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Substrate consumption and beta-galactosidase production by *Saccharomyces fragilis* IZ 275 grown in cheese whey as a function of cell growth rate

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ABSTRACT

Microfiltrated cheese whey permeate (CWP) was used as substrate by *Saccharomyces fragilis* IZ 275 yeast for the production of beta-galactosidase. Yeast growth kinetics was evaluated by lactose consumption, biomass production and, enzyme production. Lactose was almost depleted within 12 h of cultivation, only 11% of the substrate was left in the culture medium, indicating an average intake rate of 2.63 $g.L^{-1}.h^{-1}$ (lactose consumed/fermentation time). Biomass production increased with increasing cultivation time, reaching 11.06 $mg.mL^{-1}$ and a specific growth rate (μ) of 0.317.h^{-1} in 20 h of cultivation. The maximum yield of volumetric and specific beta-galactosidase activity was 14.28 $U.mL^{-1}$ and 0.039 $U.mg^{-1}$, respectively, and maximum lactose hydrolysis was obtained at 24 h of cultivation. This work evaluated the importance of the microfiltration process of cheese whey in *Saccharomyces fragilis* IZ 275 fermentation for beta-galactosidase production.

1. Introduction

Cheese whey (CW) is a byproduct of the dairy industry generated during cheese production and despite its low added value, has become an important component of many formulated products (Baldasso et al., 2011). The reuse of the byproduct as a raw material has garnered interest among researchers due to its nutritional, functional, economic, and biotechnological potential for metabolite production (Saini et al., 2017). The estimated world production of CW is over 160 million tons per year, with an estimated increase of 1–2% per year, along with dairy production (Sharma et al., 2018). The high organic content of CW makes it difficult to biodegrade and can be of concern to the environment if disposed of incorrectly (Yadav et al., 2015). Many processes for obtaining CW from natural products are being carried out, including powdered CW, concentrated CW proteins, and cheese whey permeate (CWP), which results in the production of liquid without proteins. The CWP is rich in organic and inorganic compounds, including lactose, which is a disaccharide composed of glucose and galactose (Santos et al., 2017). An alternative use of CWP is as a substrate in fermentation processes employing microorganisms such as bacteria, filamentous fungi, and yeast, to obtain commercially valuable products such as bioethanol, beta-galactosidase enzyme and galactooligosaccharide (Murari et al., 2019; Fai et al., 2014). The beta-galactosidase enzyme from yeast grown in CWP is an intracellular enzyme and its extraction with acceptable yields is difficult and costly. The use of permeabilized cells as biocatalysts is a viable alternative to the use of a purified enzyme (Panesar et al., 2007). Kinetic models can define the technical and economic viability of the fermentation process. They play an important role in monitoring and predicting the fermentation process related to cell growth, substrate consumption (lactose) and obtaining products of interest, as it provides information that predicts biomass production and the generation of products such as beta-galactosidase (Ariyanti and Hadiyanto, 2013).

Lactose intolerant individuals do not produce or produce insufficient amounts of beta-galactosidase in their bodies (Dzialanski et al., 2016; Ianiro et al., 2017). The increase in lactose intolerant population and the need for the production of lactose free products necessitates the production of the beta-galactosidase enzyme using fermentation medium

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with economical viability and that guarantees expressive production of the enzyme (Dekker et al., 2019). In this context, membrane microfiltration technology appears to be an alternative for obtaining microfiltrated CWP. The microfiltrated CWP is high in lactose and used as a substrate to produce beta-galactosidase enzyme. Microfiltration is employed for the separation of compounds, such as protein and lactose, and for the production of dairy derivatives (Hinkova et al., 2016). Thus, the objective of this study was to use microfiltrated CW as a culture medium and to evaluate beta-galactosidase enzyme production through yeast growth rate and lactose consumption.

2. Material and methods

2.1. Materials

Potato Dextrose Agar were purchased from Acumedia®. The cheese whey *in natura* were donated by Laticínio Volpato® (Arapongas, Parana - Brazil). The substrate o-nitrofenil-β-D-galactopiranosido (ONPG), was purchased from Sigma-Aldrich® (St. Louis, MO). All the other reagents were of analytical grade.

2.2. Microorganism and inoculum

The *Saccharomyces fragilis* IZ 275 yeast from the Andre Tosello Foundation Collection of Tropical Cultures – Collection of Tropical Cultures was used in the experiments. The stock culture was maintained at 4 °C in threaded tubes containing agar PDA (Potato Dextrose Agar, Acumedia®).Yeast colonies *Saccharomyces fragilis* IZ 275 were transferred to 250 mL Erlenmeyer flasks containing 50 mL of malt extract (15 $g.L^{-1}$). The flasks were incubated on an orbital shaker (Tecnal®, TE-420 – Brazil) at 35 °C and 150 rpm for 24 h. Optical density (O.D.) was determined at a wavelength of 570 nm using a spectrophotometer (Fento® 600 plus - Brazil) and at O.D. adjusted to 0.6.

2.3. Culture medium

Microfiltered cheese whey (CW) was used as a culture medium for fermentation. The CW *in natura* was filtered in a pilot-scale tangential – flow filtration process, adapted, with a microfiltration membrane of coupled ceramic of 1.4 μm porosity (TIA®, Araraquara, Brazil). The pH of the microfiltrated whey permeate was adjusted to 5.0 (Tecnal TEC-3MP – Sao Paulo – Brazil) with addition of 0,1 M HCl. Subsequently, it were distributed in 250 mL Erlenmeyer flasks with 100 mL of culture medium and then pasteurized at 65 °C for 30 min. After addition of 10% (v/v) inoculum (OD 670 nm = 0.6 OD) of *Saccharomyces fragilis* IZ 275 and incubated an orbital shaker (Tecnal®, TE – 420 – Brazil) at 35 °C, 150 rpm 24 h. Every 2 h, the erlenmeyer corresponding to the fermentation time was withdrawn and reserved for the analysis.

2.4. Cellular concentration and cell growth

The cell concentration was evaluated in terms of biomass or cell mass per volume of medium. Biomass determination after fermentation was done by withdrawing aliquots of 2 mL of the culture. Samples were centrifuged at 5000 rpm for 10 min over 3 cycles. The precipitate was resuspended in 0.85% (w/v) saline and centrifuged again. This operation was repeated twice (for complete washing of the cells). Biomass analysis was quantified gravimetrically with the dry weight of the cells. The centrifuged material was resuspended in 2 mL of 0.85% (w/v) saline and transferred to pre-weighed porcelain vials and stoved at 105 °C for 4–5 h until constant weight was reached. The value ($mg.mL^{-1}$) was given by the initial weight difference of the previously tared porcelain flasks.

The kinetic parameters of cell growth (biomass) were described according to the number of generations (n), the generation time (Tg) and the specific growth rate (μ), represented by equations (1)–(3), respectively.

$$= 3.33.(Log\ Nt - Log\ No) \quad (1)$$

where, n is the number of generations, No = number of initial cells and Nt = number of cells at time t

$$Tg = \Delta t / n \quad (2)$$

where, Δt = process time/culture (h) and n = number of generations.

$$\mu = \ln 2/Tg \quad (3)$$

where, μ = specific growth rate, Tg = generation time.

2.5. Cell permeabilization

Cell permeabilization was performed according to Morioka et al. (2016), with modifications. Aliquots with 40 mL of fermentation medium were centrifuged for 10 min, 5000 rpm at 4 °C. The supernatant was reserved for analysis of the lactose content and the biomass was permeabilized with the addition of a 35% (v/v) ethanol solution in 0.1 M potassium phosphate buffer pH 6.8 in a final volume of 10 mL. Permeabilization was performed for 20 min at 20 °C. The permeabilized biomass was resuspended in 10 mL of 0.1 M phosphate buffer, pH 6.8 and reserved for the enzymatic activity analysis through hydrolysis of lactose.

2.6. Analytical methods

2.6.1. Lactose content

The ability of the yeast to utilize the lactose present in the microfiltrated cheese whey was studied by determining the amount of initial

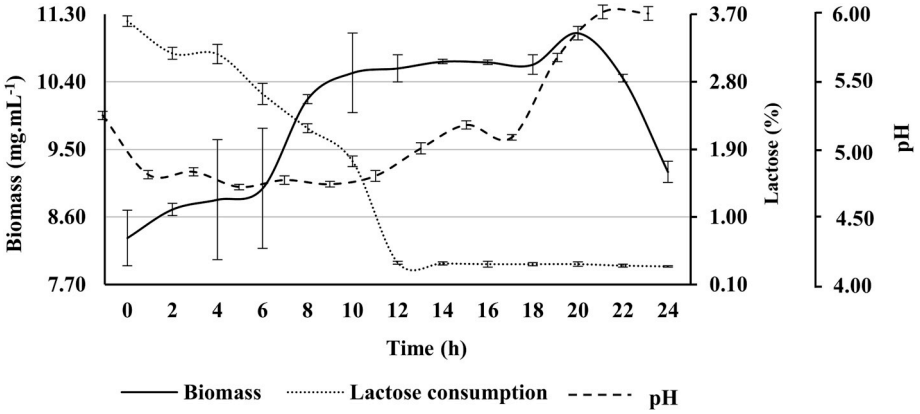

Fig. 1. Kinetics of biomass production, lactose consumption and pH during fermentation process *Saccharomyces fragilis* IZ 275 in CWP.

Table 1
Production of biomass ($mg.mL^{-1}$), volumetric enzymatic activity ($U.mL^{-1}$) and lactose hydrolysis (%) using microfiltrated cheese whey permeate.

Fermentation Time (h)	Biomass Mean± (dpm)	Volumetric enzymatic activity Mean±(dpm)	Lactose hydrolysis Mean±(dpm)
0	8.30±(0.36)	0.00±(0.00)	0.00±(0.00)
2	8.70±(0.10)	0.76±(0.00)	3.96±(0.35)
4	8.86±(0.64)	1.08±(0.07)	4.56±(0.75)
6	9.00±(0.64)	2.54±(0.04)	5.78±(0.87)
8	10.16± (0.00)	6.55±(0.20)	8.92±(0.61)
10	10.56± (0.30)	10.85±(0.04)	13.27±(1.58)
12	10.56± (0.15)	12.01±(0.42)	18.00±(1.55)
14	10.70± (0.00)	13.49±(0.18)	32.37±(1.87)
16	10.66± (0.57)	13.77±(1.48)	33.35±(1.18)
18	10.66± (0.15)	13.86±(0.65)	34.25±(0.18)
20	11.06± (0.11)	13.97±(0.34)	34.45±(0.62)
22	10.46± (0.05)	14.03±(0.28)	34.71±(0.24)
24	9.23±(0.15)	14.28±(0.23)	35.65 ±(0.09)

Mean standard deviation (dpm) of three replicates.

lactose prior to inoculation and after the incubation period. Quantitative determination of lactose in whey was performed by the method of Nickerson et al. (1975).

2.6.2. Specific enzymatic activity via ONPG

The specific enzymatic activity of *Saccharomyces fragilis* IZ 275 permeabilized cells was determined using the o–nitrophenyl–β–D-galactopyranoside substrate (ONPG), following the methodology described by Inchaurrondo et al. (1994). In a test tube containing 2 mL of 1.25 mM ONPG, prepared in 50 mM KH_2PO_4 phosphate buffer with 0.1 mM $MnCl_2.4H_2O$ pH 6.6, 50 µL of the enzyme solution was added. The solution was maintained at 37 °C for 5 min in a heating bath (Fisatom® 550 – Sao Paulo – Brazil), and then by adding 0.5 mL of 1 M Na_2CO_3. The absorbance in spectrophotometer (Femto® 600 Plus – Sao Paulo – Brazil) was then determined at 420 nm. The enzymatic activity was calculated from a calibration curve of o–nitrophenol. In order to verify the effect of cell concentration on enzymatic activity, the unit of activity was expressed per milligram of cell mass, that is, $U.mg^{-1}$. Thus, the specific enzymatic activity ($U.mg^{-1}$) was defined as 1 µmol o–nitrophenol produced per minute of reaction per milligram of dry biomass under the conditions tested.

2.6.3. Enzymatic activity via lactose hydrolysis

For lactose hydrolysis evaluation, 1% of the permeabilized cells (biocatalysts) were added to whole milk and taken in a heating bath at 37 °C for 4 h. Then the permeabilized cells were taken in a water bath for 5 min to stop the hydrolysis reaction. The percentage of hydrolysis was determined by the initial rate of the lactose hydrolysis reaction, through the glucose oxidase method, using the Glucose Kit (Bioliquid®). The absorbance measured in spectrophotometer (Fento® 600 plus – Brazil) at 505 nm. Calculation of enzymatic activity was determined by the method of initial rates of hydrolysis reaction of lactose using Equation (4).

$$EA = \frac{GC}{180} \times 1000 \div HT \quad (4)$$

where: EA corresponds to volumetric enzymatic activity ($U.mL^{-1}$); GC, glucose concentration ($mg.dL^{-1}$) obtained after the hydrolysis of lactose; 180, molecular weight of glucose; 1000 factor to convert to micromol of glucose and HT, hydrolysis time (h). The unit of the enzymatic activity (U) can be defined as micromoles of glucose produced per hour at 37 °C, pH 6.5 and initial lactose concentration (5%).

After that, the percentage of hydrolysis was calculated according to equation (5):

$$\%\ hydrolysis = (GC \times 2 \div 1000) \div Lac \times 100 \quad (5)$$

where: % hydrolysis; GC, sample glucose concentration; number 2 corresponds to a glucose molecule after the hydrolysis of lactose; 1000 to convert mg to g; LAC, initial concentration of lactose and 100, convert to percentage.

3. Results and discussion

The growth kinetics for biomass production, lactose consumption, and beta–galactosidase enzymatic activity were evaluated from *Saccharomyces fragilis* IZ 275 fermentation in microfiltrated CWP.

Fig. 1 shows that the biomass production gradually increases with the increase in the cultivation time. The delay phase occurs immediately after inoculation, the cells take approximately 2 h to adapt to the new culture condition. After the adaptation period, the cells reached the exponential growth phase with a specific growth (µ) of $0.317.h^{-1}$ which is slightly above the maximum specific growth rate recorded in other studies, such as those obtained by Lukondeh et al. (2005) and Ariyanti and Hadiyanto (2013) where the µ was $0.27.h^{-1}$ and $0.133.h^{-1}$, respectively. The decrease of nutrient content in the culture medium and yeast metabolism may explain the difference of the results found.

Of the initial lactose concentration in the culture medium, $3.56\ g.L^{-1}$ was almost completely consumed by the yeast in 12 h, with 0.39% remaining at the end of the process. Indicating an approximate lactose consumption of 89% (w/v). This data can be confirmed from the lactose intake curve, which shows a decline from beginning to the end of fermentation. Only 11% of lactose was detected in the culture medium at the end of the process, indicating an average consumption rate of $2.63\ g.L^{-1}.h^{-1}$ (lactose consumed/fermentation time). During this same fermentation time, $10.56\ mg\ mL^{-1}$ biomass was produced, an increase of 79% (w/v) from the initial biomass and calculated specific enzymatic activity of $0.03\ U.mg^{-1}$. Kumari et al. (2019) studied the production of beta–galactosidase in CW by *Kluyveromyces* sp. yeast through statistical modelling and concluded that lactose was fully consumed within 35 h of cultivation. Anvari and Khayati (2011) in a study using ultrafiltered CW showed that the biomass produced by *Kluyveromyces marxianus* also increased with cultivation time ($12.68\ mg.L^{-1}$), and the lactose concentration was reduced by 98.2% after 28 h of fermentation. Kundu et al. (2012) evaluated the growth of *K. marxianus* MTCC 4059 and confirmed that with the cultivation time, the cell concentration increases while the lactose concentration decreases. The maximum biomass production obtained by the researchers was $8.38\ mg.L^{-1}$ after 29 h of fermentation. In this case, the enzymatic activity of lactose metabolism beta-galactosidase ($U.mg^{-1}$), $YP.S^{-1}$ was 0.012 $mgP.mgS^{-1}$, while the maximum biomass yield ($YX.S^{-1}$) from initial lactose was 0.86 $mgX.mgS^{-1}$ at 20 h of fermentation (P = The yeild of the product, S = Consumed substrate). Lukondeh et al. (2005) tested different lactose concentrations contained in CW (40 and 60 $g.L^{-1}$) and obtained the same values for µmax of $0.35.h^{-1}$ and biomass yield ($YX.S^{-1}$) of 0.41 $g.g^{-1}$. The difference between the results obtained in this study is related to the different conditions of cultivation and modelling.

Santiago et al. (2004) performed fermentations with *K. marxianus* ATCC 46537 and observed a beta–galactosidase enzyme activity of 28 $U.mL^{-1}$ and a maximum cell concentration of $5.3\ mg.mL^{-1}$. Differences in biomass and/or metabolite concentrations of interest may be due to variations in the initial substrate concentration. In high lactose concentrations, the cells are less able to assimilate carbon source, probably due to the change in the lactose permease system. The growth kinetics of *Saccharomyces fragilis* IZ 275 yeast, showed that the cell concentration and specific enzymatic activity during 12–20 h fermentation ranged

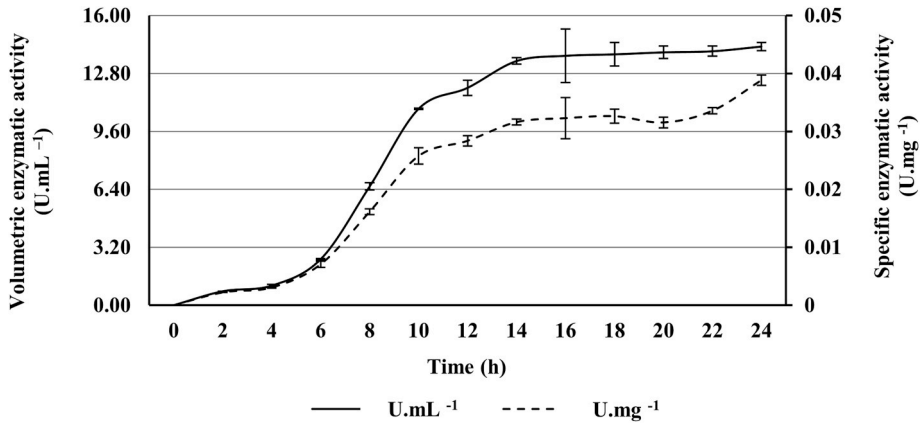

Fig. 2. Batch growth kinetics of *Saccharomyces fragilis* IZ 275 in cheese whey permeate. Enzymatic activity of volumetric beta–galactosidase ($U.mL^{-1}$) and specific ($U.mg^{-1}$). The data points represent the mean values of samples collected at time (h) indicated from the culture.

between 10.56 and 11.06 $mg.mL^{-1}$ and 0.028–0.039 $U.mg^{-1}$, respectively. The lactose medium was completely consumed and remained at a low level at the end of 24-h fermentation. Thus, it may be suggested that beta–galactosidase enzymatic activity is a process associated with cell growth.

The pH change of the culture medium was monitored throughout the fermentation. Fermentation started at pH 5.0, and after the addition of the inoculum during the adaptation phase, it decreased to pH 4.67 and remained almost unchanged until 14 h of fermentation. Then a gradual increase in pH was observed, reaching 5.80 at the end of fermentation. Santiago et al. (2004) also observed a decrease in pH during fermentation of CW with *K. marxianus* ATCC 46537. Decreasing pH may indicate an increase in acidity of fermentation medium, possibly due to the production of organic acids. Schneider et al. (2010) stated that the decrease in pH might be associated with the accumulation of CO_2 from the metabolism of the microorganism.

Table 1 shows the correlation between biomass production, volumetric enzymatic activity and lactose hydrolysis. A similar trend is observed for all three responses.

Lactose enzymatic activity and hydrolysis gradually increased with fermentation time, reaching 12.01 $U.mL^{-1}$ and 18%, respectively, within 12 h of fermentation. In 20 h of fermentation, these parameters are observed to be directly related to biomass production. As the biomass concentration increased, the lactose enzymatic and hydrolysis activity also increased. Previous results obtained from this research showed that yeast was able to hydrolyse 90% of lactose using 12% (v/v) permeabilized cells in 4 h of hydrolysis reaction (data not shown). In the fermentation medium with lactose as the sole source of carbon, synthesis of *Saccharomyces fragilis* beta–galactosidase enzyme is associated with population growth. The results show higher enzymatic activity in the late exponential and the steady growth phases. According to Guimarães et al. (2008), higher the cell growth, higher the production of beta–galactosidase enzyme, which explains the higher enzymatic activity and the percentage of hydrolysis achieved with higher growth and substrate conversion by the cells. Ornellas et al. (2008) stated that the increase in beta– galactosidase enzymatic activity is related to the ideal concentration of lactose contained in the culture medium, and the decrease in activity is related to physiological factors, such as response to nutritional stress and secondary metabolism of products such as ethanol.

Manera et al. (2008) selected *Kluyveromyces marxianus* CCT 7082 yeast for the production of beta–galactosidase enzyme using lactose P.A. as carbon source and obtained maximum response after 50 h of cultivation. The authors observed that maximum beta–galactosidase production is directly related to biomass production, total lactose consumption and lower pH. It should be noted that the difference in results reported by different authors is due to the difference in the strains and the composition of the culture medium used in each study.

The kinetics of permeabilized *Saccharomyces fragilis* IZ 275 for lactose hydrolysis (%), in the shake flask batch culture were examined by growing in the cheese whey with 3.56 $g.L^{-1}$ of initial lactose. As seen in Fig. 2, specific enzyme activity curve exhibited two distinct sharp points at the beginning, corresponding to the exponential growth phase, and at the end of the growth phase, corresponding to the stationary phase. Both the volumetric ($U.mL^{-1}$) and specific activity ($U.mg^{-1}$) remained low at the beginning of the cultivation and then increased giving a peak at the exponential growth phase. Fig. 2 shows the enzymatic activity profile of volumetric ($U.mL^{-1}$) and specific ($U.mg^{-1}$) beta-galactosidase over 24 h of fermentation. It was observed from the volumetric enzyme activity curve (Fig. 2) that the enzyme production was associated with the biomass production and the maximum volumetric activity (14.28 $U.mL^{-1}$) was obtained at the 24 h of fermentation. During the exponential growth phase, volumetric activity increased reaching a value of 12.01 $U.mL^{-1}$ at the early stationary phase and increased continuously throughout the stationary growth phase reaching a value of 14.28 $U.mL^{-1}$, see Table 1. Flores et al. (1994) studied the permeabilization of *Kluyveromyces lactis* cells by chloroform, toluene and ethanol in relation to beta–galactosidase activity. They found that the performance of those solvents was dependent on the incubation time, the incubation temperature and the concentration of both cells and solvents.

Within 12 h of fermentation, volumetric and specific enzymatic activity was 12.01 $U.mL^{-1}$ and 0.028 $U.mg^{-1}$, respectively with 18% lactose hydrolysis (see Table 1). Within 24 h of fermentation, the volumetric and specific enzymatic activity was 14.28 $U.mL^{-1}$ and 0.039 $U.mg^{-1}$, respectively, with approximately 36% lactose hydrolysis. The peak activity of beta–galactosidase near the late exponential growth phase agreed with the presence of the inducer (lactose) in the culture medium. However, the peak of beta– galactosidase activity occurred during the stationary phase when lactose was fully consumed (see Fig. 1.). Manera et al. (2008) found that the maximum activity of beta–galactosidase occurred after 50 h of cultivation at a pH of 5.5 to 4.2 after 12 h of fermentation by *Kluyveromyces marxianus* CCT 7082, reaching values greater than 2.8 $U.mL^{-1}$. Martins et al. (2002) studied the production of beta–galactosidase enzyme from *Kluyveromyces marxianus* and observed that the specific enzyme activity increased and attained a maximum after 4 h of incubation. They found a second phase of high specific activity after 16 h of incubation where the culture entered the stationary phase of growth. Rajoka et al. (2003) studied the production of beta–galactosidase enzyme from *Kluyveromyces marxianus* in shake flask cultures and reported that the bulk beta– galactosidase production reached a maximum activity after 30–40 h and the enzyme production was apparently growth associated.

4. Conclusion

Microfiltrated CW is been shown to be an efficient culture medium for the production of beta–galactosidase. *Saccharomyces fragilis* IZ 275 yeast is able to metabolize 89% lactose present in the culture medium within 12 h of fermentation with enzymatic production of 12.01 U. mL^{-1}. It is very important the kinetics studies about fermentative process due to the productivity and economic importance of beta-galactosidase.

Conflicts of interest

The authors declare that they have no conflict of interest.

Submission declaration

The present work has not been published previously in any form and is not under consideration for publication elsewhere.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101335.

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