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Original paper

Microcalorimetric growth evaluation of Candida albicans in different conditions

MIHNEA GABRIEL POPA[1,2], ANDREI-ALEXANDRU MUNTEAN[1,3], VLAD TUDOR POPA[4]*, CERASELLA CRISTIANA DRAGOMIRESCU[1,3], IRINA EREMIA[1,2], SILVIA NICA[2], MIRCEA IOAN POPA[1,3] CĂTĂLIN CÎRSTOIU[1,2]

[1]"Carol Davila" University of Medicine and Pharmacy, Bucharest, Romania
[2]Emergency University Hospital Bucharest, Romania
[3]"Cantacuzino" National Medico-Military Institute for Research and Development, Bucharest, Romania
[4]"Ilie Murgulescu" Institute of Physical Chemistry, Bucharest, Romania

Abstract The aim of this study was to investigate the growth pattern of a lyophilized *Candida albicans* strain using microcalorimetry. Microcalorimetry is a method that can record the thermal flow released by the basal metabolism of a microorganism. Using a dedicated data acquisition and processing software, the method can provide a growth curve, which is specific to every microorganism. We have obtained the growth pattern of *Candida albicans* in different conditions with the objective of determining various growth "fingerprints" that we can afterwards use within a diagnostic method. Experiments where undertaken at five different temperatures 26°C, 30°C, 34°C, 37°C and 40°C and at different suspension volumes in the microcalorimetric cell: 200 µl, 400 µl, 600 µl, 800 µl. Our experiments provided real-time, reproducible information about fungal growth, with expected differences due to the various changes in growth conditions.

Keywords Microcalorimetry, *Candida albicans*, diagnostic method, *real time growth*.

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✉ *Corresponding author: POPA VLAD TUDOR, "Ilie Murgulescu" Institute of Physical Chemistry, 202 Splaiul Independentei st., 060021, Bucharest, Romania
E-mail: vtpopa@icf.ro Tel: (004)0213163101

Introduction

The number of infections with *Candida* spp. has increased significantly over the past decades. *Candida* spp. are commensal organisms which most frequently infect patients with immunosuppression. Indwelling devices, total parenteral nutrition, use of intravenous drugs and antibiotic use or general immunocompromised status are all risk factors for *Candida* spp. infections. *Candida albicans* is the most common pathogen isolated from serious fungal infections, responsible for over 90% of cases [1-4]. Alongside this, other species, such as *Candida auris* have recently been implicated in severe infections, making this an emerging fungal pathogen.

The advancement of the medical field, spurred on by modern technology, has increased life expectancy, with more complex diagnostic and treatment devices being used in different medical specialties: cardiology (vascular catheters, cardiac devices, prosthetic valves), orthopaedics – different joint prosthesis, urology (hemodialysis fistulas of grafts, urinary catheters), neurology (central nervous system devices), etc. The increasing number of implantable devices and the aging of the population generate a rise of complications [5-6]. In 1957 Elek and Conen showed that the risk of infection is significantly higher when a foreign body is present; almost 50% of all nosocomial infections are associated with medical devices in different situations [7-9].

C. albicans is commonly found in the gastrointestinal tract of healthy adults, as a commensal organism that can become pathogenic under different conditions. This happens most commonly in immunocompromised patients such as those that undergo immunosuppression medical therapy after organ transplant, patients infected with HIV or those under chemotherapy for various types of cancers [10]. *C. albicans* are very common nosocomial pathogens, predominantly in patients who have undergone surgery or are in intensive care units [2, 7].

Strains of *Candida* spp. may produce biofilm, facilitating further adhesion of the pathogen. Studies show that biofilm producing strains are relatively refractory to medical therapy and the medical consequences of this device-related infection may be disastrous. Device malfunction or systemic infections may necessitate device removal, frequently complicated with extensive tissue destruction. Management of these pathologies is very complex and may lead to exacerbated morbidity and mortality if not swiftly diagnosed after the onset of the infection and if the correct treatment protocol is not followed. Treatment is very expensive for the healthcare systems because the medical devices oftentimes have to be removed. Treatment must be carried out until healing and new devices are reimplanted afterwards [11, 12, 13, 14].

Clinical symptoms of surgical site infection after a total joint arthroplasty or an open fracture reduction, can develop either early (less than 3 months after surgery) or late (3 months and up to 2 years after surgery). Frequently, early infections present more acute signs and symptoms (fever, edema, erythema, slow wound healing, and pain) as compared with late ones (mild chronic pain and no fever). In chronic infections early loosening of the implant will be described on the radiography. Risk factors include prior surgery, rheumatoid arthritis, poor nutritional status, diabetes mellitus, skin ulcers, obesity, immunocompromised state, psoriasis, and advanced age. Because the incidence of *Candida* spp. orthopedic surgical site infections is rare, relevant medical literature is scarce, with only a few cases reported. In general, treatment of *Candida* prosthetic infection consists of both prosthesis removal and systemic antifungal therapy [15, 16, 17]. Faced with such a difficult pathological entity, novel diagnostic and therapeutic strategies must constantly be considered, to alleviate the morbidity, mortality and cost burden [18-19].

Modern calorimetry has begun to develop since the early nineteenth century. The first differential scanning microcalorimeter appeared around 1970. Today, computer technology has allowed faster data processing, allowing for the continued evolution of the technique. With this device it is possible to analyze heat flow produced in various conditions by some microorganisms within the medical field, but not only. To achieve this, there must be an alteration in heat flow generated by changes in the basal metabolism of the studied pathogen. Besides changes in growth temperature, the environment used, and the addition of an antibiotic can help us in different studies [20, 21].

Isothermal microcalorimetry measures heat flow produced by the growth of microorganisms. This is done by means of converting the electrical signal received from the device (*Seebeck Effect)*, with appropriate calibration and special software, to a real time bacterial growth curve. This method has already been used to analyze heat produced by bacteria, mammalian cells and worms [22]. The detection time depends primarily on the sensitivity of the instrument, the number of viable cells initially introduced, and the amount of heat generated by them [23, 24, 25, 26].

The aim of this paper is to analyze the microcalorimetric features of *Candida albicans* growth upon variation of the following parameters: (**i**) inoculated volume (and thus available oxygen in unfilled cell space) and (**ii**) incubation (growth) temperature.

Materials and Methods

Fungal population

In the performed experiments, we used a lyophilized *Candida albicans* species – which we obtained with the goodwill of the medical staff of the "Cantacuzino" National Institute for Research and Development.

For the growth of bacteria, both liquid and solid Sabouraud medium without chloramphenicol was used. The medium was autoclaved before use and tested for microbiological purity. Solid Sabouraud medium was used

both to isolate cultures and to verify purity. In order to maintain viability of the isolated culture, re-culture was performed at least once a month from the lyophilized stock.

Sample preparation

Experiments were carried out using special microcalorimetric cells (with a real maximum capacity of 850 µl) and hermetically sealed using a cap with a silicone o-ring. These microcalorimetric cells are passed through several cleaning steps after an experiment. Initially, sonication is performed to remove the remaining microscopic fragments, then autoclaved at 121°C and finally left for 1 hour in the laminar flow cabinet under ultraviolet radiation before use.

We used isolated colonies from *Candida albicans* grown on solid Sabouraud medium, before inoculating the liquid medium. Liquid Sabouraud medium was used for fungal growth and for the preparation of microcalorimetric samples. For the growth of fungi, the seeded liquid medium was left in the thermostat. After overnight growth, samples were prepared in a BSL2 microbiology hood. After homogenization, liquid cultures were transferred into 1.5 mL Eppendorf tubes, spun down in a benchtop centrifuge at 3000 rpm, for 5 minutes and resuspended in fresh Sabouraud medium. This washing step was carried out in triplicate. Sterile Sabouraud medium was read using a nephelometer and baseline McFarland index was evaluated. A delta McFarland index (dMFI = actual McFarland index – baseline McFarland index) of 0.1 was prepared by pipetting from the Eppendorf tube into the fresh medium. This was then transferred into the microcalorimetric cells.

Microcalorimetry

Two differential scanning microcalorimetry (µDSC) devices were used: Setaram MicroDSC III and MicroDSC VII. Gaseous N_2 flow of 50 ml/min (99.99% SIAD – TP) was used to protect the 3D sensor array. For data acquisition and processing the dedicated software was used (Calisto v 1.077).

The sample cell was prepared using different volumes (200 µl, 400 µl, 600 µl, 800 µl) of the dMFI. The reference was filled with sterile Sabouraud medium, to which 50 µL of Gentamicin 3:1 solution was added to eliminate the risk of contamination. Volume of the reference medium matched the one of the sample cell (fungal suspension).

Both cells were introduced in the microcalorimeter and experiments were carried out at different temperatures: 26°C, 30°C, 37°C, 40°C.

Data analysis

Data analysis was carried out using Calisto and a two peak decomposition was generated for each of the experimental conditions. To account for small baseline variability, thermograms were baseline integrated by using the horizontal last point method (end-of-experiment baseline).

Results

For the reported microcalorimetric study of *C. albicans* growth we used the same quantity of fungal inoculum and changed in subsequent experiments the temperature or the volume of the sample (inoculated liquid medium) used. We changed only one parameter per experimental run, and we were able to identify some patterns of the fungal growth depending on both temperature of the experiment and volume of inoculated medium.

C. albicans has been defined as a "versatile opportunistic" pathogen [27] due to its ability to grow in various media and conditions, including aerobic and anaerobic environments. Temperature, pH and medium composition were proven to exert a sizable influence on both growth rate and morphology of fungal population [28]. Its morphogenesis alteration by gymnemic acids was convincingly demonstrated [29]. Biofilm formation of *C. albicans* and non-albicans Candida species was proven to be governed by "hydrodynamic conditions and ambient oxygen gradients" [30]. Anaerobic growth was proven slower (doubling time 248 min) than the aerobic one (doubling time 98 min), as reported by Biswas and Chaffin [31].

The sample volume within the micro calorimetric cell influences the *C. albicans* thermogram, regarding its intensity, as the thermograms reveal. Higher volume involves more nutrients and space for the bacteria to grow, so this parameter increased maximum heat flow and generated larger total duration of growth, i.e. higher overall growth heat.

In agreement with the fact that *C. albicans* develops at temperatures close to living organisms' ones, the performed experiments evidenced best growths between 30-37°C. Both below and above this temperature range growth is either slower or, to a certain extent, inhibited.

Discussion

Microcalorimetric growth of *C. albicans*

Standard microbiological practice recommends the incubation of fungal species at 35 ± 2°C.

Growth of *C. albicans* was initially assessed under normal laboratory conditions, a 600 µl volume of of inoculated medium incubated at 34°C. Using an accepted microcalorimetric cut-off for biological growth (positive displacement of 0.1 mW from baseline).

Effects of temperature on the growth of *C. albicans*

An illustration of the temperature effect on the growth of *C. albicans*, for a 400 ml sample growth volume is shown in Figure 1. As can be seen, the "thermal fingerprint" of *C. albicans* growth is sensitive to temperature variation within the narrow range investigated: (**i**) from 26°C to 37°C thermograms become less extended in time (sharper); (**ii**) within the same range the signal amplitude gradually increases with the growth temperature

(As previously reported [32], since heat flow scales with the momentary growth rate, this is equivalent to a maximum growth rate increase with temperature.); (**iii**) there is a clearly evidenced "biphasic" growth pattern, roughly corresponding to aerobic / anaerobic conditions; (**iv**) all the above growth features are attenuated at 40°C: thermogram sharpness and amplitude decrease, the two growth stages are less obvious.

In general, the above-mentioned characteristics may be observed for other sample volumes investigated.

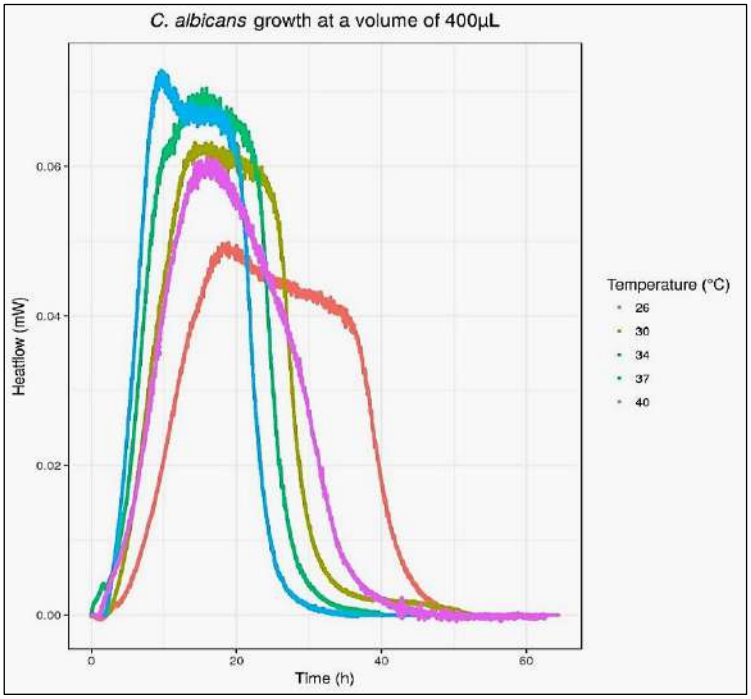

Figure 1. Microcalorimetric growth of *C. albicans* as a function of temperature.

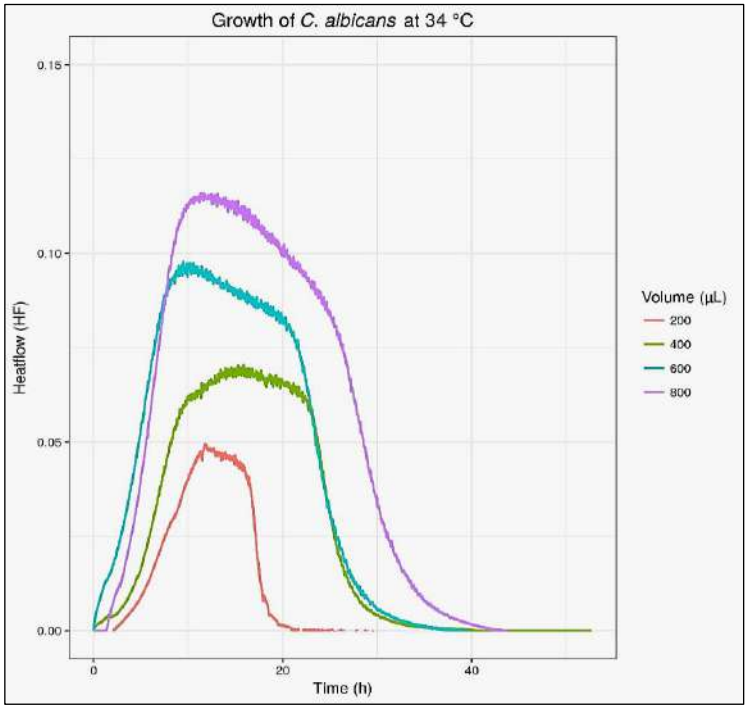

Figure 2. Microcalorimetric growth of *C. albicans* as a function of volume.

Effects of volume on the growth of *C. albicans*

Our previous studies have shown [33] that microcalorimetric growth of bacteria in "oxic conditions" exhibits a two peak thermal signal in which the first peak was ascribed to growth due to dissolved oxygen in the medium while the second peak relates to the growth at the air-liquid interface. Thus, we investigated various sample volumes of fungi to assess the standard/optimal microcalorimetric parameters. An example of the effect of volume on the growth of *C. albicans*, at a constant temperature of 34°C, is shown in Figure 2. The expected cumulative behavior is observed. The volume within the microcalorimetric cell influences the *C. albicans* thermogram, with respect to its amplitude (with increasing volumes inducing higher maximum heat flow) and extension of the total duration of growth. The increasing abundance of nutrients seems to sustain growth for longer periods of time. While the concentration of CFU/mL is constant, the total number of initial CFUs differ, which may explain the differences in the slope of growth. Whatever the sample volume, the bi-phasic feature of growth is clearly preserved at this temperature and best evidenced at samples of 400 ml and 600 ml.

Overview of growth patterns of *C. albicans* with regards to volume and temperature

As in bacterial growth microcalorimetry, the volume and the incubation temperature play an important role in the microcalorimetric patterns of fungal growth. This can be seen in Figure 3 where growth patterns of different sample volumes of *C. albicans* (depicted as plot facets) are presented in function of incubation temperature. For all investigated sample volumes growth is faster within the 30-37°C temperature range, while the biphasic feature is attenuated with increasing incubation temperature.

Plots of growth pattern dependence on sample volume, at different temperatures, are presented in Figure 4. There is a clear increase of the thermal growth with sample volume, for all temperatures investigated. On the other hand, excepting the 200 µL sample, 34°C emerges as the optimal growth temperature for all investigated volumes.

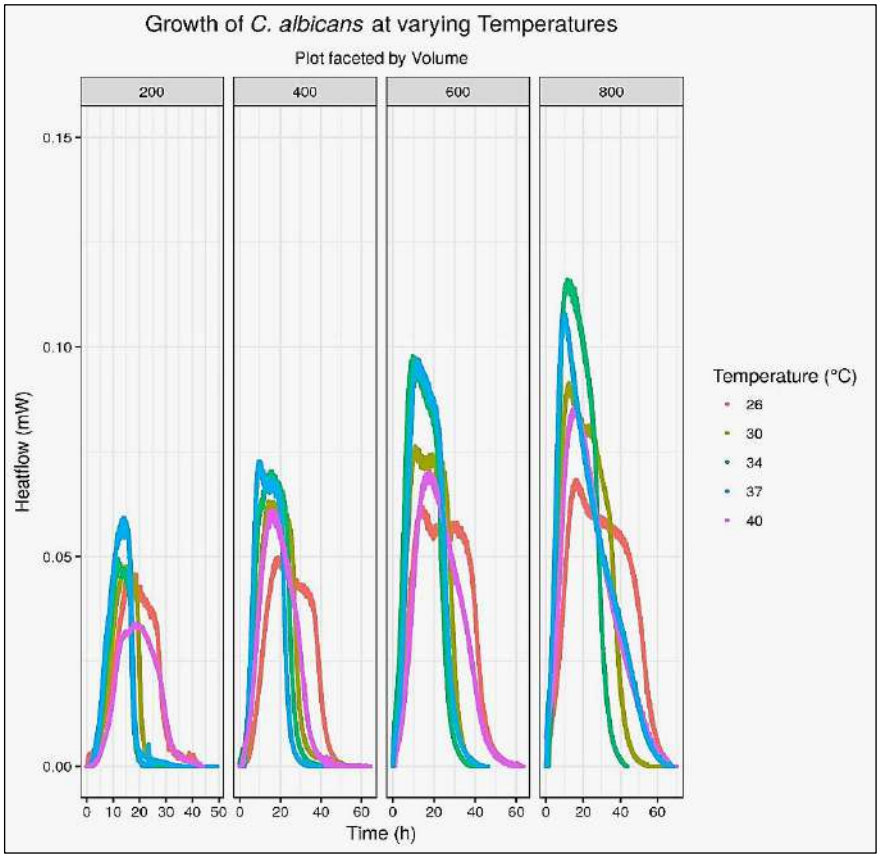

Figure 3. Overview of varying temperatures on different volumes of *C. albicans* growth.

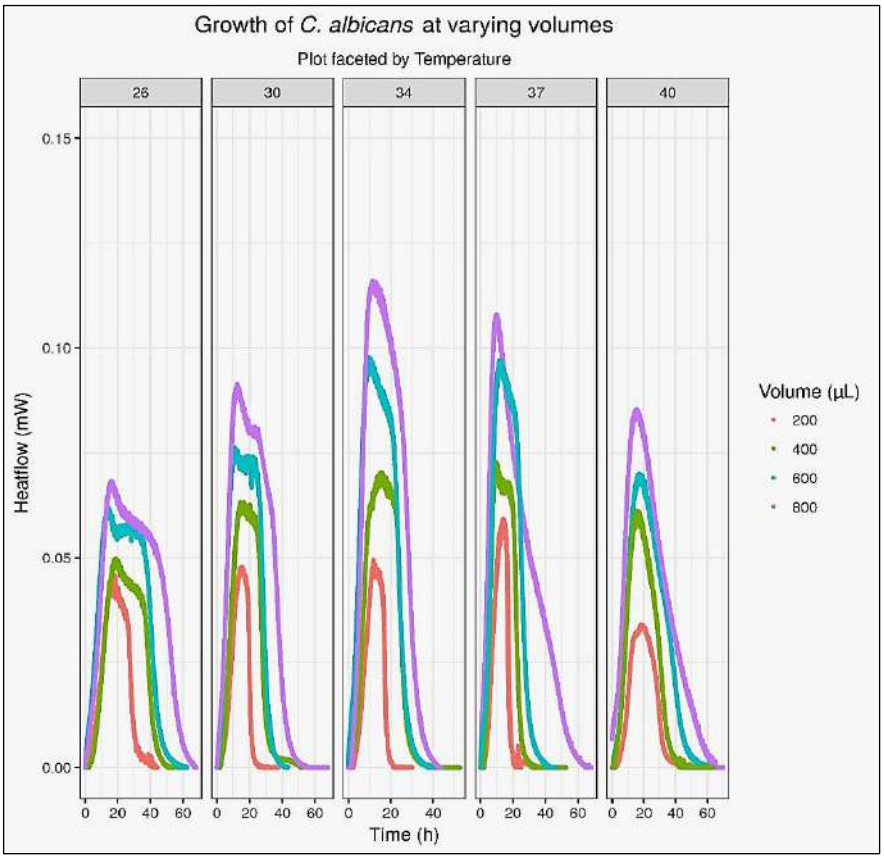

Figure 4. Overview of varying volumes and incubation temperature on *C. albicans* growth.

As previously demonstrated [32], the total thermal effect (obtained through heat flow integration) of the thermogram scales with the total bacterial (fungal) population developed within an experiment. This was defined by Monod [34] as "total growth" and in calorimetric terms as "total thermal growth" [33]. Accordingly, Fig. 5 presents an overview of the total thermal growth, which scales with the total fungal population present in the sample cell at the end of each experiment, whereas Fig. 6 depicts the variation of its sample volume normalized counterpart, termed "specific total thermal growth". The last, "specific", quantity scales with the fungal suspension concentration in the sample cell at the end of each experiment.

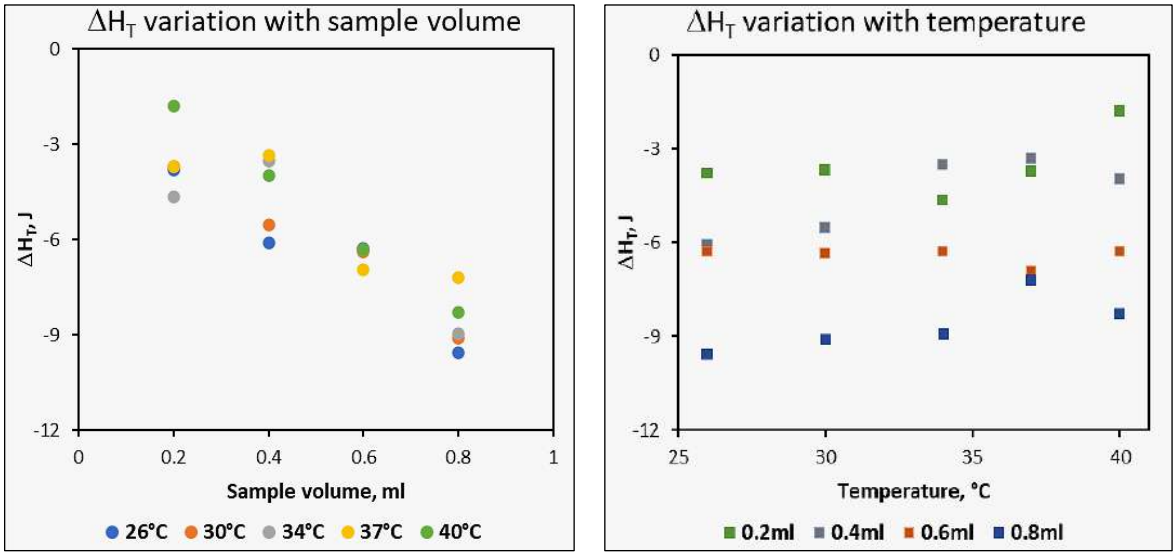

Figure 5. Variation of total thermal growth, ΔH_T: with sample volume, at various temperatures (left); with temperature, at various sample volumes (right).

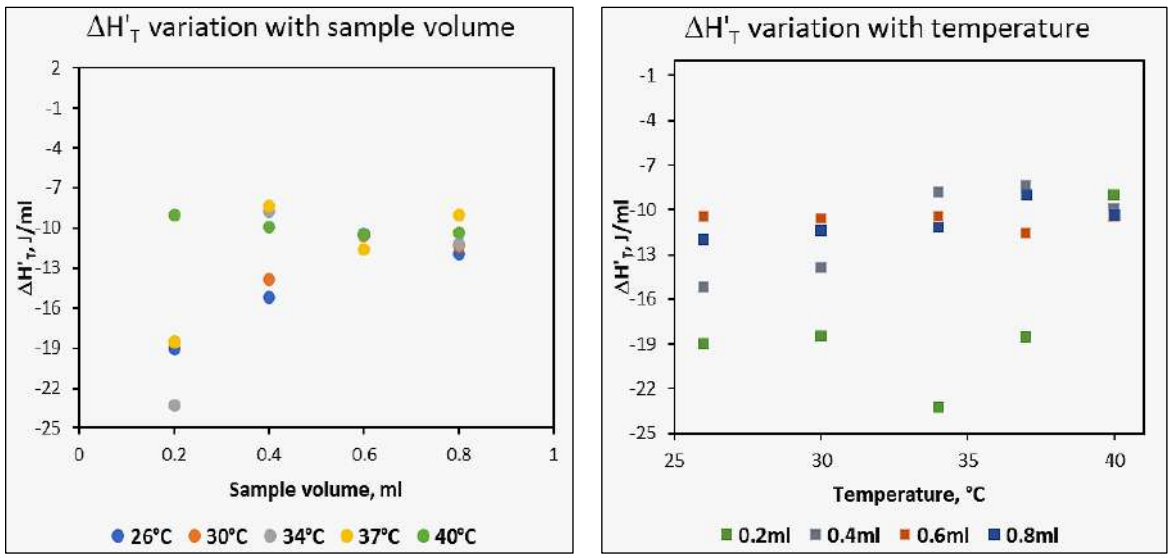

Figure 6. Variation of total specific thermal growth, $\Delta H'_T$: with sample volume, at various temperatures (left); with temperature, at various sample volumes (right).

The total thermal growth variation is rather complex, as illustrated in Fig. 5. This is due to the intricate dependence of the two phases of fungal development on both sample volume and temperature. There is a general decreasing trend of ΔH_T with increasing of the sample volume, while the opposite, increasing trend with the growth temperature is less evident. For an "optimal" sample volume of 0.6 ml the variation of ΔH_T with T is minimal, indicating a compensation between the two growth phases.

The total specific thermal growth variation, presented in Fig. 6, confirms the complexity of *C. albicans* microcalorimetric growth. On one hand, the "optimal" growth volume of 0.6 ml is preserved, as the variation of $\Delta H'_T$ is minimal for this sample volume. On the other

hand, 40°C emerges as an "optimal" growth temperature, as the fungal concentration at the end of each experiment, proportional to $\Delta H'_T$, is similar for all sample volumes utilized. Otherwise, the total specific thermal growth evidence a less regular behavior with both sample volume and temperature, a clear sign of imbalance between the two phases of fungal growth.

Conclusions

The incidence of infectious pathology is increasing due to the emergence of multidrug resistant bacteria that cannot be treated with common antibiotics. A well-known case is methicillin-resistant staphylococcus, which, in addition to antibiotic resistance, can withstand disinfectants used in hospitals [35]. An even more difficult situation is represented by multi-resistant gram-negative pathogens for which the therapeutic resources are extremely limited [36].

The multidrug resistance of *C. albicans* is less discussed, but an incriminating factor in the occurrence of bacterial and fungal resistance is the rather extended time lag before the antibiogram is obtained. In the present study, we demonstrated that the fungal presence may be detected in half of the time required for a classical culture. Additional tests published by our team [26] and by other researchers [37] have proven the feasibility of the "microcalorimetric antibiogram". By means of microcalorimetry, a versatile technique, the real-time evolution of complex biological structures such as fungi, bacteria, parasites, biofilms, or tumor microtissues [38-40] may be investigated, yielding important information.

The herein presented data on *C. albicans* have proven the complex biphasic character of its Sabouraud medium microcalorimetric growth. This corresponds approximately to aerobic – anaerobic growth modes, with 34°C and 600 ml as "best" temperature and sample volume experimental parameters, respectively.

Conflicts of Interest

This manuscript does not contain texts from previously published materials and it is not offered simultaneously for consideration/ publication entirely or in part elsewhere, regardless language.

This manuscript is conform with Uniform Requirements for Manuscripts Submitted to Biomedical Journals (ICMJE), and this paper has been conducted according to internationally accepted ethical standards (COPE).

All authors have read/ approved the final version of the paper.

All authors understood that the full responsibility for all presented data in the article belongs to authors, who are responsible for any conflict of interest (related to subjects included in their work, financial, consultant, institutional and other aspects).

The authors have no conflict of interest to disclose.

All authors have equally contributed to the writing and editing of the manuscript.

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