Characterization of two novel yeast strains used in mediated biosensors for wastewater

Steve P. Trosok, John H.T. Luong, David F. Juck, and Brian T. Driscoll

Abstract: After isolation from a pulp mill wastewater treatment facility, two yeast strains, designated SPT1 and SPT2, were characterized and used in the development of mediated biochemical oxygen demand (BOD) biosensors for wastewater. 18S rRNA gene sequence analysis revealed a one nucleotide difference between the sequence of SPT1 and those of *Candida sojae* and *Candida viswanthii*. While SPT2 had the highest overall homology to *Pichia norvegensis*, at only 73.5%, it is clearly an ascomycete, based on BLAST comparisons and phylogenetic analyses. Neighbor-joining dendrograms indicated that SPT1 clustered with several *Candida* spp., and that SPT2 clustered with *Starmera* spp., albeit as a very deep branch. Physiological tests, microscopic observations, and fatty acid analysis confirmed that SPT1 and SPT2 are novel yeast strains. Physiological tests also indicated that both strains had potential for use in mediated biosensors for estimation of BOD in wastewater. The lower detection limits of SPT1- and SPT2-based $K_3Fe(CN)_6$-mediated biosensors for a pulp-mill effluent were 2 and 1 mg BOD/L, respectively. Biosensor-response times for effluents from eight different pulp mills were in the range of 5 min. Reliability and sensitivity of the SPT1- and SPT2-based biosensors were good, but varied with the wastewater.

Key words: yeast characterization, 18S rRNA gene sequence, pulp-mill wastewater, BOD_5, mediated BOD biosensor.

Résumé : Deux nouvelles souches de levure, désignées SPT1 et SPT2, ont été isolées dans un service de traitement des eaux résiduaires de moulin de pâte à papier, caractérisées et utilisées dans le développement de biosenseurs de demande biochimique en oxygène (DBO) pour l'eau usagée. L'analyse de la sequence de gène 18S rARN a indiqué des différences d'un nucléotide entre l'ordre de SPT1 et ceux de *Candida sojae* et *Candida viswanthii*. SPT2 s'alignait le plus a *Pichia norvegensis*, avec seulement une homologie de 73,5%, elle est évidemment une ascomycete, par comparisons BLAST et analyses phylogénetiques. Les dendrogrammes « neighbour-joining » ont indiqué que SPT1 groupait avec plusieurs espèces de *Candida*, tandis que SPT2 groupait avec des espèces *Starmera*, quoique comme branche plus profound. Les tests physiologiques, les observations microscopiques et l'analyse d'acide gras ont confirmé que SPT1 et SPT2 ont des nouvelles souches de levure. Les tests physiologiques ont également indiqué que les deux souches ont offert des possibilités intéressantes pour l'usage dans les biosenseurs d'évaluation de la DBO d'eau usagée. Les limites de détection inférieures des biosenseurs $K_3Fe(CN)_6$ à base de SPT1 et SPT2 pour un effluent de moulin de pâte à papier étaient 2 et 1 mg DBO/L, respectivement. Les temps de réponse de biosenseur pour des effluents de huit moulins de pâte à papier différents étaient dans l'intervalle de 5 min. La fiabilité et la sensibilité des biosenseurs à base de SPT1 et de SPT2 étaient bonnes, mais variable avec l'eau usagée.

Mots clés : caractérisation de levure, sequence de gène 18S rARN, eaux résiduaires de moulin de pâte à papier, DBO_5, biosenseur de DBO.

Introduction

A standard water-quality test, 5-day biochemical oxygen demand (BOD_5), was designed to relate microbial oxygen consumption to the concentration of biodegradable compounds in a target sample (Greenberg et al. 1992; Chan et al. 1999). The BOD_5 test is not useful, however, for rapid determination of oxygen demand imposed by a given wastewater (Li and Chu 1991; Riedel et al. 1998; Chee et al. 1999). Rapid determination of BOD has been attempted using biosensors containing microorganisms retained on the surface of Clark-type oxygen electrodes (Racek 1995; Riedel 1998). This type of biosensor is limited, however, by the solubility of oxygen in water (~0.3 mM), and is prone to poor reproducibility and sensitivity caused by fluctuations in oxygen concentration (Greenberg et al. 1992; Riedel 1998).

These problems are not characteristic of biosensors that use mediators (low molecular weight redox couples), such as potassium ferricyanide ($K_3Fe(CN)_6$), because oxygen concentration is not directly measured. A recent report also suggests that mediated electrodes produce higher response

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S.P. Trosok,[1] J.H.T. Luong, and D.F. Juck. Biotechnology Research Institute, National Research Council of Canada, 6100 Royalmount Avenue, Montreal, QC H4P 2R2, Canada. **B.T. Driscoll.[2]** Department of Natural Resource Sciences (Microbiology Unit), McGill University, 21 111 Lakeshore Road, Ste-Anne-de-Bellevue, QC H9X 3V9, Canada.

[1]Present address: Elitra Canada Ltd., 225 President Kennedy West, Suite 2250, Montreal, QC H2X 3Y8, Canada.
[2]Corresponding author (e-mail: driscoll@nrs.mcgill.ca).

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signals than oxygen-based electrode sensors (Ge et al. 1998). The mediators in whole-cell sensors are reduced by electrons produced during aerobic cellular metabolism that would have normally shuttled along the electron-transport chain to ultimately reduce O_2 to H_2O (Rawson et al. 1989). For example, $Fe(CN)_6^{-3}$ is reduced by NADPH dehydrogenase (Atkinson and Haggett 1993). The rate of electron flow from cell to electrode via mediator, measured as a current increase, is proportional to the concentrations of oxidizable organic and nitrogenous compounds (Fig. 1; Hobson et al. 1996).

The selection of microorganism(s) to be used as the biocatalyst in a wastewater BOD biosensor is important for optimizing performance. As wastewaters are generally of complex composition (Dence and Reeve 1996), the substrate assimilation ranges of the biosensor microbes must be relatively broad (Suntio et al. 1988) and they must be robust. Metabolic breadth is related to sample affinity and, therefore, to electrode sensitivity (Racek 1995; Sangeetha et al. 1996; Reiss et al. 1998; Riedel et al. 1998; Chan et al. 1999).

Biocatalyst metabolic breadth may be achieved by the use of microbial consortia (Karube et al. 1977; Strand and Carlson 1984; Tan et al. 1992; Tan and Wu 1999), and substrate affinity may be improved if the consortia are isolated from the target wastewater (Princz and Oláh 1990; Li and Chu 1991; Liu et al. 2000). Microbes isolated from the wastewater to be monitored are also less likely to be inhibited or killed during biosensor operation. The major disadvantage in the use of microbial consortia, however, appears to be due to changes in the microbial populations with time, which reduce biosensor stability and reproducibility (Chan et al. 1999; Tan and Wu 1999; Liu et al. 2000).

A better solution is to select a single microbial species from the wastewater of interest for use as biocatalyst. The broad measuring ranges and long-term viabilities of many yeast strains make them good candidates for use as biocatalysts (Racek 1995; Riedel 1998). Several related reports have demonstrated the usefulness of *Arxula adeninivorans* in a Clark oxygen electrode biosensor (Riedel et al. 1998; Chan et al. 1999; Lehmann et al. 1999). This biosensor was sensitive to 3 mg BOD/L for wastewater, yielded reproducible results (<10% coefficient of variation), and was stable for 40 days.

Toward the development of a biosensor for monitoring BOD in pulp-mill effluents, this study focussed on the characterization of two yeast strains isolated from pulp-mill effluent and the response characteristics of BOD biosensors using these strains. The construction and optimization of BOD biosensors using these strains was previously reported (Trosok et al. 2001).

Methods

Strain isolation and growth conditions

An effluent sample obtained from a primary clarifier at a pulp-mill plant in Thurso, Que., was kept on ice until it was returned to the laboratory for analysis. Effluent (5 mL) was inoculated into a 250-mL flask containing 50 mL of Bacto yeast malt extract (YM) broth (Difco Laboratories, Detroit, Mich.) supplemented with 40% (w/v) glucose and adjusted to pH 3.5. The culture was grown with rotary shaking at 240 rpm for 3 days at 15°C, then streaked for single colonies, first on YM agar plates, and then on glucose – peptone – yeast extract (GPY) agar plates (van der Walt and Yarrow 1984). Two isolates, designated SPT1 and SPT2, were single colony purified and used for further study. Plate growth was on YM or GPY agar at ambient temperature, unless otherwise indicated. Other growth media used were prepared as described (van der Walt and Yarrow 1984). Frozen permanents of both strains made by mixing equal volumes of liquid cultures with 20% (v/v) glycerol were stored at –80°C.

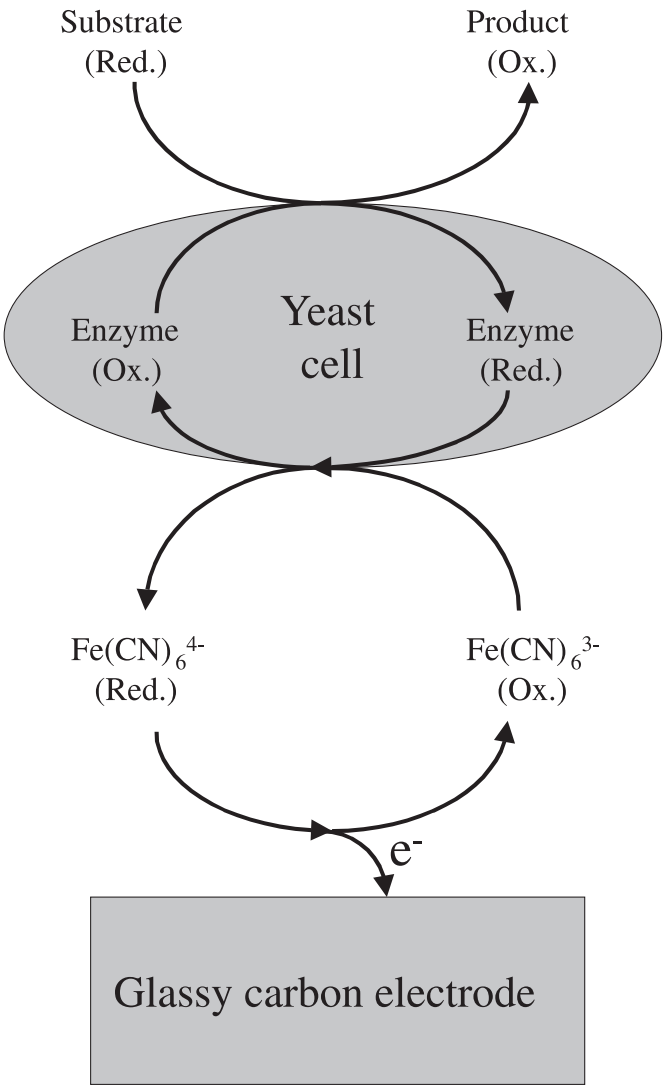


Fig. 1. Schematic representation of a biosensor showing electron mediation via $K_3Fe(CN)_6$ between yeast cellular enzymes, including the electron-transport chain, and an electrode; abbreviations: Red., reduced; Ox., oxidized.

Phenotypic characterization

Standard yeast cytological staining, microscopic, morphological, and physiological characterization methods were as described (van der Walt and Yarrow 1984), except that potato-dextrose agar (Difco Laboratories, Detroit, Mich.) was used to test for ballistospore formation and D-tryptophan (Sigma, St. Louis, Mo.) replaced DL-tryptophan in the test for vitamin-free growth. Fermentation, assimilation, and

growth tests were done in liquid media at ambient temperature (22–24°C), unless otherwise indicated. Mycelial structure was examined using the Dalmau plate technique (Wickerham 1951). Fatty acid composition analyses were done by Microcheck (Northfield Falls, Vt.), using 48-h YM agar plate cultures. Similarity index (SI) scores were based on fatty acid derivative retention times and mean percentages.

Molecular biology techniques

Yeast genomic DNA was extracted from YM broth cultures using the method of Hoffman (1997), except that cell lysis was facilitated by four cycles of freezing and thawing (–70 to 37°C). DNA was extracted with phenol and chloroform and then dissolved in buffer containing 10 mM Tris and 1 mM EDTA (pH 7.8). Agarose-gel electrophoresis of DNA was performed according to standard methods (Sambrook et al. 1989).

The central portions of the SPT1 and SPT2 18S rRNA genes were amplified using primers NS3, NS4, and NS6 (White et al. 1990) on a PTC-100 PCR thermal cycler (MJ Research, Waltham, Mass.). The cycling parameters (25 cycles) were initial denaturation for 2.5 min at 95°C; annealing for 30 s at 55°C; extension for 1.5 min at 72°C; and denaturation for 30 s at 95°C. This was followed by a final extension for 10 min at 72°C. PCR reactions (100 µL) contained 5 ng template DNA, 1 µL (50 µM) each primer, 10 µL amplification buffer, and 10 µL dNTP mixture (Roche, Laval, Que.), and 0.5 µL (5 U/µL) Taq polymerase (Gibco BRL, Life Technologies, Burlington, Ont.). PCR products were purified via phenol–chloroform extraction and ethanol precipitation, and were dissolved in sterile deionized distilled water prior to DNA sequencing.

The same primers were used to directly sequence the PCR products on both strands. Automated DNA sequencing was done by MOBIX (McMaster University, Hamilton, Ont.). The SPT1 (510 nucleotides) and SPT2 (711 nucleotides) sequences were compiled manually and deposited in GenBank under accession numbers AF247474 and AF247475, respectively.

Phylogenetic analysis of 18S rRNA gene sequences

The SPT1 and SPT2 DNA sequences were compared with the non-redundant (nr) nucleotide databases using the standard nucleotide–nucleotide BLAST (blastn) search algorithm (Altschul et al. 1997). Phylogenetic analysis was done using MacVector v. 7.0 (Oxford Molecular Ltd., Genetics Computer Group, Madison, Wis.) and selected algorithms therein. Nucleotide sequences from SPT1 and SPT2 were aligned with those of other yeast species using the CLUSTAL W algorithm (Thompson et al. 1994). The ends of the sequences obtained from GenBank for phylogenetic comparisons were trimmed after alignment to correspond to the ends of the SPT1 (510 nucleotides) sequence. All sequences were identical for the final 10 nucleotides at the 3′ end of the alignment, and for 8 out of 10 nucleotides at the 5′ end. This region corresponds to nucleotides 592–1113, inclusive, of the *Saccharomyces cerevisiae* 18S rRNA gene sequence (Mankin et al. 1986). Phylogenetic trees were reconstructed by the neighbor-joining method (Saitou and Nei 1987), using the distance matrix from the alignment. Distances were calculated using both the Kimura (Kimura 1980) and Tamura–Nei (Tamura and Nei 1993) methods. Gaps were ignored, no gamma correction shape was specified, and for the Kimura method, the transition:transversion ratio was estimated by the algorithm (average = 1.18). Phylogenetic trees were subjected to bootstrap analysis with 1000 replications (Felsenstein 1985). The 18S rDNA sequences (accession numbers in parentheses) retrieved from GenBank for comparison were *Candida guilliermondii* (*Pichia guilliermondii*) JCM 1539 (AB013587); *Candida zeylanoides* JCM 1627 (AB013509); *Candida viswanathii* (AB013589); *Candida tropicalis* (M55527); *Candida sojae* (AB013549); *Candida albicans* (AF114470); *Saccharomyces cerevisiae* (J01353); *Candida inconspicua* (AF201301); *Candida krusei* (M55528); *Pichia norvegensis* (AF201302); *Starmera amethionina* var. *amethionina* (AB017897); *Starmera caribaea* (AB017899); *Candida valdiviana* (AB015910); and the out-group, the basidiomycete *Rhodosporidium dacryoidum* (D13459).

Mediated microbial biosensor

Electrochemical experiments were performed using a model CV-1B (Bioanalytical Sciences (BAS), West Lafayette, Ill.) cyclic voltammograph in combination with a 3 mm i.d. glassy carbon working electrode, a platinum wire counter-electrode, and an Ag/AgCl (saturated KCl) reference electrode (all electrodes from BAS). Yeast cultures were grown in YM broth to late exponential phase (~28 h) and prepared as previously described (Trosok et al. 2001) for use as biocatalysts.

The model wastewater stock solution, GGA (Riedel et al. 1990), consisted of glucose (100 g/L) and glutamic acid (100 g/L), and had a BOD_5 value of 147 g/L (Trosok et al. 2001). Wastewater samples were obtained from seven different Quebec pulp mills and one paper mill (Madawaska; see Table 2). Samples were immediately placed on dry ice and transported to the laboratory. BOD_5 measurements were done using the standard protocol (Greenberg et al. 1992). Wastewater samples and GGA solutions were kept on ice during experiments and were stored at –20°C. All measurements (20-mL jacketed reaction vessel, 10-mL final volume), sensor preconditioning, calibration, and optimization were done as described (Trosok et al. 2001). All measurements were made under optimized parameters (30°C, 100 mM PBS, pH 6.0, 20 mM $K_3Fe(CN)_6$, +0.45 V, with mixing (400 rpm)), unless otherwise indicated. Sensor response was determined by the difference in current (µA) after addition of substrate, as previously described (Trosok et al. 2001). Mean sensor responses were calculated by subtracting sample electroactivity (bare electrode) from the electroactivity of the sensor-containing biocatalyst. Reproducibility for each sample was determined by calculating the coefficient of variation for five effluent measurements.

Results

Isolation of yeast strains from pulp-mill effluent

Our goal was to isolate yeast strains from pulp-mill effluent and then use them to construct BOD biosensors to monitor that type of effluent. Two distinct yeast colony types, designated SPT1 and SPT2, were observed on GPY plates

following enrichment culture of effluent from a primary clarifier from a pulp-mill plant in Thurso, Que. After 4 weeks on malt extract agar (van der Walt and Yarrow 1984), both colony types appeared off-white, waxy, and dull, with a yeasty aroma. SPT1 colonies had convex to umbonate elevations and filamentous configurations, while the SPT2 colonies had convex to pulvinate elevations and round configurations with radiating margins. The two strains were characterized morphologically, to determine if they were likely to be distinct species. SPT1 cells were larger (5.3 × 6.7 μm) than SPT2 cells (2.6 × 3.3 μm), and examination of mycelia revealed that SPT1 produced mycotoruloides, while SPT2 produced mycocandida pseudomycelia. For both strains, tests for fruiting bodies, ballistospores, asci, and ascospores were negative. Differentially staining circular bodies were observed within the vegetative cells, however, and germinating spore-like cells, which appeared to produce long mycelia-like threads upon germination, were observed under all culture conditions.

Analysis of 18S rRNA gene sequences

To determine if SPT1 and SPT2 were novel species, the central portion of their 18S rRNA genes were amplified by PCR, sequenced, and analyzed. BLAST comparison with nucleotide databases and analysis by CLUSTAL W revealed that the 510-nucleotide SPT1 sequence had the highest homology to *C. sojae* (99.8%), *C. viswanathii* (99.8%), and *C. tropicalis* (99.6%). While the 711-nucleotide SPT2 sequence was found to have no very close matches, the three closest matches were to the ascomycetes *C. krusei* (76%), *C. inconspicua* (74.6%), and *Pichia norvegensis* (73.5%).

Nucleotide sequences from SPT1 and SPT2, including the hypervariable regions V2–V4 identified by Sogin and Gunderson (1987), were aligned with those of other yeast species (not shown). The ends of the alignment were defined by the limits of the SPT1 sequence. The number of nucleotides aligned per sequence ranged from 495 (SPT2) to 528 (*R. dacryoidum*), with an average of 512. The SPT1 sequence differed in the V2 region by one nucleotide (a transversion at position 119, with respect to the SPT1 sequence) from that of *C. sojae*. *Candida viswanathii* had an insertion of one nucleotide, relative to SPT1, at position 79.

The SPT2 sequence has extensive variability, especially in the V2 region, compared with any known ascomycete sequence. When the 495 nucleotides of SPT2 used in the alignment were compared with sequence databases using BLAST, the highest homology observed was to *C. valdiviana*, a deeply branching ascomycete. The most striking difference between SPT2 and other sequences in the alignment was the absence of certain stretches of nucleotides in SPT2. SPT2 lacked two more nucleotides between positions 89 and 90 and seven more between positions 116 and 117 than any other sequence. In the latter region, the closest sequences to SPT2 in terms of length were those of the two *Starmera* spp. examined, which were two nucleotides shorter than the others.

A neighbor-joining dendrogram was generated using the partial 18S rRNA gene sequences from the SPT1 and SPT2 strains and representative ascomycete sequences (Fig. 2). As expected, SPT1 clustered with *C. viswanathii* (76% bootstrap support) and other closely related *Candida* spp. (*C. tropicalis*, *C. sojae*, and *C. albicans*). The phylogenetic relationships between the other *Candida*, *Saccharomyces*, and *Pichia* spp. were reconstructed as previously reported from an analysis of complete 18S rDNA sequences (Suzuki et al. 1999), with *C. valdiviana* being the most deeply branching sequence. SPT2 clustered with the *Starmera* spp., as a very deep branch with no closely related sequences. The *Starmera* sequences, which were not included in the phylogenetic analysis of Suzuki et al. (1999), branched off between *C. valdiviana* and the rest of the ascomycete sequences. High bootstrap values were obtained for all major nodes of the phylogenetic tree. For example, the separation of the *C. albicans* – *C. viswanathii* cluster from the *C. guilliermondii* – *C. zeylanoides* cluster was supported by a bootstrap value of 99%, and the separation of the *Starmera*–SPT2 cluster from the rest of the ascomycete sequences had 100% support. The separation of SPT2 from the *Starmera* sequences was also supported by a bootstrap value of 100%.

The same tree topology and high bootstrap values were observed when the Tamura–Nei distance method was used (not shown). When phylogenetic trees were constructed from an alignment of the entire 711-nucleotide SPT2 sequence with sequences from the other ascomycetes (not including SPT1), SPT2 again clustered with the *Starmera* sequences (not shown). Finally, phylogenetic analyses using many more fungal sequences from various divisions and with 0.5 gamma-corrected Kimura or Tamura–Nei distances also clustered SPT2 with the *Starmera* sequences (not shown).

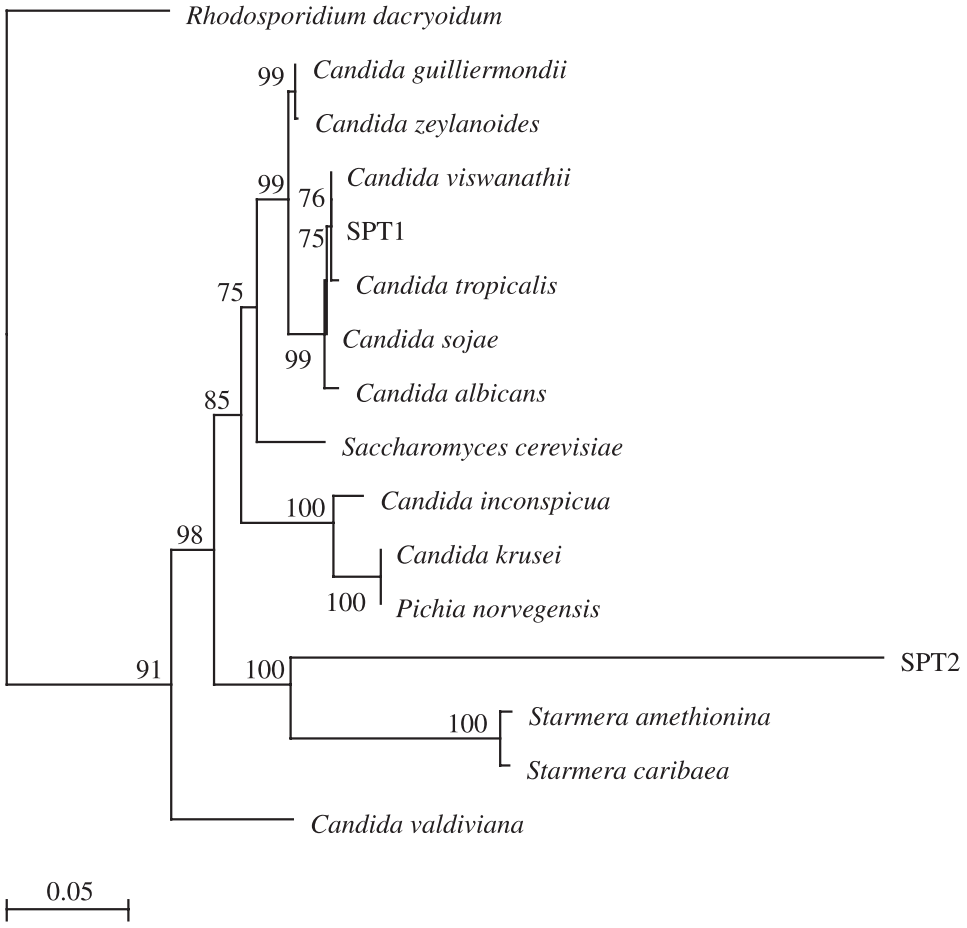
Phenotypic characterization

More than 60 diagnostic tests were done to more fully characterize SPT1 and SPT2. The results for these strains were compared with those of several reference strains using the general keys for identification of yeast species and genera not possessing characteristics of sexual reproduction (Barnett et al. 1983; Kreger-van Rij 1984). Some of the growth-test comparisons are summarized in Table 1.

Starmera amethionina was the closest match to SPT2 using the general key. However, unlike *S. amethionina*, SPT2 was able to ferment D-glucose, assimilate D-glucitol but not DL-lactic acid as sole carbon sources, and utilize cadaverine and L-lysine as sole nitrogen sources. In addition, we were unable to observe the production of definitive ascospores by SPT2. While the physiological profile of SPT1 was nearly identical to that of *C. tropicalis*, SPT1 was unable to metabolize arbutin, soluble starch, and xylitol. Also unlike *C. tropicalis*, SPT1 was able, albeit weakly, to utilize creatine and creatinine as sole nitrogen sources (Table 1). The differences between SPT1 and *C. viswanathii* were much greater. SPT1, unlike *C. viswanathii*, was able to use cellobiose, D-glucitol, DL-lactate, and L-sorbose, but not L(+) arabinose, glycerol, salicin, and soluble starch, as sole carbon sources (Kreger-van Rij 1984).

Analysis of cellular fatty acids from SPT1 and SPT2 failed to yield identities that were either conclusive or consistent with the above analyses. The highest calculated matches for SPT1, based on SI scores (in parentheses), were to the *C. albicans* GC subgroup A (0.688), *Candida famata*

Fig. 2. Phylogenetic relationships between SPT1, SPT2, and representative ascomycete species, based on partial 18S rDNA sequences. The dendrogram was generated by the neighbor-joining method, with Kimura distances, and is rooted to the out group, *Rhodosporidium dacryoidum*. Nodes with greater than 70% bootstrap support (1000 replications) are indicated. The scale bar represents 0.05 nucleotide substitutions per site. Accession numbers are reported in Methods.



(0.419), and *C. guilliermondii* (0.372). The highest SI scores for SPT2 were to *C. zeylanoides* (0.617), *C. famata* (0.487), and *C. krusei* (0.400). These results were only consistent with the other characterization methods in that they indicate that SPT1 and SPT2 are novel strains.

Mediated-biosensor measurement of wastewater

We previously reported the optimization and use of SPT1 and SPT2 in $K_3Fe(CN)_6$-mediated BOD biosensors with a simulated wastewater. However, only SPT1 was used to determine BOD of pulp-mill wastewaters, and only a limited number of wastewaters were tested (Trosok et al. 2001). In this study, the response characteristics of SPT1 and SPT2 biosensors to wastewaters from several pulp mills were compared with BOD_5 values.

Biosensors were calibrated to BOD by measuring responses to several concentrations of model wastewater GGA (Fig. 3). The SPT1 biosensors provided linear response over a range of 2–100 mg GGA/L, while the SPT2 biosensors provided linear response over a somewhat broader range, 1–200 mg GGA/L. The BOD_5 values of the pulp-mill effluents tested in this study ranged from 3 to 11.3 mg/L, well within the linear response ranges of both biosensors (Table 2). The lower detection limits of the SPT1 and SPT2 biosensors to diluted Thurso effluent were 2 and 1 mg BOD/L, respectively (not shown).

The major limit to the stability of these biosensors appeared to be related to the retention of yeast cells on the electrode for long periods of time. Loss of the biological component of the biosensors via leakage resulted in failure of the biosensors to respond to GGA or wastewater concentration. The stability of the SPT2 biosensor, under conditions identical to those used for the SPT1 biosensor (Fig. 2*b* in Trosok et al. 2001), is shown in Fig. 4. Both biosensors provided stable responses to injections of Thurso effluent for 11 days after construction when maintained in PBS buffer (starvation conditions). Both biosensors performed best when preconditioned by starvation for 4 days prior to use. In a separate experiment, the SPT1 and SPT2 biosensors provided stable responses to injections of GGA (10 mg BOD/L) for 22 and 14 days, respectively (data not shown). Therefore, the SPT1 and SPT2 biosensors provided best results when used between 4 and 11 days after construction.

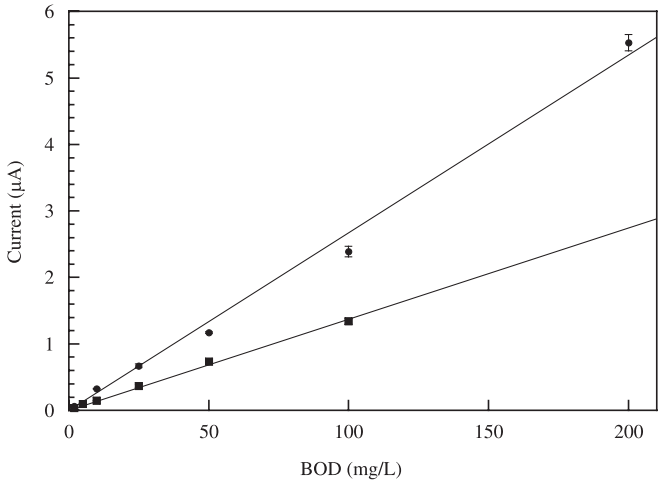
The relative responses between the two sensors varied according to the effluent being measured. For all wastewaters, mean sensor response coefficients of variation ranged from a

Table 1. Comparison of growth-test results for SPT1 and SPT2 with those of selected reference strains.

	SPT1	*Candida tropicalis*	*Candida albicans*	*Candida sojae*	SPT2	*Starmera amethionina*	*Candida zeylanoides*	*Pichia norvegensis*
Assimilation of nitrogenous compounds								
Substrate								
Cadavarine	+	+	+	+	+	–	*v*	+
Creatine	–, *w*	–	–	na	–, *w*	–	–	–
Creatinine	–, *w*	–	–	na	–, *w*	–	*v*	–
Ethylamine	+	+	+	+	–, *w*	–	*v*	+
L–Lysine	+	+	+	+	+	–	*v*	+
Nitrite	–	–	–	–	–	–	*v*	–
Nitrate	–	*v*	–	–	–	–	–	–
Growth under various conditions								
25°C	+	+	+	na	+	+	+	+
30°C	+	+	+	na	+	+	+	+
35°C	+	+	+	na	+	+	+	+
37°C	+	+	+	na	+, *w*	+	*v*	+
42°C	+	+	+	–[a]	–	–	–	+, *w*
10% NaCl, 5% D–Glucose	+	na	na	na	–	–	na	+
50% D–Glucose	+	+, *d*	–	–	–	–	+, *d*	–
60% D–Glucose	–	–	–	–	–	–	–, *d*	–
Without vitamins	–	*v*	–	–	–	–	*v*	–
Cycloheximide								
0.01%	+	+	+	na	–	–	+	–
0.1%	+	+	+	na	–	–	+	–
Additional characteristics								
Diazonium blue B	–	–	–	–	–	–	–	–
Acid production	–	–	–	+, *w*	+, *w*	–	–	–
Starch formation	–	–	–	–	–	–	–	–

Note: Abbreviations used: +, positive (growth in 1 week); –, negative (no growth in 3 weeks); *d*, delayed (growth after 1 week); *v*, variable response (some replicates weak, some negative); *w*, weak (little growth in 3 weeks); na, data not available. Data for *C. tropicalis*, *C. albicans*, *C. sojae*, *S. amethionina*, *C. zeylanoides*, and *P. norvegensis* were obtained from the literature (Barnett et al. 1983; Kreger van Rij 1984; Nakase et al. 1994).
[a]Maximum growth temperature for *C. sojae* is 39–40°C (Nakase et al. 1994).

Fig. 3. Linear ranges of SPT1 and SPT2 biosensor calibration curves to BOD (mg/L) of model wastewater (GGA). Data are the mean value (±SE) of a minimum of three measurements for SPT1 (■) and SPT2 (●).



low of 2.5% to a high of 20.9% (Table 2). Inherent electroactivity of the wastewater samples, measured using electrodes to which cells had not been added (bare electrode), varied greatly from sample to sample. These values were, however, always lower than those of electrodes containing SPT1 or SPT2 cells. This indicated that the responses of electrodes containing cells were due to the detection, via electron mediation, of oxidation of substrates in the wastewaters by the cells. Wastewater injections (200 μL) achieved response plateaus in approximately 5 min. Mean sensor responses (see Trosok et al. 2001) were converted to "biosensor BOD" values by subtracting inherent sample electroactivity and using GGA as a standard. As expected, the calculated BOD values varied from the actual BOD_5 determinations, indicating that the biosensors need to be standardized with each wastewater for practical application.

Discussion

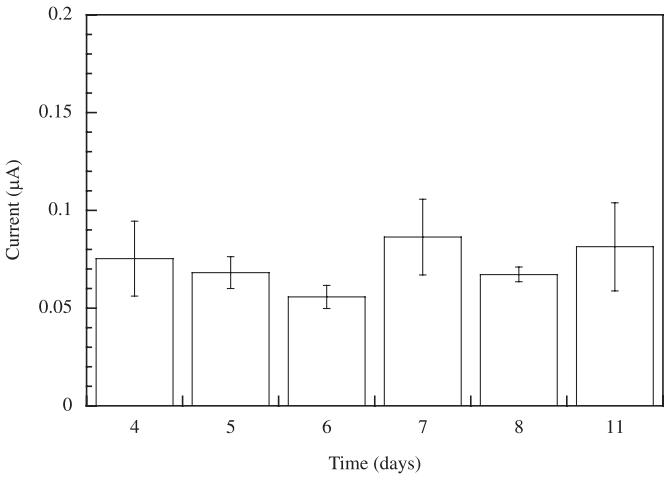
As new yeast isolates are often difficult to identify, we chose to use a combination of molecular and conventional methods to characterize SPT1 and SPT2. This type of approach is discussed in a recent review (Valente et al. 1999). 18S rRNA gene sequence analysis, standard metabolic characterization methods, and fatty acid analysis each indicated

Table 2. Biosensor responses to pulp-mill and paper-mill (Madawaska) effluents.

	Mean sensor response (nA)[a]			BOD value (mg/L)[b]		
Facility sampled	Bare electrode	SPT1	SPT2	SPT1	SPT2	BOD_5
Beaupré	13.7 (9.4)	306 (20.9)	280 (17.6)	22.3	10.2	11.3
Donnacona	13.0 (5.0)	60.0 (6.44)	61.9 (7.39)	4.37	2.25	7.68
Edmundston	10.7 (6.0)	59.3 (2.51)	160 (7.31)	4.32	5.82	7.00
Fraser	11.1 (0)	51.8 (8.89)	74.9 (13.1)	3.78	2.72	3.68
Grand Mère	25.2 (9.2)	41.9 (13.9)	87.9 (4.58)	3.06	3.20	13.3
Kenogami	10.7 (6.0)	66.9 (2.47)	49.5 (4.59)	4.88	1.80	3.50
Madawaska	–8.90 (0)	53.8 (4.83)	33.6 (11.2)	3.93	1.22	3.00
Thurso	6.30 (20.4)	72.9 (7.39)	41.7 (6.42)	5.32	1.52	4.58

[a]Calculated by subtracting the average (n = 5) bare electrode value (inherent sample electroactivity) from the average (n = 5) biosensor reading. Values in parentheses are coefficient of variation.
[b]Mean sensor response was converted to BOD using the slope of the linear response ranges of the SPT1 (13.7) and SPT2 (27.5) biosensors to BOD concentrations, using GGA as standard.

Fig. 4. Stability of SPT2 biosensors preconditioned by starvation (100 mM PBS, pH 7.2) for the indicated time. Data are the mean value (±SE) of a minimum of three measurements, using 20 mg BOD/L (Thurso effluent), at 22°C.



that SPT1 is a *Candida* species, but not a previously characterized strain. Analysis of the central 18S rRNA gene sequence of SPT1 indicated a close relationship to both *C. viswanthii* and *C. sojae*, each of which differed from it by only one nucleotide.

As the 18S rDNA sequences of *C. viswanathii*, *C. tropicalis*, *C. sojae*, and *C. albicans* are so similar, the bootstrap values at the nodes separating these sequences on the phylogenetic tree (Fig. 2) were not very high. This was expected, as similar results were previously reported for these species when the entire 18S rDNA sequences were compared (Suzuki et al. 1999), and indicates that branching order within this cluster is not highly significant. However, the bootstrap values were very high for the major nodes separating the clusters of sequences, which branched as was reported for the complete 18S rDNA sequences (Suzuki et al. 1999). As the nodes separating the major clusters containing the SPT1 and SPT2 sequences had bootstrap values of 99–100%, they appear to be significant.

Candida sojae is a well characterized yeast (Nakase et al. 1994) and differs from SPT1 in many ways, including colony morphology. Unlike *C. sojae*, SPT1 was unable to assimilate glycerol and soluble starch, ferment maltose, and grow at 42°C. Physiological characterization suggested that SPT1 was closest to *C. tropicalis*, which also clustered reasonably closely with the SPT1 sequence (Fig. 2). However, differences in carbon metabolism and dissimilar pseudomycelial structures between SPT1 and *C. tropicalis* suggested that SPT1 was distinct. Numerous differences in carbon metabolism suggested that SPT1 was also distinct from *C. viswanathii*, with which it also clustered closely. The closest match obtained via fatty acid analysis, *C. albicans*, again clustered reasonably closely with the SPT1 sequence; however, this result was supported neither by the physiological tests (cellobiose, gluconate, starch, xylitol assimilation, and sucrose fermentation) nor by the apparent absence in SPT1 of the pseudomycelia (mycotorula) characteristic of *C. albicans*. These results, taken together, indicate that SPT1 is a novel *Candida* strain; however, they do not support the assignment of this isolate to a previously described species.

The three different identification approaches revealed that SPT2 is quite unlike other characterized yeasts. None of the species that most closely matched SPT2 via 18S rRNA gene sequence analysis (*P. norvegensis*), physiological testing (*S. amethionina*), or fatty acid analysis (*C. zeylanoides*), clustered with the SPT2 sequence (Fig. 2), and sequence homology was no higher than 73.5%. Besides stretches of highly variable sequence, one of the most obvious differences between the SPT2 and other ascomycete sequences, in the V2–V4 region (Sogin and Gunderson 1987), is the length of the sequence. The SPT2 sequence has two regions in which a total of seven nucleotides that are found in other species are not present. It is in the shared absence of two of these nucleotides that SPT2 and the *Starmera* spp. are different from all other species. This seems to explain why SPT2 clustered with the *Starmera* sequences (71% homology) rather than with other species with which it shared somewhat higher overall homology in pairwise alignments or shorter stretches of higher homology, as revealed by BLAST comparisons.

SPT2 also differed, with respect to several important characteristics, from *P. norvegensis* (e.g., glucose fermentation, utilization of cellobiose, glucosamine, and mannitol), *S. amethionina* (e.g., glucose fermentation, definitive ascospores), and *C. zeylanoides* (e.g., colony morphology, pseudomycelial structure, cycloheximide resistance). It ap-

pears, therefore, that SPT2 is a novel species. A finer resolution of the phylogenetic relationship between SPT2 and other yeasts will be possible as new strains are characterized and their 18S rDNA sequences are deposited in the databases.

The biocatalytic characteristics of SPT1 and SPT2 were tested in mediated biosensors for pulp and paper wastewater. Both biosensors responded reliably to GGA and to different pulp-mill effluents (Table 2). Sensor response coefficient of variation (CV) was less than 10% in six (of eight) wastewaters for the SPT1 biosensor and five for the SPT2 biosensor. All but one determination was found to have a lower than 20% CV. The SPT1 biosensor appeared to be more sensitive than the SPT2 biosensor, as the BOD values obtained with SPT1 were generally higher. This result is consistent with previous findings (Trosok et al. 2001) and may be related to the finding that SPT1 was able to assimilate more carbon compounds than SPT2.

Others have also found that broad carbon-assimilation capacity is an important characteristic of biocatalysts used in biosensors for complex wastewaters such as pulp-mill effluent (Racek 1995; Sangeetha et al. 1996; Riedel et al. 1998). However, when comparing the responses of the two biosensors to the different wastewaters, it was observed that the SPT2 biosensor BOD values were closer than those of the SPT1 biosensor to the BOD_5 values for three of the effluents. This seems to indicate that substrate-assimilation profiles and responses to simulated wastewaters do not always predict the utility of a biocatalyst for a particular effluent. Other adaptation factors, such as cellular resistance to inhibitory compounds in the wastewater and the ability to oxidize compounds present in the wastewater but not assayed as part of standard physiological tests, are also likely to affect biocatalyst performance (Trosok et al. 2001).

Calibration of the biosensors using the GGA solution (Fig. 3) resulted in calculated BOD values that differed from BOD_5 values (Table 2). For practical applications, the biosensors could be calibrated to a specific wastewater using the BOD_5 data from that source. Our results, with the two biosensors calibrated to GGA to facilitate direct comparisons with each other, demonstrate the relative responses of the biosensors to the different wastewaters. As expected, the SPT1 and SPT2 biosensors performed differently with the different wastewaters; however, overall sensitivity and reliability (Trosok et al. 2001; Fig. 4) were good for both. These biosensors were used to determine BOD values for wastewater samples in approximately 5 min, as opposed to the 5 days required to determine BOD_5.

In conclusion, we have characterized two novel ascomycetes isolated from a pulp-mill effluent using a combination of molecular and conventional methods. SPT1 appears to be a *Candida* species, while SPT2 appears to be a very deeply branching ascomycete, with no previously reported close relative. As part of the characterization of these strains, we demonstrated that biosensors containing them responded well to several wastewaters similar to that from which they were isolated.

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