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Identification of *Zygosaccharomyces* yeasts isolated from honeybee environment

Identifikace kvasinek rodu *Zygosaccharomyces* izolovaných z prostředí včely medonosné

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ABSTRACT

While the bee microbiome has been relatively well studied at the bacterial level, information about the bee fungal communities is still sparse and does not reflect their importance. From the bee larvae of honey bee (*Apis mellifera*), bee bread and a mix of corbicular pollen, twenty yeast isolates belonging to the genus *Zygosaccharomyces* were obtained. Based on the D1/D2 region sequencing, the yeast isolates were identified as *Z. rouxii* (larvae), *Z. favi* (bee bread) and *Z. mellis* (mix of corbicular pollen). For the specific PCR-based detection of the most abundant yeast *Z. rouxii*, two species-specific primer pairs targeting the ITS region were designed. Because yeasts and their metabolites can play an important role in bee development, further investigation was focused on the production of ergosterol, an ecdysone precursor. HPLC detection of ergosterols was used and it has been found that ergosterol levels are highly variable across the monitored species and isolates and do not correlate with biomass production. The highest production of ergosterol in the *Z. rouxii* isolate was 6.2 mg/g dry biomass, in *Z. mellis* 2.3 mg/g dry biomass.

Keywords: *Apis mellifera*, bee-associated microorganisms, molecular identification, ergosterol

ABSTRAKT

Bakteriální část včelího mikrobiomu již byla relativně značně prostudována, ovšem informace týkající se společenství hub doprovázejících včely jsou náhodné a neodrážejí jejich důležitost. Z larev včely medonosné (*Apis mellifera*), plástového pylu a směsi rouskového pylu bylo získáno dvacet izolátů patřících do rodu *Zygosaccharomyces*.Na základě sekvenování oblastí D1/D2 byly izoláty identifikovány jako *Z. rouxii* (larvy), *Z. favi* (plástový pyl) a *Z. mellis* (směs rouskového pylu). Pro specifickou PCR detekci nejpočetnějšího druhu kvasinky *Z. rouxii* byly navrženy dva druhově-specifické primery zaměřující se na ITS oblasti. Protože kvasinky a jejich metabolity mohou hrát významnou roli ve vývoji včel, další zaměření bylo na produkci ergosterolu, prekursoru hormonu ekdyson. Byla použita HPLC detekce ergosterolu, která prokázala vysokou variabilitu obsahu ergosterolu mezi jednotlivými druhy a izoláty a zároveň obsah ergosterolu nekoreloval s produkcí biomasy. Nejvyšší produkce biomasy byla u izolátu *Z. rouxii* (6,2 mg/g sušiny), v případě *Z. mellis* pak 2,3 mg/g sušiny.

Klíčová slova: *Apis mellifera*, včelí mikroorganismy, molekulární identifikace, ergosterol

INTRODUCTION

Honey bees (*Apis mellifera*) are an important part of the biosphere, and as pollinators, they are involved in the pollination of many wild plants and agriculturally important crops. However, in recent years, a significant decrease in honey bee colonies has been reported. Mainly due to increased pressure of stressors, a number of diseases and pesticides (Gill et al., 2012; McMenamin et al., 2018; Hristov et al., 2020). Recent studies highlight the great importance of bee-associated microorganisms found in the hive environment (Saccà and Manici, 2021; Vocadlova et al., 2023). The examination of bee microbiomes may therefore offer an interesting view of bee immunity (Raymann and Moran, 2018).

Compared to the large number of bacteria, the intestinal microflora of bees contains less than 1% of yeasts and fungi from the whole microbiome (Tauber et al., 2019). Not only are those present in the microbiome, but yeasts and fungi are also present in the bee bread (Detry et al., 2020). While the microbiome of bees has been relatively well studied at the bacterial level, information on the occurrence of fungi and yeasts in hives is not very common and relates practically only to floristic data. Information on the role of these microorganisms in the development and functioning of the bee colony is not yet available. According to current research, fungi are important, especially in the early stages of bee development (Menezes et al., 2015). They are an important source of ergosterol for larvae, which they cannot produce on their own (Paludo et al., 2018). Paludo et al. (2018) in a study on a larval model of the stingless bee *Scaptotrigona depilis* demonstrated that ergosterol is an initial component in the production of ecdysteroids, which are key hormones that control the transformation of immature stages of bees to adults.

The spectrum of ecdysteroids varies in different insect species, depending on the sterol substrate and the enzymatic apparatus (Lavrynenko et al., 2015). While the metabolic pathway of C_{27} 20E, one of the most important ecdysteroids, begins with cholesterol (Huang et al., 2008), honey bees cannot use this biosynthetic pathway because they are unable to produce cholesterol (Clark and Bloch, 1959). Honey bees use the main hormone C_{28} ecdysteroid MaA, makisterone A, which is synthesized from the plant steroid campesterol (Feldlaufer et al., 1985).

The dependence of *S. depilis* larvae on the supply of sterols by the yeast *Zygosaccharomyces* shows the importance of the link between the bees and associated microbiota. Yeasts from the genus *Zygosaccharomyces* have been previously isolated from other bees, mainly bumblebees (Dharampal et al., 2020), honey bees (Yun et al., 2018) and other stingless bees (Rosa and Lachance, 2005), but their role has not yet been explained and clarified. Thus, other similar cases of symbiosis between fungi and other bees are likely to be discovered (Paludo et al., 2018).

This study was focused on yeasts of the genus *Zygosaccharomyces* associated with honey bees, *Apis mellifera*. The aim of this study was the isolation and molecular characterization of *Zygosaccharomyces* yeast and the determination of ergosterol production in the yeast isolates. A better understanding of the importance of yeasts in the honey bee development can help to design an optimal diet, enhancing the fitness, performance and productivity of the bee colony.

MATERIAL AND METHODS

Yeast isolation

Larvae, bee bread and mix of corbicular pollen were aseptically collected in Kamenný Malíkov, Czech Republic, during 2019. Samples were homogenized in saline solution with 1% Tween 80 (Sigma-Aldrich, Missouri, USA) and aseptically plated on a selective medium for high osmophilic yeasts (glucose agar with 30% (30G) and 50% (50G) of glucose). Medium consisted of (g/l): glucose (300/500), yeast extract (30), malt extract (30), agar (20), pH 6.0. Cultures representing each colony type were isolated and purified by repeated streaking on 30G media. The plates were incubated at 28 °C for 7 days. As a reference strain, *Saccharomyces cerevisiae* CCDM 275 (CCDM Laktoflora®, Czech Republic) was used.

Molecular characterization

DNA was extracted from isolated yeasts based on the protocol published by Harju et al. (2004) and quantified using BioSpec-nano (Shimadzu, Nakagyo-ku, Kyoto, Japan). Samples were stored in -20 °C until use. For the molecular characterization, the ITS region was to be used, but due to difficulties discussed further, the D1/D2 domain was used instead. The ITS regions of some *Z. rouxii* isolates were amplified by using primers ITS1 (5´-TCCGTAGGTGAACCTGCGG) and ITS4 (5´-TCCTCCGCTTATTGATATGC) (White et al. 1990). The D1/D2 domains of all isolates were amplified by using primers NL-1 (5´-GCATATCAATAAGCGGAGGAAAAG) and NL-4 (5´-GGTCCGTGTTTCAAGACGG) (Kurtzman and Robnett,1997. Amplicons were sequenced by a commercial sequencing facility, SEQme, Czech Republic (https://www.seqme.eu).

The PCR amplification was performed identically for both reactions in the total volume of 20 µl consisted of: 10 µl Gotaq® Green Master Mix (Promega, Madison, WI, USA), 6 µl PCR-grade Water, 1 µl primers (10 pmol/µl) each and 2 µl DNA (50 ng/µl). The temperature profile of the PCR reaction for ITS region amplification was as follows: the initial denaturation for 2 min at 95 °C, 30 cycles: 45 s at 95 °C, 45 s at 53 °C and 1 min at 72 °C and termination with a final extension step of 7 min at 72 °C. The PCR reaction for D1/D2 domain amplification was as follows: the initial denaturation of 3 min at 94 °C, 30 cycles: 1 min at 94 °C, 1 min at 58.5 °C and 1 min at 72 °C and termination with a final extension step of 5 min at 72 °C. The PCR products were visualized by 1% agarose gel electrophoresis and stained with ethidium bromide solution (Merck Life Science, Darmstadt, Germany). Prior to sequencing, PCR products were purified using ExoSAP-IT™ (Applied Biosystems, supplied by ThermoFisher Scientific, Loughborough, UK) according to the manufacturer's instructions.

The obtained sequencing records were processed in the software Geneious 8.1.9. (https://www.geneious.com) and then compared to the GenBank sequence database using the BLAST® database search program (Zhang et al., 2000). Sequencing the ITS region in selected samples resulted in unreadable chromatograms, and it was not possible to accurately identify the yeast species based on its DNA sequence. For this reason, the ITS PCR fragments were cloned into the pCR-XL-2-TOPO™ vector (Invitrogen, supplied by ThermoFisher Scientific, Loughborough, UK) according to the manufacturer's instructions. Plasmid DNA was isolated and purified for subsequent sequencing by using Roti®-Prep Plasmid Mini (Carl Roth GmbH, Karlsruhe, Germany). The plasmids were sequenced by a commercial sequencing facility SEQme, Czech Republic (https://www.seqme.eu) using universal sequencing primers M13F (5´-TGTAAAACGACGGCCAGT) and M13R (5´CAGGAAACAGCTATGACC). The obtained sequencing records were processed as previously described. Based on the alignment of four sequences of the cloned ITS region, which was created in Geneious 8.1.9. (https://www.geneious.com), new primers in this region were designed in Primer3 (Koressaar et al., 2018). Their specificity was verified with Primer-BLAST (Ye et al., 2012) and in PCR experiments. PCR experiments were first run with all twenty isolates and then with reference strains of *Z. rouxii* CCDM 276, *Z. bailii* CCDM 2041, *S. cerevisiae* CCDM 275 and *S. cerevisiae* CCDM 2001.

Content of ergosterol

To determine the concentration of ergosterol in the isolates, all samples cultivated in 30G media were first pre-screened (data are not part of the publication). Then, based on the production of this metabolite, two samples with the highest production were selected. The production of ergosterol in these isolates was measured in duplicates and compared in two different media – 30G and YEPD (30G medium consisted of (g/l): glucose (300), yeast extract (30) and malt extract (30), pH 6; YEPD medium consisted of (g/l): peptone (20), yeast extract (10), dextrose (20), pH 6.5. The comparison of ergosterol production was also performed with the reference strain CCDM 275 (*Saccharomyces cerevisiae*).

The obtained isolates of *Zygosaccharomyces* and the reference *Saccharomyces* strain CCDM 275 were cultivated in submersion conditions. The cultivation medium was prepared as follows: 50 ml of 30G/YEPD medium in a 250 ml Erlenmeyer flask was inoculated with a small amount of cells from one-week-old cultures and incubated at 30 °C, 180 rpm for 48 hours. After two days, the medium was centrifuged (12,000 g) and the supernatant was discarded. The sediment was dried out at 105 °C for 6 hours and weighed. The dried biomass was extracted with 10 ml of methanol, sonicated (S70 H Elmasonic, Elma, Germany) for 20 minutes, and left overnight at room temperature. Prior to analysis, the sample was filtered through a 45 µm filter.

The content of ergosterol was measured by HPLC (Shimadzu, Nakagyo-ku, Kyoto, Japan) using C18, 250 x 4.6 mm, 5 µm column at 210 nm with UV-VIS detector. The mobile phase was a) H_3PO_4, pH 3 with b) methanol in ratio a:b = 1:99, isocratic, flow 1ml/min and injection volume 10 µl. As standard solutions, ergosterol and cholesterol (Sigma-Aldrich, Missouri, USA) were prepared. The standard solutions were measured under the same conditions. Run time of the analysis was 20 minutes with retention time (RT) of ergosterol 11.8 minutes and RT for cholesterol 14.2 minutes.

The ergosterol concentration in the samples was evaluated according to the calibration curve in the LabSolutions program (Shimadzu, Nakagyo-ku, Kyoto, Japan). The presence of cholesterol was, due to the nature of the sample, not expected.

RESULTS AND DISCUSSION

Isolation and molecular characterization

A total of twenty isolates were obtained by culturing samples of honey bee larvae, bee bread and a mix of corbicular pollen on the high glucose media. Medium 50G was used in the initial step as selective media for the isolation of osmophilic microorganisms. The medium was used as an alternative to the concentration of glucose in honey, which is on average around 30 % (Bogdanov et al., 2008), and to "simulate" the osmotic pressure in cultivation medium, as *Z. rouxii* and *Z. mellis* belong to the group of osmophilic yeasts (Munitis et al., 1976; Čadež et al., 2015). However, the yeast grew very slowly under these highly selective conditions and formed very small colonies. Therefore, for further passage and cultivation, the 30G medium, which is more in line with the glucose concentration of honey, was used.

For precise identification, the isolates obtained were subjected to molecular analysis. For these purposes, amplification and sequencing of the ITS region of ribosomal DNA is very often used (Pincus et al., 2007; Walters et al., 2015), but due to difficulties discussed further, the D1/D2 domain was used instead. These domains are also often used for the identification of microorganisms and can overcome the bias caused by insufficient polymorphism of ITS regions (Caporaso et al., 2012; Gilbert et al., 2014; Abellan-Schneader et al., 2021). The list of isolated samples, as well as their characteristics, is shown in Table 1.

ITS region and D1/D2 domain sequencing

Based on the sequencing of the D1/D2 domain, it was possible to determine all yeast isolates to the species level. The similarity of *Z. rouxii* and *Z. favi* isolates with GenBank reached 100 %, and the similarity of *Z. mellis* reached 99.82 %. Sequences were grouped according to the species: *Z. rouxii* (17 isolates from bee larvae), *Z. favi* (two isolates from bee bread) and *Z. mellis* (one isolate from a mix of corbicular pollen).

The D1/D2 domain has been used for yeast characterization long before the concept of DNA barcoding (Scorzetti et al., 2002; Hebert et al., 2003). Although the D1/D2 domain is sufficient for the yeast characterization on species level sufficient as suggested by Kurtzman and Robnett (1998) and Fell et al. (2000), they also recommended using more markers to get better results, especially for closely related species. Nevertheless, some studies on *Zygosaccharomyces* use a combination of the ITS region and D1/D2 domain (Detry et al., 2020), some of them use either the D1/D2 domain (Liu et al., 2016) or the ITS fragment (Yun et al., 2018) alone.

Table 1. Identification of the yeast isolates based on the D1/D2 domain of 26S rDNA

No.	Source	Identity (%)	Species based on the sequence	GenBank ID
1-17	Larvae	100	*Z. rouxii*	MK341570.1
18-19	Bee bread	100	*Z. favi*	KJ825951.1
20	The mix of corbicular pollen	99.82	*Z. mellis*	KC692234.1

The ITS region is the official barcode for fungal identification (Snosch et al., 2012). However, in this study, characterization based on the ITS region alone would not be sufficient. The obtained sequences of the ITS region did not reach an adequate quality for characterization, even after purification of the yeast culture and modification of the PCR cycle. The reason for the ITS characterization failure was dual and multiple peaks on the chromatograms (Figure 1), probably due to the presence of different ITS copies as described earlier for *Zygosaccharomyces* species by Solieri et al. (2007), Saksinchai et al. (2012) and Čadež et al. (2015).

Egli and Henick-Kling (2001) proposed cloning prior to sequencing as the only solution to this problem. The mentioned solution was applied by Čadež et al. (2015) and was also used in this study. To test the method, ITS amplicons were cloned from two samples (No. 1 and No. 2) into the pCR-XL-2-TOPO™ vector. As a result, high-quality full-length sequences were obtained (Figure 1). Alignment of these samples' sequences served as a template for designing new primers to facilitate the characterization of the isolates as described earlier by Maleita et al. (2021), who followed a similar procedure.

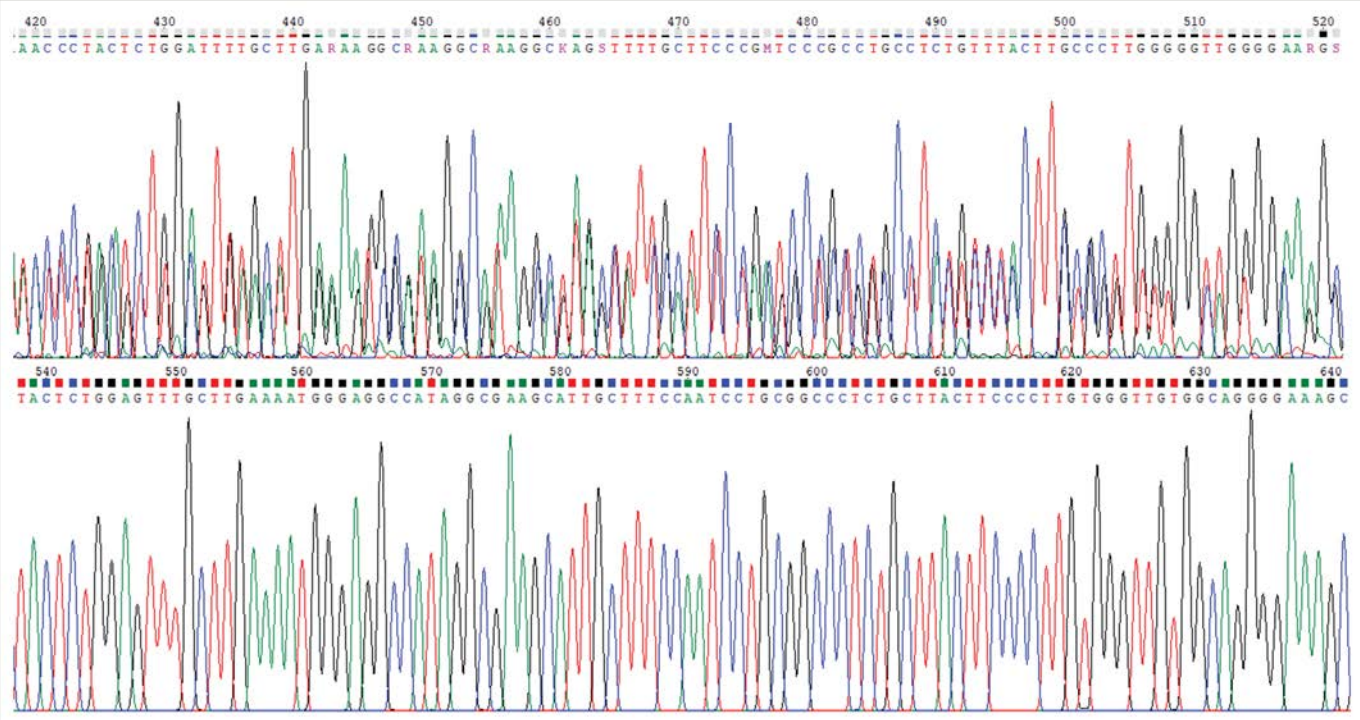

Figure 1. Comparison of the obtained sequences ITS1 primer sequencing (top) and M13F primer sequencing (bottom) after molecular cloning

Primers design

Based on the sequence analysis and the consensus of four plasmid DNA records with the cloned ITS region of *Z. rouxii*, two primer pairs were designed. Their names, orientations, sequences, and annealing temperatures (TA) used for the PCR amplification are listed in Table 2.

The PCR amplification was performed similarly to the one for ITS and D1/D2 domain with different annealing temperatures (mentioned in Table 2). Both primer pairs ZrxA and ZrxB specifically amplified only *Z. rouxii* samples (Figure 2). Amplicon sequencing was performed with selected samples to verify the specificity of the primers, and all *Z. rouxii* samples were identified in accordance with the D1/D2 domain sequencing results.

Confirmation of primer specificity was also successful with reference strains (Figure 3), these making designed primers optimal for the specific determination of *Z. rouxii*.

Ergosterol production by Zygosaccharomyces isolates

The concentration of ergosterol in yeasts was measured using HPLC. No cholesterol concentration was measured, which, as expected, is consistent with bees not being able to produce cholesterol (Clark and Bloch, 1959).

Preliminary screening revealed the highest ergosterol concentration in *Z. mellis* and one isolate of *Z. rouxii*. The production of these species was further measured with the reference *Saccharomyces cerevisiae* strain CCDM 275 in a comparative measurement (Table 3). *Z. favi* was not growing in submersion under the same conditions as *Z. mellis* and *Z. rouxii*, and no data are mentioned below.

Cultivation of *Zygosaccharomyces* isolates confirmed that biomass production in 30G medium was higher than in YEPD medium, and the increase in biomass was also matched by higher ergosterol production.

Table 2. Sequence of new primers

Primer name	Orientation	Sequence 5´-3´	TA [°C]
ZRxA_F	Forward	GCAAGGCCTGCGCTTAATTG	59.5
ZRxA_R	Reverse	CCTCTTCACTTTCGCCGAGT	59.5
ZRxB_F	Forward	CGCATCGATGAAGAACGCAG	58.5
ZRxB_R	Reverse	CCTCTTCACTTTCGCCGAGT	58.5

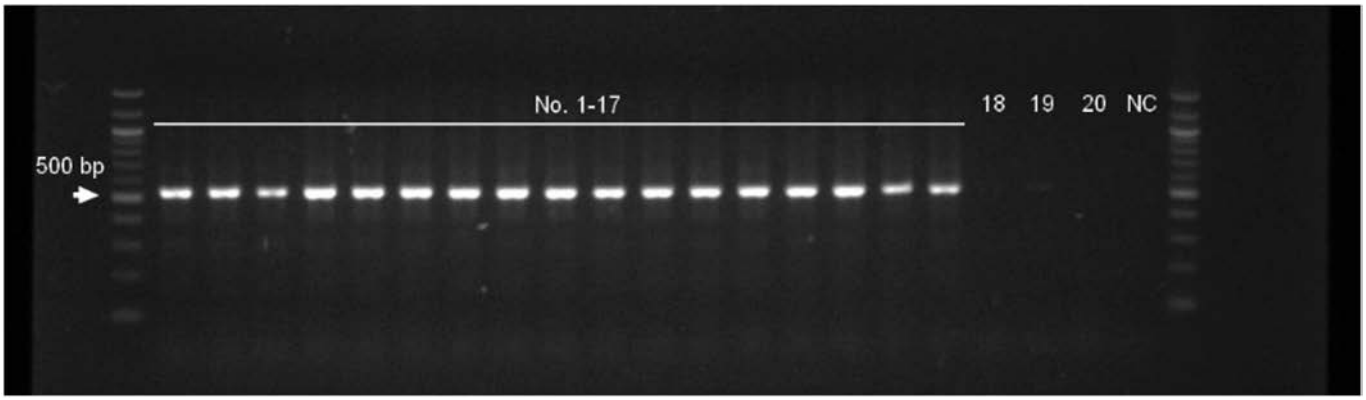

Figure 2. Illustrative output of the PCR amplification with primers ZRxA (approximately 500 bp amplicons). Positive results were shown with samples No. 1-17 (*Z. rouxii*), no results obtained with No. 18-19 (*Z. favi*) and No. 20 (*Z. mellis*) samples. NC – negative control

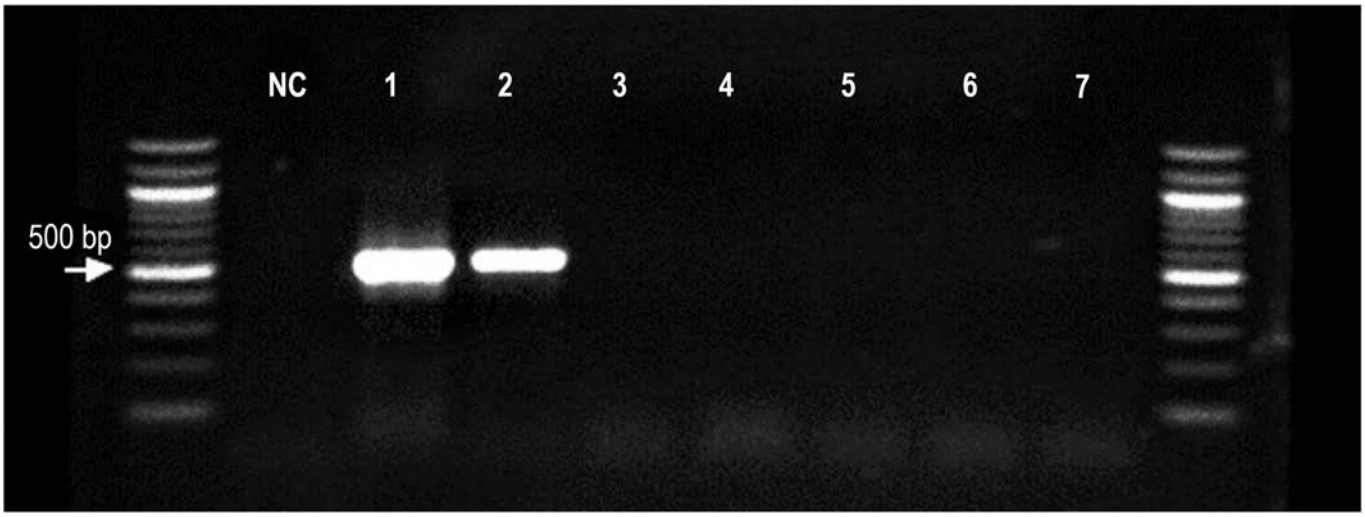

Figure 3. Illustrative output of the PCR amplification with primers ZRxA (approximately 500 bp amplicons). NC – negative control; 1 - *Z. rouxii* No. 7; 2 - *Z. rouxii* CCDM 276; 3 - *Z. mellis* No. 20; 4 - *Z. bailii* CCDM 2041; 5 - *Z. favi* No. 18; 6 - *S. cerevisiae* CCDM 275; 7 - *S. cerevisiae* CCDM 2001

This is evident from the comparison of the obtained results – the ergosterol content in YEPD medium was quite the same for both species (2.1 mg/g in case of *Z. mellis* and 1.8 mg/g in case of *Z. rouxii*), as well as the biomass production (5.2 mg/ml in case of *Z. mellis* and 3.4 mg/ml in case of *Z. rouxii*). Different behaviour of *Zygosaccharomyces* isolates was observed on 30G medium. Compared to YEPD medium, the biomass production on the 30G medium was significantly increased (biomass production in *Z. rouxii* increased to 13 mg/ml and in *Z. mellis* it reached 14.3 mg/ml), comparable for both species, and the two species were fundamentally different in ergosterol production (6.2 mg/g in dry biomass of *Z. rouxii* (nearly 3x) and 2.3 mg/g of *Z. mellis* (nearly 1.5x). Since 30G medium simulates the natural food source for osmophilic yeasts, it can be assumed that similar growth intensities and sterol production will occur in their natural environment of the beehive. Production of sterols (as an ergosterol) by two strains of *Z. rouxii* was analyzed in studies of Ushio et al. (1991). The level of ergosterol in the wild strain was 2.9 mg/ g of dry biomass and 4 mg/g of dry biomass in the mutant strain. This is comparable with the obtained results, although the high glucose medium reached a concentration reached 6.2 mg/g of dry biomass in analyzed isolate *Z. rouxii*.

Table 3. Production of dry biomass and ergosterol in different media

Isolate	Medium	Dry biomass [mg/ml]	Ergosterol in dry biomass [mg/g]
Z. rouxii No. 2	YEPD	5.2	2.1
	30G	13.0	6.2
Z. mellis No. 20	YEPD	3.4	1.8
	30G	14.3	2.3
S. cerevisiae CCDM 275	YEPD	3.2	0
	30G	no growth	-

The comparative measurement consisted of comparing selected isolates for ergosterol production in different cultivation media. However, culturing in 30G medium was not optimal for *S. cerevisiae*, resulting in complete suppression of growth. Glucose rich 30G medium is suitable for cultivation of osmophilic yeasts and not for *S. cerevisiae*. Biomass yields comparable to the species of the genus *Zygosaccharomyces* were obtained in YEPD medium (as a complex rich medium for yeast cultivation), but without the detected production of ergosterol. The absence of ergosterol was unexpected and could be caused by the way of extraction (methanol extraction without modification to alkaline or acidic pH). About 2 % of ergosterol from *S. cerevisiae* dry biomass was reported by Náhlík et al. (2017). Although they used UV mutated *S. cerevisiae* and performed alkaline hydrolysis of cells. Production of about 0,1 % reported by Tan et al. (2003), who also performed alkaline hydrolysis.

Paludo et al. (2018) reported that ergosterol is an important source of sterols for *S. depilis*, and such ergosterol comes from *Zygosaccharomyces* sp. cells growing as a pseudomycelium inside the brood cells. They also described the possibility of bee-*Zygosaccharomyces* symbiosis existence. Furthermore, *Zygosaccharomyces* sp. are also found inside the honey bee worker and queen gut as an abundant part of the microbiota (Yun et al., 2018). Since those species were found in honey bee larvae and also in bee bread and mix of corbicular pollen is therefore expected, and as was also described by Hosono (1992), it will produce ergosterol. The variability in the production of obtained isolates of *Z. rouxii* may be associated with its susceptibility to different stressors, such as glucose and NaCl, due to its osmotolerant and halotolerant nature. Song et al. (2022) mention that these factors highlight the functional differences in ergosterol under sugar stress.

CONCLUSIONS

Twenty yeast isolates from different honey bee environments were identified. The most frequently isolated species was *Zygosaccharomyces* rouxii from larvae, while *Z. mellis* and *Z. favi* were predominantly found in bee bread and mixed corbicular pollen, respectively. Because of its importance, ergosterol production of *Z. rouxii* and *Z. mellis* was evaluated in different culture media. It is evident that the ergosterol production is dependent on the genotype of the producer. Similarly, the culture medium significantly affects the production of ergosterol. Among the tested media, YEPD provided comparable results of both biomass and ergosterol production. Notably, *Z. rouxii* exhibited nearly a threefold increase in ergosterol production when cultured in 30G medium, while *Z. mellis* showed a mild increase under the same conditions. Considering that these media simulate aspects of the beehive, it can be assumed that the yeast-derived ergosterol might be produced and furthermore utilized by the honey bee brood under natural conditions. This study is a first step in a better understanding of the importance of ergosterol-producing *Zygosaccharomyces* associated with honey bees. Further research will focus on elucidating the potential utilization of yeast-derived ergosterol by developing honey bee larvae.

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REFERENCES

Abellan-Schneyder, I., Matchado, M.S., Reitmeier, S., Sommer, A., Sewald, Z., Baumbach, J., List, M., Neuhaus, K. (2021) Primer, pipelines, parameters: issues in 16S rRNA gene sequencing. mSphere, 6, e01202-20.
DOI: https://doi.org/10.1128/msphere.01202-20

Bogdanov, S., Jurendic, T., Sieber, R., Gallman, P. (2008) Honey for Nutrition and Health: A Review. Journal of the American College of Nutrition, 27, 677-689.
DOI: https://dx.doi.org/10.1080/07315724.2008.10719745

Čadež, N., Fülöp, L., Dlauchy, D., Péter, G. (2015) *Zygosaccharomyces favi* sp. nov., an obligate osmophilic yeast species from bee bread and honey. Antonie Van Leeuwenhoek, 107, 645-654.
DOI: https://doi.org/10.1007/s10482-014-0359-1

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Huntley, J., Fierer, N., Owens, S.M., Betley, J., Fraser, L., Bauer, M., Gormley, N., Gilbert, J.A., Smith, G., Knight, R. (2012) Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. ISME Journal, 6, 1621-1624.
DOI: https://doi.org/10.1038/ismej.2012.8

Clark, A.J., Block, K. (1959) The absence of sterol synthesis in insects. Journal of Biological Chemistry, 234, 2578-2582.
DOI: https://doi.org/10.1016/S0021-9258(18)69741-8

Detry, R., Simon-Delso, N., Bruneau, E., Daniel, H.M. (2020) Specialisation of Yeast Genera in Different Phases of Bee Bread Maturation. Microorganisms, 8, 1789. DOI: https://doi.org/10.3390/microorganisms8111789

Dharampal, P.S., Diaz-Garcia, L., Haase, M.A.B., Zalapa, J., Currie, C.R., Hittinger, C.T., Steffan, S.A. (2020) Microbial Diversity Associated with the Pollen Stores of Captive-Bred Bumble Bee Colonies. Insects, 11, 250. DOI: https://dx.doi.org/10.3390/insects11040250

Egli, C.M., Henick-Kling, T. (2001) Identification of *Brettanomyces/Dekkera* Species Based on Polymorphism in the rRNA Internal Transcribed Spacer Region. American Journal of Enology and Viticulture, 52, 241-247. DOI: https://dx.doi.org/10.5344/ajev.2001.52.3.241

Feldlaufer, M.F., Herbert, E.W., Svoboda, J.A., Thompson, M.J., Lusby, W.R. (1985) Makisterone A: The major ecdysteroid from the pupa of the honey bee, *Apis mellifera*. Insect Biochemistry, 15, 597-600. DOI: https://doi.org/10.1016/0020-1790(85)90120-9

Fell, J.W., Boekhout, T., Fonseca, A., Scorzetti, G., Statzell-Tallman, A. (2000) Biodiversity and systematics of basidiomycetous yeasts as determined by large-subunit rDNA D1/D2 domain sequence analysis. International Journal of Systematic and Evolutionary Microbiology, 50, 1351-1371. DOI: https://doi.org/10.1099/00207713-50-3-1351

Gilbert, J.A., Jansson, J.K., Knight, R. (2014) The Earth Microbiome project: successes and aspirations. BMC Biology 12, 69. DOI: https://doi.org/10.1186/s12915-014-0069-1

Gill, O., Ramos-Rodriguez, O., Raine, N. (2012) Combined pesticide exposure severely affects individual- and colony-level traits in bees. Nature, 491, 105-108. DOI: https://dx.doi.org/10.1038%2Fnature11585

Harju, S., Fedosyuk, H., Peterson, K.R. (2004) Rapid isolation of yeast genomic DNA: Bust n´ Grab. BMC Biotechnology, 4, 8. DOI: https://doi.org/10.1186/1472-6750-4-8

Hebert, P.D., Cywinska, A., Ball, S.L., deWaard, J.R. (2003) Biological identifications through DNA barcodes. Proceedings of the Royal Society London B, 270, 313-321. DOI: https://doi.org/10.1098/rspb.2002.2218

Hosono, K. (1992) Effect of salt stress on lipid composition and membrane fluidity of the salt-tolerant yeast *Zygosaccharomyces rouxii*. Journal of General Microbiology, 138, 91-96. DOI: https://doi.org/10.1099/00221287-138-1-91

Hristov, P., Shumkova, R., Palova, N., Neov, B. (2020) Factors Associated with Honey Bee Colony Losses: A Mini-Review. Veterinary Sciences 7 (4), 166. DOI: https://doi.org/10.3390/vetsci7040166

Huang, X., Warren, J.T., Gilbert, L.I. (2008) New players in the regulation of ecdysone biosynthesis. Journal of Genetics and Genomics, 35 (1), 1-10. DOI: https://doi.org/10.1016/s1673-8527(08)60001-6

Koressaar, T., Lepamets, M., Kaplinski, L., Raime, K., Andreson, R., Remm, M. (2018) Primer3_masker: integrating masking of template sequence with primer design software. Bioinformatics, 34, 1937-1938. DOI: https://doi.org/10.1093/bioinformatics/bty036

Kurtzman, C.P., Robnett, C.J. (1997) Identification of clinically important ascomycetous yeasts based on nucleotide divergence in the 5′ end of the large-subunit (26S) ribosomal DNA gene. Journal of Clinical Microbiology, 35 (5), 1216-1223. DOI: https://doi.org/10.1128/jcm.35.5.1216-1223.1997

Kurtzman, C.P., Robnett, C.J. (1998) Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. Antonie Van Leeuwenhoek, 73 (4), 331-71. DOI: https://doi.org/10.1023/a:1001761008817

Lavrynenko, O., Rodenfels, J., Carvalho, M., Dye, N.A., Lafont, R., Eaton, S., Shevchenko, A. (2015) The ecdysteroidome of *Drosophila*: influence of diet and development. Development, 142 (21), 3758-3768. DOI: https://doi.org/10.1242/dev.124982

Liu, G., Tao, C., Zhu, B., Bai, W., Zhang, L., Wang, Z., Liang, X. (2016) Identification of *Zygosaccharomyces mellis* strains in stored honey and their stress tolerance. Food Science of Biotechnology, 25, 1645-1650. DOI: https://doi.org/10.1007/s10068-016-0253-x

Maleita, C., Cardoso, J.M.S., Rusinque, L., Esteves, I., Abrantes, I. (2021) Species-Specific Molecular Detection of the Root Knot Nematode *Meloidogyne luci*. Biology, 10 (8), 775. DOI: https://doi.org/10.3390/biology10080775

McMenamin, A.J., Daughenbaugh, K.F., Parekh, F., Pizzorno, M.C., Flenniken, M.L. (2018) Honey Bee and Bumble Bee Antiviral Defense. Viruses, 10 (8), 395. DOI: https://doi.org/10.3390/v10080395

Menezes, C., Vollet-Neto, A., Marsaioli, A.J., Zampieri, D., Fontoura, I.C., Luchessi A.D., Imperatriz-Fonseca V.L. (2015) A Brazilian social bee must cultivate fungus to survive. Current Biology, 25 (21), 2851-2855. DOI: https://doi.org/10.1016/j.cub.2015.09.028

Munitis, M.T., Cabrera, E., Rodriguez-Navaro, A. (1976) An Obligate Osmophilic Yeast from Honey. Applied and Environmental Microbiology, 32 (1), 320-323. DOI: https://doi.org/10.1128/aem.32.3.320-323.1976

Náhlík, J., Hrnčiřík, P., Mareš, J., Rychtera, M., Kent, C.A. (2017) Towards the design of an optimal strategy for the production of ergosterol from *Saccharomyces cerevisiae* yeasts. Biotechnology Progress, 33 (1), 838-848. DOI: https://doi.org/10.1002/btpr.2436

Paludo, C.R., Menezes, C., Silva-Junior, E.A., Vollet-Neto, A., Andrade-Dominguez, A., Pishchcany, G., Khadempour, L., Nascimento, F. S., Currie, C.R., Kolter, R., Clardy, J., Pupo, M. T. (2018) Stingless Bee Larvae Require Fungal Steroid to Pupate. Scientific Reports, 8, 1122. DOI: https://doi.org/10.1038/s41598-018-19583-9

Pincus, D.H., Orenga, S., Chatellier, S. (2007) Yeast identification — past, present, and future methods. Medical Mycology, 45 (2), 97-121. DOI: https://doi.org/10.1080/13693780601059936

Raymann, K., Moran, N.A. (2018) The role of the gut microbiome in health and disease of adult honey bee workers. Current Opinion in Insect Science, 26, 97-104. DOI: https://doi.org/10.1016/j.cois.2018.02.012

Rosa, C.A., Lachance, M.A. (2005) *Zygosaccharomyces machadoi* sp. n., a yeast species isolated from a nest of the stingless bee *Tetragonisca angustula*. Lundiana International Journal of Biodiversity, 6, 27-29. DOI: http://dx.doi.org/10.35699/2675-5327.2005.22112

Saccà, M.L., Manici, L.M. (2021) Honey bee-associated bacteria as producers of bioactive compounds for protecting hives. A biosynthetic gene-based approach. Microbiological Research, 252. DOI: https://doi.org/10.1016/j.micres.2021.126860

Saksinchai, S., Suzuki, M., Chantawannakul, P., Moriya, O., Lumyong, S. (2012) A novel ascosporogenous yeast species, *Zygosaccharomyces siamensis*, and the sugar tolerant yeasts associated with raw honey collected in Thailand. Fungal Diversity, 52, 123-139. DOI: https://doi.org/10.1007/s13225-011-0115-z

Scorzetti, G., Fell, J.W., Fonseca, A., Statzell-Tallman, A. (2002) Systematics of basidiomycetous yeasts: a comparison of large subunit D1/D2 and internal transcribed spacer rDNA regions. FEMS Yeast Research, 2 (4), 495-517. DOI: https://doi.org/10.1016/S1567-1356(02)00128-9

Solieri, L., Cassanelli, S., Giudici, P. (2007) A new putative *Zygosaccharomyces* yeast species isolated from traditional balsamic vinegar. Yeast, 24 (5), 403-417. DOI: https://doi.org/10.1002/yea.1471

Song, N., Xia, H., Yang, Q., Zhang, X., Yao, L., Yang, S., Chen, X., Dai., J. (2022) Differential analysis of ergosterol function in response to high salt and sugar stress in *Zygosaccharomyces* rouxii. FEMS Yeast Research, 22 (1). DOI: https://doi.org/10.1093/femsyr/foac040

Tan, T., Zhang, M., Gao, H. (2003) Ergosterol production by fed-batch fermentation of *Saccharomyces cerevisiae*. Enzyme and Microbial Technology, 33 (4), 366-370. DOI: https://doi.org/10.1016/S0141-0229(03)00132-7

Tauber, J.P., Nguyen, V., Lopez, D., Evans, J.D. (2019) Effects of a Resident Yeast from the Honeybee Gut on Immunity, Microbiota, and *Nosema* Disease. Insects 10 (9), 296. DOI: https://doi.org/10.3390/insects10090296

Ushio, K., Ohtsuka, H., Nakata, Y. (1991) Lipid composition of an obligate osmophilic mutant in *Zygosaccharomyces rouxii*. Journal of Fermentation and Bioengineering, 72 (3), 210-213. DOI: https://doi.org/10.1016/0922-338X(91)90219-7

Vocadlova, K., Lüddecke, T., Patras, M.A., Marner, M., Hartwig, Ch., Benes, K., Matha, V., Mraz, P., Schäberle, T.F., Vilcinskas, A. (2023) Extracts of *Talaromyces purpureogenus* Strains from *Apis mellifera* Bee Bread Inhibit the Growth of *Paenibacillus* spp. *In Vitro*. Microorganisms, 11 (8), 2067. DOI: https://doi.org/10.3390/microorganisms11082067

Walters, W., Hyde, E.R., Berg-Lyons, D., Ackermann, G., Humphrey, G., Parada, A., Gilbert, J.A., Jansson, J.K., Caporaso, J.G., Fuhrman, J.A., Apprill, A., Knight, R. (2015) Improved Bacterial 16S rRNA Gene (V4 and V4-5) and Fungal Internal Transcribed Spacer Marker Gene Primers for Microbial Community Surveys. mSystems, 1 (1), e00009-15. DOI: https://doi.org/10.1128/mSystems.00009-15

White, T.J., Bruns, T.D., Lee, S.B., Taylor, J.W. (1990) Amplification and direct sequencing of fungal ribosomal RNA Genes for phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.H. (eds.), PCR Protocols: A Guide to Methods and Applications, Academic Press, Inc., New York, pp. 315-322.

Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., Madden, T. (2012) Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. BMC Bioinformatics, 13, 134. DOI: https://doi.org/10.1186/1471-2105-13-134

Yun, J.H., Jung, M.J., Kim, P.S., Bae, J.W. (2018) Social status shapes the bacterial and fungal gut communities of the honey bee. Scientific Reports, 8, 2019. DOI: https://doi.org/10.1038/s41598-018-19860-7

Zhang, Z., Schwartz, S., Wagner, L., Miller, W. (2000) A greedy algorithm for aligning DNA sequences. Journal of Computational Biology, 7 (1-2), 203-214. DOI: https://doi.org/10.1089/10665270050081478