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A colorimetric assay using tetrazolium salts with an electron mediator to evaluate yeast growth in opaque dispersed systems

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Research

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Aldana Lourdes Zalazar[a,b], Malena Mariana González[a,b], María Fernanda Gliemmo[ac] and Carmen Adriana Campos[ac*]

a. **Departamento de Industrias, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Int. Güiraldes 2160, Ciudad Universitaria (1428), Buenos Aires, Argentina.**
b. **Research fellow from Consejo Nacional de Investigaciones, Científicas y Técnicas de la República Argentina.**
c. **Member of Consejo Nacional de Investigaciones, Científicas y Técnicas de la República Argentina.**

CORRESPONDENCE AUTHOR
Carmen Adriana Campos
E-mail address: carmen@di.fcen.uba.ar

CONFLICTS OF INTEREST
There are no conflicts of interest for any of the authors.

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ABSTRACT:

Background: Traditional microbiological enumeration techniques such as colony count are time consuming. Visual evaluation of turbidity growth lacks objectivity and precision. On the other hand, the accuracy of the spectrophotometric readings can be limited by media opacity and/or additives. In this context, colorimetric methods based on tetrazolium salts are an alternative to traditional methods due to their ability to generate endpoints based on color change. The objective of this study was to develop a colorimetric assay based on two different tetrazolium salts and an electron mediator in order to replace the conventional method of viable plate count or absorbance for microbial growth detection in opaque dispersed systems.

Methods: A colorimetric method to evaluate yeast growth in broth and opaque dispersed systems using two tetrazolium salts, 2,3,5-triphenyl-2H-tetrazolium chloride (TTC), 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (INT) and an electron mediator, 2-methoxy-1,4-Naphthoquinone (MNQ) was developed. In addition, the minimum inhibitory concentrations against *Zygosaccharomyces bailii* for natamycin and lemongrass essential oil were determined by the proposed method and compared with those obtained using the conventional broth microdilution and plate count methods.

Results: Showed that the mixture of INT/MNQ, was more effective than TTC/MNQ. In opaque structure systems, the later showed to be more sensitive than absorbance However, in broth it was as sensitivity as absorbance to detect yeast growth. Besides, the INT/MNQ reagent functioned adequately to determine the minimum inhibitory concentrations of antimicrobials in opaque structured systems.

Conclusions: These results demonstrated that the proposed method functioned adequately to determine the minimum inhibitory concentrations of antimicrobials in opaque structured systems showing an excellent agreement with the plate count method and the proposed technique allowed the evaluation of yeast growth with lower population levels than the ones required through absorbance readings. Moreover, this colorimetric method is easy to perform and less time consuming.

Keywords: antimicrobials, minimum inhibitory concentration, naphthoquinone, nontransparent media, redox technique, tetrazolium chloride.

INTRODUCTION

Determination of microbial growth-no growth interface is important to evaluate microbiological stability and antimicrobial effectiveness. Microbial growth can be measured by plate count, direct microscopic count, dry weight, absorbance, visual examination of turbidity, etc. [1]. Traditional microbiological enumeration techniques such as colony count employing selective media are time consuming. Visual evaluation of turbidity growth lacks objectivity and precision. On the other hand, the accuracy of the spectrophotometric readings can be limited by media opacity, additives that affect the spectral characteristics of culture media, bacterial aggregation, or microbial pigments [2]. Therefore, few conventional methods of antimicrobial susceptibility testing are accurate and reproducible when applied to microorganisms that have specific growth requirements or slow growth rates [3]. In this context, colorimetric methods could represent an alternative approach due to their ability to generate endpoints based on a visually detectable color change. Different resazurin microtiter-plate assays were proposed to evaluate antifungal activity of essential oils and other natural antimicrobials [4, 5]. However, these assays cannot be used in acid media since the orange color of resazurin in this condition does not allow the evaluation of color change due to the redox process [6]. Therefore, other growth indicators, such as tetrazolium salts, have to be used to facilitate measurements [1, 7]. These salts are soluble in water and colorless in their native form but form insoluble, colored salts (intracellular formazan crystal) as they are reduced. The formazan can be quantified by measuring the absorbance or by visually detectable color change [1, 8]. The 2, 3, 5-triphenyl-2Htetrazolium chloride (TTC), used to test the effectiveness of antimicrobials, has been modified over the years by the addition of nitro, methoxy and iodine groups to the phenyl rings. For example, the monotetrazolium salt, 2-(4iodophenyl)-3-(4nitrophenyl)-5-phenyl tetrazolium chloride (INT), has been used effectively to study mitochondrial electron transport [9]. Furthermore, to increase the sensitivity of these indicators, intermediate electron acceptors are used since they facilitate reduction reactions [10]. The 2-methoxy-1, 4-naphthoquinone (MNQ) was successfully used as an electron mediator to evaluate effectiveness of antimicrobials [11].

In the present study, a colorimetric assay was developed to replace the conventional methods of viable plate count or absorbance for microbial growth detection in opaque dispersed systems based on two different tetrazolium salts, TTC and INT and MNQ as electron mediator. We also evaluated antimicrobial minimum inhibitory concentrations (MICs) endpoint by the proposed method.

MATERIALS AND METHODS

Materials and preparation of detection reagents

2,3,5-Triphenyl-2H-tetrazolium chloride (TTC), 2-(4-iodophenyl)-3-(4nitrophenyl)-5-phenyl-2H-tetrazolium chloride (INT), 2-methoxy-1,4-naphthoquinone (MNQ) were obtained from Sigma Aldrich, USA. Reagent grade citric acid was from Anedra (Argentina). Food grade xanthan gum was from Cargill (Argentina), natamycin was from Handary S.A. (Belgium) and lemongrass essential oil (*Cymbopogon citratus*), LEO, was from EUMA SA. (Argentina). All culture media used (SB, Sabouraud broth; SA, Sabouraud agar) were from Biokar Diagnostics (Beauvais, France).

Tetrazolium salts TTC and INT were dissolved in distilled water at concentrations of 11.1 mM and 2.8 mM, respectively. The electron mediator was dissolved in dimethylsulfoxide at concentrations of 1.0 mM (C1) and 5.0 mM (C2). Then, all solutions were sterilized by passing them through 0.22 □m filter. The colorimetric indicator solutions were mixed with the electron mediator at a ratio of 9:1.

These prepared detection reagents contained 10.0 mM of TTC with C1 (TTC1) and C2 (TTC2) mediator levels or 2.5 mM of INT with C1 (INT1) and C2 (INT2) mediator levels. Detection reagents concentrations were selected taking into account those proposed by [1, 6, 10].

Yeast strains, inoculums preparation and viability determination

Zygosaccharomyces bailii NRRL 7256, *Zygosaccharomyces rouxii* ATCC 28.253 and *Saccharomyces cerevisiae* MI (obtained from the culture collection of Laboratory of Microbiología Industrial, Facultad de Ingeniería, and Buenos Aires*)* inocula were prepared separately. The strains were stored at -30.0 ± 0.5 ºC in SB broth plus 10.0 g/100 g glycerol. Before their use, they were grown twice in SB at 25.0 ± 0.5 ºC for 24 h. After that, each inoculum was diluted in peptone water to reach 0.5 McFarland units, corresponding to a population of approximately 10^6 CFU/ml. Different dilutions of the inoculum were performed according to the objective of the assay. Yeast viability was determined by surface plating on SA and then incubated at 25°C. Colonies were counted after 5 days.

Model systems tested

Transparent liquid and opaque structured model systems were assayed. All of them were formulated in SB. The structure of systems was achieved adding different concentrations of xanthan gum (0.000, 0.250 and 1.000 wt%). Xanthan gum was finely dispersed into SB and agitated for 24 h at 25ºC to assure complete hydration. Then, the systems were sterilized for

30 min at 100°C. The pH was adjusted to 3.50, 4.00, and 4.50 by adding sterilized citric acid solution (250 g/L).

Colorimetric microbial assays using tetrazolium salts and an electron mediator

The colorimetric method is based on the use of tetrazolium salts and MNQ as electron mediator to increase the sensitivity of indicators for yeast growth detection. Assays were performed in sterile microplates. Negative and positive controls were tested in parallel, being the former no inoculated SB (pH 5.7), and the latter, inoculated SB (10^8 CFU/ml), both without adjusted pH and containing the indicator in the levels studied. Microplates were incubated at 25°C ± 0.5°C. The indicators turn was checked every 30 minutes for 3 hours. The visual detection of color change in the wells, as compared with the negative and positive controls, was considered as positive. To check inoculum level in each system, two microplate columns per system were left without adding the indicators to measure yeasts growth by viable plate count.

Assays were performed three times and each system was replicated three to six times.

a) *Assays performed in broth*

Four mixtures of TTC or INT with two levels of MNQ were prepared. The efficiency of MNQ was evaluated in SB at different pHs (3.50, 4.00, 4.50 and without adjusting pH) and at different levels of yeasts populations (10^5, 10^6, 10^7 and 10^8 CFU/ml). The yeast inocula were diluted in SB at pHs studied to adjust microbial cell density. Microbial suspensions (190 µl) were dispensed in the 96-wells sterile microplate. Then, 10 µl of dye mixtures was added to each well. This assay allowed the selection of INT1 mixture as detection reagent, which was used in subsequent assays.

b) *Assays performed in structured media*

The ability of INT1 mixture to determine the growth of yeast strains in an opaque model system with 0.250% of xanthan gum was evaluated. The effect of pH (3.50, 4.00, 4.50 and without adjusting pH) and the level of yeast population (10^4, 10^5, 10^6, 10^7 and 10^8 CFU/ml) on turn of INT1 mixture were studied. For these purposes, 850 µl of systems was dispensed into 48-wells sterile microplates, to which 100 µl of different inocula and 50 µl of INT1 were added. The indicator color change was evaluated after 2 h of incubation at 25.0ºC ± 0.5°C. This assay was also done adding a step of incubation of inoculated systems at 25.0ºC ± 0.5°C for 24 h and then INT1 was added.

Application of the colorimetric assay selected to evaluate antimicrobial effectiveness

To evaluate the performance of the colorimetric assay to determine the effectiveness of natamycin and LEO against *Z. bailii* growth, the following systems were prepared: i) *natamycin:* a stock solution of 1500 ppm was made in SB. For that, natamycin was finely dispersed over SB. Then, pH was adjusted to 3.50 with citric acid and the system was sterilized by filtration; ii) *LEO:* an oil in water emulsion was formulated. A level of 30000 ppm of LEO, 9000 ppm of Tween® 80 and distilled water at pH 4.00 (adjusted with citric acid) were mixed under sterile conditions in an Ultraturrax homogenizator (IKA, Germany). Then, this coarse emulsion was sonicated for 5.0 min with an ultrasonic processor (Model VCX 750, Vibra Cell Sonics, United States of America) at 20 kHz and amplitude of 20% with a 13 mm titanium alloy probe. The emulsion was stored at 25°C ± 0.5°C for 7 days in order to stabilize its droplet size.

The antimicrobials stock solutions were used to determine MICs of each antimicrobial against *Z. bailii*. The MIC values were determined colorimetrically by broth microdilution assays that were performed in microplates using INT1 reagent to detect the end point. The MIC was determined visually as the lowest concentration of the antimicrobial at which no color change occurred. Negative and positive controls were tested in parallel, being the former no inoculated SB, and the latter inoculated SB free of antimicrobials, without adjusted pH but containing INT1.

a) *Determination of MICs of natamycin and LEO on Z. bailii growth in broth*

The MICs of each antimicrobial were determined in 96 well-round bottomed sterilized microplates. Serial dilutions of each antimicrobial were prepared in SB adjusted to each pH. Aliquots of 180 µL of the diluted antimicrobials were pipetted into the microplate wells together with 20 µL of *Z. bailii* inocula achieving a final population of 10^5 CFU/ml. The concentrations tested were: 0.00, 1.56, 3.13, 6.25, 12.50, 25.00 and 50.00 ppm for natamycin; 0.00, 234.00, 468.00, 937.00 and 1875.00 ppm for LEO. Microplates were incubated at 25°C ± 0.5°C for 48 hours. Then, 10 µl of INT1 was added to each well. The MIC was read after 2 h of incubation with INT1 reagent as the lowest concentration of the antimicrobial at which no indicator turns occurred after 48 h of incubation at 25ºC in [11-14].

In parallel, MICs were determined by the broth microdilution method and absorbance readings. The procedure was the same as used above but without the addition of the detection reagents. During microplate storage, growth was measured by recording the absorbance at 600 nm every 1 h with a microplate reader

(BioTek Instruments, ELx808, USA). After incubation, the MICs were determined as the lowest concentration of antimicrobials at which absorbance variation was less than 0.1 units [15]. The controls were the same as used above but without adding the indicator and the absorbance of the negative controls was used as blank. Each microplate was made in triplicate, using different inocula, and containing six replicates of each system.

b) *Determination of MICs of natamycin and LEO on Z. bailii growth in structured media*

The MICs in model systems containing 0.250% and 1.000 % xanthan gum were determined in 48 well-round bottomed sterilized microplates. The concentrations tested were: 0.00, 1.56, 3.13, 6.25, 12.50, 25.00 and 50.00 ppm for natamycin; and 0.00, 234.00, 468.00, 937.00 and 1875.00 ppm, for LEO. In addition, the effect the time required for detection of the indicator (INT1) turn was estimated.

For natamycin system preparations, the stock solution was dispersed separately in SB in necessary amounts to achieve the desired concentrations. Xanthan gum was dispersed and agitated for 24 h at 25ºC to assure complete hydration. Then, the systems were thermally treated in a water bath for 15 min at 95°C to avoid antimicrobial degradation. The pH was adjusted to 3.50 with sterilized citric acid solution.

In the case of LEO, the emulsion was prepared, as mentioned in section 2.6.

Then, in aseptic conditions, aliquots were added to SB containing xanthan gum to achieve the desired concentrations and the systems were mixed with an Ultra-turrax homogenizator (IKA, Germany).

Subsequently, the formulated systems were inoculated with *Z. bailii (*10^4 CFU/ml) and 950 µl was dispensed into microplates obtaining increasing concentrations of the antimicrobials in each row. The microplate was incubated 100 h at 25.0°C ± 0.5°C. This time was selected to ensure growth inhibition since in previous tests it was found out that antimicrobials extend yeasts lag phase. At the end of incubation, 50 µl of INT1 mixture was added and the microplate was incubated for 2 h at 25.0°C ± 0.5°C.

RESULTS AND DISCUSSION

Colorimetric microbial assays using tetrazolium salts and an electron mediator

Since the production of formazan depends on inoculum size [3], different inoculum levels of yeast strains were tested in systems studied. Furthermore, tetrazolium salts were added after yeasts incubation to avoid their toxic effects on cells [16].

a) *Assays performed in broth*

The color change began to appear after 2 h of addition of indicators and INT showed a more intense color than TTC. Moreover, INT was more sensitive than TTC since 10^6 CFU/ml was needed for INT1 turn and 10^7 CFU/ml for TTC turn (Figure 1, panel A, as an example). This trend is linked with the fact that INT enters easily into cells, then is reduced by cells NAD (P)H-dependent oxido-reductases and dehydrogenases, producing formazans that can be more effectively solubilized. For these reasons, INT has been used as a vital dye and as an indicator of cellular redox activity [9]. On the other hand, increasing the MNQ concentration from 0.1 to 0.5 mM did not improve the optical analysis, since no differences were observed on color intensity or on time necessary for indicator turn (Figure 1, panel A).

Regarding pH, it was observed that as pH increased, color was intensified and the indicator turned in a shorter incubation time, independently of the yeast strain used. The most intense color was obtained in samples without adjusting pH (Figure 1, panel B).

In conclusion, regardless of pH and strain, a minimum population of 10^6 CFU/ml is required for the turn of the INT1 mixture. This population level is coincident with the minimum level needed to detect growth by absorbance evaluation [17].

b) *Assays performed in opaque structured media*

The effectiveness of INT1 dye to evaluate yeasts growth in an opaque aqueous model system at pH 3.50, 4.00, 4.50 and without adjusting pH was evaluated. Besides, the effect of yeast inoculum levels was also evaluated. It was observed that turn of the indicator took more than 6 h for inocula levels of 10^5 and 10^6 CFU/ml. However, when the same levels were reached (checked by plate count) after an incubation of 24 h, the indicator turn was observed in less than 2 h. These results suggested that yeast growth within the structure media increased the sensibility of detection of the color change. Probably the growth in colonies and the opacity of the system could improve color visualization. Furthermore, color intensity was enhanced in systems without adjusting pH. However, within the range of 3.50-4.50 color could be easily appreciated demonstrating that this detection reagent can be used in all pH range.

In conclusion, a population of 10^5 CFU/ml developed in the structured media were selected for next assays. Moreover, the proposed technique allowed the evaluation of yeast growth with lower population levels than the ones required through absorbance readings, as previously mentioned.

Application of the colorimetric assay selected to evaluate antimicrobial effectiveness

The MICs of antimicrobials to inhibit the development of *Z. bailii*, a yeast responsible for the deterioration of dressings and acid sauces were determined. The pHs values were chosen taking into account that pH of acid sauces are within the range of 3.50-4.00 [18].

a) *Determination of MICs of natamycin and LEO on Z. bailii growth in broth*

In systems containing natamycin, the indicator turned on at 3.13 ppm and no growth was detected at 6.25 ppm. Yeast growth curves were constructed with the absorbance data (Figure 2, panel A) observing no growth for the same natamycin concentration, therefore the MIC was 6.25 ppm. This value is similar to the ones previously reported [19]. Natamycin is a potent inhibitor at very low concentrations and its activity depends on its binding to ergosterol, the major sterol of fungal membranes, making them permeable [20].

In systems containing LEO, the indicator changed color in all the wells except those containing levels greater than or equal to 467 ppm LEO. Yeast growth curves constructed with absorbance data showed that at concentrations greater than or equal to 467 ppm growth was inhibited (Figure 2, panel B). In previous studies, a MIC of 1130 ppm of LEO against *Z. bailii* growth has been reported by the broth dilution method by direct mixing of the oil with Tween®80 [21]. This value is higher than the one obtained here. Probably, the emulsification process improved the contact of the oil with the aqueous medium resulting in a lower effective concentration.

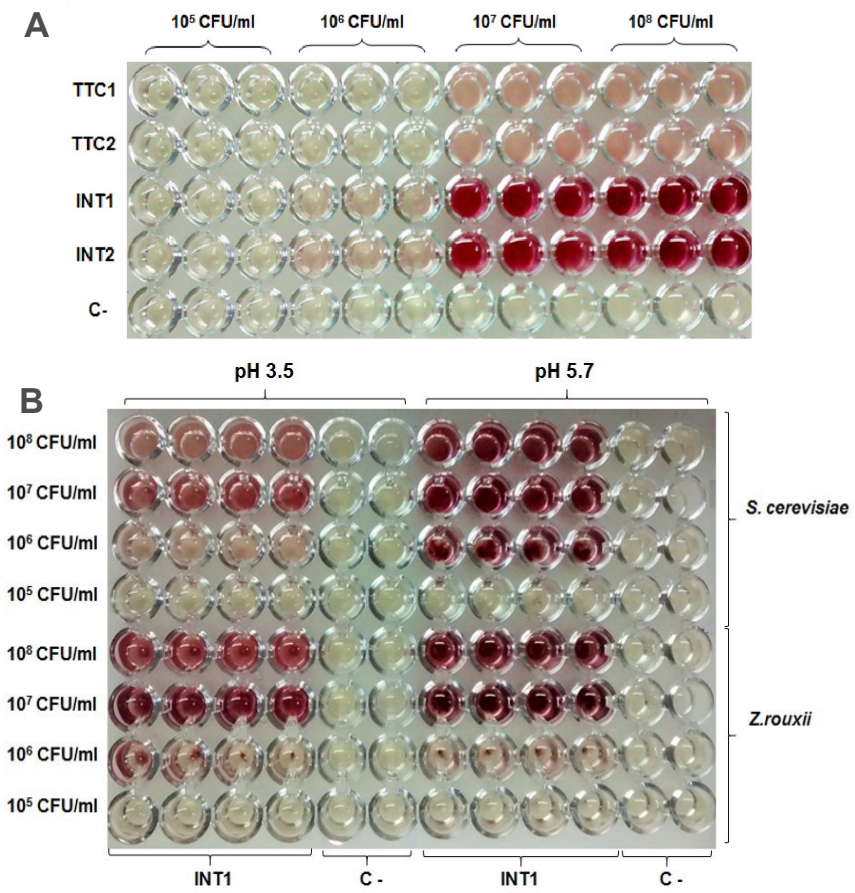

Figure 1. Detection of yeasts growth in Sabouraud broth using TTC/MNQ or INT/MNQ as detection reagents. Panel A: *Z. bailii* at pH 3.50. Panel B: *S. cerevisiae* and *Z. rouxii* at pH 3.50 and without adjusting the pH (5.70) using INT1 as detection reagent. 1: Indicator with the lowest level of the MNQ (1mM) and 2: indicator with the highest level of the MNQ (5mM). C-: CB uninoculated containing the detection reagents.

Figure 2

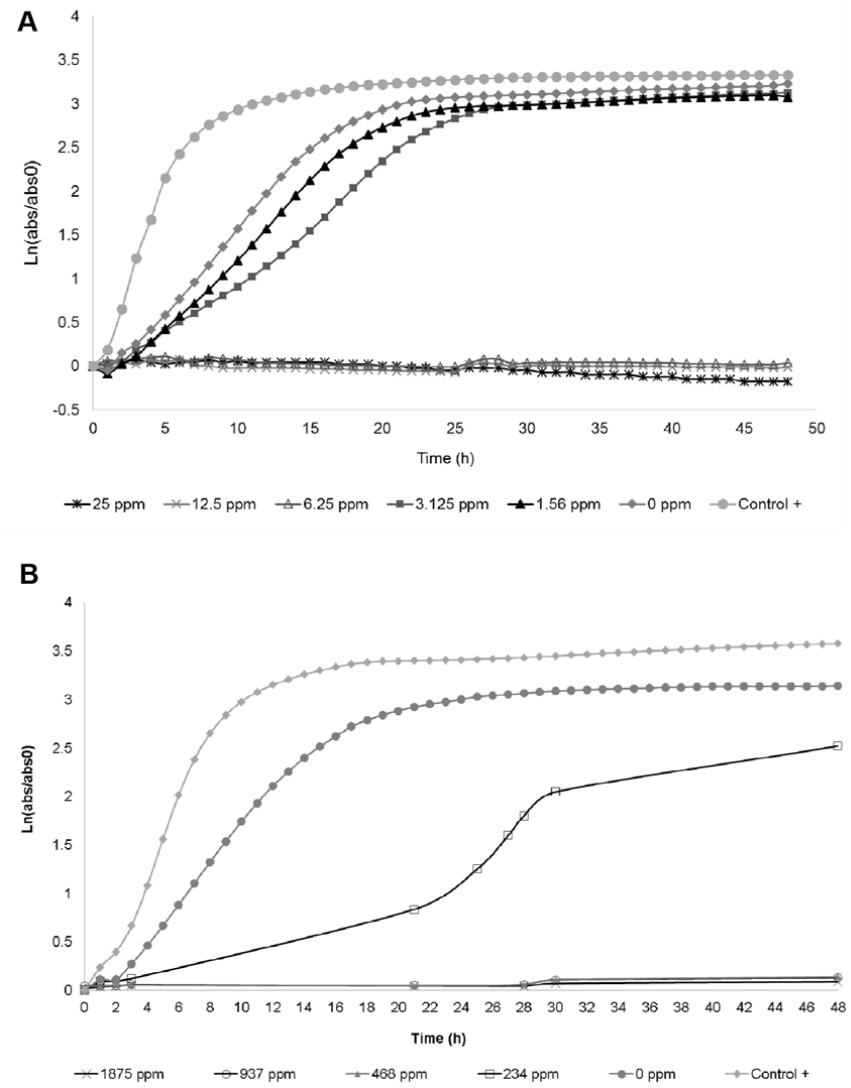

Figure 2. Growth curves of *Z. bailii* in Sabouraud broth after 48 h of storage at 25°C. Panel A: different concentrations of natamycin at pH 3.50. Panel B: different concentrations of LEO at pH 4.00.

b) *Determination of MICs of natamycin and LEO on Z. bailii growth in structured media*

Concentrations of antimicrobials greater than those required to inhibit growth in liquid media were tested since the systems were formulated with xanthan gum, which favors growth under certain conditions, according to previous studies [22]. In the case of natamycin, after 24 h of incubation and 2 h later of INT1 addition, it turned only in the antimicrobial-free system, which reached 2.10^6 CFU/ml. In all systems with natamycin, the counts were lower than 10^4 CFU/ml at 24 h, for this reason no color change was observed. After 100 h of incubation, an intense color was obtained in the systems with 3.00 ppm of natamycin and a soft color in the systems with 6.00 ppm (Figure 3, panel A). In addition, growth in the form of colonies in the stained yeasts could be observed. The microplate was incubated for 168 h and no further changes were observed. These results showed that natamycin probably extended yeast lag phase, requiring a longer incubation time to achieve the indicator turn. Considering this trend, an incubation time of 100 h was selected. This time allowed to differentiate between the concentrations analyzed, with color being observed in all the wells of 0 and 3.00 ppm and in some wells with 6.00 ppm natamycin (3 positive/9 total in the systems containing 0.250% xanthan gum and 7 positive/9 total in the systems containing 1.000%). Since at a concentration close to 6.00 ppm in some cases there was yeast inhibition and in others no, the MIC value of *Z. bailii* for natamycin for structured aqueous system was 12.00 ppm.

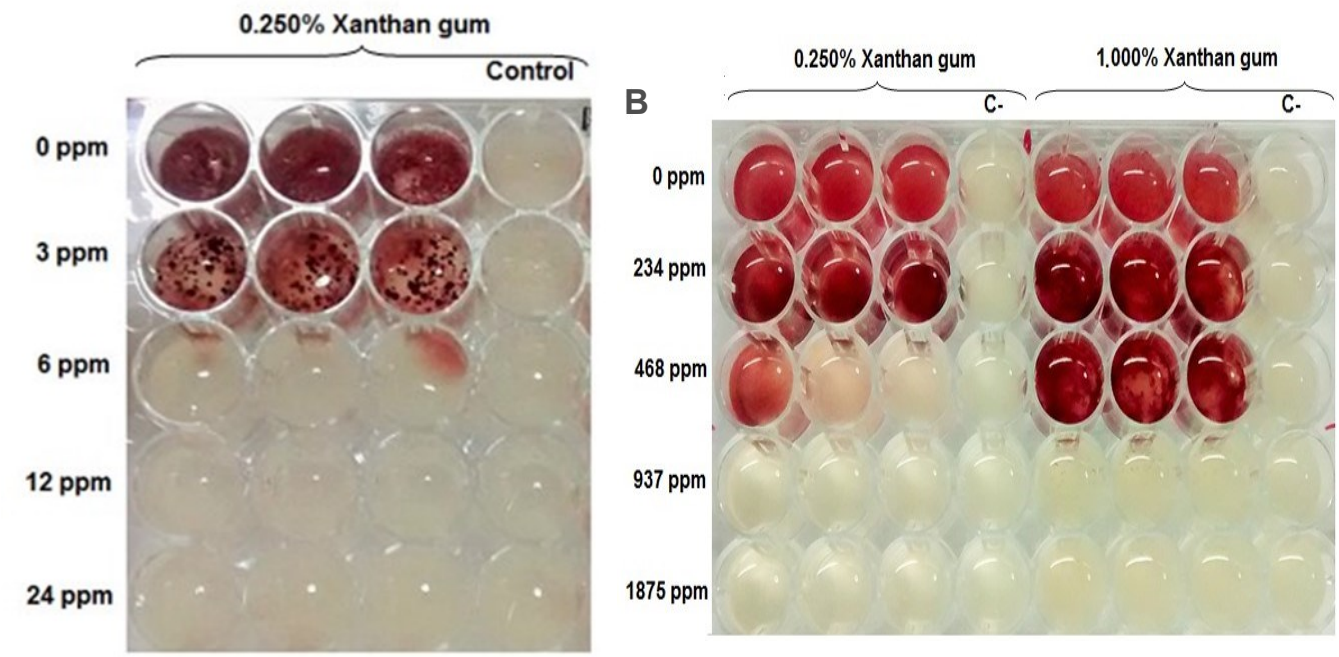

Figure 3. Detection of *Z. bailii* growth, after 100 h of incubation and 3 h later of the addition of the dye mixture (INT1) in opaque aqueous model systems. Panel A: different concentrations of natamycin at pH 3.50. Panel B: different concentrations of LEO at pH 4.00.

Regarding LEO, after 24 h of incubation and 2 h later of INT1 addition, the indicator only turned in the antimicrobial-free system, which reached a count of 10^6 CFU/ml. In all systems with LEO, a count lower than 10^4 CFU/ml of the yeast was obtained at 24 h, that is why the indicator did not turn. Furthermore, after 100 h of incubation and 2 h later of INT1 addition, an intense color also was obtained in the systems with 234 ppm and 468 ppm (Figure 3, panel B). In systems with the lower xanthan gum content (0.250%) and 468 ppm LEO, color was less intense and not all wells were colored (5 positive/9 total). In contrast, in the systems with the highest xanthan gum content (1.000%), the color changed in all the wells. This trend is linked with the fact that xanthan gum can improve growth since it can be used as a carbon source by the yeast [22]. In the systems where the color was presented, the population of *Z. bailii* was equal or greater than 10^6 CFU/ml and in systems where color change was not observed the counts were lower than 10^4 CFU/ml, for this reason no turning was achieved. The microplate was allowed to incubate for 168 h and no changes were observed. From these results, it was possible to conclude that the MIC value of LEO against *Z. bailii* growth in structured media was 937 ppm.

In the structured media used, it was necessary an extension of the incubation time to determine properly the susceptibility of natamycin and LEO on *Z. bailli*. Tsukatani et al. [11] reported the same trend in the case of bacteria resistant to antibiotics. These results stressed the importance of the adequate selection of test conditions.

CONCLUSIONS

The evaluation of TTC and INT together with MNQ, for detecting yeasts growth in broth and opaque media allowed concluding that: i) INT/MNQ was most effective than TTC/MNQ; ii) in broth, INT1 was as sensitivity as absorbance to detect yeast growth; iii) in opaque structure systems, a population of 10^5 CFU/ml developed in the media allowed the turn of indicator; iv) the INT1 reagent functioned adequately to determine the MICs of antimicrobials in opaque structured systems showing an excellent agreement with plate count method. Moreover, proposed method is easy to perform and less time consuming.

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