Biomass and Bioenergy 99 (2017) 11–20

Contents lists available at ScienceDirect

Biomass and Bioenergy

journal homepage: http://www.elsevier.com/locate/biombioe

Research paper

Rice straw hydrolysis using secretomes from novel fungal isolates from Vietnam

George E. Anasontzis [a, b, *], Nguyen Thanh Thuy [c], Dinh Thi My Hang [c], Han Thu Huong [c], Dang Tat Thanh [c], Dinh Duc Hien [c], Vu Nguyen Thanh [c], Lisbeth Olsson [a, b]

[a] Department of Chemical and Biological Engineering, Industrial Biotechnology, Chalmers University of Technology, Kemivägen 10, 41296 Gothenburg, Sweden
[b] Wallenberg Wood Science Center, Chalmers University of Technology, Gothenburg, Sweden
[c] Centre for Industrial Microbiology, Food Industries Research Institute, Thanh Xuan, Ha Noi, Viet Nam

ARTICLE INFO

Article history:
Received 12 October 2016
Received in revised form
15 January 2017
Accepted 9 February 2017

Keywords:
Aspergillus
Trichoderma
Rice-straw saccharification
Biomass degrading capability
Cellulase
Feruloyl esterases

ABSTRACT

With a production of 39 million metric tons each year, rice is one of the main agricultural products of Vietnam. Thus, rice straw is a significant by-product, whose use in a biorefinery process would contribute to the bio-based transformation of the Vietnamese and South East Asian economy. In order to find novel efficient enzyme mixtures for the hydrolysis of rice straw and other agricultural residues, we took advantage of the rapidly evolving biodiversity of Vietnam and screened 1100 new fungal isolates from soil and decaying plant tissues for their CMCase activity. We selected 36 strains and evaluated them for their cellulases, xylanases, and accessory enzymes activities. Most of these isolates belonged to the genera *Aspergillus* and *Trichoderma*. We identified a few promising isolates, such as *A. brunneoviolaceus* FEC 156, *A. niger* FEC 130 and FEC 705, and *A. tubingensis* FEC 98, FEC 110 and FEC 644, whose produced enzyme mixtures released a mass fraction of the sugar content of alkali-treated rice straw higher than 20%, compared to 10% for *Trichoderma reesei* RUT C-30. We verified that the black Aspergilli are particularly efficient in their saccharification ability. We also identified strains that although they produced low amounts of cellulases and xylanases, their enzyme mixtures had high saccharification efficiencies, indicating the importance of the synergy effect, rather than the amount of enzymes available. Our results highlight the intra-species variation, especially in the *Trichoderma* genus, regarding the biomass degradation characteristics and the associated range of enzymatic activities.

1. Introduction

Lignocellulosic material will have a key role as a raw material in the bio-based economy concept, as it is *de facto* renewable and its use may well be sustainable. As a by-product of agriculture and forestry, lignocellulose could be used without ethical constraints for the production of biofuels and other biochemicals. In addition, and only if regulated properly, it could be a sustainable alternative to the dwindling forestry sector in Europe and North America [1]. Lignocellulose is recalcitrant, due to the interweaving of cellulose within the hemicellulose and lignin matrix [2].

According to the United Nations Environment Programme [3], five billion metric tons of lignocellulosic biomass is generated every year from agriculture. The energy that is stored in its chemical bonds has the thermal equivalent of 1.2 billion metric tons of oil, which is one fourth of the current production [3]. Rice, as an important part of human diet, contributes greatly to these amounts. Almost 700 million metric tons were produced in 2014 and a mass fraction of it higher than 90% originated from Asian countries [4]. This can be translated to 700–1050 million metric tons of rice straw [5]. Due to its high silicon concentration, rice straw has low digestibility compared to other straws [6] and is normally burnt or left to degrade naturally. Consequently, it is reasonable to assume that it could function as a readily available

Abbreviations: CMC, Carboxymethylcellulose; PDA, Potato Dextrose Agar; MFA, Methyl ferulate; MSP PCR, Micro Satellite primed PCR; ITS, Internal Transcribed Spacer; BLAST, Basic Local Alignment Search Tool; MCL, Maximum Composite Likelihood; HPAEC-PAD, High Performance Anion-Exchange Chromatography equipped with Pulsed Amperometric Detection; FAE, Feruloyl acid esterase.

* Corresponding author. INRA, Aix Marseille Univ, UMR1163, Biodiversité et Biotechnologie Fongiques, Marseille, France.
E-mail addresses: georgios.anasontzis@univ-amu.fr, g.anasontzis@gmail.com (G.E. Anasontzis).

http://dx.doi.org/10.1016/j.biombioe.2017.02.008

raw material in a biorefinery concept, provided an efficient degradation is achieved.

The hydrolysis of biomass to monosaccharides is still considered to be a major bottleneck from the point of view of efficiency and applicability [7–10]. Rice straw hydrolysis has been approached from various angles; the process has been optimized with dilute acid at high temperature and pressure [11], while successful efforts to improve the enzymatic hydrolysis efficiency with alkaline pretreatment have been reported [12]. Enzymatic hydrolysis, which is often presented as a sustainable approach for conversion of cellulose to glucose, must be preceded by other types of pretreatment in order to be efficient and it is burdened by the high enzyme cost [13]. Pretreatment modifies the structural characteristics of lignocellulose by decreasing crystallinity and by increasing the surface area and the volume of the pores [14], thus improving the enzymatic digestibility of the plant cell [15].

In nature, filamentous fungi are the main decomposers of lignocellulosic biomass. This ability has been attributed to the development of (a) growth through hyphae, which enabled them to colonise the soil and penetrate the bulky plant tissues, and of (b) new enzymatic pathways that allowed degradation of the inherently recalcitrant structural compounds of vascular plants [2]. *Trichoderma reesei* QM6a was isolated (and initially described as *T. viride*) during the World War II at the Solomon Islands. It was reported as a good producer of cellulases [16]. It was then subjected to several rounds of UV and chemical mutations and screening in order to enhance its cellulase yields and reduce its catabolite repression on enzyme production, resulting in the *T. reesei* RUT-C30 strain [17]. This strain is considered to be a model fungal one due to its biomass-degrading ability and it is commonly used for cellulase production. Even so, it may not be the ideal microorganism for the complete degradation of cellulose and hemicelluloses present in plant biomass. Actually, a mass fraction of 80–85% of the proteins secreted under cellulase-inducing conditions are considered to be cellobiohydrolases, and CBHI—the major cellobiohydrolase—accounts for a fraction of about 60% of all the proteins that are secreted [18]. In 2D-gel analysis of the secretome of *T. reesei* grown on xylose and lactose, the most abundant proteins have been also found to be cellobiohydrolases [19]. Moreover, MS/MS shotgun proteomics analysis of the secretome of RUT-C30 grown on corn stover revealed a clear predominance of CBHI in terms of spectral counts [20].

Considering the renewed interest in hemicellulose degradation, together with the ever-increasing interest in cellulose degradation for the use of plant biomass, it is important to screen for efficient degraders, which could be more specialized than *T. reesei* for the saccharification of common agricultural and forestry waste products. With the intention to identify enzymatic mixtures that could be applied in a biorefinery that utilises rice straw as raw material, we screened a large number of new isolates from northern Vietnam for cellulases and hemicellulases production. Being in the tropical zone, Vietnam has a rich microbial biodiversity. This has been recently attributed to an increased rate of formation of new species, colonisation by temperate species and a lower rate of extinction of species [21]. The biodiversity of fungi in Vietnam has not been studied extensively. Our work was a thorough investigation of 200 sampling points with a view to isolate strains with high biotechnological potential regarding biomass degradation. We aimed at the identification of strains that would be worth to investigate further for the hydrolysis of rice straw and other plant feedstocks in a biorefinery perspective.

We selected 36 strains that showed higher Carboxymethyl Cellulose (CMC) and xylan hydrolytic capacity in the initial screening and we also examined their respective enzyme activities when grown on two different carbon sources. We finally investigated their ability to hydrolyse alkali-treated and untreated rice straw from Vietnam.

2. Materials and methods

2.1. Sampling

Fungi were isolated from soil samples and decaying plant debris samples collected in northern Vietnam on modified Czapek with cellulose (cellulose filter paper 10 g L^{-1}) as the sole carbon source, agar (20 g L^{-1}) and mineral composition (g L^{-1}): $(NH4)_2SO_4$, 2; K_2HPO_4, 1; KCl, 0.5; $MgSO_4.7H_2O$, 0.5; $FeSO_4.7H_2O$, 0.01.

2.2. Cultures

For initial screening, the isolated strains were cultured for seven days at 30 °C on solid medium containing rice bran (1.5 g, Hadong market, Hanoi, Vietnam), brewer's spent grain (1.5 g, from a pilot beer brewery at the Food Industries Research Institute (Hanoi, Vietnam), sugarcane bagasse (1.5 g, Lam Son Sugar JSC, Thanh Hoa, Vietnam), and 10 mL of the salt solution described above. Crude enzyme mixtures were extracted by addition of 0.1 mol L^{-1} sodium acetate, pH 5.0, incubation for 2 h at 30 °C with shaking at 3.3 Hz, and centrifugation at 10,000×*g* for 10 min at 4 °C.

Each selected strain was used to inoculate potato dextrose agar (PDA) plates and was incubated for 3–5 d at 30 °C. Spores suspensions were prepared by adding 10 mL of Tween 80 (0.5 mL L^{-1}) and by filtration through sterile Miracloth (Millipore, Darmstadt, Germany). The liquid cultures were performed for the selected strains, as well as for *Trichoderma reesei* RUT-C30 for comparison. Two different substrates were used: Avicel PH-101 (11365; Sigma-Aldrich, St. Louis, MO, USA) and wheat bran (Vetekli, Kungsörnen, Stockholm, Sweden). The carbon source was added in the medium at a concentration of 20 g L^{-1}. The composition of the medium was as follows (in g L^{-1}): KH_2PO_4, 4; $(NH_4)_2SO_4$, 13.6; $CaCl_2.2H_2O$, 0.8; $MgSO_4.7H_2O$, 0.6; and Bacto Peptone (Becton, Dickinson and Company; NJ, USA), 6. The concentrations of trace elements were (in mg L^{-1}): $FeSO_4.7H_2O$, 10; $MnSO_4.H_2O$, 3.2; $ZnSO_4.7H_2O$, 2.8; and $CoCl_2.6H_2O$, 4. Tween 80 was added to the medium at a final concentration of 200 μL L^{-1}.

All cultures were performed in duplicates in 100 mL flasks with 20 mL medium at 30 °C and shaking at 3 Hz. The initial spore concentration was approximately 5×10^6 spores mL^{-1}. Samples of 1 mL were collected at 24, 72, 120, and 168 h. The samples were centrifuged at 12,000×*g* for 5 min at 4 °C and the supernatant was filtered and maintained in aliquots at −20 °C for further use.

2.3. Enzymatic assays

For initial screening, 50 μL of the crude enzyme solution collected from the solid cultures was placed in 8-mm diameter wells on agar plates containing 20 g L^{-1} agar (LP0028B; Oxoid, Thermo Fisher Scientific, Waltham, MA, USA) and 5 g L^{-1} low viscosity CMC (C5678; Sigma-Aldrich, St. Louis, MO, USA) in 0.1 mol L^{-1} sodium acetate buffer, pH 5.0, and incubated for 16 h at 50 °C. The plate was then stained with a solution of 1 g L^{-1} Congo Red (C6277; Sigma Aldrich), for 30 min and destained with 1 mol L^{-1} NaCl for 10 min. The diameter of the clearing zone was measured as an indication of the corresponding enzymatic activity. Celluclast (10 g L^{-1}; Novozymes, Denmark) was used as a control.

Cellulase and xylanase activities of the liquid culture crude extracts were determined using dinitrosalicylic acid assay [22], by measuring the release of reducing sugars from filter paper (Whatman Chromatography paper 3001–931; GE Healthcare, Little Chalfont, UK) and xylan from birchwood (X0502; Sigma-Aldrich).