ORIGINAL ARTICLE

Extraction and purification of antibiotic principles from the culture medium of selected microbial isolates from sea cucumber

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Abstract

Introduction: Three broad-spectrum antibiotic-producing microbes (named IF_{32}, IF_{52}, and CF_{42}) were identified and isolated from intestinal region and coelomic fluids of sea cucumber species collected from Kanyakumari district, Tamil Nadu. Biochemical characterization of the isolated microbes and optimization of culture characteristic (for maximum antibiotic productivity) were performed. The present study focuses on extraction and purification of antibiotic principles from the culture medium of the selected isolates. **Methodology:** The antibiotic fermentation was carried out in the optimized fermentation medium under optimum conditions separately for the isolated species. After separation of the cells by filtration, the fermented culture media were subjected to solvent extraction using 1-butanol, chloroform, ethyl acetate, and hexane. Obtained residues were subjected to antibiotic screening against *Staphylococcus aureus* (MTCC 1430) by disc plate method. Purification of the selected residues which showed antibiotic activity was done by column chromatography using silica gel as packing material. Collected fractions of the three antibiotic residues were subjected to bioautography to ensure the purification of the antibiotics. **Results:** The results show that chloroform, 1-butanol, and ethyl acetate are found to be suitable solvents for the extraction of antibiotics from the fermented cultures of IF_{32}, IF_{52}, and CF_{42}, respectively. Bioautography performed using fractions obtained from column chromatographic separation ensured the purification of the antibiotic principles.

Key words: Bioautography of residue, extraction of antibiotic, purification of antibiotic

INTRODUCTION

Search for newer antibiotic is an ever-ending process due to the development of resistance in the microbial species. Isolation of antibiotics from the natural sources and synthesis of antibiotic process are paralleling going on to come out with better antibiotics. As antibiotic isolation from soil microbes came to end due to repetitive occurrence of existing molecules rather than newer ones, other natural sources such as marine and plant microbes become prime choice.

Reports show that sea cucumber is rich in microbial flora and several molecules including antibiotic were isolated from them. As several reports show fishermen used sea cucumber to treat minor wounds during fishing, attempt has been made to explore the antibiotic potential of the microbial flora of sea cucumber of Indian region.[1-5]

Sea cucumbers were collected from Kanyakumari District, Tamil Nadu, and three microbial isolates (named IF_{32}, IF_{52}, and CF_{42}) were found to produce a broad spectrum of antibiotics among the microbial flora collected from intestinal and

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coelomic fluids. After performing the susceptibility studies, biochemical characterization of the isolates and optimization of cultural parameters (for maximum productivity) were performed. The isolated microorganisms were used for the production of antibiotics. This article focuses on the extraction and purification of antibiotics from the culture medium of the isolates (IF_{32}, IF_{52}, and CF_{42}).

METHODOLOGY

Fermentation of Antibiotic Principles

About 1% of 24 hours old cultures (IF_{32}, IF_{52} and CF_{42}), having optical density (OD) of 1 at 600 nm, were separately inoculated in Erlenmeyer flasks containing 400 ml of designed media. To carry out extraction of antibiotic principles using four different solvents, four set of Erlenmeyer flasks were used for fermentation for each isolated culture (IF_{32}, IF_{52} and CF_{42}). Evaluated fermentative condition profiles were followed for fermentative production of antibiotic principles during incubation. In this study, the fermented cultures were subjected to the extraction process to purify the antibiotic principles.[6]

Extraction of Antibiotic Residues

The fermented culture broths (IF_{32}, IF_{52}, and CF_{42}) were subjected to centrifugation at 4000 RPM for 20 min. Supernatant fluid was collected separately. Liquid-liquid extraction method was performed separately for each fermented culture using 1-butanol, chloroform, ethyl acetate, and hexane. Equal volume of solvent was mixed with fermented medium and vigorously shaken for 1 h. Organic layer was separated and evaporated to dryness by heating under reduced pressure using rotary evaporator. The obtained residues were named [Table 1], weighed, and checked for antibiotic activity against *Staphylococcus aureus* (MTCC 1430) by disc diffusion method.[6,7]

Evaluation of Antibiotic Activity

The obtained residues were reconstituted in sterilized water. Six-millimeter diameter of sterile discs were prepared and impregnated in the reconstituted residue solutions for overnight and dried aseptically in room temperature. Twenty-four hours old cultures of *S. aureus* (MTCC 1430) were prepared and OD of the organism was checked and adjusted to 1 at 600 nm. After adjusting the absorbance, the organism was inoculated on Mueller-Hinton agar media by spread plate method. Residue loaded dried 6-mm disc was placed over the inoculated Petri plates along with penicillin standard disc and incubated at 37°C for 24 h in inverted position. Zone of inhibition (ZOI) surrounding the discs was noted in millimeters. The residues which showed antibiotic activities were considered for purification process.[8,9]

Purification of Antibiotic Principles

The residues which showed antibiotic activity were loaded in silica gel column using chloroform, 1-butanol, and ethyl acetate, respectively, for CIF_{32}, BIF_{52}, and $EACF_{42}$ and eluted with the solvent systems separately [Table 2] for different antibiotic residues.

The obtained fractions were evaporated to dryness using rotary evaporator. The residues were reconstituted in dimethyl sulfoxide and tested for the presence of purified antibiotic principle by bioautography.[10]

Bioautography

Contact bioautography was performed to ensure the purification of antibiotic principle. Thin-layer chromatography (TLC) plates were run using methanol:acetone (9:1) solvent system for fractional residues obtained from CIF_{32}. Fractional residues of BIF_{52} were run on the TLC plates using the solvent system of acetone:water (8:2) and for $EACF_{42}$ fractional residue, methanol:water (5:5) was used. The developed TLC plates were dried

Table 1: Naming of extracted residues of isolates

Solvent used	Name of residue from IF_{32}	Name of residue from IF_{52}	Name of residue from CF_{42}
1-Butanol	BIF_{32}	BIF_{52}	BCF_{42}
Chloroform	CIF_{32}	CIF_{52}	CCF_{42}
Ethyl acetate	$EAIF_{32}$	$EAIF_{52}$	$EACF_{42}$
Hexane	HIF_{32}	HIF_{52}	HCF_{42}

Table 2: Solvent system used for elution

Name of the residue	Solvent system
CIF_{32}	Chloroform:methanol (95:5, 90:10, 85:15, and so on up to 0.00:100 ratio)
BIF_{52}	Acetone:1-butanol (5:95, 10:90, 15:85, and so on up to 100:0.0 ratio)
$EACF_{42}$	Methanol:ethyl acetate (5:95, 10:90, 15:85, and so on up to 100:0.0 ratio)

and observed under ultraviolet at 366 nm. Mueller-Hinton agar Petri plates were prepared and inoculated with 24 h old culture of *S. aureus* (MTCC 1430), having OD of 1 at 600 nm, by spread plate method. Then, the developed TLC plates were placed over the Mueller-Hinton agar Petri plates so that the developed chromatogram would be in contact with inoculated media. After 15 min, the TLC plates were removed and Petri plates were incubated at 37°C for 24 h in an upright position.[11]

RESULTS

Antibiotic Activity of Obtained Extracts

The following Table 3 shows the ZOI produced by the residues obtained from the solvent extraction of fermented culture mediums.

Table 3: Zone of inhibition produced by obtained extracts

Name of residue	ZOI in mm
BIF_{32}	0
CIF_{32}	31
$EAIF_{32}$	0
HIF_{32}	0
BIF_{52}	20
CIF_{52}	0
$EAIF_{52}$	0
HIF_{52}	0
BCF_{42}	0
CCF_{42}	0
$EACF_{42}$	27
HCF_{42}	0

ZOI: Zone of inhibition, mm: Millimeter

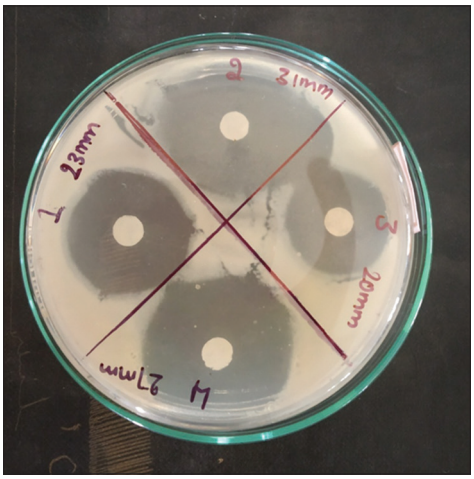

Figure 1: Zone of inhibition of penicillin, CIF_{32}, BIF_{52}, and $EACF_{42}$. (1) Penicillin. (2) CIF_{32}. (3) BIF_{52}. (4) $EACF_{42}$

The following Figure 1 was taken after performing the disc plate method only using the activity having residues along with standard disc of penicillin to get exact comparison

Figure 2: Bioautography of chloroform:methanol fraction of CIF_{32} in 60:40 ratio

Figure 3: Thin-layer chromatography of chloroform:methanol fraction of CIF_{32} in 60:40 ratio

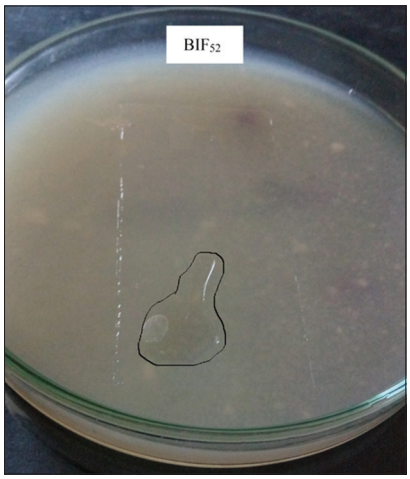

Figure 4: Bioautography of acetone:1-butanol fraction of BIF_{52} in 85:15 ratio

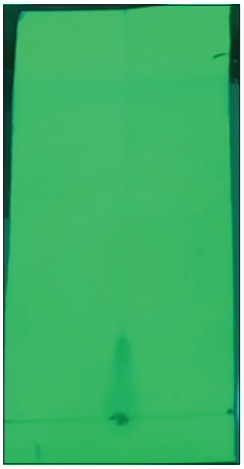

Figure 5: Thin-layer chromatography of acetone:1-butanol fraction of BIF_{52} in 85:15 ratio

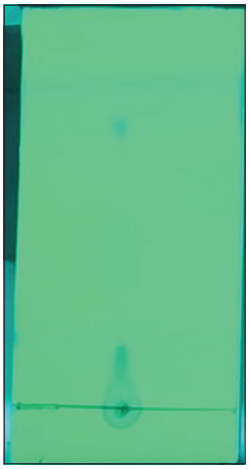

Figure 6: Thin-layer chromatography of methanol:ethyl acetate fraction of $EACF_{42}$ in 35:65 ratio

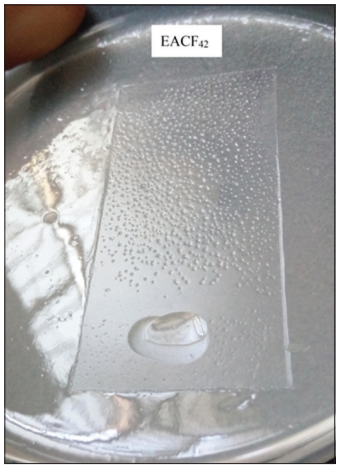

Figure 7: Bioautography of methanol:ethyl acetate fraction of $EACF_{42}$ in 35:65 ratio

of antibiotic activities of the residues. ZOI produced by penicillin, CIF_{32}, BIF_{52}, and $EACF_{42}$ is marked as 1, 2, 3, and 4, respectively.

Purification of Antibiotic Principles

Twenty fractional residues were obtained for each of CIF_{32}, BIF_{52}, and $EACF_{42}$ residues out of column chromatographic separation. They were all separately subjected to bioautography. The antibiotic principle presents in the 60:40 ratio fraction of chloroform:methanol from CIF_{32} found to be separated at TLC plate and produced ZOI surrounding the replica plated region of the spot ($R_f = -0.87$) in the inoculated plate [Figures 2 and 3]. The acetone:1-butanol fraction with the ratio of 85:15 found to contain the antibiotic principle from IF_{52} but not separated in the TLC plate as ZOI was observed at the reciprocated region of starting point of the TLC itself [Figures 4 and 5]. Though the antibiotic principle of $EACF_{42}$ was present in the methanol:ethyl acetate eluted fraction with the ratio of 35:65, but not separated in TLC plate [Figure 6]. This is evident that the ZOI was observed only at starting point of the Bioautography [Figure 7].

DISCUSSION

The results of this study reveal that chloroform, 1-butanol, and ethyl acetate are found to be suitable solvents for the extraction of antibiotic principle from fermented media of IF_{32}, IF_{52}, and CF_{42}, respectively. Purification of antibiotic principle from IF_{32} could be achieved out of this work. For rest of the two isolates, it was only partially successful.

CONCLUSION

The present work successfully isolated the antibiotic principle from isolate IF_{32}. As antibiotic principles were present in the extracted residues of IF_{52} and CF_{42} fermented medium, further attempt will be made to purify the antibiotic principles using different elutants or using different chromatographic methods.

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REFERENCES

1. Alipiah NM, Ramli NH, Low CF, Shamsudin MN, Yusoff FM. Protective effects of sea cucumber surface-associated bacteria against *Vibrio harveyi* in brown-marbled grouper fingerlings. Aquacu Environ Interact 2016;8:147-55.
2. Sha Y, Liu M, Wang B, Jiang K, Sun G. Gut bacterial

diversity of farmed sea cucumbers *Apostichopus japonicus* with different growth rates. Microbiology 2016;85:109-15.
3. Bogatyrenko E, Buzoleva L. Characterization of the gut bacterial community of the Japanese sea cucumber *Apostichopus japonicus*. Microbiology 2016;85:116-23.
4. Gao ML, Hou HM, Zhang GL, Liu Y, Sun LM. Bacterial diversity in the intestine of sea cucumber *Stichopus japonicas*. Iran J Fish Sci 2016;16:318-25.
5. Pringgenies D, Yudiati E, Djunaedi A, Santosa GW, Koesoemadji, Explorations of symbiotic microbe from sea cucumber gut as an anti-multi-drug resistant microbe agent for utilization in hand sanitizer products. AACL Bioflux 2019;12:737-47.
6. El-Naggar N, El-Bindary A, Abdel-Mogib M, Salah N. *In vitro* activity, extraction, separation and structure elucidation of antibiotic produced by *Streptomyces anulatus* NEAE-94 active against multidrug-resistant *Staphylococcus aureus*. Biotechnol Biotechnol Equip 2017;31:1-13.
7. Singh V, Haque S, Khare S, Tiwari AK, Katiyar D, Banerjee B, *et al*. Isolation and purification of antibacterial compound from *Streptomyces levis* collected from soil sample of North India. PLoS One 2018;13:e0200500.
8. Pudi N, Varikuti GD, Badana AK, Gavara MM, Singh SK, Malla R. Studies on optimization of growth parameters for enhanced production of antibiotic alkaloids by isolated marine actinomycetes. J Appl Pharm Sci 2016;6:181-8.
9. Al-Ghazali LH, Omran R. Optimization production conditions of antibacterial metabolite from *Streptomyces* sp. Asian J Pharm Clin Res 2017;10:386-91.
10. Kumar PS, Duraipandiyan V, Ignacimuthu S. Isolation, screening and partial purification of antimicrobial antibiotics from soil *Streptomyces* sp. SCA 7. Kaohsiung J Med Sci 2014;30:435-46.
11. Mounyr B, Sadiki M, Saad I. Methods for *in vitro* evaluating antimicrobial activity: A review. J Pharm Anal 2015;6:71-9.

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