Integrated Master in Chemical Engineering

Mixing characterization in novel high throughput minibioreactors: Scale-down modeling from bench scale

Master Thesis

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Abstract

Mixing time and circulation are two important parameters in fluid characterization and mixing behavior in a wide range of industries and researches, mainly in bioprocesses field. Mixing time is also a useful criterion for the scale-up and the scale-down of bioreactors, because it affects the mass transfer.

A visual method for determining mixing times in vessels was developed. Its method involves the addition of a fluorescence dye excited by UV light. This new technique was validated in a bench scale reactor with a potentiometric method (pH based). But when it was applied this visual technique in a model stirred minibioreactor, and tried to validate with the laser induced fluorescence (LIF) technique the results show significant statistical difference between both methods.

Experimental correlations were established for mixing time in bench scale reactor and in minibioreactor. In the latter, two different impeller type (i.e. paddle and pitched blade) were tested. The correlation obtained in this study describes the mixing time dependency with Reynolds number and height of liquid over vessel diameter ratio (H/T). As expected it was observed that independently of the system/configuration, the mixing time decreases when the Reynolds number increases. About the effect of H/T ratio, when it is increased, mixing time increase in bench scale and in minibioreactor equipped with paddles, but its effects are not evident in minibioreactor equipped with pitched blades.

Finally, mixing study results it is proposed a new stir speed for the minibioreactor that matches mixing time in the bench-scale bioreactor. This could provide a useful physical scale-down parameter between both scales.

Keywords: mixing time, circulation time, bioreactors, visual method
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Notation and Glossary

\[ B \] Blue brightness level
\[ C_c \] Distance between bottom of the shaft and the center of lower impeller (mm)
\[ C_b \] Distance between the centers of the impellers (mm)
\[ D \] Impeller diameter (mm)
\[ G \] Green brightness level
\[ H \] Liquid height (mm)
\[ I \] Brightness level
\[ K_C \] Number of revolutions required for a fluid element to complete a loop
\[ L \] Distance between wall and dye addition point (mm)
\[ N \] Rotation speed (s\(^{-1}\))
\[ P \] Power consumption (W)
\[ R \] Red brightness level
\[ Re \] Reynolds number
\[ s \] Baffle width (W)
\[ T \] Bioreactor diameter (cm)
\[ V \] Volume of Liquid (mL)
\[ V_a \] Volumetric of air flow rate (cm\(^3\)s\(^{-1}\))
\[ W \] Width of the impeller (mm)

Acronym

BCR Bubble column reactor
BIOSTAT Bench scale reactor
CAST Center for Advanced Sensor Technology
DOT Dissolved Oxygen
HTBR High-throughput minibioreactor
ISFET Ion-selective field effect transistor
LIF Laser induced fluorescence
MBCR Miniature bubble column reactor
MBR Microbioreactor
MSBR Miniature stirred reactor
MTP Microtitre plate
Mixing characterization in novel high throughput minibioreactors: Scale-down modeling from bench scale

**OD** Optical density  
**PMT** Photomultiplier tubes  
**RPM** Rotations per minute  
**STR** Stirred tank reactor  
**UV** Ultra violet

**Greek letters**

- $\varepsilon_T$ Dissipated energy $(Wm^3)$  
- $\mu$ Liquid viscosity $(Pa\cdot s)$  
- $\mu_w$ Difference between mixing times obtained by different methods  
- $\theta_c$ Circulation time $(s)$  
- $\theta_M$ Mixing time $(s)$  
- $\theta_{MC}$ Mean circulation time $(s)$  
- $\rho$ Liquid density $(Kg/m^3)$

**Subscript**

- $i$ Counter
1. Introduction

1.1 Guidelines and project presentation

Traditionally, bioprocess technology is implemented in pharmaceutical industry (Lee et al. 1991), especially in development of new drugs and vaccines (Kostov et al. 2001).

Nowadays, there is a wide range of applications of bioprocesses from food industry (Żyta 1992), ecology (Scott et al. 1994), water treatment (López-López et al. 1999), fuels production (Sommer et al. 2004) and biopharmaceuticals (Brecht 2010). In addition, the decoding of human genome and the first steps of comprehension in correspondence between gene and proteins boost the development of bioreactors and fermentors (Lamping et al. 2003).

To improve the yield of bioreactor, it is of critical importance to establish the relationship between physical parameters (mixing time, mass transfer coefficient) and biological parameters (such as the growth rate, product yield and quality). As a result, the design and optimization of biological processes requires the control of several parameters involving the experimental investigation of a large combination of variables (Gill et al. 2008).

Carrying out these experiments in manufacturing scale is not plausible (Papagianni et al. 2003) and even at the bench-scale the operation costs are very expensive and time consuming (Kostov et al. 2001). To face this, several authors proposed use mathematical models (Tholudur et al. 1999) (Schubert et al. 1994) but even in this case, there is a needed for running some experiments to validate them (Berkholz and Guthke 2002). For decades spinner flasks and shake flasks have been used for bioprocess development due to their relative small size that increase their high throughput capabilities reducing bioprocess optimization costs. However, these cell culture devices do not provide relevant process information (such as pH or Dissolved Oxygen) making them not too useful for bioprocess optimization (Chen et al. 2009). Also, the application of parallel instrumented shake flasks remains limited by challenges of high parallelization and cost (Amanullah et al. 2010). In addition, large-scale bioreactors are typical stirred tanks and the agitation-type difference between the small scale (shake) and the manufacturing scale (stirred) could negatively affect the biological system when scaling up. As a result, minireactors with agitation are a promising system because they can accomplish

- Low cost for raw material/culture media;
- High parallelization enabling faster timelines and quickly development;
Introduction

- Easily possibility of scale up comparing with the other small scale systems;
- Possibility of incorporation of economical, non invasive sensors.

However, despite this significant progress on minibioreactors engineering there are still some questions that need to be addressed.

Summarizing, stirred minibioreactors equipped with impellers and non-invasive optical sensing could be a real alternative to the existing bench-scale development bioreactors. Consequently, minibioreactors represent a paradigm shift in bioprocess development.

This research project is inserted in an ambitious project, which main goal is implementing high-throughput stirred mini-bioreactors with feedback control to validate them as an alternative to the existing bench-scale systems. These mini-bioreactors could then be use to perform process development studies and eventually predict results at manufacturing scale.

The specific objective of this Master Thesis is to characterize liquid mixing and establish mixing comparability between mini-bioreactors and existing bench-scale reactor. The goal is to further develop correlations that model liquid mixing as a function of reactor and operations variables as impeller type, Reynolds number and liquid height over vessel diameter ratio. Whenever the acquisition method allows the detection of circulation peaks, circulation time will be analyzed.

To get these goals, it will be used a visual technique to evaluate the mixing time. Visual technique will be improved by fluorescence process. After validate, this method it will be applicable in distinct systems (5L and 30mL reactors) to eliminate the influence of different techniques, each one used in each system until now.

1.2 Work contributions

First of all, it is intended validate visual method of measuring mixing time. The innovation will be not only in the acquisition system, but instead of that, it will concern an objective digital data processing and analyzing. It is an easy handling and economical technique that could be useful in glass made vessels mainly at lab scale. As a visual method, it will provide information about entire vessels, but at the same time it can also analyze specific regions inside of it. Moreover, the images provide some information that it is not available on other methods which the answer is given by only a plot. With these latter, it is
impossible to check the distinct behavior of the liquid in different areas of the vessel and clearly found as dead-zones, segregation zones. The quality of the images, in terms of high contrast and definition of the images are the result of a fluorescence technique.

It was shown that mixing time and even the type of the impellers affects the bioreactor product yields (Lee et al. 2000; Zhong et al. 2002). Apart biological aspects, this project will help to understand how variation in some parameters can influence or not the mixing. This study will be useful in scale down-scale up bioreactors, once that the combining the correlation from both bench scale reactor and minibioreactor, setting the parameter on one of the systems, it will be easily predict what happens in the other one about mixing time concerns.

In addition, understanding the mixing time will be helpful in future projects such as mass transfer and cell culture studies.

1.3 Thesis organization

To become easily reading the thesis is organized in Chapters.

Chapter 2 and Chapter 3 concern theoretical parts of this work. Chapter 2 focuses the fundamentals behind this project: mixing and fluorescence. In Chapter 3 it is reviewed the state of art in terms of minibioreactors, as well mixing time and circulation time assessment techniques.

In Chapter 4 it is described detailing used methods as well used materials.

Chapter 5 is the core section of this thesis. In chapter 5 it is showed the main results as well it is discussed and explained the causes and the consequences of them. It includes the modeling and the comparing between both studied systems.

Finally, in chapters 6 and 7 it is synthesized the main points of this project by means of the conclusion, final appreciation and it is identified the possible improvements of the methods used.
2. Theory

2.1 Mixing

The mixing time, $\theta_M$, is one of the most useful criterion for mixing intensity of fermentation broths and for scale-up of biosynthesis processes (Oniscu et al. 2002). It is defined as time needed to achieve a given degree of homogenization, when a perturbation is induced and it is used to characterize the mixing performance of stirred vessel (Cabaret et al. 2007). That perturbation is a tracer such as a acidic, alkaline or salts solutions, heated solutions, colored solutions (Oniscu et al. 2002). For practical purposes, the degree of homogenization is typically considered 95% or 99% of the final equilibrium value (Bonvillani et al. 2006).

Mixing time depends on a huge number of parameters whether geometric (dimensions of mixing system, dimensions of the reactor) as well technological factors (type of impeller or existence of baffles fermentation conditions, physical properties of the medium). The general correlation that describes the mixing time is of the type of Equation 1 (Oniscu et al. 2002).

\[
\theta_M = f(T, D, N, \mu, \rho, V, V_a, P, \varepsilon_T, s)
\]  
(1)

With:
- $T$: Bioreactor diameter
- $D$: Impeller diameter
- $N$: Rotation speed
- $\mu$: Liquid viscosity
- $\rho$: Liquid density
- $V$: Volume liquid
- $V_a$: Volumetric of air flow rate
- $P$: Power consumption
- $\varepsilon_T$: Dissipated energy
- $s$: Baffle width

Another important parameter helpful in mixing characterization is circulation time. Circulation time, $\theta_C$, is a parameter related to the pumping capacity of an impeller (Guérin et al. 1984). It is the time for a fluid element to complete a closed loop. However, the
probability of this is very low and in practice it is taken as the time interval between two
crossings, in the same direction, of an appropriate reference plane (Guérin et al. 1984).
Actually, its definition is closely related to the experimental technique used to measure it. If
the passage of a freely suspended particle in a vessel is analyzed by a flow follower
technique, circulation time is the time necessary for a fluid element to complete a closed
loop (Delaplace et al. 2000). If the measurement technique is a probe method (Delaplace et
al. 2000) or a sensor method (Vallejos et al. 2006), the circulation time correspond to the
time between two subsequent circulation peaks on the response curve.

Numerous authors (Delaplace et al. 2000; Holmes et al. 1964; Roberts et al. 1995)
established a correlation between circulation speed and rotational speed of the impeller
(Equation 2), as in laminar as in turbulent regimes with different impellers (helical ribbons
and turbines). Also a recent study (Vallejos et al. 2006) in transitional regime with paddles
supports this correlation.

\[
\theta_c = \frac{K_C}{N} 
\]  

Which \( K_C \) is a represents the number of revolutions required for a fluid element to
complete a loop (Delaplace et al. 2000). Equation 2 shows that circulation time is inversely
proportional to the impeller speed.

Actually, as the circulation time varies inside each experimental, for practical
proposes it is used the mean circulation time, \( \theta_{MC} \), defined in Equation 3, where \( n \) represents
the number of circulation peaks.

\[
\theta_{MC} = \frac{\sum_{i=0}^{n} \theta_C}{n} 
\]  

Mixing in stirred tank reactors is often characterized by some additional dimensionless
groups. One of the most common and useful of them is Reynolds number (Equation 4). The
numerator represents the inertia forces, while dominator expresses viscous forces.

\[
Re = \frac{ND^2 \rho}{\mu} 
\]
Three different regimes have been identified with Reynolds number:

Turbulent regime - Above the critical Reynolds number, Re_{crit}, of about 10000 for water like fluids. At this values or Reynolds inertial forces overwhelm viscous forces. It is characterized by the existence of eddies.

Laminar regime - Laminar regime is in general found at Re< 10, flow is laminar with the uniform flow profile and the absence of random eddies.

Transitional regime - This regime is found between 10 and 10000 Reynolds approximately. In the transitional regime there is coexistence of uniform flow and eddies.

Mixing in agitated vessels is considered to occur at three scales: micromixing, mesomixing and macromixing, but in terms of mixing time only this last is relevant. (Houcine et al. 2000).

Macromixing concerns the motion fluid at largest scale generating an average homogenization of the total volume blended and it is often studied for reactor and in particularly for bioreactors.

### 2.2 Fluorescence

Luminescence is a phenomenon in which the molecular absorption of energy causes emission of a photon with a longer wavelength (and obviously lesser energy), during molecular relaxation from electronic excited to the ground state (Shahzad et al. 2010). Depending on the nature of excited state luminescence is split into two categories: phosphorescence and fluorescence. The difference between both is in the spin orientation acquired by the excited electron. If after excitation, the electron is promoted to a higher energy keeping the same spin orientation it is called a singlet. Consequently, return to the ground state is spin allowed and occurs rapidly by emission of a photon. So, lifetime, that represents the time between the excitation and the return to the ground, is short (about 10 ns). That happens in polyatomic fluorescent molecules designated fluorophore (Lakowicz 2006). Otherwise, if after excitation, the promoted electron acquires the opposite spin orientation, it forms and triplet. Because that, transitions to the ground state are forbidden and the emission rates are slow carrying out longer life times from milliseconds to seconds, but even till several minutes (Lakowicz 2006). This represents a phosphorescence process. In figure 1 it is shown the spin of the different states.
In fluorescence processes not all the absorbed energy is released by radiative emission. Three nonradiative deactivation processes are also significant: internal conversion, external conversion and the intersystem crossing (So and Dong 2007).

Internal conversion is a process where the electronic energy is converted to the vibrational energy of the fluorophore. Hence, the fluorophore is relaxing to the lowest vibrational energy level of the first excited state (So and Dong 2007).

External conversion describes the process where the fluorophore loses electronic energy to its environment through collision with other solutes. Upon collision, the fluorophore is deexcited nonradiatively. Quenching by oxygen is one important process of external conversion (Lakowicz 2006).

Intersystem crossing happens when molecules in the $S_1$ state can also undergo a spin conversion to the first triplet state $T_1$ (Lakowicz 2006).

In Figure 2 it is present a Jablonsky diagram that illustrates various electronic processes that can occur in excited states and between them and ground state.
Introduction

According to the principle of energy conservation if there is some of the absorbed that is lost by internal conversion, external conversion or intersystem crossing, fluorescence emission is lower energetic and its wavelength is longer than absorption that origin it. That is designated Stokes shift. (Lakowicz 2006)

Fluorescence applications are widespread, especially as a medical diagnostic or analytical tool (So and Dong 2007) in bioprocess sensor (Rao et al. 2002) and in fluid analysis (Arratia and Muzzio 2004).

**Figure 2** - The Jablonski diagram of fluorophore excitation, radiative decay and nonradiative decay pathways (So and Dong 2007)
3. Background

Fluid mixing is a unit operation carried out to reduce temperature or concentration gradients in fluids, whereby it is a common operation in industry. Although it is widespread, there still is a need for mixing optimization. Enhanced mixing can allow to savings of several billions of dollars resulting from higher yield and selectivity, better product quality, reduced cost of separating impurities and faster product delivery to market (Dickey 2010). In several industries, namely in biochemical, mixing plays one of the most important roles, depending by itself the feasibility and the profitability of the process industry. Thus, optimization of degree of mixing in bioreactors is one of the main issues in bioprocessing (Vallejos et al. 2005).

In fact, the production and accumulation of biomass provoke the appearance of concentration gradients in the bioreactor. The formation of those zones will decrease the cell yield and modified the metabolism of the micro-organism in cultivation (Bylund et al. 1999). It was proven that in a fermentation process that cells in regions with high substrate concentration instantaneously metabolize glucose while the remainder starved (Hansford and Humphrey 1966). Thus, it is of critical important to increase the agitation to homogenize the broth as fast as possible. However it has been observed that high agitation can be lethal to animal cells (Petersen et al. 1988). So, there is a tradeoff, which the main question is finding the rotational speed that allows quickly mixing, but simultaneously avoids cells damage and death. Furthermore, it is recognized that from some rotational speed, increasing it speed will not affect significantly the mixing time in transitional regime (Vallejos et al. 2006).

3.1 Minibioreactors

Mimic bioprocesses at small scale, by means of minibioreactors, could improve exponentially the biochemical experiments and so the quickly discovery of new products. Then, these results could be scaled up to industrial level. Several types of minibioreactors systems capable of parallel operation have been tested in last couple years. Betts and Baganz (2006) did an excellent review and compiled the several studies about minibioreactors and microbioreactors systems. It is present in Table 1.
<table>
<thead>
<tr>
<th>Device/Reference</th>
<th>Device/Reference</th>
<th>Volume (ml)</th>
<th>Agitation/Aeration</th>
<th>pH, DOT and OD instrumentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fedbatch-Pro</td>
<td>Shake flask</td>
<td>50-500</td>
<td>Orbital shaker; Surface</td>
<td>pH via sterilisable probe</td>
</tr>
<tr>
<td><a href="http://www.dasgip.de">http://www.dasgip.de</a></td>
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<tr>
<td>MicroReactor</td>
<td>MTP microfabrication</td>
<td>3-5</td>
<td>Orbital shaker (up to 800 RPM); Gas sparging</td>
<td>pH and DOT via optical probes</td>
</tr>
<tr>
<td><a href="http://www.applikonbio.com">http://www.applikonbio.com</a></td>
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</tr>
<tr>
<td>SimCell,</td>
<td>Microfluidic chip</td>
<td>0.3-0.7</td>
<td>Rotation of MBR chips; Surface via membrane</td>
<td>pH, DOT and OD at-line via cell-reading station</td>
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<tr>
<td><a href="http://www.bioprocessors.com">http://www.bioprocessors.com</a></td>
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<tr>
<td>MBR array</td>
<td>MTP microfabrication</td>
<td>0.25</td>
<td>Orbital shaker (175 RPM); electrochemical O₂ generation</td>
<td>pH(ISFET sensor) and OD optically</td>
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<td>(Maharbiz et al. 2004)</td>
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<tr>
<td>Polymer-MBR</td>
<td>Microfabrication</td>
<td>0.15</td>
<td>Magnetic stirrer bar (200-800 RPM), Surface via membrane</td>
<td>pH, DOT and OD via optical probes</td>
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<td>(Szita et al. 2005)</td>
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<td>Stirrer-Pro flask</td>
<td>STR</td>
<td>200-275</td>
<td>Magnetic stirrer bar (10 - 1000 RPM); Sparger</td>
<td>pH and DOT via sterilisable probes</td>
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<td>Xplorer,</td>
<td>STR</td>
<td>Up to 100</td>
<td>Single turbine impeller (100 - 2000 RPM); Sparger</td>
<td>pH, DOT and OD probes</td>
</tr>
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<td>Cellstation,</td>
<td>STR</td>
<td>Up to 35</td>
<td>Dual paddle impeller (10 - 1000 RPM); Sparger</td>
<td>pH, DOT and OD via optical probes</td>
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<td>MSBR</td>
<td>STR</td>
<td>18</td>
<td>Triple turbine impeller (up to 7000 RPM); Sparger</td>
<td>pH and DOT via optical probes</td>
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<tr>
<td>(Betts et al. 2006)</td>
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<tr>
<td>Bioreactor block</td>
<td>STR</td>
<td>8-12</td>
<td>Gas-inducing single impeller (up to 4000 RPM)</td>
<td>DOT optically; pH and OD via plate reader</td>
</tr>
<tr>
<td>(Puskeiler et al. 2005)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parallel BCR</td>
<td>Bubble column</td>
<td>200</td>
<td>Gas-sparging</td>
<td>pH and DOT probes</td>
</tr>
<tr>
<td>(Weuster et al. 2001)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MBCR</td>
<td>Bubble column</td>
<td>2</td>
<td>Gas-sparging</td>
<td>pH and DOT via optical probes</td>
</tr>
<tr>
<td>(Doig et al. 2005)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.2 Mixing time and circulation time measuring techniques

In the last years, several physical and chemical methods have been developed to assess mixing time, although there is not a standardized technique for measurement of mixing time. Indeed, each method has its own advantages and limitations (Cabaret et al. 2007).

Roughly, according on the assessed liquid volume, mixing time measuring methods can be divided into two groups: local methods and global methods (Cabaret et al. 2007). The first provide information only about a specific point of the vessel and usually required a probe (thermal methods (Szoplik and Karcz 2008), conductometric methods (Kasat and Pandit 2004) and pH based methods (Oniscu et al. 2002)). Among this group there are also some methods that do not need probes, such as confocal optical systems (Vallejos et al. 2005), planar-laser induced methods (Arratia and Muzzio 2004) and radiotracer techniques (Domínguez et al. 1999). Global methods, they allow mixing evaluation in the entire vessel making possible to identify unmixed or segregation zones. Among global method there are chemical methods involving fast reactions and visual observation (acid-base (Melton et al. 2002) and redox color change methods (Kawase and Moo-Young 1989)), liquid-crystal thermography (Nere et al. 2003) and computer tomography with a coherent light (Zlokarnik 2001).

A review of the mixing techniques (Cabaret et al. 2007; Nere et al. 2003; Paul et al. 2004; Vallejos et al. 2005; Zlokarnik 2001) was done and it is presented in Table 2. It includes the main backwards and advantages of each one of most used techniques.
### Table 2 - Summary of the currently most used mixing time techniques

<table>
<thead>
<tr>
<th>Technique</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visual</td>
<td>Allow identifying different regions of mixing: dead zones, stagnation zone;</td>
<td>Seldom applicable at industrial scale; Subjective; Need transparent vessel;</td>
</tr>
<tr>
<td></td>
<td>Easy application;</td>
<td>Offline measurements;</td>
</tr>
<tr>
<td></td>
<td>Cheap and versatile.</td>
<td></td>
</tr>
<tr>
<td>Conductivity pH methods</td>
<td>Online measurement;</td>
<td>Local measurements;</td>
</tr>
<tr>
<td></td>
<td>Cheap and easy to use;</td>
<td>Probes affect the mixing;</td>
</tr>
<tr>
<td></td>
<td>Objective measurement;</td>
<td>Not applicable to high temperatures and organic materials;</td>
</tr>
<tr>
<td></td>
<td>Data analysis is easy.</td>
<td></td>
</tr>
<tr>
<td>Laser induced fluorescence</td>
<td>Objective measurement;</td>
<td>Requires transparent vessels;</td>
</tr>
<tr>
<td></td>
<td>Mixing time clearly monitored;</td>
<td>Local measurement;</td>
</tr>
<tr>
<td></td>
<td>Online measurement.</td>
<td></td>
</tr>
<tr>
<td>Electrical impedance tomography</td>
<td>Non invasive;</td>
<td>High cost of instrumentation;</td>
</tr>
<tr>
<td></td>
<td>Applicable to industrial scale</td>
<td>Different sensibility in wall and center of the vessel;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Time-consuming;</td>
</tr>
<tr>
<td>Electrical resistance tomography</td>
<td>Allows 3D representation of the system;</td>
<td>Not applicable to high temperatures;</td>
</tr>
<tr>
<td></td>
<td>Non-invasive.</td>
<td></td>
</tr>
<tr>
<td>Radioactive liquid tracer</td>
<td>Do not disturbs the flow;</td>
<td>Health hazard;</td>
</tr>
<tr>
<td></td>
<td>Tolerate high temperatures;</td>
<td>Transportation difficult;</td>
</tr>
<tr>
<td></td>
<td>Applicable in non transparent reactors;</td>
<td>Availability limitations.</td>
</tr>
<tr>
<td>Liquid crystal tomography</td>
<td>Non intrusive;</td>
<td>Requires transparent vessels;</td>
</tr>
<tr>
<td></td>
<td>Allows analyzing whole the vessel;</td>
<td>Complex Image analysis;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Requires a careful calibration.</td>
</tr>
<tr>
<td>Computer tomography with a coherent light</td>
<td>Provides the concentration among whole the vessel;</td>
<td>Requires transparent vessels;</td>
</tr>
<tr>
<td></td>
<td>Non invasive.</td>
<td>Not applicable to industrial scale;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Requires a powerful computing system;</td>
</tr>
<tr>
<td>Confocal optical system</td>
<td>Provides real time information;</td>
<td>Local measurement;</td>
</tr>
<tr>
<td></td>
<td>Applicable at industry scale;</td>
<td>Requires the installation of various units to accurate measurement of mixing time.</td>
</tr>
<tr>
<td></td>
<td>Able to detect dead and stagnant zones.</td>
<td></td>
</tr>
<tr>
<td>Laser Doppler velocimetry</td>
<td>High degree of accuracy;</td>
<td>Assesses only a simple point</td>
</tr>
<tr>
<td></td>
<td>Not invasive.</td>
<td>Expensive.</td>
</tr>
<tr>
<td>Particle image velocimetry</td>
<td>Ability to measure flow velocity at many locations simultaneously;</td>
<td>Cost and processing speed</td>
</tr>
<tr>
<td></td>
<td>Not intrusive</td>
<td>Not easy to apply.</td>
</tr>
</tbody>
</table>

**Background**
At least at lab scale for glass made cell culture devices, visual methods could be an excellent solution for mixing time measurement. The bottleneck remains in the subjectivity associated on this kind of techniques. Indeed, in recent years, several authors have focus on achieving an objective criterion for both global/visual methods. By image processing and analysis, a global method technique has been suggested (Cabaret et al. 2007) to improve the evaluation of mixing time.

Cabaret refers that in 1985, Müller (Müller 1985) monitored the homogenization process of methylene blue in clear liquid through black and white photographs along the homogenization of the tracer. The photos with 60,000 individual points per image were manually analyzed. The criterion was the grey value of the photos. As this technique implied manual processing of the images and hence time consuming, it was not developed.

In 1997, it was shown (Lee and Yianneskis 1997) that a thermography technique based in color change with temperature, allows establish the mixing time of water in turbulent regime. The tracer is a high temperature liquid containing liquid crystals. It is injected and the color of the whole tank is analyzed by image processing. The mixing time is evaluated by the hue distribution of the colors and it is determined when 95% of the pixels in the image have the same hue value. The drawback of this method is to require the same fluid density as the liquid crystal microcapsules.

More recently, it was tested the digital process of the images based in RGB level of brightness (Delaplace et al. 2004). The images extracted from a video represent a chemical color change using two acid-base indicators in mixing time analysis for highly viscous fluids. In case, mixing time was determined as the time to achieve 90% of brightness of green color at the final state. This method provides a whole vessel analysis with an objective criterion inexistent previously for this kind of techniques. Removing this main issue, visual techniques with digital process and analysis of the images presents a potential technique able to standardize the mixing time measurements, at least in lab scale.

Concerning circulation time, some techniques to evaluated it are: Radio Pill (Van Barneveld et al. 1987), flow visualization of a suspended particle (Takahashi et al. 1994), thermal method (Brito-De La Fuente et al. 1991), conductivity (Holmes et al. 1964) and magnetic flow follower (Roberts et al. 1995).
4. Methods and Materials

Mixing time is evaluated in two different systems: a 30 mL high-throughput minibioreactor (HTBR) and a 5L bench scale reactor (BIOSTAT), each one by two different methods. Visual method was used in both systems. Moreover, in minibioreactor it was used a Laser induced Fluorescence (LIF) method while in bench scale reactor it was a potentiometric method (pH based method).

4.1 High-throughput minibioreactor - HTBR

The vessel is a glass vial with a stainless cap that contains two holes. One of those fits the shaft and the other allows releasing the dye. The shaft is stainless steel made and supports two impellers. Figure 3 presents the design of the vessel and geometric parameters. The rotation of the shaft is provided by a motor on its top. It is linked to a processor which is plugged to an USB data acquisition (Labjack U12, www.labjack.com). This is connected to a PC where, by means of the VI Software BhargaviMotor, the motor is controlled.

![Minibioreactor geometrical parameters](image)
**Label:**

$C_c$  Distance between bottom of the shaft and the center of lower impeller

$C_b$  Distance between the centers of the impellers

$D$  Impeller diameter

$T$  Bioreactor diameter

$L$  Distance between wall and dye addition point

$W$  Width of the impeller

$H$  Liquid height

### 4.1.1 Fluorescence method

To assess both mixing time and circulation time in minibioreactor, it used fluorescence, which dye is fluorescein sodium salt (Sigma USA - Part number 49690) solution ($C_{\text{Fluorescein Sodium}} = 3.1 \text{ g/L}$). The fluorometer (Cary Eclipse Fluorescence spectrophotometer, www.varianinc.com) operated at 494 nm and 521 nm for excitation and emission respectively. As it is shown in Figure 4 these wavelengths are close to maximum fluorescein sodium absorbance and emission wavelengths and they are used until now in the Center for Advanced Sensor Technology (CAST).

*Figure 4 - Emission and absorbance wavelength of fluorescein sodium spectra at various concentrations (Doughty 2010)*
The fluorometer allows some parameters variation. After several tests with different conditions, the best configuration was founded and it is present in Table 3.

Table 3 - Fluorometer parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value / state</th>
</tr>
</thead>
<tbody>
<tr>
<td>Excitation wavelength (nm)</td>
<td>494</td>
</tr>
<tr>
<td>Emission wavelength (nm)</td>
<td>521</td>
</tr>
<tr>
<td>Excitation Slit (nm)</td>
<td>2.5</td>
</tr>
<tr>
<td>Emission Slit (nm)</td>
<td>5</td>
</tr>
<tr>
<td>Acquisition Time (s)</td>
<td>0.1</td>
</tr>
<tr>
<td>Emission filter</td>
<td>Open</td>
</tr>
<tr>
<td>Excitation Filter</td>
<td>Auto</td>
</tr>
<tr>
<td>PMT voltage (V)</td>
<td>580-650(^1)</td>
</tr>
</tbody>
</table>

The assessed region is above the first impeller and distance 42 mm from the vessel bottom and about 2 mm form the impeller shaft. That section is a 0.5 mL volume parallelepiped that results from the intersection between emission and excitation beams (information provided by manufacturer). Appendix 1 presents a schematic diagram of the intersection of both areas.

Following next procedure, a total of 10 runs per each rotational, ranging from 20 to 1000 RPM (108<Re<5380) speed were performed.

**Experimental Procedure**

1. Fill the reactor with distillated water.
2. Turn on the impeller with RPM previously defined.
3. Turn on the fluorometer.
4. Add carefully one drop (7 µL) of dye on the surface of the water.
5. Keep running until achieved steady-state.

The most relevant geometrical parameters are presented in Table 4.

\(^1\) With exception of the 1000 RPM trials that operate at 650 V, all the other run at 580 RPM. This difference is because at higher RPM the intensity of signal decreases.
### Table 4 - Minibioreactor geometric parameters in fluorescence method

<table>
<thead>
<tr>
<th>Reactor capacity (mL)</th>
<th>Impeller type</th>
<th>Cc (mm)</th>
<th>Cb (mm)</th>
<th>D (mm)</th>
<th>T (mm)</th>
<th>L (mm)</th>
<th>W (mm)</th>
<th>V (mL)</th>
<th>H (mm)</th>
<th>H/T</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>Flat paddle</td>
<td>9.5</td>
<td>13.5</td>
<td>18</td>
<td>26</td>
<td>6</td>
<td>6</td>
<td>30</td>
<td>57</td>
<td>2.2</td>
</tr>
</tbody>
</table>

#### 4.1.2 Visual method

Main advantage and difference from this visual method, which is intended to develop and to apply, is the introduction of computerized data analysis by ImageJ™, a powerful image processing and analysis Software. With this software it is intended to get an objective criteria inexistent till now in visual methods.

In addition, instead to use absorbance, it resorts to fluorescence and this allows a higher contrast and hence a more sensitivity analysis.

Two different roles from this new method are expected:
- Get the mixing time in the same condition as fluorometer tests and compare the results with both methods;
- Built a model which mixing time is a function of impeller type, rotation speed and H/T ratio.

The setup of this experiment is shown in Figure 5. It consists in the same vessel as referred in previous chapter. The experimental setup is like the fluorescence setup with two main differences: instead the fluorometer it was used a camcorder and there is needed to use a UV light, under the vessel, to excite the dye, the same as used in fluorescence experiments.

The UV light is a Fotodyne transilluminator, model FOTO/UV 21 ([www.fotodyne.com](http://www.fotodyne.com)), defined as 312 nm working single wavelength, very far from the sodium fluorescein absorption peak (about 490 nm). Despite these discrepancies, the dye excitation provided by it is enough to get good images in the high mode.

The data acquisition is provided by a Sony Camcorder HDR-CX110/L ([www.sony.com](http://www.sony.com)). It is a domestic camcorder able to record video in Full High definition mode (1920 pixels x 1080 pixels) at 60 interlaced frames per second (1080/60i). Though it can record at 24 Megabites per second (Mbps) as maximum bit rate, it was only work at 17 Mbps. After some tests, it was concluded there are not significant advantages to film at highest bit rate to compensate the computational resources required in manipulation and data analysis as RAM.
In front of camera lens it was set a 500 nm long pass filter. In Appendix number 2 it is possible to see with detail the filter used, as well a plot absorbance versus wavelength, which, in case, the interest is 500 nm dotted line. Analyzing that, it can assure that light with wavelengths lesser than 470 nm is almost all absorbed, although from 500 nm it is almost totally transmitted (remember that fluorescein sodium maximum emission wavelength is about 520 nm). Between these limits there is a hybrid region, where some light is absorbed and other is transmitted. It must be used this filter to remove stray light that became the movies too bright and impossible to analyze.

Unlike the fluorescence method, in visual method, it does not assess only one zone, but as many as intended. In this project four distinctive regions were defined: two (left and right) above the higher impellers and two (left and right) below them. Those zones, for the minibioreactor are presented in Figure 6. Each one of them has a 50 pixels x 50 pixels area.
After some test runs it was observe that two higher regions present similar values of brightness level, $I$, between them. The same happens with the lower regions. Actually, as it is proof in Appendix 3, brightness level depends on horizontal plane. As higher is the plane, lesser is the brightness level. Therefore, evaluated zones can be put together in to groups: one contains the regions above the impellers and the other the regions below them. Inside each one of these groups it was considerate the average of the two regions included. As a final result, there are two different values of mixing time, corresponding at each one of the groups.

It was used a Sony laptop VPCS111FM (www.bestbuy.com) were all the movies were trimmed with PMB™, split in frames by means of Free Video to JPG Converter™ 1.5.1.54 and finally analyzed by ImageJ™ (rsbweb.nih.gov/ij/). ImageJ™ stacks all the video frames which RGB pixels are convert to brightness level (Cabaret et al. 2007) values using the Equation 5.

$$I = \frac{R + G + B}{3}$$

Which $R$, $G$ and $B$ represent, by this order, the brightness of red, green and blue pixels and ranging from 0 to 255. Videos were trimmed in order that the time zero corresponding exactly the time which the drop contacts the fluid inside the vessel. The final point is the time enough to the vessel content is well mixed. It was defined as a function of the rotation speed. As larger is the rotational speed, shorter is the video. In Appendix 4 it is present the
time final time considered, as well the frames frequency for the different rotation speeds in HTBR.

Five runs were performed for each rotational speed. They were carrying out in the dark and with a black background to maximize the UV light effect.

Experimental procedure is similar to used in florescence method, being that it was performed 4 runs per each rotational speed, volume and impeller type.

As it was previously referred, the study of the mixing time in the minibioreactor by visual methods aimed two different purposes. On the one hand it is intended compare this method with the fluorescence method. So, the geometrical parameters of the minibioreactor keep exactly the same as used in fluoresce method (Table 4). On other hand, to build a model, the geometric parameters were slightly different from the mentioned before. These are the same as used up to now in CAST research and are presented in Table 5.

<table>
<thead>
<tr>
<th>Reactor capacity (mL)</th>
<th>Impeller type</th>
<th>Cc (mm)</th>
<th>Cb (mm)</th>
<th>D (mm)</th>
<th>T (mm)</th>
<th>L (mm)</th>
<th>W (mm)</th>
<th>V (mL)</th>
<th>H (mm)</th>
<th>H/T</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>Flat paddle</td>
<td>7.5</td>
<td>16</td>
<td>18</td>
<td>6</td>
<td>30</td>
<td>57</td>
<td></td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>45° Pitched blade</td>
<td>13</td>
<td></td>
<td>26</td>
<td>6</td>
<td>30</td>
<td>54</td>
<td>2.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

About the impellers type it should notice that flat paddle only provide radial flow, whereas pitched blade supply both radial and axial flows. In Appendix 5 it is shown pictures of both impellers.

4.2 Bench scale reactor - BIOSTAT

The BIOSTAT (www.solutions4biotech.com) is a compact benchtop fermentor with a 4.5 culture vessel. As the minibioreactor, bench scale reactor is a glass made vessel with a stainless steel cap. The cap supports the shaft with two 45° pitched impeller (Appendix 6) as well the sparging system. Also impellers and sparging system are stainless steel made. The cap contains three cavities where ordinary fit the thermometer, pH and dissolved oxygen

\[\text{Pumping down.}\]
(DOT) probes. Along these experiments thermometer and DOT probes were withdrawn. One of those holes became the releasing dye spot.

The rotation of the shaft is provided by a motor on its top that is driven by BIOSTAT control system. In Figure 7 it is presented the bench scale system.

![Bench scale reactor experimental setup](image)

**Figure 7 - Bench scale reactor experimental setup**

The dimensions of the most important geometric parameters of vessel and impellers are shown in Table 6. Except the volume, that was variable, the configuration of this system was kept fixed and it is the same as used in previous studies in CAST.

<table>
<thead>
<tr>
<th>Reactor capacity (mL)</th>
<th>Impeller type</th>
<th>Cc (mm)</th>
<th>Ch (mm)</th>
<th>D (mm)</th>
<th>T (mm)</th>
<th>L (mm)</th>
<th>W (mm)</th>
<th>V (mL)</th>
<th>H (mm)</th>
<th>H/T</th>
</tr>
</thead>
<tbody>
<tr>
<td>5000</td>
<td>45° Pitched blade³</td>
<td>30</td>
<td>76</td>
<td>72</td>
<td>159</td>
<td>35</td>
<td>30</td>
<td>4480</td>
<td>219</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3830</td>
<td>187</td>
</tr>
</tbody>
</table>

³ Pumping up.

Methods and Materials
4.2.1 Potentiometric method

Potentiometric methods, under form of pH per example, are widespread as a mixing time measurement technique (Hadjiev et al. 2006) (Godo et al. 1999). As established method it will be a reference for the visual method that is intended to validate.

To evaluate the solution potential, one electrochemical probe was set at about 40 mm from the bottom of the vessel. The electrode is made by Mettler Toledo with the model 405-DPAS-SC-K8S (us.mt.com) and it is constituted by an Argenthal (Ag/AgCl) as reference system and by a pressurized gel electrolyte as reference electrolyte.

The probe is connected to BIOSTAT that is linked to an USB data acquisition, which transmits the digital signal to a computer equipped with Labview Software. On Labview, it is converted in a plot which milivots are a time function. The acquisition time is 0.05 s.

The potentiometric experiments run at the same time as the visual experiments. Actually, it is the same experience which mixing time was analyzed by two different methods.

Following the next experimental procedure, it was performed 3 run per each rotational speed and volume, ranging from 60 to 596 RPM, corresponding to Reynolds number between 5164 and 51300.

**Experimental Procedure**

1. Fill the reactor with distilled water.
2. Turn on the impeller with RPM previously defined.
3. Turn on the camcorder.
5. Turn on ultraviolet light.
6. Add 1 mL of solution A on the surface of the water. (releasing time considered 1 s)
7. Keep running until achieved steady-state.

**Solution A** - These solution acts simultaneously as base as dye. It composition includes sodium hydroxide (8 g/L) and fluorescein sodium (3.1 g/L).
4.2.2 Visual method

To compare the results between the minibioreactor and the bench scale reactor, in both cases, it must evaluate the mixing time by the same technique. It is the visual technique. So, the setup is similar to the minibioreactor visual method setup with a small difference in the UV light. Now, instead a transilluminator, it is used a vertical UV lamp in the long wave position (356 nm). Also the image caption and the analysis procedures are the same as referred in minibioreactor.

As in the minibioreactor, the mixing time was evaluated in four different zones of the big vessel, but in this case the area of each one is 40 pixels by 40 pixels. Those zones are shown in Figure 8.

In Appendix 7 it is present the time final time considered, as well the frames frequency for the different rotation speeds in BIOSTAT.

![Figure 8 - Bench scale reactor assessed regions in visual method](image)

The experiments that allow measuring the mixing time by visual methods are the same as the experiments evaluated by potentiometric techniques so the experimental procedure is already descript in Chapter 4.1.2.
5. Results and Discussion

5.1 Visual method validation

5.1.1 Minibioreactors

As it was described in previous chapters, the validation of the new method to measure mixing time requires a comparability study with a method already tested and validate. For minibioreactors we used the Laser induced fluorescence method as our comparing method.

The data obtained from the fluorescence method is in the form of a plot, whereas the abscissas axle corresponds to the time and the ordinates axle represents the signal intensity. Figures 9 and 10 depict examples of the typical plots from two different rotation speeds. It is also represented the initial time (violet line), the 95% attenuation of the signal (green line) and its corresponding mixing time (red line). Furthermore, it is highlighted two consecutive circulation peaks (black circles). The distance between them represents the circulation time.

Figure 9 is an example at low rotational speed - 60 RPM.

In Figure 9 it is presented one example of the fluorometer response for a high rotational speed - 180 RPM.
It is clear that, after the releasing of the dye, the intensity of the signal varies within a large range. It is justified for the existence of concentration gradients in vessel. When that variation is attenuated by 95%, the vessel was reached the mixing time. After red line, almost there are not concentration gradients and the signal keeps constant.

Comparing both Figures 9 and 10, for the same 30 ml liquid volume, it can see that at 60 RPM time between two consecutive peaks is larger than in 180 RPM. This means that the fluid elements at 180 RPM takes less time to complete a closed loop, so circulation time is lower than at 60 RPM. In addition the steady state is achieved quickly at 180 RPM (11.8s at 180 RPM versus 23.9s at 60 RPM).

With the results get form 10 runs for each one of the 12 different rotational speeds were performed, which provides different mixing times it is possible plotting a graph and establishing a correlation between mixing time and Reynolds number (proportional to rotational speed). Figure 11 shows the dependence of mixing time with Reynolds number for 30mL reactor volume (H/T=2.2), with agitation provided by two flat paddles and the mixing time assessment by planar Laser induced fluorescence. Also the standard deviation for each different rotational speed is plotted.

As it was predictable as larger is the Reynolds number (consequence of increasing speed rotation) shorter is the mixing time. This is in agreement with previously results (Vallejos et al. 2006). The experimental data, excluding the 20 RPM point, are well fitted by a
power a power curve: $\theta_M = 5339 \ Re^{-0.924}$. The excluded point presents abnormal reduced mixing time possibly caused by diffusion at this speed rotation. Though the system, at that rotational speed, it is in transitional regime, Reynolds number is so lower ($Re=108$) that the behavior of the fluid should be closer from the laminar regime than from the turbulent regime.

Figure 11 - Dependence of the mixing time with Reynolds number obtained by fluorescence method in HTBR

As a negative power function, it is obvious that, as of some point, increasing the rotational speed, and so the Reynolds number, it will not reduce significantly the mixing time. Notice, per example, that mixing time for the Reynolds range 538-850 (4th to 7th blue points on the graph) is almost constant and equals to 13 s.

As it was described in Chapter 4.1.2, get the mixing with a visual method requires four steps: filming the video, trimming the movie, splitting the frames and finally analyzing the frames. Now, it will focus in two last steps.

In Figure 12 it is presented a sequence of frames taken at different times from an experiment at 221 RPM. After adding the dye, it is mixed, firstly above the higher impeller and then between the impellers. These images shows, that, apparently, it exists two different mixing regions: one above the top impeller and another below it (border marked by two red arrows in Figure 12 for t=3 s). Furthermore, part of dye still involving the shaft and it is mixed...
only later. Even at $t=7$ s, there is a little bit of dye that is not completely blended (marked by a red arrow.

![Image sequence from frames taken from HTBR at 221 RPM](image)

After removing the frames, it follows the analyzing stage. All the frames are evaluated concerning to the mean brightness level and its standard deviation inside each one of the four areas defined earlier. In Figure 13 it is plot the mean brightness from the same trial as the preceding image sequence. The different lines represent the four assessment sections defined.
The initial great oscillation, mainly in up zones, correspond to high concentration of the dye after its injection on the top of the vessel.

As it is shown is Figure 13 and referred in Chapter 4.1.2, the tendency of the lines allows putting them together in pairs. One pair corresponds to the zone above the first impeller and other the section underneath. The plot of the group mean brightness is presented in Figure 14.
Applying the criteria to 95% (or 105%) of mixing time (horizontal lines), it results in two different mixing times:
- Above the first impeller: $\theta_M = 4.67$ s
- Between the impellers: $\theta_M = 6.73$ s

In relation to the brightness standard deviation inside each one of the section, it is an auxiliary tool that allows analyzing if the brightness level, and thus the concentration of dye, are uniform and the mixing is effective. The mixing time was determinate according the mean brightness level, but the standard deviation was used as a check tool.

![Figure 15 - Evolution of standard deviation of the brightness with the time for a 221 RPM run in HTBR](image)

Checking the standard deviation brightness level (Figure 15) for the mixing times found previously, it is low, so the initial mixing time values are confirmed.

Similar analysis were done for each one of the four trials performed along the Reynolds number range 323-5380, corresponding to rotational speed range 60-1000 RPM. Comparing with the runs analyzed by laser induced fluorescence technique, the Reynolds range was reduced: it was not performed runs at 20 and 40 RPM, because at so low rotational speed the regime is too closer from the transitional regime and it must account with diffusion considerations, which are not an issue for this project. Also the 500 RPM runs were discarded. Obtained values allow plotting the graph presented in Figure 16. It represents the dependence of mixing time with Reynolds number for 30mL reactor volume, with agitation provided by two flat paddles, but in this case the mixing times are assessed by visual method.
The plot shows the mixing time obtained by visual method only for the region above the impellers. Since the fluorescence method evaluates only a region above the impellers, it just makes sense to compare values get from similar zones.

Like in the LIF method, it was fitted to the experimental results by a power function. The results were reasonably adjust by the function $\theta_M = 2019 \cdot Re^{-0.840}$.

In order to compare the results from the two methods previously defined and to get statistical validation the geometrical parameters were kept constant. In LIF method ten runs were carried out versus the four runs performed in the visual method. So, inside the LIF method results, four run were randomly chosen. Figure 17 depicted the mixing time for different methods evaluated at rotational speed.

Figure 16 - Dependence of the mixing time with Reynolds number obtained by visual technique in HTBR

![Graph showing mixing time vs Reynolds number](image)

$$y = 2019x^{-0.840}$$

$R^2 = 0.9199$

Figure 17 - Mixing time versus RPM for different evaluation methods in HTBR

![Graph showing mixing time vs RPM](image)
Figure 17 suggests that there is a difference between the results obtained by different techniques.

To test if the results obtained from both techniques do not diverge significantly, it was used a paired T-test. It was defined a 5% significance level.

It was tested the null hypothesis:

$$H_0: \mu_w = 0$$  \hspace{1cm} (6)

Against the alternative hypothesis:

$$H_1: \mu_w \neq 0$$  \hspace{1cm} (7)

Which \( \mu_w \) represents the difference between the mixing times obtained by different methods.

Running in Microsoft Excel a two sides the T-test with 5% of significance level and 9 degrees of freedom, the results are:

- P-value = 0.003;
- Critic T = 2.262;
- T-Test = 4.017.

As the p-value is lesser than significance level (0.003 < 0.05) and the value of the T-test is out of the range -2.262 - 2.262, null hypothesis is reject and it is proof that there is differences between the mixing time measurement techniques in minibioreactor case.

The results get from the tests in the minibioreactor, as by visual method as by LIF method present widespread between themselves. One of the main contributions for this fact could be improving the method of injection of the dye inside the vessel. Actually, it is impossible to release the drop of dye always in the same point of the vessel. Moreover, the major probability is in releasing the dye at different positions. Since the results compared before were come from different runs, this is different methods were applied in different trials, part of the difference between methods could be justified by the difference releasing points in different runs. Furthermore, the region assessed by the fluorometer is closer the impeller than the regions analyzed by the video method, where it looks it is harder to get well mix (Figure 12). This statement can justify why the results from the LIF method are in almost cases superior.
One last factor responsible for the differences found could be the great oscillation of the signal, which does not allow an accurate determination of the blending time. The oscillation should be caused by the shadow of the impeller.

5.1.2 Bench scale reactor

To validate the visual method in bench scale reactor the results were compared with the results from a potentiometric method. In this last, the data provided by the acquisition system are in form of a plot, which the voltage is a function of the time. Figure 18 presents a plot for 180 RPM run. The vertical lines represent different times: purple - initial time; red - 95% of mixing time; yellow - time equals 1 second corresponding to the limit of the dye releasing time. The green line represents 95% of the final voltage. From its intersection with the voltage curve, it results value of 95% of mixing time.

![Figure 18 - Potentiometric electrode response to dye injection at 180 RPM in BIOSTAT](image)

The mixing time is determined by the time difference between the red line and the yellow line. It was considered the yellow line instead the purple line because it was assumed the injection time as 1 s. In the case plotted, mixing is around 5.5 s.

Apparentely, the potentiometric response presents low oscillation, what is an advantage, but simultaneously, it carries out lower sensibility. This can be state since, as it is
Mixing characterization in novel high throughput minibioreactors: Scale-down modeling from bench scale

Results and Discussion

possible to observe in Figure 18, the potentiometric curve is split in 0.3-0.4 s steps, tough the
time acquisition be 0.05 s. Indeed, even at higher rotational speed (596 RPM) the time step is
about 0.2-0.3 s.

The data obtained from three runs at ten different rotational speeds, from 60 to 596
RPM, corresponding at Reynolds number range 5164 - 51300 (essentially turbulent regime),
are plotted in Figure 19.

Figure 19  – Dependence of the mixing time with Reynolds number obtained by potentiometric method in BIOSTAT

![Graph showing mixing time vs Reynolds number](image)

The data allow establishing a correlation between mixing time and Reynolds number.
For a 4.5 L reactor volume (H/T=1.4), with agitation provided by two 45° pitched blade, and
the mixing time evaluated by a potentiometric method the data are well fitted by a power a
power curve: \( \theta_M = 683.6 \times Re^{0.518} \).

Regarding the three last points of the graph, although the Reynolds increases 75%, the
mixing time only decreases 11%. As in the minibioreactor, it looks from some point, increasing
the rotational speed will not reduce significantly the mixing time. As discussed before, these
results can be affected by low sensitivity of potentiometric probe and the real difference
could be greater.

About the visual method, all the same considerations from the minibioreactors are
applied to bench scale reactor. The visual evolution with the time from the same 180 RPM run
as in potentiometric method is presented in Figure 20.
Results and Discussion

Figure 20 - Image sequence from frames taken from BIOSTAT at 221 RPM.

Figure 21 shows the brightness level evolution with the time for a 180 RPM run. In addition, it is present the points considered as the 95% mixing time for each one of the two sections defined previously.

Figure 21 - Brightness level evolution with the time for a 180 RPM trial in BIOSTAT.
Analyzing Figure 21 it is possible to check that there is not so high signal oscillation comparing with the minibioreactor plots. Additionally, it can observe that, after an initially disturbance provoked by releasing the dye, both up and down curves presents similar behavior and closer mixing times.

Thus, mixing time correspond to the intersection between 95% or 105% intensity of the steady-state intensity with the each intensity curve. Thus, the mixing time founded for each one of the section is:

- Above the first impeller: $\theta_M = 3.97$ s
- Near the second impeller: $\theta_M = 4.33$ s

As well, in previous analysis, it was plotted two graphs referring to the different regions defined above, which mixing time is a function of Reynolds number. The data were well fit by a power curve: $\theta_M = 7099 \text{ Re}^{-0.783}$ ($R^2=0.938$) for data above the impeller, and $\theta_M = 32173 \text{ Re}^{-0.936}$ ($R^2=0.967$) for data acquired near the second impeller.

Finally, both potentiometric and visual methods are compared. Figure 22 depicted in different series, the mixing time for different methods at different rotational speed. As the potentiometric probe in near the bottom of the vessel, the results obtained from this technique will be compared with the results get from the region near to the vessel bottom in visual method.
At naked eye, the previous plot can be split in two different sections: one until 140 RPM (including it), which the visual method provides higher mixing time values than potentiometric method; The second section from 180 RPM, which mixing time evaluated by visual method are always lesser than by potentiometric method. Part of this different could be explained, as it was referred before, by low sensitivity of the potentiometric method. Indeed, step times found (0.3-0.4s), at higher rotational speeds representing 15-20% of the mixing time.

Just like in minibioreactor case, to comparing the results get from difference mixing time techniques, it was used a paired T-test to assess if the results get from different techniques do not diverge significantly with 5% significance level. The null hypothesis is defined as:

\[ H_0: \mu_w = 0 \]  
\[ (8) \]

Against the alternative hypothesis:

\[ H_1: \mu_w \neq 0 \]  
\[ (9) \]

Which \( \mu_w \) represents the difference between the mixing times obtain by different methods.

Running in Microsoft Excel a two sides the T-test with 5% of significance level and 9 degrees of freedom, the results are:
- P-value = 0.059;
- Critic T =2.262;
- T-Test =2.165.

As the p-value is greater than significance level (0.059 > 0.05) and the value of the T-test is in the range -2.262 - 2.262, null hypothesis is accept and it concludes there is not significant differences between the mixing time obtained by both measurement techniques in bench scale reactor.
5.2 Modeling

The modeling will allow predicting the future response of both systems, as well to provide relationships between them. As the global goal of this project is to simulate in minibioreactor, the bench scale reactor behavior, this modeling could answering to the questing: what is the rotational speed in the minibioreactor that provides the same mixing time as the bench scale reactor, for given volumes/heights of liquid?

To model the mixing time in different kind of reactors, two variables have been change: rotational speed volume of liquid. These variables do not appear explicitly, but under form of Reynolds number and height of liquid over vessel diameter (H/T). It was defined a power fit like as referred in Equation 10.

$$\theta_M = a \cdot Re^b \cdot \left(\frac{H}{T}\right)^c$$

(10)

Although it had not been found any model including these, and only these variables, it was found that each one of the independent terms influences the mixing time in a power form. Thus can explain why it was chosen a power fitting.

As Equation 10 is in a non-linear form, to easily perform the modeling it is needed to linearize it. So, it is applied, resulting Equation 11.

$$log(\theta_M) = log(a) + b \cdot log(Re) + c \cdot log\left(\frac{H}{T}\right)$$

(11)

As the expression is already linearized, now it is possible to get the model with regressions tool in Microsoft Excel. This tool adjusts the data based on least squares method, that is, the minimization of sum of the square differences between the model and experiments.

5.2.1 Minibioreactors

Despite the visual method had not been validate, it will be use to modeling the minibioreactors. Visual method is the common method in HTBR and BIOSTAT and it will be used to compare results from both systems.
Concerning the minibioreactors, it was built four different models corresponding at two different zones (above and below the first impeller) per each one of the two impellers type: flat paddle and 45° pitched blade.

In flat paddles case the parameters found, as well the upper and lower intervals with 95% of confidence level are exposed in Table 7. Also the coefficient of determination, $R^2$ is presented. The model adjusts the points in the Reynolds number range 538-1189, corresponding to a rotational speed ranging 100-221 RPM.

| Table 7 - Fitting parameters concerning flat paddles in HTBR |
|-------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|
|                  | Below |       |       | Above |       |       |       |       |       |
| Value            | a    | b    | c    | $R^2$| a    | b    | c    | $R^2$|
| Upper limit      | 4.064| -1.162| 0.907| 4.428| -1.264| 0.253|
| Lower limit      | 4.702| -0.948| 1.342| 5.013| -1.068| 0.652| 0.760|

Analyzing Table 7, it is clear a huge difference between different sections, concerning the parameter c that corresponds to the H/T term. Tough, in both cases, the mixing time increases with the raise of the height of liquid (vessel diameter is constant), its effect is so much greater below the impeller. It is in agreement with the visualization of the video, which is possible to watch the first impeller actuating like a barrier to the dye, causing a high mixing time below the impeller. At higher H/T, the drop of the dye will disintegrate completely before achieving the first impeller. At lower H/D the space from liquid surface and the impeller is shorter and it is not enough to highly deform the drop, so by gravity it keeps falling and it is able to get the region bellow the impeller.

About the effect of the Reynolds (proportional to the rotational speed), as it was expect, the mixing time decreases as it increases (Vallejos et al. 2006).

The models present lower coefficient of determination because there is a widespread of the results into each setup, that is, keeping the same conditions, the results varying significantly. In Figure 23, it is possible to observe the experimental results, as well the models obtained for the minibioreactor above the paddles. Similar plot was obtained in case of the evaluation below the first impeller.
Figure 23 - Mixing time as a Reynolds function for different H/T ratios in HTBR with flat paddles, concerning the section above the impeller.

Now, it is shown the influence of the assessment area. In Figure 24, it is plotted the four models obtained for minibioreactor equipped with flat paddles.

Figure 24 - Effect of the assessment area in mixing time measurements in HTBR equipped with flat paddles.

Observing Figure 24, it can be concluded that, in the minibioreactor with flat paddles, the area of measurements can carrying out huge influence in mixing time measurements.
In relation to the 45° pitched blade impellers, the results for the fitting parameters, in both section below and above of the first impeller, are shown in Table 8. The model adjusts the points in the Reynolds number range 281-620, corresponding to a rotational speed ranging 100-221 RPM.

Table 8 - Fitting parameters concerning 45° pitched blades in HTBR

<table>
<thead>
<tr>
<th></th>
<th>Below</th>
<th></th>
<th>Above</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Value</td>
<td>a</td>
<td>b</td>
<td>c</td>
<td>R²</td>
</tr>
<tr>
<td></td>
<td>3.708</td>
<td>-1.074</td>
<td>-0.271</td>
<td></td>
</tr>
<tr>
<td>Upper limit</td>
<td>4.396</td>
<td>-0.818</td>
<td>0.222</td>
<td>0.576</td>
</tr>
<tr>
<td>Lower limit</td>
<td>3.020</td>
<td>-1.330</td>
<td>-0.764</td>
<td></td>
</tr>
</tbody>
</table>

In Figure 25, it is shown the experimental results, as well the models obtained for the minibioreactor between two pitched blades. Similar plot was to get in case of the evaluation above the top blade.

Figure 25 - Mixing time as a Reynolds function for different H/T ratios in HTBR with 45° pitched blade, concerning the section above the impeller.
As in the flat paddles case, also in the agitation provided by 45° pitched blades, the dependence of the mixing time on Reynolds number presents the same behavior: increase the Reynolds number carry out a reduction in mixing time. Actually, both systems (flat paddles and 45° pitched blade), either in section above or below the impellers, present similar $b$ parameter values in the range between -1.074 and -1.264. With respect to the dependence of mixing time with the H/T ratio, the results are in opposition to the ones found in paddles system. In pitched blade system, considering the region above the impeller, it was found that that ration almost do not influence the mixing time ($c=0.046$). Below the impeller, the results are unexpected - the mixing time reduces with the increase of the H/T ratio. Indeed, this could be explained by the effects of axial flow pumping down providing. Moreover, these last results should not be too accurate, once the coefficient of determination is too low ($R^2=0.576$).

Like in minibioreactor equipped with flat paddles, also in pitched blades system, it is reasonable to study the influence of the area analyzed. In Figure 26, it is expose the various models get from different zones for each one of the H/T ratios.

![Figure 26 - Effect of the assessment area in mixing time measurements in HTBR equipped with 45° pitched impellers](image)

Like in the paddle setup, in minibioreactors with agitation provided by 45° pitched blade, there is too difference in mixing times between the two both assessed regions, but in this setup is not so evident like in flat paddles case. Hence, this last should be deprecated in
relation with pitched blades that present lesser discrepancies in mixing times in different zones of the vessel.

5.2.2 Bench scale reactor

Concerning to the bench scale reactor like in minibioreactor, the values considered to modeling are the values get by visual method. The modeling parameters are presented in Table 9. The model adjusts the points in the Reynolds number range 5164-51300, corresponding to a rotational speed ranging 60-596 RPM.

<table>
<thead>
<tr>
<th>Value</th>
<th>Below</th>
<th>Above</th>
</tr>
</thead>
<tbody>
<tr>
<td>Value</td>
<td>a</td>
<td>b</td>
</tr>
<tr>
<td>Upper limit</td>
<td>3.710</td>
<td>-0.997</td>
</tr>
<tr>
<td>Lower limit</td>
<td>4.429</td>
<td>-0.832</td>
</tr>
</tbody>
</table>

Consulting Table 9, there is a parameter that immediately is highlighted - the coefficient of determination. Indeed, it is greater than in any setup in minibioreactor. As it was referred before, minibioreactor behavior is large affected by the dye injection. In opposition, bench scale reactor is not so sensible at slightly varying while the dye releasing. Therefore, it is achieved some reproducibility that is not possible in minibioreactor system.

As in the minibioreactor, the zones below the impeller are more influenced by the H/T ratio (parameter c). The other parameters, a and b, presents similar values in both regions evaluated.

In Figure 27, it is shown the experimental results, as well the models founded for the bench scale equipped with 45° pitched blades near the second impeller.
As it was expected, increasing the H/T ratio, the mixing time follows the same tendency. As well in HTBR, mixing time behavior is opposite to the Reynolds number evolution.

In addition, it was studied the influence of the region evaluated. Figure 28 shows various models get from different zones for each one of the H/T ratios.
Observing previous graph, it is possible to see that the model from the same H/T ratio are similar behavior in the studied Reynolds number. That means the region of evaluation of mixing time almost do not change its values.

5.3 Systems comparability

5.3.1 Paddles versus 45° Pitched blade

Two different types of impellers were tested in the minibioreactor. It was investigated what impeller provides the lesser mixing time. Figure 29 helps in understanding the different between impellers.

Figure 29 - Frames taken at different times after dye injection in minibioreactor equipped with different impellers, RPM=180

Figure 29 shows the different behaviors on the fluids caused by the impellers in the minibioreactor. On the one hand the flat top paddle keeps the tracer from reaching the lower section of the vessel. On the other hand, with pitched blades, it allows the tracer to get into the middle of the two impellers from where it is pumped and distributed to the remaining
regions of the vessel. In other words, Figure 29 shows that the top paddle acts like a barrier to the dye creating the formation of two different mixing zones, each one with different mixing times. The interface of these two regions is marked with red arrows.

These results suggest that the minibioreactor equipped with pitched blade impellers achieves the homogenization faster than when operated with flat paddles. To check this statement, it is plotted the mixing time at different rotation speeds for each one of the impellers. The results, for the region between the impellers, are present in Figure 30.

Figure 30 shows clearly the effect of the impeller type in mixing time - the mixing times obtained with flat paddles are undoubtedly superior to the ones get in minibioreactor equips with pitched blades, excepting at 1000 rpm. It represents a difference of 40% at 158 rpm, that is close to the rotational speed at which the minibioreactor is currently been operated. These differences will be even superior, if the impeller diameter were the same. Actually, while paddles diameter is 1.8 cm, pitched blade diameter is only 1.3 cm. As the mixing time decrease with the increase of Reynolds, increasing the pitched blade diameter and consequently the Reynolds number, it would reduce the mixing time even more and the differences comparing with the flat paddles would be greater.
5.3.2 Current operation condition versus experimental results

The main question that is intended to be answered is the determination of the rotational speed that should be applied in minibioreactor to get the same mixing time as in the bench scale reactor. Table 10 shows the current configuration of the studied systems.

Table 10 - Current operation condition of the bioreactors

<table>
<thead>
<tr>
<th></th>
<th>Volume (mL)</th>
<th>H/T</th>
<th>RPM</th>
<th>θₘ (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HTBR</td>
<td>30</td>
<td>2.2</td>
<td>150</td>
<td>9.93</td>
</tr>
<tr>
<td>Bench scale</td>
<td>4500</td>
<td>1.4</td>
<td>220</td>
<td>3.15</td>
</tr>
</tbody>
</table>

Mixing times in the last column of the Table 9 were obtained by Equation 12 to HTBR and by Equation 13 to BIOSTAT. These equations correspond to the section between the impellers, because here it was found higher mixing times.

\[ \theta_M = 4.064 \cdot Re^{-1.162} \cdot \left( \frac{H}{T} \right)^{0.907} \] (12)

\[ \theta_M = 4.070 \cdot Re^{-0.914} \cdot \left( \frac{H}{T} \right)^{2.321} \] (13)

As it is shown in Table 10, there are huge differences concerning the mixing times in different reactors. Using Equation 12 in minibioreactor with flat paddle and Equation 14 in pitched blade case, it is possible to find, keeping the same H/T ratio, the rotational speed in each one of the configurations that will provide the same mixing time as in the bench scale reactor.

\[ \theta_M = 3.708 \cdot Re^{-1.074} \cdot \left( \frac{H}{T} \right)^{-0.271} \] (14)

The rotational speed founded is:

Minibioreactor equipped with flat paddles - 419 rpm
Minibioreactor equipped with pitched blade - 300 rpm

As this study does not consider the impact on a biological system it is recommended to evaluate for example the shear stress caused by these stir speeds on mammalian cells (i.e. hybridomas) physiology.
5.4 Mean circulation time

In addition to the mixing time evaluation, also circulation time was studied. It was assessed only in minibioreactor by fluorometer technique. Actually, the data to circulation time calculation are the same as the mixing time. For this reason, it is not plotted again the response of the fluorometer.

Figure 31 presents a plot of mean circulation time as a function of the inverse of the impeller speed.

\[
y = 0.4147x + 0.2102 \\
R^2 = 0.9593
\]

In the mean circulation time studies it was not consider the data from 20 RPM because it was not possible to identify clearly the peaks. Instead there appear large bands. As it is shown in Figure 31, mean circulation time is inversely proportional to increasing rotational speed, apart from the points obtained for superior rotation speeds (red points in Figure 6). It is in agreement with previous publications (Holmes et al. 1964). Unlike the expectation, the mean circulation time at higher rotational speeds are superior than some of the points get a lower RPM’s. Probably, it happens because cycle time of data acquisition (0.05 sec) is not short enough to get all the circulation peaks. This statement can explain why these points were excluded from the fitting curve.
6. Conclusions

Concerning to the mixing time analysis method, the visual technique provides some advantages comparing with the other used methods:
- Versatility - It can be applied readily to minibioreactors and the bench scale vessels.
- Inexpensive - As measurements required only a regular camcorder, the price is not an issue. Furthermore the Software is freeware or it is supplied with the camcorder.
- Global method - As a global method it allows analyzing the entire vessel. It is particularly important in minibioreactors. Actually, it was observed significant differences between the region above and below the top impeller.

On the other hand, the main disadvantages are impossibility of online measurements and the time consuming analysis.

It was observed that the effects of Reynolds number on mixing time are similar in both the minibioreactor and bench scale reactor independently of the impeller type.

While in HTBR equipped pitched blades the H/T ratio almost does not affect the mixing time, its increase will increase the mixing time in minibioreactor with paddles and in the bench scale reactor. But, H/T ratio effects are more relevant in bench scale reactor.

Studying the impeller type in the minibioreactor, it was shown that the pitched blade impeller, even with a smaller diameter than the paddle impeller, provides lower mixing time values. The results suggest that the direction of the flow (radial versus upward/downward) should also be evaluated.

Finally, it was also observed that the minibioreactor and bench scale reactor are currently being operated at un-matched mixing time.
7. Evaluation

7.1 Achieved goals

The validation of the visual method to analyze the mixing time was the first task of this project. Tough, it was not a task totally fulfilled (the method was not validate in minibioreactors), it was the first step on the development of a new technology in mixing time determination. Certainly, if the recommendations referred in chapter 7.3 are followed, the technique will be certified also in minibioreactors.

About the modeling, all the correlation for both systems respecting the mixing time as a function of Reynolds number, H/T ratio were achieved. Also, correlations concerning different impellers in minibioreactors were established and compared.

The main question in this project was answered, founding the rotational speeds to run the minibioreactor at matched mixing time with bench scale bioreactor. Thus, it was proposed new operation conditions to the minibioreactor equipped with paddles, as well with the 45° pitched blade.

7.2 Limitations and Future Work

A limitation regarding the video capture was observed. Though the camera operate at 60imode (60 snapshots per second) actually, it correspond to 30 frames per second, which each frame results from the combination of two fields (odd an even horizontal lines of the video). As the Free Video to JPG Converter extracts the fields and not the frames, it will cause the appearance of black vertical lines as it is shown on right side of Figure 32.

The black lines will affect the measurement of the brightness intensity and mainly the standard deviation of brightness intensity inside each one of the regions of assessment. As all the analyses were performed in the same conditions the effect of the black lines will be avoided. Anyway, to delete this black lines, it is recommendable to get the videos with a camcorder that provide filming in 30p or even 60p, that means 30 or 60 progressive frames per second.
A disadvantage of the visual technique is the time-consuming of processing and image analysis. Despite it is not a hard process and all the tasks are performed by computer, at this moment it is indispensable a full dedicated person to run each one of the steps of the analysis for each experimental run. So, it is spent too much time doing the same procedure innumerous times. By this, maybe it would be of researchers’ behalf, built a program as macro in Visual Basic to execute automatically all the steps of analysis. It will provide a considerable time saving.

Concerning the analysis, essentially in minibioreactor system, the brightness intensity presents a great, but constant oscillation caused impellers shadow. It becomes the determination of the mixing time harder and less accurate. To face this bottleneck, it is proposed, instead considerer the brightness of each point, present the brightness of each point as the average of five points: the brightness at that time, as brightness of the two times immediately before and two after. As it is present in Figure 33 it would reduce considerably the oscillation of the signal.
Last, but not least recommendation concerns the releasing point of the dye in minibioreactor. As the injection spot of the dye in this type of reactor is larger than the tip of the micropipette it is impossible to release the dye always in the same point. Probably, it affects the results, widespread them. To fix this constraint, it is suggested to decrease the spot hall in the cap of the vessel or, even better, built a tube that would cross the cap and would fit the tip. Certainly, it will provide more reproducibility of the results and consequently better coefficients of determination, $R^2$.

### 7.3 Final balance

Despite it had not been the main goal of this project, through it there was needed to create a common method of evaluation of mixing time - the visual method. Tough it is not totally developed technique, after some improvements I think it will be a very useful tool, not only in CAST but also in the laboratories over the world. Particularly important in the bioengineering field, it can be applied in all the research areas in fluid analysis mixing.

This project established mixing time correlations using key physical and geometrical parameters, allowing prediction of the mixing times in both studied reactors. In future work, this study will help in understanding the mass transfer in the studied systems.

From a personal perspective, this assignment allows to further develop technical and other skills such as autonomy or critical thinking, fomenting a research curiosity unknown till now.

Thus, I consider it was an important project to science, but also to me personally and professionally.
8. References


Appendixes

Appendix 1 - Fluorometer assessed region

Figure 34 - Fluorometer assessed region
Appendix 2 - Long pass filter

Figure 35 - 500 nm Long pass filter

Figure 36 - Absorbance as wavelength for long pass filters
Appendix 3 - Effect of position in brightness level

Figure 37 represents the brightness level at different horizontal planes in the HTBR after a complete dye dilution and with the agitator turned off.

![Figure 37 - Effect of the region assessed in brightness level](image-url)
Appendix 4 - Trimming and frame extracting parameters

Table 11 - Video final time and frame frequency in BIOSTAT

<table>
<thead>
<tr>
<th>RPM</th>
<th>FPS</th>
<th>Final time (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>3.75</td>
<td>120</td>
</tr>
<tr>
<td>60</td>
<td>5</td>
<td>85</td>
</tr>
<tr>
<td>80</td>
<td>7.5</td>
<td>50</td>
</tr>
<tr>
<td>100</td>
<td>15</td>
<td>36</td>
</tr>
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Table 12 - Video final time and frame frequency in BIOSTAT

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Appendix 5 - HTBR impellers

Figure 38 - Detail of minibioreactor 45° pitched blade impellers

Figure 39 - Detail of minibioreactor paddles impellers
Appendix 6 - BIOSTAT impellers

Figure 40 - Detail of BIOSTAT impellers