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Valorizing recycled paper sludge by a bioethanol production process with cellulase recycling

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HIGHLIGHTS

- The recycling of cellulases on the hydrolysis of recycled paper sludge was assessed.
- Cellulases and yeast cells showed good performances towards the substrate.
- Both cellulase fractions were shown to be easily recovered with good efficiencies.
- The hydrolysis of RPS was successfully conducted over 4 cycles.
- Cellulase recycling enabled enzyme savings between 53 and 60%.

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ABSTRACT

The feasibility of cellulase recycling in the scope of bioethanol production from recycled paper sludge (RPS), an inexpensive byproduct with around 39% of carbohydrates, is analyzed. RPS was easily converted and fermented by enzymes and cells, respectively. Final enzyme partition between solid and liquid phases was investigated, the solid-bound enzymes being efficiently recovered by alkaline washing. RPS hydrolysis and fermentation was conducted over four rounds, recycling the cellulases present in both fractions. A great overall enzyme stability was observed: 71, 64 and 100% of the initial Cel7A, Cel7B and β-glucosidase activities, respectively, were recovered. Even with only 30% of fresh enzymes added on the subsequent rounds, solid conversions of 92, 83 and 71% were achieved for the round 2, 3 and 4, respectively. This strategy enabled an enzyme saving around 53–60%, while can equally contribute to a 40% reduction in RPS disposal costs.

1. Introduction

The economic feasibility of second-generation bioethanol relies on two major cost factors: the substrate and the enzymes. The identification of a cheaper, abundant and easily hydrolysable material has assumed a critical role for a more economic production of fermentable sugars. Recently, an increased utilization of different kinds of residues came as an interesting alternative to the traditional lignocellulosic substrates, enabling a considerable reduction on substrate cost, and also an additional valorization for some of these otherwise useless materials.

Recycled paper sludge (RPS) is a residue originated from the paper recycling process, more specifically, from the treatment of the liquid effluents generated in that process. It is mostly composed of small fibers with approximately 40% of carbohydrates that cannot anymore be incorporated on recycled paper (Marques et al., 2008a). Also, due to the chemical contamination, namely with ink particles, this residue has high environmental impact being usually disposed on landfills, which represents a considerable expenditure for these companies. Considering an approximate production of this waste around 300 kg per ton of recycled paper (Balwaik and Raut, 2011) and taking into account an estimated 47 millions tons of recycled paper produced only in Europe by the year of 2005 (Monte et al., 2009), this corresponds to around 14 million tons of RPS that need to be discarded. In spite of the notable potential of this material, coupled with a high worldwide availability, only few studies have been conducted so far exploring its further valorization (Presetyo and Park, 2013). Some examples refer to Lark et al. (1997) who have studied RPS hydrolysis and subsequent fermentation to ethanol by *Kluyveromyces marxianus*. Also Marques et al. studied its potential for bio-ethanol production by *Pichia stipitis* (Marques et al., 2008a) and lactic acid production by *Lactobacillus rhamnosus* ATCC 7469 (Marques et al., 2008b).

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In addition to the substrate cost, the cost of the enzymes required to hydrolyze lignocellulosic materials (cellulases and/or hemicellulases) represents one of the biggest obstacles for their economically viable conversion, due the competition from the less expensive fossil fuels. Great debate has been established concerning the exact cost of cellulases, with distinct values being pointed out by different authors. Klein-Marcusschamer et al. (2012) estimated a cost on ethanol production around $ 0.68 per gallon, close to $ 0.5 per gallon recently suggested by Novozymes (http://novozymes.com/en/news/news-archive/Pages/45713.aspx). However, Aden and Foust (2009) have also already reported a value around $ 0.1 per gallon, close to $ 0.3 reported by Lynd et al. (2008) and $ 0.32 reported by Dutta et al. (2010). Independently of the exact figure, it is consensually recognized that the enzymes cost is a major determinant of the cellulosic ethanol competitiveness, driving in the last years intense efforts to reduce the loading employed in the process. The reduction of the cost associated to enzymes has been commonly pursued following three main strategies: increasing the efficiency of enzymes; reducing enzymes production cost; and reutilizing the enzymes (Pribowo et al., 2012). Over the last years (even decades), most of the attention has been given to the first two strategies, through intense and constant research operated by both industry (e.g. Novozymes; DSM; Genencor) and academia. Through a close collaboration with Novozymes and Genencor, NREL (USA) conducted a joint project that resulted in a reduction of cellulase cost up to 10-fold (http://www.nrel.gov/docs/fy13osti/59013.pdf). Nevertheless, some authors have already admitted that such strategies will not allow pushing down cellulases cost much further. In this context, the recovery (and posterior reutilization) of cellulases has recently emerged as a very promising concept, as using enzymes multiple times will allow a natural reduction on its consumption.

Numerous studies have been conducted for some years now in what concerns the mechanisms of enzyme adsorption/desorption (Lindedam et al., 2013; Pribowo et al., 2012; Rodrigues et al., 2014; Tu et al., 2007), addressing the complexity associated to different enzymes and substrates. In a similar way, possible strategies to facilitate and/or conduct the recovery of these enzymes have already been individually studied. According to Gomes et al. (2015), enzymes remaining in the liquid fraction are usually recovered either by ultrafiltration or by addition to fresh substrate (and posterior separation), while solid-bound enzymes normally require a change of pH or the addition of specific chemical compounds (that interfere with solid-enzyme interaction). Nevertheless, very few studies were conducted so far presenting an integrated approach of such strategies to the hydrolysis of a specific lignocellulosic material over multiple rounds.

Here we conduct an overall study regarding the feasibility of using RPS as substrate for 2G-bioethanol production in a system of multiple rounds of hydrolysis with cellulase recycling. The conservation of enzymatic activity and its final partition between solid and liquid fractions is initially accessed followed by an evaluation regarding the recovery efficiency of solid-bound enzymes. Afterwards, a process with multiple rounds of hydrolysis and enzymes recycling was implemented, monitoring the activity levels and the degree of solids conversion over the entire process.

2. Material and methods

2.1. Enzymes and substrate

Enzymatic hydrolysis were conducted through the combined action of the commercial cocktail Celluclast (Sigma-Aldrich, C2730), complemented with the commercial β-glucosidase preparation Novozyme 188 (Novozymes). The activities of these preparations were determined to be 45 FPU/mL and 611 IU/mL, respectively.

The recycled paper sludge (RPS) was kindly provided by RENOVA (Torres Novas, Portugal). This refers to a solid (with approx. 53% (w/v) water) obtained from the wastewater treatment of paper recycling effluents generated by this company. This material contains high carbonates content, which results on an alkaline solid. Similarly to Marques et al. (2008a), prior to its utilization RPS material was treated with hydrochloric acid 37% and then washed, first with water and then with buffer (0.1 M acetic acid/sodium acetate). This process rendered a neutralized RPS (nRPS), which was used in all tests of the current work.

2.2. Hydrolysis and fermentation

Enzymatic hydrolysis of *n*RPS material were conducted under a standardized system with variable times and temperatures according to the purpose of each study. After RPS neutralization (and washing), the wet neutralized solid (with approx. 85% (w/v) water) was resuspended in 0.1 M acetic acid/sodium acetate buffer (pH 4.8) to a consistency of 5% (w/v) (dry weight basis). After sterilization and cooling to room temperature, enzymes were added on a small volume of the abovementioned buffer, being filter-sterilized (sterile Polyethersulfone (PES) syringe filters; 0.22 μm) into the mixture of solids. Unless otherwise stated, enzymes were added in a dosage of 20 FPU/g cellulose of Celluclast and with a β-glucosidase/Celluclast activity ratio of 5. This ratio was defined aiming to attenuate any limitation of β-glucosidase activity in order to ensure that cellulase action would be the limiting element. Solid suspension was then incubated at 200 rpm on an orbital shaker at variable times and temperatures (35/50 °C).

When a further fermentation was conducted, this mixture was inoculated with cells of *Saccharomyces cerevisiae* PE-2 strain (Basso et al., 2008; Pereira et al., 2014; collected on the beginning of the stationary phase) and the temperature reduced to 30 °C. After harvested from the culture medium, yeast cells were resuspended on ice-cold 0.9% (w/v) NaCl and then added to the solids suspension in a ration of 8 g/L (fresh biomass). Periodic sampling was conducted accordingly with the purpose of each study. A minimum of 2 independent replicates was always conducted for every test of this work.

2.3. Recovery of solid-bound enzymes

Enzymes adsorbed to the solid were recovered by a process of alkaline elution, as described previously by Rodrigues et al. (2012, 2014). Briefly, the final hydrolysate was centrifuged for 15 min at 2710*g*, after which the supernatant was collected (discarded or stored). The harvested solid was resuspended on an equal volume of freshly prepared 0.1 M Tris-HCl buffer (pH 9–10) and mixed for 2 h on a turning wheel (Rotator SB3-Stuart) at room temperature. At the end, the solids mixture was once again centrifuged and the supernatant (containing the eluted enzymes) collected and stored accordingly (at 4 °C) for future use.

2.4. Multiple rounds of hydrolysis with enzyme recycling

Enzymatic hydrolysis in the context of cellulase recycling were conducted on a similar way comparatively to the single-round experiments. Some modifications were however introduced concerning the solid preparation as described below.

For the first round, hydrolysis was performed according to the common procedure employed so far. The solids suspension (5% (w/v)) was mixed with 20 FPU/g cellulose of Celluclast