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Research article

Use of solid digestate for lignocellulolytic enzymes production through submerged fungal fermentation

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ABSTRACT

Studies were performed on the use of the solid fraction of digestate (D) for the production of lignocellulolytic enzymes (endo- and exo-glucanase, xylanase, β-glucosidase and laccase) by fungi, in comparison with wheat straw (benchmark) (W). To date, this is the first report on the use of such an inexpensive substrate in a liquid environment. Submerged instead of solid state fermentation was applied to overcome pH inhibition and increase surface accessibility. A total of 21 fungal strains were tested: the most performing ones were *Irpex lacteus* DSM1183 for both β-glucosidase (52 IU/g with D, + 400% compared to W) and endo-glucanase (236 IU/g with D, + 470% compared to W), *Schizophyllum commune* CBS30132 for xylanase (715 IU/g with W, + 145% compared to D) and *Pleurotus ostreatus* ATCC96997 for laccase (124 IU/g with D, +230% compared to D).

Cultures from *S. commune* and *P. ostreatus* were analyzed at the beginning and at the end of the growth test to determine soluble COD, total (TS) and volatile (VS) solids. COD was always lower at the end of the test suggesting a faster uptake than hydrolysis. *P. ostreatus* evidenced a higher VS reduction (−11% rather than −32%), suggesting a more effective growth of this strain on D.

Results may open up new avenues for the utilization of solid digestate, an inexpensive agricultural by-product, for the production of value-added products as well as to increase biodegradation of lignocellulosic materials.

1. Introduction

Anaerobic digestion (AD) is a biological process that converts organic substrates into biogas and digestate, this last being considered a byproduct produced in large amount. Digestate contains microbial biomass and residues of those materials that could not be gasified within the biogas plant. Digestate is generally separated by mechanical processes, such as centrifuging and screw pressing, into a liquid and a solid fraction (Monlau et al., 2015). The solid fraction, rich in residual fibers and phosphorous, is valorised as soil improver (Risberg, 2015). Nonetheless, its oligoelements and recalcitrant natural organics (humic and fulvic acids) content makes the solid fraction a potential culturing substrate to produce value-added products of microbial origin (Kuhad et al., 2016).

In this context, the production of microbial lignocellulosic enzymes has received a growing attention due to their vast industrial applications (Yadav and Yadav, 2015). Enzymes involved in the degradation of lignocelluloses, i.e. xylanase (EC 3.2.1.8), cellulases and ligninases, are applied as alternatives or in combination to chemical treatments (Lopez et al., 2007; Rollini et al., 2014). Cellulases is a family of at least 3 groups of enzymes, endo-(1,4)-β-D-glucanase (EC 3.2.1.4) exo-(1,4)-β-D-glucanase (EC 3.2.1.91), and β-glucosidases (EC 3.2.1.21) (Zhang et al., 2006). The exo-glucanase acts on the ends of the cellulose chain and releases β-cellobiose; endo-glucanase acts in the amorphous areas of cellulose on the internal *O*-glycosidic bonds and generates new reducing and non-reducing glucan chains of different lengths; β-glycosidase acts on β-cellobiose or cello-oligosaccharides producing glucose. Both exo-glucanases and β-glucosidases are inhibited by their own reaction products, namely cellobiose and glucose, respectively (Kuhad et al., 2011). Ligninolytic enzymes, such as laccase (EC 1.10.3.2), are also of interest in bio-bleaching and bio-pulping processes (Senthivelan et al., 2016). Laccases have also been demonstrated to favor micropollutants removal in wastewaters, opening up interesting

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perspective on the use of these enzymes for bioremediation (Margot et al., 2013). An interesting alternative could therefore be to grow fungi on digestate in order to produce lignocellulosic enzymes (Fernandez et al., 2004; Isikhuemhen et al., 2009; Santi et al., 2015a) while favoring the accessibility of residual polysaccharides inside the solid fraction of digestate, thus enhancing the anaerobic degradability of this residue and potentially increasing the final biogas yield of the biogas plant (Kuhad et al., 2011; Sambusiti et al., 2015; Santi et al., 2015b).

To date, the use of digestate as substrate for microbial processes has always been performed by solid state fermentations (SSF), in which microbial growth and consequent product formation are carried out within a solid medium having a reduced water activity (Santi et al., 2015a; Singh et al., 2010).

Isikhuemhen et al. (2009) reported that a maximum of 6.4 U/g of laccase, 15.7 U/g endo-glucanase, 11.4 U/g exo-glucanase, 22.9 U/g β-glucosidase and 14.87 U/g xylanase were obtained by growing *Agrocybe aegerita* on the solid fraction of digestate from anaerobic digestion of poultry manure. Moreover, by supplying the solid fraction of digestate to the culturing medium (up to 20% w/w) higher endo-glucanase and xylanase production was obtained. Four white-rot fungal strains (*Agrocybe aegerita* SMR 206, *Pleurotus ostreatus* SMR 684, *Pleurotus columbinus* SMR 688 and *Pleurotus eryngii* SMR 151) were screened for their ability to grow on solid digestate samples, using wheat straw (WS) as reference substrate (Santi et al., 2015a). The best results were obtained by employing *P. ostreatus*, with endo-glucanase and xylanase activities higher on solid digestate than on WS (2.5 *vs.* 1 IU/mg protein and 3 *vs.* 1.5 IU/mg protein, respectively).

The use of digestate in submerged fermentation (SmF) presents several advantages over SSF. Indeed, SSF processes still lack of a proper scale-up as for purification of the end products and biomass estimation (Farinas, 2015). Since digestate is characterized by a relatively high pH level (7.5–8.1), which can represent a limiting factor in fungal cultures, typically set-up in acidic environment (Risberg, 2015), its dilution coupled with the buffering effects of a SmF can overcome pH inhibition. Moreover, the enhanced turbulence improves substrate accessibility.

In the present study, the use of the solid fraction of digestate (D) as substrate in SmF trials for the production of several enzymatic activities is reported, employing wheat straw (W) as benchmark substrate. A total of 21 fungal strains were screened belonging either to white- and brown-rot fungi, focusing on endo- and exo-glucanase, xylanase, β-glycosidase and laccase production levels. To the best of our knowledge, this is the first report on the use of such an inexpensive substrate in a liquid environment: even if SSF is undergoing an extensive development to improve operating conditions, SmF systems still represents the current dominant biotechnology for industrial fermentation processes.

2. Materials and methods

2.1. Microorganisms and maintenance

Table 1 lists all the 21 fungal strains used in the research. Excluding *Psylocybe subcubensis* (brown-rot), they are all classified as white-rot fungi and belong to the internal collection of the DeFENS; some of them were deposited in official collections and the corresponding code is also given. Strains were inoculated in 5 cm plates containing PDA (potato dextrose agar) culture medium (PDB, Formedium, England, supplemented with 15 g/L agar), by depositing on the surface a quarter (around 5 cm^2) of an older (max 2 months) solid culture plate, taken off with a sterile scalpel. Plates were then incubated at 25 °C in the dark. When the mycelium had covered at least 70% of the solid culture, plates were tightly closed with Parafilm and then stored for a maximum of 2 months at 4 °C until use.

Table 1
Chemical composition of the solid digestate (D) and of wheat straw (W).

Parameter	D	W
TS (% w/w)	20	94
VS (g/kgVS)	800	897
Cellulose (g/kgVS)	440 ± 6	523 ± 7
Hemicelluloses (g/kgVS)	117 ± 4	247 ± 8
Insoluble lignin (g/kgVS)	288 ± 10	93 ± 3

2.2. Origin of solid digestate and wheat straw

Digestate originated from a full scale anaerobic digester producing 1 MW_{el} located in a cattle farm in Northern Italy (Lombardy Region, province of Cremona) breeding up to 1050 cows. The anaerobic digester is made of two digesters in parallel (2100 m^3 each) and two post-fermenters in series (2700 m^3 each) and it is fed on a mixture of cow manure (27%VS), cheese whey (15%VS), poultry manure (23%VS), olive pomace (2%VS) and corn silage (33%VS). The solid separated fraction of digestate (D) came from the solid/liquid separation facility (screw press) available on site.

Wheat straw (*Aubusson*) (W) was sampled from a farm near Cremona (Lombardy Region, Italy). After collection, samples were oven dried at 60 °C for 2 days to a moisture content of less than 10%, ground by a kitchen blender (average size 3–10 mm), and finally stored at ambient temperature in air-tight containers prior to use.

2.3. Submerged fermentation trials

Fungi grown on PDA solid culture were first inoculated in a liquid pre-culture prepared in 500 mL Erlenmeyer flasks containing 100 mL of modified Reyes liquid medium, of the following composition (g/L): glucose (Duchefa, Haarlem, the Netherlands) 20, soybean peptone (Costantino, Favria, Italy) 5, K_2HPO_4 1, KCl 0.5, yeast extract (Costantino) 2, $MgSO_4$ 0.5, microelement solution 1 mL, distilled water, pH 5.8. Microelement solution (g/L): $Na_2BO_7 \times 10\ H_2O$ 0.1, $ZnSO_4 \times 7\ H_2O$ 0.07, $FeSO_4 \times 7\ H_2O$ 0.05, $CuSO_4 \times 5\ H_2O$ 0.01, $MnSO_4 \times 4\ H_2O$ 0.01, $(NH_4)_6Mo_7O_{24} \times 4\ H_2O$ 0.01, distilled water. After sterilization for 30 min at 112 °C, each flask was inoculated with 5 cm^2 of a PDA solid culture and then incubated at 25 °C on a rotary shaker (150 rpm) in the dark for at least 5 days to obtain a visible growth (presence of pellets).

Pre-cultures grown on glucose were then employed as inoculum (10% v/v) of liquid media in which glucose was replaced with D or W. Substrates were directly added to each flask (20 gTS/L) and pH set at 6.0 before sterilization. After inoculation, flasks were incubated on a rotary shaker (150 rpm) for 7 days at 25 °C in the dark. Mycelia and residual substrate (solid fractions) were then separated from culture supernatants by filtration onto cotton cloth, and supernatants analyzed to quantify the enzymatic activities.

2.4. Determination of enzymatic activities

Endo-glucanase (CMCase) was determined by measuring the amount of glucose released from carboxymethylcellulose (CMC) using the Somogyi-Nelson method (Somogyi, 1952) with glucose as standard. For this purpose, an aliquot of diluted sample (0.5 mL) was mixed with 0.5 mL of a CMC suspension (Serva, Heidelberg, Germany) (10 g/L) in citrate buffer (0.05 M, pH 5). Reaction mixtures were incubated at 55 °C for 30 min, and then boiled to stop the enzymatic activity. Sugars release was then determined with glucose as standard.