Screening of *Aspergillus terreus* for antifungal drug susceptibility

Project report submitted in partial

Fulfilment of the requirement for the degree

Of

BACHELOR OF TECHNOLOGY

In

BIOTECHNOLOGY

By
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UNDER THE GUIDANCE OF

Dr Jata Shankar

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY,

WAKNAGHAT

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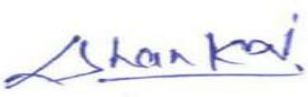
STUDENTS' DECLARATION

I hereby declare the work presented in the Project report entitled "Screening of *Aspergillus terreus* for antifungal drug susceptibility" submitted in the partial fulfilment of the requirements for the degree of Bachelor of Technology in Biotechnology at Jaypee University of Information Technology; Waknaghat is an authentic record of my work carried out under the supervision of Dr Jata Shankar, Associate Professor. This work has not been submitted elsewhere for the reward of any other degree/diploma. I am fully responsible for the contents of this project report.

Shiuli Samaddar, 161817

This is to certify that the above statement made by the candidates is correct to the best of my knowledge.



Dr Jata Shankar

Associate Professor
Department of Biotechnology and Bioinformatics
Dated: 14-07-2020

CERTIFICATE

This is to certify that the work which is being presented in the project report titled "Screening of *Aspergillus terreus* for antifungal drug susceptibility" submitted in partial fulfilment of the requirements for the degree of Bachelor of Technology in Biotechnology at Jaypee University of Information Technology, Waknaghat is an authentic record of work carried out by Shiuli Samaddar (161817) under the supervision of Dr Jata Shankar, Associate Professor, Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Waknaghat. The above statement made is correct to the best of our knowledge.

ACKNOWLEDGEMENT

Department of Biotechnology and Bioinformatics at Jaypee University of Information Technology, Waknaghat for providing all the students of biotechnology an opportunity to choose desirable individual study and assigning well knowledgeable faculty to guide the students throughout their project work

.

I extend my deep sense of gratitude towards my project guide Dr Jata Shankar, Associate Professor, Department of Biotechnology and Bioinformatics at Jaypee University of Information Technology for his invaluable guidance, keen interest and inspiration which helped me in carrying out the project work. He steered me in the right direction whenever I came across any difficulty throughout the work.

I would also like to thank Mrs Sonia Shishodia (PhD Scholar) of the Department of Biotechnology at Jaypee University of Information Technology for assisting us throughout the work of the project. It would not have been possible to get the results without her immense help, encouragement and guidance.

I am grateful to my parents for providing continuous encouragement throughout our period of study. Lastly, I thank all those who were involved directly or indirectly in the completion of present work.

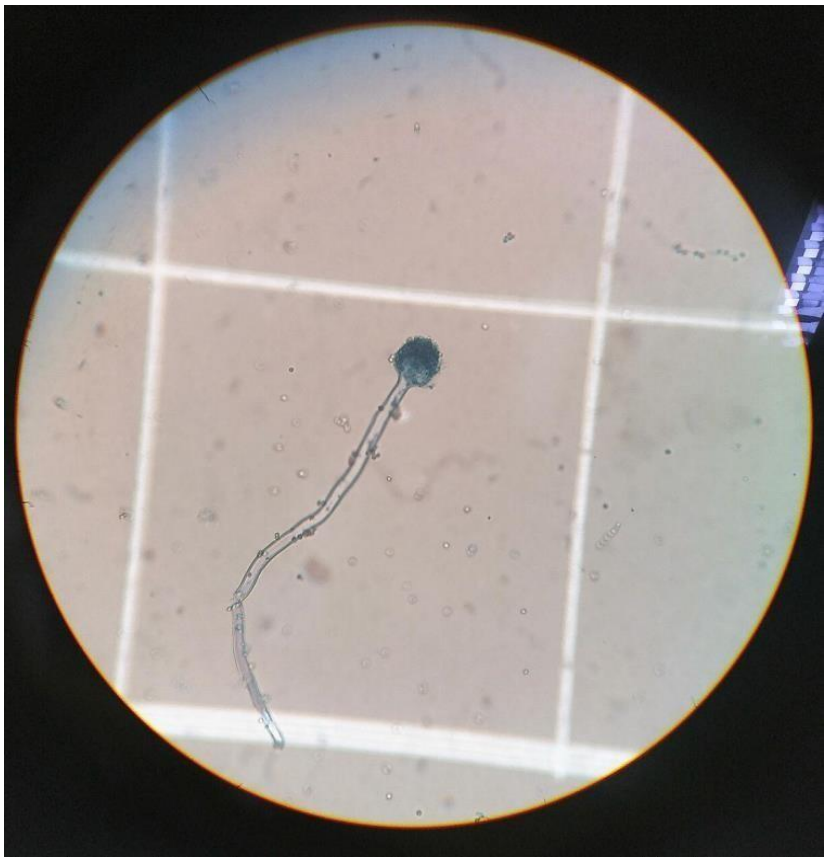
ABSTRACT

Inhalation of spores present in the environment often responsible for number of diseases. Immune status of the individual is critical to the outcome of inhaled spores, host will succumb to the disease or not. One of the most important members of these spores is from the group of *Aspergillus* species [1]. The treatment given to the patients who are infected with such type of spore cause diseases is nowadays does not work properly and the patients are still remaining untreated or effected from the disease. The reason behind this may be the increase in the resistance of the spores. That is the spores are now being able to generate such type of mechanism which is making them strong or resistance to the fungicides or the other medical treatment given to the patients [2]. Heat shock protein Hsp90 has been regulated in response to the antifungal drug (Amphotericin B) [3] and thus suggested that Hsp90 not only provide resistance to the drugs but also in maintain the cellular function in fungi [4].

This may have arises due to the increase in use of fungicides in the fields were farmers want their crop to be protected from the fungal diseases and due to which they are using fungicides very frequently and this made the spores resistance to those particular fungicides. *Aspergillus terreus* is nowadays the major cause of aspergillosis [5]. Previously, it has been evident from the reports that *Aspergillus terreus* isolates are naturally resistant to AmB. Also, *Aspergillus terreus* shows high in vitro and in vivo MICs for AmB which proves AmB resistance. To determine the resistance pattern of the isolates different methods are used like AST, MTT assay, PCR, etc. *Aspergillus terreus* isolates were successfully cultured. It was microscopically confirmed as *A. terreus* isolated. Genomic DNA extracted from mycelial mat of *A. terreus* isolated showed good quality DNA. The DNA sample was for species specific gene terrylysin for amplification and standardization. *Aspergillus terreus* conidia were harvested followed by MTT assay and followed susceptibility test.

INTRODUCTION

Aspergillus terrestris is another name for *Aspergillus terreus*, it is a fungus (mold) found everywhere in the soil. Before it was considered to be strictly asexual, but now it has seen to also reproduce by sexual reproduction. It is saprotrophic and found in tropical and also sometimes in subtropical regions. Other than soil it is also found in habitat such as decomposing and dusty area.



Morphotypes of *Aspergillus terreus* (Isolate-2)
Stained with Lactophenol blue (40 X view)

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It has its brown colour and after getting aged in media it gets darker. Its conidial heads are compact, biseriate, and densely columnar, *A. terreus* can be clinically observed by checking the elevated inflammatory responses.

Production of aleurioconidia and its cinnamon-brown colony colouration helps to easily distinguish it from other species of *Aspergillus*. *A. terreus* is a thermo tolerant species. It has optimal growth in temperatures between 35–40 °C (95–104 °F), and maximum growth within 45–48 °C (113–118 °F). *Aspergillus terreus* can cause opportunistic infection in immunocompromised patients. A total of 340 species are found in the genus *Aspergilli*. In the environment they show different types of biological functions. Forty species among them are responsible for health related problems [1, 6].

Aspergillosis is caused by the *Aspergillus* species which is server clinical especially in immunocompromised patients, because of which it falls into the category of opportunistic fungus [7].

The conidia of *Aspergillus* are very small in size i.e. 2–5 µm [8], due to which it became the main source for *Aspergilli* conidia distribution in the environment [9, 10]. Also because of its small size it remains in the environment for longer time duration [10]. It is also inhaled by the human beings very easily because of its small size and there is a chance of germination of conidia into hyphae inside the respiratory mucosa, if it is not cleared by the phagocytic cells [11].

Also the threat due to *Aspergillus* is increasing within the humans day by day due to the increase in drug resistance isolates of *Aspergillus* species [12, 13]. There are three class of antifungal drugs which currently being used forth for treating the infections which are caused by *Aspergillus* species and are common among the people; **polyenes, triazoles and echinocandin** [14, 15]. Thus, there is a need to focus investigations on to screen environmental samples that are resistant to azoles.

Present treatment strategy for IA

- There are three major classes of antifungal which are nowadays used as a treatment such as- triazoles, echinocandin and polyenes
- On the basis of MIC value, azoles resistant ***Aspergillus*** isolates were categorised; ITC > 2 μg/μl, VRC > 2 μg/μl and posaconazole (> 0.5 μg/μl). Data as per guidelines in the CLSI reference method [16]
- There was a minimal toxic effect of liposomal AmB in IA patients. They showed very long persistency against the azole resistant *Aspergillus* species [17]. But AmB is not recommended for aspergillosis that is caused by ***A. terreus*** and also AmB-resistant *A.* species. Here the alternate approach is the combination therapy with a synergistic response [18, 19].

Major two reasons for drug resistance

Aspergillus terreus produces a large amount of spores that distributes in the environment very efficiently over a wide range like other *Aspergillus* species. This fungus has a kind of morphology which helps it to distribute its spores in the air current globally.

i. Clinical practise, due to repeated use of azole drugs for diseases like aspergillosis becomes the major reason for the azole resistance [12].
ii. Also the increased use of fungicides in the field of agriculture the resistance for azole drugs in the *Aspergillus* species is also increasing [20, 21].

Also, the cause of drug resistance in *A.* Species is due to the development of biofilms. Biofilms protects the fungus in the hostile environment and provide temporary resistance to fungicide. i.e., It provide antifungal activity [22-24]; the spores are allowed to distribute in the air current like

wind. In fact *A. terreus* can disperse its stores in a vast geographical area. Therefore it explains the world wide existence of the fungus.

REVIEW OF LETERATURE

Aspergillus terreus is major cause of aspergillosis, which is reported in University and Hospital of Innsbruck in Austria and in medical centres of Houston, Texas [25, 26]. There are 26 different proteins; there expression level is affected by more than two antifungal drugs, which suggested more general response to the stress induced by the antifungal agents [2].

The analysis also showed that the enzymes from the cell wall remodelling, oxidative stress response and energy metabolism are the responsible factors for providing resistance against antifungal drugs in *Aspergillus* species and could be explained further in clinical isolates [2].

There are the two major toxins produced by *Aspergillus* i.e. Gliotoxin and Aflatoxin respectively. Also *A. fumigatus* allows invasion in the most of the tissues because of the production of Gliotoxin in in-vivo condition, particularly during germination of conidia further it is involved in immunosuppression of the host contribution to pathogenesis. Aflatoxin produced by *A. flavus* and *A. parasiticus* are carcinogenic in nature and major contaminants in food crops. Regulatory genes AflR and AflS involved Aflatoxin productions are under the influence of temperature. *A. flavus* isolates not able to produce are known as atoxigenic *A. flavus* [27, 28]. Other toxin producing *Aspergillus* species is *A. terreus*, it produces geodicin and terrotin as major toxin and are involved in pathogenesis [5].

On exploring activity of phytochemicals such as quercetin, shikonin, coumaric gallic, acid and cis-9-Hexadecenal against *Aspergillus species*, it was observed that shikonin showed the significant inhibition at MICs; 2 μg/ml, it was further tested in SEM which showed delayed

swelling and distorted cell wall organisation in the *A. terreus* conidia which were treated with shikonin [29, 30].

A cascade of reactions integrated into the signalling pathway is responsible for the germination of dormant conidia. Hence the effect of phytochemical quercetin on *A. flavus* during germination of conidia was done using scanning electron microscopy (SEM) [31]. Another study under phytochchemical stress regulated genes showed a clear response encoding for calcineurin-Crz1/ MAPK pathway signalling pathways. Therefore it is useful in screening the antifungal agents. [32].

More than 300 fungal metabolites have been reported as mycotoxins responsible to contaminate more than 25% of world's food crop. *A. parasiticus* is closely related to *A. flavus*, because both are responsible for the production of aflatoxins (B1, B2, G1 and G2). These two fungal pathogens are the main source of contamination in cotton, corn, peanuts and other oil-seed crops therefore it produces toxins both in the field and during storage.

According to the recent studies in India

i. In a referral Chest Hospital in Delhi, in most of the aspergillosis cases, 6.6% of the *A. terreus* isolates are responsible [21].

ii. Only 8% of *A. terreus* isolates were susceptible to AmB with MICs (0.5–1 mg/L), they don't show any particular genotypic pattern [33].

According to the previous reports

i It was observed, that ***A. terreus*** isolates had a capability to naturally resist AmB [25, 34].

ii It was further observed, A. *terreus* has high in vitro and in vivo values of MICs for AmB, therefore it confirms the resistance for AmB [35, 36, 37].

Resistance of *A. terreus* for different drugs

i ***A. terreus*** has even showed resistance against azole drug treatment which was observed in Danish clinical samples in a city hospital [38].

ii They have also reported that *A. terreus* is developing resistance against ITC can be associated with the M217I Cyp51A mutation.

iii Approximately 5% of *A. terreus* were found to show resistance for posaconazole in in-vitro studies.

iv Now, 10% which is a high percentage of *A. terreus* which were resistance to posaconazole were found in Austria, Germany and UK [39]. Therefore, *A. terreus* had become an infection threat in the immunocompromised patients day by day, because of its lack of response to AmB and azole (VRC) [40].

Other studies on *A. terreus*

i Caffein inhibits aflatoxins G1 and G2. Therefore, it inhibits growth and mycotoxin productivity in a no. of *Aspergillus* and *Penicillium* species.

ii Against *Magnoporthe grisea* and *Erysiphe graminis* a good antifungal activity is shown by Gallic acid.

iii The study says that 14.3% inhibition was shown by Ascorbic acid against *A. flavus*.

iv Other emerging area is Bio pesticides, they have a disfigured target of action which made them advantageous for showing antimicrobial effects and they are called phytochemicals,

which are present in plants. It is not practiced at commercial levels, though studied extensively.

- v Hence this method can become an alternative approach for an economically feasible and eco-friendly way of controlling the contamination in the crops and therefore increases the yield.

OBJECTIVES

1. **To explore *Aspergillus terreus* isolates for antifungal drug susceptibility**

2. **To determine MIC value of antifungal drug against *Aspergillus terreus* isolates.**

METHEDOLOGY

Media preparation to culture the *A. terreus* environmental isolates - (fig no. 1)

1. PDA (Potatoes infusion from 200g/l, Dextrose 20g/l) was poured in the plates and were kept for drying for 15mins
2. *A. terreus* environmental isolates, confirmed morphologically and using *Terrelysin* gene specific PCR, were used. The strains were streaked on the media
3. These culture plates were kept in the incubator at 32˚C for 5 or 6 days
4. The plates were taken inside the LAF and 3µl of PBST solution was poured into it (this leads to the fungal cell to get detached from the media).
5. PBST was kept for 1 min
6. With the help of tips the cells were scraped from the media very carefully.
7. Viscous solution was transferred with the help of pipette (note: the tip should be cut a little from the top)
8. The solution was taken in the eppendorfs.
9. It (eppendorfs) was centrifuged at 10000rpm for 2mins in 4˚C
10. Supernatant was discard very carefully such that the pellet does not get disturbed
11. The pellet was suspended in 1µl of PBS solution and was mixed well
12. This mixture was again centrifuged at 10000rpm for 2mins at 4˚c
13. Steps were repeated thrice (step no.-10,11and 12)
14. The pellet obtained was stored with 1µl PBS in -20˚C

Culturing the isolates in PDB (Potatoes infusion from 200g/l, Dextrose 20g/l)

1. A flask of 500µl was taken and according to the volume of the flask, PDB was added into the 500µl distilled water (**fig. no. 3 and fig no. 4**)
2. The strains harvested in the PBS was inoculated in the broth and were kept in the incubator at 32˚C for 5 days

Harvesting cells in muslin cloth: (**fig. no. 5**) [41]

1. Flask containing the cultures of the strains was taken
2. Media was discarded very carefully so that the cell remain in the flask
3. Cells were transferred on muslin cloth using the forceps and kept for drying
4. Cells from the muslin cloth were taken and crushed in liquid nitrogen (this step is done so that the cells get ruptured and the genetic material can come out easily from the cell during phenol chloroform method)
5. Crushed cells were suspended using lysis buffer.

Phenol chloroform method for DNA extraction (**fig. no. 5 and fig no. 6**)

1. Frozen mycelium was taken and grinded into fine powder in liquid nitrogen using pre cooled motor pestle.
2. The mycelium was resuspended in lysis buffer until the viscosity of the suspension significantly reduces and the formation of froth indicates the detachment of DNA polysaccharide.
3. 500µl of NaCl solution was added and the components were mixed by inverting the tube several times.
4. The suspension was centrifuged at 13000rpm for 20min at 4˚C.
5. It (suspension) was immediately transferred to a fresh tube and equal volume of phenol and chloroform was added.
6. Solution was mixed gently by inverting the tube until it become milky.

7. It was centrifuged for 20mins at 4˚C and the aqueous phase was transferred to a new centrifuge tube.
8. DNA in the aqueous supernatant was precipitated with two volume of ethanol.
9. Precipitated DNA was washed 3 times with 70% ethanol at maximum speed for 5mins
10. DNA pellet was air dried and dissolved in 50µl of TE buffer, stored at 20˚C.

Electrophoresis of genomic DNA was carried out using 1% agarose gel (**fig. no. 7**)

1. 0.5g of agarose was added in 500µl of 1% TAE buffer
2. It was boiled in the microwave for 1 min and was allowed to cool down (lukewarm) so that EDTA can be added.
3. 5µl of EDTA was added to the lukewarm buffer.
4. Gel was poured into the casting tray
5. It (gel) was allowed to solidify.
6. The comb was removed and 5µl of the 1kb ladder was transferred into one of the well using the pipette
7. 3µl of samples were mixed with 2µl of the loading dye
8. These samples were transferred in the well accordingly.
9. Than the gel was electrophoresed in the buffer at 100volt for 50mins.
10. The gel was observed in the gel doc.

Polymerization of the DNA using the primers Terrelysin and Tubulin (**fig. no. 8**)

1. 40µl of master mix was prepared in the PCR vials

1. The master mix contains –Water - 28µl
   Buffer - 4µl
   Taq polymerase - 2µl
   DNTP mix - 2µl
   Forward primer - 2µl
   Reverse primer - 2µl
2. Now 20µl of master mix was added to the 1µl of the sample
3. Than it was mixed very well in the vortex
4. Now the PCR was set to optimum conditions- Initial denaturation at 95˚C for 3min, and 39 cycles of 95˚C for 10s, 49˚C for 45s, 72˚C for 30

Serial dilution of the cell stock (**figure no 9**)

1. 10 eppendorf were taken and labelled from 1 to 10.
2. 900 µl of pbs was pipetted into the eppendorf.
3. 100 µl of stock solution was transferred into the eppendorf and was labelled 1
4. mixed well.
5. Now 100 µl of solution from the eppendorf 1 was transferred to eppendorf 2 and mixed well.
6. 100µl of solution from eppendorf 2 was added to the eppendorf 3 and mixed well and continued up to the 10^{th} dilution. Here 10^{-10} dilutions were made.

Cell counting using hemocytometer (**figure no.10**)

1. The cell suspension from the dilution was taken and mixed with a little amount of lactophenol blue dye.
2. Now 15-20 µl of cell suspension was added (from 10^{-1} dilution) between the hemocytometer.
3. It was covered with the cover slip.
4. The number of cells in the four outer square was counted and divided by four.
5. (the mean number of cells per square) [42].
6. The cell counting was done for every dilution until we get a cell count of 10^{-6}

Calculation

Cells in 1^{st} square =160 Cells in 2^{nd} square =128

Cells in 3^{rd} square =96 Cells in 4^{th} square =112

Mean number of cells per square =124

Mean number of cells per square × Dilution =1.24×10^{6} cells/µl

PDA plates preparation and disk preparation **(figure no. 11)**

1. 6.8 g of PDA was mixed with 50µl of distilled water.
2. The disks were cut from the filter paper.
3. These disks were wrapped with the silver foil.
4. Now the PDA flask and the disks both were autoclaved.

Drug was diluted to 1µg/µl **(figure no. 12)**

1. 5µg/µl drug was diluted in the DMSO.
2. 1µl of the concentrated drug was added to 5µl of DMSO.
3. And was mixed well.

Adding drugs to the plates **(figure no.12)**

1. The media was then poured into the plates and was kept for solidifying.
2. Now the dilution with cell count of 10^{-6} was spread on the agar (40µl) and was kept for drying.
3. Now the disk was placed in the centre of the plates.
4. 10µl of the drug was added to the disks using pipette.
5. The plates were kept in the incubation at 37°C.

WORK DONE AND RESULTS

Culture flask and tubes were sterilized. Potato dextrose agar plates were prepared followed by plating. PDA (**Potato infusion**: 200 gm/l, **Dextrose**: 20 gm/l, **Agar** : 20 gm/l) plates were inoculated with *A. terreus* spores. Two isolates of *A. terreus* were used in these experiments.

The two isolates were streaked separately on the PDA plates and the plates were made in triplets.

These plates were kept under the optimum condition of 32˚C in the incubator for 5 days.

Then the growth was observed.

Figure no. 1- Growth of *Aspergillus terreus* in PDB plate after 3 days

These colonies of the strains were further harvested using PBS and PBST method and stored in the PBS.

Then the stalks were prepared in the small eppendorf centrifuge tubes. These stalks were taken for the stalk cell count.

Calculating the stalk cell count

- Cell count or number of viable cells present in the stalk was calculated by using hemocytometer.
- Formula used-

$$\text{Cells}/\mu l = \text{cell count}/\text{number of chambers counted} \times \text{dilution} \times 10^4$$

- RESULT-

1. no. of cells in isolate -2 = 1×10^8 cell/µl

2. no. of cell in SH_7 = 6.32×10^7 cell/µl

Now the stalks prepared were stored in PBS in 4˚C

Figure no. 2-Broth Culture of *Aspergillus terreus* isolate (1) in PDB

The culture flask was prepared with potato dextrose broth and the sample or the inoculum was taken from the stalk which has been prepared and stored. After inoculation the flask was kept in incubator at 32˚C for 7 to 8 days.

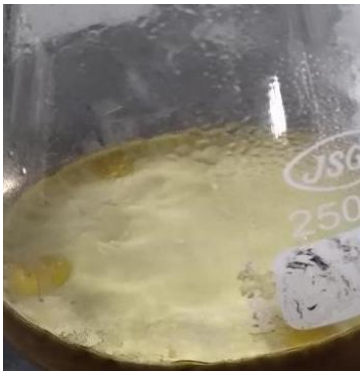
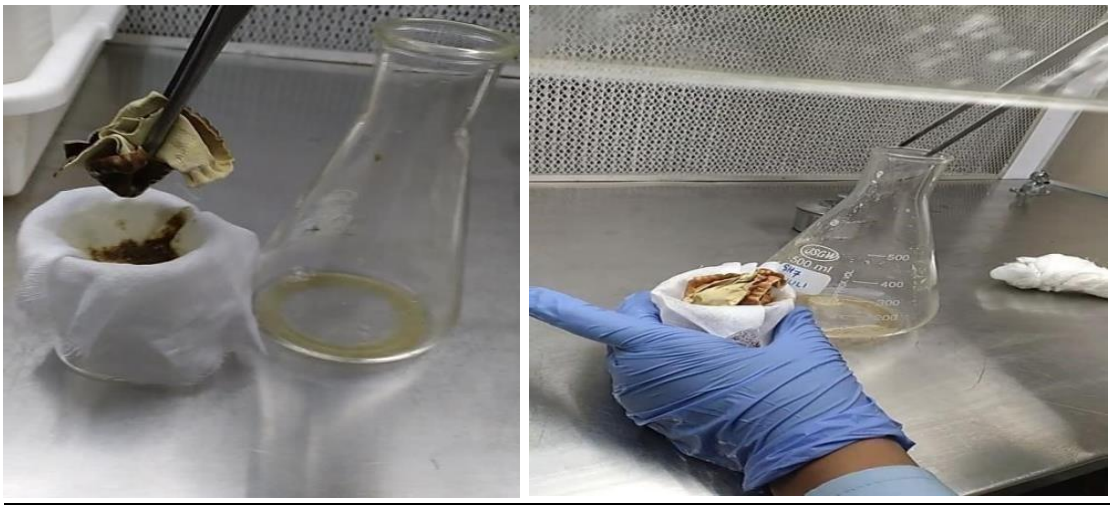


Figure no. 3- Broth Culture of *Aspergillus terreus* isolate (2) in PDB

The broth culture was kept for 7 to 8 days of incubation at 32˚C and the full cell growth was observed this was further used for DNA extraction.



Isolate-2 **Isolate-1**

Figure no. 4- The cells were harvested in the muslin cloth

The cells of the isolates were allowed to dry on the muslin cloth, followed by crushing of the cell in the liquid nitrogen using pestle motor.

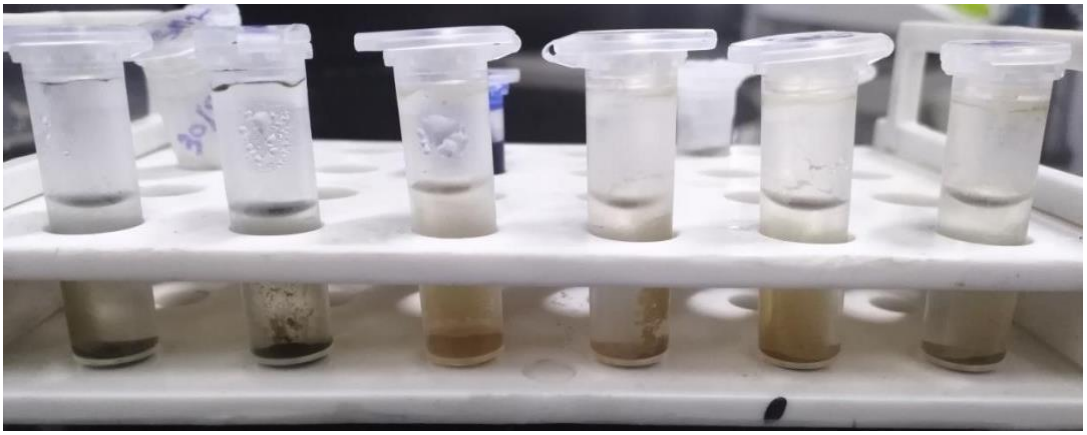


Figure no. 5- Stalks of *Aspergillus* prepared in PBS by using PBST method

These samples were taken for DNA extraction of the strains using phenol chloroform and pestle motor method of DNA extraction using liquid nitrogen.

The DNA was extracted successfully.

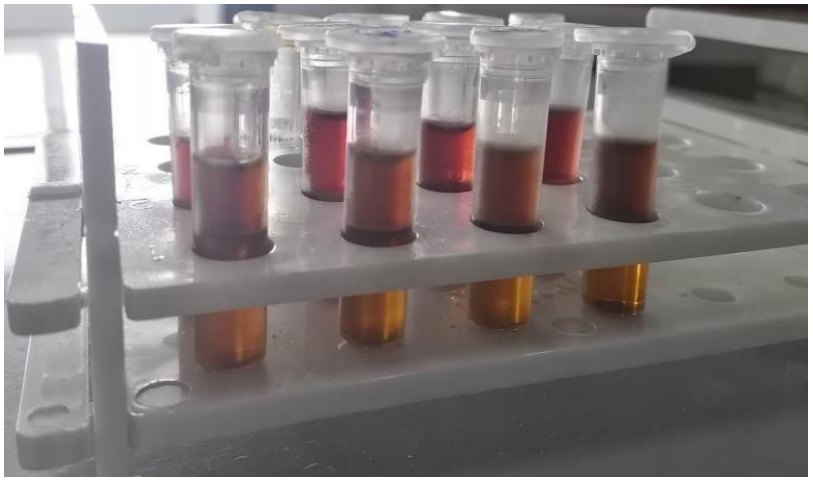


Figure no. 6- The DNA was extracted using the phenol chloroform method

The figure shows the two phases organic phase and aqueous phase separated by chloroform

Ladder I-2 I-2 I-1 I-1 I-1

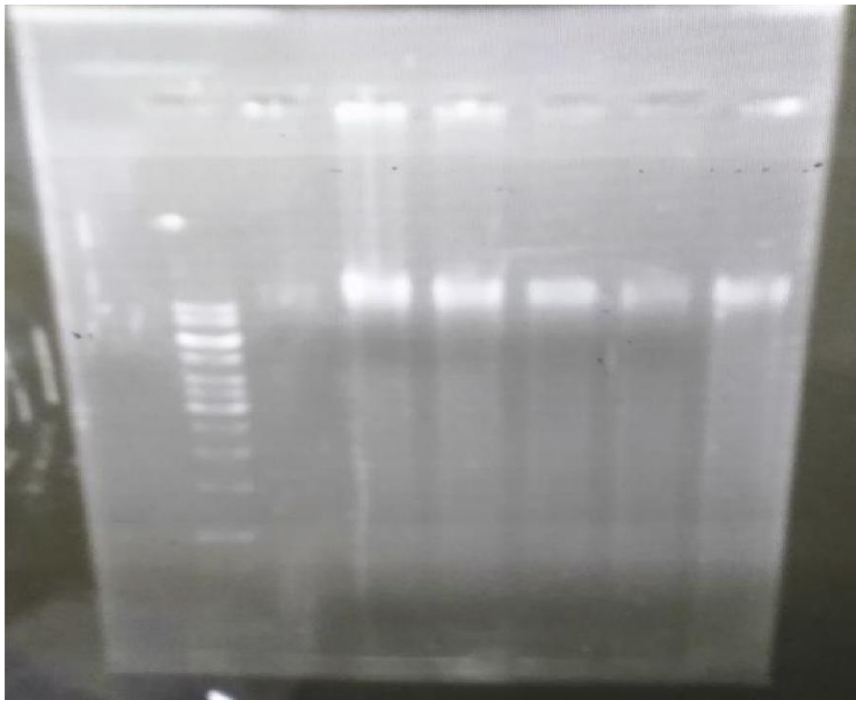
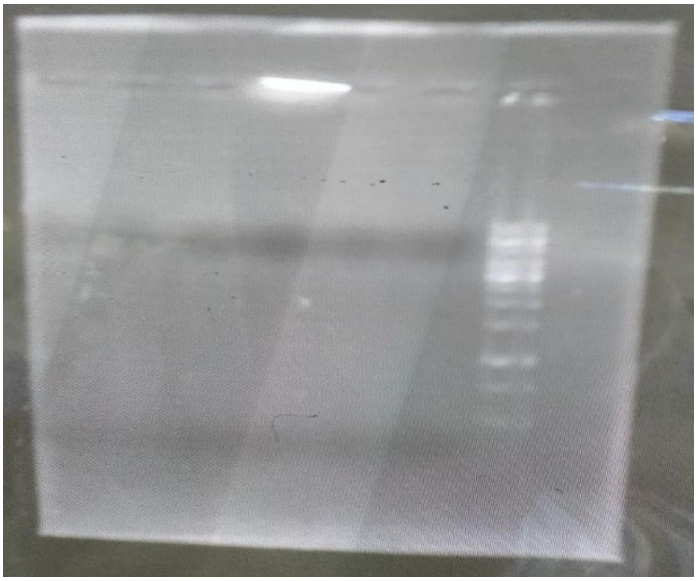


Figure no. 7 - The genomic DNA of the two isolate (2 & 1)

The extracted DNA was electrophoresed on the gel with the loading dye and observed under the gel doc.

The genomic DNA was observed very clearly above the 10,000bp band of the ladder.

Ntb Ntr 2- tb 2-tr 1-tb 1- tr Ladder



:

Ntb- Negative control *Tubilin*

Ntr- Negative control *Terrelysin*

2-tb; strain of *A. terreus* with *Tubulin*

2tr;strain of *A terreus* with *Terrelysin*

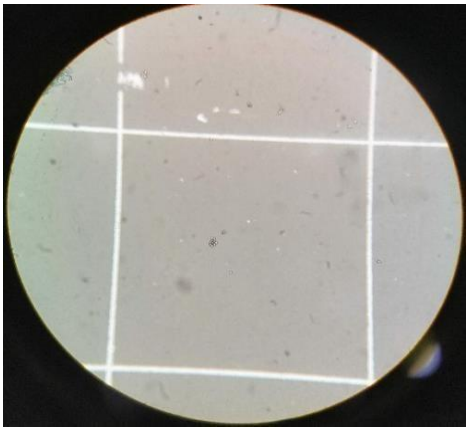
1-tb; strain of *A. terreus* with *Tubulin*

1- tr; strain of *A. terreus* with *Terrelysin*

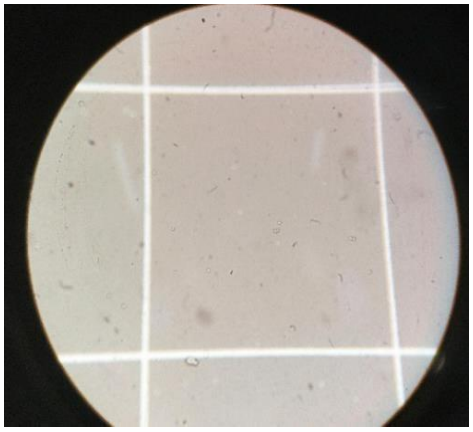
Figure no.8- The genomic DNA was replicated using primers *Terrelysin* and *Tubulin*. But the results were obtained negative

The amplification of DNA was done in PCR along with the primers *terrelysin* and *tubulin* (*Aspergillus terreus* specific primers) and was again electrophoresed in the 1% agarose gel, to reconfirm whether the replication carried out was successfully or not.

But the results were unsatisfactory because the bands were not visible. This showed that there is no amplification, this may be due to the fault in the parameters of the PCR which may be not optimum for the isolates also may be the time given for the different steps of replication and denaturation may not be suitable or the master mix prepared was wrong or the sample added to the master mix was not added properly. The other reasons can be that the gel prepared was wrong or the gel doc machine may be faulty. Experiment needs repetition.



“2”



“1”

Figure no. 10- cell counting using Hemocytometer

For getting the cell count of 10^{-6} all the dilutions from 10^{-1} to 10^{-10} were counted under the hemocytometer of both the strains 2 and 1. In ‘’2’’ the dilution of 10^{-8} was appropriate for the cell count of 10^{-6} and in “1’’ the dilution of 10^{-7} was appropriate for

getting the cell count of 10^{-2}

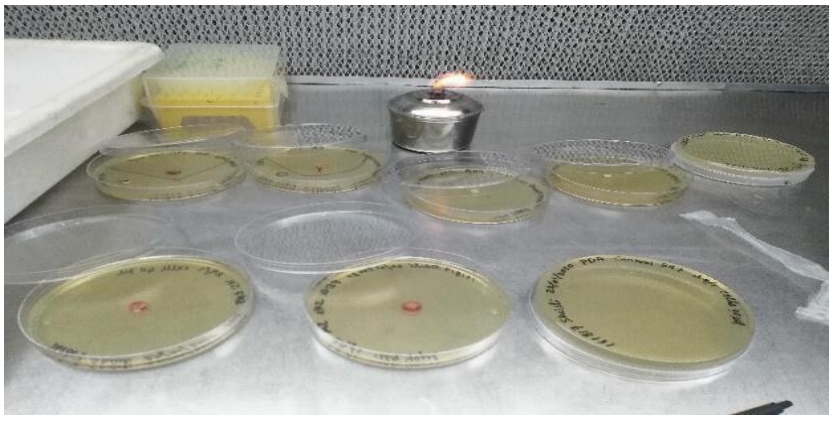
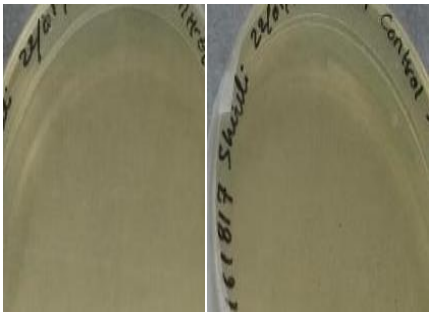
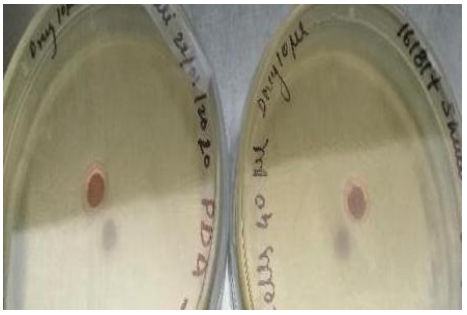


Figure no. 11- PDA plate preparation

The PDA plates were prepared inside the LAF and was labelled properly.



The cell from the suspension was spread



Now the disk were placed and allowed to dry

Figure no. 12- The cell suspension was spread and was kept for 2 min, after letting it dry the drugs were pipetted on the disk. The disk were kept in the centre so that the drug can spread evenly and MIC could be calculated easily.

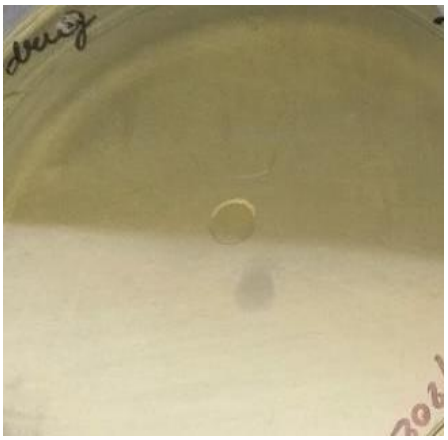
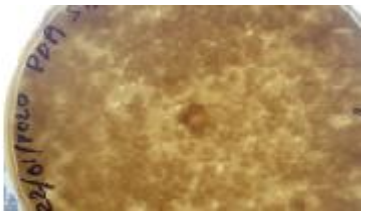


Figure no. 13- The plates were kept in the incubator at 37°C and were observed upto 7 days. This is of day zero**.**

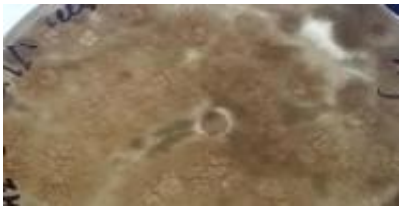
Figure no. 14- Cells in the 3rd day of incubation



I-2-AmB **I-1-AmB**

Figure no. 15- Cells in the 6th day of incubation

The two strains 2 and 1 are showing resistance to Amphotericin B (AmB),which can be seen because of the absence of inhibition zone around the disk



2-Sk **1-Sk**

Figure no. 16 - Cells in the 7th day of incubation.

RESULTS

The *Aspergillus terreus* isolates-1 and -2 both showed modest sensitivity towards shikonin (Sk), which can be observed by the presence of small zone of inhibition around the disk

DISCUSSION

Both the isolates can further be studied with different concentrations and combinations of drugs and with range of inoculums size, which will help to collect some more data for the Susceptibility testing. According to the above experimental data both the Isolate-1 and -2 were showed not much effect of AmphotericinB drug but a minor zone of inhibition can be seen in the plates containing Shikonin. Both the strains are comparatively showing some area of inhibition around the disk of Shikonin. Further detail studies are required to confirm whether these isolates are sensitive/resistance to the drugs.

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