Research Article

ISOLATION, PURIFICATION AND DETECTION OF ANTIMICROBIAL ACTIVITY OF BETA-LACTAM ANTIBIOTICS FROM FEW FUNGAL STRAINS

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ABSTRACT

An Antibiotic is a chemical substance derivable from a mold or bacterium that can kill microorganisms and cure infections. They exhibit a wide spectrum of activity against an array of bacteria. The present study describes the various methods for the isolation of β-lactam antibiotic producing fungi as well as the processes for the production of penicillin and cephalosporin using *Penicillium chrysogenum* and *Cephalosporium acremonium* respectively. Different methods were described for the purification of antibiotics and their antimicrobial activity against test microorganisms i.e *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae* and *Bacillus cereus* followed by thin layer chromatography for the identification of the purified compound. The crude and purified antibiotics exhibited antimicrobial activity against the test organisms. The R_f values obtained for crude and purified penicillin were 0.71 &0.75 and cephalosporin were 0.83 and 0.98 respectively, which were close to their standard values 0.73 (pencillin) and 0.9 (cephalosporin).

Keywords: β- lactam, *Penicillium chrysogenum*, *Cephalosporium acremonium*, TLC.

INTRODUCTION

An Antibiotic is a chemical substance derivable from a mold or bacterium that can kill microorganisms and cure bacterial infections. It can be purified from microbial fermentation and modified chemically or enzymatically for basic research[1,2]. Several hundred naturally produced antibiotics have been purified, but only a few have been sufficiently non-toxic to be of value in the treatment of infectious disease. Those that are currently of greatest use have been derived from a relatively small group of microorganisms belonging to the genera *Penicillium*, *Streptomyces, Cephalosporium, Micromonospora* and *Bacillus*[3]. More than 5000 different antibiotics have been isolated from cultures of bacteria, fungi and plant cells, 60% of them are contributed by the genus Streptomyces[4,5].

The semisynthetic penicillins like the parent drug penicillin G are remarkably safe drugs. However, a significant number of patients have been reported with the cases of neutropenia. This is a side effect of these drugs not appreciated by a significant number of physicians[6]. In 1997 Risk of administering cephalosporin antibiotics to patients with histories of penicillin allergy were observed[7]. Cephalosporin compounds were first isolated from cultures of *Cephalosporium acremonium* from a sewer in Sardinia in 1948 by Italian scientist Giuseppe Brotzu.[8] In 2003 Kelkar and Li recommended against prescribing third generation cephalosporins to patients allergic to penicillin.

New antibiotics can still be discovered by the development of novel screening procedures. Notable success has been achieved over the last few years include the monobactams, beta-lactamase inhibitors (clavulanic acid) and new glycopeptides in the antibacterial field; antiparasitic agents such as avermectins; and herbicidal antibiotics like bialaphos.

A hybrid (prokaryotic-eukaryotic) enzyme system leading to the production of benzylpenicillin has been developed. The first description of an *in vitro* assay that, using enzymes of different microbial origin, mimics the three last enzymatic steps leading to the biosynthesis of penicillin G in *P. chrysogenum*[9]. The purification to homogeneity of some of the proteins as well as their biochemical characterization has allowed some of them to be used for synthesizing many different penicillins and cephalosporin-like products *in vitro*. Thus, most of the known natural penicillins, many of the semisynthetic variants and others, which until now can only be obtained chemically, have been synthesized enzymatically from their natural precursors[9].

Semi-synthetic penicillins and cephalosporins both derive from their respective chemical nuclei, 6-aminopenicillanic acid (6-APA) and 7-aminocephalosporanic acid (7-ACA). Work leading to their isolation was being carried out in parallel, but following very different pathways, during the last half of the 1950s[10]. The Isopenicillin N synthetase (IPS) gene from *Penicillium chrysogenum* was isolated from a recombinant bacteriophage A library using the *Cephalosporium acremonium* IPS (cIPS) gene as a heterologous hybridization probe. The transformed cells were also shown to contain an abundant protein accounting for about 10% of total cell protein which reacted strongly with anti-cIPS antiserum[11].

In recent years recombinant technologies have contributed significantly to improve the capacities of production and also allowed to design genetically modified strains for production and expression of various

antibiotic molecules. The better understanding of fundamental genetic processes in model organisms has resulted in the design and generation of new experimental transformation strategies to manipulate specific gene expression and function in diverse filamentous fungi, including those having a biotechnical significance.

MATERIALS AND METHODS

Isolation of *Penicillium* and *Cephalosporium* sp.

Nutrient agar and Rose Bengal Agar media plates were exposed to air for 15 minutes for isolation of *Penicillium* sp. and for the isolation of *Cephalosporium* sp sea water samples (Gopalpur) were subjected to 10 fold serial dilution and aliquots (0.1 ml) were plated on Nutrient agar and Rose Bengal agar media. Plates were incubated at room temperature for 3-4 days[12].

Characterization of isolated strains

Morphological identification of the isolated strains were studied by high resolution microscopy[13].

Screening of the strains for the production of β-Lactam antibiotics

Isolated fungal strains were inoculated separately into seed medium and incubated at room temperature for 3-4 days. The culture was then transferred into production medium and incubated for 12 days at room temperature. The culture was then filtered aseptically to remove the mycelium and solid particles of medium and filtrate was centrifuged at 6000rpm for 10 min. Antimicrobial activity of the supernatant was determined by cup plate method on nutrient agar plates. The test organisms used for Penicillin were *Bacillus cereus, klebsiella pneumonia and Escherichia coli* and for cephalosporin *Escherichia coli, Klebsiella pneumoniae, and Staphylococcus aureus* were used. The plates were incubated at 37°C for 24hrs[14] and zone of inhibition was measured.

Sub surface Fermentation

The sub surface fermentation for Penicillin production in seed medium was carried out with corn steep liquor, sucrose and $CaCO_3$, $NaNO_3$, KH_2PO_4, and $MgSO_4.7H_2O$ in 250 ml flask. The medium was autoclaved at 121°C for 15 minutes. The medium was inoculated with 3days old well grown cultures of the isolates. After inoculation the flasks were incubated at 37°C on an orbital shaker at 150rpm. After 72 hrs of incubation, 1ml of the fermentation broth was transferred to production medium containing corn steep liquor, lactose, Glucose, KH_2PO_4, $CaCO_3$ and olive oil in 250ml flask. After inoculation the flasks were incubated at 37°C on an orbital shaker at 150rpm. After 12 days of incubation, the fermentation broth was filtered and centrifuged at 6000 rpm for 15 minutes and supernatants were assayed for its antimicrobial potency. Similarly production of Cephalosporin was carried on sucrose, trace metals solution, KH_2PO_4 and $MgSO_4$ (seed medium) and soyabean meal, sucrose and methionine in production medium.

Penicillin and Cephalosporin Assay The collected supernatant was tested for antimicrobial activity against the test pathogens viz: *Bacillus cereus, klebsiella pneumoniae, Escherichia coli* for Penicillin while *Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus* for Cephalosporin on nutrient agar plates incubated at 37°C for 24hrs and zone of inhibition was measured.

Purification of Penicillin and Cephalosporin extracted from sub surface fermentation

Solvent extraction method

An equal volume of chloroform was added to aqueous concentrate in a separating funnel and shaken vigorously until the mixture was separated into layers immediately after shaking. The Upper layer was darker as it contains impurities from the aqueous chamber. The lower aqueous layer which contains the active material was decanted and the extraction was repeated. The extraction was allowed to stand for ten minutes so as to ensure proper separation. The lower layer was decanted each time, collected and stored at 4°C. The antimicrobial assay of the collected sample was assayed and zone of inhibition was measured.

Ion exchange chromatography

A series of sodium phosphate buffers were prepared with pH 6, 6.5, 7, 7.5, 8, 8.5 having an ionic strength of 0.42M, 0.46M, 0.48M, 0.5M, 0.52M, 0.54M respectively by the addition of NaCl. The carboxy methyl cellulose buffer solution was poured into column and was allowed to stand for 30 min. The excess buffer was removed without disturbing the bed. The bed was washed with phosphate buffer of pH 7, then buffer was removed and samples (Penicillin & Cephalosporin separately) were added followed by elution with buffer of increasing pH range. The antimicrobial assay of the eluted samples was performed against different test organisms viz: *Bacillus cereus, klebsiella pneumoniae, Escherichia coli* for Penicillin while *Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus* for Cephalosporin and zone of inhibition was measured.

Identification of Penicillin and Cephalosporin in the given sample using Thin layer chromatography

Silica gel was prepared with distilled water in 2:5 ratio and immediately transferred over the glass slide uniformly, air dried and kept in the oven at 105°C for 30 mins. The solvent was prepared with butanol, acetic acid and water in the ratio of 4:1:1 for penicillin and 5:2:4 (V/V) for cephalosporin. The reference line was drawn on the silica gel plate at a distance of 1.0cm from the lower end. Two spots were marked on the end and the test samples were applied on the spots (10-20µl) the silica gel plate was suspended in the solvent. The plate was taken out when the migration of the solvent nearly equals to 70% of the length of the plate.

RESULTS AND DISCUSSION

The present study revealed that the isolated strains having circular, filamentous, and velvety format forming a ring like structure in the centre and the colonies that were initially white, green, bluish green, gray green and the reverse side was pale to yellowish in color (Fig:1) and brush like clusters microscopically was found out to be *Penicillium sp*. While the colonies that were circular, growing in wavy format forming a ring like structure, light brown in structure with spores surrounding the structure and the mass of conidia at the apex of the branched hyphae were found to be *Cephalosporium sp.*

Figure 1: Pure culture plate of *Penicillium chrysogenum*

Table 1: Antimicrobial assay of crude Penicillin from sub surface fermentation

Test organism	Zone of inhibition (in mm)
Escherichia coli	20
Bacillus cereus	9.5
Klebsiella pneumoniae	22

The crude antibiotic Pencillin exhibited antimicrobial activity against the test organisms viz: *Bacillus cereus, klebsiella pneumoniae, Escherichia coli* while *Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus* were used for Cephalosporin (Table-1) and both the crude samples showed a larger zone of inhibition against *Klebsiella pneumoniae* (22mm and 15mm respectively) which confirms that β-lactam antibiotics are not only active against gram positive bacteria but also on some gram negative bacteria. After purification of crude samples by solvent extraction method (Chloroform) and ion exchange chromatography the antimicrobial activity of purified penicillin and cephalosporin showed higher zone of inhibition compared to the crude ones. Amongst the elutes of ion exchange chromatography, eluent of pH 7.5 showed higher zone against all the test organisms (Table 2 and 3). The R_f values obtained for crude and purified penicillin were 0.71 and 0.75 and cephalosporin were 0.83 and 0.98 respectively, which were close to their standard values 0.73 (pencillin) and 0.9 (cephalosporin) (Tables-5, 6, Fig: 3,4).

Table 2: Antimicrobial assay of crude Cephalosporin from sub surface fermentation

Test organism	Zone of inhibition (in mm)
Staphylococcus aureus	9
Klebsiella pneumoniae	15
Escherichia coli	11

Table 3: Antimicrobial assay of pure Penicillin (by solvent extraction method)

Microorganism	Zone of inhibition (in mm)
Escherichia coli	8
Bacillus cereus	11.5
Klebsiella pneumoniae	26.5

Table 4: Antimicrobial assay of pure Cephalosporin (by solvent extraction method and ion-exchange chromatography)

Sample	Zone of inhibition (in mm)		
	Staphylococcus aureus	*Klebsiella pneumoniae*	*Escherichia coli*
Pure Cehalosporin (solvent extraction method)	21	9.5	11.5
Elutes at pH 6	9	12	10
pH 6.5	10.5	11	12
pH 7	11	10	8.5
pH 7.5	12	12	14
pH 8	11	12	13.5
pH 8.5	11	9	13

Table 5: Thin layer chromatography of the crude, purified and standard Penicillin

Sample	Distance travelled by the sample (in cm)	Distance travelled by the solvent (in cm)	R_f value
Crude sample	4.2	5.9	0.71
Pure sample	4.5	6	0.75
Standard	3.6	5.7	0.73

Table 6: Thin layer chromatography of the crude, purified and standard Cephalosporin

Sample	Distance travelled by the sample (in cm)	Distance travelled by the solvent (in cm)	R_f value
Crude	4.6	5.5	0.83
Purified water	5.5	5.6	0.98
pH 6	4.7	5.9	0.79
6.5	4.4	6	0.73
7	4.3	5.6	0.76
7.5	4	5.5	0.72
Standard	4.5	5	0.9

Figure 2: TLC of standard sample

Figure 3: TLC of crude, purified penicillin in UV transilluminator

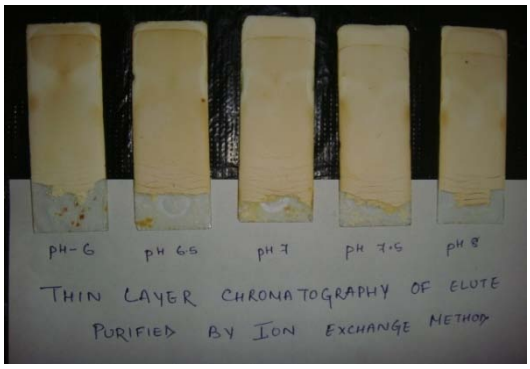

Figure 4: TLC of elutes purified by ion exchange chromatography

CONCLUSION

β-lactam antibiotics have a wide range of applications in medical field so a thorough knowledge of the fungus morphology and biological characteristics is needed to enhance its use in medicine era. The above study revealed that the β-lactam antibiotics produced during stress conditions shows antimicrobial activities against many gram positive and gram negative bacteria. Thus further testing of their activity and the genes responsible for them is required to understand the complete molecular mechanism and make ample use of it for human health and biomedical research.

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