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El Salvadorian Medicinal Plants with Specific Activity Against Trypanosoma cruzi

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ABSTRACT

Diabetes mellitus is a common metabolic illness. Chronic hyperglycemia is a hallmark of diabetes mellitus, Penicillium sp. 8, which presents serious health issues. PDB, phytochemicals, and anti-diabetic medications are available worldwide. The goal of this work was to optimize growth factors in order to increase the anti-diabetic efficacy of Penicillium sp8, which was isolated from paddy soil

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OVERVIEW: Diabetes mellitus, a complicated metabolic disease characterized by persistently high blood sugar, has become a global pandemic. Diabetes, which is characterized by insulin resistance or insufficiency, causes serious side effects include nephropathy, neuropathy, retinopathy, and cardiovascular disease. 1. The International Diabetes Federation estimates that 537 million persons worldwide had diabetes in 2021, and by 2030, that number is expected to rise to 643 million 3. This increasing pattern emphasizes how urgently new and efficient treatment strategies are needed to control and maybe even cure diabetes 4. Oral hypoglycemic medications and insulin therapy are the mainstays of diabetic treatment today. But since these therapies often have drawbacks and side effects, people are looking for safer and more efficient alternatives. 6. Natural substances obtained from a variety of sources, such as microbes and plants, are being investigated more and more for their potential to provide novel treatment alternatives with fewer adverse effects. 8. Particularly, fungi contain a wealth of bioactive substances with possible medical uses. 10. Species of the fungus Penicillium have attracted a lot of interest because of their capacity to generate a wide range of secondary metabolites with possible therapeutic uses. 12. Among these metabolites are substances that block important glucose metabolism-related enzymes, including α-amylase and α-glucosidase, which lowers postprandial hyperglycemia and may help control diabetes 13.

Studies showing these fungal metabolites' efficacy in reducing blood glucose and enhancing insulin sensitivity provide further evidence of their medicinal potential. 15.
To maximize the synthesis of these beneficial metabolites, Penicillium species' growing conditions must be optimized. According to research, metabolite production and activity may be greatly impacted by variables such temperature, pH, inoculum concentration, and nutritional content (18). In contrast to other media, Penicillium species cultivated in Potato Dextrose Broth (PDB) have shown increased synthesis of anti-diabetic compounds (20). Likewise, metabolite synthesis and activity 22 may be further improved by adjusting pH and temperature.
The significance of certain growth conditions in optimizing the synthesis of bioactive substances has been emphasized by further research. For example, studies showed that species of Penicillium cultivated under ideal circumstances produced more anti-diabetic metabolites 25. Furthermore, the effectiveness of fungal extracts was enhanced by careful temperature and pH control 26. The production and activity of anti-diabetic substances were greatly affected by varying the inoculum concentrations 27. These results are consistent with other studies showing that improving growth circumstances may result in substantial increases in the synthesis of metabolites 28.
The characterisation and quantification of bioactive chemicals in fungal extracts have been made easier by recent developments in analytical methods. The identification and measurement of anti-diabetic substances 32 have been made possible using chromatographic and spectroscopic techniques. These techniques have aided the development of fungal metabolites as effective therapies for diabetes 34 and confirmed their therapeutic potential. The importance of this study is increased by the dual potential of fungal metabolites to produce antibacterial and anti-diabetic drugs. Fungi's significance in drug discovery and development is highlighted by their capacity to create molecules with a variety of biological functions 36. In order to improve the synthesis of anti-diabetic chemicals and advance the area of fungal biotechnology, this research attempts to optimize growth conditions for Penicillium sp. 38.
This study aims to increase the production and effectiveness of anti-diabetic chemicals obtained from fungi by methodically enhancing growing conditions and using cutting-edge analytical methods. It is anticipated that the results will help to the continuous attempts to create safe and efficient remedies for this worldwide health issue and provide insightful information about the potential of Penicillium species as a source of innovative therapeutic molecules for diabetes control. 41.

RESOURCES AND METHODS:

Materials: antibiotics and media We bought the following products from Hi Media Pvt. Ltd. in Bangalore, India: Sabouraud Dextrose Broth (SDB), Potato Dextrose Broth (PDB), Czapek Yeast Autolysate Broth (CYB), and Malt Extract Broth (MEB).
Chemicals: Dinitrosalicylic acid (DNSA), potassium sodium tartrate tetrahydrate, sodium hydroxide (NaOH), sodium dihydrogen phosphate ($NaH_2PO_4•H_2O$), sodium chloride (NaCl), ethanol, sodium hydrophosphate ($Na_2HPO_4•2H_2O$), sodium hydroxide (NaOH), Ethyl acetate, petroleum ether, chloroform, Ammonia (NH_3) solution, ferric chloride ($FeCl_3$), concentrated hydrochloric acid (HCl), The reagents of Mayer and Wagner, Benedict's reagent, gelatin, zinc chloride ($ZnCl_2$), ammonium hydroxide, Acetic anhydride, concentrated sulfuric acid (H_2SO_4), Acetate of lead, Fehling's A and B solutions, DMSO, phosphate buffer saline (PBS) solution, potassium hydroxide (KOH) flakes, copper sulfate ($CuSO_4$), powdered starch, Sigma Aldrich in Bangalore, India, supplied the amylase enzyme, metformin, 3,5-Dinitrosalicylic acid (DNS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, and 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT).
The following tools are used: GC-MS, column chromatography, autoclave, water bath, pH meter, weighing balance, fungal incubator, and thin-layer chromatography.
We bought the enzymes α-amylase and acarbose from Sigma Aldrich in Bangalore, India.
Glassware and Plastic: SRV Scientific, Bangalore, India, supplied the borosilicate test tubes, petri plates, conical flasks, and beakers.
Penicillium sp. 8, a fungus, was isolated from soil samples.

Techniques:

Soil Sample Collection: Samples of soil were taken from a number of sites in the Anantapur area, especially from crops that are high in sugar, such sugarcane, bananas, and rice. To avoid contamination, the samples were put in sterile plastic bags. Since the topsoil layer is thought to be where the majority of microorganisms are normally located, soil was collected down to a depth of 15 cm.

Isolation of Fungi from Soil Samples: Using the serial dilution approach, fungi were isolated from soil samples and cultured on a variety of media, such as Sabouraud Dextrose Agar (SDA) and Potato Dextrose Agar (PDA).

Soil Dilution Plate Method: One gram of soil and one milliliter of distilled water were combined to create a microbiological suspension. The solution was diluted in various ways in order to separate the fungus. Twenty milliliters of sterile Sabouraud Dextrose Agar (SDA) were placed on sterile Petri plates, and one milliliter of each dilution was added. According to Waksman's (1927) instructions, the Petri plates were incubated at 28°C and observed every day for three days.

Choosing the Best Media for Anti-Diabetic Action: A variety of growth media were created and sterilized, such as Malt Extract Broth (MEB), Czapek Yeast Extract Agar (CYA), Potato Dextrose Agar (PDA), and Sabouraud Dextrose Agar (SDA). After being infected with 10^6 conidia/mL, the fungus was shaken and incubated for 15 days at 30°C. The fungal mats were filtered, cleaned, and dried after incubation. Ethyl acetate was used to extract the metabolites, and their anti-diabetic properties were examined.

Optimizing Inoculum Concentration: 2.5×10^4 and 2.5×10^5 conidia concentrations were inoculated into sterile Potato Dextrose Broth (PDB) medium, which was produced with a pH of 6.5.

Conidia/mL of 2.5×10^6, 2.5×10^7, and 2.5×10^8. For 15 days, the infected flasks were incubated at 30°C. The fungal mats were dried after incubation, and metabolites were taken out for testing against diabetes. The ideal conidial concentration of 2.5×10^7 conidia/mL was put into PDB medium (pH 6.5) in order to determine the ideal temperature for anti-diabetic activity.

For fifteen days, the medium was incubated at a range of temperatures: 25°C, 30°C, 35°C, 40°C, and 45°C. Following incubation, metabolites were collected for anti-diabetic testing, and the fungal mat was recovered and dried.

pH Optimization for Anti-Diabetic Activity: 2.5×10^7 conidia/mL were injected into PDB medium after it had been adjusted to a range of pH values (3.0 to 10.0). For 15 days, the flasks were incubated at 30°C. The fungal mats were dried after incubation, and their metabolites were separated and examined for anti-diabetic properties.

A fresh culture of Penicillium sp. 8 was subcultured on Potato Dextrose Agar (PDA) and incubated for seven days at 30°C in order to prepare the seed culture for inoculation in mass cultivation. Ten milliliters of sterile water and 0.5% Tween 20 were combined to create a spore suspension. A hemocytometer was used to measure the number of spores, and the suspension was adjusted to 2.31×10^8 conidia/mL. 10 mL of spore suspension was used to inoculate a 100 mL seed culture that had been produced with PDB (pH 3.5).

Production of Anti-Diabetic Molecules via Mass Cultivation: A 2 L conical flask with 1.5 L of sterile PDB (pH 3.5) was used for mass culture. After inoculating the flask with 150 mL of seed culture, it was incubated at 30°C until the highest level of anti-diabetic activity was seen. Ten milliliters of the broth were taken out at regular intervals for anti-diabetic tests in order to check the growth medium.

Crude Anti-Diabetic Molecule Extraction by Successive Solvent Extraction: The fungal mat was isolated from the growing media as soon as the anti-diabetic activity reached its peak.

Petroleum ether, ethanol, chloroform, and ethyl acetate were used in order to extract the mat. A rotary evaporator was used to evaporate the solvents, and the dry extracts were weighed and their anti-diabetic properties evaluated.

Assessment of Anti-Diabetic Activity (α-Amylase Inhibition Assay): The dinitrosalicylic acid (DNS) technique was used to test the α-amylase inhibition. In short, 0.5 mL of fungal extract and 0.5 mL of α-amylase solution (1 mg/mL) were combined, and the mixture was incubated for 10 minutes at room temperature. After adding

0.5 mL of a 1% starch solution, the reaction was incubated for ten more minutes. After adding 1 mL of DNS solution to halt the process, the tubes were incubated for five minutes at 100°C. At 540 nm, absorbance was measured.

(Accontrol−Assample)/Ac control x 100 = %inhibitionofα−Amylase

As-sample denotes the sample solution, whereas Ac-control denotes the absorbance of the control.

RESULTS: In the present study, mass production was conducted using the optimized medium to increase the synthesis of secondary metabolites, and media optimization and other factors were examined. Of the three soil types—banana, sugar cane, and paddy—paddy soil is thought to be the most effective in inhibiting the metabolites that cause diabetes.

Choosing the Best Media for Anti-Diabetic Action: Penicillium sp8 was cultivated on a variety of growth medium, including Malt Extract Broth (MEB), Czapek Yeast Autolysate Broth (CYB), Potato Dextrose Broth (PDB), and Sabouraud Dextrose Broth (SDB). Comparing these PDB Media to other media, a significant increase in antidiabetic efficacy was seen (Fig. 1). The fungus that was cultured on MEB medium then shown significant anti-diabetic action. PDB medium was thus further chosen for growth parameter tuning in order to provide further anti-diabetic action.

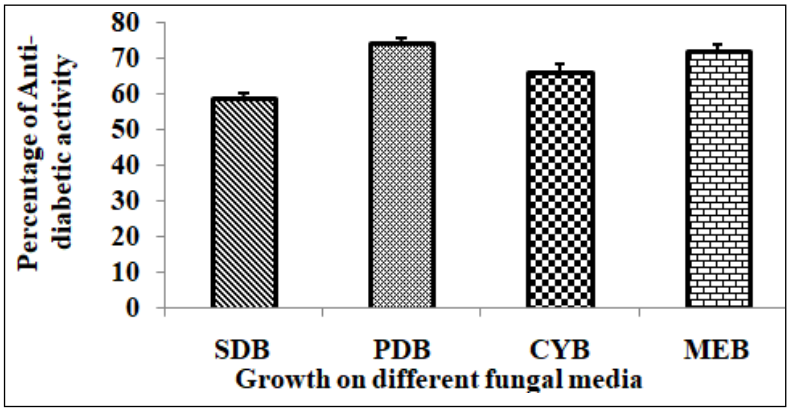
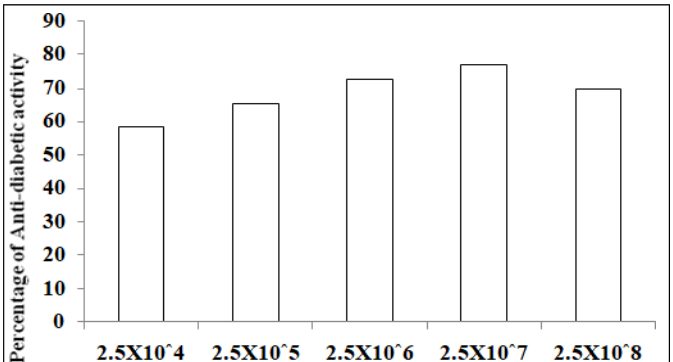


FIG.1:SCREENINGOFPOTENTFUNGALISOLATEFORANTI-DIABETICACTIVITYONDIFFERENTMEDIA

Optimization of Inoculum Concentration: PDB medium was used to ascertain the ideal inoculum concentration for optimizing anti-diabetic action. As seen in Fig. 2, the maximum activity was recorded at a conidial concentration of 2-5x10^7 conidia/ml, attaining 76.8% anti-diabetic activity. The anti-diabetic activity of the medium infected with 2-5x106 conidia/ml was 72.5%. The fungus's anti-diabetic efficacy peaked at a conidial concentration of 2–5x107/ml, and decreased activity was seen at



Both 2-5x104/ml and 2-5x108/ml had anti-diabetic activity of 58.6% and 69.7%, respectively. FIG. 2: FUNGAL INOCULUM CONCENTRATION OPTIMIZATION FOR ANTI-DIABETIC ACTIVITY Finding the Ideal Temperature: Temperature is a key physical growth element for fungi, and the temperature at which they live has a significant impact on the biochemical events that take place inside them. Significant activity at 30°C and 35°C, or 76.3% and 73.6% of anti-diabetic activity, respectively, was found in the investigation on the impact of temperature on fungal anti-diabetic activity. But when the temperature rose, the activity decreased even further, showing 46.33% anti-diabetic activity at 45ºC. The fungus cultured at 25ºC showed moderate anti-diabetic efficacy.

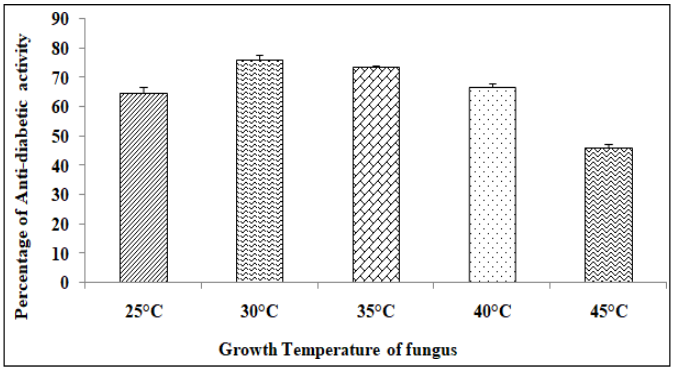


FIG. 3: STUDY OF EFFECT OF GROWTH TEMPERATURE ON ANTI-DIABETIC ACTIVITY OF SOIL FUNGUS

Optimization of pH: Using the optimum growth temperature, the optimum pH for anti-diabetic activity of fungus was determined. The fungus inoculated in sterile PDB media adjusted to different pH showed significant anti-diabetic activity at acidic pH and decreased with increased pH. Substantial anti-diabetic activity was observed atextremeacidicpH3.5andpH4.5attributedto 78.8% and 75.2% respectively as shown Fig. 3. However, the anti-diabetic activity was noticed declining at alkaline pH from pH 7.5 to 10.5.

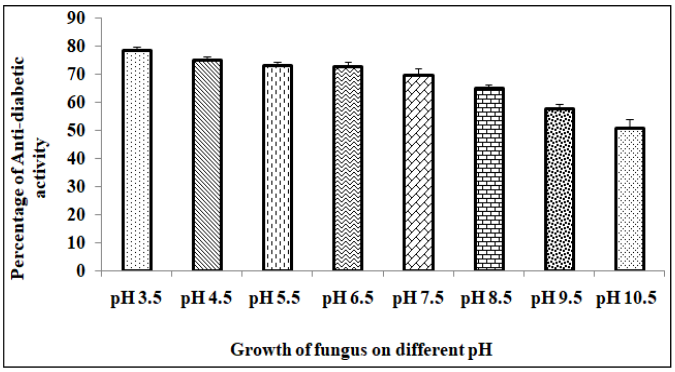


FIG.4:EFFECTOFDIFFERENTPHONANTI- DIABETIC ACTIVITY OF SOIL FUNGUS

Anti-diabetic molecule mass cultivation and production: seed culture preparation Penicillium species were subcultured on potato dextrose agar and incubated for seven days at 30ºC in a fungal incubator. A spore suspension was made in 10 milliliters of sterile water with 0.5% tween 20 after seven days. Spore suspension was adjusted to 2.5x107 conidia/ml and spores were counted using a hemocytometer (Fig. 4 & Fig. 5). In the meanwhile, 10 ml of fresh spore suspension was added to 100 ml of sterile potato dextrose broth that had been adjusted to the ideal pH of 3.5. This resulted in 2.5x107 conidia/ml of seed culture media. After being incubated for seven days at 30 degrees Celsius, the seed culture was utilized to inoculate mass cultivation.

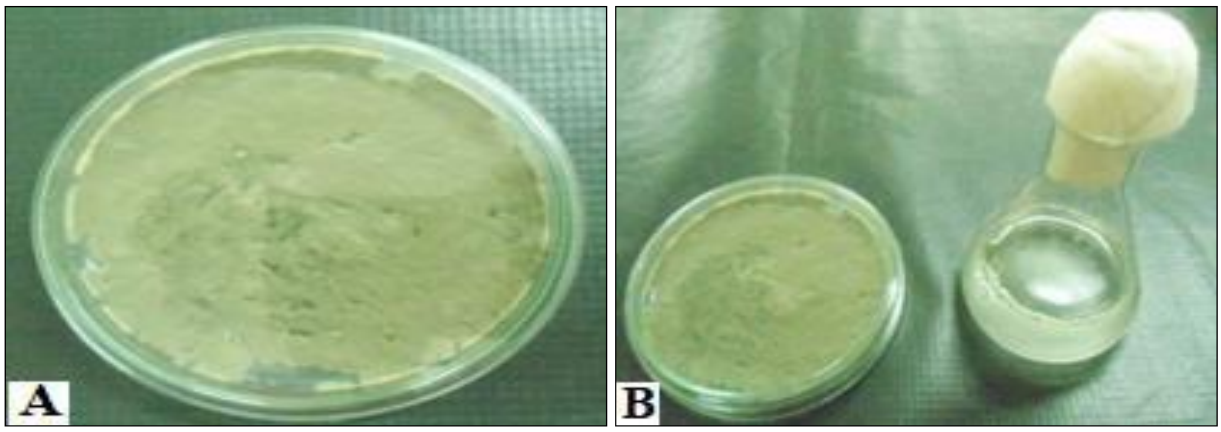


FIG.5:PENICILLIUMSPECIESGROWNONPLATE(A)ANDSPORESUSPENSIONPREPAREDINSALINE CONTAINING TWEEN 20 (B)

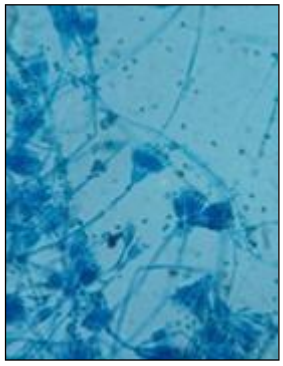


FIG.6:MICROSCOPICIMAGEOFPENICILLIUMSP8 STAINED WITH LPCB

Mass Cultivation and Monitoring: An improved potato dextrose broth was used to produce an anti-diabetic compound in large quantities. The 1.5 liters of sterile PDB in a 2-liter conical flask

(pH 3.5), 150 milliliters of Penicillium species seed culture were added. To create the most anti-diabetic compounds, the flask was incubated at 30ºC for the creation of anti-diabetic metabolites (Fig. 6). By removing 10 milliliters of growing media and conducting anti-diabetic activity, the production of an anti-diabetic molecule was observed. The fungus broth was removed and separated as soon as the highest level of anti-diabetic action was seen. Additionally, mat extract was tested for anti-diabetic properties and contrasted with broth (Fig. 7). Mat was chosen for a further solvent extraction step because it had the strongest anti-diabetic efficacy.

FIG.7:MASSPRODUCTIONOFANTI-DIABETICMOLECULEFROMSOILFUNGUS

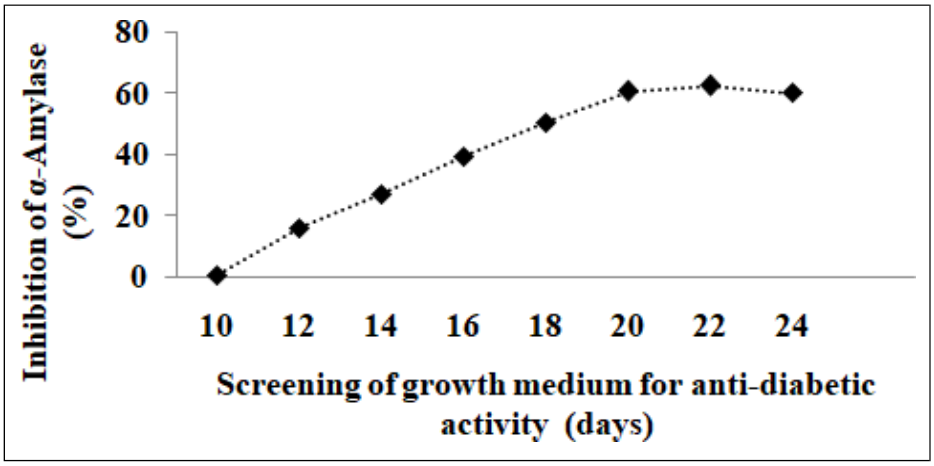


FIG. 8: MONITORING OF GROWTH MEDIUM FOR PRODUCTION OF ANTI-DIABETIC MOLECULE DURING MASS CULTIVATION OF SOIL FUNGUS

Fungal Mat Extracts' Anti-Diabetic Action: Fungi were removed from the growing media by decanting it in a different container when the growth medium's maximum anti-diabetic efficacy was noted. First, petroleum ether was used to remove the fungal mat for two days, after which petroleum ether extract was collected. Mat was once again extracted and dissolved in chloroform until all of the metabolites were found. Likewise, all of the crude fungal metabolites were recovered by extraction using ethyl acetate. The rotary evaporator was used to dry all of the crude extracts until the solvent was completely gone. Weighing each crude extract and repeating and comparing its anti-diabetic effect are shown in Figures 8 and 9. Using a variety of analytical methods, the extract with the strongest anti-diabetic activity was chosen further for anti-diabetic molecule purification.

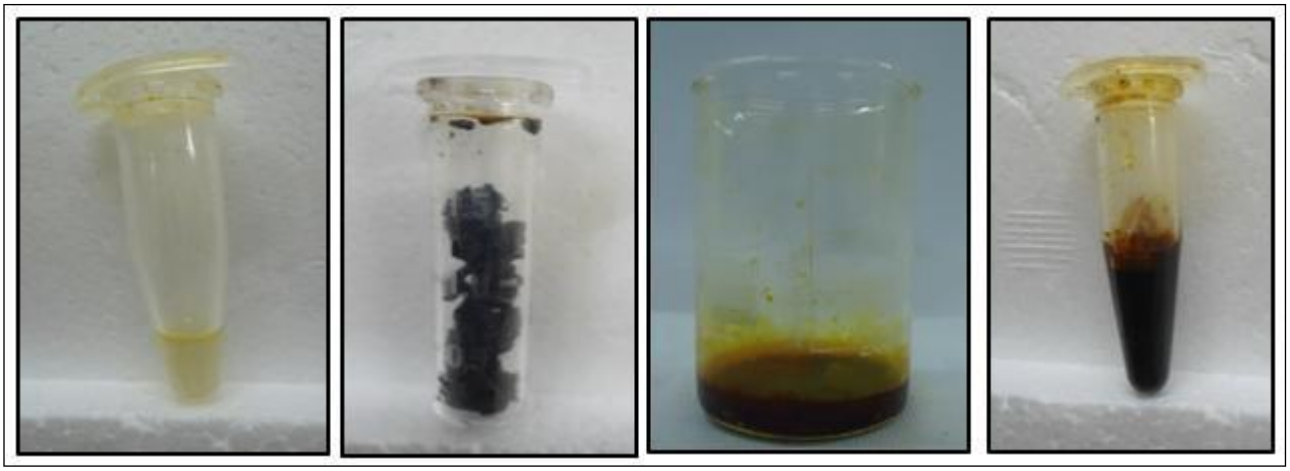


FIG.9:PET.ETHERCHLOROFORMETHYLACETATEETHANOL

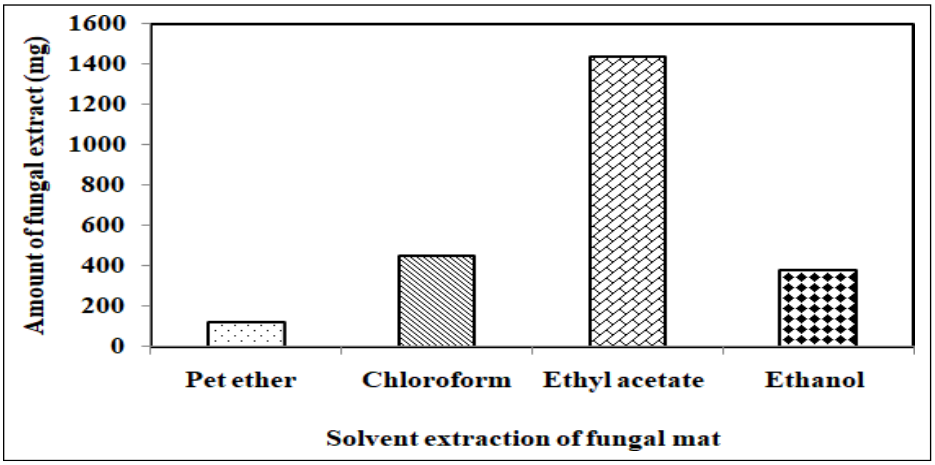


FIG.10:COMPARISONOFDIFFERENTYIELDOFFUNGALMATEXTRACTS

Assay for Anti-Diabetic Activity: The inhibition of α-amylase, a crucial enzyme in the conversion of starch to glucose, was measured to ascertain the anti-diabetic activity. The following are the findings of the α-amylase inhibition test for several fungal extracts:
Petroleum Ether Extract: With the lowest α-amylase inhibition of 2.24%, the extract showed little anti-diabetic effect.
Chloroform Extract: A moderate 19.81% α-amylase inhibition was observed in the chloroform extract. Ethyl Acetate Extract: This extract showed the strongest α-amylase inhibition, measuring 65.70%, indicating that it has strong anti-diabetic properties.
Ethanol Extract: Compared to the ethyl acetate extract, the ethanol extract exhibited a modest but noticeably lower α-amylase inhibition of 24.88%.
Inhibition Calculation: The following formula was used to determine the inhibition of α-amylase: (Ac−AsAc)×100\% %Inhibition ofα-Amylase= \text{Inhibition of α-Amylase} = \left(\frac{A_c - A_s}{A_c}\right)\times100 The inhibition of α-amylase is equal to (AcAc−As)×100. where AcA_cAc is the absorbance of the extract-free control and AsA_sAs is the absorbance of the extract-containing sample.
The ethyl acetate extract demonstrated the most promising results according to the α-amylase inhibition test. opposed to

diabetic action, as seen in Table 1 and Figure 10, and was chosen for further purification and examination.

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TABLE 1: SCREENING OF FUNGAL MAT EXTRACTS FOR ANTI-DIABETIC ACTIVITY

Fungalextract (1mg/ml)	α-amylaseInhibition(%)
Pet.ether	2.24
Chloroform	19.81
Ethylacetate	65.70
Ethylacetate	24.88

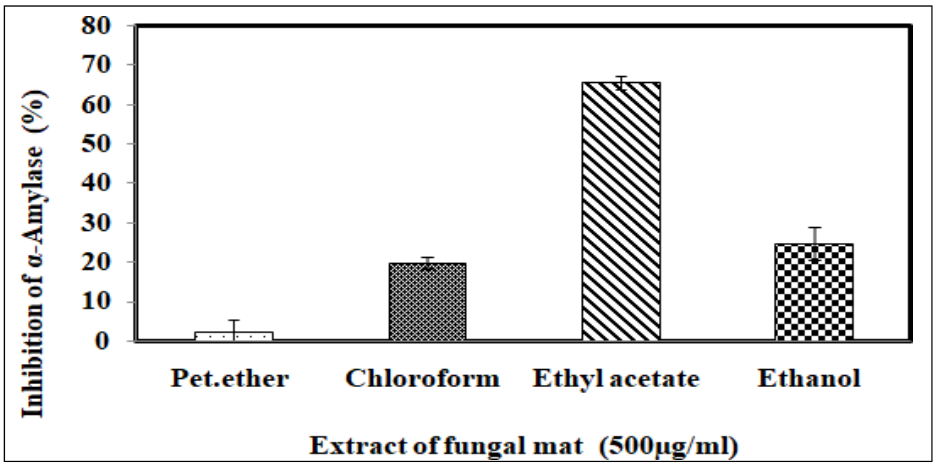

FIG.11:ANTI-DIABETICACTIVITYOFFUNGALMATEXTRACTS

Analyzing the Fungal Mat Ethyl Acetate Extract Phytochemically: The ethyl acetate extract of the fungal mat contains a variety of phytochemicals, including phenols, flavonoids, alkaloids, carbohydrates, proteins, glycosides, oils, and terpenoids, which may be the cause of the observed anti-diabetic benefits. However, no tannins nor saponins were present in the extract. The presence of these bioactive components suggests that the fungal extract may have therapeutic value in the treatment of diabetes, however further investigation is needed to isolate and identify specific active compounds.

Sl.no.	QualitativeTest	Results
1	DetectionofPhenols	Ferricchloridetest:Positive,Leadacetatetest:Positive
2	TestforTannins	Braymer'stest:Negative,Gelatinetest:Negative
3	Testfor Flavonoids	Alkalinereagenttest:Positive,Leadacetatetest:Positive
4	TestforOilsandFats	Positive
5	Testfor Alkaloids	Mayer'stest:Positive,Wagner'stest:Positive
6	Testfor Carbohydrates	Fehling'stest:Positive,Benedict'stest:Positive
7	DetectionofSaponins	Negative
8	DetectionofProteins	Positive
9	Testfor Glycosides	Positive
10	DetectionofTerpenoids	Positive

DISCUSSION: To increase the synthesis of anti-diabetic metabolites, the medium and growing conditions for Penicillium sp. were optimized in this work. With the strongest anti-diabetic action, the findings showed that Potato Dextrose Broth (PDB) was the most beneficial medium. This result is consistent with earlier research that shown that PDB's nutrient-rich makeup promotes significant outputs of secondary metabolites. 6, 7. The improved performance of PDB over other media, including Czapek Yeast Autolysate Broth (CYB), Malt Extract Broth (MEB), and Sabouraud Dextrose Broth (SDB), highlights how crucial it is to choose the

right growing medium in order to maximize metabolite synthesis. The effect of inoculum concentration on anti-diabetic action was also investigated in this research. With a noteworthy anti-diabetic effect of 76.8%, the ideal concentration was found to be 2-5 × 10^7 conidia/ml. This result is in line with findings from previous investigations that highlight how crucial inoculum concentration is for fungal fermentation. 3, 8. Higher concentrations may cause conidia to compete with one another, which would hinder development and output, while lower concentrations would leave insufficient biomass for efficient metabolite synthesis. Temperature was a key factor in Penicillium sp.'s anti-diabetic effects. The best temperatures were discovered to be 30°C and 35°C, which are known to promote the development of fungi and the generation of secondary metabolites 9,4. Higher temperatures (45°C) significantly reduce anti-diabetic action, which implies that too much heat damages enzyme function and metabolic processes 5. This result emphasizes how important it is to keep the temperature at the ideal level to maximize bioactivity.

It was also shown that acidic pH values (3.5 and 4.5) had the greatest impact on metabolite synthesis. This is in line with previous studies showing that certain fungal secondary metabolites are more likely to be produced in acidic environments10,6. Enzyme activity and metabolite stability are disrupted in less favorable pH settings, which is probably why activity decreases at alkaline pH values.

For mass culture, Penicillium sp. was cultivated on a bigger scale under the optimal circumstances. After being exposed to solvent extraction, the fungal mat showed the strongest anti-diabetic efficacy. The greatest anti-diabetic action was shown by the ethyl acetate extract (65.70% α-amylase inhibition), which is in line with research indicating that ethyl acetate is useful for extracting bioactive substances. 9, 7. The solvent's efficacy is probably influenced by its capacity to dissolve a broad variety of organic molecules while keeping out more polar ones. A phytochemical study of the ethyl acetate extract showed that it included phenols, flavonoids, alkaloids, and carbohydrates, among other bioactive substances. These substances are well-known for their anti-diabetic and other medicinal qualities. 5, 6. Flavonoids and phenolic substances, for example, have been shown to enhance insulin sensitivity and block enzymes that hydrolyze carbohydrates 4,8. These substances' presence in the ethyl acetate extract demonstrates its potential as a medicinal agent and supports its strong anti-diabetic action.

Overall, the research was effective in optimizing the circumstances for Penicillium sp. to produce the most anti-diabetic metabolites. Enhancing metabolite synthesis required the use of PDB medium, the ideal inoculum concentration, and certain pH and temperature levels. Rich in bioactive components, the ethyl

acetate extract showed strong anti-diabetic effects, suggesting that it may be developed further as a treatment for diabetes.

RESULTS: Enhancing the synthesis of anti-diabetic metabolites has advanced significantly as a result of Penicillium sp. growing conditions and medium modification. In contrast to other media including Sabouraud Dextrose Broth (SDB), Czapek Yeast Autolysate Broth (CYB), and Malt Extract Broth (MEB), this research showed that Potato Dextrose Broth (PDB) was the most efficient medium, exhibiting a considerable increase in anti-diabetic activity. The anti-diabetic action was shown to be maximized at an optimal inoculum concentration of 2–5 × 10^7 conidia/ml, highlighting the significance of exact inoculum levels. The manufacturing process was further optimized by temperature and pH, with the best temperatures being 30°C and 35°C and the maximum anti-diabetic action being provided by pH 3.5 and 4.5.
The largest output of anti-diabetic chemicals was demonstrated by mass cultivation under these ideal circumstances. With significant α-amylase inhibition, the ethyl acetate extract showed the most anti-diabetic effect among the extracted fractions. This extract's phytochemical examination identified many important bioactive substances, including as phenols, flavonoids, alkaloids, and glycosides, which are probably what cause the effects that have been seen.
The study's result emphasizes how important it is to maximize the generation of anti-diabetic compounds from Penicillium sp. by optimizing growing conditions and medium. With possible ramifications for the treatment of diabetes, the results provide insightful information for further investigation into the discovery and isolation of new anti-diabetic drugs. The medicinal potential of bioactive substances generated from fungi may be further investigated thanks to this investigation.

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