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Conversion of recycled paper sludge to ethanol by SHF and SSF using *Pichia stipitis*

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ABSTRACT

The purpose of the present work was to evaluate the possibility of converting recycled paper sludge (RPS), an industrial residue stream with strong environmental impact, into valuable products. The approach used was based on the enzymatic conversion of major sludge components (cellulose and xylan) and the simultaneous (simultaneous saccharification and fermentation—SSF) or sequential (separate hydrolysis and fermentation—SHF) fermentation of the resulting sugars to ethanol. In the enzymatic hydrolysis step using Celluclast® 1.5 L supplemented with Novozym® 188, a degree of saccharification of 100% was achieved. In relation to ethanol production using the yeast *Pichia stipitis* CBS 5773, SHF and SSF process efficiencies were compared. A slightly higher conversion yield was attained on SHF, corresponding to an ethanol concentration of 19.6 g L^{-1}, but 179 h were needed. The SSF process was completed after 48 h of incubation allowing the production of 18.6 g L^{-1} of ethanol from 178.6 g L^{-1} of dried RPS, corresponding to an overall conversion yield of 51% of the available carbohydrates on the initial substrate. These results demonstrate that the biological conversion of sludge to ethanol is efficient even with no pre-treatment or substrate supplementation.

1. Introduction

New difficulties emerging from the large increase in paper recycling rates impose urgent solutions. Wastewater sludge from paper recycling mills contains high levels of heavy metals from waste paper inks. Thus it cannot be used for land application as soil amendment and has to be disposed off to landfills, a prohibitively expensive and environmentally harmful end solution [1,2]. Interest has therefore grown in finding novel value-added uses for this residue from the paper industry [3–5].

Recycled paper sludge (RPS) is basically made up of secondary poor-quality non-recyclable paper fibres (fibres too short to be retained on fibre screens and paper machines). The high lignocellulosic content of this sludge material offers therefore an opportunity as feedstock for bio-products [6]. Moreover, paper sludge is believed to be one of the most promising feedstock for near-term commercial application of technology for converting cellulosic raw materials into commodity products [7]. In fact, this substrate has some distinctive advantages among cellulosic feedstocks including negative cost at many locations and the potential availability of pre-existing facilities [8,9].

The feasibility of biotechnological recovery of this potentially attractive substrate requires the conversion of all of its major components (cellulose and hemicellulose) to fermentable sugars, which could be further converted to fuels and chemicals, such as ethanol, organic acids or biodegradable plastics [10]. Among these possible products, ethanol possesses a rapidly expanding market, either as an octane enhancer or primary fuel reducing the harmful effects of gasoline consumption [11]. Thereby, the purpose of the

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present study was the conversion of RPS into ethanol using *Pichia stipitis*. This yeast has been described to possess the ability to convert both glucose and xylose into ethanol [12], an essential aspect for the economic feasibility of the production process. The proposed biological approach will bring the potential reduction of the residue volume with the consequent reduction on the cost of ultimate disposal of the remaining lignin/inorganic fraction, together with the upgrading of the carbohydrate component of RPS.

Hydrolysis of lignocellulosic materials to soluble products can be carried out by chemical (mild acid/alkali) or enzymatic action. Enzymatic saccharification is advantageous when compared to chemical methods, since it is by nature a more specific and cleaner process. It also allows milder operation conditions, leading to reduced formation of biological inhibitory compounds (such as sugar- and lignin-degradation products) and the catalyst is potentially reusable [13]. In fact, because of the higher ethanol yield and lower by-product formation, economic evaluation of a future full-scale plant for production of ethanol from various lignocellulosic raw materials is frequently based on enzymatic hydrolysis instead of conventional acid hydrolysis [14]. Since sludge has already been subject to an extensive mechanical and chemical processing previously imposed on the paper raw material through pulping (during refining, bleaching and drying), polysaccharides in RPS should be much more amenable to enzymatic hydrolysis, as already noted by several authors [8,9,15–17]. Therefore, this substrate might require no pretreatment, commonly performed to overcome lignocellulosics recalcitrancy [18].

The two-step process can be run as separate hydrolysis and fermentation (SHF) or as simultaneous saccharification and fermentation (SSF) [19]. SSF has been regarded as the major option because it usually results in higher overall yields and shorter residence times along with the process integration achieved (both steps performed in one reactor) [20]. In the present work, both approaches (SHF and SSF) for RPS conversion to ethanol were implemented and compared for process efficiency in terms of product yield and production rate and total residence time.

2. Materials and methods

2.1. Substrate

The present study used pressed RPS consisting of the solids resulting from the wastewater treatment facility of a local paper recycling mill (Renova, Torres Novas, Portugal). The as-received sludge contained calcium carbonate that rendered the resulting suspensions alkaline. Therefore, RPS was neutralised with hydrochloric acid (0.3 g HCl g^{-1} RPS) prior to use and it was chemically characterised.

RPS was analysed gravimetrically for water (by oven drying at 105 °C to constant weight) and ash (by igniting at 575 °C for 5 h) contents. Protein content was estimated by the Kjeldahl method [21] using a nitrogen-to-protein conversion factor of 6.25. Fat was determined by extraction with petroleum ether using conventional Soxhlet glassware and gravimetric extract analysis. Hemicellulose, cellulose and lignin contents were assayed by means of a quantitative hydrolysis with sulphuric acid according to the method described by Browning [22]. The quantification of the monosaccharides was carried out by high-performance liquid chromatography (Section 2.2). The acid-insoluble residue was considered as Klason lignin, after correction for the acid-insoluble ash.

The composition of RPS was determined to be (on a dry weight basis): 34.1% cellulose, 29.3% ash, 20.4% Klason lignin, 7.9% xylan, 4.8% protein and 3.5% fat.

2.2. Analytical methods

The commercial enzyme preparations applied were previously characterised for catalytic activity at the conditions used for the enzymatic saccharification of sludge.

Endo-β-1,4-xylanase (1,4-β-D-xylan xylanhydrolase; EC 3.2.1.8) activity was assayed using 1% (w/v) oat spelts xylan (Sigma, St. Louis, USA) as substrate. Filter paper activity (FPase), describing the cellulolytic activity, was assayed using Whatman number 1 filter paper as substrate. Enzyme activities were expressed in international units (U) as the amount of enzyme required to release 1 μmol per minute of either xylose (endo-β-1,4-xylanase) or glucose (FPase) reducing equivalent under the assay conditions. Reducing sugars were estimated by the dinitrosalycilic acid method [23].

Ethanol, sugars and sugar-degradation products were measured by HPLC using a Waters LC1 module 1 plus (Millford, LA) equipped with a two-serial differential refractive index/ultraviolet detector, the latter being set at a fixed wavelength of 280 nm (for hydroxymethylfurfural and furfural). An Aminex HPX-87 H column (Bio-Rad, Hercules, CA, USA) was used, operating at 50 °C with 5 mM H_2SO_4 as mobile phase at a flow rate of 0.4 mL min^{-1}.

2.3. Enzymatic hydrolysis trials

A sample of RPS was suspended in 0.05 M sodium citrate buffer, pH 5.5, for an initial consistency of 3% or 7.5% (w/v), expressed in terms of total carbohydrate mass, and it was steam sterilised by autoclaving (at 121 °C, 1 atm, for 15 min). Sludge on 3% (w/v) suspension consistency was incubated with the filter-sterilised enzyme solution at 50 or 35 °C in an orbital shaker (150 rev min^{-1}) for 144 or 72 h, respectively. Sludge on 7.5% (w/v) consistency was incubated at 35 °C during 120 h. Aseptic conditions were maintained throughout the experiments.

Enzymatic hydrolysis was performed with a previously selected mixture of two commercial enzyme preparations (cellulolytic and xylanolytic, from Novozymes, Denmark): Celluclast® 1.5 L (exhibiting an FPase activity of 14.7 U mL^{-1} and an endo-β-1,4-xylanase activity of 228.7 U mL^{-1}) and Novozym® 188 (exhibiting an FPase activity of 0.6 U mL^{-1} and an endo-β-1,4-xylanase activity of 854.9 U mL^{-1}). This mixture was applied at different enzyme loadings containing: Celluclast® 1.5 L on a dosage of 120 (for 3% consistency at 50 °C), 25 (for 3% consistency at 35 °C) or 10 (for 7.5% consistency) U (FPase) g^{-1} carbohydrate plus 0 or 1.0 (for 3% consistency) or 0.4 (for 7.5% consistency) mL of Novozym® 188 g^{-1} carbohydrate on sludge. For each enzyme tested, a