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Maltose biosensing based on co-immobilization of α-glucosidase and pyranose oxidase

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ABSTRACT

A new bi-enzymatic system was designed by co-immobilization of α-glucosidase (AG) and pyranose oxidase (PyOx) for maltose analysis. The immobilization was carried out by cross-linking enzyme mixture, chitosan (CHIT) and carbon nanotube (CNT) via glutaraldehyde. The structure of biosensor including enzyme, CHIT, glutaraldehyde and CNT amount together with operational conditions like pH, temperature and applied potential were optimized. Then analytical characterization was performed. A fast linear response of the biosensor was observed for maltose in the concentration range from 0.25 to 2.0 mM at 35 °C and pH 6.0. The effect of CNT addition into the immobilization matrix was also investigated. The linear relationships between sensor response (y; μA/cm^2) and substrate concentration (x; mM) were defined by the equations of $y = 0.844x + 0.029$ ($R^2 = 0.999$) and $y = 0.882x + 0.0625$ ($R^2 = 0.996$) for AG/PyOx/CHIT and AG/PyOx/CHIT–CNT biosensors, respectively. All other data were also given as comparison of two systems one with CNT-modified and CNT-free. Finally, for the sample application, maltose was analyzed in beer samples. As a result, it has been found that; complex matrix of natural beer samples had no influence on the biosensing response. Also the results were in good agreement with those obtained by spectrophotometric measurements.

1. Introduction

Starch hydrolysis is widely engaged in the brewing and food industries. When using malt, which is rich in β-amylase, to treat starch in co-ordination with other enzymes, e.g. α-amylase, maltose is one of the major products [1]. Maltose (4-O-α-D-glucopyranosyl-D-glucose), which is commonly present in various fermented foods, is mainly used as a sweetening agent [2]. Determination of maltose is a routine analysis especially in beer manufacture. Though methods like HPLC [3], paper chromatography [4] and gas chromatography [5] have been utilized for maltose detection, the necessity of expensive chemicals and instrumentation [6] for application of these techniques restricts their usage. The spectrophotometric determination of maltose can be carried out by combining the enzymatic hydrolysis of maltose using α-glucosidase and glucose determination by the glucose oxidase (GOx) [7]. Moreover three enzymatic methods, the α-glucosidase: hexokinase: glucose-6-phosphate dehydrogenase [8], maltose phosphorylase: glucose oxidase [9], and maltose 1-epimerase: maltose phosphorylase: β-phosphoglucomutase: glucose-6-phosphate dehydrogenase [2] enzyme systems have been reported previously for the maltose analysis. On the other hand, oligosaccaride dehydrogenase biosensor has been developed for maltose as well as other sugars [10]. Usage of biosensors has had a major impact in numerous scientific disciplines, and is being exploited commercially in agriculture, food, environmental control and biotechnology industries [11].

α-Glucosidase (AG, α-D-glucoside glucohydrolase, EC 3.2.1.20), also called maltase, is a membrane-bound enzyme at the epithelium of the small intestine that catalyzes the cleavage of glucose from disaccharides. Maltose is the main substrate for α-glucosidase [12,13]. Pyranose oxidase (PyOx; pyranose: oxygen 2-oxidoreductase, E.C 1.1.3.10), also called glucose-2-oxidase, is relatively large flavoprotein [14,15] which produces hydrogen peroxide from molecular oxygen by regioselective oxidation of various mono- and disaccharides, especially D-glucose, at the C2 position [16–18]. PyOx is an intracellular enzyme found in several white-rot fungi such as *Tricholoma matsutake* [19], *Peniophora gigantea* [20], *Trametes multicolor* [21], and *Coriolus versicolor* [22]. The particular advantages of using PyOx instead of GOx are as follows: (1) Mutarotation equilibrium about 36% of D-glucose occurs as the corresponding alpha anomer that is not oxidizable by GOx, but PyOx can catalyze oxidation of α-D-glucose. Due to the lack of anomeric specificity of PyOx, combination of mutarotase with PyOx based systems is not required [23], and (2) the affinity of PyOx for D-glucose is higher than GOx (Km ~ 1 mM) [24].

Studies including immobilization of enzymes on the electrode surface by means of various natural polymers have been published before. Chitosan (CHIT) which is one of the suitable polymers for enzyme immobilization is an excellent material for immobilization of several carbohydrate degrading enzymes, since it exhibits increased thermostability compared to the free enzyme [25]. Chemical modification of natural CHIT could be done for the further functionalization with various nanomaterials such as carbon nanotubes (CNT) [26], iron oxide nanoparticles (IONP) [27], and gold nanoparticles (AuNPs) [28]. CNTs promote electron-transfer reactions in electrochemical biosensors. CHIT has been used to solubilize CNTs and the resulting

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CHIT–CNT dispersions bring new capabilities for bio-electrochemical devices.

The purpose of this work is to develop a new electrochemical biosensor for maltose analysis by using both AG and PyOx activities. This novel biosensing system including use of PyOx instead of anomer specific GOx prevents the requirement of mutarotase enzyme in the bioactive layer to obtain the proper anomeric form of glucose for the next enzymatic step [2]. As well as optimization studies, characterization of AG/PyOx/CHIT biosensor was carried out and effect of CNTs on the biosensor response was searched. Finally optimized and characterized biosensor was applied for maltose detection in beer samples.

2. Experimental

2.1. Reagents

α-Glucosidase (From *Saccharomyces cerevisiae*, recombinant, 84 U/mg), pyranose oxidase (From *Coriolus* sp., recombinant; expressed in *E. coli*, 9.4 U/mg), glucose oxidase (GOx; β-D-glucose: oxygen 1-oxidoreductase, E.C 1.1.3.4, Type II-S: from *Aspergillus niger*) chitosan (from crab shells, minimum 85% deacetylated), glutaraldehyde solution (Grade II, 25%), D-glucose, D(+)-mannose, D(+)-galactose, D(+)-xylose, β-lactose were purchased from Sigma Chem. Co. (St. Louis, MO, USA), graphite powder (<20 μm, synthetic), multi-walled carbon nanotube (MWCNT; diameter: 110–170 nm, length: 5–9 μm, 90+ %) were obtained from Aldrich (Dorset, UK), D(−)-fructose, N-acetyl D-glucosamine, maltose monohydrate were purchased from Fluka (Steinheim, Germany). All other chemicals were analytical grade.

1.0 wt.% chitosan solution was prepared by dissolving 1.0 g of chitosan, flakes into 100 ml of 1.0% acetic acid and stirred for 3 h at room temperature until complete dissolution. The chitosan solution was stored in refrigerator when not in use [29].

To prepare CHIT–CNT solutions, different amounts of MWCNTs were dispersed in CHIT solutions and the blends were treated under an ultrasonic field for 10 min. This CHIT–CNT blend was then stirred for 1 h. During this step, chitosan were adsorbed on the surface of the CNTs thereby acting as polymer cationic surfactants to stabilize the CNT structures [26].

2.2. Instrumentation

Chronoamperometric measurements were carried out with the use of Radiometer electrochemical measurement unit (Lyon, France) where the modified spectrographic graphite rods (Ringsdorff Werke GmbH, Bonn, Germany, 3.05 mm diameter and 13% porosity) were used as a working electrode. The experiments were carried out with a Ag/AgCl electrode (with 3 M KCl saturated with AgCl as the internal solution, Radiometer Analytical, REF321) and platinum electrode (Metrohm, Switzerland) were used as reference and counter electrodes, respectively. The electrodes were inserted into a conventional electrochemical cell through its Teflon cover. Spectrophotometric measurements were carried out with Perkin Elmer, Lambda 35 UV/VIS Spectrometer (Shelton, USA).

2.3. Construction of AG/PyOx/CHIT bi-enzymatic sensor

For the construction of the bioactive layer, the graphite electrodes were polished and washed thoroughly with distilled water and sonicated for 2 min [30]. 20 μl of CHIT or CHIT–CNT solutions (1.0%), 10 μl of AG solution (0.6 U) and 10 μl of PyOx solution (9.0 U) were placed on the graphite electrode and allowed to dry at +4 °C for 18 h. Then 20 μl of glutaraldehyde solution (1.0%; in potassium phosphate buffer, pH 7.5, 50 mM) spread evenly onto the CHIT modified graphite electrode and allowed to stand at +4 °C for 30 min [29]. Then, it was washed with distilled water to remove excess of glutaraldehyde and unbound enzymes. Protein analysis was carried out in washing solution to check the availability of unbound protein amount by Bradford assay [31]. During the experimental steps daily prepared enzyme sensors were used to obtain more reproducible data.

2.4. Measurements

The measurements were done at steady-state conditions. There is an interval surface between bioactive layer and electrode surface. Sensor responses were defined as the difference between first and second steady-state signals obtained before and after substrate addition and registered at current density ($\mu A/cm^2$). The changes in signals due to the substrate addition were recorded at 60th seconds. After each measurement, the electrodes were washed with distilled water and kept in working buffer solution for 2 min. The decrease of the reduction current of molecular oxygen by the addition of maltose through the subsequent enzymatic reaction was used to determine the maltose concentration. As given below; initially AG hydrolyses maltose to 2 mol D-glucose. Then PyOx catalyzes the regioselective C-2 oxidation of D-glucose to 2-keto-D-glucose, concomitant with the reduction of O_2 to H_2O_2. Calculations were made by using standard curves between the current signals of consumed oxygen and maltose concentration.

$$\text{Maltose} + H_2O \xrightarrow{AG} 2\,\text{D}-\text{Glucose} \quad (1)$$

$$\text{D}-\text{Glucose} + O_2 \xrightarrow{PyOx} 2-\text{keto}-\text{D}-\text{glucose} + H_2O_2 \quad (2)$$

On the other hand, oxygen concentration of the medium could be mainly effected by the temperature so that a constant temperature and oxygen saturated buffer were used to get reproducible current signals in each measurement. Additionally, since high potential values as the working potential cause the interference of electroactive species, oxygen reduction at −700 mV was followed during our experiments.

2.5. Maltose analysis in beer samples

Maltose concentration was determined in two brands of beer samples. The only sample treatment required was a proper dilution process with working buffer solution. The results obtained with the proposed biosensor were also validated by 3.5-dinitrosalicylic acid (DNS) method [32]. Spectrophotometric DNS method was performed by addition of 0.5 ml DNS solution (0.04 M of 3,5-dinitrosalicylic acid, 1.0 M potassium sodium tartarate, and 0.4 M NaOH) to 0.5 ml of samples and allowed to stand in boiling water bath for 10 min. After that, the reaction was stopped by cooling it immediately. Distilled water (5.0 ml) was added to each test tube and the optical density was measured at 546 nm against the blank. Maltose solutions ranging from 1.0 to 20.0 mM were used as standard solution. Furthermore, samples were also spiked with known amounts of standard maltose solution to test the sample matrix effect on the analysis.

3. Results and discussion

In our previous studies, various electrochemical biosensing systems based on PyOx have been designed for glucose and some other sugars [30,33,34]. Also, an inhibition based PyOx-biosensing system was developed for glutathione (GSH) and ethanol (EtOH) as PyOx inhibitors [35]. In this work, design and characterization of a bi-enzymatic system based on AG/PyOx/CHIT were carried out and proposed system was applied for maltose detection in beer samples.

3.1. Optimization of AG/PyOx bi-enzymatic biosensor

3.1.1. Enzyme loading

As the magnitude of the sensor signal is related to the amount of biological material in the bioactive layer, the dependence of signal

amplitude on the activities of immobilized enzymes was studied. The effect of PyOx activity on the amperometric signal was initially tested in the range of 0.3 and 0.9 U while AG activity was fixed on 9.0 U. Then, the effect of AG activity was investigated in the range of 6.0 and 12.0 U (PyOx activity was kept constant at 0.6 U). Fig. 1 shows the effect of PyOx (A) and AG (B) activities on the response in phosphate buffer (pH 6.0, 50 mM) and at 35 °C. For further studies, 0.6 U and 9.0 U of PyOx and AG activities were chosen as the optimum amount.

3.1.2. CHIT and glutaraldehyde amount

In the immobilization procedure, glutaraldehyde was used as a bifunctional reagent to provide cross-linkages between the amino groups on both matrix and the biomolecules. It is also known that these interactions are pH dependent and the most efficient binding yield between proteins and amino groups of CHIT matrix was reported to be 7.0–7.5. Hence pH 7.5 was used for the enzyme immobilization [36–38]. In order to check the effect of CHIT and glutaraldehyde amounts on AG/PyOx/CHIT biosensor, different concentrations of CHIT and glutaraldehyde solutions were tested. Fig. 2 depicts the effect of CHIT (A) and glutaraldehyde (B) amount on the biosensor response. Maximum response was obtained when 1.0% of CHIT and glutaraldehyde concentrations were used. As can be seen in the figure, further increments caused lower signals due to the possible diffusion problems as a result of higher crosslinkage amounts.

3.1.3. CNT amount

Since its discovery in 1991, CNTs have been used in various applications. Combination with electrode materials for fabricating chemical sensors or biosensors is a growing area. CNTs have an outstanding ability to mediate fast electron-transfer kinetics for a wide range of electroactive species, such as hydrogen peroxide, NADH or mediators [39,40]. In this work, the influence of the CNT amount on the amperometric biosensor response was tested between 0.2 and 0.7 mg/ml of CNT solutions in 1.0% of CHIT. The response of the bi-enzymatic biosensor was almost constant with 0.5 mg/ml of CNT modification. When the concentration of CNT was increased, a sharp drop in the response was observed. This might be due to the restricted diffusion of oxygen towards the electrode as well as restricted diffusion of substrate from the electrode (data not shown).

3.1.4. pH and temperature

The effect of pH on the amperometric response of both AG/PyOx/CHIT and AG/PyOx/CHIT–CNT biosensors was examined by using acetate buffer at pH 5.5 and phosphate buffer solutions between 6.0 and 7.5 in the presence of 2.0 mM of maltose. It can be seen (Fig. 3A) that the biosensor response increases with pH values between 5.5 and 6.0. The lower response in acetate buffer at pH 5.5 could be due to the inhibition effect of acetate ions to PyOx enzyme as reported before [41]. On the other hand, a decrease in the response was observed

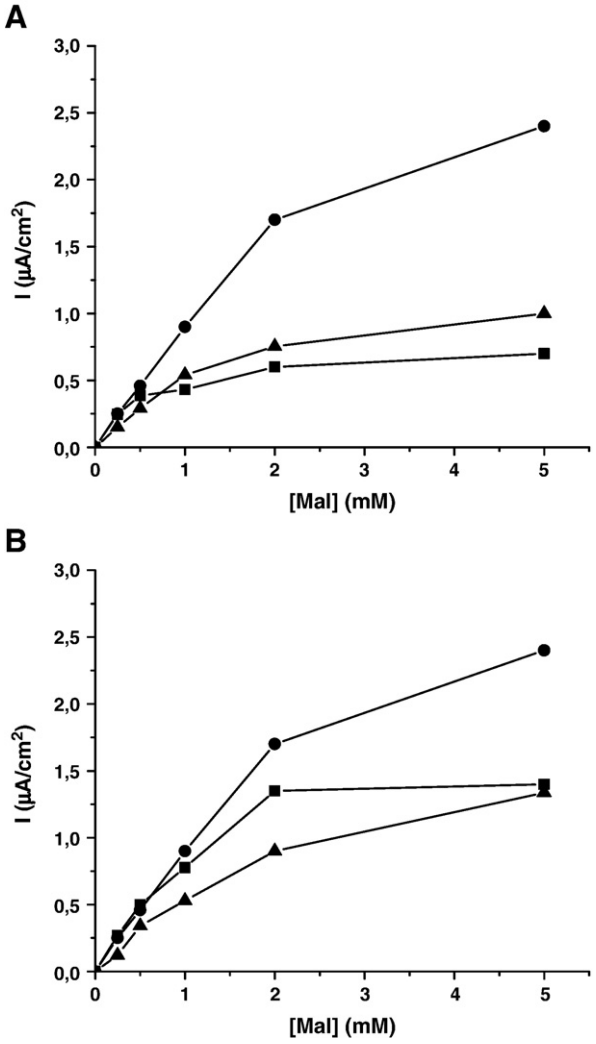

Fig. 1. Effect of enzyme loading (A: Effect of AG activity; ■ 0.3 U, ● 0.6 U, ▲ 0.9 U, B: Effect of PyOx activity; ■ 6 U, ● 9 U, ▲ 12 U; in pH 6.0, 50 mM phosphate buffer; 35 °C; −0.7 V vs. Ag/AgCl(3 M KCl)).

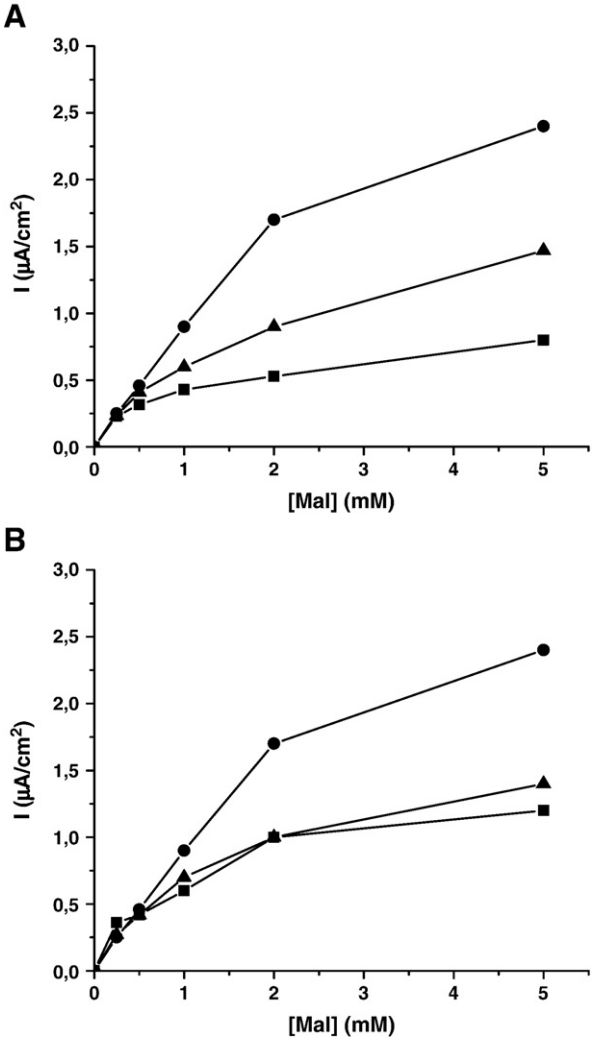

Fig. 2. Optimization of CHIT and glutaraldehyde amounts (A: Effect of CHIT amounts; ■ 0.5%, ● 1.0%, ▲ 2.0%, B: Effect of glutaraldehyde amounts; ■ 0.5%, ● 1.0%, ▲ 1.5%; in pH 6.0, 50 mM phosphate buffer; 35 °C; −0.7 V Ag/AgCl(3 M KCl)).

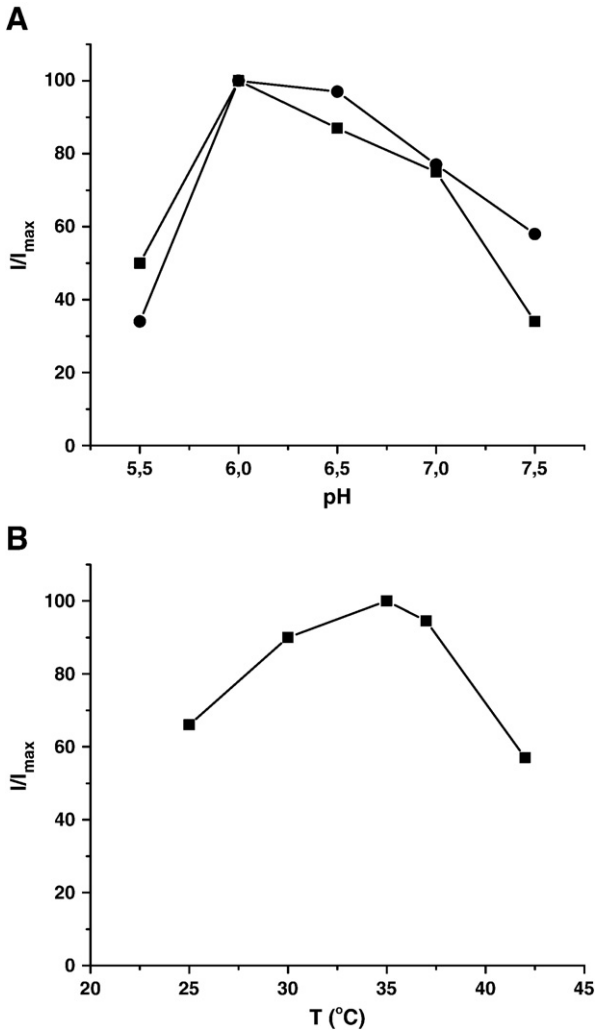

Fig. 3. Response of AG/PyOx biosensor as a function of pH and temperature in the presence of maltose (2.0 mM) (A: Effect of pH; pH 5.5; 50 mM acetate buffer, pH: 6.0–7.5, 50 mM PBS; 35 °C; −0.7 V Ag/AgCl(3 M KCl); ■ AG/PyOx/CHIT, ● AG/PyOx/CHIT–CNT and B: Effect of temperature; in pH 6.0, 50 mM phosphate buffer; −0.7 V; I: Current, I_{max}: Maximum current).

while moving towards the alkaline side. It was reported that free PyOx and AG enzymes have the maximum activity at pH of 7.0–7.5 [42] and 6.5–6.8 [43], respectively. In our case, the optimum pH for bi-enzymatic biosensor was shifted towards the acidic side compared to that of free enzymes. This situation can be attributed to the cationic characteristics of chitosan matrix. The tertiary structure of an enzyme is produced by intramolecular interactions that include hydrogen bonding, electrostatic interactions etc. Among these interactions, electrostatic interactions are sensitive to the pH of the surrounding environment where pH changes can make variations in the pattern of charges on the enzyme. These differences in enzyme pattern can result with a change in the tertiary structure of the enzyme which can affect the active site in such a way that an increase or decrease in the biocatalytic activity is obtained. Moreover, immobilization can change the enzyme's micro environment and this can cause a shift in optimum pH of the enzyme [44]. According to the data, it could also be stated that CNT modification did not affect the pH optima of these systems. As a result, further studies were conducted at pH 6.0

The effect of temperature on biosensor response was also examined for 2.0 mM maltose and results were presented in. Fig. 3B. From the figure it is obvious that, the activity of the enzyme system depends on the temperature. The decrease on the biosensor response was obtained with increasing the temperature beyond 35 °C. This can be explained mainly by the possible denaturation of enzyme at higher temperatures.

3.1.5. Applied potential

It is clear that the biosensor response was dependent on the applied potential. In this part, we explored the effect of the various potentials between −0.55 and −0.9 V (with the increments of 0.05 V) on the responses of AG/PyOx/CHIT and AG/PyOx/CHIT–CNT bi-enzymatic biosensors. The current response increases when applied potential value was increased and reaches to the maximum value when the potential is −0.70 V. Further increase in the potential caused lower signals in both types of biosensor system (Fig. 4). Hence −0.70 V was chosen as the optimum working potential.

3.2. Analytical characteristics

The response of the AG/PyOx/CHIT biosensor exhibits a linear dependence of the current density for the maltose concentration between 0.25 and 2.0 mM. The linearity was defined by the equation of $y = 0.844x + 0.029$ where x and y show concentration in mM and current density as μA/cm^2, respectively and R^2 was calculated as 0.999. In the case of AG/PyOx/CHIT–CNT biosensor, the concentration range was linear in the same concentration interval and this linearity was defined by the equation of $y = 0.882x + 0.0625$, (x and y show concentration in mM and current density as μA/cm^2, respectively) while $R^2 = 0.996$. When higher maltose concentrations than 2.0 mM were applied, current density stayed constant because of reaching to saturated substrate amount. Obvious difference cannot be found between responses of AG/PyOx/CHIT and AG/PyOx/CHIT–CNT. It is clear that the presence of CNT didn't affect the response characteristics at the defined conditions. Similar observations were also obtained in our previous works in which CNT modification only provided more stable immobilization platform with higher surface area as other nanostructured materials [45,46]. It is also well known that charged polymers are more efficient dispersing agents allowing quicker dispersion of nanotubes compared to solvents and offer higher stability of dispersion. On the other hand, other maltose biosensors including amyloglucosidase/glucose oxidase enzymes were described by Feng Ge et al. The linearity was detected in the potassium ferricyanide mediated and non-mediated conditions in the range of 5–20 mM [1] and 10–35 mM [47]. In compare to these systems, the sensitivity of our system is higher than other maltose

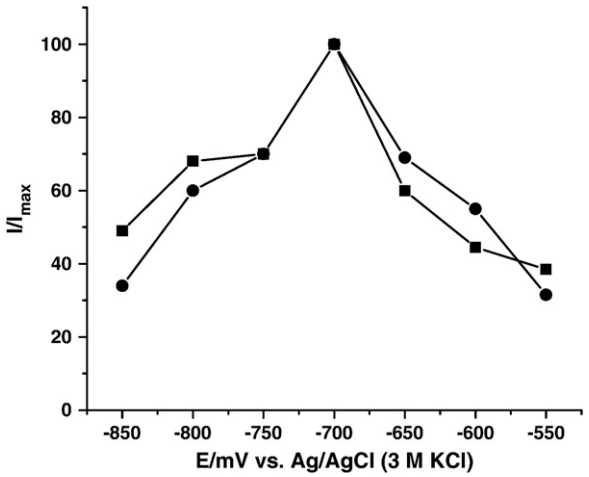

Fig. 4. Hydrodynamic voltammograms of CHIT and CHIT–CNT based AG/PyOx biosensor (pH 6.0, 50 mM, phosphate buffer; 35 °C; −0.7 V Ag/AgCl(3 M KCl); [Mal]: 2.0 mM; I: Current, I_{max}: Maximum current; ■ AG/PyOx/CHIT, ● AG/PyOx/CHIT–CNT).

biosensors; even dilution was required for maltose analysis in beer samples.

The precision of the method was evaluated by injecting 10 replicates of the standard maltose solution (1.0 mM) and standard deviations (S.D) and variation of coefficient (c.v %) were calculated as ±0.051 mM and 4.4% for AG/PyOx/CHIT and ±0.023 mM and 1.75% for AG/PyOx/CHIT–CNT biosensors.

Operational stability is an important consideration for immobilized enzymes. Stability of biosensors was investigated at operational conditions (in working buffer solution, 35 °C) for 5 h and 15% and 13% decrease in biosensor responses for AG/PyOx/CHIT and AG/PyOx/CHIT–CNT biosensors were observed. Since, PyOx was reported to be very stable enzyme in previous works [30,33], this drop could be due to the drop in AG activity at described conditions.

In addition, the shelf life of AG/PyOx/CHIT and AG/PyOx/CHIT–CNT was tested during 20 days. Biosensors were stored at 4 °C when not in use and no decrease in their responses was observed.

3.3. Comparison of PyOx and GOx in the bioactive layer

In order to investigate the advantageous of using PyOx in the bioactive layer instead of GOx, another bi-enzymatic system was prepared based on AG/GOx/CHIT in which GOx activity was equal to PyOx which was 9.0 U and maltose concentration was estimated using AG/GOx/CHIT biosensor at the same working conditions. Linear range for maltose was found to be as 0.25 to 2.0 mM with $y = 0.488x + 0.044$ ($R^2 = 0.993$, x and y show concentration in mM and current density as μA/cm^2, respectively). While similar linear ranges were obtained for both systems, approximately two times lower slope value was observed by AG/GOx/CHIT in compared with AG/PyOx/CHI in which this value was calculated as 0.844. This is an expected result and due to from the lack of anomer specificity of PyOx so the both β- and α-forms of glucose could be used as the substrate by this enzyme unlike GOx.

3.4. Substrate specificity

The substrate specificity of PyOx is rather broad, D-glucose, D-xylose, and L-sorbose are oxidized at high rates and enzymatic oxidation of many other sugars is performed at slower rates [17]. For this reason the influence of some carbohydrates on the biosensor response has been studied and results were given in Table 1. As can be seen from the table, no response was observed for mannose, fructose, N-acetyl D-glucosamine, lactose, cellobiose in our operational conditions.

The effect of the presence of various carbohydrates in the reaction medium on maltose analysis was also tested and results were summarized in Table 2. For this aim, different concentrations of the compounds were added to the reaction cell containing 0.5 mM of maltose substrate in working buffer solution. Our findings showed that presence of these compounds didn't interference the biosensor response to maltose. Apart from various substrates, the response of some compounds which could interfere with the measurements such as paracetamol, ascorbic acid and uric acid were also tested and no effect was observed to the signals.

Table 1
Carbohydrate analysis using AG/PyOx biosensors.

	Linear range (mM)	AG/PyOx/CHIT		AG/PyOx/CHIT–CNT	
		Equation[a]	R^2	Equation[a]	R^2
Glucose	0.025–0.20	$y = 10.337x + 0.008$	0.998	$y = 10.769x + 0.090$	0.994
Galactose	0.05–0.5	$y = 3.8025x + 0.036$	0.994	$y = 6.142x + 0.056$	0.999
Xylose	0.05–0.5	$y = 4.867x + 0.063$	0.990	$y = 5.840x + 0.080$	0.996
Sucrose	0.5–2.0	$y = 0.258x + 0.003$	0.999	$y = 0.321x + 0.002$	0.999

No response was observed for mannose, lactose, cellobiose, fructose, N-acetylglucoseamine (< 2.0 mM).
[a] x and y show concentration in mM and current density as μA/cm^2, respectively.

Table 2
Effects of other carbohydrates to electrode response in the presence of maltose.

Substrate	AG/PyOx/CHIT		AG/PyOx/CHIT–CNT	
	Current* (μA/cm^2)	Recovery %	Current* (μA/cm^2)	Recovery %
Mal (0.5 mM)	0.455	–	0.545	–
Glc (0.1 mM)	1.1	–	1.23	–
Mal + Glc	1.565 ± 0.007	100	1.789 ± 0.015	101
Gal (0.1 mM)	0.4	–	0.7	–
Mal + Gal	0.862 ± 0.002	101	1.252 ± 0.006	101
Man (0.5 mM)	–	–	–	–
Mal + Man	0.455 ± 0.007	100	0.545 ± 0.008	100
Fru (0.5 mM)	–	–	–	–
Mal + Fru	0.457 ± 0.009	100	0.544 ± 0.006	100
Xyl (0.1 mM)	0.5	–	0.688	–
Mal + Xyl	0.961 ± 0.001	101	1.234 ± 0.009	100
GlcNAc (0.5 mM)	–	–	–	–
Mal + GlcNAc	0.455 ± 0.008	100	0.545 ± 0.007	100
Lac (0.5 mM)	–	–	–	–
Mal + Lac	0.482 ± 0.025	106	0.544 ± 0.006	100
Suc (0.5 mM)	0.160	–	0.170	–
Mal + Suc	0.632 ± 0.011	103	0.724 ± 0.005	101
Cel (0.5 mM)	–			
Mal + Cel	0.457 ± 0.010	100	0.541 ± 0.001	99

Glc: Glucose, Gal: Galactose, Man: Mannose, Fru: Fructose, Xyl: Xylose, GlcNAc: N-acetyl D-glucosamine, Lac: Lactose, Sac: Saccarose, Mal: Maltose, Cel: Cellobiose.
*All measurements were repeated three times and reported as average ± standard deviation.

3.5. Sample application

In beer samples the main disaccharides are maltose (ca. 14% of total wort carbohydrate) and sucrose (ca. 5% of total wort carbohydrate). Much of the sucrose emanates from malt itself, whilst ca. 97% of the maltose present is produced in the mash tun. Typical levels of maltose found in beer are 0.7–3.0 g/l [48]. Table 3 depicts maltose amounts analyzed by using both biosensing and spectrophotometric DNS method in two brands of beers. Data were given as the average of three measurements and found to be in good agreement with DNS method. Additionally, standard addition method was used to test the effect of matrix nature and no influence was noticed on the response (Table 4).

4. Conclusion

In the present study, design of a new bi-enzymatic system was described and applied for maltose analysis in beer samples without requiring any pretreatment. The construction procedure is simple and fast. Proposed biosensor exhibits good sensitivity and accuracy which are necessary for any analytical methodologies. It is clear that the system may provide a useful bio-monitoring procedure also for the beer fermentation process.

Table 3
Application of biosensors to beer samples.

Sample	Maltose (g/L)		
	DNS method*	AG/PyOx/CHIT*	AG/PyOx/CHIT–CNT*
Beer, Brand I	1.335 ± 0.049	1.435 ± 0.007	1.490 ± 0.042
Beer, Brand II	0.945 ± 0.021	0.973 ± 0.003	0.970 ± 0.017

*All measurements were repeated three times and reported as average ± standard deviation.

Table 4
Maltose detection in beer samples by standard addition method.

Biosensor	Sample	Maltose in beer* (C_0) (g/L)	Added maltose* (C_{add}) (g/L)	Found maltose* (C_{tot}) (g/L)	Recovery % ($C_0 + C_{add}/C_{tot}$)
AG/PyOx/CHIT	Beer, Brand I	1.435 ± 0.007	1.890 ± 0.040	3.320 ± 0.042	100
	Beer, Brand II	0.973 ± 0.003	1.890 ± 0.040	2.875 ± 0.062	100
AG/PyOx/CHIT–CNT	Beer, Brand I	1.490 ± 0.042	1.930 ± 0.028	3.640 ± 0.014	94
	Beer, Brand II	0.970 ± 0.017	1.930 ± 0.028	2.910 ± 0.056	100

*All measurements were repeated three times and reported as average ± standard deviation.

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