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Effects of Inoculum Size of *Afifella marina* in the Bioprocessing of *Sargassum* spp. Meal

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Abstract

Brown seaweed, *Sargassum* spp. is also used as an alternative feed ingredient for aquaculture in the form of seaweed meal. Nutritional values seaweed meal with bioprocess technology using microbes such as *Lactobacillus*, *Bacillus* or *Streptococcus*, are improved with different inoculum sizes. Purple non-sulphur bacterium, *Afifella marina* could be one of the alternative and potential candidates. This study aims to determine the optimum inoculum level and period for the improvement of nutritional values in *Sargassum polycystum* meal. *Sargassum polycystum* was collected from the Sepanggar Bay. Dried seaweed was grinded into fine particles (<400 μm). Inoculum sizes of 10% (v/v), 20% (v/v) and 30% (v/v) of *A. marina* were used with 20 g *Sargassum polycystum* powder in one liter bottle with autoclaved 112 synthetic media. Bottles were incubated at 2500 lux light intensity at 30˚C ± 1˚C for 8 days. Every two days destructive samples were taken for the determination of nutritional values of bioprocessed seaweed meal. The measured analytical parameters were crude protein (%), crude ash (%), crude fiber (%) and crude lipids (%). Nutritional value of processed seaweed meal powder with *A. marina* has improved with the increase of inoculum size. The crude protein percentage of 14.70% + 0.40% was significantly high ($p < 0.05$) with 30% (v/v) inoculum size and obtained on the 8th of bioprocessing product. Consistent decrease in crude fiber values was observed with increase of inoculum sizes, as 19.34% of crude fiber decrement was determined with 30% (v/v) inoculum level on 6th day. The performance of crude lipids was observed insignificant in all inoculum levels. No significant differences ($p > 0.05$) were observed among the values of crude protein, ash, lipids and fiber within 6th and 8th day. *A. mariana* with 30% (v/v) inoculum sizes on 6th of day has capability in improving the nutritional values of *Sargassum polycystum* seaweed meal during bioprocessing.

Keywords

Afiffella mariana, *Sargassum*, Nutritional Values, Inoculum Sizes and Period

1. Introduction

There are over 400 species of *Sargassum* species distributed in the temperate and warm waters of Indo-West Pacific region which included China, Japan, Malaysia, Indonesia and Australia [1]. Seaweed meal of these species has potentiality for its use as an alternative feed ingredient in feed industry [2]. In addition to that, seaweed meals are also used as a feed additive for cattle, sheep, horses, dogs, goats, cats, poultry and aquaculture species [3]. Fishmeal is considered as an important ingredient in feeds for most of the aquaculture species. The rising cost of imported fish feed ingredients has called for an intensive research towards the production of high nutritional fish feed using cheap and natural nutrients sources [4]. Eventually, *Sargassum* sp. was used to produce seaweed silage and used as an alternative fish diet to feed rotifers through the process of decomposition using probiotic bacteria [5]. Among various seaweeds, *Sargassum thungernii* and *Sargassum polycystum* are traditionally considered the best sources as they are easily available [6]. In general, *Sargassum* spp. contains 46.59% to 50.98% carbohydrate, 0.94% to 1.99% fat, 6.44% to 10.64% protein, 22.56% to 24.76% ash, and 16.19% to 19.64% water. The composition of seaweed typically varies with the variations of different species, season, geographical location, growth environments and temperature. The major issue in the seaweed meal is the presence of high crude fiber and low protein, because these inclusion levels need to optimize while seaweed meal used in aquafeeds [7]. Bioprocess is a simple and suitable method, which might considerably decrease crude fiber content and increase protein value [8]. Conversion of seaweed meal with processing technology possibly can give potential economic values especially added more values while in bioprocess. Bioprocess are capable of increasing the nutritional value of the substrate used [9]. Value of the seaweed meal can be improved in bioprocess with some microbes such as *Lactobacillus*, *Bacillus* or *Streptococcus* [10]. Thus, the product from bioprocess seaweed meal will be highly potential to be further used in feed supplements. The microbes released several additional compounds during the bioprocess which range from peptides, antibiotics, enzymes, and growth factors [11]. The purple non-sulphur bacterium (PNSB) species of *Afifella marina* has the capability to produce extracellular nucleic acids that being used as aquaculture feed additive as growth enhancers, chemotherapeutic agents, and immune system enhancer [12]. Strain of *Afifella marina* is also a good proteolytic enzyme producer in hydrolyzing large molecular organic compounds [13]. So, purple non-sulphur bacteria could be one of the alternative and potential candidates to improve the nutritional values of seaweed meal during the bioprocess processes. The inoculum sizes for *Afifella marina* are necessary in order to optimize the yield and production of *Sargassum polycystum*. The inoculum size influences the estimated growth parameters of microbes that needed to be considered in quantitative risk assessments for the design challenge tests and experiments to gather data for predictive growth models [14]. The growth rate in a large inoculum size was greater than that of a small inoculum size indicating that growth rate is affected by inoculum size. There is lack of study on the potential of *Afifella marina* in the production of nutritionally enriched biomass through bioprocess. Thus, this study is conducted in order try to gain knowledge on improving the nutritional values of seaweed by using *Afifella marina* with different inoculum levels.

2. Materials and Methods

2.1. Preparation of *Sargassum polycystum* as Substrate

The brown seaweed *Sargassum polycystum* was collected from Sepanggar Bay, Kota Kinabalu and then was brought to the laboratory. In the laboratory, they were washed with tap water, chopped into smaller pieces and dried in oven at 65˚C for 48 hours. The dried seaweed was grinded into powder and fine particles (<400 mm) by using high speed blender. The powdered seaweed (seaweed meal) was stored in air-tight containers until further use.

2.2. Purple Non-Sulphur Bacteria, *Afifella marina*

The purple non-sulphur bacterium (PNSB) species of *Afifella marina* was used in this research was isolated from Kingfisher, Kota Kinabalu mangrove shady area and also in other mangrove microhabitats [15]. The *Afifella marina* was obtained from the culture stock available in Borneo Marine Research Institute (BMRI) and was used for this study. The 112 synthetic media were used as substrate for the development of inoculum and the bioprocess.

2.3. Preparation of Inoculum

The universal bottles contained 112 media were autoclaved at 121˚C for 15 minutes for inoculums preparation. Initially 5 ml liquid culture of *Afifella marina* from the culture stock was added into universal bottle. These bottles then were incubated anaerobically at 2500 lux light intensity at a temperature of 30˚C ± 1˚C for 48 hours. After 48-h culture were transferred into 100 ml Schoot bottles containing autoclaved 112 media. Subsequently inoculum upscale into 2 L bottles. After 48 hours, three different types of inoculum sizes, such as 10% (v/v), 20% (v/v) and 30% (v/v) of *Afifella marina* were used in the experimentation.

2.4. Bioprocess of *Sargassum polycystum* with *Afifella marina*

Three different sizes of inoculum, such as 10% (v/v), 20% (v/v) and 30% (v/v) of *Afifella marina* were used in this experiment (**Table 1**). Experiment was conducted in a 100 mL Schott bottle. The amount (ml) of inoculum and *Sargassum polycystum* powder used in each of the treatment are as follows:

All the bottles were incubated anaerobically under 2500 lux of light intensity at temperature of 30˚C ± 1˚C for the period of eight days.

2.5. Sampling Method

The bioprocess was conducted for eight days and every 2-day interval, two bottles

Table 1. Amount of *Sargassum polycystum* (g) and three different levels of inoculum sizes for *Afifella marina* used in the bioprocess.

Inoculum	112 Synthetic Media (ml)	Amount of *A. marina* Inoculum (ml)	*Sargassum polycystum* Powder (g)
10%	900	100	20
20%	800	200	20
30%	700	300	20
Control	1000	No inoculum	20

from each inoculum and one control bottle were taken to harvest the biomass by centrifugation (4.0 × 1000 rpm for 20 minutes). After that, the biomass was dried in the oven at the temperature 65˚C overnight. The values of crude protein (%), crude lipids (%), crude fiber (%) and total ash (%) of the bioprocessed product were determined with standard methods [16].

2.6. Statistical Analysis

One-Way ANOVA (Analysis of Variance) to compare the significant differences composition of nutritive value among the days of bioprocessed samples. The Post-hoc test was done to find out whether there were variations between the proximate compositions within days. All of these statistical analyses were conducted by using SPSS for windows (Window version, Chicago, IL, USA).

3. Results

Crude protein (%)

The crude protein content with 30% inoculum size shows gradual increases from the day 2 till end of experiment, with maximum values of 14.7% of protein in day 8 (**Figure 1**). Although maximum values of protein obtained on day 8, but there observed no significant differences between the values protein obtained in day 6 and day 8. The increment of proximate composition crude protein for all samples in the three different inoculum sizes, 10%, 20% and 30% from Day 0 until Day 6 were significantly ($p < 0.05$) different but no significant differences observed between day 6 and day 8 ($p > 0.05$).

Crude Fiber (%)

The crude fiber content for each different size of inoculum sizes showed a decreased pattern from Day 0 until Day 8 (**Figure 2**). Although the highest decrement 23.25% ± 0.7% of crude fiber was obtained in 8th days followed by 23.21 ± 0.6 of crude fiber during bioprocess. The decrease of proximate composition crude fiber for all samples in the three different inoculum sizes, 10%, 20% and 30% from Day 0 until Day 6 were significantly ($p < 0.05$), but no significant differences were observed within crude fiber values determined in 6th and 8th days after bioprocess.

Composition of Ash (%)

The level of crude ash (%) in 30% inoculum size showed a gradual increase from Day 2 until Day 8 with the value of 48.36%, 48.35%, 49.10% and 49.15% respectively (**Figure 3**). The proximate composition of crude ash (%) in *Sargassum*

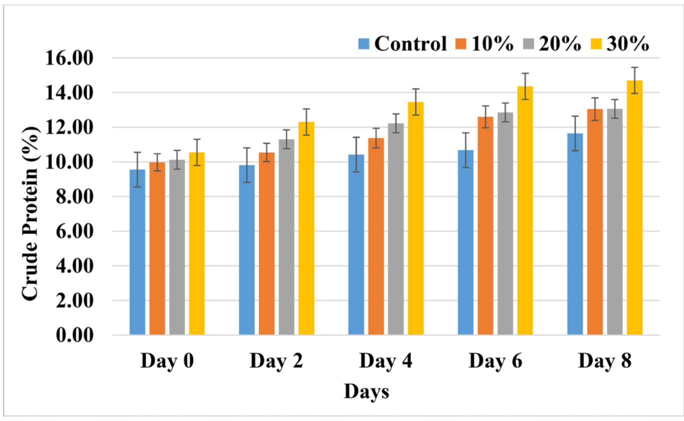

Figure 1. Comparison of crude protein (%) content in *Sargassum polycystum* biomass derived from bioprocess in three inoculum sizes with and without *Afifella marina* (control) during the experimental period.

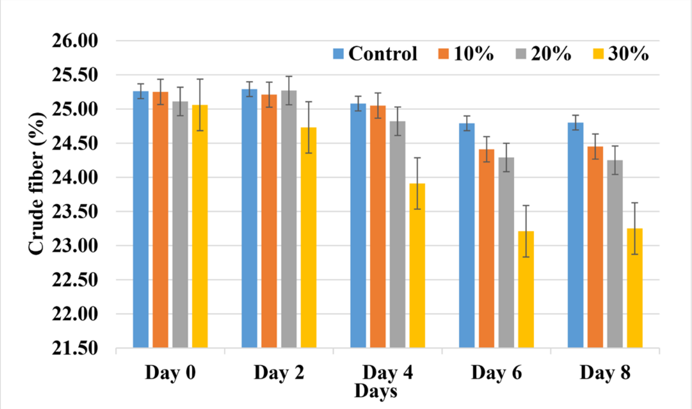

Figure 2. Comparison of crude fiber (%) content in *Sargassum polycystum* biomass derived from bioprocess in three inoculum sizes with and without *Afifella marina* (control) during the experimental period.

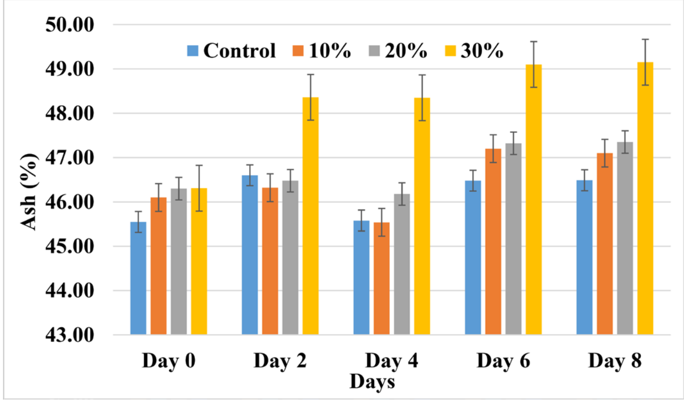

Figure 3. Comparison of composition of ash (%) content in *Sargassum polycystum* biomass derived from bioprocess in three inoculum sizes with and without *Afifella marina* (control) during the experimental period.

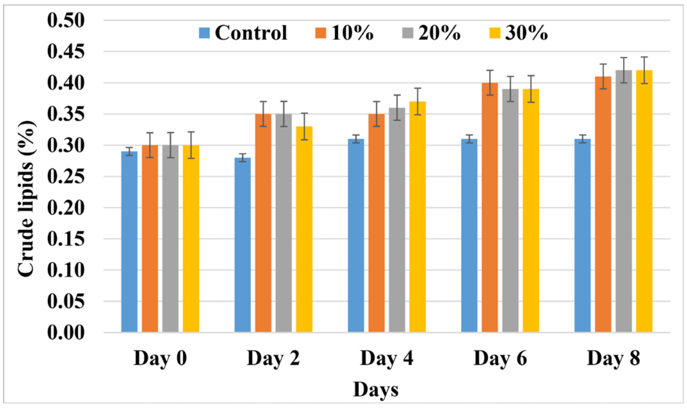

Figure 4. Comparison of content of crude lipids in *Sargassum polycystum* biomass derived from bioprocess in three inoculum sizes with and without *Afifella marina* (control) during the experimental period.

polycystum biomass with or without the presence of *Afifella marina* had shown significant difference ($p < 0.05$) in different size of inoculum, but no significant differences were observed within the values of ash composition between 6th and 8th days of bioprocessed samples.

Crude Lipids (%)

The lipid content after bioprocess of *Sargassum* with *Afifella marina* seaweed had shown little increment when compare within the days (Figure 4). Lipid content in all the inoculum sizes observed to increased, but the highest crude lipids of 0.42% ± 0.02% was determined with 20% and 30% of inoculum at from 8th days of sample. No significant differences ($p > 0.05$) were observed in the content of crude lipids within 20% and 30% of inoculum sizes bioprocess samples and also between the 6th days and 8th days of samples.

4. Discussion

The nutritional values of seaweed possessed differ in percentage as it depends on the difference of species and seasonal variations [17]. The differences are also explained between species and intra-species depending on species, habitat and season [18]. In general, dried seaweed meal contains only naturally occurring protein, fiber and lipid, hence directly use might not be economical because of poor nutritional values. Nutritional composition of *Sargassum polycystum* by the percentage dry weight of sample for crude protein was 5.40 ± 0.07 meanwhile for crude ash was 42.40 ± 0.41 [19]. On the other hand, the highest protein level of 14% dry weight was determined in *Sargassum* sp., however this brown seaweed species also recorded the lowest level of lipid which is 0.2% - 0.5% [20]. Typically, biochemical composition of *Sargassum polycystum* exhibited high nutritional potential of protein (14.2%), carbohydrate (25.0%), lipid (7.6%), fiber (21.3%), and ash (29.0%) than that in terrestrial plants and animal products [21]. Among the two seaweeds, *S. cristaefolium* contained highest protein and carbohydrates content (8.54% and 7.25%, respectively) and *S. crassifolium* possessed highest ash, fat and crude fiber (41.52%, 0.30%, and 24.54%, respectively) [22].

The chemical composition of seaweed can vary due to several environmental factors which include temperature, water, salinity, light, and nutrition. The chemical composition of seaweed can be influenced not only by concentration of aquatic nutrients but also temperature of the waters and the depth of the waters which are also influenced by seasonal variation and geographical location [22]. The nutritional composition of seaweed increases with biotechnological process. Bioprocess is one of the potential options that can change nutritional profiles in seaweed meal.

In this study, the purple non-sulphur bacterium, *Afifella marina* was grown in the 112 synthetic media with *Sargassum polycycstum* powder as its substrate has displayed overall improvement in its nutritional value in terms of crude ash and crude protein after a process. Unfortunately there was no past research that has been conducted on the bioprocess of seaweed especially brown seaweed *Sargassum polycystum* by using the purple non-sulphur bacteria. However several researchers conducted experimentation, basically on the improvement of nutritional value by fermentation with microbes [23] [24] [25]. Bioconversion of vegetable waste with fungi *Aspergillus niger* $S_1$4 and *Aspergillus niger* NCIM 616 through solid fermentation, shown the highest level of crude protein 18.26% ± 1.17% content on Day 8th and Day 7th, which was higher than the protein of 17.70% obtained in this study. On the other hand the highest protein were obtained on day 8th in both the experiments assume that the bacterial activity was at the best state during stipulated period. In addition, the purple non-sulphur bacterium *Afifella marina* in present study significantly improved the crude protein content in the seaweed *Sargassum polycystum* in 10%, 20% and 30% inoculum sizes with 13.04%, 13.06% and 14.70% respectively on the Day 8th compared to the initial protein content in seaweed meal. Increase in crude protein content of the fermented seaweed may be due to the secretion of certain extracellular enzymes which are proteinous in nature into waste during their breakdown and its subsequent metabolism [26].

Other than the increment of crude protein, nutritional values like fiber and ash can also be improved in the bioprocess of seaweed. The present study shows that the crude ash content of the bioprocess product had increased rapidly from Day 0 until Day 8th, while inoculated with 30% inoculum level. Ash content increased throughout the bioprocess period with 10% and 20% levels of inoculum. Increase of ash content due to loss in organic matter obtained through the process [23]. The contents of crude ash can be highly benefit in animal feeds and it is also necessary in determined the content of minerals [25]. Besides that, the highest crude ash of 49.15% was observed in 30% inoculum at Day 8. The crude fiber (%) content of the *Sargassum polycystum* after the bioprocess showed a reduction after day 8th. The decreasing of the crude fiber content could be due to the activities of cellulolytic bacteria [27]. This can be proven as during the bacterial growth part of the cell wall was converted into soluble sugars to provide energy [27]. These phenomena could be responsible for decrease in major fiber which are cellulose and hemicellulose components and can also be degraded into some monosaccharide and polysaccharides which are necessary to animals. The trend in the decrease in the crude fiber observed in all the inoculum levels with the days, but the rapid decrease was observed while inoculated with 30% level of inoculum. In the aquaculture industry, it is essential to lower down the crude fiber content in seaweed meal before uses in feed for easy digestibility.

The inoculum level play vital role in the improvement of nutritional values of *Sargassum* meal. Seed culture is important in the initiation for the faster growth of cell, propagation and enriches the substrate that used in bioconversion process. The inoculum size of 30% (v/v) that used in this experiment quite high compared to the 6% - 10% inoculum generally used in the production of bacterial biomass. The high inoculum level triggered in the consumption of substrate and also could be high proportion of mataboliaable polysaccharides in substrate [28]. PNSB *Rhodovulum sulfidophilum* cultivated in sardine processing wastewater with 15% - 20% inoculum resulted in bacterial biomass and pigments production with the reduction of chemical oxygen demand [29]. The inoculum size of 20% (v/v) used by *R. sphaeroides* cultivation and growth in soybean wastewater [30]. *R. faecalis* cultivation in chicken manure consist of 20% (v/v) and 30% (v/v) inoculum sizes resulted in the better production of bacterial cell and carotenoids than 10% inoculum size [31]. Purple non-sulfur bacterium, *Rhodobacter sphaeroides* strain UMS2 play a prominent role in improving the nutritional value of fish hatchery waste during in the bioprocess. The harvesting of bioprocess product from fish hatchery waste could be improved only with the optimum inoculum size and incubation period, 30% (v/v) and six days incubation not suitable to increase the nutritional values [32].

The present study had showed that the higher the inoculum size, the higher the nutritive value of the *Sargassum polycycstum* biomass. Using higher inoculum level has certain advantages over bioprocess, as capable to suppress other heterotrophic microbes. The inoculum size of the bacteria must be in optimum quality in order to suppress the growth of heterotrophic microbes [33]. Inoculum size greatly affects the length of lag time [34] and shorter lag phase [14]. The major improvement in the content of crude ash and crude protein were achieved by using 30% inoculum.

5. Conclusion

The purple non-sulphur bacterium, *Afifella marina* has the potentiality to improve the nutritional value of *Sargassum polycystum* in bioprocess. The optimum period for bioprocess using *Afifella marina* is within six days, as the nutritional values are statistically insignificant with day 8th. On the other hand, the higher levels of inoculum rapidly improve the nutritional values in bioprocess, but need to conduct a further experiment with the use of more than 30% inoculum level.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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