International Journal of Pharmacy and Biological Sciences
ISSN: 2321-3272 (Print), ISSN: 2230-7605 (Online)
IJPBS™ | Volume 8 | Issue 3 | JUL-SEPT | 2018 | 752-757
Research Article | Biological Sciences | Open Access | MCI Approved| ज्ञान-विज्ञान विमुक्तये
|UGC Approved Journal |

ANTIFUNGAL ACTIVITY EVALUATION OF *BACILLUS SUBTILIS* AND OTHER THREE SOIL ISOLATES

Joshi V. A.[1]* and Panchal H. K.[2]

[1, 2]Dolat Usha Institute of Applied Sciences and Dhiru Sarla Institute of Management and Commerce, Valsad, Gujarat, India

***Corresponding Author Email: vaibhuajoshi@gmail.com**

ABSTRACT

In the last decade invasive mycosis has become a public health problem. Fungi cause diseases like Aspergillosis (lung disease), fungal eye infection, skin infection and many more including deep mycosis. Candida spp. causes disease like candidiasis (in mouth, vagina and blood). Therefore, screening of antifungal agents is significant for development of therapeutic antifungal drug and suppression of fungal and yeast colonization. This study was carried out to isolate antifungal antibiotic producing microorganisms from soil sample collected from garden area of village Khergam, South Gujarat. Antifungal activity screening was done using agar diffusion method on R2A agar medium. Total 4 bacterial isolates with antifungal activity were successfully isolated and purified. Out of 4 isolates AF1 and AF3 showed good antifungal activity against test organisms Aspergillus spp., Penicillium spp. and Candida albicans. Antifungal antibiotics were produced by inoculating bacterial isolates in R2A broth and after incubation extraction and partial purification was done. AF1 was identified as Bacillus subtilis by 16s rDNA sequencing and bioinformatics approach. In the conclusion, Bacillus subtilis, AF2, AF3 and AF4 could inhibit the growth of pathogenic fungi and yeast therefore, these 4 isolates serve as potential antifungal agents and can be explored further to isolate antifungal compound.

KEY WORDS

Antifungal antibiotic, R2A agar medium, agar diffusion assay, Bacillus subtilis

INTRODUCTION

The occurrence of fungal infections in humans has increased significantly in the past 30 years[1]. An antifungal agent is a drug that selectively eliminates fungal pathogen from a host with minimal toxicity to the host. Of the different fungal species affecting human, *Candida species* and *Aspergillus species* are the most prevalent opportunistic pathogen reported to be resistant to numerous antifungal agents. Compared to antibacterial agents, the number of antifungal compounds approved for use in human is quite limited because of high toxicity of these substances to the hosts. However, the emergence of new diseases and reemergence of multiple-antibiotic resistance pathogens have rendered the existent used antibiotics ineffective. This problem has squared the needs for the discovery of new antibiotics. Antifungal agents can be applied to the skin, nails, or hair; vaginally; or inside the mouth to treat either fungal or yeast infections. They are available as creams, gels, lotions, nail lacquers, ointments, powders, shampoos, sprays, and tinctures. Antifungal agents can be used as bio-control agents in agriculture. They can be used as therapeutic agents in pharmaceuticals.

Antifungal agents can be used to inhibit the growth of fungi. Bioactive substances are low molecular compounds exhibit various activities and microorganisms and plants have been important sources of natural medicinal substances[2]. There is a fast emergence of newer infections and the organisms developing resistance which render already existing

antibiotics less effective. Therefore, a constant search for new antibiotics to overcome these problems is a matter of necessity. Among the diverse sources of antibiotics, soil is the most important target for scientists in the discovery of novel antibiotics[3]. Soil microorganisms have continually been screened for their useful biologically active metabolites such as antibiotics since long ago. Therefore, this study is an attempt to discover novel antibiotics from soil samples from garden area of Khergam Gujarat.

MATERIALS AND METHODS

Sample collection and Isolation of bacteria

The soil Sample was collected from garden area of village Khergam, South Gujarat. Soil sample was taken from the profile of 5-12cm in depth by using spatula. Collected soil Sample was stored in Polythene bag and maintained at laboratory for further study. One gram of soil sample was added into 9ml of sterilized water. Soil sample was then serially diluted and 0.1 ml of each dilution was plated on R2A agar plates and incubated at room temperature for 2 to 3 days. Fungal Growth inhibition was observed in R2A plates. Bacterial isolates giving zone against fungi in crowded plates were selected for further study, purified and preserved.

Production of antibiotics

Selected bacterial isolates were inoculated in 50 ml R2A broth in 250ml flasks and incubated at room temperature on shaker at 100 rpm for 2 days. After incubation, the production medium was centrifuged at 3000 rpm for 15 minutes and then the supernatant was filtered with membrane filter to remove bacterial cells completely.

Agar diffusion assay

Antifungal activity of isolated bacterial cultures was determined using agar well diffusion method[4]. In this technique fungal test organisms *Aspergillus spp*., *Penicillium spp*. and *Candida albicans* were streaked by sterile swab and further plates were divided into four parts and with the help of sterile cup-borer four wells of 6 mm were made at the center of different parts in plate. The supernatant obtained from fermentation with different isolates were carefully placed in the wells and were subjected to diffusion for 30 minutes at 4^0C. The plates were then incubated at room temperature for 2 to 3 days for antifungal activity study. The zones were measured with the help of zone meter.

Extraction and partial purification of antibiotics

Antibiotics was separated by centrifugation and filtrations. An activated charcoal was added to the liquid medium. The charcoal containing the absorbed antibiotic was separated from the liquid medium by centrifugation and washed with alcohol to remove impurities. It was then washed with acid alcohol to elute the antibiotic. The acid alcohol solution containing antibiotic was neutralized and filtered to remove precipitated impurities[5]. Agar diffusion assay was performed from the same mentioned as above.

Dual culture method

Antifungal activity of bacterial isolates was also screened by dual culture method[6]. The antifungal activity of isolates was tested against *Aspergillus spp.* Bacterial isolates AF1, AF2, AF3 and AF4 were streaked on R2A plates. Four streaks were drawn on petri plates in a square pattern. Each streak was drawn 3cm away from the center of the plate. *Aspergillus spp.* culture was inoculated in the center of R2A plates, separately. The petri plates having targeted fungal pathogens serve as control plate. The plates were incubated at room temperature, till the complete growth was observed in control plates. After a week of incubation, the growth of targeted fungal pathogen towards and away from the bacterial isolate was recorded.

Identification by 16S rDNA sequencing

Potent organism was identified by 16s rDNA sequencing and by bioinformatics approach. For the 16s rDNA sequencing bacterial culture was sent to Saffron Life-sciences. In Saffron Life-sciences DNA was isolated from the culture. Its quality was evaluated on 1.0% Agarose Gel, a single band of high-molecular weight DNA has been observed. Fragment of 16S rDNA gene was amplified by PCR. A single discrete PCR amplicon band was observed when resolved on agarose gel. The PCR amplicon was purified by column purification to remove contaminants. DNA sequencing reaction of PCR amplicon was carried out with 8F & 1492Rprimers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer.

The 16S rDNA sequence was used to carry out BLAST with the database of NCBI genbank[7]. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software programs[8] and phylogenetic tree was also obtained.

RESULTS AND DISCUSSION

ISOLATION AND SCREENING OF BACTERIA:

The collected soil sample was tested to isolate antifungal activity possessing bacteria on R2A medium with crowded growth of microorganisms. In crowded plates total 4 isolates were obtained, which were inhibiting fungal growth in vicinity of colonies. All 4 isolates were gram positive rods. Remarkably isolate AF2 was a gram positive filamentous actinomycete bacterium. Out of 4 bacterial isolates, AF1 showed highest activity and zone (Figure 1).

AGAR DIFFUSION METHOD:

The production of antibiotics was carried out in the Reasoner's 2A medium and antifungal activity was determined by agar well diffusion method. Supernatant of AF1, AF2, AF3 and AF4 showed Antifungal activity against all test fungi as depicted in figure 2 and table 1. Exceptionally AF3 and AF4 were not found to have anti-candidal activity. *Candida albicans* is resistance towards different antibiotics[9]. *C. albicans* has been involved in broad studies by researchers due to its property that can resist different antimicrobial drugs. Antifungal activity was found to increase after partial purification of supernatant. AF1 was found best among all 4 isolates with highest antifungal activity against all test organisms both before and after partial purification.

DUAL CULTURE METHOD:

The antifungal activity of bacterial isolates was screened by dual culture method. The 4 isolates were tested against *Aspergillus spp*. All 4 isolates inhibited the mycelial growth of test fungus (Figure 3). Many investigators have suggested that antagonistic bacteria, specially belonging to the *Bacillus* genus are among the most used biological agents and chitinase production has been shown to play an important role in the suppression of various disease[6].

16S RIBOSOMAL DNA SEQUENCING AND IN SILICO ANALYSIS OF AF1:

AF1 was identified by 16s rDNA sequencing and by bioinformatics approach. Partial rDNA sequence was obtained for AF1 from Saffron Life-sciences. Sequence of AF1 was analyzed with an *in silico* approach. In NCBI mega BLAST of nucleotide sequences, it has shown about 100 related sequences for AF1 (Figure 4). The results of NCBI BLAST have shown significant alignment and list of sequences with significant an alignment was obtained shown in Figure 5.

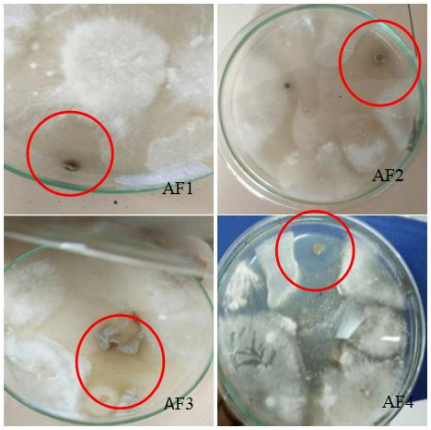
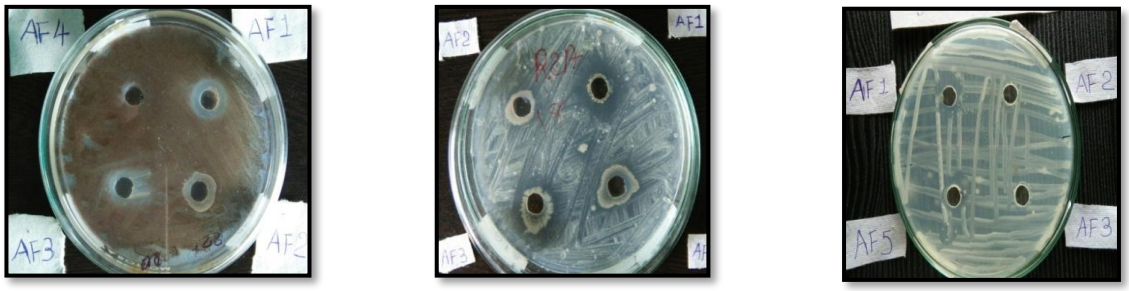


Figure 1: Isolates obtained on R2A medium with antifungal activity



A **b** **c**

Figure 2: Antifungal activity given by isolates against a) *Aspergillus spp.* b) *Penicillium spp.* and c) *Candida albicans* by agar diffusion method

Table 1: Antifungal activity of culture free supernatant before and after partial purification

Isolate	Zone of Inhibition (mm±SD)					
	Aspergillus spp.		*Penicillium* spp.		*Candida albicans*	
	Before	After	Before	After	Before	After
AF1	**18±0.34**	**20±0.33**	**17±0.17**	**19±0.13**	**14±0.64**	**16±0.61**
AF2	13±0.12	15±0.16	13±0.21	13±0.46	13±0.11	14±0.43
AF3	14±0.56	17±0.55	14±0.38	16±0.51	0±0.00	0±0.00
AF4	12±0.13	14±0.15	11±0.41	12±0.22	0±0.00	0±0.00

Figure 3: Inhibition of mycelial growth of *Aspergillus spp.* by isolate AF1 in Dual culture method

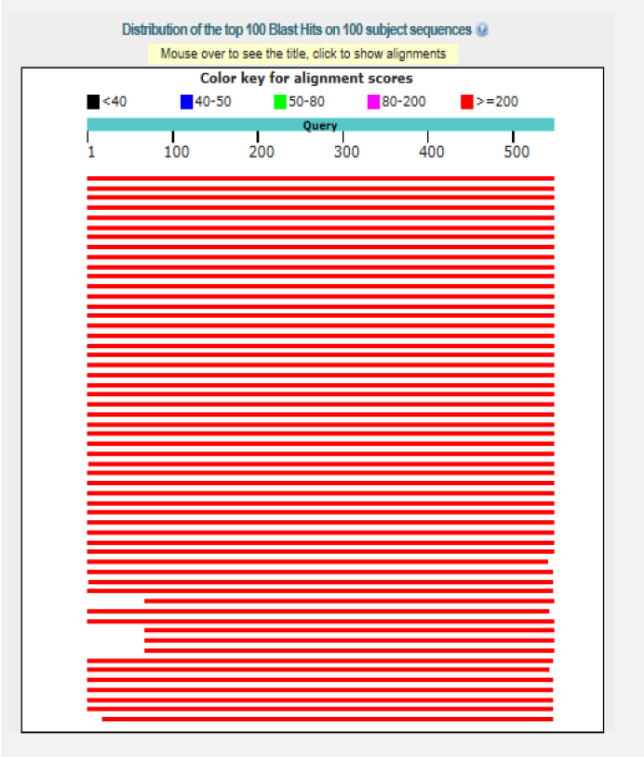


Figure 4: NCBI BLAST of partial 16s rDNA sequence of AF1

Sequences Producing Significant Alignment:

Description	Max score	Total score	Query cover	E value	Ident	Accession
Bacillus subtilis subsp. inaquosorum strain BGSC 3A28 16S ribosomal RNA gene, partial sequence	996	996	100%	0	99%	NR_104873.1
Bacillus subtilis strain IAM 12118 16S ribosomal RNA, complete sequence	990	990	100%	0	99%	NR_112116.2
Bacillus mojavensis strain NBRC 15718 16S ribosomal RNA gene, partial sequence	990	990	100%	0	99%	NR_112725.1
Bacillus subtilis strain JCM 1465 16S ribosomal RNA gene, partial sequence	990	990	100%	0	99%	NR_113265.1
Bacillus subtilis strain NBRC 13719 16S ribosomal RNA gene, partial sequence	990	990	100%	0	99%	NR_112629.1
Bacillus subtilis subsp. spizizenii strain NBRC 101239 16S ribosomal RNA gene, partial sequence	990	990	100%	0	99%	NR_112686.1
Bacillus subtilis strain BCRC 10255 16S ribosomal RNA gene, partial sequence	990	990	100%	0	99%	NR_116017.1
Bacillus subtilis strain DSM 10 16S ribosomal RNA gene, partial sequence	990	990	100%	0	99%	NR_027552.1
Bacillus mojavensis strain IFO15718 16S ribosomal RNA gene, partial sequence	990	990	100%	0	99%	NR_024693.1
Bacillus subtilis subsp. spizizenii strain NRRL B-23049 16S ribosomal RNA gene, partial sequence	990	990	100%	0	99%	NR_024931.1
Bacillus subtilis strain NCDO 1769 16S ribosomal RNA gene, partial sequence	987	987	100%	0	99%	NR_118972.1
Bacillus mojavensis strain ifo 15718 16S ribosomal RNA gene, partial sequence	985	985	100%	0	99%	NR_118290.1
Bacillus tequilensis strain 10b 16S ribosomal RNA gene, partial sequence	985	985	100%	0	99%	NR_104919.1
Bacillus halotolerans strain DSM 8802 16S ribosomal RNA, partial sequence	985	985	100%	0	99%	NR_115063.1
Bacillus axarquiensis strain LMG 22476 16S ribosomal RNA gene, partial sequence	985	985	100%	0	99%	NR_115929.1
Bacillus malacitensis strain CR-95 16S ribosomal RNA gene, partial sequence	985	985	100%	0	99%	NR_115282.1
Bacillus nakamurai strain NRRL B-41091 16S ribosomal RNA, partial sequence	979	979	100%	0	99%	NR_151897.1

Figure 5: Sequences with significant alignment

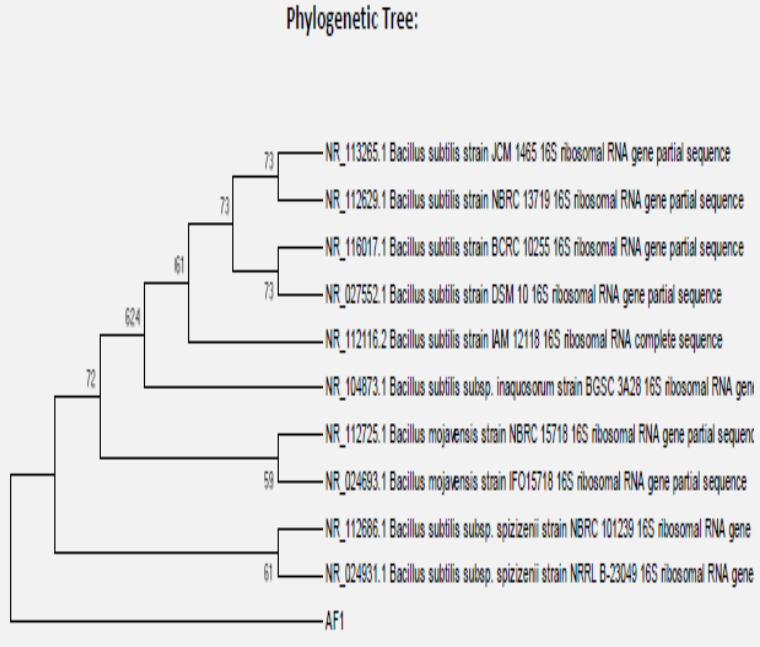


Figure 6: Evolutionary relationships with taxa

Sequences with high degree of identity were retrieved from NCBI for further multiple sequence alignment and they were having accession numbers NR 104873.1, NR 112116.2, NR 112725.1, NR 113265.1, NR 112629.1, NR 112686.1, NR 116017.1, NR 027552.1, NR 118290.1, NR 104919.1, NR 115063.1, NR 115929.1, NR 115282.1 and NR 151897.1. As a result of multiple sequence alignment AF1 was found close to organism with accession number NR 024931.1. It is a Sequence of *Bacillus subtilis* subsp. spizizenil strain NRRL B-23049.

From the above result of 16s rDNA sequence the organism AF1 was identified as *Bacillus subtilis* subsp. spizizenil strain NRRL B-23049. The phylogenetic tree analysis is shown in figure 6. Results of this study are consistent with those of previous studies, where *Bacillus subtilis* exhibited remarkable antagonistic activity against various fungi. Most *Bacillus spp.* produces much kind of antibiotics, including bacillomycin, fengycin, mycosubtilin, and zwittermicin, which are effective for suppression of the growth of target Pathogen[10]. *Bacillus subtilis* inhibition of *Aspergillus spp* with clear zone of inhibition agreed with earlier work presented by Islam et al, 2012. Activity of *Bacillus subtilis* against the pathogenic fungi is probably due to the production of metabolites into the culture plate. *B. subtilis* have been identified to produce more than 20 volatile antifungal compounds capable of inhibiting germ tube elongation and spore germination of fungi in vitro[11].

CONCLUSION

From the overall results of the present study it can be emphasized that, AF1, AF2, AF3 and AF4 isolated from soil have Antifungal activity. AF1 organism gave highest antifungal activity and it was also increased after partial purification. AF1 was identified as *Bacillus subtilis* based on the 16S rDNA sequencing and by bioinformatics approach. *Bacillus subtilis,* AF2, AF3 and AF4 isolated from soil are promising source of antifungal agents. The chemical nature, properties, purification of antibiotics can be studied further to isolate novel or more efficient antibiotics. Discovery of novel antifungal compound can satisfy the demand of effective drugs required to treat fungal infections.

Received:06.05.18, Accepted: 07.06.18, Published:01.07.2018

REFERENCES

1. Lass-Flörl C., Zygomycosis: conventional laboratory diagnosis. Clinical Microbiology and infection, 15: 60-65, (2009)
2. Bull, P. C., Berriman, M., Kyes, S., Quail, M. A., Hall, N., Kortok, M., & Newbold, C. I. Plasmodium falciparum variant surface antigen expression patterns during malaria. PLoS pathogens, 1(3): e26, (2005).
3. Dulmage, H. T., & Rivas, R. A survey of soil microorganisms, with particular reference to the actinomycetes as sources of substances toxic to Heliothis virescens. J. of Invertebrate Pathology, 31(1): 118-122, (1978).
4. Bonev, B., Hooper, & Parisot. Principles of assessing bacterial susceptibility to antibiotic using the agar diffusion method. J. of antimicrobial chemotherapy, 61(6): 1295-1301, (2008).
5. Prescott S. & dunn C. Industrial microbiology, jodhpur agrobios publisher: 781, 796, (2002).
6. Shaikh & Sahera. Antifungal Activity of Pseudomonas aeuroginosa and Bacillus subtilis against Pathogens of Cucurbitaceous Fruits. Int J of innovative reseaech in science, Engineeering and Technology, 5(3): 3320-3324, (2016)
7. National center for Biotechnology information, basic local alignment search tool: http://www.blast.ncbi.nlm.nih.gov/Blast.cgi
8. Eeuropean Bioinformatics Institute, Multiple sequence allignment: http://www.ebi.ac.uk/Tools/msa/clustalw2
9. Gulsan, K. and Moye-Rowley, W.S., Multidrug resistance in fungi. Eucaryotic cell 6, 6(11): 1933-1942, (2007)
10. Islam, M., Jeong, Y. T., Lee, Y. S., & Song, C. H. Isolation and identification of antifungal compounds from Bacillus subtilis C9 inhibiting the growth of plant pathogenic fungi. Mycobiology, 40(1): 59-66, (2012)
11. Arrebola, E., Jacobs, R., & Korsten, L. Iturin A is the principal inhibitor in the biocontrol activity of Bacillus amyloliquefaciens PPCB004 against postharvest fungal pathogens. J of Applied Microbiology, 108(2): 386-395. (2010).

***Corresponding Author:**
Joshi V. A.*
Email: vaibhuajoshi@gmail.com