https://doi.org/10.31861/biosystems2023.01.009

УДК 616.594.171.2; 615.371; 616-097; 615.371; 616-097

THE EXPERIMENTAL DETERMINATION OF THE NUMBER OF FREEZING STAGES OF CANDIDA TROPICALIS FUNGAL CELLS FOR THEIR DESTRUCTION AND OBTAINING OF PROTEINS AND POLYSACCHARIDES

M.V. RYBALKIN, O. P. STRILETS, L. S. STRELNIKOV

National University of Pharmacy of the Ministry of Health of Ukraine,
Faculty of Pharmaceutical Technologies and Management, Department of Biotechnology
ribalkin.nikolay@gmail.com

Candidiasis is a disease that occurs due to excessive growth of the *Candida* fungus. Several types of this pathogen are known, each of which can cause damage to various organs and systems. Recently, there have been reports of the loss of sensitivity of *Candida* fungi to most of antifungal drugs that have been used for more than 40 years. An alternative to antifungal drugs for candidiasis can be the use of a vaccine for the prevention and treatment of candidal infection. In our opinion, it is promising to use a candidal vaccine based on a subunit vaccine that contains fragments of a microorganism without ballast substances. Cell fragments of *Candida* fungi that have antigenic properties include proteins and polysaccharides. To isolate antigenic substances from *Candid*a fungal cells, we chose freezing as the method of destruction. The aim of this work was to experimentally substantiate the number of freezing stages for the destruction of *C. tropicalis* ATCC 20336 fungi cells and the subsequent production of proteins and polysaccharides. To determine the optimal number of freezing stages in the temperature range from (25 ± 2)°C to (-25 ± 2)°C, in order to destroy the cells of *C. tropicalis* fungi, the studies were conducted with freezing stages 3, 4, 5, and 6. Centrifugation was used to separate the excess components. Then the preliminary and sterilizing filtration was performed on membrane filters with pore diameters of 0.45 μm and 0.22 μm. The content of protein, polysaccharides and monosaccharides were determined in each case. The protein content was determined according to the State Pharmacopoeia of Ukraine (SPhU). To determine polysaccharides, a reaction with phenol and sulfuric acid was performed. Chromatographic studies of monosaccharides were conducted using paper chromatography according to the SPhU. Based on the results of the research, it was found that solutions obtained at stages 5 and 6 of freezing the biomass of *C. tropicalis* fungi cells at a temperature from (25 ± 2) °C to (-25 ± 2) °C contained the highest amount of proteins and polysaccharides. It is likely that with these numbers of stages for freezing the biomass of *C. tropicalis* fungi cells, active substances are released from all layers of *Candida* fungi cells. Thus, it can be concluded that five stages of freezing are the optimal number that ensures the maximum extraction of polysaccharides and proteins quickly and economically.

Ключові слова: Candida tropicalis, freezing, candidiasis, proteins, polysaccharides, monosaccharides, antigen, vaccine.

Introduction. Candidiasis is a disease that occurs due to excessive growth of the *Candida* fungus (Polesello V. at al., 2017). Several types of this pathogen are known, each of which can cause damage to various organs and systems. The disease occurs against the background of the weakened immunity (Mercer D.K. at al., 2020).

Recently, there have been reports of the loss of sensitivity of *Candida* fungi to most antifungal drugs that have been used for more than 40 years (Edwards J.E at al., 2018; Howley M.M. at al., 2016). Instead of antifungal drugs for candidiasis a vaccine against *Candida* infection can be used (Tarang S at al., 2020; Nami S. at al., 2019). Research on the development of a vaccine against *Candida* infection is being conducted by various biotechnological companies in many countries around the world (Wang X. at al., Da Silva at al., 2020). In our opinion, it is promising to use a candidal vaccine based on a subunit vaccine that contains fragments of a microorganism without ballast substances (Piccione D. at al., 2019). Fragments of Candida fungal cells with antigenic properties include proteins and polysaccharides (Tso G.H.W. at al., 2018).

The most affordable, effective, and inexpensive physical methods were selected for the destruction of fungal cells of *C. albicans* and *C. tropicalis*: ultrasound, grinding of cells with solid materials, and freezing - thawing. All these methods have their advantages and disadvantages, but to finally determine which one is the most effective, it is necessary to conduct research with each of these methods. In this article, to obtain antigenic

substances, namely proteins and polysaccharides, from fungal cells of *Candida* genus, we chose such method of destruction as freezing - thawing in several stages. Different numbers of freezing - hawing stages 3, 4, 5, and 6 were studied at temperatures from (25 ± 2) °C to (-25 ± 2) °C.

The aim of this work was to study the method of disintegration at different numbers of freezing - thawing stages 3, 4, 5, and 6 at temperatures from (25 ± 2) °C to (-25 ± 2) °C for the destruction of fungal cells of *C. tropicalis* in order to obtain antigens – proteins and polysaccharides.

Materials and methods. Fungal cells of *C. tropicalis* ATCC 20336 were cultivated on Sabouraud agar at a temperature of (25 ± 2) °C. Then cells of *C. tropicalis* fungi were washed with saline solution and centrifuged at a rotation speed of 3000 rpm for 10 min. Fungal cells were standardized to (8.5-9)x10^8 in 1 mL. To determine the optimal number of freezing stages in the temperature range from (25 ± 2)°C to (-25 ± 2)°C, in order to destroy cells of *C. tropicalis* fungi, the studies were conducted with freezing stages 3, 4, 5, and 6. A refrigerating chamber was used for freezing cells of *C. tropicalis* fungi, and a water bath was used for thawing. Centrifugation was used to separate the excess components. Then the preliminary and sterilizing filtration was performed on membrane filters with pore diameters of 0.45 μm and 0.22 μm. In each case, the content of polysaccharides, proteins and monosaccharides was determined.

The protein content was determined according to the State Pharmacopoeia of Ukraine (SPhU). To determine polysaccharides, a reaction with phenol and sulfuric acid was performed. The reaction proceeds with the formation of red-brown colored compounds: 1.0 mL of the polysaccharide solution was transferred to a test tube, and 1.0 mL of 5.0% phenol solution and 5.0 mL of the concentrated sulfuric acid were sequentially added. The reaction solution was heated, and in a few seconds, a red-brown color appeared.

Chromatographic studies of monosaccharides were conducted using paper chromatography according to the SPhU. The experimental data were processed using mathematical statistics methods in accordance with the requirements of the SPhU using the MS Excel application computer program and chi-square statistics.

Results and discussion. Based on the results of the research conducted, it was found that solutions obtained at stages 5 and 6 of freezing - thawing the biomass of *C. tropicalis* fungal cells at a temperature from (25 ± 2) °C to (-25 ± 2) °C contained the highest amount of proteins and polysaccharides (Table 1).

It is likely that at these number of stages during freezing thawing of the biomass of *C. albicans* fungal cells there is a release of active substances from the cell membranes and cells of *Candida* fungi.

Table 1.

The concentration of proteins and polysaccharides depending on the number of freezing-thawing cycles during the disintegration of C. tropicalis fungi cells (M±m, n = 10, p < 0.05)

Substances	The number of freezing-thawing cycles			
	3	4	5	6
	Concentration, mg / mL			
Proteins	0.10 ± 0.01	0.11 ± 0.01	0.13 ± 0.01	0.14 ± 0.02
Polysaccharides	0.71 ± 0.06	0.73 ± 0.06	0.74 ± 0.07	0.75 ± 0.08

Solutions obtained at the number of cycles 3 and 4 during freezing - thawing at a temperature of (–25 ± 2) °C to (25 ± 2) °C in the technology of disintegration of cells of *C. tropicalis* fungi contained less polysaccharides and proteins. It should be noted that the amount of polysaccharides in *Candida* fungal cells is significantly higher than the amount of proteins, but proteins have greater antigenicity against polysaccharides. Therefore, it is necessary to take into account all antigenic components of *Candida* fungal cells.

In all the samples studied, polysaccharides of *C. tropicalis* fungi were represented by several monosaccharides. The qualitative determination and quantitative comparison of monosaccharides were performed by the color intensity and the spot size during paper chromatography (Table 2).

Polysaccharides of solutions obtained at stages 5 and 6 of freezing - thawing at a temperature from (-25 ± 2) °C to (25 ± 2) °C in the technology of disintegration of *C. tropicalis* fungal cells were represented by monosaccharides: mannose, glucose, and two unidentified monosaccharides. Polysaccharides of solution obtained at stages 3 and 4 during freezing - thawing at a temperature of (-25 ± 2) °C to (25 ± 2) °C in the technology of

disintegration of *C. tropicalis* fungal cells were represented by the same spectrum of detected monosaccharides, but the spots had a less saturated color and slightly smaller size, indicating smaller amount of monosaccharides in these solutions.

According to the research results obtained, it has been found that all the numbers of freezing - thawing cycles proposed at temperatures from (-25 ± 2) °C to (25 ± 2) °C in the technology of disintegration of *C. tropicalis* fungal cells provide the release of polysaccharides with the same monosaccharide composition. However, the highest amount of proteins and polysaccharides was obtained when using freezing - thawing cycles 5 and 6.

Table 2

The monosaccharide composition of polysaccharides depending on the number of freezing thawing cycles during the disintegration of C. tropicalis fungi cells (M±m, n = 10, p < 0.05)

Monosaccharides	**The number of freezing-thawing cycles**			
	3	**4**	**5**	**6**
	The content monosaccharides, %			
Glucose	54 ± 4	56 ± 4	60 ± 5	60 ± 5
Monose	36 ± 3	34 ± 3	30 ± 0.2	30 ± 0.2
Unidentified monosaccharide 1	5 ± 0.4	5 ± 0.4	5 ± 0.5	5 ± 0.4
Unidentified monosaccharide 2	5 ± 0.5	5 ± 0.3	5 ± 0.4	5 ± 0.4

To substantiate the optimal number of disintegration cycles during freezing - thawing of *C. tropicalis* fungal cells, in addition to comparing the yield of proteins and polysaccharides in each case, it is necessary to take into account the energy costs for each freezing and thawing cycle. Disintegration of *C. tropicalis* fungal cells at freezing - thawing cycles 6 at temperatures from (-25 ± 2) °C to (25 ± 2) °C is characterized by a higher energy consumption. Regarding the disintegration of *C. tropicalis* fungal cells with freezing - thawing cycles 5 at temperatures from (-25 ± 2) °C to (25 ± 2) °C, it can be concluded that this number of cycles is more rational and economical as it requires less resources. Since in both cases the amount of proteins and polysaccharides is approximately the same, it is rational to choose a more economical option for further research, that is, the disintegration of *C. tropicalis* fungal cells at freezing - thawing cycles 5 at temperatures from (-25 ± 2) °C to (25 ± 2) °C, while an increase of the number of freezing - thawing stages is impractical. Based on the studies conducted to substantiate the number of freezing - thawing cycles at temperatures from (-25 ± 2) °C to (25 ± 2) °C in the technology of disintegration of *C. tropicalis* fungal cells by the concentration of proteins and polysaccharides obtained, and monosaccharide composition, it can be concluded that five stages are the optimal number.

Conclusion. A complex of studies has been conducted to substantiate the number of freezing - thawing cycles in the technology of disintegration of *C. tropicalis* fungal cells. The temperature conditions of the experiments ranged from (-25 ± 2) °C to (25 ± 2) °C. It has been found that the release of the highest amount of proteins and polysaccharides possessing antigenic properties was observed during freezing - thawing cycles 5 and 6. It should be noted that the disintegration of *C. tropicalis* fungal cells conducted in six cycles requires more energy. Therefore, based on the results of the experimental studies, the optimal mode of disintegration of *C. tropicalis* cells during freezing - thawing has been chosen; it is freezing at temperatures from (-25 ± 2) °C to (25 ± 2) °C during five cycles.

References:

1. Da Silva L.B.R., Taborda C.P., Nosanchuk J.D. Advances in Fungal Peptide Vaccines. *J Fungi (Basel)*. 2020; 6 (3): 119. https://doi.org/10.3390/jof6030119.
2. Edwards J.E.Jr., Schwartz M.M., Schmidt C.S., et al.. A fungal immunotherapeutic vaccine (ndv-3a) for treatment of recurrent vulvovaginal candidiasis-a phase 2 randomized, double-blind, placebo-controlled trial. *Clin Infect Dis.* 2018; 66 (12): 1928-1936. https://doi.org/10.1093/cid/ciy185.
3. Howley M.M., Carter T.C., Browne M.L., at al. Fluconazole use and birth defects in the National Birth Defects Prevention Study. *Am J Obstet Gynecol.* 2016; 214 (5): 657. https://doi.org/10.1016/j.ajog.2015.11.022.
4. Mercer D.K., O'Neil D.A. Innate Inspiration: Antifungal Peptides and Other Immunotherapeutics From the Host Immune Response. *Front Immunol.* 2020; 17 (11): 2177. https://doi.org/10.3389/fimmu.2020.02177.
5. Nami S., Mohammadi R.., Vakili M. at al. Fungal vaccines, mechanism of actions and immunology: A comprehensive review. *Biomedicine &*

Pharmacotherapy. 2019; 109: 333-344. https://doi.org/10.1016/j.biopha.2018.10.075.
6. Piccione D., Mirabelli S., Minto, N., Bouklas T. Difficult but not impossible: in search of an anti-Candida vaccine. *Current Tropical Medicine Reports*. 2019; 6: 42–49. https://doi.org/10.1007/s40475-019-00173-2.
7. Polesello V., Segat L.,Crovella S., Zupin L. Candida infections and human defensins. *Protein Pept. Lett*. 2017; 24 (8): 747-756. https://doi.org/10.2174/0929866524666170807125245.
8. Tarang S., Kesherwani V., LaTendresse B. at al. In silico design of a multivalent vaccine against Candida albicans. *Scientific Reports*. 2020; 1066 (2020): https://doi.org/10.1038/s41598-020-57906-x.
9. Tso G.H.W., Reales-Calderon J.A., Pavelka N. The elusive anti-Candida vaccine: lessons from the past and opportunities for the future. *Front Immunol*. 2018; 9: 897. https://doi.org/10.3389/fimmu.2018.00897. eCollection 2018.
10. Wang X., Sui X., Yan L. at al. Vaccines in the treatment of invasive candidiasis. *Virulence*. 2015; 6 (4): 309-315. https://doi.org/10.4161/21505594.2014.983015.

ЕКСПЕРИМЕНТАЛЬНЕ ВИЗНАЧЕННЯ КІЛЬКОСТІ ЕТАПІВ ЗАМОРОЖУВАННЯ КЛІТИН ГРИБІВ CANDIDA TROPICALIS ДЛЯ ЇХ РУЙНУВАННЯ ТА ОТРИМАННЯ БІЛКІВ І ПОЛІСАХАРИДІВ

М. В. Рибалкін, О. П.Стрілець, Л. С. Стрельников

Кандидоз – захворювання, яке виникає при надмірному зростанні грибка кандиди. Відомо кілька видів цього збудника, кожен з яких може викликати ураження різних органів та систем. В останні часи стали з'являтися повідомлення про втрату чутливості грибів Candida до більшості протигрибкових препаратів, які використовується вже понад 40 років. Альтернативою протигрибковим лікарським засобам від кандидозу може бути використання вакцини для профілактики та лікування кандидозної інфекції. На нашу думку, перспективно використовувати кандидозну вакцину на основі субодиничної вакцини, яка містить фрагменти мікроорганізму, без баластних речовин. До фрагментів клітин грибів роду Candida, які володіють антигенними властивостями, відносять білки та поліцукри. Для виділення антигенних речовин з клітин грибів роду Candida нами було обрано метод руйнування – заморожування. Метою даною роботи було експериментальне обґрунтування кількості етапів заморожування для руйнування клітин грибів C. tropicalis ATCC 20336 та подальшого отримання білків і поліцукрів. Для визначення оптимальної кількості етапів заморожування при діапазоні температури від (25 ± 2)°С до (-25 ± 2) °С, щоб зруйнувати клітини грибів C. tropicalis були проведені дослідження з 3, 4, 5, та 6 етапами заморожування. Використовували центрифугування для відокремлення зайвих компонентів. Далі проводили попереднє та стерилізуюче фільтрування на мембранних фільтрах з діаметром пор 0,45 мкм та 0,22 мкм. У кожному випадку було проведене визначення білку, поліцукрів та моноцукрів. Визначення білка проводили згідно ДФУ. Для визначення поліцукрів проводили реакцію з фенолом та сірчаною кислотою. Хроматографічні дослідження моноцукрів проводили за методом паперової хроматографії згідно ДФУ. За результатами проведених досліджень встановлено, що розчини, які одержано при 5 та 6 етапах заморожування біомаси клітин грибів C. tropicalis при температурі від (25 ± 2) °С до (-25 ± 2) °С містили найбільшу кількість білків та поліцукрів. Ймовірно, що при цих кількостях етапів заморожування біомаси клітин грибів C. tropicalis відбувається виділення діючих речовин з клітинних стінок та клітин грибів Candida. Таким чином можна зробити висновок, що 5 етапів заморожування є оптимальною кількістю, яка забезпечує максимальне виділення поліцукрів та білків швидко та економічно.

Keywords: Candida tropicalis, заморожування, кандидоз, білки, поліцукри, моноцукри, антиген, вакцина.

Отримано редколегією 28.10.2022 р.