**Research Article** | **Open Access**

# Adjunctive Application of Solid-State Culture Products from *Aspergillus Oryzae* for Semi-Hard Cheese

Haruto Kumura[1]*, Chiharu Saito[1], Yuko Taniguchi[1], Taiki Machiya[1], Yutaroh Takahashi[1], Ken Kobayashi[1] and Akira Kimura[2]

[1]*Laboratory of Dairy Food Science, School of Agriculture, Hokkaido University, Kita 9, Nishi 9, 060-8589, Sapporo, Japan*

[2]*Milk Science Research Institute, Megmilk Snow Bland Co. Ltd., 1-1-2 Minamidai, Kawagoe, 350-1165, Saitama, Japan*

*****Corresponding author:** Haruto KUMURA, Laboratory of Dairy Food Science, School of Agriculture, Hokkaido University, Kita 9, Nishi 9, 060-8589, Sapporo, Japan, Tel: +81-11-706-3642; E-mail: kumura@anim.agr.hokudai.ac.jp

## Abstract

To develop a novel type of cheese using *Aspergillus oryzae*, four strains (AHU 7139, AHU 7140, AHU 7141 and AHU 7146) were tested with respect to their extracellular protease production on solid-state media containing heat-denatured whey protein under various temperatures and pH levels. Proteolytic activity of the crude enzyme extracts was evaluated under acidic condition considering the circumstance of cheese ripening. Zymographic analysis revealed that several kinds of proteases were secreted, which were dependent on the strain, incubation temperature and initial pH value. Using three strains, six types of culture products were selected as adjunctive materials in combination with an incubation temperature of 15 or 20ºC and an initial pH condition of 4.0 or 6.5. Gouda-type cheese curd was prepared, and the curd particle received 1% (w/w) of the culture product, followed by pressing, cooling, salting and ripening for 3 months at 11.5ºC. An increase in water extractable nitrogen and free fatty acid was observed in some experimental cheeses, depending on the strain and culture history. In particular, incorporation of culture products from *A. oryzae* AHU 7139, incubated at pH 6.5 and 20ºC affected these parameters. As a novel dairy technology, this study propose new insights into the potential use of *A. oryzae* for providing an enzyme cocktail to develop palatable cheese flavor when suitable strains and culture conditions offer products with an optimum balance of proteases and lipases.

**Keywords:** *Aspergillus oryzae*; Cheese; Protease; Solid culture products

## Introduction

*Aspergillus oryzae* is a pivotal filamentous edible fungus that has been used in traditional Japanese fermentation products such as sake (rice wine), shochu (spirits), shoyu (soy sauce), and miso (soybean paste) for more than 1,000 years [1,2]. The long history of its extensive use in the food industry has prompted the United States Food and Drug Administration to place *A. oryzae* on the list of generally regarded as safe (GRAS) organisms. The safety of *A. oryzae* is also supported by the World Health Organization [1,3,4].

In sake brewing, *A. oryzae* is seeded on the steamed rice and fermented to allow starch saccharification by fungal amylases. The products are used for sequential alcohol fermentation by concomitant yeast. In contrast, in shoyu or miso processing, *A. oryzae* is seeded on steamed soybean and fermented to promote proteolysis, which generates umami, in part due to the action of glutaminase. Traditional fermentation of *A. oryzae* on steamed rice, wheat or soybean generally occurs at 30~35ºC and pH 6.0~6.5 [5,6] and suitable strains have been selected depending on their purpose. In general, solid-phase media is superior to submerged culture for enzyme production by *A. oryzae* [7-9], and some enzymes are exclusively produced by solid-state cultivation [10].

In terms of dairy usage, trials that use *A. oryzae* as an additional cheese starter [11] or that incorporate the commercial enzyme from *A. oryzae* into curd, have been limited; however, the addition of this protease tends to cause a bitter flavor [12-15]. However, genomics research on *A. oryzae* has shown that 135 of the secreted proteinases are encoded by approximately 12,074 genes [16,17], which implies that the previous studies used a limited number enzymes prepared on an industrial scale. The potential of this species has not been fully exploited despite the fact that it inherently possesses enzyme molecules to enforce flavor development.

In our previous study, Czapek-Dox medium, known as a minimal medium for fungi, was prepared by replacement of sodium nitrate with whey protein isolate as an exclusive nitrogen source [18]. The solution was autoclaved to solidify the whey protein by heat-denaturing, and *A. oryzae* AHU 7146 was seeded on the solid substrate. Because the proteases responsible for whey protein were successfully induced, we proposed that the resulting culture of protease cocktail may be applicable to promote cheese ripening if it is mixed with curd prior to pressing. Whey protein concentrate is a convenient dairy product for preparing the solid-phase substrate because autoclaving the solution solidifies it due to heat-denaturation. If necessary, it is possible to supply other additives and/or adjust the pH prior to autoclaving.

In this study, several strains of *A. oryzae* were incubated under different culture conditions, and their protease production was compared. After selecting the strains and culture conditions, the culture products were used to make the cheese. Here, an analysis of some of the components in experimental cheese is made, and the potential of *A. oryzae* for dairy application is discussed.

## Materials and Methods

### Strain and growth media

*A. oryzae* AHU 7139, 7140, and 7141 were originally isolated from Japanese rice wine, and AHU7146 was isolated from miso. These strains were obtained from the culture collection of Hokkaido University and were grown in potato dextrose agar (Merck KGaA, Darmstadt, Germany) at 25ºC for 10 days. The conidia were isolated and dispersed in 0.9% NaCl solution [18].

Whey protein concentrate 80 (WPC80; Fonterra, Auckland, NewZealand) was dissolved in 3-fold weight of de-ionized water and adjusted to the desired pH value with lactic acid or tri-sodium phosphate. The solution was divided (10 g) in an Erlenmeyer flask (100 ml) and autoclaved to prepare the solid-phase medium. Each media-containing Erlenmeyer flask (100 ml) received $3.5 \times 10^4$ spores and was maintained at a 15, 20, or 25ºC for 14 d. Spore counting was carried out using a hemocytometer.

### Preparation of the crude enzyme

Extraction of the enzyme was performed as follows: the resulting culture was harvested and homogenized. The material was transferred to a polyethylene bag and received an equal weight of distilled water. The sample was treated by a stomacher for 5 min, and then transferred to a centrifugal tube. Following centrifugation at $21,130 \times g$ and 4ºC for 10 min, the supernatant was recovered and used as a crude enzyme [18].

### Measurement of protease activity

The proteolytic activity was determined as previously described with some modifications [19]. The substrate, 0.2% casein dissolved in 0.05 M sodium citrate buffer pH 5.5 (700 µl), was incubated with a crude enzyme solution (50 µl) at 30ºC for 1 h. The reaction was terminated by adding 750 µl of TCA reagent (trichloroacetic acid: sodium acetate: acetic acid=0.11 M: 0.22 M: 0.33 M), and further incubation was carried out for 15 min. The resulting preparation was centrifuged at $21,130 \times g$ and 25ºC for 10 min. The supernatant (1 ml) was recovered and mixed with 2 ml of 0.625 M $Na_2CO_3$ followed by the addition of 1.8 N Folin reagent (Nakarai Tesque, Kyoto Japan). The mixture was held at 30ºC for 30 min, and its absorbance at 660 nm was determined. The value was corrected by subtracting that obtained from the blank, which was subjected to the same treatments except that TCA reagent was added to the enzyme prior to the addition of the substrate. Enzyme activity was expressed as micrograms of released tyrosine per h at 30ºC extracted from one gram of the resulting culture.

### Measurement of whey protein hydrolysis during cultivation

The value calculated from the blank of the protease activity measurement described above represents the accumulated amino acids during cultivation due to the proteolysis of whey protein in the solid culture. The degree of whey protein hydrolysis was expressed as micrograms of released tyrosine extracted from one gram of the resulting culture.

### Zymography

Zymography was carried out using separating gel that contained 0.2% casein [20]. Crude enzyme (10 µl) was loaded on the lane, and after electrophoresis the gels were incubated in a 0.2 M acetate buffer (pH 5.5) with mild shaking overnight at ambient temperature. Finally, the gels were stained with Coomassie blue R-250 (Nacalai Tesque, Kyoto, Japan).

### Preparation of adjunct materials for cheese making

Culture products used as adjunct materials are listed in Table 1. As the control, WPC80 was dissolved in 3-fold weight of de-ionized water, followed by autoclaving. For cultivation, WPC80 was dissolved and adjusted to a pH value of 4.0 or 6.5 and divided (55 g) in a petri dish (diameter 16 cm) and autoclaved. Filter paper NO.5C (ADVANTEC, Tokyo Japan) or a glass filter GA100 (ADVANTEC, Tokyo Japan) was gently attached to the solid culture. After seeding of $1.0 \times 10^4$ spores on the sheet, incubation was carried out at a defined temperature for 14 d. When incubation was terminated, the filter was carefully removed, and the remaining fraction was recovered for treatment with a food processor to obtain small particles.

| Sample name | Strain | Temperature (°C) | Initial pH |
|---|---|---|---|
| control | - | | |
| I | *A. oryzae* AHU 7139 | 15 | 4.0 |
| II | *A. oryzae* AHU 7139 | 15 | 6.5 |
| III | *A. oryzae* AHU 7139 | 20 | 6.5 |
| IV | *A. oryzae* AHU 7140 | 20 | 6.5 |
| V | *A. oryzae* AHU 7146 | 15 | 6.5 |
| VI | *A. oryzae* AHU 7146 | 20 | 6.5 |

**Table 1:** Adjunct material prepared for cheese making. Incubation temperature and pH value of culture substrate before autoclave are shown.

### Cheese making

Cheese making was carried out three times in August and September 2015 according to the conventional procedure of Goda type cheese making. Raw milk was obtained from the experimental farm in the Field Science Center for Northern Biosphere, Hokkaido University. Whole milk was standardized with skim milk and heated at 72ºC for 15 sec. After cooling to 31ºC, the milk was transferred to a vat with 1/1000 volume of 1.5 M $CaCl_2$ solution and 2% volume of bulk starter (BD culture CH N-01; Chr. Hansen, Denmark) prepared in sterilized skim milk. After incubation for 60 min, calf rennet (ca. 5 g for 100 kg of cheese milk) dissolved in 0.1 M NaCl solution was added to induce coagulation of the milk. After curd formation, it was cut into cubes 10 $mm^3$ in size and left for 15 min. Subsequently, gentle stirring was performed at 31ºC for 30 min and whey (35%) was discarded. Then, the same volume of hot water (60ºC) was added to reach 40ºC, followed by 15 min of agitation at this temperature. After the drainage, the curds were recovered and weighed to be mixed with 1% weight of adjunct materials. Following brief pressure, inversion and a second stage of pressure were performed. The curd was cooled in water overnight and immersed in 25% NaCl solution for 15 h. The curds were matured at 11.5ºC for 13 wk with a relative humidity of 90%. Procedures for preparation of the experimental cheese were summarized in Figure 1.
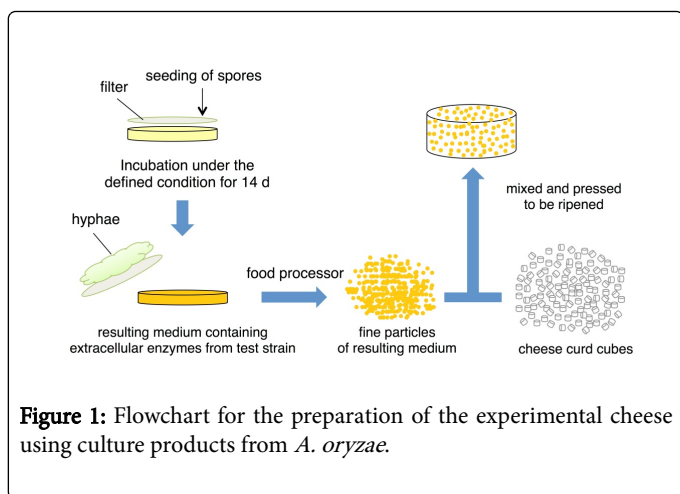
**Figure 1:** Flowchart for the preparation of the experimental cheese using culture products from *A. oryzae*.

## Analysis of Cheeses

All assays were performed in triplicate. The moisture was determined according to the IDF recommendation [21]. Fat, protein, and salt calculated from chloride content were measured by the AOAC method [22].

Water-soluble nitrogen (WSN) was determined according to the method of Kuchroo and Fox [23] with some modifications: a sample (5 g) was added to 15 ml of deionized water and heated at 75ºC for 15 min to inactivate microbial proteases. After treatment with a stomacher for 10 min, the sample was heated once again at 75ºC for 15 min. Insoluble materials were removed by filtration, and the filtrate was subjected to micro Kjeldahl method [24].

After extraction of fat according to the AOAC protocol [25], free fatty acids (FFA) were solubilized in MeOH. The sample was examined using a commercial fatty acid determination kit (NEFA C-test; Wako, Osaka, Japan) according to the manufacturer's instructions. Oleic acid was used as the standard and converted to content of oleic acid (mmol) in 100 g of cheese.

## Statistical Analysis

Percentage of WSN in total nitrogen and FFA content in cheeses were analyzed by Tukey-Kramer's multiple comparison test using JMP software (version 11.0; SAS Institute, Inc., Tokyo, Japan). Differences were considered to be statistically significant at $P<0.05$.
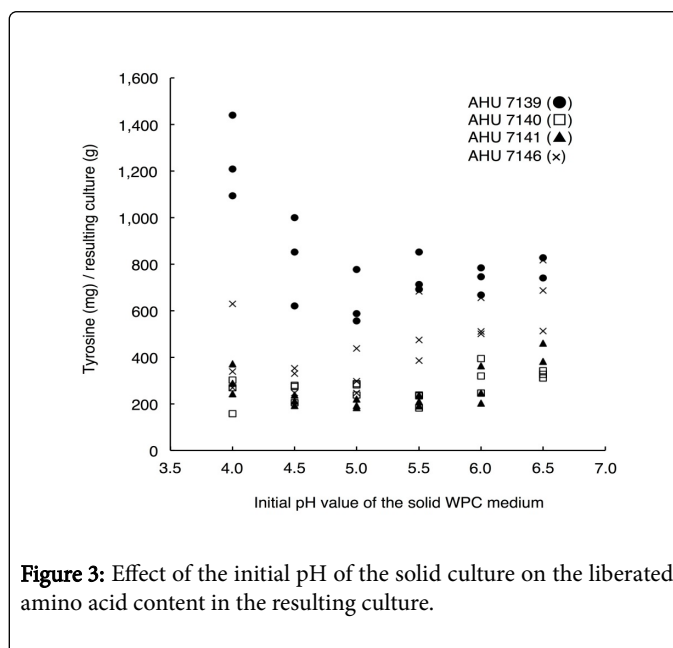
## Results

### Effect of initial pH of the culture on protease activity and culture substrate degradation

Figure 2 shows the effect of the initial pH value of the solid medium on protease activity after two weeks of cultivation at 20ºC. The average value of the duplicates in each experiment was plotted, and the experiment was repeated three times. Across the tested range of pH values, protease activity was highest for *A. oryzae* AHU 7139 and lowest for AHU 7141. The results of the protease activity in AHU 7140 and AHU 7146 were intermediate.
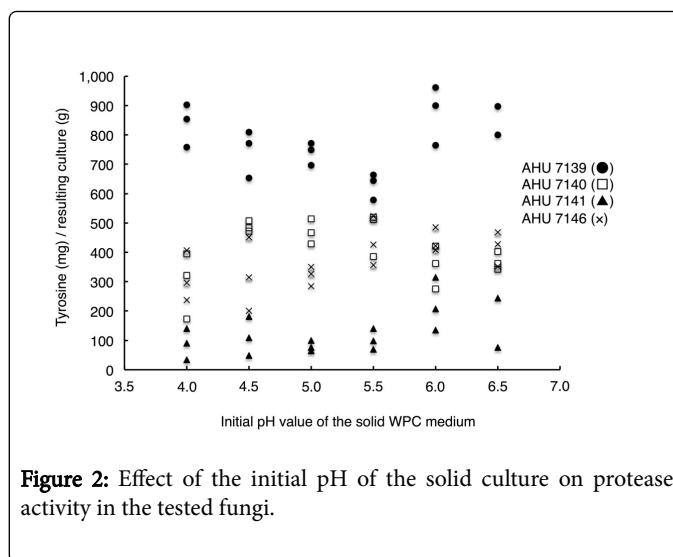


**Figure 2:** Effect of the initial pH of the solid culture on protease activity in the tested fungi.

Figure 3 shows the accumulation of liberated amino acids in the culture media due to the degradation of the solid whey protein. Incubation was carried out at 20ºC for 14 d. The blank value of the protease assay repeated three times was plotted. The amount of amino acid accumulation was highest for *A. oryzae* AHU 7139, specifically at the initial cultivation pH value of 4.0. Amino acid accumulation was much lower for AHU 7140 and AHU 7141 compared with AHU 7139. The amino acid content for AHU 7146 was intermediate.



**Figure 3:** Effect of the initial pH of the solid culture on the liberated amino acid content in the resulting culture.

Except for AHU 7139, the initial pH value of the solid media seemed to have little effect on the protease activity for the other three strains (Figure 2). In the following study for AHU 7139, initial pH values of 4.0 and 6.5 were selected based on the amount of amino acid accumulation in the culture media (Figure 3). To compare AHU 7140 and AHU 7146 with AHU 7139, AHU 7140 and AHU 7146 with an initial cultivation pH of 6.5 were selected because reproducibility was more stable at pH 6.5 than pH 4.0 (Figures 2 and 3). Considering the lowest protease activity, AHU 7141 was omitted.

## Effect of temperature and the initial pH of the culture on protease activity and culture substrate degradation

Figure 4 shows the effect of temperature on protease activity after two weeks of cultivation. The average value of the duplicates in each experiment was plotted, and the experiment was repeated three times. Protease activity in *A. oryzae* AHU 7139was higher than in the other two strains, particularly at the lower temperature. Maximum protease activity in AHU 7139 and AHU 7140 at pH 4.0 was observed at 20°C. Although the protease activity of AHU 7139 and AHU 7146 initiated with pH 6.5 at 20°C was higher than it was at 15°C, protease activity became unstable when incubation was carried out at 25°C.
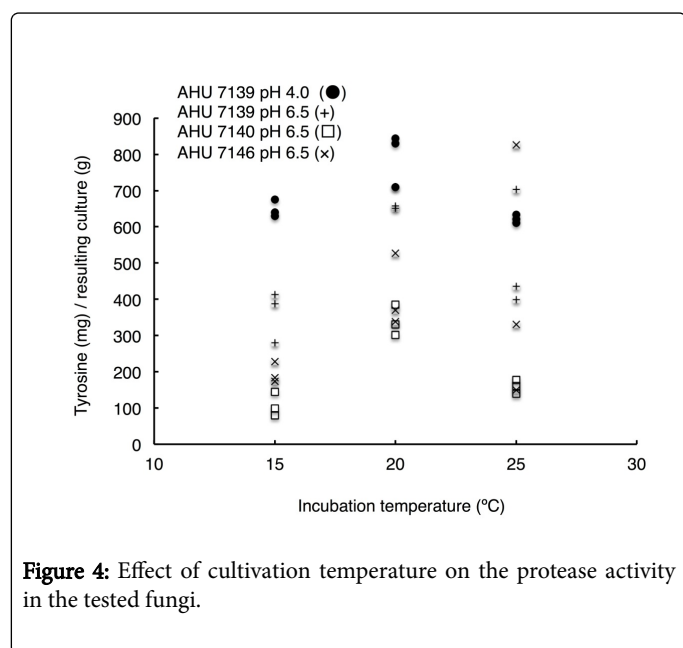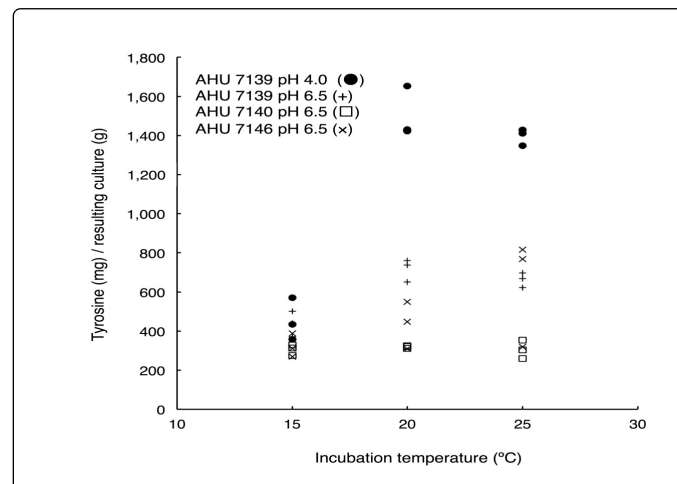


**Figure 4:** Effect of cultivation temperature on the protease activity in the tested fungi.

Figure 5 shows the amount of liberated amino acid in the culture media at the end of the two weeks cultivation. The blank value of the protease assay repeated three times was plotted. The amount of amino acid in *A. oryzae* AHU 7139, whose cultivation was initiated at pH 4.0 above 20°C, was abundant. However, when the initial pH value was set at 6.5, the amount of amino acids decreased considerably. In contrast, no remarkable difference was observed in AHU 7140. For AHU 7146, the amount of amino acids was positively correlated with incubation temperature.

## Analysis of the protease profile using zymography

Figure 6 shows the results of the zymography loaded on the crude enzymes. When the initial culture pH was adjusted to 4.0 or 6.5 at 15°C, the zymogram obtained from *A. oryzae* AHU 7139 was different. Furthermore, in both the initial cultivation pH environment, the migration pattern observed at 15°C differed from that at 20 and 25°C. The zymograph pattern seemed to be consistent when *A. oryzae* AHU 7139was cultured at 20°C or 25°C. The migration pattern obtained from AHU 7140 and AHU 7146 seemed to be similar, irrespective of incubation temperature. However, for the latter strain, the predominant molecule showing a lower extent of migration was more distinct from the other two molecular species that appeared around the center of the gel at 15°C than 20°C and 25°C.

Considering the zymography results, strains and incubation conditions were decided as shown in Table 1, and the resulting culture was used as adjunct materials for cheese making.



**Figure 5:** Effect of cultivation temperature on the liberated amino acid content in the resulting culture.
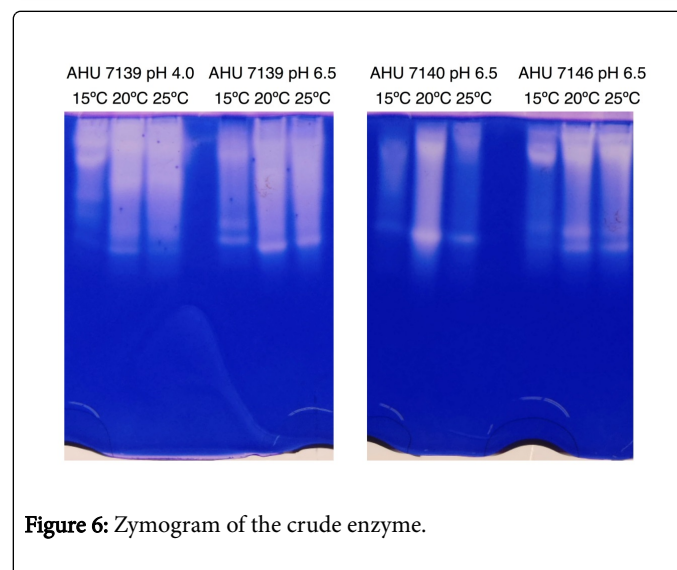


**Figure 6:** Zymogram of the crude enzyme.

## Cheese composition

Table 2 shows the composition of the control cheese from the three batches.

| Characteristic | Batch 1 | Batch 2 | Batch 3 |
|---|---|---|---|
| Moisture (%) | 34.5 | 34.4 | 37.4 |
| MNFS (%) | 51.2 | 52.0 | 52.2 |
| FDM (%) | 49.8 | 51.7 | 45.2 |
| SM (%) | 5.8 | 5.5 | 4.8 |

**Table 2:** Moisture and other characters of the control cheese obtained from three batches. Moisture nonfat substance (MNFS), fat in dry matter (FDM) and salt in moisture phase (SM) are shown.

Although batch No. 3 differed from the other two batches, chemical analysis was carried out using all batches, and values obtained from the three batches were submitted to the statistical analysis.

The percentage of WSN in the total nitrogen was significantly higher in cheeses II and III than in the control cheese (Table 3). In terms of FFA content, significant accumulation was observed in cheeses II, III, and VI (Table 3). In particular, the adjunct material prepared from AHU 7139, whose incubation was initiated at pH 6.5 at 20ºC (cheese III), remarkably promoted lipolysis.

| Sample | Percentage of water soluble N in total N (%) | | FFA (mmol) in 100 g cheese | |
|---|---|---|---|---|
| | Mean | SE | Mean | SE |
| Control | 12.5a | 0.36 | 1.1 | 0.13 |
| I | 13.6ab | 0.20 | 2.3 | 0.48 |
| II | 14.0bc | 0.19 | 10.6a | 0.37 |
| III | 14.7b | 0.25 | 23.4b | 1.41 |
| IV | 13.4ab | 0.21 | 3.1 | 0.37 |
| V | 13.1ac | 0.59 | 2.2 | 0.12 |
| VI | 13.5ab | 0.20 | 7.5c | 0.26 |

**Table 3:** Chemical analysis of cheeses. Means not sharing a common letter differ significantly (P<0.05).

## Discussion

The present study was conducted under the hypothesis that the profile of inducible enzymes of *A. oryzae* would change depending on the environmental condition. It could be possible that several protease species were produced with some of them having an optimal reaction pH in the neutral or alkaline region. However, the proteolytic activity of the crude enzyme was evaluated at a pH value of 5.5 in consideration of the pH at the onset of the Gouda-type cheese ripening. Furthermore, it was examined whether the addition of the resulting culture to cheese curd affects the character of the ripened cheese.

The effect of initial pH environment on protease production at 20ºC was investigated three times considering the relatively poor reproducibility, often encountered in solid-state fermentation. Among the four strains used in this study, *A. oryzae* AHU 7141 had the lowest proteolytic activity, whereas *A. oryzae* AHU 7139 had the highest. Additionally, the initial incubation at a pH of 5.5 resulted in lower protease activity than that of 4.0 and 6.5. Although the protease activity of *A. oryzae* AHU 7139 that was initiated at a pH of 4.0 was comparable to that at 6.0 and 6.5, the accumulation of amino acids in the substrate was more abundant when initiated at pH 4.0, which suggests a higher degree of proteolysis towards whey protein in the culture substrate.

It is interesting to note that incubation of *A. oryzae* AHU 7139 at 15ºC with pH 4.0 resulted in abundant protease activity despite the fact that the amount of amino acids released in the resulting culture, which was incubated at 15ºC, was much lower than that at 20 and 25ºC. In addition, the difference between 15ºC and 20/25ºC was also observed in the zymogram. These results demonstrated that the incubation temperature affected the profile of the proteases when incubation of *A. oryzae* AHU 7139 was initiated at pH 4.0. In contrast, protease activity

of *A. oryzae* AHU 7139 at 15ºC initiated at pH 6.5 was half that of the activity at pH 4.0, but the amino acid content of the resulting cultures was comparable. The zymogram depended on the initial incubation pH even under the same incubation temperature, which supports the idea that both the incubation temperature and initial pH value influenced the protease profiles.

For *A. oryzae* AHU 7146, the presence of three or more protease molecules was predicted when *A. oryzae* AHU 7146 was cultured at 20 and 25ºC. In our previous study, *A. oryzae* AHU 7146 was cultured at 25ºC for 14 d on solid-media at pH 6.0 using Czapek-Dox medium with the whey protein isolate replacing sodium nitrate as the restricted nitrogen source, and three molecular species of proteinases were produced [18]. Further characterization revealed that two acidic proteases were dominant, and one alkaline protease was found as a minor component. Although it remains unclear whether the molecular profile of the proteases is identical to the previous results because the carbon source is different; glucose was used in the previous study, whereas glucose-free lactose and lactic acid were included in the present study, it could be concluded that *A. oryzae* AHU 7146 has the ability to secrete multiple protease molecules on the solid whey protein substrate.

In case of *A. oryzae* AHU 7140, the highest protease production was found at 20ºC, however, the amount of amino acid in the substrate was comparable between 15 and 25ºC, which implied that the protease from *A. oryzae* AHU 7140 was less active with whey protein in the culture substrate.

Because *A. oryzae* grew at 15ºC, it was suggested that this species may proliferate under temperatures appropriate for cheese ripening, if whole culture products are used as adjunct materials being attached with mycelium or spore. Thus, filter paper was placed on the solid whey substrate followed by seeding of the fungal spore at the onset of

adjunct material preparation to exclude the mycelium from the solid substrate at the end of the culture. Introduction of the filter paper reduced protease diffusion to the substrate fraction, and only 10~35% of protease activity was recovered compared to the filter-free culture (data not shown). A similar observation was reported in α-amylase production when *A. oryzae* was incubated on sterilized wheat flour in the presence of a polycarbonate membrane [26]. This previous study demonstrated a modification of the fungal physiology by the membrane with respect to the biomass and maximum respiration rate. Therefore, certain effects of the filter were possible; however, we considered that the influence from the living mycelium during ripening should be canceled.

Cheese making was performed according to the procedure for making Gouda type cheese, and no fungal growth was recognized in any experimental cheese products. In spite of the addition of adjunct materials, a significant increase of WSN in experimental cheeses was exclusively observed in cheese II and III, which were prepared using culture products of *A. oryzae* AHU 7139, whose incubation was initiated at pH 6.5. For this reason, some speculations can be made. Adjunct materials were quantitatively insufficient in part due to the use of filter paper, which reduced enzyme diffusion from mycelium to the substrate, and protease activity might be much lower under the ripening temperature of 11.5°C than the experimental measurement at 30°C. In addition, it is conceivable to ascribe flow out of proteases with drained whey when curd received pressing. Moreover, the methodology of WSN extraction should be considered. In this study, WSN was determined based on Kuchroo and Fox's recommendation [23], where the incubation procedure at 40°C for 1 h was included between the sample homogenization and isolation of insoluble substances by centrifugation. However, in order to exclude additional enzyme reactions due to the microbial protease's involvement in the adjunct materials, incubation at 40°C for 1 h was replaced with a heat treatment at 75°C in our study, to inactivate the microbial enzymes. According to Kuchroo and Fox [23], gel filtration of water soluble cheddar cheese extract resolved into 4 fractions, and the former two fractions that represented 30% of the total recovered substances were shown to be aggregates of peptides having higher molecular weights. Therefore, the heat treatment we conducted may cause an interaction between hydrophobic peptides from the casein matrix and promote intermolecular aggregation, which could influence the recovery of WSN-related components.

In terms of flavor, Kataoka et al. [14] found an increase of WSN with a slightly bitter flavor when the Gouda-type cheese matured with commercial protease preparation from *A. oryzae*, whose optimum pH was 5~6. Fedrick et al. [13] found that the addition of neutral protease, Rhozyme 11, extracted from *A. oryzae* could accelerate the ripening of cheddar cheese. However, bitterness and structural defects were noted when an excess amount of enzyme was mixed. Law and Wigmore [12] reported the effect of microbial proteinase on the development of flavor in cheddar cheese and concluded that the addition of Neutrase, neutral proteinase from *Bacillus subtilis*, to curds accelerated the development of typical flavor, in contrast, use of Proteinase R, a commercial acid proteinase from *A. oryzae*, to curds produced a bitter defect even at a low concentration. When the acid protease from *A. oryzae* was applied to Feta cheese, a slight bitter characteristic was recognized during the early stage of ripening, but bitterness disappeared in the following maturation period probably due to the supply of endopeptidases by cell lysis of concomitant lactic microflora [15]. In this study, the control cheese was evaluated to possess typical flavor characteristics of Gouda-type cheese and no bitter flavor was noticed in the 6 experimental cheeses. However, cheese III was rancid and cheese II was noted to be somewhat rancid. In fact, the FFA content was most abundant in cheese III, and its pH value was 5.20~5.30, whereas that of the control cheese was 5.56~5.63 (data not shown) likely due to the accumulation of FFA. Because bovine milk triglycerides contain volatile fatty acids, the release of these molecular species by lipase has a significant influence on the flavor. It is of interest to note that FFA content was remarkably high in *A. oryzae* AHU 7139, which suggested that gene expression related to lipolysis occurred in *A. oryzae* AHU 7139. In contrast to cheese II, there was no significant difference between cheese I and the control cheese in terms of FFA content. Considering these culture conditions, synthesis of the lipolytic enzyme in AHU 7139 might have been regulated by the pH environment. It is unclear how much of the lipase was diffused to the culture fraction in the presence of filter paper, however, the lipolytic effect was more significant than the proteolytic contribution. Because hydrophobic properties of lipase molecules are predicted due to its substrate specificity, lipase might be readily retained in the curd matrix rather than removed with the whey drainage during pressing.

In conclusion, this study demonstrated that both the strain and the culture conditions affect the enzyme production profile of *A. oryzae* in developing the cheese flavor. Traditional fermentation of *A. oryzae* on steamed rice, wheat or soybean generally occurs at 30~35°C and pH 6.0~6.5. However, as shown in the present study, it is worthwhile to expose this species to a low temperature and/or acidic pH condition. Although amylase and protease have been known as pivotal extracellular enzymes in *A. oryzae*, this study indicated that it is also important to monitor lipolytic activity, before cheese-making protocols are applied to this species. Further studies finding suitable strains and culture conditions to obtain products with an optimum balance of proteases and lipases will lead to new insights regarding the potential use of *A. oryzae* to provide enzymes for developing palatable cheese flavor in dairy technology.

## Conflict of Interest

The authors declare that they have no conflict of interest.

## Acknowledgement

## References

1. Machida M (2002) Progress of Aspergillus oryzae genomics. Adv Appl Microbiol 51: 81-106.
2. Yu J, Proctor RH, Brown DW, Abe K, Gomi K, et al. (2004) Genomics of economically significant Aspergillus and Fusarium species. In: Khachatourians GG, Arora DK (eds) Fungal genomics. Applied mycology and biotechnology, Elsevier, Amsterdam 4: 249-283.
3. Tailor MJ, Richardson T (1979) Applications of microbial enzymes in food systems and in biotechnology. Adv Appl Microbiol 25: 7-35.
4. Barbesgaard P, Heldt-Hansen HP, Diderichsen B (1992) On the safety of Aspergillus oryzae: a review. Appl Microbiol Biotechnol 36: 569-572.
5. Ebine H (1986) Miso-kouji In Murakami H (ed) Kouji-gaku (in Japanese) The brewing society of Japan, Tokyo, pp: 256-266.

6.  Hirose Y (1986) Shoyu-kouji. In Murakami H (ed) Kouji-gaku (in Japanese) The brewing society of Japan, Tokyo, pp: 364-369.

7.  Kitano H, Kataoka K, Furukawa K, Hara S (2002) Specific expression and temperature-dependent expression of the acid protease-encoding gene (pepA) in Aspergillus oryzae in solid-state culture (Rice-Koji). J Biosci Bioeng 93: 563-567.

8.  Te Biesebeke R, van Biezen N, de Vos WM, van den Hondel CA, Punt PJ (2005) Different control mechanisms regulate glucoamylase and protease gene transcription in Aspergillus oryzae in solid-state and submerged fermentation. Appl Microbiol Biotechnol 67: 75-82.

9.  Barrios-González J (2012) Solid-state fermentation: Physiology of solid medium, its molecular basis and applications. Process Biochem 47: 175-185.

10. Oda, K, Kakizono D, Yamada O, Iefuji H, Akita O, et al. (2006) Proteomic analysis of extracellular proteins from Aspergillus oryzae grown under submerged and solid-state culture conditions. Appl Environ Microbiol 72: 3448-3457.

11. Nakanishi T, Nakazawa Y (1963) Studies on the cheese flavors II. The relation between the content of the free volatile fatty acids and the degree of ripening of cheese curd with Aspergillus oryzae. (in Japanese) Jpn J Dairy Sci 12: A148-A154.

12. Law BA, Wigmore A (1982) Accelerated cheese ripening with food grade proteinases. J Dairy Res 49: 137-146.

13. Fedrick IA, Aston JW, Nottingham SM, Dulley JR (1986) The effect of a neutral fungal protease on cheddar cheese ripening. N Z J Dairy Sci Technol 21: 9-19.

14. Kataoka K, Nakae T, Ueno M, Nukada K, Otani K (1987) Accelerated cheese ripening by added enzymes with special reference to chemical properties. Nihon Chikusan Gakkaiho (in Japanese) 58: 107-115.

15. Vafopouloua A, Alichanidisa1 E, Zerfiridisa G (1989) Accelerated ripening of Feta cheese, with heat-shocked cultures or microbial proteinases. J Dairy Res 56: 285-296.

16. Machida M, Asai K, Sano M, Tanaka T, Kumagai T, et al. (2005) Genome sequencing and analysis of Aspergillus oryzae. Nature 438: 1157-1161.

17. Kitamoto K (2015) Cell biology of the Koji mold Aspergillus oryzae. Biosci Biotechnol Biochem 79: 863-869.

18. Kumura H, Ishido T, Shimazaki K (2011) Production and partial purification of proteases from Aspergillus oryzae grown in a medium based on whey protein as an exclusive nitrogen source. J Dairy Sci 94: 657-667.

19. Kumura H, Mikawa K, Saito Z (1991) Influence of concomitant protease on the thermostability of lipase of psychrotropic bacteria. Milchwissenschaft 46: 144-149.

20. Raser KJ, Posner A, Wang KK (1995) Casein zymography: a method to study mu-calpain, m-calpain, and their inhibitory agents. Arch Biochem Biophys 319: 211-216.

21. IDF (2004) Cheese and Processed Cheese. Determination of the total solid content. : ISO 5534/IDF 4: 2004. International Dairy Federation, Brussels.

22. AOAC (1995) Chloride in cheese in Official Methods of Analysis of AOAC International, 16th ed Official Method 983.14. Washington, DC.

23. Kuchroo CN, Fox PF (1982) Soluble nitrogen in cheddar cheese: Comparison of extraction procedures. Milchwissenschaft 37: 331-335.

24. AOAC (1995) Nitrogen in cheese in Official Methods of Analysis of AOAC International, 16th ed Official Method 920.123. Washington, DC.

25. AOAC (1995) Fat in cheese in Official Methods of Analysis of AOAC International, 16th ed Official Method 933.05. Washington, DC.

26. Rahardjo YS., Korona D, Haemers S, Weber FJ, Tramper J, et al. (2004) Limitations of membrane cultures as a model solid-state fermentation system. Lett Appl Microbiol 39: 504-508.