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RESEARCH ARTICLE

Enhancement and bioavailability of phenolic content in *Kappaphycus alvarezii* through solid substrate fermentation

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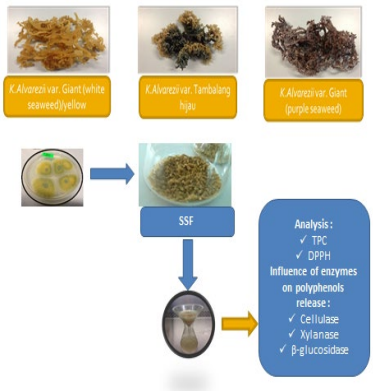
Graphical abstract



Abstract

Known as carrageenan, *Kappaphycus alvarezii* is a popular species of red edible seaweed which is mainly cultivated for its hydrocolloids. *K. alvarezii* is a good source of phenolics which are considered to be natural antioxidants that promote myriads of health benefits. Extraction of phenolic compounds using conventional solvent extraction may result in hazards and toxicological effect and low yield of bound phenolics. Therefore, this study was conducted to increase the release of bound phenolics through solid substrate fermentation. In the present study, three different varieties of *K. alvarezii* were fermented with *Aspergillus oryzae* through solid substrate fermentation (SSF) at 30 ℃ for 2 to 6 days. The changes of total phenolic content and antioxidant activity due to the effects of SSF were investigated. Results obtained revealed that the highest total phenolic content for green (10.022 mg GAE/g) and purple (4.037 mg GAE/g) varieties of *K.alvarezii* were on the fourth day of fermentation while for yellow (4.479 mg GAE/g) variety was on the sixth day of fermentation. A remarkable enhancement of antioxidant activity was discovered through DPPH radical scavenging activity on the fourth day of fermentation for all varieties of *K. alvarezii* tested. Total phenolic content also showed a significant correlation with antioxidant activity of fermented seaweed. Cellulase, xylanase, and β-glucosidase enzymes produced by *A.oryzae* during SSF were also investigated to see the relationship with the release of phenolic compounds after fermentation. The highest cellulase activity was observed on the fourth day of fermentation. The similar observation was also found for xylanase enzyme in yellow and purple varieties of *K. alvarezii*, except for green variety where the highest xylanase activity was on the sixth day of fermentation. The maximum β-glucosidase activity in yellow and green varieties was observed on the fourth day of fermentation while purple variety exhibited the maximum β-glucosidase activity on the sixth day. There are positive correlation between the enzymes studied and the polyphenols content in all varieties of *K. alvarezii*. This study demonstrated that fermented *K. alvarezii* contained more phenolic content compared to non-fermented seaweed.

Keywords: *Kappaphycus alvarezii, Aspergillus oryzae*, total phenolic content, DPPH radical scavenging activity, enzyme

INTRODUCTION

Seaweeds are rich in polysaccharides, minerals, proteins, vitamin, dietary fibers, and phenolics. *Kappaphycus alvarezii* is an economically important red seaweed which is cultivated in many tropical countries including Malaysia. Locals usually consume seaweed extract which is prepared by using hot water extraction. Unfortunately, this conventional method may result in the loss of some nutritional properties due to high temperature used during preparation.

Phenolic content in seaweed contributes to its antioxidative property and lipid peroxidation activity (Moubayed *et al.,* 2017; Yuan *et al.,* 2005). Phenolic compounds present in plants can be divided into free and bound form. Free phenolic compounds are available in the vacuoles of plant cells, while bound phenolic compounds are linked to cell wall structures through covalent bonds (Yadav *et al.,* 2013). Organic solvent extraction can be applied to extract phenolic compounds, but the disadvantage of this method is the low yield of bound phenolics. Fermentation has been justified as one of the best environmental-friendly technique to gain high quality phenolic extracts (Martin *et al.,* 2011). Previously, total phenolic contents in *K. alvarezii* extracted by using methanol was reported in the range of 30–49.04 mg GAE/100g sample while DPPH free radical scavenging activity (EC_{50}) was reported between 18.2–60.93 mg/ml (Ling *et al.,* 2013).

Solid substrate fermentation (SSF) can be applied to enhance antioxidant activity and phenolics bioavailability in solid substrates by increasing its total phenolic content (Dey *et al.,* 2016). Phenolic compounds in bound forms will be released through fermentation process. The constituents of seaweed are altered during the fermentation by the action of microbial enzymes produced, thereby affecting their structure, bioactivity, and bioavailability. SSF was

reported to increase the phenolic compound in black bean (Lee *et al.,* 2005), soybean (Lin *et al.,* 2006), cranberry pomace (Vattem and Shetty, 2002), and brown algae (Eom *et al.,* 2011).

During SSF, various hydrolytic enzymes could be secreted directly by microorganisms from solid substrate and simultaneously be utilized to release bound phenolic compounds. In this study, *Aspergillus oryzae* was used because of high activity of hydrolytic enzymes secreted. Other than that, they have quick development and high resistance to contamination. These are the key reasons why *A. oryzae* is broadly utilized in soy sauce fermentation industry (Liang *et al.,* 2009). Furthermore, since *A. oryzae* lacks expressed sequence tags genes for the production of aflatoxin, it is generally regarded as safe to be used in food production (Machida *et al.,* 2008). A few hydrolyzing enzymes are produced during fermentation with *A. oryzae* such as xylanase, cellulase, β-glucosidase, and pectinase (Huynh *et al.,* 2014). Therefore, in this study, *K. alvarezii* was fermented by using *A. oryzae*, with the aim to increase phenolic content and antioxidant activity. Cellulase, xylanase, and β-glucosidase enzymes produced during SSF were also investigated.

EXPERIMENTAL

Sample preparation

Kappaphycus alvarezii were purchased from Tawau, Sabah. The samples were rinsed with tap water and soaked in two volumes of water for 1 h. The samples were cut into smaller pieces before being dried for five days. In this study, three varieties of *K. alvarezii* were studied which were yellow, purple, and green. Different drying methods were employed to obtain different varieties of seaweed. A "sauna-dried" method was performed for yellow variety where a clear plastic bag was used to dry the seaweed under the sun until the seaweed turn into yellow-white color. To produce purple variety, the seaweed was hung under the sun until it turns into purplish color. Green variety was produced by drying under the sun. The drying process was conducted until constant weight was obtained. The dried samples were then blended by using Waring blender. The samples were kept at cold temperature in airtight bottles.

Inoculum preparation

Food grade *A. oryzae* was supplied by MARDI culture collection, Serdang, Selangor. The strain was cultured on PDA plates for 4 days at 30 °C. A hockey stick was used to collect the spores by evenly poured 100 ml of sterile distilled water on 4 PDA plates containing 4 days old culture. Whatman filter paper No. 1 was used to filter the suspended fungal cultures. The filtrate obtained was used as inoculum and stored at 4 °C.

Solid substrate fermentation (SSF)

Solid state fermentation was conducted according to the modified method by Ellaiah *et al.* (2002). Sucrose was used as a supplementary carbon source while yeast extract was used as a supplementary nitrogen source. Sterilized substrates with 70 % initial moisture content was inoculated with 1 ml of inoculum and incubated at 30 °C for 2 to 6 days. Samples were harvested by adding 100 ml of distilled water into shake flasks containing biomass. The mixture was agitated thoroughly on a rotary shaker for 1 hour at 180 rpm. The whole content was filtered, and then centrifuged at 8000 rpm for 10 minutes. The clear supernatant was used as the fermented seaweed extract.

Extraction of enzyme mixtures

Extraction of enzymes was done according to Botella *et al.* (2005). The whole content of fermented seaweed (approximately 10g working volume) was added with 50 ml of distilled water and then was agitated for 30 minutes at 120 rpm by using rotary shaker at room temperature. The content of fermented matter was then centrifuged at 8000 rpm for 10 minutes. The supernatant obtained was filtered by using Whatman filter paper No. 1 and was then kept at -20 °C for further enzyme analysis.

Determination of total phenolic content

Determination of total phenolic content was conducted based on the method described by Ganesan *et al.* (2009). Briefly, 2 ml of 2 % Na_2CO_3 was added to 100 µl of sample. The mixture was left at room temperature for 2 minutes before 100 µl of 50 % Folin-Ciocalteau's reagent was added. The reaction mixture was mixed thoroughly prior to 30 minutes incubation in the dark at room temperature. Spectrophotometer was used to measure the absorbance of samples at 720 nm. Phenolic contents determined was expressed as Gallic acid equivalent per gram (GAE/g).

DPPH radical scavenging activity

DPPH analysis was conducted based on the method described by Bhuiyan *et al.* (2009) with minor modifications. Approximately 1 ml of 0.1 mM DPPH solution was added to 1 ml of sample. The mixture was shaken well before incubated at room temperature for 30 min. The absorbance was read at 517 nm by using spectrophotometer. Positive reference was prepared by diluting ascorbic acid in distilled water at different concentration ranged from 0 to 0.7 mg/ml. A control was prepared by mixing 1 ml of methanol and 1 ml of 0.1 mM DPPH solution. The radical scavenging activity was expressed as the percentage of radical scavenging activity using the following equation;

$$\% \text{ Scavenging} = [(Ac-As)/Ac] \times 100\% \quad (1)$$

where Ac= absorbance of control, As = absorbance of the sample solution

Cellulase assay

Assay for cellulase enzymes produced during fermentation was done according to the method described by Ncube *et al.* (2012). Approximately 0.9 ml of 1 % CMC prepared in 0.05 M acetate buffer (pH 5) was added to 0.1 ml of sample. The mixture was then incubated for 15 minutes at 50 °C. Three ml of DNS reagent was added to the mixture and was boiled for 10 minutes. The absorbance was measured at 540 nm after the sample was cooled at room temperature. The same procedure was used to prepare glucose standard curve ranging from 0–3.5 mg/ml. One unit of enzyme activity (IU) was defined as the amount of enzyme that released 1 µmol of reducing sugar (glucose) per minute under assay condition.

Xylanase assay

Xylanase activity was determined according to the modified method described by Thomas *et al.* (2016). Approximately 0.5 ml of sample was mixed with 0.5 ml of 1 % Birchwood xylan prepared in 0.05 M citrate buffer (pH 5). The mixture was incubated at 50 °C for 30 minutes. Following incubation, 3 ml of DNS reagent was added to terminate the reaction and the mixture was boiled for 10 min. After cooling, the absorbance of mixture was measured at 540 nm. Xylose standard curve was constructed at concentration ranging from 0–0.8 mg/ml to quantify the amount of reducing sugar liberated. One unit of xylanase activity was defined as the amount of enzyme that liberates 1 µmol of reducing sugars (xylose) per minute under the assay conditions.

β-glucosidase assay

β-glucosidase activity towards p-nitrophenyl-b-D-glucopyranoside (pNPG) was determined with the use of p-nitrophenol (pNP) as standard (Cai *et al.,* 1998). The reaction mixture contained 0.5 ml of 2 mM pNPG in 50 mM sodium acetate buffer (pH 5) and 0.125 ml of enzyme extract. The incubation of reaction mixture was performed at 45 °C for 10 min before 1.25 ml of 1 M Na_2CO_3 was added to stop the reaction. Yellow color developed as a result of pNP liberation from pNPBG was measured by using spectrophotometer at 410 nm. One unit of β-glucosidase activity was defined as the amount of enzyme required to liberate 1 mmol of pNP per minute under the assay conditions.

RESULTS AND DISCUSSION

Total phenolic content

Seaweeds contained secondary metabolites which correlated with some biological activities including antioxidant, anti-inflammatory, antifungal, antiviral, and antitumor activities (Machu *et al.,* 2015; Lee *et al.,* 2003). Fig. 1 shows the total phenolic content (mg GAE/g) of different varieties of *Kappaphycus alvarezii* at different fermentation days.

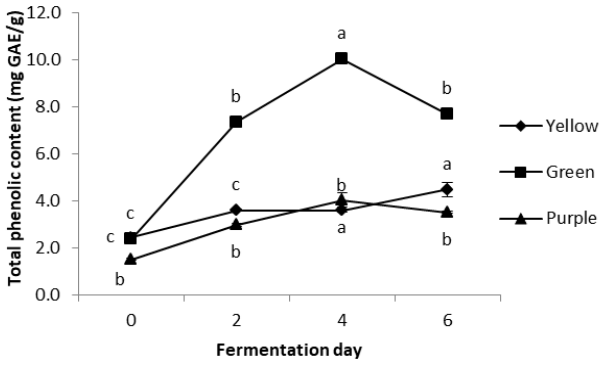


Fig. 1 Total phenolic content (mg GAE/g) of different varieties of *K.alvarezii* at different fermentation days.

Referring to Fig. 1, the maximum phenolic content is observed on the fourth day of fermentation for green (10.022 mg GAE/g) and purple (4.037 mg GAE/g) varieties of *K. alvarezii*. This result is in accordance to a study by Bae and Kim (2010) where the maximum total phenolic content of sea tangle extract obtained through submerged fermentation by using *A. oryzae* was obtained on the fourth day. However, the maximum total phenolic content for yellow varieties was observed on the sixth day (4.479 mg GAE/g). Based on the findings, total phenolic content for green varieties was enhanced 319.33 % by SSF on the fourth day of fermentation compared to non-fermented seaweed, whereas for purple varieties, total phenolic content was enhanced 166.29 % by SSF. For yellow variety, the enhancement of phenolics by SSF was low compared to green and purple varieties which is 46.93 %. The differences in the nature of substrates might be responsible for the different increment of phenolic compounds after fermentation.

The finding from this study was similar to research conducted by Bhanja *et al.* (2009) where the maximum phenolic content was detected on the fourth day of wheat koji fermentation with *A. oryzae*. Therefore, it is suggested that *A.oryzae* may be able to mobilize and increase phenolic content during solid substrate fermentation.

Antioxidant activity

The antioxidant capacity of the fermented seaweed extracts was determined based on the reduction of stable DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical antioxidants in a methanolic solution. In the presence of antioxidants, the purple color of the DPPH radical solution will change to bright yellow. Fig. 2 shows the DPPH radical scavenging activity of different varieties of *K. alvarezii* at different fermentation day.

Referring to Fig. 2, the same trend for DPPH radical scavenging activity is observed for all varieties of *K. alvarezii* tested, where the scavenging activity gradually increases as the fermentation progressed but then decreases after four days of fermentation. In comparison to all varieties used in this study, green variety showed the highest DPPH radical scavenging activity which was 72.47 % on the fourth day of fermentation. There were strong correlation between total phenolic content and DPPH scavenging activity (R^2 = 0.951 and R^2 = 0.823) at $p < 0.01$ for green and purple varieties, respectively. However, for yellow variety, low correlation (R^2 = 0.441) was observed between total phenolic content and DPPH radical scavenging activity. The higher antioxidative activities of fermented seaweed extracts could thus be connected to their high total phenolic contents. According to Ragan and Glombitza (1986), the radical scavenging activity of seaweeds is mostly related to their phenolic content. Lu and Foo (2000) also reported a strong correlation between DPPH radical scavenging activity and total polyphenols.

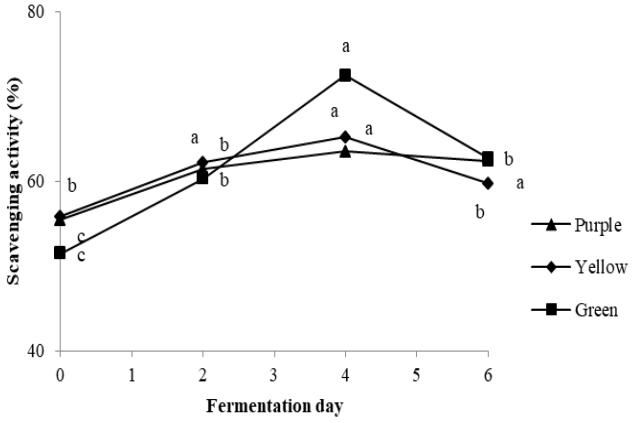


Fig. 2 DPPH radical scavenging activity (%) of different varieties of *K. alvarezii* at different fermentation days.

Influence of enzyme activities on polyphenol release

The relationship between cellulase, xylanase, and β-glucosidase produced during solid substrate fermentation of *K. alvarezii* with the enhanced release of polyphenols was investigated in this study.

Cellulase activity

In general, there are three major enzymes; endo-1,4-glucanase, cellobiohydrolase, and cellobiase in cellulase multi-component system (Pandey *et al.,* 1999; Prasad *et al.,* 2007). Hydrolysis of substrates containing crystalline cellulose can be achieved through the action of these enzymes (Dutta *et al.,* 2008).

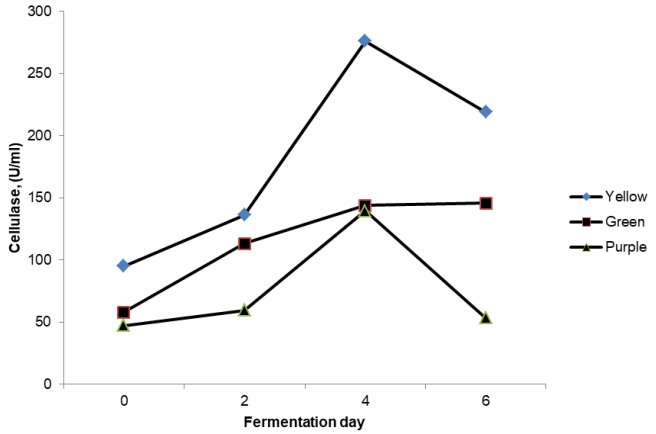


Fig. 3 Cellulase activity during seaweed fermentation by using *A. oryzae*.

Fig. 3 shows that cellulase activity for all *K. alvarezii* varieties increase as the fermentation progressed but decrease on the sixth day of fermentation. A significant correlation at $p < 0.05$ was observed between total phenolic content produced in all varieties of *K. alvarezii*. Green variety showed a strong correlation between total phenolic content and cellulase activity (R^2 = 0.939), while yellow and purple varieties showed a moderate correlation (R^2 = 0.633 and R^2= 0.688, respectively).

Xylanase activity

In many microbial systems, xylanase activity has been discovered in association with cellulases and other hydrolytic enzymes. Xylanase breaks down the main carbohydrate found in hemicellulose, known as xylan to produce xylose.

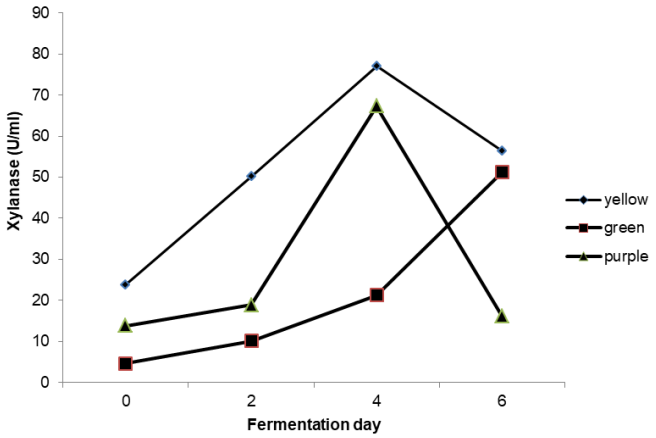


Fig. 4 Xylanase activity during seaweed fermentation by using *A. oryzae*.

Referring to Fig. 4, xylanase activity increases up to the fourth day and then decreases on the sixth day for yellow and purple varieties. However, xylanase activity in green variety increases as the fermentation progressed which showed maximum activity on the sixth day of fermentation. A positive correlation between xylanase activity and total phenolic content produced were observed. Yellow and purple varieties showed a moderate correlation ($R^2 = 0.640$ and $R^2 = 0.669$ respectively) while green variety showed low correlation ($R^2 = 0.463$). This observation might be due to the polyphenols produced in green varieties was maximum at day four of fermentation and then decreased on the sixth day, while xylanase activity was increased as the fermentation progressed.

β-glucosidase activity

β-glucosidases are responsible in the hydrolysis of the glycosidic bond between two or more carbohydrates or between a carbohydrate and non-carbohydrate moiety (Jean-Emmanuel and Ziya, 2004). The degradation of biomass (Coughlan, 1985), fuel ethanol production from cellulosic residue (Bothast and Saha, 1997), and the release of aromatic compounds in flavor industry (Gueguen *et al.,* 1995) are achieved by the applications of β-glucosidases.

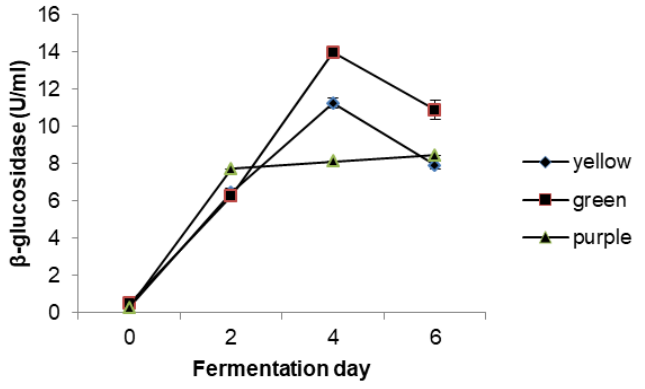


Fig. 5 β-glucosidase activity during seaweed fermentation by using *A. oryzae*.

β-glucosidase activity during solid substrate fermentation of *K. alvarezii* is shown in Fig. 5. β-glucosidase activities for yellow and green varieties increase up to day four and then decrease on the sixth day of fermentation. For purple variety, β-glucosidase activity increases with fermentation day. β-glucosidase activities in green and purple varieties showed a very strong correlation with polyphenolic contents at $p < 0.01$ ($R^2 = 0.954$ and $R^2 = 0.924$, respectively). A good correlation between polyphenols produced and β-glucosidase activity in yellow variety was also observed ($R^2 = 0.717$). The entire β-glucosidase activity peak correspond with an initial increase in phenolic content, suggesting that it might be involved in the release of soluble polyphenols. Lee *et al.* (2008) proposed that β-glucosidase enzyme produced by *A. awamori* during fermentation of black bean was responsible for polyphenol content enrichment.

CONCLUSION

This study showed that solid substrate fermentation of *K.alvarezii* by using *A. oryzae* was effective in improving the total phenolic content and DPPH free radical scavenging activity. Cellulase, xylanase, and β-glucosidase enzymes produced during fermentation were suggested to be involved in the enhancement and the release of polyphenols from non-fermented seaweed. Therefore, fermented seaweed can be added as value-added ingredients in the preparation of different seaweedbased food products.

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