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Production of a generic microbial feedstock for lignocellulose biorefineries through sequential bioprocessing

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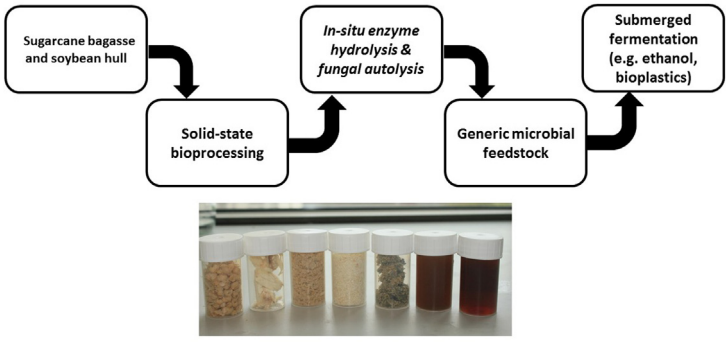
HIGHLIGHTS

- Sugarcane bagasse and soybean hulls make excellent solid state co-substrates.
- Fungal pre-treatment deconstructs ligno-cellulosic material and produces enzymes.
- Simultaneous *in-situ* enzyme hydrolysis and fungal autolysis produce nutrient medium.
- Ethanol from bagasse is possible without the need for external enzymes or chemicals.

GRAPHICAL ABSTRACT



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ABSTRACT

Lignocellulosic materials, mostly from agricultural and forestry residues, provide a potential renewable resource for sustainable biorefineries. Reducing sugars can be produced only after a pre-treatment stage, which normally involves chemicals but can be biological. In this case, two steps are usually necessary: solid-state cultivation of fungi for deconstruction, followed by enzymatic hydrolysis using cellulolytic enzymes. In this research, the utilisation of solid-state bioprocessing using the fungus *Trichoderma longibrachiatum* was implemented as a simultaneous microbial pretreatment and *in-situ* enzyme production method for fungal autolysis and further enzyme hydrolysis of fermented solids. Suspending the fermented solids in water at 50 °C led to the highest hydrolysis yields of 226 mg/g reducing sugar and 7.7 mg/g free amino nitrogen (FAN). The resultant feedstock was shown to be suitable for the production of various products including ethanol.

1. Introduction

Biorefining is the sustainable processing of biomass into a spectrum of marketable products and energy (FitzPatrick et al., 2010). Among several sources of biomass residues that can be employed in energy generation, sugarcane bagasse is one of the most used in the world. Sugarcane bagasse is the residue produced by cane sugar mills after juice is extracted from the cane. It is a fibrous lignocellulosic material, which is easily combusted. Most of this bagasse, 75%, is used as fuel for power generation or as raw material for low-value products such as mulch or ceiling tiles. The remaining 25% is considered as solid waste and is dumped to landfill (Dawson and Boopathy, 2008). Similarly, Soybean hull, the main by-product of the soybean processing industry, is also a lignocellulosic material but containing only a small proportion of lignin when compared to sugarcane bagasse (Gnanasambandam and Proctor, 1999; Hickert et al., 2014). Due to their low cost and relatively large abundance, sugarcane bagasse and soybean hull are

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potential alternative sources to satisfy the demands of biorefinery development.

Lignocellulosic materials could be used as more accessible fermentation feedstocks after an effective pretreatment process. An efficient pretreatment must set free the highly crystalline structure of cellulose and extend the amorphous areas for enzyme digestion. Based on these above requirements there are different techniques that are well-established for lignocellulose pretreatment including; (1) Physical; (2) physico-chemical; (3) chemical and (4) microbial.

Microbial pretreatment has recently received attention as an alternative to the prevalent physicochemical pretreatment processes due to its potential advantages of lower environmental impact, process simplification and reduced energy (Shi et al., 2008). The filamentous fungi from *Trichoderma*, *Aspergillus*, *Phlebia* and *Pleurotus* genera are some of the microorganisms that have been used to deconstruct ligno-cellulosic materials directly (Shi et al., 2008; Wang et al., 2002). However, no microorganism has the capacity to degrade cellulose, hemicellulose and lignin directly and efficiently into metabolites of interest.

Webb and Wang (1997) developed a process based on submerged fungal bioconversion for the production of a nutrient-rich fermentation feedstock from wheat. The process minimised the number of conversion steps, avoiding unnecessary separation, using *in-situ* enzymes and preventing loss of nutrients. At the Satake Centre for Grain Process Engineering (SCGPE), researchers have published a wide range of biorefining strategies based on fungal fermentation for fuels, platform chemicals, and biodegradable plastics production. Cereal crops or rapeseed meal were enzymatically converted into a generic fermentation feedstock, enriched in amino acids, peptides and various micro-nutrients, using *in-situ* crude enzymes produced via solid state cultivation of fungal strains such as *Aspergillus oryzae* and *Aspergillus awamori*. The studies showed that on-site fungal fermentations could provide not only enzymes for the hydrolysis of starch, cellulose or hemicellulose, but also a nutrient-rich solution from the further hydrolysis of fermented residues (Du et al., 2008; Koutinas et al., 2004, 2007; Wang et al., 2010).

The residues of solid-state cultivation contain large amounts of fungal cells, enzymes and undigested polymers. These can be broken down by endogenous (autolysis) and exogenous (hydrolysis) enzymes to generate nutrient-rich solutions under suitable conditions. Not only *Aspergillus* species could be employed in this nutrient generation process, but also other fungi such as *Mucor indicus* and *Grifola frondosa* (Asachi and Karimi, 2013; Xu et al., 2012).

It is possible that a similar concept to that used by Webb and Wang (1997) could be employed in lignocellulose-based processes, producing not only sugars but also nutrients (amino acids, peptides, nucleotides, phosphorous and vitamins). As well as reducing the overall number of process steps, the result would be a richer feedstock than is obtained using current industrial practices for sugar production through physico-chemical pretreatment followed by enzymatic saccharification.

The work presented in this paper is aimed at the simultaneous *in-situ* deconstruction of lignocellulosic substrates and production of enzymes by solid state bioprocessing (SSB). Application of autolysis and further hydrolysis of whole fermented solids, containing the *in-situ* enzymes and mycelia, for the production of generic microbial feedstocks is also investigated. The novel process concept is represented schematically in Fig. 1.

In this process, a bed of the solid lignocellulosic raw material is inoculated with a filamentous fungus, which grows throughout and excretes the hydrolytic enzymes necessary to degrade the substrate. The secreted enzymes attach to the surface of the substrate (cellulose, hemicellulose) as the mycelium penetrates into bundles of lignocellulosic fibres during the solid state cultivation. Following the SSB, the fermented materials, along with their associated fungal mycelia, are transferred directly to the hydrolysis step where, unlike with the use commercial enzymes, the required enzymes are already absorbed into the substrate. In the example given in this paper, the fungus *Trichoderma longibrachiatum* is used to degrade mixtures of sugarcane bagasse and soybean hull, by simultaneously secreting enzymes and breaking down recalcitrant lignocellulose. This consolidated fungal cultivation not only eliminates the expensive pretreatment stage but also provides abundant enzymes (cellulase, beta-glucosidase, xylanase etc.) for further hydrolysis.

2. Materials and methods

2.1. Microorganisms

Trichoderma longibrachiatum DSMZ 16517, obtained from the German Collection of Microorganisms and Cell Cultures (Germany). Prior to experimental work, *T. longibrachiatum* spores were purified and incubated in petri dishes for 5 days, containing 20 g/L dextrose, 4 g/L potato extract and 15 g/L agar (Sigma, UK) at natural pH was used to purify, maintain and proliferate the strain. Inoculum size to all fermentations was controlled at about one million spores to each gram of rapeseed meal on a dry basis (db). The resultant spores were suspended in 10% (v/v) glycerol solution and stored in 2 ml cryo-vials at −30 °C.

Yeast fermentations were carried out using *Saccharomyces cerevisiae* ATCC 22602 for the production of ethanol. Yeast cells were cultivated in standard yeast extract dextrose xylose (YDX) medium (5 g/L yeast extract, 10 g/L xylose, and 5 g/L glucose) for inoculum preparation.

2.2. Raw materials

Sugarcane bagasse was kindly provided by Dr. R.F. Chang, Taiwan, packaged in vacuum-packed bags to maintain freshness. To adjust the different particle sizes for experiments, sugarcane bagasse was ground using a kitchen blender then passed through sieves (1.4–0.85 mm) and the fractions were stored in air-tight plastic containers. Soybean hulls were kindly supplied by Cargill Plc, Liverpool, United Kingdom. These were also ground using a kitchen blender then passed through a 2 mm sieve to remove other debris and stored in an air-tight plastic container. The ground sugarcane bagasse and soybean hulls were stored in a cold room (4 °C) until use.

2.3. Solid-state cultivation by T. longibrachiatum

The impact of the mixed-substrate on microbial feedstock production was examined. Sugarcane bagasse (SB) was mixed with soybean hull (SH) in a 250 ml Duran bottle to adjust the SB:SH ratios to 1:0, 8:2, 6:4, 5:5, 2:8 0:1 (w/w), respectively. Mineral salt solution ($CaCl_2{\cdot}2H_2O$: 0.5 g/L; KH_2PO_4: 3 g/L; $MgSO_4$: 0.5 g/L) was added to improve the fungal growth. The final moisture content was 65% on a wet basis (wb). The Duran bottles were sterilised at 121 °C for 30 min. SSB experiments were commenced by adding spore suspension (1×10^6 spore/g of dry substrate) into each Duran bottle. A sterilised spatula was used to mix the mash in the Duran bottle to enable distribution of the inoculum. Around 5 g (wb) of inoculated substrates were distributed to each Petri dish and then incubated in a static incubator at 30 °C for up to 7 days.

In the environmental humidity experiments, the relative humidity of the incubator was regulated by placing an open container filled with sterilised water at the bottom of the incubator.