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Operation and performance of analytical packed-bed reactors with an immobilised alcohol oxidase

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Abstract

Hansenula polymorpha alcohol oxidase (AOX) was immobilised on propylamino-derivatised controlled pore glass (CPG) by covalent attachment using glutaraldehyde (GA) as cross-linker. Different pore and particle sizes were used as well as different experimental conditions (GA concentration, buffer type, pH, time of activation) to optimise the enzymatic productivity and operational stability of the immobilised enzyme. The best results were obtained by activating CPG with 6.5% GA in phosphate buffer, pH 7, for 1 h. The highest activity (0.45 U/mg) and productivity (6.2 mol/m^3 min) were obtained with a CPG support with 120–200 mesh and 550 Å pore size. Mini packed-bed bioreactors (9.4–69 mm^3) with the immobilised AOX, were used to monitor ethanol. The performance of the bioreactors was simulated using a plug flow model. External mass transfer limitations were observed for residence times higher than 2 s. The bioreactors operated continuously at 32 °C for more than 14 h without significant loss of performance (less than 5%). Ethanol in real samples such as beer, brandy and fermentation media was also successfully monitored. Bi-enzymatic bioreactors containing AOX and HRP were further constructed and displayed a similar performance.

Keywords: *Hansenula polymorpha*; Alcohol oxidase; Immobilisation; Glutaraldehyde; Controlled pore glass; Operational stability; Immobilised enzyme reactors; Mass transfer limitations; Horseradish peroxidase

1. Introduction

Immobilised enzyme reactors have been widely used in analytical chemistry, offering many advantages over homogeneous enzyme systems, especially when incorporated into adequately designed flow systems that minimise reagent consumption and allow the handling of small sample volumes [1].

Enzymes have been immobilised onto solid supports, either by physical adsorption, covalent bonding, cross-linking and entrapment [2]. The immobilisation of enzymes enables their continuous use in analytical assays, reactors, sensors and industrial processes with high retention of activity. Of the methods commonly used, covalent binding onto inert supports favours a long-time stability and the re-use of the immobilised enzyme, although the activity may become drastically reduced. Controlled pore glass (CPG) is one of the most popular solid supports for covalent immobilisation. CPG is a macroporous high-silica glass, obtained from alkali-borosilicate glass, a material which has excellent mechanical properties and can be prepared with a wide range of porosities and pore sizes [3].

The obtention of high activities and stabilities during operational conditions usually requires an optimisation of the enzyme immobilisation protocol. On the other hand, the particle and pore size can have a major impact on the activity and stability of the immobilised enzyme. The larger the particle, the greater the effect of diffusion control. Hence, the smallest particle size is usually the best choice, although in this case pressure drop issues become important if the use of a packed bed is envisaged. Regarding the support pores, the smaller their size, the higher the surface area available for enzyme loading. Nevertheless, extremely small pores may exclude the target enzyme [4].

Alcohol oxidase (AOX; E.C. 1.1.3.13; alcohol:O_2 oxidoreductase) is an oligomeric flavoprotein with eight identical sub-units arranged in a quasi-cubic orientation, each containing a non-covalently bound flavin adenine dinucleotide molecule (FAD) as a cofactor [5]. AOX catalyses the oxidation of low molecular weight alcohols by molecular

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oxygen (O_2) into the corresponding aldehydes with the concomitant production of hydrogen peroxide (H_2O_2). One of the most relevant applications of AOX has been the monitoring of ethanol in the beverages and fermentation industries, clinical chemistry and forensic analyses. In all these applications, enzyme stability is fundamental.

A strategy to enhance the operational stability of immobilised AOX bioreactors has been recently reported, which is based on the in situ consumption of the H_2O_2 produced during the oxidation of ethanol by the enzyme horseradish peroxidase (HRP) and its reducing substrates phenol-sulfonic acid (PSA) and 4-aminoantipiryne (4-AAP) [6].

The main objective of this work is to optimise the coupling of alcohol oxidase using a homofunctional crosslinker, glutaraldehyde, to CPG supports with different particle and pore sizes and to study the performance of AOX mini packed-bed bioreactors during the oxidation of ethanol.

2. Materials and methods

2.1. Materials

Alcohol oxidase from the methylotrophic yeast *Hansenula polymorpha* was obtained from Applied Enzyme Technology (Leeds, UK) as a 25 mg/ml solution in 0.1 M sodium 3-(*N*-morpholino)propanesulfonate (MOPS) buffer, pH 7 with an activity of 500 U/ml versus the Clark Oxygen Electrode. Peroxidase (HRP; E.C. 1.11.1.7; donor:H_2O_2 oxidoreductase) from *Armoracia rusticana* (horseradish) was purchased from Biozyme, UK, freeze-dried and with a specific activity of 252 U/mg versus pyrogallol. Alkylamine controlled pore glass with different sizes were obtained from Sigma and Aldrich. Absolute ethanol and glutaraldehyde was obtained from Merck and the reducing substrates, phenol-4-sulfonic acid sodium salt and 4-aminoantipyrine were purchased from Fluka and Sigma, respectively.

2.2. Methods

2.2.1. Preparation of immobilised enzyme reactors

2.2.1.1. Enzyme immobilisation. AOX was immobilised onto aminopropyl-derivatised CPGs in a two-step procedure/process. First, the supports were activated with functional aldehyde groups. Aqueous solutions of glutaraldehyde (GA) were added to the aminated CPGs in a volume ratio of 10:1 (Table 1). The reaction was performed at room temperature during 1 h, unless otherwise stated. At the end of this step, the supports were thoroughly washed with 0.1 M phosphate buffer pH 7.0 to remove unbound GA. Secondly, the buffer was removed and an equivalent volume (i.e. equal to the washed supports) of 25 mg/ml AOX in 0.1 M MOPS buffer pH 7.0, was added. The coupling of the enzyme to the support was first performed without stirring for 14 h at 7 °C, and then with top-over-top mixing for an additional 7 h at 22 °C.

HRP was immobilised in CPG with 120–200 mesh and 550 Å. The activation of the support was performed with 6.5% GA in phosphate buffer pH 7 during 1 h. The support was thoroughly washed with phosphate buffer and an equivalent volume of 20 mg/ml HRP in 0.1 M phosphate buffer pH 7.0 was added [7]. The co-immobilisation of HRP and AOX was performed in a similar way but a double volume of enzyme solution was added, which contained 10 mg/ml HRP and 12.5 mg/ml AOX.

2.2.1.2. Packed-bed bioreactor. The CPG supports with immobilised enzyme were packed into small glass columns with 25 mm length, 2 mm i.d. and 0.5 mm wall thickness (for more details see [6]). Different amounts of beads were loaded to the columns originating packed-bed bioreactors with different volumes, from 9.4 to 69 mm^3.

2.2.2. Protein determination

Protein concentration was determined using the Folin phenol reagent [8]. A known amount of support (approximately 10 mg) was completely hydrolysed in 2N NaOH during 20 min at 100 °C. The samples were cooled down and diluted to 0.5N NaOH. The hydrolysed protein was then determined using the Lowry method. Albumin was used as a standard.

2.2.3. Activity studies

The activity of AOX was determined using a bi-enzymatic colorimetric assay comprising HRP and its reducing substrates PSA and 4-AAP. Ethanol oxidation by molecular oxygen catalysed by AOX yields acetaldehyde and hydrogen peroxide, which is further reduced by HRP with the concomitant formation of a quinoneimine dye. This dye has a characteristic magenta colour with maximum absorption around 490 nm ($\varepsilon_{490\,nm} = 5.56\,mM^{-1}\,cm^{-1}$ determined experimentally towards H_2O_2 [6]). The activity of AOX

Table 1
Experimental conditions used during the activation of CPG supports with 120–200 mesh and 550 Å pore diameter and respective protein loading and yield

CPG_{100_550} ID	GA (%)	$T_{activation}$ (h)	Buffer type (pH)	Protein (mg/g)	Yield (%)
$GA_{10\%\_2\,h}$	10	2	Water (4.0)	75	75
$GA_{10\%\_1\,h}$	10	1	Water (3.7)	84	73
$GA_{6.5\%\_pH5}$	6.5	1	KH_2PO_4 (4.8)	100	85
$GA_{6.5\%\_pH7}$	6.5	1	Phosphate (6.9)	84	67
$GA_{6.5\%\_pH8}$	6.5	1	Bicarbonate (8.5)	90	72

was determined by monitoring the increase in absorbance at 490 nm with a Jencons spectrophotometer. One unit of activity (U) was defined as the number of μmol of H_2O_2 produced per minute at 25 °C. 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.

2.2.3.1. Free AOX activity assay. 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 of 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 AOX activity assay. Immobilised AOX was assayed colorimetrically at 490 nm using a recirculation system, which comprises a 20 cm^3 stirred glass tank thermostatised at 32 °C, a Watson–Marlow peristaltic pump, a Hellma quartz flow cell incorporated in a Jencons spectrophotometer and the AOX immobilised bioreactor, all connected in series using Omnifit teflon tubing [6]. The piping system was completely filled with the standard assay reaction mixture, 10 ml of this reaction mixture was added to the stirred tank and the reaction was initiated by the addition of 250 μl of 6.5 mM ethanol in 0.1 M phosphate buffer pH 7, under a constant flow rate of 1 ml/min.

2.2.4. Stability studies

The operational stability of immobilised AOX was studied during the in situ ethanol oxidation by O_2. The AOX bioreactors were continuously fed with 6.5 mM ethanol in the standard assay reaction mixture at a constant flow rate of 1 ml/min. The production of H_2O_2 was followed at 490 nm through the formation of the quinoenimine dye. In order to accelerate the deactivation process, all stability experiments were pursued at 32 °C.

2.2.5. Flow injection analysis (FIA) system

The packed bed bioreactors containing the immobilised AOX were connected to a Gallamp peristaltic pump and a Rheodyne rotatory PTFE injection valve, controlled by an Omnifit Universal switching module. Absorbances were measured in a Hellma 18 mm^3 glass flow cell in a Jencons spectrophotometer and the output recorded on a Gallenkamp Datatrace flat-bed recorder. Omnifit tubing with 0.8 mm i.d. was used.

3. Results and discussion

3.1. Optimisation of the immobilisation protocol

The covalent immobilisation of proteins onto a support usually involves two steps: the activation of the support with reactive/functional groups and the covalent binding of the protein to those groups. The yield of immobilisation and the final enzymatic activity are usually affected by the conditions used in these steps.

3.1.1. Activation with glutaraldehyde

Many different techniques can be used to couple proteins to amine derivatised supports. Glutaraldehyde is one of the most widely used cross-linkers, notwithstanding the lack of complete understanding of the reaction mechanism between GA and proteins. GA reacts predominantly with free amino groups from proteins, especially with the ε-amino group of lysine residues, through the formation of a Schiff base. This assumption, however, is not unambiguously supported by several facts observed during the reaction of GA with proteins [9]. Besides the monomeric form, commercial aqueous preparations of GA also contain a mixture of oligomeric and even polymeric material that are also able to participate in the binding of proteins to an aminated support. This binding is believed to occur through a different mechanism. Hence the concentration of GA and the time of reaction affect the yield of immobilisation and the final activity of the enzyme.

The effect of the conditions used in the GA activation on the immobilisation yield and on the kinetic properties of the enzyme was investigated. High enzyme loadings per gram of support were obtained with all experimental protocols tested, corresponding to more than 70% of immobilisation of the initial protein (Table 1). The activation of the support with a solution of 6.5% GA at pH 4.8 led to the highest enzyme loading. Nevertheless, a large protein loading does not necessarily translate into a high specific activity or operational stability, as described next.

3.1.1.1. Kinetic studies. The different preparations of immobilised AOX obtained were packed inside 69 mm^3 mini-reactors and the enzymatic activity was measured under a constant flow rate of 0.5 ml/min, i.e., at a nominal residence time of 8.3 s (Table 2). The specific activity of the supports was measured at two different ethanol nominal concentrations, one higher (1.1 mM) and another lower (0.16 mM) than the dissolved O_2 concentration at 25 °C (0.25 mM), and the corresponding percentage of activity expressed was calculated. A striking feature was that the expressed activity at higher ethanol concentrations was remarkably lower than at lower ethanol concentrations. This particular behaviour suggested an inhibition of the immobilised enzyme at high ethanol concentrations, probably by end-products (acetaldehyde or the quinoneimine dye) or external mass transfer limitations. To check this hypothesis, the specific activity of the supports was determined in smaller bioreactors (31 mm^3) at a higher flow rate (1 ml/min), in order to decrease the contact time between the products and the immobilised enzyme (Table 2). At this lower nominal residence time ($\tau = 1.9$ s), the differences between the expressed activity at 1.1 and 0.15 mM ethanol were not as significant as those observed at 8.3 s, suggesting a reduction on the inhibition by the end-products or on mass

Table 2
Effect of GA activation protocol on the apparent Michaelis–Menten parameters and on AOX specific activity (U/mg protein), determined under two nominal residence times (τ) and at two distinct ethanol concentrations (0.16 and 1.1 mM), and the respective activity yield upon immobilisation in brackets

CPG_{100_550} ID	Specific activity (U/mg protein)				K_M^{app} (mM)	V_{max}^{app} (U/mg)
	$\tau = 8.3$ s		$\tau = 1.9$ s			
	0.16 mM (%)	1.1 mM (%)	0.16 mM (%)	1.1 mM (%)		
Free AOX	0.55 (100)	3.03 (100)	0.55 (100)	3.03 (100)	3.6	15
$GA_{10\%\_2\,h}$	0.10 (18)	0.18 (5.9)	0.09 (16)	0.41 (14)	0.81 ± 0.09	0.63 ± 0.07
$GA_{10\%\_1\,h}$	0.07 (13)	0.11 (3.6)	0.10 (18)	0.39 (13)	0.67 ± 0.02	0.62 ± 0.16
$GA_{6.5\%\_pH4}$	0.08 (15)	0.13 (4.3)	0.09 (15)	0.38 (13)	0.49 ± 0.02	0.52 ± 0.05
$GA_{6.5\%\_pH7}$	0.09 (16)	0.12 (4.0)	0.12 (19)	0.45 (15)	0.47 ± 0.03	0.59 ± 0.06
$GA_{6.5\%\_pH8}$	0.07 (13)	0.11 (3.6)	0.10 (18)	0.41 (14)	0.45 ± 0.02	0.52 ± 0.05

transfer limitations. Nevertheless, all the supports exhibited less than 20% of the specific activity of the free enzyme, which is probably a consequence of conformational changes in the 3-D structure of the protein caused by the covalent binding of AOX to the supports. This low specific activity may be also due to steric factors, since the enzyme may be coupled to the support in a way that hinders the access of substrates to the active centre. The specific activity exhibited by the different immobilised AOX preparations was very similar although the highest value was obtained when the support was activated with a solution of 6.5% GA in phosphate buffer pH 7.

The overall kinetics of immobilised AOX was studied using a nominal residence time of 1.8 s in order to minimise end-products inhibition (i.e. in 31 mm^3 bioreactors at a constant flow rate of 1 ml/min). All immobilised preparations follow a typical Michaelis–Menten kinetics. The values of the apparent Michaelis–Menten constant, K_M^{app}, of the supports activated with 6.5% GA (around 0.47 mM) were very similar, but higher values were obtained with the supports activated with 10% GA, suggesting higher conformational changes of AOX upon immobilisation (Table 2). Among these supports, an activation time of 2 h, led to an even higher K_M^{app} value. The values of the apparent maximum velocity are essentially the same for the different immobilised preparations, within the experimental error (Table 2). Nevertheless, the V_{max}^{app} are almost two orders of magnitude lower than the value observed for the free enzyme (15 U/mg). This drastic decrease in V_{max}^{app} arises most likely from mass transfer restrictions to the transport of both substrates and products to and from the site of the catalytic reaction.

3.1.1.2. Stability studies. The stability of the immobilised bioreactors was tested by injections of 25 μl of 6.5 mM ethanol standard in a flow injection analysis (FIA) system. Reproducible peak heights and shapes were obtained with all bioreactors and in the course of successive injections during several weeks. In order to simplify and speed up the evaluation of the operational stability of the bioreactors, further studies were performed using a continuous set-up. Thus, instead of analysing the decrease in peak height after successive injections of an ethanol standard, the standard was continuously fed to the bioreactors and the decrease in conversion recorded.

The operational stability of the 31 mm^3 bioreactors prepared with the different immobilised preparations was studied at 32 °C. The bioreactors were continuously fed with 6.5 mM ethanol in the standard assay reaction mixture at a constant flow rate of 1 ml/min and the rate of ethanol up-take was evaluated along the time. The initial conversion of the limiting substrate (O_2) was higher than 80% in all bioreactors, and the highest value was observed for the support activated with 6.5% GA in phosphate buffer, pH 7.0 (Table 3). The initial volumetric productivity (q_p), i.e. the initial production of H_2O_2 per unit of volume of the bioreactor, was also determined and again the highest value was obtained for the support activated with 6.5% GA in phosphate buffer. Since the volumetric productivity is

Table 3
Effect of GA activation protocol on the initial O_2 conversion (χ_{O_2}), initial volumetric productivity (q_p) and enzymatic productivity (TOF) after different operational times

CPG ID	χ_{O_2} (%)	q_p (mol/m^3 min)	TOF ($mol_{substrate}/mol_{enzyme}$ min)		
			$t = 0$ h (%)	$t = 12$ h (%)	$t = 30$ h (%)
$GA_{10\%\_2h}$	82	5.9	162 (100)	121 (75)	79 (49)
$GA_{10\%\_1h}$	84	6.0	147 (100)	133 (90)	121 (82)
$GA_{6.5\%\_pH4}$	82	5.8	127 (100)	111 (87)	N.D.
$GA_{6.5\%\_pH7}$	86	6.2	143 (100)	138 (97)	122 (85)
$GA_{6.5\%\_pH8}$	85	6.0	138 (100)	125 (90)	N.D.

N.D., not determined.

dependent on protein concentration and enzyme activity, the enzymatic productivity, defined as the number of moles of substrate that are converted by one mole of biocatalyst per time unit (i.e. the turnover efficiency of the enzyme, TOF), was also determined. The enzyme preparation that exhibited the highest initial TOF was the one which was activated with a 10% GA solution in water for 2 h. Nevertheless, the TOF decrease with this enzyme preparation was the fastest. Although the support activated with 6.5% GA in phosphate buffer pH 7 displayed a slightly higher retention of productivity after 30 h, all other enzyme preparations exhibited similar profiles of productivity decay. The turnover efficiencies obtained with all immobilised preparations are lower than the published turnover, 220 min^{-1} [10]. This discrepancy is partly due to the fact that the reported turnover value was measured towards methanol, the natural substrate of AOX. Nevertheless, considering that the activity of AOX towards ethanol is 67% of the activity towards methanol [10], the turnover towards ethanol should be 147 min^{-1}.

At the end of the stability experiments, the bioreactors were thoroughly washed with phosphate buffer pH 7 and the remaining activity was evaluated. This confirmed that the loss in activity correlated well with the observed loss in productivity.

According to the previous studies, an activation time of 2 h lead to the lowest retention of productivity, with 50% of activity lost after 30 h of continuous ethanol oxidation. Moreover, the activation time shows up as a key parameter for the stability of the immobilised AOX. In fact, after 12 h of operation, the support activated for 2 h had already lost 25% of its initial activity while the others had only lost about 10%. This decrease in stability can be related to the higher probability of intramolecular crosslinking occurrence, between nearby $–NH_2$ groups present in the CPG surface. This intra-crosslinking leads to a decrease on the concentration of free carbonyl groups responsible for the binding of the enzyme, and consequently, leads to lower protein loading (Table 1). Simultaneously, it could reduce the possibility of multipoint attachment of the enzyme to the support.

An activation protocol was therefore adopted in which the supports were activated for 1 h with a 6.5% GA solution prepared in phosphate buffer pH 7, since this was the support that exhibited the highest retention of activity and at the same time the highest initial conversion and specific activity.

3.1.2. Effect of particle and pore size

AOX was immobilised onto CPG with different particle size and pore size distribution. Studies with glucose oxidase and catalase have shown that the pore diameter should be at least twice the major axis of the unit cell of the enzyme [11], the so-called spin diameter [12]. In order to estimate the spin diameter of AOX, the Stokes radius of each of the globular eight 80 kDa monomers in the native AOX was first calculated using the equation $r(Å) = 0.38 \times MW^{0.4}$ [13] and found to be 35 Å. By further assuming a cubic arrangement of the eight subunits, AOX's major axis, which coincides with the internal diagonal of a 70 Å sided cube, was estimated at 240 Å. The effect of pore size was then studied using pore diameters higher than the AOX's spin diameter (480 Å), namely 550, 700, 875 and 1275 Å. Particle diameters of 100, 150 and 300 μm were also evaluated.

As Table 4 shows both particle and pore sizes had a strong effect on the protein loading. For the same particle diameter (150 μm), an increase in the support pore size led to a decrease in the amount of protein bound per weight of support, as a consequence of the decrease in the specific surface area. A drastic and unexpected decrease in the protein loading was observed when the particle size increased from 100 to 300 μm. This decrease can be partially attributed to an incomplete glutaraldehyde activation of the amino groups in the 300 μm support, brought about by diffusional limitations during the activation step.

3.1.2.1. Kinetic studies. The different supports were packed into 31 mm^3 bioreactors and the AOX activity was determined at 1.1 mM of ethanol under a constant flow rate of 1 ml/min, i.e. under a nominal residence time of 1.8 s. A decrease in the activity was observed with an increase in both pore and particle size (Table 4). Nevertheless the specific activity of all the supports is almost the same with the exception of the support with 300 μm, which is probably due to the low protein loading. The bioreactor packed with this support also presented the lowest initial conversion and initial volumetric productivity values (Table 5).

Table 4
Characterisation of the different supports used to immobilise AOX, regarding particle size (mesh numbers) and respective nominal average diameter (d), pore size, surface area, protein yield and loading after immobilisation, activity (U/ml bioreactor), specific activity (U/mg protein), at a nominal residence time (τ) of 1.9 s, and respective activity yield upon immobilisation in brackets

CPG ID	Particle size		Pore size (Å)	$A_{surface}$ (m^2/g)	Protein			Activity	
	Mesh	d (μm)			Yield (%)	(mg/g)	(mg/m^2)	(U/ml)	(U/mg)
CPG_{100_550}	120–200	100	550	37	67	84	2.3	11.8	0.45 (15%)
CPG_{300_550}	40–80	300	550	37	27	40	1.1	3.5	0.28 (9%)
CPG_{150_700}	80–120	150	700	29	95	96	3.3	N.D.	N.D.
CPG_{150_875}	80–120	150	875	24	51	74	3.1	8.1	0.41 (13%)
CPG_{150_1275}	80–120	150	1275	16	37	52	3.2	7.3	0.45 (15%)

N.D., not determined.

Table 5
Effect of particle and pore size on the initial O_2 conversion (χ_{O_2}), initial volumetric productivity (q_p) and in the enzymatic productivity (turnover efficiency) at different operational times

CPG ID	χ_{O_2} (%)	q_p (mol/m^3 min)	Turnover ($mol_{substrate}/mol_{enzyme}$ min)		
			$t = 0$ h (%)	$t = 12$ h (%)	$t = 30$ h (%)
CPG_{100_550}	86	6.2	143 (100)	138 (97)	122 (85)
CPG_{300_550}	46	3.3	171 (100)	156 (91)	107 (63)
CPG_{150_700}	90	5.8	124 (100)	117 (94)	N.D.
CPG_{150_875}	82	5.9	163 (100)	147 (90)	118 (72)
CPG_{150_1275}	82	5.9	233 (100)	187 (80)	107 (46)

N.D. = not determined.

Nevertheless, the initial enzymatic productivity (turnover) is higher than the one observed for the smaller particle support (100 μm). The initial enzymatic productivity also increased with an increase in the pore size. Both increases in the turnover value are probably correlated with the decrease of the protein loading of these supports.

3.1.2.2. Stability studies. The stability of the bioreactors is also dependent on the particle and pore size. Despite an increase observed in the initial enzymatic productivity, an increase in both particle and pore size leads to a decrease in the retention of the initial productivity (Table 5). It seems that the smaller the particle and the pore of the support, the higher is the retention of the bioreactor performance. In fact, the highest retention was achieved in 100 μm average diameter particles with 550 Å pore size. This observation can reflect the effect of the pore and particle size in the protein content but can also be due to an increase in the protein rigidity in smaller pore sizes. Moreover, due to the large number of lysine residues (35 [14]), and hence a large number of free amino groups, present in each AOX sub-units the possibility of achieving a multipoint binding of the enzyme to the support is enhanced in the supports with 550 Å, whose pore size is very close to the AOX spin diameter.

3.2. Operational performance of AOX bioreactors

The CPG support with 100 μm average diameter and 550 Å pore size was chosen for further studies. Different amounts of this support were packed inside the bioreactors, leading to volumes of 9.4, 19 and 31 mm^3. The effect of the flow rate on the conversion of these bioreactors was investigated. The bioreactors were fed with 6.5 mM of ethanol in the standard assay reaction mixture at different flow rates and the conversion of ethanol was evaluated at 490 nm throughout the formation of the quinoneimine dye formation. Fig. 1a shows that the 31 and 19 mm^3 bioreactors display a similar behaviour. There is an increase in the initial O_2 conversion for low flow rates, which can be attributed to a decrease in both diffusional resistance and

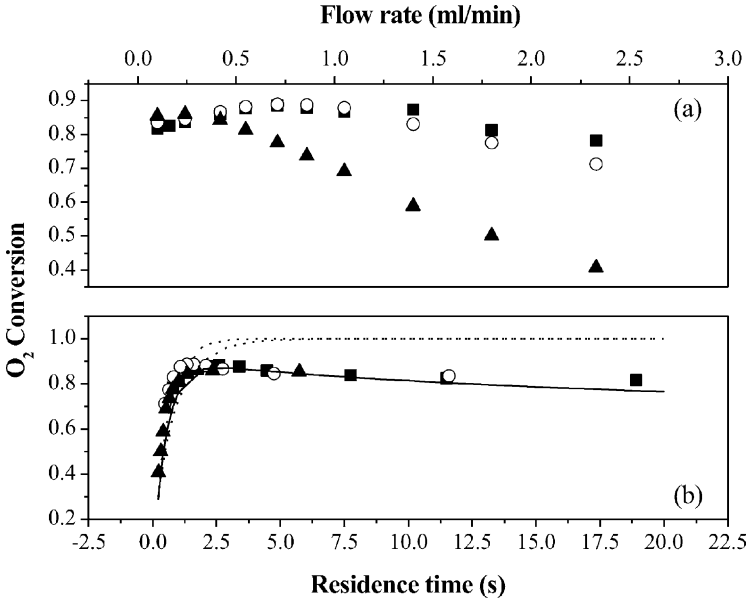

Fig. 1. Effect of flow rate (a) and residence time (b) on the initial O_2 conversion for different bioreactor volumes: 9.4 (▲), 19 (○) and 31 mm^3 (■). The data was fitted with different models: ideal plug flow (black dotted line), with product inhibition (grey dotted line) and with external mass transfer limitations (black solid line).

Table 6
Initial O_2 conversion (χ_{O_2}), initial volumetric productivity (q_p) and enzymatic productivity (TOF) at different operational times of different AOX and AOX/HRP bioreactors

Bioreactor ID	$V_{bioreactor}$ (mm^3)	χ_{O_2} (%)	q_p (mol/m^3 min)	TOF (min^{-1})	
				$t = 0$ h	$t = 12$ h (%)
Ethanol source					
Analytical	31	86	6.1	149	147 (99)
Analytical	19	87	10.2	251	237 (95)
Analytical	9.4	68	15.9	389	302 (78)
Beer	19	85	10.0	246	170 (69)
Brandy	19	87	10.2	251	248 (86)
Fermentation	19	63	7.3	180	166 (92)
Bi-enzymatic reactors (analytical ethanol)					
Mixed AOX HRP	19	80	9.5	233	223 (96)
Co-imm. AOX HRP	19	82	9.8	272	244 (90)

inhibition by co-products, followed by a decrease in conversion at higher flow rates, probably due to an insufficient residence time. The conversion attained in both reactors is practically the same at low flow rates while slightly lower values are observed in the 19 mm^3 bioreactors at high flow rates. This suggests that there is an excess of enzyme in the 31 mm^3 immobilised bioreactor, considering the extremely low concentration of the reaction limiting substrate, the dissolved oxygen (0.25 mM). Regarding the smaller bioreactor (9.4 mm^3), the decrease in the initial O_2 conversion is observed at much lower flow rates (0.4 ml/min) and is due to the low residence times attained in this bioreactor. The initial volumetric and enzymatic productivity (q_p and TOF, respectively) of these bioreactors at 1 ml/min decreases with an increase in the bioreactor volume (Table 6), showing that only a small part of these bioreactors has been used during the bioconversion of ethanol to acetaldehyde.

The operational stability of the three bioreactors was studied during 14 h of continuous conversion of 6.5 mM ethanol fed in the standard assay reaction mixture at 32 °C and under a constant flow rate of 1 ml/min (Fig. 2). Both 19 and 31 mm^3 bioreactors exhibited a similar decay in conversion, with less than 5% lost during the first 12 h of bioconversion (Table 6). The smaller bioreactor presented however a lower retention in conversion, probably due to a lower amount of enzyme available.

3.2.1. Bioreactor modelling

If the initial O_2 conversion is plotted as a function of the residence time of the reagents inside each bioreactor (Fig. 1b), a high correlation of the data is obtained. This data was modelled using the equation that describes the behaviour of an ideal plug flow reactor [15]. It was assumed a first order kinetics towards O_2 ($r = k_1 C_{O_2}$), since the initial

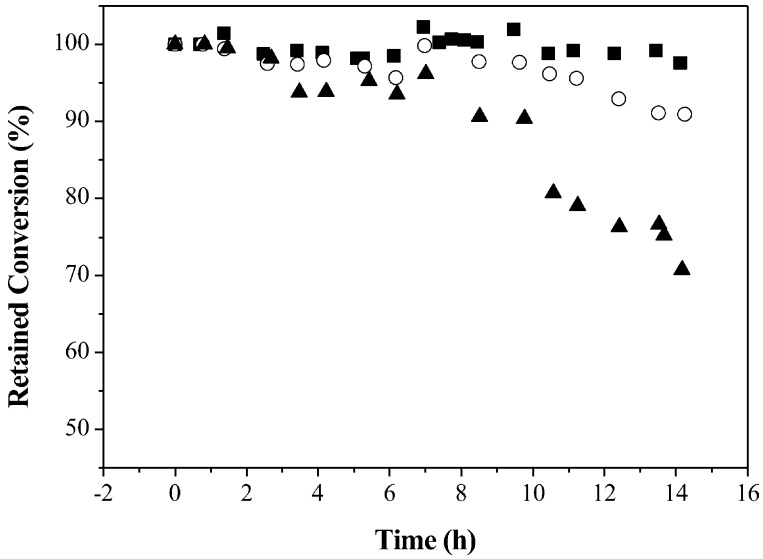

Fig. 2. Operational stability at 32 °C of AOX immobilised into CPG with 120–200 mesh and 550 Å pore size, under continuous oxidation of 6.5 mM ethanol, fed in the standard assay reaction mixture at 1 ml/min, for different bioreactor volumes: 9.4 (▲), 19 (○) and 31 mm^3 (■).

concentration of O_2 (0.25 mM) is lower than the published Michaelis–Menten constant for O_2 (0.4 mM [16]).

$$\tau = \int_0^X \frac{dX}{k_1(1-X)} \Leftrightarrow X = 1 - \exp(-k_1\tau) \quad (1)$$

where τ is the residence time, X the O_2 conversion and k_1 the first order kinetics constant. The value of k_1 ($1.8\,s^{-1}$) was determined using the reaction rate value obtained at 6.5 mM ethanol for the O_2 dissolved concentration at 25 °C (0.25 mM). The experimental values are well fitted using Eq. (1), for residence times lower than $2\,s^{-1}$ (Fig. 1b). For higher residence times, the conversion of O_2 is probably being limited by mass transfer effects or by product inhibition. Introducing in the reaction rate expression, a product inhibition term ($1 + C_P/K_P$), the following equation is obtained:

$$\tau = -\left(\frac{1}{k_1} + \frac{C^0_{O_2}}{k_1 K_P}\right)\ln(1-X) - \frac{C^0_{O_2}}{k_1 K_P} X \quad (2)$$

where C_P is the product concentration, K_P the product inhibition constant and $C^0_{O_2}$ is the initial O_2 concentration. The value of K_P was estimated by minimising the difference between the experimental points and the data originated from Eq. (2); a value of 0.23 mM was obtained. Eq. (2) is also plotted in Fig. 1b but again it does not adequately describe the experimental data for τ higher than $2\,s^{-1}$.

Conversion of O_2 must then be limited by mass transfer effects, which are known to affect the kinetics of immobilised enzymes since the substrates have to diffuse from the bulk solution to the biocatalyst. In the case of AOX, external and internal mass transfer effects can affect the performance of the bioreactors because AOX is immobilised on and within the matrix of the support. Nevertheless, the deviations observed only occur at high residence times, i.e., at low flow rates, suggesting that the external mass transfer limitations are probably more important than the internal mass transfer effects, which are dependent upon the diffusivity of O_2 within the CPG matrix rather than the flow rate. External mass transfer effects are quantitatively expressed by the external effectiveness factor (η_e), defined as the ratio of the observed reaction rate to the rate that would be observed if all the biocatalyst was surrounded by the bulk concentration [17]. Assuming a first order reaction, η_e can be determined using Eq. (3) in which k_L is the liquid-phase mass transfer coefficient and D_p is the support diameter and r_{obs} is the reaction rate expressed per volume of catalyst (determined from the Michaelis–Menten kinetics) and C_B is the substrate bulk concentration [17].

$$\eta_e = 1 - \frac{D_p}{6}\frac{r_{obs}}{k_L C_B} \quad (3)$$

The liquid-phase mass transfer coefficient, k_L, can be determined using the correlation described in Eq. (4), in which F_v is the linear flow velocity, ε is the void fraction (0.6), *Re* is the Reynolds number, *Sc* is the Schmidt number and D_{O_2} is the molecular diffusivity of O_2 ($2.64 \times 10^{-9}\,m^2/s$) [18].

$$k_L = \frac{1.09 F_v D_P}{\varepsilon(Re)^{2/3}(Sc)^{2/3} D_{O_2}} \quad (4)$$

For each flow rate evaluated, the value of k_L was determined (ranging from 1.3×10^{-4} to 3.7×10^{-4} m/s), allowing the calculation of the external effectiveness factor (ranging from 0.77 to 0.92). As expected, at lower flow rates, i.e., at high residence times, the value of k_L decreases leading to an increase in mass transfer limitations (i.e., a decrease in the effectiveness factor). When the conversion values originated by Eq. (1) were multiplied by the effectiveness factor, the experimental data was well fitted (Fig. 1b).

3.2.2. Bioconversion of ethanol from real samples

Ethanol from different real samples, such as beer (5%, v/v), brandy (38%, v/v) and fermentation media was continuously fed, at a constant flow rate of 1 ml/min, to 19 mm^3 bioreactors. Beer and brandy samples were diluted in the standard assay reaction mixture solution in order to originate an ethanol concentration of 6.5 mM. Fermentation medium components from concentrated stock solutions, previously sterilised at 121 °C during 20 min, were added to the standard assay reaction mixture. The nominal concentration of the fermentation substrates and metabolites were 1.7 g/l pyruvate, 0.4 g/l succinate, 4.2 g/l yeast extract, 8.3 g/l glucose, 0.4 g/l glycerol and 6.0 g/l acetate.

The initial conversion of ethanol from beer and brandy samples was not affected by the presence of their media but a decrease of 25% was observed in the presence of fermentation medium additives (Table 6). This decrease is probably due to an inhibition of both enzymes (AOX and HRP) by these additives. The operational stability of these bioreactor was also investigated. A drastic decrease in conversion was observed after 10 h in the bioreactor fed with beer, which was due to an occasional microbial contamination of this bioreactor. The bioreactors fed with brandy and fermentation media exhibited a decay in conversion similar to the bioreactor fed with analytical ethanol.

3.2.3. Bi-enzymatic bioreactors

Up to now HRP has been used free and dissolved in the standard assay reaction mixture. In order to minimise HRP consumption, bi-enzymatic bioreactors containing both AOX and HRP were constructed using two different approaches. As a first approach, equal amounts of CPG with immobilised AOX and HRP (19 mm^3) were packed into the bioreactor originating a mixed bioreactor with 37 mm^3. In the second approach both enzymes were co-immobilised in the same CPG particles. Table 6 summarises the behaviour of these bi-enzymatic bioreactors. There is a slight decrease in the conversion and volumetric productivity when using the immobilised HRP. The retention of the productivity when using a mixed bioreactor is not affected but when both enzymes were co-immobilised there was a small decrease in

the retention of productivity probably due the lower protein loading obtained with this immobilisation protocol.

4. Conclusions

Alcohol oxidase was covalently immobilised in controlled pore glass using glutaraldehyde. In the optimised immobilisation protocol, CPG beads were activated during 1 h with 6.5% GA prepared in phosphate buffer pH 7. The highest activities and stabilities were obtained with a CPG support with 100 μm average diameter (120–200 mesh) and 550 Å pore size.

Mini packed-bed bioreactors constructed with this support allowed the monitoring of ethanol in both FIA and continuous mode, in which ethanol uptake was indirectly detected by measuring H_2O_2 with free HRP in a colorimetric system.

The bioreactors operated for more than 14 h at 32 °C with a continuous addition of 6.5 mM ethanol in the standard assay reactin mixture without significant loss of performance (<5%). This corresponds to an equivalent number of 33,600 injections of 25 μl of 6.5 mM ethanol in a FIA mode. AOX bioreactors can be successfully used to monitor ethanol from real samples, such as beer, brandy and fermentation media, diluted in the carrier stream of the flow system, with high operational stability.

Bi-enzymatic bioreactors containing AOX and HRP were also constructed which displayed a similar performance but with a significant reduction in the amount of HRP used.

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