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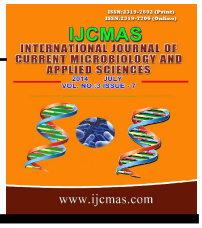

Original Research Article

Crude protein extract of Actinobacteria exhibits antibacterial activity against *Salmonella typhi*

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A B S T R A C T

Actinomycetes have proven to be a prominent source of natural products especially antibiotics. Many pathogens have developed resistance to an array of broad spectrum antibiotics, which requires extensive study on determining an alternative source from natural products. Our study focused on the screening of actinobacterial isolates for extra cellular protein extract that inhibits clinical isolate of *S. typhi*. Four out of twenty isolates were identified as *Streptomyces* sps. All isolates were subjected to extracellular protein extraction. Among four isolates the crude protein extract of isolate BR-13 exhibited prominent antibacterial activity against *S. typhi*. The molecular weights of the protein which shows activity against Salmonella were in the range of 40-65 KDa for BR-13 strain.

Keywords

Actinomycetes, Microbial peptide, SDS-PAGE, Antimicrobial activity

Introduction

Antibiotics, once hailed as the bedrock of modern medicine, may not work on infections in near future as their indiscriminate usage had spawned drug resistant bacteria, which motivated the pursuit of new biomolecules with novel mechanisms of action in the place of existing antibiotics.

Microbial enzymes are routinely used in many environmentally friendly and economic industrial sectors. Environmental pollution is no longer accepted as inevitable in technological societies. Over the past century, there has been a tremendous increase in awareness of the effects of pollution, and public pressure has influenced both industry and government. There is increasing demand to replace traditional chemical processes with biotechnological processes involving micro-organisms and enzymes such as pectinases (Bajpai 1999; Bruhlmann *et al*. 2000). Xylanases (Beg *et al*. 2000). Cellulases (Bajpai 1999), mannanase (Montiel *et al*. 2002), agalactosidase (Clarke *et al*. 2000), and raccases and ligninases (Bajpai 1999; Onysko 1993), which not only provide an economically viable alternative but are also more environmentally friendly (Viikari *et al*. 2001). Actinomycetes which are isolated from different types of soil are rich sources

of bioactive natural products with a great functional diversity, which are extensively used as pharmaceuticals and agrochemicals. The present work would be useful for the identification and production of effective antimicrobial biomolecules from actinomycetes, for treating drug resistant bacteria. The present work deals with the identification of actinobacterial crude protein extract for antibacterial activity against clinical isolate of *Salmonella typhi.*

Materials and Methods

Isolation of Actinomycetes from Reservoir Sediments

Soil samples were collected from different sites of fish cultivating reservoirs in Coimbatore district, Tamil Nadu India. The soil samples were collected from 5-25 cm depth in sterile plastic bags and transported aseptically to the laboratory, and air-dried, for one week at room temperature. Dried soil samples were finely grinded using a mortar and pestle. One gram of soil was subjected to heat treatment by heating at 60˚C for about 10 to 15 minutes. Isolation and enumeration of actinomycetes were performed by serial dilution and spread plate technique (Elliah *et al*, 2004). The plates were incubated at 37 ºC for 10 days. Based on the colony morphology, the actinomycetes cultures were selected on SCA medium.

Morphological characterization of isolates

The Actinomycetes colonies were characterized morphologically by following methods (Locci, 1989). The morphology of the isolated Actinomycetes were observed under the light microscope Gram staining as described in Bergey's Manual. The growth, aerial mycelium color, substrate mycelium, reverse side pigmentation and spore morphology were observed for identification of the isolates.

Slide culture method

This technique is used to find out the spore morphology and type of mycelium produced by the isolated strains. Actinomycetes agar plates were prepared. 4 x 4 mm of agar was sliced and placed over the surface of sterile glass slide on moist chamber with 60% ethanol. Isolates were inoculated on the four corners and incubated at 37 °C for 2-3 days. The plates were observed after three days of incubation and the cover glasses were stained using 0.4% crystal violet. Then, the cover glasses were air dried; mounted on a clean glass slide and observed under light microscope (Kozo Optics; Model: XJS900T) using 40x objective and the visible spore morphology was documented.

Biochemical analysis

Various Biochemical tests were performed for the identification of potent isolates are as follows Indole, methyl red and vogues Proskauer, citrate utilization and Hydrolysis of starch. (Berd, 1973;Kurup and Fink 1975;Gordan *et al*., 1966).

Analysis of Carbohydrate metabolism

The sugar utilization medium were prepared and sterilized. Six different sugar compounds (arabinose, inositol, lactose, mannitol, mannose, and xylose) were taken and 1% concentration of sugar was taken and added to their labeled medium respectively. The isolated Actinomycetes were inoculated in their respective medium. After inoculation the tubes were incubated at 37 ˚C for 4 days. After incubation the tubes were observed and the results were tabulated.

Crude Protein precipitation

Isolation of extra cellular protein from BR-13 grown on SCA medium:

Different methods were used to isolate the protein from fermented SCA medium, the broth was centrifuged at 5000 rpm to remove the cells and mycelium and the supernatant was filtered through Whattman filter paper.

a) TCA method

Methodology: 10 volumes of 10% TCA in acetone was mixed with culture filtrate sample and kept overnight at refrigerator. 10% of cold acetone was added, vortex and stored at -20 °C for 10 minutes. The mixture was centrifuged at 15000 rpm for 5 minutes. Remove supernatant allowed pellet to dry. Prevent complete desiccation of the protein pellet.

b) Methanol chloroform method

Methodology: 4 volume of methanol was added and vortex well. 1 volume of chloroform was added and vortex. 3 volume of distilled water was added and vortex. This mixture was centrifuged for 2 minutes at 15000 rpm the protein should be at the liquid interface, removed aqueous top layer and added 4 volume of methanol vortex. Centrifuged 2 minutes at 15000 rpm, remove as much liquid as possible without disturbing precipitates, speed-vac samples to dryness (or) dry under nitrogen.

c) Acetone method:

Methodology: Equal volumes of acetone and the filtrate extract was added and vortex. This mixture was kept overnight at 4°C and then centrifuged at 15000 rpm for 15 minutes. Remove supernatant allowed pellet to dry. Prevent complete desiccation of the protein pellet.

Total protein estimation by Lowry's method (Lowry *et al*., 1951)

20-100 µg/ml of standard bovine serum albumin was prepared. 200µl of standard solution was taken and mixed with 1ml of alkaline copper sulphate reagent and incubated room temperature for 10minutes. 100 µl of folin-ciocalteu reagent are added to each tube and incubated for an hour. The unknown sample was diluted and a known volume of sample was treated with alkaline copper and folin-ciocalteu reagent. The O.D values for all the samples were observed at 660 nm.

Molecular weight determination by SDS PAGE (Laemmli, 1970**)**

The crude protein samples were run on SDS PAGE to determine the molecular weight of the enzyme samples.

In gel digestion

Protein spots of interest were excised from gels using spot picker. The gel plugs were destained and dehydrated by washing three times (~10 minutes) with 25 mM NH4HCO3-50% acetonitrile (ACN) (1:1). Dried gel plugs were treated with freshly prepared 10 mM DTT in 50 Mm NH4HCO3 for 45 minutes at 56°C. After incubation, the DTT was replaced quickly by the same volume of freshly prepared 55 mM iodoacetamide in 50 mM NH4HCO3 for 30 minutes and then dehydrated with 100% ACN. The dried gel pieces were incubated for 12 hours at 37°C with 25 mM NH4HCO3 containing 0.02 µg/µl of mass spectrometry grade trypsin. The resulting peptides were extracted twice from the gel pieces, using peptide extraction buffer [1:1 mixture of 70% ACN and 0.1% trifluoroaceticacid (TFA)].

Antibacterial activity of proteins

About 20 µg of isolated protein was loaded on sterile disc and allowed to air dry overnight at chamber. Nutrient agar plates for test organism were prepared and the respective test pathogens were swabed over the surface of agar plates. The protein disc was placed over the surface of test organism swabbed plates. Nutrient agar plates were incubated at 37°C for 24 hrs and the plates were kept for 3 weeks. The zone of inhibition was recorded.

Result and Discussion

The isolates share their morphological similarities to the genus *Streptomyces*

The actinomycetes bacteria isolated from the fish cultivating reservoir exhibited different morphologies on the third day of incubation, the colonies appeared as chalky white spots, and the aerial mycelium arose from the surface of the agar plate in the form of single hyphae that subsequently branched heterogeneously. After five days they showed distinct differences in their aerial mycelia color, and some of the grey and white colonies showed fine droplets of extracellular exudates on their surface. 38 different isolates of actinomycetes with white pale pink brown and grey colonies were isolated from the fish cultivating reservoir. The aerial and substrate mycelia of all these colonies were observed by light microscopy and they were well branched and non-fragmentary.

Fig.1 Isolation plate showing BR-13 strain which inhibits human pathogens

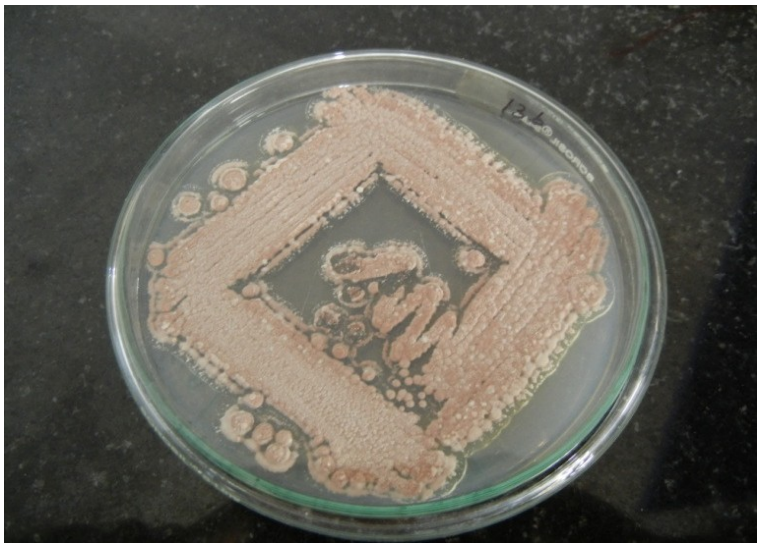

Table.1 Biochemical characterization of Actinomycetes isolates

Actinomycetes Isolates				
Biochemical Test	**BR-11**	**BR-12**	**BR-13**	**BR-14**
Hydrolysis of casein	-	-	-	-
Hydrolysis of Starch	-	-	-	-
Hyrdrolysis of xanthine	+	+	+	+
Nitrate Reduction.	-	+	+	-
Indole	-	-	-	-
MR	-	-	-	-
VP	-	-	-	+
Citrate	+	+	+	+
TSI	+	+	+	+

Note: + (Positive) – (Negative).

Protein estimation & Molecular Weight determination of BR-13 strain:

Table.2 Estimation of protein by Lowry's method

Samples							
	Standard					**Test**	
Absorbance at 660nm	**S_1(20 µg/ml)**	**S_2 (40 µg/ml)**	**S_3 (60 µg/ml)**	**S_4 (80 µg/ml)**	**S_5(100 µg/ml)**	**T_1**	**T_2**
	0.178	0.279	0.364	0.388	0.406	3.691	3.514

From the standard value, the concentration of the protein sample was observed to be 1.2 mg/ml.

Molecular weight determination by SDS-PAGE:

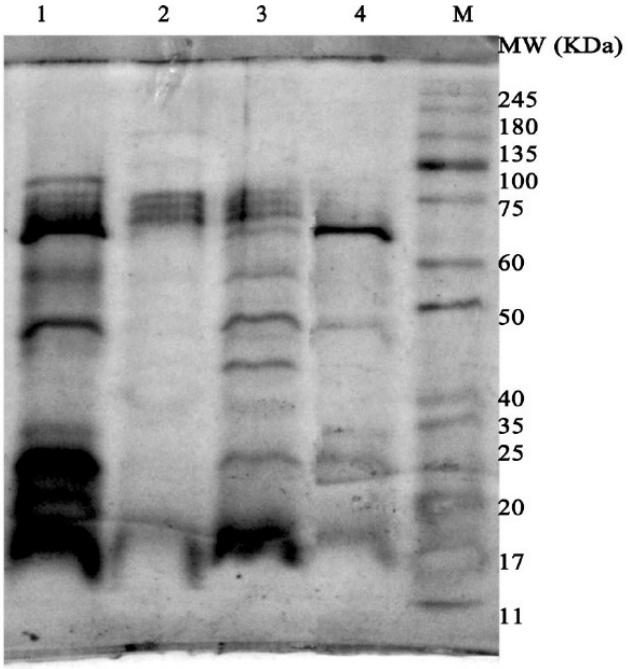

Fig 2 SDS-PAGE analysis of crude extracellular extract of actinomycete isolate Lane 1: Extract of BR-13, Lane 2: Biomass of BR-13, Lane 3: Crude extracellular protein of BR-13, Lane 4: Crude intracellular protein of BR-13.

The protein which is showing activity against *Salmonella* appears to be a high molecular weight peptide of 49 & 65 KDa based on SDS-PAGE gel. Cyclic depsipeptide has been reported from *Streptomyces* sp. Effective against Vancomycin resistant *Enterococci* (Rhee K H 2002).

Fig.3 Antibacterial activity of protein:

Fig.3 Antibacterial activity of protein. Extracellular protein from BR-13 with molecular weight of 65 & 49 KDa inhibits *Salmonella typhi.* The maximum zone of inhibition was observed on wells incorporated with 49 KDa protein. Whereas other wells showed very minimal zone of inhibition. The current strain BR-13 demonstrated antibacterial activity for *Salmonella* which may have significant potential as a possible therapy for a broad range of microbial infections. As the organism is a potent antibiotic producer but slow growing, further modification of fermentation medium to improve yield is validated.

The morphological characteristics like musty odor, spore formulation, dimorphic mycelia forms such as aerial and substrate mycelium, and the gram positive non-motile nature of the colonies indicated that they belong to the genus *Streptomyces* of the bacterial community. All the isolates could be grown on starch casein agar medium supplemented with 0.1% NaCl and they display proliphic mycelium formation and spore production within two days of incubation. The organisms also grow well on the other growth medium tryptone-yeast extract medium (ISP medium 1), yeast extract-malt extract agar (ISP medium 2) and inorganic starch agar (ISP medium 4). They utilized a wide range of carbon sources glucose, fructose, sucrose, lactose, starch, mannitol, arabinose, raffinose and all xylose. The utilization of starch, tributryn and casein showed that these isolates produce extracellular enzymes amylase, lipase and protease to metabolize the polymeric components of the nutrient mixture. Positive reaction for the catalase enzyme revealed that the isolates could withstand the stress conditions generated by reactive oxygen spp. The test on triple sugar iron agar revealed that these organisms would not produced gas and acid when incubated in carbon sources such as glucose, sucrose and lactose.

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Extracellular protein from BR-13 with molecular weight of 65 & 49 KDa inhibits *Salmonella typhi.* The maximum zone of inhibition is shown by 49 KDa protein. The current strain BR-13 demonstrated antibacterial activity for Salmonella which may have significant potential as a possible therapy for a broad range of microbial infections. As the organism is a potent antibiotic producer but slow growing, further modification of fermentation medium to improve yield is warranted.

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