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Protease and lipase activities of fungal and bacterial strains derived from an artisanal raw ewe's milk cheese

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ABSTRACT

We previously identified the microbiota present during cheese ripening and observed high protease and lipase activity in Divle Cave cheese. To determine the contribution of individual isolates to enzyme activities, we investigated a range of species representing this microbiota for their proteolytic and lipolytic ability. In total, 17 fungal, 5 yeast and 18 bacterial strains, previously isolated from Divle Cave cheese, were assessed. Qualitative protease and lipase activities were performed on skim-milk agar and spirit-blue lipase agar, respectively, and resulted in a selection of strains for quantitative assays. For the quantitative assays, the strains were grown on minimal medium containing irradiated Divle Cave cheese, obtained from the first day of ripening. Out of 16 selected filamentous fungi, *Penicillium brevicompactum, Penicillium cavernicola* and *Penicillium olsonii* showed the highest protease activity, while *Mucor racemosus* was the best lipase producer. *Yarrowia lipolytica* was the best performing yeast with respect to protease and lipase activity. From the 18 bacterial strains, 14 and 11 strains, respectively showed protease and lipase activity in agar plates. *Micrococcus luteus, Bacillus stratosphericus, Brevibacterium antiquum, Psychrobacter glacincola* and *Pseudomonas proteolytica* displayed the highest protease and lipase activity. The proteases of yeast and filamentous fungi were identified as mainly aspartic protease by specific inhibition with Pepstatin A, whereas inhibition by PMSF (phenylmethylsulfonyl fluoride) indicated that most bacterial enzymes belong to serine type protease. Our results demonstrate that aspartic proteases, which usually have high milk clotting activity, are predominantly derived from fungal strains, and therefore fungal enzymes appear to be more suitable for use in the cheese industry. Microbial enzymes studied in this research might be alternatives for rennin (chymosin) from animal source because of their low cost and stable availability. Future studies will aim to purify these enzymes to test their suitability for use in similar artisanal cheeses or in large scale commercial cheeses.

1. Introduction

Proteases catalyze the hydrolysis of peptide bonds in proteins molecules resulting in peptides and aminoacids. Proteases are among the industrially important enzymes and account for over 65% of the total global sales of industrial enzymes (Hsiao et al., 2014; Rao et al., 1998). They have applications in detergent, pharmaceutical and biotechnological industries, as well as food and dairy industry (Sabotic and Kos, 2012; Yegin et al., 2011). During cheese manufacture, proteases are commonly used as milk-clotting agents and affect cheese quality by improving the taste, flavor and functional properties (Yegin et al., 2011).

Proteases can be categorized depending upon the amino acids present in their active sites such as aspartic, serine and cysteine, or as metalloprotease if a metal ion is required for catalytic activity (Sumantha et al., 2006). They are present in all living beings and are widely distributed in nature (Sumantha et al., 2006), while mainly secreted by fungi and bacteria (Quintero and Bermudes, 2014), in a variable ratio from one strain to another (Boutrou et al., 2006b).

Aspartic proteases are the most important protease type in cheese production because of their milk-clotting property. Chymosin is an aspartic protease and a common milk-clotting enzyme that specifically cleaves κ-casein (cleaves the bond at Phe105-Met106) and results in milk coagulation for the manufacture of cheese (Merheb-Dini et al., 2010; Majumder et al., 2015). Almost all protease enzymes are capable of clotting milk but chymosin-like proteases are more suitable due to their specificity and purity (Merheb-Dini et al., 2010).

Lipases are another important group of enzymes with a considerable physiological significance and industrial potential. Lipases mainly catalyze the hydrolysis of fats and oils to glycerol and fatty acids (Mohamed et al., 2011). They are produced by various plants, animals and microorganisms. Lipases from microbial origin, bacterial and fungal,

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are most commonly used in biotechnological applications (Ülker and Karaoğlu, 2012). The most commonly used bacterial genera for lipase applications are *Bacillus, Arthrobacter* and *Pseudomonas*. Filamentous fungi are able to produce higher amounts of extracellular lipases (Saxena et al., 1999) and the most commonly used fungal genera in applications are *Mucor, Penicillium, Aspergillus, Geotrichum* and *Rhizopus* (Ghosh et al., 1996). The most commonly used species from the genus *Penicillium* are *P. cyclopium, P. brevicompactum, P. chrysogenum, P. crustosum, P. roqueforti* and *P. verrucosum* (Li and Zong, 2010). Similar to proteases, lipases have also been widely applied in detergent, pharmaceutical, food and dairy industries, especially during cheese ripening (Gupta and Rathi, 2004; Hasan et al., 2006). Lipases are the third largest group of enzymes based on total sales market, following proteases and carbohydrases (Liu et al., 2008).

The unique character of artisanal dairy products is partly due to the large number of microorganisms and enzymes associated with raw milk. Specific microorganisms and natural starters derived from raw milk contribute to the sensory properties and quality of cheeses (Chaves-Lopez et al., 2006). Raw milk and associated microorganisms were analysed as a source of proteases and lipases. Specifically, psychrotrophic bacteria isolated from raw milk were examined to determine their spoilage potential during storage and refrigeration (Baur et al., 2015; Champagne et al., 1994; Hantsis-Zacharov and Halpern, 2007; Munsch-Alatossava and Alatossava, 2006).

Many studies addressed the production, purification and characterization of an individual protease or lipase produced by a specific bacterial or fungal strain. The objective of these studies was generally to determine the potential of the enzyme for specific applications. In relation to dairy applications, microbial sources are evaluated to find substitutes for animal enzymes, because of their greater potential stability and relatively lower cost. Moreover, these enzymes show heat sensitivity and industrially desirable characteristics for cheese production (Kumar et al., 2010). There are several reports that fungal proteases are efficient in milk-clotting enzymes, such as from *Mucor* sp. and *Rhizomucor* sp. (Kumar et al., 2005; Merheb-Dini et al., 2010; Yegin et al., 2011; Leite Júnior et al., 2015). Moreover, the protease activity of *P. roqueforti* strains were confirmed to contribute to the texture and organoleptic properties of different varieties of Blue cheeses (Fernandez-Bodega et al., 2009). Also, synergistic protease acivities of *Geotrichum candidum* and *P. camamberti* were demonstrated in mixed cultures of Camambert cheese whey, which showed the contribution of protease activities of these strains in soft cheese ripening (Boutrou et al., 2006a). In addition, the presence of protease and lipase enzymes produced by different yeast strains such as *Candida zeylanoides* and *Y. lipolytica* were reported in dairy products and *Y. lipolytica* was found to show the highest lipase activity among the yeasts isolated from milk and dairy products (Corbo et al., 2001). However, proteases and lipases also have applications in other industrial processes. Proteases from *Debaryomyces hansenii* have a particular role in food fermentation processes and accelerated the proteolysis in fermented meat products (Bolumar et al., 2008), while purified proteases from *Bacillus cereus* were applied in laundry detergents (Banik and Prakash, 2004).

Divle Cave cheese is an artisanal raw milk cheese ripened in a cave in goat skin bags and during ripening a variety of yeasts, filamentous fungi and bacteria are observed (Ozturkoglu Budak et al., 2016a). These cheese microorganisms derived from raw milk and the cave environment develop and contribute to the sensory properties such as aroma and flavor of the cheese (Ozturkoglu-Budak et al., 2016b), in part by releasing extracellular enzymes such as proteases and lipases. Fungi growing on the surface of goatskin bags usually secrete their enzymes into the inner part of cheese.

The aim of this study was to determine protease and lipase production by yeasts, filamentous fungi and bacteria previously isolated from Divle Cave cheese. To simulate the real ripening conditions, the enzyme activities were quantified in submerged cultivations of the isolates in medium made from irradiated cheese at conditions adapted from cheese ripening. The effects of protease inhibitors were also monitored in terms of the inhibition of the protease activities of strains to determine the class of proteases.

2. Materials and methods

2.1. Strains and growth conditions

17 filamentous fungi, 5 yeast and 18 bacteria which are most frequently isolated from Divle Cave cheese (Ozturkoglu Budak et al., 2016a) were used to examine their proteolytic and lipolytic activities. Yeast and fungal strains were grown on Malt Extract Agar (Merck, Darmstadt, Germany) and incubated at 28 °C for 5–7 days until good sporulation had occurred. Bacterial strains were grown on Tryptic Soya Agar (Merck, Darmstadt, Germany) at 35 °C for 48 h.

Fresh yeast cells and fungal spores were harvested by gentle agitation in 10 ml ACES (acid buffer). Cell and spore counts of suspensions were determined using a haemocytometer (Burker-Turk, Brand, Germany) under a microscope (Axioplan, Zeiss, Jena, Germany). Microbial density of bacterial cells were also measured at 660 nm using a UV–visible spectrophotometer (Perkin-Elmer, Massachusetts, US). Cell and spore suspensions of yeast and fungal strains were adjusted to 5.10^5 cells/ml and spores/ml with ACES. Bacterial cells were adjusted to 1.10^6 cfu/ml with physiological saline.

Utilization of carbohydrates by fungal strains were performed by growth tests using minimal medium (MM) prepared according to de Vries et al. (2004). Carbon sources were added to a final concentration of 25 mM for D-glucose and lactose and 1% for casein (Sigma-Aldrich, Germany). Plates of all media contained 1.5% agar. Strains were inoculated with 2 μl of spore suspensions (500 spores/μl) and incubated at 15 °C for 10 days.

2.2. Qualitative enzymatic activities in plates

2.2.1. Screening for protease activity

The qualitative evaluation of protease activities of all strains were determined on skim-milk agar plates, prepared with 10% skim-milk powder (Oxoid, Hampshire,

UK), 1% yeast-extract (Merck, Darmstadt, Germany) and 2% agar (Merck, Darmstadt, Germany). Yeast, fungal and bacterial strain suspensions were prepared as 5.10^5 cells/ml, 5.10^5 spore/ml and 1.10^6 cells/ml, respectively. 2 μl spore suspension was inoculated in the middle of plate for yeast and fungal strains and a loopfull sample of cell suspension were spreaded onto the plate for bacterial strains. They were incubated at 15 °C for 10 and 7 days, respectively. A lower temperature (15 °C) than for normal cultivation (30 °C) was used to mimic the low temperature of cave in which the cheese is produced. The activity was evaluated after incubation by the formation of a transparent halo around the colonies, as a result of the hydrolysis of milk proteins (Mayerhof et al., 1973). The strains showing such a halo were selected for the quantitative protease assays. *Mucor miehei* (CBS 182.67), the protease of which is commonly used as a chymosin substitute during cheese production, was used as positive control.

2.2.2. Screening for lipase activity

Both fungal and bacterial strains were evaluated in terms of their lipase activities on Spirit Blue agar (Sigma-Aldrich, St. Louis, USA) which includes the pancreatic digest of casein (1%), yeast extract (0.5%), agar (2%) and spirit blue (0.02%). Lipase reagent (15 ml) (Sigma-Aldrich, St. Louis, USA) containing tributyrin, polysorbate 80 and triglycerides was added aseptically in 500 ml of sterilized medium. Plates were inoculated with the same amounts of spore and cell suspensions for fungal and bacterial strains and incubated at the same conditions as in the protease plate assay. The lipolysis was assessed by observation of halos on the plate indicating that microorganisms metabolized the lipids. *Mucor miehei* (CBS 182.67) was used as a positive control for lipase activity since it is known to secrete a number of extracellular lipases.