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Full Length Research Paper

Investigation of enzyme modified cheese production by two species of *Aspergillus*

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Enzymatic biotransformation of dairy protein and fat is the basis of most commercial cheese flavour ingredient processes; such products are commonly referred to as enzyme modified cheese (EMC). EMCs have approximately 15 - 30 times the flavor intensity of natural cheese. They are available as pastes or spray-dried powders. *Aspergillus oryzae* and *Aspergillus niger* are two kinds of molds that were used in this study for production of enzyme modified cheese. The results showed that *A. niger* and *A. oryzae* have lipase enzyme activities of about 43.3 and 10 U/g (U = 1 µmol/min), respectively, while the proteolytic activity was 143 U/g for *A. oryzea* and 38 U/g for *A. niger*. The EMC produced using both *A. oryzae* and *A. niger* had the best score of flavor and odor after 3 days of storage; however the cheese produced by only *A. oryzea* had good flavor after this period of time and the cheese treated with *A. niger* only just had a strong odor. The results of this study showed that the mixture of *A. oryzea* and *A. niger* can be used to produce EMC in much shorter ripening period and with better flavor.

Key words: Enzyme modified cheese, *Aspergillus niger*, *Aspergillus oryzae*, proteolytic enzyme, lipolytic enzyme.

INTRODUCTION

Cheese curd which has been treated with enzymes to generate a concentrated cheese flavor is considered to be enzyme-modified cheese (EMC). EMCs provide the food manufacturer with a cost-effective source of cheese flavor to enhance an existing cheese taste or to confer a specific cheese character on a food product (Kamini et al., 2000; Kilcawley et al., 2000). Commercial production of EMC typically involves the simultaneous hydrolysis of cheese curd by proteolytic and lipolytic enzymes. This process is relatively unsophisticated as it is not optimized for any specific enzyme type and may result in the digestion of one enzyme type by another. Additionally, final product quality is unpredictable due to variation in the biochemical and compositional properties of the curd substrate (Kilcawley et al., 2006). To produce a consistent EMC product, it is necessary to have a highly controlled process; therefore, the enzymatic reactions under the condition used must be fully understood before any attempt is undertaken to produce EMCs (Kilcawley et al., 1998). In this study, we investigated the potential of using *Aspergillus oryzae* and *Aspergillus niger* as starter cultures for production of enzyme modified cheese with very low ripening time.

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MATERIALS AND METHODS

Materials

A. oryzae and *A. niger*, was purchased from the Persian Type Culture Collection (PTCC). Whole casein, Arabic gum, sodium chloride, hexane and toluene were obtained from Merck Company (Darmstadt, Germany). All other reagents were reagent grade and were generally commercially available.

Preparation of an inoculum culture

The inoculum culture was prepared by inoculating mycelium of the molds with an inoculating needle from a stock culture into a potato dextrose agar slant medium in a tube and incubating at 30°C for about 1 week until the spores would be visible. After sporulation, the spores were suspended in distilled water by a bacteriological loop and the concentration of spores was adjusted at 10^6/ml using a hemocytometer.

Wheat bran was used as the substrate for cultivation of the molds and enzymes production. 10 g of wheat bran was mixed with 5 - 10 ml distilled water and autoclaved for 20 min at 121°C. 1 ml of spore suspension was uniformly dispensed on to the wheat bran which was then incubated at 30°C for 3 days. For investigation of the effect of moisture content, it was adjusted at 30 and 60% in two treatments. After the incubation time, the substrate was moistened with distilled water which was added at amount of 5 - 10 times of media weight and then was shaked for 30 min at 30°C. The enzyme extract was obtained by centrifugation of the substrate at 10,000 × g and 4°C for 10 min. This enzyme extract was used for the rest of the experiments. This procedure was performed when both *A. oryzae* and *A. niger* were used.

Protease activity

A mixture of enzyme solution (0.1 ml), 2% casein solution (2 ml) and 0.6 ml of glycine-NaOH buffer (0.2 M, pH 8.0) were incubated at 37°C for 30 min. The reaction was terminated and unhydrolyzed casein precipitated out with 5 ml trichloroacetic acid (5%) and 0.2 ml HCl (1 N). The peptide content of the clear filtrate was estimated by the Lowry method (Lowry et al., 1951). One unit (U) of enzyme activity was defined as the amount of the enzyme that liberates peptides equivalent to 1 mg of bovine serum albumin (BSA) under the assay conditions (Aikat and Bhattacharyya, 2000).

Lipase assay

Determination of lipase activity in the raw enzyme extract was performed using the procedure described by Freire et al. (1997) and Gombert et al. (1999). According to this procedure, 18 ml of an arabic gum emulsion (5% w:v) and olive oil (5% w:v) (prepared in 50 mM phosphate buffer, pH 7.0) were mixed with 2 ml enzyme extract and allowed to react for 60 min at 37°C. The reaction was then stopped through the addition of 20 ml of a 1:1 acetone: ethanol mixture. After further agitation for 10 min for total extraction of fatty acids, titration was performed with 0.05 N NaOH in a pH-stat (Mettler DL 21) until end-point 11.0. Blank assays were done by adding the acetone: ethanol mixture prior to the enzyme sample. One unit of lipase activity was defined as the amount of enzyme, which produced 1 mmol of fatty acids equivalent per minute under the assay conditions. The results finally were reported as units per gram of substrate (U/g).

Cheese making

For preparation of the cheese curd, cows' milk of 3.4% fat was pasteurized at 63°C for 30 min. When the temperature reached to 42°C, 2% yogurt starter (commercial plain yogurt as yogurt starter was used for making traditional cheese) was added. Rennet addition (1%) was performed at 30°C and the mixture was maintained at this temperature for 45 min. After coagulation, curd cutting (1.5 × 1.5 × 1.5 cm), holding the curd for 10 min, curd landing in plastic hoops, curd draining under pressure and curd cutting into pieces were performed, respectively. The produced curd was used for production of the EMC. Curd was analyzed for total solids, protein, and fat contents according to the methods of IDF (1982, 1986, and 1996, respectively). A sample was considered as control, which was brine salted and was kept for one month as the ripening period.

For production of EMC, the cheese curd (65%), distilled water (29.8%), NaCl (3.5%), trisodium citrate (1%), trisodium phosphate (0.5%) and potassium sorbate (0.2%) were mixed with each other. Production was performed in 300 g capped jars. After pasteurization at 70°C for 30 min, 10 ml of the mold enzyme extracts (with certain enzyme activity) were added (three samples were inoculated with *A. oryzae* extract (O), three with *A. niger* extract (N) and three with both of the extracts (ON)). The inoculated samples were incubated at 30°C for 1 and 3 days as the ripening period. After incubation, the mixture was heated to 80°C to inactivate the enzymes.

SDS-PAGE analysis

Pure whole casein and 3 days cheese samples were analyzed by SDS-PAGE as described by Andrews (1983). Electrophoresis was performed using 10% acrylamide separating gel in a vertical slab unit (Gel electrophoresis apparatus, GE-2/4, Pharmacia, Sweden) with 180 × 140 × 1.5 mm slabs, equipped with a Hetofrig cooling bath type CB 60 and an Electrophoresis Power Supply (EPS 500/ 400, Pharmacia, Sweden). The zones of pure whole casein samples of the corresponding milk were used in electrophoresis for the identification of different bands.

Sensory evaluation

Each produced cheese was examined after 1 and 3 days of ripening. Duplicate cheese samples were placed on petri dishes, tempered for 1 h and presented to the panel members in a neutral environment. Panels of 12 postgraduate students familiar with expected properties of an EMC were provided with a descriptive vocabulary. The panelists were asked to score for flavor (odor and taste) by marking a line from nil to extreme for each characteristic.

Statistical analysis

The data were analyzed using analysis of variance (ANOVA) of the general linear models procedure of the SAS program (SAS Institute, Inc., Cary, NC, USA). The level of significance was set for $P < 0.05$.

RESULTS AND DISCUSSION

Cheese flavors are derived from three main pathways, that is, proteolysis, lipolysis and glycolysis, the extent of which varies according to cheese variety. Proteolysis is the most complex of the three primary events during cheese ripening (Kilcawley et al., 1998) The proximate analysis showed that the produced cheese curd contained 32.7% total solids, 13.8% protein, and 14.5% fat.

The protease activity of *A. oryzae* and *A. niger* are compared in Table 1 which shows the protease activity of *A. oryzae* is about 3.5 times higher than that of *A. niger*. The measurements of enzyme activity at two moisture contents of 30 and 60% showed that at higher moisture content (60%), the growth rate of molds were higher. However in lower moisture content (30%) the enzyme production increased, as the enzyme activity of *A. oryzae* in 30% moisture content was 2.4 folds higher than that of 60% (data are not shown). These results are in good agreement with the findings of Kamini et al. (1998). Also, Zadrazil and Brunnert, (1981) have indicated that a low moisture level leads to suboptimal growth and a lower degree of substrate swelling which might decrease enzyme production. A moisture content of 1:1 (substrate:

Table1. Comparison of lipase and protease activities of *A. oryzae* and *A. niger*.

Mold species	Lipase activity (U/g)*	Protease activity (U/g)
A. oryzae	10.0	143.0
A. niger	43.3	38.0

* 1U/g is equivalent to release of 1 µmol fatty acid or peptide per minute per 1 g of substrate.

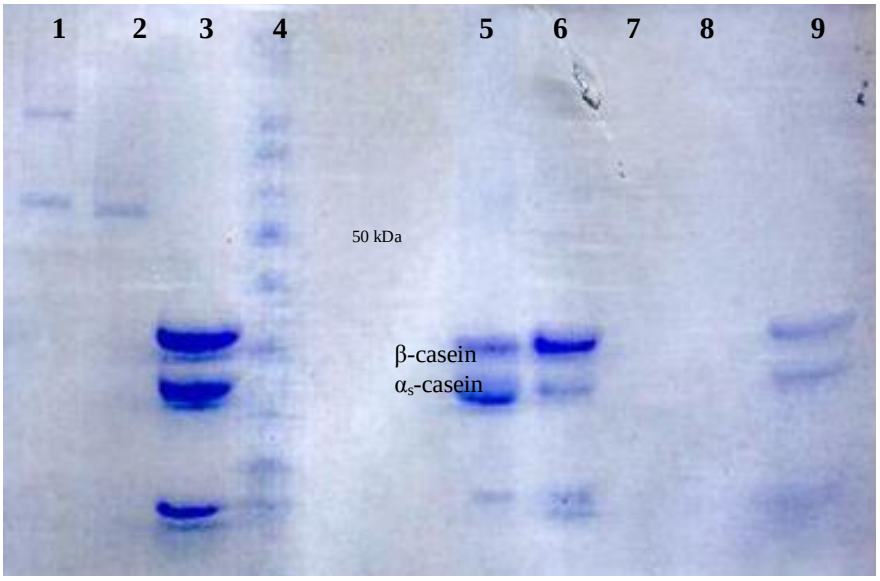

Figure 1. SDS-PAGE pattern of enzyme modified cheeses. Lane 1: *A. niger* extract; lane 2: *A. oryzea* extract; lane 3: Control after one month ripening; Lane 4: Molecular weight markers (10 - 200 kDa); lane 5: pure casein; lane 6: Control after 3 days storage; lane 7: EMC produced by *A. oryzea*; lane 8: EMC produced by *A. oryzea* and *A. niger*; lane 9: EMC produced by *A. niger*.

water, w/v) was optimal for lipase production by *Candida rugosa.*

Lipolysis refers to the hydrolysis of triglycerides, diglycerides and monoglycerides to yield free fatty acids and flavor precursors by lipolytic enzymes. The degree and contribution of lipolysis to cheese flavor varies considerably between cheese varieties (Kilcawley et al., 1998). In some cheeses 20% of fat may be hydrolyzed to medium and long chain fatty acids (between C_8 and C_{14}) which are oxidized to methyl ketones and in turn are reduced to secondary alcohols.

In Iranian Lighvan cheese, lipolysis has an important role in the final flavor of the product. Measurement of the lipase activity showed that the enzyme activity of *A. niger* was 43.3, which was 4.3 times more than that of *A. oryzae* (Table 1).

According to the enzyme activities of the extracts, four batches of cheese (control without starter culture, with *A. oryzae*, with *A. niger*, with mixture of *A. niger* and *A. oryzae*) were made and the electrophoretic pattern and sensory properties of the produced cheeses were evaluated. Intact caseins and high molecular mass peptides (derived from the proteolysis of caseins) can be separated into various protein bands on an electrophoresis gel, based only on their molecular weight using SDS-PAGE. As it is shown in Figure 1, in *A. oryzae* extract, the bands which seem to belong to the proteolytic enzymes are obvious. Two bands, which showed the proteolytic and lipolytic enzymes, are apparent in the *A. niger* extract. These results, confirm the more lipolytic activity of *A. niger* compared to that of *A. oryzae*. The presence of the caseins bands (α_s-casein, β casein) in *A. niger* inoculated cheeses confirms the weak proteolytic activity of this culture. However, these bands were disappeared in the *A. oryzae* inoculated cheeses, which implied the hydrolysis of these bands by the proteases. However, the rate of caseins hydrolysis was the same in this culture, as their bands were disappeared after three days. In the *A. niger* and *A. oryzae* inoculated cheeses, these bands were not seen, referring to the action of proteases of *A. oryzae* extract.

The results for the sensory evaluation of cheeses after

Table 2. Sensory evaluation of enzyme modified cheeses produced by *A. niger*, *A. oryzea* and a mixture of *A. niger* and *A. oryzea.*

EMCs	Sensory evaluation	
	After 1 day	After 3 days
Control	Very weak	Very weak
A. niger	Weak	Medium
A. oryzea	Medium	Relatively good
A. niger and *A. niger*	Relatively good	Very Good

ageing for 1 day and 3 days are given in Table 2. The sensory evaluation of the cheeses showed that after one day of ripening, the sensory properties of the cheeses were not established yet. However, the cheeses inoculated with the mixture of molds obtained the highest score, which was followed by *A. oryzae* inoculated cheeses and then *A. niger* inoculated cheeses. Controls without inoculation received the lowest scores.

After 3 days of ripening, the flavor of *A. oryzae* and *A. niger* inoculated cheeses were developed completely. These cheeses had a high intensity flavor (good taste with a strong odor). Such property was not seen in the other samples. The *A. oryzae* inoculated cheeses, had poor odor, but a good cheese flavor. Such cheese flavor was not sensed in the *A. oryzae* inoculated cheeses; however, these cheeses had a very strong smell.

Conclusion

The results of this study showed that the mixture of *A. oryzae* and *A. niger* can result in the production of a cheese with strong flavor and so shorten the ripening time. These cultures are potent media for production of enzyme modified cheeses, which can be used as flavoring agents in other food products.

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