Biobased Chemistry and Technology

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Sustainable co-production of H_2O_2 and chemicals by oxidases

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Summary

Hydrogen peroxide is a powerful and versatile oxidant and is considered an environmentally friendly compound because its only degradation product is water, but the methods that are used to produce it are not as "green" as the compound itself.

This report presents the development of a new route for a more sustainable production of hydrogen peroxide (H_2O_2) together with chemicals in a tandem reaction. In the first part of the reaction alcohol oxidase (AlOx) oxidases ethanol with O_2 producing H_2O_2 and acetaldehyde. In the second part H_2O_2 is consumed by vanadium chloroperoxidase (VCPO) within the oxidative decarboxylation of amino acids to nitriles.

Three commercially available alcohol oxidases from *Pichia pastoris, Candida boidinii* and *Hansenula polymorpha* were tested at different pH and at different buffer, substrate and oxygen concentration in order to find the most suitable conditions to perform the tandem reaction with VCPO.

The enzymatic activity was followed by H_2O_2 detection by using a coupled enzymatic assay of AlOx with a peroxidase from *Horseradish*. Oxidative decarboxylation of amino acids with VCPO was tested and the amino acid and the nitrile were analysed by HPLC. Furthermore the tandem reaction was tested with MCD assay and the acetaldehyde production was investigated by acetaldehyde assay procedure.

The optimal conditions to couple the AlOx with VCPO are AlOx from *H. polymorpha* oxidising 100 mM ethanol in 20 mM sodium citrate buffer pH 5.6.

The tandem reaction works successfully with monochlorodimedone as substrate for VCPO. The reaction was also performed with glutamic acid as substrate, but due to inhibition of AlOx by glutamic acid no conversion to nitriles was detected. The same inhibition effect is observed for aspartic acid, but no inhibition is caused by the aliphatic amino acid, valine.

Furthermore it was assessed that acetaldehyde can be easily removed from the reaction mixture, therefore, an easy downstream process should be enough to recover the by-product from the solution.

Hence oxidative decarboxylation by VCPO combined with *in situ* production of H_2O_2 by AlOx was assessed and further research is needed to find suitable amino acids to be converted into nitriles for industrial applications.

Introduction

Oxidation processes play an essential role in chemical industry for the conversion of many kinds of hydrocarbon feedstock, such as olefins, alkanes and aromatics, to industrially important oxygenated products (organic acids, aldehydes, anhydrides, etc.)[1].

Molecular oxygen is usually the oxidant of choice for these processes, but because of selectivity problems related to the radical nature of the reaction involved and to the severe conditions required, a wide use of it in synthetic chemistry is not easily applicable[2].

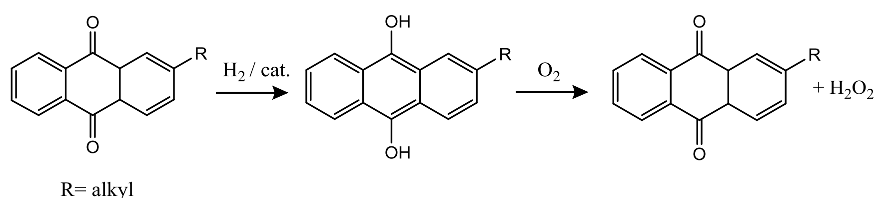
The most attractive alternative to molecular oxygen is hydrogen peroxide.

The global production of H_2O_2 is about 2.2 million metric tons per year and besides the usage in chemical synthesis it has wide applications in textile industry, paper bleaching, wastewater treatment and production of detergents[3].

As oxidant, it presents the following advantages:

- water is the only by-product of the oxidation reactions
- hydrogenation of oxygen is a well know process and several methods are available such as catalytic hydrogenation with Pd or Au and the antraquinone process[2,3].
- the chemistry of hydrogen peroxide has been well developed over the years[2]
- it has a high oxidation potential over the all pH range (E_0 =1.763 V at pH 0, E_0 =0.878 V at pH 14)
- it is a strong oxidant of organic and inorganic substrate under very mild reaction conditions[3].

The common production method, which covers more than 95% of the global demand, is the anthraquinone oxidation process, a reaction cycle in which hydrogen, atmospheric oxygen and anthraquinone derivatives are employed[3]. The mechanism involves 2-alkyl-9,10-anthraquinones, which react with H_2 in presence of a catalyst (e.g. palladium) leading to formation of hydroquinones. Then O_2 oxidises the hydroquinones to quinones and H_2O_2 is simultaneously formed and extracted with water afterwards, while the quinones are recycled[4]. The mechanism is shown in Scheme 1.



Scheme 1. Anthraquinone oxidation reactions.

The main advantages of the anthraquinone oxidation process are the high yield, the possibility of continuous production at mild temperatures and the possibility to avoid direct contact between O_2 and H_2, which causes an explosive reaction[3].

However, this method presents many drawbacks, among which there is the formation of several by-products leading to consumption of the catalyst, which needs to be regenerated. Moreover formation of organic and metal impurities occurs and separation steps need to be added to the downstream process. Also, the process is energy intensive because the product is extracted at a concentration of 30% H_2O_2, concentrated up to 70%[3] and diluted again at the application step if employed for laboratory purposes where concentrations in the mM range are needed. Regarding the high concentration of the product, a main disadvantage is also the storage and transportation risk, because presence of catalytic impurities in the product can cause decomposition of H_2O_2, a highly exothermic reaction (-98.3 kJ/mol in solution) that leads to serious explosions and special materials and conditions are required to avoid it. Concentrations up to 8% are safe to transport without special restrictions[4].

Some alternatives to anthraquinone autoxidation have been studied, such as direct synthesis, catalysed by palladium, gold or other metals, which produces H_2O_2 directly from hydrogen and oxygen. This process needs further optimization to avoid direct contact between hydrogen and oxygen. Furthermore side reactions can lead to fast decomposition of hydrogen peroxide[5]. Photocatalysis, production with fuel cells or synthesis from $CO/O_2/H_2O$ mixtures have also been investigated, but none of them can be considered as a valuable substitution to anthraquinone approach so far.

In situ enzymatic production of H_2O_2 by oxidases is a further alternative for production of H_2O_2 and is the one which is investigated in this research. By using the enzymatic approach some of the downsides of anthraquinone process, such as transportation and storage issues, cross-contaminations and side reactions could be avoided or bypassed because the reaction occurs in mild conditions and it does not produce by-products nor catalytic impurities[3]. Furthermore the broad range of substrates that oxidases can use allows an oxidation of substrate into product which generate equimolecular amounts of H_2O_2 with a selectivity that is close to 100%[3]. The broad range of substrates convertible by enzymes also allows to focus on the sustainability of the process, with special attention on the choice of the substrate, preferably contained in biobased material coming from rest streams of other biobased processes. The economic feasibility of the process is also a main criterion in this research and it can be reached with the biocatalytic approach by choosing an enzyme which can generate a valuable product for industrial applications as intermediate for other chemicals or as final saleable product.

Production of nitriles with vanadium chloroperoxidase

The practical necessity to investigate an enzymatic approach has risen from the need of producing H_2O_2 *in situ* to use it as an oxidant in the reaction of oxidative decarboxylation of amino acids to nitriles by the enzyme vanadium chloroperoxidase (VCPO).

Nitriles are important compounds for chemical industry and they are characterised by the presence of a triple bound carbon-nitrogen as functional group[6]. Nitriles are employed as starting material or intermediate in the synthesis of polymers (nitrile butadiene rubber), adhesives (cyanoacrylate), solvents (acetonitrile), pharmaceuticals, pesticides and resins[7]. Many synthetic pathways are employed in industry for production of nitriles, such as the widely used ammoxidation with ammonia and the minor dehydrogenation of amines and amides and electrohydrodimerisation[6].

However, with the prospective of transition of the chemical industry from a petrochemical industry to a biobased economy, production of nitriles has been studied also considering renewable starting materials which already contain nitrogen in their molecule, such as the non-essential amino acids contained, for example, in the rest streams from bioethanol (dried distiller's grains with soluble known as DDGS and vinasse from sugar beet or sugar cane) and biodiesel (meals or seedcakes of rapeseed, soybean and *Jatropha*) production[8]. These compounds are not necessary for production of food or feed so they can be a suitable starting material for the production of valuable chemicals[9].

Conversion of amino acids into nitriles requires an activated halogenating species (X^+), which is formed by the *in situ* oxidation of a halogen source (X^-: bromide or chloride salt). The conversion is well known in literature and it has been performed by chemical approach, electrochemical approach, heterogeneous catalytic approach and biocatalytic approach. The chemical approach uses a combination of NaOCl, NaBr and low temperature, which requires high energy consumption for cooling and high amount of salt[10]. This is not an issue in case of the electrochemical approach, where bromide is oxidised continuously to BrO^- at the anode of an anode-cathode system. However, electricity has to be supplied continuously and methanol is used as main solvent[11]. Both catalytic approaches have the advantages that they can be performed at room temperature, only catalytic amounts of the halogen source are required, water is used as solvent and only electricity for stirring has to be supplied. The catalysts serve to generate the activated halogenating species *in situ*. Both catalytic approaches require the use of hydrogen peroxide for the regeneration of the hypohalous acid after reacting with the amino acid. In the heterogeneous catalytic approach, the catalyst is peroxotungstate immobilised on a layered double hydroxide and prepared by co-precipitation of Ni^{2+} and Al^{3+} nitrates followed by anion exchange with Na_2WO_4[12]. The biocatalytic approach does not involve such a complex procedure and involves the enzyme haloperoxidase[13].

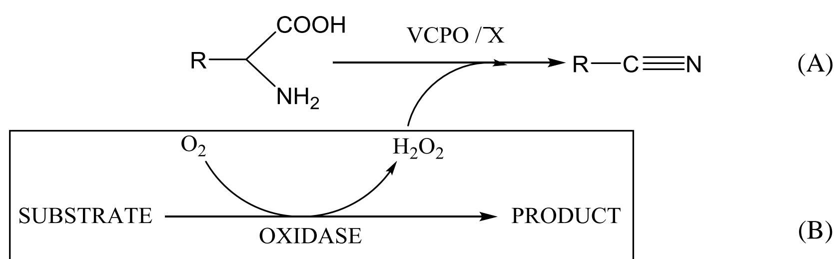
Due to its simplicity this last method was chosen as approach to convert amino acids into nitriles and the enzyme of interest is VCPO because it has a wide substrate range, high termostability (90ºC), high storage stability at -20 ºC, high resistance towards oxidation, stability in presence of organic solvents as well as aqueous solvents[13]. The reaction of VCPO combined with the halogen source and H_2O_2 can be seen in Scheme 2-A.

Normally H_2O_2 is added to the solution and it has been shown that if it is supplied in too high concentration it can lead to deactivation as it is in the case of Fe-Heme chloroperoxidase[14]. Furthermore there are unpublished results demonstrating that also VCPO is inhibited by high concentration of H_2O_2.

To overcome the problem of VCPO deactivation and to obtain H_2O_2 *in situ* without facing the issues regarding the current industrial production explained above, the solution can be to perform a tandem reaction together with oxidases, which produce hydrogen peroxide by reduction of oxygen and

conversion of a substrate into a product[15] (Scheme 2-B). Besides having the advantage of maintaining a lower local hydrogen peroxide concentration[16], this reaction could be exploited for the production of both H_2O_2 and a valuable product, depending on the substrate of choice, the enzyme that is used and the reaction conditions.

The overall mechanism of the tandem reaction is shown in Scheme 2.



Scheme 2. Overall reaction for nitrile production. (A) conversion of amino acids into nitriles by vanadium chloroperoxidase (VCPO), coupled to (B) H_2O_2 production by oxidases.

Oxidases

Oxidases are enzymes from the subclass of oxidoreductases that catalyse an oxidation-reduction reaction. Typically the oxidation of a substrate into a product is taking place by using molecular oxygen as electron acceptor and reducing it in most cases to hydrogen peroxide[17].

There are two main oxidases families based on type of cofactor: one utilises copper in various oxidation forms, the second one utilises flavin-based cofactors such as FAD (flavin adenine dinucleotide). The flavin-containing oxidases are the most abundant in nature, especially the major group of the FAD-based enzymes, which have the advantage that the cofactor is regenerated during the catalytic cycle[15]. Therefore, this research was focus on FAD-oxidases.

Based on their substrate, these enzymes can be classified in glucose-methanol-choline oxidoreductases, which include various alcohol oxidases such as short-chain alcohol oxidases and aryl alcohol oxidases, vanillyl alcohol oxidases, amine-oxidases, sulfhydryl oxidases and acyl-CoA oxidases[15].

In order to meet both the desired reaction conditions and the economic feasibility of production of H_2O_2, the following criteria were formulated to select the most suitable oxidases for this research:

- SUBSTRATE
  - It should come from biomass rest streams or biobased materials
  - It should be efficiently converted by the enzyme
- PRODUCT
  - It should be obtained from the reaction mixture through an easy downstream processing
  - It should not have negative or even positive effect on reaction kinetics (working at V_{max}, no product inhibition, etc.)
  - It should have valuable application
- OXIDASE CHARACTERISTICS
  - It should work together with VCPO in a tandem reaction
  - It should work in mild working conditions: room temperature, aqueous solution and pH around 5.6 (similar to VCPO)
  - It should have a K_m and k_{cat} values similar to VCPO
  - The cofactor should be easy to regenerate

Some attempts of combining production of nitriles from amino acids and production of H_2O_2 by oxidases have been made over the last years.

For example, an hybrid system has been designed in which glucose oxidase was co-immobilised with a chloroperoxidase onto carbon nanotubes for the *in situ* production of hypochlorous acid (HOCl) with decontamination purposes. This experiment was successful and showed the tandem reaction was able to generate the product HOCl[18].

In addition, another work described co-immobilisation of chloroperoxidase (CPO) from *C. fumago* with glucose oxidase from *A. niger* to test the production of 2-oxindole from indole using H_2O_2 generated *in situ*. This experiment had positive outcomes and it was found that in situ formation of H_2O_2 by glucose oxidase can avoid deactivation of the CPO by high concentration of H_2O_2. Furthermore it was established

that the reaction is strongly pH dependent, with maximum indole conversion and H_2O_2 formation at pH 5.5 and it is not dependent on the concentration of the substrate glucose[16].

Based on the criteria mentioned above and after analysing the literature and BRENDA database to get information about the main oxidases families, three main groups have been selected: glucose oxidases, aryl-alcohol oxidases and short-chain alcohol oxidases. Among the several oxidases they have the advantages that they need only FAD as cofactor, which is regenerated during the catalytic cycle[15] and they have a K_m that in the same range as VCPO.

Glucose oxidase has the advantage that is the most known oxidase and the reaction in tandem with VCPO has already been performed but it needs glucose as a substrate, which is not contained in biomass rest streams but is found in biomass rich in carbohydrates. Furthermore the product of the oxidation is a lactone which is rapidly hydrolysed to gluconic acid in presence of H_2O, a compound that is difficult to separate from the reaction mixture and causes lowering of pH[19].

Aryl-alcohol oxidases have the advantage of producing aromatic aldehydes such as benzaldehyde which is an important building block for synthesis of natural compounds and drugs[20], but the boiling point of this compound is high (179 °C), therefore is not easy to separate it from the reaction mixture. Furthermore the optimal pH for these enzymes is slightly higher than the optimum for VCPO and the turnover frequency is quite low[21]. Moreover, the aromatic alcohols are contained in lignin, which is a widely investigated source for biorefinery, but lignin depolymerisation and product isolation is challenging and at the moment the lignin fraction obtained from lignocellulosic material is burnt or gasified[22].

Short-chain alcohol oxidases convert short chain alcohols such as methanol, ethanol, propanol into their corresponding aldehydes but the longer the carbon chain the lower the reaction rate[23]. Thus methanol (MeOH), due to the low number of carbons, is the primary alcohol which oxidases have the highest affinity for[23], but the product formaldehyde is highly toxic and is associated with carcinogenic effects in case of inhalation[24].

Ethanol (EtOH) is already worldwide produced as fuel from fermentation of glucose[25]. As MeOH, it is efficiently recovered and recycled, but a little amount can still be found in the rest stream and considered as a substrate for our reaction[26].

The product derived from oxidation of EtOH is acetaldehyde, widely used as precursor of acetic acid[25], as intermediate for resins and fertilisers and for chemicals such as butadiene, vinyl acetate, acrolein and pyridine[27]. Furthermore acetaldehyde does not present safety problems for the usage required in this research. A main advantage is also that acetaldehyde is highly volatile (boiling point +20 °C)[28], so it can be easily separated from the solution with several available methods such as distillation, adsorption, condensation and pervaporation. In this way the cost of the downstream process to extract acetaldehyde can be consistently lowered[29]. In addition, K_m is still in the same order of magnitude as VCPO.

Short-chain alcohol oxidases (AlOx) are thus the oxidases of choice for performing production of H_2O_2 and EtOH is used as a substrate.

Aim of the thesis

The aim of this research was to develop a new route for a more sustainable production of hydrogen peroxide together with chemicals by *in situ* formation and consumption in the following tandem reactions:

- The production of H_2O_2 by AlOx
- The conversion of amino acids to nitriles using VCPO/NaBr and the H_2O_2 produced in the first step

Different commercially available AlOxs were tested in this research. The goal consisted in determining the optimum working conditions for the production of hydrogen peroxide and products, in this case acetaldehyde, in relation with further oxidation reactions and downstream processing. It is known that AlOxs from *O. angusta* have a temperature range between 25 and 80 °C at which they are active[30]. According to the experiments of Jung et al. (2008) the H_2O_2 formation rate is strongly affected by pH and it reaches a maximum around pH 5.5[14]. If AlOx will behave similarly, it would be an advantage for performing the tandem reaction with VCPO, since its optimum pH is 5.6. From this the first research question is formulated:

How do the reaction conditions (temperature, pH, solvent) affect the production of H_2O_2?

It is known from literature that many oxidases are inhibited by their product H_2O_2[30]. The hypothesis is that this issue could be overcome if H_2O_2 is consumed by a second reaction meanwhile is produced by the oxidases, so that the concentration in the solution is kept low. The second research question is thus arisen:

Is it possible to produce H_2O_2 in situ and consume it by a second reaction to avoid product inhibition?

Previous experiments with glucose oxidases showed that variations of glucose concentration have only little effect on the H_2O_2 formation rate and thus glucose concentration does not limit the H_2O_2 production[16]. It is necessary to establish if EtOH has the same effect on the AlOx, so the third research question is:

How does the concentration and the addition of substrate affect the production of H_2O_2 with AlOxs? How is substrate inhibition related to H_2O_2 production?

Regarding the product acetaldehyde, it is known that it can be recovered from aqueous solutions through well-established methods such as distillation, pervaporation, condensation, adsorption[29]. These techniques require specific materials and conditions that have to be considered in order to make the reaction feasible. Therefore the forth research question is:

Is it possible to remove the by-product, acetaldehyde, from the reaction mixture to avoid downstream processing?

Once the conditions for the production of H_2O_2 with AlOxs are established, the tandem reaction of oxidase together with VCPO will be tested to check if the overall system works. As explained before, tandem reaction with chloroperoxidase and glucose oxidase has already been performed for oxidation of indole[14]. A last research question needed to be investigated to see the feasibility of the reaction in the context of this research:

Is it possible to have a tandem reaction with an AlOx together with vanadium chloroperoxidase to convert amino acids into nitriles?

Approach

Three different commercially available AlOx from *Pichia pastoris, Candida boidinii* and *Hansenula polymorpha* were tested at different pH, buffer concentration, substrate concentration and oxygen concentration. The enzymatic activity was followed by H_2O_2 detection by using a coupled enzymatic assay of AlOx with a peroxidase from *Horseradish*[31].

Oxidative decarboxylation of amino acids with VCPO was tested and the amino acid and the nitrile were analysed by HPLC. Furthermore the tandem reaction was tested with MCD assay[32] and the acetaldehyde production was investigated by acetaldehyde assay procedure[33].

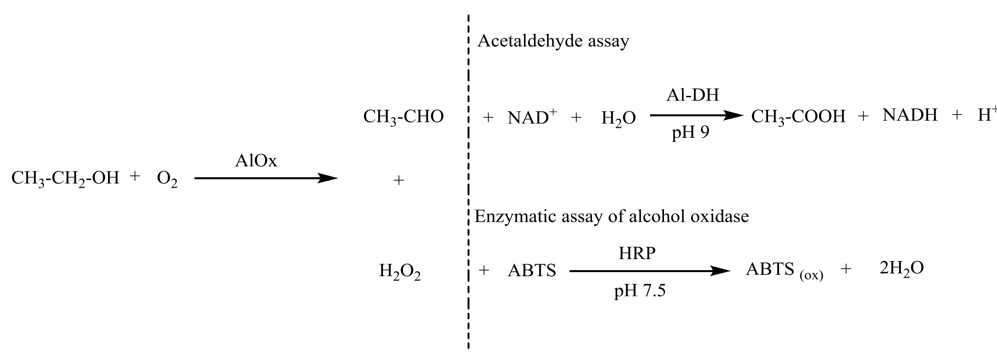
Results and discussion

I. Reaction parameters screening for alcohol oxidase

In order to be able to use an AlOx in tandem with VCPO it is necessary to find the AlOx which can work at similar conditions as the vanadium chloroperoxidase. Several parameters have been tested for this purpose:

- pH of the reaction
- buffer concentration
- substrate concentration
- oxygen concentration

The main experiments were carried out by using an acetaldehyde assay to analyse the production of acetaldehyde and an enzymatic assay of AlOx to analyse the activity of AlOx and thus the H_2O_2 production. An overview of the correspondent reactions is shown in Scheme 3. In the first assay acetaldehyde is converted by Aldehyde dehydrogenase and NAD^+ to acetic acid and NADH and in the second assay H_2O_2 is oxidizing ABTS (2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)) by Peroxidase from *Horseradish* (HRP). NADH and the oxidized ABTS formation is monitored by spectrophotometric analysis (see experimental section for more details).



Scheme 3. EtOH conversion into acetaldehyde and H_2O_2 by AlOx and O_2 and the coupled assays to determine the formation of the products.

pH of the reaction

In order to be able to use an AlOx in tandem with VCPO it is necessary to find an enzyme which can work at similar conditions as the vanadium chloroperoxidase. The first criterion is the pH of the reaction. VCPO activity is optimal at pH 5.6 and it is preferable to maintain this condition to ensure good performance for the nitriles production. The commercially available AlOxs which have been tested have an optimal pH between 7 and 9, depending on the organism of provenience.

Figure 1 shows the results of a pH screening (7.5, 6.6 and 5.6) of the activity of three different AlOxs (*P. pastoris*, *C. boidinii* and *H. polymorpha*).

The activity decreases for all the enzymes with lowering of pH. The activity of AlOx from *P. pastoris* shows a peak at pH 6.6 but at pH 5.6 – the optimal pH for VCPO – the activity decreases by 60% compared to pH 6.6. The activity of AlOx from *C. boidinii* is progressively decreasing with the decrease of the pH until no activity is registered at pH 5.6. Lastly, the activity of AlOx from *H. polymorpha* drops dramatically between 6.6 and 5.6 by losing half of its activity, but nevertheless it remains the one that performs the best in all the conditions.

According to the protocol, the activity should result 0.1 U/mL when MeOH is used as substrate. Because EtOH is used less efficiently[34,35], the activity can be lower. To overcome this issue an assay based on MeOH should be performed to asses internally the activity of each AlOx.

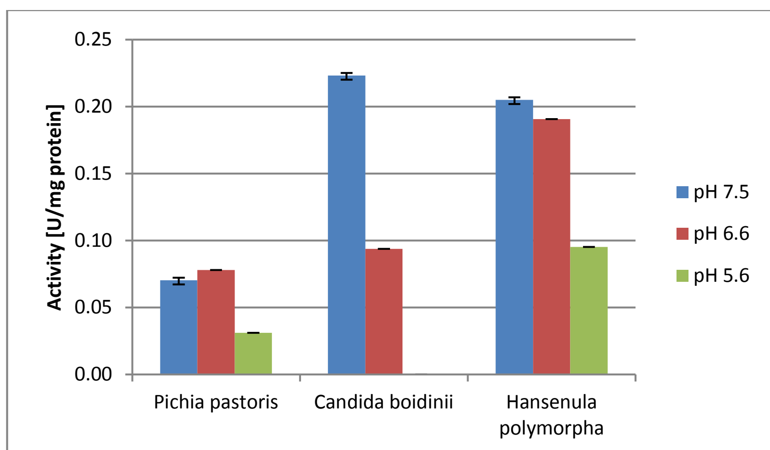


Figure 1. pH screening of the activity of the AlOx from *P. pastoris*, *C. boidinii* and *H. polymorpha*. 0.1 U/mL of AlOx based on MeOH assay were used in each experiment. The results represent the average of duplicates. A Bradford assay was used to determine the concentration of the protein in solution and the results are shown in Table 3 and Figure 20 in Appendix.

It is known that AlOxs are active over a pH range from 6 to 9[36,35] but it can be seen that AlOx from *P. pastoris* and *H. polymorpha* can still work also at slightly lower pH.

It is necessary to point out that the buffer concentration was not the same for the three pHs (see experimental section). Due to wrong choice of the buffer, the results do not have the maximum accuracy, therefore a pH screening only for the AlOx from *H. polymorpha* was performed with 100 mM sodium citrate buffer at pH 6.6 and 5.6. The other two enzymes were not considered for this experiment due to low activity at acidic pH.

The results can be seen in Figure 2, where the activity is compared to the previous experiment. It can be observed that at the same pH the lower the buffer concentration, the higher the activity is and this will be demonstrated with further results.

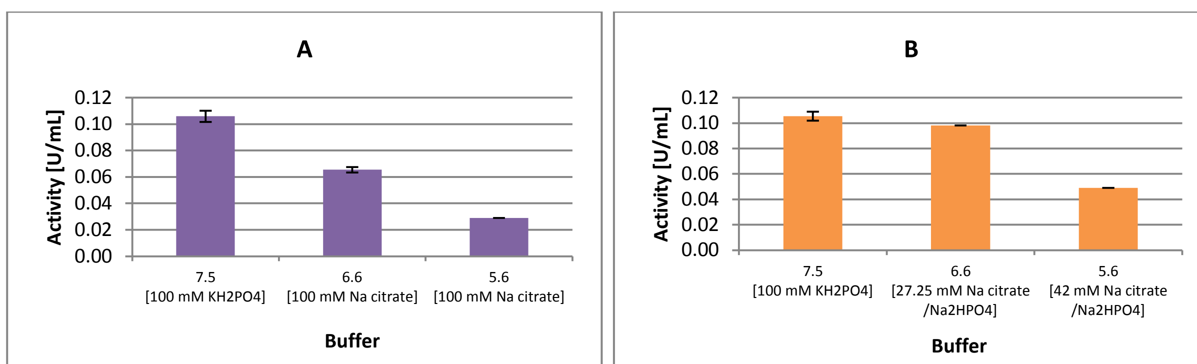


Figure 2. pH screening of the activity of AlOx from *H. polymorpha* **(a)** with 100 mM buffer and **(b)** with three different buffer concentrations (100 mM, 27.25 mM and 42 mM respectively). The results represent the average of duplicates.

Although the drop in activity at pH 5.6 was 72% of the initial value at pH 7.5, AlOx from *H. polymorpha* showed the best performance among the three enzymes, so it was chosen to go on with other experiments.

Buffer concentration

The activity of an enzyme is also influenced by the concentration of buffer which is used in the reaction[37]. Based on previous results and to match the requirements for the VCPO activity which works at 20 mM sodium citrate buffer it was necessary to see how the AlOx performs at different concentrations of buffer.

Figure 3 shows the results of a screening of the AlOx activity at different sodium citrate buffer concentrations at pH 5.6.

It can be seen that the lower the buffer concentration, the higher the activity. A concentration of 20 mM ensures the best performance of the enzyme, almost double than with a buffer concentration of 100 mM

when EtOH is used as a substrate. With MeOH as substrate the performance is even higher and with 20 mM of buffer three times more H_2O_2 is produced with respect to EtOH at the same buffer concentration (results not shown).

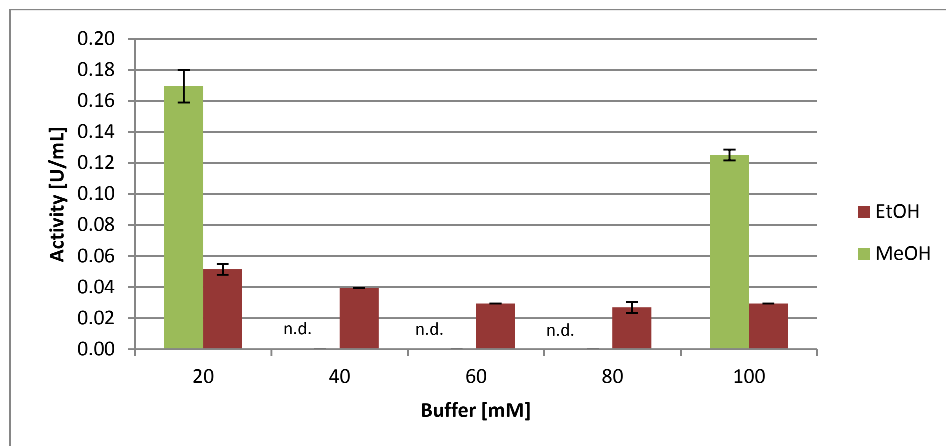


Figure 3. Activity of AlOx from *H. polymorpha* at different sodium citrate buffer concentration at pH 5.6 with EtOH or MeOH as substrate. The results represent the average of duplicates.

Previous experiments regarding a highly purified AlOx from *H. polymorpha* used as immobilised biosensor show a 65% decrease in activity by going from a 5 mM to a 50 mM phosphate buffer pH 7.7, but the mechanism has not been elucidated yet[38].

20 mM seems to be the best option, but was necessary to check whether the higher activity observed was also due to the fact that ABTS, which has 2 sulfonyl groups, is changing the pH of the solution now that the buffer concentration is lower and thus the buffering capacity is less strong. Further investigation has been done by measuring the pH in the solution containing ABTS, at time zero and after 15 minutes from the addition of the enzyme. No change in pH was observed, so it can be concluded that the higher activity is connected only to the lower buffer concentration.

Substrate screening

In order to see the difference between alcohols with different chain length, MeOH and EtOH were used as substrate and the activity of AlOx from *H. polymorpha* was measured at different pH values and the concentration of H_2O_2 produced was calculated (Table 1). The activity in presence of MeOH is higher than in presence of EtOH and this is reflected also on the concentration of H_2O_2 produced by the enzyme.

Table 1. Comparison between activity and H_2O_2 production of AlOx from *H. polymorpha* in presence of either MeOH or EtOH at different reaction pH. The results represent the average of duplicates.

Alcohol	pH	Activity [U/mL]	Activity [%]	Conc. H_2O_2 [mM/min]
MeOH	7.5	0.243	100	0.008
MeOH	6.6	0.211	86.83	0.007
MeOH	5.6	0.128	52.51	0.004
EtOH	7.5	0.106	43.62	0.003
EtOH	6.6	0.066	27.16	0.002
EtOH	5.6	0.029	12.12	0.001

According to literature, the longer the carbon chain of the alcohol, the lower the reaction rate[34]. In the case analysed here, there is a decrease of 56.38% at pH 7.5 and 77.34% at pH 5.6 for AlOx from *H. polymorpha*, showing a more dramatic drop at low pH. This phenomenon is confirmed by literature, which reports that the activity of AlOx from *P. pastoris* in presence of EtOH at 37 $^{\circ}$C and in 100 mM potassium phosphate buffer pH 7.5 is 82% of the activity in presence of MeOH at the same conditions[35].

In conclusion, AlOx from *H. polymorpha* is the selected enzyme for production of H_2O_2 in the tandem reaction with VCPO because it maintains a relative good activity at pH 5.6. MeOH is the preferred substrate, but due to high toxicity of its product formaldehyde it was not chosen as reagent for this research. However it will be further monitored together with the chosen substrate EtOH to keep an overview on the comparison between the two.

Substrate concentration screening

The concentration of the substrate is another important parameter which needs to be optimised in order to ensure the best performance of the AlOx. Figure 4 shows the different EtOH and MeOH concentrations that were tested to see the change in activity of the enzyme.

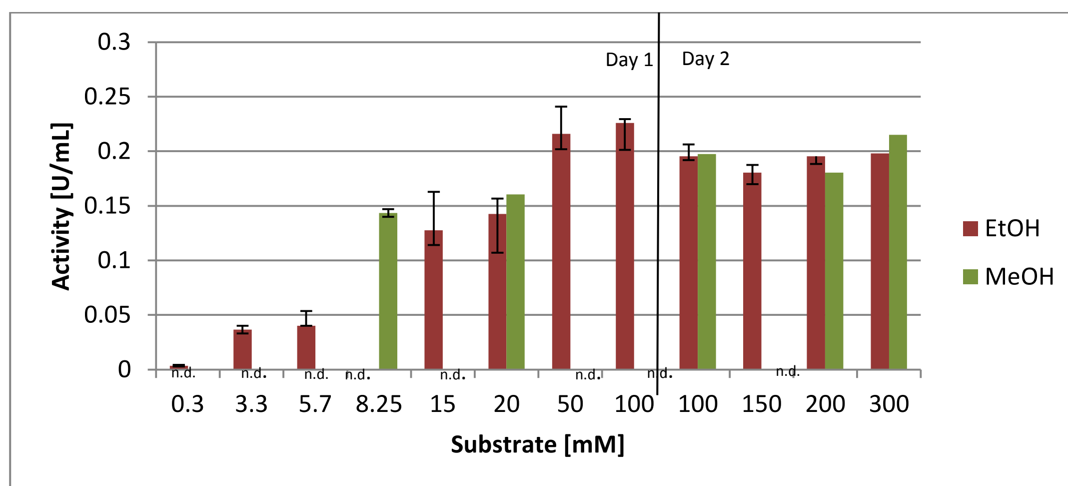


Figure 4. Activity of AlOx from *H. polymorpha* at different EtOH and MeOH concentrations. The black line indicates the separation in two different days of the data collection. The lower activity in case of concentration >100 mM is due to the experimental setup change, as explained in the experimental session. The results represent the average of duplicates.

The data follow Michaelis-Menten kinetics presenting a V_{max} at around 100 mM for both MeOH and EtOH meaning that the saturation level for the enzyme is reached with 100 mM of substrate[37] and a K_m of about 5 mM for MeOH and 12 mM for EtOH, confirming that the affinity of AlOx is higher for MeOH compared to EtOH[34]. The fact that kinetics of alcohol oxidation by AlOx follows Michaelis-Menten kinetic is confirmed by literature, where AlOx from *H. polymorpha* has a K_m for MeOH around 2 mM at pH 7.5[35].

It is notable that the difference in activity between the two substrates is very small from a concentration above 100 mM, meaning that at this conditions the activity of the enzyme is not influenced by the choice of the substrate.

100 mM EtOH is thus the selected concentration of substrate for the tandem reaction with VCPO.

Oxygen concentration

Oxygen is the second substrate for the AlOx reaction and is crucial to analyse how much is needed for the AlOx reaction and what are the effects of variations in concentration. Table 2 shows the results obtained from measuring the O_2 dissolved in the reaction solution by an O_2 electrode.

There is no significant difference among the values, so it can be concluded that after 5 minutes the solution is saturated with oxygen, therefore, longer time of bubbling air through the reaction mixture is not necessary.

These results are in accordance with literature, where it can be found that the solubility of oxygen in water is about 7.6 mg/L at room temperature (20 °C) and at this value the solution is saturated[39].

Table 2. Oxygen concentration of a solution containing ABTS in citrate buffer. The values are the result of a single experiment.

Time [min]	**O_2 [mg/L]**	**O_2 (mM)**
5	8.15	0.25
10	8.15	0.25
15	8.31	0.26
20	8.26	0.26

To sum up the first part of this research, the best conditions for performing the tandem reaction with VCPO and AlOx have been set and they are:

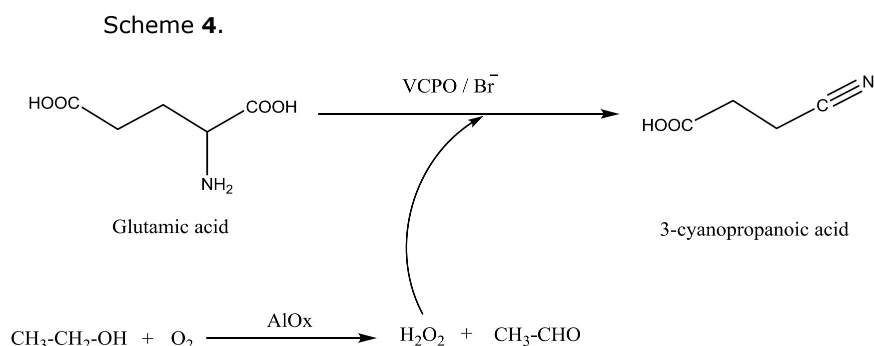
- AlOx from *H. polymorpha*
- 20 mM sodium citrate buffer at pH 5.6
- 100 mM EtOH

At this conditions 0.0033 U/mL of AlOx produce 0.008 mM/min of H_2O_2, the same amount produced at initial best conditions (100 mM potassium posphate buffer pH 7.5 and 8.25 mM MeOH as substrate). Compared to the 0.0033 U/mL of AlOx used so far, 30 times more enzyme should be added to the reaction in order to reach 0.27 mM/min of H_2O_2 necessary to convert 5 mM of amino acid to 5 mM of nitrile.

II. Tandem reaction of VCPO and AlOx

In order to start working on the tandem reaction of VCPO and AlOx it was necessary to choose the most suitable amino acid. In previous studies it was found that the amino acid which can reach complete conversion and the highest selectivity towards the corresponding nitrile with the broadest range of NaBr concentrations is glutamic acid (Glu)[40]. This amino acid can be fully converted using NaBr in concentration ranging from 0.2 mM up to 20 mM. The concentration of NaBr that was planned to be used is 0.5 mM. This value was chosen because it is known that concentrations of NaBr higher than 10 mM can inhibit the VCPO[40] and also AlOx can be affected by high concentrations of this compound[41]. Furthermore, Glu is not converted to side-products at 0.5 mM NaBr. At this concentration the other amino acids have a lower conversion towards nitriles[40]. Therefore, Glu has been used for the further experiments to produce 3-cyanopropanoic acid. The global reaction is schematised in

Scheme **4**.



Scheme 4. Overall reaction of production of 3-cyanopropanoic acid from Glu via tandem reaction of VCPO with AlOx from *H. polymorpha.*

EtOH inhibition test for VCPO

First of all, it was necessary to exclude any kind of inhibition towards VCPO, especially by EtOH. Figure 5 shows that all the Glu is converted to the corresponding nitrile in presence of 100 mM of EtOH. The reaction rate (0.153 mM/min of Glu converted to GluCN) is similar to the one observed in absence of EtOH[40], so there is no inhibition occurring in presence of EtOH. This is confirmed by literature where it was shown that the stability of this enzyme is not affected by incubation in solution containing 40% MeOH (10 M) or EtOH (6.8 M)[42].

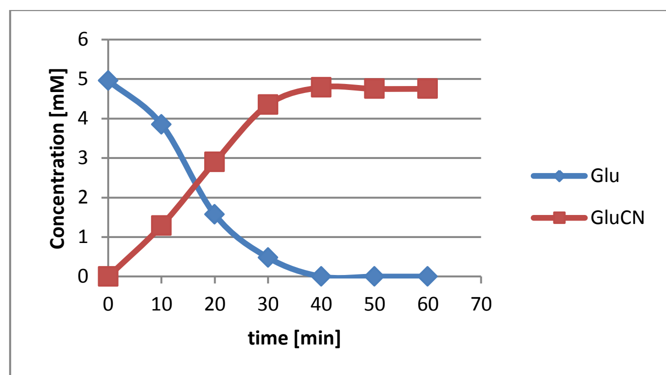


Figure 5. Formation of 3-cyanopropanoic acid (GluCN) and depletion of Glu in time in presence of 100 mM EtOH. The values are the result of a single experiment.

Tandem reaction of VCPO with AlOx for Glu conversion – first trial

After having confirmed that EtOH is not inhibiting VCPO the next step was to perform the the tandem reaction of VCPO with AlOx from *H. polymorpha* with the reaction conditions described in the experimental section.

As it can be seen in Figure 6, no conversion of Glu was observed. Depletion of Glu did not occur and neither formation of nitriles was detected. The slight fluctuation of the concentration values is probably due to the sensitivity limits of the HPLC machine.

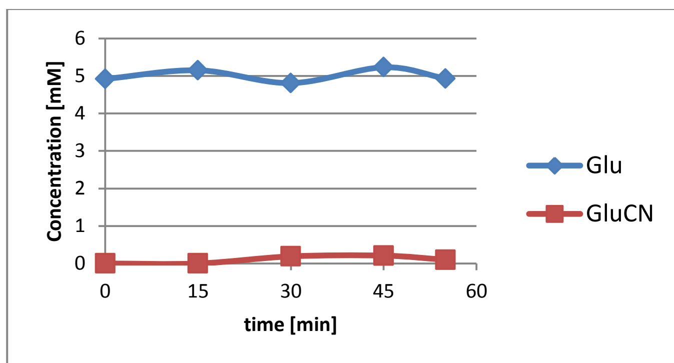


Figure 6. Formation of 3-cyanopropanoic acid (GluCN) and depletion of Glu in tandem reaction of VCPO with AlOx. The values are the result of a single experiment.

Several parameters that could influence the AlOx performance in the tandem reaction were tested:

- The air bubbling through the reaction have been analysed to check if it causes loss of volatile compounds or denaturation of enzymes by foaming.
- The presence of NaBr has been tested for inhibition of the AlOx in presence of this compound.
- The presence of Glu on the activity of AlOx from *H. polymorpha* has been analysed as well.

Air bubbling influence on AlOx activity

Influence of bubbling air into the reaction vial to provide oxygen was tested because formation of foam was observed on the top of the solution during the tandem reaction, thus denaturation of the enzyme could have occurred[43].

Figure 7 presents a comparison between the reaction of AlOx with and without air bubbling.

No foam was observed and the activity of the AlOx was still good.

A 27% loss in activity was registered, but further investigation was needed to find something that affects the reaction at a higher extent.

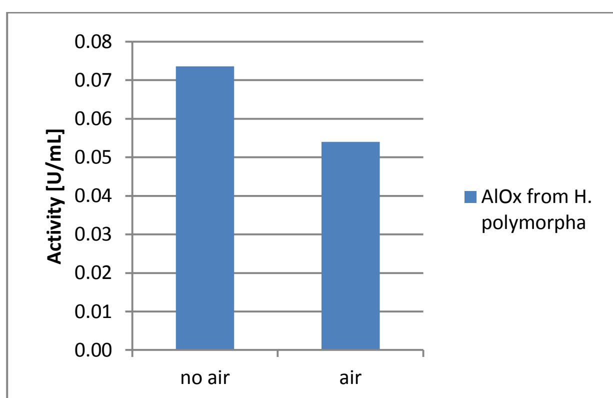


Figure 7. Comparison between activity of AlOx from *H. polymorpha* in absence and in presence of air bubbling. The second column is 73% of the first column. The values are the result of a single experiment.

NaBr influence on AlOx activity

In literature it was found that AlOx is inhibited by KBr and KCN[34]. This mechanism is known for different families of oxidases like AlOxs, glucose oxidases and D-amino acid oxidases in which KBr and KCN cause removal of the cofactor FAD from the holoenzyme.

Figure 8 shows the activity of the AlOx from *H. polymorpha* and *P. pastoris* measured in presence of different NaBr concentrations. There is a slight decrease in activity of AlOx for both the enzymes in presence of NaBr but the difference in activity is not significant between low concentration of NaBr (0.05 mM) and high concentration of NaBr (5 mM).

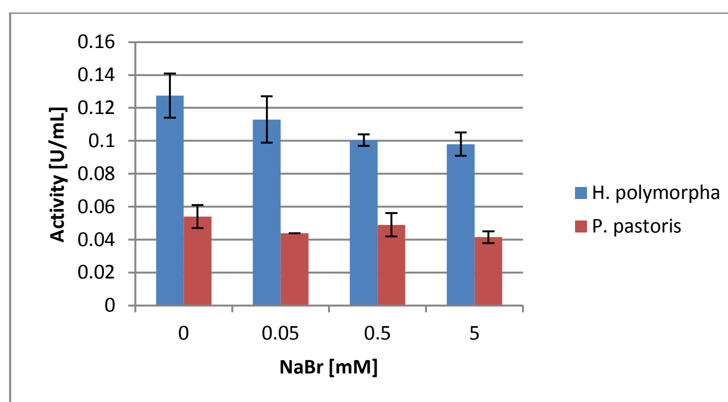


Figure 8. Activity of AlOx from *H. polymorpha* and *P. pastoris* in presence of different concentrations of NaBr. The results represent the average of duplicates.

According to the literature the concentrations causing inhibition of the AlOx are 3.5 M of KBr or 6 mM of cyanide compounds[41]. These concentrations are much higher than the 0.5 mM of NaBr used in the tandem reaction of VCPO with AlOx.

In conclusion the results obtained do not mean that NaBr has no effect on the AlOx, but they confirm that a concentration of bromide of 0.5 mM in the reaction mixture is within the limit in which the enzyme is still active.

Glutamic acid influence on AlOx activity

The same procedure that has been used for analysing the effect of NaBr on AlOx was used to investigate the role of Glu.

Figure 9 shows the change in reaction rate of AlOx in presence of different concentration of Glu. It can be seen that the higher the concentration of Glu in the solution, the stronger the decrease of the observed activity.

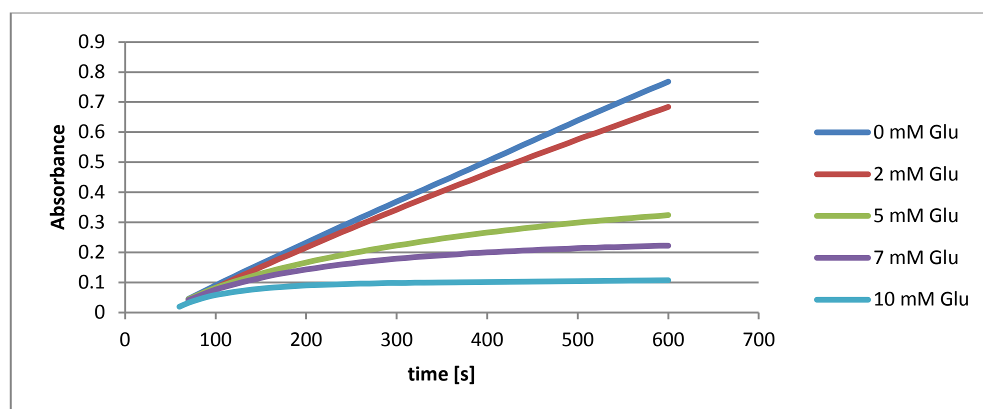


Figure 9. Absorbance in time of the reaction of AlOx from *H. polymorpha* in presence of different concentrations of Glu. The results represent the average of duplicates.

The blue line in the graphs corresponds to the observed activity without amino acid. The stronger decrease is registered between 2 mM Glu (red line) and 5 mM Glu (green line) and the AlOx activity is progressively decreasing until zero with 10 mM Glu, which cause total inhibition in less than one minute. At a concentration of 5 mM of amino acid, which was used in the tandem reaction of VCPO

with AlOx, the activity of *H. polymorpha* AlOx is 47% of the initial one, where Glu is absent. Differences in activity are shown in

Figure **18** in Appendix.

Glutamic acid influence on HRP activity

To check whether the HRP –the coupled enzyme used for determining the AlOx activity- is inhibited by presence of Glu, the activity of the AlOx was tested after either HRP or AlOx were incubated with 5 mM Glu. Figure 10 shows that the absorbance of oxidised ABTS in time. The blue line represents the activity of AlOx without inhibitor and the red line corresponds to the activity in presence of 5 mM Glu. The green, purple and light blue lines represent the activity of AlOx with 5 mM Glu with the following conditions respectively: HRP incubated for 3 min in 5 mM Glu, AlOx incubated for 3 min in 5 mM Glu and AlOx incubated for 5 min in 5 mM Glu. The activity in case of incubation of HRP is the same with the activity without incubation. Instead incubation of AlOx in presence of Glu gives a dramatic drop in activity proportional to how long the incubation lasts. After 5 minutes of exposure to Glu, AlOx is completely inactivated. Differences in activity are shown in Figure 19 in Appendix.

These results suggest that AlOx is strongly inhibited by Glu whereas HRP is not affected by presence of the amino acid.

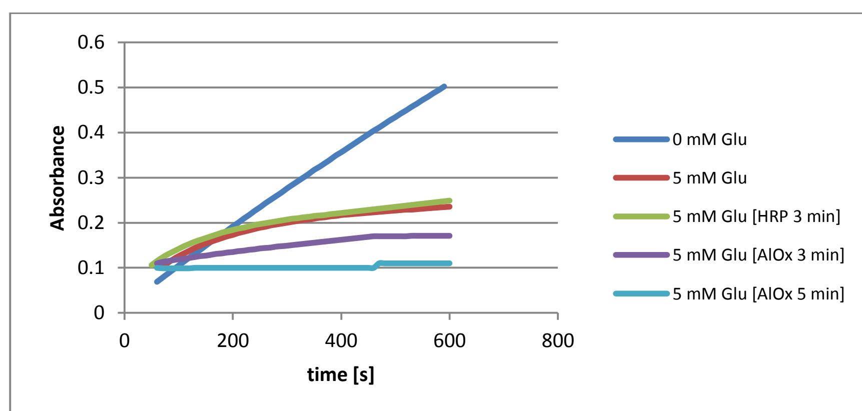


Figure 10. Absorbance in time of the reaction of AlOx from *H. polymorpha* in presence of different concentrations of 0 or 5 mM Glu and different incubation times for AlOx and HRP. The first two results represent the average of duplicates, the others are single experiments.

Kinetic study of AlOx in presence of glutamic acid

Another necessary step was analysing what type of inhibition Glu causes to the AlOx in order to see if it can be overcome.

To test if Glu is a competitive, non-competitive or uncompetitive inhibitior[37], AlOx activity has been tested at different concentration of EtOH and different concentrations of Glu and a Lineweaver-Burk plot was built (Figure 11).

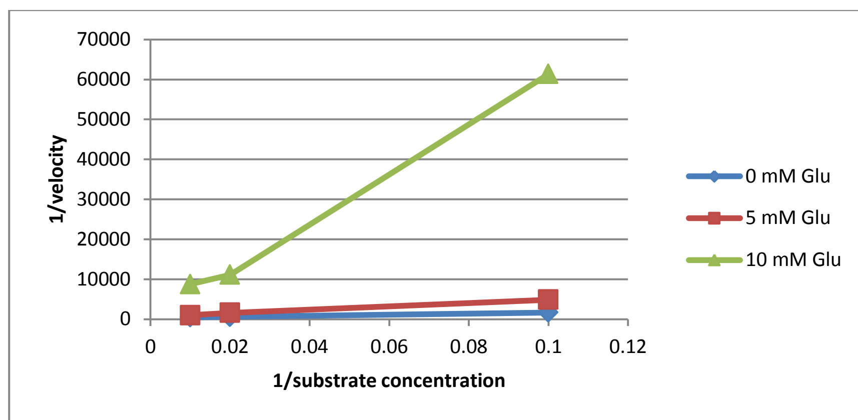


Figure 11. Lineweaver-Burk plot for the inhibition of AlOx by Glu. The values are the result of a single experiment.

According to the interception among the three lines, which occurs neither on the x-axis (as in case of non-competitive inhibition) nor on the y-axis (as in case of competitive inhibition), it might be a mixed inhibition. Therefore Glu binds an allosteric site of the enzyme and reduces its reaction rate[44]. However it would be necessary to repeat the experiment with more data to have a confirmation of the results.

Tandem reaction of VCPO with AlOx for Glu conversion – second trial

So far it was shown that AlOx is inhibited by concentration of Glu in the range of mM, but no experiments have been performed in which AlOx was exposed at lower concentration of amino acid. In order to check if AlOx could be active with lower concentration of Glu, the tandem reaction was performed by gradually adding Glu in the reaction mixture in order to maintain a small concentration of the inhibitor (0.5 mM each aliquot). The reaction has been performed as explained in the experimental part and the results are shown in Figure 12. No formation of 3-cyanopropanoic acid was detected. The values corresponding to Glu depletion present high fluctuations, probably due to complications related to the addition of Glu in small aliquots. Indeed a small volume could have been lost because of air bubbling or pipetting.

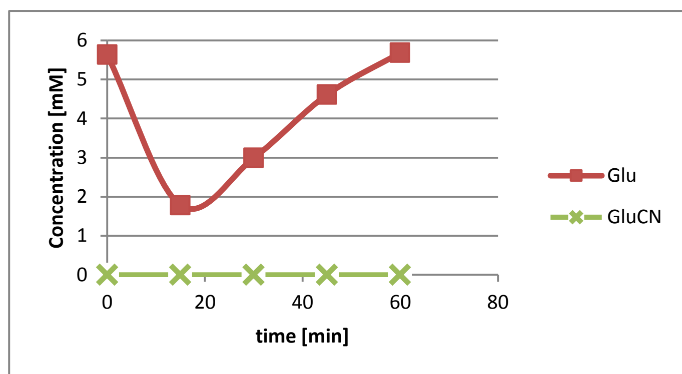


Figure 12. Formation of 3-cyanopropanoic acid (GluCN) and depletion of Glu in time in presence of 100 mM EtOH. The values are the result of a single experiment.

Influence on acetaldehyde production by AlOx in presence of glutamic acid

After having analysed the effect of Glu on the activity of the AlOx, it was necessary to test if the inhibition is reflected on the production of acetaldehyde, which is the result of the oxidation of EtOH by the AlOx enzyme. Conversion of EtOH into acetaldehyde was performed and the concentration of the product in the reaction mixture was measured by an enzymatic assay presented in the experimental section.

In Figure 13 the results show that acetaldehyde production is slowed down if Glu is present in the reaction mixture.

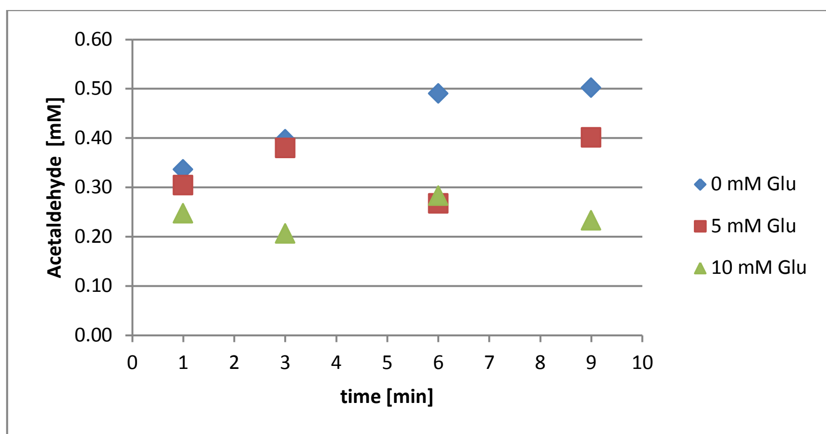


Figure 13. Acetaldehyde production in time by AlOx in presence of different concentrations of Glu. Acetaldehyde concentration was determined by a separate enzymatic assay at each time point. The values are the result of a single experiment.

Excluding the point at 6 min with 5 mM Glu a decrease of acetaldehyde production is register with increasing amount of Glu. This is probably an effect of AlOx inhibition by the presence of Glu as observed before when H_2O_2 was monitored by HRP.

The values at 3 and 6 minutes are fluctuating, probably due to difficulties in keeping the same reaction conditions for all the samples. Some recommendations for future trials are given later in the report.

Influence on acetaldehyde evaporation during air bubbling

The acetaldehyde content in the tandem reaction was also measured to test if acetaldehyde was formed from the reaction of AlOx but no acetaldehyde was detected (results not shown). This could be related to the evaporation of acetaldehyde, which has a boiling point of 20 °C,[28] due to the bubbling of air into the solution. Figure 14 shows the results of air bubbling in a solution containing only acetaldehyde in water. It can be seen that evaporation is occurring at a high extent and the effect gets stronger with lower concentration of acetaldehyde. After 30 minutes (Figure 14-A) 84% and (Figure 13-B) 55% of acetaldehyde is lost through evaporation. The evaporation rate is 0.03 mM/min and 0.08 mM/min respectively. These results are clear that acetaldehyde is very volatile, so it is not possible to measure the production of acetaldehyde by taking samples from the reaction solution.

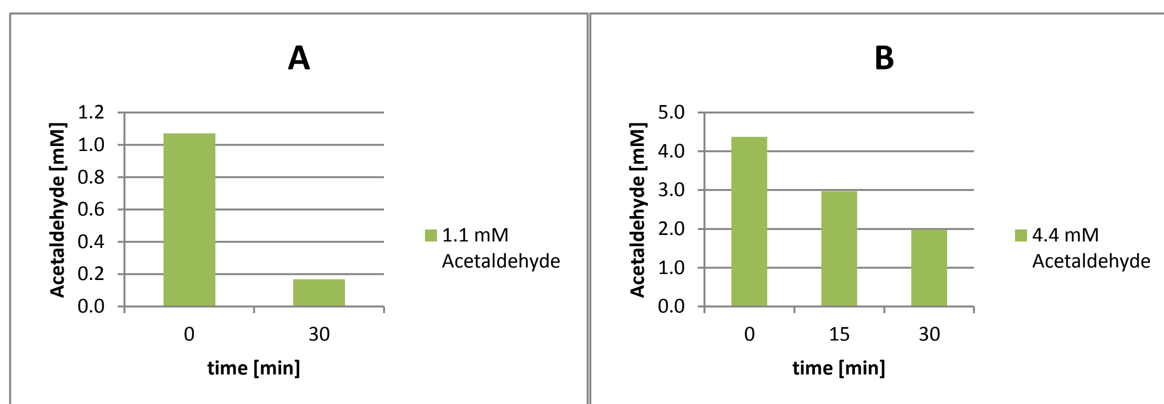


Figure 14. Concentration of acetaldehyde present in the solution after air supply through bubbling. The values are the result of a single experiment.

The absence of product in the tandem reaction is definitively due to evaporation, because acetaldehyde is produced gradually and the little amount present in solution immediately evaporates. However the contribution of Glu inhibiting the AlOx cannot be excluded.

The positive aspect of these results is the possibility of the easy downstream processing characterising the final product acetaldehyde. When thinking about a large scale reaction in which acetaldehyde has to be separated from the mixture containing the nitriles, the fact that a simple distillation can be enough to reach complete separation is promising[29].

Tandem reaction of VCPO with AlOx for monochlorodimedone conversion

In order to check other substrates different than Glu affect the activity of AlOx, a tandem reaction using MCD as substrate for VCPO was performed and the results are shown in Figure 15.

Activity of VCPO is registered with and without AlOx producing H_2O_2. The blue line represents the decrease in absorbance corresponding to the decrease in MCD concentration and from this slope an activity of 80.30 U/mL was calculated. The red line represents the decrease in MCD concentration in the tandem reaction when EtOH is added in the MCD mixture and air is supplied through. The activity of VCPO in the tandem reaction is 77% of the activity when H_2O_2 is externally added and this is the first proof that the tandem reaction works.

The green line is also a result of the tandem reaction, but when EtOH is added in the cuvette after the MCD mixture has been saturated with air. These results also help in affirming that air bubbling does not cause EtOH evaporation, because loss in activity does not occur if EtOH is added in the MCD mix where air is bubbled through (B). The lower activity in (C) is probably due to pipetting error due to the small volume of EtOH supplied in the cuvette.

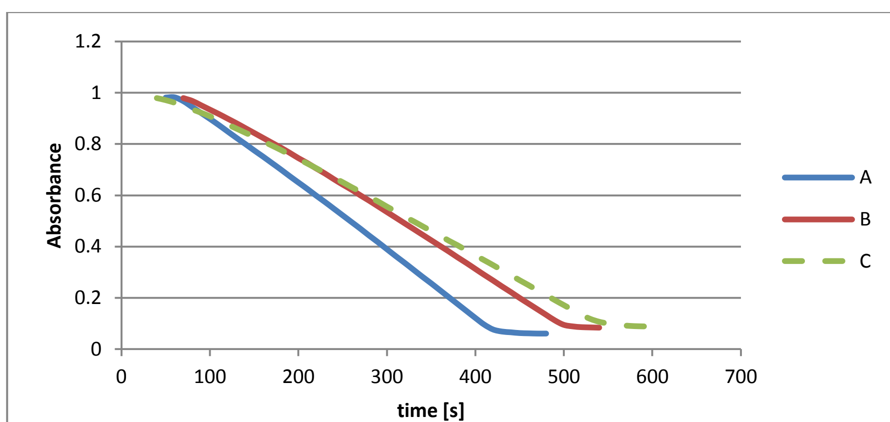


Figure 15. MCD absorbance in time for VCPO activity in A) MCD mix + VCPO, B) 1st (MCD mix + EtOH) + 2nd air bubbling + VCPO + AlOx , C) 1st (MCD mix + air bubbling) + 2nd EtOH + VCPO + AlOx . The results represent the average of duplicates.

Other amino acids influence on AlOx activity

Even if it was demonstrated that the tandem reaction works in presence of another substrate, the fact that Glu is inhibiting the AlOx is still an essential issue in this research because it is considered the most suitable amino acid for obtaining nitriles with VCPO. The impossibility of using this particular amino acid opens the possibility of reconsidering other ones which were not taken into account before, after proving they do not inhibit the AlOx.

An inhibition test was performed with 5 mM of either Glu or aspartic acid or valine. The structures of these amino acids are shown below in Figure 16.

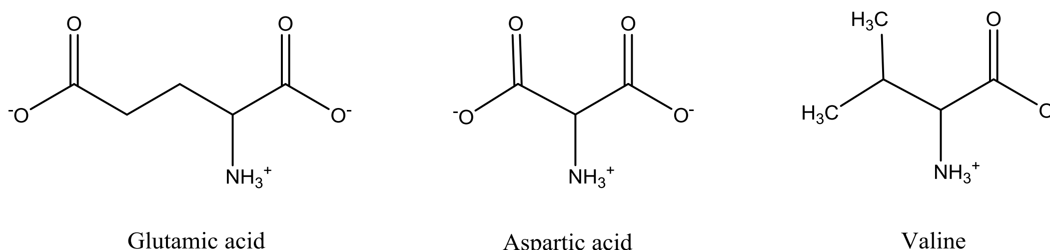


Figure 16. Structure of L-glutamic acid, L-aspartic acid and L-valine.

Both Glu and aspartic acid have a side chain ending with a carboxylic group, which is deprotonated at pH 5.6[28]. Valine is a branched amino acid with an aliphatic side chain.

The comparison of the activity of AlOx from *H. polymorpha* in presence of the different amino acids is shown in Figure 17. On one hand it can be observed that aspartic acid and Glu inhibit the

enzyme dramatically to a similar level. On the other hand, valine does not influence the activity of the AlOx, which remains the 99% of the original one.

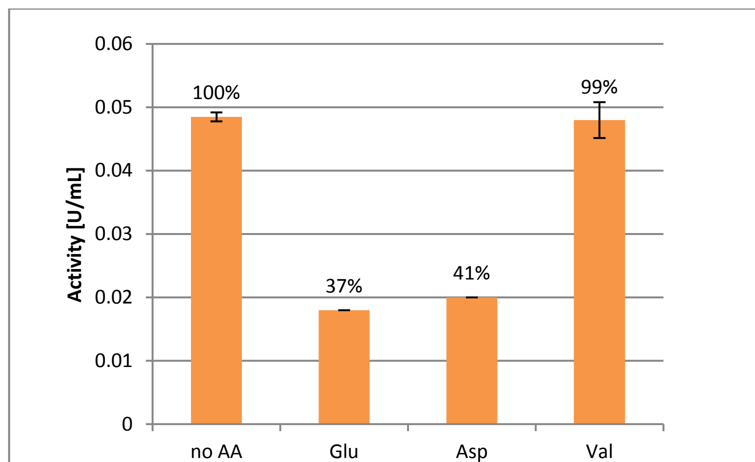


Figure 17. Activity of AlOx from *H. polymorpha* in presence of 5 mM of Glu (Glu), aspartic acid (Asp) or valine (Val). The percentage is shown with respect to the activity in absence of AA. The results represent the average of duplicates.

These results can be a starting point for further experiments in which other amino acids are analysed as well to establish why some of them act as inhibitors and others do not. Further research is needed to obtain more information and see if some other amino acids could be used to be converted into nitriles in a tandem reaction with VCPO.

Conclusions

I. Production of H_2O_2 by short chain alcohol oxidases

The first part of the research was focused on finding an AlOx suitable for the tandem reaction with VCPO, matching the reaction conditions of AlOx with those of VCPO and optimising these conditions to reach the maximum production of H_2O_2.

- After testing the activity of three commercially available AlOx at 3 different pH values, the AlOx from *H. polymorpha* was chosen as best option because it was the only one maintaining a relatively high activity at pH 5.6, which is the optimal value for VCPO. With ethanol as substrate the activity of the AlOx from *H. polymorpha* at pH 5.6 was about 28% of the activity at pH 7.5.
- The buffer concentration screening showed that the AlOx from *H. polymorpha* has an optimal activity in 20 mM of sodium citrate buffer, the same optimal buffer concentration of VCPO.
- The substrate concentration at which AlOx from *H. polymorpha* reached V_{max} was found to be 100 mM ethanol. At V_{max} in 20 mM sodium citrate buffer pH 5.6 the enzyme is producing 0.008 mM/min H_2O_2, the same amount of H_2O_2 which is produce at initial assay conditions (methanol as substrate and 100 mM potassium phosphate buffer at pH 7.5).

II. Conversion of amino acids to nitriles using VCPO/H_2O_2/NaBr

The second part of the research consisted in performing the tandem reaction with alcohol oxidase and VCPO.

- It was shown that the tandem reaction of VCPO with AlOx from *H. polymorpha* was successful for the conversion of monochlorodimedone – the main substrate used for VCPO activity assay -.
- AlOx and VCPO were coupled for the conversion of glutamic acid into 3-cyanopropanoic acid but no formation of the nitrile or depletion of glutamic acid was detected by HPLC analysis.

To determine what are the factors responsible for the success of the tandem reaction on the conversion of MCD but not of Glu, several tests were made on different reaction parameters and the conclusions are listed below.

- The enzyme VCPO is not inhibited by the presence of ethanol in the reaction mixture. This was tested in literature before on chlorination of monochlorodimedone by VCPO/H_2O_2/HCl and now it can be confirmed also for the oxidative decarboxylation of Glu by VCPO/H_2O_2/NaBr in presence of ethanol.
- The enzyme AlOx is inhibited by the presence of glutamic acid in the reaction mixture. This was tested for H_2O_2 detection and also for acetaldehyde.
- The mechanism of inhibition of AlOx by Glu is probably a mixed inhibition based on the kinetic measurements of AlOx in presence of different concentrations of substrate (ethanol) and inhibitor (Glu)
- The inhibition of AlOx by Glu in the tandem reaction was not overcome by the fed-batch reaction setup where Glu was added in small aliquots during the course of the reaction.
- Enzymatic assay of AlOx from *H. polymorpha* revealed:
  - slight inhibition of AlOx due to air bubbling,
  - no inhibition in present of NaBr,
  - strong inhibition in presence of acidic amino acids (glutamic acid and aspartic acid),
  - no inhibition in presence of aliphatic amino acid (valine).
- Acetaldehyde evaporation occurs at room temperature thanks to air bubbling through the solution. Therefore, a simple distillation is probably enough to collect the by-product acetaldehyde and no further downstream steps are required to extract it.

Recommendations for future work

Firstly, a pH screening of AlOx from H.polymorpha, C. boidinii and P. pastoris should be performed in presence of MeOH as substrate at the conditions given by the protocol to to asses internally the activity of each AlOx

Secondly, it is crucial to find an alternative way to supply oxygen to the tandem reaction. By using an oxygen supplying pipe instead of an air supplying pipe, the flow needed to reach the necessary amount of oxygen in the reaction could be reduced and the bubbling flow will decrease by approx. 5 times.

Thirdly, the mechanism of inhibition of Glu towards the AlOx has to be analysed by:

1) Building a more accurate Lineweaver-Burk chart by performing the activity of the AlOx with a broader range of EtOH concentrations (2 mM, 5 mM, 7 mM and 10 mM). Furthermore duplicates are necessary to have more reliable results. When the nature of the inhibition is known, it will be easier to analyse how to solve it. If the inhibition results to be competitive a higher concentration of substrate can help to overcome it because of lower possibility for Glu to bind the active site. Also in case the inhibition is mixed, like it results from the experiment, a higher concentration of substrate could help in reducing the effect of Glu. In case of non-competitive, uncompetitive and irreversible inhibition this solution cannot be applied[37,45]. In this case a solution could be to perform the production of H_2O_2 by AlOx in a separate reactor and transfer the product into the solution containing VCPO to convert Glu into nitriles.
2) Analysing the influence of Glu on acetaldehyde production. With the assay performed in this research it was difficult to have an accurate measurement of acetaldehyde concentration because of short reaction time and low amount of acetaldehyde present in solution. By trying lower concentrations of aldehyde dehydrogenase the conversion of acetaldehyde to acetic acid would be extended in time and easier to analyse. Furthermore it could be useful to try a different assay in which a broader range of acetaldehyde concentration can be analysed, like a colorimetric or a fluorometric assay[46].
3) Testing the inhibition effect of other amino acids on AlOx. After seeing that valine does not cause inhibition of AlOx some hypothesis have risen: the difference could be due to the COO^- group which could interfere somehow with the activity of the enzyme (see point 4). Another reason could be the fact that valine is a hydrophobic amino acid and the other two are hydrophilic, so they can have different affinity for the active site. Furthermore the branched structure of valine could play a role in avoiding the amino acid to enter in contact with the active site of the enzyme. In order to obtain an answer, different amino acids with different structures and properties such as alanine, which is hydrophobic and non-branched or phenylalanine, which has hydrophobic features and has a bulky side chain should be tested for the inhibition towards AlOx.
4) Performing an inhibition assay in which the activity of AlOx is measured in presence of different concentration of carboxylic acids, such as acetic acid, formic acid, etc., to check if the carboxylic group is the cause of the inhibition.

Experimental section

Materials

Acetaldehyde assay kit K-ACHYD was purchased from Megazyme, KH_2PO_4 and EtOH (100%) from Merck KGaA, MeOH (100 %) from Biosolve and Na_2HPO_4 from Riedel-De Haën. ABTS – 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid), NAD^+, NaBr (99% pure), H_2O_2 (35 wt%), citric acid, L-Glu (≥99%), L-aspartic acid (≥98%), L-valine (≥98%), Na_3VO_4 were provided by Sigma-Aldrich and monochlorodimedone by BioResource Products.

Enzymes

AlOxs (EC 1.1.3.13) from *P. pastoris, C. boidinii* and *H. polymorpha* and peroxidase from *Horseradish* (HRP) 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.

Enzymatic assay of AlOx

The protocol was taken from Sigma-Aldrich and was used to measure the activity of AlOx in the desired samples.

Standard procedure[31]

In a plastic cuvette were put 2.80 mL of 2 mM ABTS prepared in 100 mM phosphate buffer pH 7.5 and 0.01 mL of 250 U/mL *Horseradish* peroxidase stock solution. After reading the absorbance at 405 nm, 0.10 mL of 1% (v/v) MeOH (8.25 mM) were added and the absorbance was read again. The reaction was started by addition of 0.10 mL of 0.1 U/mL AlOx and the oxidation of ABTS by H_2O_2 and HRP was followed in time at 405 nm for appoximatively 15 minutes at about 21 ºC on a Beckman Coulter DU®720 UV/Vis spectrophotometer. The final volume in the cuvette was 3.01 mL and the solution contained: 96 mM potassium phosphate, 2 mM ABTS, 0.19 mM MeOH, 0.83 U/mL HRP and 0.0033 U/mL AlOx. The absorbance was measured also for the blank solution where the 0.10 mL of AlOx were substituted by 0.10 mL of buffer.

Variation from standard procedure

1) *pH screening of P. pastoris, C. boidinii and H. polymorpha AlOx and substrate screening*

- Substrate: 5.7 mM EtOH or 8.25 mM MeOH (1% alcohol, v/v).
- Enzymes:

AlOx from P. pastoris: from the bottle (630 U/mL) a solution of 3.15 U/mL was prepared in 100 mM potassium phosphate buffer pH 7.5. Aliquots of 64 μL were stored in freezer. Before use 2 mL of 0.1 U/mL stock solution were prepared by adding 1936 μL of 100 mM potassium phosphate buffer pH 7.5.

AlOx from C. boidinii: from the bottle (0.7 U/mg solid) a solution of 1.4 U/mL (2 mg/mL) was prepared in 100 mM potassium phosphate buffer pH 7.5. Aliquots of 143 μL were stored in freezer. Before use 2 mL of 0.1 U/mL stock solution were prepared by adding 1857 mL of 100 mM potassium phosphate buffer pH 7.5.

AlOx from H. polymorpha: from the bottle (0.6 U/mg solid) a solution of 1.2 U/mL (2 mg/mL) was prepared in 100 mM potassium phosphate buffer pH 7.5. Aliquots of 167 μL were stored in the freezer. Before use 2 mL of 0.1 U/mL stock solution were prepared by adding 1833 μL of 100 mM potassium phosphate buffer pH 7.5.

- Buffer: 100 mM potassium phosphate buffer pH 7.5 (13.6 mg/mL solution of potassium phosphate monobasic in ultrapure water adjusted with 1 M KOH to reach pH 7.5). 27.25 mM sodium citrate/Na_2HPO_4 buffer pH 6.6 (27.25 mL of 0.1 M citric acid and 72.75 mL of 0.2 M Na_2HPO_4). 42 mM sodium citrate/Na_2HPO_4 buffer pH 5.6 (42 mL of 0.1 M citric acid and 58 mL of 0.2 M Na_2HPO_4).

2) *H. polymorpha* AlOx pH screening (2):

Buffer: 100 mM potassium phosphate buffer (pH 7.5) prepared as above. 100 mM sodium citrate buffer (pH 6.6 or 5.6) prepared from a stock solution of 0.5 M sodium citrate buffer pH 4.6 by dilution in ultrapure H_2O and 5M NaOH to adjust the pH.

3) *Buffer concentration screening*
- Buffer: different amounts of 0.5 M sodium citrate buffer pH 4.6 are used to obtain sodium citrate buffer pH 5.6 in the concentration of 100, 80, 60, 40, 20 mM.
- Substrate: EtOH 5.7 mM tested in the all range of buffer concentrations. MeOH 8.25 mM tested only in 100 and 20 mM buffer.

4) *Substrate concentration screening*
- Buffer: 20 mM sodium citrate buffer pH 5.6.
- Substrate: a solution of 100% EtOH was diluted in demineralised H_2O to obtain concentrations of 0.3, 3.3, 5.7, 15, 20, 50, 100, 150, 200 and 300 mM in cuvette. A solution of 100% MeOH was diluted to obtain concentrations of 8.25, 20, 100, 200, 300 mM in cuvette.
- Volume: the experiment was performed in two different days. The values corresponding to a concentration of EtOH between 0.3 and 100 mM were prepared first and the final volume in the cuvette was 3 mL. The values higher than 100 mM were prepared one day later based on a final volume of 1 mL, so the volume of alcohol was lower and the pipetting error caused a difference among the results of the two different days together with the decrease in activity of the AlOx stored in 100 mM potassium phosphate buffer pH 7.5 (Figure 4).

5) *Influence of air bubbling on alcohol oxidase from H. polymorpha*

The assay was performed both in a cuvette in the spectrophotometer and in a vial in which air was supplied by bubbling through a needle. The absorbance was measured 15 minutes after the start of the reaction for both samples. The final volume was 2 mL.

6) *Influence of NaBr on alcohol oxidase from H. polymorpha and P. pastoris*

0.02 mL of 2.57, 25.7 and 257 mM NaBr stock solution in demineralised H_2O was added into the cuvette to rich a final concentration of 0.05, 0.5 or 5 mM of NaBr respectively. The final volume was 1.04 mL.

7) *Influence of glutamic acid on AlOx from H. polymorpha*

0.22, 0.55, 0.77 and 1.1 mL of 50 mM Glu stock solution prepared in 20 mM sodium citrate buffer pH 5.6 were added to the ABTS solution in 20 mM sodium citrate buffer pH 5.6 to reach a final volume of 5 mL. The final concentration in the cuvette was 2, 5, 7 and 10 mM of Glu respectively. The final volume was 1 mL.

8) *Influence of aspartic acid and valine on AlOx from H. polymorpha*

1.6 mL of 17 mM Asp stock solution in 20 mM sodium citrate buffer pH 5.6 or 3 mg of Val (solid) were added to to the ABTS solution in 20 mM sodium citrate buffer pH 5.6 to reach a final volume of 5 mL. the final concentration in the cuvette was of 5 mM of Asp or Val. The final volume was 1 mL.

9) *Incubation of AlOx and HRP in glutamic acid*

To test the effect of incubation of AlOx in Glu the same experiment was carried out by changing the order of the reagents in the cuvette. AOx or HRP were incubated first in the mixture of 20 mM sodium citrate buffer pH 5.6 + ABTS + Glu and the reaction was started by addition of the substrate (EtOH). Glutamic acid concentration was 5 mM for the all samples. HRP was incubated for 3 minutes and AlOx was incubated for 3 and 5 minutes.

10) *Kinetic study of AlOx in presence of glutamic acid*

The AlOx enzymatic assay was performed at three different substrate concentrations (10, 50 and 100 mM EtOH) each with three different concentration of inhibitor (0, 5 and 10 mM Glu).

Acetaldehyde assay procedure

Standard procedure[33]

In a plastic cuvette were put 2 mL of demineralised H_2O, 0.10 mL of sample solution or of standard solution of acetaldehyde ammonia trimer (1.1 mM acetaldehyde), 0.20 mL of concentrated buffer solution pH 9.0 (provided in the kit) and 0.20 mL of 20 mM NAD^+ (concentration determined based on absorbance at 260 nm of a solution containing NAD^+ (provided in the kit) in demineralised H_2O and on ε_{NAD+}=18 $mM^{-1}*cm^{-1}$) After reading the absorbance at 340 nm, the reaction was started by adding 0.05 mL of aldehyde dehydrogenase (Al-DH). The final volume was 2.55 mL and this solution contained: 0.04 mM of acetaldehyde and 1.6 mM of NAD^+. The absorbance was measured also for the blank solution where the 0.10 mL of sample solution was substituted by 0.10 mL of demineralised H_2O. The formation of NADH was followed in time at 340 nm for approximatively 10 min. The assay is suitable for acetaldehyde concentrations between 0.11 and 4.5 mM.

Variation from standard procedure

1) *Influence of glutamic acid on acetaldehyde production*

To test the effect of Glu on acetaldehyde production two separated reactions were carried out. In the first one 100 mM ethanol (33 µL) was converted to acetaldehyde. 20 µL of a stock solution of 4.8 U/mL AlOx from *H. polymorpha* stored in 100 mM potassium phosphate buffer at pH 7.5 were added. The reaction was performed in 20 mM sodium citrate buffer pH 5.6. According to the final Glu concentration different volumes were prepared: 947 µL of buffer (0 mM Glu), 780 µL of buffer + 167 µL Glu stock solution (5 mM Glu) and 614 µL buffer + 333 µL Glu stock solution (10 mM Glu). The stock solution consists in 30 mM Glu in 20 mM sodium citrate buffer pH 5.6. The final volume was 1 mL. The reaction was stopped after 1, 3, 6, 9 minutes. The second reaction followed the acetaldehyde standard procedure with different reagents volumes: 1 mL of demineralised H_2O, 0.1 mL buffer pH 9, 0.1 mL NAD^+, 0.05 mL acetaldehyde sample solution, 0.025 mL Al-DH. the final volume was 1.25 mL.

2) *Acetaldehyde evaporation*

A 2 mL solution containing either 1.1 or 4.4 mM acetaldehyde in demineralised H_2O was put in a vial and air was bubbled through. Samples were taken after 15 and/or 30 minutes. The final volume was 1.25 mL.

3) *Acetaldehyde concentration in product of tandem reaction of VCPO with AlOx.*

To determine the acetaldehyde content, every 15 minutes a sample (0.05 mL) was taken from the tandem reaction mixture of VCPO with AlOx, mixed with the acetaldehyde assay solutions (buffer, H_2O and Al-DH) and measured as described in the acetaldehyde standard procedure. in the by. The final volume in the assay was 1.25 mL.

MCD assay for bromination reaction

Standard procedure

In a disposable UV cuvette were added 1 mL of MCD mix (50 µM MCD, 1 mM H_2O_2, 0.5 mM NaBr, 100 µM Na_3VO_4, 20 mM citrate buffer pH 5.6) and 10 µL of 0.92 U/mL VCPO. The bromination of monochlorodimedone (MCD) was followed in time at 290.

Tandem reaction

In a UV disposable cuvette were added 1 mL of MCD mix without H_2O_2 (50 µM MCD, 0.5 mM NaBr, 100 µM Na_3VO_4, 20 mM citrate buffer pH 5.6), 10 µL of 0.92 U/mL VCPO and 0.1 mL of 0.6 U/mL of AlOx from *H. polymorpha*. In the first sample (Figure 14-B) 584 µL of 8.56 M EtOH were included in the MCD mix replacing part of the buffer. The final volume in the cuvette was 1.110 mL. In the second sample (Figure 14-C) 12 µL of 8.56 M EtOH were added into the cuvette. In both cases the final concentration of EtOH was 100 mM. The final volume in cuvette was 1.122 mL.

Oxygen concentration measurement

An oxygen electrode was used to measure the oxygen content of a solution containing citrate buffer 20 mM at pH 5.6 and 2 mM ABTS. Air was supplied through a needle and measurement were taken every 5 minutes for a total duration of 20 minutes. The calibration of the electrode was done against air.

Oxidative decarboxylation of amino acids by VCPO

Standard procedure

In a glass vial 5 mM of amino acid, 0.5 mM NaBr, 0.36 U/mL of VCPO and 20 mM citrate buffer at pH 5.6, contained in a total volume of 2 mL, are stirred at 400 rpm, at room temperature (21°C). To this reaction mixture, 16 mM H_2O_2/h (66 µL of 0.5 M H_2O_2/h) is added continuously, using a NE-1600 syringe pump from ProSense. The reaction is stopped at the different time points by adding $Na_2S_2O_3$. Samples were taken from the reaction mixture for HPLC analysis.

Variation from standard procedure

1) *EtOH inhibition test*

12 µL of EtOH 100% (17.12 M) were included in the reaction mixture to reach a final concentration of 100 mM EtOH. Six individual reaction vials have been prepared and they were stopped with $Na_2S_2O_3$ after 10, 20, 30, 40, 50 and 60 minutes respectively to test the formation of nitriles together with consumption of Glu in time with HPLC analysis.

2) *Tandem reaction*

12 µL of EtOH 100% (17.12 M) and 125 µL of a stock solution of 4.8 U/mL AlOx from *H. polymorpha* in 20 mM sodium citrate buffer pH 6.3 were added to the reaction mixture to reach a concentration of 100 mM EtOH and 0.3 U/mL AlOx. The solution was stirred at room temperature and air was supplied via a needle connected to a pipe. The final volume was 2 mL and the reaction was started with addition of AlOx. To stop the reaction $Na_2S_2O_3$ was added a tip of a small spatula). Samples (0.4 mL) were taken every 15 minutes during one hour for HPLC analysis.

3) *Tandem reaction with Glu added in fed-batch*

The same procedure as the tandem reaction, except for Glu addition. 33.3 µL of a solution containing 30 mM Glu in 20 mM sodium citrate buffer pH 5.6 was added in aliquots every 6 minutes to reach a final concentration of 5 mM after 1 hour of reaction. Four individual reaction vials have been prepared and they were stopped with $Na_2S_2O_3$ after 15, 30, 45 and 60 minutes respectively to test the formation of nitriles together with consumption of Glu in time with HPLC analysis.

HPLC

Amino acids were analysed by derivatisation as previously described[13].

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.

Bradford – Protein assay[48]

Bovine serum albumin (BSA): 1 mg/mL of BSA stock solution was prepared by mixing 0.9 mL of 2 mg/mL BSA with 0.9 mL of water. 0.5 mL of standards were prepared in different concentration (0.9, 0.75, 0.5, 0.25, 0.1 mg/mL) in water. 1 mL of Bradford Reagent (BFR) was put in all the cuvettes. 33.4 µL of every standard were added in the different cuvettes. After 5 min the absorbance was read at 595 nm for each of them. In the blank 33.4 µL of demineralised H_2O were added on the top of BFR. A calibration line was build and used for the calculation of the concentration of the samples.

Samples (AlOx): 33.4 µL of AlOx solution from *P. pastoris*, *C. boidinii* and *H. polymorpha* (see pH screening method) were added on the top of BFR.

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Appendix

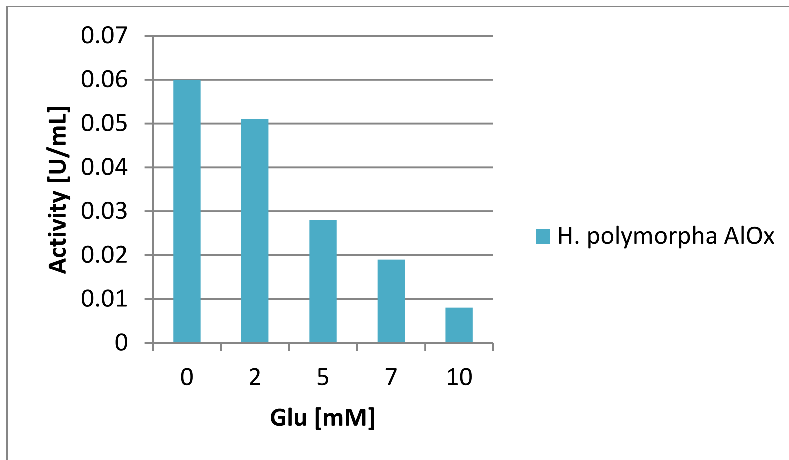

Figure 18. Activity of AlOx from *H. polymorpha* in presence of different concentrations of Glu. The results represent the average of duplicates. *The values were taken based on absorbance registered at time = 400 s.

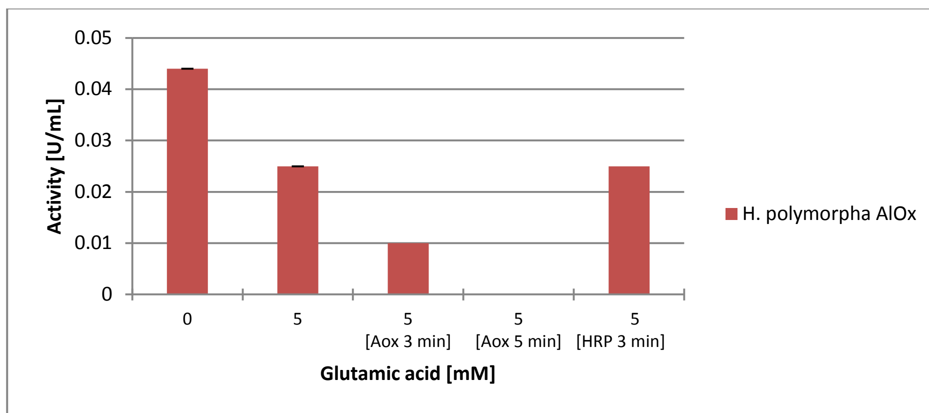

Figure 19. Activity of *H. p.* AlOx in different conditions. The first column is the activity with 0 mM of Glu and the second column with 5 mM. The third and the fourth ones represent the experiments in which AlOx has been incubated for 3 or 5 minutes and the last column corresponds to the activity when HRP is incubated for 3 minutes. the first two results represent the average of duplicates, the other three are single experiments.

Table 3. Concentration of the AlOx in the stock solution used for the pH screening (column 2 and 3) and in the bottle supplied by Sigma-Aldrich. The results were obtained after a Bradford assay on the stock solution.

Organism	**U/mL SS**	**mg $_{protein}$/mL SS**	**Concentration bottle**
P. Pastoris	3.15	0.314	62.820 mg $_{protein}$/mL
C. boidinii	1.40	0.209	0.105 mg $_{protein}$/mg $_{solid}$
H. polymorpha	2.40	0.515	0.515 mg $_{protein}$/mg $_{solid}$

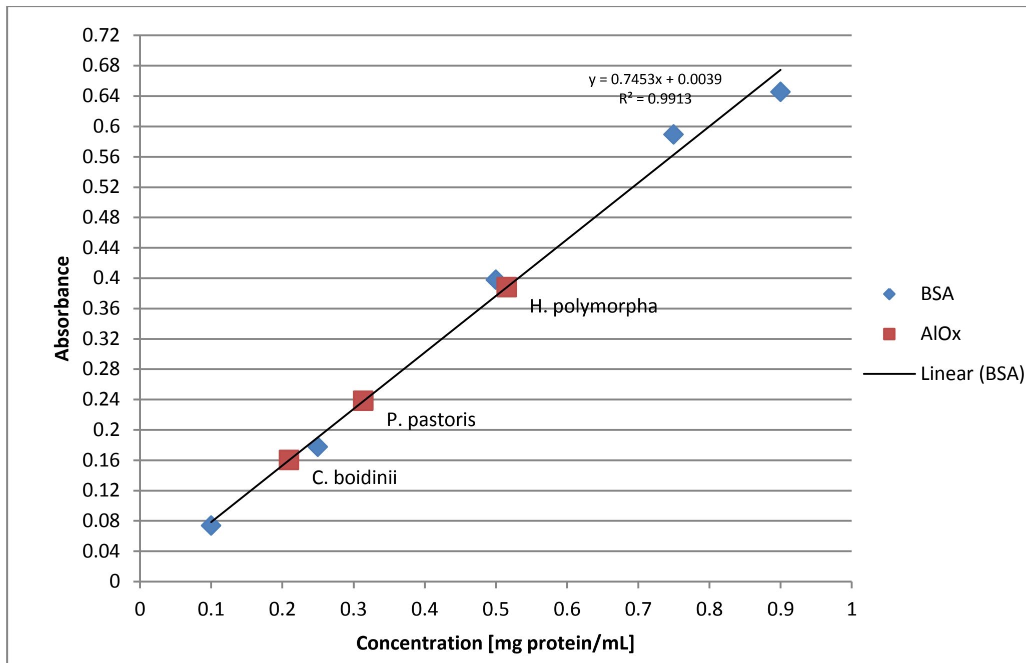

Figure 20. Absorbance against concentration in mg $_{protein}$/mL stock solution of bovine serum albumin (BSA) and AlOx from *C. boidinii, P. pastoris* and *H. polymorpha*. The calibration line (linear (BSA)) is built according to the absorbance of BSA at different concentration. The values are obtained by performing a Bradford assay. The values of BSA are the result of single experiment, whereas AlOx values represent the average of triplicates.