Study on the applicability and precision of a new adaptation of optical mixing time measurement method in a *ReadyToProcess* WAVE 25 bioreactor

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Abstract: A new adaptation of the optical mixing time measurement method has been developed. The method relies on the neutralization reaction and computer image analysis. The procedure was proved to have significantly higher precision than the commonly applied methodology based on the sensor method for mixing time measurement.

Keywords: single-use bioreactors, wave mixed bioreactors, mixing time, optical method, computer image analysis, characterization of bioreactors.

Introduction

Single-use wave mixed bioreactors are a group of devices widely used in modern areas of basic research in biotechnology and bioengineering, and applied in the biopharmaceutical industry. The devices are used to scale up in vitro cultures of biomass of isolated plant cells and organs, as well as for bioprocessing of animal and human cells. In recent years, numerous scientific reports have been published on the use of single-use wave-type mixing bioreactors for the production of biotic agents for the formulation of vaccines against viral diseases (Demirden et al., 2022) (including against COVID-19), human mesenchymal stem cells (Tsai et al., 2017), banks of modified malaria germ cells (Pawliw et al., 2018), human hematopoietic HL-60 cells (Wierzchowski et al., 2020), or dendritic cells for immunotherapy (Meng et al., 2018).

Single-use bioreactors with wave-type mixing became commercially available only at the turn of the 20th century, much later than the conventional stirred tank bioreactors. Therefore, the body of knowledge regarding the impact of the values of parameters characterizing process conditions (such as the volumetric mass exchange rate, mixing time, mixing power) on the values of non-typical wave mixing parameters (such as the amplitude and frequency of oscillatory motion, liquid volume, acceleration profile of the oscillatory motion) is limited and non-systematized (Bartczak et al., 2022). An important issue in completing the characteristics of processes occurring in wave mixed bioreactors is the development of effective and accurate methods for precise measurement of values of parameters characterizing the hydrodynamic conditions inside the disposable culture containers. The aim of this work was to develop an adaptation of the optical method for measuring mixing time in *ReadyToProcess*™ WAVE 25 bioreactor equipped with Cellbag^{TM} culture vessels. The motivation for the work in this area was the experience of measuring mixing time using a sensor method with a conventional pH electrode in earlier studies, i.e. significant drawbacks of pH electrode use related to its long response time, the invasiveness of the measurement, and errors in final results due to the manual dosing of the tracer by the person performing the measurement.

Materials and methods

The optical mixing time measurement method was adapted with the *ReadyToProcess*™ WAVE 25 (Cytiva, USA) bioreactor system equipped with disposable Cellbag™ polymer culture vessels. The bioreactor system consists of a central unit with a rocking, thermostatted platform and a *ReadyToProcess*™ CBCU control and measurement unit responsible for regulating the composition and flow rate of the gas mixture supplied to the culture vessel. The bioreactor's operating parameters, such as the frequency and amplitude of the platform's oscillating motion or

the temperature of the liquid in the vessel, were controlled and adjusted using dedicated UNICORN software.

The starting solution in the measurement procedure was a hydrochloric acid solution of 0.001 mol/dm^3 (pH = 3). Bromothymol blue was introduced into the starting solution to act as an indicator. A 1 mol/dm^3 sodium hydroxide solution (pH = 14) was used as a tracer at 2 cm^3 per 1 dm^3 of the starting solution. The addition of the tracer caused a change in the pH of the tank mixture to pH = 11 and a simultaneous change in the indicator's colour from yellow to blue.

The space in the bioreactor tank above the liquid phase was filled with atmospheric air supplied at a rate of 0.55 dm^3/min via the CBCU and an external compressor. The gas was supplied to the tank using dedicated lines equipped with filters. The gas supply allowed the bioreactor tank to maintain the proper shape during the experiments.

In order to eliminate the errors associated with the manual addition of the tracer solution to the liquid in the tank, an automatic dosing system was developed (Pilarek & Bartczak, 2023) based on a rack-and-pinion mechanism moved by a stepper motor controlled using an Allegro A4988 controller and a microcontroller on an Arduino board. The system allows precise and repetitive tracer dosing at the selected phase of the platform's oscillating motion and adjustment of the volume of the dosed liquid portion. The system's mechanical components were self-made by 3D printing with a Prusa i3 MK3S+ printer and allow the filled syringe to be attached to the bioreactor platform at the connection point with the syringe port of the polymer vessel (Figure 1).

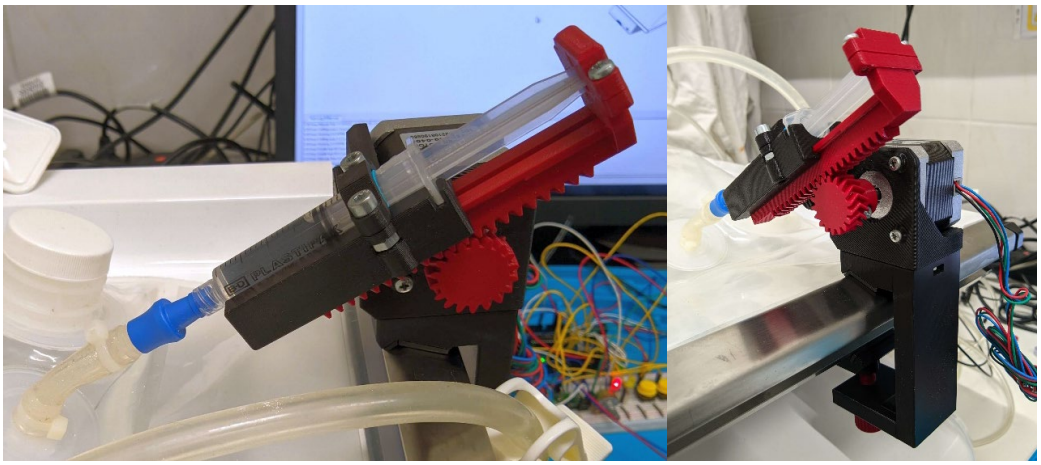

Fig 1. Mechanical components of the automatic tracer dosing device.

The determination of mixing time values is based on footage showing the process of mixing a portion of the tracer in the liquid inside the vessel. The footage is recorded using a GoPro Hero 10 digital camera. The camera is placed above the bioreactor tank, stationary relative to the rocking platform, using a dedicated mount (Figure 2). The mount was designed using Autodesk Fusion 360 CAD software and fabricated using 3D printing. The tripod also includes mounting locations for LED floodlights and reflective panels to provide shadow-free illumination of the platform. The use of the mount makes it possible to acquire high-quality footage without external interference.

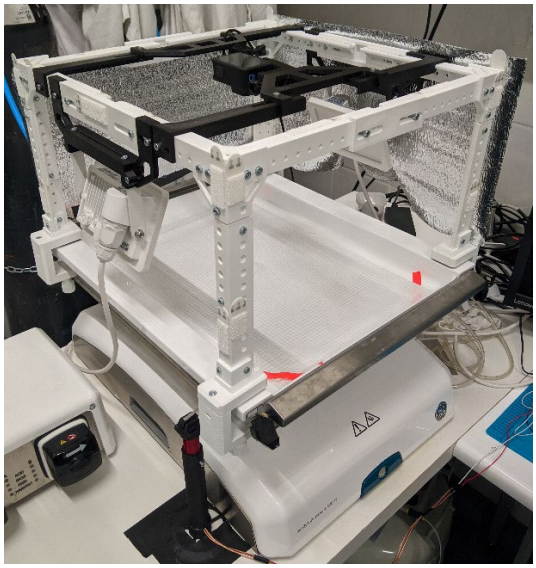

Fig 2. Setup for mounting the digital camera, LED lighting and reflective panels (some of the reflective panels were removed for taking this photograph).

The footage was recorded at 3840 by 2160 pixels at 50 frames per second, translating to a spatial resolution of 0.13 mm/pixel and a temporal resolution of 0.02 Hz. Selected frames from one of the recordings of the mixing process are shown in Figure 3.

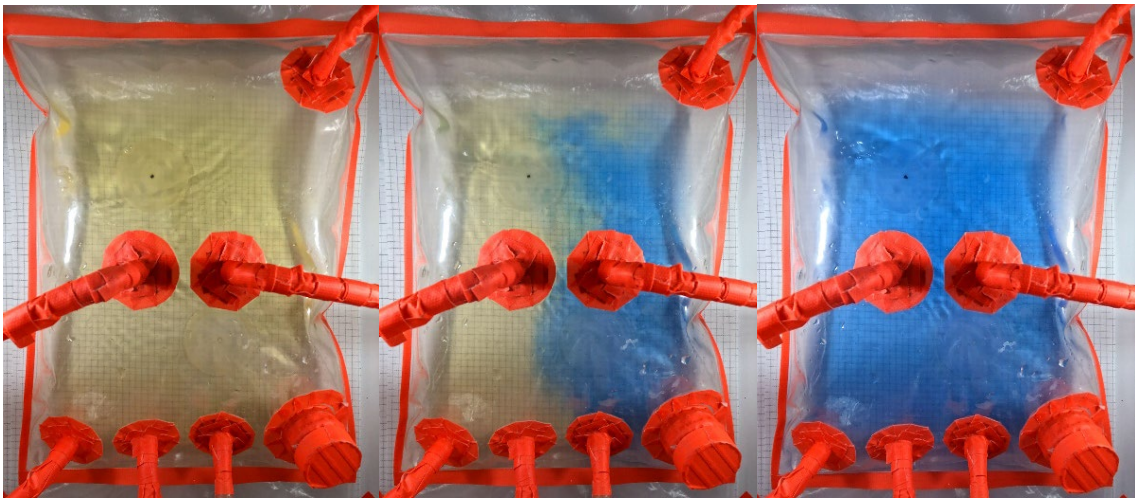

Fig 3. Selected frames from the recording of a mixing process inside the disposable container. A change in the indicator's colour is visible.

Video files containing data from the experiments were processed using an algorithm written in Python, which utilizes functions from the open-source OpenCV library. The image analysis algorithm is adapted to use the computing power of the processor (CPU) or graphics card (GPU). The algorithm performs the following:

- reading movie frames,
- filtering the source images to reduce noise,
- converting pixel colour data from *RGB* space to *L*a*b** space,
- extracting the area corresponding to the interior of the polymer container,
- masking and delineating the area of areas corresponding to the selected colour of the pH indicator (yellow or blue),
- generating result files and images.

The *L*a*b** colour space was chosen as the target representation of pixel colour data because of the separation of blue and yellow colours on the *b** coordinate axis. A typical range of values on the *b** axis is from -128 to 127. A value of the *b** coordinate in the range of -128 to -1 means that a given pixel is blue. On the other hand, a value of the *b** coordinate in the range from 0 to 128 means that a given pixel is yellow. The intensity of the colour in both cases is greater the farther the coordinate value is from the value of 0.

The resulting file containing data on the area of the areas corresponding to the selected colours of the pH indicator is taken for further analysis using the MATLAB environment. Analysis of the resulting file is based on determining the moving average of the area of image parts of a given colour and then determining the value of mixing time. The mixing time is defined as the time required to permanently reach 95 % of the total change of colour in the area occupied by blue pixels, corresponding to the final colour of the indicator.

Results and discussion

Experiments were conducted to determine the precision of the developed adaptation of the optical method for measuring mixing time and to compare it with the previously used sensor method using a conventional pH electrode. A series of 7 experiments at the same values of wave mixing parameters (rocking amplitude of 7°, rocking frequency of 21 min^{-1}), the same volume of the liquid phase in the tank equalled 0.6 dm^3 and at the same temperature equalled 25 °C were performed. The obtained mixing time values were compared with those obtained from a series of 9 experiments conducted under the same conditions using the sensor method.

A comparison of the data obtained using the two measurement methods is shown in Figure 4. For the sensor method, the mean value of mixing time was 13.9 s, while the standard deviation was 4.3 s. For the sensor method, the mean value of mixing time was 13.96 s, while the standard deviation was 1.03 s. Based on the data shown in Figure 4, it can be variation in the mixing time values from adjacent measurements is much more significant for the sensor method. The value of the standard deviation of the results obtained using the optical method is more than four times smaller than that obtained using the sensor method.

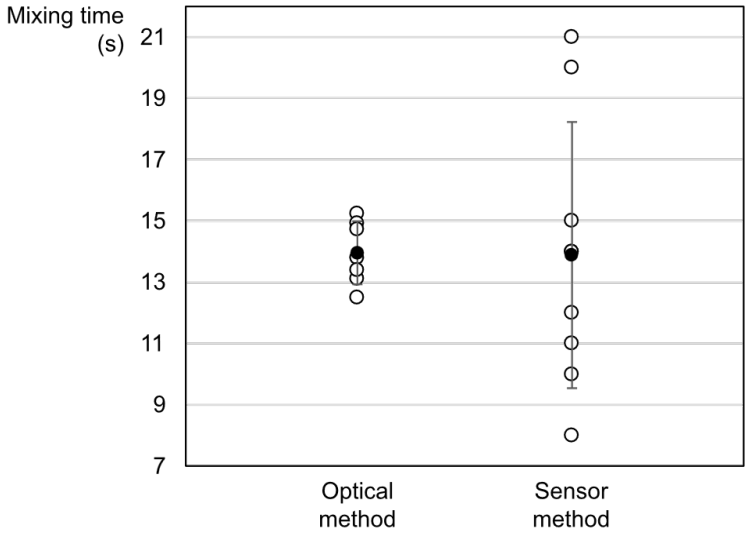

Fig 4. Comparison of the precision between the optical method and the sensor method.

The significantly higher precision of the optical method compared to the sensor method can be attributed to several properties of the new procedure:

- The measurement of mixing time in the newly developed adaptation of the optical method is carried out without introducing any additional objects, such as measuring probes, into the culture vessel. As a result, the velocity distributions and flow patterns of the liquid phase remain unchanged and are not subject to any errors that are difficult to be quantitatively characterized.
- Using an automatic tracer dosing system with repetitive dosing at a specific phase of the platform oscillation cycle eliminates the errors associated with manual dosing of the reagent by the person performing the measurement.
- Mixing time measurement is not subject to error due to sensor response time. With the sensor method, the response time of the pH electrode is typically 1 to 5 seconds (Sohanghpurwala et al., 2009), a significant fraction of the final mixing time value.
- The time resolution of measurements in the optical method is much higher than that of the sensor method. In the case of the optical method, the temporal resolution is equal to the interval between the registration of successive image frames and in the present case was equal to 0.02 seconds, while in the case of the sensor method, the maximum temporal resolution was limited by the frequency of the pH meter reading and was equal to 1 second.

Conclusions

The developed adaptation of the optical method for measuring mixing time will serve as a tool for quantitative characterization of the mixing process in wave mixed disposable bioreactor containers. The optical method has several significant advantages over the previously used sensor method, such as non-invasiveness of the measurement, elimination of interference associated with manual tracer dosing and high electrode response time, and high temporal resolution of the measurement. In addition, an essential advantage of the optical method is the ability to observe the mixing process in the entire volume of the fluid and to determine local values of mixing time in selected areas of the liquid phase based on the high spatial resolution of the acquired images.

The developed measurement procedure is characterized by independence from external factors that can affect the quality of the acquired film material. The mechanical components of the camera mounting and lighting mount, as well as the automatic dosing device, were designed to apply the procedure to the study of mixing time characteristics in polymer tanks of different total volumes. Mixing time values can be measured using the developed adaptation of the optical method for the full range of bioreactor operating parameters.

It was shown that the precision of the developed adaptation of the optical method is several times higher than that of the previously used procedure using a conventional pH electrode, so it will be possible to reduce the number of necessary experimental runs in future studies.

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