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Control of Vulvovaginal Candidiasis by used Antimicrobial Compound Produced by Saccharomyces Cerevisiae-Based probiotic

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Abstract

This research aims to study the antimicrobial activity (AMA) of Saccharomyces cerevisiae against Candida spp. associated to vulvovaginal candidiasis and compared their efficacy to other therapeutic alternatives. Clinical samples were collected from 70 women aged 40-50 years old, married with children and referred for chronic vulvovaginal complaints. Drug sensitivity test were carried out on 25 Candida isolates used disk diffusion method. Agar well diffusion method was used to evaluate the antifungal activity of vaginal douching solutions and the AMA of S. cerevisiae. Growth kinetic of antimicrobial agent was monitored by measuring optical density at 530 nm. Microdillution method was applied to determine MIC. In current study, Candida spp. distribution was (35.70%), Enterobacteriaceae (25.70%), Staphylococcus spp. (14.20%) and Lactobacillus spp. (14.20%). Identification of Candida isolates reveled that C. albicans was higher (52.0%) then C. glabrata (36.0%) and C. tropicalis (12.0%). Drug susceptibility testing showed that C. albicans YCI13, C. grabrata YCI15, C. tropicalis YCI23 and C. tropicalis YCI25 were high resistant to antibiotics and resist relatively to antifungals. Candida spp. were resistant to citric acid, acetic acid and H2O2, but sensible to NaHCO3 and EDTA. S. cerevisiae showed effective antifungal activity against C. albicans YCI13, C. grabrata YCI15, C. tropicalis YCI23 and C. tropicalis YCI25. The AMA peaked at the early latency phase and high level was produced during exponential phase 24 h of fermentation. MIC and MBC values of S. cerevisiae antimicrobial agent are 0.25 µg / ml and 3.104 cfu / ml against C. tropicalis YCI25.

Keywords: Candidiasis; Candida spp; drugs resistance; antiseptics; Saccharomyces cerevisiae; antimicrobial activity,

Introduction

Candida is a microscopic fungus that is usually harmless and found in the genital tract, digestive tract, mouth, and on the skin. It can sometimes become pathogenic by releasing toxins Candidiasis is a disease caused by the yeast Candida, and it is spread, especially in hot, humid places.

Vulvovaginal candidosis is among the most common human mycoses causing significant gynaecological and obstetric morbidity3, 4. Studies have found that this affection accounted for 75% of all infections vaginal in women. 95% of cases are result of Candida albicans and incidence rate of Non-C. albicans is 5%.

Some factors favor the appearance of this condition including antibiotics use, long term steroid treatment, sexual intercourse, diabetes mellitus, immunosupression, pregnuncy, and cunnilingus, use of vaginal contraceptive sponges and intrauterine device, and miscellaneous factors [1].

Women with vulvitis caused by VVC may respond best to a combination of intravaginal and topical vulval therapy. Conventional antifungal Imidazole, Triazole, Polyene, and Nystatin agents are used to treat VVC. Non albicans Candida spp. infection was treated with amphotericin, flucytosine, boric acid and crystal violet (gentian), depending on the susceptibility profile of the isolates.

Increasing incidence of antifungal resistance over time lead to research for other alternatives as natural derivatives with antifungal properties from plants extracts, animals products and microbial substances.

Saccharomyces cerevisiae is unicellular microscopic yeast widely distributed in the natural environment, it belonging to Saccharomycetaceae family and used in industrial applications, genetic studies, and clinical researchs. Many studies showed that Saccharomyces genus characterized by angonistic properties and probiotic with various health promoting benefits. Therefore, the objective of this study was to evaluate the antimicrobial activity of S. cerevisiae against Candida species associated to VVC and compared their efficacy to other therapeutic alternatives such as standard antibiotics, antiseptics, and vaginal douching solutions.

Materials and Methods

Vaginal swabs collection

Clinical samples were collected from 70 women aged 40-50 years old, married with children and referred for chronic vulvovaginal complaints to Hospital-Maternity Service of Mascara, Algeria during 2018. Vaginal secretions were collected

using a moisten swab with sterile saline (0.9% NaCl, Institute Pasteur, Algeria). The samples were transferred to the laboratory for study [2].

Isolation and identification of microbial strains

Brain heart broth (BHI, Merck, Germany) was used as an enrichment medium for all bacterial strains. Several selective media (Merck, Germany) were used for isolation and purification of cultures including Chapman Agar for Staphylococcus spp., Salmonella-Shigella Agar (SS), Hektoen Agar and Eosine Blue Methylene Agar for Enterobacteriaceae. MRS agar (De Man Rogosa and Sharpe Agar) for Lactobacillus spp. Yeast Potato Dextrose Agar (YPD) and Sabouraud Agar for yeast Candida spp. Clinical yeast isolates were identified through morphological, cultural, physiological and biochemical tests in Medical Laboratory of Hospital-Maternity Service of Mascara, Algeria. Identification of the bacterial strains was carried out by Gram staining followed by conventional biochemical tests.

Candida spp. drug susceptibility test

A total of 63 recent clinical isolates (bacteria and yeast) were isolated from the vaginal discharge samples. Drug sensitivity test were carried out on 25 Candida isolates and by disk diffusion method on Muller Hinton Agar plates (MH, Merck, Germany) in the presence of selected antibiotics divided into four (04) antifungal that are active against a VVC infections and include Metronidazole 500 mg, Neomycin Sulfate 65000 UI, Nystatin 100000 UI, and Polymyxin Sulfate 35000 UI; and four (04) antibacterial antibiotics include Piperacillin 75 µg, Streptomycin 10 µg, Cloxacillin 5 µg, and Trimethoprim 5 µg. MH agar plates are inoculated with yeast inoculum 0.5-2.5×105 cfu / ml (0.5 Mc Farland) and incubated for 16–24 h at 35°C and then the diameters of inhibition growth zones are measured and results were compared to CLSI standard documents.

Antifungal activity of vaginal douching on Candida spp.

5% acetic acid, 5% citric acid, 1 gr/ 10 ml sodium bicarbonate NaHCO3, 1 ml/ 10 ml disodium EDTA and 1 ml/ 10 ml hydrogen peroxide H2O2 were dissolved in distilled water (v/v) and sterilized by Millipores 0.22 µm filter (Millex-GV, Renner D-67125/GMBH Germany) to eliminate contamination. Evaluation of antifungal activity is performed using agar well diffusion method. Agar plate surface is inoculated by spreading the yeast inoculum (0.5–2.5 × 105 cfu/ ml) over the entire agar surface. 100 µl of each antifungal solution is introduced into wells (5 mm in diameter) and plates were first placed at 4°C for 30 min in order to diffusion of antifungal and then incubated for 16-24 h at 35°C. After incubation, the antimicrobial interactions are analyzed by observing the inhibition zone size [3].

Antimicrobial activity of S. cerevisiae

Inoculum preparation and cultural conditions

S. cerevisiae was taken from the Commercial Baker Yeast (Pakmaya, SARL VITA ferme, Elharrach- Alger, 2018). The yeast strain was characterized based on their cultural characteristics (Colony shapes, pigment, elevation, edge and surface appearance). Morphological and biochemical characterization of the isolated yeast was performed according to Boboye and Dayo-Owoyemi.

Antimicrobial spectrum

Inhibitory activity of S. cerevisiae against the high resistant Candida spp. was assayed by the agar well diffusion assay. The cells of S. cerevisiae were grown as 2% in YPD broth at 30°C, collected after 24 h at early exponential phase by centrifugation at 10,000 xg for 20 min at 4oC. The cell supernatant CS was passed through membrane filters with a pore diameter of 0.22 µm to eliminate contamination and stored in the refrigerator at 4°C until use. A volume of 100 µl of CS is induced into wells, after incubation the diameters of inhibition zones were scored in mm.

Detection of antimicrobial agent production during growth kinetic

Test strain S. cerevisiae (0.5–2.5 × 105 cfu / ml, 2% v/v) is cultivated into 100 ml of YPD broth and incubated at 30°C for 24 h. Growth is evaluated every 2 hours on basis of 530 nm turbidimetry measurements. Then, cells were removed from the broth culture by centrifugation at 10,000 xg for 20 min at 4oC Avery 2 h. Pathogen indicator strain C. tropicalis YCI25 (0.5–2.5 × 105 cfu / ml) was added to the culture supernatant CS and incubated at 35 °C for 16-24 h. Percentage of inhibition was expressed as inhibition (%) of indicator strain growth relative to the control (C. tropicalis YCI25 cultevated in YPD broth without CS) [4].

Evaluation of antifungal activity by broth microdilution

For quantitative tests to determine MIC, serial dilutions from culture supernatant CS (taken in the exponential phase growth 24 h) were made with MH broth. 100 µl of CS was added in each well of 96-well microtiter plate and inoculated with 100 µl of the diluted suspension of C. tropicalis YCI25. For each test plate, two controls were included, one with the medium alone (sterile control) and the other with 100 µl of medium plus 100 µl of inoculum suspension (growth control). The microdilution plates were incubated at 35°C and were read visually after 24 h of incubation. Sample well producing negative microbial growth was inoculated by spreading on YPD plate surface and incubated at 35°C for 24 h to determine the minimum bactericidal concentration MBC in cfu/ ml.

Statistical analysis

The experiments were repeated three times and data were expressed as mean ± standard deviation.

Results and Discussion

Distribution of vaginal discharge isolates

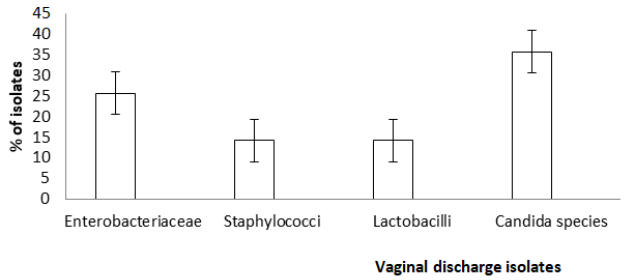
Results show the high rate of yeasts Candida spp. (35.70%), followed by Enterobacteriaceae (25.70%), then Staphylococcus spp. (14.20%) and Lactobacillus spp. (14.20%), Figure 1.

In previous study, it was found that Candida spp. is the most common germ causing infection in married and pregnant women, as well as elderly women and those with chronic diseases. As for bacterial vaginosis enterobacteriaceae,

they are in a few cases and are caused by poor hygiene and genital practices.

Many research papers show that VVC is an infectious disease transmitted to women, especially when their sexual activity increases and affects about 70 to 75% of women in reproductive age. Our results are consistent with many studies that have been shown that incidence of bacterial vaginosis occurs in non pregnant women (30.76%) and women with childbearing age (30.0%); whereas Candidiasis was more common in pregnant women (61.53%) [5].

Figure1: Percentage of vaginal discharge isolates

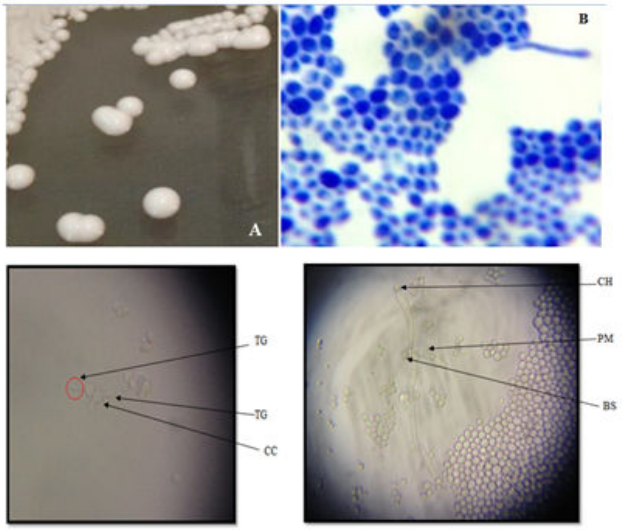


Present of pathogens in high concentration in the vagina is the result of the decrease of protective resident microorganisms (Döderlein Flora) as Lactobacillus spp. (14.20%), which is in normal condition 90.0 to 95.0%. Lactobacilli were present at a low relative abundance in women that have bacterial vaginosis infections and become opportunistic pathogens when the normal vaginal microflora was imbalance.

According to many research, some symptoms were seen during diagnosis as vaginal discharge, pruritus and burning sensation. The vaginal infections is associated with several factors as site, age of the patient and hormonal background (circulating oestrogen levels), pregnancy, phase of menstrual cycle, and use of oral contraceptive device, sexual contact, shared bathrooms and hygiene behaviors.

Gram staining and phenotypic characteristics findings of 25 Candida isolates on Sabouraud Dextrose Agar and Chrome Agar showed that C. albicans was higher distrubition (52.0%) then C. glabrata (36.0%) and C. tropicalis (12.0%). Results were shown in Table 1. Candida species are most frequently isolated from the vulvovaginal and are detected in approximately 31–55% of healthy individuals40,41. In our study, the overall prevalence of C. albicans (Figure 2) was found to be 52.0%, C. albicans is considered as a vaginal mycoflora and the main causative agent of vaginal candidiasis, non-albicans species have increased during last decades [6].

Figure2: Methylene blue staining of C. albicans. Blastese test : TG : germinative tube/ CC : Corps cellulaire/ CH: Chlamydospore/ PM: Pseudomycelium/ BS: Blaspospore.



Several authors have believed that 75% of women are affected at least once during lifetime. Furthermore, chronic vaginitis and recurrent VVC were more reported among several groups of women. Both forms of diseases are problematic conditions for patients. The healthy women vagina is containing several normal microflora including C. albicans, and patients associated factors (patient physiology, hormone balance, and decrease in immune function), organism pathogenic factors and external factors are interference in involving disease [7].

Table1: Percentage of distribution and some morphological, phenotypic and virulence characteristics of Candida species isolated from vaginal discharge samples

Candida isolates	Species and Distrubition %	Color on Sabouraud agar	Color on Chrome agar	Germ Tube Test	Growth at 450C	Chlamydospores	Growth at 30°C and 35°C
YCI1	52.00 %	Creamy	Light green	+	+	+	+
YCI2	C. albicans	Creamy	Light green	+	+	+	+
YCI3		Creamy	Light green	+	+	+	+
YCI4		Creamy	Light green	+	+	+	+
YCI5		Creamy	Light green	+	+	+	+
YCI6		Creamy	Light green	+	+	+	+
YCI7		Creamy	Light green	+	+	+	+
YCI8		Creamy	Light green	+	+	+	+
YCI9		Creamy	Light green	+	+	+	+
YCI10		Creamy	Light green	+	+	+	+

YCI11		Creamy	Light green	+	+	+	+
YCI12		Creamy	Light green	+	+	+	+
YCI13		Creamy	Light green	+	+	+	+
YCI14	36.00 %	Creamy	White Ppurple/ pink	-	-	-	+
YCI15	C. glabrata	Creamy	White Ppurple/ pink	-	-	-	+
YCI16		Creamy	White Ppurple/ pink	-	-	-	+
YCI17		Creamy	White Ppurple/ pink	-	-	-	+
YCI18		Creamy	White Ppurple/ pink	-	-	-	+
YCI19		Creamy	White Ppurple/ pink	-	-	-	+
YCI20		Creamy	White Ppurple/ pink	-	-	-	+
YCI21		Creamy	White Ppurple/ pink	-	-	-	+
YCI22		Creamy	White Ppurple/ pink	-	-	-	+
YCI23	12.00 %	Creamy	Metalic blue	-	-	-	+
YCI24	C. tropicalis	Creamy	Metalic blue	-	-	-	+
YCI25		Creamy	Metalic blue	-	-	-	+

Antibiotics susceptibility

The results of the antibiotics were shown in Tables 2 and 3. Interpretation criteria were determined following the CLSI standards. All Candida spp, were resistant to Piperacillin, Sreptomycin, Cloxacillin, Trimethoprim; whereas C. albicans showed a resistant inferior to Metronidazole 23.07%, Neomycin Sulfate 38.40%, Nystatin 15.30 %, and Polymyxin Sulfate 23.07%. Resistance of C. grabrata to these antifungal was lower 11.11% to 33.33%, but C. tropicalis showed relative resistance superior of 66.0%. Four isolates C. albicans YCI13, C. grabrata YCI15, C. tropicalis YCI23 and C. tropicalis YCI25 were high resistant to both antibacterials and antifungals respectively.

Candida albicans is the most prevalent species isolated from the stools of women with candidiasis, while the ratio of non-albicans Candida spp. is increasing over the past several years, such as Candida glabrata, Candida tropicalis, Candida parapsilosis and Candida krusei. Azoles, polyenes, echinocandins and allylamines are major classes of available antifungals against fungal infection in human. Growing fungal resistance, however, limits their favorable therapeutic efficacies, ultimately making the treatment of fungal infection disease more intractable. Effective strategies to cope with fungal resistance issues are therefore urgently needed [8].

Recently, some researchers have been dedicating to searching for non-antifungals that can enhance the efficacy of conventional antifungals against Candida spp. such as antibiotics. Interestingly, researchers found that some of them displayed potential antifungal activities when used alone or in combination with antifungals but there are a series of reports regarding that antibiotics have become ineffective in treating the VVC, especially if they are used for treatment for a long time, as the germs become more resistant to these drugs, especially when the patients suffer from other diseases.

Table2: Antibiotic susceptibility and screening of resistant Candida species

Candida isolates	Pi	Str	Cl	Tri	M 500 mg	NS	Nys 100000UI	PS
	75 µg	10µg	5µg	5µg		65000UI		35000UI
YCI1	NZR	NZR	NZR	NZR	13.0 I	NZR	13.0 I	15.0 S
YCI2	NZR	NZR	NZR	NZR	12.0 I	12.0 I	11.0 I	12.0 I
YCI3	NZR	NZR	NZR	NZR	NZR	17.0 I	15.0 S	16.0 I
YCI4	NZR	NZR	NZR	NZR	15.0 I	NZR	15.0 S	16.0 S
YCI5	NZR	NZR	NZR	NZR	13.0 I	32.0 S	10.0 I	18.0 I
YCI6	NZR	NZR	NZR	NZR	23.0 S	26.0 S	18.0 S	15.0 I
YCI7	NZR	NZR	NZR	NZR	15 I	NZR	14.0 I	16.0 I
YCI8	NZR	NZR	NZR	NZR	NZR	12.0 I	10.0 I	NZR
YCI9	NZR	NZR	NZR	NZR	12.0 I	22.0 S	NZR	14.0 I
YCI10	NZR	NZR	NZR	NZR	19.0 I	NZR	13.0 I	NZR
YCI11	NZR	NZR	NZR	NZR	16.0 I	11.0 I	10.0 I	11.0 I
YCI12	NZR	NZR	NZR	NZR	16.0 I	25.0 S	18.0 S	17.0 I
YCI13*	NZR	NZR	NZR	NZR	NZR	NZR	NZR	NZR

YCI14	NZ R	NZ R	NZ R	NZ R	16.0 I	15.0 I	14.0 I	12.0 I
YCI15*	NZ R	NZ R	NZ R	NZ R	NZ R	NZ R	NZ R	NZ R
YCI16	NZ R	NZ R	NZ R	NZ R	16.0 I	17.0 I	14.0 I	10.0 I
YCI17	NZ R	NZ R	NZ R	NZ R	15.0 I	15.0 I	17.0 S	15.0 S
YCI18	NZ R	NZ R	NZ R	NZ R	20.0 S	NZ R	23.0 S	NZ R
YCI19	NZ R	NZ R	NZ R	NZ R	17.0 I	20.0 S	18.0 S	22.0 S
YCI20	NZ R	NZ R	NZ R	NZ R	30.0 S	NZ R	23.0 S	17.0 S
YCI21	NZ R	NZ R	NZ R	NZ R	20.0 S	18.0 I	17.0 S	NZ R
YCI22	NZ R	NZ R	NZ R	NZ R	NZ R	18.0 I	19.0 S	15.0 I
YCI23*	NZ R	NZ R	NZ R	NZ R	NZ R	NZ R	NZ R	NZ R
YCI24	NZ R	NZ R	NZ R	NZ R	12.0I	15.0 I	15.0 S	13.0 I
YCI25*	NZ R	NZ R	NZ R	NZ R	NZ R	NZ R	NZ R	NZ R

Legends: YCI: yeast Candida isolate, R: resistant, S: sensitive, I: intermediate. NZ: no zone, Pi: Piperacillin 75µg, Str: Streptomycin 10µg, Cl: Cloxacillin 5µg, Tr: Trimethoprim 5µg, M: Metronidazole 500mg, NS: Neomycin Sulfate 65000UI, Nys: Nystatin 100000UI, PS: Polymyxin Sulfate 35000UI, *: Candida species high resistant to all antibiotics. The zones diameter of growth inhibition was in mm.

Table3: Percentage % of resistant Candida species to antibiotics

Candida isolates	Pi75 µg	Str 10µg	Cl 5µg	Tri 5µg	M 500 mg	NS 65000UI	Nys 100000UI	PS 35000UI
C. albicans	100	100	100	100	23.07	38.40	15.30	23.07
C. glabrata	100	100	100	100	22.22	33.33	11.11	33.33
C. tropicalis	100	100	100	100	66.66	66.66	66.66	66.66

Legends: YCI: yeast Candida isolate, Pi: Piperacillin 75µg, Str: Streptomycin 10µg, Cl: Cloxacillin 5µg, Tr: Trimethoprim 5µg, M: Metronidazole 500mg, NS: Neomycin Sulfate 65000UI, Nys: Nystatin 100000UI, PS: Polymyxin Sulfate 35000UI.

Table4: Antimicrobial activity of vaginal douching solutions against Candida species

	Acetic acid	Citric acid	Sodium bicarbonate	Hydrogen peroxide	Disodium EDTA
Candida species	5%	5%			
	Inhibition diameter zone mm*				
C. albicans YCI13	04.0 R	00.0 R	15.0S	00.0 R	22.0 S
C. glabrata YCI15	06.0 R	00.0 R	10.0 R	00.0 R	26.0 S
C. tropicalis YCI23	08.0 R	08.0 R	22.0 S	08.0 R	25.0 S
C. tropicalis YCI25	15.0 S	10.0 R	00.0 R	10.0 R	26.0 S

Legends: R: resistant, S: sensitive, I: intermediate.

As shown in Table 4, in antifungal susceptibility testing using five vaginal douching solutions, the four Candida species were sensible to disodium EDTA with average diameter inhibition zone ranged between 22.0 mm and 26.0 mm. In addition, C. albicans YCI13 and C. tropicalis YCI23 were sensible to sodium bicarbonate with diameter inhibition zone 15.0 mm and 22.0 mm respectively, but C. glabrata YCI15 and C. tropicalis YCI25 were resistant. Whereas, acetic acid sensibility was observed in C. tropicalis YCI25 (15.0 mm) and the rest species were more resistant. Citric acid, acetic acid and H2O2 resistance was observed against all strains in compared with sodium bicarbonate and sodium EDTA [9].

On the basis of various research papers, the chelator sodium EDTA shows a high effectiveness as anticandidiasis, but it use for long time lead to the imbalance in the vaginal microbiota and irritation of vagina52. Chemicals vaginal solutions as citric acid, acetic acid, H2O2 and NaHCO3 showed a significant inhibitory effect against Candida spp. when they are mixed with others antiseptics ingredients such as purified water, diazolidinyl urea, sodium citrate, vinegar, octoxynol-9, Benzoic acid, edetate disodium, and lysol. But water mixed with vinegar give good result and is harmless for vagina and Döderlein Flora53,54. Although vaginal douching is effective, according to many studies, it has side effects, among them inflammation, risk reproductive tract infections and remove normal vaginal flora.

Antimicrobial activity of S. cerevisiae

Antifungal effect of S. cerevisiae was better against C. tropicalis YCI23 and YCI25 with diameter inhibition zone 10 to 17 mm than C. albicans YCI13 06 mm and C. glabrata YCI15 08 mm. Culture supernatant showed least antifungal activity compared to disodium EDTA and standard antifungal drugs (Figure 3 and 4) [10,11].

Many studies reported that S. cerevisiae possesses antagonistic activity against other yeasts and microbial pathogens negative and positive bacteria. According to,

yeast produces a large range of secondary metabolites as killer toxins (mycocins), organic acids, antibiotic factors, volatile acids, and hydrogen peroxide which support the inhibitory mechanisms. The antagonistic properties of S. cerevisiae enabled in the medical field, food and feed, agriculture, veterinary medicine and environmental protection [12,14].

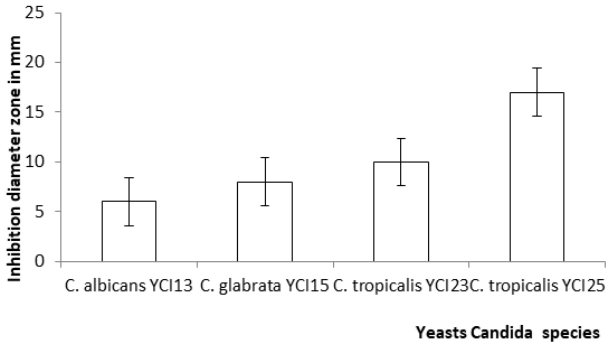


Figure3: Antifungal activity of S. cerevisiae against Candida species.

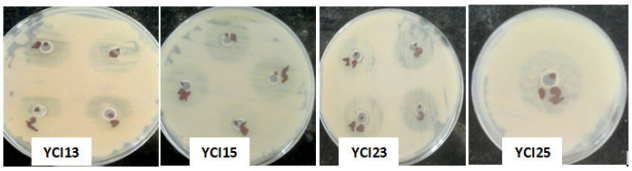
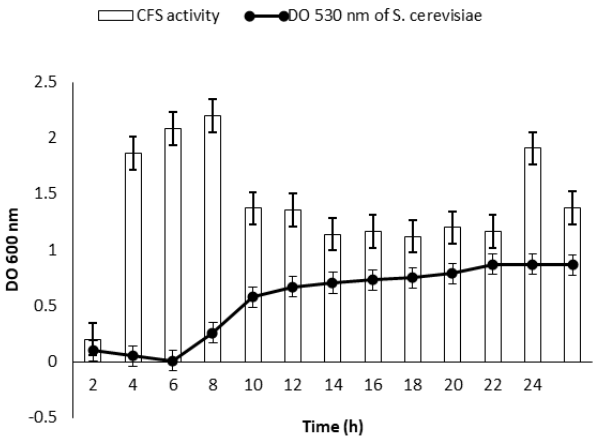


Figure 4: Inhibition zone of S. cerevisiae CFS activity against Candida spp. performed on YPD agar

Detection of antimicrobial agent derived by S. cerevisiae during growth

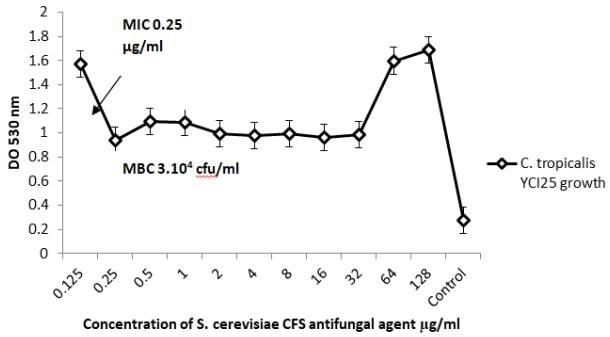
High antimicrobial agent was recorded during the latency phase at zero time to 10 h and the S cerevisiae biomass amount was OD 0.668. Figure 5 depicts high antifungal activity against C. tropicalis YCI25 yeast at zero time, and maximal antifungal activity was achieved at the exponential phase for 24 h. The biomass concentration was OD 0.870 at this phase (Figure 5). According to previously published data, S. cerevisiae produce high level of antimicrobial agent in the end of the exponential growth phase, and those compounds are peptides of 2-10 kDa that are active against several yeasts and bacteria [15].

Figure5: Optical density (OD) of S. cerevisiae in the antimicrobial test against C. tropicalis YCI25 performed with YPD broth



MIC and MBC of S. cerevisiae antimicrobial agent

Figure6: MIC and MBC values of cell supernatant of against S. cerevisiae against C. tropicalis YCI25.



MIC is 0.25 µg/ ml and MBC is 3.104 cfu/ ml for C. tropicalis YCI25 that shows a significant sensibility (Figure 6). According to CLSI interpretive criteria S. cerevisiae antimicrobial agent shows a good effect on C. tropicalis YCI25

Conclusion

Yeast S. cerevisiae has shown promising antimicrobial activity and a broad spectrum of action against infectious and virulent Candida spp. It is considered an alternative antimicrobial agent in treatment VVC and the field medicine.

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