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Development of Fermented Teff-Based Probiotic Beverage and Its Process Monitoring Using Two-Dimensional Fluorescence Spectroscopy †

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Abstract: This study aims to evaluate a teff-based substrate (hereinafter substrate) for its suitability to carrying probiotics, *Lacticaseibacillus rhamnosus* GG (LCGG) and *Lactiplantibacillus plantarum* A6 (LPA6). Also, a two-dimensional (2D) fluorescence spectroscopy was applied to monitor the fermentation process by analyzing its spectral data using a partial least-squares regression (PLSR) and an artificial neural network (ANN). The fermentation process parameters time and inoculum were optimized to 15 h and 6 log cfu/mL, respectively. During a fermentation run by using the optimized parameters, cell counts of LPA6 and LCGG were increased from 6 to 8.42 and 8.11 log cfu/mL, respectively. Values of pH, titratable acidity (TA), lactic acid, and acetic acid were measured in ranges of 6.13–3.92, 0.37–1.5 g/L, 0–1.7 g/L, and 0.04–0.23 g/L, respectively. Glucose were progressively consumed throughout fermentation. For the prediction of cell counts of LPA6 and LCGG, relative root mean square error of predictions (pRMSEP) were measured between 0.25 and 0.37%. Also, for lactic acid prediction, a pRMSEP values of 7.6 and 7.7% were obtained. Findings of this research showed that cell counts of LPA6 and LCGG, and content of lactic acid could be predicted accurately by a 2D fluorescence spectroscopy coupled with PLSR and ANN. Moreover, whole teff flour alone would be served as a substrate to develop a probiotic rich beverage.

Keywords: functional beverage; *Lactiplantibacillus plantarum*; *Lacticaseibacillus rhamnosus*; probiotic; teff flour; 2D fluorescence spectroscopy

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1. Introduction

The recent consumers' health awareness is increasing and it has changed their food preferences to functional foods [1]. Functional food contains a biologically active ingredients such as probiotics with an adequate amount to provide a health benefit to the consumers [2]. Probiotic containing foods are considered the most important functional foods to meet the current consumers demand [1]. Beverages can be used as an important medium to incorporate the desired bioactive components like probiotics since they are suitable in handling and refrigerated storage [3]. Also, acceptance of non-dairy-based products have been increasing due to the uprising of vegetarianism and the limitations related to dairy-based products. Specially, gluten free cereal products are getting attention in worldwide since they are suitable to gluten sensitive consumers and they are vegetarian

friendly [4]. For this aim, teff would be a good alternative as it is one of the most important gluten free cereal, which is a staple food ingredient in Ethiopia [5].

Fermentation process is a vital unit operation to produce probiotic beverage, which is usually using high performance liquid chromatography for the analysis of the process. However, this way of analysis is time consuming, labor intensive, and costly. Likewise, plate count agar is using commonly for viable microbial counting, which is a tedious work. Recently, an actual time and effective control of a fermentation process is considered as an essential way to increase efficiency and improve quality of the final products [6]. For this intention, an alternative would be the usage of 2D fluorescence spectroscopy, which is a nondestructive way of analysis [7]. In fluorescence measurements, usually important information can't be collected in specific part of the spectrum, but distributed throughout the spectroscopic data set. So, for the extraction of important information, multivariate data analysis are always applied [8].

Then, this research aims the application of 2D-fluorescence spectroscopy for the online monitoring of a teff-based substrate fermentation inoculated with co-culture strains of LPA6 and LCGG. For the fluorescence spectral data evaluation, PLSR and ANN were used. The analysis target components were cell counts of LPA6 and LCGG, and contents of lactic acid and glucose.

2. Materials and Methods

2.1. Starter Culture

Starter cultures of LPA6 (LMG 18053, BCCM, Gent, Belgium) and LCGG (LMG 18243, BCCM, Gent, Belgium) were prepared according to the method used by [9]. Strains of LPA6 and LCGG were incubated for approximately 24 h in a refrigerated incubator (BINDER GmbH, KB 115, Tuttlingen, Germany) in sterile MRS (DE MAN, ROGOSA and SHARPE) broth. Starter culturs were put in a refrigerator (4–6 °C) until utilization within 48 h.

2.2. Off-Line Measurement of Cell Count and Fermentation Condition

Samples containing LPA6 were prepared with a serial ten-fold dilutions using a saline solution. Then, with a calibrated micropipette a prepared dilutions were spread on MRS agar plates, and put in an inubator for 24 h at 30 °C [10]. Similar procedure was followed for cell count determination of LCGG, but incubated for 48 h. Moreover, the selective enumeration of mixed strains of LPA6 and LCGG was conducted according to the method described by ST Alemneh, SA Emire and B Hitzmann [9]. A 6 log cfu/mL starter co-cultures of LPA6 and LCGG were inoculated to the fermentation medium, which was prepared by mixing 7 g whole grain teff flour within 100 mL distilled water. Samples for the analysis of cell counts of LPA6 and LCGG, glucose, and lactic acid were taken in 2 h interval since from the first 3 h fermentation.

2.3. Off-Line Measurement of Glucose and Lactic Acid

Analysis of glucose and lactic acid were performed using high performance liquid chromatography (HPLC). The collected samples' supernatant after centrifugation (3000× *g*, 4 °C) for 15 h was filtered using 0.45 μm polypropylene membrane (VWR, Darmstadt, Germany). Then, the supernatant was analyzed by using HPLC (ProStar, Variant, Walnut Creek, CA, USA), which was connected with refractive index detector. A 20 μL supernatant samples were injected into a Rezex ROA-organic acid H^+ (8%) column (Phenomenex, Aschaffenburg, Germany) worked at 70 °C. A 5 mM H_2SO_4 was used as a solvent with a flow rate of 0.6 mL/min. For the calculation of the lactic acid and glucose contents, a chromatography software Galaxie™ (Varian, Walnut Creek, CA, USA) was used.

2.4. On-Line Measurement Using 2D-Fluorescence Spectroscopy

A 2D-fluorescence spectra were collected using a BioView sensor (DELTA Lights & Optics, Venlighedsvej 4, 2970, Horsholm, Denmark). A sensor measured multi-wavelength combinations of excitation (270–550 nm) and emission (310–590 nm). A resulted spectra consisted of 120 wavelength combinations with their corresponding intensity values. For the evaluation of the spectral data PLSR and ANN were used. Software Unscrambler X version 10.3 (CAMO Software AS., Oslo, Norway) and MATLAB R2019a version 9.6 (The MathWorks Inc. 2019, Natick, MA, USA) were utilized to build a calibration and prediction models.

3. Results and Discussion

3.1. Optimization of Fermentation Process

Nelder-Mead simplex method was followed for the optimization of fermentation process using the influencing variables time from 9 to 15 h and inoculum from 5 to 7 log cfu/mL. Objective of the optimization process was to obtain a maximum value of the quality function (QF) (Equation (1)).

$$\mathrm{QF} = \left(\frac{\text{Cell count in } \log \frac{\mathrm{cfu}}{\mathrm{mL}}}{8 \log \frac{\mathrm{cfu}}{\mathrm{mL}}} \right) + \left(\frac{4}{\mathrm{pH}} \right) \quad (1)$$

Here, in our previous work [9], the denominator 8 log cfu/mL and numerator 4 were chosen since cell counts of LPA6 arrived at stationary phase when their counts were determined of about 8 log cfu/mL with approximately 4 pH. Results of the quality function were measured from 1.8 to 2.0. In total, eight experiments were executed among which the sixth experiment was taken as the maximum value of quality function. Among the eight experiments, results of the quality function at the 3rd, 4th, 7th, and 8th experiments didn't show a significant difference ($p < 0.05$). The obtained optimum parameters were time (15 h) and inoculum size (6 log cfu/mL). At the point where the optimum variables taken, values of quality function, cell counts of LPA6, and pH were documented as 2, 8.4 log cfu/mL, 4.2, respectively.

3.2. Co-Culture Strains Fermentation

Using the optimized parameters, the fermentation of substrate inoculated with co-culture strains of LPA6 and LCGG was performed. Results of cell counts of LPA6 and LCGG, pH, and titratable acidity (TA) during the 15 h fermentation are displayed in Figure 1.

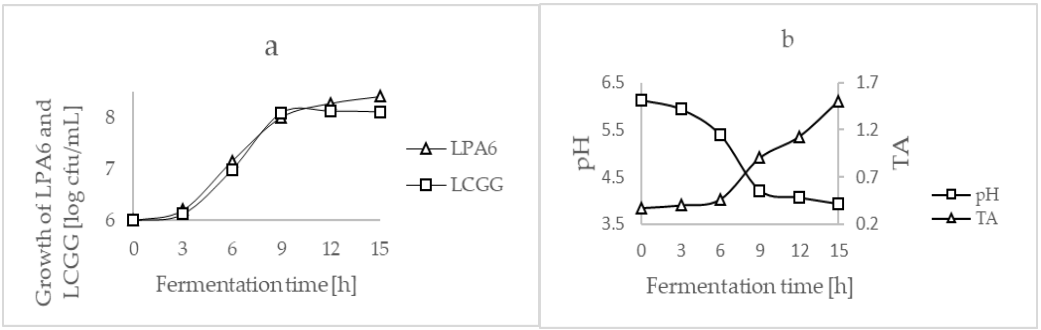

Figure 1. (**a**) Growth of *Lactiplantibacillus plantarum* A6 (LPA6) and *Lacticaseibacillus rhamnosus* GG (LCGG), (**b**) pH and TA measurements; TA, titratable acidity.

In the 15 h fermentation, cell counts of LPA6 and LCGG were registered from 6 log cfu/mL to 8.42 and 8.11 log cfu/mL, respectively. Cell counts of LPA6 increased significantly ($p < 0.05$) from the start of the fermentation up to the 9 h fermentation. But, from 12 to 15 h fermentation, its cell counts didn't increase significantly. Also, cell counts of LCGG

increased significantly from the start of fermentation to the 9 h fermentation. Then, its cell counts didn't show a significant increase until the end of fermentation time. Growth of LCGG showed longer lag phase and turned into stationary phase sooner as compared to LPA6. During the 15 h fermentation both microbial cells counted above 8 log cfu/mL, which is more than the minimum required number. To give a probiotic effect, a food should have a minimum probiotic cell counts of 6–7 log cfu/mL in time of consumption [11].

In the 15 h fermentation, pH and TA values were measured from 6.13 to 3.92 and from 0.37 to 1.5 g/L, respectively (Figure 1). Values of pH decreased significantly ($p < 0.05$) throughout the fermentation time. However, values of TA didn't show a significant increase for the first 3 h fermentation. But, a significantly increased TA values were observed from 3 to 15 h fermentation. Values of pH dropping and TA increasing in time of fermentation can be due to production of organic acids such as lactic and acetic acids. In this study, the observed pH of 3.92 was in the range of 3.5–4.5, which is a confort pH zone for probiotics in the gastrointestinal tract. Also, in this pH range values, the survivability of the consumed probiotics are increased [12].

Progressively glucose was completely consumed from 1.5 to 0 g/L. However, contents of lactic and acetic acids were increased from 0 to 1.7 g/L and from 0.04 to 0.23 g/L, respectively.

3.3. Prediction of Lactic Acid, Glucose and Cell Counts

An SNV preprocessed fluorescence data were used for the models calibration of ANN and PLSR with the aim to predict glucose, and cell counts of LPA6 and LCGG. But, for lactic acid prediction the original data were used. For the model calibration and prediction, the fluorescence spectral data and off-line measured data were used. The predicted cell counts of LPA6 and LCGG, lactic acid, and glucose are displayed in Figures 2 and 3. Predicted values were compared with the actual results to see the prediction ability of the models.

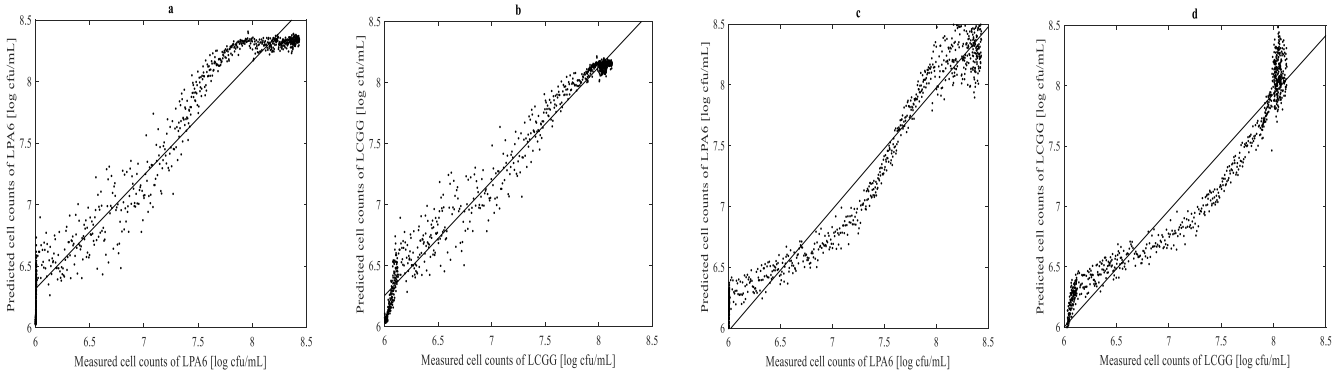

Figure 2. Measured vs. predicted cell counts using ANN (**a**) and (**b**), and using PLSR (**c**) and (**d**); ANN, artificial neural network; PLSR, partial least squares regression; LPA6, *Lactiplantibacillus plantarum* A6; LCGG, *Lacticaseibacillus rhamnosus* GG.

Cell counts of LPA6 and LCGG were measured from 6 log cfu/mL to 8.49 and 8.29 log cfu/mL, respectively. The prediction of cell counts of LPA6 and LCGG with ANN using one hidden neuron showed a pRMSEP of 3.6 and 2.7%, respectively. Similarly, the prediction of cell counts of LPA6 and LCGG with PLSR using five principal components resulted 2.5 and 2.6%, respectively (Figure 2).

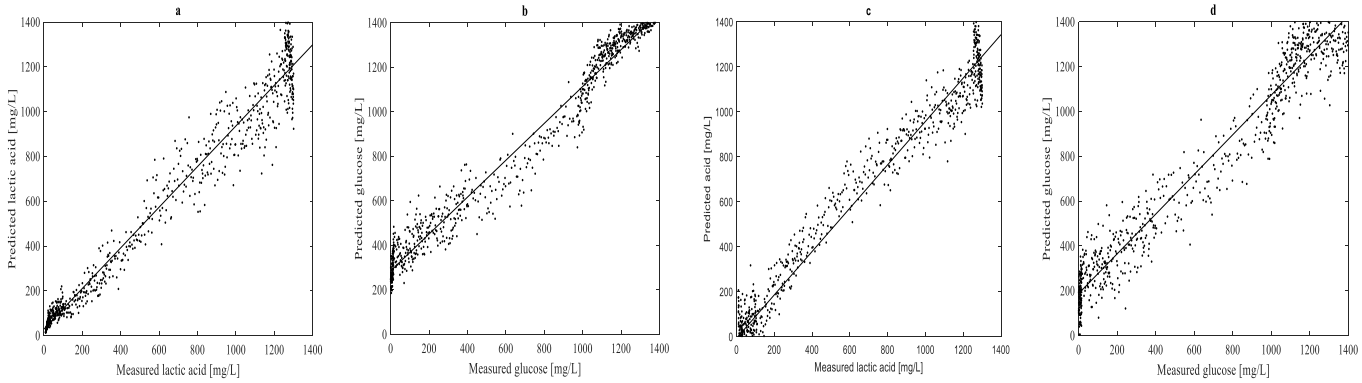

Figure 3. Measured vs. predicted lactic acid and glucose using ANN (**a**) and (**b**), and using PLSR (**c**) and (**d**); ANN, artificial neural network; PLSR, partial least squares regression.

Experimentally measured values of lactic acid and glucose were observed between 0.01 and 1.8 g/L and 1.4 and 0 g/L, respectively. A pRMSEP of 7.6% was found for the prediction of lactic acid with PLSR using seven principal components. Whereas, 11.6% of pRMSEP was measured for glucose prediction with PLSR using seven principal components. Likewise, the prediction of lactic acid and glucose with ANN using one hidden neuron showed a pRMSEP of 7.7 and 14%, respectively (Figure 3). Overall, high values of coefficient of determination (R^2), which measured from 0.89 to 0.96 and low values of pRMSEP showed that lactic acid, and cell counts of LAP6 and LCGG could be predicted accurately with ANN and PLSR.

4. Conclusions

A 2D-fluorescence spectroscopy is an ideal instrument for the on-line supervision of the fermentation process without destruction. Once PLSR and ANN models developed, it is possible to obtain values of cell counts, lactic acid, and glucose in short time by using 2D-fluorescence spectroscopy. However, it took long time to obtain similar results by using the conventional methods of plate count agar and high performance liquid chromatography. This study has shown that a 2D-fluorescence spectroscopy coupled with PLSR and ANN can be applied to predict cell counts of LPA6 and LCGG, and lactic acid during the fermentation of a teff-based substrate.

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Conflicts of Interest: The authors declare that there is no conflict of interest.

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