

The Optimization Procedure for the Attachment of *Saccharomyces cerevisiae* on the Surface of a Carbon Paste Electrode

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Abstract: Various kind of organic dyes (*Amido black*, *Phenol red*, *Rhodamine B*, *Neutral red*, *Eriochrome black T*, *o-cresol*, *Crystal blue*, and *fuchsine*) were studied on the purpose of indirectly evaluating the attachment process of *Saccharomyces cerevisiae* on the surface of a carbon paste electrode (CPE). Appropriate conditions for adsorption of the individual organic dyes onto the CPE (concentration, accumulation time, stirring speed) have been optimized. Besides, it has been observed that the current derived from the oxidation of *Amido black*, which was adsorbed onto the CP-surface after attaching the yeast cells, decreases along with time.

Keywords: Carbon paste electrode; Surface attachment process; Adsorption; Organic dyes; *Saccharomyces cerevisiae*.

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Introduction

In principle, biological materials can be attached to an electrode surface [1]. There have been many investigations about attachment of biological materials for analytical purpose, such as electro-analytical application of enzymatic reactions to measure the concentration of its substrate [2] and attachment of bacterial cells to carbon electrodes [3]. These results indicate that the bacterial-attachment process can be analyzed by measuring the electric current derived from the dye (Hoechst) instead of counting the number of attached cells. However, little has been reported about *Saccharomyces* in general and *Saccharomyces cerevisiae* in particular on with respect to their adsorption onto the electrode surfaces.

Carbon paste electrodes (CPEs) belong to promising electrochemical or bioelectrochemical sensors of wide applicability. Nowadays, CPEs represent one of the most frequent types of working and indicator electrodes. Among others, carbon paste also serves as almost ideal material for preparation of various electrodes and sensors in stripping analysis [4].

In this study, we have attempted to optimize the adsorption process of various organic dyes onto the surface of a CPE instead of using the Hoechst test. The results from this optimization will be used for interpretation of the attachment process of *Saccharomyces cerevisiae* on the surface of CPE.

Experimental

Chemicals and Reagents

The stock solutions of different organic dyes (*Amido black*, *Phenol red*, *Rhodamine B*, *Neutral red*, *Eriochrome black T*, *o-cresol*, *Crystal blue*, fuchsin) were prepared in phosphate buffer (pH 7.2) and stored in a dark place until used. All the reagents were purchased in Sigma-Aldrich (Czech Republic). Distilled water purified by deionized water system was used in this study ($G \leq 0.055 \mu\text{S}$). The soluble oxygen was removed from all the solutions by purging with argon for 15 min (purity 99.99%, Linde Technoplyn, Prague, Czech Rep.).

Organisms and Culture Condition

Saccharomyces cerevisiae CCM 8191 (Czech Collection of Microorganisms, Masaryk University, Brno) stock cultures were prepared as follows: the freeze-dried cells of *S. cerevisiae* were activated by transfer into malt extract broth (model MALT; HiMedia, Bombay, India) at 30°C for 24 h followed by an appropriate incubation in MALT agar plate. Stock culture was kept in refrigerator and refreshed each two weeks.

For the purpose of the study, a fresh culture was prepared from the stock culture by incubating inoculum in freshly prepared MALT (30°C, 24 h). Thereafter, a cell suspension was prepared in phosphate buffer saline (pH 7.2). The concentration of the cell suspension was adjusted to 1.5 according to McFarland turbidimetric standard.

The Preparation of Carbon Paste Electrode

The carbon paste electrodes were prepared as following: 0.5 g of graphite powder (with particle size of 5.5-7.0 μm ; product "CR-5", Maziva Týn n. L., Czech Republic) and 130 μl of mineral oil ("M5904", Sigma-Aldrich) was exhaustively hand mixed together in ceramic mortar by ceramic pestle. The resulting paste was packed into the *Teflon*[®] piston holder ([5], with inner diameter of 2 mm).

Electrochemical Measurements with Organic Dyes

A three-electrode system consisting of (working) carbon paste electrode, Ag/AgCl/3M KCl reference and a Pt-wire as the counter electrode were connected to electrochemical analyzer (model "PalmSens"; Ivium Technologies, Netherland) and used for measurements. The surface of CPE was regenerated by renewing and polishing it on wet filter paper before each experiment.

Each dye solution (10^{-6} M) was scanned using cyclic voltammetry (CV) with the following parameters: potential range from -0.4 to 1.0 V vs. ref., potential step 0.025 V, and scan rate 0.1 Vs^{-1} . The oxidation of each organic dye at a model concentration of 1×10^{-6} M in phosphate buffer (pH 7.2) was examined with the aid of square-wave voltammetry (SWV) after appropriate accumulation of the dye onto the surface of CPE (for 60s, in stirred solution at *ca.* 400 rpm). In the SWV mode, the following parameters were applied: potential range from 0.0 to 1.0 V vs. ref., SW-frequency of 25 Hz, potential step (increment) of 0.025 V, and pulse amplitude of 0.025 V.

Free Adsorption of Organic Dye and Microorganisms on the CPE Surface: An Optimization

The effects of various times of accumulation (30-150 s), stirring speeds (100-600 rpm), and the concentrations of the organic dye ($5.00 \times 10^{-7} - 2.25 \times 10^{-6}$ M) on its oxidation current were examined when using SVW. The optimized parameters were used in further experimentation.

The working electrode was immersed into 4 ml of cell suspension (1.5×10^8 cfu ml^{-1}) in phosphate buffer saline (pH 7.2) for 30, 60, 120, and 180 s at 400 rpm. After the attachment process, CPE an organic dye was allowed to accumulate using optimized parameters. CPE with adsorbed cells and organic dye was dipped into the fresh phosphate buffer (pH 7.2) and the oxidation current of amido black ($I_0(\text{cells})$) was recorded in the SWV mode as described above

The extent of free cell adsorption ($I_{0(\text{extent})}$) to the surface of CPE was calculated using the following equation:

$$I_{0(\text{extent})} = I_0 - I_{0(\text{cells})} \quad (1)$$

where " I_0 " is the oxidation current of the adsorbed dye (μA), " $I_{0(\text{cells})}$ " the oxidation current of the dye adsorbed after accumulation of the cells of microorganisms (μA). It is accomplished that the more cells attached to the surface of the electrode the greater the value of $I_{0(\text{extent})}$.

All experiments had been made in ten replicates and the results obtained represented an average with standard deviation. The statistical differences were calculated using analysis of variance at the probability factor of $\alpha = 0.05$.

Results and Discussion

Electrochemical Measurement of Organic Dyes

According to the results presented in Table I, *Amido black*, *Eriochrome black T* and *Crystal blue* gave statistically highest oxidation current peaks with the values: 2.890, 2.109 and 1.912, respectively. Among these organic dyes, *Amido black* seemed to be suitable for adsorption process since its response gave rise to a significantly higher oxidation peak and lower standard deviation in comparison with the rest of organic dyes. In addition, the peak potential of *Amido black* (0.669 V) was far from peak potential of the oxygen oxidation.

Table I. Oxidation current peak and oxidation peak potential of various organic dye by SWV ($n = 10$)

Organic dye	E[V]	I [μ A]	σ [μ A]
<i>Amido black</i>	0.669	2.890	0.077
<i>Eriochrome black T</i>	0.219	2.109	0.263
<i>Phenol red</i>	0.679	0.083	0.016
Fuchsine	0.819	0.249	0.045
<i>Crystal blue (1)</i>	0.559	0.231	0.066
<i>Crystal blue (2)</i>	0.839	1.912	0.334
<i>Neutral red</i>	0.849	0.417	0.076
<i>o</i> -Cresol	0.669	0.061	0.035
<i>Rhodamin B</i>	0.249	0.212	0.056

Legend: E(V) ... oxidation peak potential of organic dye, I [μ A] ... oxidation current peak, σ [μ A] ... the standard deviation.

The result depicted in Fig. 1 indicates that *Amido black* is an irreversible system giving one region of oxidation with no reduction current observed in cyclic voltamogram. Moreover, the oxidation peak height decreased with the number of cycling. It could be interpreted that this organic dye was destroyed at a certain degree after the oxidation process and the resulted fragments are not electrochemically active. These results were in good agreement with the previous study on degradation of azo-dye *Amido black 10B* in aqueous solution by Fenton oxidation process [6].

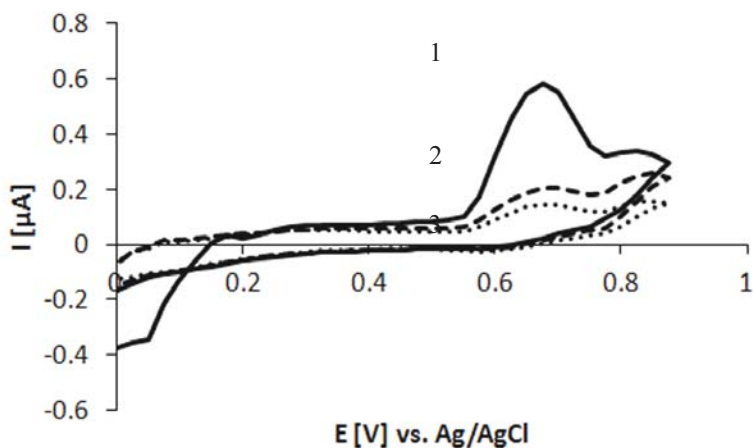


Fig. 1: Cyclic voltammogram of Amido black in phosphate buffer (pH 7.2). Legend: 1) 1st cycle, 2) 2nd cycle, 3) 10th cycle. Experimental condition: potential range from 0.0 to 0.9 V, potential step 0.025 V, scan rate 0.1 V.s⁻¹.

It was confirmed that the initial concentration had significant effect on the adsorption process of organic dye before it reached equilibrium at the concentration of 1×10^{-6} M. As shown in Fig. 2, the oxidation current of dye adsorbed at the concentration of 10^{-6} M was significantly higher ($p < 0.05$) than those determined in lower concentration while insignificant difference was found when compared with higher concentration ($p > 0.05$).

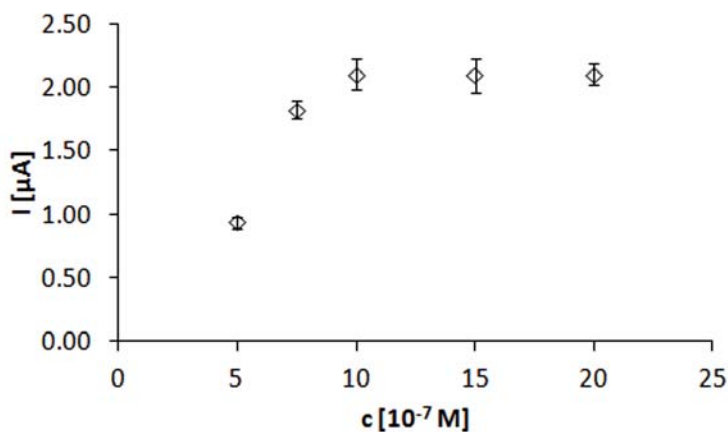


Fig. 2: The effect of the concentration of Amido black on its oxidation current (phosphate buffer solution pH 7.2). Experimental condition of SWV: time of accumulation 120 s, stirring speed 400 rpm, frequency 25 Hz, potential range 0.0-1.0 V, potential step 0.025 V, potential pulse 0.025 V. Mean values with standard deviations ($n = 10$).

Similar to the concentration, the adsorption process of organic dye was significantly influenced by stirring speed of accumulation (Fig. 3). The process reached to its equilibrium at the stirring speed 400 rpm since the oxidation current was significantly higher than those determined in slower speed ($p < 0.01$). Practically, the stirring speed higher than 400 rpm is not recommended because higher speed showed inconvenience resulted in formation of air bubbles inside the solution and even damage of electrodes.

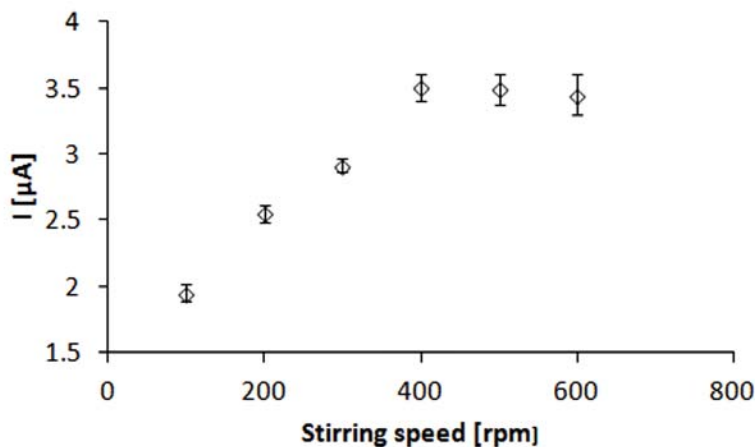


Fig. 3: The effect of stirring speed on the oxidation current of Amido black solution (10^{-6} M in phosphate buffer, pH 7.2). Experimental condition of SWV: time of accumulation 120 s, frequency 25 Hz, potential range 0.0-1.0 V, potential step 0.025 V, potential pulse 0.025 V. Mean values with standard deviations ($n = 10$).

The adsorption process of *Amido black* was also significantly influenced by time of accumulation as shown in Fig. 4. The oxidation current increased with increase of accumulation time up to 120 s where the equilibrium has been reached. A further increase of accumulation time did not affect the oxidation current of *Amido black* ($p > 0.05$).

It was found that the time of adsorption had an effect on the amount of the accumulated cells of *S. cerevisiae* cells up to 60 s. As shown in Fig. 5, the oxidation current of dye adsorbed after 30 s on the surface of CPE was significantly lower than those determined after 60 s of accumulation ($p < 0.001$). Further increase of accumulation time had no significant effect on the attachment of *S. cerevisiae* cells.

It should be noted that the absolute value of the oxidation current of *Amido black* dye strongly depend on the composition of the carbon paste electrode. Even prepared by the same person and using identical chemicals, the characteristics may differ.

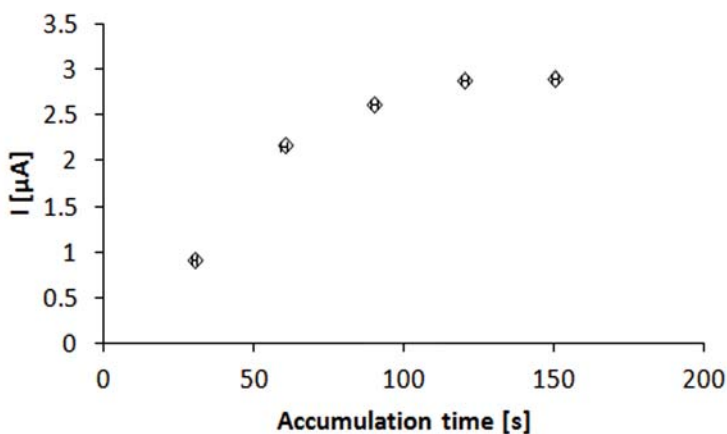


Fig. 4: The effect of accumulation time on the oxidation current of Amido black dye solution (10^{-6} M in phosphate buffer pH 7.2). Experimental condition of SWV: stirring speed 400 rpm, frequency 25 Hz, potential range 0.0-1.0 V, potential step 0.025 V, potential pulse 0.025 V. Mean values with standard deviations (n = 10).

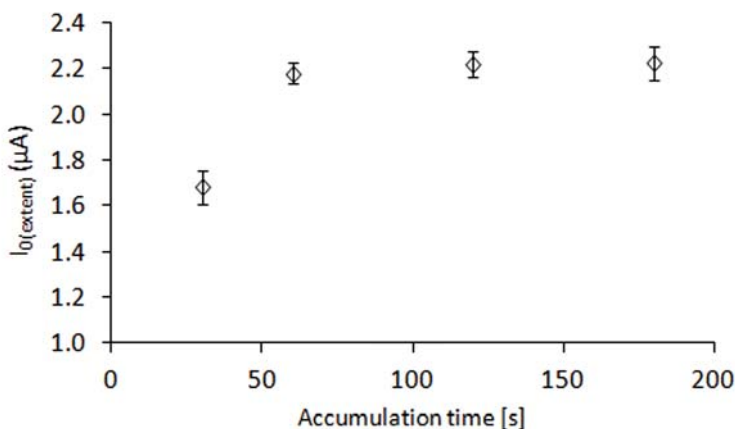


Fig. 5: Increase in the anodic peak current of Amido black along with the attachment time of *Saccharomyces cerevisiae*. Experimental condition of SWV: stirring speed 400 rpm, frequency 25 Hz, potential range 0.0-1.0 V, potential step 0.025 V, potential pulse 0.025 V. Mean values with standard deviations (n = 10).

It is evident from the table II, where value I_0 and $I_{0(\text{cells})}$ of CPE no. 3 is different from those determined using CPE no. 1 or 2 ($p < 0.05$). Therefore, we used a relative values to describe the extent of free attachment/accumulation of yeast cells on the surface of carbon paste electrode (eq. 1). These values are then not statistically different ($p > 0.05$).

Table II. Oxidation current peak obtained at different CPEs

No. CPE	I_0 [μA] ¹	$I_{0(\text{cells})}$ [μA] ²	$I_{0(\text{extent})}$ [μA] ³
CPE 1	2.98 ± 0.06	2.22 ± 0.01	0.77 ± 0.06
CPE 2	2.99 ± 0.06	2.22 ± 0.02	0.76 ± 0.06
CPE 3	3.54 ± 0.08	2.80 ± 0.05	0.74 ± 0.08

Legend: ¹ I_0 the oxidation current of *Amido black* after 120 s accumulation at stirring speed 400 rpm; ² $I_{0(\text{cells})}$ the oxidation current of amido black after free attachment of microbial cell; ³ $I_{0(\text{extent})}$ the extent of free cells adsorption calculated according to eq. 1.

Conclusions

In this study, the attachment process of yeast cells to the surface of CPE was performed during magnetic stirring without applying the potential and we have found the optimal conditions for measuring the attachment of yeast cells onto the carbon paste electrode. Although the adsorption process had reached an equilibrium, it was previously observed that the cells did not cover the entire surface of CPE [7].

According to Morisaki et al. [3], the bacterial-attachment process can be analyzed by measuring the electric current derived from the dye instead of counting the number of attached cells. In our study, since the relationship between the number of attached cells and oxidation peak was not observed, further investigations should be executed. The negative charge of the cell wall of yeast resulted from phosphate, pyruvate or glucuronic acid which can be linked to mannoproteins [8]. The attachment of *S. cerevisiae* cells could be enhanced by the application of positive potentials during the process.

Activated carbon which has been processed to make it extremely porous and thus to have a very large surface area available for adsorption or chemical reactions could a suitable material for modifying working carbon paste electrode in the purpose of study adsorption process of organic dyes or attachment process of biological materials. In some previous studies, mechanism of adsorption on activated carbons and carbon derivatives has been described [9,10]. Adsorptive capacity of activated carbon has been examined, establishing adsorption isotherms and changes in zeta potential resulting from interaction between the dye and the carbon adsorbent.

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