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Antimicrobial and antioxidative potential of free and immobilised cellobiose dehydrogenase isolated from wood degrading fungi

Justyna Sulej [a, *], Monika Osińska-Jaroszuk [a], Magdalena Jaszek [a], Marcin Grąz [a], Jolanta Kutkowska [b], Anna Pawlik [a], Agata Chudzik [a, 1], Renata Bancerz [a]

[a] Department of Biochemistry, Maria Curie-Sklodowska University, Akademicka 19, 20-033 Lublin, Poland
[b] Department of Genetics and Microbiology, Maria Curie-Sklodowska University, Akademicka 19, Lublin 20-033, Poland

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ABSTRACT

Cellobiose dehydrogenase (CDH, EC 1.1.99.18) is a glycoprotein having many biotechnological applications. In the present study, CDHs isolated from *Phlebia lindtneri* (PlCDH), *Phanerochaete chrysosporium* (PchCDH), *Cerrena unicolor* (CuCDH), and *Pycnoporus sanguineus* (PsCDH) were studied the first time for their ability to generate antioxidant and antimicrobial agents. The aim of the research was to evaluate the antioxidant and antimicrobial activity of systems composed of four CDHs and lactose or cellobiose as a reaction substrate. The free radical scavenging effect of free and immobilised enzymes was evaluated using the DPPH method. The lowest values of EC_{50} (10.04 ± 0.75 μg/ml) was noted for *Pl*CDH/lactose and for *Pl*CDH/cellobiose (12.06 ± 1.35 μg/ml). The EC_{50}value reached 12.6 ± 1.51 μg/ml in the case of *Ps*CDH/lactose and 15.96 ± 1.35 for *Ps*CDH. The CDH preparations were also effectively immobilised in alginate (the immobilisation efficiency expressed as a protein yield ranged from 61.6 to 100 %). The operational stability expressed as a scavenging effect showed the possibility of using the alginate beads 4 times. Both the free and immobilised CDHs as well as the CDH/substrate were tested against Gram-negative *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and Gram-positive *Staphylococcus aureus* ATCC 25923 bacteria. All samples, except PlCDH, were potentially effective in suppression of bacterial growth. The highest percentage of inhibition (100 %) was obtained for *S. aureus* bacteria using PsCDH and PchCDH with lactose as a substrate, whereas a slightly lesser effect was observed for *E. coli* and *P. aeruginosa* bacterial cells, i.e. 64.1 % and 86.5 % (PsCDH) and 94.1 % and 41.4 % (PchCDH), respectively. Furthermore, the concentrations of the reaction products (aldonic acids and hydrogen peroxide) were quantified and the surface morphology of the alginate beads was analysed using SEM visualisation.

1. Introduction

Cellobiose dehydrogenase (CDH; EC1.1.99.18; CAZy: AA3.1 and AA8) is produced by a number of fungi belonging to the dikaryotic phyla of Basidiomycota (Class I) and Ascomycota (Class II and Class III) (Harreither et al., 2011; Zámocký et al., 2004). The CDH enzyme family is a heterogeneous group of proteins, very important in the modern biotechnology, with sequence homology as low as 35 % (Cameron and Aust, 2001). Therefore, CDH produced by various cultures of fungi varies widely in substrate specificity and properties. In the secretome of wood-degrading fungi, CDH constitutes a considerable fraction of lignocellulolytic enzymes, e.g. 0.5 % in *Phanerochaete chrysosporium* (Samoïlenko et al., 1983), up to 1.2 % in *Trametes* spp (Barbosa et al., 2005), 2.4 % in *Ceriporiopsis subvermispora* (Harreither et al., 2009), 2.2 % in *Sclerotium rolfsii* (Ludwig and Haltrich, 2002), and even 12 % in *Corynascus thermophilus* (Harreither et al., 2011; Sygmund et al., 2012). Cellobiose dehydrogenase is an extracellular flavocytochrome containing two cofactors in its molecule, i.e. a flavin and a heme located in separate domains: a flavodehydrogenase domain (CAZy: AA3.1) carrying a non-covalently bound FAD cofactor and a heme b containing a cytochrome domain (CAZy: AA8) connected via a flexible proteolysis-sensitive linker (Ludwig et al., 2013; Tangthirasunun

* Corresponding author. Fax: +48 81 537 51 02.
E-mail address: justyna.sulej@poczta.umcs.lublin.pl (J. Sulej).
[1] Chair and Department of Neurosurgery and Paediatric Neurosurgery, II Faculty of Medicine with English Language Division, Medical University, Racławickie 1 Street, 20-059 Lublin, Poland.

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et al., 2017). CDH catalyses the oxidation of cellobiose as well as most di- or oligosaccharides linked by beta-1,4-glycosidic bonds, sometimes also monosaccharides, to the corresponding aldono-1,5-lactones with the concomitant reduction of FAD to $FADH_2$ (Bao and Renganathan, 1992; Henriksson et al., 1998). Subsequently, lactones are hydrolysed spontaneously to aldonic acids (such as cellobionic or lactobionic acid) in aqueous solutions. $FADH_2$ is reoxidised by either two-electron acceptors (e.g. quinones, 2,6-dichloroindophenol) or by one-electron acceptors (e.g. polysaccharide monooxygenases, cytochrome c, ferricyanide, and ferrocenium), either directly or via the cytochrome domain (Henriksson et al., 2000; Sygmund et al., 2013). Electrons produced as a result of the described reactions can also be reduced by molecular oxygen and form hydrogen peroxide (H_2O_2) (Tegl et al., 2015).

Both carboxylic acids and hydrogen peroxide are the products of a reaction catalysed by CDH, which is important for application in many biotechnological fields such as pharmaceutical, cosmetics, and food industry or recently in different clinical applications (Nyanhongo et al., 2017). Hydrogen peroxide is a well-known reactive oxygen species with a confirmed antimicrobial potential. It can disturb the structure and permeability of the cell wall or cytoplasmic membrane causing enzyme inhibition, disruption of protein synthesis, impaired energy production, and damage to bacterial DNA (Finnegan et al., 2010; Nyanhongo et al., 2017; Samoĭ;lenko et al., 1983). Available reports have shown that antimicrobial systems based on CDH activity combined with cellobiose and in-situ H_2O_2 generation can be applied for medical uses such as wound dressings for the treatment of chronic wounds (Huber et al., 2017) or as an antibiofilm factor with potential incorporation in different biomaterials such as urinary catheters (Thallinger et al., 2016). Carboxylic acids (cellobionic or lactobionic) produced via cellobiose or lactose oxidation in the presence of CDH are often described as antioxidant, chelating, humectant, and emulsifying effectors (Gutiérrez et al., 2012). Lactobionic acid (LBA) is one of the products derived from lactose oxidation, with high potential applications as a bioactive compound in the pharmaceutical, food, cosmetic, and chemical industries. LBA can also be used as a stabilising component in organ preservation solutions, an anti-ageing and keratinising ingredient of skin care cosmetics, a gelling agent in dessert products, or an acidifier agent in fermented milk products (Minal et al., 2017). Given the capability of CDH of simultaneous synthesis of antimicrobial and antioxidant bioactive molecules, construction of an effective system involving the properties of both described substances seems to be reasonable. Immobilisation of the enzyme seems to be one of the good proposals. In the case of immobilisation of CDH, it is important to preserve the intact enzyme–substrate system for H_2O_2 and carboxylic acid production, which requires the use of an appropriate method of immobilisation and support matrix. There are many immobilisation techniques based on both physical and chemical interactions. However, the advantages of physical methods include the preservation of the enzyme structure and the ease of carrying out the immobilisation process. Among the physical methods, there are entrapment techniques in which a given substance is suspended in a natural or synthetic gel, after which the mixture is cross-linked. Considering their low cost, biocompatibility, biodegradability, and low toxicity, the use of natural polysaccharides as a support matrix seems to be very promising. In the present study, alginate was chosen as a carrier for the CDH immobilisation. Alginate is a favourable, non-toxic biopolymer material with high functionality, porosity for product diffusion, and a relatively simple procedure of immobilisation (Lee and Mooney, 2012). From the practical point of view, the use of alginate as a carrier is beneficial because it is not expensive and provides mild conditions, such as scaffolds in tissue engineering (Barbosa et al., 2005), controlled-release drug delivery systems (Sun and Tan, 2013), a thickening and gelling agent, a stabiliser in food and feed industry (Brownlee et al., 2009), and an emulsifying agent to prepare edible coatings and films (Galgano et al., 2015). Although many reports on cellobiose dehydrogenase immobilisation are available (Ludwig et al., 2013; Öhlknecht et al., 2017; Tegl et al., 2015), there are no data on the entrapment of the CDH/substrate mixture in alginate beads presented in these studies.

The experimental plan implemented in this work assumed the following stages: (i) determination of the yield of CDH immobilisation in alginate beads and (ii) evaluation of the antioxidant and antimicrobial properties of the free enzyme and the immobilised enzyme–substrate complex, and (iii) quantitative analysis of the products obtained in the reaction catalysed by fungal cellobiose dehydrogenase. The CDHs used in the work were isolated from wood degrading fungi: *Phlebia lindterii*, *Pycnoporus sanguineus*, and *Cerrena unicolor*, which were previously described in our research group (Sulej et al., 2013a, 2013b, 2015), and *P. chrysosporium* CDH as a reference enzyme.

2. Materials and methods

2.1. Materials and microorganisms

All chemicals used in the work were of the highest purity available and analytical grade. Media components and other chemicals were procured from Sigma–Aldrich (Steinheim, Germany), Merck (Darmstadt, Germany), VWR (Vienna, Austria), Bio-Rad (Warsaw, Poland), or BioMaxima (Lublin, Poland). Deionised water was used for making aqueous solutions during the experiments.

The cellobiose dehydrogenase producing white rot fungi *P. chrysosporium* (FCL236) and *P. sanguineus* (FCL199) were obtained from the culture collection of the University of Agriculture in Tokyo, *P. lindtneri* (FCL22) from the culture collection of the Agriculture Academy in Cracow, and *C. unicolor* (FCL139) from the culture collection of the Regensburg University in Germany. All strains were deposited in the fungal collection at the Department of Biochemistry of Maria Curie-Sklodowska University (Poland). All fungi were identified genetically and their nucleotide sequences were deposited in the GenBank with the accession numbers: FJ594058 (FCL236), JF308951 (FCL199), FJ594063 (FCL22), and DQ056858 (FCL139).

Escherichia coli ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and Gram-positive *Staphylococcus aureus* ATCC 25923 bacteria used in the experiments were acquired from the culture collection of the Department of Genetics and Microbiology of Maria Curie-Sklodowska University (Poland). Due to the screening character of the antimicrobial analyses, the commonly used reference strains of bacteria were selected for testing. These three taxonomically unrelated bacteria are a serious cause of a variety of community- and hospital-acquired infections and can also be present in industrial food processing.

2.2. Culture conditions and purification of CDH

CDHs from *P. sanguineus*, *P. lindtneri*, and *C. unicolor* were produced and isolated as described in our previously published works (Sulej et al., 2013a, 2013b, 2015). In the case of *P. chrysosporium*, a proper medium supplemented with 5 g/l Avicel, 1 g/l $(NH_4)_2HPO_4$, 1 g/l KH_2PO_4, 0.3 g/l $MgSO_4 \cdot 7H_2O_2$, 0.08 g/l $CaCl_2$, 5 mg/l $ZnSO_4 \cdot 7H_2O$, 1.5 mg/l $MnSO_4 \cdot 4H_2O$,1.5 mg/l $CoCl_2 \cdot 6H_2O$, 5 mg/l $FeSO_4 \cdot 7H_2O$, 100 mg/l yeast extract, and 0.1 mg/l thiamine was used for CDH production. The pH was

adjusted to 4.5 with 5 M HCl and cultured at 28 °C for 7 d in an incubator shaker Multitron (Infors, Bottmingen, Switzerland) at 120 rpm. The culture supernatant was clarified by centrifugation at 4 °C for 30 min at 12 000×*g* on a 6K15 device (Sigma, Osterode am Harz, Germany) and used as a source of the enzyme to carry out the purification and characterisation. The purification strategy for the supernatants of all fungi was similar and the first step involved concentration via ultrafiltration using a Prep/Scale TFF Cartridge with a 10-kDa cut-off polyethersulphone (PTGC, 0.09 m^2) membrane (Millipore, Bedford, MA). The protein concentrate was subjected to ammonium sulphate fractionation in the ranges of 40–90 % (*P. sanguineus*), 30–50 % (*P. lindtneri*), 15–85 % (*C. unicolor*), and 20–80 % (*P. chrysosporium*) saturation at 0 °C. The protein pellet obtained by centrifugation was solubilised in deionised water and desalted by diafiltration through centrifugal concentrators (Vivaspin Turbo 15) in polyethersulphone (PES) with a cut-off of 30 kDa (Sartorius, Göttingen, Germany). Protein purification procedures were carried out using an AKTA-Prime purification system (GE Healthcare, Uppsala, Sweden) operated at 24 °C. The diafiltrated sample was applied to a DEAE-Sepharose (fast flow) column (GE Healthcare, Uppsala, Sweden) previously equilibrated with 50 mM sodium acetate buffer (pH 5.0). Elution was performed by a linear NaCl gradient from 0 to 0.5 M in the same buffer at a flow rate of 3 ml/min. The fraction containing cellobiose dehydrogenase activity (intact protein with flavin and heme domains) were pooled and concentrated using a 50 kDa cut-off PES Vivaspin Turbo 15 (Sartorius, Göttingen, Germany). The purified proteins were stored at −20 °C until further use.

2.3. Enzyme activity assay and protein determination

Cellobiose dehydrogenase activity was determined by the lactose-dependent reduction of 2,6-dichloroindophenol (DCIP) (Sigma ChemicalCo., St. Louis, MO, USA) at 520 nm (ε_{520} = 6.8 $mM^{-1}cm^{-1}$), pH 4.5, and 30 °C. A modification of the method developed by Baminger et al. (1999) was used. Assays were conducted in 96-well microplates using a Tecan Infinite M200 Pro (Tecan, Zürich, Switzerland) plate reader. The assay mixture (200 µl) contained 20 µl of a properly diluted CDH sample, 20 µl lactose (300 mM in 100 mM sodium acetate buffer, pH 4.5), and an appropriate amount of the same buffer. The mixture was incubated at 30 °C for 10 min. After that time, the reaction was initiated by addition of 10 µl of 3 mM DCIP (solution in water containing 10 %, v/v ethanol) and the decrease in absorbance was monitored during the first 60 s. One unit of the enzyme (U) was defined as the amount of the enzyme reducing 1 µmol DCIP per minute in the described assay conditions. This assay was used for determination of the activity of the native enzyme as well as for the catalytically active flavin domain. Alternatively, the activity of the intact protein containing both the flavin and heme domains was determined by monitoring the reduction of cytochrome c (20 µM) at 30 °C in 100 mM sodium acetate buffer, pH 4.5 (Sigma Chemical Co., St. Louis, MO, USA) in the presence of lactose (30 mM). The extinction coefficient (ε_{550}) was 19.6 $mM^{-1}cm^{-1}$ (Canevascini et al., 1991). The increase in absorbance at 550 nm after 10 min incubation at 30 °C was associated with the oxidation of the substrate (lactose) and was measured with a microplate reader (Tecan Infinite 200 PRO). One unit of CDH activity was defined as the amount of enzyme that reduces cyt *c* at a rate of 1 µmol min^{-1} in the selected assay conditions (pH 4.5, 30 °C).

The protein concentration was determined using the Bradford method (Bradford, 1976) with crystalline bovine serum albumin (BSA) as a standard or by monitoring ultraviolet (UV) absorbance at 280 nm.

2.4. Enzyme immobilisation in alginate and determination of activity and protein yields

Cellobiose dehydrogenase from *P. lindtneri* (*Pl*CDH), *C. unicolor* (*Cu*CDH), *P. sanguineus* (*Ps*CDH), and *P. chrysosporium* (*Pch*CDH) was immobilised in alginate. Four experimental variants were made for each enzyme: 1) control – 1.5 % sodium alginate was added to 0.06M $CaCl_2$ in a ratio of 1:3; 2) CDH immobilised in alginate – the enzyme was mixed separately with 0.06M $CaCl_2$ and 1.5 % sodium alginate in a ratio of 1:9 and next the CDH-1.5 % sodium alginate mixture was dropped into the CDH-0.06M $CaCl_2$ mixture in a ratio 1:3; 3) CDH with 100 mM lactose substrate immobilised in alginate – the lactose substrate was added to 1.5 % sodium alginate in a ratio of 1:4 and next mixed with CDH in a ratio of 1:9. The prepared mixture of CDH with the lactose substrate and 1.5 % sodium alginate was dropped into the CDH-0.06M $CaCl_2$ mixture in a ratio of 1:3; 4) CDH with 10 mM cellobiose substrate immobilised in alginate - performed as in point 3 with replacement of lactose with the cellobiose substrate. The initial concentration of protein in the tested enzymes was as follows: PlCDH 4.6 ± 0.02 mg/ml; CuCDH 2.1 ± 0.02 mg/ml; PsCDH 5.5 ± 0.05 mg/ml, and PchCDH 3.9 ± 0.05 mg/ml. The resulting granules of the immobilised enzyme were left for 24 h in a $CaCl_2$ solution at 2-8 °C for hardening. Then, the alginate beads were drained from $CaCl_2$ and washed with distilled water. The average wet mass of the beads was obtained. Before measuring the weight of the beads, the beads were drained on a Miracloth filter.

Protein immobilisation efficiency were calculated as a ratio of the immobilised protein to the introduced protein in the solution used for the immobilisation process. These efficiency were expressed as a percentage. The protein efficiency were evaluated with a Modified Lowry (Lowry et al., 1951) Protein Assay Kit (Thermo Scientific) using BSA as a standard.

2.5. Antimicrobial activity assay

Antimicrobial activities of CDH were evaluated using Gram-negative *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and Gram-positive *S. aureus* ATCC 25923 bacteria. Broth microdilution tests were performed with 96-well microtitre plates. Mueller–Hinton (MH) broth medium (100 µl) was pipetted into each well; subsequently, 50 µl of CDH supplemented with 5 mM cellobiose or 50 mM lactose were added. The wells were inoculated using 10 µl of the tested bacterial strain to obtain a final concentration of 10^4 CFU/ml. MH medium samples from each experiment were tested for bacterial growth by measuring the optical density (OD) at 550 nm. The optical density for each replicate at T_0 was subtracted from the optical density for each replicate at T_{24}. The percentage of bacterial growth inhibition was determined using the following formula:

$$\%\ bacterial\ growth\ inhibition = 100 - (OD_{tested} / OD_{control}) \times 100$$

2.6. Antimicrobial activity of immobilised CDH

The alginate beads with immobilised CDH were placed on Muller-Hinton agar inoculated with 10^4 CFU/ml of the tested strains. Microbial growth was determined after 18 h incubation at 37 °C and calculated as described above.

2.7. Determination of H_2O_2 concentration

The concentration of H_2O_2 formed in the reaction of CDH with cellobiose and lactose was determined by the chemiluminescence

of luminol catalysed by Co (II) (Perez and Rubio, 2006). After an appropriate time of incubation of CDH (50 μl) with the substrate (50 μl of 10 mM of cellobiose and 100 mM of lactose), 100 μl of a diluted mixed reagent solution containing luminol and cobalt ions were added. The emitted photons were counted with the microplate reader (TECAN Infinite M200 PRO). The formation of H_2O_2 was compared with the calibration curve and expressed in micromoles.

2.8. Antioxidant properties of cellobiose dehydrogenase

The antioxidant properties of free cellobiose dehydrogenases (CDH) and those encapsulated with the alginate beads were investigated using the 1,1-diphenyl-2-picrylhydrazyl (DPPH·) assay, described by Paduch et al. (2008). The analysed compounds (0.1 ml) at concentrations ranging from 12.5 to 800 μg/ml were added to 0.1 ml of a DPPH· solution (0.2 mg/ml in ethanol). Standards (Trolox and Vitamin C) that are well known for their strong antioxidant activity were used as a positive control. Absorbance at 515 nm was determined at room temperature after 10 min (optimal time of incubation). The percentage of reduction of the DPPH oxidation rate was calculated according to the formula:

$$DPPH\ scavenging\ effect\ (\%) = [(X_0 - X_1)/X_0] \times 100$$

where X_0 is the absorbance of the control sample and X_1 is the absorbance of the tested compound/standard.

The inhibition curves were prepared and EC_{50} values were obtained. The antioxidant activity of the non-immobilised enzyme was expressed as EC_{50}. The EC_{50} value was defined as the concentration (in μg/ml) of the sample leading to 50 % reduction in absorbance of the initial DPPH concentration. The effective 50 % concentrations (EC_{50}) were determined by fitting the data to a sigmoidal dose–response curve using OriginPro 2018 software (OriginLab, Northampton, USA).

2.9. LBA and CBA determination

The concentration of substrates (cellobiose or lactose) and products (cellobionic or lactobionic acids) was quantified by high-performance liquid chromatography (HPLC, Agilent Infinity 1260 equipped with RID and DAD detectors). The enzyme samples (CDHs) with appropriate concentrations were incubated at room temperature with the substrate (100 mM lactose or 10 mM cellobiose) in a ratio of 1:1. After incubation (3 h, 6 h, and 24 h), the samples were subjected to ultrafiltration through centrifugal concentrators (Vivaspin 500) in the polyethersulphone membrane (PES) with a cut-off of 10 kDa (Sartorius, Göttingen, Germany). The disaccharides and aldonic acids present in the low molecular weight fraction were analysed. The HPLC system fitted with a Bio-Rad Aminex HPX-87H column was operated at 50 °C with 0.45 mM H_2SO_4 as the mobile phase at the flow rate of 0.7 ml/min and injection time set to 20 s.

2.10. Antioxidant stability studies

2.10.1. Stability in time

Antioxidant stability of all studied samples was determined after 1 and 7 d of incubation at 4 °C. Free CDHs and CDHs encapsulated with the alginate beads with and without lactose (100 mM) and cellobiose (10 mM) as a substrate were investigated. Additionally, Trolox (600 μg/ml) and alginate beads without CDH were used as positive markers. The antioxidant properties were determined in standard conditions as described for the antioxidant DPPH assay.

2.10.2. Operational stability

Antioxidant operational stability of the immobilisation CDH was assessed by incubating the alginate beads (~16 mg) in 0.1 ml of a DPPH· solution (0.2 mg/ml in ethanol). After 30 min. of incubation, DPPH absorbance in the solution was measured. The alginate beads were washed five times with the buffer and suspended in a new solution of DPPH to begin the next cycle of activity measurement.

2.11. Scanning electron microscopy (SEM) of alginate beads

The surface morphologies of freeze-dried alginate beads with CDH were observed by scanning electron microscopy (SEM) at 30 kV accelerating voltage. The alginate beads without CDH served as the control. The samples were sputter-coated with gold and observed under VEGA 3 LMU SEM (TESCAN, Czech Republic) in standard conditions. Magnification ×10 000 was used to observe the control beads as well as the beads after the CDH immobilisation procedure. Ten different areas covered with alginate beads were used to observe morphological changes caused by the enzyme immobilisation process.

2.12. Statistical analysis

The presented results are expressed as mean ± SD from three independent experiments ($n = 3$). The mean values and standard deviation were calculated using one-way ANOVA (Statgraphics Online) and next the means were compared using Tukey's multiple range test. Excel program (Microsoft Office 2010 package) was used for the calculation of the data. Values of $p \leq 0.05$ were considered statistically significant.

3. Results and discussion

3.1. Antimicrobial properties of free and immobilised cellobiose dehydrogenase

Fungi are widely known for their production of natural bioactive compounds with antimicrobial activities. The main mechanisms responsible for the antibacterial activity of bioactive substances involve chemical interference with the synthesis or function of vital components of bacteria and/or evasion of the conventional mechanisms of antibacterial resistance (Khameneh et al., 2019).

Many enzymes oxidising sugars such as glucose oxidase and cellobiose dehydrogenase show the ability to inhibit bacterial growth through naturally produced hydrogen peroxide. The general mechanism of the action of hydrogen peroxide towards bacterial cells is the formation of radicals, which attack essential cell components important for the viability of the microorganism, including proteins, lipids, and DNA (Linley et al., 2012). The generation of radicals (OH·) is very often associated with Fenton's reaction initiated with reduction of Fe^{3+} to Fe^{2+} by such agents as the superoxide radical ($\cdot 0^{2-}$). H_20_2 produced for example by enzymes react with Fe^{2+} to provide a hydroxyl anion, hydroxyl radical, and Fe^{3+}. Either the higher-valency ions or the hydroxyl radicals formed might be responsible for bacterial cellular damage (Juven and Pierson, 1996). It should be underlined that, in contrast to the glucose oxidase system which uses glucose as a substrate for the production of H_2O_2 and finds widespread use as an antimicrobial system in food packaging and wound dressings (Dobbenie et al., 1995), the catalysis conducted by CDH is much more attractive because cellobiose and lactose are more hardly assimilated by microorganisms (Nyanhongo et al., 2017). The antibacterial activity of CDH may also be associated with the presence of lactobionic acid formed in the enzymatic reaction. The mechanism of action of this agent is based on breaking down the structure of the bacterial cell

wall and membrane, thereby releasing the cellular contents as well as inhibiting protein synthesis, which ultimately leads to cell death (Cao et al., 2019).

3.1.1. Free cellobiose dehydrogenase system

Based on the obtained results, it can be proposed that the antimicrobial activity of CDH is mainly related to the production of hydrogen peroxide (Fig. 1). In the present study, the antimicrobial effects of free CDHs and the CDH/substrate systems from four fungi (*P. chrysosporium, P. lindtneri, P. sanguineus*, and *C. unicolor*) were tested against two strains of Gram-negative bacteria (*E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853) and one strain of Gram-positive bacteria (*S. aureus* ATCC 25923). Evaluation of the antibacterial activity of the tested CDHs and the CDH/substrate systems is presented in Table 1. The results revealed the lowest effectiveness of *Pl*CDH towards of all tested organisms. A slight level of inhibition was achieved in the case of *S. aureus* (7.4 %) and *P. aeruginosa* (3.1 %) only in the presence of lactose as a substrate. The other enzymes were effective in suppressing the growth of bacteria with variable potency. The highest percentage growth inhibition (100 %) was obtained for *S. aureus* when *Ps*CDH and *Pch*CDH with lactose as a substrate were applied, whereas slightly lower inhibition was achieved in the case of *E. coli* and *P. aeruginosa* bacteria, i.e. 64.1 % and 86.5 % (*Ps*CDH) and 94.1 % and 41.4 % (*Pch*CDH), respectively. The growth of *S. aureus* was inhibited by *Ps*CDH at the same level (100 % and 98.6 %) with both substrates (lactose and cellobiose) used. The percentage of *S. aureus* survival in the experiments with cellobiose as a substrate for *Cu*CDH and *Pch*CDH was 62.7 % and 55.2 %, respectively. Substantially lower effectiveness was observed in the case of *E. coli* and *P. aeruginosa* bacteria incubated with *Cu*CDH (10.4 % and 4.3 %) and *Pch*CDH (4.6 % and 0 %) with cellobiose as a substrate. The results of the antimicrobial activity of the four CDHs with different substrates may suggest that *S. aureus* was the most CDH-sensitive strain, in contrast to *E. coli* and *P. aeruginosa*. Evidence for the effective operation of the CDH/cellobiose system on bacterial cells was provided by Thallinger et al. (2014). The authors demonstrated the capability of the CDH/cellobiose system of complete inhibition of the growth of *E. coli* and *S. aureus* as well as other microorganisms colonising urinary catheters (Thallinger et al., 2014). Studies were conducted on a recombinantly produced CDH from *Myriococcum thermophilum*, i.e. an Ascomycota fungus, whose properties and substrate specificity are different from those of the enzymes analysed in our study. Despite the differences, the results obtained in our research team proved to be equally promising. Three of the four investigated enzymes exerted a strong antimicrobial effect (almost 100 % inhibition) depending on the substrate (lactose or cellobiose).

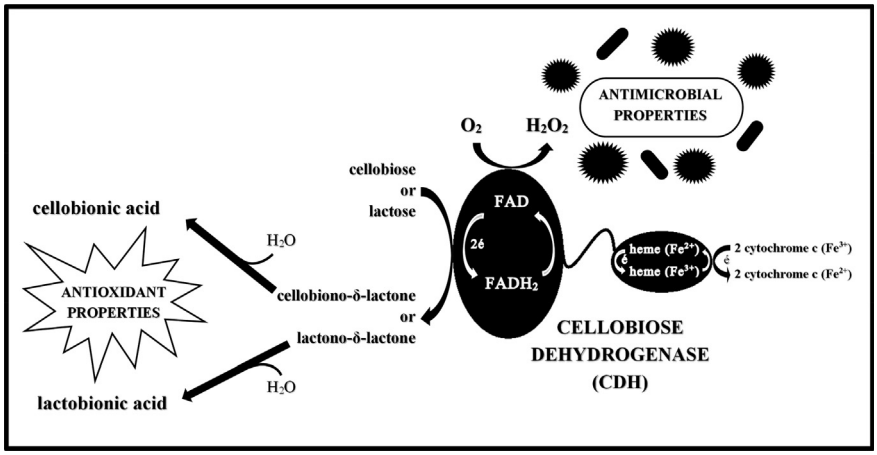

Fig. 1. Schematic representation of the pathway of electron transfer of cellobiose or lactose and production of appropriate carboxylic acids with antioxidant properties and hydrogen peroxide with antimicrobial properties (adopted from Fujita et al., 2009).

Table 1
Antimicrobial properties of CDHs (free and with cellobiose or lactose substrates) isolated from *P. lindtneri, C. unicolor, P. sanguineus*, and *P. chrysosporium* submerged cultures.

Enzymes	% bacterial growth inhibition		
	S. aureus ATCC 25923	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853
*Pl*CDH	*n.d.*	*n.d.*	*n.d.*
*Pl*CDH/cellobiose	*n.d.*	*n.d.*	*n.d.*
*Pl*CDH/lactose	$7.4 \pm 0.62_a$	*n.d.*	$3.1 \pm 1.28_b$
*Cu*CDH	*n.d.*	$19.6 \pm 1.44_a$	$6.4 \pm 1.81_b$
*Cu*CDH/cellobiose	$62.7 \pm 1.12_a$	$10.4 \pm 2.32_b$	$4.3 \pm 1.18_c$
*Cu*CDH/lactose	$97.5 \pm 0.82_a$	$63.2 \pm 1.64_b$	$58.2 \pm 1.73_c$
*Ps*CDH	37.6 ± 1.14	*n.d.*	*n.d.*
*Ps*CDH/cellobiose	$98.6 \pm 1.26_a$	$2.2 \pm 1.34_b$	$23.6 \pm 1.62_c$
*Ps*CDH/lactose	$100 \pm 0.74_a$	$64.1 \pm 1.84_b$	$86.5 \pm 1.23_c$
*Pch*CDH	1.16 ± 0.71	*n.d.*	*n.d.*
*Pch*CDH/cellobiose	$55.2 \pm 1.34_a$	$4.6 \pm 0.82_b$	*n.d.*
*Pch*CDH/lactose	$100 \pm 0.78_a$	$94.1 \pm 1.98_b$	$41.4 \pm 0.99_c$

n.d. - not detected.
Mean values of three replicate assays (n = 3) with standard deviations (S.D.), ANOVA, Tukey.
Lowercase letters (a, b, c) in the same row indicate statistically significant differences ($P < 0.05$) in the growth inhibition bacterial strains in the presence of tested enzymes.

In our study, CDHs without an additional substrate inhibited the growth of bacteria as well. These interesting results may be connected with the possibility to use exopolysaccharides from bacterial biofilm as a substrate for production of hydrogen peroxide. The results described by Thallinger et al. (2016) confirmed the possibility of using CDHs as antimicrobial and antibiofilm agents applied in functionalised urinary catheters. Furthermore, the use of bacterial biofilm as a substrate for CDH was also investigated (Thallinger et al., 2016).

In the next step, the concentration of *in situ* produced hydrogen peroxide in the CDH/substrate system was investigated. It was observed that the antimicrobial effect was distinctly correlated with the amount of H_2O_2 produced during the reaction catalysed by CDH. In our study, the enzymatic production of hydrogen peroxide was measured with the chemiluminescence assay with luminol catalysed by Co (II). The analysis was performed for three different concentrations of the tested enzymes (50 μg/ml, 200 μg/ml, 600 μg/ml). The measurement was carried out after 3, 6, and 24 h of incubation. Significant differences in the concentration of H_2O_2 were detected depending on the tested enzyme preparation. The results are shown in Fig. 2. CDH from *P. lindtneri* produced the lowest quantities of hydrogen peroxide (below 1 μM with cellobiose as a substrate). The maximum concentration of hydrogen peroxide for this enzyme was achieved after 6 h of incubation. These results explain the absence of antimicrobial properties of *Pl*CDH. On the other hand, Miyasaki et al. (1986) and Watts et al. (2003) demonstrated that exposure of bacteria to 0.7 μM H_2O_2 caused bactericidal effects. The most effective producer of H_2O_2 was the enzyme isolated from *C. unicolor* cultures. The highest concentration of hydrogen peroxide was obtained during the reaction of *Cu*CDH

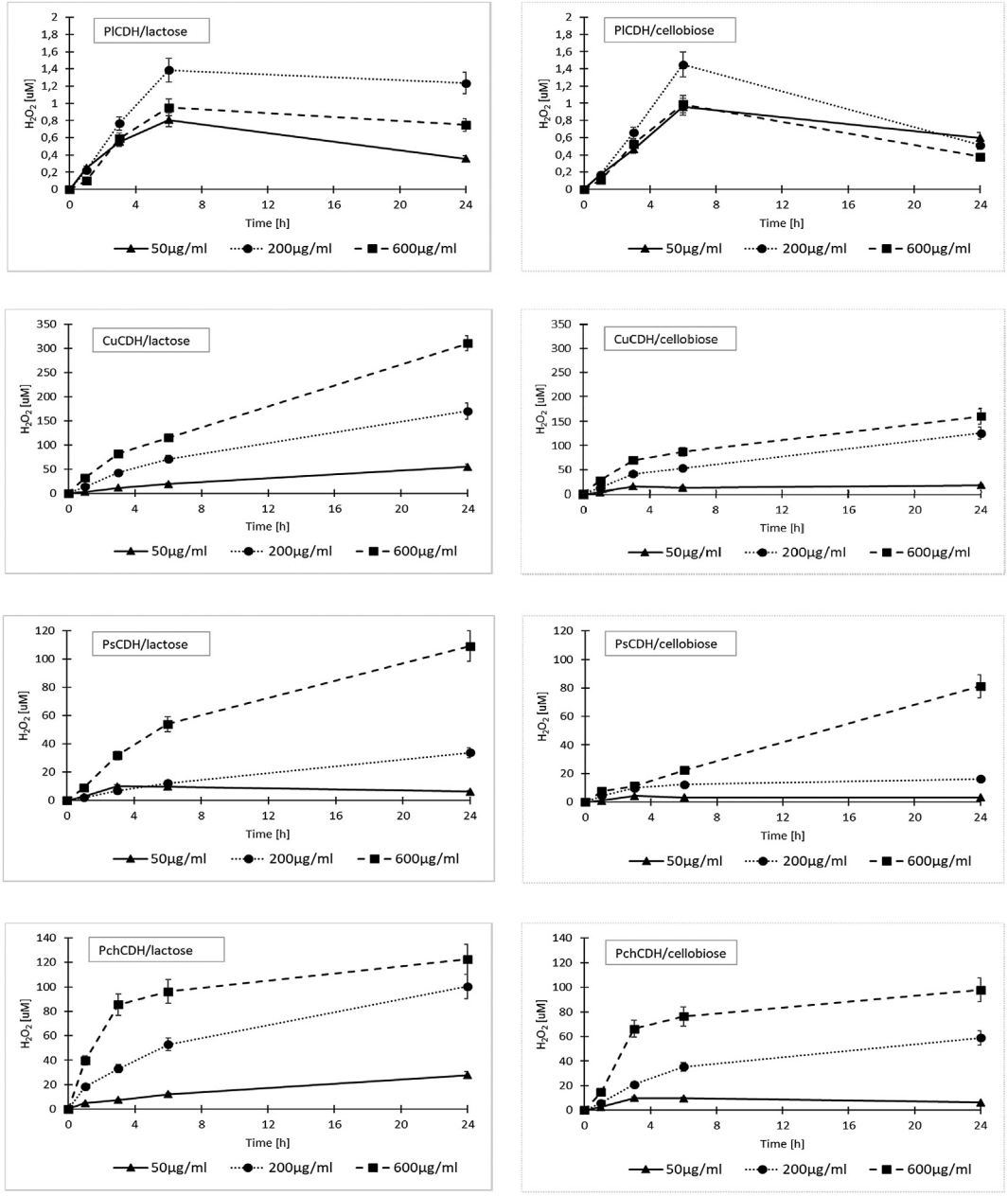

Fig. 2. Time course of hydrogen peroxide generation from different amounts of CDHs from *P. lindtneri* (*Pl*CDH), *C. unicolor* (*Cu*CDH), *P. sanguineus* (*Ps*CDH), and *P. chrysosporium* (*Pch*CDH) using lactose (50 mM) or cellobiose (5 mM) as a substrate.

with lactose as a substrate (310 μM after 24 h of incubation). Hence, the inhibition of bacterial growth in this case was at high levels. The other CDH preparations effectively inhibited the growth of bacteria in the presence of 5 mM cellobiose or 50 mM lactose, with hydrogen peroxide production in the range of 80–120 μM (*Ps*CDH and *Pch*CDH) after 24 h of incubation. These results are similar to other findings reported by several research teams dealing with the use of the CDH/cellobiose system for biomedical application (Tegl et al., 2015; Thallinger et al., 2014, 2016). Tegl et al. described an antimicrobial system based on *in situ* release of hydrogen peroxide by cellobiose dehydrogenase immobilised on chitosan particles. After 24 h of incubation, the concentration of H_2O_2 in the system based on free CDH (control of the immobilisation process) and cellobiose was around 200 μM (Tegl et al., 2015). However, in the studies carried out by Sina Pricelius on the potential of CDH from *M. thermophilum* for *in situ* H_2O_2 generation for bleaching purposes in detergents, the hydrogen peroxide level was significantly higher (30 mM after 7 h of incubation) but only in the presence of desferrioxamine mesylate as a chelant (Pricelius et al., 2011).

3.1.2. Immobilised cellobiose dehydrogenase system

The use of enzymatic systems in practice is related to the immobilisation process on an appropriate support in order to release bioactive substances at a specified concentration and time. Natural biodegradable polysaccharides such as starch, chitosan, carrageenan, and alginate, which have widespread uses especially due to their ability to form hydrogels, beads, fibres, or films, are interesting carriers for food industrial applications. For this reason, we decided to immobilise CDHs in alginate beads. This polymer was also used by Nyanhongo et al. to develop a hydrogel based on gelatin and alginate with CDH, cellobiose, and catechol for the healing of chronic wounds (Nyanhongo et al., 2013). Some biomedical applications of CDH were investigated with the use of chitosan particles (Tegl et al., 2015), while the electrochemical properties of CDH were tested using nanotubes as a support (Ludwig et al., 2010).

In our study, immobilisation of CDHs from *P. chrysosporium, P. lindtneri, P. sanguineus*, and *C. unicolor* in the alginate beads was checked for the first time. With their unique physicochemical properties and versatile biological activity, alginates are an interesting material for binding enzymes. Using the common procedures, CDH was immobilised in alginate beads and the results are shown in Table 2. As a result of immobilisation, beads with the same diameter of 3 mm were obtained, but different weights of the alginate beads were observed depending on the type of enzyme and substrate used (from 12.7 mg/bead for *Ps*CDH/Alg/Cel to 21.7 mg/bead for *Pch*CDH/Alg). The immobilisation efficiency expressed as a protein yield ranged from 61.6 to 100 %. Similar yields of immobilisation in alginate beads was demonstrated for fungal laccase and manganese peroxidase preparations (Bilal and Asgher, 2015; Phetsom et al., 2009).

In the research of the antimicrobial properties of CDH/substrate systems, chitosan was often used as a support material for enzyme immobilisation (Öhlknecht et al., 2017; Tegl et al., 2015). Experiments conducted by Tegl et al. showed strong inhibition of the growth of *E. coli* and *S. aureus* for three CDH functionalised chitosan preparations (Tegl et al., 2015). In our study, CDHs immobilised in the alginate beads were also tested for their ability to inhibit the growth of selected bacterial strains. Surprisingly, no preparations with immobilised CDHs showed antimicrobial properties. One of the causes of these results can probably be associated with the too low diffusion of CDHs immobilised in the alginate beads.

In the next step, the bead surface morphology was analysed using Scanning Electron Microscopy (SEM). Recently, SEM has become a routine technique for characterisation of support materials for biocatalyst immobilisation and for observation of immobilised cells, especially cell colonisation and cell support interaction (Toldra and Lequerica, 1991). The SEM studies conducted in the present work were performed using alginate beads modified by CDH immobilisation. The results are presented in Fig. 3a,b. It was detected that the immobilisation procedure distinctly changed the alginate bead surface in all the experimental variants in comparison to the control samples. Generally, the beads retained their spherical shape, but many morphological changes were observed on the modified carrier. Contrarily to untreated alginate, the immobilisation of CDH alone or with the substrate (Lac and Cel) caused considerable shrinkage of the alginate beads. The most visible morphological changes were observed in the case of *Pl*CDH + Lac,

Table 2
Immobilisation yield and catalytic capability of *P. lindtneri, C. unicolor, P. sanguineus*, and *P. chrysosporium* CDHs entrapped within Ca-alginate.

Strain/type of bead	Average bead diameter [mm]	Average weight mg/bead	Activity before immobilisation Cyt.c/DCIP [U/ml]	Protein [mg/ml]	Protein immobilisation efficiency [%]
(Alginate) Control	3.3 ± 0.02	19.6 ± 0.08	–	–	–
Phlebia lindtneri					
(*Pl*CDH) free enzyme	–	–	5.3/4.9	4.6 ± 0.02[a]	–
*Pl*CDH + Alg	3.1 ± 0.02	16.3 ± 0.08		4.6 ± 0.03[b]	100
*Pl*CDH + Alg + Lac	3.0 ± 0.07	13.7 ± 0.11		4.6 ± 0.02[b]	100
*Pl*CDH + Alg + Cel	3.3 ± 0.02	19.0 ± 0.08		4.1 ± 0.07[b]	88.9
Cerrena unicolor					
(*Cu*CDH) free enzyme	–	–	8.2/7.5	2.1 ± 0.02[a]	–
*Cu*CDH + Alg	3.1 ± 0.03	16.5 ± 0.08		2.1 ± 0.01[b]	100
*Cu*CDH + Alg + Lac	3.1 ± 0.02	17.5 ± 0.06		1.3 ± 0.03[b]	61.6
*Cu*CDH + Alg + Cel	3.0 ± 0.02	14.1 ± 0.12		1.9 ± 0.02[b]	93.6
Pycnoporus sanguineus					
(*Ps*CDH) free enzyme	–	–	2.5/10.2	5.5 ± 0.05[a]	–
*Ps*CDH + Alg	3.0 ± 0.02	13.9 ± 0.08		5.0 ± 0.02[b]	91.9
*Ps*CDH + Alg + Lac	3.1 ± 0.04	16.7 ± 0.07		4.4 ± 0.06[b]	79.9
*Ps*CDH + Alg + Cel	3.0 ± 0.01	12.7 ± 0.13		5.2 ± 0.08[b]	94.5
Phanerochaete chrysosporium					
(*Pch*CDH) free enzyme	–	–	3.4/8.4	3.9 ± 0.05[a]	–
*Pch*CDH + Alg	3.3 ± 0.04	21.7 ± 0.05		3.9 ± 0.06[b]	100
*Pch*CDH + Alg + Lac	3.1 ± 0.02	15.1 ± 0.07		3.8 ± 0.06[b]	97.7
*Pch*CDH + Alg + Cel	3.1 ± 0.02	16.7 ± 0.11		3.8 ± 0.07[b]	96.1

[a] Protein before immobilisation.
[b] Protein immobilized in the beads.

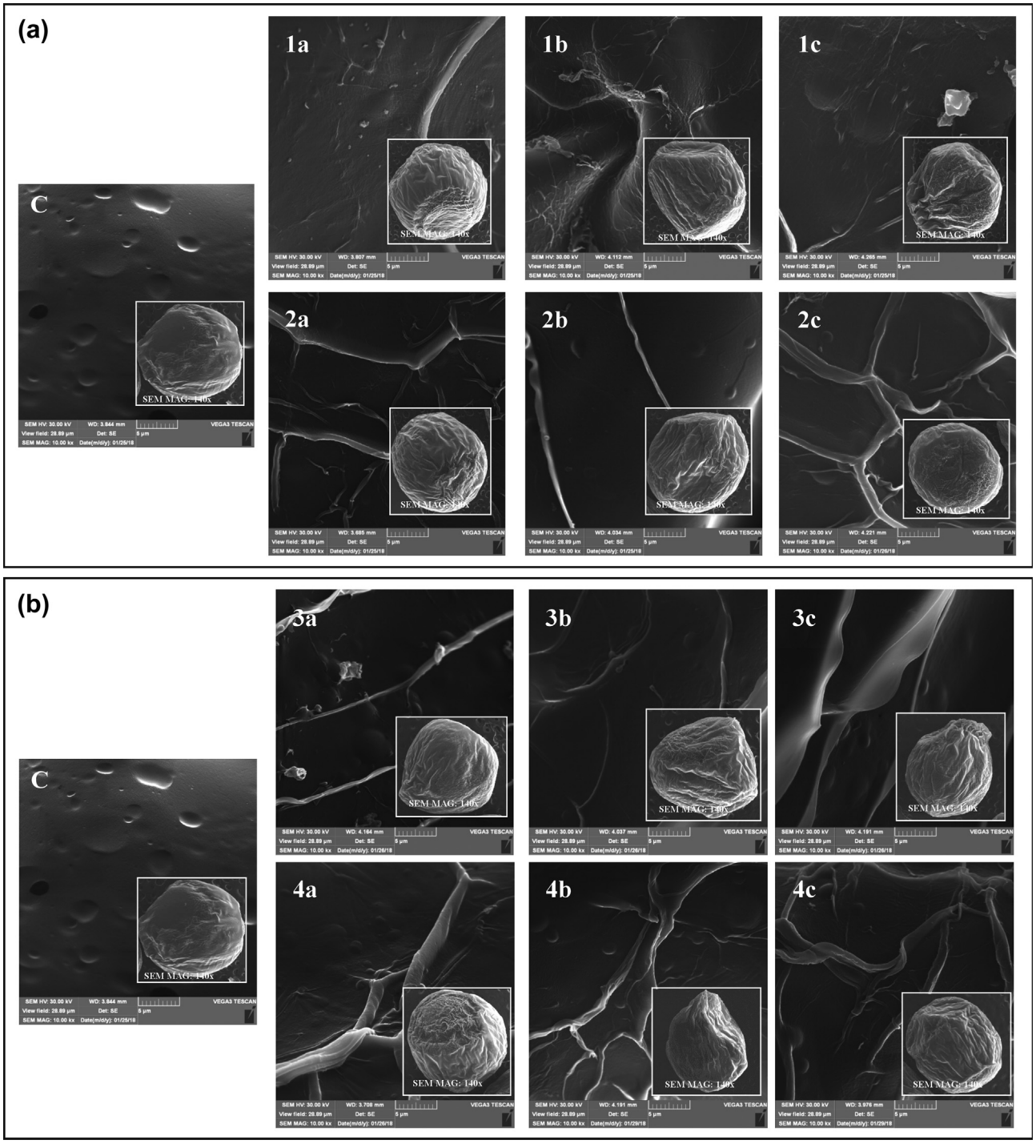

Fig. 3. (a) SEM micrographs of the surface of unfilled alginate beads (C-control) and alginate beads containing CDHs isolated from *P. lindtneri*: *Pl*CDH + Alg (1a), *Pl*CDH + Alg + Lac (1b), *Pl*CDH + Alg + Cel (1c) and from *C. unicolor*: *Cu*CDH + Alg (2a), *Cu*CDH + Alg + Lac (2b), *Cu*CDH + Alg + Cel (2c). (b) SEM micrographs of the surface of unfilled alginate beads (C-control) and alginate beads containing CDHs isolated from *P. sanguineus*: *Ps*CDH + Alg (3a), *Ps*CDH + Alg + Lac (3b), *Ps*CDH + Alg + Cel (3c) and from *P. chrysosporium*: *Pch*CDH + Alg (4a), *Pch*CDH + Alg + Lac (4b), and *Pch*CDH + Alg + Cel.

*Pl*CDH + Cel, *Ps*CDH, *Ps*CDH + Lac, *Ps*CDH + Cel, and *Pch*CDH + Cel. Immobilisation creates many artefacts by the destruction of the alginate bead pore walls. In some samples, a sponge-like structure on the carrier surface was observed. Fortunately, besides these changes, no destruction of the alginate beads was detected after the CDH immobilisation procedure. Differences in the appearance of beads were noticed by Li et al. (2017), who investigated alginate combined with two types of cellulose, starch, or xylan (Li et al., 2017). Similarly, Noreen et al. studied the morphology of dried alginate bead surfaces with immobilised laccase using SEM (Noreen et al., 2015).

3.2. Antioxidant properties of free and immobilised cellobiose dehydrogenase

A wide range of mushrooms have been reported to have significant antioxidant properties due to their bioactive compounds, such as polyphenols, polysaccharides, tocopherols, vitamins,

carotenoids, glycosides, ergothioneine, and ascorbic acid. Some proteins with enzymatic activity may also have antioxidant properties in the presence of specific substrates. In the case of CDH, these properties are probably related to the production of cellobionic or lactobionic acids (Fig. 1).

3.2.1. Free cellobiose dehydrogenase system

In the earlier studies, we have shown the strong antioxidant capability of the fungal CDHs from *P. sanguineus* and *C. unicolor* in the presence of cellobiose dehydrogenase substrates (lactose or cellobiose) in the reaction mixture (Sulej et al., 2013b). Considering the strong biotechnological potential of enzymes with antioxidant properties, we examined the antioxidant activities of two additional cellobiose dehydrogenases isolated from *P. lindtneri* and *P. chrysosporium* fungi. The antioxidant properties of the enzymes were evaluated using the DPPH method with appropriate control model systems and were presented as EC_{50} normalised values (Table 3). The lowest values of EC_{50} amounted to 10.04 ± 0.75μg/ml for the *Pl*CDH/lactose system, 12.06 ± 1.35μg/ml for the *Pl*CDH/cellobiose system, 12.60 ± 1.51 for the *Ps*CDH/lactose system, and 15.96 ± 1.35 for *Ps*CDH/cellobiose system. In the case of the *Pch*CDH/lactose and *Pch*CDH/cellobiose systems, the EC_{50} values were 159.90 ± 1.88μg/ml and 67.69 ± 0.91μg/ml, respectively. Our earlier experiments proved that the DPPH method seems to be the best for estimation of antioxidative capacity in the case of dehydrogenase cellobiose. Because dehydrogenase cellobiose reacts with the 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonate) radical cation (ABTS.+), the antioxidant capacity (TEAC) assay is not suitable for this enzyme. We demonstrated that the enzyme without substrates did not exhibit antioxidant properties. Via the enzymatic reaction of CDHs with the substrates (lactose or cellulose), reaction products (cellobionic or lactobionic acids) were formed. These reaction products, i.e. cellobionic or lactobionic acids, are probably responsible for the antioxidant properties observed in the study (Fig. 1). Although in the research conducted by Nyanhongo et al. catechol was used as an antioxidant, CDH in

Table 3
EC_{50} values (expressed in μg/ml) for the DPPH radical scavenging assay of CDHs isolated from *P. lindtneri, C. unicolor, P. sanguineus*, and *P. chrysosporium* submerged cultures in comparison to Trolox and Vitamin C.

Name of the fungi	EC_{50} (μg/ml)[a]					References
	Trolox	Vitamin C	CDH	CDH/lactose	CDH/cellobiose	
P. lindtneri (*Pl*CDH)	62.80 ± 0.96	41.36 ± 1.22	405.45 ± 1.73	10.44 ± 0.75	12.06 ± 1.35	data not shown
C. unicolor (*Cu*CDH)	28.42 ± 0.72	25.14 ± 0.61	–	39.83 ± 1.42	48.51 ± 1.56	Sulej et al. (2015)
P. sanguineus (*Ps*CDH)	62.82 ± 1.14	41.25 ± 0.62	71.35 ± 1.46	12.60 ± 1.51	15.96 ± 0.87	data not shown
P. chrysosporium (*Pch*CDH)	24.12 ± 1.32	35.96 ± 0.36	–	159.90 ± 1.88	67.69 ± 0.91	data not shown

[a] EC_{50} (μg/ml): effective concentration at which 50 % of DPPH radicals are scavenged.

Table 4
Time course of the conversion of lactose to lactobionic acid (A) and cellobiose to cellobionic acid (B) by different amounts of CDHs from *P. lindtneri* (*Pl*CDH), *C. unicolor* (*Cu*CDH), *P. sanguineus* (*Ps*CDH), and *P. chrysosporium* (*Pch*CDH).

A

Enzymes	Time (h)	Concentration of CDH (μg/ml)			Concentration of CDH (μg/ml)		
		50	200	600	50	200	600
		Lactose (mM)			Lactobionic acid (mM)		
*Pl*CDH	3	46.7 ± 0.03	47.9 ± 0.02	48.3 ± 0.01	0.0 ± 0.00	0.0 ± 0.00	0.3 ± 0.02
	6	48.6 ± 0.02	48.1 ± 0.04	47.9 ± 0.02	0.0 ± 0.00	0.0 ± 0.00	0.3 ± 0.01
	24	49.8 ± 0.02	49.0 ± 0.01	47.7 ± 0.01	0.0 ± 0.00	0.3 ± 0.00	1.0 ± 0.01
*Cu*CDH	3	48.1 ± 0.04	46.6 ± 0.02	38.7 ± 0.01	0.2 ± 0.01	1.2 ± 0.04	2.7 ± 0.03
	6	47.8 ± 0.01	47.2 ± 0.02	38.7 ± 0.03	0.3 ± 0.01	1.4 ± 0.02	3.4 ± 0.03
	24	47.5 ± 0.03	39.8 ± 0.03	35.6 ± 0.02	0.6 ± 0.02	2.8 ± 0.03	7.1 ± 0.03
*Ps*CDH	3	48.3 ± 0.02	47.8 ± 0.01	47.0 ± 0.04	0.0 ± 0.00	0.2 ± 0.01	0.5 ± 0.02
	6	48.4 ± 0.01	47.3 ± 0.01	46.0 ± 0.02	0.0 ± 0.00	0.3 ± 0.02	0.7 ± 0.02
	24	48.0 ± 0.04	45.9 ± 0.03	42.3 ± 0.02	0.0 ± 0.00	0.6 ± 0.02	1.6 ± 0.01
*Pch*CDH	3	49.1 ± 0.02	48.2 ± 0.02	47.3 ± 0.03	0.0 ± 0.00	0.4 ± 0.01	0.9 ± 0.01
	6	48.9 ± 0.01	48.3 ± 0.03	47.2 ± 0.01	0.2 ± 0.01	0.5 ± 0.03	1.2 ± 0.02
	24	49.2 ± 0.03	48.2 ± 0.03	41.9 ± 0.01	0.3 ± 0.01	1.0 ± 0.01	2.9 ± 0.03

B

Enzymes	Time (h)	Concentration of CDH (μg/ml)			Concentration of CDH (μg/ml)		
		50	200	600	50	200	600
		Cellobiose (mM)			**Cellobionic acid (mM)**		
*Pl*CDH	3	4.2 ± 0.01	3.7 ± 0.02	1.7 ± 0.04	0.0 ± 0.00	0.0 ± 0.00	0.1 ± 0.01
	6	4.1 ± 0.01	3.1 ± 0.02	1.2 ± 0.01	0.0 ± 0.00	0.0 ± 0.00	0.2 ± 0.03
	24	3.4 ± 0.03	1.4 ± 0.03	0.2 ± 0.01	0.0 ± 0.00	0.1 ± 0.02	0.3 ± 0.01
*Cu*CDH	3	4.3 ± 0.01	1.5 ± 0.01	0.1 ± 0.03	0.1 ± 0.02	0.3 ± 0.01	0.9 ± 0.02
	6	4.0 ± 0.01	1.2 ± 0.04	0.0 ± 0.02	0.1 ± 0.01	0.3 ± 0.03	1.0 ± 0.02
	24	1.6 ± 0.02	0.3 ± 0.01	0.0 ± 0.00	0.1 ± 0.01	0.4 ± 0.01	0.9 ± 0.01
*Ps*CDH	3	5.0 ± 0.02	4.9 ± 0.01	3.6 ± 0.02	0.0 ± 0.00	0.1 ± 0.01	0.2 ± 0.01
	6	5.0 ± 0.01	4.8 ± 0.02	3.1 ± 0.03	0.0 ± 0.00	0.1 ± 0.02	0.1 ± 0.01
	24	5.0 ± 0.03	4.7 ± 0.01	2.9 ± 0.01	0.0 ± 0.00	0.1 ± 0.01	0.3 ± 0.03
*Pch*CDH	3	5.0 ± 0.02	3.3 ± 0.03	2.5 ± 0.01	0.0 ± 0.00	0.1 ± 0.01	0.4 ± 0.04
	6	5.0 ± 0.01	3.3 ± 0.03	2.3 ± 0.02	0.0 ± 0.00	0.1 ± 0.03	0.4 ± 0.02
	24	4.8 ± 0.04	2.9 ± 0.04	1.5 ± 0.04	0.1 ± 0.01	0.2 ± 0.02	0.6 ± 0.01

combination with cellobiose created a CDH-antioxidant regeneration system (Nyanhongo et al., 2013). Due to the potentially toxic effects of synthetic antioxidants, the search for new natural free radical scavengers has increased in recent years (Rather et al., 2016). The production of natural molecules such as lactobionic acid in the enzymatic oxidation process seems to be very important for many industrial applications (Minal et al., 2017).

The concentration of the CDH reaction products (LBA and CBA) was determined in the work. Analysis of the products of the reaction catalysed by cellobiose dehydrogenase was performed for three different concentrations of the enzymes, which exhibited different levels of antioxidant properties. The time course of the conversion of lactose to lactobionic acid (LBA) and cellobiose to cellobionic acid (CBA) by the different concentrations of CDHs are shown in Table 4. The results obtained in these assays confirmed the accumulation of different concentrations of LBA and CBA in the reaction medium, depending on the enzyme quantities and incubation time. The highest concentration of LBA and CBA during enzymatic oxidation of disaccharides was achieved after 24 h of incubation at the CDH level of 600 μg/ml. As shown in Table 3, increasing amounts of products were found at 24 h of incubation, as the initial product concentration increased from 0.3 to 7.1 mM for LBA and from 0.1 to 1 mM for CBA. The most effective enzyme was the *Cu*CDH with lactose as a substrate at the concentration of 600 μg/ml, which produced more than 7 mM lactobionic acid after 24 h incubation. The maximum conversion yields in relation to the initial substrate concentration were around 20 % of cellobionic acid and 14 % of lactobionic acid when cellobiose and lactose were used as substrates, respectively. The productivity of this study is significantly lower efficient as previous studies (Baminger et al., 2001; Tian et al., 2018). In recent years, considerable research efforts have focused on the enzymatic production of LBA. Enzymatic systems used to oxidise lactose are specific biocatalytic cascades employing enzymes such as oxidoreductases (Nordkvist et al., 2007; Van Hecke et al., 2009). A study carried out by Baminger et al. (2001) revealed 100 % lactose conversion after only 150 min of incubation with a cellobiose dehydrogenase from *S. rolfsii*, laccase from *Trametes pubescens*, and a redox mediator (ABTS) (Baminger et al., 2001). Saha et al. (2008) reported the total conversion of 150 μM lactose to lactobionic acid within 5 h by CDH from *Termitomyces clypeatus* (Saha et al., 2008). In turn, the native system of *Neurospora crassa* cellobiose dehydrogenase catalysed the oxidation of cellobiose to cellobionic acid using the CDH-redox mediator-laccase configuration (Hildebrand et al., 2015). Microbial and enzymatic conversion of sugars to sugar acids by various biocatalysts can be used for the industrial synthesis of aldonic acids. However, many scientific papers describe the use of CDH for the enzymatic production of cellobionic or lactobionic acids (Ludwig et al., 2004; Tian et al., 2018). In the recent years, considerable attention has been devoted to natural antioxidant and antimicrobial agents. In our study, for the first time, simultaneous antioxidant and antimicrobial properties of the CDH/substrate system have been demonstrated and the concentration of substances responsible for this effect has been quantified.

3.2.2. *Immobilised cellobiose dehydrogenase system*

In order to compare the properties of the CDHs immobilized in the alginate beads with those of the native enzyme stability, antioxidant properties were examined. As presented in Fig. 3, in the presence of lactose or cellobiose as substrates, all the native CDH preparations had very good antioxidant properties (about 80–90 % of scavenging effect) after 7 d of incubation. In turn, a decrease in the antioxidant properties was observed for the preparations with CDH immobilised in the alginate beads (about 5–30 % of scavenging effect) after 7 d. Generally, the antioxidant properties of the immobilised enzyme had a lower value than that of the free enzyme. The reduction of the antioxidant properties of the immobilised CDH may have been caused by steric effects and structural changes in the enzymes after the reaction of immobilisation processes. It might also be related to the lower

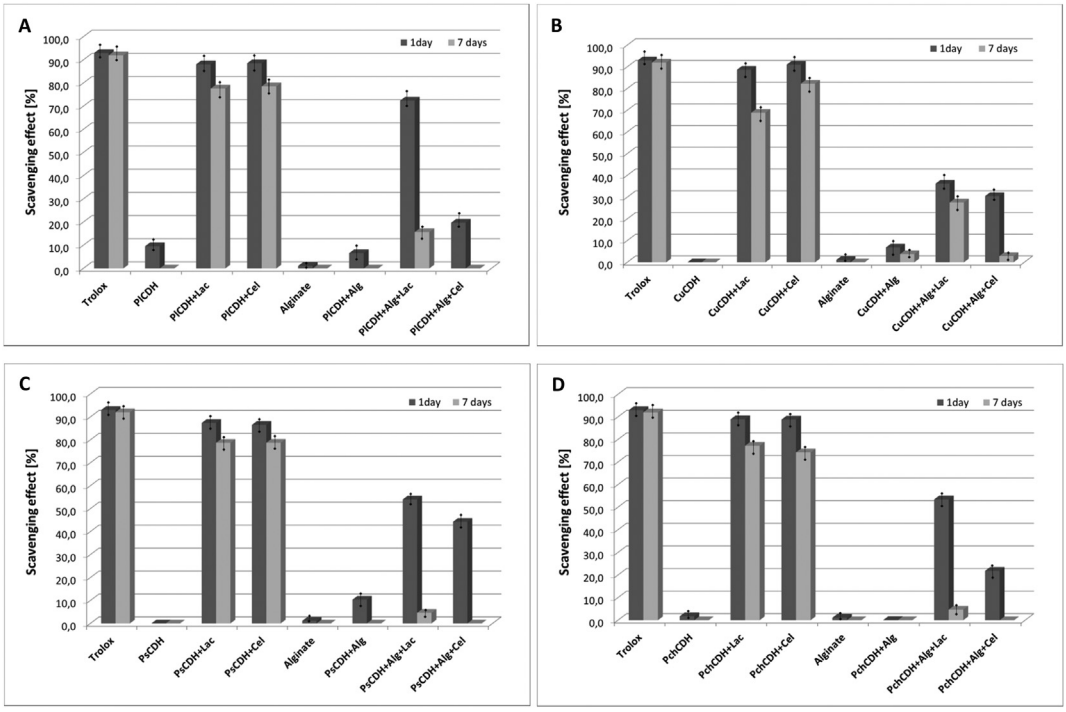

Fig. 4. Stability in time of alginate beads containing CDHs isolated from *P. lindtneri Pl*CDH (A), *C. unicolor Cu*CDH (B), *P. sanguineus Ps*CDH (C), and *P. chrysosporium Pch*CDH (D) after 1 and 7 d of incubation.

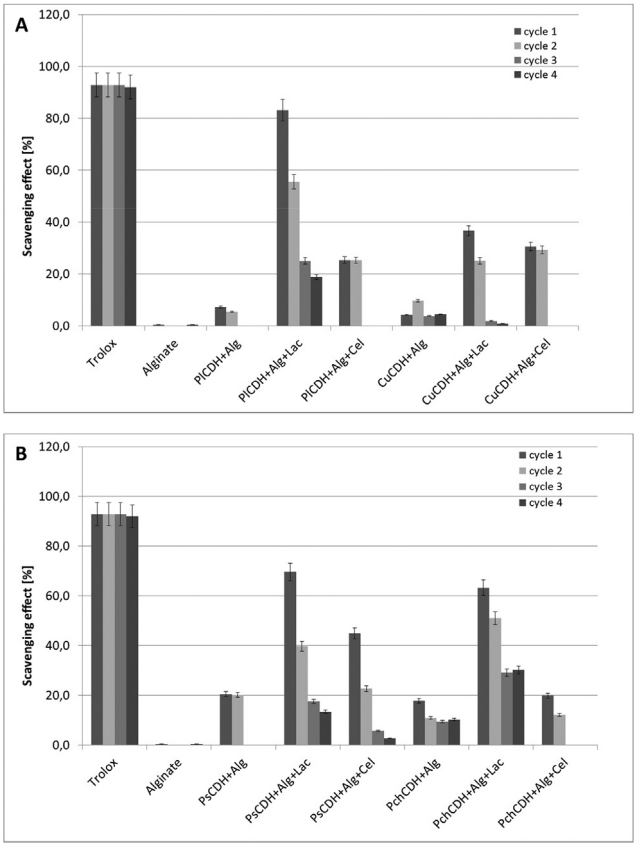

Fig. 5. Operational stability for alginate beads containing CDHs isolated from *P. lindtneri* (*Pl*CDH), *C. unicolor* (*Cu*CDH) (A) and *P. sanguineus* (*Ps*CDH), *P. chrysosporium* (*Pch*CDH) (B).

accessibility of the substrate to the active site of the immobilised enzyme or diffusional limitations of released products (Zdarta et al., 2018).

In our study, we also tested the antioxidant operational stability of the immobilised CDH, which specifies the time after which half of the initial activity of the enzyme was lost and immobilised CDHs simultaneously lost their antioxidant properties. This parameter is very important for preparative and industrial use of immobilised enzymes and allows evaluation of the suitability of the immobilisation method. Among the tested alginate beads containing CDH, the best results were obtained for preparations containing lactose as a substrate. The highest results in the fourth cycle of determinations, (about 35 % and 20 % of scavenging effect) were obtained for *Pch*CDH/Alg/Lac and *Pl*CDH/ALG/Lac (Fig. 4). The operational stability of the immobilised enzyme is a very important parameter. Immobilised enzymes may be easily separated from the reaction solution and reused, which greatly decreases the costs of the enzyme and increases its significance for practical application (Guzik et al., 2014). The operational stability of CDH in our research is low (Fig. 5), compared to the values obtained by laccase immobilised in alginate-gelatin gel used for decolourisation of synthetic dyes (Mogharabi et al., 2012).

4. Conclusions

The conducted research indicated that the antioxidant and antimicrobial properties of the CDH/substrate system are most likely associated with secretion of sugar acids and hydrogen peroxide as products of the enzymatic reaction catalyzed by CDH. This research describes for the first time the differences between the properties of enzymes obtained from four different fungi and the correlation with the concentration of the products. All tested enzymes showed strong antioxidant properties and almost all of them (except *P. lindtnerii*) inhibited the growth of both Gram-negative and Gram-positive bacteria. The obtained results showed the real possibility to use CDHs in the antioxidant and antimicrobial agent generation systems useful in many biotechnological applications e.g. food packaging industry.

All of the enzyme preparations were successfully immobilised in the alginate beads with a protein yield ranging from 61.6 to 100 %, but the biological properties of the immobilised CDH/substrate systems are not satisfactory at present and need further research.

Conflicts of interest

The authors declare that there is no conflict of interest.

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