

## Minibioreactor-gas collector for determining bacteria-produced hydrogen sulfide

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**Abbreviations:** rpm: revolutions per minute.

**A simple and economical minibioreactor-gas collector system for determination of hydrogen sulfide produced by a microorganism was designed. The detection of hydrogen sulfide was based on the reaction between the hydrogen sulfide in the gas stream from the culture, and a lead acetate solution 0.090 mol/L, contained in a tube gas collector; the conductimetric back titration of lead excess was made, and hydrogen sulfide was quantified indirectly, being the detection limit 0.5  $\mu\text{mol}$ . The developed system was applied using *Tsukamurella paurometabola* DSM 20162 as a model, being the amount of hydrogen sulfide produced, 1.2  $\mu\text{mol}$  in 24 hrs.**

There is a great diversity of microorganisms that produce hydrogen sulfide (Aiking et al. 1982; Kim and Olson, 1989; Barton and Tomei, 1995; Cooney et al. 1996; Levine et al. 1998; Wang et al. 2000), from organic (*i.e.* sulfur-containing amino acids) and inorganic sources (*i.e.* sulfates). Although hydrogen sulfide is a toxic pollutant gas generally occurring in wastewater, it has been used to precipitate metals in wastewater treatment reactors and has been proposed for stabilization of metals in soils and for formation of metal sulfide “quantum” particles for microelectronics applications (Holmes et al. 1997).

When a novel hydrogen sulfide producer bacterial strain is isolated, it is necessary the physiological and biochemical characterization of such strain, in order to optimize the hydrogen sulfide production for its further application (Fortin et al. 1994; Peyton et al. 1995; White and Gadd, 1996; White and Gadd, 1998; Smith, 2000). Although the experimental determination of this compound has been done through different methods, and the analytical methods for H<sub>2</sub>S detection and quantification have ranged from spectrophotometric analysis (Cline, 1969; Acree et al. 1971; Siegel, 1965; Chu et al. 1997) to gas chromatography (GC), either with distinct features in terms of accuracy, precision and detection limit (Heida et al. 1995; Chinivasagam et al. 1998; Mestres et al. 1999); the study at small scale is a suitable and economical option for the achievement of a such objective.

In this paper a minibioreactor-gas collector system designed for characterization and study of hydrogen sulfide production from bacteria, is described. In addition, a simple analytical method, based on H<sub>2</sub>S indirect conductimetric determination as a modification of the method of lead acetate for detection of hydrogen sulfide (Hunter and Crecelius, 1938), was developed. The gas stream from the minibioreactor passes through a lead acetate solution

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contained in the tube (gas collector), thus  $\text{H}_2\text{S}$  reacts with  $\text{Pb}^{2+}$ , producing lead sulfide; then the  $\text{Pb}^{2+}$  excess is determined by conductimetric titration with sodium phosphate.

## MATERIALS AND METHODS

### Development of the Analytical Method

**$\text{H}_2\text{S}$  standard preparation.** In order to develop a method of analysis, a  $\text{H}_2\text{S}$  standard was prepared, from the reaction of 1 g of  $\text{Na}_2\text{S}$  (Merck) with 11 mL of 0.1 N  $\text{H}_2\text{SO}_4$  (BDH), in a tightly closed 100 mL bottle. The bottle was sealed immediately after the  $\text{Na}_2\text{S}$  was added, then  $\text{H}_2\text{SO}_4$  was injected into the bottle.

**Method description.** 10  $\mu\text{L}$ , 100  $\mu\text{L}$ , 300  $\mu\text{L}$ , 500  $\mu\text{L}$ , 700  $\mu\text{L}$  and 1000  $\mu\text{L}$  of the  $\text{H}_2\text{S}$  previously prepared standard were injected into different sealed bottles containing 2 mL of 0.090 mol/L  $\text{Pb}(\text{CH}_3\text{COO})_2$  (Merck) solution, which produced a black  $\text{PbS}$  precipitate. The content in each bottle was filtered with Whatman paper 40, and transferred to 250 mL volumetric flasks and filled up. Each point had 5 replicas.

Volumes of 100 mL were taken from the volumetric flasks and titrated with a  $\text{Na}_2\text{HPO}_4$  solution (Merck) 4 mM; conductivity was measured for each volume of added titrant with a Copenhagen CDM 80 Radiometer Conductivity Meter. The conductimeter was calibrated using standard solutions of KCl (Radiometer) with conductivities 20  $\mu\text{S}/\text{cm}$  and 100  $\mu\text{S}/\text{cm}$  respectively at 25°C. In each particular case, the equivalence point was determined from the conductimetric titration curve (Figure 3). Once the curves were adjusted by linear regression (descending and ascending straight lines) the equivalence point was the interception between them.

By determining the  $\text{Pb}^{2+}$  ion final concentration after reaction with  $\text{H}_2\text{S}$ , the amount of absorbed  $\text{H}_2\text{S}$  was indirectly determined, according to:

$$n(\text{H}_2\text{S}) = V_i(\text{Pb}^{2+}) \Delta C(\text{Pb}^{2+})$$

Where,

$n(\text{H}_2\text{S})$ : Amount of absorbed  $\text{H}_2\text{S}$  substance ( $\mu\text{mol}$ )

$V_i(\text{Pb}^{2+})$ : Initial  $\text{Pb}^{2+}$  volume (mL)

$\Delta C(\text{Pb}^{2+})$ :  $\text{Pb}^{2+}$  concentration variation (mM).

1 mL of hydrogen sulfide standard (1.27 nmol/mL) was injected (in triplicate) into a gas chromatograph Varian 300 (Flame Photometric Detector with a filter for determination of sulfur-containing compounds, Column DB-5, Temperature 35°C); and the retention time was determined.

The standards were analyzed too by classical iodometric method according to Ayres (1968).

**Minibioreactor-gas collector design.** The minibioreactor with a rubber cap (Figure 1), was made from a culturing tube (50 mL) functioning as the vessel of the bioreactor (A). Air input was through a 2 mm glass tube (B), with one of its ends coupled to the air filter (C) for sterile air input (Midisart, 0.2  $\mu\text{m}$ ). The fermentation gases pass through the exhaustion pipe (D) and were absorbed in the gas collector (E). The samples of culture were taken through the sample valve (F).

Gas collector was a two-way burette modified, containing 10 mL of lead acetate 90 mM. The gases from culture pass through the lead acetate solution in one position of stopcock (G); in the other position of stopcock, the samples were taken, as the way to evaluate  $\text{Pb}^{2+}$  ion excess.

**Development of culture and inocula.** Inocula were grown in preculture tubes with 5 mL of LB medium, for 12 h; 500  $\mu\text{L}$  from *Tsukamurella paurometabola* DSM 20162 cell bank, cryopreserved at  $-70^\circ\text{C}$  in glycerol at 20% had been previously inoculated into each tube.

Each fermentation experiment (in triplicate) was made using 3 minibioreactors like the above described (labeled as minibioreactor 1, minibioreactor 2, and minibioreactor 3). Each minibioreactor was inoculated with 2 mL of inoculum. The culture medium was composed by M9 medium supplemented with cysteine 1.5 mmol/L, and the effective volume was 30 mL. The air flow at input of minibioreactors was 30 mL/min and the culture temperature was 28°C. The culture samples and lead acetate samples were taken at 6, 10, 12, 17, 20 and 24 hrs alternately from minibioreactors 1, 2 and 3 to avoid errors due to volume variation. In minibioreactor 1 were taken the samples corresponding to 6 and 12 hrs; in minibioreactor 2 were taken the samples corresponding to 10 and 20 hrs; and in minibioreactor 3 were taken the samples corresponding to 17 and 24 hrs.

Three additional fermentations were done to determine the chromatographic profile of sulfur-containing gases by GC.

**Determination of bacterial growth and hydrogen sulfide production.** The bacterial growth was determined measuring optical density at 530 nm.

The gaseous samples were collected in gas sampling bulbs (Konik). The chromatographic profile of sulfur-containing gases from culture was obtained by GC using a gas chromatograph Varian 300 (Flame Photometric Detector with a filter for determination of sulfur-containing compounds; Column DB-5; Temperature 35°C); 1 mL of sample was injected for each time.

The analysis of hydrogen sulfide chemically absorbed in the gas collector was made using the conductimetric method above described. 500  $\mu\text{L}$  were taken for each determination, centrifuged at 10000 rpm during 10 min., and 300  $\mu\text{L}$  of the corresponding supernatant were diluted

up to 100 mL to be analyzed.

## RESULTS AND DISCUSSION

In relation to the developed system, its novelty is in the whole as a hydrogen sulfide determination tool. The minibioreactor-gas collector system is shown in [Figure 1](#). The device has the advantage that it is possible to study the hydrogen sulfide production either by aerobic or anaerobic microorganisms; in the case of anaerobic, sulfate is the main source for hydrogen sulfide production (Barton and Tomei, 1995) and Nitrogen should be injected into the system in place of oxygen (Afshar et al. 1990; Chu et al. 1997; ter Linde et al. 1999). Notice that the minibioreactor is a kind of air-lift bioreactor, using the energy of pressurizing gas as the power source for agitation.

One of the main advantages of the designed system is the possibility of working at small scale, making it more attractive economically, because it decreases the volumes of necessary reagents in order to prepare the corresponding culture medium. Another advantage is that the agitation takes place without use of impeller, which reduces the energy expenses; achieving, with the flow of the pressurizing gas, an appropriate agitation for the growth of the studied microorganism.

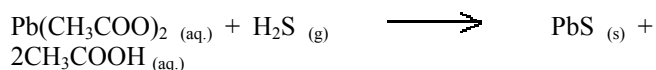
In spite of the simplicity of the design, it is possible to maintain the constant temperature if an external thermostat is used. Several minibioreactors have been designed with diverse purposes (Man Bock et al. 1999; Jiho Min et al. 2000; Man Bock et al. 2001). However, until date, there are not reports in relation to the use of this type of device for the study of bacteria-produced hydrogen sulfide.

On the other hand, the gas collector, due to its shape, avoid the possible contamination of the culture; also the stopcock permits ([Figure 1](#)) in a position the pass of fermentation gases, and in the other position to take the sample from the absorbent solution for trapping gases, giving flexibility to the system.

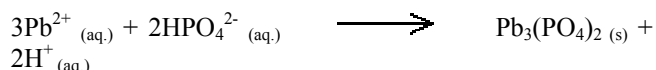
The chromatographic profile of sulfur-containing gases produced during fermentation was determined by GC. In [Figure 2](#) the chromatograms corresponding to the hydrogen sulfide standard, and a running sample corresponding to 17 hrs of fermentation time, are shown. The retention time for hydrogen sulfide standard was  $1.93 \pm 0.14$  min. When sample was chromatographically processed, the retention time was 1.86 min, being this value between the confidence interval of the standard. Then, peak 1 in the running sample ([Figure 2B](#)), corresponds to hydrogen sulfide. Another sulfur-containing gas was detected (peak 2, [Figure 2B](#)), having a retention time of 6.96 min; this gas was not identified.

In each analyzed sample the levels of hydrogen sulfide were higher than the amounts of the unknown sulfur-containing gas.

For the quantitative determination of hydrogen sulfide produced in the minibioreactor, a conductimetric method was developed as a modification of the classical method for determination of hydrogen sulfide in bacterial cultures (Hunter and Crecelius, 1938). The method has two stages: first, the hydrogen sulfide released during a bacterial culture, is collected in a 0.090 mol/L lead acetate solution, via the reaction:



In the second stage, excess  $\text{Pb}^{2+}$  is determined conductimetrically by titration with a 4 mmol/L sodium hydrogenphosphate solution, via the reaction:



In this way, the hydrogen sulfide formed in the fermentation process is indirectly quantified.

The conductimetric titration curve at 25°C is shown in [Figure 3](#), being a typical titration curve of precipitate formation (Ayres, 1968). Once the ascending and descending curves were adjusted by linear regression, the equivalence point was the interception between them.

[Figure 4](#) shows the correlation between volumes of  $\text{H}_2\text{S}$  standards injected into the recipients containing  $\text{Pb}(\text{CH}_3\text{COO})_2$  0.090 mol/L, and the amounts of detected substance, being the detection limit 0.5  $\mu\text{mol}$ . This value of detection limit is higher than values obtained when other methods such as gas chromatography are evaluated (Heida et al. 1995; Chinivasagam et al. 1998; Mestres et al. 1999) however it is low enough to detect hydrogen sulfide amounts produced by strains that are low-level hydrogen sulfide producers (Barton and Tomei, 1995; Chinivasagam et al. 1998; Levine et al. 1998).

In case of producing other volatile substances that contain the  $-\text{SH}$  group, reaction to  $\text{Pb}^{2+}$  ions could occur and consequently,  $\text{PbS}$  precipitate is produced; therefore these other volatile sulfur compounds could cause interference in the analysis. If this happened, we could then expect that this conductimetric method could be able to detect total released volatile sulfide, which would allow characterizing the fermentation system in general terms regarding production of sulfur-containing gas. In our experiments, the content of the unknown sulfur-containing gas, using GC, was very low; around 10% of peak 1 height ([Figure 2B](#)). The lowest amount of volatile sulfide detected in gas stream produced during bacterial culture, by the conductimetric method, was 0.9  $\mu\text{mol}$  ([Figure 5](#)). Taking into account the GC chromatogram ([Figure 2B](#)), around 10 % (0.09  $\mu\text{mol}$ ) of this amount could be associated to the unknown sulfur-containing gas. The detection limit of the developed method was 0.5  $\mu\text{mol}$ , therefore it was not possible to detect the

amount of 0.09  $\mu\text{mol}$  corresponding to the unknown compound.

In order to make a validation of the developed method, it was compared to classical iodometric method (Ayres, 1968). [Table 1](#) summarizes the comparison between both methods for the analysis of hydrogen sulfide standards. It was found that all observed t-values were lower than tabulated t-value (95% confidence, 4-degree of freedom); showing that both methods do not differ in accuracy. On the other hand all observed F-values were lower than tabulated F-value (95% confidence, 4-degree of freedom), so it is concluded that there are not significance differences in precision.

The developed system was applied in detection and quantification of hydrogen sulfide levels produced by *Tsukamurella paurometabola* DSM 20162, grown in a culture medium containing cysteine 1.5 mM as a sole carbon source. [Figure 5](#) shows the kinetic behaviour of the system, where minimum  $\text{H}_2\text{S}$  amount detected in the gas collector was 0.9  $\mu\text{mol}$  at 17 hrs, and maximum amount was 1.2  $\mu\text{mol}$  at 24 hrs. The kinetic behaviour showed a hydrogen sulfide production growth-associated, which indicates that *T. paurometabola* uses cysteine as a carbon source for growing; and due to catabolism produces hydrogen sulfide, which has been reported for other microorganisms (Acree et al. 1971; Kim and Olson, 1989; Chu et al. 1997).

## CONCLUDING REMARKS

A minibioreactor-gas collector system was developed for studying the biological production of hydrogen sulfide in microorganisms. This device promises to be useful in studies at small scale, being simple and economical. In addition, an analytical method for indirect determination of hydrogen sulfide chemically absorbed in the gas collector was developed, as a modification of the method of Hunter and Crecelius (1938); detecting hydrogen sulfide levels higher than 0.5  $\mu\text{mol}$ , which allows to determine the levels of hydrogen sulfide produced by such strains that produce small amounts of this gas.

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## APPENDIX

## Tables

Table 1. Comparison between proposed method and official method (iodometric).

Amount of H <sub>2</sub> S Detected by iodometric method (μmol)	Amount of H <sub>2</sub> S Detected by conductimetric method (μmol)	Confidence Interval for Conductimetric method (μmol)	t-value (Critical t-value <sup>a</sup> = 1.860)	f-value (Critical F-value <sup>b</sup> = 6.388)
0.500	0.502	± 0.02	0.144	2.031
5.060	5.078	± 0.03	1.068	0.844
15.028	15.048	± 0.07	0.338	3.969
25.136	25.164	± 0.03	1.667	1.431
35.260	35.300	± 0.08	1.195	0.287

<sup>a</sup> The critical t-value with 95% confidence at 4-degrees of freedom.

<sup>b</sup> The critical F-value with 95% confidence at 4-degrees of freedom in numerator and denominator.

## Figures

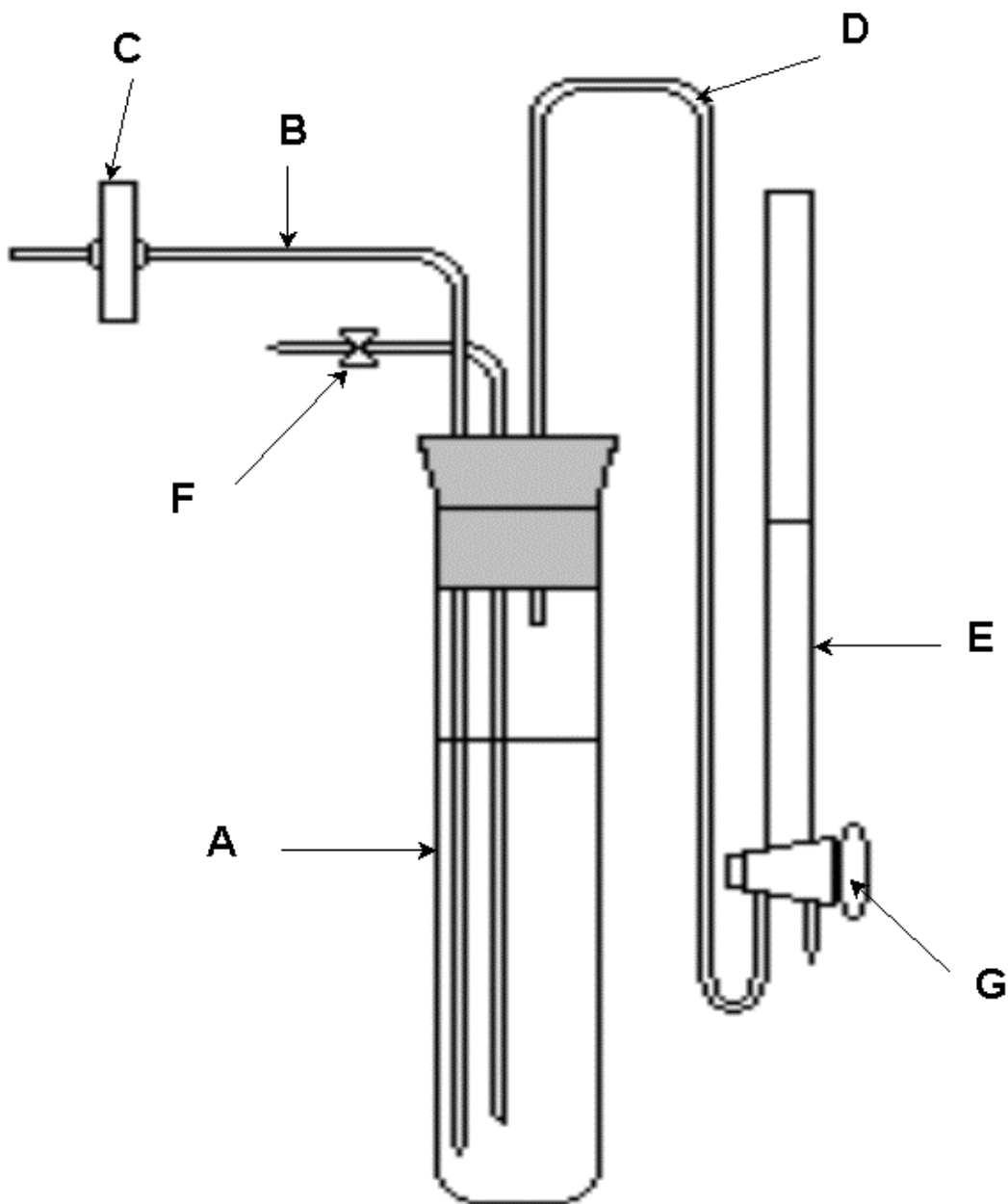


Figure 1. Minibioreactor-gas collector system designed for determination of bacteria-produced hydrogen sulfide. (A) Vessel; (B) Air Input; (C) Air Filter; (D) Exhaustion Pipe; (E) Gas Collector; (F) Sample Valve; (G) Stopcock.

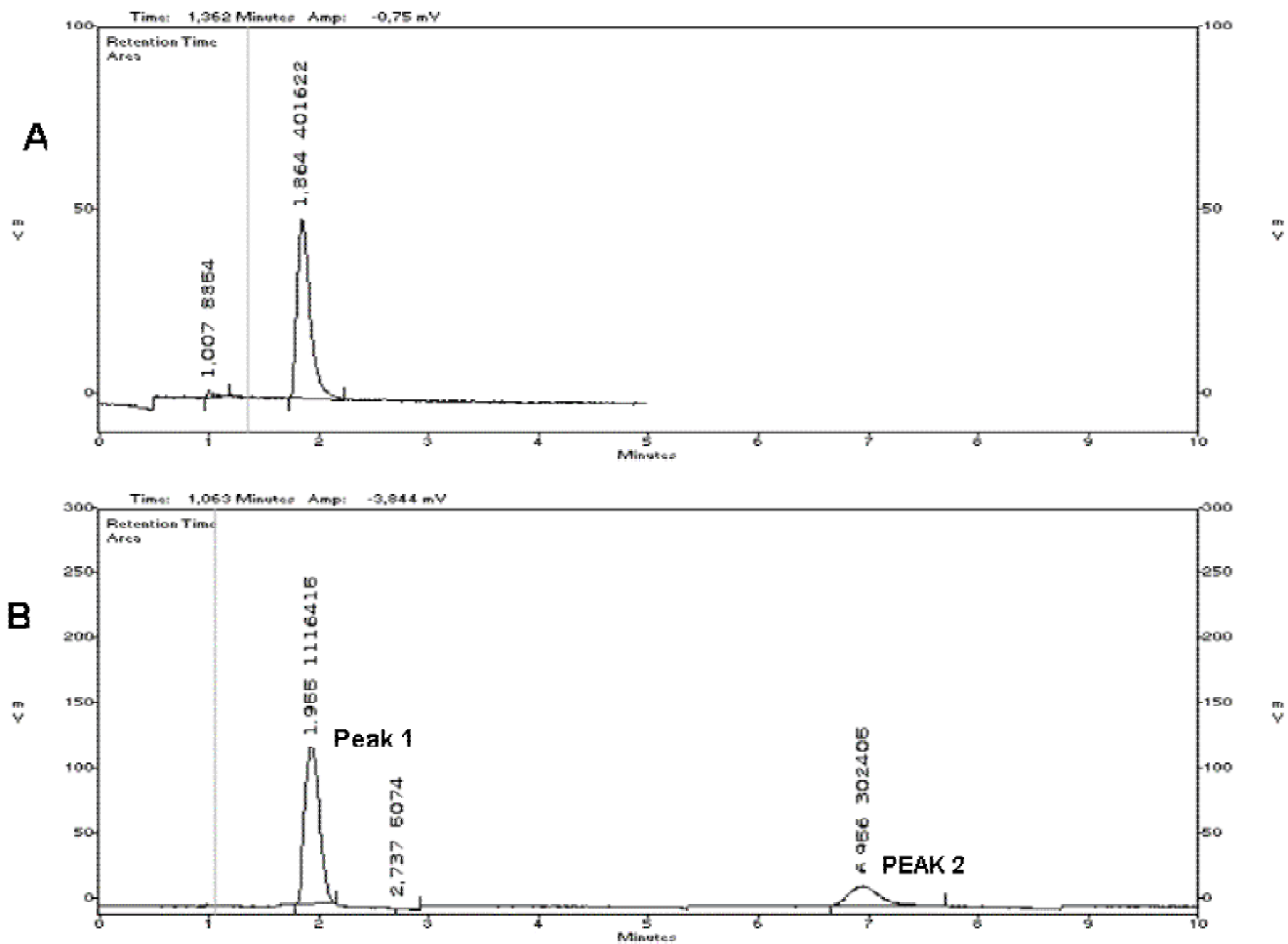


Figure 2. Gas Chromatography of samples for characterization of retention times and qualitative composition. (A) Hydrogen sulfide standard; (B) Running sample of fermentation gases taken at 17 hrs.



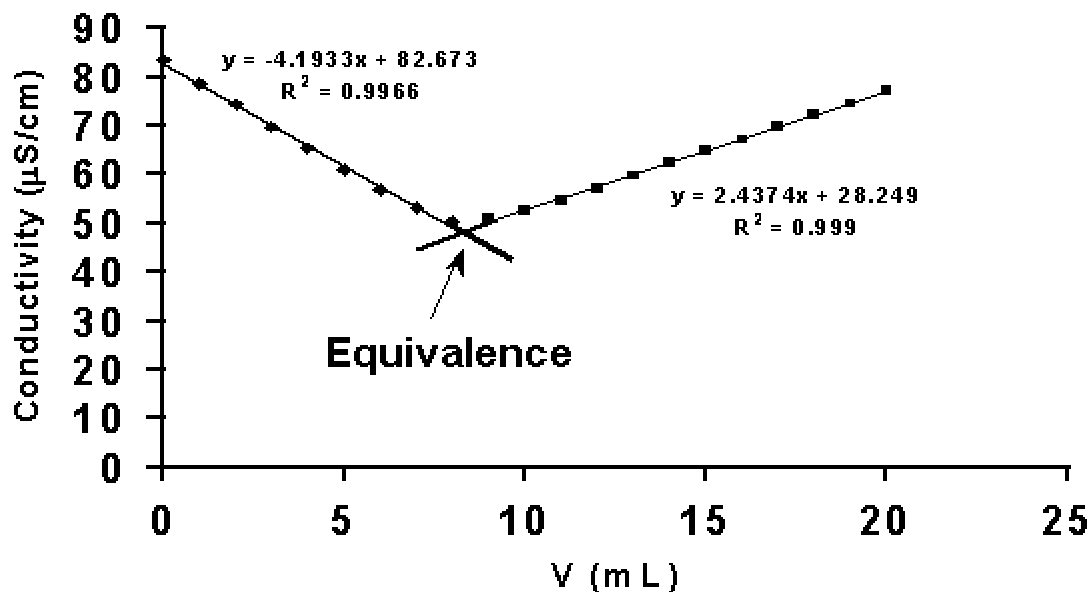


Figure 3. Conductimetric titration curve obtained while  $\text{Pb}^{2+}$  excess reacts with titrant  $\text{Na}_2\text{HPO}_4$  to form  $\text{Pb}_3(\text{PO}_4)_2$ , at  $25^\circ\text{C}$ .

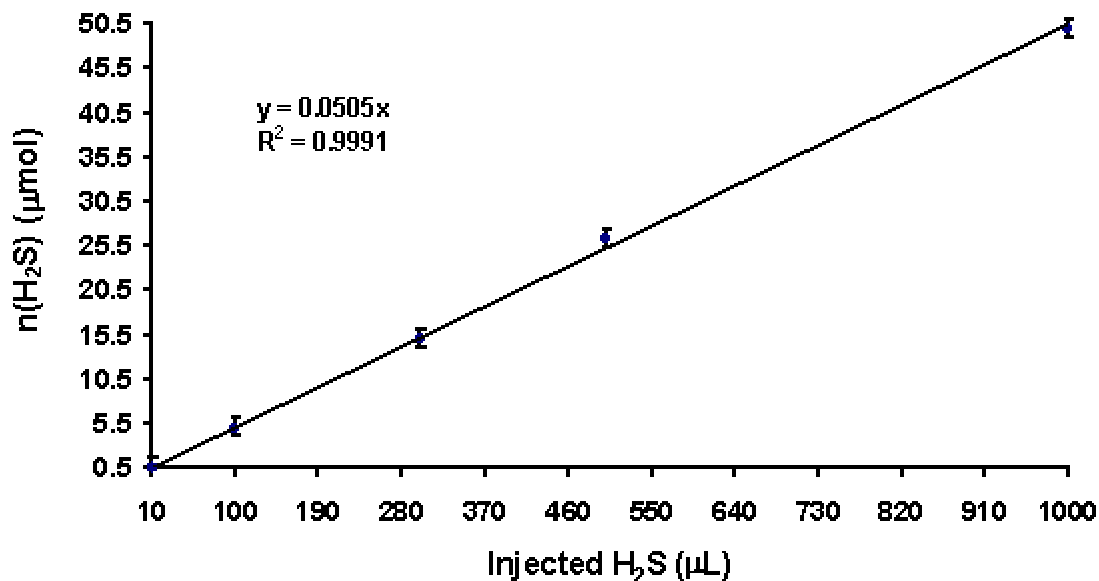


Figure 4. Correlation between volume of  $\text{H}_2\text{S}$  injected, and amount detected by conductimetric titration.

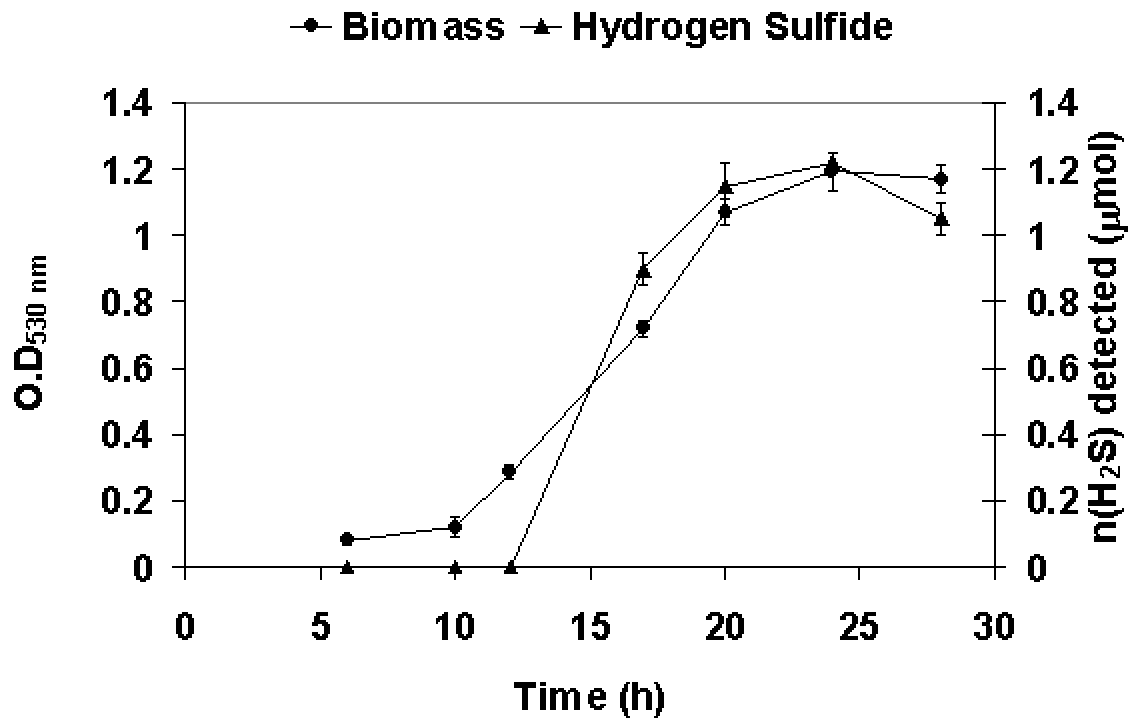


Figure 5. Hydrogen sulfide produced by *Tsukamurella paurometabola* DSM 20162 and Growth curve at 28°C. Levels of H<sub>2</sub>S were determined by the described method.