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Extraction and purification of hydrolytic enzymes from activated sludge

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ABSTRACT

A major proportion of the organic matter contained by domestic wastewater is mainly formed by lipids, proteins and carbohydrates. Hydrolytic enzymes like proteases and lipases are produced by microorganisms to degrade this organic matter. In the present study protease and lipase were extracted from activated sludge using ultrasound disintegration combined with a non ionic detergent. It was observed that the concentration of Triton X100 has a strong influence for the extraction of protease, while it has a negligible effect for the extraction of lipase. Samples obtained after ultrasound disintegration using 0% and 2% (v/v) Triton X100 were further purified by precipitation with ammonium sulphate and dialysis. The samples were frozen and lyophilised to recover them in powder form. Lipase activity was tested after all the purification steps, finding that the optimal process was a combination of ultrasound treatment (without any detergent) followed by dialysis and lyophilisation. This process allowed recovering up to 23 lipase units/g solid.

1. Introduction

The final disposal way of excess sludge generated by wastewater treatment is becoming a serious issue in Europe, mainly due to the growth of population and its accumulation in large cities, and the increment in the amount and complexity of the industrial activity. For this reason, it is necessary to reduce the amount of sludge generated attacking the problem in two ways: improving the efficiency of the treatment methodologies and implementing new technologies able to use sludge as raw material to generate biomolecules and energy. The recovery of valuable products from sludge that could be used in the sludge degradation itself or for other industrial applications is promising.

Organic matter in domestic wastewater consists of complex compounds, from which 60–70% is formed by lipids, proteins and polysaccharides. A huge fraction of this organic matter (30–85%) is formed by particles larger than 0.1 μm that cannot be directly assimilated by the microorganisms. These microorganisms degrade the organic matter by producing hydrolytic enzymes that are released into the media. However, it was demonstrated that the free enzymatic activity present in the liquid phase of activated sludge is almost negligible, being immobilised on flocs (connected to the polymeric extracellular substances) or attached to the cellular wall by ionic and hydrophobic interactions (Cadoret et al., 2002; Gessesse et al., 2003).

From the several types of enzymes that have been detected in activated sludge, the most common ones are aminopeptidases, dehydrogenases, galactosidases, glucosidases, lipases and phosphatases. Ultrasonication of activated sludge has been used previously to treat activated sludge by reducing its amount, achieving a better dewaterability, increasing the soluble chemical oxygen demand and destroying the flocs (Appels et al., 2008; Dewil et al., 2006a,b). Besides this, ultrasonication alone or combined with detergents or ion exchange resins was one of the methods allowing recovering these enzymes maintaining them active (Cadoret et al., 2002; Frolund et al., 1995, 1996; Gessesse et al., 2003; Jung et al., 2001, 2002; Yu et al., 2007, 2008a,b, 2009). The purification procedure employed to purify enzymes and proteins depends mainly on the required purity for a specific application, and on the cost of the overall process. The basic steps in any purification process normally include centrifugation, selective precipitation, dialysis and separation in column (Scopes, 1994).

Extracted enzymes from activated sludge have limited use until now. Some studies have been carried out to evaluate the influence of an enzymatic pretreatment step prior to the anaerobic digestion of domestic or industrial wastewater. The results showed that it was possible to remove solids, decrease the COD level and improve the biogas production during the anaerobic digestion. However, commercial enzymes (like lipases) have been used for almost all the cases (Barjenbruch and Kopplow, 2003; Cammarota et al., 2001;

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Table 1
Characteristics of the activated sludge collected from biological reactor.

Parameter	Activated sludge
TS (g/L)	1.5–2.7
VSS (g/L)	0.5–1.2
VSS/TS	0.4
Organic load (kg BOD_5/(kg TSS day)	0.6
Sludge age (days)	3.9–4.4

Jeganathan et al., 2007; Jordan and Mullen, 2007; Leal et al., 2006; Parmar et al., 2001; Roman et al., 2006), and just in some studies crude enzymes extracted from sludge were used to treat and improve the degradation of phthalate esters in wastewater (Zhang et al., 2006). Lipases are also the most common enzymes used industrially together with proteases, being applied in detergents, surfactants, oleochemistry, pulp and paper, dairy and agrochemistry, and also in cosmetics and pharmaceutics.

In previous studies, the extraction of protease and lipase by ultrasonication and stirring was optimized (Nabarlatz et al., 2008a,b, 2010). The most promising method for the recovery of both types of enzymes was ultrasonication using Triton X100 as additive (for the recovery of protease) and without Triton X100 (for the recovery of lipase). The present study evaluates the extraction of lipase and protease using ultrasonication (with and without Triton X100) followed by the purification of lipase. To this purpose, a preliminary purification procedure will be evaluated for the recovery of lipase to give us an idea of the difficulties of the overall process and the possibilities of further applications in the industry, where a certain degree of purity is required.

2. Materials and methods

2.1. Sludge collection and handling

The activated sludge was collected from the biological reactor (aerated basin) of the Wastewater Treatment Plant in Reus which processes almost 20,000 m^3 of domestic wastewater per day (the city has 105,000 inhabitants). The samples of activated sludge were taken and transported to the laboratory in 30 min. All the disintegration experiments were carried out on the same day, preserving the sludge under aeration at room temperature. The sludge was used as received, and it was analysed in order to determine the total solids content (TS) and volatile suspended solids content (VSS) according to standard methods (APHA, 1999). Table 1 shows the characteristics of the activated sludge used in this study.

2.2. Ultrasonic disintegration of activated sludge (UD)

The ultrasonic disintegration experiments were carried out using an ultrasonic disintegrator UP200S (Hielscher Ultrasonics GmbH, Germany). The experiments were carried out at 24 kHz working frequency, 3.9 W/cm^2 ultrasonic power intensity and 30 min of disintegration time (in duplicate). The ultrasonic power intensity was calculated as the ultrasonic power supplied to the liquid (W) divided by the submerged area of the sonotrode in the liquid (cm^2). In all the experiments the temperature was maintained constant at $5 \pm 1\,^{\circ}C$ using a water–ice bath to control it. The sludge volume used in each test was 200 mL using 400 mL vessels. The extraction experiments were carried out using sludge alone or combined with a non-ionic detergent called Triton X100 (TX100, Sigma–Aldrich) at concentrations from 0.1% (v/v) to 2% (v/v). The conditions were chosen taking into account preliminary experiments for the recovery of protease and lipase (Nabarlatz et al., 2008a,b, 2010). Samples of sludge were taken before and after the disintegration process and were centrifuged for 10 min at 10,100 × *g* prior to the analysis. The supernatant was used as the source of enzyme for the determination of enzymatic activity and the amount of protein.

2.3. Purification of the enzymes

In order to purify the fraction of enzymes present in the supernatant recovered after the centrifugation step, several purification procedures like dialysis, precipitation with ammonium sulphate and lyophilisation were tested.

2.3.1. Dialysis

For the dialysis process (D), a 12–14 kDa dialysis tubular membrane (Medicell International) was used. The membrane was cut to the desired volume, soaked in deionised water for 1 h and filled with the enzyme solution (25 mL). The membrane was placed in a container having deionised water as solvent (100 mL) stirred at 500 rpm. The equipment was placed in a water bath which was maintained at $5 \pm 1\,^{\circ}C$. To control the progress of the dialysis, the conductivity of the diffusate was measured over the time. The solvent was changed at least 3 times during 24 h, and the process was repeated until the conductivity remained constant and close to that of the deionised water. The samples obtained after dialysis were stored at 4 °C in the fridge for analysis or frozen (−20 °C) for lyophilisation.

2.3.2. Precipitation with ammonium sulphate

The precipitation with ammonium sulphate process (P) was carried out by adding slowly 25 mL of 100% saturated (761 g/L) ammonium sulphate solution to 50 mL of enzyme solution (253.6 g/L, 41.6% saturated solution) as described elsewhere (Scopes, 1994). The mixture was placed in a water bath at $5 \pm 1\,^{\circ}C$ under stirring (500 rpm). After 24 h the mixture was centrifuged for 10 min at 10,100 × *g* and the supernatant discarded. The solids (pellet) were redissolved in 4 mL of 50 mM potassium phosphate buffer (pH 7.5 at 37 °C). The samples were stored at 4 °C in the fridge for analysis or frozen (−20 °C) for lyophilisation.

The purification factor for each purification process (D and P) is calculated dividing the specific enzyme activity (lipase units/mg protein) obtained after the separation step by the specific enzyme activity of the crude extract before purification.

2.3.3. Lyophilisation

The samples after the disintegration, precipitation and dialysis were lyophilised (L) until dryness using an Armfield Lyophilisation Equipment (purchased from Prodel S.A., Spain) equipped with a high vacuum pump JAVAC (Brook Crompton Betts Pty.). The conditions used were a vacuum pressure of 0.5 mbar and a temperature of −40 °C.

2.4. Determination of the amount of protein and enzymatic activity

The enzymes identified and quantified were lipase and protease. The methods for determining the enzymatic activity were based on standard protocols from Sigma–Aldrich. A UV-VIS DINKO spectrophotometer (purchased from DINTER S.A., Spain) was used for all the experiments.

2.4.1. Lipase activity

The lipase activity was determined using *p*-nitrophenyl butyrate as substrate. This method measures the release of *p*-nitrophenol by continuous spectrophotometric rate determination. For these experiments, 0.2 mL of enzyme solution were placed in a suitable cuvette, adding 1.8 mL of a buffer solution (100 mM NaH_2PO_4 + 150 mM NaCl + 0.5%, v/v, TX100, pH 7.2 at 37 °C). The