Enzymatic production of alcohols by valorisation of volatile fatty acids embedded in anaerobic digestate

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Converting carbon dioxide, the organic fraction of municipal solid waste and other types of waste, agricultural or industrial, into high value-added molecules in a sustainable manner is the main challenge to be overcome worldwide to facilitate a transition to a circular economy model. In particular, by fully exploiting the organic fraction of municipal solid waste and the numerous agricultural by-products, carboxylic acids, biohydrogen, and biomethane can be produced through an anaerobic fermentation process (Ruggeri et al., 2015).

Carboxylic acids, after a first phase of concentration and purification, can be further enhanced to produce molecules with higher added value, for example, alcohols, which have countless applications in the chemical industry and can be used as fuels, fragrances, emollients, and plasticizers (Kalim Akhtar et al., 2013).

There are several techniques to enhance carboxylic acids, for example using inorganic catalysts or different fungal species. In this work, an enzymatic strategy using two enzymes in series was proposed. In the first step of the reaction, carboxylic acids are reduced to aldehydes, using the enzyme Aldehyde Dehydrogenase (AldDH), and then the aldehydes to alcohols, using the enzyme Alcohol Dehydrogenase (ADH). Both the enzymes were obtained from *Saccharomyces cerevisiae*.

The enzyme Carboxylic Acid Reductase (CAR) is widely used to perform the first reaction (Kalim Akhtar et al., 2013). However, this enzyme uses at the same time two cofactors to perform the reduction, ATP and NADH. It was therefore decided to use the AldDH enzyme. This enzyme catalyzes much faster the reverse reaction, that is the oxidation of aldehydes to carboxylic acids. However, the use in series with the enzyme ADH could shift the reaction to the right, as shown in Figure 1, as the aldehydes produced would be immediately converted to alcohols. Similar behaviour is found in the case of the production of methanol from formic acid, although in this case the enzyme Formaldehyde Dehydrogenase is used (Luo et al., 2015). The second enzyme of the series, on the other hand, catalyzes very well the reaction of interest, that is, the reduction of aldehydes to alcohols (Luo et al., 2015). Both enzymes, therefore, use only NADH as a cofactor and a high concentration is necessary to obtain high quantities of desirable product. NADH is a very expensive molecule and it is, therefore, necessary to carry out a process of regeneration of the cofactor to make the process more sustainable and feasible its scalability (Marpani et al., 2017).

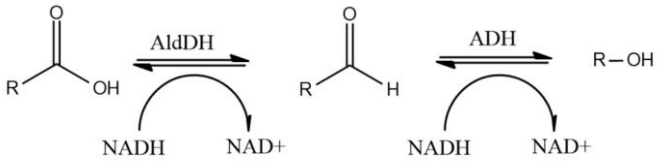

Figure 1- Reduction of carboxylic acid using AldDH and ADH enzymes in cascade

In order to reuse the enzymes and increase their stability, it was decided to immobilize the two enzymes covalently. The choice of support is crucial to optimize the immobilization of each enzyme. In particular, the surface area and the characteristic pore size may vary. Several different types of mesoporous silica have been tested as supports, varying for average pore diameter and surface area. In particular, two different commercial mesoporous silica (MSU-H and MSU-F) and one synthesized silica ($MCF_{0.75}$), highly suitable for enzymatic immobilization (Pietricola et al., 2021), were used. $MCF_{0.75}$ has been synthesized with an average pore diameter of 25nm. The MSU-H and MSU-F have an average pore diameter of 7 and 20 nm respectively. Using different pore diameters, it is possible to evaluate the diameter and optimal support for the two enzymes. The supports have been characterized with X-ray Diffraction (XRD), Field Emission Scanning Electron Microscopy (FESEM), and N_2 physisorption at -196 C. Subsequently a functionalization has been carried out in order to covalently bind the enzyme to the support. In particular, to immobilize the enzyme AldDH was carried out a functionalization with (3-Glycidyloxypropyl)trimethoxysilane (GPTMS) (1% v/v in toluene), to create glyoxyl groups after three

reactions in series. Subsequently, it is possible to perform the immobilization by working in a 100 mM carbonate buffer at pH 10 for 3h at 4º C. Retained enzymatic activity, obtained by measuring over time the absorbance variation of NADH at 340 nm and comparing it to the free enzyme, of 100% has been obtained. To immobilize the enzyme ADH it has been observed that this immobilization technique does not guarantee a good retained enzymatic activity, as at pH 10 this enzyme is quickly deactivated. It was then chosen to functionalize the support with the molecule (3-Aminopropyl)triethoxysilane (APTES) (1% in toluene), to create amino groups on the support, and perform immobilization at pH 7 in 5 mM phosphate buffer and using glutaraldehyde as a linker. In both cases were reached yields of immobilization of 100%. In the latter case the immobilization was carried out at 4 ºC for 3h. As for the retained activity, the first enzyme of the series has been obtained a value of 100% with all the supports used while the ADH has been obtained a maximum value of 23% using the support MSU-H. In all cases, an enzymatic load of $4mg_{enz}/g_{supp}$ was used.

The activity was then evaluated with varying pH and temperature for each enzyme immobilized on the different supports, comparing it with that of free enzymes. It has been observed that the immobilized enzyme is much more stable when the optimal conditions vary compared to the free enzyme, both in terms of temperature and pH. However, no optimal pH or T variation of the various immobilized enzymes was observed. The thermal stability was also evaluated by incubating the free and immobilized enzyme at 50 ° C and evaluating the residual activity at different times. Free AldDH after 72 h has no more activity, while immobilized on MSU-H has even more than 80% of activity and on MSU-F about 20% after 120 h to 50 C. Free ADH after 24h to 50 C presents no more activities. Immobilized on MSU-H after 120 h has more than 40% of residual activity while on MSU-F has not been observed a decrease of activity. This shows that these types of supports are very suitable for these enzymes and immobilization allows obtaining a high stability factor. Subsequently, the two reduction reactions were carried out. The first reaction was carried out using propionic acid and NADH and the second using propionaldehyde and NADH. To evaluate the conversion, the decrease in absorbance of NADH over time was measured using a UV-VIS spectrophotometer. An NADH decrease of more than 10% with AldDH and 30% with ADH was achieved in all cases, working at pH 7 and T=30 °C. The actual production of propionaldehyde using the first enzyme and propanol using the second enzyme should be evaluated using a GC-MS headspace. Finally, a reuse test was carried out, evaluating the residual activity after several reaction cycles. In all cases, the activity after 5 reaction cycles is greater than 30%.

To obtain higher conversion values the two immobilized enzymes should be used at the same time. These supports and these types of immobilization can also be used to immobilize other types of enzyme, even of different size compared to those used having the possibility to use supports with different average pore diameters. To increase the production of aldehydes obtained from the first step of realization could be used the enzyme CAR, extractable from different microorganisms. In this case, however, a process should be developed in which to regenerate ATP in addition to the cofactor NADH.

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