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***Research paper***

# The effects of microbial fermentation on antibacterial activity of seaweed (*Kappaphycus alvarezii*) extracts

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**Abstract**

In the present study, *Kappaphycus alvarezii* one of the most abundant red algae in Malaysia was fermented with *Aspergillus oryzae* at 30°C for six days with 70% initial moisture content. Total phenolic content and antibacterial activity of fermented seaweed extracts was determined and compared to the non-fermented counterparts. It is found that total phenolic content increased as the fermentation progressed. Green variety fermented for four days exhibited the highest phenolic content (10.022 mg GAE/g). Meanwhile, fermented seaweed extracts tested for antibacterial activity showed that antibacterial activity of fermented seaweed against six different bacteria increased as the fermentation day increased. Green and Yellow variety fermented for six days demonstrated the highest antimicrobial activity (7 mm) against MRSA and *Salmonella* respectively. pH of fermented seaweed extracts increased with the increase of fermentation day and it might be related with the antimicrobial susceptibility which greater at increasing pH value.

***Keywords***: Antibacterial, *Aspergillus oryzae*, *Kappaphycus alvarezii*, microbial fermentation, red algae

## 1. Introduction

Nowadays, there is growing interest in marine organisms, especially algae in seeking therapeutic drugs from natural sources. Seaweed can be considered as futuristically promising plants forming one of the important living marine organisms of high nutritional value and nutraceutical potentials [1]. They produce complex secondary metabolites as a response to ecological pressure, such as competition for space, predation and tide variations. Seaweed provides a great variety of metabolites and natural bioactive compounds such as polysaccharides, polyunsaturated fatty acids, phlorotannins and other phenolic compounds [2]. Some of these compounds are antimicrobials that inhibit or limit the development and growth of other competitive microorganisms. *Kappaphycus alvarezii (K.alvarezii)* is a type of edible red seaweed which is abundant in tropical Asia such as Malaysia, India and Philippines. The cultivation cycle for *K.alvarezii* is every 45 days. The fast growing properties and its relatively high antioxidants, vitamins and minerals provide the impetus to study the medical uses such as antimicrobial activity [3], offering a rich source of new drugs with potentially lower toxicity.

A number of processes have been developed to increase the synthesis of secondary metabolites. Since seaweeds are known to contain valuable nutrients which can support the growth of microorganisms, exploitation of the nutritive potential of this marine organism for the production of high value compounds is a promising and attractive strategy. Microbial fermentation through solid state fermentation (SSF) is recognized as simple biotechnology process that can be used to improve bioactive compounds particularly polyphenols in many agricultural wastes by products. Most phenolic compounds occur in conjugated form, linked to sugar moieties and other compounds such as organic acid, amines and lipids. These conjugations reduce their ability to function as a good antioxidant and antimicrobial, due to the lower free hydroxyl groups on the phenolic rings [4]. During SSF, carbohydrate degrading enzymes produced by fungi will hydrolyze the phenolic conjugates, making it an attractive strategy to enhance the concentration of phenolics in agricultural by products [5].

Fungi have been known for their ability to produce enzyme that can degrade plants cell wall. *Aspergillus oryzae* which used in this study is widely used in soy sauce fermentation industry. One of the best features of *A.oryzae* is the ability to secrete high activity of hydrolytic enzymes. Besides that, they have fast growth and most importantly high resistance to contamination. Furthermore, *A. oryzae* is a safe organism to be used in food production because it lacks of expressed sequence tags genes for the production of aflatoxin [6].

To date and to the best of our knowledge, there is no report on the study of solid state fermentation of *K.alvarezii* by using *A.oryzae* with the purpose to enhance the phenolic compounds. Therefore, the present study aimed to investigate the antimicrobial activity of *K.alvarezii* fermented with *A.oryzae*. These functional properties of fermented *K.alvarezii* were assessed to evaluate their potential as food ingredients for applications in food industry to extend the shelf life of foods.

## 2. Materials and Method

### 2.1 Sample Preparation

*Kappaphycus alvarezii* was purchased from Tawau, Sabah. Samples were rinsed with tap water to remove dirts and soaked in two volumes of water for 1 h. Samples were then cut into smaller size, dried at room temperatures for five days and then grounded by using Waring blender. Dried samples were kept at 4°C in air- tight bottles.

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(a) (b) (c)

**Fig. 1:** *Kappaphycus alvarezii*; (a) *K.alvarezii* var. Giant (yellowish-white) seaweed (b) *K.alvarezii* var. Tambalang hijau (Green) (c) *K.alvarezii* var. Giant (Purple) seaweed

### 2.2 Inoculum Preparation

*Aspergillus oryzae* used in this study was obtained from MARDI culture collection centre, Serdang, Selangor. It was growth and maintained on potato dextrose agar (PDA) media. A hockey stick was used to collect the spores by evenly poured 100 ml of sterile distilled water on 4 PDA plates containing fungi culture at their active sporulating stage, which was 4 days old culture. Then the suspended fungal cultures were filtered using Whatman filter paper No. 1 and the filtrate was used as inoculum. The inoculum was stored at 4$^{o}$C and can be used within one month of storage.

### 2.3 Microbial Fermentation

Microbial fermentation was performed according to the method used by [7] with some modifications. Sucrose and yeast extract were used as supplementary carbon and nitrogen sources respectively. Sterilized medium with 70% initial moisture content was inoculated with 10% of inoculum and the incubation was done at 30$^{o}$C for 6 days. Sampling was done every two days by adding100 ml distilled water to the flasks containing the biomass and the whole content was agitated thoroughly on a rotary shaker for one hour at 180 rpm. The whole content was centrifuged at 8000 rpm for 10 minutes. The supernatant obtained was further filtered by using Whatman filter paper no 1. The filtrate obtained was used as the crude fermented seaweed extract.

### 2.4 pH analysis

pH of fermented samples were determined by using pH meter.

### 2.5 Determination of total phenolic content

The Folin-Ciocalteu method was used to determine the total phenolic content in fermented seaweed extracts. Briefly, 100 µl of samples was allowed to react with 2 ml of 2% $Na_2CO_3$ for 2 min. Then, 100 ml of 50% Folin-Ciocalteu reagent was added and incubated at room temperature for 30 min in the dark. Absorbance was measured at 720 nm using a spectrophotometer and the results were expressed as mg gallic acid equivalent (GAE)/gram sample.

### 2.7 Microbial isolates preparation

Tested organisms used consists of three Gram +ve bacteria (*Staphylococcus aureus, Bacillus subtilis, MRSA*) and three Gram –ve bacteria (*Escherichia coli, Pseudomonas aerugenosa, Salmonella*). The test pathogens were obtained from Microbiology Laboratory, Faculty of Applied Sciences, UiTM. Isolates were kept fresh on nutrient agar plates and on nutrient broth except for *Pseudomonas aerugenosa* on Lysogeny Broth (LB). One mL of bacterial suspension was transferred into peptone water and was incubated at 37$^{o}$C until it achieves or exceeds the turbidity of the 0.5 McFarland standard. This results in a suspension containing approximately 1 to 2 x $10^8$ CFU/mL.

### 2.8 Antibacterial assay

Antibacterial activity test was carried out in vitro by using the disc diffusion method [8]. One gram of freeze-dried sample was suspended in the 9 mL of 100% dimethyl sulfoxide (DMSO). Approximately, 100 mg of stock sample was then diluted further in 5% DMSO to a final concentration of 100 mg/mL. Sterile paper discs (Oxoid) were prepared by pipetting 40 µl of sample to each disc and then was left to dry under the laminar air flow for few minutes. An amount of 100 µl inoculum suspension was pipetted on the Muller Hinton agar (MHA) plates and a sterile cotton swab was used to spread the test microorganism evenly on the MHA plates. Similarly swabbing was done separately for each test microorganism on the MHA plates and left for few minutes to allow complete absorption of the inoculum on the MHA agar [9]. The plate was left for about three to five minutes, but no longer than 15 minutes for any excess surface moisture to be absorbed before applying the discs [10]. Sterile forceps were used to place the disc on the Mueller Hinton Agar (MHA) plates. The discs were placed such that they have complete contact with the agar surface by touching the discs with forceps. Plates were kept for some time until the extract diffuses into the medium. The prepared discs were placed on the agar Mueller-Hinton and incubated at 37°C for 24h [11]. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test organism. Mean diameter values were calculated from duplicate runs of each assay. Chloramphenicol antibiotic (50µg) was used as a positive control.

## 3. Results and Discussion

### 3.1. pH changes during fermentation

pH has influence over efficacy of antibacterial activity against pathogenic bacteria [12]. pH changes recorded throughout the fermentation was shown in Table 1.

**Table 1:** pH value of different variety of *K.alvarezii* at different fermentation days

| Fermentation Days | *K.alvarezii* | | |
|---|---|---|---|
| | Yellow | Green | Purple |
| 0 | $7.500^{b} \pm 0.014$ | $7.500^{d} \pm 0.028$ | $7.250^{c} \pm 0.014$ |
| 2 | $7.545^{b} \pm 0.007$ | $7.750^{c} \pm 0.028$ | $7.300^{c} \pm 0.014$ |
| 4 | $7.645^{b} \pm 0.035$ | $7.975^{b} \pm 0.035$ | $7.650^{b} \pm 0.028$ |
| 6 | $7.905^{a} \pm 0.064$ | $8.265^{a} \pm 0.049$ | $8.035^{a} \pm 0.078$ |

pH value was expressed as mean of two replications ± standard deviation at P<0.05. Values bearing similar superscript(s) letters (a, b, c and d) denotes significantly different at (P < 0.05).

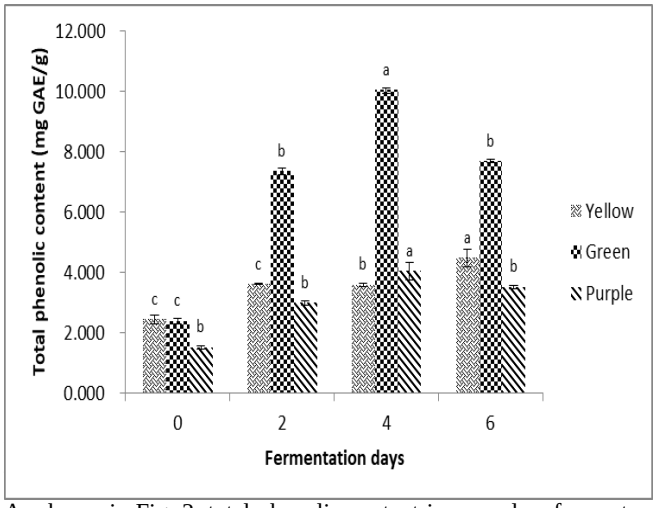
In general, pH value of fermented seaweed extracts increased as the fermentation progressed. The reason for this finding was unclear, since *A.oryzae* has been previously shown to generate organic acids during the fermentation of carbohydrates. [13] reported that pH of fermented sea tangle extract also increased as the fermentation progressed and they assumed that *A.oryzae* may generate basic compounds during the fermentation of sea tangle extract. The similar findings was reported by [14], where pH of soybean koji fermented with *A.oryzae* was decreased after 24h of cultivation but it further increased until the end of cultivation. [15] suggested that the increasing of pH of fermented matter was due to the microbial metabolic activities especially various extracellular proteins production.

Antibiotic susceptibility is greater with increasing pH value as shown in previous research by [16], at pH 5.9, the bactericidal activities of amoxycillin, clarithromycin and erythromycin were poor, but at pH 7.2 and 7.9, the bacterial susceptibility was increased. Therefore, the inhibition zone is expected to increase with longer period of fermentation since 6 day fermentation shows the highest pH compared to other days.

### 3.2 Total phenolic content

Phenolic compounds are usually found in conjugated forms through hydroxyl groups in plants. According to [17], phenolic compounds may acts as antioxidants by several mechanism in-

cluding free radical scavenging, chelation of metal ions and the inhibition of metal ions.




As shown in Fig. 2, total phenolic content increased as fermentation progressed. The increase of phenolic content in fermented seaweed extracts is suggested to be attributed with hydrolytic enzymes produced by *A.oryzae* during fermentation. This finding is in line with [18] which stated that hydrolytic enzymes produced may act upon the substrates and increase the availability of free hydroxyl groups on the phenolic structure, thus increasing the content of free phenolics and subsequently antioxidant activity of the substrate. In this study, Green variety demonstrated the highest phenolic content with the value of 10.022 mg GAE/g samples and was significantly increased by 319.33% from 0 day to day 4 of fermentation.

### 3.4 Antibacterial activity

Synthesis of different metabolites from seaweeds is an indicator of the presence of antimicrobial active compounds [19]. Different types of algae family was reported to contain variant of properties; for example: brown seaweed tends to possess higher activity against antimicrobial than red and green seaweed extracts [20,21]. Inhibition zone (mm) for different varieties of *K.alvarezii* were displayed in Figures 3-5.

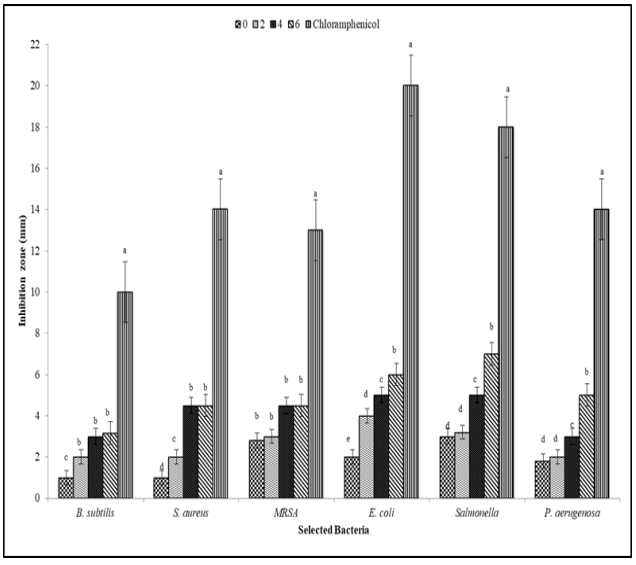



**Fig. 3:** Inhibition zone (mm) for Yellow variety against selected bacteria at different fermentation day

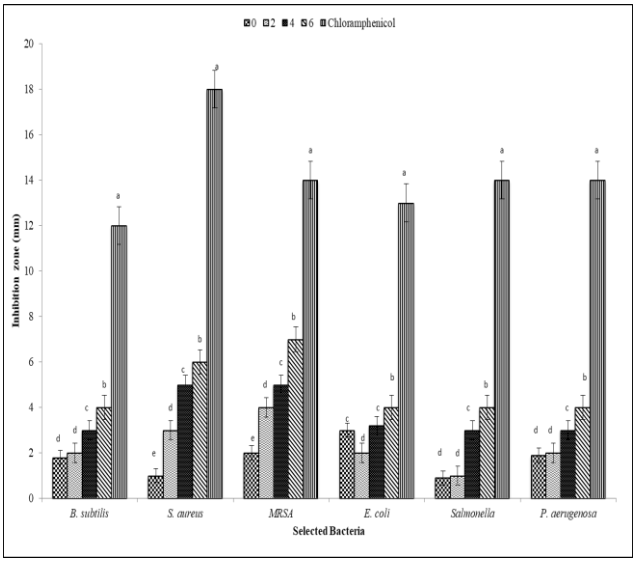



**Fig. 4:** Inhibition zone (mm) for Green variety against selected bacteria at different fermentation day

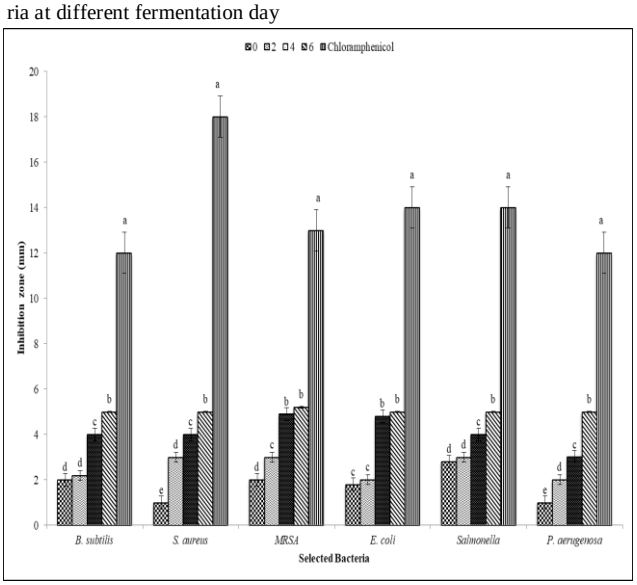



**Fig. 5:** Inhibition zone (mm) for Purple variety against selected bacteria at different fermentation day

In general, it can be seen that fermented seaweed shows higher inhibition zone compared to unfermented seaweed. Based on the results obtained, it is suggested that Green and Yellow variety which were fermented for 6 day, exhibited the highest antibacterial activity (7 mm) against MRSA and *Salmonella* respectively, see Fig. 6 and Table 2. Chloramphenicol which represented positive control in this research exhibited larger inhibition zone compared to the seaweed on all six bacteria. Chloramphenicol exhibited the largest inhibition zone 18 mm against *Staphylococcus aureus* and the smallest inhibition zone 12 mm against *Bacillus subtilis.*

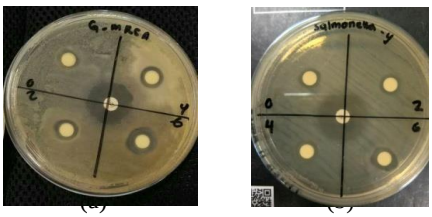



**Fig. 6:** Inhibition zone for (a) Green variety against MRSA and (b) Yellow variety against *Salmonella*

**Table 2:** Zone-size interpretation based on NCCLS standard for disc diffusion technique

| | Fermentation Day | Yellow | Purple | Green |
|---|---|---|---|---|
| ***B. subtilis*** | **0** | R | R | R |
| | **2** | R | R | R |
| | **4** | R | R | R |

| | | | | |
|---|---|---|---|---|
| | **6** | R | R | R |
| **Chloramphenicol** | | R | R | R |
| ***S. aureus*** | **0** | R | R | R |
| | **2** | R | R | R |
| | **4** | R | R | R |
| | **6** | R | R | R |
| **Chloramphenicol** | | I | S | S |
| ***MRSA*** | **0** | R | R | R |
| | **2** | R | R | R |
| | **4** | R | R | R |
| | **6** | R | R | R |
| **Chloramphenicol** | | I | I | I |
| ***E. coli*** | **0** | R | R | R |
| | **2** | R | R | R |
| | **4** | R | R | R |
| | **6** | R | R | R |
| **Chloramphenicol** | | S | I | I |
| ***Salmonella*** | **0** | R | R | R |
| | **2** | R | R | R |
| | **4** | R | R | R |
| | **6** | R | R | R |
| **Chloramphenicol** | | S | I | I |
| ***P. aerugenosa*** | **0** | R | R | R |
| | **2** | R | R | R |
| | **4** | R | R | R |
| | **6** | R | R | R |
| **Chloramphenicol** | | I | R | I |

*R: Resistant I:Intermediate S:Susceptible

The type of pathogen used in this research affects the antibacterial activity of all *K.alvarezii* varieties tested. In general, Gram positive bacteria can be distinguished by its thick peptidoglycan whereby Gram negative bacteria contains thin peptidoglycan which sandwiched between an outer membrane and inner cytoplasmic cell membrane. On top of that, Gram negative bacteria contained its major component present on the outer cell membrane, known as Lipopolysaccharides (LPS) [22]. Although all bacteria have an inner cell membrane, gram-negative bacteria have a unique outer membrane. This outer membrane excludes certain drugs and antibiotics from penetrating the cell, partially accounting for why gram-negative bacteria are generally more resistant to antibiotics than are gram-positive bacteria [23]. Therefore, it is believed that Gram negative bacteria tested in this experiment were more resistance than Gram positive bacteria may be due to the presence of LPS on its outer membrane, serving as physical-barrier from its surrounding [24,25]. However, based on the results obtained from this study, there is no specific trend in inhibition zone between Gram positive and Gram negative bacteria among all varieties of fermented *K.alvarezii* tested but it is apparently showed that inhibition zone increased as the fermentation progressed.

The inhibition zone of *K. alvarezii* exhibited during the fermentation day 4 and 6 resulted in the highest antibacterial activity against pathogens. As discussed in the study by [26], fermentation enhances release of phenolic content. Thus, in relation to that, plant phenols are known to possess antimicrobial properties [27]. Based on the results obtained, total phenolic content was maximum on day 4 of fermentation, however antibacterial activities showed the highest at day 6 of fermentation. It is suggested that, the compounds that contributed in antibacterial properties may not only phenolic compounds, but may also involve other compounds such as polysaccharides and peptides. The antibacterial activity of seaweeds may be influenced by some factors such as the habitat and the season of algal collection, different growth stages of plant and also the experimental methods adopted for the investigation [28]. Furthermore, [29] stated that antibacterial activities of algal extracts depend on algal species, the efficiency of the extraction method and concentration of the extract.

## 4. Conclusion

The present study demonstrated that the enrichment of phenolic compounds in *K.alvarezii* can be carried out by SSF using *A.oryzae*. Total phenolic content increased by over 319.33% from day 0 to day 4 of fermentation. Inhibition zones of fermented seaweed extracts also increased with the increased of fermentation day.

## Acknowledgement

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