

IN VITRO ANTIMICROBIAL ACTIVITY AND MOLECULAR CHARACTERIZATION OF *BACILLUS AMYLOLIQUEFACIENS* ISOLATED FROM SIMLIPAL BIOSPHERE RESERVE, ODISHA, INDIAIPSITA DAS¹, MRUNMAYA KU PANDA¹, CHANDI C.RATH^{2*}¹Department of Botany, Microbial Biotechnology Laboratory, North Orissa University, Baripada, Odisha, India. ²Department of Life Sciences, Rama Devi Women's University, Bhubaneswar, Odisha, India. *Email: chandicharanrath@yahoo.com

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ABSTRACT

Objective: The purpose of our study was to isolate and identify the bacteriocinogenic strain exhibiting broad range antimicrobial activity and to analyze the effect of different culturing conditions on the production of an antimicrobial metabolites isolated from the soil of Simlipal Biosphere Reserve, India.

Methods: In the current study, bacterial strains were screened for antimicrobial activity from soil samples of five different regions. The effect of varying culture conditions such as pH, incubation period, and temperature along with carbon and nitrogen sources with and without certain salts was studied. The characterization of the potent strain was studied by morphological, biochemical, and 16S rRNA genetic sequencing. A phylogenetic affiliation of the strain was studied.

Results: A total of 31 out of 245 strains isolated from soil were screened on the basis of antimicrobial results against the test pathogens. On the basis of bacteriocin-like inhibition studies method, one potential isolate that exhibited the highest inhibition against all the pathogens was selected. The optimization of highest antimicrobial metabolite production by the isolate with the influence of physical parameters was found as the incubation period of 3 days with 37°C temperature at pH 8 and for the chemical parameters dextrose was showed the most effective carbon sources when implemented with salts and yeast extract as the best sources of nitrogen with salts. The crude metabolite showed an absorbance peak value of 1.234 with optimum λ -max at 214 nm. The potent isolate showed maximum identity with *Bacillus amyloliquefaciens* (99% similarity) with highest query coverage on basic local alignment search tool search analysis of the 16S rDNA sequence. Phylogenetic analysis revealed close affiliation of the isolate with *B. amyloliquefaciens* (KC494392.1) having antimicrobial activity.

Conclusion: The findings revealed that the incubation period, temperature, pH, and the culture medium have a direct influence on the production of metabolites. These parameters can be modified for the improvement of the fermentation process.

Keywords: *Bacillus amyloliquefaciens*, Secondary metabolites, Antimicrobial activity, 16S rDNA sequencing, Phylogenetic study.

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INTRODUCTION

Rapidly rising strains of bacteria resistant to most advanced antibiotics have been an important public health concern. In response to the critical need for new drugs, for treatment of various infections and infectious diseases of humans and animals very few new antibiotics are being developed. There is a dire need to produce a remarkable range of antibiotics to solve the growing health problems in response to microbial resistance by the pharmaceutical industry [1]. Microbes are seen as a potential source for therapeutic uses with unlimited novel chemical structures [2], and they have the potentiality to grow in unique and extreme habitats, with the capability to produce unique and unusual metabolites. Many of these metabolites are believed to have certain chemicals to defend the microbes that compete for their survival [3]. Although a myriad of compounds with antimicrobial activity had been reported from various microorganisms over the decades, a few of them have determined to be clinically beneficial [4]. Till now, only 1% of the microorganisms had been studied while the advances in culturing techniques for microbes and extraction of their genes from various habitats have given scope for accessing an untapped reservoir of genetical and Metabolical diversity [5]. A large part of earth's surface is covered with soil. A large part of earth's surface is covered with soil. It is the natural habitat for all organisms and is the center of important ecological processes. It is a major reservoir of microbial diversity due to its extreme complexity, a result of multiple interacting parameters such as shape, size, texture, structure, porosity, pH, climatic variations, and biotic activity.

Bacteria are adaptive to diverse soil environments, and they produce secondary metabolites due to ecological stress. These secondary metabolites are invoked as special chemotherapeutic agents for lethal or inhibitory effects on other microbes in therapeutic concentrations with little or no toxic effect [6-7]. The temporal nature of secondary metabolite formation is genetic, but its expression can be influenced by manipulating different environmental parameters. The synthesis of antimicrobials is sought on by adding an inducer or by a decreasing the growth rate or by exhausting a nutrient [8].

The production of the antimicrobial metabolite depends on the basal medium required for their optimum growth, various concentrations of different nutrients in the medium, temperature, and pH [9]. Carbon and nitrogen are the important nutrients required for bacterial growth and enhancing the antimicrobial metabolite production. Continuous studies on the effect of the carbon and nitrogen sources for improved production of antimicrobial metabolites have been the subject by various industry and research groups for synthesis of newer drugs [10].

Thus, the objective of the study was to isolate a *Bacillus* spp. having antimicrobial activity from the soils of Simlipal Biosphere Reserve and to explore the influence of the physical and chemical conditions on the culture medium on biosynthesis of bioactive molecules.

METHODS

Chemicals

The chemicals and media used in the current study were procured from HiMedia, Mumbai and Sigma-Aldrich, USA.

Bacterial strains and growth conditions

All the test pathogens (*Escherichia coli*, Enteropathogenic *E. coli*, *Bacillus sp.*, *Shigella dysenteriae*, *Staphylococcus aureus*, *Vibrio cholerae* 0139, and *V. cholerae* (Inaba) and four *Candida* species *Candida albicans*, *Candida tropicalis*, *Candida parasilopsis*, and *Candida glabrata*) were procured from the Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India used in the current study. All bacteria were cultured in nutrient broth (NB) at 37°C for 16–18 h.

Sample collection and bacteriological analysis for source organism

Composite soil samples were collected randomly from each plot collected from different sites of Simlipal Biosphere Reserve, India, according to the method of Baruah and Barthakur, [11]. 1 g of soil was mixed with 9 ml of autoclaved distilled water, homogenized using a vortex mixture and serially diluted with autoclaved water. Subsequently, 100 µl of each dilution was spread onto Nutrient Agar (NA) agar plate (Hi-media), and the plates were incubated at 37°C for 24 h. Well-isolated colonies were inoculated into fresh nutrient broth for stock preparation. Individual bacterial isolates were sub-cultured on NA medium to obtain pure culture and preserved at 4°C for further characterization. It was one of the isolates obtained by culturable method following dilution plate technique on NA medium. Further, the isolates were identified. The isolate was identified by series of morphological and biochemical characteristics up to generic level using Bergey's Manual of Determinative Bacteriology. Species confirmation of the isolate was carried out by 16S rDNA sequence analysis.

Determination of antimicrobial activity

The bacterium was cultured in NB medium and incubated at 37°C, for 24 h. Preliminary antimicrobial activity was examined by spot inoculation and by disk diffusion method (Bauer *et al.*) [12] against *S. aureus*, *Bacillus subtilis*, *Shigella dysenteriae*, *V. cholerae* (Inaba), *C. albicans*, and *E. coli*. All the test pathogens used in the study were procured from the MTCC, IMTECH Chandigarh, India.

Secondary screening by bacteriocin-like inhibition studies (BLIS) assay

The isolate showed positive antimicrobial activity both by disc and spot inoculation methods and was again subjected to secondary screening by BLIS assay. An overnight culture of the isolates was taken, and BLIS activity was done by the diametric-streak technique of Mayr-Harting *et al.*, [13]. The test isolate was lawn cultured (1 inch) at the center of the NA plate by the help of a sterile cotton swab and incubated at 37°C, for 24 h. After the incubation period, the bacterial mass was swept out from the plate aseptically, and the plate was exposed to chloroform vapour for 40 min followed by exposure to ultraviolet (UV) radiation for 1 h, to ensure the death of the cells. Then, freshly grown cultures of the test pathogens were streaked across the plates and incubated at 37°C for 24 h. Plates were observed for growth on either side of the lawn, and no growth at the center represents potent antimicrobial activity by the isolate against the test pathogens.

Statistical analysis

The evaluation of results was done by taking mean data of two independent experiments with three replicates of different characterization studies. Correlation analysis was done to find out the linear association and to compare the factor level difference among the variables. All the analysis was carried out using SPSS software for windows release 19.0 version (SPSS Inc., IBM, New York, USA).

Extraction of crude metabolites and antimicrobial study

The isolate was cultured in the conditions that yield maximum metabolites. The filtrate which is cell-free (500 ml) was neutralized

with 1 N HCl, and resultant filtrate was extracted with an equal volume (500 ml) of ethyl acetate thrice by vigorously shaking in a separating funnel. The ethyl acetate extracts were combined dried to obtain the crude extract. The antibacterial study was determined by the paper disk diffusion assay method [14].

Metabolites determination by UV Analysis

The λ-max in ethyl acetate of the crude metabolites was scanned using a UV spectrophotometer (Specord AU-2701, Systronics). For the presence of bioactive components at wavelength ranging from 190 nm to 550 nm, and peak absorbance values were determined.

Bioassay of the antimicrobial properties of the bioactive compounds of the isolate

The selected isolate was incubated in a broth medium with required nutrients and was incubated at the rotary shaker at 120 rpm, and the compounds present in the broth was extracted by adapting liquid extraction method using ethyl acetate. The solvent portion was collected and concentrated by evaporation. The extract was then tested for the antimicrobial assay by agar well diffusion method, and the result was recorded by measuring the zone of inhibition produced.

PCR amplification and 16Sr DNA sequencing

Sequencing of 16S rDNA of the isolate and amplification of the target gene was done using Big Dye Chemistry and performed as per the manufacturer's protocols (Applied Biosystems, USA). Universal bacterial primer 1492R (5'- TAC GGY TAC CTT GTT ACG ACT T-3') and the domain bacteria-specific primer 27F (5'- AGA GTT TGA TCM TGG CTC AG-3') were used for 16S rDNA amplification. The PCR product was purified using QIA quick PCR purification kit (Qiagen). Purified 16S rDNA was sequenced partially using ABI PRISM big dye terminator cycle sequence reading reaction kit (Applied Biosystems) under the following conditions: Initial denaturation at 94°C for 5 min; 30 cycles of denaturation at 94°C for 45 s, annealing at 48°C for 45 s, extension at 72°C for 90 s, and a final extension at 72°C for 5 min. The purified sequencing reaction mixtures were electrophoresed using an Applied Biosystems model 310 automatic DNA sequencer (Perkin Elmer, Massachusetts USA). The sequence was annotated and submitted to GenBank.

Homology search and phylogenetic analysis

The 16S rDNA sequence of the isolate was been compared with the non-redundant databases in all GenBank + EMBL + DDBJ + PDB sequences (but no EST, STS, GSS, environmental samples or phase 0, 1, or 2 HTGS sequences) using the program Basic Local Alignment Search Tool(N) by selecting the optimization parameter to highly similar sequences (Megablast). The phylogenetic study was conducted by considering 16S rDNA sequences of 20 bacterial isolates with antibacterial properties. The tree was generated by the neighbour joining method using MEGA 6.0 [15].

RESULTS AND DISCUSSION

Isolation and identification of strain

It is of interest to isolate the *in vitro* antagonistic potential of natural isolate against several pathogenic microorganisms. This study revealed the presence of a thermostable and barostable antimicrobial metabolite in the isolated bacterial strain. In our study, soil samples collected from selected sites of Simlipal Biosphere Reserve, Odisha, India, were screened for bacteria with antimicrobial activity against test pathogens. The bacterial strains that showed antagonistic activity on spread plate were isolated and tested by Petri plate assay. The strain (B5) exhibited good antimicrobial activity against selected Gram-positive, Gram-negative, and *Candida* strains. The Gram staining of the strain revealed it as Gram-positive, aerobic, motile, and rod-shaped as shown in Fig. 1 and spores were found in the later period, which was mid-born and Elliptical.

The morphological analysis of strain B5 showed the colony as milky white, opaque with smooth, and moist surface on NA plates adhering the medium tightly. Its colony surface becomes hilly and wrinkled in a later period, and its border gets transformed into a smooth white

biofilm layer when cultured in liquid medium. The morphology of the strain was pinkish on HiCrome *Bacillus* Agar (Fig. 2). These studies suggested that strain B5 was *Bacillus* sp. and based on 16S rRNA gene sequence analysis, strain B5 was most likely *Bacillus amyloliquefaciens*.

The biochemical and physiological analysis of the strain showed the similarity of percentage identity with *B. subtilis*, which estimates the close proximity of the strain corresponding to the taxon relative to all other microorganisms. For further confirmation of the strain, 16S rDNA sequencing was done. The sequencing of the strain showed the maximum identity value of 99% with query coverage 99% and maximum score of 2667 with *B. amyloliquefaciens* (Fig. 3).

The phylogenetic analysis was conducted in MEGA6 [15], and the neighbour joining method was used to infer the evolutionary history [16]. The maximum composite likelihood method was followed to compute the evolutionary distances [17]. The analysis involved 20 nucleotide sequences. The rate variation among sites was modeled with a gamma distribution (shape parameter=0.2). The selected isolate *B. amyloliquefaciens** (KM384034.1) clustered with *B. amyloliquefaciens* (KC494392.1) supported by bootstrap 67. Therefore, the isolated strain was confirmed as *B. amyloliquefaciens* based on the results of the morphological, biochemical, and 16S rDNA sequence analysis along with the phylogenetical study.

Antimicrobial activity

A total of 245 isolates showing a zone of inhibition around them were selected from the soil samples by crowded plate techniques and were preliminarily screened by disk diffusion and spot inoculation method for antimicrobial activity against test pathogens. About 31 isolates showed a zone of inhibition in both the methods followed they were then subjected to the BLIS method. This potent isolate B5 was found to exhibit good activity against the entire pathogens. A clear zone in the center of the NA plates with pathogens on both sides as shown in Fig. 4 indicated the antimicrobial potential of the isolate.

We studied the effects on the antimicrobial metabolite production by the strain B5 isolated from the soils of Simlipal Biosphere Reserve,

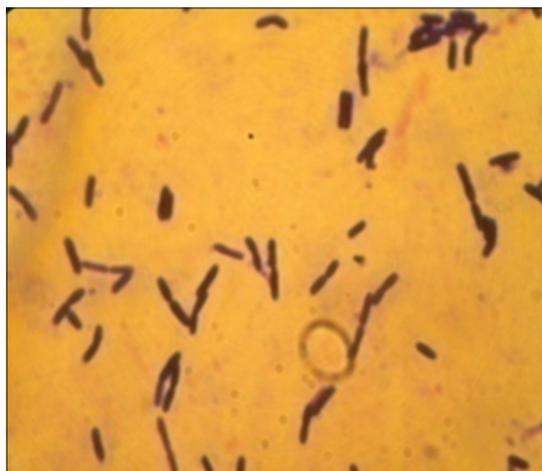


Fig. 1: Microscopic structure of B5



Fig. 2: (a) Isolate B5 on slant (b) Isolate on B5 NA plate (c) Isolate B5 on HiCrome *Bacillus* Agar medium

Odisha, India. Under different culturing conditions with a different array of species-specific features of varied physical and chemical factors were taken for developing efficient fermentation processes for the production of secondary metabolites in optimum media. Carbohydrate and nitrogen are essential for different structural and energy compounds in cells. Thus, various carbon and nitrogen sources along with certain salts as precursors were used in our study for determining the optimal medium for antimicrobial metabolite production by the strain B5. Different carbon and nitrogen sources at a concentration of 0.5 g/100 ml were added to the production media. A high degree of variation in the production of antimicrobial activity was found when different carbon and nitrogen sources were tested in the medium. The results showed that the most effective fermentative medium is with carbon sources as dextrose and the best source of nitrogen as yeast extract along with the addition of certain salts as precursors (Figs. 5 and 6) as compared the media without salts (Figs. 7 and 8).

Nutrients play a key role in the onset and intensification of secondary metabolites production in microorganisms. The results of our study showed a direct influence of incubation time, temperature, and the

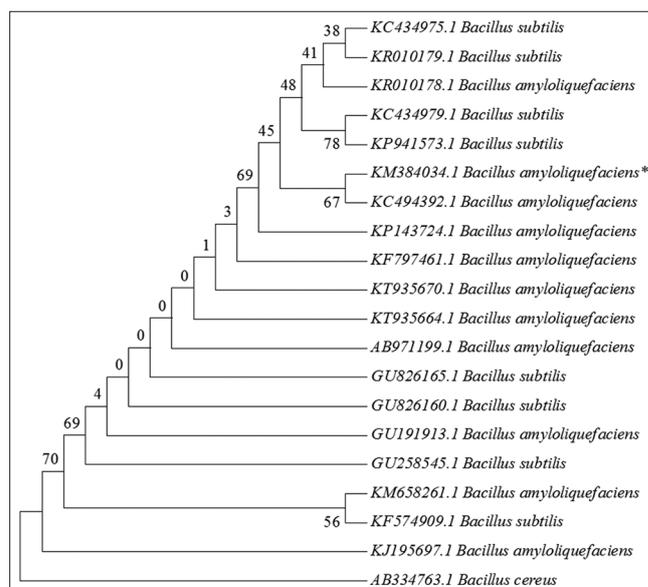


Fig.3: Phylogenetic analysis: Tree generated by neighbour joining method showed that the selected isolate clustered with *Bacillus amyloliquefaciens* (KC494392.1) supported by bootstrap 67



Fig. 4: Antagonistic activity of isolate B5 against different human pathogens by Bacteriocin Like Inhibition Studies method

culturing medium on the production of secondary metabolites. These parameters can be modified for the optimizing and improving the fermentation process for increasing the production of the compound of interest. Agar diffusion tests were done to study the antimicrobial activity of the culture filtrates under optimized conditions and for monitoring the time course for the production of the antimicrobial substances in batch culture, usually, in *Bacillus*, the time of antibiotic activity varies in between 24 h and 72 h of incubation. The time at which the maximum antibiotic activity occurs changes depending on the particular species of *Bacillus* as different species have different metabolic pathways [18]. The bacterial biomass and antibiotic activity of our isolate reached maximal levels in the medium containing dextrose with salts. The highest zone of inhibition was approximately 18 mm. Studies that have investigated the effects of different concentrations of carbon and nitrogen sources on the activity of antibiotics show different results. The results usually depend on the particular type of microorganism used and its interaction with components of the medium, as differences in biosynthetic pathways can affect the activity of antibiotics. The change in pH affects many cellular processes as in the regulating the biosynthesis of secondary metabolites. The effect of pH on the antibiotic activity of our isolate was studied by adjusting the initial pH range (4.0–12.0) of the active medium. The optimal pH for maximal antibiotic activity from *Bacillus* sp. B5 was 8.0, and the zone of inhibition was 19 mm at this pH (Fig. 9).

The calibration for λ -max of the crude extract dissolved in ethyl acetate was studied. The scanning of the metabolite was done at a wavelength ranging from 190 nm to 550 nm. The metabolites showed two peaks, but the optimum λ -max was shown at 214 nm with an absorbance value of 1.589 (Fig. 10).

B. amyloliquefaciens is a species of the genus *Bacillus* was discovered in soil 1943 [19]. The genomics comparison of *B. amyloliquefaciens* reveals it to be closely related to *B. subtilis* with the similarity of 50% genes in the whole genome [20] and also as a synthesizer of several CLPs, such as iturins. [21]. The crude metabolites produced by the isolate B5 showed strong activity against a variety of Gram-positive bacteria, yeasts by agar diffusion test. The multifunctional process of antibiotics biosynthesis was demonstrated to be in control of limiting nutrients, which affects the metabolism. However in this present study addition of certain salts as precursors to the culture media increased the metabolite production and enhanced antimicrobial activity.

DISCUSSION

In our present study for antimicrobial metabolites producing microorganisms, an efficient antagonistic soil microorganism, B5, isolated from Simlipal Biosphere Reserve and identified as *Bacillus amyloliquefaciens*. It exhibited remarkable antagonistic activity against different pathogenic test organisms. This species of *Bacillus* is an aerobic spore former found commonly in soil, groundwater, plants and animals during harvest or slaughter [22]. Studies based on the past decade analysis on antibiotic screening revealed most members of the genus *Bacillus* to have a contribution in the search for new antibiotics [23]. Different kinds of antibiotics that are found to be effective in suppressing the growth of target pathogens are being produced by many *Bacillus* spp. [24-25]. Similar results have also been reported by Pannapa and Pattra [26]. The antagonistic activity of the selected isolate B5 against several pathogens can be attributed due to the antibiotic effect. In this work, the composition of various carbon and nitrogen sources along with certain salts as precursors and environmental conditions such as pH and temperature influence

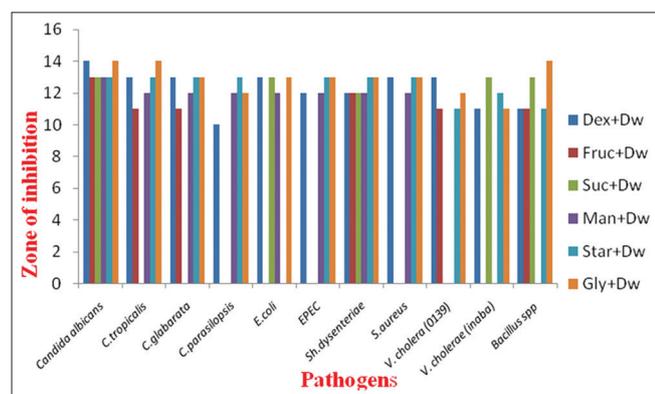


Fig. 5: Effect of different carbon sources (0.5 g/100 ml) on the activity of secondary metabolite of isolateB5 without salts

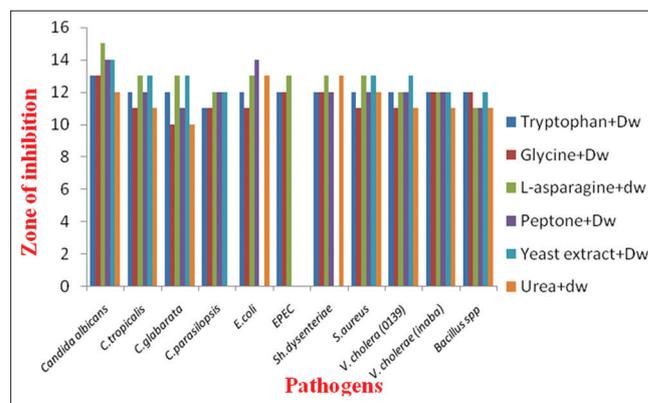


Fig. 7: Effect of different nitrogen sources (0.5 g/100 ml) on the activity of secondary metabolite of isolateB5 without salts

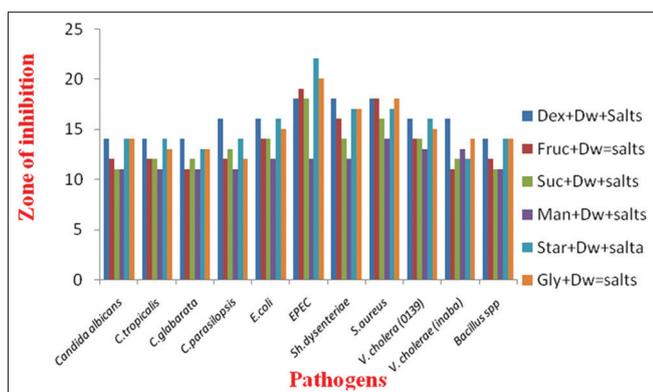


Fig. 6: Effect of different carbon sources (0.5 g/100 ml) on the activity of secondary metabolite of isolateB5 with salts

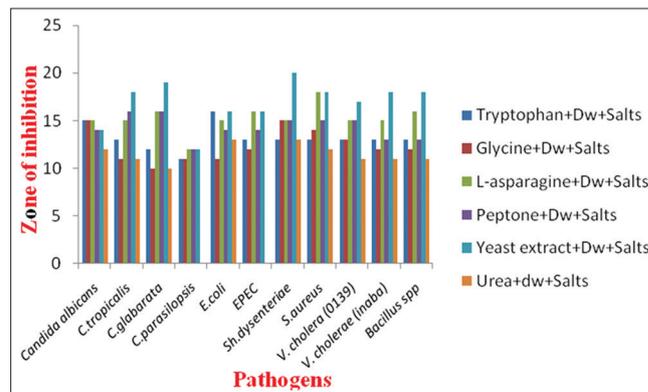


Fig. 8: Effect of different nitrogen sources (0.5 g/100 ml) on the activity of secondary metabolite of isolateB5 with salts

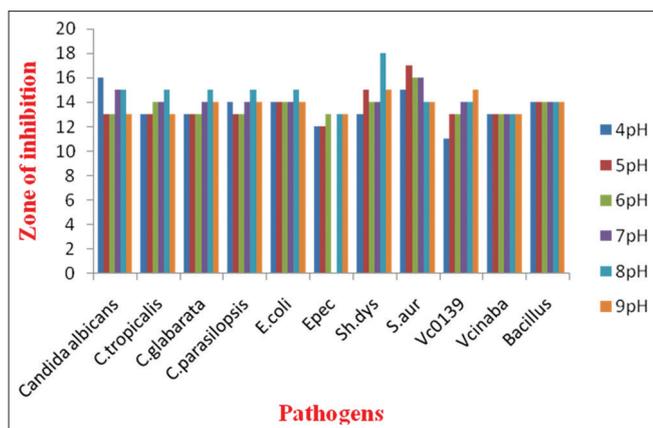


Fig. 9: Effect of pH on the activity of antimicrobial metabolite produced by isolates B5

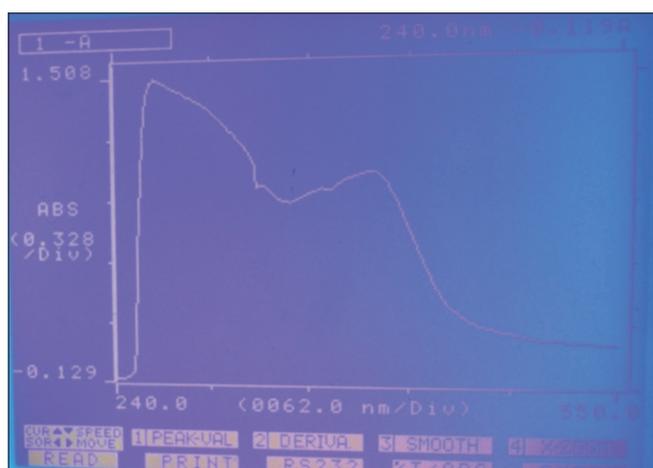


Fig. 10: Ultraviolet k-max in ethyl acetate of the crude metabolites produced by strain B5 (ABS: 1.234 at 214 nm)

isolate B5 for use in the production of antibiotic substances with antimicrobial activity. In the present study, dextrose (0.5%) and yeast extract (0.5%) along with certain salts used as a fermentative medium showed the potential activity of the antimicrobial metabolite produced by the isolate. The pH along with the incubation period is the critical factors for microbial growth and metabolic biosynthesis, which affect antibiotic production. The present study revealed 8.0 and 37°C as the optimum pH and temperature for production of antibiotic substances from isolate B5. The ethyl acetate extract of the isolate B5 significantly induced the inhibition of growth of test pathogenic microbes used in the study. In batch culture, the growth phase (trophophase) is followed by a production phase (idiophase) in the production of antibiotics [27]. The potent isolate (B5) seems to produce the antimicrobial substances in a fair amount in the culture broth. Under the conditions studied, the active substance is found to be accumulating late in the growth cycle, i.e., (in stationary phase), in liquid media reaching a maximum at 72 h. Naclerio *et al.* [28] had reported the isolation of antibiotics of *Bacillus* spp. from the culture filtrates. The time required for fermentation for maximum yield of the antimicrobial metabolites production is different among different bacterial strains [9,29-32]. The antimicrobial spectrum of the active substances isolated from culture filtrate B5, determined by agar diffusion method, exhibited strong activity against a variety of Gram-positive bacteria, and yeasts, whereas less activity was detected against tested Gram-negative bacteria. El-Banna [33] had reported that the antimicrobial spectra of the active substances isolated from culture filtrates of *Bacillus* spp. such as *B. cereus* NB-4, *B. cereus* NB-5, and *B. circulans* NB-7 to have exhibited potent antifungal activity against filamentous fungi and yeasts. Carbon source plays an important role

as a source of precursors and energies for the synthesis of biomass building blocks and secondary metabolite production [34,35]. The influences of medium components and environmental conditions are an initial and important step to improve the metabolite production of the genus *Bacillus*. Hence, the role of different carbon and nitrogen sources was studied for their influence and antibiotic production by this *Bacillus* strain. The results indicated that the production of the antimicrobial compound was maximum in the medium containing dextrose as the carbon source with salts. El-Banna [36] reported that glycerol and fructose were the best carbon sources for antimicrobial substances production. In our study on B5, lactose, maltose, sucrose, fructose, starch, glucose, and galactose showed less effect on antibiotic production. Several examples of secondary metabolites are reported to be suppressed by the presence of the carbon source. Glucose and ribose as carbon sources have been reported to interfere with the synthesis of secondary metabolites [37]. The antimicrobial activity of strain B5 was greatly influenced by nitrogen sources. Vahidi *et al.* [38] reported a high level of antifungal activity of *Mycena leptoccephala* when yeast extract was used as a nitrogen source and lower antifungal activity when NH_4Cl and NaNO_3 were used as nitrogen sources. The antimicrobial metabolite synthesis is often repressed by nutrients that favours rapid cellular growth, such as glucose (catabolite repression) and ammonium ions (nitrogen repression). However, when these nutrients level is low, the cellular growth rate is slowed, and the synthesis is decompressed [39]. The antimicrobial substance produced from any *Bacillus* species is believed to have the potency of an antimicrobial agent [40]. The strain B5 is found to be potent and effective against different test-organisms studied *in vitro*. Further, research is required in determining additional characteristics regarding purification, characterization, and identification of the active antimicrobial compound for it to become a good potent in pharmaceutical and biotechnological approaches.

CONCLUSION

Development of multiple drug resistance among pathogens is of global concern today. There is a constant pressure mounting among the researchers and academicians for the search of new antimicrobial compounds from microorganisms as well as from medicinal and aromatic plants. Although it is a preliminary endeavor, here we report the bacterial isolates of selected soil samples collected from soil samples of Simlipal Biosphere Reserve, producing antimicrobial compounds through both primary and secondary methods. Further, characterization would lead an alternate for development/discovery of newer antimicrobials and studies such as this are a prerequisite for tapping the biotechnological potential of these microorganisms.

AUTHORS' CONTRIBUTIONS

The first author performed the procedure, data collection, and analysis, manuscript preparation. The second author contributed toward statistical and phylogenetical analysis, and the last author contributed in study design and concept development. All the authors discussed and contributed to the final manuscript.

CONFLICTS OF INTEREST

We declare that we have no conflicts of interest.

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