RESEARCH ARTICLE

Minibioreactor-gas collector for determining bacteria-produced hydrogen sulfide

Armando Hernández García*
Centro de Ingeniería Genética y Biotecnología de Camagüey
P.O. Box 387, Camagüey 70100, Cuba
Tel: 5332261014/5332261024
Fax: 5332261587
E-mail: armando.hernandez@cigbcam.cigb.edu.cu

Eulogio Pimentel Vázquez
Centro de Ingeniería Genética y Biotecnología de Camagüey
P.O. Box 387, Camagüey 70100, Cuba
Tel: 5332261014/5332261024
Fax: 5332261587
E-mail: eulogio.pimentel@cigb.edu.cu

Jesús Mena Campos
Centro de Ingeniería Genética y Biotecnología de Camagüey
P.O. Box 387, Camagüey 70100, Cuba
Tel: 5332261014/5332261024
Fax: 5332261587
E-mail: jesus.mena@cigb.edu.cu

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 µmol. The developed system was applied using *Tsukamurella paurometabola* DSM 20162 as a model, being the amount of hydrogen sulfide produced, 1.2 µmol 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_2S 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_2S 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

---

*Corresponding author

contained in the tube (gas collector), thus H_2S reacts with Pb^{2+}, producing lead sulfide; then the Pb^{2+} excess is determined by conductimetric titration with sodium phosphate.

MATERIALS AND METHODS

Development of the Analytical Method

H_2S standard preparation. In order to develop a method of analysis, a H_2S standard was prepared, from the reaction of 1 g of Na_2S (Merck) with 11 mL of 0.1 N H_2SO_4 (BDH), in a tightly closed 100 mL bottle. The bottle was sealed immediately after the Na_2S was added, then H_2SO_4 was injected into the bottle.

Method description. 10 µL, 100 µL, 300 µL, 500 µL, 700 µL and 1000 µL of the H_2S previously prepared standard were injected into different sealed bottles containing 2 mL of 0.090 mol/L $Pb(CH_3COO)_2$ (Merck) solution, which produced a black 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 Na_2HPO_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 µS/cm and 100 µS/cm respectively at 25ºC. 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 Pb^{2+} ion final concentration after reaction with H_2S, the amount of absorbed H_2S was indirectly determined, according to:

$n (H_2S)=V_i (Pb^{2+}) \Delta C (Pb^{2+})$

Where,

$n (H_2S)$: Amount of absorbed H_2S substance (µmol)

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

$\Delta C (Pb^{2+})$: 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 compunds, Column DB-5, Temperature 35ºC); 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 µm). 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 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 µL from *Tsukamurella paurometabola* DSM 20162 cell bank, cryopreserved at –70ºC 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 28ºC. 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 35ºC); 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 µL were taken for each determination, centrifuged at 10000 rpm during 10 min., and 300 µL 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 ± 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:

$$Pb(CH_3COO)_2 \text{ (aq.)} + H_2S \text{ (g)} \longrightarrow PbS \text{ (s)} + 2CH_3COOH \text{ (aq.)}$$
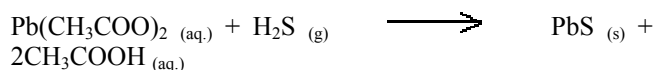
In the second stage, excess Pb^{2+} is determined conductimetrically by titration with a 4 mmol/L sodium hydrogenphosphate solution, via the reaction:

$$3Pb^{2+} \text{ (aq.)} + 2HPO_4^{2-} \text{ (aq.)} \longrightarrow Pb_3(PO_4)_2 \text{ (s)} + 2H^+ \text{ (aq.)}$$
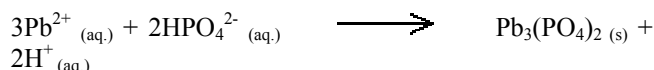
In this way, the hydrogen sulfide formed in the fermentation process is indirectly quantified.

The cnductimetric titration curve at 25ºC 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 H_2S standards injected into the recipients containing $Pb(CH3COO)_2$ 0.090 mol/L, and the amounts of detected substance, being the detection limit 0.5 µmol. 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 –SH group, reaction to Pb^{2+} ions could occur and consequently, 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 µmol (Figure 5). Taking into account the GC chromatogram (Figure 2B), around 10 % (0.09 µmol) of this amount could be associated to the unknown sulfur-containing gas. The detection limit of the developed method was 0.5 µmol, therefore it was not possible to detect the

amount of 0.09 μmol 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, grownin a culture medium containing cysteine 1.5 mM as a sole carbon source. Figure 5 shows the kinetic behaviour of the system, where minimum H_2S amount detected in the gas collector was 0.9 μmol at 17 hrs, and maximum amount was 1.2 μmol 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 μmol, which allows to determine the levels of hydrogen sulfide produced by such strains that produce small amounts of this gas.

REFERENCES

ACREE, T.E.; SONOFF, E. P. and SPLITTSTOESSER, D.F. Determination of hydrogen sulfide in fermentation broths containing sulfur dioxide. *Applied Microbiology*, 1971, vol. 22, no. 1, p.110-112.

AIKING, H.; KOK, K.; VAN HEERIKHUIZEN, H. and VAN´T RIET, J. Adaptation to cadmium by *Klebsiella aerogenes* growing in continuous culture proceeds mainly via formation of cadmium sulfide. *Applied and Environmental Microbiology*, 1982, vol. 44, p. 938-944.

AFSHAR, A.S.; VAZ ROSELL, C.E. and SCHALLER, K. Bacterial conversion of molasses to acetone and butanol. *Applied Microbiology and Biotechnology*, 1990, vol. 34, p. 168-171.

AYRES, G.H. *Quantitative Chemical Analysis*. 2nd ed. New York, Harper & Row Publishers, 1968, 710 p.

BARTON, L.L. and TOMEI, F. Characteristics and activities of sulfate-reducing bacteria. In: BARTON, L.L. ed. *Sulfate-reducing bacteria*. New York, Plenum Press, New York, 1995, p. 1-32. ISBN 0-306-44857-2.

CLINE, J.D. Spectrophotometric determination of hydrogen sulfide in natural waters. *Limnology and Oceanography*, 1969, vol. 14, p. 454-458.

COONEY, M.; ROSCHI, E.; MARISON, I.; COMMINELLIS, C. and VON STOCKAR, U. Physiologic studies with the sulfate-reducing bacterium *Desulfovibrio desulfuricans*: evaluation for use in a biofuel cell. *Enzyme and Microbial Technology*, 1996, vol. 18, no. 5, p. 358-365.

CHINIVASAGAM, H.N.; BREMMER, H.A.; WOOD, A.F. and NOTTINGHAM, S.M. Volatile components associated with bacterial spoilage of tropical prawns. *International Journal of Food Microbiology*, June 30, 1998, vol. 42, no. 1-2, p.45-55.

CHU, L.; EBERSOLE, J.L.; KURZBAN, G.P. and HOLT, S.C. Cystalysin, a 46-Kilodalton Cysteine Desulfhydrase from *Treponema denticola*, with Hemolytic and Hemoxidative Activities. *Infection and Immunity*, August 1997, vol. 65, no. 8, p. 3231-3238.

FORTIN, D.; SOUTHAM, G. and BEVERIDGE, T.J. Nickel sulfide, iron-nickel sulfide and iron sulfide precipitation by a newly isolated *Desulfotomaculum* species and its relation to nickel resistance. *FEMS Microbiology and Ecology*, 1994, vol. 14, no. 2, p. 121-132.

HEIDA, H.; BARTMAN, F. and VAN DER ZEE, S.C. Occupational exposure and indoor air quality monitoring in a composting facility. *American Industrial Hygiene Association Journal*, January 1995, vol. 56, no. 1, p. 39-43.

HOLMES, J.D.; RICHARDSON, D.J.; SAED, S.; EVANS-GOWING, R.; RUSELL, D.A. and SODEAU, J.R. Cadmium-specific formation of metal sulfide `Quantum particles´ by *Klebsiella pneumoniae. Microbiology*, 1997, vol. 143, no. 8, p. 2521-2530.

HUNTER, C.A. and CRECELIUS, H.G. Hydrogen sulfide studies: detection of hydrogen sulfide in cultures. *Journal of Bacteriology*, 1938, vol. 35, p. 185.

JIHO, M.; CHANG, W. L.; SEUNG-HYEUN, M.; LAROSSA, R. A. and MAN BOCK, G. Detection of radiation effects using recombinant bioluminescent *Escherichia coli* strains.*Radiation and Environmental Biophysics*, 2000, vol. 39, no. 1, p. 41-45.

KIM, S. and OLSON, N. Production of hydrogen sulfide in milkfat-coated microcapsules containing *Brevibacterium linens* and cysteine. *Journal of Microencapsulation*, October-December1989, vol. 6, no. 4, p. 501-513.

LEVINE, J.; ELLIS, C.; FURNE, J.; SPRINGFIELD, J. and LEVITT, M. Fecal hydrogen sulfide production in ulcerative colitis. *American Journal of Gastroenterology*, January 1998, vol. 93, no. 1, p. 83-87.

MAN BOCK, G.; BYOUNG, C. K.; JAEWEON, C. and HANSEN, P. D. The Continuous Monitoring of Field Water Samples With a novel multi-channel two-stage mini-bioreactor system.*Environmental Monitoring and Assessment*, 2001, vol. 70, no. 1-2, p. 71-81.

MAN BOCK, G.; GEUN CHEOL, G. and JOONG HYUN, K.. A two-stage minibioreactor system for continuous toxicity monitoring.*Biosensors and Bioelectronics*, 1999, vol. 14, no. 4, p. 355-361.

MESTRES, M.; MARTI, M. P.; BUSTO, O. and GUASCH, J. Simultaneous analysis of thiols, sulphides and disulphides in wine aroma by headspace solid-phase microextraction-gas chromatography. *Journal of Chromatography A*, July 16 1999, vol. 849, no. 1, p. 293-297.

PEYTON, B.M.; TRUEX, M.J.; TOTH, J.J. Biogenic sulfide precipitation of heavy metals to recycle electroplating and electronics manufacturing process streams. Richland, WA, Batelle, Pacific Northwest Division, 1995, PNWD-2315.

SIEGEL, L.M. A direct microdetermination for sulfide. *Analytical Biochemistry*, 1965, vol. 11, p. 126-132.

SMITH, A. Harnessing nature to clean up Doñana-scientists recommend a biological solution. *European Science Foundation*, Strasbourg, France, 2000.

TER LINDE, J.J.; LIANG, H.; DAVIS, R.W.; STEENSMA, H.Y.; VAN DIJKEN, J.P. and PRONK, J.T. Genome-wide transcriptional analysis of aerobic and anaerobic chemostat cultures of Saccharomyces cerevisiae.*Journal of Bacteriology*, December 1999, vol. 181, no. 24, p. 7409-7413.

WANG, C. L.; MARATUKULAM, PRIYA D.; LUM, A. M.; CLARK, D. S. and KEASLING, J.D. Metabolic engineering of an aerobic sulfate reduction pathway and its application to precipitation of cadmium on the cell surface.*Applied and Environmental Microbiology*, 2000, vol. 66, no. 10, p. 4497-4502.

WHITE, C. and GADD, G.M. Accumulation and effects of cadmium on sulfate-reducing bacterial biofilms. *Microbiology*, 1998, vol. 144, no. 5, p. 1407-1415.

WHITE, C. and GADD, G.M. Mixed sulphate-reducing bacterial cultures for bioprecipitation of toxic metals: factorial and response-surface analysis of the effects of dilution rate, sulphate and substrate concentration. *Microbiology*, 1996, vol. 142, no. 8, p. 2197-2205.

APPENDIX

Tables

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

Amount of H_2S Detected by iodometric method (μmol)	Amount of H_2S Detected by conductimetric method (μmol)	Confidence Interval for Conductimetric method (μmol)	t-value (Critical t-value[a] = 1.860)	f-value (Critical F-value[b] = 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

[a] The critical t-value with 95% confidence at 4-degrees of freedom.
[b] The critical F-value with 95% confidence at 4-degrees of freedom in numerator and denominator.
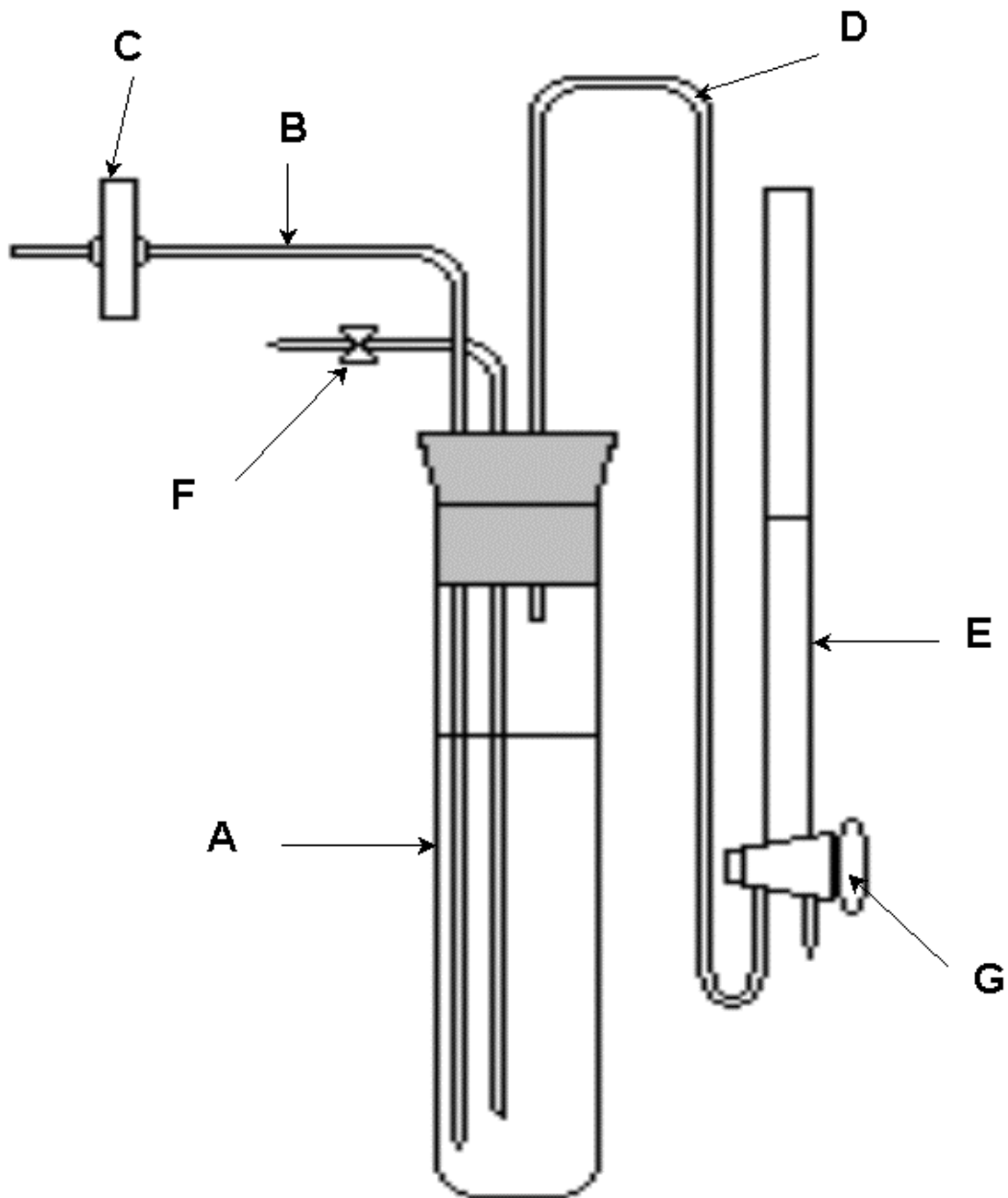
Figures

Figure 1. Minibioreactor-gas colector 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.
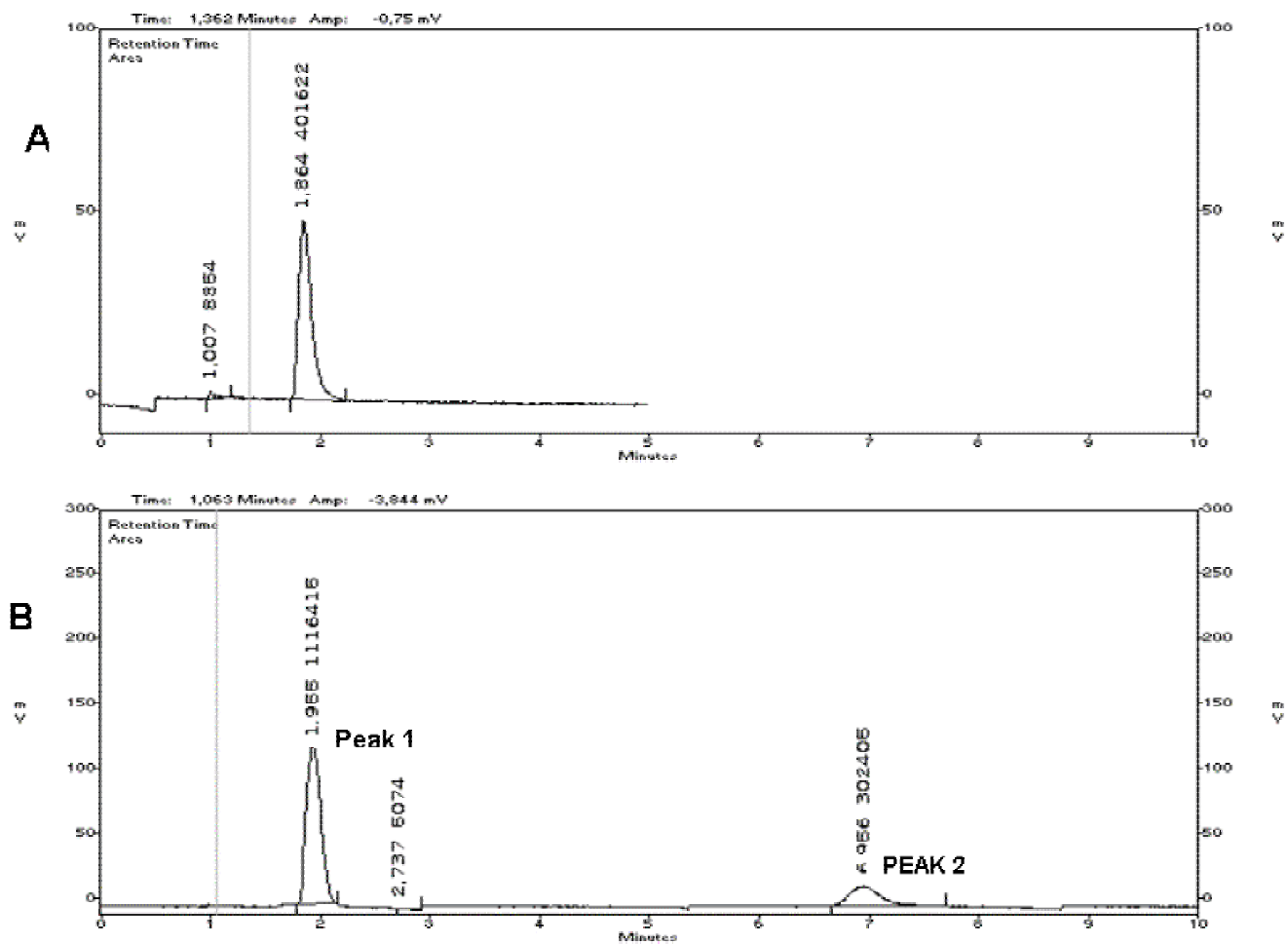
Figure 2. Gas Chromatography of samples for characterization of retention times and qualitative composition. (A) Hydrogen sulfide standard; (B) Running sample of fermentation gases taken at 17 hrs.
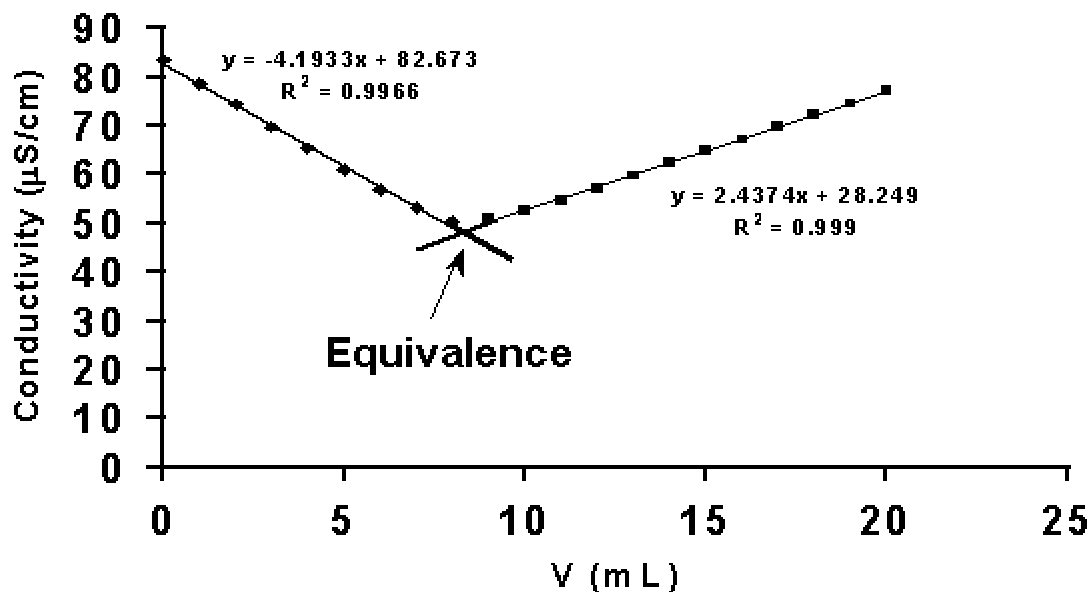
Figure 3. Conductimetric titration curve obtained while Pb^{2+} excess reacts with titrant Na$_2$HPO$_4$ to form Pb$_3$(PO$_4$)$_2$, at 25°C.
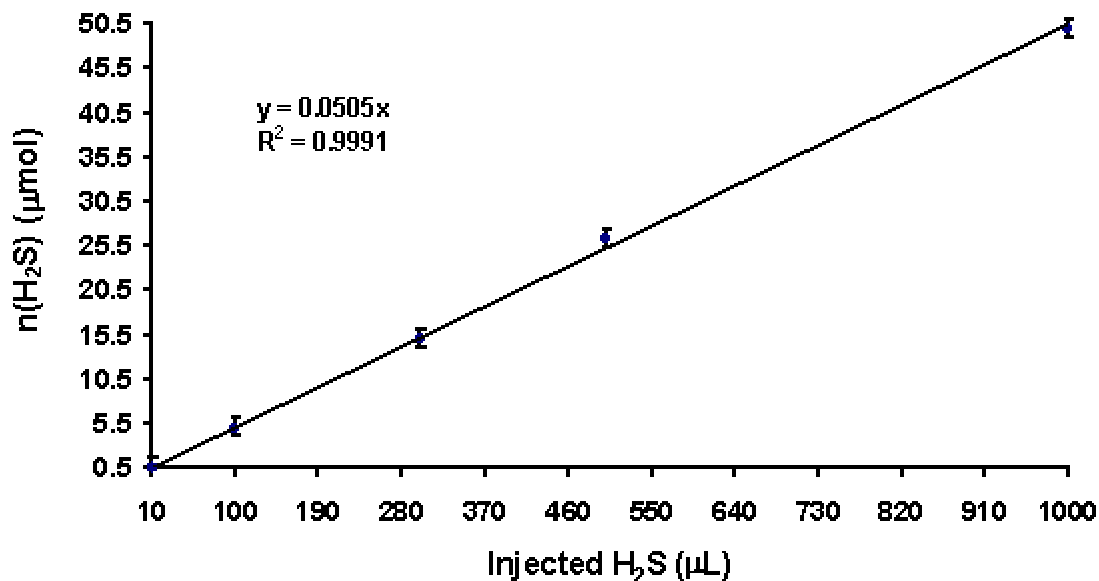

Figure 4. Correlation between volume of H$_2$S injected, and amount detected by conductimetric titration.
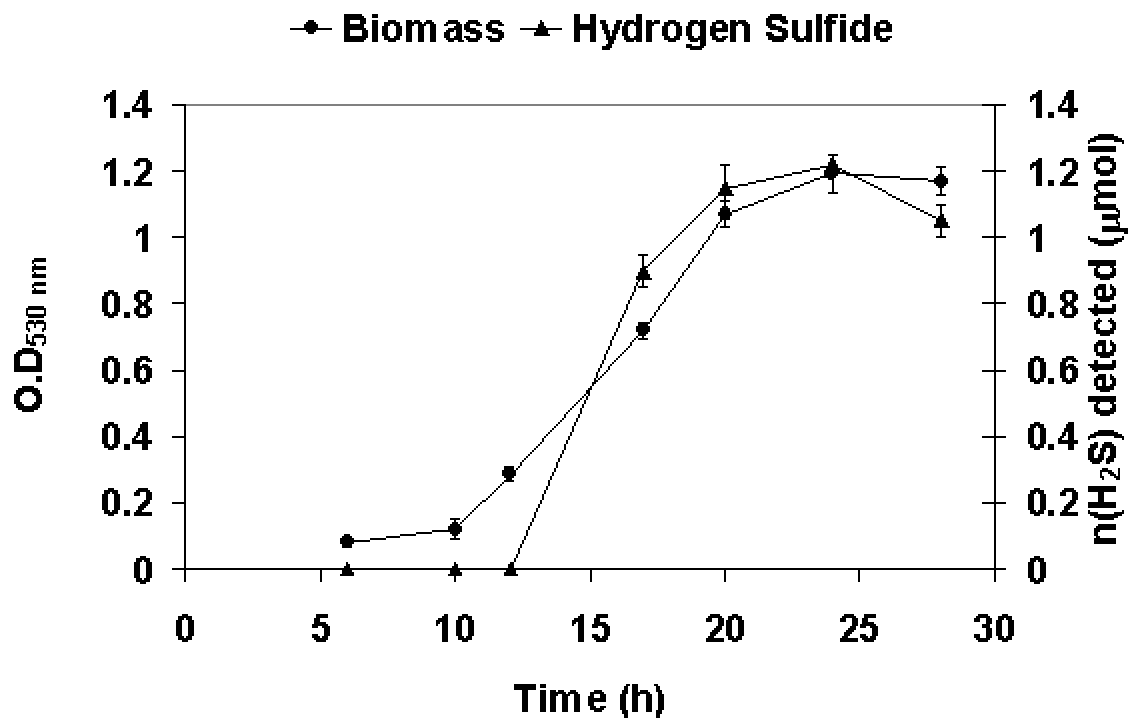
Figure 5. Hydrogen sulfide produced by *Tsukamurella paurometabola* DSM 20162 and Growth curve at 28ºC. Levels of H$_2$S were determined by the described method.