Development and Automation of Microturbulence Intensifying Systems and Biosynthesis Conditions in Bioreactors

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Received: August 24, 2007 Accepted: September 27, 2007

Abstract: Results of the investigation applying newly developed methods: a Stirring Intensity Measuring Device (SIMD), a counterflow mixing system, identification of the state of turbohypobiosis, counterflow bioreactor’s design and a process controller BIO-3 have been analysed. Special attention has been given to the phenomena on the liquid-cell interface area. A combination of the above-mentioned methods with conventional ones does not provide the scale up and scale down of bioreactors when geometrical dissimilarity occurs. Special adjustment is necessary for each case and culture.

Keywords: Bioreactors, Mixing intensity, Turohypobiosis, Process control.

Introduction

There are hundreds of publications issued annually showing a considerable influence of the stirring system’s geometry and mixing intensity on the population growth and product synthesis characteristics. Many authors have dealt with profiles of rpm (stirrer rotational speed) and pO₂ (also consumed power, oxygen uptake, etc.). They always come to conclusions regarding the optimum conditions for the system (bioreactor with a mixing device, media, aeration, culture and technology) under investigation. Different approaches for scaling up and down for such defined (optimised) systems are described (mainly for geometrically similar systems), although the problem is still unsolved, especially for geometrically dissimilar systems.

To go into details and possibly to solve the scaling up / down problems in dissimilar systems, we have developed:

- a special stirring intensity measuring device, based on the piezo-effect;
- a special counterflow mixing system;
- a method for determining the mixing unevenness;
- the definition of the turbohypobiosis state as phenomena explaining the suppressed metabolism and even cell damages;
- determination of the liquid cell interface area and, respectively, specific O₂ consumption in terms of g/m²·h, g/g·h;
- a special process control system BIO-3.

The goal of the present review article is summarising of the conclusions based on the results gained using the above-mentioned comparatively new methods and approaches.
Methods
In order to utilise new measurements and to obtain more informative results, we have developed a special Stirring Intensity Measuring Device (SIMD) methodology for measurements of the kinetic energy $E$, J/m$^3$ of flow fluctuations [17, 18, 20].

To simulate the dissimilarity of the systems, two different types of mixing systems were used, namely, the turbine mixing system (TMS) and the specially developed counterflow mixing system (CMS) [16]. Both the mixing systems had double-tier impellers. The turbine impellers consisted of a disc (38 mm in diameter) with 6 blades (25 x 25 mm) with a total impeller diameter of 76 mm.

The counterflow impellers consisted of 4 wing-shaped blades. The blades of the upper and lower impellers were bent at equal angles ($\alpha = 28^\circ$) but in correspondingly opposite directions from the horizontal plane of reference. During the impeller rotation, the flow that was generated by the lower impeller was directed upwards, while the flow of the upper impeller was directed downwards. The diameter of the counterflow impellers was 87.5 mm. The volume of the bioreactor was 5 L.

To determine the maximum unevenness of energy distribution, $K_{D\text{max}}$, in bioreactors with TMS and CMS, the energy $E$ distribution after the process was analysed using water and fermentation broth [8]. The criterion of $K_{D\text{max}}$ was determined as follows:

$$K_{D\text{max}} = \frac{2\pi}{V} \iint r \left[ \left( \frac{\delta E}{\delta r} \right)_{E=E_{\text{max}}}^2 + \left( \frac{\delta E}{\delta h} \right)_{E=E_{\text{max}}}^2 \right] \, dr \, dh$$

where $V$ – the operating volume of the bioreactor, $r$ and $h$ – radial and height coordinates.

The turbohypobiosis state has been defined [12, 15] as slowing down of metabolic processes and biomass growth as a result of increased power input (mixing intensity). After this point, the mechanical crashing of cells starts, i.e. the stirring device of the reactor works as a disintegrator.

For the analysis of specific $O_2$ consumption, the gas balance method has been applied, while the interface area has been determined by the image analysis method [9] or by a special microscope connected with a television system [2].

The cultures used: *Brevibacterium flavum*, *Rhodotonela gracilis*, *Saccharomyces cerevisiae*, *Aspergillus niger*, *Trichoderma viride*, *Fusarium moniliforme*, *Fusarium amygdali* covered the whole spectrum of commercially used strains – bacteria, yeast, and mycelia forming cultures.

Experiments have been made during the recent 10 years, particularly the methods and results published and discussed at conferences. In the present work, an attempt was made to summarise the main conclusions without methodological details and calculations.

Careful analysis of the literature had been performed when planning experiments. The reference list of the present article comprises mainly our works on the basis of which this comprehensive summarising has been carried out.
Summarising of results

There is enormous literature, including our own studies [3, 7, 9, 10, 16, 18], on mass exchange problems in general and O₂ transfer in particular in gas-liquid-cell-liquid systems, hence, on the bioreactor / mixing design. The problem of scaling up / down has been more or less solved in geometrically similar systems when cultivating a definite type of producers such as single cell cultures, mycelial-pelleted or filamentous cultures, etc.

When dissimilarity occurs, individual optimisation and adjustment provide the main means for the harmonisation of systems (culture / mixing / inner design of bioreactor / aeration). As has been mentioned above, the goal of the present work is not the analysis of the results of new experiments in the field. We are summarising the results of our investigation as follows.

Conventional methods for hydrodynamic analysis not always allow to obtain valid results for optimising of mixing, therefore, it is necessary to look for new approaches. We have developed a specialised unit, the Stirring Intensity Measuring Device (SIMD), which allows the measurement of the kinetic energy $E$ of flow fluctuations in multiphase coloured media and could be a solution in the analysis of the flow energy character in a bioreactor.

Since the sensitivity of a particular fungal culture to mixing and flow distribution conditions varies due to the different mycelial morphology as well as broth viscosity and physico-chemical effects, the performance of the fermentations under study and energy $E$ patterns varied substantially. Axial counterflow systems, creating a more even mixing, were favourable for fungi, forming large agglomerates (Trichoderma viride) or soft pellets (A. niger), while the turbine mixing system was more effective for F. moniliforme growing in the form of small clumps and freely dispersed hyphae.

It was observed that the growth and synthesis intensity of metabolites decreased at very high total energy input values, which was obviously connected with the state of turbohypobiosis and further – with the effect of irreversible shear stress on mycelial morphology.

For single cell cultures, morphological damages were less important phenomena. However, intensive modes of aeration and mixing inevitably led to turbohypobiosis.

The experiments carried out, for example, with the lysine producing bacterium Brevibacterium flavum show that several stages of the physiological state of bacterial cultures can be achieved by different stirring regimes:

A – cells with a complete tricarbon acid cycle characterised by a high lysine synthesis rate and a high value of carbon conversion into lysine;

B – cells with an incomplete tricarbon acid cycle and the crabtree effect with an increased RNA content, when the specific growth rate approximates its maximum;

C – cells with a low activity of metabolic enzymes as well as decreased values of the substrate uptake rate, specific growth rate, lysine synthesis rate, and other characteristics for the state of turbohypobiosis;

D – damaged morphology;

E – obvious disintegration.
Discussing the correlations of the growth (also product synthesis) and hydrodynamic characteristics in bioreactors, authors very rarely consider the liquid-cells interface area (cell surface) and the interaction phenomena occurring on it.

We have carried out such an experiment with *Rhodotorula gracilis* yeast (Table 1). The hypothesis was that the oxygen consumption per unit of cell surface or per unit of cell mass could be better parameters in comparison with the well-known volumetric mass exchange quotient, $K_{Vp}, \text{g/l}\cdot\text{h}.$

However, the results have shown extremely high deviations of the specific interface area $F_k,$ specific oxygen consumption $L_{O2},$ depending on mixing, continuous cultures dilution rate $D = \mu$ and other cultivation variables. Therefore, the O$_2$ balance method still remains the basic tool for process control both when pO$_2$ is limiting or not.

Table 1. Change in the specific growth rate, respiration intensity and surface of the yeast *Rh. Gracilis Mn* cells in the transition regime depending on their cultivation conditions

<table>
<thead>
<tr>
<th>n, rpm</th>
<th>Q, vvm</th>
<th>pO$_2,$ %</th>
<th>X, g/l</th>
<th>$S_K,$ %</th>
<th>$\int,$ kg/l</th>
<th>$\mu,$ h$^{-1}$</th>
<th>$K_{Vp},$ gO$_2$/l-h-atm</th>
<th>$F_{g}/X,$ m$^2$/g</th>
<th>$L_{O2},$ mgO$_2$/m$^2$h</th>
<th>$I_{O2}/,$ g/g-h</th>
</tr>
</thead>
<tbody>
<tr>
<td>500</td>
<td>1</td>
<td>6</td>
<td>2.68</td>
<td>4.0</td>
<td>1.048</td>
<td>0.12</td>
<td>1.63</td>
<td>6.20</td>
<td>18.6</td>
<td>0.120</td>
</tr>
<tr>
<td>700</td>
<td>1</td>
<td>6</td>
<td>3.76</td>
<td>4.0</td>
<td>1.050</td>
<td>0.17</td>
<td>2.49</td>
<td>5.40</td>
<td>24.2</td>
<td>0.131</td>
</tr>
<tr>
<td>1000</td>
<td>1</td>
<td>6</td>
<td>11.22</td>
<td>3.3</td>
<td>1.053</td>
<td>0.23</td>
<td>8.35</td>
<td>4.83</td>
<td>30.4</td>
<td>0.147</td>
</tr>
<tr>
<td>1000</td>
<td>1</td>
<td>27</td>
<td>7.66</td>
<td>3.4</td>
<td>1.055</td>
<td>0.33</td>
<td>8.20</td>
<td>4.55</td>
<td>36.1</td>
<td>1.164</td>
</tr>
</tbody>
</table>

where $\mu$ – specific growth rate; n – stirrer’s rotational speed; Q – gas supply intensity; pO$_2$ – partial pressure of dissolved oxygen; $K_{Vp}$ – real mass transfer coefficient of oxygen; X – biomass concentration; $S_K$ – concentration of reducing substances in the medium; $\int$ – mean density of cells; $F_{g}/X$ – specific cell surface recalculated to the absolute dry biomass; $L_{O2}$ – oxygen consumption rate per unit of cell surface; $I_{O2}$ – specific oxygen consumption rate per unit of cell mass.

Certainly, not the above-mentioned characteristics of the interface area alone should be considered, but also its quality, e.g. solute binding proteins responsible for transmembrane transport.

Regardless the fact that the mycelial fungi population has a very complicated morphology, by using the image analysis and flow structure identification methods, it would be possible to determine the interface area also for such complicated structures, and thus to explore more thoroughly the phenomena occurring on it.

It could be expected that, by ensuring a higher mixing evenness, as it is with CMS (after $K_{Dmax}$), better results should be obtained. It was really so with, for example, *Aspergillus niger*. However, this time, it should be regarded that CMS ensures a better energy supply to the smallest eddies. Hence, it should be assumed that, in our case, this microstirring intensity was too high for optimum growth of the population and the product synthesis. Evidently, the state of turbohypobiosis set in. Essential differences in the morphology of both hyphae and clumps are observed. However, it is impossible yet to tell exactly, which of the image analysis indices correlates most directly with the productivity of the given culture, hence, which should be optimised by the flow structure (mixing system design and power input mode) variables.
Presently, it is clear that such a correlation exists. Besides, evidently, it would not be enough also with the morphology mechanical character indices.

The properties of hyphae and clump surfaces, e.g. transport proteins, should be studied. It is most likely that just through the changes of these structures, which occur as a result of the interaction between the cell and the liquid microeddies, the system reaches the effect, which is known in the literature as shear stress or, according to the terminology we offer – the state of turbohypobiosis.

The deformation damage of cells in intensively mixed zones proved to be much more dangerous than the insufficient mass exchange in the so-called dead zones of bioreactors. We even have proved that the reasonable limit of O₂, controlled by substrate feeding, could be tolerable and even effective from the viewpoint of O₂ supply and heat removal [19].

A special bioreactor design was performed to provide mycelial (Aspergillus, Fusarium) and other producers sensitive to deformation forces with even mixed cultivation conditions. Axial mixing systems, e.g. the counterflow system, were most promising in this context. Axial mixing systems were also more efficient in terms of energy consumption for mass exchange in fermentations.

The cultivation of Trichoderma lignorum in geometrically dissimilar bioreactors was investigated in production at different stages of growth. The producer growth was much better in the axial mixing system, compared to the turbine mixing system. Morphological changes in the strain were investigated during batch fermentation at different mixing regimes. Characteristic dynamics of morphology were considerably better for the axial system.

There is no unambiguous correlation among the activity of certain biochemical reactions, medium mixing intensity, substrate concentration and ingredient mass transfer rate in liquid-cell or gas-liquid-cell systems.

It can be assumed that the shear effect (state of turbohypobiosis) plays a role mainly when alternative mechanisms in cells cannot ensure a normal physiological state under stress conditions.

With Saccharomyces cerevisiae [4, 5], different types of energy input were investigated:

- pulsing aeration and mixing,
- vibromixing with two shafts,
- conventional mixing system.

The main difference observed was in the efficiency of energy consumption, but not very much in the processes of liquid-cells interactions.

Generally, the results allow to make conclusions that practically any system (inner constructions of a bioreactor, producer and cultivation conditions, including mixing) requires its own optimisation to achieve the final goal, i.e. the maximum yields Y_P/S or Y_X/S, where P – synthesised product, S – substrate, raw material, carbon source consumed, X – cell mass grown. Data on the biotechnological performance of cultivation as well as power input, kinetic energy E of flow fluctuations, air consumption rate, stirrer rotational speed, tip speed, etc. do not correlate directly if the mixing systems (impellers-baffles) are dissimilar.
Even the widely used specific power consumption cannot be regarded as a criterion for scaling up the cultivation performance using dissimilar mixing systems. A biochemical explanation of the substrate and product transport via cell walls, carbon pathways, energy (ATP) generation and utilisation, etc. furnishes insight into the cells interactions and are described by turbulence of different origin (scale) for different types of microorganisms (single cells, mycelia forming cells, etc.).

Taking into consideration the above peculiarities of the mycelial system for bioconversion, special bioreactors [1, 2, 6, 11, 13, 14, 22] having a counterflow (axial) mixing system, a novel electromagnetic drive, which ensures convenient servicing application and good aseptic conditions, have been developed and implemented for targeted customers as well as the process control systems BIO-3 and others [21, 23]. The main advantage is that BIO-3 is based on the Basic programmable multitasking microprocessor (Wilke Technology, GmbH), which allows easy corrections in the controller and its expanding. The bioprocess controller BIO-3 ensures the following functions in the user-friendly way: process control according to PID algorithms for temperature, oxygen pressure pO\textsubscript{2} and foam, adjusting of set point parameters and PID coefficients, digital calibration of sensors, data communication with PC through RS-232 or RS-485. It is possible to use BIO-3 also in complex with chemical reactors, because the process control program can be easily adapted to special needs.

Conclusions
1. The energy $E$ values were substantially higher in the fermentation broths of $F.\ amygdali$ and $A.\ niger$, which had a higher apparent viscosity in comparison with the low viscosity of the $F.\ moniliforme$ broths. In TMS, where the energy $E$ in the broths analysed was higher than in CMS, the intensive agitation of the $F.\ amygdali$ and $A.\ niger$ broths caused unfavourable conditions for the culture growth and product synthesis. The achievement of the critical level of energy $E$ was obviously accompanied by irreversible changes in morphology. In CMS, even at higher agitation rates, energy $E$ values for $F.\ amygdali$ and $A.\ niger$ broths were lower than in TMS, and better growth and metabolite production parameters were achieved. Using the $F.\ moniliforme$ broths, better culture growth and Gibberellic acid production were achieved in TMS in comparison with CMS, where sufficiently intensive mixing was not obviously achieved at higher agitation rates.
2. The limiting substrate concentrations and the mixing intensity are alternative variables within reasonable limits.
3. Shear stress as a result of the interaction between the liquid (including gas bubbles) – cells – moving inner constructive elements creates the state of turbohypobiosis resulting in slowing down of growth and product synthesis and even irreversible cell damages. Intensively mixed zones (in contrast to the well-known dead zones) appear, which induce the state of turbohypobiosis and cell damages.
4. SIMD is a new tool, which helps to study the mixing phenomena, especially in coloured gassed culture liquids.
5. The volumetric O\textsubscript{2} transfer coefficient is a process characteristic in the absence of oxygen limitation and an equipment characteristic if it is limited.
6. Even using constant pO\textsubscript{2}, power consumption, O\textsubscript{2} assimilation, stirrer rotational speed, different parameters measured by SIMD and their combinations (certainly, optimum $t^\circ$, pH and other profiles), we did not manage to scale up and down the microbial synthesis in dissimilar bioreactors.
7. Hence, the optimum regimes (profiles of the parameters under control) for the given system (the bioreactor, mixing device, media, aeration, culture, technology) should be found for each particular case. This could be exemplified by BIO-3 as an appropriate tool.
Acknowledgements

The present work was supported by the Latvian Council of Sciences, Project 05.1558.

References


