Culturing Cellulolytic Fungi in Sea Water

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Abstract- This study aim to find the colonizing ability of cellulose degrading fungi which were derived from terrestrial sources, in sea water with salinity of 34‰, which can be used in degradation of marine algal debris in order to use as fertilizer.
Algal debris is rich in minerals but due to its salt content it takes relatively longer time to degrade by microbes. Here the fungus *Cladosporium* sp. depicted the highest activity of 64.01 *FPU/ml*. which was 20.74 FPU/ml in CBM medium on the same substrate; this shows their preference as well as tolerance to marine environment. While *Helminthosporium* sp. showed the activity of 60.65 FPU/ml which is also relatively higher than that of CBM medium, activity of fungal crude enzymes was evaluated in different temperature and varying pH medium, the optimum temperature for all given strains was 50^0C while pH vary.

Index Terms- Biodegradation, cellulose, Fpase, fungi, enzyme, sea water, halophilic, salinity.

I. INTRODUCTION

Fungi are highly insubordinate microorganisms as they can prevail in many vast rage of environments. As in insects presence of chitin in their cell wall gives more rigidity for their cell surface. In addition fungal enzymes also can tolerate considerably broader temperature variations, as decomposers their enzymes are extracellular and have the ability to function any relevant substrate. Moreover many terrestrial fungal species have shown the ability to retain the same efficiency of degrading in marine environments; many of the species of the genera are facultative halophiles such as *Aspergillus* sp. and *Cladosporium* sp.and *Trichoderma* sp. [1, 3]. Even in hyper saline environment like Great Salt Lake and Dead Sea many of the genera of terrestrial origin exists. In a study among 100 samples of water from dead sea 68 were positive [2]. It is also reported *Cladosporium* sp. was found on sub merged pine at Great salt lake at high salt concentration of 290-360‰ (parts per thousand) which is ten times higher than the salinity of sea water used here and *Cladosporium cladosporioides* was found in dead sea ecosystem where salinity is 300‰ [4]. Main purpose of the work is to culture the cellulose degrading fungi from terrestrial sources in sea water and examine their enzyme activity which can be used in production of compost using the algal debris from the sea, since algae is rich in minerals, but their degradation is relatively slow due to its high salt content.

II. Research Elaborations

2.1 Source of fungi and initial culturing

Samples were collected from sawdust, straw dust and sprinkled soil (garden, beach, Mud). Decaying wood particles and decaying leaf collected from the surroundings. Samples were collected into sterile containers and stored separately. Potato dextrose agar medium was used to grow the initial cultures, where samples were cultured by streak plate method and sprinkle method (Figure 1).

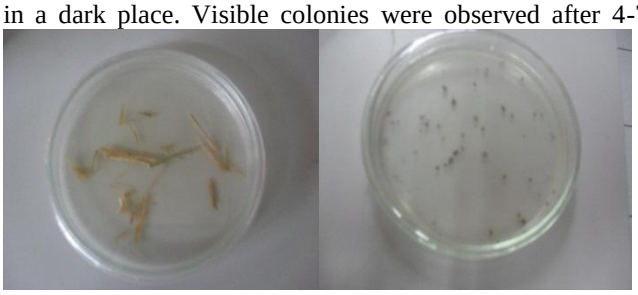
2.2 Methods of inoculation

2.2.1 Streak plate method

In order to isolate the fungi this method is best in practice. Initial streak is made with the sample then all other streaks are continuum of the previous strike using separate sterile tooth peck for each strike.

2.2.2 Sprinkled method

This is more suitable for the soil samples and sawdust. Particles were sprinkled on the medium. Well-spaced sprinkled particles would result in separation of colonies. Finally, petri-dishes were sealed with Para film, labeled and inverted dishes were incubated in a dark place. Visible colonies were observed after 4-7days.



Straw sprinkled Sawdust sprinkled

Figure 1: Inoculation of samples for the first time on PDA medium by sprinkle method

2.3 Media used for sub culturing

2.3.1 Selective water agar medium

To separate the fungi causing cellulose digestion, a medium consisting cellulose as a sole carbon source was prepared. For cellulose whatman no. 1 filter paper made of 100% cellulose was used. Water agar medium was prepared by dissolving 4g of agar in 250ml of distilled water. Autoclaved pieces of filter paper (1cm×1cm) were used for inoculation.
After pouring the water agar medium on petri dishes, paper strips were carefully placed on top of the agar bed by a sterile forceps. After the inoculation, sealed dishes kept for 4-7 days of incubation. To avoid bacterial growth, antibiotic was added to the medium. This made the medium more selective to fungi.

Table 1: Antibiotics and their concentrations that used in the culture

Antibiotic	Ampicillin	Tetracycline
In a ml of stock solution	50mg/ml	100mg/ml

Final concentration in a ml of water Agar medium	50µg/ml	100µg/ml

Table 1 depicts the amount of antibiotics that were added for the preparation of selective water agar medium. Sub culturing continued until the pure culture was available.

2.4 Culturing fungi in liquid cellulose basal medium on filter paper substrate and preparation of crude enzymes.

In order to extract the secreted cellulolytic enzymes by each fungal colony, it is important to culture them in a liquid medium. Cellulolytic basal medium (CBM) was chosen for this purpose. Cellulolytic basal medium (g /250ml in distilled water) was prepared [7].

Diammonium tartrate ($C_4H_{I2}N_20_6$) 1.5

Potassium dihydrogen phosphate (KH_2P0_4) 0.25
Yeast extract 0.02
MgS04• 7H20 0.15
CaC12.2H20 0.0002

CBM medium was autoclaved and 10ml aliquots were transferred to sterile 20ml bottles.

Cotton wool strips of same amount were submerged into the CBM medium aseptically inside the laminar airflow. Samples which were previously obtained from pure culture were inoculated by streaking on the submerged cotton strip aseptically by sterile tooth peck. Always a control bottle was kept without inoculation. Caps of the bottles were loosely fitted to allow the adequate air exchange. All the bottles were incubated for 4 days at room temperature (25^0C). After the incubation, observations were made, and the liquid medium, which contains the crude fungal enzymes, was collected.

Each bottle contains the cellulolytic enzymes that were secreted as extra cellular enzyme by each fungus colony. At the time of isolation, which is after a period of incubation it is better to vortex in slow speed in order to ensure the distribution of enzymes all over the liquid medium. About 1ml of aliquot was taken by sterile micro pipet and placed into the 1.5ml sterile centrifuge tubes. Centrifugation was done at 12,000 r.p.m for 15 minutes. The resulted supernatant consists of proteins that are mostly the fungal crude enzymes. Centrifugation is important for the separation of fungal spores since spores could not be allowed in the later filter paper assay.

2.5 Filter paper Assay

International Union of Pure and Applied Chemists recommended filter paper assay (FPA) as the standard measure of cellulase activity. Enzymatic reactions often occur in the presence of buffer, which helps to keep the reaction environment stable. This is obtained by maintaining the ionic balance and the pH unchanged. 2ml 0.05M of Trisodium citrate dihydrate (C6H5Na3O7•2H2O) buffer was used with the crude enzymes and filter paper strips (0.5cm×0.1cm) were used as substrate.

0.1ml of crude enzyme of each fungus was added with 0.15 ml of Trisodium citrate dihydrate solution, while the pH was maintained at 4.8. Always a blank was maintained in one tube without adding any fungal enzymes. Instead, it was replaced by same volume of sterilized distilled water. Then the Whatman no. 1 filter paper strip (0.5cm×0.1cm) was added as the substrate. Each tube was then incubated in 50^0C shaking incubator running at 100 r.p.m [5].

2.6 Measuring the activity of cellulolytic enzymes from liquid cellulose basal medium

The activity of extracted fungal enzymes can be quantitatively measured. Here the concentrations of reducing sugars (products of enzyme activity) were measured using DNS reagent test since the optical absorbance can be more accurately measured numerically using spectrophotometer at 540nm.

2.6.1 Dinitrosalicylic acid method.

Dinitrosalicylic acid reagent was prepared by adding 1g 3, 5-dinitrosalicylic acid in 50ml of distilled water. 200mg crystalline phenol (optional) and 30g of Sodium potassium tartrate were added to the solution, which turns the solution into yellow colour. To this, 20ml of 2N NaOH was added. This turns the colour of the solution into transparent orange yellow. Finally, the stock was made into 200ml by adding distilled water. Stock was stored at 4°C in refrigerator, to prevent deterioration [9].

After the incubation, filter paper strips were carefully removed from the tubes using a glass rod. Then 0.5ml of DNS reagent was pipetted into each tube. This terminated all enzymatic reactions occurred in the tube. Then the lids of tubes were tightly closed, and placed in a water bath at 95-100^0C for 10 minutes. After this, tubes were immediately transferred into an ice cold bath and kept for few minutes. 1ml of distilled water was pipetted into each tube before measuring the optical absorbance.

Colour change in each tube was measured by using UV spectrophotometer at 540nm wavelength. Finally, the optical absorbance readings were compared and plotted with the standard glucose curve to find the glucose (product) concentrations [9].

From each glucose and buffer mixture, 0.1ml of solution was added to 0.15ml of Trisodium citrate dihydrate buffer solution. Then each centrifuge tube was transferred into a water bath where tubes were incubated at 50^oC temperature for an hour, same as the conditions given for the enzyme filter paper assay. After the incubation, 0.5ml of DNS reagent was pipetted into each tube and the lids of all tubes were tightly closed. Then the temperature in the water bath was raised to 95-100^oC and kept for 10 minutes. Finally, the tubes were immediately transferred into an ice cold bath for few minutes and 1ml of distilled water was pipetted to each tube before measuring the absorbance of optical absorbance, and the samples were examined for the colour change.

Colour change in each tube including the control blank was measured by using UV spectrophotometer at 540nm wavelength. Finally, the optical absorbance readings were plotted against the concentration of glucose.

Table 2: Glucose concentration vs. Optical absorption at 540nm.

Glucose concentration	Optical absorption
3.35mg/0.5ml	0.766
2.50mg/0.5ml	0.580
1.65mg/0.5ml	0.378
1.00mg/0.5ml	0.228

As given in the table 2 optical absorbance differ according to the concentration of glucose, this is ranging from 1.00mg/0.5ml to 3.35mg/0.5ml resulted in optical absorbance ranging from 0.228 to 0.766 respectively.

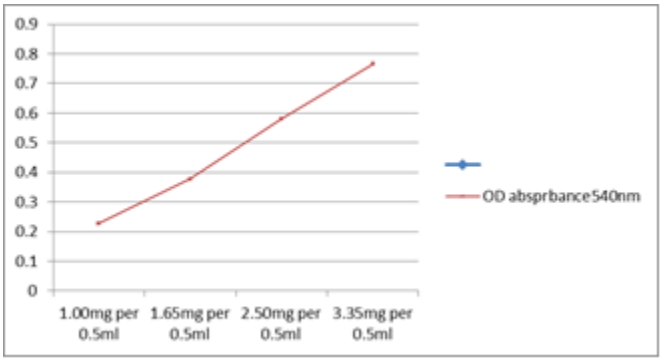


Figure 2: Glucose concentrations vs. Optical absorbance (540nm): - standard glucose plot.

This standard curve (Figure 2) was used to find the unknown concentrations of reducing sugars in all samples, dilutions used were translated into enzyme concentrations. Concentration of enzyme which would have released exactly 2.0 mg/ 0.5ml of glucose by means of a plot of glucose liberated against the logarithm of enzyme concentration was estimated.

Filter paper unit was calculated according to IUPAC-FPU. As given below:

$$\text{FPU} = \frac{0.37}{\text{Enzyme concentration to release 2mg glucose}} \text{units} \bullet \text{ml-1}$$

This quantitatively shows the activity of cellulolytic enzymes as given in Adney and Baker, 1996.

Percentage of saccharification of cotton wool by each enzyme is calculated by using given formula.

Saccharification(%) ={Glucose (mg/0.5 ml) / Substrate (mg/0.5 ml)}x 100

Since Whatman no.1 filter paper consist of 98% cellulose, substrate concentration in 0.5ml can be derived as 49mg (cellulose). By applying the product (glucose) concentration retrieved from the standard glucose curve the percentage of saccharification was calculated.

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2.7 Culturing fungi in sea water medium

Fungi samples were cultured in sea water medium using Whatman no. 1 filter paper strips (1cm ×2cm) as substrate to check its ability to grow in marine environment that is highly saline. Here the CBM medium was replaced by seawater which is autoclaved and aseptically transferred into 20ml sterile bottles. Then autoclaved filter paper strips were carefully submerged inside the medium of sea water and inoculations were made by using sterile toothpicks. Lids of the bottles were loosely closed to ensure the airflow. After 4 days of incubation at room temperature (25^{o}C) crude enzyme extracts were assayed on filter paper at 50^{o}C for an hour and products were measured using DNS reagent test. Concentrations of reducing sugar were obtained from the standard glucose curve and, finally activity was calculated as FPU/ml. (Figure 3).

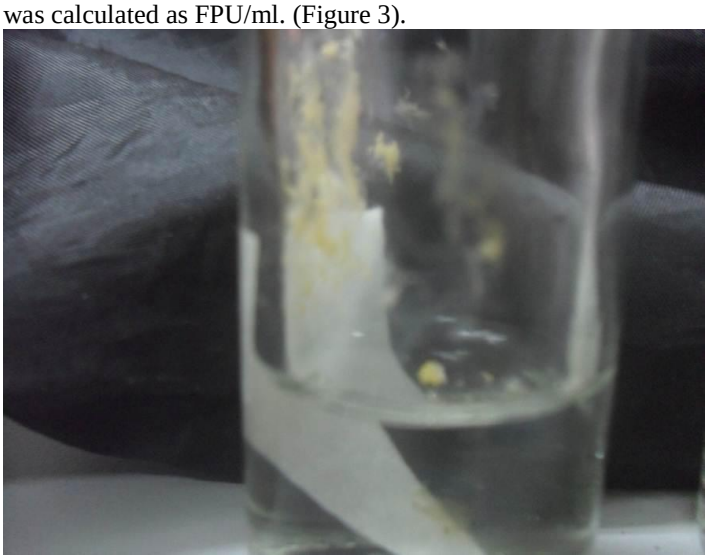
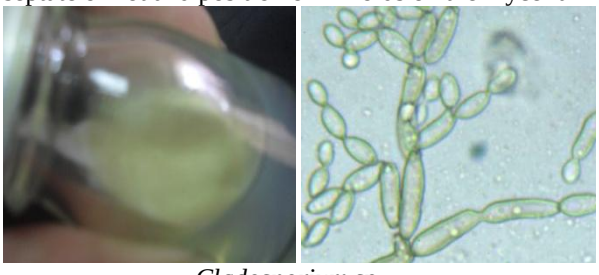


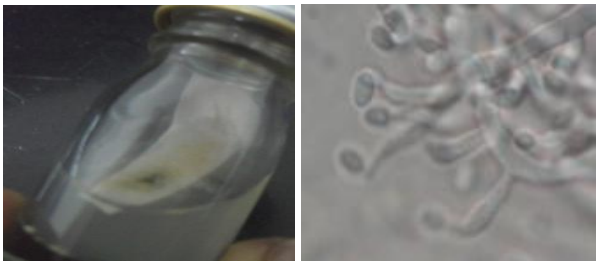
Figure 3: Colony of *Trichoderma* sp on filter paper strip immersed in Sea water.

2.8 Identification of fungi

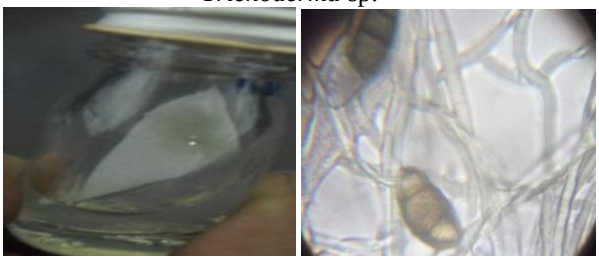
In order to identify the fungal colonies colony colour, shape, border, and spots (if the spores are available) were recorded as given in table 4.2. Microscopic visuals were observed under high power (40×10) oil immersion objective. Spores and the mycelia were observed so clearly (Figure 4), and the data were recorded and used in classification. Fungi were classified up to the genus level by their morphological features. Classification was based on microscopic observation of mycelia as well as reproductive structures such as spores and fruiting bodies, if available. Characters used in classification were compared by considering mycelial characters such as presence of septa, whether mycelium was branched or not, on mature colonies the presence of reproductive structures such as sporangia, conidia and their morphology, types of spore they generate, whether spores are septate or not and position of rhizoids on the mycelium etc.



Cladosporium sp.



Trichoderma sp.



Helminthosporium sp.

Aspergillus sp.

Figure 4: Fungal samples

2.9 Effects of pH and Temperature on fungal enzyme activity.

Enzyme activity related to variation in temperature and pH were measured separately. Filter paper assays of each fungal crude enzyme were kept in water baths at temperatures of 37^0, 50^0 and 60^0C. After an hour of incubation DNS reagent test was done.
Similarly, pH of the each buffer solution was changed to 3, 6, 8, and 13 by adding either dil NaOH or dil HCl. Then crude enzyme of each fungus was added and filter paper strips were placed. Assay was incubated at 50^0C for an hour and products were measured using DNS reagent test. Concentrations of reducing sugars were obtained from the standard glucose curve and, finally activity of enzymes was calculated as FPU/ml.

III. Results and discussion

3.1 Comparison of the enzyme activity of fungi in Sea water medium with that of CBM.

According to the results (Figure 5), *Cladosporium sp.* showed an increased level of activity of 64.04 FPU/ml which is vastly higher than its previous observation that is 20.74 FPU/ml in CBM medium on the same substrate. And comparatively *Cladosporium* sp. showed the lowest activity among all four samples in CBM but in sea water it showed the highest enzyme activity. This showed it may have some preference to salinity of the sea as well as tolerance, It is also reported *Cladosporium* sp. was found on sub merged pine at Great salt lake at high salt concentration of 290-360‰ (parts per thousand) which is ten times higher than the salinity of sea water used here [4] and *Cladosporium cladosporioides* was found in dead sea ecosystem where salinity is 300‰ [8]. In addition, *Helminthosporium* sp. and *Trichoderma* sp. also depicted higher tolerance as their enzyme activity was 60.65 and 53.24 FPU/ml respectively, whereas *Aspergillus* sp. depicted relatively lowest activity of 41 FPU /ml. None of the sample is sensitive to the salinity of sea water.

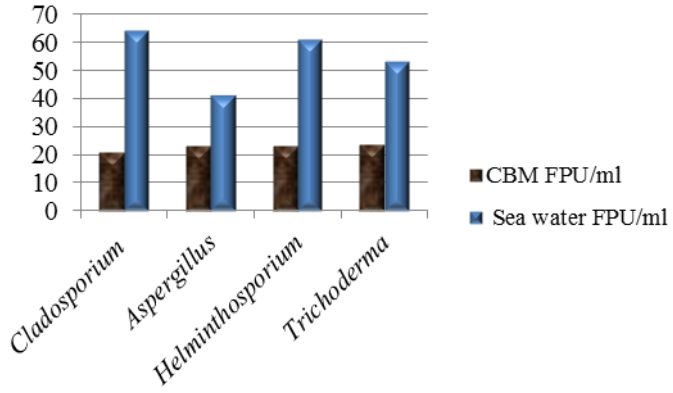


Figure 5: Comparison of the activity of fungi in Sea water with that of CBM.

3.2 Comparison of the percentage of saccharification of cellulose in Sea water with that of CBM media.

As shown in the comparison of enzyme activity in 3.1 saccharifying percentage of the cellulose filterpaper also resulted in similar manner. According to the results (Figure 6) where *Cladosporium* sp. was topping with 2.29% in sea water, which was denoted by the lowest saccharification of 0.775% in CBM medium. Further *Helminthosporium* sp., *Trichoderma* sp., and *Aspergillus* sp. also depicted increased levels of saccharification compare to that of in CBM medium.

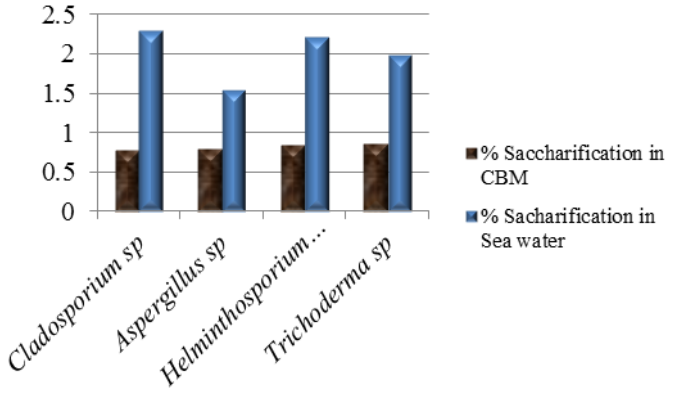


Figure 6: Comparison of the % of Saccharification of cellulose in Sea water with that of CBM.

3.1 Effect of environmental factors on saccharification of cotton.

(Based on examining derived enzyme activity by filter paper assay).

Environmental parameters are one of the major factors in saccharification of fungal enzymes. Mainly during the storage of cotton humidity, temperature, and even pH of the substances come to contact may facilitate colonization of fungi. However in case of temperature, there is no proved relationship between temperature of colonization and optimal temperature of their enzymes. In addition preference of such parameters by each fungal species may differ.

3.1.1 Effect of temperature on the activity of cellulolytic fungal enzymes (FPU/ml).

The rate of an enzyme catalyzed reaction increases as the temperature has risen. Variations in reaction temperature by 1 or 2 degrees may introduce changes of 10 to 20% in the results. In this experiment, enzymatic reaction of given fungi (Figure 5) showed a peak at 50^0C by reaching a peak of 32.5 FPU/ml. This shows that the temperature for the cellulolytic enzymes of three given fungi was 50^0C. However, if further high temperatures are tested it is possible to find the point they get denature. Normally animal enzymes get denatured even at 40^0C. Nevertheless, for fungi it is higher. It is also possible to observe the tolerance as well as the preference of high temperature (since 50^0C as optimum) by fungal enzymes. According to the graph, the fungus *Trichoderma* sp. shows an increase in the rate of reaction until 50^0C followed by decline afterwards.

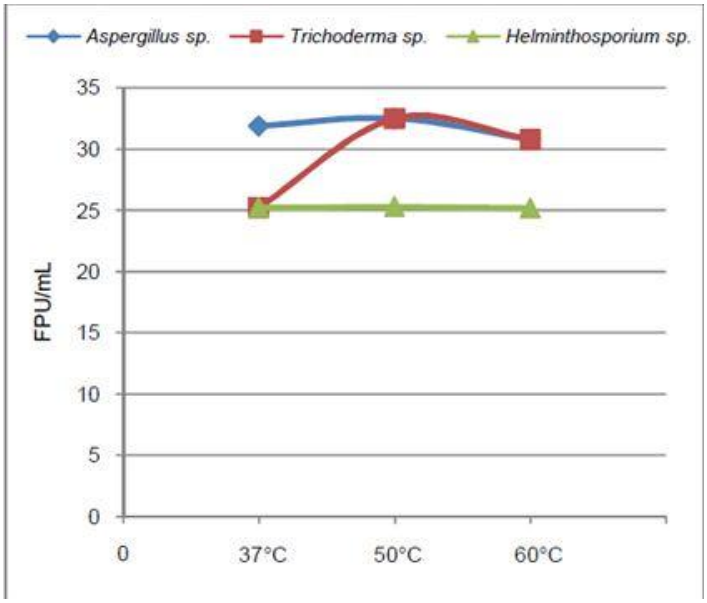


Figure 7: Temperature vs. activity of fungal enzymes (FPU/ml).

3.1.2 Effect of pH on the activity of cellulolytic fungal enzymes (FPU/ml)

Since enzymes are proteins, they are very sensitive to changes in pH. Each enzyme has its own optimum range for pH, where it is most active, and the result is determined by the effect of pH on a combination of factors such as binding of the enzyme to substrate, catalytic activity of the enzyme, ionization of the substrate, and the variation of protein structure. The initial rates for many enzymatic reactions exhibit bell-shaped curves. The most favourable pH value (optimum pH) may vary among enzymes of different fungi. In this experiment (Figure 6) the optimum pH for fungus *Trichoderma sp.* and *Fusarium* sp. was closer to neutral, and for *Helminthosporium sp.* it is 3. It means *Helminthosporium sp.* prefers slightly acidic medium. For *Aspergillus sp.* the curve was peaking at very low pH that showed the preference of *Aspergillus sp.* towards acidic environment.

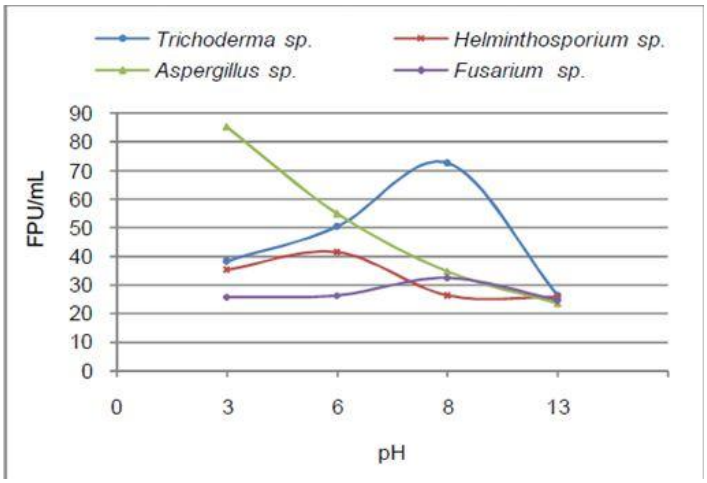


Figure 8: Effect of pH on the activity of cellulolytic enzymes from fungi.

IV. Conclusion and related works

Based on the results, the cellulose saccharifying ability of each fungus in sea water medium is increased. This shows the halophilic nature of them, though the fungi are terrestrial origin they have an individual preference as well as tolerance to marine environment. Some of the samples are facultative halophiles such as *Cladosporium* sp. and some *Aspergillus* sp., even though they have no exposure to saline environment in any part of their life cycle as they are belongs to terrestrial ecosystem their potential metabolic activity is higher in marine environment. It is possible to use the extracted fungal enzymes in degradation of algal debris from sea in the production of fertilizer, since algae are rich in minerals but due to its salt content it takes longer time to decompose by microbes.

In a similar work done in Goa, India. Colonizing ability in higher concentration of salt solution was evaluated by the growth rate of the colony, and few species belong to *Aspergillus* sp. which is a facultative halophile [3]. In another work a total of 17 species were screned soil samples isolated from a manmade solar saltern in Ban Laem, Thailand, many of the isolated species were belongs to genera *Aspergillus* sp.[6] Even in hyper saline environment like Great Salt Lake and Dead Sea many of the genera of terrestrial origin exists. In a study among 100 samples of water from Dead Sea 68 were positive, isolated strains include *Aspergillus* sp. [2].

Acknowledgement

It is my honor and perquisite to offer sincere thanks to Dr. Preminda Samaraweera, Department of Molecular Biology and

Biotechnology, University of Peradeniya, Srilanka for supervising this work.

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