Gharieb *et al. Annals of Microbiology* (2024) 74:23
https://doi.org/10.1186/s13213-024-01764-9

Annals of Microbiology

UNIVERSITÀ DEGLI STUDI DI MILANO

ORIGINAL ARTICLE

Open Access

Anticandidal activity of a wild *Bacillus subtilis* NAM against clinical isolates of pathogenic *Candida albicans*

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Abstract

Background Resistance to antifungal medications poses a significant obstacle in combating fungal infections. The development of novel therapeutics for *Candida albicans* is necessary due to the increasing resistance of candidiasis to the existing medications. The utilization of biological control is seen as a more advantageous and less hazardous strategy therefore the objective of this study is to identify the antifungal properties of *Bacillus subtilis* against pathogenic *C.* albicans.

Results We conducted a study to evaluate the antifungal properties of three bacterial isolates against the human pathogen *Candida albicans*. One of the bacterial isolates exhibited a potent antifungal activity against this fungal pathogen. This bacterium was identified as *Bacillus subtilis* based on the 16Sr RNA gene sequence. It exhibited inhibitory efficacy ranging from 33.5 to 44.4% against 15 *Candida* isolates. The optimal incubation duration for achieving the maximum antifungal activity was determined to be 48 h, resulting in a mean inhibition zone diameter of 29 ± 0.39 mm. The Potato Dextrose agar (PDA) medium was the best medium for the most effective antifungal activity. Incubation temperature of 25°C and medium pH value of 8.0 were the most favorable conditions for maximum antagonistic activity that resulted fungal growth inhibition of 40 ± 0.16 and 36 ± 0.94 mm respectively. Furthermore, the addition of 10.5 mg/ml of bacterial filtrate to *C. albicans* colonies resulted in 86.51%. decrease in the number of germinated cells. The fungal cell ultrastructural responses due to exposure to *B. subtilis* filtrate after 48 h were investigated using transmission electron microscopy (TEM). It revealed primary a drastic abnormality that lead to cellular disintegration including folding and lysis of the cell wall, total collapse of the yeast cells, and malformed germ tube following the exposure to the filtrate. However, the control culture treatment had a characteristic morphology of the normal fungal cells featuring a consistently dense central region, a well-organized nucleus, and a cytoplasm containing several components of the endomembrane system. The cells were surrounded by a uniform and intact cell wall.

Conclusion The current study demonstrates a notable antifungal properties of *B. subtilis* against *C. albicans* as a result of production of bioactive components of the bacterial exudate. This finding could be a promising natural antifungal agent that could be utilized to combat *C. albicans*.

Keywords Antifungal compounds, *Bacillus subtilis*, *Candida albicans*, Chemical profiling, GC/MS, TEM

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Introduction

The majority of human fungi infections are spread by *Candida* species (Lopes and Lionakis 2022). Numerous *Candida* species can colonize skin and mucosal surfaces, however in healthy individuals, this colonization does

not cause disease (Kühbacher et al. 2017). This yeast can invade the body and cause chronic infections if the mucosal or skin barrier is damaged or if the immune system is poor (De Groot et al. 2021). Few species, such as *Candida albicans*, *Candida glabrata*, *Candida tropicalis*, *Candida parapsilosis*, and *Candida krusei*, are responsible for the vast majority of candidiasis infections over the world (Singh et al. 2020). Around 70% of fungal infections worldwide are caused by *Candida albicans*, which is the most prevalent species to cause mucosal and systemic infections (Talapko et al. 2021). The commensal fungus called *C. albicans* typically lives on the skin and gastrointestinal tract of individuals., can cause both serious mucosal and fatal invasive infections in those who have impaired immune systems as a result of AIDS or cancer chemotherapy (Jia et al. 2019). The majority of *C. albicans* infections result in high rates of morbidity and mortality due to the development of a biofilm on the surface of the host or on abiotic surfaces (implants) (Tsui et al. 2016). Currently, azoles, polyenes, allylamines, echinocandins, and 5-fluorocytosine are the most often used antifungal medications. The prevalence of opportunistic pathogen infections has gradually increased since the widespread use of broad-spectrum antifungal medications (Jia et al. 2019). Therefore, research into new and more powerful antifungals is necessary for preventing fatal candidiasis.

Biological control is an alternative method for controlling microbial infections, that employing antagonistic organisms or its products to stop the propagation of harmful pathogens (Elfeky et al. 2023; Zayed et al. 2022; Li et al. 2022). Numerous microorganisms have been investigated as potential antagonistic organisms for the treatment of *C. albicans*. These microorganisms include fungus (such as non-toxic *Aspergillus*, *Trichoderma*, and *Penicillium*), yeast strains, and bacteria (Li et al. 2022). *Bifidobacterium* was used to restrict the expansion of infectious microbes (Fukuda et al. 2011; Lau et al. 2014). This bacterium is a critical component of the normal human gut microbiota and can be used as probiotics in food, medicine and feed (Abou-Kassem et al. 2021; Kadja et al. 2021). Also, Lactic acid bacteria are used as probiotics and normally found in the oral cavities, gastrointestinal tracts and vagina of human (Bulgasem et al. 2017). There are certain strains were used as antifungal against *Candida* spp. *Lactobacillus acidophilus* was found to produce compounds that potentially have an impact on *C. albicans* (Bulgasem et al. 2017). *Lactobacillus paracasei subsp M3* had an antifungal effect on *C. albicans*, *C. pseudointermedia* and *C. blankie* (Strus et al. 2005; Kariptas et al. 2010). *Lactobacillus johnsonii* had an effect on both biofilm and planktonic condition of *Candida albicans* (Vazquez-Munoz et al. 2022). *Bacillus spp.* are known with its superior biosafety, powerful resistance and widely used for antifungal action (Wang et al. 2022). *Bacillus subtilis* is a Gram-positive bacterium, able to survive in different environment (Li et al. 2022). It has a broad antibacterial spectrum, an accelerated rate of reproduction, a resistance to stress, the ability to form endophytic spores, and not hazardous to individuals or animals (Wang et al. 2022).

B. subtilis is considered as an extremophilic microorganism due to adaptation to severe environmental circumstances, as high or low temperature, pH, salinity, and pressure, all of which are unfavourable to the majority of living species (Etemadzadeh and Emtiazi 2021). It can release a huge number of metabolites (Harwood et al. 2018). A large number of these metabolites classified as secondary metabolites, since they are not necessary for the organisms' growth, development, or reproduction (Kai 2020). The two categories of secondary metabolites are volatile and non-volatile based on their physicochemical characteristics. The non-volatile secondary metabolites of *B.subtilis* include polyketides, non-ribosomal peptides, and lipopeptides (such as the classes of surfactin, iturin, and fengycin) (Harwood et al. 2018; Caulier et al. 2019). In general, bacteria release a wide range of secondary metabolites, such as terpenes, alcohols, ketones, sulphur- and nitrogen-containing chemicals, and hydrocarbons (Lemfack et al. 2018; Elmahmoudy 2021). While their main function is to facilitate interactions within and between species through long- and short-range information molecules, these metabolites can also possess antibacterial or antifungal activities (Schmidt et al. 2017; Schulz- Bohm et al. 2017). The four strains of *Bacillus A16* (*B. sphaericus*), *M142* (*B. circulans*), *M166* (*B. brevis*) and *T122* (*B. brevis*) which were isolated by researchers from soil samples, showed extensive inhibition of *C. albicans* (Ghai et al. 2007). *Bacillus* spp. isolated from soil and marine samples exhibited an antifungal activity against *C. albicans* (Li et al. 2022). The cell-free supernatant of *B. subtilis spizizenii DK1-SA11*, that was isolated from bay of yellow sea in China, had an extensive inhibitory impact on *C. albicans* (Khan et al. 2017). *Bacillus velezensis* was common in the surrounding environment and produced a lot of lipopeptides with effective bacteriostatic properties (Li et al. 2022). Devi et al. (2019) reported that *Bacillus velezensis* DTU001 has been tested by certain researchers for its ability to suppress 20 different kinds of human and/or plant pathogenic fungi. They showed that this bacteria produced lipopeptide (iturin and fengycin) and significantly inhibited *C.albicans* proliferation. Li et al. (2021) also showed *C. albicans* growth inhibition in vitro by *Bacillus velezensis* 1B-23. Li et al. (2016) observed that the cell- free supernatant of *Bacillus amyloliquefaciens* SYBC H47,

which was isolated from honey, had an extensive inhibitory effect on *C. albicans*.

The purpose of this research was to identify the potential antifungal activity of a wild *Bacillus subtilis* against *Candida albicans.* The effects of temperature, pH, incubation duration, medium composition, and metal salts on these antifungal activities were investigated. The active bacterial metabolite probably implicated in the antifungal activity as well as the consequent ultrastructural responses of *Candida* cells were also conducted.

Materials and methods

Organisms and cultivation media

Three bacterial strains that were isolated from our laboratory were uniformly spread across the surface of nutrient agar (NA) that consisted of the following components (in g/l): peptone (5.0), beef extract (1.5), yeast extract (1.5), NaCl (5.0), and agar (15.0). The pH was set to 7.2. The purity of the isolates was assessed, and they were subsequently stored in a mixture of 50% glycerol and 50% potato dextrose (PD) broth media at a temperature of -20 °C for further investigations.

Fourteen pathogenic strains of *Candida albicans* (*C. albicans*) were generously supplied by the Laboratory of Mycology at the Institute of National Liver, Menoufia University, Egypt. These strains are designated as C.1 to C.14. The reference strain used in this study was *C. albicans* NCPF3179/ATCC (SC Tody Laboratories INT. SRL, Romania), which was designated as C.15. The yeast strains were commonly cultured on Potato Dextrose Agar (PDA, that consisted of the following ingredients in (g/L): Potato extract (200), dextrose (20) and agar (15) at a temperature of 28 °C and preserved in Potato Dextrose Broth (PDB) supplemented with 50% glycerol at a temperature of -20 °C for subsequent analysis.

Bacterial supernatant (cell-Free supernatant) preparation and evaluation its in Vitro Inhibitory Activity

To prepare cell-free supernatant (CFS), the bacterial seed culture (BSC) was initially prepared by introducing an agar disc with evenly distributed bacterial biofilm to sterile PDB (100 ml) and incubated for 15 h (approximately 0.8 optical density (OD_{600}). Then 5 ml of seed culture was inoculated in a new sterile medium (100 ml), and incubated for two days at 30 °C and 120 rpm. Under aseptic conditions, the suspensions were centrifuged at 6000 rpm for 10 min, and the supernatant was then collected and filtered through bacterial filter (0.22 µm).

The antifungal activity of CFS was assessed using the well diffusion method, as described by El Barnossi et al. (2020). Wells with a diameter of 6 mm were created using a sterile cork borer on PDA media that had been inoculated with *C. albicans*. Subsequently, these wells were filled with 100 µl of cell-free supernatant (CFS). Three replicas were made for each bacterial isolate. The petri dishes have been incubated at 37 °C for 24 h. The appeared inhibition zone was measured for each individual isolate. Itraconazole (50 µg/ml) were used as a positive control. A sterile, fresh medium devoid of bacteria was employed as the negative control.

Biochemical and molecular identification of the active bacterial isolate (BAC3)

The bacterial isolate, designated as BAC3 exhibited the most effective antagonistic activity against *C. albicans*, was selected for further experimentations. The detection of bacterial cell type, shape, and arrangement was accomplished through Gram stain technique (Moyes et al. 2009). The chemical characteristics of the bacterial cells were determined using the VITEK system at the Animal Health Research Institute, Cairo, Egypt. The identification was genetically confirmed by 16s rRNA sequencing. Macrogen, Korea carried out the molecular identification of the isolated bacterium. The process of DNA extraction and purification was carried out using InstaGene Matrix (BIO-RAD, cat. no. 732–6030), following the methodology provided by the provider. The purity of the purified DNA was deemed satisfactory when the ratio of OD260/OD280 was approximately 1.8, and the ratio of OD260/OD230 was approximately 2.0. In order to determine the sequence of the gene encoding 16 S RNA, the polymerase chain reaction (PCR) was conducted. This involved the utilization of the Taq polymerase Dr. MAX DNA Polymerase (manufactured by Doctor Protein, Korea, catalogue number DR00302) and the universal primers 27 F/1492R (Tables 1 and 2). The PCR process was carried out using the DNA Engine Tetrad 2 Peltier Thermal cycler (manufactured by Applied Biosystems, Foster City, CA, USA). The PCR product underwent purification using the Multiscreen Filter Plate manufactured by Millipore Corp. in Darmstadt, Germany. Subsequently, the sequencing process was conducted utilizing Sanger (dideoxy) technique, employing the BigDye (R) Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Vilnius, Lithuania), and employing the universal primers

Table 1 Sequences of the universal primers used for PCR amplification and sequencing

	Primer	Sequence
PCR Amplification	**27 F**	5` (AGA GTT TGA TCM TGG CTC AG)3`
	1492R	5` (TAC GGY TAC CTT GTT ACG ACT T)3`
Sequencing	**785 F**	5` (GGA TTA GAT ACC CTG GTA)3`
	907R	5` (CCG TCA ATT CMT TTR AGT TT)3`

Table 2 PCR cycle conditions

Step		Temperature (°C)	Time (min)	Cycles
Initial Denaturation		95	5	1
Cycling	Denaturation	95	0.5	30
	Annealing	55	2	
	Extension	68	1.5	
Final Extension		68	10	1

785 F/907R (Tables 1 and 2). The ABI PRISM 3730XL Analyzer (96 capillary type) was employed for sequencing purposes. Following the process of sequencing assembly and subsequent elimination of low-quality sections, the acquired sequences were compiled into contigs, and subsequently, the sections of low quality were eliminated. The resulting sequence was submitted to the GenBank database and assigned the accession number OQ026817.

The bacterial strain has been preserved at the Moubasher Mycological Centre (Assiut University in Egypt) with the identification number AUMC B-542.

Evaluation of the susceptibility of the *Candida* isolates to antifungal drugs

Using the previously described well diffusion approach, the *Candida* species isolates were examined to determine their susceptibility to antifungal drugs; fluconazole and itraconazole at concentrations 100 µg/ml and 50 µg/ml, respectively. The antifungal drugs chosen for this investigation were based on those commonly used in medical practice and health therapy (Bulgasem et al. 2016).

Culture conditions affecting Anticandidal activity of *Bacillus subtilis*

PDB was inoculated with 5 ml of bacteria seeding culture (BSC) ($OD_{600}=0.8$) and incubated at a temperature of 30 °C with shaking at 120 rpm for 18, 24, and 48 h periods, then the CFS was prepared and tested against *C. albicans* strains was conducted as described above. Three replicates were created for each treatment that were incubated at the previously mentioned conditions, after which the diameter of the zone of inhibition was assessed. In order to investigate the influence of culture media on the antagonistic activity of bacterial isolates, 5 ml aliquite of the BSC (bacterial suspension culture) were inoculated into different microbial growth media; Luria Bertani (LB) containing in (g/l): tryptone (10), yeast (5), and NaCL(10), nutrient broth (NB) containing in (g/l): peptone (5.0), beef extract (1.5), yeast extract (1.5), NaCl (5.0), and potato dextrose broth (PDB). The flasks were subjected to incubation for a duration of 48 h at a temperature of 30 °C and 120 rpm. The aforementioned steps were employed to assess the antagonistic activity of the cell-free supernatant (CFS) derived from the bacterial cultures against *Candida albicans* strains. In order to examine the effect of incubation temperature on the antagonistic activity of the bacterial isolate, triplicates of inoculated PDB were subjected to incubation at 25, 30, and 35 °C for 48 h with shaking at 120 rpm. The same medium and conditions were used to investigate the influence of initial pH value, being 4, 6, 7, 8, 10 and 12, were used.

Administration of PDB medium with different metallic salts, including sodium selenite, mercuric chloride, zinc sulphate, ferrous sulphate, and copper sulphate at two concentrations of 0.1 µm and 1 µm was executed to measure the extent of inhibitory zone. In each experiment, a sterile, fresh medium devoid of bacteria was employed as the negative control.

Effect of CFS on germ tube formation of *C. Albicans*

The influence of CFS on the process of germ tube formation by *Candida albicans* was conducted using human serum, following the methodology outlined by Moya et al. (2018). In this experiment, a solitary colony of *Candida albicans* was introduced into 20 ml of human serum and thoroughly mixed. Subsequently, the resulting mixture was divided into individual Eppendorf tubes, with each tube containing 0.5 ml of the mixture. A total volume of 300 ml of cell-free supernatant (CFS) underwent the process of lyophilization. Subsequently, the resulting lyophilized product was reconstituted in sterilized distilled water to get a stock solution with a concentration of 30 mg/ml. After that, various concentrations were manipulated within the infected serum to generate solutions with final concentrations of 1.5 mg/ml, 4.5 mg/ml, 7.5 mg/ml, 9 mg/ml, and 10.5 mg/ml. The inoculated tubes with no CFS were used as controls. Following a 24-hour incubation period at a temperature of 37 °C, the enumeration of germinated cells was conducted using a hemocytometer. Itraconazole (50 µg/ml) was used as a positive control.

Transmission electron microscopy (TEM) JEOL – JSM-1400 PLUS

To investigate how the filtrate of *Bacillus subtilis* affected the germinated cells of *Candida albicans*, fresh treated and untreated cells of *C. albicans* were cut into small pieces and fixed by immediately submerging them in 1 ml of 2.5% glutaraldehyde to 24 ml of either 4% paraformaldehyde in phosphate buffer solution (pH = 7.2) at 4 °C for three hours. After that, specimens were post-fixed for two hours at 4 °C in the same buffer containing 2% OsO_4. Therefore, samples were then dehydrated at 4 °C using a graded series of acetone after being cleaned in the

buffer. Samples were then divided into portions that were roughly 90 angstroms thick after being placed in resin to polymerize. Assemble parts on the grid copper. Finally, Lead citrate is added after five minutes of uranyl acetate staining (Tahmasebi et al. 2015). The images were captured by transmission electron microscope (JEOL – JSM-1400 PLUS) at faculty of science, Alexandria University, Egypt.

Chemical profiling of CFS using gas chromatography / mass spectrometry (GC-Ms)

GC/MS analysis was done using Shimadzu GCMS-QP2020 (Tokyo, Japan). The GC was equipped with Rtx-1MS fused bonded column (30 m × 0.25 mm i.d. × 0.25 µm film thickness) (Restek, USA) and a split-splitless injector. The initial column temperature was kept at 45 °C for 2 min (isothermal) and programmed to 300 °C at a rate of 5 °C/min, and kept constant at 300 °C for 5 min (isothermal). Injector temperature was 250 °C. Helium carrier gas flow rate was 1.41 ml/min. All the mass spectra were recorded applying the following condition: (equipment current) filament emission current, 60 mA; ionization voltage, 70 eV; ion source, 200 °C. Diluted samples (1% v/v) were injected with split mode (splite ratio 1: 15). The ions were found between 5000 and 55,000 m/z. The GC ran for 60 min in total. The gases produced by *Bacillus subtilis* were then identified by comparing the mass spectra of the VOCs obtained with those in the NIST/EPA/NIH Mass Spectrometry Library in relation to the spectra in the Mainlib and Replib databases (Yuan et al. 2012).

Results and discussion

Evaluation of the Anticandidal activity of the bacterial isolates

The antagonistic activity of three bacterial isolates against *C. albicans* are depicted in Fig. 1. In present study, three bacteria isolate were inoculated onto PDA plates and incubation at 30°C for 24 h. Then, growing bacteria were introduced against *C.15* by agar plug method and zone of inhibition was measured after 24 h at 30°C. The strain Bac 3 demonstrates a strong inhibitory effect on the pathogenic yeast in comparison to the other bacterial isolates. This is evident by the observation of an inhibition zone measuring around 30 mm, which is greater than the inhibitory effect of the commercially available antifungal agent, itraconazole (13.67 mm). While Bac 1 and Bac 2 show inhibition zone 19 and 14 mm respectively. In contrast to earlier research, Bulgasem et al. (2016) found that lactic acid bacteria isolated from honey samples suppressed the growth of *C. albicans* by 6 ~ 10 mm using the dual agar overlay method after 24 h and at 30°C of incubation, and by 10.0 and 17.2 mm using the well diffusion method after 24 h at 37°C. El Barnossi et al. (2020) found that *Bacillus* sp. Gn-A11-18 isolated from solid green household waste (banana, pomegranate and tangerine waste) had significant inhibitory effects on the development of *C. albicans*. The inhibition zone measured 44.66 mm when agar plugs were used. While

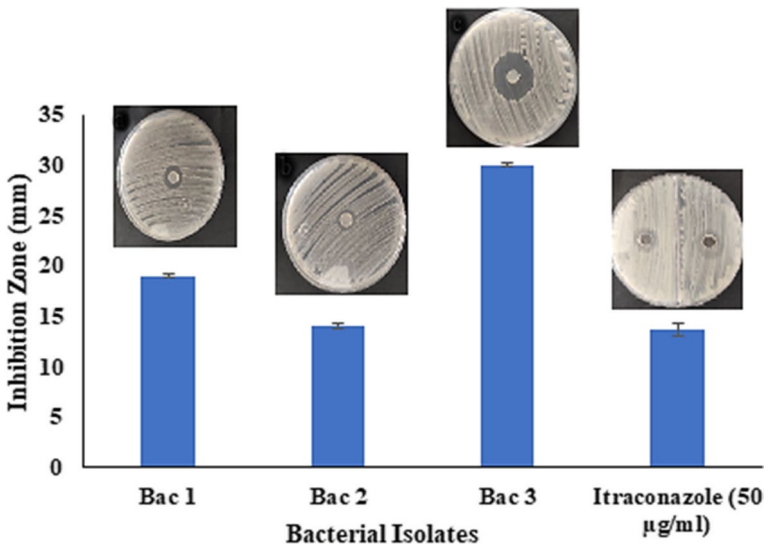


Fig. 1 Inhibition zone (mm) of *C.albicans* produced by the bacterial isolates (Bac 1, Bac 2, and Bac 3). Itraconazole was used as a positive control. The results are represented by the means of three replicates. The *C. albicans* cells were evenly distributed across the surfaces of the PD agar plates, and bacteria were introduced as an agar plug in the center of the plates. Itraconazole was applied as a positive control using the well diffusion method. Standard deviation is represented as an error bar

filtrate of *Bacillus* sp. Gn-A11-18 had strong antifungal activity against *C. albicans* with inhibition diameter of 31.33 mm and 42.33 mm when disc and well diffusion methods were employed, respectively. Furthermore, the *Gn-A11-18* isolate's filtrate, which was autoclaved for 30 min at 120 °C, demonstrates strong activity against *Candida albicans* by 27.33 and 41.00 mm using disk and well diffusion method respectively, indicating that the bioactive material exhibiting antifungal activity in the isolate is thermoresistant (El Barnossi et al. 2020). Khan et al. (2017) showed that CFS of marine *Bacillus* which isolated from marine invertebrate samples could hinder the growth of *C. albicans*, resulting in a 19 mm zone of inhibition by using Oxford Cup method. Moussaid et al. (2019) have reported the isolation of an *F27* isolate, whose filtrate has an antifungal activity with an inhibition diameter of 14.7 mm against *C. albicans*. These studies are among the many that have focused on the control of *C. abicans* by the use of essential oils and substances of bacterial and fungal origin. *Bacillus spp.* isolated from *Calotropis procera* rhizosphere have demonstrated antifungal activity against *C. albicans ATCC 102,031* with an inhibition diameter of 36.33 mm on the YM (Yeast and Malt extract) medium, according to research by Balouiri et al. (2015). The six *lactic acid bacteria* strains exhibited varied degrees of *anti-Candida* activity, according to the study by Bamidele et al. (2019). *Pediatric pentosaceus BTA 51 cucumber*, in particular, displayed the largest inhibitory zone, measuring 14 mm at neutral pH. According to research by Bulgasem et al. (2016), the free cell supernatant of *L. plantarum* isolates possesses strong antifungal activity against *C. albicans*, with a 25 mm inhibitory zone. Upon comparing our findings with the literature, it is evident that the current Bac3 strain is among the most competitive and efficient biocontrol agents documented for combating *C. albicans*.

Biochemical and molecular identification of the bacterial isolate (bac 3)

Due to the significant antagonistic activity of Bac 3 isolate it was chosen for further experimentations. The Gram stain was carried out to detect the shape, arrangement, and type of bacterial cells. As indicated in Fig. 2a, this bacterium is gram positive, rod shape, and arranged as mono bacillus and diplo bacillus (Kai, 2020). The biochemical features shown in Table 3 revealed its complete similarity with *Bacillus subtilis.* The identified strain has the ability to ferment glucose, but it does not have the same capability for maltose or lactose (Lu et al. 2018). The results of the Catalase, Indole, and Urease tests are negative, indicating the absence of certain enzymes (Lu et al. 2018). However, the oxidase test is positive, indicating the presence of the Oxidase enzyme (Al-Dhabaan, 2019). Di et al. (2023) demonstrated that *Bacillus subtilis B9* characterized with positive gram stain, hydrolysis of starch, formation of indole, catalase test, phospholipase, sucrose, Voges-Proskauer test, Hydrolysis of gelatin, Urease test, Glucose, and Mannitol reaction, while negative for KOH reaction, Methyl red test, Utilization of citrate, and H2S production. Lee et al. (2020) reported that, biochemical test of *Bacillus subtilis* by vitek was positive reaction for Beta-xyloxidase, L-Aspartate arylamidase, Leucine arylamidase, Phenylalanine arylamidase, L-Pyrrolydonyl-arylamidase, Alpha-galactosidase, Alanine arylamidase, Tyrosine arylamidase, Ala-Phe-Pro arylamidase, Myo-inositol, D-Mannose, D-Melezitose,

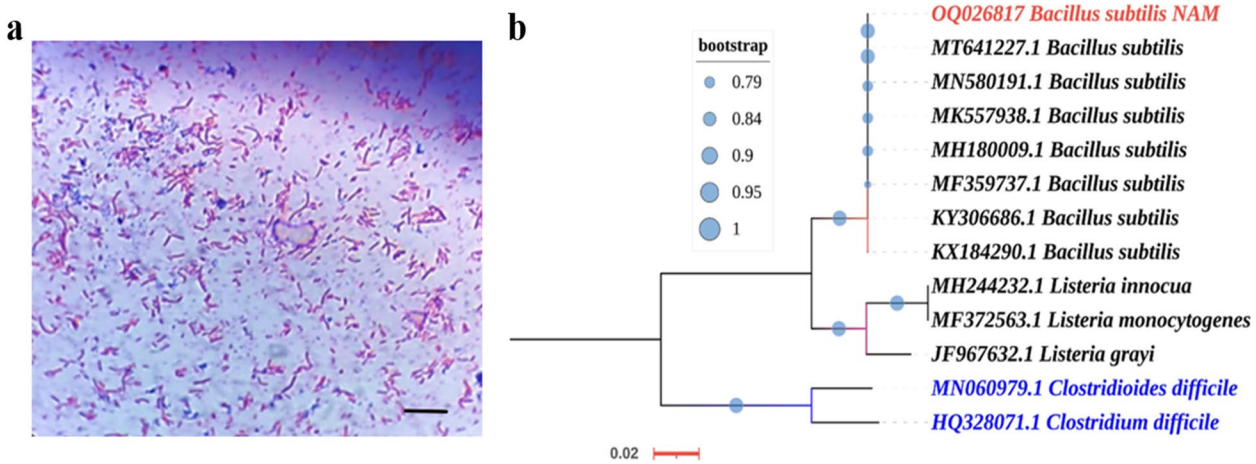


Fig. 2 Morphological and molecular identification of the bacterial isolate Bac 3; **a** microscopic image of Gram-stained bacterial cells, the gram positive bacillus shape bacteria are detected, the scale bar = 10 µm. **b** Constructed phylogenetic tree based on the 16 S rDNA sequences of numerous bacterial strains. The computation of evolutionary distances was performed using the Kimura 2-parameter technique (Kimura 1980)

Table 3 Biochemical characteristic of Bac 3 as detected by VITEK system

Test	Result	Test	Result	Test	Result	Test	Result
BXYL	+	LysA	-	AspA	-	LeuA	-
BGAL	+	PyrA	+	AGAL	+	AlaA	+
APPA	+	CDEX	-	dGAL	-	GLYG	+
ELLM	-	MdX	-	AMAN	-	MTE	+
Dmna	+	dMLZ	-	NAG	-	PLE	+
BMAN	-	PHC	-	PVATE	+	AGLU	-
INU	+	dGLU	+	dRIB	+	PSCNa	-
OLD	+	ESC	+	TTZ	+	POLYB I	+
PheA	-	INO	+	IRHA	-	NaCl 6.5%	+
TyrA	+	GlyA	-	dTAG	-	ProA	-
BNAG	-	MdG	+	Dman	+	BGLU	+
dTRE	+	KAN	-				

Palatinose, Beta-glucosidase, Beta-mannosidase, Pyruvate, and Alpha-glucosidase. While, negative reaction for L-Lysine-arylamidase, L-Proline arylamidase, Beta-galactosidase, Cyclodextrine, D-Galactose, Glycogene, Alpha-mannosidase, Maltotriose, Glycine arylamidase, D-Mannitol, N-Acetyl-D-glucosamine, L-Rhamnose, and Phosphoryl chloline.

The genotypic identity of the organism was determined using 16S rDNA sequencing and afterwards analyzed using BLAST. As shown in Fig. 2b, the top branches display the results of the bootstrap test (1000 repetitions), indicating the proportion of duplicate trees in which the linked taxa formed clusters together (Felsenstein 1985). The depicted tree has been accurately scaled, ensuring that the lengths of its branches are consistent with the evolutionary distances utilized in the inference of the phylogenetic tree. The computation of evolutionary distances was performed using the Kimura 2-parameter technique (Kimura 1980), with the resulting values expressed in units of base substitutions per site. The present study encompassed a total of 13 nucleotide sequences. Any locations that had less than 95% site coverage were removed. This means that any positions with fewer than 5% alignment gaps, missing data, or ambiguous bases were not allowed and were partially deleted. The final dataset consisted of a total of 703 locations. Evolutionary analyses were conducted in MEGA11 (Tamura et al. 2021). The results of this analysis revealed a 100% similarity with *Bacillus subtilis*, as depicted in Fig. 2b. Accordingly, the studied strain was named *Bacillus subtilis* NAM.

Susceptibility of the *Candida* isolates to antifungal agents

Depending on the species, different antifungal drugs had different effects on *Candida spp*. sensitivity. According to breakpoints laid down by CLSI guidelines, we classified the studied *Candida spp* into sensitive when the inhibition zone (IH) was ≥20 mm, dose-dependent sensitive (IH ranged between 9 and 19 mm), and resistant (IH ≤ 8 mm) for fluconazole, while for itraconazole the sensitive ones have IH ≥ 18 mm, the dose-dependent sensitive ones have IH ranged between 12 and 17 mm, and the resistant ones have IH ≤ 11 mm. As a result, *C.4*, *C.5*, and *C.6* were sensitive to itraconazole (50 µg/ml) while *C.1* and *C.2* were resistant and *C.3*, *C.7*, *C.8*, *C.9*, *C.10*, *C.11*, *C.12*, *C.13*, *C.14*, and *C.15* were dose-dependent sensitive (Table 4). While *C.1, C.2, C.3, C.4, C.7, C.11, C.12, C.13, C.14* were resistant to fluconazole (100 µg/ml). *C.5, C.6* were dose-dependent sensitive, and *C.8, C.9, C.10, C.15* were sensitive (Table 4).

On the same pattern, Bulgasem et al. (2016) found that *C. glabrata* ATCC2001 and *C. tropicalis* ATCC750 exhibited resistance to itraconazole (50 µg). However, *Candida albicans* ATCC14053 was susceptible to fluconazole (100 µg), amphotericin B (20 µg), and nystatin (100 U). Nystatin, amphotericin B, and fluconazole shown no efficacy against *C. tropicalis* ATCC750, *C. parapsilosis* ATCC22019, or *C. krusei* ATCC6258. Furthermore, it was observed that *C. glabrata* had susceptibility to nystatin, but demonstrated significant resistance to itraconazole and amphotericin B when tested using the disc diffusion method. Magaldi et al., (2004) reported that, for all antifungal drugs tested with *Candida* spp., there was no significant difference ($p > 0.05$) between the well diffusion method and the National Committee for Clinical Laboratory Standards' recommended (NCCLS) microdilution method. As a result, this straightforward well diffusion test is highly reproducible for pathogenic yeasts and strongly provides. The authors used NCCLS breakpoints to analyze the results for each test and

Table 4 Activity of antifungal drugs against different isolates of *Candida* spp as expressed by diameter of inhibition zone (mm). The data are the mean of three replicates ± standard deviation (SD)

Candida strains	Inhibition Zone (mm)	
	Fluconazole (100 µg/ml)	Itraconazole (50 µg/ml)
C.1	≤ 6	≤ 6
C.2	≤ 6	≤ 6
C.3	≤ 6	17.22 ± 0.77
C.4	≤ 6	24.12 ± 0.38
C.5	9.880 ± 0.19	24.22 ± 0.19
C.6	19.89 ± 0.20	24.78 ± 0.39
C.7	≤ 6	17.88 ± 0.19
C.8	21.78 ± 0.39	14.67 ± 0.58
C.9	29.88 ± 0.19	14.89 ± 0.20
C.10	29.55 ± 0.38	16.33 ± 0.58
C.11	≤ 6	12.44 ± 0.38
C.12	≤ 6	12.32 ± 0.58
C.13	≤ 6	12.32 ± 0.58
C.14	≤ 6	14.55 ± 0.19
C.15	28.33 ± 0.58	13.67 ± 0.58

Table 5 Inhibition zone (mm) of *Candida spp.* due to *B. subtilis* NAM activity and itraconazole. The results are the mean of three replicates ± SD (Standard Deviation)

Candida spp.	B. subtilis Inhibition Zone (mm)	Itraconazole Inhibition Zone (mm)
C1	38.00 ± 0.58	≤ 6
C2	31.00 ± 1.15	≤ 6
C3	38.00 ± 0.58	17.22 ± 0.77
C4	38.00 ± 0.58	24.12 ± 0.38
C5	≤ 6	24.22 ± 0.19
C6	27.00 ± 1.15	24.78 ± 0.39
C7	≤ 6	17.88 ± 0.19
C8	29.00 ± 0.58	14.67 ± 0.58
C9	34.00 ± 0.58	14.89 ± 0.20
C10	30.00 ± 0.47	16.33 ± 0.58
C11	≤ 6	12.44 ± 0.38
C12	24.00 ± 0.58	12.32 ± 0.58
C13	39.00 ± 1.15	12.32 ± 0.58
C14	34.00 ± 1.15	14.55 ± 0.19
C15	30.00 ± 0.16	13.67 ± 0.58

categories into susceptible (IH ≥ 19 mm), susceptible-dose dependent (IH = 18 –13 mm), and resistant (IH ≤ 12 mm) for fluconazole and itraconazole using well diffusion method. While for NCCLS method, the susceptible ones had IH ≤ 0.8 µg ml^{-1}, The susceptible-dose dependent (IH = 16–32 µg ml^{-1}), and resistant (IH ≥ 64 µg ml^{-1}) for fluconazole, but for itraconazole susceptible (IH ≤ 0.125 µg ml^{-1}), susceptible-dose dependent (IH = 0.25–0.5 µg ml^{-1}), and resistant (IH ≥ 1 µg ml^{-1}) (Espinel-Ingroff et al. 1998, 2000). Chongtham et al. (2022) used disc diffusion method to evaluate susceptibility of *Candida* to antifungal agent, with fluconazole 25 µg, itraconazole 10 µg, amphotericin B 20 µg, voriconazole 1 µg and ketoconazole 30 µg. In their studies, breakpoints zone diameter was determined and classified into susceptibility (≥ 19 mm), susceptibility-dose dependent (15–18 mm), and resistant (≤ 14 mm) for fluconazole, but for itraconazole, susceptibility (≥ 17 mm), susceptibility-dose dependent (14–16 mm), and resistant (≤ 13 mm). Yassin et al., (2020) also reported that, *C. albicans* was resistant to the antifungal drug fluconazole and this might be as a result of its long-term therapeutic use while the yeast strain was sensitive to itraconazole and terbinafine.

Generally, azole medicines exert their pharmacological effect by inhibiting the formation of ergosterol, a vital constituent in yeast cells (Vanreppelen et al. 2023). Azoles attach to Erg11p, a protein known as 14α–demethylase, resulting in a significant decrease in the cell's ergosterol levels. This reduction leads to the production of a toxic sterol called 14α methylergosta 8–24 (28) dienol, which is formed by various enzymes in the pathway, namely Erg6p, Erg25p, Erg26p, Erg27p, and Erg3p (Bhattacharya et al. 2018, 2020). In addition, azoles also contribute to the elevation of reactive oxygen species (ROS) levels (Delattin et al. 2014). The production of toxic sterols and elevated levels of reactive oxygen species (ROS) hinder the growth of the infected fungus (Bhattacharya et al. 2020). The increased resistance of specific strains of *Candida* sp. to fluconazole and itraconazole can be related to the overexpression of the ERG11/CYP51A/CYP51B genes. These genes are responsible for the production of ergosterol, a compound essential for the growth and survival of *Candida* sp. (Houšť et al. 2020). In addition, the increased expression of ABC (adenosine triphosphate binding cassette) transporters and specific factors responsible for increased drug efflux are the secondary mechanism that leads to acquired resistance to azole drugs (Revie et al. 2018). The diverse impact of antifungal medicines on various *Candida* spp. investigated may be attributed to distinct genetic mutations resulting from prolonged and frequent use of these drugs, leading to the development of drug-resistant strains.

Efficacy of *Bacillus subtilis* NAM against various *Candida* strains

Table 5 and Fig. 3 represent the inhibitory effectiveness of *B. subtilis* NAM against several isolates of *Candida* sp.

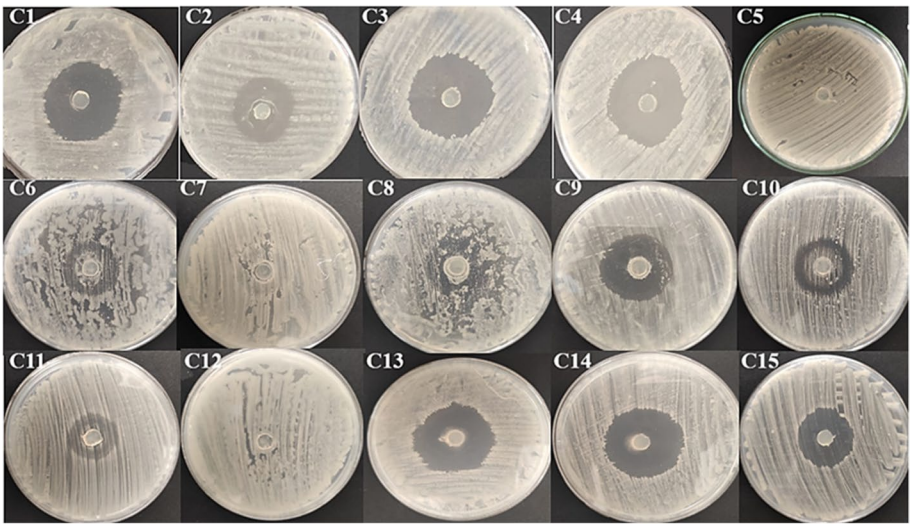


Fig. 3 Agar plug diffusion methods; *B. subtilis* agar plugs were placed in the center of plates swapped by different isolates of *Candida* species ranging from *C1* to *C15*. The plates incubated at 30 °C for 48 h. The diameter of inhibition zone was measured to detect the anticandidal activity of *B. subtilis* against various *Candida* isolates

obtained from diverse biological specimens. Out of the fifteen *Candida* isolates, only three showed resistances to the bacteria. The inhibitory effectiveness of the studied bacteria against the remaining isolates varied from 24 to 39 mm. This implies that our strain exhibits strong inhibitory effects against *Candida spp* on PDA media at 30°C for 24 h. The possible antifungal mechanism of *Bacillus spp*. filtrate toward *Candida spp* may be related to the destroying and lysis of the lipid membrane. It has the potential to alter the fungal cell membrane's surface tension, leading to micropore development, ion leakage (including K+), and ultimately cell death (Lima et al. 2018; Lei et al. 2019; Banerjee et al. 2022). The antifungal action of cell wall lyases produced by *Bacillus* species also inhibits pathogenic fungus. Lyases (include glucanase, cellulase, protease, and chitinase) are particularly effective against fungus due to the fact that chitin and glucan make up the majority of the fungal cell wall (Gomaa and El-Mahdy, 2018). In addition, it can produce bacteriocin which include nisin A, subtilin, and lanthionine (Caulier et al. 2019). Nisin A inhibits fungal growth by impeding the synthesis of newly formed cell walls and perforating cell membranes (Wang et al. 2022). *Bacillus subtilis* releases a variety of volatile secondary metabolites, which are believed to function as long- and short-range infochemical signals that facilitate interactions within and between different species. In addition, they often demonstrate antifungal or antibacterial characteristics (Kai, 2020).

Yuliani et al. (2018) found that *Bacillus subtilis* C19, a marine bacteria from Indonesia, has the ability to produce surfactin, a substance that can inhibit the reproduction of *Candida albicans*. Liu et al. (2019) showed that *Bacillus amyloliquefaciens* fmb60 lipopeptide C16-fengycin A can be extracted to obtain C16-fengycin A, which exhibits potent anti-*Candida albicans* activity. López et al. (2009) reported that *B. subtilis* utilizes cannibalism as a defense mechanism to slow its sporulation process. The cannibal cells secrete two toxins, skf and sdp, that possess the capability to impede the proliferation of other bacteria. The variation in inhibitory action against *Candida* isolates may be attributed to the presence of distinct *Candida* strains. There was variation in inhibitory effect of *Bacillus subtilis* against the *Candida* isolates may be due to specific genetic mutation of *Candida* which led to make some strain able to resist the extracellular metabolites of *B. subtilis* while the other cannot resist.

For our subsequent experiments, we chose the *Candida* strains *C13* and *C15.* Strain *C13*, which is inhibited by azoles, had the greatest sensitivity to our CFS compared to the other tested *Candida* strains while *C15* was the reference strain.

Culture conditions affecting Anticandidal activity of *Bacillus subtilis*

The accumulation of any secondary metabolite is influenced by the surrounding environmental circumstances and the unique properties of the microbial strain being produced (Abada et al. 2014; Yi et al. 2015). The composition of the medium, acidity, temperature, and cultivation period are some of the most important factors

influencing how bacteria display their antibiotic properties (Volova et al. 2014; Tumbarski et al. 2015). The current work aimed to examine the antifungal characteristics of the cell-free supernatant (CFS) obtained from *Bacillus subtilis* cultures at various time intervals (18 h, 24 h, and 48 h) against two strains of *C. albicans*. Based on the results provided in Fig. 4a, it is evident that there was a significant increase in the size of the inhibition zone as the duration of incubation increased. The measurements of the inhibitory zones for *C. albicans 13* and *C. albicans 15* were recorded as 29 ± 0.39 and 28 ± 0.39, respectively, after a 48-hour incubation period. Furthermore, the measurements of the inhibitory zones for *C. albicans 13* and *C. albicans 15* after a 24-hour incubation period were recorded as 27 ± 0.39 and 26 ± 0.58 respectively, and were recorded as 23 ± 0.58 and 25 ± 0.00 for *C.13* and *C.15* respectively, after a 18-hour incubation period. The increase of the inhibition zone with increasing incubation period may be depending on the growth phase. Most antibiotics are secondary metabolites, which are produced as the organism shifts from the active growth phase to the stationary phase (Demirkan et al. 2013). Our results align with Demirkan et al. (2013) findings, which indicated that the concentration of the inhibitory component in *Bacillus subtilis* MZ-7 rose during the post-exponential phase, reaching its maximum at 48 h in the stationary phase. Furthermore, in accordance with Pang et al. (2021), it was found that the optimal incubation time occurred after 48 h.

In order to examine the influence of various media components on the antimicrobial activity of *B. subtilis* against *Candida albicans*, three different types of media were utilized: potato dextrose broth (PDB), Luria-Bertani (LB) broth, and nutrient broth (NB) Fig. 4b. The findings of the study indicate that the PDB exhibited superior performance as a culture medium for *B. subtilis*, resulting in the largest inhibition zone (40 ± 0.31 and 45 ± 0.47 for *C13* and *C15*, respectively). In contrast, the use of NB as a culture medium only yielded inhibition zones of 18 ± 0.00 and 21 ± 0.94 for *C13* and *C15*, respectively Fig. 4b. The results obtained from LB culture did not demonstrate any inhibitory efficacy against *C. albicans*.

PDA media is a semi-synthetic media that contains a substantial quantity of sugar, minerals, and vitamins. These components can potentially increase the formation of antifungal metabolites by the antimicrobial agents (Fiddaman et al. 1993). Pang et al. (2021) propose that deliberate adjustments to the medium and nutrients can be systematically employed to stimulate the synthesis of particular desired metabolites. Our results are consistent with Fiddaman et al. (1993), who found that PDA was the most effective medium for producing antifungal chemicals by *Bacillus subtilis* against *Rhizoctonia solani* and *Pythium ultimum*. Likewise, Binmad et al. (2022) employed PDA medium to assess the inhibitory impact

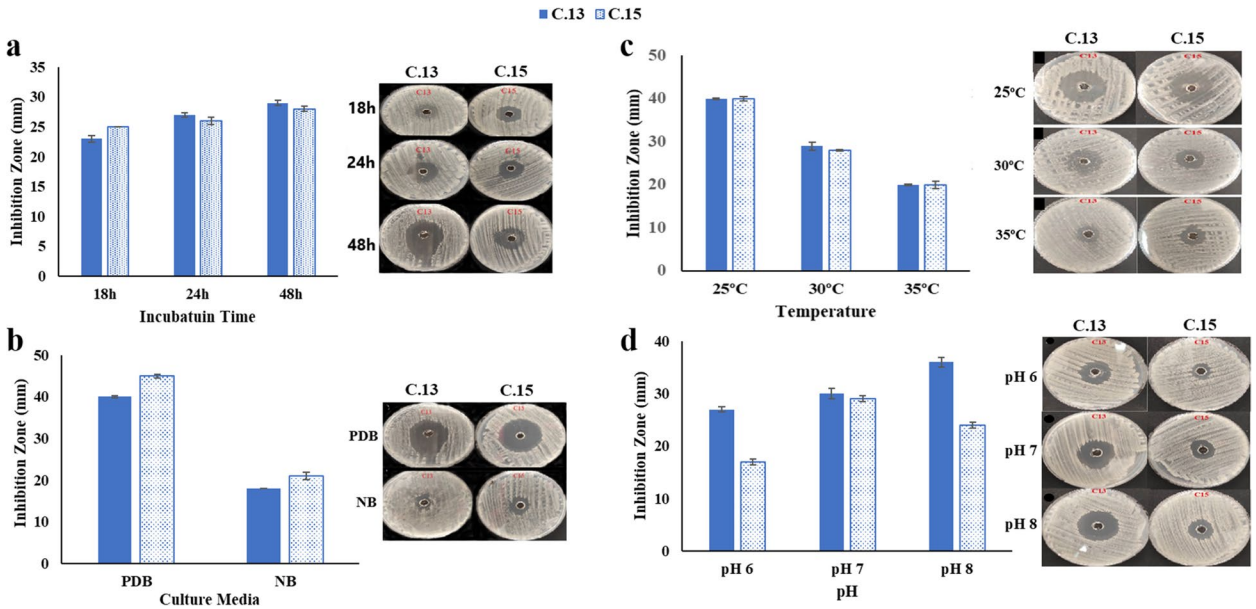


Fig. 4 Effect of different culture conditions on the anticandidal activity of *B. subtilis* CFS against two strains of *C. albicans C.13&C.15*; **a** Incubation time, **b** Temperature degrees, **c** Culture media, PDB (Potato Dextrose Broth), NB (Nutrient Broth), **d** pH degrees. The result is the mean of three replicates. The standard deviation was calculated and represented as error bar. The agar plates display the inhibition zone generated by *B. subtilis* CFS, which was prepared under various culture conditions, against *C. albicans* (C13 and C15)

of extracellular polymeric compounds evaluated from *Bacillus velezensis P1* against *Bipolaris oryzae NPT0508* and *Curvularia lunata SPB0627*.

The influence of different incubation temperatures on the inhibitory efficiency of *B. subtilis* against two strains of *C. albicans* is depicted in Fig. 4c. The highest inhibitory efficacy was seen at a temperature of 25°C, resulting in inhibition zones measuring 40 ± 0.16 mm and 40 ± 0.47 mm for *C13* and *C15*, respectively. While further increase in the incubation temperature led to the decline in the inhibition zone. Therefore, at temperature of 30 °C resulting in inhibition zone measuring 29 ± 0.94 mm and 28 ± 0.16 mm for *C13* and *C15*, respectively. While, at temperature of 35 °C resulting in inhibition zone measuring 20 ± 0.16 mm 20 ± 0.78 mm for *C13* and *C15*, respectively. Temperature is considered as one of the important physiological factors that effect on the growth and biocontrol activity of *Bacillus* sp (Jiménez-Delgadillo et al. 2018). Khan et al. (2017) reported that 25 °C was the best temperature for production antimicrobial compounds from *B. subtilis subsp. spizizenii DK1-SA11* against *salmonella typhimurium, E. coli O157:H7, C. albicans, Klebsiella pneumoniae, Listeria monocytogenes, Vibrio parahaemolyticus, E. coli, Pseudomonas fluorescens, Vibrio cholerae* and methicillin-resistant *Staphylococcus aureus* because this temperature was associated with the greatest and most stable production over an extended period of time. Sidorova et al. (2020) reported that the inhibition rate of *Fusarium oxysporum var. Orthoceras* by *Bacillus subtilis* was shown to be negatively impacted by both higher and lower culture temperatures, suggesting that the generation of antifungal metabolites was temperature-dependent.

On the other hand, Oyedele et al. (2014) observed that the optimal temperature for antifungal activity of *Bacillus subtilis* against four pathogenic fungi including *Aspergillus niger, Fusarium oxysporum, Aspergillus flavus*, and *Rhizopus stolonifer* was at 37°c. Also Pang et al. (2021) demonstrated that rate of inhibitory effect was high at 31 °C when used *Bacillus amyloliquefaciens* as antimicrobial agent against *Botryosphaeria dothidea*.

To examine the influence of pH on the anticandidal activity of *B. subtilis*, the pH of the culture media was progressively varied within the range of 5 to 12, Fig. 4d. Based on the experimental results, it was seen that a pH value of 8 was identified as the optimal condition for the inhibition of *C. albicans* (*C13*) by the culture supernatant of *B. subtilis*. The present scenario resulted in the formation of an inhibition zone of 36 ± 0.94 mm. On the other hand, it was noted that a pH of 7 displayed the most favorable conditions for emergence of anticandidal activity by *B. subtilis* against *C. albicans* (*C15*). The present scenario resulted in the formation of an inhibition zone of 29 ± 0.58 mm (as shown in Fig. 4d). While the acidic pH degrees did not show any anticandidal activity.

Fluctuations in the external pH have a significant influence on various biological processes, such as the regulation of secondary metabolite synthesis (Demirkan et al. 2013). Hence, modifying the pH level has the potential to influence the quantity of *Bacillus* antifungal activity generated against the infection (Wang et al. 2002). According to Demirkan et al. (2013), *Bacillus* sp. EA62 exhibited the highest antibiotic activity when the pH was 7.5. The studies conducted by Khan et al. (2017) and Rafanomezantsoa et al. (2022) revealed that the ideal pH range for both growth and production of antimicrobial activity is between 5.7 and 8.0. Sidorova et al. (2020) reported that the metabolic composition, both in terms of quality and quantity, is affected by changes in the acidity of the nutritional medium and these changes are strain specific.

Trace metals are crucial for bacterial growth since they serve as cofactors for enzymes that facilitate vital metabolic activities necessary for cellular energy production and development (Pajarillo et al. 2021). The detection of the influence of metals on the antibacterial activity of *B. subtilis* against *C. albicans* was observed, Fig. 5. In comparison to the control group, the addition of 0.1 µm of mercury chloride resulted in an augmentation of the anticandidal activity of *B. subtilis*, specifically against strain *C13* Fig. 5a. The addition of zinc sulphate and ferric sulphate at a concentration of 1 µm resulted in an increase in the anticandidal activity of *B. subtilis* against strain *C15*, as shown in Fig. 5b.

The production of secondary metabolites (SMs) has been found to be influenced by trace metals, as indicated by numerous studies (Chiang et al. 2011; Ochi and Hosaka 2013; Dubey et al. 2019). SMs are typically generated under specific cultural settings, as stated by Dubey et al. (2019). Hence, to effectively stimulate the generation of these substances, it is crucial to have the specific trace metal present, as it plays a pivotal role in regulating metabolic functions by exerting control over gene expression on a global scale (Ochi and Hosaka, 2013). In addition, proteins biosynthesis may also affect gene expression in response to different concentrations of metal ions, such as copper and zinc (Dubey et al. 2019). Supplementing scandium in the culture media of *Bacillus subtilis* effectively enhances antibiotic synthesis, particularly bacilysin (Ochi and Hosaka, 2013). Similarly, a recent study carried by (Shatnawi et al. 2021) revealed that the methanolic and aqueous extracts of *Paronchia argentea*, which were cultivated under lead (Pb), copper (Cu), or cobalt (Co) stress, had notable inhibitory effects against the tested pathogenic fungus and bacteria when cultivated in vitro. The detected inhibitory zones ranged from 6.7 to 30.0 mm. According to our findings,

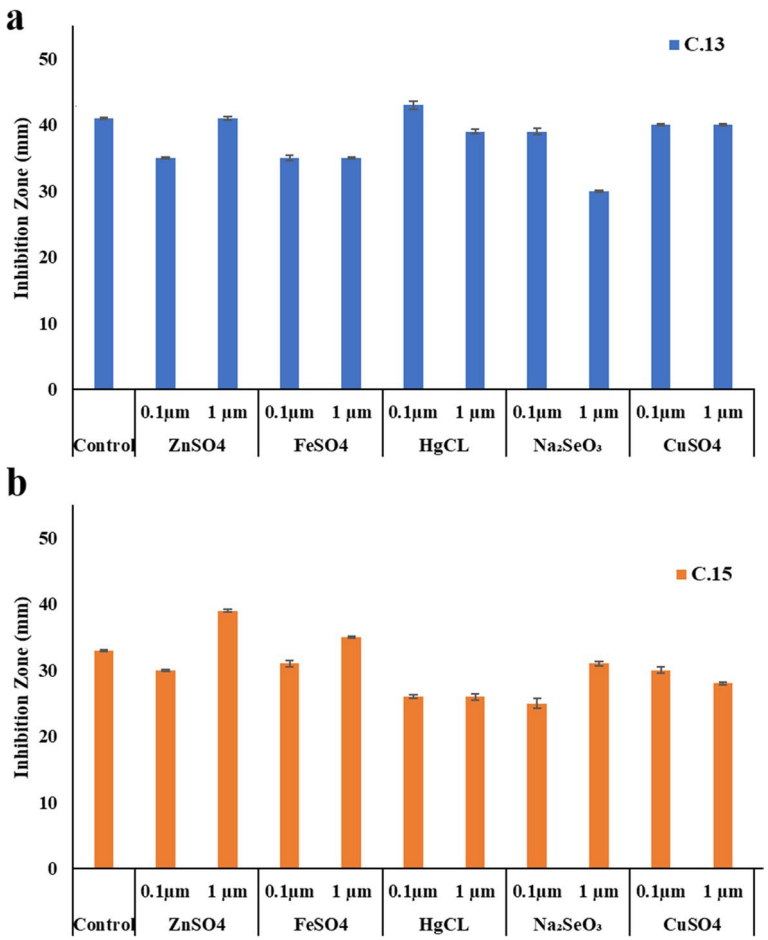


Fig. 5 Inhibition zone of *C. albicans* strains (*C13* and *C15*) due to treating with the CFS of *B. subtilis* culture supplemented with different metal salts at concentrations of 0.1 and1.0 µM. The data are the mean of three replicates. The standard deviation is calculated and represented as error bar

the growing conditions for *B. subtilis* antifungal metabolite production could be modified to increase production efficiency. *B. subtilis*' anticandidal activity can be improved by using PDA as a growth medium and adding trace metals such as 0.1 µm mercury chloride, 1 µm zinc sulphate, and ferric sulphate. Set the pH to 8 and incubate at 25°C for 48 h.

Effect of CFS on germ tube formation by *C. Albicans*

One potential method for managing candidiosis is by inhibiting cell germination, as the pathogenicity of *Candida* is highly dependent on the development of germ tubes (Sudbery et al. 2004). In our investigation, we combined the *Candida* cells with serum that had varying concentrations of CFS. The mixture was then placed in an incubator at a temperature of 37 °C in order to facilitate the creation of germ tubes. The findings demonstrated the effectiveness of CFS in suppressing the germination of *Candida* cells, as indicated in Table 6. CFS was obtained by inoculating bacteria into potato dextrose broth media and was incubated in shaker for 48 h at 30°C and 120 rpm. Then, inoculating media was centrifuged at (6000 rpm, for 10 min). After that, *Bacillus* supernatant was filtered used (0.45 µm-pore-size filter; Millipore, Darmstadt, Hesse, Germany) (Ogunbanwo, 2005). The germination of *C.15* and *C.13* cells was suppressed by 86.5% and 70.80% correspondingly at a concentration of 10.5 mg/ml. However, utilizing a concentration of 0.05 mg/ml of itraconazole as a positive control, there was a reduction of germ tube production by 66.8% and 50.00% correspondingly.

In a similar pattern, Palande et al. (2015) found that *Bacillus* spp. effectively blocked the germ tube of *Candida albicans* by 99%. *Pediococcus acidilactici* inhibited the germination of *Candida albicans* cells by 77% (Zareshahrabadi et al. 2020). The suppression of germ tube formation can be linked to the suppression of gene expression that encodes virulence factors, such as the hyphae wall proteins HWP1 and ALS3, which are associated with the growth of hyphae (Tsang et al. 2012). *B.*

Table 6 Effect of different concentrations of *B. subtilis* CFS on germ tube formation by *C. albicans* isolates C15 and C13, Itraconazole was used as a positive control. The data are the mean of three replicates. The standard deviation is calculated and represented as error bar

		C.15		C.13	
		Germinated cell	Inhibition %	Germinated cell	Inhibition %
Control		2.69E + 07 ± 1.03	-	2.40E + 07 ± 1.15	-
CFS (mg/ml)	1.5	2.63E + 07 ± 0.75	2.33	1.90E + 07 ± 0.11	20.83
	4.5	2.48E + 07 ± 0.35	7.91	1.30E + 07 ± 0.58	45.83
	7.5	9.50E + 06 ± 1.09	64.65	1.10E + 07 ± 0.11	54.17
	9	4.00E + 06 ± 0.29	85.12	8.50E + 06 ± 0.57	64.58
	10.5	3.63E + 06 ± 0.23	86.51	7.00E + 06 ± 0.60	70.83
Itraconazole (0.05 mg/ml)		8.93E + 06 ± 0.42	66.79	1.20E + 07 ± 1.36	50.00

subtilis has the ability to down-regulate the expression of the genes ALS3, HWP1, BCR1, EFG1, and TEC1which are responsible for the generation of biofilm and filament by *C. albicans* (Silva et al. 2019). Additionally, some *Lactobacillus* species have the ability to block the biphasic transition between yeast and hyphae through reduced expression of filament-related genes (TEC1 and UME6) in *C. albicans* (de Barros et al. 2018). By suppressing the TEC1 and UME6 genes, which are necessary for the development of mycelial cells, *Lactobacillus paracasei* can decrease the in vitro filamentation of *Candida albicans* (de Barros et al. 2018). Huang et al. (2019) reported that, a potential mechanism is that yeasts' suppression of cAMP-Efg1p which is a well-known regulatory gene EFG1 responsible for the transcription of genes specific to hyphae. Similarly, HWP1, which is necessary for the development of *C. albicans*' mycelial form and attachment to host cells, was down-regulated in *Candida* cells treated with probiotics (Sharkey et al. 1999; Orsi et al. 2014).

Transmission Electron Microscopy (TEM)

The untreated *C. albicans* cell, as observed through TEM Fig. 6 exhibited characteristic features of the yeast. These included a consistent central density, a uniformly structured nucleus (N), a cytoplasm containing various endomembrane system (ES) components, and an intact cell wall (CW) that remained attached to the plasma membrane (PM) and exhibited a uniform thickness across the entire fungal cell Fig. 6a. Also, Fig. 6b demonstrates the formation of an endotrophic germ tube by the endogenous germination of a *C. albicans* yeast cell. The germ tube exhibits walls that are parallel and lacks any constriction at its point of origin within the blastospore mother cell. It has been proposed that it plays a role in the degree of virulence of *C. albicans* (Fazly et al. 2013). Following administration of the *B. subtilis* supernatant Fig. 6c–f, there was a discernible reduction in cytoplasmic volume, accompanied by significant modifications to the cell membrane and cell wall. Notable structural disorganization within the cytoplasm of the cell resulted in a greater reduction in cytoplasmic volume with the formation of the ghost cells (GC) Fig. 6c.

In the initial stage of autolysis, the formation of ghost cells, the yeast cells displayed notable features like the existence of periplasmic space, pyknosis processes, and cytoplasmic vacuolization (Mazzoni and Falcone 2008). A specific subset of cells appeared to have undergone profound alterations in morphology, and the cell wall was breached at a particular location (black arrow), resulting in the protoplast protruding externally, as evidenced by the malformed germ tube or the cells themselves Fig. 6d-f. It appears that the bacterial CFS caused cell wall dysfunction, including loss of cellular metabolic functions and germ tube formation.

The yeast-hyphae morphological transition plays a crucial role in determining the severity of fungal infections. Multiple studies have established connections between the development of physical form and the ability to cause disease in dimorphic fungi that affect humans (Rooney and Klein 2002; Zhai et al. 2013). Hence, the conversion from the yeast morphology to the filamentous morphology plays a pivotal role in the progression of fungal diseases (Rooney and Klein 2002). According to reports, mutant strains of *C. albicans* that are unable to produce hyphae are generally not dangerous in animal models of experimental disseminated or mucosal candidiasis (Lo et al. 1997; Zhai et al. 2013). The results of this investigation regarding the exposure of *C. albicans* to the bacterial supernatant could have important consequences for the development of future antifungal therapies.

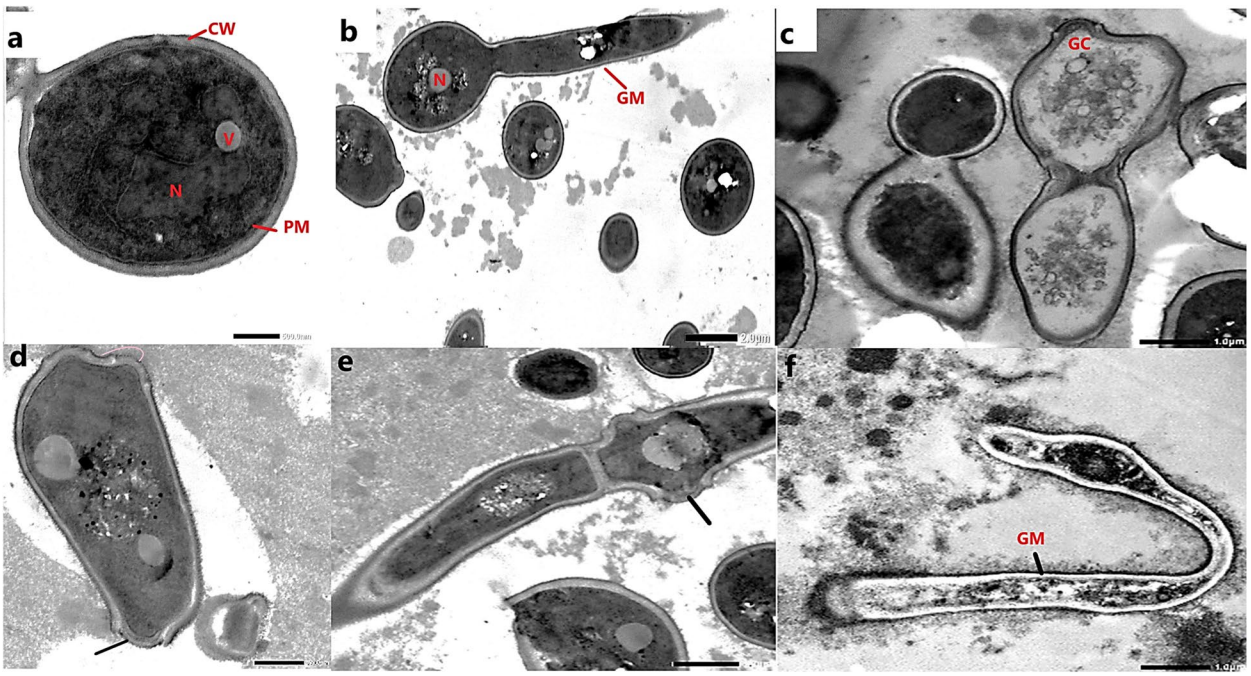


Fig. 6 Ultrastructural variation after exposure of *C. albicans* to the *B. subtilis* supernatant (**a**, **b**) untreated cells, (**c**-**f**) treated cells. The scale bars (black lines) are indicated at the bottom of each photo. V, Vacuole; N, nucleus; CW, cell wall; PM, plasma membrane; GC, ghost cell; GM, germ tube. The distortion of the outermost layers of the cell wall and cytoplasmic membrane is indicated by the black arrow

GC/MS analysis of bacterial metabolite

The potential basic metabolic components that might be present in the lyophilized CFS of *Bacillus subtilis* were identified utilizing the GC/MS method. The principal constituents identified in lyophilized CFS of *Bacillus subtilis*, as shown in Table 7, include the following: 13-Docosenamide, 3-Allyl-6-methoxyphenol, Phenol, 2-methoxy-4-(2-prpenyl)-acetate (eugenol acetate), E,E, Z-1,3,12-Nonadecatriene-5,14-diol, Bicyclo [5.2.0]0.2-methylene-4,8,8-trimethyl-4-vinyl nonane and hexamethyl cyclotrisiloxane.

13-Docosenamide, (z) has been documented to exhibit antifungal and anticancer properties. It has been identified in biosurfactants derived from halophilic *Bacillus* sp. BS3 when analyzed using gas chromatography-mass spectrometry (GC-MS) Donio et al. in 2013. The chloroformic extract of *Bacillus isolate LMB3093* was found to contain 13-docosenamide (Z) according to research by Nas et al. in 2021. The compound 9-Octadecenamide, (z)- was identified in the active crude extract of actinomycetes *Nocardiopsis dassonvillei* MAD08 using GC-MS analysis. This extract was tested against *Candida* strain and showed notable anticandidal characteristics, as reported by Selvin et al. (2009).

Furthermore, the compound 9-Octadecenamide, (z)- was identified in the ethyl acetate fraction of intracellular metabolites of *Bacillus subtilis* BS-01 during GC-MS analysis. The culture filtrate supernatant (CFS) exhibited antifungal properties against *Alternaria solani* (Awan et al. 2023). In their study, Bharose and Gajera (2018) identified the presence of 9-Octadecenamide and its derivative 9-Octadecenamide, (z)- in the crude extract of *Bacillus subtilis*. This extract exhibited antifungal properties against *Aspergillus* sp. GC-MS study of crude extract from *Pseudomonas* isolates revealed the presence of 9-Octadecenamide (Bharose and Gajera 2018).

The acidified supernatant of *Paenibacillus* sp. (Raj et al. 2014) was found to contain 3-Allyl-6-methoxyphenol and Phenol, 2-methoxy-4-(1-propenyl)- (Eugenol acetate) as identified by GC-MS. The bacterium *Streptomyces* sp. LS1 synthesized a crimson pigment including eugenol acetate and phenol, 2-methoxy-3-(2-propenyl). The pigment was extracted utilizing ethanol and examined employing gas chromatography-mass spectrometry (GC-MS). These compounds exhibited diverse biological properties, including strong antioxidant, antiviral, antifungal, and antibacterial activity (Hemeda et al. 2022). In other study, Ghanem et al. (2022) detected the occurrence of phenol-2-methoxy-4-(2-propenyl) in the volatile organic compounds (VOCs) component of *Streptomyces* sp. In addition, they conducted an in vitro bioassay which demonstrated that the volatile organic compounds (VOCs) derived from the three strains of *Streptomyces* had potent fungicidal activity against *Botrytis cinerea*,

Table 7 Main indicative chemical compounds in the CFS through using GC/MS

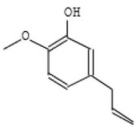
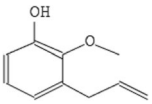
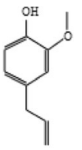
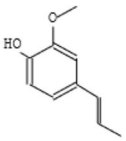
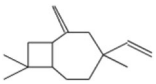
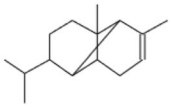
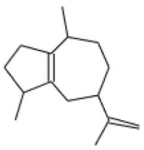
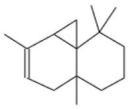
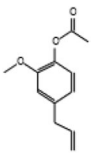
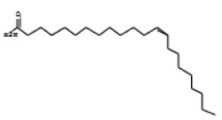
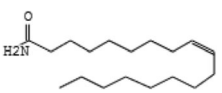
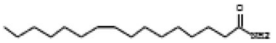
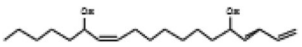
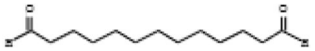
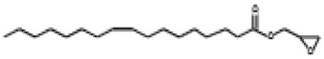
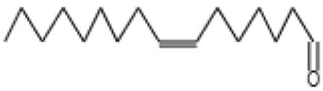
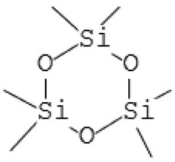
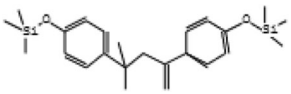
RT(min)	Hint	Name	Molecular Formula	Molecular Weight	RetIndex	Structure
19.61	1	3-Allyl-6-methoxyphenol	C10H12O2	164	1392	
	2	Phenol, 2-methoxy-3-(2-propenyl)-	C10H12O2	164	1392	
	3	Phenol, 2-methoxy-4-(2-propenyl)-	C10H12O2	164	1392	
	4	Phenol, 2-methoxy-4-(1-propenyl)-, (E)	C10H12O2	164	1410	
21.945	1	4,8,8-Trimethyl-2-methylene-4-vinylbicyclo[5.2.0]nonane	C15H24	204	1407	
	3	Ylangene	C15H24	204	1221	
	4	alpha.-Guaiene	C15H24	204	1490	
	5	Thujopsene	C15H24	204	1416	
23.85	1	Phenol, 2-methoxy-4-(2-propenyl)-, acetate	C12H14O3	206	1552	
41.215	1	13-Docosenamide, (Z)-	C22H43NO	337	2625	
	2	9-Octadecenamide, (Z)-	C18H35NO	281	2228	
	3	9-Hexadecenamide, (Z)-	C16H31NO	253	2029	
	4	9-Octadecenamide	C18H35NO	281	2228	

Table 7 (continued)

RT(min)	Hint	Name	Molecular Formula	Molecular Weight	RetIndex	Structure
43.395	1	E,E,Z-1,3,12-Nonadecatriene-5,14-diol	C19H34O2	294	2241	
	2	Tridecanedial	C13H24O2	212	1690	
	3	Glycidyl (Z)-9-Heptadecenoate	C20H36O3	324	2275	
	4	7-Hexadecenal, (Z)-	C16H30O	238	1808	
61.835	1	Cyclotrisiloxane, hexamethyl-	C6H18O3Si3	222	620	
	2	4-Methyl-2,4-bis(p-hydroxyphenyl)pent-1-ene, 2TMS derivative	C24H36O2Si2	412	2442	

Macrophomina phaseolina, and *Sclerotinia sclerotiorum* fungi. In addition, independent research conducted by Jha et al. (2022) and Foss et al. (2023) have discovered the presence of phenol, 2-methoxy-4-(1-propenyl), in the metabolic extract of *Streptomyces* M1 and in the volatile organic compounds (VOCs) of red beetroot juice that has undergone fermentation by *Lactobacillus*. Phenols possess the ability to undergo proton exchange due to the presence of an unbound hydroxyl group. This capability enhances their efficacy in altering the composition of cell membranes in microorganisms (Ben Arfa et al. 2006). In addition, Tian et al. (2022) asserted that the precise positioning of the hydroxyl group on the benzene ring could impact the antibacterial efficacy of the molecule. The lipophilic nature of eugenol enhances its antifungal properties by disrupting the structure of the fungal membrane (Olea et al. 2019). The primary cause of this disturbance is the accumulation of eugenol within the phospholipid bilayer (Olea et al. 2019). The interaction modifies the permeability of fungal membranes and influences their flexibility. Proteins or enzymes that are linked to the membranes also undergo alterations in their functioning (Wang et al. 2010).

The compound 2-methylene-4,8,8-trimethyl-4-vinyl bicyclo [5.2.0] nonane was discovered in GC-MS analysis of volatile oils of *Muscodor fengyangensis*, and *Fusarium tricinctum* which has been identified as effective antimicrobial drugs in studies conducted by Zhang et al. (2010), and Ahmed et al. (2023), respectively.

Conclusion

In present study, three bacteria were screened against *Candida albicans* to evaluate their antifungal properties, one of bacterial isolates exhibited strong antifungal activity that was identified as *Bacillus subtilis* based on the 16Sr RNA gene sequence. The optimum culture conditions were found to be temperature 25°c, pH 8 and media PDA. Also, this bacteria exhibited tolerance to low concentrations of heavy metals. Filtrate from *Bacillus* had impact inhibitory effect on germ tube formation and when 10.5 mg/ml of bacterial filtrate was added, the percentage of germinated cells reduced to 86.51%. The investigative GC/MS analysis of the CFS of *Bacillus subtilis* revealed the presence of the following compounds: 13-Docosenamide, (z), 3-Allyl-6-methoxyphenol, Phenol, 2-methoxy-4-(2-Propenyl)- acetate, E, E, Z-1,3,12-Nonadecatriene-5,14-diol, and 2-methylene-4,8,8-trimethyl-4-vinyl bicyclo[5.2.0] Nonane.

Transmission Electron microscopy demonstrated drastic cellular effects due to administration of bacterial metabolite. This result could suggest a promising novel alternative anticandidal drugs.

Overall, the strain *B. subtilis* NAM shows potential as a bacterium that can effectively restrict the growth of drug-resistant pathogenic yeasts.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13213-024-01764-9.

Supplementary Material 1.

Supplementary Material 2.

Acknowledgements
We would like to extend our appreciation to the Laboratory of Mycology at the Institute of National Liver, Menoufia University, Egypt for generously providing our laboratory with fourteen clinical pathogenic *Candida* specimens for the purpose of conducting this study.

Authors' contributions
Conceptualization and design the study, Mohamed Gharieb (MG) and Nora Elfeky (NE); Performed experiments, Aya Rizk (AR); Validated and analyzed the developed data, MG and NE; wrote the original manuscript, NE and AR. Revising the manuscript MG and NE. All authors have read, agreed, and approved the final version of the manuscript.

Funding
Open access funding provided by The Science, Technology & Innovation Funding Authority (STDF) in cooperation with The Egyptian Knowledge Bank (EKB).

Availability of data and materials
All the data and materials used for the preparation of the manuscript are presented in it. The datasets used or analyzed during the preparation of the manuscript are available from the corresponding author at reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
All listed authors consented to the submission of this manuscript for publication.

Competing interests
The authors declare no competing interests.

Received: 19 January 2024 Accepted: 13 May 2024
Published online: 20 July 2024

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