POLITECNICO DI MILANO

The Protein Factory

Towards the production of an industrial amino acid oxidase: stabilization of a thermophilic L-aspartate oxidase

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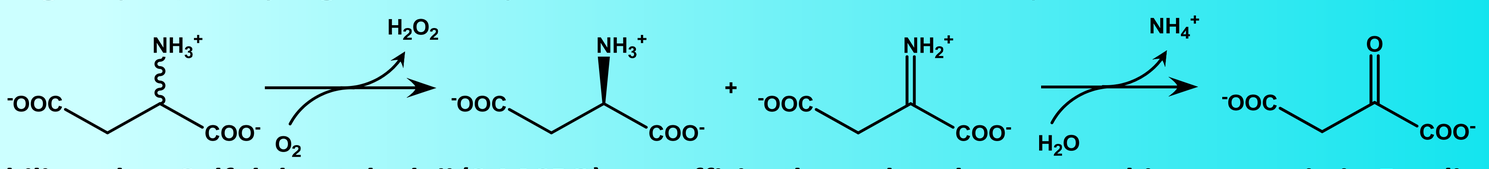
Introduction

L-Aspartate oxidase is a FAD containing enzyme, catalyzing the stereospecific oxidative deamination of L-aspartic acid to oxalacetate, ammonia and hydrogen peroxide.

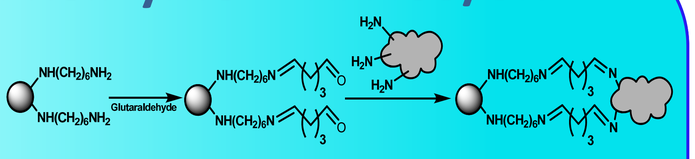


This flavoenzyme from the thermophilic archea *Sulfolobus tokodaii* (StLASPO) was efficiently produced as a recombinant protein in *E. coli* and has been deeply characterized.[1] StLASPO presents very distinctive features such as: high thermostability, stable activity over a broad range of pH values, low K_m for dioxygen and tight binding of the FAD cofactor, making it very attractive for biotechnological applications . Immobilization studies of this novel biocatalyst on different solid supports such as resins or in the form of cross-linked enzyme aggregates (CLEA) were performed.[2]

A recently developed approach is represented by the immobilization of a flavooxidase on magnetic Fe_3O_4 nanoparticles (NP@RgDAAO)[3], which now paves the way to the immobilization of StLASPO.

Immobilization on Relizyme™ HA403/SR

- 200 mg of resin HA 403/ S R
- 10 mL of 0.125% (v/v) glutaraldehyde solution in water
- 1 mL StLASPO solution (2 U)
- 0.1 M phosphate buffer, pH 7.5



Immobilization yield : 100 % (2 U)
The preparation showed an activity of 10 U/g wet matrix.

Best Result:

Resolution of 50 mM D,L-Aspartate at pH 10:

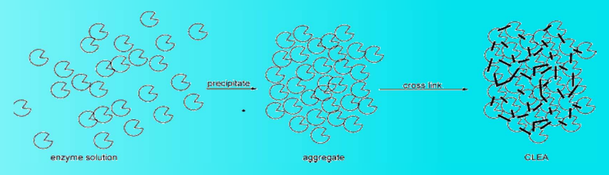
- 0.5 mL of D,L-Aspartate solution at pH 10
- 2 U of immobilized StLASPO
- 70 °C in a thermomixer (600 rpm)

Cycle number	Time (h)	e.e. (%)
1	1	> 99.5
2	1	> 99.5
3	1	> 99.5
4	1	87
	2	> 99.5
5	1	77
	2	> 99.5

Immobilization as CLEA

- 4.5 mL $(NH_4)_2SO_4$ precipitating solution are added to 0.5 mL of enzyme solution
- 0.4 % (v/v glutaraldehyde solution/total precipitation solution)

The mixture was incubated on a rotary shaker for 2.5 h at room temperature



Immobilization yield : 100%
The preparation showed an activity of 4 U/g wet matrix

Best Result:

Resolution of 50 mM D,L-Aspartate at pH 10:

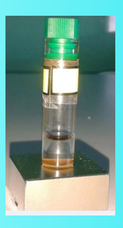
- 0.5 mL of D,L-Aspartate solution at pH 10
- CLEA-StLASPO preparation (1.1U)
- 70 °C in a thermomixer (600 rpm)

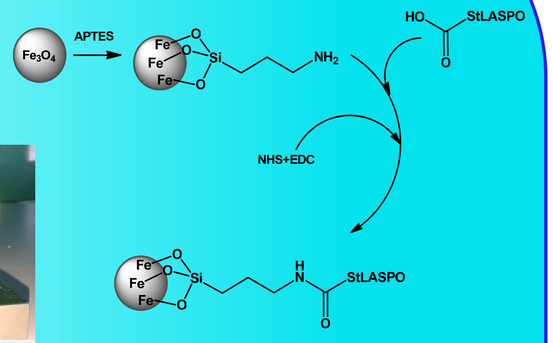
Cycle number	Time (h)	e.e. (%)
1	4	> 99.5
2	4	> 99.5
3	4	> 99.5
4	4	68
	6	> 99.5
5	4	69
	6	95

Immobilization on Fe_3O_4 NPs

- 150 mg of Fe_3O_4 NP
- 5 mL APTES (2% v/v) in H_2O left under stirring for 5 h at 50 °C. Fe_3O_4 NP-APTES were then separated from unbound APTES by using a magnet .
- 4 mg of Fe_3O_4 NP@APTES
- 2 mg of EDC
- 3 mg of NHS
- 0.037 U of StLASPO

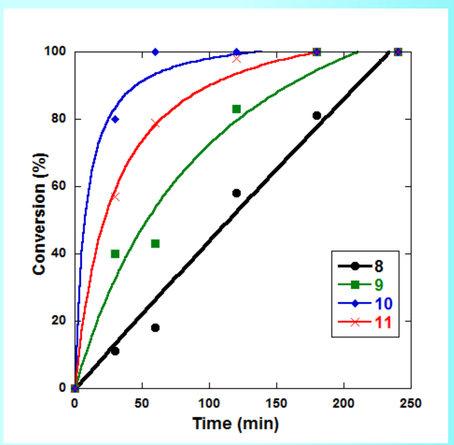
The reaction was carried out for 2 h at r.t.





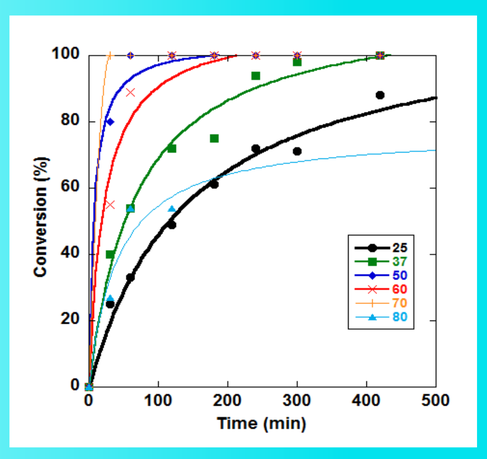
Immobilization yield: 95% (0.035 U)

Effect of pH



-Fe_3O_4NP@APTES-StLASPO (0.2699 U on 16 mg NP)
-100 µL of 50 mM L-Aspartate in water at different pH values (8, 9, 10, 11)
-100 µL of water (adjusted to the desired pH with NaOH 0.5 M)
-70 °C on a thermomixer (600 rpm)

Effect of temperature

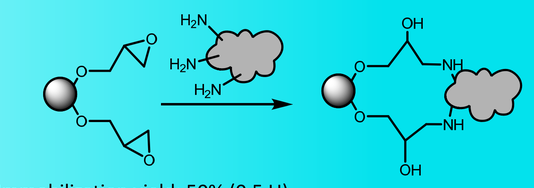


-Fe_3O_4NP@APTES-StLASPO (0.322 U on 16 mg NP)
-100 µL of a 50 mM L-Aspartate solution at at pH 10
-100 µL of water (adjusted at pH 10 with NaOH 0.5 M)
These reactions were conducted at different temperatures (25, 37, 50, 60, 70, 80 °C) on a thermomixer (600 rpm) .

Immobilization on SEPABEADS® EC-EP/S

- 0.5 mL (1 U) StLASPO
- 60 mg of SEPABEADS ® EC-EP/S
- 0.3 mL 1.25 M potassium phosphate buffer at pH 8

The mixture was incubated for 18 h at 25 C



Immobilization yield: 50% (0.5 U)
The preparation showed an activity of 8.3 U/g dry matrix.

Best Result:

Resolution of 50 mM D,L-Aspartate at pH 10 :

- 0.5 mL of D,L Aspartate solution at pH 10
- 0.5 U of immobilized StLASPO
- 70 °C in a thermomixer (600 rpm)

Cycle number	Time (h)	e.e. (%)
1	4	> 99.5
2	4	> 99.5
3	4	> 99.5
4	4	81
	6	> 99.5
5	4	45
	10	> 95

Conclusions

- **Resin HA 403/SR** presents a high yield of immobilization; moreover the resulting catalyst can be used for 5 cycles yielding complete conversion in < 2 hours.
- The **CLEA-StLASPO** preparation maintains its activity for 5 cycles, with complete conversion in < 8 hours.
- The immobilization of **StLASPO on magnetic NPs** presents very promising results in terms of stability and potential reusability of the biocatalyst. The recovery of the reaction products and of the immobilized enzyme NP@StLASPO is very simple. Indeed, the removal of the catalyst from the reaction medium can be easily performed with a magnet; this is a very attracting aspect helpful for the scaling up of the bioconversion.

[1] D. Bifulco, L. Pollegioni, D. Tessaro, S. Servi, G. Molla Appl. Microbiol. Biotechnol. 97 (2013) 7285-7295.
[2] P. D'Arrigo, C. Allegretti, A. Fiorati, L. Piubelli, E. Rosini, D. Tessaro, M. Valentino, L. Pollegioni Catal. Sci. Technol. 5 (2015) 1106-1114.
[3] A. Bava, R. Gornati, F. Cappellini, L. Caldinelli, L. Pollegioni, G. Bernardini Nanomed. (Lond.) 11 (2013) 1797-1806.

Acknowledgment: Resindion S.r.l. A Subsidiary of MITSUBISHI CHEMICAL

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