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Enhancement of phenolics extraction from red algae (*Kappaphycus* spp.) using solid-state fermentation

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Abstract

The effects of solid-state fermentation (SSF) on microbial growth contribute to the bio-enrichment and availability of phenolic compounds in different varieties of *Kappaphycus* spp. red algae through the action of hydrolytic enzymes produced are investigated. Three different red algae samples were used; *K. striatum* var. green flower (GF), *K. alvarezii* var. white giant (WG) and *K. alvarezii* var. purple giant (PG). SSF was performed using *A. oryzae* for 0 to 6 days at 30°C. Results obtained demonstrated that the highest ($p<0.05$) extraction of phenolics (10.022 mg GAE/g and 14.90 mg CE/g), and antioxidant properties (72.47% activity of DPPH radical scavenging and 18.23 mM/g FRAP value respectively) was obtained for GF sample at day 4 of fermentation. Cellulase, β-glucosidase, and xylanase were found to be responsible for enhancing phenolics and antioxidant activity of WG and GF varieties by releasing bound phenolics. However, for the PG sample, β-glucosidase showed a significant relationship with TPC and antioxidant activity.

1. Introduction

The bioavailability of phenolics varies as they exist inbound (chemically bound in the cell walls elements, mostly polysaccharides) or liberated (positioned in the vesicles) form (Yadav *et al.,* 2013). Difficulties in the extraction of phenolics are due to several conjugated phenolics with at least one sugar residue being attached to the hydroxyl groups, and other chemicals including organic acid, lipids, amines, and carboxylic. As cited in Kumar and Goel (2019), extraction relies primarily on the type of the sample matrix, as well as the chemical features of the desired phenolic, such as the number of aromatic rings and hydroxyl groups in its concentration, structure and polarity. The majority of phenolic compounds are present as glycosides or esters rather than as free molecules, therefore their extraction procedure must be carefully designed as it impacts the recovery, selectivity and yield of the bioactive chemical of interest.

During SSF, microorganisms are developed on the solid surface materials with little water. Hence the fermentation medium should include all nutrients required for microbial growth and product synthesis. Enzymes produced by microorganisms depend on the substrate used. Among the other substrates, including rice bran, green gram bran, maize bran, black gram bran, corn flour, jowar flour, barley flour and wheat rawa, wheat bran had the greatest glucoamylase activity (Ellaiah *et al*., 2002). SSF using filamentous fungi has been used to improve the Total Phenolic Content (TPC) of different foods to improve their nutritional value (Cai *et al.,* 2011). SSF has been reported to improve the TPC in wheat koji (Bhanja *et al.,* 2009), plum fruit (Dulf *et al*., 2016), pearl millet (Salar *et al*., 2017), rice bran (Schmidt *et al*., 2014), fig (Buenrostro-Figueroa *et al*., 2017), and pineapple waste (Correia *et al*., 2004). The profiles of phenolic compounds during SSF are influenced by the action of various hydrolytic enzymes

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generated by microorganisms directly from solid substrates. These hydrolytic enzymes efficiently break down plant cell wall constituents and hydrolyze ester bond connections, which then helps to free bonded phenolics from the plant matrix (Dulf *et al.*, 2018).

Lignocellulolytic enzymes consist of xylanases and cellulases, which are hemicellulose and cellulose-degrading enzymes (Ncube *et al.*, 2012). Cellulase catalyses the hydrolysis of glycosidic linkages, thereby depolymerising cellulose to fermentable sugar. In many biological systems, xylanase activity is often associated with hydrolytic enzymes, especially cellulases (Ncube *et al.,* 2012). Xylanases convert xylan (the predominant carbohydrate in hemicellulose) to xylose. In industry, xylanase has been used to convert lignocellulose to xylitol to obtain clear fruit juice, improve the nutritional properties of hay and green feed, and de-ink waste papers.

β-glucosidase catalyses the hydrolysis of glycosidic bonds in aryl or alkyl β-glucosides, and glucosides containing only carbohydrate residues. This enzyme is relatively prevalent in all living species although it is secreted in large amounts by fungi in SSF of lignocellulosic biomass. The enzyme is responsible for hydrolysing phenolic glycosides and producing extractable free aglycones, which may improve their antioxidant properties (Vattem and Shetty, 2003).

Studies have shown that the cellulolytic enzymes generated by tea fungus can enhance the breakdown of plant tissues, resulting in a greater release of cathechins, oxidizing enzymes, and phenolic substrates, which lead to a greater rate of product synthesis (Murugesan *et al.*, 2002). A previous study on fermented cranberry pomace using *Lentinus edodes* has implicated β-glucosidase in phenolic mobilisation (Vattem and Shetty, 2003). However, no studies have investigated the effect of enzymes produced during fermentation on the phenolics in red algae. Therefore, the objective of this study was to examine the impact of SSF on microbial growth, which contributes to the bio-enrichment and availability of phenolic compounds in different varieties of *Kappaphycus* spp. through the action of hydrolytic enzymes produced.

2. Materials and methods

2.1 Sample preparation

Dried samples of red algae were purchased from Tawau, Sabah. Three different red algae samples used; *K. alvarezii* var. Giant, locally known as white seaweed (WG), *K. striatum* var. Green Flower (GF), and *K. alvarezii* var. Giant, locally known as purple seaweed (PG). Sample preparation was carried out according to Norakma *et al*. (2022). Samples were washed with tap water and immersed for one hour in twice the amount of water compared to algae weight. This was followed by cutting into smaller sizes, drying for five days at room temperature, and further mixing using a Waring blender. Then the samples were stored at chiller temperature in a tight-fitting cap bottle. The moisture content of samples was 8.90±0.001%.

2.2 Inoculum preparation

A. oryzae was acquired from the MARDI culture collection, Serdang, Selangor. The isolate was grown at 30°C on PDA plates for four days. The spores were collected using a hockey stick by evenly pouring 100 mL of sterile distilled water over four PDA plates containing a four-day-old culture. Then, the precipitated fungal cultures were filtered using No. 1 Whatman filter paper. The supernatant was utilized as an inoculum and stored at 4°C.

2.3 Solid-state fermentation

Solid-state fermentation was carried out according to Ellaiah *et al.* (2002) with some modification. Sucrose and yeast extract were utilized as additional nitrogen and carbon sources. Sterilised medium with 70% initial moisture content was inoculated with 1 ml of inoculum and the incubation process was carried out for 2 to 6 days at 30°C. The biomass-containing flasks received 100 ml of distilled water at the conclusion of the fermentation process, and the entire mixture was thoroughly stirred on a rotary shaker for 1 h at 180 rpm. After that, the entire contents were centrifuged at 8000 rpm for 10 minutes. Using Whatman filter paper no. 1, the resulting supernatant was further filtered. As a crude fermented seaweed extract, the filtrate was employed.

2.4 Extraction of enzyme

Enzymes were extracted in according to the method described by Norakma *et al.* (2019). Fermented seaweed (10 g) was added to 50 mL of distilled water, followed by 30 minutes of rotational shaking at 120 rpm at room temperature. The mixture was centrifuged for 10 minutes at 8,000 rpm. The supernatant was filtered using a Whatman filter paper No. 1. The resultant filtrate was stored at -20°C for further enzyme analysis.

2.5 Chemical analysis

2.5.1 Total phenolic content (TPC)

The total phenolic content was determined according to Johari and Khong (2019). The sample (100 µL) was blended with 2% Na_2CO_3 (2 mL), and the mixture was left to remain at room temperature for 2 minutes prior to addition of 50% Folin-Ciocalteau's reagent (100 µL). After completely mixed, the reaction mixture was kept at room temperature in the dark for 30 minutes. A

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spectrophotometer was used to determine the sample absorbance at 720 nm. Gallic acid (concentration range of 0.1 to 1 mg/mL) was used as the standard reference to draw a calibration curve and TPCs were represented as Gallic acid equivalent per gram (GAE/g) sample.

2.5.2 Total flavonoid content (TFC)

The aluminium chloride colorimetric technique as described by Singleton and Rossi (1965) was used to assess the total flavonoid content of fermented seaweed extracts. The crude extract (1 mL), methanol (1 mL), 10% aluminium chloride (0.5 mL), and 0.5 mL potassium acetate (1 M) were combined. A spectrophotometer was used to determine the absorbance at 415 nm after incubation at room temperature for 30 minutes. Using an equation derived from the calibration curve, the amount of TFC was expressed as mg of catechin equivalents (CE)/g sample.

2.5.3 Determination of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

DPPH analysis was carried out as reported by Baliyan *et al.* (2022) with modification. Sample (1 mL) and 1 mL DPPH solution (0.1 mM) were mixed. After thorough mixing, the mixture was left to stand at room temperature for 30 minutes. A spectrophotometer was used to measure the absorbance at 517 nm. Ascorbic acid was dissolved in distilled water to give concentrations ranging from 0 to 0.7 mg/mL and utilised as a positive reference. As a control, 1 mL of methanol and 1 mL of DPPH solution 0.1 mM were used. The following equation was used to calculate the radical scavenging activity as a %:

$$\%\ Scavenging\ activity = \left[\frac{Ac-As}{Ac}\right] x\ 100\%$$

Where Ac = absorbance of the control, As = absorbance of the sample solution.

2.5.4 Ferric reducing antioxidant power assay (FRAP)

The FRAP analysis was carried out according to Naji *et al.* (2020). Then 2.5 mL of 20 mM $FeCl_3.6H_2O$, 2.5 mL of a 10 mM 2,4,6-tris (1-pyridyl)-5-triazine (TPTZ) solution in 40 mM HCl and 25 mL of 0.3 M acetate buffer (glacial acetic acid 16 mL, sodium acetate trihydrate 3.1 g and distilled water 16 mL) at pH 3.6 were mixed to produced freshly FRAP reagent at a ratio 1:1:10.

Seaweed extract (150 µL) and FRAP reagent (2,850 µL) were mixed. A UV spectrophotometer was utilised to determine the mixture absorbance at 593 nm after incubation at 37°C for 4 minutes. The aqueous solution of $FeSO_4.7H_2O$ with concentrations ranging from 0.1 to 1.0 mM was used to produce the calibration curve. The FRAP value was computed using the following equation:

$$FRAP\ value = \frac{raw\ FRAP\ value\ x\ dilution\ factor\ x\ volume\ of\ the\ solvent}{weight\ of\ seaweed}$$

2.6 Enzymes produced during SSF

2.6.1 Cellulase assay

The procedure described in Ncube *et al*. (2012) was used to determine cellulase activity during SSF. The reaction mixture included 0.1 mL of sample, and 0.9 mL of 1% carboxymethylcellulose (CMC), made with 0.05 M acetate buffer (pH 5). The mixture was incubated at 50°C for 15 minutes, and 3 mL DNS reagent was added. The liquid was heated to boiling for 10 minutes, then cooled in an ice bath to room temperature before measuring the absorbance at 540 nm. The same method was applied to produce standard glucose curves with sucrose concentrations used between 0 and 3.5 mg/mL. The amount of enzyme required to release 1 µmol of reducing sugar (glucose) per minute was referred to as one unit (IU) of cellulase activity.

2.6.2 Xylanase assay

The method described by Thomas *et al.* (2016) was used to determine xylanase activity with some modifications. A mixture of the sample (0.5 mL) and 0.5 mL 1 % Birchwood xylan made in 0.05 M citrate buffer (pH 5) was incubated at 50°C for 30 minutes. This was followed by boiling the mixture for 10 minutes, and 3 mL of DNS reagent was added to stop the reaction. The mixture absorbance was examined at 540 nm after cooling at room temperature. A xylose standard curve was constructed using xylose concentrations ranging from 0 to 0.8 mg/mL, and the quantity of released reducing sugar was measured. The quantity of enzyme that released 1 µmol of reducing sugars (xylose) per minute under the test conditions was referred to as one unit of xylanase activity.

2.6.3 β-glucosidase assay

Using p-nitrophenol (pNP) as a reference, the β-glucosidase activity on p-nitrophenyl-b-D-glucopyranoside (pNPG) substrate was assessed (Gao *et al.,* 2012). The enzyme extract (0.125 mL) and 2 mM pNPG (0.5 mL) in 50 mM (pH 5) sodium acetate buffer were combined and incubated at 45°C for 10 minutes. A solution of 1 M Na_2CO_3 (1.25 mL) was added to the mixture to halt the reaction. A spectrophotometer was used to measure the yellow colour produced as a result of pNP release from pNPBG at 410 nm. The quantity of enzyme necessary to release 1 mmol of pNP per minute under the test conditions was used to define one unit of β -glucosidase activity.

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2.7 Microbial growth

The growth of *A. oryzae* on seaweed was enumerated using the plate count technique. One mL of fermented seaweed extract from each sampling day was mixed with 9 mL of peptone water in a test tube. Serial dilution was carried out up to 10^{-10}. Approximately 1 mL of the diluted extract was used to enumerate the growth of *A. oryzae* by pour plate technique using potato dextrose agar (PDA). The number of colonies on PDA was calculated, and the growth of *A. oryzae* was expressed as log CFU/mL. The following equation was used to determine the colony-forming units (CFU/mL):

$$\frac{CFU}{ml} = \frac{no\ of\ colonies\ x\ dilution\ factor}{volume\ of\ a\ culture\ plate}$$

2.8 Statistical analyses

Data obtained from triplicate samples were analysed. The mean and standard deviation were used to represent the results. One-way analysis of variance (ANOVA) SPSS 16.0 was carried out to determine this significance exists between the independent variables at $p<0.05$. Tukey's post hoc tests were carried if significance existed to determine differences between mean values of samples. The Pearson's correlation test was used to examine the relationship between TPC, antioxidant activity, generated enzymes, and microbial growth. result

3. Results and discussion

3.1 The changes in polyphenols, antioxidant activity and enzymes released during SSF

The majority of phenolics present in plants are found conjugated via hydroxyl groups attached to sugar molecules in glycosides. Since the availability of a free hydroxyl group on the phenolic is used for free radical conversion, this condition lowers antioxidant activity. This research investigated how the phenolic content changed both before and after fermentation. The variations in polyphenols, antioxidant activity, and enzymes during SSF for WG are summarised in Table 1.

As seen in Table 1, phenolic content was low at the initial fermentation stage. The result can be explained by the predominance of distinct chemically bound forms of these biomolecules during the early stage of fermentation. TPC for WG increased significantly on day 2 of SSF, and was significantly the highest on day 6 of fermentation. Based on the findings, TPC was enhanced by 83.57% after six days of fermentation. TFC in WG indicated a slightly different pattern from TPC. Total flavonoid content and DPPH free radical scavenging activity increased significantly as the fermentation progressed from day 0 to day 2 with no significant differences between days 2, 4 and 6 samples. There was an increase of 70.25% TFC by day 4 and a similar trend was seen for DPPH radical scavenging activity and FRAP values, showing that SSF boosted antioxidant activity. Furthermore, the FRAP values for WG significantly increased after day 2 of fermentation.

The ability of seaweed extracts to reduce the ferric cyanide complex into ferrous form could be attributed to the presence of phenolic chemicals in seaweed. The synthesis of reductants during fermentation may result in increased reducing power by reacting with free radicals to rebalance and end radical chain processes. Starter organism, peptides, intracellular antioxidants and hydrogen-donating capacity may all influence the increasing reducing ability. The FRAP and DPPH tests use distinct reaction mechanisms. The FRAP test examines the extracts' capacity to reduce, while the

Table 1. The changes in polyphenols, antioxidant activity and enzymes released during SSF for *K. alvarezii* var. Giant (white seaweed) (WG) samples.

	Fermentation (day)			
	0	2	4	6
TPC (mg GAE/g)	$2.44^{c}\pm0.16$	$3.61^{b}\pm0.02$	$3.59^{b}\pm0.06$	$4.48^{a}\pm0.29$
TFC (mg CE/g)	$4.45^{b}\pm0.55$	$6.85^{a}\pm0.30$	$7.58^{a}\pm0.05$	$6.95^{a}\pm0.79$
DPPH (% scavenging activity)	$55.81^{b}\pm1.59$	$62.17^{a}\pm3.18$	$65.17^{a}\pm1.59$	$59.74^{a}\pm1.32$
FRAP value (mM/g)	$5.54^{b}\pm0.06$	$5.94^{b}\pm0.17$	$6.87^{a}\pm0.12$	$7.16^{a}\pm0.40$
Enzymes released:				
Cellulase (U/mL)	$94.95^{d}\pm0.72$	$136.24^{c}\pm0.72$	$276.15^{a}\pm1.08$	$219.06^{b}\pm0.36$
β-glucosidase (U/mL)	$0.27^{d}\pm0.01$	$6.47^{c}\pm0.22$	$11.25^{a}\pm0.27$	$7.92^{b}\pm0.21$
Xylanase (U/mL)	$23.67^{c}\pm10.89$	$50.129^{b}\pm0.68$	$77.071^{a}\pm0.68$	$56.384^{b}\pm2.72$
Microbial growth (log CFU/mL)	$5.70^{c}\pm0.01$	$8.08^{b}\pm0.02$	$11.54^{a}\pm0.01$	$11.40^{a}\pm0.02$

[a,b,c] Results were expressed as the mean of three replications ± standard deviation. Mean with a different letter for each row showed significantly different at $p<0.05$.

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DPPH analysis determines the capability of radical chemicals to scavenge. This supports the different trends between DPPH and FRAP assay with increasing fermentation time.

The enhancement of polyphenols and antioxidant activity was due to cell wall degradation by microbial enzymes released during SSF. During SSF, different types of enzymes are produced by microorganisms. The ability of SSF to enhance polyphenols and antioxidant activity of *K. alvarezii* is mostly caused by the release of conjugated phenolics as a result of cell wall destruction by microbial enzymes produced during SSF (Norakma *et al.*, 2019).

In this study, three types of enzymes were investigated to relate the liberation of bound phenolics and enhancement of antioxidant activity. In general, all the enzymes examined (cellulase, β-glucosidase, xylanase) showed a significant increase in enzyme activity up to day 4 that significantly decreased on day 6 of fermentation. The reduction in enzyme activity might indicate the presence of more polyphenols, which may cause the liberated enzymes to be promptly feedback repressed or controlled (Bhanja *et al.,* 2009). Increasing enzyme activity was similarly correlated with *A. oryzae* growth. The growth of *A. oryzae* on WG was significantly increased up to day 4 of fermentation. There was no significant difference in microbial growth on days 4 and 6 of fermentation. When seaweed is inoculated with *A. oryzae*, the growing culture will pass through several phases. At the beginning of fermentation, low microbial growth was found, which might be due to microbial adaptation to the environment, also known as the lag phase of microbial growth. Then, microbial growth increased with the increase of fermentation day, and this stage is defined as the log phase. The cells divide at a constant and maximum rate at this stage, which is also described as the exponential phase. On the day 6 of fermentation, results showed no further increase in microbial growth. It might be because the medium (seaweed) is depleted of nutrients and enriched with metabolites. Polyphenols are secondary metabolites that will be produced at the end of the microbial growth stage.

The changes in polyphenols, antioxidant activity, and enzymes released during SSF for GF are shown in Table 2. Based on the results found, the maximum TPC and TFC were obtained (10.02 mg GAE/g and 14.90 mg CE/g respectively) on the day 4 of fermentation. Similarly, DPPH value with maximum scavenging ability was obtained on day 4 of fermentation (increased by 72.47%). TPC was enhanced by 319.33% after four days of fermentation, while TFC was enhanced by 184.93%. The lowest FRAP value for GF was observed at day 0, and this value increased as the fermentation progressed. A previous study (Bae and Kim, 2010) reported that the extract obtained from submerged fermentation sea tangle with *A. oryzae* day 4 had high TPC. Another research also showed that the highest TPC was obtained on day 4 fermentation of wheat koji with *A. oryzae* (Bhanja *et al.,* 2009), and whole seed fermentation with *A. oryzae* and *A. awamori* (Sadh *et al.,* 2017)

The growth of *A. oryzae* during fermentation influenced the release of phenolics that are produced by the fermentation process. The existence of hyphae that are shorter and heavily branched helps to promote the generation of pellets, which ultimately results in an enhancement SSF performance (Punia *et al.,* 2021). The

Table 2. The changes of polyphenols, antioxidant activity and enzymes released during SSF for *K. striatum* var. Green Flower (GF).

	Fermentation (day)			
	0	2	4	6
TPC (mg GAE/g)	2.39 ±0.10	7.35^{b}±0.11	10.02^{a}±0.09	7.67^{b}±0.05
TFC (mg CE/g)	5.23^{c}±0.30	9.59 ±0.30	14.90^{a}±0.60	14.23^{b}±0.45
DPPH (% scavenging activity)	51.50^{c}±0.79	60.30^{b}±0.53	72.47^{a}±1.32	62.73^{b}±3.71
FRAP (mM/g)	14.23^{c}±0.92	$16.41^{b,c}$±1.61	$18.23^{a,b}$±0.23	19.16^{a}±0.34
Enzymes released:				
Cellulase (U/mL)	58.06^{c}±0.36	113.05^{b}±1.08	143.88^{a}±3.60	145.67^{a}±0.36
β-glucosidase (U/mL)	0.48^{d}±0.00	6.27^{c}±0.04	13.97^{a}±0.19	10.90^{b}±0.55
Xylanase (U/mL)	4.64^{c}±0.14	10.12^{c}±1.63	21.28^{b}±5.17	51.21^{a}±3.40
Microbial growth (log CFU/mL)	6.22^{c}±0.02	8.35^{b}±0.03	11.54^{a}±0.02	11.45^{a}±0.02

^{a,b,c} Results were expressed as the mean of three replications ± standard deviation. Mean with a different letter for each row at $p<0.05$ are significantly different.

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increase in the number of spores contained inside the inoculum makes certain that fast biomass proliferation and enzyme production will occur, which in turn quickens the pace at which nutrients will be consumed (Rodrigues *et al.,* 2008). As nutrients depleted, microorganisms began to use phenolics as substrates, which ultimately led to a reduction in TC (Jamal *et al.,* 2011).

The secretion of cellulase and xylanase into the fermentation medium showed a similar pattern. Both enzymes increased as the fermentation progressed. However, β-glucosidase significantly increased up to day 4 of fermentation. There was no significant difference between samples on day 4 and day 6 of fermentation. The growth of *A. oryzae* on GF was similar to that of *A. oryzae* in WG.

Table 3 shows the changes in polyphenols, antioxidant activity, and enzymes released during SSF of PG. TPC, TFC and DPPH activity increased significantly from day 0 to day 4 of fermentation and then significantly decreased on day 6 of fermentation. TPC and TFC were increased by 131.86% and 18.36% respectively, after four days of fermentation. However, the FRAP value significantly increased with day 4 fermented sample. There was no significant difference between day 4 and day 6 fermented samples.

The production of β-glucosidase by *A. oryzae* in PG significantly increased with the increase in fermentation time. On the other hand, cellulase and xylanase were significantly increased up to 4 days of fermentation and then significantly reduced on day 6 of fermentation. *A. oryzae* growth in PG significantly increased until day 4 of fermentation before significantly decreasing on day 6. The low moisture content allowed fungal mycelium to penetrate through solid substrates, makes SSF is an ideal method for the development of fungi during the conventional process of fermentation. Fungal mycelium can penetrate the solid substrate in a process that involves four levels of mycelium penetration. The initial layer consists of aerial hyphae, then aerobic wet hyphae and anaerobic wet hyphae, and lastly, the penetrative hyphae layer completes the structure (Rahardjo *et al*., 2002). When there is a low level of humidity, the microorganisms that are present in SSF are increasingly able to create specific enzymes and metabolites, which are normally not formed during submerged fermentation.

The enhanced pattern of phenolic components in the various species of *Kappaphycus* may be related to substrate changes. The different types of *Kappaphycus* sp. are the result of various drying processes, which create seaweeds of various hues (Mei Ling *et al.*, 2013). The WG was formed by placing seaweeds in a translucent plastic bag and exposing them to the light until they developed a yellow-white hue. The seaweed was then exposed to the sun until a steady weight was achieved. This method of drying is known as "sun-dried." PG was made by hanging the seaweed in the sun until it took on a purple hue. The seaweed was sun-dried until a consistent weight was achieved. As for GF, it was sun-dried until a consistent weight was reached.

The progressive increase in detectable free phenolics throughout the SSF process may be due to the ability of *A. oryzae* to secrete significant amounts of cellulolytic enzymes in the solid-state system, which may catalyze the hydrolysis of β-glucosidic bonds. During fermentation, the structural breakdown of the cell wall may take place, resulting in the release and/or production of several bioactive chemicals (Adebo *et al.*, 2020).

Table 3. The changes in polyphenols, antioxidant activity and enzymes released during SSF for *K. alvarezii* var. Giant (Purple) Seaweed (PG).

	Fermentation (day)			
	0	2	4	6
TPC (mg GAE/g)	$1.52^{b}\pm0.05$	$2.98^{b}\pm0.06$	$4.04^{a}\pm0.30$	$3.52^{b}\pm0.05$
TFC (mg CE/g)	$6.70^{b}\pm0.25$	$6.85^{b}\pm0.10$	$7.94^{a}\pm0.40$	$2.87^{c}\pm0.30$
DPPH (% scavenging activity)	$55.43^{c}\pm2.12$	$61.42^{b}\pm1.59$	$63.48^{a}\pm1.32$	$62.36^{b}\pm6.09$
FRAP (mM/g)	$5.37^{b}\pm0.86$	$6.25^{a,b}\pm0.49$	$6.73^{a,b}\pm0.03$	$7.52^{a}\pm0.17$
Enzymes released:				
Cellulase (U/mL)	$47.04^{b}\pm3.60$	$59.53^{b}\pm3.96$	$139.55^{a}\pm42.17$	$53.42^{b}\pm9.73$
β-glucosidase (U/mL)	$0.29^{d}\pm0.01$	$7.73^{c}\pm0.01$	$8.13^{b}\pm0.01$	$8.47^{a}\pm0.00$
Xylanase (U/mL)	$13.78^{d}\pm0.27$	$18.88^{b,c}\pm0.14$	$67.28^{a}\pm4.35$	$16.18^{c,d}\pm1.22$
Microbial growth (log CFU/mL)	$6.20^{c}\pm0.02$	$8.40^{b}\pm0.02$	$11.48^{a}\pm0.04$	$11.40^{a}\pm0.03$

[a,b,c] Results were expressed as the mean of three replications ± standard deviation. Mean with a different letter for each row at $p < 0.05$ are significantly different.

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Table 4. Relationship between TPC, TFC, DPPH, FRAP and microbial growth with the enzymes produced during SSF of *K. alvarezii* var. white giant (WG), *K. striatum* var. green flower (GF) and *K. alvarezii* var. purple giant (PG) samples.

	TPC (mg GAE/g)	DPPH (%)	TFC (mg CE/g)	FRAP (mM/g)	Microbial growth (log CFU/mL)
WG					
Cellulase	$R^2 = 0.633$ [a]	$R^2 = 0.710$ [a]	$R^2 = 0.815$ [a]	$R^2 = 0.881$ [a]	$R^2 = 0.953$ [a]
β-glucosidase	$R^2 = 0.717$ [a]	$R^2 = 0.862$ [a]	$R^2 = 0.952$ [a]	$R^2 = 0.813$ [a]	$R^2 = 0.929$ [a]
Xylanase	$R^2 = 0.640$ [a]	$R^2 = 0.894$ [a]	$R^2 = 0.882$ [a]	$R^2 = 0.776$ [a]	$R^2 = 0.898$ [a]
GF					
Cellulase	$R^2 = 0.939$ [a]	$R^2 = 0.862$ [a]	$R^2 = 0.981$ [a]	$R^2 = 0.938$ [a]	$R^2 = 0.972$ [a]
β-glucosidase	$R^2 = 0.954$ [a]	$R^2 = 0.948$ [a]	$R^2 = 0.986$ [a]	$R^2 = 0.885$ [a]	$R^2 = 0.976$ [a]
Xylanase	$R^2 = 0.463$ [a]	$R^2 = 0.383$ [b]	$R^2 = 0.741$ [a]	$R^2 = 0.420$ [a]	$R^2 = 0.778$ [a]
PG					
Cellulase	$R^2 = 0.688$ [a]	$R^2 = 0.483$ [b]	$R^2 = 0.544$ [b]	$R^2 = 0.218$ [b]	$R^2 = 0.625$ [a]
β-glucosidase	$R^2 = 0.924$ [a]	$R^2 = 0.828$ [a]	$R^2 = -0.237$ [b]	$R^2 = 0.800$ [a]	$R^2 = 0.855$ [a]
Xylanase	$R^2 = 0.669$ [a]	$R^2 = 0.471$ [b]	$R^2 = 0.558$ [b]	$R^2 = 0.207$ [b]	$R^2 = 0.583$ [a]

[a]significant at $p<0.05$
[b]not significant at $p>0.05$

The elevated amount of TPC in fermented samples can be attributed to three main factors. First, after colonization by a fungus, the cell wall structure was broken down, resulting in the release of phenolics. Second, the bonded phenolics were released due to the mechanism of various hydrolytic and ligninolytic enzymes secreted by microorganisms in the fermentation process. Hence, the third factor is that there exists a possibility that the microbe will produce some soluble phenolic chemicals (Bhanja *et al.,* 2016).

3.2 Correlation between enzymes produced and the release of total phenolic content and antioxidant activity

The changes in antioxidant activity and TPC during the fermentation process are caused by the activity of hydrolytic enzymes mostly generated through the growth of the microorganisms. Table 4 shows the relationship between TPC, TFC, DPPH, FRAP, and microbial growth with the enzymes produced during SSF for all samples used. TPC, TFC, DPPH, FRAP, and microbial growth of WG showed a positive correlation with all enzymes investigated (Table 4). Therefore, it is suggested that all enzymes investigated in this study played a key role in the release of phenolics, which subsequently increased the antioxidant activity. The production of cellulase, β-glucosidase, and xylanase were shown to have a positive correlation with the microbial growth of *A. oryzae* in WG.

In terms of GF samples, TPC, TFC, DPPH, and FRAP showed a strong correlation with cellulase and β-glucosidase produced. On the other hand, there was no correlation between DPPH and xylanase enzymes produced, while TPC, TFC, and FRAP showed moderate correlation only. Hence only cellulase and β-glucosidase played a substantial role in the phenolic mobilisation of GF.

In PG samples, TPC showed a significant correlation with the production of cellulase, β-glucosidase and xylanase. On the other hand, DPPH and FRAP only showed a strong correlation with β-glucosidase produced during fermentation. Hence in PG, only β-glucosidase was correlated to mobilisation of the phenolics that increased antioxidant activity.

Microbes are responsible for the liberation of conjugated phenolic in the cell wall degradation by microbial enzymes. Fungi are responsible for producing hydrolytic enzymes that promote the release of aglycone from the substrate. As a result, the TPC and antioxidant activity of the substrate are increased.

4. **Conclusion**

This study showed that the highest extraction of phenolics and increase in antioxidant properties were obtained for sample GF at day 4 of fermentation. Hydrolytic enzymes generated by fungus catalyze the liberation of aglycone from the substrate, increasing TPC and antioxidant activity consequently. Antioxidant

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activity and phenolic compounds were shown to be correlated with the increase in β-glucosidase, cellulase, and xylanase activities. This suggests that enzymes may play a significant role in releasing conjugated phenolics from seaweed. Further study is needed to understand how enzymes (β-glucosidase, cellulase and xylanase) can be incorporated in seaweed products to enhance release of phenolics.

Conflict of interest

The authors declare no conflicts of interest to disclose.

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