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STUDY OF FUNGAL DIVERSITY IN DIFFERET SOIL SAMPLES AND ASSESSMENT OF THIS FUNGAL FLORA FOR THEIR POTENTIAL ANTI-DIABETIC PROPERTIES

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ABSTRACT

Fungi are associated with immense diversity and versatility, and they grow in extreme conditions which are instigating factors to mycosynthesize medical therapeutics. Therefore, they play a significant role in medicine a wide variety of bioactive therapeutics is biosynthesized them. Although the majority of biomolecules are from other than soil flora such as endophytic fungi, only a few molecules are reported from soil fungi. Henceforth, isolation, screening, and anti-diabetic prospective of soil fungi isolated from banana, sugarcane, and paddy field was investigated in the present study. Physical parameters analysis of soils the showed normal temperature of 28-30ºC and pH 6.1-8.2 in contrast to recommended level pH of 6.5 – 8.5. Banana soil showed a high amount of Potassium and low content of nitrogen whereas, sugarcane soil showed high NPK and elementary carbon. On contrary, in the paddy field except for Phosphorous, all other constituents are found in low quantity compared to the recommended level. Totally, 186 fungal isolates belonging to different genera were isolated from three fields in which the highest fungal population were noticed for the sugar cane field, followed by banana and paddy fields. Fungi isolated for three soils showed significant diversity indicating several unknown fungal species indicating some of the dominant species of *Aspergillus*, *Penicillium*, *Fusarium*, *Cladosporium*, and *Rhizopus*species. Among the antidiabetic activity screened from these isolates, the highest activity was observed from the fungi isolated from the paddy field *i.e.,* 73.5% and 70.5% antidiabetic activity of unknown and *Penicillium* sp8 respectively. Hence in the

present study fungi isolated shows significant diversity and antidiabetic activity. The study establishes a base for future scope of novel antidiabetic drug discovery and establishes their mechanism of action.

Keywords: Soil collection,Soil analysis,Fungal Diversity, Sugarcane field, Paddy field, banana field, isolation of soil flora, Anti-diabetic activity

INTRODUCTION

Currently,it has been estimated that approximately 2.2 – 3.8 million species of fungi are included in Kingdom fungi revealing their hugediversity. The diversity in fungal species plays significant functions in communities (as symbionts of animals and plants) as well as in ecosystems (as decomposers to recycle nutrients) which are two stages of ecological organization [Hawksworth and Lucking, 2017].Several factors such as climatic conditions *i.e.*, temperature zones, seasons, plant diversity, and soil fungal community fluctuate the fungal diversity [Li *et* al 2022]. In addition,the type of crop treatment and cultivation also varies the fungal community of the soil [Cloutier, *et al.*, 2020].

Molecular-based identification revealed that Sugarcane bagasse associated with different fungal populations including*Cladosporiumcladosporioides*, *Penicilliumglabrum*, *Aspergillusniger*, *A. terreus*, and *A. fumigatus*[Cortés-Espinosa *et al.*,2006]. The study of fungal communities in different layers of paddy fields indicated high dominance of *Ascomycota, Zygomycota,* and *Basidiomycota*accounting for 46.45%, 31.58%, and 14.35% respectively across all soil layers. However, with an increase in depth, the abundance of *Chytridiomycota*, *Ascomycota*, and*Basidiomycota*decreasedwhile the reverse trend was observed with*Zygomycota*. *Neocallimastigomycota*and*Glomeromycota*are distributed equally in each layer of soil [Li *et al.*, 2020]. On contrary,the soil collected from the banana field was dominantly prevailing *Dueteromycetes,Zygomycotina,Ascomycotina,*and *Mastigomycotina*with 63.63%, 18.18%, 13.63%, and 4.54% respectively.The predominant species were *Penicillium, Aspergillus, Fusarium, Trichoderma, Rhizopus, Alternaria, Mucor, Rhizoctonia,* and *Curvularia* [Shitole *et al.*, 2019].

Several features of fungi such as vast biodiversity, a wide range of inhabitant and survival mechanisms, ease of growth, and more economical production of bioactive molecules optedthem as more trustable organisms for medical applications [Hyde et al., 2019]. In medicine, fungal metabolites are reported to be effective against diabetes, cancer, coronavirus 2019 (COVID-19), cardiovascular diseases, hyperlipidemia, chronic inflammation, and hypertension [Kitagaki et al., 2012].In this view, a fewendophytic fungi example *Guignardia sp*. and insect-origin*Isariafumosorosea* have been reported to synthesize Guignardins A–F and Fumosorinoneanti-diabetic metabolites. However, the anti-diabetic metabolites isolated from fungi are very rare and extensive research needs to be undertaken in this arena.

On the other hand, Owing to the rapid spread of type 2 Diabetes all over the globe, it has become the major concern in health care. About 6% of the global community *i.e.*, 462 million people were suffering from diabetes type 2 indicating 6059 affected individuals in a lakh which is expected to reach7079 cases by 2030 per 1,00,000.Being the ninth leading cause of death, the disease is also responsible for about 1 million-annual mortality and the incidences are more common in low and middle income nations [Khan *et al.*,

2020]. Moreover, the recent rise in Pediatric diabetes from 2.2 to 2.5 per lakh annually is furthermore alarming [Wang *et al.*.2022]. Hence, in the current study effort has been made to establish the relationship between fungal communities isolated from soil cultivated with sugar-rich crops like sugarcane, banana, and paddy and anti-diabetic activity.

Materials

Chemicals: Ethanol, Disodium hydrogen phosphate ($Na_2HPO_4.2H_2O$), Sodium dihydrogen phosphate ($NaH_2PO_4.H_2O$), Sodium Chloride (NaCl), Dinitrosalicylic acid (DNSA), Potassium sodium tartrate tetrahydrate, Sodium hydroxide (NaOH), and Dextrose are laboratory grade purchased from HimediaPvt Ltd. Bangalore.

Media and Antibiotic: Malt Extract, Peptone, Potato dextrose agar (PDA), Malt Extract Broth (MEB)Streptomycin were purchased from HimediaPvt Ltd. Bangalore.

Enzymes: α- amylase and acarbose were purchased from Sigma Aldrich, Bangalore.

Plastic and glasswares: Test tubes, Petri plates, conical flasks, and beakers were borosilicate make purchased from company SRV Scientific Bangalore.

Soil samples: Sugarcane field soil (SF), banana field (BF) soil, and Paddy field (PF) soil

Methods

Collection and physical and chemical characterization of soil samples

Soil samples collected from different fields grown with sugar-rich crops *viz.*,BF, SF, and PF aseptically were analyzed for various physical and chemical parameters using standard procedure. After clearing the top vegetation, the soil was dug for 15 to 20 minutes and thermometer was inserted into the soil, and the temperature of the soil was recorded. Followed by this, the soil was collected in a sterile container and immediately brought to the laboratory. The pH of soil samples was determined using soil slurry prepared by dissolving 10 grams of soil in 50 ml of distilled water.Analyses of physical and chemical characters of soil were carried out on the Agriculture Research campus, Gangavathi, Centre of Agricultural Science, University of Raichur, Karnataka, India.

Isolation of fungal flora from soil samples and their identification

Fungal flora was isolated from soil collected from BF, SF, and PF by soil dilution technique [Waksman, 1927]. Briefly, 1gof soil sample was weighed aseptically and dissolved in the sterile test tube containing 10ml saline. The soil mixture serially diluted was added to the sterile Petri plates and sterile Sabouraud Dextrose Agar (SDA) containing streptomycin antibiotic at 100mg/liter concentration was added. Plates were allowed to solidifyand after solidification plates were incubated at room temperature (30°C) for 7 days. After incubation, the fungal colonies were counted and results were recorded.

Study of fungal diversity through morphological and microscopic identification

Based on the colony morphology, fungi were subcultured in slants and incubated for 7 days at 28°C.The fungi from the slant were stained with lactophenol cotton blue staining and observed under the microscope. Fungi were identified to genus and species level based on colony morphology, the structure of spores, mycelium, conidiophores, and other features with help of the Gilman manual [Gilman, 1966]. The fungal diversity of all soil samples collected fromdifferent fields was studied.

Preparation of soil fungal extracts and screening for anti-diabetic activity

Soil fungi isolates grown for seven days on potato dextrose agar medium were inoculated in Malt extract broth and incubated for 15 days at 30ºC in a rotary shaker at 200 rpm/minute. After incubation, fungal mats were recovered by filtration and washing with sterile water twice and mats were dried. Fungal metabolites were extracted by dissolving dried mats in ethyl acetate for 3days and on the 4th day mats were crushed in a pestle and mortar at 4°C on ice. Mat extracts were centrifuged for 5 minutes at 2000rpm at 4°C and supernatants collected was dried. The dried extracts from the supernatant were re-dissolved in 100µl of 10% DMSO at 1mg/ml concentration by adding 900µl of sterile water and used for anti-diabetic activity [Khan *et al.*, 2019].

All species of fungal isolated were screened for anti-diabetic potential using standard protocol [Shettar*et al.*, 2017]. In the sterile test tube, 0.5ml of the fungal extract was mixed with 0.5ml of α-amylase prepared at 0.5mg/ml in 0.02M sodium phosphate buffer of pH 6.9. The tubes were incubated for 10 min at room temperature (30°C) and 0.5ml of 1% starch solution prepared in 0.02M sodium phosphate buffer of pH 6.9 was added and the reaction mixtures were again incubated at 30°C for 10 min. The reaction was stopped by adding 1ml of DNS (3, 5-dinitrosalicylic acid). The tubes were further incubated again in the pre-set water bath at 100°C for 5 minutes and thereafter the mixtures in tubes were set to cool at 40°C. After cooling the mixture at room temperature, 10ml of deionized water was added in each tube. Along with the test sample, blank containing only buffer in place of extract and amylase, control containing only buffer, and positive control containing Acarbose as a standard drug in place of extract was maintained. Absorbance was recorded for test tubes at 540 nm using a spectrophotometer. The inhibition of α-amylase was calculated using the following.

$$\%\text{inhibition of } \alpha\text{−Amylase} = (\text{Ac control} - \text{As sample}) / \text{Ac control} \times 100$$

Where Ac- control corresponds to the absorbance of the control,

As- sample corresponds to the sample solution

Results and Discussion

In the current investigation, the isolation of fungi from soil cultivated with different sugar-rich crops like BF, SF, and PF was carried out and their diversity was studied. The anti-diabetic potential of these fungi was assessed in vitro and the relationships offungal strains of different soils with potent anti-diabetic activity were established in the present study.

Analysis of physical and chemical parameters of soil samples

The soil composition of agricultural land plays important role in supporting the growth of fungal populations. Since fungi are a major community that anticipates in biodegradation of organic materialin indirectly supplies nutrients for the growth of plants. In the present study, prior isolation of fungi from three soils, all soil samples were analyzed for physicochemical analysis and results are tabulated in **Table 1**. BF soil analyzed for physical parameters showed atemperature of 28ºC and pH of 8.2. Chemical parameters analysis of BF soil showed a high concentration of potassium, a low level of nitrogen. However, the amount of phosphorus and elemental carbon was present in the normal range as compared to recommended concentration. The soil collected from SF contained a high level of NPK and elementary carbon and the pH of the soil was found to be in the normal range. On contrary, NK concentration in PF soil was present at a very low concentration *i.e.*, both at 50kg/acre and phosphorous was found at very high level. The temperature of paddy field soil was 30ºC with acidic pH in nature *i.e.*, pH 6.1.

Properties of paddy field soil are subjected to vertical variation and the chemical constituents of paddy soil at different depths of the soil change. It has been reported that available and total nitrogen and phosphorous, C/N ratio, organic carbon, and the capacity of cation exchange capacity substantially declined with an increase in the depth even though Fe and pH had a reverse trend. In contrast to the subsoilof the paddy field, environmental variations are common in the top soil layer of 0 to 10 or 10 to 20 cm depth [Li *et al.*, 2020].

Table 1.0 Physico-chemical properties of soil samples

Property of Soil	BF Soil	SF Soil	PF Soil	Recommended level in the soil
Temperature (˚C)	28	29	30	-
pH	8.2	7.7	6.1	6.5 – 8.5
Elementary Carbon (EC)	1.6	2.7	1.6	2 – 4
Nitrogen (kg/acre)	65	300	50	112-224
Phosphorous as P_2O_2 (kg/acre)	13	45	43	09- 23
Potassium as K_2O (kg/acre)	320	325	50	56 - 134

Isolation and identification of fungi from soil samples

The fungi population of three soil samples was studied using soil dilution method. From all three soil samples total of 186 fungal isolates *i.e.*, 57, 102, and 27 fungi colonies from BF, SF, and PF soil respectively were isolated as shown in **Figure 1.0**. Further, these fungal isolates were categorized into 5 different genera based on colony morphology and microscopic characteristics. In addition,the majority of these fungi were found to be unknown species.

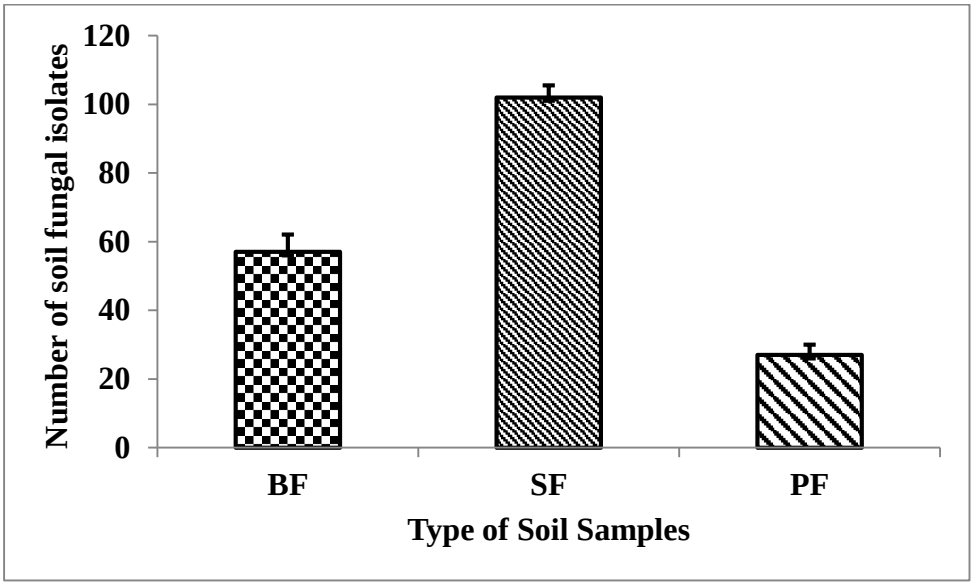

Figure 1 Fungal population of three types of soils grown with sugar-rich crops

The highest fungal population was observed in SF soil compared to BF and PF soil. Furthermore, BF soil was dominated by*Aspergillus* species both in number and diversity contributing around 36% of the total population. Among them, *Aspergillusoryzae*(17%), *A. niger*(9%),*A. ochraceus*(7%), and other *Aspergillus*species werepredominantly present in the soil. Followed by this *Cladosporium*species accounted for about 29% of BF soil fungal flora. Followed by this, *Penicilliumexpansum* and *Fusarium* sp1 are present at 14% and 10% respectively. Only 3.44% of fungal flora was attributed to*Rhizopus*sp [**Figure 2**]. Fungi are very versatile organisms that adapt to any kind of unfavorable and adverse situations and possess high plasticity. As fungi produce a wide variety of enzymes, fungal diversity plays a significant role in the recycling of the nutrients like carbon and nitrogen, the breakdown of organic matter and converting it into biomass, and the bioasorption of toxic metals [*Frac et al.*, 2018]. Hence, fungi diversity is a very important factor in the enhancement of soil fertility due to various activities of soil fungi and maintaining soil health.

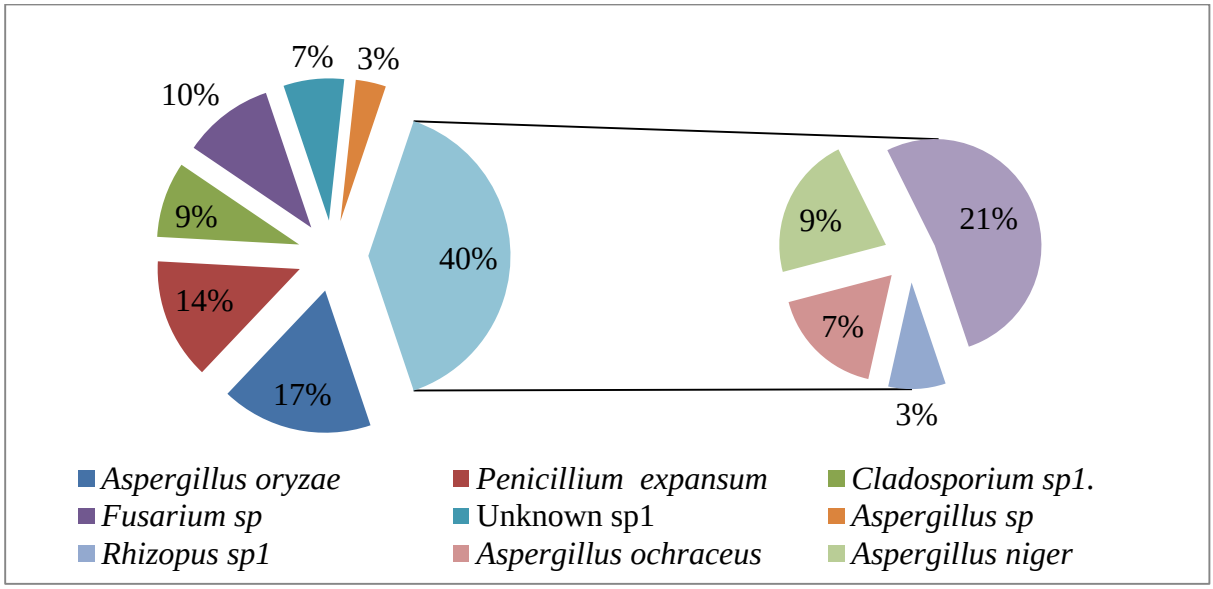

Figure 2 Graphical representation of the percentage of the fungal population in BF soil

Soil collected from the SC field also revealed the highest percentage of *Aspergillus* sp. and *Penicillium* sp. representing 33.66% and 20% of the total fungal community of SC field soil. Interestingly, around 13.26% and 12.24% of total fungi were dominated by unknown sp4 and unknown sp5. Unknown sp6 and Unknown sp7 were present at 6.12% and 8.16% respectively. The remaining 8.16% of fungal flora was populated by unknown sp8 [**Figure 3**].

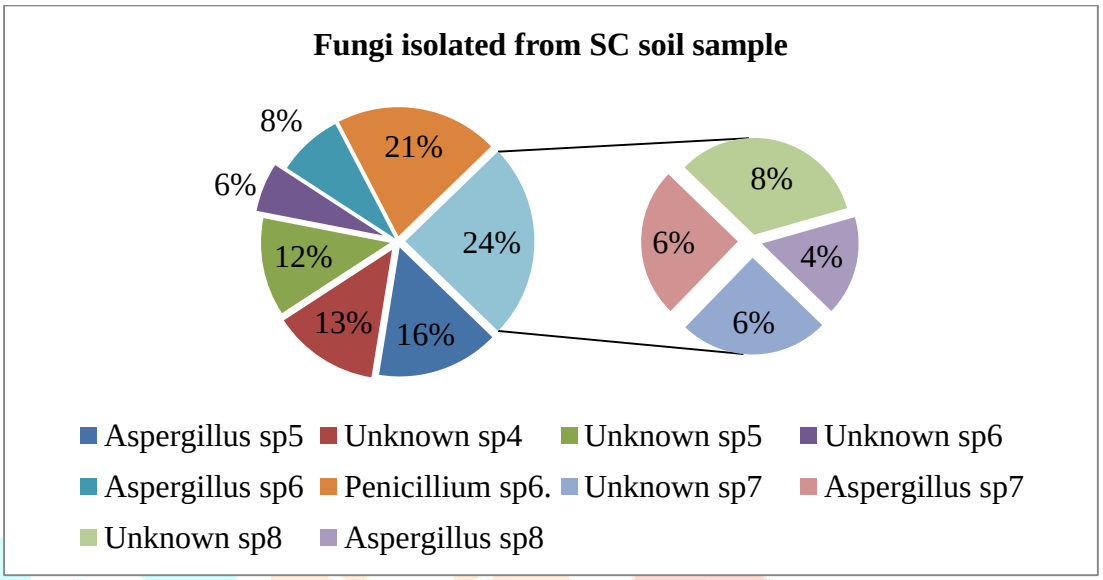

Figure 3 Percentage of fungal species indicating a total fungal community of SC field soil

The fungal community of PF field soil majorly consisting of unknown sp13 indicating 33.3%. Followed by this, 16.67% and 13.3% of the fungal population were contributed by the unknown sp11 and unknown sp16 respectively. Both *Penicillium*sp 7 and sp8 were present at 3.33% and remaining all fungi were found to be unknown species. Unexpectedly, the soil of PF field also not indicated the presence of any *Aspergillus* species which are dominantly present in most of the PF field soil [**Figure 4**].

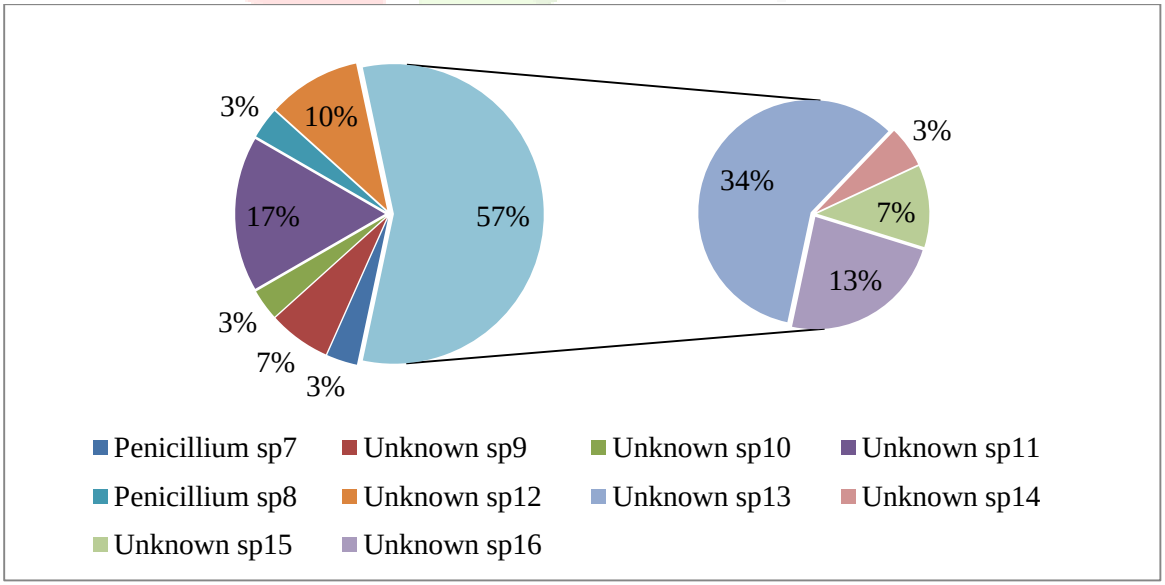

Figure 4 Percentage of fungal population isolated from PF field soil

Assessment of anti-diabetic activity of fungi isolated from BF, SC, and PF field soil

Fungi isolated from soils BF, SC, and PF fields were screened for their potential anti-diabetic activity and the results were compared with the standard drug Acarbose. The results of the anti-diabetic activity of soil fungi were shown in **Figure 5.**In soil fungi isolated from the PF field significant anti-diabetic activity was observed by Unknown sp14 revealing 73.5% activity and followed by this, *Penicillium* sp8 showed 70.5% of anti-diabetic activity. However, in contrast Acarbose used at 250µg/ml indicated 80.6% of anti-diabetic activity. Among the fungal flora of the SC field, the fungi Unknown sp8 and unknown sp4 revealed 69.2% and 68% of anti-diabetic activity determined as α-amylase inhibition potential. Unknown sp7 and *Aspergillus* sp7 also indicated 63.8% and 56.3% of anti-diabetic activity. The remaining fungi isolates of the SC field showed moderate to low activity [**Figure 6**]. *Aspergillus*sp 3 and sp4 isolated from BF soil also showed 69.4% and 67% of anti-diabetic activity and other species such as *Penicillium* sp1 and *Rhizopus* sp1 showed 52.2% and 53.2% of anti-diabetic activity [**Figure 7**].

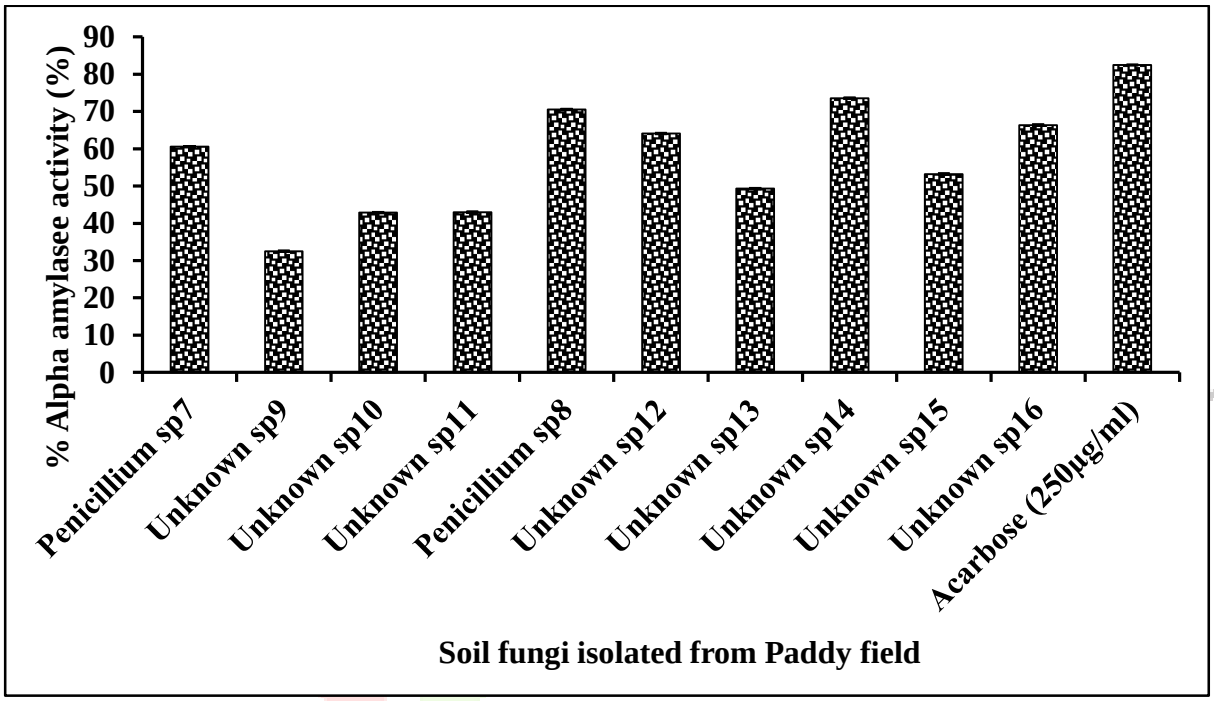

Figure 5. The anti-diabetic potential of soil fungi isolated from PF field

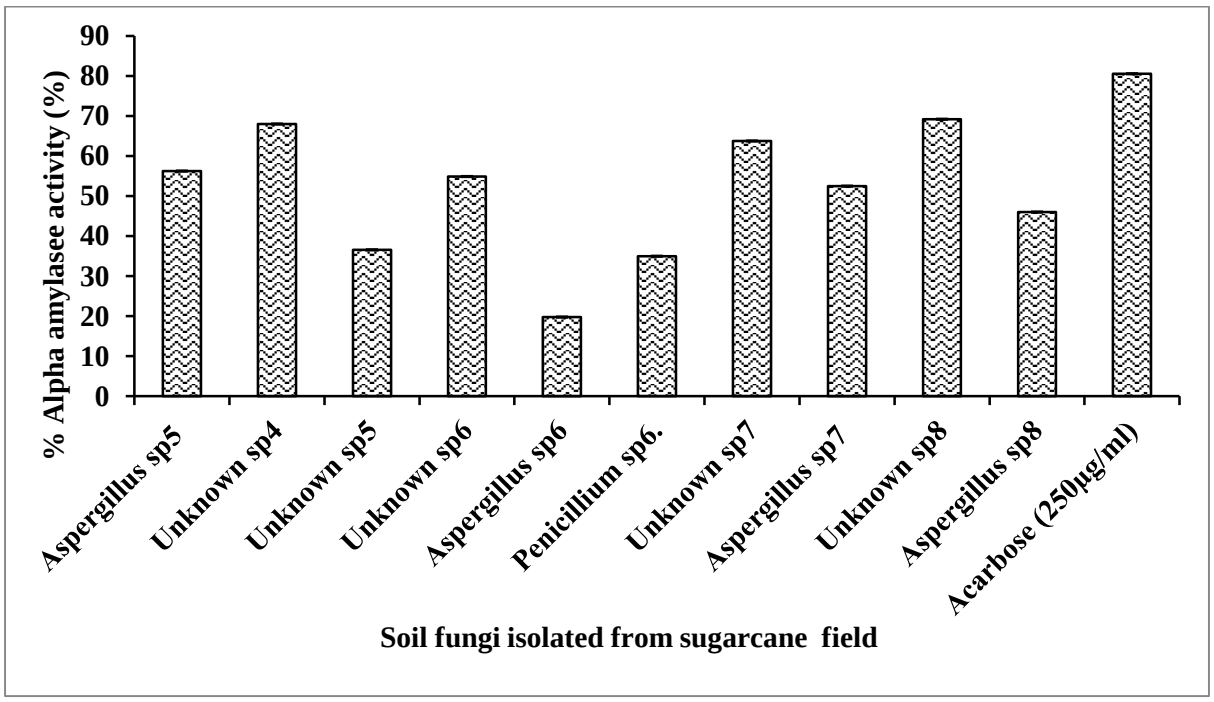

Figure 6. Isolation and anti-diabetic properties of fungi of SC field

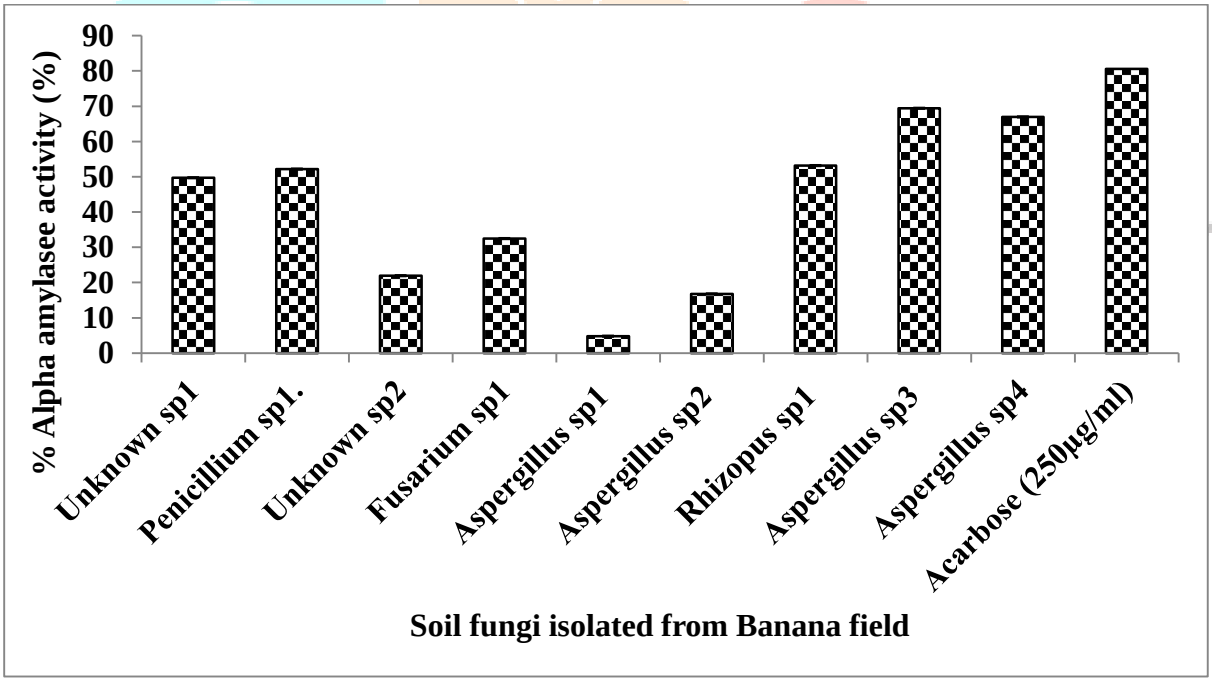

Table 7. Screening of anti-diabetic activity of soil fungi isolated from BF field

Discussion

The biosynthesis of bioactive molecules and a wide array of fungal enzymes that are medically important are strongly interconnected with huge diversity of soil fungi. Increased diversity in the fungal community is always associated enlarged probability of synthesizing novel bioactive substances. Additionally, extreme conditions like osmophilic (due to high sugar levels), barophilic, elevated temperature, and pH (extremelyacidic or basic) also create inexorable pressure on fungi to biosynthesize unique set of enzymes and bioactive molecules.As consequence of diversity and inevitable stress, fungi synthesize the vast

majority of secondary metabolites including antioxidants, anti-inflammatory, anticancer, and antimicrobial (antibacterial and antiviral) agents [Roy *et al.*, 2021].Fungi are also recognized as potential resources for vast number of anti-diabetic therapeutics. Currently, more than 128 anti-diabetic metabolites have been isolated including fumosorinone A, nordivaricatic acid, the divarinyldivarate, and 6-O-methylalaternin which are potent lead for developing anti-diabetic drugs and novel PTP1B (protein-tyrosine phosphatase 1B) inhibitors [Deshmukh et al., 2022]. Although currently several anti-diabetic drugs are proposed they possess various disadvantages including severe side effects.

In the currently study, the fungal diversity of soils cultivated with sugar-rich crops such as sugarcane, banana, and paddy field studies and their anti-diabetic activity was screened to establish the relationships of fungal diversity, anti-diabetic potential, and sugar-rich crops. It has been noticed that the highest fungal species were found in the sugarcane followed by the banana and paddy field soil. However, the fungus isolated from paddy field showed the highest anti-diabetic activity compared to the fungal population isolated from other sources. The fungi isolated from different agriculture fields showed permissible anti-diabetic activity which can be exploited for detailed anti-diabetic activity. Hence, the present investigation proposes the preliminary screening of fungi for anti-diabetic activity leaving the huge future scope of the study. The study is fundamental in future antidiabetic drug discovery which involved the isolation, *invitro*,and *invivo*studies of a potent anti-diabetic molecule.

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