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Short communication

Spectrophotometric flow injection monitoring of sulfide during sugar fermentation

Claudineia R. Silva, Valdemir A.F. Barros, Luiz C. Basso[1], Elias A.G. Zagatto*

Center for Nuclear Energy in Agriculture, University of S. Paulo, Av. Centenario 303, Piracicaba-SP 13400-970, Brazil

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ABSTRACT

A spectrophotometric flow injection procedure involving N,N-dimethyl-*p*-phenylenediamine (DMPD) is applied to the sulfide monitoring of a sugar fermentation by *Saccharomyces cerevisiae* under laboratory conditions. The gaseous chemical species evolving from the fermentative process, mainly CO_2, are trapped allowing a cleaned sample aliquot to be collected and introduced into the flow injection analyzer. Measurement rate, signal repeatability, detection limit and reagent consumption per measurement were estimated as 150 h^{-1}, 0.36% (n = 20), 0.014 mg L^{-1} S and 120 μg DMPD, respectively. The main characteristics of the monitoring record are discussed. The strategy is worthwhile for selecting yeast strain, increasing the industrial ethanol production and improving the quality of wines.

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1. Introduction

During sugar fermentation by *Saccharomyces cerevisiae* aiming at industrial ethanol or wine production, several volatile compounds are generated [1], and sulfide plays a relevant role in the context [1,2]. Excess of sulfide may alter the yeast metabolism thus affecting the fermentative process [2,3]; moreover, this excess may impart undesirable aromas associated with off-odors of beverages as *e.g.* wines [4].

The presence of sulfide in the yeast cells is primarily due to the enzymatic reduction of sulfate or sulfite in excess of cell requirements; sulfide is routinely formed in controlled amounts as an intermediate for the synthesis of yeast essential sulfur amino acids such as cysteine and methionine [5,6]. Nevertheless, the sulfide production is influenced by yeast strains and fermentation conditions, such as the sulfur precursor compounds, the culture growth rate and yeast assimilable nitrogen content in the fermented medium [4]. Depending on the relative rates of the sulfate/sulfite reduction, amino acid synthesis and release towards the environment, sulfide can be accumulated in the yeast cells, thus affecting the fermentation [3].

Influence of the main parameters associated with the sulfide formation and accumulation should be investigated for an efficient control of the sugar fermentation by specific yeast strains under different conditions. Moreover, efforts aiming at a better sensory quality of fermented beverages [7] are recommended.

To this end, analytical procedures yielding reliable results in a fast manner are needed for on-line sulfide monitoring in order to permit an improved industrial ethanol production, a better-quality wine production and the selection of yeasts leading to lower sulfite formation.

Due to its favorable characteristics of versatility, efficiency, ruggedness, easy implementation and low cost, the flow injection analyzer has been widely accepted for industrial process monitoring [8]. Regarding sulfide, a flow-based analytical procedures involving the oxidative coupling of sulfide with N,N-dimethyl-p-phenylenediamine (DMPD) in the presence of ferric ions yielding the colored methylene blue was already applied to water analysis [9], The aim of the present work was then to develop a flow injection procedure for sulfide monitoring during the sugar fermentation under laboratory conditions.

2. Experimental

2.1. Reagent, standard and sample solutions

The solutions were prepared with chemicals of analytical grade quality and distilled-deionized water.

The chromogenic reagent (R_1, Fig. 1) was a weekly prepared 5.0 mmol L^{-1} DMPD plus 1.0 mol L^{-1} HCl solution. The R_2 reagent was a 50 mmol L^{-1} Fe^{3+} (as $FeCl_3 \cdot 6H_2O$) plus 1.0 mol L^{-1} HCl

* Corresponding author.
E-mail address: ezagatto@cena.usp.br (E.A.G. Zagatto).
[1] [1]Present address: Agricultural College Ëuiz de Queiroz, University of S. Paulo. Av. Padua Dias 9, Piracicaba-SP 13418-900, Brazil.

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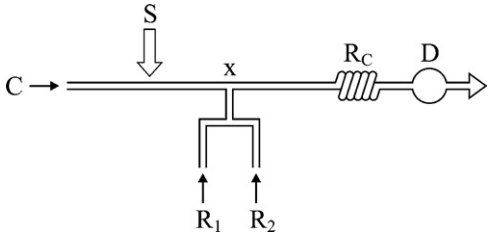

Fig. 1. Flow diagram. S = sample; C = sample carrier stream (0.025 mol L^{-1} NaOH at 3.9 mL min^{-1}); x = confluence site; R_1 and R_2 = DMPD and Fe(III) reagent streams (0.42 mL min^{-1}); R_C = coiled reactor; D = detector.

solution. The C chemically inert sample carrier stream was a 0.025 mol L^{-1} NaOH solution.

The T_1 trapping solution (Fig. 2) was a 0.10 mol L^{-1} Ca (as $CaCl_2$) plus 0.01 mol L^{-1} $Na_2B_4O_7$ solution (pH = 11), and the T_2 trapping solution was a 0.10 mol L^{-1} NaOH solution.

The sulfide stock solution (1000 mg L^{-1} S, also 0.1 mol L^{-1} NaOH) was based on $Na_2S{\cdot}9H_2O$ The working standard solutions (0.10–1.0 mg L^{-1} S, also 0.025 mol L^{-1} NaOH) were daily prepared.

The fermentation medium was established by combining 5.0 g of yeast (*S. cerevisiae*), 30 mL of wort and 15 mL of water (20% total reducing sugars), the same proportions as used in a typical industrial fermentation [1].

2.2. The flow injection system

The flow set-up comprised a model 482 Femto UV–vis spectrophotometer provided with a glass flow-cell (80-μL inner volume, 10-mm optical path), a model 111 Kipp & Zonen strip-chart recorder, a model 7618-40 Ismatec peristaltic pump, a manually operated injector [10], Perspex connectors and accessories. The manifold was built up with 0.8-mm i.d. polyethylene tubing of the non-collapsible type. Wavelength was set as 668 nm. The gas diffusion unit used in the preliminary experiments was similar to that already described [11] and included a commercial strip of Teflon thread sealing as the semi-permeable membrane.

The flow injection system in Fig. 1 was operated as follows. The sample aliquot was introduced into the chemically inert carrier stream and the established sample zone converged with the previously mixed R_1 and R_2 reagents at the x confluence point, allowing the methylene blue formation inside the following coiled reactor. Passage of the sample zone through the detector resulted in a transient modification in absorbance, which was recorded as a peak. Height of this recorded peak constituted itself as the measurement basis.

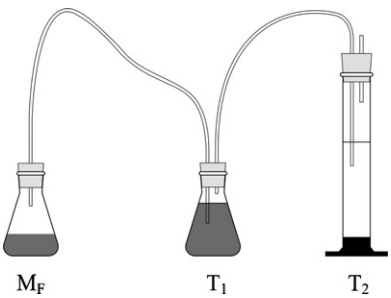

Fig. 2. The trapping system. M_F, T_1 and T_2 = fermentation medium, first trapping solution and second trapping solution. Tube at T_2 stopper needed for gas outlet and sample aliquot collection. For details, see text.

Table 1
Investigated parameters, variation range and selected values after optimization of flow injection system.

Parameter	Variation range	Selected value
Reaction coil length (cm)	25–100	75
Sample volume (μL)	120–800	500
R_1 and R_2 flow rates (mL min^{-1})	0.42–1.0	0.42
Fe(III) concentration (mmol L^{-1})	20–100	50
DMPD concentration (mmol L^{-1})	2.0–10	5.0
R_1 acidity (mol L^{-1} HCl)	0.20–1.0	1.0
R_2 acidity (mol L^{-1} HCl)	0.20–1.0	1.0

2.3. Procedure

The flow injection system was dimensioned by the univariate method: the working standard solutions were run in triplicate after each parameter variation. Table 1 indicates the parameter variation ranges and the selected values. The main analytical figures of merit were then evaluated. Repeatability was expressed as the relative standard deviation of the results estimated after twenty successive measurements of a typical sample aliquot (0.63 mg L^{-1} S and sampling rate was calculated as the inverse of the washing time [12].

3. Results and discussion

3.1. Monitoring strategies

Two strategies for sulfide monitoring were investigated namely: (i) the direct analysis of the fermentate with a flow injection system involving gas diffusion similar to that already described [11]; and (ii) the analysis of sample aliquots with the flow injection system in Fig. 1 after a sample clean-up with the trapping system in Fig. 2.

Sample aliquots directly taken from the fermentate medium could not be analyzed by the flow injection system due to the excessive gas release, mainly the CO_2 formed in large amounts during the fermentative process. This aspect resulted in the formation of gas bubbles inside the analytical path which affected the sample dispersion and impaired detection [13]. It should be recalled that CO_2 was able to cross the semi-permeable membrane. It was then decided to trap the released CO_2 and to analyze the cleaned sampled aliquot.

In order to investigate the feasibility of this strategy, the fermentation was accomplished inside a 500-mL Erlenmeyer as the process reactor, which was connected to two trapping flasks (Fig. 2). The first one contained 250 mL of the T_1 solution and retained the evolved CO_2 as the slightly soluble calcium carbonate, whereas the second one contained 70 mL of the T_2 solution and retained the H_2S as the HS^-. The sample aliquots (about 2 mL) were then taken from the second flask. Sulfide trapping inside the T_1 solution was minimized due to combined influence of several phenomena: the large excess of formed CO_2 relatively to H_2S (mol L^{-1}, mmol L^{-1}), the higher CO_2 solubility in water relatively to H_2S (3.8 and 1.7 g kg^{-1} in water at 20 °C [14]); the high solubility of calcium hydrogen carbonate in the T_1 solution; the H_2S carrying by CO_2, the higher alkalinity of T_2 relatively to T_1 and the kinetic aspects involved. The pH of T_1 solution decreased during fermentation monitoring and this is another favorable aspect in relation to the H_2S release towards T_2 solution.

As the alkalinity of the T_2 solution also underwent a slight decrease during the fermentation, and the working standard solutions and sample carrier stream of the flow injection system were prepared in 0.025 mol L^{-1} NaOH and the flow injection system in Fig. 1 was dimensioned accordingly. The alkalinity variations in the sample zone were not relevant: preliminary experiments revealed no modifications in recorded peak height when the alkalinity of the

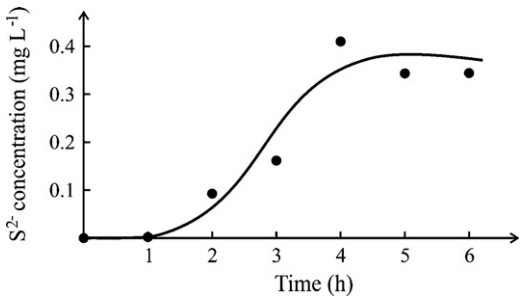

Fig. 3. Graphical record of a monitored fermentation. Figure refers to the flow-injection system in Fig. 1. Ordinate = sulfide concentrations in the T_2 solution. For details, see text.

test-sample was varied between 0.01 and 0.1 mol L^{-1}, and the effect is due to the high acidity involved in the methylene blue formation.

3.2. System dimensioning

Experiments involving switching the peristaltic pump OFF when the sample zone was flowing through the flow cell pointed out the fast methylene blue formation. Length of the main reactor was then selected as short as possible (75 cm) yet long enough for attaining good mixing conditions. The carrier stream flow rate was set as 3.9 mL min^{-1} as a compromise between sampling rate and hydrodynamic pressure.

The R_1 and R_2 flow rates were selected as 0.42 mL min^{-1} in order to minimize sample dilution at the confluence site; too low flow rates were not used, as the involved concentrations might be increased accordingly, thus impairing mixing conditions [15].

Regarding concentrations of the R_1 and R_2 reagents, slight modifications (usually < 2%) in recorded peak height were noted when the Fe(III) or DMPD concentrations were varied between the limits specified in Table 1. Also, no modifications in baseline were noted by varying the reagent concentrations. These aspects are especially relevant in terms of system ruggedness. The concentrations of R_1 and R_2 streams were then set as 50 and 5 mmol L^{-1}, respectively.

Increasing the injected volume from 120 to 800 μL increased the recorded peak height following an asymptotical tendency. Beyond 500 μL, the increase in recorded peak height was too low to compensate the concomitant decrease in sampling rate. Length of the sampling loop was then selected as 100 cm (*ca* 500 μL).

3.3. Analytical characteristics

The flow injection system in Fig. 1 yields precise results, as repeatability was estimated as 0.36% ($n = 20$). The washing time is 24 s, meaning a sampling rate of was 150 h^{-1}. In this situation, 0.17 mL DMPD (120 μg) are needed per determination. This very low reagent consumption matches the present tendency towards a clean chemistry [16].

A linear analytical curve is verified for the 0.10–1.0 mg L^{-1} S range, and a typical equation is:

$$h = 0.0110\,(\pm 0.0006) + 0.3983\,(\pm 0.0023)\,C\,(n = 6, r = 0.99918)$$

where h = recorded peak height, in absorbance; C = concentration in mg L^{-1} S.

The detection limit was estimated [17] as 0.014 mg L^{-1}. The mean available time for reaction development was 0.16 s, enough for reaching the involved chemical equilibria.

Regarding sulfide monitoring during fermentation, analysis of Fig. 3 permits one to infer that sulfide production is not evident during the first 50 min, probably because of the solubility of hydrogen sulfide [14] in the fermentation medium and its consumption by the cells for amino acid synthesis [5]. Thereafter, sulfide formation becomes evident, and increases during the next 3 h, approaching the maximum value after about 5 h.

The ordinate in Fig. 1 refers to sulfide content in the sample aliquot taken from the T_2 solution. The instant sulfide concentration in the fermentation medium is then not straightforward evident. It could be determined by adding a known sulfide amount to the fermentation medium which would act as an external standard. This strategy was not however implemented, as analysis of Fig. 3 permits to evaluate the speed of the fermentation as well as the relative production of sulfite at the end of several fermentative processes.

4. Conclusions

The proposed strategy demonstrated to be useful for evaluating the speed of the fermentation process and the total accumulated sulfide, and this information is worthwhile for controlling the fermentation process and selecting the yeast strain.

With the designed trapping system for sample clean-up, cumbersome steps of gas diffusion are not required, thus simplifying the design of the flow injection system, and this aspect is relevant for the system applicability in routine analysis.

Investigations on the feasibility a similar flow-based strategy for micro-scale (~ few mL) monitoring of a fermentative process is presently in progress.

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