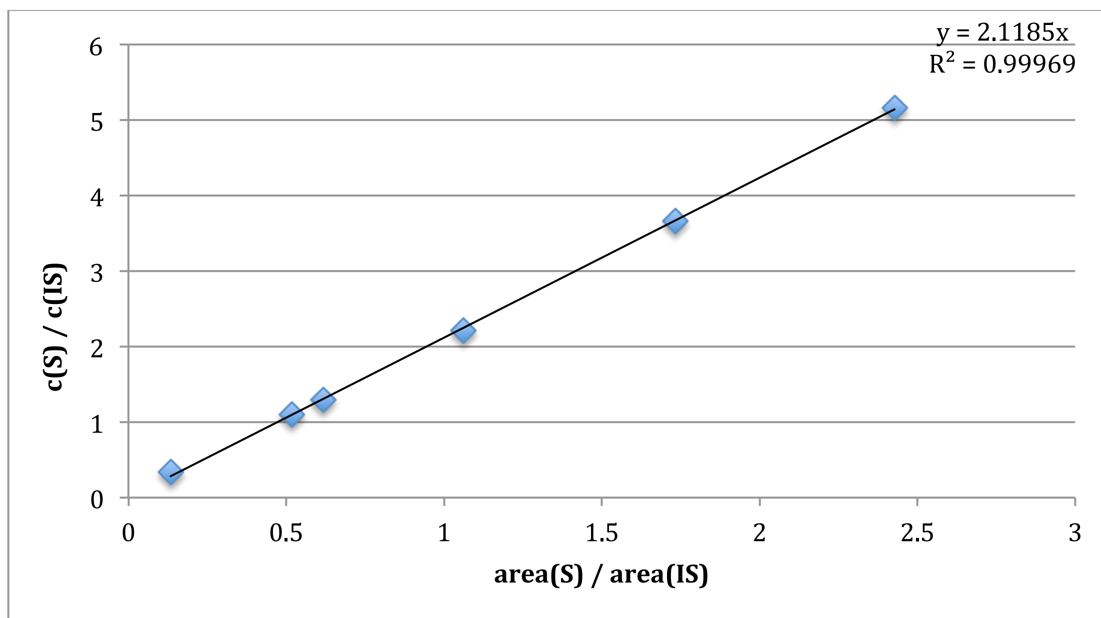

Figure 5.1-3: Calibration of ethyl 6-hydroxyhexanoate (**1d**).

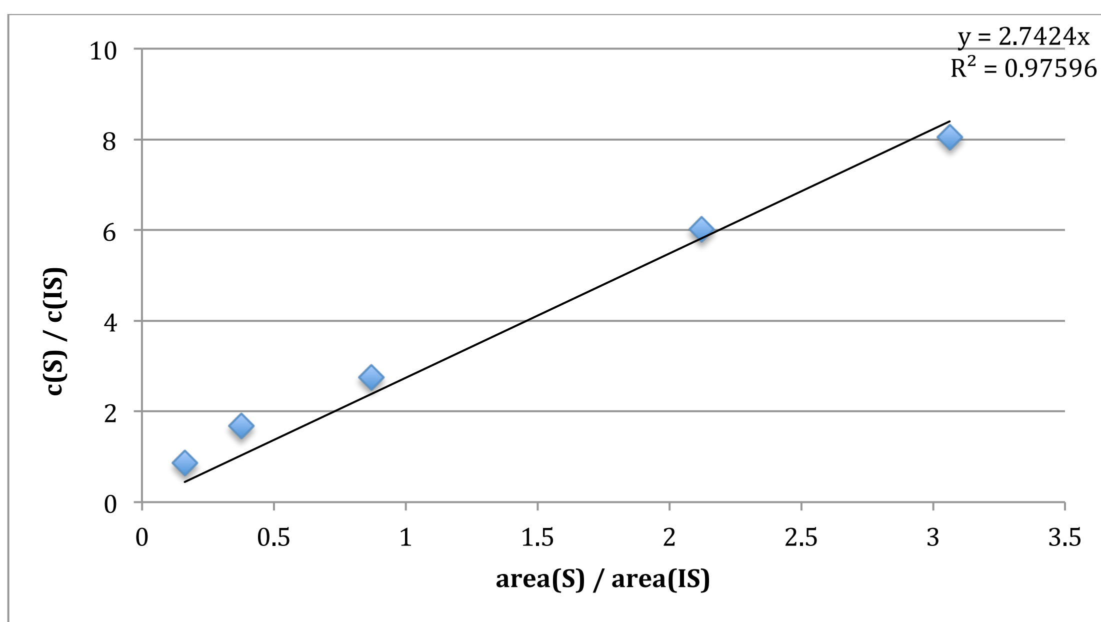

Figure 5.1-4: Calibration of 6-hydroxyhexanoic acid **(1e).**

5.2 Calibration of 7-Methyoxepan-2-one (2b), methyl 6-hydroxyheptanoat (2c) and 6-hydroxyheptanoate (2e)

6-Methyl-ε-caprolactone (**2b**), methyl-6-hydroxyheptanoat **(2c)** and 6-hydroxyheptanoate (**2e**) were weighted and dissolved in phosphate buffer (894 µl, 200 mM Na_2HPO_4/KH_2PO_4, pH 7.5) and MeOH (100 µl) was added. The mixture was saturated with NaCl, external standard [dodecane (2.25 µg, 13 µmol) dissolved in 20 µl toluene] was added and samples were extracted with EtOAc (3 x 400 µl). The combined organic layers were dried over Na_2SO_4 and analyzed by GC.

DB 1701 capillary column (30 m, 0.25 mm, 0.25 µm film) was used to analyze lactones and corresponding esters. Temperature program: 100 °C hold 1 min, ramp 1: 20 °C/min – 170 °C – hold 4 min , ramp 2: 30 °C/min – 250 °C hold 1 min postrun 280 °C.

To extract the corresponding carboxylic acid the aqueous layer was acidified with HCl (35%, 50 µl), standard [dodecane (2.25 µg, 13 µmol) dissolved in 20 µl toluene] was added and the acid was extracted with 2-PrOH (3 x 400 µl). The combined organic layers were dried over Na_2SO_4 and analyzed by GC.

Agilent HP-5 capillary column (30 m, 0.32 mm, 0.25 µm film) was used to analyze carboxylic acid. Temperature program: 80 °C hold 1 min, ramp 1: 20 °C/min – 140 °C hold 4 min, ramp 2 – 40 °C/min – 300 °C hold 1 min, postrun 300 °C.

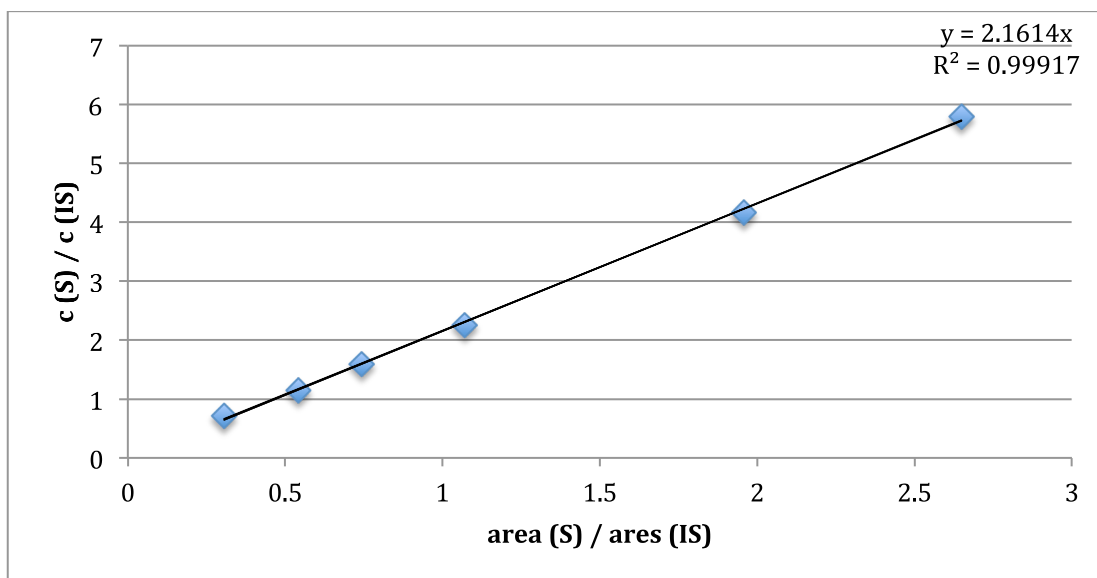

Figure 5.2-1: Calibration of 6-methyl-ε-caprolactone (**2b**).

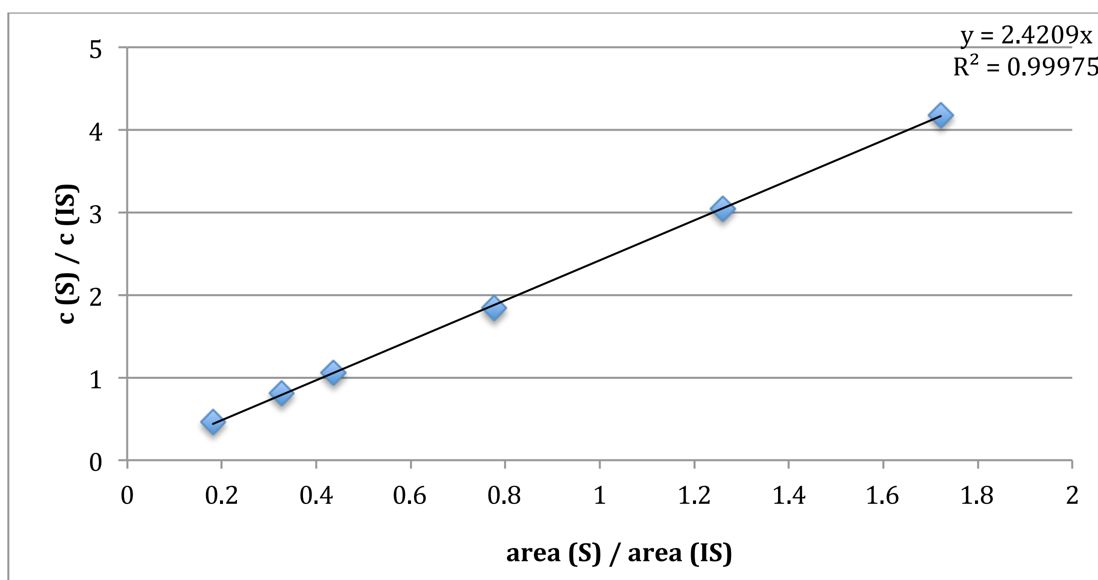

Figure 5.2-2: Calibration of methyl 6-hydroxyheptanoate (**2c**).

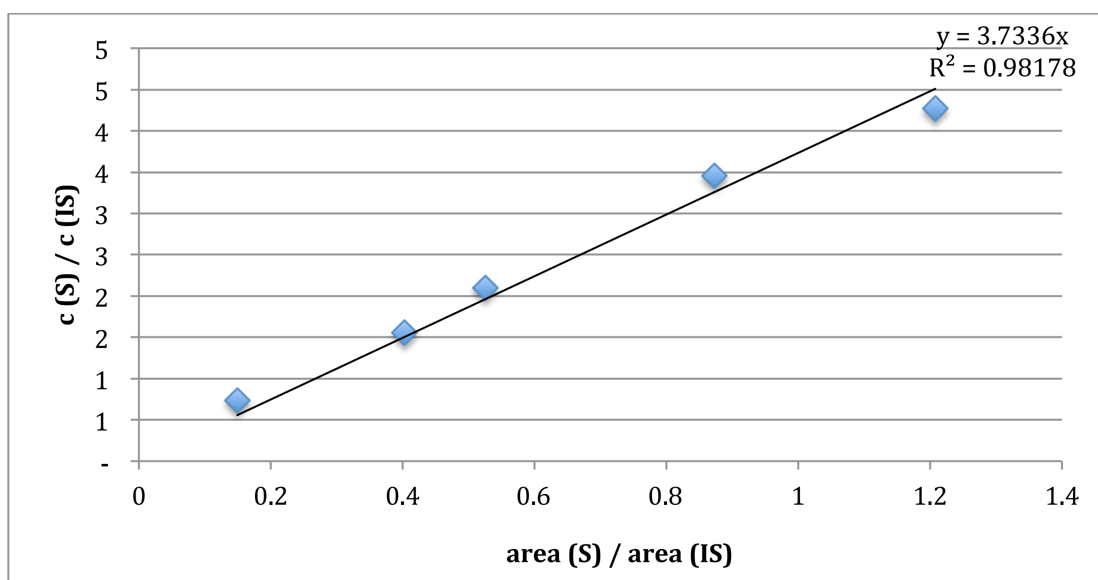

Figure 5.2-3: Calibration of 6-hydroxyheptanoic acid (**2c**).

1.1 Protein and DNA sequence of characterized HLE isoenzymes.

HLE isoenzyme 1_1 with signal sequence: Amino acid sequence

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DQRTATSLLQNSSTLLNIPEELTPVAIEKYLGGTDDPVKKKDLFLDLMGDVMFGVPSVTVARLHRDAGASTFMYEFQYRPSFSSAMRPKTVIG
DHGDEIFSVFGAPFLKEGASEEEIKLSKMVMKFWANFARTGSPNGEGLPHWPVYDQKEGYLQIGVTTQAAQKLKDKEVAFWTELLAKKAAEKQ
QQTEHVEL

HLE isoenzyme 1_1 with signal sequence: Nucleotide sequence (restriction sites are marked in bue)

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CCTCTGGGTAGCCTGCGTTTTGCACCGCCTCAGCCTGCAGATCCGTGGCCGTTTGTTAAAAATGC
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Sequence name: HLE1_1_mS

Sequence type: DNA

Sequence name / optimized for

HLE1_1_mS/ Escherichia coli

ORF	Protected sites	Protected areas	Motifs to avoid
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```
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  70. TGGGGTCATCCGAGCAGCCCTCCGGTTGTTGATACCGCACAGGGTAAAGTTCTGGGTAAACATGTGAGC
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 208. CTGCGTTTTGCACCGCCTCAGCCTGCAGATCCGTGGCCGTTTGTTAAAAATGCAACCAGCTATCCGCCT
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 277. ATGTGTAGCCAGGATCCGGTTGCCGGTCAGATTGCAAGCGACCTGTTTACCATTCGCAAAGAAAATATT
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 346. CCGGTGCAGTTTAGCGAAGATTGTCTGTATCTGAACATTTATACACCGGCAGACCTGACCAAAAAAAGT
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 415. CGCCTGCCGGTTATGGTTTGGATTCATGGTGGTGGTCTGATGGTTGGTGGTGCAAGCACCTATGATGGT
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 484. CTGGCACTGAGCGCACATGAAAATGTTGTTGTTGTGACCATTCAGTATCGTCTGGGTATTTGGGGTTTT
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      G E S V S V L V L S P L A K N L F H R A I S E
 691. GGTGAAAGCGTTAGCGTTCTGGTTCTGAGTCCGCTGGCAAAAAACCTGTTTCATCGTGCAATTAGCGAA
      S G V A F T A G L V Q K D S K A A A Q Q I A V
 760. AGCGGTGTTGCATTTACCGCAGGTCTGGTTCAGAAAGATAGCAAAGCAGCAGCACAGCAGATTGCCGTT
      F A G C K S T T S A V I V H C L R Q K T D D E
 829. TTTGCAGGTTGTAAAAGCACCACCAGTGCAGTTATTGTTCATTGTCTGCGCCAGAAAACCGATGATGAA
      L L E L S L K M K F L S L D L L G E P R E S H
 898. CTGCTGGAACTGAGCCTGAAAATGAAATTTCTGAGTCTGGATCTGCTGGGTGAACCGCGTGAAAGCCAT
      P L L P T V V D G V L L P K M P E E I L A E K
 967. CCGCTGCTGCCGACCGTTGTTGATGGTGTTCTGCTGCCTAAAATGCCGGAAGAAATTCTGGCAGAAAAA
      T F N T V P Y I V G I N K Q E F G W I I P T M
1036. ACCTTTAATACCGTGCCGTATATTGTGGGCATCAACAAACAAGAATTTGGCTGGATTATTCCGACCATG
      M G Y P L S E G K M D Q R T A T S L L Q N S S
1105. ATGGGTTATCCGCTGAGCGAAGGTAAAATGGATCAGCGTACCGCAACCAGCCTGCTGCAGAATAGCAGC
      T L L N I P E E L T P V A I E K Y L G G T D D
1174. ACCCTGCTGAACATTCCGGAAGAACTGACACCGGTTGCAATTGAAAAATATCTGGGTGGCACCGATGAT
      P V K K K D L F L D L M G D V M F G V P S V T
1243. CCGGTTAAAAAAAAAGACCTGTTCCTGGACCTGATGGGTGATGTTATGTTTGGTGTTCCGAGCGTTACC
      V A R L H R D A G A S T F M Y E F Q Y R P S F
1312. GTTGCACGTCTGCATCGTGATGCCGGTGCCAGCACCTTTATGTATGAATTTCAGTACCGTCCGAGCTTT
      S S A M R P K T V I G D H G D E I F S V F G A
1381. AGCAGCGCAATGCGTCCGAAAACCGTTATTGGTGATCATGGTGATGAAATCTTTAGCGTTTTTGGTGCA
      P F L K E G A S E E E I K L S K M V M K F W A
1450. CCGTTTCTGAAAGAAGGTGCAAGCGAAGAAGAAATCAAACTGAGCAAAATGGTTATGAAATTTTGGGCC
      N F A R T G S P N G E G L P H W P V Y D Q K E
1519. AATTTTGCACGTACCGGTAGCCCGAATGGTGAAGGCCTGCCGCATTGGCCTGTTTATGATCAGAAAGAA
      G Y L Q I G V T T Q A A Q K L K D K E V A F W
1588. GGCTATCTGCAAATTGGTGTTACCACCCAGGCAGCACAGAAACTGAAAGATAAAGAAGTTGCCTTTTGG
      T E L L A K K A A E K Q Q Q T E H V E L *
1657. ACCGAGCTGCTGGCCAAAAAAGCAGCCGAAAAACAGCAGCAGACCGAACATGTTGAACTGTAACTCGAG

1726. CCACTGAGATCCGGC
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HLE isoenzyme 1_1: Amino acid sequence

MSSPPVVDTAQGKVLGKHVSLEGFAQPVAVFLGVPFAKPPLGSLRFAPPQPADPWPFVKNATSYPPMCSQDPVAGQIASDLFTIRKENIPVQF
SEDCLYLNIYTPADLTKKSRLPVMVWIHGGGLMVGGASTYDGLALSAHENVVVVTIQYRLGIWGFLSTGDEHSPGNWGHLDQVAALHWVQDNI
ANFGGDPGSVTIFGESAGGESVSVLVLSPLAKNLFHRAISESGVAFTAGLVQKDSKAAAQQIAVFAGCKSTTSAVIVHCLRQKTDDELLELSL
KMKFLSLDLLGEPRESHPLLPTVVDGVLLPKMPEEILAEKTFNTVPYIVGINKQEFGWIIPTMMGYPLSEGKMDQRTATSLLQNSSTLLNIPE
ELTPVAIEKYLGGTDDPVKKKDLFLDLMGDVMFGVPSVTVARLHRDAGASTFMYEFQYRPSFSSAMRPKTVIGDHGDEIFSVFGAPFLKEGAS
EEEIKLSKMVMKFWANFARTGSPNGEGLPHWPVYDQKEGYLQIGVTTQAAQKLKDKEVAFWTELLAKKAAEKQQQTEHVEL

HLE isoenzyme 1_1: Nucleotide sequence (restriction sites are marked in bue)

GTGCCGCGCGGCAGCCATATGAGCAGCCCTCCGGTTGTTGATACCGCACAGGGTAAAGTTCTGGG
TAAACATGTTAGCCTGGAAGGTTTTGCACAGCCGGTTGCAGTTTTTCTGGGTGTTCCGTTTGCAA
AACCGCCTCTGGGTAGCCTGCGTTTTGCACCGCCTCAGCCTGCAGATCCGTGGCCGTTTGTTAAA
AATGCAACCAGCTATCCGCCTATGTGTAGCCAGGATCCGGTTGCCGGTCAGATTGCAAGCGACCT
GTTTACCATTCGCAAAGAAAATATTCCGGTGCAGTTTAGCGAAGATTGTCTGTATCTGAACATTT
ATACACCGGCAGACCTGACCAAAAAAAGTCGCCTGCCGGTTATGGTTTGGATTCATGGTGGTGGT
CTGATGGTTGGTGGTGCAAGCACCTATGATGGTCTGGCACTGAGCGCACATGAAAATGTTGTTGT
TGTGACCATTCAGTATCGTCTGGGTATTTGGGGTTTTCTGAGCACCGGTGATGAACATTCACCGG
GTAATTGGGGTCATCTGGATCAGGTTGCAGCACTGCATTGGGTTCAGGATAACATTGCAAATTTT
GGTGGTGATCCGGGTAGCGTTACCATTTTTGGTGAAAGTGCCGGTGGTGAAAGCGTTAGCGTTCT
GGTTCTGAGTCCGCTGGCAAAAAACCTGTTTCATCGTGCAATTAGCGAAAGCGGTGTTGCATTTA
CCGCAGGTCTGGTTCAGAAAGATAGCAAAGCAGCAGCACAGCAGATTGCCGTTTTTGCAGGTTGT
AAAAGCACCACCAGTGCAGTTATTGTTCATTGTCTGCGCCAGAAAACCGATGATGAACTGCTGGA
ACTGAGCCTGAAAATGAAATTTCTGAGCCTGGATCTGCTGGGTGAACCGCGTGAAAGCCATCCGC
TGCTGCCGACCGTTGTTGATGGTGTTCTGCTGCCTAAAATGCCGGAAGAAATTCTGGCAGAAAAA
ACCTTTAATACCGTGCCGTATATTGTGGGCATCAACAAACAAGAATTTGGCTGGATTATTCCGAC
CATGATGGGTTATCCGCTGAGCGAAGGTAAAATGGATCAGCGTACCGCAACCAGCCTGCTGCAGA
ATAGCAGCACCCTGCTGAACATTCCGGAAGAACTGACACCGGTTGCAATTGAAAAATATCTGGGT
GGCACCGATGATCCGGTTAAAAAAAAAGACCTGTTCCTGGACCTGATGGGGGATGTTATGTTTGG
TGTTCCGAGCGTTACCGTTGCACGTCTGCATCGTGATGCCGGTGCCAGCACCTTTATGTATGAAT
TTCAGTACCGTCCGAGCTTTAGCAGCGCAATGCGTCCGAAAACCGTTATTGGTGATCATGGTGAT
GAAATCTTTAGCGTTTTTGGTGCACCGTTTCTGAAAGAAGGTGCAAGCGAAGAAGAAATCAAACT
GAGCAAAATGGTTATGAAATTTTGGGCCAATTTTGCACGTACCGGTAGCCCGAATGGTGAAGGCC
TGCCGCATTGGCCTGTTTATGATCAGAAAGAAGGCTATCTGCAAATTGGTGTTACCACCCAGGCA
GCACAGAAACTGAAAGATAAAGAAGTTGCCTTTTGGACCGAGCTGCTGGCCAAAAAAGCAGCCGA
AAAACAGCAGCAGACCGAACATGTTGAACTGTAACTCGAGCCACTGAGATCCGGC

Sequence name: HLE1_1
Sequence type: DNA

Sequence name / optimized for

HLE1_1/ Escherichia coli

ORF	Protected sites	Protected areas	Motifs to avoid
19-1659 [ATG...TAA]	16-21 NdeI [CATATG] 1660-1665 XhoI [CTCGAG]		NdeI [CATATG] XhoI [CTCGAG]

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                         M  S  S  P  P  V  V  D  T  A  Q  G  K  V  L  G  K
   1.  GTGCCGCGCGGCAGCCATATGAGCAGCCCTCCGGTTGTTGATACCGCACAGGGTAAAGTTCTGGGTAAA
       H  V  S  L  E  G  F  A  Q  P  V  A  V  F  L  G  V  P  F  A  K  P  P
  70.  CATGTTAGCCTGGAAGGTTTTGCACAGCCGGTTGCAGTTTTTCTGGGTGTTCCGTTTGCAAAACCGCCT
       L  G  S  L  R  F  A  P  P  Q  P  A  D  P  W  P  F  V  K  N  A  T  S
 139.  CTGGGTAGCCTGCGTTTTGCACCGCCTCAGCCTGCAGATCCGTGGCCGTTTGTTAAAAATGCAACCAGC
       Y  P  P  M  C  S  Q  D  P  V  A  G  Q  I  A  S  D  L  F  T  I  R  K
 208.  TATCCGCCTATGTGTAGCCAGGATCCGGTTGCCGGTCAGATTGCAAGCGACCTGTTTACCATTCGCAAA
       E  N  I  P  V  Q  F  S  E  D  C  L  Y  L  N  I  Y  T  P  A  D  L  T
 277.  GAAAATATTCCGGTGCAGTTTAGCGAAGATTGTCTGTATCTGAACATTTATACACCGGCAGACCTGACC
       K  K  S  R  L  P  V  M  V  W  I  H  G  G  G  L  M  V  G  G  A  S  T
 346.  AAAAAAAGTCGCCTGCCGGTTATGGTTTGGATTCATGGTGGTGGTCTGATGGTTGGTGGTGCAAGCACC
       Y  D  G  L  A  L  S  A  H  E  N  V  V  V  V  T  I  Q  Y  R  L  G  I
 415.  TATGATGGTCTGGCACTGAGCGCACATGAAAATGTTGTTGTTGTGACCATTCAGTATCGTCTGGGTATT
       W  G  F  L  S  T  G  D  E  H  S  P  G  N  W  G  H  L  D  Q  V  A  A
 484.  TGGGGTTTTCTGAGCACCGGTGATGAACATTCACCGGGTAATTGGGGTCATCTGGATCAGGTTGCAGCA
       L  H  W  V  Q  D  N  I  A  N  F  G  G  D  P  G  S  V  T  I  F  G  E
 553.  CTGCATTGGGTTCAGGATAACATTGCAAATTTTGGTGGTGATCCGGGTAGCGTTACCATTTTTGGTGAA
       S  A  G  G  E  S  V  S  V  L  V  L  S  P  L  A  K  N  L  F  H  R  A
 622.  AGTGCCGGTGGTGAAAGCGTTAGCGTTCTGGTTCTGAGTCCGCTGGCAAAAAACCTGTTTCATCGTGCA
       I  S  E  S  G  V  A  F  T  A  G  L  V  Q  K  D  S  K  A  A  A  Q  Q
 691.  ATTAGCGAAAGCGGTGTTGCATTTACCGCAGGTCTGGTTCAGAAAGATAGCAAAGCAGCAGCACAGCAG
       I  A  V  F  A  G  C  K  S  T  T  S  A  V  I  V  H  C  L  R  Q  K  T
 760.  ATTGCCGTTTTTGCAGGTTGTAAAAGCACCACCAGTGCAGTTATTGTTCATTGTCTGCGCCAGAAAACC
       D  D  E  L  L  E  L  S  L  K  M  K  F  L  S  L  D  L  L  G  E  P  R
 829.  GATGATGAACTGCTGGAACTGAGCCTGAAAATGAAATTTCTGAGCCTGGATCTGCTGGGTGAACCGCGT
       E  S  H  P  L  L  P  T  V  V  D  G  V  L  L  P  K  M  P  E  E  I  L
 898.  GAAAGCCATCCGCTGCTGCCGACCGTTGTTGATGGTGTTCTGCTGCCTAAAATGCCGGAAGAAATTCTG
       A  E  K  T  F  N  T  V  P  Y  I  V  G  I  N  K  Q  E  F  G  W  I  I
 967.  GCAGAAAAAACCTTTAATACCGTGCCGTATATTGTGGGCATCAACAAACAAGAATTTGGCTGGATTATT
       P  T  M  M  G  Y  P  L  S  E  G  K  M  D  Q  R  T  A  T  S  L  L  Q
1036.  CCGACCATGATGGGTTATCCGCTGAGCGAAGGTAAAATGGATCAGCGTACCGCAACCAGCCTGCTGCAG
       N  S  S  T  L  L  N  I  P  E  E  L  T  P  V  A  I  E  K  Y  L  G  G
1105.  AATAGCAGCACCCTGCTGAACATTCCGGAAGAACTGACACCGGTTGCAATTGAAAAATATCTGGGTGGC
       T  D  D  P  V  K  K  K  D  L  F  L  D  L  M  G  D  V  M  F  G  V  P
1174.  ACCGATGATCCGGTTAAAAAAAAAGACCTGTTCCTGGACCTGATGGGGGATGTTATGTTTGGTGTTCCG
       S  V  T  V  A  R  L  H  R  D  A  G  A  S  T  F  M  Y  E  F  Q  Y  R
1243.  AGCGTTACCGTTGCACGTCTGCATCGTGATGCCGGTGCCAGCACCTTTATGTATGAATTTCAGTACCGT
       P  S  F  S  S  A  M  R  P  K  T  V  I  G  D  H  G  D  E  I  F  S  V
1312.  CCGAGCTTTAGCAGCGCAATGCGTCCGAAAACCGTTATTGGTGATCATGGTGATGAAATCTTTAGCGTT
       F  G  A  P  F  L  K  E  G  A  S  E  E  E  I  K  L  S  K  M  V  M  K
1381.  TTTGGTGCACCGTTTCTGAAAGAAGGTGCAAGCGAAGAAGAAATCAAACTGAGCAAAATGGTTATGAAA
       F  W  A  N  F  A  R  T  G  S  P  N  G  E  G  L  P  H  W  P  V  Y  D
1450.  TTTTGGGCCAATTTTGCACGTACCGGTAGCCCGAATGGTGAAGGCCTGCCGCATTGGCCTGTTTATGAT
       Q  K  E  G  Y  L  Q  I  G  V  T  T  Q  A  A  Q  K  L  K  D  K  E  V
1519.  CAGAAAGAAGGCTATCTGCAAATTGGTGTTACCACCCAGGCAGCACAGAAACTGAAAGATAAAGAAGTT
       A  F  W  T  E  L  L  A  K  K  A  A  E  K  Q  Q  Q  T  E  H  V  E  L
1588.  GCCTTTTGGACCGAGCTGCTGGCCAAAAAAGCAGCCGAAAAACAGCAGCAGACCGAACATGTTGAACTG
       *
1657.  TAACTCGAGCCACTGAGATCCGGC
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HLE isoenzyme 1_2: Amino acid sequence

MSSPPVVDTAQGKVLGKHVSLEGFAQPVAVFLGVPFAKPPLGSLRFAPPQPADPWPFVKNATSYPPMCSQDTVAGQMLSDLFTNRKENIPVQI
SEDCLYLNIYTPADLTKKSRLPVMVWIHGGGLMVGGASTYDGLALSAHENVVVVTIQYRLGIWGFLSTGDEHSPGNWGHLDQVAALRWVQENI
ANFGGDPGSVTIFGESAGGESVSVLVLSPLAKNLFHRAISESGVTYTAGLVQKDSKAAAQQIAVFAGCKTTTSAVIVHCLRQKTEDELLETSL
KMKFLSLDLFGEPRESHPFLPTVVDGVLLPRMPEEILAEKTFNTVPYIVGINKQEFGWIIPTMMGYPFSEGKMDQRTATSLLQNSSTLLHIPE
ELTPVAIEKYLGGTDDPVKKKDLFLDLMGDVMFGVPSVTVARLHRDAGASTFMYEFQYRPSFSSAMRPKTVIGDHGDEIFSVFGAPFLKEGAS
EEEIKLSKMVMKFWANFARTGNPNGEGLPHWPVYDRKEGYLQIGVTTQAAQKLKDKEVAFWTELLAKEAAEKQQQTEHVEL

HLE isoenzyme 1_2: Nucleotide sequence (restriction sites are marked in bue)

GTGCCGCGCGGCAGCCATATGAGCAGCCCTCCGGTTGTTGATACCGCACAGGGTAAAGTTCTGGG
TAAACATGTTAGCCTGGAAGGTTTTGCACAGCCGGTTGCAGTTTTTCTGGGTGTTCCGTTTGCAA
AACCGCCTCTGGGTAGCCTGCGTTTTGCACCGCCTCAGCCTGCAGATCCGTGGCCGTTTGTTAAA
AATGCAACCAGCTATCCGCCTATGTGTAGCCAGGATACCGTTGCAGGTCAGATGCTGAGCGACCT
GTTTACCAATCGTAAAGAAAATATTCCGGTGCAGATTAGCGAAGATTGTCTGTATCTGAACATTT
ATACACCGGCAGACCTGACCAAAAAAAGTCGCCTGCCGGTTATGGTTTGGATTCATGGTGGTGGT
CTGATGGTTGGTGGTGCAAGCACCTATGATGGTCTGGCACTGAGCGCACATGAAAATGTTGTTGT
TGTGACCATTCAGTATCGTCTGGGTATTTGGGGTTTTCTGAGCACCGGTGATGAACATTCACCGG
GTAATTGGGGTCATCTGGATCAGGTTGCAGCACTGCGTTGGGTTCAAGAAAACATTGCAAATTTT
GGTGGTGATCCGGGTAGCGTTACCATTTTTGGTGAAAGTGCCGGTGGTGAAAGCGTTAGCGTTCT
GGTTCTGAGTCCGCTGGCAAAAAACCTGTTTCATCGTGCCATTAGCGAAAGCGGTGTTACCTATA
CCGCAGGTCTGGTTCAGAAAGATAGCAAAGCAGCAGCACAGCAGATTGCCGTTTTTGCAGGTTGT
AAAACCACCACCTCAGCAGTTATTGTTCATTGTCTGCGCCAGAAAACCGAAGATGAACTGCTGGA
AACCAGCCTGAAAATGAAATTTCTGAGCCTGGACCTGTTTGGTGAACCGCGTGAAAGCCATCCGT
TTCTGCCGACCGTTGTTGATGGTGTTCTGCTGCCTCGTATGCCGGAAGAAATTCTGGCAGAAAAA
ACCTTTAATACCGTGCCGTATATTGTGGGCATCAACAAACAAGAATTTGGCTGGATTATTCCGAC
CATGATGGGTTATCCGTTTAGCGAAGGTAAAATGGATCAGCGTACCGCGACCAGCCTGCTGCAGA
ATAGCAGCACCCTGCTGCATATTCCGGAAGAACTGACACCGGTTGCAATTGAAAAATATCTGGGT
GGCACCGATGATCCGGTTAAAAAAAAAGACCTGTTCCTGGATCTGATGGGTGATGTTATGTTTGG
TGTTCCGAGCGTTACCGTTGCCCGTCTGCATCGTGATGCCGGTGCCAGCACCTTTATGTATGAAT
TTCAGTACCGTCCGAGCTTTAGCAGCGCAATGCGTCCGAAAACCGTTATTGGTGATCATGGTGAT
GAAATCTTTAGCGTTTTTGGTGCACCGTTTCTGAAAGAAGGTGCAAGCGAAGAAGAAATCAAACT
GAGCAAAATGGTTATGAAATTTTGGGCCAATTTTGCCCGTACCGGTAATCCGAATGGTGAAGGCC
TGCCGCATTGGCCTGTTTATGATCGCAAAGAAGGTTATCTGCAAATTGGTGTTACCACCCAGGCA
GCACAGAAACTGAAAGATAAAGAAGTTGCCTTTTGGACCGAGCTGCTGGCCAAAGAAGCAGCAGA
AAAACAGCAGCAGACCGAACATGTTGAACTGTAACTCGAGCCACTGAGATCCGGC

Sequence name: HLE_CES1_2
Sequence type: DNA

Sequence name / optimized for

HLE_CES1_2/ Escherichia coli

ORF	Protected sites	Protected areas	Motifs to avoid
19-1659 [ATG...TAA]	16-21 NdeI [CATATG] 1660-1665 XhoI [CTCGAG]		NdeI [CATATG] XhoI [CTCGAG]

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                        M  S  S  P  P  V  V  D  T  A  Q  G  K  V  L  G  K
   1. GTGCCGCGCGGCAGCCATATGAGCAGCCCTCCGGTTGTTGATACCGCACAGGGTAAAGTTCTGGGTAAA
      H  V  S  L  E  G  F  A  Q  P  V  A  V  F  L  G  V  P  F  A  K  P  P
  70. CATGTTAGCCTGGAAGGTTTTGCACAGCCGGTTGCAGTTTTTCTGGGTGTTCCGTTTGCAAAACCGCCT
      L  G  S  L  R  F  A  P  P  Q  P  A  D  P  W  P  F  V  K  N  A  T  S
 139. CTGGGTAGCCTGCGTTTTGCACCGCCTCAGCCTGCAGATCCGTGGCCGTTTGTTAAAAATGCAACCAGC
      Y  P  P  M  C  S  Q  D  T  V  A  G  Q  M  L  S  D  L  F  T  N  R  K
 208. TATCCGCCTATGTGTAGCCAGGATACCGTTGCAGGTCAGATGCTGAGCGACCTGTTTACCAATCGTAAA
      E  N  I  P  V  Q  I  S  E  D  C  L  Y  L  N  I  Y  T  P  A  D  L  T
 277. GAAAATATTCCGGTGCAGATTAGCGAAGATTGTCTGTATCTGAACATTTATACACCGGCAGACCTGACC
      K  K  S  R  L  P  V  M  V  W  I  H  G  G  G  L  M  V  G  G  A  S  T
 346. AAAAAAAGTCGCCTGCCGGTTATGGTTTGGATTCATGGTGGTGGTCTGATGGTTGGTGGTGCAAGCACC
      Y  D  G  L  A  L  S  A  H  E  N  V  V  V  V  T  I  Q  Y  R  L  G  I
 415. TATGATGGTCTGGCACTGAGCGCACATGAAAATGTTGTTGTTGTGACCATTCAGTATCGTCTGGGTATT
      W  G  F  L  S  T  G  D  E  H  S  P  G  N  W  G  H  L  D  Q  V  A  A
 484. TGGGGTTTTCTGAGCACCGGTGATGAACATTCACCGGGTAATTGGGGTCATCTGGATCAGGTTGCAGCA
      L  R  W  V  Q  E  N  I  A  N  F  G  G  D  P  G  S  V  T  I  F  G  E
 553. CTGCGTTGGGTTCAAGAAAACATTGCAAATTTTGGTGGTGATCCGGGTAGCGTTACCATTTTTGGTGAA
      S  A  G  G  E  S  V  S  V  L  V  L  S  P  L  A  K  N  L  F  H  R  A
 622. AGTGCCGGTGGTGAAAGCGTTAGCGTTCTGGTTCTGAGTCCGCTGGCAAAAAACCTGTTTCATCGTGCC
      I  S  E  S  G  V  T  Y  T  A  G  L  V  Q  K  D  S  K  A  A  A  Q  Q
 691. ATTAGCGAAAGCGGTGTTACCTATACCGCAGGTCTGGTTCAGAAAGATAGCAAAGCAGCAGCACAGCAG
      I  A  V  F  A  G  C  K  T  T  T  S  A  V  I  V  H  C  L  R  Q  K  T
 760. ATTGCCGTTTTTGCAGGTTGTAAAACCACCACCTCAGCAGTTATTGTTCATTGTCTGCGCCAGAAAACC
      E  D  E  L  L  E  T  S  L  K  M  K  F  L  S  L  D  L  F  G  E  P  R
 829. GAAGATGAACTGCTGGAAACCAGCCTGAAAATGAAATTTCTGAGCCTGGACCTGTTTGGTGAACCGCGT
      E  S  H  P  F  L  P  T  V  V  D  G  V  L  L  P  R  M  P  E  E  I  L
 898. GAAAGCCATCCGTTTCTGCCGACCGTTGTTGATGGTGTTCTGCTGCCTCGTATGCCGGAAGAAATTCTG
      A  E  K  T  F  N  T  V  P  Y  I  V  G  I  N  K  Q  E  F  G  W  I  I
 967. GCAGAAAAAACCTTTAATACCGTGCCGTATATTGTGGGCATCAACAAACAAGAATTTGGCTGGATTATT
      P  T  M  M  G  Y  P  F  S  E  G  K  M  D  Q  R  T  A  T  S  L  L  Q
1036. CCGACCATGATGGGTTATCCGTTTAGCGAAGGTAAAATGGATCAGCGTACCGCGACCAGCCTGCTGCAG
      N  S  S  T  L  L  H  I  P  E  E  L  T  P  V  A  I  E  K  Y  L  G  G
1105. AATAGCAGCACCCTGCTGCATATTCCGGAAGAACTGACACCGGTTGCAATTGAAAAATATCTGGGTGGC
      T  D  D  P  V  K  K  K  D  L  F  L  D  L  M  G  D  V  M  F  G  V  P
1174. ACCGATGATCCGGTTAAAAAAAAAGACCTGTTCCTGGATCTGATGGGTGATGTTATGTTTGGTGTTCCG
      S  V  T  V  A  R  L  H  R  D  A  G  A  S  T  F  M  Y  E  F  Q  Y  R
1243. AGCGTTACCGTTGCCCGTCTGCATCGTGATGCCGGTGCCAGCACCTTTATGTATGAATTTCAGTACCGT
      P  S  F  S  S  A  M  R  P  K  T  V  I  G  D  H  G  D  E  I  F  S  V
1312. CCGAGCTTTAGCAGCGCAATGCGTCCGAAAACCGTTATTGGTGATCATGGTGATGAAATCTTTAGCGTT
      F  G  A  P  F  L  K  E  G  A  S  E  E  E  I  K  L  S  K  M  V  M  K
1381. TTTGGTGCACCGTTTCTGAAAGAAGGTGCAAGCGAAGAAGAAATCAAACTGAGCAAAATGGTTATGAAA
      F  W  A  N  F  A  R  T  G  N  P  N  G  E  G  L  P  H  W  P  V  Y  D
1450. TTTTGGGCCAATTTTGCCCGTACCGGTAATCCGAATGGTGAAGGCCTGCCGCATTGGCCTGTTTATGAT
      R  K  E  G  Y  L  Q  I  G  V  T  T  Q  A  A  Q  K  L  K  D  K  E  V
1519. CGCAAAGAAGGTTATCTGCAAATTGGTGTTACCACCCAGGCAGCACAGAAACTGAAAGATAAAGAAGTT
      A  F  W  T  E  L  L  A  K  E  A  A  E  K  Q  Q  Q  T  E  H  V  E  L
1588. GCCTTTTGGACCGAGCTGCTGGCCAAAGAAGCAGCAGAAAAACAGCAGCAGACCGAACATGTTGAACTG
      *
1657. TAACTCGAGCCACTGAGATCCGGC
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HLE isoenzyme 1_3: Amino acid sequence

MSSPPVVDTAQGKVLGKHVSLEGFAQPMAVFLGVPFAKPPLGSLRFAPPQPADPWPFVKNTTSYPPMCSQDTVAGQMLSDLFTNRKENISVQI
SEDCLYLNIYTPADLTKKSRLPVMVWIHGGGLMIGGASTYDGLALSAHENVVVVTIQYRLGIWGFFSTGDEHSPGNWGHLDQVAALRWVQENI
ANFGGDPGSVTIFGESAGGESVSVLVLSPLAKNLFHRAISESGVAFTAGLVQKDSKAAAQQIAVFAGCKTATSAVIVHCLRQKTEDELLETSV
KMKFLSLDFRGDSRESHPFLPAVVDGVLLPKMPEEILAEKTFNTVPYIIGINKQEFGWVIPMMMGYPLSEGKLDQKTATSLLQKSCPILNIPE
EVTPVATEKYLGGTEDPVKKKDLFLDLIGDVMFGVPSVTVARLHRDAGASTFMYEFQYRPSFSSVMKPKTVIGDHGDEIFSVFGAPFLKEGAS
EEEIKLSKMVMKFWANFARNGNPNGEGLPHWPAYDQKEGYLQIGVTTQAAQKLKDKEVAFWTELLAKEAAEKQQQTEHVEL

HLE isoenzyme 1_3: Nucleotide sequence (restriction sites are marked in bue)

GTGCCGCGCGGCAGCCATATGAGCAGCCCTCCGGTTGTTGATACCGCACAGGGTAAAGTTCTGGG
TAAACATGTTAGCCTGGAAGGTTTTGCACAGCCGATGGCAGTTTTTCTGGGTGTTCCGTTTGCAA
AACCGCCTCTGGGTAGCCTGCGTTTTGCACCGCCTCAGCCTGCAGATCCGTGGCCGTTTGTGAAA
AATACCACCAGTTATCCGCCTATGTGTAGCCAGGATACCGTTGCAGGTCAGATGCTGAGCGACCT
GTTTACCAATCGTAAAGAAAACATTAGCGTGCAGATTAGCGAAGATTGTCTGTATCTGAACATTT
ATACACCGGCAGACCTGACCAAAAAAAGTCGCCTGCCGGTTATGGTTTGGATTCATGGTGGTGGT
CTGATGATTGGTGGTGCAAGCACCTATGATGGTCTGGCACTGAGCGCACATGAAAATGTTGTTGT
TGTGACCATTCAGTATCGTCTGGGTATTTGGGGTTTTTTTAGCACCGGTGATGAACATTCACCGG
GTAATTGGGGTCATCTGGATCAGGTTGCAGCACTGCGTTGGGTTCAAGAGAATATTGCAAATTTT
GGTGGTGATCCGGGTAGCGTTACCATTTTTGGTGAAAGTGCCGGTGGTGAAAGCGTTAGCGTTCT
GGTTCTGAGTCCGCTGGCAAAAAACCTGTTTCATCGTGCCATTAGCGAAAGCGGTGTTGCATTTA
CCGCAGGTCTGGTTCAGAAAGATAGCAAAGCAGCAGCACAGCAGATTGCCGTTTTTGCAGGTTGT
AAAACCGCAACCAGCGCAGTTATTGTTCATTGTCTGCGTCAGAAAACCGAAGATGAACTGCTGGA
AACCAGCGTGAAAATGAAATTTCTGAGCCTGGATTTTCGCGGTGATAGCCGTGAAAGCCATCCGT
TTCTGCCTGCAGTTGTTGATGGTGTTCTGCTGCCGAAAATGCCGGAAGAAATTCTGGCAGAAAAA
ACCTTTAATACCGTGCCGTATATTATCGGCATCAACAAACAAGAATTTGGCTGGGTTATTCCGAT
GATGATGGGTTATCCGCTGAGCGAAGGTAAACTGGATCAGAAAACAGCGACCAGCCTGCTGCAGA
AAAGCTGTCCGATTCTGAATATTCCGGAAGAAGTTACACCGGTTGCCACCGAAAAATATCTGGGT
GGCACCGAAGATCCGGTTAAAAAAAAAGACCTGTTCCTGGATCTGATCGGTGATGTTATGTTTGG
TGTTCCGAGCGTTACCGTTGCCCGTCTGCATCGTGATGCCGGTGCCAGCACCTTTATGTATGAAT
TTCAGTACCGTCCGAGCTTTAGCAGCGTTATGAAACCGAAAACCGTTATTGGTGATCATGGCGAC
GAAATCTTTAGCGTTTTTGGTGCACCGTTTCTGAAAGAAGGTGCAAGCGAAGAAGAAATCAAACT
GAGCAAAATGGTGATGAAATTTTGGGCCAATTTTGCCCGTAATGGTAATCCGAATGGTGAAGGCC
TGCCGCATTGGCCTGCCTATGATCAGAAAGAAGGCTATCTGCAAATTGGTGTTACCACCCAGGCA
GCACAGAAACTGAAAGATAAAGAAGTTGCATTTTGGACCGAGCTGCTGGCCAAAGAAGCAGCAGA
AAAACAGCAGCAGACCGAACATGTTGAACTGTAACTCGAGCCACTGAGATCCGGC

Sequence name: HLE1_3

Sequence type: DNA

Sequence name / optimized for

HLE1_3/ Escherichia coli

ORF	Protected sites	Protected areas	Motifs to avoid
19-1659 [ATG...TAA]	16-21 NdeI [CATATG] 1660-1665 XhoI [CTCGAG]		NdeI [CATATG] XhoI [CTCGAG]

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                        M  S  S  P  P  V  V  D  T  A  Q  G  K  V  L  G  K
1.    GTGCCGCGCGGCAGCCATATGAGCAGCCCTCCGGTTGTTGATACCGCACAGGGTAAAGTTCTGGGTAAA
      H  V  S  L  E  G  F  A  Q  P  M  A  V  F  L  G  V  P  F  A  K  P  P
70.   CATGTTAGCCTGGAAGGTTTTGCACAGCCGATGGCAGTTTTTCTGGGTGTTCCGTTTGCAAAACCGCCT
      L  G  S  L  R  F  A  P  P  Q  P  A  D  P  W  P  F  V  K  N  T  T  S
139.  CTGGGTAGCCTGCGTTTTGCACCGCCTCAGCCTGCAGATCCGTGGCCGTTTGTGAAAAATACCACCAGT
      Y  P  P  M  C  S  Q  D  T  V  A  G  Q  M  L  S  D  L  F  T  N  R  K
208.  TATCCGCCTATGTGTAGCCAGGATACCGTTGCAGGTCAGATGCTGAGCGACCTGTTTACCAATCGTAAA
      E  N  I  S  V  Q  I  S  E  D  C  L  Y  L  N  I  Y  T  P  A  D  L  T
277.  GAAAACATTAGCGTGCAGATTAGCGAAGATTGTCTGTATCTGAACATTTATACACCGGCAGACCTGACC
      K  K  S  R  L  P  V  M  V  W  I  H  G  G  G  L  M  I  G  G  A  S  T
346.  AAAAAAAGTCGCCTGCCGGTTATGGTTTGGATTCATGGTGGTGGTCTGATGATTGGTGGTGCAAGCACC
      Y  D  G  L  A  L  S  A  H  E  N  V  V  V  V  T  I  Q  Y  R  L  G  I
415.  TATGATGGTCTGGCACTGAGCGCACATGAAAATGTTGTTGTTGTGACCATTCAGTATCGTCTGGGTATT
      W  G  F  F  S  T  G  D  E  H  S  P  G  N  W  G  H  L  D  Q  V  A  A
484.  TGGGGTTTTTTTAGCACCGGTGATGAACATTCACCGGGTAATTGGGGTCATCTGGATCAGGTTGCAGCA
      L  R  W  V  Q  E  N  I  A  N  F  G  G  D  P  G  S  V  T  I  F  G  E
553.  CTGCGTTGGGTTCAAGAGAATATTGCAAATTTTGGTGGTGATCCGGGTAGCGTTACCATTTTTGGTGAA
      S  A  G  G  E  S  V  S  V  L  V  L  S  P  L  A  K  N  L  F  H  R  A
622.  AGTGCCGGTGGTGAAAGCGTTAGCGTTCTGGTTCTGAGTCCGCTGGCAAAAAACCTGTTTCATCGTGCC
      I  S  E  S  G  V  A  F  T  A  G  L  V  Q  K  D  S  K  A  A  A  Q  Q
691.  ATTAGCGAAAGCGGTGTTGCATTTACCGCAGGTCTGGTTCAGAAAGATAGCAAAGCAGCAGCACAGCAG
      I  A  V  F  A  G  C  K  T  A  T  S  A  V  I  V  H  C  L  R  Q  K  T
760.  ATTGCCGTTTTTGCAGGTTGTAAAACCGCAACCAGCGCAGTTATTGTTCATTGTCTGCGTCAGAAAACC
      E  D  E  L  L  E  T  S  V  K  M  K  F  L  S  L  D  F  R  G  D  S  R
829.  GAAGATGAACTGCTGGAAACCAGCGTGAAAATGAAATTTCTGAGCCTGGATTTTCGCGGTGATAGCCGT
      E  S  H  P  F  L  P  A  V  V  D  G  V  L  L  P  K  M  P  E  E  I  L
898.  GAAAGCCATCCGTTTCTGCCTGCAGTTGTTGATGGTGTTCTGCTGCCGAAAATGCCGGAAGAAATTCTG
      A  E  K  T  F  N  T  V  P  Y  I  I  G  I  N  K  Q  E  F  G  W  V  I
967.  GCAGAAAAAACCTTTAATACCGTGCCGTATATTATCGGCATCAACAAACAAGAATTTGGCTGGGTTATT
      P  M  M  M  G  Y  P  L  S  E  G  K  L  D  Q  K  T  A  T  S  L  L  Q
1036. CCGATGATGATGGGTTATCCGCTGAGCGAAGGTAAACTGGATCAGAAAACAGCGACCAGCCTGCTGCAG
      K  S  C  P  I  L  N  I  P  E  E  V  T  P  V  A  T  E  K  Y  L  G  G
1105. AAAAGCTGTCCGATTCTGAATATTCCGGAAGAAGTTACACCGGTTGCCACCGAAAAATATCTGGGTGGC
      T  E  D  P  V  K  K  K  D  L  F  L  D  L  I  G  D  V  M  F  G  V  P
1174. ACCGAAGATCCGGTTAAAAAAAAAGACCTGTTCCTGGATCTGATCGGTGATGTTATGTTTGGTGTTCCG
      S  V  T  V  A  R  L  H  R  D  A  G  A  S  T  F  M  Y  E  F  Q  Y  R
1243. AGCGTTACCGTTGCCCGTCTGCATCGTGATGCCGGTGCCAGCACCTTTATGTATGAATTTCAGTACCGT
      P  S  F  S  S  V  M  K  P  K  T  V  I  G  D  H  G  D  E  I  F  S  V
1312. CCGAGCTTTAGCAGCGTTATGAAACCGAAAACCGTTATTGGTGATCATGGCGACGAAATCTTTAGCGTT
      F  G  A  P  F  L  K  E  G  A  S  E  E  E  I  K  L  S  K  M  V  M  K
1381. TTTGGTGCACCGTTTCTGAAAGAAGGTGCAAGCGAAGAAGAAATCAAACTGAGCAAAATGGTGATGAAA
      F  W  A  N  F  A  R  N  G  N  P  N  G  E  G  L  P  H  W  P  A  Y  D
1450. TTTTGGGCCAATTTTGCCCGTAATGGTAATCCGAATGGTGAAGGCCTGCCGCATTGGCCTGCCTATGAT
      Q  K  E  G  Y  L  Q  I  G  V  T  T  Q  A  A  Q  K  L  K  D  K  E  V
1519. CAGAAAGAAGGCTATCTGCAAATTGGTGTTACCACCCAGGCAGCACAGAAACTGAAAGATAAAGAAGTT
      A  F  W  T  E  L  L  A  K  E  A  A  E  K  Q  Q  Q  T  E  H  V  E  L
1588. GCATTTTGGACCGAGCTGCTGGCCAAAGAAGCAGCAGAAAAACAGCAGCAGACCGAACATGTTGAACTG
      *
1657. TAACTCGAGCCACTGAGATCCGGC
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HLE 1_4: Amino acid sequence

MSSPPVVDTAQGKVLGKHVSLEGFAQPVAVFLGVPFAKPPLGSLRFAPPQPADPWPFVKNTTSYPPMCSQDAVMGQMLSDLVTNRKEKIALKF
SEDCLYLNIYTPADLTKKSRLPVMVWIHGGGLVVGGASTYDGLPLSAYENVVVVTIQYRLGIWGFFSTGDEHSPGNWGHLDQVAALKWVQENI
ANFGGNPGSVTIFGESAGGESVSVLMLSPLAKNLFHRAISESGVALTTAVCKKDSKAAAQKIAVFSGCKTTTSAVIVHCLRQKTEDELLETSL
KMKFFTLDFHGDPRESYPFLPTVVDGVLLPKMPEEILAEKKFNTVPYIVGINKQEFGWFLPTILGYPLSEGKLDQKKATSLLWKSYPIVNIPE
ELTPVAIEKYLGGTNDPVKKKDLFLDLMADVVFGVPTVTVARYHRDAGAPTYVYEFQHYPSFSSDRRPKTVIGDHMDELFPLFGAPFLKGGAS
EEEINFSKMVMKFAANFARNGNPSGKGVPHWPVYDQKEAYLQVGVTTQVAQKLKDKEVAFWTELLAKGAAEKRQETEHVEL

HLE isoenzyme 1_4: Nucleotide sequence (restriction sites are marked in bue)

GTGCCGCGCGGCAGCCATATGAGCAGCCCTCCGGTTGTTGATACCGCACAGGGTAAAGTTCTGGG
TAAACATGTTAGCCTGGAAGGTTTTGCACAGCCGGTTGCAGTTTTTCTGGGTGTTCCGTTTGCAA
AACCGCCTCTGGGTAGCCTGCGTTTTGCACCGCCTCAGCCTGCAGATCCGTGGCCGTTTGTGAAA
AATACCACCAGTTATCCGCCTATGTGTAGCCAGGATGCAGTTATGGGTCAGATGCTGAGCGATCT
GGTTACCAATCGTAAAGAAAAAATCGCCCTGAAATTTAGCGAGGATTGTCTGTATCTGAACATTT
ATACACCGGCAGACCTGACCAAAAAAAGTCGCCTGCCGGTTATGGTTTGGATTCATGGTGGTGGT
CTGGTTGTTGGTGGTGCAAGCACCTATGATGGTCTGCCGCTGAGCGCCTATGAAAATGTTGTTGT
TGTGACCATTCAGTATCGTCTGGGTATTTGGGGTTTTTTTAGCACCGGTGATGAACATTCACCGG
GTAATTGGGGTCATCTGGATCAGGTTGCAGCACTGAAATGGGTTCAAGAAAACATTGCAAACTTT
GGTGGTAATCCGGGTAGCGTTACCATTTTTGGTGAAAGTGCCGGTGGTGAAAGCGTTAGCGTTCT
GATGCTGAGTCCGCTGGCAAAAAACCTGTTTCATCGTGCAATTAGCGAAAGCGGTGTTGCACTGA
CCACCGCAGTTTGTAAAAAAGATAGCAAAGCAGCAGCACAGAAAATTGCCGTTTTTAGCGGTTGT
AAAACCACCACCTCAGCAGTTATTGTTCATTGTCTGCGTCAGAAAACCGAAGATGAACTGCTGGA
AACCAGCCTGAAAATGAAATTTTTCACCCTGGATTTTCATGGCGATCCGCGTGAAAGCTATCCGT
TTCTGCCGACCGTTGTTGATGGTGTTCTGCTGCCGAAAATGCCGGAAGAAATTCTGGCAGAGAAA
AAATTCAATACCGTGCCGTATATTGTGGGCATCAACAAACAAGAATTTGGTTGGTTTCTGCCTAC
CATTCTGGGTTATCCGCTGAGTGAAGGTAAACTGGATCAGAAAAAAGCGACCAGCCTGCTGTGGA
AAAGTTATCCGATTGTTAATATTCCGGAAGAACTGACACCGGTGGCCATTGAAAAATATCTGGGT
GGCACCAATGATCCGGTGAAAAAAAAAGACCTGTTCCTGGATCTGATGGCCGATGTTGTTTTTGG
TGTTCCGACCGTTACCGTTGCACGTTATCATCGTGATGCGGGTGCACCGACCTATGTTTATGAAT
TTCAGCATTATCCGAGCTTTAGCAGCGATCGTCGTCCGAAAACCGTTATTGGTGATCACATGGAT
GAACTGTTTCCGCTGTTTGGTGCACCGTTTCTGAAAGGTGGTGCCAGCGAAGAAGAAATCAATTT
TAGCAAAATGGTGATGAAATTTGCAGCCAACTTTGCCCGTAATGGCAATCCGAGCGGTAAAGGTG
TTCCGCATTGGCCTGTTTATGATCAGAAAGAGGCATATCTGCAGGTTGGTGTTACCACCCAGGTT
GCACAGAAACTGAAAGATAAAGAAGTTGCCTTTTGGACCGAGCTGCTGGCCAAAGGTGCAGCAGA
AAAACGTCAAGAAACCGAACATGTTGAACTGTAACTCGAGCCACTGAGATCCGGC

Sequence name: HLE1_4

Sequence type: PROTEIN

Sequence name / optimized for

HLE1_4/ Escherichia coli

ORF	Protected sites	Protected areas	Motifs to avoid
22-1659 [AGC...TAA]	16-21 NdeI [CATATG] 1660-1665 XhoI [CTCGAG]		NdeI [CATATG] XhoI [CTCGAG]

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                          S  S  P  P  V  V  D  T  A  Q  G  K  V  L  G  K
1.    GTGCCGCGCGGCAGCCATATGAGCAGCCCTCCGGTTGTTGATACCGCACAGGGTAAAGTTCTGGGTAAA
      H  V  S  L  E  G  F  A  Q  P  V  A  V  F  L  G  V  P  F  A  K  P  P
70.   CATGTTAGCCTGGAAGGTTTTGCACAGCCGGTTGCAGTTTTTCTGGGTGTTCCGTTTGCAAAACCGCCT
      L  G  S  L  R  F  A  P  P  Q  P  A  D  P  W  P  F  V  K  N  T  T  S
139.  CTGGGTAGCCTGCGTTTTGCACCGCCTCAGCCTGCAGATCCGTGGCCGTTTGTGAAAAATACCACCAGT
      Y  P  P  M  C  S  Q  D  A  V  M  G  Q  M  L  S  D  L  V  T  N  R  K
208.  TATCCGCCTATGTGTAGCCAGGATGCAGTTATGGGTCAGATGCTGAGCGATCTGGTTACCAATCGTAAA
      E  K  I  A  L  K  F  S  E  D  C  L  Y  L  N  I  Y  T  P  A  D  L  T
277.  GAAAAAATCGCCCTGAAATTTAGCGAGGATTGTCTGTATCTGAACATTTATACACCGGCAGACCTGACC
      K  K  S  R  L  P  V  M  V  W  I  H  G  G  G  L  V  V  G  G  A  S  T
346.  AAAAAAAGTCGCCTGCCGGTTATGGTTTGGATTCATGGTGGTGGTCTGGTTGTTGGTGGTGCAAGCACC
      Y  D  G  L  P  L  S  A  Y  E  N  V  V  V  V  T  I  Q  Y  R  L  G  I
415.  TATGATGGTCTGCCGCTGAGCGCCTATGAAAATGTTGTTGTTGTGACCATTCAGTATCGTCTGGGTATT
      W  G  F  F  S  T  G  D  E  H  S  P  G  N  W  G  H  L  D  Q  V  A  A
484.  TGGGGTTTTTTTAGCACCGGTGATGAACATTCACCGGGTAATTGGGGTCATCTGGATCAGGTTGCAGCA
      L  K  W  V  Q  E  N  I  A  N  F  G  G  N  P  G  S  V  T  I  F  G  E
553.  CTGAAATGGGTTCAAGAAAACATTGCAAACTTTGGTGGTAATCCGGGTAGCGTTACCATTTTTGGTGAA
      S  A  G  G  E  S  V  S  V  L  M  L  S  P  L  A  K  N  L  F  H  R  A
622.  AGTGCCGGTGGTGAAAGCGTTAGCGTTCTGATGCTGAGTCCGCTGGCAAAAAACCTGTTTCATCGTGCA
      I  S  E  S  G  V  A  L  T  T  A  V  C  K  K  D  S  K  A  A  A  Q  K
691.  ATTAGCGAAAGCGGTGTTGCACTGACCACCGCAGTTTGTAAAAAAGATAGCAAAGCAGCAGCACAGAAA
      I  A  V  F  S  G  C  K  T  T  T  S  A  V  I  V  H  C  L  R  Q  K  T
760.  ATTGCCGTTTTTAGCGGTTGTAAAACCACCACCTCAGCAGTTATTGTTCATTGTCTGCGTCAGAAAACC
      E  D  E  L  L  E  T  S  L  K  M  K  F  F  T  L  D  F  H  G  D  P  R
829.  GAAGATGAACTGCTGGAAACCAGCCTGAAAATGAAATTTTTCACCCTGGATTTTCATGGCGATCCGCGT
      E  S  Y  P  F  L  P  T  V  V  D  G  V  L  L  P  K  M  P  E  E  I  L
898.  GAAAGCTATCCGTTTCTGCCGACCGTTGTTGATGGTGTTCTGCTGCCGAAAATGCCGGAAGAAATTCTG
      A  E  K  K  F  N  T  V  P  Y  I  V  G  I  N  K  Q  E  F  G  W  F  L
967.  GCAGAGAAAAAATTCAATACCGTGCCGTATATTGTGGGCATCAACAAACAAGAATTTGGTTGGTTTCTG
      P  T  I  L  G  Y  P  L  S  E  G  K  L  D  Q  K  K  A  T  S  L  L  W
1036. CCTACCATTCTGGGTTATCCGCTGAGTGAAGGTAAACTGGATCAGAAAAAAGCGACCAGCCTGCTGTGG
      K  S  Y  P  I  V  N  I  P  E  E  L  T  P  V  A  I  E  K  Y  L  G  G
1105. AAAAGTTATCCGATTGTTAATATTCCGGAAGAACTGACACCGGTGGCCATTGAAAAATATCTGGGTGGC
      T  N  D  P  V  K  K  K  D  L  F  L  D  L  M  A  D  V  V  F  G  V  P
1174. ACCAATGATCCGGTGAAAAAAAAAGACCTGTTCCTGGATCTGATGGCCGATGTTGTTTTTGGTGTTCCG
      T  V  T  V  A  R  Y  H  R  D  A  G  A  P  T  Y  V  Y  E  F  Q  H  Y
1243. ACCGTTACCGTTGCACGTTATCATCGTGATGCGGGTGCACCGACCTATGTTTATGAATTTCAGCATTAT
      P  S  F  S  S  D  R  R  P  K  T  V  I  G  D  H  M  D  E  L  F  P  L
1312. CCGAGCTTTAGCAGCGATCGTCGTCCGAAAACCGTTATTGGTGATCACATGGATGAACTGTTTCCGCTG
      F  G  A  P  F  L  K  G  G  A  S  E  E  E  I  N  F  S  K  M  V  M  K
1381. TTTGGTGCACCGTTTCTGAAAGGTGGTGCCAGCGAAGAAGAAATCAATTTTAGCAAAATGGTGATGAAA
      F  A  A  N  F  A  R  N  G  N  P  S  G  K  G  V  P  H  W  P  V  Y  D
1450. TTTGCAGCCAACTTTGCCCGTAATGGCAATCCGAGCGGTAAAGGTGTTCCGCATTGGCCTGTTTATGAT
      Q  K  E  A  Y  L  Q  V  G  V  T  T  Q  V  A  Q  K  L  K  D  K  E  V
1519. CAGAAAGAGGCATATCTGCAGGTTGGTGTTACCACCCAGGTTGCACAGAAACTGAAAGATAAAGAAGTT
      A  F  W  T  E  L  L  A  K  G  A  A  E  K  R  Q  E  T  E  H  V  E  L
1588. GCCTTTTGGACCGAGCTGCTGGCCAAAGGTGCAGCAGAAAAACGTCAAGAAACCGAACATGTTGAACTG
      *
1657. TAACTCGAGCCACTGAGATCCGGC
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HLE 1_5: Amino acid sequence

MSSPPVVDTAQGKVLGKHVSLEGFAQPVAVFLGVPFAKPPLGSLRFAPPQPADPWPFVKNTTSYPPMCSQDAVMGQMLSDLVTNRKEKIALKF
SEDCLYLNIYTPADLTKKSRLPVMVWIHGGGLVVGGASTYDGLPLSAYENVVVVTIQYRLGIWGFFSTGDEHSPGNWGHLDQVAALKWVQENI
ANFGGNPGSVTIFGESAGGESVSVLMLSPLAKNLFHRAISESGVALTTAVCKKDSKAAAQKIAVFSGCKTTTSAVIVHCLRQKTEDELLETSL
KMKFFTLDFHGDPRESYPFLPTVVDGVLLPKMPEEILAEKKFNTVPYIVGINKQEFGWFLPTQILGYPLSEGKLDQKKATSLLWKSYPIVNIP
EELTPVAIEKYLGGTNDPVKKKDLFLDLMADVVFGVPTVTVARYHRDAGAPTYVYEFQHYPSFSSDRRPKTVIGDHMDELFPLFGAPFLKGGA
SEEEINFSKMVMKFAANFARNGNPSGKGVPHWPVYDQKEAYLQVGVTTQVAQKLKDKEVAFWTELLAKGAAEKRQETEHVEL

HLE isoenzyme 1_5: Nucleotide sequence (restriction sites are marked in bue)

GTGCCGCGCGGCAGCCATATGAGCAGCCCTCCGGTTGTTGATACCGCACAGGGTAAAGTTCTGGG
TAAACATGTTAGCCTGGAAGGTTTTGCACAGCCGGTTGCAGTTTTTCTGGGTGTTCCGTTTGCAA
AACCGCCTCTGGGTAGCCTGCGTTTTGCACCGCCTCAGCCTGCAGATCCGTGGCCGTTTGTGAAA
AATACCACCAGTTATCCGCCTATGTGTAGCCAGGATGCAGTTATGGGTCAGATGCTGAGCGATCT
GGTTACCAATCGTAAAGAAAAAATCGCCCTGAAATTTAGCGAGGATTGTCTGTATCTGAACATTT
ATACACCGGCAGACCTGACCAAAAAAAGTCGCCTGCCGGTTATGGTTTGGATTCATGGTGGTGGT
CTGGTTGTTGGTGGTGCAAGCACCTATGATGGTCTGCCGCTGAGCGCCTATGAAAATGTTGTTGT
TGTGACCATTCAGTATCGTCTGGGTATTTGGGGTTTTTTTAGCACCGGTGATGAACATTCACCGG
GTAATTGGGGTCATCTGGATCAGGTTGCAGCACTGAAATGGGTTCAAGAAAACATTGCAAACTTT
GGTGGTAATCCGGGTAGCGTTACCATTTTTGGTGAAAGTGCCGGTGGTGAAAGCGTTAGCGTTCT
GATGCTGAGTCCGCTGGCAAAAAACCTGTTTCATCGTGCAATTAGCGAAAGCGGTGTTGCACTGA
CCACCGCAGTTTGTAAAAAAGATAGCAAAGCAGCAGCACAGAAAATTGCCGTTTTTAGCGGTTGT
AAAACCACCACCTCAGCAGTTATTGTTCATTGTCTGCGTCAGAAAACCGAAGATGAACTGCTGGA
AACCAGCCTGAAAATGAAATTTTTCACCCTGGATTTTCATGGCGATCCGCGTGAAAGCTATCCGT
TTCTGCCGACCGTTGTTGATGGTGTTCTGCTGCCGAAAATGCCGGAAGAAATTCTGGCAGAGAAA
AAATTCAATACCGTGCCGTATATTGTGGGCATCAACAAACAAGAATTTGGTTGGTTTCTGCCTAC
CCAGATTCTGGGCTATCCGCTGAGTGAAGGTAAACTGGATCAGAAAAAAGCGACCAGCCTGCTGT
GGAAAAGTTATCCGATTGTTAATATTCCGGAAGAACTGACACCGGTGGCCATTGAAAAATATCTG
GGTGGCACCAATGATCCGGTGAAAAAAAAAGACCTGTTCCTGGATCTGATGGCCGATGTTGTTTT
TGGTGTTCCGACCGTTACCGTTGCACGTTATCATCGTGATGCGGGTGCACCGACCTATGTTTATG
AATTTCAGCATTATCCGAGCTTTAGCAGCGATCGTCGTCCGAAAACCGTTATTGGTGATCACATG
GATGAACTGTTTCCGCTGTTTGGTGCACCGTTTCTGAAAGGTGGTGCCAGCGAAGAAGAAATCAA
TTTTAGCAAAATGGTGATGAAATTTGCAGCCAACTTTGCCCGTAATGGCAATCCGAGCGGTAAAG
GTGTTCCGCATTGGCCTGTTTATGATCAGAAAGAGGCATATCTGCAGGTTGGTGTTACCACCCAG
GTTGCACAGAAACTGAAAGATAAAGAAGTTGCCTTTTGGACCGAGCTGCTGGCCAAAGGTGCAGC
AGAAAAACGTCAAGAAACCGAACATGTTGAACTGTAACTCGAGCCACTGAGATCCGGC

Sequence name: HLE1_5

Sequence type: PROTEIN

Sequence name / optimized for

HLE1_5/ Escherichia coli

ORF	Protected sites	Protected areas	Motifs to avoid
22-1662 [AGC...TAA]	16-21 NdeI [CATATG] 1663-1668 XhoI [CTCGAG]		NdeI [CATATG] XhoI [CTCGAG]

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                            S  S  P  P  V  V  D  T  A  Q  G  K  V  L  G  K
   1.  GTGCCGCGCGGCAGCCATATGAGCAGCCCTCCGGTTGTTGATACCGCACAGGGTAAAGTTCTGGGTAAA
       H  V  S  L  E  G  F  A  Q  P  V  A  V  F  L  G  V  P  F  A  K  P  P
  70.  CATGTTAGCCTGGAAGGTTTTGCACAGCCGGTTGCAGTTTTTCTGGGTGTTCCGTTTGCAAAACCGCCT
       L  G  S  L  R  F  A  P  P  Q  P  A  D  P  W  P  F  V  K  N  T  T  S
 139.  CTGGGTAGCCTGCGTTTTGCACCGCCTCAGCCTGCAGATCCGTGGCCGTTTGTGAAAAATACCACCAGT
       Y  P  P  M  C  S  Q  D  A  V  M  G  Q  M  L  S  D  L  V  T  N  R  K
 208.  TATCCGCCTATGTGTAGCCAGGATGCAGTTATGGGTCAGATGCTGAGCGATCTGGTTACCAATCGTAAA
       E  K  I  A  L  K  F  S  E  D  C  L  Y  L  N  I  Y  T  P  A  D  L  T
 277.  GAAAAAATCGCCCTGAAATTTAGCGAGGATTGTCTGTATCTGAACATTTATACACCGGCAGACCTGACC
       K  K  S  R  L  P  V  M  V  W  I  H  G  G  G  L  V  V  G  G  A  S  T
 346.  AAAAAAAGTCGCCTGCCGGTTATGGTTTGGATTCATGGTGGTGGTCTGGTTGTTGGTGGTGCAAGCACC
       Y  D  G  L  P  L  S  A  Y  E  N  V  V  V  V  T  I  Q  Y  R  L  G  I
 415.  TATGATGGTCTGCCGCTGAGCGCCTATGAAAATGTTGTTGTTGTGACCATTCAGTATCGTCTGGGTATT
       W  G  F  F  S  T  G  D  E  H  S  P  G  N  W  G  H  L  D  Q  V  A  A
 484.  TGGGGTTTTTTTAGCACCGGTGATGAACATTCACCGGGTAATTGGGGTCATCTGGATCAGGTTGCAGCA
       L  K  W  V  Q  E  N  I  A  N  F  G  G  N  P  G  S  V  T  I  F  G  E
 553.  CTGAAATGGGTTCAAGAAAACATTGCAAACTTTGGTGGTAATCCGGGTAGCGTTACCATTTTTGGTGAA
       S  A  G  G  E  S  V  S  V  L  M  L  S  P  L  A  K  N  L  F  H  R  A
 622.  AGTGCCGGTGGTGAAAGCGTTAGCGTTCTGATGCTGAGTCCGCTGGCAAAAAACCTGTTTCATCGTGCA
       I  S  E  S  G  V  A  L  T  T  A  V  C  K  K  D  S  K  A  A  A  Q  K
 691.  ATTAGCGAAAGCGGTGTTGCACTGACCACCGCAGTTTGTAAAAAAGATAGCAAAGCAGCAGCACAGAAA
       I  A  V  F  S  G  C  K  T  T  T  S  A  V  I  V  H  C  L  R  Q  K  T
 760.  ATTGCCGTTTTTAGCGGTTGTAAAACCACCACCTCAGCAGTTATTGTTCATTGTCTGCGTCAGAAAACC
       E  D  E  L  L  E  T  S  L  K  M  K  F  F  T  L  D  F  H  G  D  P  R
 829.  GAAGATGAACTGCTGGAAACCAGCCTGAAAATGAAATTTTTCACCCTGGATTTTCATGGCGATCCGCGT
       E  S  Y  P  F  L  P  T  V  V  D  G  V  L  L  P  K  M  P  E  E  I  L
 898.  GAAAGCTATCCGTTTCTGCCGACCGTTGTTGATGGTGTTCTGCTGCCGAAAATGCCGGAAGAAATTCTG
       A  E  K  K  F  N  T  V  P  Y  I  V  G  I  N  K  Q  E  F  G  W  F  L
 967.  GCAGAGAAAAAATTCAATACCGTGCCGTATATTGTGGGCATCAACAAACAAGAATTTGGTTGGTTTCTG
       P  T  Q  I  L  G  Y  P  L  S  E  G  K  L  D  Q  K  K  A  T  S  L  L
1036.  CCTACCCAGATTCTGGGCTATCCGCTGAGTGAAGGTAAACTGGATCAGAAAAAAGCGACCAGCCTGCTG
       W  K  S  Y  P  I  V  N  I  P  E  E  L  T  P  V  A  I  E  K  Y  L  G
1105.  TGGAAAAGTTATCCGATTGTTAATATTCCGGAAGAACTGACACCGGTGGCCATTGAAAAATATCTGGGT
       G  T  N  D  P  V  K  K  K  D  L  F  L  D  L  M  A  D  V  V  F  G  V
1174.  GGCACCAATGATCCGGTGAAAAAAAAAGACCTGTTCCTGGATCTGATGGCCGATGTTGTTTTTGGTGTT
       P  T  V  T  V  A  R  Y  H  R  D  A  G  A  P  T  Y  V  Y  E  F  Q  H
1243.  CCGACCGTTACCGTTGCACGTTATCATCGTGATGCGGGTGCACCGACCTATGTTTATGAATTTCAGCAT
       Y  P  S  F  S  S  D  R  R  P  K  T  V  I  G  D  H  M  D  E  L  F  P
1312.  TATCCGAGCTTTAGCAGCGATCGTCGTCCGAAAACCGTTATTGGTGATCACATGGATGAACTGTTTCCG
       L  F  G  A  P  F  L  K  G  G  A  S  E  E  E  I  N  F  S  K  M  V  M
1381.  CTGTTTGGTGCACCGTTTCTGAAAGGTGGTGCCAGCGAAGAAGAAATCAATTTTAGCAAAATGGTGATG
       K  F  A  A  N  F  A  R  N  G  N  P  S  G  K  G  V  P  H  W  P  V  Y
1450.  AAATTTGCAGCCAACTTTGCCCGTAATGGCAATCCGAGCGGTAAAGGTGTTCCGCATTGGCCTGTTTAT
       D  Q  K  E  A  Y  L  Q  V  G  V  T  T  Q  V  A  Q  K  L  K  D  K  E
1519.  GATCAGAAAGAGGCATATCTGCAGGTTGGTGTTACCACCCAGGTTGCACAGAAACTGAAAGATAAAGAA
       V  A  F  W  T  E  L  L  A  K  G  A  A  E  K  R  Q  E  T  E  H  V  E
1588.  GTTGCCTTTTGGACCGAGCTGCTGGCCAAAGGTGCAGCAGAAAAACGTCAAGAAACCGAACATGTTGAA
       L  *
1657.  CTGTAACTCGAGCCACTGAGATCCGGC
```

HLE 1_6: Amino acid sequence

MSSPPVVDTAQGKVLGKHVSLEGFAQPVAVFLGVPFAKPPLGSLRFAPPQPADPWPFVKNTTSYPPMCSQDPVIMEMTSDVATFRKEKIAFQF
SEDCLYLNIYTPADLTKKSRLPVMVWIHGGGLMVGRASDYDGLALSAYENVVVVTIQYRLGIWGFFSTGDEHSPGNWGHLDQVAALRWVQENI
ANFGGDPSSVTIFGESAGGESVSVLVLSPLAKNLFHRAISESGVALTTCLVKKDSKAEAQQIAILAGCKTTTSAVIVDCLRQKTEDELLETSL
KMKFFTLDFHGDPRESHPFLPTVVDGVLLPKMPEEILAEKTFNTVPYIVGFNKQEFGWILPTQLMGYPLSEGKLDQKTATSLLWKSYPIVNIP
EELTPAATEKYLGGTDDPVKKKDLFLDLIGDVMFGVPSVTVARLHRDAGAPTYMYEFQYRPSFSSDMKPKTVIGDHGDDVYSVFGVPLIKEGA
SEEEIKLSKMVMKFWANFARNGNPNGEGLPHWPVYDQKEGYLQIGVPTQAAQKLKDKEVAFWTKLLAEAVEKPLQTEHIEL

HLE isoenzyme 1_6: Nucleotide sequence (restriction sites are marked in bue)

GTGCCGCGCGGCAGCCATATGAGCAGCCCTCCGGTTGTTGATACCGCACAGGGTAAAGTTCTGGG
TAAACATGTTAGCCTGGAAGGTTTTGCACAGCCGGTTGCAGTTTTTCTGGGTGTTCCGTTTGCAA
AACCGCCTCTGGGTAGCCTGCGTTTTGCACCGCCTCAGCCTGCAGATCCGTGGCCGTTTGTGAAA
AATACCACCAGTTATCCGCCTATGTGTAGCCAGGATCCGGTTATTATGGAAATGACCAGTGATGT
TGCAACCTTCCGCAAAGAAAAAATCGCATTTCAGTTTAGCGAGGATTGCCTGTATCTGAACATTT
ATACACCGGCAGATCTGACCAAAAAAAGCCGTCTGCCGGTTATGGTTTGGATTCATGGTGGTGGT
CTGATGGTTGGTCGTGCAAGCGATTATGATGGTCTGGCACTGAGCGCCTATGAAAATGTTGTTGT
TGTGACCATTCAGTATCGTCTGGGTATTTGGGGTTTTTTTAGCACCGGTGATGAACATTCACCGG
GTAATTGGGGTCATCTGGATCAGGTTGCAGCACTGCGTTGGGTTCAAGAAAACATTGCAAATTTT
GGTGGTGATCCGAGCAGCGTTACCATTTTTGGTGAAAGTGCCGGTGGTGAAAGCGTTAGCGTTCT
GGTTCTGAGTCCGCTGGCAAAAAACCTGTTTCATCGTGCAATTAGCGAAAGCGGTGTTGCACTGA
CCACCTGTCTGGTTAAAAAAGATAGCAAAGCAGAAGCACAGCAGATTGCAATTCTGGCAGGTTGT
AAAACCACCACCTCAGCAGTTATTGTTGATTGTCTGCGTCAGAAAACCGAAGATGAACTGCTGGA
AACCAGCCTGAAAATGAAATTTTTCACCCTGGATTTTCATGGCGATCCGCGTGAAAGCCATCCGT
TTCTGCCGACCGTTGTTGATGGTGTTCTGCTGCCGAAAATGCCGGAAGAAATTCTGGCCGAAAAA
ACCTTTAATACCGTGCCGTATATTGTGGGCTTTAACAAACAAGAATTTGGCTGGATCCTGCCGAC
CCAGCTGATGGGTTATCCGCTGAGCGAAGGTAAACTGGATCAGAAAACAGCGACCAGCCTGCTGT
GGAAAAGCTATCCGATTGTTAATATTCCGGAAGAACTGACTCCGGCAGCAACCGAAAAATATCTG
GGTGGCACCGATGATCCGGTGAAAAAAAAAGACCTGTTCCTGGATCTGATTGGCGACGTTATGTT
TGGTGTTCCGAGCGTTACCGTTGCACGTCTGCATCGTGATGCGGGTGCACCGACCTATATGTATG
AATTTCAGTACCGTCCGAGCTTTAGCAGCGATATGAAACCGAAAACCGTTATTGGTGATCATGGT
GATGATGTGTATAGCGTTTTTGGTGTGCCGCTGATTAAAGAAGGTGCCAGCGAAGAAGAAATCAA
ACTGAGCAAAATGGTGATGAAATTCTGGGCCAATTTTGCCCGTAATGGTAATCCGAATGGTGAAG
GTCTGCCGCATTGGCCTGTTTATGATCAGAAAGAAGGTTACCTGCAGATTGGCGTTCCGACCCAG
GCAGCACAGAAACTGAAAGATAAAGAAGTTGCCTTTTGGACCAAACTGCTGGCAGAAGCAGTTGA
AAAACCGCTGCAGACCGAACATATTGAACTGTAACTCGAGCCACTGAGATCCGGC

Sequence name: HLE1_6

Sequence type: DNA

Sequence name / optimized for

HLE1_6/ Escherichia coli

ORF	Protected sites	Protected areas	Motifs to avoid
19-1659 [ATG...TAA]	16-21 NdeI [CATATG] 1660-1665 XhoI [CTCGAG]		NdeI [CATATG] XhoI [CTCGAG]

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                        M  S  S  P  P  V  V  D  T  A  Q  G  K  V  L  G  K
   1. GTGCCGCGCGGCAGCCATATGAGCAGCCCTCCGGTTGTTGATACCGCACAGGGTAAAGTTCTGGGTAAA
      H  V  S  L  E  G  F  A  Q  P  V  A  V  F  L  G  V  P  F  A  K  P  P
  70. CATGTTAGCCTGGAAGGTTTTGCACAGCCGGTTGCAGTTTTTCTGGGTGTTCCGTTTGCAAAACCGCCT
      L  G  S  L  R  F  A  P  P  Q  P  A  D  P  W  P  F  V  K  N  T  T  S
 139. CTGGGTAGCCTGCGTTTTGCACCGCCTCAGCCTGCAGATCCGTGGCCGTTTGTGAAAAATACCACCAGT
      Y  P  P  M  C  S  Q  D  P  V  I  M  E  M  T  S  D  V  A  T  F  R  K
 208. TATCCGCCTATGTGTAGCCAGGATCCGGTTATTATGGAAATGACCAGTGATGTTGCAACCTTCCGCAAA
      E  K  I  A  F  Q  F  S  E  D  C  L  Y  L  N  I  Y  T  P  A  D  L  T
 277. GAAAAAATCGCATTTCAGTTTAGCGAGGATTGCCTGTATCTGAACATTTATACACCGGCAGATCTGACC
      K  K  S  R  L  P  V  M  V  W  I  H  G  G  G  L  M  V  G  R  A  S  D
 346. AAAAAAAGCCGTCTGCCGGTTATGGTTTGGATTCATGGTGGTGGTCTGATGGTTGGTCGTGCAAGCGAT
      Y  D  G  L  A  L  S  A  Y  E  N  V  V  V  V  T  I  Q  Y  R  L  G  I
 415. TATGATGGTCTGGCACTGAGCGCCTATGAAAATGTTGTTGTTGTGACCATTCAGTATCGTCTGGGTATT
      W  G  F  F  S  T  G  D  E  H  S  P  G  N  W  G  H  L  D  Q  V  A  A
 484. TGGGGTTTTTTTAGCACCGGTGATGAACATTCACCGGGTAATTGGGGTCATCTGGATCAGGTTGCAGCA
      L  R  W  V  Q  E  N  I  A  N  F  G  G  D  P  S  S  V  T  I  F  G  E
 553. CTGCGTTGGGTTCAAGAAAACATTGCAAATTTTGGTGGTGATCCGAGCAGCGTTACCATTTTTGGTGAA
      S  A  G  G  E  S  V  S  V  L  V  L  S  P  L  A  K  N  L  F  H  R  A
 622. AGTGCCGGTGGTGAAAGCGTTAGCGTTCTGGTTCTGAGTCCGCTGGCAAAAAACCTGTTTCATCGTGCA
      I  S  E  S  G  V  A  L  T  T  C  L  V  K  K  D  S  K  A  E  A  Q  Q
 691. ATTAGCGAAAGCGGTGTTGCACTGACCACCTGTCTGGTTAAAAAAGATAGCAAAGCAGAAGCACAGCAG
      I  A  I  L  A  G  C  K  T  T  T  S  A  V  I  V  D  C  L  R  Q  K  T
 760. ATTGCAATTCTGGCAGGTTGTAAAACCACCACCTCAGCAGTTATTGTTGATTGTCTGCGTCAGAAAACC
      E  D  E  L  L  E  T  S  L  K  M  K  F  F  T  L  D  F  H  G  D  P  R
 829. GAAGATGAACTGCTGGAAACCAGCCTGAAAATGAAATTTTTCACCCTGGATTTTCATGGCGATCCGCGT
      E  S  H  P  F  L  P  T  V  V  D  G  V  L  L  P  K  M  P  E  E  I  L
 898. GAAAGCCATCCGTTTCTGCCGACCGTTGTTGATGGTGTTCTGCTGCCGAAAATGCCGGAAGAAATTCTG
      A  E  K  T  F  N  T  V  P  Y  I  V  G  F  N  K  Q  E  F  G  W  I  L
 967. GCCGAAAAAACCTTTAATACCGTGCCGTATATTGTGGGCTTTAACAAACAAGAATTTGGCTGGATCCTG
      P  T  Q  L  M  G  Y  P  L  S  E  G  K  L  D  Q  K  T  A  T  S  L  L
1036. CCGACCCAGCTGATGGGTTATCCGCTGAGCGAAGGTAAACTGGATCAGAAAACAGCGACCAGCCTGCTG
      W  K  S  Y  P  I  V  N  I  P  E  E  L  T  P  A  A  T  E  K  Y  L  G
1105. TGGAAAAGCTATCCGATTGTTAATATTCCGGAAGAACTGACTCCGGCAGCAACCGAAAAATATCTGGGT
      G  T  D  D  P  V  K  K  K  D  L  F  L  D  L  I  G  D  V  M  F  G  V
1174. GGCACCGATGATCCGGTGAAAAAAAAAGACCTGTTCCTGGATCTGATTGGCGACGTTATGTTTGGTGTT
      P  S  V  T  V  A  R  L  H  R  D  A  G  A  P  T  Y  M  Y  E  F  Q  Y
1243. CCGAGCGTTACCGTTGCACGTCTGCATCGTGATGCGGGTGCACCGACCTATATGTATGAATTTCAGTAC
      R  P  S  F  S  S  D  M  K  P  K  T  V  I  G  D  H  G  D  D  V  Y  S
1312. CGTCCGAGCTTTAGCAGCGATATGAAACCGAAAACCGTTATTGGTGATCATGGTGATGATGTGTATAGC
      V  F  G  V  P  L  I  K  E  G  A  S  E  E  E  I  K  L  S  K  M  V  M
1381. GTTTTTGGTGTGCCGCTGATTAAAGAAGGTGCCAGCGAAGAAGAAATCAAACTGAGCAAAATGGTGATG
      K  F  W  A  N  F  A  R  N  G  N  P  N  G  E  G  L  P  H  W  P  V  Y
1450. AAATTCTGGGCCAATTTTGCCCGTAATGGTAATCCGAATGGTGAAGGTCTGCCGCATTGGCCTGTTTAT
      D  Q  K  E  G  Y  L  Q  I  G  V  P  T  Q  A  A  Q  K  L  K  D  K  E
1519. GATCAGAAAGAAGGTTACCTGCAGATTGGCGTTCCGACCCAGGCAGCACAGAAACTGAAAGATAAAGAA
      V  A  F  W  T  K  L  L  A  E  A  V  E  K  P  L  Q  T  E  H  I  E  L
1588. GTTGCCTTTTGGACCAAACTGCTGGCAGAAGCAGTTGAAAAACCGCTGCAGACCGAACATATTGAACTG
      *
1657. TAACTCGAGCCACTGAGATCCGGC
```

Esterase from *Equus przewalskii*: Amino acid sequence

VPRGSHMSSPPVVDTAQGKVLGKHVSLEGFAQPVAVFLGVPFAKPPLGSLRFAPPQPADPWPFVKNATSYPPMCSQDTVAGQMLSDLFTNRKE
NISVQISEDCLYLNIYTPADLTKKSRLPVMVWIHGGGLMIGGASTYDGLALSAHENVVVVTIQYRLGIWGFLSTGDEHSPGNWGHLDQVAALR
WVQENIANFGGDPGSVTIFGESAGGESVSVLVLSPLAKNLFHRAISESGVAFTAGLVQKDSKAAAQQIAVFAGCKTATSAVIVHCLRQKTEDE
LLETSVKMKFLSLDFRGDSRESHPFLPAVVDGVLLPKMPEEILAEKTFNTVPYIVGINKQEFGWIIPTMMGYPLSEGKLDQKTATSLLQKANP
ILNIPEEVTPVATEKYLGGTEDPVKKKDLFLDLIGDVMFGVPSVTVARLHRDAGASTFMYEFQYRPSFSSVMKPKTVIGDHGDEIFSVFGAPF
LKEGASEEEIKLSKMVMKFWANFAWNGNPNGEGLPHWPAYDQKEGYLQIGVTTQAAQKLKDKEVAFWTELLAKEAAEKQQQTEHVEL*LEPLR
SG

Esterase from *Equus przewalskii*: Nucleotide sequence (restriction sites are marked in bue)

GTGCCGCGCGGCAGCCATATGAGCAGCCCTCCGGTTGTTGATACCGCACAGGGTAAAGTTCTGGG
TAAACATGTTAGCCTGGAAGGTTTTGCACAGCCGGTTGCAGTTTTTCTGGGTGTTCCGTTTGCAA
AACCGCCTCTGGGTAGCCTGCGTTTTGCACCGCCTCAGCCTGCAGATCCGTGGCCGTTTGTTAAA
AATGCAACCAGCTATCCGCCTATGTGTAGCCAGGATACCGTTGCAGGTCAGATGCTGAGCGACCT
GTTTACCAATCGTAAAGAAAACATTAGCGTGCAGATTAGCGAAGATTGTCTGTATCTGAACATTT
ATACACCGGCAGACCTGACCAAAAAAAGTCGCCTGCCGGTTATGGTTTGGATTCATGGTGGTGGT
CTGATGATTGGTGGTGCAAGCACCTATGATGGTCTGGCACTGAGCGCACATGAAAATGTTGTTGT
TGTGACCATTCAGTATCGTCTGGGTATTTGGGGTTTTCTGAGCACCGGTGATGAACATTCACCGG
GTAATTGGGGTCATCTGGATCAGGTTGCAGCACTGCGTTGGGTTCAAGAGAATATTGCAAATTTT
GGTGGTGATCCGGGTAGCGTTACCATTTTTGGTGAAAGTGCCGGTGGTGAAAGCGTTAGCGTTCT
GGTTCTGAGTCCGCTGGCAAAAAACCTGTTTCATCGTGCCATTAGCGAAAGCGGTGTTGCATTTA
CCGCAGGTCTGGTTCAGAAAGATAGCAAAGCAGCAGCACAGCAGATTGCCGTTTTTGCAGGTTGT
AAAACCGCAACCAGCGCAGTTATTGTTCATTGTCTGCGCCAGAAAACCGAAGATGAACTGCTGGA
AACCAGCGTGAAAATGAAATTTCTGAGCCTGGATTTTCGCGGTGATAGCCGTGAAAGCCATCCGT
TTCTGCCTGCAGTTGTTGATGGTGTTCTGCTGCCGAAAATGCCGGAAGAAATTCTGGCAGAAAAA
ACCTTTAATACCGTGCCGTATATTGTGGGCATCAACAAACAAGAATTTGGCTGGATTATTCCGAC
CATGATGGGTTATCCGCTGAGCGAAGGTAAACTGGATCAGAAAACAGCGACCAGCCTGCTGCAGA
AAGCAAATCCGATTCTGAATATTCCGGAAGAAGTTACACCGGTTGCCACCGAAAAATATCTGGGT
GGCACCGAAGATCCGGTTAAAAAAAAAGACCTGTTCCTGGATCTGATCGGTGATGTTATGTTTGG
TGTTCCGAGCGTTACCGTTGCCCGTCTGCATCGTGATGCCGGTGCCAGCACCTTTATGTATGAAT
TTCAGTACCGTCCGAGCTTTAGCAGCGTTATGAAACCGAAAACCGTTATTGGTGATCATGGCGAC
GAAATCTTTAGCGTTTTTGGTGCACCGTTTCTGAAAGAAGGTGCAAGCGAAGAAGAAATCAAACT
GAGCAAAATGGTGATGAAATTTTGGGCCAATTTTGCCTGGAATGGTAATCCGAATGGTGAAGGCC
TGCCGCATTGGCCTGCCTATGATCAGAAAGAAGGCTATCTGCAAATTGGTGTTACCACCCAGGCA
GCACAGAAACTGAAAGATAAAGAAGTTGCCTTTTGGACCGAGCTGCTGGCCAAAGAAGCAGCAGA
AAAACAGCAGCAGACCGAACATGTTGAACTGTAACTCGAGCCACTGAGATCCGGC

Sequence name: HLE_przewalskii
Sequence type: DNA

Sequence name / optimized for
HLE_przewalskii/ Escherichia coli

ORF	Protected sites	Protected areas	Motifs to avoid
19-1659 [ATG...TAA]	16-21 NdeI [CATATG] 1660-1665 XhoI [CTCGAG]		NdeI [CATATG] XhoI [CTCGAG]

```
                       M S S P P V V D T A Q G K V L G K
1.    GTGCCGCGCGGCAGCCATATGAGCAGCCCTCCGGTTGTTGATACCGCACAGGGTAAAGTTCTGGGTAAA
      H V S L E G F A Q P V A V F L G V P F A K P P
70.   CATGTTAGCCTGGAAGGTTTTGCACAGCCGGTTGCAGTTTTTCTGGGTGTTCCGTTTGCAAAACCGCCT
      L G S L R F A P P Q P A D P W P F V K N A T S
139.  CTGGGTAGCCTGCGTTTTGCACCGCCTCAGCCTGCAGATCCGTGGCCGTTTGTTAAAAATGCAACCAGC
      Y P P M C S Q D T V A G Q M L S D L F T N R K
208.  TATCCGCCTATGTGTAGCCAGGATACCGTTGCAGGTCAGATGCTGAGCGACCTGTTTACCAATCGTAAA
      E N I S V Q I S E D C L Y L N I Y T P A D L T
277.  GAAAACATTAGCGTGCAGATTAGCGAAGATTGTCTGTATCTGAACATTTATACACCGGCAGACCTGACC
      K K S R L P V M V W I H G G G L M I G G A S T
346.  AAAAAAAGTCGCCTGCCGGTTATGGTTTGGATTCATGGTGGTGGTCTGATGATTGGTGGTGCAAGCACC
      Y D G L A L S A H E N V V V V T I Q Y R L G I
415.  TATGATGGTCTGGCACTGAGCGCACATGAAAATGTTGTTGTTGTGACCATTCAGTATCGTCTGGGTATT
      W G F L S T G D E H S P G N W G H L D Q V A A
484.  TGGGGTTTTCTGAGCACCGGTGATGAACATTCACCGGGTAATTGGGGTCATCTGGATCAGGTTGCAGCA
      L R W V Q E N I A N F G G D P G S V T I F G E
553.  CTGCGTTGGGTTCAAGAGAATATTGCAAATTTTGGTGGTGATCCGGGTAGCGTTACCATTTTTGGTGAA
      S A G G E S V S V L V L S P L A K N L F H R A
622.  AGTGCCGGTGGTGAAAGCGTTAGCGTTCTGGTTCTGAGTCCGCTGGCAAAAAACCTGTTTCATCGTGCC
      I S E S G V A F T A G L V Q K D S K A A A Q Q
691.  ATTAGCGAAAGCGGTGTTGCATTTACCGCAGGTCTGGTTCAGAAAGATAGCAAAGCAGCAGCACAGCAG
      I A V F A G C K T A T S A V I V H C L R Q K T
760.  ATTGCCGTTTTTGCAGGTTGTAAAACCGCAACCAGCGCAGTTATTGTTCATTGTCTGCGCCAGAAAACC
      E D E L L E T S V K M K F L S L D F R G D S R
829.  GAAGATGAACTGCTGGAAACCAGCGTGAAAATGAAATTTCTGAGCCTGGATTTTCGCGGTGATAGCCGT
      E S H P F L P A V V D G V L L P K M P E E I L
898.  GAAAGCCATCCGTTTCTGCCTGCAGTTGTTGATGGTGTTCTGCTGCCGAAAATGCCGGAAGAAATTCTG
      A E K T F N T V P Y I V G I N K Q E F G W I I
967.  GCAGAAAAAACCTTTAATACCGTGCCGTATATTGTGGGCATCAACAAACAAGAATTTGGCTGGATTATT
      P T M M G Y P L S E G K L D Q K T A T S L L Q
1036. CCGACCATGATGGGTTATCCGCTGAGCGAAGGTAAACTGGATCAGAAAACAGCGACCAGCCTGCTGCAG
      K A N P I L N I P E E V T P V A T E K Y L G G
1105. AAAGCAAATCCGATTCTGAATATTCCGGAAGAAGTTACACCGGTTGCCACCGAAAAATATCTGGGTGGC
      T E D P V K K K D L F L D L I G D V M F G V P
1174. ACCGAAGATCCGGTTAAAAAAAAAGACCTGTTCCTGGATCTGATCGGTGATGTTATGTTTGGTGTTCCG
      S V T V A R L H R D A G A S T F M Y E F Q Y R
1243. AGCGTTACCGTTGCCCGTCTGCATCGTGATGCCGGTGCCAGCACCTTTATGTATGAATTTCAGTACCGT
      P S F S S V M K P K T V I G D H G D E I F S V
1312. CCGAGCTTTAGCAGCGTTATGAAACCGAAAACCGTTATTGGTGATCATGGCGACGAAATCTTTAGCGTT
      F G A P F L K E G A S E E E I K L S K M V M K
1381. TTTGGTGCACCGTTTCTGAAAGAAGGTGCAAGCGAAGAAGAAATCAAACTGAGCAAAATGGTGATGAAA
      F W A N F A W N G N P N G E G L P H W P A Y D
1450. TTTTGGGCCAATTTTGCCTGGAATGGTAATCCGAATGGTGAAGGCCTGCCGCATTGGCCTGCCTATGAT
      Q K E G Y L Q I G V T T Q A A Q K L K D K E V
1519. CAGAAAGAAGGCTATCTGCAAATTGGTGTTACCACCCAGGCAGCACAGAAACTGAAAGATAAAGAAGTT
      A F W T E L L A K E A A E K Q Q Q T E H V E L
1588. GCCTTTTGGACCGAGCTGCTGGCCAAAGAAGCAGCAGAAAAACAGCAGCAGACCGAACATGTTGAACTG
      *
1657. TAACTCGAGCCACTGAGATCCGGC
```

Hydrolase from *Bacillus subtilis* (pdb code: 2R11): Amino acid sequence

```
MSNHSSSIPELSDNGIRYYQTYNESLSLWPVRCKSFYISTRFGQTHVIASGPEDAPPLVLLHGALFSSTMWYPNIADWSSKYRTYAVDIIGDK
NKSIPENVSGTRTDYANWLLDVFDNLGIEKSHMIGLSLGGLHTMNFLLRMPERVKSAAILSPAETFLPFHHDFYKYALGLTASNGVETFLNWM
MNDQNVLHPIFVKQFKAGVMWQDGSRNPNPNADGFPYVFTDEELRSARVPILLLLGEHEVIYDPHSALHRASSFVPDIEAEVIKNAGHVLSME
QPTYVNERVMRFFNAKTGISR
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Hydrolase from *Bacillus subtilis* (pdb code: 2R11): Nucleotide sequence (coding region is marked in grey

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CTGATAAAATTCATCATCATCATCATCACGAAAACCTGTACTTCCAGGGCATGTCAAACCATTCATCTAGTATTCCCGAATTA
AGTGACAACGGTATCCGCTATTATCAAACTTATAATGAAAGCCTTAGTCTTTGGCCGGTCCGTTGTAAATCATTCTATATATC
TACTCGTTTTGGTCAAACACATGTGATTGCAAGCGGCCCAGAGGATGCCCCGCCGCTTGTATTACTCCACGGAGCATTATTCA
GCTCGACGATGTGGTATCCCAACATCGCCGATTGGAGCAGTAAATACAGAACTTATGCAGTTGATATCATAGGTGATAAAAAC
AAGAGTATTCCTGAGAATGTAAGCGGTACAAGAACGGATTACGCCAATTGGCTTCTTGATGTGTTTGACAATCTGGGGATCGA
AAAGTCCCACATGATCGGACTTTCGCTTGGCGGTCTCCATACGATGAATTTCCTTTTACGTATGCCTGAGAGAGTAAAAAGCG
CAGCTATACTGAGTCCGGCAGAAACGTTTTTGCCATTTCATCACGATTTCTACAAATACGCTCTTGGCCTTACAGCGTCAAAT
GGAGTTGAAACATTCTTAAATTGGATGATGAATGATCAGAATGTGCTGCACCCGATTTTTGTGAAGCAGTTTAAGGCAGGGGT
AATGTGGCAGGATGGATCAAGAAATCCAAATCCTAATGCCGACGGATTTCCGTATGTTTTTACCGATGAGGAATTACGTTCAG
CAAGAGTTCCTATCCTATTATTACTTGGTGAACATGAAGTCATCTATGATCCCCACTCAGCCCTGCACCGAGCCTCTTCATTC
GTTCCAGATATTGAGGCGGAAGTCATTAAAAATGCCGGACATGTTTTATCGATGGAACAACCCACTTACGTAAATGAACGTGT
AATGCGTTTTTTCAATGCAAAAACAGGCATTTCACGGTAACGCGACTTAATTAA
```

967bp (953bp)

5.3 GC Chromatograms

In this section GC-chromatograms of the performed biotransformations are shown. Also chromatograms of synthesized chiral compounds **2b** and **2c** are shown. **Table 4.2-1** summarizes the retention times of each compound involved and methods used for analysis.

Table 5.3-1: Retention times and methods used for conversion analysis

Compound	t_r [min]	Method
1b	6.4	Modified Gradient DB 1701
1c	6.2	Modified Gradient DB 1701
1d	6.8	Modified Gradient DB 1701
1e	6.4	HP5 modified Säure inj200
2b [a]	6.9/7.5	Modified Gradient DB 1701
2c [b]	6.5/7.0	Modified Gradient DB 1701
(*S*)-2b	5.6	Template DEX CB
(*R*)-2b	5.8	Template DEX CB
(*S*)-2c_*a*	6.5	Template DEX CB
(*R*)-2c_*a*	6.7	Template DEX CB
2e	6.4	HP5 modified Säure inj200

[a] two retention times are given because peaks shifted 0.5 min due to shortening of the column. Solvent peaks shifted as well

[b] two retention times are given because peaks shifted 0.5 min due to shortening of the column. Solvent peaks shifted as well.

5.3.1 Biotransformation of ε-caprolactone (1b)

Method: Modified Gradient DB 1701
Column: DB 1701 capillary column (30 m, 0.25 mm, 0.25 µm film)
Temperature Program: 100 °C (1 min), ramp 1: [20 °C/min] – 170 °C (4 min), ramp 2: [30 °C/min] – 250 °C (1 min), postrun 280 °C
Carrier Gas Flow: (H_2) 0.9 mL/min
Split Ratio: 50:1
Injector Temperature: 250 °C
FID Detector Temperature: 250 °C

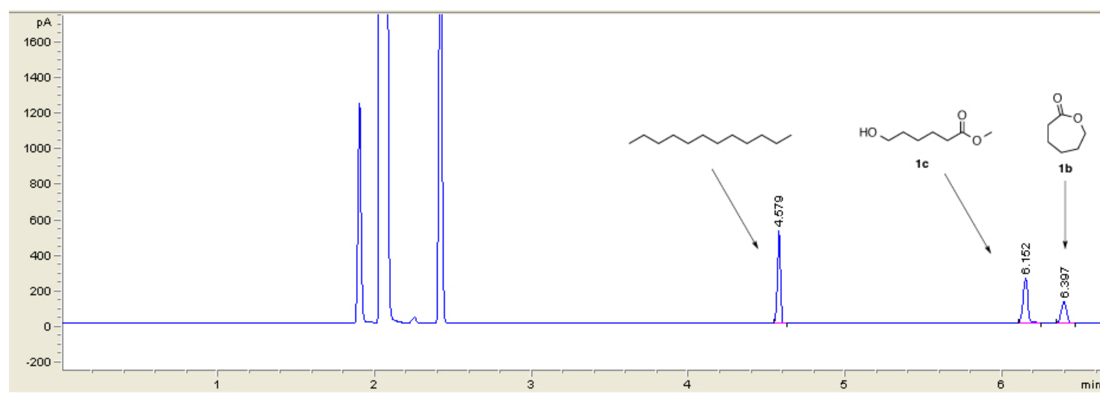

Figure 5.3-1: Products obtained from biotransformation of ε-caprolactone **(1b)** employing HLE crude preparation.

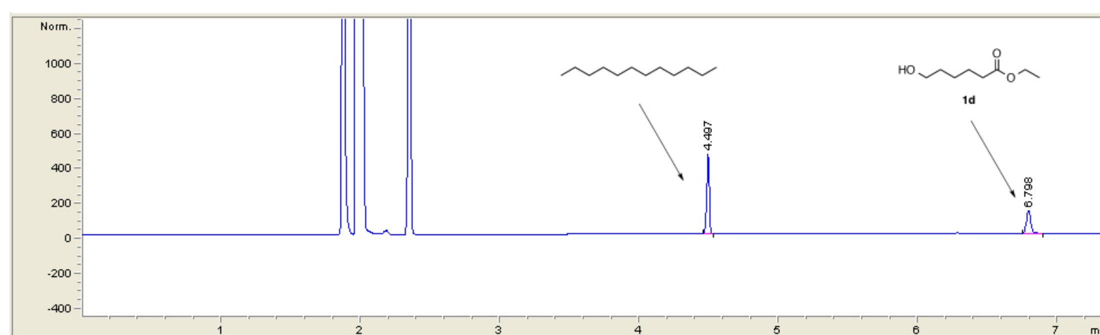

Figure 5.3-2: Product obtained from biotransformation of ε-caprolactone (**1b**) employing HLE crude preparation.

Method: HP5 modified Säure inj200
Column: HP-5 capillary column (30 m, 0.32 mm, 0.25 µm film)
Temperature Program: 80 °C (1 min), ramp 1: [20 °C/min] – 140 °C (4 min), ramp 2: [40 °C/min] – 300 °C (1 min), postrun 300 °C
Carrier Gas Flow: (H_2) 1.5 mL/min
Split Ratio: 50:1
Injector Temperature: 200 °C
FID Detector Temperature: 250 °C

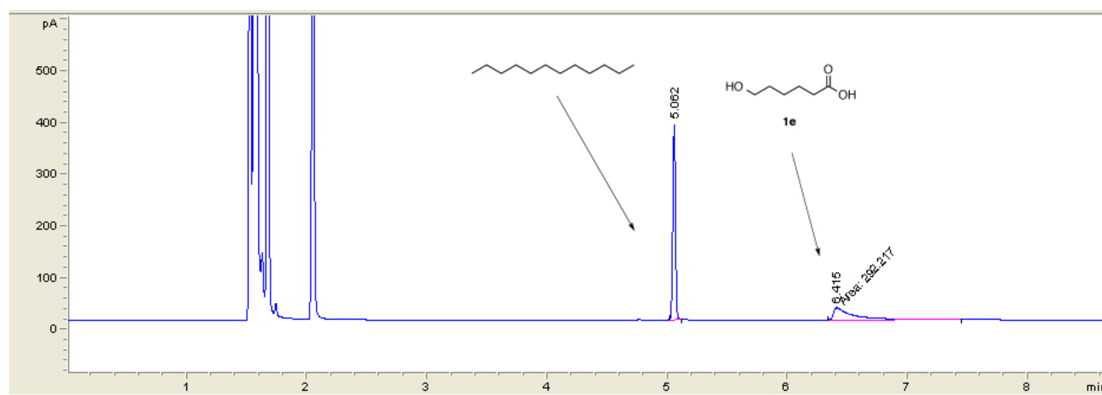

Figure 5.3-3: Product obtained from biotransformation of ε-caprolactone (**1b**) employing HLE crude preparation.

5.3.2 Biotransformation of 6-methyl-ε-caprolactone (2b)

Method: Modified Gradient DB 1701
Column: DB 1701 capillary column (30 m, 0.25 mm, 0.25 µm film)
Temperature Program: 100 °C (1 min), ramp 1: [20 °C/min] – 170 °C (4 min), ramp 2: [30 °C/min] – 250 °C (1 min), postrun 280 °C
Carrier Gas Flow: (H_2) 0.9 mL/min
Split Ratio: 50:1
Injector Temperature: 250 °C
FID Detector Temperature: 250 °C

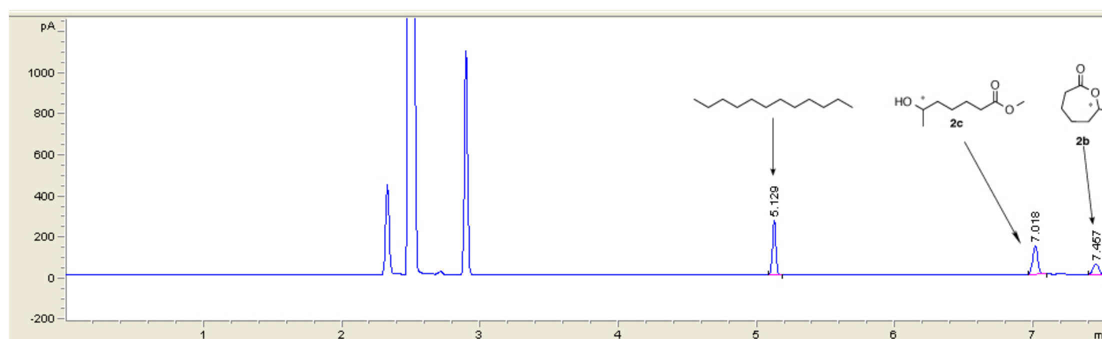

Figure 5.3-4: Products obtained from biotransformation of 6-methyl-ε-caprolactone (**2b**) employing HLE isoenzyme 1_2.

Method: HP5 modified Säure inj200
Column: HP-5 capillary column (30 m, 0.32 mm, 0.25 µm film)
Temperature Program: 80 °C (1 min), ramp 1: [20 °C/min] – 140 °C (4 min), ramp 2: [40 °C/min] – 300 °C (1 min) – postrun 300 °C
Carrier Gas Flow: (H_2) 1.5 mL/min
Split Ratio: 50:1
Injector Temperature: 200 °C
FID Detector Temperature: 250 °C

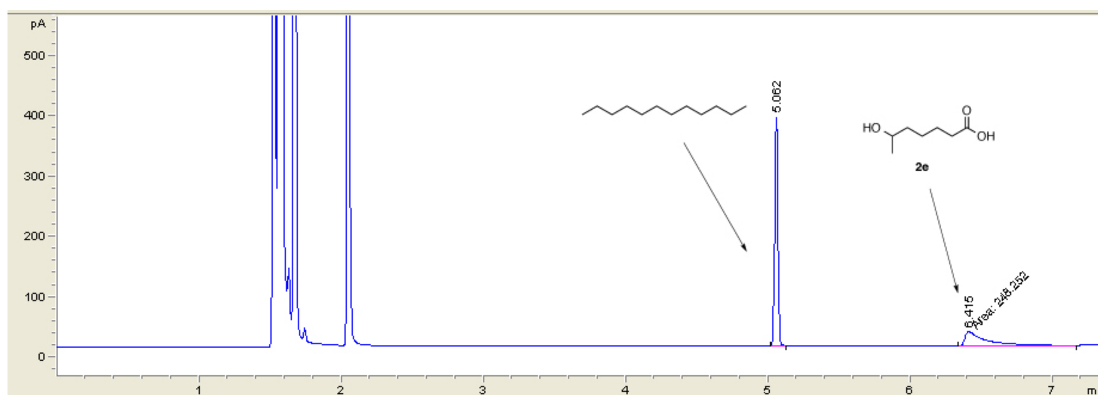

Figure 5.3-5: Product obtained from biotransformation of 6-methyl-ε-caprolactone (**2b**) employing HLE isoenzyme 1_1.

5.3.3 Chiral analytic of methyl-ε-caprolactone (2b) and derivatized methyl 6-hydroxyheptanoate (2c)

Method: Template Dex CB
Column: Varian Chirasil Dex CB column (25 m x 0.32 mm x 0.25 µm film).
Temperature Program: 100 °C (1 min), ramp: [10 °C/min] – 180 °C (1 min), postrun 180 °C
Carrier Gas Flow: (H_2) 1.3 mL/min
Split Ratio: 50:1
Injector Temperature: 250 °C
FID Detector Temperature: 250 °C

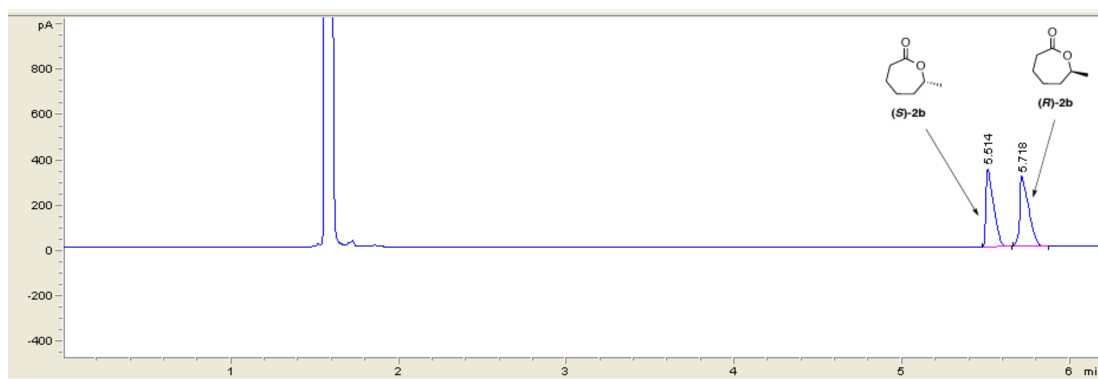

Figure 5.3-6: Product obtained from synthesis of racemic 6-methyl-ε-caprolactone (**2b**).

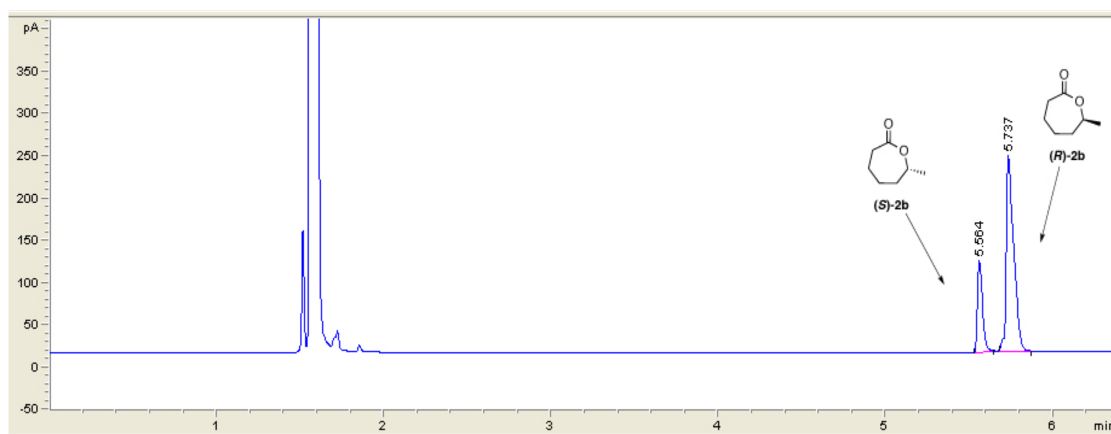

Figure 5.3-7: Product obtained from enzymatic resolution of 6-methyl-ε-caprolactone (**2b**) employing HLE crude preparation.

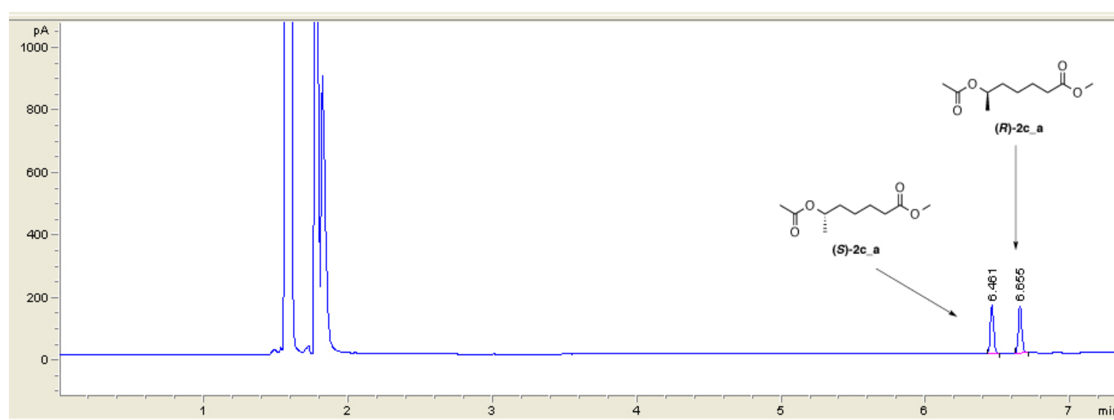

Figure 5.3-8: Product obtained from synthesis of racemic methyl 6-hydroxaheptanoate (**2c**). For the analysis on GC **2c** was derivatized.

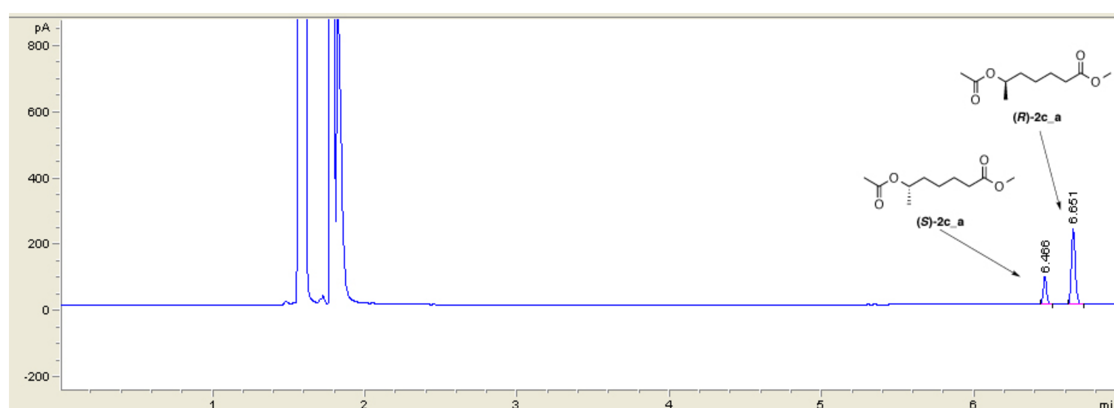

Figure 5.3-9: Product obtained from synthesis of (*R*)-methyl 6-hydroxaheptanoate (**2c**). For the analysis on GC **2c** was derivatized.

5.3.4 Chiral analytic: Biotransformation of methyl-ε-caprolactone (2b)

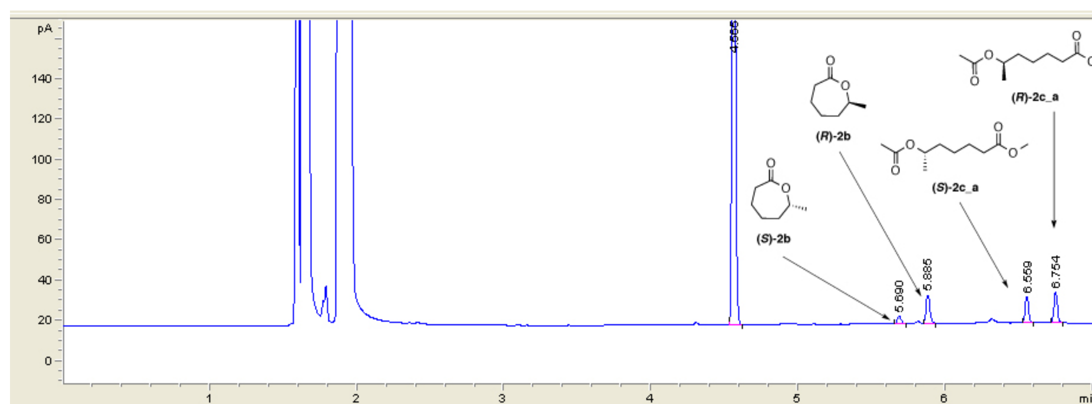

Figure 5.3-10: Products obtained from biotransformation of 6-methyl-ε-caprolactone (**2b**) employing HLE isoenzyme 1_2.

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Publikationen

'Biocatalytic C=C double bond cleavage by a Mn(III) dependent enzyme: Gene sequence identification & beyond'
Aashrita Rajagopalan*, Markus Schober, Lucas Hammerer, Anita Emmersdorfer, Francesco. G. Mutti, Harald Pichler, Wolfgang Kroutil: Poster, Gordon conference on Biocatalysis, RI, USA, July **2012**

Enzymatic Aerobic Alkene Cleavage Catalyzed by a Mn3+-Dependent Proteinase A Homologue
Aashrita Rajagopalan*, Markus Schober, Anita Emmerstorfer, Lucas Hammerer, Anna Migglautsch, Birgit Seisser, Silvia M Glueck, Frank Niehaus, Juergen Eck, Harald Pichler, Karl Gruber, Wolfgang Kroutil; ChemBioChem 12/**2013**; 14(18): 2427-2430

Gemeinnützige Arbeit

09-10.2011 Trashore: Projekt der EU bei welchem Jugendliche, mit unterschiedlichem kulturellem Hintergrund, die Küste Europas bereisen und Strände vom Müll reinigen.

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EDV-Kenntnisse

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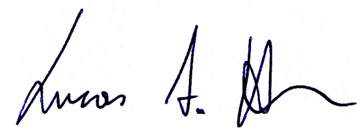
- Excel
- PowerPoint
- Lightroom
- Final Cut Pro

Interessen

Freizeit

- Sport
- Reisen
- Photo- & Videographie



Datum: 09.11.2015

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