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Antifungal potential of human pathogenic *Blastobotrys* sp. by *Bacillus licheniformis* strain S10 identified using 16S rRNA and first time cultivated on Glycerol Asparagine Agar with implications for NCBI database enhancement

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ABSTRACT
A soil sample collected from the rhizosphere of *Ocimum tenuiflorum* (tulsi), Karad, Maharashtra, India, was diluted to 10^{-2} and spread on Glycerol Asparagine agar. The isolate was catalase positive, able to utilize starch and ferment the sugar. Molecular identification through partial 16S ribosomal RNA gene sequencing confirmed the isolate as *Bacillus licheniformis* strain S10, with only 95.22% identity with NCBI sequence database suggesting the implications for NCBI database enhancement. Phylogenetic analysis using the Maximum Likelihood method revealed its closest relationship to the strain KX946197.1:370-1248. The strain showed antifungal activity against human pathogenic *Blastobotrys* sp.

Key words: *Bacillus licheniformis, Ocimum tenuiflorum*, Phylogenetic tree, *Blastobotrys* sp. Glycerol Aspargine Agar

INTRODUCTION
Blastobotrys adeninivorans, Blastobotrys proliferans, Blastobotrys serpentis, Blastobotrys raffinosifermentans cause diseases to humans; especially, to patients with cystic fibrosis as well as immunocompromised patients[1]. In addition, the *Blastobotrys* cause invasive mycosis in immunocompromised people with lower susceptibility to antifungal agents[2]. *Blastobotrys mokoenaii* causes the invasive fungal infection in the immunocompromised patient who have acute myeloid leukemia[3]. In addition, *Blastobotrys* causes the infection to the cystic fibrosis patients, although it is treatable[4,5]. *Blastobotrys* fungal peritonitis was reported in the travelers who received the peritoneal dialysis[6].
Bacillus licheniformis MH48 showed the activity against the plant pathogens *Phytophthora capsici, Colletotrichum gloeosporioides* and *Rhizoctonia solani*[7]. *Bacillus licheniformis* inhibits growth of different fungi[7] including phytopathogenic fungi[8]. They indicated activity against phytopathogenic fungi, for example, *Fusarium oxysporum, Botryotinia fuckiliana, Verticillium dahlia, Phytophthora cinamomi, Phytophthora cactorum, Colletotrichum acutatum, Botrytis cinerea, Aspergillus niger* and *Aspergillus flavus*[9].
Besides, *Bacillus licheniformis* reported the antifungal activity against *Blastobotrys cinerea*[10,11,12]. *Bacillus licheniformis* OE-04 produces antifungal metabolites *Colletotrichum gossypii*[13]. *Bacillus licheniformis* BC98 produces antifungal molecules[14]. To add, the bacterial strain *Bacillus licheniformis* EF 617325 revealed the antifungal activity against *Ophiostoma flexuosum, O. polonicum, O. tetropii* as well as *O. ips*[15]. *Bacillus licheniformis* HS 10 produces antifungal proteins[16]. *Bacillus licheniformis* KMU-3 is antifungal against crop pathogenic fungi.
Less work has been reported on the antifungal activity of *Bacillus licheniformis* against *Blastobotrys* species, the potential human pathogenic agent. In addition, Glycerol Aspargine agar was not tested yet for the growth of *Bacillus licheniformis*. Further, NCBI has reference sequence database that has popular issues including wrong and unspecific taxonomic labelling, underrepresentation and overrepresentation of taxons, incorrect exclusion or inclusion of non-microbial, host, and vector taxa from the database, contamination of the partitioned and chimeric sequence, reference sequence with poor quality, masking with low complexity, errors/delay in database updating and maintenance[17]. In addition, the NCBI database based identification of species is not strong and reliable as there is variation in the similarities of the sequences obtained from organisms of variant geographical locations[18].
In this study, the antimycotic potential of the bacteria under study has been investigated against the *Blastobotrys* sp. in addition with confirmation of NCBI 16S rRNA sequence database reliability for *Bacillus licheniformis* strain S10 identification and ability of the bacteria under study for its cultivation on Glycerol Aspargine agar.

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MATERIALS AND METHODS

Sampling:

To isolate *Bacillus licheniformis* strain S10, a soil sample was collected from the rhizosphere of *Ocimum tenuiflorum* from Karad, Maharashtra, India.

Isolation of *Bacillus licheniformis*

Initially, 1 gram of the soil sample was diluted in 100 ml of distilled water, followed by serial dilutions up to 10^{-2}. The diluted sample was then spread on Glycerol Asparagine Agar plates[19] by the spread plate technique. Plates were incubated at 28°C[20] for 4–6 days[21]. Colonies exhibiting morphological characteristics consistent with *Bacillus licheniformis* strain S10 were isolated and purified. Molecular identification of the purified isolates was performed at Progenome Life Science, Sambhajinagar, Maharashtra, India.

Molecular Identification

Genomic DNA was extracted from the isolated culture by the Nucleospin Microbial DNA Kit. The quality was checked by agarose gel electrophoresis (on 1% agarose gel). The gel was visualized by UV Transilluminator). PCR amplification of the 16S ribosomal RNA gene was performed with primers 27_F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492_R(5′-TACGGYTACCTTGTTACGACTT-3′)[40]. The following conditions were maintained for the PCR: initial denaturation at 95^0C for 3 minutes with 1 cycle, priming at 95^0C for 45 seconds, extension at 52^0C for 50 seconds, termination at 72^0C for 70 seconds (35 cycles), final extension at 72^0C for 10 minutes (1 cycle) and the final storage at 4^0C. The resulting PCR product, a single distinct band, was visualized on a 1.2% agarose gel by UV transilluminator.

Unincorporated nucleotides were removed from the PCR product. The residual primers were removed by Exonuclease-I and Shrimp Alkaline Phosphatase enzymes. With help of BigDye® Terminator v.3.1 Cycle Sequencing Kit, cycle sequencing reaction was performed. Ethanol precipitation method was used to perform the sequencing cleanup. The template was dissolved in HiDi formamide. It was then bidirectionally sequenced in ABI 3730 Genetic analyzer. PCR products were then processed for direct bi-directionally sequencing using ABI PRISM 3730 × 1 Genetic Analyzer. The resulting DNA sequences were aligned, manually trimmed and edited using CodonCode Aligner to obtain complete sequences. Homology analysis was performed using the BLASTn tool in the NCBI reference sequence database to identify the obtained 16S rRNA gene sequence.

Biochemical and Microscopic Characterization

Biochemical characterization of the isolated *Bacillus licheniformis* strain S10 culture included a series of tests, specifically the Triple Sugar Iron test[22], catalase activity[23], and starch hydrolysis[24]. Additionally, microscopic analysis and Gram staining[25] were conducted for further confirmation of isolates' identification.

Phylogenetic Analysis

NCBI Submission

Partial sequence of 16S rRNA gene of *Bacillus licheniformis* was studied for the matching with other sequences in NCBI using Blastn tool[26] and submitted in the database of National Center for Biotechnology Information (NCBI) with accession number PQ361285.

Evolutionary analysis by Maximum Likelihood method

The phylogenetic analysis of the obtained partial 16S rRNA gene sequence of *Bacillus licheniformis* was performed. The Maximum Likelihood method as well as Tamura-Nei model were used to analyze the evolutionary relationship[27] with the bootstrap support 500 replicates[28]. The branches related to partitions reproduced in less than 50% bootstrap replicates were collapsed. The % of replicate trees in which the associated taxa grouped collectively in the bootstrap analysis, 500 replicates were displayed next to the branches[28]. The initial tree(s) were received for the heuristic search by application of BioNJ algorithm along with Neighbor-Joining to a matrix which was related with the pairwise distances

estimated by Tamura-Nei model. The selection of topology based on the superior log likelihood was performed. This analysis involved 27 nucleotide sequences. There were a total of 859 positions in the final dataset. Evolutionary analyses were conducted in MEGA 11[29].

Antifungal activity

At colony level

20 ml of Sabouraud Dextrose agar was prepared in the 100 ml capacity Erlenmeyer flask, autoclaved at 121^{0}C at 15 psi (pounds per square inch)[30] and cooled. The fresh grown culture of *Blastobotrys* sp. was then inoculated (18%) in the medium. The agar was then poured in the plate and allowed to solidify. Further, the colony of *Bacillus licheniformis* was spread at the center of the plate. It was then kept for incubation at 28^{0}C[31]. After incubation, the average zone of inhibition was calculated.

At broth level

The culture of *Bacillus licheniformis* was inoculated in the Glycerol Aspargine broth and incubated at 28^{0}C for 8 days in the rotary shaker incubator. Then, the broth was centrifuged at 5,000 rpm for 20 minutes. The supernatant was collected in a separate tube and the cell pellet was collected in another tube. Two Petri plates were prepared with Sabouraud Dextrose agar mixed with freshly grown *Blastobotrys* sp fungal culture. The wells of 7mm size were made at the center of each plate by a cork borer. 50 µl of supernatant (200 µl/ml) was added in one well and another well was kept as a control in which no supernatant was added. This process was performed to check the presence of antifungal compound in the supernatant.

Poisoned Food Technique

Poisoned food technique was performed for evaluation of the inhibition of growth of mycelium in percentage[32]. 100 µl of Sabouraud Dextrose agar was prepared in the separate 250ml capacity Erlenmeyer flask and autoclaved at 121^{0}C at 15 psi[30] for 20 minutes. The medium was then allowed to cool and it was then distributed in five 50 ml Erlenmeyer flasks, as 20 ml in each flask. Further, the supernatant was added in each flask by maintaining its concentration as 1%, 2.5%, 5%, and 7% by adding the supernatants in each flask as 0.2ml, 0.5ml, 1ml, 1.4ml in each flask, respectively (table 1). The medium was then poured in the plates with respective labels and allowed to solidify. Afterwards, the wells of 7mm size were bored with a cork borer at the center of each plate. The fungal discs of the same size were placed in the wells and the plates were then incubated at 28^{0}C for 8 days. After incubation, the percent inhibition of the growth of mycelia was evaluated as follows:

Inhibition of growth of mycelium (%)= dc-dt/dc x100

Where, dc = Colony growth in relation with fungi in the control plate (cm), dt = Colony growth in relation with the fungi in the experimental plate (cm).

RESULT AND DISCUSSION

Morphological and Gram characteristics with biochemical properties

The isolated *Bacillus licheniformis strain S10* culture produced distinctive grey-colored colonies on the starch agar medium[33] (Figure 1) as a pure culture. The similar finding was presented by Kumar et al., (2023)[33]. Microscopic examination at 45X magnification revealed thin, thread-like structures with cells attached to each other (Figure 1b). This observation was supported by Ojima, Y., et al. (2023)[35] who stated that *Bacillus licheniformis* showed the formation of aggregation in the suspension culture when the carbon was limited. Gram staining confirmed the Gram-positive nature of the culture (Figure 2) as reported by Jamali, N., et al. (2023)[36]. Biochemically, the Triple Sugar Iron (TSI) test indicated the development of a reddish-black coloration without gas production, in contrast to the colourless control (Figure 3). The catalase test showed a positive result (Figure 4). This finding was supported by de Jesús Barreras-Bojórquez, M., et al. 2023[37] who claimed that *Bacillus licheniformis* ferments the sugar with no gas production and it produces catalase enzyme. To add, the bacteria under study showed starch untilization potental (figure 5) as reported by Božić, N., et al. 2011[38]. The starch hydrolysis test was positive, indicating the culture's ability to hydrolyze starch (Figure 5).

Phylogenetic Analysis

The 899 bp partial 16S rRNA gene sequence of *Bacillus licheniformis* strain S10 showed 95.29% sequence similarity with the NCBI database sequences (figure 6a). Similarly, Barreras-Bojórquez et al., (2023)[37] reported that 1413 bp 16S rRNA sequences of *Bacillus licheniformis* showed 99% identity with various GenBank sequences. The phylogenetic analysis of the obtained sequence of *Bacillus licheniformis* strain S10 (represented as T421 in figure 6b) showed closest match with KX946197.1:370-1248 *Bacillus licheniformis* strain GJ2-1 16S ribosomal RNA gene partial sequence forming a separate clade (figure 6b). In contrast, de Jesús Barreras-Bojórquez, M., et al. (2023)[37] reported the single clade with 15 *Bacillus licheniformis* 16S rRNA sequences whereas, our study showed a cluster with a single 16S rRNA *Bacillus licheniformis* sequences from NCBI database.

Antifungal activity

The centrally placed colony of *Bacillus licheniformis* strain S10 showed average zone of inhibition as 1.8 cm (figure 4;table 2). Likewise, Kim, H. J., et al. (2007)[10], Lee, J. P., et al. (2006)[11], Lim, J. H., and Kim, S. D. (2010)[12] proposed the antifungal potential of the bacteria under study against *Botrytis cenerea.* The supernatant obtained by the centrifugation of a broth (figure 8a) showed the zone of inhibition against *Blastobotrys* sp. figure (8b). The poisoned food technique analysis of the supernatant (extract) produced by *Bacillus licheniformis* strain S10 against *Blastobotrys* sp. showed potential antifungal activity (figure 9; table 3)[34].

NOVELTY OF THE WORK

Bacillus licheniformis strain S10 was first time isolated on Glycerol Aspargine Agar and broth. This has provided the substitution for the growth medium used for the growth of bacteria under study. For example, Luria-Bertani (LB) broth was used by Zhu H, et al. (2024)[39] that required shaking of the medium with the culture at 180 rpm. In case of Glycerol Aspargine agar/broth no shaking was required saving the expenses of electricity for the researchers. Our work has contradicted the idea proposed by Yang MQ et al. (2024)[40] who proposed that 16S rRNA analysis is a choice of microbial identification. In our case, comparison of 16S rRNA gene sequence of *Bacillus licheniformis* strain S10 with NCBI reference sequence database showed only 95.22% identity. Thus, the proposed find is a novel that appeals combination of two or more DNA based methods for species level identification instead of one.

CONCLUSION

Bacillus licheniformis strain S10, identified on the basis of its 899bp partial 16S rRNA sequnce, may have a potential of inhibition of human pathogenic *Blastobotrys* sp. to reduce the fungal infections across globe. Glycerol Aspargine Agar supports the growth of *Bacillus licheniformis* which is not a regular media for its growth. NCBI 16S rRNA partial sequence database of NCBI is not completely reliable database for the accurate species level identification and needs up gradation. Further research is invited in this area for purification, identification and confirmation of the antifungal compound produced by the strain under study against *Blastobotrys* sp.

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CONFLICT OF INTEREST

Authors declare that no conflict of interest exists among them.

FUNDING SOURCES

No fund received for the conducted research work.

ETHICAL STATMENT

No ethical guidelines were violated during the research.

AUTHOR CONTRIBUTIONS

All authors have considerable contributions for concept development as well as design, data acquisition, analysis, along with the interpretation of the data; involved in the process of drafting and revising of the article; agreed for the submission to the current journal; provided the final approval of the article to be published; and agree for the accountability of all dimensions of the work. All included authors are eligible for authorships as per the International Committee of Medical Journal Editors (ICMJE) guidelines or requirements.

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Table 1: Preparation of different concentrations (1%, 2.5%, 5%, and 7%) of the supernatant with the Sabouraud Dextrose agar.

Growth medium (ml)	Supernatant (ml)	Concentration of supernatant (%)
20	0.2	1
20	0.5	2.5
20	01	5
20	1.4	7

Fig 1a: Grey colored colony of *Bacillus licheniformis* strain S10 cultured on the Starch Agar medium as a pure culture.

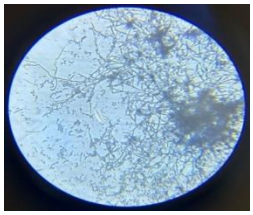

Fig 1b: The culture of *Bacillus licheniformis* strain S10 showed thread like structure under 45X objective.

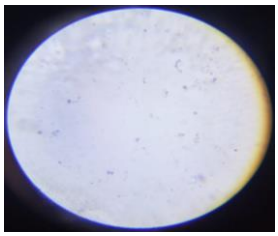

Fig 2: Gram staining of *Bacillus licheniformis* strain S10 under 100X oil immersion objective.

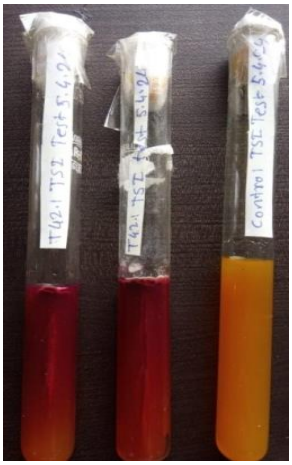

Fig 3: Result of Triple Sugar Iron Utilization Test by *Bacillus licheniformis* S10. left two tubes: experimental, right tube-control).

Fig 4: Catalase test of *Bacillus licheniformis* strain S10 was found to be positive (left two slides-experimental, right one slide-control).

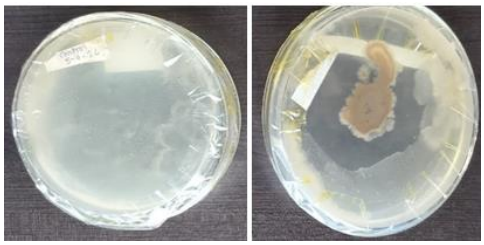

Fig 5: The *Bacillus licheniformis* strain S10 showed the potential of starch hydrolysis (right) against control (left).

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Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Bacillus licheniformis strain S10 16S ribosomal RNA gene, partial sequence	Bacillus licheniformis	1628	1628	100%	0.0	100.00%	899	PQ361285.1
Bacillus licheniformis strain 55L2-1 16S ribosomal RNA gene, partial sequence	Bacillus licheniformis	1393	1393	97%	0.0	95.22%	1459	JN366759.1
Bacillus licheniformis strain HBUAS71381 16S ribosomal RNA gene, partial sequence	Bacillus licheniformis	1393	1393	96%	0.0	95.29%	1472	OR144791.1
Bacillus licheniformis strain BDGO-A4 16S ribosomal RNA gene, partial sequence	Bacillus licheniformis	1393	1393	97%	0.0	95.10%	1000	OM864638.1
Bacillus haynesii strain FC9 16S ribosomal RNA gene, partial sequence	Bacillus haynesii	1393	1393	96%	0.0	95.42%	1435	MW217207.1
Bacillus licheniformis strain HBUAS77088 16S ribosomal RNA gene, partial sequence	Bacillus licheniformis	1393	1393	96%	0.0	95.29%	1472	PQ312803.1
Bacillus licheniformis strain S10 16S ribosomal RNA gene, partial sequence	Bacillus licheniformis	1393	1393	96%	0.0	95.29%	1423	MT270822.1
Bacillus licheniformis strain QSB3 16S ribosomal RNA gene, partial sequence	Bacillus licheniformis	1391	1391	96%	0.0	95.41%	1412	ON004998.1
Bacillus haynesii strain 24005 16S ribosomal RNA gene, partial sequence	Bacillus haynesii	1389	1389	96%	0.0	95.28%	1086	OR430200.1
Bacillus haynesii strain 24033 16S ribosomal RNA gene, partial sequence	Bacillus haynesii	1389	1389	96%	0.0	95.28%	984	OR430218.1
Bacillus licheniformis strain HBUAS56297 16S ribosomal RNA gene, partial sequence	Bacillus licheniformis	1389	1389	96%	0.0	95.31%	1472	MT270436.1
Bacillus licheniformis strain HBUAS64164 16S ribosomal RNA gene, partial sequence	Bacillus licheniformis	1389	1389	96%	0.0	95.31%	1472	OP358117.1
Bacillus haynesii strain HBUAS56375 16S ribosomal RNA gene, partial sequence	Bacillus haynesii	1389	1389	96%	0.0	95.31%	1472	MZ959499.1
Bacillus licheniformis strain GJ2-1 16S ribosomal RNA gene, partial sequence	Bacillus licheniformis	1389	1389	98%	0.0	94.90%	1298	KX946197.1
Bacillus sonorensis strain T1 16S ribosomal RNA gene, partial sequence	Bacillus sonorensis	1389	1389	96%	0.0	95.18%	1452	PP267324.1
Bacillus licheniformis strain 1-27 16S ribosomal RNA gene, partial sequence	Bacillus licheniformis	1389	1389	96%	0.0	95.31%	1430	PP593750.1

Fig 6a: The 899 bp sequence of 16S rRNA gene of *Bacillus licheniformis* strain S10 showed only 95.22% matching with NCBI sequence database except the first match. Note: The first match was 16S rRNA partial gene sequence of the strain obtained in our study which was a submitted in the NCBI, hence, it was not included in the matching analysis.

Fig 6b: Phylogenetic analysis of *Bacillus licheniformis* strain S10 with NCBI sequences from NCBI database.

Figure 7: *Bacillus licheniformis* strain S10 showed antagonistic activity against *Blastobotrys* sp.

Table 2: Measurements of zones of inhibitions of by *Bacillus licheniformis* strain S10 against *Blastobotrys* sp.

Test Organism	Tested against	Average zone of inhibition (cm)
Bacillus licheniformis	*Blastobotrys* sp.	1.8

strain S10		

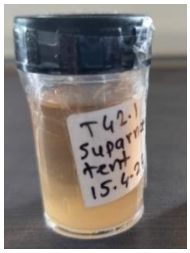

Fig 8a: Supernatant produced after growth of *Bacillus licheniformis* strain S10 in the Glycerol Aspargine broth.

Fig 8b: **Antifungal activity of supernatant against *Blastobotrys* sp. obtained after centrifugation after growth of *Bacillus licheniformis* strain S10 in Glycerol Aspargine broth.**

Fig 9: Confirmation of *Bacillus licheniformis* strain S10 produced supernatant for its activity against *Blastobotrys* sp. by Poisoned Food Technique.

Table 3: Observation table of poisoned food technique.

Concentration of supernatant (%)	Average mycelial growth (cm)	Inhibition of the mycelial growth (%)
1	05	12.65-5/12.65X100=60.47
2.5	3.9	12.65-3.9/12.65 X 100=69.16
5	5.4	12.65-5.4/12.65 X 100=57.31
7	9.1	12.65-9.1/12.65 X 100=28.06
No supernatant	12.65	No inhibition of growth