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RESEARCH ARTICLE

Nutritional and environmental optimization of antifungal potential of *Bacillus* strains

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Abstract

An analysis of the number of pathogenic bacteria and fungi indicated that they become resistant to antibiotics in common use. These antibacterial and antifungal resistances are presently an urgent focus of research and new antibiotics are necessary to combat these pathogens. Members of the genus *Bacillus*, abundantly present in soil, have the ability to produce various antibiotics this can be used as antifungal compounds. The present study aimed to isolate bioactive *Bacillus* sp. against fungal pathogens from environmental conditions and optimized the cultural condition for prolific growth condition. Altogether five bioactive *Bacillus* strains were isolated from different environmental conditions of Jabalpur (M.P.). They all were screened for antagonistic activity against the pathogenic fungi such as *Aspergillus niger*, *Curvularia lunata*, *Alternaria solani*, *Fusarium solani* etc. After screening, a single strain of *Bacillus* showing remarkable antagonistic activity against tested fungal pathogens. This strain further identified as *Bacillus carotarum* on the basis of morphological and biochemical characteristics and by PIB computer kit. The effect of different temperature, pH, and various carbon and nitrogen sources on the bacterial growth in a fixed volume of culture broth was studied. The prolific growth was recorded at pH 9 after incubation at 37^0C for 24 hrs. Fructose (1%) and peptone (1%) were the best carbon and nitrogen sources respectively for optimum growth and production of active metabolites by the isolate. The results of the present investigation indicated that cultural conditions as well as physical factors greatly affected the growth and production of bioactive metabolites by *Bacillus* sp.

Introduction

Treatment of infectious diseases caused by pathogenic bacterial and fungal strains is one of the most traditional problems in the clinical field (Mehrgan *et al*., 2008; Fazly Bazzaz *et al*., 2005; Calvin, 1993). This necessity encouraged the investigators to synthesize novel and more potent inhibitory compounds against such pathogens (Emami *et al*., 2008; Shafiee *et al*., 2008). Screening of fungal and bacterial strains producing inhibitory compounds is the first step in the discovery of novel antibiotic compounds (Imada *et al*., 2007).

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The search for new, safer, broad-spectrum antifungal antibiotic with greater potency has been progressing slowly (Gupte *et al*., 2002). In the course of screening for new antibiotics, several research studies are currently oriented towards isolation of new microorganism's species from different soils and ecosystems (Mellouli *et al*., 2003; Errakhi *et al*., 2007). Within microorganisms, the *Bacillus* species are one of the largest sources of bioactive natural products. Various studies have confirmed that *Bacillus* species have a wide range of antimicrobial activities since they are used as antifungal (Milner *et al*., 1995), antibacterial (Yilmaz *et al*., 2006; Gupta,

2013), antiviral (Steller *et al*., 1999), antiameobocytic (Galvez *et al*., 1994) and antimycoplasma agents (Peypoux *et al*., 1999). These are often complex secondary metabolites with great potential for biotechnological and biopharmaceutical applications.

The gram-positive spore forming bacterium *Bacillus cereus* is a widely distributed organism in the environment and can be easily isolated from a variety of foods, including dairy products, meats, spices, and cereals. Many *Bacillus* sp. are known to suppress fungal growth in vitro by the production of one or more antifungal antibiotics (Katz and Demain, 1977). The potential of *Bacillus* sp. to synthesize a wide variety of metabolites with antibacterial and antifungal activity has been intensively exploited in medicine and industry (McKeen *et al*., 1986; Silo-suh *et al*., 1994; Leifert *et al*., 1995).

Biosynthesis of antibiotics from microorganisms is often regulated by nutritional and environmental factors. It was reported that antimicrobial substances produced by bacterial species were greatly influenced by variation of carbon sources (El-Banna, 2006). Several abiotic factors, such as pH and temperature, have been identified as having an influence on antibiotic production from bacteria (Raaijmakers, 2002). Therefore, the present study was undertaken to screen a number of *Bacillus* strains isolated from the various environmental conditions of Jabalpur for their antifungal potential.

Materials and methods:

Test pathogens:

Pathogenic fungal strains used in the present study were *Aspergillus niger* (MTCC 404), *Curvularia lunata* (MTCC 2030), *Alternaria solani* (MTCC 2101) and *Fusarium solani* (MTCC 3004). All the strains were procured from Microbial Type Culture Collection (MTCC), IMTECH, Chandigarh, India.

Sampling and Isolation:

Various soil samples were collected from different locations of Jabalpur (M.P.) and to isolate strains of *Bacillus* sp. by standard Heat Enrichment method (Chan *et al*., 2007).

Screening:

The 5 isolated *Bacillus* strains were screened for their antifungal activity against test pathogens by Lawn spot technique (Edward and Richard, 2003). The cell free extract of the *Bacillus* strain with the maximum inhibition of test pathogens was prepared by centrifugation of overnight cultures grown in Luria broth at 10,000rpm for 10min and subsequent microfiltration by using 0.2µ nitrocellulose filter paper. This cell free extract was screened for antifungal activity by Agar well diffusion method (Sen *et al*., 1995).

Identification of bacterial isolates:

All the isolated *Bacillus* strain were tested to first confirm their belonging to the genera *Bacillus* and thereafter, the most active *Bacillus* isolate was identified on the basis of its biochemical characteristics by PIB computer kit (Bryant, 2004) using the *Bacillus* identification matrix (Priest and Alexander, 1988).

Optimum conditions for antifungal compound production:

The growth parameters were optimized to obtain prolific growth and production of antifungal compounds. The parameters used were source of carbon (glucose, lactose, fructose, sucrose and galactose) and nitrogen (glutamic acid, ammonium sulphate, peptone, glycine and aspargine), pH values (5, 6, 7, 8, 9 adjusted by 0.1N HCl and NaOH), and temperature (25, 30, 37, 45 and 50^{o}C)

Results and Discussion

Isolation and screening the bacterial isolates for antifungal potential:

Five *Bacillus* isolates were obtained from the soils collected from different localities of Jabalpur (M.P.). Out of these isolates, the two strains (I-2 and I-4) showed the remarkable inhibitory effect against all or some of the test pathogenic fungi (Table 1 and Fig 1). The isolate I-2 revealed broad spectrum antifungal activity against the test pathogenic fungi. It was observed that of antifungal activity was the highest in case of *Curvularia lunata,* with the inhibition zone of 16.0mm (Table 2 and Fig 2). Previously, the inhibitory activity of *Bacillus* sp. was also observed against several yeast cultures such as *Saccharomyces cerevisiae, S. diastaticus, Candida albicans, C. guillermondii* and other molds cultures as *Aspergillus niger, Fusarium oxysporum and Trichoderma lignorum* (Kumar *et al.,* 2005). *B. cereus* X16, a halophilic strain isolated from salty soils, inhibited the growth of *F. roseum var. sambucinum* on solid medium as well as on wounded potato tubers (Sadfi *et al*., 2001).

Table 1: Antifungal activity of bacterial isolates by Lawn-spot Technique

Bacterial isolates	Zone of inhibition (diameter in mm)			
	Aspergillus niger	*Curvularia lunata*	*Alternaria solani*	*Fusarium solani*
I-1	2.0	2.0	<1	<1
I-2	10.0	20.0	12.0	9.0
I-3	3.0	<1	<1	4.0
I-4	8.0	12.0	9.0	<1
I-5	5.0	<1	<1	2.0

Table 2: Antifungal activity of bacterial isolates by Agar well diffusion technique

Bacterial isolates	Zone of inhibition (diameter in mm)			
	Aspergillus niger	*Curvularia lunata*	*Alternaria solani*	*Fusarium solani*
I-2	14.0	16.0	9.0	10.0
I-4	5.0	10.0	6.0	<1

Table 3: Morphological characterization of bacterial isolate

S.No.	Name of the test	Result for isolate I-2
1	Gram staining	Gm +ve
2	Motility	+
3	Pigment production	-
4	Spore formation	+

Table 4: Biochemical characterization of bacterial isolate

S. No.	Name of the test	Result for isolate I-2
1	Voges-proskauer	_
2	Citrate utilization	+
3	Starch hydrolysis	+
4	Urea hydrolysis	+
5	Casein hydrolysis	+
6	Nitrate reduction	+
7	Catalase	+
Carbohydrate fermentation		
8	Fructose fermentation	+
9	Galactose fermentation	-
10	Lactose fermentation	-
11	Raffinose fermentation	-
12	Salicin fermentation	-
13	Xylose fermentation	-
14	Growth in 10% NaCl	-
15	Growth in anaerobic condition	-
16	Growth at 50^{o}C	+

Identification:
The isolate I-2 was identified as *Bacillus carotarum* (Table 3 and 4).

Optimum conditions for antifungal compound production:
Maximum growth was recorded in the media having fructose (1% w/v) and peptone (1% w/v) as source of carbon and nitrogen, respectively at 9.0 pH and at 30^{o}C for 24h (Fig 3, 4, 5 and 6). Fructose was the best carbon and peptone was the best nitrogen source for antifungal compound production and optimum temperature was 30^{o}C at pH 9. Similar findings were also reported previously (Abd and Abada, 2008).

Fig 1: Antifungal activity of *Bacillus carotarum* (Isolate I-2) against *Curvularia lunata*

Fig 2: Antifungal activity of cell free extract of *Bacillus carotarum* (Isolate I-2) against *Curvularia lunata*

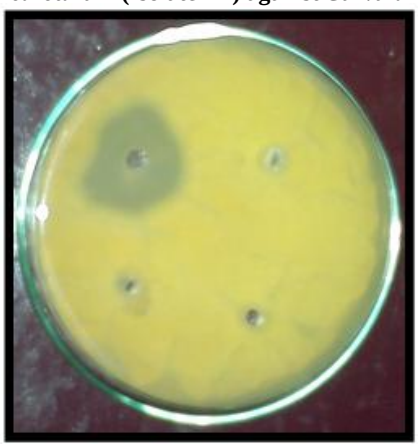


Fig 3: Effect of different carbon source

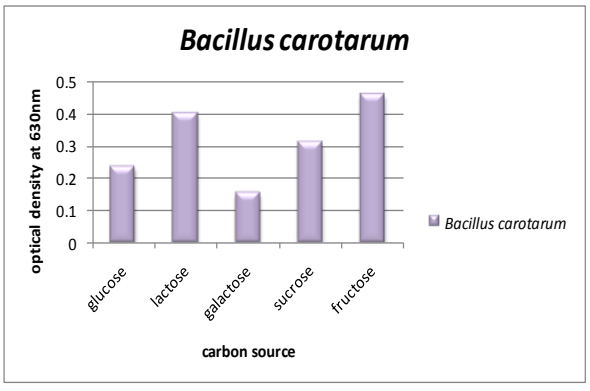


Fig 4: Effect of different nitrogen source

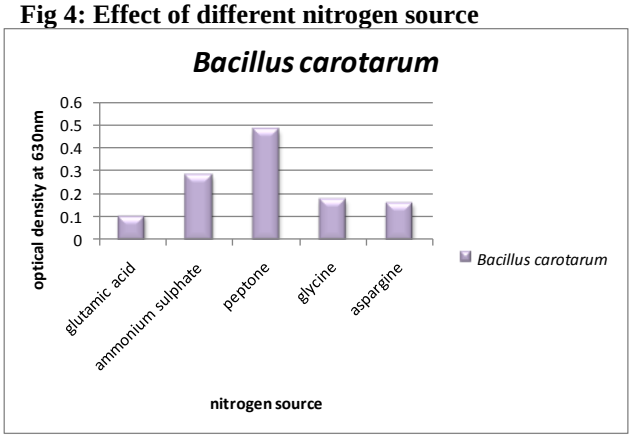


Fig 5: Effect of different pH value

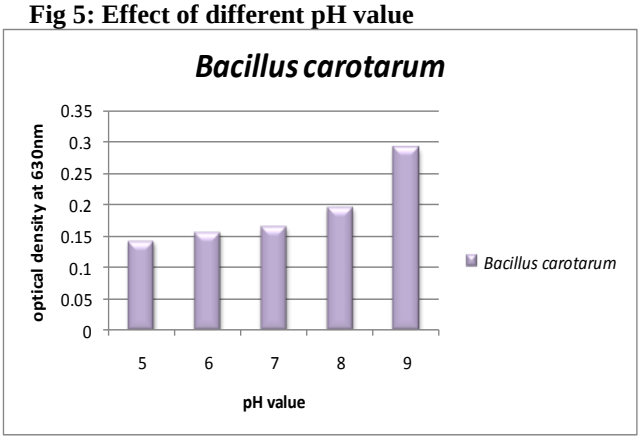
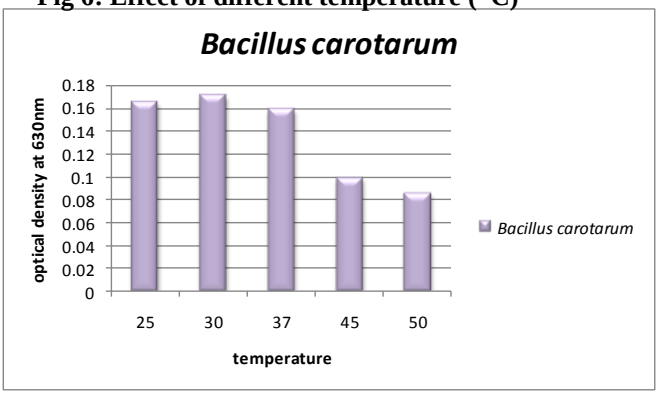


Fig 6: Effect of different temperature (^{O}C)



Conclusion:

We reported here a soil *Bacillus* isolate, identified as *Bacillus carotarum*, which inhibited growth of the common pathogenic fungal strains. We also optimized the nutritional and environmental conditions for the prolific growth of the isolate and the maximum antifungal compound production by optimizing various parameters like pH of the medium, temperature, carbon and nitrogen sources. We are presently working on partial purification of antifungal compounds by using TLC, SDS-PAGE and ammonium sulphate precipitation.

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