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Antimicrobial activity and amylase production by *Rhizophus microsporus* strain isolated from germinating maize seed

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ABSTRACT

The study was aimed to determine the antimicrobial activity and enzyme production of Rhizophus microsporus isolated from the maize seed germination experiment. Antifungal and antibacterial activities of the Rhizophus species were studied against Pythium ultinum, Rhizoctonia solani, Fusarium oxysporium, Bacillus sp, Escherichia coli, Pseudomonas and Streptococcus. Rhizophus microspore showed marked antimicrobial activity against Rhizoctonia solani, Bacillus sp and Pseudomonas sp. Bioassays of Rhizophus microsporus on fungal, bacterial, protozoan and nematode were done using the crude extracts of Rhizophus microsporus in methanol and distilled water. Methanol extract exhibited antifungal activity whereas the water extract displayed antibacterial activity. The organism produces volatile and water soluble antibiotics and shows significant inhibition on the formation of sclerotia of Rhizoctonia solani. Antiprotozoan activity was also observed but there was no effect on the motility of nematode. The enzyme amylase which is widely used in the food industry was also produced by this organism. The pH optimum for the amylase enzyme was 8.0 at 40^{o}C and the temperature optimum was 50^{o}C at pH 8.0.

Key words: *Rhizophus microsporus*, antimicrobial activity, amylase, antibiotics, bioassay

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INTRODUCTION

Discovery of an antibiotic is the greatest breakthrough in fighting against pathogens. Before the invention of antibiotics, plants and their extracts played an important role in curing diseases. However, the exploration of the microbes and their metabolic products as source of therapeutically useful compounds, arguments the usage of plants and the extracts. Antibiotic producing microbes found in nature are not only useful for medical purposes, but very useful in agricultural disease management, enzyme production, etc [1]. Most of them have been isolated from bread, starchy food and nutritive soil. These fungal antibiotics comprise of many semisynthetic penicillins, the biosynthetic penicillin V, the semi synthetic cephalosporins and the natural penicillin G. It has been confirmed that large numbers of fungal extracts or extracellular fungal products have been confirmed to have antimicrobial activity, mainly from the filamentous fungi, e.g. *Penicillium* species [2].

Amylases are a group of enzymes produced by different bacteria and fungi [16]. Amylases are widely used for processing starch to produce sugar syrups containing glucose, maltose and higher oligosaccharides in the industrial level [3]. Amylases contribute for about 30% towards the world's enzyme production [15]. Generally *Rhizopus* sp. is considered good producers of amylase enzymes [13]. *Rhizopus oryzae* MUCL 28168, MUCL 28627, ATCC 34612 produced alpha amylase and glucoamylase when raw and cooked cassava was used as the substrate [12]. *Rhizopus microsporus* var. rhizopodiformis was used for the production of α-amylase and glucoamylase using agricultural residues such as wheat bran, cassava flour, sugar cane bagasse, rice straw, corncob and crushed corncob as carbon sources in solid state fermentation system [9].

Rhizophus species is one of the most common genera of fungi and diverse species of this genera found in nature and they are widely distributed all over the world. This genus includes several plant saprophytic fungi and specialized animal parasites [10]. *Rhizophus* occurs nearly in all the carbohydrate substrates and other natural habitats consisting of organic matter. *Rhizophus* is a member of Kingdom fungi and classified under phylum zygomycota. *Rhizohus microsporus* is a fungal plant pathogen infecting maize, sunflower and rice. It can cause an infection and necrosis to the infected area, particularly prevalent in pre-term infants. This fungus has a bacterial endosymbiont *Burkholderia rhizoxinica* that produces the antitumor drug named as rhizoxin [8]. *Rhizophus* species are widely used in the production of antibiotics and for the bio-control of plant diseases. There have been no reports identifying the antibiotic activity and the enzyme producing capacity other than lipase, of this species. Since the characteristic features of this extracted fungal species are unknown, it was planned to investigate the important properties of this fungi. Therefore, the objective of the study was to determine the antimicrobial activity and capacity of enzyme production of *Rhizophus microsporus* and to characterize the enzyme produced by this mold.

MATERIALS AND METHODS

Microorganism

The fungi, isolated from decaying maize seed and characterized as *Rhizohus microsporus*, was used in this study. Fungi used as targets in the antibiotic bioassay were *Pythium ultimum*, *Rhizoctonia solani* and *Fusarium oxysporium*. Inoculum was taken from colony margin of actively growing cultures. The following bacteria were used as target in the antibacterial bioassay. *Escherichia coli*, *Bacillus* sp., *Pseudomonas* sp and *Streptococcus* sp. Overnight broth cultures of these bacteria were used in the bioassay. *Entamoeba histolytica*, *Crithidia tapiculata* and *Trichomonas varginalis* were used as target for antiprotozoan bioassay. A soil nematode was used to study the anti-nematode activity of the isolated organism.

Chemicals and media

All the chemicals used were of analytical grade. Potato Dextrose Agar (PDA) media was used for fungal growth and tryptic soy agar (TSA) was used for bacterial growth. Physiological culture media were provided for protozoan and nematode bioassay.

Preparation of Inoculum and Cultivation of the Strain

The fungus was grown in 1% PDA plates at 28-30°C for 48 h. The inocula were prepared by making hyphal discs (0.5 cm diameter). Each disc was used to inoculate 10 ml of medium [3]. The strain was cultivated in LSF (Liquid State Fermentation) in 100 mL Erlenmeyer flasks each containing 20 mL Basal Medium (BM) composed of (g L^{-1}): peptone 0.9; $(NH_4)_2 HPO_4$ 0.4; KCl 0.1; $MgSO_4.7H_2O$ 0.1 and starch 0.25. (pH: 8.0).

Enzyme Assay

In SSF, after the addition of sterile water and thorough mixing, the broth was filtered through filter paper (Whatman No. 1); the filtrate was centrifuged at 10,000 x g for 5 min and the supernatant was used as the crude enzyme [18]. To measure the activity of alpha amylase, the assay mixture (1 mL) containing an equal volume of enzyme and 1% (w/v) starch dissolved in 0.1(M) phosphate buffer (pH5.0) was incubated at 55°C for 5 min. The reducing sugar released was measured by the dinitrosalicylic acid method [20], taking glucose as standard. Blanks were prepared with inactivated enzymes. One unit of alpha amylase was defined as that amount of enzyme that liberated one millimole of glucose per milliliter per minute of reaction.

Antimicrobial activity of *Rhizophus*

Antifungal activity

Rhizohus microsporus was placed with the target fungi. 5mm diameter cork bored inocula of target and test fungi were placed in opposite sites of the PDA plates and incubated in dark at 25°C. Control was set up by placing the cork bore inoculum of target fungus only on PDA plate. Measurements were made by marking the growth of target fungus, every day, in the presence and absence of test fungus up to 5 days. % of inhibition on growth of target fungus was calculated in the following manner:

$$\frac{\text{Distance traveled by the target fungus in the absence of test fungus} \times 100}{\text{Distance traveled by the target fungus in the presence of test fungus}}$$

Antibacterial activity

TSA plates were spread with 200µL of overnight culture of target bacteria and let to dry. 5mm core bore inocula of fungi were then placed on the plates separately. Plates were then incubated at 30°C for 2-5 days. Clear zone around the cork bore inoculum was taken as (+) result.

Preparation of crude extract

Methanol extraction

6-10 days old culture of the *Rhizohus microsporus* in PDA was used for crude extract preparation. Pieces of agar with the culture was mixed with 20mL of methanol and kept at room temperature for an hour. Agar pieces were then removed and the suspension was centrifuged at 10,000 rpm for 5 minutes to remove hyphal fragments and spores. The methanol fraction was then concentrated on a rotary evaporator at 20^{o}C to give final volume of 1mL.

Water extraction

Potato dextrose broth was inoculated with a loop full of inoculum and incubated at 25^{o}C on an orbital shaker in the dark for a week. The culture was then filtered through a whatmann filter paper in a funnel. Filtrate was then centrifuged at 10,000 rpm for 5 minutes to remove hyphal fragments and spores. Extract was then concentrated to make the final volume of 1mL.

Fungal bioassay

5mm diameter cork bore inoculum of target fungus was placed centrally on a PDA plate. Antibiotic discs loaded with 20µL of methanol extract were placed away from the fungal inoculum. Control was set up by loading the antibiotic disc with 20µL of methanol. Solvent was let to evaporate before placing the disc into plates. Plates were then incubated in dark at 20^{o}C and radial growth of the target fungus towards the antibiotic discs was observed.

Bacterial bioassay

200µLof overnight culture of bacteria was spread on TSA plates by using sterile glass spreader. The plates were allowed to dry. Antibiotic discs loaded with 20µL of water and methanol extract were placed away from each other. Solvent was let to evaporate before placed the disc into plates. Controls were set up by loading the antibiotic discs with appropriate solvent. Plates were incubated at 30^{o}C for 24h. Diameter of the clear zone developed around the discs, were measured.

***Entamoeba histolytica* and nematode bioassay**

20µL of cells and 170µL of medium were mixed with increasing 2 fold dilution of 10µL crude methanol extract. Control was set up by adding 10µL of methanol. These were then incubated at 37^{o}C for 48h. After the incubation, medium with extract was tipped off. Cells were then washed and mixed with tetrasolium salt. Absorbances of the solutions were measured at 620nm. Nematodes isolated from soil grown on substrate were mixed with 10mL of sterile water and centrifuged at 1300rpm for 3minutes. Supernatant was decanted and the pellet was resuspended in sterile water. This was again centrifuged at 1600rpm for 5 minutes. Pellet was then mixed with sterile water and used for bioassay. 50µL of worm suspension and 1mL of sterile water were mixed with 5, 10, 15 and 20 Optical density of the solutions was measured at 620nm of crude methanol extract. Controls were set up by adding same amount of methanol. These were incubated at 20^{o}C for 2 days. Worm movement was scored by looking under the microscope.

Enzyme production by the isolated strain

Production of enzymes

Plates with gelatin agar were inoculated with 5mm diameter cork bore inoculum of the selected isolate. Protease production was detected by the development of clear zone with acid $HgCl_2$. Starch and xylan agar plates were prepared separately and inoculated with 5mm diameter cork bore inoculum of the selected isolate. Amylase production was detected by the development of clear zone with iodine.

Effect of temperature on amylase activity

The effect of temperature on amylase activity was determined by incubating the appropriately diluted enzyme (0.01M phosphate buffer at pH 7.0) for optimized amount of time with 0.5mL of soluble starch at pH 7.0 (20gL^{-1}) and at different temperatures.

Effect of pH on amylase activity

The effect of pH on amylase activity was measured by preparing 2gL^{-1} soluble starch in buffers at different pH values (pH 3-6 Citrate-phosphate buffer; pH 8.0 Tris- aminomethane buffer; pH 9.0 Glycine-NaOH buffer and pH 10 to 12 Phosphate buffer) and incubated at optimized conditions.

RESULTS AND DISCUSSION

Antimicrobial activity of *Rhizophus* isolates

Eight different *Rhizophus microsporus* colonies were purified and designated as 1-8 in numbers. Table 1 illustrates their activity with target fungus and bacteria. Among the eight *Rhizophus microsporus* isolates, the isolate no 5 showed greater antifungal and antibacterial activities than the others. Therefore the isolate no 5 was selected for further studies.

Table 1: Antimicrobial activity of *Rhizophus microspores*

Target organisms	*Rhizophus microsporus*							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Pythium ultimum	-	+	-	N.D	-	N.D	-	-
Rhizoctonia solani	-	-	-	N.D	+	N.D	-	N.D
Fusarium oxysporium	N.D	-	-	-	-	N.D	+	-
Bacillus sp	-	-	-	-	+	N.D	-	-
Streptococcus sp	N.D	-	-	-	-	-	-	N.D
Pseudomonas sp	N.D	-	-	N.D	+	-	-	N.D
Escherichia coli	N.D	-	-	N.D	-	+	N.D	N.D

N.D – Not determined.

Morphological features of *Rhizohus microsporus*

Colonies grew rapidly and mycellial formed at first a smooth-surfaced, watery white mycelial mat. The conidial areas were whitish green but later turned to dull colour. The reverse of the colony remained uncoloured. In the PDB culture, colonies formed yellowish pigment and turned the culture media to yellowish green. The hyphae are branched, smooth walled and colourless. Conidiophores are long sylinder like, much branched.

The effect of *Rhizophus microsporus* on antifungal activity

On dual plating assay, *Pythium ultimum* has grown over the test fungus after 7 days of incubation. *Fusarium oxysporium* is insensitive to the test fungus. When grown, *Rhizoctonia solani* with test fungus, it stopped growth after met the test fungus. On further incubation test fungus started to grow over *Rhizoctonia solani.*

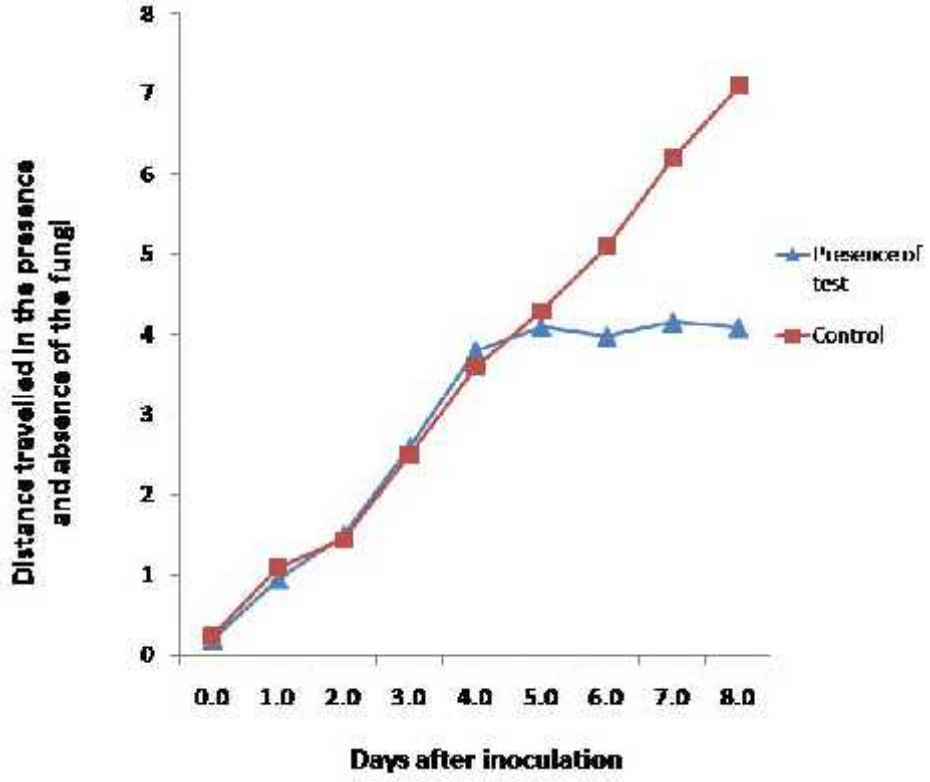

Figure 1: Effect of *Rhizophus microsporus* on the growth of *Rhizoctonia solani*

Figure 1 shows the inhibition on *Rhizoctonia solani* by test fungi. It seems after four days of growth, test fungus start to produce inhibitory substances and inhibit the growth of *Rhizoctonia solani* .On further incubation, *Rhizoctonia solani* formed sclerotia.

Antimicrobial activity of crude extract of test fungus

Antifungal activity

The results of the antifungal activity are summarized in Table 2. The growth of *Rhizophus microsporus* was inhibited by 52% compare to the control after 8 days on incubation with the test fungus. Spore formation was

markedly disrupted around the antibiotic discs. As the cultures matured, spore initials in the controls continued to develop. After 15 days, spores had formed only at the surfaces of the colonies treated with crude extract. *Rhizophus* overcome the inhibitory effect and eventually overgrown the antibiotic discs, but no spore formation around the antibiotic discs.

Table 2 : Antifungal activity of the crude extract on the growth of *Rhizophus microsporus*

Days after inoculation	Percentage of growth compared with the control
3	94
5	84
8	56
10	47

Antibiosis can play a major role in microbial antagonism. The present study also suggests that the mechanism of antagonism by *Rhizophus microsporus* on *Rhizoctonia solani* can be explained by antibiosis as well as mycoparasitism. Alkyl pyrones produced by the *Rhizophus microsporus* might have created a condition which changes the spatial distribution of the organism's biomass but not its rate of production. Here inhibition on the growth of *Rhizoctonia solani* may be due to the lysis of the mycelium. The capacity of *Rhizophus microsporus* to produce volatile compounds has an advantage over non volatile inhibitors, since organisms remote from the site of production are likely to be affected. This study clearly shows significant inhibition on the formation of sclerotia of *Rhizoctonia solani.* If the inhibition is due to the volatile compounds produced by the *Rhizophus microsporus*, then exploitation, formulation and usage of these compounds may give an advantage over the use of inoculum of *Rhizophus microsporus* as a biological control agent.

Antibacterial activity

Results of antibacterial assays are summarized in Table 3. Clear zones were not developed with methanol crude extract. Water extraction gave most marked zone of inhibition of bacterial growth around the antibiotic disc. *Pseudomonas*, *Streptococcus* and *E.coli* appeared to be resistance whilst *Bacillus* sp was sensitive. Antibacterial activity in methanol is not effective as in water. The mechanisms of antibacterial action of Rhizophus microspores like species is not very clear [5]. Different hypothesis suggest different mechanisms. One suggests that hydrophobic and hydrogen bonding of phenolic compounds to membrane proteins, followed by partition in the lipid bilayer; perturbation of membrane permeability consequent to its expansion and increased fluidity causing the inhibition of membrane embedded enzymes; membrane disruption; destruction of electrons transport systems and cell wall perturbation [7].

Table 3: Antibacterial activity of crude water extract on bacterial growth

Target organism	Diameter of clear zone (mm)
Escherichia coli	---
Bacillus sp	28
Pseudomonas	06
Streptococcus	---

Antiprotozoan activity

The effect of different concentrations of methanol extract on the growth of *Entamoeba histolytica* is shown in Figure 2. Absorbance of the medium decreased with the increase concentration of crude extract. 58% of absorbance reduction was observed with the extract compared to the control.

Nematode bioassay

Crude methanol extract has no effect on the motility of the nematode worm even after 2 weeks of incubation.

Enzyme production by *Rhizophus microsporus*

Rhizophus microsporus did not produce either protease or xylanase type of enzymes. But amylase production was notified by the formation of clear zone in the starch agar plates.

Effect of pH and temperature on amylase activity

When the pH was varied from 4.0 to 12.0 the activity of amylase was increased up to pH 8.0 and further increase of pH decreased the enzyme activity (Figure 3). Amylase showed activity between pH 4.0 to 12.0. However the enzyme showed 75% of its original activity at pH values between 6.0 and 10.0. Since the highest activity was obtained at pH 8.0, it was selected for further studies. The amylase activity was assayed at different temperatures ranging from 30 - 75^{o}C at pH 8.0. The activity of amylase increased up to 50^{o}C and further increase in temperature decreased the enzyme activity. Amylase from the strain exhibited a temperature profile with a sharp peak of maximal activity at 50^{o}C and showed activity between 30 - 75^{o}C (Figure 4). The optimum temperature for the

activity depends on the type of organisms [6]. The dependence of enzyme activity on pH is a consequence of the amphoteric properties of proteins [17]. Different ionizable groups with different pK_a values are present on the surface of the protein molecule and surface charge distribution on the enzyme molecule varies with the pH of the environment. These changes in charges may affect the enzyme activity either by changing the structure or by changing the charge on a residue functional in substrate binding or catalysis [11]. The optimum temperature for the activity depends on the type of organisms [10,19]. Most of the thermophilc organisms produce thermostable enzymes. If the enzyme activity is in alkaline range this can also act at higher temperature of above 50^{o}C [1]. In order to use the antibiotic compounds and enzymes in this extract, they will have to be purified and the physical and chemical characteristics as well as mode of inhibitory effect and action should be further studied [4].

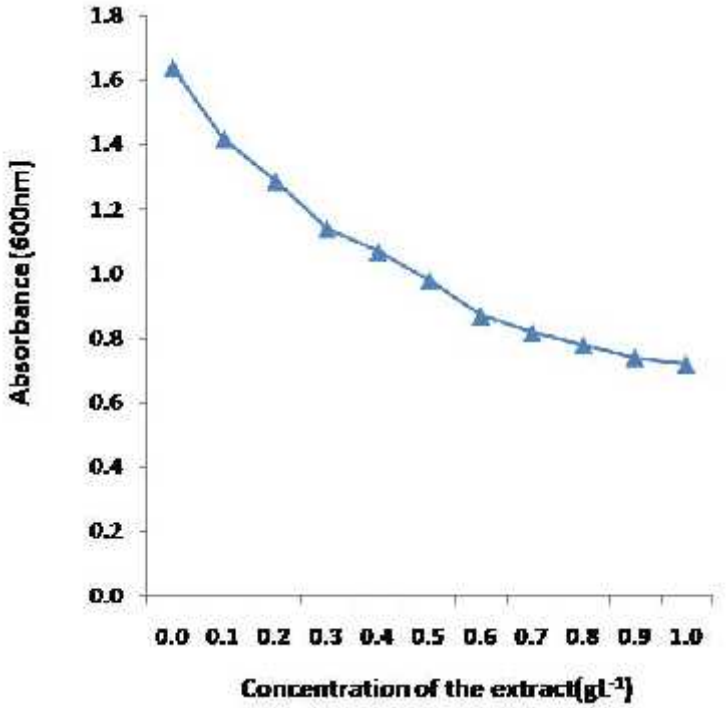

Figure 2: Effect of methanol crude extract on the growth of *Entamoeba histolytica*

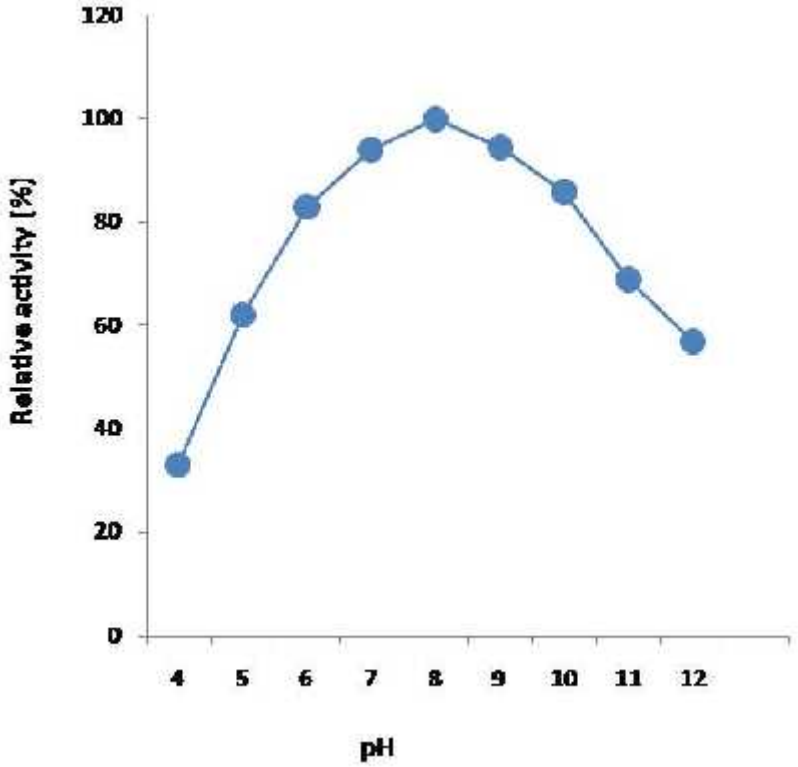

Figure 3: Effect of temperature on amylase activity at pH 7.0 and at 20gL^{-1} starch

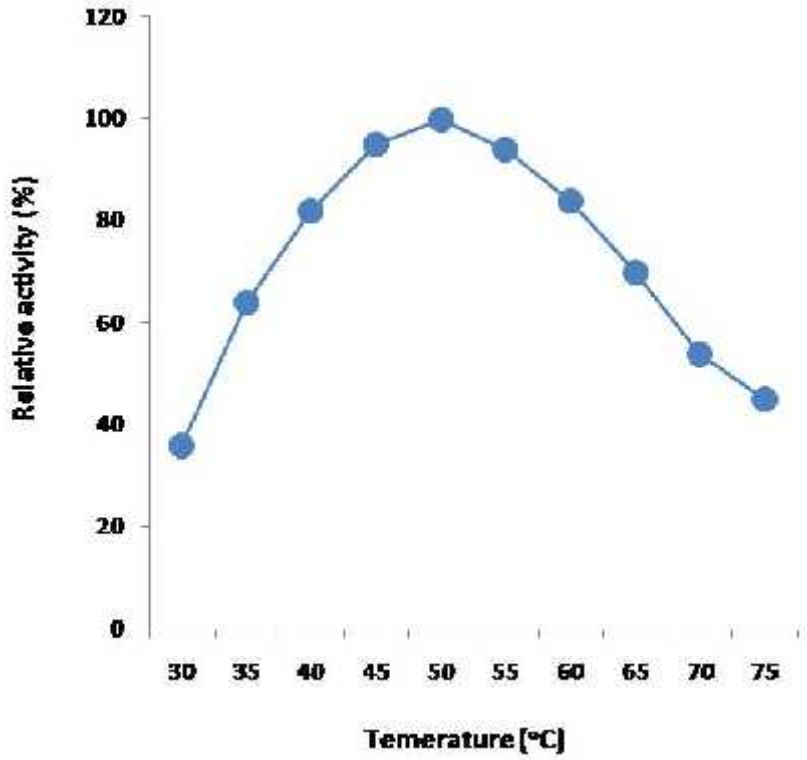

Figure 4: Effect of pH on amylase activity at temperature 50°C and at 20gL^{-1} starch

CONCLUSION

Rhizophus microsporus has marked antimicrobial activity against *Rhizoctonia solani*, *Bacillus* sp and *Pseudomonas* sp. The fungi can produce alpha amylase and not xylanase and protease. Methanol extract exhibited antifungal activity whereas the water extract displayed antibacterial activity. The organism produces volatile and water soluble antibiotics and shows significant inhibition on the formation of sclerotia of *Rhizoctonia solani*. Antiprotozoan

activity had no effect on the motility of nematode. The enzyme amylase was also produced by this organism but xylanase and protease were not. The pH optimum for the amylase enzyme was 8.0 at 40^{o}C and the temperature optimum was 50^{o}C at pH 8.0.

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