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

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Introduction
Wastewater valorization

1. Thermochemical conversion
2. Microbial conversion
3. Enzymatic conversion

Carboxylic acids embedded in anaerobic digestate → Bio-alcohols

- Renewable Catalysts
- Possibility to operate with environmental P and T
- High selectivity
- Cost
- Stability
- Often dependent on a cofactor

- Fuels;
- Fragrances;
- Emollients;
- Thickeners;
- Plasticizers.
Carboxylic acid reduction

- Use of two enzymes in series;
- Use of the NADH cofactor as a reducing agent.

1. Enzymatic stability
2. Reuse of enzymes

Immobilization process

Vidal et al., 2018, BBA - Proteins and Proteomics, 1866, 327-347.
Experimental part
Specific enzyme activity

IU (international activity unit): amount of enzyme necessary to catalyze the transformation of a μmol of substrate per minute in conditions of:

- Concentration of saturating substrate;
- pH = 7;
- T = 30 °C.

For the activity of the free and immobilized enzyme the change in the absorbance of NADH at 340nm is measured, under stirring.

\[
\text{UI}_{\text{FE}} = \frac{\Delta A}{\Delta t} \cdot \frac{1}{\varepsilon} \cdot \frac{V_{\text{tot}}}{\text{mg}_{\text{enz}}}
\]

\[
\text{UI}_{\text{IE}} = \frac{\Delta A}{\Delta t} \cdot \frac{1}{\varepsilon} \cdot \frac{V_{\text{tot}}}{\text{mg}_{\text{supp}}}
\]
Functionalization with amino groups

Amino groups formations:

- APTES 1% v/v in Toluene
- 105°C, 5h.

The reaction is carried out in a boiling flask with refrigeration column.

Lazghab et al., 2010, Chemical Engineering Research and Design, 88, 686-692.
Functionalization with glyoxyl groups

**Epoxy groups formation:**
- GPTMS 1% v/v in Toluene
- 105°C, 5h.

**Hydrolysis of epoxy groups:**
- H₂SO₄ 0.1M
- 85°C, 2h

**Formation of glyoxyl groups:**
- NaIO₄ 0.1M
- T_{amb}, 2h


Immobilization with amino groups

1st step reaction:
- Amino functionalized support
- Enzyme
- Phosphate buffer 5 mM pH 7
- 4 °C
- 3 h

2nd step reaction (after filtration)
- Phosphate buffer 25 mM pH 7
- Glutaraldehyde 0.1% v/v as linker to stabilize bond
- 4 °C
- 30min

Formation of ionic bonds between amino groups on the support and enzyme

Bolivar et al., 2006, Enzyme and Microbial Technology, 40, 540-546
Immobilization with glyoxyl groups

1st step reaction
- Functionalized support
- Enzyme
- 0.1 M carbonate buffer pH 10
- 4 °C
- 3 h

2nd step reaction (without filtration)
- NaBH₄;
- Glycerol.

Formation of weak bonds between support and enzyme

NaBH₄ as a reducing agent for the formation of the covalent bond, using glycerol as protecting agent

Results
Materials characterization

- Physisorption at -196 °C
  - Specific area greater than 500 m²/g, in all cases
  - Average pore diameter ranging from 7 to 25 nm
- FESEM analysis
  - MSU-H → Cylindrical shape
  - MSU-F, MCF₀.75 → Spongy-like structure

<table>
<thead>
<tr>
<th>Sample</th>
<th>$S_{BET}$ (m²/g)</th>
<th>$V_p$ (cm³/g)</th>
<th>$D_p$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSU-H</td>
<td>750</td>
<td>0.91</td>
<td>7</td>
</tr>
<tr>
<td>MSU-F</td>
<td>562</td>
<td>2.31</td>
<td>15</td>
</tr>
<tr>
<td>MCF₀.75</td>
<td>600</td>
<td>1.40</td>
<td>25</td>
</tr>
</tbody>
</table>
## Immobilized enzyme activity and immobilization yield

- Higher immobilization yields with materials with higher average pore diameter;
- Higher specific activities with materials with smaller average pore diameters;
- Enzymatic load: $4 \text{mg}_{\text{enz}}/\text{g}_{\text{supp}}$;
- ADH immobilized on amino support;
- AldDH immobilized on glyoxyl support.

### Sample activity and immobilization yield

<table>
<thead>
<tr>
<th>Sample</th>
<th>$D_p$ (nm)</th>
<th>$\text{IU/g}_{\text{supp}}$</th>
<th>IY (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADH/MSU-H</td>
<td>7</td>
<td>$48.6 \pm 1.32$</td>
<td>82$\pm6.2$</td>
</tr>
<tr>
<td>ADH/MSU-F</td>
<td>15</td>
<td>$12.6 \pm 0.89$</td>
<td>100$\pm3.1$</td>
</tr>
<tr>
<td>ADH/MCF$_{0.75}$</td>
<td>25</td>
<td>$3.6 \pm 0.53$</td>
<td>100$\pm4.2$</td>
</tr>
<tr>
<td>AldDH/MSU-H</td>
<td>7</td>
<td>$1.3 \pm 0.37$</td>
<td>50$\pm5.1$</td>
</tr>
<tr>
<td>AldDH/MSU-F</td>
<td>15</td>
<td>$1.01 \pm 0.45$</td>
<td>96$\pm4.6$</td>
</tr>
<tr>
<td>AldDH/MCF$_{0.75}$</td>
<td>25</td>
<td>$0.97 \pm 0.33$</td>
<td>100$\pm3.4$</td>
</tr>
</tbody>
</table>

### AldDH activity:
- 1,8 mL phosphate buffer 100 mM;
- 0,125 mL propionaldehyde 7,5 mM;
- 0,125 mL $\text{NAD}^+$ 50 mM;
- 30°C, pH 7;
- 5 mg of support or 0,025 mg of AldDH.

### ADH activity:
- 2 mL ethanol 250 mM in phosphate buffer 100mM;
- 0,1 mL $\text{NAD}^+$ 50 mM;
- 30°C, pH 7;
- 5 mg of support or 0,005 mg of ADH.
Comparison of thermal stability

Stability tests carried out at 50 °C for soluble enzyme and immobilized enzyme
Deactivation of the first order:

\[ \text{E}_{\text{nat}} \xrightarrow{k_d} \text{E}_{\text{den}} \]

Active enzyme concentration:

\[ A(t) = A_0 e^{(-k_d \cdot t)} \]

\[ A(t) = A_0 [(1 - \alpha)e^{(-k_d \cdot t)} + \alpha] \]

Stability factor:

\[ F_s = \frac{t_{1/2}^{IE}}{t_{1/2}^{FE}} \]

<table>
<thead>
<tr>
<th>Sample</th>
<th>k_D (h^{-1})</th>
<th>( \alpha ) (-)</th>
<th>( t_{1/2} ) (h)</th>
<th>( F_s ) (-)</th>
<th>( R^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free ADH</td>
<td>0.280</td>
<td>-</td>
<td>2.47</td>
<td>-</td>
<td>0.99</td>
</tr>
<tr>
<td>ADH/MSU-F</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ADH/MSU-H</td>
<td>0.015</td>
<td>0.3</td>
<td>83.5</td>
<td>33.8</td>
<td>0.95</td>
</tr>
<tr>
<td>Free AldDH</td>
<td>0.050</td>
<td>-</td>
<td>13.86</td>
<td>-</td>
<td>0.96</td>
</tr>
<tr>
<td>AldDH/MSU-F</td>
<td>0.025</td>
<td>-</td>
<td>27.73</td>
<td>2.0</td>
<td>0.98</td>
</tr>
<tr>
<td>AldDH/MSU-H</td>
<td>0.015</td>
<td>-</td>
<td>46.21</td>
<td>3.3</td>
<td>0.98</td>
</tr>
</tbody>
</table>
pH and T profile of AldDH derivates

- Activity variation between soluble and immobilized enzyme, with varying pH and T
- No variation of optimal pH
- Good increase in optimal T of AldDH/MSU-F derivates.
pH and T profile of ADH derivates

- Activity variation between soluble and immobilized enzyme, with varying pH and T
- There is a slight increase in optimal pH of ADH/MSU-H derivates.
- No variation of optimal T
1\textsuperscript{st} reaction step: very low conversion, the ATP cofactor is probably also needed.

2\textsuperscript{nd} reaction step: good result obtained with MSU-H; probably with MSU-F the enzyme occludes all the pores and the reagents cannot reach the enzyme site.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Conversion yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>AldDH/MSU-H</td>
<td>&lt;0.1%</td>
</tr>
<tr>
<td>AldDH/MSU-F</td>
<td>&lt;0.1%</td>
</tr>
<tr>
<td>ADH/MSU-H</td>
<td>14%</td>
</tr>
<tr>
<td>ADH/MSU-F</td>
<td>&lt;0.1%</td>
</tr>
</tbody>
</table>

Conditions:
- NADH 50mM;
- Substrate (propionic acid or propionaldehyde) 50mM;
- 30°C;
- pH 7.
Reusability test

- Residual activity is measured after several batch reaction.
- In both cases, residual activity greater than 20% is observed after 5 cycles.
Conclusions

- Good increase in enzymatic stability after immobilization;
- Residual activity of 20% after 5 batch reactions;
- Need to use another enzyme, more effective in the first reaction step (e.g. CAR enzyme);
- Need to implement cofactor regeneration processes.
Thanks for your attention!

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