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Physicochemical and Retrogradation Properties of Fermented Melinjo (*Gnetum gnemon*) Seed Flour

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Abstract

The fermentation effects of melinjo (*Gnetum gnemon*) seed flour on the physicochemical and retrogradation properties were investigated. Fermented melinjo seed flour (FMF) was prepared using a *Lactobacillus fermentum*. The DSC and RVA were used to evaluate the properties of the samples. The results indicated that FMF had a higher significant ($P<0.05$) value for gelatinization temperature and enthalpy but lower values in the starch-lipid temperature, enthalpy, and viscosity peak than the unfermented sample (UMF). X-ray diffraction demonstrated that fermentation resulted had little effect and the crystalline pattern cannot be changed (type A), but it was shown that a crystalline region ratio to region amorphous increased from 30.9 to 36.66%. The FTIR spectra showed that FMF and UMF were identical. Furthermore, the observation of the FMF using a SEM showed that starch granules had slight superficial corrosion, but UMF has a smooth outer surface. The retrogradation rate (k) of UMF was slowed (0.63 day^{-1}) when compared with the FMF (0.68 day^{-1}). Thus, fermentation may alter starch amorphous region and chemical components, eventually transforming the physicochemical and retrogradation characteristics of melinjo seed flour.

Keywords

Fermentation

Gelatinization

Lactobacillus fermentum

Starch-lipid

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Introduction

Melinjo (*Gnetum gnemon* L.) is an ancient plant and a species in the family Gnetaceae that grows in tropical areas. The plant is cultivated non-intensive in Southeast Asia. In Indonesia, all parts (leaves, seeds, and flowers) of this plant are edible, and the seeds can be made for a crisp snack (emping). The various nutritional analysis of melinjo seed showed that the seed contains 9-11% protein, 64.1% carbohydrates, and 16.03% fatty acids and involves adequate amounts of essential amino acids, dietary fiber, and minerals (Bhat & Binti Yahya, 2014). The melinjo seeds have been reported that the extract contains various stilbenoids which have the ability to be antimicrobial, antioxidant (Kato *et al.*, 2009), angiogenesis-inhibitory (Kunimasa *et al.*, 2011), anti-aging, and tyrosinase-inhibitory activities (Yanagihara *et al.*, 2012). In accordance to recent findings, it has been found that the seed contains bioactive compounds, such as gnetin C, gnemonoside A and gnemonoside D (resveratrol dimers) and trans-resveratrol and its glucoside (Kato *et al.*, 2009). Trans-resveratrol has been shown to have anti-aging (Baur & Sinclair, 2006), cardiovascular and cerebrovascular protective (Lemeshow *et al.*, 1998; Renaud & de Lorgeril, 1992), anti-dementia (Kennedy *et al.*, 2010), and estrogenic (Lu & Serrero, 1999; Mitchell *et al.*, 1999) properties. Because melinjo seeds contain trans-resveratrol and its derivatives, consumption of melinjo seeds is expected to contribute to human health (Tatefuji *et al.*, 2014). Furthermore, Bhat and Binti Yahya (2014) reported that melinjo seed flour has a high potential to be used as a basic raw material for food products and formulations in developing new nutritious foods. In its application, technology is still needed to improve the physicochemical properties. The technology of fermentation has long been employed to improve the properties of products. Many researchers have reported that microorganisms can be used to modify the constituents of a plant during fermentation (Katina *et al.*, 2007). Fermentation-based flour process could be a potential technique not only to increase the content of active ingredients, nutritional and antinutritional, and extend the shelf life (Nkhata *et al.*, 2018; Siswoyo *et al.*, 2017; Vlassa *et al.*, 2022) but also to alter

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physicochemical characteristics of the flour(Lu *et al.*, 2022; Park *et al.*, 2021; Shi *et al.*, 2016). The application of the fermentation method for flour production and food formulation has been established to produce various food forms, which include bread, beverages, and porridge from the raw materials of several cereals origin, including rice, wheat, corn, and sorghum (De Vuyst *et al.*, 2023; Li *et al.*, 2018); legumes and tubers including seeds (Adebo *et al.*, 2022), where these fermentation techniques were mainly used to produce a physicochemical and functionally better flour. However, there has been no information explaining how fermentation affects the physicochemical and retrogradation properties of melinjo seed flour. It is credible information to determine the potential use of this flour in the formulation of food. The study was aimed at determining the fermentation effect of melinjo seed flour on the physicochemical and retrogradation flour properties.

Materials and methods

Materials

The *Gnetum gnemon* L. (melinjo) seeds used in this study were harvested in March-April 2017 from plants in the collection of the Agriculture Faculty, University of Jember, Jember, Indonesia. *Lactobacillus fermentum* used in this study was obtained from the Microbiology Lab. of the Faculty of Life Sciences, Brawijaya University, Indonesia. All the reagents used in this study were analytical grade.

Preparation of Melinjo Flour

Melinjo seeds were peeled and done in three repeated washes using sterile water and dried (Froilabo-AC120, France) at 40-50 °C for 24 h. The dried samples were ground to pass a 50-250 μm sieve and collected. At the fermentor scale, fermentation of melinjo seed flour was accomplished by suspending 300 g of flour sample in 1 L of sterile distilled water prepared aseptically. The suspension of the sample was fermented using inoculation using 10% (v/v) inoculum, representing 108 cells/mL of *L. fermentum* at 37 °C for 48 h, in a 1000 mL fermentor stirred (Winpact FS-01, USA) at 150x g. The fermented samples were freeze-dried (VirTis-freezemobile™ FM25XL, USA) and stored at 4 °C until further analysis.

Thermal Analysis

Thermal properties were analyzed using differential scanning calorimetry (DSC-60, Shimadzu, Japan) by connected to a thermal analyzer and controlled using TA-60 WS software described by Siswoyo and Morita (2002). About 4 to 5 mg of the samples flour were weighed accurately into a dried aluminum DSC pan, and double-distilled water and sample were combined by sample to water ratio (2:1, w/v). The pan sample was sealed and left at 20-22 °C for 16 h. The pan samples were firstly scanned from 30 to 125 °C at a rate of 5 °C/min under nitrogen gas. Liquid paraffin was used as the reference. Thermal properties data was obtained from DSC thermograms such as outset temperature (To) and peak temperature (Tp) of samples, the enthalpy values for starch gelatinization (ΔHg), and starch-lipid complexes (ΔHs-l).

Starch retrogradation was determined on the same gelatinized samples after stored for zero to 7 days at 22 °C, and then the sample was scanned for the second time through in the DSC from 30 to 125 °C at a rate of 5 °C/min to determine the temperature and enthalpy of retrogradation. The retrogradation kinetic thermal was predicted using a DSC thermogram and calculated using the Avrami model, as explained by Baker and Rayas-Duarte (1998).

Rapid Viscoelasity Analysis

Pasting properties consisting of pasting temperature, peak viscosity, breakdown, setback and final viscosity of samples were through Rapid Visco Analyzer (RVA, Instrument of TecMaster instruments, Hägersten, Sweden) following previous methods (Meng *et al.*, 2014). At the dried aluminum RVA sample canister, the sample was conducted through the suspension of 3 g of flour a sample (14% mb) in 25 mL of double-distilled water. A thorough, proper, mixed suspension was carried out so that lumps remained undetected with the canister attached to the rapid visco analyzer. Immediately after the paddle was put into the canister, the test proceeded, and thus the characteristic curve was automatically plotted. Some features such as peak viscosity, setback viscosity, final viscosity, trough, and breakdown viscosity which past temperature and time to obtain peak time and reported as the average of 5 samples.

X-ray Diffractometry and FTIR Analyses

X-ray powder diffraction (X'pert model Philips, Almelo, Netherlands) was used to obtaining of diffractogram X-ray data of the sample. X-ray diffractograms were determined from 5°2θ to 50°2θ with a scanning step of 0.017°2θ and a scanning step time of 10 s/step. Relative crystallinity of the sample was through calculation, considered to be the ratio of crystalline area and X-ray diffractogram amorphous regions as described by Hulleman *et al.* (1999). Fourier Transform Infrared (FTIR) spectra of samples were obtained in the wavelength region between 400 to 4,000 cm^{-1} at room temperature (21-23 °C) with a FTIR spectrometer (Bruker Alfa, Germany). Powdered samples (pulverized to pass 150 mesh) were equilibrated at 23 °C and 45% relative humidity for 2 days before analysis.

Scanning Electron Microscopy Analysis

The morphological characteristics of UMF and FMF were observed using a Hitachi scanning electron microscopy (SEM) model TM3000. Starch granule samples were sprinkled onto double-sided tape on the surface of silver paste on SEM metal stubs. Thin-layer coated (~150 μm of palladium/platinum) samples were viewed at 10 kV with 100 s/picture speed photograph and with 17 mm working distance to scan electron microscope.

The Analysis of Statistics

Each data point represents the means of 5 determinations of sample ± standard deviation (SD). Data was applied to the analysis of variance (ANOVA) followed by Duncan's test and a significant differences level of $P < 0.05$ was employed.

Results and discussion

Thermal properties

The DSC results of melinjo seed flour (UMF and FMF) are presented in Table (1), which include the thermal transition temperatures (To and Tp) and enthalpy value (ΔHg). Melinjo seed starch showed a typical gelatinization endotherm, with a thermal transition temperature (To and Tp) in the range of 70.10-74.05 and 79.03-81.53 °C, respectively, and also was showed significant differences ($P < 0.05$). The higher temperature value of FMF had indicated that a high-thermo-stable granular structured sample is requiring more energy to destabilize the amylopectin and amylose molecules. Furthermore, the lower UMF temperatures of the gelatinization transition showed more amorphous and less crystalline material, resulting in less gelatinization resistance. The differences are due to the fermentation effect detected in the starch structure. Elsewhere, observation of the variation in the transition temperatures of the melinjo seed flour sample can depend on the starch composition, moisture content and by the organization of amylose and amylopectin chains which affect the swelling process rate and extension and crystallinity loss (Hoover, 2010; Siswoyo & Morita, 2003). The enthalpy gelatinization of UMF and FMF were varied from 3.27 to 5.6 J/g (Table 1). This is a parameter measure of the order of molecular with the structure of double helices and crystalline structure of amylopectin in the granule (Alonso-Gomez *et al.*, 2016; Fredriksson *et al.*, 1998; Hong *et al.*, 2022). The higher values of To, Tp and ΔHg were observed in FMF, indicating that the sample may have highly crystalline of amylopectin; it was required more energy to destroy the double-helical and deconstruct the structure of starch. Consequently, the more resistant granules required higher energy to disorganize their structure (Liu *et al.*, 2005; Siswoyo & Morita, 2002).

Table 1. The Physical Properties of UMF and FMF*

	UMF	FMF
Thermal (DSC)		
Gelatinization		
T_o (°C)	70.10 ± 0.34^a	74.05 ± 0.22^b
T_p (°C)	80.03 ± 1.28^a	81.53 ± 0.70^a
ΔH_g (J/g)	3.27 ± 0.89^a	5.60 ± 1.23^b
Starch-lipid complex		
T_o (°C)	103.23 ± 3.45^a	100.47 ± 1.16^a
T_p (°C)	112.67 ± 0.70^b	111.10 ± 0.45^a
$\Delta H_{s\text{-}l}$ (J/g)	1.18 ± 0.44^b	0.91 ± 0.28^a
Pasting (RVA)		
Pasting Temp. (°C)	81.60 ± 0.30^a	78.75 ± 0.70^b
Peak viscosity (cP)	2683.00 ± 35.00^a	1231.00 ± 10.00^b
Breakdown (cP)	778.00 ± 0.50^a	780.00 ± 1.00^a
Setback (cP)	482.00 ± 3.00^a	385.00 ± 2.10^b
Final viscosity (cP)	2387.00 ± 2.50^a	836.00 ± 2.30^b

*Data are mean ± SD. Each value is a mean of triplicate determinations. a, b Mean values in each horizontal row followed by the different superscripts are significantly different as determined by Duncan's Multiple Range Test ($P < 0.05$).

Furthermore, RVA data has shown an increase in pasting temperature by 2.85 °C (78.75 to 81.60 °C) after fermentation. The degradation could be caused by micro-organisms that fermented lipid, protein, or/and starch modification processed by enzymatic and acid. The contents of protein and lipid of melinjo seed flour decreased during fermentation as protein or/and lipid are naturally combined with starch and released through that process, consistent with the reported finding of fermented rice flour (Park *et al.*, 2021). Melinjo seed starch, through the impact of fermentation can even absorb water more quickly than UMF control and can gelatinize more efficiently when heated. The phenomenon also can be seen in the second peak of the DSC thermograph, where the enthalpy value (ΔHs-l) changes from 1.18 ± 0.44 to 0.91 ± 0.28 J/g. The weak form of starch-lipid complexes indicated the lower enthalpy change for FMF. The fermentation effect of melinjo seed flour, especially on the pasting properties, is shown in Table (1). The pasting temperature, peak viscosity, and final viscosity greatly decreased, from 81.60 to 78.75 °C, 2683 to 1231 cP, and 2387 to 836 cP, respectively, and the FMF showed significantly different ($P < 0.05$) from the UMF. The viscogram of UMF and FMF melinjo seed flour is shown in Fig. (1). The peak and final viscosity and pasting temperature decreased, indicating a weak granular structure, lower thickening power, and lower amylose content of melinjo seed flour after fermentation. It looked similar to the results of starch of fermented rice, cassava starch, cocoyam flour and sweet potato flour (Alvarado *et al.*, 2013; Lu *et al.*, 2005; Oke & Bolarinwa, 2012; Yuliana *et al.*, 2023).

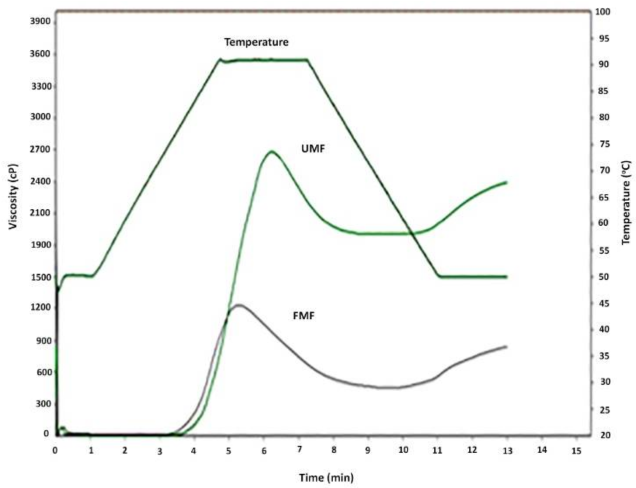

Fig. 1. Viscogram of unfermented melinjo seed flour (UMF) and fermented melinjo seed flour (FMF).

Fourier Transform Infrared (FTIR) and X-ray Diffraction

The novel technique for analyzing constituent function was evaluated using FTIR spectroscopic. The infrared (IR) absorbance spectrum of the FMF and UMF showed typical peaks with similar intensity, and no obvious difference occurred between them (Fig. 2). It can be observed that a -O-H stretching band was shown at 3200-3700 cm^{-1}, -CH_2- at 2927 cm^{-1}, C=O at 1651 cm^{-1} and C-O at 1018 cm^{-1}, they indicated the similarity of results as reported by (Lu *et al.*, 2005) on fermented rice starch, although the O-H absorption peak changed from 3326.12 (UMF) to 3307.05 (FMF) cm^{-1}. The FMF showed similar leading bands as presented in the UMF, but it can be monitored that fermentation showed higher relative intensities of the starch bands concerning unfermented melinjo seed starch. This phenomenon could be because of some degree of modification of unfermented melinjo seed flour and structural changes that may occur during fermentation.

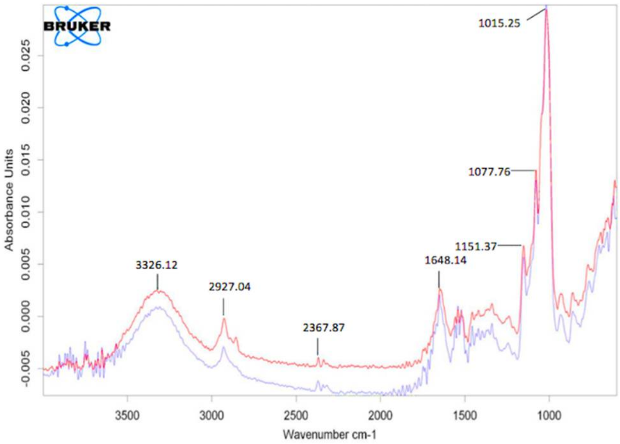

Fig. 2. Fourier transform infrared spectroscopy of unfermented melinjo seed flour (UMF/red line) and fermented melinjo seed flour (FMF/blue line).

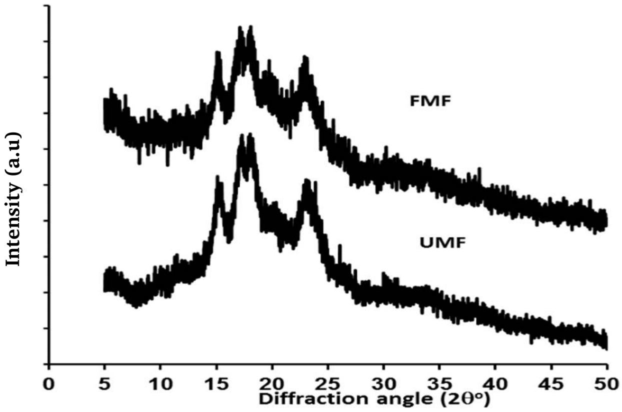

Fig. 3. X-diffraction spectra of unfermented melinjo seed flour (UMF) and fermented melinjo seed flour (FMF).

The X-ray diffraction patterns of melinjo seed starch of FMF and UMF have presented in Fig. (3). The melinjo seed starch sample appeared to have a typical pattern of the A-type of crystal starch with maximum d-spacing = 5.79, 5.12, and 3.86 (Å) as suggested by Zobel (1964). Likewise, the FMF showed a typical pattern of the A-type crystal starch with maximum d-spacing = 5.87, 5.28, and 3.86 (Å). The pattern of FMF crystalline cannot be changed (type A), and the typical cereal starch pattern can still be detected. The unchanged A-type crystalline pattern of the FMF indicates that fermentation did not modify the crystalline properties of melinjo seed flour. The 2θ peaks of A-type characteristic at 15.3°, 17.1°, 18.2°, and 23.5° still cannot be changed.

The fermented sample was shown a crystalline region ratio to region amorphous increased from 30.9 to 36.66% (Fig. 3). The percent crystallinity of melinjo seed flour was increased after fermentation, and it might be due to the hydrolysis of the amorphous region after fermentation. A similar increase in the degree of crystallinity was reported for fermented rice flour (Lu *et al.*, 2005).

Scanning electron microscopy observation

Scanning electron micrographs of UMF and FMF are shown in Fig. (4). The results of the inspection showed the form of granular melinjo seed starches in oval, round, and large and axial diameters of 5-20 μm. The surface layer of FMF (Fig. 4B) is a smooth outer surface and looks different from UMF (Fig. 4A). UMF starch seemed rough, and hollow particles showed a perforated or lacey structure.

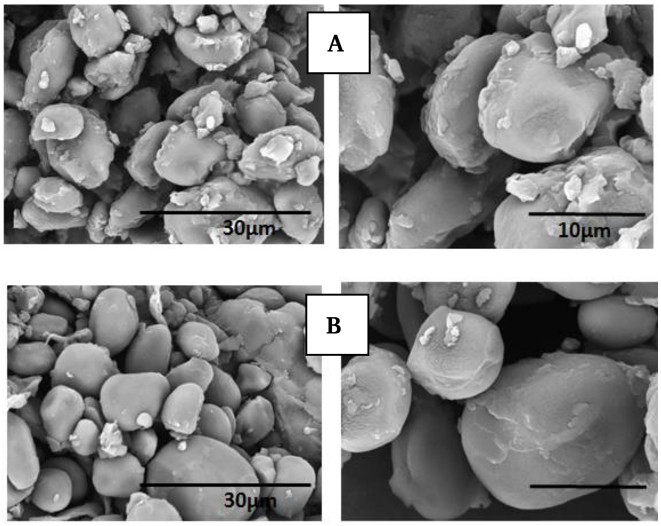

Fig. 4. Scanning electron microscopy images of UMF (A); and FMF (B). Images were taken at 10 kV and photographed at a speed of 100 s/picture at 17 mm working distance.

The divergences may occur due to the interaction between starch and lipid in the form of protein on the starch surface that disappears before fermentation. A similar structure was also found in fermented and unfermented rice flour and sorghum flour (Elkhalifa & Bernhardt, 2013; Ilowefah *et al.*, 2015; Lu *et al.*, 2005). They reported that cellulolytic, proteolytic, hydrolytic, and lipolytic enzymes were mainly produced during the growth of microorganisms, and this influenced the starch-protein or lipid interaction coat, which disappeared in fermented of seed flour. This phenomenon may be related to weak starch-starch or starch-lipid interactions during fermentation (Singh *et al.*, 2011; Siswoyo *et al.*, 2017).

Retrogradation properties

The enthalpy value of re-gelatinization of melinjo seed flour samples stored at 22 °C during 0-7 days was monitored in the range from 50 to 75 °C. That range temperature was identified as retrogradation-related endotherm, gelatinization, and retrogradation enthalpies (Siswoyo & Morita, 2010; Zhang & Jackson, 1992). The ΔHt at zero days all samples on the test could not be detected after the 1st scan. The enthalpy value of the melinjo seed sample showed a logarithmical increase with time of storage (Fig. 5A). The retrogradation rate of FMF and UMF increased in ΔH commonly at the 4th day of storage and resulted in ΔH maximal on the 7th day. However, the UMF showed deficient ΔH compared to FMF after being stored for 7 days. During storage, fermentation did not indicate a significant value reduction of ΔH when compared to UMF. This result confirms that the melinjo seed sample retrogradation was not retarded by fermentation. The result of the Avrami model showed that Table (2) and Fig (5B) was achieved for a reasonable description of melinjo seed starch retrogradation during the starch gelatinization starch with 0.97-0.99 regression coefficient, and ΔH_∞ values were around 5.38 (UMF) and 5.49 (FMF) J/g.

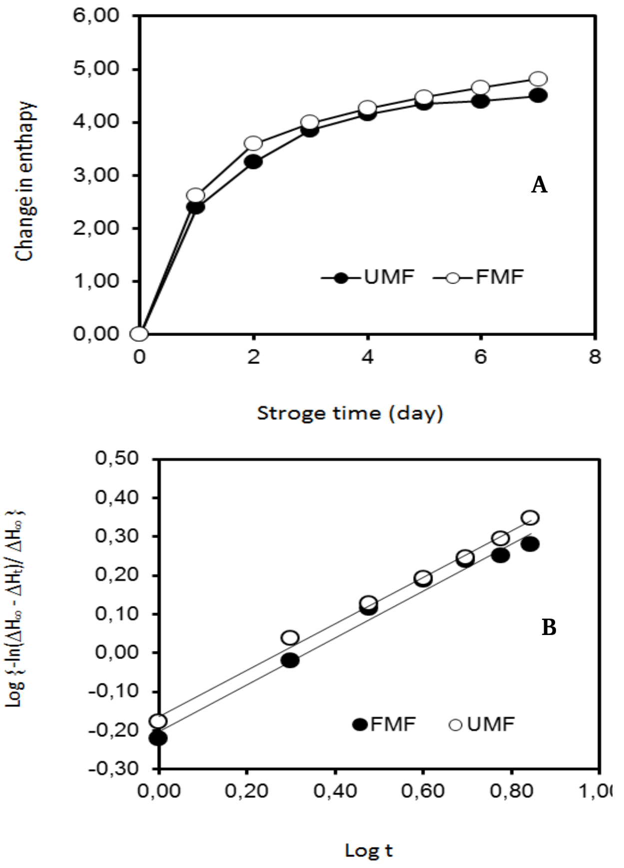

Fig. 5. The function of gelatinization enthalpy (A) and plot of the logarithmic fraction of crystallization Log {-ln(ΔH∞-ΔHt)/ΔH∞} against logarithmic time (log t) of UMF and FMF (B) retrogradation during storage at 22 °C.

Meanwhile, the Avrami exponent (n) for kinetics retrograde has the same value (0.60) between UMF and FMF. The same in values between the UMF and FMF in melinjo seed starch proposes that the starch recrystallization mechanism might not be different as the effect of fermentation on the intermolecular formation of lipid or protein, and the n value of melinjo seed starch perfectly coincided with n value of wheat starch ranging 0.60-1.26 (Zhang & Jackson, 1992). For the rate constant (*k*), the fermentation is faster (0.68 day^{-1}) than the retrogradation rate concerning the control (0.63 day^{-1}). These results suggest that fermentation results accelerate the occurrence of starch retrogradation during storage rather than control. Fermentation may lead to lower intermolecular formation between starch-lipid or starch-protein complexes, where the complex may prevent the reorganization of starch molecules during storage (Lai *et al.*, 2000).

Table 2. Avrami Parameter for UMF and FMF

	Avrami parameter*				
	ΔH_{∞} (J/g)	$\Delta H_{at\ 7days}$ (J/g)	*n*	*k* (day^{-1})	r^2
UMF	5.38	4.50	0.60	0.63	0.99
FMF	5.49	4.80	0.60	0.68	0.98

*ΔH_{∞} is the limiting enthalpy change, *k* is the rate constant, and *n* is the Avrami exponent.

Conclusion

Based on the resulted of this study, it can be concluded that fermentation had an effect on the gelatinization temperature (Tp) and RVA peak viscosity of melinjo seed flour decreased, and the gelatinization enthalpy (ΔH) increased after fermentation. Starch crystallinity had little effect, but the ratio of the crystal region to the amorphous region increased. Starch granules showed slight superficial corrosion after fermentation. The results revealed that fermentation might alter starch amorphous region and chemical components and eventually transform the physicochemical and retrogradation characteristics of melinjo seed flour.

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Author contributions

Tri Ardyati: Presenting the research idea and study design, Data analysis; **Keizo Hosokawa**: Writing the draft of the manuscript, Data analysis and interpretation; **Akihiro Ohara**: Writing the draft of the manuscript, Data analysis and interpretation; **Tri Agus Siswoyo**: Presenting the research idea and study design, Data analysis, Writing the draft of the manuscript, Data analysis and interpretation, Revising and editing the manuscript, Supervising the study, Approval of the final version.

Conflict of interest

There is no conflict of interest based on the writers.

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