Thesis Biobased Chemistry and Technology and Organic Chemistry

Co-production of hydrogen peroxide and nitriles by coupled enzymatic reactions

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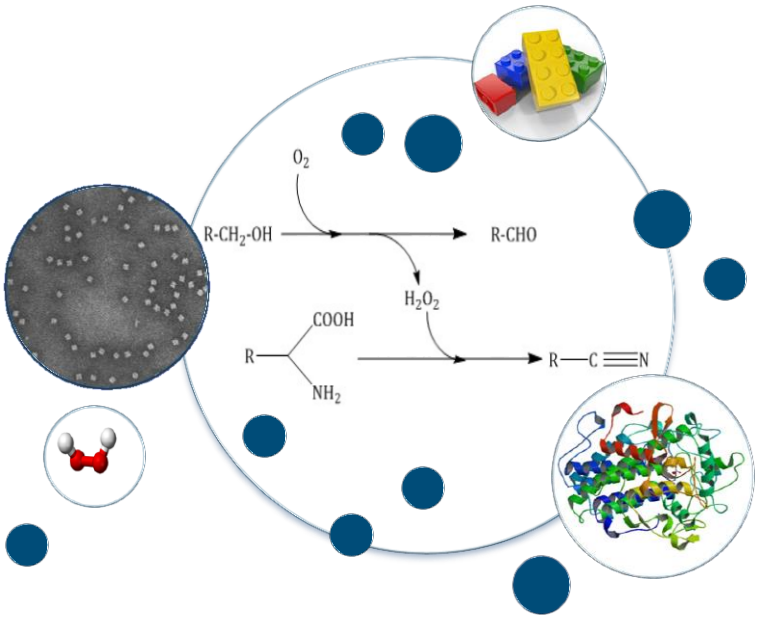
September 2015 – February 2016



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Co-production of hydrogen peroxide and nitriles by coupled enzymatic reactions

Vanadium Chloroperoxidase and Alcohol Oxidase

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Abstract

Nitriles are important materials for polymers, adhesives and solvents and are industrially produced by the reaction of nitrogen-free precursors with ammonia. The production of ammonia however, is really energy intensive as is the subsequent reaction to the nitrile. As an alternative for the current production method, sustainable enzymatic production methods for nitriles from amino acids are being developed. In this research, the enzymatic conversion of glutamic acid to 3-cyanopropanoic acid with Vanadium Chloroperoxidase (VCPO) is investigated. This enzyme however, uses hydrogen peroxide (H_2O_2) as substrate, which is not a sustainable compound due to product distillation and the hazardous properties of the chemical. In this research, *in situ* H_2O_2 production was investigated by the enzyme Alcohol Oxidase (AOX) in a coupled enzymatic reaction with VCPO for nitrile production. The aim of this thesis is to find the optimal reaction conditions for this enzymatic tandem reaction for the sustainable production of nitriles using *in situ* generated hydrogen peroxide.

The optimal reaction conditions of the coupled enzyme reaction were determined using monochlorodimedone (MCD) as substrate. The operational ratio between the two enzymes was determined by performing the reaction at different VCPO:AOX ratios. Furthermore, the MCD assay was scaled up in order to reach similar conditions as for glutamic acid conversion. The tandem reaction with glutamic acid as substrate was performed under different operational conditions; at high and low VCPO:AOX ratios, at changing glutamic acid concentrations and in different experimental configurations. To increase the AOX stability in the tandem reaction, the enzyme was covalently immobilized onto two different epoxy activated polymethacrylate supports (Relizyme^{TM} HFA403 and EP403). Moreover, the inhibitory effect of glutamic acid, 3-cyanopropanoic acid and Br^+ on AOX was investigated.

An optimal ratio of VCPO:AOX in the coupled enzymatic reaction was found to be 1:5 and under gentle oxygen bubbling where a full conversion of 2.5 mM MCD was achieved. Immobilization of AOX on HFA beads was most promising, resulting in a immobilization yield of 100% and 53% recovered activity. The AOX activity was shown to be inhibited by Br^+, but not by glutamic acid nor 3-cyanopropanoic acid. However, under these conditions the reaction of VCPO with AOX has still not been successfully demonstrated with glutamic acid.

It is suggested that a competitive reaction with the tandem reaction causes the unsuccessful reaction. Br^+ is known to react with acetaldehyde forming either an carboxylic acid or a halogenated compound. When Br^+ reacts with the aldehyde formed by AOX in a faster reaction than with glutamic, no conversion into 3-cyano propionic acid will occur. Moreover, if a halogenated compound is produced, the halogen source will be depleted due to the incorporation of this compound in the end-product.

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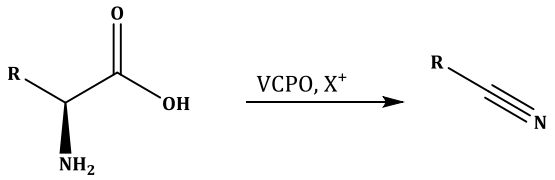
Background

Most carbon based products from the chemical industry are derived from naphtha. As a result of fluctuating oil prices, the diminishing availability of oil and the growing awareness for sustainable sources of energy and raw materials, alternative resources are being explored. Polysaccharides, lignin, triglycerides and proteins from biomass have gained a lot of attention as natural resources for bio-based chemicals and materials.[1] Protein-rich waste streams for example, are substantially produced by the food industry. Non-essential amino acids from these rest streams can be used for the production of chemicals without competing with the food- or feed industry. Chicken feathers, for example, contain 75% (w/w) proteins of which 57% are non-essential amino acids.[2] Those amino acids have an amino functionality that can be used for the production of nitrogen containing chemicals, like nitriles.

Nitriles, organic compounds containing a carbon-nitrogen triple bond (-C≡N), are important starting materials for polymers (nitrile butadiene rubbers), adhesives (cyanoacrylates) and solvents (e.g. acrylonitrile).[3] Nitriles are industrially produced by the reaction of nitrogen-free precursors, such as propylene, with ammonia.[4] The production of ammonia however, is energy intensive, with an average of 11 MWh energy consumption per tonne ammonia produced.[5] In addition to the energy use for the ammonia production, the subsequent reaction to incorporate the nitrogen and the nitrile functionality in the product by e.g. ammoxidation is also really energy intensive. The use of amino acids for nitrogen containing chemicals circumvents the energy intensive processes of nitrogen fixation and incorporation of the nitrogen into the product. Amino acids can be derived from protein-rich rest streams for example, which are substantially produced by the food industry. A broad range of nitriles can be produced from these rest streams depending on the starting amino acid. For example, glutamic acid will give access to 3-cyanopropanoic acid which is a precursor of acrylonitrile and succinonitrile.[6,7]

Oxidative decarboxylation of amino acids to nitriles

Amino acids can be converted into nitriles by oxidative decarboxylation.[7] This reaction is dependent on an activated halide (X^+) for which both bromide[8] and chloride[9] are suitable. The activated species are formed *in situ* by either a chemical or a catalytic reaction.



X^+: Br^+ or Cl^+

Scheme 1. Schematic representation of the conversion of amino acids into nitriles.

In the chemical approach, amino acids are oxidatively decarboxylised by a hypohalite to form nitriles. This hypohalide can be added e.g. NaOCl[9] or can be produced *in situ* by reagents such as trichloroisocyanuric acid,[10] N-bromosuccinimide[11] In this approach stoichiometric amounts of reagent are used and large amounts of salts are formed. Due to the exothermic nature of the reaction cooling is required, making these processes energy intensive. Another method for the production of the activated halogenated species is by electrogeneration of bromide.[12] This electrochemical approach has the advantage over the chemical approach that only catalytic amounts of the halide are used, as the bromide is continuously to oxidised to BrO^-. The disadvantage of this approach is that a continuous electrical current is needed and methanol is used as main solvent. To overcome the disadvantages stated above, catalytic methods are being developed.

In the catalytic approach the halide is continuously oxidised to the activated form by a heterogeneous[13] or biological catalyst[14] solely requiring catalytic amounts of the halogen source. Moreover, both catalytic methods can proceed under ambient conditions at room temperature in aqueous solutions. The heterogeneous catalyst, consisting of tungstate immobilized on a solid support, produces hypohalites using hydrogen peroxide as terminal oxidant. The biocatalytical approach involves a halogenating enzyme that oxidises a halide into a hypohalite.

Three types of halogenating enzymes are known: peroxide dependant haloperoxidases, oxygen-dependent halogenases and nucleophilic halogenases.[15] Peroxide dependant haloperoxidases can be subdivided into two classes based on their prosthetic group: heme-dependent haloperoxidases and vanadium-dependent haloperoxidases (VHPO). The latter group, VHPOs, have been shown to convert amino acids into nitriles.[7,14]

VHPOs indirectly catalyse the oxidative decarboxylation of an amino acid via the production of a activated halide (X^+) from hydrogen peroxide (H_2O_2) and a halide. For the nature of X^+ multiple forms are proposed depending on the halide: XOH, X_2, X_3-, Enzyme-X.[16] The XOH, X_2,X_3-, are proposed to be equilibrium in aqueous solutions. It is suggested that X^+ species can react with the amino group glutamic acid,[14] inducing oxidative decarboxylation. A second X^+ molecule reacts with the amine functionality in the amino acid, forming the nitrile. The focus in this research will be on the conversion of glutamic acid into its corresponding nitrile, as glutamic acid is an abundant non-essential amino acid which can be separated from rest streams by electrodialysis.[17] However, other research points to the mechanism wherein the α-carbon is halogenated instead of the amine.[11]

Vanadium Chloroperoxidase (VCPO) from *Curvularia inaequalis* is the most versatile VHPO, due to a wide substrate range, high thermostability (90°C), low activity loss at -20°C, low K_m in bromide oxidation (less than 10 µM) and high stability against oxidising agents (up to 100 mM).[18] A crystal structure of VCPO is known, as well as the structure of the active site.[18] In the active site, the cofactor vanadium is covalently attached to a histidine residue, stabilized by the neighbouring residues. (Figure 1)

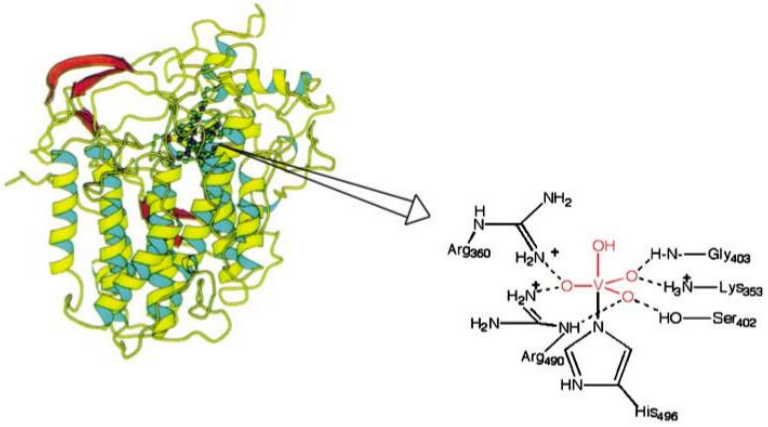
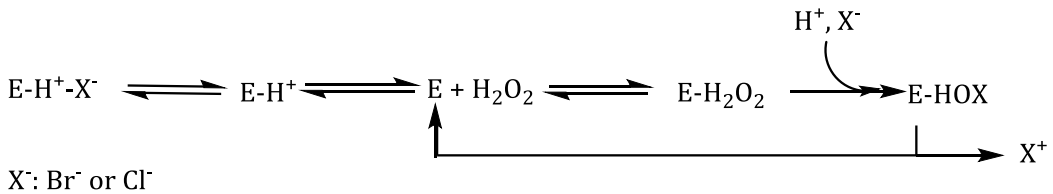


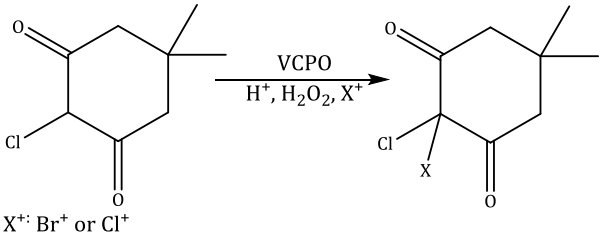
Figure 1. Vanadium chloroperoxidase from *Curvularia inaequalis* and its active site. The vanadate cofactor is shown in red.[18]

Hydrogen peroxide binding is dependent on an ionisable group and is only possible at low pH, when deprotonated. At higher pH, the enzyme will thus be inactive. After formation of the peroxo-intermediate, the halide (X^-) reacts with the intermediate forming an HOX-enzyme complex. In case of Cl^- oxidation an additional proton (H^+) is needed to protonate the peroxo-intermediate before Cl^- can react with this intermediate. X^+ is subsequently released in the rate determining step. [19] The proposed mechanism is depicted in Scheme 2.



Scheme 2. Schematic reaction mechanism of vanadium chloroperoxidase. For Br^- oxidation a H^+ is not needed to for the reaction with the peroxo-intermediate.[19]

A common procedure to monitor Br^+ formation by haloperoxidases is the monochlorodimedone (MCD) assay:[20, 21, 22] In this assay, MCD is brominated into a dihalogenated product, (Scheme 3) which has a different absorption spectrum than the monohalogenated form. The conversion can be monitored spectrophotometrically by a decrease in UV absorption at 290 nm.



Scheme 3. Schematic representation of the halogenation of MCD.

Hydrogen peroxide production

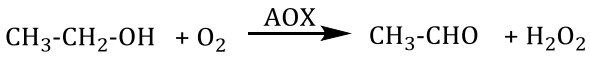
VCPO uses hydrogen peroxide as oxidising agent for the production of nitriles.[7] Currently, hydrogen peroxide is produced industrially by anthraquinone oxidation process, which includes among others distillation from 30% up to 70% (wt%) and regeneration of the antraquinone by hydrogenation.[23] However, side reactions, energy intensive distillation and the explosion hazard during transportation of the hydrogen peroxide in concentrations above 28 wt% or if contaminated with combustible material, make this process far from environmentally friendly.[24] Therefore, alternatives for this production process have been studied. For example direct synthesis from H_2 and O_2 by noble metal catalysts[25] and electrochemical fuel cells[26] but also enzymatic *in situ* hydrogen peroxide production by oxidases is possible.

Oxidases are enzymes that catalyse redox reactions, using oxygen as electron acceptor. Based on the co-factor, two oxidases families are classified: one family uses copper as cofactor in various forms, while the other comprises the flavin-containing oxidases.[27] The latter family is divided in subfamilies based on their sequence homology and structure. One of the subfamilies is the glucose-methanol-choline flavoprotein family (GMC) that act on primary or secondary alcohols. Examples of GMC-oxidases are Choline Cehydrogenases (CHD), Glucose Oxidases (GO) and Alcohol Oxidases (AOX).

Short chain alcohol oxidases

Short chain alcohol oxidases (SCAOX) are oxidases that catalyse the oxidation of lower chain length alcohols in the range of C1-C8 carbons. In this research SCAOX were chosen due to their ability to convert ethanol into acetaldehyde, see Scheme 4. Ethanol can be produced in a bio-based way by fermentation of glucose from sugarcane[28] and the acetaldehyde formed during the reaction is an important intermediate in the production of e.g. acetic acid.[29] Acetaldehyde can be easily removed from the reaction mixture due to its low boiling point (20.16 °C). Therefore, this enzyme was selected for this research as well.

Of the three most common sorts of AOX (*Pichia pastoris*, *Candida boidinii* and *Hansenula polymorpha*) the AOX from *H. polymorpha* was the most active at lower pH, which is important as the optimum pH of VCPO is 5.6.[30] This is why AOX from *H. polymorpha* was selected for this research.



Scheme 4. Schematic representation of H_2O_2 production from primary alcohols by alcohol oxidase (AOX).

Alcohol oxidases consist of eight identical subunits, each containing one non-covalently bound flavin adenine dinucleotide (FAD). The three dimensional structure of alcohol oxidases have not been fully elucidated, but electron microscopy and image analysis of alcohol oxidase from *H. polymorpha* were performed.[31] These analyses showed that the enzyme consists of two layers of four subunits connected by small stalks, each subunit facing in the opposite direction. A sequence analysis of different GMC-oxidases showed that the structure of the enzymes can be divided into five distinct domains: three major (FAD-binding domain, flavin attachment loop and substrate-binding domain) and two minor (FAD covering lid and extended FAD-binding domain).[32] The FAD-binding domain is the most conserved domain containing a βαβ-fold close to its amino terminus, which binds the ADP-moiety of the FAD molecule.[33] FAD binding plays a major role in assembly and

stability of the enzyme as AOX with specific point mutations in the FAD-binding domain resulted in reduced enzyme activity and lower protein levels.[34]

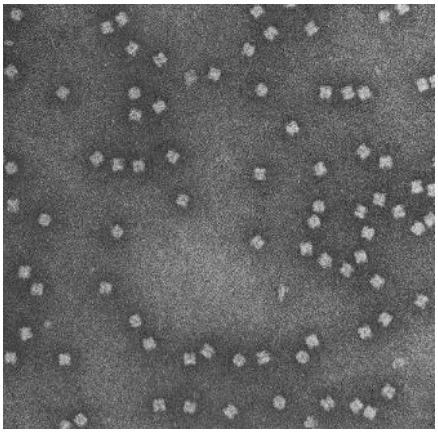
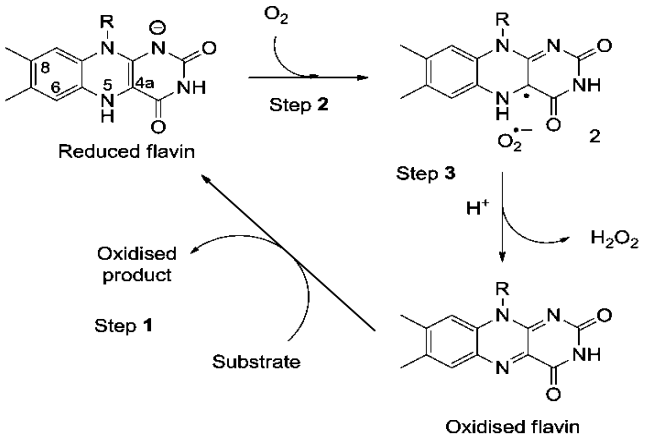


Figure 2. Single molecules of AOX: Electron rmcrograph of AOX negatively stained with 2% SST[31]

During the catalytic cycle of flavoenzymes, FAD is reduced by a two electron transfer whilst oxidising the substrate.[35] The cofactor is regenerated by two single-electron transfers in a stepwise process. First, the oxygen is reduced resulting in a superoxide anion and an one electron semi reduced flavin. The radical pair subsequently undergoes a second electron transfer forming hydrogen peroxide and an oxidised flavin. The whole process is depicted in Scheme 5.



Scheme 5. Catalytic cycle of flavoenzymes.[27]

Immobilization of enzymes might be beneficial, because this enables reuse and might improve properties like stability and activity[36]. The immobilization of AOX has often been studied in relation with in biosensors for enhancement of the enzyme stability.[37,38] Immobilization on supports like DEAE-cellulose particles[39] and epoxy-containing beads[40] have shown promising results, but also non carrier bound immobilization as cross-linked enzyme aggregates was shown to stabilize the enzyme. [41] Relizime™ produced two types of epoxy activated polymethacrylate beads; HFA403 (a long linker amino epoxide) and EP403 (short linker epoxide), see Figure 3.

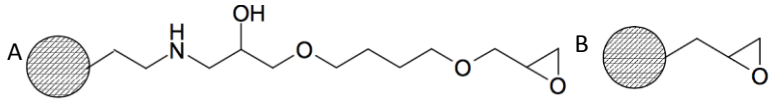


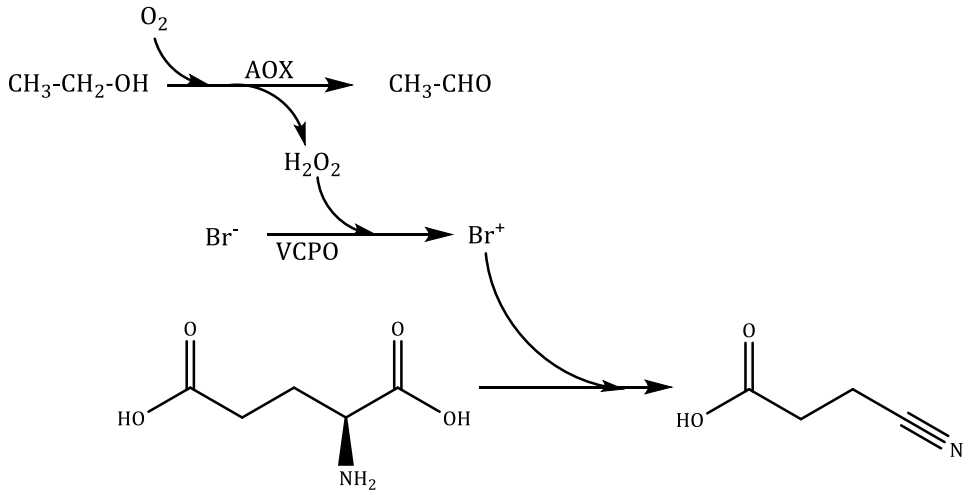
Figure 3. Schematic representation of Relizime™ A) HFA403 and B) EP403.[42]

The epoxy groups of the beads are able to react with the nucleophilic ones, e.g. amino, hydroxyl and thiol moieties, on the protein surface. The immobilization is thought to proceed via a two-step mechanism. [4343] The first step involves the adsorption of the enzyme to the epoxy beads surface, increasing the enzyme density near the epoxy groups. In the second step, the epoxy groups react covalently with the nucleophilic groups on

the surface of the enzyme. Both epoxy beads have a highly hydrophobic polymethacrylate matrix and contain reactive epoxy groups for covalent binding of the enzyme. EP epoxy beads have a one carbon linker, Figure 3, making the support highly hydrophobic. The first step of immobilization, adsorption of the enzyme to the support, requires high concentrations of salts to stimulate hydrophobic interaction of the enzyme with the bead. The effect of various salts on the immobilization of enzymes corresponds to the position of the ions in the Hofmeister series. [44] With increasing salt concentration, the content of the bound enzyme increases till the enzymes are precipitated out of the solution. HFA has a long linker with polar groups, including an amino group. The amino group can bind the enzyme ionically, bringing the protein in the proximity of the reactive epoxy group. Due to the heterofunctionallity of the HFA linker, immobilization on this type of bead can take place under mild conditions at low ionic strength. [43]

Tandem reactions combining chloroperoxidase (CPO) with glucose oxidase (GOX) was previously shown to be to be successful using both free and immobilized enzymes. The coupled enzyme reaction with the free form of CPO and GOX was tested on thianosol as models substrate, yielded a racemic sulfoxide.[45] In addition to the reaction with free enzymes, both enzymes were also co-immobilized onto multi-walled carbon nanotubes yielding HOCl as final product.[46] AOX has also been used in coupled enzymatic reactions with *Horseradish peroxidase* for application in biosensors.[47] In all of the above mentioned tandem reactions, H_2O_2 was produced *in situ* by enzymatic oxidation and used as substrate in the subsequent enzymatic conversion.

The coupled enzyme reaction between VCPO and AOX (Scheme 5) however, was partly successful in the previous project.[48] The tandem reaction was successful for the conversion of monochlorodimedone (MCD), but showed no nitrile formation when glutamic acid was used as substrate.



Scheme 6. Coupled enzyme reaction between VCPO and AOX.

Aim of the thesis

The aim of this thesis is to investigate and find the reaction conditions for the tandem reaction between VCPO and AOX for the sustainable production of nitriles using *in situ* generated hydrogen peroxide.

Immobilization of enzymes might be beneficial, because this enables reuse and might improve properties like stability and activity.[36] The research will be focused on the immobilization of AOX from *H. polymorpha* on two different Relizyme^{TM} polymethacrylate supports (HFA and EP); the enzyme activity and stability of the immobilized AOX will be monitored. As immobilisation could lead to changes in the optimum pH of the enzyme,[49] a pH screening will be performed.

The production of nitriles from amino acids by VCPO is proven to be successful,[7] but the tandem reaction including both VCPO and AOX has not yet been successful on amino acids.[48] It was suggested that glutamic acid and air bubbling were inhibiting AOX. The extent of the inhibition of those inhibitors were determined during the research, and more inhibitors were identified. Based on the extend of the shown inhibition, changing operational conditions of the tandem reaction were essayed.

The AOX activity will be monitored using the coupled enzymatic assay with *Horseradish peroxidase*.[50] VCPO activity will be analysed using the MCD assay.[21] Glutamic acid and 3-cyanopropanoic acid will be monitored by HPLC to determine the activity of the tandem reaction.

Results and discussion

To investigate and to find the optimal reaction conditions for the two enzyme reaction with VCPO and AOX, multiple tandem reactions were performed. The nitrile production of VCPO in a single enzyme reaction, was monitored as comparison for the enzymatic activity of the tandem reaction. Different possible inhibitors were investigated to identify the cause of the presently unsuccessful tandem reaction.

In this study, immobilization of AOX) from *Hansenula polymorpha* was investigated. Immobilization of enzymes enables reuse of the enzyme, easy downstream processing and might improve properties such as stability and activity[36] Because immobilization might influence the optimum pH, a pH screening was performed.

Analysis

For analysis of the tandem reaction by HPLC, the activity of both VCPO and AOX reactions need to be quenched. In this research, a combination of $Na_2S_2O_3$ and a pH change resulting in a final pH of pH=4 was used. The slope in Figure 4 shows the activity of the single VCPO reaction in **A** and the activity of the tandem reaction in **B**. As can be seen, after the change in pH to 4 VCPO is still active, but the tandem reaction is ceased. Upon addition of $Na_2S_2O_3$, the VCPO reaction and possible accumulation of Br^+ are quenched and the reaction does not proceed. The difference in adsorption observed at time point 2, is due to a volume change: the total volume of the reaction is doubled while changing the pH to 4.

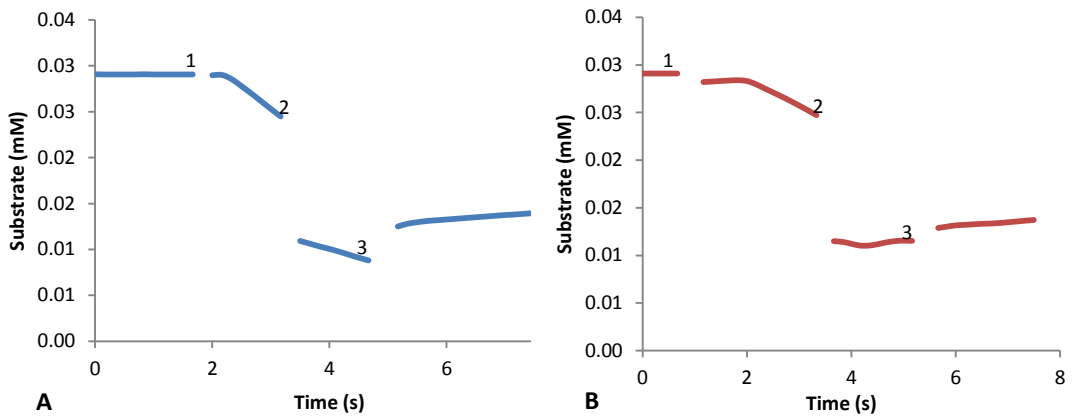


Figure 4. MCD assay: quenching A) VCPO reaction and B) the tandem reaction by a combination of Na2S2O3 and a final pH of 4. 1: enzyme addition. 2: pH change to pH=4. 3: addition of $Na_2S_2O_3$.

In this research coupled enzymatic reaction of AOX with *Horseradish peroxidase* (HRP) is used for the determination of the activity of AOX. The H_2O_2 produced by AOX is assayed by its reaction with ABTS in a HRP catalysed reaction. The accuracy of the ABTS assay was determined by comparing the activity of AOX in the ABTS assay with the oxygen consumption of the enzyme with an oxygen electrode. (Table 1) As the activity of the enzyme stock is similar in both assays, the ABTS assay was determined to be accurate.

Table 1. Comparison of activity of the AOX stock measured by ABTS assay and O_2 electrode.

	Activity enzyme stock (U/mL)
ABTS	1.3
O_2 electrode	1.2

Tandem reaction with MCD as substrate

In previous research, the tandem reaction of VCPO and AOX on MCD has been shown to be successful.[48] The ratio between the two enzymes is crucial in the tandem reaction.[51] If too much AOX is present, H_2O_2 will accumulate in the reaction mixture, inactivating AOX.[52] When H_2O_2 production is not high enough, VCPO will not reach its maximum conversion rate and will inactivate. The inactivation is due to the irreversible dissociation of vanadium from VCPO at acidic pH, which is used in this experiment.[30] VCPO has a higher affinity for the pervanadate than for the vanadate itself,[22] making the enzyme more stable if H_2O_2 is present in the reaction mixture. To determine the operational ratio between VCPO and AOX in the reaction, a MCD assay was performed using different ratios between the enzymes. Furthermore, the MCD assay was scaled up in order to reach similar conditions as for glutamic acid conversion.

Operational ratio between VCPO and AOX

In Figure 5, the activity of the tandem reaction is shown. The reaction of VCPO in presence of H_2O_2 was taken as reference, VCPO having an activity of 0.006 U/mL in this assay. Three different operational ratios between VCPO and AOX were selected 1:1, 1:2 and 1:5.[51] The highest activity was reached when five times more AOX than VCPO was used, reaching an activity of 0.006 U/mL equal to the activity of VCPO in presence of H_2O_2. The same operational ratio of 1:5 was also found to be the optimum ratio for a chloroperoxidase–glucose oxidase coupled enzymatic reaction.[51]

To reach full conversion of MCD, theoretically an equimolar amount of H_2O_2 is required. The optimum operational ratio of VCPO and AOX is 1:5, see Figure 5, which might be explained by partial inactivation of one of the two enzymes. Vanadium is known to reversibly dissociate from VCPO at acidic pH in the absence of H_2O_2.[22] In the tandem reaction it takes some time for AOX to produce a viable concentration of H_2O_2, giving VCPO the opportunity to dissociate. More AOX present in the reaction mixture will overcome this problem as more H_2O_2 can be produced in a shorter amount of time decreasing the possibility of vanadate to dissociate from VCPO.

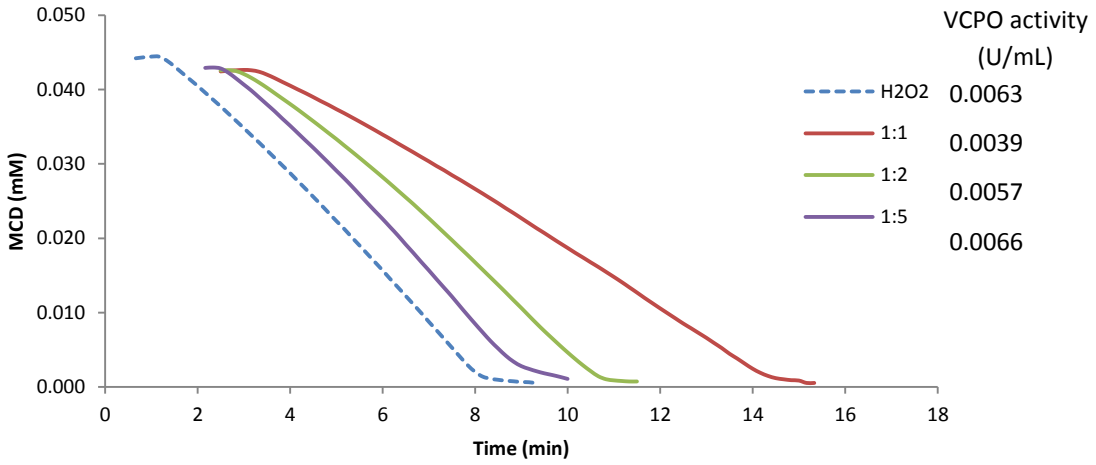


Figure 5. MCD assay of VCPO in tandem reaction with AOX for H_2O_2 production. 50µM MCD was used as substrate at pH 5.6.

In all activity assays of VCPO with MCD as substrate, a lag phase is present. The total length of the lag phase differs between 30 and 50 seconds and represents the time between the moment of addition of the final reagent to the moment when the substrate is consumed. VCPO is kept at pH 8 for storage, according to personal correspondence with R. Wever to maintain its catalytic activity and when added to the acidic reaction mixture (pH 5.6) a protonation change will take place. As protons are involved in substrate binding and activation of VCPO, the protonation state of the enzyme is of great consequence for its activity, explaining the lag phase in this MCD assay.

MCD assay: scaling up

First, the operational stability of the coupled enzyme reaction was tested by determining the activity of the enzyme during a longer reaction time using the standard MCD assay. The operational stability test showed that the tandem reaction can proceed for at least 80 minutes without losing all its activity (Figure 6). This creates

the opportunity to test the tandem reaction at higher substrate concentrations, as longer reaction times are to be expected if more substrate has to be converted.

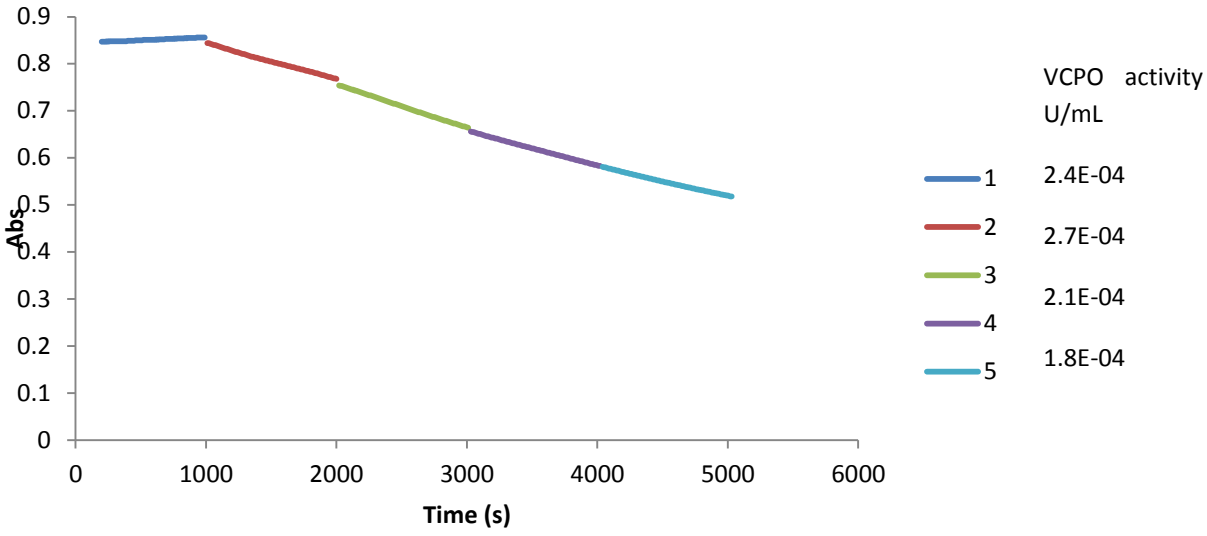


Figure 6. MCD assay of VCPO in tandem reaction with AOX for H_2O_2 production during 85 min. 50 µM MCD and an operational VCPO:AOX ratio of 1:2 were used. The reaction was started at t=2 min by the addition of AOX.

To convert 2.5 mM MCD, an equimolar amount of H_2O_2 and therefore O_2, is need to be available for AOX and VCPO. Water at 21°C only contains 0.28 mM dissolved O_2 so the amount of oxygen needs to be replenished during the reaction.[53] For this purpose different reactions setups regarding the supply of oxygen were tested for the tandem enzyme reaction. The tandem reaction was tested at ambient pressure with and without stirring, at a slight O_2 overpressure with and without stirring and with O_2 bubbling.

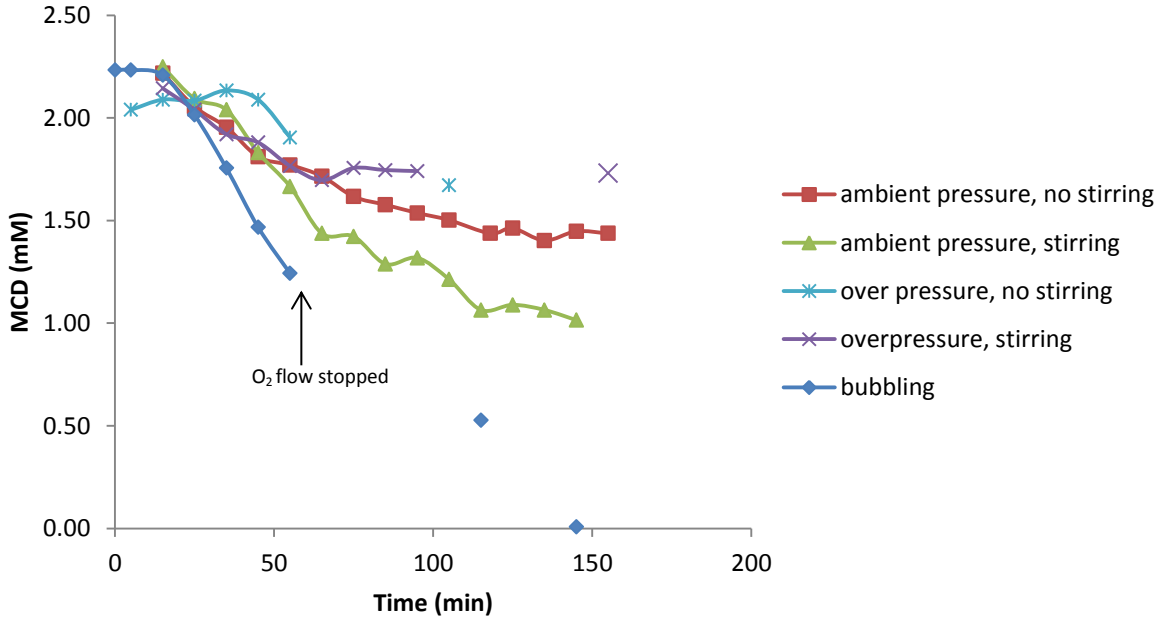


Figure 7. MCD assay of VCPO in tandem reaction with AOX for H_2O_2 production under different reaction conditions regarding oxygen supply. 2.5 mM MCD was used as substrate, a VCPO:AOX ratio of 1:5 was used. The points represent data of a single experiment and the lines were drawn to guide the eye.

In the control reaction at ambient pressure and without stirring only 35% of the substrate was converted, revealing O_2 limitations after about 100 min. The observed inhibition might be due to oxygen limitation for with stirring, the reaction proceeded faster and longer. Stirring promotes the diffusion of oxygen into the reaction mixture by decreasing the liquid diffusion layer and increasing the surface area.[54]

Next, to increase the amount of oxygen in the reaction mixture, a balloon filled with O_2 was used to put a slight O_2 overpressure to the head space of the reaction. The activity of the enzymes was slowed down for both stirred and non-stirred reaction compared to the stirred reaction at ambient pressure. In contrast to the reactions at ambient pressure, the reaction vial was closed, preventing the evaporation of acetaldehyde. As aldehydes are known inhibitors for AOX,[55] the reaction activity might have slowed down by product inhibition of AOX. Moreover, it might be possible that the slight oxygen overpressure influenced the quaternary structure of the AOX, supposedly leading to subunit dissociation. In contrast to the reactions at ambient air pressure and a slight oxygen overpressure, a full conversion of MCD was reached using gentle bubbling of pure oxygen to keep the reaction mixture from O_2 depletion. O_2 supply was stopped during the reaction due to technical problem, as indicated in Figure 7, but even then the reaction reached completion.

The activity plot shows clear trends, but some fluctuations are registered perhaps due to due to difficulties in sampling and dilution protocol. Samples were taken every 5 minutes and diluted to adapt the absorption value to the accuracy range of the spectrophotometer. All the samples were treated separately, increasing the error per measurement. The dilution of the samples also caused a delay in time, because the reaction in the sample could not be stopped without interfering with the assay. Moreover, all the samples were taken from the same reaction mixture, decreasing the total volume per sample. To decrease the total volume as little as possible, only single measurements were taken per data point.

To summarise, the tandem reaction of VCPO and AOX using higher concentration of MCD was found to be succesful using an operational ratio between the two enzymes of 1:5 and using a gently O_2 bubbling for the supply of oxygen to the reaction mixture.

Tandem reaction with glutamic acid as a substrate

VCPO can catalyse the production of nitriles from amino acids by oxidative decarboxylation.[7] H_2O_2 is used as substrate in this reaction (Scheme 1). However, this leads to issues with process unsustainability. Alcohol oxidase (AOX) from *H. polymorpha* can produce this substrate *in situ* increasing the sustainability of the process. AOX is able to oxidise ethanol into acetaldehyde, forming H_2O_2 as by-product. VCPO can in turn use this by-product to convert glutamic acid into 3-cyanopropionic acid in the presence of bromide.

To investigate and to find the optimal reaction conditions for the two enzyme reaction with VCPO and AOX, multiple tandem reactions were performed using glutamic acid as substrate. The nitrile production of VCPO in a single enzyme reaction, was monitored as comparison for the enzymatic activity of the tandem reaction.

Single enzyme reaction

The conversion of glutamic acid into 3-cyanopropanoic acid by VCPO in the presence of H_2O_2 was monitored as a control reaction for the tandem reaction of VCPO with AOX. Instead of H_2O_2 production by AOX, the reaction mixture was supplied with a continuous flow of H_2O_2. The activity of VCPO was determined to be 0.14 U/mL and this is in accordance with previous findings.[56]

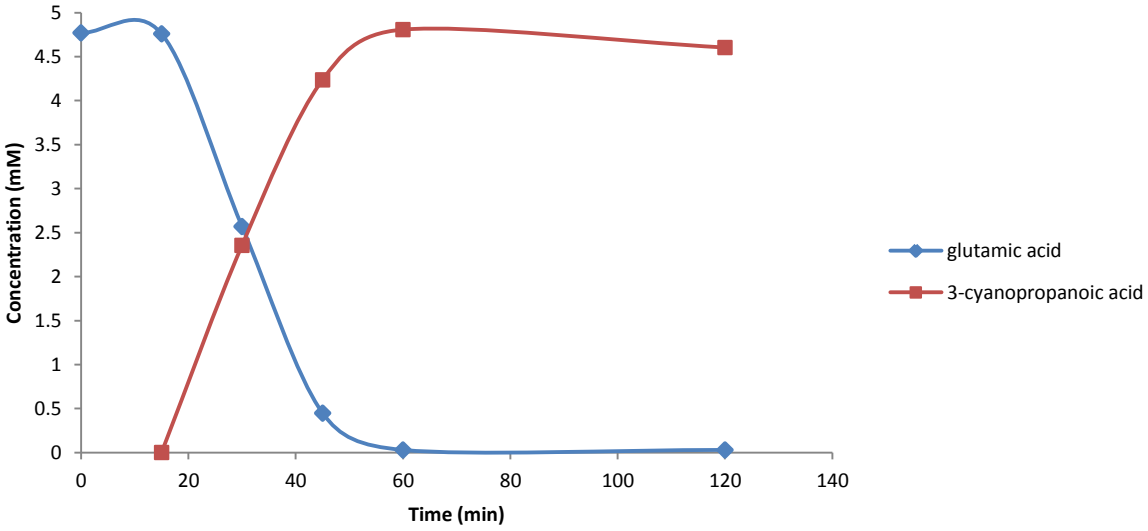


Figure 8. Activity VCPO on glutamic acid. Activity of 0.14 U/mL is based on both glutamic acid decrease and on 3-cyanopropanoic increase. The lines were drawn to guide the eye.

Tandem reaction VCPO and AOX

The tandem reaction was tested for the conversion of glutamic acid to 3-cyanopropanoic acid using a VCPO:AOX ratio of 1:1 and supplying the oxygen to the reaction mixture by gentle O_2 bubbling. As can be seen in Figure 9A, neither glutamic acid was consumed nor any 3-cyanopropanoic acid was produced.

The optimum ratio VCPO to AOX on MCD was 1:5 (Figure 6). As a lag phase is present during the tandem reaction, dissociation of vanadium from the VCPO is possible as explained previously. To reduce the inactivation of VCPO, the tandem reaction was repeated, using a higher AOX concentration (Figure 8B). This reaction showed no improvement compared to the tandem reaction with a 1:1 VCPO:AOX ratio, as no glutamic acid was consumed nor any 3-cyanopropanoic acid was produced.

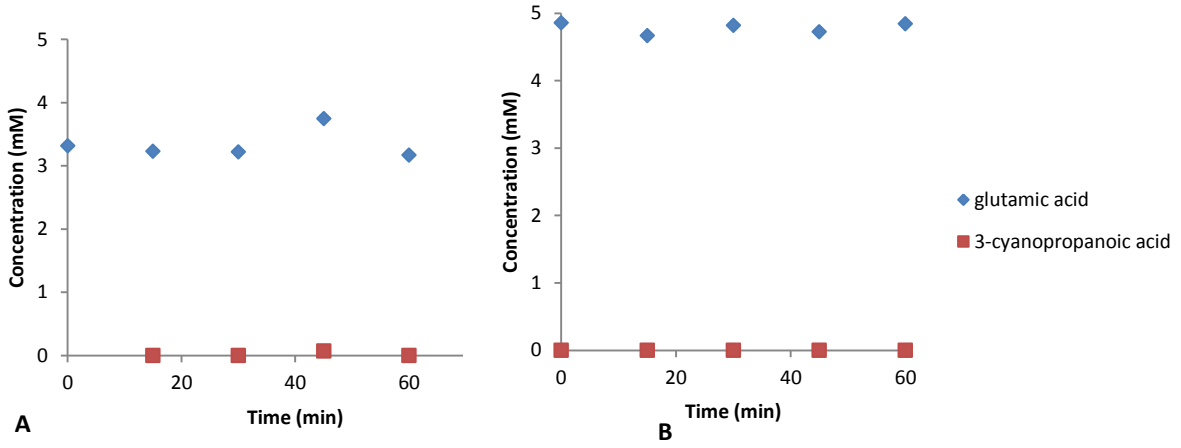


Figure 9. Coupled VCPO and AOX reaction for the production of 3-cyanopropanoic acid. Glutamic acid was used as substrate. A) a ratio VCPO:AOX of 1:1 was used. The data point at 45 min for glutamic acid could be due to an experimental error. B) a ratio VCPO:AOX of 1:5 was used.

During the reaction with a VCPO:AOX ratio of 1:5, the oxygen flow was harsh and irregular and a foam developed on top of the reaction mixture, suggesting that protein denaturation took place. A phenol red test showed qualitatively that after one hour VCPO was still active,[57] therefore it can be suggested that denaturation of AOX happened, resulting in a ceased tandem reaction. AOX can be denatured by the action of air bubbles, as reported in previous research.[48] However, an oxygen flow of 10 mL/min were reported to be suitable for supplying oxygen to the reaction mixtures,[58] which is comparable to the flowrate used in this reaction setup. To prevent AOX from dissociating, the octameric structure has to be stabilized: increased AOX stability was reported on the immobilization of AOX. [36]

Immobilization of alcohol oxidase from *H. polymorpha*

Alcohol Oxidase (AOX) stability is a major problem in its application and as shown above in the tandem reaction as well.[36] The inactivation is suggested to be caused by subunit dissociation of the octamer.[40] Immobilization of AOX is demonstrated to improve the stability of the enzyme and also enables reuse of the enzyme and facilitates downstream processing. Compared to other immobilization techniques, epoxy beads allow easy immobilization protocols under mild conditions and a large amount of enzyme can be immobilized on the support.[43]

In this research, the immobilization of AOX was performed on two types of epoxy beads: one with a long linker amino epoxide, Relizyme™ HFA403, and the other one with a short linker epoxide, Relizyme™ EP403. EP beads require high concentrations of salts to stimulate hydrophobic interaction of the enzyme with the bead, whereas the immobilization of the enzyme on HFA, due to the heterofuncionality of the linker, can proceed at low ionic strength. As subunit dissociation of multimeric enzymes has been reported at high ionic strength,[59] a low ionic strength buffer was used for immobilization of AOX for both supports, which is suboptimal for immobilization on EP supports.

Immobilization yield, protein yield and activity recovery

During the immobilization procedure (see Experimental) the protein content and the enzyme activity in the supernatant were monitored. The protein content was determined by Bradford protein assays and the residual AOX activity was tested by the standard activity assay. The AOX activity in the washing liquids were also tested for residual activity, as it could contain the non-covalently bound enzymes. The immobilization yield and the activity recovery were calculated using equation (1) and (2).[60]

$$\text{Yield}_{immobilization}\ (\%) = \frac{\text{starting activity}_{supernatant} - \text{final activity}_{supernatant}}{\text{starting activity}_{supernatant}} \cdot 100 \quad (1)$$

$$\text{Activity recovery}\ (\%) = \frac{\text{observed activity}_{beads}}{\text{starting activity}_{supernatant}} \cdot 100 \quad (2)$$

The activity in the supernatant at the beginning and at the end of the immobilization was tested, as well as the observed activity of the immobilized enzyme. The protein yield was calculated based on the protein concentrations at the beginning and at the end of the immobilization procedure, using equation (1) where the activity was replaced by protein concentration.

Table 2. Immobilization yield, activity recovery and protein yield AOX from *H. polymorpha*. immobilised on Relizyme™ supports.

Type of Relizyme™ support	Immobilization yield (%)	Activity recovery (%)	Protein yield (%)	Protein load (%)
EP	76	3	28	0.05
HFA	100	53	95	0.02

The immobilization yield, or the enzyme activity that is immobilized based on the loss of enzyme activity in the supernatant, is 100% for HFA and 76% for EP. The protein yield of immobilization on HFA beads, was similar to the immobilization yield. The protein yield on EP beads was 28%. Despite the high yield, the activity recovery shows that the activity of the enzyme immobilized on HFA is half the activity of the free enzyme. High immobilization yields of AOX were reported in literature on HFA beads, whereas no results are known for EP.[40] Literature reports a 80% activity recovery of AOX on amino-epoxy Sepabeads, which is higher than the 53% presented in this experiment.

The difference in immobilization and protein yield between the two beads could be due to several factors. First of all, as explained earlier, EP beads need a high ionic strength buffer to be activated. High protein immobilization on methacrylate beads has been reported using 1 to 1.5 M phosphate buffer,[44] confirming that the 10 mM potassium phosphate buffer used in this research was not saline enough to induce hydrophobic adsorption.

The discrepancy between the immobilization yield and the activity recovery can partly be explained by steric hindrance of the enzyme by the beads. Studies revealed that a structural change follows substrate binding,

showing a closer subunit association of the enzyme-substrate complex[61]. Covalent binding of the enzyme to the epoxy bead will complicate conformational changes of the enzyme, in particular the EP beads due to the short linker length. The restricted mobility of the enzyme on the EP beads might also cause impaired access to the active site of the protein. Due to the rigid protein coordination on the bead, orientation of the active site plays an important role in activity recovery: only if the active site of enzyme is exposed to the medium, it will be able to catalyse the reaction.

Another reason for the difference in immobilization yield and activity recovery can be subunit dissociation. AOX stability has been shown to be dependent on the protein concentration in the solution, suggesting that subunit dissociation is involved in the inactivation of the enzyme. [40,62] Because immobilization is not specific and because its octameric structure consists of two layers of four subunits,[31] it is possible that not all eight subunits are covalently bound, resulting in a partially stabilized quaternary structure.[40] During the immobilization procedure, the concentration of proteins in solution decreases and the enzymes are less stabilized by protein-protein interactions.

The difference in activity recovery between HFA and EP might be caused by the different mechanism of adsorption of the enzyme to the bead. Adsorption of the enzyme on EP is dependent on hydrophobic interaction with the surface of the bead, whereas HFA uses ionic interaction to physically bind the enzyme to the linker.[43] The difference in absorption mechanism implies that the enzyme interacts with the bead via different areas, resulting in dissimilar enzyme coordination. As the orientation of the enzyme is important for enzyme activity, activity recovery might be affected.

The activity recovery is based on the observed activity of the immobilized enzyme. This measured value however, is not accurate. Because an unsuitable stirring bar was used for the homogenisation of the reaction mixture, some beads were crushed during the reaction, resulting in losses of immobilized enzyme. Moreover, the crushed beads floated in the reaction mixture interfering with the spectrophotometric measurement. This could increase the observed absorbance and therefore, yield a higher activity recovery.

There is a large difference between the immobilisation yield and protein yield concerning the EP beads. Where only 28% of the protein was immobilized, nearly 75% yield was obtained. This might be due to inactivation of the enzyme in the supernatant during the immobilization. In case of HFA, no such decrease was measured, possibly indicating an error in the activity measurement of the supernatant during AOX immobilization on EP beads.

In the rest of this research only enzymes immobilised on HFA were used.

Storage stability of immobilized AOX

To determine if the immobilized AOX is stabile during storage, its activity was tested. After 35 days of storage at 4-10 °C, the activity of the immobilized beads halved (Table 3). It must be noticed that the beads were taken out of the fridge repeatedly, increasing the temperature of the stock temporarily. This might lead to lower measured stability as enzyme activity can possibly be decreased at higher temperatures over time.

Table 3. Sorage stability at 4-10°C of AOX immobilised HFA after 35 days .

Time (days)	Activity (U/g)
0	0.53
35	0.27

pH screening

It is known in literature that immobilisation could lead to changes in the optimum pH of enzymes.[63] To assess if this occurs with the AOX immobilised on HFA support, a pH screening was performed. As can be observed in Figure 10, the pH activity profile of the immobilised enzyme is similar to the one of the free enzyme, displaying an increased activity at higher pH. At higher pH values the activity of the enzymes have reached a plateau. AOX shows no activity up to pH 4, not even after immobilisation. This is not in agreement with the pH activity profile observed for AOX from *H. polymorpha* immobilized on DEAE-cellulose particles,[39] which does show activity at pH 4. Moreover, a pH profile of the AOX reported by John R. Woodward shows a steep increase in activity between pH 5 and 6 from reaching its maximum at 6.5.[64] Woodward used a 100 mM sodium citrate buffer, as

opposed to the 20 mM in this research. The difference in pH profiles might be caused by the ionic strength of the buffer used.[36] At higher pH values, the free enzyme shows higher activity than the immobilized one which can be explained by a higher affinity of the free enzyme for the substrate. This phenomenon has been previously reported in literature on AOX immobilization.[39] The lower substrate affinity of the immobilized enzyme can be explained by the decreased availability of the active site.

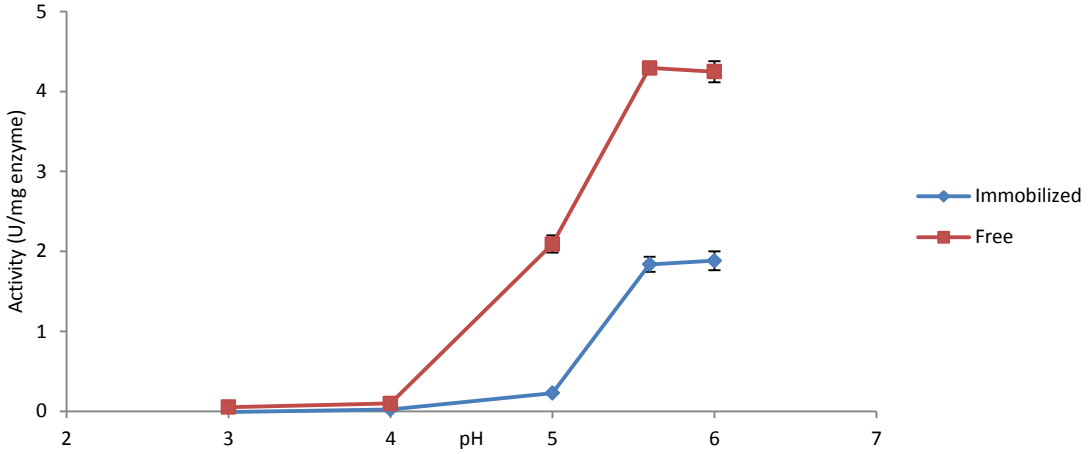


Figure 10. pH screening of free and immobilized AOX on ABTS at different pH. 100 mM ethanol is used as substrate. The error bas represent standard deviation, the lines were drawn to guide the eye.

Tandem reaction with immobilized AOX using glutamic acid as substrate

The coupled enzyme reaction of VCPO and AOX was suggested to be ceased by AOX denaturation. After stabilization of the enzyme on HFA beads, the tandem reaction was performed using immobilized AOX instead of the free enzyme. As can be seen in Figure 11, no glutamic acid was consumed during the reaction. Although foam formation was difficult to determine due to the mixing and bubbling of the reaction mixture with enzyme immobilized beads, it was possible to observe the absence of protein smear on top of the reaction mixture. This is in contrast to the observation of foam formation in previous tandem reaction, (Figure 9) indicating a possible stabilization of AOX upon immobilization. Partial inactivation of AOX might still be possible, but it cannot explain the total lack of activity in the coupled enzyme reaction. Other mechanisms of inhibition or inactivation of AOX are likely to play a role as well.

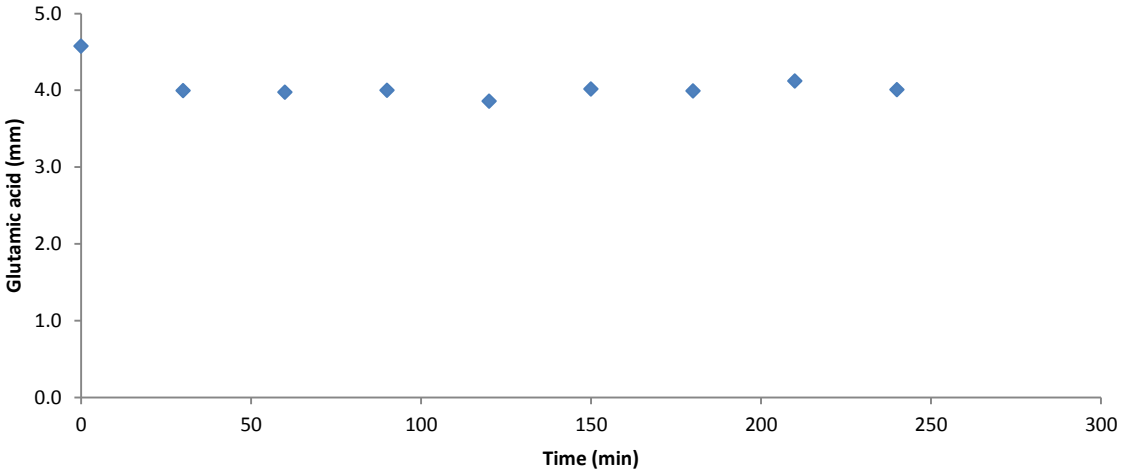


Figure 11. Coupled VCPO and immobilized AOX reaction for the production of 3-cyanopropanoic acid. 5 mM glutamic acid was used as substrate. A ratio VCPO:AOX of 1:2 was used.

Inhibition of the tandem reaction might be caused by several factors, e.g. VCPO inhibition by ethanol and NaBr influence on AOX, which were excluded in previous research.[48] Glutamic acid however, was shown to have an inhibitory effect on AOX, but no explanation was provided concerning the mechanism of this phenomenon. Here, in addition to the above mentioned study, the influence of glutamic acid and 3-cyanopropanoic acid on AOX activity was investigated.

Influence of glutamic acid on AOX activity

To assess the effect of glutamic acid on AOX, standard AOX activity assays (see Experimental) were performed under different conditions. (Figure 12A). First, the activity of AOX in the absence of glutamic acid was tested as reference for the inhibition assays. Then, the activity of free AOX was tested in the presence of glutamic acid at pH 5.6. Under those conditions no inhibition was observed. Furthermore, AOX activity was essayed without correction of the pH of the glutamic acid solution resulting in a final pH value of the reaction mixture of 4.9. The activity of AOX in the presence of glutamic acid at pH 4.9 shows inhibition of the enzyme, similar to the inhibition curve observed in the previous research.[48] The activity of AOX from *H. polymorpha* is shown to drop dramatically between pH 4 and 6, with total inactivation at pH 4 (Figure 10). This observation explains the difference in activity of AOX when a low pH was used. The pH of the glutamic acid solution used in the above mentioned tandem reactions was 5.6 (Experimental) excluding glutamic acid as inhibitor for the idle tandem reaction.

In addition to the inhibition assay with glutamic acid, the effect of 3-cyanopropanoic acid on AOX activity was investigated. 3-cyanopropanoic (5 mM) appeared to have no inhibitory effect on AOX activity as can be seen in (Figure 12B)

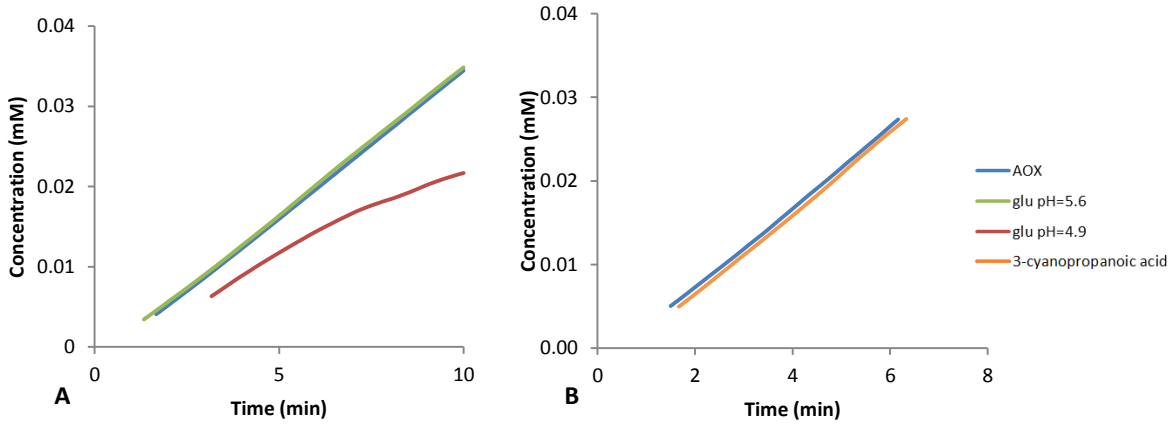


Figure 12. AOX activity based on ABTS assay. A) 5 mM glutamic acid is used as inhibitor. The glutamic acid solution added to the ABTS assay differed in pH. The lines representing the activity of AOX in the presence and absence of glutamic acid overlap B) 5 mM 3-cyanopropanoic acid was used as inhibitor.

Tandem reaction at low glutamic acid concentration

To determine if oxygen was the limiting reagent for the tandem reaction, the reaction between VCPO and the free AOX enzyme was tested using an amount of glutamic acid (0.14 mM) below the saturation limit of O_2 in water. In the tandem reaction, to convert one equivalent of glutamic acid by oxidative decarboxylation reaction, two H_2O_2 equivalents are used requiring two moles of oxygen. According to literature,[53] at 21 °C and atmospheric pressure the saturation concentration of oxygen in water is 0.28 mM , therefore, if 0.14 mM Glu are converted no O_2 limitation should occur during this reaction. As depicted in Figure 13, the glutamic acid concentration is decreased by 20% after 20 minutes of reaction, but the conversion reaches an early stop after 30 minutes.

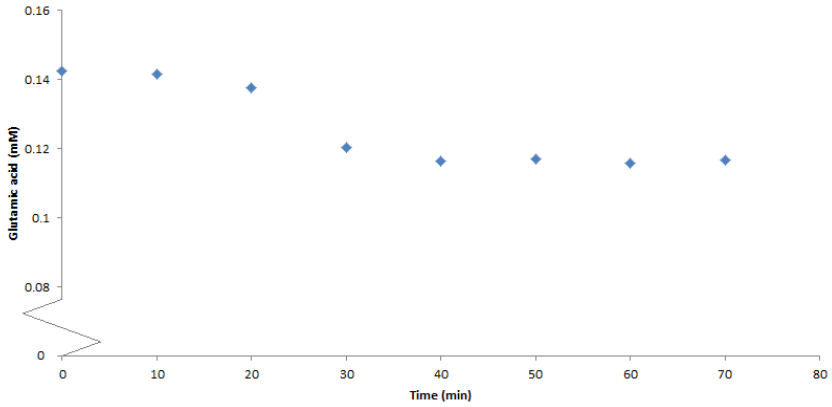


Figure 13. Coupled VCPO and AOX reaction for the production of 3-cyanopropanoic acid. 0.14 mM glutamic acid was used as substrate, the VCPO:AOX ratio of 1:5 was used. The conversion of glutamic acid was performed in the HPLC with direct injection from the reaction mixture.

A lag phase is present in this reaction and is approximately 20 minutes long. The lag phase in coupled enzyme reactions is calculated to be the sum of the lag phases of both enzymes,[65] so is expected to be longer than the lag phase observed for VCPO on MCD (Figure 5). Especially with VCPO, the protonation the change in protonation state between the buffer and the reaction mixture has a major influence on the activity of the enzyme. Moreover, it is suggested that a slight Br^+ accumulation is needed to start oxidative decarboxylation.

As can be seen, a low VCPO activity (0.0017 U/mL) is reached between 20 and 30 minutes after the start of the reaction. This activity is quickly slowed down, converting just 26 µM in total. The discontinuation of the reaction is probably caused by inactivation of one or both of the enzymes These results could be explained by the contamination of the reaction mixture with the derivatisation agents, o-phthaldialdehyde and ethanethiol, used in the HPLC analysis of glutamic acid. The reaction was performed in the HPLC at 20 °C and samples were taken by autosampler directly from the reaction mixture. The HPLC method involves mixing the derivatisation agent with a sample of the reaction mixture in the injection loop of the HPLC, with a wash of the needle only

after the injection in the column. This causes a contamination of the reaction mixture with o-phthaldialdehyde, which is quite reactive towards amines or thiols; and with ethanethiol, which can react with cysteine residues in the enzyme, resulting in possible inactivation of the two enzymes. The derivatisation agents are likely to inactivate the enzyme by reacting with enzyme residues involved in substrate binding. The cofactors in the active site are not likely to be affected because they are shielded against reactive agents in both enzymes: the FAD of AOX is buried in the protein matrix[66] and the active site of VCPO has a hydrophobic channel leading to the vanadate.[67] Another explanation for the impaired reaction might be inhibition of AOX by acetaldehyde.[55] The reaction was performed in a closed HPLC vial, hindering the evaporation of acetaldehyde.

The derivatisation program of the HPLC was changed for another tandem reaction, adding a wash step between the sampling of the o-phthaldialdehyde and the sample. In this reaction no glutamic acid consumption was measured, favouring the suggestion of acetaldehyde inhibition in this assay. Due to these technical impediments no clear conclusions can be drawn regarding the oxygen limitation for the tandem reaction with glutamic acid.

Influence of Br^+ on AOX activity

VCPO induces oxidative decarboxylation by producing Br^+ as highly reactive reaction intermediate. Scheme 1. In the MCD assay, the Br^+ production is thought to be the rate limiting step.[68] The reaction with glutamic acid however, proceeds much slower denoting different reaction kinetics. It might be possible that the rate-limiting step shifted from Br^+ production to the oxidative decarboxylation. Consequently, Br^+ can accumulate in the reaction mixture, inactivating AOX. To determine the effect of Br^+ on the activity of AOX immobilized beads, the beads were incubated for two minutes with a Br^+ solution and the activity was subsequently measured. (Figure 14) Br^+ was produced *in situ* by the reaction of NaOCl with NaBr, however this reaction is exothermic and energy is released into the reaction mixture as heat. The heat is not likely to cause inactivation of the enzyme as the temperature stability of AOX is reported to be stable up to 40 °C.[64] Although milled beads interfered with the spectophotometric assay, a clear difference is seen between the beads incubated with Br^+ and the blank.

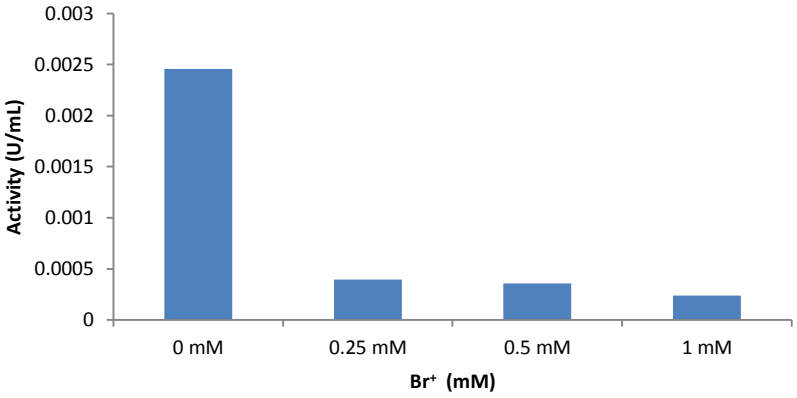


Figure 14. AOX activity on ABTS. 110 mM ethanol is used as substrate. The beads with immobilised AOX are first incubated with a 1:1.5 NaOCl:NaBr solution; incubations were performed with 0, 0.25, 0.5 and 1 mM NaOCl. After 2 min incubation, the Br^+ solution was removed and the remaining activity of AOX was determined.

Compartmentalized tandem reaction

To prevent Br^+ inhibition during the tandem reaction, a setup was made in which the two enzymes were physically separated. The immobilized AOX was placed in a packed bed above the reaction mixture containing VCPO. The reaction mixture passed through the packed bed in about 100 min resulting in a flow rate of 1.4 mL/min. This flow rate would ensure a theoretical production rate of H_2O_2 of 0.006 U. The reaction mixture containing H_2O_2, acetaldehyde and unreacted ethanol was supplied in drops to the fed-batch reactor containing the reaction mixture for the conversion of glutamic acid.

Despite the physical separation of the two enzymes, the conversion of glutamic acid to 3-cyanopropanoic acid was not successful. This could be caused by various factors such as AOX inhibition by H_2O_2 and acetaldehyde, O_2 limitations or Br^- depletion.

The activity of VCPO was measured qualitatively by phenol red [57] assay after 100 minutes and showed to be present. AOX inhibition in the reaction mixture could have been caused by the accumulation of H_2O_2 due to low flow rates. However, according to the manufacturer of the AOX, the K_i value for H_2O_2 is in the range of 5-10 mM [21]and the calculated maximum concentration of H_2O_2 in the packed bed at the end of the reaction would have been about 4 mM. Even if the enzyme was inhibited by the production of higher concentrations of H_2O_2, the conversion of glutamic acid to 3-cyanopropanoic acid should have occurred at the beginning of the reaction

Oxygen was supplied to the reaction mixture with AOX by bubbling. Measuring the amount of oxygen present in the mixture is difficult due to small working volumes compared to the size oxygen electrode. However, oxygen limitation is not likely to be the inhibitor of the reaction as the tandem reaction with 0.14 mM glutamic acid was also unsuccessful.

Another plausible explanation for the idle tandem reaction is the consumption of Br^+ by a competitive reaction with the oxidative decarboxylation of glutamic acid. A known reaction with Br^+ and acetaldehyde is an acid catalysed halogenation reaction. The acetaldehyde is first tautomerized into to give an enol (vinyl alcohol). A brominated end-product is subsequently formed via a stabilized cation. [69] In addition to the halogenation, a reaction of Br^+ with acetaldehyde is known converting the aldehyde into a carboxylic acid.[70] (Figure 15)

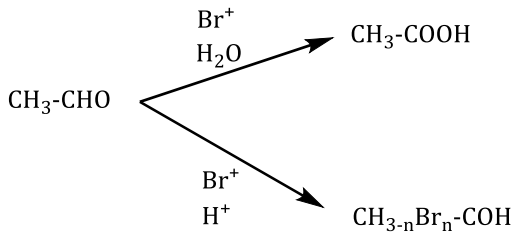


Figure 15. Suggested competitive reaction mechanism of the tandem reaction.

Assumed that alpha halogenation takes place, a depletion of the halogen source from the reaction mixture will take place as the halide will be incorporated into the brominated acetaldehyde. When Br^+ reacts with the aldehyde formed by AOX, in a faster reaction than with glutamic no conversion into 3-cyano propionic acid will occur. This reaction also explains the unsuccessful tandem reaction in closed vials. If acetaldehyde in unable to evaporate from the reaction mixture, it will react with the Br^+ species. A physical separation of the two enzymes did not result in 3-cyanopropanoic acid production, which accords with the hypothesis as the separation will not circumvent the reaction of acetaldehyde with the reactive bromide species.

Conclusions

The aim of this thesis was to investigate and find the reaction conditions for the tandem reaction between VCPO and AOX for the sustainable production of nitriles using *in situ* generated hydrogen peroxide. For this purpose multiple tandem reactions were performed under different conditions. In order to stabilize the AOX quaternary structure, the enzyme was immobilized on epoxy activated beads.

The operational ratio between VCPO and AOX has been found to be optimal at a 1 to 5 ratio. Hydrogen peroxide stabilizes VCPO and as a consequence of accumulation of this compound in the early reaction phase will keep VPCO from inactivation. Owing to the stability of the tandem reaction over 80 min, higher substrate concentrations could be investigated. The coupled enzyme reaction was shown to be working on higher concentrations of MCD. To reach full conversion of the substrate, gentle oxygen bubbling was used to keep the reaction mixture from oxygen depletion.

The immobilization of AOX was most successful on HFA beads, the immobilization yield was 100% and the protein yield was 95%. The recovered activity of the immobilized enzyme was approximately half the activity of the free enzyme. The steric hindrance of the enzyme by the bead can attributed to the difference in immobilized yield and recovered activity. If the enzyme is covalently bound to the bead on multiple points, structural changes upon substrate binding might be hampered. The difference between the immobilization yield and activity recovery may also be explained by subunit dissociation of the octamer, as possibly not all subunits are stabilized by the covalent attachment to the bead. The activity of the immobilized enzymes is shown to be halved over 35 days at storage conditions of 4-10 ^{o}C, The pH screening showed that the immobilized enzyme had a similar pH profile to the free enzyme.

The coupled enzyme reaction was performed multiple times under different conditions. The influence of enzyme concentration, oxygen limitation and compartmentalization on the reaction was determined. Under none of those conditions was glutamic acid produced or 3-cyanopropanoic acid consumed. Glutamic acid was excluded as inhibitor on AOX, but Br^{+} did inhibit the enzyme. However, when the tow enzymes were physically separated, thereby preventing inhibition of AOX by Br^{+}, the reaction still did not proceed. The reaction is suggested to be ceased by competitive reaction of Br^{+} with acetaldehyde, the end-product of AOX. Two reactions of Br^{+} with aldehydes exist: an acid catalysed alpha halogenation yielding a halogenated end-product and a reaction yielding a carboxylic acid. In both cases the Br^{+} will react with the acetaldehyde in a competitive reaction to the tandem reaction, ceasing oxidative decarboxylation.

It was shown that immobilization was successful and could result in better stability of the enzymes under operational conditions. The reuse and downstream are facilitated by the use of immobilized enzymes, which can potentially make it more feasible for large scale productions. If this can be achieved on a large scale, the use of ammonia for the production of nitriles could be significantly reduced. The coupled enzyme reaction was not successful, but a competitive reaction of the activated halide with acetaldehyde is suggested to inhibit the tandem by competitively reacting with Br^{+}.

Recommendations

Immobilization

The AOX stability has been improved by immobilization of the enzyme on HFA amino epoxy supports. After immobilization of the enzyme on HFA beads, possible subunit dissociation took place because not all subunits were attached to the support. It would be interesting to immobilize the enzyme by cross linking the enzyme to form aggregates (CLEA)[41] to stabilize the quaternary structure of the enzyme. The enzymes are cross-linked via reaction of lysine and arginine residues on the enzyme surface with a bi-functional chemical cross-linker. The disadvantage of the system is that end-product inhibition occurs. However, this might be overcome by the elimination of acetaldehyde by evaporation.

Relizyme^{TM} beads are crosslinked polymethacrylate spheres. A reaction of Br^+, the reaction product of VCPO, with the beads might take place if there are double bonds present in the support. Polymethacrylate does not contain double bonds, but the crosslinker might. Information regarding this subject is difficult to obtain, due to protection of product specifications by the manufacturer. Further research should be done regarding the influence of Br^+ on the methacrylate beads.

Stirring of the reaction mixture containing enzyme immobilized beads poses the problem of grinding. An unsuitable stirring bar was used, leading to grinding of the beads. Upon grinding, the enzymes are pulled apart, leading to partial inactivation of the enzyme. Moreover, the grounded beads floated around in the reaction mixture, interfering with the spectrophotometrical assay. An ellipsoidal stirring bar was used, where as a triangular bar was preferred. The contact area of the triangular bar is a flat plane, so no beads can be milled between the stirring bar and the bottom of the reaction vial. To improve the activity assay of immobilized enzyme, a more suitable stirring method is recommended.

In the activity assay of immobilized AOX on ABTS, stirring was needed to distribute the beads over the reaction mixture. Stirring can only take place outside the spectophotometer making time courses difficult to execute. Quenching of the AOX reaction is done using saturated thiosulphate. In the ABTS assay, the thiosulphate will react with the ABTS leading to substrate depletion for the activity test. Due to the continuation of the reaction between sampling and the measurement, a time deviation between the samples was created during time course reactions. In addition to the time error, stirring of the reaction caused milled beads to float in the reaction mixture. Both these causes give rise to an inaccuracy in activity assays of enzyme immobilized beads. Another activity assay might be interesting to develop, making accurate time courses of immobilized AOX possible. In literature a recircular system, consisting of a 20 ml glass stirred tank, a peristaltic pump, continuous flow optical cell and a packed-bed bioreactor connected in series, is used for activity determination of immobilized AOX. The volume of the reaction however, was 20 mL which might be too large for our application.[36]

Tandem reaction

The AOX enzyme is sensitive towards air bubbling. 10 mL/min was reported to be suitable for supplying oxygen to reaction mixtures containing the free AOX.[58] In this research, a flowmeter was used to regulate the oxygen flow to the reaction. The meter however, was not accurate at flowrates lower than 25 mL/min. To regulate a suitable airflow in future research, it is advised to use a flowmeter that is more accurate at low flowrates.

In unsuccessful tandem reactions, it is possible to qualitatively determine VCPO activity. The activity of AOX in the reaction mixture might also be interesting to test. It is possible to qualitatively and quantitiatively The qualitative determine the presence of H_2O_2 with a chromium pentoxide test.[71] The sample of the acidic reaction mixture is mixed with a little diethyl ether, amyl alcohol or amyl acetate and a few drops potassium dichromate solution. After gentle shaking the upper organic layer will be coloured blue in the presence of H_2O_2. To qualitiatively determine the amount of H_2O_2, a potassium permanganate titration can be performed.[72,73]

The tandem reaction is suggested to be inhibited by the reaction of Br^+ with acetaldehyde. To determine if either a carboxylic acid or a halogenated compound is formed in the competitive reaction, the reaction mixture can be tested on its pH to observe acidification of the reaction mixture or analysed by mass spectrometry for the presence of brominated compounds. A compartmentalized tandem reaction might be interesting to test, using air stripping of the reaction mixture between the separated AOX and VCPO reactors. It is not possible to evaporate acetaldehyde from the reaction mixture containing AOX, as the enzyme will be inactivated by the air bubbling.

Experimental section

Materials

ABTS – 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (≥98%), Bradford reagent, BSA (98%), Citric acid (99%), L-Glutamic acid (≥98%), H_2O_2 (35 wt%), NaBr (99%), NaOCl (5-10 wt%) were purchased from Sigma-Aldrich; Na_2HPO_4 and NaH_2PO_4 from Riedel-De Haën. EtOH - ethanol (100%) was bought from VWR Chemicals and $Na_2S_2O_3$ from Merck. 3-Cyanopropanoic acid (95.9%) was provided by Interchim and monochlorodimedone by BioResource Products.

Alcohol Oxidase from *Hansenula polymorpha* (EC 1.1.3.13) Horseradish Peroxidase from *Horseradish* (EC 1.11.1.7) were purchased from Sigma-Aldrich. Vanadium chloroperoxidase (EC 1.11.1.B2) plasmid was expressed in *E. coli* using a protocol described elsewhere[47]. The obtained VCPO (40-60% purified) was concentrated and stored in 100 mM Tris/H_2SO_4 buffer pH=8.2 containing 100 µM Na_3VO_4 at -20°C and has an activity of 92 U/mL (based on MCD assay). VCPO was supplied by the group of Biobased Chemistry and Technology, Wageningen University.

Relizyme™ EP403 (epoxide) and HFA403 (epoxide on an amino linker) used in this study are epoxy activated methacrylate beads with pore sized in the range of 200-500µm. Both were kindly donated by Alessandra Carminati from Resindion S.R.L. (Mitsubishi Chemical, Milan, Italy)

Standard procedure alcohol oxidase activity assay

Free alcohol oxidase activity assay

The reaction solution (2 mM ABTS in 20 mM sodium citrate buffer pH 5.6) was saturated with O_2 by gentle bubbling for 5 min before the reaction. In a 2 mL disposable cuvette a reaction mixture containing 2 mM ABTS, 20 mM sodium citrate, 110 mM EtOH and 0.83 U/mL HRP (from a stock of 3.0 mg HRP/mL in 20 mM sodium citrate buffer pH 5.6) was prepared to a total volume of 1.51 mL. The reaction was started by addition of 30 µL AOX solution (4.0 mg/mL of 20 mM sodium citrate buffer pH 5.6), was mixed and followed in time by spectrophotometer (DU® 720 UV/Vis spectrophotometer; Beckman Coulter) at 405 nm.

Immobilized alcohol oxidase activity assay

1490 µL oxygen saturated reaction buffer was added to 10 mg of beads in a 4 mL disposable cuvette and after addition of 0.83 U/mL HRP the absorbance was measured by spectrophotometer (DU® 720 UV/Vis spectrophotometer; Beckman Coulter) at 405 nm. The reaction was started with 10 µL absolute EtOH and stirred at 100 rpm. After 2 minutes a 1 mL sample was taken and transferred to a 2 mL disposable cuvette: the absorbance was determined

Standard assay VCPO with monochlorodimedone as substrate

1.025 mL of monochlorodimedone (MCD) reaction mixture (50 µM MCD, 5 mM NaBr in 20 mM sodium citrate buffer pH 5.6) saturated with O_2 and 10 µL of VCPO (32U/mL in 100 mM Tris/SO_4 +VO_4Na_3 buffer pH 8.3) were added to a 2 mL disposable cuvette. The reaction was started upon addition of 66 mM H_2O_2 to the reaction mixture.

Immobilization procedure

1.00 gram dry weight of both beads (Relizyme™ HFA403 and EP403) was transferred to a 10 mL polystyrene screw-cap tube, duplicates were made per type of beads. A 0.25 mg/mL alcohol oxidase solution was prepared in a 10 mM phosphate buffer (pH 7.0). To each tube 4.5 mL enzyme solution was added. 2 mL enzyme solution was transferred to a 5 mL screw-cap tube to serve as blank. All tubes were fastened to a rotator (Stuart rotator SB3) and incubated for 24 hours at room temperature (21°C) at 5 rpm.

After 24 hours, the beads were filtered (filter pore size 25-50 µm) with reduced pressure. The beads were washed twice with 4 mL 20 mM sodium citric acid buffer (pH 5.6) and the washing waters were collected. The beads were stored filter-dry at 4-10°C in a 10 mL screw-cap tube. The protein content was determined with Bradford protein assays (Sigma-Aldrich). Residual activity was tested with AOX activity assay for the free enzyme.

pH screening of the immobilised and free AOX

The pH screening was performed according to the standard assay for AOX activity (for free and immobilized enzyme) at different pH values of the oxygen saturated reaction solution (pH 3; 4; 5; 5.6 and 6) per assay.

Tandem reaction VCPO-AOX

Was done according to the standard procedure for VCPO with MCD assay. However, the reaction was started by the addition of 45 µL AOX and 100 mM EtOH was used as substrate. The bromination of MCD was followed in time at 290 nm.

Operational ratio VCPO:AOX

Different VCPO:AOX ratios were tested according to the standard VCPO assay, except the AOX concentration was changed (1:1; 1:2 and 1:5) by changing the dilution factor of AOX to 10, 5 and 2 respectively.

Stability test

The stability test was done using the same 50 µM reaction buffer, but a 0.00063 U/mL VCPO and 0.00126 U/mL AOX was used.

MCD assay: scaling up

In a 4 mL cuvette, 930 µL MCD reaction mixture (2.5 mM MCD, 5 mM NaBr and 100 mM EtOH in 20 mM sodium citrate buffer pH 5.6) and 8 µL of VCPO was added. The reaction was started by addition of 62 µL AOX. At 10 minutes interval samples were taken, diluted 100 times in a 2 mL disposable cuvette and measured by spectrophotometer (DU® 720 UV/Vis spectrophotometer; Beckman Coulter) at 290 nm. Activity tests were performed with an oxygen supply by bubbling of approximately 1 mL/min, a slight oxygen overpressure of the headspace by a balloon filled with O_2 and no oxygen feed. In the cases of slight O_2 overpressure by balloon and no oxygen feed, both stirred and non-stirred reaction were tested.

Standard tandem reaction VCPO-AOX with glutamic acid as substrate

A reaction mixture containing 5 mM glutamic acid, 5 mM NaBr, 100 mM EtOH and 0.14 U/mL VCPO with various amounts of AOX stirred at 200 rpm for 1 hour at room temperature (21 ^{o}C), with an air supply of ca. 1 mL/min. Samples were taken directly from the reaction mixture, quenched with 10 µL saturated $Na_2S_2O_3$ and diluted with 40 µL 20 mM citric acid pH 2.5 to a total dilution factor of 2. For HPLC analysis of glutamic acid samples were further diluted to a total dilution factor of 10 with 400 µL MeOH absolute, 400 µL 20 mM citric acid buffer (pH 4) and 100 µL internal standard. 3-cyanopropanoic acid was analysed without other dilutions by HPLC.

Fed-batch reactor

The single enzyme reaction of VCPO was done, using 16 mM M H_2O_2 as substrate instead of EtOH and AOX in the standard reaction mixture. A solution of 0.5 M H_2O_2 was added to the reaction mixture at a rate of 66 µL/hour using a NE-1600 syringe pump from ProSense.

Enzyme concentration

The tandem reactions VCPO:AOX were performed at a 1:1 ratio (0.13 U/mL AOX) and a 1:5 ratio (0.72 U/mL AOX). The tandem reaction with immobilized AOX was performed over 4 hours (stirred at 90 rpm), using enzyme concentrations of 0.0048 U/mL VCPO and 0.0096 U/mL AOX (VCPO:AOX 1:2).

Compartmentalized setup

The compartmentalization was done using a packed bed AOX reactor on top of a fed-batch VCPO reactor. The packed bed reactor consisted of a 2 mL Discardit™ syringe containing a 1 cm cotton wool (flushed with 20 mM sodium citrate buffer pH 5.6). 300 mg HFA beads with immobilized AOX (0.06 U/mL) were weighed and added on top of the cotton wool and flushed with buffer. The syringe was equipped with a 0.20 µm filter and a needle and placed above the fed-batch reactor containing 1 mL reaction mixture (5 mM of glutamic acid, 5 mM of NaBr) and 0.098 U/mL VCPO. The reaction was started by the addition of 2 mL reaction buffer (100 mM EtOH in 20 mM sodium citrate buffer pH 5.6) to the packed bed. The final volume of the fed-batch reactor after 100 min was 2.4 mL.

Analysis

Amino acids were analysed by derivatisation as previously described[7]. Nitriles were analysed without dilution or derivatisation by using an UltiMate 3000 from Thermo Scientific. Detection was achieved using a RI-101 detector from Shodex set at 35°C. The columns used were a Rezex ROA Organic acid H+ (8%) column (7.8 × 300 mm) from Phenomenex, at 35°C with a flow of 0.5 mL/min. The elution was carried out using 12 mM H_2SO_4 and the quantification was performed by external standard method.

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