

An automatic turbidimetric method to screen yeast extracts as fermentation nutrient ingredients

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Abstract

Yeast extracts (YEs) are frequently used as fermentation nutrient ingredients. However, lots from the same manufacturing process gave biomass and growth rate (μ) levels that could vary by almost 50%. Establishing growth curves with shake flasks, or in fermenters under external pH control, is tedious and labour-demanding. An automated turbidimetry (AT) system (BioscreenTM) was thus used with the aim of screening various YEs in a growth medium optimization process. Biomass production of *Lactobacillus plantarum* MA18/5-U was evaluated by optical density (OD) at 600 nm. Media destined for the AT system were diluted 20 fold, and the data obtained were multiplied by the dilution factor, while media in flasks and fermenters were not diluted for the fermentation, but samples were diluted for the biomass measurement. Biomass data of the AT system underestimated the levels obtained in shaker flasks and pH-controlled fermenters by 30 to 50%. However, good correlations were obtained between the various systems (R^2 values of between 0.94 and 0.96). Therefore, AT was found to be useful in predicting the potential biomass level in this particular YE-supplemented medium. The μ_{\max} data obtained in the AT unit were approximately half of those obtained in flasks, and a third of those in fermentations conducted under external pH control (fermenters). A good correlation between AT and flask μ_{\max} data was obtained ($R^2=0.98$). However, a rather low correlation ($R^2=0.45$) was reached between AT and fermenter μ_{\max} data. The AT system could be adapted to effectively replace shake flask cultures in the evaluation of the biostimulatory activity of YEs on *L. plantarum* MA18/5-U. It could also be useful as a YE screening tool in the development of media destined for fermentations under external pH control. © 1997 Elsevier Science B.V.

Keywords: Activity; Culture medium; *Lactobacillus*; Lot; Spectrophotometry

1. Introduction

The world wide production of yeast extracts (YEs) in 1991 was 3500 tons for an estimated market of

190 million \$US [1]. Yeast extracts are registered as natural products by the Food and Drug Administration (USA) and find many applications in the food industry as flavour components [2]. They are also frequently used as a rich fermentation nutrient ingredient in the cheese starter and pharmaceutical/biotechnology industries.

The starting material for YE production is general-

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ly brewers' yeast or bakers' yeast (*Saccharomyces cerevisiae*) [3]. The autolysis process releases soluble intracellular components and hydrolyses insoluble material, such as proteins and nucleic acids. The extraction level depends on pH, temperature, time of the process and the type of yeast [4–6], as well as on plasmolytic agents (ethanol, ethyl acetate, sodium chloride) or other external enzymes (nucleases, glucanases) [7,8]. Consequently, depending on the process and the raw material used, the final composition of a product can be quite different. As an example, six different commercial autolysates were shown to have free amino acid contents varying from 45 to 78% [2]. It is our industrial experience that lot-to-lot variations also occur.

Yeast extract biostimulation on microbial growth of the lactic acid bacteria has frequently been demonstrated [9–14]. Smith et al. [15] indicated that nitrogen is the main stimulatory factor in YEs. Lactic acid bacteria are fastidious and require different vitamins, amino acids and other growth factors [16,17]. However, the requirements for peptides and free amino acids vary between strains [18,19], thus indicating that different hydrolytic levels would be appropriate for the various strains. Some commercial suppliers have indeed shown that there is a strain–product interaction [20,21].

In order to rapidly identify the best suited product for one specific application and to maintain the reproducibility, a rapid, simple and automatic screening method is needed. The aim of this study is to evaluate an automated turbidimetry system (BioscreenTM) as a screening tool for the determination of the appropriateness of various YEs as nutritional supplements for the growth of lactic cultures. Automated turbidimetry has been used for the determination of bacteria in food samples [22,23], as well as for the construction of growth kinetic models [24,25]. In this study, we report on the use of AT for the determination of growth rates (μ) and biomass concentrations in fermentation media supplemented with YEs.

2. Materials and methods

2.1. Strain

An industrial strain of *Lactobacillus plantarum*

MA18/5-U was kindly provided by Equipharm S.A., Lallemand's subsidiary in Aurillac, France. *L. plantarum* mother cultures were prepared by mixing 25 ml of *Lactobacillus* MRS broth (Difco, Detroit, MI, USA) to 25 ml of a 15% glycerol solution and 10 ml of a freshly MRS-grown (16 h/25°C) culture. This bacterial suspension was distributed in aliquots of 1 ml into sterile cryogenic vials (Nalgene, Rochester, NY, USA) and kept frozen at –70°C for no more than 3 months. The inocula were prepared by adding 0.5 ml of a frozen ampoule (mother culture) to 100 ml of MRS medium (Difco), adjusted at pH 6.45, and incubating the mixture for approximately 9 h at 30°C, until its pH reached 4.5. The culture was then put on ice for 30 min to stop the acidification process.

2.2. Media

To study the biostimulatory effects of YEs on growth, a base medium derived from the MRS broth was prepared. The composition was, per 1 of medium: glucose, 20 g; K₂HPO₄, 3 g; sodium citrate, 3 g; (NH₄)H₂PO₄, 3 g; Tween 80, 1 g; MgSO₄·7H₂O, 0.2 g; MnSO₄·2H₂O, 0.05 g, and different YEs at set concentrations. Glucose and magnesium sulphate were mixed together at twice the concentration (40 and 0.4 g/l, respectively) and were sterilized for 15 min at 121°C. The rest of the medium was also prepared at double the concentration and was sterilized in the same way. Both solutions were mixed following sterilization. Six commercial yeast extract lots from the same autolytic process were obtained and were coded C1, C2, C3, C4, C5 and C6. The extracts were produced from bakers' yeast.

2.3. Fermentation conditions

As a control method, cultures were prepared in 250 ml erlenmeyer flasks containing 100 ml of sterile medium and 2% of the inoculum. This represents traditional fermentations with internal buffering agents (phosphates and citrates). The flasks were incubated at 30°C with agitation (150 rpm; LabLine Instruments, Melrose Park, IL, USA) and samples were removed periodically. Some fermentations were carried out under external pH control (5.8) in 2-l Braun (Melsungen, Germany) Biostat M units, using

2% NaOH as the neutralizing agent. Agitation was also set at 150 rpm.

2.4. Bioscreen

The AT unit, Bioscreen™, was purchased from Labsystems, Helsinki, Finland. The apparatus is basically a running read-through spectrophotometer that can hold 2 plates, each containing 100 mini-wells. The incubation temperature (30°C), shaking frequency (20 min) and intensity (high), wavelength (600 nm), time of the fermentation (18 h) and optical density reading frequency (1 h) for a number of preselected wells were set automatically at the beginning of the run. Sterile media were prepared in flasks and inoculated. Some samples were then diluted to the desired level in sterile bacteriological peptone (0.1%). These inoculated–diluted media were used for shaker flask parallel trials, and aliquots of 400 µl were added aseptically to each mini-well. Up to 200 conditions or trials could be processed at one time. The plates were then installed into the Bioscreen incubation chamber and the experiment was initiated. Growth curves were then transferred to Sigmaplot (Jandel, San Rafael, CA, USA) spread sheets for regression analyses.

2.5. Analyses

In the control samples, the biomass in shaker flasks was monitored through optical density measurements at 600 nm on a Beckman DU-7 spectrophotometer. If optical density (OD) values were over 1.0, samples were diluted in water to give an optical density reading of between 0.2 and 0.8, and the values obtained were corrected for their dilution factor. A non-inoculated media at the proper dilution factor was also monitored and its optical density reading was subtracted from that of the sample, to precisely estimate the absorbance value related to biomass. Non-inoculated wells were also used in the Bioscreen to estimate the baseline under each condition. Quadruple replicates of a given condition indicated that results were reproducible to within 5% on the AT unit. The pH was read on a Corning 140 meter. The biomass values (non-diluted equivalents) in shaker flasks and with the AT system were transformed into Ln values of biomass and plotted against time for the exponential growth part of the

curve (Sigma Plot software, Jandel). The slope of the curve (maximum growth rate) was obtained by a simple linear regression.

2.6. Replicates

Three independent replicates were carried out for each fermentation, and data are the average of these three assays.

3. Results and discussion

3.1. Flask and fermenter assays

Many cultures are propagated without external pH control, and the fermentations carried out in shake flasks enable a laboratory reproduction of such fermentations. However, with some lactic acid bacteria, fermentation without external pH control in shake flasks would result in growth being limited by pH rather than by the nutrient composition of the medium [26]. Therefore, both types of fermentation were conducted.

Comparative growth curves in base media supplemented with 2.5% YE (lot C5) are shown in Fig. 1. Growth was faster in the fermenter under pH control, but the total biomass was similar after 18 h of fermentation. With this particular medium (at 2%

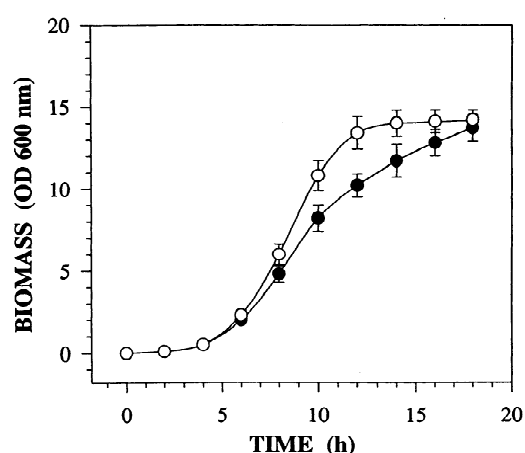


Fig. 1. Growth curves of *L. plantarum* MA18/5-U in shaker flasks (●), or in 2 l fermenters under external pH control (○). Growth medium: Base medium supplemented with 2.5% of lot C5 yeast extracts. Error bars represent the standard deviation.

glucose), it thus appeared that pH was not the limiting growth factor and that the buffering agents added to the medium (phosphates and citrates) were effective in preventing complete pH inhibition. Thus, shake flasks were used as controls for some experiments on the determination of the value of the AT system. One must keep in mind, however, that lower growth rates are to be expected in the flasks (no external pH control) as compared to those in fermenters (external pH-control).

3.2. Yeast extracts as fermentation nutrient ingredients

Yeast extracts are a key component in growth media for *L. plantarum*. When the base medium was supplemented with 2% ammonium phosphate, an OD (600 nm) of 0.8 was observed in shaker flasks after 18 h of incubation at 30°C. Addition of 1% YE (lot C5) to the base medium gave values that were 10 times higher (Table 1). Supplementation with 1% proteose peptone (Difco) was slightly less effective, giving an OD₆₀₀ of 6.2, while a combination of both YEs and proteose peptone gave the highest OD₆₀₀ (10.4).

Table 1 also shows the effect of concentration and YE lot on the biomass obtained in shaker flask cultures. Lots from the same manufacturing process gave biostimulatory activities that could vary by almost 50%. Thus, in the optimization of a growth

Table 1

Maximum biomass (OD at 600 nm) obtained in shaker flask cultures of *L. plantarum* MA18/5-U after 18 h of growth in a base medium supplemented with different lots and concentrations of yeast extracts

Yeast extract lot	Yeast extract concentration			
	0.1%	0.5%	1.0%	2.5%
C1	2.25	6.00	8.00	12.00
C2	2.15	5.50	7.50	10.00
C3	2.75	6.50	8.00	11.00
C4	2.00	6.00	9.25	12.50
C5	2.70	8.00	10.50	15.00
C6	3.80	9.50	10.50	12.50
<i>Other assays:</i>				
1. NH ₄ Cl	0.80			
2. 1% Peptone	6.20			
3. 0.5% Peptone +0.5% YE	10.40			

medium for a given strain, both the source and the concentration of YEs must be considered. Strain specificity seems important, as trials showed that a concentration of 2.5% rather than 0.5% of a known product provided a 5-fold increase in the final biomass concentration of one lactic acid bacteria strain but only a 1.5-fold increase with another strain (data not shown). However, establishing growth curves with shaker flasks, or in fermenters under external pH control, is tedious and labour-demanding because of the need to sample regularly, dilute the samples and proceed to OD measurements. Attempts were thus made to determine if AT could be used to replace flasks or fermenters in the medium optimization process.

3.3. Dilution factor and optical density measurements

A first attempt to use the AT system to follow the growth curves is presented in Fig. 2. The AT curves show similar growth patterns under the conditions

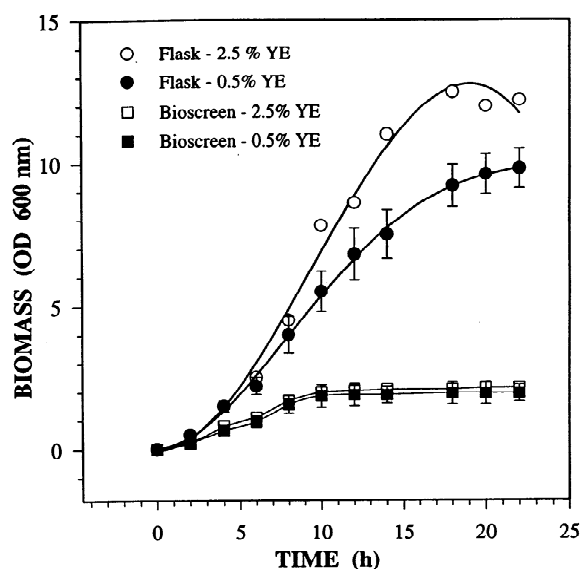


Fig. 2. Biomass levels and optical density (OD) at 600 nm of *L. plantarum* MA18/5-U cultures grown in shaker flasks or in an AT unit (Bioscreen). Growth medium: Base medium supplemented with 0.5 or 2.5% of lot C6 yeast extracts. OD readings in flasks were corrected for the dilution required for spectrophotometric analysis of the sample. Bioscreen data are uncorrected. The scope of variations (error bars of standard deviation) are presented on one bioscreen (■) and one flask (●) treatment.

tested with a final OD value of 2.0. The reference method (i.e. shaker flasks with appropriate dilution corrections) clearly shows that the biomass concentration reaches a much higher final value after 18 h, and that the YE concentration affects both final biomass and growth rate. It is well established that the direct proportionality between absorbancy and biomass concentration is only valid at optical densities below 1.0. Over this critical value, the correlation is no longer linear with most spectrophotometers and is completely out of range for high biomass values. Thus, direct use of the AT unit method was inappropriate and unreliable if applied in this fashion with this strain. Since it was impossible to dilute the samples during a fermentation in order to correct readings during the AT operation, we determined if diluting the sample prior to inoculation into the wells would be useful and could be mathematically correlated with reference methods.

3.4. Growth in diluted media

An inoculated medium was diluted 5, 10 or 20 fold and added to the AT plate wells. Diluted-media trials in flasks were run in parallel. Samples in flasks were again diluted for OD values exceeding 1.0, in order to obtain the real absorbancy values, whereas AT unit values correspond to observed readings from the method. Similar growth curves were obtained between flasks and the AT system when the sample was diluted 20 times (Fig. 3). It is to be expected that this dilution factor would only apply to this particular strain and medium combination. With other cultures, preliminary trials in undiluted flasks should give an order of magnitude for the dilution factor to be used. Although similar flask/AT growth curves could be obtained in the diluted media, it was necessary to determine if the data obtained in the AT diluted samples could be used to predict growth patterns in fermenters with undiluted media. Two variables defining the growth curve were studied: Maximum growth rate (μ_{\max}) and maximum biomass (OD at 600 nm).

3.5. Maximum biomass estimation

In order to generate different growth rates and final biomass levels, the following lot/concentration

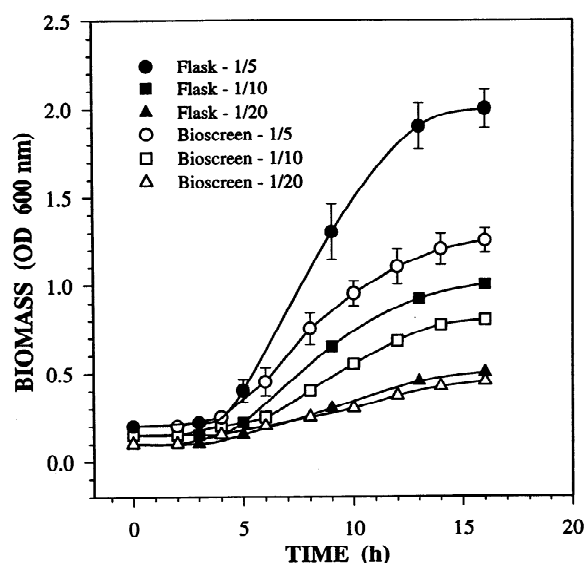


Fig. 3. Effect of dilution of the growth medium on biomass levels, represented by optical density (OD) at 600 nm, of *L. plantarum* MA18/5-U cultures propagated in flasks or in an AT unit (Bioscreen). Growth medium: Base medium supplemented with 0.5% of lot C6 yeast extracts. OD readings in flasks were corrected for the dilution required for spectrophotometric analysis of the sample. Bioscreen data are uncorrected. The scope of variations (error bars of standard deviation) are presented on one bioscreen (○) and one flask (●) treatment.

combinations were used: (1) lot C6 at 0.5%, (2) lot C6 at 2.5%, (3) lot C2 at 0.5% and (4) lot C5 at 2.5%. Media kept for the AT system were diluted 20 fold, and the data obtained were multiplied by the dilution factor. Media in flasks and fermenters were not diluted for the fermentation, but samples were diluted for the OD₆₀₀ measurements. The fermentations were conducted at 30°C for 18 h under agitation, as described previously. Fig. 4 shows that the 18 h biomass data for the AT system underestimated the biomass obtained in flasks or fermenters by 30 to 50%. However, good correlations were obtained between the various systems (Fig. 4; R^2 values of 0.94 and 0.96). Therefore, AT was found to be useful in predicting the potential biomass level in this particular YE-supplemented base medium.

3.6. Maximum growth rate (μ_{\max})

Since bacterial growth is exponential, values of Ln OD₆₀₀ were plotted against time to give a first order

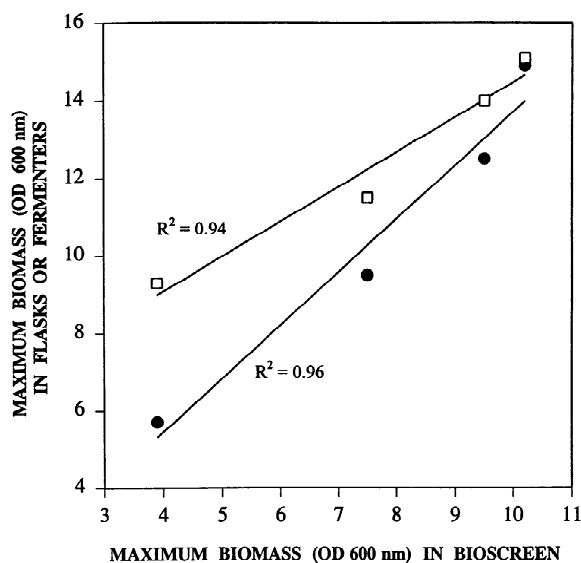


Fig. 4. Relationship between biomass levels and optical density (OD) at 600 nm of *L. plantarum* MA18/5-U cultures obtained with AT (Bioscreen) readings and those of cultures grown in shaker flasks (●) or fermenters under external pH control (□). OD readings in flasks or fermenters were corrected for the dilution required for spectrophotometric analysis of the sample. Bioscreen data are corrected for the 1/20 dilution of the growth medium.

relation where the slope equals the maximum μ value obtained during the exponential growth phase. This relation was established in all three systems (AT, fermenter and flask), but only OD₆₀₀ values below 1.0 in the exponential growth phase were used to calculate the μ_{\max} value. The μ_{\max} data obtained in the AT unit were approximately half of those obtained in flasks, and a third of those in fermenters (Fig. 5). Growth rates obtained in the absence of external pH control (flasks) were lower than when external pH control was applied (fermenters), which confirmed previous data (Fig. 1). A good correlation was obtained between AT and flask μ_{\max} data ($R^2=0.98$). However, a rather poor correlation ($R^2=0.45$) was reached between AT and fermenter. The μ_{\max} values observed in this study on *L. plantarum* are in the 0.3–0.5 h⁻¹ range that the literature reports for the MRS medium [27], which is a widely used medium for the culture of lactobacilli. Therefore, the minimum medium used for this study was effective in providing conditions that enabled the evaluation of the biostimulatory effectiveness of YEs.

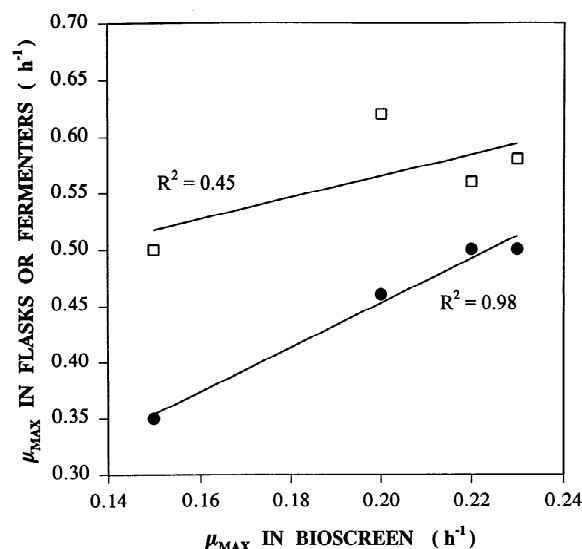


Fig. 5. Relationship between maximum growth rates (μ_{\max}) of *L. plantarum* MA18/5-U cultures obtained with AT (Bioscreen) readings and those of cultures grown in shake flasks (●) or fermenters under pH control (□). Conditions as in Fig. 4.

3.7. Application of AT in YE selection

Trials with YE concentrations varying by only 0.1% demonstrated that the AT system could discriminate between narrow margins, but significant differences ($P<0.05$) were only observed between media having 0.2% variations in YE.

The two growth parameters (μ_{\max} and maximal biomass) are not necessarily related and both should be considered when evaluating the biostimulatory activity of YEs. For instance, lots C6 and C5 (at 2.5%) are very similar in terms of their maximum growth rate with *L. plantarum* MA18/5-U (μ_{\max} of 0.49 and 0.50 h⁻¹, respectively, in flasks), but the final biomass concentration was 20% higher with lot C5.

When industry evaluates the appropriateness of a given ingredient lot for a large-scale fermentation, it obviously carries out its evaluation in small volumes. Results show that under these experimental conditions, the AT system could be adapted to effectively replace shake flask cultures in the evaluation of the biostimulatory activity of YEs on *L. plantarum* MA18/5-U. It is also a good screening method in a

preselection step for the evaluation of YEs for fermentations under external pH control. In this instance, the rather low correlation between μ_{\max} values in AT and external pH control (fermenter) conditions point to the limit of AT data in the selection of YEs for this particular criterium. It would thus seem appropriate to use the AT unit to screen the various YEs available, and to conduct small scale fermentations under pH control on products that gave the best results with the AT system. Although the AT would not completely replace small scale fermentation trials under external pH control for the selection of YEs, it will greatly limit their range, as AT appears to be effective as a screening tool.

An important feature of the AT procedure described in this study relies on dilution of the complete media. Although it was shown to be successful, it has its drawbacks. It is well known that water activity and ionic strength affect growth of bacteria [28,29], and it could be argued that the method could be improved by diluting only the growth supplement. Attempts are thus under way to determine if diluting only the YE fraction can improve the usefulness of this AT method, particularly in predicting developments in fermentations under external pH control.

This study shows that the AT system could thus help the fermentation industry to develop its media. Furthermore, it would also be useful to the YE supplier, who could accurately identify the biostimulation value of his products for a given client. Present experience reveals that there is no ideal YE, and that the bioactivity value is often species- and even strain-related. Up to 200 different conditions can be screened at one time, within 24 h, provided the dilution factor is adjusted to keep the OD values below 1.0. Using the AT methodology, suppliers of YEs are thus in a position to rapidly screen different lots to better suit the needs of specific clients/strains.

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