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Detection of *ALS3* and *EAP1* gene expresssion in *Candida albicans* and *Candida maltosa* biofilms by FISH

Danitza Xiomara Romero[1], Oscar Víctor Cárdenas[2], María Teresa Álvarez[3]

1. Instituto de Investigaciones Fármaco Bioquímicas – IIFB, Universidad Mayor de San Andrés. La Paz, Bolivia.
danitzaxiomara@gmail.com
2. Instituto de Investigaciones Fármaco Bioquímicas – IIFB, Universidad Mayor de San Andrés. La Paz, Bolivia.
bioscar@gmail.com
3. Instituto de Investigaciones Fármaco Bioquímicas – IIFB, Universidad Mayor de San Andrés. La Paz, Bolivia.
mariateresa.alvarezaliaga@gmail.com

ABSTRACT

Biofilm is regarded as universal forms of microorganism life in aquatic and industrial wastewater systems as well as in a large number of environments and medical devices relevant for public health, where the exact mechanisms by which biofilm-associated microorganisms elicit infection diseases are still poorly understood.

Candida biofilm formation is regulated by different mechanisms where adhesins play a clue role in the yeast attachment to certain surfaces. These adhesins are encoding by *ALS3, HWP1* and *EAP1* genes among others and they are also considered as *Candida* virulence factors.

Methodologies use to study biofilm productions are intended to verify the biofilm composition, formation steps, tridimensional structure and might facilitate the monitoring of biofilm regarding, antibiotic resistance, degradations, inhibitors, enhanciement biofilm formation and other features.

Here, FISH expression a modified method to detect gene expression *in situ* was used in order to detect *ALS3, HWP1* and *EAP1* in *C. albicans* and *C. maltosa* biofilms, constituting a useful tool to monitor biofilm formations. In this regard, *ALS3* expression was identified in *C. albicans* and *C. maltosa* biofilms.

Indexing terms/Keywords

Biofilm, FISH expression, *Candida albicans*, *Candida maltosa, ALS3, EAP1.*

Academic Discipline And Sub-Disciplines

Biotechnology.

SUBJECT CLASSIFICATION

Molecular Biology.

TYPE (METHOD/APPROACH)

Experimental.

INTRODUCTION

During the last three decades the conception about microbial life is changing, in part due to emerging scientific evidence of microbial biofilms [1, 2, 3]. Biofilm is commonly defined as colonies of microbial cells included in a self-produced organic polymeric matrix and is the most common microbial growth in nature. Microbial biofilms are difficult to eradicate, producing many clinical and economic consequences [4]. They were described as clue factors in microbial resistance to antibiotic compounds, in bioremediation process and microbial quorum sensing, among others.

Recent research into the pathogenicity of *Candida* was focused on the prevention and management of biofilms which may contribute to its versatility, in adapting to a variety of different habitats and also to its virulence [6]. The National Institutes of Health, all over the world, reported that pathogenic biofilm is responsible, directly or indirectly, of 80% of all microbial infections approximately [4, 5, 6].

In most individuals with a healthy immune system *C. albicans* is an innocuous commensal that exists in harmony with other members of the microbiota. However, variations in the local environment such as pH shifts, nutritional changes, use of antibiotics, alterations in immune system, can disorder the balance favoring *C. albicans* biofilm formation [7, 8, 9, 10, 11]. *C. albicans* biofilm can colonize mucosal surfaces as well as implanted medical devices, such as prosthetics heart valves, and catheters, producing systemic infections in humans. *C. albicans* is one of the most important microorganisms involved in hospital-acquired infections constituting 40% of bloodstream infections and 15% of all sepsis in clinics [12, 13, 14].

On the other hand, there are several *Candida* species with non-pathogenic features and their ability to form biofilm are studied in bioremediation processes [15]. For instance, *Candida viswanathii* TH1 isolated from oil-polluted sediments collected in coastal zones in Vietnam can transform branched aromatic hydrocarbons in biofilm than in planktonic form [16]. In addition, *Candida maltosa* biofilm was used to degrade and remove phenol with efficiency 7 times more efficiently than without biofilm [17]. Moreover, previously unpublished studies described *C. maltosa* as a strong biofilm forming yeast that could increase the removal of hexadecane [18, 19].

A biofilm typically develops over four sequential steps: 1) the adhesion of a microorganism to a surface, 2) discrete colony formation, and cells organization, 3) secretion of EPS (Extracellular polymeric substances) and maturation into a three-dimensional structure and, 4) dissemination of planktonic cells from mature biofilm. Main features that establishes the first step in biofilm development is the presence of more complex surface structures such as pili, secreted extracellular matrix material and expression of specific cell surface proteins (adhesion molecules or adhesins), which facilitate stronger adhesion [20, 21, 22].

Some *Candida* species biofilm formation are regulated by different factors such as adhesion proteins located at the cell surface (glycosylphosphatidylinositol-modified protein anchors - GPI) with various functions, involved in cell wall biosynthesis and remodeling. These proteins determine surface hydrophobicity and antigenicity, and they are thought to have a role in adhesion and virulence. Some of them are Hifal Wall protein (Hwp1), Aglutinine like sequence 3 (Als3) ,and Polystyrene and epithelial cell (Eap1) identified as critical elements for biofilm adhesion and virulence in *C. albicans* and *Candida* species [23, 24, 25, 26, 27, 28].

Many methodological attempts were proposed to study *Candida* biofilm formation in different fields, for example scanning electron microscopy, fluorescence microscopy, and confocal scanning laser microscopy are used to describe the biofilm development as such, while to study the glycoprotein expression related to biofilm formation, Real time PCR, Southern Blot, Gene sequencing, immunoblotting and others were set up. All these techniques were established to verify the biofilm composition, formation steps and tridimensional structure among others, their uses and applications could facilitate the monitoring of biofilm characteristic such as antibiotic resistance, degradation, inhibitors and other features [21, 22, 25, 29, 30, 31].

The aim of the present study was to identify *ALS3*, *HWP1* and *EAP1* genes in *C. albicans* and *C. maltosa* during biofilm formation by sequencing and identified their expression *in situ* by FISH. *ALS3*, *HWP1* and *EAP1* gene expressions can be used as molecular markers to monitor formation, stability and viability of biofilms. Considering that biofilm is a biodegradable matrix, it is crucial to identify these transcripts using a novel molecular process detecting gene expression *in situ* for clinical and industrial applications.

MATERIALS AND METHODS

Strains, culture conditions and kinetic growth determination

The strains *Candida maltosa SM4* and *Candida albicans S14,* from the microbial culture collection of IIFB (Instituto de Investigaciones Fármaco-Bioquímicas, La Paz-Bolivia) was used in this study. Three Erlenmeyer Flasks (100 mL capacity) containing 50 mL of Yeast Nitrogen Base – Glucose (YNB-Glc) culture medium were inoculated with 1 mL of cryopreserved culture of *C. maltosa* and then incubated at 30 °C on an orbital shaker (100 rpm) for two days. The YNB-Glc culture medium consisted on 0.002 gL^{-1} YNB (SIGMA ALDRICH) and 100 mM glucose [32]. In order to prepare YNB-Glc culture medium, the YNB solution was sterilized by membrane filtration (0.22 µm pore size), then mixed with the autoclaved solution of glucose and finally pH adjusted to 7 adding 1 M NaOH.

One mL sample of culture media was collected daily to determine the absorbance at 620 nm. These determinations were used to establish the kinetic growth, doubling time and specific growth rate.

Biofilm determination

BFC index

Among several described methods to determine Biofilm forming capacity (BFC) of microorganism [33, 34, 35], the tubing method was used in this study [18, 36]. The BFC of *C. maltosa* was evaluated using Falcon 50 mL conical centrifuge tubes containing a glass coverslip (22 x 22 mm) per tube and autoclaved. 7.5 mL of inoculated culture medium were taken at stationary phase and then, added to each conical centrifuge tube. They were incubated for 24, 48, 72, and 96 h independently at 30 °C. Subsequently, the coverslips were transferred to another conical tube containing 7.5 mL of 0.1% crystal violet to stain them for 45 min, then planktonic cells (supernatant) have measured at 630 nm. After that the coverslips were rinsed carefully with deionized water and dried, then transferred to another conical centrifuge tube containing 7.5 mL of absolute ethanol for 10 min. Finally, the absorbance was determined at 570 nm with a spectrophotometer (SpectroMaster). The BFC was determined according to the formula described in Table 1 and also the classification in four categories according to the biofilm attachment to a glass surface.

Table 1. **Semi-quantitative classification of biofilm formation in four categories**

Formula	Strong	Moderate	Weak	No forming
BFC=(ODb-ODc)ODC^{-1}(*)	≥1.10	0.70-1.09	0.35-0.69	<0.35

(*) BFC: Biofilm Forming Capacity, ODb: OD 570 nm =bacterial adherence, ODc: OD 570 nm =medium without inoculum ODC: OD 630 nm =bacterial growth [22].

Biofilm microscopic analysis

EPS matrix of biofilm was observed by microscopy. In this sense, another coverslip incubated for 90 h at 30 °C, was stained with crystal violet (0.1%) for 45 min. The excess of staining was removed by consecutive washes with distilled water. Finally, the coverslip was placed onto a slide and observed at 100x using an optical microscope (Olympus) [36].

Extracellular Polymeric Substances Determination (EPS)

Biofilm was collected in conical centrifuge tubes of 15 ml and centrifuged at 1000 x g for 20 min at 4°C. Then was centrigutated, supernatant was filtered through a 0.22 µm membrane to be used as the EPS sample. After that, dialysis was carried out using 5 KDa cut off, to be finally followed by a freeze-drying treatment during a week. Subsequently, Chemical Oxygen demand (COD) determination [37], total carbohydrates quantification by anthrone method described by Rodriguez [38] and the determination of total proteins according to Lowry [39] were carried out in order to determine the EPS composition. Culture medium without yeast cells and biofilm was used as negative culture.

Molecular identification of genes involved in biofilm formation

Genetic material isolation

One mL of *C. maltosa* and *C. albicans* growth culture in stacionary phase was centrifugated at 4 800 x *g* for 5 min, washed three times with 0.9 M NaCl, subsecuently 500 µL of lysis solution (0.5 M Tris pH 7, 0.1M NaCl, 5% SDS) were added to the pellet, after that, it was homogenized and incubated for 1 h at 65°C. Then, 50 µL of Tris (2M, pH 7) and 150 µL of 5 M NaCl were added, has been shaken and centrifugated at 4 800 x *g* for 5 min. Afterward, 500 µL of lysis buffer (0.5 M Tris pH 7, 0.1M NaCl, 5% SDS) were added, incubated at 65°C for 1h, centrifugated at 6 700 x *g* for 10 min. Supernadant was collected in a eppendorf tube, then 800 µL of absolute ethanol were added, homogenizated and incubated on ice for 20 min. Subsequently, suspension was centrifugated at 11 300 x *g* for 15 min. Pellet was washed three times with 100 µL of 70% etanol, dried at 55°C, then 100 µL of bidistilled water was added. Finally, nucleic acid was cuantified using an espectrometrophometer (SpectroMaster).

Primers designs

Primers BLAST [40] and Primers3plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/) were used to primers design. *ALS3, EAP1* and *HWP1* identification in *C. albicans* was done by NCBI accession numbers AY223551.1, XM_709573.1 and XM_441625.1, respectively.

The oligonucleotide sequences specifity and homology was analized by BLAST (Basic Local Aligment Search Tool) (blast.ncbi.nlm.nih.gov). Primers synthesis were done by IDT (*In vitro* DNA Technology Company, USA). Table 2 shows the primers sequences obtained using the program.

Table 2. Primers sequences designed to identify microbial gene expression used in this study

Microorganism	N° of Access	Probe	Sequence	Amplicon size
Candida albicans	AY223551.1	*ALS3*	5′- CCACTT CAC AAT CCCCATC-3′ 5′-CAGCAGTAGTAG TAACAG TAGTAG TTTCATC -3′	342 pb
	XM_709573.1	*EAP1*	5′-GTCACCAAATGTGATGGCGG -3′ 5′-AGCTGTCTCAGTGGATGCAG -3′	465 pb
	XM_441625.1	*HWP1*	5′-CCATGTGATGATTACCCACA -3′ 5′-GCTGGAACAGAAGATTCAGG -3′	271 pb

PCR amplification of *ALS3, EAP1* and *HWP1* genes

DNA extracted from *Candida albicans* was used as a positive control and bidistilled water as a negative control. *ALS3* and *HWP1* genes were amplified with 1 µM primers, 0.2 mM dNTPs, and 1.5 mM $MgCl_2$ and 1x Colorless GoTaq® Flexi Buffer, 0.03 $UµL^{-1}$ GoTaq® Flexi DNA Polymerase (Promega Kit - GoTaq® Flexi DNA Polymerase).

PCR was established under the following conditions: for *ALS3* were 94°C 10', 94°C 1', 47°C 1.30', 72°C 1.30' for 30 cycles and final extention of 72°C 10' and for *HWP1* were: 94°C 10', 94°C 1', 49**°**C 1.30', 72°C 1.30', for 25 cycles and a final extention of 72°C 10'. The PCR products were electrophoresed on 1% (wt/vol) of agarose gel.

The PCR reaction mixtures (20 µl) of *ALS3* and *HWP1* genes contained, dNTPs each 0.2 mM; 1X PCR Colorless GoTaq® Flexi Buffer ; each primer 1 µM; GoTaq® Flexi DNA Polymerase 0.03 $UµL^{-1}$ and bacterial DNA 10 µg mL^{-1}. The *ALS3* gene PCR mixture in a final volume of 20 µl was carried out with initial denaturation of 94 °C for 10 min followed by thirty cycles of program (94 °C for 1 min, 47 °C for 1 min and 30 s, 72°C for 1 min and 30 s) and ending with a 10 min extension at 72 °C. The *HWP1* gene PCR also was done in a final volume of 20 µl was carried out with initial denaturation of 94 °C for 10 min followed by twenty five cycles of program (94 °C for 1 min, 49 °C for 1 min and 30 s, 72°C for 1 min and 30 s) and ending with a 10 min extension at 72 °C. PCR reactions were run on a 1.5 % agarose gel in 1X TAE.

Sequencing of *ALS3* gen and phylogenetic analysis

PCR products were purified according to Applied Biosystems method, using the primers described above. sequencing reactions consisted of 2 ul of BigDye Terminator v. 3.1 mix (Applied Biosystems), 3 ul of dilution buffer (Applied Biosystems), 5 pmol of primer, and 0.2 ug of template DNA in a final reaction volume of 10 ul. Cycle conditions were an initial denaturation at 96° for 2', then 35 cycles of 96° for 10", 52° for 15", 60° for 3', followed of 1' at 72° [41].

Nucleotide sequencing was carried out with an automatic sequencer (3100 genetic analyzer; Applied Biosystems). Homology was determined by BLAST search, oligonucleotide sequences were aligned with CLUSTAL W, Bioedit [42, 43] and phylogenetic tree was building by neighbor-joining method of MEGA 6 [44].

Identification of molecular expression of genes comprised in biofilm formation

Probes design

Designing Antisense Oligonucleotides program was used to design the probes. This program is proposed to design antisense oligonucleotides, and iRNA in eukaryotes [45].

Identification of *ALS3, EAP1* and *HWP1* expression in *C. maltosa* biofilm was done using *C. albicans* as positive control, since they were previously described on it. The syntheses of the designed probes (table 3) which were labeled with two fluorophores, was done at IDT (*In vitro* DNA Technology Company, USA).

Table 3. Probe sequences designed to determine *ALS3, EAP1* and *HWP1* genes expression in *Candida albicans*

Strain	N° of Access	Probe	Sequence	Fluorophores
Candida albicans	AY223551.1	*ALS3*	5' GTGTGGTTTGGTGGTTCTCT 3'	6 Fam (*)
	XM_709573.1	*EAP1*	5' GTGTCAGTCGTGTAGGAGGT 3'	Cy3 (**)
	XM_441625.1	*HWP1*	5' GTTCTTGTGGTTGTTGTGGGT 3'	Cy3 (**)

(*) , 6 Fam : 6 - carboxyfluorescein (**) Cy3 : Cyanine 3

Fluorescent *in situ* hybridization expression (FISH expression)

FISH RNA or FISH expression is a molecular method used to identify mRNA *in situ* through DNA – RNA hybridization which is detected via fluorescence [46]. In the present study, some modifications to this method were established using RNA protect™ Bacteria Reagent (QIAGEN) to protect bacterial RNA, and also the probe design was done through the bioinformatics program (Designing Antisense Oligonucleotides).

Samples containing only biomass or biofilm were obtained from the growing cultures, correspondingly. A pretreatment was established consisting of 800 µL sample mixed with 500 µL of ARN protect™ Reagent (QIAGEN) and incubated for 15 min at room temperature. After this time and also after each of the following steps, this suspension was kept on ice. The suspension was centrifuged at 4800 x g for 8 min and washed with PBS three times. Once It was washed, 850 µL of absolute ethanol were added and incubated at 4 °C for 16 h. Subsequently, 8 µL of the suspension were fixed onto a slide and dehydrated with ethanol at 50, 80 and 96%. After that, 8 µL of hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl, 0.01% SDS, pH 7.2) and 8 µL of probe were added over the treated sample. The slide was incubated in a humid chamber at 45 °C for two hours. Then, the slide was treated using the Washing Buffer (5 M NaCl, 0.5 mM EDTA, 10% SDS, 1 M Tris-HCl pH 7.0) and incubated at 45 °C for 10 min. Finally, the slides were observed at 10x and 100x using a fluorescence microscope (Olympus BX-40) [47, 48].

RESULT AND DISCUSION

Biofilm Forming Capacity

The kinetic growth of planktonic cells of *C. maltosa* was determined, while they were growing in YNB-Glc medium at 30 ºC for 96 h. The specific growth rate, doubling time, and start–time of stationary phase were 0.041 h^{-1}, 17h and 48h, respectively. These parameters were useful to determine the BFC index, to establish DNA isolation and for FISH analysis. Acording to BFC index determination, *C. maltosa* was classified as a strong biofilm forming yeast with an average index of 1.820 ± 0.832. Previous reports have also determined the BFC index, above 1 for *C. albicans* and *C. maltosa* growing in YNB-Glc medium [17].

According to Hee *et al.* [2002], considerable differences in biofilm production existed among Candida species as *C. albicans*, *C. tropicalis*, *C. glabrata*, and *C. parapsilosis* when growing in high-glucose medium and Sabouraud dextrose broth (SDB) medium. Biofilm formation occurred most frequently in isolates of *C. tropicalis,* followed by *C. parapsilosis, C. glabrata*, and *C. albicans* [49]. *Candida* non-*albicans* species were more prolific biofilm producers than *C. albicans* and that the source of isolates might influence the biofilm production [50].

In contrast, Hawser and Douglas [1994], reported that isolates of *C. parapsilosis* and *C. glabrata* were significantly less biofilm formers than the more pathogenic species such as *C. albicans* [51, 52]. However, these studies examined only a few selected strains of different *Candida* species grown in yeast nitrogen base medium containing 50 nM glucose. Additionally, *C. albicans* also produced biofilm in yeast peptone dextrose (YPD) and in yeast nitrogen base (YNB) medium supplemented with 100 mM glucose [53, 54].

Moreover, in this study, COD (445.2 mg O_2L^{-1}), total carbohydrates (53.1 mg mL^{-1}) and proteins (0.1 mg mL^{-1}) were determined in EPS. On the other hand, Fattani and Doulglas [2006] described that *C. albicans* and *C. tropicalis* were grown in yeast nitrogen base (YNB) medium (Difco) containing 50 mM glucose, at 37 °C for 24 h in an orbital shaker at 60 r.p.m. The *C. albicans* biofilm composition were carbohydrates (396 mg mL^{-1}) and proteins(50 mg mL^{-1}) . In adittion, in *C. tropicalis* the concentration of carbohydrates and proteins were 33 mg mL^{-1}, and 33 mg mL^{-1}, respectively [55, 56, 57].

The initial attachment of yeast cells was followed by germ tube formation within 3 to 6 h. After 24 to 48 h of incubation, the fully mature *C. albicans* biofilms consisted of a dense network of yeasts, hyphae, and pseudohyphae, and extracellular polymeric material was visible on the surfaces of some of these morphological forms [52].

Identification of *ALS3* gene in *C. albicans* and *C. maltosa*

Adhesion is the first key step for biofilm formation. Cell adhesion may be mediated by non-specific factors, including hydrophobicity and electrostatic forces of the cell surface, or by specific adhesins on the surface of ***C. albicans.***

The biofilm phenotype can be described in terms of the genes expressed by biofilm-associated cells. A complex transcriptional network controlling the development of *C. albicans* biofilms is comprised of six “master regulators” (Efg1,Tec1, Bcr1, Ndt80, Brg1, and Rob1) that are required for normal biofilm development, *in vitro* and *in vivo* conditions [4].

Bcr1, as well as some of its downstream targets such as the cell wall proteins Als1, Als3, and Hwp1, are required for adherence during biofilm formation [4]. The hyphal adhesins Als1, Als3 and Hwp1 appear to establish heterotypic interactions that are necessary for maintaining cell-cell contacts in *C. albicans* within the biofilm and their genes are induced strongly during hyphal growth [26, 27, 28, 29, 30, 31, 58, 59, 60].

In this work *ALS3* and *HWP1* genes were identified in *C. albicans,* however *ALS3* was only identified in *C. maltosa* by PCR and its amplification product was 370 pb approximately. Furthermore, *EAP1* was not identified in *C. maltosa* neither in *C. albicans* by DNA sequencing, may be due to a not well primer design.

The sequence alignment analysis of *ALS3* gene of *C. albicans* shown 98% of homology and *C. maltosa* shown 99% of homology (E-value: 5e-146 <1e-5 and E-value: 4e-142 <1e-5 respectively, both sequences corresponding to NCBI Gen Bank Accession No. AY223552.1). In addition, there is not information about the *ALS3* gene sequence described for *C. maltosa* in other gene banks such as European Bioinformatics Institute (EBI) and the DNA Data Bank of Japan (DDBJ). Multiple aligment analysis of *ALS3* gene sequences from *C. albicans* (obtained from NCBI GenBank) and *C. maltosa* shown substitutions, transversions and transitions dispersed in different fragments of the analyzed sequence (figure 1).

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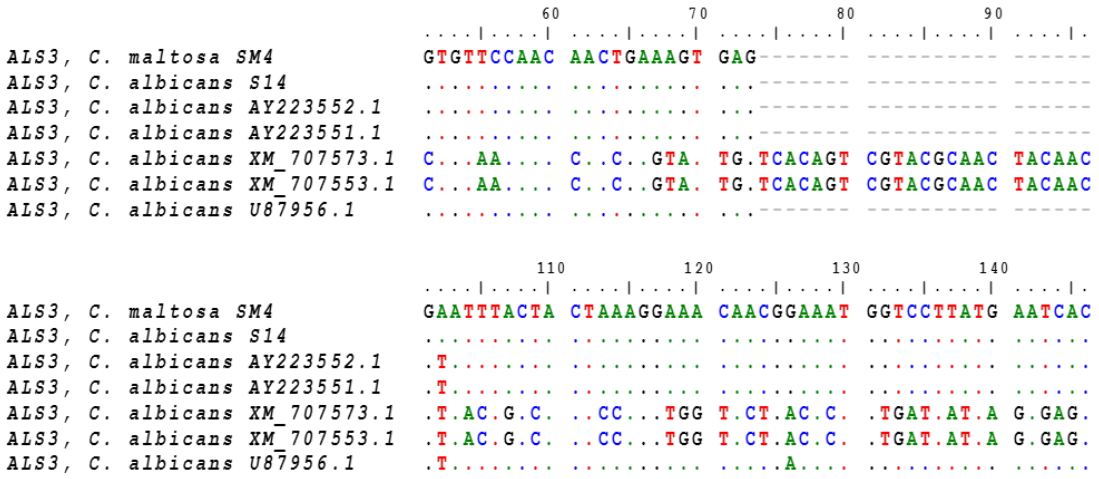

Figure 1. Multiple aligment of *ALS3* sequences in *C. maltosa* and *C. albicans* (this study) and *ALS3* sequences of *C. albicans* from NCBI GenBank.

According to phylogenetic analysis using neighbor-joining method, *ALS3* gene sequence of *C. maltosa* SM4 is on the same clade of *ALS3* gene sequence of *C. albicans* S14. Furthermore, threre is a common node between this gene and the others, which might indicate that the *ALS3* gene sequences of *C. maltosa* and *C. albicans* show high homology (figure 2).

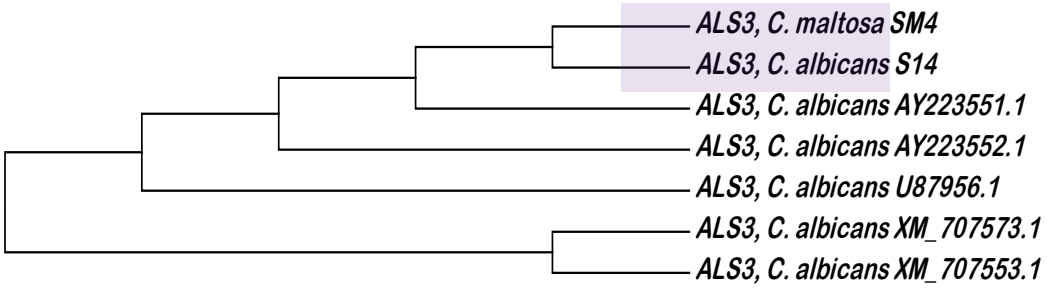

Figure 2. Phylogenetic analysis by Neighbor-Joining method building from *ALS3* gene sequences from *C. albicans* and *C. maltosa* (this study) and *ALS3* gene sequence of *C. albicans* from GenBank.

Fluorescent *in situ* identification of *ALS3* and *EAP1* gene expression

In this study, *C. maltosa* was classified as strong biofilm forming according to BFC index and also sessil cells agregations and EPS matrix on a glass surface was observed through optical microscopy (figure 3a). Furthermore, gene expression of *ALS3* and *EAP1* was identified during biofilm formation in *C. maltosa* using Fluorescent *in situ* hybridyzartion (figure 3b and d). Interestingly, *ALS3* was detected in yeast and hiphal forms, while *EAP1* was only detected in yeast forms. On the other hand *HWP1* expression was not detected, It could indicate that this gene is transcribed at early stages of biofilm formation [61, 62]. Overexpression of *ALS3* in *C. albicans* was observed in initial stages of biofilm formation, that maximal expression is associated with formation of germ tubes and hyphae forms [50].

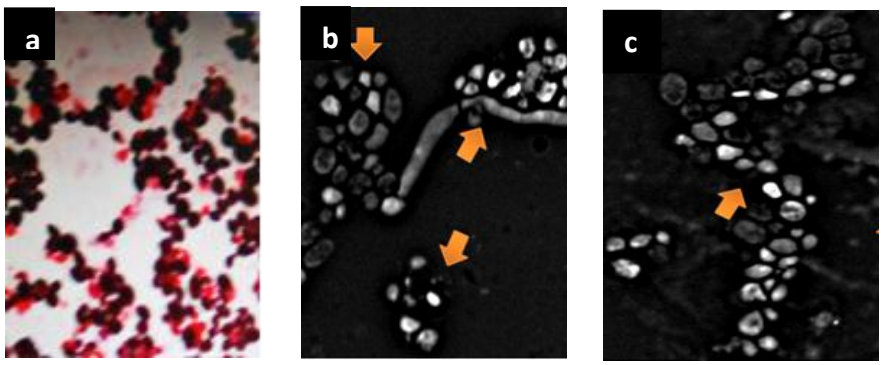

Figure 3. Sessil cells agregations of *C. maltosa* . a) Crystal violet stain. Oranges arrows shown b) ALS 3 and c) *EAP1* gene expression in *C. maltosa.*

Other reports suggest that *ALS3* gene was expressed in hyphae and pseudohyphae phase of *C. albicans* but not in yeast forms. Furthermore, It was determined that there is a defective biofilm formation when the strain of *C. albicans bcr1* Δ / Δ having *ALS3* gene and other mutated adhesins, was used in *in vitro* and *in vivo* experiments. The study also suggested

that there is a complementary binding of Hwp1 to Als1 and Als3 during biofilm formation [63, 64]. Nevertheless, other work determined that while multiple adhesins participate in biofilm formation *in vivo*, Als3 had a central role in this process [60]. Als proteins were also identified in other *Candida* species such as *Candida tropicalis* and *Candida dubliniensis* and Eap 1 adhesin was described in *Candida glabrata* [28].

In this work, the expression of *ALS3* during biofilm formation was determined using FISH expression, and the presence of *ALS3* gene was verified with DNA sequencing. On the other hand *EAP1* expression was only identified by FISH expression. In spite of this method let to monitor gene expression when there are enough target to stablish hibrydization and signal detection [65, 66, 67, 68, 69, 70, 71], the viabilility of sessil and planktonic cells might be observed.

CONCLUSIONS

The present study verified that *C. maltosa* is a strong biofilm forming yeast supporting previous not published studies, where this microorganism was used in bioremediation purposes.

ALS3 expression was identified in *C. albicans* and *C. maltosa* biofilms in terms of FISH expression. Furthermore, sequence analysis of *ALS3* gene of *C. maltosa* showed 99% of homology to *ALS3* gene of *C. albicans*. Probable expression of *ALS3* and *EAP1* in *C. maltosa* could be related to biofilm formation.

Therefore, a modified method to detect gene expression *in situ* (FISH expression) was used in order to detect *ALS3, HWP1* and *EAP1 in C. albicans* and *C. maltosa* biofilms, constituting a useful tool to monitor biofilm formations.

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Author' biography

Danitza Xiomara Romero Calle was born in October of 1990 in La Paz city, Bolivia. In2006 she get High school grade, the next year began to study Biochemistry in Facultad de Ciencias Farmacéuticas y Bioquímicas (FCFB) of the Universidad Mayor de San Andres (UMSA). She was speaker in a Biochesmistry festival of FCFB in 2009. She has worked in typification of *Leishmania spp*. at Instituto de Investigaciones Farmaco Bioquímicas (IIFB) to get the grade of Bachelor Biochemistry in 2012, she co worked of Biology in pre university courses from FCFB. She studied at Maestría en Ciencias Biológicas y Biomédicas UMSA to get a Master Scientiarum degree in Biology Molecular mention in was 2014, the same year she was working identifying probiotic bacteria by molecular tools in PROBIOQUINUA Project at IIFB

In 2015 she was researcher of Analyses of bacteria community from Milluni basin, La Paz city project at FCFB and get a Higher Education Diploma course, now she is teaching researcher at IIFB.