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High-throughput pH monitoring method for application in dairy fermentations

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Optimization of dairy fermentation processes often requires multiplexed pH measurements over several hours. The method developed here measures up to 90 samples simultaneously, where traditional electrode-based methods require a lot more time for handing the same number of samples. Moreover, the new method employs commonly used materials and can be used with a wider range of fluorescence readers than commercial 96-well plates with optical pH sensors. For this application, a milk-like transparent medium is developed that shows acidification properties with dairy starters that are similar to milk. Combination of this milk-like medium and 3 fluorescent indicators allow precise measurements of pH in a range of 4·0–7·0. The new method showed much higher throughput compared to the benchmark electrode systems while being as accurate, as shown by successful application for a comparison of various dairy starter cultures and for optimizing the inoculation rate.

Keywords: Dairy starter cultures, pH monitoring, fluorescence indicators, acidification curve, milk-like medium, multi well plate reader.

Production processes at many dairy plants rely heavily on fermentation of milk. Starter cultures play crucial roles in these dairy production processes. Dairy producers should, therefore, know the performance of the starter. pH change of fermentation media is one of the main parameters that has to be monitored and/or controlled during screening and performance tests with given starter cultures. Lactic acid fermentation is one example of such processes, where pH-drop is an important indicator of the starter culture activity (Carminati et al. 2010).

It is not possible to measure pH in multiple samples simultaneously with a single electrode. To overcome this limitation, systems like iCINAC (iCINAC, AMS Alliance) are quite common in modern fermentation laboratories and dairy plants. iCINAC-like systems typically have a limit of 16–32 samples to test at the same time. As a consequence, the main drawbacks of such systems are high manual labor requirements as well as the need for connecting and calibrating electrodes, sterility of electrodes and required desk space. All electrode-based pH measurement systems are also prone to measurement errors caused by a protein layer build-up on the electrode, electrode contamination, temperature effects and changes in ionic strength (McMillan, 1991).

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Compact commercial analysis kits with 96-well plates containing fluorescent dye, for example the HydroPlate HP96U (PreSens Precision Scanning), give an option to measure pH in all 96-wells with a 96-well plate reader (**WPR**) (John et al. 2003). HydroPlate like systems often require using a specific type of WPR. Additionally, such specific commercial 96-well plates are often not available for laboratories with limited access to a highly developed supply chain. WPR measuring optical properties of the analyzed liquids are quite common equipment in most analytical labs. WPR runs preprogrammed cycles of measurements including mixing and inoculation steps. However, WPR is not very applicable for color detection in turbid media such as milk (Wolfbeis, 2008).

Fluorescent indicators **(FI)** are a great tool to measure biological changes of transparent media. Besides measuring intensity of the scattered light from growing microorganisms (Kensy et al. 2009), many FI are changing emission intensity with changing pH of the growth medium, which allows for the creation of high-throughput screening systems (Kermis et al. 2002; Arain et al. 2006; Tian et al. 2010). However, an accurate and specific high-throughput method of pH measurements in 4·0–7·0 range, using FI in milk and/or milk-like medium is not known to date.

In this study, we aimed at creating a high-throughput and accessible method to monitor pH change in multiple

samples over time using a microplate reader and a specifically designed milk-like medium. This medium should particularly well represent milk in fermentations with dairy starter cultures.

Material and methods

Briefly, the method includes following steps: (1) Preparation of milk-like medium; (2) Handling of starter culture; (3) Mixing milk-like medium, starter culture and fluorescent indicators; (4) Loading samples into 96-well plate; (5) Running fermentation in the fluorescence reader; (6) Translation of raw fluorescence data to pH values based on a calibration curve. The steps are discussed in more details below.

Measurements of pH values, fluorescence and optical absorbance were done in quadruplicates. Data shown and plotted on figures represent means of 4 measurements per each value ($n = 4$).

Selection of detection system

A detection system has to be selected in order to fit requirements for the fluorescence measurements, sample incubation temperature and mixing. A WPR (Synergy Mx Microplate Reader, BioTek) was used to perform all measurements of the optical absorbance and top-fluorescence in transparent flat-bottom 250 µl 96-well plates (disposable for cell cultures, Nunclone Delta Surface, Nunc A/S). Similar but black-colored 96-well plates were used to run samples and calibration solutions (BRAND plates®, cellGrade™, BRAND GMBH). Transparent film (MicroAmp™, Applied Biosystems) was applied on top of the plates to avoid evaporation. The media in the 96-well plates were automatically mixed before each measurement.

Preparation and application of a milk-like fermentation medium

Skimmed milk powder (Arla, instant skimmed milk powder HP) was dissolved (10% w/w) in deionized water. The casein fraction of the milk proteins was removed by addition of 0·1 ml of stock rennet (CHY-MAX, Chr.Hansen) and 0·1 g $CaCl_2$ (anhydrous, Sigma-Aldrich) per 1000g of 10% reconstituted milk, continuous stirring at room temperature for 15 min, followed by heating to 60 °C in a water bath for 30 min. The resulting casein curd was separated from the whey with paper filter (No. 1, Whatman). 3·5% (w/w) of casein hydrolysate (Bacto Tryptone pancreatic digest of casein, Becton Dickinson and Co.) was added to the filtered whey. The suspension was centrifuged at $17\,105 \times \boldsymbol{g}$ to remove all remaining curd particles and possible solid components of the casein hydrolysate. The centrifuged solution was sterile filtered using 0·45 µm syringe filters (Q-Max cellulose acetate membrane, Frisenette). Optical properties of the milk-like medium (**MLM**) were studied in transparent 96-well plates using the WPR by measuring the optical absorbance every 10 nm in the range of 350–800 nm.

Suitability of MLM for starter culture growth was tested using Flora Danica with an inoculation rate of 0·02% and incubation at 30 °C. Measurements of the optical density at 600 nm were taken using WPR every 5 min for 12 h to follow the fermentation process.

Fermentation properties of MLM were compared with two standard fermentation media used to test activity of Flora Danica starter culture – 10% reconstituted skim milk (**RSM**) and 0·1% fat commercial UHT milk from 1 l pack (Arla). Inoculation rate was 0·02% and incubation temperature 30 °C. Fermentation curves were obtained with the iCINAC system, using triplicates and pre-calibrated glass electrodes with measurements of pH every 1 h.

Handling of the starter cultures

The two mesophilic dairy starter cultures – Flora Danica (Chr.Hansen) and C-160 (Chr.Hansen) used for acidification tests were stored at −45 °C. Shortly before inoculation, starter cultures were thawed in 10 °C water until liquid.

The mesophilic starter culture Probat505 (DuPont) is a bulk starter culture. Therefore, additional preparation steps were included before running the acidification test with this culture. Freeze-dried powder of Probat505 was mixed with 50 ml of MLM at a rate of 0·02% w/v and fermented for 12 h at 30 °C to grow enough live starter cells. MLM was chosen over RSM due to the easier cell harvesting in the absence of coagulated casein. Growth medium and cells were centrifuged at $13\,684 \times \boldsymbol{g}$ for 10 min. Pellets were collected by removing the liquid and subsequently resuspended in 5 ml of 10% sterile RSM.

Choice and optimization of the right pH-sensitive/insensitive fluorescent indicators

Several pH-sensitive FI are known to match the required range of pH 4·0–7·0. Fluorescein isothiocyanate isomer I (**FITC**) (suitable for protein labeling, ≥90% (HPLC), Sigma-Aldrich) and 5(6)-Carboxy-2′.7′-dichlorofluorescein (**CDCF**) (BioReagent, suitable for fluorescence, ≥95% (TLC) Sigma-Aldrich) were chosen for the tests. It is known that the maximal excitation wavelengths for FITC and CDCF are 492 and 504 nm. Maximal emissions are at 518 nm for FITC and 529 nm for CDCF (Nedergaard et al. 1990; Kim et al. 1998). For the mixture of two FI, wavelength parameters were set to ex490/em520.

As for the pH-insensitive fluorescent indicator, sulforhodamine 101 (**SRH**) (powder, Sigma-Aldrich) was chosen for the application. Its maximum wavelength of excitation is 586 nm and emission is 605 nm, which are not overlapping with those of FITC and CDCF (Sipior et al. 1995). Therefore, SRH is suitable for applications in combination with FITC and CDCF.

Working solutions of the pH-sensitive indicators were prepared by dissolving dry powder in 10 mL of 96%

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ethanol (Sigma-Aldrich) in the case of FITC to produce a 5·14 mM (2 mg/ml) solution and similarly CDCF was dissolved in 10 ml of deionized water for a 4·49 mM (2 mg/ml) solution. The pH-insensitive indicator SRH was dissolved in 70% (v/v) ethanol to reach a concentration of 1·64 mM (1 mg/ml). Working solution of SRH was added to the tested media at the rate of 0·75 µl per every 1 ml of medium used, reaching a final concentration of 1·24 µM in the media. Applicability of FITC and CDCF (final concentrations respectively 5·14 and 4·49 µM) at pH range of 4·0–7·0 was tested by measuring fluorescence in phosphate saline buffer (PBS tablet, Sigma-Aldrich) at 7 calibration points of pH 4·0; 4·5; 5·0; 5·5; 6·0; 6·5 and 7. pH of the PBS was adjusted to the values of calibration points using 23% w/w water solution of lactic acid (90%, VWR). Measurements of the pH values during preparation of PBS solutions were made using a glass electrode pH meter (Type 1140, Mettler Toledo).

It is important to know the best ratio of indicators to be added to the medium to achieve optimal results. The best applicable ratio of FITC and CDCF was determined by applying working solutions of FITC and CDCF to the following final concentrations (µM FITC to µM CDCF): 5·14 to 4·49; 6·43 to 4·49; 7·71 to 4·49; 5·14 to 3·37 and 5·14 to 5·61. Calibration curves for the linearity coefficients (r^2) were based on the 7 calibration points of PBS in the pH range of 4·0–7·0. After finding the optimal ratio, optimization of indicator concentrations was done to avoid over- and underexposure. The following final concentrations of µM FITC to µM CDCF were tested: 3·86 to 2·53; 5·14 to 3·37; 6·43 to 4·22 and 7·71 to 5·06.

A calibration curve of FITC, CDCF and SRH (5·14, 3·37 and 1·24 µM, respectively) in MLM was made in the same way as the calibration curves for FITC and CDCF in PBS. SRH was used to calculate reduction of fluorescence due to the cell multiplication. Thus, values of fluorescence obtained at 520 nm emission (fluorescence of FITC and CDCF) were corrected by those obtained at 605 nm following the formula '$Em605_0/Em605_x \times Em520_x$' with $Em605_0$ the fluorescence at 605 nm measured at time-point zero, $Em605_x$ the fluorescence at 605 nm measured at time-point x, and $Em520_x$ the fluorescence at 520 nm measured at time-point x.

Testing application of MLM together with FITC, CDCF and SRH mixture on a dairy starter

Before applying combination of fluorescence indicators and MLM to real-life samples, it is important to prove that combination of fluorescent indicator (FITC, CDCF and SRH) mixture and MLM (**FIM**) allows monitoring of acidification by fermentation.

Since every 96-well plate with samples has to have a separate calibration curve, FIM was pH-adjusted with lactic acid to 4·0, 4·5, 5·0, 5·5, 6·0 and 6·5. The solutions were filter sterilized before pipetting a volume of 250 µl into the 96-well plate. The rest of the wells were filled with FIM inoculated with Flora Danica (0·02% v/v), Probat 505 (0·02% v/v) and C-160 starter cultures (0·01% v/v). Overall, 88 wells were filled with tested FIM and starter cultures (29 wells per each culture), 2 wells were not inoculated to act as negative controls (sterility of the FIM) and 6 were used for pH-adjusted calibration points (1 well per point). Fluorescence at ex490/em520 and ex586/em605 was recorded with the WPR every 5 min for 12 h. After finalization of the reading, values of the fluorescence were recalculated into pH values using the calibration curve.

Comparing FIM performance in acid fermentations with a benchmark iCINAC system

Performance of the FIM for ability to track an acid fermentation progress was compared to the benchmark iCINAC system.

500 ml of the inoculated FIM with Flora Danica starter culture (0·02% v/v), was divided into 4 Schott bottles (100 ml each). The rest of the inoculated medium was used to fill a 96-well plate. The bottles were placed in the water bath of the iCINAC reader with pre-calibrated electrodes and initialized system. The 96-well plate was simultaneously placed into the reader. After 12 h of incubation and measurements, data from the iCINAC system and the 96-well plate reader were collected.

Application of FIM to compare inoculation rates of the Flora Danica starter culture

To test applicability of the new method in the real-life scenario, the method was used to select the optimal inoculation rate for the Flora Danica starter culture. Taking an aim to reach a pH of 5·5 in 6 h, Flora Danica was inoculated into FIM in 0·01; 0·02; 0·03; 0·04; 0·05% v/v. FIM inoculated at mentioned rates was quickly pipetted into the prepared plate with all 6 calibration and 2 sterility check wells ready. Fluorescence values at ex490/em520 and ex586/em605 were recorded every 5 m and measuring time was set to 11 h. After finalization of the readings, values of the fluorescence were recalculated into pH values using the linear calibration.

Results

Milk-like fermentation medium and its properties

The main purpose of the MLM is to minimize opacity leading to reduction of fluorescence of the pH sensitive indicators as would be the case in normal milk. The goal was to create a medium similar to milk with respect to fermentation properties, but transparent at the same time. Prepared MLM resulted in a yellow transparent solution with pH 6·55–6·7. The light absorbance spectrum of the MLM was measured in the 350–800 nm range in order to assure minimal interference with the emission and excitation spectra of the FI. MLM absorbs visible light with wavelengths below 550 nm, reaching a maximum in the ultraviolet region. Hence, measuring

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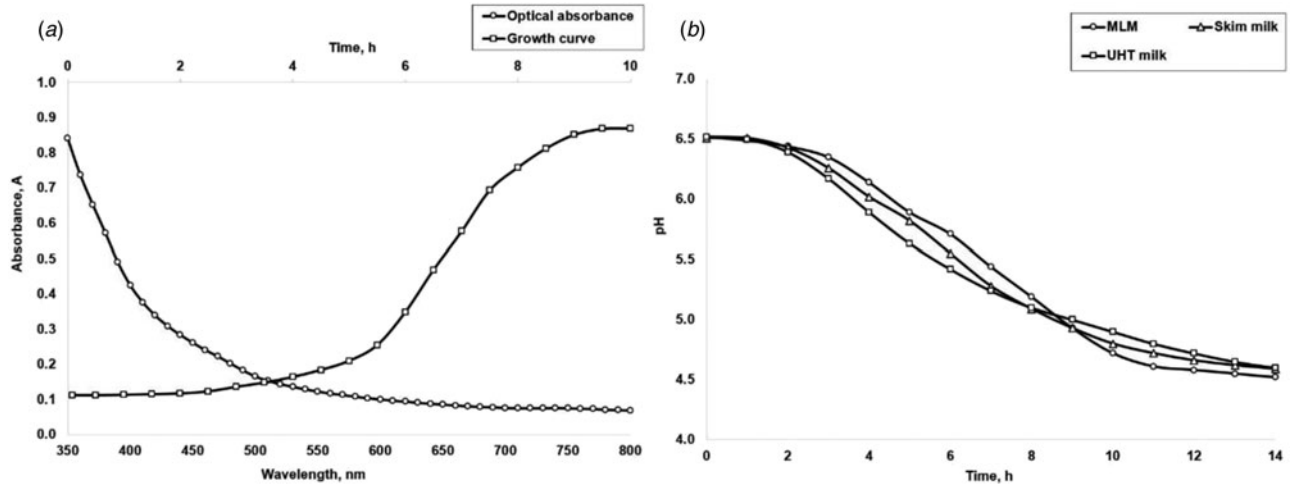

Fig. 1. Physical and fermentation properties of MLM. (a) Optical absorbance spectrum of the MLM for the wavelength between 350 and 800 nm and 10 h cell growth curve of the starter culture in MLM measured at 600 nm absorbance. (b) Acidification curves of Flora Danica starter culture in MLM, 10% reconstituted skim milk and 0·1% commercial UHT milk using iCINAC as a pH measuring system. S.D. = 0·016, $n = 4$.

absorbance at 600 nm gives a good indication of the cell growth process with an absorbance of only 0·1 from the medium itself. Increase of the optical density at 600 nm of the MLM during the fermentation process was clearly tracked using Flora Danica as a starter culture (Fig. 1a).

Acidification curves with the iCINAC as a monitoring system for MLM, UHT milk and 10% RSM were made using Flora Danica as a starter culture. The resulting curves are presented in Fig. 1b. All three media follow a similar acidification trend, indicating that MLM can be used instead of milk as a fermentation medium.

Choice and optimization of pH-sensitive/insensitive fluorescent indicators

For reliable calculations of pH in the medium, suitable pH-sensitive indicators have to be chosen. FITC and CDCF at equal concentrations show similar fluorescence at pH 7·0 (Fig. 2). The observed properties of FITC and CDCF gave a good indication that CDCF is capable of compensating low fluorescence of FITC in the pH range between 5·5 and 4·0. The calibration curve for 5·14 to 4·49 μM mixture of FITC and CDCF is much more linear ($r^2 = 0{\cdot}997$) compared to the individual FI.

The next step was to determine the best ratio of both pH-sensitive indicators in the mixture. Following calibration curve results from different FITC to CDCF μM ratios in PBS were obtained: 5·14 to 4·49 $r^2 = 0{\cdot}9970$; 6·43 to 4·49 $r^2 = 0{\cdot}998$; 7·71 to 4·49 $r^2 = 0{\cdot}997$; 5·14 to 3·37 $r^2 = 0{\cdot}999$ and 5·14 to 5·61 $r^2 = 0{\cdot}953$. Based on the results, the optimal ratio is 5·14 μM FITC and 3·37 μM of CDCF. Increase of FITC or CDCF part in the mixture decreases linearity of the curve.

After finding the right ratio of two FI, it is important to know the best applicable concentration. Despite of the fact that the Biotech fluorescence reader can adjust measured fluorescence to a required range, excessive or insufficient light emission from the sample medium could affect parameters of the calibration curve. From the fluorescence results (data not shown), concentrations of 5·14 μM of FITC and 3·37 μM of CDCF are the optimal among the tested ones, giving the calibration curve with r^2 value of 0·999 in PBS.

SRH as the pH-insensitive fluorescent indicator was tested for its ability to retain its fluorescence intensity when the pH of PBS is changing. Changes of the pH in the range of 4·0 to 7·0 do not lead to the significant change in fluorescence (Fig. 2). The best-found concentrations of the FITC and CDCF mixture were applied together with SRH. The resulting calibration curve using 3 FI (FITC, CDCF and SRH) is linear with an r^2 of 0·9982.

Testing MLM together with FI mixture

To test the real-life applicability of the pH monitoring system, FIM and Flora Danica starter culture (0·02%) were applied to a 96-well plate, loaded into the WPR and measured at 30 °C for 12 h. To compensate the effect of increasing biomass and associated reduction in measured fluorescence, values of the pH-sensitive indicators (ex490/em520) were recalculated according to the level of the fluorescence loss of the pH insensitive SRH (ex586/em605). The corrected values represent the decrease of the fluorescence of FITC and CDCF solely due to the acid-related pH drop of the MLM.

Figure 3 indicates how FITC and CDCF fluorescence loss due to biomass growth is compensated by the known reduction of SRH fluorescence. At time 0 of the fermentation process, there is no reduction of the fluorescence due to the cell growth. At 3·3 h, when starter cells are starting to grow exponentially, emission at 605 nm (SRH) indicates

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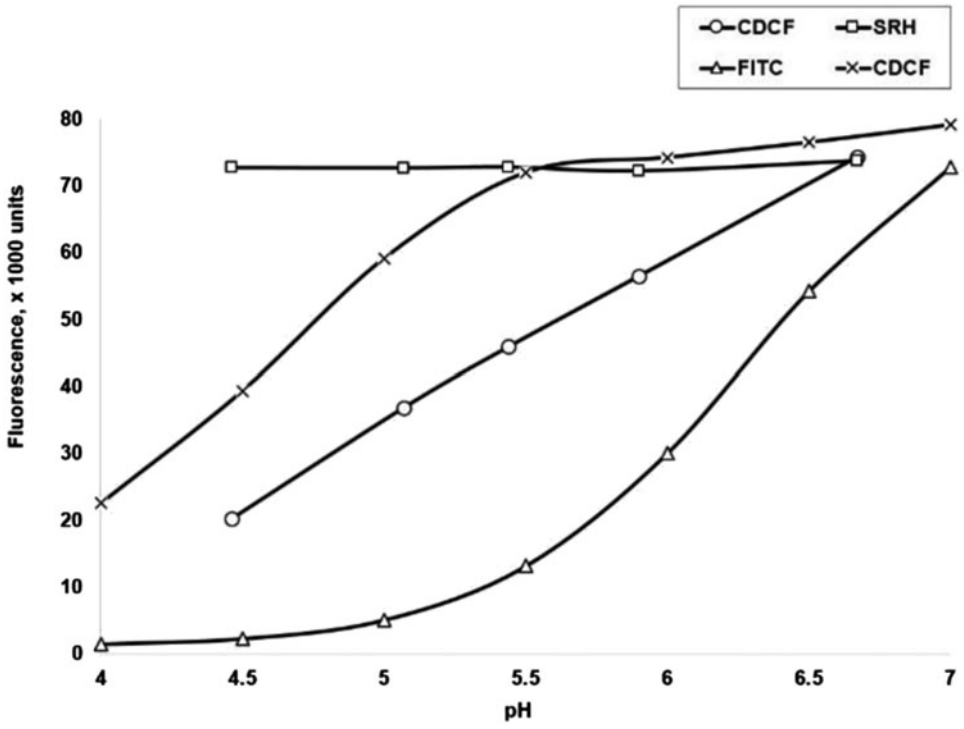

Fig. 2. Selecting FI and optimizing their mixture. Curves of the fluorescent indicators – FITC (r^2 = 0·880), CDCF (r^2 = 0·853), combination of FITC + CDCF (r^2 = 0·997) and SRH.

that there is 5·38% loss of the fluorescence. Therefore, the value of fluorescence from FITC and CDCF together is recalculated by increasing it by 5·69%, resulting in a pH of 6·29 instead of 6·13. After 10 h of fermentation, the reduction of fluorescence based on SRH measurements is 21·25%. Accordingly, the measured fluorescence of FITC and CDCF was increased by 26·98%, giving pH 4·49 instead of the incorrect 4·21.

Comparing FIM performance in acid fermentations with a benchmark iCINAC system

Performance of the FIM system was compared to the iCINAC system. Figure 4a shows the acidification process of Flora Danica starter culture tracked by both systems. pH values obtained by the FIM system are well comparable with iCINAC measurements. The difference of pH readings did not exceed 0·07 (iCINAC *vs.* FIM) throughout the fermentation. Standard deviation of pH values between parallel samples measured with FIM system did not exceed 0·03 for any measurement. This was also the case in the other tests.

Application of FIM for comparison of different starter cultures and inoculation rates

One of the main goals for development of the FIM system was to test industrial dairy starter cultures for their acidification activities. Figure 4a illustrates that Flora Danica (0·02% v/v), Probat 505 (0·02% v/v) and C-160 starter culture's (0·01% v/v) acidification curves can be easily distinguished using the FIM system. Even at lower concentration, C-160 showed higher acidification rate than the other 2 cultures, while Probat 505 was by far the slowest one.

Identification of the most optimal inoculation rate is a very important part of the starter assessment process. In the example of identifying the best inoculation rate for Flora Danica, the aim was to match a rate that allows to reach a pH of 5·5 in 6 h. The FIM system was applied with Flora Danica at rates 0·01, 0·02, 0·03, 0·04 and 0·05% v/v. As it can be seen in Fig. 4b, the closest inoculation rate to reach pH 5·5 in 6 h is 0·04%. The curve for 0·04% inoculation rate reaches pH 5·5 at 5 h 55 min after the initiation of fermentation.

Discussion

Successful application of starter cultures at dairy sites is heavily dependent on the right inoculation rate and performance of the starter culture. To monitor pH, indirect analytical setups such as iCINAC are commonly used. The main drawbacks of multi-electrode systems like iCINAC are the need to recalibrate multiple electrodes and their maintenance. Additionally, measuring large amounts of samples is rather complicated, labor intensive and expensive due to the high use of consumables and electrodes. As an alternative, 96-well plate kits with integrated optical sensors are easier to use and allow running up to 90 samples at the same time. However, if there is no possibility to buy 96-

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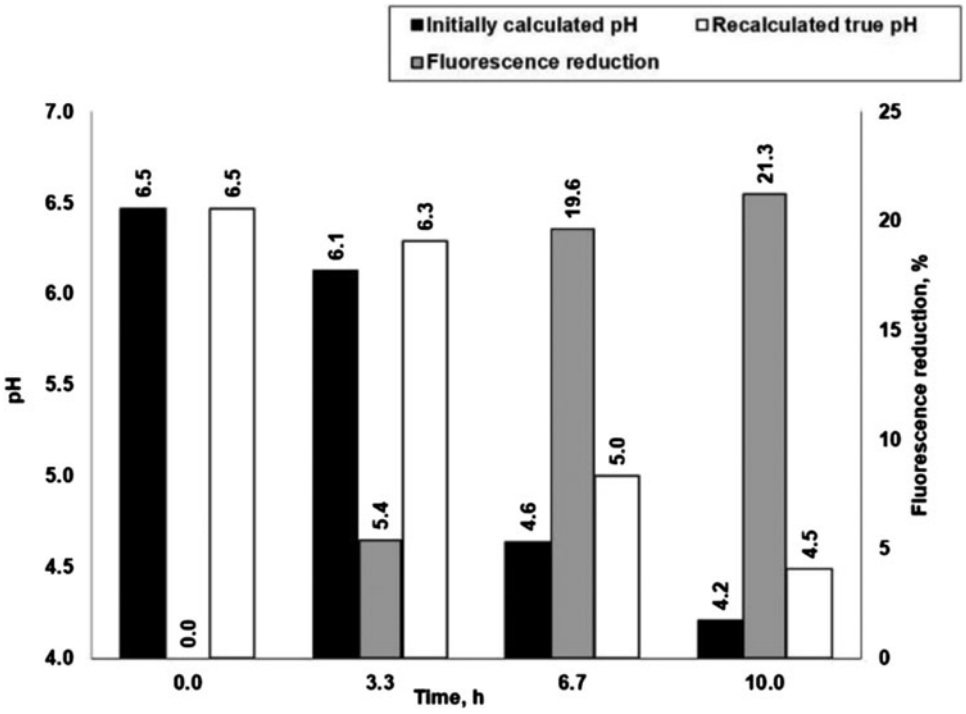

Fig. 3. Compensation of the initial calculated pH values (black) based on the cell growth-related fluorescence reduction at 605 nm (grey) to the recalculated true pH values (white).

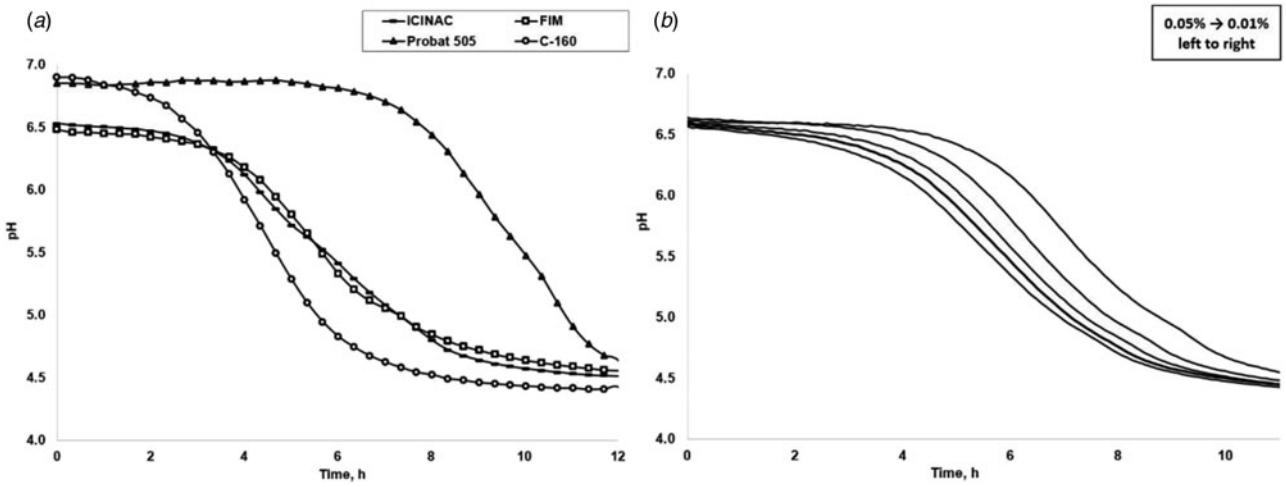

Fig. 4. Benchmarking and application of the FIM. (a) Comparison of fermentation curves from iCINAC and FIM systems using Flora Danica starter culture as well as curves obtained from following the fermentation process of C-160 and Probat 505 starter cultures using the FIM system. S.D. = 0·020, $n = 8$. (b) Comparison of fermentation curves from FIM systems with the different inoculation rates 0·05; 0·04; 0·03; 0·02 and 0·01% (left to right) of Flora Danica starter culture. S.D. = 0·024, $n = 8$.

well plates with optical sensors and/or a WPR that does not fit the requirements for these special 96-well plates, use of such kits is not possible.

The goal of the current research was to develop an easy to use and reliable 96-well plate based pH monitoring system. It is very hard to monitor optical changes in milk-based media using optical instruments such as plate readers. To overcome such difficulty, it was decided to develop a transparent milk-like medium that allows detection of fluorescence of dissolved pH-sensitive indicators. One of the ways to make such medium is to remove the colloidal caseins from the milk using rennet. To restore the nitrogen content of the MLM in replacement of the removed proteins, casein hydrolysate was added. The resulting clear MLM has minimal

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absorption above 550 nm (Fig. 1a). MLM was shown suitable for tracking cell growth of the Flora Danica starter culture at 600 nm (Fig. 1a). MLM together with WPR gives a high-throughput opportunity to measure cell growth based on the optical density measurements in medium that is very similar to milk. Additionally, the acidification curve of MLM inoculated with Flora Danica was similar to that of the culture growing in low-fat UHT milk and 10% RSM (Fig. 1b). Thus, MLM can be used as an alternative medium to milk in acidification tests with typical dairy starters.

The initial hypothesis that addition of CDCF can compensate lack of FITC fluorescence at pH below 5·5 was confirmed. Addition of SRH as a pH insensitive indicator to the FITC and CDCF mixture had minor effect on the calibration curve and good linearity was maintained ($r^2 = 0{\cdot}9982$ instead of 0·9991). The small loss of linearity could be due to the minor overlap in excitation\emission spectra of the indicator mixture.

Application of the FIM together with a Flora Danica starter culture showed that 3 indicators can successfully track changes in the fluorescence of the fermentation medium as a result of acidification. Reduction of fluorescence due to the growth of bacterial biomass was measured by pH insensitive SRH and values of FITC and CDCF were corrected accordingly. It is obvious that in later stages of the fermentation the difference between compensated and uncompensated fluorescence values become even greater as a result of increased opacity by increased biomass. In the current tests, cell multiplication caused reduction of fluorescence up to 21·25% based on the SRH readings, giving a difference in calculated pH values of up to 0·28 (Fig. 3). Thus, using SRH, a mistake in calculated pH values can be avoided.

A comparison of the results of the FIM system and benchmark iCINAC system showed comparable performances of both methods. Thus, with a higher throughput and similar performance the new method is a good alternative to the iCINAC system when it is necessary to run many simultaneous acidification tests. Such property can be very helpful, when there is a need to compare acidification potentials of several different starter cultures and when tests need to be performed in replicates in order to provide statistical significance. The FIM system was used for comparing Flora Danica, C-160 and Probat 505 starter cultures (Fig. 4a). The obtained acidification curves show clear differentiation between 3 cultures.

Comparison of different inoculation rates (0·01–0·05% v/v) of Flora Danica starter culture was successfully performed using the FIM system (Fig. 4b). Therewith it is confirmed that the system gives the necessary information on starter culture performance and can be useful for choosing the right inoculation rate for a variety of dairy starter culture.

Conclusion

The developed method for high throughput pH monitoring of dairy fermentation processes is simple and does not require any highly specific equipment. The method allows monitoring of the acidification processes in the range of pH 4·0–7·0 in 90 samples simultaneously. MLM as a transparent fermentation medium shows a very similar acidification curve for typical dairy starter cultures when compared with milk. Thus, it can be successfully used for cell growth essays based on turbidity measurements as well as for essays with FI such as those being pH sensitive. A mixture of 3 FI and MLM was successfully used for pH monitoring of the acidification process for 3 different dairy starter cultures, clearly showing their differences. The acidification process of a mixed dairy starter culture – Flora Danica – was tracked, showing a standard deviation within the 96-well plate that is similar to that of pH electrodes. Besides allowing to track pH though the whole acidification process, the new method was useful in finding the right inoculation rate for Flora Danica starter culture by showing differences between several inoculation rates.

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