Means and methods for selective double diol oxidation.

Fraaije, Marco Wilhelmus; Trajkovic, Miloš; Martin, Caterina

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
15 April 2021 (15.04.2021)

WIPO | PCT

(10) International Publication Number
WO 2021/071356 A1

(51) International Patent Classification:
C12P 7/42 (2006.01) *C12P 11/00* (2006.01)

(21) International Application Number:
PCT/NL2020/050618

(22) International Filing Date:
07 October 2020 (07.10.2020)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
19201979.2 08 October 2019 (08.10.2019) EP

(71) Applicant: **RIJKSUNIVERSITEIT GRONINGEN** [NL/NL]; Broerstraat 5, 9712 CP Groningen (NL).

(72) Inventors: **FRAAIJE, Marco Wilhelmus**; University of Groningen, Faculty of Science and Engineering, GBB, Molecular Enzymology, Nijenborgh 4, 9747 AG Groningen (NL). **TRAJKOVIC, Miloš**; University of Groningen, Faculty of Science and Engineering, GBB, Molecular Enzymology, Nijenborgh 4, 9747 AG Groningen (NL). **MARTIN, Caterina**; University of Groningen, Faculty of Science and Engineering, GBB, Molecular Enzymology, Nijenborgh 4, 9747 AG Groningen (NL).

(74) Agent: **WITMANS, H.A.**; V.O., P.O. Box 87930, 2508 DH Den Haag (NL).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, IT, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*

WO 2021/071356 A1

(54) Title: MEANS AND METHODS FOR SELECTIVE DOUBLE DIOL OXIDATION.

(57) Abstract: The invention relates to the field of enzyme engineering and biocatalysis, in particular to alcohol oxidases and their application in the selective oxidation of diols for the production of industrially useful oxidation products thereof, such as precursors for biodegradable polyesters. Provided is a method for the selective oxidation of a diol substrate, comprising subjecting a diol substrate of the formula HO-CH_2-CH_2-X-CH_2-CH_2-OH, wherein X = O, S or CH_2, to a FAD-containing alcohol oxidase (AOX) enzyme of the class EC 1.1.3.13 or EC 1.1.3.17. or a mutant thereof, under conditions allowing lor the double oxidation of one hydroxyl moiety of the diol substrate into an oxidation product.

Title: Means and methods for selective double diol oxidation.

The invention relates to the field of enzyme engineering and biocatalysis. In particular, it relates to alcohol oxidases and their application in the selective oxidation of diols for the production of industrially useful oxidation products thereof, such as precursors for biodegradable polyesters.

Poly(1,4-dioxan-2-one) [poly(*p*-dioxanone); PPDO] is a biocompatible aliphatic poly(ether-ester) with good flexibility and tensile strength. It has been used to make monofilament sutures with good tenacity and knotting, and it can be applied clinically in place of multifilament sutures made from poly(glycolic acid) and poly(lactide) (PLA). Because of its good biocompatibility and physical properties, PPDO is a candidate not only for medical use but also for universal uses such as film, molded products, laminates, foams, nonwoven materials, adhesives, and coatings.

PPDO is typically prepared by the ring-opening polymerization of 1,4-dioxan-2-one (PDO) with a metallic catalyst such as Sn(II) 2-ethylhexanoate or $Al(Et)_3$ or derivatives of Ti, Zr,Cd, and Hg. Sn(II) 2-ethylhexanoate has been approved for surgical and pharmacological applications by the U.S. Food and Drug Administration. The metallic catalyst should be removed before use, particularly for medical applications.

Oxygen-containing heterocycles (O-heterocycles) form a class of compounds proven to be relevant in the polymers, fuel and in the medical fields [1],[2],[3],[4]. Some of these O-heterocycles such as lactones and in particular 1,4-dioxan-2-one can be used for synthesis of biodegradable polyesters that find countless clinical applications thanks to their biocompatibility and strength properties [5],[6]. Bioabsorbable polymers derived from 1,4-oxathian-2-one possess similar characteristics to polydioxanone, but they are not as widely studied and commercially used, probably due to the poor yield of synthesis of 1,4-oxathian-2-one[7]. The

chemical routes to synthetize some of these compounds often require expensive transition metal catalysts (Au or Pd catalysts) that increase the production costs [8]. Moreover, biodegradable polymers for biomedical applications need to be metal free [9].

Enzymatic synthesis of lactones as building blocks for polyesters recently gained great interest as environmentally sustainable alternative to current chemical methods. The most common biocatalysts to produce lactones are Baeyer-Villiger monooxygenases (BVMOs) and alcohol dehydrogenases (ADHs) [10],[11],[12],[13]. These two classes of enzymes require cofactor NAD(P)H regeneration systems if used as cell-free system. However, this cofactor requirement makes these strategies less suitable for commercial applications.

Nishida *et al.* (J. Polymer Science Part A Polymer Chemistry 38(9):1560-1567 · May 2000) reported that metal-free polyesters useful for medical applications can be prepared by enzyme-catalyzed polymerization. More in particular, it was found that the harmful effects of metallic residues in PPDO for medical applications can be avoided by carrying out the enzymatic polymerization of 1,4-dioxan-2-one (PDO) at 60°C for 15 h with 5 wt % immobilized lipase CA derived from *Candida antarctica*. However, the PDO monomers used in this enzymatic ring-opening polymerization were still obtained in a chemical fashion by the oxidative dehydrogenation of diethylene glycol involving highly dispersed copper metal supported on silica.

The present inventors therefore set out to develop a method for the enzymatic synthesis of compounds that can serve as building blocks for medical grade polymers such as PPDO. More specifically, they aimed at the provision of a biocatalytic process for converting organic starting compounds that are cheap and widely available into precursors for the (enzyme-catalyzed) production of metal-free PPDO and polymers related thereto.

It was surprisingly found that these goals could be met by the use of specific alcohol oxidase (AOX) enzymes to produce a hydroxyl acid by the selective double oxidation of a single hydroxyl group of a 1,5-diol compound as starting material. Diols are cheap and easy accessible feedstock that can be transformed to lactones or hydroxy acids, which are precursors for biodegradable polymers. The hydroxyl acid product can serve as building block for e.g. biodegradable polymers including PPDO.

As is shown herein below, AOX from *Phanerochaete chrysosporium* (PcAOX; EC 1.1.3.13) or a mutant thereof, and a mutant choline oxidase from *Arthrobacter chlorophenolicus* (AcCO; EC 1.1.3.17) comprising mutations S101A, D250G, F235R, V355T, F357R and M359R showed an unexpected promiscuity for different long chain diols and selectively oxidises these compounds to lactones or hydroxy acids. In contrast, (sequence-)related AOX enzymes from other sources including alcohol oxidase from *Hansenula* sp., *Pichia pastoris* or *Candida boidinii*, did not show any double diol oxidation activity under the same conditions.

Accordingly, the invention provides a method for the selective oxidation of a diol substrate, comprising subjecting a 1,5-diol substrate of the formula HO-CH_2-CH_2-X-CH_2-CH_2-OH, wherein X = O, S or CH_2, to a FAD-containing alcohol oxidase (AOX) enzyme of the class EC1.1.3.13 or EC 1.1.3.17, or a mutant thereof, under conditions allowing for the double oxidation of one hydroxyl moiety of the diol substrate into an oxidation product, wherein said AOX is AOX from *Phanerochaete chrysosporium* (PcAOX; EC 1.1.3.13) or a mutant thereof, or wherein said AOX is a mutant choline oxidase from *Arthrobacter chlorophenolicus* (AcCO; EC 1.1.3.17) comprising mutations S101A, D250G, F235R, V355T, F357R and M359R (AcCO6).

This approach can replace expensive processes, because alcohol oxidases are stable and contain a tightly bound flavin cofactor for which no cofactor recycling system is needed. For regenerating the oxidized flavin cofactor, dioxygen is used. The only side product in this reaction is hydrogen peroxide, which easily degrades into water and dioxygen upon the addition of catalase. The reaction process is clean, there are no other side products other than water and dioxygen, and it can be coupled to further enzymatic or chemical process(es) without prior purification of the oxidation product.

A method for the enzyme-catalyzed selective diol oxidation as herein disclosed is not taught or suggested in the art. Desymmetrization of diols is a challenging problem and selectively double oxidation of diols on one hydroxyl group has never been done enzymatically by alcohol oxidases for industrial interesting compounds.

Nguyen *et al.* (2018, Biochemistry. 57, 43, p. 6209-6218; see also WO2019/212351) reported that alcohol oxidase from *Phanerochaete chrysosporium* (PcAOX; EC1.1.3.13) is a very promising enzyme for glycerol biotransformation. They describe the comprehensive structural and biochemical characterization of from the white-rot basidiomycete. Steady-state kinetics revealed that PcAOX is highly active towards methanol, ethanol, and 1-propanol, but showed very limited activity towards glycerol. Based on the crystal structure of the homo-octameric PcAOX, several mutants with significantly improved activity towards glycerol were designed, including the F101S variant having a high k_{cat} value of 3 s^{-1} and retaining a high degree of thermostability. Furthermore, the generated mutant enzymes were found to also accept other small aliphatic alcohols such as (*R*)-(−)-1,2-propanediol, yielding the corresponding lactaldehyde. However, the documents are silent about activity towards a 1,5-diol of the above formula, let alone that it teaches or suggests the formation of a double oxidation product as disclosed in the present invention.

In a study by Kawashima (Biosci. Biotech. Biochem., 59 (5), 934-935, 1995), a screening of more than hundred micro-organisms revealed that six strains, among which resting cells of *Candida rugose* IFO 1364, are able to oxidize thiodiglycol (TOG) and produce[(2-hydroxyethyl)thio] acetic acid (HETA). However, the identity of the responsible enzyme(s) was not investigated or otherwise revealed. Furthermore, nothing is disclosed about *P. chrysosporium* or *A. chlorophenolicus*, let alone the AOX enzymes thereof.

The invention provides a method for the selective oxidation of a diol substrate, comprising subjecting a 1,5-diol substrate of the formula HO-CH_2-CH_2-X-CH_2-CH_2-OH, wherein X = O, S or CH_2, to a FAD-containing alcohol oxidase (AOX) enzyme, wherein said AOX is PcAOX or a mutant thereof, or wherein said AOX is AcCO6.

According to the invention, a 1,5-diol of the formula HO-CH_2-CH_2-X-CH_2-CH_2-OH is contacted with a FAD-containing alcohol oxidase (AOX) enzyme of the class EC1.1.3.13 or EC1.1.3.17, or a mutant thereof as defined herein above, under conditions allowing for the double oxidation of one hydroxyl moiety of the diol substrate into an oxidation product.

In one embodiment, X is O or S. For example, the 1,5-diol substrate is diethylene glycol (X is O) to yield the oxidation product (2-hydroxyethoxy)acetic acid. In another embodiment, the diol substrate is thiodiethanol, (also known as thiodiglycol; X is S) to yield the oxidation product [(2-hydroxyethyl)thio]acetic acid. In a still further embodiment, the diol substrate is 1,5-pentanediol (X is CH_2) and wherein the oxidation product is 5-hydroxypentanoic acid.

The reaction can also be performed using a mixture of two or more different diols of the formula HO-CH_2-CH_2-X-CH_2-CH_2-OH, thereby providing a mixture of different oxidation products. The conversion can reach full conversion for 25 mM of substrates during 24 hours, but these

enzymes can convert also higher concentrations of substrates upon prolonged reaction time.

A method of the invention is based on the surprising discovery that a FAD-containing alcohol oxidase (AOX) enzyme of the class EC1.1.3.13 or EC1.1.3.17, or a mutant thereof as defined herein above has the unique capacity to catalyze a double oxidation reaction on a single hydroxyl moiety of a linear 1,5-diol substrate of the above formula.

In one embodiment, the AOX is AOX from *Phanerochaete chrysosporium* (PcAOX; EC1.1.3.13) or a mutant thereof. For example, wildtype PcAOX was shown to efficiently convert a 1,5-diol to the corresponding 5-hydroxy acid as a single oxidation product. The k_{cat}/ K_M observed was in the range of about 1 to 16 $M^{-1}s^{-1}$, depending on the substrate used. In another embodiment, the AOX is a mutant PcAOX showing a desired catalytic activity towards the 1,5-diol. For example, the mutant comprises a substitution of the amino acid corresponding to Phe at position 101 into Gln, Asn, His or Ser. Very good results are obtained with mutant Phe101Ser PcAOX, which was previously described in relation to glycerol conversion[21]. As shown herein below, Phe101Ser PcAOX (F101S) showed selective double oxidation of all 1,5-diol substrates tested, with a higher overall yield as compared to the wild-type enzyme.

In another embodiment, a method according to the invention uses a mutant AOX wherein said AOX is an engineered choline oxidase from *Arthrobacter chlorophenolicus* (AcCO; EC 1.1.3.17). Preferably, the mutant AcCO comprises the mutations S101A, D250G, F235R, V355T, F357R and M359R. This six-amino-acid variant, herein referred to as AcCO6, was previously disclosed [20] in relation to the oxidation of primary alcohols to aldehydes.

Suitable reaction conditions can be determined by a person skilled in the art. Typically, the reaction mixture comprises a buffer in the range pH 6.5-8, for example a 100 mM potassium phosphate buffer pH 7.5 is used. The

oxidation can be performed at any suitable temperature, preferably in the range of room temperature to about 40°C, for example around 35°C. Conversions can be done at atmospheric oxygen concentrations. The concentration of the diol substrate can be varied according to needs, and may range from e.g. 1 to 100 mM, preferably 2 to 50 mM. The AOX enzyme used is suitably obtained as recombinant polypeptide, as was previously described.[21]

Once the double oxidation product is formed, a method of the invention may further comprise the step of subjecting the oxidation product to a subsequent enzymatic or chemical reaction. Preferably, said subsequent reaction is a polymerization reaction. For example, the polymerization reaction comprises the synthesis of a polymer that is biocompatible and/or biodegradable. In a specific aspect, it comprises the synthesis of poly(*p*-dioxanone) (PPDO) or a product or precursor related thereto.
In one embodiment, the oxidation product(s) are used in the enzymatic synthesis of biodegradable polymers. For example, it is used as precursor in the lipase-catalyzed (bulk) esterification of linear aliphatic hydroxyacids as described by Mahapatro *et al.* (Biomacromolecules, 2004, **5**, 62-68).

Alternatively, hydroxyl acids resulting from a method of the invention can be easily chemically transformed to lactone with sodium bicarbonate, and then lactones are used as monomers (Grablowitz *et al.*, J. Mater. Chem. 2007, **17**, 4050-4056).

The invention therefore also relates to the use of a FAD-containing alcohol oxidase (AOX) enzyme of the class EC1.1.3.13 or EC1.1.3.17, or a mutant thereof, in the enzymatic conversion of a diol to an oxidation product that finds application in the synthesis of a biodegradable polymer. Provided is the use of a FAD-containing alcohol oxidase (AOX) enzyme in the enzymatic conversion of a diol to an oxidation product that finds application in the synthesis of biodegradable polymer, wherein said AOX is AOX from

Phanerochaete chrysosporium (PcAOX; EC 1.1.3.13) or a mutant thereof, or wherein said AOX is a mutant choline oxidase from *Arthrobacter chlorophenolicus* (AcCO; EC 1.1.3.17) comprising mutations S101A, D250G, F235R, V355T, F357R and M359R (AcCO6).

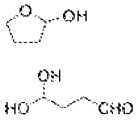
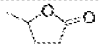
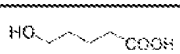
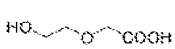
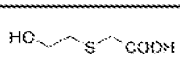
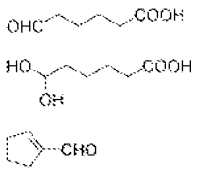
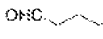
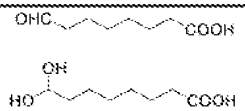
EXPERIMENTAL SECTION

Example 1: Diol substrate specificity

To determine the substrate specificity of AOX, a series of diol substrates was incubated with wild-type or variant F101S of alcohol oxidase from *Phanerochaete chrysosporium* (PcAOX; EC1.1.3.13). Table 1 shows the list of tested substrates and the conversion according to 1H NMR. NMR analysis proved to be the best method for analysis and evaluation for this type of compounds. This approach is fast and it allows for a quantitative and qualitative analysis of the products.

PcAOX showed activity towards the shortest diol tested (1,3-propanediol), but a mixture of products was obtained in this reaction (very complex NMR spectra without any defined product). Most likely, the enzyme converts this substrate to very active aldehyde or dialdehyde species. Substrates **1** and **2** (1,4-diols) were oxidized by PcAOX in a similar manner. In both cases the product was obtained in the γ-butyrolactone form. Under these conditions, the reaction for substrate **1** was not completed (26 % of substrate remained in the reaction). The intermediate was the main component for this substrate and exists in a buffer environment as mixture of hemiacetal and gem-diol (ratio 3/1). Substrate **2** was instrumental in understanding the reaction mechanism. For substrate **2**, complete conversion was reached and the enzyme showed preference for the primary alcohol group in presence of secondary alcohol groups. The product of the double oxidation was obtained as a mixture of the lactone form and the hydrolyzed form 4-hydroxypentanoic acid (ratio 4:1) .

Table 1. Results of AOX F101S catalysed conversion of diols [a].

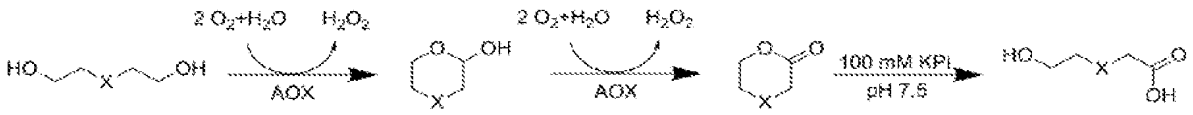
Entry	Substrate	Intermediate	Product	Intermediate [%]	Product [%]
1*				64	10
2*				-	>99[b]
3				-	>99
4				-	>99
5				-	>99
6*				-	67[c] 33 (aldol)
7*				-	>99[c]

* denotes comparative example

[a] Reaction condition: substrate (20 mM), enzyme (40 µM), 100 mM potassium phosphate buffer pH 7.5, 48 h, 35 °C. Yields were determined based on ^{1}H NMR. NMR was performed after the addition of D_2O (15% v/v). [b] Cyclic to open product ration 4:1. [c] same ratio of *gem*-diol and aldehyde form of carboxylic acid.

Surprisingly, for the 1,5-diol substrates **3**, **4** and **5**, the conversion was complete and the product of double oxidation for these substrates was obtained as single product in the form of the corresponding 5-hydroxy acid. Any other products (lactone, diacid or aldehyde acid) were not observed by ^{1}H NMR. These results were opposite from those obtained for substrates **1** and **2**, which were obtained in the lactone form.

Additional experiments were performed to explain the different outcome for these substrates. Initially, we tested the stability of commercially available 1,4-dioxan-2-one, which is the lactone form of the oxidation product of substrate **4**. This compound was found to be susceptible to hydrolysis when dissolved in buffer (Scheme 1). In less than 30 minutes, the lactone compound completely hydrolyzed to hydroxy acid in the buffer environment (100 mM KPi buffer pH = 7.5). The lactone was more stable in pure D_2O, wherein complete hydrolysis was observed after some hours. Presumably, if lactone is formed during the oxidation of 1,5-diols (substrates **3**, **4** and **5**), it is hydrolyzed very fast to yield a stable hydroxy acid. We also tracked the oxidation reaction of substrate 4 over time, and observed that the hemiacetal form exists as intermediate after a single oxidation step under this condition. According to this data, we suppose that product of double oxidation is mainly formed via the hemiacetal towards lactone and then lactone is hydrolyzed to 5-hydroxy acid.



Scheme 1. Catalytic route for oxidation of 1,5-diol substrates by AOX. X can represent a carbon, oxygen or sulphur atom.

The 1,6-diol substrate **6** was included in the studies, because the corresponding product lactone (ε-caprolactone) has a relevant and established commercial value in the polymer industry [22]. Unfortunately, and quite unforeseen, the catalytic oxidation by PcAOX resulted in the oxidation of both hydroxyl groups to aldehyde groups with the production of adipaldehyde. Adipaldehyde could spontaneously undergo to product of aldol condensation in buffer environment (non-enzymatic reaction) or can be further oxidized to 6-oxohexanoic acid. The remaining aldehyde group on the other terminal of the aliphatic chain is in equilibrium with the gem-diol

form (ratio ~1:1). At any rate, this 1,6-diol does not yield a single reaction product like the 1,5-diol substrates **3**, **4** and **5**.

Similar to substrate **6**, 1,8-octanediol (substrate **7**) undergoes oxidation on both hydroxylic groups yielding suberaldehyde, which then is further oxidized on one aldehyde group resulting in a oxocarboxylic acid. The remaining aldehyde group is also in equilibrium with the *gem*-diol form (ratio ~ 1:1).

For selective oxidation of only one hydroxyl group, the formation of a very stable hemiacetal intermediate is essential. If the hemiacetal form is not stable, then the PcAOX preferably oxidises the other hydroxyl group to dialdehyde, and at the end one of the aldehyde group is oxidized to carboxylic acid via gem-diol. Once the carboxylic acid is obtained, the enzyme cannot accept this substrate for the further oxidation towards diacid.

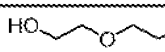
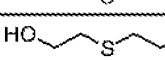
Example 2: Kinetic parameters of diol conversion by PcAOX

The initial reaction rates (Table 2) were measured and fitted to the Michealis-Menten equation to extrapolate k_{cat} and K_M values. To that end, a HRP(horse radish peroxidase)-coupled assay was used. This assay measures the reaction rate thanks to the hydrogen peroxide generated by AOX during the substrate conversion. Hydrogen peroxide is used by HRP (40 U/mL) to catalyze the oxidative coupling reaction of 4-aminoantipyrine (0.1 mM) and 3,5-dichloro-2-hydroxybenzenesulfonic acid (1 mM). This reaction results in the formation of a pink quinoid product that can be detected using a spectrophotometer (ε515 =26 mM−1cm−1).

The values obtained for substrate **2** are in support of the catalytic mechanism of the double oxidation going through the lactol intermediate. Plausibly, the rate limiting step is the second one (lower k_{cat}): the oxidation of the hydroxylic group of the lactol. This is confirmed by substrate **1** and by

shorter conversion experiments that showed accumulation of the lactol as intermediate (same trend was observed for substrates **2**-**5**). Indeed, Substrate **2** compared to substrates **3**, **4** and **5**, showing kinetic parameters in the same order of magnitude. However, the conversion reaction after a 48 h time period does not show the same amount of product formed, which implies that the kinetics observed are relative to the first oxidation step, and the second step occurs with a lower rate.

Table 2. Steady-state kinetic parameters of wild-type and AOX F101S.

Entry	Substrate	Wild-type PcAOX			F101S PcAOX		
		K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)
3		>600	>2.2	3.7	69.5	3.5	50
4		308	4.9	16	12.3	0.73	59
5		>400	>0.4	1	119	3.6	30

Values obtained using the HRP-coupled assay in 50 mM potassium phosphate, pH 7.5.

Example 3: 1, 5-diol conversion by other oxidative enzymes

The 1,5-diol compounds **4** and **5** were also evaluated as substrates for other oxidative enzymes: alditol oxidase (HotAldO) from *Acidothermus cellulolyticus* 11B [24], chitooligosaccharide oxidase (ChitO) from *Fusarium graminearum* [25], 5-hydroxymethylfurfural oxidase (HMFO) wild type and variant HMFO8b from *Methylovorus* sp. strain MP688 [17][19], AOX alcohol oxidase from *Hansenula* sp. (EC 1.1.3.13), glucose oxidase form *Aspergillus niger* (EC 1.1.3.4) [26], and choline oxidase (AcCO) wild type and an engineered variant (AcCO6) from *Arthrobacter chlorophenolicus* [20] .

No oxidation products of substrates **4** and **5** were detected in any of these cases, except for incubation with AcCO6. The six-fold mutant of choline oxidase (EC 1.1.3.17) also proved to be able to perform the double oxidation of 1,5-diols **4** and **5**, albeit with lower yields: 5% of product 4 (38% of intermediate) and 28% of product 5 (68% of intermediate) using the same reaction conditions of AOX.

In conclusion, this study has revealed the potential of selected AOX enzymes to selectively double oxidize one hydroxylic group in diol-substrates that can form a stable hemiacetal *in situ*. The products obtained (e.g. γ-butyrolactone and γ-valerolactone) find a direct application in the polymer industry [4] [27]. Very advantageous is the conversion by AOX of diethylene glycol and thiodiethyleneglycol to their corresponding hydroxy acids, which are interesting building blocks for biodegradable polymers. The versatility of the alcohol oxidases to perform these conversions provides a promising industrial application.

Example 4: Activities of Related AOX enzymes

In order to demonstrate the unique properties of PcAOX, two sequence-related alcohol oxidases, also known as methanol oxidases, were evaluated with respect to their substrate usage.

Two commercial AOXs from Sigma Aldrich (Alcohol Oxidase from *Pichia pastoris* and Alcohol Oxidase from *Candida boidinii*) were tested under the same experimental conditions as herein above. This involved the following reaction mixture: substrate (20 mM), enzyme (40 μM), 100 mM potassium phosphate buffer pH 7.5, 48 h, 35 °C.

Enzyme activity was tested towards 1,5-diol substrates **3** (1,5-pentanediol), **4** (diethylene glycol) and **5** (2,2'-thiodiethanol). Neither of the *Pichia pastoris* and *Candida boidinii* enzymes showed any conversion of these three diol compounds. Only the starting material was present after 48 h of incubation.

PcAOX wt and PcAOX F101S were included as positive control, and complete conversion for all three substrates to the corresponding hydroxy acid products was confirmed. Furthermore, the two commercial AOXs were also tested for activity towards methanol: both enzymes showed activity towards methanol, showing that they were catalytically active but not capable of performing the double diol oxidation reaction.

REFERENCES

[1] P. H. PLESCH, in *Polym. Heterocycles (Ring Opening)* (Ed.: S. Penczek), Pergamon, **1977**, pp. 287–293.

[2] P. K. Singh, O. Silakari, **2018**, 1071–1087.

[3] Z. Lu, **2017**, 6567–6574.

[4] M. Gagliardi, A. Bifone, *PLoS One* **2018**, *13*, 1–15.

[5] N. Goonoo, R. Jeetah, A. Bhaw-luximon, D. Jhurry, **2015**, *97*, 371–391.

[6] J. A. Von Fraunhofer, R. S. Storey, I. K. Stone, B. J. Masterson, **1985**, *19*, 595–600.

[7] A. C. Company, M. Street, **1985**, *23*, 843–849.

[8] A. Deiters, S. F. Martin, **2004**, DOI 10.1021/cr0200872.

[9] H. Nishida, M. Yamashita, M. Nagashima, T. Endo, Y. Tokiwa, **2000**, 1560–1567.

[10] F. Hollmann, S. Kara, D. J. Opperman, Y. Wang, *Chem. - An Asian J.* **2018**, DOI 10.1002/asia.201801180.

[11] C. D. Dithugoe, J. van Marwijk, M. S. Smit, D. J. Opperman, *ChemBioChem* **2018**, DOI 10.1002/cbic.201800533.

[12] S. Kara, D. Spickermann, J. H. Schrittwieser, A. Weckbecker, C. Leggewie, I. W. C. E. Arends, F. Hollmann, *ACS Catal.* **2013**, *3*, 2436–2439.

[13] L. Huang, G. V. Sayoga, F. Hollmann, S. Kara, *ACS Catal.* **2018**, *8*, 8680–8684.

[1] P. H. PLESCH, in *Polym. Heterocycles (Ring Opening)* (Ed.: S. Penczek), Pergamon, **1977**, pp. 287–293.

[2] P. K. Singh, O. Silakari, **2018**, 1071–1087.

[3] Z. Lu, **2017**, 6567–6574.

[4] M. Gagliardi, A. Bifone, *PLoS One* **2018**, *13*, 1–15.

[5] N. Goonoo, R. Jeetah, A. Bhaw-luximon, D. Jhurry, **2015**, *97*, 371–391.

[6] J. A. Von Fraunhofer, R. S. Storey, I. K. Stone, B. J. Masterson, **1985**, *19*, 595–600.

[7] A. C. Company, M. Street, **1985**, *23*, 843–849.

[8] A. Deiters, S. F. Martin, **2004**, DOI 10.1021/cr0200872.

[9] H. Nishida, M. Yamashita, M. Nagashima, T. Endo, Y. Tokiwa, **2000**, 1560–1567.

[10] F. Hollmann, S. Kara, D. J. Opperman, Y. Wang, *Chem. - An Asian J.* **2018**, DOI 10.1002/asia.201801180.

[11] C. D. Dithugoe, J. van Marwijk, M. S. Smit, D. J. Opperman, *ChemBioChem* **2018**, DOI 10.1002/cbic.201800533.

[12] S. Kara, D. Spickermann, J. H. Schrittwieser, A. Weckbecker, C. Leggewie, I. W. C. E. Arends, F. Hollmann, *ACS Catal.* **2013**, *3*, 2436–2439.

[13] L. Huang, G. V. Sayoga, F. Hollmann, S. Kara, *ACS Catal.* **2018**, *8*, 8680–8684.

[14] P. N. R. Vennestrøm, C. H. Christensen, S. Pedersen, J. Grunwaldt, J. M. Woodley, **2010**, 249–258.

[15] E. Romero, J. R. Gómez Castellanos, G. Gadda, M. W. Fraaije, A. Mattevi, *Chem. Rev.* **2018**, acs.chemrev.7b00650.

[16] M. Pickl, M. Fuchs, S. M. Glueck, K. Faber, K. Faber, **2015**, 6617–6642.

[17] W. P. Dijkman, M. W. Fraaije, *Appl. Environ. Microbiol.* **2014**, *80*, 1082–1090.

[18] W. P. Dijkman, C. Binda, M. W. Fraaije, A. Mattevi, *ACS Catal.* **2015**, *5*, 1833–1839.

[19] C. Martin, A. Ovalle Maqueo, H. J. Wijma, M. W. Fraaije, *Biotechnol. Biofuels* **2018**, *11*, 56.

[20] R. S. Heath, W. R. Birmingham, M. P. Thompson, A. Taglieber, L. Daviet, N. J. Turner, **2019**, 276–281.

[21] Q.-T. Nguyen, E. Romero, W. Dijkman, S. P. de Vasconcellos, C. Binda, A. Mattevi, M. W. Fraaije, *Biochemistry* **2018**, *57*, acs.biochem.8b00918.

[22] A. L. Sisson, D. Ekinci, A. Lendlein, *Polymer (Guildf).* **2013**, *54*, 4333–4350.

[23] A. Rouchaud, J. C. Braekman, *European J. Org. Chem.* **2009**, *5*, 2666–2674.

[24] R. T. Winter, D. P. H. M. Heuts, E. M. A. Rijpkema, E. Van Bloois, H. J. Wijma, M. W. Fraaije, *Appl. Microbiol. Biotechnol.* **2012**, *95*, 389–403.

[25] A. R. Ferrari, M. Lee, M. W. Fraaije, *Biotechnol. Bioeng.* **2015**, *112*, 1074–1080.

[26] I. J. van der Klei, L. V Bystrykh, W. Harder, in *Hydrocarb. Methylotrophy*, Academic Press, **1990**, pp. 420–427.

[27] M. Danko, J. Mosnázek, *Polimery/Polymers* **2017**, *62*, 272–282.

Claims

1. A method for the selective oxidation of a diol substrate, comprising subjecting a diol substrate of the formula HO-CH_2-CH_2-X-CH_2-CH_2-OH, wherein X = O, S or CH_2, to a FAD-containing alcohol oxidase (AOX) enzyme under conditions allowing for the double oxidation of one hydroxyl moiety of the diol substrate into an oxidation product, wherein said AOX is AOX from *Phanerochaete chrysosporium* (PcAOX; EC 1.1.3.13) or a mutant thereof, or wherein said AOX is a mutant choline oxidase from *Arthrobacter chlorophenolicus* (AcCO; EC 1.1.3.17) comprising mutations S101A, D250G, F235R, V355T, F357R and M359R (AcCO6).

2. Method according to claim 1, wherein X is O or S.

3. Method according to claim 2, wherein the diol substrate is diethylene glycol (X is O) and wherein the oxidation product is (2-hydroxyethoxy)acetic acid.

4. Method according to claim 2, wherein the diol substrate is thiodiethanol, (thiodiglycol) (X is S) and wherein the oxidation product is [(2-hydroxyethyl)thio]acetic acid.

5. Method according to claim 1, wherein the diol substrate is 1,5-pentandiol (X is CH_2) and wherein the oxidation product is 5-hydroxypentanoic acid.

6. Method according to any one of the preceding claims, wherein said AOX is AOX from *Phanerochaete chrysosporium* (PcAOX; EC 1.1.3.13) or a mutant thereof.

7. Method according to claim 6, wherein the mutant is Phe101Ser PcAOX.

8. Method according to any one of claims 1 to 5, wherein said AOX is a mutant choline oxidase from *Arthrobacter chlorophenolicus* (AcCO; EC 1.1.3.17) comprising mutations S101A, D250G, F235R, V355T, F357R and M359R (AcCO6).

9. Method according to any one of the preceding claims, further comprising the step of subjecting the oxidation product to a subsequent enzymatic or chemical reaction.

10. Method according to claim 9, wherein said subsequent reaction is a polymerization reaction.

11. Method according to claim 10, wherein said polymerization reaction comprises the synthesis of a biocompatible and/or biodegradable polymer.

12. Method according to claim 11, comprising the synthesis of poly(*p*-dioxanone) (PPDO).

13. The use of a FAD-containing alcohol oxidase (AOX) enzyme in the enzymatic conversion of a diol to an oxidation product that finds application in the synthesis of biodegradable polymer, wherein said AOX is AOX from *Phanerochaete chrysosporium* (PcAOX; EC 1.1.3.13) or a mutant thereof, or wherein said AOX is a mutant choline oxidase from *Arthrobacter chlorophenolicus* (AcCO; EC 1.1.3.17) comprising mutations S101A, D250G, F235R, V355T, F357R and M359R (AcCO6).

14. Use according to claim 13, in the enzymatic conversion of a diol diol substrate of the formula HO-CH_2-CH_2-X-CH_2-CH_2-OH, wherein X = O, S or CH_2.

INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2020/050618

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12P7/42 C12P11/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS, COMPENDEX, EMBASE, FSTA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	WO 2019/212351 A2 (UNIV GRONINGEN [NL]; UNIV DEGLI STUDI DI PAVIA [IT]) 7 November 2019 (2019-11-07) abstract; claim 14 page 11, lines 1-19	13
A	HIDEKI KAWASHIMA: "Production of [(2-Hydroxyethyl)thio] acetic Acid from Thiodiglycol (2,2'-Thiodiethanol) by Resting Cells of Candida rugosa IFO 1364", BIOSCIENCE, BIOTECHNOLOGY AND BIOCHEMISTRY, vol. 59, no. 5, 1 January 1995 (1995-01-01), pages 934-935, XP055679861, JP ISSN: 0916-8451, DOI: 10.1271/bbb.59.934 the whole document	1,2,4, 9-11,13, 14

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 December 2020	12/01/2021

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Schröder, Gunnar

1

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PICKL MATHIAS ET AL: "The substrate tolerance of alcohol oxidases", APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, SPRINGER BERLIN HEIDELBERG, BERLIN/HEIDELBERG, vol. 99, no. 16, 8 July 2015 (2015-07-08), pages 6617-6642, XP035521108, ISSN: 0175-7598, DOI: 10.1007/S00253-015-6699-6 [retrieved on 2015-07-08] abstract Schemes 3, 4, 6; page 6617 - page 6621; table 1	1,13
A	ERIK W. VAN HELLEMOND ET AL: "Exploring the Biocatalytic Scope of Alditol Oxidase from Streptomyces coelicolor", ADVANCED SYNTHESIS & CATALYSIS, vol. 351, no. 10, 1 July 2009 (2009-07-01) , pages 1523-1530, XP055679885, DE ISSN: 1615-4150, DOI: 10.1002/adsc.200900176 abstract; table 1 page 1526; table 2	1,13
A	NISHIDA A ET AL: "Formation, purification, and partial characterization of methanol oxidase, a hydrogen peroxide-producing enzyme in Phanerochaete chrysosporium", BIOTECHNOLOGY AND APPLIED BIOCHEMISTRY, vol. 9, no. 4, 1987, pages 325-338, XP009519564, ISSN: 0885-4513 abstract; table II	1-3,6,13
A	QUOC-THAI NGUYEN ET AL: "Structure-Based Engineering of Phanerochaete chrysosporium Alcohol Oxidase for Enhanced Oxidative Power toward Glycerol", BIOCHEMISTRY, vol. 57, no. 43, 1 October 2018 (2018-10-01), pages 6209-6218, XP055626075, ISSN: 0006-2960, DOI: 10.1021/acs.biochem.8b00918 abstract	1

-/--

1

INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2020/050618

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	RACHEL S. HEATH ET AL: "An Engineered Alcohol Oxidase for the Oxidation of Primary Alcohols", CHEMBIOCHEM, vol. 20, no. 2, 10 December 2018 (2018-12-10), pages 276-281, XP055760781, ISSN: 1439-4227, DOI: 10.1002/cbic.201800556 abstract; tables 1-3 -----	1,8

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/NL2020/050618

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2019212351 A2	07-11-2019	NONE	