Original Paper

The effect of sugar concentration on growth of yeast associated to floral nectar and honey

Efecto de la concentración de azúcar en el crecimiento de las levaduras asociadas con el nectar floral y la miel

César Canché-Collí [1], Felipe Barahona [1], Luis A. Medina-Medina [2], Azucena Canto [1]

[1] Centro de Investigación Científica de Yucatán A.C. Calle 43 número 130, Col. Chuburna de Hidalgo, C.P. 97205, Mérida, Yucatán, México.
[2] Facultad de Medicina Veterinaria y Zootecnia, Universidad Autónoma de Yucatán. Km. 15.5 carretera Mérida-Xmatkuil, Apartado Postal Itzimna, C.P. 97100, Mérida, Yucatán, México.

RESUMEN

Antecedentes: El gradiente de concentración de azúcares entre el néctar floral y la miel varía de baja a alta. Esto determina el crecimiento de levaduras y puede conducir a su especialización de su nicho ecológico.

Objetivos: Evaluar el efecto de un gradiente de concentración de glucosa en el crecimiento y tamaño celular de levaduras del néctar floral y miel de *Melipona beecheii*.

Métodos: Cepas identificadas como *Metschnikowia koreensis* y *Sympodiomycopsis paphiopedili*, aisladas del néctar floral, y *Starmerella apicola* y *Starmerella apicola* 2, aisladas de miel de *Melipona beecheii*, se cultivaron en medios artificiales con un gradiente de 2, 10, 20, 40 y 60 % de glucosa. Se evaluó la densidad de células, la velocidad de crecimiento y tamaño celular.

Resultados y Conclusiones: Las cepas de la miel tuvieron un crecimiento alto en mayores concentraciones de glucosa, mientras que las cepas del néctar floral crecieron mejor a concentraciones bajas. El tamaño celular disminuyó a medida que aumentó la concentración de glucosa en todas las cepas. Los datos respaldan la hipótesis del gradiente de concentración de azúcares como un filtro ecológico que modifica el crecimiento y morfología de levaduras asociadas a flores y miel.

Palabras clave: abejas, tamaño celular, filtro ambiental, *Melipona beecheii*, cepa de miel, cepa de néctar, levaduras

ABSTRACT

Background: The sugar concentration gradient of the nectar-honey continuum varies from low in nectar to high concentration in honey. Variation in sugar concentration is a gradient determining yeast growth and leading to its ecological niche specialization.

Objective: Evaluate the effect of a sugar concentration gradient on the growth kinetics and cell size of yeasts isolated from the floral nectar and honey of *Melipona beecheii*.

Methods: Four strains identified as *Metschnikowia koreensis* and *Sympodiomycopsis paphiopedili*, isolated from floral nectar, and *Starmerella apicola* and *Starmerella apicola* 2, isolated from the honey of *Melipona beecheii* were grown in artificial media with a gradient of 2, 10, 20, 40, and 60 % glucose. We evaluated cell density, growth, and cell size in each strain.

Results and Conclusions: Strains isolated from honey had high growth rates at the highest glucose concentrations, while strains isolated from floral nectar grew best at low concentrations. In addition, cell size decreased as glucose concentration increased in all yeasts. Thus, the data support the hypothesis that sugar concentration gradient is an ecological filter that modifies the growth and morphology of yeasts associated with flowers and honey and leads to niche specialization in yeasts that colonize plant-bee environments.

Keywords: bees, cell size, environmental filter, *Melipona beecheii*, honey strain, nectar strain, yeasts

CORRESPONDING AUTHOR
✉ Azucena Canto, azucanto@cicy.mx
ORCID: 0000-0002-0712-2241

INTRODUCTION

A group of microorganisms, mainly yeasts and bacteria, tends to colonize bee environments such as the floral nectar and honey. They have specialized in exploiting environments rich in sugars, deficient in other nutrients such as lipids, proteins, vitamins, and amino acids, and extreme in their conditions of high or low water availability (Nicolson *et al.* 2007; Herrera *et al.* 2009; Álvarez-Pérez and Herrera 2012; Pozo *et al.* 2012). The yeasts that inhabit floral nectar interact with various floral visitors, mainly insects, which disperse them among flowers and into other sugar-rich environments that may drastically differ in terms of chemistry and nutrients (Lachance *et al.* 2001; Canto *et al.* 2008; Starmer and Lachance 2011; Pozo *et al.* 2015; Madden *et al.* 2018). The specific associations established by the yeasts with their vectors, such as bees, enable them to disperse and colonize environments beyond floral nectar. Yeasts proliferate in environments with contrasting conditions with the floral nectar, including for example bee gut, honey, and bee bread in the bee nests (Hajsig 1958; Gilliam 1997; Rosa *et al.* 2003; Teixeira *et al.* 2003; Brysch-Herzberg 2004). This implies, for example, that yeasts should move through environments with differing sugar concentrations. When moving from flowers to bee nests, yeasts must cope with environments of increasing sugar concentrations (Spencer and Spencer 1997; Belisle *et al.* 2012; Lievens *et al.* 2015; Pozo *et al.* 2015; Madden *et al.* 2018). Sugar concentration in the floral nectar of tropical plants can range from about 1 to 50 % (Nepi *et al.* 2009; Canto *et al.* 2017), but in honey stored in bee nests, it can vary from 60 to 85 % (Snowdon and Cliver 1992; De-Melo *et al.* 2018). This sugar gradient is salient because, at medium to high sugar concentrations, water availability for metabolic processes (quantitatively expressed as water activity, a_w) declines (Grant 2004; Buzzini *et al.* 2018). Environments such as honey have lower water activity (a_w = 0.56-0.62 according to Pascual-Maté *et al.* 2018), than floral nectar (a_w = 0.93-0.99 according to Lievens *et al.* 2015), which can be a limiting factor for yeast growth.

For yeasts to move and survive in environments such as floral nectar and honey is a challenge because they must face an environmental gradient determined by water activity and osmotic pressure. These variables determine yeast growth kinetics in any habitats to which bees transport them (Spencer and Spencer 1997; Pozo

et al. 2015). In their movement and dispersal between flowers and nests, yeasts move from an environment that provides an osmotic pressure conducive to growth, to one that imposes a high osmotic pressure on cells. High osmotic pressure is one of the main limitations on cell growth and size in general (Stevenson *et al.* 2015). A gradient of increasing sugar concentration in the medium causes a gradual rise in osmotic pressure, affecting yeast cell growth as well as cell size since water loss due to osmosis reduces cell volume and the amount of water available for cellular processes (Rose 1975; Mager and Siderius 2002; Grant 2004; Raspor and Zupan 2006; Lievens *et al.* 2015; Stevenson *et al.* 2015). For example, yeast strains of *Saccharomyces cerevisiae* and *Zygosaccharomyces rouxii* exhibit decreased cellular density and biomass as well as slower growth rates when medium sugar concentrations gradually increase (e.g., 40 to 60 %) (Membré *et al.* 1999; Arroyo-López *et al.* 2009). In yeast strains isolated from flower and bee environments, the effect of the increasing sugar gradient on the kinetic growth and cell size is currently unexplored. This phenomenon is important because the sugar concentration gradient can determine growth kinetics and the ability of a yeast strain to exploit new ecological niches and acts as a selective force to yeast specialization in extreme sugary environments (Herrera *et al.* 2012; Chappell and Fukami 2018). The objective of the study was to quantify growth kinetics of four yeast strains isolated from floral nectar and honey of *Melipona beecheii*, in response to increments in the sugar concentration of the growth medium, similar to the concentration gradient that is experienced by yeasts when transported from floral nectar to honey stored in bee nests. The increase in sugar concentration causes declines in yeast cell growth and size. We hypothesize that the yeast strains isolated from the floral nectar will grow better in low or medium concentrations of sugar in the growth medium; however, yeast strains isolated from honey will grow better at high concentrations of sugar in the medium.

MATERIAL AND METHODS

Strains
We studied four strains of yeasts, two of them were isolated from the floral nectar of *Tecoma stans* (L.) Juss. ex Kunth (Bignoniaceae) and *Distimake aegyptius* (L.) Simões and Staples (Convolvulaceae), and

the other two, from the honey stored in nests of the native stingless bee *Melipona beecheii* (Apidae: Meliponini) isolated in two previous works (Lizama 2011; Canto and Herrera 2012). All strains were deposited in the microbial collection of the Centro de Investigación Científica de Yucatán, A.C. (CICY), and preserved in sterile distilled water at 10 °C until the experiments were carried out. Taxonomical identity of the strains was *Metschnikowia koreensis*, strain CICY-RN-035 and *Sympodiomycopsis paphiopedili*, strain CICY-RN-209, both isolated from the floral nectar; *Starmerella apicola*, strain CICY-RN-364, and *Starmerella apicola* 2, strain CICY-RN- 354, isolated from the honey. The criteria to select the strains were their frequency in nectar, or honey samples, percentage of identical sites (nucleotides; approximately 99 %), and coverage sequence (approximately 99 %) observed among strains and reported in two previous studies (Lizama 2011; Canto and Herrera 2012). The criteria denote the association of the strains with their source (nectar and honey). Appendix is shown the nucleotide sequences for the D1-D2 region of the large subunit ribosomal DNA and GenBank accessions of the tested strains.

Inoculum, cell count, and viability

Prior to the experiment, the strains were reactivated by incubation in cell culture dishes containing YPD media agar (yeast extract peptone dextrose) with (w/w) 2 % glucose, 1 % peptone, 0.5 % yeast extract, and 2 % agar (Sigma Aldrich). Glucose was the carbohydrate source used in the culture medium (Rodrigues *et al.* 2006). The sown yeasts incubated for 5-12 days at 24 °C until colony growth was observed. Then, all colonies on each plate were suspended in 3 mL sterile distilled water and diluted again in sterile distilled water (1:100) and finally diluted in methylene blue solution (1:200) to identify living (colorless) and dead (blue) cells (Kwolek-Mirek and Zadrag-Tecza 2014). One aliquot (10 µL) of each cell suspension was placed in an improved Neubauer chamber (Marienfeld®, 0·1 mm depth), and examined under a microscope to record living cell count, dead cell count and total cell count in ten quadrants (0.04 mm^3). These cell counts were used to estimate two parameters: a) culture density per microliter of growth medium (equation 1, Canto *et al.* 2017) and, b) the viability of the cells in suspension (equation 2, Kwolek-Mirek and Zadrag-Tecza, 2014):

Cell density = "total cell count x dilution factor x 0.025

(volume of the chamber)" $\qquad$ (1)

Cell viability = "total live cell count" / "total dead cell count" x 100 $\qquad$ (2)

A final solution with a culture density of 9 x 104 living cells / µL was obtained for each strain. This solution was the initial culture density for the calibration curves and the inoculum for growth experiments.

Calibration curves

For estimation of culture density, growth curves for each strain were generated by correlating the absorbance values with culture density values produced by direct microscopic counts (Valero *et al.* 2006). Inocula (culture density of 9 x 104 living cells/ µL) of strains were cultured in triplicate in 100 mL YPD broth containing (w/w) 2 % glucose, 1 % peptone (Sigma Aldrich) and, 0.5 % yeast extract. Strains were incubated in an orbital agitator (Zhicheng® ZHWY-200B) at 150 rpm and 24 °C and yeast cells counted in triplicate (10 µL aliquots) with a microscope (Olympus® CX31) and improved Neubauer chamber (Marienfeld®, 0·1 mm depth) at a 10-40x magnification. Absorbance was measured in triplicate for accuracy (3 mL aliquots) with a spectrophotometer (Jenway 6405 UV-Vis) calibrated to 590 nm. The total experiment duration was 76 h, and absorbance measurements were taken every four hours. Time intervals were used in each species to provide an accurate characterization of the three growth phases: 1) latency, when cells have adapted to initial environmental and nutritional conditions; 2) exponential, when cells have adapted to the medium and attain maximum multiplication rate; and 3) stationary, during which multiplication rate becomes fairly similar to mortality rate. Calibration curves were fit using regression models to produce an equation that best described growth. Absorbance was the response variable and culture density was the causal variable. Statistical analyses were run with the SPSS ver. 17 software. Finally, the variable of absorbance was used to estimate cell growth in experiments with different glucose concentrations.

Culture cell density

Culture density (cells / µL) in each strain at different glucose concentrations was assessed in triplicate using YPD broth (w/w) containing one of the glucose concentration tested (2, 10, 20, 40 or 60 %) plus 1 % peptone, and 0.5 % yeast extract (Sigma Aldrich). Each re-

plicate corresponded to an Erlenmeyer flask (250 mL) containing 100 mL YPD medium and inoculum, which incubated at 150 rev min^{-1} and 24 °C. The sugar concentration of 2 %, used in conventional microorganism growth trials, was designated as the low concentration treatment in this study. The moderate concentrations were 10 and 20 % glucose, commonly found in floral nectar, and the high concentrations were 40 and 60 %, frequently observed in the honey (Souza *et al.* 2006; Da Silva *et al.* 2016; De-Melo *et al.* 2018). We also registered the time in hours to which each strain achieved every growth phase.

Given that the culture medium turbidity affects absorbance measurement, adjustments were applied to absorbance measurements by using the averaged value of absorbance of another two replicates of not inoculated YPD broth under the same experimental conditions set for the strains. Each replicate for the absorbance adjustment consisted of an Erlenmeyer flask (250 mL) containing 100 mL of culture medium but no added yeast. Additionally, we used a cuvette that contained water as blank (absorbance value with no medium) before measure absorbance in samples for each medium type, using a spectrophotometer (Jenway® 6405 UV-Vis) calibrated to 590 nm. Cell density in samples for each combination of strain and sugar concentration treatment, were calculated with the equation derived from calibration curves.

Growth parameters

For each strain, we estimated and compared two growth parameters, the rate of the maximum specific growth (μmax), which is the maximum multiplication rate attained by microbial cells (Salvesen and Vadstein 2000; Maier 2009), and the duplication time (t_d), which is the time in hours required by microbial cells to duplicate themselves and produce a new generation (Sprouffske and Wagner, 2016; Giannuzzi, 2018). These parameters calculated using equation 3 and 4 as follow:

$$\mu max = \frac{LnX_t - LnX_0}{t_t - t_0}$$

LnX_t: natural logarithm of final culture density in the exponential phase.
LnX_0: natural logarithm of initial culture density in the exponential phase.

t_t and t_0: onset and termination of the exponential growth phase (h).
Ln2: natural logarithm of the number 2
μmax: the maximum multiplication rate.

Cell length

The effect of increased sugar concentration on cell size in each strain was analyzed. At stationary phase onset in each glucose concentration level, an aliquot (10 μL) was taken, and lactophenol blue (10 μL) stain added. Images were taken of the cells using an improved Neubauer chamber in a microscope (×1000 magnification) and a photographic camera (Infinity 1®). Measurements of length were taken of approximately 90 randomly selected cells from each replica of strains and glucose concentrations, using the Infinity Analyze ver. 5.0.2 software (Lumenera Corp.) calibrated to the 1000 x magnification.

Statistical analysis

The effect of glucose concentration on culture density was analyzed by fitting a generalized mixed model to the data. The glucose concentration and the strain were fixed factors, and the time interval (h) was a random variable. This analysis conducted for each yeast strain used only data from the exponential growth phase since this period tent to be linear, and therefore allows comparisons between the different yeasts and sugar concentrations. Data for *S. paphiopedili* were square-root transformed to achieve linearity. The analyses were conducted with the MIXED procedure of the SAS ver. 9.1.3 statistical software (Statistical Analysis System. Institute, Cary, NC, USA). To test the effect of glucose concentration on cell size (length), we conducted a Kruskal-Wallis nonparametric test in the SPSS ver. 17 software.

RESULTS

Culture density

We observed differences both between the different strains and between the sugar concentrations in the culture medium (Figure 1). Slower growth and a lower maximum density are observed with increasing sugar concentrations above 20 % (Figure 1a). For *M. koreensis* strain, the model used was significant (χ^2 = 5.20; P < 0.05), explaining a relatively high amount of the data variance. The significant factors were glucose concentra-

tion ($F_{4, 8}$ = 6181.68; P < 0.0001), time ($F_{6, 12}$ = 2877.74; P < 0.0001) and the interaction between glucose concentration and time ($F_{24, 28}$ = 112.67; P < 0.0001). Culture density in this strain decreases with increasing glucose concentrations (P < 0.0001, Bonferroni test). The YPD broths with 2, 10 and 20 % glucose concentrations had the highest cell growth, almost three times that observed in the broth 60 % glucose concentration (1.06 × 10⁶ cells / µL). In this strain, the latency phase lasted only 8 h at low and moderate glucose concentrations (2, 10, and 20 %) but up to 16 h at higher concentrations (40 and 60 %). The longest exponential phase (14 h) was in the 10 % sugar concentration.

In the *S. paphiopedili* strain, the model was significant (χ^2 = 4.37; P < 0.05), but the growth was pronouncedly slower than the growth of the other strains studied here (Figure 1b). Growth of the strain was affected by the glucose concentration ($F_{4, 8}$ = 4789.81; P < 0.0001), time ($F_{7, 14}$ = 4001.04; P < 0.0001), and also by the interaction between glucose concentration and time ($F_{28, 56}$ = 101.25; P < 0.0001). The growth was similar between glucose concentrations, very low in latency, until 40 hours of cultivation; after approximately 40 hours, the culture density decreases with increasing

glucose concentrations (P < 0.0001, Bonferroni test). After 40 hours, culture density was highest in the broth with 2 % glucose concentration (4.81 × 10⁶ cells / µL), exceeding the culture density at 40 % (1.49 × 10⁶ cells / µL) and 60 % (5.48 × 10⁵ cells / µL). The latency phase of growth lasting approximately 42 hours at all broth concentrations, and it was longer at 40 and 60 %.

For the *S. apicola* strain, the model was significant (χ^2 = 7.85; P < 0.05). The factors glucose concentration ($F_{4, 8}$ = 11396; P < 0.0001), time ($F_{5, 10}$ = 11250.10; P < 0.0001) and the interaction between glucose concentration and time ($F_{20, 40}$ = 482.37; P < 0.0001) were significant. Similar to the other strains (Figure 1c), we observed in this one a slower growth and a lower maximum density with increasing sugar concentrations above 20 % (P < 0.0001, Bonferroni test). Density was highest in 2 % glucose (1·35 × 10⁷ cells / µL) and lowest in 60 % (4·52 × 10⁶ cells / µL). In low and intermediate glucose concentrations (2, 10, and 20 %), the latency phase was shorter (12 h) but longer (> 16 h) at the high concentrations (40 and 60 %). The exponential phase duration decreased as glucose concentration increased; at the 2 % concentration it lasted 18 h, and at 60 % it lasted 12 h.
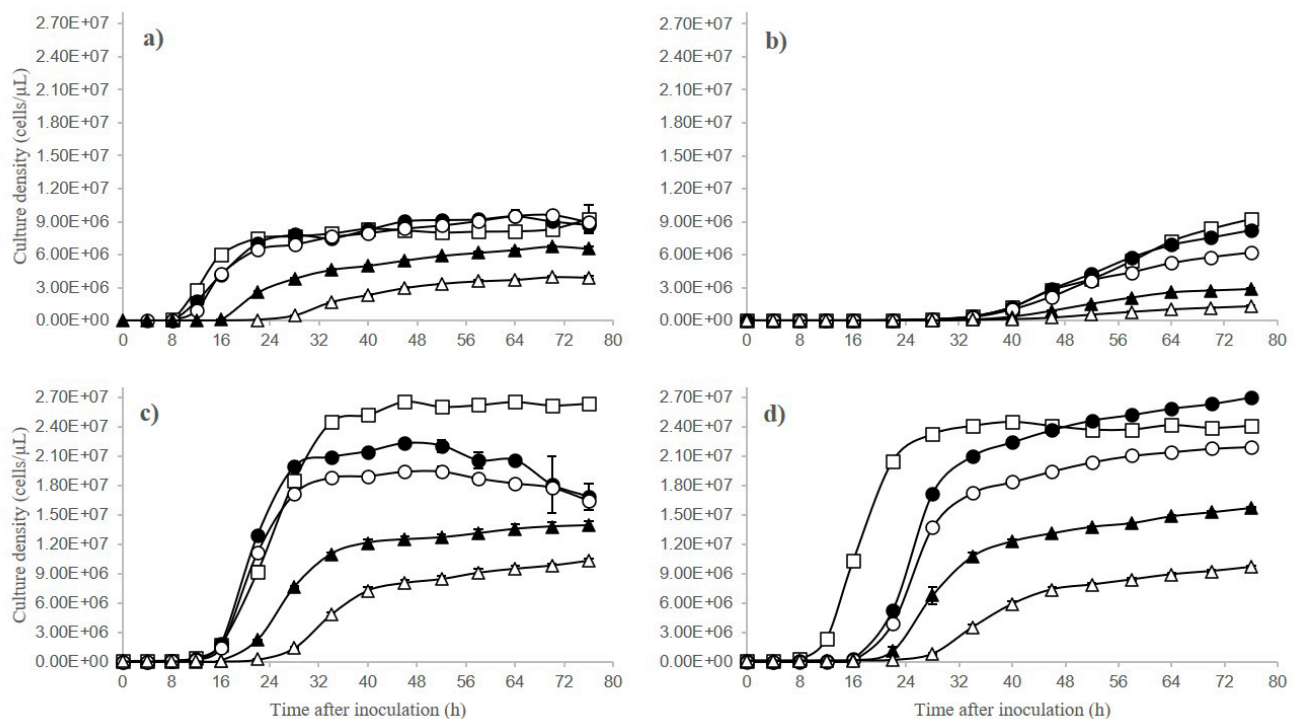

Figure 1. Culture density (cells / µL ± SD) of four yeast strains. a: CICY-RN-035 (*Metschnikowia koreensis*). b: CICY-RN-209 (*Sympodiomycopsis paphiopedili*), both isolated from the floral nectar. c: CICY-RN-364 (*Starmerella apicola*). d: CICY-RN- 354 (*Starmerella apicola* 2), isolated from the honey of *Melipona beecheii*. Growth media used was YPD with stepped increments in glucose concentration (w/w): (□)2 %, (●) 10 %, (o) 20 %, (▲) 40 %, and (Δ) 60 %.

For the *S. apicola* 2 strain, the model was significant (χ^2 = 5.88; P < 0.05). In this strain, only the factor of glucose concentration had a significant effect on culture density ($F_{4, 8}$ = 177.98; P < 0.0001). We observed (Figure 1d) a slower growth and a lower maximum density with increasing sugar concentrations above 20 % (P < 0.0001, Bonferroni test). Culture density was highest (1.12 × 10^7 cells / μL) at the 10 % concentration, and notably limited in the 40 % (6.22 × 10^6 cells / μL) and 60 % (3.41 × 10^6 cells / μL) concentrations. The latency phase lasted eight hours at the 2 % concentration and got longer to 16 h at 60 % concentration. The exponential phase also lengthened as glucose concentration increased, from ten hours at the 2 % concentration to 12 h at the 60 % concentration.

Growth parameters

The rate of the maximum specific growth (μmax) and duplication time (t_d) of each strain responded to the glucose concentration in the broth medium. In the strain of *M. koreensis*, the μmax was highest at the 2 % concentration and decreased as sugar concentration increased (the 40 % concentration was an exception), and the t_d was shortest and increased as the sugar concentration did. A similar pattern in the μmax and t_d observed for the strains CICY-RN-364 (*S. apicola*) and CICY-RN- 354 (*S. apicola* 2). The exception was the strain CICY-RN-209 (*S. paphiopedili*), which showed the lowest growth rate and the longest duplication time, no matter the glucose concentration (Table 1).

Cell length

The nonparametric test indicated that different glucose concentrations affect cell length. In general, all the study yeasts reduced in cell size due to the increment of glucose concentration in the broth medium (Figure 2). The strains exhibited a reduction in cell length as follow: strains CICY-RN-035 (*M. koreensis*) from 7.31 ± 1.57 to 5.6 ± 1.01, strain CICY-RN-364 (*S. apicola*) from 3.77 ± 0.58 to 2.53 ± 0.37, strain CICY-RN- 354 (*S. apicola* 2) from 4.13 ± 0.88 to 2.87 ± 0.48, and strain CICY-RN-209 (*S. paphiopedili*) from 7.21 ± 1.26 to 5.95 ± 1.72 (Figure 3).

Table 1. Average (± SD) of the rate of the maximum specific growth (μmax) and duplication time (t_d) in hours (h) of four yeast strains under an increasing gradient of glucose concentration in the medium

Glucose	CICY-RN-035 Metschnikowia koreensis		CICY-RN-209 Sympodiomycopsis paphiopedili		CICY-RN-364 Starmerella apicola		CICY-RN- 354 Starmerella apicola 2	
	μmax	t_d	μmax	t_d	μmax	t_d	μmax	t_d
2 %	0·52 ± 0·04	1·34 ± 0·11	0·08 ± 0·002	8·99 ± 0·28	0·15 ± 0·01	4·72 ± 0·34	0·22 ± 0·001	3·23 ± 0·02
10 %	0·41 ± 0·01	1·69 ± 0·04	0·07 ± 0·003	9·44 ± 0·43	0·20 ± 0·004	3·48 ± 0·08	0·20 ± 0·01	3·51 ± 0·11
20 %	0·38 ± 0·01	1·81 ± 0·05	0·07 ± 0·004	9·44 ± 0·55	0·21 ± 0·003	3·37 ± 0·04	0·21 ± 0·01	3·34 ± 0·09
40 %	0·51 ± 0·01	1·37 ± 0·03	0·08 ± 0·01	8·30 ± 0·49	0·13 ± 0·004	5·21 ± 0·17	0·19 ± 0·03	3·63 ± 0·51
60 %	0·22 ± 0·02	3·13 ± 0·31	0·09 ± 0·01	7·88 ± 0·42	0·14 ± 0·003	5·12 ± 0·11	0·17 ± 0·01	4·18 ± 0·22

Strains isolated from floral nectar were *Metschnikowia koreensis* and *Sympodiomycopsis paphiopedili*, and the isolated from honey were *Starmerella apicola* and *Starmerella apicola* 2.
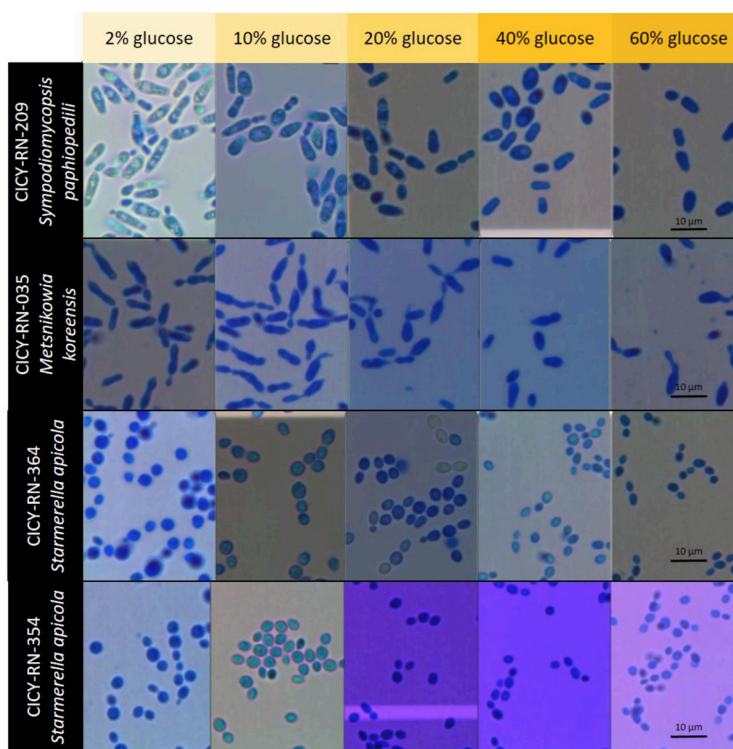
Figure 2. Cells of four yeast strains: CICY-RN-035 (*Metschnikowia koreensis*), CICY-RN-209 (*Sympodiomycopsis paphiopedili*), both isolated from the floral nectar; CICY-RN-364 (*Starmerella apicola*), and CICY-RN- 354 (*Starmerella apicola* 2), isolated from the honey of *Melipona beecheii*. Growth media used was YPD with stepped increments in glucose concentration (w/w): 2 %, 10 %, 20 %, 40 %, and 60 % glucose. Cells were stained with lactophenol blue to observe at ×1000 magnification.
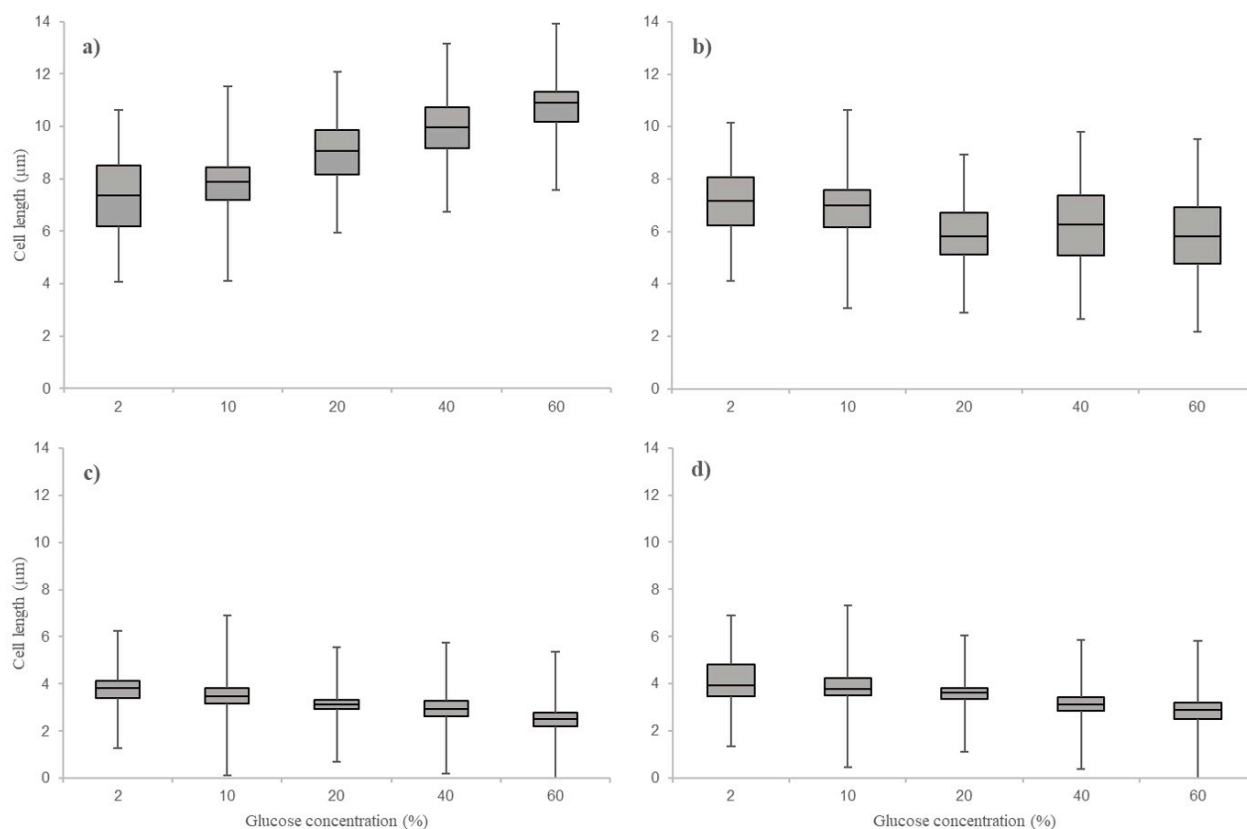

Figure 3. Cell size (µm) of four yeast strains in artificial media with 2 %, 10 %, 20 %, 40 %, and 60 % glucose (w/w). a: CICY-RN-035 (*Metschnikowia koreensis*). b: CICY-RN-209 (*Sympodiomycopsis paphiopedili*), both isolated from the floral nectar. c: CICY-RN-364 (*Starmerella apicola*). d: CICY-RN- 354 (*Starmerella apicola* 2), isolated from the honey of *Melipona beecheii*. The plot shows the 25th, 50th (median), and 75th percentiles.

DISCUSSION

Growth was different between the four evaluated yeasts in response to glucose concentration and the type of yeast strain. Higher glucose concentration reduced growth in terms of culture density per microliter, growth rate, duplication time, and cell length. The strains with the highest culture density at high glucose concentrations were the strains isolated from honey, while those isolated from floral nectar had lower culture density at high concentrations.

The ability of microorganisms to grow at high solute concentrations is called osmotolerance (Blomberg and Adler 1992; Buzzini *et al*. 2018). This capacity is vital in yeast strains colonizing nectar and honey environments because it allows them to protect membrane function and maintain intracellular biochemical processes regardless of the low or high water availability in the sugary environments (Lachance *et al*. 2001; Lachance 2006; Pozo *et al*. 2015; Mittelbach *et al*. 2016). According to the results obtained in the present study, the *M. koreensis* strain has a wide tolerance range with respect to sugar concentration in its growth medium, so it can thrive in both nectar and honey. This agrees with previous reports in which this species has been observed in the following flower environments: in the nectar of *T. stans* (Bignoniaceae) which is high in glucose (48 %), in the flowers of *Lilium* sp. which has an intermediate glucose concentration in its nectar (19 %), and in the nectar of *Ipomoea* sp., which has nectar with a high sugar concentration (67 %) (Hong *et al*. 2001; Galetto and Bernadello 2004; Stolar and Davis 2010; Canto and Herrera 2012). The genus *Metschnikowia* includes several strains related to floral nectar and flowers. For example, the strains of *M. reukaufii*, *M. gruessii*, *M. lachancei,* and *M. vanudenii* tend to associate with bees in substrates such as honey and can grow in 40-60 % glucose concentrations (Giménez-Jurado *et al*. 2003; Brysch-Herzberg 2004). Culture density data for the strain of *M. koreensis* suggests that it can grow adequately in honey or concentrated nectars, meaning that the yeast can survive in environments with low water activity such as honey (a_w = 0.53-0.67) as well as in environments with slightly higher water activity such as floral nectar (a_w = 0.93-0.99) (Lievens *et al*. 2015).

The presence of a strain of *S. paphiopedili* in sugar-rich substrates associated with bees is rare, although the yeast was isolated for the first time from the nectar of the orchid *Paphiopedilum primurinum* (Sugiyama *et al*. 1991). The strain studied here was isolated from the floral nectar of *D. aegyptius* (32 %) but was found to grow at high glucose concentrations (e.g., 60 %), albeit in a significantly reduced manner. This yeast is unable to grow at relatively high glucose concentrations (50 %) (Sugiyama *et al*. 1991), and it prefers growth conditions with a moderate glucose concentration, such as the 32 % in *D. aegyptius* (Convolvulaceae) nectar (Canto and Herrera 2012). *Sympodiomycopsis paphiopedili* is a Basidiomycota yeast, a type rarely observed in floral nectar (Lachance 2006). Basidiomycota yeasts are more sensitive than Ascomycota yeasts to low water activity (high sugar concentration) in a growth medium (Tekolo *et al*. 2010; Mittelbach *et al*. 2015).

Results show that both strains of *S. apicola* 2 and *S. apicola* are osmotolerant strains that can maintain a constant growth rate at glucose concentrations of 40 and 60 %. For the strain of *S. apicola* this ability was first reported over sixty years ago (Hajsig 1958). Our results show that strains had similar growth pattern, exhibiting accelerated growth at a relatively low glucose concentration (20 %) and moderate growth at higher sugar concentration. Both strains were isolated from *M. beecheii* honey, which has a high sugar concentration (71-76 %) (Souza *et al*. 2006; Moo-Huchin *et al*. 2015). Conventional tests evaluating a microorganism's ability to grow at high sugar concentrations (osmotolerance) only use a 60 % glucose concentration (Barnett *et al*. 2007). However, yeasts associated with sugary substrates such as floral nectar and honey confront varying sugar concentrations during movement between environments and can find themselves in concentrations gradually increasing to a maximum of up to 80 %. Although this study has the limitation of having simplified the gradient of sugar composition and concentrations that yeasts cope from floral nectar to honey environments, the most evident gradient is the stepped increase of sugar concentration due to the loss of water in the medium, similar to the sugar gradient tested here. The results contribute to elucidate how some types of strains can be found mostly in the flower substrates and others in the honey, and how the gradient of sugar concentration, from floral nectar to honey is acting as an ecological filter that leads to yeast strain specialization.

Cell length decreased as medium glucose concentration increased, independent of strain. This physiologi-

cal response is expected in microorganisms growing in environments with high solute concentrations. Under these conditions, cells maintain a balance between intracellular solute concentration and that of the surrounding environment, causing cells to lose water by osmosis and reduce their volume (Rose 1975). Strains of *Z. mellis* from *Apis mellifera* honey reduced their cell size as the amount of sugar in the culture medium increased (Marvin *et al*. 1931). In another case, the yeast *S. cerevisiae* reduced cell volume in concentrated sodium chloride solutions, which was due to the loss of water in response to high osmotic pressure (Morris *et al*. 1983). The only evaluated strain that did exhibit a considerable reduction in cell length was the strain of *S. paphiopedili*. The reduction may be the result of differences in cell membrane lipid composition between Basidiomycota and Ascomycota, which could facilitate active transport in some species but not in others (Golubev *et al*. 2004). Even sugar composition (O'Brien and Ralph 1966) and cell wall lamellar structure (Kreger-Van Rij and Veenhuis 1971) differ between these two fungal divisions. Further research on these characteristics will help to understand how the membrane and cell wall are involved in fungal response to high sugar concentrations like those in floral nectar and honey.

The results obtained indicated that yeast strains isolated from floral nectar and from honey have different levels of osmotolerance, which can be interpreted as adaptive strategies to survive, proliferate, and even migrate between sugar environments. Although each of the four evaluated strains exhibited the ability to grow in, or migrate between environments with different glucose concentrations, all exhibited reduced growth as concentration increased. The different growth trajectories observed in each yeast strain suggest that they are specialized to grow in a specific sugar concentration favoring rapid growth and high cell densities. The two strains isolated in nectar grew best at low and moderate glucose concentrations, while the strains isolated in honey did so at relatively high glucose concentrations; the latter also experienced smaller reductions in cell size. These adaptations allow the strains colonizing honey to grow better at high sugar concentrations, reinforcing the idea that they are associated with bees; in this case, native stingless bees such as *M. beecheii*. These findings help to clarify which biological aspects are involved in the association of yeast strains with native bees and their food resources, such as honey and floral nectar. To better understand the functional role of the yeast strains capable of colonizing sugary flower and bee-related environments in bee and plant life, future research involving more strains both isolated from floral nectar and honey is necessary.

REFERENCES

Álvarez-Pérez S, Herrera CM. 2012. Composition, richness and nonrandom assembly of culturable bacterial-microfungal communities in floral nectar of Mediterranean plants. FEMS Microbiology Ecology 80, 685-699. https://doi.org/10.1111/j.1574-6941.2012.01329.x

Arroyo-López FN, Orlic S, Querol A, Barrio E. 2009. Effects of temperature, pH and sugar concentration on the growth parameters of *Saccharomyces cerevisiae*, *S. kudriavzevii* and their interspecific hybrid. International Journal of Food Microbiology 13, 120-127. https://doi.org/10.1016/j.ijfoodmicro.2009.01.035

Barnett JA, Payne RW, Yarrow D. 2007. Yeasts: characteristics and identification. Cambridge University Press, Cambridge.

Belisle M, Peay KG, Fukami T. 2012. Flowers as islands: spatial distribution of nectar-inhabiting microfungi among plants of *Mimulus aurantiacus*, a hummingbird-pollinated shrub. Microbial Ecology 63, 711-718. https://doi.org/10.1007/s00248-011-9975-8

Blomberg A, Adler L. 1992. Physiology of osmotolerance in fungi. Advances in Microbial Physiology 33, 145-212. https://doi.org/10.1016/S0065-2911(08)60217-9

Brysch-Herzberg M. 2004. Ecology of yeasts in plant-bumblebee mutualism in Central Europe. FEMS Microbiology Ecology 50, 87-100. https://doi.org/10.1016/j.femsec.2004.06.003

Buzzini P, Turchetti B, Yurkov A. 2018. Extremophilic yeasts: the toughest yeasts around? Yeast 35, 487-497. https://doi.org/10.1002/yea.3314

Canto A, Herrera CM. 2012. Micro-organisms behind the pollination scenes: microbial imprint on floral nectar sugar variation in a tropical plant community. Annals of Botany 110, 1173-1183. https://doi.org/10.1093/aob/mcs183

Canto A, Herrera CM, Medrano M, Pérez R, García IM. 2008. Pollinator foraging modifies nectar sugar composition in *Helleborus foetidus* (Ranunculaceae): An experimental test. American Journal of Botany 95, 315-320. https://doi.org/10.3732/ajb.95.3.315

Canto A, Herrera CM, Rodríguez R. 2017. Nectar-living yeasts of a tropical host plant community: diversity and effects on community-wide floral nectar traits. PeerJ 5, e3517. https://doi.org/10.7717/peerj.3517

Chappell CR, Fukami T. 2018. Nectar yeasts: a natural microcosm for ecology. Yeast 35, 417-423. https://doi.org/10.1002/yea.3311

Da Silva PM, Gauche C, Gonzaga LV, Costa ACO, Fett R. 2016. Honey: chemical composition, stability and authenticity. Food Chemistry 196, 309-323. https://doi.org/10.1016/j.foodchem.2015.09.051

De-Melo MAA, Almeida-Muradian LBD, Sancho MT, Pascual-Maté A. 2018. Composition and properties of *Apis mellifera* honey: A review. Journal of Apicultural Research 57, 5-37. https://doi.org/10.1080/00218839.2017.1338444

Galetto L, Bernadello G. 2004. Floral nectaries, nectar production dynamics and chemical composition in six *Ipomoea* species (Convolvulaceae) in relation to pollinators. Annals of Botany 94, 269-280. https://doi.org/10.1093/aob/mch137

Giannuzzi L. 2018. Cyanobacteria growth kinetics. In: Wong, Y.K. (ed.), Algae. IntechOpen, London. 1-17. http://dx.doi.org/10.5772/intechopen.81545

Gilliam M. 1997. Identification and roles of non-pathogenic microflora associated with honeybees. FEMS Microbiology Letters 155, 1-10. https://doi.org/10.1111/j.1574-6968.1997.tb12678.x

Giménez-Jurado G, Kurtzman CP, Starmer WT, Spencer-Martins I. 2003. *Metschnikowia vanudenii* sp. nov. and *Metschnikowia lachancei* sp. nov., from flowers and associated insects in North America. International Journal of Systematic and Evolutionary Microbiology 53, 1665-1670. https://doi.org/10.1099/ijs.0.02470-0

Golubev WI, Kulakovskaya TV, Kulakovskaya EV, Golubev NW. 2004. The fungicidal activity of an extracellular glycolipid from *Sympodiomycopsis paphiopedili*. Microbiology 73, 724-728. https://doi.org/10.1007/s11021-005-0015-x

Grant LW. 2004. Life at low water activity. Philosophical Transactions of the Royal Society B 359, 1249-1267. https://doi.org/10.1098/rstb.2004.1502

Hajsig M. 1958. *Torulopsis apicola* nov. spec., new isolates from bees. Antonie Van Leeuwenhoek 24, 18-22. https://doi.org/10.1007/BF02548426

Herrera CM, de Vega C, Canto A, Pozo MI, 2009. Yeast in floral nectar: a quantitative survey. Annals of Botany 103, 1415-1423. https://doi.org/10.1093/aob/mcp026

Herrera CM, Pozo MI, Bazaga P. 2012. Jack of all nectars, master of most: DNA methylation and the epigenetic basis of niche width in a flower living yeast. Molecular Ecology 21, 2602-2616. https://doi.org/10.1111/j.1365-294X.2011.05402.x

Hong SG, Chun J, Oh HW, Bae KS. 2001. *Metschnikowia koreensis* sp. nov., a novel yeast species isolated from flowers in Korea. International Journal of Systematic and Evolutionary Microbiology 51, 1927-1931. https://doi.org/10.1099/00207713-51-5-1927

Kreger-Van Rij N, Veenhuis M. 1971. A comparative study of the cell wall structure of basidiomycetous and related yeasts. Journal of General Microbiology 68, 87-95. https://doi.org/10.1099/00221287-68-1-87

Kwolek-Mirek M, Zadrag-Tecza R, 2014. Comparison of methods used for assessing the viability and vitality of yeast cells. FEMS Yeast Research 14, 1068-1079. https://doi.org/10.1111/1567-1364.12202

Lachance MA, 2006. Yeast biodiversity: how many and how much? In: Rosa C, Gábor P. (eds.), Biodiversity and Ecophysiology of Yeasts. Springer-Verlag, Berlin. 1-9. https://doi.org/10.1007/3-540-30985-3

Lachance MA, Starmer WT, Rosa CA, Bowles JW, Barker JSF, Janzen DH, 2001. Biogeography of the yeasts of ephemeral flowers and their insects. FEMS Yeast Research 1, 1-8. https://doi.org/10.1111/j.1567-1364.2001.tb00007.x

Lievens B, Hallsworth JE, Pozo MI, Belgacem ZB, Stevenson A, Willems KA, Jacquemyn H. 2015. Microbiology of sugar-rich environments: diversity, ecology, and system constraints. Environmental Microbiology 17, 278-298. https://doi.org/10.1111/1462-2920.12570

Lizama B. 2011. Levaduras asociadas a dos especies de abejas sin aguijón (Apidae: Meliponini) y a tres especies de la flora nectarífera de Yucatán. Master's thesis. Centro de Investigacion Cientifica de Yucatan, Merida, Mexico.

Madden AA, Epps MJ, Fukami T, Irwin RE, Sheppard J, Sorger DM, Dunn RR. 2018. The ecology of insect-yeast relationships and its relevance to human industry. Proceedings of the Royal Society B 285, 20172733. https://doi.org/10.1098/rspb.2017.2733

Mager WH, Siderius M. 2002. Novel insights into the osmotic stress response of yeast. FEMS Yeast Research 2, 251-257. https://doi.org/10.1016/S1567-1356(02)00116-2

Maier R. 2009. Bacterial growth. In: Maier R, Pepper I, Gerba C (eds.), Environmental microbiology. Elsevier, New York. 38-56. https://doi.org/10.1016/B978-0-12-370519-8.00003-1

Marvin GE, Peterson WH, Fred EB, Wilson HF, 1931. Some of the characteristics of yeasts found in fermenting honey. Journal of Agricultural Research 43, 121-131.

Membré JM, Kubaczka M, Chéné C, 1999. Combined effects of pH and sugar on growth rate of *Zygosaccharomyces rouxii*, a bakery product spoilage yeast. Applied and Environmental Microbiology 65, 4921-4925.

Mittelbach M, Yurkov AM, Nocentini D, Nepi M, Weigend M, Begerow D. 2015. Nectar sugars and bird visitation define a floral niche for basidiomycetous yeast on the Canary Islands. BMC Ecology 15, 2. https://doi.org/10.1186/s12898-015-0036-x

Mittelbach M, Yurkov AM, Stoll R, Begerow D, 2016. Inoculation order of nectar-borne yeasts opens a door for transient species and changes nectar rewarded to pollinators. Fungal Ecology 22, 90-97. https://doi.org/10.1016/j.funeco.2015.12.003

Moo-Huchin VM, González-Aguilar GA, Lira-Maas JD, Pérez-Pacheco E, Estrada-León R, Moo-Huchin MI, Sauri-Duch E. 2015. Physicochemical Properties of *Melipona beecheii* Honey of the Yucatan Peninsula. Journal of Food Research 4, 25-32. https://doi.org/10.5539/jfr.v4n5p25

Morris GJ, Winters L., Coulson GE, Clarke KJ, 1983. Effect of osmotic stress on the ultrastructure and viability of the yeast *Saccharomyces cerevisiae*. Journal of General Microbiology 129, 2023-2034. https://doi.org/10.1099/00221287-132-7-2023

Nepi M, von Aderkas P, Wagner R, Mugnaini S, Coulter A, Pacini E. 2009. Nectar and pollination drops: How different are they? Annals of Botany 104, 205-219. https://doi.org/10.1093/aob/mcp124

Nicolson SW, Nepi M, Pacini E, 2007. Nectaries and Nectar. Springer-Verlag, Dordrecht. https://doi.org/10.1007/978-1-4020-5937-7

O'Brien RW, Ralph BJ. 1966. The cell wall composition and taxonomy of some Basidiomycetes and Ascomycetes. Annals of Botany 30, 831-843. https://doi.org/10.1093/oxfordjournals.aob.a084117

Pascual-Maté A, Osés SO, Fernández-Muiño MA, Sancho MT, 2018. Methods of analysis of honey. Journal of Apicultural Research 51, 38-74. https://doi.org/10.1080/00218839.2017.1411178

Pozo MI, Herrera CM, den Ende WV, Verstrepen K, Lievens B, Jacquemyn H. 2015. The impact of nectar chemical features on phenotypic variation in two related nectar yeasts. FEMS Microbiology Ecology 91, fiv055. https://doi.org/10.1093/femsec/fiv055

Pozo MI, Lachance MA, Herrera CM. 2012. Nectar yeasts of two southern Spanish plants: the roles of immigration and physiological traits in community assembly. FEMS Microbiology Ecology 80, 281-293. https://doi.org/10.1111/j.1574-6941.2011.01286.x

Raspor P, Zupan J. 2006. Yeasts in extreme environments. In: Rosa CA, Péter G (eds.), Biodiversity and ecophysiology of yeasts. Springer, Berlin. 371-418. https://doi.org/10.1007/3-540-30985-3

Rodrigues F, Ludovico P, Leao C. 2006. Sugar metabolism in yeasts: an overview of aerobic and anaerobic glucose catabolism. In: Rosa CA, Péter G (eds.), Biodiversity and ecophysiology of yeasts. Springer, Berlin. 101-122. https://doi.org/10.1007/3-540-30985-3

Rosa CA, Lachance MA, Silva JOC, Teixeira ACP, Marini MM, Antonini Y, Martins RP, 2003. Yeast communities associated with stingless bees. FEMS Yeast Research 4, 271-275. https://doi.org/10.1016/S1567-1356(03)00173-9

Rose D. 1975. Physical responses of yeast cells to osmotic shock. Journal of Applied Bacteriology 38, 169-175. https://doi.org/10.1111/j.1365-2672.1975.tb00517.x

Salvesen I, Vadstein O. 2000. Evaluation of plate count methods for determination of maximum specific growth rate in mixed microbial communities, and its possible application for diversity assessment. Journal of Applied Microbiology 88, 442-448. https://doi.org/10.1046/j.1365-2672.2000.00984.x

Snowdon JA, Cliver DO. 1992. Microorganism in honey. International Journal of Food Microbiology 31, 1-26. https://doi.org/10.1016/0168-1605(96)00970-1

Souza B, Roubik D, Barth O, Hearth T, Enríquez E, Carvalho C, Villas-Boas J, Marchini L, Locatelli J, Persano-Oddo L, Almeida-Muradian L, Bogdanov S, Vit P. 2006. Composition of stingless bee honey: Setting quality standards. Interciencia 31, 867-875.

Spencer JFT, Spencer DM, 1997. Ecology: Where yeast live. In: Spencer JFT, Spencer DM (eds.), Yeasts in natural habitats. Springer, Berlin. 33-58. https://doi.org/10.1007/978-3-662-03370-8

Sprouffske K, Wagner A. 2016. Growthcurver: an R package for obtaining interpretable metrics from microbial growth curves. BMC Bioinformatics 17, 172. https://doi.org/10.1186/s12859-016-1016-7

Starmer WT, Lachance MA. 2011. Yeast Ecology. In: Kurtzman C, Fell JW, Boekhout T (eds.), The yeast. A taxonomic study. Elsevier, London. 65-83.

Stevenson A, Burkhardt J, Cockell CS, Cray JA, Dijksterhuis J, Fox-Powell M, Kee TP, Kminek G, McGenity TJ, Timmis KN, Timson DJ, Voytek MA, Westall F, Yakimov MM, Hallsworth JE. 2015. Multiplication of microbes below 0.690 water activity: implications for terrestrial and extraterrestrial life. Environmental Microbiology 17, 257-277. https://doi.org/10.1111/1462-2920.12598

Stolar J, Davis AR. 2010. Floral nectary structure, nectar production, and carbohydrate composition in the *Lilium* Asiatic hybrid 'Trésor'. Botany 88, 185-205. https://doi.org/10.1139/B09-109

Sugiyama J, Tokuoka K, Suh S, Hirata A, Komagata K. 1991. *Sympodiomycopsis*: a new yeast-like anamorph genus with basidiomycetous nature from orchid nectar. Antonie Van Leeuwenhoek 59, 95-108. https://doi.org/10.1007/BF00445653

Teixeira ACP, Marini MM, Nicoli JR, Antonini Y, Martins RP, Lachance MA, Rosa CA, 2003. *Starmerella meliponinorum* sp. nov., a novel ascomycetous yeast species associated with stingless bees. International Journal of Systematic and Evolutionary Microbiology 53, 339-343. https://doi.org/10.1099/ijs.0.02262-0

Tekolo OM, Mackenzie J, Botha A, Prior BA. 2010. The osmotic stress tolerance of basidiomycetous yeasts. FEMS Yeast Research 10, 482-491. https://doi.org/10.1111/j.1567-1364.2010.00612.x

Valero A, Pérez-Rodríguez F, Carrasco E, García-Jimeno RM, Zurera G. 2006. Food microbiology and safety modeling the growth rate of *Listeria monocytogenes* using absorbance measurements and calibration curves. Journal of Food Science 71, M257-264. https://doi.org/10.1111/j.1750-3841.2006.00139.x