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Effect of Edible Laccase on Chinese Bread Texture

by Maharani Pertiwi Koentjoro

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Effect of Edible Laccase on Chinese Bread Texture

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SUMMARY

Laccase application in the food industry is an alternative and innovative solution for natural ingredients since it is able to improve the food quality especially for baking industry. Laccase is a natural catalyst which could improve the structure of gluten in dough by increasing phenol oxidation resulting a better bread texture, taste, volume, and freshness. The aim of this study was to evaluate *Trametes versicolor* laccase oxidation on physicochemical quality of Chinese bread. Laccase were pasteurized to obtain edible laccase with the final activity as 774.7 U/ml. Laccase treatment managed to reduce bread hardness until 9.55 N. The softening phenomenon was related to the laccase–mediated depolymerization of the cross–linked network. Chemical parameters of laccase treatment were 0.065 ppm lower than control. The functional group in the polymer pretreatment mixture of edible laccase observed by Fourier Transform Infrared Spectrometer (FTIR) showed the formation of disulfide group at a wavelength of 450–500 cm^{-1}. The Scanning Electron Microscope analysis revealed formation of gluten protein which has smaller and uniform pores compared to the control group. Based on the results obtained in the present study, laccase from *Trametes versicolor* can be considered a promising application to improve quality of Chinese bread at industrial level.

Keywords: laccase, *Trametes versicolor*, physicochemical quality, Chinese bread

Introduction

Laccase (EC 1.10.3.2) is a type of enzyme containing polyphenol oxidase, which can oxidize polyphenols, methoxy–substituted phenols, and diamines (Minnusi et al. 2002). Among plants, fungi, and bacteria, white–rot fungi are the major laccase producer. Laccase by *Trametes versicolor* has been produced using lignin containing materials (Shraddha et al. 2011; Imran et al. 2012; Offia–Olua 2014).

Laccase in the food industry has been implemented in dough as bread quality improver to increase volume or crumb structure and softness in the bakery products (Minnusi et al. 2002; Imran et al. 2012; Osma et al. 2010). Laccase from *T. versicolor* have been evaluated to their ability to increase the strength of the gluten structure prior oxidizing lignocellulose in flour (Kaur et al. 2014; Minnusi et al. 2002). The elastic properties of low–protein flour used in making Chinese bread has a weak gluten index therefore it needs to be improved using laccase (Gulia and Khatkar, 2015).

Generally, the baking industry uses artificial food additives such as baking soda (sodium bicarbonate) to increase volume and delighting texture. Long–term of chemicals consumption could result negative impact of human health thereby reducing serum iron and calcium levels in the body which lead to specific doses toxicity (Fakhri et al. 2016). It is well known that the addition of certain enzymes to the dough have unwanted side effects. Since the enzyme are denaturated and inactivated by heat during cooking process (Gallagher et al. 2009). Therefore, the application of laccase as an enhancer of the texture of the bread is less health risks.

The objective of this study was to determine the effects of edible laccase from *T. versicolor* on the texture of Chinese bread by measuring phenol content, functional groups, and micro visual characters.

Materials and Methods

1. *Laccase production*

1.1 *Organism and cultivation conditions*

The *T. versicolor* fungi are a culture collection of Microbiology and Biotechnology Laboratory, Department of Biology, Institut Teknologi Sepuluh Nopember (ITS) Surabaya, Indonesia. The fungi cultures were maintained on Potato Dextrose Agar (PDA) medium and incubated at 28°C, for 6 days before using as seeding. The procedures of maintaining fungi culture is briefly explained as follows. Sporulated PDA medium were dislodged and washed with PBS sterile. As much as 10 mL of 10^8 CFU fungi spores' suspension were inoculated into 100 mL of Potato Dextrose Broth (PDB) (pH 6.0) and incubated for 6 days at room temperature (25°C) with a rotary shaker at 130 rpm (Jo et al. 2010; Qin et al. 2017).

1.2 *Partially edible laccase production*

All experiments were carried out in 300–mL Erlenmeyer flasks containing 100 mL culture medium. The medium contained 4.5 g/L rice husk, 1.5 g/L yeast extract, 1 g/L $C_6H_{12}O_6$, 0.5 g/L NH_4Cl and 100 mL of salt solution. The salt solution contained 2 g/L KH_2PO_4, 0.5 g/L $MgSO_4.7H_2O$, 0.1 g/L $CaCl_2.2H_2O$ and 0.5 g/L KCl and maintained pH 5.0, (Koentjoro et al. 2016). $CuSO_4$ was added by dissolving 400 mg in 1 L of dH_2O as laccase inducer. The medium was autoclaved at 121°C for 15 min. Each flask was inoculated using an agar piece 1 cm^2 cut from an actively growing fungal culture. The flasks were incubated at 30°C on a rotary shaker (130 rpm). The culture broth was filtered, clarified by centrifugation at 10,000 rpm for 15 min, frozen, defrosted and then filtered to remove the precipitated polysaccharides. The resulting clear filtrate was used for the further experiment. Partially edible laccase was sterilized by pasteurizing (the High Temperature Short Time) (HTST) is as follows, a total of 50 mL of laccase solution was placed in a water bath heated at 72°C for 25 seconds (Ranieri et al. 2009).

2. *Characterization of laccase*

2.1 *Laccase activity*

Laccase activity was determined as the increase in absorbance at 468 nm due to the oxidation of 2,2 –azino–bis (3 ethylbenzothiazoline 6–sulfonate) (ABTS) at 30°C (Koentjoro et al, 2016). The reaction mixture contained 200 µL 10 mM ABTS, 300 µL enzyme solution in 500 µL 100 mM citrate buffer, pH 4.5.

Oxidation of ABTS was observed spectrophotometrically at 436 nm for 5 minutes. The laccase activity is calculated based on the ABTS standard curve. One unit (U) of laccase activity was defined as the amount of enzyme required to oxidize 1 µmol ABTS per minute and considered equivalent to 1 unit of enzyme activity ($\varepsilon 420 = 36000\ M^{-1}\ cm^{-1}$ and path length l = 1 cm) (Wolfenden and Wilson, 1982).

2.2 *Isoelectric point (pI)*

To investigate the isoelectric point of supernatant, as much as one mL of laccase was incubated in each tube containing a phosphate buffer with variable pH level (3.0, 4.0, 5.0, 6.0, 7.0 and 8.0) at room temperature for overnight. The occurring of precipitation was monitored and justified as the isoelectric point of laccase in particular pH level.

2.3 *Total Protein determination in enzyme solution*

Total Protein concentration in supernatant was determined by Bradford method using Bovine Serum Albumin (BSA) as standard. Reaction was started by addition of 0.1 mL of supernatant and 5 mL of Bradford reagent (10 mg of Coomassie Brilliant Blue G–250 in 5 mL of 95% ethanol, 10 mL of 85% phosphoric acid, and 85 mL of dH_2O). The changes in absorbance due to laccase content in supernatant were recorded by spectrophotometer in absorbance at 595 nm.

3. *Preparation of laccase modified Chinese bread*

Dough was made manually by mixing flour with enzymes, yeast, water and sugar. The experimental formulations set are shown in Table 1. The average of duplicate data was used in analysis. The dough in each experimental formulation was divided into 6 similar weight. The dough was placed in a bowl covered with a

Table 1 Composition of bread with different levels of edible laccase

Sample	Sample 1	Sample 2	Sample 3	Sample 4
Treatment	Negative control	Non–Edible Laccase	Edible Laccase	Baking soda
Flour wheat low protein (gr)	100	100	100	100
Non–Edible Laccase	0	1.5	0	0
Edible Laccase	0	0	1.5	0
Baking soda (gr)	0	0	0	400
Yeast (gr)	2.2	2.2	2.2	2.2
Water (mL)	50	50	50	50
Sugar (gr)	20	20	20	20

plastic foil and allowed to stand at room temperature (28°C) for 45 minutes. The dough was then steamed for 7 minutes (Laohaprasit and Sricharoenpong, 2018).

4. *Laccase modified Chinese bread analysis*

4.1 *Total phenol content*

Total phenolic content was determined according to Folin–Ciocalteu method with slightly modified. Every extraction solution of bread (10 mL) was mixed with 10 mL of 96% ethanol and 5 mL of 0.1% HCl for 10 min. The reaction mixtures were allowed shaking in a rotary shaker for 30 minutes at room temperature. The mixtures were then centrifuged for 10 minutes at 4,000 rpm and the supernatant was collected. Every extraction of supernatant (1 mL) was mixed with 100 µL of Folin–Ciocalteu reagent and 1 mL of sodium carbonate 15% (w/v). After incubation for 1 hour at room temperature, the absorbance was measured at 710 nm using a spectrophotometer. Total phenol was calculated from the calibration curve of tannic acid and expressed as milligram of tannic acid equivalent per gram of dough (mg TAE/g).

4.2 *Texture profile analysis*

Evaluation of dough properties (hardness) was carried out by texture profile analysis. The test consists of compressing a bite–piece of bread two times in a reciprocating motion that imitates the action of the jaw and extracting from the resulting force–time curve a hardness parameters. The test was applied on bread by using following procedures. Each sample was placed on the Texture Analyzer (SMS) mod.TA.HDi 500 (Stable Micro Systems, Surrey, UK) preparation table followed by applying a pressure using a piston diameter of 75 mm (Ayala–Soto et al. 2017).

4.3 *Scanning Electron Microscope (SEM)*

The micro structural of starch granules in bread with different formulation were studied using Scanning Electron Microscope. Samples were prepared by knife cutting then placed on a carbon tip followed by gold and palladium coating. The SEM images of each samples were taken at magnification of 3,000 (Wojciechowicz–Budzisz et al. 2015).

4.4 *Fourier Transform Infrared Spectroscopy (FTIR) Analysis*

The FTIR analysis in this study has been carried out in order to identify functional groups shifting in every treatment at a wavelength between 800–3600 cm^{-1} (Amir et al. 2013).

5. *Statistical analysis*

The data obtained were then analyzed using of one–way variance (ANOVA) Duncan test using Minitab 14.0 (Minitab 14 Statistical software, 2014). Meanwhile, a qualitative descriptive design was conducted for texture and functional group analysis.

Results and Discussion

1. *Production and characterization of edible laccase*

The activity of laccase before and after pasteurization were calculated as 799.4 and 774.7 U/mL, respectively (Table 2). These results demonstrated the pasteurization processes has no effect on laccase activity.

Table 2 Activity and quantitative estimation of laccase production

Laccase	Activity (U/mL)	Protein Contain (mg/mL)
Before pasteurization	799.4	0.02
After pasteurization	774.7	0.11

The pI of the non–and edible laccase was determined on pH 3.0. This information is helpful to developing purification scheme as a crucial factor in enzyme purification, the precipitation occurs is avoided (Xia, 2007). This result is consistent with the study of Afreen et al (2017) which stated that generally, laccase has an isoelectric point between at pH 3.0–3.1.

The pasteurization did not change the total protein in laccase solution as shown in Table 2. It is indicated that laccase can be pasteurized using heating with a certain temperature and time that serves to kill pathogenic microorganisms while maintaining physical properties (Verhoeckx et al. 2015).

2. *Texture Analysis (Test Hardness)*

Bread treated with edible laccase was demonstrated in deformation in cavity characters (Fig. 1). The pretreatment–control had a greater cavity compared to non–and edible laccase pretreatment. Moreover, between non–and edible laccase Chinese bread has small and uniform cavity. The result confirms the findings of Salinheimo et al (2007) and Tsegaye et al. 2018.that the bread treated with laccase has a small and uniform cavity. The small and uniform cavities, due to the ability of the laccase to form crosslinking in arabinoxilan through ferulic acid side groups allowing strengthen of gluten and more ability to retain gas (Meybodi et al. 2015). Therefore, the

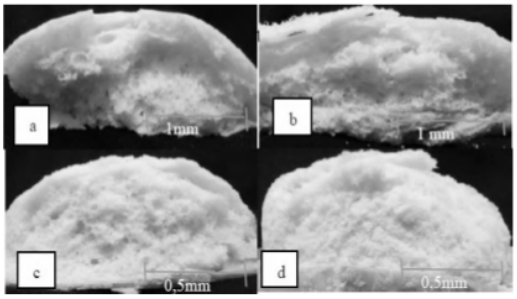

Fig. 1 Cross Section of Chinese Bread with a Variety of Pretreatment a) Control b) baking soda c) non–edible laccase d) edible laccase.

pasteurization does not affect the protein component of laccase.

3. *Total Phenol Analysis*

Analysis of total phenols in various treatments for dough are reported in Table 3. The non–pretreatment and edible laccase experimental group both of them have a lower total phenol concentration of 0.096 and 0.065 ppm respectively compared to a control of 0.168 ppm. This is because of ability of laccase to oxidize phenolic compounds such as ferulic acid that act as laccase mediator in a laccase–mediator system (LMS) reaction mode (Imran et al. 2012; Everette et al. 2014). The higher amount of oxidized phenol, it will increase the formation of disulfide groups (Linares–García et al. 2019). The ferulic acid is oxidized and formed radicals and continues to oxidize the sulfidryl (SH) groups. The oxidation can affect the natural disulfide formation (S–S) in gluten polymers and increase the rate of protein depolymerization lead to increase gluten networks (Linares–García et al. 2019).

Texture analysis on various treatments are shown in Table 4. Pretreatment of edible laccase and controls had a significantly different hardness value of 9.55 and 21.9 N respectively. These results indicated that the addition of laccase to the dough was able to reduce the hardness of the Chinese bread. This is consistent with the statement (Osma et al. 2010; Brijwani et al. 2010) that the laccase shows an oxidizing effect to improve the structure of gluten in the dough and can reduce stiffness, increase volume, improve crumb structure, and increase the softness of bread. Indeed, one good parameter of bread quality is a soft texture and low hardness (Borla et al. 2014). In addition, in Table 4 the non–and edible laccase pretreatment showed non–significantly different of hardness level which shows that pasteurization has no effect on the laccase protein component due to glycosylation. The edible laccase has a greater hardness value (9.55N) compared to non–edible one (9.15 N), this is most probably because of the different laccase activity shown in Table 2.

Table 4 A comparison of the physicochemical characteristics of bread

Pretreatment	Hardness (N)
Control	21.9 ± 0.269^{b}
Baking soda	17.97 ± 3.514^{b}
Non–Edible Laccase	9.15 ± 0.869^{a}
Edible Laccase	9.55 ± 0.262^{a}

Notes: Values with different superscript letters within a column are significantly different ($p \leq 0.05$. Data are expresses as means values of two samples ± standard deviation

4. *Scanning Electron Microscope (SEM) Analysis*

SEM analysis was taken to investigate the effect of laccase on microstructure of whole bread. This technique allowed a qualitative description of the structural cell and their distribution.

As shown in Fig. 2, that the microstructure of gluten bread was more uniform and more number of pores after adding of laccase due to higher volume of gas was released during dough fermentation.

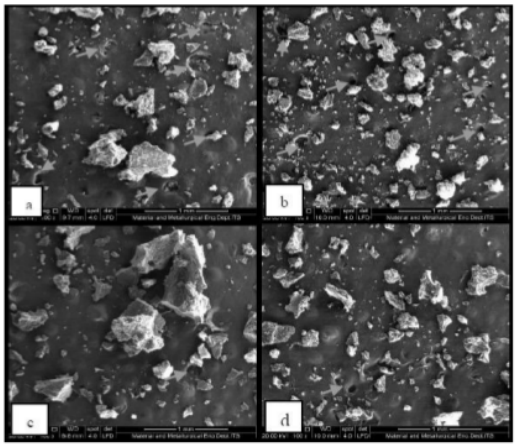

Fig. 2 SEM of a) control; b) baking soda; c) non–edible laccase; d) edible laccase at 1000X magnification Arrows show pores on gluten tissue.

The pore structure can be caused by the presence of an elastic gluten protein matrix which is able to withstand the development of gas while the cell walls was not easily broken (Ortolan and Steel, 2017).

5. *FTIR analysis*

FTIR spectroscopy of bread functional groups at various pretreatments was done in the range of 400–4000 cm^{-1} (Fig. 4). Characters of spectral bands were similar for control and laccase containing bread. Major peaks were indicated absorption of OH groups at a wavelength range of 3000–3,700 cm^{-1}.

In the range of 450–500cm^{-1} there is a change in the peak (peak) spectra at the pretreatment of non–edible laccase and edible laccase. At this wavelength range is the absorption of disulfide groups (S–S) (Amir et al. 2013). Disulfide groups are formed due to the oxidation process in sulfidryl groups which can affect the natural disulfide formation (S–S) (Linares–García et al. 2019).

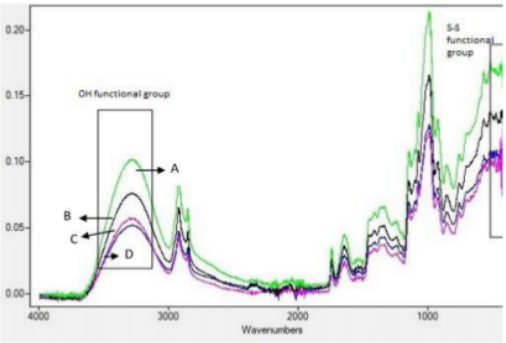

Fig. 3 FTIR spectra of bread in variety treatment

Conclusion

Laccase can be pasteurized excellently without decrease their activity. Pretreatment of edible laccase was able to reduce the value of bread hardness up to 9.55 N. The functional group in the polymer pretreatment mixture edible laccase formed a disulfide group formation at a wavelength of 450–500cm^{-1}. The structure of gluten was formed in uniform pores compared to controls. Therefore, applying laccase as natural food additives is able to improve bread texture quality.

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