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Fermentation of *Gracilaria verrucosa* to Reduce Insoluble Non-Starch Polysaccharide (iNSP) Using Cellulolytic Bacteria *Pseudomonas stutzeri* (ISO2) for a Dietary Ingredient of Golden Rabbitfish, *Siganus guttatus*

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ABSTRACT

A series of experiments were conducted to optimize *P. stutzeri* (ISO2) fermentation in reducing the non-starch polysaccharide (NSP) of *G. verrucosa* as golden rabbitfish feed ingredient. A completely randomized experimental design was used to optimize the pH of CMC medium and the duration of fermentation. Using a 3 × 3 factorial design with two factors and triplicates, the optimum substrate-inoculum interaction was determined. Nine levels combination of *G. verrucosa* (G1 = 50 g; G2 = 100 g; G3 = 150 g) and *P. stutzeri* inoculum (P1 = 5%; P2 = 10%; P3 = 15%) were investigated. Cellulolytic activity (CA) of *P. stutzeri* was measured using UV-vis spectrophotometer, meanwhile chemical compound and physical characteristics of fermented *G. verrucosa* were evaluated using Fourier Transform Infra Red (FTIR) and Scanning Electron Microscope (SEM) respectively. Data on fermentation optimization were analyzed statistically using ANOVA. The result indicated pH, duration of fermentation and substrate-inoculum ratio had a significant effect on bacteria growth and CA (P<0.05). *P. stutzeri* grew well at pH 8, the fermentation process was ideally running in four days with (150 g-10%) G:P level. Hemicellulose was the most biodegradable NSP compared to cellulose and lignin. Fermented *G. verrucosa* (FG) had FTIR vibrations which indicated the conversion of the cell wall to reducing sugar. The fermentation process resulted in surface structure changes of *G. verrucosa* based on SEM photos.

1. Introduction

Golden rabbitfish (*S. guttatus*) has become an emerging species for aquaculture in Indonesia, due to its high economic value. The aquaculture development of golden rabbitfish requires to fulfill the feed specifications, where commercial feed for rabbitfish has not been widely produced in Indonesia. Some factors considered in determining feed ingredients are the components and the sustained availability of some ingredients (Wan *et al.* 2019).

Seaweed is a potential dietary ingredient for golden rabbitfish feed development. As an herbivorous species, rabbitfish often graze in seaweed cultivation areas. Rabbitfish have high palatability to *Gracilaria* species (Cuihong *et al.* 2014). High abundance and diversity of *Gracilaria* in Indonesia make this seaweed species to be a potential ingredient for aquafeed including rabbitfish feed. Previous study reported that 10% inclusion of dry *Gracilaria* in formulated feed could improve the growth of *Labeo rohita* and *African catfish* growth, respectively (Al-Asgah *et al.* 2016; Vadher *et al.* 2016). Valente *et al.* (2006) also reported that European Seabass fed 10% *Gracilaria-bursa pastoris* had better growth than those provided by *Ulva rigida* and *Gracilaria cornea*.

Seaweed mainly contains carbohydrates, which is a cheap dietary energy source for fish in particular golden rabbitfish as herbivorous species.

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Carbohydrate content of seaweed varies by species, but generally ranges from 20-76% (Holdt and Kraan 2011). The type of carbohydrate-containing seaweed is mainly in indigestible fiber components as non-starch polysaccharides (NSPs); only a tiny portion of the carbohydrates of seaweed can be utilized by fish, namely glucose, mannose, and galactose (Rajapakse and Kim 2011). NSPs consisting of soluble NSP (sNSP) and insoluble NSP (iNSP) in a ratio that differs based on the kind and plant maturation (Gómez-Ordóñez *et al.* 2010; Deng *et al.* 2021). sNSP dissolve in water and forming viscous gels. sNSP of red algae (Rhodophyta) are composed of sulfated galactans, such as agar and carrageenan; in brown algae (Phaeophyta) are composed of alginates, fucans, and laminarans; and in green algae (Chlorophyta) are predominantly composed of Ulvan, a major structural polysaccharide found in the cell walls of *Ulva* sp. (Gómez-Ordóñez *et al.* 2010; Samarasinghe *et al.* 2021). Generally, iNSP consist of cellulose-a fundamental structural component of plant cell walls; hemicellulose-an alkali solubilized fraction; and lignin-a high molecular weight polymer of phenylpropane (Sinha *et al.* 2011).

In general, fish cannot digest and utilize NSPs at a certain level due to the lack of degradation enzymes in its intestines such as β-glucanases and β-xylanases (Kuz'mina 1996; Li *et al.* 2012). The high NSPs inclusion in feed causes decreasing on the diffusion rate of the substrate and digestive enzymes, thereby reducing the overall efficiency of nutrient absorption in the intestinal wall. The fermentability of NSPs determines their nutritional value.

sNSP is a fermentable NSP in the fish digestive tract. They bypass digestion and are easily fermented by the microbiota of the intestine. It is also related to prebiotic and health-promoting effects, as NSPs stimulate the growth of beneficial microorganisms at the intestinal level (Chen *et al.* 2021; Samarasinghe *et al.* 2021; Kang *et al.* 2022). However, iNSP is more resistant to intestinal fermentation than sNSP (Diógenes *et al.* 2018). Consequently, the dietary iNSP remains indigestible and cannot be used as an energy source, so its inclusion in a feed ingredient must be reduced. Pretreatment of *G. verrucosa* using cellulolytic microbes is necessary to reduce its iNSP before being used as a feed ingredient.

A cellulolytic bacteria of *Pseudomonas* spp. has been discovered to digest cellulose (Singh *et al.* 2015; Pathak and Navneet 2017; Sun *et al.* 2020). Recent investigations have extensively documented using *Pseudomonas* spp. as probiotics in aquaculture, including those isolated from marine fish (Zorriehzahra *et al.* 2016). Probiotics containing *Pseudomonas aeruginosa* have several benefits related to fish health, including improved immune response of *Labeo rohita* (Giri *et al.* 2012; Dawood *et al.* 2019), *Pseudomonas fluorescens* effectively suppressed the pathogenic bacteria of *Streptococcus faecium* in tilapia (Eissa *et al.* 2014), and other species *Pseudomonas* sp. have antibacterial effect preventing the bacterial pathogen of *Flavobacterium psychrophilum* in rainbow trout (Korkea-aho *et al.* 2011, 2012); In addition, *P. stutzeri* has been used as a probiotic for cultured *Artemia larvae* (Abdelkarim *et al.* 2010). Inclusion of *P. stutzeri* and *P. aeruginosa* into the diet can boost gut lactic acid bacteria and digestive enzyme activity of *Barbonymus gonionotus* (Salam *et al.* 2021). Our previous study revealed that *P. stutzeri* isolated from solid seaweed waste could be candidate agent for seaweed fermentation to be used as feed ingredient for golden rabbitfish, *S. guttatus* (Mulyaningrum *et al.* 2021). Since the appropriate condition for promoting the isolated bacteria's ability to reduce *G. verrucosa's* iNSP has not been determined, a series of experiments was conducted to optimize the *P. stutzeri* fermentation process to reduce *G. verrucosa's* iNSP content and also to evaluate chemical composition and morphological characteristics of fermented *G. verrucosa*.

2. Materials and Methods

2.1. *Gracilaria verrucosa* Seaweed Meal

G. verrucosa seaweed was selectively collected from earthen pond in Marana, Maros Regency, South Sulawesi, Indonesia, packed freshly, and sent to the Laboratory of Nutrition and Feed Technology of Research Institute for Brackishwater Aquaculture and Fisheries Extension (RIBAFE) for further treatment. The seaweed was thoroughly cleansed with tap water to remove sand, mud, or any undesirable particles before being air-dried. The dried seaweed was then ground to produce fined meal and stored in an airtight container until it used.

2.2. *Pseudomonas stutzeri* Inoculum

P. stutzeri (ISO2) bacteria isolated from solid seaweed waste were cultivated on 1% sterile Carboxy Methyl Cellulose (CMC) medium including 10 g CMC;

1.6 g KCL; 1.43 g NaCl; 0.94% KH_2PO_4; 0.15% NH_4Cl; 0.037 g $MgSO_4 \cdot 7H_2O$; 0.017 $CaCl_2 \cdot H_2O$; 10 g bacto agar and 5 g yeast extract in 1 L of sterile aquadest (Sheng *et al.* 2012). The inoculum was incubated at 37°C for 1 × 24 hours following the procedure applied in our previous study (Mulyaningrum *et al.* 2021). A loopful of bacteria isolate was put into 100 ml of 1% CMC medium and shaken for 24 hours. An inoculum stock with a density of 10^8 CFU/ml was employed for subsequent testing.

2.3. Optimization of Fermentation Process

2.3.1. Optimization of pH for *P. stutzeri* (ISO2) Growth

This experiment was designed into Completely randomized Design with six different pHs as the treatment and two replicates. The optimization of pH was done by measuring the growth of *P. stutzeri* inoculum in six different pH levels at 5, 6 7, 8, 9 and 10. A rate of 10% (v/v) inoculum was observed in 1% CMC media at each of pH levels, namely pH = 5 (adjusted using sodium acetate-acetic acid buffer), pH = 6 (adjusted using phosphate buffer), pH = 7 (adjusted using phosphate buffer), pH = 8 (adjusted using glycine-sodium hydroxide buffer), pH = 9 (adjusted using glycine-sodium hydroxide buffer), and pH = 10 (adjusted using glycine-sodium hydroxide buffer) (Sheng *et al.* 2012). The incubation process was placed in a shaker at room temperature for 24 hours, then the number of bacterial colonies was counted in a six serial dilution. Data on bacterial growth was statistically analysed using analysis of variance (one-way ANOVA) and Duncan post hoc test. The pH value with the highest inoculum density was used in the following observations.

2.3.2. Optimization of Duration of Fermentation

The dried *G. verrucosa* meal was sterilized using autoclave, and 100 g of autoclaved seaweed was fermented with a 10% (v/v) *P. stutzeri* inoculum. A loopful of *P. stutzeri* (ISO2) isolate with a density of 10^8 CFU/ml was shaken for 24 hours in 10 ml of 1% CMC media at the optimum pH. The optimum pH applied at this trial was based on the result observed in the earlier trial as explained above. After adding 90 ml of 1% CMC medium to 10 ml of active inoculum, the mixture was shaken for 24 hours to activate. The activated inoculum was employed in the next step.

An aerobic fermentation was carried out in a plastic container with dimensions 20 × 15 × 5 cm^3 at room temperature. The treatments were three durations of fermentation time of two, four, and six days in three replicates, respectively. The fermentation process was terminated by drying the fermented *G. verrucosa* using an oven at 60°C (Memmert-UNB 400). Parameter measured was cellulolytic activity (CA) of *P. stutzeri.* The CA was measured by determining CMCase activity using a modified version of (Kogo *et al.* 2017) method. Two grams of fermented seaweed were dissolved in 30 ml of citrate buffer (pH = 4) and centrifuged at 9,000 x g for ten minutes at 4°C. The resulting supernatant (crude extract of cellulolytic enzymes) was then evaluated for CMCase activity using 1% CMC as substrate. After 30 minutes at 37°C incubation, the amount of released reducing sugar was determined. CA is defined as the quantity of enzyme necessary to breakdown one mole of cellulose in one minute in order to produce one mole of glucose. The reducing sugar was determined using the DNS method using a UV-vis spectrophotometer at λ = 550 nm (Jain *et al.* 2020). A glucose standard curve was utilized to determine the concentration.

The study was completely randomized design, and the data of CA was analyzed statistically using analysis of variance (one-way ANOVA) and Duncan post hoc test.

2.3.3. *G. verrucosa* Meal Substrate-*P. stutzeri* Inoculum Interaction

The interaction of substrate-inoculum in the fermentation process was determined according to the procedure developed by Jamal *et al.* 2017 with modification. Autoclaved seaweed was fermented at room temperature in a 20 × 15 × 5 cm^3 plastic container. Fermentation was carried out at the best pH and fermentation duration obtained in the earlier experiment for bacteria growth. A 3 × 3 factorial design with two factors and triplicates was employed for this experiment. The first factor was *G. verrucosa* meal substrate dose (G), consisting of three levels which were 50 g (G1); 100 g (G2); 150 g (G3); and the second factor was *P. stutzeri* inoculum concentration, consisting of three levels which were 5% (P1); 10% (P2), and 15% (P3). The treatment was nine combinations of G and P namely: G1P1 (50:5); G1P2 (50:10); G1P3 (50:15); G2P1 (100:5); G2P2 (100:10); G2P3 (100:15); G3P1 (150:5); G3P2 (150:10) and G3P3 (150:15). The observed parameter was the CA of *P. stutzeri,* which was measured according to the previously described method. Data on CA was analyzed using two-way analysis of variance (two way ANOVA) followed by Duncan's Multiple Range Test (DMRT).

2.4. Chemical and Physical Characteristics of Fermented *G. verrucosa*

In order to produce a high amount of fermented *G. verrucosa*, an intermediate scale of fermentation was applied in two replicates by using two plastic containers (dimension 35 × 28 × 11 cm^3) as bioreactors. *G. verrucosa* of 1,500 g was fermented with 1,000 ml of 10% (v/v) *P. stutzeri* inoculum. The fermentation process was carried out at pH 8 during four days at room temperature following the results obtained from the series trial as explained earlier. The fiber component, chemical composition and morphology of fermented and unfermented *G. verrucosa* were measured and analysed descriptively.

2.4.1. iNSP and Chemical Compound Analysis Using Fourier Transform Infra Red (FTIR) Spectroscopy

Fiber content of *G. verrucosa* was analyzed using Fibertherm analyzer model EV1 Gerhadt (Germany) following the industrial procedure. iNSP (cellulose, hemicellulose and lignin) content was measured using Van Soest method (Van Soest *et al.* 1991).

Fermented and unfermented *G. verrucosa* were analyzed in duplo to determine their chemical compound using FTIR Spectroscopy (Shimadzu 8200s). The potassium bromide (KBr) pellet method was used for analysis in the wavelength range of 4,000-550 cm^{-1} in 32 seconds.

2.4.2. Determination of Structure Surface Using Scanning Electron Microscope (SEM).

The surface structure of fermented (FG) and unfermented *G. verrucosa* (UFG) was characterized using a JEOL model 6000-EDS SEM. The composite two seaweed samples were used to analyze their surface structures (Agusman and Wibowo 2021).

3. Results

3.1. Optimization of Fermentation Process

3.1.1. The best pH for *P. stutzeri* Growth

The pattern of *P. stutzeri* growth cultured at different pH levels after 24-h incubation was illustrated by Figure 1. There were significant differences (P<0.05) among the treatments, the highest bacterial density was (3.7 × 10^9 CFU/ml) at pH 8. The growth of *P. stutzeri* at pH 9 (2.1 × 10^9 CFU/ml) and pH 7 (1.4 × 10^9 CFU/ml) was lower than those that grew at pH 8, but significantly had higher growth compared to other three pH levels at 5, 6, and 10 (P<0.05). Furthermore, the growth of *P. stutzeri* had the lowest density at pH 6 (1 × 10^8 CFU/ml), which was not significantly different (P>0.05) from those at pH 5 (4 × 10^8 CFU/ml) and pH 10 (3 × 10^8 CFU/ml).

3.1.2. Duration of Fermentation

Figure 2 demonstrates the CA of *P. stutzeri* at different duration of fermentation. Statistical analysis (ANOVA) indicated the duration of fermentation had a significant effect on CA of *P. stutzeri* (P<0.05). The CA on four days (0.082±0.008 unit/ml/minute) and two days fermentation duration (0.079±0.008 unit/ml/minute) did not significantly differ (P>0.05). However, the CA detected on six days of fermentation was the lowest (0.053±0.002 unit/ml/minute) and significantly different (P<0.05) from those two fermentation durations.

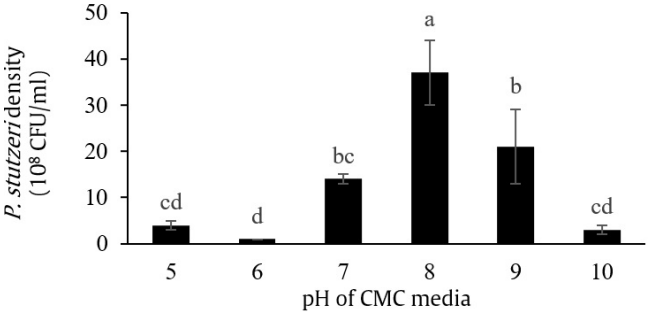


Figure 1 . Growth of *P. stutzeri* on CMC media at different pH levels. Data are presented as means ± SD (n = 2), values with different the same superscript indicating significantly different (P<0.05) in the Duncan post hoc test

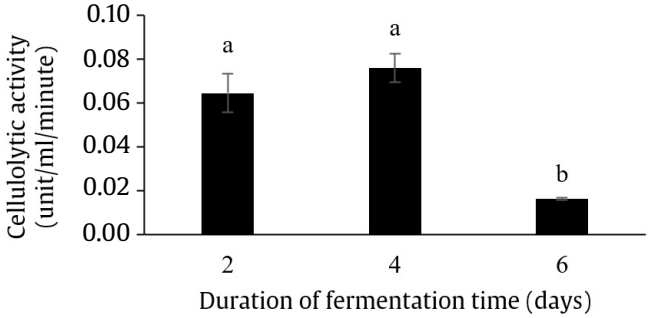


Figure 2. Cellulolityc activity of *P. stutzeri* in different fermentation periods. Data are presented as means SD (n = 3), value with different superscript means significantly different (P<0.05) in the Duncan post hoc test

3.1.3. *G. verrucosa* Meal Substrate-*P. stutzeri* (ISO2) Inoculum Interaction

Based on two-way ANOVA, both *G. verrucosa* meal substrate and *P. stutzeri* (ISO2) inoculum levels had significant differences among groups, and their interaction was also significantly different. The highest CA was found at 150:10 G:P level, while the lowest CA was found at 50:5 G:P level. Substrate-inoculum level had a very significant interaction in the affected CA of *P. stutzeri* ($P<0.01$) (Table 1). Cellulolytic activity in G:P levels of (50:5), (50:10), (50:15), and (100:5) had not significantly different ($P>0.05$), but the four levels of G:P were significantly different ($P<0.05$) compared to other G:P level. Meanwhile, G:P levels of (100:10), (100:15), (150:5), and (150:15) were not significantly different ($P>0.05$). In contrast, those four G:P levels were significantly different ($P<0.05$) with G:P level of (150:10).

3.2. Chemical Compound and Physical Characteristics of Fermented *G. verrucosa*

3.2.1. iNSP and Chemical Compound Analysis Using Fourier Transform Infra Red (FTIR) Spectroscopy

The fermentation process resulted in a decreasing content of three types of iNSP contained in *G. verrucosa* measured in this study which was cellulose, hemicellulose, and lignin. The most degradable iNSP was hemicellulose (53.20±3.71%), followed by cellulose (23.20±0.42%) and lignin (11.87±0.75%) as illustrated by (Figure 3).

Table 1. Cellulolytic activity (CA) of *P. stutzeri* (means ± SD) in different *G. verrucose* meal substrate-*P. stutzeri* inoculum level

G:P level		CA (unit/ml/minute)
50:5		$0.028±0.011^{A}$
50:10		$0.033±0.010^{AB}$
50:15		$0.034±0.001^{ABC}$
100:5		$0.041±0.008^{ABCD}$
100:10		$0.075±0.007^{E}$
100:15		$0.087±0.005^{EFGH}$
150:5		$0.083±0.010^{EF}$
150:10		$0.109±0.003^{I}$
150:15		$0.084±0.011^{EFG}$
Two Way ANOVA		
Substrate	Inoculum	Substrate*Inoculum
**	**	**

Values are expressed as means ± SD (n = 3); * = significant ($P<0.05$), ** = very significant ($P<0.01$), NS = no significant ($P>0.05$). Values with a different superscript in the same column mean a significantly different ($P<0.05$)

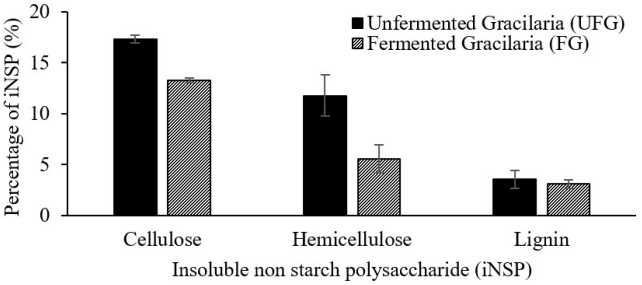


Figure 3. Insoluble non-starch polysaccharide (iNSP) of fermented and unfermented *G. verrucosa.* Data are presented as means ± SD (n = 2)

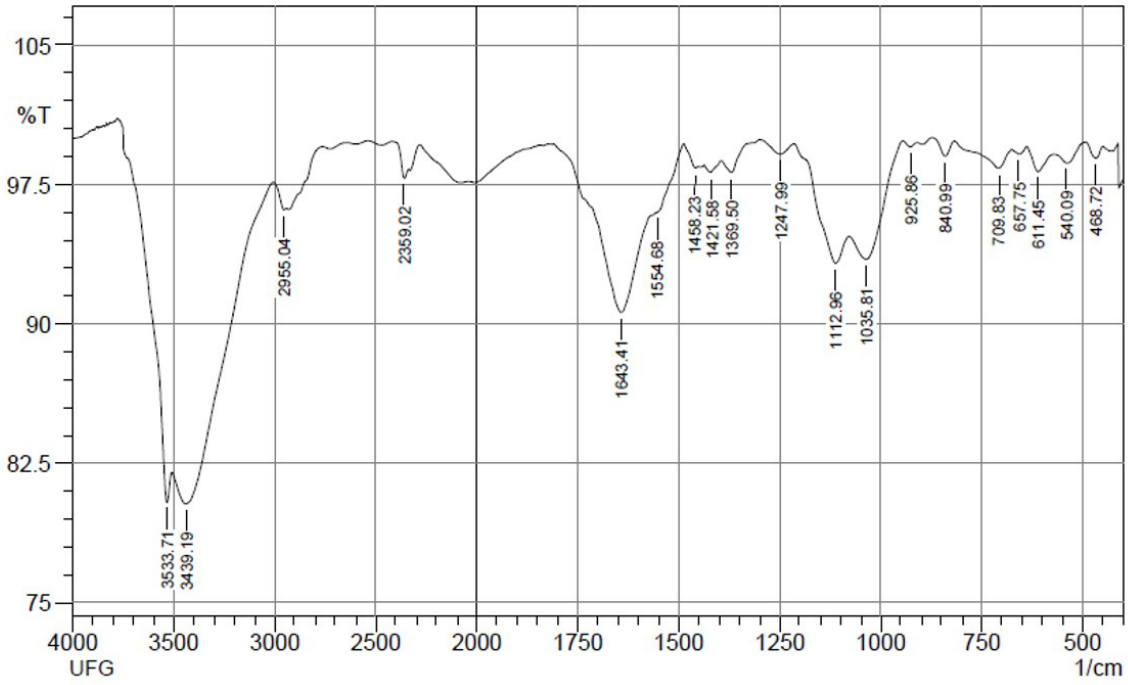
Figure 4 illustrates the findings of the FTIR analysis of both UFG (Figure 4A) and FG samples (Figure 4B). Carbohydrates were the most compound both in UFG and FG, indicated by a broad absorption band in 3533.71-3439.19 cm^{-1} (UFG) and 3529.85-3425.69 cm^{-1} (FG) followed by some peaks on 1554.68-1247.99 cm^{-1} (UFG) and 1543.10-1251.84 cm^{-1} (FG) respectively. The fermentation process resulted ketone (1836.29 cm^{-1}) and aldehyde (1737.92 cm^{-1}) FTIR spectra on FG, a carbonyl functional group of reduction sugar.

Both UFG and FG revealed iNSP peaks in the β-glycosidic bond, as evidenced by peaks on 952-709 cm^{-1} (UFG) and 893-748.81 cm^{-1} (FG). Peak 1112.96 of UFG indicated cellulose, while peaks 893 cm^{-1} and 1109.11 cm^{-1} of FG indicated cellulose deformation. The presence of hemicellulose was revealed by peaks at 1247.99 cm^{-1} (UFG) and 1251.84 cm^{-1} (FG), which suggested hemicellulose C-O stretching. Peaks of 1421.58 cm^{-1} demonstrated the presence of lignin; 1554.68 cm^{-1} on UFG and 1425.44 cm^{-1}; 1543.10 -1521 cm^{-1} on FG.

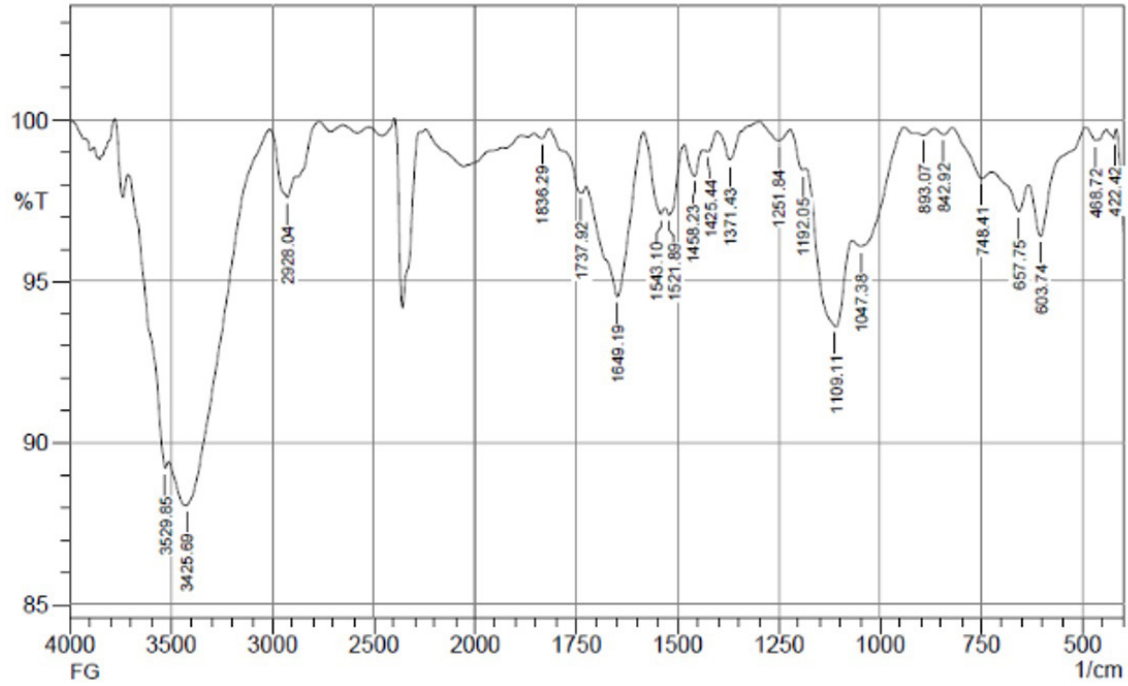
Proteins were discovered in the FTIR spectra both in UFG (2359.02 cm^{-1}; 1643.41 cm^{-1}) and FG (2350 cm^{-1}; 1649 cm^{-1}). In addition, the sulfate-containing agar was defined by the presence of S-S bonds on peak 657.75-468.72 cm^{-1} (Figure 4A) of UFG and 657.75-422.42 cm^{-1} of FG (Figure 4B).

3.2.2. Surface Structure by SEM

The SEM photos of the surface structure of two UFG and FG groups are demonstrated in Figure 5. UFG had a smoother, nonporous, and well-structured texture (5A-B) compared to the FG, which showed more cracks on its surface (5C-D). The surface disruption of the UFG revealed that *P. stutzeri* performed enzymatic hydrolysis.



A



B

Figure 4. FTIR spectra of *G. verrucosa*: (A). unfermented *G. verrucosa* (UFG) and (B) fermented *G. verrucosa* (FG)

4. Discussion

Pseudomonas sp. is a Gram-negative staining bacteria that can be isolated from a variety of habitats, including but not limited to soil, sewage, and the digestive tracts of insects, and is frequently employed as a probiotic (Nayak 2010; Korkea-aho *et al.* 2012; Lakshmi *et al.* 2013). Several studies reported *Pseudomonas* sp. as a probiotic such as Alavandi *et al.* (2004) reported using *Pseudomonas* sp. as a probiotic candidate in tiger shrimp culture. Lazado *et al.* (2015) also used *Pseudomonas* sp. as a probiotic to overcome the attack of *Vibrio anguillarum* and *Aeromonas salmonicida* on Atlantic cod. The fermentation process by microorganisms is strongly influenced by several factors, including pH, substrate concentration, inoculum concentration, and the duration of fermentation (Shajahan *et al.* 2017). Acid-base conditions of bacterial growth media are one factor that affects bacterial growth. In this study, the best bacterial growth was obtained at pH 8. The findings of this study are consistent with those of Silva *et al.* (2020), who found that *P. stutzeri*

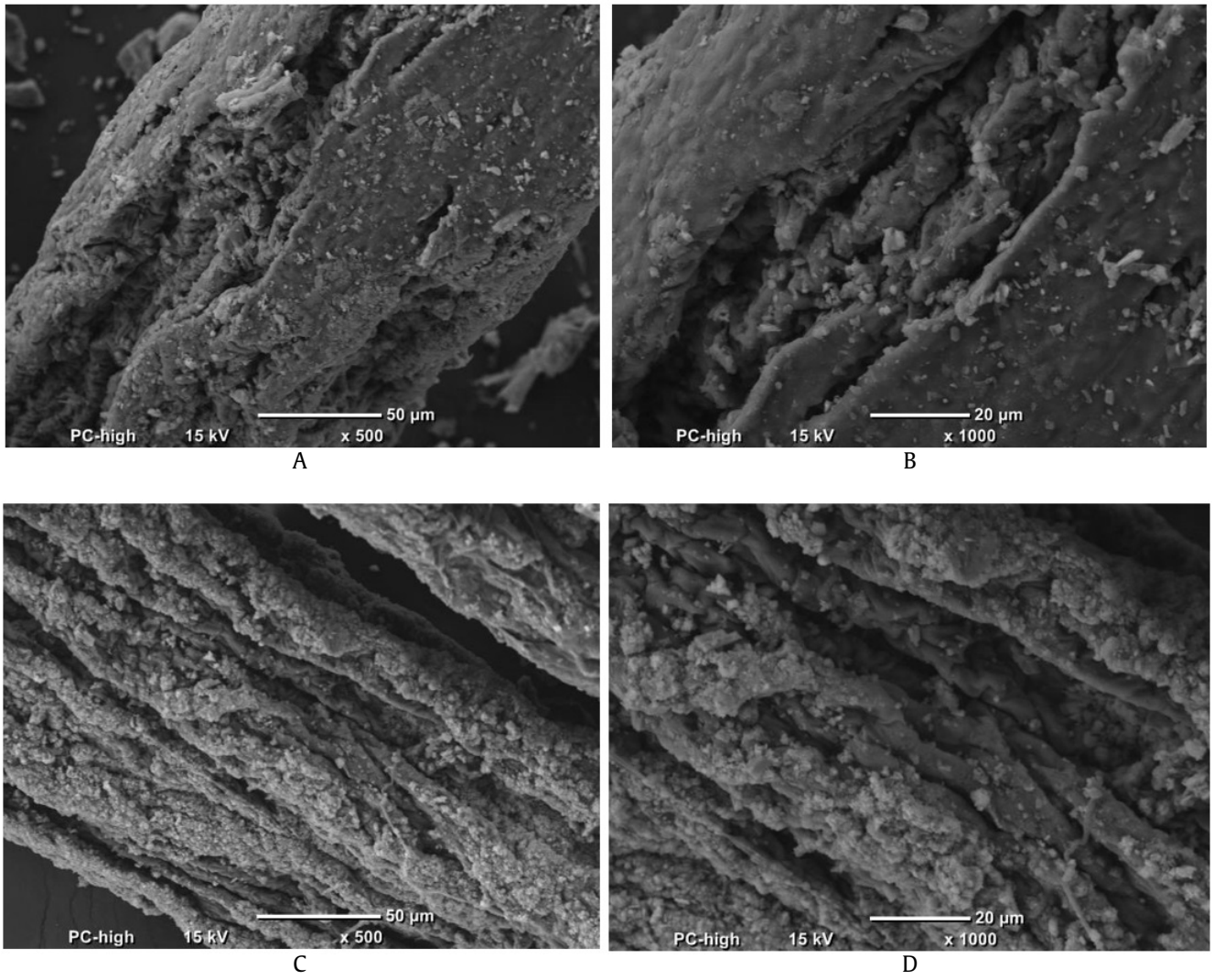


Figure 5. Scanning electron microscope (SEM) image of *G. verrucosa* surface structure in various magnifications; UFG in 500x magnification (A) and 1,000x magnification (B); FG in 500x magnification (C) and 1,000x magnification (D)

bacteria thrived best at pH 7-9, but not at lower pH levels. Lalucat *et al.* (2006) reported *P. stutzeri* was not acid-tolerant; they did not survive at a pH of 4.5. Jin and Kirk (2018) reported pH impacts microbial metabolisms and could influence the composition and activity of microbial communities if it changes. Significant interaction between *G. verrucosa* meal substrate and *P. stutzeri* (ISO2) inoculum revealed that these two factors influenced the performance of cellulolytic enzymes of *P. stutzeri* (ISO2) in degrading seaweed's iNSP. Okoro-Shekwaga *et al.* (2020) reported that the ratio of inoculum to substrate affects the kinetics of the fermentation process and the stability of product formation. The highest enzyme activity was obtained at 150:10 level, it related to the lower substrate moisture level of the treatment. The *P. stutzeri* bacteria used in this study originated from solid waste of seaweed processing to work optimally in low humidity. The result of this study showed the duration of fermentation had a significant effect on CA of *P. stutzeri* agreed with Rosyida *et al.* (2015) who reported significant effect of fermentation time of straw substrate using cellulolytic bacteria *Trichoderma reesei*. In general, the production of the enzyme depends on biomass accumulation, but only during the exponential phase of cell growth, since cellulase is considered a primary metabolite whose production begins during the exponential phase and declines during the death phase.

Hemicellulose was the highest iNSP which could be reduced by *P. stutzeri* (ISO2) bacteria. The complex structure of cellulose and lignin might be the reason of the low degradation process of both fiber components. Zanchetta *et al.* (2021) reported *G. verrucosa* had a bi-layered cell wall structure, with an agar inner layer and a cellulose outer layer that contains a small amount of xylose and mannose. The presence of lignin is also one of the reasons for the degradation of a tiny fraction of cellulose. Lignin is a cell wall component that must be eliminated through pretreatment before the cellulose degradation process (Siddhanta *et al.* 2013). Another factor that influenced the higher degrading of hemicellulose was the fermentation process under alkaline conditions (pH 8). The most effective pretreatment in hemicellulose degradation was the alkaline pretreatment which was considered the most effective in breaking the ester bonds between cell wall polysaccharides (Jmel *et al.* 2018). Baghel *et al.* (2021) explained that seaweed cell walls are composed of fibrillar skeleton, whereas red seaweeds mainly comprise cellulose and hemicellulose microfibrils. *G. verrucosa* contains cellulose in the range of 14-18% dry weight, which in the fermentation process will be broken down into glucose and cellobiose (Kumar *et al.* 2013).

FTIR analysis showed both FG and UFG had high-intensity vibrations at 3,500-3,400cm^{-1}, related to the O–H stretch for the hydroxyl group, which is a carbohydrate functional group that is the major component of seaweed (Figure 4A and B). IR area spectra over 3,000 cm^{-1} were shown to be associated with H-bond vibration (Corgié *et al.* 2011). The fermentation process resulted in some peak intensities differences, such as vibrations at 2955.04 cm^{-1} of UFG and 2928.04 cm^{-1} of FG suggesting the presence of C-H for aldehydes (Dave *et al.* 2021). The different intensities of those two peaks and the presence of vibration at 1836.29 cm^{-1} and also 1737.92 cm^{-1} (C = O stretching of aldehyde) in fermented *G. verrucosa* verified the hydrolysis process and conversion of the cell wall to reducing sugar. The presence of thiols on the 657.75-422.42 cm^{-1} vibration both UFG and FG related to the agar yield of *Gracilaria* which is a sulfactan galactan compound. Praiboon *et al.* (2006) reported sulfate compounds at various positions in polymers isolated from Thai and Japanese species of *Gracilaria*.

The fermentation process resulted in surface structure changes of *G. verrucosa*. There was some cracks and disruption on fermented compared to unfermented *G. verrucosa*. The structure of some seaweed species has been identified in enzymatic, chemical, and thermal hydrolysis. Park and Jeong (2021) reported that physio-chemical pretreatment using ultrasonic and acid catalyst resulted in torn and holed surface structure of *G. verrucosa*. Fermented *Ulva rigida* showed surface roughness and some cracks compared to unfermented ones, revealing a compact and smooth surface (Fernandes *et al.* 2019). Hii *et al.* (2015) revealed surface structural changes on *Sargassum binderi* after treatment with sulfuric acid and heating. Jmel *et al.* (2018) also discovered alterations in the cell wall structure of *Ulva lactuca* after pretreatment with ethanol organic solvents, hot water solvents, and ionic solvents.

We concluded that *P. stutzeri* (ISO2) grew effectively as a fermentation agent at pH 8. *G. verrucosa* fermentation ran ideally for four days with a (150 g-10%) SI level. Hemicellulose was the

most degradable iNSP (53.20±3.71%) compared to cellulose (23.20±0.42%) and lignin (11.87±0.77%). The fermentation process resulted FTIR vibrations which indicated the conversion of the cell wall to reducing sugar and surface disruption indicating seaweed's fiber degradation.

Conflict of interest

The author(s) declare that we have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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