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RESEARCH ARTICLE

BOD biosensors for pulp and paper industry wastewater analysis

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Abstract
Introduction Two semi-specific microbial biosensors were constructed for the analysis of biochemical oxygen demand (BOD) in high-cellulose-content pulp and paper industry wastewaters. The biosensors were based on living cells of *Bacillus subtilis* and *Paenibacillus* sp. immobilized in an agarose gel matrix. Semi-specific microorganisms were isolated from various samples (decaying sawdust and rabbit manure) and were chosen based on their ability to assimilate cellulose.
Materials & methods The biosensors were calibrated with the Organization for Economic Cooperation and Development synthetic wastewater, and measurements with different wastewaters were conducted.
Results The response time of biosensors using the steady-state method was 20–25 min, and the service life of immobilized microorganisms was 96 days. Detection limit was 5 mg/l of BOD_7 while linear ranges extended up to 55 and 50 mg/l of the BOD_7 for *B. subtilis*- and *Paenibacillus* sp.-based biosensors, respectively. Repeatability and reproducibility of both biosensors were within the limits set by APHA—less than 15.4%. In comparison, both biosensors overestimated the BOD_7 values in paper mill wastewaters and underestimated the BOD_7 in aspen pulp mill wastewater.
Conclusions The semi-specific biosensors are suitable for the estimation of organic pollution derived from cellulose, while the detection of pollution derived from tannins and lignins was minor. Better results in terms of accuracy and repeatability were gained with *Paenibacillus* sp. biosensor.

Keywords Biochemical oxygen demand · BOD · Biosensor · Semi-specific biosensor · *Bacillus subtilis* · *Paenibacillus* sp. · Pulp and paper industry · Industrial wastewater

1 Introduction

The pulp and paper industry has been considered to be a major consumer of natural resources (wood, water) and energy (fossil fuels, electricity) and a significant contributor to pollutant discharges into the environment. The paper manufacturing generates significant quantities of wastewater, as high as 60 m^3/t of paper produced (Thompson et al. 2001). This kind of industrial wastewater is very heterogeneous and can potentially be very harmful to nature (Maximova and Dahl 2007). The major water pollutants are wood polymers, fillers, process and auxiliary chemicals, and their reaction products in the form of suspended solids and colloidal and dissolved matter. Concentrations of wood extractives in discharged wastewaters from kraft pulp mills and mechanical pulp mills vary between 0.4 and 11 kg per ton of pulp. Therefore, monitoring of pollution in pulp and paper industry wastewater is essential.

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Organic pollution in wastewaters, effluents, and polluted waters can be characterized using oxygen demand of water samples. A widely used universal parameter for this purpose is biochemical oxygen demand (BOD). The BOD measures the amount of oxygen required for the biochemical degradation of organic material and oxygen used to oxidize inorganic material. The determination of BOD is an empirical test in which standardized laboratory procedures are used to determine the relative oxygen demand of wastewater samples. The test requires 5 or 7 days of incubation under specified conditions, and BOD is computed on the basis of dissolved oxygen concentration initially and after incubation of the sample (APHA 1985). The method is a universal method to analyze various types of samples and requires no expensive equipment. However, due to prolonged incubation time, it is not suitable for the monitoring of wastewater treatment systems where fast feedback is necessary.

The faster and more effective method for BOD measurements, based on microbial biosensors, was first reported in 1977 (Karube et al. 1977). Since then, different types of BOD biosensors have been developed (Kwok et al. 2005; Nakamura et al. 2007; Oota et al. 2010; Wang et al. 2010), but the biofilm-type biosensors have remained the main type of BOD sensors. These biosensors generally utilize Clark-type dissolved oxygen sensor as an electrochemical transducer and a membrane containing immobilized microorganisms as a biological recognition element (Thévenot et al. 2001). The biosensor measures the respiration of immobilized microorganisms as the dissolved oxygen and substrate that diffuse into the microbial membrane. As the substrate is added into the system, the respiration rate of microorganisms increases and oxygen concentration measured with the dissolved oxygen sensor decreases. The change in biosensor output signal can be correlated with substrate concentration, and on the basis of that, BOD can be calculated.

The BOD values as observed by the BOD biosensor reflect the concentrations of the dissolved organic substances which are assimilated/metabolized by the immobilized microbes (Rastogi et al. 2003b). Within the short measurement time of a biosensor, only fast and easily degradable compounds are assimilated and detected by the biosensor. In case of specific industrial wastewater samples, the microorganisms used in the biosensor might not be able to utilize high molecular weight and refractory compounds. On the other hand, these compounds can be measured with BOD_7 analysis. This kind of difference in methods causes disagreement between sensor-measured BOD and BOD_7 values. Earlier studies on BOD biosensors for wastewaters containing high molecular weight substances have used specific enzymes or sample pretreatment to enhance the poor biodegradability of the sample (Chee et al. 2001, 2007; Kwong et al. 1998; Reiss et al. 1998; Tag et al. 2000). In addition, better results have been obtained by selecting microorganisms with low selectivity and wide substrate spectrum (Rastogi et al. 2003a) or microorganisms adapted to certain pollutants present in wastewater (Raud et al. 2010). The importance of pre-testing and selection of microorganisms for the construction of reliable BOD biosensors have been emphasized in previous studies (Dhall et al. 2008; Liu and Mattiasson 2002; Rastogi et al. 2003a).

Biosensors based on a well-chosen microorganism with semi-specific properties have a potential for more precise analysis of specific industrial wastewaters. In addition to universal substrate spectra, semi-specific microorganisms can assimilate some refractory compound or group of compounds specific to that particular microorganism. Therefore, these microorganisms are able to oxidize refractory compounds found in industrial wastewaters. Thus, better correlation of sensor BOD with BOD_7 can be achieved. On the other hand, universal microorganisms use a limited substrate spectrum common to a majority of aerobic heterotrophic microorganisms. On the contrary to semi-specific microorganisms, these microorganisms are not able to assimilate specific refractory compounds. For that reason, these compounds would remain undetected by universal biosensors, and lower BOD values than the actual level will be gained. The application of semi-specific biosensors for the analysis of specific industrial wastewaters has been demonstrated in earlier publications (Raud et al. 2010; Raudkivi et al. 2008), but little attention has been paid to the analysis of pulp and paper industry wastewater.

The objective of this study was to construct a BOD biosensor based on semi-specific microorganisms for the rapid and precise analysis of wastewaters generated in paper production. Since cellulose is the major pollutant in paper industry wastewater (Thompson et al. 2001), pure cultures of cellulose-degrading microorganisms were isolated from various samples and used as a biosensing element in biosensor. Two biosensors based on pure cultures of *Bacillus subtilis* and *Paenibacillus* sp. were assembled, and their suitability for wastewater analysis was tested.

2 Experimental

2.1 Isolation and identification of microorganisms

Samples of decaying sawdust and rabbit manure were collected to isolate microorganisms able to degrade cellulose. Using serial dilutions, the samples were spread on the selective cellulose-containing agar medium (yeast extract, 2 g/l; K_2HPO_4, 1 g/l; $MgSO_4 \times 7\ H_2O$, 5 g/l; carboxylmethyl cellulose (CMC), 5 g/l; NaCl, 2 g/l; agar, 15 g/l). The agarose plates were incubated at room temperature (22±2°C) until colonies developed. Subsequently,

the colonies that were able to degrade CMC and grew rapidly on the given medium were chosen and restreaked on a fresh agar plate. This procedure was repeated until pure bacterial strains were gained.

The isolates were grown in a minimal agar plate (yeast extract, 2 g/l; KH_2PO_4, 1 g/l; $MgSO_4 \times 7\ H_2O$, 1 g/l; CMC, 2 g/l; agar, 15 g/l) for 2 days to screen for cellulolytic organisms. These plates were then flooded with an aqueous solution of Congo red (1 mg/ml) for 15 min and washed with 1 M NaCl to visualize the hydrolysis zones (Apun et al. 2000). *B. subtilis* CE 22.1 (isolated from decaying sawdust) and *Paenibacillus* sp. HTK 158a (isolated from rabbit manure) were chosen due to active digestion of CMC indicated by the clear zone around the colonies.

2.2 Cultivation of microorganisms

Cultivation and immobilization of microorganisms were conducted similarly to our previous work (Raud et al. 2010; Raudkivi et al. 2008). The microorganisms were cultivated under aerobic conditions in a rotating shaker (Sanyo Orbital Incubator, Sanyo) at 210 rpm and 30°C in a Luria-Bertani liquid medium (tryptone, 10 g/l; yeast extract, 5 g/l; NaCl, l, 5 g/l). Two milliliters of culture medium was inoculated and incubated for 14 h; subsequently, the cell suspension was subcultured into 150 ml of culture medium and incubated for 14 h until the late exponential growth phase of the microorganisms. Optical density of the bacterial suspension was measured with a spectrophotometer (HP 4853) to assure that the derived cells were in the late exponential phase of growth (*B. subtilis* OD_{600}=7.63 and *Paenibacillus* sp. OD_{600}=2.83).

The bacterial suspension was centrifuged (Jouan CR3) at 4,000 rpm for 15 min at room temperature, and the supernatant was decanted. To circumvent the culture medium from getting into the membrane, the cells were washed twice with phosphate buffer (pH 6.86; Na_2HPO_4, 6.90 g/l; $NaH_2PO_4\ H_2O$, 7.04 g/l). The washed bacterial paste was immediately used to prepare the microbial membranes.

2.3 Immobilization

The microorganisms were immobilized into an agarose gel matrix to gain microbial membranes used in biosensor construction. The mixture of 180 mg agarose (Type I-A, Low EEO, Sigma-Aldrich, Spain) and 7.5 ml phosphate buffer was heated up to 70°C until complete melting of the agarose. The homogeneous agarose mixture was cooled down to 45°C, and 900 μl of the previously prepared bacterial suspension was added. After mixing, the agarose suspension was spread on three polypropylene net (Scrynel, PP 500 HD) disks. To gain certain and even thickness of membranes, the membranes were placed between two glass plates, and even force was applied until the formation of a homogeneous gel.

2.4 Standard solution

Synthetic wastewater prepared according to the recipe of the Organization for Economic Cooperation and Development (OECD) (peptone, 1.6 g/l; meat extract, 1.1 g/l; urea, 0.3 g/l; K_2PO_4, 0.28 g/l; NaCl, 0.07 g/l; $CaCl_2\ \cdot 2H_2O$, 0.05 g/l; $MgSO_2\ 7H_2O$, 0.02 g/l) was used as a standard solution for the calibration of BOD biosensors (OECD 1984). The BOD_7 of the solution was measured as 2,000 mg/l.

2.5 Pre-conditioning

Studies have shown that pre-conditioning of a newly prepared BOD sensor is necessary for stable and reproducible measurements (Tan and Wu 1999). For pre-conditioning, the freshly prepared membranes were placed in a phosphate buffer solution dosed with OECD synthetic wastewater until the sensor response to the standard solution remained steady at constant value. The pre-conditioning time was 14 days in a solution with 30 mg/l of BOD_7. After pre-conditioning and between measurements, membranes were maintained at 4°C in a phosphate buffer solution.

2.6 Experimental setup of BOD measurements

The BOD biosensor consisted of a Clark-type dissolved oxygen sensor (WTW, CellOx 325, Germany) used as an electrochemical transducer and a membrane containing immobilized microorganisms. The microbial membrane was attached to a dissolved oxygen sensor with a specially designed mounting collar and a holder as shown in Fig. 1b. The biosensor signal was detected using measuring module

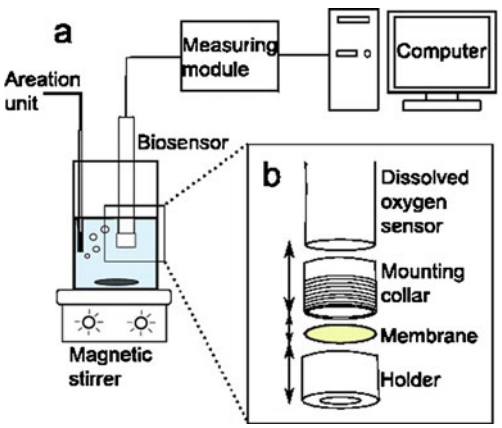

Fig. 1 **a** Schematic presentation of a biosensor measuring system. **b** Biosensor design

InoLab 740 (WTW, Germany) and transformed and saved in a computer using MultiLab Pilot program. An aeration unit and a magnetic stirrer (Magnetmix 2070) were used in order to ensure saturation of the measuring solution with oxygen during the measurement.

2.7 BOD sensor measurements

The measurements were carried out in room temperature (22±2°C) in a beaker containing 50 ml of previously aerated phosphate buffer. Before the biosensor assembly and measurements, the membranes were held in room temperature for 1 h in order to adapt the immobilized microorganisms with room temperature. The biosensor was inserted into an aerated phosphate buffer, and after the initial steady-state signal (I_0) was reached, a certain amount of substrate solution was added. When the biosensor output signal reached new steady state (I_s), the new steady-state signal was recorded. Time between substrate addition and reaching into the new steady state was marked as a response time. Subsequently, the substrate was added to the measuring solution using step-by-step method in order to increase the BOD_7 of the measuring solution gradually. The substrate was added until the output signal of the biosensor reached zero or did not change after the addition of substrate.

The biosensor response at a certain concentration was calculated as a difference between the initial and final steady-state signal ($\Delta I = I_0 - I_s$). The results were normalized by dividing biosensor response at a certain concentration with an initial output signal ($NSR = \Delta I / I_0$). To a certain extent, the normalized signal response is proportional to the BOD_7 of the sample and therefore can be used to compile calibration graphs and conduct measurements.

2.8 Wastewater samples

Experiments with synthetic and industrial wastewater samples were conducted to characterize the suitability of biosensors to analyze pulp and paper industry wastewater. Real industrial wastewater samples were taken from paper mill (Räpina, Estonia) and aspen pulp mill (Kunda, Estonia) outflows. The synthetic wastewater sample consisted of OECD synthetic wastewater and an additional refractory compound, and its consistency simulated cellulose-containing wastewater generated in pulp and paper industries. Combined wastewater dosed with CMC was prepared by adding 1 g CMC into 1,000 ml of OECD synthetic wastewater.

2.9 Conventional BOD_7 analyses

Conventional BOD_7 analysis according to APHA (1985) were conducted with all samples.

3 Results and discussion

3.1 Calibration of BOD biosensors

3.1.1 Linear range and sensitivity

Calibration measurements for all biosensors were conducted using the steady-state method and OECD synthetic wastewater as a standard solution (see Fig. 2). Calibration parameter linear range and sensitivity were used to characterize the biosensors. The linear range of the sensor is defined as the substrate range that gives a signal directly proportional to the concentration. The linearity of a BOD biosensor over a certain concentration range is a measure of detection capacity to analyze samples with varying substrate concentrations (Liu et al. 2004). The linear range extended up to 55 and 50 mg/l of BOD_7 for *B. subtilis*- and *Paenibacillus* sp.-based biosensors, respectively. The detection limit of biosensors in the current study was 5 mg/l. Sensitivities measured as a slope in the calibration graphs were 0.01557 (1/(mg/l BOD_7) and 0.01669 (1/(mg/l BOD_7) in the case of the *B. subtilis* and *Paenibacillus* sp. biosensors, respectively.

Linearity and sensitivity are related to the thickness of the microbial membrane and the type and density of the cells immobilized in the microbial membrane. Higher-cell-density microbial membranes are generally more sensitive but have a narrower linear range. Highly sensitive BOD biosensors can be applied for the analysis of very low BOD values while a broad linear range is necessary for BOD assessment over a broad concentration range. Relatively high sensitivity of studied biosensors enables precise measurements, but the selection of proper dilutions in the case of unknown samples with high BOD is essential due to a shorter linear range.

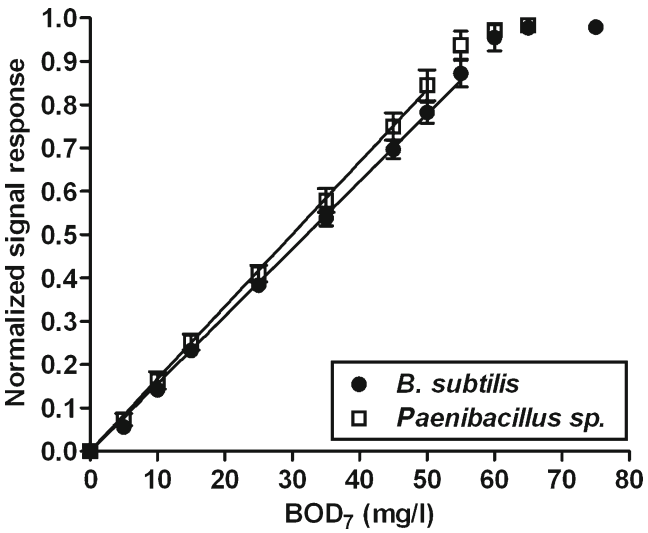

Fig. 2 Calibration graphs of *B. subtilis* and *Paenibacillus* sp. biosensors when OECD synthetic wastewater was used as a standard solution

3.1.2 Response time

Response time of biosensors depends primarily on the applied measuring method. In the case of the steady-state method, response time is defined as the time it takes for the biosensor to reach steady state after substrate addition into the measurement solution. In the initial steady state (BOD_7=0), the diffusion of oxygen through the microbial membrane and consumption of oxygen is in equilibrium, and it is a measure of the endogenous respiration rate of immobilized microorganisms. After substrate addition, the substrate diffuses into the microbial membrane and is assimilated by microorganisms; thereby, the consumption rate of oxygen increases and the output signal of the biosensor decreases. The output signal of the biosensor decreases until a new equilibrium—final steady state—is established. Utilizing the given method in BOD measurements resulted in the response time of 20–25 min.

3.1.3 Stability and service life

Operational stability and service life were evaluated by calibration measurements with OECD synthetic wastewater at BOD_7 value of 25 mg/l. Calibration measurements were carried out with three parallel membranes during service life at least once a week or after every two measurements with wastewater samples (Fig. 3). The stable period was defined as a period where deviation from average sensor response was within the control limits of 15.4% specified by APHA (1985). The stable period allowing reproducible measurements began 15 days after immobilization. The service life of biosensors was 96 days after which an unstable sensor signal was observed, and the membranes were discarded. The sensor signal was reproducible during a stable period of service life with standard deviation of ±5.57% and ±8.85% for *B. subtilis* and *Paenibacillus* sp. biosensors, respectively.

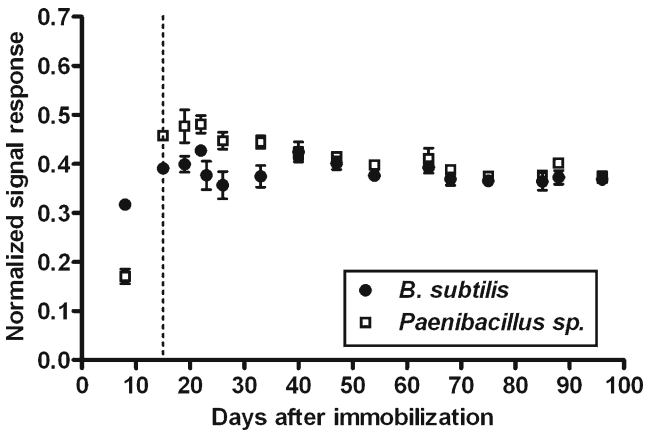

Fig. 3 Stability and service life of biosensors observed by calibration with OECD synthetic wastewater at 25 mg/l BOD_7. The *points* and *error bars* represent the average and standard deviation of three parallel measurements

Experimental results indicated that both biosensors had a sufficiently long service life and good stability for utilization in practical applications. The operational stability and service life of BOD biosensors are primarily related to the stability of the immobilized microorganisms. Therefore, the immobilized bacteria need to be kept well conditioned. Constant supply of oxygen and nutrients can be provided during the measurements. Thus, the stability of biosensors was best when frequent measurements were carried out.

3.2 BOD measurements

3.2.1 Repeatability and reproducibility

Precision of the biosensor depends on two parameters: repeatability and reproducibility. Repeatability is defined as a standard deviation of a sensor tested by one operator under the same conditions, while reproducibility is as a standard deviation of a series of sensors tested by more than one operator under different conditions (Wang et al. 2010). According to the conventional APHA method for BOD measurements using a standard GGA solution (150 mg/l glucose and 150 mg/l glutamic acid), with BOD_5 value of 199.4 mg/l, the relative standard deviation of results—15.4%—is intended for the reference for every laboratory (APHA 1985).

Repeatability and reproducibility of biosensors were evaluated during measurements with OECD synthetic wastewater. Repeatability of *B. subtilis* and *Paenibacillus* sp. biosensors was ±3.2% and ±4.7% (N=5), respectively. In the case of synthetic and industrial wastewater samples, repeatability varied between ±6.8% and ±10.8% (N=5) and between ±4.5% and ±8.6% (N=5) for *B. subtilis* and *Paenibacillus* sp. biosensors, respectively. The reproducibility of biosensors was evaluated conducting measurements with OECD synthetic wastewater using six parallel biosensors. The standard deviation of different biosensors were ±5.3% and ±3.7% (N=6) for *B. subtilis*- and *Paenibacillus* sp.-based biosensors, respectively. The results show that the repeatability and reproducibility of the biosensors used in the current study remained within the control limit of 15.4% set by APHA.

3.2.2 Correlation of BOD_7 and sensor BOD

BOD biosensors were used to analyze the BOD of different synthetic and industrial wastewaters at different BOD_7 values. The synthetic wastewater consisted of OECD synthetic wastewater (also used as a standard solution) which was spiked with CMC. The composition of this wastewater resembled industrial wastewater containing cellulose residues

generated during paper production. The industrial wastewater samples were collected from the circulating water of the paper mill (Räpina, Estonia) and outflow of the aspen pulp mill (Kunda, Estonia).

Figure 4 shows the results of sensor BOD and conventional BOD_7 analyses for different samples. Correlation between measured BOD values using different methods was best in the analysis of synthetic wastewater spiked with CMC. Sensor BOD measured with *B. subtilis* and *Paenibacillus* sp. biosensors overestimated the BOD_7 values by 11.5% and 3.1%, respectively. It has been shown that BOD values lower than the actual level will be gained when non-specific seeding material is used in conventional BOD analysis. Lower BOD values were caused by the partial assimilation of refractory organic compounds which remain undetected (Kumar et al. 2010). CMC added to the OECD synthetic wastewater elevated the BOD_7 of the solution only to a small extent, and therefore, it can be concluded that CMC did not degrade during the 7-day incubation period of BOD_7 analysis and remained undetected. Meanwhile, biosensors based on carefully chosen microorganisms, as in current study, were capable of degrading cellulose and thereby detected CMC added to the solution. Overestimation of BOD_7 indicates that the pollution resulting from dissolved cellulose was detected by the studied biosensors, and better estimation of the pollution was achieved.

In the analysis of industrial wastewater samples, the sensor BOD values of the *Paenibacillus* sp. biosensor were closer to conventional BOD_7 than the sensor BOD gained with the *B. subtilis* biosensor (Fig. 4b and c). *B. subtilis* and *Paenibacillus* sp. biosensors overestimated the BOD_7 of paper mill wastewater by 26.3% and 21.6% while underestimating the BOD_7 of pulp mill wastewater by 13.6% and 4.7%, respectively. The difference in sensor BOD results in pulp and paper mill wastewater analysis can be explained by the different composition of the wastewater samples. Significant differences in the quality and composition of the wastewaters from the pulp- and paper-making operations are due to dissimilarity of the processes. The wastewater generated during paper making contains a substantial quantity of cellulose fines and other additives (up to 50% of the total mass) (Thompson et al. 2001) while the main pollutants in pulp industry wastewater are dissolved wood-derived substances mainly tannins and lignins (Kumar et al. 2010; Maximova and Dahl 2007). The cellulose in paper mill wastewater was detected better with studied semi-specific biosensors than with conventional BOD_7 analysis causing the overestimation of BOD_7. On the other hand, semi-specific microorganisms used in this study had difficulties assimilating tannins and lignins. The rate of oxidation for these substrates was slower than that of the substrates in standard solution; therefore, biosensors underestimated BOD_7.

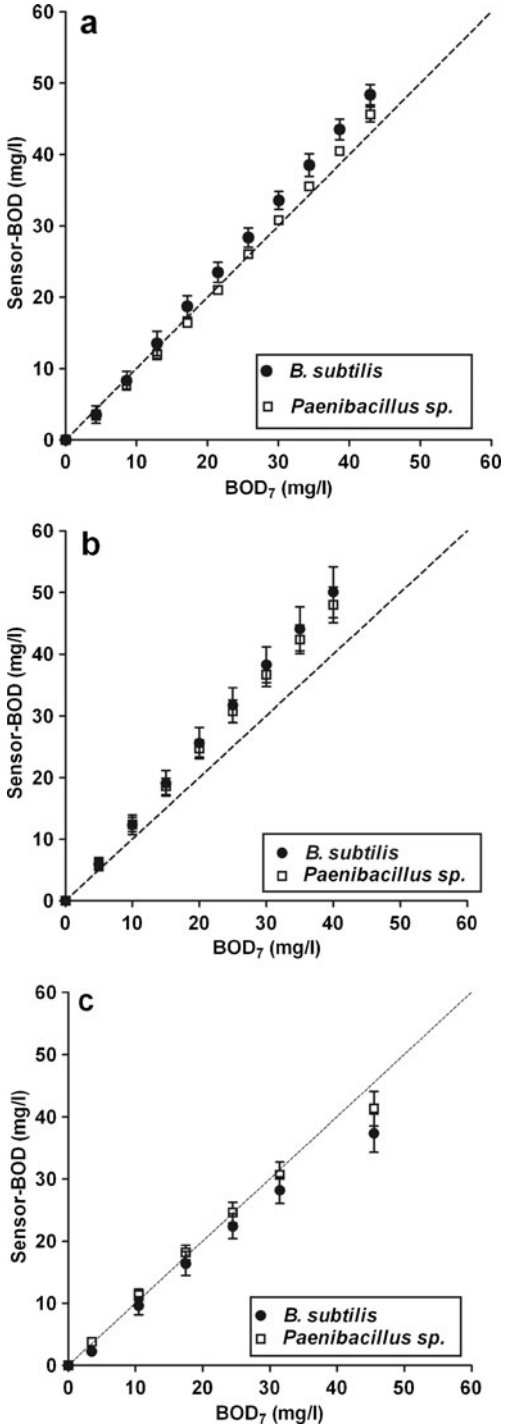

Fig. 4 Correlation between BOD_7 and sensor BOD for *B. subtilis* and *Paenibacillus* sp. biosensors in wastewater samples: **a** OECD synthetic wastewater spiked with CMC, **b** paper mill wastewater, and **c** aspen pulp mill wastewater

4 Conclusion

Simple and reliable semi-specific BOD biosensors were constructed to analyze high-cellulose-content wastewaters generated in paper production. The biosensors were based

on semi-specific bacterial cultures of *B. subtilis* and *Paenibacillus* sp. which were chosen due to their ability to assimilate cellulose.

The results indicate that both developed biosensors are useful tools for the monitoring of the wastewaters generated during paper production. Repeatability and reproducibility of both biosensors were within the limits set by APHA. Better accuracy in the analysis of actual industrial wastewaters was gained with *Paenibacillus* sp. In comparison, both biosensors overestimated the BOD_7 values in paper mill wastewaters. This indicates that a better estimation of organic pollution derived from cellulose is possible using biosensors based on cellulose-degrading microorganisms.

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