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Antibacterial Potency of Bacterial Metabolites against ESKAPE Pathogens

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Abstract: The current study focused on the isolation and characterization of bacterial strains from industrial effluent outfall channel collected from Vashi, Navi Mumbai, Maharashtra, India, with the aim to explore their secondary metabolites against ESKAPE pathogens. The effluent sample, with a pH of 6.5 and a temperature of 34°C, was subjected to serial dilution, and seven bacterial isolates were identified on nutrient agar plates, which include *Bacillus*, *Escherichia coli*, *Acinetobacter*, *Pseudomonas*, *Staphylococcus*, *Proteus*, and *Micrococcus*. The isolates were purified and their morphological and biochemical characteristics were determined. Broth fermentation of each isolate was performed, followed by centrifugation and liquid-liquid extraction using ethyl acetate for secondary metabolite extraction. The antibacterial activity of the extracts was evaluated using the disc diffusion method against ESKAPE pathogens, which include *Enterococcus faecium*, *Streptococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species. The results revealed that *Bacillus* spp. exhibited the highest antibacterial activity among the isolates, showing broad-spectrum inhibition against both Gram-positive and Gram-negative bacteria.

Keywords: Bacterial secondary metabolites, Liquid-liquid extraction, Antibacterial, ESKAPE pathogens

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Introduction

When antibiotics were first introduced over half a century ago, they were hailed as "miracle drugs." However, their widespread use has quickly led to overuse, diminishing their efficacy as pathogens have evolved resistance. Over the past decade, it has become increasingly evident that antibiotics are losing their potency due to the rapid emergence of drug-resistant pathogens. This challenge is further exacerbated by the slow rate at which new antibiotics are developed and brought to market. Bacteria can acquire resistance through various mechanisms, making it difficult to address this growing problem.

Pharmaceutical companies have recently renewed efforts to develop novel antibiotics, driven by the urgent need to combat resistant

strains such as methicillin-resistant *Staphylococcus aureus* (MRSA). Natural products remain a critical source of chemical diversity and are essential components of modern pharmaceuticals (Baker *et al.*, 2007). However, many currently available antibacterial and antifungal agents are associated with undesirable toxicity, and their extensive use has accelerated the emergence of drug-resistant strains, leading to treatment failures in both clinical and agricultural settings (Saleem *et al.*, 2010).

While several thousand microbial products with potential medicinal applications have been discovered, many remain unexplored. The rise of multi-drug-resistant pathogenic bacteria poses a significant threat to the treatment of infectious diseases, necessitating the urgent discovery of new, effective drugs. Despite this need, no new antibiotic compounds have been introduced into clinical practice in the past two decades, further emphasizing the critical demand for novel therapies (Butler and Buss, 2006; Luzhetskyy *et al.*, 2007; Awad *et al.*, 2012).

Industrial wastewater is characterized by a high microbial load and diversity due to the presence of substantial total suspended solids, toxic and inhibitory substances, salts, heavy metals, as well as oils and greases (Porwal *et al.*, 2015; Karn and Kumar, 2019). The elevated biological oxygen demand (BOD) in industrial effluents indicates a significant microbial diversity. Predominant microbial species found in the activated sludge of such effluents are gram-negative bacteria, including genera such as *Achromobacter*, *Alcaligenes*, *Bacillus*, *Flavobacterium*, *Micrococcus*, and *Pseudomonas* (Ventola, 2015; Silva-bedoya *et al.*, 2016; Abdulsalam *et al.*, 2020). These bacteria actively degrade industrial waste, converting it into various by-products. During this process, they also produce secondary metabolites (SMs), some of which possess pharmaceutical properties. The extraction and study of these pharmaceutically active compounds from industrial wastewater are becoming increasingly important (Couto *et al.*, 2019).

Secondary metabolites (SMs) produced by various microbial strains are complex compounds with diverse biological activities, giving them significant industrial relevance. The production of SMs by specific microbes is critical, as these metabolites often act as inhibitors against competing microorganisms (Lewis, 2013; Kelsic *et al.*, 2015). Antibiotics, one of the most well-known classes of SMs, are derived from microbial sources (Al-maadheed *et al.*, 2019). However, a decline in efforts to search for and isolate new antibiotics has created a significant research gap, especially as bacterial resistance continues to rise, presenting a major global challenge in infection control (Wright, 2014). The growing issue of antibiotic resistance has driven researchers to intensify the search for novel antibiotics, although only a few have been approved for clinical use in the past two decades (Wright, 2014; WHO, 2015, 2022).

This study aimed to isolate and identify bacteria from industrial effluents and extract SMs capable of inhibiting the growth of pathogenic microorganisms, including *Enterococcus faecium*, *Streptococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species.

Materials and Methods

Sample collection

100 ml of industrial effluent outfall sample was collected in sterile stoppered bottle (which was previously cleaned with non-ionic detergent) from Vashi (lat, lon: 19°05'31.4"N, 73°00'56.6"E), Navi Mumbai, Maharashtra, India and sample was stored at 4ºC for further study.

Isolation and identification of bacteria

Serial dilution of the sample was carried out under aseptic conditions. From each dilution, 0.1 ml of diluted sample was plated on nutrient agar (NA). The plates were further incubated at 37ºC for 24 h. After incubation each single colony was selected and separated from the plates and sub-cultured on

to the fresh nutrient agar media for isolation and purification. Each bacterial isolate was studied for morphological and biochemical characteristics for identification. The purified colonies were further preserved in vials with 20% glycerol at -80ºC.

Test bacteria

Test organisms such as *Enterococcus faecium* MTCC 2763, *Streptococcus aureus* ATCC 33591, *Klebsiella pneumonia* MTCC 432, *Acinetobacter baumannii* ATCC 19606, *Pseudomonas aeruginosa* ATCC 27853, and *Enterobacter species* MTCC 2296 were obtained from MTCC, Chandigarh, Punjab, India.

Fermentation and extraction of metabolites

All bacterial isolates were inoculated into Nutrient Broth and incubated at 30°C on a shaker for seven days. Following fermentation, the culture broth was centrifuged at 5000 rpm for 10 min to separate the filtrate, which was then passed through a Millipore filter. The sterile filtrate was aseptically transferred into a glass bottle and stored at 4°C for further analysis (Remya and Vijayakumar, 2008; Khattab *et al.*, 2017).

A 200 ml portion of the cell-free filtrate was extracted three times using ethyl acetate, with a 1:1 (v/v) solvent-to-filtrate ratio. The mixture was vigorously shaken for 20 min, and the ethyl acetate layer, containing the metabolite, was separated from the aqueous phase using a separating funnel. The ethyl acetate phase was evaporated at 40°C to dryness, yielding a brown crude extract, which was collected in a vial (Jayne *et al.*, 2019; Abdel-nasser *et al.*, 2023).

Antibacterial activity of the extract by disc diffusion method

The antibacterial activity of the extract was evaluated using the disc diffusion method. Test bacterial cultures were standardized to a 0.5 McFarland turbidity and evenly spread onto Mueller-Hinton agar plates using a sterile swab moistened with an 18-hour-old bacterial suspension. After allowing the plates to dry for 15 min, discs impregnated with the crude metabolite extract were placed on the agar surface (Bauer *et al.*, 1966). Positive and negative controls were used for reference. The plates were subsequently incubated at 37°C for 24 h. Following incubation, the zones of inhibition were observed and measured in millimetres.

Results

Sample

The industrial effluent outfall sample was collected from Vashi, Navi Mumbai, Maharashtra, India. The pH of the sample was found to be 6.5 and temperature of the sample was 34ºC.

Identified bacterial isolates

A total of 7 bacterial isolates were identified from industrial effluent sample. They were isolated in pure culture on N.A. agar media. The morphological and physiological characteristics of the bacterial isolates are given in Tables 1 and 2. Identified isolates were *Bacillus*, *Escherichia coli*, *Acinetobacter*, *Pseudomonas*, *Staphylococcus*, *Proteus*, *Micrococcus* and they were named alphabetically as A, B, C, D, E, F, G, respectively as shown in Table 1. Genus level biochemical identification of these bacterial isolates has been mentioned in Table 2.

Fermentation and metabolite extraction

Broth fermentation was conducted for all seven bacterial isolates, designated as A, B, C, D, E, F and G. The cell-free filtrate was successfully obtained through centrifugation and filtration techniques. Liquid-liquid solvent extraction was performed using ethyl acetate as the extraction solvent. A brown crude extract containing the metabolites was collected in a vial. The yield of the metabolites was quantified and recorded in Table 3.

Antibacterial activity of bacterial secondary metabolites

The antibacterial activity of the extract against ESKAPE test organisms was evaluated by measuring the inhibition zones. The activity was tested at metabolite concentrations ranging from 2-10 µg/ml. The inhibition zones and corresponding antibacterial activity are detailed in

Table 1: Colony characteristics of bacterial isolates

Characteristics	Shape	Size	Margin	Colour	Opacity	Elevation	Surface	Consistency
A	Circular	1mm	Regular	Brownish-white	Translucent	Raised	Smooth	Watery
B	Round	Pinpoint	Regular	Colourless	Transparent	Flat	Smooth	Watery
C	Round	Pinpoint	Regular	Colourless	Transparent	Raised	Glistening	Mucoid
D	Round	Pinpoint	Regular	Off-white	Opaque	Convex	Rough	Brittle
E	Circular	1mm	Regular	Yellowish-white	Translucent	Flat	Smooth	Viscid
F	Round	Pinpoint	Regular	White	Opaque	Raised	Rough	Brittle
G	Rhizoid	2-3mm	Irregular	Orangish	Opaque	Raised	Rough	Brittle

Table 2: Biochemical characteristics of isolated bacteria

Characteristics	A	B	C	D	E	F	G
Indole	-	+	-	-	-	-	-
Methyl Red	-	+	-	-	+	+	-
Voges-Proskauer	+	-	-	-	+	-	-
Citrate utilization	+	-	+	+	+	+	-
TSI slants	+	+	+	-	+	-	-
Mannitol	+	+	-	+	+	-	+
Sucrose	+	+	-	-	+	-	+

+ positive; - negative

Table 3: Yield of ethyl acetate metabolite extract obtained by broth fermentation

Metabolite extract	Yield mg/200 ml
A	180.6
B	78.4
C	102.5
D	58.6
E	104.2
F	68.9
G	70.4

Table 4 and Figure 1. Among the seven bacterial isolates obtained from the industrial effluent, *Bacillus spp*. exhibited the highest antibacterial activity against both Gram-positive and Gram-negative bacteria compared to the other strains.

Discussion

The present study successfully isolated and characterized bacterial strains from industrial effluent outfall channel collected from Vashi, Navi Mumbai, Maharashtra. The effluent sample exhibited a neutral pH (6.5) and a moderately high temperature (34°C), which likely influenced the microbial diversity. A total of seven bacterial isolates were identified, including species of *Bacillus*, *E. coli*, *Acinetobacter*, *Pseudomonas*, *Staphylococcus*, *Proteus*, and *Micrococcus*, suggesting a diverse microbial community capable of thriving in industrial effluent conditions. This diversity is consistent with previous studies, where industrial effluents have been shown to harbour bacteria with adaptive capabilities due to

Table 4: Antibacterial activity of crude metabolites extracted by broth fermentation

Bacterial isolates from IE	Concentration in µg/ml	*Enterococcus faecium*	*Streptococcus aureus*	*Klebsiella pneumonia*	*Acinetobacter baumannii*	*Pseudomonas aeruginosa*	*Enterobacter species*
A	2	20	12	10	12	10	20
	4	22	12	18	24	12	26
	6	24	20	20	26	12	28
	8	32	24	22	26	26	32
	10	32	32	26	32	28	32
B	2	-	-	-	-	-	-
	4	18	-	-	-	-	20
	6	20	16	-	20	-	22
	8	22	18	18	22	24	24
	10	24	20	20	24	24	24
C	2	-	-	-	-	-	-
	4	20	-	20	-	-	26
	6	22	18	20	24	20	26
	8	22	18	22	24	24	28
	10	24	20	22	26	24	32
D	2	-	-	-	-	-	-
	4	18	16	-	18	-	20
	6	20	18	18	20	22	22
	8	24	18	22	20	22	24
	10	26	20	22	24	24	24
E	2	-	-	-	-	-	-
	4	20	20	18	24	22	26
	6	24	24	20	24	24	28
	8	26	28	20	26	26	32
	10	28	28	22	30	26	32
F	2	-	-	-	-	-	-
	4	18	14	-	20	-	20
	6	18	16	18	22	20	22
	8	20	16	20	22	22	22
	10	22	18	20	24	22	24
G	2	-	-	-	-	-	-
	4	18	16	-	20	-	24
	6	20	18	18	20	22	24
	8	22	18	18	22	24	26
	10	24	20	20	24	24	26

the presence of high levels of organic matter and pollutants. The morphological and physiological analysis of these isolates provided crucial insights into their taxonomy, which was further confirmed through biochemical identification. Each strain was isolated in pure culture on nutrient agar, indicating the robustness of these bacteria in surviving and proliferating in potentially toxic environments.

The fermentation of these isolates and subsequent extraction of secondary metabolites (SMs) using ethyl acetate yielded crude metabolites, with quantification results presented in Table 3. The choice of ethyl acetate for liquid-liquid solvent extraction is widely accepted for its efficiency in isolating metabolites from aqueous solutions, particularly in studies focusing on antibiotic discovery. The production of brown crude extract, containing these SMs, further supports the idea that industrial effluent bacteria can serve as reservoirs for bioactive compounds.

The antibacterial activity of the metabolite extract was assessed against the ESKAPE pathogens, a group of clinically relevant bacteria known for their role in multidrug-resistant infections. Among the seven isolates, *Bacillus spp*. exhibited the most significant antibacterial activity, demonstrating broad-spectrum inhibition

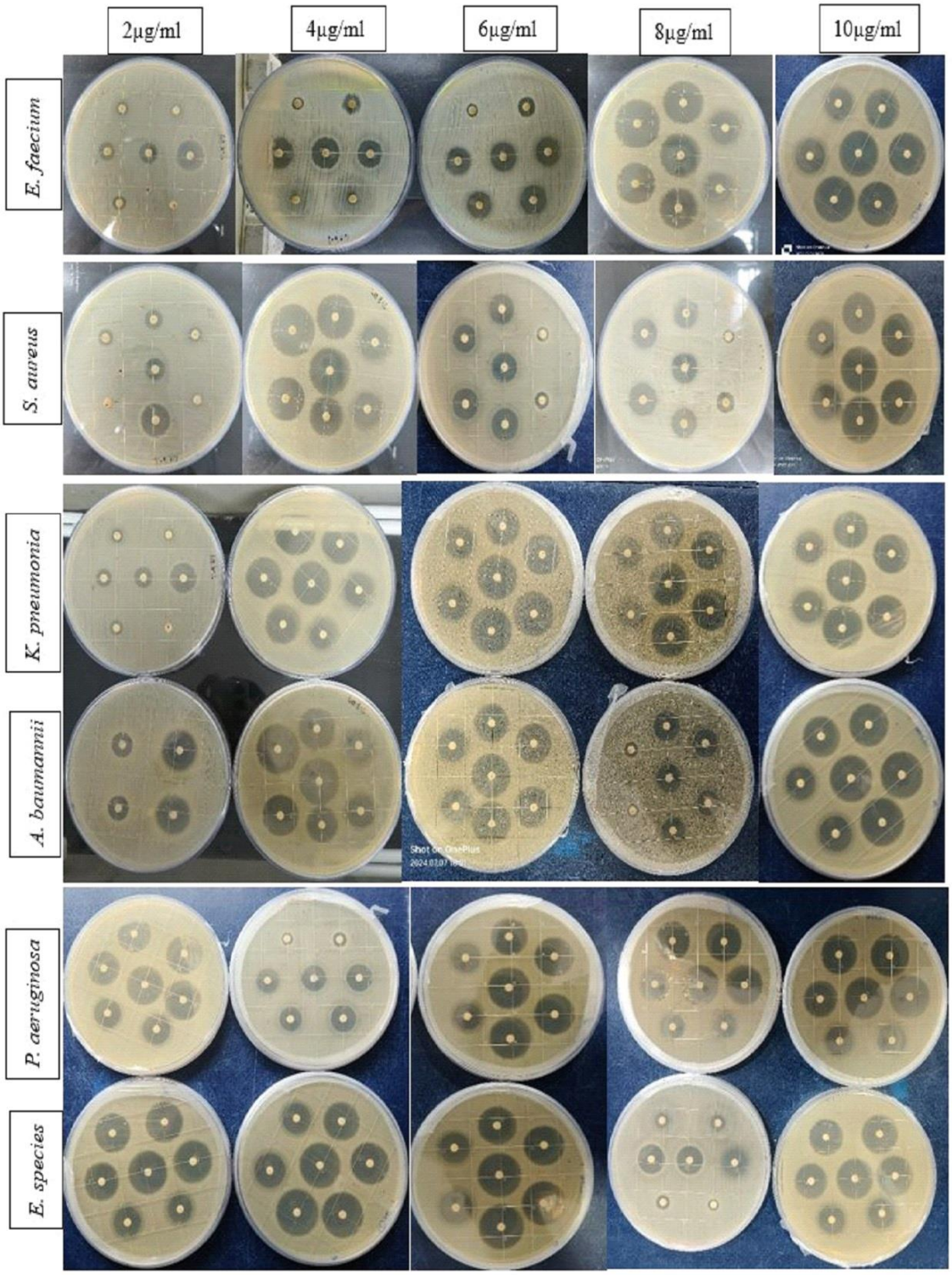

Fig. 1: Antibacterial activity of metabolite solvent extracts against ESKAPE test organisms.

against both Gram-positive and Gram-negative bacteria. This finding is particularly important as *Bacillus* species are known producers of various antibiotics, such as bacitracin and polymyxin (Sansinenea and Ortiz, 2011). Previous studies on *Bacillus* species have documented similar antibacterial effects, although targeting different bacterial species (Kumar *et al.*, 2011). For example, Esikova *et al.* (2002) reported that thermophilic *Bacillus* strains VK2 and VK21 are involved in the production of bacteriocins, peptides and other antibacterial compounds (Esikova *et al.*, 2002). Secondary metabolites produced by bacteria, particularly those with antimicrobial or antifungal activity, such as peptides, have gained increasing significance in recent years. Given the growing number of clinical challenges, their therapeutic potential has become increasingly important (Kiranmayi *et al.*, 2011).

The ability of *Bacillus* to inhibit *ESKAPE* pathogens underscores the potential of effluent-derived bacteria as a source of novel antibiotics, which is critical in the current context of rising antibiotic resistance. The variation in antibacterial activity observed among the different isolates could be attributed to differences in the types and quantities of secondary metabolites produced. While *Bacillus* showed the highest inhibition, other isolates like *Pseudomonas* and *Acinetobacter* demonstrated lower antibacterial activity. This suggests that further optimization of fermentation conditions or extraction methods could enhance metabolite yield and efficacy for these strains (Martínez-Cardeñas *et al.*, 2012; Katalin, 2022).

In light of these results, the study highlights the importance of industrial effluents as untapped sources of microbial diversity and bioactive compounds. The identification of bacteria capable of producing secondary metabolites with potent antibacterial properties reinforces the need for further exploration in this field. This approach not only contributes to the discovery of new antibiotics but also promotes the sustainable reuse of industrial waste. Future studies could focus on optimizing the fermentation conditions, scaling up metabolite production, and elucidating the molecular structure of the active compounds

for potential clinical application.

Conclusion

The study revealed the antibacterial potential of bacterial metabolites against both Gram-negative and Gram-positive bacteria. While the precise nature and quantity of the active compounds or secondary metabolites produced by bacteria isolated from industrial effluent remains unclear, they show significant results and could contribute to the development of novel, natural drugs to address the growing issue of drug resistance. Further metabolomic analysis is required to fully characterize these bioactive compounds.

Ethical Statement

This study does not involve experiments on animals or human subjects.

Author Contributions

Each author has contributed equally to the study's planning, analysis, writing, and editing. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest

The authors declare no conflicts of interest.

References

Abdel-Nasser A, Hathout AS, Badr AN, Barakat OS and Fathy HM. (2023) Extraction and characterization of bioactive secondary metabolites from lactic acid bacteria and evaluating their antifungal and antiaflatoxigenic activity. Biotechnol Rep. 38: e00799.

Abdulsalam M, Che H, Faezah K, Zainal Z, Isma A and Hazwan M. (2020) Augmented yeast-extract and diary-waste for enhancing bio-decolourization of palm oil mill effluent using activated sludge. J Water Process Engineer. 36: 101263.

Al-Maadheed S, Goktepe, I., Binti, A., Lati, A., & Shomar, B. (2019) Antibiotics in hospital effluent and domestic wastewater treatment plants in Doha, Qatar. J Water Process Engineer. 28: 60-68.

Awad M, Aziz R and Sarmidi MR. (2012) Antibiotics as microbial secondary metabolites : Production and application. J Teknologi 59: 101-111.

Baker DD, Chu M, Oza U and Rajgarhia V. (2007) The value of natural products to future pharmaceutical discovery. Natural Prod Rep. 24(6): 1225-1244.

Bauer AW, Kirby WM, Sherris JC and Turck M. (1966) Antibiotic susceptibility testing by a standardized single disk method. American J Clin Pathol. 45(4): 493-496.

Butler MS and Buss AD. (2006) Natural products--the future scaffolds for novel antibiotics? Biochem Pharmacol. 71(7): 919-929.

Couto CF, Lange LC and Amaral MCS. (2019) Occurrence, fate and removal of pharmaceutically active compounds (PhACs) in water and wastewater treatment plants - A review. J Water Process Engineer. 32: 100927.

Esikova TZ, Temirov YV, Sokolov SL and Alakhov YB. (2002) Secondary antimicrobial metabolites produced by thermophilic *Bacillus* spp. strains VK_2 and VK_{21}. Prikl Biokhim Mikrobiol. 38(3): 261-267.

Jayne M, Rajaofera N, Wang Y, Yaseen G, Jin P and Fan L. (2019) Volatile organic compounds of Bacillus atrophaeus HAB-5 inhibit the growth of *Colletotrichum gloeosporioides*. Pesticide Biochem Physiol. 156: 170-176.

Karn SK and Kumar A. (2019) Protease, lipase and amylase extraction and optimization from activated sludge of pulp and paper industry. Indian J Exp Biol. 57(3): 201-205.

Katalin B. (2022) The role of physical support in secondary metabolite production by *Streptomyces species*. Biochem Engineer J. 185(2): 108495.

Kelsic ED, Zhao J, Vetsigian K, Kishony R. (2015) Counteraction of antibiotic production and degradation stabilizes microbial communities. Nature 521(7553): 516-519.

Khattab AI, Babiker EH and Saeed HA. (2017) Streptomyces : isolation, optimization of culture conditions and extraction of secondary metabolites. Int Curr Pharmaceut J. 5(3): 27.

Kiranmayi Usha M, Sudhakar P, Sreenivasulu K and Vijayalakshmi M. (2011) Optimization of culturing conditions for improved production of bioactive metabolites by *Pseudonocardia sp*. VUK-10. Mycobiology 39(3): 174-181.

Kumar S, Woo H, Oh J and Park H. (2011) Occurrence and removal of antibiotics , hormones and several other pharmaceuticals in wastewater treatment plants of the largest industrial city of Korea. Sci Total Environ. 409(20): 4351-4360.

Lewis K. (2013) Platforms for antibiotic discovery. Nature Rev Drug Discover. 12(5): 371-387.

Luzhetskyy A, Pelzer S and Bechthold A. (2007) The future of natural products as a source of new antibiotics. Curr Opin Invest Drugs 8(8): 608-613.

Martínez-Cardeñas JA, de la Fuente-Salcido NM, Salcedo-Hernández R, Bideshi DK and Barboza-Corona JE. (2012) Effects of physical culture parameters on bacteriocin production by Mexican strains of *Bacillus thuringiensis* after cellular induction. J Ind Microbiol Biotechnol. 39(1): 183-189.

Porwal HJ, Mane AV and Velhal SG. (2015) Biodegradation of dairy effluent by using microbial isolates obtained from activated sludge. Water Resources Industry 9: 1-15,

Remya M and Vijayakumar R. (2008) Isolation and characterization of marine antagonistic actinomycetes from west coast of India. Facta Universitatis Med Biol. 15(1): 13-19

Saleem M, Nazir M, Ali MS, Hussain H, Lee YS, Riaz N, Jabbar A. Antimicrobial natural products: an update on future antibiotic drug candidates. Nat Prod Rep. 2010 Feb;27(2):238-54.

Sansinenea E and Ortiz A. (2011) Secondary metabolites of soil *Bacillus spp*. Biotechnol Letts. 33(8): 1523-1538.

Silva-bedoya LM, Sánchez-pinzón MS, Cadavid-restrepo GE and Moreno-Herrera CX. (2016) Bacterial community analysis of an industrial wastewater treatment plant in Colombia with screening for lipid-degrading microorganisms. Microbiol Res. 192: 313-325.

Ventola CL. (20150 The antibiotic resistance crisis: part 1: causes and threats. P T. 40(4): 277-283.

WHO. (2015) Global antimicrobial resistance surveillance system: Manual for early implementation. https://iris.who.int/bitstream/handle/10665/188783/9789241549400_eng.pdf

WHO. (2022) Global antimicrobial resistance and use surveillance system (GLASS). https://www.who.int/initiatives/glass

Wright GD. (2014) Something old, something new: revisiting natural products in antibiotic drug discovery. Can J Microbiol. 60(3): 147-154.