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ISOLATION AND IDENTIFICATION OF NEW YEAST STRAINS FROM BEE BREAD

Marek Kieliszek[1]✉, Vitaliy Kolotylo[1], Anna Mikołajczuk-Szczyrba[2], Liviu Giurgiulescu[3], Anna M. Kot[1], Stanisław Kalisz[4], Katarzyna Pobiega[1], Andrzej Cendrowski[4]

[1]Institute of Food Sciences, Department of Food Biotechnology and Microbiology, Warsaw University of Life Sciences − SGGW, 159C Nowoursynowska St., 02-776 Warsaw, Poland;
[2]Prof. Wacław Dąbrowski Institute of Agricultural and Food Biotechnology, Department of Microbiology, 36 Rakowiecka St., 02-532 Warsaw, Poland;
[3]Chemistry-Biology Department, Technical University of Cluj Napoca, North University Center of Baia Mare, 26 Victoriei St., Romania
[4]Institute of Food Sciences, Department of Food Technology, Warsaw University of Life Sciences − SGGW, 159C Nowoursynowska St., 02-776 Warsaw, Poland
✉*marek_kieliszek@sggw.edu.pl*

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ABSTRACT

Bee bread is a preserved bee pollen, which is a mixture of plant pollen, nectar, secretions from the digestive system of bees, and a layer of honey. It has a very high nutritional value. Bee bread can be used as dietary supplements due to their rich protein content and the presence of essential amino acids, fatty acids, mineral salts, and vitamins. This work carried out the isolation and identification of yeast strains from bee bread. The obtained strains displayed the phenotypic characteristics of *Rhodotorula* yeast. The DNA electrophoretic analysis showed a band size of 640 bp. Sequencing analysis of the internal transcribed spacer regions of the 5.8S rRNA gene confirmed the presence of the yeast *Rhodotorula mucilaginosa* (MK1).

1. Introduction

One of the least known and studied bee products is bee bread (Khalifa *et al*., 2020). During winter and early spring, this product is the only source of food for the bee colony, following honey (Babarinde *et al*., 2018). It is formed from pollen and provides protein, lipids, vitamins, and minerals for bees. Water constitutes the largest part of the mass of bee feathers, which is present in the amount of 20–30% of the mass of the fresh product. Proteins (up to 30%) constitute the second ingredient with the highest content in the bee bread. Carbohydrates are present at an amount of 13–55% in the bee bread, with most of them being simple sugars—mainly glucose and fructose (Kieliszek *et al*., 2018). Fatty acids and vitamins are considered as other significant ingredients. The rich composition of bee bread contributes to its nutritional and health-promoting properties. The presence of various ingredients, characteristic of natural origin, indicates that bee bread can be successfully used as a supplement and a dietary component (Tomás *et al*., 2017).

The use of bee bread and its derivatives poses some health risks. The unprocessed bee bread, obtained from the natural environment, may contain microbiological contaminants (Janashia *et al*., 2018). Insects and various microorganisms are a threat to beekeeping related to the acquisition and processing of bee bugs. The very transformation of pollen into

bee bread occurs under the influence of biochemical and metabolic processes carried out by bacteria and yeast in the product. Lactic acid bacteria *Lactobacillus* and *Bifidobacterium* are the dominant microflora in bee bread, while yeasts (*Candida*, *Saccharomyces*, *Cryptococcus*, *Zygosaccharomyces*) and molds (including *Penicillium*, *Rhizopus*, *Trichothecium*) (Gilliam *et al*., 1989; Nogueira *et al*., 2012) constitute the second significant group of microorganisms. The results of various studies suggest that the microorganisms may be derived partly from pollen and partly from the bees (Gilliam, 1997; de Arruda *et al*., 2017).

This study aimed to identify and characterize new yeast strains isolated from bee bread using classical microbiology and molecular methods such as polymerase chain reaction (PCR).

2. Materials and methods

2.1. Materials

2.1.1. Bee bread

A natural product originating from bees—bee bread—was used as the biological material. The study used bee brood obtained from Lubelskie Voivodeship (Poland). Bee bread was crushed under sterile conditions from honeycombs (Fig. 1).

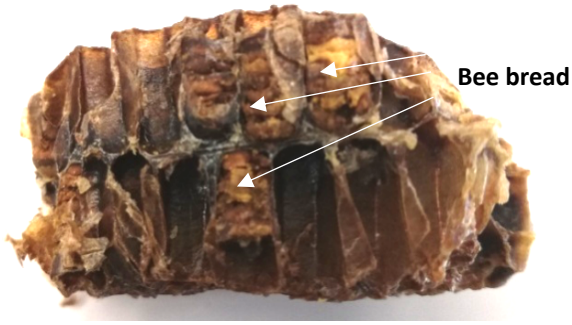

Figure 1. Bee bread in honeycomb.

2.2. Methods

2.2.1. Culture Conditions

YPD (Yeast Extract–Peptone–Dextrose) liquid medium was used to multiply yeasts from the biological material (bee bread) and isolate them. After adding 3 grains of bee bread to the liquid medium, the mixture was homogenized by vortexing (1 min). Multiplication of the yeast microflora was allowed to occur at 28°C for 48 h. Approximately 30 inoculations were performed.

After 48 h, a reduction inoculation of the liquid medium was performed on solid YPD (Petri dishes). Cultures were developed at 28°C for 48 h, and those representing each type of colony were isolated and purified by repeated streaking on YPD agar. The obtained pure yeast colonies were used as the basic material for further studies and stored at 4°C on YPD slants with 2% agar.

2.2.2. Microscopic observations

The isolated yeast was observed under the OPTA-VIEW optical microscope with Vision 7 software (Poland).

2.2.3. DNA isolation from yeast cells

DNA was isolated from yeast cells according to a modified method of Bzducha-Wróbel *et al*. (2013). Briefly, liquid yeast cultures were centrifuged (10 min, 5000 rpm), then washed twice in sterile water, and centrifuged again. The obtained pellet was suspended in 0.1 mL of lysis buffer (200 mM Tris–HCl, pH 7.5; 0.5% SDS; 30 mM EDTA) and incubated at 70°C for 15 min.

Furthermore, 0.1 mL of a 2.5 M potassium acetate solution was added and incubated at –20°C for 20 min. Subsequently, 0.3 mL of phenol:chloroform:isoamyl alcohol mixture (25:24:1) was added and vortexed for 1 min, and the sample was centrifuged once again (10 min, 13,000 rpm).

The top layer was gently pipetted from the sample and transferred to new Eppendorf tubes. 2-Propanol was added to the mixture and centrifuged (20 min; 13,000 rpm). Subsequently, the supernatant was decanted, and the remaining pellet was rinsed with 70%

ethanol and centrifuged again (20 min, 13,000 rpm). After centrifugation, ethanol was decanted, and the isolated DNA was suspended in 0.03 mL of sterile nuclease-free water. The genetic material was stored at 4°C for further analysis.

2.2.4. Yeast identification using API tests

The precise identification system API 20 C AUX (bioMérieux, France) was used for the identification of the obtained yeast strains, in accordance with the manufacturer's instructions.

2.2.5. DNA amplification and agarose gel electrophoresis

Specific primers ITS1 (5′-CGGGATCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-CGGGATCCTCCGCTTATTGATATG-3′) (a concentration of 20 pmol each) were used in the PCR process to amplify internally transcribed gene regions (ITS) and 5.8S rDNA. $MgCl_2$ (1.5 mM/L), 0.25 mmol/L dNTP, 0.5 U Taq polymerase (Fermentas, Lithuania) were added to the template DNA at a concentration of 300 ng/μL (3 μL).

The amplification reaction was carried out in a Mastercycler gradient thermocycler (Eppendorf, Germany) according to the following program: predenaturation at 94°C for 120 s, followed by 34 cycles: at 94°C for 30 s, at 53°C for 30 s, at 72°C for 60 s, and at 72°C for 30 s.

The obtained PCR products were separated by using 1.5% agarose gel added with Midori Green DNA Stain reagent (2 μL) in 1× TAE buffer (40 mM Tris–HCl, 20 mM acetic acid, and 1 mM disodium edetate). The GeneRuler 1 kb DNA Ladder molecular weight marker (ready-to-use; Thermo Fisher Scientific, USA) was used to determine the size of the resulting bands.

The parameters of the electrophoresis process were 70 mA and 120 V. During visualization, Quantity One 4.2.1 program (Bio-Rad, Poland) was used to read the molecular masses of the amplified DNA.

2.2.6. Sequencing of genetic material

The PCR products were sequenced at Genomed S.A. (Warsaw, Poland) by enzymatic synthesis (Sanger's dideoxy). The yeasts were identified by sequence comparison with the Genbank® genetic sequence database, NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi), using the BLAST sequence analysis tool.

3. Results and discussion

3.1. Macro- and microscopic evaluation of yeast isolated from bee bread

It is highly necessary to conduct a microbiological analysis on bee products in order to ensure consumer safety and for the development of new technologies. Numerous scientific publications (DeGrandi-Hoffman *et al*., 2013; Kaplan *et al*., 2016; Kieliszek *et al*., 2018) confirm the variability of the contaminating microflora depending on the origin of the bee bread (botanical and geographical).

The present study found only one strain of yeast belonging to the genus *Rhodotorula*. A uniform growth pattern of both yeast strains was noted on the YPG solid medium after 2 days of cultivation at 28°C. The colonies exhibited pink-salmon color with a dull surface and a compact structure (Fig. 2A). The yeast cells were spherical and ovoid in shape with a vacuole located at the center of the cell. *Rhodotorula* MK1 yeasts had an average length of 3.50 ± 0.11 μm and a width of 3.07 ± 0.09 μm (Fig. 2B).

Owing to the diversity and proven ability to produce potential metabolites, *Rhodotorula* yeast is used for producing many commercially bioactive compounds and natural pigments, such as carotenoids (Kot *et al*., 2016). The identified *Rhodotorula* strains were found to belong to red yeast as they were characterized by a pink-colored colony. A typical feature of this type of microorganism is the production of carotenoids. These metabolites are naturally occurring lipid-soluble pigments (Ram *et al*., 2020) and can exhibit yellow, orange, and red colors (Yoo *et al*., 2016).

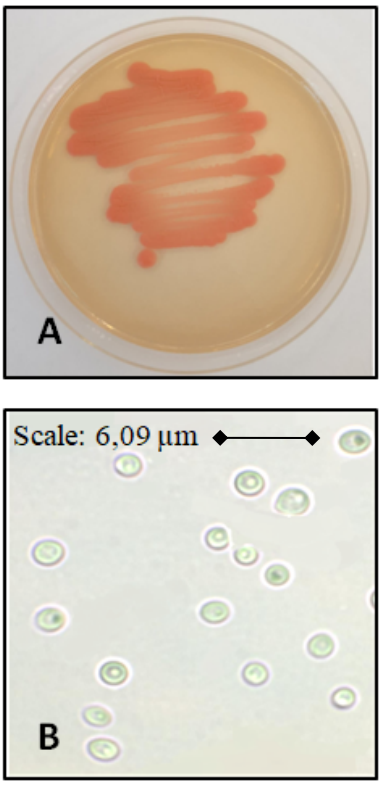

Figure 2. Yeast biomass on YPD agar medium (**A**); Exemplary microscopic photographs of *Rhodotorula* (**B**).

Since the beginning of the 21st century, the diversity of microorganisms (including yeasts) in bees and their food sources has been increasingly studied. The work of Thai scientists (Saksinchai *et al*., 2012) confirmed the validity of yeast studies in bee products. The authors managed to isolate as many as 186 yeast strains. Based on the morphological and physiological characteristics, 55 representative strains were identified (including *Zygosaccharomyces siamensis*).

Bee bread is a source of various species of yeast used in the production of mead. The study conducted by Silva *et al.* (2020) showed that bee products can contain the microorganisms used for the fermentation of alcoholic beverages. The following yeast species were isolated during the research: *Papiliotrema flavescens*, *Rhodotorula mucilaginosa*, *Saccharomyces cerevisiae*, and *Starmerella meliponinorum*. Similar dependencies were reported by Čadež *et al*. (2015). The authors isolated five new strains of *Zygosaccharomyces* from Hungarian bee bread. It is noteworthy that the obtained microorganisms cannot grow on ordinary yeast nutrients; however, they develop well on media with a high content of sugar. Research by Brysch-Herzberg *et al*. (2019) showed the presence of *Schizosaccharomyces* strains of osmophilic yeast in bee bread (originating from Germany).

3.2. Strain identification (API 20C)

After conducting the API 20 C AUX test, the obtained results allow the conclusion that the first identified strain was R. mucilaginosa MK1. The tested strain was able to metabolize i.a. glucose, arabinose, sucrose, galactose (Table 1).

It is noteworthy that *Rhodotorula* yeast is widely used in industries owing to its potential to produce several interesting compounds such as carotenoids and lipids in significant amounts (Kot *et al*., 2019). From their research, Prabhu *et al*. (2019) found that the strain of *R. mucilaginosa* belongs to the class of oily microorganisms, because, when grown in a bioreactor, it accumulates lipids up to 25% of dry substance.

The outstanding biotechnological properties of the strain *R. mucilaginosa* are widely mentioned in the scientific literature. The yeast species *R. mucilaginosa* is considered as a valuable source of carotenoids and has a potential commercial value. However, the low carotenoid production limits its commercial use (Wang *et al*., 2017).

Further research aimed at determining the biochemical properties of the isolated strain of bee yeast is required. In this study, molecular identification of the isolated yeast species was performed for a more detailed analysis.

Table 1. Biochemical results for the yeast strain.

Sugars	*Rhodotorula* MK1
Glucose	+
Glycerol	–
Calcium 2-keto-gluconate	–
Arabinose	+
Xylose	+
Adonitol	+
Xylitol	+
Galactose	+
Inositol	–
Sorbitol	+
Methyl-αD-glucopyranoside	–
N-acetyl-glucosamine	–
Cellobiose	–
Lactose	–
Maltose	+
Saccharose	+
Trehalose	+
Melezitose	+
Rafinose	+

3.3. Molecular identification of yeast

The DNA amplification products with primers ITS1 and ITS4 were analyzed to confirm that a given strain of yeast isolated from bee belongs to the *Rhodotorula* genus. The PCR products separated by agarose gel electrophoresis are shown in Fig. 3.

A band with a mass of about 640 bp was obtained from the analyses. By comparing the results with the current scientific literature, it can be concluded that the mass of the PCR product bands is characteristic of the species *R. mucilaginosa*. Esteve-Zarzoso *et al.* (1999) determined the band weight of various yeast strains of *Rhodotorula* genus: *R. acuta*, 675 bp; *R. minute*, 660 bp; and *R. mucilaginosa* 640 bp. The obtained yeast strain was deposited in the Culture Collection of the Department of Food Biotechnology and Microbiology of the Warsaw University of Life Sciences (SGGW) as MK1. In addition, the information of the obtained yeast was documented in a file with GenBank (NCBI) under number LC527461.1. BLAST analysis showed 100% identity, including the following sequences: MT465994.1, MK805510.1, and MT328138.1.

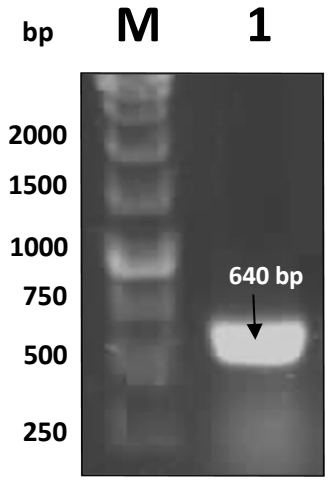

Figure 3. PCR gel electrophoresis of yeast from the bee bread (M—marker, 1—*Rhodotorula* MK1).

Of note, various scientific publications state that *R. mucilaginosa* yeast was isolated not only from fermented milk but also from various extreme ecosystems, for example, sea and glacial shores, products of plant origin, and industrial areas polluted with hydrocarbons (Deligios *et al.*, 2015). Observations by Tartor *et al.* (2018) confirmed our results showing that the DNA amplification product of the yeast *R. mucilaginosa* isolated from diseased freshwater fish was approximately 640 bp.

In conclusion, the yeasts isolated from different natural environments exhibit outstanding biochemical features that can be applied in various branches of the biotechnology industry.

4. Conclusions

The results confirm the need for research on the microflora contaminating bees. In our attempts to determine the presence of yeast in bee bread, we isolated the strain *Rhodotorula* MK1. In the future, particular attention will be paid to the determination of new strains of

microorganisms in other bee products (e.g., propolis) and their biochemical characteristics.

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