Research Paper Biotechnology

Antibacterial, Antialgal and β-lactamase Inhibition Activity of Penicillium Purpurogenum var Rubrisclerotium

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ABSTRACT

One of the biggest challenges encountered by the medical world is antibiotic resistance posed by the superbugs. Similarly, algae pose a threat by growing in swimming pools, aquariums, causing toxicity in potable waters etc. In this study, a fungal strain was isolated from algal source and studied for its antibiotic activity. The strain was identified as Penicillium purpurogenum var rubrisclerotium and was tested for its antibacterial activity against S.aureus, B.subtillis, E.coli and P.aeruginosa; antialgal activity against Chlorella vulgaris and β-lactamase inhibitor activity against Penicillinase enzyme (B. cereus) using sulbactum as a control for β-lactamase inhibition, and Penicillin as a substrate. The fermented broth exhibited antibacterial, antialgal activity against test cultures along with a significant β-lactamase inhibitory activity, measured by iodometric and agar gel diffusion bioassay methods. The broth was further concentrated by treating it with butyl acetate and tried against test organisms which resulted in many fold increase in the activities.

Keywords: Antibiotic, Antibacterial, Antialgal, β-lactamase.

Introduction

The production of β-lactamases by pathogens is the common major cause of bacterial resistance to β-lactam antibiotics [2]. On administration of an antibiotic drug, the defense mechanism of pathogenic bacteria against β-lactam antibiotics becomes active and they produce β-lactamases that hydrolyze the β-lactam ring of antibiotics, rendering it to loosen its activity. Clavulanate, sulbactam and tazobactam are some of the known irreversible inhibitory molecules of many β-lactamases that forms covalent complexes with the β-lactamase which subsequently resist their hydrolysis [3]. The development of β-lactamase inhibitors has allowed clinicians to rely on the well-tolerated, clinically effective antibiotics against a variety of gram-positive and gram-negative bacterial super infections [10]. Due to the limitations of these inhibitors to specific class of β-lactamases, it has become indispensible to search for novel compounds with antibiotic properties that can fight against deadly superbug infections on a broader spectrum and at the same time restore the activity of existing antibiotics by making use of newer inhibitors.

On the other hand, the undesirable growth of microalgae as a weed at places like swimming pools, aquariums, drainage systems, pipelines etc are known to cause severe blocking and toxicity problems in the potable water systems [1, 7]. Not many effective, naturally occurring antialgal compounds have yet been identified that can act as an algaecide.

The current study explores isolation and identification of a fungus, *P. purpurogenum;* extraction of its active fraction that possess effective antibacterial activity against some gram positive microbe's viz. *S.aureus* and *B.subtilis*, with a mild activity against some gram-negative organisms like *E coli,* antialgal activity against *Chlorella vulgaris* and β-lactamase inhibitory activity against penicillinase enzyme.

Materials & Methods

(1) Isolation of fungal strain and Inoculum Preparation: A fungal culture, found co-existing with microalgal flora was isolated by streaking on czapek agar dox plates, incubated at 25 0 C for 7 days. The isolates were studied for their morphology, mode of reproduction, and pigment and sclerotia production for identification of the strain. A 10 % germinating spore suspension grown at 25 0C, 200 rpm for 24 hrs on a rotary shaker was used as an inoculum.

(2) Production Medium: A 250 ml of Erlenmeyer flask, containing 50 ml czapek dox broth was used as production medium, incubated for 7 days at 25 0C pH 7.0 on a rotary shaker at 200rpm. The broth was harvested; the supernatant was analyzed for its antibiotic activity against test micro-organisms. The broth was concentrated by solvent – solvent extraction method using butyl acetate. Escherichia coli (ATCC- 6538), Staphylococcus aureus (ATCC- 6633), Pseudomonas aeruginosa (NCTC- 9002) and Bacillus subtillis (NCTC- 6750) were used as test micro- organisms [6] for antibacterial assay.

(3) Antialgal Activity: Antialgal activity was determined against microalgae test culture Chlorella vulgaris (DPU 0012), cultured in 250 ml Erlenmeyer flask containing 100 ml Bold Basal Medium, incubated at 25 0C for 7 days, under 40w fluorescent lamp with 12 hours of illumination. Varied concentrations of fungal supernatant (1.0 to 5.0 ml) were added to the above test culture. For control, a set of flask with only test culture and without the addition of fungal supernatant was used. The algal growth rate was determined as cell count by haemocytometer / O.D at 540nm.

(4) β-lactamase Inhibition Activity: Agar gel diffusion bioassay methods (in vivo) and Iodometric (in vitro) were employed to measure the β-lactamases inhibitory activity.

(4.1) Agar Gel Diffusion Bioassay Method: After appropriate dilution of the basic

Solutions of: Benzyl penicillin sodium (5000U/ml) as a substrate [4]; Penicillinase enzyme (2.5U/ml) as β-lactamase; sulbactum (3.5mg/ml) as a positive control (as standard β-lactamase inhibitor) and sterile distilled water (as the negative control), were used. *S.aureus* was used as the test organism. A 100 *µl* of test mixture containing 50µl penicillinase enzyme, 50µl inhibitor (sulbactum/ fungal extract) was incubated at 30 ^{0}C for 15 min and then 1 ml of penicillin was added and again incubated at 30 ^{0}C for 15 min. From this reaction mixture, 100 µl was added to each of the wells of agar gel diffusion bioassay plates, incubated at 37 ^{0}C for 20 hrs. The zone of inhibition obtained from (a) penicillin, (b) penicillin + enzyme, (c) penicillin + enzyme + sulbactum and (d) penicillin + enzyme + fungal extract, were measured in mm.

(4.2) Iodometric Assay: This method depends upon the reduc-

tion of iodine by the hydrolyzed substrate. When determined experimentally, 1 mol of hydrolyzed penicillin consumes 3.4 to 4.0 mol of iodine that can be determined colorimetrically using starch as an indicator. The method is useful for routine assay of β-lactamase activity with various substrates [9]. Incorporating the same principle, a modified method was designed to determine the β-lactamase inhibition activity of the fungal broth:

(A) A control tube was prepared with 2.0 ml gelatin, followed by a 3 drops of starch and 1.0 ml of Penicillin solution, incubated at 30 ^{0}C for 15 minutes. After the incubation, 2.0 ml iodine solution was added.

(B) 2.0 ml of 1 % gelatin solution was added to each of the following reaction glass test tubes along with 3 drops of 1 % starch solution, and the given test tubes were prepared in the order as given below:

(i) Penicillinase tube-50 µl enzyme solution was added followed by 1.0 ml of penicillin solution, incubated at 30 ^{0}C for 15 minutes. After the incubation, 2.0 ml iodine solution was added. (ii) Inhibitor tube with sulbactam- 50 µl enzyme solution was added followed by 1.0 ml of the sulbactum (inhibitor) and incubated for 15 min at 30^{0}C. The incubation time period would enable the enzyme to bind with the inhibitor. After incubation, 1.0 ml Penicillin solution was added, followed by quick addition of iodine reagent. (iii) Inhibitor tube with fungal extract -To 50 µl enzyme solution, 1.0 ml fungal extract was added, incubated for 15 min at 30 ^{0}C. After incubation, 1.0 ml Penicillin was added followed by quick addition of 2.0 ml iodine reagent. The time taken for decolorization from blue to colourless was recorded for all the sets of test tubes.

Results & Discussion

The fungal strain was isolated from microalgal culture on czapek dox agar medium and was identified to be *P. purpurogenum var rubrisclerotium* [8].

Table 1: Antibacterial Activity of Fungal Broth

Test Organism	Penicillin		Filtrate-Crude		Filtrate-Concentrated	
	Growth Inhibition Activity	Zone of Inhibition (mm)	Growth Inhibition Activity	Zone of Inhibition (mm)	Growth Inhibition Activity	Zone of Inhibition (mm)
B.subtillis	+++	7	+++	10	+++	20
S.aureus	+++	26	+++	27	+++	30
E.coli	_	0	++	5	++	9
P. aeruginosa	_	0	++	4	++	8

+++ Good activity ++ Moderate activity - no activity

Antialgal Activity

The antibiotic produced by *P. purpurogenum* also proved to have a strong inhibitory activity against the growth of *C.vulgaris*. A healthy algal growth was observed in the control flask. Mild inhibition of growth was observed with the addition of 1.0 ml of fungal broth. By increasing the concentration of fungal extract from 1.0 to 5.0 ml, there was a steep fall in the growth of algae. Flasks with 4ml and 5ml of fungal extract showed no growth of algae (Figure 1). Probably the antibiotic compound produced by the fungi during the fermentation has an inhibitory affect against the growth of microalgae as already being discussed by researchers in past [5].

β-lactamase Inhibitory Activity

In Agar gel diffusion bioassay method, the zone of inhibition obtained from (a) penicillin was 53 mm; (b) penicillin + enzyme was 0 mm; (c) penicillin + enzyme + sulbactum was 47.5 mm and (d) penicillin + enzyme + fungal extract was 34.5 mm (Figure 2), indicating good inhibitory activity by crude fungal broth in comparison to sulbactam, a known inhibitor [3].

In Iodometric assay, in the control tube (A), colour change from blue to colorless did not occur and it remained dark blue permanently indicating there was no absorption of iodine and penicillin remained intact and active.

In the assay tube (B, i) the time for decolourization was recorded to be 5 sec indicating that penicillin was hydrolyzed by penicillinase, converting it into penicllinoic acid, absorbing the required amount of iodine [4,9].

In the tube (B, ii) the decolourization time was beyond 1 hour (1 hour, 20 minutes). It is sulbactum that has blocked the enzyme molecule, not allowing it to react with penicillin. Thus, the iodine is not absorbed and the colour has remained blue for a long period. Sulbactum results in complete irreversible inhibition of certain enzyme molecules [3].

In the tube (B, iii) the enzyme activity of crude broth was 15 % as compared to the activity of sulbactum. The concentrated broth showed a 5 fold increase in the enzyme activity. The presence of inhibitor is evident in the fermented broth, which has blocked the active site of penicillinase rendering it inactive for a long time, not allowing the iodine to be absorbed and the blue colour has remained for a long time.

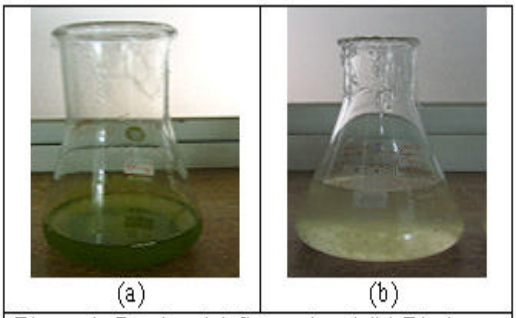

Figure 1: Depicts (a) Control and (b) Flask with algae and fungus.

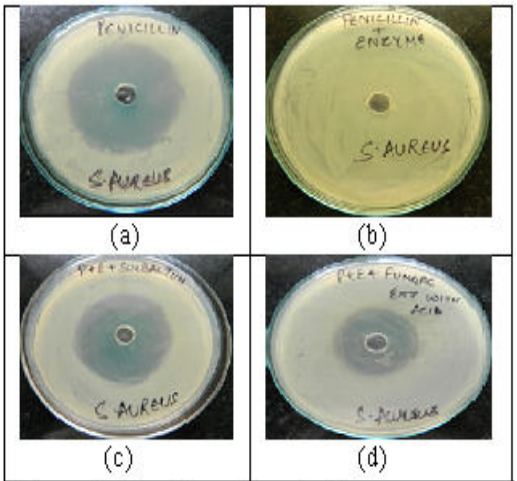

Figure 2: Depicts well containing (a) penicillin, (b) penicillin + enzyme, (c) penicillin + enzyme + sulbactum and (d) penicillin + enzyme + fungal extract in Agar Gel Diffusion Bioassay.

Conclusion

The species of Penicillia are well known to produce antibiotics and many other useful metabolites. The species discussed in this study, seems to be of greater importance as it has a high potential for producing antibiotic compound having antibacterial, antialgal along with a significantly high activity of β-lactam inhibitors.

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