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Antimycotic Potential of Bacterial Ethanolic Extract Isolated from Acid Soil of Mahishapat, Odisha

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In view of the large tract of acid soil in India, an attempt was made in the present investigation to study the antimicrobial potential of bacterial flora from acid soil of Mahishapat, Dhenkanal, Odisha. Four bacteria isolated from the soil sample (pH 5.3 ± 0.10) showed wide range of pH tolerance (pH 5.0 to 8.0) were maintained and selected for further study. After biochemical characterizations of the four isolates were identified as Gram positive rods viz., *Bacillus subtilis*, *Bacillus brevis*, *Bacillus azotofermans* and *Bacillus licheniformis*. Two of the isolates, *Bacillus subtilis* and *Bacillus azotofermans* were found to have antimycotic activities against dermatophytes like on *Candida albicans* MTCC No.854 and *Cryptococcus neoformans* MTCC No. 1347 respectively. When the organism showed an inhibition zone of 22 mm and 17 mm while the ethanol extract showed 26mm and 21 mm against the test pathogens respectively. It was larger than the medicines recommended by physicians.

Key words: Acid soil, Bacteria, Ethanol extract, Antimycotic activity, Dermatophytes.

Acid soil is a matter of great concern not only in India but also on global basis. Out of 157 million hectors of cultivable land in India, 49 million hectors of land are acidic, of which 26 million hectares of land having soil pH less than 5.6 and rest 23 million hectares of land having soil pH range of 5.6 to 6.5. In Odisha, acid soil is estimated to be 12.5 Mha, about 80% of the total geographical area ranging between a pH of 4.5 to 5.5. Soil pH not only affects chemical, physical properties and soil microbial diversity, but also reduces crop productivity[1]. Soil pH is probably at least as important as soil C and N concentration in influencing the microbial biomass, which has acclimatized in such area, can have some novel properties[2]. Acidophilic bacteria being nitrogen reducer or sulphur reducer may have some antimycotic activity[3].

Antibiotics are low molecular-weight (non-protein) molecules produced as secondary metabolites, mainly by microorganisms that live in the soil[4]. The large hitherto unexplored reservoir of microbial flora is being tapped as a source of various natural products[5]. Several bacterial species produce antibiotics, mainly polypeptides or active metabolites from *Bacillus* sp. that are active against gram positive pathogens[6], yeast and mold[7]. In view of this an attempt has been made in the present investigation to study antimycotic activities of bacteria and ethanolic extract of bacteria isolated from the lateritic group acid soil of Dhenkanal as well.

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MATERIALS AND METHODS

Soil sampling

The soil sample was collected from Mahishapat, Dhenkanal at an elevation of 80 meters (20° 67' N latitude, 85° 6' E longitude). The parents of the soil are mainly the mountain with the river Bramhani as the main water supplying body. Soil sampling was done from the field ploughed for the cultivation of sugarcane during early spring season. The crop field at Mahishapat is significant being acidic in nature (pH 5.0-5.5). Top 0–15 cm of soil was collected aseptically, covered with plastic bags. Then air dried for 48 hrs and powdered for soil pH measurement. 10gms soil sample was mixed with double distilled water (soil: water = 1:2 w/v).

Isolation

The bacteria were isolated by dilution plate technique using HiVeg Nutrient Agar(NA) [8]. The colonies were identified following Bergey's Manual of Systematic Bacteriology.

Acid Tolerance

Each organism was inoculated in test tubes containing broth with varying pH from 2.0 to 12.0 for 24hrs and growth was observed after incubation for 24hrs. Four out of 10 isolates showing tolerance to pH 5.0 to 8.0 were taken for antimycotic potential.

Microscopic examination

Gram staining

A loopful of bacterial suspension (young culture) was used for Gram staining of the isolates.

Physiological and biochemical profile

Various physiological and biochemical characters of the organisms were checked following the standard methods for identification of the isolates[9].

NaCl tolerance

Growth of the organisms on NA medium supplemented with 1 to 11% NaCl was checked. Highly diluted suspensions of the organisms were spot inoculated on the plates, and after incubation for 72 hrs, growth was recorded.

Catalase test

One drop of 30% hydrogen peroxide was placed on a slide. One loop full of fresh bacterial culture was taken by a sterile needle and placed on the drop of hydrogen peroxide previously taken on the slide. Bubble production indicates positive result.

Urease test

Urease splits urea in to ammonia and carbon dioxide, again was detected by inoculating the bacterial cultures in to tubes containing urease broth having filter sterilized urea, and incubated for 72 hrs. Purplish pink coloration of the medium indicates a positive reaction.

Indole production test

One loop full of fresh bacterial culture (24 hrs old) was inoculated in tryptone broth and incubated at 37°C for one to three days. After incubation, Kovac's reagent was added(1:1 v/v) and shaken vigorously for 1 minute. A pink ring at the interface of two solutions indicates positive result.

Nitrate reduction test

Nitrate reduction test was carried out in nitrate broth(nb). The freshly prepared cultures were incubated in sterile nb containing tubes and incubated at 37°C for 24 hrs. After incubation sulphanillic acid and napthyl amine mixture (1:1 v/v) was added. The appearance of deep pink color showed that bacterial isolates reduced nitrate to nitrite.

Voges Proskauer(VP) test

VP test carried out in one ml of fresh bacterial culture grown in MRVP broth. After incubation, a mixed solution of α-napthol and potassium hydroxide were added. Development of crimson red color indicating acetyl methyl carbinol was present and the organism is positive for VP test.

Hydrolysis of starch

Hydrolysis of starch was carried out by taking 1% soluble starch on NA plates and spot inoculating the bacterial culture over that plate. After incubation for 24 hrs at 30°C, the plates were flooded with iodine solution for 5 minutes. Hydrolysis of starch was indicated by a clear zone around the growth and unchanged starch gave a blue color.

Cultural revive of fungal pathogens

On the basis of the availability the human fungal pathogens i.e. human dermatophytes were maintained in Sabouraud Dextrose Agar(SDA) slants by periodic subculturing. The revive culture done on *Candida albicans* MTCC 854 and *Cryptococcus neoformans* MTCC 1347.

Colony characteristics

C. albicans MTCC 854 grows readily

(24 to 48 hrs) at both room and incubator temperatures on SDA. The colonies are of moderate size, smooth, pasty and have a characteristics yeasty odour with small, oval, budding like fungus in morphology. While *C. neoformans* MTCC 1347 have white mucoid, glistening colonies with round yeast like cells, thin walled, oval to spherical, budding cell, surrounded by wide gelatinous capsule morphologically.

Evaluation of Antimycotic activity

The efficacy of the bacteria against fungal pathogens was studied by disc diffusion and agar well diffusion method. Each of the fungal pathogens were seeded over SDA plates as a lawn culture. The sterile filter paper discs dipped in bacterial broth were placed on the plates as 1 disc per plate likewise with the help of a cork borer well was made on the plate and ethanolic extract were loaded on the plate. These plates were incubated at 28ºC for 48 hrs. The inhibition zone diameter was measured. Antifungal activities of the isolates were measured in terms of percent inhibition (P.I.) of fungal growth.

$$P.I. = \frac{C - T}{C} \; x \; 100$$

where 'C' is the diameter of fungal growth in control plate and 'T' is diameter of fungal growth in test plate[10,11]. The total P.I.(TPI) can be calculated from the below formula

$$[TPI = 100 - P.I.]$$

Study of antifungal antibiotic discs on fungal pathogens

The relative efficacy of some commonly used antifungal antibiotics was studied. The antifungal discs used are Clotrimaoxazole, Fluconazole, Ketconazole, and Itraconazole. Using a sterile swab, the culture of each organism was seeded over each SDA plate. The antibiotic discs were placed on SDA plates as 3 discs on each plate. These plates were incubated at 28ºC for 48 hrs. The diameter of the inhibition zone was recorded as indication of antifungal activity.

Extraction of antifungal compounds

For the isolation of antifungal compounds the bacteria were cultured in NA slants 24hrs prior to transferred to synthetic medium containing 20g of Hiveg-Bacto dextrose, 5g of L-Glutamic acid (HiMedia, India), 1.02g of $MgSO_4$ $7H_2O$, 1.0g of K_2HPO_4, 0.5g of KCl and 1ml of Trace element solution(0.5g of $MnSO_4$, 0.16g of $CuSO_4$ $5H_2O$ and 0.015g of $FeSO_4$ $7H_2O$ in 100ml distilled water) per liter of sterile distilled water[12]. The pH maintained at 6.0-6.5. The culture was grown in batch culture in 500ml conical flask contain 200 ml of the medium and It was incubated for 3days in a shaker incubator (Satyam Ltd., India) with 150rpm at 31°C.

After incubation the supernatant was obtained by centrifuging the culture at 14500 g for 27 mins at 4°C. The supernatant were precipitated by standard acid precipitation method followed by centrifuging at 14500 g for 15 mins. The pellet containing the active fraction were suspended in absolute ethanol (1:4 w/v)(Hong Yang Chem. Cropn., China) and air dried at room temperature for 24 hrs. The crude bacterial ethanolic extract pronounced as Bext-e was further tested for its activity against dermatophytes. Bext-e stored at 4°C for further analysis.

RESULTS

Ten bacteria isolated from the soil were Gram positive spore forming rod shaped (Fig. 1 & 2). Out of 10 only 4 organisms showed high range pH tolerance between 5.0 to 8.0. These organisms were numbered as BBM-4, BBM -6, BBM -8 and BBM-9. Through physiological and biochemical identification they were identified as *B. subtilis*, *B. brevis*, *B. azotoformans* and *B. licheniformis* (Table 1). These isolates were bioassayed for antimycotic activity against human dermatophytes through disc diffusion and well diffusion method as well (Fig. 3 & 4). The Zone of inhibition (ZI) was measured in millimeter on culture plates through agar well diffusion method (Table 2). Commonly available medicine against the test pathogens were

Table 1. Organisms showed wide pH tolerance

Organisms	Identification (Physiologically and Biochemically)
BBM-4	*Bacillus subtilis*
BBM -6	*Bacillus brevis*
BBM -8	*Bacillus azotoformans*
BBM-9	*Bacillus licheniformis*

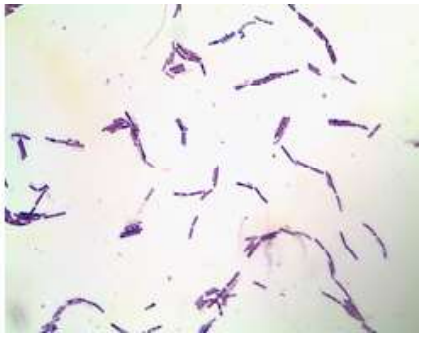

Fig. 1. Micrograph of *Bacillus subtilis*

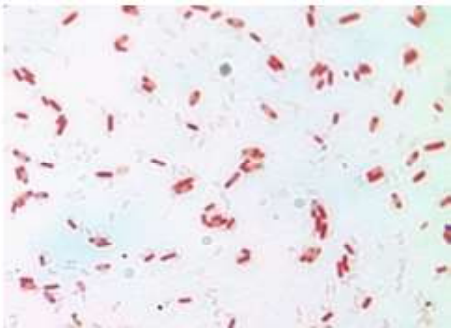

Fig. 2. Micrograph of *Bacillus azotoformans*

Fig. 3. *B. subtilis* against *C albicans*

Fig. 4. *B. azotoformans* against *C. neoformans*

Fig. 5. Antibiotics against *C. albicans*

Fig. 6. Antibiotics against *C. neoformans*

Fig. 7. Bext-e against *C. albicans*

Fig. 8. Bext-e against *C. neoformans*

Table 2. Zone of Inhibition (ZI) against Human Dermatophytes

Bacillus sp.	Diameter of ZI(in mm)	
	C. albicans MTCC 854	*C. neoformans* MTCC 1347
Subtilis	22	-
Brevis	-	-
Azotoformans	-	17
Licheniformis	-	-

Table 3. Inhibition of test dermatophytes against antibiotics

Antibiotic discs	ZI of *C. albicans* MTCC 854	ZI of *C. neoformans* MTCC 1347
Clotrimaoxazole	-	-
Ketconazole	13	14
Itraconazole	-	9

Table 4. Percentage of inhibition by bacterial isolates, antibiotics and Bext-e

Human dermatophytes	Percentage of Inhibition(%)		
	Bacterial isolates	Antibiotics	Bext-e
Candida albicans MTCC 854	25.88	15.29	30.58
Cryptococcus neoformans MTCC 1347	20.00	16.47	24.70

also bioassayed (Table 3, Fig. 5 & 6). Presence of antimycotic substances was indicated in *B. subtilis* and *B. azotoformans* tends to collection of metabolites by standard precipitation method from cell free supernatant and its ethanolic extract. The ZI of Bext-e from *B. subtilis* was 26mm against *C. albicans* MTCC 854 is more than the antifungal medicines prescribed by physicians and cultural broth (Fig. 7). The ZI by Bext-e from *B. azotoformans* against *C. neoformans* MTCC 1347 was 21mm is more than the medicine and cultural broth (Fig. 8). The Total percentage of inhibition was determined. Bext-e of *B. subtilis* inhibited 30.58% growth of *C. albicans* MTCC 854 in comparison to 15.29% by medicine. *B. azotoformans* Bext-e inhibit the growth of *C. neoformans* MTCC 1347 by 24.7% against 16.47% by medicine (Table 4).

DISCUSSION

The genus *Bacillus* consists of a large number of diverse, aerobic, rod shaped gram positive endospore forming bacteria that are highly resistant to unfavorable environmental conditions. Many species of genus *Bacillus* synthesize relatively abundant antimicrobial compounds[13,14]. The potential of *B. subtilis* to produce antibiotics has been recognized for 50 years. Members of the genera *Bacillus*, *Streptomyces* and *Pseudomonas* are soil bacteria that produce a high proportion of agriculturally and medically important antibiotic[15,16]. Out of the ten gram positive rod shaped bacteria isolated from acid soil(pH 5.3 ± 0.10), four showed acid tolerance viz. 5.0 to 8.0 amongst *B. subtilis* and *B. azotoformans* showed good zone of inhibition against human dermatophytes. Antimicrobial substances production by *B. subtilis* is pH dependent[17]. Bioassay of *B. subtilis* Bext-e results ZI of 26 mm. against *C. albicans* MTCC 854. However, there is little or no information on antimicrobial substances produced by *B. azotoformans* and it is still in a primary stage of research. Bext-e of *B. azotoformans* showed ZI of 21mm. against *C. neoformans* MTCC 1347 in vitro. As reported in this investigation the Bext-e of both the organisms are better than medicines availed in market(Ketconazole and Itraconazole). Thus the presence of active constituent/(s) in ethanolic extract was clearly indicates which could be utilized further for treatment of dermal infections caused by *C. albicans*. and *C. neoformans* but only after conclusive extensive clinical trial.

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