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ORIGINAL RESEARCH PAPER

THE ANTIMICROBIAL PROPERTIES OF ENZYMATIC HYDROLYSATES OF GOAT MILK FAT

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Goat milk fat is known for the higher content of short- and medium- chain fatty acids, which have antibacterial and antifungal properties. By solid state enzymatic hydrolysis of goat milk fat, using different *Candida lipolytica* strains as lipase sources, were obtained hydrolysate products with antimicrobial activity. The antimicrobial properties of hydrolysates against some spoilage microorganisms, bacteria (*Bacillus subtilis, Bacillus cereus*) and fungi (*Saccharomyces cerevisae, Candida mycoderma, Rhodotorula glutinis, Aspergillus niger, Penicillium* spp. and *Geotrichum candidum*) were evaluated. Hydrolysate extracts produced by hydrolysis activity of *Candida lipolytica* G.01.3.1strain exerted inhibitory effect against bacteria, as well as fungi. The results of this study could be considered promising for developing new natural preservatives that could be used in food industry in order to reduce food contamination, increase the shelf life and for food safety assurance.

Keywords: *Candida lipolytica,* goat milk fat, solid state hydrolysis, free fatty acids, antimicrobial activity

Introduction

It is known that components of foods can also represent nutrients for wild microbiota which modify the nutrional value and have influence on food safety (Kompan and Komprej, 2012). Fat is one of the most important components of goat milk with technological and nutritional value. The concentration of fat in goat milk is dependent on factors such as: breed, species, diet, age, period of lactation and season (Bisig *et al.*, 2007; De-La-Fuente *et al.*, 2009; Serafeimidou *et al.*, 2012; Serafeimidou *et al.*, 2013). Gennerally, goat milk contains approx. 3.25%-4.2% of fat (Markiewicz-Keszycka *et al.*, 2013).

The major compounds from the goat milk fat are short and medium chain fatty acids which have been shown to possess antimicrobial properties. According to literature, the saturated fatty acids, capric, caprylic, lauric, myristic acids as well as the unsaturated fatty acids, linoleic and linolenic acids, have a known antimicrobial impact against food spoilage microorganisms with incidence in food spoilage and

food safety assurance, and its specific composition may result in the increased antimicrobial compounds production (Slacanac *et al.*, 2004; Thormar *et al.*, 2006; Liu *et al*., 2008; Desbois and Smith, 2010; Thormar *et al.*, 2011; Desbois and Lawlor, 2013; Paiwan *et al.*, 2013; Mwenze, 2015).

In the composition of goatmilk fat, a higher proportion of short and medium-chain fatty acids especially butyric (C4:0) (2.18%), caproic (C6:0) (2.4%), caprylic (C8:0) (2.73%), capric (C10:0) (10%), lauric (C12:0) (5%), myristic (C14:0) (9.81%), palmitic (C16:0) (28.2%), linoleic (C18:2) (3.19%) and linolenic acid (C18:3) (0.42%) can be found (Park *et al.*, 2007; Park *et al.*, 2009). Among these fatty acids, only three, such as: caproic, caprylic and capric acids, have actually been named after goats, due to their predominance in goat milk (Haenlein, 2004; Ceballos *et al.*, 2009; Tilahun *et al.*, 1014).

Bacteria, yeasts and moulds are recognized as extracellular lipase producers (Treichel *et al.*, 2010). Several studies reported the use of yeasts in solid state fermentation (SSF) as a new area to be discovered and elucidated. Hence, *Y. lipolytica* is a very interesting yeast to be used in this type of process. Therefore, Farias *et al.* (2014) showed that *Y. lipolytica* was able to grow and produce lipase in SSF, using cottonseed and soybean cake as the solid substrat. Moreover, the use of highly active lipase from *C. rugosa* was studied for the purpose of fat enzymatic hydrolysis (Ting *et al.*, 2006). The advantages of the solid state fermentation can be used to perform fat enzymatic hydrolysis in a simple, low cost as well as energy consumption and controlled condition in order to release antimicrobial fatty acids (Singhania *et al.*, 2009; Farias *et al.*, 2014).

During the enzymatic hydrolisis of native fat with lipases or cells producing lipases, in controlled biotechnological conditions, a great quantity of fatty acids from triglycerides could be released (Hurley, 2009). Therefore, the enzymatic hydrolysis of fat by SSF technique, with selected *Yarrowia lipolytica* strain, was used efficiently for fatty acids production (Parfene *et al.*, 2013). This technique is defined as the bioconversion process, which involves fat biotransformation, by cultivation of yeast on solid medium, which contains appropriate nutrient sources for microorganism growth (Parfene *et al.*, 2013; Farias *et al.*, 2014). The microorganisms growth can occur inside or at the solid medium surface. According to SSF definition, the fermentation of solids occurs in the absence of free water, but the substrate must possess enough moisture to support the growth and metabolism of microorganism (Babu and Rao, 2007; Singhania *et al.*, 2009).

Free fatty acid derivates from goat milk fat can act for increasing the shelf-life of food. Nowadays, bio preservation has become an interest topic. The discovery of new sources of bio preservatives has contributed to the development of food bio preservation methods (Zenebe *et al.*, 2014). However, there is only limited report concerning the production of antimicrobial fatty acids from goat milk fat, and their utilisation in order to obtain new natural preservatives. Immanuel *et al.*, (2011), Fischer *et al.*, (2011) point out the antimicrobial properties of fatty acids, most research has focused on medium chain fatty acids.

The aim of the present study was to evaluate the potential of three *Candida lypolitica* strains to hydrolyse raw goat milk fat by solid state cultivation and evaluation of antimicrobial properties of hydrolysates enriched in fatty acids.

Materials and methods

Reagents, media and microorganisms

Fresh goat milk was obtained from a goat farm from a geographical area near Galati. Fat from fresh milk was extracted using Disc Bowl Centrifuge (Armfield, U.K.), at 45°C for 5 minutes. After separation, from 2.5 L goat milk were obtained 15 g fat. The fat was kept in refrigerated conditions, until the analysis time.

The chemical reagents (diethyl ether, heptane, Na_2SO_4, H_2SO_4, NaCl, DMSO) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). The commercial culture media, broth meat with agar (BMA), meat extract agar (MEA), yeast extract, peptone, dextrose (YPD) broth, purchased from Sigma–Aldrich (Germany) and Spirit Blue Agar (SBA) purchased from DIFCO Laboratoires (Detroit Mi, USA), were used as basal media for microorganisms cultivation.

The three *Candida lipolytica* strains F. 03.3.3., F. 08.3.6., G. 01.3.1. used in this study, as lipases sources, and the indicator microorganisms, *Bacillus subtilis, Bacillus cereus, Saccharomyces cerevisae, Candida mycoderma, Rhodotorula glutinis, Aspergillus niger, Penicillium* spp. and *Geotrichum candidum*, belong to the Collection of Microorganisms of Bioaliment Research Platform of Faculty of Food Science and Engineering, "Dunărea de Jos" University of Galati, Romania. The yeast strains taxonomic affiliation was confirmed by API biochemical test (Biomerieux). Stock cultures were preserved in 20 % glycerol at -70 °C. All cultures were reactivated by cultivation in specific conditions.

Candida lipolytica inoculum preparation

Candida lipolytica strains were first cultivated on YPD agar medium in Petri dishes at 28 °C for 72 h. Then, one colony of? yeast biomass was further transferred in 8 mL fresh YPD broth and cultivated again in the same conditions. The number of viable cells of inoculum was established by the plate count method proposed by Oblinger and Koburger (1975).

Candida lipolytica extracellular lipase production assay

The lipases specificity from *Candida lipolytica* strains to goat milk fat was tested according to the method elaborated by Bullerman (1993). For this method, the culture media Spirit Blue Agar (SBA) was used. It was prepared by adding 7 g of the powder of comercial medium into 200 mL of purified water. Afterwards, 3 % NaCl was added , to obtain a value of the activity water 0.98, and it was mixed throughly. The water activity was measured with an Aqua Lab Series 3 instrument (Decagon, USA), as in the previous study by Parfene *et al.* (2011). The media was autoclaved at 121 °C, for 15 minutes and afterwards was cooled to 50-55 °C. Afterwards, 5 g of goat milk fat was aseptically added and mixed thoroughly. The medium was casted in Petri dishes and dried for 20 minutes. Then, 30 µL of every inoculum 10^6 CFU mL^{-1} were put in the central well of plates. The control plates

with medium without added fat were uninoculated. All the plates were incubated at 28 °C, for 96 hours. The fat hydrolysis is confirmed by medium color change, from blue to yellow. Every 24 hours, the yeast colony diameter and the diameter of the hydrolysis zone were measured. The evaluation of the lipase activity was determined by making the difference of these two values. The samples was done in triplicate. The incubation duration was 96 hours for every sample.

Solid-state enzymatic hydrolysis of goat milk fat and fatty acids extraction

The enzymatic hydrolysis of the goat milk fat was carried out by solid state fermentation (SSF). The method was adapted from similar methods described in the literature (Bullerman, 1993; Parfene *et al.*, 2013; Farias *et al.*, 2014). Thus, 200 mL of SBA medium were prepared and supplemented with 200 ppm chloramphenicol. After sterilization, 5 g of raw goat milk fat were added and mixed properly. These SBA plates with homogenized goat milk fat were inoculated with 1mL yeast inoculum (10^6 CFU mL^{-1}). The plates were incubated at 28 ºC, for 72 hours. The enzymatic hydrolysis of the fat was performed at the interface between the aqueous phase, represented by the yeast cell suspension (inoculum) which produces extracellular lipase, and goat milk fat phase (substrate). The monitoring of the hydrolysis process was carried out by medium color change, from blue to yellow. After incubation, the samples were collected for fatty acids extraction. The method of fatty acids extraction was described by Hiroyuki (1992). Hence, 2 g sample (SSF medium), 6 g of Na_2SO_4 and 0.6 mL 2,5M H_2SO_4 were mixed thoroughly. To this mixture were added 3 mL of a diethyl ether-heptane (1:1) and kept at room temperature for 30 minutes. The mixture was centrifugated for 5 minutes at 3000 rpm. The supernatant was collected in sterile tubes, then kept at -20 °C.

Antimicrobial susceptibility test

The method elaborated by Bauer *et al.* (1996) was used for antimicrobial susceptibility testing. Suspensions of indicator microorganisms were prepared by transfering bacteria, yeast or mold cells from pure cultures into tubes containing sterile physiological solution (SFS). The inoculum of test microorganisms was prepared using 24 h growing cultures, and suspensions were adjusted to 0.5 McFarland standard turbidity. 1 mL of indicator microorganism suspension was distributed in sterile Petri plates, and then, the culture medium (BMA for bacteria, MEA for yeasts and molds) cooled to 42 ºC was added. The medium was distributed in sterile Petri dish and after solidification, on the surface of the medium were placed discs of filter paper immersed in extract of hydrolysates of fat solubilised in 10% DMSO. After that, the Petri dishes were incubated at 37 °C for 48 hours for bacteria (*Bacillus subtilis, Bacillus cereus*) and at 25 ºC for 3-5 days for yeasts (*Saccharomyces cerevisiae, Rhodotorula glutinis, Candida mycoderma*) and the molds (*Aspergillus niger, Penicilliu requeforti, Geotrichum candidum*). The evaluation of antimicrobial activity of hydrolysate extracts was carried out by measuring the diameters of zones of inhibition around the disc. Control samples were prepared in the same conditions, except that the discs were immersed in 10 % DMSO solution.

Statistical analysis

All the experiments were done in triplicate and the data presented here represents the mean of these replicates. The data related to the zone of inhibition due to the extract of hydrolysates of goat milk fat were subjected to analysis of variance (one way ANOVA) in Duncan multiple range test using SPSS v10 statistical software. The differences with $p<0.05$ were considered significant.

Results and discussion

Evaluation of lipolytic potential of Candida lipolytica strains in solid state hydrolysis of goat milk fat

The enzymatic hydrolysis of the goat milk fat was carried out according to the specificity of the yeast strains to the fat substrate that influences both the growth of yeast strains and the extracellular lipase biosynthesis (Figure 1).

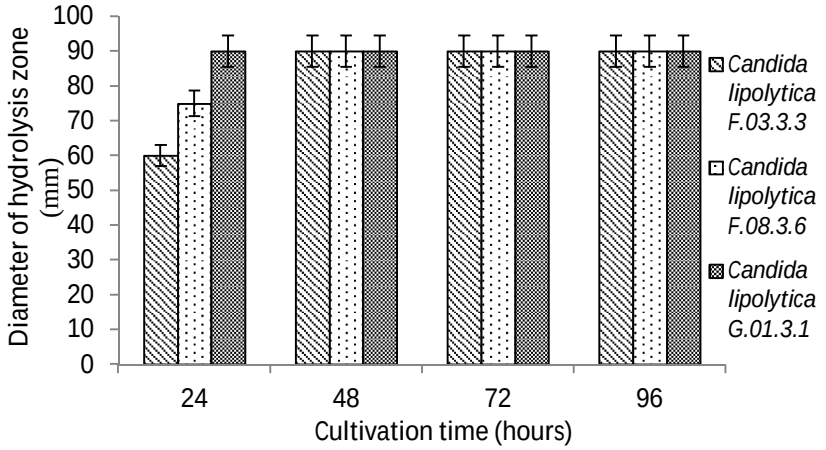

Figure 1. *In situ* enzymatic hydrolysis of goat milk fat with *Candida lipolytica* strains

The enzymatic hydrolysis was evident after 24 hours, when the appearance of hydrolysis zone with significant size ($p<0.05$) was observed. Thus, *Candida lipolytica* F.03.3.3 strain had produced an hydrolysis zone with a diameter of 60 mm, and after 48 hours, the hydrolysis zone had reached 90 mm; *Candida lipolytica* F.08.3.6 strain, after 24 hours, had presented a hydrolysis zone with a diameter of 75 mm and up to 90 mm after 48 hours. The most effective source of lipase was the strain *Candida lipolytica* G.01.3.1, which presented the largest hydrolysis zone, 90 mm after only 24 hours, compared to the other strains. This was influenced by the hydrolysis temperature (25ºC), which coincided with the optimum temperature for yeast growth. Because yeast was grown under optimal conditions, it was recorded the optimum biosynthesis of extracellular lipases, which were found to have a higher specificity for the goat milk fat hydrolysis. The results of the enzymatic hydrolysis of goat milk fat are according to the other studies such as the hydrolysis of babassu fat and canola oil (Castro, 2010), that were used to produce lipase by *Yarrowia lipolytica* and filamentous fungi in SSF (Gutarra *et al.*, 2009; Souza *et al.*, 2013).

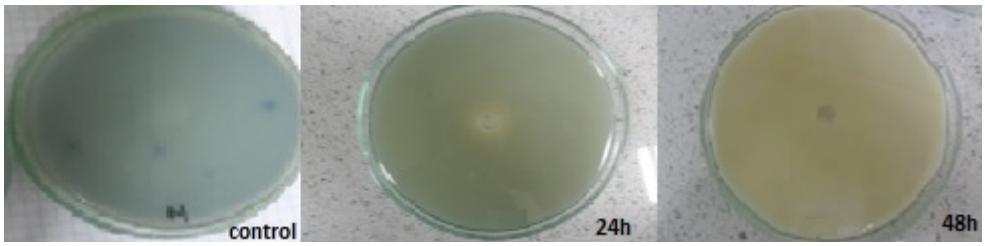

Figure 2. Solid state enzymatic hydrolysis of goat milk fat by *Candida lipolytica* G.01.3.1

The enzymatic hydrolysis of goat milk fat was visually observed by the color change of the medium, from blue to yellow (Figure 2). Thus, the control samples, consisting of non-hydrolyzed fat and medium Spirit Blue Agar, have a blue color until the end of the hydrolysis, while for the inoculated samples, the enzymatic hydrolysis was carried out, and, finally, the color was changed. This confirmed the enzymatic hydrolysis of goat milk fat (Parfene *et al.*, 2013).

Antimicrobial activity of hydrolysates of raw goat milk fat

The antimicrobial properties of extracts obtained by hydrolysis of goat milk fat were investigated. The tested extracts were: E_1 – hydrolysis with *Candida lipolytica* G.01.3.1; E_2 - hydrolysis with *Candida lipolytica* F.08.3.6 and E_3 - hydrolysis with *Candida lipolytica* F.03.3.3.

The antimicrobial activity of these extracts was determined by measuring the inhibition zone around the paper discs impregnated with the fatty acids extracts.

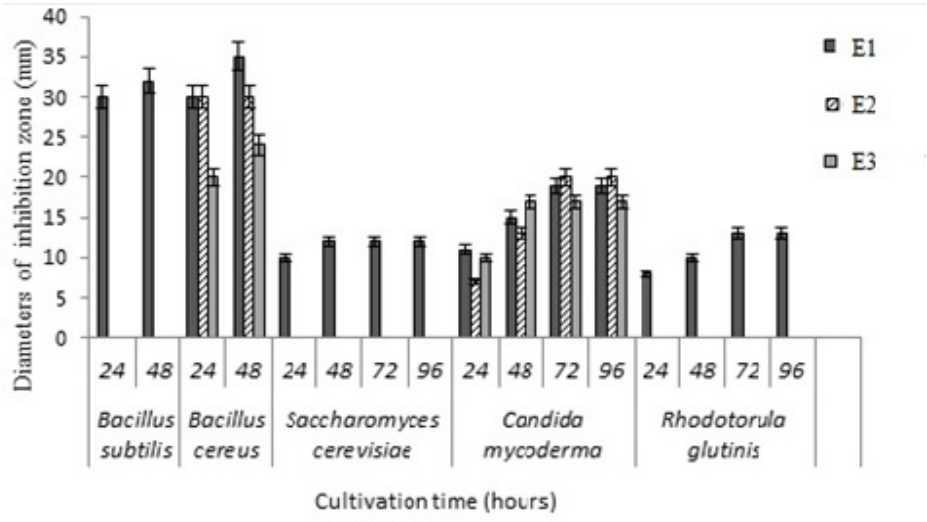

Figure 3. The antimicrobial activity of fatty acids extracts obtained by the hydrolysis of goat milk fat

The fatty acids extract (E_1) showed the highest inhibitory activity on the bacteria, which presented the highest antimicrobial activity against the *Bacillus subtilis* and *Bacillus cereus* (Figure 3), with an inhibition zone diameter ranged between 30 and 35 mm (Figure 4A). For the other extracts (E_2 and E_3), the inhibition zones were observed only for *Bacillus cereus*, that ranged between 20 - 30 mm (Figure 4A).

The literature data reported for the ciprofloxacin (5 µg/disc) zones of inhibition that ranged from 31 to 36 mm against *Bacillus subtilis, Bacillus pumilus, Micrococcus luteus, Pseudomonas aeruginosa, Klebsiella pneumonia* (Agoramoorthy *et al.*, 2007). Moreover, Batovska *et al.* (2009) reported the antimicrobial activity of medium chain fatty acids, such as caprylic (C8:0), capric (C10:0), lauric (C14:0), myristic (C14:0) acids, against *Listeria monocytogenes*, *Bacillus cereus*, *Staphylococcus aureus* and *Streptococcus pyogenes*. Huang *et al.* (2011) also showed a higher antimicrobial activity of short-chain fatty acids (SCFAs), medium-chain fatty acids (MCFAs) against *Streptococcus* sp., *Candida albicans*, *Fusobacterium nucleatum*, and *Porphyromonas gingivalis*. Similarly, the capric (C10:0) and caprylic (C8:0) acids were reported to be active against *Clostridium difficile*, the leading cause of diarrhea diseases worldwide (Shilling *et al.*, 2013).

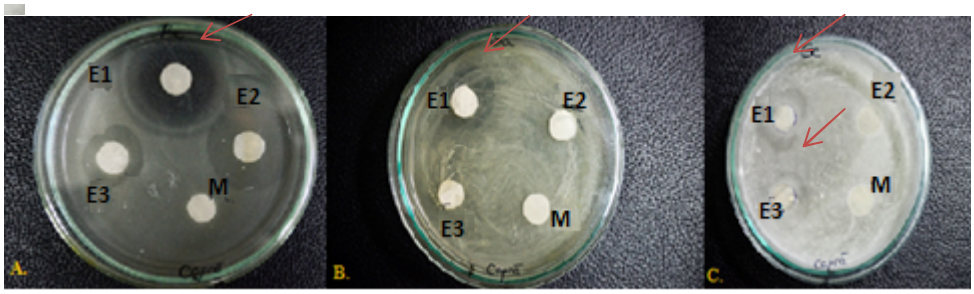

Figure 4. Antimicrobial activity of hydrolysate extracts against: A. *Bacillus cereus*; B. *Saccharomyces cerevisae*; C. *Geothricum candidum*

The antifungal activity of the extracts was also investigated. Hence, it was observed that the highest mean zones of inhibition up to 15, 20 and 10 mm were produced by E_1 extract against *Saccharomyces cerevisae*, *Candida mycoderma* and *Rhodotorula glutinis*. Analyzing the image presented in Figure 4B, one can see the growth-inhibiting effect of *Saccharomyces cerevisae*, produced by E1. On the other hand, the extracts E_2, E_3 produced inhibition zones that ranged from 15 to 20 mm against *Candida mycoderma*, but were unable to inhibit the *Saccharomyces cerevisae* and *Rhodotorula glutinis*. The E_1 and E_3 extracts were found to be effective in inhibiting the sporulation of *Aspergillus niger* and *Geothricum candidum* more than *Penicillium* spp. Otherwise, the extract E_2 had no effect on molds growth (Figure 4C). These differences could be due to the nature and level of the antimicrobial fatty acids present in the extracts and their mode of action on different test microorganisms (Huang *et al.*, 2011).

Several studies reported the antifungal activity of fatty acids and their derivatives against *Aspergillus flavus, Aspergillus ochraceus* and *Aspergillus ochraceus*, which

appears to be promising to food spoilage in food industry field (Abdelillah *et al.*, 2013). Moreover, Altieri *et al.*, (2009) presented the antifungal effect of lauric, myristic and palmitic acids and their monoglycerides against *Fusarium oxysporum* and F*usarium avenaceum*. Moreover, the preservative effect of fatty acids, to inhibit or to control the growth of *Fusarium* spp. on food surface and in laboratory media was described by Altieri *et al.* (2009).

Furthermore, Mucchetti and Neviani (2006) showed that the fatty acids and their monoglycerides have inhibited the growth *of Aspergillus* spp., *Penicillium* spp. and *Fusarium* spp. on cheese surface, to prevent changes in colour, flavour and texture. Therefore, the fatty acids contained in different matrix could be natural preservatives with important effect in inhibiting spoilage microbiota and in increasing the shelf life of food (Altieri *et al.*, 2009).

Conclusions

This paper attempted to demonstrate the efficiency of enzymatic hydrolysis of raw goat milk fat with strain *Candida lipolytica* G.01.3.1 in order to release free fatty acids with antimicrobial activity by *in situ* solid state cultivation. The obtained results would be useful in future studies to find new sources of antimicrobial compounds for obtaining natural preservatives, as natural ingredients for commercial products for biochemical and microbiological stability and safety assurance.

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Conflict of Interest: No conflict of interest declared.

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