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Characterization of a monolignol oxidoreductase from *Arabidopsis thaliana* for biocatalytic applications

Master Thesis

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2 Abstract

Flavoproteins are a large and diverse group of proteins which use either flavin mononucleotide or flavin adenine dinucleotide as cofactor for their catalysis. They can be classified due to their binding of the cofactor. The flavin cofactor can either be covalent or non-covalent attached to the enzyme. In rare cases the cofactor is even bi-covalently attached to the enzyme. An common example for a bi-covalently attached enzyme is the berberine bridge enzyme (BBE) form *California poppy*. In various plants BBE-like enzymes were identified in recent years. Till know their specific function is not known.

In this master thesis a flavin-dependent BBE-like monolignol oxidoreductase from *Arabidopsis thaliana* (*At*BBE-like 15) was characterized to identify its potential as biocatalyst for oxidation reactions. As a dehydrogenase the enzyme shows sluggish activity towards oxygen. The resulting slow reaction velocities hamper the use of this enzyme as biocatalyst. The exchange of a single amino acid resulted in a higher activity of *At*BBE-like 15 towards oxygen, which makes this enzyme interesting for industrial applications. The enzyme was tested under various conditions, the pH and the temperature optimum of the enzyme was determined. Furthermore the stability in organic co-solvents and the substrate scope was measured.

The enzyme shows the highest activity at pH 7 and 50°C. Between pH 5 and 10 it retains more than 50% of its activity. In the presence of acetonitrile, 2-propanol, 1,4-dioxane, THF, 1-butanol, DMSO and ethanol the enzyme shows an increased activity in comparison to the activity in buffer. Long-term stability in the presence of various organic solvents was tested. The highest long term stability is given in DMSO. As substrate primary and secondary allylic alcohols and primary benzylic alcohols are accepted by the enzyme. The investigation of the enantioselectivity of the enzyme towards secondary allylic alcohols lead to good results with an E-value > 34 to > 200. This results make the enzyme a possible biocatalyst for oxidation reactions and enantioselective conversions which cannot be

performed by organic chemical synthesis. Parts of this work have been published by Pils *et al.* [1].

3 Zusammenfassung

Flavoproteine sind eine große und vielfältige Gruppe von Proteinen, welche entweder FMN oder FAD als Kofaktor für ihre Katalyse nutzen. Man kann sie nach der Art ihrer Kofaktorbindung einteilen. Der Flavin-Kofaktor kann entweder kovalent oder nicht-kovalent an das Enzym gebunden sein. Selten kommt es vor, dass der Kofaktor bikovalent and das Enzym gebunden ist. Ein bekanntes Beispiel für ein Enyzem, welches den Flavinkofaktor bikovalent bindet ist das Berberine Bridge Enyzme (BBE) aus dem kalifornischen Goldmohn. In vielen verschiedenen Pflanzen konnten Enzyme identifiziert werden welche BBE ähnlichen Strukturen aufweisen. Deren genaue Funktion ist noch unklar.

In dieser Masterarbeit wurde eine flavin-abhängige BBE ähnliche Monolignoloxidoreduktase aus *Arabidopsis thaliana* (*At*BBE-like 15) charakterisiert, um ihr Potential für die Anwendung als Biokatalysator für Oxidationsreaktionen zu bestimmen. *At*BBE-like 15 ist eine Dehydrogenase und zeigt daher eine geringe Aktivität gegenüber Sauerstoff. Die daraus resultierenden langsamen Reaktionsraten hemmen den Einsatz des Enzyms als Biokatalysator. Durch den Austausch von einer einzigen Aminosäure konnte die Reaktivität in Bezug auf Sauerstoff stark erhöht werden und das Enzym kann jetzt für die Anwendung als Biokatalysator in Betracht gezogen werden. Das Enzym wurde unter unterschiedlichsten Bedingungen getestet. Es wurde das pH - und das Temperaturoptimum, die Lösungsmitteltoleranz und das Substratspektrum des Enyzms untersucht.

Das Enyzm hat sein Aktivitätsmaximum bei pH 7 und 50°C. Zwischen pH 5 und pH 10 behält es mehr als 50% seiner maximalen Aktivität bei. In der Gegenwart von Aceonitril, 2-Propanol, 1,4-Dioxan, THF, 1-Butanol, DMSO und Ethanol weist das Enyzm eine höhere Aktivität als in Puffer auf. Die höchste Langzeitstabilität wurde in DMSO bestimmt. Primäre und sekundäre allylische Alkohole und primäre benzylische Alkohole wurden von dem Enyzm umgesetzt. Die Untersuchung der Enantioselektivität des Enzyms bezüglich sekundärer allylischer Alkohole lieferte gute

Ergebnisse mit E-Werten zwischen >34 to > 200. Die erhaltenen Ergebnisse zeigen, dass das Enzym gute Voraussetzungen als Biokatalysator für Oxidationsreaktionen und enantioselektive Umsetzungen hat, welche mit organisch-chemischer Synthese nicht machbar sind. Teile der Arbeit wurden von Pils *et al.* [1] veröffentlicht.

Eidesstattliche Erklärung

Ich erkläre an Eides statt, dass ich die vorliegende Arbeit selbstständig verfasst, andere als die angegebenen Quellen/Hilfsmittel nicht benutzt und die den benutzten Quellen wörtlich und inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

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Glossary

ATP adenosine triphosphate

BBE berberine bridge enzyme

Cys cysteine

DMAP 4-dimethylaminopyridine
DMF dimethyl formamide
DMSO dimethyl sulfoxide
DNA desoxyribonucleic adid

e.e. enantiomeric excess

FAD flavin adenine dinucleotice
FMN flavin mononucelotide

GC-FID gas chroatography with flame ionization detector
GC-MS gas chromatography with mass spectroscopy
GOOX glucooligosaccaride oxidase

HCl hydrocloric acid
His histidine

Leu leucine
Lys lysine

m/z mass divided by charge number
MES 2-(N-morpholino)ethanesulfonic acid

rpm revolutions per minute
RT retention time

THF tetrahydrofurane
Tyr tyrosine

v/v volume per volume
VAO vanillyl-alcohol oxidase

Contents

4 Introduction

4.1 Flavoproteins

Flavoproteins are a large group of proteins which are able to catalyse a wide range of cellular reactions. Up to 3% of all genes in eukaryotes and prokaryotes are predicted to encode for flavoproteins. As the name indicates flavoproteins use either FMN (flavin mononucelotide) or FAD (flavin adenine dinucleotice) as cofactor for their catalysis [2] [3].

FMN and FAD derived from vitamin B_2 which is also called riboflavin. While mammals have to take up vitamin B_2 via their nutrition, bacteria and plants are able to synthesize vitamin B_2 themselves [4]. FMN and FAD are then build up of vitamin B_2 in a two step reaction. The first step of the synthesis is carried out by the riboflavin kinase resulting in an phosphorylation of the ribityl side chain which is located at the N10-position of the flavin isoalloxazine ring. The second step, an adenylation, is catalysed by the FAD-synthetase. Both reactions are ATP dependent.

FAD is more frequently used as cofactor for catalysis as FMN. FAD depending proteins are more likely to bind the cofactor in a Rossman fold. For FMN depending proteins adoption of a $(\beta\alpha)8$-TIM barrel or a flavodoxin-like fold is preferred [5].

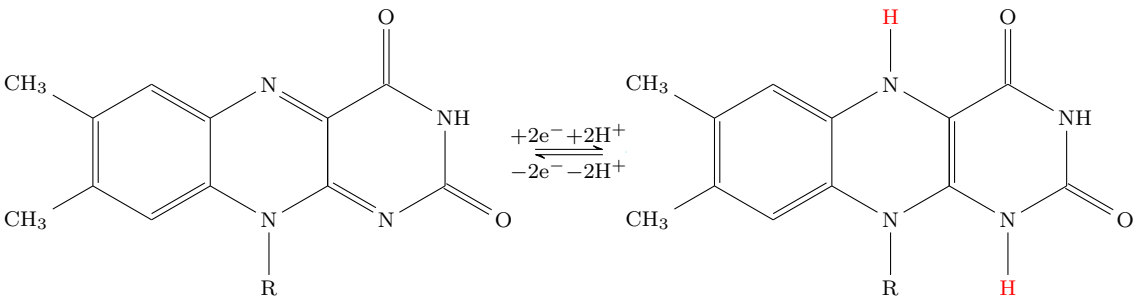


Figure 1: Flavin cofactor

Most of the flavoproteins are oxidoreductases and are involved in redox reactions. Around 10% of all flavoproteins are involved in non-redox re-

actions like halogenations, light sensing and emission and DNA repair [3]. Due to their wide applicability in different catalytic processes flavoproteins can be set apart from other cofactor depending enzymes which only catalyse one type of reaction.

Flavoproteins catalyse reactions by transferring one- or two-electrons [6]. During the reaction electron transfer between the substrate of the enzyme and the flavin cofactor occurs [7]. The reaction can be divided into two half reactions. In the first step, the reductive half reaction, the flavin cofactor is in the oxidized ground state and gets reduced by the substrate. The substrate gets oxidized. In the second step of the reaction a adequate electron acceptor must be present. By passing the electrons further to the electron acceptor, the flavin gets back into its oxidized ground state. It is now able to enter another catalytic cycle [3].

Depending on their reactivity towards oxygen flavin-dependent oxidoreductases can be divided in dehydrogenases and oxidases. Enzymes which use molecular oxygen as electron acceptor are called oxidases while enzymes which react slowly with oxygen and hence require another oxygen acceptor for electron transfer are called dehydrogenase [8].

The flavin cofactor can either non-covalently or covalently bind in the active side of the enzyme [1]. In approximately 90% of all flavoproteins the cofactor is non-covalently attached to the enzyme. Covalent attachment of the flavin cofactor happens either by forming a bond via the 8α-position or via the 6-position of the isoalloxazine ring system [5]. The covalent bond can either be formed to a histidine (via the 8α-position), cystein (via the 8α- or the 6-position) or to a tyrosin (via the 8α-position) side chain.

In some cases the cofactor is bicovalently attached. The first structural characterization of a bi-covalently linked flavoprotein was performed in

2005 with the gluccooligosaccaride oxidase (GOOX) [9]. The biocovalent attachment is achieved by binding of the amino acid side chain via the 8α-position and via the 6-position of the isoalloxazine ring. Biocovalent attachment is suggested to be beneficial for the stability of the protein, for the saturation of the active side, for the prevention of unwanted modifications of the flavin, for the oxygen activity and for balancing the flavin redox potential. It also might facilitate the transfer of electrons [10].

4.2 Berberine bridge enzyme

One example for a well characterized bicovalently linked flavoprotein is the berberine bridge enzyme (BBE) from California poppy (*Eschscholzia california*). BBE catalyses the oxidative ring closure from *(S)*-reticuline to *(S)*-scoulerine at the N-methyl group by forming the so called "berberine bridge". During the reaction hydrogen peroxide is released. The reaction marks an important branch point in isoquinoline alkaloid biosynthesis of plants [11] [12]. Recently a flavoprotein from the frustosyl amino acid oxidase (fsqB) from *Aspergillus fumigatus* was found to catalyse a similar reaction although no structural similarity to BBE can be found [13]. The formation of the ring is shown in Figure 2.

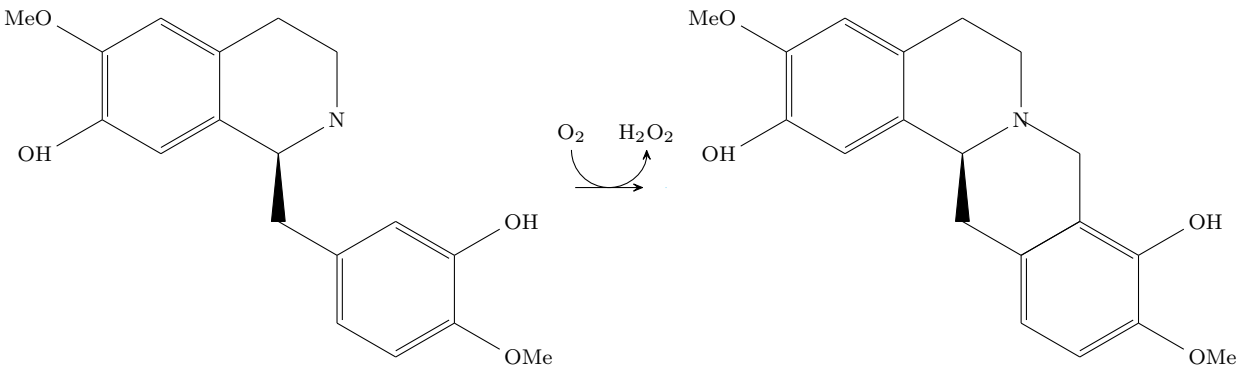


Figure 2: BBE catalyzes the reaction from (s)-reticuline to (S)-scoularine.

4.3 BBE-like enzymes

In recent years in many plant genes which encode for BBE-like enzymes (pfam 08031) have been identified. In the course of their evolution land plants have acquired a significant set of BBE-like proteins. For example in the moss *Physcomitrella patens* oneBBE-like enzyme has been identified whereas in the model plant *Arabidopsis thaliana* 28 BBE-like enzymes have been found. The plant with the highest amount of genes encoding for BBE-like enzymes was found to be *Populus trichocarpa* with 57 BBE-like enzymes [14]. As mentioned in section 4.2 BBE catalyses an important reaction in the alkaloid biosynthesis of plants. In nature plants have been found that have genes for the expression of BBE-like enzymes in their genomes but those plants do not accumulate alkaloids. So the BBE-like enzymes in those plants must have a different metabolic function. The specific functions of most of the BBE-like enzymes today is not know and further investigations have to be done. For this thesis *At*BBE-like 15 from the model plant *Arabidopsis thaliana* was chosen for further studies on the function.

4.4 *At*BBE-like 15

Recent studies by Daniel *et al.* [2] showed that *At*BBE-like 15 is a monolignol dehydrogenase. The overall structure of *At*BBE-like 15 is shown in Figure 3.

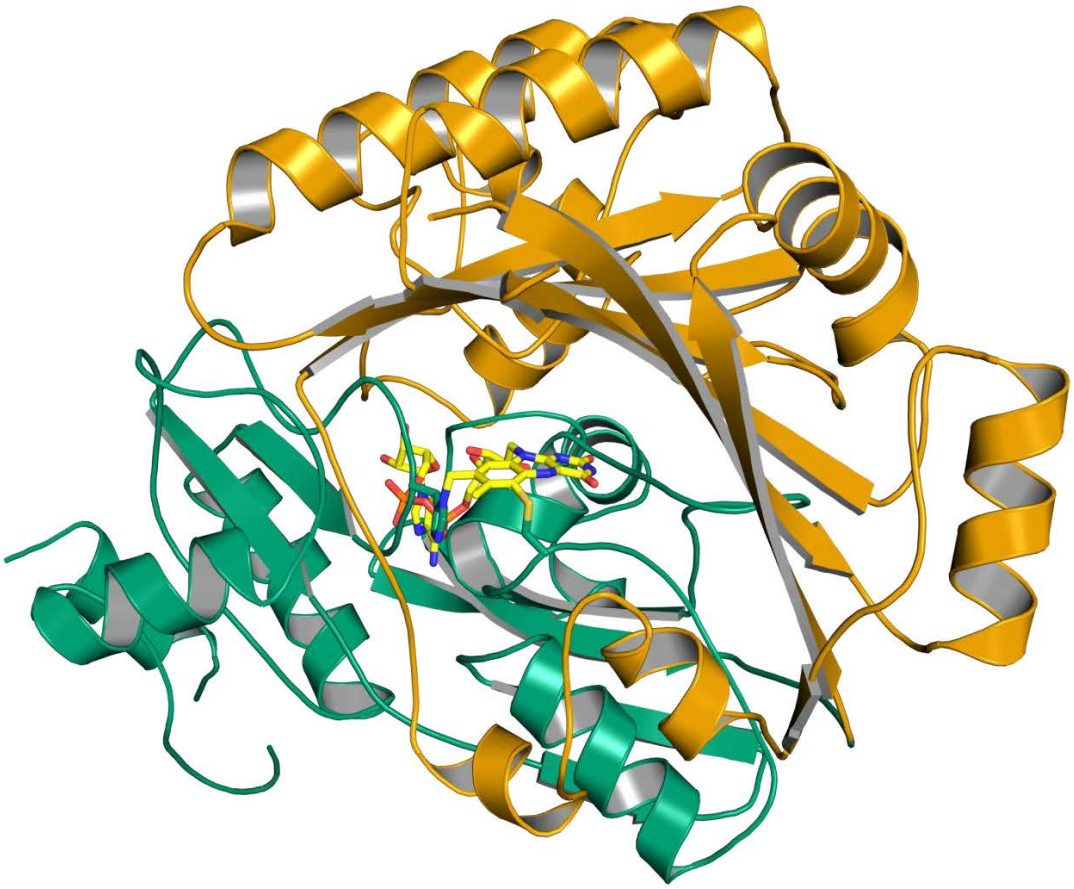


Figure 3: Structure of *At*BBE-like 15 (PDB code: 4UD8). The flavin cofactor is shown in yellow in the middle. The substrate binding domain is shown in yellow whereas the flavin binding domain is shown in green.

In the middle of the figure the flavin cofactor is shown in stick presentation in yellow. The enzymes consists of a substrate binding domain shown in orange and a flavin binding domain shown in green [2].

The enzyme is able to oxidize coniferyl alcohol, p-coumaryl alcohol and sinapyl alcohol to their corresponding aldehydes. Those three alcohols are the building blocks of the lignin polymer. Due to that fact it was suggest that *At*BBE-like 15 plays an important role in the lignin biosynthesis of plants. Lignin is found in the cell walls of plants. It gives the plants strength and rigidity and form a barrier to prevent the plant from microbial attack. Lignin also plays a role in the transport of water and nutrients

through the plants [15].

The structure of p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol are shown in Figure 4. They can be distinguished in the number of methoxy groups they possess.

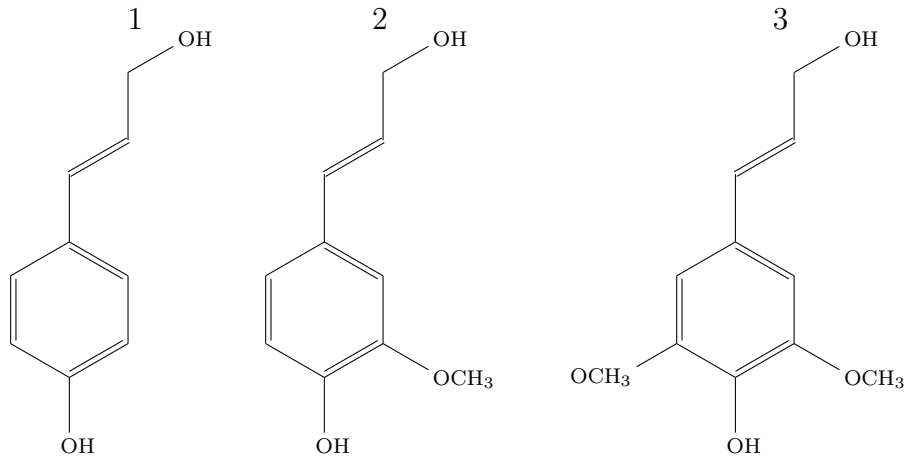


Figure 4: 1: p-coumaryl alcohol, 2: coniferyl alcohol, 3: sinapyl alcohol

As *At*BBE-like 15 reacts sluggishly with oxygen it is defined as an monolignol dehydrogenase [2]. In Figure 5 the suggested reaction mechanism for the oxidation reaction performed by *At*BBE-like 15 by Daniel *et al.* [2] is shown.

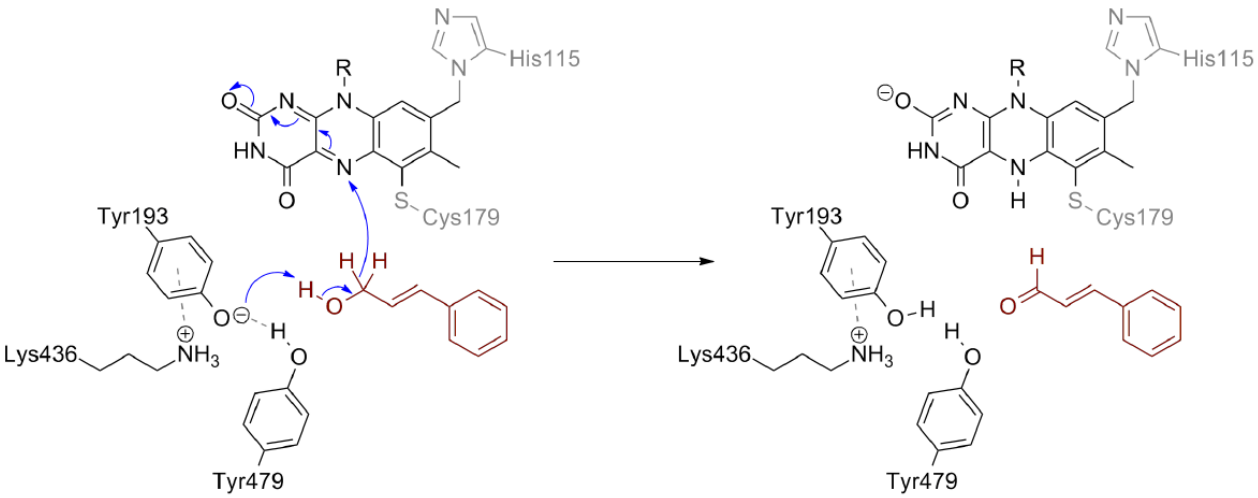


Figure 5: Reaction mechanism of AtBBE-like 15

In the upper part of the figure the flavin cofactor is shown. It is bicovalently bound via His115 and Cys179. The substrate which was chosen to be cinamyl alcohol is shown in dark red. Tyr193, Lys436 and Tyr479 form the catalytic base site. Tyr193 is suggested to abstract a proton from the alcoholic group of the substrate and a hydride transfer to the N5-position of the flavin isoalloxazine ring occurs. The oxidized substrate is released in its aldehyde form. Tyr193 is stabilized in its deprotonated form by Tyr479. Lys436 interacts with the aromatic ring of Tyr193 via a π-interaction [2].

4.5 Engineering *At*BBE-like 15 L182V

*At*BBE-like 15 is a dehydrogenase and the natural electron acceptor for this enzyme is currently not known. This hampers the usage of the enzyme in biocatalysis. Recent research showed that for BBE-like enzymes a single amino acid acts as gatekeeper which controls the reactivity of the enzyme towards oxygen [16]. To overcome the problem of the unknown electron acceptor an enzyme engineering was performed. For *At*BBE-like 15 this gatekeeper is Leu182. It was changed to a valine which is one C-atom smaller than leucine. The alteration with the smaller amino acid makes the whole of the oxygen binding pocket wider and oxygen can now enter the cavity. The enzyme behaves now like an oxidase and reacts more than 400 times faster with oxygen in comparison to the wild type enzyme.

In Table 1 the reactivity towards oxygen for the BBE, for *At*BBE-lik 15 and for the variant which was engineered towards higher oxygen reactivity is shown. Although *At*BBE-like 15 L182V reacts much faster than the wild type enzyme it is still 5 times slower than the berberine bridge enzyme [2].

	k_{OX} [10^4 M^{-1} s^{-1}]
*At*BBE-like 15	0.027
*At*BBE-like 15 L182V	1
*Ec*BBE	5

Table 1: k_{OX}-values for different BBE species

In Figure 6 the oxygen binding pocket of *At*BBE-like 15 is shown in two different views. The pocket is located in the FAD-binding domain and is analogous to the oxyanion hole of serine proteases.

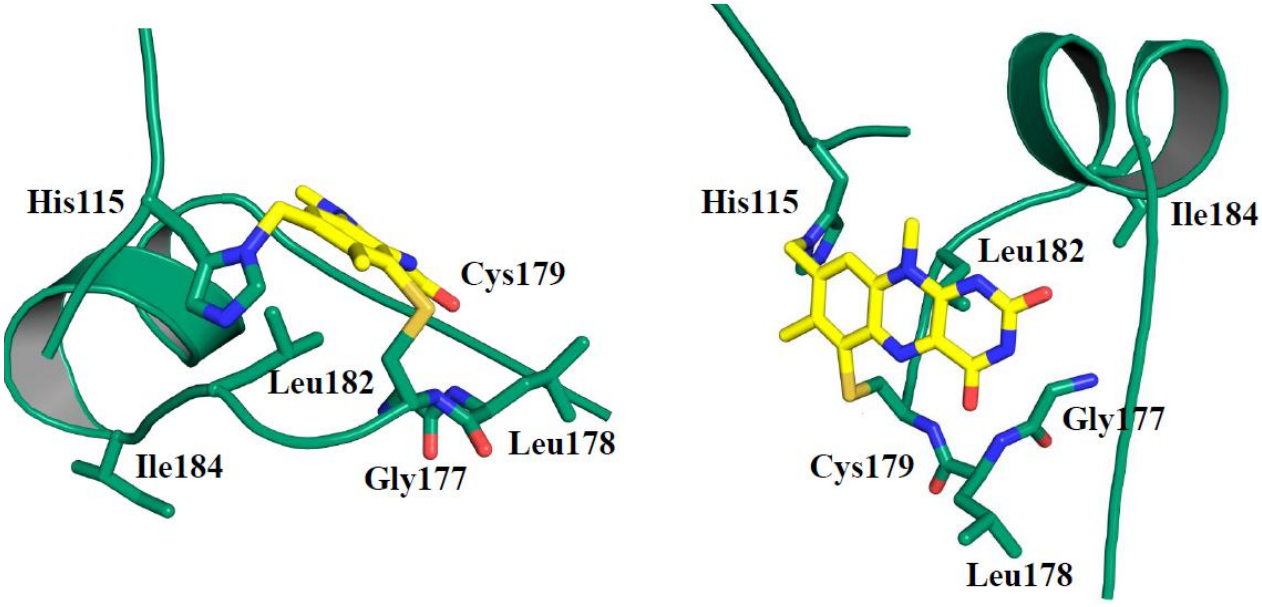


Figure 6: AtBBE-like 15 L182V Oxygen binding pocket

His115 and Cys179 are responsible for the bicovalent attachment of the flavin cofactor which is shown in yellow. The oxygen is complexed in the pocket by two amino acids, Leu178 and Cys179. In this position the oxygen can interact with the reduced flavin. A C(4a)-hydroperoxyflavin is formed that continuously spends hydrogen peroxide to yield the flavin in its oxidized form. Leu182 was found the be the single gatekeeper residue for the reactivity towards oxygen. It points into the oxygen binding pocket and controls the entering of the oxygen molecule. For the oxidase the leucine

is altered to a valine which enables the oxygen to bind in the pocket and further to improve the reactivity of the reduced flavin with oxygen. Ile184 is also suggested to play a role in the formation of the oxygen binding pocket but its special function is currently unknown [16] [2].

4.6 Aim of this thesis

The aim of this master thesis was to characterize the performance of the engineered variant *At*BBE-like 15 L182V to determine the applicability of the enzyme as a biocatalyst. Therefore the influence of important process parameters on the enzyme were tested.

At first the pH-optimum and the temperature optimum of the enzyme were determined.

Most of the substrates used in biotechnological processes are highly unpolar and have to be solved in unpolar organic solvents. Hence it is important for an industrial application of an enzyme to show adequate stability and activity in organic solvents. For this reason the enzyme was tested towards a set of different solvents. With the solvents in which the enzyme showed an increased activity further experiments were conducted. Test series with increasing solvent concentrations were performed.

Due to the fact that an industrial enzymatic reaction is often run over a few hours it is important that the enzyme shows stability and activity throughout the whole implementation [17]. For that *At*BBE-like 15 L182V was tested for its long-term stability in organic solvents.

Following *At*BBE-like 15 L182V was screened towards various substrate classes to get an overview about the substrate range of the enzyme. Primary and secondary allylic alcohols, primary and secondary benzylic alcohols, aliphatic alcohols and amines were tested. The obtained products

were identified with GC-MS analysis.

At last the enantioselectivity of the enzyme was determined. Enzymes which are able to convert a demanded substrate enantiselectively are very useful because often only for one enatiomer a specific function can be found. A separation of the enantiomers after the bioreaction is most of the time and cost expensive. For the determination of the enantioselecivity four chiral substrates were tested.

5 Materials and methodes

All chemicals used for the following procedures were acquired from Sigma Aldrich or Merck. All solvents were acquired from Roth.

5.1 pH optimum

The pH optimum of the enzyme was determined via the consumption of oxygen during the reaction of the enzyme. Therefore an optical oxygen meter FireSting O_2 (Pyro Science GmbH, Aachen, Germany) combined with a retractable needle-type oxygen sensor (Pyro Science GmbH, Aachen, Germany) were used.

Before starting the measurements the electrode was calibrated via a two-point calibration employing air saturated buffer and a saturated sodium sulfide solution. As for the identification of the pH optimum a variety of buffers was used an own calibration for the application in each buffer has to be done. All buffers had a concentration of 50 mM. In Table 2 the buffers tested are listed.

pH 4	acetate buffer
pH 5	acetate buffer
pH 6	MES
pH 7	MES
pH 8	TRIS/HCl
pH 9	TRIS/HCl
pH 10	TRIS/HCl

Table 2: Buffers employed for the determination of the pH optimum of *At*BBE-like 15 L182V

The reaction was performed in a measuring cell with an integrated magnetic stirrer in triplicate at 25°C and 500 rpm. The total reaction volume was 600 μL. The substrate used for the reactions was sinapyl alcohol with

an concentration of 0.2 mM in the measuring cell. *At*BBE-like 15 L182V had a concentration of 1.05 μM. A master mix of the enzyme containing buffer was made. 575 μL buffer containing the enzyme were equilibrated in the measuring cell. Then the calibrated oxygen electrode was placed in the solution. The solution was stirred until a stable oxygen level was reached. The recording of the oxygen level was started and at last 25 μL substrate solution were added with a Hamilton syringe. The reaction was recorded over 300 seconds. The consumption of the oxygen reflects the conversion of the substrate during the reaction.

5.2 Temperature optimum

Due to the fact that sinapyl alcohol is not stable at high temperatures coniferyl alcohol was used for the assay. The temperature optimum of the enzyme was identified based on the absorption of the resulting product coniferyl aldehyde at around 402 nm. The absorption was measured via NanoDrop 2000 spectrometer (Eppendorf, Hamburg, Germany). As someone can see from the previous experiment in section 5.1 the enzyme is most active at pH 7. So for the measurement of the temperature optimum MES buffer pH 7 50 mM was used.

The extinction coefficient ε of coniferyl aldehyde was determined. Therefore the absorption maximum of a 0.125 mM coniferyl aldehyde solution in TRIS/HCl buffer pH 11 was measured via NanoDrop 2000 spectrometer (Eppendorf, Hamburg, Germany). The absorption maximum and the concentration of the coniferyl aldehyde are then use to calculate the extinction coefficient ε with the Lambert Beer law.

The reaction was performed at 20, 25, 30, 40, 50, 60 and 70°C. Before starting the reaction ten plastic reaction tubes each containing 10 μL 1 M TRIS/HCl buffer pH 11 were provided. The reaction was performed in 1.5 mL plastic reaction tubes on a Thermomixer (Eppendorf, Hamburg,

Germany). 500 μL 50 mM MES buffer pH 7 containing 0.2 μM *At*BBE-like 15 were preheated for 10 min at 500 rpm to reach the required reaction temperature. A 2 μL sample was taken and measured on the NanoDrop to get a blank value of the reaction. Then the reaction was started by adding 100 μL coniferyl alcohol to reach a final concentration of 3.33 mM. The reaction was followed over 10 minutes whereby every 60 seconds a sample was taken. Therefore 20 μL of the reaction solution were mixed with 10 μL 1M TRIS/HCl buffer pH 11 to stop the reaction. In 2 μL of this mixture the coniferyl aldehyde concentration was determined immediately and by plotting the absorption at 402 nm against the time the reaction velocity was calculated.

5.3 Enzyme activity in the presence of 10% (v/v) of organic co-solvent

The enzyme activity in the presence of different buffers employing the analytics described in section 5.2 with light modifications. At first the extinction coefficient of coniferyl aldehyde in various organic solvents was determined to see if there are any differences in comparison to the coefficient in pure buffer. Following organic solvents were tested:

- methanol
- DMF
- acetone
- acetonitril
- 2-propanol
- 1,4-dioxane
- THF
- 1-butanol
- DMSO
- ethanol

The total volume of the measuring cell was 600 μL. To achieve an end concentration of 10% organic solvent in the sample 440 μL 50 mM MES buffer pH 7 were mixed with 60 μL of the solvent. To this solution 100 μL 2.5 mM coniferyl aldehyde were added. The samples were mixed on the thermocycler. Then 2μL of each sample were measured on the NanoDrop. The absorption and the concentration of the coniferyl aldehyde were used to calculate the extinction coefficient for coniferyl aldehyde in the solvents.

As for the temperature optimum the enzyme stability in organic solvent was identified by measuring the absorption of the product coniferyl aldeyhde at 402 nm. The reaction was performed in an a 1.5 mL plastic reaction tube on a Thermomixer (Eppendorf, Hamburg, Germany) at 25°C and 500 rpm. 60 μL of the organic solvent in 240 μL buffer were provided. *At*BBE-like 15 L182V was dissolved in buffer. Therefore per 3 mL 50 mM MES buffer pH 7 20 μL of the enzyme were added. Then 200 μL of the enzyme stock solution were added to the provided solvent solution in the 1.5 mL plastic reaction tube on the thermocycler to reach an enzyme concentration of 0.28 μM. The reaction was started by adding 100 μL of coniferyl alcohol to reach a final concentration of 3.33 mM. After every 60 seconds a 20 μL sample was taken and mixed with 10 μL of 1M Tris buffer pH 11 like it has been done for the identification of the temperature optimum. Thereof 2 μL were measured with the NanoDrop spectrometer. The activity of *At*BBE-like 15 L182V found in buffer was set to 100%. The activity of *At*BBE-like 15 L182V found in the different organic solvents were compared with the activity of the enzyme in buffer.

5.4 Enzyme activity in the presence of 10–50% (v/v) organic co-solvent

The experimental set up was conducted as described in section 5.3 in the presence of 10-50% (v/v) co-solvent. For the experiment solvents were chosen in which the enzyme showed an higher activity than in buffer (ethanol,

2-propanol, 1-butanal, acetonitril, THF, DMSO and 1,4-dioxane). The only difference was the amount of organic solvent used for the reaction. Depending on the percentage of solvent required different solvent volumes in MES buffer 50 mM pH 7 were provided:

- 20% solvent: 120 μL solvent in 180 μL buffer
- 30% solvent: 180 μL solvent in 120 μL buffer
- 40% solvent: 240 μL solvent in 60 μL buffer
- 50% solvent: 300 μL solvent

To those mixtures 200 μL enzyme stock solution were added and the reaction was started with 100 μL coniferyl alcohol to reach a concentration of 3.33 mM.

5.5 Long-term stability of the enzyme in the presence of 30% (v/v) organic co-solvent

The experiment in the presence of 30% (v/v) co-solvent was set up as described in section 5.4 though for each solvent two preparations were provided. Following to one preparation 100 μL 20 mM coniferyl alcohol were added to start the reaction and the reaction was monitored with NanoDrop (Eppendorf, Hamburg, Germany). To the second preparation of each solvent no substrate was added to see if after a longer incubation of the enzyme with the solvent still an activity can be found. The sample was incubated for 24 h at 25°C and 500 rpm on a Thermocycler. After 24 hours 100 μL 20 mM coniferyl alcohol were added before measuring the absorption over 10 minutes.

5.6 Substrate screening

The reactivity of *At*BBE15-like L182V towards different substrates was tested. Substrates with different alcoholic groups were tested. As the retraceable needle – type sensor, which was used for the identification of the

temperature optimum, is not stable in organic solvent an optical oxygen meter FireSting O_2 (Pyro Science GmbH, Aachen, Germany) equipped with a solvent resistant oxygen probe (OXSOLV, Pyro Science GmbH, Aachen, Germany) was used.

The electrode is calibrated by a two point calibration. The first point of calibration "% air" is measured in ambient air. Therefore it is important that the electrode is absolutely dry. The second point "0% O2" is measured in a saturated sodium sulphite solution in water.

The substrates were dissolved in a concentration of 50 mM in absolute DMSO. 200 μL of the substrate solution were mixture with 800 μL buffer to achieve a final concentration of 10 mM substrate and 20% DMSO. The buffer which was used was 50 mM MES buffer pH 7. The preparations were incubated for 3 hours at 25°C and 500 rpm in a 1.5 mL reaction tube in a Thermomixer (Eppendorf, Hamburg, Germany) prior to the addition of the enzyme. The lid of the plastic reaction tube was let open to achieve an equilibrium of oxygen in the reaction solution to have a stable oxygen concentration for the following measurement with the oxygen probe. The reaction was started by the addition of 5 μL enzyme to achieve a final concentration of 0.6 μM. The course of the reaction was followed over 300 seconds by the by measuring the decreasing oxygen partial pressure within the solution. To get an overview about the substrate acceptance of *At*BBE-like 15 L182V representatives of different substance classes were screened: p-coumaryl alcohol, 4-phenyl-3-buten-2-ol, benzyl alcohol, piperonyl alcohol, 2-cyclohexen-1-ol, 3-octen-2-ol, 3-penten-2-ol, trans-2-hexen-1-ol, cis-2-hexen-1-ol and cis/trans-crotyl alcohol, 1-phenylethanol, 3-phenyl-1-propanol and geranylamine.

5.7 Product identification

For the substances which were mentioned in section 5.6 the product of the reaction was determined using GC-MS analysis with an authentic standard. Beside the substrates mentioned 3-phenylprop-2-yn-1-ol and (Z)-2-fluor-3-phenyl-prop-2-en-1-ol were identified as substrates for *At*BBE-like 15 L182V by GC-MS measurements. The reactions were carried out in 10 mL glass reaction tubes with screw caps to avoid evaporation of the DMSO during the reaction and to prevent the diffusion of substances out and also into the plastic layer of the plastic reaction tube.

For the GS-MS analysis as well as for the measurements with the oxygen probe the unpolar alcoholic substrates were first dissolved in pure DMSO. 400 μL substrate solution were then mixed with 1590 μL 50 mM MES buffer pH 7. The total volume of each preparation was 2 mL. To each preparation 10 μL *At*BBE-like 15 L182V were added to reach a final concentration of 50 mM substrate and 1.25 μM *At*BBE-like 15 L182V. The samples were mixed for 24 hours at 30°C on an orbital shaker. After 24 hours all samples except the one with crotylalcohol were extracted with ethyl acetate. For the samples containing crotylalcohol the extraction was done with dietyhlether. The products were extracted two times with 500 μL solvent. The extraction phase was dried over potassium sulfate. The potassium sulfate was separated with a table top centrifuge. The samples were measured with GC-MS analyses.

For every substrate a control experiment without enzyme was performed. Additionally an authentic standard was used as reference.

All GC-MS analyses were carried out on an Agilent 7890A GC system. An Agilent J&W HP-5ms capillary column (30 m x 0.25 mm x 0.25 μm; stationary phase: bonded & cross-linked 5%-phenyl-methylpolysiloxane) was used which was coupled with an Agilent 5975C mass-selective detector

(electron impact ionistaion, 70 eV; quadrupole mass selection). Helium was used as carrier gas.

In Table 3 the three different GS-MS methods used for the substrate screening are shown.

	Method M1	Method M2	Method M3
Inlet temp.	250°C	250°C	250°C
Split ration	90:1	90:1	90:1
Injection volume	1 µL	1 µL	1 µL
Column flow rate	0.7mL/min	0.7mL/min	0.7mL/min
Oven program	100°C for 0.5min	40°C for 2min	40°C for 5min
	10°C/min to 300°C	10°C/min to 180°C	5°C/min to 100°C
		180°C for 1min	25°C/min to 200°C
			200°C for 1min
MS transfer line temp.	300°C	300°C	300°C
MS source temp.	230°C	230°C	230°C
MS quadropole temp.	150°C	150°C	150°C
MS scan range	m/z = 33-400	m/z = 33-400	m/z = 33-400

Table 3: GC-MS methods

The employed method for each screened substrate is shown in Table 4.

substrate	method
p-coumaryl alcohol	M1
piperonyl alcohol	M2
benzyl alcohol	M2
cinnamyl alcohol	M2
trans-2-hexen-1-ol	M2
cis-2-hexen-1-ol	M2
cis/trans crotyl alcohol	M3
(Z)-2-fluoro-3-phenyl-pro-2-en-1-ol	M1
3-phenylprop-2-yn-1-ol	M1
1-phenylethanol	M1
3-phenyl-1-propanol	M1
geranylamine	M2

Table 4: Employed methods for product identification

The products were identified according to their retention times which are summarized in Table 5.

substrate	RT substrate	RT bioreaction	RT standard
p-coumaryl alcohol			
piperonyl alcohol	14.4	13.8	13.9
benzyl alcohol	3.4	7.5	8.0
cinnamyl alcohol	13.4	12.9	12.9
trans-2-hexen-1-ol	6.3	6.0	6.0
cis-2-hexen-1-ol	6.3	6.0	-
cis/trans-crotyl alcohol	3.2	3.0	3.0
(Z)-2-fluoro-3-phenyl-prop-2-en-1-ol	6.2	5.7	-
3-phenylprop-2-yn-1-ol	6.2	5.1	5.1
1-phenylethanol	3.6	3.6	-
3-phenyl-1-propanol	12.3	12.3	11.3
geranylamine	12.3	12.3	12.8

Table 5: Retention times non-chiral GC-MS

5.8 Kinetic resolution

The kinetic resolution of the four chiral substrates 3-Penten-2-ol, 4-Phenyl-3-buten-2-ol, 3-Octen-2-ol and 2-Cyclohexenol was estimated with GC - FID analyses. Therefore the sample preparation and the extraction was done equal as for the substrate identification in 20% DMSO and with an enzyme concentration of 1.25 μM. Except of 3-Penten-2-ol were diethyl ether was used as extracting agent ethylacetate was used for the extraction.

Achiral GC-FID analyses were carried out on an Agilent 7890A GC system with an Agilent J&W DB-1701 capillary column (30 m x 0.25 mm x 0.25 μm; stationary phase: bonded & cross-linked 14%-cyanopropylphenyl-methylpolysiloxane). Helium was used as carrier gas. The exact method is shown in Table 6.

Inlet temperature	220°C
Split ration	50:1
Injection volume	1 µL
Column flow rate	1mL/min
Oven program	60°C for 1min
	10°C/min to 280°C
Detector temperature	300°C

Table 6: Method for achiral GC-FID analyses

The chiral GC-FID analyses for 3-Penten-2-ol, 3-Octen-2-ol and 2-Cyclohexen-1-ol were carried out on an Agilent 7890A system equipped with a Varian CP-Dex capillary column (25 m x 0.32 mm x 0.25 μm; stationary phase: β-cyclodextrin bonded to dimethylpolysiloxane). Hydrogen was used as carrier gas. The specific methods which were used for the ciral GC-FID analyses are shown in Table 7.

	3-Penten-2-ol	3-Octen-2-ol; 2-Cyclohexen-1-ol
Inlet temperature	220°C	220°C
Split ratio	90:1	90:1
Injection volume	1 µL	1 µL
Column flow rate	1mL/min	1mL/min
Oven program	60°C for 1 min	60°C for 1 min
	20 °/min to 70 °C	20 °/min to 95 °C
	70°C for 5 min	95°C for 9 min
	20 °/min to 180 °C	20 °/min to 180 °C
	180°C for 1 min	180°C for 1 min
Detector temperature	250°C	250°C

Table 7: Chiral GC-FID methods for 3-Penten-2-ol, 2-Cyclohexen-1-ol and 3-Octen-2-ol

4-Phenyl-3-buten-2-ol was converted into the corresponding acetate derivative prior to chiral-phase GC analysis by reaction with acetic anhydride (20 μL/mL sample) and DMAP (4-dimethylaminopyridine; 1 mg/mL sample)

at room temperature for 3 h.

Chiral GC-FID analyses for the resulting 4-Phenyl-3-buten-2-yl were carried out on an Agilent 7890A GC system equipped with a Restek-βDEXse capillary column (25 m x 0.32 mm x 0.25 μm; stationary phase: 2,3-di-O-ethyl-6-O-tert butyldimethylsilyl-β-cyclodextrin added into 14%-cyanopropylphenyl/86% dimethylpolysiloxane). Hydrogen was used as carrier gas. The exact methode is shown in Table 8.

	4-Phenyl-3-penten-2-ol
Inlet temperature	220°C
Split ration	50:1
Injection volume	1 µL
Column flow rate	2mL/min
Oven program	60°C for 1min
	5 °/ min to 180 °C
Detector temperature	250 °C

Table 8: Chiral GC-FID method for 4-Phenyl-3-penten-2-ol

The retention times which were observed for the chiral substrates are shown in Table 9.

substrate	RT alcohol 1	RT alcohol 2	RT ketone
3-octen-2-ol	10.2	10.5	9.0
cyclohexen-2-en-1-ol	9.8	10.2	7.6
3-penten-2-ol	5.4	5.6	4.1
4-phenylbut-3-en-2-ol	21.7	22.0	19.3

Table 9: Retention times for chiral GC-FID

6 Results and discussion

6.1 pH optimum

The pH optimum was determined using 0.2 mM sinapyl alcohol and 1.05 μM *At*BBE-like 15 L182V. During the reaction the enzyme consumes oxygen. This consumption of oxygen was recorded throughout the first 300 seconds of the reaction. The measured activities for each pH value are shown in Figure 7. The value on the y-axis equals the initial rate in mol product per mole enzymes per second.

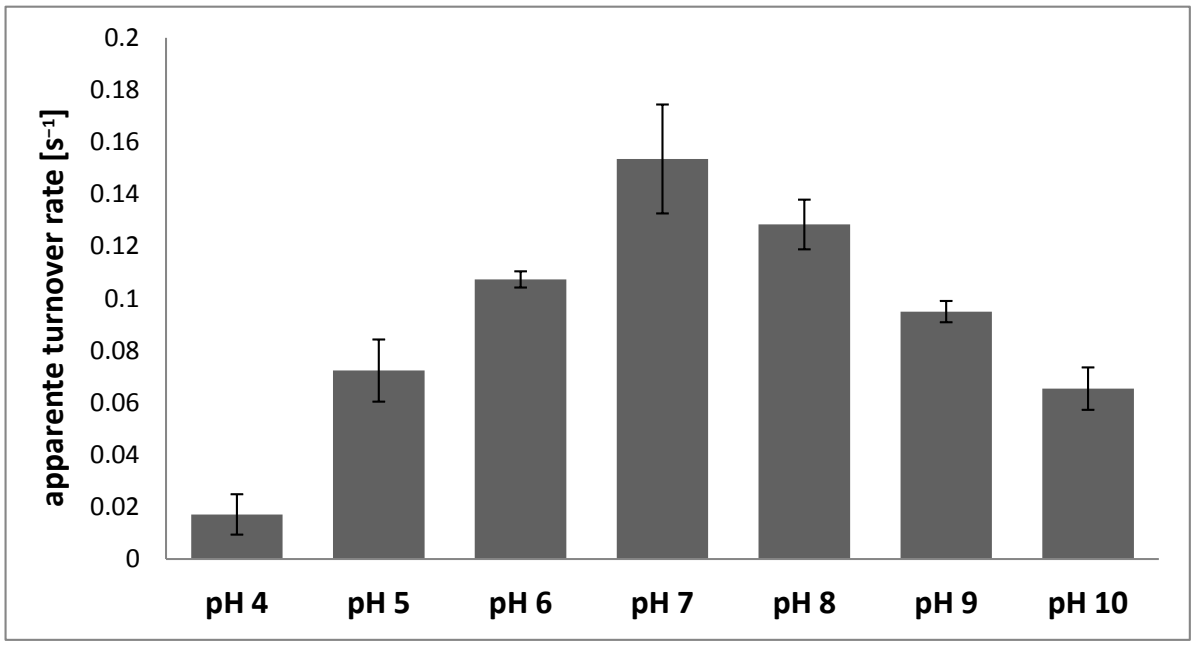


Figure 7: pH optimum of AtBBE-like 15 L182V

The enzyme remains active over a broad pH range. The temperature were the highest activity for *At*BBE-like 15 L182V was obtained was at at pH 7. Except for pH 4, for every pH value an activity of at least 50% or even more than 50% could be obtained.

6.2 Temperature optimum

The temperature optimum of the enzyme was determined using 0.2 μM *At*BBE-like 15 L182V. As substrate coniferyl alcohol with a concentration of 3.33 mM was used. The course of the reaction was followed spectrophotometrically. The measured activities for each temperature are shown in Figure 8.

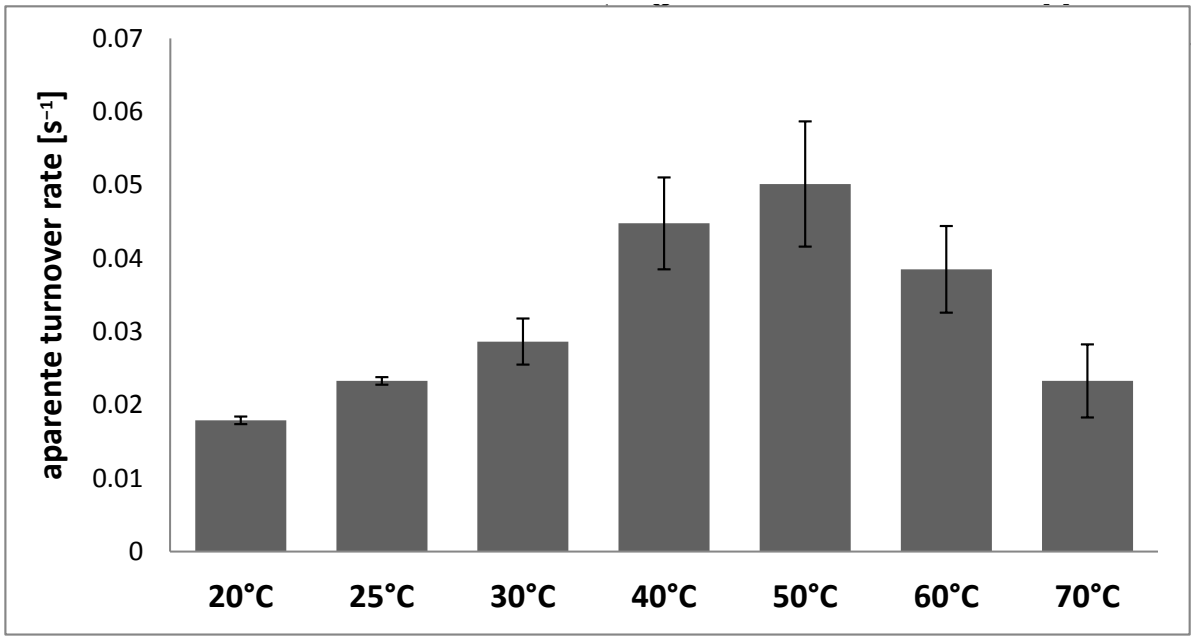


Figure 8: Temperature optimum of AtBBE-like 15 L182V

The reaction velocities are given equals one mole product per mole enzyme per second. The temperature with the highest activity of AtBBE-like 15 L182V was 50°C. This result is in agreement with previous research, where for the wild type enzyme the melting point was found to be at 56°C.

6.3 Activity in the presence of 10% (v/v) co-solvent

In nature enzymes are always found in aqueous systems. Industrial used substrates are often unpolar and their solubility is limited in aquas environment. This problem can be overcome by the addition of co-solvents to the reaction solution. By the addition of co-solvents to the buffer not only the substrate solubility can be increased it can also lead to an increased oxygen solubility. As *At*BBE-like 15 L182V is an oxidase and oxygen is the electron acceptor the solubility of oxygen in the reaction solution is an important factor [18] [19]. To get information on solvents effects to the enzyme the activity in the presence of 10% (v/v) co-solvent was measured. The results are summarized in Figure 9.

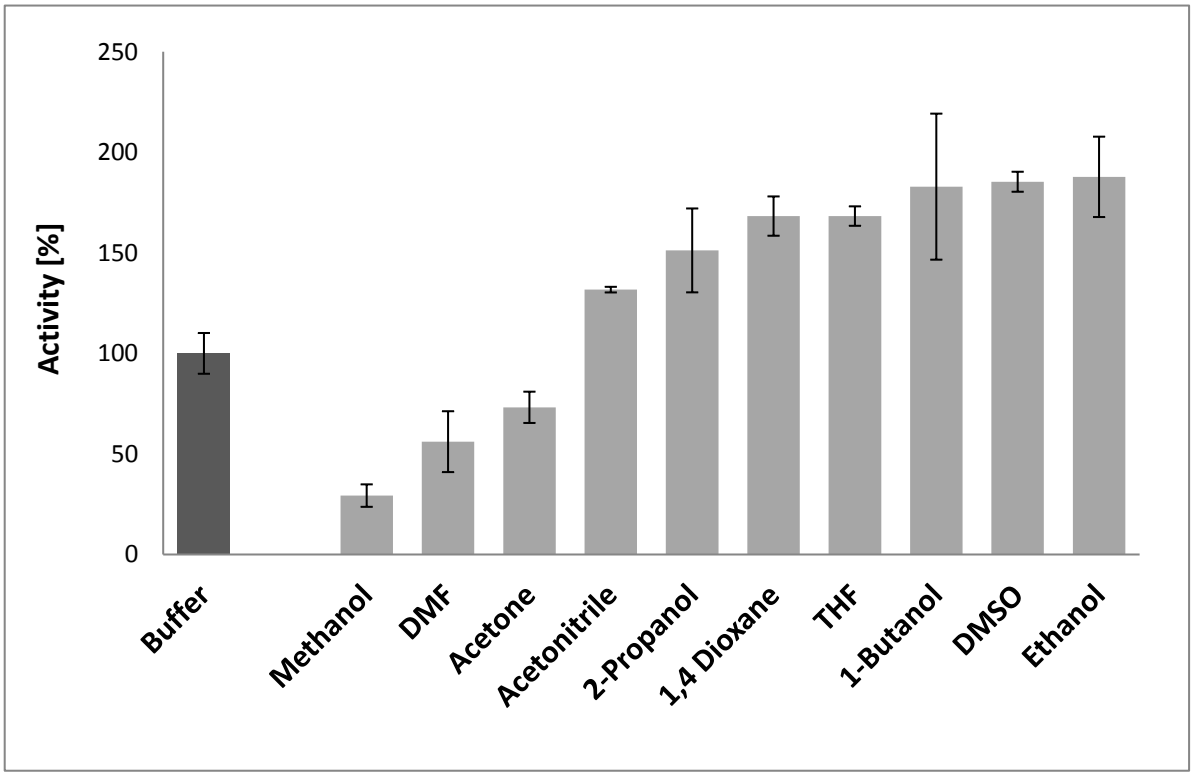


Figure 9: Activity in the presence of 10% (v/v) organic solvents of AtBBE-like 15 L182V

The activity in buffer was set to 100%. The enzyme shows high solvent tolerance. For acetonitrile, 2-propanol, 1,4-dioxane, THF, 1-butanol, DMSO and ethanol an increased activity in comparison to the buffer was found. The highest activity was found for ethanol. Here the activity is

increased to 188% in comparison to the buffer. For methanol, DMF and acetone an decrease in activity was found. The lowest activity of all tested co-solvents was found for methanol. Here the activity is only 29% of the activity in buffer.

6.4 Activity in the presence of 10 to 50% (v/v) co-solvent

Due to the fact that the enzyme is tolerant towards various co-solvents further experiments with increased solvent concentrations were performed. In the present of 10% (v/v) acetonitrile, 2-propanol, 1,4-dioxane, THF, 1-butanol, DMSO and ethanol the enzymes shows an increased activity in comparison to the activity in buffer. For that reason only those co-solvents were consulted for spectrometric activity measurements in 10%, 20%, 30%, 40% and 50% co-solvent. The measured activities are shown in Figure 10.

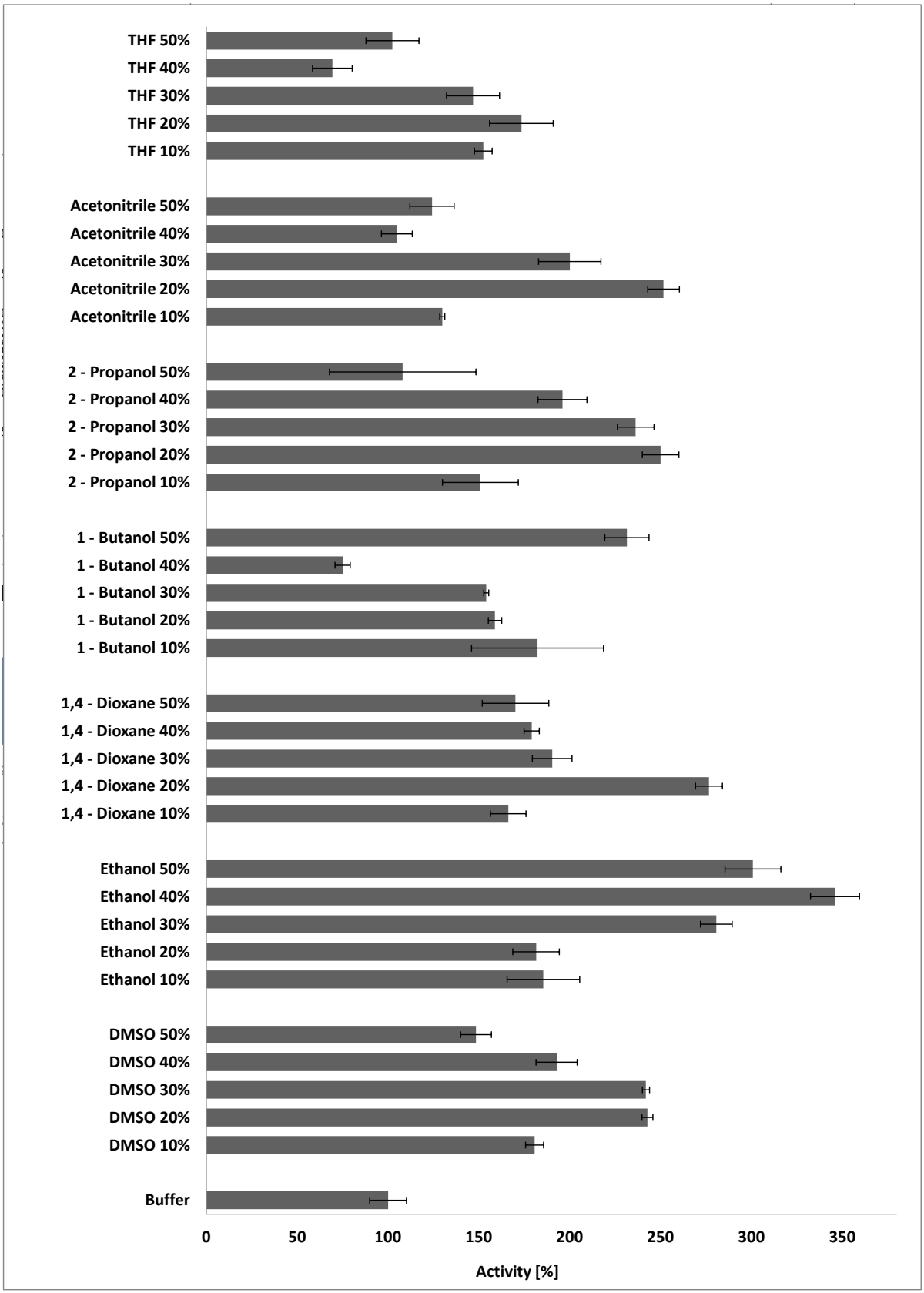


Figure 10: Activity of AtBBE15-like L182V in the presence of 10 to 50% (v/v) co-solvent.

Again the activity in buffer was set to 100% and all the other activi-

ties were compared to this value. Except for THF an higher co-solvent concentration leads to an increase of activity. In 40% ethanol the highest degree of activity was found with 346% of the activity found in buffer. The results show that the enzyme is tolerant to solvents and that a higher solvent concentration can increase the activity of the enzyme. High solvent concentrations can however lead to denaturation and deactivation of the enzyme. Therefore a good long-term stability of the enzyme in the presence of buffer is necessary.

6.5 Long-term stability of AtBBE15 - like L182V in the presence of 30% co-solvent

The long-term stability of the enzyme was determined by incubation the enzyme with 30% (v/v) of the respective co-solvent for 24 hours (t2). Then the reaction was started. In Figure 11 the results for the long-term stability measurement are shown. The activity which was found in buffer after an incubation time of 0 min was set to 100%.

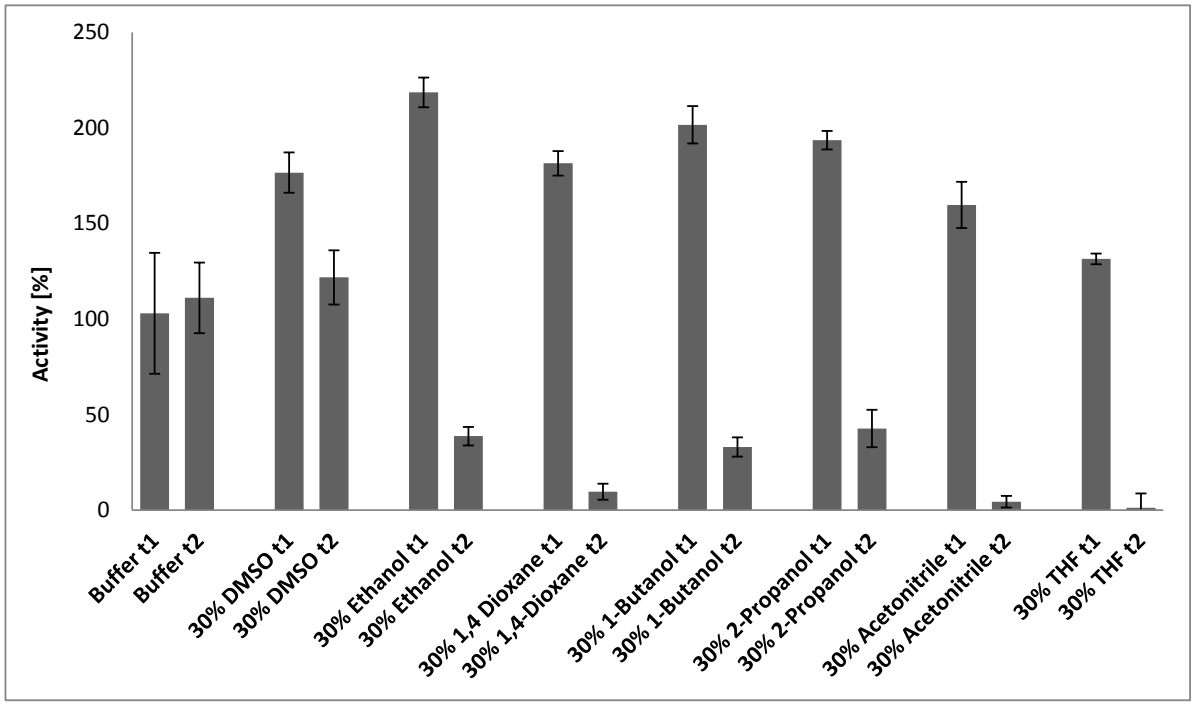


Figure 11: Long-term stability of AtBBE-like 15 L182V with t1 activity after 0 min and t2 activity after 24 h.

The long term incubation of the enzyme in buffer showed no decrease in activity after 24 hours. In all co-solvents the activity of the enzyme was highly decreased after 24 hours. Only for DMSO an acceptable activity could be found after the incubation time. Here the activity after 24h decreased only from 177% to 122% in comparison to the buffer. The biggest loss in activity was found for THF. Here after 1 day no activity could be found. The addition of co-solvents leads on the one hand to an increased activity of the enzyme but on the other hand the life time of the enzyme get shorted.

6.6 Substrate screening

To get information about the substrate scope of *At*BBE-like 15 L182V a screening with different alcohols was performed. Tested were aliphatic, allylic, benyzlic alcohols as well as amines. The enzyme showed the best stability and activity in the presence of DMSO as co-solvent. For that reason the substrate screening was performed in a reaction solution containing 20% (v/v) DMSO. The substrates had a concentration of 10 mM. The reaction was followed by the recording of the oxygen partial pressure in the reaction solution. In Figure 12 only the oxygen consumption rates for substances which were successfully oxidized by *At*BBE-like 15 L182V are shown.

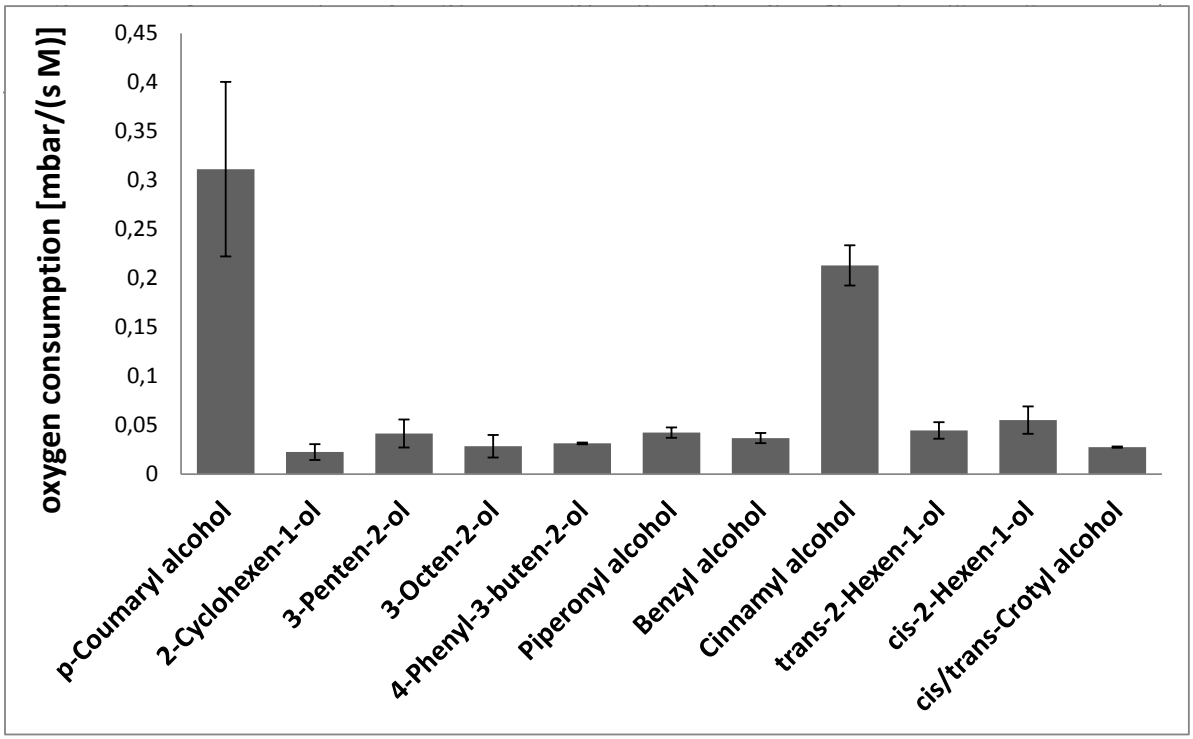


Figure 12: Substrate screening for AtBBE-like 15 L182V

The highest remarkable conversion velocities found were 0.31 mbar/(s M) for p-coumaryl alcohol followed by 0.21 mbar/(s M) for cinamyl alcohol. All the other substances showed slower velocities in a range of 0.023 mbar/(s M) to 0.055 mbar/(s M). 1-phenylethanol, 3-phenyl-1-propanol and geranylamine were not accepted by the enzyme.

In Figure 13 the molecular structures of the substances that are accepted as substrates for *At*BBE-like 15 L182V are shown and in Figure 14 the reaction equation is shown.

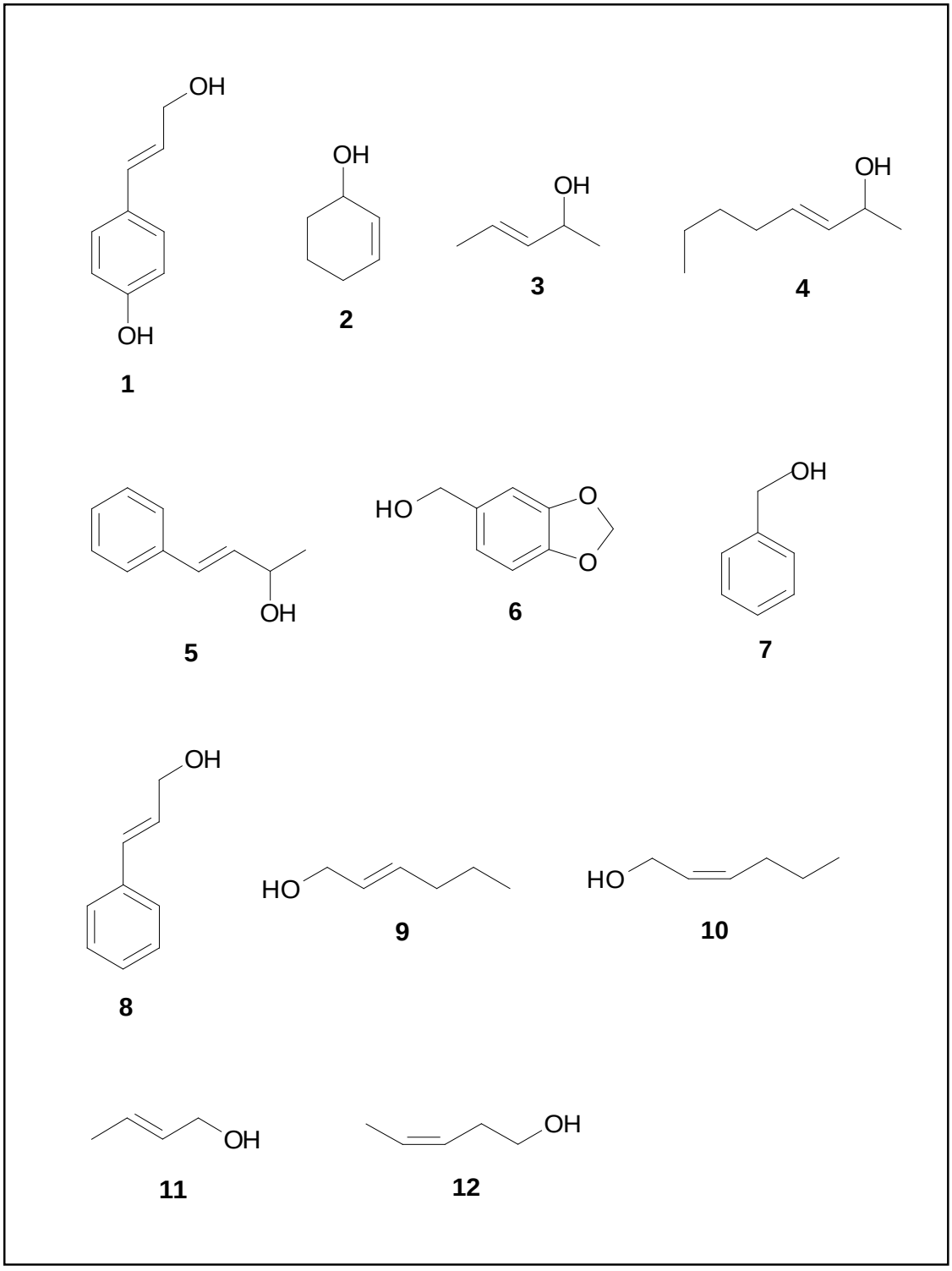


Figure 13: Substrates converted by *At*BBE15-like L182V: **1** p-coumaryl alcohol, **2** 2-cyclohexen-1-ol, **3** 3-penten-2-ol, **4** 3-octen-2-ol, **5** 4-phenyl-3-buten-2-ol, **6** piperonyl alcohol, **7** benzyl alcohol, **8** cinnamyl alcohol, **9** trans-2-hexen-1-ol, **10** cis-trans-2-hexen-1-ol, **11** trans-crotyl alcohol, **12** cis-crotyl alcohol

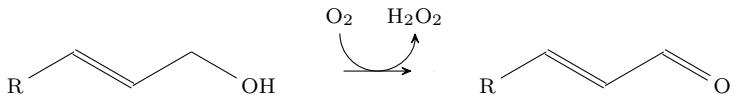


Figure 14: Reaction equation

P-coumaryl alcohol and cinnamyl alcohol showed the highest reactivity in the substrate screening. Both are primary allylic alcohols. 4-phenyl-3-buten-2-ol has the same structure as cinnamyl alcohol but it has an additional methyl group on the alcohol carbon atom. This turns 4-phenyl-3-buten-2-ol into a secondary alcohol and results in a 7 times smaller oxygen consumption rate of 0.031 mbar/(s M). It seems like the additional methyl group of 4-phenyl-3-buten-2-ol due to steric demands does not fit so well into the active site of the enzyme. The two tested benylic alcohols benzyl alcohol and piperonyl alcohol and the tested primary allylic alcohols show oxygen consumption rates in the same range. A benzyl ring beside the alcohol group seems not beneficial for the enzyme.

6.7 Product identification

All alcohols were oxidized to the corresponding aldehydes by *At*BBE-like 15 L182V as confirmed by GC-MS analysis. For the oxidation of primary alcohols by other flavin-dependent oxidases the formation of carboxylic acids have been found. This overoxidation has not been found for *At*BBE-like 15 L182V. In Table 10 the conversions observed by GC-MS are shown.

substrate	conversion [%]
piperonyl alcohol	97
benzyl alcohol	> 99
cinnamyl alcohol	97
trans-2-hexen-1-ol	47
cis-2-hexen-1-ol	28
cis/trans-crotyl alcohol	15
(Z)-2-fluoro-3-phenyl-prop-2-en-1-ol	> 99
3-phenylprop-2-yn-1-ol	19
1-phenylethanol	n.d.
3-phenyl-1-propanol	n.d.
geranylamine	n.d.

Table 10: Substrate conversion determined by GC-MS

To sum everything up the enzyme accepts substrates which own a primary allylic alcohol group like it can be found for p-coumaryl alcohol and cinamyl alcohol but also secondary allylic alcohols like 3-penten-2-ol are accepted. Furthermore primary benzylic alcohols were converted like for example benzylic alcohols but no secondary benzylic alcohol is accepted by the enzyme. For all aliphatic and secondary benzylic alcohols as well as amines no conversion could be detected (n.d.).

6.8 Kinetic resolution

The structure, the *e.e.* value and the E-value for the tested ciral substrates 3-octen-2-ol, cyclo-2-en-1-ol, 3-penten-2-ol and 4-phenylbut-3-en-2-ol are shown in Table 11. The enatioselectivity (E) was calculated according to Rakels *et al.* [20].

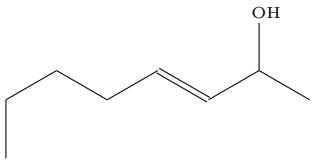
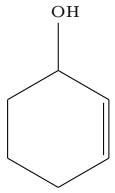
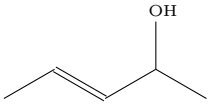
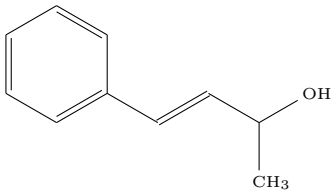
chiral substance	structure	*e.e.*	E-value
3-octen-2-ol		100%	> 47
cyclo-2-en-1-ol		53%	> 200
3-penten-2-ol		20%	> 200
4-phenylbut-3-en-2-ol		100%	> 34

Table 11: Kinetic resolution

For 3-octen-2-ol and 4-phenylbut-3-en-2-ol the kinetic resolution is good. The absolute configuration of the remaining alcohol are currently under investigation.

7 Conclusion

In this thesis the applicability of the BBE-like 15 variant *At*BBE-like 15 L182V for an implementation in biotechnological processes was investigated.

Therefore the pH - and the temperature optimum, the co-solvent tolerance and the long-term stability of the enzyme were tested. Furthermore the substrate scope and the enantioselectivity of the enzyme were determined.

*At*BBE-like 15 L182V was found to be most active at pH 7. The enzyme retains more than 50% of its activity between pH 5 and pH 10. The temperature where the enzyme was found to be most active was found to be at 50°C. The enzymes remains active in various organic solvents. The highest activity which remained even after 24 hours was found in DMSO. This makes the enzyme favourable for processes which are performed in the presence of a co-solvent.

The enzyme was found to convert primary and secondary allylic alcohols to the corresponding aldehyde. Especially for cinnamyl alcohol derivative high reaction velocities were found. Furthermore primary benzylic alohols where accepted by the enzyme. It seems like the steric demand of the benzolic ring is beneficial for the enzyme. Aliphatic alcohols and amines were not converted.

The high enantioselectivity of the enzyme towards secondary chiral alcohols could be proved and was found to be good to excellent [21].

The research shows that *At*BBE-like 15 L182V is active under various conditions. This makes the enzymes a possible useful biocatalyst for industrial applications.

8 Outlook

In this thesis the biocatalytic potential of the variant *At*BBE-like 15 L182V could be shown. It could be demonstrated that the enzyme is sufficiently stable in organic solvents. By immobilizing the enzyme to a solid carrier its solvent tolerance possibly be improved [22].

In oxidation reactions often an overoxidation to the carboxylic acid occurs. This is a problem because no aldehyde can be obtained. *At*BBE-like 15 L182V catalyses the specific oxidation to the aldehyde and the reaction stops at this point. This is important as the enzyme is able to selectively convert benzylic alcohols to the corresponding aldehydes.

A feasible application is to use the enzyme in cascade reactions in combination with VAO (vanillyl-alcohol oxidase). VAO is active with a broad variety of *p*-substituted phenols. Even 4-alkylphenols are accepted by the enzyme. The resulting 4-hydroxycinnamyl alcohols are perfect substrates for *At*BBE-like 15 L182V and can so be further oxidised to the particular aldehydes [23] [24].

Another possible application is to use the enzyme in combination with an enzyme which has cytochrome P450 oxidase activity. Cytochrome P450 oxidase is used to convert toluol into benzyl alcohol via a hydrogen peroxide consuming reaction. Benzylalcohol is than further transformed to benzyl aldehyde with *At*BBE-like 15 L182V. Thereby hydrogen peroxide gets released which can be recycled in the first step of the cascade.

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Characterization of the monolignol oxidoreductase *At*BBE-like protein 15 L182V for biocatalytic applications

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ABSTRACT

Monolignol oxidoreductases from the berberine bridge enzyme-like (BBE-like) protein family (pfam 08031) catalyze the oxidation of monolignols to the corresponding aldehydes. In this report, we explore the potential of a monolignol oxidoreductase from *Arabidopsis thaliana* (*At*BBE-like protein 15) as biocatalyst for oxidative reactions. For this study we employed a variant with enhanced reactivity towards oxygen, which was obtained by a single amino acid exchange (L182V). The pH and temperature optima of the purified *At*BBE-like protein 15 L182V were determined as well as the tolerance toward organic co-solvents; furthermore the substrate scope was characterized. The enzyme has a temperature optimum of 50 °C and retains more than 50% activity between pH 5 and pH 10 within 5 min. The enzyme shows increased activity in the presence of various co-solvents (10–50% v/v), including acetonitrile, 2-propanol, 1,4-dioxane, and dimethyl sulfoxide. Primary benzylic and primary or secondary allylic alcohols were accepted as substrates. The enantioselectivity *E* in the oxidation of secondary alcohols was good to excellent (*E*>34 to >200).

1. Introduction

Flavoproteins are a diverse protein class employing either flavin mononucleotide (FMN) or the flavin adenine dinucleotide (FAD) for catalysis [1,2]. Among them, the BBE-likes (pfam 08031) can be distinguished due to their bicovalent cofactor tethering [3]. The namesake of this protein family is the berberine bridge enzyme (BBE) from California poppy (*Eschscholzia californica*), which catalyzes the oxidative ring closure from (*S*)-reticulin to (*S*)-scoulerine during isoquinoline alkaloid biosynthesis [4,5].

The majority of flavoproteins, including the BBE-likes, catalyze redox reactions, and they are involved in a plethora of biological processes [6]. The catalytically active moiety of the flavin cofactor is the isoalloxazine ring, whose properties are modulated by the protein environment in which it is embedded. The isoalloxazine ring is capable of one or two electron exchange reactions: In addition to the fully reduced (hydroquinone) or oxidized (quinone) state, also stable radical species (semiquinone) can be formed. A comprehensive overview of the function and mechanism of different flavoproteins has been provided by Fagan et al. [6].

The catalytic cycle of a flavoprotein can be divided into two half reactions: In the resting state the flavin cofactor is oxidized. In the reductive half reaction, the flavin is reduced by a given substrate. In the oxidative half reaction, the flavin reacts with an appropriate electron acceptor, thus it becomes reoxidized and is subsequently able to enter another catalytic cycle. The nature of the final electron acceptor is a crucial attribute of flavoproteins. Enzymes that promote the reaction of the flavin cofactor with oxygen are considered to function as oxidases, while enzymes that inhibit the reaction of the reduced flavin with oxygen are defined as dehydrogenases. For BBE-likes it has been shown that a single gatekeeper residue controls the oxygen reactivity of the enzyme [7] (compare Fig. 1 panel A). The rationale behind the engineering of the *At*BBE-like 15 L182V variant is described in [7], creation of the *At*BBE-like variant is published in [8]. We have recently identified two BBE-likes from *Arabidopsis thaliana* as monolignol oxidoreductases (*At*BBE-like protein 13 and *At*BBE-like protein 15) [8]. These enzymes were heterologously expressed in *Komagataella phaffii* (formerly *Pichia*

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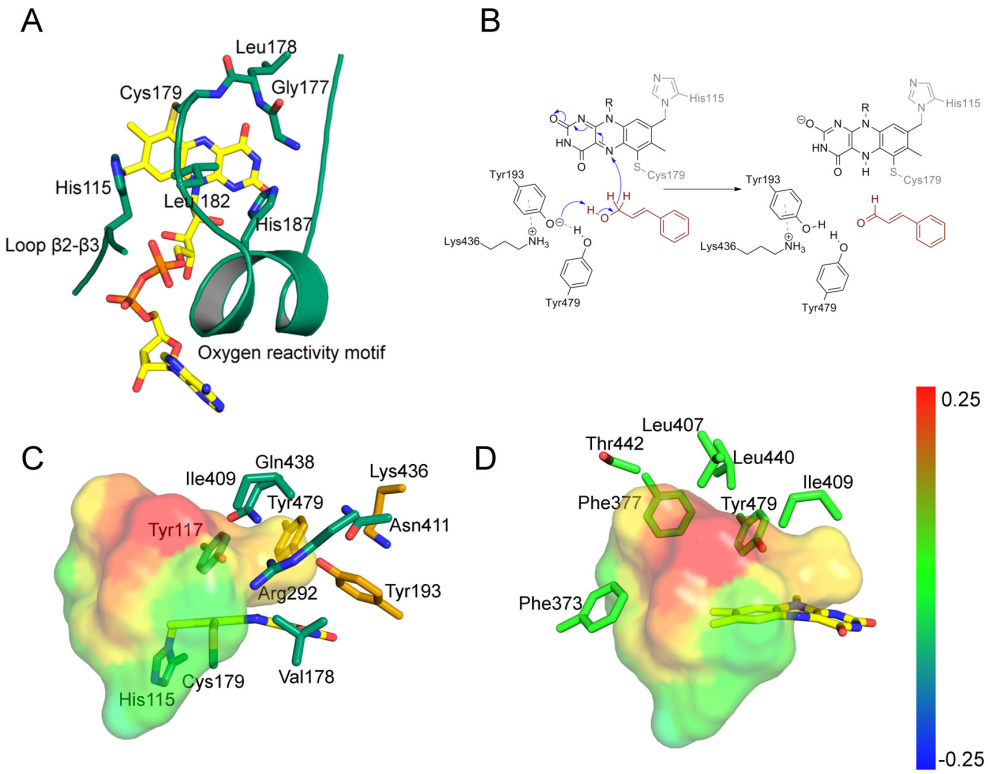

Fig. 1. A: Oxygen reactivity motif of *At*BBE-like protein 15; gate keeper residue Leu182 was changed to valine to turn the enzyme from a dehydrogenase to an oxidase; B: Proposed reaction mechanism; C: Active site and substrate binding pocket colored by hydrophobicity (red: hydrophobic, blue: hydrophilic). Green residues determine the shape of the active site, orange residues also act as a catalytic base. D: Substrate binding pocket; the green residues are responsible for the hydrophobic properties of the substrate binding pocket.

pastoris), and a biochemical and structural characterization has been conducted in our laboratory. Both enzymes were found to oxidize monolignols (*p*-coumaryl **1a**, coniferyl **1b** and sinapyl alcohol **1c**) to the corresponding aldehydes while reacting only sluggishly with molecular oxygen and were hence considered as monolignol dehydrogenases. The physiological electron acceptor for *At*BBE-like proteins 15 and 13 is not known yet; therefore, the sluggish oxidative rate hampers the potential application of *At*BBE-likes as biocatalysts as the turnover rate is determined by the slowest reaction rate. This means that it is virtually impossible to achieve significant turnover rates with a dehydrogenase in the absence of an appropriate electron acceptor. Moreover, while *At*BBE-like 15 was found to be stable and expressed with good yields, expression of *At*BBE-like 13 was very cumbersome and the enzyme was found to be unstable. To overcome these limitations, the oxygen gatekeeper residue in *At*BBE-like protein 15 was changed from leucine to valine to turn the enzyme into an oxidase. The resulting enzyme variant *At*BBE-like protein 15 L182V reacts more than 400 times faster with oxygen compared to the wild type enzyme [8]. This enables the usage of this enzyme for steady state kinetics and the putative application of the enzyme as biocatalyst in oxidative reactions. The structure of the oxygen reactivity motif harboring the variation L182V is shown in Fig. 1A, the proposed reaction mechanism of *At*BBE-like protein 15 is represented in panel B. A representation of an imprint of the active site and the substrate binding site is shown in panel C and D. Figure one is created using the crystal structure of the wild type enzyme (pdb entry: 4UD8).

The structure of the enzyme can be divided in a FAD-binding domain and a substrate binding domain [8]. The oxygen reactivity motif is located in the FAD-binding domain and creates an oxygen pocket that is analogous to the oxyanion hole of serine proteases. In this pocket oxygen is complexed by two backbone amides (compare Fig. 1 panel A: Leu178 and Cys179). From this position the oxygen reacts with the reduced flavin to form a hydroperoxy adduct at the C4a position that will subsequently eliminate hydrogen peroxide to yield oxidized flavin. The accessibility of this pocket is sterically controlled by a single gate keeper residue corresponding to Leu182 in *At*BBE-like 15 (compare Fig. 1, panel A). In order to promote the reaction of the reduced flavin with oxygen oxidases feature a valine in this position, which will allow oxygen to bind in the pocket. In contrast, dehydrogenases sterically block access to the pocket with a larger amino acid side chain and thus prevent the reoxidation of the reduced flavin cofactor [7,9]. The isoalloxazine ring and the active site are located at the interface of both domains. The active site forming residues are shown in Fig. 1C. Tyr 193 is proposed to act as catalytic base. It is stabilized in the deprotonated form by Tyr479 and is positioned by Lys436 via a cation-π interaction (compare Fig. 1B). Tyr193 deprotonates the allylic alcohol to facilitate the hydride transfer to the flavin. The enzyme possesses a wide surface accessible cavity harboring the substrate binding site and the active site [8]. This funnel-like cavity was visualized and analyzed using the CASoX tool (Fig. 1C and D) [10,11]. Residues with a major contribution to the binding site are shown in green in Fig. 1D, also the flavin cofactor shown in yellow contributes to the binding site. In the vicinity of the binding site is the active site. In Fig. 1C in dark green the residues that contribute to the active site are shown. In dark green Tyr117, Gln438, Ile409, Arg292, Val178 and Cys179 are contracting the cavity and thereby form the entrance to the active site. The catalytic base motif (orange residues Fig. 1 panel C), formed by Tyr193, Lys436 and Tyr479, is located opposite to

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this entrance [8]. Asn411 also contributes to the properties of the active site. On the one hand it is involved in the complexation of a water molecule together with Gln438, on the other hand it is a putative binding partner to anchor the monolignols prior to oxidation. His115 and Cys179 are responsible for the bicovalent attachment of the FAD, which confers a very tight steric control of the position of the cofactor to the enzyme (Fig. 1C) [3].

The application of flavin-containing enzymes as oxidative catalysts is highly desirable as they combine the advantage of using molecular oxygen as the most environmentally benign oxidant with the substrate-, regio-, and enantioselectivity of an enzyme [12]. Therefore we initiated a feasibility study to determine the potential of *At*BBE-like protein 15 L182V as a biocatalyst. In particular, the substrate scope, the pH and temperature optima, solvent tolerance, and enantioselectivity were in the focus of our study.

2. Experimental

All chemicals were from Sigma Aldrich or Acros Organics, respectively and were used as received. All solvents were from Roth. NMR spectra were recorded with a Bruker NMR unit at 300 (^{1}H) and 75 (^{13}C) MHz, shifts are given in ppm and coupling constants (*J*) are given in Hz. The *At*BBE-like L182 V was expressed and purified as described previously [8]; one Unit (U) is defined as the amount of enzyme that forms one μmol substrate per minute. The molecular mass of the catalyst is 60 kDa. Catalase from *Micrococcus lysodeikticus* (CAS 9001-05-2) was from Sigma Aldrich.

All GC–MS measurements were carried out with an Agilent 7890A GC system, equipped with an Agilent 5975C mass-selective detector (electron impact, 70 eV), Helium was used as carrier gas at a flow rate of 0.55 mL/min. HPLC analysis was performed with a Shimadzu HPLC system using the chiral stationary phase as indicated. Optical rotation values were measured with a Perkin Elmer Polarimeter 341.

Analytical thin layer chromatography (TLC) was carried out on Merck TLC silica gel 60 F254 aluminium sheets and spots were visualized by UV light (λ = 254 and/or 366 nm) and/or by staining with potassium permanganate (0.3 g $KMnO_4$, 20 g K_2CO_3, 5 mL 5 % aqueous NaOH in 300 mL H_2O) which were ultimately heated for development of the stains. For preparative product purification a 50 to 100 fold excess of silica gel was used with respect to the amount of dry crude product, depending on the separation problem. The dimensions of the column were selected in such a way that the required amount of silica gel formed a pad between 10 cm and 25 cm. The column was equilibrated first with the solvent or solvent mixture, and the crude product diluted with the eluent was applied onto the top of the silica pad.

2.1. Influence of pH

2.1.1. Activity of At*BBE-like 15 L182V at different pH values*

The pH optimum was determined with an optical oxygen meter FireSting O2 (Pyro Science GmbH, Aachen, Germany) equipped with a retractable needle-type oxygen sensor (Pyro Science GmbH, Aachen, Germany). The reaction was performed in a measuring cell with an integrated magnetic stirrer in triplicate at 25 °C and 500 rpm. 575 μL 50 mM MES buffer pH 7 containing 1.05 μM *At*BBE-like 15 L182 V was stirred until a stable oxygen level was reached. The reaction was started by the addition of 25 μL sinapyl alcohol (**1c**) solution to reach a final substrate concentration of 0.2 mM. The reaction was recorded for at least 5 min. After the initial consumption of 30 μM of oxygen data was not taken in account any more. The consumption of oxygen reflects the conversion of the substrate during the reaction.

2.1.2. Melting point of At*BBE-like protein 15 at different pH values*

Thermofluor experiments were performed using a Biorad® CFX Connect Real time PCR system (BioRad, Hercules, CA, USA). The experiments were performed using Sypro® Orange as fluorescent dye in 50 mM MES buffer pH 7.0. The total volume in each well was 25 μL with a protein concentration of 0.4 mg/mL. The starting temperature of 20 °C was kept for 5 min and then the temperature was increased at a rate of 0.5 °C/min to 95 °C. Melting temperatures were determined using the program Biorad CFX Manager 3.0.

2.2. Influence of temperature

2.2.1. Temperature optimum

The temperature optimum of *At*BBE-like 15 L182V was determined spectrophotometrically by measuring the formation of coniferyl aldehyde (**2b**) from coniferyl alcohol (**1b**). The reaction was performed in 1.5 mL plastic reaction tubes in a thermomixer (Eppendorf, Hamburg, Germany) in a temperature range of 20 °C to 70 °C. 500 μL 50 mM MES buffer pH 7 containing 0.2 μM *At*BBE-like 15 L182V was preheated for 10 min at 500 rpm. The reaction was started by the addition of 100 μL coniferyl alcohol (**1b**) solution to reach a final concentration of 3.33 mM. The reaction was followed over 10 min, whereby every 60 s a sample was taken. To this end, 20 μL of the reaction solution was mixed with 10 μL 1 M TRIS-buffer pH 11. 2 μL of this solution was immediately used to determine the product formation with a NanoDrop 2000 spectrometer (Thermo Fisher Scientific, Waltham, USA). The coniferyl aldehyde (**2b**) concentration was measured at 402 nm, the extinction coefficient of 37120 M^{-1} cm^{-1} was determined with an authentic standard under the given conditions.

2.2.2. Long-term stability of At*BBE-like 15 L182 V at different temperatures*

The enzyme was diluted in 50 mM TRIS/HCl buffer pH 8.0 to a final concentration of 0.125 μM. 950 μL was incubated in plastic reaction tubes in a thermomixer (Eppendorf, Hamburg, Germany) at 20 °C, 30 °C, 40 °C, 50 °C, 60 °C, and 70 °C. After 24 h the solutions were cooled to 20 °C and residual enzyme activity was determined employing a Specord 200 Plus spectrophotometer (Analytik Jena, Jena, Germany). The enzyme solution was transferred to a Hellma SUPRASIL® cuvette (Hellma GmbH & Co. KG, Müllheim, Germany) and the reaction was started by the addition of coniferyl alcohol (**1b**) to a final concentration of 0.5 mM. Product formation was followed for 5 min at 20 °C, the extinction coefficient of coniferyl aldehyde (**2b**) under reaction conditions of 21000 cm^{-1} M^{-1} was determined by an authentic standards. The experiments were performed with and without the addition of glucose to a final concentration of 5 mM.

2.3. Influence of co-solvents

2.3.1. Activity of At*BBE-like 15 L182V in the presence of 10% co-solvents*

The measurements were conducted as described in Section 2.2.1 in the presence of 10% (v/v) of the respective co-solvents. Methanol, ethanol, 2-propanol, 1-butanol, acetone, acetonitrile, tetrahydrofuran (THF), *N*,*N*-dimethylformamide (DMF), dimethyl sulfoxide (DMSO) and 1,4-dioxane were employed. The activity determined in pure buffer was set to 100%. The presence of co-solvents was not found to influence the extinction coefficient of the coniferyl aldehyde.

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Table 1
GS-MS analytics: Retention times employing a non-chiral stationary phase.

Substance	Method	Retention time starting material [min]	Retention time product [min]	Retention time aldehyde (authentic standard) [min]
Cinnamyl alcohol (**1d**)	M2	13.4	12.9	12.9
Benzyl alcohol (**1g**)	M1	3.4	7.55	8.0
Piperonyl alcohol (**1h**)	M2	14.4	13.8	13.9
trans-2-Hexen-1-ol (**1j**)	M2	6.3	6.0	6.0
cis-2-Hexen-1-ol (**1k**)	M2	6.3	6.0	
Crotyl alcohol (**1i**)	M3	3.2	3.0	3.0
3-Phenyl-2-propyn-1-ol (**1l**)	M1	6.2	5.1	5.1
(*Z*)-2-Fluoro-3-phenyl-prop-2-en-1-ol (**1e**)	M1	6.2	5.7	
1-Phenyletanol (**1p**)	M1	3.6	3.6	
3-Phenyl-1-propanol (**1q**)	M1	12.3	12.3	11.3
Geranylamine (**3a**)	M2	12.3	12.3	12.8

2.3.2. Activity in the presence of 10–50% (v/v) of organic co-solvents

The measurements were conducted as described in Section 2.3.1 in the presence of 10–50% (v/v) co-solvent. Only these solvents were investigated which were found to increase the activity of the enzyme in the experiments described in Section 2.2.1 (ethanol, 2-propanol, 1-butanol, acetonitrile, tetrahydrofuran, dimethyl sulfoxide, and 1,4-dioxane).

2.3.3. Long-term stability in the presence of 30% (v/v) of organic co-solvents

The measurements were conducted as described in section 2.3.1 in the presence of 30% co-solvents. The initial enzyme activity and the residual activity were determined after 24 h incubation at 25 °C and 500 rpm in a thermomixer (Eppendorf, Hamburg, Germany).

2.4. Substrate screening

Reactions were carried out in 50 mM MES buffer pH 7.0 in the presence of 20% (v/v) DMSO. A total volume of 2 mL reaction mixture was incubated in 11 mL glass test tube with screw cap (Pyrex, Darmstadt, Germany) at 30 °C in an orbital shaker at 110 rpm. The reaction was started by the addition of 10 μL enzyme to achieve a final enzyme concentration of 1.2 μM. After 24 h the reaction mixture was extracted with ethyl acetate (2 × 1 mL), the combined organic phase was dried with Na_2SO_4 and subjected to GC analysis to identify putative products.

2.4.1. GC–MS

GC–MS analyses were carried out on an Agilent 7890A GC system equipped with an Agilent J&W HP–5 ms capillary column (30 m × 0.25 mm × 0.25 μm; stationary phase: bonded & cross-linked 5%-phenyl-methylpolysiloxane) (Agilent Technologies, Santa Clara, USA) and coupled to an Agilent 5975C mass-selective detector (electron impact ionisation, 70 eV; quadrupole mass selection) using helium as carrier gas.

2.4.1.1. Method M1. Inlet temperature: 250 °C; split ratio: 90:1; injection volume: 1 μL; column flow rate: 0.7 mL/min; oven program: 100 °C for 0.5 min, 10 °C/min to 300 °C; MS transfer line temperature: 300 °C, MS source temperature: 230 °C, MS quadrupole temperature: 150 °C; MS scan range: m/z = 33–400.

2.4.1.2. Method M2. Inlet temperature: 250 °C; split ratio: 90:1; injection volume: 1 μL; column flow rate: 0.7 mL/min; oven program: 40 °C for 2 min, 10 °C/min to 180 °C, 180 °C for 1 min; MS transfer line temperature: 300 °C, MS source temperature: 230 °C, MS quadrupole temperature: 150 °C; MS scan range: m/z = 33–400.

2.4.1.3. Method M3. Inlet temperature: 250 °C; split ratio: 90:1; injection volume: 1 μL; column flow rate: 0.7 mL/min; oven program: 40 °C for 5 min, 5 °C/min to 100 °C, 25 °C/min to 200 °C, 200 °C for 1 min; MS transfer line temperature: 300 °C, MS source temperature: 230 °C, MS quadrupole temperature: 150 °C; MS scan range: m/z = 33–400.

The products were identified by their retention times determined with authentic standards, which are summarized in Table 1. If no standard was available the products were identified by the MS-spectrum.

2.4.2. Substrate-specific activity of At*BBE-like 15 L182V*

Substrate solutions (2 mM) were provided in MES buffer pH 7.00 in a measuring cell with a magnetic stirrer at 1000 rpm and 25 °C. The reaction was started by the addition of 25 μL enzyme solution. The substrates and the final enzyme concentrations are summarized in Table 2. The course of the reaction was followed by the oxygen consumption determined with an optical oxygen meter FireSting O2 (Pyro Science GmbH, Aachen, Germany) equipped a retractable needle type oxygen probe (OXR50-UHS, PyroScience, Germany). The probe was calibrated with the oxygen saturated buffer 50 mM MES pH 7,00 and a saturated Na_2SO_3-solution.

2.5. Stereo specificity

Kinetic resolutions were carried out with substrates **1l, 1 m 1n** and **1o** as described in Section 2.4. The products were identified by coinjection with authentic samples on GC–FID.

2.5.1. GC–FID (chiral stationary phase)

GC–FID analyses for the optical purity of 3-penten-2-ol (**1n**), 3-octen-2-ol (**1m**), and cyclohex-2-en-1-ol (**1o**) were carried out on an Agilent 7890A GC system equipped with a Varian CP-Chirasil Dex-CB capillary column (25 m × 0.32 mm × 0.25 μm; stationary phase: β-cyclodextrin bonded to dimethylpolysiloxane) (Agilent Technologies, Santa Clara, USA) using hydrogen as carrier gas.

Method **M5** (used for **1n**): Inlet temperature: 220 °C; split ratio: 90:1; injection volume: 1 μL; column flow rate: 1 mL/min; oven program: 60 °C for 1 min, 20 °C/min to 70 °C, 70 °C for 5 min, 20 °C/min to 180 °C, 180 °C for 1 min; detector temperature: 250 °C.

Method **M6** (used for **1o** and **1m**): Inlet temperature: 220 °C; split ratio: 90:1; injection volume: 1 μL; column flow rate: 1 mL/min; oven program: 60 °C for 1 min, 20 °C/min to 95 °C, 95 °C for 9 min, 20 °C/min to 180 °C, 180 °C for 1 min; detector temperature: 250 °C.

4-Phenyl-3-buten-2-ol (**1l**) was converted into the corresponding acetate derivative prior to chiral-phase GC analysis by reaction with acetic anhydride (20 μL/mL sample) and

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Table 2
Reaction mixtures.

Substrate	Enzyme concentration [μM]
Sinapyl alcohol (**1c**)	0.125
Coniferyl alcohol (**1b**)	0.125
p-Coumaryl alcohol (**1a**)	0.125
Cinnamyl alcohol (**1d**)	0.625
Piperonyl alcohol (**1h**)	2.5
4-Phenylbut-3-en-2-ol (**1l**)	2.5
Crotyl alcohol (**1i**)	2.5

Table 3
GC-FID analytics: Retention times employing a chiral stationary phase.

Substance	Method	Retention time alc. 1 [min]	Retention time alc. 2 [min]	Retention time ketone [min]
4-Phenylbut-3-en-2-ol (acetyl derivative) (**1l**)	M7	21.7	22.0	19.3
3-Octen-2-ol (**1m**)	M6	10.2	10.5	9.0
3-Penten-2-ol (**1n**)	M5	5.4	5.6	4.1
Cyclohex-2-en-1-ol (**1o**)	M6	9.8	10.2	7.6

4-dimethylaminopyridine (DMAP; 1 mg/mL sample) at room temperature for 3 h.

Chiral GC–FID analyses of the resulting 4-phenyl-3-buten-2-yl acetate (**1l** acetate) were carried out on an Agilent 7890A GC system equipped with a Restek Rt®-βDEXse capillary column (25 m × 0.32 mm × 0.25 μm; stationary phase: 2,3-di-*O*-ethyl-6-*O*-*tert*-butyldimethylsilyl-β-cyclodextrin added into 14%-cyanopropylphenyl/86%-dimethylpolysiloxane) using hydrogen as carrier gas.

Method **M7** (used for 1l): Inlet temperature: 220 °C; split ratio: 50:1; injection volume: 1 μL; column flow rate: 2 mL/min; oven program: 60 °C for 1 min, 5 °C/min to 180 °C; detector temperature: 250 °C.

The retention times of the observed in these measurements are summarized in Table 3.

2.5.2. HPLC–MS (chiral stationary phase)

HPLC analysis was performed with a Shimadzu HPLC system using equipped with a CHIRALCEL OD-H column (Chiral Technologies Inc., West Chester, United States). Heptane/2-PrOH = 90/10 was employed as mobile phase at flow rate of 0.7 mL/min and 25 °C. For the respective retention times were observed; t_{ret}[(*R*)-enantiomer] = 11.9 min, t_{ret}[(*S*)-enantiomer] = 17.6 min, and t_{ret}(ketone) = 10.5 min.

2.5.3. Scale up kinetic resolution

The substrate **1l** (100 mg, 0.68 mmol) was solubilized in DMSO (14 mL), mixed with MES buffer (50 mM, pH = 7.0, 56 mL) and incubated for 3 h at 30 °C in a shaker at 120 rpm. The oxidase *At*BBE-like 15 (350 μL, 140 μM) and the catalase from *Micrococcus lysodeikticus* (1050 μL, 170000 U/mL) were added and the reaction was incubated for 20 h at 30 °C and 120 rpm. The reaction mixture was extracted with diethyl ether (4 × 40 mL), the combined organic phase was dried over with Na_2SO_4, filtered and concentrated in vacuo. The crude product was purified by flash chromatography (hexane/diethyl ether 5:1). The fractions were collected and concentrated in vacuo to give compounds **1l** (white solid, 35 mg, 0.24 mmol, 35%) and **2l** (yellow oil, 41 mg, 0.28 mmol, 41%) with following physical properties:

(*R*)-**1l**: $[\alpha]_D^{20}$ = +14.7 (c = 1.00, MeOH); lit. :[1] $[\alpha]_D^{20}$ = −17.8 [c = 0.32, MeOH, (*S*)-enantiomer]; ^{1}H NMR (300 MHz, $CDCl_3$): 7.40-7.21 (m, 5H), 6.57 (d, *J* = 15.9, 1H), 6.26 (dd, J_1 = 15.9, J_2 = 6.4, 1H), 4.49 (dquint, J_1 = 6.4, J_2 = 1.0, 1H), 1.70 (bs, 1H), 1.37 (d, *J* = 6.4, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): 136.8, 133.7, 129.5, 128.7, 127.8, 126.6, 69.1, 23.5; MS (EI): *m*/*z*: 148 (54), 130 (47), 115 (66), 105 (100), 91 (60), 77 (45);

HPLC analysis on chiral stationary phase {Daicel Chiralcel OD-H, *n*-heptane/2-propanol 90/10, 0.7 mL/min, 25 °C, t_{ret}(enantiomer 1) = 11.5 min, t_{ret}(enantiomer 2) = 16.8 min}: t_{ret}(major isomer) = 11.5 min, ee = 94%; the physical data is in consistency with literature [12].

2l: ^{1}H NMR (300 MHz, $CDCl_3$): 7.56-7.49 (m, 3H), 7.42-7.39 (m, 3H), 6.72 (d, *J* = 16.3, 1H), 2.39 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): 198.4, 143.4, 134.4, 130.5, 129.0, 128.3, 127.2, 27.6; MS (EI): *m*/*z*: 146 (60), 145 (67), 131 (71), 103 (100), 77 (50); the physical data is in consistency with a commercial sample.

2.6. Synthesis of (Z)-3-fluoro-3-phenyl-prop-2-en-1-ol (1e)

2.6.1. Synthesis of ethyl (Z)-3-fluoro-3-phenylacrylate

The synthesis of ethyl (*Z*)-3-fluoro-3-phenylacrylate was performed according to the work of Li et al. [14]. In a 5 mL round bottom flask AgF (140 mg, 1.10 mmol, 1.9 eq.) was added to a solution of 2 mL acetonitrile and 0.1 mL H_2O. Ethyl 3-phenylpropiolate (94.5 μL, 0.57 mmol, 1.0 eq.) was then added to the brownish suspension and the reaction was heated to 90 °C for 22 h. The reaction mixture was then cooled to room temperature and the solvents were removed under reduced pressure. The crude product was taken up in 10 mL H_2O and washed with Et_2O (3 × 10 mL). The combined organic layers were dried over Na_2SO_4, filtered and concentrated under reduced pressure to give the crude product which was purified via silica column chromatography with cyclohexane/ethyl acetate 50:1 to 20:1 as eluent. The yield was 133 mg (0.685 mmol, 60%) colorless oil (R_f = 0.24, cyclohexane/ethyl acetate 18:1).

^{1}H NMR (300 MHz, $CDCl_3$): δ 7.65 (d, $^3J_{H,H}$ = 6.7 Hz, 2H, H4 + H6), 7.52–7.37 (m, 3H, H1-3), 5.90 (d, $^3J_{H,F}$ = 33.3 Hz, 1H, H8), 4.26 (q, $^3J_{H,H}$ = 7.1 Hz, 2H, H10), 1.33 (t, $^3J_{H,H}$ = 7.1 Hz, 3H, H11).

^{13}C NMR (76 MHz, $CDCl_3$): δ 166.40 (C_q, d, $J_{C,F}$ = 277.6 Hz, C7), 164.18 (C_q, d, $J_{C,F}$ = 2.2 Hz, C9), 131.60 (CH, C1 + C3), 130.81 (C_q, d, $J_{C,F}$ = 26.2 Hz, C5), 128.98 (CH, d, $J_{C,F}$ = 1.9 Hz, C4 + C6), 125.78 (CH, d, $J_{C,F}$ = 7.9 Hz, C2), 97.35 (CH, d, $J_{C,F}$ = 6.9 Hz, C8), 60.55 (CH_2, C10), 14.41 (CH_3, C11).

2.6.2. Reduction of ethyl (Z)-3-fluoro-3-phenylacrylate to (Z)-3-fluoro-3-phenylprop-2-en-1-ol (**1e**)

In a 10 mL Schlenk-tube ethyl (*Z*)-3-fluoro-3-phenylacrylate (100 mg, 0.515 mmol, 1.0 eq.) was dissolved in 3 mL CH_2Cl_2 and cooled to −78 °C via an acetone/dry ice bath. 1.2 mL (1.192 mmol, 2.3 eq.) DIBAL-H (1.0 M in toluene) were slowly added to the colorless solution at −78 °C. The reaction mixture was then warmed to 0 °C over a period of 2 h. Subsequently, the reaction was transferred into an 80 mL Schlenk-tube, diluted with CH_2Cl_2 (15 mL) and quenched by the addition of H_2O (1 mL). Rochelle-salt (sat.) (20 mL) was added and the two phases were stirred vigorously for 19 h. The aqueous phase was then washed with CH_2Cl_2 (3 × 20 mL) and the combined organic layers were washed with 1 M HCl (1 × 50 mL) and brine (1 × 50 mL), dried over Na_2SO_4, filtered and concentrated under reduced pressure. The crude product was purified via column chromatography with cyclohexane/ethyl acetate 5:1 as eluent. The yield was 71 mg (0.466 mmol, 91%) colorless oil (R_f = 0.18, cyclohexane/ethyl acetate 5:1).

^{1}H NMR (300 MHz, $CDCl_3$): δ 7.58–7.30 (m, 5H, H1-6), 5.66 (dt, $^3J_{H,H,F}$ = 36.6, 7.1 Hz, 1H, H8), 4.45 (dd, $^3J_{H,H,F}$ = 7.1, 1.9 Hz, 1H, H9), 1.82 (s, 1H, OH).

^{13}C NMR (76 MHz, $CDCl_3$): δ 158.22 (C_q, d, $J_{C,F}$ = 251.2 Hz, C7), 131.87 (C_q, d, $J_{C,F}$ = 28.6 Hz, C5), 129.44 (CH, C1 + C3), 128.66 (CH, d,

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$J_{C,F}$ = 2.0 Hz, C4 + C6), 124.55 (CH, d, $J_{C,F}$ = 7.2 Hz, C2), 104.92 (CH, d, $J_{C,F}$ = 15.3 Hz, C8), 56.23 (CH_2, d, $J_{C,F}$ = 7.7 Hz, C9).

3. Results and discussion

*At*BBE-like protein 15 L182V was found to oxidize allylic and benzylic alcohols to the corresponding aldehydes. In Scheme 1 the reaction equation and the structure of substances that were used for enzymatic assays are shown.

3.1. pH dependency of the activity of AtBBE-like protein 15 L182V

The pH profile of the enzyme was investigated in a range of 4 to pH 10 (Fig. 2a). Thermofluor experiments were conducted to determine the melting point on the enzyme in a pH-range from 5 to 10 (Fig. 2b).

The enzyme is active over a broad pH range, with highest activity at pH 7, and between pH 5 and pH 10 the enzyme retains more than 50% activity (5 min). The highest melting point of the enzyme was found to be in HEPES buffer pH 7.0 (60 °C).

3.2. Temperature dependent performance of AtBBE-like 15 L182V

The initial enzyme activity was measured in a temperature range of 20 to 70 °C (Fig. 3A). The residual activity of the enzyme was determined after 24 h incubation at temperatures ranging from 20 °C to 70 °C in the presence and absence of glucose (Fig. 3B).

The highest activity was found at 50 °C. The findings are in good agreement with the melting point of the wild type enzyme of 60 °C.

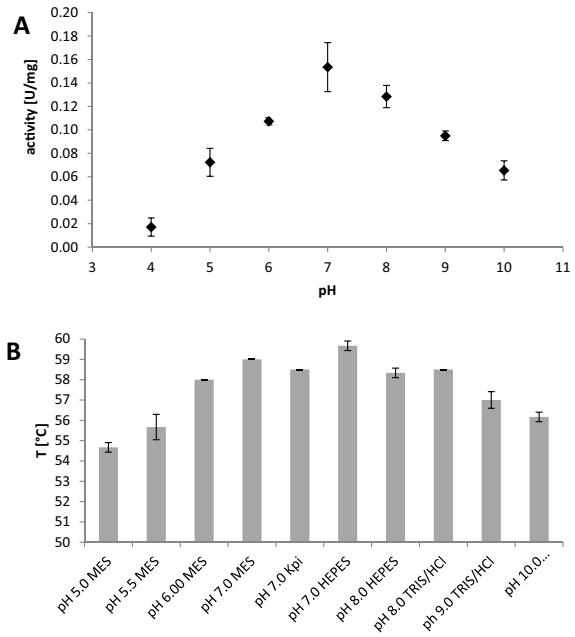

Fig. 2. A: pH optimum of *At*BBE-like 15 L182V. Plotted is the observed consumption of μmol oxygen per mg enzyme per minute. B: Melting point of *At*BBE-like protein 15 at different pH values.

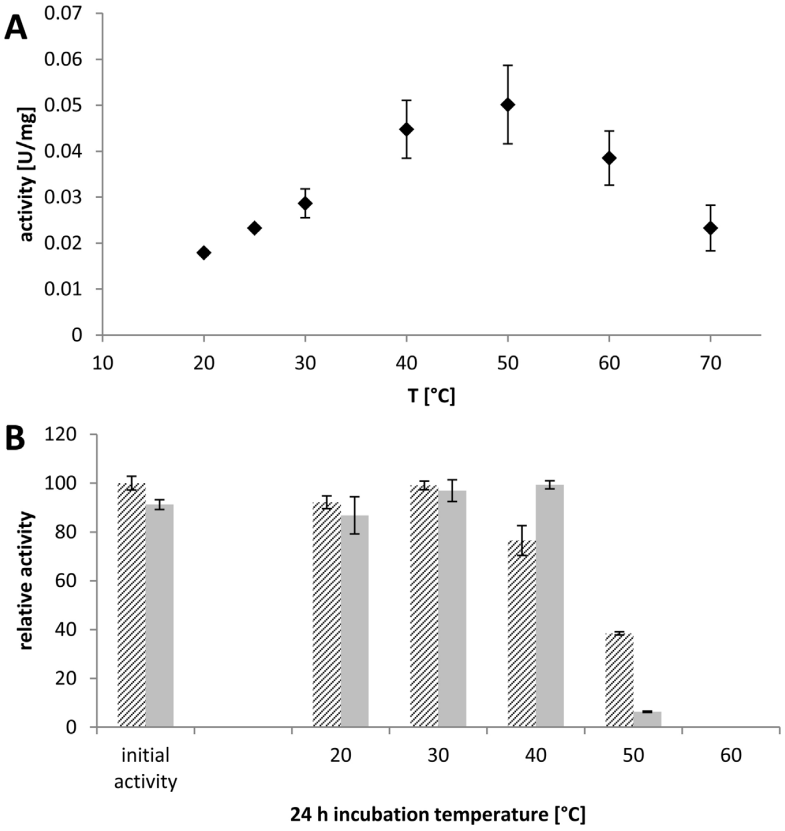

Fig. 3. A: Temperature optimum of *At*BBE-like 15 L182V. B: Residual enzyme activity after incubation at different temperatures for 24 h in the presence (grey bars) and absence (striped bars) of 5 mM glucose. The initial activity determined prior incubation without glucose was set to 100%.

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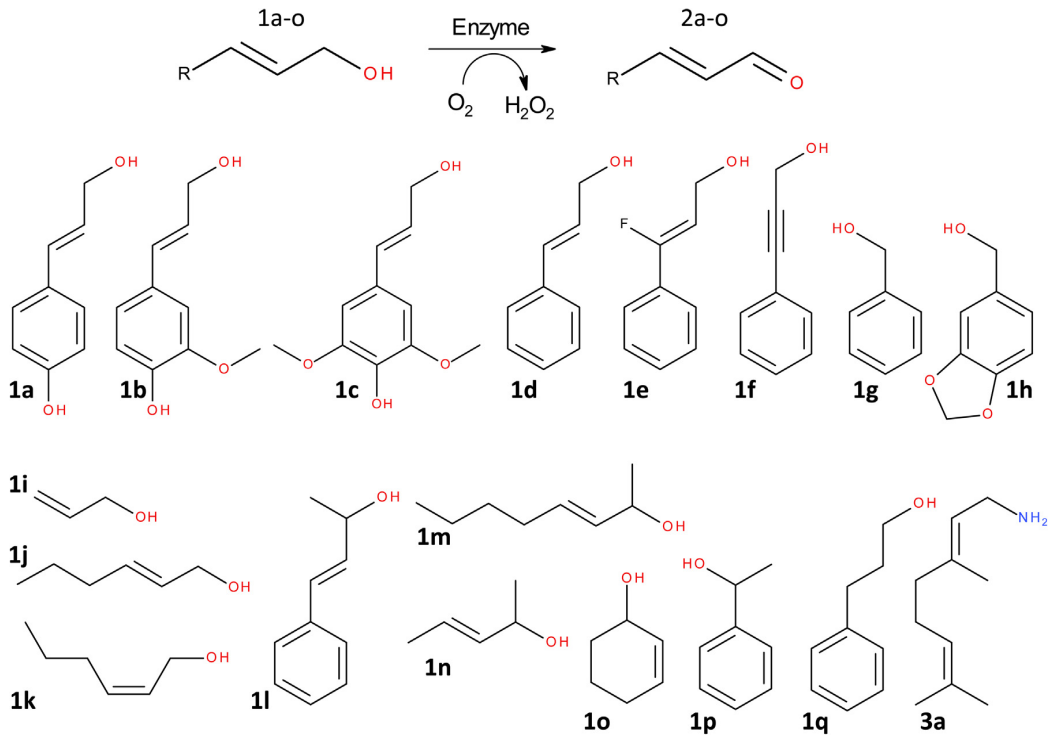

Scheme 1. Reaction equation and substrates used for enzymatic assays.

No loss of activity after 24 h can be observed at 30 °C. At 40 and 50 °C a residual activity of 77% and 38% are found, respectively. Glucose was shown to increase the melting temperature of the enzyme by 15 °C; in the long term experiment, a stabilizing effect of glucose at 40 °C can be observed, while at 50 °C the residual enzyme activity is decreased by glucose [8].

3.3. Activity in the presence of 10% (v/v) of organic co-solvents

While an aqueous system is the natural environment of enzymes the solubility of nonpolar substrates is limited under purely aqueous conditions. This limitation can be overcome by the addition of co-solvents. A high tolerance of the enzyme towards organic solvents is also required if a thermomorphic solvent system is supposed to be applied for enzyme recycling [15]. As oxygen is the final electron acceptor of *At*BBE-like protein 15 L182V, also the oxygen solubility is an important factor for the overall performance of the catalyst. The addition of co-solvent is not only beneficial for substrate solubility but can also enhance the oxygen solubility [16]. To determine the effect of organic solvents on the enzyme stability, measurements in the presence of 10% (v/v) of various organic solvents were conducted. The course of the reaction was followed spectrophotometrically. The enzyme activity in buffer was set to 100%. In Fig. 4 the activities found in the presence of organic solvents are shown.

An increased activity of the enzyme was found in acetonitrile, 2-propanol, 1,4-dioxane, tetrahydrofuran, 1-butanol, dimethyl sulfoxide and ethanol. The highest degree of activation was found for ethanol with 188% of the activity observed in buffer. A decreased activity was determined in the presence of methanol, dimethylformamide and acetone, with the lowest activity found with of methanol (29%).

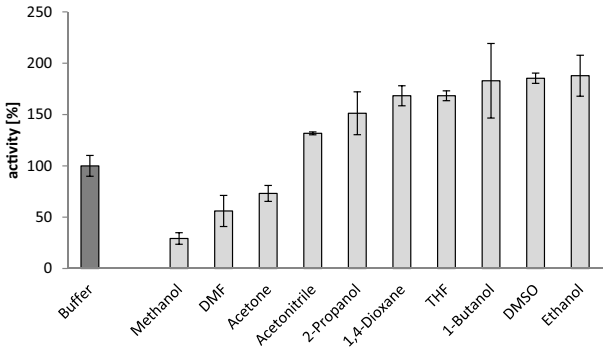

Fig. 4. Activity of *At*BBE-like protein 15 L182V with 10% (v/v) of organic co-solvents.

3.4. Activity in the presence of 10–50% (v/v) of organic co-solvents

The high solvent tolerance of the enzyme encouraged us to further increase the co-solvent concentration to probe the enzyme stability. Experiments were conducted with all solvents that were found to increase the enzyme activity. The enzyme activity was determined with acetonitrile, 2-propanol, 1,4-dioxane, tetrahydrofuran, 1-butanol, dimethyl sulfoxide, and ethanol at concentrations of 10%, 20%, 30%, 40% and 50% (v/v). The measured activities are shown in Fig. 5.

An increasing solvent concentration led to an increased enzyme activity in all cases except for tetrahydrofuran, which was not tolerated at higher concentrations. The highest acitivity was found in 40% ethanol with 346% of the activity measured in buffer. The enzyme was shown not only to tolerate high solvent concentrations, also the activity can be enhanced. Still, high solvent concentrations can lead to the denaturation and thereby deacti-

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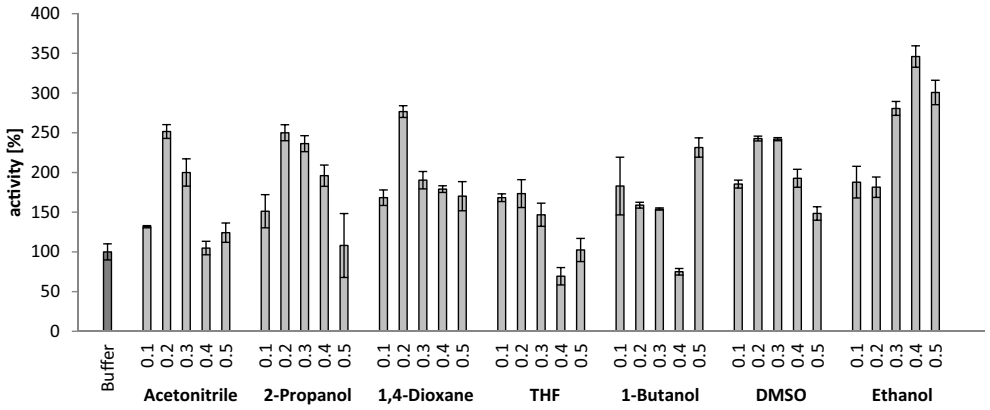

Fig. 5. Effect on the enzyme activity of different solvents at concentrations between 10% and 50% (v/v).

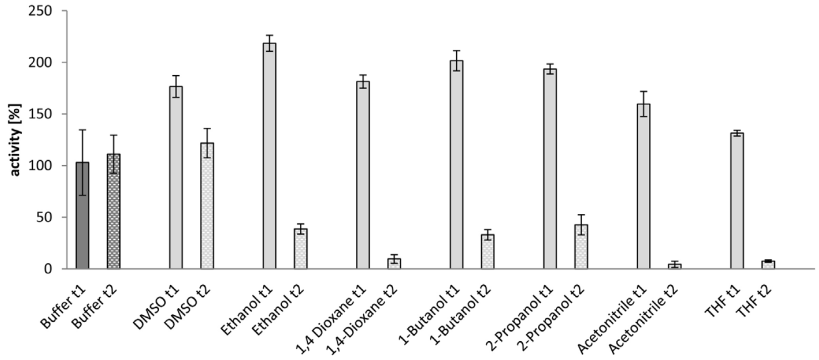

Fig. 6. Long-term activity of *At*BBE-like protein 15 L182V with 30% (v/v) of organic co-solvents with t1 = 0 h, t2 = 24 h.

vation of the enzyme. A good long-term stability of the enzyme under the given reaction conditions is essential to achieve a high space/time yield.

3.5. Long-term stability in the presence of 30% (v/v) of organic co-solvents

The presence of various organic solvents was found to enhance the activity of the enzyme. To test the long-term stability of the enzyme, it was incubated with 30% (v/v) of the respective solvents for 24 h (t2) before the reaction was started. The activity in buffer was set to 100%, the starting and residual activities are depicted in Fig 6.

While no deactivation of the enzyme in buffer was detectable, the presence of organic solvents led to enhanced activity but also a decreased life-time of the enzyme. The highest activity after 24 h incubation was determined in 30% DMSO. The enzyme activity in DMSO was 177% initially and dropped to 122% within 24 h. Also for other solvents a lower but still acceptable retention of the initial activity was determined. The initial activity dropped in ethanol from 218% to 38%, in 2-propanol from 194% to 47% and in 1-butanol from 201% to 33%. Especially the long-term stability in 2-propanol creates interesting options with regard to coupled reactions with NADH dependent alcohol dehydrogenases [17]. 2-Propanol is frequently used to regenerate NADH from NAD^+. In a one-pot process, *At*BBE-like 15 L182V could be employed for kinetic resolutions of secondary allylic alcohols, while an alcohol dehydrogenase with NADH regeneration by 2-propanol could be employed to reduce the allylic ketone formed *in situ* to reach a theoretical yield of 100% of the desired enantiopure alcohol.

Table 4
Substrate conversion determined by GC.

Substance	Conversion [%]
Cinnamyl alcohol (**1d**)	97
(*Z*)-3-Fluoro-3-phenyl-prop-2-en-1-ol (**1e**)	>99
3-Phenyl-2-propyn-1-ol (**1f**)	19
Benzyl alcohol (**1g**)	>99
Piperonyl alcohol (**1h**)	49
Crotyl alcohol (**1i**)	15
trans-2-Hexen-1-ol (**1j**)	47
cis-2-Hexen-1-ol (**1k**)	28
1-Phenyl-ethanol (**1p**)	n.d.
3-Phenyl-propanol (**1q**)	n.d.
Geranylamine (**3a**)	n.d.

3.6. Substrate screening

In order to elucidate the substrate scope of *At*BBE-like 15 L182V, the enzyme was screened for activity with various alcohols. Experiments were conducted in HEPES-buffer pH 7.0 containing 20% DMSO with a substrate concentration of 10 mM.

The substances **1a**-**o** were converted to the corresponding aldehydes, as confirmed by GC–MS analysis. The formation of carboxylic acids, which has been reported as a follow-up reaction in the oxidation of primary alcohols by other flavin-dependent oxidases, has never been observed in our experiments. The conversions observed by GC–MS are summarized in Table 4.

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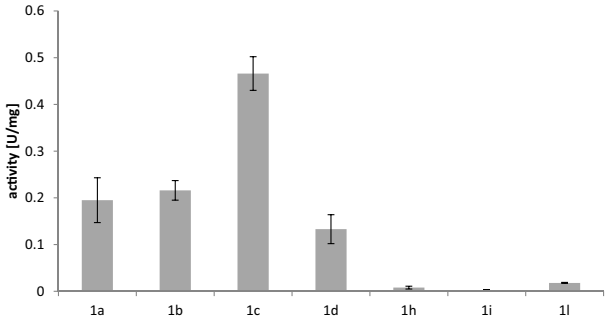

Fig. 7. Specific activity of *At*BBE-like protein 15 L182V with p-coumaryl alcohol (**1a**), coniferyl alcohol (**1b**), sinapyl alcohol (**1c**), cinnamyl alcohol (1d), piperonly alcohol (**1h**), crotyl alcohohl (**1i**) and 4-Phenylbut-3-en-2-ol (**1l**).

Table 5
Kinetic resolutions with *At*BBE-like protein 15 L182V.

Substance	Ketone [%]	Sum alcohols [%]	*e.e.* [%]	*E*
4-Phenylbut-3-en-2-ol (acetyl derivative) (**1l**)	58	42	>99 (*R*)	>34
3-Octen-2-ol (**1m**)	55	45	>99 (*R*)	>47
3-Penten-2-ol (**1n**)	12	88	20 n.d.	>200
Cyclohex-2-en-1-ol (**1o**)	35	65	53 (*R*)	>200

All allylic and primary benzylic alcohols that were tested were accepted as substrate. No conversion was detected (n.d.) for aliphatic and secondary benzylic alcohols (**1p-q**) as well as genanylamine **3a**.

3.6.1. Substrate specific activity of At*BBE-like 15 L182V*

To evaluate the activity toward the different substance categories that were found to be converted, the specific activity of the enzyme was assayed employing cinnamyl alcohol derivatives (**1a-d**, **1l**), a benzylic alcohol (piperinyl alcohol **1 h**), and an allyl alcohol (crotyl alcohol **1i**). The determined activities are summarized in Fig. 7.

The activities displayed in Fig. 7 are in good agreement with the conversions summarized in Table 4. The highest activity is found employing cinnamyl alcohol derivatives, ranging from 0.47 U/mg for sinapyl alcohol (**1c**) to 0.13 U/mg for cinnamyl alcohol. The activity towards a benzylic alcohol (piperonyl alcohol **1 h**, 0.008 U/mg) and towards crotyl alchohol (**1i**, 0.002 U/mg) is one and two orders of magnitude lower, respectively.

3.7. Kinetic resolutions

As secondary allylic alcohols were found to be converted by the enzyme, this substance class was chosen for testing the enantioselectivity of the enzyme. Kinetic resolutions were conducted starting from racemic alcohols. Enantiomeric excess (*e.e.*) of the remaining substrate as well as conversion were determined by GC analysis on chiral phases, and the enantioselectivity (*E*) was calculated according to Rakels et al. [18]. The results are summarized in Table 5.

The absolute configuration of the remaining alcohols **1l**, **1 m** and **1o** was determined according to [13], [19] and [20], respectively. The results indicate that the enzyme shows a high enantioselectivity in the oxidation of secondary allylic alcohols. While the (*S*)-enantiomer is converted, the (*R*)-enantiomer remains untouched. This is in good agreement with a productive docking mode we have published previously, in that the pro-S proton of the allylic alcohol was found to be oriented towards the N5 position of the flavin, while the pro-R proton was not [8].

4. Conclusions

In the present study we have probed the applicability of a monolignol oxidoreductase for biocatalytic transformations. We have employed the *At*BBE-like 15 L182V variant that was engineered previously toward higher oxygen reactivity. We have elucidated the pH- and temperature optima of the enzyme, the substrate scope and tolerance towards organic co-solvents. *At*BBE-like protein 15 L182V retained more than 50% of activity in a broad pH range between 5 and 10 for 5 min. The highest activity was reached at 50 °C, no deactivation of the enzyme was found after incubation at 30 °C for 24 h. The enzyme exhibits an enhanced activity in the presence of organic co-solvents. The most suitable co-solvent was found to be DMSO: in 30% DMSO the enzyme Exhibits 177% of the activity determined for an aqueous system; after 24 h at 20 °C 122% of enzyme activity is remained. Various allylic and benzylic alcohols were accepted as substrates and converted to the corresponding aldehydes. The enzyme preferably converts *S*-alcohols and was successfully applied in a kinetic resolution in preparative scale. Aliphatic alcohols were not converted by the enzyme. Therefore *At*BBE-like protein 15 L182V could be useful as a chemo- and enantioselective enzyme for future biocatalytic applications.

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