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RESEARCH

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Estimating the expression levels of genes controlling biofilm formation and evaluating the effects of different conditions on biofilm formation and secreted aspartic proteinase activity in *Candida albicans* and *Saccharomyces cerevisiae*: a comparative study

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Abstract

Background Characterization of yeast virulence genes is an important tool for identifying the molecular pathways involved in switching yeast virulence. Biofilm formation (BF) and secreted aspartic proteinase (SAP) activity are essential virulence factors that contribute to yeast pathogenicity.

Results Four *Candida albicans* and two *Saccharomyces cerevisiae* strains were tested for BF and SAP activity under optimum conditions, and the expression levels of several genes controlling BF were quantified under the optimal conditions. Biofilm formation was assessed by the microplate method at different pH values, incubation times and culture media. Similarly, SAP activity was assessed at different pH values and incubation periods. The expression levels of nine genes were determined via qRT-PCR technique. All tests were carried out in triplicate, and the values presented as the means ± standard deviations and were analysed with the SPSS programme. Only *C. albicans* (1), *C. albicans* (2) and *S. cerevisiae* 43 formed biofilms. The optimal BF was obtained after culture in sabouraud dextrose broth with 8% glucose at pH 7.5, 4 and 6, respectively, for 48h. *Candida albicans* biofilm production was more significant than that of *S. cerevisiae* 43. Moreover, the SAP activity was estimated under the optimum conditions. All yeasts showed optimal SAP activity at pH 4, but astonishingly the SAP activity of *S. cerevisiae* 44 was higher than that of *C. albicans*. The expression levels of *EFG1* and *ZAP1 (*transcription factors); *ALS3, HWP1*and *YWP1* (adhesion genes); *SAP1* and *SAP4* (aspartic proteinase) in *C. albicans* (1); and *FLO11* (adhesion gene) and *YPS3* (aspartic proteinase) in *S. cerevisiae* 43 were quantified during biofilm development at different time intervals. The expression levels of *EFG1*, *ALS3*, *YWP1*, *SAP1*, *SAP4*, *FLO11* and *YPS3* were upregulated at 8 h, while that of *ZAP1* was upregulated at 48 h. Only *HWP1* was downregulated.

Conclusions The findings of the present study may provide information for overcoming yeast BF and pathogenicity by regulating specific genes at specific times. Additionally, this study revealed the virulence of the commensal *S. cerevisiae*, which may take the pathogenicity direction as *C. albicans*.

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Keywords Biofilm formation, *Candida albicans* and *Saccharomyces cerevisiae*, Secreted aspartic proteinase activity, Yeast virulence genes expression

1 Background

Fungal infections have frequently increased in recent years [1]. Fungal infections have emerged as a major important public health challenge [2]. The most opportunistic fungal pathogen that causes mucosal to systemic infections is *C. albicans*. It is responsible for about 70% of fungal infections worldwide. In recent decades, *C. albicans* has become linked to fatal invasive infections [3, 4].

The yeast pathogenicity mechanism is associated with many virulence factors. *Candida albicans* undergoes cellular differentiation and transcriptional reprogramming inside the host under the control of different genes to adapt to new habitats with various nutritional elements, pH values, and CO_2 and O_2 levels [4, 5]. Additionally, *Candida albicans* pathogenicity is attributed to adhesions to the cell surface, the production of hydrolytic enzymes and biofilm formation, which increase the opportunity of *C. albicans* to cause infection in various host habitats [6, 7].

On the other hand, *Saccharomyces cerevisiae* is a safe (nonpathogenic) fungus. It is the most explored microorganism in industrial aids and genetic research [8, 9]. *Saccharomyces cerevisiae* has traditionally been generally regarded as a safe (GRAS) microbe for nutritional purposes without considering its unfavourable attributes. However, this concept has changed because of the increasing number of infections associated with *S. cerevisiae* [10]. There is no doubt that these infections are opportunistic. However, studies have revealed that not all strains can cause infection even in the presence of favourable conditions [11]. The morphological change of *S. cerevisiae* from the yeast form to multicellular pseudohyphae promotes invasive growth [12, 13]. The virulence of *S. cerevisiae* is related to its ability to grow at high temperatures and to form pseudohyphae [14].

Many microbes, including yeasts, form biofilms as one of the major virulence factors. A biofilm is a community of microbes attached to biotic or abiotic surfaces embedded within an extracellular matrix (ECM) [15]. *Candida albicans* is one of the most common biofilm-forming yeasts [16, 17]. *Candida albicans* biofilm development includes four phases: adhesion, proliferation, maturation, and dispersion [18]. Likewise, *S. cerevisiae* has also been found to form biofilms but in only two stages (adhesion and maturation) [12, 19]. Additionally, *S. cerevisiae* has been explored as an attractive model for biofilm formation due to its short cell growth cycle, genetic tractability, and easy culture [12, 19].

The morphological transformation between yeast, pseudohyphae and hyphae during biofilm development is associated with pathogenicity and is controlled by many regulatory genes [20, 21]. In *C. albicans*, several genes are involved in biofilm formation; *HWP1* (hyphal wall protein) and *ALS3* (agglutinin-like sequence) are associated with biofilm formation by acting as complementary adhesions to biotic and abiotic surfaces [6, 22] and play roles in cell morphology [18, 23]. Additionally, *SAP1* and *SAP4* (secreted aspartic proteinase) are associated with the adherence process [24]. Moreover, secreted aspartic proteinase promotes the invasion of host tissues through the breakdown of cell surface proteins [25]. *EFG1* (enhanced filamentous growth protein) is the central transcription regulator of biofilm formation in *C. albicans* [26]. Additionally, it is involved in the colonization process. Its expression is related to the host's immune system state and confers to *Candida albicans* ability to switch from a commensal status to an opportunistic pathogen status [27, 28]. *ZAP1* (zinc-responsive transcription factor) is involved in biofilm maturation by regulating the production of ECM [17, 29]. Additionally, *YWP1* (yeast wall protein) plays a vital role in the dispersion phase [30]. The *Saccharomyces cerevisiae* gene *FLO11* (flocculins) is associated with adhesion and biofilm formation, enabling yeast cells to adhere to surfaces and to each other [31, 32]. Moreover, the FLO11 protein forms a part of the ECM of *S. cerevisiae* mature biofilms [33].

Secreted aspartic proteinase activity is another major virulence factor due to its role in host defense protein hydrolysis and yeast adhesion during biofilm development [34, 35]. The expression of *SAP* genes leads to biofilm development and raises *C. albicans* pathogenicity. The variety of *SAP* genes in host tissues enables the utilization of various kinds of nitrogenous compounds in the host. Hence, the presence and expression of *SAP* genes provide certain adaptive advantages for *Candida* spp., especially in response to the selective pressure of antifungal agents [36]. In *C. albicans,* secreted aspartic proteinase is encoded by ten genes (*SAP1* to *SAP10*) [24, 37]. *Saccharomyces cerevisiae* has yapsin gene homologues to *SAP* genes in *C. albicans* [38, 39]. Yapsin protein 3 (Yps3) shares the most peptide sequence like SAPs in *C. albicans* [39, 40].

Studying the molecular mechanism of yeast pathogenicity during virulence stages is very important for accurate diagnosis and improvement of therapeutic

strategies. Therefore, the objective of this study was to quantify biofilm formation and secreted aspartic proteinase activity under optimum conditions for both *C. albicans* and *S. cerevisiae* and following up the temporal profiles of different gene expression levels during biofilm development: *EFG1, ZAP1, ALS3, HWP1, YWP1, SAP1* and *SAP4* in *C. albicans* and *FLO11* and *YPS3* in *S. cerevisiae* at different time intervals to determine the specific time required for gene activation and to investigate the relationship between biofilm formation stages and switching time of their virulence genes.

2 Methods

2.1 Yeast cultures

2.1.1 Candida albicans

Four identified *Candida albicans* strains, *C. albicans* (1), *C. albicans* (2), *C. albicans* (3) and *C. albicans* (4) were used in this study. They were obtained from the Nephrology Department at Theodor Bilharz Research Institute Hospital, Giza, Egypt [41]. They were identified using CHROM agar *Candida*, Cornmeal-Tween 80 agar and germ tube formation tests as well as biochemical characterization according to Kurtzman and Fell [42].

2.1.2 Saccharomyces cerevisiae

Two strains, *S. cerevisiae* 43 and *S. cerevisiae* 44, were obtained from the Mersin Culture Collection. The working cultures were kept at 4 °C on universal agar slants [43].

2.2 Estimation of biofilm formation

Biofilm formation was quantified by using a microplate method as described by Mohammed et al. [22] with some modifications. Yeasts grown overnight on sabouraud dextrose agar (SDA) (Oxoid) were suspended in sabouraud dextrose broth (SDB) with 8% glucose. Suspensions equivalent to 0.5 McFarland were prepared and diluted to 1:20 by SDB with 8% glucose after which 200 µl of each yeast suspension was pipetted into three wells. After 24 h of incubation at 37 °C, the broth medium was removed, and the wells were rinsed with 200 µl of phosphate-buffered saline (PBS). Adhered biofilms were stained with 200 µl of 0.1% crystal violet for 20 min followed by washing with PBS. Crystal violet was solubilized by the addition of 200 µl of acetone: ethanol mixture (20:80 v/v) for 10 min and the absorbance was determined using an ELISA reader (Bio Tek ELx808, USA) at a wavelength of 450 nm. Biofilm formation was detected as reported by Rodrigues et al. [44]. Uninoculated SDB was used as a negative control.

2.3 Optimization of some environmental conditions affecting biofilm formation

The optimization of pH, incubation time and culture media for biofilm formation was determined for the biofilm-forming yeast strains by utilizing SDB with 8% glucose over a wide pH range (4–8 with 0. 5 intervals) and different incubation periods (90 min and 8, 24 and 48 h) and two culture media, SDB with 8% glucose and Roswell Park Memorial Institute 1640 (RPMI 1640) (Caisson laboratories Inc., USA) as mentioned before.

2.4 Detection of secreted aspartic proteinase activity

The secreted aspartic proteinase activity of the tested yeast strains was estimated according to AKçağlar et al. [45] with slight modifications. Overnight-grown yeast on yeast extract peptone dextrose (YEPD) agar [46] at 37 °C was inoculated into a flask containing YEPD broth, and a suspension equivalent to 0.5 McFarland was prepared. Then sterile filter paper disks were dipped into this suspension and applied to bovine serum albumin agar (BSAA) plates. Dipped filter paper disks into uninoculated YEPD broth were used as negative controls. After incubation for 7 days at 28 °C, enzyme activity was recorded according to AKçağlar et al. [45].

2.5 Optimization of some environmental factors affecting secreted aspartic proteinase activity

The optimal environmental conditions, such as pH and incubation time, for SAP production were determined by utilizing BSAA over a wide pH range (4–8 with 0. 5 intervals) and different incubation periods (2, 3, 4, and 5 days) as mentioned before.

2.6 Gene expression analysis

From the previous procedures, *C. albicans* (1) and *S. cerevisiae* 43 strains were chosen to evaluate the temporal profile of biofilm-forming genes, as *C. albicans* (1) formed the strongest biofilm and *S. cerevisiae* 43 was the only *S. cerevisiae* strain that formed a biofilm. Gene expression analysis was carried out for the biofilm-forming cells under optimum conditions. Yeast suspensions equivalent to 0.5 McFarland were prepared and diluted to 1:20 using SDB with 8% glucose. A volume of 0.5 ml of yeast suspension was added to polystyrene tubes containing 4.5 ml of SDB with 8% glucose according to Shin et al. [47] and incubated for 90 min and 8, 24 and 48 h at 37 °C. Following incubation, the broth was aspirated, and the adhering biofilms were rinsed with PBS. Biofilm cells were recovered from the tubes according to the methods of Samaranayake et al. [48]. Ribonucleic acid was extracted using a TRIzol kit (Invitrogen Life Technologies, Catalogue # 15596-026)

according to the manufacturer's protocol [49]. Ribonucleic acid quantity and quality were assessed with a NanoDrop 2000c Spectrophotometer (Thermo Scientific, Q992, USA).

The extracted RNA (1µg) was treated with DNase I and 10X reaction buffer with $MgCl_2$ (Thermo Fisher Scientific Inc. kit) followed by complementary DNA (cDNA) synthesis using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc.) according to the manufacturer's instructions [50] and utilized directly in the qRT-PCR assay. qRT-PCR was done utilizing Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Fisher Scientific Inc.) as described by Longo et al. [51]. Briefly,10 µl of Maxima SYBR Green/ROX qPCR Master Mix (2X), 0.25 µl for each primer, 2 µl of cDNA solution and 7.5 µl of nuclease-free water were added to each gene. Then, the microtubes were subjected to qRT-PCR (Bio-Rad, CFX Connect, Singapore). The amplification programme was run on a two-step cycling protocol: 1 cycle of initial denaturation at 95 °C for 10 min, 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 60 s. The primers used were obtained from Macrogen, Korea (Table 1). The qRT-PCR products were analysed by amplification and melting curve analysis. The expression levels of genes were analysed for *C. albicans* and *S. cerevisiae* using *CEF3* and *ACT1* (actin) as housekeeping genes for *C. albicans* and *S. cerevisiae*, respectively. Gene expression levels were calculated in relation to $2^{-\Delta\Delta ct}$ according to Livak and Schmittgen [52].

2.7 Statistical analysis

Each test was carried out in triplicate, and the acquired values are presented as the mean ± standard deviation (SD) according to SPSS version 16.0 [59]. A t test was used to compare biofilm formation between the positive yeast strain and negative controls and the expression levels of virulence-related genes. A p value < 0.05 indicated a statistically significant difference.

3 Results

3.1 Biofilm formation and optimization of factors affecting biofilm formation

Three out of six yeast strains, *C. albicans* (1), *C. albicans* (2) and *S. cerevisiae* 43, were able to form biofilms while *C. albicans* (3), *C. albicans* (4) and *S. cerevisiae* 44 were unable to form biofilms. *Candida albicans* (1) showed strong biofilm formation (OD 0.987 ± 0.02) and *C. albicans* (2) formed moderate biofilm (OD 0.257 ± 0.01), while *S. cerevisiae* 43 formed weak biofilm (OD 0.091 ± 0.01) (Fig. 1).

The optimization results of the three positive strains revealed that *Candida albicans* (1) and *C. albicans* (2) showed optimal and marked significant biofilm

Table 1 Primers used for qRT-PCR of genes involved in biofilm formation in *Candida albicans* and *Saccharomyces cerevisiae*

Strain	Gene	Sequence (5'–3')	Function	References
C. albicans	*EFG1*	F- TGCCAATAATGTGTCGGTTG R- CCCATCTCTTCTACCACGTGTC	Transcription regulator	[53, 54]
	ZAP*1*	F- CGACTACAAACCACCAGCTTCATC R- CCCCTGTTGCTCATGTTTTGTT	Zinc response regulator	[53, 55]
	ALS3	F- CAACTTGGGTTATTGAAACAAAAACA R- AGAAACAGAAACCCAAGAACAACCT	Cell surface protein for adhesion	[28, 56]
	YWP1	F- GAATCCGGTTCTGGTTCT R- CAACGGTGGTTTCTTGAC	Yeast wall protein	[57]
	HWP1	F- CGGAATCTAGTGCTGTCGTCTCT R- CGACACTTGAGTAATTGGCAGATG	Cell wall-related protein	[53, 54]
	SAP1	F- GAACCAAGGAGTTATTGCCAAGA R- TTTGTCCAGTGGCAGCATTG	Aspartic proteinase	[48, 53]
	SAP4	F- AGCGGCTCTTTAGTTGATTTGC R- AGAATCTAAGAGGACACCAGCGTT	Aspartic proteinase	[24]
	CEF3	F- CAACCCAAGACGAATGTAAAACC R- GTCAAACCAACTTCACCATCTTCA	Housekeeping gene	[24]
S. cerevisiae	*FLO11*	F-CCGCTGGTAAGACGACAACT R-TGGTACGGCATTAGTGGCAG	Cell surface protein for adhesion	[58]
	YPS3	F- AGCAGTCTTAACTAGTCCGG R- TCGATCTCTTGCTGAGTTCA	Aspartic proteinase	[39]
	ACT1	F- GAAATGCAAACCGCTGCTCA R- TACCGGCAGATTCCAAACCC	Housekeeping gene	Primer's sequences obtained from NCBI database as reference

F forward, *R* reverse

formation (ODs of 1.416 ± 0.07 and 0.298 ± 0.01, respectively) at pH 7.5 and pH 4, while *S. cerevisiae* 43 showed optimal biofilm formation (OD of 0.123 ± 0.02) at pH 6 (Fig. 2). The optimum incubation time for biofilm formation by the three strains was 48 h (Fig. 3). Additionally, SDB with 8% glucose was the optimum medium

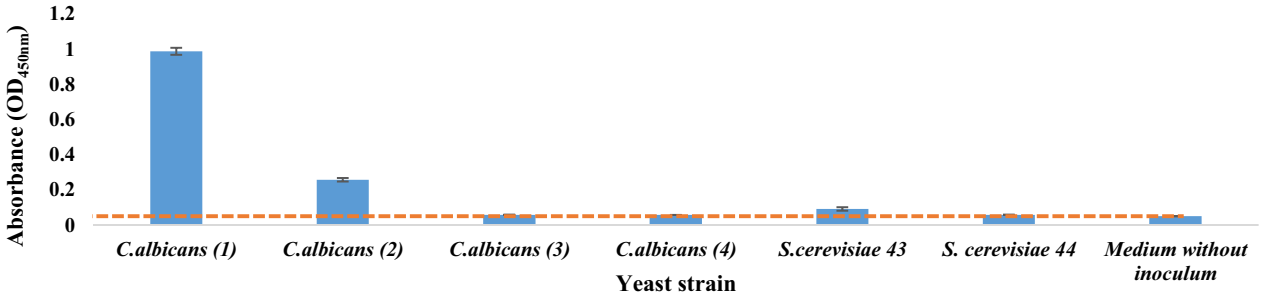

Fig. 1 Quantitative estimation of biofilm formation by different yeast strains a using microplate method. *C. albicans* (1), *C. albicans* (2) and *S. cerevisiae* 43 are positive for biofilm formation. The OD of sabouraud dextrose broth (SDB) medium with 8% glucose without inoculum (0.05) was considered the cut-off for determining positive strains for biofilm formation. Error bars show standard deviations

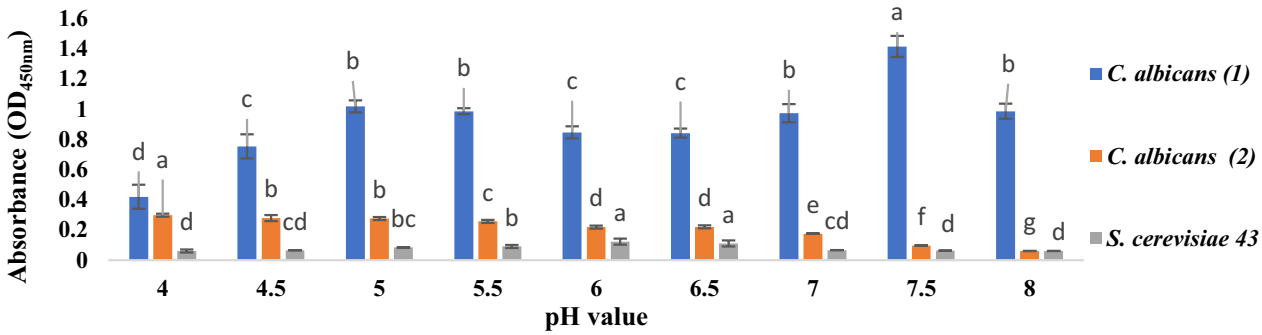

Fig. 2 Effect of pH on biofilm formation. Nonidentical letters for the same strain indicate significant differences among the means according to Duncan's test. Error bars show standard deviations

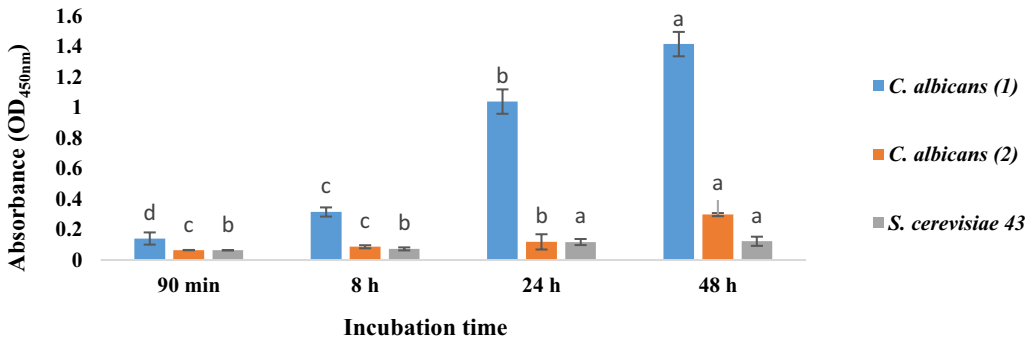

Fig. 3 Effect of incubation time on biofilm formation. Nonidentical letters for the same strain indicate significant differences among the means according to Duncan's test. Error bars show standard deviations

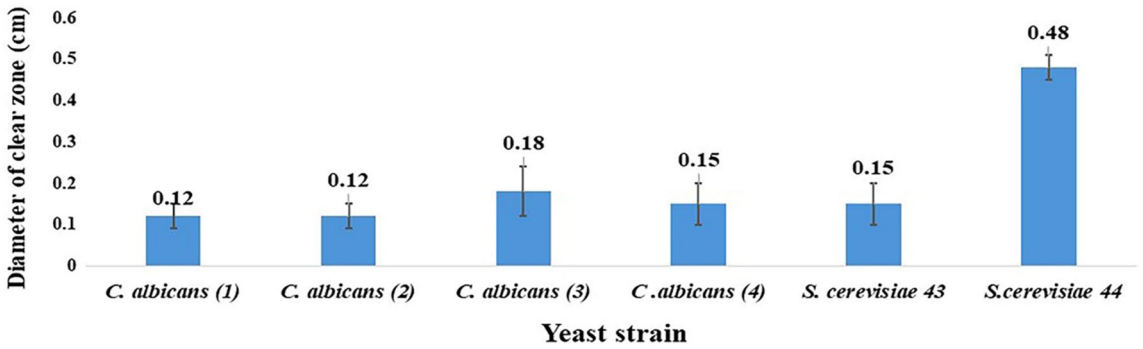

Fig. 4 Secreted aspartic proteinase activity of different yeast strains on bovine serum albumin agar. Error bars show standard deviations

for biofilm formation by *C. albicans* (1) and *C. albicans* (2) strains (ODs of 1.416 ± 0.07 & 0.445 ± 0.03 and 0.298 ± 0.01 & 0.183 ± 0.03 in SDB with 8% glucose and RPMI 1640, respectively), while for *S. cerevisiae* 43, there was no significant difference in biofilm formation between the two media (ODs of 0.123 ± 0.03 and 0.129 ± 0.03 in SDB with 8% glucose and RPMI 1640, respectively).

3.2 Secreted aspartic proteinase activity and optimization of some factors affecting secreted aspartic proteinase activity

All the tested yeast strains were positive for SAP production. The diameter of the clear zones reflecting SAP activity among *C. albicans* strains ranged from 0.12 ± 0.03 to 0.18 ± 0.06 cm, while in *S. cerevisiae*, it ranged from 0.15 ± 0.05 to 0.48 ± 0.03 cm (Fig. 4). Secreted aspartic proteinase activity was detected at pH values ranging from 4 to 6 with 0.5 intervals. All yeast strains showed optimal SAP activity at pH 4. Notably, the highest secreted aspartic proteinase activity (1.02 ± 0.08 cm) was detected with *S. cerevisiae* 44 in comparison with *C. albicans* (3) (0.38 ± 0.08 cm) which showed the highest SAP activity among *C. albicans* strains (Fig. 5). Moreover, there was no clear measurement of SAP activity after 2, 3, 4 or 5 days of incubation.

3.3 Gene expression during different phases of biofilm formation in *Candida albicans* (1) and *Saccharomyces cerevisiae* 43

Since *C. albicans* (1) formed the strongest biofilm and *S. cerevisiae* 43 was the only *S. cerevisiae* strain that formed a biofilm, these strains were selected to estimate the temporal profile of biofilm-forming genes. The expression levels of *EFG1* and *ZAP1* which are transcriptional regulatory genes; *ALS3, YWP1* and *HWP1*, which are adhesion genes; and *SAP1* and *SAP4*, which are aspartic proteinase genes, were quantified in *C. albicans* (1), while the expression levels of the adhesion gene *FLO11* and the aspartic proteinase gene *YPS3* were quantified in *S. cerevisiae* 43. All these genes were quantified at 90 min, 8, 24 and 48 h of biofilm development in relation to the expression levels of the housekeeping genes *CEF3* for *C. albicans* (1) and *ACT1* for *S. cerevisiae* 43 using qRT-PCR. Cells grown for 90 min were used as a control since no biofilm had formed.

In *C. albicans* (1), the expression level of the *EFG1*gene showed a fluctuating pattern in which it

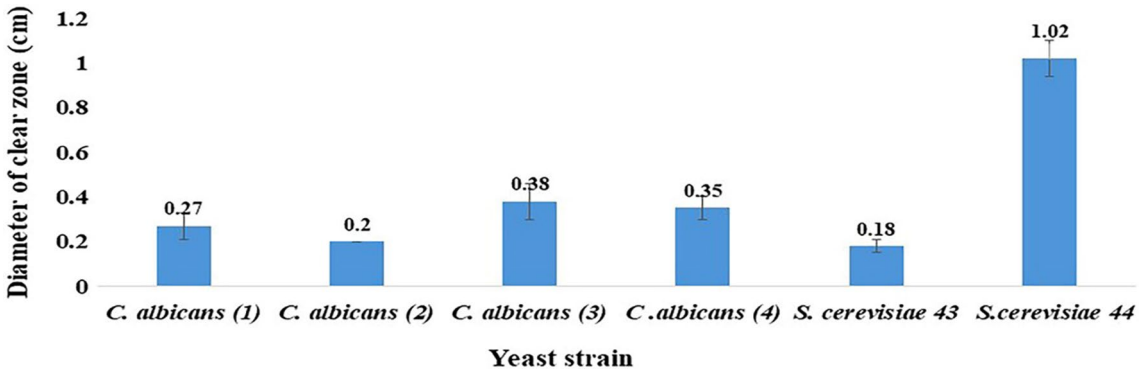

Fig. 5 Secreted aspartic proteinase activity of yeast strains on bovine serum albumin agar at optimum pH. Error bars show standard deviations

was highly significantly upregulated by sevenfold at 8 h (7.53 ± 1.006) ($p = 0.003$) compared with the control, and a highly significant increase at 48 h was observed. However, after 24 h, there was no significant difference in gene expression compared with that at 8 h. For the *ZAP1* gene, the expression level was directly proportional to the incubation time. The highest expression level was achieved at 48 h (1.73 ± 0.248) but this upregulation was not significant.

For the adhesion genes *ALS3* and *YWP1*, 8 h had the highest expression level. The *ALS3* gene expression level was positively regulated at 8 h (2.51 ± 0.247), but the

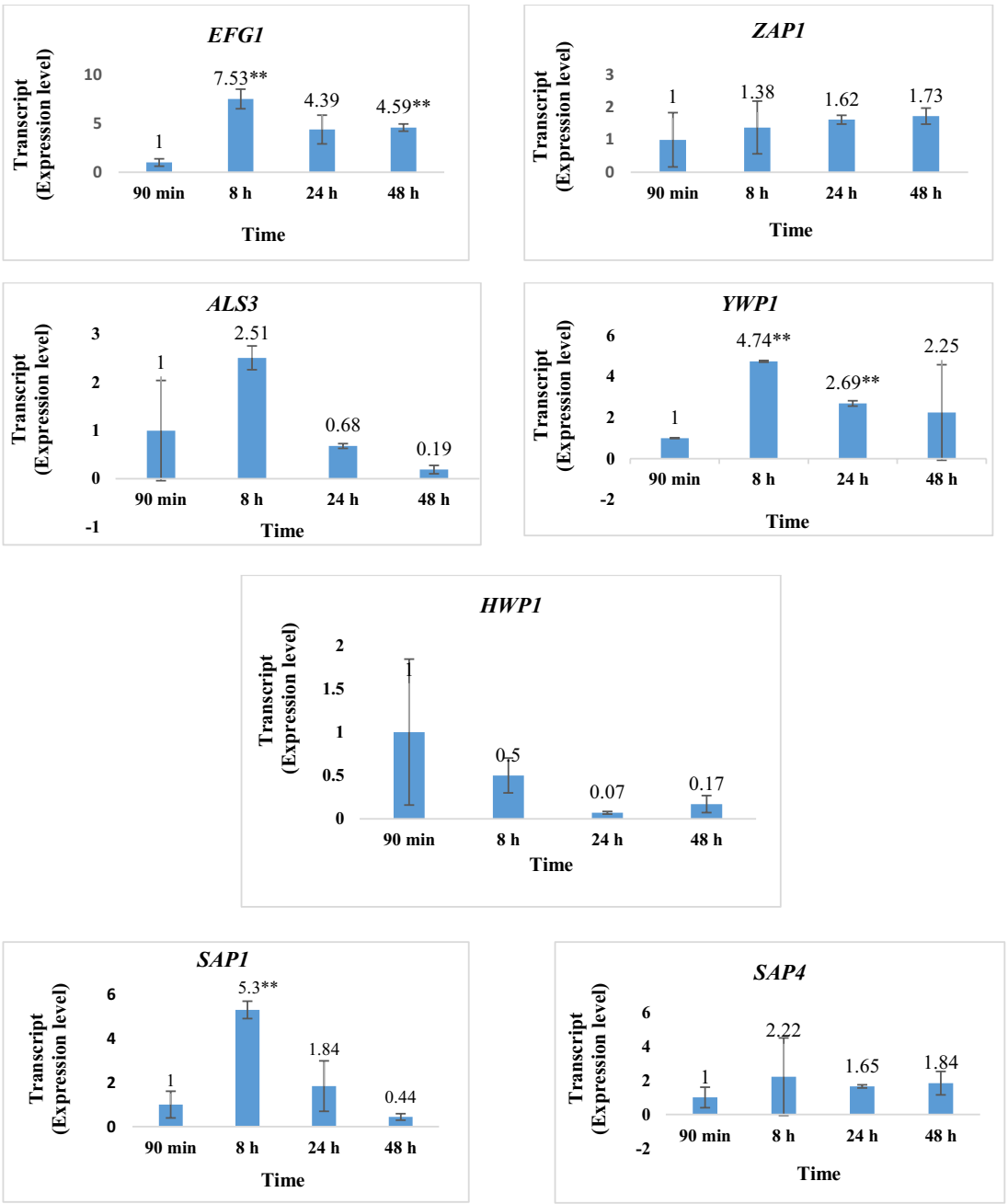

Fig. 6 Temporal expression levels of the transcription factors *EFG1* and *ZAP1*; the adhesion genes *ALS3, YWP1* and *HWP1;* and the aspartic proteinase *SAP1* and *SAP4* during biofilm formation in *C. albicans* (1). 90 min was used as a comparative control. ** indicates high significance at $p < 0.01$. Error bars show standard deviations

difference was not significant. The *YWP1* gene expression level was significantly upregulated at 8 h (4.74 ± 0.041) ($p = 0.004$) compared with that in the control group and gradually decreased with increasing time but was still greater than that in the control group. However, the *HWP1* gene expression level gradually downregulated, and its expression level was less than that of the control, with no significant difference (Fig. 6).

In addition, the expression level of the *SAP* genes (*SAP1* and *SAP4*) showed the highest expression level at 8 h. The *SAP1* gene expression level was significantly upregulated at 8 h (5. 30 ± 0.394) ($p = 0.003$) compared with those in the control group and these values gradually downregulated with increasing time. However, the expression level of *SAP4* fluctuated. It exhibited the highest level at 8 h (2.22 ± 2.273), but this difference was not significant (Fig. 6).

On the other hand, in *S. cerevisiae* (43), the expression level of the adhesion gene *FLO11* was upregulated twofold, reaching the highest level at 8 h (2.67 ± 2.645), but it was not significant, and there was significant downregulation after 24 and 48 h (Fig. 7). However, the expression level of *YPS3* fluctuated, as it was upregulated at 8 h and increased at 48 h to1.34 ± 2.336, with no significant difference, whereas after 24 h, there was a decrease in the gene expression level, which was less than the expression level of the control (Fig. 7).

4 Discussion

Biofilm formation is a main virulence trait for numerous microorganisms [15]. The National Institute of Health demonstrated that biofilms are the cause of 80% of all infections [18]. The formation of biofilms by yeast has gained attention, and many species can grow into communities [60, 61]. The development of biofilms is under the control of different genes [62].

The current study revealed that out of the six yeast strains, two *C. albicans* strains and one *S. cerevisiae* strain were able to form biofilms, while the remaining strains were negative for biofilm formation. *Candida albicans* (1) showed strong biofilm formation and *C. albicans* (2) formed moderate biofilm while *S. cerevisiae* 43 formed weak biofilm. These findings are similar to the findings of Ranjith et al. [53], who stated that 4 out of 7 *C. albicans* isolates were positive for biofilm formation. In addition, Speranza et al. [61] noted that *S. cerevisiae* strains had the ability to form biofilm. These findings are also supported by the study of Tefiani et al. [1], who found that 53.33% of *C. albicans* isolates displayed high biofilm formation, while 46.66% exhibited moderate biofilm formation. Like the present results, Chandra et al. [63] revealed that *S. cerevisiae* is a poorer biofilm producer than *C. albicans*.

Biofilm formation is affected by nutrient availability, pH, species, and other factors [64]. The influence of these parameters on adhesion could vary from one species to another [61]. *C. albicans* can adapt to a broad range of pH values and this characteristic is essential for modifying gene expression and morphological changes [65]. Interactions as hydrophobic and hydrogen bonding, covalent bonding, and ionic bonding are involved in adhesion and biofilm development. Thus, it is reasonable to assume that the pH of the medium influences the cell surface properties involved in adhesion and biofilm development [66].

The present study demonstrated that the three biofilm-forming yeast strains had the ability to form biofilms at a wide range of pH values (from 4 to 8). These findings are in agreement with the findings of Mba and Nweze [62], who found that changes in pH promote signalling pathways that enable the microbes to adapt to various pH values. Furthermore, the optimization results revealed that *C. albicans* (1), *C. albicans* (2) and *S. cerevisiae* 43 had optimal pH values of 7.5, 4 and 6, respectively, which indicates that each strain had its own optimal pH for biofilm formation. These findings agree with the findings of Speranza et al. [61], who found that the effect of pH

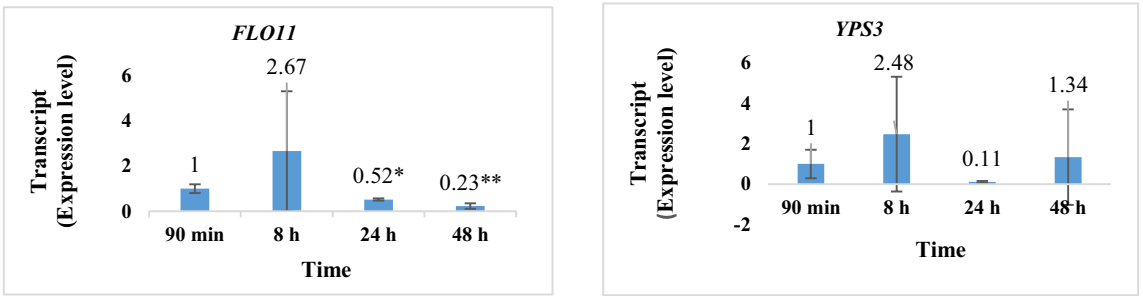

Fig. 7 Temporal expression levels of the adhesion gene *FLO11* and the aspartic proteinase gene *YPS3* during biofilm formation in *S. cerevisiae* 43. 90 min was used as a comparative control. ** indicates high significance at $p < 0.01$ and * indicates significance at $p < 0.05$. Error bars show standard deviations

depends on the strain, as they reported that *S. cerevisiae* strain 4 formed the highest level of biofilm at pH 4–5, while *S. boulardii* (subtype of *S. cerevisiae*) formed biofilm at pH 6. The effect of pH on biofilm development is strain dependent, which may be explained by the ability of various strains to utilize various pathways for morphogenesis transition under nutritional and environmental conditions [67]. Previously, Shih et al. [68] stated that nutrient consumption may result in the production of organic acids or other metabolic products and subsequently change the pH of the medium. *Candida albicans* (1) showed optimal biofilm formation at pH 7.5. Alshanta et al. [65] and Davis et al. [69] stated that *C. albicans* grows as a yeast at acidic pH and in a filamentous form at alkaline pH. The result for *C. albicans* (2) at pH 4 is in accordance with those of de Vasconcellos et al. [70] and Ferreira et al. [71], who observed that an acidic pH had a significant positive effect on biofilm development by *Candida* species.

The present results showed a fluctuating pattern of biofilm formation by *C. albicans* (1) at different pH values, as the OD representing biofilm formation of *C. albicans* (1) was high at pH 5–5.5, decreased at pH 6–6.5 and increased again at pH 7 to reach the highest OD at pH 7.5 (Fig. 2), which may be explained as suggested by Verstrepen et al. [72],who stated that surface proteins (involved in adhesion) may be inactive at certain pH values as a result of conformational changes that takes place when the electrostatic charge of surface proteins changes.

The incubation period is very important for the formation of mature biofilms. Adhesion is the first stage of biofilm formation; it takes approximately11h, and it is characterized by adherence and development into distinct microcolonies. The second stage (proliferation) takes 12-30h and is characterized by the formation of a bilayer of yeast, germ tubes and hyphae in addition to ECM production. In the maturation stage, the accumulation of a thick layer of ECM in which yeasts and hyphae are embedded is observed. Dispersion is the last stage in biofilm formation in which nonadherent cells are released to develop other biofilms [63, 73]. The present results showed that the incubation period had a significant effect on biofilm formation and that 48 h was the optimum time for biofilm formation by the three yeast strains compared to shorter incubation periods, revealing that biofilm production by *C. albicans* and *S. cerevisiae* strains increased with increasing incubation time. These results are supported by the study of de Barros et al. [28], who concluded that the biofilms produced by *C. albicans* at different incubation periods, 12, 24 and 48 h, increased with increasing time. Moreover, Serrano-Fujarte et al. [74] noted that biofilm formation significantly increased after a longer incubation time (48 h). Chandra et al. [63] found that in comparison with *C. albicans* biofilms, *S. cerevisiae* biofilms exhibited much less growth at 0, 12, 36, 48, 60 and 72 h.

Culture media are vital for the growth and development of microorganisms [75]. The availability of nutrients in growth media has been found to affect microbial adhesion to surfaces; thus, an increase in nutrient levels increases the microbial adhesion rate [76]. This study proved that SDB with 80 g/l glucose was the most suitable medium for *C. albicans* biofilm development. SDB with 8% glucose contains a high amount of glucose that provides energy for microorganism growth and contains peptone, which supplies vitamins, nitrogen, amino acids, minerals, and growth factors, as stated by Porfírio et al. [77]. Moreover, Jastrzębska et al. [78] observed that biofilm formation increased with increasing sugar content in culture media. Furthermore, Santana et al. [79] stated that the ability of *C. albicans* to produce polysaccharides increases in response to high glucose levels, which modulates biofilm formation and matrix composition. In a related study, Van Nguyen et al. [80] stated that glucose regulates biofilm formation by modulating cell adhesion and allowing planktonic cells to be released from biofilm cells.

This study also revealed that there was no significant difference in *S. cerevisiae* 43 biofilm formation between SDB with 80 g/l glucose and RPMI 1640 media. RPMI 1640 medium contains 2 g/l glucose and high concentration of Arginine, Asparagine, Glutamine, vitamins, and inorganic salts, as described by Weerasekera et al. [81]. These findings are in agreement with Speranza et al. [61], who reported that *S. cerevisiae* formed the greatest amount of biofilm when it was grown in rich media containing yeast extract, glucose and peptone and formed the lowest amount of biofilm when it was grown in poor media containing only carbohydrate or nitrogen sources.

All yeast strains were positive for SAP production with optimal activity at pH 4, but their proteolytic activity differed from one strain to another, and surprisingly, *S. cerevisiae* 44 showed greater proteolytic activity than *C. albicans* strains. According to several studies, low pH is the optimum pH for the activity of Sap1-Sap3, and high pH is the optimum pH for the activity of Sap4–Sap6 in *C. albicans* [1, 82]. The Yps1 and Yps3 yapsins family of aspartic proteinases in *S. cerevisiae* are functional at pH 5–6 [39, 40, 83]. These results are in accordance with those of Germaine and Tellefson [84], who reported that pH 3.8 to 4.0 was the optimum pH for proteinase activity. The results of Younes et al. [85] support the present results, as *S. cerevisiae* has the ability to secrete proteinase at low pH. The present results also indicate that after 2, 3, 4 and 5 days of incubation, no detectable SAP activity was detected. This result confirmed that seven days

is the optimal incubation period for SAP production, as stated by AKçağlar et al. [45].

The development of biofilms is controlled by the expression of different genes and takes place in a sequential process over a period of 24–48 h. Although the expression of genes linked to virulence in biofilms has been extensively examined in bacterial diseases, less is known about the expression of these genes in fungal biofilms [48].

The current results revealed that different genes are responsible for initial adherence and adhesion maintenance. Furthermore, the expression levels of genes vary depending on the gene and the time of biofilm development. This work studied and quantified the temporal expression level of the genes; *EFG1*, *ZAP1*, *ALS3, YWP1*, *HWP1***,** *SAP1*, *SAP4*, *FLO11* and *YPS3*, which are involved in the development of biofilm under optimum conditions. To form mature biofilms, cells must remain adhered to surfaces for a long time. The ability of *C. albicans* to form biofilms has been associated with the presence of transcriptional regulatory genes [28, 81], including *EFG1* and *ZAP1* [53]. The upregulation of these two genes is related to the increase in the incubation time which in turn caused an increase in biofilm formation by *C. albicans*, as reported above. Previously, de Barros et al. [28] studied the morphological progression of biofilm formation by *C. albicans.* A gradual increase in the biomass of the biofilm was detected by increasing the time from 0 to 48 h. Therefore, incubation time is a very important factor for the formation of mature biofilms. The early phase of biofilm formation (adhesion phase) takes approximately 11 h and is characterized by the adherence and development of distinct microcolonies [73]. In this respect, the current results showed that the *EFG1, ALS3* and *YWP1*genes exhibited the greatest upregulation after 8 h. Additionally, biofilm formation by *C. albicans* is associated with the presence of the adhesion genes *ALS3, YWP1* and *HWP1* [30, 86]. These genes are also associated with the morphological transition from yeast to the hyphal form [30, 87]. The adherence gene *EFG1* regulates the expression of *HWP1* and *ALS3*, which function in cell invasion and biofilm formation [88]. Additionally, *EFG1* regulates the expression of *YWP1*, which is an adhesion maintenance protein [89]. *ALS3* is the most important member of the agglutinin-like sequence family involved in biofilm formation because it promotes cell adhesion [62]. The *YWP1* gene is responsible for adherence to surfaces during the growth stage [89], and *HWP1*, which encodes the cell wall's mannoprotein, also plays a critical role in the development of biofilms [86]. The present findings revealed that 8 h showed the highest expression level for *ALS3.* However, downregulation of *HWP1* expression was observed throughout all time periods. These findings agree with the findings of de Barros et al. [28], who reported that *ALS3* gene expression in *C. albicans* was upregulated at 12 h, and a significant gradual decrease was found at other time intervals (24 and 48 h).

Moreover, Bonfim-Mendonça et al. [24] found that *ALS3* expression level in *C. albicans* isolated from a patient with recurrent vulvovaginal candidiasis increased from 2 to 6 h but decreased in *C. albicans* isolated from a patient with asymptomatic vaginal candidiasis at 6 h. Additionally, *YWP1* gene expression was upregulated at 8 h, which agrees with the findings of McCall et al. [89], who noted that *C. albicans* strains with *YWP1* exhibited a greater initial attachment rate than the wild-type strain and that *YWP1*had dual functions, as initial attachment, and adhesion maintenance. This result proved the importance of the *YWP1* gene in the adhesion process. Moreover, Samaranayake et al. [48] observed that *HWP1* expression was upregulated at 90 min and downregulated at 24 and 48 h on silicon biomaterial discs with or without serum. In contrast, Bonfim-Mendonça et al. [24] found that *HWP1* expression in recurrent vulvovaginal candidiasis isolate increased from 2 to 6 h. *ALS3* and *HWP1* expression levels varied according to the isolate's characteristics [24]. Furthermore, Pokhrel et al. [90] found that *ALS3* and *HWP1* were upregulated at 8 h. The ability of *S. cerevisiae* to form pseudofilaments, adhesion and biofilms is not related to the expression of these genes but is associated with the presence of *FLO* genes [13]. The *FLO11* gene is among the *FLO* gene family in *S. cerevisiae,* and it is important for cell–cell and cell–surface adhesion by pathogenic yeast to adhere to abiotic surfaces [13]. The present findings revealed that the *FLO11* gene was expressed at the highest level in *S. cerevisiae* after 8 h, *w*hich proves the significance of the *FLO11* gene in the adhesion phase of biofilm formation. This result was previously reported by Yang et al. [91], who found that the *FLO11* gene was upregulated throughout biofilm formation. Moreover, the absence of this gene prevents biofilm formation, as reported by McCall et al. [89], who found that *S. cerevisiae* with a deleted *FLO11* gene fails to make initial attachments.

The maturation stage of biofilm formation is characterized by the accumulation of a thick layer of ECM in which yeasts and hyphae are embedded, which takes 38-72h (Cavalheiro and Teixeira, [73]. In this respect, the current findings revealed that the expression level of *ZAP1* gene increased with increasing time and was positively regulated after 48 h. These findings are in accordance with the findings of Jastrzębska et al. [78] and Weerasekera et al. [81] who stated that *ZAP1* regulates biofilm maturation and ECM accumulation. These findings are also in accordance with Ranjith et al. [53] who found that *EFG1*

is required for the maturation phase and that the *ZAP1* gene expression level increases throughout the biofilm formation stages, with greater increases occurring at 48 and 72 h of biofilm development, and that *ZAP1* gene may be needed for maturation.

Following biofilm maturation, many hydrolases are produced to facilitate host cell penetration and efficient nutrient acquisition. Secreted aspartic proteinase genes (*SAP1* and *SAP4*) are among the *SAPs* family in *C. albicans* and have several functions during infection, such as hydrolysis of host tissue barriers, proteins during invasion, distortion of host defense molecules or nutrient acquisition [24, 92]. The present results showed that 8 h of treatment upregulated the expression of *SAP1, SAP4* and *YPS3.* These findings confirm a previous report, showing that the upregulation of *SAP1* expression in asymptomatic vaginal candidiasis isolate and of *SAP4* in recurrent vulvovaginal candidiasis isolate was observed at 6 h and was associated with the adhesion step [24]. Therefore, *SAP* genes are associated with virulence processes, such as adhesion, invasion, and immune evasion.

The *YPS3* gene is a member of the yapsin family in *S. cerevisiae* and contributes to proteolytic activity at the cell surface [39, 93]. Additionally, Mercurio et al. [39] stated that *S. cerevisiae* Yps3 protein has the highest peptide sequence like that of *C. albicans* SAPs. These results indicate that *YPS3* might play a vital role in biofilm formation in *S. cerevisiae* as *SAP*s genes function in *C. albicans*. These findings revealed that the temporal changes in gene expression in response to the commensal *S. cerevisiae* occurred in the same way as those in response to the pathogenic *C. albicans* and may be involved in the pathogenesis.

5 Conclusions

This work may serve as a prospective objective for inhibiting the virulence process and yeast pathogenicity by downregulating the studied genes at specific times. Further studies will be crucial to determine whether *S. cerevisiae* uses biofilms and aspartic proteinase to initiate pathogenicity or accomplish other biochemical processes.

Abbreviations

ACT1	Actin 1
ALS3	Agglutinin-like sequence 3
BF	Biofilm formation
BSAA	Bovine serum albumin agar
CEF3	Cassette protein translation elongation factor
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EFG1	Enhanced filamentous growth protein 1
ELISA	Enzyme-linked immunosorbent assay
FLO11	Floculins 11
HWP1	Hyphal wall protein 1
OD	Optical density
PBS	Phosphate-buffered saline
qRT-PCR	Quantitative real-time polymerase chain reaction
RPMI 1640	Roswell park memorial institute 1640
RNA	Ribonucleic aid
SAP	Secreted aspartic proteinase
SDA	Sabouraud dextrose agar
SDB	Sabouraud dextrose broth
SPSS	Statistical package for the social science
YEPD	Yeast extract peptone dextrose
YPS3	Yapsins
YWP1	Yeast wall protein 1
ZAP1	Zinc-responsive transcription factor

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Author contributions
Conceptualization, supervision, data collection and resources were produced by SAT. Experimental design, data collection, methodology, interpretation of the results and writing original draft of the genetic work were achieved by ShSS. Experimental design, data collection, providing the tested yeast strains, methodology, interpretation of the results and writing original draft of the microbiology work were done by SSZ. Data collection, methodology and analysis were done by RGE. All authors read and approved the final manuscript.

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