Application of glucose to prepare *Aspergillus oryzae* koji as an adjunct to prevent rancidity in cheese products

A short title: Koji adjunct preparation to prevent rancidity in cheese

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Abstract

Koji made using *Aspergillus oryzae* shows potential for application as a cheese adjunct; however, flavor defects resulting from volatile free fatty acid (FFA) accumulation should be avoided. Hence, a modified glucose-containing whey solid medium was used to culture *A. oryzae* AHU 7139, and the triacylglycerol (TG) lipase activity and lipase gene (*tglA* and *mdlB*) expression were compared with those of a culture using a conventional whey solid medium. The results showed that TG lipase activity and the expression of both lipase genes were reduced in the modified medium. Moreover, the expression level of *farA*, a positive transcription factor of the lipase genes, was also reduced. The cheese adjunct prepared by culturing the AHU 7139 strain in the modified medium lowered the FFA content in the cheese products, resulting in comparable FFA levels with those in the adjunct-free cheese. Thus, adding glucose is recommended to prepare the koji adjunct for cheesemaking.

Keywords: *Aspergillus oryzae*, lipase, gene expression, free fatty acids, rancidity

Introduction

Aspergillus sp. is an edible filamentous fungus with a long history of use in Japanese fermented food because of its enzyme production and safety. In traditional koji-making, *Aspergillus* is seeded on solid materials such as moistened rice, soybean, and wheat because solid cultures are considered superior to submerged cultures for enzymes produced by filamentous fungi (Oda *et al*, 2006).

In contrast to Japanese traditional foods, there is little information on the application of *Aspergillus* to dairy products. Taking advantage of koji's high proteolytic potential, we prepared koji of *A. oryzae* from whey protein base solid culture products (CPs) as an adjunct for cheese flavor enrichment (Kumura *et al*, 2017; Chintagavongse *et al*, 2022). Proteolysis and lipolysis are stimulated in cheese products during ripening. Although lipolysis generates volatile compounds that improve cheese flavor, excess amounts of FFA, particularly volatile short-chain fatty acids derived from milk fat lipolysis, may cause off-flavors known as rancidity. Thus, lipase activity should be monitored to avoid rancidity when koji from *A. oryzae* is used as a cheese adjunct.

The regulation of *tglA* and *mdlB* expression may improve the quality of the CPs of *A. oryzae* AHU 7139 prepared as adjuncts because triacylglycerol lipase (TglA) and mono-, diacylglycerol lipase (MdlB) are key lipases for rancidity development (Chintagavongse *et al*, 2024). Many factors can affect enzyme production in fungi. Regarding the effects of medium components on lipase production in *A. oryzae*, Garrido *et al* (2012) showed that replacing oleic acid with glucose as the sole carbon source in a liquid Czapek-Dox (CD) minimal medium substantially reduced the expression of the lipase genes (*tglA* and *mdlB*) of *A. oryzae* RIB40. In addition, the expression of three lipase genes (*cutL1*, *tglA*, and *mdlB*) was hampered in the mutant

63 showing *farA* disruption, suggesting that FarA functions as the positive transcription
64 factor for these lipase genes. Accordingly, downregulation of *farA* under certain
65 culture conditions may suppress *tglA* and *mdlB* expression (Tanaka and Gomi, 2021).

66 Considering Garrido's results, a quantitative evaluation of lipase activity with its
67 corresponding gene expression (including that of *farA*) in the presence of glucose
68 should be conducted. As lactose was the carbon source of the whey protein base solid
69 culture mentioned above, we introduced an alternative one to use glucose instead of
70 lactose. Then, the expression levels of lipase genes (*tglA* and *mdlB*) were compared
71 with that of *farA* to determine glucose's effect. In addition, lipase and protease
72 activities were evaluated because our goal was to find a favorable culture condition
73 for the cheese adjunct with proteolytic qualities.

74 In this study, the adjunct was prepared by culturing *A. oryzae* AHU 7139 on a heat-
75 denatured whey protein base solid substrate containing glucose. This adjunct was
76 compared to that prepared using lactose, which was conventionally used in previous
77 studies. The effect of glucose on lipase production and its regulation by FarA was
78 investigated. Then, the adjunct was applied to cheesemaking to evaluate its potential
79 to reduce rancidity development. This study is expected to provide valuable
80 information for preparing *A. oryzae* CP as an adjunct for cheese flavor enrichment and
81 decreased rancidity development.

Materials and methods

Strain and culture condition

The strain used in this study was *A. oryzae* AHU 7139 from the culture collection of Hokkaido University. Spore preparation was performed as previously described (Chintagavongse *et al*, 2024).

Two types of whey protein-based solid media were prepared: one with 20% whey protein isolate (WPI) in simulated ultrafiltrate (SMUF) buffer (Jenness and Koops, 1962) at pH 4.0 or 6.5, containing 5% glucose (WPI + glucose); and another with whey protein concentrate (WPC) dissolved in deionized (DI) water, adjusted to an initial pH of 4.0 or 6.5 using lactic acid or NaOH (Chintagavongse *et al*, 2022). WPI + glucose consists of 18.6% protein, 5% glucose, 0.1% lactose, 0.04% fat, 1% minerals, and 75% moisture, while WPC consists of 20% protein, 2% lactose, 2% fat, 1% minerals, and 75% moisture. WPI + glucose and WPC were dispensed into at least two Erlenmeyer flasks each (10 g per flask) and autoclaved at 121°C for 15 min to prepare the solid medium. The spore suspension (150 µL) with a concentration of 2.5×10^5 spores mL^{-1} was inoculated on the medium and cultivated at 20°C. The CPs were collected at 0, 100, 120, 140, and 160 h for WPI + glucose cultures and at 100, and 160 h for WPC cultures.

Preparation of the extracellular enzymes

The CPs were mixed with an equal weight of DI water for glucose assay and protease activity measurement. For lipase activity, CPs were mixed with an equal weight of 250 mmol L^{-1} Tris-maleate buffer pH 5.5. These samples were treated by Exnizer® 400 homoginizer (Sansyo, Tokyo, Japan) at 260 rpm for 5 min. The mixtures were then

transferred to centrifugae tubes and centrifuged at 21,130 ×*g*, 4°C for 10 min. The supernatant was collected to used as the extracellular enzyme.

Glucose assay

The samples extracted with DI water were used to evaluate the remaining glucose content in the CPs using a commercial Glucose assay kit-WST (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. Glucose standard solutions (0-0.5 mmol L^{-1}) or samples were prepared and transferred to 96-well plate and measured at OD 450 using a microplate reader (Infinite F200 PRO, Tecan, Switzerland). The glucose concentration in the samples were calculated from the standard glucose curve.

Measurement of enzyme activity

Proteolytic activity was determined as previously described (Chintagavongse *et al*, 2020) with some modifications. The casein substrate dissolved in 0.05 mol L^{-1} Tris-maleate buffer pH 5.5 (700 µL), was incubated with 50 µL enzyme at 30ºC for 1 h. Protease activity was expressed as micrograms of tyrosine released per h at 30ºC by the extracts equivalent to that obtained from one gram of the CPs (1 protease unit: PU).

The lipase activity was determined as previously described with some modifications using purified triolein as the substrate (Chintagavongse *et al*, 2024). Here, 300 µL of the prepared enzyme in 250 mmol L^{-1} Tris-maleate buffer pH 5.5 was used. Lipase activity was expressed as µmol of oleic acid released from the substrate per 1 h at 30°C by the extracts, equivalent to that obtained from one gram of the CPs (1 lipase unit: LU).

Total RNA isolation

The solid CPs were recovered and immediately freeze-dried and ground with a mortar and pestle to obtain freeze-dried powder (FDP). The FDP (0.05 g) was transferred into 1 mL of RNA extraction solution (4 mol L^{-1} guadinine thiocyanate; 2 mol L^{-1} NaCl; pH 9.0) and mixed well. The sample was centrifuged at 10,000 ×*g*, 4℃ for 15 min and the supernatant was transferred to a new tube followed by the addition of neutral phenol/chloroform:isoamylalcohol (49:1; CIA) (1:1). After centrifugation at 10,000 ×*g*, 4℃ for 10 min, the upper phase was transferred to a new tube. Then, total RNA sample was precipited by conventional isopropanol precipitation.

The recovered sample was incubated with DNase I. The solution containing total RNA and 1 U RNase-free DNase I (Nippon Gene, Tokyo, Japan) was incubated at 37℃ for 20 min. Then DNase I reaction was stopped by adding acidic phenol/CIA followed by conventional isopropanol precipitation. The sample was re-dissolved in formamide and RNA concentration was determined by measuring absorbance at 260 nm using a spectrophotometer (Shimadzu UV-1800, Kyoto, Japan).

Quantitative reverse-transcription PCR (qRT-PCR)

The mixture containing 1 μg of total RNA, random primer (Promega, Wisconsin, US), Oligo dT primer and dNTP was heated at 65ºC for 5 min and immediately placed on ice. Then, the mixture containing 5×RT buffer, RNase inhibitor (Toyobo, Osaka, Japan) and reverse transcriptase (M-MLV, Nippon Gene, Tokyo, Japan) was added and incubated at 42ºC for 30 min followed by 70ºC for 15 min according to the manufacturer's instructions.

The standard curve of the targeted genes for quantitation was prepared as follows. The diluted cDNA sample (0.5 μL) was used as a template and mixed with the designed

primers (1.5 µL), nuclease-free water (3 µL), and 5 µL of the GoTaq® PCR mixture solution (Promega, Wisconsin, US). The PCR amplification involved denaturation at 95ºC for 2 min, followed by 40 cycles reactions at 95ºC for 45 s, annealing at 63ºC for 30 s, and an extension at 73 ºC for 1 min. A final extension at 73ºC for 10 min was followed by cooling to 4ºC. All the primers used in this study are listed in Table 1. The PCR products were loaded on 1.8% agarose gel and the target band was cut out to extract DNA using GenElute™ Agarose Spin Columns (Sigma-Aldrich Japan, Tokyo, Japan). The standard curve was prepared using known amount of the target genes from 10^{-3} to 10^{-5} ng/µL.

Quantitative RT-PCR was performed using the CFX Connect™ Real-time system (Bio-Rad, USA), using the primers shown in Table 1. Reactions and subsequent analyses were performed with the CFX Maestro software (Bio-Rad, USA). The protocol involved denaturation at 95ºC for 3 min, followed by 40 cycles of two-step reactions at 95ºC for 5 seconds and annealing with elongation at 63ºC for 30 seconds. The relative mRNA levels were normalized to histone H4 as a reference gene at the same sampling point.

Preparation of adjunct for cheesemaking

The spore suspension of *A. oryzae* AHU 7139 was inoculated on WPI + glucose and WPC medium at initial pH of 4.0, incubated at 20℃ for 5 days in WPI + glucose, and 7 days in WPC. The CPs were freeze-dried and then ground with a mortar and pestle to obtain FDP. On average, 0.27 g of FDP was obtained from 1 g of both types of media.

Cheesemaking

Cheesemaking was carried out according to the conventional procedure for Gouda-type cheesemaking as previously described (Chintagavongse *et al*, 2020). Raw milk was obtained from the experimental farm at the Field Science Center for Northern Biosphere, Hokkaido University, and 108.2 kg of standardized milk (3.0% fat) was used in this study. After whey drainage, each 1.2 kg portion of the recovered curds was mixed with each FDP adjunct (two blocks of 0.25% WPC-FDP and three blocks of 0.25% WPI-FDP), while adjunct-free three curd blocks were used as the control cheese. Following brief pressing (1.0 kg/cm^2 for 15 min), inversion and the second stage of pressing (1.2 kg/cm^2 for 50 min) were performed. The cheese blocks were cooled in water overnight then subjected to brine salting (25% NaCl, w/w) for 15 h. The cheeses were matured at 11.5ºC with a relative humidity of 85% for 12 weeks. After 10 days of the production, the cheese was vacuum-packed in bags and left for ripening.

Chemical analysis of cheese

After the ripening period, the cheese samples were individually collected and shredded using a food processor. The moisture, salt, lipid, protein, FFA and water soluble nitrogen (WSN) were determined as previously described (Chintagavongse *et al*, 2020). All assays were performed in duplicate in each cheese block.

Volatile compound analysis in cheese

The shredded cheese samples (4 g) were weighed 4 g in 20 mL vials and sealed with a PTFE septa and magnetic cap. Then, volatile organic compounds (VOCs) in the cheese were extracted using a headspace solid-phase micro-extraction (SPME) technique as previously described (Chintagavongse *et al*, 2022).

Statistical analysis

Enzyme activities and chemical components in the cheese products were analyzed using Tukey-Kramer's multiple comparison test. Data were analyzed using JMP software (Pro 17; SAS Institute, Inc., Tokyo, Japan). Differences were considered to be statistically significant at $p < 0.05$. The differences in the amounts of VOCs in cheese samples were analyzed using a principal component analysis (PCA).

Results and discussion

Enzymatic activities and glucose consumption during the culture of the fungus on WPI + glucose and comparison with those of conventional WPC cultures

Lipase activity in the *Aspergillus* culture initiated at pH 4.0 on WPI + glucose was 3.0 LU at 100 h (Fig. 1A) and was not significantly different from that of WPC (2.9 LU). Moreover, when the culture on WPI + glucose was initiated at pH 4.0, the LU between 100 h and 160 h was not significantly different. The lipase activity of WPC at 160 h was 3.3 LU and was not significantly different from that of WPI + glucose (4.1 LU). Fig. 1B depicts the lipase activity of the culture initiated at pH 6.5. At 100 h, the lipase activity of the WPC culture was 27.6 LU, 12.7-fold higher than that of the WPI + glucose culture (2.2 LU). Between 100 h and 160 h, the lipase activity of the WPI + glucose culture ranged between 1.7 and 3.0 LU. However, a remarkable increase in the lipase activity of WPC was observed at 160 h (50.5 LU), 27.4-fold higher than that of WPI + glucose (1.8 LU). These findings agree with a previous study (Kumura et al, 2017) that showed higher lipase activity when the strain was cultured on WPC at an initial pH of 6.5 compared to 4.0.

Fig. 2A shows the protease activity of the culture initiated at pH 4.0. At 100 h, the protease activity of WPC was 340.7 PU, significantly lower than that of WPI + glucose

(997.9 PU). Then, at 160 h, the protease activity increased to 1665.3 PU and 1502.5 PU for WPC and WPI + glucose, respectively, with insignificant differences. The protease activity of the WPI + glucose culture reached a maximum at 120 h and showed a plateau until 160 h. Fig. 2B presents the protease activity of the culture initiated at pH 6.5. At 100 h, the protease activity of WPC was 596.2 PU, lower (although not significantly) than that of WPI + glucose (953.4 PU). At 160 h, the protease activity of WPC increased to 1750.4 PU, significantly higher than that of WPI + glucose (1050.0 PU). The protease activity of the WPI + glucose culture reached a maximum at 140 h. However, the difference in protease activity between the two substrates at the end of the culture process was not as remarkable as that observed for lipase at an initial pH of 6.5.

In addition, glucose consumption was monitored during the culture process. The results showed that the glucose amounts were comparable from 0 h to 100 h at both initial pHs (Figs. 1 and 2). Then, the glucose amount gradually declined until 160 h, where 20.8% and 1.8% of glucose remained in the media with initial pHs of 4.0 and 6.5, respectively. Regardless of the presence of glucose, *A. oryzae* AHU 7139 grew well on both WPC and WPI + glucose media (Fig. S1).

Carbon catabolite repression (CCR) is a process in which the supply of glucose lowers extracellular enzyme production (Magasanik, 1961). In this study, glucose suppressed lipase production, especially for cultures at an initial pH of 6.5, compared with the WPC culture with lactose. By contrast, glucose stimulated the production of acidic protease at the culture's early stage (100 h) although there was a report described the inhibitory effect of glucose on protease production in *Aspergillus* sp. (Katz *et al*, 1994).

Overall, glucose decreased lipase production when the initial pH was pH 6.5; however, glucose had a limited effect on acidic protease production in the CPs of *A. oryzae* AHU 7139.

Quantitative analysis of gene expression during culture

The expression levels of the *tglA*, *mdlB*, and *farA* genes in *A. oryzae* AHU 7139 cultured on WPI + glucose or WPC at different time points were evaluated by quantitative PCR (qPCR).

At initial pH 4.0, the expression of *tglA* in the WPC culture was 2.8- and 3.2-fold higher than that in the WPI + glucose culture at 100 and 160 h, respectively. At initial pH 6.5, these values were 1.8- and 2.3-fold higher, respectively (Fig. 3A). The highest expression of *tglA* was observed at 160 h, particularly when AHU 7139 was grown on WPC at pH 6.5, which is similar to the conventional koji preparation conditions. Despite the insignificant difference in TG lipase activity obtained with an initial pH of 4.0 (Fig. 1A), glucose inhibited *tglA* expression, suggesting that other lipase molecules are generated rather than TglA at an initial pH of 4.0 when glucose is present.

MdlB is the lipase that hydrolyzes the diacylglycerol (DG) generated by TglA action on milk TG; thus, MdlB plays a crucial role in releasing FFA during lipolysis (Chintagavongse *et al*, 2024). The expression of *mdlB* was higher in the WPC cultures than in the WPI + glucose cultures at 100 h (6.7- and 2.4-fold higher in pH 4.0 and pH 6.5, respectively) (Fig. 3B). At both initial pHs, the relative expression of *mdlB* gradually increased as the culture of the fungus on WPI + glucose progressed. However, at 160 h, the expression of *mdlB* significantly decreased for WPC at pH 4.0 but increased for WPC at pH 6.5. Nonetheless, the expression levels were still higher

than those of the WPI + glucose culture at 160 h (1.8-fold and 4.9-fold higher in pH 4.0 and 6.5, respectively).

The *farA* gene was analyzed to elucidate the relationship between FarA as a transcription factor and the lipase genes. The relative expression of *farA* was similar to that of *tglA* (Fig. 3C). Moreover, the expression of *farA* in the WPC culture was higher than in the WPI + glucose culture at both initial pHs. However, a higher expression level was observed at an initial pH of 6.5. At 160 h, WPC cultures showed higher *farA* expression than WPI + glucose cultures, with 2.0- and 2.4-fold increases at pH 4.0 and pH 6.5, respectively. The correlation coefficient (R) between *tglA* and *farA* was 0.98 at the initial pH of 6.5 and 4.0 while that between *mdlB* and *farA* was 0.91 and 0.47 at the initial pH of 6.5 and 4.0, respectively. Thus, the expression of *mdlB* under acidic condition is likely to be influenced by other transcription factors.

Despite the correlation between *farA* and *tglA* expression, extracellular TG lipase activity did not directly align with *tglA* expression levels (Fig. 1). This suggests the involvement of other lipases such as *cutL*, annotated lipase gene AO090012000690, and AO090005001319 because their knockout strains showed reduced TG lipase activity compared to the parent strain (AHU 7139) under acidic initial conditions but not under neutral initial conditions (Chintagavongse *et al*, 2024). Because the TG lipase activities were noticed when the Δ*tglA* knockout strain was under acidic conditions, other TG lipases are likely to be involved.

According to a previous study (Garrido *et al*, 2012), using glucose as the sole carbon source in a liquid culture did not induce the expression of *tglA* and *mdlB* in *A. oryzae* RIB40. On the other hand, the deletion of *farA* hampered the expression of lipase genes including *cutL*, *tglA*, and *mdlB* in *A. oryzae*. FarA is highly conserved in aspergilli

291 including *A. oryzae* (Vongsangnak *et al*, 2010) and is similar to the cutinase
292 transcription factor Ctf1 of *Fusarium solani* that binds to the cutinase gene promoter.
293 FarA induces gene expression related to fatty acid metabolism by binding to the
294 'CCTCGG' motif, which is found at -313 and -324 for *tglA* and *mdlB*, respectively,
295 and *farA* deletion destroys the ability to use short- and long-chain fatty acids (Hynes
296 *et al*, 2006). FarA regulation of the cutinase gene is affected by CCR in *A. nidulans*
297 (Bermúdez-García *et al*, 2019). Since the expression of *farA* in the WPI + glucose
298 culture was suppressed, FarA regulation of lipase genes in solid cultures of AHU 7139
299 is likely to be regulated by CCR.

300 Moreover, the possibility of other transcription factors besides FarA influencing lipase
301 gene expression in *A. oryzae* cannot be ruled out. For example, CreA and FlbC are
302 known to regulate specific gene expressions under certain conditions. CreA mediates
303 α-amylase gene expression with the synergistic action of CreB (Tanaka and Gomi,
304 2021). The consensus motif of CreA binding has been proposed as 5'-SYGGRG-3'
305 (Takashima *et al*, 1996) and it is located upstream of *tglA* (-566) and *mdlb* (-588) genes.
306 However, since these motifs are more distal than the potential FarA binding sites, FarA
307 may have a greater influence on the regulation of *tglA* and *mdlB* than CreA. On the
308 other hand, FlbC has been reported to regulate some solid-state culture-specific gene
309 expressions (Tanaka *et al*, 2016) and influences *glaB* expression with AmyR, another
310 essential transcription factor (Ishida *et al*, 2000). Although FarA can be considered
311 one of the dominant regulators for *tglA* and *mdlB*, further investigation on other
312 transcription factor(s) for these lipases is needed to regulate the gene expression in the
313 solid culture of *A. oryzae*.

Chemical components of cheese products supplied with koji adjuncts

Table 2 presents the results of the chemical analysis of the experimental cheeses after 12 weeks of ripening. The moisture, salt, and lipid content of the three types of cheese were not significantly different; however, the protein content in the WPC cheese (29.0 ± 0.2%) was significantly higher than that in the non-adjunct control cheese (25.7 ± 0.7%). A significant increase in FFAs was observed when using the WPC adjunct, whereas both adjuncts increased the WSN content compared to the control. Thus, replacing lactose with glucose decreased rancidity development while maintaining the WSN level.

Although the TG lipase activity of AHU 7139 cultured on WPI + glucose and WPC at an initial of pH 4.0 was comparable during 100–160 h (Fig. 1A), the FFA levels in the cheese products made with the WPC adjuncts were significantly higher than those made with the WPI + glucose adjuncts. We found that TglA is the most relevant lipase to rancidity among those from *A. oryzae* AHU 7139 (Chintagavongse *et al*, 2024), suggesting that some, like CutL are not involved in FFA accumulation in cheese. Accordingly, evaluation based solely on TG lipase activity may include lipase activity that is not influential in rancidity. Hence, TG lipase activity measurement alone could not predict the FFA accumulation in cheese. Despite the low TglA production under acidic culture conditions, TglA still contributes to rancidity. Considering that several TG lipases are produced in solid cultures, the gene expression of *tglA*, obtained via qRT-PCR (Fig. 3), was better correlated with the FFA levels in cheese products than the TG lipase activity that might include. Furthermore, adding glucose to the solid culture reduced *mdlB* expression (Fig. 3), potentially preventing the degradation of DG by MdlB. In addition, screening of the TglA dysfunction strain is an alternative option to find a candidate strain for use as a cheese adjunct. Recently, the KC43 strain

was found to have a single nucleotide insertion on the exon 2 region of *tglA,* accompanied by codon frameshifting, resulting in early stop codon generation on the exon 2 of *tglA* transcription while the putative active site of *tglA* is coded on the exon 3 region (Kawakami, unpublished data).

Proteolysis in both cheeses prepared with adjuncts was enhanced by increasing the WSN levels. The protease activity of AHU 7139 cultured on WPC at an initial pH of 4.0 for 160 h was comparable to that cultured on WPI + glucose at the same initial pH for 120–160 h (Fig. 2A). The comparable WSN levels in the cheese products made using these adjuncts correlated with the protease activity. Thus, in contrast to lipolysis, the protease activity can be used to predict the degree of proteolysis during ripening.

Volatile organic components

The PCA showed the relative amounts of VOCs in the cheese samples (Fig. 4). Thirty-six volatile compounds were detected in the cheeses prepared for this study, with different profiles (Table S1). The dominant VOCs differed in each type of cheese. Rancidity-related compounds (volatile FFAs) were abundant in conventional WPC cheeses. However, the volatile profile of the WPI + glucose cheese was close to that of the control cheese, except for the presence of fatty acid ethyl ester forms (FAEEs), which were detected in the adjunct cheeses but not in the control. Thus, *A. oryzae* adjuncts obtained from a glucose-added medium did not contribute to rancidity development but yielded a moderate aroma profile that differed from that of the control cheese. Therefore, adding glucose to the whey protein medium to prepare AHU 7139 koji as a cheese adjunct prevented rancidity in the cheese products by reducing FFA levels.

CONCLUSION

Triacylglycerol (TG) lipase activities and the expression levels of *tglA* and *mdlB* genes were reduced in the WPI + glucose solid medium, compared with the WPC medium, which was previously used to prepare koji for cheesemaking. FarA was downregulated by glucose and, in turn, the expression of *tglA* and *mdlB* was decreased. The culture products (CPs) of *A. oryzae* AHU 7139 grown on the WPI + glucose or conventional WPC media were applied as cheese adjuncts. The chemical analysis of the ripened cheese products revealed that the adjuncts boosted proteolysis. Furthermore, glucose addition to the WPI medium reduced the free fatty acid (FFA) levels compared to the WPC medium. The FFA levels in the former were comparable to those in the control cheese. Therefore, adding glucose to the medium to prepare *A. oryzae* CP as an adjunct was proven worthwhile as it prevented rancidity in cheese products.

Supplementary material

Supplementary material is available at *Bioscience, Biotechnology, and Biochemistry* online

Data availability

The data underlying this article are available in the article and in its online supplementary material.

Author contribution

N.C. and H.K. wrote the manuscript, handled editing, and were responsible for conceptualization. N.C. and T.M. performed the experiments. N.C., T.M., K.T., and H.K. analyzed the data. T.H., J.W., and H.K. provided suggestions. H.K. was the

supervisor and managed funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Disclosure statement

The authors declare no conflicts of interest.

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439 **Tables**

Table 1. PCR primers used for qPCR analysis		
Gene accesion no.	Primer name	Sequence (5'→3')
AO090012000496 (*histone H4*)	hisFw	TCTTGCGTGACAACATCCA
	hisRv	AGATACGCTTGACACCACCA
AO090003001507 (*tglA*)	tglAFw	CAAATGTCCTGACACTAAGC
	tglARv	AAGAGGAGGACGCTGCTGA
AO090701000644 (*mdlB*)	mdlBFw	CACATCAGCCCTGAATACTA
	mdlBRv	TGGAACGCAAGGAGGTCA
AO090011000961 (*farA*)	farAFw	ACAGGTGTTCGGAATGGAGA
	farARv	GCTATTACTCCAGGTGTCAT

440

Table 2. The chemical analysis of experimental cheeses

Cheese sample	Moisture (%)	Salt (%)	Lipid (%)	Protein (%)	FFA (mmol/ 100 g cheese)	WSN (%)
Control	40.5 ± 1.6	0.9 ± 0.0	27.5 ± 0.3	25.7 ± 0.7b	3.0 ± 0.0b	15.7 ± 0.4b
WPC	39.2 ± 0.1	1.2 ± 0.0	25.5 ± 1.0	29.0 ± 0.2a	12.5 ± 0.7a	21.8 ± 0.1a
WPI+glu	38.7 ± 0.1	0.9 ± 0.0	27.6 ± 0.4	25.7 ± 0.5b	3.2 ± 0.0b	21.9 ± 0.8a

Values are mean ± SE, n = 2-3.
a, b within a column indicates a significant difference between the samples in each component using Tukey-Kramer test at $p < 0.05$.

441

Figure legends

Fig.1. Time course of lipase production according to the amount of glucose in WPI + glucose cultures from 0 to 160 h and lipase production in WPC at 100 and 160 h at initial pHs 4.0 (A) and 6.5 (B). Different letters indicate significant differences in LU levels according to the Tukey-Kramer test ($p < 0.05$) (n = 2).

Fig.2. Time course of protease production according to the amount of glucose in WPI + glucose cultures from 0 to 160 h and protease production in WPC at 100 and 160 h at initial pHs 4.0 (A) and 6.5 (B). Different letters indicate significant differences in PU levels according to the Tukey-Kramer test ($p < 0.05$) ($n = 2$).

Fig. 3. qRT-PCR relative expression of *tglA* (A), *mdlB* (B), and *farA* (C) genes in *A. oryzae* AHU 7139, cultured on whey protein base solid cultures. The relative expression was normalized to the histone H4 gene ($n = 2$).

Fig. 4. Principal component analysis (PCA) biplot (PC 1 and 2) of volatile organic compound (VOC) attributes of experimental cheeses. C = control (non-adjunct) cheese, W = conventional WPC adjunct cheese, WG = modified WPI + glucose adjunct cheese.

Graphical abstract caption

Koji preparation by modified whey protein (WPI + glucose) reduces lipase gene expression along with its activity, resulting in lower FFA levels in ripened cheese.

Tables

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	farARv	GCTATTACTCCAGGTGTCAT

Table 2. The chemical analysis of experimental cheeses

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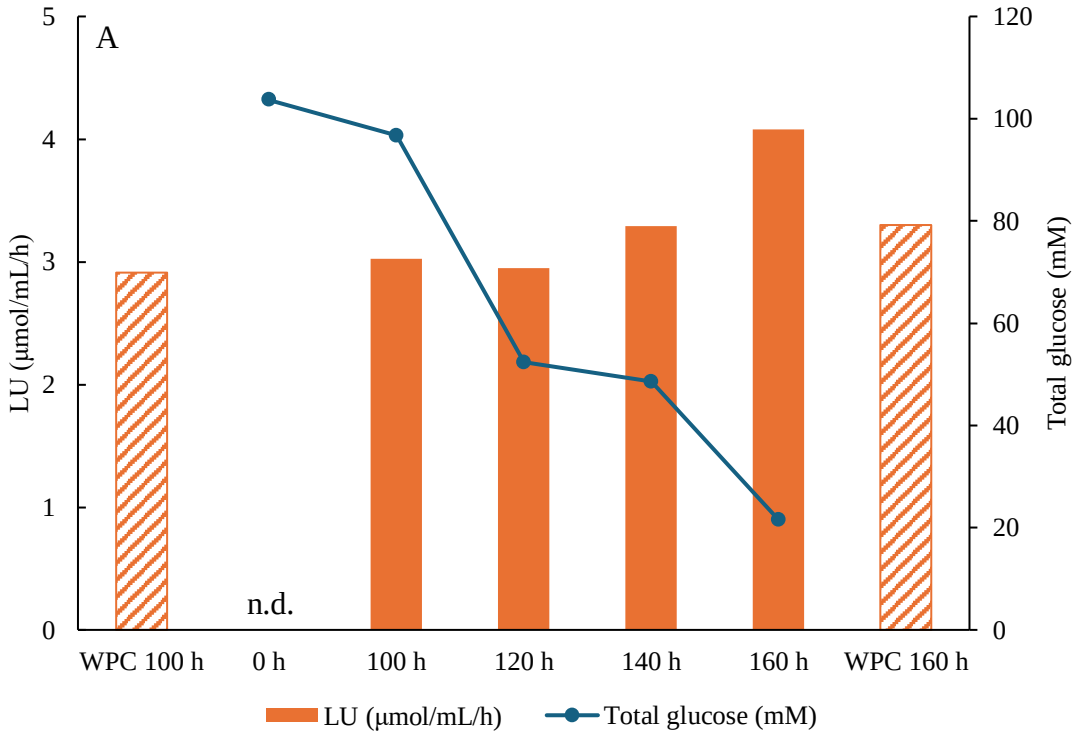
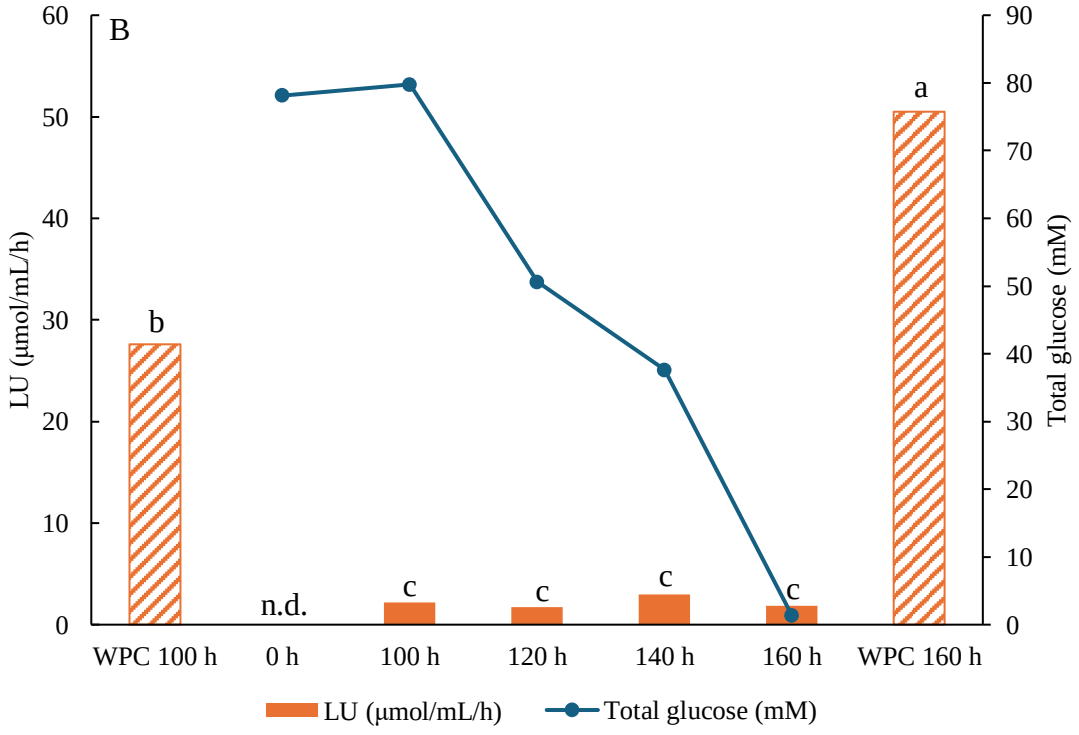
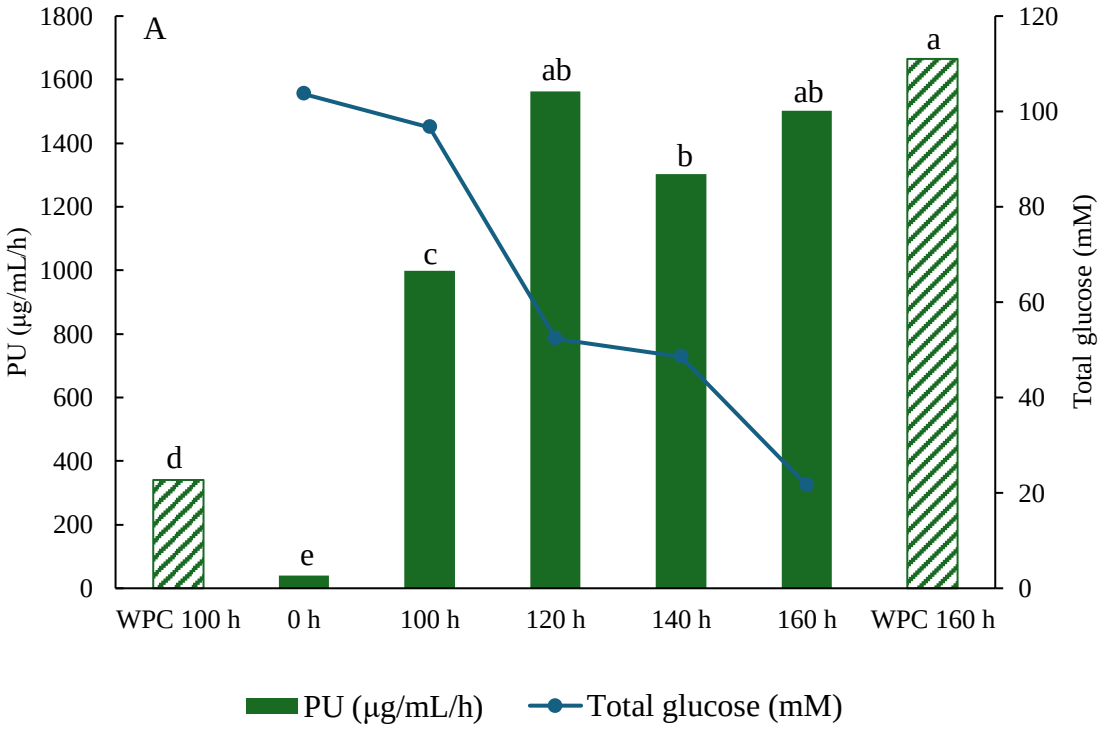
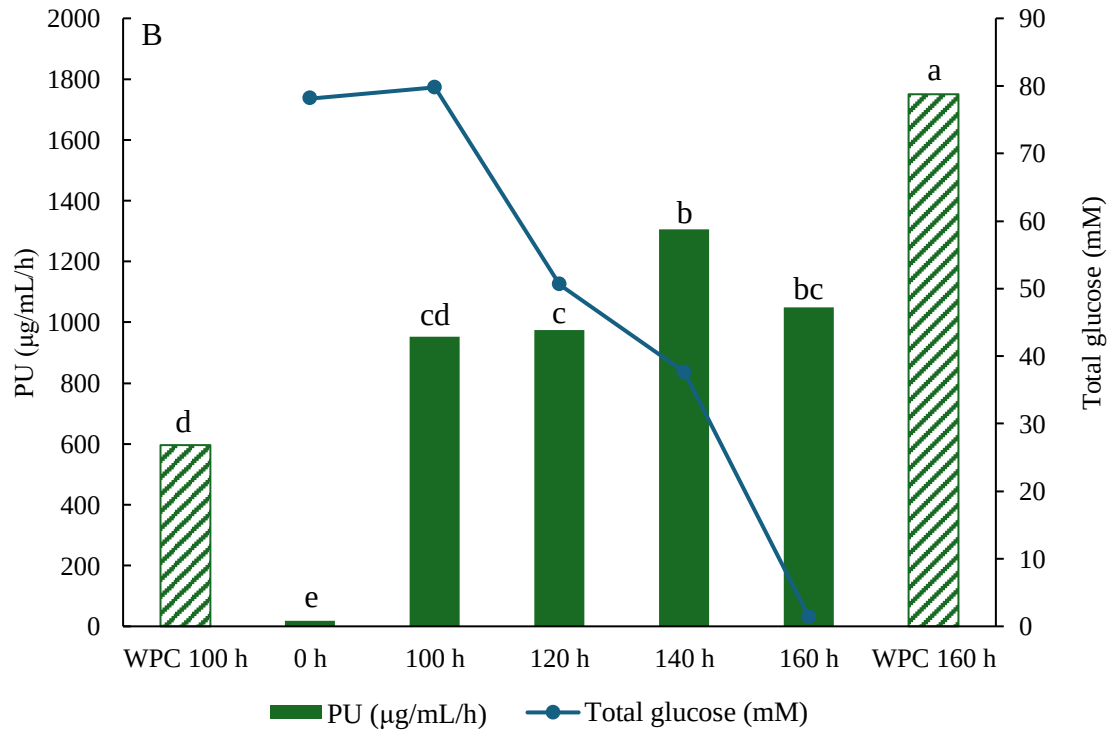
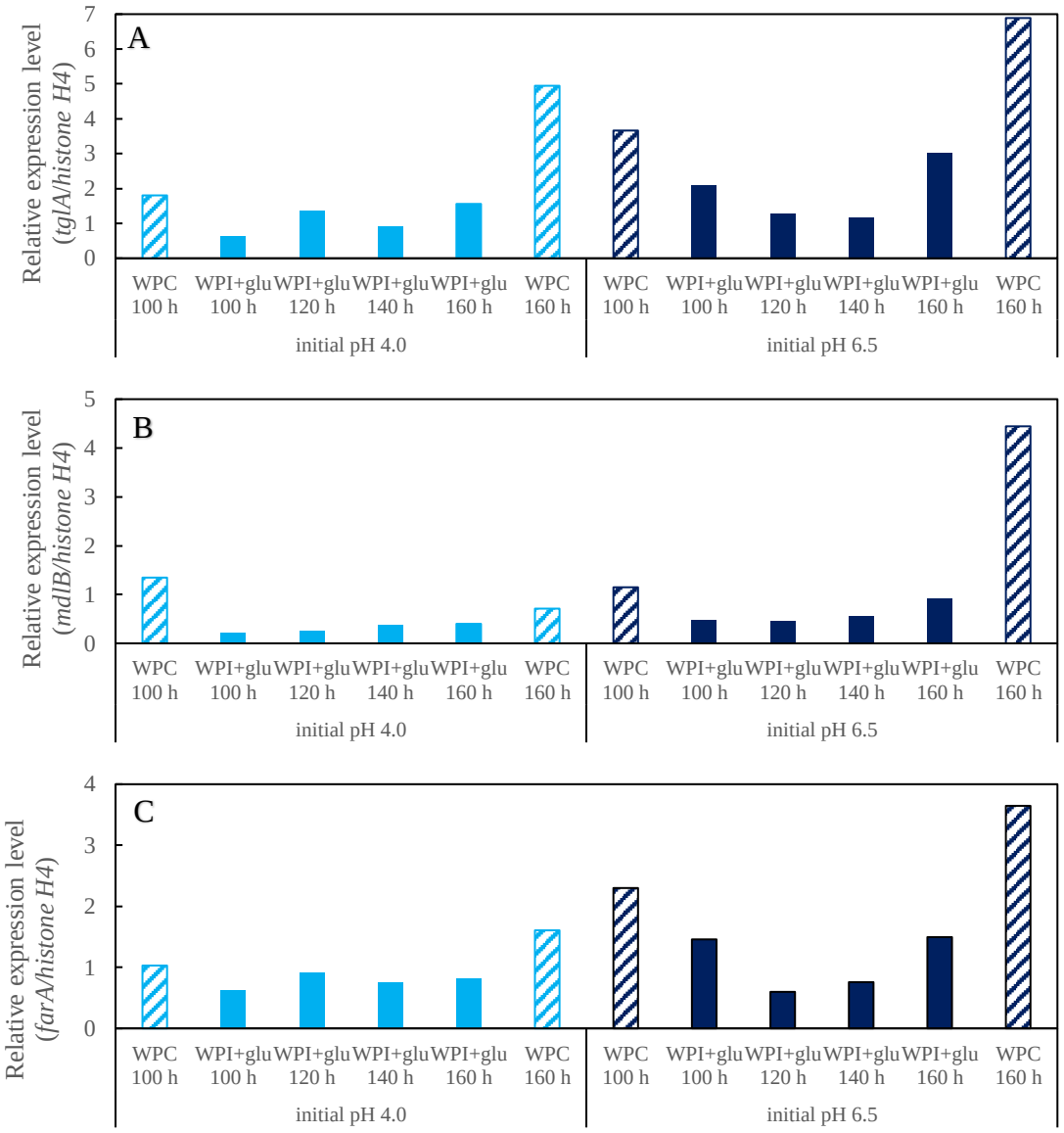
Values are mean ± SE, n = 2-3.
a, b within a column indicates a significant difference between the samples in each component using Tukey-Kramer test at $p < 0.05$.

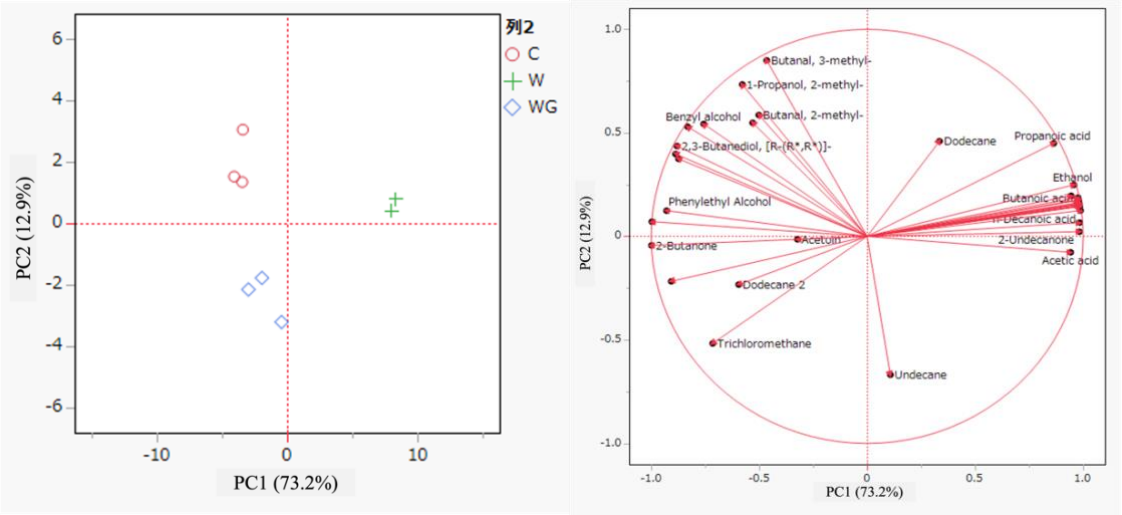

A
LU (µmol/mL/h)
Total glucose (mM)
n.d.
WPC 100 h
0 h
100 h
120 h
140 h
160 h
WPC 160 h
LU (µmol/mL/h)
Total glucose (mM)


B
LU (µmol/mL/h)
Total glucose (mM)
b
n.d.
c
c
c
c
a
WPC 100 h
0 h
100 h
120 h
140 h
160 h
WPC 160 h
LU (µmol/mL/h)
Total glucose (mM)


A
PU (µg/mL/h)
Total glucose (mM)
1800
1600
1400
1200
1000
800
600
400
200
0
120
100
80
60
40
20
0
d
e
c
ab
b
ab
a
WPC 100 h
0 h
100 h
120 h
140 h
160 h
WPC 160 h
PU (µg/mL/h)
Total glucose (mM)


B
PU (µg/mL/h)
Total glucose (mM)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
90
80
70
60
50
40
30
20
10
0
d
e
cd
c
b
bc
a
WPC 100 h
0 h
100 h
120 h
140 h
160 h
WPC 160 h
PU (µg/mL/h)
Total glucose (mM)


A
Relative expression level (tglA/histone H4)
0
1
2
3
4
5
6
7
WPC 100 h
WPI+glu 100 h
WPI+glu 120 h
WPI+glu 140 h
WPI+glu 160 h
WPC 160 h
initial pH 4.0
initial pH 6.5
B
Relative expression level (mdlB/histone H4)
C
Relative expression level (farA/histone H4)


列2
C
W
WG
PC2 (12.9%)
PC1 (73.2%)
6
4
2
0
-2
-4
-6
-10
0
10
Butanal, 3-methyl-
1-Propanol, 2-methyl-
Butanal, 2-methyl-
Benzyl alcohol
2,3-Butanediol, [R-(R*,R*)]-
Dodecane
Propanoic acid
Ethanol
Butanoic acid
1-Decanol
2-Undecanone
Acetic acid
Phenylethyl Alcohol
2-Butanone
Acetoin
Dodecane 2
Trichloromethane
Undecane
1.0
0.5
0
-0.5
-1.0
PC2 (12.9%)
PC1 (73.2%)

1 **Supplementary table 1**

Table S1. Volatile component assessment of the experimental cheese by SPME-GC/MS method			
VOCs	**Cheese sample**		
	Control	**WPC**	**WPI + glucose**
FFA			
Acetic acid	280952.3b	3219011.0a	779771.7b
Propanoic acid	27872.3b	62610.0a	13865.0b
Propanoic acid, 2-methyl- (Isobutyric acid)	91526.3a	N.D.	34158.3b
Butanoic acid	614710.0b	11624696.0a	463361.0b
Butanoic acid, 3-methyl- (Isovaleric acid)	176880.3a	N.D.	87770.3ab
Pentanoic acid	8255.0b	446434.5a	8322.7b
Hexanoic acid	137231.0b	15665589.0a	179885.0b
Heptanoic acid	N.D.	166595.5a	3852.3b
Octanoic acid	28071.3b	3287363.0a	72832.3b
n-Decanoic acid	10758.0b	699042.5a	42069.7b
FAEE			
Butanoic acid, ethyl ester	N.D.	472053.0a	12241.7b
Hexanoic acid, ethyl ester	N.D.	204546.0a	2352.3b
Octanoic acid, ethyl ester	N.D.	91641.5a	2820.7b
Decanoic acid, ethyl ester	N.D.	93350.5a	2887.3b
Ketone			
Acetone	2694528.7a	1354705.5b	2408940.0a
2-Butanone	316704.3a	156372.0b	297115.0a
2-Pentanone	555751.0b	2160722.5a	571067.3b
2-Heptanone	66702.0b	718088.0a	80439.7b
Acetoin	4686540.7	3313826.0	4219615.0
2-Nonanone	23486.3b	96504.0a	21461.3b
2-Undecanone	N.D.	28177.0a	4406.3b
Alcohol			
Ethanol	227917.3b	1168947.5a	145117.0c
1-Propanol, 2-methyl- (Isobutanol)	19990.3	N.D.	N.D.
1-Butanol, 3-methyl- (Isoamyl alcohol)	348681.7a	5991.0b	390621.3a
2,3-Butanediol	118615.0a	N.D.	59137.0ab
2,3-Butanediol [R-(R*,R*)]	889597.3a	N.D.	393206.0b
Benzyl alcohol	26807.0a	N.D.	7558.7b
Phenylethyl alcohol	90185.0a	5365.5b	72386.7ab

2
3
4
5

Table S1. (cont.) Volatile component assessment of the experimental cheese by SPME-GC/MS method			
VOCs	**Cheese sample**		
	Control	**WPC**	**WPI + glucose**
Aldehyde			
Butanal 2-methyl-	33707.7	18565.0	18338.3
Butanal 3-methyl-	167622.3a	86080.5b	68714.7b
Benzaldehyde	3746.3b	29558.0a	5566.3b
Alkane			
Undecane	59881.7	64290.5	67459.7
Dodecane	521607.0	536376.5	439226.7
Dodecane 2	102483.3	75195.5	98703.7
Haloalkane			
Trichloromethane	120763.3	60562.5	156245.7
Ether			
2-propanol, 1-methoxy- (PGME)	14661	4756.5	7873.7
Values are mean (n = 2-3) a, b, c within a row indicates a significant difference between the samples in each volatile compound using Tukey-Kramer test at $p < 0.05$.			

6

Supplementary figure 1

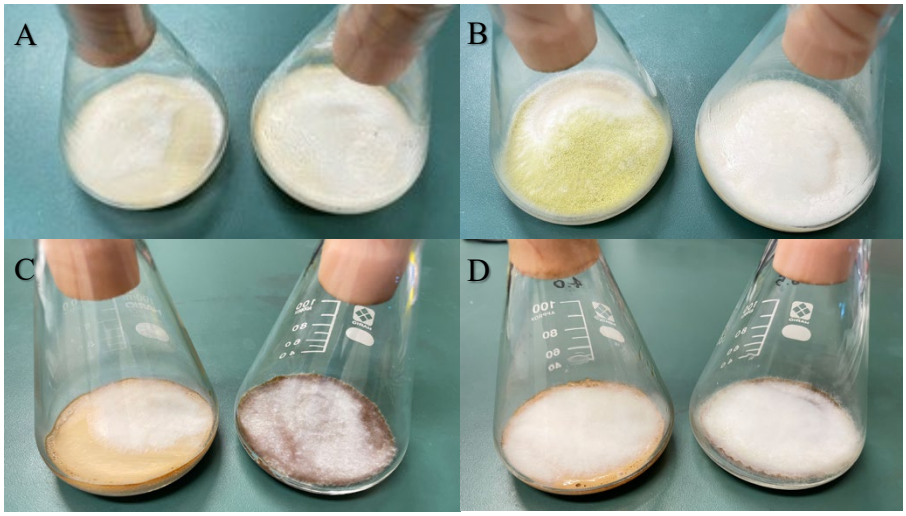


Fig. S1. Growth appearance of *A. oryzae* AHU 7139 inoculated on conventional WPC medium incubated for 100 h (A) and 160 h (B) and that of inoculated on WPI + glucose medium incubated for 100 h (C) and 160 h (D) with initial pH 4.0 (left flask) and pH 6.5 (right flask).