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# Pyranose oxidase: A versatile sugar oxidoreductase for bioelectrochemical applications

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ABSTRACT

Pyranose oxidase (POx) is an FAD-dependent oxidoreductase, and like glucose oxidase (GOx) it is a member of the glucose-methanol-choline (GMC) superfamily of oxidoreductases. POx oxidizes several monosaccharides including D-glucose, D-galactose, and D-xylose, while concurrently oxygen is reduced to hydrogen peroxide. In addition to this oxidase activity, POx shows pronounced activity with alternative electron acceptors that include various quinones or (complexed) metal ions. Even though POx in general shows properties that are more favourable than those of GOx (e.g., a considerably higher catalytic efficiency ($k_{cat}/K_m$) for D-glucose, significantly lower Michaelis constants $K_m$ for D-glucose, reactivity with both anomeric forms of D-glucose) it is much less frequently used for both biosensor and biofuel cell applications than GOx. POx has been applied in biosensing of D-glucose, D-galactose, and D-xylose, and in combination with α-glucosidase also maltose. An attractive application is in biosensors constructed for the measurement of 1,5-anhydro-D-glucitol, a recognised biomarker in diabetes. Bioelectrochemical applications of POx had been restricted to enzymes of fungal origin. The recent discovery and characterisation of POx from bacterial sources, which show properties that are very distinct from the fungal enzymes, might open new possibilities for further applications in bioelectrochemistry.



**Contents**



*Abbreviations:* 1,4-BQ, 1,4-benzoquinone; 1,5-AG, 1,5-anhydro-D-glucitol; *An*GOx, glucose oxidase from *Aspergillus niger*; *As*POx, pyranose oxidase from *Arthrobacter siccitolerans*; BFC, biofuel cell; CNT, carbon nanotube; CPE, carbon paste electrode; *Cs*POx, POx from *Coriolus* sp.; DCIP, dichlorophenolindophenol; DET, direct electron transfer; DNS, 3,5-dinitrosalicylic acid; EC, enzyme coating; EPC, enzyme precipitation coating; FAD, flavin-adenine dinucleotide; Fc, ferrocene; $Fc^+$, ferrocenium ion; FcMeOH, ferrocenemethanol; GCE, glassy carbon electrode; GMC, glucose-methanol-choline superfamily of oxidoreductases; GNP, gold nanoparticle; GOx, glucose oxidase; *Cs*POx, pyranose oxidase from *Coriolus* sp.; HRP, horseradish peroxidase; *Ka*POx, pyranose oxidase from *Kitasatospora aureofaciens*; MET, mediated electron transfer; *Pc*POx, pyranose oxidase from *Phanerochaete chrysosporium*; POx, pyranose oxidase; *To*POx, pyranose oxidase from *Trametes ochracea*.

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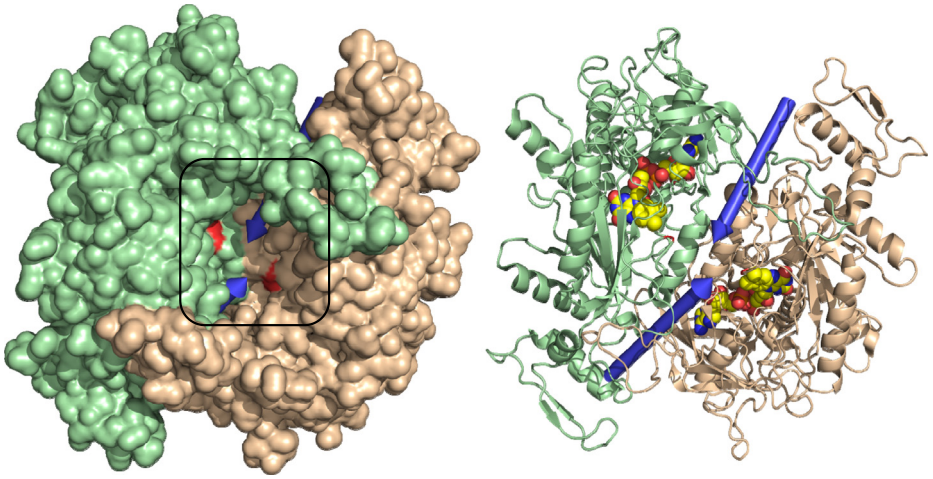


**Fig. 1.** Two subunits of the tetrameric pyranose oxidase from *Trametes ochracea* (1TT0) showing the central void (black frame) that isolates the active site entrances from the exterior. Blue arrows indicate the substrate channels leading from the exterior to the central void. Left panel: The active-site entrances with its active-site loop (residues 452–456) in its closed confirmation is marked (Ser455 in red). Right panel: the FAD is highlighted in this cartoon representation to indicate the position of the active site.

## 1. About the enzyme

Pyranose oxidase (POx; pyranose 2-oxidase, glucose 2-oxidase; EC 1.1.3.10, pyranose:oxygen 2-oxidoreductase) is a flavin-dependent oxidoreductase of the glucose-methanol-choline (GMC) superfamily of oxidoreductases. It oxidizes D-glucose as well as other monosaccharide substrates at the C2 position while reducing molecular oxygen to hydrogen peroxide. The GMC superfamily includes other well-characterized sugar oxidoreductases such as glucose oxidase, FAD-dependent glucose dehydrogenase, cellobiose dehydrogenase or pyranose dehydrogenase [1]. POx is typically found in wood-degrading fungi, where it is extracellularly associated with membrane-bound vesicles or other membrane structures in the periplasmic space of hyphae [2]. As a producer of hydrogen peroxide, POx is considered to fuel other enzymes for lignocellulose degradation, and as such it is also a member of the auxiliary activity family 3 (AA3_4) in the database of Carbohydrate-Active enZYmes (CAZy; http://www.cazy.org/) [3]. First reports of the enzyme go back to 1968, when a carbohydrate oxidase was found in mycelia of *Spongipellis unicolor* (formerly *Polyporus obtusus*) [4]. POx from fungal sources is a biochemically well-studied enzyme with crystal structures available from *Trametes ochracea* (synonym *Trametes multicolor*; PDB 1TT0), *Peniophora* sp. (PDB 1TZL), and *Phanerochaete chrysosporium* (PDB 4MIF). In addition, structures from a number of POx variants have been determined [5–7]. Like other members of the GMC family, POx consists of two domains, a flavin-binding and a substrate-binding domain. The flavin-binding domain contains the canonical Rossmann fold or βαβ mononucleotide-binding motif, and is highly conserved throughout flavoproteins. The substrate-binding domain shows more sequence variations, reflecting preference for different electron-donor substrates. A fungal POx is formed by four identical monomers, each of which harbours a flavin-adenine dinucleotide (FAD) in the active site that is covalently linked to a histidine residue [8]. The tetrameric structure of fungal POx shows a big central void that is connected to all four individual active sites (Fig. 1). It is only through this central void that substrates can enter the active sites via narrow substrate channels (Fig. 2) [5,6]. The active sites in POx are therefore deeply buried within the tetrameric structure and isolated from the ambient environment, and this restricted access of the active site through the substrate channel and the void seems to limit activity of POx to monosaccharides. In addition, an active-site loop in direct vicinity of the isoalloxazine, which can shift between an open

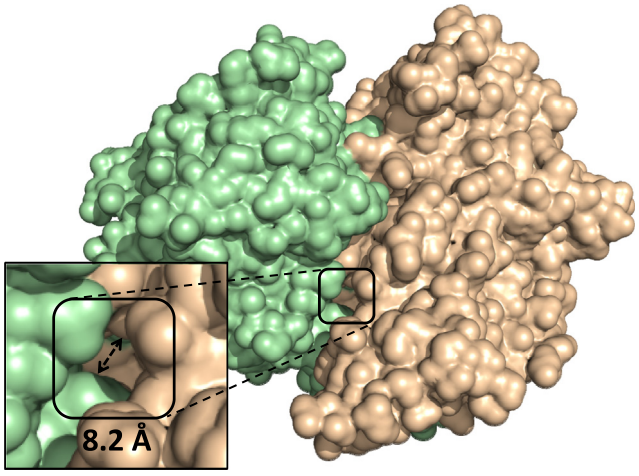



**Fig. 2.** Close-up of the entrance to channel leading into to interior void of pyranose oxidase from *Trametes ochracea* (1TT0).

(electron-donor binding) and a closed (electron-acceptor binding) confirmation, is important for substrate binding and specificity of POx [6,9]. Interactions between the single subunits to form the homo-tetramer are made by oligomerisation structures that include an oligomerisation arm stretching out to embrace a neighbouring subunit as well as a loop providing subunit-subunit interactions and a small domain termed head domain [6].

In addition to fungi, POx is also found in bacterial species of actinobacteria, proteobacteria and bacilli [10]. Although a bacterial origin of the fungal *pox* gene was already suggested in 2008 by Kittl et al. [11], it was only recently shown that POx sequences are also frequently found in bacteria with sequence identities of 39–24% to fungal POx. A recent phylogenetic study showed that POx is the only GMC enzyme that is shared among both fungi and bacteria [12]. This is evident from the close relationship of bacterial POx genes with the clade of fungal POx sequences and a maximal (100%) bootstrap support for this relation (Fig. 3). This phylogenetic tree of POx sequences shows that putative pyranose oxidase-encoding genes are mainly found in the phylum of *Firmicutes* and particularly in *Actinobacteria.* The first bacterial POx biochemically characterized is from *Arthrobacter siccitolerans* (*As*POx) [13]. Bacterial *As*POx showed some properties that are distinctly different from its fungal counterparts, for example the FAD is

non-covalently bound, and recombinant *As*POx produced in *E. coli* was monomeric in solution [13].

One actinobacterial sequence that is closely related to fungal POx was shown to be from *Kitasatospora aureofaciens* (formerly *Streptomyces aureofaciens*). Comparison of the sequence of *K. aureofaciens* POx (*Ka*POx) and that from *Trametes ochracea* (*To*POx), which is the most thoroughly characterized fungal POx to date, revealed a sequence identity of 38.7% and a query match of 545 out of 623 *To*POx residues. *Ka*POx was recombinantly produced in *E. coli* and biochemically studied, and again some distinct differences to fungal POx were shown. Recombinant *Ka*POx forms active dimers in solution while the FAD is covalently attached [10].

The overall reaction mechanism of POx is similar to that of other FAD-dependent GMC oxidoreductases and consists of two half-reactions. Sugar oxidation includes a direct hydride transfer from C2 of the substrate to the N5 atom of the isoalloxazine moiety of FAD [14] and results in reduced $FADH_2$ (reductive half-reaction). $FADH_2$ is subsequently re-oxidized by a suitable electron acceptor (oxidative half-reaction). The overall reaction mechanism follows the Ping Pong Bi Bi type [14]. The preferred electron donor substrate of POx as judged from the catalytic constants is D-glucose, which is oxidized in both the α- and β-anomeric form [15] giving 2-keto D-glucose (2-dehydro-D-glucose, D-*arabino*-hexopyranos-2-ulose) as the product. This reactivity with both anomers of D-glucose is in contrast to glucose oxidase (GOx), which is much more frequently used in bioelectrochemistry but only reacts with the β-anomer. Other monosaccharides in their aldopyranose form can also be oxidized by POx at the C2 position to the corresponding keto-sugars [16,17]. These additional sugar substrates include for example D-xylose, D-galactose, L-sorbose, and L-arabinose (Table 1). POx oxidizes compounds lacking a C2 OH-group or have a restricted access to this position, such as 2-deoxy-D-glucose, 2-keto-D-glucose or methyl β-D-glucopyranoside, at the C3 position, albeit with significantly lower rates [18]. The Michaelis constant for D-glucose $K_{m,Glc}$ of different pyranose oxidases ranges from 0.74 to 5.0 mM, which is significantly lower and therefore more favourable than the values reported for *Aspergillus niger* glucose oxidase (*An*GOx), 22–28 mM [19] or 33–248 mM [20]. Turnover numbers for D-glucose ($k_{cat,Glc}$) of POx from different fungal sources range from 1.48 to 111 $s^{-1}$. The bacterial POx from *A. siccitolerans* differs from its fungal counterparts in this respect as it is only shows a $k_{cat,Glc}$ of 0.152 $s^{-1}$ while bacterial *Ka*POx has a $k_{cat,Glc}$ of 15.4 $s^{-1}$. These catalytic constants are lower than those measured for recombinant wild-type *An*GOx ($k_{cat,Glc}$ of 55–190 $s^{-1}$; [19]), yet overall, most pyranose oxidases are characterized by a catalytic efficiency ($k_{cat}/K_m$) for D-glucose that is considerably higher than for *An*GOx. A recent report specifically compared POx from *T. ochracea* and *An*GOx, and showed that the former enzyme gives a more than ten-fold higher catalytic efficiency for D-glucose under identical reaction conditions (oxygen from air, 30 °C, pH 6.5) [21].

The presumed natural electron acceptor of POx is oxygen, which can take up two electrons, and is thus reduced to hydrogen peroxide. It has been shown though that various quinones, complexed metal ions and radicals can also be utilized as electron acceptors by POx. Indeed, some of these electron acceptors show significantly better catalytic efficiencies than oxygen (Table 2). This increase is caused both by more favourable Michaelis constants as well as turnover numbers for the alternative electron acceptors. The catalytic efficiencies $k_{cat,O2}/K_{m,O2}$ for *To*POx and POx from *P. chrysosporium* (*Pc*POx) are 790 and 89.2 $mM^{-1}$ $s^{-1}$, while catalytic efficiencies for 1,4-benzoquinone (1,4-BQ), the ferrocenium ion ($Fc^+$) and dichlorophenolindophenol (DCIP) for these two enzymes under optimal conditions are 900 and 11,990 $mM^{-1}$ $s^{-1}$, 574 and 1,890 $mM^{-1}$ $s^{-1}$, and 1,600 and 2,150 $mM^{-1}$ $s^{-1}$, respectively [16,17].

The redox potential of *To*POx was determined by the xanthine/xanthine oxidase method to be −105 mV vs NHE at pH 6.5. Removal of the covalent link between the FAD and the histidine residue (His167 in *To*POx) resulted in a decrease of the redox potential, and a value of −150 mV vs NHE was measured at pH 6.5 for the H167A variant of *To*POx [22]. The bacterial *As*POx, which lacks the covalent His attachment, exhibited a redox potential of −50 mV vs NHE at pH 7.6 [13]. For comparison, *An*GOx shows redox potentials of −51 mV and −99 mV vs NHE at pH 6.5 and 7.6, respectively [23].

## 2. Previous and current use in biosensors

The development of improved POx-based biosensors, which includes the discovery of novel enzymes, production of variants with desirable improved properties, enhancement of electron transfer between the redox site and the electrode, as well as efficient immobilization on electrodes, has been the subject of increasing research over the past years. The interest is mainly due to a growing array of applications for the detection of various biomolecules by POx-based devices, with applications found in food or beverage industries, diagnosis of diseases, bioprocess monitoring and screening of environmental pollutants [24,25]. The effectiveness of POx as a biocomponent for biosensors has been evaluated and proven on various types electrodes including glassy carbon, gold, platinum, and screen-printed electrodes, and is currently still being evaluated on other platforms for the improvement of the overall performance of the biosensor.

The interest in POx as a biosensor is mainly due to its reaction with glucose. However, since POx can also detect other sugars, application of POx as biosensor is extended to the detection of other carbohydrates as well. Exclusively different POx of fungal origin have been used in these biosensor studies since their properties and structures had already been extensively studied. One of the first studies on the application of POx in a biosensor was performed by Lidén et al. [26]. In their study, POx from *P. chrysosporium* (*Pc*POx) was co-immobilized with horseradish peroxidase (HRP) on a carbon paste electrode (CPE). The constructed bi-enzymatic biosensor detected both $H_2O_2$ and glucose. POx reacts with glucose and forms $H_2O_2$, which is then taken up by HRP, followed by direct electron transfer between HRP and the graphite electrode. This bi-enzymatic biosensor demonstrated good responses with various carbohydrates such as D-glucose (response of 840 nA and sensitivity of 30.2 μA $cm^{-2}$ $mM^{-1}$), D-xylose (80 nA), and D-galactose (36 nA). In contrast, the biosensor had only marginal or no response with δ-gluconolactone, D-mannose, L-arabinose, and cellobiose. Both the sensitivity of the bi-enzymatic biosensor for the detection of glucose and its stability were improved with the combined effects of lactitol and polyethyleneimine additives. This bi-enzymatic biosensor was incorporated into a liquid chromatograph (LC) system for the detection of a standard mixture of glucose, xylose, and galactose. Because of the high selectivity of the HRP-POx electrode in the LC system, it was used for monitoring substrate concentrations in fermentation processes.

A decade later, POx-based biosensors were mainly studied for food and beverage analyses and monitoring of various food processes. POx from *Coriolus* sp. (*Cs*POx) immobilized on a carbon paste electrode was first applied to detect glucose (sensitivity of 0.429 μA $mM^{-1}$), galactose (sensitivity of 0.050 μA $mM^{-1}$), mannose (sensitivity of 0.005 μA $mM^{-1}$), xylose (sensitivity of 0.084 μA $mM^{-1}$), and maltose (sensitivity of 0.010 μA $mM^{-1}$). The POx-based biosensor was also used to determine the glucose content of red wine samples. However, it gave values of glucose that were somewhat lower (max. 7%) compared to the standard DNS (3,5-dinitrosalicylic acid) method, which may be due to

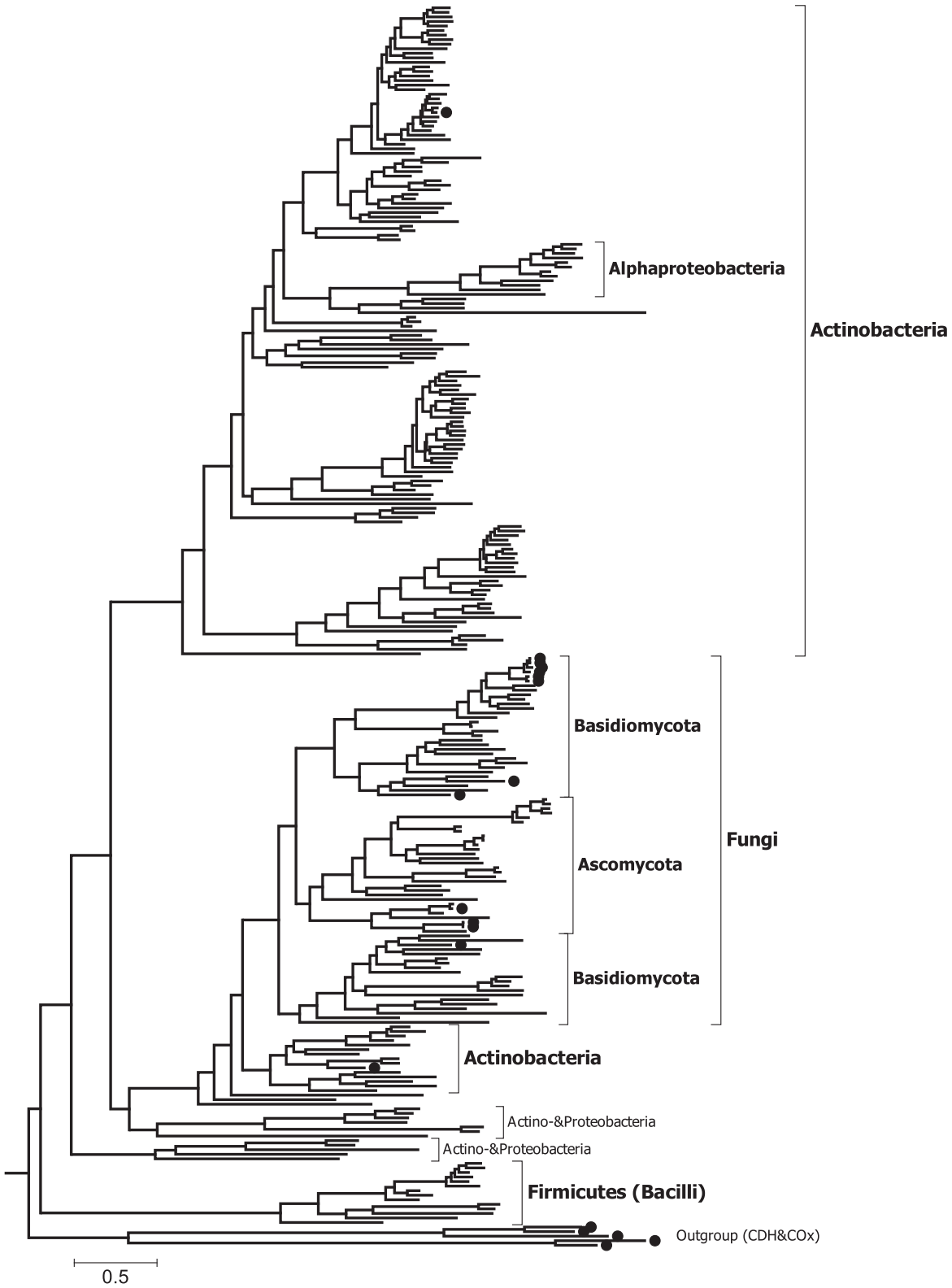



**Fig. 3.** Maximum likelihood tree of the currently known sequence space of POx, occurring in fungi and bacteria. Black circles indicate biochemically and/or structurally characterized sequences. A fungal cellobiose dehydrogenase (CDH) and a bacterial cholesterol oxidase (COx) were used for the outgroup. The topology was inferred by PhyML from a MAFFT G-INS-i alignment, trimmed for positions with >95% gaps. Node support is given by SH-like support (not displayed).

the specificity of the enzyme [27]. Shortly after, *Cs*POx was immobilized on glassy carbon electrodes (GCE) for the analysis of glucose in different juices (pomegranate, peach, orange, and mixed fruit), green tea, energy drinks, soft drinks, and wines. The biosensor detected concentrations of glucose comparable with those measured by the Trinder reaction, which was used as the reference method. Although the Trinder reaction is a fast and effective glucose assay used since 1969, the POx-based biosensor has the

**Table 1**
Apparent steady-state kinetic constants of pyranose oxidase from different sources for selected sugar substrates. Kinetic constants were measured with oxygen as electron acceptor at a constant concentration (oxygen from air and ambient pressure).

| Organism | D-glucose | | | D-galactose | | | D-xylose | | | Conditions (pH, T) | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | $K_m$ (mM) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($mM^{-1}$ $s^{-1}$) | $K_m$ (mM) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($mM^{-1}$ $s^{-1}$) | $K_m$ (mM) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($mM^{-1}$ $s^{-1}$) | | |
| *Arthrobacter siccitolerans* | 1.1 | 0.153 | 0.140 | n.r. | n.r. | n.r. | n.r. | n.r. | n.r. | 6.5, 37 °C | [13] |
| *Aspergillus nidulans* | 1.77 | 35.44 | 20.0 | 19.2 | 1.95 | 0.1 | 66.9 | 1.39 | 0.02 | 6.5, 30 °C | [60] |
| *Aspergillus oryzae* | 2.86 | 1.48 | 0.52 | 13.0 | 0.02 | 0.002 | 18.1 | 0.27 | 0.015 | 6.5, 30 °C | [60] |
| *Irpex lacteus* | 0.74 | 33.8 | 45.6 | 7.73 | 13.1 | 1.70 | 25.9 | 25.4 | 0.98 | 6.5, 25 °C | [62] |
| *Kitasatospora aureofaciens* | 1.50 | 15.4 | 10.0 | 2.7 | 11.9 | 4.4 | 32.4 | 6.8 | 0.21 | 7.0, 30 °C | [10] |
| *Lyophyllum shimeji* | 0.314 | 6.92 | 22.1 | 3.84 | 1.02 | 0.265 | 6.30 | 5.48 | 0.867 | 6.5, 30 °C | [61] |
| *Peniophora gigantea* | 1.1 | 55.5 | 50.5 | 8.2 | 2.80 | 0.342 | 29.4 | 22.7 | 0.772 | 6.5, 30 °C | [63] |
| *Peniophora* sp. | 5.0 | 9.4 | 1.88 | n.r. | n.r. | n.r. | 40 | 1.3 | 0.033 | 6.5, 30 °C | [64] |
| *Phanerochaete chrysosporium* | 0.84 | 83.1 | 98.9 | 2.94 | 4.87 | 1.66 | 20.9 | 44.9 | 2.15 | 6.5, 30 °C | [17] |
| *Phlebiopsis gigantea* | 1.2 | 40.5 | 33.8 | n.r. | n.r. | n.r. | 22.2 | 17.7 | 0.8 | 6.5, 30 °C | [65] |
| *Trametes ochracea* | 0.76 | 33 | 43 | 6.1 | 1.7 | 0.27 | n.r. | n.r. | n.r. | 6.5, 30 °C | [9] |
| | 0.74 | 54 | 73 | 9.2 | 3.1 | 0.33 | 30 | 30 | 1.0 | 6.5, 30 °C | [16] |
| *Tricholoma matsutake* | 1.28 | 111 | 87 | 45.0 | 22.5 | 0.5 | 45.8 | 55.8 | 1.2 | 7.0, 37 °C | [66] |

n.r. = not reported.

**Table 2**
Apparent steady-state kinetic constants of pyranose oxidase from different sources for its electron acceptors 1,4-benzoquinone, ferrocenium ion ($Fc^+$, used as $FcPF_6$), and 2,6-dichlorophenolindophenol. Kinetic constants were measured with D-glucose used at saturating concentrations.

| Organism | 1,4-Benzoquinone | | | | Ferrocenium ion | | | | 2,6-Dichlorophenolindophenol | | | | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $K_m$ (mM) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($mM^{-1}$ $s^{-1}$) | pH, T | $K_m$ (mM) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($mM^{-1}$ $s^{-1}$) | pH, T | $K_m$ (mM) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($mM^{-1}$ $s^{-1}$) | pH, T | |
| *Arthrobacter siccitolerans* | 0.12 | 0.39 | 3.25 | 6.5, 37 °C | n.r. | n.r. | n.r. | – | n.r. | n.r. | n.r. | – | [13] |
| *Aspergillus nidulans* | 0.15 | 186 | 1240 | 4.5, 30 °C | 0.67 | 158 | 235 | 8.0, 30 °C | 0.39 | 13.1 | 33.6 | 6.5, 30 °C | [60] |
| *Aspergillus oryzae* | 0.32 | 3.04 | 9.4 | 4.5, 30 °C | 1.12 | 1.7 | 1.5 | 8.0, 30 °C | n.r. | n.r. | n.r. | – | [60] |
| *Irpex lacteus* | 0.045 | 228 | 5085 | 6.5, 25 °C | n.r. | n.r. | n.r. | – | 0.16 | 269 | 1695 | 6.5, 25 °C | [62] |
| *Kitasatospora aureofaciens* | 0.08 | 24.9 | 311 | 7.5, 30 °C | 1.03 | 214 | 208 | 7.5, 30 °C | 0.03 | 9.4 | 313 | 7.5, 30 °C | [10] |
| *Lyophyllum shimeji* | 0.033 | 92.3 | 2760 | 6.5, 30 °C | 0.187 | 39.9 | 213 | 6.5, 30 °C | 0.187 | 67.3 | 361 | 6.5, 30 °C | [61] |
| *Phanerochaete chrysosporium* | 0.11 | 400 | 3640 | 6.5, 30 °C | 0.33 | 228 | 691 | 6.5, 30 °C | 0.051 | 108 | 2150 | 6.5, 30 °C | [17] |
| | 0.042 | 477 | 11,900 | 4.5, 30 °C | 0.29 | 549 | 1890 | 8.0, 30 °C | n.r. | n.r. | n.r. | – | [17] |
| *Trametes ochracea* | 0.253 | 225 | 895 | 6.5, 30 °C | 0.507 | 291 | 574 | 6.5, 30 °C | 0.413 | 42 | 102 | 6.5, 30 °C | [61] |
| | 0.31 | 210 | 690 | 6.5, 30 °C | n.r. | n.r. | n.r. | – | 0.065 | 20 | 300 | 6.5, 30 °C | [16] |
| | 0.3 | 270 | 900 | 4.5, 30 °C | n.r. | n.r. | n.r. | – | 0.094 | 150 | 1,600 | 4.5, 30 °C | [16] |

n.r. = not reported.

advantage of being compatible with online monitoring of food and beverage processes [28]. *Cs*POx was also co-immobilized with α-glucosidase to detect maltose or maltodextrins in beer. The bi-enzymatic biosensor consisting of POx and α-glucosidase, which cleaves malto-oligosaccharides to glucose, was constructed by immobilizing both enzymes on graphite electrodes with the aid of chitosan and glutaraldehyde. The bi-enzymatic biosensor detected D-glucose (sensitivity of 10.77 μA $mM^{-1}$), D-galactose (sensitivity of 6.14 μA $mM^{-1}$), D-xylose (sensitivity of 5.84 μA $mM^{-1}$), and sucrose (sensitivity of 0.321 μA $mM^{-1}$). When tested on beer samples, the bi-enzymatic biosensor indicated concentrations of glucose that were 3–12% higher than those measured by the standard DNS method [29].

An additional application was found for *Cs*POx-based biosensors on gold electrodes in monitoring of bioreactor cultivations of *Saccharomyces cerevisiae*. The analytic results correlated well with the glucose assay performed using HPLC as the reference method. However, the biosensor was not particularly stable, with a 72% loss of the initial activity after one month when stored in buffer solutions [30]. Subsequent studies reported the construction of further *Cs*POx-based biosensors, which were all used for analysis of glucose in several beverages such as soft drinks, iced tea, energy drinks, milk and fruit juices. All of these biosensors showed results that compared well with the reference method applied [28,31,32].

While POx has a good affinity towards glucose and other carbohydrates, only second-generation biosensors based on mediated electron transfer (MET) can be constructed with this enzyme. Direct electron transfer (DET) between the redox-active prosthetic group and the electrode is not achievable since the active site is embedded in the interior of the protein and shielded from the electrode [33]. Mediators typically used together with POx include various quinones such as 1,4-benzoquinone (1,4-BQ) or different complexed metal ions, such as ferrocenes (Fc). Fig. 4 shows the bioelectrocatalytic response (voltammograms) of a glassy carbon electrode and immobilized *To*POx in the absence or presence of three different mediators, 1,4-BQ, DCIP and ferrocenemethanol (FcMeO), the clear lack of any response of the electrode in the absence of a mediator and the presence of only glucose, and the efficient oxidation of glucose in the presence of the mediators. To improve MET, recent studies on POx-based biosensors employed various conductive polymers as mediators. Conducting polymers provide a three-dimensional matrix for biomolecule deposition, which enhances charge transfer between the enzyme and the electrode, while protecting the three-dimensional structure and hence stability of the enzyme. Moreover, they promote stable adsorption of the enzyme to the electrode [31]. Thereby, significant amounts of active enzyme are deposited or incorporated on the electrode, resulting in higher response values and a wide concentration range of

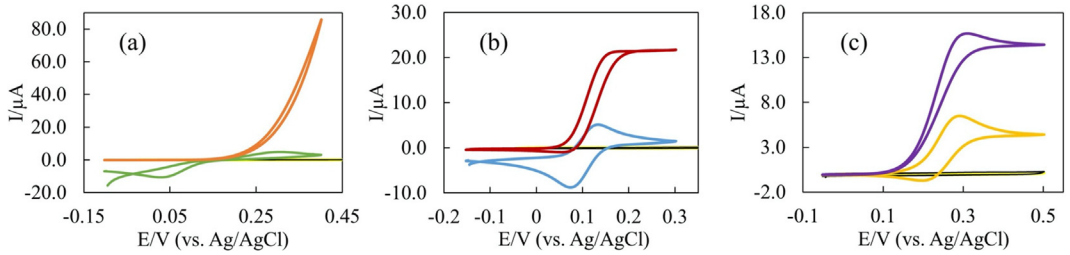



**Fig. 4.** Bioelectrocatalytic response (voltammograms) of *Trametes ochracea* POx (*To*POx) immobilized on a glassy carbon electrode in the absence and presence of the mediators 1,4-benzoquinone (1,4-BQ), dichlorophenolindophenol (DCIP) and ferrocenemethanol (FcMeOH). D-glucose was added as the electron donor substrate as indicated. Five μL of an enzyme mixture containing 8 mg $mL^{-1}$ *To*POx in 50 mM sodium citrate buffer pH 5.0 were immobilized on the electrode by using chitosan and glutaraldehyde. (a) 1,4-BQ as mediator: black, reaction blank of 100 mM sodium phosphate buffer and *To*POx; yellow, *To*POx and 50 mM glucose; green, *To*POx and 1.0 mM 1,4,-BQ; orange, *To*POx, 1.0 mM 1,4,-BQ and 50 mM glucose. (b) DCIP as mediator: black and yellow, as above; blue, *To*POx and 1.0 mM DCIP; red, *To*POx, 1.0 mM DCIP and 50 mM glucose. (c) FcMeOH as mediator: black and yellow, as above; dark yellow, *To*POx and 1.0 mM FcMeOH; purple, *To*POx, 1.0 mM FcMeOH and 50 mM glucose and 50 mM glucose.

analytes detected. The incorporation of osmium redox polymer I, poly(1-vinylimidazole)$_{12}$-[osmium (4,4′-dimethyl-2,2′-bipyridyl)$_2$ $Cl_2]^{2+/+}$, in a *Cs*POx -based biosensor on spectrographic graphite rods allowed detection of several different carbohydrates. Specifically, higher detection was observed for D-glucose (64.7 nA $mM^{-1}$), cellotriose (30.6 nA $mM^{-1}$), and D-xylose (14.2 nA $mM^{-1}$). The use of the osmium redox polymer II, a poly (vinylpyridine)-[osmium-(*N,N*′-methylated-2,2′-biimidazol e)$_3]^{2+/3+}$ complex, increased the sensitivities for D-glucose (710 nA $mM^{-1}$) and D-xylose (329 nA $mM^{-1}$). The stability of the POx-based biosensor was also improved by osmium redox polymer II, with only about 6% of the original response lost after 18 h of measurements. The use of another conducting polymer, HKCN [4-ami no-N-(2,5-di(thiophen-2-yl)-1H-pyrrol-1yl)benzamide] in the fabrication of a *Cs*POx-based biosensor on graphite electrodes led to a sensitivity for D-glucose of 102 nA $mM^{-1}$. Good operational stability of the fabricated biosensor with no decrease in activity with glucose after 81 measurements was additionally observed [34]. The use of the selenium-based polymer poly(4,7-bis(thieno[3,2-b ]thiophen-2-yl)benzo[c][1,2,5] selenadiazole; poly(BSeTT)) in the fabrication of a *CsPOx*-based biosensor on gold electrodes gave a high sensitivity of 1390 nA $mM^{-1}$ for D-glucose. The polymer also contributed to the stability of the POx-based biosensor with only 11% decrease in activity after 5 h of measurements [31]. Other mediators such as FcMeOH contributed to an increase in the sensitivity of *Cs*POx-based biosensor for glucose from 0.9 ± 0.06 to 31 ± 5.6 μA $mM^{-1}$ $cm^{-2}$ (Nazaruk and Bilewicz, 2007) as well. 1,4-BQ increased the sensitivity of a *Cs*POx-based biosensor for D-glucose from 3.7 to 59 mA $M^{-1}$ $cm^{-2}$ [35].

A significant improvement as judged by considerably higher sensitivity was achieved when a commercial preparation of *Coriolus* sp. POx (*Cs*POx) was replaced by an experimental, highly purified preparation of recombinant POx from *T. ochracea* (*To*POx) in the fabrication of a biosensor on graphite electrodes together with osmium redox polymer I, which gave a sensitivity of 2480 nA $mM^{-1}$ for D-glucose. The *To*POx-based biosensor incorporated with osmium redox polymer I was found to be stable under dry storage conditions at 4 °C with only a 10% loss in activity after 7 days [36]. In a similar fashion, the incorporation of osmium redox polymer III ($[Os(bpy)_2(PVI)_{10}Cl]^{2+/+}$) into a *To*POx-based biosensor resulted in maximum currents of 1917 nA $mM^{-1}$ and 820 nA $mM^{-1}$ with D-glucose and D-galactose as substrates, respectively [37].

Throughout the years, nanotechnology played an increasingly important role in the improvement of the performance of biosensors. Hence, different nanomaterials – carbon nanotubes, nanowires, nanostructures, gold nanoparticles, and metal oxides – contributed to more efficient and stable POx-based biosensors as well [24]. Carbon nanotubes (CNT) have been intensively researched for the improvement of POx-based biosensors since they are known to contribute to enhanced electron transfer, high electrical conductivity, good mechanical properties, as well as the construction of nanoscale devices. One of the first reported studies on the use of CNT in the fabrication of a *Cs*POx-based biosensor is the work of Odaci et al. [27]. A POx-based, CNT-modified electrode was prepared by mixing multi-walled CNT, graphite powder, and mineral oil. Cyclic voltammetry measurements showed that the electrocatalytic activity of a CNT-modified POx-based electrode is higher than that of the unmodified carbon paste electrode. Higher current values were obtained with these CNT-modified, POx-based electrode (2.3 μA) compared to the unmodified enzyme electrode (0.732 μA) in the presence of D-glucose (4 mM), indicating improvement in the efficiency of electron transfer. The CNT-modified, POx-based biosensor can also be used as a multi-analyte detector since it showed improved response as indicated by its sensitivities with different analytes compared to the unmodified biosensor when tested with substrates such as D-mannose (increase from 0.005 to 0.020 μA $mM^{-1}$), D-xylose (from 0.084 to 0.294 μA $mM^{-1}$), and D-galactose (from 0.050 to 0.194 μA $mM^{-1}$). This type of biosensor was also stable when kept in the reaction cell at 35 °C for 8 h. In another study by Odaci and colleagues [29], CNT were added to the bi-enzymatic system of *Cs*POx/α-glucosidase, aiming at an improvement of the efficiency of the biosensor. It was found that the presence of CNT only contributed to stability of the enzyme by providing a more stable platform, while the amperometric response of the biosensor was not improved. Incorporation of gold nanoparticles (GNP) in the fabrication of POx-based biosensors on GCE produced higher signals, increasing from 1.25 μA to 1.75 μA in the presence of 0.8 mM glucose. GNP together with the incorporation of gelatin, a conducting polymer (polyaniline), and an inorganic nanocomposite (AgCl) contributed to the stabilisation of the biosensor, which showed a 12% drop in the biosensor response after 6 h of measurement [28]. Use of mesoporous carbon (MSU-F-C) in the fabrication of a *Cs*POx biosensor on a GCE support displayed a sensitivity of 590 nA $mM^{-1}$ for glucose [38]. Pt nanoparticles electrodeposited on a $MnO_2$ film on GCE enhanced the performance of the *Cs*POx-based biosensor, exhibiting a sensitivity of 6.10 μA $mM^{-1}$. The stability of the biosensor was also improved with approx. 5% loss in activity after>200 measurements [32].

During the past decade, a number of studies have been performed to elucidate optimal immobilization techniques for POx-based biosensors. Enzyme immobilization is a crucial step since it greatly affects sensitivity, selectivity, and operational stability of enzyme biosensors [39]. In 2007, a monoolein-based liquid crystalline cubic phase was used to immobilize *Cs*POx on Pt electrodes. The *Cs*POx-based biosensor was stable on this matrix, not

showing a change in response for at least 6 days, and gave a sensitivity of 900 nA $mM^{-1}$ for D-glucose. However, it was observed that the turnover number (56 ± 10 $s^{-1}$) was somewhat lower than that obtained by spectroscopic measurements of the free enzyme (71 $s^{-1}$; [16]) [40]. A recent study comparing different immobilization techniques showed that enzyme precipitation coating (EPC) is a better method for a POx-based biosensor on CNT compared to covalent attachment (CA) or enzyme coating (EC) as shown by a wider layer of enzyme on the electrode observed by field emission-scanning electron microscopy (FE-SEM). EPC POx-based biosensors displayed a sensitivity of 3.7 mA $M^{-1}$ $cm^{-2}$, which is 2.3 times better than with the biosensors prepared by CA or EC [35].

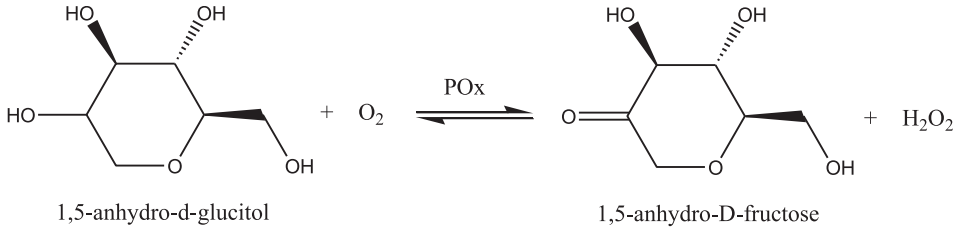
The broad substrate specificity of POx can be exploited for other applications aside from sugar monitoring, such as the measurement of serum 1,5-anhydro-D-glucitol (1-deoxy-D-glucopyranose, 1,5-anhydroglucitol, 1,5-AG) as a marker for the glycemic index and diabetes. 1,5-AG is a major polyol in human serum with a structure resembling that of glucose. With a half-life of 1 to 2 weeks, 1,5-AG is secreted in the urine but reabsorbed in the kidney without being metabolized. When glucose levels become high, reabsorption of 1,5-AG is suppressed, and it is excreted in the urine causing its level in serum to decrease. Thus, its measurement is an indirect indication of periods of hyperglycemia. Interpreting 1,5-AG levels is a useful and sensitive marker of acute and postprandial hyperglycemia episodes in diabetology and cardiabetology [41], providing a unique perspective of a patient's recent hyperglycemic excursions, which may not be evident from standard glycemic markers. POx catalyzes the oxidation of the C-2 hydroxyl group of 1,5-AG forming 1,5-anhydro-D-fructose, with the reduction of oxygen to $H_2O_2$ (Scheme 1). Enzymic tests kits based on POx are nowadays commercially available for the measurement of 1,5-AG and have been approved by the FDA [42,43]. It was furthermore found that 1,5-AG can also be detected in saliva and that it mirrors 1,5-AG levels in blood, indicating that it could be used as a noninvasive marker of short-term glycemic control [44].

An enzymatic assay based on POx was used for the analysis of 1,5-AG, which was also compared with gas-liquid chromatography (GLC) and HPLC measurements. Although the chromatographic methods gave more precise results, they are time-consuming and unsuitable for the determination of 1,5-AG levels in numerous samples in routine clinical practices. Fabrication of an improved POx-based biosensor to measure 1,5-AG levels still poses a better alternative since it is easier, faster and inexpensive [45]. Recently, an organic field-effect transistor (OFET)-based biosensor was used as a potentiometric electrochemical sensor measure both 1,5-AG and glucose. An OFET-based biosensor, combined with a Prussian blue electrode modified with CsPOx, was utilized and both glucose and 1,5-AG could be measured in the concentration range of 1 mM and up [46]. Hence, the establishment of a fast and reliable electrochemical method to measure 1,5-AG in blood or saliva might offer potentials in clinical analysis of diabetes patients.

## 3. Other uses in e.g. biofuel bells, bioelectrocatalysis

Pyranose oxidase has a promising role as a biocomponent in biofuel cells (BFCs) for small-scale energy production. This is based on its ability to efficiently oxidize various carbohydrates (Table 1) typically found in lignocellulose hydrolysates [47]. Even though it is classified as an oxidase, POx has high activities with alternative electron acceptors such as the ferrocenium ion ($Fc^+$), other complexed metal ions or quinones (Table 2), which facilitates shuttling of electrons from the active site to the electrode in mediated electron transfer [35]. This is for example illustrated in the study of Kwon et al. [38], in which the performance of a POx-based electrode was evaluated and compared with that of a GOx-based one, both enzymes immobilized on mesoporous carbon (MSU-F-C). This study showed that, regardless of the absence or presence of 1,4-BQ as the mediator, POx-based anodes produced 30–40% higher maximum power densities than the GOx-based anodes. The POx-based electrode has a maximum power density of 11.3 and 40.7 mW $cm^{-2}$, while the GOx-based electrode showed lower values of 8.4 and 31.4 mW $cm^{-2}$ in the absence and presence of the 1,4-BQ mediator, respectively. Although the POx-based electrode had a lower activity compared to the GOx-based one, it produced a higher maximum power current, possibly due to higher electron transfer rates and/or higher mass transport rates. Sahin et al. [48] also illustrated that *To*POx variant T169G generated a higher current output in the presence of a mediator over GOx in a BFC. The electrodes were prepared by immobilizing the enzymes on screen-printed electrodes (SPE). Fc was used as the mediator because of its low toxicity, while multi-walled CNT were used to enhance the electron transfer properties of the Fc-Nafion mixture. Chronoamperometric measurements showed that GOx produced a higher initial current of 55 μA compared to POx T169G (initial current of 10 μA). However, the former lost most of its activity (about 90%) during the first hour of the total operation period of 12 h. POx T169G, on the other hand, maintained > 70% of its initial current during the first hour. With respect to their fuel cell performance, both anodes showed a similar initial open circuit voltage (0.442 V for POx T169G and 0.444 V for GOx) but POx T169G showed 25% higher power output (maximum value of 1.06 μW) than GOx (0.8 μW) in aerated solutions. In another study, Spadiut et al. [49] reported *To*POx variants having higher maximum current outputs compared to the wild-type enzyme. *To*POx variant H450G produced 0.610 and 28.8 μA, while V546C produced 0.862 and 30.7 μA, in the absence and presence of 1,4-BQ, respectively. Wild-type *To*POx only produced 0.285 and 12.62 μA, again without and with 1,4-BQ, respectively.

Efficient immobilization on the electrodes also enhanced the efficiency of POx as a component in BFCs. The maximum power density of a POx-based anode prepared by enzyme precipitate coating (53 μW $cm^{-2}$) was larger than that prepared by covalent attachment (41 μW $cm^{-2}$) and enzyme coating (47 μW $cm^{-2}$). SEM images confirmed the increase in enzyme loading, which apparently led to the higher maximum power densities. This




**Scheme 1.**

possibly resulted in a higher number of electrons generated per unit of geometrical surface area of the POx-based anodes for the BFC. The maximum power output for all these POx-based anodes was further increased in the presence of the mediator 1,4-BQ (500, 330 and 260 μW $cm^{-2}$ for EPC, EC, and CA, respectively) [35].

## 4. Greatest benefits of the enzyme and the biosensor

POx has gained more interest as a component of biosensors during the past years, and it is slowly emerging as an attractive alternative to GOx, which is still employed in most of the studies on BFC. In contrast to fungal GOx, POx is typically not glycosylated [50,51], which both facilitates the recombinant overproduction of the enzyme and supports the electron transfer from the enzyme to the electrode, presumably because of short distances that can be achieved. This later aspect was for example also shown for *An*GOx, which was studied in both the native, glycosylated and the enzymatically deglycosylated forms [52]. In further comparative studies, non-glycosylated *Cs*POx and glycosylated *An*GOx were used to fabricate biosensors on GCE with the aid of MSU-F-C. The glucose sensitivity of the POx-based biosensor was 0.59 ± 0.04 μA $mM^{-1}$, which is six-fold higher compared to the GOx biosensor [38]. *Cs*POx biosensors on CNT showed a glucose sensitivity of 3.7 mA $M^{-1}$ $cm^{-2}$, which is five-fold higher than for the BFC employing glycosylated GOx on CNT. In addition, an improvement in the electron transfer rates was shown, increasing from 0.031 $s^{-1}$ for the GOx-based biosensor to 0.046 $s^{-1}$ for the POx biosensor [35]. This more efficient electron transfer is presumably the result of the shorter distance from the redox-active prosthetic group in the active site to the electrode surface, which is 11 Å for (non-glycosylated) POx as opposed to 13–15 Å shown for glycosylated GOx [6,52]. This superiority of POx over GOx in bioelectrochemical applications was corroborated in other comparative studies as well. A POx-based biosensor on gold electrodes showed better sensitivity for D-glucose than a biosensor using GOx and fabricated in the same manner (sensitivity of 3.36 and 1.54 μA $mM^{-1}$ for POx and GOx, respectively) [30].

POx can thus efficiently detect D-glucose and can produce higher power output from the oxidation of glucose. It can effectively reduce both the α- and β-anomers of D-glucose as opposed to GOx, which only catalyses the oxidation of β-D-glucose [53]. As already stated above, both the Michaelis constants $K_m$ and the catalytic efficiencies $k_{cat}/K_m$ are more favourable for POx as exemplified by a comparative study on *To*POx and *An*GOx, showing a ten times higher catalytic efficiency for the former enzyme under identical conditions [21]. Taken together, these data suggest that *To*POx can generate a higher power output than *An*GOx with D-glucose as a substrate in BFC applications. POx catalyses the oxidation of its sugar substrates at position C-2 yielding keto-glucose, which does not cause any pH shift in contrast to GOx and its final reaction product gluconic acid [18]. This shift to lower pH values caused by the GOx-catalysed reaction may be detrimental for biosensors and BFCs [54]. Furthermore, a POx-based biosensor has the potential to be applied as a multi-analyte detector since it has excellent reactivities with sugars other than D-glucose, such as D-galactose, sorbose, mannose, D-xylose, and δ-gluconolactone [47]. Again this highlights the advantageous properties of this enzyme compared to GOx.

## 5. Greatest problems of the enzyme and the biosensor

Although POx has a lower turnover rate for oxygen than GOx, it shows considerable activity with oxygen, which is reduced to hydrogen peroxide during catalysis. This can interfere with its sensitivity especially when operating at low potentials. Although oxygen is readily available, the reaction with oxygen is not a desired property for biosensor and BFC applications as it may cause electron leakage. In addition, $H_2O_2$ produced from $O_2$ can cause oxidative damage and hence inactivation of the enzyme [55]. One promising approach to this problem can be enzyme engineering, and it has been shown that oxygen reactivity of POx can be altered by this technique. The *To*POx variants T166R, L545C, Q448H, L545C, and N593C were identified in an engineering approach that aimed at reducing reactivity of the enzyme with oxygen. To achieve this goal, various amino acid residues in the direct vicinity of the isoalloxazine were targeted by saturation mutagenesis, and subsequently screened for reduced $O_2$ activity and conserved reactivity with the alternative electron acceptor DCIP [56]. Electrochemical studies of a biosensor constructed with SPE and the *To*POx variant N593C showed complete suppression of the reaction with oxygen as illustrated in cyclic voltammograms. The activity towards glucose is still preserved with $K_M$ of 2.5 ± 0.5 mM for glucose of N593C, which is comparable to that of wild-type *To*POx ($K_M$ = 2.3 ± 0.1 mM) [57]. Another *To*POx variant, T169G, with reported reduced oxygen activity compared to the wild-type was used as the component of an enzymatic fuel cell. The T169G BFC generated maximum power output of 1.06 μW in 5.5 mM aerated glucose solution, which is a 25% higher power output than a corresponding GOx-based BFC [48].

## 6. Outlook on the possible improvements and alternative use

POx, even though it is a fungal enzyme, can be easily expressed and produced in the bacterial host *Escherichia coli* in high yields. A comparative study by Spadiut et al. [58], evaluating three different *E. coli*-based expression systems, concluded that the highest yields can be obtained in batch cultivation mode by using the pET21d$^+$ system (productivity of 31 U/L h, ~1000 U per L medium corresponding to 125 mg POx/L), while in fed-batch mode the pCOLD system was superior (productivity of 206 U/L h, 3300 U/L, ~400 mg/L). This ease of recombinant production in the expression system *E. coli* makes the enzyme amendable to various techniques of molecular biology and can serve as the basis for a number of approaches to engineer POx towards desired properties. POx shows good reactivity towards quinones and complexed metal ions such as $Fc^+$ currently used as mediators. Rational and semi-rational protein design can be employed to produce POx variants with high reactivity towards mediators commonly used in bioelectrochemical applications and various sugar substrates to improve the performance of POx-based biosensors or BFCs. Recent examples in the literature show that these engineering approaches can indeed be very successful. POx has been engineered towards improved activity with D-galactose, and three variants of *To*POx in which four to six crucial amino acid positions had been modified showed significantly improved activity with galactose. These variants were compared with wild-type *To*POx for biosensor applications. While the wild-type could not be applied for galactose sensing, amperometric biosensors based on graphite and wired with various Os polymers showed a performance that was comparable to other galactose biosensors with a limit of detection in the low micromolar range, a limit of quantification of around 10 μM, and a linear range between approx. 0.1 to 10 mM galactose [37]. In a similar way, semi-rational engineering of *To*POx results in variants that showed considerably decreased reactivity with oxygen while the dehydrogenase activity, *i.e.*, reactivity with different alternative electron acceptors and glucose, was maintained or even increased. The *To*POx variants L545C and T166R seem to be the most interesting candidates for bioelectrochemical application when an improved activity with the mediator is desired. When compared with wild-type *To*POx on the basis of the catalytic efficiency $k_{cat}/K_m$, L545C shows only 19% relative activity with oxygen while

the $k_{cat}/K_m$ values with DCIP, 1,4-BQ and $Fc^+$ are 184, 413, and 202% relative to wild-type *To*POx. Relative $k_{cat}/K_m$ values for *To*POx variant T166R with $O_2$, DCIP, 1,4-BQ and $Fc^+$ are 39, 334, 192 and 48% [56]. Even though these variants are attractive biocatalysts based on their improved properties they have not been evaluated for bioelectrochemical applications. Yet they clearly show the feasibility of engineering approaches to tailor POx towards desired properties and applications.

In addition to the kinetic properties of an enzyme their stability is of high interest for various applied aspects. Depending on the source of the enzyme, POx shows moderate to good thermostability as indicated by the melting temperature $T_m$. This value was for example determined for the fungal enzymes *To*POx (60.7 °C; [59]), POx from *A. niger* (67 °C; [60]) and *PcPOx* (75.4 °C; [61]), as well as for the bacterial enzymes *As*POx (43 °C; [13]) and *Ka*POx (56.1 °C; [10]). The feasibility of enzyme engineering was again shown to improve thermostability of POx. The single-mutant *To*POx E542K showed a $T_m$ value of 74.7 °C, a 14° improvement compared to wild-type *To*POx, while the half-life activity of E542K at 70 °C $T_{½,70}$ was 55 min (less than 1 min for the wild-type). A further stabilisation could be achieved by introducing an addition mutation, L537W/E542K. This double mutant showed a two-phase melting behaviour, so its melting temperature could not be determined, while its $T_{½,70}$ values was 105 min [59].

Probably the most significant potential for future applications and improvements can be found in the diversity of different fungal and bacterial pyranose oxidases. POx displays a widespread taxonomic distribution and it also shows an exceptionally high level of sequence variation when compared with other GMC oxidoreductases [12]. Currently, approximately ten fungal POx have been studied biochemically, yet most of these are closely related. Bacterial POx offer an even greater untapped resource, with only two biochemically characterised members, none of which have been tested for an application in biosensors or BFC. Especially these latter bacterial enzymes might show bioelectrochemical properties that are very different from those of their fungal counterparts. All fungal POx studied to date show a tetrameric geometry with a restricted access to the active site, while the two bacterial representatives are monomeric (*As*POx) and dimeric (*Ka*POx) in solution. Hence, the active site could be more open and better accessible, resulting in an improved communication with an electrode or a mediator.

## Declaration of Competing Interest

The authors declared that there is no Conflict of Interest.

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