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Exploration of possible antimycotic activity of *Pseudomonas stutzeri* against human pathogenic *Aspergillus sp.*

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Abstract

Human pathogenic fungi cause serious skin infections that persist longer. Human pathogenic fungi, namely, *Aspergillus* sp., was isolated from 35 years old patient suffering from fungal skin infections. Isolated fungi was identified by 18S rRNA sequencing with 1200 bp fragment. The bacteria isolated from soil sample collected from Hadapsar region of Pune, Maharashtra, India and identified as *Pseudomonas stutzeri* by 16S rRNA sequencing showing zone of inhibition against the isolated pathogenic *Aspergillus* sp. by dual culture method. The bacteria under study showed 86.66% inhibition of *Aspergillus* sp. This study may lead to isolation of antifungal compounds which can be utilized for treating human fungal infections.

Keywords: *Aspergillus* spp., *Pseudomonas stutzeri*, fungal skin infections, antimycotic activity.

1. Introduction

Aspergillus is a group of conidial fungi (fungi in asexual state). *Aspergillus* species are highly aerobic and are found in almost all oxygen rich environments. Some *Aspergillus* species cause serious diseases in humans and animals. The most common pathogenic species are *Aspergillus flavus* and *Aspergillus fumigatus*. *Aspergillus flavus* produces aflatoxin which is both a toxin and carcinogen (cancer causing), which is harmful to humans. *Aspergillus fumigatus* causes allergic diseases. *A. fumigatus* is one of the most clinically relevant and frequently associated with human infections (https://en.wikipedia.org/wiki/*Aspergillus_fumigatus*). Fungi cause severe infections in human health. *Aspergillus flavus* produces aflatoxin (Alshannaq A. F. et al.2018) that causes considerable hazards to the human health (Waliyar F. et al. 2015).

In addition, aflatoxin B1 (AFB1) causes not only liver but also lung cancer with acute death of people (Asters M. C. et al. 2014). To add, pathogenic funigi *Aspergillus flavus* produces toxic and carcinogenic aflatoxins threatening human life. As a result, this leads to need of proposing potential antifungal agent against this species (Gong AD et al. 2022). This highlights importance of the work against *Aspergillus* sp. control. *Pseudomonas stutzeri* (Burri R. and Stutzer A. (1895); Van Niel, C. B. and Allen, M. B. (1952)) is a Gram-Negative soil bacterium that is motile in nature and it has a single polar flagellum. The bacterial endophytes *Pseudomomonas stutzeri* produces antifungal diffusible compounds (Rojas-Solís, D., and Santoyo, G. 2018). In this work, *Pseudomonas stutzeri* was isolated from the skin care hospital soil sample from Hadapsar, Pune Maharashtra, India and its antifungal potential against *Aspergillus* sp. obtained from infected skin of a 35 years old patient was reported.

2. Materials and Methods

Isolation of *Pseudomonas stutzeri* and *Aspergillus* sp.

For the isolation of *Pseudomonas stutzeri*, a soil sample was collected from a skin care hospital in Hadapsar, Maharashtra, India **(figure 1).**

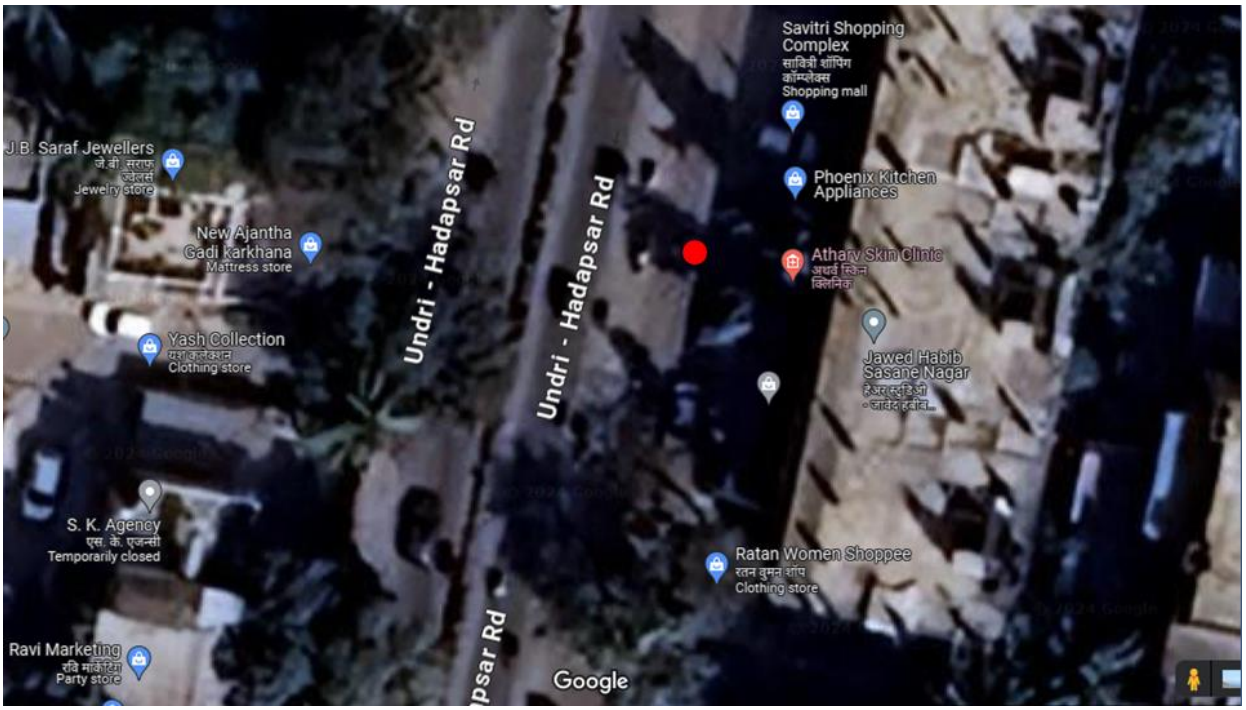

Figure 1: *Sampling location of soil collected for Pseudomonas stutzeri isolation. (Legend: ● Collection location) (Available at https://www.google.com/maps/@18.4876395,73.9397541,131m/data=!3m1!1e3?entry=ttu)*

The collected sample was diluted in distilled water at a ratio of 1 gram per 100 milliliters, and serial dilutions were subsequently performed up to 10^{-2}. The diluted test sample was then spread onto Starch Casein Agar plates using the spread plate technique. Following this, the plates were incubated for a period of 7 days. Colonies displaying morphological characteristics such as dry rigid colonies clinging together tightly (Rojas-Solís D. and Santoyo G. 2018) were isolated, purified, and subjected to further identification. Additionally, for the isolation of *Aspergillus* sp., the infected skin scrap of 35 years old patient was spread onto Potato Dextrose Agar plates using the spread plate technique. These plates were then incubated for a duration of 8 days at 25^0C.

Extraction of DNA from bacterial culture and its identification

The identification of isolates was carried out at the sequencing facility of National Centre for Microbial Resource (NCMR), National Centre for Cell Science, Pune. At the facility, genomic DNA was isolated by the standard phenol/chloroform extraction method (Maniatis T., Fritsch E., and Sambrook J., 1989) followed by PCR amplification of the 16S rRNA gene using universal primers 16F27 [5'-CCA GAG TTT GAT CMT GGC TCA G-3'] and 16R1492 [5'-TAC GGY TAC CTT GTT ACG ACT T-3']. The amplified 16S rRNA gene PCR product was purified by PEG-NaCl precipitation and directly sequenced on an ABI® 3730XL automated DNA sequencer (Applied Biosystems, Inc., Foster City, CA) as per manufacturer's instructions. Essentially, sequencing was carried out from both ends using additional internal primers so that each position was read at least twice. Assembly was carried out using Lasergene package followed by identification using the EzBioCloud database (Yoon, S. H., et al. 2017). In addition, the sequence was identified by NCBI BLAST.

Extraction of DNA from fungal culture and its identification

The identification of isolates was carried out at the sequencing facility of National Centre for Microbial Resource (NCMR), National Centre for Cell Science, Pune. At the facility, genomic DNA was isolated by the standard phenol/chloroform extraction method (Maniatis, T., Fritsch, E., & Sambrook, J. 1989), followed

by PCR amplification of the SSU regions using universal primers NS1 [5'-GTAGTCATATGCTTGTCTC -3'] and NS8 [5'-TCCGCAGGTTCACCTACGGA-3']. The amplified SSU PCR product was purified by PEG-NaCl precipitation and directly sequenced on an ABI® 3730XL automated DNA sequencer (Applied Biosystems, Inc., Foster City, CA) as per manufacturer's instructions. Essentially, sequencing was carried out from both ends so that each position was read at least twice. Assembly was carried out using Lasergene package followed by NCBI BLAST against sequences from type material for tentative identification (Boratyn, G. M. et al. 2013). As well, the fungi was morphologically observed by Lactophenol blue staining **(figure 2)** method for wet mount (SC P., 1995).

NCBI Submission

16S rRNA and 18S rRNA genes sequences of bacterial and fungal isolates were deposited in the National Center for Biotechnology Information (NCBI) database with accession numbers PP510642 and PP510873, respectively.

Phylogeny Analysis

The phylogenetic analysis of the obtained 16S rRNA sequence of *Psedomonas stutzeri* **(figure 3)** and 18S rRNA sequence of *Aspergillus* spp. **(figure 4)** was conducted with Neighbour-Joining (NJ) method of phylogeny construction (Saitou, N. and Nei M., 1987) with a bootstrap support of 500 replicates. The phylogenetic tree was constructed by Molecular Evolutionary Genetic Analysis 11 (MEGA 11) Software (Tamura, K. et al. 2021).

Antifungal Activity and its confirmation by Poisoned Food Technique

The antifugal activity of *Pseudomonas stutzeri* was studied by dual culture (Gratia A. 1936) against *Aspergillus* sp. **(figure 5)**. For this, the *Aspergillus* species was spread on the Starch Casein Agar and two spots of *Pseudomonas stutzeri* culture were made opposite to each other **(figure 6a, 6b)**. The plates were incubated for 7 days at 28^0C. To confirm the antifungal activity further, the poisoned food technique (Euloge S. A. et al., 2012) was implemented by allowing the fungal culture to grow against the antifungal agent produced by *Pseudomonas stutzeri* **(figure 7a, 7b)**. For this, the *Pseudomonas stutzeri* culture was inoculated in the Starch Casein broth and incubated for at 28^0C for 8 days in the shaker incubator **(figure 5)**. The broth was then centrifuged at 5000 Revolutions Per Minute (rpm) for 10 minutes at 4^0C. The supernatant was collected in a separate test tube to check the presence of antifungal agent in it.

The Starch Casein Agar plates were prepared with increasing concentrations of antifungal agent as 75 µl/mL, 100 µl/mL, 125 µl/mL, 150 µl/mL **(table 1).** One plate was prepared as a control in which supernatant was not added. The fungal discs were placed at the centers of all plates in the wells prepared by a cork borer. The plates were placed for incubation at 28^0C for 8 days (Mehra, S. K. and Jaitly A. K. 1995) (**figure 7a, 7b**). The percentage of inhibition was calculated by the formula:

Inhibition rate (%) = [(mycelium diameter in control sample × mycelium diameter in experimental sample)/mycelium diameter in control sample] × 100 (Gong A. D. et al. 2022).

Table 1: Serial dilutions and percentage inhibition of (*Aspergillus* sp.) of antifungal broth

Stock with antifungal compound (ml)	**Distilled water (ml)**	**Concentration of antifungal compound (ul/ml)**	**Total Volume (ml)**	**% inhibition of *Aspergillus* sp.**
01	09	10	10	00
02	08	20	10	00
03	07	30	10	00
04	06	40	10	38
05	05	50	10	34
06	04	60	10	86.66

3.Result

Bacterial and fungal identification

The bacteria under study was identified as *Pseudomonas stutzeri* by National Center for Biotechnology Information Basic Local Alignment Search Tool (NCBI BLAST) (Samal K. C. 2021) with 100% sequence identity with *Pseudomonas stuzeri* or *Stutzerimonas stuzeri* in the NCBI nucleotide database **(figure 8)**. Similarly, the 18S sequence of fungal specimen showed 100% identity with *Aspergillus flavus* and *Aspergillus oryzae* **(figure 9)** in the same database. The fungal specimen appeared with the blue coloured mycelial threads under 45X objective of the microscope **(figure 2).**

Phylogeny Analysis

Our sequence of the unknown bacteria labeled as 'B SEP 22 166' revealed close relationship with *Pseudomonas stutzeri* strain DQ1 16S ribosomal RNA gene partial sequence with NCBI accession number KF850545.1 and *Pseudomonas stutzeri* strain HMGM-7 16S ribosomal RNA gene partial sequence **(figure 3)**. In addition, the isolated fungal culture entitled B_SEP_22_165 identified as *Aspergillus* sp. showed a separate clade with closest match with CP051025.1 2862856-2864286 *Aspergillus flavus* strain AF36 chromosome 7 **(figure 4).**

Confirmation of antifungal activity

In the control plate, the *Aspergillus* sp. showed luxuriant growth on whole plate whereas in the experimental plate, the *Pseudomonas stutzeri* showed zones of inhibitions against the fungal culture under study.

Production of antifungal agent

Pseudomonas stutzeri showed growth in the Casein Starch broth with whitish appearance. The culture also got attached to the walls of the flask. In the control flask, the culture was not added, hence no growth was reported. On the other hand, in the experimental flask, the culture was added. Hence, growth was reported **(figure 5).**

Antifungal Activity and its confirmation by Poisoned Food Technique

In the first plate with the concentration of supernatant as 75 µl/mL, maximum growth of *Aspergillus* sp. was reported. The growth declined in the second and third plates with increasing antifungal compound concentrations as 100 µl/mL and 150 µl/mL, respectively (**figure 7b**, **from left to right).**

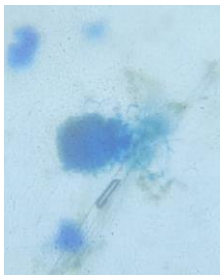

Figure 2: *Human pathogenic fungi under 45X objective*

Descriptions | Graphic Summary | Alignments | Taxonomy

Sequences producing significant alignments Download Select columns Show 100

select all *100 sequences selected* GenBank Graphics Distance tree of results MSA View

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Aspergillus flavus strain AF36 chromosome 7	Aspergillus flavus	2907	4103	100%	0.0	100.00%	2870405	CP051025.1
Aspergillus flavus strain K49 chromosome 7	Aspergillus flavus	2907	2907	100%	0.0	100.00%	2996299	CP051081.1
Aspergillus flavus strain Tox4 chromosome 7	Aspergillus flavus	2907	3224	100%	0.0	100.00%	2903554	CP051049.1
Aspergillus oryzae voucher LCWU AA3 small subunit ribosomal RNA gene, partial sequence	Aspergillus ory...	2907	2907	100%	0.0	100.00%	1698	MK371712.1
Aspergillus sp. isolate J3 small subunit ribosomal RNA gene, partial sequence	Aspergillus sp.	2907	2907	100%	0.0	100.00%	1649	MN995535.1
Aspergillus sp. isolate M09 small subunit ribosomal RNA gene, partial sequence	Aspergillus sp.	2907	2907	100%	0.0	100.00%	1623	MN906958.1
Aspergillus flavus strain NRRL 3357 chromosome 7	Aspergillus flavus	2907	26169	100%	0.0	100.00%	3097510	CP044617.1
Aspergillus sp. isolate F115 small subunit ribosomal RNA gene, partial sequence	Aspergillus sp.	2907	2907	100%	0.0	100.00%	1688	MN240474.1
Aspergillus oryzae RIB40 18S ribosomal RNA (AO090206r00001), rRNA	Aspergillus ory...	2907	2907	100%	0.0	100.00%	1799	XR_002735719
Aspergillus oryzae strain KSS2 chromosome 7, complete sequence	Aspergillus ory...	2907	1.163e+05	100%	0.0	100.00%	3249718	CP031440.1
Aspergillus oryzae strain KBP3 chromosome 7, complete sequence	Aspergillus ory...	2907	1.160e+05	100%	0.0	100.00%	4314074	CP031432.1
Aspergillus oryzae isolate MP4 small subunit ribosomal RNA gene, partial sequence	Aspergillus ory...	2907	2907	100%	0.0	100.00%	1655	MN744413.1
Aspergillus flavus strain AF INIFAP 2021 chromosome 7	Aspergillus flavus	2907	26169	100%	0.0	100.00%	3032964	CP082260.1
Aspergillus oryzae strain JMET 15 small subunit ribosomal RNA gene, partial sequence	Aspergillus ory...	2907	2907	100%	0.0	100.00%	1770	MZ892606.1

Figure 8: *Sequence alignment of Human pathogenic fungi (Aspergillus sp.*

Sequences producing significant alignments Download Select columns Show 100

select all *100 sequences selected* GenBank Graphics Distance tree of results MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Stutzerimonas stutzeri strain ER36 16S ribosomal RNA gene, partial sequence	Stutzerimonas stutzeri	2595	2595	100%	0.0	99.86%	1430	MT124561.1
Stutzerimonas stutzeri ATCC 17588 = LMG 11199 16S ribosomal RNA gene, partial sequ...	Stutzerimonas stutzeri ATCC 17588 ...	2595	2595	100%	0.0	99.86%	1472	MT027239.1
Stutzerimonas stutzeri strain PaKu14 16S ribosomal RNA gene, partial sequence	Stutzerimonas stutzeri	2595	2595	100%	0.0	99.86%	1466	MN067779.1
Pseudomonas sp. strain SBk13 16S ribosomal RNA gene, partial sequence	Pseudomonas sp.	2595	2595	100%	0.0	99.86%	1486	MH191156.1
Pseudomonas sp. strain GZ2 16S ribosomal RNA gene, partial sequence	Pseudomonas sp.	2595	2595	100%	0.0	99.86%	1459	MK217489.1
Pseudomonas stutzeri strain NCTC10450 genome assembly, chromosome: 1	Stutzerimonas stutzeri	2595	10377	100%	0.0	99.86%	4438731	LR134319.1
Stutzerimonas stutzeri strain APB-6 16S ribosomal RNA gene, partial sequence	Stutzerimonas stutzeri	2595	2595	100%	0.0	99.86%	1479	MH667647.1
Stutzerimonas stutzeri strain SD-7 16S ribosomal RNA gene, partial sequence	Stutzerimonas stutzeri	2595	2595	100%	0.0	99.86%	1499	MF555700.1
Stutzerimonas stutzeri strain 1W1-1A chromosome, complete genome	Stutzerimonas stutzeri	2595	10371	100%	0.0	99.86%	4454378	CP027664.1
Stutzerimonas stutzeri strain BKGP002 16S ribosomal RNA gene, partial sequence	Stutzerimonas stutzeri	2595	2595	100%	0.0	99.86%	1479	KY287931.1
Stutzerimonas stutzeri strain IHBB 9574 16S ribosomal RNA gene, partial sequence	Stutzerimonas stutzeri	2595	2595	100%	0.0	99.86%	1484	KU921576.1
Stutzerimonas stutzeri strain 1005 16S ribosomal RNA gene, partial sequence	Stutzerimonas stutzeri	2595	2595	100%	0.0	99.86%	1476	KU749990.1
Stutzerimonas stutzeri strain NA3 16S ribosomal RNA gene, partial sequence	Stutzerimonas stutzeri	2595	2595	100%	0.0	99.86%	1468	KU708861.1
Stutzerimonas stutzeri strain MN1 16S ribosomal RNA gene, partial sequence	Stutzerimonas stutzeri	2595	2595	100%	0.0	99.86%	1488	KU708859.1
Pseudomonas sp. strain BAB-5900 16S ribosomal RNA gene, partial sequence	Pseudomonas sp.	2595	2595	100%	0.0	99.86%	1468	KX609741.1

Figure 9: *Sequence alignment of pseudomonas stutzeri*

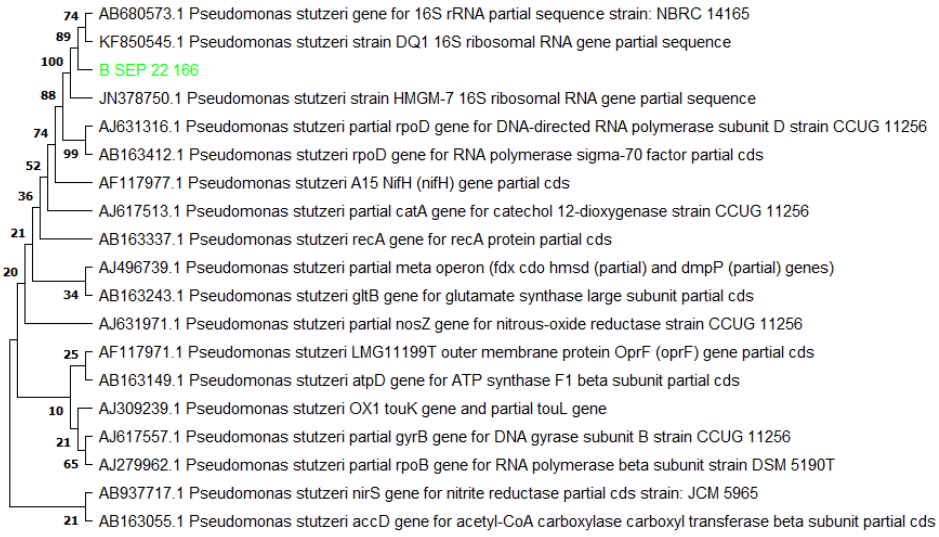

Figure 3*: *Phylogenetic analysis of Pseudomonas stutzeri with NCBI sequences from NCBI database

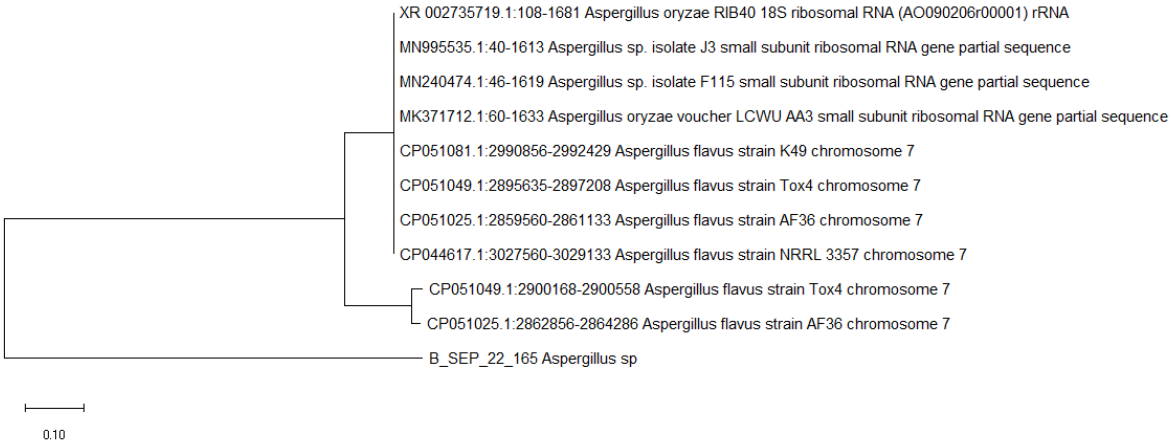

Figure 4: *Phylogenetic analysis of Aspergillus sp. with NCBI sequences from NCBI database*

Figure 5: *Pseudomonas stutzeri was cultured in Casein Starch broth for antifungal metabolite production. Left- Control, Right- Experimental*

Figure 6a: *Zone of inhibition by Pseudomonas stutzeri against Aspergillus spp (dorsal view; Left-Control, Right-Experimental)*

Figure 6b: *Zone of inhibition by Pseudomonas stutzeri against Aspergillus spp (Ventral view)*

Figure 7a: *Inhibition of fungal growth by the antifungal agent present in the supernatant (Control)*

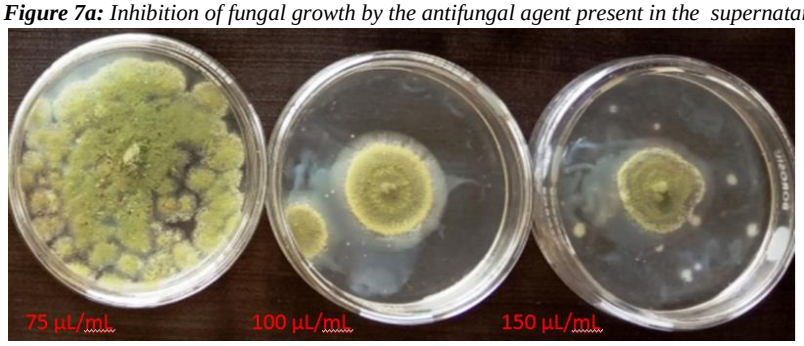

Figure 7b: *Inhibition of fungal growth by the antifungal agent present in the supernatant with concentrations as 75 µl/mL, 100 µl/mL, 125 µl/mL, 150 µl/mL (from left to right)*

4.Discussion

Isolation of *Pseudomonas stutzeri* and *Aspergillus* sp.

The *Pseudomonas stutzeri* culture was isolated from the soil of near the skin care hospital using starch casein agar at 30^0C with the incubation period of 48 hours whereas Gong AD et al. (2022) isolated the strain YM6 (*Pseudomonas stutzeri*) using nutrient agar at 37^0C with incubation period of 24 hours from the sediment of sea belonging to the yellow sea of China. The fungal culture *Aspergillus* was cultivated with potato dextrose agar (PDA) at 28^0 C (Gong A. D. et al. 2015) with the incubation period of 8 days.

Bacterial and fungal identification

The bacteria under study was identified as *Pseudomonas stutzeri* by National Center for Biotechnology Information Basic Local Alignment Search Tool (NCBI BLAST) (Samal K. C. et al. 2021) with 100% sequence identity with *Pseudomonas stuzeri* or *Stutzerimonas stuzeri* in the NCBI nucleotide database. Similar result was reported by Kumar A., Kumar S. P. J., (2022) who stated that 16S rRNA based identification led to nomenclature of the endophytic bacterium ***Pseudomonas stutzeri.*** However, the authors relied on the Kit based DNA extraction from the bacterium under study in contrast to our DNA extraction

method that did not affect the final result. In addition, we isolated the bacteria under study from the soil sample near skin care hospital, Hadapsar, Pune, Maharashtra, India in contrast to Lim HS et al. (1991) who isolated the same bacteria from the rhizosphere in ginseng root rot-suppressing soil from Yeyngpung-gun, Korea. In contrary to this, Asters M.C., Williams W.P. (2014) isolated *Pseudomonas stutzeri* YM6 from the marine habitats. It showed that the bacterium under study is present in the soil as well as marine water.

Similarly, the 18S sequence of fungal specimen showed 100% identity with *Aspergillus flavus* and *Aspergillus oryzae* in the same database. Identification of the obtained bacterial sequence was as *Pseudomonas stutzeri* with 99.86% sequence similarity with NCBI sequence database at species level. In contrary to this, the 16S rDNA sequnce belonging to the YM6 strain showed homology with *P. stutzeri, P. putida* and *P. xanthomarina* belonging to *Pseudomonas* spp.

Phylogeny Analysis

Our sequence of the unknown bacteria labeled as 'B SEP 22 166' revealed close relationship with *Pseudomonas stutzeri* strain DQ1 16S ribosomal RNA gene partial sequence with NCBI accession number KF850545.1 and *Pseudomonas stutzeri* strain HMGM-7 16S ribosomal RNA gene partial sequence **(figure 3).** In addition, the isolated fungal culture entitled B_SEP_22_165 identified as *Aspergillus* species showed a separate clade with closes match with CP051025.1 2862856-2864286 *Aspergillus flavus* strain AF36 chromosome 7 **(figure 4).** YM6 identified as *Psuedomonas stutzeri* and *P. stutzeri* 28a42 (AJ312165.1) revealed high homology with respect to clade level (Gong A. D. et al. 2022). However, *Pseudomonas stutzeri* under study was not be able for grouping into the single clade with other homologous bacterial sequences from the NCBI database **(figure 3).**

Confirmation of antifungal activity

In dual culture method, in the control plate, the *Aspergillus* sp. showed luxurient growth on whole plate where as in the experimental plate, the *Pseudomonas stutzeri* showed zones of inhibitions against the fungal culture in question. Similar results were reported by Kumar A et al. (2022) who stated that in the dual culture assay, the *Pseudomonas stuzeri* PSIISS-1 inhibited the growth of *Fusarium oxysporum* var *ciceri* as well as *R.solani* with 61.0% and 53.3%, respectively. Likewise, antifungal agent produced by *Pseudomonas stutzeri* showed 86.66% zone of inhibition against *Aspergillus* sp **(table 1)** which was near to 100% as reported by Gong AD et al. (2022) against *Aspergillus flavus* by the strain YM6 which was further identified as a *Psuedomonas stutzeri*. The control plate made by Gong AD et al. (2022) showed branched hyphae growing outward with extensive network on the Potato Dextrose Agar (PDA) medium. This report was similar with our control study. This finding was further supported by Gong AD et al. (2022) who reported that the bacteria strain YM6 identified as *Pseudomonas stutzeri* was effective for control of *Aspergillus flavus* owing to antifungal volatiles' production.

5.Conclusion

DNA sequence database in NCBI is needed to be updated for producing uniform results at species level identification. Furthermore, the study highlights antimycotic capacity of *Pseudomonas stutzeri* against human pathogenic *Aspergillus* sp. suggesting its applicability as a culture that is used for the treatment of *Aspergillus* based human fungal diseases. Future research efforts may focus on exposure of the molecular mechanism of anti *Aspergillus* agent production by *P. stutzeri* and evaluation of its efficacy in the clinical setting. This may offer promising values for the treatment of fungal infections and improvement in the health care system.

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Conflict of Interest

Authors declare that no conflict of interest exists among them.

Grant Support Details

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Ethical Statement

No ethical guidelines were violated during the research.

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