Amino acid biosynthetic and antioxidant activity of Brevibacterium sp. InaCC B46 using glucose substrate produced by saccharification of oil palm trunk biomass

Emma Riftyan[1*], *Yusmarini* Yusmarini[1], *Shanti* Fitriani[1], *Usman* Pato[1], *Evy* Rossi[1], and *Novianti Monica* Nadapdap[1]

[1]Faculty of Agricultural, Universitas Riau, Kampus Bina Widya KM 12,5, 28293, Indonesia

Abstract. Brevibacterium sp. is commercially essential due to its production of amino acids, particularly glutamic acid and lysine. This work investigates the effect of Brevibacterium sp. concentration on L-amino acid synthesis from glucose hydrolysed from oil palm trunk biomass as a substrate. The study employed a two-factorial, completely randomised experimental design. Factor A consisted of concentrations of Brevibacterium sp. (1%, 3%, and 5%), while factor B was incubation duration (24 and 48 hours). The study measured the concentrations of four amino acids (alanine, glycine, tyrosine, and glutamic acid) using the colourimetric ninhydrin reaction. Additionally, the viability and antioxidant activity of Brevibacterium sp. during incubation time was measured with spectrophotometry (UV-VIS). The results indicate no significant interaction between factors ($P>0.05$) but a significant effect ($P<0.05$) depending on the concentration and incubation time of Brevibacterium sp. InaCCB46 for all parameters. The study found that the biosynthesis of amino acids was most efficient with a 5% concentration of bacteria during 24-hour incubation, resulting in the highest output of alanine, glycine, tyrosine, and glutamic acid at 6.46, 4.58, 6.16, and 6.67 μg/mL, respectively. However, it should be noted that bacterial viability was higher after 24 hours of incubation, ranging from 144.18–216.99% and had strong antioxidant activity at 22.43 ppm.

1 Introduction

Indonesia is one of the largest producers of palm oil in the world. According to Statistics Indonesia, the production of the oil palm plant area in 2021 is about 8.579.000 ha. In 2022, oil palm plantations produced 23,5 million tonnes of crude palm oil (CPO). Indonesia has the potential to generate over 40 million tonnes of crude palm oil (CPO) per year by 2023 while also expanding its oil palm sector [1]. The recycling biomass from oil palm trunks biomass (OPTB) will contribute to the sustainability and economic success of the oil palm industry and Indonesia's 2060 net zero target [2]. OPTB is acknowledged as a highly potential source

* Corresponding author: emma.riftyan@lecturer.unri.ac.id

of lignocellulosic materials derived from oil palm plantation (replanting), which comprises around 29–45% cellulose, 12–29% hemicellulose, and 18–23% lignin [3].

The composition of OPT indicates that an auspicious material will be used as biomass to convert to any valued product. Hemicellulose is a polysaccharide composed of a linear backbone of a β-1,4-linked homopolymer of sugar, which can potentially be converted to xylose, rhamnose, arabinose, glucose, mannose, and galactose [3]. Saccharification-related enzymatic degradation of OPTB is exceedingly difficult due to its lignocellulosic composition. As a result, pretreatment is required to deconstruct the lignocellulosic components effectively. Lignocellulosic biomass is high in sugar-convertible cellulose and hemicellulose, with glucose being the predominant constituent. The cellulase enzyme is typically necessary and utilised to accomplish the conversion objective. By enzymatic saccharification, high cellulose digestibility of 86–87% of raw OPTB solids [2]. In addition, according to [4], 43.5 g of glucose per 100 g of dry OPTB was obtained, corresponding to 81.3% of the theoretical glucose yield. It is very important to research the use of monosaccharides from oil palm trunk (OPT) biomass as the substrate for microbial growth media and biosynthesised amino acids and other functional metabolites, including superoxide dismutase (SOD), catalase (CAT), and peroxidases (PODs) as antioxidants.

Brevibacterium sp. is an obligate, aerobic, non-motile, non-spore-forming, Gram-positive bacterium. It has a growth temperature range of 4–42°C, with an optimum temperature of 21–28°C. The bacteria are rod-shaped and can be found in single, paired, or short chains. After incubation for approximately 48 hours, the cell size of these bacteria will begin to enlarge [5]. Several taxonomically related genera, such as Brevibacterium, Micrococcus, Corynebacterium, and Microbacterium, could produce glutamic acid from their metabolic products. It is suggested that *Brevibacterium lactofermentum* and *Brevibacterium flavum* are subspecies of *Corynebacterium glutamicum*. The bacteria commonly found to have the Embden-Meyerhof-Parnas (EMP) glycolytic pathway, the hexose monophosphate (HMP) pathway, the TCA cycle (Krebs cycle), and the glyoxylate bypass pathway to produce amino acids [6]. Furthermore, according to [7], Brevibacterium sp. significantly increases antioxidant defences to eliminate reactive oxygen species (ROS) and avoid oxidative damage by excess activity of the antioxidant system by expressing the psbA gene, which might block the electron transport chain. This study focused on the use of monosaccharides obtained from the biomass of oil palm trunks (OPT) as possible substrates for microbial growth medium and amino acid biosynthesis **(**glutamic acid, alanine, glycine, and tyrosine) and the activity of antioxidants produced by Brevibacterium sp. InaCC B46.

2 Material and methods

2.1 Bacterial strain and cultures

Brevibacterium sp. InaCC B46 was screened to identify four amino acid products. These bacteria were obtained from the National Research and Innovation Agency, Indonesia (ELSA-BRIN)—Brevibacterium sp. InaCC B46 was cultured in nutrient broth and agar at 37°C for 48 h.

2.2 Pretreatment of oil palm trunk biomass

The oil palm trunk biomass (OPTB) used in this research was obtained from the palm oil variety Tenera, Bandar Sikijang District, Pelalawan Regency, Riau Province. The physicochemical pretreatment method of OPTB conducted in this study was alkaline peroxide combined with an autoclave. The OPTB raw materials were powdered with the

following steps: size reduction, washing, drying, grinding, and screening with a sieve 80 mesh. The OPTB powder was then soaked with a solution of 5% NaOH and 5% H_2O_2 at pH 11.5 in a ratio of 1:10 for 5 days. Then, the palm trunk waste powder was oven-dried at 50°C for 60 minutes. Furthermore, after soaking for 5 days, the samples were heated in an autoclave at 121°C with a pressure of 1 atm for 15–20 minutes [8].

2.3 Saccharification of OPTB

One percent of the OPTB powder (b/v) was mixed with 0.5 M sodium acetate and acetate buffer (pH 5) in a beaker glass to reach a volume of 100 ml. Then, 1% (v/v) cellulase enzyme was added. The mixture of sample solution and cellulase enzyme was then placed in a water bath shaker that was set at a speed of 150 rpm and a temperature of 50ºC for 5 hours, and this process is the saccharification stage. After the saccharification process was complete, the sample solution was filtered with filter paper, and then each ml volume of sample was added with 0.5 N NaOH in a ratio of 1:9 (v/v). The sample was then used as the main ingredient in the formulation of bacterial growth media as a carbon source of OPTB (monosaccharide) [9].

2.4 Viability of lactic acid bacteria

The viability of lactic acid bacteria, which grew with medium formulation using glucose of OPTB, was measured at a wavelength of 400 nm (OD_{400}) at 0 hours (initial absorbance value) and 48 hours after the incubation period (final absorbance value) at 37°C. Wavelength measurements using UV-Vis spectrophotometer (Shimadzu UV-1800). Furthermore, the percentage of bacterial viability was calculated using the following formula [10]:

$$\text{Viability}(\%) = 100 - \left(\frac{\text{Sample Abs}}{\text{Control Abs}} \times 100\right)$$

2.5 Total amino acid analysis

A standard solution of amino acids, namely glutamic acid, alanine, glycine, and tyrosine, was prepared with concentrations of 0.1%, 0.3%, 0.5%, 0.7%, and 1% (2 mL each). A ninhydrin solution (0.5 mL) was added to this solution in a ratio of 4:1 (v/v). The mixture was then placed in test tubes and heated in a boiling water bath at 100°C for 10 minutes per the predetermined time. After heating, the tubes were immediately cooled in an ice bath. The reaction mixture's absorbance (550 nm) was measured with a spectrophotometer UV-VIS (Shimadzu UV-1800). Then, a standard curve was made for the resulting regression linear equation to determine the total amino acid in the cell-free supernatant of Brevibacterium sp. InaCC B46. The step for analysis of total amino acid (glutamic acid, alanine, glycine, and tyrosine) in the cell-free supernatant was conducted similarly to the amino acid solution standard before. However, the volume sample was 4 mL with 1 mL ninhydrin solution (4:1) v/v [11].

2.6 Scavenging ability of DPPH radicals

The ability of DPPH radicals to scavenge was assessed using the methods described by [12]. To prepare the DPPH solution, dissolve 0.1 g of DPPH in 100 ml of methanol—Additionally, 1.3 ml of culture supernatant from Brevibacterium sp. InaCC B46 was used at various concentrations (1%, 3%, and 5%). The cells were incubated at room temperature for 24 hours and 48 hours. 1.3 ml of culture supernatant bacteria was transferred to a test tube containing 5 ml of DPPH solution at concentrations of 500 ppm, 250 ppm, 125 ppm, and 62.5 ppm in

the dark room and homogenised using a vortex mixer. The mixture was homogenised using a vortex mixer and incubated in the dark for 30 minutes. The solution's absorbance was measured at a wavelength of 517 nm using a spectrophotometer [13]. The DPPH radical inhibition was then calculated using the following formula:

$$\text{Inhibition}(\%) = 100 - \left(\frac{\text{Abs Control} - \text{Abs Sample}}{\text{Abs Control}} \times 100\right)$$

The percent inhibition and absorbance values data were then processed and analysed using the linear regression equation y = ax + b. This equation was used to calculate the IC_{50} value.

2.7 Data analysis

The experimental design in this study was a two-factor factorial, completely randomised design (CRD). Factor A consisted of concentrations of Brevibacterium sp. (1%, 3%, and 5%), while factor B was incubation duration (24 and 48 hours). All the assays were performed in triplicate. The GraphPad PRISM version 10.1.0 was used to analyse the interaction between two factors using analysis of variance (ANOVA). Post hoc tests (Duncan's Multiple Range tests*)* were approached to show the significance at P<0.05, and the software was also used to demonstrate the result with the graph.

3 Result and discussion

This study demonstrates Brevibacterium sp. InaCC B46 can grow in OPTB glucose-modified media. ANOVA analysis of Brevibacterium sp. B46 viability showed significant differences for each treatment (concentrations of Brevibacterium sp. 1%, 3%, and 5% v/v; and incubation periods of 24 and 48 h) and interaction between factors (P<0.05)—the viability range for Brevibacterium Sp. InaCC B46 after 24 and 48 hours of incubation is between 144.18–216.99% and 115.57–126.69%, respectively, with the highest viability observed at a 1% concentration starter (v/v) after 24 hours and showing a significant increase at 48-hour incubation at 5% concentration.

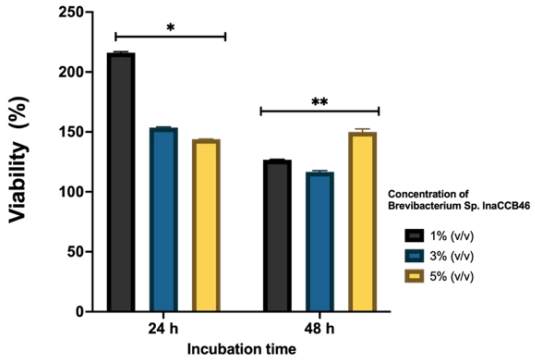

Fig. 1. Viability of Brevibacterium sp. InaCCB46 at different concentrations and incubation time.

Bacterial growth is dependent on an appropriate biochemical and biophysical environment. ANOVA analysis of viability Brevibacterium sp. B46 showed significant differences for each factor (concentrations of Brevibacterium sp. 1%, 3%, and 5% v/v; and incubation periods of 24 and 48 h), but no significant result of the interaction between factors (P>0.05) (Fig 1). A culture medium containing the necessary nutrients in appropriate amounts provides this environment. Microorganisms require a carbon source for growth and

development [14]. Brevibacterium, like most bacteria, uses glucose as a carbon source. According to [15], most Brevibacterium strains cannot consume lactose. Nevertheless, certain strains classified under the phylogenetic groupings Aurantiacum, Sandarakinum, Antiquum, Linens, Siliguriense, and Iodinum possess the genetic information for producing four specific enzymes involved in the Leloir pathway, which is responsible for the metabolism of galactose. The whole metabolic pathway for the breakdown of D-galactonate is found in strains belonging to the phylogenetic families Aurantiacum, Sandarakinum, Antiquum, Linens, Siliguriense, Iodinum, and Casei. The genes are organised in a cluster that contains the instructions for producing transcriptional regulators, 2-dehydro-3-deoxyphosphogalactonate aldolase [EC 4.1.2.21], 2-dehydro-3-deoxygalactonokinase [EC 2.7.1.58], galactonate dehydrase [EC 4.2.1.6], and one or two D-galactonate importers.

However, developing an efficient growth medium for cultivating bacteria remains a challenge. However, commercial media, including nutrient broth (NB), contain several growth-essential components such as carbohydrates, amino acids, peptides, vitamins, and Mg/Mn salts. The selective nature of the nutrient standard medium towards common microorganisms may require using an alternative medium to accumulate biomass and isolate a broad spectrum of microorganisms. Compared to other carbon sources, the strain presented rapid growth in the case of these two carbon sources. Lactose, fructose, and mannose is also well metabolised by the strain. In conclusion, the sources of carbohydrates contained in the saccharification product of OPTB not only consist of glucose but also some other monosaccharides or disaccharides.

The pathway for synthesising amino acids is mainly produced by the metabolic activities of bacteria in the growth medium (substrate). Glutamine and glutamate are direct products of ammonia and the assimilation of nitrogen, which synthesise other substrates. Amino acids are essential as protein precursors and precursors for other vital compounds, including polyamines, S-adenosylmethionine, pantothenic acid, and nucleotides [6]. Microorganisms can generate substantial quantities of amino acids through fermentation procedures in biotechnology. The findings about the interaction between the concentration of Brevibacterium Sp. InaCCB46 and the levels of amino acid biosynthesis (L-glutamate, L-alanine, L-glycine, and L-tyrosine) with 24 h and 48 h incubation times are presented in Figure 2.

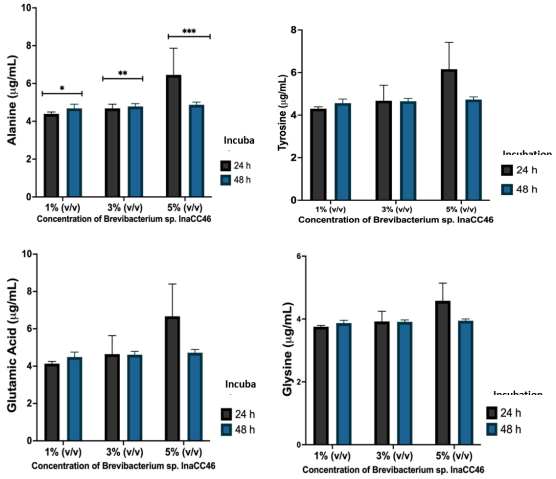

Fig. 2. Amino acid production of Brevibacterium Sp. InaCCB46 at different concentrations and incubation time.

ANOVA analysis of amino acid production (L-glutamate, L-alanine, L-glycine, and L-tyrosine) by Brevibacterium InaCCB46 showed no significant differences for each factor and

interaction between factors (P>0.05). However, there was a significant difference in incubation time (24 and 48 h) in alanine production results (P<0.05). It is hypothesised that higher bacterial concentrations lead to improved amino acid production. Furthermore, the incubation duration significantly influenced the overall yield of amino acids synthesised, leading to elevated concentrations of glycine, glutamic acid, alanine, and tyrosine compared to a 48-hour incubation period. Figure 3 shows that the Brevibacterium InaCCB46 strain in the medium displays a similar correlation pattern for each concentration of amino acids, including L-glutamate, L-alanine, L-glycine, and L-tyrosine. After 24 hours of incubation, the concentration of amino acids ranges from 3.76 µg/mL to 6.67 µg/mL among the Brevibacterium InaCCB46 concentrations. After 48 hours, the range is 3.87–4.87 µg/mL. At 24 hours of incubation, the highest amino acid concentration produced was glutamic acid (6.67 µg/mL), followed by alanine (4.87 µg/mL). Brevibacterium sp, *Corynebacterium glutamicum*, and *Escherichia coli* are capable of producing a wide range of amino acids, including commercially significant ones such as glutamic acid, methionine, tryptophan, lysine, tyrosine, phenylalanine, leucine, valine, arginine, and histidine [16]. Brevibacterium sp. is of great industrial interest because it produces various products such as amino acids (especially glutamic acid and lysine) and enzymes that are also important in cheese processing (ripening) [17].

In contrast, L-glycine produced the lowest concentration by Brevibacterium InaCCB46. In some methylotrophic bacteria, glycine is produced naturally by the SHMT reaction to assimilate C-1 compounds. The SHMT reaction fuels the serine cycle to produce C-3 compounds for anabolism and C-1 fixation. The acceptor glycine is the product of the glyoxylate regeneration cycle [18]. The pathway of glycine production from the serine cycle caused the level of glycine production in this research to be low.

Currently, alanine is produced from aspartate using enzymatic methods. However, microbial fermentation could be a promising alternative, using bacteria as a renewable and inexpensive raw material. Alanine dehydrogenase requires NADH to reduce pyruvate to L-alanine, making it the primary biosynthetic enzyme. NADH-dependent alanine dehydrogenase catalyses the synthesis of L-alanine from pyruvate. L-alanine is the end product of the EMP pathway. The alanine dehydrogenase enzymes are encoded by ald1 and ald2. As a result, bacterial strains that produce alanine tend to have these genetic codes. The biosynthesis of L-alanine was studied in detail in the bacterium Coryneform Sp. L-alanine can be synthesised from pyruvic acid via alanine transaminase (EC 2.6.1.2, L-alanine-2-oxoglutarate aminotransferase), which is considered to be the primary pathway, or from L-valine via valine-pyruvate transaminase (EC 2.6.1.66, L-valine-pyruvate aminotransferase) [19].

This study demonstrates the antioxidant activity of Brevibacterium sp. InaCC B46 to scavenging DPPH. ANOVA analysis of Brevibacterium sp. B46 showed significant differences for each treatment (concentrations of Brevibacterium sp. 1%, 3%, and 5% v/v; and incubation periods of 24 and 48 h) and interaction between factors (P<0.05) (Fig 3)—the scavenging DPPH (IC_{50} value) of Brevibacterium Sp. InaCC B46 after 24 and 48 hours of incubation is 22.43 ppm (powerful antioxidant) and 87.87 ppm (strong antioxidants), respectively. Reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), superoxide anion radicals (O_2*), and hydroxyl radicals (OH*), are formed during normal and pathological consumption of molecular oxygen. These free radicals may target many biological components, including DNA, resulting in harmful consequences and cell death. Living cells, including bacteria, defend themselves from oxidative harm through many defensive mechanisms, including the enzymatic conversion of ROS into less toxic molecules and detoxification by interaction with antioxidants.

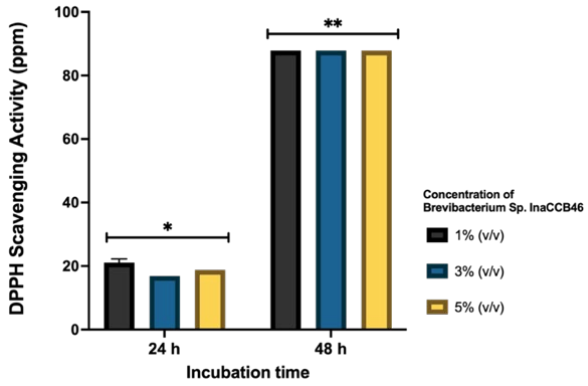

Fig. 3. DPPH Scavenging Activity (IC_{50}) of Brevibacterium Sp. InaCCB46 at different concentrations and incubation time.

Polysaccharides from some microorganisms can potentially possess antioxidant properties among other naturally occurring compounds. Brevibacterium are Gram-positive, aseptate, unbranched rods. The antioxidant properties of the exopolysaccharide produced by *Brevibacterium otitidis* have been documented [20]. The antioxidant capacity of the primary polysaccharide (BSMA) derived from *Brevibacterium otitidis* BTS 44 was examined. BSMA exhibited DPPH radical-scavenging activity in the in vitro antioxidant experiment, with an IC_{50} value of 120 µg/ml (estimated 12.000 ppm) [20]. According to [21], the natural compounds have powerful antioxidants if the IC_{50} value is <50 ppm, strong 50–100 ppm, moderate 100–150 ppm, and weak 150–200 ppm.

4 Conclusion

The study demonstrates that a 5% concentration of Brevibacterium sp. during a 24-hour incubation period is the most efficient condition for amino acid synthesis from glucose hydrolysed from oil palm trunk biomass. These findings provide strong evidence for the effectiveness of this method for amino acid synthesis. The results show that this condition resulted in the highest output of amino acids. Additionally, bacterial viability and antioxidant activity were higher after 24 hours.

We appreciate the research grant from LPPM Universitas Riau with contract number 8229/UN19.5.1.3/AL.04/2023.

References

1. Directorate General of Estates, Tree crop estate statistics of Indonesia 2018-2020 (2022)
2. R. Nabila, W. Hidayat, A. Haryanto, U. Hasanudin, D. A. Iryani, S. Lee, S. Kim, S. Kim, D. Chun, H. Choi, H. Im, J. Lim, K. Kim, D. Jun, J. Moon, and J. Yoo, Renewable and Sustainable Energy Reviews, **176**, 113193 (2023)
3. F. M. Said, N. F. Hamid, M. A. Razali, and N. F. S. Daud, Lignocellulosic of oil palm biomass to chemical product via fermentation in elaeis guineensis. IntechOpen (2022).
4. I. Y. Eom, J. H. Yu, C.D Biotechnol Biofuels, **8**,1 (2015)
5. M. P. Forquin and B. C. Weimer, Brevibacterium in Encyclopedia of Food Microbiology, Elsevier, (2014)

6. L. Reitzer, Encyclopedia of Microbiology, Elsevier (2009)
7. H. Zhang, X. An, Y. Zhou, B. Zhang, S. Zhang, D. Li, Z. Chen, Y. Li, S. Bai, J. Lv, W. Zheng, Y. Tian, and T. Zheng, PLoS One, **8**, 5 (2013)
8. M. Zhang, F. Wang, R. Su, W. Qi, and Z. He, Bioresour Technol, **101**, 13 (2010)
9. A. L. F. Santos, K. Y. F. Kawase, and G. L. V. Coelho, J Supercrit Fluids, **56**, 3 (2011)
10. S. Kamiloglu, G. Sari, T. Ozdal, and E. Capanoglu, Food Front, **1**, 3 (2020)
11. E. W. Yemm, E. C. Cocking, and R. E. Ricketts, Analyst, **80**, 948 (1955)
12. M. G. Shehata, M. M. Abu-Serie, N. M. Abd El-Azi, and S. A. El-Sohaimy, Inter J of Pharma, **15**, 6 (2019)
13. U. Pato, Y. Yusuf, E. Riftyan, E. Rossi, A. Syahrul, F. Muhammad, C.A. Ramadhana, L.M. Lestari S.P. Lasari, *Probiotic properties of Lactobacillus fermentum and Pediococcus pentosaceus encapsulated with or without hydrogel of cellulose microfiber from oil palm leaves*, in Proceedings V. Balkan Agricultural Congress, September 2023 Edirne, Turkey (2023)
14. S. A. Hayek, R. Gyawali, S. O. Aljaloud, A. Krastanov, and S. A. Ibrahim, J. of Dairy Res **86**, 4 (2019)
15. N. P. Pham, S. Layec, E. Dugat-Bony, M. Vidal, F. Irlinger, C. Monnet, BMC Genomics, **18**,1 (2017)
16. A. Iram, A. Ozcan, I. Turhan, and A. Demirci, Processes, **11**, 6 (2023)
17. J. Mounier, M. C. Rea, P. M. O'Connor, G. F. Fitzgerald, and T. M. Cogan, Appl Environ Microbiol, **73**, 23 (2007)
18. J. Z. Ye, M.L. Xiang, Z. X. Cheng 3, J. Proteomics, **183**, (2018)
19. L. H. Melkonyan, G. Y. Avetisova, A. A. Hambardzumyan, A. Kh. Chakhalyan, and A. S. Saghyan, Appl Biochem Microbiol, **49**, 2 (2013)
20. M. M. S. Asker and B. T. Shawky, Food Chem, **123**, 2 (2010)
21. R. Batubara, T. Surjanto, T.I. Hanum, A. Handika, O. Affandi, Biodiv **21**, 4 (2020)