



BIODIVERSITY EVALUATION OF *GEOTRICHUM CANDIDUM* LINK. IS ARTHROSPORIC NUCLEUS NUMBER IN *GEOTRICHUM CANDIDUM* RELATED TO THE FUNGUS BIODIVERSITY?*

M. Koňuchová, V. Lehotová, A. Šípková, E. Piecková, Ľ. Valík

Slovak University of Technology in Bratislava, Faculty of Chemical and Food Technology, Bratislava, Slovak Republic

Geotrichum candidum species exhibits properties of both moulds and yeasts and its affiliation to one of the groups has been intensively discussed. It is because this filamentous microscopic fungus is displaying substantial morphological variability and wide phenotypic diversity. The present study assesses the variability of arthrosporic nucleus number of twelve isolates of *G. candidum* originating from artisanal manufacturing and ripened traditional Slovak cheeses. Results showed that arthrospores of the studied isolates contained on average 1.5 ± 0.7 (on the Gorodkova medium) and 1.5 ± 0.6 (on the McClary medium) Hoechst 33258-stained nuclei (range 1–4 nuclei on both agars) after a 7-day cultivation at 25°C. Majority of arthrospores comprised one nucleus, irrespective to the used nutrient-limited medium. Generally, a higher relative nucleus number per arthrospore was exhibited in yeast-like isolates with microscopic structure composed predominantly of spores, while it was lower in vegetative hyphae. These isolates originated from ewe's lump cheese. Our study reveals that the arthrosporic nucleus number of the *G. candidum* strains is closely related to morphotype and origin of this yeast.

arthrospores, nuclei per spore, food safety



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INTRODUCTION

Geotrichum candidum Link possesses several atypical characteristics that make its taxonomic classification rather complicated, keeping it evolving on. It displays wide morphological variability and large phenotypic diversity, and owns many attributes generally associated with filamentous fungi (Marcellino, Benson, 2014). The genus *Geotrichum* was described by Johann Heinrich Friedrich Link in 1809 to accommodate the species *G. candidum* found on decaying leaves. As early as 1850, this eukaryotic microorganism was isolated from milk by Fresenius (Wouters et al., 2002) and was initially classified as yeast (Kurtzman, Fell, 1998; Barnett et al., 2000), although later reclassified as yeast-like micromycete (Wouters et

al., 2002). Nowadays, *G. candidum* is accepted as the yeast with moldy tendencies by the major taxonomic monography (de Hoog et al., 2014). Over the past 15 years, advances in molecular methods have led to revision of its classification. *G. candidum* is the asexual microorganism belonging to the *Saccharomycotina* subphylum (phylum *Ascomycota*) and it is considered to be the mitotic state of *Galactomyces geotrichum* Redhead and Malloch (Marcellino, Benson, 2014).

It appears to be an intermediate between the filamentous fungus which grows in a non-fragmentary mycelal form and the yeast showing predominantly unicellular bodies. From the microscopic point of view, *Geotrichum* species form septate branching hyphae, which can be broken down into chains or

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individual arthrospores during the fragmentation process. Arthrospores are usually of cylindrical shape or may become barrel-like with flat or rounded ends (to $5\text{--}6 \times 5\text{--}17 \mu\text{m}$). The predominant branching pattern exhibited by fungal hyphae is dichotomous branching of the peripheral hyphae. At this type of branching, the main branch forks the way that one hyphae, which is a little bit thinner than the main branch, buds as the first, and after that, another branch starts to bud in the same plane as the previous one, while this one is again slightly thinner than the previous (mother) one. The main branches are $7\text{--}12 \mu\text{m}$ wide, with the lateral one of $2.5\text{--}4 \mu\text{m}$ with early disarticulation into cubic arthrospores (Kurtzman et al., 2011).

G. candidum is a yeast species naturally found in milk and dairy products such as cream, curd cheese, soft cheeses (Camembert, Pont-L'Évêque), and semi-fresh cheese, including the goat's and ewe's cheeses. In addition, *G. candidum* is desirable on the surface of smeared soft cheeses (Münster, Livarot), mould-ripened and semi-hard cheeses (St. Nectaire, Reblochon; Marcellino, Benson, 2014). Due to the production of many volatile compounds important for the flavour, such as phenyl compounds, lactones, esters, and volatile sulphur compounds, *G. candidum* is used as an adjunct culture. It grows in early stages of the ripening process (Montel et al., 2014) and stimulates the development of other microbiota in the next stages of cheese maturation (Boutrou, Guéguen, 2005). *G. candidum* is responsible for uniform white and velvety coat on the surface of many soft-ripened cheeses (Marcellino, Benson, 2014).

If *G. candidum* is present in excessive quantities on the surface of the raw soft ripened cheeses (camembert type), it is considered to be responsible for many defects such as unstable or slippery rind or unequal covering of cheese surface (Boutrou, Guéguen, 2005; Laurenčík et al., 2008). It can cause degradation of the fresh cheeses and dairy products like cottage cheese resulting also in economic losses. In addition, *G. candidum* can also contribute to degradation of milk fat and proteins that may result in unpleasant organoleptic properties and appearance of the cheese. Species of the genus *Geotrichum* cause spoilage of some cream cheeses and are able to grow on the surface of butter, cream and cream products, where are considered to be contaminants leading to off-flavours (Marcellino et al., 2001; Hudcová et al., 2011; Koňuchová et al., 2016).

G. candidum, also known as milk yeast, tolerates a wide range of environmental conditions, notably temperature and pH values. It can grow in a wide pH interval (3–11) and at temperatures of $5\text{--}38^\circ\text{C}$, with optimum around 25°C (at pH 5.0–5.5) (Boutrou, Guéguen, 2005). Generally, it is considered to be salt sensitive, however, the property is strain dependent (Hudcová et al., 2010). According to van den Tempel, Nielsen (2000), the majority of

strains tolerate 1.0–2.5% (w/v) of salt in the growing medium. But they are usually not able to grow on the medium containing above 4% of salt.

According to the study of Marcellino et al. (2001), there is a tremendous amount of biochemical and genetic diversity present within the *G. candidum* clade. This fungus is an important component of the microbiota of many cheeses, which provide an excellent source of the fungus regional diversity.

As the average number of nuclei formed in arthrospores could vary between the monitored *G. candidum* strains, the aim of this study was to perform technological characterization of twelve *G. candidum* isolates from Slovak traditional products and to assess the number of nuclei per spore cell.

MATERIAL AND METHODS

Culture conditions

Twelve studied representatives (A–L) of *G. candidum* had been isolated from Slovak ewes' lump cheese or fresh cheese using the yeast extract glucose chloramphenicol agar (YGC; Biokar Diagnostics, Beauvais, France). The obtained isolates were purified and maintained on slant skim milk agars (SMA) (Merck, Darmstadt, Germany) at $5 \pm 1^\circ\text{C}$.

Yeast identification and characterization

Three simultaneous approaches have been followed for the identification: classical morphological tests, biochemical tests according to Kurtzman et al. (2011), and molecular biological techniques. The isolates were identified based on morphological and cultural aspects (shape and cell dimensions) of each strain cultivated for 10 days on malt extract agar (MEA) at 25°C . Physiological characteristics were determined by (i) zymograms in yeast extract medium enriched with 2% of the particular sugar tested, and (ii) carbon and nitrogen auxanograms on the agar media with mineral salts and vitamins. In order to complete the taxonomic characterization, PCR identification (in cooperation with Pangallo et al., Institute of Molecular Biology, Slovak Academy of Sciences, Bratislava) was performed. Primers ITS1 and ITS4 were then used for amplification of the internal transcribed spacer (ITS) region of isolates.

Cultivation and staining of cell nuclei

To perform nuclear counting experiments, spores of *Geotrichum* isolates were collected by inoculation hook from 7-day-old cultures that were grown on plates with McClary's acetate agar and the Gorodkova agar (Kurtzman et al., 2011) at 25°C .

Table 1: Frequency of nuclear number per an arthrosporic cell of *G. candidum* isolates

Isolate	Gorodkova agar		McClary acetate agar	
	interval of nuclear Nr.	relative Nr. of nuclei	interval of nuclear Nr.	relative Nr. of nuclei
A	1-4	2.0 ±0.9	1-4	1.9 ±0.7
B	1-2	1.3 ±0.5	1-3	1.6 ±0.6
C	1-4	1.8 ±0.8	1-3	1.7 ±0.6
D	1-3	1.6 ±0.5	1-3	1.4 ±0.6
E	1-2	1.2 ±0.4	1	1.1 ±0.3
F	1-2	1.2 ±0.4	1	1.0 ±0.0
G	1-2	1.1 ±0.3	1-2	1.2 ±0.4
H	1-3	1.7 ±0.5	1-3	1.6 ±0.6
I	1-3	2.2 ±0.7	1-4	2.1 ±0.6
J	1-2	1.5 ±0.5	1-2	1.5 ±0.5
K	1-3	1.7 ±0.6	1-2	1.5 ±0.5
L	1-3	1.7 ±0.6	1-2	1.4 ±0.5

Initially, microbial cells were fixed by the addition of 70% ethanol for 1 h at room temperature. This chemical fixative creates chemical bonds between proteins to increase their rigidity. Fixed cells were removed from solution by centrifugation (Eppendorf Minispin® centrifuge, Eppendorf AG, Hamburg, Germany) at 3000 rpm for 10 min, McIlvain buffer (pH 7.4), and a stock solution of Hoechst 33258 dye (Sigma Aldrich, St. Louis, USA) (final concentration of Hoechst solution $10 \mu\text{g ml}^{-1}$) were added into the pellet. Then, stained cell suspension was incubated in the dark for 30 min. Cells of isolates were repeatedly centrifuged at 3000 rpm and pellets were re-suspended in the small volume of McIlvain buffer with optimal dye binding at pH 7.4. Stained microbial cells on coverslip were analyzed under a fluorescent microscope Imager.1A (Carl Zeiss, Jena, Germany) at a wavelength of 350 nm. Relative nucleus number per compartment of *G. candidum* (arthrospore) was calculated as the average number of nuclei per arthrospore from four microscopic fields.

RESULTS

Based on the morphological, biochemical, and molecular tests, all studied isolates were identified as *G. candidum* (data not shown). In the next step, we were interested in studying if the origin of *G. candidum* isolates affected the multinuclear character of yeast cells (Shlezinger et al., 2014).

Visualization of nuclei of the cells by fluorescent compounds provides a wide variety of information for the analysis of cell functions. Hoechst dyes interact with nucleotides to emit fluorescence and its molecules attach at the minor groove of the DNA double helix.

These fluorescent dyes are positively charged under physiological conditions and are permeable through the cell membranes of viable cells. Hoechst 33258 excites in the near UV (350 nm) and emits in the blue region (450 nm) (Merok et al., 2002; Zhou et al., 2004) (Fig. 1).

Two solid media (the Gorodkova agar with sodium chloride and the McClary agar with sodium acetate), necessary to maintain adequate sporulation, were used for our microscopic studies (Kurtzman et al., 2011). The number of nuclei per compartment varied between isolates. We observed distribution in the range of 1–4 (Table 1) with an average of 1.5 ± 0.7 (mean \pm standard deviation) nuclei per compartment after 7 days of incubation on the Gorodkova agar. The number of nuclei in the arthrospore formed on the McClary medium varied from 1 to 4 (Table 1), 1.5 ± 0.6 on average. A lower relative number of nuclei in differentiated parts of mycelium were noticed in isolates cultivated on the McClary medium (except the isolates B and G). However, in general, number of nuclei per compartment did not depend on the medium composition.

Fig. 2 and Fig. 3 show a small part of section image containing the cell nuclei. Cells were predominantly of cylindrical shapes (arthrospore), some of them clustered, containing 1–4 (with the average of

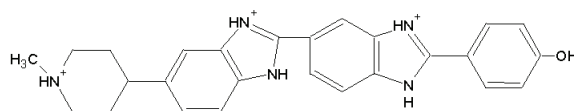


Fig. 1: Structure of the Hoechst 33258 dye

1.5 ± 0.6 on the McClary agar or 1.5 ± 0.7 on the Gorodkova agar, respectively) stained nuclei.

The nuclei of all isolates grown on the two nutrient-limited media were distributed throughout arthrospores and there was no obvious correlation to the positions of nuclei. On the Gorodkova agar containing sodium chloride, arthrospores prevalently (54%) comprised one nucleus and on the McClary medium with sodium acetate 52% of isolates were mononuclear. Only 1% of randomly analyzed arthrospores exhibited four nuclei on the both nutrient-limited agars. Based on observed results it can be concluded that medium composition had no obvious impact on multinuclear character of arthrospores.

The lowest average number of nuclei was encountered in isolates E, F, and G, regardless of medium composition. These three isolates formed white felting colonies spreading out on agar plates, with microscopic structure composed mainly of vegetative hyphae and few arthrospores. Isolates E, F, and G were isolated from fresh cheeses (cottage cheese and quark), where presence of *G. candidum* is considered as a contaminant imparting off-flavours.

At the contrary, relative nucleus number per arthrospore was the highest in the isolates A and I and ranged from 1.9 (isolate A on the McClary agar, SD ± 0.7) to 2.2 (isolate I on the Gorodkova agar, SD ± 0.7). From macroscopic point of view, these isolates exhibited cream-coloured yeast-like colonies and under microscope they predominantly produced abundant arthrospores. These isolates originated from unripened ewes' lump cheese manufactured in the laboratory (isolate A) or a farm premise in North Slovakia (isolate I).

Other isolates under study represented a borderline between two groups mentioned and their average nucleus numbers varied from 1.3 to 1.7. They could grow in the filamentous as well as yeast tiny cell mode similarly and originated from traditional dairy products, such as ewes' lump cheese or soft Slovakian traditional 'Bryndza' cheese from Central and North Slovakia (Dolná Lehota, Zázrivá, and Rojkov localities).

The results indicate that there is a possibility of grouping *G. candidum* strains into morphotype

ensembles in accordance with arthrosporic nucleus number. It is noticeable that morphotype and origin of *G. candidum* is correlated to the properties that are important in cheese industry, e.g. proteolytic, lipolytic, acidifying/alkalizing activity, and growth potential (Boutrou, Guéguen, 2005). However, these findings need to be confirmed by analyzing more isolates of various origin.

DISCUSSION

The ubiquitous *Geotrichum* species is known for its enormous biodiversity which was empirically selected through the centuries by different traditional manufacturing techniques (Marcellino et al., 2001). There are two commonly known processes, rapidly generating biodiversity that may be important in adaptation to particular environments: gene duplication and horizontal gene transfer. A genome analysis of the yeast *G. candidum* by Morel et al. (2015) reveals a novel mechanism which contributes to understanding the genetic basis of the functional diversity, specific gene retention that significantly contributes to yeast biodiversity. The relative number of nuclei is specific to certain yeasts. Nuclei of fungi are divided between compartments, each containing a number of nuclei, which can also migrate between the compartments (Shlezinger et al., 2014).

In studies from the 1960s based on microscopic methods, *G. candidum* was described as multinucleated fungus (Caldwell, Trinci, 1973). Fiddy, Trinci (1976) stained fixed cells cultivated on DM medium omitting vitamins according to Giemsa and obtained the count of nuclei in the *G. candidum* isolate FI in the range from 1 to 7. In isolates used in our work, maximally 4 nuclei per arthrospore were found, irrespective to the cultivation medium used.

The majority of arthrospores in the forementioned work cited contained two nuclei (44% out of all the studied arthrospores). We obtained comparable results, since 41% of arthrospores of the isolates cultivated on the McClary minimal agar showed two nuclei. However, in the same study only 3% of randomly

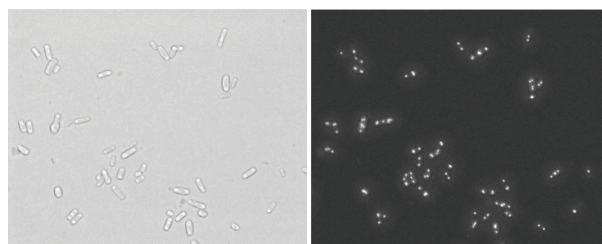


Fig. 2: Fluorescent staining of nuclear DNA in the isolate K cultivated on the McClary medium (magnification 63x10x1)

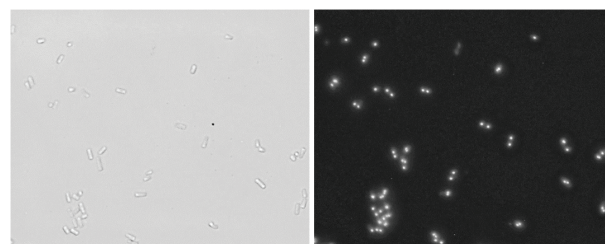


Fig. 3: Fluorescent staining of nuclear DNA in the isolate I cultivated on the Gorodkova medium (magnification 63x10x1)

analyzed arthrospores consisted of 1 nucleus. In our experiments more than a half of the arthrospores observed (52%) were mononuclear. Slight differences can be explained e.g. by different geographical origin of *G. candidum* FI strain. Thus, the results exhibit possible correlation between the average number of nuclei per an arthrosporic cell and the micromycete isolate origin.

Another studies by Kaminskyj (2000) revealed that cells of *Aspergillus nidulans hypA1* mutant, after 8.5 h of growth at 28°C, had contained 11.7 ± 3.7 Hoechst 33258-stained nuclei (range from 6 to 18) on average. The number of nuclei per compartment in filamentous fungi can grow up to hundreds (e.g. in *Neurospora crassa*). On the contrary, yeast cells of *Dipodascopsis tothii* and *D. uninucleata* are usually uninucleate (Kurtzman et al., 2011). In general, yeast cells are mostly uninucleate although binucleate, trinucleate, and multinucleate cells may occur sporadically. In accordance with our results presented, the current taxonomic classification of *G. candidum* as the yeast seems appropriate.

CONCLUSION

G. candidum is somewhat unusual yeast and its taxonomy has undergone many changes since the first description by Link in 1809. Its microscopic appearance with the both single cell stage (arthrospores) and the mycelium (septate hyphae) makes the classification uneasy. *Geotrichum* exhibits high biodiversity in different traditional cheese ripening environments that has been selected by different traditional manufacturing techniques. In order to better define diversity and variability, we studied if the average number of nuclei formed in arthrospores of twelve *G. candidum* isolates cultivated on two nutrient-limited media (Gorodkova and McClary agars) was the subject of changes.

We proved that the number of nuclei per arthrospore was isolate-dependent. Counting the relative nucleus number (stained by Hoechst 33258) per arthrospore in twelve *G. candidum* isolates showed a variety range from 1 to 4 nuclei. The obtained results suggest that the average number of nuclei per arthrospore (between 1.0 ± 0.0 and 2.2 ± 0.7) did not depend on the composition of nutrient-limited medium but was affected by some particularity of *G. candidum* isolates, e.g. their natural habitat.

Overall, a lower relative nucleus number per arthrospores (1.0–1.2) was observed during the mycelial growth of *G. candidum* isolates with typical white felt-like colonies; on a microscopic level, the vegetative hyphae dominated and sporulation was very sparse. A higher arthrosporic nucleus number (1.9–2.2) was reported in isolates which formed cream-coloured yeast-like colonies; from microscopic point of view, they produced generous arthrospores by breaking the

hyphae. Other isolates were intermediate between the mentioned two clades and their average nucleus numbers varied from 1.3 to 1.7. They could grow in the filamentous as well as yeast tiny cell mode similarly.

It can also be suggested that there might be some correlation expected between the average arthrosporic number and the morphotype of *G. candidum* strain. Consequently, clustering into various morphotype groups is important, because they are related to physiological requirements of micromycetes. In addition, grouping of *G. candidum* strains into ensembles might be significant in terms of the relationship between the arthrosporic nucleus number and pathogenicity or virulence of the strains. With respect to the findings mentioned and employment of *G. candidum* in the cheese-making industry as a complementary culture component, resulting in a loss of its biodiversity and desirable biochemical properties, the study of diversity of *G. candidum* strains is an important research task.

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Corresponding Author:

Ing. Martina Konu chová, Slovak University of Technology in Bratislava, Faculty of Chemical and Food Technology, Department of Nutrition and Food Quality Assessment, Radlinského 9,812 37 Bratislava, Slovak Republic, phone: +421 259 325 524, e-mail: martina.konuchova@stuba.sk
