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Customized design of electronic noses placed on top of air-lift bioreactors for in situ monitoring the off-gas patterns

Pablo E. Rosi · Silvia A. Miscoria · Delia L. Bernik · R. Martín Negri

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Abstract A specially designed electronic nose was coupled to an air-lift bioreactor in order to perform on-line monitoring of released vapors. The sensor array was placed at the top of the bioreactor sensing the headspace in equilibrium with the evolving liquor at any time without the need of aspiration and pumping of gases into a separated sensor chamber. The device was applied to follow the off-gas of a bioreactor with *Acidithiobacillus thiooxidans* grown on beds of elemental sulfur under aerobic conditions. Evolution was monitored by acid titration, pH and optical density measurements. The electronic nose was capable to differentiate each day of reactor evolution since inoculation within periods marked off culture medium replacements using multivariate data analysis. Excellent discrimination was obtained indicating the potentiality for on-line monitoring in non-perturbed bioreactors. The prospects for electronic nose/bioreactor merging are valuable for whatever the bacterial strain or consortium used in terms of scent markers to monitor biochemical processes.

Keywords Electronic nose · Bioreactors · Sensor array · Gas monitoring · Non-invasive control · Real-time measurement

P. E. Rosi · S. A. Miscoria · D. L. Bernik · R. Martín Negri (✉)
LAMS, Departamento de Química Inorgánica, Analítica y Química Física. Instituto de Química Física de los Materiales, Medio Ambiente y Energía (INQUIMAE), Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, Pabellón II. INQUIMAE, C1428EGA Buenos Aires, Argentina
e-mail: rmn@qi.fcen.uba.ar

S. A. Miscoria
Departamento de Química, Facultad de Ciencias Naturales, Universidad Nacional de la Patagonia, San Juan Bosco, Argentina

Introduction

The monitoring of reactors at industry and at R&D laboratories using low-cost, highly sensitive and on-line methods of easy operation is one of the present challenges in the field of analytical instrumentation, remote control and automation. Optimization of processes in large-scale reactors applied to the synthesis, remediation, fermentation or even energy production must be achieved by implementing fast and reliable methods of monitoring instead of the traditional approach based on off-line extraction techniques and laboratory analysis [1].

Reactors using microorganisms has become relevant due to the permanent advances in genetic engineering which allow obtaining bacterial strains with wide spectra of catalytic abilities. Bacterial metabolism and digestion are complex processes which require stable operation conditions. The traditional approach for monitoring is based on sampling the system at given times and a subsequent analysis by means of different equipments in order to evaluate the process evolution and take decisions. This procedure is time consuming and involves human resources devoted to such regular controls. Therefore, industries and research laboratories demand a permanent renewing and improvement of methods for monitoring and automation of reactors in real time.

Within the spectrum of new techniques proposed for this purpose, the utilization of methods based on arrays of sensors is expanding fast, where electronic tongues (ET) and electronic noses (EN) using electrochemical, semiconductor or other solid-state sensors take the lead with some relevant examples. There are no previous reports using optical sensor arrays for monitoring bioreactor's headspace, although Bendriaa et al. [2] used a single photodetector for sensing the luminescence emitted from

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engineered bacteria in the bulk of a bioreactor for biomass monitoring purposes.

EN and ET are arrays of sensors coupled to multivariate data analysis methods for pattern recognition [3]. The application of electronic tongues (ET) for monitoring the bulk of a bioreactor is relatively advanced in comparison with the use of electronic noses and brings very valuable information regarding liquor composition [4–7].

However, in some cases, monitoring of volatile production patterns has great potential as a tool for early diagnosis of bioreactor's efficiency. For instance, anaerobic digesters require fast measurements of methane, carbon dioxide and volatile fatty acids, among other compounds [8, 9]. In this regard, electronic noses permit bioreactor control using real-time sampling methods with low instrumental cost.

Although there are some articles concerning the use of electronic noses for control of fermentation processes [10–13], the application for in situ monitoring of bioreactors is in its beginnings [14–16]. In all the cited studies, the same protocol was performed, consisting of sampling vapors from bioreactor's headspaces by means of aspiration and pumping with a carrier gas at a constant flow into an electronic nose chamber where sensors are placed, which in some cases is located some meters away from the body of the bioreactor [17].

Instead, we have developed a model of EN with different protocol of sampling based on placing the EN inside the bioreactor's structure but separated by a valve. Indeed, different EN models have been developed in our group and applied to the analysis of complex composition systems used in detection of pollutants, perfumery, food and agro sciences [18–26].

In this work we describe an alternative and distinctive methodology of using a gas sensor array: we specifically designed an electronic nose mounted on the top of the bioreactor directly sensing headspace gases. This type of gas detection uses the concept of static headspace monitoring. The difference is not subtle because the measurement gives direct information of the gases in equilibrium with the bioreactor liquor. The type of EN designed in this work is truly non-invasive in the sense that gives a pattern of headspace composition without altering the pre-existing balance. We called static EN, and it was developed in our laboratory and applied previously to other systems with particular success [23, 24, 26].

In the present case the studied system is an air-lift bioreactor inoculated with *Acidithiobacillus thioxidans* (AT), a chemolithotrophic bacteria used for biotechnological leaching and heavy metal bioremediation [27, 28]. AT biofilms can easily grow on a bed of tiny bits of elemental sulfur in air-lift bioreactors. The biofilm grows by oxidation of elemental sulfur to sulfate, in a process that increases the acidity of the medium and generates sulfur chains generically named as polythionates (S_nO_6, $n > 3$).

The specific objective is to explore whether this tailor-made EN device can discriminate the released gases along several days of bacterial growth, empathizing the evolution of the pattern developed in the headspace. The approach is based on processing the signals obtained from the sensor array with pattern recognition methods and can be applied to any other bioreactor, irrespective of the bacteria strain or consortia used. The whole set of signals is considered as a fingerprint of the system at a given time, whose changes can be recognized and discriminated using multivariate data analysis.

Materials and methods

Chemicals

K_2HPO_4, $(NH_4)_2SO_4$ and KCl were purchased from Anedra. $MgSO_4{\cdot}7H_2O$ was provided by Carlo Erba and $CaCl_2{\cdot}2H_2O$ was from Merck AG. All chemicals were reagent grade and used without further purification. Ultrapure water ($\rho = 18$ MΩ) from a Millipore-MilliQ system was used for preparing all the solutions.

Culture medium and bacterial cells

Acidithiobacillus thiooxidans (DSM11478) was from Minas Gerais (Brazil). The cell culture medium 0 K consisted of: $CaCl_2{\cdot}2H_2O$ 0.01 g/L, KCl 0.10 g/L, $MgSO_4{\cdot}7H_2O$ 0.50 g/L, K_2HPO_4 0.50 g/L and $(NH_4)_2SO_4$ 2.00 g/L, pH = 7.35 in ultrapure water.

Bioreactors

Two identical air-lift bioreactors were designed and built in glass (Fig. 1). The bioreactors consist of two concentric glass cylinders, the inner cylinder (IC) of 4.5 cm diameter and the external one (EC) of 9.0 cm diameter. The IC was filled up to 30.0 cm of elemental sulfur chips of 5 mm maximum length and it was fixed in its place by two specially designed plastic sieves. Air stream was injected from the bottom of the IC and the reactor was filled with 0 K and kept at 29 °C with an external heating pad.

One reactor was inoculated with pH ≤ 1.5 grown culture. Every time culture medium had to be replaced, it was drained from the bottom of the reactor. A 250-mL volume of old culture always remained and served itself as a new inoculum for fresh 1.5 L of medium 0 K poured till it covers 4 cm over the top sieve.

A control reactor was assembled under the same conditions but was not inoculated.

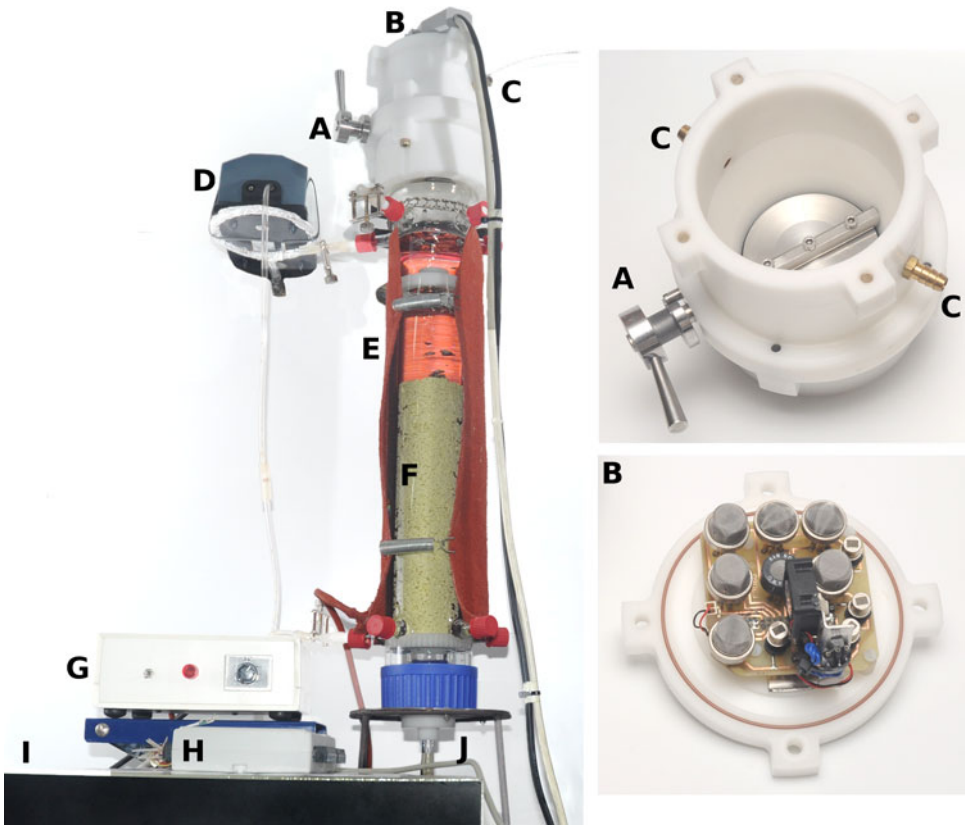

Fig. 1 Designed system, coupling an electronic nose (EN) to an air-lift bioreactor. *A* Butterfly valve to insulate gases in close position to the EN/Bioreactor adapter. *B* EN sensors and platform. *C* Pure air inlet. *D* Ambient air pump. *E* Heating blanket. *F* Sulfur chips. *G* Heating blanket control. *H* Data acquisition platform. *I* EN signal transferring module, where wires are connected to (not observed in the picture). *J* Bioreactor air inlet

Both the acidity and optical density were measured every day for samples taken from the bioreactors. Acidity was determined by titration with a 0.02 N NaOH using phenolphthalein as indicator. Optical density at 600 nm (OD600 nm) was recorded with a T60-PG Instrument spectrophotometer using 3 mL cells.

Electronic nose

The EN system designed for monitoring the release of gases from the bioreactor consist of three parts: (1) a multiple output voltage supply which provides different voltages and currents for sensor's heating and polarization, (2) a circular-plate with suitable dimensions in order to be placed at the top of the bioreactor as a support for the array of sensors and (3) a signal acquisition platform which acquires, digitalizes and stores the sensor's signals. The array of sensors platform and the multiple voltage-current sources were designed and implemented in our laboratory. A NI6218 DAQmx card (National Instruments) was used as a signal acquisition platform, the rest of the EN system was designed similarly to those previously designed parts in our group. An array of 11 conductimetric gas sensors was implemented, which signals are referred as S1, …, S11. The signals of each sensor are primary registered as voltages on loading resistances (the platform was designed with the possibility of easily selection of different loading resistance for the circuit of each sensor). The eleven gas sensors that compose the array (Si, Figaro Inc, Osaka, Japan) and the family of compounds to which its selectivity is enhanced are indicated as follows: S1: TGS826 (amines), S2: TGS825 (sulfur volatiles), S3: TGS2611 (hydrocarbons), S4: TGS2610 (hydrocarbons), S5: TGS813 (combustible gases), S6: TGS2620 (alcohols and solvent vapors), S7: TGS884 (alcohols), S8: TGS2611 (hydrocarbons), S9: TGS285 (sulfur volatiles), S10: TGS2620 (alcohols and solvent vapors), S11: TGS880 (organic vapors).

Sensors were heated following the current suggestions of the manufacturers (Figaro Inc). A 5Vdc was applied to supply the sensors, each one connected in series with a respective loading resistance, over which the voltage drops were registered and digitalized to obtain the group of signals S_1, …, S_{11} in each measurement. Data analysis (see "Multivariate data analysis: principal components analysis (PCA) and cluster analysis (CA)") was performed using the described not only voltages as inputs but also the conductance of the sensors in all cases. No differences were observed, that is, discrimination was not improved by converting voltage into conductance.

The assembly of the EN at the top of the bioreactor was implemented as shown in Fig. 1. The sensor plate can be

Table 1 Cluster analysis (CA)

Period	Days associated to the different periods	ASW	% of right assignations (hits)
A	1–5	0.62	97
B	22–26	0.65	100
C	43–50	0.66	100
D	50–54	0.73	100

Discrimination between inoculated and non-inoculated bioreactors in different periods. Input data are the signals of the sensors which composes the electronic nose. The number of clusters is $k = 2$ (inoculated and non-inoculated)

isolated from the bioreactor headspace using a butterfly valve which delimits sensors' area. The EN design has a lateral inlet which allows a fast cleaning of the sensors by means of a flow of pure synthetic air in order to set a baseline previous to the measurements. This design with the butterfly valve is particularly appropriated for continuous monitoring preventing the system components from potential corrosion by the bioreactor gases. A continuous flow of air (air stream) is continuously supplied from the bottom of the bioreactor, which is air-saturated. The headspace atmosphere sensed by the E-Nose is a mixture of the gas spontaneously released by the bacteria during its metabolism (mainly volatile sulfur gases) and the supplied air. The air flux is high enough to cover the microorganisms' demand and be in sufficient excess to ensure a proper functioning of the sensors along the measurement period (5 min). After measurements, the sensor's chamber is isolated from the bioreactor by closing by the butterfly valve and cleaned by passing a flow synthetic air over the surface of the sensors until the sensor's signals recover the original baseline values. In this manner the sensors are maintained clean and no poisoning was observed at least for a period of 1 year. No drift of the signals was observed within the periods of 5–7 days as those indicated in Tables 1 and 2 and Fig. 5.

When the device is programmed to record signals, synthetic air passes through until recovering the original baseline, as mentioned. Then air stream is stopped, the butterfly valve is opened and the system is left to evolve freely for 5 min in order to ensure signals became stable to be recorded. Each record consisted of a set of 11 independent values. This procedure was followed each day by triplicate.

Table 2 Cluster analysis intra-period

Period	Days	Clusters	ASW	% of right assignations (hits)
A	1–5	5	0.57	93
B	22–26	5	0.47	80
C	43–50	6	0.42	72
D	50–54	5	0.42	60

Multivariate data analysis: principal components analysis (PCA) and cluster analysis (CA)

Two unsupervised methods, PCA and CA, which are the most commonly used methods for analyzing sensors' array data, were performed [29, 30]. In PCA, each measurement is considered as an N-dimensional vector, where here N is the number of sensors (11 in our case) of the array and which components in a canonical base are the sensors' signals. The projections of these vectors into the orthonormal base defined by the directions of the maxima data variance are referred as the so-called principal components. Frequently, the projections into the first two or three directions of the new base of the measurement space contains more than 90% of the total data variance, thus investigation of these reduced subset of components provides a substantial dimensional reduction, extract the relevant information for variance analysis and improves the ability for grouping data for discrimination purposes. Hence, the results of PCA are 2D or 3D plots, commonly referred as PCA maps or score plots, representing the relevant principal components for each measurement. The samples are grouped by similarities in these PCA maps and groups are observed by visual inspection. The criterion for deciding to represent 2D or 3D PCA plots is to account no less than 95% of the data variance in the PCA map.

Cluster analysis is also an unsupervised method [30]. A difference with respect to PCA is the ability to separate the data information held in each sample in a number of groups or "clusters". CA can be performed using different algorithms being the partitioning around medoids (PAM), the one used in the present work [31]. CA-PAM is based on searching k representative objects among the data set ($k =$ number of target clusters) called medoids. The clusters are calculated such that the total distance between all elements and their nearest medoid is minimal. Each element is then assigned to the cluster corresponding to the nearest medoid. Therefore, PAM indicates which data input are the clusters composed by; hence the number of correctly grouped samples can be determined. Thus, the parameter "% of hits" (percentage of correct assignations) is indicated in CA. Additionally, CA-PAM provides also the so-called Average Silhouette Width (ASW). Following the criteria for the goodness of the clusterization indicated by Struyf et al. [30] the ASW must be >0.25 for a good assignation and clusterization is perfect when ASW is equal to one; the higher the ASW the better the fit.

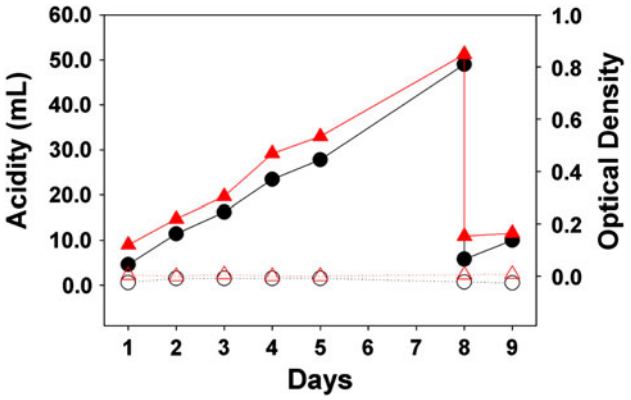

Fig. 2 Variation of acidity (measured as mL of 0.02 N NaOH solution) (*circles*) and optical density (*triangles*) along one representative period. *Filled symbols* inoculated reactor. *Hollow symbols* non-inoculated

Results and discussion

Acidithibacillus thiooxidans growth in the bioreactor

We defined a period as a set of consecutive days in which the reactor evolved with the same medium culture. A period finishes when the culture media is partially replaced by fresh 0 K culture. The whole experiment covered 64 days gathered in 9 periods. Both acidity and optical density at 600 nm (OD600 nm) increased daily within every period for the inoculated bioreactor, while no changes were observed for the non-inoculated bioreactor. This behavior was observed in all the periods and it is illustrated in Fig. 2. Acidity and optical density increased in a highly correlated way in the inoculated reactor, indicating increasing sulfuric acid concentration and bacterial growth in each period. The sudden changes in acidity and optical density observed after 5 days in Fig. 2 correspond to medium culture change. The saw tooth pattern repeated after medium culture change along 9 periods.

Comparison of the electronic-nose response between inoculated and non-inoculated bioreactors

Electronic-nose (EN) experiments were performed in two identical bioreactors, one inoculated with AT and other non-inoculated. Four representative periods were chosen to study. Figure 3 shows PCA results when considering as inputs all the measurements performed in both bioreactors for two selected periods (an early period, Fig. 3a, and a late period, Fig. 3b). It is clearly observed by visual inspection in these PCA maps, that two groups of data points are well separated coinciding with the inoculated and non-inoculated bioreactors. CA for the four periods was performed in order to quantitatively confirm the excellent grouping obtained by visual inspection in PCA (Table 1). In fact, about 100% of correctly clusterized samples were obtained by CA (the percentage depends on the period, see Table1). This means that, for each period, almost all of the EN measurements for the inoculated bioreactor were grouped (clusterized) into the same cluster, and vice versa for the non-inoculated bioreactor. The ASW was higher than 0.6 in each period, confirming the excellent goodness of the clusterization.

These results demonstrate that the EN is sensing the gases released during the bacterial growing showing that the system can perfectly discriminate between inoculated and non-inoculated bioreactors.

Electronic-nose response for the inoculated reactor

Figure 4a shows the sensors' signals of the EN for the inoculated bioreactor in different days of the first period of 5 days (inoculation was done at day 1). Figure 4b, c and d show radar plots for different periods, respectively. In these plots, each vertex represents a sensor of the array. Only four sensors and 3 days for each period are considered, (signals of sensors 7, 9, 10 and 11, which values were relative to the signal of sensor 8, just for clarity). It is observed in each figure how the signals of the four sensors increase from day 1 to day 5 in the corresponding period;

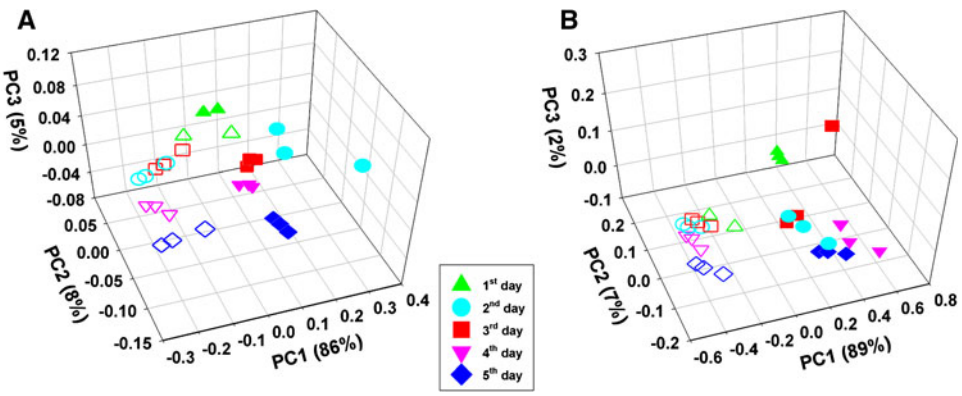

Fig. 3 Electronic nose PCA maps (3D) including measurements for non-inoculated (*hollow symbols*) and inoculated (*filled symbols*) bioreactors. **a** For an early period (days 1–5). **b** for a late period (days 50–54)

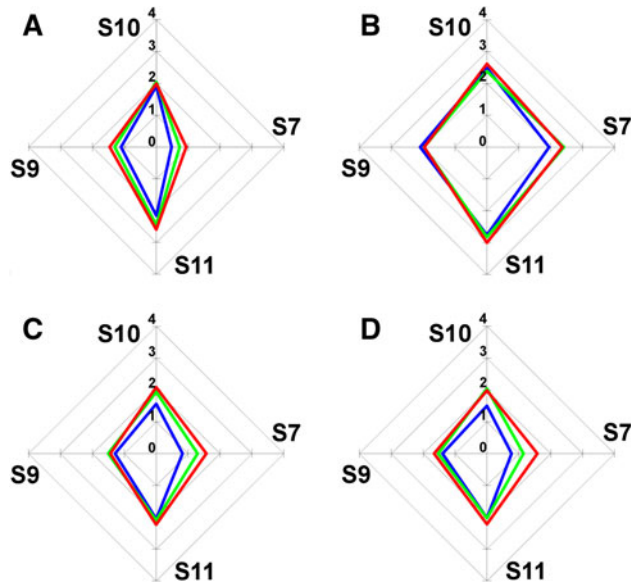

Fig. 4 Electronic nose (EN) gas sensors signals during AT growth in the inoculated bioreactor. **a** Radar plot for a given period associated to four selected sensors of the EN (arbitrary divided by the signal of sensor 8), where each vertex is associated to a sensor, and each contour line correspond to measurements performed in a given day of the period A (see Tables 1 and 2) *inner line* day 1; *middle line* day 3; *outer line* day 5. **b**, **c** and **d** the same than in **a** but for the periods B, C and D indicated in the tables, respectively. **b** *Inner line* day 22; *middle line* day 24; *outer line* day 26. **c** *Inner line* day 43; *middle line* day 45; *outer line* day 47. **d** *Inner line* day 50; *middle line* day 52; *outer line* day 54. *Inner line*: *blue*; *middle line*: *green*; *outer line*: *red* (color figure online)

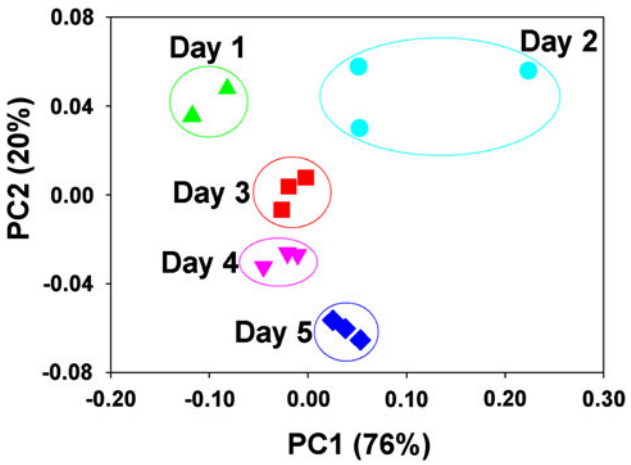

Fig. 5 Electronic nose PCA map (2D) for measurements with the inoculated bioreactor through five consecutive days of the early period (days 1–5)

this indicates an increasing generation of volatiles through days. In addition, the "shape" of these radar plots shows slight variations through the days in the periods (radars are not perfectly concentric), this fact indicates a change in the compounds pattern distribution in addition to the odor intensity variation. These variations are recognized by the statistic methods used (PCA and CA), driving to discrimination among days within each period. When comparing Fig. 4b, c, and d, it is observed that differences among the sensors' signals diminish in correlation with the bioreactor aging.

CA was performed in different periods in order to quantify the goodness of clusterization among days; non-normalized signals were used in order to take into account changes in odor intensity. The number of clusters k (see "Multivariate data analysis: principal components analysis (PCA) and cluster analysis (CA)") was the number of days considering in each period (usually $k = 5$). The results, shown in Table 2, indicate an excellent discrimination among days was obtained for the first period (93% of hits). This excellent discrimination was also observed in the 2D-PCA plot (Fig. 5). The percentage of right cluster assignations (hits) decreases systematically when considering more advanced periods, that is, when the bacterial system grows and stabilizes, in agreement with observations noted when discussing Fig. 4b, c, and d. Also in concordance, ASW follows the same tendency as shown in Table 2, it must be noted that ASW <0.6 in this case indicating data dispersion, a non-surprising result considering the complexity of the biological system under study.

Electronic nose coupled to bioreactors, advantages and perspectives

The results discussed in previous sections show that the implemented EN is able to clearly discriminate among days along the whole bioreactor operation. The discrimination is statistically better for early periods when the bacterial colonies are growing fast probably due to progressive colonization of the sulfur chips, in agreement with the expected evolution of the bioreactor.

In previous reports other type of electronic noses were used to follow up bioreactor's evolution. Nevertheless, it is worth to note that in those works the sampling was done by aspiration of the off-gas mixture from bioreactor's headspace: the gases were extracted from the bioreactor chamber and pumped with a reference gas carrier to an external EN chamber [32]. This action produces a perturbation of the system until the equilibrium of the headspace gases with the underlying liquid is restored. Therefore, the interaction of these types of EN with bioreactors is not strictly non-invasive. Instead, the EN designed in this work named "static ENose" measures the off-gases without altering equilibrium. After measurement, the butterfly valve is closed and the sensors are cleaned by a flux of pure air getting ready for the next measurement. This can be an advantage when some target off-gases need to be regularly detected in, for example, anaerobic bioreactors [33, 34].

There is another relevant characteristic of this type of "static" EN: during the measurements the gases are in contact with the gas sensors for periods of minutes until sensors signals reach a plateau. With this procedure a steady plateau of every sensor signal is obtained at early times and, at equilibrium, produce a different fingerprint pattern than the one obtained with EN based on aspiration and pumping with a carrier gas passing through a sensor chamber (manuscript in preparation), procedure which yields an on–off sensor response kinetics [32].

Finally, considering that nowadays bioengineered bacteria are itself real "whole cell biosensors" able to produce a scent as a response to an elicited stimulus (Fernández and Bernik [35] and references therein), the perspectives of this static EN fused in a closed bioreactor setup allows to work both aerobically or anaerobically in bioremediation processes, in addition to any other industrial or research application with reactors, fermenters, etc., expanding enormously the potential industrial and environmental applications.

Conclusions

The coupled electronic nose/bioreactor system was able to monitor the evolution of gases released from *Acidithiobacillus thiooxidans* (AT) supported on sulfur chips, demonstrating the usefulness of the system following up the maturation of air-lift bioreactors. The methodology of detecting the released gases by placing an array of gas sensors as an integrated part of bioreactors avoiding aspiration of the vapors to an external chamber had not been previously reported and constitutes an innovation in the area of on-line sensor analysis. It opens a wide perspective in helping the early detection of reactors departure from normal working operation, thus allowing fast decision-making.

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