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Potential applications of enzymes on the extraction of vitexin from dried Mas Cotek leaves

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Mas Cotek or *Ficus deltoidea* var. *deltoidea* leaves extracts contain various beneficial properties such as antidiabetic, antihypoglycemic and antioxidant activities, which can be enzymatically extracted in water. Vitexin is one of the antioxidants and remarkable compound of flavone C-glycosides found in Mas Cotek leaves. Cellulase is one of the essential enzymes applied to hydrolyze cellulose into fermentable sugars and therefore, this enzyme was used to extract vitexin in this study. Single parameter approach was used to determine factors influencing the enzymatic-mediated extraction. These factors were enzyme concentration, sample-to-water ratio, temperature, incubation time and agitation. The maximum yield of vitexin found was 0.547% under the optimum conditions: sample-to-water ratio 1:10 g/mL, enzyme concentration 0.4%, incubation time of 240 min, temperature of 50 ± 0.02°C and stirring speed of 200 rpm. The highest amount of reducing sugar obtained was 4.039 mg/mL with the use of 0.4% cellulose. A correlation was also observed between the yield of vitexin and reducing sugars.

Key words: Cellulose, enzymatic assisted extraction, vitexin, reducing sugars, cellulose, Mas Cotek, *Ficus deltoidea* var. *deltoidea*, antioxidants.

INTRODUCTION

Mas Cotek or *Ficus deltoidea* var. *deltoidea* leaves extracts contain various beneficial properties such as anti-osteoporotic properties, anti-bacterial activity (Abdullah et al., 2018), and antidiabetic activity (Misbah et al., 2013; Papitha et al., 2018). Also, Mas Cotek extracts are found to be natural anti-oxidants (Sin et al., 2017). However, Mas cotek extraction is a challenge as it has low efficiency of aqueous extraction. In order to overcome the low efficiency of aqueous extraction process including potential loss of bioactive compounds, high energy and time consuming (Azwanida, 2015), the use of hydrolytic and cell-wall degrading enzymes as

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eco-friendly alternatives (Sharma and Gupta, 2006) are introduced which help to release the active compounds in the plant cell wall and increase the extraction yield. Rosenthal et al. (1996) had listed some advantages regarding the use of enzymatic extraction in terms of lowering the investment cost and energy requirement. The development of this method is also to fulfil the needs towards a clean environment especially the increasing awareness about volatile organic compounds (VOCs) caused by solvent emissions and safety concerns. In this study, cellulase enzyme was used to catalyse the hydrolysis of cell wall in Mas Cotek leaves.

Cellulase (EC 3.2.1.4) commonly used in the food industry to catalyse a wide variety of hydrolytic reactions and a high percentage act on cell wall polymers improving extraction yield of compounds, juices, oils and sugars (Salina et al., 2013). It is used for the extraction of oil from some oil-bearing seeds/fruits has been attempted in the laboratory or at the pilot industrial scale level (Domínguez et al., 1993; Rosenthal et al., 2001). The high cost of cellulase application had become the main limitation in the industrial processes, but with latest biotechnology advances it is already possible to obtain enzymatic formulations with lower costs and better quality (Zuniga et al., 2003).

Trichoderma reesei is a mesophilic and filamentous fungus. It is one of the good sources of cellulase enzymes. It produces 2-exo-β-D-glucanases, 5-endo-β-D-glucanases and 2-β-glucosidases. The whole hydrolysis process of cellulose can be divided into primary hydrolysis, that involves depolymerisation step by the action of endo-β-D-glucanase and exo-β-D-glucanase on the solid surface of substrate releasing soluble sugars and secondary hydrolysis, which involved the hydrolysis of cellobiose to glucose by β-glucosidase (Binod et al., 2011).

Cellulase is one of the thermostable enzyme that have several advantages including higher specific activity and higher stability which improve the overall hydrolytic performance (Viikari et al., 2007). As the catalytic efficiencies of enzymes improved, the cost of hydrolysis will be reduced eventually by enabling lower enzyme dosage (Dashtban et al., 2009). Based on the cellulase enzyme action, in this study, the enzymatic-assisted extraction was carried out to investigate the capability of cellulase enzyme in the optimum release of bioactive compounds (vitexin and isovitexin) from Mas Cotek leaves extracts, assisting the conventional aqueous extraction.

Antioxidant flavonoids like vitexin and isovitexin can be found in Agati et al. (2012), located in the intercellular plant cell. Chloroplast is a plant organelle that is responsible for the photosynthesis activity of a plant cell. It also carried out the synthesis of lipids and pigments and performed starch metabolism process. Beside the good health effects to human consumption, antioxidant flavonoids may effectively control the growth of plant cell and regulate the development of the whole plant and individual organs (Brunetti et al., 2013). As the leaves cell wall are predominantly made up of cellulose, hemicellulose and lignin, cellulase enzymes will assist in the digestion of lignin and bind to their cellulose substrate to generate glucose products in the extracts. The digestion of cell wall also released the intercellular components and active compounds into the extracts. Therefore, the objectives of this study were to investigate the effects of cellulase concentration and sample-to-water ratio on the extraction of vitexin compound from dried Mas Cotek leaves. Furthermore, to analyse active compounds and the amount of glucose released from the reaction of cellulase enzyme and plant cell activity were also determined.

MATERIALS AND METHODS

Chemicals and plant material preparation

Cellulase from *T. reesei* ATCC 26921 (lyophilized powder, ≥1 unit/mg solid) was purchased from Sigma-Aldrich (Missouri, USA). Vitexin standard (CODE: 1232 S) was ≥99% purity purchased from Extrasynthese (Genay Cedex, France). HPLC grade of methanol, formic acid (98 to 100%), acetonitrile, 50 mM acetate buffer and 0.1 M hydrochloric acid were purchased from Merck (Darmstadt, Germany). Dried leaves of Mas Cotek (*F. deltoidea* var. *deltoidea,* 4 kg) were obtained locally from Muadzam Shah, Pahang, Malaysia. Samples were further dried, cleaned and ground into small particles and kept in a freezer at -4°C until experiments were done. The uniformity of particle size was determined by sieving the leaves powder through an 850 µm (ASTM No. 20, 20 Mesh) standard sample sieve and a sieve shaker (Fritsch, Germany). Figure 1 shows the flow chart for the whole experimental work of the present study.

Cellulase-mediated extraction

Cellulase was quantified accurately and dispersed in deionized water to obtain enzyme solutions at various concentrations of 0.1 to 0.6% (g E/g substrate). Leaf powder samples of Mas Cotek were immersed in a mixture of deionized water and 50 mM acetate buffer (Merck, Darmstadt, Germany) at different sample-to-water ratio of 1:10, 1:20 and 1:30 g/mL (Badr and Sitohy, 1992; Ghose, 1987; Murashima et al., 2003). The solution pH was adjusted to 4.8 to 5.0 using certain amount of 0.1 M hydrochloric acid (Merck, Darmstadt, Germany). The mixture was put together in a 500 mL flask and incubated in a water bath (BS-21, Lab. Companion, Jeio Tech, Seoul, Korea) at 50°C with constant shaking at 200 rpm for 8 h. Cellulase solution was added after the sample solution achieved the optimum conditions for the cellulase to take action. A control sample (without cellulase addition) was prepared under the same condition in each ratio studied.

Five millilitres of samples were taken out in two separate test tubes at every 60 min time interval for active compound analysis and reducing sugar determination as will be described subsequently. Then, the samples taken were boiled at 100°C for 5 min to allow the denaturation of enzyme in the sample solution. After the enzyme deactivated, the extracts were centrifuged. Then, after 8 h extraction, the remaining solution in the beaker was filtered. The obtained residue was collected into another beaker

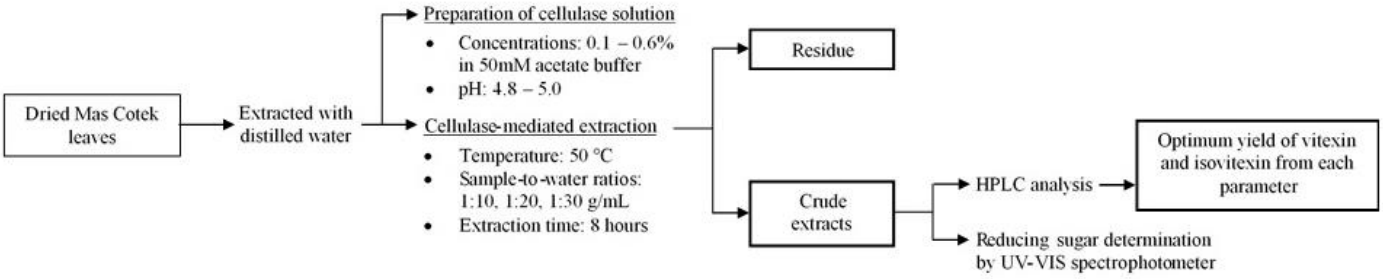

Figure 1. Flow chart for the whole experimental work of enzymatic-mediated extraction of vitexin from Mas Cotek leaves.

while the filtered solvent was discarded. The same proportion of distilled water was added into the sample residue according to the initial ratio. The aqueous extraction (without the enzyme) was done at 100°C for another 4 h. The extract was also sampled at every hour for further analysis. When the extraction process was accomplished, samples were cooled to room temperature and centrifuged and analysed using HPLC (Agilent, CA, USA) and UV-VIS Spectrophotometer (U-1800, Hitachi, Tokyo, Japan) for active compound and reducing sugar determination, respectively.

Reducing sugar determination

Glucose concentration was determined using 1% (w/v) solution of dinitrosalicyclic acid (DNS) based on Miller (1959) method. The sample taken every hour from enzymatic-mediated extraction of Mas Cotek leaves was centrifuged and appropriately diluted with deionized water up to 10 dilution factor. The diluted sample (1 mL) was mixed with 1 mL of DNS reagent and heated for 5 min on a boiling water bath. The absorbance of the cooled solution was measured at 575 nm against a blank that had been prepared using deionized water instead of the sample. The absorbance was converted to glucose concentration using a standard curve.

The standard curve had been prepared using glucose solutions of known concentrations (Shu et al., 2013; Zhang et al., 2009). One unit of cellulase will liberate 1.0 µmole of glucose from cellulose in 1 h at pH 5.0 and 37°C.

HPLC analysis

Samples of Mas Cotek leaves extracts from each extraction method were first analysed using HPLC once the extraction was done. Vitexin compound in this fine extract were identified and quantified by using a reversed-phase Phenomenex (Torrance, CA, USA) column (Prodigy, C_{18}, 5 µ, 250 × 4.60 mm i.d.). Calibrating curves of vitexin (ranging between 0.01 and 0.10 mg/ml) were prepared by diluting stock solution with the ultrapure water (Merck Millipore, USA). The HPLC consisted of a computer-controlled system with G1379A Degasser, G1311A QuatPump and G1321A fluorescence detector. Data acquisition was performed by Agilent ChemStation B.04.03 (Murashima et al., 2003). The column temperature was maintained at 25°C. Isocratic HPLC elution consisted of [A] 1.0% formic acid and water and [B] methanol were used. The mobile phases were filtered through a 0.2 µm nylon membrane and degassed using an ultrasonic bath (Crest Ultrasonics, Trenton, New Jersey) prior to use. The flow rate was set at 1.0 mL/min. Fluorescence detection was conducted at 335 nm wavelength. The concentration of compound in Mas Cotek leaves extracts were calculated by using the calibration equation of reference compounds. Based on the obtained concentration, actual percentage weight (%w/w) of vitexin was calculated by using the following formula (Equation 1):

Vitexin

$$\left(\%\frac{w}{w}\right)=\frac{[(Conc\,of\,compound\,from\,HPLC)(Volume\,of\,solution,L)(Dilution\,Factor)]}{(Weight\,of\,solute,mg)}\times 100 \quad (1)$$

Statistical analysis

The observations were replicated thrice for each parameter. Mean values were grouped and standard error (SE) was calculated. Statistical analysis was carried out using analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Cellulase-mediated extraction

In the enzymatic-assisted or cellulase-assisted or mediated extraction (EnAE) of Mas Cotek leaves, 18 samples with replicate were prepared consisting of 6 different concentrations of hydrolytic enzyme (cellulase) ranging from 0.1 to 0.6% (g E/g substrate) in different sample-to-water ratios of 1:10, 1:20 and 1:30 g/mL. The solution pH was adjusted at 4.8 ± 0.05 and the solution temperature was kept constant at 50 ± 0.02°C throughout the experiment in a shaking water bath (200 rpm). Both pH and temperature were in the optimum conditions for cellulase enzyme to take action in the extraction process.

Effect of enzyme concentration and sample to water ratio

Cellulase enzyme concentration of 0.4% (g E/g substrate) gave the highest vitexin value of 0.547% with sample-to-water ratio of 1:10 g/mL at the 4th hour of extraction (Figure 2). This is where the enzyme activity reaches the peak and the catalytic efficiency is also the highest (Duan et al., 2015).

Compared to the highest vitexin yield obtained for control sample, the highest yield of vitexin from EnAE

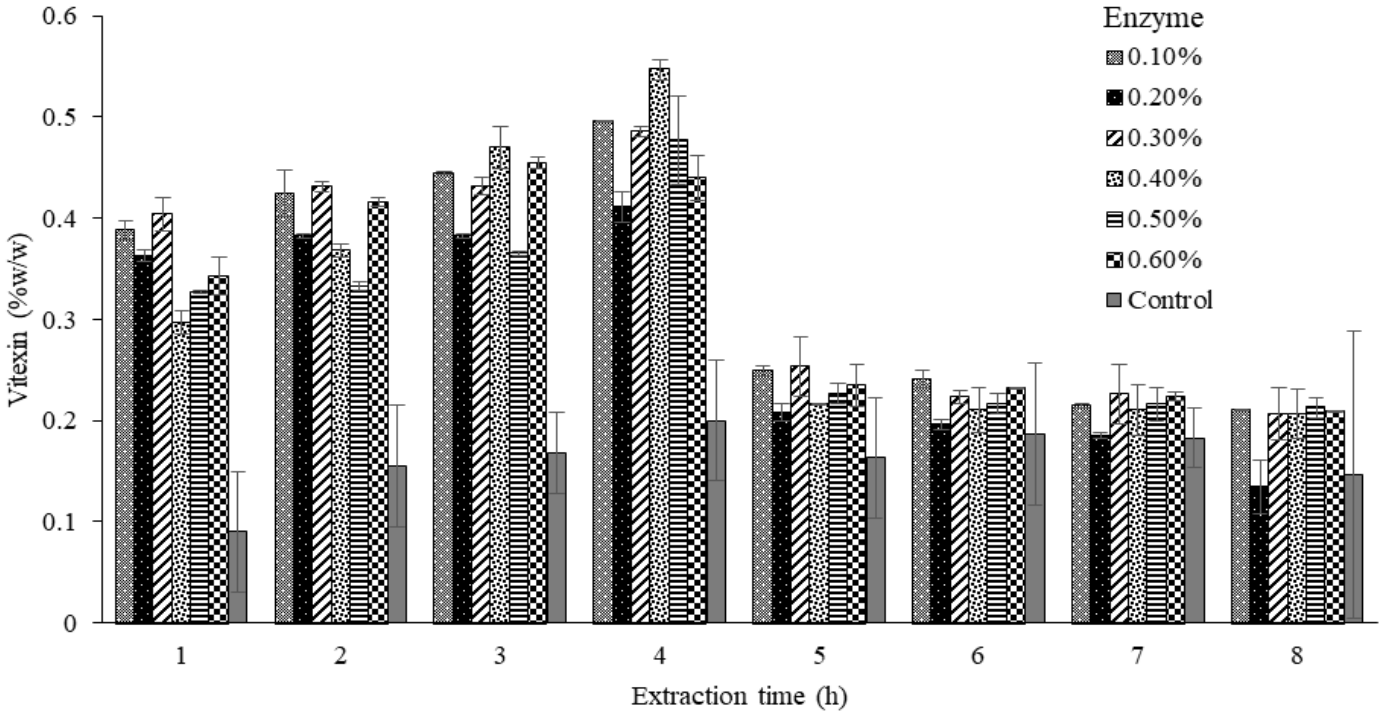

Figure 2. The effects of different cellulase concentrations (0.1, 0.2, 0.3, 0.4, 0.5, 0.6%) in the yield of vitexin compound from Mas Cotek leaves extract in sample-to-water ratio of 1:10 g/mL compared to control sample (50°C; pH 4.8; 8 h; n=6).

differed by 93% increase in the same ratio. The higher yield of vitexin compound from this method is due to the ability of hydrolytic enzyme to degrade or disrupt cell walls and membranes, thus enabling better release and more efficient extraction of bioactive compounds (Puri et al., 2012). It is also observed that at all concentrations applied the yield of vitexin drops for about 50 to 70% from 4 to 5th hour of extraction in this ratio. Parameter of time gives important effects in hydrolytic enzymes activity.

The longer the hydrolysis time, the more sufficient was the degeneration of Mas Cotek plant cells, and the more complete was the enzymatic hydrolysis. Thus, as the hydrolysis time increased, the quality of compounds will be decreased. This pattern is however consistent through all cellulase concentrations from 0.1 to 0.6% where from the 1st to 4th hour of extraction, the yield appeared to incline gradually but levelled off at the 5th hour, leaving a uniform trend until the end of THE experiment. Therefore, 4 h was taken as the best time of enzymatic mediated extraction in 1:10 (g/mL) sample-to-water ratio. Cellulase loading with 0.4% gave the best performance in this ratio. Compared to the lower concentrations, the increase in the cellulase dosage to 0.4% could be more helpful in the extraction of vitexin compounds in this ratio. However, as cellulase activity decreased during the hydrolysis process, the falloff pattern in the graph can be observed from the 4th to the end of extraction time. This deactivation is partially caused by the irreversible adsorption of cellulase on cellulose; in this study referred to as plant cell. Also, increasing the cellulase concentration to 0.6% did not contribute to the higher yield of vitexin in this ratio.

The second batch of EnAE was studied using 1:20 (g/mL) sample-to-water ratio as shown in Figure 3. In this ratio, cellulase concentration of 0.1% gave the highest yield of vitexin (0.530%) at the 4th hour of extraction.

A steep increase was observed in the pattern of yield from the 1st to 4th hour of extraction at all concentrations and then decreased gradually at the 5th hour until the 8th hour of extraction process. The same pattern was observed in the previous ratio as the recovery of active compounds from the plant sample influenced the extraction time and reflects the conflicting actions of solubilization and analyte degradation by oxidation with an increasing time. It was also found that increasing amounts of cellulase concentration to 0.6% lowered the yield of vitexin compared to other concentrations in this ratio. Other than the chances of phenolic compound oxidation at longer extraction time, at some point, the hydrolysis process is more adequate with the increasing of enzyme amount and caused limited amount of substrates. In average, the yield of vitexin in 1:20 (g/mL) ratio decreased by 20% compared to the average yield in 1:10 (g/mL) ratio.

The low yield of vitexin from EnAE in enzyme concentration of 0.6% at 7 and 8th hour of extraction

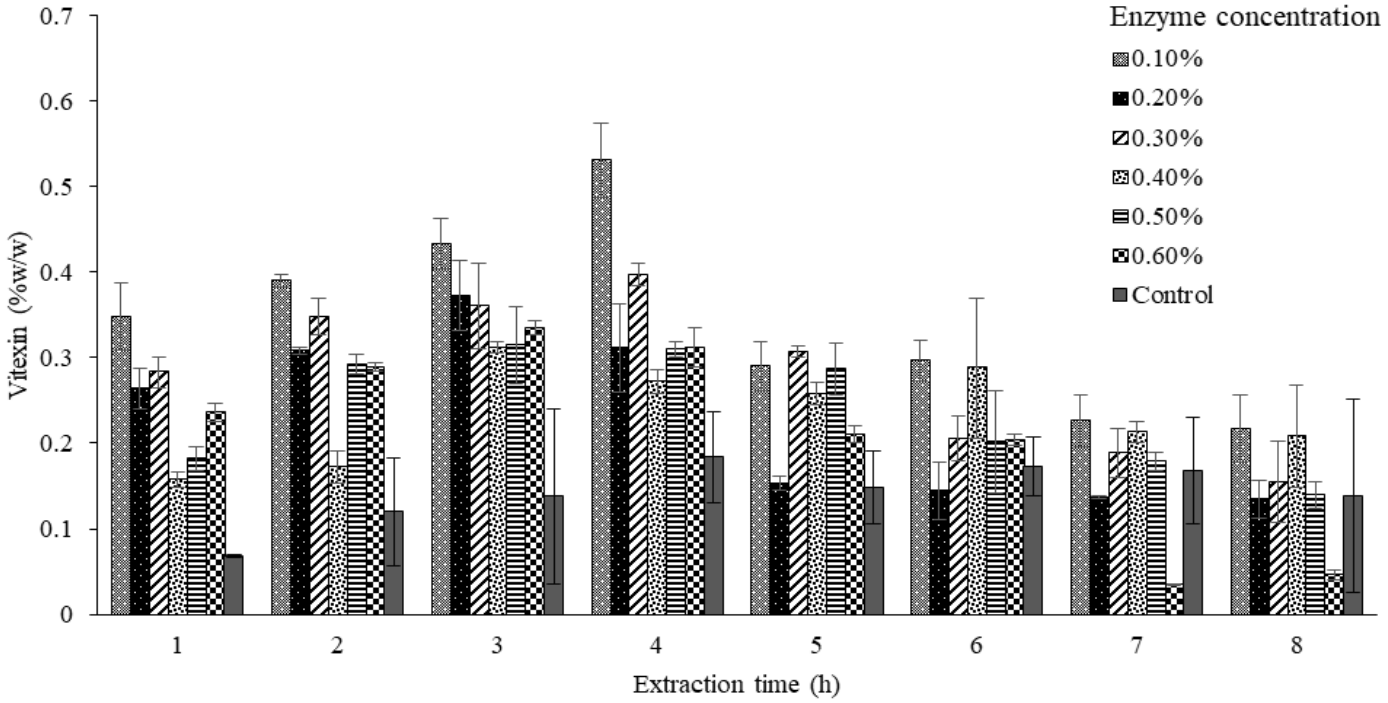

Figure 3. The effects of different cellulase concentrations (0.1, 0.2, 0.3, 0.4, 0.5, 0.6%) in the yield of vitexin compound from Mas Cotek leaves extract in sample-to-water ratio of 1:20 g/mL compare to control sample (50°C; pH 4.8; 8 h; n=6).

compared to the control sample were also observed in this ratio with 0.79 and 0.67-fold decreased, respectively. There are some factors that might affect the low yield from enzymatic-assisted extraction of vitexin compound. As more cellulosic cell wall digested by cellulase enzyme, more compounds will migrate into the surrounding medium. The released of unwanted and inactive compounds will cause the low detection of compound of interest (Patindol et al., 2007; Zhao et al., 2016).

The last batch of EnAE was carried out using 1:30 (g/mL) sample-to-water ratio as shown in Figure 4. The highest yield of vitexin (0.521%) was recorded from 0.2% cellulase concentration at the 3rd hour of extraction. This was however contrary to the findings obtained in the earlier two tested ratios. However, in respect of average yield, the value of vitexin from ratio 1:30 (g/mL) was lower than the value of vitexin from ratio 1:10 (g/mL) and the decrease was recorded by 11%. The percentage yield of vitexin dropped significantly with further increase in sample-to-water ratio, indicating that the reduced vitexin yield is likely to be resulted from the lowest solid content. As the highest average of vitexin obtained with the lowest ratio (1:10 g/mL) with the highest solid content, this ratio is the best choice and more acceptable due to lesser water requirement.

At the 7 and 8th hour of extraction, the yield of vitexin from 0.3 and 0.2% cellulase concentration observed were 67 and 11%, respectively. During the enzymatic-mediated extraction process, chemical reactions might happen on the complicated chemical composition in vitexin compound. Flavonoids (with phenolic hydroxyl group) and triterpenoids (with carboxyl group) present in the Mas Cotek leaves extracts may increase acidity of the extraction solution as it was already at pH 4.8. Under acidic condition, there may be a series of chemical reactions to cleave the glucosidic bonds in vitexin, resulting in different extraction values during the process at different sample-to-water ratio and cellulase concentrations (Bansal et al., 2009).

In terms of monetary aspect, increasing the dosage of cellulase enzyme in the process to a certain extent can enhance the yield and rate of the hydrolysis, but would significantly increase the cost of the process (Sun and Cheng, 2002). Cellulase concentration of 0.4% was chosen as the best concentration in assisting the extraction of vitexin compound from Mas Cotek leaves sample. Different sample-to-water ratio gave different effects on the hydrolysis of plant cells at different cellulase concentrations. The differences might be due to the substrate (sample) as main factor, which caused the increase in yield and reaction rate at low substrate level and substrate inhibition at higher substrate concentration that leads to the low rate of hydrolysis.

When compared with the control sample (without cellulase-mediated), it was observed that even with small ranges of cellulase concentration, there are small differences in the optimum extraction time from both methods. The optimum time for control sample can be

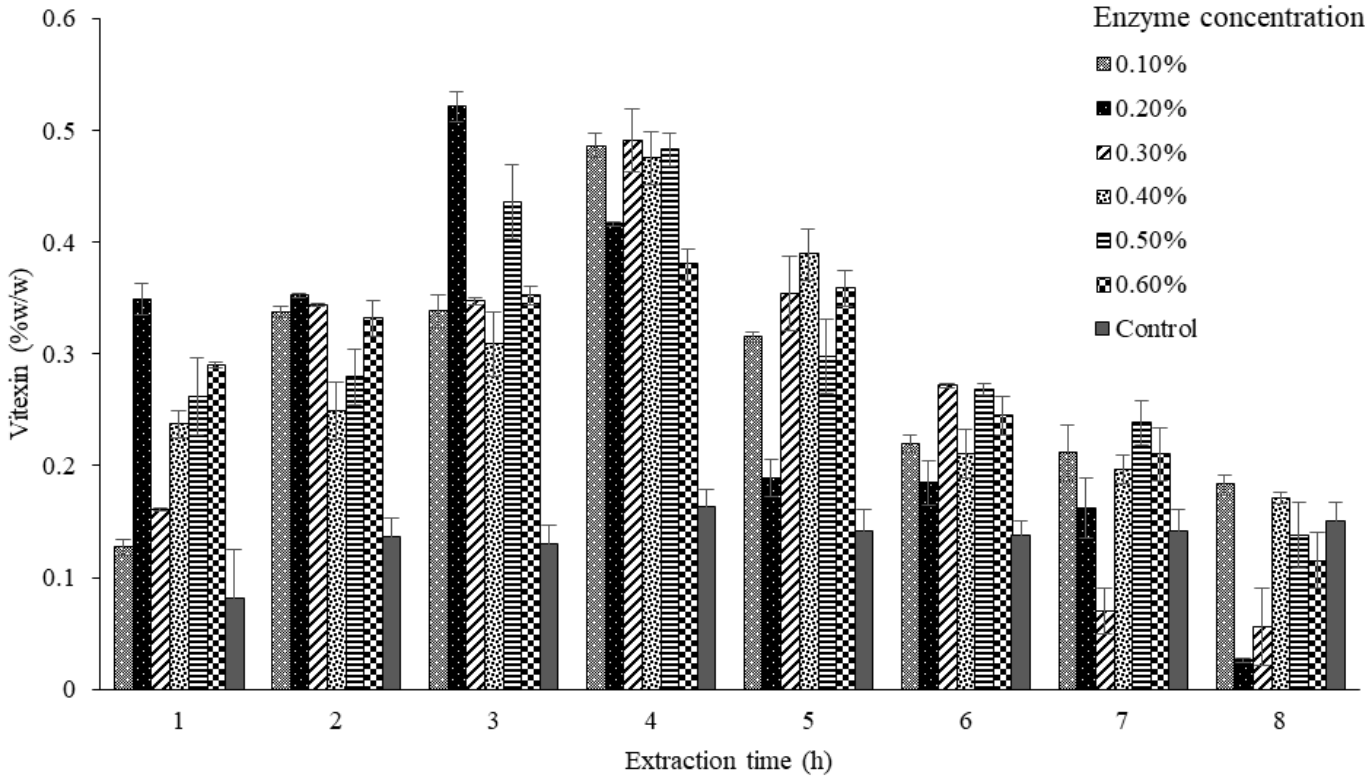

Figure 4. The effects of different cellulase concentrations (0.1, 0.2, 0.3, 0.4, 0.5, 0.6%) in the yield of vitexin compound from Mas Cotek leaves extract in sample-to-water ratio of 1:30 g/mL compare to control sample (50°C; pH 4.8; 8 h; n=6).

observed at the 4 and 5th hour of extraction while the 3th or 4th hour can be observed from EnAE. It showed that the use of enzyme significantly increased the rate of extraction by promoting the release of vitexin compound at shorter time ranges. The significance of using small ranges of cellulase concentration also can be justified with the unbalanced distribution of active compounds yield. This might be seen as a good finding because the used of more amount of enzyme will be more costly in terms of economic value.

The significant differences in the yield of vitexin achieved from enzymatic-assisted extraction using cellulase enzyme compared to yield obtained from control samples was the result of plant cellulose hydrolysis. Cellulose exists at nanometer scale in native plant cell walls as a microfibril network embedded in matrices of hemicelluloses, pectins and lignin (Liu et al., 2010). Cellulase is a class of enzyme that has the synergistic reaction to efficiently break down this chemically and physically complex plant cell wall polymeric network. This type of enzyme however plays different roles cooperatively in the hydrolysis of cellulose where some cleave to the cellulose chain from the middle into fragments containing 4 to 5 glucoses, some breakdown these fragments into smaller units of two glucoses, and some finally turn these small units into single glucose (Kumar et al., 2008). When more cellulose turns into simple glucose, more bioactive compound contained in the plant cell wall will be released into the surrounding medium without the aid of high temperature and energy. In summary, compared to control sample, cellulase-assisted extraction can break the cell wall under mild conditions to accelerate the dissolution of active compounds, which also reduce the extraction time, avoid the heat damage to compounds, improve the extraction yield of compounds and reduce the use of organic solvents and costs (Cao et al., 2014).

Glucose concentration liberated from sample

Another approach to indicate the efficiency of cellulase enzyme to cellulose degradation during the extraction is to calculate the amount of sugar released at every hour the experiment was conducted. Enzymatic hydrolysis provides a method to convert cellulose into glucose at high yields without reducing sugar product degradation. Cellulase enzyme used converted the hydrolysed cellulosic cell wall of Mas Cotek leaves into small particles of glucose. A good recovery of active compounds indicates that the Mas Cotek cell wall was more effectively degraded by the cellulase enzyme,

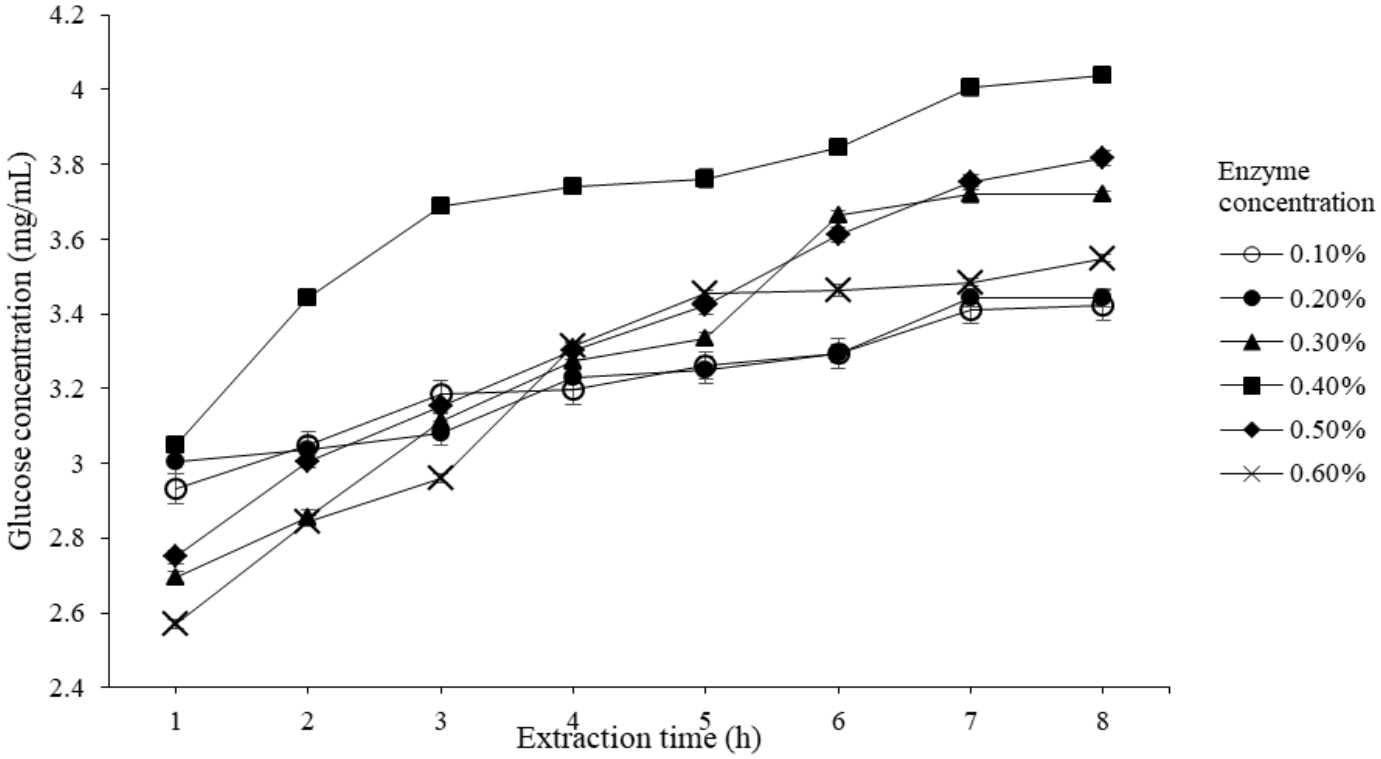

Figure 5. Glucose concentration determined in Mas Cotek leaves extract with different cellulase concentrations of 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6% (50°C; pH 4.8; 8 h; n=6).

leading to the release of most of the compounds and other materials enmeshed within the cells into the aqueous medium. Therefore, the extracted solutions were sampled every hour to measure the concentration of glucose content in each sample. Figure 5 shows the glucose content in EnAE.

The reducing sugars released in all enzyme concentrations in this study were always determined by linear regression as glucose equivalents. The highest concentration of glucose was 4.039 mg/mL recorded from 0.4% cellulase concentration at the 8th hour extraction. The graph shows an increasing trend from 1st to 8th hour of extraction for all cellulase concentration. It was also observed that 0.4% cellulase concentration gave the highest glucose concentration compared to other concentration by 6%. The reducing sugars of the treated samples increase with cellulase concentration from 0.1 to 0.4%. However, a decrease is verified at higher cellulase concentration (0.5 and 0.6%) due to the removal of the broken chain by excess of enzyme and less accessibility of cellulose (Cavaco-Paulo and Almeida, 1994). Thus, any further increment in the cellulase concentration of more than 0.4% may not favour the release of reducing sugar into the extracts.

Other cellulase concentrations gave slight difference in glucose concentration throughout the extraction. The high glucose concentration in 0.4% cellulase concentration supported the findings in EnAE as more compounds were extracted in the same cellulase concentration compared to others. Enzymatic breakdown of cellulose to reducing sugars was done by enzymatic hydrolysis of the glucosidic bond. As more cellulose cell wall were digested by the enzyme, more active compounds escaped to the surrounding medium. Also, more production of glucose was determined in the sample. These findings were contributed by the mechanism of hydrolysis by cellulase enzyme complex. Beta-glucosidase hydrolyzes cellobiose, a disaccharide of beta-glucose and the product of cellulose hydrolysis by cellulase, into glucose (Sousa Jr. et al., 2011).

Different cellulase concentrations gave different amount of glucose production. Even though the range of concentrations used was small, there are a lot of factors that may contribute to these differences. In concentrations other than 0.4%, pectin hinders the hydrolysis of cellulose in the Mas Cotek leaves cell wall, and must be hydrolysed for cellulase to further hydrolyze other cell wall polysaccharides. Steric hindrance of cellulose hydrolysis by pectin is supported by the cell wall model for flowering plants proposed by Carpita and Gibeaut (1993), which stated that a pectin matrix surrounds cellulose fibers coated with xyloglucan, a hemicellulose. It was also found that the hydrolysis is thus a two-substrate reaction involving both cellulose and

water. While there has been considerable interest in the cellulose-enzyme interactions as well as on the cellulose composition, limited attention has been paid to the role of water in the process. During the enzymatic hydrolysis, cellulose-water interactions occurred. As the action of the enzyme system is to breakdown and loosen the cellulose, more water will be introduced into the structure and provided better access for the enzymes.

To date, there are no specific data on the use of dinitrosalycilic acid (DNS) colorimetric method to determine the amount of reducing sugars released as a result of hydrolysis of cellulase-mediated extraction of Mas Cotek leaves. In the present study, the obtained optimum values of glucose concentrations and the yield of active compounds was complementary to one another. In this process, cellulase enzyme (endoglucanse, exoglucanase and β-glucosidase) was used to convert cellulose into sugars. Due to the specific role of enzyme, for example while endoglucanase cleaves the internal bonds of cellulose, the exoglucanase and β-glucosidase give complete conversion of cellulose into sugars, a mixture of these enzymes is required (endoglucanases and exoglucanses for the hydrolysis of cellulose into cellobiose and β-glucosidases for the conversion of cellobiose to final product of glucose) (Bhaumik and Dhepe, 2005). It is useful to demonstrate the relative contribution of endoglucanase, exoglucanases and β-glucosidases during a time course hydrolysis of this cellulosic substrate (Silveira et al., 2014). Furthermore, this procedure gave a new insight into the synergy that exists among different components of the cellulase system.

Conclusion

In this study, cellulase enzyme at six different concentrations was tested to improve the release of vitexin yield from Mas Cotek leaves. The optimal conditions regarding the enzyme concentration and sample-to-water ratio were identified. The yield of vitexin compound was much higher in cellulase-mediated extracts of Mas Cotek leaves compared to control. This creates new opportunities for potential applications of this enzyme-assisted process. However, the long extraction time limits the economic potential. Further improvement might be required by coupling cellulase hydrolysis to other techniques such as ultrasonic extraction.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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