Journal of Microbiological Methods 127 (2016) 28–40

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Development of three specific PCR-based tools to determine quantity, cellulolytic transcriptional activity and phylogeny of anaerobic fungi

Veronika Dollhofer [a,*], Tony Martin Callaghan [a], Samart Dorn-In [b,1], Johann Bauer [b], Michael Lebuhn [a]

[a] Department for Quality Assurance and Analytics, Bavarian State Research Center for Agriculture, Lange Point 6, 85354 Freising, Germany
[b] Chair of Animal Hygiene, WZW, TUM, Weihenstephaner Berg 3, 85354 Freising, Germany

ARTICLE INFO

Article history:
Received 26 January 2016
Received in revised form 20 May 2016
Accepted 20 May 2016
Available online 21 May 2016

Keywords:
Anaerobic fungi
Biogas production
Lignocellulosic biomass
Neocallimastigomycota
Phylogeny
Real-time quantitative PCR

ABSTRACT

Anaerobic fungi (AF) decompose plant material with their rhizoid and multiple cellulolytic enzymes. They disintegrate the complex structure of lignocellulosic substrates, making them more accessible and suitable for further microbial degradation. There is also much interest in their use as biocatalysts for biotechnological applications. Here, three novel polymerase chain reaction (PCR)-based methods for detecting AF and their transcriptional activity in *in vitro* cultures and environmental samples were developed. Two real-time quantitative PCR (qPCR)-based methods targeting AF were developed: AF-SSU, was designed to quantify the 18S rRNA genes of AF. AF-Endo, measuring transcripts of an endoglucanase gene from the glycoside hydrolase family 5 (GH5), was developed to quantify their transcriptional cellulolytic activity. The third PCR based approach was designed for phylogenetical analysis. It targets the 28S rRNA gene (LSU) of AF revealing their phylogenetic affiliation. The *in silico*-designed primer/probe combinations were successfully tested for the specific amplification of AF from animal and biogas plant derived samples. In combination, these three methods represent useful tools for the analysis of AF transcriptional cellulolytic activity, their abundance and their phylogenetic placement.

1. Introduction

Anaerobic fungi (AF)[2] represent a basal phylum of the Kingdom Fungi, the Neocallimastigomycota. To date, five monocentric genera, *Neocallimastix*, *Piromyces*, *Caecomyces*, *Buwchfawromyces*, and *Oontomyces*, and three polycentric genera, *Anaeromyces*, *Cyllamyces*, and *Orpinomyces*, have been described (Callaghan et al., 2015; Dagar et al., 2015; Griffith et al., 2010; Gruninger et al., 2014; Haitjema et al., 2014). Their flagellated zoospores use chemotaxis to swim towards plant material and attach to the plant surface (Lowe et al., 1987; Orpin and Bountiff, 1978). Depending on the genus, they develop a filamentous rhizoid (*Neocallimastix*, *Piromyces*, *Anaeromyces*, *Orpinomyces*, *Buwchfawromyces* and *Oontomyces*) or bulbous holdfasts (*Caecomyces* and *Cyllamyces*), both growing into the plant matter and rupturing the plant structure. During growth, the AF excrete a plethora of enzymes that enables them to digest different plant sugars and also to liberate cellulose and hemicellulose from their lignin coats (Borneman et al., 1990; Teunissen and Op den Camp, 1993). The lignocellulolytic enzymes are secreted individually or are found combined in multi-enzyme complexes called cellulosomes (Fontes and Gilbert, 2010; Haitjema et al., 2014). Cellulosomes were first described for cellulolytic bacteria from the family Clostridiaceae. AF are the only eukaryotes hitherto known equipped with this unique feature. The combination of enzymes in cellulosomes mediates their synergistic attack and thereby enhances cellulolytic efficiency (Gruninger et al., 2014). With their ability to break down recalcitrant substrates mechanically and enzymatically, AF are ideal candidates for the anaerobic microbial pretreatment of lignocellulose-rich wastes (Kazda et al., 2014; Nagpal et al., 2011; Procházka et al., 2012) useful for several biotechnological approaches.

However, AF are notoriously difficult to cultivate and to preserve; and the lack of a centralized culture collection has hampered research (Griffith et al., 2010; Gruninger et al., 2014). Moreover, for the identification of promising AF strains, up-to-date molecular biology tools applicable for the screening of environmental samples are a necessity. To our knowledge only three unique quantitative polymerase chain reaction (qPCR) based quantification methods for AF have been reported, probably due to the handling issues mentioned above and the still relatively small number of sequences deposited in online databases. The first one is targeting a 120 bp region, at the 3′ end of the 18S rRNA gene (small ribosomal subunit, SSU) and the 5'end of the internal transcribed spacer region 1 (ITS1) (Denman and McSweeney, 2006). The second one is

* Corresponding author.
E-mail addresses: veronika.dollhofer@lfl.bayern.de (V. Dollhofer), neocallimastix@gmail.com (T.M. Callaghan), s.dorn-in@ls.vetmed.uni-muenchen.de (S. Dorn-In), Johann.M.Bauer@t-online.de (J. Bauer), michael.lebuhn@lfl.bayern.de (M. Lebuhn).
[1] Present address: Chair of Food Safety, Faculty of Veterinary Medicine, LMU, Schönleutnerstr. 8, 85764 Oberschleißheim, Germany.
[2] Abbreviations: AF = anaerobic fungi; BS = bootstrap value; GH5 = glycoside hydrolase family 5; LoB = limit of blank; LoD = limit of detection; LoQ = limit of quantification; LSU = large ribosomal subunit; rRNA = ribosomal ribonucleic acid; SSU = small ribosomal subunit; TCA = transcriptional cellulolytic activity.

http://dx.doi.org/10.1016/j.mimet.2016.05.017
0167-7012/

targeting a 110 bp region of the 5.8S rRNA gene and was developed and validated as being more specific than the previous method (Edwards et al., 2008). The third one is a qPCR based method using a 433 bp rRNA region, starting in the 18S rRNA gene, spanning the ITS1 and parts of the 5.8S rRNA gene. It was first used by Li and Heath (1992) and was adapted by Kittelmann et al. (2012) for the qPCR based quantification of AF. Since a longer amplicon than that of Edwards et al. (2008) with higher internal sequence variability presents advantages, *e.g.* to design group-specific qPCR hydrolysis probes, possibly in a multiplexing approach, we designed primers and a probe (AF-SSU) specific for an 475 bp AF 18S rRNA segment on the basis of currently available sequences and used the AF-SSU primer/probe combination for quantification of the multicopy AF 18S rRNA genes. The primer sites for the mentioned qPCR assays, referring to an *Orpinomyces* sp. reference sequence (AJ864475) are shown in Fig. A1 in the Supplementary data. It has been attempted to relate qPCR DNA quantity to AF biomass using *in vitro* prepared standards (Denman and McSweeney, 2006; Edwards et al., 2008). However, this approach is limited by several issues, such as differences in *in vitro* and *in vivo* growth and inter-generic variations in growth morphology, DNA content and even potentially rRNA copy numbers in different developmental stages. Therefore, we limited the determination of the abundance of AF to the quantification of 18S rRNA genes.

Knowledge of the quantity of AF present in environmental samples is interesting, but this is not the sole element. What is also important for identifying a strain that is suitable for biotechnological purposes (*e.g.* pretreatment) is the cellulolytic activity of a particular AF isolate. To date, activity testing of AF is typically based on fermentation (Paul et al., 2010) or enzymatic screening techniques (Aylward et al., 1999). However, a RT-qPCR-based activity measurement approach could be more time saving and would be independent of difficult cultivation techniques. Endoglucanases cleave glucosidic bonds in cellulose at amorphous non-crystalline sites, liberating oligosaccharides (Lynd et al., 2002). The expression of endoglucanases belonging to glycoside hydrolase family 5 (GH5) were upregulated during the degradation of plant material in an *Orpinomyces* species (Youssef et al., 2013). The gene sequences of cellulolytic enzymes of AF were thus screened in online databases in this study, and the gene sequences of endoglucanases belonging to glycoside hydrolases family 5 (GH5) were selected as a target of the novel primer pair AF-Endo. If AF are actively degrading cellulosic substrates, endoglucanases should be produced and the associated mRNA upregulated. For the quantitative assessment of GH5 specific transcriptional cellulolytic activity (TCA) of AF in samples, experiments were thus conducted at the mRNA level.

In addition to quantitative approaches, a tool for the phylogenetic characterization of AF populations was needed to be developed. The ITS1 region has been proposed as a standard marker for fungal taxonomy (Schoch et al., 2012), and initial research has used this locus for identification of AF isolates (Brookman et al., 2000; Li and Heath, 1992). More recently it has been applied to study the environmental abundance of the AF in pyrosequencing studies (Liggenstoffer et al., 2010) and clone libraries (Kittelmann et al., 2012; Koetschan et al., 2014). However, the ITS1 region confesses limitations attributable to high intra-genomic sequence variability. In a *Buwchfawromyces eastonii* strain, *e.g.* up to 12.9% divergence among ITS1 clones was observed (Callaghan et al., 2015). ITS1 also shows significant size polymorphism among the AF. This has been exploited in ARISA based community finger printing studies (Edwards et al., 2008). The variability present makes phylogenetic assignments uncertain, and in next generation sequencing studies, false-positive clustering of AF (Eckart et al., 2010; Gruninger et al., 2014) was observed. Using the 18S rRNA gene as a phylogenetic marker is limited for AF, too, as this region is too conserved and does not allow differentiation between individual species (Eckart et al., 2010). The 28S rRNA (large ribosomal subunit, LSU) gene, however, was recently reported as a suitable marker for the differentiation of *Orpinomyces* spp. (Dagar et al., 2011). Tests with isolates from all known AF genera showed better differentiation than ITS1 (Kumar, 2014). Accordingly, we developed a PCR based method, AF-LSU, which targets specifically the AF large ribosomal subunit.

2. Material and methods

2.1. Isolates and samples

Isolates used for validation of PCR-based approaches were provided by the Academy of Sciences (Prague, Czech Republic), the University of Aberystwyth (Wales, UK) and by the Rowett Institute for Nutrition and Health, University of Aberdeen (Scotland, UK). The isolate names, their phylogenetical affiliation, and the source institutions are shown in Table 1. Cultivation of the isolates followed the method described by Callaghan et al. (2015).

Cattle rumen fluid, used for specificity testing of primer pairs AF-Endo and AF-LSU, was derived from fistulated cows maintained by the Chair of Animal Nutrition at Technische Universität München (TUM). DNA samples for specificity testing of primer pair AF-SSU were supplied by the Chair of Animal Hygiene, TUM. These samples comprised two types of pig forage (FM1, FM2), two samples of perennial ryegrass (G1 and G2), and a maize sample (G3).

Biogas plant 21 (PB 21, numbered to mask the associated operators identity) was operated at a temperature between 45 °C and 46 °C and fed with a mixture of 68.6% grass silage, 2% grain, 21.6% cattle manure, and 7.8% cattle slurry. Samples of fermenter 1, the post-digester, maize and grass silage were analyzed by PCR, cloning and sequencing to prove specificity of assay AF-SSU. For the showcase analysis (see Section 3.3), the fermenter of biogas plant 25 (PB 25) was sampled. It was maintained at a temperature of 40 °C and fed with a mixture of 2.4% shredded grain, 6.7% sugar beets, 8.8% grass silage, 22% whole plant silage, 35.1% maize silage and 44.9% cattle slurry.

All samples were collected in 1 l polyethylene bottles and transported at an ambient temperature to the laboratory where nucleic acids were extracted immediately. Subsamples were collected and stored at −20 °C. In addition, samples of cattle slurry used as substrates of biogas plants PB 14 and PB 22 were collected. The biogas plants were part of a monitoring study by the Institute for Agricultural Engineering and Animal Husbandry at the Bavarian State Research Center for Agriculture (Ebertseder et al., 2012).

2.2. Nucleic acid extraction

Following the method of Lebuhn et al. (2003), 500 μl of the samples were transferred to a 2 ml reaction tube using a 1000 μl pipette with a tip cut at its end to facilitate flowthrough of viscous samples and solids. The filling line was marked. Each sample was mixed with 1 ml of sterile 0.85% KCl by shaking. After centrifugation at 13.200 rpm for 5 min, the supernatant was discarded, the washing step was repeated and the original volume was reconstituted with 0.85% KCl. Soluble putative inhibitors, particularly humic compounds, were discarded with the supernatant and solids retained in the reaction tube.

If nucleic acids were extracted from fungal cultures, the whole cultures were transferred into 50 ml centrifugation tubes. AF cells were pelleted by centrifugation at 5000 rpm for 10 min. The AF cell pellet was washed twice with 5 ml of sterile 0.85% KCl and processed for nucleic acid extractions.

2.2.1. DNA extraction

Washed samples (40 μl) were processed with a Fast-DNA Spin Kit for soil (MP Biomedicals) in a FastPrep-24 system (MP Biomedicals; bead beating for 40 s, at speed 6.0 m/s). The extraction was performed according to a previously published protocol (Lebuhn et al., 2003), yielding 100 μl of DNA-containing eluate. In a previous study, approximately 90% of spiked DNA was recovered by performing DNA extraction with this method (Lebuhn et al., 2016). At this high recovery