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Isolation and Characterization of a Bacterium Exhibiting Excellent Antifungal Potential

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Abstract: ***The current study reports isolation and characterization of a bacterium, named SCPS1, from the garden soil of a college campus from University of Delhi in New Delhi, India. Garden soil was used for bacterial isolation using standard isolation procedures of serial dilution and plating. Out of several 100 isolates, 6 morphologically distinct colonies were picked up and screened for antifungal potential. SCPS1 showed excellent activity against Aspergillus, Mucor and Rhizopus. It was selected for further study and characterization was done using biochemical tests and 16S rRNA based molecular approach. Gram positive rods with capsules were observed microscopically and SCPS1 was found to be a member of Bacillus group, Bacillus amylo liquifaciens, upon phylogenetic analysis.***

Keywords: Bacterium, Antifungal Potential

1. Introduction

Fungal infections have been on a rise across the globe. They include invasion of tissues by fungal strains and are also known as Mycosis with respect to animal and human hosts. These infections are common among people but are particularly dangerous for patients already suffering from diseases such as cancer, AIDS and diabetes. Infections caused by opportunistic fungi, such as *Candida*, *Aspergillus*, *Cryptococcus* etc., have proved lethal in patients with compromised immune system. Also, on agricultural fronts, a large proportion of our annual crop production is lost to an increasing population of phytopathogens. To add to the adversities we have new problems such as harmful side effects of commercial anti-fungal drugs, emergence of drug resistant pathogens, pharmacological limitations etc. (Jensen et al, 2005; Hakvag et al, 2008; Frieri et al., 2017; Aslam et al., 2018). Due to human carelessness, antibiotics have found ways to enter almost all of our ecosystems, either through excretion or through improper waste discard methods. These may persist and accumulate over time (Larsson, 2014; Gothwal and Shashi dhar, 2015). Continuous exposure to these antibiotics can led to development of resistance in resident microbial flora.

A few decades back, fungal infections were not as serious as they are at present and were considered curable with chemotherapy including potassium iodide and two polyenes, nystatin and amphotericin B (Vicente et al., 2003). Since the discovery of flucytosine, there has been little development in the area. Numerous antibacterial antibiotics have been researched into but reports about antifungal antibiotics are still limited. A very common antibiotic producer providing us with antibiotics such as Streptomycin, Amphotericin B, and Nystatin is actinomycete. They have as much as 70% contribution to the current antibiotic lot with *Streptomyces* contributing the most (Watve et al, 2001; Berdy, 2005). The role of an isolated protein baciamin has been observed in broad spectrum antifungal potential of *Bacillus amylo liquefaciens (*Wong et al. 2008). Another member of *Bacillus sp., Bacillus cereus* was also found to possess activity against *Saccharomyces cerevisiae (*Qazi et al., 2009). Several other members of *Bacillus* have been shown to possess excellent antimicrobial activity. Some of them include *Bacillus subtilis, B. amylo liquefaciens, B. cereus,* and *B. licheniformis*. These not only exhibited strong antibacterial activity but also showed excellent antifungal and anti-algal potential. These strains produced a combination of peptides such as surfactins, iturins, fengycins, subtilin and subtilosin. *sboX*gene and subtilosin and *sboX*were observed for the first time in *Bacillus amylo liquefaciens* (Al-Ajlani & Hasnain, 2010). Bacteria with antimicrobial potential have been isolated from regions of extreme weather conditions such as Antarctic and Arctic samples. These psychro-tolerant bacterial possess antimicrobial potential showed remarkable antifungal activity against MDR-yeast strain *Candida albicans* NCIM3471 (Shekh et al. (2011). *Bacillus amylo liquefaciens* isolated from Chinese medicinal plant *Ginkgo biloba* inhibited growth of *Lasio diplo diarubropurpurea, L. crassispora* and *L. theobromae* when using crude broth filtrate and ethyl acetate extract. On further analysis, the ethyl acetate extract was found to consist of lipopeptides such as fengycin, surfactin and bacillomycin which contributed to antifungal potential of the strain (Yuan et al. (2012).

Molecular characterization based on 16S rRNA gene sequence is one of the most frequently used methods for microbial identification. The sequence thus obtained is used for bioinformatics analysis. It is compared to other pre-defined sequences deposited in various databases, such as Genbank, to establish the identity of the test organism. This bioinformatic analysis helps determine the phylogenetic relationships among unknown strain and known reported strains and hence, helps in defining bacterial identity.

2. Material and Methods

Soil samples from garden of a College in New Delhi (28.5547° N, 77.2183° E) were collected and bacterial isolation was carried out using standard techniques of serial dilution and plating. The resultant colonies were

purified and screened for antifungal potential using the method of dual culture (Xiaoning et al., 2014).For morphological characterization, size; mass colour, colony shape, margin, surface, elevation, pigment production and broth consistency were noted. Biochemical characteristics were noted by performing starch hydrolysis test, casein hydrolysis test, gelatin hydrolysis test, carbohydrate utilization test, triple sugar iron test, catalase test, oxidase test, urease test, IMViC tests, NaCl and pH tolerance following standard procedures.

Genomic DNA isolation was performed using genomic DNA extraction kit (Bhat Biotech India Pvt. Ltd., Bangalore) and 16S rRNA gene was amplified. Sequencing of both the strands of purified PCR product was then performed using automated DNA sequencer - 3037*xl* DNA analyzer using BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems). Sequences so obtained were analyzed to generate the final contig. NCBI BLASTN was performed and most similar neighbours were selected, aligned using Clustal W2 and Mega 5 and used for identification of the organism.

3. Results and Discussion

Upon isolation and screening, an isolate (SCPS1) was found to inhibit growth of a large number of fungi including yeasts. Figure 1 shows inhibition of *Aspergillusniger*, *Mucor* and *Rhizopus*by this bacterium (Figure 1). The isolate was purified and subjected to standard morphological analysis and biochemical tests were performed, the results of which are summarized in Table 1. Morphologically, SCPS1 gave off white, flat, glistening, irregular colonies with concentric rings in the center and lobate margins. No pigments were observed on solid media as well as in liquid culture and broth consistency was uniformly turbid. Staining established the presence of gram positive rods with capsules and spores. Biochemically, it showed a prominent amylase and protease activity and led to acid production from almost all the sugars tested. Negative indole production test indicates the absence of tryptophanase enzyme responsible for degradation of tryptophan in SIM agar to indole. Absence of indole means that Kovac's reagent is unable to give a cherry red color. A negative MR test indicates oxidation of glucose to neutral end products. Production of acetyl methyl carbinol, a non acidic end product, synthesized from organic acids produced after glucose fermentation indicates a positive VP test. Furthermore, due to the absence of enzyme citrase, the isolate gave no change in the color of the medium from green to a deep Prussian blue, as it did not make the medium alkaline by utilizing citrate bromothymol blue (pH indicator) present in the citrate agar slant. Reduction of nitrate to nitrite by the bacterium was observed as cherry red color appeared after addition of reagent I and reagent II in overnight incubated nitrate broth tubes. Positive catalase test suggested aerobic nature of the isolate. When compared to standard tables and related studies, the isolate SCPS1 was found to be similar to *Bacillus* genera. A biochemical profile similar to that obtained for SCPS1 has been reported for various *Bacilli* strains by Ivanova et al. (1999) and Parvathi et al. (2009).

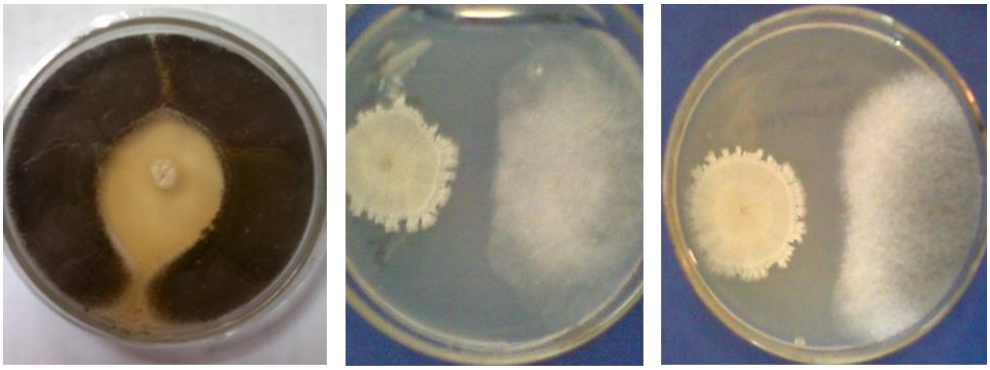

Figure 1: Inhibition of *Aspergillus, Mucor and Rhizopus* by the bacterial strain SCPS1

The sequence analysis of the 16SrRNA gene showed that the organism SCPS1 bears a great level of similarity with the *Bacillus amylo liquefaciens* 16SrRNA. Thus, from the results of conventional biochemical tests and genetic analysis, it could be concluded that the isolateSCPS1is very closely related to *Bacillus amyloliquefaciens.* This strain *Bacillus amyloliquefaciens* NBRC 15535 has been listed in the UNIPROT database and is reported to be producing chitosanase. The enzyme catalyzes the endo hydrolysis of beta-1, 4-linkages between N-acetyl-D-glucosamine and D-glucosamine residues in a partly acetylated chitosan (UniProtKB Database).

Bacillus group of bacteria has been extensively studied for their antimicrobial potential, isolation and identification of responsible biomolecules (Ehling-Schulz et al., 2019). The biomolecules responsible for antimicrobial potential of other *Bacillus* strains include lipopeptide surfactants and several antibiotics such as iturin, bacilysin, bacillomycin, fungycin, surfactin, subtilin, and subtilosin. Furthermore, the authors have also reported the presence of multiple genes coding for these bioactive compounds in one single strain (Sarwar et al., 2018; Vinod kumar et al., 2017; Saravana kumar et al., 2019).

4. Conclusion

The present study reports successful isolation and screening of *Bacillus amyloliquifaciens* isolate SCPS1 for excellent antifungal activity. The isolate was characterized using traditional biochemical methods and 16S rRNA based molecular approach. SCPS1 showed excellent inhibition of *Aspergillus*, *Mucor* and *Rhizopus*. However, characterization of the biomolecule secreted by the bacterium still needs to be done to establish its identity. Identification of novel isolates and antimicrobial compounds can help overcome the problem of antibiotic resistance and can also help in bioremediation of contaminated sites. This study could be further pursued for the development of potential antifungal agent.

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Table 1: Morphological and biochemical profile of SCPS1

Morphological Test	**Results**
Gram's staining	Gram positive
Acid fast staining	Negative
Capsule staining	Present
Spore staining	Spores in chain and in intercalary position
Physiological Tests	
Growth at Temperatures	
28°C	+
37°C	+
Growth at pH	
pH 4.0	+/-
pH 5.5	+
pH 7.0	+
Growth in presence of NaCl	
2%	+ +
4%	+ +
6%	+ +
8%	+ +
10%	+
12%	+
14%	-
Utilization of sugars	
Glucose	Acid+ gas-
Lactose	Acid- gas-
Mannitol	Acid + gas -

Sucrose	Acid + gas -
Fructose	Acid + gas -
Xylose	Acid + gas -
Maltose	Acid – gas -
Triple sugar iron	Yellow butt by yellow slant, no gas, no H_2S
IMViC Series of tests	
Indole	-
Methyl red	-
Vogue's Proskauer	+
Citrate	-
Nitrate reduction	+
Enzyme production	
Amylase	+
C1 cellulase	-
Cx cellulase	-
Protease	+
Gelatinase	-
Catalase	+
Oxidase	+

Table 2: Zones of inhibition of *A. niger* by organic extracts of culture supernatant

Solvent	**Zone of inhibition for *A. niger* (mm)**
Methanol extract	27
Chloroform extract	24
Ethyl acetate extract	19