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Food Science and Nutrition Technology
ISSN: 2574-2701

Fortification of Modified Cassava Flour through Application of Fermented Food Containing Poly-Glutamic Acid

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Research Article

Volume 2 Issue 4

Received Date: December 02, 2017

Published Date: December 12, 2017

Abstract

Present study was designed to investigate properties of fortified cassava flour produced from co-processing of modified cassava flour with poly-glutamic acid (PGA) derived from protein of beans that had been fermented by *Aspergillus oryzae* and *Bacillus natto*. Proximate analysis, cyanide content, swelling power, solubility and viscosity of modified cassava flour (MCF) which was fortified with PGA was found to indicate improvements as compared to native cassava flour (NCF). The protein content of fortified cassava flour (FCF) showed significantly increased (5.29 ± 0.01^{b} to 13.98 ± 0.79^{d} and 4.72 ± 0.06^{c} to 12 ± 006^{e} using PGA derived from *A. oryzae* and *B. natto*, respectively) as compared to the NCF (1.08 ± 0.02^{a} and 1.06 ± 0.11^{b}) and crude fiber of FCF showed significantly increased as well (1.55 ± 0.01^{c} to 3.31 ± 0.04^{e} and 2.31 ± 0.11^{c} to 3.08 ± 010^{e} as compared to crude fiber of NCF (1.25 ± 0.57^{b} and 1.58 ± 0.05^{a}). Swelling power of NCF (20.62 ± 4.04^{b} and 21.75 ± 0.12^{e}) was significantly decreased as compared to FCF (11.59 ± 0.09^{a} to 11.02 ± 0.20^{a} and 14.34 ± 0.19^{c} to 9.48 ± 0.11^{a} using PGA derived from *A. oryzae* and *B. natto*, respectively), meanwhile solubility of NCF (11.26 ± 0.11^{a} and 11.57 ± 0.25^{a}) was significantly increased as compared to FCF (16.42 ± 0.30^{c} to 23.09 ± 0.18^{e} and 16.68 ± 0.17^{c} to 23.48 ± 0.20^{e}. Observation by scaning electron microscope (SEM) indicated that starch granules of MCF and FCF were depolymerized by enzymatic hydrolysis lead to cause change and degrade exterior surface of the granules within corrosion via pores of small granules.

Keywords: Modified cassafa flour; Fortification; Polyglutamic acid; Physicochemical properties

Abbreviations: PGA: poly-glutamic acid; MCF: modified cassava flour; NCF: native cassava flour; FCF: fortified cassava flour

Introduction

Cassava (*Manihot esculenta* crantz) is one of the leading food plants in the world; it ranks fourth among staple crops with a global production of about 160 milllion tons per year [1]. Cassava is often castigated as an “inferior food crop” and “poor people crop” [2]. These labels on cassava were due to some limitations of the crop including low quality and quantity of protein Cooke and Coursey [3] and have major drawbacks of poor starch and protein digestibilities that undermine its nutritional

value and thus it has been underutilized compared to wheat [4].

Modification of native cassava starches using various chemical reagents makes significant variation in the structural, physicochemical, thermal and rheological properties of starch. Demiate and Cereda [5] proposed a chemical modification of cassava starch that resulted in high degree of expansion of the product. Modified starch is more resistant to acid, heat and shearing than is native starch [6,7] and therefore modified starch is suitable for canned food and other applications [8]. In recent years, substantial progresses have been made in obtaining starches from non-conventional sources and studying their functional and physicochemical properties [9] where the characteristics of modified starch can be used for industrial applications. Thus, modifying starch is important to provide the following properties such as thickening, gelatinization, adhesiveness or to improve water retention, enhance palatability and to remove or add opacity as well as to modify cooking characteristics, reducing retrogradation, reducing paste's tendency to gelatinize, increasing paste's stability when cooled or frozen, increasing transparency of pastes and gels, improving texture of pastes and gels, improving adhesiveness between different surfaces [10].

Akingbala, et al. reported about a 95-98% decrease in HCN content after fermentation of cassava during manufacture of fermented cassava. Since food processing usually includes heating, HCN produced is likely to evaporate completely [4]. However, boiling whole cassava root can result in toxicity as the hydrolytic enzymes are denatured by heat without hydrolyzing the HCN. However, cassava and its products are low in protein, deficient in essential amino acids and therefore have poor protein quality [10]. Thus, continuously dependence on fermented cassava without supplementation with protein-rich sources would result in protein deficiency. However, because of the high cost of animal proteins, a protein-rich legume such as soybean with good essential amino acid profile is potentially the most useful protein source for complementing and enhancing the nutritional value of fermented cassava [11].

Supplementation of vegetable proteins to fermented cassava is therefore expected to enhance its protein quantity and quality as well as improve its health promoting benefits [12,13] that is reported to lower cholesterol levels in the blood and its amino acid content which is considered key in its ability to control blood pressure, and this appears to be related to calcium conservation [12], while isoflavone have been reported to play essential roles in preventing certain types of cancers and in reducing the risk of cardiovascular diseases [14,15]. This study is undertaken to modify cassava flour using microbial starter culture and fermented product containing PGA to compensate for lack of gluten and leads to changes its physicochemical properties to the search for alternatives to gluten in the manufacture of MCF which is improved in its protein content.

Material and Methods

Modification Procedure

Cassava chips (1.0 kg) were mixed with 2.0% (w/v) of enzymatic starter derived from culture of *A. oryzae* and *B. natto* respectively. The mixture was stirred and then incubated at room temperature for 24-48h, and then dried using oven at 50˚C for 24-48h to reduce moisture content up to lower than 10% [16,17].

Measurement of HCN Content

HCN content of cassava flour samples were measured by using alkali titration method [18]. Five % (w/v) starch slurry was prepared by soaking 10 g of cassava flour in 200 ml of distilled water for 4h in a distillation flask. The solution was then distilled into 20 ml of 0.625M NaOH using steam distillation until its volume became 150 ml. Distilled water was added and filled up to 250 ml. Eight ml of 5% KI was added into 100 ml of solution and titrated using 0.02N silver nitrate solution. End point of titration was determined as color of the solution turned to bright yellow.

Proximate Analysis

Proximate composition of the cassava flour including moisture, ash, fat, and protein contents were determined using Association of Official Analytical Chemists methods [19]. Total carbohydrate content was determined by subtracting the ash, protein, and fat percentages from 100%.

Measurement of Solubility

Solubility was examined by dissolving 2 gram cassava powder into 40 ml of distilled water, then heated in a water bath at 60°C for 30 min. Supernatant and paste were formed, and thus it separated using a centrifuge at 3000 rpm for 20 min. Furthermore approximately 10 ml supernatant was dried using oven and weight of its precipitated was recorded.

Swelling Power Determination

Swelling power was examined by dissolving 0.2 g cassava powder into 20 ml distilled water and heated at 60°C for 30 min. Supernatant was separated by centrifugation at 2500 rpm for 15 min. The slurry was heated at 70°C for 30 min, cooled to room temperature and furthermore centrifuged at 1200*g* for 15 min. The supernatant was decanted in the centrifuge tube was dried at 50°C for 25 min and weighed prior to adding with distilled water.

Measurement of Viscosity

Viscosity of cassava flour samples were measured using a viscometer as described by Reddy and Bhotmange [20]. Cassava flour at concentration 1 to 4% was prepared using distilled water. Each solution was heated for 20 min and then cooled at room temperature and reading toward their viscosities of each solutions were the recorded.

Observation using Scaning Electron Microscope

Characteristics of cassava flour granules were observed under Scanning Electron Microscopy (SEM). The respective cassava flour samples i.e. NCF, MCF and FCF were sent to the Faculty of Science and Natural Resources, Universiti Malaysia Sabah for SEM analysis services. Observation by using SEM was conducted to distinguish differences in the structure and morphology of starch granules before and after modification and fortification.

Results and Discussion

(Table 1) and (Table 2) showed the results of proximate analysis of the respective cassava flour. Statistical analysis showed a significant difference ($p<0.05$) among of the cassava flour samples. FCF prepared with 10, 20 and 30% PGA of *B. natto* showed the highest protein content (4.74%±0.06^{c}, 8.67%±0.09^{d}, 12.79%±0.06^{e}, respectively) and with 10, 20 and 30% PGA of *A. oryzae* (5.29%±0.01^{b}, 9.51%±0.01^{c}, 13.98%±0.79^{d}, respectively) compared with NCF (1.06%±0.11 and 1.08%±0.02) and MCF prepared with *B. natto* and *A. oryzae* (0.77%±0.07^{a} and 0.82%±0.00^{a}, respectively).

	Average ± Standard Deviation (%)				
Analysis	**NCF**	**MCF**	**FCF-10**	**FCF-20**	**FCF-30**
Moisture	3.73±0.12^{a}	6.08±0.06^{b}	7.16±0.10^{c}	8.44±0.05^{d}	9.81±0.09^{a}
Ash	1.77±0.08^{d}	1.07±0.12^{a}	1.30±0.05^{b}	1.59±0.07^{c}	1.91±0.17^{e}
Crude fat	0.68±0.04^{b}	0.50±0.15^{a}	2.35±0.09^{c}	4.44±0.08^{d}	6.64±0.11^{e}
Crude protein	1.06±0.11^{b}	0.77±0.07^{a}	4.74±0.06^{c}	8.67±0.091^{d}	12.79±0.06^{e}
Crude fiber	1.58±0.05^{a}	1.19±0.04^{b}	2.31±0.11^{c}	2.68±0.02^{d}	3.08±0.10^{e}
Carbohydrate	91.18±0.19^{e}	90.40±0.05^{d}	82.15±0.08^{c}	74.18±0.17^{b}	65.77±0.14^{a}

Table 1: Proximate analysis of NCF compared to MCF prepared with B. natto and FCF prepared with PGA of B. natto.

	Average ± Standard Deviation (%)				
Analysis	**NCF**	**MCF**	**FCF-10**	**FCF-20**	**FCF-30**
Moisture	7.13±0.04^{a}	8.41±0.06^{c}	8.04±0.01^{b}	8.06±0.08^{b}	8.17±0.04^{b}
Ash	1.77±0.03^{cd}	1.12±0.02^{a}	1.43±0.14^{ab}	1.55±0.18^{bc}	2.00±0.01^{d}
Crude fat	1.03±0.40^{a}	1.29±0.24^{ab}	2.54±0.16^{b}	5.07±0.60^{c}	7.05±0.12^{d}
Crude protein	1.08±0.02^{a}	0.82±0.00^{a}	5.29±0.01^{b}	9.51±0.01^{c}	13.98±0.79^{d}
Crude fiber	1.25±0.57^{b}	0.96±0.01^{a}	1.55±0.01^{c}	2.96±0.00^{d}	3.31±0.04^{e}
Carbohydrate	87.73±0.32^{d}	87.40±0.33^{d}	81.16±0.19^{c}	72.86±0.32^{b}	66.00±0.19^{a}

Table 2: Proximate analysis of NCF compared to MCF prepared with A. oryzae and FCF prepared with PGA of *A. oryza.*

According to Tonukari [21] and Charles et al [22], the protein content of cassava flour was 1-2% or no more than 4% [23], and due to low protein content causing flour cassava is only served as a starchy food. Protein content of modified cassava flour was lower than that of the native flour. It might be due to enzymatic activity of *Aspergillus oryzae* used for incubation of cassava chips. According to Papagianni 2004 [24], *A. oryzae* potential to produce some other enzymes including protease other than amylase and cellulase. It has catalytic function to

hydrolyze and degrade peptide bond between amino acids in cassava flour, resulting in lower protein content of modified cassava flour compared with native cassava flour.

Enzymatic modification of cassava flour might encourage a reduction in protein content of cassava flour. According Richana, et al. [19], most of protein contains in cassava flour might be dissolved during modification process. However, fortified cassava flour showed much higher protein content compared to modified and native cassava flour. It is caused by a protein fortification using protein source derived from fermented bean containing PGA. The process of fortification is aimed to increase the protein content in cassava flour while improving quality of its nutrition value. Transglutaminase was added dealing with fortification where this enzyme might play a role on binding of cross-linking a series of protein covalent in cassava flour granules [13].

According to (Tables 3 and 4), statistical analysis showed there was a significant difference ($p<0.05$) for lipid content of NCF ($0.68\%\pm0.04^{b}$ and $1.03\%\pm0.40^{a}$) compared to MCF prepared with *B. natto* and *A. oryzae* ($0.50\%\pm0.15^{a}$ and $1.29\%\pm0.24^{ab}$, respectively) and FCF prepared with 10, 20 and 30% PGA of *B. natto* ($2.35\%\pm0.09^{c}$, $4.44\%\pm0.08^{d}$ and $6.64\%\pm0.11^{e}$, respectively) and with 10, 20 and 30% PGA of *A. oryzae* ($52.54\%\pm0.16^{b}$, $5.07\%\pm0.60^{c}$, $7.05\%\pm0.12^{d}$, respectively).

Cassava Flour	Swelling Power	Solubility	Viscosity (Cp)	HCN (mg/kg)
NCF	21.75 ± 0.12^{e}	11.57 ± 0.25^{a}	14480 ± 105.83^{e}	3.71 ± 0.06^{e}
MCF	18.52 ± 0.22^{d}	13.61 ± 0.10^{b}	11493 ± 100.66^{d}	2.65 ± 0.05^{d}
FCF-10	14.34 ± 0.19^{c}	16.68 ± 0.17^{c}	9140 ± 280.00^{c}	1.63 ± 0.10^{c}
FCF-20	12.36 ± 0.13^{b}	19.33 ± 0.21^{d}	8690 ± 81.85^{b}	1.22 ± 0.06^{b}
FCF-30	9.48 ± 0.11^{a}	23.48 ± 0.20^{e}	7747 ± 128.58^{a}	1.01 ± 0.06^{a}

Table 3: Physicochemical properties of NCF compared to MCF prepared with *B. natto* and FCF prepared with PGA of *B. natto.*

Cassava Flour	Swelling Power	Solubility	Viscosity (Cp)	HCN (mg/kg)
NCF	20.62 ± 0.04^{b}	11.26 ± 0.11^{a}	18960 ± 678.82^{c}	1.72 ± 0.01^{d}
MCF	19.04 ± 0.28^{b}	13.55 ± 0.13^{b}	14185 ± 417.19^{b}	0.65 ± 0.00^{c}
FCF-10	11.59 ± 0.09^{a}	16.42 ± 0.30^{c}	8250 ± 155.56^{a}	0.43 ± 0.00^{b}
FCF-20	11.16 ± 0.28^{a}	19.23 ± 0.47^{d}	7840 ± 452.55^{a}	0.22 ± 0.00^{a}
FCF-30	11.02 ± 0.20^{a}	23.09 ± 0.18^{e}	6660 ± 197.99^{a}	0.22 ± 0.00^{a}

Table 4: Physicochemical properties of NCF compared to MCF prepared with *A. oryzae* and FCF prepared with PGA of *A. oryzae.*

According to Charles, et al. [22], lipid content of native cassava flour is approximately 0.1% to 0.4% based on dry weight of raw material. According to Papagianni [24], *A. oryzae* potential to produce some other enzymes including lipase other than amylase and cellulase since crude enzyme was used as starter culture that has catalytic function to hydrolyze and degrade lipid content of cassava flour to be a simpler molecule including fatty acid and glycerol. As a hydrolysis product it was furthermore dissolved into incubation media and drained with enzyme solution before process of drying.

Statistical analysis showed there was a significant difference ($p<0.05$) for cyanide (HCN) content among of cassava flour samples. NCF showed the highest HCN content ($3.71\text{mg/kg}\pm0.06^{e}$ and $1.72\text{mg/kg}\pm0.01^{d}$) compared to MCF prepared with *B. natto* and *A. oryzae* ($2.65\text{mg/kg}\pm0.05^{d}$ and $0.65\text{mg/kg}\pm0.00^{c}$, respectively) and FCF prepared with 10, 20 and 30% PGA of *B. natto* showed the lowest HCN content ($1.63\ \text{mg/kg}\pm0.10^{c}$, $1.22\ \text{mg/kg}\pm0.06^{b}$, $1.01\ \text{mg/kg}\pm0.06^{a}$, respectively) and with 10, 20 and 30% PGA of *A. oryzae* ($0.43\text{mg/kg}\pm0.01^{d}$, $0.22\text{mg/kg}\pm0.00^{a}$, $0.22\text{mg/kg}\pm0.00^{a}$, respectively) Table 3 and 4.

According to standard of WHO these three cassava flour were actually safe to be consumed, since the HCN content did not exceed than 10 ppm as determined by using HPLC [25,26]. Akindahunsi, et al. [27] reported that reducing amount of HCN in cassava flour to be safety level was due to linamarin and lotaustralin in cassava had been sequentially destroyed and degraded to be free cyanide

during flour producing process including enzymatic process. Thus, the process involved in the production of cassava flour may be as detoxification process to make it safe for consumption and possibly for manufacturing of food products [28,29].

The value of swelling power, solubility, and viscosity of the cassava flour samples have been summarized in Table 3 and 4 which shown there was significant difference ($p>0.05$) in the swelling power among of these cassava flour. The swelling power of MCF modified with starter culture of *B. natto* (18.52%±0.22^{d}) was slightly decrease compared to swelling power of NCF (21.75%±0.12^{e}) and significantly decreased compared to FCF prepared with 10, 20 and 30% PGA of *B. natto* (14.34%±0.19^{c}, 12.36%±0.13^{b} and 9.48%±0.11^{a}, respectively). The statistical analysis showed there was no significant ($p<0.05$) difference in the swelling power between NFC (20.62%±0.04^{b}) and MCF modified with starter culture of *A. oryzae* (19.04%±0.28^{b}) and there was only slightly significant differences between NCF compared to FCF prepared with 10, 20 and 30% PGA of *A. oryzae* (11.59%± 0.09^{a}, 11.16%±0.28^{a} and 11.02%±0.20^{a}, respectively).

Swelling properties is a capacity of flour to bind water molecules through hydrogen bonding [21]. Swelling of cassava starch granules proves that there was interaction among of the starch chains with amorphous and crystalline regions [30]. The MCF showed the highest swelling power since there was a rejection among of negative charged of phosphate groups on amylopectin chains. It would weaken hydrogen bonds inside the chain led to cause hydration and high swelling power [31,32].

As shown in Table 3 and 4, statistical analysis showed there was a significant difference ($p<0.05$) for solubility among of cassava flour samples. NCF showed the lowest solubility (11.57%±0.25^{a} and 11.26%±0.11^{a}) compared to MCF prepared with *B. natto* and *A. oryzae* (13.61%± 0.10^{b} and 13.55%± 0.13^{b}, respectively) and FCF prepared with 10, 20 and 30% PGA of *B. natto* showed the highest solubility (16.68%±0.17^{c}, 19.33%±0.21^{d} and 23.48%±0.20^{e}, respectively), as well as prepared with 10, 20 and 30% PGA of *A. oryzae* (16.42%±0.30^{c}, 19.23%± 0.47^{d} and 23.09%±0.18^{e}, respectively).

Both of MCF and FCF showed higher solubility due to hydrolysis by enzyme amylase on starch granules containing internal bond of α-1, 4-glycosidic linkages to break it down into glucose [33]. The solubility properties are a capacity of starch granule to bind water by formation of hydrogen bonds with water molecules [34]. The hydrolysis of starch granules have increased motility of granules in which breaking of granules bonds will increase the spread of the granules in water molecules when it is heated. More water molecules would penetrate and form hydrogen bonds with the starch granules and thus the solubility of MCF and FCF was increased [35]. In addition, amount of amylose contained in cassava flour was a major factor that affected the swelling power of starch granules [35]. Amylose was an obstacle for granules to swell and served to maintain integrity of swelling [31]. Cassava flour that has more amylose content has a lower swelling power [33].

As shown in Table 3 and 4, there was significant difference ($p>0.05$) in viscosity of MCF with starter culture of *B. natto* and *A. oryzae* (11493cp±100.66^{d} and 14185cp± 417.19^{b}, respectively), compared to viscosity of NCF (14480cp±105.83^{e} and 18960cp± 678.82^{c}) and significantly decreased compared to FCF prepared with 10, 20 and 30% PGA of *B. natto* (9140cp±280.00^{c}, 8690cp±81.85^{b} and 7747cp±128.58^{a}, respectively), however, there was only slightly significant different compared to FCF prepared with 10, 20 and 30% PGA of *A. oryzae* (8250cp±155.56^{a}, 7840cp±452.55^{a} and 6660cp±197.99^{a}, respectively).

Overall, NCF exhibited the highest viscosity compared to MCF and FCF. Viscosity properties may be defined as resistance of a substance to flow and it will increase when temperature decreases and vice versa, the resistance of flow will decrease as the temperature rises. The viscosity of flour will increase or decrease depending on type of modification being performed [36]. The difference among of viscosity of the three cassava flour may be depended on degree of hydrolysis towards granules of starch, total content of starch and fiber content that is contained in cassava flour [20]. The higher process on enzymatic hydrolysis towards starch granules will contribute to degrading of granules and reduction in amount of available granules in cassava flour and reducing amount of starch will directly lead to decreasing in the viscosity of flour, and therefore MCF and FCF had a lower viscosity.

Observing of structure of the cassava starch granules using SEM showed that cassava flour granules revealed a different morphology after modification as shown in Figure1 and 2. According to Putri, et al. [30] diameter of cassava starch granules were varied in the range of 3-10μm and exhibited irregular shape with longitude and ellipsoid truncated shape with one side containing a cone hole where the hole was very deep for some starch granules. Hydrolysis that occurred toward cassava flour

granules could be categorized based on intensity and the way in which granules eroded and diminished [16]. The degree of hydrolysis that occurred over modified starch granules could be identified by the shape and size of each granule. It showed that modification process indicated different morphology of starch granules compared unfermented starch granules.

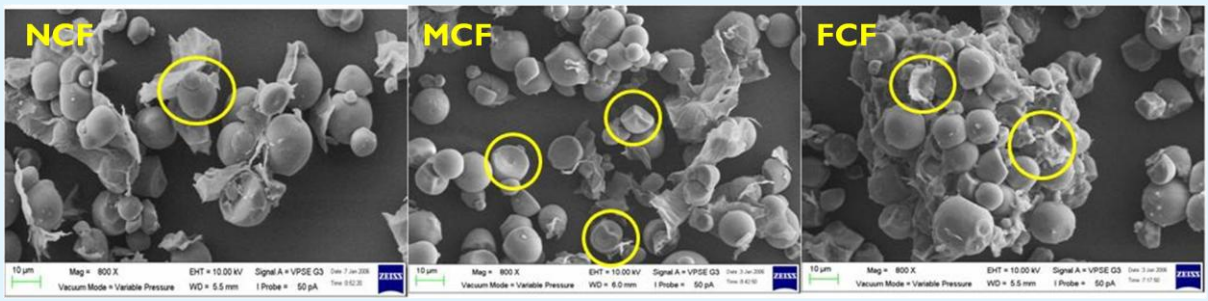

Figure 1: SEM observation on starch granules of NCF (A, left) compared to MCF (B, middle) prepared with *B. natto* and FCF prepared with PGA of *B. natto* (C, right).

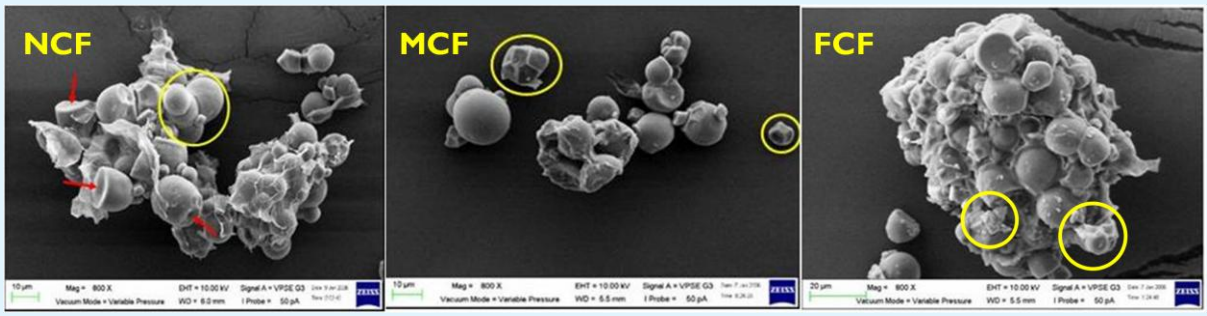

Figure 2: SEM observation on starch granules of NCF (A, left) compared to MCF (B, middle) prepared with *A. oryzae* and FCF prepared with PGA of *A oryzae* (C, right).

From the SEM micrographs, the process of corrosion and enzymatic hydrolysis on cassava starch granules occurred mainly on surface of starch granules as shown in Figure 1B and 2B as well as 1C and 2C. Qualitatively, rough surface and eroded starch granules could be observed in Figure 1B and 2B as well as 1C and 2C of that MCF was prepared with starter culture of *B. natto* and *A. oryzae*, respectively and FCF was fortified with PGA of *B. natto* and *A. oryzae*, respectively, compared to Figure 1A and 2A of the respective NCF. Figure 1A and 2A showed smooth surface of starch granule with irregular sections while Figure 1B and 2B as well as 1C and 2C of the respective MCF and FCF exhibited that some of starch granules had been broken with rough and eroded surface. Refer to the Figure 1B and 2B as well as 1C and 2C, size of some granules had become smaller and the amount of the granules had become increasingly decreased. According to Putri, et al. [30], the granule molecules that were reside in amorphous region had been depolymerized by enzymatic hydrolysis process. According to Shariffa, et al. [16], enzymatic hydrolysis by amylase had taken place in which the amylase was able to cause change and degrade exterior surface of the granules by exo and endo-corrosion occurence. Through small pores of the granules subjected to enzyme penetration process into the starch granules to hydrolyze inside part of starch granules of modified and fortified cassava flour [37-43].

Aknowledgement

This work was financially supported by Fundamental Research Grant Scheme Phase 1/2014, under the Ministry of Education Malaysia in cooperation with Universiti Malaysia Sabah.

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