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Full Length Research Paper

The inhibitory effect of *Streptomyces chromofuscus* on β-lactamase of *Pseudomonas aeruginosa* ATCC-10145

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Seventy un-repeated *Streptomyces* isolates were isolated from different agricultural regions in Riyadh city. Amoxicillin-resistant *Pseudomonas aeruginosa* ATCC-10145 was obtained with minimum inhibitory concentration (MIC) > 1000 µg.ml^{-1}. *Streptomyces* isolates were screened for producing β-lactamase inhibitory effect against *P. aeruginosa* ATCC-10145. There were ten *Streptomyces* isolates which had inhibitory effect. Although, one *Streptomyces* isolate has been considered the most potent, this was identified by using biochemical characteristics as *Streptomyces chromofuscus*. Optimization factors for maximum yield of β-lactamase inhibitory protein were studied. The best incubation period at 7th day, incubation temperature at 28°C, pH value at 6.8, the best carbon source was galactose and the best nitrogen source was prolin. The highest amount of β-lactamase inhibitory protein was precipitated at 40% of saturated ammonium sulphate. The purification was carried out by using both diethyl-aminoethyl-cellulose and sephadex G-200 column chromatography, respectively. The β-lactamase inhibitory protein was separated at 32 KDa. The purified β-lactamase inhibitory protein was characterized as tazobactam. The combination of tazobactam at 128 mg.L^{-1} and amoxicillin at 125 µg.ml^{-1} (*in vitro*) leads to growth inhibition of amoxicillin-resistant *P. aeruginosa* and make it very sensitive to the amoxicillin.

Key words: Amoxicillin**,** enzyme inhibitors, protein purification, tazobactam.

INTRODUCTION

Beta-lactam compounds account for more than 60% of worldwide consumption of antibiotics as a result of the high selectivity, low toxicity and versatility of these compounds. However, the chemotherapeutic application of β-lactam antibiotics has been continually threatened by the development of bacterial resistance. The most common mechanism of resistance among both Gram-positive and Gram-negative bacteria involves the production of β-lactamases, enzymes which clear the β-lactam ring. Amoxicillin is a moderate-spectrum and bacteriolytic β-lactam antibiotic which has a molecular weight 419.45 and used to treat bacterial infections caused by susceptible microorganisms. Amoxicillin is susceptible to degradation by β-lactamase-producing bacteria, and so may be given with clavulanic acid or other β-lactamase inhibitors to decrease its susceptibility (Doran et al., 1990).

The acquisition of β-lactam antibiotics resistance genes by bacterial pathogens is currently a worldwide phenomenon. It is not difficult to imagine that antibiotic-resistance could become a particular severe problem in some developing countries in the setting of poverty especially when large groups of people live together in extremely crowded quarters under poor sanitary condition. Further, lack of regulation of antibiotic use, such as frequent self-prescribing and the availability of drugs without prescription local pharmacies or open air markets, has compounded this situation (Kunin, 1993).

The most common mechanism of β-lactam antibiotics resistance among both Gram-positive and Gram-negative bacteria involves the production of various β-lactamases, which clear the β-lactam ring of β-lactam antibiotics (Kim and Lee, 1994). There has been a significant increase in the incidence of β-lactamases-producing bacteria isolated from both hospital and community environments (Sutherland, 1990). Beta–lactamases inactivate β-lactam

antibiotics by covalently binding the carbonyl moiety of β-lactam ring and hydrolyzing its amino bound (Medeiros, 1997). An alternative approach to the β-lactamase problem has been the search for inhibitors of these enzymes which combined with a β-lactamase labile antibiotic and protect it from degradation and allow the β-lactam antibiotics to exert its antibacterial effect (Russell and Quesnel, 1983).

It is now clear that drug-resistance genes can also be transferred by conjugation in various species of Gram–positive bacteria, including *Streptococcus, Staphylocccus, Bacillus* and *Clostridium*. The genes conjugative activities are found on plasmids, prophages and transposons. The transfer of genetic information between bacteria by bacteriophage (bacterial virus) transduction occurs in Gram-positive and Gram-negative bacteria. Transformation is confined essentially to Gram-positive bacteria and works in this way: DNA released upon cell lysis may be absorbed by competent cells and integrated into their genomes. Furthermore, *Pseudomonas aeruginosa* as Gram-negative bacteria presents an especially intractable chemotherapeutic problem as it has unusually high intrinsic resistance to many antibiotics. This inherent resistance of Gram-negative bacteria seems to be associated with the impermeability of the complex outer layers of the cell envelope to some drugs, which prevents the attainment of an inhibitory concentration within the cell (Hugo and Russell, 2000). Acquired resistance is the acquired ability of bacteria to grow and multiply in the presence of antibiotics, which present in certain strains of a species or of a genus (Quintiliani and Courvalin, 1995).

The genetic characteristics and the physiological or the environmental condition depended of recombinant protein. The environmental conditions such as temperature, pH and dissolved oxygen of the host cell, especially on the stability of the chimeric plasmid-encoding extracellular β-lactamase in *Streptomyces. lividans* were evaluated in relation to the extracellular production of β-lactamase (Lee and Lee, 1994). A new type of β-lactamase has been isolated and characterized in *Streptomyces griseus* NRRL B-2682; the enzyme has membrane – bound and extracellular forms. Biochemical characterization of some of the properties of the enzyme showed that it belongs to the class A group of penicillinases. Comparison of the membrane – bound and extracellular forms of the β-lactamases suggests that they seem to be differently processed forms of the same enzyme (Deak et al*.,* 1998).

Tazobactam is a derivative of penicillinic acid sulfone and functions as a mechanism based inhibitor of several β-lactamases, blocking the effects of non-group 1 β-lactamase mediated resistance. Tazobactam is hypoallergenic and can function either irreversibly or in a transient manner. The offending agent in an allergic reaction to β-lactam/β-lactamase inhibitor combinations is always the β-lactam component. Piperacillin/tazobactam is inactive *in vitro* against Gram-negative bacterial isolates that harbor AmpC β-lactamases, however the combination drug retains its activity against broad spectrum β-lactamase producing and some extended spectrum β-lactamase producing enterobacteriaceae (Sanders and Sanders, 1996).

MATERIALS AND METHODS

Isolation of actinomycetes from soil

It has been described by Johnson et al. (1972). A suspension of the soil sample was prepared by shaking 10 g of the air dried soil in 100 ml sterile saline solution for about 20 min. Serial dilutions were made in the range of 10^{-1} to 10^{-8}. One ml of each dilution was transferred to plates of starch nitrate agar medium (Waksman, 1961), and spread evenly on the surface of agar using sterile glass spreader under aseptic conditions. Duplicate plates were prepared for each dilution. The plates were then incubated for 7 days at 30°C. Actinomycetes colonies were picked up and purified by streaking on the agar surface of the same medium.

Determination of minimum inhibitory concentration (MIC)

The MIC was determined according to Jennifer (2001).

Preparation of the McFarland standard

Firstly, 0.5 ml of 0.048 M $BaCl_2$ (1.17% w/v $BaCl_2.2H_2O$) was added to 99.5 ml of 0.18 M H_2SO_4 (1 % v/v) with constant stirring. The standard was distributed into screw cap tubes of the same size and with the same volume as those used in growing the broth cultures. The tubes were sealed tightly to prevent loss by evaporation. They were stored protected from light at room temperature. The turbidity was agitated vigorously on a vortex mixer before use. Standards were stored for up to 6 months, after which time they should be discarded. Alternatively, prepared standards can be purchased (Leslie et al., 1990).

Growth method

P. aeruginosa ATCC-10145 growth was transferred into ISB or equivalent that has been shown not to affect the performance of the test, and incubate broth with shaking at 35-37°C until the visible turbidity is equal to or greater than the 0.5 McFarland standard. Alternatively, an overnight broth culture can be used (Leslie et al., 1990).

Screening test

Actinomycete isolates were screened for their ability to produce β-lactamase inhibitory protein against *P. aeruginosa* ATCC-10145. Disks of 10 ml diameter of seventh day old cultures of actinomycetes isolates grown starch nitrate agar medium (Waksman, 1961) were cultured using sterile cork-borer and placed onto surface of seeded nutrient agar medium with an amoxicillin–resistant bacteria. Petri dishes were kept in a refrigerator for 1 h to permit diffusion of β-lactamase inhibitory protein. Inoculated plates were incubated at 37°C. The ability was determined by measurement of inhibition zone (mm) after 24 h.

Preparation of general (standard) inoculums

Stoppard test tubes containing 3-5 ml of sterile distilled water were prepared. The spores were transferred from a stock culture slant to one of the tubes of sterile distilled water. Sufficient spore material was transferred to make a very turbid suspension in the distilled water. The mycelia in the distilled water were triturated with a sterile glass rod or the tip of a sterile pipette.

Preparation of washed inoculum

Five ml of turbid suspension of spores or mycelium was prepared in sterile distilled water as described for general inoculum. 4–5 ml of this suspension were transfer to 50 ml of medium (Tryptone Yeast Extract Broth) in a 250 ml conical flask. The flask was incubated for 48 h at 25–28°C on shaking incubator. Vigorous agitation was used by sterile glass beads to break up the growth during 48 h. 5–10 ml of this fragmented broth culture were transferred into sterile centrifuge tubes equipped with sterile caps. Centrifugation was carried out and the supernatant broth was decanted. Sterile distilled water was added to restore the original volume in the centrifuge tube. The washed sediment was mixed and re-suspended with a sterile rod.

Identification of the most potent actinomycete isolate

The morphological and cultural characteristics of the most potent isolate were examined according to Shirling and Gottlieb (1966). For electron microscopy ISP4 agar was inoculated with spores of actinomycete isolate and incubated for 7 days at 28°C. A plug of the culture was removed and fixed in glutaraldehyde (2.5 % v/v), washed with water and post-fixed in osmium tetraoxide (1% w/v) for 1 h. The sample was washed twice with water and dehydrated in ascending ethanol before drying in a critical point drying apparatus (Polaron E3000) and finally coated in gold and examined in a JEOLISM 541OLV scanning electron microscope at 15 kv. B-lactamase inhibitor activity of culture supernatants was determined by removing a plug of agar from a plate inoculated with the test organism and introducing β-lactamase inhibitor containing suspension (200 µl) into the well. The plate was left at room temperature for 2 h to allow the liquid to diffuse into the media and was then incubated at 28°C for 18 h. The β-lactamase inhibitor activity of the supernatant was assessed as the zone of clearing (mm).

Optimization for maximum yield of the antifungal compound(s)

Effect of incubation period

Erlenmeyer flasks (250 ml) containing 100 ml sterile starch-nitrate broth, each were inoculated with the selected actinomycete isolate and incubated on rotary shaker (160 rpm) at 30 ± 2°C for various incubation periods (3 to 14 days). At each incubation period, 5 ml of the culture filtrate were then taken aseptically and the antifungal activity was measured using the inhibition zone method described earlier. Three plates were used within each incubation period foreach fungus (Isenberg, 1992).

Effect of incubation temperature

Erlenmeyer flasks (250 ml) containing 100 ml sterile starch-nitrate broth, each were inoculated with the selected actinomycete isolate and incubated on rotary shaker (160 rpm) for the optimum incubation period (7 days), at different temperatures (20 to 44°C). For each, 5 ml of the culture filtrate were then taken aseptically and the antifungal activity was measured using the inhibition zone method described earlier. Three plates were used for each temperature incubation for each fungus (Isenberg, 1992).

Effect of pH

Erlenmeyer flasks (250 ml) containing 100 ml sterile starch-nitrate broth, each were adjusted at various levels of pH (5 to 9) using a phosphate buffer before the sterilization and then inoculated with the selected isolate and incubated for the optimum incubation period (7 days) at the optimum temperature (28°C. For each, 5 ml of the culture filtrate were then taken aseptically and the antifungal activity was measured using the inhibition zone method described earlier. Three plates were used for each pH level for each fungus (Isenberg, 1992).

Effect of carbon source

In this experiment, glucose, fructose, sucrose, lactose, manose and galactose were tested as substitutive carbon sources. Starch of starch-nitrate medium was substituted with one of the tested carbon sources. Erlenmeyer flasks containing starch substituted starch-nitrate medium were inoculated with the selected isolate. The initial pH of the various media was adjusted at 6.8, before sterilization and the flasks were incubated for 7 days at 28°C on a rotary shaker (160 rpm). For each, 5 ml of the culture filtrate were then taken aseptically and the antifungal activity was measured by the inhibition zone method described earlier. Three plates were used for each carbon source for each fungus (Vanderzant and Splittstoesser, 1992).

Effect of nitrogen source

In this experiment, ammonium nitrate, ammonium sulphate, prolin, alanine, and histidin were tested as substitutive nitrogenous sources. Nitrogenous source of starch-nitrate medium was substituted with one of the tested sources. Erlenmeyer flasks containing starch-nitrate medium (potassium nitrate substituted with one of the tested sources) were inoculated with the selected actinomycete isolate. The initial pH of the various media was adjusted at 6.8, before sterilization and the flasks were incubated for 7 days at 28°C on a rotary shaker (160 rpm). For each, 5 ml of the culture filtrate were then taken aseptically and the antifungal activity was measured by the inhibition zone method described earlier. Three plates were used for each nitrogenous source for each fungus (Vanderzant and Splittstoesser, 1992).

Preparation of cell free extract

The modified starch nitrate broth medium (where the optimum factors were applied) was inoculated by most potent actinomycete isolate and then incubated at 28°C as a best incubation temperature for 7 days as a best incubation period on shaking incubator at 180 rpm for good aeration. After incubation period, the filtrate was centrifuged at 10,000 g for 10 min for precipitation of the mycelia. The supernatant (cell free extract) which contains β-lactamase inhibitory protein was taken and subjected to precipitation by ammonium sulphate.

Precipitation by ammonium sulphate

A range of ammonium sulphate was used (10 to 90%). In each step

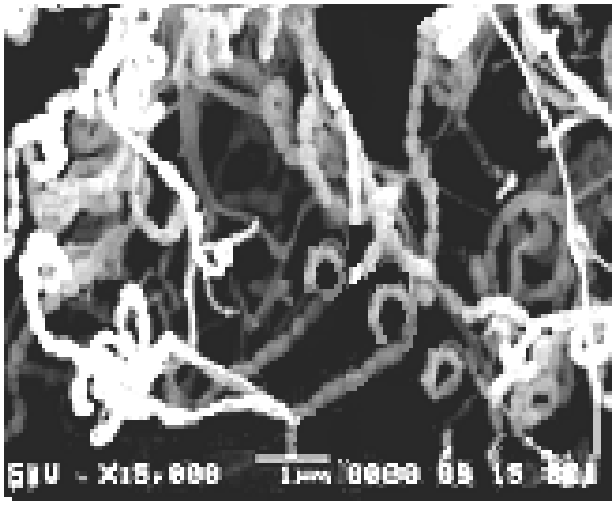

Figure 1. Scanning electron micrograph of *Streptomyces chromofuscus* (X 15,000).

with at definite concentration the mixture was left for 2 h at 4°C and was followed by centrifugation at 8000 g for 20 min at 4°C. The obtained precipitate was dissolved in 10 ml phosphate buffer (pH 7.5), the buffered extract was heated at 70°C for 10 min, cooled immediately and centrifuged at 8000 g for 20 min at 4°C. The resulting supernatant was brought to definite concentration saturation with ammonium sulphate. Further centrifugation and the obtained precipitate dissolved in 10 ml phosphate buffer (pH 7.5).

Quantitative estimation of total protein content

Firstly, 1 ml of the protein containing fraction was pipette then 5 ml of working alkaline copper reagent was added, standing was allowed at room temperature for 15 min (Lowery et al., 1951). Immediately, 0.5 ml of diluted folin reagent was mixed and allowed for standing at room temperature for 30 min. The colour intensity of the sample was measured by spectrophotometer at 280 nm. Blank is 5 ml of copper reagent with 0.5 ml of diluted folin reagent.

Ion-exchange chromatography

Only 100 g of DEAE-cellulose was placed in 1 L conical flask and then washed with distilled water followed by using 1 N HCl and water till the pH of the suspension was about 6.5. It was then washed several times with 0.5 M NaOH until no more colour was removed after the lat alkaline wash the resin was rinsed with distilled water until it was free from alkalinity. The washed resin was then suspended in about three volume of the phosphate buffer pH 7.5. At this stage, the supernatant fluid was almost clear indicating the removal of the fine particles; this precipitate was used for column packing.

Gel filtration

Ten gram of sephadex G-200 were dissolved in 400 ml phosphate buffer pH 7.5, boiled in water bath for 6 h, then cold to 50°C and packed in column (2.5 × 20 cm). Gel filtration was done basically according to Andrews (1969). Active fractions were pooled and dialyzed from ion-exchange column were applied to a sephadex G-200 column which was pre-equilibrated with phosphate buffer pH 7.5, at a flow rate of 5 ml/ 25 min.

Separation of β-lactamase inhibitor by SDS-PAGE

The β-lactamase inhibitory protein was subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis, which carried out with 10% polyacrylamide gel as described by Blackshear (1984), in the presence of 0.1% sodium dodecyl sulphate at pH 8.8 and 1 mM – dithiothreitol. Gel and gel buffers were prepared as described by Laemmli (1970) and See and Jackowski (1990). Gel was stained for showing the formed bands clearly by Coomassie Blue dye as described by Fairbanks et al. (1971).

Susceptibility test

Preparation of amoxicillin stock solutions

A suitable range of amoxicillin concentrations was chosen against *P. aeruginosa* (31.25 - 1000 µg.ml^{-1}). Stock solutions were prepared using equation W = C × V × 1000/P; where W is the weight of amoxicillin (mg), C is the concentration of amoxicillin (mg.L^{-1}), V is the volume of distilled water as a solvent (ml) and P is the potency of amoxicillin which equal 600 µg.mg^{-1} (Michael et al., 1993).

Preparation of β-lactamase inhibitory protein stock solutions

A suitable range of β-lactamase inhibitory protein concentrations was chosen (0.015 - 128 mg.L^{-1}), stock solutions were prepared using the same equation which mentioned before.

Preparation of agar dilution plates

Nutrient agar medium was prepared and cooled at 50°C. Amoxicillin concentrations (39.06 - 1000 µg.ml^{-1}) were added to each concentration of β-lactamase inhibitory protein (0.015 - 128 mg.L^{-1}). Then plates were poured (each one contains 20 ml) and left for solidification, and then inoculated by *P. aeruginosa*, and incubated at 30°C for 24 h. Examine the appearance of growth.

RESULTS

There are seventy *Streptomyces* isolates were isolated from different areas on Riyadh city and then purified by using all recommended media which mentioned in International Streptomycete Project (ISP). All these isolates were screened against *P. aeruginosa* ATCC-10145 which has MIC > 1000 µg.ml^{-1} for producing β-lactamase inhibitory protein. There were ten isolates which have an inhibitory effect against *P. aeruginosa* ATCC-10145. Nevertheless, one *Streptomyces* isolate was considered most potent where the highest activity of β-lactamase inhibitory protein (diameter of inhibition zone = 40 mm). According to morphological and cultural characteristics (Figure 1) and (Table 1) this isolate was identified as *S. chromofuscus*, as well as biochemical characteristics which also were studied.

Optimum factors were studied for maximum yield of β-lactamase inhibitory protein produced by *S. chromofuscus*

Table 1. Cultural characteristics of *Streptomyces chromofuscus*.

Medium	Growth	Color of aerial mycelia	Color of substrate mycelia	Color of diffusible pigment
TY extract broth	Good	263. L. Gray light gray	79.l.gy.YBr light gray yellowish brown	76-l.yBr light yellowish brown
YM extract agar	Poor	263. L. Gray light gray	90-gy.Y grayish yellow	No
OM extract agar	Good	263-L. Gray light gray	60-l.gy.Br light grayish brown	61-gy.Br grayish brown
ISS agar	Poor	10-PK. Gray pinkish gray	76-l.yBr light yellowish brown	No
GA agar	Good	264.L. Gray light gray	79.l.gy.YBr light gray yellowish brown	No
PY extract iron agar	Moderate	263-White white	73-p.oy pall orange yellow	No
T agar	Good	9-PK. White pinkish white	76-l.yBr light yellowish brown	79-l.gy.yBr light gray yellowish brown

TY = Tryptone Yeast; YM = Yeast Malt; OM = Oat Meal; ISS = Inorganic Salt Starch; GA = Glycerol Asparagine; PY = Peptone Yeast; T = Tyrosine

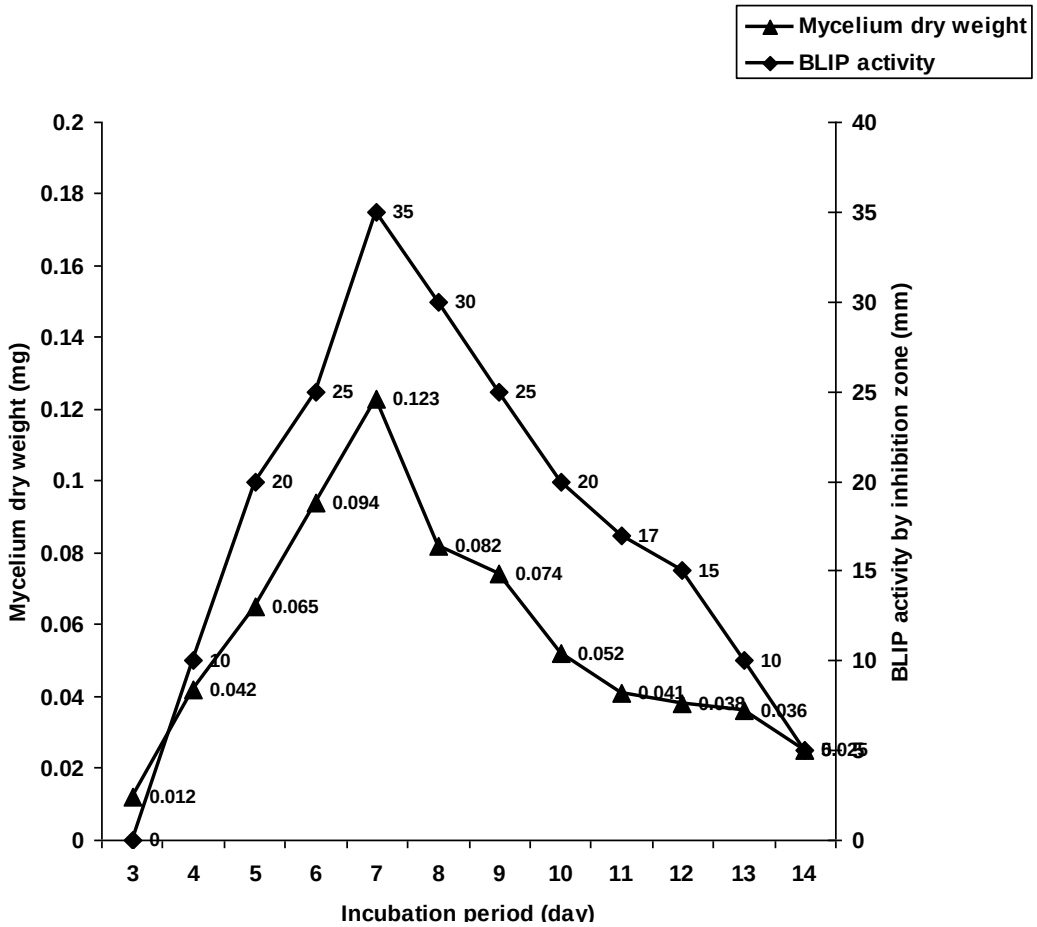

Figure 2. Effect of different incubation periods on data lactamase inhibitory protein activity of *Streptomyces chromofuscus* against *Pseudomonas aeruginosa* ATCC-10145.

included incubation period, incubation temperature, pH values, carbon sources and nitrogen sources. Optimization study resulted in 7th day is a best incubation period (Figure 2), 28°C is a best incubation temperature (Figure 3), 8.6 is a best pH value (Figure 4), galactose is a best carbon source (Figure 5) and prolin is a best nitrogen source (Figure 6).

Based on the results of optimization study, *S. chromofuscus* was inoculated in 10 L of starch nitrate broth medium for production of an inhibitor. The protein inhibitor was precipitated by saturated ammonium sulfate with a wide range of concentrations (10 to 90%), it was

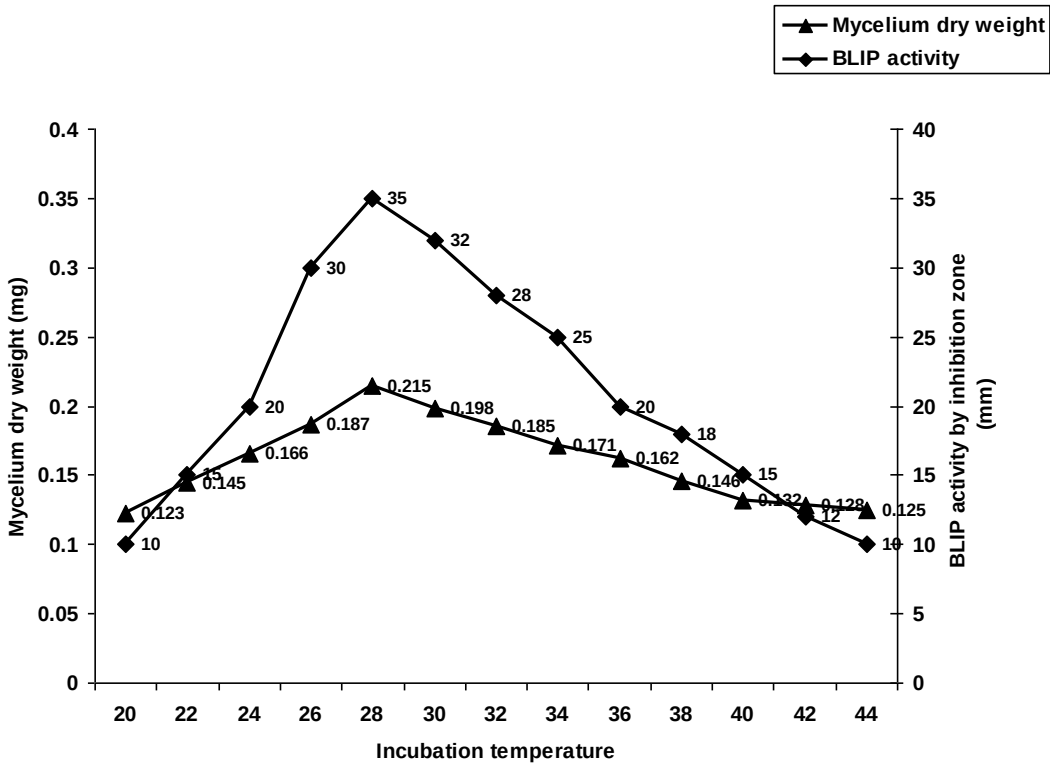

Figure 3. Effect of different incubation temperature on beta lactamase inhibitory protein activity of *Streptomyces chromofuscus* against *Pseudomonas aeruginosa*.

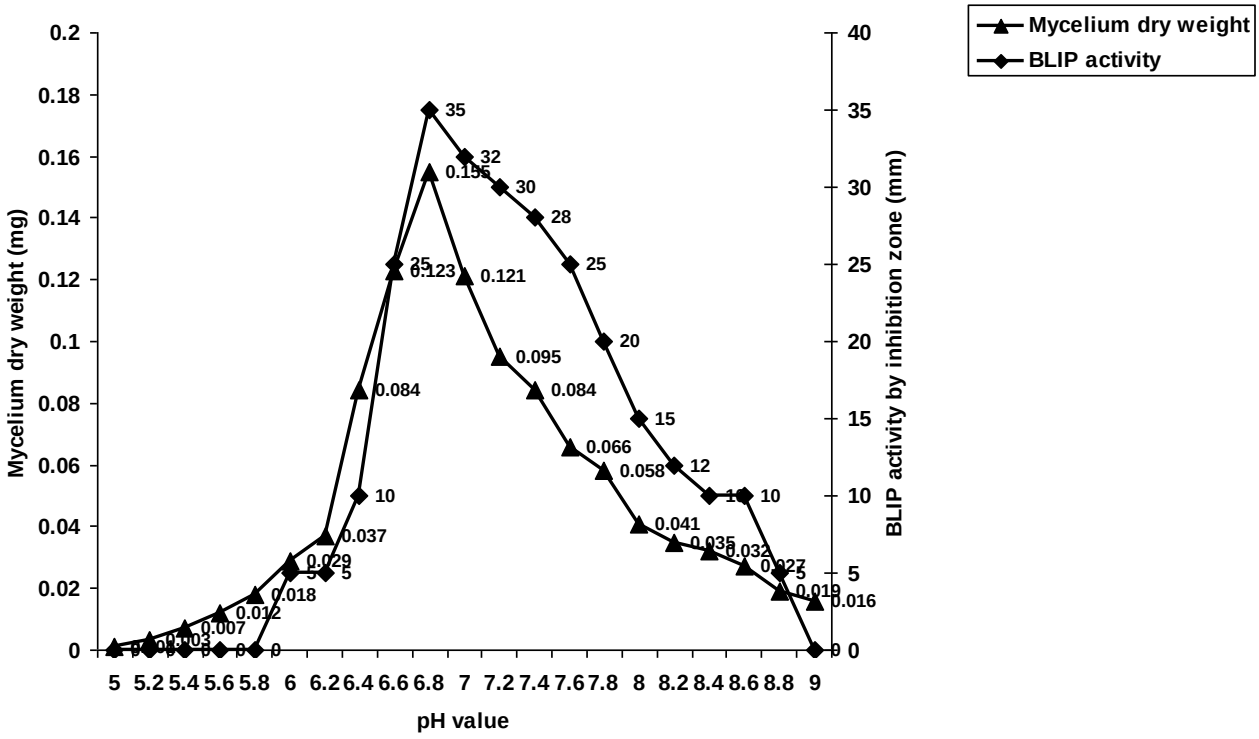

Figure 4. Effect of different pH values on beta lactamase inhibitory protein activity of *Streptomyces chromofuscus* against *Pseudomonas aeruginosa*.

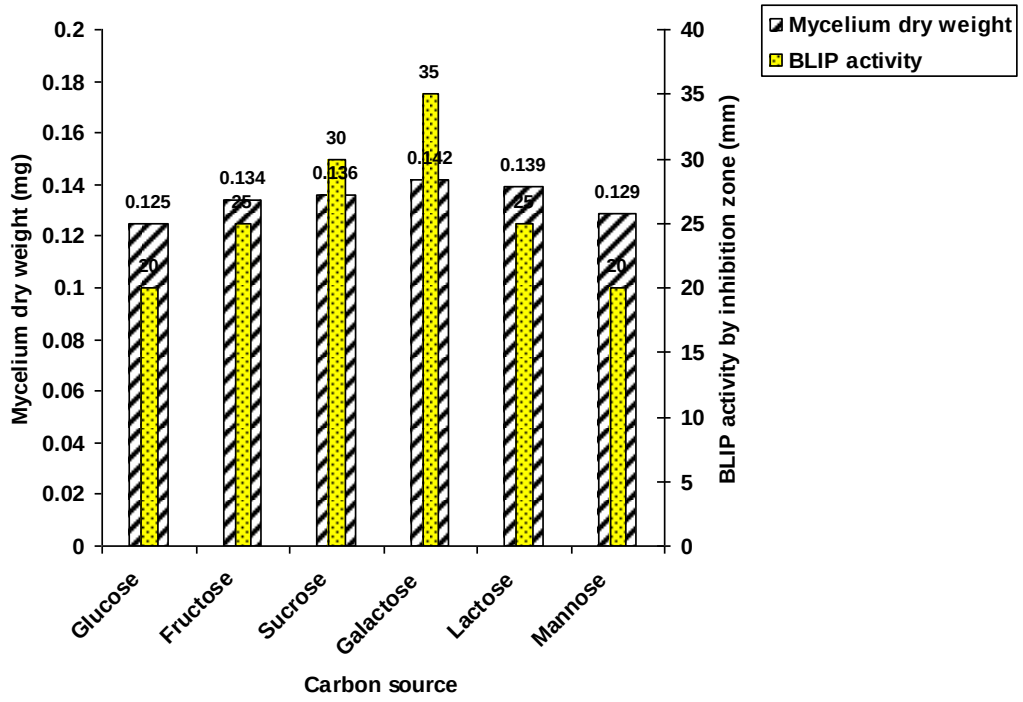

Figure 5. Effect of different carbon sources on beta lactamase inhibitory protein activity of *Streptomyces chromofuscus* against *Pseudomonas aeruginosa* ATCC-10145.

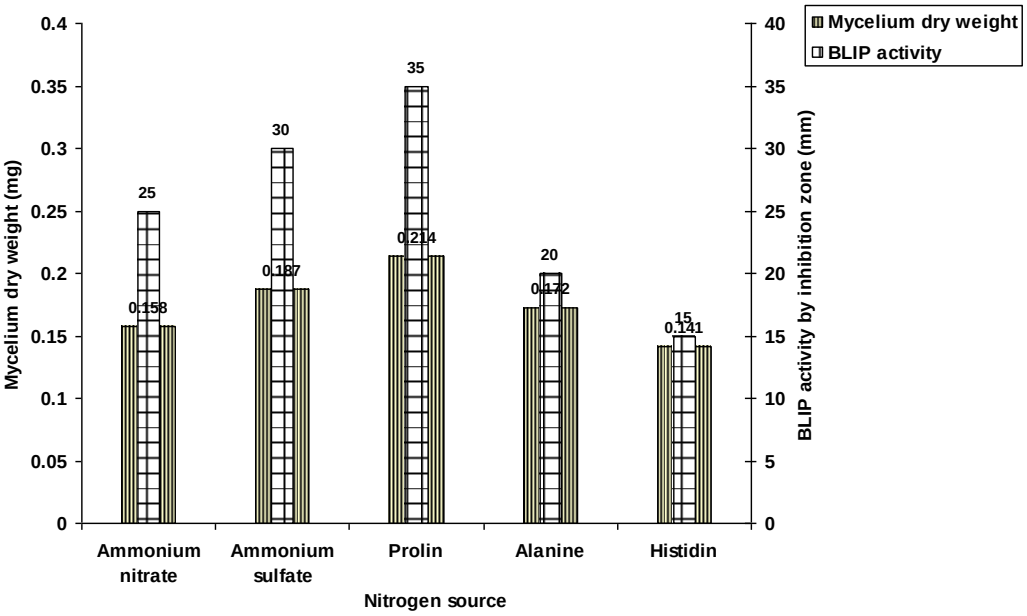

Figure 6. Effect of different nitrogen sources on beta lactamase inhibitory activity of *Streptomyces chromofuscus* against *Pseudomonas aeruginosa* ATCC-10145.

precipitated only at three fractions (30-50%), but the highest activity was presented at 40%. Total activity of β-lactamase inhibitory protein (u), total protein content (mg), specific activity (u/mg), fold purification and yield per cent were calculated with each fraction to determine the highest purification which correlated with the highest

Table 2. Precipitation and purification of β-lactamase inhibitory protein

$(NH_4)2SO_4$	Ac (mm)	TA of BLIP (u)	TPC (mg)	SA (u/mg)	FP	Yield (%)
Control	40.0	200.0	180.0	1.1	1.0	100.0
10.0	0.0	0.0	4.0	0.0	0.0	0.0
20.0	0.0	0.0	6.0	0.0	0.0	0.0
30.0	20.0	170.0	6.8	25.0	22.7	85
40.0	35.0	195.0	7.6	25.6	23.2	97.5
50.0	30.0	185.0	7.8	23.7	21.5	92.5
60.0	0.0	0.0	6.3	0.0	0.0	0.0
70.0	0.0	0.0	5.8	0.0	0.0	0.0
80.0	0.0	0.0	5.3	0.0	0.0	0.0
90.0	0.0	0.0	4.2	0.0	0.0	0.0

Ac = Activity; TA = Total Activity; BLIP = Beta Lactamase Inhibitory Protein; TPC = Total Protein Content; SA = Specific Activity; FP = Fold Purification.

Table 3. Susceptibility of *P. aeruginosa* ATCC-10145 to amoxicillin and tazobactam inhibitor.

Concentration of amoxicillin (µg/ml)	Concentration of clavulanic acid (mg/L)													
	128.0	64.0	32.0	16.0	8.0	4.0	2.0	1.0	0.5	0.25	0.125	0.062	0.032	0.015
1000	-	-	-	-	-	+	+	+	+	+	+	+	+	+
500	-	-	-	+	+	+	+	+	+	+	+	+	+	+
250	-	-	+	+	+	+	+	+	+	+	+	+	+	+
125	-	+	+	+	+	+	+	+	+	+	+	+	+	+
62.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+
31.25	+	+	+	+	+	+	+	+	+	+	+	+	+	+

+ = bacterial growth, - = no bacterial growth.

value of specific activity, fold purification and yield per cent (Table 2).

The active fractions of saturated ammonium sulfate (30-40-50 %) were pooled and exposed to ion (anion) exchange column chromatography using diethylaminoethyl cellulose G-25 (DEAE-cellulose). Elution was done by phosphate buffer at pH 7.5 and active fractions were dialyzed overnight against the same buffer (Figure 7). Also, active fractions were pooled and exposed to gel filtration column chromatography using sephadex G-200 and so dialyzed (Figures 8). Purified β-lactamase inhibitory protein was separated electrically by gel protein electrophoresis through one band at 32 KDa molecular weight (Figure 9).

The purified β-lactamase inhibitory protein was characterized as tazobactam inhibitor by using nuclear magnetic resonance (NMR), infrared (IR) and elemental analysis (data not shown). Susceptibility test was done according to Checkerboard method against amoxicillin-resistant *P. aeruginosa* ATCC-10145 (Table 3). Tazobactam inhibitor at 128 $mg.L^{-1}$ acted synergistically with amoxicillin at 125 $\mu g.ml^{-1}$ to inhibit the growth of amoxicillin-resistant *P. aeruginosa* ATCC-10145.

DISCUSSION

This study was begun with isolation of seventy *Streptomyces* isolates from different soils on Riyadh city. It is worth mentioning that, the genus of *Streptomyces* was chosen in particular due to its high metabolic ability for production of secondary metabolites which serving in many areas. These isolates were screened to inhibit the growth of amoxicillin- *P. aeruginosa* ATCC-10145. Screening test resulted in presence of only ten active isolates. The most potent isolate was identified as *S. chromofuscus*. Identification process had been carried out according to Shirling and Gottlieb (1966). Similar results had been recorded by various workers; (Andrews, 1969; Butterworth et al., 1979; Doran et al., 1990; Kim and Lee, 1994).

The optimum factors for producing a maximum yield of β-lactamase inhibitory protein were studied. Environmental factors included incubation period, incubation temperature and pH value, but nutritional factors included utilization of different carbon and nitrogen sources. The nutritional sources like carbon and nitrogen, as well as the environmental factors such as

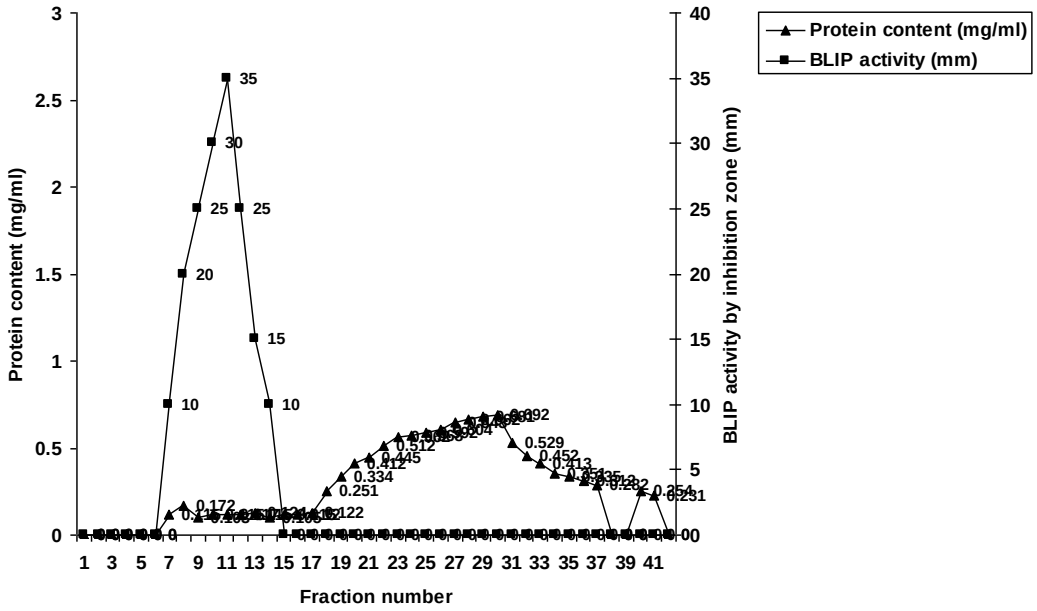

Figure 7. Ion exchange column chromatography of beta lactamase inhibitory protein of *Streptomyces chromofuscus*.

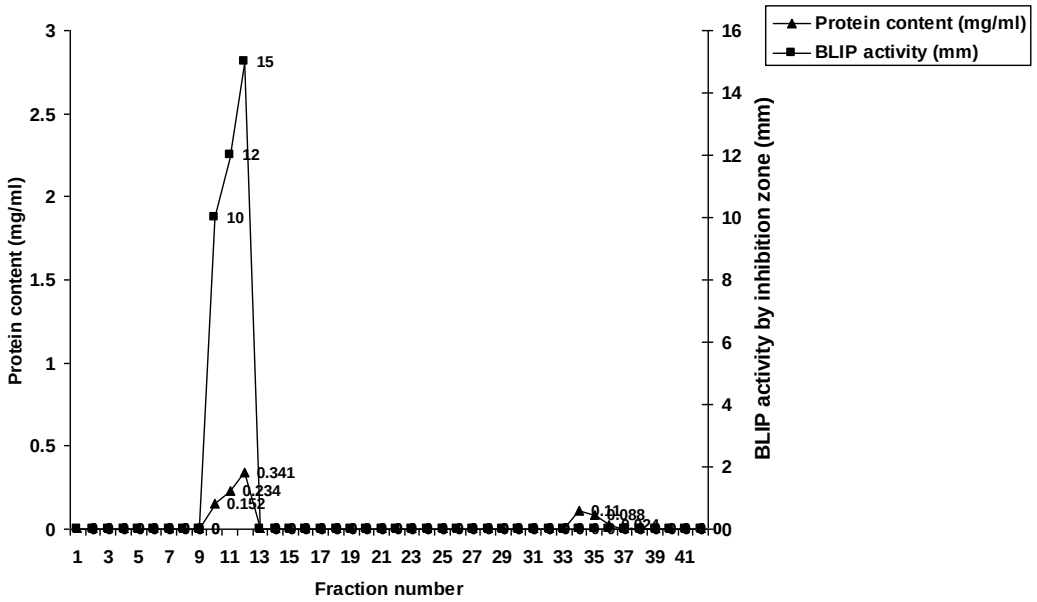

Figure 8. Gel filtration column chromatography of beta lactamase inhibitory protein of *Streptomyces chromofuscus*.

incubation period, pH and temperature are known to have a profound effect on antibiotic production by actinomycetes (Himabindu and Jetty, 2006). The β-lactamase inhibitory protein was precipitated by ammonium sulphate (40%), precipitated inhibitor was dissolved in 50 ml of 0.2 M phosphate buffer at pH 7.5. The enzyme inhibitor solution (50 ml) was subjected to dialysis in cellophane tubing for 18 h at 4°C. Similar results were obtained by (Orosz et al., 2005; Spencer et al., 2005).

The purification process was done through two steps. The first step was carried out by using DEAE–Cellulose(diethyl-aminoethyl cellulose) (2 × 70 cm) which considered anion exchange column chromatography. The

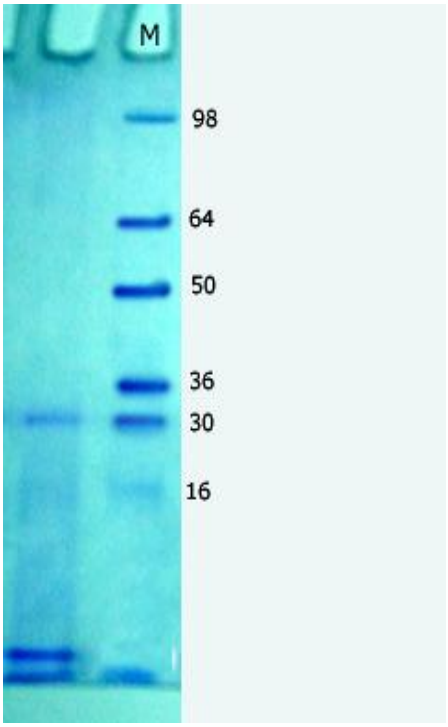

Figure 9. SDS-PAGE of beta lactamase inhibitory protein.

elution was exerted by phosphate buffer at pH 7.5. Sodium chloride was supplemented through this step by certain gradient (0.1 to 0.2 M) to activate the particles of cellulose. One peak illustrated due to presence of one type of β-lactamase inhibitory protein. The second step was carried out by using sephadex G-200 column chromatography (2 × 70 cm). Also one peak illustrated due to presence of purified inhibitory protein. Similar results were obtained by (Dale and Smith, 1971; Huang et al., 1996; Spencer et al., 2005).

A purified protein was dialyzed through overnight against phosphate buffer at pH 7.5. A purified protein was separated by polyacrylamide gel electrophoresis (10%) in the presence of sodium dodecyl sulphate (SDS). Protein was localized by staining with coomassie blue. The active protein was homogenous in disc gel electrophoresis and gave only one band of protein at 32 KDa molecular weight. Similar results were recorded by (Black-Shear, 1984; Huang et al., 1996).

The β-lactamase inhibitor was characterized as tazobactam (data not shown). The combination of tazobactam at MIC 128 $mg.L^{-1}$ and amoxicillin at MIC 125 $\mu g.ml^{-1}$ leads to inhibition the growth of amoxicillin-resistant *P. aeruginosa* ATCC-10145. Accordingly, the MIC of amoxicillin-resistant *P. aeruginosa* ATCC-10145 (> 1000 $\mu g.ml^{-1}$) decreased at 125 $\mu g.ml^{-1}$ in the presence of both tazobactam as β-lactamase enzyme inhibitor and amoxicillin as β-lactam antibiotic at concentrations mentioned earlier, respectively.

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