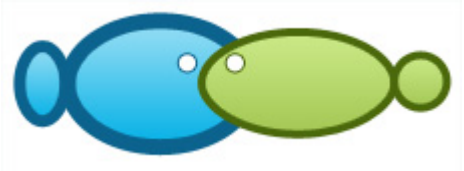

Development of aquafeed ingredient by solid state fermentation of the crinklegrass, *Rhizoclonium riparium* on a laboratory scale

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Abstract. Solid state fermentation (SSF) of the crinkle grass *Rhizoclonium riparium* var. *implexum* was done to develop it as an aquafeed ingredient. A bacterial strain, *Micrococcus flavus* (L11) or a fungal strain *Kluyveromyces* sp. (F2) or their combination (LF) were inoculated on the seaweed base material for 14 days. During the fermentation period, changes in pH, lactic acid, ethanol and total protein content were determined. Results showed that the bioconversion of crinklegrass into a fermented *Rhizoclonium* meal (FRM) was best processed by an enzyme pre-treatment using 375 U cellulase and α-amylase followed by inoculation with *Kluyveromyces* sp. (F2) and supplementation of 10% substrate under SSF conditions. At the end of the 14-day incubation, protein content significantly ($p < 0.05$) increased from 15% to 22%, accounting for 41% protein improvement. Results of the present study show that SSF of crinkle grass *Rhizoclonium implexum* can increase its protein content thus making it a potential protein supplement for aquafeeds.

Key Words: solid state fermentation, lactic acid bacteria, yeast, *Rhizoclonium implexum*, marine silage.

Introduction. Presently, macroalgae are used worldwide as an alternate protein source replacing fishmeal successfully. Several feeding trial studies in shrimps showed that many types of macroalgae have been used for increased growth (protein accretion), feed utilization, physiological activity, stress response, starvation tolerance, disease resistance, and carcass quality (Aquino et al 2014; Declarador et al 2014; Santizo et al 2014; Roy et al 2011).

Algae such as *Rhizoclonium riparium* var. *implexum* (Chlorophyceae) is a potential ingredient in aquafeeds despite the fact that it is often considered a nuisance species in fishponds because it removes dissolved oxygen at times, reduces space for cultured animals and hinders their growth (Chao et al 2005; Caffrey 1992). Studies show that it has significant amount of proteins, vitamins, and minerals (Chakraborty & Santra 2008) and has shown potential for large scale culturing year-round (Chao et al 2005).

Marine silage (MS) is a fish dietary material prepared from algae by enzymatic and microbial fermentation processes (Uchida & Miyoshi 2010). Enzymatic hydrolysis of the seaweed cell wall is considered to improve protein digestibility. Cellulase can decompose raw materials in the natural cellulose into fermentable sugars and can decompose the cell wall to release protein, starch and other nutrients in it (Han et al 2012; Behera 2013). It also makes available the degraded raw fiber into the glucose which can be digested and absorbed by livestock (Han et al 2012). Amylase, on the other hand, aids in the utilization of starch and complex polysaccharide by penetrating and

digesting them more effectively after the breakdown of cellulose (Behera 2013). Uchida et al (2004) also combined lactic acid bacteria and yeast suitable for the preparation of marine silage. Furthermore, solid state fermentation (SSF) has gained renewed interest and fresh attention of researchers due to its importance in biomass energy conservation, in solid waste treatment and in its application to produce secondary metabolites (Grover et al 2013; Elliaiah et al 2002). SSF is described as the cultivation of microorganism on moist solid substrate in the absence of free water, mimicking the natural environment of the cultivated microorganisms (Zepf & Jin 2013; Mitchell et al 2000). *Rhizoclonium* sp. can be fermented by a simple and cheap method to increase its protein content and decrease its crude fiber content (Felix & Brindo 2014; Zepf & Jin 2013). This study aims to develop a protocol for the bioconversion of crinkle grass *Rhizoclonium riparium* var. *implexum* as an aquafeed ingredient using solid state fermentation (SSF).

Material and Method

Collection and preparation of seaweed powder. This research was conducted from November 2015 until February 2016. *Rhizoclonium riparium* var. *implexum* were collected from the brackish water ponds of the Brackishwater Aquaculture Center (BAC) of the Institute of Aquaculture, College of Fisheries and Ocean Sciences (CFOS-IA), University of the Philippines Visayas (UPV) in Leganes, Iloilo. The samples were transported, cleaned from debris and washed with freshwater in the Multi-Species Hatchery of CFOS-IA, UPV in Miagao, Iloilo. The seaweeds were air-dried for 48 h, subsequently oven dried for 24 h at 60^{o}C and hammer milled into fine powder.

For the preparation of the base material for SSF, a preliminary study was conducted to determine the optimum pre-treatment conditions that will yield highest total number of particles (TNP). α-amylase from *Bacillus amyloliquefaciens* and cellulase from *Trichoderma reesei* were procured from the NIMBB-Biotech Laboratory of the University of the Philippines Los Baños. The α-amylase has an enzyme activity of 500 U mL^{-1} and activated at 40^{o}C while the cellulase 500 U mL^{-1} activity and activated at 50^{o}C. Results showed that a combination of cellulase and α-amylase enzymes at 375 U concentrations for 60 min at 45^{o}C activation condition was found to be optimal in producing the highest total number of particles in marine silage of this alga (6.08 x 10^{7} TNP mL^{-1}).

Microorganisms. The bacterial and fungal strains were isolated from the indigenous microbiota of a 14-day fermented unsterilized *Rhizoclonium* powder as the starter microorganisms in the SSF process. Isolates were presumptively identified based on biochemical tests for lactic acid bacteria (LAB) (Bergey et al 1974; Bergey 2009) and visual observation of colony shape and microscopic observation using slide culture method for fungi (Ebabhi et al 2013). *M. flavus* L11 was cultured on Difco^{TM} Lactobacilli deMan, Rogosa & Sharpe Agar, while *Kluyveromyces* sp. F2 in Malt Extract Agar. The cultures were maintained at 4^{o}C as seed culture for further use and were re-cultivated periodically.

Standardization of substrates. To standardize sugar and protein substrates, a combination of potato and soya powder, respectively, at various concentrations i.e., 2%, 5% and 10 % were added to 10mL of the base material in a 50 mL beaker keeping enzyme concentration, microbial and fungal load constant (Felix & Pradeepa 2012). L11, F2 and a co-culture of L11-F2 (LF) isolates were individually inoculated on the base material containing varied substrate concentrations. The samples were drawn from the container periodically i.e., 2nd, 4th and 6th day to monitor change in pH (HM Digital) as indicator of fermentation.

Fermentation process. The method as described by Felix & Pradeepa (2012) was adopted. The development of a fermented *Rhizoclonium* meal using solid state fermentation is shown in Appendix 1. Briefly, the present method involved two phases: (1) cellulolytic enzymatic treatment of seaweed which led to single cell units. In a 250 mL Wheaton bottle the base material, mixture of 90 mL seawater and 10g powdered

seaweed (9:1), was treated enzymatically using 375 U cellulase and α-amylase for 60 min. After digestion, the samples were autoclaved at 121°C for 15 min to deactivate the enzymes, and cooled down to room temperature. And (2) fermentative phase wherein the enzymatic digest was further treated with bacteria or yeast or their combination. Isolates were raised in their respective broths for 18-24 h and standardized with a concentration of 1.0 OD at 660 nm (Ultra-Visible Spectrophotometer), were aseptically and individually added to the base material. The optimum substrate concentration for each strain(s) was added on the base material. The same SSF set-up but without any bacterial or yeast inoculation served as control. Culture bottles were then incubated in an anaerobic jar (BBL™ GasPak™ 150 Anaerobic System, USA) supplied with an oxygen scavenger pack (AneroPack- MicroAero, Mitsubishi Gas Chemical Co., Inc, Japan). The fermentation contents were homogeneously mixed and incubated statically at room temperature for 14 days (Zepf & Jin 2013).

Chemical analysis: pH, titrable acidity, ethanol and protein content detemination. The samples were evaluated on the initial and 14th day for the fermentative phase. In every sampling period, one gram of sample was diluted with 10 mL distilled water, sonicated for 2 min and centrifuged at 2912 g for 10 min. The samples were subjected to pH determination, lactic acid concentration using determination of titrable acidity (Sanchez & Dizon 1992) and determination of ethanol concentration (Caputi et al 1968). Total protein was determined using crude protein analysis by Kjeldahl method (AOAC 2000).

Statistical analysis. Statistical analysis was performed using SPSS version 16. Data were presented as mean ± standard error of the mean (SEM) with triplicate trials for each treatment. Data were subjected to one-way analysis of variance (ANOVA) at $\alpha=0.05$. When ANOVA result showed significant difference, Tukey's test was performed to determine the differences between the treatment means.

Results and Discussion. Results of the optimization of substrate concentration is found in Table 1. The substrate (sugar and protein) concentration was found effective at 5% for *M. flavus* L11 and mixed culture LF and 10% for *Kluyveromyces* sp. F2 for the preparation of marine silage. Bubble formation and spaces in-between fermented medium were noticeable in all treatments and increased with increased substrate concentration and incubation time. Gas formation was also evident upon opening the containers in almost all treatments. The optimum substrate concentration for each microbial isolate was based on the concentration that yielded the lowest pH level.

Table 1

Effect of sugar and protein substrate concentration on pH level in fermented *R. riparium* meal

Microbial/ fungal strain	*Substrate concentration*		
	2%	*5%*	*10%*
Micrococcus flavus	5.60^{b}	5.37^{a}	5.65^{b}
Kluyveromyces sp.	5.49^{c}	5.33^{b}	5.19^{a}
Mixed	5.55^{b}	5.35^{a}	5.42^{a}

Different letters in the same row indicate significant differences ($p < 0.05$).

Characteristics of the marine silage (MS) after 14 days of fermentation are shown in Table 2. The pH value decreased from 7.0 at initial to 4.4 after 14 days of incubation, which was inversely proportional with lactic acid production, demonstrated that fermentation occurred on the fermented media. The present study showed that a single inoculation of LAB and yeast with enzyme saccharification on the base material was found effective in lactic acid fermentation/ethanol fermentation, respectively, as well as in protein enrichment yield than a co-culture of both. Usually, an increase in lactic acid or ethanol production signifies an increase in microbial/fungal growth. The SSF set-up inoculated with *M. flavus* isolate (0.90%) obtained a significantly higher ($p < 0.05$) lactic

acid production value than the set-up containing the mixed culture (0.76%). Similarly, the fermented algae with yeast strain (0.77%) reached significantly superior ethanol production than that of the LAB-Fungi co-culture (0.67%) ($p < 0.05$). It can be inferred that in the co-culture of strains, the LAB isolate might have some inhibitory factors towards the yeast isolate since both competed for the same resources or nutrients for growth. Uchida et al (2004) showed that a single use of LAB is suitable for the preparation of marine silage. However, Manpreet et al (2005) reported that of all the groups of fungi, the filamentous fungi are the major group of microorganisms which predominate in the SSF process because of their ability to spread over and to penetrate inside the solid-substrate and their fungal mycelia can synthesize and release large quantity of extracellular hydrolytic enzymes. In contrast, some studies have shown that yeast and LAB can coexist in a same medium and could even enhance the fermentation process (Felix & Pradeepa 2012; Felix et al 2005; Uchida et al 2004). LAB can produce lactic acid with high efficiency and retard the growth of contaminating bacteria while yeast helps in fermentation apart from its unique bioremediatory effect in aquaculture system (Felix & Pradeepa 2012; Felix et al 2005; Uchida et al 2004). In these combinations yeast usually grows on solid substrate as a minor member and the dominant species are bacteria (Manpreet et al 2005; Moo-Young et al 1983). In addition, the MS inoculated with bacteria, yeast or their combination was positive for gas formation on the fermented media after 14 days. This further indicates that the fermentation process occurred.

Table 2

Characteristics of fermented *Rhizoclonium* meal (FRM) prepared with different compositions of starter microorganisms after 14 days of culture

Strain code	*Starter composition*	*Characteristics of marine silage*				
		pH	*Lactic acid production (% titrable acidity)*	*Ethanol (%)*	*Gas*	*Crude protein (% dry basis)*
None	Initial	7.0	-	-	-	15.55%±0.0
L11	*Micrococcus flavus*	4.4[a]	0.90[b]	-	+	19.27±0.09[b]
F2	*Kluyveromyces* sp.		-	0.77[b]	+	21.87±0.12[c]
LF	*Kluyveromyces* sp. & *M. flavus*	4.4[a]	0.76[a]	0.67[a]	+	16.76±0.13[a]

Crude protein analysis (AOAC 1990); Different letters in the same column indicate significant differences ($p < 0.05$).

The best bioconversion performance in terms of protein enrichment was noted in the algae fermented using the yeast strain which yielded the significantly highest crude protein content in the seaweed followed by the fermented product of the bacteria and mixed culture, respectively. A 41% crude protein increase on the fermented *Rhizoclonium* meal (21.87% crude protein) using *Kluyveromyces* sp. was achieved in comparison to the meal form of this macroalgae (15.55%; Bunda et al 2015). This study showed that an increased lactic acid/ethanol production through increased microbial growth also increased protein enrichment yield on the 14th day. Microbial fermentation is capable of concentrating the protein content of algae by degrading its crude fiber content into some monosaccharides and polysaccharide prebiotics, necessary to animals, aside from being itself an added protein source in the fermented algal biomass (Han et al 2012). In the present study, microbial metabolism entered gradually into a stable period which concurs with the findings of Liu et al (2012). In their study, crude protein level of seaweed waste inoculated by microbial agents reached its peak after 15 days and gradually declined on the 20th day of fermentation because the microorganisms were in a decline phase and the number of them began decreasing. The single inoculation of yeast isolate in the present study yielded the highest protein content enrichment and the low amount of water in the fermented media due to its high substrate concentration at 10%. The type of microorganism chosen for fermentation on solid-substrates has a crucial role in SSF. Manpreet et al (2005) observe that the low amount of free liquid in the substrate affects the whole process of SSF and is the most important feature of SSF. All the factors in SSF

depend upon the amount of water present in the substrate. Due to the less availability of free water in SSF process than the majority of liquid fermentations, most of the SSF processes involve fungi, although there are number of reports involving bacteria and yeast. Bacteria are mainly involved in processes like composting, ensiling and yeasts have been used for ethanol and food or feed production (Mitchell et al 1992; Manpreet et al 2005).

Conclusions. Our findings showed that the bioconversion of crinklegrass into a fermented *Rhizoclonium* meal was best processed using a single inoculation of a yeast strain (*Kluyveromyces* sp.) with 375 U cellulase and α-amylase, 10% substrate concentration and incubated for 14 days under SSF conditions. Evaluation on the crude protein yield and the proximate composition of a seaweed meal form could be a good indicator for quick evaluation of a novel protein source until the data on ingredient digestibilities and its biological value (the result of a complete feeding trial) becomes available. It is generally recommended that a feed ingredient should have at least 20% protein for it to be considered a protein supplement while those below are considered energy sources. Enhancing the content value of the seaweed by bioconversion resulted in a 41% increase in protein. FRM contains crude protein value of 21.87% that makes it a potential protein supplement for aquafeeds.

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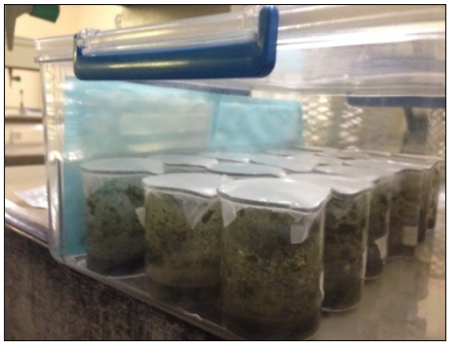
Appendix 1

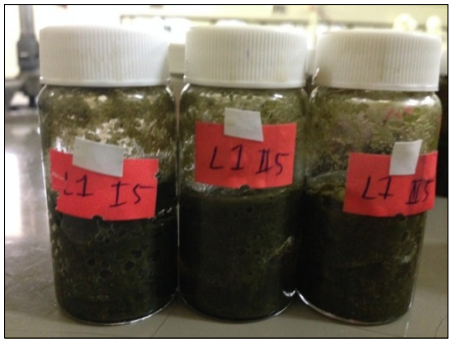
Development of protocol for fermented *Rhizoclonium* meal using solid state fermentation

Air-drying of *Rhizoclonium riparium* for 48 hrs

Rhizoclonium raw meal in powdered form



Rhizoclonium riparium powder soaked in water (1:9, seaweed: seawater) and enzyme for 2 weeks



Fermented *Rhizoclonium* with 5% protein and sugar substrate

Fermented *Rhizoclonium* samples during pre-treatment optimization

Fermentation Proper: 10 g base material incubated for 14 days in an anaerobic jar