

Lipase Production by Some Aquatic Phycomycetes in Maiduguri a Semi-Arid Area of Nigeria

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Abstract

Studies on the abilities of three species of aquatic phycomycetes isolated from the semi arid zone of Nigeria in Maiduguri, Borno State to produce lipase was carried out. The isolates included *Allomyces anomalus*, *Allomyces arbuscular* and *Brevilegnia diclina*. These three test aquatic phycomycetes were isolated using baiting technique with hemp seeds and pure cultures maintained on malt extract agar for lipase production. Plate assay and culture broth assay methods using mineral salts medium with tween 80 in conjunction with Olive oil and Mustard seed oil as lipase inducers were used in assessing the abilities of the isolates in producing lipase. The three isolates of aquatic phycomycetes showed via the plate assay method abilities to produce lipase with evidence of halo formation around colonies without stains and separately with intensification of halo formation with methyl red and sudan III indicators. *Allomyces anomalus* recorded the highest growth and halo diameter followed by *Allomyces arbuscular* and least by *Brevilegnia diclina*. Lipase level was not detectable using the culture broth method. Lipase production by aquatic phycomycetes have not been encountered from available literature, hence this preliminary result needs to

be optimised fully to exploit new opportunities of lipase production from these group of aquatic fungi

Keywords: Aquatic phycomycetes, Lipase, Olive oil, Mustard seed oil, Tween 80

Introduction

Lipases are valuable biocatalysts which are widely used in the detergent, food, dairy and pharmaceutical industries. Enzyme lipase hydrolyses the breakdown of triacylglycerols to diacylglycerols and monoacylglycerol with free fatty acids as co-products (Beisson *et al.*, 2000).

There are ubiquitous natural sources of lipases, including animals (Gargouriet *al.*, 1986), plants (Kapronchikovet *al.*, 2004 and Fuchs *et al.*, 1996), fungi and bacteria (Gupta *et al.*, 2004). Modern biotechnology has also developed various lipases for application in foods, detergents and pharmaceuticals (Gupta *et al.*, 2004). Fungi are a diverse group of microorganisms ranging from Yeasts and rusts to mushrooms and include many organisms of industrial and medical importance. Aquatic phycomycetes are a group of aquatic fungi that mainly belong to members of Chytridiomycetes and Oomycetes, though mainly aquatic, some species are found on moist soil. Chytridiomycetes and Oomycetes are separated on the basis of flagella being unflagellate and biflagellate respectively (Khulbi, 2001). Production of enzymes, particularly lipase by aquatic phycomycete have not been encountered in available literature but Some filamentous fungi like several species of *Rhizopus* have found many applications in industrial processes including enzyme production (Semeriva and Dufour, 1972). Microbial enzymes like lipases are produced by a wide range of microorganisms. Microbial lipase acts on a variety of substrates which have been shown to be hydrolysed by *Rhizopus* species and other filamentous fungi, these substrates include olive oil, tributyrin, triacetin and polyester molecules (Semeriva and Dufour, 1972; Tokiwa and Suzuki, 1977). Fan *et al.* (2013) also used optimised mineral salt medium supplemented with olive oil and Tween 80 to increase the lipase activity of *Rhizopus stolonifer* by 2.04 fold. Apart from lipase producing abilities of *Rhizopus* species, recently fungal lipases were reviewed and included *Aspergillus*, *Penicillium*, *Rhizopus*, *Candida* etc (Singh and Mukhopadhyay, 2012). This recent review of fungal lipase are all filamentous fungi, reports of enzyme lipase production by aquatic phycomycetes species have not been captured in reviews of fungi that have the abilities to produce enzyme lipase. Microorganisms have existed on the planet Earth over millions of years and are a source of biotechnological possibilities due to their genetic plasticity and adaptation. The isolation of new species from several and different

habitats, such as saltwater and freshwater, soils, hot springs, contaminated water bodies, soils, caves and hostile arid environments is therefore required. Microorganisms adapted to these conditions may have great biotechnological potentials (Izabelet *al.*,2012). This study is about assessing the potentials of aquatic phycomycetes from an unexploited arid zone environment of North Eastern Nigeria to produce enzyme lipase which available literature have not reported coming from these species of fungi.

Materials and methods

Sampling sites

The water samples were collected from three sampling sites in the Maiduguri area of Borno State namely Alau dam, River Ngadda by Coca-cola company area and River Ngadda by Lagos bridge. Maiduguri is the capital city of Borno State located in the north- east geopolitical zone of Nigeria and shares borders with Niger, Cameroon and Chad.

Water Sample Collection, Transportation And Storage

Water sample from experimental sampling sites (Alau dam, River Ngadda by Coca-cola area and River Ngadda by Lagos bridge) were collected in sterile 100mls and 200mls bottles at the littoral zones (shorelines) of these water bodies in the month of April, 2017. The sampling bottles were opened inside the water and immediately capped before bringing them out of the water to avoid contamination. All collected water samples were labelled with different code for analysis purpose. Water samples were then transported to the laboratory in Biological Sciences department of University of Maiduguri, in a plastic cooler with ice packs maintained at a temperature of 4°C and were analysed as soon as practicable on the day of collection between 2-6 hours.

Isolation and identification of aquatic phycomycetes

Aquatic phycomycetes species were isolated from the water samples collected from the three sampling sites above using hemp seeds (*Cannabis sativa*) as bait as used by Marano *et. al.*, (2008) and Nascimentol *et. al.*, (2011). Observations of growth of the baited aquatic phycomycetes were made for four to seven days and in some cases for up to 2 weeks. Colonies appearing on baited seeds were separated based on closely observed morphological differences like colour, length of the hyphae, concentration of hyphae around the seed and in conclusion differentiation into species was based on morphological differences of zoosporangia and hyphae. Baited seeds with aquatic phycomycetes isolated showing similar morphological characteristic were pulled together into Petri dishes containing sterile distilled water to which anti-biotic had been added to suppress bacteria

growth and observed directly under the compound light microscope for identification (Trifa and Adiba, (2011),. Marano *et. al.*, (2008) and Nascimento *et. al.*, (2011), alternatively, observation of a pin head of hyphae stained with lactophenol cotton blue was also made under a compound light microscope to support identification. Result as seen from the microscope under appropriate magnification were then compared for similarity with pictures and description found in the manual for identification of aquatic phycomycetes by Khulbi, (2001) and to existing stock cultures in some Nigerian Universities laboratory and also Ali, (2007). Three aquatic phycomycetes isolated baited on hemp seeds separated based on observed morphological characteristics and purified in sterile distilled water had their hyphae point inoculated onto malt extract agar medium and subsequently maintained as pure culture. Four day old pure cultures of the three isolates were used for the preliminary assay of enzyme lipase by plate method and liquid broth method using tween 80 in conjunction with olive oil and mustard seed oil as inducers. The three test aquatic phycomycetes isolates included *Allomyces anomalus* isolated from River Ngadda by Lagos bridge, *Allomyces arbuscular* isolated from River Ngadda by Coca-cola area and *Brevilegnia declina* isolated from Alau dam, all in Maiduguri metropolis of Borno State.

Enzyme lipase plate assay

The mineral salt media (MSM) used for the enzyme lipase plate assay was prepared according to the composition used by Maria *et al.*, (2015) and was composed of Peptone 10g, Sodium Chloride (NaCl) 5g, Hydrated calcium chloride ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$) 0.1g, Agar powder 16g and Distilled water 1000ml. The mineral salt medium (MSM) prepared above was sterilized in a laboratory auto clave at 121 °C for 15 minutes and was divided into 100ml amounts and supplemented with separately sterilized tween 80 in conjunction with olive oil and mustard seed as follows; Control mineral salt medium without incorporation of tween 80, olive oil or mustard seed oil, 1% olive oil MSM - 100ml of MSM supplemented with 1ml olive oil and 0.5ml tween 80, 1% mustard seed oil MSM- 100ml of MSM was supplemented with 1ml of mustard seed oil and 0.5ml of tween 80. Olive oil, mustard seed oil and tween 80 were separately sterilized gradually with Millipore filter paper before incorporation into the mineral salt medium.

Each of the three four day old test aquatic phycomycetes were grown separately on the three different mineral salt medium incorporated with tween 80 in conjunction with either olive oil or mustard seed oil by centrally inoculating the hyphae with an inoculating needle and incubated at 28 °C and observed for growth and development of halo. A clear zone (Halo) around the colony indicated lipase -positive aquatic phycomycetes. The second set

of experiment incorporated two indicators (stains) as used by Mohammed *et al.*, (1988) to intensify the clear zone or halo formed by the isolates. Drops of the stains, methly red and sudan III reagent were separately incorporated into the MSM media supplemented with olive oil and mustard seed oil before point inoculation of the three test aquatic phycomycetes isolates and incubated at 28 °C and also observed daily for growth and halo development. A clear zone around the colony also indicated lipase positive aquatic phycomycetes isolates.

Enzyme lipase broth assay

Production of enzyme lipase using nutrient broth supplemented separately with olive oil and mustard seed and both seeded with tween 80 was also carried out according to the method of Sumathy *et al.*, (2012). Nutrient broth was prepared according to manufacturers instruction and 50mls was dispensed into sets of 100ml conical flasks and stopped with cotton wool and aluminium foil. 0.5ml (1%) of tween 80 was added to each of the conical flasks containing 50mls of nutrient broth except the control conical flask. The conical flasks with the nutrient broth and tween 80 were then divided into two sets . One set was supplemented with 1%olive oil while the second set was supplemented with 1% mustard seed oil while the control without tween 80 was not supplemented with either olive oil or mustard seed oil. The nutrient broth supplemented with tween 80 and either olive oil and mustard seed oil and the control conical flask were then separately inoculated with four day old culture of the three test aquatic phycomycetes isolates and properly covered with cotton wool and aluminium foil. The culture broths were then incubated at 28 °C for seven days.

Enzyme extraction and purification

The seven days old culture broths were then used for enzyme extraction, purification and assay of lipase activity. The culture broth was centrifuged at 6,000 rpm at 4°C for 40 minutes. The supernatant was collected from the culture broth and subjected to cold acetone precipitation. Finally the protein pellet was dissolved in proper buffer (20mM Tris-Hcl buffer, pH 7.2)

Assay of Lipase activity

Lipase activity was estimated by titrimetric assay

$$\text{Units/ml of enzyme} = \frac{\text{Final burette reading} \times \text{Normality of NaOH}}{\text{X Time of Incubation} \times \text{Dilution factor}}$$

 volume of enzyme used

Result

Occurrence and selection of test aquatic phycomycetes

Ten species of aquatic phycomycetes were isolated from the three sampling sites and they included *Allomyces anomalus*, *Allomyces arbuscular*, *Allomyces recurvus*, *Brevilegnia declina*, *Saprolegnia bhargavi*, *Saprolegnia ferax*, *Protoachyla oryzae*, *Protoachyla paradoxa*, *Blastocladiella variabilis* and *Aphanomyces helicoides* but three isolates were selected based on their highest frequency of occurrence in each of the three sampling sites. *Allomyces anomalus* selected from River Ngadda by Lagos bridge are shown as micrographs in Plates 4 and 5. The Plates 4 and 5 shows fertile terminal hyphae and irregular surface captured on microscopic slide stained with lactophenol blue. The micrographs of *Allomyces anomalus* also shows abundant resting sporangia capable of producing zoospores after rest. Plate 5 also shows almost at the left hand base of the micrograph, a fertile terminal branch showing globular zoosporangia. *Allomyces arbuscular* isolated from River Ngadda by Coca-cola area as shown in micrograph of Plate 6 reveals a dichotomously branched and pseudoseptate hyphae. *Brevilegnia declina* isolated from Alau dam in Maiduguri is shown in Plate 7 as terminal matured zoosporangium with liberation of zoospores. Alau dam is the source of water treatment for supply of portable water to the inhabitants of Maiduguri.

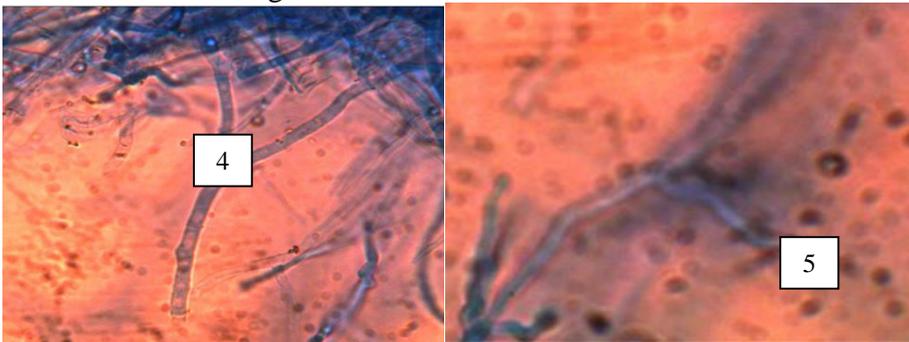


Plate 4 :*Allomyces anomalus* ($\times 10\times 40$) showing fertile terminal branching of hyphae and irregular surface captured on microscope slide stained with lactophenol cotton blue

Plate 5: *Allomyces anomalus* ($\times 10\times 40$) showing fertile terminal branching of hyphae, irregular surface and resting abundant sporangia captured on microscope slide stained with lactophenol cotton blue.

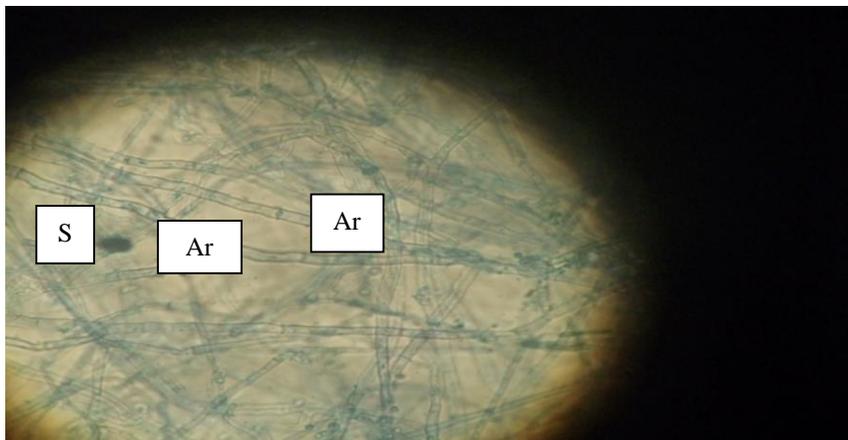


Plate 6: Micrograph of *Allomyces arbuscular* (Ar) ($\times 10\times 40$) showing branching pattern of hyphae, thick walled resting sporangium (S) and pseudoseptate hyphae.



Plate 7: Micrograph of *Brevilegnia diclina* (B) ($\times 10\times 40$) showing terminal matured zoosporangium with liberation of zoospores

Enzyme lipase production by aquatic phycomycetes via plate assay without stain

The result of abilities of three species of aquatic phycomycetes to produce enzyme lipase using plate assay without stains showed clearly positive results with visible halo surrounding the colonies of all the test species of aquatic phycomycetes. These halo (clear zone around colony) however showed little or no observable difference. The control experiment of the three test species was negative for halo formation. Table1 shows the highest colony diameter for *Allomyces anomalus* grown on MSM supplemented with mustard seed (29.5mm) with a slightly higher halo diameter of 2.0mm. Same Table 1 shows that *Allomyces anomalus* grown on MSM supplemented with olive oil had the next highest colony diameter of 27.5 with a halo diameter of 1.5mm. *Allomyces anomalus* showing clear zone around colonies of isolates grown on MSM supplemented with olive oil

and mustard seed oil are shown in Plate 8. *Allomyces arbuscular* showed colony diameters of 23.5mm and 24.2mm with corresponding halo diameters of 1.5mm each for growth on MSM supplemented with olive oil and mustard seed oil respectively. Plate 9 also shows clear zone as halo around the colony of *Allomyces arbuscular* indicating enzyme lipase production. *Brevilegnia diclina* showed the least colony diameters of 20.2mm and 20.0mm with a corresponding halo diameter of 1.0mm for growth on MSM supplemented with mustard seed oil and olive oil respectively.

Enzyme lipase production by aquatic phycomycetes via plate assay with stain

The result of the experiment on the abilities of species of aquatic phycomycetes to produce enzyme lipase via plate method to intensify enzyme production with two stains methyl red and sudan III reagent showed methyl red stain intensifying halo formation for *Allomyces anomalus* grown on MSM supplemented with mustard seed oil as shown in Table 2. Table 2, also shows halo produced by *Allomyces arbuscular* supplemented with mustard seed oil intensified by sudan III reagent while *Brevilegnia diclina* grown on MSM supplemented with Olive oil also had its halo intensified by methyl red stain as also shown in Table 2. Table 2 also confirms production of enzyme lipase with evidence of halo or clear zone formation around some of the colonies grown on MSM supplemented with two enzyme lipase inducers namely olive oil and mustard seed oil, However, the halo diameters did not show clear observable differences in terms of diameter measurements.

Table 1: Enzyme lipase production by aquatic phycomycetes via plate assay without stain

Test aquatic phycomycetes	Inducer	Conc of inducer (ml)	Conc of surfactant tween 80 (ml)	Colony diameter (mm)	Halo diameter (mm)
Isolate A <i>Allomyces anomalus</i>	Olive oil	1.0	0.5	27.5	1.5
	Mustard seed oil	1.0	0.5	29.5	2.0
Isolate B <i>Allomyces arbuscular</i>	Olive oil	1.0	0.5	23.3	1.5
	Mustard seed oil	1.0	0.5	24.2	1.5
Isolate C <i>Brevilegnia diclina</i>	Olive oil	1.0	0.5	20.0	1.0
	Mustard seed oil	1.0	0.5	20.2	1.0
Isolates A, B &	Olive oil	0.0	0.0	25.5	0

C (mean)	Mustard seed	0.0	0.0	26.0	0
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Table 2: Halo/colony diameter of aquatic phycomycete enzyme lipase plate assay with stains (methylred stain and sudan III reagent)

Isolate	Inducer	Stain or indicator	Colony diameter (mm)	Halo diameter (mm)
<i>Allomyces anomalus</i>	Olive	Methyl red	-	-
<i>Allomyces anomalus</i>	Mustard	Methyl red	62	2.0
<i>Allomyces arbuscular</i>	Olive	Methyl red	-	-
<i>Allomyces arbuscular</i>	Mustard	Sudan III	14.8	1.5
<i>Brevilegnia declina</i>	Olive	Methyl red	2.1	1.5
<i>Brevilegnia declina</i>	Mustard	Methyl red	-	-
Control (mean)	none	None	32.6	0

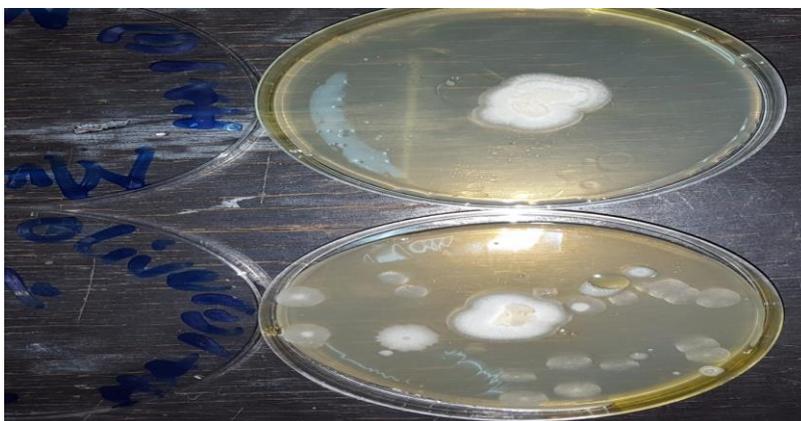


Plate 8: Enzyme lipase produced by *Allomyces anomalus* as halo on olive and mustard seed oil MSM without stain



Plate 9: Enzyme lipase produced by *Allomyces arbuscular* as halo on mustard seed oil MSM without stain

Enzyme lipase production by aquatic phycomycetes via liquid broth method

Since enzyme lipase is usually produced extracellularly, the extraction was considered successfully completed by the centrifugation of the culture broth. The purpose of the cold acetone precipitation was to purify the enzyme lipase produced.

The result of the growth of test species of aquatic phycomycetes isolates after seven days in broth culture did not show fungal mycelia mat but rather a colloidal appearance of the culture broth.

The result of enzyme lipase production via broth culture determined by titrimetric method were all inconclusive since detectable levels of enzyme lipase was not recorded as shown in Table 3.

Table 3 : Enzyme lipase production by aquatic phycomycetes via liquid broth assay

Test aquatic phycomycetes isolates	Inducers	Concentration of inducer (%)	Titrimetric values (unit/ml)
<i>Allomyces anomalus</i>	Olive oil	1%	In conclusive (nil)
	Mustard seed oil	1%	In conclusive
<i>Allomyces sarbuscular</i>	Olive oil	1%	Inconclusive
	Mustard seed oil	1%	Inconclusive
<i>Brevilegnia declina</i>	Olive oil	1%	Inconclusive
	Mustard seed oil	1%	inconclusive

Note: inconclusive means that no detectable level of enzyme lipase was observed.

Discussion

Enzyme lipase has found a wide industrial application for the past two decades. The isolation of lipase by sources such as plants and animals are cost effective and yield is also very less. Due to this low yield of the enzyme from plants and animals sources, attention have been focused on microbial enzymes. Of all microbes, fungal strains are preferred for lipase production since their enzymes are secreted extracellularly which makes extraction technique easy. Production of enzyme lipase by the group of water fungi known as aquatic phycomycetes is rare or even nonexistent from available literature. Hence the result obtained from this research effort is discussed in the light of production of enzyme lipase by fungi particularly filamentous fungi. Literature on production of enzyme lipase by filamentous fungi (e.g *Aspergillus*, *Penicillium*, *Rhizopus* and *Candida* spp) abounds due to the high economic value of this very important enzyme (Singh and Mukhopadhyay, 2012). Aquatic phycomycetes occurrence have not been reported in published literature from the war ravaged arid zone North Eastern Nigeria, it is therefore novel that a variety of aquatic phycomycetes were isolated from three fresh water bodies in Maiduguri. Three of these isolates namely *Allomyces anomalus*, *Allomyces arbuscular* and *Brevilegnia declina* occurred commonly in these freshwater bodies in Maiduguri and were used

as test organisms for their abilities to produce enzyme lipase. In line with this study that found amongst others, the occurrence of three aquatic phycomycetes in the harsh arid zone freshwater environment of Maiduguri, North eastern Nigeria, aquatic phycomycetes are found throughout the world according to a review by Shearer *et al.* (2007). In Nigeria, Alabi (1971) investigated the distribution of Saprolegniaceae in Ibadan (Western part of Nigeria), while many ecological studies of aquatic phycomycetes have been studied worldwide (Willoughby, 1974; Steiow, 1998; Marano *et al.*, 2008). Some studies have equally confirmed them as parasites of fish. Czezug *et al.* (2002) found that some of these species may be potential source of infection and are able to grow and cause infection on muscles of fish and their eggs. In Nigeria, Ogbonna and Alabi (1991) also worked on fungi associated with mycotic infection of fish and isolated twenty four (24) species belonging to six genera of aquatic phycomycetes from infected fishes. Thus similar species of aquatic phycomycetes from this study have been isolated in Nigeria but no study has been recorded with aquatic phycomycetes on their abilities to produce enzymes or enzyme lipase in particular. The results of this study showed that the three test isolates of aquatic phycomycetes were able to produce enzyme lipase in the preliminary studies using plate assay method and two inducers, olive oil and mustard seed oil. The tween 80 incorporated is a surfactant which increases the surface contact between substrate and organism. The three test aquatic phycomycetes namely *Allomyces anomalus*, *Allomyces arbuscular* and *Brevilegnia dictina* showed clear halos around their colonies as evidence of enzyme lipase production but there were no great observable differences in the diameter of the halo produced by the three isolates even though *Allomyces anomalus* seemed to have recorded the highest colony diameter and slightly higher halo diameter. In line with the result of this study, Fan *et al.* (2013) used an optimized medium for lipase production in *Rhizopus stolonifer* which had some mineral salts with olive oil and tween 80 at the same time. They reported that the optimized medium increased the lipase activity nearly two fold from 0.46 to 1.40 μ /ml. Tween 80 was used in these study to achieve better stimulation of enzyme lipase in aquatic phycomycetes as was used by Fan *et al.* (2013). In line with the results of this study Mohammed *et al.* (1988) used tween 80 as substrate with different stains or indicators including Victoria blue and Methyl red as indicator. They determined lipolytic activity using a method of plate assay as used in this study by observing the formation of the zone of intensification of the indicator colour after 24 hours. *Allomyces anomalus* in this study produced enzyme lipase with evidence of halo around the colony which was intensified by Methyl red indicator which proved better than the Sudan III reagent.

The study of enzyme lipase production in broth culture could not yield a detectable level of enzyme lipase from aquatic phycomycetes using titrimetric method. This is as opposed to similar study by Sumathy *et al.* (2012) who produced lipase activity from three filamentous fungi, *Aspergillus niger*, *Rhizopus oryzae* and *Fusarium oxysporium* using three enzyme lipase inducers in broth culture namely Olive oil, Gingerly oil and Mustard seed oil. They found that *Aspergillus niger* with Olive oil as inducer produced the maximum lipase activity as compared with other strains and concluded from their results that the concentration of the inducers used for the study did not affect the production of lipase. Even though Sumathy *et al.*(2012) concluded that the filamentous fungi produced lipase efficiently in the chosen inexpensive substrates as used in this study, they also stated that several other factors like temperature, pH, fermentation method, environmental factors, etc, may also affect the enzyme production. In line with these, it is worthy to note that the mineral salts used in this study were different from those used by Sumathy *et al.*(2012). It is therefore very important to analyse the above stated parameters in detail to optimize the lipase production by commonly available fungal strains. Thus further research may be needed that will incorporate some other factors in assaying for enzyme lipase production by culture broth method particularly considering the arid zone environmental factor of Maiduguri, North eastern Nigeria with its attendant high temperature regimen. Sabiha and Jadhav (2013) studied lipase production of some fungal species and their application in oil spillage degradation. They studied the role of three lipolytic fungal species (*Aspergillus niger*, *Aspergillus paraciticus* and *Aspergillus aculeatus*) activity on various oil spillages (Soyabean, Mustard and Coconut oil) degradation. Their result revealed that all three fungal strains were able to degrade Soyabeans, mustard and coconut oil and they affirmed that the lipolytic fungi have ability to degrade other oil spillage and hydrocarbons as also supported by Kavitha *et al.* (1997). This is in line with the result of this research effort which equally showed that three species of aquatic phycomycetes were able to produce enzyme lipase by evidence of halo production when grown on mineral salt medium supplemented with two lipase inducers namely Olive oil and Mustard seed oil.

Conclusion

The implication of these findings is that since these aquatic phycomycetes were isolated from polluted freshwater environment (River Ngadda by Lagos bridge and Cocacola areas of Maiduguri in particular), they have the abilities to degrade pollutants like vegetable oils and oil of hydrocarbon origin from automobile washing and domestic activities within the water bodies and in degrading these oils, they also have abilities to

produce enzyme lipase that breaks the oil to fatty acids and glycerol as simpler subunits. Aquatic phycomycetes species should therefore be further surveyed in future work for their role in biodegradation of oil pollutants and commercial production of enzyme lipase.

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