Asian Journal of Chemistry; Vol. 28, No. 10 (2016), 2261-2266

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2016.19962

Characterization and Application of Polygalacturonase from Trinitario (*Theobroma cacao* L.) Pulp

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Received: 10 March 2016; *Accepted*: 13 May 2016; *Published online*: 30 June 2016; AJC-17978

Destruction of cacao (*Theobroma cacao* L) pulp is an important part in cacao beans drying process. Ripening of the fruit pulp that cause softening of cacao beans will be followed by formation of polygalacturonase enzyme. In this study, polygalacturonase was extracted using ammonium sulphate at 80 % saturation of pulp cacao beans that have been cured for 3, 5 and 7 days. Activity of polygalacturonase has been evaluated in term of its pH, temperature and content of different substrates. All of the polygalacturonase with different curing time has optimum activity at pH 5.5, temperature of 50 °C and 0.9 % of substrate. Application of polygalacturonase on the fermentation of cacao beans have been evaluated through DMRT test ($P \leq 0.05$) and showed significant difference in organoleptic and catechin content of beans fermentation for 3, 5 and 7 days.

Keywords: Enzyme, Cacao, Polygalacturonase, Pulp.

INTRODUCTION

Enzyme is a protein group that is responsible for the regulation of various chemical reactions that occur within plant tissues, especially fruit. The whole series of reactions inside the fruit, for example ripening process, is also controlled by enzyme. Enzymes involved in ripening fruit come from pectolytic enzyme group [1]. Proteolytic enzyme is an enzyme group whose activity in pectin degradation into D-glucuronic acid [2]. Pectin is a polymer cell wall constituent that is contained in the primary cell walls and intercellular spaces of the plant. Pectin residues consist of 1,4-α-D-galacturonic acid that is partially esterified (more than 75 %) and rhamnose residue that is linked together with D-galacturonic residue through β-1,2 and α-1,4 bonds [3]. Some of the galacturonase residues also undergo acetylation (about 5 %) in their C-2 and C-3 positions. Polygalacturonase (PG) is a group of enzymes that has depolymerization activity of pectin acid to break the glycoside through the hydrolysis reaction [4].

Polygalacturonase was produced from plants [5-7] and microorganisms *i.e.* bacteria, fungi [8-10] and a few species of yeast [11,12]. Based on polygalacturonase activity in hydrolyzing -α-(1,4) glycosidic bond from non-esterification residues, polygalacturonase was classified as endo-polygalacturonase (EC 3.2.1.15) and exo-polygalacturonase (EC 3.2.1.67). Prefixes endo and exo show random or terminal cleavage activity. Endo-polygalacturonase is distributed in fungi, bacteria and yeast and posses characteristics namely the molecular weight of 30-80 kD, optimum pH 2.5-6.0 and optimum temperature at 30-50 °C [13,14]. On the other hand, exo-polygalacturonase (E.C.3.2.1.67) has molecular weight range of 30-50 kD. Polygalacturonase can be found in many types of fruit such as peaches, citrus, apples, mango and carrots [15-17]. Utilization of polygalacturonase in industry has long application history such as in clarification of grape juice [18], extraction of essential oils, flavours and pigments from plants [19], fermentation of coffee and tea, degumming and waste water treatment [20].

Polygalacturonase is found in plant tissue is a group of pectin methyl esterase (PME) and polygalacturonase (PG). Polygalacturonase enzymes are generally involved in the process of softening in some fruits such as tomato, pear, avocado, mango and cacao. Up to now, many researchers reported that polygalacturonase enzymes have been isolated from various microorganisms such as fungi and bacteria. Anuradha *et al.* [21] reported the isolation of polygalacturonase from *Aspergillus awamori* MTCC 9166 isolate obtained from waste orange peel. Martin *et al.* [22] have isolated polygalacturonase from *Thermomucor indicaeseudaticae*, while the pectinase enzyme has been reported that it could be isolated from various sources like *Carica papaya* [23,24], *Lycopersicum esculentum* [25], *Vitis vinifera* [26], *Prunus malus* [27], *Citrus* sp. [28], *Pouteria sapota* [29], *Malpighia glabra* L [30].

Southeast Sulawesi is a cacao-producing region in Indonesia. However, the post-harvest handling in the region is not maximized yet, thus enabling polygalacturonase enzyme research of cacao pulp is needed to be done. Polygalacturonase enzyme can be applied to the fermentation of cacao beans for seed pectin depolymerization resulting in cleaner and easier process to dry cacao beans, which increase the economic value of cacao. Fermentation is an important step in the production of cacao beans because production of chocolate aroma precursors are formed in the fermentation phase [31,32]. Internal and external fermentation stage are also occur during cacao fermentation. External fermentation bean pulp primarily involves catabolism, whereas internal fermentation involves biochemical changes in the seed cotyledons [31]. Chocolate that is produced from non-fermentation process has low cacao flavour and bitter taste [33]. The level of bitterness and astringency can be reduced during fermentation [34-36].

EXPERIMENTAL

All materials used in this work were analytical grade. Sodium bisulphite (39.0 % solution in water), sodium acetate (≥ 99.99 %), ammonium sulphate (≥ 99.5 %) and disodium hydrogen phosphate dihydrate (≥ 99.0 %) were purchased from Merck, Germany. Polyethylene glycol (PEG 4000), pectin citrus (85.0 %), acetic acid (≥ 99.7 %), sodium dihydrogen phosphate dihydrate ($NaH_2PO_4 \cdot 2H_2O$, ≥ 99.0 %), 3,5-dinitrosalicylic acid (≥ 98. %), sodium hydroxide (NaOH, ≥ 98. %), sodium potassium tartrate (≥ 99 %), Copper (II) sulphate dihydrate ($CuSO_4 \cdot 5H_2O$, ≥ 98. %), galacturonic acid monohydrate (97.0 %) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich, Singapore. Cacao (*Theobroma cacao* L) was taken from Konawe Selatan, Kendari, Southeast Sulawesi, Indonesia.

Curing of cacao beans: Curing of cacao fruit aims to obtain uniformity on ripeness of the fruit and facilitates the issuance of beans from cacao fruit. Curing was performed for 3 (H3), 5 (H5) and 7 (H7) days. After ripening is complete, isolation of polygalacturonase was conducted on cacao pulp.

Isolation of polygalacgturonase: 500 g result of bean curing was extracted using 400 mL acetate buffer (50 mM, pH 5) for 10 min. 40 g aliquot of the extract was added with 80 mL PEG 4000 12 % and 80 mL sodium-bisulphite 0.2 %. The mixture is stirred for 10 min at 4 °C and centrifuged (5000 rpm, 4 °C for 20 min). The 75 mL (pH 5) of acetate buffer was then added to the obtained residue and was incubated for 1 h at 4 °C. The mixture was re-centrifuged (5000 rpm, 4 °C for 20 min) in order to obtain crude extract of polygalacturonase enzyme [37,38].

Characterization of polygalacturonase enzyme

Determination of activity and protein content: Polygalacturonase activity was determined at 37 °C for 60 min using reaction mixture of 4 mL substrates containing 0.75 % citric pectin and 1 mL sample of enzyme polygalacturonase. The reaction was stopped by heating at 100 °C for 5 min. 2 mL of DNS reagent was added as method for determining reducing sugar [39]. One unit of enzyme activity was defined as the amount of enzyme producing 1 μM of galacturonic acid per minute under experimental conditions. The protein content is determined by the method of Bradford [40] using bovine serum albumin (BSA) as standard.

Determination of optimum substrate content: The influence of the substrate on enzyme activity is determined by concentration variations of substrate which contain 0.1-1.0 % citrus pectin, with intervals of 0.1 % in buffer solution [24,41]. Furthermore, the activity and protein content of enzyme were calculated by spectrophotometry.

Determination of optimum pH: Determination of the pH effect on polygalacturonase enzyme activity was conducted by reacting 1 mL crude polygalacturonase enzyme and 4 mL substrate containing pectin 0.9 %, 1 mL of acetate buffer 50 mM (pH 4.0; 4.5; 5.0) and 1 mL phosphate 50 mM (pH 5.5; 6.0). The mixture was incubated at 35 °C for 60 min [41]. 2 mL of DNS reagent was added to determine the reducing sugars. Activities of polygalacturonase was calculated by spectrophotometry.

Determination of optimum temperature: Determination of the optimum temperature on the polygalacturonase activity is carried out by reacting 1 mL crude polygalacturonase enzyme, 1 mL optimum buffer pH 50 mM and 4 mL substrate containing 0.9 % pectin. The mixture was incubated for 60 min at various temperatures of 30, 40, 50 and 60 °C. 2 mL DNS reagent was added to determine the reducing sugars.

Purification of polygalacturonase (precipitation and dialysis): Semi-pure enzyme was obtained by precipitating the crude extract using ammonium sulphate with saturation level of 20-100 %. The precipitation is performed by adding ammonium sulphate into the supernatant or crude extract gradually. Enzyme was obtained by centrifugation at 8000 rpm for 30 min. The resulting precipitates were dissolved in 20 mM phosphate buffer at pH 5.5 with a ratio of 1:2 (w/v). While the further purification process is carried out using a dialysis membrane of 12 kDa cut-off (MWCO). Before using, the membrane is processed to remove contaminants such as glycerol, heavy metal ions or micro components that contain sulfur. Dialysis preparation was carried out by boiling the membrane in EDTA (1 % Na_2CO_3 in 0.003 M EDTA). Boiled membrane is cooled in distilled water with addition of 0.02 % sodium azide. Each obtained enzyme in every purification steps was analyzed by standard test to determine the level of purity of the enzyme.

RESULTS AND DISCUSSION

Isolation of polygalacturonase enzyme: Polygalacturonase (PG) is a group of enzymes that have activity for depolymerization of pectin acid. According to Fox [42], proteolytic enzymes, in addition to present in the plant tissue, can also be produced by microbial secretion. The principles of isolation process in this research were including extraction, filtration and centrifugation. The initial process of this study is the extraction of cacao beans pulp that have been fermented for 3, 5 and 7 days using acetate buffer with agitation speed of 120 rpm for 10 min. Crude enzyme was obtained by reacting extracts with PEG 4000 12 % and sodium bisulphite. The extraction process is followed by centrifugation to separate the crude enzyme and the remain pulp. Every 500 g of cacao

beans that is extracted produces crude enzyme extract as much as 600 mL. Determination of enzyme activity and protein levels was conducted to determine the effect of curing time on the optimum enzyme activity. One unit of enzyme activity is defined as the amount of enzyme required to release 1 µmol galacturonic acid/min. Fig. 1. shows the relationship between duration of curing and polygalacturonase activity.

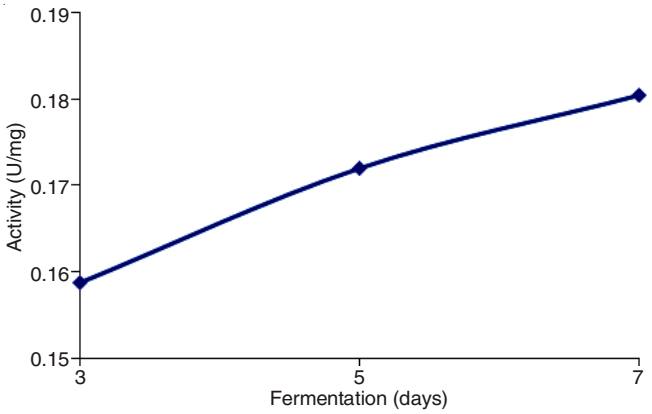

Fig. 1. Activity of polygalacturonase on curing of cacao for 3, 5 and 7 days

Based on Fig. 1, polygalacturonase enzyme as the result of 7 days curing period possess optimum activity of 0.180 U/mg. The different results have been reported by Anuradha *et al.* [21] that polygalacturonase enzyme extracted from *Aspergillus awamori* MTCC 9166 using orange peel, jack fruit rind, carrot peel and beet root peel as the substrate pectin possessed greater activities of 16.8, 38, 36 and 24 U/mL, respectively. Bird *et al.* [43] reported that polygalacturonase enzyme is produced during the ripening process of cacao fruit and is active during the ripening process. Curing cacao fruit of more than 7 days can cause cacao beans become media for fungi growth hence it can degrade the quality of the cacao beans and affects the quality of the product.

Characterization of polygalacturonase enzyme

Optimum of substrate concentration: One of the factors that affects of the enzyme activity is the substrate concentration. In an enzymatic reaction, the enzyme binds the substrate to form the enzyme-substrate complex [ES], then it will break down into complex enzyme [E] and the product [P]. The more complex [ES] is formed, the faster the reaction takes place up to the limit of saturation [ES]. At the time of substrate concentration [S] beyond the limits of saturation, the rate of reaction will be constant. In such condition the whole enzyme complex has been in the form of E-S. The addition of the substrate does not increase the number of complex E-S. Polygalacturonase substrates used in this study is citric pectin as a standard reaction. Concentration substrate was made at range of 0.1-1.0 %. The optimum substrate concentration of cacao pulp is shown in Fig. 2.

Fig. 2 shows that the polygalacturonase extracts obtained from fermentation of cacao for 3, 5 and 7 days have optimum activity on substrate containing 0.9 % pectin citrate. Linear response to the concentration of the substrate is shown by polygalacturonase H3. The larger of substrate concentrations of 0.9 %, the less activity of the three polygalacturonase. The different results on polygalacturonase *Aspergillus aculeatue*

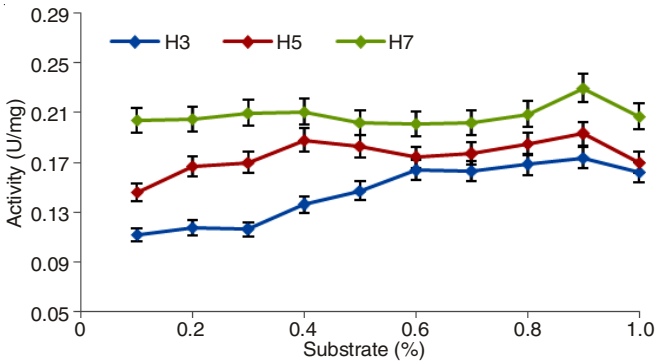

Fig. 2. Optimum substrate concentration of polygalacuturonase

and *Mucor pusillue* show the strong inhibition to the polygalacturonic acid and pectin 1.6 % substrate [44]. Polygalacturonase *B. licheniformis* KIBGE IB-21 has optimum activity at a concentration of 1 % pectin substrate [45]. Saturation limit of substrate to the enzyme active site needs to be known, so that the application of enzymes can be optimized.

Optimum pH: Determination of the effect of pH on polygalacturonase activity is carried out by reacting polygalacturonase extract with the substrate at various pH levels. In general, each enzyme has maximum activity at given pH. The pH activity profile of an enzyme illustrates the pH when proton or acceptor donor groups on active side of the enzyme are in the desired degree of ionization. Effect of pH on the polygalacturonase activity was performed in the range of pH 4-6. Effect of pH on the polygalacturonase activity in the three samples 3H, 5H and 7H are shown in Fig. 3.

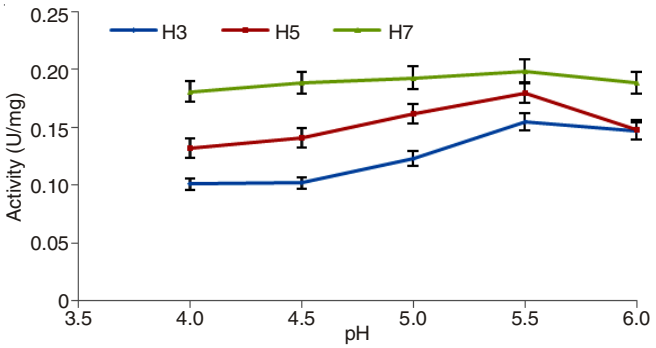

Fig. 3. Effect of pH on the activity of polygalacturonase

The optimum pH of polygalacturonase enzyme shows uniform value at pH 5.5; with a curing time of 7 days and possesses the highest activity of 0.199 U/mg. The different results has been reported previously by Nitinkumar *et al.* [46]. They found that the optimum activity of exo-polygalacturonase from *Paecilomyces variotii* NFCCI 1769 is found in pH 6.0. Serrat *et al.* [47] reported similar results that polygalacturonase of *Kluyveromyces marxianus* strain isolated from coffee wet-processing wastewater possesses optimum activity at pH 5. In general, the optimum pH of the polygalacturonase enzyme are in a narrow range from pH 5 to 6 [46,48-50].

Effect of temperature: Increasing of temperature will increase the kinetic energy of the reaction. The kinetic energy is proportional to the frequency of collision between the substrate and active site of enzyme, so that the catalytic properties of

the enzyme will be the function temperature. The too high energy can damage the reactivity of hydrolyzing enzymes in the substrate. Increasing of the temperature causes the enzyme protein to be denatured. Determination of the optimum temperature of crude polygalacturonase, which is isolated from cacao bean pulp shows optimum uniform polygalacturonase activity at 50 °C. The optimum temperature of the polygalacturonase cacao beans pulp is shown in Fig. 4. Polygalacturonase enzyme is an enzyme that is responsible for the maturation phase of fruit by means of hydrolyzing the pectin fiber into D-polygalacturonic acid monomer. In this research, the longer of the fermentation period, the more polygalacturonase enzyme content in the cacao bean pulp.

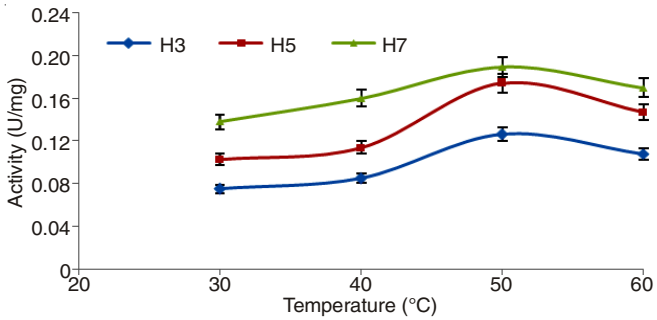

Fig. 4. Effect of temperature on the activity of polygalacturonase

Polygalacturonase that isolated after 7 days of fermentation (H7) produces the highest activity of 0.189 U/mg. Activity H7 samples is higher than polygalacturonase H3 and polygalacturonase H5, which possess activity of 0.015 U/mg and 0.063 U/mg, respectively. The optimum temperature reported on polygalacturonase *Penicillium viridicatum* (Rfc3 strains, fraction of Sephadex G50), polygalacturonase *Aspergillus fumigatus* Fres. MTCC 4163 and polygalacturonase *Burkholderia cepacia* are in the 50 °C [48,49,51]. In general, polygalacturonase enzyme has the optimum temperature in the range of 30-50 °C [44,45,52,53].

Purification of enzyme (precipitation and dialysis): Crude polygalacturonase obtained by extraction is purified by precipitation method using ammonium sulphate. The aim of this purification step is to separate the desired enzyme protein from other compounds. Precipitation of crude polygalacturonase cacao is carried out on the saturation level of 20-100 %. Activities of cacao polygalacturonase obtained from precipitation of ammonium sulphate is shown in Fig. 5. Precipitation process of enzyme using salt is a common process in isolation stage of protein or enzyme. Precipitation with ammonium sulphate at 80 % saturation levels produces the optimal of polygalacturonase activity of 0.318 U/mL (Fig. 5). The saturation level of 80 % indicates that the most of proteins in extracts of cacao pulp has been settled (salting out).

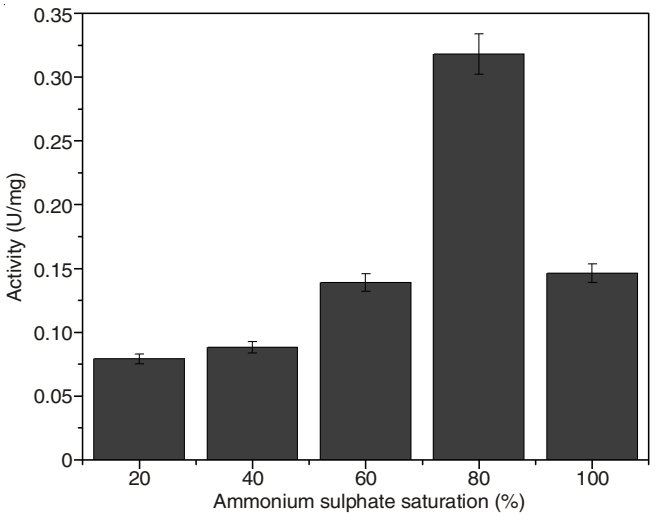

Fig. 5. Polygalacturonase activity with precipitation of ammonium sulphate

Generally, various polygalacturonases from different sources have been purified by pretreatment of ammonium sulfate precipitation. Polygalacturonase *Bacillus licheniformis* KIBGE-IB2 was precipitated with ammonium sulphate at 50 % saturation [45], polygalacturonase *Mangifera indica* cv. Dashehari has been precipitated at the saturation level of 30-90 % [54] and polygalacturonase from tomatoes was deposited at the saturation level of 60 % [55]. Because of salt content is relatively high in protein deposits, the dialysis process is then performed using D0655 dialysis bag. Dialysis is performed using phosphate buffer at pH 5.5. The results of purification step of cacao polygalacturonase is shown in Table-1.

Purification step in Table-1 shows that ammonium sulphate precipitation could increase the specific activity of polygalacturonase up to 0.138 U/mg and fold purification up to 1.76 times. While the result of dialysis process does not show significant effect on the specific activity and fold purification of polygalacturonase. The result of dialysis only gives different value with ammonium sulfate fraction of 0.001 and 0.01 for a specific activity and fold purification. To produce the polygalacturonase with higher purity, further purification step *i.e.* chromatography column should be conducted. Polygalacturonase *Mangifera indica* cv. Dashehari has been purified using DEAE cellulose and Sephadex G-200 and can improve the fold purification up to 26 times [54]. Polygalacturonase *T. harzianum* that purified using *Sephacryl* S200 and exo-polygalacturonase from *Oryza sativa* roots purified using Sephacryl S-ZOOHR were obtained with fold purification of 16 and 15 times [53,56].

Application (fermentation of cacao beans using polygalacturonase extracts): Polygalacturonase pulp extracted from cacao beans is then used in the fermentation of cacao beans. Fermentation of cacao beans is carried out using extracted polygalacturonase pulp of polygalacturonase H3, H5

TABLE-1
PURIFICATION STEP OF POLYGALACTURONASE OF CACAO BEANS PULP

Purification step	Total activity (Unit)	Total protein (mg)	Specific activity (Unit/mg)	Fold purification	Recovery (%)
Crude	15.724	87.197	0.180	1	100
$(NH_4)_2SO_4$ 80 %	2.432	7.650	0.318	1.76	15.53
Dialysis	1.024	3.21	0.319	1.77	6.54

and H7. Variation of fermentation time is equal to the variation of curing, those are 3, 5 and 7 days. The effect of fermentation is determined by the evaluation of organoleptic and catechin content in cacao beans and the result is shown in Tables 2 and 3.

TABLE-2
ORGANOLEPTIC OF FERMENTED CACAO POWDER POLYGALACTURONASE

Fermentation (days)	Organoleptics	
	Colour (%)	Flavour (%)
3	3.92[b]	3.91[b]
5	4.29[a]	3.93[b]
7	4.05[b]	4.05[a]

Means with different letters in the same column indicate significant differences according to DMRT test ($P \leq 0.05$).

TABLE-3
CATHECIN CONTENT OF FERMENTED CACAO POLYGALACTURONASE

Curing (days)	Fermentations (days)		
	3	5	7
3	6.45[a]	3.65[e]	5.74[b]
5	6.41[a]	5.57[b]	5.34[bc]
7	5.02[c]	4.43[d]	3.78[e]

Means with different letters in the same column indicate significant differences according to DMRT test ($P \leq 0.05$).

According to Table-2, the organoleptic colour and flavour is directly proportional to fermentation time. Fermentation is a decisive step for the quality of processed cacao. Colour formed as a result of oxidation of polyphenols into tannins that are insoluble during fermentation is responsible for the formation of flavour precursors in chocolate [57]. Chocolate that is produced from non-fermented beans possesses bitter taste and does not possess the chocolate aroma [33]. Fermentation of 7 days, which produce the highest scores for the desired aroma is formed on the phase of the fermentation time. Stark *et al.* [58] have reported that catechins, including epicatechin, catechin, procyanidin B2, B5 procyanidin and procyanidin C1 and amino acids are the compounds that are responsible for bitter and astringent taste of cacao. In this work, the catechin content decrease as the fermentation time increase as shown in Table-3. The content of polyphenols in the fresh cacao beans are ranged from 12-18 % [59]. The decrease is caused by the diffusion of catechin content of polyphenols on the cell fluids during fermentation and then undergoes oxidation process resulting condensed tannin which largely insoluble. The process is catalyzed by polyphenol oxidase [36]. Cacao beans from Ecuador has the highest amount of polyphenols, followed by cacao beans from Ghana and Trinidad [60]. Othman *et al.* [61] and Krawczyk [62] reported that the cacao beans from different countries have different polyphenol content and they found that the highest phenolic content are in the cacao beans originated from Sulawesi, Malaysia, Ghana and Ivory Coast. The content of flavonoids in chocolate is strongly influenced by the fermentation phase. The too much loss of flavonoids should be avoided and it must be controlled in every cacao processing.

Conclusion

In cacao, there is polygalacturonase that is responsible for fruit ripening and pulp softening during of curing beans. The activity of polygalacturonase increased with the longer curing time. Polygalacturonase showed optimum activity at pH 5.5 and temperature of 50 °C. Purification step of polygalacturonase in this research did not show significant different of fold purification yet, thus it is necessary to perform further purification. Polygalacturonase from the pulp of cacao beans could potentially be applied to fermentation of cacao beans. DMRT test based on the level of ($P \leq 0.05$) produces significant different in organoleptic and content of catechins from fermentation of beans based on polygalacturonase extract.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the financial support from Ministry of Education and Culture, Indonesia, under "MP3EI" research.

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