Research Article

Antifungal Potential of Bioactive Metabolites Produced by *Pseudomonas aeruginosa* against *Candida* Species

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Abstract: Background: The frequency of fungal infections targeting immune-suppressed patients has been prominently rising day by day. Among these infections, Candidiasis is one of the most common life-threatening systemic fungal infections in recent years. These infections are challenging to treat because of the inconvenience of effective antifungal drugs. Therefore, the insufficiency of current drug regimens coupled with constant mutations by the fungi to develop drug resistance can pose a potential problem for future anti-fungal treatments. This frequent increase in drug-resistant fungi has directed attention toward the use of alternative therapy from natural sources.

Objective: This study aimed to evaluate *Pseudomonas aeruginosa* for its antifungal potential against *Candida* species.

Materials and Methods: This study was conducted at the Federal Urdu University of Arts, Science and Technology, Karachi, Pakistan during 2021-2022. For the detection of the antifungal compound, a clinical isolate *P. aeruginosa* HS 28 (identified conventionally as well as by 16S r RNA analysis) was screened for its bioactivity against *Candida* species by using agar well diffusion technique. The growth kinetics as well as the effect of different physical and chemical factors were also determined in the study. The compound was also partially purified by ammonium sulfate precipitation.

Result: This antifungal compound showed good inhibitory activity against *Candida* species such as *Candida albicans*, *C. glabrata*, *C. tropicalis*, *C. krusei*, etc. This compound retained its stability at a high range of temperatures, and varying pH. Moreover, its bioactivity was also conserved when treated with organic solvents, chloroform vapors, metal salts, and different surfactants/detergents. The growth kinetics analysis illustrated that the maximum production of antifungal compounds occurred in the log phase of growth and extended supreme until the late log phase. Furthermore, the highest saturation of this protein was achieved at the concentration of 60% ammonium sulfate.

Conclusion: The overall results indicated the promising antifungal potential of the compound produced by *Pseudomonas aeruginosa* HS 28 against *Candida* species.

Keywords: Antifungal compound, *P. aeruginosa*, Antifungal activity, *Candida* species, Cytotoxic chemotherapies, Antibiotics.

INTRODUCTION

The prevalent use of antibiotics, cytotoxic chemotherapies, and transplantations are some of the few factors that have resulted in the increased frequency of fungal infections, especially in immunocompromised patients [1]. Among them, Candidiasis is a well-known fungal infection that occurs worldwide caused by the yeast fungi *Candida* which is also a common resident of the human body like the skin, vagina, mouth, and gastrointestinal tract. The quickly acquiring resistance to the available antifungals makes treatment of these infections challenging [2]. The family *Candida* comprises over 150 pathogenic and non-pathogenic species. Among them, the most commonly isolated species in human infections include *C. albicans*, *C. tropicalis*, *C. parapsillosis*, *C. krusei*, *C. kefyr*, *C. glabrata*, and *C. guillermondii*. These fungi have become increasingly drug resistant to current drug regimens which has become a reason for serious concern when it comes to treating infections caused by these pathogens. This failure of the current drug regimens is attributed to factors including the emergence of multi-drug resistant fungi that have decreased the effectiveness of the current drugs alongside a potent increase in toxicity of these antifungal agents [3].

The increased frequency of drug-resistant fungi and the toxicity of existing antifungal compounds have drawn attention to the antimicrobial activity of certain natural metabolites produced by bacteria. In this connection, *Lactobacillus* species display favorable anti-Candidal activity that can participate in low *candida* burdens and prevent fungal infections. Plantaricin (a class II b bacteriocin from *Lactobacillus plantarum*) is the purified bacteriocin whose effect on *C. albicans* physiology and morphology has been described. This bacteriocin kills *C. albicans* by disrupting the cell membrane integrity due to depolarization and leakage of essential ions [4]. Different studies also reported

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anti-candidal activity produced by certain other bacterial species such as *Lactococcus*, *Streptococcus*, and *Enterococcus* [5]. *Pseudomonas aeruginosa* is also known to produce different metabolites which are bio-active against many microorganisms [6]. This specie not only exhibits antagonistic activity against closely related species but is also reported to inhibit other human pathogenic bacteria species such as *Staphylococcus aureus* particularly Methicillin-resistant *S. aureus* [7]. Moreover, this bioactivity is no more limited to prokaryotes, it was also reported against eukaryotes such as fungal species *Aspergillus niger*, *Helminthosporium* spp., *Fusarium* spp., and *Candida* spp. [8].

To survive in stressful conditions, *P. aeruginosa* produces various metabolites which are antibacterial as well as antifungal in nature including bacteriocins, toxins, bacteriolytic enzymes, antibiotics, and different extracellular pigments like pyocyanin and fluorescein as competitive strategy [9-11]. Keeping in view the significance of *P. aeruginosa* in the production of novel antimicrobial substances, the present study was designed to screen *P. aeruginsa* for its antifungal potential against *Candida*, particularly non-*albicans Candida* species.

MATERIALS AND METHODS

Collection of *Pseudomonas aeruginosa* Strains and *Candida* Species

This study was conducted during December 2021 to December 2022 at Lab of Applied Microbiology and Clinical Mycology (LAMCM), Department of Microbiology, Federal Urdu University of Arts, Science and Technology, Karachi, Pakistan. A total of 30 clinical strains of *P. aeruginosa* were collected from various clinical laboratories in Karachi. These isolates were identified by utilizing standard indicative procedures [12]. To investigate the anti-Candidal potential of *P. aeruginosa*, about 120 *Candida* strains comprising *Candida albicans*, *C. tropicalis*, *C. glabrata*, *C. krusei*, *C. rugosa*, *C. parapsillosis*, *C. zeylanoides*, *C. guillermondii*, *C. kefyr*, *C. lipolytica*, *C. apicola* were gathered from the Lab of Applied Microbiology and Clinical Mycology (LAMCM), Department of Microbiology, Federal Urdu University of Arts, Science, and Technology. All the *Candida* spp.. were re-identified by standard fungal identification methods [13-15].

Screening of *P. aeruginosa* Strains for their Anti-Candidal Potential

Preliminary, all the clinical (30) strains of *P. aeruginosa* were screened for their bioactivity against *C. albicans* and *C. glabrata* (non-*albicans Candida*) by using agar well diffusion assay. For this purpose, the *P. aeruginosa* strains were grown in Brain heart infusion agar at 37°C for 24 hours. After incubation, centrifuged at 4000 rpm for 30 min and the pellet was discarded while the cell-free supernatant (CFS) was collected carefully. This CFS of *P. aeruginosa* strains were considered a crude antifungal metabolite preparation and was used for the determination of their bioactivity against *Candida* species [16, 17].

To determine the antifungal activity of *P. aeruginosa*, the agar well diffusion technique was utilized [16, 17]. Concisely, for this purpose, the *Candida* strains (indicator strains) were transferred to Sabouraud dextrose broth and incubated at room temperature for 2 hours. After the vortex, a uniform lawn of *Candida* sp. was prepared (as per 0.5 McFarland index) by using a sterile cotton swab on Sabouraud Dextrose Agar plates. Then wells of size 5mm diameter were cut by using a sterile borer. The 100µl CFS of *P. aeruginosa* strains (producers) were poured into each well. The plates were incubated at 37°C for 24 hrs. The following day, the zone of inhibition around the wells was measured in millimeters [18, 19].

Selection of the Best Producer Strain of *P. aeruginosa* and its Identification by Molecular Approaches

According to the results of antifungal screening, the clinical strain *P. aeruginosa* HS 28 was selected as the most potent producer strain as it showed good inhibitory potential against *Candida* species. This strain was selected for further studies and its identification was confirmed by PCR amplification by using forward and reverse primers sequence FD1 (16S rRNA) 5' AGA GTT TGA TCC TGG CTC AG 3' and RP1 (16SrRNA) 5' CGG TTA CCT TGT TAC GAC TT 3', respectively [9, 16].

Determination of Anti-Candidal Potential of *P. aeruginosa* HS 28 against Different Species of *Candida*

After the selection of a potent producer strain, *P. aeruginosa* HS 28 was tested against different *Candida* species including fifty different strains of *C. albicans*, twenty different clinical strains each of *C. tropicalis*, *C. glabrata*, *C. krusei*, *C. rugosa*, *C. parapsillosis*, *C. zeylanoides*, *C. guillermondii*, *C. kefyr*, *C. lipolytica*, *C. apicola* for its bioactivity by using agar well diffusion technique [20].

Optimization of *P. aeruginosa* HS 28 for Antifungal Metabolite Production

To attain maximum antifungal metabolite production from *P. aeruginosa* HS 28, the producer strain was grown in different broth media such as Nutrient broth (NB), Brain heart infusion broth (BHI), and Cetrimide broth. After incubation at a temperature of 37°C overnight, CFS of grown cultures were obtained and their bioactivity against different species of *candida* was investigated [21] (Fig. **1**).

Characterization of Antifungal Metabolite

The antifungal metabolite produced by *P. aeruginosa* HS 28 was also characterized in this study. For this purpose, the crude preparation of antifungal metabolite produced by *P. aeruginosa* HS 28 was exposed to different physical and chemical agents, and the effect on the bioactivity was observed. Among physical agents, the temperature stability of antifungal metabolite was determined by treating CFS to different ranges of temperature i.e. from 40°C to 80°C for 30 minutes and 100°C to 121°C for 15 minutes. After treatment, the residual activity was determined

by agar well diffusion assay [14, 22]. Further, the crude antifungal metabolite was stored at low temperatures 0°C and 4°C for different time duration and then the residual activity was determined by agar well diffusion assay to observe the effect of low temperature on storage [23] . To determine the impact of different pH on the bioactivity of antifungal metabolite, crude metabolite was adjusted to different pH ranging between 2 to 14 (by treating with 1N NaOH or 1N HCL). The effect of different chemicals on the bioactivity of *P. aeruginosa* HS 28 was also investigated in the study. The CFS was treated at different concentrations (1%, 5%, and 10%) of organic solvents such as methanol, ethanol, butanol, propanol, and acetone [24], and with chloroform vapors at different time intervals such as 10 min, 20 min, and 30 min [25]. Moreover, the CFS was also exposed to different metal salts such as $BaCl_2$, $CaCl_2$, $MgSO_4$, $FeSO_4$, $NiSO_4$, and $ZnSO_4$ in the concentration of 1mM [26], and with different surfactants/detergents including Tween 20, Tween 80, and EDTA at a concentration of 1%. All the samples were incubated for 2 hours at 37°C. Then bioactivity was detected by the agar well diffusion method [16, 27] (Figs. **2** and **3**).

Antifungal Metabolite Production and their Growth Kinetics

The kinetics of antifungal metabolite produced by the strain of *P. aeruginosa* HS 28 was observed by growing the bacterial culture for 24 hours at 37°C in BHI broth. After every 2 hours, OD600 of broth was observed. After that, the sample was centrifuged and the cell-free supernatant was analyzed to check the bioactivity by using the agar well diffusion method [14].

Partial Purification of Crude Antifungal Metabolite

The crude antifungal metabolite produced by *P. aeruginosa* HS 28 was partially purified. For this purpose, the CFS was purified partially by using ammonium Sulfate precipitation [22]. In this method, the salt was added gently with constant agitation to achieve 40%, 60%, and 80% (w/v) concentration, and then precipitates were recovered by centrifugation. The supernatant was discarded and the pellet was dissolved in 50 mm sodium phosphate buffer (pH 7.0) and denoted as" Partially purified antifungal metabolite". Agar well diffusion was applied to check the bioactivity of partially purified metabolite [23].

RESULT

Screening of *P. aeruginosa* Strains for their Anti-Candidal Potential

This study mainly focused on the assessment of the anti-candidal potential of clinical isolates of *P. aeruginosa* strains. For this purpose, thirty clinical isolates of *P. aeruginosa* were screened for their bioactivity against *C. albicans* and *C. glabrata* (non. *albicans Candida*). On the basis of screening, *P. aeruginosa* HS 28 was selected as the best producer strain and used for further studies (Table **1**).

Table 1. Screening of *P. aeruginosa* Strains for their Anti-Candidal Potential.

Strains of *P. aeruginosa*	**Zone of Inhibition**		**Strains of** *P. aeruginosa*	**Zone of Inhibition**	
	C. albicans **GS 12**	*C. glabrata* **GS 34**		*C. albicans* **GS 12**	*C. glabrata* **GS 34**
P. aeruginosa HS01	00mm	00mm	*P. aeruginosa* HS16	20mm	12mm
P. aeruginosa HS02	21mm	24mm	*P. aeruginosa* HS17	00mm	18mm
P. aeruginosa HS03	20mm	18mm	*P. aeruginosa* HS18	00mm	15mm
P. aeruginosa HS04	20mm	17mm	*P. aeruginosa* HS19	00mm	20mm
P. aeruginosa HS05	00mm	08mm	*P. aeruginosa* HS20	00mm	00mm
P. aeruginosa HS06	16mm	13mm	*P. aeruginosa* HS21	21mm	00mm
P. aeruginosa HS07	15mm	10mm	*P. aeruginosa* HS22	00mm	00mm
P. aeruginosa HS08	16mm	12mm	*P. aeruginosa* HS23	00mm	18mm
P. aeruginosa HS09	12mm	00mm	*P. aeruginosa* HS24	00mm	20mm
P. aeruginosa HS10	00mm	00mm	*P. aeruginosa* HS25	21mm	20mm
P. aeruginosa HS11	20mm	28mm	*P. aeruginosa* HS26	00mm	00mm
P. aeruginosa HS12	15mm	00mm	*P. aeruginosa* HS27	00mm	00mm
P. aeruginosa HS13	15mm	00mm	*P. aeruginosa* HS28	29mm	21mm
P. aeruginosa HS14	23mm	18mm	*P. aeruginosa* HS29	00mm	10mm
P. aeruginosa HS15	00mm	00mm	*P. aeruginosa* HS30	00mm	00mm

This strain was identified preliminary by conventional methods but later it was also identified by using the 16S rRNA gene amplification process.

The bioactivity of crude extract of *P. aeruginosa* HS 28 was further tested for its anti-Candidal potential against different species of *Candida*. The results revealed that this antifungal metabolite

was bioactive against 50 % of strains of C. *albicans*, *C. guillermondii*, *C. lipolytica*, while 40 % of the C. parapsilosis were also found sensitive. However, the remaining strains of the *Candida* species were moderately sensitive to this metabolite (Table **2**).

Optimization of Growth Media for the Maximum Production of Antifungal Metabolite

For the maximum production of anti-Candidal metabolite, the growth media for the cultivation of *P. aeruginosa* HS 28 was optimized by growing this producer strain in different media. On the basis of a maximum zone of inhibition, Brain Heart Infusion (an enriched medium) was revealed as the best media for the production of antifungal metabolites. Therefore this media was used for the growth of producer strain in all the further studies.

Characterization of Antifungal Metabolite

The diversity of physical and chemical factors may have a vital effect on the bioactivity of antifungal metabolites. According to the observations, the antifungal activity against *C. albicans* remained stable at temperatures 30°C, 37°C, and 40°C exposed for 30 minutes. However, the bioactivity was completely lost at higher temperatures above 60°C. This finding suggests that the antifungal compound produced by *P. aeruginosa* HS 28 was heat-labile therefore it lost its activity above 60°C (Table **3**). The effect of pH on bioactivity was also determined and it showed that the antifungal metabolites remained stable at neutral pH [7] while losing their activity at acidic and alkaline pH ranges. Furthermore, various chemical agents may also influence the bioactivity of antifungal metabolites. In this study, the exposure of antifungal metabolites to different concentrations of organic solvents and metal salts did not influence the bioactivity of the metabolite. Similarly, with the exposure of antifungal metabolite to surfactants such as EDTA, Tween 20, and Tween 80, the bioactivity remains constant however exposure to chloroform vapors enhances its bioactivity (Table **3**).

Partial Purification of Crude Antifungal Metabolite

In this study, the crude antifungal compound was partially purified by ammonium sulfate precipitation and the results revealed that the crude antifungal metabolite was partially purified at 60% and 80% precipitation of ammonium sulfate (Table **3**).

DISCUSSION

Currently, the mortality and morbidity ratio of fungal infections is rapidly increasing due to low efficacy, high toxicity, heavy cost, and limited availability of antifungal drugs. Moreover, their extensive use has led to the emergence of numerous multi-drug-resistant fungal strains that have rendered the current antifungal drugs useless making it difficult to treat and cure fungal infections. Therefore, there is a need to design new antifungal drugs and focus on some alternative sources such as the use of natural products or antimicrobial compounds which are secreted from microbial cells to overcome fungal resistance. Several bacteria can produce antimicrobial compounds having both antibacterial and antifungal potentials. These antifungal metabolites have efficient therapeutic potency against numerous infectious fungal diseases, particularly Candidiasis [2]. Due to the increasing impact of new antifungal compounds, different studies were conducted earlier and this study also gives an insight into the antifungal potential of clinically isolated strains of *P. aeruginosa*.

In this study, different strains of *P. aeruginosa* were screened for their activity against a well-known opportunistic pathogen *Candida*. Interestingly, almost 50% of all the *P. aeruginosa* strains revealed bioactivity against *C. albicans* as well as *C. glabrata* which is a non-*albicans Candida* specie. These NAC are emerging opportunistic species which are known for their more antifungal resistance as compared to *C. albicans* [24]. The most potent producer (*P. aeruginosa* HS 48) of this activity also demonstrated good activity against appreciable numbers of strains belonging to *Candida* genus. In previous studies, the commonly found bacteria, *P. aeruginosa*, have been reported to produce certain compounds having antifungal activity [25]. They can inhibit a wide range of plant pathogenic fungi and helps to control the spread of various plant diseases [26]. Previously it has been studied that the phenazine pigment of *P. aeruginosa*, also known as 'pyocyanin', can inhibit the growth of *A. flavus* and other fungal species such as *Candida* [27]. The maximum production of antifungal metabolite from *P. aeruginosa* HS 28 was also optimized and Brain Heart Infusion medium was observed as the best growth media for this purpose. This is in accordance with another study where BHI was found as the best growth media for *Francisella tularensis* [28]. Another study also demonstrated BHI as the most favorable media among other media for studying in vitro biofilm formation and planktonic growth kinetics of *P. aeruginosa* mainly in co-culture [28].

Table 2. Determination of Anti-Candidal Potential of *P. aeruginosa* HS 28 against Different Species of *Candida*.

Candida spp.	***P. aeruginosa* HS 28**	
	No.	**%**
C. albicans (n= 50)	25/50	50
C. tropicalis (n= 20)	05/20	25
C. glabrata (n= 20)	05/20	25
C. krusei (n= 05)	01/05	20
C. rugosa (n=03)	01/03	33
C. parapsillosis (n= 05)	02/05	40
C. zeylanoides (n= 05)	01/05	20
C. guillermondii (n= 04)	02/04	50
C. kefyr (n=05)	01/05	20
C. lipolytica (n= 02)	01/02	50
C. apicola (n= 01)	00/01	00

Table 3. Physicochemical Characteristics of Antifungal Metabolite.

Treatments	Zone of Inhibition (mm)	Treatments	Zone of Inhibition (mm)
Temperature treatment		**Organic Solvents 1%**	
0°C (06 months)	0	Ethanol	20
4°C (06 months)	8.6	Methanol	10
Control (CFS without any treatment)	13.1	Butanol	15
30°C (30 min)	10.2	Propanol	10
37°C (30 min)	12.3	Acetone	10
40°C (30 min)	13.2	+ve Control (CFS without any treatment)	30
60°C to 121 °C (30 min)	0		
pH		**Organic Solvents 5%**	
2 – 6	0	Ethanol	0
7	13.5	Methanol	10
8-13	0	Butanol	15
Chloroform		Propanol	10
10 to 20 min	0	Acetone	10
30 min	40	+v Control (CFS without any treatment)	25
Surfactants (1%)			
EDTA	0	**Metal Salts (1mM)**	
Tween 20	30	$BaCl_2$	25
Tween 80	30	$MgSO_4$	25
Ammonium Sulfate Precipitation		$HgCl_2$	35
40%	0	$NiSO_4$	30
60%	20	$ZnSO_4$	20
80%	15	+ve Control (Pyocin without any treatment)	30

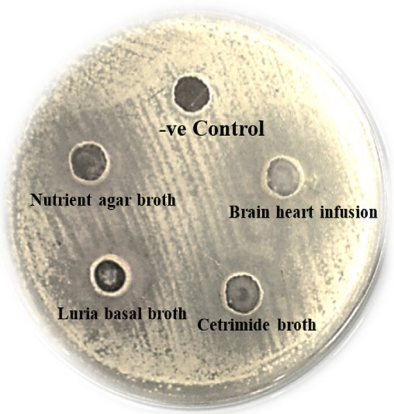

Fig. (1). Optimization of Growth Media for the Production of Antifungal Metabolite.

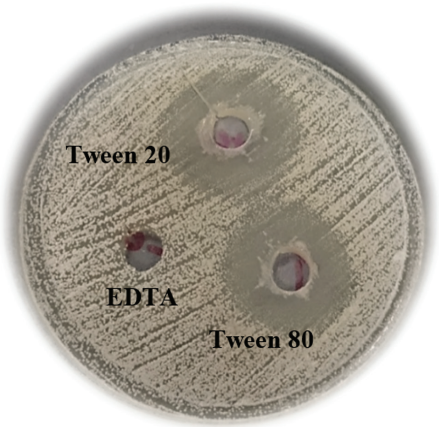

Fig. (2). Effect of Surfactant/Detergents on the Bioactivity of Antifungal Metabolite.

The bio-stability of the antifungal metabolite produced in this study was also determined by exposing the cell-free supernatant of *P. aeruginosa* HS 28 to different physical and chemical conditions. The effect of high and low temperatures and pH variations was analyzed in physical characterization. Whereas in chemical characterization, effects of organic solvents, chloroform, surfactants or detergents, and metal ions were assessed. According to the observations, the antifungal activity against

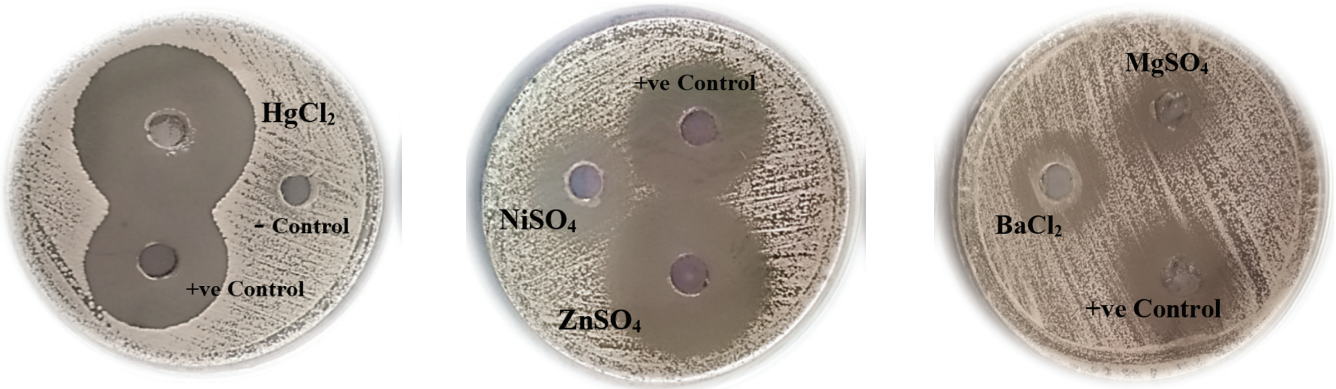

Fig. (3). Influence of Metal Salts on the Bioactivity of Antifungal Metabolite.

Candida spp. remained stable at the temperatures 30°C, 37°C and 40°C exposed for 30 minutes. However, the bioactivity was completely lost at higher temperatures such as 60°C for 30 minutes, 80°C for 30 minutes, 100°C for 15 minutes and 121°C for 15 minutes. This finding suggests that the antifungal compound produced by *P. aeruginosa* HS 28 was heat-labile therefore it lost its activity above 60°C. However, in contrast to our findings, some studies reported certain antifungal metabolites which were thermo-tolerant and did not lose their activity at high temperatures [28]. As per observation, *Bacillus methylotrophicus* produced a thermostable bacteriocin 'BM47' in Bulgaria which exhibited antifungal potential while retaining its biostability in diverse ecological niches. These factors made the bacteriocin applicable in agriculture as a bio-control agent as well as bio-preservative against the microbes who spoil food [28].

The effect of low temperature on the efficacy of antifungal metabolite was also determined and the results revealed that the bioactivity against *Candida* spp. was not retained at 0°C. However, the bioactivity was retained when the CFS was stored at 4°C for six months. This finding coincides with a study where the bioactivity of antifungal metabolite produced by *P. aeruginosa* was also found preserved at 4°C. Variation in pH values has an influence on the bioactivity of antifungal products. The antifungal metabolite produced by *P. aeruginosa* HS 28 when exposed to varying pH, the activity was only retained at neutral pH (pH 7) while the bioactivity was lost after exposure to acidic and alkaline pH. This finding deviated from previous studies where the antifungal activity of bacteriocin obtained from *Bacillus methylotrophicus* BM47, remained stable at the 3-11 pH range whereas maximum activity was observed at pH 7 and 8 [22].

The effects of different chemicals on the bioactivity of CFS of *P. aeruginosa* HS 28 were also determined. For this purpose, the CFS having antifungal metabolites was treated by diverse concentrations such as 1% and 5% of organic solvents such as ethanol, methanol, butanol, propanol, and acetone. Results declared no alteration in the bioactivity after such exposure, except for only 5% ethanol. Furthermore, in the case of surfactants, Tween 20 and Tween 80 did not affect the antifungal activity of CFS of *P. aeruginosa* HS 28. However, the treatment with EDTA (1%), resulted in the loss of this antifungal activity. These findings were consistent with a previous study where the partial loss of activity was reported after treatment with 50% methanol and acetone. However, the bioactivity was lost after exposure to Tween 20, Tween 80, and EDTA [22]. In this study, the effect of metal ions such as $BaCl_2$, $CaCl_2$, $MgSO_4$, $FeSO_4$, $NiSO_4$, and $ZnSO_4$ on antifungal activity was also estimated. According to our findings, metal ions did not alter the bioactivity of antifungal metabolites. An exclusive finding of our study is that no effect on the antifungal metabolite was observed when exposed to chloroform for any period of time under 30 minutes. However, after 30 minutes, an antifungal effect was induced. This study can be further extended to the purification of the bioactive protein through size exclusion and ion exchange chromatography, etc. Moreover, SDS PAGE analysis can be done for the complete identification followed by characterization of the compound using advanced techniques.

CONCLUSION

All bacteria can naturally produce various antimicrobial metabolites for survival; this phenomenon does not need any specific environment. This was also observed in this study where the *P. aeruginosa* of clinical origin revealed good antifungal potential against *Candida* species, particularly non-*albicans Candida* species *in vitro*. There is a need to purify this antifungal compound and further determine its antifungal potential in experimental animal models to present this compound as a candidate for future alternatives to anti-candidal agents.

AUTHORS' CONTRIBUTION

- **Hamna Hanif**: Methodology, Investigation, Data collection, Writing-original draft.
- **Sehar Afshan Naz**: Conceptualization, Funding acquisition, Project administration.
- **Nusrat Jabeen**: Formal analysis, Resources.
- **Maryam Shafique**: Resources, Critical review, Editing.
- **Waleed Ahmed**: Formal analysis, Critical review, Editing.

CONFLICT OF INTEREST

Declared none

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