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Assessing the xylanolytic bacterial diversity during the malting process

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ABSTRACT

The presence of microorganisms producing cell wall hydrolyzing enzymes such as xylanases during malting can improve mash filtration behavior and consequently have potential for more efficient wort production. In this study, the xylanolytic bacterial community during malting was assessed by isolation and cultivation on growth media containing arabinoxylan, and identification by 16S rRNA gene sequencing. A total of 33 species-level operational taxonomic units (OTUs) were found, taking into account a 3% sequence dissimilarity cut-off, belonging to four phyla (*Actinobacteria*, *Bacteroidetes*, *Firmicutes* and *Proteobacteria*) and 25 genera. Predominant OTUs represented xylanolytic bacteria identified as *Sphingobacterium multivorum*, *Stenotrophomonas maltophilia*, *Aeromonas hydrophila* and *Pseudomonas fulva*. DNA fingerprinting of all xylanolytic isolates belonging to *S. multivorum* obtained in this study revealed shifts in *S. multivorum* populations during the process. Xylanase activity was determined for a selection of isolates, with *Cellulomonas flavigena* showing the highest activity. The xylanase of this species was isolated and purified 23.2-fold by ultrafiltration, 40% ammonium sulfate precipitation and DEAE-FF ion-exchange chromatography and appeared relatively thermostable. This study will enhance our understanding of the role of microorganisms in the barley germination process. In addition, this study may provide a basis for microflora management during malting.

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1. Introduction

Malting is the limited, controlled germination of cereal grains, aiming at the production of various enzymes capable of degrading the grain macromolecules into soluble compounds (Laitila et al., 2006). During germination, cell wall degradation is the first step in a number of hydrolytic processes, generally referred to as modification (Noots et al., 2003). Barley starchy endosperm cells have relatively thin cell walls with (1 → 3), (1 → 4)-β-D-glucan (approximately 71%) and arabinoxylan (approximately 19%) as major polysaccharide components (Fincher, 1975; Ballance and Manners, 1978). Both compounds may cause viscosity problems, resulting in a retarded mash filtration. However, it has been recognized that arabinoxylans contribute more to viscosity problems since they are not degraded as extensively as β-glucans during malting and mashing (Han and Schwarz, 1996; Evans et al., 1998; Sadosky et al., 2002; Kanauchi et al., 2011).

Complete degradation of arabinoxylan requires a large variety of cooperatively acting enzymes, including endo-1,4-β-D-xylanase, β-D-xylosidase, α-D-glucuronidase, α-L-arabinofuranosidase, acetylxylan esterase, ferulic acid esterase, and *p*-coumaric acid esterase (Biely, 1985; Collins et al., 2005). Among them, endo-1,4-β-D-xylanase (EC 3.2.1.8) (hereafter referred to as xylanase) is the key enzyme that generates short xylooligosaccharides of varying lengths from the hydrolysis of xylan (Biely, 1985). During malting,

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endogenous xylanases are produced late in the germination process, the maximum of xylanase activity occurring only after 72 h of germination (Slade et al., 1989; Banik et al., 1997; Li et al., 2005).

In addition to the germinating grains, these enzymes may also be produced by a diverse microbial community including various types of bacteria, yeasts and filamentous fungi, representing a second metabolically active compound in the malting ecosystem (Petters et al., 1988; Noots et al., 1998; Flannigan, 2003; Laitila et al., 2006, 2007; Laitila, 2007; Justé et al., 2011). For example, Van Campenhout (2000) reported that approximately 75% of malt xylanase activity was derived from the microbial community and 25% from the germinating grains. In the past, most attention has been paid to xylanases produced by fungi (Van Campenhout, 2000; Polizeli et al., 2005). For example, a starter technology based on the use of a *Rhizopus* strain was developed for malting applications with a particular aim to compensate for deficiencies in malt cell wall degrading enzymes (Coppens et al., 1998; Noots et al., 2001b; Dufait and Coppens, 2003; Noots et al., 2003). Likewise, addition of heat-stable exogenous xylanases from microbial origin at mashing and during germination was found to be an effective strategy to degrade arabinoxylan, resulting in an increased wort filtration rate (Malfliet et al., 2010). Nevertheless, although bacterial populations show great potential to be exploited in industrial applications (Hammes et al., 2005; Justé et al., 2008b), surprisingly, so far very little is known about the presence and activity of xylanolytic bacteria during malting.

In this study, we investigated the arabinoxylan-degrading bacterial community during malting by isolation and cultivation on different media enriched with arabinoxylan. Obtained isolates were identified by sequencing part of the 16S ribosomal RNA (rRNA) gene. In addition, isolates belonging to *Sphingobacterium multivorum*, the most predominant xylanase-producing bacterial species found in this study, were subjected to genetic fingerprinting to assess intraspecific variation throughout the malting process. Furthermore, xylanase activity was determined for a selection of isolates found in this study. In addition, the xylanase produced by an isolate showing the highest activity (identified as *Cellulomonas flavigena*) was partly purified and characterized in respect of temperature stability.

2. Material and methods

2.1. Study samples

Samples were obtained from an industrial malting of the barley variety Sebastian (French harvest 2011). Samples were obtained from two different malting houses exhibiting a different kilning regime (i.e. M1 with a two floor kiln and M2 with a single floor kiln), exploited by the same malting company and malting the same batch of barley. Samples were taken at different steps of the malting process, i.e. from barley, 1 day germinated barley, 5 days germinated barley, and kilned malt. In each malting step, multiple samples were taken, combined (about 100 g in total) and homogenized.

2.2. Bacterial isolation, culturing and DNA extraction

For each sample, subsamples of 5 g were mixed with 45 mL sterile physiological water. Following homogenization with a Stomacher lab blender, serial dilutions of the homogenate were plated (100 μL) on two isolation media, composed of 0.7% K_2HPO_4, 0.3% KH_2PO_4, 0.01% $MgSO_4$, 0.2% peptone, 1.5% agar, and 0.1% Azo-arabinoxylan from wheat (dyed with remazol brilliant blue; Megazyme, Bray, Ireland) (Noots et al., 2001a) or 0.5% arabinoxylan from oat (Sigma–Aldrich, Bornem, Belgium), and aerobically incubated at 30 °C for 72 h. Following incubation, the latter plates were flooded with 0.1% Congo Red solution (Sigma–Aldrich, Bornem, Belgium) for 5 min and excess dye was rinsed off with 1 M NaCl (Laitila et al., 2006; Chassard et al., 2007). For both methods, clear halos around the colonies indicate hydrolysis of the substrate. In addition to direct plating, samples were also enriched in broth prior to plating. More specifically, 50 μL of the 10 times diluted homogenate was inoculated in four enrichment broths, including a De Man Rogosa Sharpe broth (MRSB), and three selective broths differing in arabinoxylan source, composed of 0.7% K_2HPO_4, 0.3% KH_2PO_4, 0.01% $MgSO_4$, 0.2% peptone, and 0.5% arabinoxylan from wheat (Megazyme, Bray, Ireland), oat (Sigma–Aldrich, Bornem, Belgium) or barley. After 72 h of incubation at 30 °C, serial dilutions of the enrichment cultures were plated and incubated as mentioned above. All colonies producing a clearing zone on countable plates (for the barley sample: dilution 10^4–10^5; for the samples during germination and after kilning: dilution 10^6–10^7; for the enrichment cultures: dilution 10^7–10^9) were further subcultivated on the isolation medium with blue arabinoxylan from wheat to achieve purity and to confirm xylanase activity. For each purified culture, genomic DNA was extracted from three-day old cultures by the phenol–chloroform extraction method described by Lievens et al. (2003). DNA extracts were stored at −20 °C. Isolates were stored at −80 °C in the selective broth with 0.5% arabinoxylan from oat containing 37.5% glycerol.

2.3. Identification

Obtained isolates were identified by grouping into species-level operational taxonomic units (OTUs) based on 16S rRNA gene sequence similarity followed by BLAST analysis and placement in phylogenetic trees. To this end, first DNA samples were amplified in a total volume of 25 μL, containing 0.1 μM of each primer, 0.25 mM of each dNTP, 2.5 mM $MgCl_2$, 0.625 U Go*Taq* DNA polymerase, 1× Go*Taq* Flexi buffer (Promega, Madison, USA), and 1 ng genomic DNA (as measured by a Nanodrop spectrophotometer). Amplification was performed using the universal bacterial primers 516F (5′-TGCCAGCAGCCGCGGTA-3′) (Justé et al., 2008a) and 1541R (5′-AAGGAGGTGATCCAGCC-3′) (Liu et al., 2000), or 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) (Edwards et al., 1989) and 1387R (5′-GGGCGGWGTGTACAAGGC-3′) (Marchesi et al., 1998) in case the first primer pair failed. Before amplification, DNA samples were denatured at 94 °C for 2 min. Subsequently, 30 cycles were run of 45 s at 94 °C, 45 s at 58 °C, and 45 s at 72 °C, with a final extension step at 72 °C for 10 min. Next, sequencing was performed using the forward primer 516F or the reverse primer 1387R. Subsequently, sequences were grouped into OTUs based on a DNA similarity cut-off value of 3% using Mothur v.1.23.1 (Schloss et al., 2009), and subjected to a BLAST search (Altschul et al., 1990). Additionally, a phylogenetic analysis was performed. To this end, representative sequences for each OTU (deposited in GenBank under the accession numbers KC455507-KC455539) were aligned using MEGA5 (Tamura et al., 2011; http://www.megasoftware.net) against reference sequences of type strains retrieved from GenBank showing the highest sequence homology to our sequences. Phylogenetic trees were calculated by the neighbor-joining method (Saitou and Nei, 1987) implemented in Clustal X and displayed by TreeView version 1.6.6 (Page, 1996). Finally, a rarefaction curve was generated using the Vegan package (version 2.0-7) for R (R Development Core Team, 2012), assessing the adequacy of sampling as well as OTU richness. In this analysis, OTU occurrence data from the entire malting process were analyzed together, yielding a rarefaction curve assessing overall OTU richness. The rarefaction curve was generated by sampling without replacement from the original OTU table and was repeated 1000 times for 100 different sample sizes.

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