Laser biosensor use for the microbial metabolic activity assessment of kefir vinegar

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Abstract – The objective of this work was to verify the use of a laser biosensor for the microbial metabolic activity assessment of kefir vinegar. Kefir grains were inoculated in apple must and were analyzed daily using the biospeckle technique. The initial biological activity was of 14.21 pixels per absolute value of differences (AVD). The biological activity of kefir grains decreased in the early days of fermentation and increased on the fourth day to 11.51 pixels per AVD; however, on the last day, the biological activity of kefir grains decreased to 7.12 pixels (alcoholic fermentation) and to 6.77 pixels per AVD (acetic fermentation). This new biospeckle methodology facilitates the microbiological control in fermentation processes.

Index terms: biosensor, biospeckle, kefir grains, vinegar.

Uso de biossensor a laser para avaliação da atividade metabólica microbiana do vinagre de kefir

Resumo – O objetivo deste trabalho foi verificar o uso de biossensor a laser para a avaliação da atividade metabólica microbiana do vinagre de kefir. Os grãos de kefir foram inoculados em mosto de maçã e analisados diariamente pela técnica de biospeckle. A atividade biológica inicial foi de 14,21 pixels por valor absoluto das diferenças (AVD). A atividade biológica dos grãos de kefir diminuiu nos primeiros dias de fermentação e aumentou no quarto dia para 11,51 pixels por AVD; no entanto, no último dia, a atividade biológica dos grãos de kefir diminuiu para 7,12 pixels (fermentação alcoólica) e 6,77 pixels por AVD (fermentação acética). Esta nova metodologia do biospeckle facilita o controle microbiológico em processos fermentativos.

Termos para indexação: biossensor, biospeckle, grãos de kefir, vinagre.

Kefir grains consist of rich bacterial (lactic and acetic) and yeast microbiota responsible for the production of this traditional fermented milk beverage with unique flavour properties (Simova et al., 2002; Magalhães et al., 2010; Puerari et al., 2012; Corona et al., 2016; Roos & Vuyst, 2018). They are a mixed culture of various yeast species of the genera *Kluyveromyces, Candida, Saccharomyces*, as well as lactic acid bacteria of the genus *Lactobacillus*, and acetic acid bacteria of the genus *Acetobacter,* combined in a matrix of proteins and 'kefiran' polysaccharide, which are formed during cell growth under aerobic conditions (Simova et al., 2002; Magalhães et al., 2010; Corona et al., 2016; Cho et al., 2018; Roos & Vuyst, 2018).

An optical technique with potential use in biological metrology, particularly in biological activity, is the dynamic laser biosensor or biospeckle, associated to biological material (Braga Jr et al., 2003; Guedes et al., 2014). When a laser beam is scattered by a biological sample, the scattered waves generated in the illuminated sample create the speckle pattern that changes its image in accordance with the changes in the monitored material. Thus, the surface appears to be covered with tiny bright dots that fluctuate in a

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seemingly random way as for a boiling liquid. Many efforts have been devoted to characterize quantitatively the biological material activity (Zdunek et al., 2014) for seed viability (Braga Jr et al., 2003) and for meat maturation (Amaral et al., 2013).

The objective of this work was to evaluate the laser biosensor in the microbial metabolic activity assessment of kefir vinegar.

Brazilian kefir grains from the stock-culture of the microbiology laboratory of the Universidade Federal de Lavras, Lavras, MG, Brazil, and apple fruit obtained from the market of the city were used in the experiments. Apple pulps were extracted using an automatic pulping machine (Itametal 0.5 DS, Itabuna, BA, Brazil). The initial apple must was inoculated with kefir grains at 10% w/v, with three replicates. Flasks were incubated statically at 28°C, and fermentation was monitored daily to observe the end of the fermentation (consumption stability of sugars – Brix in 5 days). Fermentation must was filtered by a Kitasato filter (0.5 µm). The kefir grains were recovered. To determine the fermentation performance, the following parameters were determined: the substrate conversion factor in ethanol (Yp/s); the substrate conversion in glycerol (Yg/s); the substrate conversion in acetic acid (Yac/s); the ethanol volumetric productivity of ethanol (Qp); biomass productivity (Px); and conversion efficiency (Ef). The total sugar concentration was calculated considering the conversion for each mole of sucrose (342 g) in 1 mol of glucose (180 g) and 1 mol of fructose (180) (Duarte et al., 2010).

Fermented apple must obtained in the previous fermentation was acetified in 500 mL Erlenmeyer flasks, in a controlled temperature of 28°C and agitation of 150 rpm, with three replicates. Acetic fermentation was conducted using the following treatments: kefir grains at 10% (w/v) and kefir grains at 20% (w/v). During the acetic fermentation, samples were taken daily (6 days) in triplicate for acidity (pH meter) and alcohol (alcoholometer) analyses. The acetic fermentation finished on the sixth day when vinegar showed alcohol content below 1.0% (v/v). The yield was calculated as the acetic acid produced in relation to the theoretical yield (Maal et al., 2010). The theoretical yield was calculated as the amount of ethanol converted to acetic acid, in which 1.0 g ethanol yields 1.304 g of acetic acid (Maal et al., 2010). Kefir-apple vinegar was then filtered through diatomaceous earth, bottled, and pasteurized at 65°C for 20 min. The samples were analyzed by optical microscopy (National Optical 131 Microscope, Hamburg, Germany) to evaluate the vinegar clarity.

Daily analyses of kefir grains were performed by a laser biosensor (laser biospeckle) designed and developed at the Universitat Politècnica de València, in Spain, to observe the biological activity. Kefir grains were illuminated by a HeNe laser (Prophotonix Limited, Salem, NH, USA), at 632 nm wavelength and 10 mW power, enlarged by a plane concave lens to cover the entire sample. Interference patterns formed on them were captured by a CCD camera of 640 × 486 pixels (Systems Ltd, Chennai, India), with a 1/60 s shutter speed, at 0.08 s rate, creating a collection of 128 images. The analysis of the images, from the laser illumination, was performed according to Guedes et al. (2014). Image analyses from the laser illumination were performed by monitoring the temporal history of the speckle pattern (THSP) and its numerical output, the values of the differences (AVD) (Guedes et al., 2014):

$$\mathrm{AVD} = \sum_{ij} \frac{\mathrm{OCM}.\left|i - j\right|}{\mathrm{Normalization}},$$

in which: OCM is the occurrence matrix of the successive values in the THSP; and i and j variables are the dimensions of the OCM matrix. The normalization provides the relation between the AVD values and the summation of all occurrences. Eight sessions of illumination were performed in four grains of kefir, with three replicates, during the whole fermentation process. Each set of 128 frames were tested for homogeneity, and the cropped area was processed by AVD method.

The biological activity of kefir grains obtained 14.21 pixels per AVD. This value was considered for the initial fermentation time (0 h). The image of kefir grains is presented with an illustration of the yellow and red windows where the laser was illuminated and the images assembled (Figure 1 A and B). In the alcoholic fermentation, the biological activity of kefir grains decreased in the early days of the fermentation process (Figure 2 A). The decrease of biological activity persisted until the third fermentation day. On the fourth day, the biological activity of kefir grains in apple must increased up to 11.51 pixels per AVD. On the last day of fermentation, the biological activity of kefir grains decreased to 7.12 pixels per AVD. The acetic fermentation occurred subsequently to the alcoholic fermentation. In the acetic fermentation, the biological activity of kefir grains decreased in the early days of the fermentation process for treatments 1 and 2 (Figure 2 B). The decrease of the biological activity persisted until the third day of fermentation. On the fifth day, the biological activity of kefir grains increased up to 8.01 pixels per AVD in the treatment 1, and 8.79 pixels per AVD in the treatment 2. On the last day of fermentation, the biological activity of kefir grains decreased to 6.36 pixels per AVD in treatment 1, and 6.77 pixels per AVD in treatment 2. The biological activity values in the present study represents the kefir grains microbial development during the fermentation process. The kefir grains microorganisms showed a growth curve with defined phases (Lag = adaptation, Log = exponential growth, stationary growth, and death). This fact can be visualized in the present study, through of the biological activity analysis by laser biospeckle.

The final alcoholic must showed 3.82 pH value, and 4.37 ºBrix. The values of the kinetic parameters obtained from the alcoholic apple must are described as ethanol productivity (Yp/s) 0.47 g g^{-1}, yield glycerol

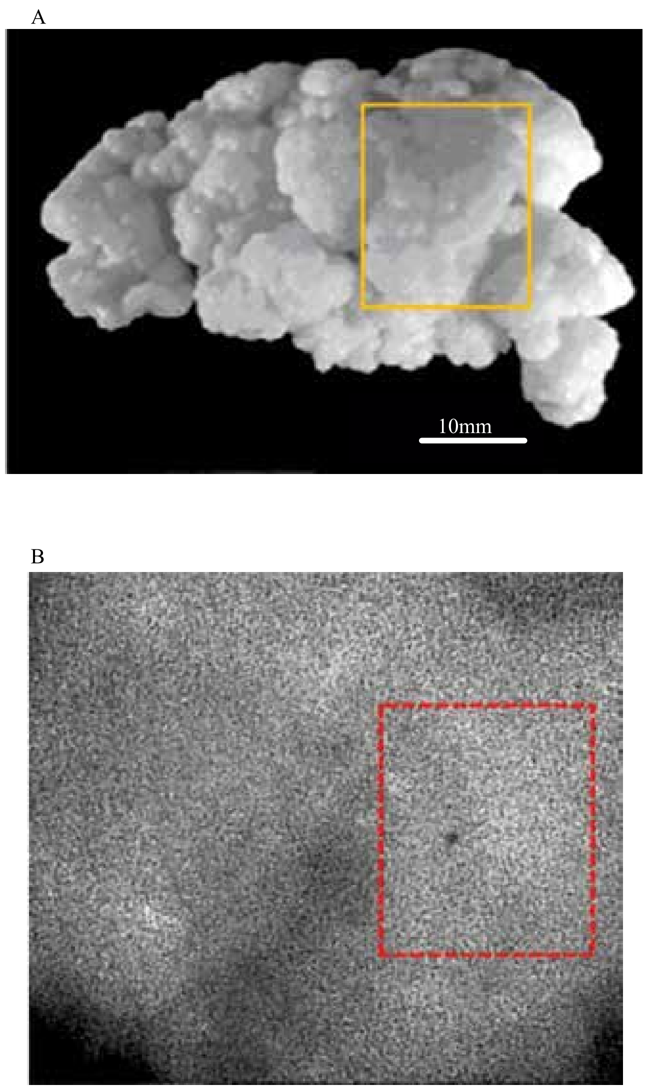

Figure 1. Biological activity of kefir grains by laser biospeckle technique: A, kefir grains; B, image formed by laser biospeckle (captured by a CCD camera of 640 × 486 pixels (Systems Ltd, Chennai, India), with a shutter speed of 1/60 s at rate of 0.08 s).

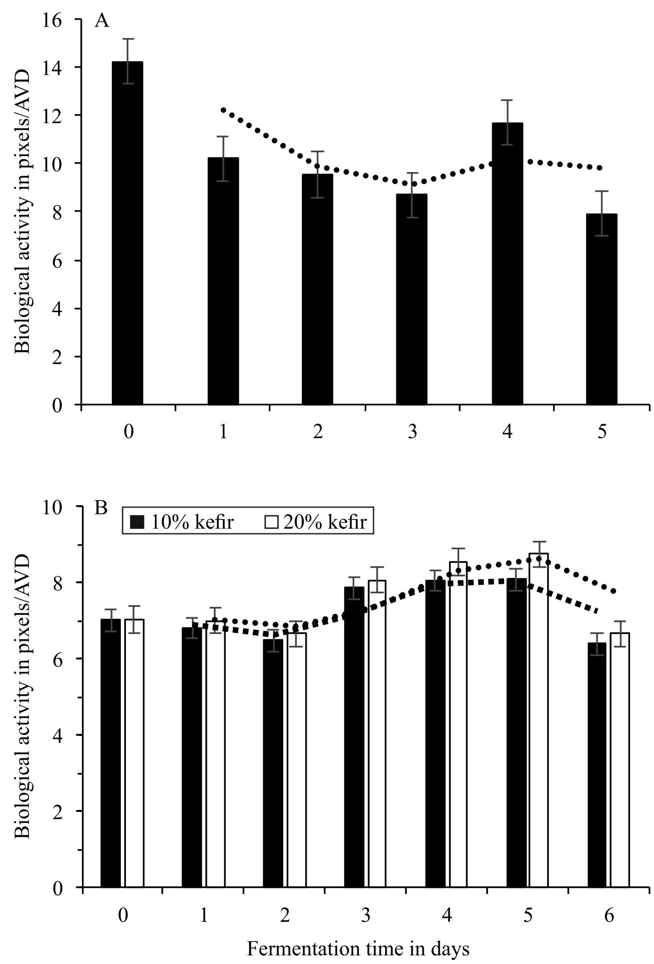

Figure 2. Biological activity of kefir grains by laser biospeckle technique: A, alcoholic fermentation; B, acetic fermentation, with 10% kefir inocula, 20% kefir inocula. AVD, absolute value of the differences.

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(Yg/s) 0.05 g g^{-1}, ethanol yield (qp) 0.37 g L^{-1} h^{-1}, and fermentation efficiency (Ef) 93.12%.

Acetic acid yielded in the kefir vinegars was ~79%. The total acetic acid concentration was ~41.00 g L^{-1}, reaching the required standard by the Brazilian law to accepted it as vinegar (4.0% acetic acid) (Brasil, 2009). Kefir vinegars showed a clear appearance and had a good color (pale yellow) according to Brazilian law (Brasil, 2009).

The biological activity of kefir grains was shown by laser biospeckle during the fermentation process. The sensitivity of the laser biospeckle technique was observed in the following of a growth curve with the defined phases Lag (adaptation) and Log (exponential growth, stationary growth and death) (Figures 2 A and 2 B). These results were similar to the those obtained by Guedes et al. (2014), whose aim was to show that the biospeckle laser is a potential methodology to assess the biological activity of kefir grains, monitoring them during the production of milk beverage. Zdunek & Herppich (2012) used biospeckle to monitor the chlorophyll biological activity in apples, to evaluate the potential relationships between the biospeckle activity and chlorophyll content in apples. The experiment showed that, for apples, the biospeckle activity linearly decreases with increasing chlorophyll content. Vinegar could be successfully produced from alcoholic apple must using kefir grains. The biospeckle technique measures the biological activity of kefir grains in the vinegar fermentation. Its key point for industrial application is the promotion of the microbial control in the fermentation process, which provides the possibility of eliminating the use of traditional methodologies that require a high-industrial investment.

The new biospeckle methodology facilitates the microbiological control in the fermentation process using kefir grains as starter cultures.

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