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Application of oxygen-independent biosensor for testing yeast fermentation capacity

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ABSTRACT

The pyrroloquinoline quinone (PQQ)-dependent soluble glucose dehydrogenase based carbon paste electrodes were investigated and applied for glucose monitoring in the oxygen deficient media. Reagentless biosensors possessing a wide linear range (up to 5 mM glucose with a detection limit of 0.12 mM) were designed. The oxygen-insensitive response of the biosensor creates the opportunity to use it as a flow-through device for continuous monitoring of glucose in media during the wine yeast fermentation process. The analysis of glucose assimilation rate by yeast strains using the developed biosensor correlated well (R^2 = 0.9938) with convenient yeast testing methods.

1. Introduction

Numerous amperometric mediated biosensors have been constructed and commercialized during the last 2–3 decades and huge efforts towards developing and improving the performance of such systems have been made. Commercially available biosensors still have drawbacks, especially in controlling fermenters. Thus, the application of glucose sensors based on glucose oxidase (GOD) is problematic, since glucose measurement is often interfered with dioxygen due to the competition of the mediator with O_2 for reduced GOD. The Michaelis constant of GOD for O_2 is approximately 0.2 mM (Nakamura et al., 1976) (this value is close to the limit of oxygen solubility) resulting in the sensor being susceptible to O_2-concentration-dependent drift.

Dehydrogenases, which do not utilize O_2 as an electron acceptor, are promising candidates as electrocatalysts for O_2-insensitive biosensors. Three types of dehydrogenases have been applied for glucose biosensors. Two of them were nicotinamide adenine dinucleotide (NAD^+) (Bremle et al., 1991) and pyrroloquinoline quinone (PQQ) (Duine, 1999) dependent glucose dehydrogenase (GDH). However, the necessity for a free-diffusing coenzyme NAD^+ or a free-diffusing redox mediator caused tight restrictions for the application of these sensors (Matsushita et al., 1980, Tang et al., 2001). There are three groups of PQQ-GDHs: a soluble nonspecific aldose sugar dehydrogenase purified from *Escherichia coli* (Southall et al., 2006), a soluble (periplasmic) glucose dedydrogenase (sPQQ-GDH) from *Acinetobacter* (Dokter et al., 1986) and membrane-bound quinoprotein glucose dehydrogenase (mPQQ-GDH) (Matsushita et al., 1980). The latter enzyme exhibits high glucose selectivity, but requires suitable detergents for solubilization. In addition, the stability of this enzyme was low due to the easy dissociation of coenzyme from the active centre (Matsushita et al., 1980). sPQQ-GDH had low substrate specificity and thermal stability, though some attempts were made to improve these features by protein engineering (Igarashi and Sode, 2003; Igarashi et al., 2004; Laurinavičius et al., 2004).

The third type of GDH is FAD-dependent glucose dehydrogenase (FAD-GDH) discovered recently, which exhibits high substrate specificity, insensitivity to O_2 and does not require additional cofactors for activity (Tsujimura et al., 2006a,b).

A direct electron transport (DET) from the enzyme active centre to the solid electrode was reported for several glucose biosensors containing nanowires as a sensing material (Yogeswaran and Chen, 2008). Recently, the carbon paste electrodes prepared from specially modified carbon containing a high amount of the ionogenic acidic functional groups and exhibiting carbon nanotubes (CNT)-like features have been developed and used for DET from mPQQ-GDH or sPQQ-GDH to the electrode surface (Razumienė et al., 2005, 2006).

In this work, we describe an application of the glucose biosensor combining the unique features of soluble PQQ-GDH (enhanced stability due to the tightly bound PQQ cofactor and high catalytic

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efficiency (Matsushita et al., 1989; Laurinavičius et al., 2003)) with the modified carbon paste demonstrating the CNTs-like features (Razumienė et al., 2005). The aim of the paper was the application of device based on specially modified carbon black electrodes and enzyme PQQ-glucose dehydrogenase for glucose analysis in specific environment. A novel composition of enzymatic membrane with prolonged linear range was employed for creating the reliable easy-to-use device for the on-line glucose monitoring. We present an optimized construction and data on the application of the biosensors in microbiological media with unstable concentration of oxygen under real conditions, as well as the comparison with the results obtained with the GOD-based sensor. The biosensor with high sensitivity was employed for the analysis of glucose assimilation in wine yeast ferment medium.

2. Materials and methods

2.1. Enzymes and chemicals

The soluble glucose dehydrogenase was extracted and purified from *Acinetobacter calcoaceticus* LMD 79.41 according to the known protocol (Matsushita et al., 1989). The activity of the purified enzyme was about 11,000 U/ml. Glucose oxidase from *Aspergillus niger*, (activity 84 U/mg), yeast extract powder and peptone were obtained from Sigma–Aldrich (Steinheim, Germany). D-Glucose was obtained from Ceretor (Germany).

Saccharomyces cerevisiae yeast strains α′1 (*MATα, leu2–2 [kil–0]*), Romanesti K100 (RomK–100) (wt, *HM/HM [kil–K2]*), M437 (wt, *HM/HM [kil–K2]*) and K28 have been described previously (Servienė and Melvydas, 2001; Meškauskienė and Melvydas, 2007). New yeast strains 1 M, 55B, I_2, 55R, N166K and 137K were isolated from spontaneous fermentation of grapes, apples, cloudberries and blackberries. All the tested strains were cultivated at the Institute of Botany, Lithuania. Standard yeast cultivation medium (YEPD) without glucose consists of 1% of yeast extract and 2% of peptone.

All aqueous solutions were prepared in bi-distilled water purified in a Purator-Bi Glass Keramik (Berlin, Germany). All experiments were carried out in 0.01 M potassium phosphate, pH 7.0, containing 0.1 M potassium chloride (phosphate buffer), or 0.01 M sodium acetate, pH 6.0, containing 0.1 M potassium chloride and 1 mM $CaCl_2$ (acetate buffer).

2.2. Preparation of the biosensors

Carbon black (CB) was synthesized from carbon monoxide and subsequently modified as described previously (Razumienė et al., 2005). The obtained batches of graphitized CB were examined by means of microscopy, acid-base titrations and nephelometry. The micrographs of the modified CB were investigated by SEM. Typical morphology of CB is presented in Fig. 1. It was determined that the modified CB sample suitable for biosensor design contains a fine fraction of 69.3% with an average diameter of the carbon particles between 20 and 100 nm. CB powder was mixed with the pasting liquid consisting of 10% polyvinyl dichloride in acetone and used for designing the screen-printed paste electrodes.

The CB based screen-printed electrodes were manufactured on polyethylene terephtalate (PETF) film using a screen printer. A silver track of MINICO M 4200 paste (10 μm thick) and insulating film were deposited according to the previously described method (Razumiene et al., 2001). The insulating film with opening window diameter 2 mm covering the carbon paste film defined the reaction area. The sensor (35 mm × 10 mm) contained the working electrode of 3.2 mm^2. The electrodes made of modified CB were denoted as carbon paste electrode (CP) hereafter.

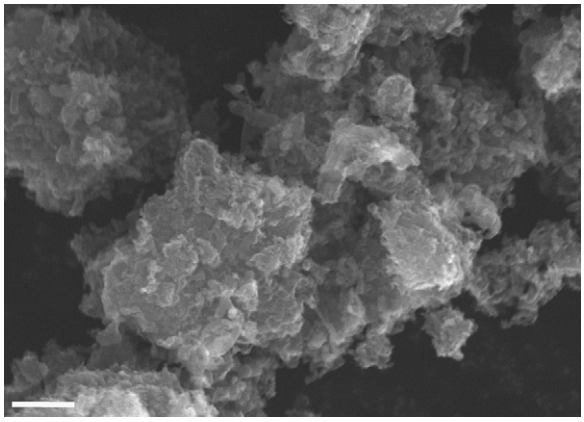

Fig. 1. SEM image of modified CB particles. White bar corresponds to 1 μm.

The preparation of a multiple film-coated electrode consisting of carbon paste, GDH and polymer mixture (polyvinyl acetate and cellulose diacetate) layers (CP-GDH/PVA-DAC) was carried out in the following way. As the protective layer lavsan film perforated by argon nucleus obtained from Dubna (Russia) was used. The film has a thickness of 10 μm and pore density of 1×10^8 pores/cm^2. Implicate holes with a diameter of about 1 μm were etched in alkaline solutions (Mchedlishvily and Flerov, 1987). The lavsan film was pre-treated by dropping 2 μl of a mixture of cellulose diacetate (DAC) (1% in acetone) and polyvinyl acetate (PVA) (50% suspension in bi-distilled water) (1:1, v/v). Finally, 3 μl of PQQ-GDH solution (1100 U/ml) prepared in acetate buffer (pH 6.0) were dropped on this layer and held under glutaraldehyde (GA) vapour (above 25% GA water solution) for 30 min. The resulting membrane was deposited on the CP electrode surface and fixed in the cell holder.

For comparison tests, PQQ-GDH was immobilized on the CP electrode surface by simple adsorption of 3 μl of enzyme solution (1100 U/ml) for 30 min. Then the electrode with the adsorbed enzyme (CP-GDH) was covered with the lavsan film.

The CP-GOD electrode was prepared by adsorbing 2–4 U of GOD from the phosphate buffer solution on the surface of the CP electrode. The lavsan membrane was also used—to cover the electrode surface.

2.3. Electrochemical measurements

All electrochemical measurements were performed using a polarographic analyzer PGZ 402 (“Radiometer Analytical”, France) equipped by a home-made three-electrode system. The biosensor was installed into the flow-through cell made from polymethyl methacrylate, where a working electrode forms the bottom of the flow cell. We used a silver wire (0.5 mm diameter and 2 cm length) in saturated KCl as a reference electrode. All potential values indicated in the text have been estimated *vs.* this reference electrode. As a counter electrode, a titanium tube (diameter 2 mm and 2 cm length) was used. 0.05 M acetate buffer (pH 6.0) containing 1 mM of Ca^{2+} and 0.1 M KCl was used as a default buffer. The currents of the biosensors were recorded using the polarographic analyzer. Dependences of the experimentally measured currents (I) on concentrations of substrates (S) were fitted as a hyperbolic function:

$$I = \frac{I_{max}^{app} S}{(K_M^{app} + S)},$$

where I_{max}^{app} denotes the apparent maximum current and K_M^{app} denotes the apparent Michaelis constant.

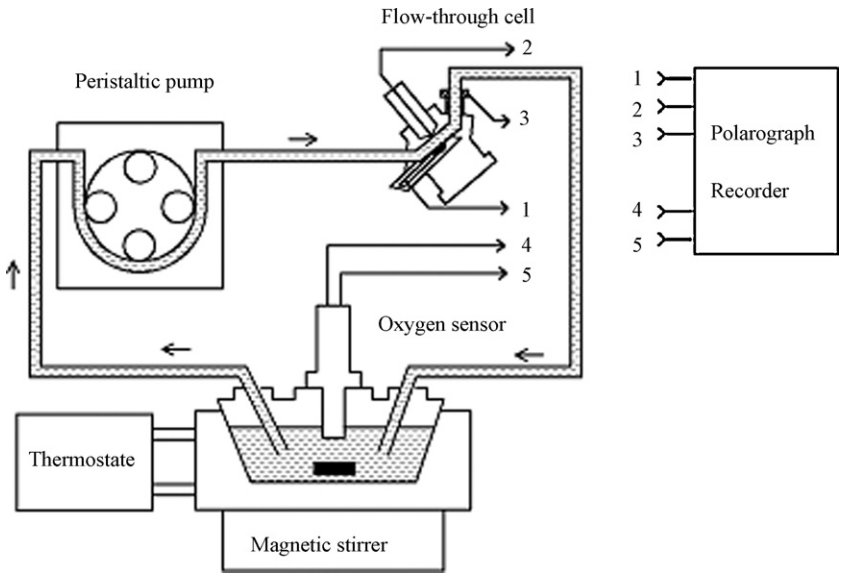

Fig. 2. Scheme of continuous glucose monitoring during wine yeast fermentation in thermostating batch. Working electrode (1) polarized at 0.4 V *vs.* reference Ag/AgCl electrode (2) and auxiliary Ti electrode (3). Pt working electrode of oxygen sensor (4) was kept at −0.6 V *vs.* reference Ag/AgCl electrode (5).

2.4. Measurements in the continuous mode

For measurements in the continuous mode, the analytical system was designed as shown in Fig. 2. The cell with the biosensor was connected via peristaltic pump to 10 ml thermostated batch equipped with a Clark-type oxygen electrode. The flow rate was 1.2 ml/min. Yeast suspension was thermostated in the batch, circulated through the biosensor and continuously analysed. The measurements were carried out at 0.4 V. Before the measurements were performed, the biosensor was allowed to settle to a stable current by a flow of the acetate buffer over a period of approximately 30 min.

2.5. Wine yeast fermentation conditions

Wine yeast culture collected from the surface of agar media was suspended in the acetate buffer (A_{600} approx. 0.5). 10 ml of the suspension was placed into the thermostated batch (Fig. 2), and 4 mM glucose was added. The signals of the sensor were registered alongside with the measurement of oxygen concentration. For the control, another sample of yeasts containing 4 mM of glucose was stored at the same temperature in an open glass vessel. Glucose concentration in the control yeast suspension was measured by reference method using a glucose analyser "Eksan-G" (Analita, Lithuania) (Kulys et al., 1989).

Fermentative efficiency tests of wine yeasts using a conventional method were accomplished in 250 ml flasks containing 150 ml of apple juice (9%, diluted concentrate), vitamins (2 μg/l biotin and 0.2 mg/l thiamin), ammonium chloride (1 g/l), at pH 3.4, and 25 °C under anaerobic conditions. The initial concentration of glucose in the medium was 86.2 mM and total sugar concentration was 9.7%. The initial optical density of yeast cell suspension was 0.4. After one week of fermentation, the residual amount of sugar in the yeast fermentation medium was determined according to the standard 3,5-dinitrosalycilic acid (DNS) method (Miller, 1959). The fermentation efficiency was also estimated visually judging by the appearance of CO_2 in ten-day period, after using the method described earlier (Melvydas et al., 2006).

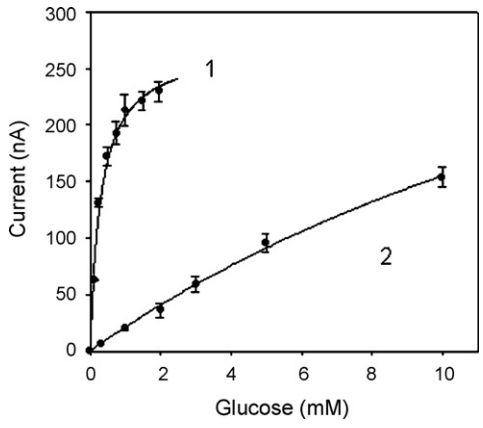

Fig. 3. Calibration curves of glucose biosensors. 1—CP-GDH biosensor, 2—CP-GDH/DAC-PVA biosensor.

3. Results and discussion

3.1. Biosensor with prolonged calibration curve formation and characterization

The current responses of the CP-GDH and CP-GDH/PVA-DAC glucose biosensors were investigated as a function of glucose concentration and typical calibration curves of both biosensors are shown in Fig. 3. When PQQ-GDH was adsorbed on the screen-printed CP electrode the very fast response of the biosensor was observed. The steady-state conditions were reached after approximately 20 s. The CP-GDH biosensor showed a short linear range (Fig. 3, curve 1), up to 0.3 mM, sensitivity of the CP-GDH biosensor in this range was 508 ± 23 nA/mM. The K_M^{app} for glucose calculated for the CP-GDH sensor was 0.3 mM, which is in the same range as Michaelis constant of the native enzyme (0.23 mM, in reaction with 2,6-dichlorophenol indophenol as the electron acceptor (Matsushita et al., 1989)). Due to the low K_M^{app}, this biosensor was not suitable for continuous monitoring of the cultivation medium where glucose concentrations might reach 10 mM. In order to extend the linear range of the biosensor response, additional layers were applied in order to create a substrate diffusion-limiting barrier. Extra cellulose diacetate and PVA layers prolonged the linear range, and the calibration curve exhibited a linear relationship with the concentration of glucose in the range from 0.33 mM to

5 mM with a detection limit of 0.12 mM (estimated at S/N = 3) and the correlation coefficient of 0.9993 (Fig. 3, curve 2).

K_M^{app} for glucose calculated for the CP-GDH/PVA-DAC sensors increased up to 12–16 mM. The repeatability of the biosensor varied in a wide range; however, employing the daily biosensor calibration, the response to glucose can be registered within 5% accuracy. Standard deviation of glucose determination during 5 h in the 3.8 mM solution was 3.23%. The freshly prepared biosensor showed a sensitivity of 450 ± 8.5 nA/mM.

Biological media often contain a number of electrochemically active compounds that might affect the performance of an electrochemical biosensor. An additional diffusive membrane restricts the mass-transport of these compounds, especially those of anionic nature, to the electrode surface and, thereby, improves the selectivity of the biosensor. The tests showed that a blank signal of the developed sensors did not exceed 2 nA in the YEPD yeast cultivation medium without glucose. Moreover, a random noise does not exceed 5% of the working signal in the presence of 4 mM glucose.

It should be stressed that all the biosensors developed in this study worked without any additional redox mediators. As it has been shown in our previous work, some quinohemo-enzymes can directly transfer electrons to the solid electrode due to the formation of nanostructures and cones observed for this type of modified carbon (Razumienė et al., 2005). It is known that the direct electron transfer between PQQ-GDH and electrode is possible by using modified CB as the material for the screen-printed electrode, probably due to the formation of redox groups during the modification procedure. Such functional groups as phenolic ones of an amount of 0.86 ± 0.02 mM/g of CB were determined (Razumienė et al., 2006). Possibility the creation of a biosensor without any soluble mediator for electron transportation opens new opportunities to develop reagentless analytical systems for assays of untreated real samples.

3.2. The stability of the glucose biosensors

The stability of the biosensors was evaluated as both storage and working conditions.

The stability of the biosensor under storage conditions (acetate buffer, pH 6.0, 4 °C) was investigated using the same buffer containing 4 mM of glucose. Soluble PQQ-glucose dehydrogenases are less stabile than membrane-bound (Laurinavičius et al., 2004). However, soluble enzymes possess much higher activity. It means, that biosensor will operate in kinetic mode of action. This assumption was confirmed experimentally. The response of the biosensor changes exponentially, with a rate constant 0.0015 h^{-1} (R^2 = 0.9892). It means, that proper conditions of the immobilization procedure increases stability of the enzyme more than 30 times (Laurinavičius et al., 2004). The half-life of the biosensor is about 460 h, even so, after 48 days the response to 4 mM of glucose was higher than 100 nA (approximately 15% of the initial current). Kinetic mode of the biosensor action, thereby exponential decrease of the biosensor stability, is more predictible than diffuse mode of the biosensor action and better fits to the requirements of the continuous application in real samples. During daily biosensor calibration, glucose readings can be registrated up to three months. The observed decrease of the response current is related to the decrease of the enzyme activity during storage and the fouling of the enzyme electrode. The leakage of PQQ from the active centre of the enzyme is a most believable reason of the fast initial inactivation of these biosensors (Oubrie et al., 1999).

A very important parameter of biosensor applicability is the operational stability. The performance of the biosensor might be affected by the formation of gluconic acid, which can accumulate under the negatively charged membrane, and which can change the pH under the membrane or exhibit a product inhibition behaviour.

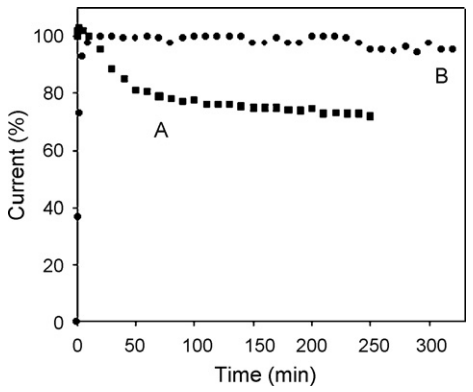

Fig. 4. Operation stability of glucose biosensor at room temperature. Continuous monitoring of YEPD medium containing about 5 mM of glucose with CP-GDH biosensor (A) and CP-GDH/DAC-PVA biosensor with diffusion layer (B).

Both effects can reduce the activity of the enzyme. In the case of the CP-GDH biosensor without any protective membrane, various chemical compounds including proteins may as well affect the stability of the biosensor (Linke et al., 1999). In order to elucidate this in our set-up, a YEPD medium sample containing approximately 5 mM of glucose was monitored in the continuous mode using both types of biosensors with and without the polymeric diffusion layer. The obtained data are shown in Fig. 4. During the first hours of exploitation, the sensitivity of the CP-GDH sensor decreased exponentially, with the constant of inactivation 0.0022 ± 0.003 h^{-1}, probably due to the desorption of a weekly bound enzyme. The immobilized PQQ-GDH-based biosensor showed much better initial stability. The rates of inactivation of both sensors were similar: 1.8 and 1.2% per hour (Fig. 4A and B, respectively). As could be expected, the protecting diffusion layer improved the operational stability of the biosensor.

3.3. Application of the biosensor for continuous wine yeast medium monitoring

In order to demonstrate the applicability of the CP-GDH/PVA/DAC biosensor for the continuous glucose measurement in solutions with unstable concentrations of oxygen, several experiments were carried out. Wine yeast *S. cerevisiae* fermentation medium was used as a model. Yeast suspension (optical density at 600 nm—0.5) was stirred in the presence of 6 mM glucose in acetate buffer at 25 °C as shown in Fig. 2. In order to analyse glucose and oxygen concentration, the glucose biosensor and O_2 sensor were applied on a closed loop flow-through cell.

Simultaneously, the glucose uptake was controlled by the reference method. The results depicted in Fig. 5 demonstrate that the analysis of glucose by the biosensor was independent of O_2 concentration in the chamber. During the first two hours, oxygen concentration dropped about 5 times. The rates of glucose consumption measured with the biosensor and using the reference method were close (0.03 and 0.026 mM/min, respectively). It means that the PQQ-GDH-based biosensor can be directly integrated into the reactor and used to control the level of glucose on-line.

To demonstrate the limitations of glucose-oxidase-based biosensors, the CP-GOD-based biosensor was installed into the close-loop system. In aerated buffer a calibration plot of the CP-GOD biosensor is linear to up to 4 mM of glucose. The signal of the CP-GOD biosensor registered in media with reduced oxygen concentrations decreased distinctly in a few minutes (Fig. 5), and there were no correlation between glucose concentration in the reactor and CP-GOD biosensor response. These data confirm the conclusion, as was known and pointed out in many publications before,

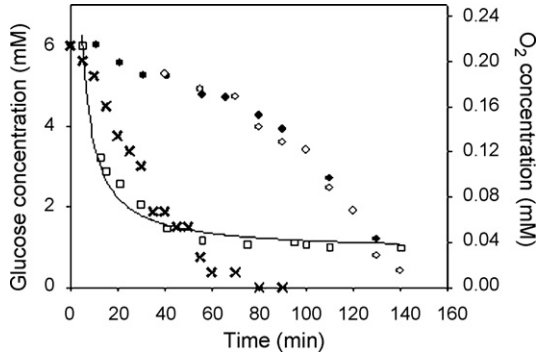

Fig. 5. On-line monitoring of glucose decrease in yeast (Rom K-100) suspension with 6 mM of glucose initial concentration in 0.05 M acetate buffer, pH 6.0 containing 0.1 M of KCl. Closed circles—glucose decrease measured with glucose analyzer; open circles—response of CP-GDH/DAC-PVA biosensor; crosses—response of CP-GOD biosensor; rectangle—changes of oxygen electrode response.

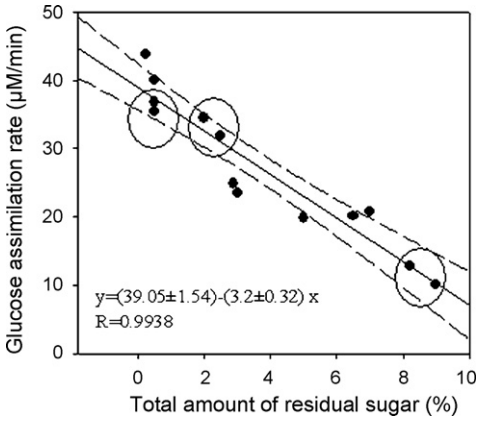

Fig. 6. Correlation between CP-GDH/DAC-PVA biosensor data and residual sugar concentration in fermented apple juice determined by 10 different yeast strains. Data joined with circle—the same strain of yeast.

that oxygen-dependent biosensors are not suitable for the direct operation in reactors with unstable oxygen concentration.

3.4. Evaluation of fermentative capability of killer yeast strains

The primary selection criteria for fermentable industrial yeasts are good conversion of sugar into alcohol and carbon dioxide, total time and low temperature of fruit juice fermentation (17–21 °C), and rather high ethanol tolerance. Particular attention was paid to the use of killer yeasts as selected starters in the fermentation processes. Killer strains guarantee competitive advantages over the starter ethanol making yeast. Many fermentative processes use non-pasteurised media, which can allow the predominance of wild yeast strains coming from the raw material outnumbering the starter yeast. Those contaminations can hamper the fermentation and decrease ethanol productivity. The killer system may be a way to avoid the effects caused by undesirable yeasts in the fermentative processes (Palpacelli et al., 1991; Chen et al., 2000). While screening of yeast strains suitable for wine and ethanol production, it is very important to have a quick and easy method for yeast fermentation capacity tests.

Fermentation efficiency of yeast was commonly estimated by several methods: on the basis of CO_2 volume produced in a ten-day period, and estimating the residual glucose concentration or amount of total sugar remaining after the fermentation for one week. Both methods are out of date and consume more time. We investigated the possibility of yeast strains selected from naturally occurring communities of Lithuanian fruits and berries (strains 1 M, 55B, I_2, 55R, N166K, 137K) and the standard *S. cerevisiae* killer strains Rom K-100, M437, α′1 and K28 to ferment apple juice. After one week of fermentation, the residual amount of sugar in the yeast fermentation medium was determined according to the standard 3,5-dinitrosalycilic acid (DNS) method (Miller, 1959).

The same yeast strains were tested for fermentation efficiency on the basis of the rate of glucose assimilation measured with the PQQ-GDH-based biosensor. The yeast suspension was thermostated at 25 °C in a flow-through batch and circulated through the glucose biosensor (Fig. 2) during 30–40 min. The data obtained by the measurement of glucose uptake by the yeast suspension in the presence of 5 mM glucose were compared with the same yeast strain fermentation efficiency measured by common methods. The data are summarized in Fig. 6. Glucose consumption rate in the modelling batch correlates with the residual sugar concentration in the fermented juice. The results show affirmative correlation between the rate of glucose assimilation and fermentation efficiency ($R = 0.9938$). According to the results, the best strains 1 M and K28 were selected for a field trial. It has been shown that 1 M strain in apple juice and sugar mixture produce ethanol up to 18% and is a promising strain to be used in ethanol industry. Therefore, the results imply that this biosensor-based method may be helpful to quickly and easily evaluate yeast fermentation possibilities.

4. Conclusions

The reagentless glucose biosensor combining the PQQ-dependent soluble glucose dehydrogenase, carbon black-modified paste electrode and the polymer membrane for extension of linearity have been designed. The biosensor exhibited linear response range up to 5 mM of glucose and considerably improved both the sensitivity and stability. It was revealed, that the signal of the biosensor was independent of oxygen concentration in the sample.

It was demonstrated, that the biosensor can be used for prolonged continuous monitoring of glucose in yeast fermentation media under reduced or unstable concentration of oxygen. The enzyme immobilized under mild conditions was stable during several months. The analysis of glucose assimilation rate by yeast strains using the developed biosensor correlated with convenient yeast testing methods.

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