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Determination of The Optimum pH and Enzyme Ratio for Starch Hydrolysis Test and Characterization of Steamed, Baked, and Fried Wheat Doughs

Setya Budi Muhammad Abduh[1]*, V. Priyo Bintoro[1], Vitus Dwi Yunianto[2], Yoyok Budi Pramono[2]

[1]Department of Food Technology, Faculty of Animal and Agricultural Sciences, Universitas Diponegoro, Indonesia
[2]Professional Engineer Education Program, Faculty of Engineering, Universitas Diponegoro, Indonesia

*Corresponding author (setya.abduh@live.undip.ac.id)

Abstract

Digestibility is the most important nutritional functionality of starchy foods. Unfortunately, in Indonesia, this functionality has not been extensively studied due to the relatively challenging methods involving enzymes. This research aims to determine the optimal pH and enzyme ratio of α-amylase (AA) and glucoamylase (GA) for starch hydrolysis tests and apply them to characterize steamed, baked, and fried wheat doughs. For optimization, hydrolysis was carried out at 37 °C with an enzyme-to-substrate ratio (E/S) of 100 mL/g for 120 min. Samples of wheat dough obtained from three major dough producers in Banyumanik, Semarang, Indonesia was then tested for starch hydrolysis, texture, chemical analyses, sensory, and hedonic test. The collected data were analysed using principal component analysis (PCA). Under the determined conditions, the optimal pH and AA/GA ratio were found to be 6.6 and 0.5. Regarding quality of processed doughs, the steamed dough exhibited a cohesive texture, a soft sensory profile with a pleasant aroma, sweet taste, and was most preferred by the panellists. The baked dough was hard in texture, high in ash content, and brown in colour. The fried dough poses high starch hydrolysis, chewy texture, adhesion, solid particles, protein content, and relatively high lipid. The sensory evaluation indicated that the fried dough poses an oily, rancid, foreign aroma, and bitter and salty taste. It can be concluded that at a temperature of 37 °C, an enzyme-to-substrate ratio of 100 mL/g, and a reaction time of 120 min, the optimal starch hydrolysis of processed wheat dough can be achieved at a pH of 6.6 and an AA/GA ratio of 0.5. Furthermore, steaming resulted in a unique property of dough compared to the baked and fried, while the latter two yielded products with similar properties.

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Introduction

Starch digestion is the process that converts starch into simple sugars that can be digested by the body. This process is carried out by the enzymes α-amylase and amyloglucosidase or glucoamylase, which are secreted by the human digestive system. The first enzyme breaks down the starch polymer into oligosaccharides at the α-(1-4) linkages present in both amylose and amylopectin, while the second enzyme breaks down the oligosaccharides into glucose monomers at the α-(1-4) linkages at the ends, resulting in glucose monomers. The glucose produced can then be utilized by the body as an energy source (Dhital *et al*., 2017).

The hydrolysis of starch into glucose is indeed an enzymatic reaction. This reaction can be carried out in vitro using the appropriate enzymes. Therefore, under standardized conditions, the rate of hydrolysis occurring in starch can serve as an indication of the potential digestibility of starch in the body (Goñi *et al*., 1997; Abduh *et al*., 2019; Brodkorb *et al*., 2019)

There are available α-amylase and glucoamylase enzymes that have catalytic similar to the enzymes used in standardized in vitro digestibility tests. However, the optimal reaction conditions of these commercially available enzymes need to be determined. To note, depending on its source, enzyme α-amylase can hydrolyse glucose polymers within a temperature range of 30 – 40 °C and a pH range of 0 –110 (Schomburg *et al*., 2017). For glucoamylase, depending on its source, can hydrolyse oligosaccharides within a temperature range of 3.5 – 100 °C and a pH range of 1 – 12 (Schomburg *et al*., 2017).

Every enzymatic reaction requires optimal

conditions considering the specific objective. For digestibility, the conditions within the digestive tract of the human body need to be considered. For example, a temperature around 37 °C, pH within the range of 4 – 9, enzyme-to-substrate ratio, and the concentration of minerals as ligands. These conditions have been accommodated in an in vitro digestibility assay protocol that has become a global consensus (Minekus *et al*., 2014; Brodkorb *et al*., 2019) despite minor modifications is necessary in some cases (Abduh *et al*. 2019).

Apart from the enzymes, the microstructure of starch granules can impact the speed and efficiency of starch digestion (Tian *et al*., 2019). When heated, starch granules undergo swelling (Tako *et al*., 2014), experience surface damage and become more accessible to enzymes (Wang & Copeland, 2013). The presence of water swells and gelatinizes the starch granule and serves as a medium for α-amylase to access the glucose polymers of starch and cleaves them into oligosaccharides (Slaughter *et al*., 2001). Doughs are thermally processed by steaming, baking, and frying whose differentiating in the heat transfer medium and behaviour, differently affect to the starch microstructure and lead to different properties of the final products (Shao *et al*., 2022; Zhang & Datta, 2006; Mondal & Datta, 2008; Oke *et al*., 2018; Langton & Hermansson, 1989; Hug-Iten *et al*., 1999).

The problem to be addressed through this research is the unknown optimal conditions for enzymatic hydrolysis of starch in vitro using commercially available enzymes. Additionally, the digestibility of wheat doughs undergoes different thermal processes that are available in the market is also unknown. Therefore, this research aims to determine the optimal conditions for enzymatic hydrolysis of starch using commercially available α-amylase and glucoamylase enzymes in the Indonesian market, and to characterize the digestibility potential of thermally processed wheat doughs and correlate it with their physical, chemical, sensorial, and hedonic properties. This research provides a method for starch hydrolysis using enzymes that currently available in the Indonesia market.

Materials and Methods

The research was conducted in the Laboratory of Food and Agricultural Products Engineering and the Laboratory of Food Chemistry and Nutrition, Department of Agriculture, Faculty of Animal and Agricultural Science, Universitas Diponegoro. The materials used in this research comprised α-amylase enzyme (Novozyme, Denmark), glucoamylase enzyme (Novozyme, Denmark), distilled water, pH buffer (Hanna Instruments, Italy), 1 M HCl (hydrochloric acid) and 1 M NaOH (sodium hydroxide), glucose oxidase-peroxidase kit (All Medicus Co., Ltd, Korea). The equipment used included an electronic balance (Shimadzu ATX 224, Japan), pH meter (Hanna HI98103, Italy), incubator (Temperature Controller XHW3001, China), orbital shaker mixer (Wincom KJ-201BS, China), glucose sensor (GlucoDr, All Medicus Co., Ltd, Korea), texture analyser (Brookfield CT3, USA), and oven (Memmert UN30, Germany).

The research was conducted through stages of 1) determination of the optimal pH for starch hydrolysis; 2) determination of the optimal enzyme ratio for starch hydrolysis; 3) selection of research samples; 4) starch hydrolysis experiments on the samples; 5) texture testing on the samples; 6) chemical quality testing on the samples; 7) sensory and preference testing on the samples; and 8) data analysis. The flowchart of the research can be seen in Figure 1.

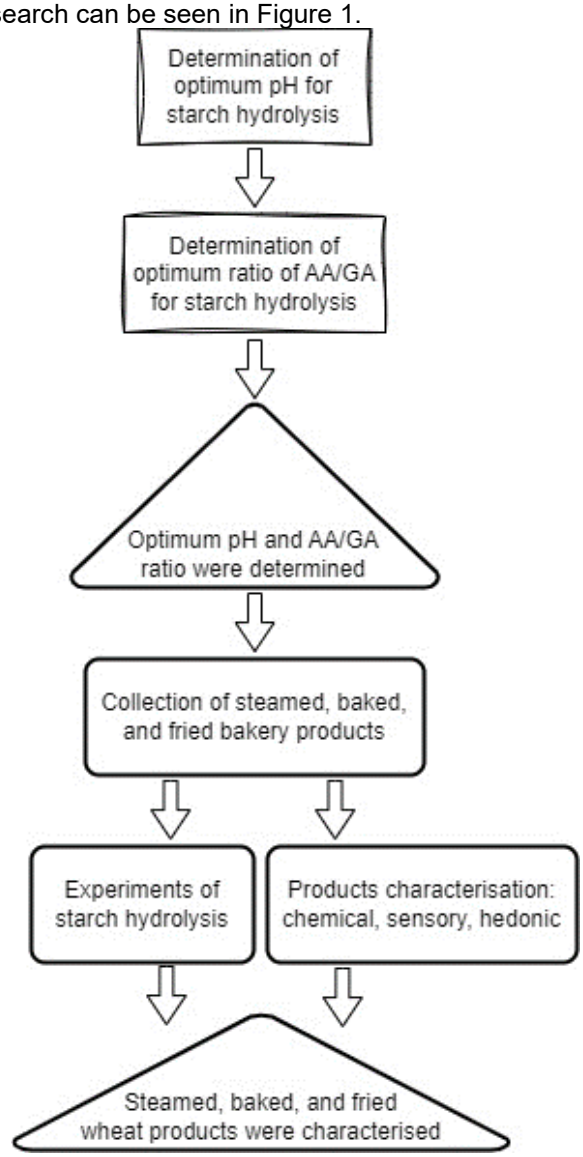

Figure 1. Flow diagram of the study

Determination the optimal pH for starch hydrolysis

To determine the optimal pH for starch hydrolysis, several pH conditions were tested, namely 4.2, 4.8, 5.4, 6.0, 6.6, and 7.2. The pH values were adjusted in the mixture of α-amylase and glucoamylase enzymes. The pH in the enzyme mixture was adjusted using 1 M HCl solution to decrease the pH and 1 M NaOH solution to increase the pH. The enzyme mixture was prepared by mixing 3.3 ml of α-amylase enzyme (Novozyme) and 6.7 ml of glucoamylase enzyme (Novozyme) in a beaker. Figure 2 shows the experimental procedure for determining the optimal pH for starch hydrolysis. Finely ground bread samples weighing 0.1 g were added to the enzyme mixture. After adjusting the pH to 4.2, 4.8, 5.4, 6.0, 6.6, and 7.2, the mixture was incubated at 37 °C with agitation for 2 hours in a shaking incubator. The range of pH, the temperature, and the length of duration considers the condition along the human digestive tract from gastric to entire small intestine. The glucose produced was

tested using a glucose oxidase-peroxidase strip test system (Allmedicus, Korea). If necessary, the hydrolysis solution was diluted before testing its glucose content. This experiment was repeated three times.

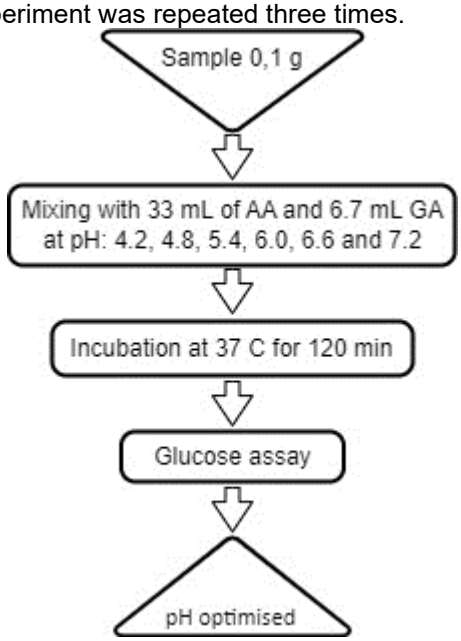

Figure 2. Flow diagram of determination of optimum pH for starch hydrolysis

Determination the optimum enzyme ratio

To determine the optimal ratio of α-amylase and glucoamylase for starch hydrolysis, an experiments condition was set up (Figure 3). In this experiment, the pH was adjusted to 6.7, and the ratio of α-amylase and glucoamylase enzymes was varied at 0.25, 0.50, 1.00, 1.50, and 2.00, with a total volume of 10 mL for both enzymes.

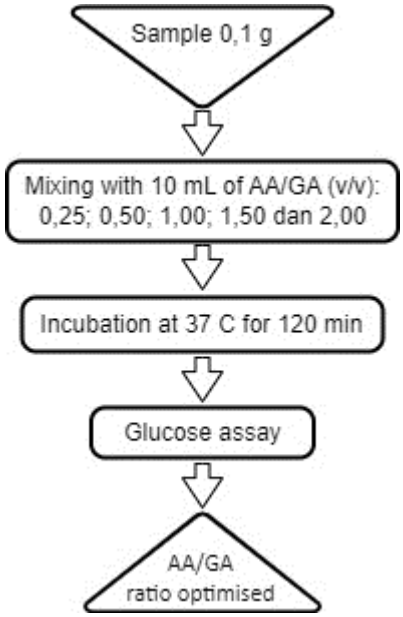

Figure 3 Flow diagram of determination optimum ratio of AA/GA for starch hydrolysis

Sample collection and preparation

The samples of steamed, baked, and fried wheat doughs were obtained from several renowned bread manufacturers in Banyumanik, Semarang, Indonesia. The chosen products included sweet bread, steamed sponge cake, and donuts. For quality testing purposes, the selected samples were cleaned, removing any additional components such as sprinkled chocolate toppings or banana fillings to focus only on the main body of the products.

Starch hydrolysis test

The hydrolysis of starch in the processed wheat products was tested using the preoptimized conditions.

Texture analysis

The texture of the products was tested using the Brookfield CT3 Texture Analyzer (America) with the standard method (Castell-Perez, 2014) to obtain values for hardness, cohesiveness, springiness, and adhesion. The TA10 probe (diameter: 12.7 mm, length: 35 mm) was used, with a trigger setting of 4.5 g, deformation of 20 mm, and a speed of 1.0 mm/s.

Total solid analysis

The total solids content of the product was determined gravimetrically by calculating the ratio of the difference between the initial weight of the sample and the weight of the sample after drying in an oven at 105 °C until a constant weight was obtained, relative to the initial sample weight. A sample of 2 g was placed in a pre-dried empty dish and dried in an oven at 105 °C for a period of time until a constant weight was achieved. The total solids content was calculated using the following equation, where x is the weight of the dish and sample before drying (g), y is the weight of the dish and sample after drying (g), and a is the weight of the empty dish (g):

$$\text{Total Solid } (\%) = 100 - \left(\frac{(x-y)}{(y-a)} \text{ x } 100\%\right)$$

Total protein analysis

Protein analysis was performed on the product using the Kjeldahl method. This method involves several steps, starting with the sample destruction using sulfuric acid. The sample is then subjected to distillation to separate the two liquid phases that are mixed. The process concludes with titration of the resulting acidic distillate using sodium hydroxide (NaOH). The titration allows for the determination of the nitrogen content, which is used to calculate the protein content of the sample.

Total lipid analysis

Lipid analysis was performed on the product by extracting the lipid content using a non-polar solvent, hexane, in a Soxhlet flask. The sample is subjected to repeated extraction cycles, where the solvent extracts the lipids from the sample. The weight difference before and after extraction is calculated as the total lipid content of the sample. This method allows for the separation and quantification of the lipid components present in the product.

Ash content analysis

Ash content indicates the mineral content of a product. The ash content is determined by calculating the weight remaining after burning the product at a temperature of 600 °C for 4 hours. During this process, organic matter is completely combusted, leaving behind the inorganic mineral components, referred to as ash. The weight of the ash residue is measured and used to

determine the ash content, which provides an estimation of the mineral content present in the product.

Sensory and hedonic tests

A panel of at least 25 individuals is involved in evaluating the sensorial properties and obtaining their hedonic perception of the product. The quantification of panellists' assessments and hedonic is done using the Semantic Differential Scale, which ranges from 1 to 10. This scale allows panellists to provide numerical ratings based on specific sensory attributes or characteristics of the product. By using this scale, the panellists expressed their opinions and preferences by selecting a point on the scale that best represents their perception, with higher values indicating a more positive evaluation. The ratings provided by the panellists were then analysed statistically to assess the sensorial properties and hedonic of the product.

Data analysis

The obtained data were tabulated and presented as descriptive statistics. Subsequently, the data were processed using the chemometrics technique called Principal Component Analysis (PCA) to simplify the data dimensionality from multiple observed variables and obtain a visualization of the correlations among all the observed variables simultaneously. In the conducted PCA, data cleaning and preliminary treatments was applied comprising mean-cantering, autoscaling, and normalization, to obtain a PCA model that explains the relationships between the various variables and samples with a sufficiently high variance. The PCA was conducted using Chemoface software (Nunes *et al*., 2003).

Results and Discussion

Optimum pH and enzyme ratio

Starch hydrolysis is a process that breaks down starch molecules into simpler components such as dextrin, maltose, and glucose. In Figure 4 part a is shown the changes in glucose concentration in the reaction mixture after bread samples undergo hydrolysis for 120 minutes under various pH conditions. At the pH conditions tested, it was observed that at pH 4.2, there was a negative difference value (-1350 mg/dL), indicating that the glucose concentration at the beginning of hydrolysis was higher compared to after 120 minutes of hydrolysis. At a higher pH of 4.8, the negative difference value decreased (-450 mg/dL), indicating that hydrolysis at pH 4.8 successfully produced glucose in higher concentrations than at pH 4.2.

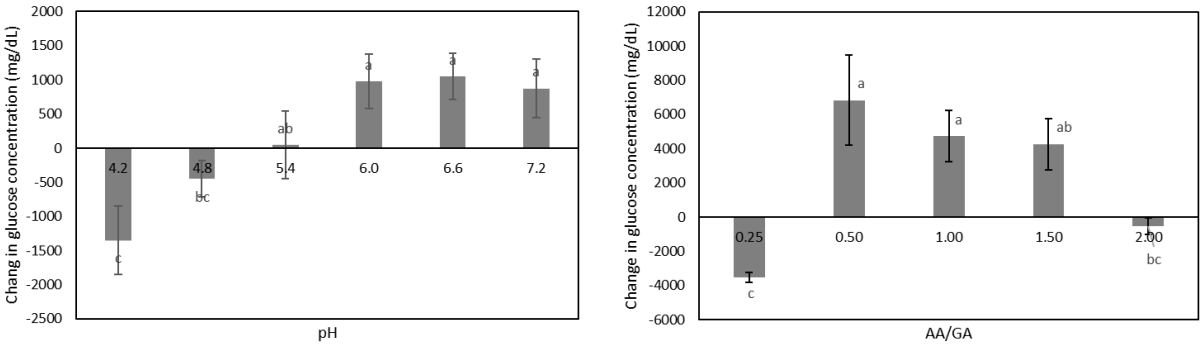

Figure 4. The changes in glucose concentration in the reaction mixture after bread samples undergo hydrolysis at 37 °C for 120 minutes with an enzyme-to-substrate ratio (E/S) of 100 mL/g. The changes are observed under different pH conditions (part a) and different ratios of α-amylase (AA) and glucoamylase (GA) enzymes (part b).

Under pH conditions ranging from 5.4 to 7.2, the difference between glucose concentrations after and before hydrolysis for 120 minutes showed positive values. This indicates that hydrolysis with an E/S ratio of 100 mL/g for 120 minutes at these pH ranges successfully broke down starch into glucose and produced a higher glucose yield compared to hydrolysis at pH 4.8 and 4.2. Although not statistically significant, glucose concentrations appeared to be higher at pH 6.6 with a smaller standard error compared to hydrolysis at pH 6.0 and 7.2. The high glucose concentration after hydrolysis at pH 6.6 suggests that at this pH, starch hydrolysis by both enzyme mixtures occurs optimally, which is consistent with Maulani *et al*. (2018). On the other hand, the low glucose concentration in hydrolysis at pH 4.2 and 4.8 could be attributed to suboptimal reactions, possibly due to enzyme denaturation and/or protonation of the enzymes (Mardiah, 2011).

Regarding the enzyme ratio of α-amylase and glucoamylase in the mixture, hydrolysis at enzyme ratios of 0.25 and 2.00 at 37 °C, E/S ratio of 100 mL/g, pH 6.6 for 120 minutes resulted in negative values. Positive values were obtained at enzyme ratios of 0.5, 1.0, and 1.0 in the mixture. Among these three ratios, the 0.5 ratio yielded a relatively large average difference in glucose concentration. Therefore, it was concluded that the optimal enzyme ratio for starch hydrolysis under the given conditions of 37 °C, pH 6.6, and E/S ratio of 100 mL/g is 0.5.

Properties of steamed, baked, and fried wheat doughs

The starch hydrolysis, textural and chemical properties of steamed, baked, and fried doughs are presented in Table 1, while the sensorial and hedonic properties are presented in Table 2. In Tables 1 and 2, the average values and standard errors of the observed variables are presented with an adequate number of replications. With a total of 20 variables the data were not analysed using univariate analysis of variance (ANOVA). The reason is that ANOVA and its subsequent post-hoc tests such as Duncan Multiple Range Test (DMRT), Tukey's Honestly Different, and other advanced tests analyse variance differences one by one for each observed variable, leading to difficulty in drawing the

conclusions when characterize the samples. Therefore, a chemometrics tool, namely principal component analysis (PCA), was used in the data analysis for this study.

Principal Component Analysis (PCA) is a technique used to reduce the dimensionality of a dataset by projecting it onto a lower-dimensional space. This process is performed by determining the eigenvectors of the data's covariance matrix, which are then used to transform the data into a new coordinate system (principal components). PCA can be useful for visualizing high-dimensional data, reducing noise in the data, and improving the performance of machine learning algorithms (Abdi & Williams, 2010). Eigenvectors, on the other hand, are vectors that, when multiplied by a matrix, result in the scalar multiplication of the original vector. The scalar value is known as the eigenvalue. For example, if A is a matrix and x is an eigenvector of A, then Ax = λx for some scalar λ (Shlens, 2014).

Figure 5 displays the PCA biplot of the data on starch hydrolysis, textural, chemical, sensorial, and hedonic properties of steamed, baked, and fried wheat doughs. It can be observed that a total variance of 44.87% can be explained by PC1 and PC2. The samples of products form three distinct clusters, indicating that the three types of products have different characteristics. However, some samples of baked are grouped with the fried product cluster, and vice versa. For the steamed product, a cluster form separately from the baked and fried. Nevertheless, the higher proximity of the steamed to the baked compared to the fried suggests that the steamed product is more similar to the baked than the fried.

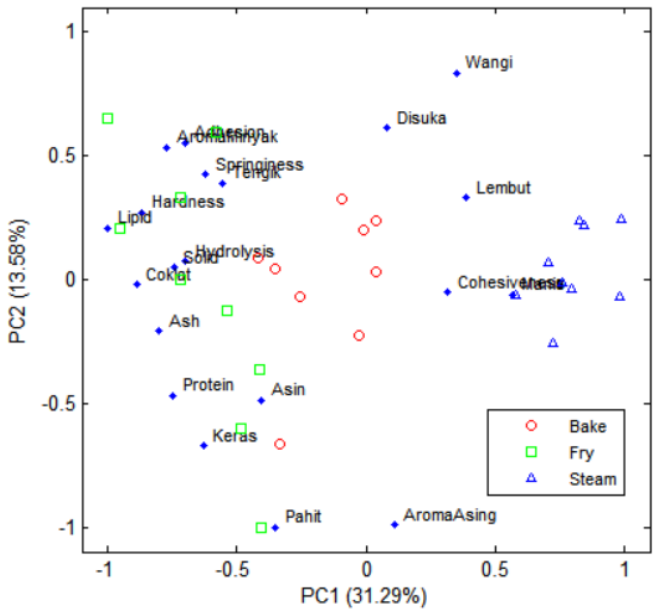

Figure 5. PCA biplot that integrates starch hydrolysis, textural, chemical, sensorial, and hedonic properties of steamed, baked, and fried wheat doughs.

The steamed samples form a compact cluster, indicating that their properties are relatively consistent compared to fried and baked. The consistent property of steamed dough is due to stable temperature of steaming compared to that of frying and baking. In steaming, the heat is derived from boiling water vapor and is limited to atmospheric pressure. The steamed doughs pose a cohesive texture and a soft sensory texture, with a pleasant aroma, a sweet taste, and they were most preferred by the panellists.

The properties of the fried doughs are relatively inconsistent compared to the steamed and baked. This is indicated by the scattered plot of samples and the formation of non-compact clusters. The relatively inconsistent quality of fried products can be attributed to the use of different types of oil, resulting in variations in the types and distribution of fatty acids (Li *et al*., 2019). Another possibility is that the frying oil has been used multiple times, leading to varying degrees of thermal degradation and oxidation (Tadesse Zula & Fikre Teferra, 2022).

This study found that fried doughs exhibit a wide range of properties comprising high starch hydrolysis, foreign aroma, bitter taste, chewy texture, adhesion, solid content, protein content, ash content, and relatively high lipid content compared to the steamed and baked. In terms of sensorial properties, the fried doughs are characterized by an oily aroma, rancid aroma, foreign aroma, bitter and salty taste, and a hard texture.

The high protein content in fried doughs is suggested to be a result of their high solid content due to water evaporation during frying. The phenomenon of high starch hydrolysis in fried dough is interesting because it suggests that the presence of oil in fried products is not sufficient to hinder enzyme access to starch. According to the findings of Kapcum *et al*. (2022), the relatively high intensity of hydrolysis in fried products is suspected to occur in the fraction of resistant starch. It should be noted that the hydrolysis test was conducted for up to 120 min, which certainly includes resistant starch. Furthermore, this phenomenon is likely to occur because the microstructural characteristics of the product matrix formed during frying (Wang *et al*., 2021) have a greater impact on facilitating enzyme access to the starch structure compared to steamed and baked products.

The presence of foreign aroma and a relatively strong brown colour in fried doughs can be associated with the frying oil whose fatty acids and glycerol can generate foreign aroma and cause it to darken due to degradation and thermal oxidation when heated (Vaskova & Buckova, 2015; Erickson *et al*., 2022).

Baked products exhibit relatively consistent quality, as indicated by compact clusters formed by sample plots. Baked products have a characteristic hard texture, high ash content, and a brown colour. The hard texture of baked products is formed by the crust on the product's surface (Vanin *et al*., 2009). The brown colour in baked products is the result of the Maillard reaction between amino acids and reducing sugars that occurs at high temperatures, high pH, and low moisture content (Hellwig & Henle, 2014; Purlis & Salvadori, 2009). The similarity between steamed, baked, and fried products is depicted in Figure 6.

Figure 6 confirms the unique quality of steamed products compared to baked and fried products. As shown in Figure 5, steamed products form a separate cluster from the clusters of baked and fried samples. On the other hand, some baked and fried products overlap, with some baked samples being grouped with fried

products and vice versa, where some fried samples are grouped with baked products. In terms of data analysis, the use of PCA in this study has successfully provided a more comprehensive and conclusive understanding in characterizing wheat-based processed products that were prepared using steaming, baking, and frying methods based on all the tested variables. Such an outcome is considered valuable compared to univariate data analysis, which involves comparing variances per variable across different sample groups of thermal processing methods.

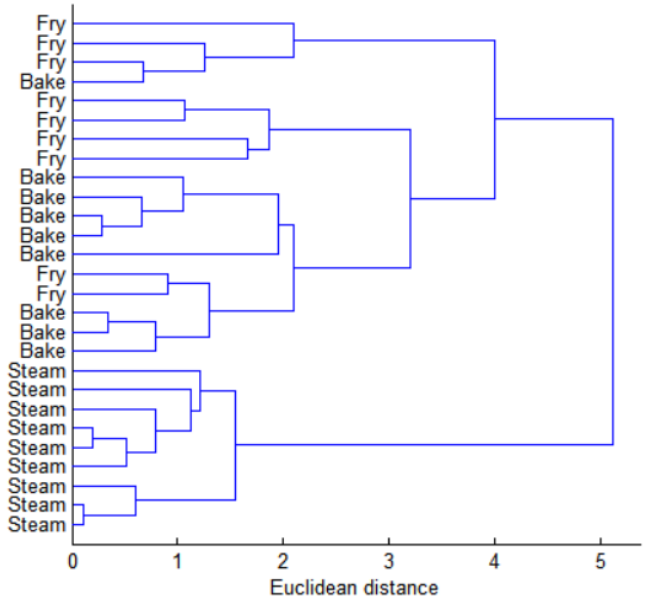

Figure 6. Dendrogram of PCA to illustrate the similarity of baked, steamed, and fried wheat doughs based on starch hydrolysis, textural, chemical, sensorial, and hedonic properties.

Conclusion

It can be concluded that at a reaction condition of 37 °C, reaction time of 120 min, enzyme-to-substrate ratio of 100 mL/g, the optimum ratio of AA/GA is 0.5 and optimum pH is 6.6. This optimized condition is suggested to be implemented for further studies at the corresponding condition. Based on starch hydrolysis, chemical, textural, sensorial, and hedonic properties, steamed wheat dough exhibited consistent and unique quality compared to those baked and fried. Among the various properties evaluated, baked wheat dough was preferred the most by the panellists.

Acknowledgments

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