ISOLATION OF PIGMENTED FUNGUS FROM *LOBOPHORA VARIEGATA* (J.V.LAMOUROUX) AND EXTRACTION OF ANTIBACTERIAL ACTIVITY AGAINST ON *ESCHERICHIA COLI* AHU5436

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Abstract

In this investigation, the isolation of pigmented fungus has been conducted from brown seaweed. The samples of brown seaweed species, *Lobophora variegata* (J.V.Lamouroux) was collected the natural beds from the rocky shore of Minn- Land coastal area, Ngwe- Saung region, Pathein township. The isolation of pigmented fungus was carried out by Washing method. During the preliminary study of antibacterial activity on *Escherichia coli* AHU5436, the paper disc diffusion assay method was used to check the activity of the fermented broth. The filter paper and four solvents such as 20% NH_4CL, water saturated n-butanol, n-butanol- acetic- water (3:1:1) and water saturated ethyl acetate were used for Paper chromatography according the method of Tomita,1988. Antibacterial metabolite was extracted with ethyl acetate from the fermented broth based on the results of (R_f value) paper chromatography.

Keywords: antibacterial activity, *Escherichia coli*, extracted, fermented broth, *Lobophora variegata*, pigmented fungus.

Introduction

Marine ecosystems cover about 70% of the planet surface and are still an under exploited source of useful metabolites. Among marine organisms, marine algae and seaweeds are rich sources of bioactive and their value as a source of novel substances has grown rapidly in recent years. Seaweeds are one of the major producers of marine ecosystem, found almost in all part of the coastal regions around the globe. They are defined as evolutionarily diverse assemblages of marine photosynthetic, non-vascular macro-algal forms, inhabiting the littoral zone in sea, which vary in their color, shape and size. The size of marine algal forms may be very small (few mm) or up to several centimeters. Seaweeds fall under three different categories including green (chlorophyceae), red (rhodophyceae), and brown (phaeophyceae). The characteristic colors of seaweeds are due to different pigmentation. Seaweeds are macroscopic benthic algal forms, different from microscopic algae and constitute one of the highest productive ecosystems. Macroalgae, also known as seaweeds, are conspicuous and dominant features in marine ecosystems. They differ from other plants, in that algae lack roots, leafy shoots, flowers, and vascular tissues (Suryanarayanan, T.S, 2012).

Seaweeds are the most primitive group of vegetation and they have gained great importance as a promising source of bioactive compounds that can be used for health-promoting substances such as polysaccharides, proteins, peptides and polyphenols, pigments, dietary fiber, omega-3 fatty acids, and vitamins with antibacterial, antiviral, and antifungal properties. (Hamed et. *al*., 2015). Bioactive compound in the seaweeds, algae, and fungi can be procured by employing many different processes and methods (Belattmania et. *al*., 2016). In this study period, marine algae especially brown algae *Lobophora variegata* (J.V.Lamourous) has been used for the isolation of marine pigmented fungus and its antibacterial activity on test organism *Escheria coli* AHU 5436. The present study was also focused on the investigation of fermentation period and the extractions of antibacterial activity were also studied.

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Materials and Methods

Sample Collection

Plants of the species *Lobophora variegata* (J.V.Lamourous) was collected in the form of live materials growing the natural beds from the rocky shore of Minn Land coastal area (Lat 16˚ 54.7564″ N ,Long 094˚ 89.754″ E), Ngwe - Saung, Pathein Township, Ayeyarwady Region. The samples were carried by ice box with natural seawater of salinity30 **‰**.The samples were identified based on the external morphology, Herbarium specimens, literatures and other related voucher specimens. The collected samples were photographed by using digital camera and the fresh samples were used for the isolation.

Figure 1 Map showing the location of sample collecting area

Isolation of Pigmented Fungus

In this study, the isolation method was carried out with the following scheme (Inaba and Ando 2004). In the first step, prepare the GPY agar medium, glucose 1.0g, polypeptone 0.5g, yeast extract 0.5g and agar 2.0 g and seawater 1ml were placed in a conical flask. Then 100ml of sterile distilled water was added to the medium. The flask was plugged with cotton wool and sterilized by autoclaving. After this the medium was poured into the plate near the flame of a spirit burner in the culture chamber. In the second step, the plant samples were washed with tap water for removing sands and then cut into smaller pieces. After cutting pieces, the samples were washed again with sterile distilled water and dipped into 70% methanol in 1minute for removing external dusts including unwanted microbes. After 1 minute, the pieces were rinsed with sterile distilled water and dry on tissues paper. After drying, they are plated on agar medium and incubated for 7 days. The isolated fungus was stored in PDA medium (Potato 20g, Dextrose 1.5g, Agar 1.8g and DW 100mL).

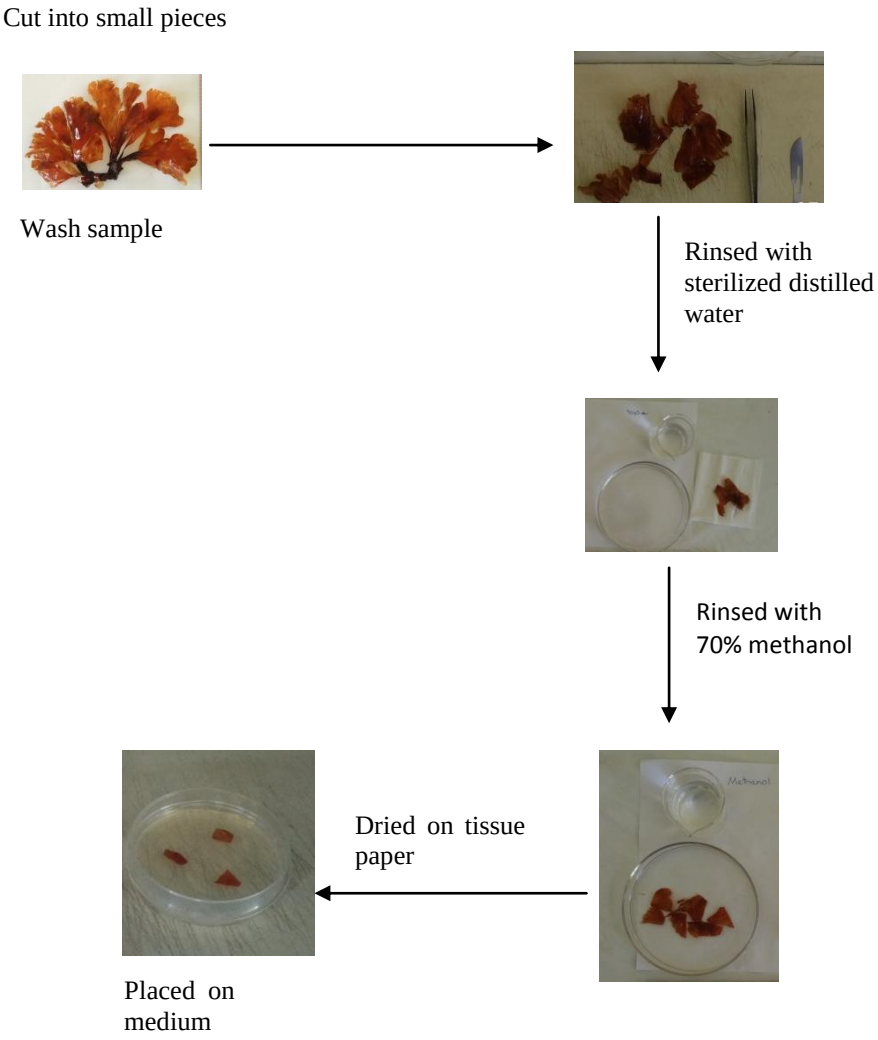


Figure 2 Preparation of isolation for pigmented fungus.

Preparation of fermented broth cultures for screening of antibacterial activity

The seed medium was prepared by mixing glucose 1.0g, polypeptone 0.3g, KNO_3 0.1g and K_2HPO_4 0.01g in a conical flask. Then 100 ml of sterile distilled water was added. After this, every 25 ml of the medium was poured into the 50ml conical flask. Then the flask was plugged with cotton wool and sterilized by autoclaving. The isolated fungus was grown on GSY medium in 7 days for sporulation. Then isolated fungus was inoculated on seed medium in the culture chamber for 3 days. After incubation for 3 days, the seed culture was transferred into the conical flask containing the fermentation media glucose 1.0g, yeast extract 0.5g ,$CaCo_3$ 0.1g, K_2 HPO_4 0.001g and $MgSO_4$ 0.001 were put in a conical flask with 100 ml distilled water . The fermentation period took 10 days. During the fermentation, the fermented broth was used for the paper disc diffusion assay.

Screening the antibacterial activity of the fermented broth by paper disc diffusion assay

The paper disc diffusion assay method (Tomitta, 1988) was used to check the activity of the obtained fermented broth. Test organisms *Escheria coli* AHU 5436 were used to test the activities. These test organisms were inoculated in 20ml of assay broth in conical flasks and incubated overnight. To get assay media, glucose1.0g, yeast extract 0.3and agar 2.0g were placed

in a conical flask and then 100 ml distilled water was added to obtain complete assay medium. The flask was plugged with cotton wool and sterilized by autoclaving. After this the assay medium was cooled. The one percent of test organisms was added to the assay medium, and then poured into plates in the culture chamber. After solidification, paper disc impregnated with samples were applied on the agar plates and the plates were incubated at room temperature for 24hours. Clear zones (inhibitory zones) around the test discs indicate the presence of antibiotic (bioactive compounds) which inhibits the growth of the test organisms selectively.

Preliminary Characterization of Metaboliteas by Using Paper Chromatography

Paper chromatography was carried out by the method of Tomita, 1988. The filter paper (Toyo Advantec, Japan) and four solvents such as 20%NH_4Cl, water saturated n-butanol, n-butanol- acetic acid- water (3:1:1) and water saturated ethyl acetate were used for preliminary characterization of metabolites. The fermented broth samples (100µl) were applied on the papers and allowed to dry. The papers were chromatographed in each solvent. Then, bioautography was done to check the antibacterial activity of each. Each paper was placed on assay agar plates. After one hour the paper was taken out, and then the plates were incubated for 24 hours. According to the inhibitory zone, R_f value was calculated for the corresponding metabolite.

$$R_f = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent}}$$

The Effects of pH and the Extraction of Antibacterial Metabolite

Fermented broth (each 10mL) was adjusted at pH (5,6,7 and 8) using sodium hydroxide (NaOH) and dilute Hydrochloric acid (HCl). By using paper disc diffusion assay method, the inhibitory zones were determined to obtain the effects of pH for the extraction. Antibacterial metabolite was extracted from the fermented broth based on the results (R_f value) of paper chromatography -bioautography.

Figure 3 Extraction of antibacterial compound

Results

Scientific classification

Phylum Phaeophyta
Class Phaeophyceae
Order Dictyotales
Family Dictyotaceae
Genus *Lobophora*
Species *Lobophora variegata* (J.V. lamouroux,1817)

Figure 4 External morphology of *Lobophora variegata*

Description- Plants orangy to dark brown colour, 7-10 cm broad, prostrate, overlapping in clusters, upper potions entire, lobate with variegated markings in concentric zones, thalli circular to fan shaped, attached by rhizoids arising from the basal parts of the fronds, grows intertidally or in shallow water in tropical and warm temperate seas.

Isolation of Marine Pigmented fungus

The morphological characteristic and photomicrograph of pigmented fungus isolated from *L.variegata* was shown in figures. The front view of isolated fungus is white color and the back view is red.

front view back view

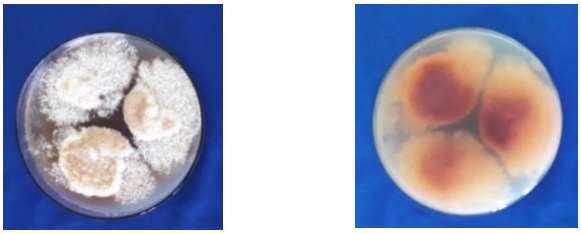


Figure 5 Morphological characteristic of front view and back view of isolated fungus from *L. variegata*

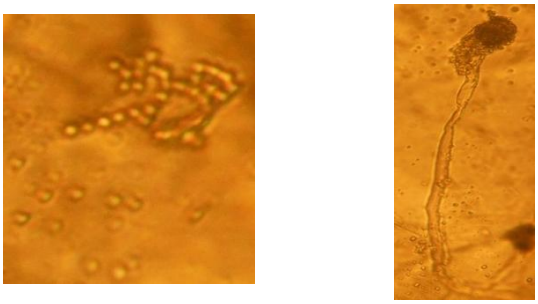


Figure 6 Photomicrograph of isolated fungus from *L. variegata*

Antibacterial Activity Against on *E.Coli* AHU5436

In the investigation of antibacterial activity, fermentation period 5, 6, 7 and 8days showed the activity against on test organism *E.coli* AHU5436. The 6 days of fermentation period showed the best activity on *E.coli* AHU 5436. The detail results were shown in Table 1 and Figure 7.

Table 1 Antibacterial activity of ten days fermentation period

Fermentation period	Clear zone(mm)
1-4 days	no activity
5day	18.42
6day	**20.06**
7day	15.48
8day	13.74
9-10days	no activity

Test organism----- *E.coli* AHU5436.

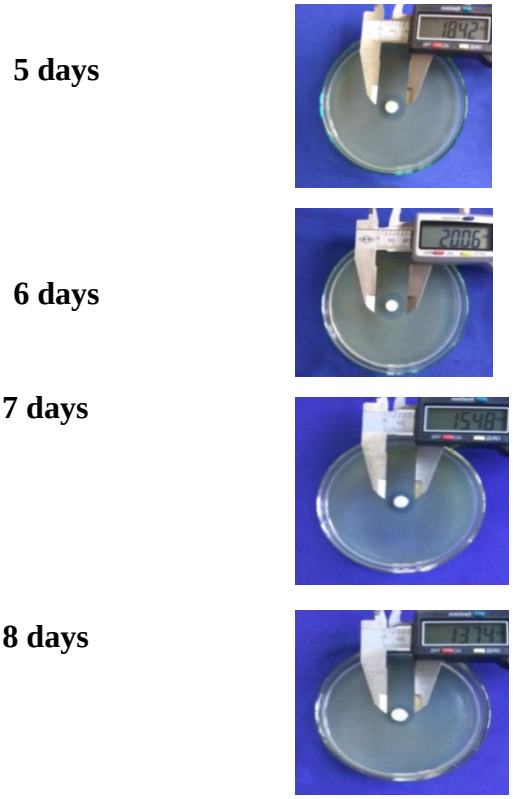


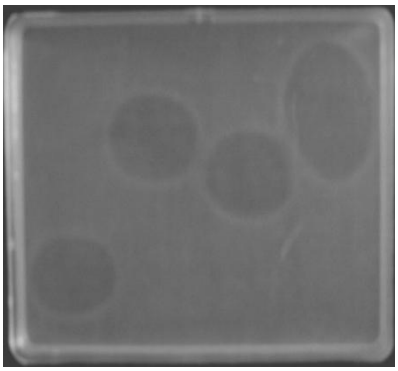
Figure 7 Fermentation period of antibacterial activity on *E.coli* AHU5436

Preliminary Characterization of Metabolites by using Paper Chromatography

According to the R_f values, it was considered that antibacterial metabolite is soluble in solvent No.4, ethyl acetate. Therefore, solvent No.4, ethyl acetate is suitable for the extraction of antibacterial metabolite. The results were showed in Figure 7.

Table 2 Solvent system and the result of R_f value

No	Solvent	R_f value
1	20% NH_4Cl	0.16
2	Water saturated *n*-BuOH,	0.6
3	n-BuOH-acetic acid- water	0.5
4	Water saturated ethyl acetate	0.8

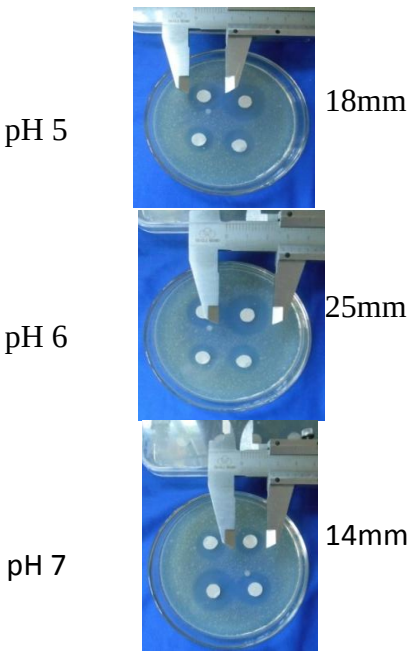


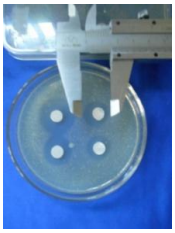
1 2 3 4

Figure 8 Bioautographic overlay assay

The Effects of pH and the Extraction of antibacterial metabolite

In the study of the effects of pH for the extraction with ethyl acetate, it was found that the fermented broths with adjusted pH exhibited the activities. However, pH 6.0 condition showed the best activity. Therefore, it was determined that pH 6.0 was suitable for the extraction of metabolite from the fermented broths with ethyl acetate. Five liters fermented brotwas used for the extraction of antibacterial metabolite from the isolated fungus.



pH 7  15mm

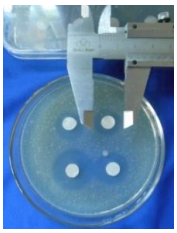
pH 8  14mm

Figure 9 The effects of pH for the extraction with ethyl acetate

Discussion

Marine fungi are potential producers of bioactive compounds that may have pharmacological and medicinal applications. The marine environment is extremely complex and contains a broad spectrum of fungal diversity. Fouillaud, M. et.*al*, 2016 state that the variety of new marine-derived fungal genera have been identified and evaluated, numerous marine fungi are identical to terrestrial fungi, e.g., *Aspergillus* spp., *Cephalosporium* spp, *Fusarium* spp, or *Penicillium* spp. Mayer et.*al*, 2013, report that the significant involvement of fungi in the industry, through the production of various useful substances, such as antibiotics, immune suppressants, anti-cancer drugs, plant hormones, enzymes, acids and also natural pigments. Zhang et.*al*, 2012 mentioned that marine derived fungi are regarded as a potential bright sources of new molecules with likely application in pigment production. Many genera producing pigments have been isolated from water, sediments, and decaying organic residues, or from living organisms such as invertebrates, plants or algae. This study initiated the search for pigmented fungi from marine brown algae *L.variegata* from Minn- Land coastal area, Ngwe-Saung region, Pathein township. The studied of antibacterial activity against on *E.coli* 5436 and the extraction of antibacterial metabolites with ethyl acetate for the results of Paper Chromatography bioautographic overlay assay.

Conclusion

In the screening of pigmented fungi, only one species was isolated from marine brown algae *L. variegata.* During the study of preliminary antibacterial activity, fermentation period was carried out 10 days by using on test organism *E.coli* 5436. The starting of fermentation period 1, 2, 3 days and the ending of fermentation period 9, 10 days do not showed the activity. The fermentation period 5, 6, 7 and 8 days showed the activity against on *E.coli* 5436. The fermentation period 6days activity showed the best in this study. In the using of four solvents for Paper chromatography method, solvent No.4 ethyl acetate showed the best R_f value. Therefore this solvent was used for the extraction. In the extraction of antibacterial metabolite, the effects of pH were checked by using sodium hydroxide (NaOH) and dilute hydrochloric acid (HCL). According to the pH results, pH 6.0 was more suitable for the extraction of antibacterial metabolites with ethyl acetate.

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References

Ando, K. (2004), **Isolation and identification of fungi**, *Workshop , BRBDC- Pathein University.*

Belattmania, Z.; Engelen, A.H.; Pereira, H.; Serrão, E.A.; Barakate, M.; Elatouani, S.; Zrid, R.; Bentiss, F.;Chahboun, N.; Reani, A.; et al**.** (2016), **Potential uses of the brown seaweed Cystoseira humilis biomass: 2-Fatty acid composition, antioxidant and antibacterial activities**. *J. Mater. Environ. Sci*, *7*, 2074–2081

Coppejans, E., Prathep, A.; Leliaert, F.; Lewmanomont, K.; Clerk, O.D, ;(2010) **Seaweeds of Mu Ko Tha Lae Tai (Se Thailand);** Methodologies and field guide to the dominant species

Fouillaud, M.; Venkatachalam, M.; Girard-Valenciennes, E.; Caro, Y.; Dufossé, L. (2016) **Anthraquinones and derivatives from marine-derived fungi: Structural diversity and selected biological activities.** *Mar. Drugs* , *14*, 64.

Hamed, I., Özogul, F., Özogul, Y., and Regenstein, J.M. (2015). **Marine bioactive compounds and their health benefits:** a review. Compr. Rev.Food Sci. Food Saf. 14: 446–465.

Inaba, S and K. Ando. (2004): **Washing method for the isolation of endophytes**.

Mayer, A.M.; Rodríguez, A.D.; Berlinck, R.G.; Fusetani, N. Marine pharmacology in (2008) **Marine compounds with antibacterial, anticoagulant, antifungal, anti-inflammatory, antimalarial, antiprotozoal, antituberculosis, and antiviral activities; affecting the immune and nervous system, and other miscellaneous mechanisms of action.** *Comp. Biochem. Physiol. C Toxicol. Pharmacol.***2011**, *153*, 191–222.

Soe-Htun, U. (2005). **Distribution and potential uses of marine algae found along the Coastal Regions of Myanmar**, Department of Marine Science, Mawlamyine University. *Tech. Rep*., 5pp.

Suryanarayanan, T.S. (2012) **Fungal endosymbionts of seaweeds**. In *Biology of Marine Fungi*; Springer: Berlin, Germany,.

Tomita, F. (1988), **Fermentation method and paper chromatography**, Hokkaido University, Japan.

Zhang, C.; Kim, S.K. (2012) **Application of marine microbial enzymes in the food and pharmaceutical industries**. Adv. Food Nutr. Res., 65, 423–435.