SHORT COMMUNICATION

Preservation and extracellular enzyme production of myxomycetes from Lubang Island, Occidental Mindoro, Philippines

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In vitro culture of myxomycetes offers potential application for the mass production of their natural products. We grew *in vitro* 18 species of myxomycetes on a modified, solid, semi-defined medium (SDM) for up to 4 weeks. Of these, 10 developed into amoeboflagellates after germination on diluted semi-defined medium (dSDMA) and/or sterile water, while 7 grew into plasmodia. The amoeboflagellates were preserved in 15% glycerol and stored for 3 months at 5°C. Revival of the preserved amoeboflagellates resulted in a 90% success rate. This is the first attempt to store and preserve amoeboflagellates of myxomycetes. *In vitro*-grown plasmodia of *P. compressum* and plasmodia derived from sclerotia of unidentified myxomycetes were also tested for their ability to produce extracellular enzymes. Results showed the species excreted amylase and protease. This finding suggests an alternative mode of nutrition for myxomycetes in addition to the phagotrophic mode of nutrition known for these organisms.

KEYWORDS
amoeboflaggelate preservation, extracellular enzymes, laboratory culture, mode of nutrition, slime molds

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INTRODUCTION

Myxomycetes are good sources of natural compounds with bioactivities. Dembitsky et al. (2005) listed almost 100 natural compounds from myxomycetes. Interestingly, some of these metabolites exhibited bioactivities (Ishibashi 2005). However, these metabolites were extracted directly from fruiting bodies which entail the need for a massive amount of fruiting bodies of the same species. Alternatively, *in vitro* cultivation of myxomycetes presents a solution to this dilemma. *In vitro* culture of most, if not all, microorganisms can pave the way for the mass production of secondary metabolites. Among myxomycetes, most *in vitro* cultured species belong to the genera *Physarum* and *Didymium*. Although these two genera have served as excellent model systems for study, they give only a partial view of the biology of the myxomycetes (Aldrich and Daniel 1982; Everhart and Keller 2008). There is an imperative need to get a wide range of myxomycetes into agar culture. The success of culture methods can lead to better manipulation of the possible various products which can be derived from myxomycetes. In one of the studies about the relationship between plasmodia of myxomycetes and their bacterial associates, Ali and Kalyanasundaram (1991) found that the plasmodia of *Physarum flavicomum* and *Stemonitis herbatica* were able to degrade starch in starch agar plates, while their bacterial associates *Flavobacterium breve* and *Paracoccus denitrificans* in pure culture could not do so, proposing a dependence of the bacterial associate on the plasmodium for the utilization of a complex substrate. In this research, we report for the first time the *in vitro* culture and enzyme production of plasmodial myxomycetes from Lubang Island, Philip-

pines. We also present a method for the preservation and revival of amoeboflaggelates derived from myxomycetes.

MATERIALS AND METHODS

Myxomycetes used in the study

The following are the 18 species of myxomycetes used: *Arcyria cinerea*, *Craterium atrolucens*, *C. concinnum*, *Diderma effusum*, *D. hemisphaericum*, *Didymium ochroideum*, *D. squamulosum*, *D. leucopodia*, *Lamproderma scintillans*, *Oligonema schweinitzii*, *Perichaena microspora*, *Physarum bivalve*, *P. cinereum*, *P. compressum*, *P. echinosporum*, *P. melleum*, *Stemonitis fusca*, and *S. pallida*. These were collected from moist chambers prepared from ground leaf litter and twigs substrates collected along the coastal forests of Lubang Island and from the two slopes of Mt. Gonting in Lubang Island, Occidental Mindoro, Philippines. The complete checklist of myxomycetes collected from Lubang Island can be found in Macabago et al. (2012).

Culture of myxomycetes

Eighteen species of myxomycetes were cultivated following slight modification of Dee et al. (1997). Initially, fruiting bodies were obtained from moist chambers. Spores were then transferred to sterile tubes containing 0.5 mL sterile distilled water and were allowed to germinate for 24 hours. Upon germination, 200 µL were transferred to 1:1/17 dilution of Semi-Defined Medium (g/L composition: 10 Glucose, 10 Bacto-casitone, 2 KH_2PO_4, 1.35 $CaCl_2.6H_2O$, 0.6 $MgSO_4.H_2O$, 0.039 $FeCl_2.4H_2O$, 0.034 $ZnSO_4.7H_2O$, 3.54 Citric Acid, 0.224 Na_2.EDTA, 0.005 Biotin, and 0.04 Thiamin) supplemented with 1.5% (w/v) agar (dSDMA). The cultures were then incubated at room temperature in the dark for up to 3 days to allow the development of the amoeboflagellates. The amoeboflagellates were then purified following subculture to fresh dSDMA and cultivated under similar conditions as described previously for up to 4 weeks. After a pure culture of myxamoeba was obtained, harvesting was done by adding 5 mL sterile Bonner's salt solution (g/L composition: 0.75 KCl, 0.60 NaCl, 0.30 $CaCl_2$) onto the culture plates. The amoebae were gently scraped off from the culture media using a flame-sterilized glass L-rod. The harvested amoebae suspension was aseptically transferred to sterile Eppendorf tubes. Pure cultures of myxamoebae were further cultured on dSDMA plates for up to 4 weeks until the formation of plasmodia.

Preservation of myxomycete amoeboflagellate cultures

Following culture of myxomycetes at room temperature in the dark for 1 to 3 days as previously described, amoebae were harvested by adding 5 mL sterile 15% glycerol (v/v). The amoebae were gently scraped off, transferred aseptically to sterile Eppendorf tubes and the harvested amoebae suspension stored at 5^{o}C for up to 3 months or longer. To revive the stored amoeboeflagellate, 200 µL of the stored amoebae suspension was aseptically transferred to plates containing fresh dSDMA and incubated at room temperature in the dark for 3-5 days. After the 5th day of incubation, the culture plates were then further incubated at room temperature under diffused light. The cultures were checked every other day until the 6th week for the formation of plasmodium.

Screening of enzymatic activity of selected myxomycetes

In vitro-grown plasmodia of *Physarum compressum* and the plasmodia from sclerotia of unidentified myxomycetes were assayed for the production of extracellular enzymes. To test for agarase, 0.5% (w/v) Soft Water Agar (SWA) was prepared in 10x150 mm test tubes. To test for amylase, cellulase and protease, culture plates pre-filled with 1.5% (w/v) Water Agar (WA) supplemented with 0.5% (w/v) of either one of the following substrates: Soluble starch Agar (SA), CarboxyMethylcellulose Agar (CMA), and non-fat Skim Milk Agar (SMA), were prepared. Then, agar blocks (~0.5 mm^2) of the plasmodium were cut and aseptically placed on the center of the culture media. The inoculated culture plates and tubes were incubated at room temperature under diffused light for 5 days to allow plasmodial growth. After incubation, the SWA was observed for liquefaction indicating the production of agarase. SA and CMA were flooded with 0.1% Lugol's solution and 1% aqueous congo red solution for 1 minute, respectively. Clearing zones around the plasmodial growth indicate production of amylase and cellulase. Clearing zones on the SMA also indicate production of protease. The absence of clearing zones or liquefaction indicates negative results.

RESULTS AND DISCUSSION

Spore germination

Of the 18 species of myxomycetes tested, only 13, i.e. *C. atrolucens*, *C. concinnum*, *D. effusum*, *D. ochroideum*, *D. squamulosum*, *D. leucopodia*, *L. scintillans*, *O. schweinitzii*, *P. microspora*, *P. bivalve*, *P. cinereum*, *P. compressum*, and *P. melleum*, showed evidences of spore germination in sterile distilled water and/or dSDMA culture plates (Table 1). Ten species germinated in both sterile distilled water and in dSDMA. Only two species germinated in dSDMA alone. *O. schweinitzii* was the earliest documented species to germinate which showed disintegration of the spore wall after 18-20 hours on dSDMA. This was followed by *D. squamulosum* observed after 20 hours in distilled water, and *P. microspora* on dSDMA. Most species germinated in distilled water after 22-24 hours such as *P. compressum*, *C. atrolucens*, *P. melleum,* and *D. ochroideum*. *P. bivalve* and *P. cinereum* germinated the latest, i.e. after 24-26 hours in distilled water.

Amoeboflagellate formation

After 48-72 hours, 10 of these 13 species exhibited amoeboflagellate development (Table 1). These species were *C. atrolucens*, *D. effusum*, *D. ochroideum*, *D. squamulosum*, *O. schweinitzii*, *P. microspora*, *P. bivalve*, *P. cinereum*, *P. compressum*, and *P. melleum.* Two species were documented for the first time to show myxamoebae, i.e., *O. schweinitzii* and *P. microspora*. Brightly-colored amoebae of *P. bivalve* and *P. melleum* were also seen from the ruptured spores within 24-48 hours (Figure 1). The release of amoeboid-forms of *D. ochroideum* was observed after 36-48 hours in distilled water. In general, evidences of amoebae for all the cultured species of myxomycetes were seen upon germination up to 22-56 hours. High motility and aggregation of amoebae were seen mostly between 36 and 72 hours after incubation.

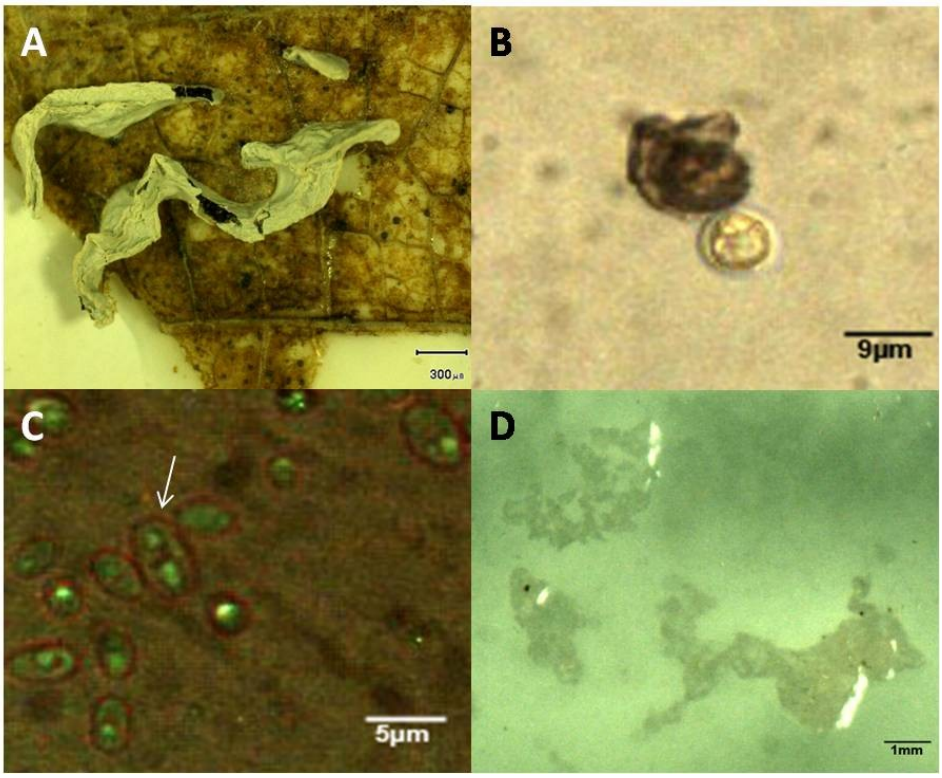

Figure 1. *Physarum bivalve* showing laterally-compressed fruiting bodies with black spore mass (A), germinating spore releasing a myxamoeba (B), growing myxamoeba (C), and developing plasmodia (D).

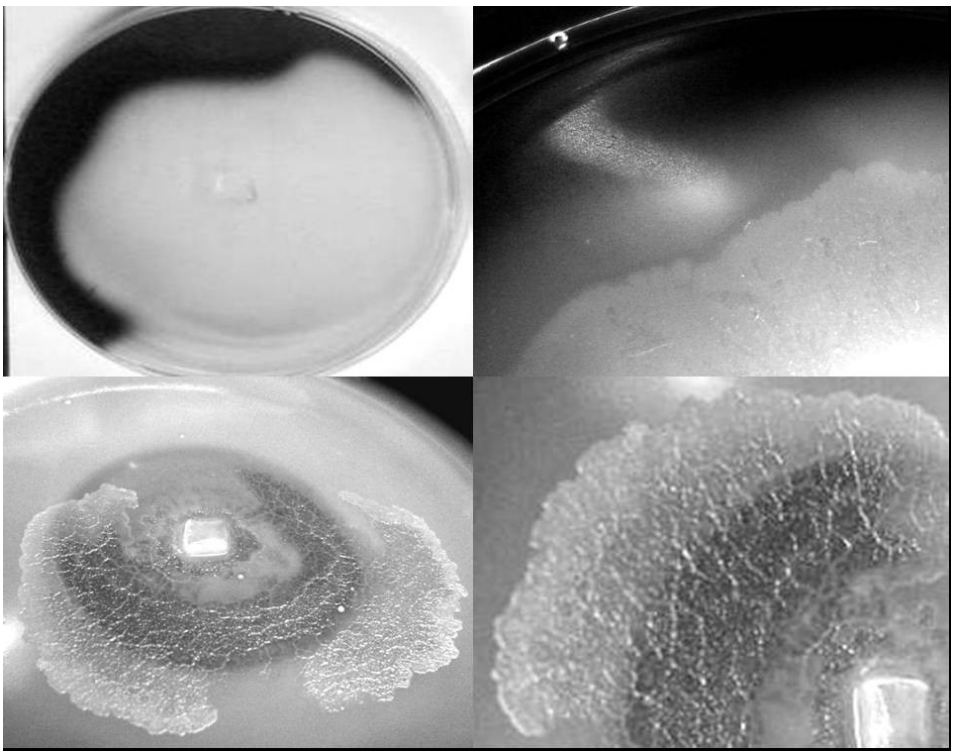

Figure 2. *P. compressum* plasmodia showing: (A) positive amylase production on starch agar plates, and (B) starch clearance around the plasmodia revealing exoenzyme production. Phaneroplasmodia of *P. compressum* showing: (C) evident clearing zone implying protein degradation, and (D) rigorous fan-like growth on SMA plate.

Table 1. Selected species of myxomycetes grown *in vitro*.

Myxomycetes	Germination	Amoeboflagellate	Plasmodia	Fruiting body
Arcyria cinerea	-	-	-	-
Craterium atrolucens	+	+	+	-
Craterium concinnum	+	-	-	-
Diderma effusum	+	+	-	-
Didymium ochroideum	+	+	-	-
Diachea leucopodia	+	-	-	-
Diderma hemisphaericum	-	-	-	-
Didymium squamulosum	+	+	+	-
Lamproderma scintillans	+	-	-	-
Oligonema schweinitzii	+[a]	+	+	-
Perichaena microspora	+[a]	+	+	-
Physarum bivalve	+	+	+	-
Physarum cinereum	+	+	-	+[b]
Physarum compressum	+	+	+	-
Physarum echinosporum	-	-	-	-
Physarum melleum	+	+	-	-
Stemonitis fusca	-	-	-	-
Stemonitis pallida	-	-	-	-

[a] These species germinated on dSDMA alone.
[b] This species formed typical fruiting body structures. However, the fruiting bodies appeared immature, and thus, could not be confirmed at the moment.

Preservation of amoeboflagellates

Preservation of the 10 species which formed amoeboflagellates (Table 1) resulted in a 90% revival rate after 3 months in 15% glycerol at 5°C. Harvested amoeboflagellates were subcultured on dSDMA plates and were observed to be viable, and further developed to plasmodia. Only *D. effusum* was not successfully revived.

Screening of enzyme production

P. compressum and 5 sclerotia from unidentified myxomycetes were induced to form plasmodia *in vitro* and tested for extracellular enzymes. Of these, only 2 (GL140C, GL173B) and *P. compressum* successfully formed plasmodia after 24-48 hours of incubation in Water Agar (WA) seeded with heat-killed *Escherichia coli* as food bacterium. Results also showed weak activity for amylase by 1 of the 2 sclerotia tested, GL173B, but a better activity for *P. compressum* (Figure 2A). The presence of a "clearing zone" around the plasmodial growth indicated that the enzyme was produced extracellularly (Figure 2B). However, all three myxomycetes failed to produce the enzymes cellulase and agarase as indicated by the absence of clearing zones on CMA and the absence of liquefaction on SWA. When tested for protease, *P. compressum* and the plasmodia from GL140C showed positive protein hydrolysis. This was observed on and/or around the plasmodial growth and indicated the hydrolysis of casein in skim milk (Fig. 2C and 2D).

Of the 980 species of myxomycetes known worldwide, only about 120 have life cycles fully described in the literature. For example, Clark et al. (2004) cultured 11 species of myxomycetes and described their reproductive systems: *Didymium dubium, D. iridis, D. vaccinum, D. ovoideum, Licea biforis, Perichaena vermicularis, Physarum didermoides, P. gyrosum, P. pusillum, Semimorula liquescens*, and *Ceratiomyxa fruticulosa*. However, not all of the illustrated life cycles have been done *in vitro*. Most of the observations were from *in situ* methods or a mixture of *in situ* and *in vitro* methods. Therefore, the *in vitro* culture of the other myxomycetes can contribute significantly to the understanding of their life cycle and in assessing their possible biotechnological applications. In this study, 13 myxomycetes successfully germinated on water and/or dSDMA plates, and were grown *in vitro* for 2 months (Table 1). Only *P. cinereum* were induced to form fruiting bodies, albeit immature. *D. squamulosum* and *P. compressum* formed visible macroplasmodia while the other species grew indistinct microplasmodia which seem to resemble protoplasmodia, a type of plasmodium characteristic of the *Echinosteliales* (Keller et al. 2008). However, no studies have been reported on the protoplasmodial development of these species *in vitro*. Interestingly, the plasmodial stage of *C. atrolucens, O. schweinitzii* and *P. microspora,* is reported here for the first time. To date, this is also the first report of *in vitro* culture of myxomycetes in the Philippines. But since it could take longer to observe the complete life stages of the cultivated myxomycetes under laboratory conditions, the research study did not grow these myxomycetes for more than 8 weeks.

Myxomycetes have a phagotrophic mode of nutrition, wherein they ingest bacteria, fungal spores, yeasts and other microorganisms, thus are termed "microbial predators" (Keller et al. 2008). However, the possibility of executing other forms of nourishment is possible, e.g. myxomycete plasmodia were found to produce amylase when growing on agar with starch (Mubarak Ali et al. 1992). In this study, plasmodia of *P. compressum* and two unidentified myxomycetes showed positive substrate degradation (Figure 2). The clearance around the plasmodial growth of *P. compressum* and GL173B indicated that the enzyme was produced extracellularly. A similar result was observed with *Physarum flavicomum* and *Stemonitis herbatica* which were able

to degrade starch and form clearing zones around the plasmodia (Ali & Kalyanasundaram 1991). However, all three myxomycetes failed to produce the enzymes cellulase and agarase as indicated by the absence of clearing zones on the CarboMethylcellulose Agar (CMA) following addition of the congo red dye and the absence of liquefaction of the Soft Water Agar (SWA) tubes. When the three plasmodia were further tested for protease activity, *P. compressum* and the plasmodia formed from the sclerotia of GL140C showed positive protein hydrolysis as shown by the clearing of Skim Milk Agar (SMA) plates. Growth of *P. compressum* on milk agar showed evident net-like growth of phaneroplasmodium characteristic of this species, comparable with the diffused growth on starch agar. This suggests that protein more than sugar caters to normal plasmodial growth. This was observed on and/or around the plasmodial growth and indicated hydrolysis of casein in skim milk. The qualitative enzyme tests in this research study showed important findings in the study of myxomycete physiology, particularly for the species *P. compressum*. There is the possibility now of an alternative mode of nutrition for the myxomycetes, apart from the accepted knowledge that they have animal-like characteristics of feeding on bacteria or protozoa (Stephenson & Stempen 1994) by engulfing them. This was indicated by the fact that extracellular enzymes, in this case, amylase and protease (caseinase) were produced by *P. compressum* and the plasmodium developed from sclerotia (GL140C). If this enzyme production can be further quantified, myxomycetes may be shown to have an absorptive mode of nutrition apart from the ingestive mode generally known for its species. The finding in this research study is also the first report of extracellular enzyme activity by the species *P. compressum.*

The preservation of myxomycetes is usually done by maintaining fruiting bodies in a herbarium. But Dee et al. (1997) preserved myxamoebae in glycerol and the myxamoebae were later used for their mass cultivation in broth media. Thus, this method seems to be a reasonable way of storing myxomycetes for future studies. Rätzel et al. (2013) also preserved myxamoebae in glycerol and were later used to screen for mutants. However, no studies were ever conducted to evaluate the effectivity of the preservation techniques employed in myxomycetes. More so, there are no studies conducted yet to confirm the viability of stored myxomycetes grown *in vitro*. Therefore, the importance of effectively reviving myxomycetes after they have been maintained in the laboratory for a long time is of great significance. In this study, the success of the preservation of myxomycetes in 3 months and their successful revival mean a good feasibility of long-term storage for *in vitro*-grown myxomycetes and the continuity of *in vitro* culture studies of myxomycetes. Although some species were not successfully revived, the high turn-out of successful preservation indicated effectiveness of the method employed (Table 1). This may open doors for further improvement of the preservation techniques of myxomycetes.

CONFLICTS OF INTEREST

None.

CONTRIBUTIONS OF INDIVIDUAL AUTHORS

Dr. Thomas Edison dela Cruz and Ms. Sittie Aisha B. Macabago were involved in the planning of the research study, collection of the substrates, identification of the collected specimens, and writing the manuscript. The experiments were performed by Ms. Macabago as part of her graduate thesis. The authors read and approved the final manuscript.

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