# A study of lipolysis induced by adjuncts from edible *Aspergillus* sp. solid culture products on ripened semi-hard cheese

**Short running title: Lipolysis induced by edible *Aspergillus* sp. adjunct on ripened cheese**

Napaporn Chintagavongse[1], Hayate Takiguchi [1], Chi Ming-Hsuan[2], Koichi Tamano[3], Toru Hayakawa[1], Jun-ichi Wakamatsu[1], Tomohiro Mitani[4], Haruto Kumura[1,*]

*[1]Laboratory of Applied Food Science, Graduate School and Research Faculty of Agriculture, Hokkaido University, 060-8589, N9, W9, Sapporo, Japan; [2]National Taiwan Ocean University; [3]Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), 2-17-2-1 Tsukisamu-Higashi, Toyohira-ku, Sapporo, Hokkaido 062-8517, Japan; [4]Laboratory of Animal Production System, Graduate School and Research Faculty of Agriculture, Hokkaido University, 060-8589, N9, W9, Sapporo, Japan*

* Corresponding author: Haruto Kumura

Mailing address: Laboratory of Applied Wood Science, Graduate School and Research Faculty of Agriculture, Hokkaido University, 060-8589, N9, W9, Sapporo, Japan

Tel: +81-11-706-2817, E-mail: uraki@for.agr.hokudai.ac.jp

**ABSTRACT**

BACKGROUND: *Aspergillus* sp. has been used in traditional Japanese fermented foods. The protease-containing culture products of *A. oryzae* have been applied as the adjunct enzyme source to enrich the flavor in ripened cheese. Although proteolysis was stimulated, the increase of free fatty acids (FFA) was recognized in some products. Since an excess amount of FFA accumulation could cause rancidity in cheese products, the assessment of lipase activity was considered to be essential for the cheese adjunct preparation.

RESULT: Although an equal lipase activity from the adjunct materials of *A. kawachii* NBRC 4308, *A. luchuensis* RIB 2604, and *A. oryzae* AHU 7139 was applied to semi-hard cheese, the FFA level was significantly higher in *A. oryzae* cheese than in the others. Furthermore, the profiles of volatile components were different in experimental cheeses. Application of *in vitro* study with experimental curds demonstrated that the high FFA might not depend on the lipase retainability on curds. On the contrary, the pronounced activation of the lipases occurred in *A. oryzae* after incubation with the curds. Moreover, incubation of the insoluble lipase that had been attached to the cells with skim milk curd extracts allowed the release of lipases from the cells into the medium with remarkable activation.

CONCLUSION: *A. oryzae* AHU 7139 possessed a complex lipolytic system comprising extracellular and cell-binding lipases that were attributed to the increase in FFA in *A. oryzae* cheese.

**KEYWORDS:** lipase; *Aspergillus* sp.; curd peptides; free fatty acids; ripened cheese

## INTRODUCTION

The genus *Aspergillus* has been used in traditional Japanese fermented products such as miso, shoyu, and alcoholic beverages.[1,2] In the traditional protocol, suitable *Aspergillus* strains have been seeded on solid cereal materials such as steamed rice, soybean, and wheat for koji preparation to obtain enzyme cocktails containing amylases and proteases, which contribute to the fermentation of the corresponding products.[3] Genomic analysis of *A. oryzae*, a representative species of this genus, revealed that it encodes about 12,000 genes, 135 of which are thought to be related to proteases.[4] Focusing on this high proteolytic potential along with its food safety, we have proposed a semi-hard cheesemaking protocol with the solid culture products of *A. oryzae* as flavor-enrichment adjuncts.[5] In this protocol, an adjunct material was mixed with the fresh cheese curds followed by pressing and ripening to stimulate proteolysis in the products. Although *A. oryzae* adjuncts promoted proteolysis during cheese ripening, it led to a remarkable free fatty acid (FFA) accumulation in some cheese products. Although FFA accumulation during cheese ripening could be attributed to the action of lipases from several sources,[6] involvement of endogenous milk lipase is unlikely when the cheese milk had been pasteurized due to its heat lability.[7] Moreover, added rennet and starter culture activity had less impact of lipolysis than the additive *Aspergillus* culture products which was monitored in the control cheese.[5] Thus, the lipase from an adjunct material was thought to be the significant factor for FFA accumulation. Pronounced release of volatile fatty acids from milk fat could cause an undesirable flavor defect in the finished cheese products known as rancidity.[8] Accordingly, in order to take advantage of the proteolytic potential of *A. oryzae*, not only proteases but also lipases should be investigated when *Aspergillus* culture products are intended to use for cheesemaking adjuncts.

Based on the simple presumption, if the fungal enzyme activity levels in the adjuncts increase, their respective reaction products in cheese would be parallelly increased. In fact, Kataoka et al.[9] used several commercial proteases to accelerate cheese ripening with the similar cheesemaking procedure as we did.[5] They found that the water-soluble nitrogen (WSN), an index of cheese maturity, increased in a dose-dependent manner when they added the same enzyme source in different amounts. However, they recognized that the effect did not always correlate with the protease activity when different protease sources were used. Specifically, the WSN level of 4-month-old cheese using 0.05 U of acidic protease from *A. oryzae* was comparable to that of using 0.005 U of neutral protease from *B. subtilis*. Regarding lipase, similar inconsistency between lipase activity and FFA in the resulting products can be postulated.[10,11] Considering the hydrophobic feature of cheese curds, formed by a network of para-casein micelles, hydrophobic interaction is likely to occur between cheese curds and the lipases during the mixing procedures of the adjuncts into curds followed by pressing with whey drainage. Assuming that the entrapment of the lipase molecules in the curds specifically depends on the degree of its affinity toward cheese curds, the correlation between lipase activity in a supplied adjunct and the resulting FFA content in ripened cheese products would be unpredictable.

In this study, we prepared three adjunct materials containing an equivalent lipase activity using different species of *Aspergillus* to mix with the fresh cheese curds followed by pressing and ripening in order to compare the FFA amount in the products. Since a remarkable increase of FFA in the cheese was recognized with the adjunct of *A. oryzae*, its cause was investigated from the viewpoint of the retainability of lipases on the curds with a simulated experiment to measure the soluble lipase activity before and after incubation with the curds. Furthermore, as the prepared adjunct material was

proved to include both extracellular and intracellular lipases[12], fractionation of the lipases from *A. oryzae* adjunct materials was carried out to specify the fraction responsible for the FFA increase in *A. oryzae* cheese. Finally, we proposed the possible mechanism of FFA accumulation during cheese ripening that would take place when the adjunct materials from *A. oryzae* are supplemented.

## MATERIALS AND METHODS

### Strain and culture condition

The strains used in this study were as follows; *A. kawachii* NBRC 4308 from Biological Resource Center (Chiba, Japan), *A. luchuensis* RIB 2604 kindly gifted by National Research Institute of Brewing (Hiroshima, Japan) and *A. oryzae* AHU 7139 from the culture collection of Hokkaido University. Then the spore suspension was prepared as described in previous study[12] to a concentration of $2.5 \times 10^5$ spores $mL^{-1}$.

Twenty-five grams of WPC80 (Fonterra, Auckland, New Zealand) was dissolved in 75 g of deionized water and adjusted to pH 4.0 with lactic acid. The solution was divided (10 g) into 100 mL Erlenmeyer flask each and autoclaved at 121°C for 15 min to prepare the solid medium. The medium was inoculated with 150 μL of the spore suspension and cultivated at 20°C for 7 days.

### Preparation of adjunct materials for cheese making

The adjunct material from culture products (CP) was prepared by recovery of the 10 g CP from flasks, pooled to freeze dry and then ground with a mortar and pestle to obtain freeze-dried powder (FDP), which was regarded as the adjunct material. On average, 0.27 g of FDP was obtained from 1 g of CP. As a control, un-inoculated solid medium was prepared likewise to obtain un-inoculated freeze-dried powder (UIFDP).

## Measurement of enzyme activity in the adjunct materials

The suspension of the FDP (0.27 g) dispersed in 2 mL of 0.02 mol $L^{-1}$ sodium phosphate buffer, pH 7.0 was used. The lipase activity was determined using 1 g of butter oil emulsified with 100 mL of 2% polyvinyl alcohol (degree of polymerization 2000, Kishida Chemical, Osaka, Japan) as the substrate. The substrate was divided into 2 mL into each test tube and subjected to pre-incubation at 15ºC for 5 min, followed by an addition of the enzyme to be tested (50 µL) and 0.5 M sodium phosphate-citric acid buffer pH 5.5 (250 µL). The reaction was carried out at 15ºC for 30 min and terminated by adding 7.5 mL of extract solution (heptane : isopropanol : 0.5 mol $L^{-1}$ sulfuric acid = 48:48:4). The extracted FFA was determined by the phenol-red method.[13, 14] Enzyme activity was expressed as µmol of released oleic acid from the substrate per 1 h at the defined temperature from 1 g of the CPs (1 µmol oleic acid/h/g-CP = 1 lipase unit: LU).

The proteolytic activity was determined as previously described with some modifications.[12] The enzyme reaction was incubated at 30ºC for 1 h. Enzyme activity was expressed as µmol of released tyrosine per h at 30ºC extracted from 1 g of the CPs (1 µmol tyrosine/ h/ g-CP = 1 protease unit: PU).

## Cheesemaking

The cheesemaking was carried out according to the conventional procedure for Gouda-type cheesemaking as previously described.[12] Raw milk was obtained from the experimental farm in the Field Science Center for Northern Biosphere, Hokkaido University, and 105 kg of standardized milk (3% fat) was used in this study. After whey drainage, 1 kg of the recovered curds was mixed with each FDP and UIFDP mixture (total 2.16 g) to adjust to an equivalent lipase activity in all prepared experimental cheeses, and only UIFDP was mixed with the curds as the control cheese (Table 1). The

143 control and experimental cheeses were prepared in duplicate. Following brief pressure
144 (1.0 kg/cm$^2$ for 15 min), inversion and the second stage of pressure (1.2 kg/cm$^2$ for 50
145 min) were performed. The 1 kg cheese blocks were cooled in water overnight then
146 salted with brine (25% NaCl, w/w) for 12 h. The cheeses were matured at 11.5ºC with a
147 relative humidity of 85% for 12 weeks. After 28 days of the manufacture, the cheese
148 surface was coated with wax (Paramelt, Netherland) and left for ripening.

149 **Chemical analysis of cheese**

150 The moisture, fat, protein, and water soluble nitrogen (WSN) were determined as
151 previously described.[12] For free fatty acids (FFAs) determination, the cheese sample
152 (0.2 g) was transferred to 2 mL of 7.7 M HCl in a test tube and placed into boiling water
153 to dissolve the sample. FFAs were extracted by the same extraction reagent as was used
154 in the lipase activity measurement to determine by the phenol-red method[14] and its
155 content was expressed as (mmol) in 1 kg of cheese using oleic acid as the standard. Salt
156 calculated from chloride content were measured as previously described.[5] All assays
157 were performed in triplicate.

158 **Volatile compound analysis in cheese**

159 The cheese samples were chopped by food processor and 5 g samples were weighed
160 in 20 mL vials and closed with a PTFE septa and magnetic cap. Volatile organic
161 compounds (VOCs) in the cheese were extracted using a headspace solid-phase micro-
162 extraction (SPME) technique with an auto-sampler (AOC-5000 plus; Shimadzu Co.,
163 Kyoto, Japan). The vials were placed into a heat block at 45°C for 5 min, then absorbed
164 into SPME fiber (2 mm of 50/30 μm DVB/CAR/PDMS fiber; Supelco, Bellefonte, PA)
165 at 45°C for 20 min. Before analysis of the first sample, the fiber was baked at 250°C for
166 30 min. After absorption, the fiber was inserted into the gas chromatograph/mass

spectrometer (GCMS-QP2010 Ultra; Shimadzu Co., Kyoto, Japan). The analysis was conducted in split mode with the following conditions: injector temperature 250°C; split ratio 5:1; helium carrier gas; linear velocity 50 cm/s. The VOCs were separated on a fused silica capillary column (DB-WAX 30 m length × 0.25 mm internal diameter, Agilent Technologies Inc., Santa Clara, CA) with a temperature-rising condition (initial oven temperature at 40°C, held at 40°C for 1 min, increased by 16°C/min to 120°C, increased by 8°C/min to 200°C, and held at 200°C for 19 min, total time of 35 min).

The mass spectrometer was operated in the electron impact ionization mode at 70 eV; the mass range used was *m/z* 29 to 300. The mass spectrometer interface and ion source temperatures were 210°C and 200°C, respectively. The VOCs were identified by comparison with the mass spectra of the National Institute of Standards and Technology (NIST) 11 Mass Spectral Library (NIST, Gaithersburg, MD). The peak of the VOC was integrated from specific ions for each molecule to avoid overlapping between compounds.

**Retention of fungal lipase onto the experimental curds**

Experimental curds were prepared using skim milk, which contained neither milk lipid nor lactic starter culture. Five kilograms of skim milk were heated at 72ºC for 15 sec and cooled down to 31ºC. Lactic acid solution was then added to adjust the pH to 5.5. After the addition of 0.3 g rennet (Chr. Hansen, Hoersholm, Denmark) dissolved in 0.25 mol $L^{-1}$ NaCl, subsequent procedures were the same as the cheesemaking. The curds were stored at –20°C until use.

As illustrated in Supplementary Figure 1, two test tubes containing the FDP (40 mg) of each strain dispersed in 4 mL simulated milk ultrafiltrate (SMUF)[15] pH 5.5, were prepared and pre-incubated in a water bath at 30ºC for 10 min. Either of them received

4 g of the skim milk curds. Subsequently, both samples were homogenized (IKA® T25 disperser with S25N-8G tool, Germany) at 15,000 rpm for 3 min, followed by incubation at 30°C for 30 min. Then the tubes with curds were filtered through coarse filter paper (Azumi, Japan) and both samples were centrifuged at 21,130 × g at 25ºC for 15 min. Three hundred microliters of the recovered supernatant were added to 2 mL of the substrate and incubated at 30ºC for 30 min to determine lipase activity. In this experiment, butter oil was replaced with olive oil as the substrate.

The whole enzyme from *A. oryzae* AHU 7139 was further fractionated into extracellular and intracellular enzymes. The extracellular enzyme was extracted by adding an equal weight of deionized water to CP and centrifuged at 21,130 × g, at 4ºC for 10 min. The precipitates were dispersed in deionized water to extract the remaining extracellular enzyme. This additional extraction was performed twice, and these extracts were pooled to use as the extracellular enzyme. The precipitate was washed with deionized water once again and centrifuged at 21,130 × g at 4ºC for 10 min. Then, the precipitate was freeze-dried and ground. A suspension of the freeze-dried powder in SMUF was used as the intracellular enzyme. To fractionate the intracellular enzymes, the freeze-dried powder was dispersed in SMUF at 30ºC for 30 min and centrifuged at 21,130 × g at 4ºC for 10 min to recover the supernatant, which was used as the water-soluble intracellular enzyme. The precipitate was washed by deionized water three times to remove any remaining water-soluble intracellular enzyme. Then, the precipitate was suspended in SMUF to use as the insoluble enzyme. Thus, three enzymes, namely from the extracellular, intracellular soluble, and insoluble fractions, were used in the presence or absence of skim milk curds in the same manner shown in Supplementary Figure 1.

### Analysis of SMUF extractable peptides from skim milk curds by SDS-PAGE

To observe the curd components relevant to cell-binding lipase solubilization, 1 g of the skim milk curds was mixed with 1 mL of SMUF pH 5.5 to homogenize (IKA® T25 disperser with S25N-8G tool, Germany) at 15,000 rpm for 3 min followed by filtration. The filtrate was subjected to SDS-PAGE using 12.5% polyacrylamide gel.[16] Whole casein (0.2% casein in 0.05 mol $L^{-1}$ Tris-HCl buffer pH 7.0) and commercial protein standard (DynaMarker® MultiColor III, Biodynamics laboratory, inc., Tokyo, Japan) were also loaded, and the gel was stained with Coomassie Brilliant Blue R-250.

### Statistical analysis

The composition of water, salt, total protein, total lipid, WSN in total nitrogen, and FFA content in the cheeses were analyzed using Tukey-Kramer's multiple comparison test. The data were analyzed by JMP software (version 11.0; SAS Institute, Inc., Tokyo, Japan). Differences were considered to be statistically significant at $p < 0.05$. The difference in the amounts of VOCs in cheese samples made using different adjuncts from *Aspergillus* strains was analyzed using a principal component analysis (PCA).

## RESULTS

### Lipase and protease activity in adjunct materials

The amount of each culture FDP for 1 kg of cheesemaking was adjusted to an equivalent level of lipase activity (Table 1), so the initial protease activity was variable depending on the adjunct materials. The highest protease was supplied through the adjuncts from *A. oryzae* with 85.0 PU, which was 4.6 times more than that of *A. kawachii* (18.5 PU).

## Cheese composition

After 12 weeks of ripening, all the experimental cheeses were analyzed. The results of the chemical analysis of the experimental cheeses are shown in Table 2. The moisture, salt, lipid, and protein content were not significantly different between the four types of cheese. However, every *Aspergillus* adjunct caused a higher FFA level, in particular, the *A. oryzae* adjunct resulted in a significantly higher amount of FFA compared to the other three types. In contrast, WSN amounts increased in a dose-dependent manner with proteolytic activity in these three adjuncts.

## Volatile organic components

VOC levels were evaluated using an SPME technique and the values were expressed as the peak area. Then PCA was performed to show the relative VOC amounts in the cheese samples (Figure 1). Thirty-four volatile compounds were detected in the experimental cheeses and were categorized into 6 different compound groups, including 11 FFA, 7 ketones, 7 hydrocarbons, 4 ethyl esters, 3 alcohols, and 2 aldehydes. Although all 34 VOCs were found in each of the samples, the dominant VOC levels differed with each adjunct used. The control cheese had high amounts of acetoin, toluene, and alcohols such as ethanol, 1-octanol, and 2,3-butanediol, while the *A. kawachii* and *A. luchuensis* cheeses had higher proportions of ketones, including acetone, 2-pentanone, and 2-nonanone, and 3-methyl-butanal. Benzaldehyde, 2-undecanone, FFA, and ethyl ester amounts were higher in the *A. oryzae* cheese than in the others. Accordingly, these *Aspergillus* adjuncts provided their characteristic VOCs in the resulting cheese products.

259 **Retention of fungal lipases onto the experimental curds**

260 Table 3 shows the distribution of lipase activities in the presence or absence of the
261 skim milk curds using the whole enzyme or its fractions dispersed in SMUF. Lipase
262 activity of *A. kawachii* was reduced from the initial of 601.1 LU to 399.2 LU after
263 incubation with the curds, which implies that about 30% of the lipase was entrapped in
264 the curds. In contrast, the initial lipase activity of *A. luchuensis* (307.9 LU) was
265 maintained after incubation with the curds (313.6 LU), which shows the negligible
266 affinity of *A. luchuensis* lipase towards the skim milk curds. It was of interest to note
267 that the unexpected activation was recognized in *A. oryzae,* as its lipase activity
268 increased by 7.2 times.

269 Because of the remarkably high FFA in *A. oryzae* adjunct cheese and the pronounced
270 lipase activation after incubation with curds, *A. oryzae* was selected for further study to
271 identify the related fraction. Fractionation of *A. oryzae* FDP revealed that the
272 intracellular fraction was more obvious in terms of activation ratio after incubation with
273 the curds compared to the extracellular one (6.4 and 1.7 times, respectively). Since the
274 intracellular fraction comprises soluble and insoluble components, they were
275 fractionated into cytosolic soluble and insoluble fractions. The degree of the activation
276 of the intracellular soluble fraction was comparable to that of the extracellular one. In
277 contrast, the degree of activation of the intracellular insoluble fraction was unavailable
278 since lipase activity was negligible in the recovered supernatant of the insoluble
279 fraction. When the insoluble materials were suspended in SMUF with no incubation
280 with the curds to evaluate its lipase activity, 57.6 LU of the activity was detected, which
281 showed that the lipase activity being attached to the cell components was lower than
282 intracellular soluble lipase activity (72.0 LU). However, incubation of the insoluble
283 fraction with the curds caused the release of the lipase from the cell components into the

284 supernatant, and the recovered activity was 244.8 LU, which was as much as 4.3 times
285 of the activity on the cells (57.6 LU).

286 **Analysis of extracted skim milk curd peptides**

287 The above results strongly suggested that the release of the cell-binding lipases of *A*.
288 *oryzae* relied on the soluble components in the skim milk curds. Thus, SDS-PAGE of
289 the curd extract using SMUF was performed to observe the proteinaceous profile.
290 Despite high hydrophobicity of milk curds, numerous peptides were extracted and
291 recovered in the soluble fraction by the addition of SMUF, as shown in Figure 2.
292 Polypeptides migrated faster than the intact caseins were detected, which implied that
293 they were derived from casein degradation. Two faint bands representing higher
294 molecular mass than the intact caseins were estimated to be 66 and 70 kDa and assumed
295 to be serum albumin and lactoperoxidase, respectively.

296 **DISCUSSION**

297 Since the lipases in dairy products can cause rancidity, it is crucial to select proper
298 *Aspergillus* when they are attempted to be used as the cheese adjuncts. Despite addition
299 of equivalent lipase activity prepared from three sorts of *Aspergillus* adjunct materials, a
300 significantly higher FFA level was found in the *A. oryzae* cheese. Therefore, we
301 attempted to clarify the reason for the different levels of FFA in the resulting products
302 from the point of view of the lipase affinity with the curds using laboratory-scale
303 prepared skim milk curds. Through focusing on the lipase fractions from *A*. *oryzae*, the
304 possible mechanism behind the higher level of FFA in its adjunct cheese was postulated.
305 At the first step, the adjunct materials with an equivalent lipase activity were
306 prepared using three species of *Aspergillus* strains to produce cheese and the FFA levels
307 of the resulting products were compared. We determined the lipase activity in the three

FDP adjuncts under the acidic and low-temperature condition using butter oil as a substrate to simulate conditions close to cheese ripening. Since the higher lipolytic activity was detected in the culture products from *A. kawachii* and *A. luchuensis* than that of *A. oryzae*, the amounts of FDP adjunct for these two experimental cheeses were reduced. *A. kawachii* and *A. luchuensis* have been used as the starter koji-mold for making shochu. Accordingly, attention has been paid solely to amylase and glycosidase rather than to protease and lipase productivity.[17,18] However, comparable or even higher protease and lipase activity for *A. kawachii* have been found in rice-koji for Doenjang making, compared to *A. oryzae*.[19] Thus, the species other than *A.oryzae* were thought to be deserved to be applied for cheese adjunct preparation. As expected, the degree of WSN generated was entirely consistent with the protease activity involved in the adjunct materials showing the feasibility of these adjunct materials to promote proteolysis as was shown in our previous study.[5] *A. luchuensis* adjunct materials provided a comparable level of WSN to *A. oryzae* whereas lower FFA levels as compared to the control cheese, which suggests the potential for the development of rancidity free with higher proteolysis cheese. Although it is a beyond scope of this study, further comparison of the peptides and free amino acids profiles would clarify the traits of the two adjunct cheese more specifically because undesirable proteolysis could cause generetion of bitter taste. On the contrary, the higher impact of the *A. oryzae* adjunct material on FFA content was confirmed despite the supplement of equivalent activity of lipase. Furthermore, the volatile compound analysis clearly showed that the *A. oryzae* cheese produced FFAs in addition to ethyl esters, benzaldehyde, and 2-undecanone, whereas the major odor components found in the *A. kawachii* and *A. luchuensis* cheeses were acetone, 2-pentanone, 2-nonanone, and 3-methyl-butanal, which was similar to be found in the well-known mold cheese using *Penicillium roqueforti* and *P. camemberti*,

333 which provide a high concentration of methyl ketones, mainly 2-nonanone and 2-
334 heptanone in blue and Camembert cheese.[20] Thus, characteristic traits of cheese were
335 able to be developed using the adjunct material from these three strains in terms of
336 proteolysis, lipolysis, and odor profile.

337 Our original assumption of the high FFA accumulation in the *A. oryzae* cheese
338 product was a high level of fungal lipase retainability onto the curd matrix. In this
339 context, the lipase from *A. oryzae* was hypothesized to be more adsorbable onto the
340 curds, while that from *A. kawachii* and *A. luchuensis* was not expected as much. In this
341 cheese manufacture protocol, the adjunct materials were mixed with curds followed by
342 pressing, which drained the whey. Accordingly, we introduced *in vitro* simulation to
343 evaluate how much lipase activity would be drained away with whey. If the ratio of the
344 lipase entrapped in the curds is higher in *A. oryzae* than *A. kawachii* and *A. luchuensis,*
345 lower lipase activity should be remained in the soluble fraction after incubating with the
346 curds *in vitro*. However, high activation was contrarily recognized in *A. oryzae* lipase
347 after incubation. Even though the skim milk curds were replaced with the conventional
348 cheese curds containing milk lipids and lactic starter, this activation of *A. oryzae* lipase
349 was confirmed as well, which suggests that milk lipids and lactic acid bacteria are not
350 concerned in this activation (data not shown). Furthermore, the affinity of *A. kawachii*
351 lipase toward cheese curds was noted, despite no remarkable FFA increase having taken
352 place in the *A. kawachii* cheese. Based on these conflicting results, it might be
353 concluded that the higher accumulation of FFA in the experimental cheese might not
354 depend on an affinity of the lipase onto cheese curds, and another explanation must be
355 proposed.

356 Then, we focused on the remarkable lipase activation of adjunct material from *A.*
357 *oryzae* AHU 7139 after incubation with the curds. The lipases involved in the adjunct

358 materials from *A. oryzae* AHU 7139 were categorized into three types:, extracellular,
359 intracellular-soluble lipase, and intracellular-insoluble lipase that was regarded as the
360 cell-binding lipase. As shown in Table 3, the extracellular lipase took the highest ratio,
361 followed by the intracellular-soluble and the cell-binding fraction in the absence of
362 contact with the curds. However, incubation with the curds caused the activation of
363 every fraction, in particular, the cell-binding fraction. Furthermore, this study
364 demonstrated that the lipase originally bounded on the cells, as the insoluble form was
365 turned to be soluble through the incubation with the curds. In this regard, it might make
366 us underestimated the lipolytic potential when we decided the adjunct amount for the
367 cheese manufacture because we had not noticed such kind of release and activation of
368 the cell-binding lipase due to the curds. Several extracellular lipases of *A. oryzae* have
369 been studied,[21] and we confirmed that *A. oryzae* AHU 7139 produces at least two types
370 of extracellular lipases by the column chromatography, one of which was identified as
371 the known diacylglycerol lipase with no specificity towards triglycerides.[22] The
372 production of a diacylglycerol lipase is not specific for *A. oryzae* because *A. kawachii*
373 and *A. luchuensis* produce this type of lipase as well.[17,23] However, digestibility of the
374 adjunct material from *A. oryzae* AHU 7139 towards purified diolein was 3.3- and 3.5-
375 fold as high as that from *A. kawachii* NBRC 4308 and *A. luchuensis* RIB 2604,
376 respectively (data not shown), showing that the capability of degradation for the
377 subsequent substrate of diglycerol in adjunct metarial from *A. oryzae* AHU 7139 was
378 much higher than that from other two strains. Thus, it can be concluded that the higher
379 digestibility of diacylglycerol lipase in *A. oryzae* together with triacylglycerol lipase
380 possibly promoted the high lipolysis in the ripening cheese. In contrast, limited
381 information has been available regarding the cell-binding lipases of this species.
382 Considering its unique responsibility towards the curd components, further observation

383 of cell-binding lipases is needed to investigate their interaction with the c:and
384 subsequent release from the cells, in addition to their molecular characterization and/or
385 location in the fungal cells.

386 According to the general understanding, cell-binding protein can be solubilized with
387 surfactant. In contrast, skim milk curds contain neither surfactant nor emulsifier, while
388 it contained calf rennet comprising chymosin as the major protease and pepsin as the
389 minor one. Thus, we first assumed that the addition of rennet solution to the insoluble
390 fraction allows the release of the lipases into the soluble fraction. However, no lipase
391 activity was found after treatment of cell binding fraction with rennet solution without
392 curds (data not shown). Then, we focused on what proteinaceous components in the
393 curds were extracted accompanied by the solubilized cell-binding lipase. Chymosin
394 specifically cleaves the Phe105–Met106 bond in bovine milk κ-casein[24] in the early
395 stage of the standard cheese-making protocol. However, at the later stage, the pH of the
396 curd drops due to the growth of the lactic starter. Since chymosin exerts its activity
397 under acidic conditions of pH 3 – 6,[6] it could turn out to be active on the other casein
398 components, in addition to κ-casein. In this study, the skim milk curds were prepared at
399 pH 5.5, which is close pH circumstance at the beginning of the cheese ripening and we
400 found several peptides in their extracts on SDS-PAGE. Most of them were considered
401 to be from $\alpha_{s1}$-casein and β-casein, which are major components in the skim milk curds.
402 Green[25] reviewed cleavage sites of chymosin towards $\alpha_{s1}$-casein and β-casein under
403 acidic conditions. Both are amphiphilic,[26,27] and some of their peptides have been shown
404 to exhibit emulsifying properties.[28,29] Thus, these casein-derived peptides could work as
405 surfactants and release the lipases attached to the cells into the soluble fraction.

406 Overall, we postulated a flow of the elevated FFA in *A. oryzae* cheese, as illustrated
407 in Figure 3. In the adjunct preparation step, The pressing procedure prompted draining

the extracellular and intracellular soluble lipases with whey, while the cell-binding lipases from the FDP adjuncts were released from the fungal cell-matrix and turned into a soluble form in the cheese block due to the curd-derived peptides, which are relevant to the activation of the lipase as well. Consequently, the released and activated cell-bounded lipases along with the extracellular lipase responsible for triglycerides that remained in the curds degrade the intact milk lipids as the first step. Then, the resulting diglycerides were supplied to the subsequent substrates for diacylglycerol lipase as the second lipolysis step. These consequences are likely to be the cause of the elevated FFA in the *A. oryzae* cheese and produce an excess amount of volatile fatty acid over the threshold, leading to rancid flavor in *A. oryzae* cheese products.

In conclusion, the progressed lipolysis in the *A. oryzae* adjunct cheese was attributed to synergic effect of the predicted extracellular lipase responsible for triglycerides and the cell-binding lipases followed by diacylglycerol lipase that was lacking digestability towards triglycerides. This study uncovered the significance of cell-binding lipase when the culture products of *Aspergillu*s were applied as adjunct materials for cheese flavor enrichment. Furthermore, curd-derived peptides were proven to be concerned in FFA accumulation of the *A. oryzae* cheese. Our finding will pave the way to improve cheese adjunct materials using genus *Aspergillus* as the rancid-free cheese flavor enrichment.

**ACKNOWLEDGEMENTS**

The authors are very grateful to Yu Toba and Hiroki Matsumo, Field Science Center for Northern Biosphere, Hokkaido University, for their assistance in cheese manufacturing.

## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

## REFERENCES


1 Gomi K, *Aspergillus* | *Aspergillus oryzae*, in *Encyclopedia of Food Microbiology,* 2nd ed., ed. by Batt CA and Tortorello ML. Academic Press, US, pp. 92-96 (2014).

2 Park HS, Jun SC, Han KH, Hong SB and Yu JH, Chapter Three - Diversity, application, and synthetic biology of industrially important *Aspergillus* fungi, in *Advances in applied microbiology*, ed. by Sariaslani S and Gadd GM. Academic Press, UK, pp. 161-202 (2017).

3 Kitamoto K, Molecular biology of the koji molds. *Adv Appl Microbiol* **51**:129-153 (2002).

4 Machida M, Asai K and Sano M, Genome sequencing and analysis of *Aspergillus oryzae. Nature* **438**: 1157–1161 (2005).

5 Kumura H, Saito C, Taniguchi Y, Machiya, T, Takahashi Y and Kimura A, Adjunctive application of solid-state culture products from *Aspergillus oryzae* for semi-hard cheese. *J Adv Dairy Res* **5**: 188 (2017).

6 Garcia HS, López-Hernandez A and Hill CG, Enzyme technology - Dairy industry applications, in *Comprehensive Biotechnology,* 3rd ed., ed. by Moo-Young M. Pergamon, UK, pp. 608-617 (2017).

7 Shipe WF and Senyk GF, Effects of processing conditions on lipolysis in milk. *Int J Dairy Sci* **64**(11): 2146-2149 (1981).

8 Ray PR, Chatterjee K, Chakraborty C and Ghatak PK, Lipolysis of milk: A Review. *Int J Agric Vet Sci* **1**: 58-74 (2013).

9 Kataoka K, Nakae T, Ueno M, Nukada K and Otani K, Acceclerated chesse ripening by added enzymes with special reference to chemical properties. *Jap J Zootech Sci* **58**(2): 107-115 (1987).

10 Aminifar M and Emam-Djomeh Z, Changes of texture, microstructure and free fatty acid contents of Lighvan cheese during accelerated ripening with lipase. *J Agric Sci Technol* **16**: 113-123 (2014).

11 Wang B and Xu S, Effects of different commercial lipases on the volatile profile of lipolysed milk fat. *Flavour Fragr J* **24**: 335–340 (2009).

12 Chintagavongse N, Yoneda T, Ming-Hsuan C, Hayakawa T, Wakamatsu J, Tamano K, and Kumura H, Adjunctive application of solid-state culture products and its freeze-dried powder from *Aspergillus sojae* for semi-hard cheese. *J Sci Food Agric* **100**: 4834-4839 (2020).

13 Saito Z, Application of the phenol-red method for investigations on the lipolysis of raw milk. *Jap J Zootech Sci* **50**: 710-715 (1979).

14 Kumura H, Mikawa K, and Saito Z, Influence of concomitant protease on the thermostability of lipase of psychrotrophic bacteria. *Milchwissenschaft* **46**(3):144-149 (1991).

15 Jenness R and Koops J, Preparation and properties of a salt solution which simulates milk ultrafiltrate. *Neth Milk Dairy J* **16**: 153-164 (1962).

16 Laemmli U, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685 (1970).

17 Yamada O, Machida M, Hosoyama A, Goto M, Takahashi T and Futagami T et al., Genome sequence of *Aspergillus luchuensis* NBRC 4314. *DNA Res* **23**(6): 507–515 (2016).

18 Omori T, Takeshima N and Shimoda M, Formation of acid-labile α-amylase during barley-koji production. *J Ferment Bioeng* **78**: 27-30 (1994).

19 Kum SJ, Yang SO, Lee SM, Chang PS, Choi YH and Lee JJ et al., Effects of *Aspergillus* species inoculation and their enzymatic activities on the formation of volatile components in fermented soybean paste (doenjang). *J Agric Food Chem* **63**(5): 1401-1418 (2015).

20 Chávez R, Fierro F, García-Rico RO and Laich F, Chapter 5 Mold-fermented foods: *Penicillium* spp. as ripening agents in the elaboration of cheese and meat products, in *Mycofactories*, ed. by Leitao AL. Bentham Science Publisher Ltd., United Arab Emirates, pp. 73-98 (2011).

21 Toida J, Arikawa Y, Kondou K, Fukuzawa M and Sekiguchi J, Purification and characterization of triacylglycerol lipase from *Aspergillus oryzae*. *Biosci Biotechnol Biochem* **62**(4): 759-763 (1998).

22 Toida J, Kondoh K, Fukuzawa M, Ohnishi K and Sekiguchi J, Purification and characterization of a lipase from *Aspergillus oryzae*. *Biosci Biotechnol Biochem* **59**(7): 1199-1203 (1995).

493 23 Futagami T, Mori K, Yamashita A, Wada S, Kajiwara Y and Takashita H et al.,
494 Genome sequence of the white koji mold *Aspergillus kawachii* IFO 4308, used
495 for brewing the Japanese distilled spirit shochu. *Eukaryot Cell* **10**(11): 1586-
496 1587 (2011).
497 24 Ono W, Oka D, Hamakawa A, Noguchi T and Takano K, Effects of κ-casein
498 dissociation from casein micelles on cheese curd formation, *Food Sci. Technol*
499 **23**(5): 743-748 (2017).
500 25 Green ML, Milk coagulants. *J Dairy Res* **44**(1): 159 (1977).
501 26 Eskin NAM and Goff HD, Chapter 4 – Milk, in *Biochemistry of foods*, 3rd ed., ed.
502 by Eskin NAM and Shahidi F. Elsevier Academic Press, UK, pp. 187-214
503 (2013).
504 27 O'Connell JE, Grinberg VYa and Kruif CG, Association behavior of β-casein, *J*
505 *Colloid Interface Sci* **258**: 33-39 (2003).
506 28 Shimizu M, Lee SW, Kaminogawa S and Yamauchi K, Emulsifying properties of an
507 N-terminal peptide obtained from the peptic hydrolyzate of αs1-casein. *J Food*
508 *Sci* **49**:1117-1120 (1984).
509 29 Lee SW, Shimizu M, Kaminogawa S and Yamauchi K, Emulsifying Properties of
510 Peptides Obtained from the Hydrolyzates of β-Casein, *Agr Biol Chem* **51**(1):
511 161–166 (1987).
512

513 **TABLES**

| **Table 1.** The amount of adjunct FDP used for 1 kg cheesemaking and the initial LU and PU | | | | |
|---|---|---|---|---|
| **Adjunct recipe** | **FDP weight (g)** | **UNFDP weight (g)** | **Total LU** | **Total PU** |
| Control | 0 | 2.16 | 0 | 0 |
| *A. kawachii* | 0.70 | 1.46 | 666.6 | 18.5 |
| *A. luchuensis* | 1.09 | 1.07 | 666.6 | 50.0 |
| *A. oryzae* | 1.38 | 0.78 | 666.6 | 85.0 |
| FDP: freeze-dried powder, UIFDP: un-inoculated freeze-dried powder | | | | |

514

| **Table 2.** The chemical analysis of experimental cheeses after ripening | | | | | | |
|---|---|---|---|---|---|---|
| **Cheese sample** | **Moisture (%)** | **Salt (%)** | **Lipid (%)** | **Protein (%)** | **FFA (mmol $kg^{-1}$)** | **WSN (%)** |
| Control | 33.0 ± 0.2 | 1.3 ± 0.0 | 30.5 ± 0.1 | 29.5 ± 0.3 | 295.5 ± 0.1a | 11.9 ± 0.2a |
| *A. kawachii* | 32.5 ± 0.2 | 1.4 ± 0.0 | 31.6 ± 0.5 | 29.2 ± 0.0 | 332.9 ± 0.1b | 12.5 ± 0.2a |
| *A. luchuensis* | 32.6 ± 0.3 | 1.3 ± 0.0 | 31.8 ± 0.2 | 28.5 ± 0.2 | 331.9 ± 0.5b | 16.7 ± 0.0b |
| *A. oryzae* | 33.3 ± 0.2 | 1.3 ± 0.1 | 29.6 ± 0.2 | 29.0 ± 0.8 | 494.7 ± 0.1c | 17.8 ± 0.4b |
| Values are mean ± SE (N=2).<br>a, b and c within a column indicate a significant difference between the samples in each component at $p < 0.05$. | | | | | | |

515

516

517

518

519

520

521

522

523

524

| **Table 3.** Distribution of fungal lipase activity (LU) before and after incubation with skim milk curds. | | | | | |
|---|---|---|---|---|---|
| **Strain** | **Fraction** | | **Skim milk curds** | | |
| | | | **Before[A]** | **After[B]** | **B/A** |
| *A. kawachii* | whole | | 601.1 | 399.2 | 0.7 |
| *A. luchuensis* | whole | | 307.9 | 313.6 | 1.0 |
| *A. oryzae* | whole | | 100.5 | 720.0 | 7.2 |
| | whole | extracellular | 237.6 | 405.0 | 1.7 |
| | | intracellular | 124.2 | 799.2 | 6.4 |
| | intracellular | soluble | 72.0 | 127.8 | 1.8 |
| | | insoluble | trace | 244.8 | - |
| B/A is the ratio of lipase activity before and after incubation with skim milk curds *in vitro*.<br>1 LU= 1 μmol oleic acid/h/g-CP | | | | | |

525 **Figure Legends**

526 **Figure 1.** Principal component (PC) analysis biplot (PC 1 and 2) of VOC attributes of
527 experimental cheeses. Numbers represent: 1–2 = control cheese, 3–4 = *A. kawachii*
528 NBRC 4308 cheese, 5–6 = *A. luchuensis* RIB 2604 cheese, 7–8 = *A. oryzae* AHU 7139
529 cheese.

530 **Figure 2.** SDS-PAGE patterns of the proteins from intact acid casein and extracted
531 skim milk curds; M: molecular mass marker protein; lane 1: intact acid casein; lane 2:
532 extracted skim milk curd soluble fraction in SMUF buffer.

533 **Figure 3.** The proposed flow of free fatty acid (FFA) accumulation in *A. oryzae* cheese.
534 Freeze-drying and grinding fragmented the cell walls of *A. oryzae* mycelia. The powder
535 is mixed with cheese curds followed by a pressing step. Pressing of the cheese allowed
536 flowing out some extracellular and intracellular-soluble lipases with the whey, while the
537 cell-binding lipases can be released from cell debris by curd-derived peptides. All the
538 retained lipases on cheese curds are activated by curd-derived peptides; they allow FFA
539 accumulation due to lipolysis during ripening period.

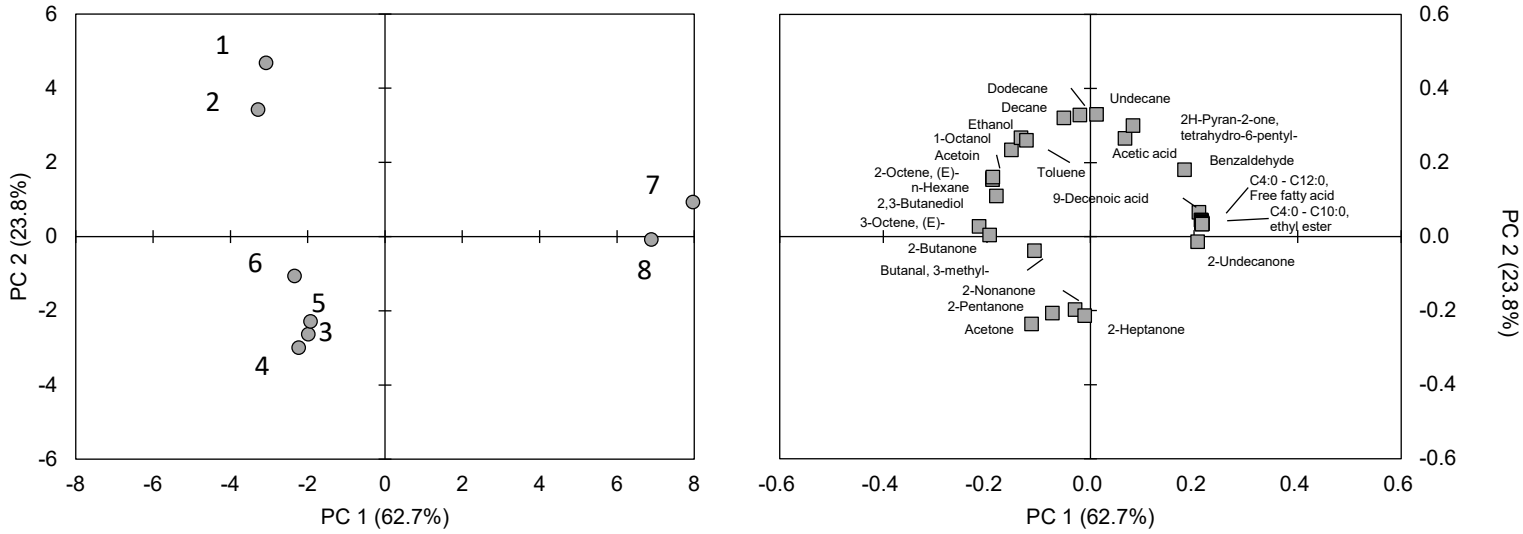



**Figure 1.** Principal component (PC) analysis biplot (PC 1 and 2) of VOC attributes of experimental cheeses. Numbers represent: 1–2 = control cheese, 3–4 = *A. kawachii* NBRC 4308 cheese, 5–6 = *A. luchuensis* RIB 2604 cheese, 7–8 = *A. oryzae* AHU 7139 cheese.

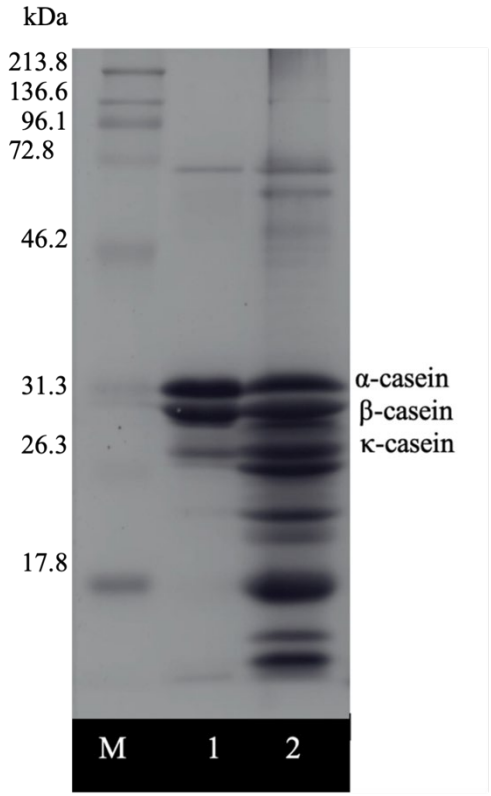



**Figure 2.** SDS-PAGE patterns of the proteins from intact acid casein and extracted skim milk curds; M: molecular mass marker protein; lane 1: intact acid casein; lane 2: extracted skim milk curd soluble fraction in SMUF buffer.

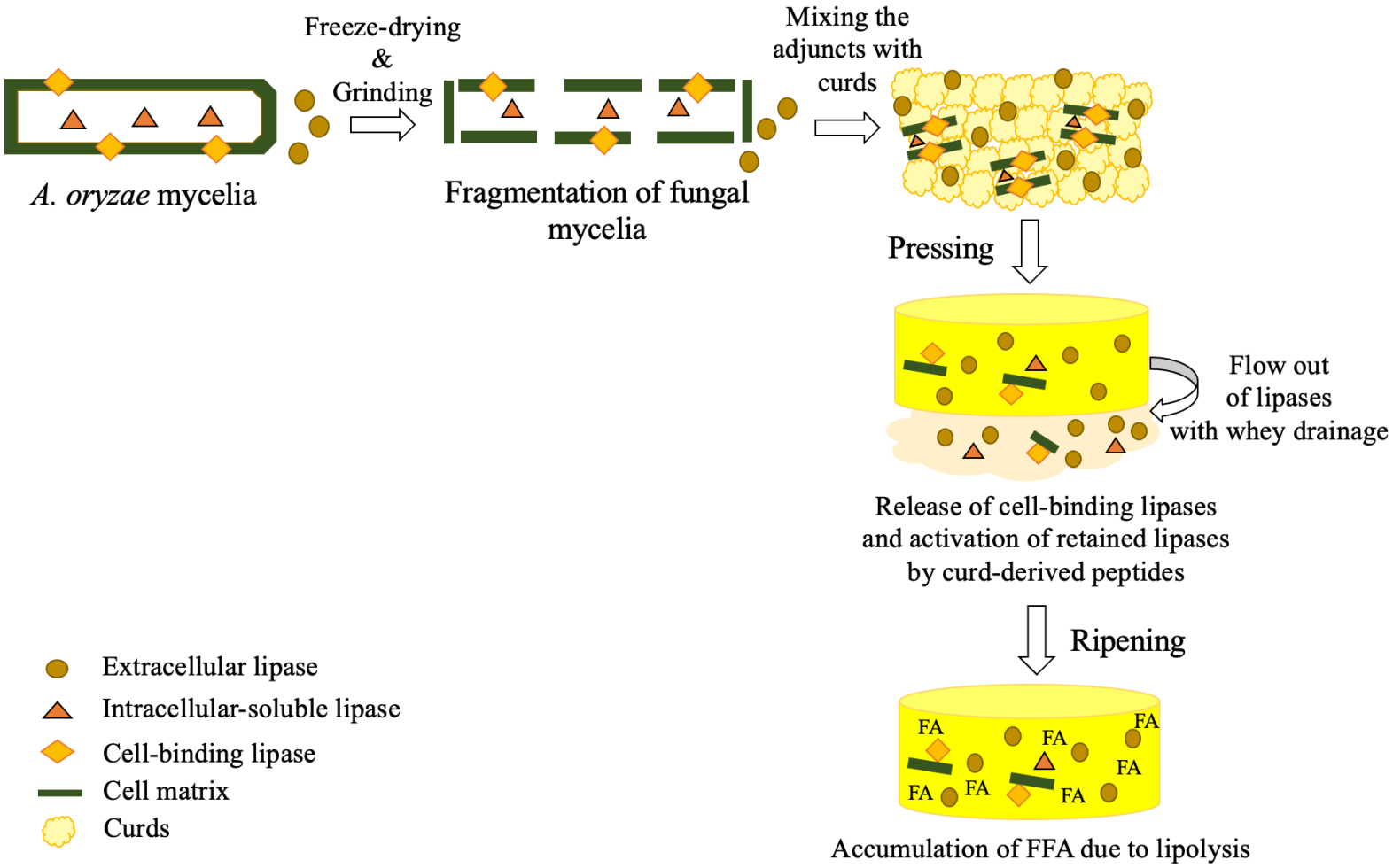
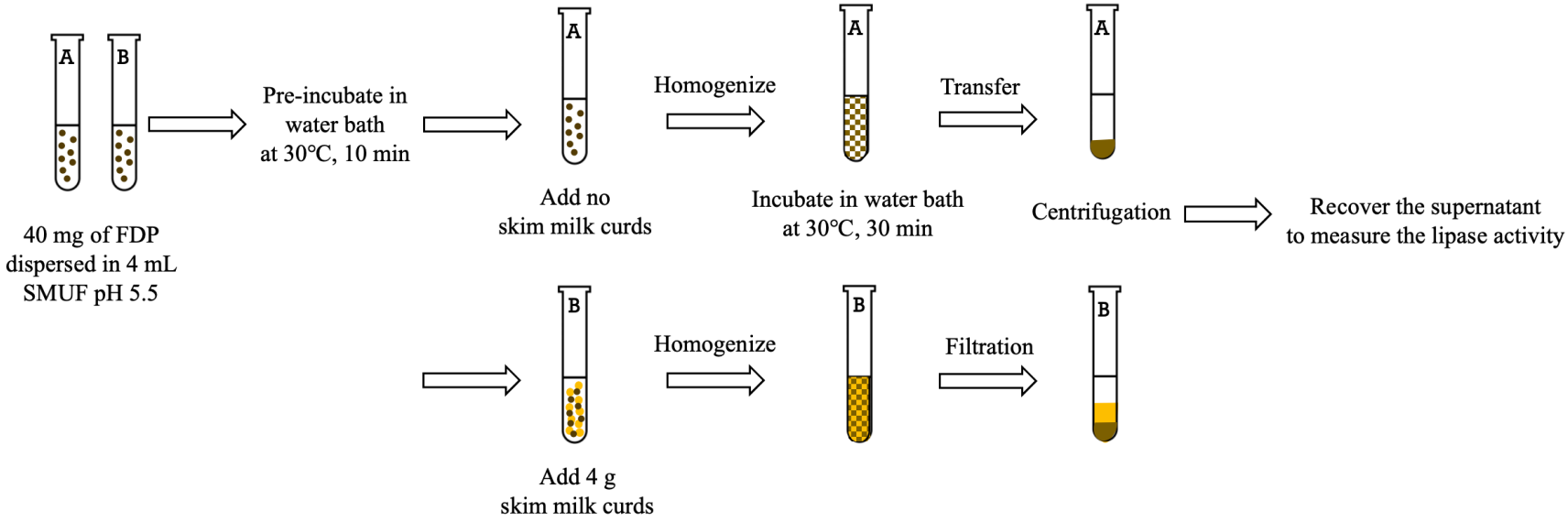



**Figure 3.** The proposed flow of free fatty acid (FFA) accumulation in *A. oryzae* cheese. Freeze-drying and grinding fragmented the cell walls of *A. oryzae* mycelia. The powder is mixed with cheese curds followed by a pressing step. Pressing of the cheese allowed flowing out some extracellular and intracellular-soluble lipases with the whey, while the cell-binding lipases can be released from cell debris by curd-derived peptides. All the retained lipases on cheese curds are activated by curd-derived peptides; they allow FFA accumulation due to lipolysis during ripening period.




**Supplementary Figure 1.** Flowchart of the evaluation of lipase distribution in the presence of experimental skim milk curds. When the fractionation was performed, FDP was replaced with fractionated enzymes and mixed with the SMUF