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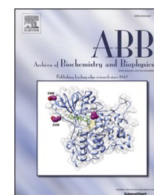

Tools to investigate oxygen-related challenges with flavin-dependent enzymes

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ABSTRACT

Enzymes have multiple applications in medicine but during the past decades interest in the application of enzymes as (bio)catalysts to produce a wide range of valuable molecules in various industries has increased. Many chemical compounds (from pharmaceuticals to bulk commodities) can be produced by a series of enzymatically-catalysed chemical steps, and in many cases one of these steps is an oxidation.

The use of molecular oxygen as an oxidising agent in biocatalytic processes is a double-edged approach. From one side, the oxygen is supplied to the reactor in the form of air bubbling, which is cheap, highly available and non-toxic. From the other side, bubbling air into the reaction media creates a gas-liquid interface which adsorbs enzymes and compromises their stability. Moreover, the oxygen is quite insoluble in water, which often results in oxygen-limited reactions.

These aspects are the main limiting factors for the stability and kinetics of enzymes that perform oxidative biocatalysis and prevent the reaction from happening at a rate that is high/competitive enough for industrial feasibility. Therefore, we need systems to mimic and understand better these factors to try and mitigate their effects upon scale-up.

In this review, we present two complementary systems to study these factors: one apparatus that ensures a constant gas-liquid interface and another one that maintains a constant oxygen partial pressure. Both can provide highly valuable information regarding the maximum rate of reaction and about the deactivation profiles of enzymes in the presence of bubbles.

1. Introduction

Conventionally, flavoenzymes have found multiple applications in medicine as therapeutics [1] and biomarkers [2,3]. However, during the past decades their application as biocatalysts has increased among major pharmaceutical and chemical companies due to their remarkable properties [4–7].

The application of enzymes in medicine has a high process cost due to the required high levels of purity, specificity and health safety the enzymes must offer. However, since reactions occur fast inside the body or in microfluidic chips, a low activity and short-term stability of enzymes do not suppose a great drawback for the application [2,8]. Enzymes have also been powerful tools to produce pharmacological products such as drug carriers [9]: application which is at the intersection *in vitro* applications and production of pharmaceutical products at small scale. The production of larger amounts of high value products (pharmaceuticals and others) requires a higher efficiency of the enzymes to reduce the overall cost of the process and product [10,11]. At larger scales, the price of the process must be kept within an acceptable range. This implies at least one of the following conditions: the reuse of enzymes (highly stable enzymes) and performing the reactions fast and completely (high enzymatic activity). These requirements are important in synthetic processes of high-value products and even more when the price of the product becomes very low such as in the synthesis of bulk commodities or in degradation processes [12–14] (Fig. 1).

Although enzymes have traditionally had many uses as degrading catalysts, more recently they have been used for synthetic organic chemistry. Today we see important examples both in degradation [15,16] as well as synthesis [17,18]. The use of enzymes as synthetic biocatalysts is desirable due to two main reasons: their excellence in performing chemical reactions in a highly efficient and selective manner, and the overall sustainable nature of the biochemical process compared to conventional chemical catalysis [19].

Chemical compounds are often produced by a series of (bio)chemical

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steps that can be either a series of enzymatic reactions or a combination of chemical and biochemical conversion steps [14,20–22]. The word biocatalyst describes the biological origin of enzymes combined with their ability to make chemical modifications to a substrate. Biocatalysis then, studies the reactions that biocatalysts perform, with the main interest on the process and their application in degrading or synthesizing compounds in industry.

It is important to note that biocatalysts are not only considered as isolated enzymes but also encompass enzyme-containing cells. Fermentation processes are also considered biocatalytic processes. However, when we talk about enzymatic reactions in industry, we are not referring to whole-cell biocatalysis, but to isolated-enzyme biocatalysis. Highly pure enzyme formulations are often the most desirable solution (often in combination with enzyme immobilization [23]), but the cost of purifying enzymes can be too high in relationship to the profit gained from the process. Therefore, enzymes are often isolated from cells and used in different degrees of purity depending on the needs of the process.

Today we can see industrial applications of enzymes as biocatalysts. For example McIntosh et al. (Merck and Co, USA) reported the synthesis of Molnupiravir, an investigational antiviral agent for the treatment of COVID-19 [5]. They reduced a 70 % the length of the synthesis and increased the yield 7-fold by using several engineered enzymes, including the flavoenzyme pyruvate oxidase. Another example is reported as a joint effort of Merck and Co (USA) and Codexis (USA) for the chemo-enzymatic synthesis of Boceprevir, a novel drug treatment for hepatitis C [6]. They engineered the FAD-containing monoamine oxidase (MAO) towards better and more selective substrate binding and a higher activity to produce an intermediate for Boceprevir. Moreover, less raw materials and water were used, resulting in a much more sustainable process.

Enormous advances have been made at the level of enzyme engineering [24] leading to higher selectivity for the substrates [25], a broader substrate scope [26], production of enantio-pure products [27], a higher selectivity for non-natural substrates and the ability to perform new-to-Nature reactions [28], or higher stability [29,30]. These features contribute greatly to unleashing the full potential of enzymes and their contribution to well-established processes in the laboratory. Yet, the acquired individual features are still not enough to fulfil the required metrics for economic feasibility and scale-up [31].

In industry, several factors (e.g. high concentrations of substrate and product, concentration and pressure gradients, multi-phase interfaces, adsorption of the enzymes to gases and solids) are present in the reactor where the enzymatic catalysis is happening. These factors can limit the stability and kinetics of the enzyme, preventing the reaction from happening at a high enough rate. As represented in Fig. 2, industrial biocatalysis, must then be studied in the laboratory where the effects of upscaling can be mimicked and well understood through scale-down experiments. Next, the new knowledge must be transferred from process engineers to experts in biochemistry and enzyme engineering who can contribute into paving the way towards understanding which parameters of the enzyme are sub-optimal (e.g. residues at the active site, residues distal from the active site, folding, hydrophobicity, tunnels …) [32,33]. This knowledge will, in turn, become very useful to engineer enzymes that fit industrial conditions and can achieve the desired metrics [19,34]. These collaborations are key to build on the vast knowledge gained in biochemistry and (computational) enzyme engineering laboratories, transfer it into industry and achieve cheaper and better processes and products that can help people every day [35,36].

2. Oxidative biocatalysis

Oxidation is one of the most widely used reactions in organic chemistry since it is part of many industrial synthetic schemes. Traditionally, oxidation reactions have been performed using metals as catalysts such as manganese dioxide, permanganate and chromium (VI)

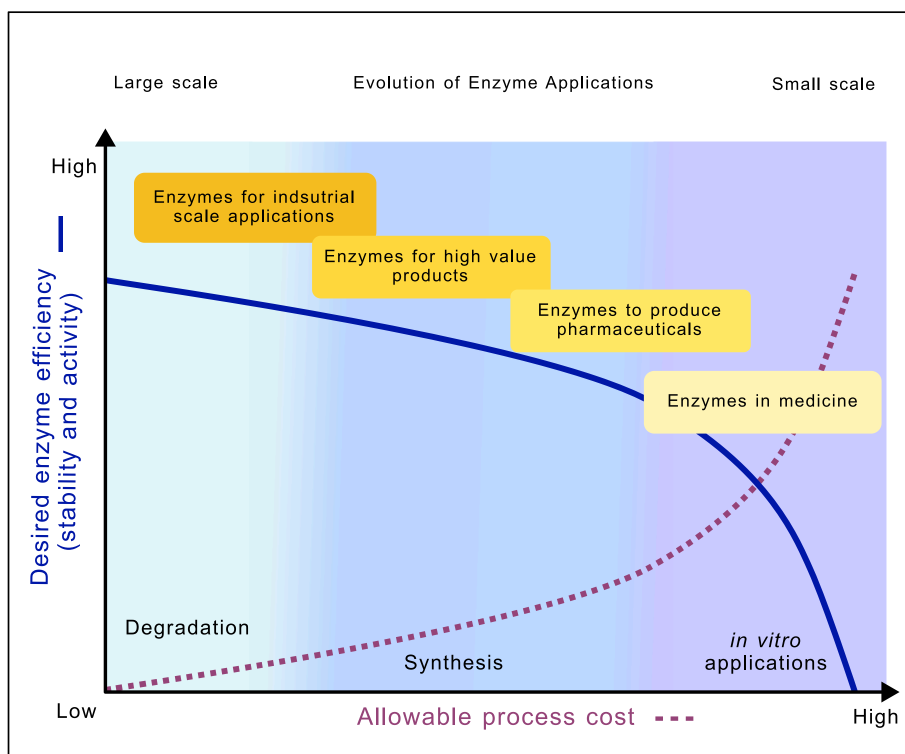

Fig. 1. Evolution of enzyme applications from medicine to biocatalysis. In the x-axis (purple, dotted) the allowable process cost of each application. In the y-axis (blue, solid), the minimum enzyme efficiency that will allow the process to become economically feasible. From right to left, the evolution of the scale of in which enzymes have been used (small to large) and the different areas to which enzymes have been applied (*in vitro* applications, synthesis of products, synthesis and degradation of products). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

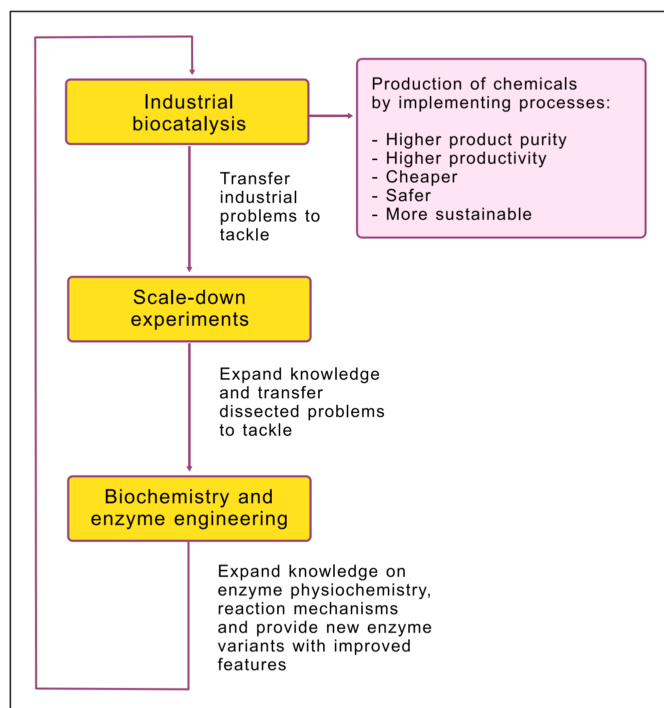

Fig. 2. Proposed workflow to achieve economically feasible production processes in biocatalysis.

under high temperature and pressure. These are strong oxidants, that can act upon virtually any radical that is susceptible to oxidation. Therefore, the protection (and deprotection) of functional groups is often essential in chemical catalysis. In such cases, large amounts of catalyst relative product often need to be used, which also creates large amounts of toxic and hazardous waste at the end of every reaction. Moreover, mineral acids (HF, H_2SO_4, H_3PO_3) and Lewis acids ($ZnCl_2$, $AlCl_3$, BF_3) are used as reagents in the reaction or in the downstream processing, which are quite difficult to dispose of and thereby contribute to the generation of chemical waste [11,19,37].

In contrast, biocatalysts are produced using only organic materials (sugar, nitrogen source, air and water) so only non-hazardous and non-toxic organic matter comes out as waste at the end of the line, also in the catalysis step. Furthermore, enzymatic reactions are generally performed in water and under mild conditions (ambient temperature and atmospheric pressure). Biocatalytic multi-step reactions (namely cascade reactions) eliminate functional group activation, protection and deprotection, some purification steps, offer the possibility to recycle expensive cofactors and improve atom efficiency. This is due to the high selectivity of enzymes for their substrates and the possibility to engineer not only the reactor and process configuration, but also other parameters such as the enzyme itself. This extra degree of freedom in the design is enormously powerful.

Biocatalytic oxidation is particularly attractive since it is an irreversible reaction and allows the use of molecular oxygen as the oxidant under mild conditions. There are numerous examples where oxidation performed by oxidases is a crucial step in the production of pharmaceuticals. In fact, examples mentioned above [5,6] include at least one oxidation step performed by an flavin-dependent oxidase. Working with flavin-dependant oxidases can imply an advantage in terms of available knowledge. This is, it has been reported that oxygen seems to interact with positions N(5) and C(4a) of the reduced flavin in the active site in some flavoenzymes [38,39]. As an alternative to flavin-dependant oxidases, metal-dependent oxidases also have huge industrial potential. They are preferred in some cases since they do not depend on (often expensive) cofactors such as FAD or FMN. However, the mechanism of oxygen-enzyme interaction is unknown for many metal-dependant oxidases with interesting applications [40]. This may make oxygen-related site-directed mutagenesis easier in flavoproteins than in metalloenzymes. Nevertheless, the oxygen-enzyme interaction might not always be the target, and other strategies can also be used but it is outside of our scope [33]. For example, Huffman and co-workers (Merk and Co, USA), succeeded in the synthesis of Islatrivir [4], an investigational drug for HIV. They reported the engineered enzymatic cascade that consisted of nine enzymes in one pot and achieved an overall yield of 51 %. One of the enzymes was a galactose oxidase that was engineered towards a better activity, a reduction in product inhibition, and a reversal of the innate enantioselectivity.

What all oxidases have in common is that they rely on molecular oxygen as an electron acceptor. This makes oxidation processes more cost-effective and simpler and thereby more suitable for industrial application [13,41].

2.1. *The oxygen dilemma*

Even if the use of oxygen could be perceived as a simple procedure *a priori*, it implies several detrimental effects that limit the process metrics greatly. The gaseous nature of oxygen leads to the need to supply it constantly into the reactor tank. This is typically done in industry by bubbling air from the bottom for the reactor through a sparger or nozzle, which makes this procedure highly standardised in the sector (Fig. 3). Moreover, the supply of air is cheap, highly available from the atmosphere and non-toxic. Hence, oxidative biocatalysis a great example of a sustainable production process.

The oxygen transfer rate (OTR) is the velocity at which the oxygen gets transfused from the gas bubble to the liquid (Fig. 3a, steps 1 and 2), it is a very important parameter in the oxygen transfer phenomena that affects directly the final dissolved oxygen [42]. It is affected by the composition of the gas (oxygen partial pressure in the bubble), the nature of the media (typically water), and the interfacial area (amount of gas-liquid interface *per* volume of the liquid) [43]. Only after being solubilized, the oxygen becomes available for the enzymes (Fig. 3a, step

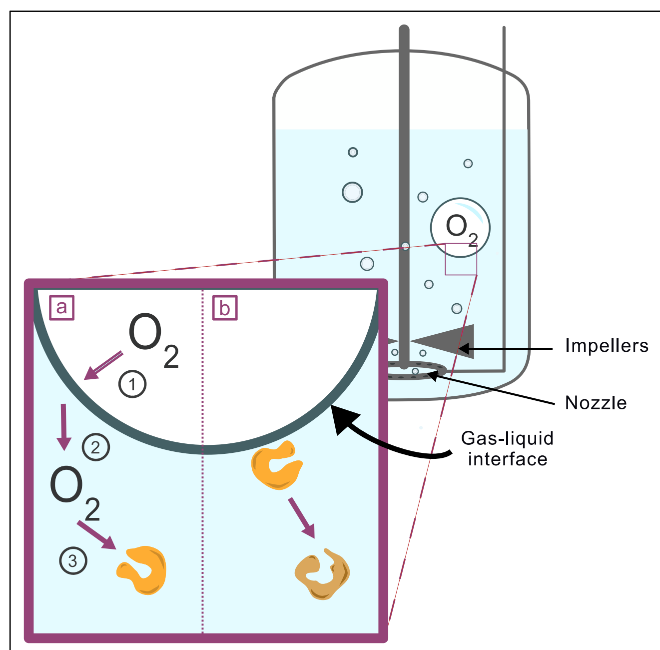

Fig. 3. Reactor with air bubbling from the bottom. Zoomed in, a) oxygen transfer process: 1. The oxygen reaches the gas-liquid interface; 2. Oxygen interacts with the water molecules to get solubilized in the liquid; and 3. Only then it becomes available to interact with the active site of the enzyme; b) the enzyme gets adsorbed to the gas-liquid interface and loses its structure.

3).

As reported by Merck and Co (USA) and Codexis (USA) [6], the first issue that has to be addressed in industrial oxidations is the limitation of oxygen availability in the aqueous medium. This is because oxygen is a hydrophobic gas, and the maximum concentration of oxygen that water can hold in equilibrium with air is 250 μM (at 30 °C and atmospheric pressure). The use of pure oxygen has been a valid alternative in some cases [6], but it poses a safety challenge as it constitutes a fire hazard when mixed with organic compounds. An increase in the pressure of the reactor also increases the solubility of oxygen, but it is not always possible for practical reasons and can also present safety issues.

The loss of stability as a result of the adsorption of the enzyme to the bubbles (Fig. 3b) is another equally important phenomenon that usually occurs in oxidative biocatalysis [44,45]. Guzman et al. shed light on the gas-liquid interface as a source of deactivation and developed a kinetic deactivation model for proteins [46]. Recently, a number of studies have also corroborated that the adsorption of enzymes to the interface (as well as model proteins) is a major source of deactivation [47–50].

Therefore, the interfacial area affects both the oxygen transfer and the stability of the enzyme. The relationship between enzymatic activity and the enzymatic stability can be expressed as the total turnover number. The total turnover number (TTN) is a dimensionless number that represents the quotient between the observed catalytic constant ($k_{cat,obs}$) and the observed first-order deactivation rate constant ($k_{d,obs}$), as shown in Eq. (1), [51].

$$\mathrm{TTN} = \frac{k_{cat,\mathrm{obs}}}{\mathrm{k}_{\mathrm{d,obs}}} \tag{1}$$

We hypothesise that the TTN will rise as a result of an increase in k_{cat} due to a larger availability of oxygen. At the same time, the increasing interfacial area will provoke an increase in k_d (enzyme destabilization) and at some point, the k_d will be so high, relative to k_{cat}, that the destabilization effect of the interface will deactivate the enzyme irreversibly, making it lose activity.

The contrary effects of the increase in interfacial area to stability and activity are governed by natural physico-chemical phenomena which cannot be avoided most of the time and seldom mitigated. Therefore, a profound understanding of these phenomena is needed to achieve a high-performing enzymatic process and reach acceptable product formation rates. These rates can only be achieved by reaching the right equilibrium between enzyme stability and activity which requires further characterisation of stability and kinetics of the enzyme under industry relevant conditions. Therefore, the recreation of industrial conditions at small scale is crucial [52].

A major challenge is failing to measure biocatalyst performance in terms of stability and kinetics separately. The lack of laboratory methods limits the study of these parameters [52]. For this reason, we need systems to mimic and understand better the physico-chemical factors that affect the enzymes to try and mitigate their effects to the reaction. Here, we present two complementary systems to study the stability and kinetics of enzymes respectively: the tuneable constant gas-liquid interface apparatus (modified bubble column) provides a tight control of the gas-liquid interface, and the constant partial pressure apparatus (Tube-in-Tube Reactor) displays a constant oxygen partial pressure. Depending on the conditions and restrictions of the system, one setup will be more relevant than the other. Fig. 4 shows a decision map that evaluates process limitations that may stem from bubbling and from oxygen transfer and the recommended actions to take in each case.

3. Tuneable constant gas-liquid interface apparatus: modified bubble column

In traditional reactors such as stirred tank reactors or regular bubble the only controlled factor regarding the air supply is the gas flow rate. Now, we present a modified bubble column setup, which enables a highly controlled supply of bubbles, which rise independently, at a constant velocity and in an undisturbed fashion unlike other bubble columns. This leads to experiments performed in defined and reproducible interfacial areas obtained under specified conditions. The main objective is to determine the loss of enzyme stability caused exclusively by the effect of bubbling a gas into a liquid.

The modified bubble column setup is a novel apparatus that has recently been used and proved to provide valuable information about the effect of surface-driven processes on enzymes [47,53].

A good example of a tuneable constant gas-liquid interface apparatus to study this phenomenon is the one described in Fig. 5. The apparatus is comprised of a glass cylinder (bubble column) that contains the solution of enzyme in water (or buffer). In a laboratory setup, a normal length of the bubble column is around 50 cm, and the inner diameter is between 0.5 and 2 cm. A needle fixed at the bottom of the bubble column operates as a nozzle and delivers the gas, in most cases, air. The pH, temperature and dissolved oxygen concentration are monitored online by a

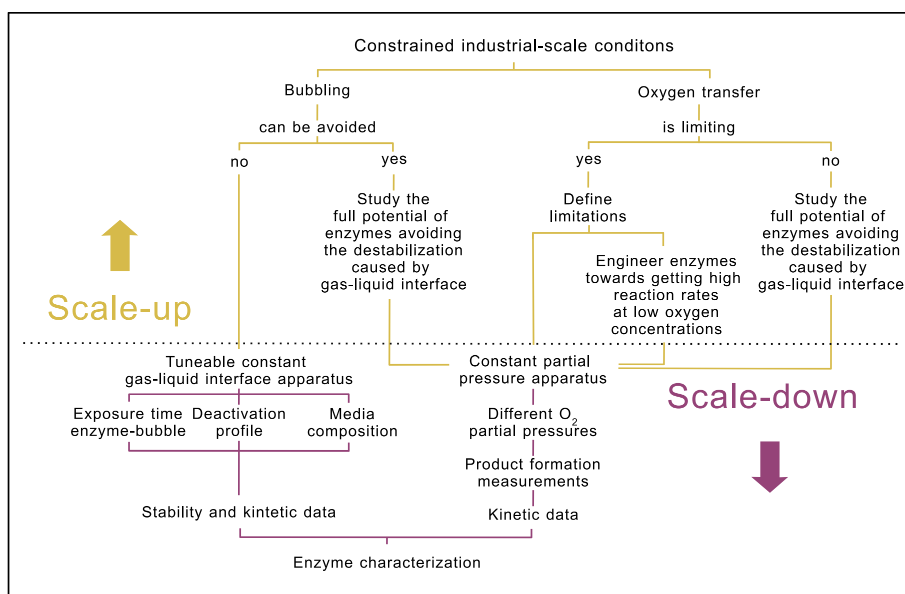

Fig. 4. Decision map to choose the right apparatus and what experiments to perform depending on the needs and challenges of the process.

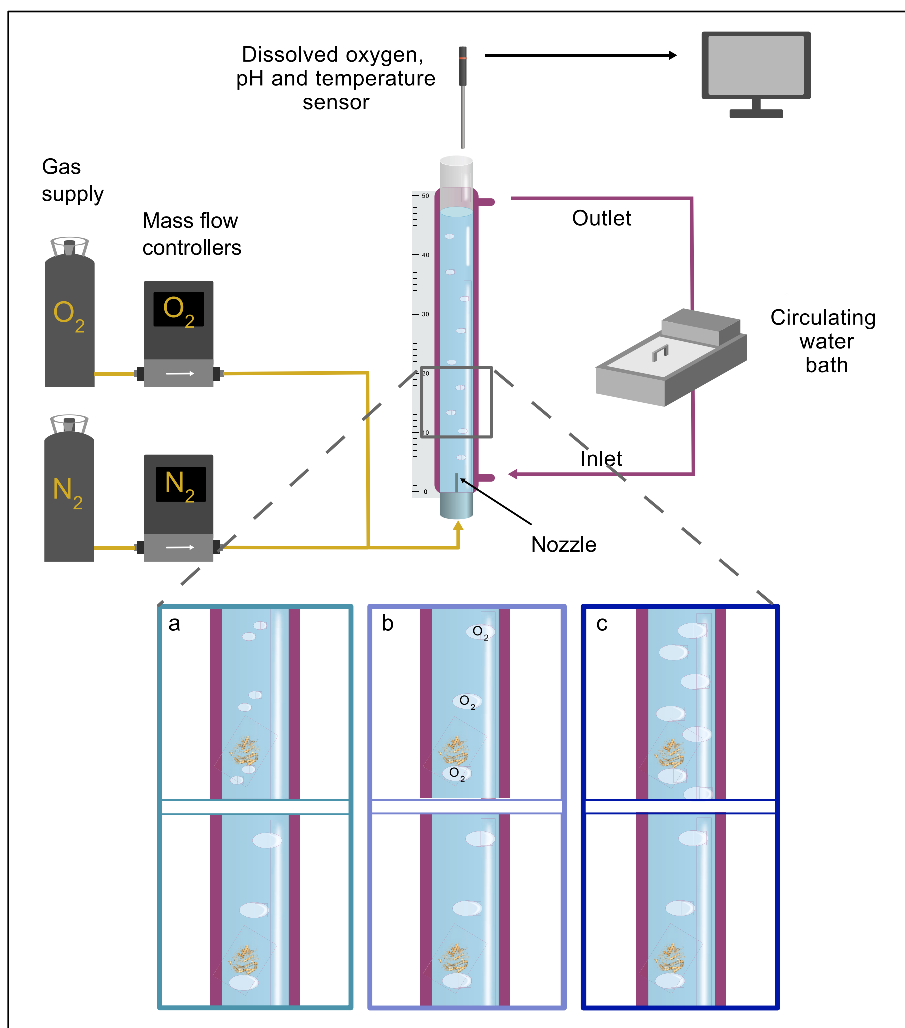

Fig. 5. Up, the modified bubble column, adapted from Ref. [54]. Down, stability test conditions. a) bubble size; b) gas composition; c) gas holdup.

sensor. Upstream from the bubble column, two mass flow controllers govern the flow rate of the gas inlet. The bubble column has an external water jacket connected to a circulating water bath for temperature control [54]. A measure is attached to the column to take bubble size and velocity-related measurements, which are done by recording the bubbles rise independently at different conditions. Furthermore, samples are taken, and multiple sorts of data can be obtained. The residual enzyme activity and protein concentration are measured by standard techniques such as spectrophotometric assays and the Bradford assay.

As shown in Fig. 5, experiments can be done where the main conditions that influence enzyme stability in a gas-water system can be tunned one at a time. a) The control of the size and shape of the bubbles contributes in a great extent to the control of the interfacial area; b) a broad range of highly defined gas compositions can be supplied; c) a higher gas flow rate contributes to increasing the number of bubbles, which can be extrapolated to a higher gas holdup (volume of gas inside the liquid) [55]. The increase in gas flow rate in case c) does not only cause a larger number of bubbles inside the column, but it also affects the shape and size of the bubbles, the speed at which they rise, the time of contact with the enzymes and the total interfacial area. This demonstrates that the isolation of parameters is a complex task, even when using such specialized apparatus. Nevertheless, the tuneable constant gas-liquid interface apparatus has been used in several cases to confirm hypotheses and create new ones.

3.1. *Interfacial bubble size*

The effect of the interfacial area was studied by using the modified bubble column setup by Anderson et al. [53]. The introduction of smaller bubbles at a constant flow rate (high interfacial area) was shown to have a greater effect on the deactivation than the introduction of larger bubbles in the same conditions.

3.2. *Gas compositions*

Wang et al. [54] have described very different kinetic deactivation profiles for the flavin-containing NAD(P)H oxidase (NOX), only by supplying different gas compositions (N_2, air, and O_2), being air the gas which caused the biggest destabilizing effect the fastest. The decrease in activity was linked to a loss of soluble protein as a result of protein aggregation rather than to a loss of specific activity (activity *per* enzyme unit).

3.3. *Alternative deactivation profiles*

Recent studies that have used the apparatus confirm a two-stage first-order deactivation trend and presented a new mechanistic model for the deactivation of enzymes [50,53,56].

3.4. Ionic strength

The effect of ionic strength on deactivation of enzymes has recently been studied in the bubble column by Vang Høst et al. [50]. Ionic strength was increased by the addition of NaCl and showed a decrease in bubble size, and a decrease in the deactivation rate of the flavin-dependant NAD(P)H-oxidase (NOX2), an enzyme of potential industrial interest for NAD(P)H regeneration. Even if previous work [53] suggested that increasing the gas-liquid interfacial area by decreasing the bubble size (controlled by changing the size of the nozzle) was detrimental to the stability of NOX, the new study [50] shows that the stabilizing effect of the increased ionic strength dominates the damaging effect of a large gas-liquid interfacial area provided by the small sized bubbles to an extent [49].

4. Constant partial pressure apparatus: tube-in-tube reactor system

There are many systems to collect enzyme kinetics data such as batch, flow and stopped-flow reactors [57], but few are able to supply high oxygen concentrations without the presence of bubbles [58].

Here we present the constant partial pressure apparatus, also previously called Tube-in-Tube Reactor [59] which is capable to achieve concentrations of dissolved oxygen up to 12 mM without the presence of gas-liquid interface. It is an automated system that has a reactor part and a detector part.

The reactor part is composed by a gas-permeable tube inserted in a gas-impermeable tube (Fig. 6a). The liquid phase is flowing though the inner tube and the gas flows between the outer and inner tubes, diffusing through the inner tube wall, which acts as a membrane, and gets solubilized in the liquid phase [60]. The reactor flows in a continuous, plug-flow way and samples are taken in-line periodically by a UV detector. The spectrophotometric absorbance of the product and/or substrate is measured correlated with the concentration of each compound. Ultimately, the enzyme kinetics can be calculated (Fig. 6b).

4.1. No interface

This instrument has several very special characteristics that allow to avoid some of the factors that create noise and limitations in kinetic measurements such as the loss of stability or the inability to achieve high concentrations of oxygen in the liquid media.

The Tube-in-Tube Reactor provides oxygen to the enzyme while introducing a physical barrier between the gas and the liquid instead of supplying the gas in the form of bubbles. The inner tube of the Tube-in-Tube reactor is made of Teflon-2400 fluoropolymer [61] characterized by a high permeability to gases [62], while showing a high chemical resistance. It is encased in a larger PTFE tube, practically impermeable to oxygen and nitrogen. A mixture of oxygen and nitrogen is supplied into the outer tube, whereby the oxygen can be transferred into the liquid reaction phase in the inner tube by diffusing through the Teflon-2400 tube and react with the enzymes.

The absence of bubbles leaves out of the equation the decrease of activity as a result of the loss of stability by avoiding direct contact with the gas-liquid interface. Hence, the real, undisturbed kinetics of the enzyme can be measured. This information is useful if we want to compare it to the kinetic measurements obtained with the presence of gas-liquid interface and assess the effect of bubbles on the enzymatic activity.

4.2. Broad range of oxygen concentrations

Another very relevant feature of the Tube-in-Tube Reactor is the possibility to achieve a broad range of dissolved oxygen concentrations in the liquid phase that are constant throughout the whole length of the tube. This way, it is possible to achieve the typical oxygen concentrations that are used in industry to study the enzyme kinetics at small scale in a controlled manner (i.e. without variations due to pressure at different points in a large reactor).

It is also possible to deliver much higher dissolved oxygen concentrations than in normal laboratory operation, which allows to test the

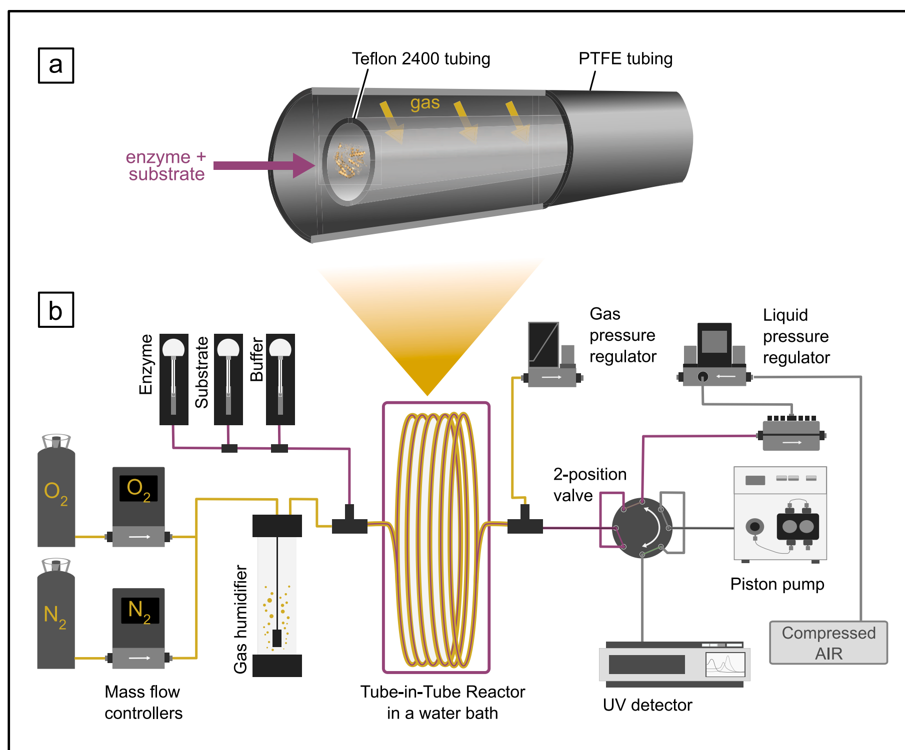

Fig. 6. Diagram of the constant partial-pressure reactor. a) Zoom in the reactor, showing the two tubes and the phases flowing in each tube, adapted from Ref. [60]. b) Diagram of the overall setup illustrating the different parts adapted from Ref. [59].

full kinetic potential of enzymes avoiding oxygen limitations. Two mass flow controllers regulate the oxygen and the nitrogen flow going into the reactor. The two gases are mixed before entering the reactor. This means the dissolved oxygen can get to a range of 0.06 mM–1.2 mM at atmospheric pressure (and 30 °C). Since the system can be pressurized up to 10 bar, the maximum limit can be ten times more, becoming a maximum dissolved oxygen concentration of 12 mM.

Somewhere in between such high oxygen concentration levels, the activity of the enzyme is expected to reach a plateau that will indicate the minimum amount of oxygen required for the enzyme to work at maximum rate (not being oxygen limited). This provides information about how small increments in oxygen concentration affect the reaction rate, which is directly related to the economics of the process. From an industrial perspective, efforts must be made to achieve higher dissolved oxygen concentrations and attempt to get higher rates. These can be changing reactor type, and tuning temperature and pressure.

4.3. New selection parameters for industrial enzyme engineering

The characterization of enzymes in industrial conditions at small scale is, therefore, essential to understand the effects of various factors on stability and kinetics of enzymes. It also opens the door for the improvement in enzyme kinetics by using the tremendous potential that enzyme engineering has to offer.

In many cases, industries are very constrained in terms of making changes in machinery and procedures and often the only option left to improve the process is engineering the enzyme structure to fit the given conditions. For instance, low oxygen availability in the reactor.

As the maximum concentration of oxygen is often fixed in an industrial setup, enzymes could potentially be engineered to use the small amounts of oxygen that they experience more effectively. This means, achieving a higher rate of reaction at lower dissolved oxygen concentrations.

Oxidases need oxygen to get re-oxidised after oxidizing the primary substrate. In a hypothetical case where the limiting step is not the interaction of the enzyme with the substrate but the reoxidation of the enzyme, the space where the enzyme interacts with the oxygen could potentially be engineered [40]. There are several techniques to do so such as directed evolution or site-directed mutagenesis. Even if it is practically very difficult to ensure zero-oxygen environments to perform directed mutagenesis and it is challenging to define which residues interact with oxygen to perform site-directed mutagenesis [63,64], improvements have been achieved in some cases [40]. The fine control of the oxygen supply in the tube-in-tube reactor system allows to test the enzyme performance at tiny differences of oxygen concentration to find the lowest concentration that still allows the highest rate of conversion.

5. Future perspectives

Enzymes are exceptional catalysts that possess remarkable and distinct characteristics. They have been used to perform meticulous and precise roles in medicine. More and more, their function as biocatalysts is gaining popularity provided by their unique attributes. Protein engineering has driven a great revolution in biocatalysis, and the development of enzymatic cascade reactions has proven their potential in the production of chemicals. Still, the full potential of this field has not yet been achieved. Further research is crucial to achieving the required metrics for economic feasibility and scale-up.

Particularly in the case of oxidation reactions, the benefits of their efficient implementation will be exceptionally relevant in terms of production cost and sustainability, creating a notable impact in society. These processes have a very concrete set of challenges that stem from the use of molecular oxygen and are yet to be overcome. The above presented effects of bubbling air into a reactor must be well understood in order to make the right choices for the industrial process. More data needs to be generated by analysing more enzymes in different conditions and the data must be accessible and transferable to other disciplines of enzyme-related science [65].

Biochemical and computational studies will be highly valuable to industry in terms of understanding how industrial scale reactor conditions influence the enzyme structure. That is: a) understanding in detail the effect of the gas-liquid interface to the enzyme structure, and b) understanding the interaction oxygen-enzyme at an atomic level. This knowledge will set the basis to engineer more stable enzymes and enzymes that work with high efficiencies even at industry-relevant oxygen levels.

The advances in related disciplines will trigger major advances in biocatalysis which will consequently contribute to broaden the use of this promising technology and contribute to more sustainable ways to produce a broad range of chemicals.

CRediT authorship contribution statement

Ariadna Pié Porta: Conceptualization, Investigation, Methodology, Resources, Visualization, Writing – original draft. **Elif Erdem:** Conceptualization, Formal analysis, Investigation, Project administration, Supervision, Validation, Visualization, Writing – review & editing. **John M. Woodley:** Conceptualization, Funding acquisition, Supervision, Validation, Writing – review & editing.

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