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BUDAPEST

Biodiversity of food spoilage *Yarrowia* group in different kinds of food

Theses of dissertation

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1. INTRODUCTION

Yarrowia lipolytica is one of the most extensively studied yeasts, playing a prominent role in the food industry and biotechnology. Its natural occurrence, beneficial and detrimental role in different kinds of food (mainly in raw meat, raw milk and milk products) are widely reported. Based on these studies *Y. lipolytica* is usually predominant, making up 15% of the yeast isolates from yoghurt, 39% from poultry stored at 5°C, and based on a study 69% of the isolates from beef.

This species is known for its pronounced lipolytic and proteolytic activities, and pigment producing capacity. Although its contribution to spoilage is widely reported, its beneficial role in the food industry is also important. *Y. lipolytica* is capable of producing important metabolites and has an intense secretory activity, which justifies efforts to use it in industry and in studies. Lipase produced by this yeast can be exploited for several applications in the food, detergent, pharmaceutical, and environmental industries. *Y.* lipolytica is used as a production host for not only lipase, but citric acid, carotenoids, mannitol, erythritol and aroma compounds.

Using conventional methods based on phenotypic characteristics for identification, some yeasts of the *Yarrowia* clade can be misidentified as *Y. lipolytica*, which was the only known member of the clade for a long time. However, using molecular biological methods, the heterogeneity of the species was recognized. *Y. lipolytica* is a member of a species complex, which - supplemented with some closely relative species - forms the *Yarrowia* group. At the time when I began my work, *Yarrowia* group comprised of the following species: *Y. lipolytica, Y. (Candida) deformans, Y. (C.) yakushimensis, Candida galli, C. phangngensis, C. oslonensis, C. alimentaria, C. hollandica, C. hispaniensis*. Since then, partly as a result of my work, the number of the members of the *Yarrowia* group increased from 9 to 13.

It can be supposed, that some of the yeast isolates identified as *Y. lipolytica* by using conventional methods, and reported as spoilage yeasts or applied in industries, may not really be *Y. lipolytica*, but an other member of the *Yarrowia* group. Thus, *Y. lipolytica* cannot be mentioned as the only important yeast of the *Yarrowia* group, as other members can play a prominent role in the industries, regardless of being beneficial or detrimental.

2. OBJECTIVES

Objectives of my PhD work were:

- to develop an efficient method to isolate food spoilage yeasts of the *Yarrowia* group from different kinds of food (raw meat, raw milk and milk products);
- to select hexadecane-assimilating strains from the isolates;
- to separate members of the *Yarrowia* group from the hexadecane-assimilating strains by using physiological tests;
- to cluster and identify the members of the *Yarrowia* group at species level, based on their DNA sequences;
- to find out if phenotypic differentiation of the identified species is possible or not;
- to reveal the biodiversity of the yeast strains of the *Yarrowia* group identified based on their DNA sequences, and to examine their product-related occurrence;
- to answer questions and solve problems emerging during the experiments.

Other objectives emerged during the experiments:

- to enhance the efficiency of the newly carried out method;
- to test the potential spoilage causing characteristics of the members of the *Yarrowia* group;
- to test the ascosporulation of the species the teleomorphic state of which was yet unknown;
- to group and identify at species level the hexadecane-assimilating yeasts, which are not members of the *Yarrowia* group, then to recognise their diversity in the examined foods.

3. MATERIALS AND METHODS

One hundred and thirty-three food samples were used; raw meat, raw milk and milk products. Eight hundred and twenty-six yeast isolates and 56 reference strains were examined.

Isolation method

Developing an efficient isolation method was necessary. First a three-step enrichment procedure was applied in liquid Yeast Nitrogen Base medium, pH 3.6, supplemented with 0.5 % (v/v) hexadecane. The reduced pH of the medium served to inhibit bacterial growth. The rationale of using hexadecane in the enrichment medium was that only about 10% of the known yeast species are able to grow with this compound as a sole carbon source (among the species tested for this character), including members of the *Yarrowia* group. Enrichments were followed by serial decimal dilutions and surface plating on Rose-Bengal Chloramphenicol agar. After incubation, cultures representing each colony type were isolated and purified by repeated streaking.

The media were prepared with phosphate-citric acid buffer, thus some non-hexadecane-assimilating strains were also isolated. To enhance the efficiency of the method, another buffer was used for preparing enrichment media, which contained hexadecane as a sole carbon source.

Selection of strains of the Yarrowia group based on physiological tests

The ability of the isolated strains to grow on hexadecane was confirmed by inoculating strains into a medium containing 0.5% hexadecane as a sole carbon source.

Strains of the *Yarrowia* group were selected based on physiological tests. Their glucose fermentation and nitrate-assimilation were tested, and the assimilation of 30 different carbon-sources by using API ID32 C tests was examined.

Grouping of the selected strains and identification at species level

A microsatellite primer was used in PCR amplification reactions of the DNA extracted from the strains. PCR products were separated by horizontal agarose gel electrophoresis. Strains were grouped based on the comparison of the resulting DNA fragments.

D1/D2 regions of the large subunit (LSU) rRNA gene (and in some cases also ITS sequences) of a few strains from each group were amplified and sequenced. For the identification sequence similarity searches were performed against the GenBank sequence database.

Possibilities of phenotypic differentiation of the identified species

After identification, efforts were made to find out if phenotypic differentiation of the identified species of the *Yarrowia* group is possible or not.
Data were obtained from the latest monography, species descriptions and the database of the Centraalbureau voor Schimmelcultures (CBS). In case of contradictions or lacking data the particular characteristics were tested by me, then a summarizing table of these data was constructed. Using these data a taxonomic key to the species was compiled.

Testing the potential spoilage causing characteristics of the members of the Yarrowia group

Potential spoilage causing characteristics of all the strains belonging to the *Yarrowia* clade and also the strains used as reference were tested, namely their lipolytic and proteolytic activity and their ability to produce brown pigments. Pigment production was tested on a media containing tyrosine; brownish discolouration around the colonies indicated positive reaction. Lipolytic activity was tested on Gorodkowa agar supplemented with 5% olive oil; positive reaction was indicated by blue discolouration of the colonies after flooding them with saturated copper-sulphate solution. Proteolytic activity was assessed by gelatine liquefaction.

Ascosporulation

Intra-species mating ability between strains of the *Yarrowia* clade, and formation of ascospores were studied in mixtures of actively growing cultures of the investigated strains. Mixtures were subcultured on 14 different media, incubated at 15 and 25 ºC, and examined regularly under the microscope. If ascosporulation was observed in the mixture of the cultures, formation of ascospores was studied individually and in their pairwise mixtures in all possible combinations, under the same culture conditions.

4. RESULTS AND DISCUSSIONS

Eight hundred twenty-six yeast strains were isolated from 133 food samples by using the newly developed method.

Fifty-five percent of the strains isolated from raw meat by using enrichment media containing McIlvaine buffer was hexadecane positive and 41% of them were members of the *Yarrowia* group. Twenty percent of the strains from raw milk were hexadecane-positive and 6% of them were members of the *Yarrowia* group. Forty-six percent of the strains from cottage cheese were hexadecane-positive and 23% of them were members of the *Yarrowia* group. Sixty-one percent of the strains from cheese were hexadecane-positive and 19% of them were members of the *Yarrowia* group.

In case of isolates originating from meat, there was no significant difference between the efficiency of the two enrichment media. In case of strains from cottage cheese, enrichment in medium containing hexadecane as sole carbon source proved to be more efficient to isolate hexadecane-assimilating strains and to isolate members of the *Yarrowia* group, respectively.

As some non-hexadecane assimilating strains also were isolated, the newly developed methods are not fully selective, but both of them are efficient to isolate strains of the *Yarrowia* group.

Based on physiological tests, 219 strains belonging to the *Yarrowia* group were selected.

These strains were assigned to seven groups based on their fingerprints obtained from the gel electrophoretic separation of their DNA fragments amplified by using microsatellite-primed PCR. Identity of the strains could be assessed by comparing their fingerprints to the fingerprints of the type strains of the *Yarrowia* group.

The DNA sequence-based identification confirmed, that the seven groups represent seven species. Using conventional methods, most of the strains could have been identified as *Y. lipolytica*, although only 51% of them represented this species. Conventional methods would not enable to differentiate 26% of them (*Y. deformans, Yarrowia divulgata* and *Yarrowia porcina)* from each other and from *Y. lipolytica*. Four of the 7 species were already known, 3 of them proved to be novel ones.

Revealing four novel species

The PCR fingerprints of 31 strains differed from the fingerprints of the known type strains of the *Yarrowia* group. After the determination of their D1/D2 sequences it became clear, that despite the phenotypic similarity to each other and to other species of the group, they represent 4 novel species within the *Yarrowia* group. Since then three of them had been described (*Y. divulgata*, *Y. porcina* and *Y. bubula*).

One hundred thirty-one isolates from raw meat were assigned to the above mentioned 7 species. Thirty-two percentage was *Y. deformans*, 21% of them was *Y. lipolytica*, 15% of them was *C. galli*, 12% of them was *Y. bubula*, 6% of them was *C. alimentaria*, 10% of them was *Y. divulgata* and 4% of them was *Y. porcina*. Conventional methods were not able to differentiate 26% of them (*Y. deformans, Y. divulgata* and *Y. porcina*) from each other and from *Y. lipolytica*.

Despite such diversity in meat, except *Y. lipolytica*, only one *C. alimentaria* strain could have been isolated from milk and milk products.

Fifty-one percent of all the strains belonging to the *Yarrowia* clade proved to be *Y. lipolytica*, 19% *Y. deformans*, 9% *C. galli*, 7% *C. alimentaria*, 7% *Y. bubula*, 4% *Y. porcina*, 3% *Y. divulgata*.

Using the newly constructed taxonomic key, 8 of 13 species can be discriminated based on some of their physiological characteristics. Reliable identification of 5 species of the *Yarrowia* clade has to be based on molecular examinations.

Potential spoilage causing characteristics of all the strains belonging to the *Yarrowia* clade and also the strains used as reference (273 strains) were tested. Only few of them could not produce brown pigments. Except few *Y. yakushimensis* strains, most of them had lipase activity. Except the type strains of *C. phangngensis* and *C. hispaniensis*, the strains had protease activity. Based on the results it can be stated, that each tested strain of the *Yarrowia* clade owns at least one of the potential spoilage-causing characteristics, and most of them mean multiple risk to the food quality.

For a long time *Y. lipolytica* was the only species of the *Yarrowia* clade with known teleomorphic state. Recently, teleomorphic state of *C. deformans* was found, thus this species was transferred to the *Yarrowia* genus. Examining the intra-species mating ability between the strains of the *Yarrowia* clade, mating and ascosporulation had been observed in the mixture of some *Y. porcina* strains, thus this species emerged as the third teleomorphic species of the *Yarrowia* clade. *Y. porcina* is the only species of the genus with ascospores embedded in

capsular material. This feature is quite rare even within the entire yeast domain. In the culture used for ascosporulation after more than three months not only asci and ascospores could be found, but also budding of the ascospores was observed and documented, proving the viability of the ascospores.

Hexadecane assimilation is a quite rare feature, only a small proportion of yeast species is known of being capable of assimilating it. This prompted me to examine the hexadecane assimilating strains that were not assigned to the *Yarrowia* clade (175 strains) based on their physiological characteristics, and their diversity was revealed. Including the members of the *Yarrowia* group, 22 species could have been isolated from the examined foods. One of them belongs to the fourth novel species.

5. NEW SCIENTIFIC RESULTS

1. A novel, efficient isolation method was developed for the isolation of yeasts of the *Yarrowia* group.
2. Members of the *Yarrowia* group were selected from eight hundred twenty-six isolated yeast strains, then they were clustered, and identified at species level.
3. Biodiversity of the *Yarrowia* group, and other hexadecane-assimilating yeasts was revealed from the examined foods.
4. Yeast strains belonging to four novel, undescribed species were isolated. Three of them have already been described. One of them, *Y. porcina* is the third teleomorphic species of the genus.
5. A taxonomic key was constructed based on physiological tests. Eight species out of 13 can be distinguished from each other by using this key.
6. It was revealed that each tested strain of the *Yarrowia* group owns at least one of the potential spoilage-causing characteristics.

6. CONCLUSION AND RECOMMENDATION

In the last few decades due to molecular methods it was unravelled that identification of microorganisms using conventional methods is not always reliable. It became clear, that diversity of yeasts occurring in food and other habitats is underestimated. This is confirmed by the numerous species described in the past few years, and by the discovery of species occurring in such small numbers, that without enrichment could not be found. Moreover, in the past, several species were misidentified, some of them with similar phenotypic characteristics were believed to be conspecific. The newly developed method is efficient for the isolation of yeasts of the *Yarrowia* group, but it is not fully selective.

At least 7 species, which is almost three quarter of the described species of the *Yarrowia* group occurs in meat, and can be isolated by the newly developed method. From cheese only *Y. lipolytica* strains were isolated, from cottage cheese except one *C. alimentaria* strain, also only *Y. lipolytica*. From raw milk numerous *Y. lipolytica* and three *Y. bubula* strains were isolated.

Although phenotypic examinations are very important, identification based on phenotypic characteristics is not always reliable, thus molecular methods are required for the identification.

Most strains of the *Yarrowia* group can be discriminated from strains not belonging to the group by using physiological tests, but they are not always sufficient for the differentiation of the strains representing different species in the group. For that microsatellite-primed-PCR method proved to be effective, because strains of different species of the *Yarrowia* group could be successfully discriminated based on their DNA fingerprints. Based on the examinations of the strains outside the *Yarrowia* group, it can be stated, that unlike in the case of strains of the *Yarrowia* clade, in some cases conspecific strains showed different fingerprints of DNA fragments separated with gel electrophoresis. Although this method was successful in case of the strains of the *Yarrowia* clade, only cautious inferences should be drawn when studying strains showing greater diversity.

Examining the sequence of the D1/D2 region of the ribosomal RNA’s large subunit coding gene proved to be sufficient in most of the cases, but in some extraordinary cases examination of the ITS regions are also necessary for the reliable identification.

Y. lipolytica is often reported as food spoilage yeast, but in most of the cases only conventional methods were used for the identification, thus it is possible, that not only *Y. lipolytica*, but other members of the *Yarrowia* clade play a prominent role in food spoilage.

Examining the physiological characteristics of strains can contribute to the recognition of their beneficial or detrimental role in the food industry, and to the assessment of their biotechnological or industrial applicability. Molecular based verification of the identity of strains in culture collections assigned as *Y. lipolytica* is necessary.

PUBLICATIONS RELATED TO THE DISSERTATION

Original Research Papers

Published in referred (IF) journals

Nagy E. Sz. (2014): Isolation and diversity of food spoilage *Yarrowia* yeast strains from meat. *Acta alimentaria*, 43 (Suppl), 101-106. p. DOI: http://dx.doi.org/10.1556/AAlim.43.2014.Suppl.15 IF=0,274 (2014)

Nagy E., Dlauchy D., Medeiros A.O., Péter G., Rosa C.A. (2014): *Yarrowia porcina* sp. nov. and *Yarrowia bubula* f.a. sp. nov., two yeast species from meat and river sediment. *Antonie van Leeuwenhoek*, 105 (4):697-707. p. DOI: http://dx.doi.org/10.1007/s10482-014-0125-4 IF=1,806 (2014)

Nagy E., Niss M., Dlauchy D., Arneborg N., Nielsen D.S., Péter G. (2013): *Yarrowia divulgata* f.a., sp. nov., a yeast species from animal-related and marine sources. *International Journal of Systematic and Evolutional Microbiology*, 63:4818-4823. p. DOI: http://dx.doi.org/10.1099/ijs.0.057208-0 IF=2,798 (2013)

Published in other journal

Nagy E. Sz. (2014): Differentiation of food spoilage yeast strains of the *Yarrowia* group by microsatellite polymerase chain reaction fingerprinting. *Journal of Universal Sciences Online*, 1(1): 6-11. p. DOI: http://dx.doi.org/10.17202/JUSO.2014.1.6

Conference Full Paper in Hungarian

Nagy E. Sz. (2013): *Yarrowia* törzsek izolálása húsokról és rendszertani azonosításuk. II. Interdisciplinary Doctoral Conference, May 15-17, 2013, Pécs, Hungary. Conference book pp. 427-434.

International Conference Proceedings

Nagy E. Sz. (2013): Isolation, identification and diversity of food spoilage *Yarrowia* yeast strains from different foods. Food Science Conference, November 7-8, 2013, Budapest, Hungary. Abstr. pp. 53.

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Conference Proceedings In Hungarian

Nagy E. (2013): *Yarrowia* törzsek izolálása húsokról és rendszertani azonosításuk. II. Interdisciplinary Doctoral Conference, május 15-17, 2013, Pécs, Hungary. Abstr. pp. 254.

Nagy E., Péter G. (2012): Élelmiszerromlást okozó *Yarrowia lipolytica* komplex csoport törzseinek szelektív izolálása. Mikológiai Közlemények Clusiana Vol.51. No.1. V. Magyar Mikológiai Konferencia, május 24-26, 2012, Budapest, Hungary. Abstr. pp. 154.