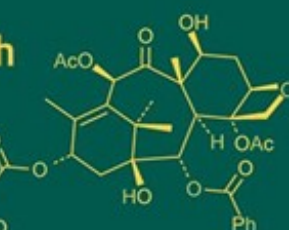


International Journal of Advanced Biochemistry Research



ISSN Print: 2617-4693
 ISSN Online: 2617-4707
 NAAS Rating (2026): 5.29
 Indexing: Embase
 IJABR 2026; SP-10(9): 2572-2576
www.biochemjournal.com
 Received: 31-07-2026
 Accepted: 30-08-2026
 Published: 29-09-2026

The names and affiliations of all authors are provided below, following the references.

Comparative analysis of effects of enzymatic hydrolysis reaction in pH controlled and non pH controlled medium on defatted ghee residue protein

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DOI: <https://www.doi.org/10.33545/26174693.2026.v10.i9Saf.10220>

ABSTRACT

Protein hydrolysis reactions are widely recognized for their importance in increasing the bioavailability and functional utility of protein rich by-products, thereby contributing to nutrient security. In this study, byproduct of dairy industry i.e defatted ghee residue (DGR), has been evaluated to study the effects of enzymatic hydrolysis process on its protein. Defatted GR has been hydrolysed using different concentrations of Alcalase enzyme in pH controlled (Phosphate buffer) as well as in non pH controlled medium (Distilled water). The effect of hydrolysis on the protein was assessed by periodically monitoring the pH changes, degree of hydrolysis and antioxidant potential of resulting hydrolysate. Reaction samples were collected at every 2 hr intervals over an 8 hrs incubation period. In enzymatic hydrolysis, Alcalase 1:50 E:S ratio in pH controlled medium for 4 hr hydrolysis has shown very good degree of hydrolysis and antioxidant scavenging capacity after breaking down of protein into smaller peptides.

Keywords: Protein denaturation, Peptides, Functional properties, Protein powder

1. Introduction

Ghee residue obtained as byproduct of dairy industry has significant amount of protein, varying as per the method of preparation as well as regional disparity in milk used and its handling. To separate this protein and harness its full potential it has been defatted however, for increasing its utility the protein characters need to be changed so as to increase its functional value. Ghee residue is originally an acidic substance due to its over handling and production of lactic acid in it. Correctly adjusting its pH suitable for hydrolysis reaction and how it changes over period of hydrolysis reaction is studied in pH controlled phosphate buffer solution and distilled water medium for evaluating changes of pH variation on reaction progression.

Enzymatic hydrolysis as a biocatalytic process has converted complex macromolecules into simple and usable structure with production of good amount of bioactive peptides in case of proteins. These biopeptides have shown very good functional properties thus converting low value byproduct industry to important supply unit for high valued food industry. Thus utilizing it for byproduct processing is popular as well as sustainable practice.

Enzymatic hydrolysis has better performance over any other hydrolysis process. It uses milder conditions for reaction and yield better results over other methods. It has very less environmental footprint and proved to be ecologically conscious concept (Carrasco-Castilla *et al.*, 2012; Juhneva-Radenkova *et al.*, 2021) [1, 2]. However instead of proving better performance, the reaction is still very sensitive to pH changes and temperature during process. The reaction shows continuous breakdown and production of ions which cause changes in pH and also affect the temperature simultaneously, thus reaction needs continuous monitoring in controlled condition. pH has important role in determining enzyme activity, substrate solubility, enzyme-substrate interactions and the stability of the enzyme throughout the hydrolysis process. In this, release and consumption of acidic and basic group leads to pH change in medium continuously. These changes alter the catalytic effect of the enzyme, thus influencing rate of hydrolysis as well as final results. For this, comparative analysis of pH changing medium with non pH changing medium is mainly focused in this study.

2. Materials and Methods

-In this experiment enzyme Alcalase (From-Bacillus licheniformis) is used.

-Activity-2.972 U/mol

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Preparation of protein hydrolysates by proteolytic enzyme (Alcalase)

Defatted powder of ghee residue with protein content of 64.63 ± 1.77 was reconstituted in distilled water and PBS at 5% level and homogenized for 15 min at 20000 RPM using homogenizer. pH of solution was adjusted to 8 using 0.1N NaOH solution. The solution was heated to 85°C for 15 mins to reduce bacterial contamination and to inactivate other enzymes present. Three enzyme substrate ratios 1:50, 1:100, 1:200 were evaluated at a temperature 55 °C. After addition of enzyme, it was incubated at 55°C in shaking water for 8 hours of hydrolysis time. At interval of every 2 hours, the sample was drawn upto 8 hrs (2h, 4h, 6h, 8h). Inactivation of enzyme was done by transferring it into water bath at 85°C for 15 min so as to denature residual enzymes. The resulting hydrolysates were analyzed for pH changes, degree of hydrolysis and antioxidant activity.

Change in pH

Change in pH of hydrolysate was recorded at every 2 hrs of hydrolysis after inactivating starter culture using digital pH meter.

Sample preparation to evaluate DH and antioxidant properties

The samples were subsequently centrifuged at 8000 rpm for 30 minutes at 4 °C, supernatant was collected for analysis

Degree of hydrolysis

The degree of hydrolysis (DH) of fermented sample was evaluated by SN-TCA method in which measurement of the protein that dissolved in a 10% (w/v) trichloroacetic acid (TCA) solution relative to the total protein content in the sample was assessed (Hoyle and Merritt, 1994). A 500µL sample of the supernatant was mixed with an equal volume of 20% (w/v) TCA solution in order to separate the soluble and insoluble fractions, resulting in a final concentration of 10% TCA. The mixture was allowed to stand at room temperature for 30 minutes before being centrifuged at 3500 rpm for 15 minutes in a refrigerated centrifuge. The soluble protein content in the supernatant was then measured using the Lowry *et al.* (1951) [3] method, with results expressed in mg of protein. Bovine serum albumin served as the reference standard. The degree of hydrolysis was calculated using equation

$$DH (\%) = \frac{\text{Solubilised protein content in 10\%TCA (mg)}}{\text{Total protein content (mg)}} \times 100$$

Antioxidant capacity

DPPH radical scavenging activity

The method used to evaluate the scavenging activity of 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) radicals by antioxidants in the sample was adapted from Brand-Williams *et al.* (1995) [4]. 9.85 mg of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) was dissolved in 250 ml methanol and kept in dark at 4°C used as reagent.

The DPPH solution in methanol acted as a stable radical, with peak absorption observed at 517 nm. 4.9 ml of 0.1 mM (millimolar) DPPH reagent in methanol was combined with 100 µl of samples in test tubes. The mixture was gently agitated and absorbance was recorded at 0 min of incubation and 20 min after incubation. Methanol served as the blank. The absorbance of the solution was measured at 517 nm using a Genesys 10S UV-Vis spectrophotometer. A reduction in absorbance at 517 nm indicated the conversion of the DPPH radical to 2,2-Diphenyl-1-Picrylhydrazine. The DPPH radical scavenging activity was determined using equation

$$\% \text{ Inhibition} = 100 - \left[\left(A_{20}/A_0 \right) \times 100 \right]$$

A₂₀ denotes absorption at 20th min

A₀ denotes absorption at 0th min

ABTS radical scavenging activity

The antioxidant properties of the hydrolysates were evaluated using the ABTS⁺ (2,2-Azinobis(3-Ethylbenzothiazoline-6-Sulfonic acid)) radical cation decolourization method, as outlined by Salami *et al.* (2009) [5]. This technique involves generating the ABTS⁺ radical through a reaction with potassium per-sulphate. Antioxidants present in the sample reduce ABTS⁺ via hydrogen or electron donation, or a combination of both mechanism and this reduction is measured spectrophotometrically at 734 nm.

Reagents

a) Potassium per-sulphate solution (2.45 mM)

3.1 mg of potassium per-sulphate was dissolved in double distilled water and the volume was made up to 5 ml, freshly prepared before use.

b) ABTS stock solution (7 mM)

19.2 mg of ABTS was dissolved in 5 ml of double distilled water and was mixed with 2.45 mM potassium per-sulphate solution. The mix-

ture was stored in an amber colour bottle in dark for 15 h for production of sufficient free radicals (ABTS⁺).

Procedure

The ABTS radical cation (ABTS⁺) was generated by reaction of a 7 mM ABTS stock solution with 2.45 mM potassium per-sulphate (final concentration) and allowing the mixture to incubate in the dark at room temperature for 15 hours. The ABTS⁺ solution was then diluted with methanol to achieve an absorbance of 0.70 (±0.02) at 734 nm at the initial time point (*t* = 0 min) and equilibrated at 30 °C exactly 6 minutes after initial mixing. A 10 µl aliquot of the hydrolysate sample was mixed with 1 ml of the diluted ABTS⁺ solution, and the absorbance was measured at 734 nm using Genesys 10S UV-Vis spectrophotometer, readings were taken at 30°C (exactly 1 min after initial mixing and up to 20 min at 1 min intervals). It showed that the dependence of the antioxidant activity on time was linear up to the first 6-min reaction time. So, 6-min reaction time was used for all hydrolysates. The ABTS⁺ scavenging activity was calculated using the formula

$$\text{ABTS activity (\%inhibition)} = \left[\left(0.7 - A_6 \right) / 0.7 \right] \times 100$$

Where, A₆ represents the absorption at the 20th minute

3. Result and Discussion

Change in pH

During enzymatic hydrolysis, the pH of the samples was initially maintained at the optimum pH of 8 for enzyme activity. However, as the hydrolysis progressed, a decrease in pH was observed in both media—PBS (phosphate-buffered saline) and DW (distilled water), as shown in Table-1 and Table-2. This decline in pH suggests ongoing biochemical changes during the hydrolysis process.

In PBS, after 2 hrs of hydrolysis significant ($p < 0.05$) fall has been recorded at all E:S ratio however different concentration of enzyme had shown insignificantly ($p > 0.05$) effect on pH fall. The trend of pH fall got somewhat stabilized after 4 hours of hydrolysis, with minor changes. However, among different enzyme concentrations E:S ratio 1:50 had shown significantly ($p < 0.05$) higher fall in pH at 4, 6 and 8 hrs of hydrolysis

In case of DW significant ($p < 0.05$) fall has been recorded over time as hydrolysis reaction progresses, however different concentration not shown many differences in pH fall at different time of hydrolysis.

The pH of samples during enzymatic hydrolysis falls significantly over time in both PBS and DW mediums. In PBS, the pH stabilizes after 4 hours, with the E:S ratio of 1:50 showing a more pronounced fall. In DW, the pH continues to fall significantly over time, with enzyme concentration having little effect on the extent of pH change.

Kumar *et al.* (2016) [6] reported similar fall in pH as hydrolysis reaction progresses for preparation casein hydrolyste using PBS and distilled water in respective studies. pH is highly dependent on extent of hydrolysis of protein and it affects the structure and activity of enzyme during hydrolysis (Kumar *et al.*, 2016; Fernández *et al.*, 2016) [6, 7] reaction which is reported in subsequent sections of this study.

It indicates that the medium (PBS versus DW) significantly influences pH changes during enzymatic hydrolysis, with DW exhibiting a greater rate of acidification. This difference signifies the importance of selecting the appropriate buffer medium for enzymatic reactions to cope up pH fall during the process, as it can substantially affect the outcomes and interpretations of the experiments which is discussed in further parameters below.

($n = 6$); Mean $\pm$ SE with different superscripts row wise (a,b,c) and column wise (A,B) differ significantly ($p < 0.01$)

Table 1. pH Change during Enzymatic Hydrolysis of Ghee Residue in PBS

Time (hrs)	Enzyme Concentration		
	1:50	1:100	1:200
0	8.010 $\pm 0.006^{Aa}$	8.010 $\pm 0.006^{Aa}$	8.010 $\pm 0.006^{Aa}$
2	7.420 $\pm 0.017^{Ab}$	7.428 $\pm 0.017^{Ab}$	7.428 $\pm 0.017^{Ab}$
4	7.302 $\pm 0.009^{Bc}$	7.375 $\pm 0.011^{ABbc}$	7.397 $\pm 0.017^{Ab}$
6	7.277 $\pm 0.040^{Bc}$	7.345 $\pm 0.011^{ABc}$	7.372 $\pm 0.014^{Ab}$
8	7.275 $\pm 0.017^{Bc}$	7.332 $\pm 0.018^{ABc}$	7.367 $\pm 0.018^{Ab}$

Table 2. pH Change during Enzymatic Hydrolysis of Ghee Residue in DW

Time	1:50:00	1:100	1:200
0 hrs	8.010 $\pm 0.005^{Aa}$	8.010 $\pm 0.005^{Aa}$	8.010 $\pm 0.005^{Aa}$
2 hrs	7.008 $\pm 0.044^{Bb}$	7.257 $\pm 0.059^{Ab}$	6.931 $\pm 0.048^{Bb}$
4 hrs	6.793 $\pm 0.063^{Ab}$	6.901 $\pm 0.044^{Ac}$	6.745 $\pm 0.063^{Abc}$
6 hrs	6.543 $\pm 0.068^{Ac}$	6.659 $\pm 0.055^{Ad}$	6.655 $\pm 0.058^{Ac}$
8 hrs	6.269 $\pm 0.061^{Bd}$	6.351 $\pm 0.063^{Be}$	6.600 $\pm 0.053^{Ac}$

($n = 6$); Mean $\pm$ SE with different superscripts row wise (a,b,c,) and column wise (A,B,C) differ significantly ($p < 0.05$)

4.3.2 Degree of hydrolysis (DH)

The results for the DH are presented in Table-3 and Table-4. In the PBS medium, as hydrolysis progressed, a significant ($p < 0.05$) increase in DH was observed after 2 hours. The highest DH was recorded in the PBS 1:50 concentrations (24.993 ± 0.714), compared to other enzyme concentrations. In contrast, the 1:200 concentrations showed the lowest DH (11.975 ± 0.710). These findings suggest that higher enzyme concentrations promote more efficient hydrolysis.

By 4 hrs, the trend continues, with PBS 1:50 reaching to highest observation in experiment for DH of 33.894 ± 0.389 , which had shown again superior hydrolysis over other two enzyme concentrations, For 6 and 8 hrs, there is a slight decrease in hydrolysis for PBS 1:50, but it remains the highest overall.

In DW medium similar trend of increasing DH with progress of hydrolysis reaction had been observed and higher concentration of enzyme had performed superior to other two concentrations.

Comparatively higher reading for DH in PBS medium over DW medium concludes that the DH is significantly affected by the choice of medium, with PBS providing a more favorable environment for enzymatic activity than DW which is due to pH change observed during the experiment where decrease in pH for PBS medium was less as mentioned in section pH change. The findings suggest that enzyme concentration as well as appropriate medium providing buffering action during reaction is crucial for enhancing enzymatic hydrolysis efficiency. Butré *et al.*, 2015 [8] reported significant change in DH on slight change in pH during enzymatic reaction which signifies the importance of pH control medium for enzymatic hydrolysis.

A decrease in the hydrolysis rate over time may occur when high enzyme concentrations deplete the available substrate for further activity. Conversely, at lower enzyme concentrations, there is less enzyme to act on the substrate (Ye *et al.*, 2014; Deng *et al.*, 2018) [10, 9]. Precipitation can impact the measurement of the DH in the experiment. This explains why the initially increasing trend in the DH began to decline after several hours of the enzymatic hydrolysis reaction. Similarly Kumar *et al.* (2016) [6] and Espejo-Carpio *et al.* (2018) [11] reported stabilization of rate of DH in subsequent hours of hydrolysis due to various reasons like depletion of enzyme activity sites and saturation in reaction rate during hydrolysis.

Table 3. Degree of hydrolysis of enzymatically hydrolysed ghee residue in PBS

Time (hrs)	1:50	1:100	1:200
0	4.595 $\pm 0.489^{Ad}$	4.595 $\pm 0.489^{Ad}$	4.595 $\pm 0.489^{Ad}$
2	24.993 $\pm 0.714^{Ac}$	17.864 $\pm 0.398^{Bc}$	11.975 $\pm 0.710^{Cc}$
4	33.894 $\pm 0.389^{Aa}$	22.418 $\pm 0.446^{Bb}$	19.983 $\pm 0.239^{Ca}$
6	28.207 $\pm 0.546^{Ab}$	26.596 $\pm 0.547^{Aa}$	15.918 $\pm 0.444^{Bb}$
8	25.874 $\pm 0.598^{Ac}$	25.814 $\pm 0.422^{Aa}$	13.853 $\pm 0.528^{Bbc}$

($n = 6$); Mean $\pm$ SE with different superscripts row wise (a,b,c,d) and column wise (A,B,C) differ significantly ($p < 0.05$)

Table 4. Degree of hydrolysis of enzymatically hydrolysed ghee residue in DW

Time	1:50	1:100	1:200
0 hrs	3.976 $\pm 0.670^{Ac}$	3.976 $\pm 0.670^{Ac}$	3.976 $\pm 0.670^{Ac}$
2 hrs	14.700 $\pm 1.697^{Ab}$	7.045 $\pm 0.597^{Bbc}$	5.704 $\pm 0.352^{Bbc}$
4 hrs	16.214 $\pm 1.123^{Ab}$	11.861 $\pm 1.043^{Ba}$	10.632 $\pm 0.906^{Ba}$

(continued on next page)

Time	1:50	1:100	1:200
6 hrs	19.256 ± 1.379 ^{Aab}	10.921 ± 0.712 ^{Ba}	4.699 ± 0.411 ^{Cbc}
8 hrs	22.580 ± 1.800 ^{Aa}	9.332 ± 0.973 ^{Bab}	7.239 ± 0.745 ^{Bb}

(*n* = 6); Mean±SE with different superscripts row wise (a,b,c,) and column wise (A,B,C) differ significantly (*p* < 0.05)

Antioxidant capacity

DPPH radical scavenging activity

DPPH radical scavenging potential of peptides obtained during enzymatic hydrolysis of GR is shown in Table-5 and Table-6.

Unhydrolysed protein had shown 5.373 ± 1.169% inhibition of DPPH radical at 0 hrs in PBS medium and 6.034 ± 0.684% inhibition in DW medium. Which was significantly (*p* < 0.05) increase as reaction progresses with highest inhibition of 15.420 ± 0.512% at 8 hrs hydrolysis in PBS medium at 1:50 E:S ratio. As time progresses for reaction, inhibition of DPPH radical is increasing which indicates as time progresses more and more peptides were generating due to hydrolysis of protein which are responsible for antioxidant activity (Al-Shamsi *et al.*, 2018) [12]. Both the tables has clearly shown that inhibition is higher with higher concentration of enzyme, similar result are obtain for DH which clearly demonstrate greater hydrolysis when more enzyme is available for substrate. It also yields better results in PBS medium compared to distilled water (DW), supporting enhanced enzyme activity for hydrolysis in PBS medium. From this observations we can say that enzymatically hydrolysed GR prepared using alcalase has potential to act as electron donor for inhibition of free radicals which highlighted its ability as potential antioxidant in food system (Mao *et al.*, 2011) [13].

This finding aligns with the results of Garg *et al.* (2024) [14], who also noted a significant initial increase in scavenging activity within the first two hours of hydrolysis of casein. While this increase continued in the following hours, the rate of change eventually got stabilized (Nascimento *et al.*, 2021) [15].

Table 5. DPPH inhibition of enzymatically hydrolysed ghee residue in PBS

Time (hrs)	1:50	1:100	1:200
0	5.373 ± 1.169 ^{Ac}	5.373 ± 1.169 ^{Ab}	5.373 ± 1.169 ^{Ac}
2	12.778 ± 0.407 ^{Ab}	11.322 ± 0.984 ^{ABa}	11.033 ± 0.710 ^{Bbc}
4	13.724 ± 1.495 ^{Aab}	12.403 ± 0.720 ^{ABa}	10.823 ± 0.816 ^{Bc}
6	15.351 ± 0.336 ^{Aa}	11.387 ± 0.664 ^{Ba}	13.087 ± 1.611 ^{Bab}
8	15.420 ± 0.512 ^{Aa}	13.091 ± 0.606 ^{Ba}	13.254 ± 1.000 ^{Ba}

(*n* = 6); Mean±SE with different superscripts row wise (a,b,c,) and column wise (A,B) differ significantly (*p* < 0.05)

Table 6. DPPH inhibition of enzymatically hydrolysed ghee residue in DW

Time	1:50:00	1:100	1:200
0 hrs	6.034 ± 0.684 ^{Ab}	6.034 ± 0.684 ^{Ac}	6.034 ± 0.684 ^{Ab}
2 hrs	14.986 ± 0.681 ^{Aa}	12.576 ± 0.905 ^{ABab}	11.603 ± 0.779 ^{Ba}
4 hrs	14.503 ± 0.848 ^{Aa}	13.607 ± 0.875 ^{Aa}	12.525 ± 0.932 ^{Aa}
6 hrs	15.102 ± 0.718 ^{Aa}	14.465 ± 0.898 ^{Aa}	6.988 ± 0.834 ^{Bb}
8 hrs	14.910 ± 0.654 ^{Aa}	10.132 ± 0.747 ^{Bb}	11.062 ± 0.787 ^{Ba}

(*n* = 6); Mean±SE with different superscripts row wise (a,b,c,) and column wise (A,B) differ significantly (*p* < 0.05)

ABTS radical scavenging activity

ABTS radical scavenging potential of peptides obtained during enzymatic hydrolysis of GR is shown in Table-7 and Table-8.

For ABTS assay trend of inhibition is varying as at 2,4,6 hrs shown significantly (*p* < 0.05) higher inhibition at lower enzyme concentration. Also significant (*p* < 0.05) variation occurred at different concentration of enzyme at different time of hydrolysis this all is possible due to influence of enzyme to substrate concentration on mechanism of hydrolysis reaction and exposure different amino acid at different time of reaction and at different E:S activity to free radicals which is important mechanism of antioxidant capacity of peptides (Deng *et al.*, 2018; Klompong *et al.*, 2007) [9, 16]. However, overall hydrolysis had performed well in PBS medium with highest inhibition occurred at 6hrs of hydrolysis by 1:50 E:S ratio in PBS medium. Unhydrolysed protein has ABTS inhibition around 39% (39.091 ± 0.893% in PBS and 39.455 ± 0.929% in DW) in both the medium which had shown significant (*p* < 0.05) increase in ABTS inhibition potential at different E:S ratio as well as different reaction time.

Maux *et al.* (2016) [17] reported better antioxidant potential for hydrolysate prepared in pH controlled medium as compare to hydrolysate prepared in medium having no pH control, in our study for we observed similar activity in DW and PBS medium.

Table 7. ABTS inhibition of enzymatically hydrolysed ghee residue in PBS

Time (hrs)	1:50	1:100	1:200
0	39.091 ± 0.893 ^{Ad}	39.091 ± 0.893 ^{Ad}	39.091 ± 0.893 ^{Ac}
2	79.687 ± 0.367 ^{Cc}	86.222 ± 0.234 ^{Bb}	92.564 ± 0.352 ^{Aa}
4	83.574 ± 0.224 ^{Bb}	92.618 ± 0.421 ^{Aa}	93.628 ± 0.644 ^{Aa}
6	96.801 ± 0.294 ^{Aa}	83.022 ± 0.418 ^{Cc}	92.311 ± 0.553 ^{Ba}
8	82.304 ± 0.446 ^{Ab}	82.271 ± 0.329 ^{Ac}	76.674 ± 0.359 ^{Bb}

(*n* = 6); Mean±SE with different superscripts row wise (a,b,c,) and column wise (A,B,C) differ significantly (*p* < 0.05)

Table 8. ABTS inhibition of enzymatically hydrolysed ghee residue in DW

Time	1:50:00	1:100	1:200
0 hrs	39.455 ± 0.929 ^{Ad}	39.455 ± 0.929 ^{Ad}	39.455 ± 0.929 ^{Ad}
2 hrs	59.436 ± 0.994 ^{Bab}	73.529 ± 0.962 ^{Aa}	71.580 ± 0.903 ^{Aa}
4 hrs	63.599 ± 0.963 ^{Ba}	69.436 ± 1.009 ^{Aa}	61.531 ± 0.924 ^{Bb}
6 hrs	50.142 ± 1.269 ^{Bc}	59.973 ± 1.128 ^{Ab}	59.547 ± 0.918 ^{Ab}
8 hrs	56.716 ± 1.061 ^{Ab}	55.547 ± 0.956 ^{Ac}	53.562 ± 0.901 ^{Ac}

(*n* = 6); Mean±SE with different superscripts row wise (a,b,c,d) and column wise (A,B) differ significantly (*p* < 0.05)

4. conclusion

Considering all the parameters in the enzymatic hydrolysis experiment, an enzyme-substrate (E:S) ratio of 1:50 performed the best overall, particularly in PBS medium. Therefore, a 4-hour hydrolysis period in PBS medium at an E:S ratio of 1:50, at 55 °C, with an initial pH of 8, is considered best.

Researchers such as Belitz *et al.* (1999) [18] and Carreira *et al.* (2003) [19] have suggested limiting enzymatic hydrolysis to a duration of up to 5 hours for various reasons, including the potential contamination of protein preparations after 5 hours and the generation of bitter pep

tides with extended hydrolysis. Additionally, for large-scale applications, economic considerations favor a shorter hydrolysis time. Based on these factors, a 4-hour hydrolysis time was chosen for this study.

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How to cite this article: Khawale AV, Chauhan G, Mishra J, Ahmed J, Sen A, Chaple PM, et al. Comparative analysis of effects of enzymatic hydrolysis reaction in pH controlled and non pH controlled medium on defatted ghee residue protein. *International Journal of Advanced Biochemistry Research.* 2026;SP-10(9):2572-2576. doi: <https://www.doi.org/10.33545/26174693.2026.v10.i9Saf.10220>

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