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Thermal and operational stabilities of *Hansenula polymorpha* alcohol oxidase

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Abstract

The thermal stability of *Hansenula polymorpha* alcohol oxidase (AOX) was evaluated at 50 °C. The stability of free AOX was dependent of pH, buffer and additives but independent of protein concentration. More than 80% of the initial activity was retained after 9 h in the presence of additives, such as lactose, dextran sulphate and PEG 400 and combinations thereof. In the specific case of 0.01% dextran sulphate and 50 mM lactose no activity was lost for 9 h. Salts (ammonium sulphate and chloride) had a strong destabilising effect on the enzyme.

The immobilisation of AOX onto controlled-pore glass (CPG) beads allowed the use of mini packed-bed bioreactors (31 mm^3) to monitor ethanol concentration. The conversion decrease (80% after 4 h) during continuous oxidation of ethanol at 32 °C in phosphate buffer was attributed to inactivation by hydrogen peroxide rather than thermal deactivation. Accordingly, an in situ stabilisation strategy was devised, which consisted in promoting the instantaneous consumption of H_2O_2, by horseradish peroxidase (HRP) and its reducing substrates, phenol-4-sulfonic acid and 4-aminoantipyrine. This strategy led to high operational stabilities (more than 10 h with no loss in conversion degree) and was successfully applied in a flow injection analysis (FIA) system for ethanol analysis.

Keywords: *Hansenula polymorpha* alcohol oxidase; Ethanol; FIA; Horseradish peroxidase; Immobilised bioreactors; Operational stability; Thermal stability

1. Introduction

Enzymes have been increasingly used in many analytical applications, offering many advantages over conventional chemical methods, which mainly stem from their intrinsic specificity, sensitivity and ability to operate under mild operational conditions.

Since the development of the first enzymatic sensor comprising the enzyme glucose oxidase, a multitude of enzyme-based sensors have been constructed. Nevertheless, their implementation into commercial successful instruments has been hampered mainly by the lack of stability of the biological component [1]. This biological component should present a high stability not only during storage but also during operation [2].

There is a large amount of information in the literature regarding the enhancement of the stability of enzymes for use in bioanalytical systems. Immobilisation by physical adsorption or covalent attachment to insoluble matrices, entrapment into polymeric films or membranes, covalent crosslinking of the protein structure and addition of additives to the enzyme preparation are some of the most used techniques to improve the thermal stability of the biological element [3,4].

Many oxidoreductase enzymes, especially peroxidase [5], oxidases [6] and dehydrogenases [7], have been the focus of intense stabilisation studies. Nevertheless few studies have assessed the stability of alcohol oxidase (AOX). Gibson and co-workers have conducted several stabilisation studies of AOX in the dry state using a combination of polyelectrolytes and sugar derivatives [2,8]. In particularly, the system diethylaminoethyl (DEAE) dextran/lactitol showed promising results in retaining the activity of dried AOX [9]. In this work, the influence of other additives in the thermal stability of free AOX is investigated in order to enhance storage stability. The operational stability of AOX during the continuous oxidation of ethanol is also addressed and different strategies are applied in order to obtain high performances.

Alcohol oxidase (E.C. 1.1.3.13; alcohol: O_2 oxidoreductase) is an oligomeric enzyme consisting of eight identical sub-units arranged in a quasi-cubic orientation, each

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containing a strongly bound flavine adenine dinucleotide (FAD) cofactor [10,11]. The binding of the cofactor to the protein depends rather on the adenine than on the flavine moiety. The adenine-moiety is believed to bind to the N-terminal domain of each monomer, which contains a typical nucleotide-binding fold [12]. It has been found that the FAD molecules are bound to the surface of the monomers rather than to the interface between sub-units [13].

Alcohol oxidase catalyses the oxidation of primary low molecular weight alcohols, e.g. methanol and ethanol, into the corresponding aldehydes. During this reaction, the AOX cofactor (FAD) is first reduced to its hydrogenated form ($FADH_2$) and then re-oxidised to its native form by molecular oxygen (O_2), with the concomitant formation of hydrogen peroxide (H_2O_2). The kinetics of AOX has been studied by two different strategies: by monitoring the consumption of oxygen, using e.g. a Clark oxygen electrode, or by monitoring the production of hydrogen peroxide [14]. The concentration of H_2O_2 in turn can be determined by different enzymatic and non-enzymatic methods. Most enzymatic methods make use of the enzyme horseradish peroxidase (HRP), which is able to oxidise many chromogenic substrates during the H_2O_2 reduction. A new system to assay H_2O_2, based on the oxidation of phenol-4-sulfonic acid (PSA) in the presence of 4-aminoantipyrine (4-AAP) catalysed by HRP has been recently characterised [15]. In this work, the applicability of this system to the study of the kinetics of oxidation of ethanol by AOX is also investigated.

2. Experimental

2.1. Materials

Hansenula polymorpha alcohol oxidase (AOX) was obtained from Applied Enzyme Technology (Leeds, UK) with an activity of 500 U/ml measured with the Clark Oxygen Electrode and supplied as a solution in 0.1 M sodium 3-(*N*-morpholino)propanesulfonate (MOPS) buffer. Horseradish peroxidase (HRP) isoenzyme C was purchased from Biozyme (catalogue name HRP 4) as a brown freeze-dried powder with a specific activity of 252 U/mg material, measured using pyrogallol as reducing substrate. Absolute ethanol was obtained from Merck and the reducing substrates, phenol-4-sulfonic acid sodium salt (PSA) and 4-aminoantipyrine (4-AAP) were purchased from Fluka and Sigma, respectively. Alkylamine controlled-pore glass (CPG) was a gift from Dr. H. Weetall. Other chemicals used were obtained with the highest grade available.

2.2. Methods

2.2.1. Immobilisation of AOX

AOX was immobilised onto controlled-pore glass beads bearing functional amino groups, with 74–125 μm (120–200 mesh) particle size and 550 Å pore size. The amino group content was determined using the 2,4,6-trinitrobenzosulfonic acid (TNBS) method [16] and a value of $48.3 \pm 0.8\,\mu mol_{NH_2}/g_{support}$ was obtained. The covalent immobilisation of AOX to the support was accomplished using glutaraldehyde as coupling agent, through the formation of Shiff's bases. Aminopropyl-CPG was suspended in a 10% (v/v) glutaraldehyde aqueous solution for 2 h, and manually agitated from time to time. The support was then thoroughly rinsed with water and phosphate buffer in order to remove excess glutaraldehyde. The buffer was removed with a pipette and an equivalent volume of enzyme solution (25 mg/ml in MOPS buffer) was added to the support. The amount of protein loaded was quantified using a modification of the Lowry method [17] and a value of $78 \pm 4\,mg_{protein}/g_{support}$ of was determined.

2.2.2. Bioreactor preparation

A mini packed-bed enzyme reactor was prepared using CPG beads with immobilised AOX. The CPG beads were packed in a glass microcolumn with 25 mm length, 2 mm internal diameter and 0.5 mm wall thickness. The ends of the column were plugged with glass wool and connected to an external Omnifit teflon tubing circuit (i.d. 0.8 mm) with silicone tubing. The length of the AOX-CPG packed bed was 10 mm, originating a total volume of 31 mm^3.

2.2.3. Alcohol oxidase activity assay

A bi-enzymatic assay comprising alcohol oxidase (AOX) and horseradish peroxidase (HRP) was used to monitor the oxidation of ethanol to acetaldehyde by AOX. A colorimetric system based on the combination of phenol-4-sulfonic acid (PSA) and 4-aminoantipyrine (4-AAP) was chosen to measure the concentration of H_2O_2 produced by AOX. In this particular system, two moles of H_2O_2 react with one mole of PSA and one mole of 4-AAP, yielding three moles of water, one mole of sodium hydrogenosulfate and one mole of a quinoneimine dye [15].

$$\begin{aligned} &\text{Ethanol} + O_2 \xrightarrow{\text{AOX}} \text{Acetaldehyde} + H_2O_2 \\ &2\,H_2O_2 + \text{PSA} + \text{4-AAP} \xrightarrow{\text{HRP}} \text{Quinoneimine dye} \\ &\qquad\qquad +3\,H_2O + NaHNO_3 \end{aligned} \tag{1}$$

This dye has a characteristic magenta color with maximum absorption around 490 nm ($\varepsilon_{490\,nm} = 5.56\,mM^{-1}\,cm^{-1}$ determined experimentally towards H_2O_2). The activity of AOX was determined by monitoring the associated increase in absorbance at 490 nm with a Hitachi U-2000 spectrophotometer. This increase is proportional to the rate of H_2O_2 production and, consequently, to the rate of ethanol consumption. All kinetic studies were performed at 25 °C using a *standard assay reaction mixture*, containing 0.4 mM 4-AAP, 25 mM PSA, and 2 U/ml HRP in 0.1 M phosphate buffer, pH 7.0, unless otherwise stated. One unit of activity (U) was defined as the number of μmol of H_2O_2 produced per minute at 25 °C.

2.2.3.1. Free alcohol oxidase. In a typical assay, 25 μl of an AOX sample was added to 1 ml of the standard assay reaction mixture. The reaction was initiated by the addition of 25 μl ethanol. The reaction was followed at 490 nm for 1 min in a 1.5 ml, magnetically stirred, quartz cell (Hellma) thermostatised at 25 °C.

2.2.3.2. Immobilised alcohol oxidase. The activity of AOX immobilised in CPG was determined using a continuous system. This system consisted of a 20 ml glass stirred tank, a Watson–Marlow peristaltic pump, a 18 μl Hellma continuous flow optical cell and a packed-bed bioreactor, all connected in series with Omnifit teflon tubing. The piping system was completely filled with the standard assay reaction mixture. A certain volume (5–15 ml) of this reaction mixture was added to the stirred tank and the reaction was initiated by the addition of 25 μl of 6.5 mM ethanol per ml of reaction mixture. The flow rate was 1 ml/min.

2.2.4. Stability assays of alcohol oxidase

2.2.4.1. Thermal stability. The thermal stability of free AOX was studied at 50 °C in 0.1 M phosphate buffer pH 7 and in the presence of a wide range of additives. Different aqueous solutions of 0.25 mg/ml AOX were placed in polypropylene micro-tubes and incubated in a thermostated water bath. Aliquots of 25 μl were taken at regular time intervals and the residual enzyme activity was determined as described earlier.

2.2.4.2. Operational stability. The operational stability of the CPG immobilised AOX during the in situ oxidation of ethanol was studied at 32 °C. The 31 mm^3 AOX packed bed bioreactor was continuously fed with 6.5 mM ethanol either in 0.1 M phosphate buffer or in the standard assay reaction mixture (enriched or not with AOX stabilisers) at a flow rate of 0.1 ml/min. When the reaction took place in buffer, the stream exiting from the bioreactor was mixed with a stream carrying a reaction mixture, with twice the concentration of the standard assay mixture (i.e. 50 mM PSA, 0.8 mM 4-AAP, 4 U/ml HRP in 0.1 M phosphate buffer pH 7) and at the same flow rate. The H_2O_2 produced in the bioreactor reacted with the assay reaction mixture in a 2 m long coil. The absorbance was measured after the coil at 490 nm in a 18 mm^3 glass flow-cell (Hellma). When ethanol was oxidised in the standard assay reaction mixture, the solution exiting the bioreactor, passed directly through the reaction coil and the spectrophotometer flow-cell, without any dilution. This methodology allowed the on-line measurement of the final reaction product concentration and thus, the H_2O_2 produced by AOX during the oxidation of ethanol.

2.2.5. Ethanol analysis in FIA system

Ethanol analysis was performed in a flow injection analysis (FIA) system with an incorporated immobilised AOX bioreactor. The FIA system was set up with Gallamp peristaltic pumps connected with Tygon tubing obtained from Elkay. Sample injection was performed with a Rheodyne rotatory PTFE valve, controlled by an Omnifit universal switching module. Absorbances were measured with a Jencons spectrophotometer, using an 18 mm^3 glass flow cell with a 10 mm path length from Hellma, and the output was recorded on a Gallenkamp data trace flat-bed recorder. Teflon tubing (i.d. 0.8 mm) and connectors were supplied by Omnifit. Connections were made using 1/4-28 thread low-pressure HPLC unions, nuts and ferules from Upchurch. The FIA system has a double reagent line (the buffer and the assay reaction mixture lines). All measurements were performed at 32 °C under a constant flow rate of 1 ml/min.

3. Results and discussion

3.1. Kinetic and activity assays

A bi-enzymatic colorimetric assay comprising alcohol oxidase (AOX) and horseradish peroxidase (HRP) was used to study the kinetics of ethanol oxidation by AOX. This colorimetric assay is based on the co-oxidation of phenol-4-sulfonic acid (PSA) and 4-aminoantipyrine (4-AAP) by HRP in the presence of H_2O_2.

3.1.1. Effect of PSA and 4-AAP in the oxidation of ethanol

The concentrations of PSA and 4-AAP, which led to optimal HRP activity for the H_2O_2 assay, have been determined to be 25 mM and 0.4 mM, respectively [15]. In order to couple this assay to a H_2O_2 producing oxidase, it is necessary to guarantee that the reducing substrates (PSA and 4-AAP) do not inhibit the oxidase activity. The activity of AOX presents exactly the same pattern of the activity of HRP in relation to PSA and 4-AAP obtained by Vojinovic et al. [15]. Thus, the changes of activity of AOX are probably linked with the activity variation of the coupled enzyme HRP with PSA and 4-AAP rather than on AOX. These results validate the use of the H_2O_2 colorimetric assay to study the oxidation of ethanol by AOX.

3.1.2. Effect of additives

Ethanol quantification is of great importance in on-line fermentation monitoring. Colorimetric systems used for the detection of ethanol should be sensitive, accurate and robust. Nevertheless many compounds present in fermentation broths are known to affect the detection of H_2O_2 and, hence, the rate of oxidation of ethanol. The effect of these type of compounds on the activity of AOX was studied. The additives chosen were typical nutrients and fermentation metabolites, namely, 5 g/l yeast extract, 10 g/l D-glucose, 10 g/l D-galactose, 2 g/l sodium pyruvate, 0.5 g/l succinate, 0.5 g/l glycerol and 7.2 g/l potassium acetate and were previously added to the ethanol standard. Additive concentrations were chosen based on a 1:4 dilution of a typical fermentation sample. None of these additives had a negative effect

on the activity of AOX, except potassium acetate, which led to a 13% decrease. This colorimetric assay can thus be used to monitor ethanol in fermentation broths.

3.1.3. Kinetic studies of AOX

Since it is known that H_2O_2 produced by oxidases, inhibits the activity of these enzymes [18], the oxidation of ethanol by AOX was studied in the presence of HRP and reducing substrates (i.e. in the standard assay reaction mixture), in order to insure that all H_2O_2 produced is immediately consumed by HRP.

The kinetics of oxidation of ethanol catalysed by *Hansenula polymorpha* alcohol oxidase in the standard assay reaction mixture follows a typical Michaelis–Menten kinetics. The kinetic parameters were determined using a Lineweaver–Burk plot and the values 14.8 ± 0.2 U/mg and 4.46 ± 0.19 mM were obtained for the maximum velocity and Michaelis–Menten constant, respectively. The fitting correlation factor was 0.9992.

3.2. Thermal stability

In order to be successfully implemented into a commercial sensing device, AOX has to exhibit a high retention of activity during storage. The thermal stability of AOX was studied at 50 °C in order to accelerate the deactivation process (up to 12 h).

An initial screening study was performed in different buffer solutions at different pH values. Table 1 summarises the influence of pH in the initial activity and in the retained activity after 4 and 8 h of incubation at 50 °C. These results show that highest activities and stabilities were achieved at physiological pH values. At acidic pH, both activity and stability are extremely low. No activity was retained after 4 h incubation at 50 °C at pH 5 and after 8 h at pH 5.5. The activity retained at pH 6.0 and 6.5 after 8 h incubation at 50 °C was <10%. At basic pH values, the decrease in activity was less pronounced than at acid pH values. Besides the pH value, the type of buffer is also crucial for AOX stability. At pH 7.0, the activity in phosphate buffer and in MOPS buffer is almost the same. Nevertheless the activity retained in MOPS buffer is much lower. At pH 8.0, a similar effect is observed in phosphate and Tris–HCl buffer, but in this case the retention of activity in phosphate is significantly lower than in Tris–HCl. These results suggest that the stability of AOX is greatly influenced by the type of buffer ions present in solution. Apparently this effect follows the lyotropic (Hofmeister) series for anions (phosphate; sulphate; acetate ∼ chloride), with the exception of Tris–HCl and assuming that MOPS (3-(*N*-morpholino)propanesulfonate) behaves as the sulphate ion. The stabilisation observed in Tris–HCl may be attributed to the presence of hydroxymethyl groups in the Tris–cation, which are analogues of the AOX in vivo substrate, methanol, and can thus stabilise the enzyme by interaction with the active centre.

Table 1
Effect of buffer and pH value on the AOX initial activity and retained activity after 4 and 8 h of incubation at 50 °C

pH	Buffer (0.1 M)	Initial activity (U/mg)	Retained activity (%)	
			4 h at 50 °C	8 h at 50 °C
5.0	Acetate	0.14 ± 0.03	0.0	0.0
5.5	Acetate	0.85 ± 0.03	23.7	0.0
6.0	Phosphate	11.5 ± 0.1	47.7	7.5
6.5	Phosphate	13.1 ± 0.3	62.4	9.9
7.0	Phosphate	14.7 ± 0.5	73.2	64.2
7.0	MOPS	14.8 ± 0.6	48.3	37.6
7.5	Phosphate	15.8 ± 0.0	95.0	79.0
8.0	Phosphate	15.0 ± 0.0	27.0	9.0
8.0	Tris–HCl	14.5 ± 0.1	46.6	28.8
9.0	Tris–HCl	13.7 ± 0.2	47.6	24.2

The native AOX is an octamer composed of eight identical sub-units each containing a FAD cofactor. Loss of activity during thermal deactivation assay can be associated to the disassembly of the octamer into the inactive monomers [19] or to the loss of the FAD cofactor [20] among other events. The deactivation of AOX was studied in detail at pH 7.0 and 7.5 in phosphate and at pH 7.0 in MOPS buffer (Fig. 1). All points represent the average of two independent measurements. At pH 7.0, phosphate buffer, three different sets of experiments were performed. One of these experiments was performed at a protein concentration 10 times lower than the other two sets. As Fig. 1 shows, the deactivation profile of AOX is not significantly affected by protein concentration.

Several authors [21–23] have proposed that protein inactivation occurs in a stepwise manner, involving different structural intermediates, which may or may not retain the same activity as the native form. For the specific case of AOX, during the early stage of the inactivation process, the stable octamer is probably converted into a more labile but still active intermediate form, which unfolds leading to an inactive form. This inactivation process is schematised in Eq. (2), where $(AOX)_{native}$ is the native octamer, $(AOX)_{labile}$ the labile intermediate and $(AOX)_d$ the deactivated AOX.

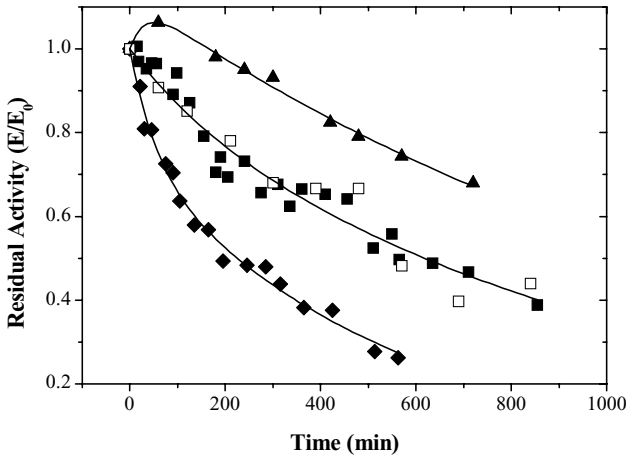

Fig. 1. Deactivation profile of AOX at 50 °C in 0.1 M MOPS buffer pH 7.0 (◆) and in 0.1 M phosphate buffer pH 7.0 (■ and □) and 7.5 (▲). Closed symbols: [AOX] = 0.25 mg/ml; open symbols: [AOX] = 0.025 mg/ml.

Table 2
One and two-step deactivation model parameters, correlation coefficient (r) and half-life ($t_{1/2}$) for AOX at 50 °C in different 0.1 M buffers and pH values

Model parameters	Phosphate buffer		MOPS buffer
	pH 7.0	pH 7.5	pH 7.0
Two-step deactivation model			
α_i	0.734	1.103	0.666
α_d	0.001	0.000	0.000
k_i (min^{-1})	5.8×10^{-3}	3.3×10^{-2}	1.9×10^{-2}
k_d (min^{-1})	9.1×10^{-4}	7.2×10^{-4}	1.8×10^{-3}
r	0.977	0.997	0.995
$t_{1/2}$ (min)	618	1129	225
One-step deactivation model			
k_d (min^{-1})	1.1×10^{-3}	4.7×10^{-4}	2.6×10^{-3}
r	0.968	0.932	0.952

$$(\mathrm{AOX})_{\mathrm{native}} \xrightarrow{k_i} (\mathrm{AOX})_{\mathrm{labile}} \xrightarrow{k_d} (\mathrm{AOX})_d \quad (2)$$

Assuming that at time zero only the native enzyme is present, then the total activity, a, can be expressed by Eq. (3), where k_i and k_d are first-order rate constants and α_i and α_d represent the relative activity of the species $(\mathrm{AOX})_{\mathrm{labile}}$ and $(\mathrm{AOX})_d$.

$$a = \alpha_d + \left(1 + \frac{\alpha_i k_i}{k_d - k_i} - \frac{\alpha_d k_d}{k_d - k_i}\right) \exp(-k_i t) - \left(\frac{\alpha_i k_i}{k_d - k_i} - \frac{\alpha_d k_i}{k_d - k_i}\right) \exp(-k_d t) \quad (3)$$

The experimental data was fitted using this two-step deactivation model (Table 2) and also with a first-order deactivation model. As was expected the first model originated a better fitting than the later. The highest half-life (18.8 h) was achieved in phosphate buffer at pH 7.5. At this pH there is an enhancement in activity prior to the onset of inactivation. This enhancement probably occurs because the labile intermediate has a higher specific activity than the native octamer (the value of α_i is higher than one). At the other experimental conditions studied, the labile octamer has a lower activity than the native one (α_i is lower than one) which reflects the effect of pH and buffer on the activity of the intermediate form. The specific activity of the denatured enzyme (i.e. the value of α_d) is as expected practically zero for all experimental conditions. The value of k_i represents the rate of formation of the labile octamer and k_d the actual deactivation rate. In accordance with the values of half-lives obtained, higher deactivations rates are observed at pH 7.0 in MOPS buffer, than in phosphate buffer and the lowest rate is attained at pH 7.5.

3.2.1. Effect of additives

To improve the thermal stability of AOX, the effect of several additives, including sugars, polymers and salts, was evaluated. The additives chosen were those that led to higher retentions of activity of the enzyme HRP (Graham et al., unpublished results).

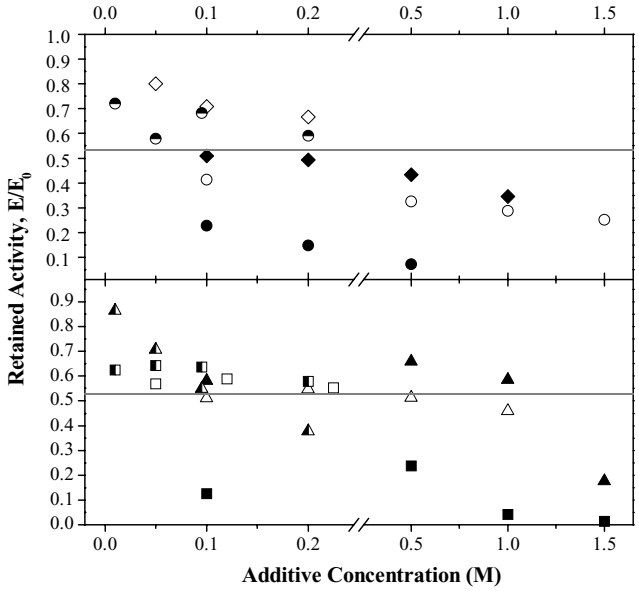

Fig. 2. Effect of reducing and non-reducing sugars in the retained activity after 9 h of incubation at 50 °C in phosphate buffer pH 7 in the presence of different additive concentrations: (□) inositol, (△) sorbitol, (◆) glucose, (●) frutose; (◇) lactose, (▲) sucrose, (○) maltose, (■) trehalose; (◭) melezitose, (◓) maltotriose, (◧) raffinose. The grey dotted lines represent the activity retained only in phosphate buffer. [AOX] = 0.25 mg/ml.

3.2.1.1. Effect of sugars. Polyols, such as sugars have long been regarded as protein stabilisers [6,24,25]. Nevertheless, it should be mentioned that each protein-additive-solvent system is unique because every protein possesses its own individual pattern of surface groups. Fig. 2 shows the effect of different sugars (mono-, di- and tri-sacharides) on the activity retained after 9 h of incubation at 50 °C and pH 7.0. Higher retentions of activity were attained at lower concentrations of additives. In the presence of 50 mM lactose and 10 mM melezitose, <20% of activity was lost after 9 h of incubation at 50 °C (compared with 54% loss in phosphate buffer).

Solvent additives have the ability to affect the equilibrium between the native and the unfolded conformational state of a protein [26]. As a general rule, additives, which stabilise the native structure at high concentrations, are preferentially excluded from the vicinity of the protein [25]. As Fig. 2 shows, increasing additive concentrations leads to a decrease in the activity, which indicated that instead of being preferentially excluded, these additives probably bind to the protein surface, accelerating the inactivation of AOX either by facilitating the cofactor loss or the protein disassembly.

3.2.1.2. Effect of salts. The influence of ammonium sulphate and ammonium chloride in the stability of AOX was studied at different salt concentrations. Increasing salt concentrations leads to a drastic decrease in the stability. Sulphate salts have long been used as protein stabilisers and are routinely used for storage purposes. Nevertheless, for concentrations higher than 0.5 M, AOX retained less than

10% of its initial activity after 9 h of incubation at 50 °C. This decrease was even more pronounced for the chloride salt, as expected from the Hofmeister series, with <1% of the activity retained. These results suggest that these ions probably bind to charged groups of the protein (or peptide bonds), reducing the number of water molecules clusters surrounding the protein. This interaction, usually described in the literature as salting-in, favors the disassembly and/or unfolding of the octamer.

3.2.1.3. Effect of polymers. The effect of different polymers on the stability of AOX at 50 °C was also investigated (Table 3). The polymers chosen included different molecular weight dextrans (dextran 9500, dextran 464000, dextran sulphate and DEAE dextran), different molecular weight polyethylene glycols (PEG 400, PEG 1000, PEG 20000 and a methoxy derivative—mPEG 2000) and polyethyleneimine (PEI). As observed with salts and sugars, an increase in the polymer concentration led to a decrease in the AOX stability, although less pronounced.

Within the PEG family a decrease in stability with the molecular weight of the polymer was observed. The introduction of a methoxy group slightly improved the stability of AOX, especially at high concentrations. Nevertheless, the highest retention of activity was obtained in the presence of 0.01% PEG 400. The enhancement of protein stability by PEGs and other neutral polymers has been attributed to the steric exclusion of the polymer from the vicinity of the protein, which promotes a preferentially hydration of the protein [25].

On the other hand, within the dextran family, AOX stability depends more on the charge of the polymer than upon its molecular weight. Dextran sulphate is a polyanionic polymer in which each glucose monomer can contain up to three sulphate groups. These negatively charged groups can interact with positively charged groups of amino acids residues (namely with the amine group of lysine and with the guanidinium group of arginine, at the working pH) on the protein surface, creating large complexes. These complexes can also be formed with polycations, such as DEAE-dextran, a polymer derived from dextran T-500 by substitution of the second position with diethylaminoethyl groups. In this case, the positive tertiary amine group of DEAE can bind to negatively charged amino acids residues (such as the carboxyl groups of aspartic and glutamic acids). The formation of such complexes stabilises AOX probably due to the formation of a network of polymer chains around the protein surface, which entangles the enzyme and restrains unfolding and dissociation of the octamer sub-units.

Polyethylenimine (PEI), a highly branched cationic polymer containing primary, secondary and tertiary amine groups in a ratio of 1:2:1, also protected AOX against thermal inactivation at 0.01% (w/v). Nevertheless, at higher concentrations, there was a strong destabilisation of the enzyme, which was probably due to the strong interaction of the charged amino groups with protein amino acids residues or bound water.

It has been reported that under certain experimental conditions (at a certain ionic strength), the enzyme/polyelectrolyte complex precipitates [9]. This precipitation was observed at 0.5% DEAE-dextran and 1% PEI.

3.2.1.4. Effect of additives combinations. It has already been reported that a combination of DEAE-dextran with a sugar alcohol, lactitol, is able to protect AOX against loss of activity during freeze-drying [9,27] and to improve biosensing characteristics of AOX electrodes [28]. The effect of using polymer/sugars mixtures to improve the stability of AOX was thus investigated (Table 4). The polymers and sugars chosen were the ones that led to higher activity retention when incubated alone with AOX, i.e. 0.01% PEG 400, 0.01% dextran sulphate, 50 mM lactose and 0.5 M sucrose. Melezitose was not used due its high cost when compared with sucrose.

Extremely high retentions of activity were obtained in the presence of the polyelectrolyte dextran sulphate and the sugar lactose (Table 4). It should be mentioned that although only the data relative to the activity after 9 h of incubation at 50 °C is presented, the inactivation of AOX was followed regularly during 12 h. A striking fact was an increase in activity (up to 15%) observed during the incubation of AOX in polymer/sugars mixtures. This enhancement in activity suggests the formation of an enzyme intermediate (probably

Table 3
Retained activity (%) after 9 h incubation at 50 °C in the present of different concentrations of polymers

Polymer		Concentration (% (w/v))			
Designation	MW (Da)	0.01	0.1	0.5	1
Dextran	9500	61.6	72.5	62.4	64.0
Dextran	464000	61.0	71.1	59.5	36.9
Dextran sulphate	10000	84.8	74.8	56.8	67.4
DEAE dextran	500000	70.6	61.1	–[a]	54.4
PEG	400	89.1	64.9	58.9	46.9
PEG	1000	72.2	76.3	55.0	45.0
PEG	20000	62.7	26.8	4.2	0.2
mPEG	2000	75.9	71.9	69.8	70.0
PEI	750000	79.0	47.4	N.D.	–[a]

Without stabilisers, the retained activity was 53.9%. N.D.: not determined.
[a] The enzyme/polymer solution got turbid.

Table 4
Activity retained after 9 h incubation at 50 °C in the present of different combinations of additives

Additives combination				Retained activity (%)
Polymer	Percentage	Sugar	mM	
Dextran sulphate	0.01	Sucrose	500	91.4
Dextran sulphate	0.01	Lactose	50	99.9
PEG 400	0.01	Sucrose	500	89.7
PEG 400	0.01	Lactose	50	87.4

Without stabilisers, the retained activity was 53.9%.

the enzyme-polymer complex), exhibiting more activity than the native enzyme. In the presence of these polymers/sugars combinations, activity loss was only observed after 6 h of incubation at 50 °C.

3.3. Operational stability of AOX

The successful integration of enzymes into analytical devices requires not only high storage stabilities but also high operational stabilities. The retention of enzymatic activity under conditions of continuous or semi-continuous substrate conversion is of extreme importance in biosensors design. The operational stability of AOX immobilised in CPG was studied at 32 °C during the continuous oxidation of ethanol fed at a flow rate of 0.1 ml/min (Fig. 3). The conversion of ethanol is however low since O_2 is the limiting substrate (0.22 mM). An ethanol conversion of 2.8% corresponds to an O_2 conversion of 82%.

When the bioreactor was fed with 300 mg/l ethanol solution prepared in 0.1 M phosphate buffer, after 3 h of operation there was a sudden decrease in the consumption of ethanol (Fig. 3). After interrupting the experiment, the bioreactor column was opened and it was possible to observe that the glass beads had lost their individuality and had aggregated into flocs of 2–3 mm of diameter. These flocs were gently stirred in a fresh buffer solution and packed again into the bioreactor. This renewed bioreactor was able to operate for 40 min more without loosing activity but after 3 h of continuous operation the residual conversion was almost zero. When ethanol was fed to the bioreactor in the standard assay reaction mixture the system was able to operate during at least 11 h without any decrease in the ethanol conversion. Furthermore, after 34 h of continuous operation the decrease in conversion was only of 25% (Table 5).

These results clearly indicate that if the H_2O_2 produced during the oxidation of ethanol is not eliminated, a strong inhibition/deactivation of AOX occurs. In fact, it is known

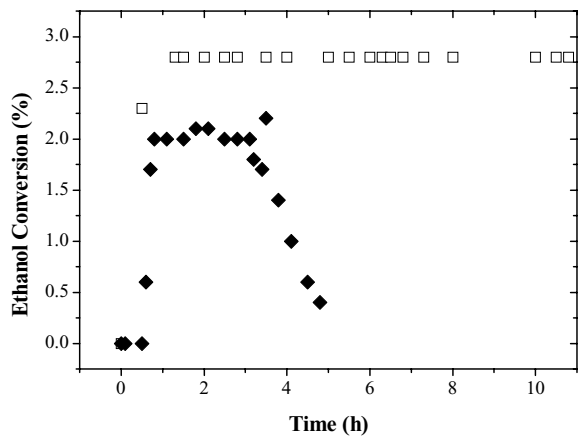

Fig. 3. Operational stability of CPG immobilised AOX during the continuous oxidation of 300 mg/l ethanol in 0.1 M phosphate buffer pH 7 (◆) and in the standard assay reaction mixture (□).

Table 5
Loss in ethanol conversion and in AOX activity after a certain time of continuous oxidation of ethanol in phosphate buffer 0.1 M pH 7 and in the standard assay reaction mixture at 32 °C at flow rate of 0.1 ml/min

System	Buffer	Standard assay reaction mixture	
	8 h	34 h	140 h
Loss in conversion (%)	79	25	73
Loss in activity (%)	31	7	30

that many enzymes deactivate under the action of H_2O_2, due to the oxidation of catalytically essential amino acids residues [18,29,30].

At the end of these stability studies, the bioreactors were unpacked, the supports were suspended in phosphate buffer and the remained enzymatic activity was evaluated according to the procedure described in the material and methods for CPG immobilised AOX. The residual activities were 69 and 93% of the initial activity for the CPG beads operating in buffer and in the standard assay reaction mixture, respectively (Table 5).

The operational stability of AOX under continuous oxidation of ethanol in the standard assay reaction mixture was further studied during 6 days at 32 °C and 0.1 ml/min. The decrease in ethanol conversion ($\eta_{ethanol}$) followed an exponential decay according to the equation ($r = 0.994$):

$$\eta_{ethanol}(\%) = 2.51e^{-0.0092t} \quad (4)$$

After 140 h of operation, the ethanol conversion had decreased to 25%. The bioreactor was then thoroughly washed with water and phosphate buffer, and AOX residual activity was evaluated (Table 5). The data in Table 5 shows that the decrease in ethanol conversion does not directly correlate with the deactivation of the immobilised enzyme. A more likely explanation for the loss of conversion is inhibition/poisoning either by the reducing substrates 4-AAP and PSA or by the reaction products, acetaldehyde or the quinoneimine dye. Acetaldehyde, for instances, has been found to be a potent end product inhibitor of alcohol oxidase [31].

In order to elucidate whether loss in conversion was mainly due to inhibition rather than deactivation, the standard assay reaction mixture was enriched with a stabilising polyelectrolyte/sugar combination. The decrease in ethanol conversion followed the same pattern as observed without the stabilisers. This clearly indicates that the loss in the bioreactor performance was not due to the deactivation of the biocatalyst but to its poisoning, probably by end-products.

3.4. Ethanol assay

The AOX immobilised bioreactor was incorporated into a FIA system and its stability was tested by multiple manual injections of 25 μl samples of 250 mg/l ethanol. The bioreactor was incorporated in both buffer and standard assay reaction mixture lines, mimicking the operational stability stud-

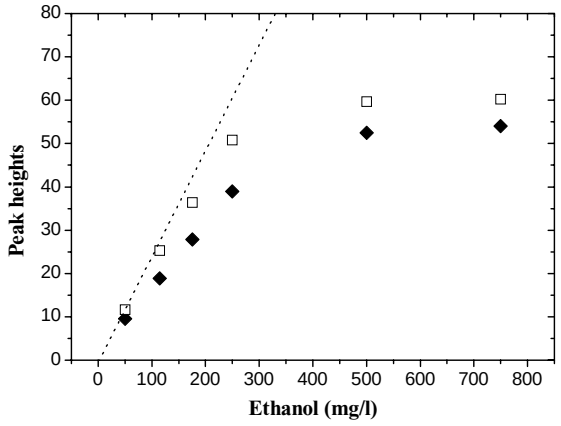

Fig. 4. Peak heights resulting from the injection of different concentrations of ethanol through an injection valve located just before the bioreactor, which was incorporated in the buffer line (◆) or in the standard assay reaction mixture line (□). The straight solid line represents a calibration with H_2O_2 standards.

ies described above. In both cases, stable and reproducible peak heights were obtained even when ethanol was oxidised in the buffer line. According to the operational stability studies performed, after 3 h of continuous ethanol oxidation in buffer, there was a decrease in the bioreactor performance. This 3 h-long period of continuous operation corresponds to the equivalent volume of more than 850 injection samples. However, the incorporation of the bioreactor in the standard assay reaction mixture line, will enable more than 3000 injections to be performed before the enzyme starts loosing performance after.

Ethanol samples of different concentrations (50–750 mg/l) were injected through an injection valve positioned upstream of the immobilised bioreactor. The peak heights obtained were directly proportional to ethanol concentration for concentrations lower than 250 mg/l, whatever the location of the bioreactor (Fig. 4). Higher peak heights are however obtained when the bioreactor is perfused with the standard assay reaction mixture (Fig. 4). This peak height difference probably relates to the inhibition of AOX by the H_2O_2 formed in the buffer line. To confirm this, different H_2O_2 standards were injected in the buffer and standard assay reaction mixture lines and no difference in peak heights were observed. These peak heights allowed the calculation of the yield of conversion of the ethanol injected and it was observed that the deviations from linearity on the plot of peak heights versus ethanol concentration are due to a decrease in the conversion yield of ethanol for concentrations higher than 250 mg/l. This problem could be overcome using a longer bioreactor.

4. Conclusions

A colorimetric assay based on the oxidation of H_2O_2 by HRP in the presence of PSA and 4-AAP was successfully used to study the kinetics of *Hansenula polymorpha* AOX and to monitor the oxidation of ethanol by this enzyme.

The AOX thermal stability was found to be rather sensitive to the type of buffer and pH value of the medium. Optimum working conditions were obtained in phosphate buffer at pH 7–7.5. AOX could be successfully protected against thermal deactivation using a combination of polyelectrolytes and sugars, such as dextran sulphate and lactose.

The operational stability of AOX immobilised on CPG beads was studied in a mini packed-bed bioreactor operating in continuous mode. AOX was strongly inactivated by the end-product, H_2O_2, during catalytic turnover. Combinations of additives, such as the ones used to stabilise free AOX were useless during continuous operation of the enzyme. The use of an in situ strategy, which consisted in the removal of H_2O_2 by HRP and reducing substrates, led to high operational stabilities.

The in situ stabilisation strategy was successfully applied in a flow injection analysis (FIA) system for ethanol monitoring. The use of the mini packed-bed bioreactor with AOX immobilised on CPG enabled the rapid quantitation of ethanol in samples with concentrations lower than 250 mg/l.

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