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Solid-State Fermentation using a Strain of *Trichoderma asperellum* Improves the Saccharification of Rice Straw

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Abstract

Rice straw is a cheap and widely available agro-waste biomass that can be used to generate renewable biofuel. However, due to its composition, it is particularly recalcitrant to enzymatic degradation. Here, prior to enzymatic hydrolysis, biological pre-treatment of rice straw for saccharification by solid-state fermentation (SSF) was performed using a new isolate of *Trichoderma asperellum* called UNIPV1. More specifically, the study aimed to investigate the effect of adding the fungus to rice straw on the temporal activity of secreted enzymes, and reducing sugar formation. As excepted, under long-term solid-state fermentation (SSF), depolymerizing enzymes such as xylanases and exo/endo-cellulases were secreted by *Trichoderma asperellum* UNIPV1 during its growth. *T. asperellum* was an excellent producer of cellulolytic enzymes; a significant peak in reducing sugars occurred between 10-20 days. *Trichoderma* initially showed a high preference for the secretion of xylanases to degrade the linear polysaccharide beta-1,4-xylan into xylose. Afterwards, endoglucanase and exoglucanase enzymes were secreted to complete the hydrolytic activity on the substrate. This result is consistent with the trend of total protein accumulated in the secretome and the fungal metabolic activity measured by CO_2 production. Overall, our findings suggest that a short fungal pre-treatment of rice straw might be useful to begin the degradation of cell-wall polymers, and can therefore effectively improve saccharification. That said, further work is required to improve the fungal pre-treatment, since alone does not entirely complete the degradation of lignocellulosic insoluble material.

Keywords: rice straw, solid-state fermentation (SSF), *Trichoderma*, saccharification, cell-wall degrading enzymes.

Резюме

Оризовата слама е евтина и широко достъпна отпадъчна биомаса от селското стопанство, която може да се използва за генериране на възобновяеми биогорива. Поради състава си, обаче, тя е трудно податлива на ензимно разграждане. В настоящото изследване, преди ензимната хидролиза на оризовата слама се извършва предварителна биологична обработка с цел озахаряване в условията на твърдо фазова ферментация (ТФФ), за която се използва новоизолиран щам от *Trichoderma asperellum*, наречен UNIPV1. По-конкретно, изследването има за цел да проучи ефекта от добавянето на гъбата към оризовата слама върху активност на секретираните ензими и образуването на редуциращи захари. Както се очаква, при дълговременна ТФФ, деполимеризиращите ензими като ксиланази и екзо/ендоцелулази се секретират от *T. asperellum* UNIPV1 по време на растеж. *T. asperellum* е високоефективен продуцент на целулолитични ензими, а пик на количеството редуциращи захари настъпва между 10 и 20 ден. В началото на процеса, новият щам Trichoderma показва ускорена секрецията на ксиланази, за да разгради линейния полизахарид β-1,4-ксилан в ксилоза. След това се секретират ендо- и екзо-глюканазните ензими, които завършват хидролитичното действие върху субстрата. Този резултат е в съответствие с установената тенденция на нат-

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рупване на общия белтък в секретомата, както и с метаболитна активност на щама, измерена чрез количеството на CO_2. В заключение, установените от нас резултати предполагат, че кратката предварителна обработка на оризовата слама с гъбния щам може да бъде полезна за започване разграждането на полимерите в клетъчната стена и следователно може ефективно да подобри озахаряването. Въпреки това, необходимо е по-нататъшно оптимизиране на предварително третиране с гъби, тъй като този процес не може да осъществи пълно разграждане на неразтворимите лигноцелулозни материали.

Introduction

Lignocellulosic biomasses such as wheat bran, sugarcane bagasse, corncob, and rice straw constitute an extremely abundant and inexpensive global resource. Via the degradation of their cellulosic material by microbial conversion, a wide variety of value-added products can be derived (Elisashvili *et al*., 2009), including renewable fuels, chemical feedstocks, and raw material for chemical reactions in an environmentally friendly fashion. Rice straw is a particularly cheap and underutilized agro-waste biomass. For instance, Italy is the largest European rice producer with 1.5 million tons annually, but only approximately 30% of the resultant rice straw is used in industrial processes (Ministry of Agriculture, Food and Forestry of Italy, 2014).

Such underutilization of agro-wastes is largely due to certain persistent practical problems. In particular, the lignocellulosic biomass in its natural form is a tough feedstock for hydrolysis due to the crystallinity of cellulose, presence of hemicellulose, and lignin in the plant material (Hu *et al*., 2013). Biomass pre-treatment processes are therefore necessary to reduce the paracrystalline cellulose structure and to make cellulose more available to the enzymes by removing hemicellulose and lignin (Cai *et al*., 2008). A number of different pre-treatment methods have been extensively investigated, including steam explosion (Nakamura *et al*., 2001), organosolv extraction (Pan *et al*., 2005), subcritical and supercritical water treatment (Schmieder *et al*., 1999; Yoshida *et al*., 2004), biological pre-treatment with white-rot fungi (Hatakka *et al*., 1983; Itoh *et al*., 2003), as well as various physical and thermomechanical processes (Chahal *et al*., 1999). However, biological pre-treatments excepted, most approaches have expensive energy requirements, albeit with some variability depending on the complexity (Wingren *et al*., 2004). Presently, the efficient depolymerization of lignocellulosic using biological pre-treatment is considered to be a safe and environmentally friendly method of breaking down the lignin and overcoming the resistance of cellulose to hydrolytic cleavage. To enhance susceptibility to enzymatic hydrolysis and thus digestibility, various pre-treatments have been exploited white-rot fungi over the years, mainly considering fungi belonging to *Basidiomycota*.

Amongst the many promising micro-organisms used for biological pre-treatment, *Trichoderma*, a genus of filamentous fungi belonging to the phylum *Ascomycota* (Druzhinina *et al*., 2016) has been extensively studied due to its diverse physiological characteristics and biotechnological applications. Within the species, *Trichoderma asperellum* stands out as a particularly versatile organism with remarkable degrading abilities (Zafra and Cortes-Espinosa, 2015). This fungus is commonly found in soils worldwide, and it is characterized by rapid growth and high rate of sporulation (Eveleigh, 1999). Interestingly, the secretome of *T. asperellum* comprises a wide range of potential useful enzymes, such as cellulases, pectinases, chitinases, and laminarinases (Eveleigh, 1999). In particular, cellulases (exo/endo-glucanases and xylanases) are a family of enzymes that act at several stages on the cellulose: firstly, through endocellulases cutting randomly inside the macromolecule, in order to generate a new end of the polysaccharide chain; secondly, these ends are subjected to the action of exocellulases that make disaccharides available. Ultimately, beta-glucosidases act on these products releasing glucose. Most fungi prefer to use xylanases to degrade hemicellulose and consequently make cellulose more accessible to the enzymatic action of cellulases (Longoni *et al*., 2015).

Another characteristic of the *Trichoderma* species is that, thanks to its hyperparasitic nature, it is well-known for its antagonistic action against pathogenic fungi, as well as for its potential use in the production of antibiotic substances and enzymes (Thrane *et al*., 1997; Dengkolb *et al*., 2003; Osbourn, 2010). For this reason, considerable attention has been devoted to the use of *Trichoderma* as biological control agents of plant diseases, with many commercial formulations available on the market. Therefore, its environmental release is not expected to pose any hazard.

Processing rice straw waste presents a particular challenge. Of its total dry weight, 40% is given by cellulose, 18% by hemicellulose, and

5.5% lignin (Takagi, 1987). This constitution, along with high silica content, makes it particularly recalcitrant to degradation. In light of the high enzymatic activity of the *T. asperellum* secretome, the aim of this paper is to evaluate how solid-state fermentation (SSF) using this fungus could contribute to the pre-treatment of rice straw with a view to its subsequent usage for biogas production by anaerobic biodigestion. Three classes of enzymes were considered: i) xylanases (EC 3.2.1.8 Endo-1,4-β-xylanase), which catalyzes the hydrolysis of β-1,4 bonds of xylan backbone in xylose thus breaking down hemicellulose, one of the major components of plant cell walls (Rasala, 2012; Gupta, 2000); ii) endoglucanases (EC 3.2.1.4), which break the intra-chain beta bonds of cellulose and belongs to the family of Hydrolases (Adlakha, 2011); and iii) exoglucanases (EC 3.2.1.91), which hydrolyze 1,4-beta-D-glucosidic bonds releasing cellobiose from both ends of the glucan chain.

Materials and Methods

Fungal strain

A strain of *T. asperellum* UNIPV1 was isolated as a hyperparasite of a *Fusarium* strain growing on a freshly harvested sample of rice seeds that were collected in northern Italy in March 2013. This strain was selected on account of its heavy growth on cellulosic materials, seeds, and agro-residues (demonstrated by previous experiments in our laboratories). The fungus was maintained in pure culture on Malt Extract Agar (MEA, Oxoid, UK) in slant tubes and stored under mineral oil at room temperature (25°C) in the mycological collection of Pavia University. It was assigned the code *T. asperellum* UNIPV1. Its optimum temperature for growth was detected at 25°C. The taxonomical identification was based on morpho-dimensional analysis following dichotomous key reported by Samuels *et al*. (1999) and Bisset (1991a, 1991b). The molecular characterization was performed by amplifying and analysing the sequences of Internal Transcribed spacer gene 1 and 2 (ITS) and translation elongation factor 1-alpha encoding gene (tef1). Identifications were made using the BLAST interface in TrichOKEY and TrichoBLAST sequence of the gene encoding for Elongation Factor-1α (eEF1α) (Druzhinina *et al*., 2005; Kopchinskiy *et al*., 2005). The nucleotide alignment of the obtained eEF1a sequence in NCBI (National Center for Biotechnology Information), Mycobank and TrichoMARK (Trichoderma genus-specific database) confirmed that the strain UNIPV1 belongs to *T. asperellum*.

Rice straw biomass

Rice straw collected in the Lombardy region (northern Italy) was air-dried and stored at room temperature for 3-6 months before starting the experimental phase. Subsequently, it was milled and sub-samples of 30 g were put in polypropylene gas-permeable bags with filters (SacO2, Microsacs, Belgium) to allow gas exchange. They were then wetted with 30 mL of sterile distilled water. Before proceeding with the experiment, the sub-samples of rice straw were pre-treated with a strong sterilization in autoclave at 120°C for 50 minutes, which was repeated after 24 hours. The materials were then stored at room temperature.

Inoculum preparation and solid-state fermentation (SSF)

UNIPV1 was grown on Potato-dextrose-agar (PDA; Oxoid) medium in sterile Petri dishes for 10 days in a dark growth chamber at 25°C. A suspension of actively growing mycelium was prepared to initiate fermentation. A mother suspension was obtained by gently excising 10 disks of 10 mm diameter of a 10-day-old colony and putting them in a sterile flask with 100 mL of agar gel prepared with sterilized water and 0.02% of agar. The suspension was shaken on a magnetic stirrer for 10 min. A volume of 30 mL of the suspension was then added to each bag containing sterile rice straw. Subsequently, for the SSF process, the plastic bags were sealed and kept in the dark growth chamber at 25°C for 0, 10, 15, 20 and 45 days. Three replicates were performed for each time condition, and three untreated samples, constituted by rice straw with 30 mL of sterile distilled water without the fungus, represented the control. All the experimental procedures were carried out under the flow cabinet to maintain aseptic conditions.

Biomass pre-treatment and enzymatic activity assay

Cellulolytic enzymes, i.e. xylanases and exo/endo-cellulases, derived from the secretome of *T. asperellum* UNIPV1 were obtained during its growth on rice straw under SSF. A sub-sample of 2.5 g from each treated sample (i.e. rice straw colonized by UNIPV1), was put in a 100 mL flask and subjected to 15 mL of extraction buffer (100 mM sodium acetate buffer, pH 5 – ratio 1:1), kept for 24 hours at 4ºC in an orbital shaker at 180 rpm. The entire volume of liquid derived by the rice straw pre-treatment (13-14 mL) was collected in 50 mL falcons and subjected to a double centrifugation for 20 min at 4ºC at 4,000 rpm. The supernatant of each falcon was collected, filtered (pore size 0.2 µm), and stored at -20ºC. The enzymatic activity assay was

performed on both untreated and treated rice straw. It was carried out in PCR tubes in a final volume of 180 µL, using 10 µL of total enzymatic extract derived from the filtered broths of the UNIPV1 secretome. Three different substrates were utilized to check the different enzymatic activities: 1% xylan from beechwood (w/w) (Sigma-Aldrich, Germany) for xylanase activity, 1% carboxymethyl-cellulose (CMC) (Panreac, Spain), for endoglucanase activity, and 4% microcrystalline cellulose (MCC) (Acros Organics, USA) for exoglucanase activity. The analyses were performed in triplicate. To determine enzymatic activity, aliquots of 10 µL of total enzymatic extract were added to 10 µL of synthetic substrate and diluted in a final volume of 70 µL sodium acetate buffer (100 mM, pH 5). Afterwards, reactions were incubated for 60 min at 55ºC in a thermal cycler (Eppendorf). At regular intervals (10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60 mins), reactions were performed by analyzing samples to evaluate the optimum reaction time between enzyme and substrate. Sterilized water was used as a control. After incubation, samples were boiled for 5 min to inactivate the enzymes. The enzymatic activity was measured by adding an equal volume (90 µL) of 3,5-dinitrosalicylic acid (DNS) reagent to the samples and then boiling the mixture at 95ºC for 5 min in a thermal cycler, following the method described by Ghose (1987). The reactions were then transferred into a 1 mL cuvette for spectrophotometric analyses, adding 180 µl of water. Absorbance of the samples was read at 540 nm (corresponding to absorbance peak of reacted DNS) and 700 nm (the absorbance peak of the sample turbidity). The net absorbance of each sample was calculated by subtracting the absorbance value at 700 nm from that at 540 nm. Using a calibration curve developed with known dilutions of glucose, absorbance values were converted to mg/mL glucose equivalents. Enzymatic activity was expressed in U/mL, where one Unit is the amount of enzyme that releases 1 µmol of reducing sugar equivalent per minute from the substrate under the assay conditions.

Saccharification and protein quantification

Saccharification experiments were required to determine the hydrolytic ability of enzymes produced by UNIPV1 growing on lignocellulosic agro-wastes. The method proposed by Jeya *et al*. (2009) was followed. Tests to evaluate the degradation of rice straw were carried out at different times of incubation with the fungus, respectively at 0, 10, 15, 20 and 45 days, both on treated (with UNIPV1) and untreated rice straw. Time courses were followed by two different assays: the DNS assay (Ghose, 1987) to evaluate the reducing sugar ends, whereas samples from three biological replicates were analyzed in triplicate, and the oxygraphic method specific for glucose. In parallel, proteins were quantified using Bicinchoninic acid (BCA) from protein assay kit (Sigma-Aldrich), followed by spectrophotometrical analysis at λ = 562 nm.

Fungal metabolic activity measured by CO_2 production

In order to monitor the metabolic activity of UNIPV1 growing on rice straw, CO_2 production was evaluated during the course of the experiments. The CO_2 flux (µmol m^{-2} s^{-1}) was measured with portable CO_2 analyser (ADCPro-sd with sample chamber). This device consists primarily of a dual-channel, non-dispersive infrared (NDIR) sensor (TelaireT6615) (General Electric Company, Billerica, MA, USA), an aspirator pump (FM1001; Chengdu, China), and a display screen. The aspirator pump pulls air from the sample through silicone pipes (2×4 mm) to the sensor; the maximum gas flow through the CO_2 sensor was 50 mL min^{-1}. After 3 min, the CO_2 concentration was recorded. Throughout, the data were directly collected from the plastic bags containing rice straw and the fungus growing on it and compared to the control.

Fiber content analysis

In order to evaluate the effect of SSF on the mobilization of recalcitrant polymers such as cellulose and lignin, a gravimetric analysis at different times following fungal addition was performed. After 10, 15, 20, and 45 days, the resulting pre-treated rice straw substrate was placed in a 70°C dryer overnight to prevent the growth of spores. Subsequently, the dried substrate was transferred into pre-weighed crucibles for fiber content analysis, and solvent extraction was used to separate the soluble from insoluble fiber according to the procedures proposed by Official Methods of analysis of AOAC International, 16th Edition, vol. II 1995.

Results and Discussion

Isolation and identification of fungal isolate T. asperellum UNIPV1

Morpho-dimensional and biomolecular analysis confirmed that the fungal strain UNIPV1 belongs to *T. asperellum* (Samuel *et al*., 1999), a cosmopolitan soil borne species with teleomorph in *Hypocrea* (*Fungi*, *Dicarya*, *Ascomycota*, *Pezi-*

zomycotina, *Sordariomycetes*, *Hypocreomycetidae*, *Hypocreales*, *Hypocreaceae*). *T. asperellum* is known to be an outstanding environmental opportunist with behaviour ranging from saprotrophy to biotrophy. It is also able to antagonize other fungi by a necrotrophic hyperparasitism. Therefore, this species is utilized as a biological control agent against a wide spectrum of plant-pathogenic fungi and oomycetes (Tondje *et al*., 2007; Mbarga *et al*., 2012; El_Komy *et al*., 2015). *T. asperellum* has also been particularly investigated for its ability to stimulate the plants defence reactions against plant pathogens (Segarra *et al*. 2007; Viterbo *et al*., 2010; Yoshioka *et al*., 2011; Herrera-Télle *et al*., 2019). Beside their biotrophic nature, species of *Trichoderma* are particularly fast decomposers of cellulose rich substrates. This feature can be exploited for many industrial perspectives such as the production of biofuels. Preliminary experiments in our laboratories (Picco A. M., unpublished data) suggested that the strain UNIPV1 may strongly degrade rice straw. Based on this, we decided to specifically investigate its possible utility to improve saccharification of rice straw in biofuel industry.

Secretome analysis

The time course of xylanase and cellulase secretion was analyzed during SSF over a period of 45 days. The results of this analysis, which are plotted in Fig. 1, show that xylanases were initially rapidly produced, reaching a peak at day 15 (25 000 U/g IDS, Initial Dry Substrate), decreasing thereafter to a very low level by day 45.

Endoglucanase and exoglucanase activities were assessed, respectively, using CMC and MCC substrates. Endoglucanase showed a specific activity of 2 600 U/g IDS after 10 days due to fungal addition which remained constant until day 20, declining thereafter. On the contrary, exoglucanases slowly increased until day 20 (2 000 U/g IDS), reaching a specific activity of 12 000 U/g IDS at day 45, possibly due to further process oligosaccharides. A similar trend was observed in the accumulation of *Trichoderma* exoglucanases in batch fermentation by Elshafei *et al*. (2014). The pattern of enzymatic activity clearly reveals that the fungus uses xylanases first, probably to facilitate the access of cellulases to cellulose. The trend observed in our experiments is consistent with that reported previously in relation to *Chaetomium globosum*, whereby xylanases were the first secreted enzymes to act upon poplar wood, followed by the action of endo- and exo-cellulases (Longoni *et al*. 2015).

The kinetic of the total soluble protein release in the secretome, meanwhile, is reported in Fig. 2. The maximum protein concentration was observed at day 20 and decreased thereafter. The pattern of total protein secretion is in accordance with cell-wall degrading enzyme activities. The release of proteins observed in the untreated sample is probably due to maceration.

Respiratory activity during SSF

The pattern of enzyme secretion was also consistent with the fungal respiratory activity as assessed by CO_2 evolution during the SSF of rice straw. As shown in Fig. 3, the peak of CO_2 occurred between 10 and 20 days, before levelling off at a slightly lower plateau value.

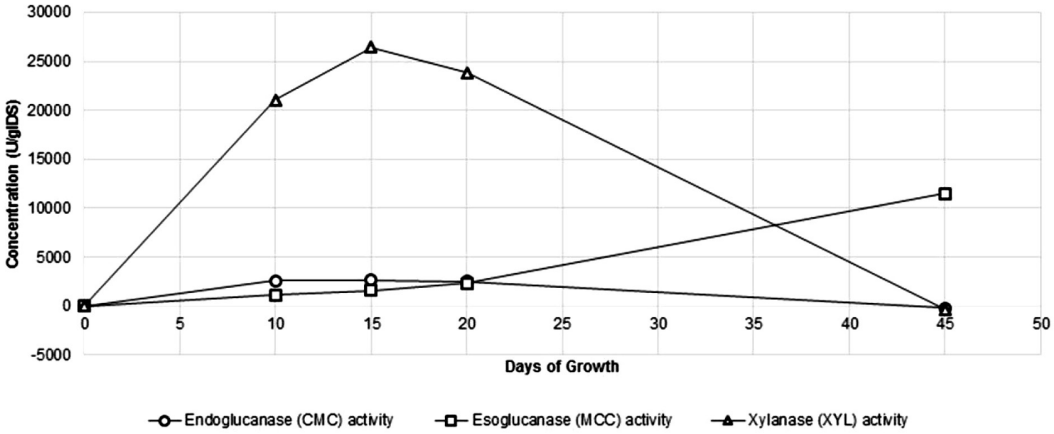

Fig. 1. Time course of enzymatic secretion of *T. asperellum* UNIPV1 grown on milled rice straw

Reducing sugar production during SSF

Figure 4 illustrates the time course of reducing sugar production, assayed by DNS, following the SSF of rice straw by *T. asperellum*. Total reducing sugars produced by the saccharification of rice straw polymers reached a peak between 10 and 15 days from the beginning of SSF. Between day 20 and the end of the experiment, these values decreased from 0.3 mg/mL to 0.1 mg/mL. Con-

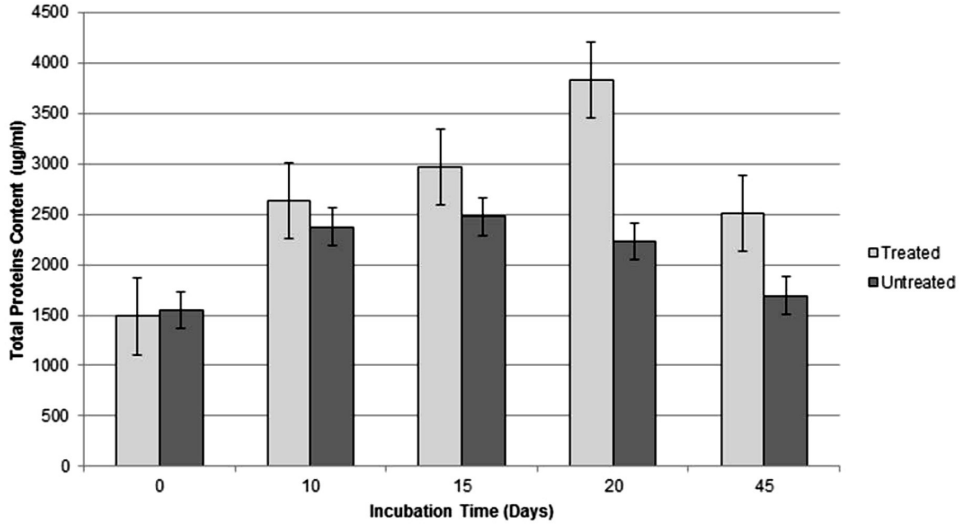

Fig. 2. Time course of total protein accumulation in the fungal secretome during SSF of milled rice straw

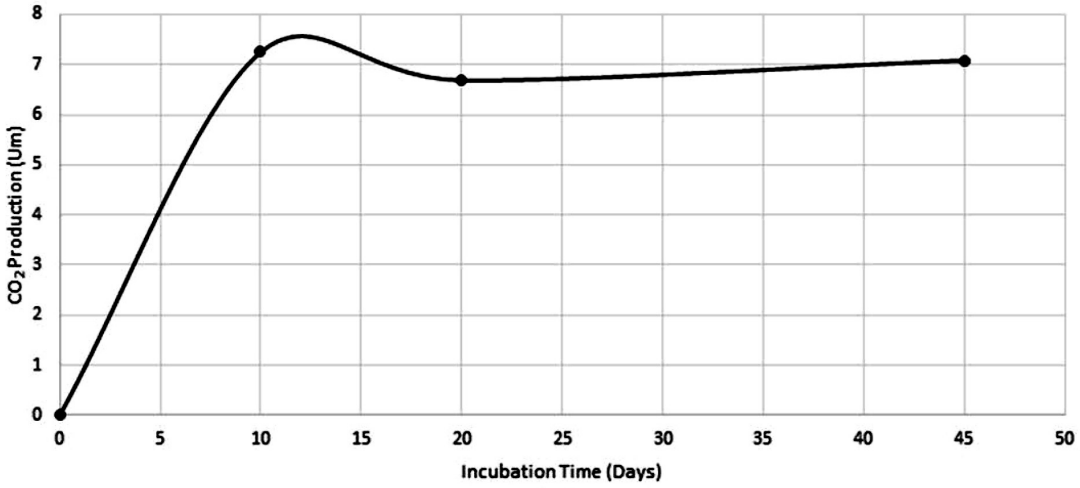

Fig. 3. Rate of CO_2 evolution during SSF of *T. asperellum* UNIPV1 on milled rice straw

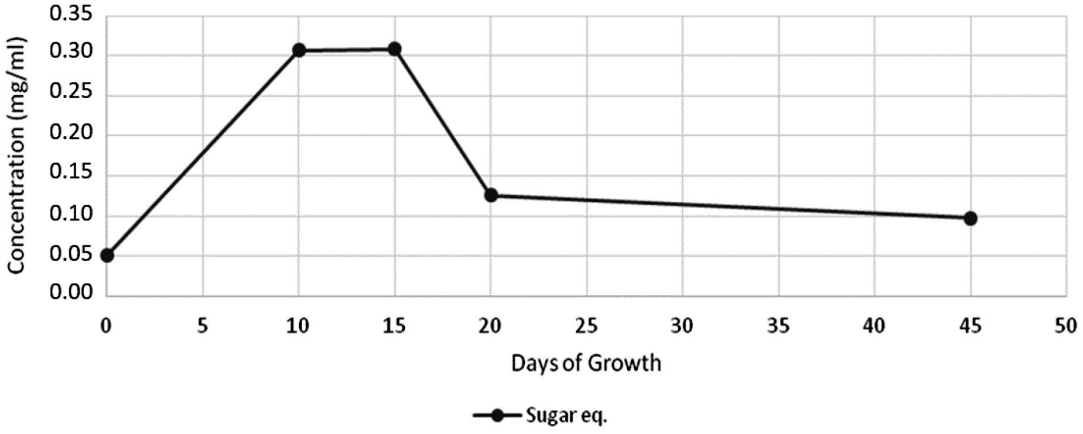

Fig. 4. Time course of reducing sugar accumulation during SSF of milled rice straw inoculated with *T. asperellum* UNIPV1

versely, CO_2 production remained high from day 20 onwards, suggesting that from this point onwards all reducing sugars released were uptaken by the fungus for growth.

Gravimetric analyses of biomass deconstruction

Results of gravimetric analysis reported in Fig. 5 show that in the sample treated with *T. asperellum*, the amount of soluble fibers started to significantly decrease ($P < 0.05$) at day 15 of SSF, and then continued to decline until day 45 when a value equal to one third of the initial soluble fiber weight was detected. In contrast, the decrease in the weight of insoluble fibers was much more delayed, with a significant decrease ($P < 0.05$) with

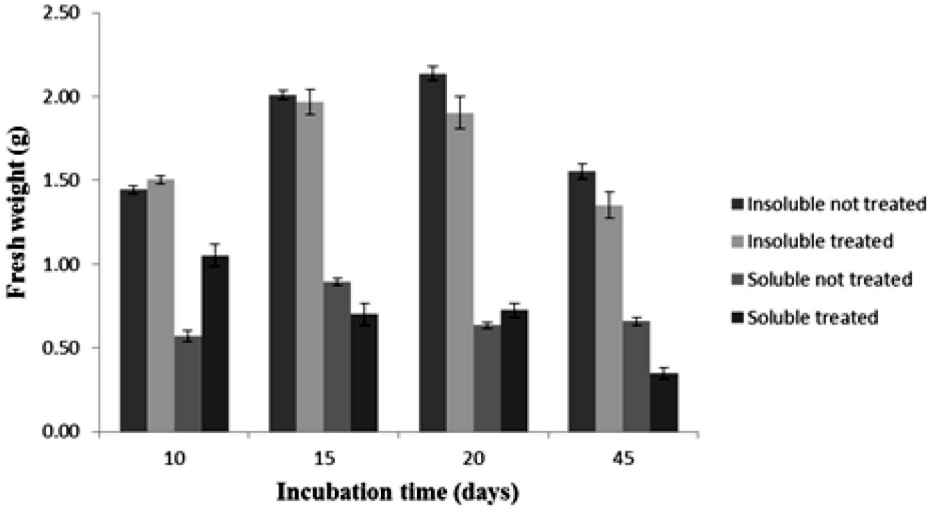

Fig. 5. Insoluble and soluble fiber content in milled rice straw following SSF

respect to the previous time point occurring only at day 45. This finding is most likely due to an initial preference of *T. asperellum* for soluble fibers, readily available for the fungal metabolism, while the more recalcitrant insoluble fibers were degraded only subsequently, when simple sugars were no longer available.

Conclusion

In this work, enzymatic tests were performed via the inoculation of *T. asperellum* UNIPV1 on sterile rice straw. The results revealed that in treated straw, i) a significant production of CO_2 occurs, ii) peak production of enzymes occurred between 10 and 20 days after inoculation, and iii) an increase in the total free reducing sugars of rice straw occurred alongside a decrease in the soluble component. These findings reveal how *T. asperellum* UNIPV1 can degrade cellulose and hemicellulose and are consistent with those of Bech *et al*. (2015). Overall, we conclude that adding the fungus to rice straw may lead to the formulation of "bioactive agro-matrices" that are more easily degradable. Accordingly, *T. asperellum* UNIPV1 seems to be an excellent candidate for future practical applications. However, use of the fungal pre-treatment alone does not appear to be sufficient to fully complete the degradation of lignocellulosic insoluble material, suggesting a need for further research in this area.

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