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Experimental development of a biosensor system to measure the concentration of phenol diluted in water using alternative sources of oxidoreductase enzymes

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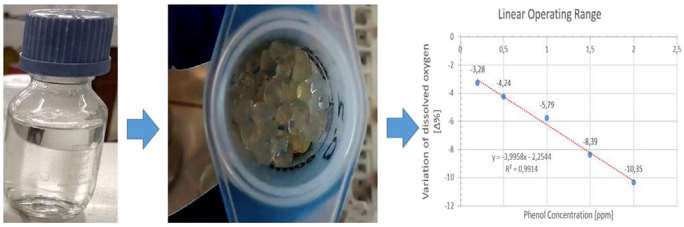
HIGHLIGHTS

- A novel biosensor system for in-situ measurement of phenol concentration in water.
- Numerous sources of oxidoreductase enzyme for the biosensor were evaluated.
- The immobilization technique used allows to the sensor taking various measurements.

GRAPHICAL ABSTRACT



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ABSTRACT

The presence of phenol in industrial wastewater is an issue of great relevance for petrochemical and energy companies, among others. The high toxicity level of this substance requires polluting industries to continuously monitor the concentration of phenol in their wastewaters so as to comply with environmental regulations and to minimize environmental impact. This research work proposes the experimental development of an analytical method for "in situ" measurement of the concentration of phenol diluted in water, with application in Oil & Gas production wastewater monitoring. The method is based on the principle of selectivity exhibited by the oxidoreductase enzymes in the presence of phenolic compounds. The differences in the performance found when using organic tissues and microorganisms, as natural alternative sources of the enzyme, are also highlighted. These alternative sources of oxidoreductase enzyme work as the recognition element of the biosensor. A dissolved oxygen sensor is used as the transducer of the chemical signal produced by the reaction between the analyte and the enzyme. The bioencapsulation technique is very adequate in this case, because it offers a nutrient medium to the microorganisms and is reusable, making it ideal for repetitive measurement applications. The results show that the biosensor exhibits an approximately linear behavior when measuring phenol concentrations from 0.2 to 2 ppm.

1. Introduction

About 40 million dollars per year are spend worldwide to

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confront the problems that arise from the disposal of their wastewaters [1]. In the case of the Oil & Gas industry, as oil wells have become more mature and depleted, water consumption in the extraction process has increased exponentially, which has a strong impact on the life quality of the communities located closer to the oil fields and on the environment in general. In fact, the oil industry globally employs an average of three barrels of water per barrel of extracted oil [1].

The water used in the oil extraction process is commonly referred to as production water. Production water contains different pollutant compounds such as hydrocarbons, oils, suspended solids, grease and phenol, among others. Permissible limit concentration values (for Oil industry) of these pollutants are shown in Table 1 [2].

Among the pollutants of wastewaters from the Oil & Gas industry presented in Table 1, phenol is of great concern to environmental authorities, due to its high toxicity and harmfulness to both humans and the environment [3].

Some of the traditional methods currently available for measuring the content of phenol in industrial wastewater include gas chromatography and spectrophotometry [4]. The process to measure phenol by means gas chromatography combined with mass-spectrometry uses liquid liquid extraction of this analyte with dichloromethane from water at pH 2, as pre-treatment [5]. This pre-treated solution is purified with gel permeation chromatography, in order to remove benzoic acids and other compounds, which can add noise to measurement [5]. Finally, the chromatograms are recorded in a selected ion monitoring mode for the analysis [5]. On the other hand, the operating principle of the spectrophotometry technique is based on colorimetry, in which the substance 4-aminoantipyrine produces a change in the color of the sample after its reaction with phenol takes place, this change can be detected by the spectrophotometer, due to the wavelength [6].

The use of these techniques is common practice in industry due to their high sensitivity, selectivity and precision; however, they do not permit on-site determination of the concentration of phenol in industrial wastewater.

In addition, these traditional techniques require long sample residence times and trained staff to carry out the measurement and data analysis, which significantly increases the cost and time required for pollutant concentration verification in an industrial process.

In response to the time and financial constraints imposed by the traditional methods available for phenol concentration measurement, new technologies such as biosensors have emerged, with the goal of providing alternative methods for measuring the concentration of many contaminating agents.

According to the International Union of Pure and Applied Chemistry [7], biosensors are analytical systems that incorporate a biological material (such as isolated enzymes, antibodies or tissues, which act as element of recognition), which exhibit great selective properties with respect to the compound of interest, and a transducer capable of turning the biochemistry interaction between the analyte and the element of recognition, into a quantifiable electrical, thermal or optical signal. In this way, on-site monitoring of various types of substances can be achieved.

Table 1
Maximum permissible concentration values of wastewater pollutants for Oil & Gas industry [2].

Parameter	Value (mg/L)
Oils	15,00
Grease	15,00
Total Suspended solids	50
Phenols	0,20
Total hydrocarbons	10,00

The diagram in Fig. 1 presents the typical elements of a biosensor.

The development of biosensor systems for phenol measurement has taken great relevance in recent years. They are based on the principle of the catalytic properties of some specific enzymes. The reactive state (Eox) of the enzyme is reduced to another form (Ered) by a phenol molecule [9]. The enzyme is regenerated by oxygen. This phenomenon can be detected by electrochemical principles, monitoring the consumption of the regenerating agent or the reaction product such as a free radical (quinone) [9], as Fig. 2 shows. Most common enzymes used are: laccase [10-12]or tyrosinase (also known as catechol oxidase or phenolase) [13-15]. These enzymes have been chosen for this application due to their ability to catalyze the oxidation process of phenol in the presence of molecular oxygen.

Table 2 shows the detection limit of biosensor systems reported in the literature, for phenol detection using tyrosinase and laccase enzymes.

The phenolase enzyme, which is responsible for the process of enzymatic browning [22], can be found in several natural sources such as potatoes, apples, mushrooms, bananas, and pears, among others.

The oxidation reaction of phenol, catalyzed by the phenolase enzyme, is presented in Fig. 2.

Otherwise, microorganisms biosensors for the detection of phenol have been developed [24-26] using *Trichosporo cutaneum*, *Rhodococcus spec.* and *Pseudomonas putida*, respectively. Furthermore, tissue based sensors for the same application have been reported in previous works using *Agaricus bisporus* [27], *Musa cavendish* [28] and *Helianthus tuberosus* [29,30].

Likewise, biosensors for phenol measurement have been developed using different transduction technologies, such as optics, which is based on the interaction between the enzyme and phenol, which generates a change in the pigmentation of the solution [31].

Other transduction technologies commonly used in biosensors for phenol measurement are the amperometric [27,32] and the electrochemical [33,34] techniques. Furthermore, phenol concentration have also been measured using a bioelectronics nose [35,36].

In this research work, the concentration of phenol in water is determined using organic tissues and microorganisms as natural sources of the oxidoreductase enzymes. A dissolved oxygen sensor is used as the transducer, based on the relationship between the oxidation of phenol, and the subsequent variation of dissolved oxygen that takes place during the reaction between the phenolase enzyme and the dissolved phenol in a sample [27,30]. Additionally,

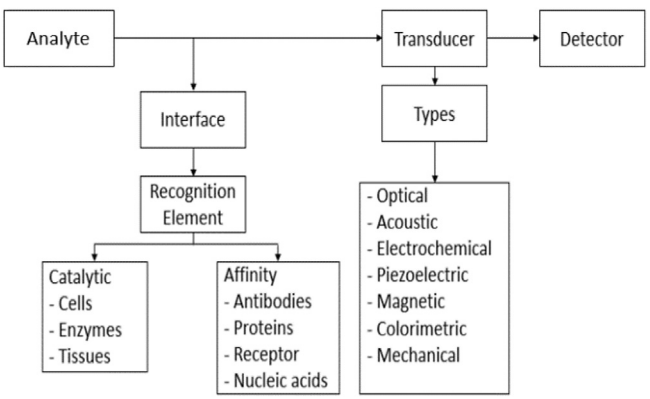

Fig. 1. Diagram of a biosensor. Adapted from Ref. [8].

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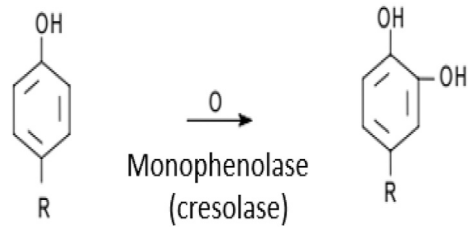

Fig. 2. Reaction of monophenol to phenolase enzyme [23].

Table 2
Detection limit of phenol biosensors based on oxidoreductase enzymes.

Enzyme	Detection Limit
Tyrosinase [16]	0.05 ppm
Tyrosinase [17]	0.0098 nM
Tyrosinase [18]	23.5 ppm
Laccase [19]	0.32 ppm
Laccase [20]	98 ppm
Laccase [21]	0.049 ppm

the feasibility of using different sources for the element of recognition is assessed, seeking to reduce the reaction time, and to improve the quality of the transducer in terms of selectivity and biosensor accuracy.

2. Materials and methods

This section presents the methodology that was followed to identify the most appropriate source of the tyrosinase enzyme for the development of a phenol biosensor. Likewise, the conditions and procedure of the experiments are described, which lead to the determination of the operating conditions of the biosensor system and its further construction.

2.1. Recognition element

The recognition element is responsible for chemically reacting with phenol to catalyze its oxidation process. Two types of alternative sources of the tyrosinase enzyme were considered: organic tissues and microorganisms.

The details of the two types of alternative sources of the tyrosinase enzyme selected are presented next.

2.1.1. Organic tissue

The organic tissues that were used as alternative sources for the phenolase enzyme were mushrooms, potatoes and apples. Samples of these tissues were cut in pieces of approximately 5 mm wide, to be used in subsequent steps of the methodology, as described in the following sections.

Agaricus bisporus, better known as mushrooms, was used based on the results from previous studies, which have demonstrated not only the presence of the tyrosinase enzyme in this organic tissue, but also its active participation in the reaction with phenol compounds. This phenomenon causes the enzymatic browning of the fungus. The last one occurs when the tissue is bruised, cut or exposed to any abnormal conditions, so it rapidly darkens on exposure to air as a result of the conversion of phenolic compounds to brown melanins [22,37,38].

Similarly, previous research have also revealed the presence of the tyrosinase enzyme in potatoes [39,40] and apples [41,42].

2.1.2. Microorganisms

The microorganisms tested in this investigation were *Saccharomyces cerevisiae* and *Aspergillus niger*.

Saccharomyces cerevisiae is a unicellular fungus that is industrially used in the production of bread, beer and wine, with a fast growth rate and easy adaptability [43].

Aspergillus niger, is a fungus that produces black mold on vegetables; very common in lettuce, tomato orchard and lemon [44].

When using microorganisms, a process of search, isolation, adaptation and microencapsulation is required to choose the recognition element with the best features for the application under study. For this reason, microbes with the capability to produce the enzyme, and to adapt in a medium containing phenol, were selected. Each of these stages is explained in detail in the Appendix section.

2.2. Experiment setup and measurement procedure

The measurement procedure consists primarily in letting the microorganism react with a phenol diluted solution for a predetermined time, recording and monitoring for reference purposes the amount of dissolved oxygen before and after the reaction. At the same time, the optimal time for the enzyme to perform its oxidation process is sought in these experiments. The conditions under which the measurements were carried out are presented next.

2.2.1. Reactor

The reactor is a cylindrical-shape device, as seen in Fig. 3, with a small opening at the bottom and a larger one on the top, through which the solution is poured in. In this vessel, the recognition material reacts with the sample (distilled water - Phenol) for a given period of time. The products of this reaction are poured into a distillation flask to perform the dissolved oxygen measurement.

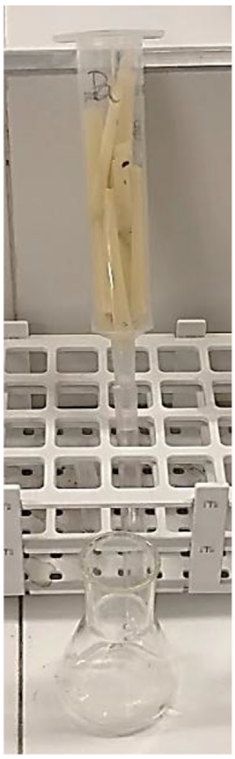

Fig. 3. Reactor with potato.

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2.2.2. Reaction time and number of repetitions

To determine the optimum reaction time, the experiment was performed during three different time intervals: 1, 3 and 5 min, respectively. Each test sample was run three times.

2.2.3. Phenol concentration

Only for exploration purposes, three phenol concentration levels were initially tested: 0.2, 1 and 2 ppm, respectively. These trial tests were aimed at evaluating the performance of the recognition elements to avoid unnecessary measurements later. These levels were selected taking into account maximum allowable limits of phenol concentration in industrial wastewater [2] and in drinking water [3].

2.2.4. Temperature

To standardize the initial conditions in all experiments, each test started with an average room temperature equal to 25 °C (77 °F). The tests were conducted in a chemical laboratory environment with automatic temperature control.

2.2.5. Amounts of recognition elements

The amount of recognition element was determined based on the total volume of the reactor, with the goal of ensuring the largest surface area for contact between the recognition element and the analyte during the reaction. The specific amounts of recognition elements can be observed in Table 3 and Fig. 4.

As for the amount of analyte, the entire volume of the reaction tube was used, which was about 40 ml of solution sample.

2.3. Transducer

The phenol oxidation catalysis and the amount of dissolved oxygen in the mixture have exhibited an approximately linear correlation in previous works [27,30], for a measurement range from 5 to 10 ppm and 0.002 to 0.010 μM, respectively. Thus, it was determined that the most suitable transducer for the determination of the phenol concentration is a dissolved oxygen sensor. Although it does not directly provide values of the concentration of the substance of interest, this kind of sensor is highly sensitive to variations in the reaction parameter of interest.

3. Results

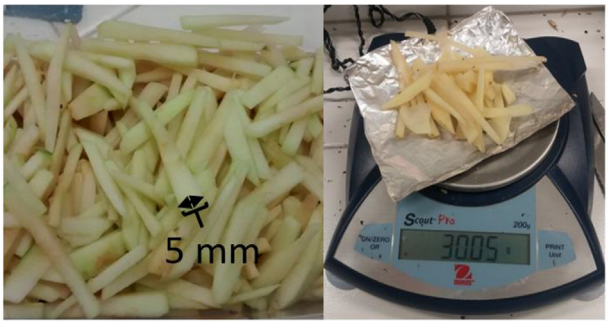
This section presents the results obtained after performing the above mentioned methodology, using the two types of recognition elements: organic tissue and microorganisms. These results serve as basis criterion to select the most suitable recognition element for the biosensor system under development.

The mean and standard deviation of the variation of dissolved oxygen (dissolved oxygen before and after the reaction) were determined, with the objective of evaluating the experiment repeatability.

In addition, the average of the standard deviation of the variation of dissolved oxygen at each time is calculated to estimate the best reaction time for the biosensor.

Table 3
Amounts of recognition elements.

Biological material	Amount
Saccharomyces cerevisiae	40 g
Aspergillus niger	40 g
Mushrooms *(Agaricus bisporus)*	15 g (Cuts 5 mm wide)
Potatoes (*Solanum tuberosum)*	30 g (Cuts 5 mm wide)
Apples (*Malus domestica)*	25 g (Cuts 5 mm wide)



(a) (b)

Fig. 4. (a) Wide of the apple used (b) Weight of the pieces of potatoes used.

3.1. Organic tissue

The collected data for the three different kinds of organic tissues used in this work are shown next.

3.1.1. Agaricus bisporus (mushrooms)

The results obtained for Agaricus bisporus are presented in Table 4.

The above table shows that the standard deviation for each of the reaction times and concentrations is high in comparison to the recorded variation of dissolved oxygen. Therefore, *Agaricus bisporus* is not a suitable biological material for the measurement of phenol concentration due to its low repeatability for the testing conditions considered in this study.

3.1.2. Solanum tuberosum (potatoes)

Table 5 shows the collected data for *Solanum tuberosum.*

From the results in Table 5, it can be observed that the mean of the standard deviations for each one of the reaction times has a relatively low value, which means that this experiment has better repeatability, as compared to the results with *Agaricus bisporus*, making the *Solanum tuberosum* a possible candidate to choose from. In addition, the percentage variation values of dissolved oxygen, for different concentrations, at 5 min, are more distanced among each other. That is to say, it exists a greater difference in the variation of dissolved oxygen between concentrations, which suggests that 5 min is the most suitable reaction time with this biological material as recognition element.

3.1.3. Malus domestica (apples)

The results obtained for Malus domestica are presented in Table 6.

The previous table shows that the standard deviation for each of the reaction times, with different phenol concentrations, has a relatively high value, as compared with the generated variation of dissolved oxygen. This means that using *Malus domestica* as biological material for the biosensor would not be adequate due to its low repeatability.

3.2. Microorganisms

The experimental results for the two different types of microorganisms (*Saccharomyces cerevisiae* and *Aspergillus niger*) used in this investigation are shown below.

3.2.1. Saccharomyces cerevisiae

Table 7 shows the obtained data for *Saccharomyces cerevisiae*.

The results in Table 7 reveal that the average of the standard

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Table 4
Average variation of dissolved oxygen percentage obtained using *Agaricus bisporus.*

Reaction Time (min)	Phenol Concentration (ppm)			Average Deviation
	0,2	1	2	
1	−3,03 ± 1,78	−3,00 ± 1,54	−2,23 ± 1,53	1,62
3	−10,70 ± 1,22	−14,27 ± 2,57	−17,00 ± 5,46	3,08
5	−14,27 ± 1,01	−17,60 ± 7,86	−21,00 ± 2,52	3,80

Table 5
Average variation of dissolved oxygen percentage obtained using *Solanum tuberosum.*

Reaction Time (min)	Phenol Concentration (ppm)			Average Deviation
	0,2	1	2	
1	−3,33 ± 0,97	−2,67 ± 1,72	−2,60 ± 0,26	0,99
3	−6,83 ± 1,26	−7,07 ± 1,52	−7,97 ± 1,02	1,27
5	−13,80 ± 1,28	−12,00 ± 1,65	−10,70 ± 1,31	1,41

Table 6
Average variation of dissolved oxygen percentage obtained using *Malus Domestica.*

Reaction Time (min)	Phenol Concentration (ppm)			Average Deviation
	0,2	1	2	
1	−0,80 ± 0,96	−0,87 ± 0,29	−0,43 ± 0,55	0,60
3	−7,63 ± 0,76	−5,30 ± 1,91	−4,47 ± 1,72	1,46
5	−5,43 ± 1,25	−4,00 ± 1,23	−3,87 ± 1,04	1,17

deviations for each of the reaction times has a relatively low value, which means that this experiment has better repeatability, making *Saccharomyces cerevisiae* an excellent candidate to be selected for the next stage of the study. Also, the values for 5 min of reaction time exhibit the best agreement with the proportional relation between the phenol concentration and the variation of the dissolved oxygen, as previous works have demonstrated [27,30] which means that this is the most suitable reaction time for the biosensor system when using *Saccharomyces cerevisiae* as the recognition element.

3.2.2. *Aspergillus niger*

The results obtained for *Aspergillus niger* are presented in Table 8.

The results in Table 8 indicate that the variation of dissolved oxygen does not change considerably with each of the different phenol concentrations; therefore, *Aspergillus niger* is not an appropriate biological material for the biosensor.

3.3. Biosensor calibration

From the experimental results presented above, it is concluded that the microorganism *Saccharomyces cerevisiae* produces the best response and repeatability of measurement among the different biological materials tested. Therefore, this microorganism was chosen as the recognition element for the biosensor. This choice is very important for the operation of the biosensor because it guarantees its ability to take accurate measurements.

The *Saccharomyces cerevisiae* microorganism has an additional advantage over organic tissues, which is a longer shelf life, i. e., it can be reused. This feature is enabled by the microencapsulation method, which provides favorable conditions to preserve the biochemical properties of the microorganism.

After the recognition element was selected, the calibration curve of the sensor was constructed using six operating points for different concentration values in an operating range from 0.2 to 2.5 ppm (0.2, 0.5, 1, 1.5, 2 and 2.5 ppm) as illustrated in Fig. 5. The test was repeated four times for each concentration value. The calibration curve demonstrates the behavior of the percentage variation of the dissolved oxygen generated by the reaction between the biological element and the analyte versus the concentration of phenol contained in the sample. The calibration curve shown in Fig. 5, which was generated with experimental data, was fitted to different types of function, as shown in Table 9 with the assistance of the software tool Microsoft Excel® [45]. In addition, the correlation coefficient was calculated, to determine which kind of function is in better agreement with the data behavior. The results are presented below in Table 9.

According to Table 9 and Fig. 5, the variation of dissolved oxygen as a function of phenol concentration can be approximated with a third degree polynomial in the operating range from 0.2 to 2.5 ppm. However, if the last data point was to be neglected (2.5 ppm), the system would have an approximately linear behavior for the operating range from 0.2 to 2 ppm of phenol concentration, as

Table 7
Average variation of dissolved oxygen percentage obtained using *Saccharomyces cerevisiae.*

Reaction Time (min)	Phenol Concentration (ppm)			Average Deviation
	0,2	1	2	
1	−2,50 ± 0,71	−0,65 ± 0,49	−2,80 ± 0,14	0,45
3	−1,95 ± 1,63	−1,15 ± 0,35	−7,35 ± 1,34	1,11
5	−3,28 ± 0,81	−5,79 ± 0,72	−10,35 ± 0,94	0,82

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Table 8
Average variation of dissolved oxygen percentage obtained using *Aspergillus niger*.

Reaction Time (min)	Phenol Concentration (ppm)			Average Deviation
	0,2	1	2	
1	−1,00 ± 1,41	−0,70 ± 0,14	−1,60 ± 0,42	0,66
3	−0,85 ± 0,21	−0,80 ± 1,84	−0,85 ± 0,92	0,99
5	−1,75 ± 1,06	−0,75 ± 0,49	−1,55 ± 0,49	0,68

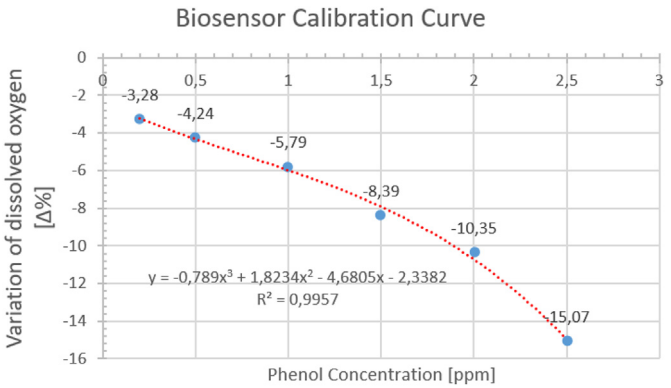

Fig. 5. Biosensor calibration curve.

Table 9
Correlation coefficients.

Approximation Function	Correlation coefficient (R^2)
Logarithmic	0.7721
Linear	0.9595
2th degree Polynomial	0.9921
3th degree Polynomial	0.9957

shown in Fig. 6. The correlation coefficient was calculated to determine the agreement of the linear approximation with the experimental data. The R2 value and the linear equation for this operating region is presented in Fig. 6.

4. Discussion

The design approach of the biosensor system proposed in this paper was based on the principle of phenol oxidation caused by oxidoreductase enzymes such as laccase or tyrosinase. Alternative sources of these kind of enzymes were tested with the goal of finding the most suitable alternatives sources of the enzyme for a phenol biosensor. The types of sources considered were samples of common organic tissues or microorganisms. This work also proposed the use of this sources without any kind of pre-process, such as lyophilisation, which is commonly employed to isolate the enzymes [11]. This approach seeks to reduce the time and cost of the construction of the biosensor system, without deteriorating its final performance.

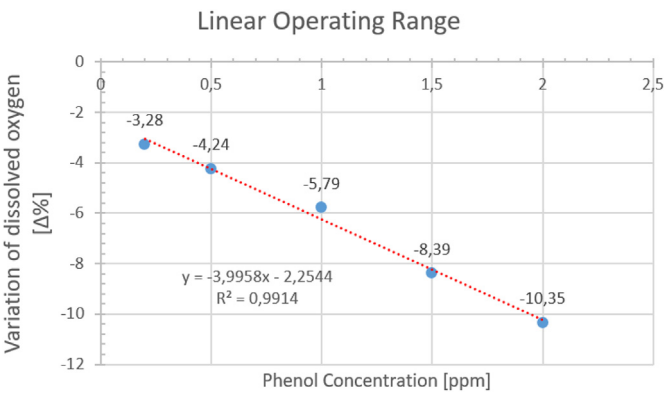

Fig. 6. Linear operating range.

The biosensor system developed in this investigation is adequate for measuring phenol concentrations typically found in industrial wastewater, which must be kept below maximum allowable legal limits [2,3]. The results found are in good agreement with data from previous works. This sensor also features an operating range that is comparable with others that incorporate isolated enzymes. For example, the operating range (0.2 ppm-2.5 ppm) determined in this article is close, or it has a detection limit that is even lower, in comparison to other similar systems found in the literature, where isolated enzymes were similarly used: laccase: (0.022−1 ppm) [10], (4−60 ppm) [11], (1 ppm-limit detection) [29] and tyrosinase (0.25−7 ppm) [13] (0.5−7 ppm) [14]. The biosensor system developed is also faster than several commercially available instruments. The method most commonly implemented by commercial instruments to measure phenol concentration is by means of spectrophotometric techniques. Typical examples of commercial instruments based on spectrometric techniques have an operating range between 0.1 and 2.5 ppm [46], 0.025−5 ppm [47] and 0.002−0.1 ppm [47], with a reaction time of 1 min [46], 10 min [47] and 30 min [47], respectively. In consequence, the system developed exhibits a shorter reaction time (5 min), as compared with two of the three most above mentioned commercial instruments, for similar concentration ranges. Although the system developed is not the fastest solution available, its reduction in speed is compensated with low production costs, since the sensor does not required costly specialized equipment for the measurement process.

This work also demonstrates a new progress in the development of microbial biosensors by the immobilization of *Saccharomyces cerevisiae* using the microencapsulation technique. This technique lets carrying out the interaction between the yeast and the analyte through the pores of the spheres, generating a decrease in the dissolved oxygen of the water sample, after the reaction time is over. Additionally, the capsules can be reused for multiple measurements. This makes the method highly suitable for industrial sensing applications, in comparison with biosensor systems that incorporate organic tissues as recognition elements, which cannot be reused. As for the kind of microorganism employed in the biosensor, The state of the art showed the use of several species of microorganisms for the measurement of phenol haven been previously reported, such as *Trichosporo cutaneum* [24], *Rhodococcus spec* [25] and *Pseudomonas putida* [26]. Thus, this work proposes a new potential application of *Saccharomyces cerevisiae*, immobilized as recognition element in a biosensor system for phenol detection. This proves a new application for *Saccharomyces cerevisiae*, which have been previously used in biosensors for the detection of vitamin B1 [48] and fatty acid [49].

The efficacy of the system relies on the stability given by the

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nutrient medium where the microorganism is immobilized, by means of the microencapsulation technique. This medium offers the possibility to the microbial to interact with the sample while remaining trapped. On the other hand, the decrease of the dissolved oxygen in the sample correlates well with the enzymatic activity that occur during the reaction time. This thanks to the containing of oxidoreductase enzyme in *Saccharomyces cerevisiae.*

5. Conclusions

The presence of the enzyme tyrosinase in three common organic tissues such as apples, potatoes and mushrooms, was verified, showing their feasibility as alternative natural sources of the enzyme for industrial applications.

The results obtained for the variation in dissolved oxygen, with the *Saccharomyces cerevisiae* microorganism, immobilized by means of the microencapsulation technique, exhibit better repeatability, as compared to the other organic substances tested in this investigation. This suggests that this microorganism is a very good source of enzymatic catalysts for the reaction with phenolic compounds. The concentration of the enzyme in natural sources is variable, due to several factors such as size and thickness of the sample, and culture, among others. It could be possible that the *Aspergillus niger* requires more reaction time with the analyte to obtain a higher percentage variation of dissolved oxygen.

The main advantage of the technique of microcapsules to identify the phenol concentration in water is that these microcapsules can be reused for multiple measurements.

The biosensor constructed has a high potential of application in the Oil & Gas industry, since it can provide reliable measurements in the typical phenol concentration ranges that are found in the wastewaters produced by this industry.

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Appendix

Methodology for the determination of the most suitable microorganism for this application

The steps for the determination of the most suitable microorganism for the measurement of phenol diluted in water are described next.

- Search. Two nutritious solid media were prepared in Petri dishes during this step. The first one with Agar-Agar and the second one with tyrosine (Aminoacid that allows oxidation of phenol). After the preparation of the media, the petri dishes were left exposed to air to capture microorganisms present in the environment.
- Seeding and isolation. The growth of the different microorganisms in the dishes was observed after three weeks of isolation. Thereafter, each of these microbes were isolated in an environment with tyrosine, in order to evaluate their capacity to produce the enzyme. Simultaneously, the same procedure was performed with a yeast (*Saccharomyces cerevisiae*) due its phenolase enzyme content. Previous works demonstrated the presence of laccase in *Saccharomyces cerevisiae* [50,51]. In addition, *Aspergillus niger* has also been used in investigations related to the biodegradation of phenol [52,53].
  At the end of this stage, the microorganisms that exhibited the best growth were selected to be used in the next step.
- Adaptation. After verifying the production capacity of the enzyme by the microorganisms, an adaptation process in an environment with phenol was carried out.
  In this step, the samples of the microorganisms are transferred from the solid medium to a liquid medium, which was made of brain-heart infusion, BHI, broth mixed with phenol at different concentrations.
  This training process was carried out gradually, i.e., the microorganism was exposed to the lowest concentration of phenol and then the concentration of phenol was slowly increased, looking carefully at the response of the microorganism to the adaptation process.
- Microencapsulation. The microencapsulation, or bioencapsulation, process refers to the immobilization of biologically active components in microcapsules [54].
  For this particular case, the microcapsules are small spheres containing the microorganisms, immobilizing them in a nutrient medium, generally coated by a protective material of polymeric nature. These spheres are formed and solidified by means of an induced change in temperature.
  Similar to the procedure proposed in the previous stage, the microencapsulated microorganisms must adapt to this new medium (microcapsules).
  The immobilized agent must have a stabilization time before it can be used. Finally, once the microorganisms were encapsulated and stabilized, they were subjected to the laboratory tests described in the main body of the article.

Fig. A.1. Microcapsules.

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