Title page

(a) Title of this manuscript

“Lipase and protease production of dairy *Penicillium* sp. on the milk-protein-based solid substrates.”

Keywords

lipase, protease, *Penicillium roqueforti*, *Penicillium camemberti*, solid substrate

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(c) Running headline

Lipase and protease production of dairy fungi

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Summary

The lipolytic and proteolytic activity of Penicillium camemberti *PTCC 033 and* Penicillium roqueforti *PRG 3, cultured on the whey solid or simulated cheese media were compared under several pH reaction conditions. Lipolytic activity was higher when both strains had been cultured on the whey medium than on the simulated cheese medium, whereas proteolytic activity was less influenced by the culture medium. The relationship between the reaction pH and these enzyme activities was dependent on the culture medium, which suggested that the expression level and balance of isozyme relies on the culture substrate.*

INTRODUCTION

Higher free fatty acid (FFA) content of the blue-veined cheese ripened by *Penicillium roqueforti* has been reported compared to that of the white-mold cheese ripened by *P. camemberti* (De la Feunte *et al*. 1993; Contarini and Toppino 1995; Thierry *et al*. 2017). Accordingly, it has been assumed that the lipase activity of *P. roqueforti* is higher than that of *P. camemberti*. During these kinds of cheese ripening processes, the *Penicillium* sp. starter germinated and assimilated milk fat and a limited amount of lactic acid as the carbon source, in

addition to casein as the nitrogen source.

On the other hand, our previous study reported the application of the culture products of *Aspergillus oryzae* for cheese making, as an adjunct enzyme cocktail, to enrich its flavour (kumura *et al*. 2017). Since solid cultivation is superior to a submerged culture in terms of the enzyme productivity of filamentous fungi, heat-denatured whey protein concentrate (WPC) was used to prepare a solid culture substrate. Applying this concept, *P*. *camemberti* AHU 8113 or *P*. *roqueforti* AHU 8056 were seeded on the WPC substrate and the culture products were mixed with fresh cheese curds that had been prepared according to the protocol of Gouda-type cheese making. In the matured cheese that was received, WPC culture products using *P*. *camemberti* showed higher FFA levels than those using *P*. *roqueforti* (Machiya unpublished results). This result implies the high lipase production capability of *P*. *camemberti* if it is seeded under certain circumstances, such as on the WPC substrate where the primary carbon and nitrogen sources are lactose and whey protein, respectively.

In addition to lipase, protease is also a pivotal enzyme in generating the cheese flavour. However, most of the previous studies concerning lipase and protease from *P*. *camemberti* and *P*. *roqueforti* were conducted using the enzymes obtained from the liquid culture system despite these species having been utilized on solid cheese curds (Eitenmiller *et al*. 1970; Gripon and

52 Hermier 1974; Gripon and Debest 1976; Lemberet and Lenoir 1976; Menassa and Lamberet
53 1982; Lamberet and Menassa 1983; Yamaguchi and Mase 1991; Chrzanowska *et al*. 1993,
54 1995; Mase *et al*. 1995; Tan *et al*. 2004). Although limited information is available on using a
55 solid culture system, Igoshi *et al*. (2007) reported that *P*. *roqueforti* KTU-11 cultured on wheat
56 bran produced two kinds of extracellular proteases, which were assumed to be metalloprotease
57 and aspartic proteinase. Because this strain secreted only metalloprotease when it was grown in
58 a liquid skim milk culture, the researchers concluded that the balance of isozyme production
59 depended on the properties of the medium. Thus, more attention should be paid to the solid
60 culture-specific properties of dairy *Penicillium* to uncover its potential.
61 In this study, we evaluated the lipase and protease activity to compare the productivity of
62 these two species grown on the milk-relevant solid substrates and demonstrated that these
63 activities were influenced by the culture media.
64
65 MATERIALS AND METHODS
66 **Strain and growth medium**
67 *P*. *camemberti* AHU 8113 and *P*. *roqueforti* AHU 8056 were obtained from the culture
68 collection of Hokkaido University. *P*. *camemberti* PCA 3 and PTCC 033, and *P*. *roqueforti* PR

69 3 and PRG 3 were purchased from Chr. Hansen (Denmark) as commercial dairy starters. These
70 fungi were grown on potato dextrose agar (Merck KGaA, Germany) at 25ºC for 12 d. The
71 conidia were isolated and dispersed in 0.9% sodium chloride solution.

72 The whey protein concentrate 80 (WPC80; Fonterra, New Zealand) was dissolved in
73 deionized water at a ratio of 1:3 (w:w) and adjusted to pH 4.0 using lactic acid. The solution (10
74 g) was placed into an Erlenmeyer flask (100 mL) and autoclaved at 121ºC for 15 min to prepare
75 the WPC solid medium. The WPC medium was composed of fat (0.7 g), whey protein as
76 protein (19.5 g), lactose as carbohydrate (2.3 g), minerals (1.0 g), and water (76.5 g/100 g of
77 medium).

78 Raw milk obtained from the experimental farm in the Field Science Center for Northern
79 Biosphere at Hokkaido University was mixed with skim milk to adjust its fat content of 2.8~3.0
80 g/100 g. Following procedures, including pasteurisation at 72ºC for 15 s, the starter addition,
81 rennet treatment and subsequent gentle stirring, were performed as previously described
82 (Kumura *et al*. 2017). Ten grams of the recovered curds in 1 cm^2 cubes were transferred into an
83 Erlenmeyer flask (100 mL) and autoclaved at 121ºC for 15 min to use as the simulated cheese
84 medium. The simulated cheese medium was composed of fat (26.7 g,) casein as protein (24.1 g),
85 negligible amount of lactose as carbohydrate, minerals (3.4 g), and water (45.8 g/100 g of

86 medium).

87 Each medium contained in the Erlenmeyer flask (100 mL) received 4.2×10^4 conidia and
88 was maintained at a 15ºC for 7 or 10 d in a plastic container with wet papers attached to the
89 inside. The incubation was repeated three times except for the initial screening test shown in
90 Table 1.

91

92 **Preparation of the crude enzyme**

93 The resulting culture was transferred to a polyethylene bag and received an equal weight of
94 deionized water. The sample was treated by a stomacher for 5 min, and then transferred to a
95 centrifugal tube. Following centrifugation at 21,130 *g* and 4ºC for 10 min, the supernatant was
96 recovered and used as a crude enzyme.

97

98 **Measurement of lipase activity**

99 The lipase activity was determined as previously described (Kumura *et al*. 1991) with some
100 modifications. As the substrate, one gram of olive oil emulsified with 2 g/100 mL (degree of
101 polymerization was 2,000, Wako, Japan) or 4 g/100 mL of polyvinyl alcohol (degree of
102 polymerization was 2,000, Kishida, Japan) containing 0.05 M sodium phosphate-citric acid

103 buffer (pH 5.5 and 7.0) or 0.05 M Tris-HCl buffer (pH 7.0 and 8.5) was used. The substrate was
104 divided into 2.25 mL into each test tube and subjected to pre-incubation at 15ºC for 5 min,
105 followed by an addition of the crude enzyme (0.05 mL). The reaction was carried out at 15ºC
106 for 30 min. The enzyme activity was expressed as μmol of released oleic acid from the substrate
107 per 1 h at 15ºC using 1 mL of the crude enzyme (1 μmol oleic acid/h = 1 lipase unit: LU).
108
109 **Measurement of protease activity**
110 Proteolytic activity was determined as previously described (Kumura *et al*. 2017). As the
111 substrate, 0.2 g of casein was dissolved in 100 mL of 0.05 M sodium phosphate-citric acid
112 buffer (pH 5.5 and 7.0) or 0.05 M Tris-HCl buffer (pH 7.0 and 8.5). The substrate (700 μL) was
113 aliquoted to the microtubes and subjected to pre-incubation at 15ºC for 5 min, followed by an
114 addition of the crude enzyme (50 μL). The reaction was performed at 15ºC for 6 h. The enzyme
115 activity was expressed as μmol of released tyrosine from the substrate per 1 h at 15ºC using 1
116 mL of the crude enzyme (1 μmol tyrosine /h = 1 protease unit: PU).
117
118 **Statistical analysis**
119 Statistical analysis was performed with JMP pro 12.0.1 (SAS Institute, Inc., Tokyo, Japan). The

analysis was performed with a Tukey-Kramer's multiple comparison test. A possibility of less than 0.05 was considered statistically significant.

RESULTS

Selection of the strain

Figure 1 shows the representative appearance of the tested strains during cultivation on the WPC medium. Although the growth of 5 strains except AHU 8056 seemed to be moderate at day 7 (Figure 1, photos A, C, E, J and L), the solid surface was almost completely covered by mycelia in all strains if the incubation was continued until day 10 (Figure 1, photos B, D, F, I, K and M).

Table 1 shows the acidic lipase activity of the crude enzyme obtained from the WPC culture products. The lipase activities of all three strains of *P. roqueforti* were lower than those of *P. camemberti*. When the lipase activity of the crude enzyme was measured at day 10, the cultures of all *P. camemberti* strains were comparable, and that of *P. roqueforti* PRG 3 was much higher than the other two strains of *P. roqueforti*.

Due to its higher lipolytic feature and commercial availability as a dairy starter, we selected *P. roqueforti* PRG 3 for further observations. Consequently, both *P. camemberti* PC

TT033 and PCA 3 can be candidates for the following studies. However, strain PC TT033 was selected because of higher proteolytic activity than PCA 3 (data not shown).

Lipase activity of *P. camemberti* PC TT033 and *P. roqueforti* PRG 3 on the different culture medium and reaction pH values

The two selected strains were seeded on the WPC substrate and on the simulated cheese medium to compare their lipolytic activities and reactions to the pH levels under different conditions. The growth of mycelia from both strains on the cheese medium at day 10 was apparently comparable to that on the WPC medium (Figure 1, photos F, G, M and N). However, the lipase activity was reduced when the strains were cultured on the simulated cheese medium as shown in Table 2 that indicated the averages and standard deviation of the three independent cultures.

Statistical analysis showed that maximal lipolytic activity of *P. camemberti* PC TT033 on the WPC medium was at the alkaline pH region, whereas that from the simulated c heese medium was relatively constant within a wide range of pH. In contrast, the lipolytic activity of *P. roqueforti* PRG 3 on the WPC medium was maintained within a wide range of pH, whereas that on the simulated cheese medium was lower at the alkaline pH region than at

neutral pH.

Protease activity of *P. camemberti* PC TT033 and *P. roqueforti* PRG 3 on the different culture medium and reaction pH values

Table 3 shows the averages and standard deviation of the protease activity of crude enzyme at different pH values obtained from the three independent culture products of *P. camemberti* PC TT033 and *P. roqueforti* PRG 3. Independent from the culture media, the proteolytic activity of *P. camemberti* PC TT033 was constant within a wide range of pH. In contrast, maximal protease activity of *P. roqueforti* PRG 3 was clearly found at the acidic region independent from the culture media. However, statistical analysis showed that the higher proteolytic activity of *P. roqueforti* PRG 3 on the simulated cheese medium was maintained from the acidic until neutral pH.

DISCUSSION

Favorable growth of the six fungal strains on the WPC solid culture was confirmed. In addition, growth of the selected two strains of *P. camemberti* PTCC 033 and *P. roqueforti* PRG 3 on the simulated cheese medium seemed to be comparable to that on the WPC medium. Among the

171 three strains of *P. roqueforti*, pigmentation was lacking or less intense during the 10 days of
172 cultivation for strain AHU 8056 and PRG 3. Strain PRG 3, which had intermediate
173 pigmentation, showed the highest lipolytic activity of the three strains at 10 d cultivation.
174 Furthermore, Knight *et al*. (1950) reported that ultra-violet-induced mutants of *P. roqueforti*
175 produced lipase and protease with no marked change of growth rate. From these observations,
176 there seems to be no direct relevance between the green pigmentation and enzyme production in
177 *P. roqueforti*.

178 To consider the pH and temperature circumstances during cheese ripening, we initiated
179 the lipolytic activity measurement under the acidic region of pH along with a lower reaction
180 temperature. Since most milk fat will be solid under this lower temperature, poor
181 reproducibility of emulsion preparation was predicted. Accordingly, we selected olive oil as the
182 substrate to facilitate stable and liquid emulsification.

183 In our subsequent investigation, it was proved that the estimation under alkaline pH was
184 more appropriate if the crude enzyme of *P. camemberti* PC TT033 cultured on the WPC
185 medium was used. Lambert and Lenoir (1976) reported on the lipase activity of *P. camemberti*
186 with an optimum pH of 9.0 and Tan *et al*. (2004) evaluated the lipase activity of *P. camemberti*
187 Thom PG-3 using olive oil emulsified with gum arabic as the substrate with a pH of 8.0.

188 However, it was possible to influence an expression balance of the lipase genes when *P*.
189 *camemberti* PC TT033 was cultured on the simulated cheese medium because the level of
190 lipase activity at pH 5.5 was comparable to that at pH 8.5. Although no information has been
191 available concerning acidic lipase from *P*. *camemberti*, at least two molecular species of the
192 secreted type of lipase are predicted according to the nucleotide analysis of *P*. *camemberti* FM
193 013, nominated as accession No. CRL24317.1 and CRL29609.1 in the DNA database
194 (https://www.ncbi.nlm.nih.gov/). Furthermore, Yamaguchi and Mase (1991) examined the
195 characteristics of mono- and diacylglycerol lipase obtained from the liquid culture of *P*.
196 *camemberti* U-150 under an acidic enzyme reaction condition. If *P*. *camemberti* PC TT033
197 produced a similar molecular species, concomitant triacylglycerol lipase would release
198 diacylglycerol, which could be the subsequent substrate for diacylglycerol lipase. Whether *P*.
199 *camemberti* PC TT033 produced acidic mono- and diacylglycerol lipase or an unknown acidic
200 triacylglycerol lipase on the simulated cheese medium should be investigated.

201 In terms of the lipolytic activity of *P*. *roqueforti* PRG 3, an alkaline pH was unfavorable
202 for reaction when the crude enzymes of the strain cultured on the cheese medium. Mase *et al*.
203 (1995) purified lipase of *P*. *roqueforti* IAM 7268 with an optimum pH value of 6.0~7.0.
204 Lamberet and Menassa (1983) found the optimum lipase activity of *P*. *roqueforti* at pH 6.0~6.5

205 with second minor optimum appeared close to pH 2.8, whereas there was a minimum activity at
206 pH 3.6. Mcsweeney and Sousa (2000) reviewed that *P. roqueforti* secretes 2 lipases with
207 optimum pH values between 6.0 and 6.5 and alkaline region (7.5–9.5). Some lipases from *P.*
208 *roqueforti* showed its maximum activity around pH 8.0 (Eitenmiller *et al*. 1970) or pH 9.0
209 (Menassa and Lamberet 1982).

210 Regarding protease from *P. camemberti*, the acid proteases, including metalloprotease
211 and aspartate proteinase, have been reported (Chrzanowska *et al*. 1995; Spinnler 2017) whereas
212 the alkaline protease has been found in endopeptidase (Gripon and Debest 1976) and
213 aminopeptidase II (Matsuoka *et al*. 1991). Proteinase profile seemed to be unaffected from the
214 culture media used in this study.

215 In contrast, metalloprotease and aspartic proteinase have been reported in *P. roqueforti*
216 (Gripon and Hermier 1974) with an optimum pH in the acidic region. This study agreed with
217 previous results that the proteolytic activity of the crude enzymes obtained from *P. roqueforti*
218 PRG 3 cultivated in both culture media decreased along with the reaction circumstance shifts to
219 the alkaline region, although the protease produced in the simulated cheese medium was more
220 favorable at neutral pH region than that in the WPC medium.

221 Overall, the impact of the culture medium replacement for both strains seemed much

222 more on the lipase activity than what was observed in the protease activity. In spite of milk lipid
223 abundance with limited amount of carbohydrate, the lipolytic activity was decreased when both
224 strains were cultured on the simulated cheese medium. Nevertheless, lipolytic activity of *P.*
225 *camemberti* PC TT033 cultured on the simulated cheese medium was still higher than the
226 activity from *P. roqueforti* PRG 3. This is contradictory to the general understanding that a
227 lower FFA content is found in white-mold cheese than in blue-veined cheese. One of the
228 possible reason of this inconsistency is that *P. camemberti* grows only on the surface of the
229 white-mold cheese and its ripening proceeds gradually from the surface to the inner part of the
230 curds, whereas the blue cheese is matured by *P. roqueforti* with a marble-like distribution
231 within the inner part the curds, which would result in the difference in biomass of the starter
232 fungi. Since bloomy-rind cheese and blue-veined cheese generally contains 2.5% and 3.5~4.5%
233 of salt, respectively (Guinee and Fox 2017), we incubated *P. camemberti* PC TT033 and *P.*
234 *roqueforti* PRG 3 on the cheese medium containing 2.5% and 4.0% sodium chloride,
235 respectively, which resulted in considerable growth reduction (data not shown). The growth of
236 these fungi along with the enzyme production should be investigated in the presence of
237 commonly applied salt concentration in cheese manufacture. Furthermore, the ripening period
238 of white-mold cheese is shorter than that of blue cheese in general and it should be taken into

account that the fate of the FFA that are released due to the action of lipase during ripening. According to the metatranscriptome analysis of ripening Camembert-type cheese, the conversion of fatty acids to acetyl-CoA was activated around day 15 of ripening (Lessard *et al*. 2014), suggesting that the released FFA would be consumed immediately by the living *P. camemberti*.

In conclusion, this study suggested that the production of multiple isozymes belonging to the lipase and protease of *P. camemberti* and *P. roqueforti* would be regulated on the milk-protein-based solid substrates and demonstrated the high potential of *P. camemberti* for lipase production compared to *P. roqueforti*. Further studies on which capabilities and potential could be exerted when dairy *Penicillium* sp. is placed under various environments using a solid culture system are still to be completed.

Figure 1 Growth and appearance of *P. camemberti* AHU 8113, d 7 (A), d 10 (B), PCA 3, d 7 (C), d 10 (D), and PC TT033, d 7 (E), d 10 (F), and *P. roqueforti* AHU 8056, d 7 (H), d 10 (I), PR 3, d 7 (J), d 10 (K), and PRG 3, d 7 (L), d 10 (M) on the WPC medium or *P. camemberti* PC TT033, d 10 (G) and *P. roqueforti* PRG 3, d 10 (N) on the simulated cheese medium incubated at 15ºC.

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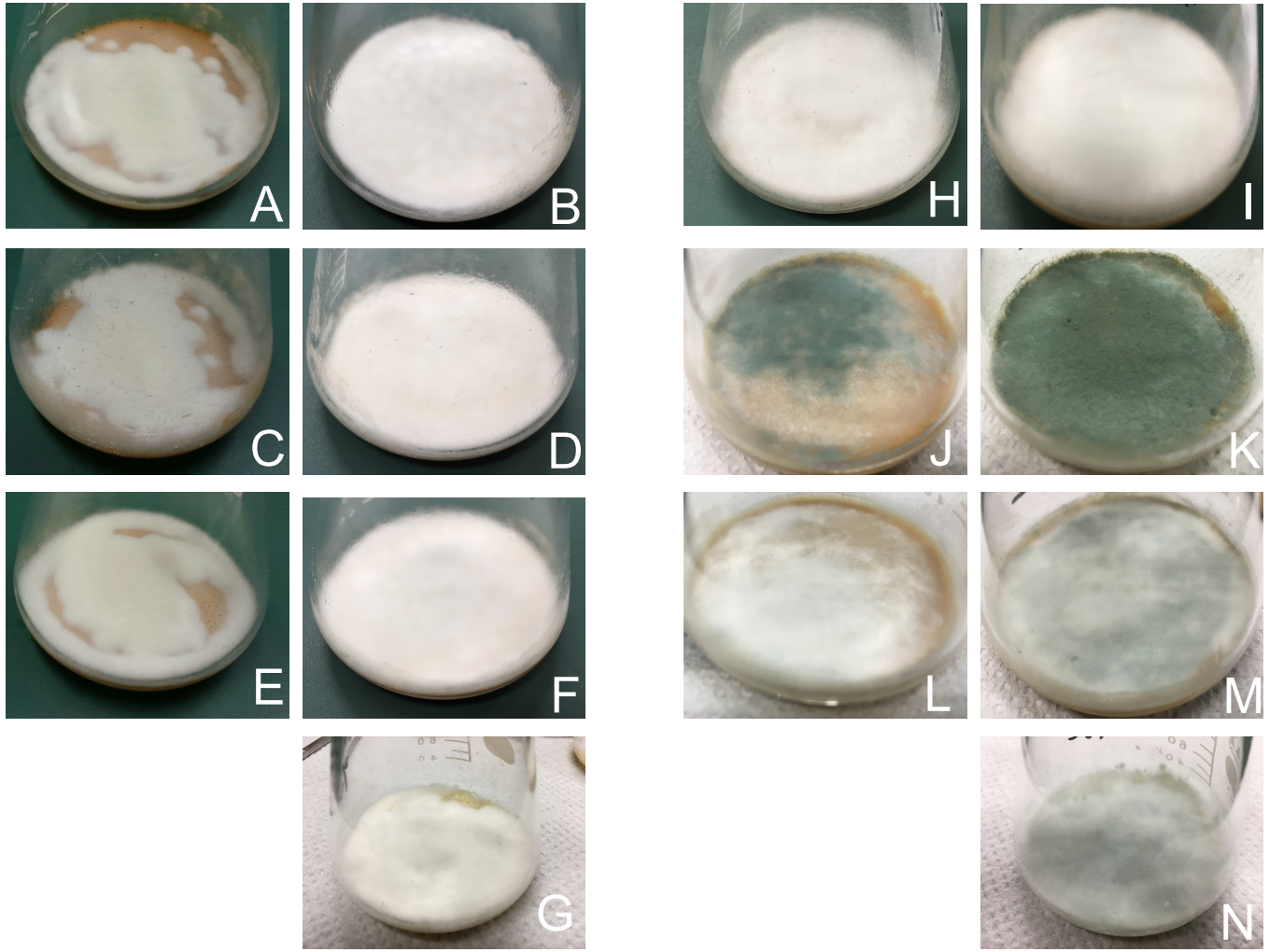

Fig. 1

Table 1 Lipase activity[1] of 6 strains *Penicillium* sp.

species	*P. camemberti*			*P. roqueforti*		
incubation (d)/strain	AHU 8113	PCA 3	PC TT033	AHU 8056	PR 3	PRG 3
7	13.4	105.1	45.1	1.5	5.0	0.4
10	154.3	151.0	156.1	3.7	8.2	23.8

Incubation was carried out at 15ºC for 7 and 10 on the WPC medium.

[1] The lipase activity was measured using emulsified olive oil in 0.05 M sodium phosphate-citric acid buffer (pH 5.5) and expressed as LU.

Table 2
Lipase activity of selected *Penicillium* sp. strains cultivated on the WPC or cheese medium.

Strain	*P. camemberti* PC TT033		*P. roqueforti* PRG 3	
	WPC medium	Cheese medium	WPC medium	Cheese medium
pH				
5.5	172.2 ± 12.5 [a]	62.3 ± 2.6 [a]	13.7 ± 11.2 [a]	2.5 ± 0.6 [ab]
7.0	216.8 ± 7.9 [b]	43.2 ± 13.0 [a]	21.2 ± 15.5 [a]	3.5 ± 0.5 [a]
8.5	307.3 ± 9.3 [c]	46.7 ± 11.9 [a]	24.2 ± 22.8 [a]	1.7 ± 1.1 [b]

Averaged LU and standard deviation were shown.
Not sharing the same letter in the same column expresses the statistical significance of enzyme activity obtained from the specific medium under different pH circumstances ($P < 0.05$).

Table 3
Protease activity of selected *Penicillium* sp. strains cultivated on the WPC or cheese medium.

Strain	*P. camemberti* PC TT033		*P. roqueforti* PRG 3	
	WPC medium	Cheese medium	WPC medium	Cheese medium
pH				
5.5	0.107 ± 0.034 [a]	0.095 ± 0.070 [a]	0.421 ± 0.081 [a]	0.314 ± 0.022 [a]
7.0	0.122 ± 0.018 [a]	0.195 ± 0.053 [a]	0.308 ± 0.042 [b]	0.279 ± 0.093 [a]
8.5	0.135 ± 0.011 [a]	0.117 ± 0.011 [a]	0.049 ± 0.019 [c]	0.103 ± 0.021 [b]

Averaged PU and standard deviation were shown.
Not sharing the same letter in the same column expresses the statistical significance of enzyme activity obtained from the specific medium under different pH circumstances ($P < 0.05$).