Omeike *et al. Beni-Suef University Journal of Basic and Applied Sciences* (2019) 8:18
https://doi.org/10.1186/s43088-019-0026-8

Beni-Suef University Journal of Basic and Applied Sciences

RESEARCH Open Access

Potential antibiotic-producing fungal strains isolated from pharmaceutical waste sludge

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Abstract

Background: Antibiotic resistance and dearth of novel compounds from natural sources warrants the need to search other environments for potential antibiotic-producing microbial species. The study investigated isolation and identification of antibiotic-producing fungi from pharmaceutical waste sludge.

Results: Seven hundred and ninety-seven isolates obtained from sludge of seven pharmaceutical industries in Sango Ota, Ogun State using several growth media, with mould isolates highest (696). Isolated species were from genera *Aspergillus* (28.55%), *Penicillium* (18.35%), *Trichoderma* (13.44%), *Rhizopus* (10.21%) and *Geotrichum* (4.01%), and *Stachybotrys* (0.13%). The CFS of strains named *Geotrichum candidum* OMON-1, *Talaromyces pinophilus* OKHAIN-12, and *Penicillium citrinum* PETER-OOA1 had high reproducible bioactivity against *Staphylococcus aureus* (32 ± 0.12 mm) and *Klebsiella pneumoniae* (29 ± 0.12 mm) while *P. citrinum* MASTER-RAA2 had activity against *K. pneumoniae* only. Active metabolites were successfully extracted using Diaion HP-20 and methanol:iso-propanol:acetone (6:3:1 v/v). Antibacterial-active fractions of fungal extract successfully eluted with 40–60% NaCl on ion-exchange chromatography using a cation column.

Conclusions: The study successfully screened antibiotic-producing fungal species from pharmaceutical waste storage facilities. Study also showed that similar species from same toxic environment could potentially produce different metabolites.

Keywords: Antibacterial screening, Pharmaceutical waste, *Geotrichum candidum*, *Talaromyces pinophilus*, *Penicillium citrinum*, Ion exchange chromatography

1 Background

Natural antibiotics are chemical substances derived from microorganisms, which destroy and/or inhibit the growth of other microorganisms [1]. Due to the constant need for antibiotics to cure infections and diseases, several antibiotics have been introduced for clinical use, from the discovery of penicillin to the various synthetically derived modifications presently in use. However, since the end of the antibiotic boom era in the 1980s, dwindling fortune is being experienced in the discovery and introduction of new antimicrobial compounds of microbial origin for clinical use. Only nine new antibiotics were approved in the first decade of the 2000s, while no new class has been discovered since the 1980s [2].

According to Hamburg's Academy of Sciences and Humanities/German National Academy of Sciences [3], 73% of antibiotics approved between 1981 and 2005 were structural modification of compounds in five antibiotic classes, with only three novel classes introduced over the past 30 years [4, 5]. Coupled with increased antibiotic resistance, there is a need for a continuous search for novel antibiotics discovery.

Over 23,000 antibiotic compounds have been sourced from microbial species isolated from natural habitats [6]; therefore, the need to seek new habitats as sources for microbial communities with possible antimicrobial properties, leading to novel antimicrobial compounds. These could include urban parks for well-known species with antibiotic scaffolds, desert, or extreme marine environment for rare microorganisms [7, 8].

Pharmaceutical dumpsites have not been explored as a possible source of microorganisms with antimicrobial properties. In Nigeria, pharmaceutical industries practice

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indiscriminate disposal of wastes (both hazardous and non-hazardous) into the environment without proper treatment [9]. Pharmaceutical waste storage facilities represent an alternative and unusual environment that could be a source of microorganisms with diverse chemical scaffolds. Incessant disposal of untreated chemical wastes into such a facility could result in the survival of microflora with specialized metabolism, leading to novel antibiotic-related properties [10]. The aim of this study was to screen fungal species isolated from pharmaceutical waste storage facilities for bioactivity and determine the antibacterial potential of extract from identified bioactive strains.

2 Methods

2.1 Location and sample collection

Sludge samples (10 g each) were aseptically collected from the base of waste storage tanks of seven drug-producing pharmaceutical industries in Sango-Ota Industrial area of Ogun State, Nigeria (6° 42′ 46.6308′′ N, 3° 10′ 11.0172′′ E). Samples were promptly transferred to the laboratory under cold storage for immediate analysis.

2.2 Isolation

One gram of each sludge sample was suspended and vortexed (REMI CM-101, India) in 9 mL of sterilized distilled water for 2 min at room temperature. One milliliter of the dissolved solution was transferred using sterile pipettes and diluted serially until reach 10^{-6} dilution. Aliquots (1 mL) were inoculated on PDA, SDA, YPDA, CSPY, TSA, CDA, MEA, and R2A media (Hi-Media, India), and plates were incubated at 30 °C for 3–7 days. Distinct colonies were sub-cultured as pure isolates and stored on agar slants at 4 °C until required for further studies.

2.3 Identification of fungal isolates

The identification of fungal isolates via morphological characteristics was done using lactophenol cotton-blue stain, then compared with standards [11]. The antimicrobial-active strains were characterized by molecular technique. Genomic DNA was isolated from 48 to 72 h old fungal broth using the ZR Fungal/Bacterial DNA mini-Prep™ Kit (Zymo Research, India) protocol. Polymerase chain reaction (PCR) amplification of purified DNA was carried using ITS 1 (5′–TCCGTAGGTGAACCTGCGG-3′) and NL4 (5′–GGTCCGTGTTTCAAGACGG-3′) as forward and backward primers respectively. Nucleotide sequence was analyzed using the Basic Local Alignment Search Tool (BLAST-N) on the NCBI database to identify the active strains [12].

2.4 Screening for antibiotic-producing fungal isolates

Fungal spore plug (6 mm) was cut from YPDA medium onto CSPY, TSB, R2A, and YPD broth media respectively in conical flasks (100 mL). Fermentation was proceeded at 30 °C in a thermostat-regulated shaker incubator at 150 rpm for 14 days. From third day onward, 1 mL broth aliquot was centrifuged to obtain the cell-free supernatant (CFS) tested for reproducible antibacterial activity against clinical strains *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus cereus*, *Klebsiella pneumoniae*, *Escherichia coli*, and *Enterococcus faecalis* which were obtained from the culture collection laboratory of the Department of Microbiology, Federal University of Agriculture, Abeokuta, Ogun State.

2.5 Antimicrobial activity of fungal isolates

Bioactivity of CFS against both *S. aureus* and *K. pneumoniae* was determined using the agar well-diffusion method described by the Clinical and Laboratory Standards Institute [13]. Briefly, bacterial inoculum (1 mL) adjusted to 10^5 cfu/mL (0.5 McPharland's standard) was seeded onto Muller-Hinton agar (MHA) medium and CFS added into appropriate wells bored onto the plate using pre-sterilized cork borer (6 mm diameter). Plates were incubated at 37 °C for 18–24 h and antibiotic activity determined by clear zones of inhibition.

2.6 Extraction and bioactivity of bioactive fungal strains

Crude extract of bioactive strains was obtained from CFS using modified methods [14, 15]. Following fermentation of antibiotic-producing strains on TSB for 8–10 days, CFS was obtained by centrifuging at 9000 rpm for 15 min, and extracted via solid-liquid extraction using activated Diaion HP-20 beads at room temperature. Bounded active compounds were eluted using methanol:iso-propanol:acetone (6:3:1 v/v) for optimum recovery. Eluted crude extract was concentrated under vacuum on a rotary evaporator (rotavap) at 60 rpm with chiller temperature 9–12 °C, water bath at 35–45 °C and vacuum pump pressure at 140 mmPa. Antibiotic activity of the extract was confirmed via agar well-diffusion method as previously described.

2.7 Partial separation of antimicrobial extracts

Separation of antimicrobial extracts attempted via Ion Exchange Column Chromatography (IECC) protocol [16]. CM-Sepharose CL-B6 beads (Pharmacia, Sweden) and 1M NaCl, buffered with 10 mM ammonium acetate (pH 5.0) were stationary and mobile phases respectively. Briefly, the crude extract was passed through stationary bed (1.5 mL/min) for binding, and compounds eluted with $NaCl_{(aq)}$ in a gradient system (0–100%, 2.5 mL/min). Collected fractions were tested for antibacterial activity to determine eluted fraction which retained bioactive compounds.

3 Results

3.1 Fungal isolation and characterization

Seven hundred and ninety-seven (797) isolates were obtained, and filamentous fungi were 696 isolates (87.3% of

the total isolates) while the rest were yeast-like colonies. The different fungal genera isolated are described in Fig. 1. *Aspergillus* (28.55%) was the most identified genus, while *Stachybotrys* (0.13%) was least observed. Other important genera identified included isolates of *Penicillium* (18.35%), *Trichoderma* (13.44%), *Rhizopus* (10.21%), and pleomorphic *Geotrichum* (4.01%). Based on 18S rRNA sequencing data, four isolates which had reproducible antibacterial activity were identified as *Geotrichum candidum* OMON-1, *Talaromyces pinophilus* OKHAIN-12, *Penicillium citrinum* PETER-OOA1, and *Penicillium citrinum* MASTER-RAA2. Their phylogenetic relationships are described in Fig. 2 and nucleotide sequences submitted to GenBank.

Table 1 and Plate 1a–f detailed the colonial and morphological characteristics of antibacterial-producing strains *G. candidum* OMON-1, *T. pinophilus* OKHAIN-12, *P. citrinum* PETER-OOA1, and *P. citrinum* MASTER-RAA2. *Geotrichum candidum* OMON-1 grows as smooth, cheese-colored colonies with entire margins and presence of white-colored aerial mycelia after 24 h. A characteristic of all growth media is its fruity smell after 24–48 h of growth. Microscopic observation of *G. candidum* OMON-1 (Plate 1b) shows septate hyphae releasing rod-shaped asexual spores known as arthrospores at either ends or other openings on the structure.

Talaromyces pinophilus OKHAIN-12, a filamentous fungus, grows as yellow colonies on SDA (Plate 1c), greenish-yellow on PDA and CDA, white with green spores on TSA and YPDA, and as white mycelium with orange spores on MEA medium, respectively. A red pigment deposit in the medium produced from 48 h onwards was observed on all growth media. Pigment production was most pronounced in MEA but poorly expressed on PDA. Mycelia grow as a mass of septate hyphae which branches into broom-like conidiophores with conidiospores given off at the tip (Plate 1d). *Penicillium citrinum* PETER-OOA1 and *Penicillium citrinum* MASTER-RAA2 showed similar growth of white filamentous colonies with green spores on all growth media. Colonies grow as crateriform and no pigmentation observed (Plate 2e). In addition, septate hyphae and conidiospores were observed.

3.2 Antibacterial activity of bioextracts

Antibacterial activity of fungal extracts against pathogenic bacteria strains following CFS extraction was presented in Table 2. *G. candidum* OMON-1 inhibited the growth of *S. aureus* highest (32 ± 0.12 mm zone of inhibition) while *T. pinophilus* OKHAIN-12 extract showed the highest activity against *K. pneumoniae* (29 ± 0.11 mm). Furthermore, *G. candidum* OMON-1 inhibited only *S. aureus* while *P. citrinum* MASTER-RAA2 extract inhibited *K. pneumoniae* only. However, *Talaromyces pinophilus* OKHAIN-12 and *P. citrinum* PETER-OOA1 inhibited both *S. aureus* and *K. pneumoniae*.

3.3 Separation of active fractions from bioextracts

Separation of crude extract of potential antibiotic-producing isolates to sequester active compound via ion-exchange chromatography is described in Table 3. The bioactive extract was separated into ten fractions and bioassay showed that fractions 4–5 of *G. candidum* OMON-1 eluted with 40–50% NaCl inhibited *S. aureus* growth and no activity against *K. pneumoniae*. Similarly, bioactive compounds of *T. pinophilus* OKHAIN-12 and *P. citrinum* PETER-OOA1 were obtained in fractions eluted with 40–60% NaCl. However, bioactive compounds of *P. citrinum* MASTER-RAA2 were eluted with 70–100% NaCl, and inhibited only *K. pneumoniae* growth.

4 Discussions

Isolation of 797 species indicates that high number of organisms were able to survive the chemically contaminated environment. Production of antibacterial activity

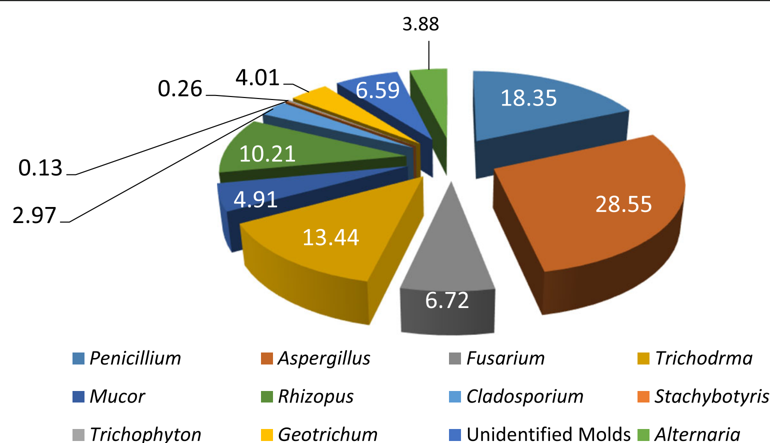


Fig. 1 Percentage distribution of fungal genera that were isolated from pharmaceutical waste sites' sludge

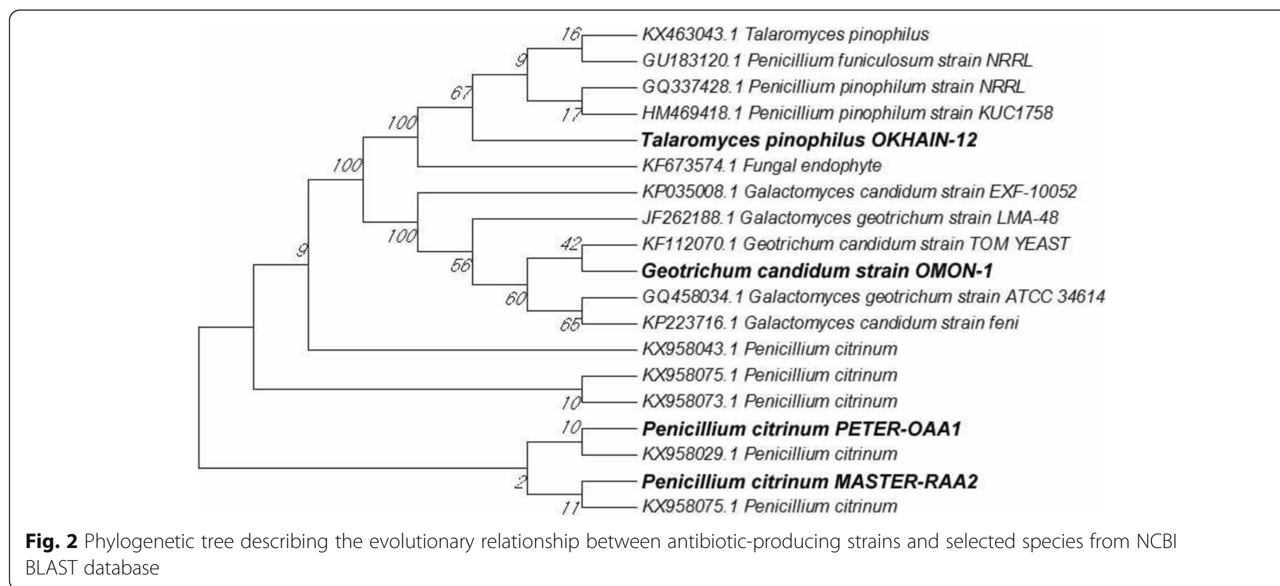


Fig. 2 Phylogenetic tree describing the evolutionary relationship between antibiotic-producing strains and selected species from NCBI BLAST database

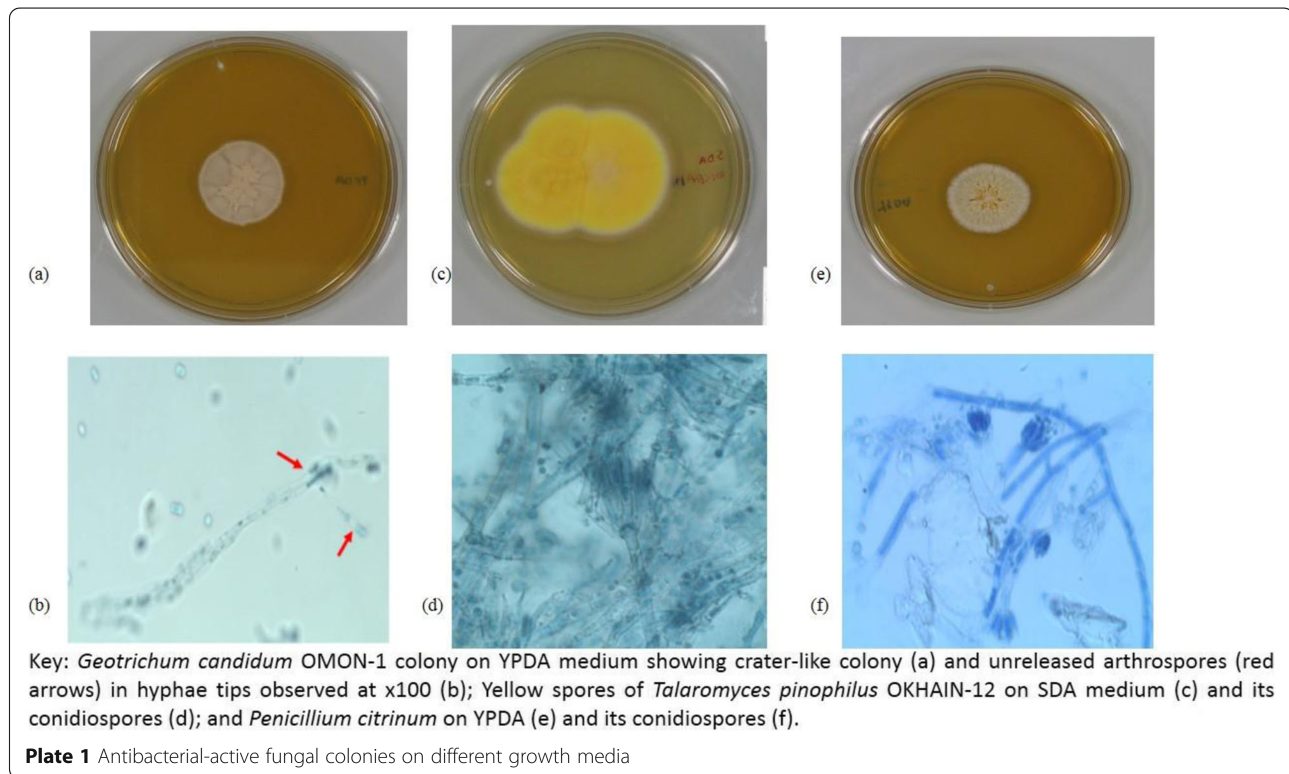
by four strains indicates their ability to use the chemical and other contaminants as substrates for growth and production of specialized metabolites. Fungi are also reported in various industrial environments for their ability to produce metabolites of economic importance, while their ability to degrade different organic and industrial wastes has been widely reported [17–19]. Similarly, fungal contamination of pharmaceutical environment and products has been reported [20], but there is paucity of information on fungi indigenous to pharmaceutical industries' wastes. Species of common fungal genera *Penicillium*, *Aspergillus*, *Fusarium*, *Trichoderma*, *Mucor*, and *Rhizopus* isolated from pharmaceutical sludge further confirms ubiquitous nature of fungi. Furthermore, Obuekwe et al. [21] also reported isolation of *P. chrysogenum*, *A. flavus*, *Candida albicans*, and *Saccharomyces* spp. in pharmaceutical products. In the same vein, *Cladosporium* spp and *Alternaria* spp. have also been reported as contaminants of antibiotics and synthetic drugs [22]. These isolates are termed indoor fungi and indigenous to most homes, and their presence could be due to the introduction by humans [23].

Geotrichum candidum OMON-1, an anamorph of *Galactomyces geotrichum*, is a strain in the *Geotrichum* genus that are borderline yeast-mold species presenting aerial mycelium on yeast-like colonies on different growth media [24]. Anamorphism describes the asexual state of a fungus, and the observation of asexual arthrospores in *G. candidum* OMON-1 hyphae supports its identification [25]. Furthermore, in spite of a few reports about *G. candidum* metabolites with antibacterial property, they have been reported as important in cheese-making [26]. However, two antimicrobial compounds were purified from *G. candidum* by Dieuleveux et al. [27], while broad-spectrum antibacterial activity of ethyl acetate extracts of *G. candidum* from root biome of date palm trees has also been reported [28].

Similarly, *Talaromyces pinophilus* OKHAIN-12 belongs to the *Talaromyces* genus, an anamorphic stage of *Penicillium*. Beside their pigment production, *Talaromyces* spp. from different habitats were reported as producers of a wide range of bioactive secondary metabolites [29, 30]. Furthermore, Silva Lima et al. [31]

Table 1 Morphological characteristics of antibiotic-producing fungal strains isolated from pharmaceutical waste sludge

Isolates	Colony Shape	Spore Color	Margin	Colony elevation	Pigmentation	Type of hyphae	Sporulation
G. candidum OMON-1	Circular	Cheese-colored smooth colony with white-colored aerial mycelia at the surface.	Entire	Raised colonies. crateriform on YPDA only	None	Septate	Arthrospores
T. pinophilus OKHAIN-12	Filamentous	Yellow spores on SDA, yellowish, and green spores on PDA and CZDA; green spores on YPDA and TSA; Orange spores on MEA	Filiform	Numbonate	Red deposit on media	Septate	Conidiospores
P. citrinum PETER-OOA1	Filamentous	White mycelia with green spores	Filiform	Crateriform	None	Septate	Conidiospores
P. citrinum MASTER-RAA2	Filamentous	White mycelia with green spores	Filiform	Crateriform	None	Septate	Conidiospores



Key: *Geotrichum candidum* OMON-1 colony on YPDA medium showing crater-like colony (a) and unreleased arthrospores (red arrows) in hyphae tips observed at x100 (b); Yellow spores of *Talaromyces pinophilus* OKHAIN-12 on SDA medium (c) and its conidiospores (d); and *Penicillium citrinum* on YPDA (e) and its conidiospores (f).

Plate 1 Antibacterial-active fungal colonies on different growth media

reported crude extract of pre-treated *T. Pinophilus* possessed in vitro antibiotic activity against *Helicobacter pylori* and *Listeria monocytogenes*.

Conversely, the genus *Penicillium* is known for antibiotic production, and *P. citrinum* are reported producers of bioactive compounds. Recently, Qader et al. [32] purified an antitumor antibiotic from an endophytic strain, while Diep et al. [33] also reported the purification of several bioactive compounds in a strain isolated from the sponge.

Based on the antibacterial activity of fungal crude extracts, it was deduced that *G. candidum* OMON-1 and *Penicillium citrinum* MASTER-RAA2 produce compounds with narrow-spectrum activity, while both *Talaromyces pinophilus* OKHAIN-12 and *P. citrinum* PETER-OOA1 possibly produce broad-spectrum compounds. Furthermore, similar antibacterial activity of *P. citrinum* against *S. aureus* and *E. coli* has previously been reported [34]. However, the difference in antibacterial activity spectrum of *P. citrinum* PETER-OOA1 and *P. citrinum* MASTER-RAA2 extracts indicates different antibiotic compounds produced. This could be attributed to production of different metabolites from a similar pathway [35].

For compound separation, ion-exchange column chromatography separates based on the presence of charged groups (polarity) on desired molecules [36]. Therefore, the antibacterial activity of specific fractions against *S. aureus* and *K. pneumoniae* indicates a successful separation of different bioactive compounds. Furthermore, successful separation of bioextract by cation-exchange column indicates elution of positively charged bioactive compound [37]. In addition, elution of bioactive

Table 2 Antibacterial activity of bioactive extracts of fungi strains screened from pharmaceutical waste sludge

Clinical isolates	Zones of inhibition (mm)			
	G. candidum OMON-1	*T. pinophilus* OKHAIN-12	*P. citrinum* PETER-OOA1	*P. citrinum* MASTER-RAA2
S. aureus	32 ± 0.12	24 ± 0.2	12 ± 0.17	–
B. cereus	14 ± 0.3	13 ± 0.1	12 ± 0.2	–
E. faecalis	18 ± 0.28	13 ± 0.2	14 ± 0.32	–
K. pneumoniae	–	29 ± 0.11	16 ± 0.35	18 ± 0.1
P. aeruginosa	–	12 ± 0.18	–	12 ± 0.3
Escherichia coli	–	15 ± 0.2	10 ± 0.2	15 ± 0.1

Table 3 Bioactivity of fractions of *G. candidum* OMON-1, *T. pinophilus* OKHAIN-12, *P. citrinum* PETER-OOA1, and *P. citrinum* MASTER-RAA2 antimicrobial extracts separated using ion-exchange chromatography

Fractions	NaCl (%, v/v)	*G. candidum* OMON-1		*Talaromyces pinophilus* OKHAIN-12		*Penicillium citrinum* PETER-OOA1		*Penicillium citrinum*	
		SA	KP	SA	KP	SA	KP	SA	KP
1	10	–	–		–	–	–	–	–
2	20	–	–	+	–	–	–	–	–
3	30	–	–	+	–	–	–	–	–
4	40	+	–	–	–	+	–	–	–
5	50	+	–	–	+	+	+	–	–
6	60	–	–	–	+	+	+	–	–
7	70	–	–	–	–	–	–	–	+
8	80	–	–	–	–	–	–	–	+
9	90	–	–	–	–	–	–	–	+
10	100	–	–	–	–	–	–	–	+

Legend: + indicates bioactive fraction; non-active fraction is denoted by –

compounds using 40–50% NaCl indicates the presence of weakly charged compounds, while 70–100% NaCl elution indicates strongly charged compounds [38].

5 Conclusion

The study successfully isolated fungal isolates from pharmaceutical waste storage facilities. Screening confirmed four strains with antibiotic production potential based on reproducible antibacterial property. Active extract of producer strains was successfully sequestered via solid-liquid extraction, while the attempted separation of bioactive fragment using ion-exchange chromatography yielded bioactive fractions with mostly weakly charged antibiotic compounds. Further purification and characterization of antibiotic compounds produced by antibacterial-active fungal strains should be investigated for possible novelty.

Abbreviations

BLAST-N: Basic local alignment search tool; CDA: Czapek-Dox agar; CFS: Cell-free supernatant; CSPY: Casein starch peptone yeast extract agar; DNA: Deoxyribonucleic acid; IECC: Ion exchange column chromatography; MEA: Malt extract agar; MHA: Muller-Hinton agar; PCR: Polymerase chain reaction; PDA: Potato dextrose agar; SDA: Sabouraud dextrose agar; TSA: Tryptone soy agar; TSB: Tryptone soy broth; YPDA: Yeast extract potato dextrose agar

Acknowledgements

Not applicable

Availability of data and material

The datasets generated during the current study are available in the GenBank repository, NCBI.These include *Geotrichum candidum* OMON-1 (MF431584), *Talaromyces pinophilus* OKHAIN-12 (MF491448), *Penicillium citrinum* PETER-OOA (MF491449), and *Penicillium citrinum* MASTER-RAA (MF491450).

Authors' contributions

OSO co-designed the study, carried out research, and wrote manuscript. KSO contributed to study design and manuscript writing. LAA advised on chemical separation design and editing manuscript. All authors have read and approved the manuscript.

Funding

Not applicable

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests

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Received: 12 September 2019 Accepted: 18 November 2019
Published online: 12 December 2019

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