Enzyme aggregates for antibiotic degradation

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Introduction

Global annual losses of USD billions are reported in milk production due to mastitis. Antibiotics are used in dairy farms for the treatment and prevention of diseases in dairy cattle. There are national and international regulations that prohibit the processing of milk with antibiotics, so it is important to study alternatives to final disposal. Veterinary drug residues are considered a danger and potential risk for public health, dairy industrialization processes and the environment.

There are different techniques for the treatment of milk contaminated with antibiotics. Some of these techniques are very expensive and have low yields, others use very toxic reagents and generate toxic by-products for the environment. The use of enzymes, specially laccases, in biodegradation processes have many advantages as they are safer, less disruptive, less expensive, require lower energy employment, considered as a green catalysis processes.

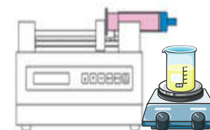
Enzyme immobilization is an enabling technology for application of enzyme in industrial biocatalytic processes. Particularly, cross linked enzyme aggregates (CLEAs) are facile and cost-effective to prepare; the general procedure consists of enzyme precipitation and subsequent cross-linking with a bifunctional agent. With this immobilization strategy we are capable to generate diverse biocatalysts. These can be reused in bioprocesses and allow continuous use, thus simplifying processes and reducing costs.

Objective

The objective of this work was to evaluate the potential of cross linked enzyme aggregates of laccase in the degradation of the model antibiotic Oxytetracycline

Metodology

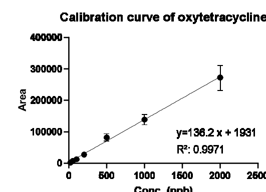
Preparation of CLEA's:
Acetone was dropped with a syringe pump to the mixture of laccase (0,3 mg/mL) and BSA (3 – 30 mg/mL) or trehalose (300 mM) at a ratio of 3:1 to precipitate proteins at 22 ºC for 30 min at 450 rpm under magnetic agitation. Glutaraldehyde was used as the cross-linking agent in 10 mM final concentration. After 16 h the mixture was centrifugated for 15 min at 13000 rpm. CLEA's were recovered and washed several times with 25 mM sodium phosphate buffer pH7.



Antibiotic degradation reaction:
500 ppb of Oxytetracycline (OTC) were incubated with CLEA's (0.3 UE/mL) in 25 mM sodium phosphate buffer pH7 and 22 ºC for 24 h with magnetic agitation (450 rpm). The final reaction volume was 2.8 mL.

HPLC Analysis:
Residual antibiotic concentrations were determined by employing a Shimadzu Nexera X2 (SIL-30AC) UHPLC with diode array detector (SPD-M30A). The analysis was carried out with LabSolutions software (Shimadzu) based on the calibration curve for oxytetracycline.

Cromatographic conditions: Column: ACQUITY UPLC BEH C18 Column, 130Å, 1.7 µm, 2.1 mm X 50 mm (Waters); Flow rate: 0.15 mL/min; λ: 365 nm; mobile phase: 10 mM ac. Oxálico : Metanol : Acetonitrilo (60 : 25 : 15); injection volume 20 µL



Thermal stability:
Thermal stability of CLEA's and soluble laccase were examined measuring the residual activity at 405 nm for 2 minutes using a spectrophotometer with kinetic mode. The assay was carried out at 60 °C in 25 mM sodium phosphate buffer pH7 using 9.5 mM ABTS as substrate. Residual activity was measured every 30 minutes

Results

CLEAs Preparation

Table 1. Study of the concentration of BSA added during CLEAs preparations.
Effect on the immobilization parameters

	CLEAs (BSA + Lac)	CLEAs (Trehalose + Lac)	CLEAs (Laccase only)
I%	86,110 ± 0,005	93,874 ± 3,995	95,467 ± 2,323
R%	24,680 ± 0,553	6,385 ± 1,172	6,512 ± 5,844

CLEAs. **BSA + Lac**: Laccase co-precipitated with BSA. **Trehalose + Lac**: Laccase co-precipitated with Trehalose.

Better results were obtained in the immobilization by co-precipitating laccase in the presence of BSA, obtaining similar values in I% and 4times more R% .

Table 2. Study of different additives during CLEAs preparation. Immobilization table
Effect on the immobilization parameters

	BSA 30 mg/mL	BSA 15 mg/mL	BSA 6 mg/mL	BSA 3 mg/ mL
I%	88,5 ± 3,5	93,0 ± 0,0	96,0 ± 2,8	98,0 ± 0,0
R%	26,0 ± 1,4	22 ± 2,8	20,0 ± 1,4	16,5 ± 10,6
Specific activity (UE/g)	12,0 ± 0,9	17,7 ± 0,8	25,9 ± 3,0	21,33 ± 1,2

The immobilized preparations showed similar I% and R%. The specific activities were also remained within the same order (Table 1). However, the best immobilized was generated using 6 mg/mL of BSA.

Thermal stability experiments

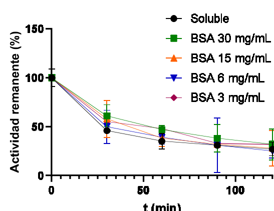


Figure 1. Thermal stability at 60 °C of different laccase preparations.
3 IU/mL in 25 mM sodium phosphate buffer pH7

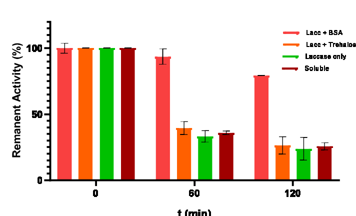


Figure 2. Thermal stability at 60 °C of different laccase preparations.
6 IU/mL in 25 mM sodium phosphate buffer pH7

No thermal stabilization was found using 3 IU/mL for the CLEAs preparation. However, thermo stabilization was obtained when using 6 IU/mL and 30 mg of BSA during laccase precipitation.
CLEAS with BSA were more stable than the other preparations, presenting 79% of its initial activity at 120 min, while the others retained 25% of its initial activity.

Antiobiotic degradation

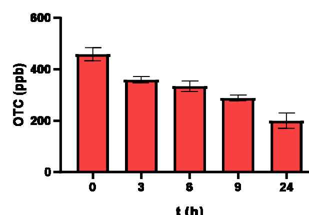


Figure 3. Degradation of OTC by CLEA
500 ppb of Oxytetracycline in 25 mM sodium phosphate buffer pH7 (Lacc +BSA 6mg/mL).

258 ppb was degraded in 24 hours, which means a degradation of 56% of the initial concentration of oxytetracycline was achieved.

Conclusions

It is concluded that **an active and stable biocatalyst of laccase capable of degrading the oxytetracycline was produced.** Our studies will focus in the future on the optimization of the degradation conditions of antibiotics and in depth characterization of the CLEAS, in order to increase their prospects for industrial implementation.

Aknowledgements

Laboratorio de Biotecnología de Universidad ORT, ANII (FSA_I_2017_1_138926, POS_FSA_2018_1_1008118) PEDECIBA