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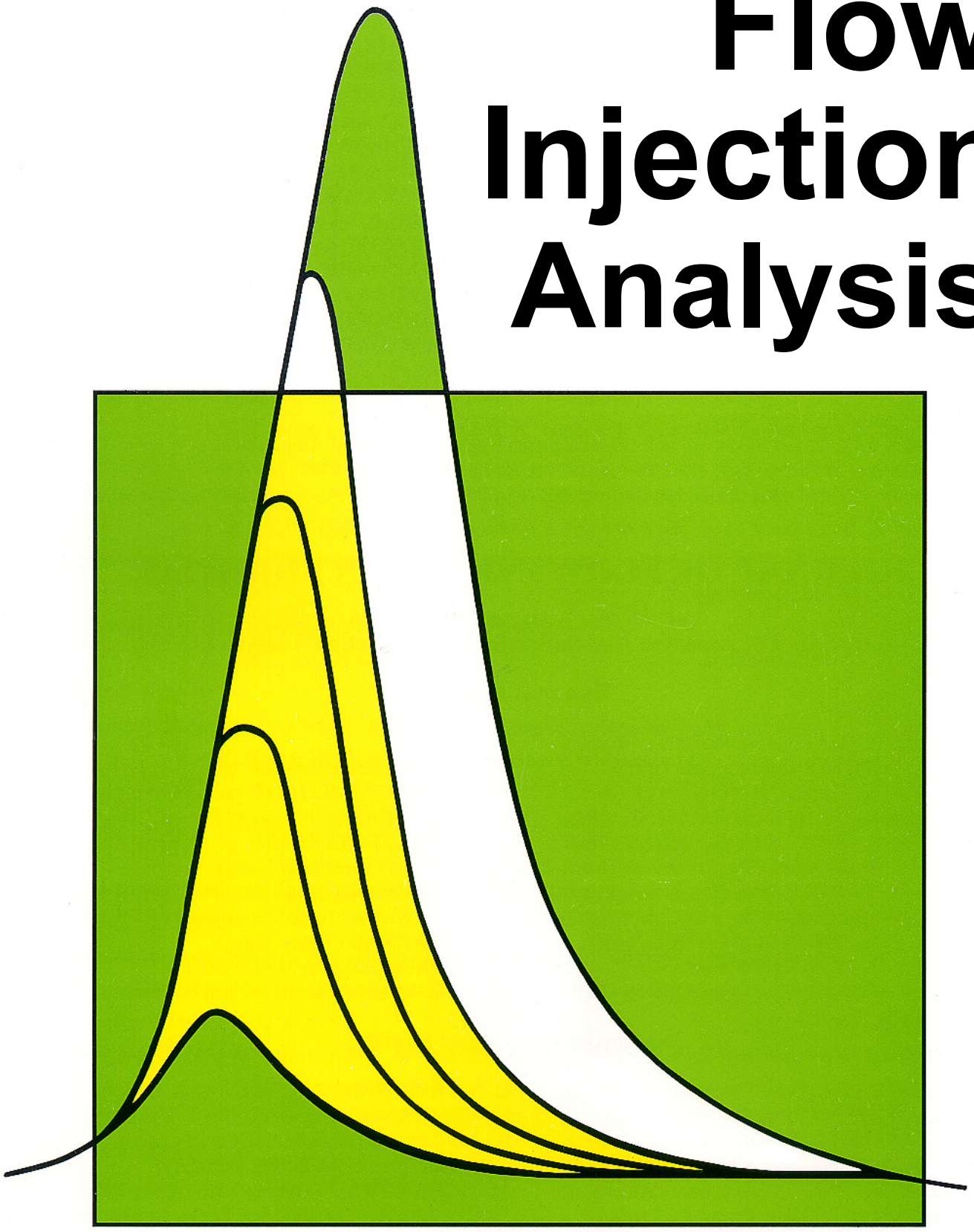

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Development of a Turbidimetric Flow Injection Analysis System for Cell Counting

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Abstract

A flow injection analysis (FIA) system with turbidimetric determination for cell counting was developed. Similar to the McFarland's method, the present work is based on the proportionality between the turbidimetric signals obtained for barium sulfate suspension and cell suspension. In spite of differences in physical properties, it was possible to establish a correlation between the two systems. The analytical curves for both species presented the following equations: $S = 0.0087C_{sulfate} + 0.0005$ for sulfate calibration and $S = 4.8 \times 10^{-11}\ C_{S.cerevisiae} - 0.0018$ for *Saccharomyces cerevisiae*, thus, the correlation between the two systems was: $C_{S.\ cerevisiae} = 1.0875\ C_{sulfate} + 0.0023$. The results obtained, using the proposed procedure, were compared with results obtained in the Neubauer Chamber and there was no significant difference at confidence level of 95% (paired t-test). The proposed technique presented a limit of detection equal to 2.37×10^8 cell L^{-1}. The main advantages of the FIA system were high sample throughput and good precision; both parameters proved to be better than conventional counting system into Neubauer Chamber. The method was also tested to monitor the cells growth and showed satisfactory results.

Keywords FIA, Neubauer Chamber, Cell count, Turbidimetry, *Saccharomyces cerevisiae*.

1. Introduction

Cell count is a procedure that has been used in various areas of science and can be executed basically by three ways: direct count [1], turbidimetric methods [2-7] and viable cells count [8]. This parameter is important, for example, to monitor microorganism growth in culture media which are used in microbiology, food technology, biotechnology and pharmaceutical industry studies [9].

Employing appropriate reagents, it is also possible to count viable and non-viable cells. One example is the Trypan Blue reagent, which penetrates into cells presenting membrane rupture, and differentiates them from the others when these are observed with a microscope [10].

Some methods, common in instrumental analytical chemistry, have also been used for cells count including both viable and non-viable cells [11]. One of the most important instrumental techniques for cells counting is turbidimetry.

Turbidimetry is the measure of the scattered radiation for particles, and in our case the radiation is scattered by cells suspension, thus, at higher cell densities, turbidity is proportional to the cell concentration [12].

Likely the most of the instrumental techniques, the turbidimetry needs calibration solutions, i.e., standard quantity of microorganisms (cells) must be used. In the present work, for the initial standards solutions (stock solution), the number of microorganisms in a liquid medium was determined in Neubauer Chamber in clear microscope field, which is considered sensible to determine cellular density.

The correlation between cell concentration and turbidity depends on the microbial species, and, sometimes, on the specific strain used. The range of concentration proportionality depends on the size and shape of the microorganism [13].

The present work was inspired in the McFarland method in which turbidity of cells suspensions was compared with standard suspensions of barium sulfate [14].

One intrinsic difficulty of the McFarland technique is the instability of the suspensions due to its precipitations. Such problem is also present in the conventional instrumental turbidimetric measurements. However, this drawback can be circumvented by using flow injection analysis (FIA) system as will be demonstrated in the present work.

Under the FIA system, sample and reagents are inserted in a carrier stream, with a previously determined flow rate, where the sample zone flows towards the detector. As main advantages it can be cited the low sample/reagent consumption, high sample throughput and, since the FIA is a dynamic system, the precipitation of particles won't occur [15]. A FIA system with turbidimetric detection would then be a very interesting and highly valuable method for cell counting.

According to the above comments, the aims of this work were to propose and evaluate a turbidimetric FIA system for *Saccharomyces cerevisiae* cell counting. An inherent difficult for preparing standard cell solutions in FIA system is the very slow process. Thus, the standard cell counting was performed through a Neubauer Chamber. Initially, analytical curves (equations) for the analytes, with the same FIA system, were obtained with barium sulfate and S. cerevisiae standards and a linear combination between linear equations, obtained for both analytes, resulted in the correlation between $C_{Saccharomyces}$ and $C_{sulfate}$. As in the McFarland technique, after the determination of the correlation between analyzed standards, only the analytical curve with barium sulfate was enough for cells count.

2. Experimental

2.1. Strain

Saccharomyces cerevisiae strains (ATCC 2601) were cultivated in Sabouraud broth with chloramphenicol

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($C_{11}H_{12}Cl_2O_5$), which is an antibiotic of wide use, thus, contaminants bacteria are eliminated. A kit for gram coloration (NEWPROV®) was used to confirm the bacteria elimination. Following that, the strains were inoculated in Sabouraud agar without antibiotic. After five days cultivation at 28º C in incubator (FANEM®), the cells were collected and inoculated in physiological solution. The cellular suspension was centrifuged and the sediment was added in 0.9% (m/v) NaCl suspension (stock solution) containing 2 x 10^{-3} w/v thimerosal ($C_9H_9HgNaO_2S$) to stop the yeast growth. All the sample treatment process was done in the flow laminar chamber (LABCONCO®).

2.2. Reagents and solutions

All the solutions used were of analytical grade, and Milli-Q water (Millipore®, MA) was used throughout, unless otherwise mentioned.

The sulfate determination is based on the reaction between sulfate and barium ions (barium chloride - Reagen) in micellar medium of polyvinylic alcohol (SIGMA®). Once the product formed precipitates itself in the spectrophotometric cell, the base line is unstable. Therefore, a solution of 0.3% (w/v) EDTA (MERCK®) in NaOH (QUIMEX®) 0.07 mol L^{-1} was employed to solubilize the precipitate [16].

Sulfate stock solution was prepared with ferrous sulfate (VETEC®) and reference solutions (5.0 to 100.0 mg L^{-1}) were prepared by appropriate dilutions from the stock solution.

Saccharomyces cerevisiae stock solution was prepared with tur bidity similar to the tube number 5 of McFarland scale [14] and reference solutions ($1.46x10^9$ to $7.34x10^9$ cell L^{-1}) were prepared by appropriate dilutions from the stock solution.

2.3. Flow Injection Analyses (FIA)

The readings for both systems were done at 410 nm in a UV-Vis (FEMTO® - model 482) spectrophotometer, with 1cm optical path flow cell. A peristaltic pump (Ismatec®- Zurich, Switzerland, 7618-40 model) was used to propel the fluids through polyethylene tubes (ɵ = 0.8mm). The data were recorded by a PCL-711 (ADAVANTECH®) AD/DA interface.

The FIA system, for sulfate signals, is shown in Figure 1. Through the proportional-commutator the standards were introduced in the carrier stream. In the first confluence the turbidimetric reaction occurs due to a mixture between standards and barium chloride in polyvinylic alcohol medium, and then the formed product was measured by the spectrophotometer. When the proportional-commutator was switched to the initial position, alkaline EDTA was inserted replacing barium chloride, and the base line of the system restablished [16].

For the best comparison the sign for cells suspensions were obtained with the same FIA system used for barium sulfate (Figure 1), but, a physiological solution was introduced into the carrier stream, R1 and R2 channels. Through the proportional-commutator the sample was introduced in the carrier stream of the physiological solution and flows towards to the detector.

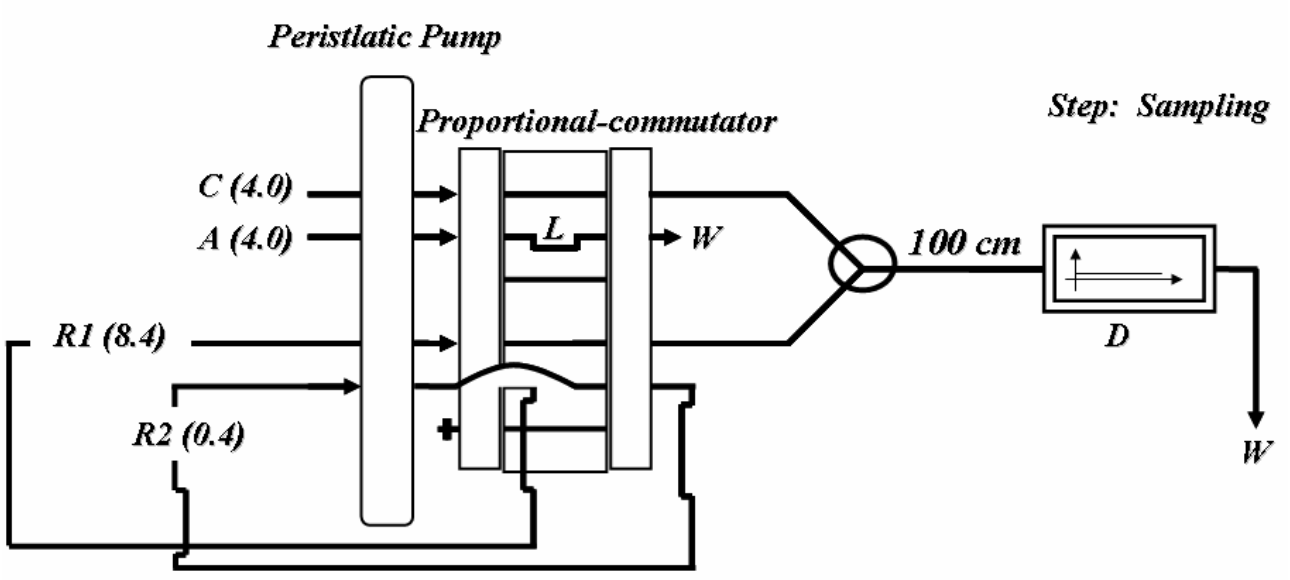

Fig. 1 FIA Manifold. A = sample; C = carrier flow (H_2O); R1 = 5.0 % (m/v) $BaCl_2.2H_2O$ in 0.05% (m/v) polyvinylic alcohol (PVA); R2 = 0.3% (m/v) EDTA in 0.07 mol L^{-1} NaOH; D = detector (spectrophotometer , 410 nm); W = waste; L = sample loop (volume = 502 µL). The values between parentheses are the flow rates (mL/min).

2.4. Cell count in Neubauer chamber

Aliquots of 10 until 15 µL of homogenized suspension were introduced into a Neubauer Chamber [10]. The count was made through the stereoscopic microscope (OLYMPUS BX41 and CX40) [17].

2.5. Interference studies

The selectivity of the proposed method was tested considering the composition of studied samples (two ferments and one probiotic samples). Thus, the first concentrations studied were: ascorbic acid (1.55 x 10^{-3} g L^{-1}), folic acid (2.01 x 10^{-8} g L^{-1}),

amide (1.01 x 10^{-1} g L^{-1}), calcium (3.61 x 10^{-3} g L^{-1}), sodium chloride (5.95 x 10^{-1} g L^{-1}), iron (1.21 x 10^{-6} g L^{-1}), phosphorous (4.20 x 10^{-5} g L^{-1}), glucose (5.01 x 10^{-1} g L^{-1}), hystidine (9.14 x 10^{-3} g L^{-1}), magnesium (2.11 x 10^{-6} g L^{-1}), proteins (4.43 x 10^{-1} g L^{-1}), pyridoxine (2.69 x 10^{-5} g L^{-1}), potassium (1.33 x 10^{-5} g L^{-1}) and thiamine (1.35 x 10^{-5} g L^{-1}). The second and third concentrations were respectively equal to ten and hundred times the first concentration.

Aliquots of solutions of those interferents were mixed with *Saccharomyces cerevisiae* solution (3.83 x 10^{9} cells L^{-1}). Three interferent concentrations were examinated: the first one was the same found in the sample and the others were ten times and a hundred times higher than the first one.

2.6. Application in real samples

The samples were prepared by adding 2.0 g of the samples to 1000 mL of physiologic solution containing thimerosal and chloramphenicol, under agitation and constant temperature. After the period of one hour, the samples were determined in the FIA system.

To check the accuracy, the determination by the optical microscope was also done, leaving an aliquot of 200 µL diluted in 800 µL of the physiologic solution. Soon after, an aliquot of 20 µL was removed and placed in the camera of Neubauer.

2.7. Cells growth studies

Saccharomyces cerevisiae strains had been inoculated into an assay tube containing 10 mL of Sabouraud broth with chloramphenicol. After 48 hours 1 mL of the Sabouraud broth was transferred to an erlenmeyer containing a sterile new broth, totalizing a final volume equal to 50 mL. Samples were kept at 25ºC under continuous shaking throughout the procedure. The microbial growth was followed by the proposed method, and for this, measurements were done each hour, until the microbial population reached the steady state of growth phase.

3. Results and discussion

3.1. Relationship between cells and sulfate turbidimetric signals

Figures 2a and 2b show records of signals obtained in triplicate by FIA system, for sulfate determination, and for *Saccharomyces cerevisiae*.

After optimization, the obtained sulfate analytical curve was: ($S = 0.0087C_{sulfate} + 0.0005$) and the *S. cerevisiae* analytical curve: ($S = 4.01 \times 10^{-11} C_{S.cerevisiae} - 0.0018$).

The analytical parameters of these curves are shown in Table 1.

a) b)

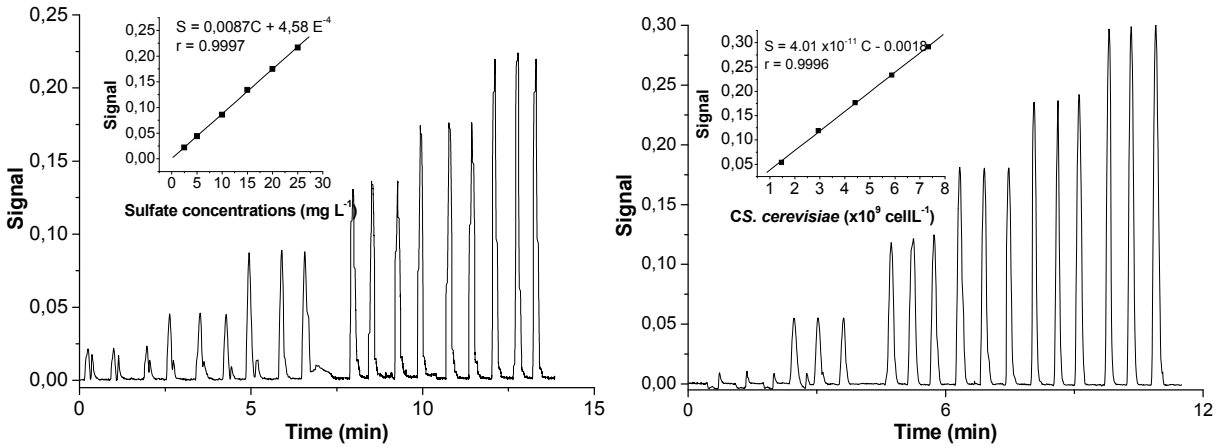

Figure 2: Signal registrations. a) Sulfate standards (2.5, 5.0, 10.0, 15.0, 20.0 and 25.0 mg L^{-1}) and analytical curve; b) *Saccharomyces cerevisiae* standards (1.47 x 10^{9}, 2.94 x 10^{9}, 4.41 x 10^{9}, 5.87 x 10^{9} and 7.34 x 10^{9} mg L^{-1}) and analytical curve.

Table 1. Analytical parameters.

Parameters	Sulfate analytical curve	*S. cerevisiae* analytical curve
Linear range	2.5 - 25 mg L^{-1}	1.47 - 7.34 x 10^{9} cells L^{-1}
m	8.7 x 10^{-3} L mg^{-1}	4.01 x 10^{-11} L $cells^{-1}$
r	0.9997	0.9993
(σ)	3.16 x 10^{-4}	3.16 x 10^{-4}
LOD[a]	1.09 x 10^{-1} mg L^{-1}	2.37 x 10^{8} cells L^{-1}
LOQ[b]	3.63 x 10^{-1} mg L^{-1}	7.90 x 10^{8} cells L^{-1}

[a] LOD = 3 * (σ /m)
[b] LOQ = 10 * (σ/m)

The linear equations of both analytical curves have been combined in order to establish proportionality between the sulfate concentration and the *S. cerevisiae* concentration. Thus, the obtained linear equation ($C_{S.\ cerevisiae} = 1.088\ C_{sulfate} + 0.0023$) can be used to determine the microorganisms concentrations in the unknown samples.

3.2. The interference studies

Table 2 shows the results of interference studies. The studied concentrations of the interferents were above mentioned (item 2.5). The most important fact is that there is no significant interference, based on reduced increase of the analytical signal (in percentage) of the *Saccharomyces cerevisiae* solution (3.83 x 10^{9} cells L^{-1}). For the concentration two and three the main interference is caused by protein, nevertheless, these

concentrations are not present in real sample, so these results indicate that analyst should be attentive with this interfering. The accuracy test, presented below, confirm the no occurrence of interference for the studied samples.

Table 2. Interference concentrations .

Interferences	Concentration 1	Concentration 2	Concentration 3
Ascorbic acid	1.50%	2.52%	3.50%
Folic acid	5.99%	5.35%	6.29%
Amide	0.55%	1.57%	9.44%
Calcium	0.25%	1.92%	4.55%
Sodium chloride	0.45%	0.46%	0.70%
Iron	4.90%	3.86%	4.55%
Phosphorous	2.45%	3.14%	4.00%
Glucose	3.18%	3.20%	4.20%
Hytidine	0.74%	1.57%	1.40%
Magnesium	5.24%	5.03%	6.29%
Total proteins*	3.85%	16.35%	54.20%
Pyridoxine	0.34%	0.63%	2.10%
Potassium	0.64%	0.09%	2.45%
Thiamine	1.50%	2.52%	3.50%

* Bovine serum albumin

3.3. Precision and Accuracy studies

In the Neubauer chamber, the most frequent causes of errors that modify the count results are: chambers or pipettes badly calibrated; bad adjustment of the cover glass, cells adsorption in the pipette, different distribution in the chamber, lack of experience of the operator and sampling not representative of cellular suspension.

Table 3. Comparison between cell count in neubauer chamber and FIA system.

Samples	Neubauer Chamber (10^9 cells L^{-1})	FIA System (10^9 cells L^{-1})
A	4.35 ± 2.41 [1]	4.73 ± 0.06
B	6.38 ± 7.02	6.63 ± 0
C	9.35 ± 7.94	10.53 ± 0.05
D	1.20 ± 1.58	1.29 ± 0.09
E	1.85 ± 0.65	1.92 ± 0.03
F	2.81 ± 0.69	2.90 ± 0.04
G	2.17 ± 0.71	2.42 ± 0.02
H	0.51 ± 1.58	0.76 ± 0.03
I	5.67 ± 1.06	5.62 ± 0.20
J	11.62 ± 4.92	10.44 ± 0.78

[1] Values are showed with confidence interval (average $\pm \frac{ts}{\sqrt{n}}$) with α=0.05, (n=3).

Due especially to wrong sampling, the determination by Neubauer chamber presented lower precision, which can be verified through the confidence interval showed in Table 3.

In the determination through FIA system advantageous characteristics were obtained such as high sample throughput (120 h^{-1}), besides the excellent precision (Table 3). It could also be mentioned that the response range for cells counting in FIA system was from $1.4x10^9$ up to $1.4x10^{10}$ cell L^{-1} and, considering the blank standard deviation (3 σ), the limit of detection was $2.53x10^8$ cell L^{-1}.

In order to check the accuracy of the proposed method samples of *Saccharomyces cerevisiae* were prepared and the results obtained with the FIA method were compared with the Neubauer Chamber results. According to results there was no significant difference at 95% of confidence level (paired t-test). The application of the proposed system into real sample also done with Neubauer chamber and confirm the accuracy of the method.

3.4. Application in real samples

Table 4 shows the results of cells counting for four real samples. Beside that complexity of samples, it can be noted that there is no difference between the results with proposed system and Neubauer chamber. Thus, the feasibility of the proposed method can be confirmed.

It can be noted (Table 3 and 4) that the proposed method always presented better precision than Neubauer chamber counting and this can be checked with the confidence interval that was always smaller by the proposed method. Another fact is that the precision for real samples were better than for the simulated sample, which occurs because the preparation for real sample is simpler: For the simulated sample preparation it is necessary an agar Saboraud solution medium for culture while real samples were done by simple dilution.

Table 4. Determination of cell concentration by turbimetry and microscopical count.

Samples	Neubauer Chamber (10^9 cells L^{-1})	FIA System (10^9 cells L^{-1})
Organonew®	0.897 ± 0.082 [1]	0.802 ± 0.017
Dona Benta®	1.35 ± 0.06	1.37 ± 0.01
Fleischmman® dry	2.10 ± 0.13	2.05 ± 0.05
Fleischmman® wet	1.01 ± 0.23	1.11 ± 0.02

[1] Values are showed with confidence interval (average $\pm \frac{ts}{\sqrt{n}}$) with α=0.05, (n=3).

Maukonen, et al. [18] evaluated different methods of counting for probiotics, among them, the methods of fluorescence microscope, flow cytometry and counting on plate. It concluded that in these study fluorescence techniques shown a rapid assessment of the quantity of cells in samples of probiotics. However the authors admit the limitations of this technique, related mainly to the need of pre-treatment of the sample.

In our work it did not need any pre-processing of samples. It may also be mentioned characteristics such as simplicity, low cost, precision and accuracy satisfactory.

3.5. Cells growth studies

The method was also used to monitor cells growth studies and a typical growth curve was obtained (Fig. 4). During the experience a yeast culture was kept under shaking at 25°C. Similar results with respect to the lag and log phases times, during the growth of yeast *Saccharomyces cerevisiae* are found in the literature [19]. Lag phase was during four hours. From that point on it exhibits a logarithmic phase up to the fourteenth hour and, from that, a stationary phase plateau of 1.98×10^{10} cells L^{-1}. Growth curves of 3 replicate yeast cultures, inoculated at an initial strain with 10^8 cells L^{-1} from the same starter culture in phase growth, are highly reproducible and have a mean average time to reach half-maximum growth of 13.9 ± 0.21 h. This repro-

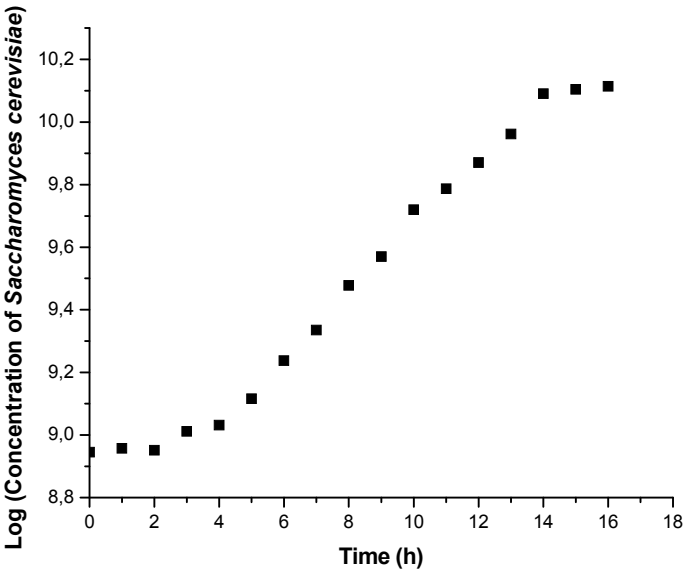

Fig. 4 Screening of growth curve.

ducibility allows precise quantitative measurement of growth parameter [19].

5. Conclusion

This paper proposes an alternative method of cell counting and presents several advantages on the Neubauer Chamber counting. The turbidimetric determination method is more practical because it requires less time for operator training. The FIA counting method is also faster, since the sample throughput is *ca.* 120 determinations per hour, about 30 times faster than the manual counting. Additionally, the precision evidenced by the confidence interval was notably better. The FIA system proposed was applied efficiently to monitor cell growth studies. Finally, the system is also simpler and presents low cost in comparison to Neubauer Chamber.

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