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MICROBIAL PRODUCTION OF β LACTAM ANTIBIOTIC AND ASSESSMENT OF β LACTAMASE PRODUCING ABILITIES OF PATHOGENS

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ABSTRACT
Studies were carried out to produce Penicillin by using *Penicillium notatum* (NCIM 741) by submerged fermentation. Bioassay was performed by using *E. coli* (NCIM 2809). β-lactamase activity of pathogens was assessed. β- lactamase activity was also checked by using starch agar method and filter paper method. It was found that SV7 is an efficient β-lactamase producer among these. Available pathogens were tested for their susceptibility to β lactamase Inhibitors *viz*. clavulanic acid and sulbactam. It was found that, growth of all isolates except SV 7 was inhibited in Clavulanic acid, Penicillin + Clavulanic acid, Sulbactam and Penicillin + Sulbactam containing medium. Growth of SV 7 was inhibited only in penicillin + sulbactam containing medium. All seven pathogenic isolates were also tested for their succeptibility to other antibiotics *viz*. tetracycline, streptomycin and meropenum. It was found that SV2, SV3 and SV4 are multi drug resistant (MDR) organisms.

KEYWORDS: *Penicillium notatum,* β- lactamase, Clavulanic acid, Sulbactam.

INTRODUCTION

Penicillin is the β-lactam antibiotic. The widespread use of antibiotics has put bacteria under tremendous selective pressure to devise mechanisms to escape the lethal action of the drugs. The increase in resistance to β-lactam antibiotics and the broad activity spectrum of β-lactamases in many pathogenic bacteria are frightening. In pathogenic bacteria, β-lactamase production is the most important contributing factor to β-lactam resistance [Medeiros, A.A. 1997]. β- Lactamases catalyze the hydrolysis of an amide bond in the β-lactam ring of penicillins and cephalosporins, rendering a species that is no longer an inhibitor of bacterial transpeptidases [Wang *et al.,* 1999]. Powers, J. H. [2013] studied that the use of multiple compounds to produce synergistic or additive effects against microbial pathogens [Powers J. H., 2004]. One such example is coamoxiclav, a combination of amoxicillin and clavulanic acid. This combination is also effective against amoxicillin-resistant bacteria because clavulanic acid is an inhibitor of β-lactamase, an enzyme which degrades β- lactam drugs including amoxicillin. Other similar combinations which are in clinical use include ampicillin/ sulbactam and piperacillin/ tazobactam. β-lactamase mediated resistance to β – lactam antibiotics emerged as a significant clinical threat to these lifesaving drugs. In response to this challenge, two strategies were advanced to preserve the utility of β -lactam antibiotics (i) discover or design β-lactam antibiotics that are able to evade bacterial enzymatic inactivation conferred by β -lactamases, or (ii) inhibit β -lactamases so the partner β -lactam can reach the penicillin binding proteins (PBPs), the target of β –lactam antibiotics Behravan and Rangsaaz [2004]. In the present study, Penicillin was produced by using *Penicillium notatum* (NCIM 741), it was then extracted and purified. This purified penicillin was used for bioassay. β lactamase activity of Pathogens was assessed, multiple drug resistance of pathogens was determined and susceptibility of pathogens to β lactamase inhibitors was assessed.

MATERIALS & METHODS

Penicillin production

The culture from slant of *P. notatum* (NCIM74) was sub-cultured on Czapedox's agar containing plate and plate was incubated at 37^0C for 24 to 48 hrs. Spores from the Czapedox's agar were then scrapped out and inoculated in Czapedox's broth and kept for incubation for 2 days. After incubation, the growth of organism was observed and this was used as an inoculum for fermentation. 100 ml fermentation medium (composition mentioned in appendix) was prepared and added to 250 ml capacity Erlenmeyer flask. 10% inoculum was added to it. The flask was then kept for incubation at 30^0C for 5days. At the end of fermentation, fermentation broth was filtered using Whatman's filter paper 1 and volume of filtrate was measured. The pH of filtrate was adjusted to 2-3 using sulphuric acid. In the filtrate, ethyl acetate (20% by volume) was added and this mixture was then transferred in separating funnel. The mixture was shaken vigorously for 20 min and the separating funnel was allowed to stand for 5 min so as to separate two layers. Lower (aqueous) layer was discarded and the upper (organic) layer was collected in a beaker. The organic layer obtained after extraction was poured in the evaporating dish and kept in an oven at 50^0C for the evaporation of ethyl acetate. After evaporation the obtained crystals were dissolved in 2 ml Ringer's solution.

Antibiotic Bio-assay

A stock of Penicillin having concentration 1000 µg/ml was prepared. The stock was then serially diluted with a range

100-1000 µg/ml. sterile filter paper discs were then dipped in individual dilutions separately. Soaked filter paper discs were then placed on nutrient agar plates previously inoculated with *E. coli* (NCIM 2809). All the plates were kept in refrigerator for 20 mins for diffusion of antibiotic. After 20 mins the plates were kept for incubation at 37^0C for 24 hrs. A sterile filter paper disc was dipped in extracted penicillin and placed on a nutrient agar plate previously inoculated with *E. coli* (NCIM 2809).

Assesment of β lactamase activity of Pathogens

100ml Nutrient agar was prepared and autoclaved at 121^0C for 15 minutes. After autoclaving the agar was allowed to cool down (approximately till 45^0C). Then 0.20gm (w/v) of penicillin was added to the medium and the medium was poured in the plates. After solidification of the plates, seven pathogenic test cultures viz., *E.coli* 2995**,** *E. coli* 2809**,** *E. coli* (Laboratory isolated culute)**,** *S. aureus* (Pure culture), *S. aureus* (Laboratory isolated culture), *S. typhi* [NCIM 2501] (pure culture) and Culture 7 (laboratory contaminant culture) were spot inoculated on each plate separately. For convenience these test cultures were labeled as SV 1, SV2, SV3, SV4, SV5, SV6 and SV7 respectively. All plates were then kept for incubation at 37^0C for 24 hrs.

β-lactamase assay

Starch agar method

Petri plates containing minimal agar + 1% starch was prepared and pathogenic test cultures were spot inoculated on it. The plates were then kept for incubation at 37^0C for 24 hrs. After incubation the plates were flooded with iodine solution (composition mentioned in appendix).

Filter paper method

A 10ml mixture of Starch and Iodine was prepared and spreaded on a filter paper strip. Test cultures were spot inoculated on the strip separately. The strips were then incubated at 37^0C for 24 hrs. After incubation the strips were observed for the zone of clearance.

Assessment of Susceptibility of Pathogens to Other Antibiotics

Tetracycline

100ml Nutrient agar was prepared and autoclaved at 121^0C for 15 minutes. After autoclaving the agar was allowed to cool down (approximately till 45^0C). Then 0.20gm (w/v) of tetracycline was added to the medium and the medium was poured in the plates. After solidification of the plates seven pathogenic test cultures viz., SV 1, SV2, SV3, SV4, SV5, SV6 and SV7 were spreaded on separate plates. Then all plates were kept for incubation at 37^0C for 24 hrs.

Streptomycin

100ml Nutrient agar was prepared and autoclaved at 121^0C for 15 minutes. After autoclaving the agar was allowed to cool down (till approximately 45^0C). Then 0.20gm (w/v) of streptomycin was added to the medium and the medium was poured in the plates. After solidification of the plates seven pathogenic test cultures viz., SV 1, SV2, SV3, SV4, SV5, SV6 and SV7 were spreaded on separate plates. Then all plates were kept for incubation at 37^0C for 24 hrs.

Meropenum

100ml Nutrient agar was prepared and autoclaved at 121^0C for 15 minutes. After autoclaving the agar was allowed to cool down (till approximately 45^0C). Then 0.20gm (w/v) of Meropenum was added to the medium and the medium was poured in the plates.After solidification of the plates seven pathogenic test cultures viz., SV1, SV2, SV3, SV4, SV5, SV6 and SV7 were spreaded on separate plates. Then all plates were kept for incubation at 37^0C for 24 hrs.

Assesment of Succeptibility of Pathogens to β lactamase Inhibitors

Using Clavulanic Acids

100ml Nutrient agar was prepared and autoclaved at 121^0C for 15 minutes. After autoclaving the agar was allowed to cool down (till approximately 45^0C). Then 0.20gm (w/v) of clavulanic acid was added to the medium and the medium was poured in the plates. After solidification of the plates seven pathogenic test cultures viz., SV 1, SV2, SV3, SV4, SV5, SV6 and SV7 were spot inoculated on these plates separately. Then all plates were kept for incubation at 37^0C for 24 hrs.

Using Sulbactum + Penicillin

100ml Nutrient agar was prepared and autoclaved at 121^0C for 15 minutes. After autoclaving the agar was allowed to cool down (till approximately 45^0C). Then 0.20gm (w/v) of Sulbactum and 0.20 gm (w/v) of Penicillin was added to the medium and the medium was poured in the plates. After solidification of the plates seven pathogenic test cultures viz., SV 1, SV2, SV3, SV4, SV5, SV6 and SV7 were spot inoculated on these plates separately. Then all plates were kept for incubation at 37^0C for 24 hrs.

Using Clavulanic acid + Penicillin

100ml Nutrient agar was prepared and autoclaved at 121^0C for 15 minutes. After autoclaving the agar was allowed to cool down (till approximately 45^0C). Then 0.20gm (w/v) of clavulanic acid and 0.20 gm (w/v) of Penicillin was added to the medium and the medium was poured in the plates. After solidification of the plates seven pathogenic test cultures *viz*., SV 1, SV2, SV3, SV4, SV5, SV6 and SV7 were spot inoculated on these plates separately. Then all plates were kept for incubation at 37^0C for 24 hrs.

Different antibiotics tested with penicillin

The plates of nutrient agar containing the antibiotics tetracycline, streptomycin and meropenum were prepared and to these plates the spots of following cultures were given:

RESULTS & DISCUSSION

Penicillin Fermentation

After incubation of the 24 hrs, the spores of *P.notatum* (NCIM 741) were observed on the czapedox agar. Then the spores were inoculated in czapedox broth. The growth obtained after incubation was used as inoculum for fermentation. Fermentation was carried out in fermentation medium for 5 days at 30^0C in rotary incubator shaker adjusted at 120 rpm. After completion of fermentation, fermentation broth was filtered using Whatman filter paper no 1 to separate biomass of *P.notatum*. The volume of filtrate obtained after filtration was found to be 100 ml. The filtrate was extracted using ethyl acetate. After extraction upper organic layer was collected and kept in evaporating dish so as to evaporate

ethyl acetate. After complete evaporation of ethyl acetate crystals of penicillin were obtained. The crystals were dissolved in 2 ml of ringer's solution. This solution was used for bioassay.

Antibiotic Bio-assay

First a stock of Penicillin having concentration 1000 µg/ml was prepared and it was then serially diluted to get a range of penicillin concentrations 100µg/ml to 1000 µg/ml. Filter paper discs were immersed in each dilution and placed on nutrient agar plate previously inoculated with *E. coli* (NCIM-2809). After incubation at 37^0C for 24 hrs, the zones of inhibition were observed and the diameters of the zones were measured and are mentioned in table.

The extracted penicillin was also tested in same way so as to estimate the concentration of extracted penicillin. After incubation it was found that a 2.8 cm zone of inhibition was obtained for the extracted penicillin. This value was interapolated with the standard graph of penicillin and it was noted that the extracted penicillin is having a concentration of 710 µg/ml

TABLE 1. Bioassay of standard penicillin

Concentration (µg/ml)	Diameter
100	1.7
200	2
300	2.2
400	2.5
500	2.2
600	2.5
700	2.4
800	2.7
900	3.5
1000	2.3
Extracted penicillin	2.8

PLATE 1. Zone of extracted penicillin

Assesment of β lactamase activity of Pathogens

β lactamase activity of pathogens was assessed by preparing Nutrient agar plates containing 0.20gm (w/v) of penicillin. After solidification of the plates were spot inoculated with seven pathogenic test cultures *viz*., SV 1, SV2, SV3, SV4, SV5, SV6 and SV7 separately. All plates were then kept for incubation at 37^0C for 24 hrs. After incubation it was observed that culture SV 2, SV 3, SV 4, SV 5 and SV 7 shows growth as well as zone of clearance on the plate. The diameter of zone of clearance for each culture was measured and mentioned in table. From the values in table it can be concluded that, isolates SV 2, SV 3, SV 4, SV 5 and SV 7 have β lactamase activity.

Confirmation of β-lactamase

By Starch agar method

Petri plates containing minimal agar + starch (1%) were prepared and seven pathogenic test cultures were spot inoculated on it. After incubation the plates were flooded with iodine solution. It was observed that SV 2, SV 3, SV 4, SV 5 and SV 7 shows zone of starch hydrolysis while SV 1 and SV 6 did not show any development of zone. The diameter of zone was measured and mentioned in table. It was concluded that SV 7 was an efficient β-lactamase producer.

TABLE 2. Detection of β-lactamase using two methods

	Diameter of zone of clearance (in cm)	
Name of culture	Starch Agar method	Filter paper method
SV1	No zone	No zone
SV2	1.8	0.7
SV3	0.5	0.2
SV4	2.5	1.0
SV5	2.5	0.9
SV6	No zone	No zone
SV7	3	1.2

PLATE 2. β lactamase producing cultures SV 2 and SV 3

By filter paper method

A 10ml mixture of Starch and Iodine was prepared and spreaded on a filter paper strips. Test cultures were spot inoculated on the strips separately. The strips were then incubated at 37^0C for 24 hrs. It was observed that SV 2, SV 3, SV 4, SV 5 and SV 7 shows zone of starch hydrolysis while SV 1 and SV 6 did not show any development of zone. The diameter of zone was measured and mentioned in table 2. It was concluded that SV 7 was an efficient β-lactamase producer.

Assessment of Susceptibility of Pathogens to β lactamase Inhibitors

Available pathogens were tested for their succeptibility to β lactamase Inhibitors. For that, two β lactamase Inhibitors were used viz. clavulanic acid and sulbactam. Nutrient agar plates supplemented with Penicillin, Clavulanic acid, Penicillin + Clavulanic acid, Sulbactam and Penicillin + Sulbactam were prepared and test organism viz. SV 1, SV2, SV3, SV4, SV5, SV6 and SV7 was spot inoculated on these plates separately. After incubation at 37^0C for 24 hrs, it was observed that the zones of inhibition were observed as in the following table (table no 3).

TABLE 3. Assesment of Succeptibility of Pathogens to β lactamase Inhibitors

Culture	Nutrient agar + penicillin	Nutrient agar + clavulanic acid	Nutrient agar + Penicillin + clavulanic acid	Nutrient agar + sulbactam	Nutrient agar + Penicillin + sulbactam
SV1	-	-	-	-	-
SV2	++	-	-	-	-
SV 3	++	-	-	-	-
SV 4	++	-	-	-	-
SV 5	++	-	-	-	-
SV 6	-	-	-	-	-
SV 7	++	+	+	+	-

+ means growth ++ means growth and zone of clearance. – means no growth and no zone of clearance

On the basis of results obtained it was concluded that, growth of all isolates except SV 7 was inhibited in Clavulanic acid, Penicillin + Clavulanic acid, Sulbactam and Penicillin + Sulbactam containing medium. Growth of SV 7 was inhibited only in penicillin + sulbactam containing medium.

Clavulanic acid alone can also have inhibitory action as mentioned by Brown *et al.*, (1976).

Assessment of Susceptibility of Pathogens to Other Antibiotics

All seven pathogenic isolates were also tested for their susceptibility to other antibiotics viz. tetracycline, streptomycin and meropenum. For that individual antibiotic was added to nutrient agar separately and all seven isolates *viz.*, SV 1, SV2, SV3, SV4, SV5, SV6 and SV7 were spot inoculated on these media. After incubation the plates were observed for the growth of organism. The results obtained are mentioned in table

TABLE 4. Growth of pathogenic isolates on antibiotic containing media

Name of culture	Tetracyclin	Streptomycin	Meropenum
SV1	-	-	-
SV2	+	+	+
SV3	+	+	-
SV4	+	-	+
SV5	-	-	-
SV6	-	-	-
SV7	-	-	+

+ Means growth - means no growh and no zone

SV2 is resistant to all three tested antibiotic viz. tetracycline, streptomycin and meropenum while SV1, SV 5 and SV 6 did not show any growth. SV 3 is resistant to tetracycline and streptomycin and SV 4 is resistant to tetracycline and meropenum. According to the results mentioned in the table it can be concluded that SV 2, SV3 and SV 4 are multi drug resistant (MDR) organism.

CONCLUSIONS

From the above result it was concluded that, the Penicillin production was carried out from *P. notatum* (NCIM 741). β-lactamase activity of pathogens was detected and it was found that SV 2, SV 3, SV 4, SV 5 and SV 7 have β-lactamase activity and SV 7 is an efficient β-lactamase producer among these. SV 2, SV3 and SV 4 are multi drug resistant (MDR) organisms. Growth of all isolates except SV7 was inhibited in Clavulanic acid, Penicillin + Clavulanic acid, Sulbactam and Penicillin + Sulbactam containing medium. Growth of SV 7 was inhibited only in penicillin + sulbactam containing medium.

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