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MEDIATED BIOCHEMICAL OXYGEN DEMAND BIOSENSORS FOR PULP MILL WASTEWATERS

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March, 2000

A Thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the degree of Master of Science

MEDIATED YEAST BOD BIOSENSORS

ABSTRACT

Mediated microbial sensors utilizing two different yeast isolates (SPT1 and SPT2) were developed for the estimation of biochemical oxygen demand (BOD). Measurements of glucose/glutamic acid (GGA) standard solution with potassium ferricyanide mediation resulted in linear ranges extending from the detection limits (i.e. 2 and 5 ppm BOD) to 100 and 200 ppm BOD for the SPT1- and SPT2- based sensors, respectively. The standard error of the mean (SEM) for 10 ppm BOD measurements was 10.1 % (SPT1) and 3.9 % (SPT2). Response reproducibility had 10.6 % error between three identically prepared SPT1 sensors. Response times for concentrations of 20 ppm BOD were within 10 minutes. For pulp mill effluent, the detection limits were 2 (SPT1) and 1 (SPT2) ppm BOD, with SEMs of 3.6 % and 14.3 % for the SPT1 and SPT2 sensors, respectively. Based on the results obtained in this study, it is concluded that SPT2 is the more suitable biocatalyst for pulp mill wastewater analysis.

While 18S rRNA gene sequence analyses, including BLAST homology searches, have suggested that isolate SPT1 is a close relative of *Candida sojae* (99.8 % homology), no close matches have been found for isolate SPT2. The closest match for SPT2 was to *Candida krusei* (76.0 % homology). Evidence from biochemical tests, fatty acid analysis, and 18S rRNA gene sequence analyses, indicates that isolate SPT2 is a novel yeast species.

RÉSUMÉ

Des isolats de levures, SPT1 et SPT2, ont été utilisés en tant qu' éléments sensibles pour la mise-au-point de biocapteurs ampérométriques. Une estimation de la demande biochimique en oxygène (DBO) d'effluents de pulperies a été possible en utilisant un composé électroactif (ferricyanure de potassium) comme médiateur entre les isolats de levure et la surface de l'electrode. Dans le cas de solutions standardisées de glucose/acide glutamique (GGA), la réponse du biocapteur (courant) en fonction de la concentration (entre 2 et 100 ppm avec SPT1, entre 5 et 200 ppm avec SPT2) est linéaire. De plus, les coefficients de variation (CV), à l'ajout de 10 ppm de DBO, pour une même électrode, sont respectivement de 10.1 % (SPT1) et 3.9 % (SPT2). Des temps de réponse inférieurs à 10 minutes ont été obtenus après addition de 20 ppm de DBO. Une reproductibilité de 10.6 % a été atteinte après montage de trois capteurs utilisant l' isolat SPT1. Dans le cas des effluents de pulperies, les limites de détection sont respectivement de 2 ppm de DBO (SPT1) et 1 ppm de DBO (SPT2) pour des CV de 3.6 % (SPT1) et 14.3 % (SPT2). A la vue des résultats, l'isolat SPT2 semble le biocatalyseur le plus approprié quant à l'analyse des eaux usées provenant de pulperies.

Les analyses de séquence génique de l'ARNr 18S, incluant des recherches d'homologie "BLAST", ont suggérées que l'isolat SPT1 est un proche parent de *Candida sojae* (homologue à 99.8 %). Quant à l'isolat SPT2, aucun rapprochement significatif n'a pu être fait. SPT2 possède seulement 76.0 % d'homologie avec son plus proche parent, *Candida krusei*. Les résultats des analyses biochimiques, des analyses de la composition des acides gras ainsi que des analyses de séquence génique de l'ARNr 18S tendent à suggérer que l'isolat SPT2 est une nouvelle espèce de levure.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to Dr. John H.-T. Luong for enabling me to undertake this research project in his laboratory at the Biotechnology Research Institute (BRI). His advice and criticism was much appreciated. Furthermore, I extend my deepest gratitude to Dr. Brian T. Driscoll for believing in me and for his continued guidance throughout the past two years. I am indebted to the members of both the Macdonald Campus laboratory (Marc Dumont, Luigi Marcangione, Sergey Dymov, and Fred D'Aoust) and the BRI biosensor group (Tracey Rigby, Abdelkader Hilmi, Keith Male, Hamid Nazari, An-Lac Nguyen, Coralie Saby, and Pierre Bouvrette) for their continued technical assistance and social persistence. I am particularly grateful to Keith Male for proof reading my thesis and making many important suggestions. A special mention must be made of - soon to be - Dr. David Juck for his suggestion of, and assistance in, 18S rDNA sequence analysis. I must also thank Wayne Levadoux and Denis Bourque for technical assistance and the supply of many chemicals; and Maria Oliver for supplying sterilized filters and free tea. I am indebted to Francis Gauthier for his kind supply of effluents and responses to questions. Both Francine Landry and Marie Kubecki were completely wonderful with me and their secretarial expertise is deeply appreciated. I also thank my many friends at BRI for making the last few years fun, even through the tough times. I wish to extend my appreciation to Dominic Frigon for introducing me to Dr. Driscoll and for being a friend. I am also grateful to Caroline Bonan for many intellectual conversations and for urging me on. My good friends David Assayag, Les Hollic, Peter Wong, Nick Kollias and Rózsa Attila are noted for their moral support throughout this venture. An extraordinary thanks goes out to my parents (without whom I would not have even attempted this endeavour) and siblings for "encouraging" me over the years. I also thank Misty for being Misty. Special recognition goes out to Pierre Bouvrette for all the laughs, the lunchtime hockey, for always listening, pour le résumé, but most of all for giving me a start. Finally, a heartfelt gratefulness is extended to Toula Xanthakos for being there for me always.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

BOD	biochemical oxygen demand
bp	base pair
CD	cyclodextrin
CV	cyclic voltammetry
DBB	diazonium blue B
DMFe	dimethylferrocene
DMFe+	dimethylferricinium
DNA	deoxyribonucleic acid
GGA	glucose-glutamic acid
GPY	glucose peptone yeast extract
Hanes plot	[substrate] / velocity versus [substrate]
HMF	hydroxymethylferrocene
HMF+	hydroxymethylferricinium
hp-ß-CD	hydroxypropyl-ß-cyclodextrin
i_{pa}	anodic peak current
i_{pc}	cathodic peak current
K_s	reaction constant: | x-intercept |
ME	malt extract
min	minute(s)
MWCO	molecular weight cut-off
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PD	potato dextrose
ppm	parts per million (mg/L)
RNA	ribonucleic acid
rRNA	ribosomal RNA
s	second(s)
SI	similarity index
TE	Tris EDTA (ethylenediamine-tetraacetic acid)
TMB	tetramethylbenzidine
TTF	tetrathiafulvalene
V1	variable region 1
V_{max}	maximum velocity (current): K_s / y-intercept
YM	yeast malt extract

LIST of TABLES

LIST OF FIGURES

INTRODUCTION

The objective of this research was to develop and optimize a mediated biosensor system which can rapidly and accurately measure the biochemical oxygen demand (BOD) of pulp mill wastewater. The biosensor must have good reproducibility, high sensitivity, broad response range, long service and storage life, and be simple to operate. Such a biosensor would allow for improved process optimization and management of effluent treatment. Currently, the standard BOD_5 analysis is laborious, time-consuming, and poorly reproducible.

There have been numerous attempts to create a BOD biosensor which can meet these criteria, based on the use of a Clark-type oxygen electrode. These BOD biosensors retain microorganisms on the surface of the electrode, and measure changes in oxygen concentration at an applied potential of approximately +600 mV. An important limitation associated with oxygen-based BOD biosensors is the lack of a suitable response range, owing to the finite concentration of oxygen present in a given sample (0.4 mM). Also, fluctuating oxygen concentrations in the measuring sample can create artifacts.

Second generation biosensors (mediated biosensors) make use of small organic compounds or mediators such as ferricyanide, ferrocene, and tetrathiafulvalene to shuttle electrons from the redox centre of reduced microbial enzymes to the surface of an electrode. Electron flow, measured as a current increase, is proportional to the substrate concentration in the sample. In addition to reducing electrochemical interference, mediated biosensors have improved reliability because the errors caused by variability in dissolved oxygen concentrations are eliminated. The biosensors developed here measured substrate metabolism rather than the change in oxygen concentration.

Two yeast strains isolated from pulp mill effluent in Thurso, Quebec, were used as the biological component of biosensors developed in this study. A secondary objective was the identification of the isolates. Characterization was done via standard physiological and biochemical tests, long-chain fatty acid analysis, as well as small subunit rRNA (18S rRNA) gene analysis.

LITERATURE REVIEW

1.1 Standard BOD_5 technique

Pulp mill wastewater is comprised of a complex mixture of hundreds of compounds (Dence and Reeve, 1996; Suntio et al., 1988). Over 400 individual compounds have been identified. While the majority of the compounds characterized have a molecular weight of less than 1000 g/mol, most compounds in wastewater have a molecular weight >1000 (Dence and Reeve, 1996). Characterization of pulp mill effluent is difficult because little is known about effluent composition and because effluent composition changes over time depending on the type of wood being processed, the pulping procedure, and the degree of wastewater treatment (Dence and Reeve, 1996; Lloyd et al., 1997). The release of untreated effluent containing the degradation products of hemicelluloses and lignin creates an increased oxygen stress on receiving waters caused by organic compounds. Most dissolved organics are generated during the pulping and bleaching treatment stages (Dence and Reeve, 1996; Lloyd et al., 1997). Carbohydrates, carboxylic acids and lignin make up 67-87 % of the dissolved organic carbon found in the processing of hardwood kraft pulp. Ristolainen and Alén (1998) found that the wood sugar xylose accounted for 93% of the carbohydrate fraction.

The biochemical oxygen demand (BOD) test is a standardized laboratory procedure designed to measure the oxygen requirements in waters receiving organic pollutants (Greenberg, 1992). It is an estimation of the amount of oxygen consumed by bacteria and protozoa during the total biodegradation of organic matter (carbonaceous) and the oxidation of inorganic reducing solutes (sulphides and ferrous iron). A common modification of this procedure called the BOD_5 is a universally accepted parameter for the determination of organic pollution concentration in wastewater and has been in use since 1936 (LeBlanc, 1974). The BOD_5 value expresses the oxygen consumption in a sample kept in the dark at 20 °C for 5 days. The decrease in oxygen concentration is directly proportional to the concentration of biodegradable organic compounds in

the effluent (Chan et al., 1999). Thus the BOD_5 test allows for an index of the concentration of biodegradable organic compounds in wastewater (Tan et al., 1993). Ideally, potable waters should have a BOD_5 value of zero, however, wastewater from pulp mill sources can have a BOD_5 value as high as 20 000 - 30 000 mg/L (Li et al., 1994).

The major disadvantage of the BOD_5 test is that the procedure requires 5 days to evaluate the oxygen demand imposed by a given wastewater, thus allowing for no feedback information for immediate wastewater process control. The 5-day BOD test was not designed for on-line monitoring, but rather to assess the effect of pollutants on the oxygen-carrying capacity of receiving waters (LeBlanc, 1974). The BOD_5 test is laborious, time-consuming, and dependent on the skill of the operator. The accuracy of BOD_5 measurements can vary from 15-50% depending on the microbial environment (i.e. temperature, oxygen concentration, toxicants, etc.) and the response of the microbial seed to it, and has an inherent error of over 10% (Chee et al., 1999; Li and Chu, 1991; Fitzmaurice and Gray 1989; Karube 1977; Riedel et al 1990). On-line estimation of organic compound concentration requires a simple and reproducible method to enable for pollution control. The standard BOD_5 test cannot meet these criteria.

1.2 Oxygen electrode-based BOD biosensors

To date, researchers have been attempting to improve upon the standard BOD_5 measurement. These attempts have included shortening the incubation time and raising the temperature; correlation of BOD_5 results with various other tests such as the chemical oxygen demand, or total organic carbon; increase of the inoculation size or using specialized inocula. Unfortunately, these modifications have all suffered from a lack of reproducibility (LeBlanc, 1974). Thus far, the most promising improvements have come from the field of biosensor technology (Riedel et al. 1998).

Biosensors are composed of a biological component (biocatalyst) and a transducer (i.e. electrode) which can convert the chemical signal of the biocatalyst into a quantifiable electronic one

(Reiss et al., 1998). In BOD biosensors, microbial cells (bacteria or yeasts) are predominately used as the biocatalyst (Racek, 1995). Whole cells are preferred over enzymes since the complex mixture of compounds found in effluent precludes the use of enzyme based biosensors. The first sensor for BOD measurement was developed in 1977 (Karube et al., 1977). Since then the vast majority of BOD biosensors have been based on amperometric oxygen electrodes. Over the past 23 years, many different microorganisms have been used as biocatalysts. A short list of some of these BOD biosensors can be found in Riedel et al. (1998) and Racek (1995).

Oxygen based BOD biosensors are all similar in design in that the biological component is sandwiched between a semipermeable outer membrane and a gas permeable inner Teflon® membrane covering the sensor (Riedel, 1998). The membranes and microorganisms are often held in place by a rubber O-ring. There are two types of measuring systems in which the oxygen based biosensor can be used. Biosensors can be used in "batch" systems where the measurement is made in a stirred chamber to which the substrate is added, or in a flow-through system which allows for continuous substrate monitoring (Riedel, 1998).

Since BOD determinations are based on the change in oxygen concentration at the surface of the electrode, precise measurement of BOD levels depends on stable and sufficient oxygen concentrations. A fluctuating oxygen concentration can introduce poor reproducibility while a low oxygen concentration can prohibit the complete measurement of oxygen consumption (Racek, 1995; Riedel 1998). Most oxygen electrodes consist of a solid platinum cathode and a silver anode. Once the potential has been applied, oxygen is selectively reduced at the cathode (Karube and Nakanishi, 1994):

$$\text{cathode: } O_2 + 2H_2O + 4e^- \rightarrow 4OH^-$$

$$\text{anode: } 4Ag + 4Cl^- \rightarrow 4AgCl + 4e^-$$

The electrode measures the oxygen concentration present at the interface between the electrode and the microbial layer. The oxygen reduced at the cathode creates an oxygen consumption current displayed as the base line or steady-state current. Upon addition of the effluent to the mixing chamber, the readily biodegradable compounds are oxidized by the microorganisms, causing an increase in their respiration rates. This causes a reduction in the oxygen concentration diffusing to the electrode, which is reflected by a rapid decrease in the current and the establishment of a steady-state. The difference between the two steady-states is proportional to the concentration of readily biodegradable compounds in the sample (Riedel, 1998). BOD biosensors are generally calibrated with a glucose and glutamic acid (GGA) standard solution. The linear portion of the calibration curve is used to calculate the BOD value of the wastewater sample. Owing to the short exposure time, the sensor BOD results represent the oxidation of only readily degradable compounds. Consequently, BOD biosensor results are not considered true BOD_5 values but rather represent a BOD index which can be correlated to BOD_5 results (Marty et al., 1997; Praet et al., 1995; Riedel, 1998; Tanaka et al., 1994).

The BOD_5 values determined for various wastewaters, including municipal and industrial sources, indicate that the best biosensor/BOD_5 correlations come from untreated wastewater emanating from fermentation and food plants. It is believed that this is due to the abundance of easily assimilated compounds (Riedel, 1998; Tanaka et al., 1994). Li and Chu (1991) suggested that in order to expand biosensor application to many different types of wastewaters, it is necessary to use biocatalysts isolated from the substrate to be measured. However it was shown that substrate specificity can be improved simply by preincubating (or preconditioning) the microbial electrode in a buffer containing some of the wastewater to be measured (Princz and Olah, 1990; Riedel, 1990; Tan et al., 1993).

There is a great variation in performance between the many biosensors developed thus far. The average linear range (determined using the standard solution) of these biosensors is approximately 10-80 ± 40 mg/L BOD (Yang et al., 1996). A good list of these results can be found

in Riedel (1998b). Recently, the yeast *Arxula adeninivorans* has been used to develop a BOD biosensor which yielded an excellent measuring range of 2-550 mg/L BOD (Chan et al., 1999; Riedel, 1998). Other authors have concentrated on developing a biosensor which is capable of detecting BOD concentrations down to 0.2 or 0.5 mg/L (Chee et al., 1999; Yang et al., 1996). One consequence of the later type of biosensor is the tendency for a short lifetime. The majority of BOD biosensors have good stability for about one month (Riedel 1998), however these low BOD biosensors lasted for only 3-10 days (depending on storage temperature). When batch measurement techniques are used, the average response time is about 20 min. Measuring the slope of the response (dynamic transient technique) allows for measuring times as low as 30 s (Yang et al., 1996). Other important factors determining the measuring time include the concentration of the sample, quantity of organisms, and the organisms used. Generally, the higher the BOD concentration, and the more microorganisms immobilized, the longer the response time. This is largely a consequence of the rate of substrate diffusion and assimilation (Chan et al., 1999; Li et al., 1994; Tanaka et al., 1994).

The choice of microorganism can have an important effect on biosensor performance. Since most wastewater is comprised of numerous compounds, it is desirable to use a microbe with a broad substrate spectrum to allow for the assimilation of the many different compounds present in wastewater. This results in an expanded working range and improved correlations with BOD_5 values (Chan et al., 1999; Racek, 1995; Reiss et al., 1998; Riedel et al., 1998; Sangeetha et al., 1996). In an attempt to achieve these qualities some researchers have used mixed populations of microorganisms as the biocatalyst (Karube et al., 1977; Strand and Carlson 1984; Tan et al, 1992). However, this has resulted in poor reproducibility due to population variation (Chan et al., 1999). In order to reduce this variability and increase the fraction of compounds measured in wastewater most researchers have focused on the use of a single species with a broad substrate range (Chan et al., 1999; Sangeetha et al., 1996). Using *Arxula adeninivorans* , Riedel's group (1998) was able to obtain a reproducibility of ± 5% for periods of over one month. A survey of several BOD

biosensors indicates that the precision can vary from 3% to 10% with a mean of 6% (Riedel 1998).

The current state of BOD biosensor technology allows plenty of room for improvement. There have been improvements in some performance factors such as detection limit (Chee et al., 1999; Yang et al., 1996), temperature range (Karube et al., 1989), pH range (Kim and Kwon, 1999), and linear response range (Riedel et al., 1998). However, current BOD biosensors still have insufficient stability (as compared to standard chemical and physical techniques) to allow for practical application (Praet et al, 1995). Ihn et al. (1992) pointed out that biosensors are not suitable for BOD measurement when wastewater is primarily comprised of high molecular weight compounds. These compounds can be assimilated over the course of 5 days via BOD_5 testing, but not by the microbes immobilized on the biosensor. In order to overcome legislative restriction preventing replacement of the standard BOD_5 technique, improvements are needed that would create a biosensor with increased reproducibility, selectivity, sensitivity, linearity as well as stability (Riedel 1998).

1.3 Mediated amperometric biosensors

Amperometric biosensors based on oxygen monitoring are classified as first generation biosensors (Moody and Thomas 1991). Major limitations associated with first generation biosensors include a limited response range owing to the low solubility of oxygen in water, and fluctuating oxygen concentrations leading to poor reproducibility. Second generation biosensors make use of low molecular weight redox couples called mediators which shuttle electrons from the redox centre of reduced enzymes to the surface of an electrode (Figure 1.1). In whole cell sensors, mediators act as electron acceptors which replace oxygen as a cofactor for many enzymes (i.e. glucose oxidase). The main reason for using mediators is to overcome the dependence on oxygen concentration for sensor response and thus to increase the linear response range beyond the point of oxygen saturation. When whole cells are used, mediators are reduced by microbial enzymes as they substitute for the natural electron acceptor during substrate metabolism. The reduced mediator is

subsequently re-oxidized after diffusing to the surface of the electrode. The electron flow is measured as a current increase, and is proportional to the organic compound concentration (Hobson et al., 1996). In addition to eliminating many electrochemical interferants, mediated biosensors operating at low potentials allow for improved reliability via the elimination of errors caused by fluctuations in the dissolved oxygen concentration. Furthermore, mediated electrodes can produce higher response signals than conventional oxygen or hydrogen peroxide based electrode sensors (Ge et al., 1998). Some important properties of good mediators used in microbial sensors include: (1) the ability to be rapidly reduced by the microorganism and re-oxidized at the electrode; (2) stable and reversible kinetics under different assay conditions (i.e. pH, temperature); (3) low oxidizing potential thus eliminating interference from oxygen and other electrochemically active compounds (Moody and Thomas, 1991; Roller et al., 1984).

The simplest form of amperometric sensor is comprised of two electrodes, a working electrode and a combined reference/counter electrode. However a three electrode system imparts higher stability and is preferred for more sensitive measurements (Hobson et al., 1996). Amperometric sensors have an external circuit and measurements are based on the change in current produced at a working electrode poised at a defined potential (Atkinson and Haggett, 1993; Hobson et al., 1996).

With the development of second generation biosensors many different mediators have been evaluated. The majority of research has been done on enzyme-based electrodes rather than those using whole cells. Benzoquinone was the first mediator used to replace oxygen. Its lower redox potential (+ 400 mV versus a saturated calomel electrode) reduced the effects of electroactive interferants (Moody and Thomas, 1991). Although initially attempted with microbial cells, the use of quinone as a mediator has since waned. This is likely a consequence of the deleterious effects lipophilic mediators, such as benzoquinone, have on the service life of biosensors (Rawson et al., 1989). Other mediators used in microbial sensors include various benzoquinone derivatives (Takayama et al., 1996); azo dyes (Keck et al., 1997); phenazine methosulfate,

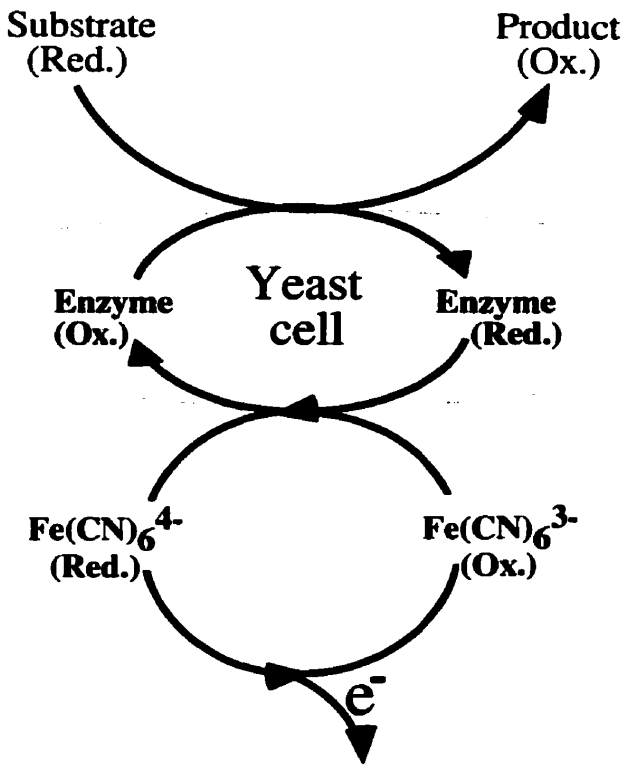

Figure 1.1 Schematic representation of electron mediation between yeast cellular enzymes and an electrode using potassium ferricyanide mediation where Red. and Ox. refer to reduced and oxidized states, respectively.

dichlorophenolindophenol, ferricyanide, ferrocene, tetrathiafulvalene, tetracyanoquinodimethane (Kaláb and Skládal, 1994); benzyl viologen, thionine, gallocyanine, phenazine ethosulphate, resorufin, etc. (Roller et al., 1984). While many different mediators have been evaluated, many different microorganisms have been used as well. A general conclusion that has been reached is that mediator-microorganism interactions are very diverse and cannot be accurately predicted, therefore making the selection of suitable combinations difficult (Roller et al., 1984).

Water soluble mediators such as organic dyes and potassium ferricyanide were initially preferred. However organic dyes do not fulfil all the required criteria for good mediation in that they can be unstable and can autoxidize, have pH dependent redox potentials, and do not exhibit good reversible electrochemistry (Hill et al., 1990). Potassium ferricyanide was one of the first mediators used successfully and exhibits good electron transfer properties (Bennetto et al., 1983). It is still in frequent used today despite the disadvantages of its high working potential and relative inefficiency (Svitel et al., 1998). Recently, the emphasis has shifted to the use of water insoluble mediators. Many of these have redox potentials well below that of most electrochemically interfering compounds (Kaláb and Skládal, 1994; Svitel et al., 1998). Water insoluble mediators must be immobilized at the surface of the working electrode instead of being added to the buffer solution. This type of heterogenous application uses immobilization schemes such as physisorption, chemisorption, polymer-film deposition and paste blending (Luong et al., 1995). These biosensors have met with limited success owing to problems of low sensitivity and limited reusability due to mediator leakage (Luong et al., 1995). Attempts at alleviating these problems by chemically derivatized mediators such as ferrocene have met with limited success. One such derivative, carboxyferrocene, exhibited improved solubility but also had a higher redox potential thus creating the possibility of interferences (Hill and Sanghera, 1990). Another drawback of using derivatized water-insoluble mediators stems from the use of potentially toxic organic solvents to solubilize them (Luong et al., 1990).

1.4 Cyclodextrins as solubilizing agents for redox mediators

Cyclodextrins (CD) are cyclic oligosaccharide structures most commonly with six (α), seven (β), or eight (γ) glucopyranose subunits (Figure 1.2). The subunits are held together via α-1,4 linkages creating a conical structure. External hydroxyl groups enable CDs to be water soluble. The internal cavity, however, is relatively hydrophobic allowing for the formation of stable inclusion complexes with normally water-insoluble compounds (Szejtli, 1982). This property enables CDs to trap and solubilize a large variety of molecules ranging up to five-ring polynuclear aromatic hydrocarbons (PAHs) in size (i.e. benzo(a)pyrene) or larger molecules possessing appropriately sized side chains (Luong et al., 1995). For example, Brown et al. (1996) has demonstrated that several PAHs (i.e. chrysene, phenanthrene, fluoranthene, pyrene, etc.) can form inclusion complexes with derivatives of CD. In aqueous environments, hydrophobic non-polar molecules are energetically favoured to form inclusion complexes with CDs (Szejtli 1982).

Water insoluble mediators such as tetrathiafulvalene (TTF), dimethylferrocene (DMFe), tetramethylbenzidine (TMB) and ferrocene have been shown to form electrocatalytically stable complexes with CD (Luong et al., 1995; Schmidt et al., 1995). A β-CD derivative, 2-hydroxypropyl-β-CD (hp-β-CD), was most suitable for mediator solubilizing since its cavity (approximately 7.8 Å in diameter and 7.8 Å in depth) readily accommodates mediators the size of TTF (0.6 nm). Using hp-β-CD, 100 mM and 88 mM solutions of both TTF and DMFe, respectively, have been achieved (Luong et al., 1995). These homogenous solutions have been used in conjunction with enzyme-based biosensors producing rapid responses, increased sensitivity and a broad linear range (Luong et al., 1995). Solutions of the TTF-hp-β-CD complex perform homogeneous enzyme reactions at voltages as low as 100 mV (Schmidt et al., 1995). Mediator oxidation at such low voltages minimizes interference from electroactive compounds while allowing for accurate measurement of glucose, hypoxanthine, lactate and glutamate (Luong et al., 1995). Wastewaters may contain numerous interfering electroactive compounds (i.e. reducing sulphurs and nitrogenous compounds) that can potentially create falsely elevated readings. Therefore, accurate

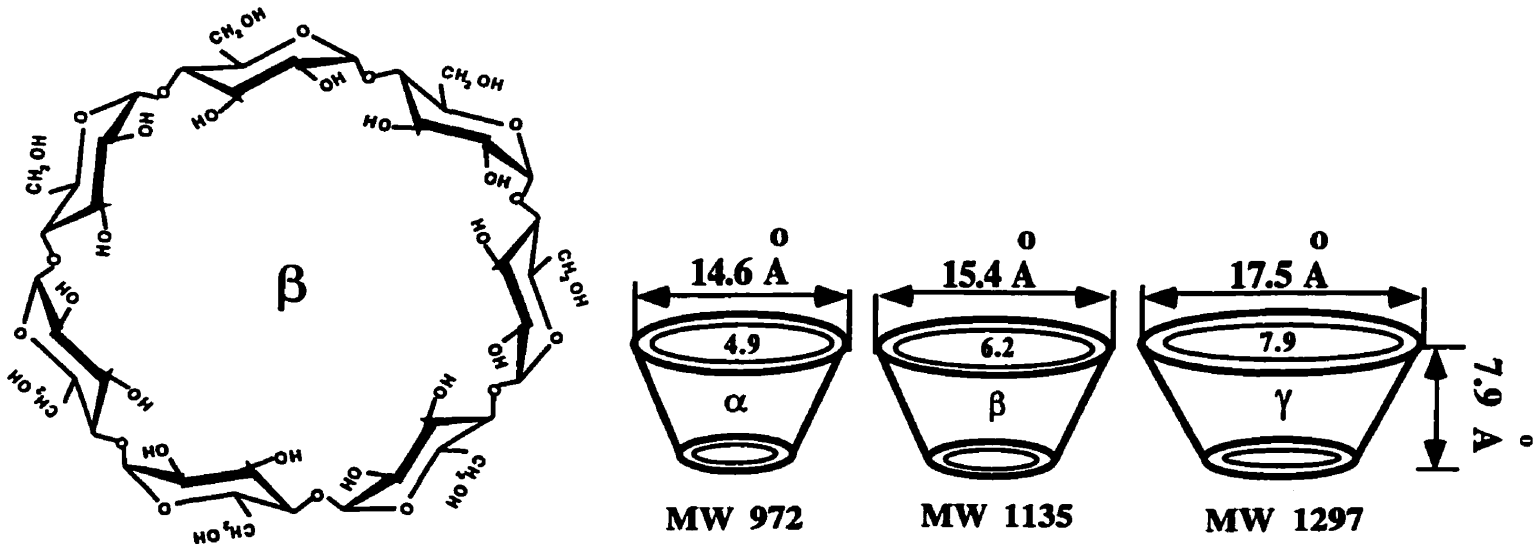

Figure 1.2 Schematic of glucopyranose subunits in the β-cyclodextrin ring structure and the relative cavity diameters for all three common cyclodextrins. Modified from Luong et al. (1995).

mediated biosensor measurements of wastewater should optimally be carried out at the lower applied potentials permissible with water insoluble mediators. Water insoluble mediators may be solubilized by CD in the presence of microbial cells without any deleterious effects.

1.5 Characterization of yeast isolates

Functional constraints in the ribosomal RNA (rRNA) gene have resulted in some of the most evolutionary conserved sequences in all living systems. These "universally conserved" regions have remained unchanged for billions of years as they are critical functional domains established in the earliest ancestors (Sogin and Gunderson, 1987). Highly conserved regions can be found in all phylogenetic domains thus leading to the identification of "universal" sequences and "signature" sequences for the Archaea, Bacteria, and Eukarya (Head et al., 1998). The ubiquity of rRNA allows for examination of phylogenetic relationships between diverse organisms via comparative sequence analysis (Head et al., 1998).

The lack of sequence variation within the highly conserved regions means that little information on evolutionary diversion can be gleaned from them. Accurate determination of both close and distant phylogenetic relationships requires random sequence changes over time. Such a molecule can be considered a chronometer. For a molecule to be a useful chronometer it must exhibit (1) random sequence changes, (2) a commensurate rate of mutations over time, (3) enough size to provide adequate information (Woese, 1987). The small subunit rRNA (18S rRNA) has become the favoured gene for phylogenetic comparison. It is approximately 13 times longer than the 5.8S rRNA subunit, ranges in size from about 1700 to 2300 base pairs, and exhibits all the traits of a good chronometer (Head et al., 1998; Sogin and Gunderson, 1987; Woese, 1987).

Rapid sequencing strategies based on the interspersion of moderate and high variability regions throughout the "universally conserved" regions have recently been developed. "Universal" primers homologous to highly conserved sequences at the 5' and 3' end of 18S rRNA permit the specific amplification of nearly complete 18S rRNA (Head et al., 1998). Amplification can be done

via the polymerase chain reaction (PCR), eliminating the need to use an M13 cloning and sequencing system (Sogin, 1990). This technique allows for the amplification of just a few nanograms of bulk genomic DNA into several micrograms of 18S rRNA/PCR product (Sogin, 1990). Errors during PCR amplification range from one to five positions per 1000 base pairs when compared to genomic clone amplification (Sogin, 1990). One strategy employed to reduce these potential errors is the direct sequencing of the PCR products via double-stranded DNA procedures. This technique makes early base pair mismatches seem ambiguous while late errors do not contribute to sequence interpretation (Sogin, 1990). Once the sequence (complete or incomplete) has been obtained, phylogenetic analysis can be easily done via sequence comparisons to thousands of sequences available at various databases.

Most authors now believe that, due to the inherent weaknesses in the procedures traditionally used for yeast identification, the present yeast taxonomy poorly reflects the true evolutionary relationships between organisms. Conventional yeast taxonomy based on morphological and physiological traits can lead to incomplete or erroneous conclusions (Valente et al., 1999). Owing to strain variability, phenotypic and metabolic characteristics are sometimes not sufficient to allow for species delineation and therefore accurate strain identification (Barns et al., 1991; Mannarelli and Kurtzman, 1998; James et al., 1998). Consequently, 18S rDNA analysis has become increasingly relied upon for accurate strain identification because it offers a higher level of resolution (close and distant relationships can be resolved) (Valente et al., 1999). The ease and rapidity of strain identification via partial sequencing has made it very common, however, its effectiveness depends heavily on the quality of the chosen region (Valente et al., 1999). One important limitation is that complete 18S rDNA sequence information for many species is not available. This can lead to false or inconclusive results for less commonly studied species (van de Peer and Wachter 1995). The objective of using 18S rDNA sequence analysis was to improve strain characterization and identification of the two newly isolated yeast strains.

MATERIALS AND METHODS

2.1 Yeast Isolation

Two yeast strains, designated as SPT1 and SPT2, were isolated from the primary clarifier effluent at a pulp mill plant in Thurso, Quebéc. Isolation was achieved by a combination of techniques described by van der Walt and Yarrow (1984). The conditions included the following: acidic medium (pH 3.5), an elevated glucose concentration (40%), and a low growing temperature (15 °C). A 250 mL flask containing 50 mL of bacto-yeast malt extract (YM) broth (Difco Laboratories, Detroit, MI) was inoculated with 5 mL effluent and subjected to rotary shaking at 240 rpm for 3 days. The resulting microbial culture was then streak purified on YM (Difco Laboratories, Detroit, MI) and glucose-peptone-yeast extract (GPY, prepared according to the recipe of van der Walt and Yarrow, 1984) agar plates. All media and glassware were sterilized in an autoclave for 20 min at 121 °C, 15 psi before use. Strains were maintained on both YM and GPY agar plates, and colonies were transferred monthly. Frozen cultures of both strains were kept at -80 °C in 10 % gylcerol.

2.2 Yeast Characterization

2.2.1 Physiological and biochemical characterization

Vegetative morphology in liquid and on solid media

Tests to characterize the vegetative morphology of both strains were based on those given by van der Walt and Yarrow (1984). Growth in both liquid and solid media was examined. For growth in liquid media the strains were grown in both malt extract (ME) and 4% glucose-peptone-yeast extract (GPY - prepared according to van der Walt and Yarrow 1984). Characteristics such as cell shape, mode of reproduction, cell encapsulation, cell size, cultural growth pattern and shine, surface growth, and odour were observed for liquid media. Growth on solid media enabled

description of colony characteristics (i.e. texture, colour, surface, elevation, margin). Potato dextrose (PD) and ME agar was used as colony morphology media.

Formation of pseudomycelium and mycelium

According to the methods given by van der Walt and Yarrow (1984), the Dalmau plate technique was used to examine the formation of pseudomycelium and true mycelium. Potato-glucose agar was used as the medium and prepared as described.

Formation of ballistospores

The Do Caromo-Sousa and Phaff technique as outlined in van der Walt and Yarrow (1984), was used to check for ballistospore formation. A modification was made in the use of PD agar in place of corn meal agar as the growth medium.

Characterization of asci and ascospores

The Schaeffer-Fulton staining procedure was used to verify asci and ascospores, as described by van der Walt and Yarrow (1984). Sporulation media included Gorodkowa agar, growth restrictive agar, potato-dextrose agar, 2% malt extract agar, McClary's acetate agar, YM agar, and V8 agar. All agars were prepared according to the recipe.given by van der Walt and Yarrow.

Carbon assimilation tests

Carbon assimilation testing was initially done in both liquid and solid media as described by van der Walt and Yarrow (1984). After initial ambiguous results with the solid media all further tests were conducted in liquid media. Liquid media was contained in sterile 150 x 16 mm test tubes (Corning Inc., Corning, NY) stoppered with polyurethane foam plugs (Identi-Plugs, Jaece Industries, Inc. North Tonawanda, NY). Test results were followed over a course of three weeks

and scored according to the criterion provided on page 82 of van der Walt and Yarrow. All tests were initially carried out in sets of two, whenever weak or ambiguous results occurred the experiments were redone in triplicate. All carbohydrates were of analytical grade. Please refer to Table 2.1 for details regarding carbon sources.

Fermentation of carbon sources

Carbohydrate fermentation tests were carried out in sterile 150 X 16 mm test tubes containing inverted Durham tubes and stoppered with polyurethane foam plugs. The fermentation tests were carried out and scored according to the method described by van der Walt and Yarrow (1984). Bromothymol blue (Anachemia, Montréal, Québec) was used as the indicator dye. Experiments were carried out in quadruplicate. Six carbohydrates of analytical grade (galactose, glucose, lactose, maltose, raffinose, sucrose) were used in this test.

Growth at high temperatures

Growth on solid media was monitored at 37 °C and 42 °C as described by van der Walt and Yarrow (1984). Petri dishes containing both YM and ME agar were used. Maximum temperature of growth was not determined.

Acid production from glucose

A 5% glucose - 0.5% calcium carbonate (Anachemia, Montréal, Québec) agar was used for qualitatively determining the amount of acid produced from glucose, as described by van der Walt and Yarrow (1984). The yeasts were grown on slants of this media and calcium carbonate dissolution was monitored for a period of 10 days. Experiments were done in sets of five.

Diazonium blue B test

The diazonium blue B (DBB) (Sigma, St. Louis, MO) test was performed on liquid and

Table 2.1 List of chemicals used in physiological characterization and their origin.

Chemical	Source
01 L(+)Arabinose	Sigma, St. Louis MO USA
02 L-Arabitol	Sigma, St. Louis MO USA
03 Arbutin	Sigma, St. Louis MO USA
04 Cadaverine	Sigma, St. Louis MO USA
05 Cellobiose	BDH Inc., Toronto Ont. Canada
06 Citric Acid	Anachemia, Montreal Qué. Canada
07 Creatine	Sigma, St. Louis MO USA
08 Creatinine	Sigma, St. Louis MO USA
09 Dulcitol (Galactitol)	Aldrich, Milwaukee WI USA
10 Ethanol	prepared in the laboratory of Dr. C. Greer
11 Ethylamine	Sigma, St. Louis MO USA
12 Erythritol	Aldrich, Milwaukee WI USA
13 ß-D-Fructose	Sigma, St. Louis MO USA
14 D(+)Galactose	Sigma, St. Louis MO USA
15 D-Gluconic Acid	Aldrich, Milwaukee WI USA
16 D-Glucitol (Sorbitol)	Fisher Scientific, Nepean Ont. Canada
17 D-Glucose	BDH Inc., Toronto Ont. Canada
18 D-Glucosamine	Aldrich, Milwaukee WI USA
19 D-Glucuronic Acid	Sigma, St. Louis MO USA
20 Glycerol	Anachemia, Montreal Qué. Canada
21 DL-Lactic Acid	Sigma, St. Louis MO USA
22 D-α-Lactose	Anachemia, Montreal Qué. Canada
23 L-Lysine	Sigma, St. Louis MO USA
24 Maltose	Sigma, St. Louis MO USA
25 D-Mannitol	Anachemia, Montreal Qué. Canada
26 Melibiose	Sigma, St. Louis MO USA
27 D(+)Melezitose	Sigma, St. Louis MO USA
28 Methanol	EM Science, Gibbstown NJ USA
29 Methyl-α-D-glucopyranoside	Sigma, St. Louis MO USA
30 myo-Inositol	Sigma, St. Louis MO USA
31 D(+)Raffinose	Sigma, St. Louis MO USA
32 L-Rhamnose	Sigma, St. Louis MO USA
33 Ribitol (Adonitol)	Sigma, St. Louis MO USA
34 D-Ribose	Sigma, St. Louis MO USA
35 Salicin	Sigma, St. Louis MO USA
36 L-Sorbose	Sigma, St. Louis MO USA
37 Starch (soluble)	Anachemia, Montreal Qué. Canada
38 Succinic Acid	Aldrich, Milwaukee WI USA
39 Sucrose	BDH Inc., Toronto Ont. Canada
40 D(+)Trehalose	Sigma, St. Louis MO USA
41 D(+)Xylose	Anachemia, Montreal Qué. Canada
42 Xylitol	Aldrich, Milwaukee WI USA

solid media as outlined by van der Walt and Yarrow (1984). *Trichosporon cutaneum* was used as a positive control.

Vitamin-free growth

The procedure described by van der Walt and Yarrow (1984) was used to determine whether the two isolates exhibited vitamin-free growth. In the vitamin free medium D-tryptophan (Sigma, St. Louis, MO) was used rather than DL-tryptophan. Tests were carried out in triplicate. Three culture transfers, into fresh vitamin-free media, were made to verify that growth was not a consequence of excreted nitrogenous compounds. Tests were scored in the same manner as carbon assimilation tests.

Growth in high osmotic pressures

Tests for growth in high osmotic pressures were carried out in both liquid and solid media according to the procedures described by van der Walt and Yarrow (1984). The liquid media contained 5% glucose and 10% sodium chloride, while the solid media was comprised of three concentrations of glucose (40, 50, 60%). All tests were conducted in sets of five and carried out at ambient temperatures (22 °C).

Starch test

The procedure and media preparation used for the monitoring of the formation of extracelluar amyloid compounds in liquid media is described in van der Walt and Yarrow (1984). The reaction of the medium was maintained at pH 3. Tests were carried out in sets of five. Reactions were allowed to run overnight to ensure there was no glycogen interference.

Cycloheximide resistance

The procedure for testing cycloheximide (Aldrich, Milwaukee, WI) resistance is described in van der Walt and Yarrow (1984). Sterile 150 x 16 mm test tubes stoppered with polyurethane foam

plugs containing 100 ppm and 1000 ppm concentrations of cycloheximide were inoculated, and incubated at 26 °C for three weeks (240 rpm). Experiments were carried out in sets of five for each concentration of cycloheximide.

Assimilation of nitrogen compounds

The ability of the two yeast strains to utilize various nitrogen sources was verified in liquid media according to the procedure described by van der Walt and Yarrow (1984). The yeasts were checked for growth in media with either nitrate or nitrite as the sole nitrogen source. Potassium nitrate (Aldrich, Milwaukee, WI) served as the nitrate source and sodium nitrite (Aldrich, Milwaukee, WI) was used as the nitrite source. The liquid media were contained in sterile 150 x 16 mm test tubes stoppered with polyurethane foam plugs. The basal carbon source (Bacto® yeast carbon base Difco Laboratories, Detroit, MI) was filter sterilized with a 0.22 µm Millex®-GP filter unit (Millipore, Mississauga, Ontario) . Blank tubes contained only carbon base and no nitrogen sources. A second set of tubes were loop inoculated from the first set to eliminate false positives due to excreted soluble nitrogen sources. Nitrate reactions were confirmed by the chemical test for nitrite. Tests were carried out in sets of three.

2.2.2 Fatty acid analysis

Fatty acid composition of two day old cultures (YM agar plates) for both strains were analyzed by Microcheck, Inc. (Northfield Falls, VT). The microbial identification system included gas chromatographic analysis (using five percent methyl phenyl silicone capillary columns) of fatty acid composition via comparison of the fatty acid profiles to the 7000 different profiles in the database.

2.2.3 18S rDNA sequence analysis

Genomic DNA preparation

Genomic DNA was extracted from both strains using a two week old culture grown in YM broth instead of the recommended YPD (yeast extract-peptone-dextrose) broth using a procedure reported by Hoffman (1997). Some procedural modifications were made in this study: Cells were broken via 4 cycles of freeze/thaw lysis with temperatures ranging from 37 °C to -70 °C, respectively, rather than by vortexing with glass beads; a second chloroform extraction (400 µl) was added in the sixth step prior to mixing with 100% ethanol; 70 % ethanol (500 µl) was added at the tenth step prior to dissolving in 10 mM Tris 1 mM EDTA (TE) buffer. The DNA was extracted with phenol (500 µl) and then twice with chloroform (500 µl). Agarose gel electrophoresis (on 0.8% agarose gel) based on the method described by Voytas (1997), was used to visualize the extracted DNA.

To ensure that the genomic DNA fragments were ready for PCR amplification the two DNA extracts were subjected to restriction analysis using *Hin*dIII, utilising a procedure described by Sambrook et al. (1989). The total volume of the reaction mixture was 10 µL, consisting of 5 µl of DNA extract, 1 µL one-Phore-all buffer, 1 µL RNAase, and 3 µL of the *Hin*dIII restriction endonuclease (Pharmacia Biotech, Baie d' Urfé, Québec). The reaction was done in a sterile 1.5 mL microcentrifuge tube for 3 h at 37 °C. After three hours, 2 µL of Tris acetate EDTA buffer was added to the reaction mixture and the sample was electrophoresed on a 0.8 % agarose gel for 30-40 min at 100 mV/cm (Sambrook et al., 1989). The DNA was quantified according to the procedure of Sambrook et al. (1989) using a Beckman DU 70 spectrophotometer (Beckman Coulter Inc., Fullerton, CA).

PCR amplification

Three universal fungal primers were used (NS3, NS4, NS6) to amplify a portion of the nuclear small rDNA. The NS3 primer (GCAAGTCTGGTGCCAGCAGCC) was a 5' primer,

while NS4 (CTTCCGTCAATTCCTTTAAG) and NS6 (GCATCACAGACCTGTTATTGCCTC) were 3' primers. Detailed information for PCR amplification can be found elsewhere (White et al., 1990).

The PTC-100 PCR thermal cycler (MJ Research, Inc. Waltham, MA) was used for amplification of the ribosomal RNA to eliminate the need for evaporation protection via mineral oil addition (as suggested by White et al., 1990). The cycling parameters were as follows: initial denaturation for 2.5 min at 95 °C; annealing for 30 s at 55 °C; extension for 1.5 min at 72 °C; denaturation for 30 s at 95 °C; and a final extension for 10 min at 72 °C. A total of 25 cycles were carried out. A 100 µL reaction volume consisted of 50 µL diluted DNA sample (5 ng) and 50 µL of working reaction mixture. The working reaction mixture consisted of: 27.5 µL sterile-distilled water, 10 µL 10X amplification buffer, 10 µL dNTP stock mixture (Roche, Laval, Québec), 1 µL excess primer, 1 µL limiting primer, 0.5 µL *Taq* polymerase (Gibco BRL, Life technologies Inc., Burlington, Ontario). Automated DNA sequencing of the PCR products using the above primers was carried out by MOBIX (McMaster University, Hamilton, Ontario).

Phylogenetic analysis

The sequences obtained from MOBIX were manually corrected against two opposite strand sequences and deposited into GenBank. Sequences were compared to the compilation of 18S rDNA sequences located in the GenBank and EMBL nucleotide libraries (www.ncbi.nlm.nih.gov/) using the BLAST search method (Altschul et al., 1997). Sequences were aligned to the closest matches determined via BLAST searching as well as the sequences of the closest identity matches as determined by the physiological, biochemical and fatty acid tests. Sequence alignment was performed by the CLUSTAL X (Thompson et al., 1997) software program. Subsequent phylogenetic analysis of the sequence data sets were done using the PHYLIP software package version 3.6 (Felsenstein, 1999). Distance matrices were produced by using the DNADIST program using the maximum likelihood algorithm option. Unrooted phylogenetic trees were created using

the neighbour-joining method of Saitou and Nei (1987) with the NEIGHBOR program. The FITCH program was used to adjust the estimated (via DNADIST) branch lengths. Bootstrapping (1000 replicates) was used to determine the statistical significance of each branch by using the SEQBOOT, DNADIST, NEIGHBOR and CONSENSE programs. The unrooted tree was built using the DRAWGRAM program. 18S rRNA sequences from *Rhodosporidium dacryoidum* and *Tremella globospora* were incorporated in the tree in an attempt to represent the basidiosporogenous yeasts. *Saccharomyces cerevisiae* was included as the representative of the ascosporogenous yeasts, while *Starmera caribaea* was used to increase branch point stability for *Starmera amethionina.* The 18S rRNA sequence of *Homo sapiens* was used as the out-group.

The 18S rRNA gene sequences retrieved from the GenBank database were: *Tremella globospora* U00976; *Rhodosporidium dacryoidum* D13459; *Starmera caribaea* AB017899; *Starmera amethionina* var. *amethionina* AB017897; *Candida sojae* AB013549; *Candida tropicalis* M55527; *Candida albicans* AF114470; *Candida zeylanoides* AB013509; *Saccharomyces cerevisiae* J01353 M27607; *Candida krusei* M55528; *Homo sapiens* U13369. The SPT1 and SPT2 sequences have the GenBank accession numbers AF247474 and AF247475, respectively.

2.3 Biosensor Design

2.3.1 Oxygen electrode-based biosensor

The effects of cyclodextrin (CD) on yeast responses to glucose/glutamic acid (GGA) was monitored via an oxygen electrode based biosensor. The objective was to determine if response heights differ in the presence and absence of CD. The BOD sensor was comprised of a Clark-type oxygen electrode (YSI model 54A, Yellow Springs, OH) set at a potential of -650 mV, connected to a LKB Bromma 2210 chart recorder (Kipp & Zonen, Delft, Holland). Each yeast strain was sandwiched between an inner Teflon membrane (supplied by YSI) and an outer dialysis membrane (6000 - 8000 Da MWCO Spectra/Por®, Spectrum® Medical Industries Inc., Houston, TX). Each dialysis membrane was secured to the dissolved oxygen probe by means of a rubber O-ring

(supplied by YSI). The yeasts were prepared according to section four of the materials and methods section with the exception that 50 µL of the yeast slurry was used rather than 20 µL. The electrode was preconditioned for one night in a stirred (400 rpm on a magnetic stir plate) solution of 100 mM phosphate buffer (PBS) containing 0.8 % NaCl at pH 7.2 in the presence of 100 ppm (BOD) of glucose/glutamic acid (GGA) standard solution. Note that since relative responses were of interest, additional preconditioning was not required.

Measurements of the response heights to multiple additions of 5 ppm (BOD) GGA were compared to responses to 5 ppm (BOD) injections of GGA in the presence of 10 mM hp-β-CD (Aldrich, Milwaukee WI). Response heights to all GGA additions were measured after five minutes had elapsed. All experiments were carried out at ambient temperature (22 °C) in a 20 mL beaker containing 10 mL of PBS buffer (100 mM, pH 7.2).

2.3.2 Mediator-based biosensor

Electrochemical experiments were carried out using a BAS CV1B voltammograph (Bioanalytical Sciences (BAS), West Lafayette, IL). A three-electrode system was used with a 3 mm diameter glassy carbon working electrode, a platinum wire counter electrode, and an Ag/AgCl (saturated KCl) reference electrode (all electrodes BAS). The measured current was digitized and displayed on a computer equipped with a DAS-8 A/D card (Computer Boards, Inc.,Mansfield, MA) and custom software.

The glassy carbon electrode was polished using Buehler® (Lake Bluff, IL) nylon polishing cloth (40-7068) first with 1 µm Buehler Metadi® II diamond polishing compound, and subsequently with 0.05 µm alumina (Buehler® Gamma Micropolish® II). Afterwards, the electrodes were subjected to 10 min of sonication in distilled water prior to use. The counter electrode was cleaned with concentrated sulphuric acid and rinsed in distilled water.

All experiments were carried out in a 20 mL reaction vessel in a final volume of 10 mL. A magnetic stirrer (400 rpm) was used in measurements taken at a set potential only. Substrates were

kept on ice during the course of measurements and stored in at -20 °C between measurements to eliminate microbial degradation.

Measurements at a set potential (amperometric measurements) were carried out at optimized parameters (i.e. 30 °C, 100 mM PBS, pH 6.0, and 20 mM potassium ferricyanide). After the baseline current had been attained, successive injections of substrate were introduced to the mixing vessel after each new baseline current was obtained. Responses were measured from the lower current after injection (i.e. the baseline or the current dip after injection) to the higher (i.e. the new baseline). Calibration of the sensor was done using a standard (GGA) solution. For substrate additions above 100 µL, the sample included 10 percent of ten-fold concentrated mediator and buffer. Measurements were performed in triplicate within all electrode measurements. Data are presented as mean ± standard deviation.

2.4 Yeast Cultivation and Electrode Preparation

A single colony from a YM agar plate was used to inoculate 50 mL YM broth in a 250 mL Erlenmeyer flask (26 °C, 240 rpm). Upon reaching late exponential phase, 1 mL of this culture was subcultured to 50 mL YM broth in a 250 ml Erlenmeyer flask. Yeasts in late exponential phase (28 h) of the subculture were harvested for biosensor use after verifying the absorbance (± 10 %) of a 100-fold culture dilution at 600 nm (i.e. SPT1= 15.5, SPT2 18.3). For one electrode, 1 mL of the subculture was placed in a microcentrifuge tube and centrifuged at 15 000 rpm for two minutes using a Brinkmann Instruments, Inc. Eppendorf centrifuge 5414 (Rexdale, Ontario). The supernatant was discarded and 1 mL of 100 mM PBS buffer, pH 7.2 was added. The cells were washed by resuspending them via vortexing at a high speed. The resulting cells were pelleted and washed once again, and the supernatant was discarded. The cells were resuspended in 10 µL of PBS buffer (pH 7.2). In a laminar flow hood, 15 µL of yeast slurry was pipetted and left to dry for 20 min on the surface of a clean glassy carbon electrode. Reproducibility of this technique was

verified by measuring the absorbance at 600 nm of the yeast slurry after washing, and by measuring the wet weight of the yeast slurry (15.0 mg ± 10 % for each strain). The yeasts were then retained on the working electrode by securing a 12 000 -14 000 Da MWCO dialysis membrane (Spectra/Por®2, Spectrum® Medical Industries, Inc., Houston, TX) over the yeasts using two rubber O-rings.

2.5 Mediator Screening via Cyclic Voltammetry

Cyclic voltammetry (CV) was used to screen mediators for use in amperometric sample measurement. All CV measurements were performed at ambient temperature (22 °C) in 100 mM PBS buffer, pH 7.2 (except for CV scans of hydroxymethylferricinium, which were carried out in 100 mM phthalate, pH 6.0), with a final mixing volume of 10 mL. The scan rate was set at 10 mV/sec. Only properly preconditioned electrodes, as indicated by GGA calibration (see section 6.3 of the materials and methods) were used for mediator screening. Cyclic voltammetry was performed in an unstirred solution using 5 mM of mediator (except for measurements taken with hydroxymethylferricinium and water soluble 1,1'-dimethylferrocene, which were only available in lower concentrations - see below). Biocatalyst - mediator interactions were studied via CV analysis by sweeping the applied potential over a broad range and measuring the oxidation current generated by the mediator before and after addition of 500 ppm (BOD) GGA standard solution (upon establishment of a stable background current). The selection of mediators was based on an increase in the oxidation current upon GGA addition and on reversible electrochemistry. The working potential of selected mediators was determined by the redox potential of the mediator.

A total of eight water soluble mediators were screened for mediation. Solutions of 100 mM were made up in double distilled water for the following mediators: potassium ferricyanide (Fisher Scientific, Nepean, Ontario), phenazine ethosulphate (Sigma, St. Louis, MO), and 2,6-dichlorophenol-indophenol (Sigma, St. Louis, MO). A 50 mM solution of EDTA (Sigma, St.

Louis, MO) was used to solubilize 100 mM of $FeCl_3$ (Aldrich, Milwaukee, WI). The solution was subsequently referred to as Fe^{III} EDTA. The Fe^{III} EDTA was prepared by stirring the mixture for 30 min under low heat. Solutions of ferrocenium tetraflouroborate and ferrocenium hexafluorophosphate were prepared in concentrations of 25 mM in 100 mM PBS buffer. Both of the latter mentioned mediators were supplied by Aldrich (Milwaukee WI). A 0.5 mM solution of water soluble 1,1'-dimethylferrocinium ($DMFe^+$) was prepared according to the procedure of Luong et al. (1995b). Similarly, hydroxymethylferricinium (HMF^+) was prepared in concentrations ranging from 4-6 mM.

Two water insoluble mediators were used in the mediator screening process. Both the 3,3',5,5'-tetramethylbenzidine (TMB) and the 1,1'- dimethylferrocene ($DMFe^+$) were purchased from Aldrich (Milwaukee, WI). Solutions of TMB and $DMFe^+$ were prepared according to the methods given by Cattaneo and Luong (1994) and Male et al. (1997), respectively. Mediator concentrations were verified spectrophotometrically (Beckman DU 640, Fullerton, CA) using the respective absorption coefficients for each mediator.

2.6 Biosensor Optimization

2.6.1 Mediator concentration optimization

The optimal mediator concentration was determined by comparing responses to 150 ppm (BOD) GGA additions under increasing mediator concentrations. Concentration optimization was done only for mediators that were chosen from the CV screening procedure (i.e. hydroxymethylferricinium and potassium ferricyanide). Only isolate SPT1 was used to determine the optimal mediator concentrations. Kinetic parameters such as K_s and V_{max} were determined from Hanes plots using experimentally measured values (i.e. substrate concentration and current) determined at saturating GGA concentrations (150 ppm BOD GGA was considered saturating since the electrodes were not preconditioned). The conditions for the potassium ferricyanide optimization

were as follows: 20 µL yeast slurry, + 400 mV applied potential, 100 mM PBS buffer, pH 7.2. Potassium ferricyanide concentrations ranged from 0.1 - 80 mM. Measurements for hydroxymethylferricinium (HMF^+) optimization were taken under the same conditions (except for the following changes: + 285 mV and 15 µL of yeast slurry). The concentrations of HMF^+ ranged from 0.1 mM to 4 mM (note that the lack of solubility did not permit the use of a HMF^+ concentration above 4 mM). The HMF was already solubilized in 50 mM phthalate buffer (pH 5.0) prior to addition while potassium ferricyanide was dissolved in double distilled waster. The 20 ppm (BOD) GGA additions were made once a steady-state background current was established.

Mediator toxicity evaluation

The standard plate count was used to determine whether potassium ferricyanide was inhibitory to either of the two yeast strains. Filter sterilized (0.22 µm Millex®-GP filter unit, Millipore, Bedford, MA) potassium ferricyanide was added to the YM agar prior to pouring to create plates containing various mediator concentrations (1 mM - 20 mM). Culture dilutions of $1:10^{-6}$ and $1:10^{-7}$ for the opaque and white strains, respectively, were used to inoculate both mediator plates and control plates (no mediator). Inhibition was indicated by no growth over a period of 10 days.

2.6.2 Optimal yeast loading

To determine the optimal quantity of yeast to use for the sensor, a range (2-35 µL) of yeast slurry was pipetted on to the surface of the working electrode and the response to GGA was monitored (150 and 20 ppm BOD GGA was used for SPT1 and SPT2, respectively). There was no preconditioning of the sensor, and measurements were taken in triplicate. The conditions for all measurements were as follows: + 450 mV applied potential, 20 mM potassium ferricyanide, 100 mM PBS buffer, pH 7.2 and ambient temperature (22 °C).

2.6.3 Preconditioning of electrode

Inconsistent or complete lack of responses after electrode preparation indicated that preconditioning was necessary in order for the sensor to function optimally. All preconditioning tests were carried out using 20 mM potassium ferricyanide as the mediator. Preconditioning times were determined for the SPT1 and SPT2 strains under two conditions: starvation in 100 mM PBS buffer pH 7.2; and incubation in a 50% PBS buffer (100 mM, pH 7.2) and 50 % effluent (from the primary clarifier in Thurso, Québec) mixture. The response to 20 ppm (BOD) additions of the primary clarifier effluent obtained from the Thurso mill were measured every day until a clear response maximum was established (or up to a maximum of 12 days). The time required to establish the response plateau was judged to be the time needed for electrode preconditioning under those conditions. All measurements were taken at ambient temperature (22 °C), + 450 mV and with 20 µL of yeast slurry retained at the surface of the electrode.

2.6.4 pH response range

The optimal pH was determined by measuring the response of fully conditioned electrodes to 20 ppm (BOD) additions of GGA at various pHs. The measuring conditions for all these experiments were kept constant at + 450 mV, 20 mM potassium ferricyanide, and 30 °C. Prior to pH optimization, the response of 5 mM potassium ferricyanide additions in the various buffer conditions using an electrode without cells was measured. This allowed for the determination of any effects of the various buffers on potassium ferricyanide mediation. The following buffers were used in the pH range of 5-9: 100 mM phthalate (pH 5-6); 100 mM phosphate (pH 5-8); 100 mM borate (pH 7-9).

2.6.5 Temperature response range

The optimum temperature was determined by recording responses of fully conditioned working electrodes to 20 ppm (BOD) additions of GGA. All measurements were made at + 450

mV using 20 mM potassium ferricyanide in 100 mM PBS buffer, pH 6.0. The detection chamber consists of a jacketed vessel attached to a Lauda RM20 constant temperature water circulator (Rexdale, Ontario). The temperature conditions ranged from 4 - 50 °C.

2.7 Standard Solution (GGA) Calibration Curve

A standard solution calibration plot was determined for both sensors using the following optimized conditions: 30 °C, 100 mM PBS pH 6.0, and 20 mM $K_3Fe(CN)_6$. A stock (GGA) solution containing 100 g/L glucose (BDH, Inc., Toronto, Ontario) and 100 g/L glutamic acid (Sigma, St. Louis, MO), with a BOD value of 146 700 ppm, was used as model wastewater according to Riedel et al. (1990). Standard solution measurements of various BOD concentrations were prepared by dilution of the stock solution. In all measurements involving potassium ferricyanide, GGA was added after the attainment of a steady-state background current at + 450 mV. When hydroxymethylferricinium (HMF^+) was used as the mediator, the aforementioned optimized conditions were used except that an applied potential of + 285 mV and 100 mM phthalate buffer at pH 6.0 were used. Phthalate was used in place of PBS buffer for HMF^+ measurements in an attempt to eliminate the possibility of increased HMF^+ precipitation in the presence of phosphate ions. Successive injections of substrate were made after each new baseline current was achieved The response was measured as the current change between the steady-state current in the presence of GGA and that which was established in the blank buffer. Measurements were taken in triplicate within one electrode. Reproducibility within one electrode was determined by calculating the standard error of the mean for a series of five or more measurements of 10 ppm BOD of GGA.

The sensor BOD values for effluent samples were calculated from the calibration curve of each isolate. Since the line of the calibration curve was forced through zero on the X and Y axis, the sensor BOD value was determined by dividing the corrected mean response height by the slope of the calibration curve. Note that the corrected mean response height was taken as the mean

response plus 10 percent of the response (due to the real sample being 90 % effluent and 10 % of ten fold PBS buffer and mediator) minus the negative control. The negative control for each effluent was determined by the response of a sensor without yeast cells to the addition of an equivalent amount of effluent to the mixing vessel.

2.8 Measurement of Pulp Mill Effluent

2.8.1 BOD_5 measurement

The standard five day BOD measurement was done according to the procedure outlined in the "Standard methods for the examination of water and wastewater" (Greenberg et al. 1992). Oxygen concentration was measured using a YSI model 54A oxygen meter (Yellow Springs, Ohio). Only the effluent from the Thurso primary clarifier was measured. The BOD_5 values for other effluents were supplied by the Pulp and Paper Research Institute of Canada, Point-Claire Quebec (Beaupré, Donnacona, Fraser, Grand Mère, Kenogami) and Noranda Technology Centre, Point-Claire Quebec (Edmunston, Madawaska). Note that all eflluents originated from pulp mills except for Madawaska (paper mill).

2.8.2 Linear response range to Thurso effluent

Primary clarifier effluent from the pulp mill located in Thurso was used to determine the linear response range to a real sample. Preconditioning of the working electrodes for both strains was done via incubation in 50 % Thurso primary clarifier effluent and 50 % 100 mM PBS buffer at pH 7.2. Dilutions of the effluent were measured (i.e. from the detection limit of the sensor, up to 60 ppm). Both potassium ferricyanide and HMF were used in determining the linear response range for the Thurso effluent. When potassium ferricyanide was the mediator, the effluent was prepared by creating a mixture that was 90% effluent and 10% PBS buffer/potassium ferricyanide (10x concentrate). The absolute response height was measured as the current increase to effluent

addition plus 10 % of the response height minus the response obtained from a bare (dialysis membrane only) electrode. A 10-fold concentration mixture was not possible with HMF^+ measurements due to the mediator insolubility problems mentioned earlier. All sensors using potassium ferricyanide had the following conditions + 450 mV, 20 mM potassium ferricyanide, 100 mM PBS buffer pH 6.0. Measurements with 3 mM HMF used were performed at + 285 and 100 mM phthalate buffer, pH 6.0. Mean response values were used to establish the linear response range. Reproducibility within one electrode was determined by calculating the standard error of the mean for a series of five or more measurements of 10 ppm BOD of Thurso effluent.

2.8.3 Measurement of various effluents

The universality of the sensors was tested by measuring their responses to the eight different effluents listed in Section 2.8.1. Working electrodes for both strains were fully preconditioned (i.e. 9 days for SPT1 and 5 days for SPT2) prior to the addition of the effluent sample, via the starvation technique. Each sample addition consisted of 200 µL of effluent that had been prepared as in Section 2.8.2. The detection chamber conditions were also the same as in Section 2.8.2 (pH 6.0 was used). A minimum of five measurements were taken for each effluent sample. The InStat statistical analysis program (GraphPad Software, San Diego, CA) was used to determine the 95% confidence intervals for measurements of response reproducibility within different effluent samples.

Sensor responses to the various effluents were correlated to the measured BOD_5 value by dividing the mean response to each effluent by the slope for their respective GGA calibration curve. The resulting value, "x", was divided by the dilution factor of the BOD_5 value and multiplied by 100 to determine the correlation value (given in percentage).

Response reproducibility between electrodes was examined by a series of three identical sensors. These measurements were conducted during the preconditioning trials of the sensors to avoid increased standard deviations due to leakage of the biocatalyst. The following conditions were used: 22 °C, 100 mM PBS pH 7.2, 20 mM $K_3Fe(CN)_6$, 20 µL yeast load.

2.9 Determination of Biosensor Operating Life

The operating life of the sensor (for both strains) was determined under optimal conditions (+ 450 mV, 20 mM potassium ferricyanide, 100 mM PBS buffer pH 6.0, 15 μL yeast slurry). Daily measurements of 10 ppm (BOD) GGA were taken until no significant response was obtained. The measurements were taken in triplicate within one electrode for each strain.

RESULTS AND DISCUSSION

3.1 Yeast Characterization

Two yeast strains, designated SPT1 and SPT2, were isolated from the primary clarifier effluent of a pulp mill (Thurso, Québec) for use in pulp mill wastewater analysis. Initial classification tests on the yeasts were based mostly on physiological characterization techniques. In addition, 18S rRNA sequencing was performed. Classification based on fatty acid composition was initiated as a means to verify the results obtained by the latter two of classification schemes.

3.1.1 Physiological and biochemical characterization

Streak purification on GPY agar revealed two distinct colony types, initially designated as white (off-white colouration) and opaque (semi-transparent appearance). Microscopic examination confirmed that these colonies were comprised of yeasts. The strains were later designated SPT1 and SPT2, respectively.

Strain identification was attempted after both isolates exhibited electron transfer on an electrode. Preliminary identification was attempted using classical techniques involving physiological and biochemical tests. Later, characterization tests were based on fatty acid analysis and 18S rRNA sequence analysis. Results of the physiological characterization tests are summarized in Table 3.1. After three days of growth in GPY broth at 22 °C, SPT1 cells were mostly ovoidal (5.3 x 6.7 μm), while the smaller SPT2 cells were round in shape (2.6 x 3.3 μm). Both strains exhibited multilateral budding in GYP broth. Only SPT2 exhibited multilateral budding in ME broth. Pellicle formation was observed for both strains.

The colony morphologies of the isolates on solid media were markedly different. Although both colony types exhibited viscous texture with off-white colouration and a matt appearance, the shape of the colonies were considerably distinct. After four weeks on ME agar, SPT1 colonies were off-white, butyrous, dull, waxy, and had convex to umbonate elevations and filamentous

Table 3.1 Morphological and cultural characterization of yeasts isolated from the mixing basin of a pulp mill effluent treatment system in Thurso Québec.

Test	SPT1	SPT2
Growth in liquid media*		
shape	ovoidal	circular
length (mean, n= 20)	6.7 µm	3.3 µm
width (mean, n= 20)	5.3 µm	2.6 µm
pellicle formation	yes	yes
odour	butyrous	butyrous
Growth on solid media†		
texture	waxy	waxy
colour	off-white	off-white
surface	dull	dull
elevation	convex	convex-pulvinate/ umbonate
configuration	filamentous	round &radiating/ lobate
mycelium	absent	absent
pseudomycelium	mycotoruloides	mycocandida
ballistospores	absent	absent
asci and ascospores	absent	absent

* GPY broth

† ME and PD agar

configurations. Four week old SPT2 cultures were off-white, butyrous, dull, and waxy. SPT2 colonies were convex to pulvinate on ME agar and umbonate on PD agar, depending on the medium used. Most strikingly, the SPT2 colonies exhibited round configurations with either radiating margins (ME agar) or lobate margins (PD agar). Visualization of the mycelial structure via the Dalmau plate technique (Wickerham, 1951) indicated that SPT1 and SPT2 produced mycotoruloides and mycocandida pseudomycelia, respectively.

The test for the formation of ballistospores was negative for both strains. Ballistospore formation is a specialized mode of reproduction largely confined to the basidiomycetous yeasts (van der Walt and Yarrow 1984). Similarly, the DBB tests were negative for both strains, suggesting that neither belonged to the basidiosporogenous family. The intense dark red colour associated with a positive DBB reaction is restricted to the basidiomycetous taxon (van der Walt and Yarrow, 1984).

Despite using eight different media to search for spore formation in the two isolates, no definite asci or ascospores were observed. The discovery of differentially staining circular bodies found within vegetative-like cells suggested they were ascospores. These circular bodies were observed in SPT1 after one month of growth on YM and PD agars. The SPT2 strain developed similar circular bodies after only three weeks. Both strains exhibited differentially staining bodies on Gorodkowa, McClary's, V8, and growth restrictive agar. Analogous circular bodies were noted in cells growing in GYP broth. It is assumed that these bodies were vacuoles, however, the reason for their differential staining is not known. The presence of circular bodies were frequently greater than four per cell, thus eliminating the possibility of them being ascospores (van der Walt and Yarrow 1984). Furthermore, a clearly distinctive fruiting body was never found for either isolate. Interestingly, what appeared to be germinating spores (small circular differentially staining bodies) were present in all media. These cells were very similar to blastospores in shape and tended to produce a long mycelial-like thread upon germination. The possibility that these were vegetative cells with unusual growth patterns resulting from physiological stresses was not eliminated. Fungi

imperfecti lack the sexual stage which allows for the formation of asci and ascospores (van der Walt and Yarrow, 1984). These findings strongly suggest that the yeasts belong to the imperfect genus *Candida.*

The results of the carbon assimilation and the fermentation tests are summarized in Table 3.2 and 3.3 for isolates SPT1 and SPT2, respectively. SPT1 most closely matched to *Candida tropicalis* using the key (in Kreger-van Rij 1984) to species and genera not requiring characteristics of sexual reproduction. SPT1 had an identical physiological and biochemical profile to *C. tropicalis* except that SPT1 was unable to metabolize soluble starch, ethylamine, L-lysine, and cadaverine. Similarly, SPT1 was able to grow at temperatures up to 42 °C; in high osmotic pressure conditions (50 % glucose); exhibited a negative starch test; was resistant to 1000 ppm cycloheximide; and was not able to grow in vitamin-free media. Morphological and cultural tests performed on SPT1 also yielded results analogous to those reported for *C. tropicalis* (Table 3.1).

The general key not requiring characteristics of sexual reproduction led to the possibility that SPT2 was *Starmera amethionina* (formerly *Pichia amethionina*) (Kreger-van Rij, 1984). Only three differences occurred in the carbon assimilation profile, SPT2 was able to utilize glucitol but not lactic acid, while conversely *S. amethionina* was able to utilize lactic acid but not glucitol. Also, SPT2 could ferment glucose, while *S. amethionina* was not able to ferment any carbohydrates. One crucial difference was the absence of ascospores in SPT2 This latter finding suggests that strain SPT2 is not likely an ascosporogenous yeast despite its apparent similarity to *S. amethionina* .

3.1.2 Fatty acid analysis

All fatty acid analysis was conducted by Microcheck, Inc. (Northfield Falls, VT). The isolates were grown overnight on Sabouraud dextrose agar at 28 °C prior to fatty acid analysis. In a dendrogram generated by a multivariate cluster analysis the two isolates were linked above the species level at an Euclidian Distance of 14.2. Based on the cellular fatty acid data analysis, both

Table 3.2 Summary of biochemical characterization of SPT1 based on the key to species and genera not requiring characteristics of sexual reproduction†.

Fermentation					
01 D-Glucose	+	04 α-D-Lactose	-	07 D(+)Trehalose	+
02 D(+)Galactose	+	05 D (+)Raffinose	-		
03 Maltose	+	06 Sucrose	+		
Carbon assimilation					
09 L(+)Arabinose	-	24 D-Glucitol (Sorbitol)	+	38 myo-Inositol	-
10 L-Arabitol	-	25 D-Glucose	+	39 D(+)Raffinose	-
11 Arbutin	-	26 D-Glucosamine	-,d	40 L-Rhamnose	-
12 Cadaverine	-	27 D-Glucuronic Acid	-	41 Ribitol (Adonitol)	+
13 Cellobiose	+	28 Glycerol	-	42 D-Ribose	-
14 Citric Acid	+,d	29 DL-Lactic Acid	+,d	43 Salicin	-,d
15 Creatine	-	30 D-α-Lactose	-	44 L-Sorbose	+
16 Creatinine	-	31 L-Lysine	-	45 Starch	-,d
17 Dulcitol (Galactitol)	-	32 Maltose	+	46 Succinic Acid	+
18 Ethanol	+	33 D-Mannitol	+	47 Sucrose	+
19 Ethylamine	-	34 Methanol	-	48 D(+)Trehalose	+
20 Erythritol	-	35 Methyl-α-D		49 D(+)Xylose	+
21 ß-D-Fructose	+	glucopyranoside	+	50 Xylitol	-
22 D(+)Galactose	+	36 Melibiose	-		
23 D-Gluconic Acid	+	37 D(+)Melezitose	+		
Additional characteristics					
51 growth at 25°C	+	57 50% D-Glucose	+	62 NO_3 assimilation	-
52 growth at 30°C	+	58 60% D-Glucose	-	63 Diazonium Blue B	-
53 growth at 35°C	+	59 0.01% Cyclo-		64 Vitamin free	
54 growth at 37°C	+	heximide	+	growth	-
55 growth at 42°C	+	60 0.1% Cyclo-		65 Acid production	
56 growth in 10% NaCl		heximide	+	from glucose	-
& 5% D-glucose	+	61 NO_2 assimilation	-	66 Starch formation	-

† Codes for responses to tests: + positive growth ≤ 1 week; - no growth ≤ 3 weeks; d delayed growth response (> 1 week); v variable response (+,-); w weak response.

Table 3.3 Summary of biochemical characterization of SPT2 based on the key to species and genera not requiring characteristics of sexual reproduction†.

Fermentation					
01 D-Glucose	+	06 α-D-Lactose	-	05 D(+)Trehalose	+
02 D(+)Galactose	-	07 D(+)Raffinose	-		
03 Maltose	-	04 Sucrose	+		
Carbon assimilation					
09 L(+)Arabinose	-	24 D-Glucitol (Sorbitol)	+	38 myo-Inositol	-
10 L-Arabitol	-,d	25 D-Glucose	+	39 D(+)Raffinose	-
11 Arbutin	-	26 D-Glucosamine	-	40 L-Rhamnose	-
12 Cadaverine	-	27 D-Glucuronic Acid	-	41 Ribitol (Adonitol)	-,d
13 Cellobiose	-	28 Glycerol	+,d	42 D-Ribose	-
14 Citric Acid	-	29 DL-Lactic Acid	-,d	43 Salicin	-
15 Creatine	-	30 D-α-Lactose	-	44 L-Sorbose	-
16 Creatinine	-	31 L-Lysine	-	45 Starch	-
17 Dulcitol (Galactitol)	-	32 Maltose	-	46 Succinic Acid	+
18 Ethanol	+	33 D-Mannitol	+	47 Sucrose	-
19 Ethylamine	-	34 Methanol	-	48 D(+)Trehalose	-
20 Erythritol	-	35 Methyl-α-D glucopyranoside	-	49 D(+)Xylose	-
21 ß-D-Fructose	+	36 Melibiose	-	50 Xylitol	-
22 D(+)Galactose	-	37 D(+)Melezitose	-		
23 D-Gluconic Acid	+,d				
Additional characteristics					
51 growth at 25°C	+	57 50% D-Glucose	-	62 NO_3 assimilation	-
52 growth at 30°C	+	58 60% D-Glucose	-	63 Diazonium Blue B	-
53 growth at 35°C	+	59 0.01% Cyclo-heximide	-	64 Vitamin free growth	-
54 growth at 37°C	+w	60 0.1% Cyclo-heximide	-	65 Acid production from glucose*	+
55 growth at 42°C	-	61 NO_2 assimilation	-	66 Starch formation	-
56 growth in 10% NaCl & 5% D-glucose	-				

† Codes for responses to tests: + positive growth ≤ 1 week; - no growth ≤ 3 weeks; d delayed growth response (> 1 week); v variable response (+,-); w weak response.

* acid production was not vigorous and may be related to citric acid and not acetic acid

SPT1 and SPT2 were judged to be *Candida* species. Similarity indices (SI) based on fatty acid retention times and mean percentages, yielded excellent matches for both isolates. Strain SPT1 was identified as *C. albicans* GC subgroup A, with an SI value of 0.688. SPT2 was most closely matched to *C. zeylanoides* and had an SI value of 0.617. *Candida krusei* was also listed as a possible match for strain SPT2 but had an SI value of only 0.400.

3.1.3 18S rDNA sequence analysis

Partial double stranded DNA sequences for the 18S rRNA genes of both SPT1 and SPT2 were determined. Accuracy was increased by sequencing in both directions and twice in the 3'-5' direction. The resulting corrected sequence did not contain any undetermined positions. These sequences corresponded to approximately one third of the small subunit rRNA gene (White et al., 1990).

Sequence comparison via BLAST (Altschul et al., 1997) searches revealed that *Candida sojae* was the closest match to SPT1. Homology (510 bp) was nearly complete (99.8 %) with only a one nucleotide difference (Figure 3.1, position 120). *Candida viswanathii* also had 99.8 % homology (with a guanine insertion at position 79 - not shown), while the 18S rDNA of *C. topicalis* was 99.6 % similar. Comparison of the 18S rRNA sequence (711 bp) of SPT2 to the compilation available in the GenBank and EMBL databases via BLAST searching did not yield any close sequence matches. BLAST searches revealed that *Candida krusei*, *Pichia membranifaciens* and *Candida mesenterica* were the nearest matches. Sequence comparisons obtained from the DNADIST program of the PHYLIP package did not yield homologies greater than 74 % for SPT2. Sequence homology for *Candida krusei* was only 71 %. Such low homology indicates that SPT2 is a previously uncharacterized species and likely an uncharacterized genus.

The sequence alignment program CLUSTAL X (Thompson et al., 1997) was used to align the sequences of the two isolates with their closest matches (as determined by the biochemical and nuclear characterization methods). All aligned sequences were 510 nucleotides long, corresponding

to the length of the 18S rDNA sequence of SPT1. Aligned sequences extended from position 592-1133 according to the *S. cerevisiae* numbering system for the 18S rRNA gene (Mankin et al., 1986). When compared to the sequence conservation histogram for eukaryotic 18S rRNA provided by Sogin and Gunderson (1987), the length of these sequences encompasses variable regions V2-V4.

The 18S rDNA sequences from *C. tropicalis, C. albicans, and C. sojae* were compared to the sequence of isolate SPT1 (Figure 3.1). The choice of these species was based on the results of the physiological and biochemical tests, fatty acid analysis, and the BLAST search, respectively. A very low frequency of sequence variation was exhibited between SPT1 and these *Candida* species. The first 46 nucleotides in Figure 3.1 are characteristic of the conserved region between variable regions V1-V2 (Sogin and Gunderson, 1987). The most variable region between the four sequences is between nucleotides 46-124. This corresponds to the location of the second hypervariable region (V2). The low sequence variability (1 mismatch at position 228) in the V2 region highlights the close relationship of these *Candida* strains.

The alignment of SPT2 to the three closest matches obtained from the physiological, biochemical, fatty acid analysis, and the BLAST results is shown in Figure 3.2. Extensive variability was observed in the V2 region (positions 65-164). The V3 regions were poorly defined and not easily comparable. Both Sogin and Gunderson (1987) and Stackebrandt and Rainey (1995) have indicated that the location of hypervariable regions differ from taxon to taxon, so the variability in Figure 3.2 was not unexpected. The lack of sequence homology strongly suggests that SPT2 is a previously uncharacterized species.

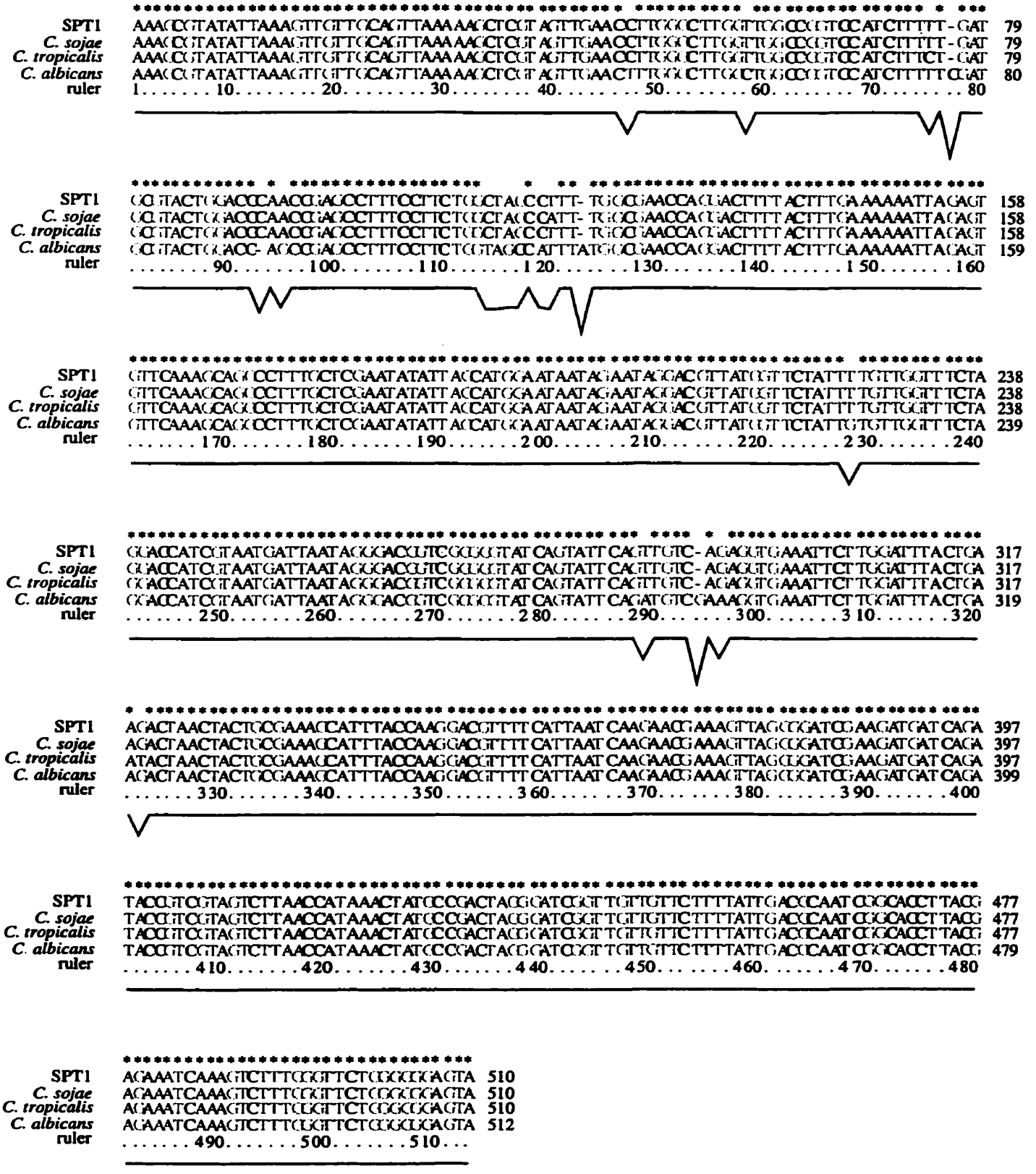

Figure 3.1 Alignment of central region of the 18S rRNA gene sequence of isolate SPT1, *C. sojae*, *C. tropicalis* and *C. albicans*. The single basepair difference between SPT1 and *C. sojae* is located at position 121. The bottom line indicates the degree of non homology.

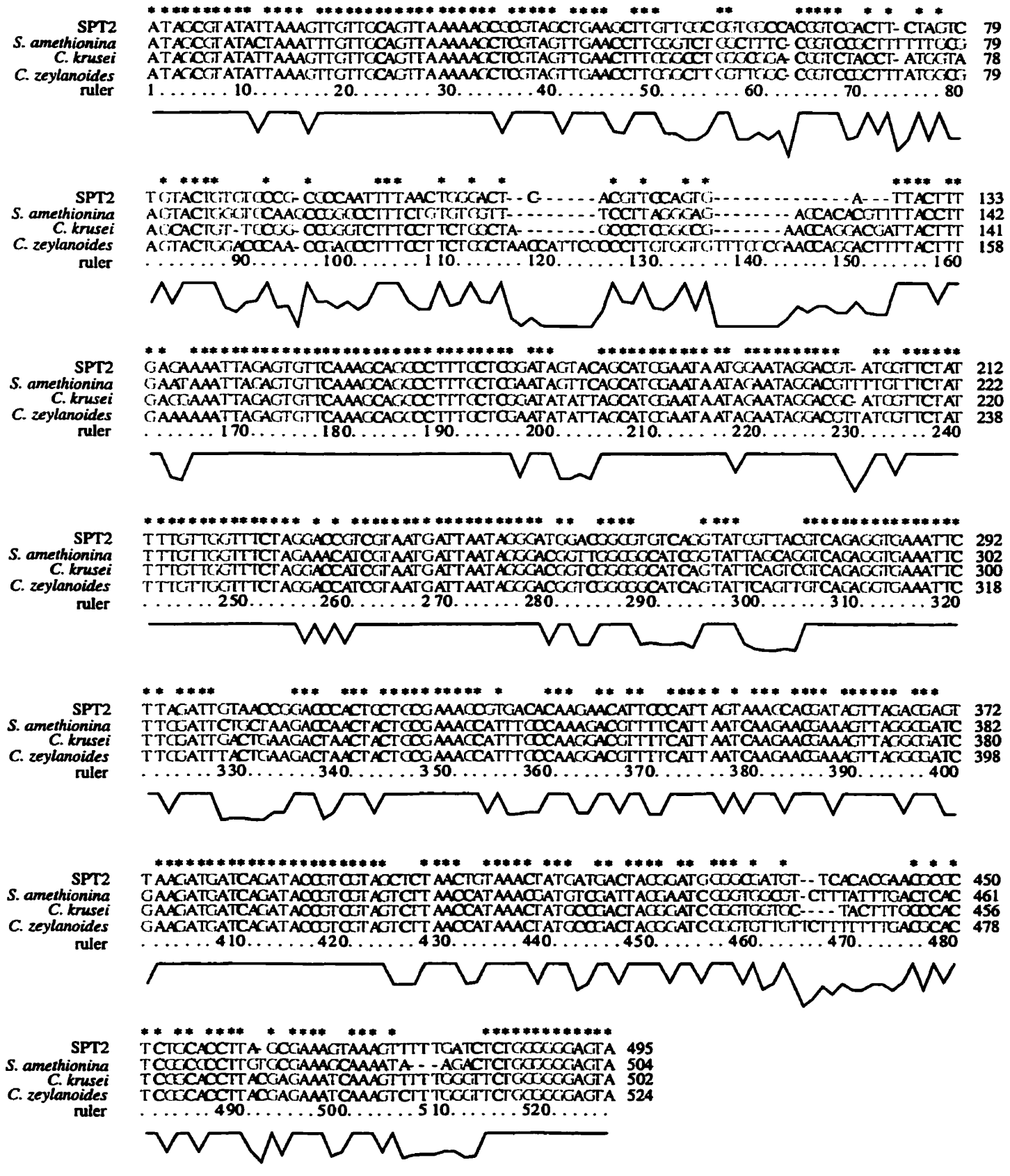

Figure 3.2 Alignment of central region of the 18S rRNA gene sequence of isolate SPT2, *S. amethionina*, *C. krusei*, *C. zeylanoides*. The bottom line indicates the degree of non homology.

A phylogenetic tree was created using the PHYLIP software package (Figure 3.3). The number of alignments in the outfile was increased to 13 to decrease the likelihood of false branching within the dendrogram (Stackebrandt and Rainey 1995). *Saccharomyces cerevisiae* was included in the alignment as a representative of the ascosporogenous yeast. Members of the basidiosporogenous yeasts used in the dendrogram included *Rhodosporidium dacryoidum* and *Tremella globospora*. *Starmera caribaea* was included in an attempt to help stabilize the branching point of *S. amethionina*. Calculations of genetic distances were performed on blocks of sequences 510 bp long.

Three distinct clusters were formed in the dendrogram. Cluster I is comprised of *Starmera amethionina* and *Starmera caribaea*, and is supported at a high level (100 %) by bootstrapping analysis. These species were observed to be highly related, displaying a sequence similarity value of 98.8 %. Cluster II was comprised of the basidiosporogenous yeasts *Rhodosporidium dacryoidum* and *Tremella globospora*, and had a branch confidence value of 96.4 %. Cluster III was the most diverse group and consisted of the *Candida* species, and *S. cerevisiae*. SPT1, and its closest matches based on the three different characterization methods, formed a highly significant subcluster (95.6 %) within cluster III. Close sequence similarities between SPT1 and *C. albicans*, *C. tropicalis*, and *C. sojae* were observed: 98.6, 99.6, and 99.8 %, respectively. The subclustering of isolate SPT1 with *C. sojae* suggested that SPT1 is a strain of *C. sojae*.

C. albicans was the identity match obtained for SPT1 via fatty acid analysis. The sequence similarity index obtained from the DNADIST program indicated that *C. albicans* was more distantly related (1.2 %) to SPT1 than *C. tropicalis*. Despite a sequence homology of 99.8 % between SPT1 and *C. sojae*, it can not be inferred that SPT1 is a strain of *C. sojae*. Important differences in

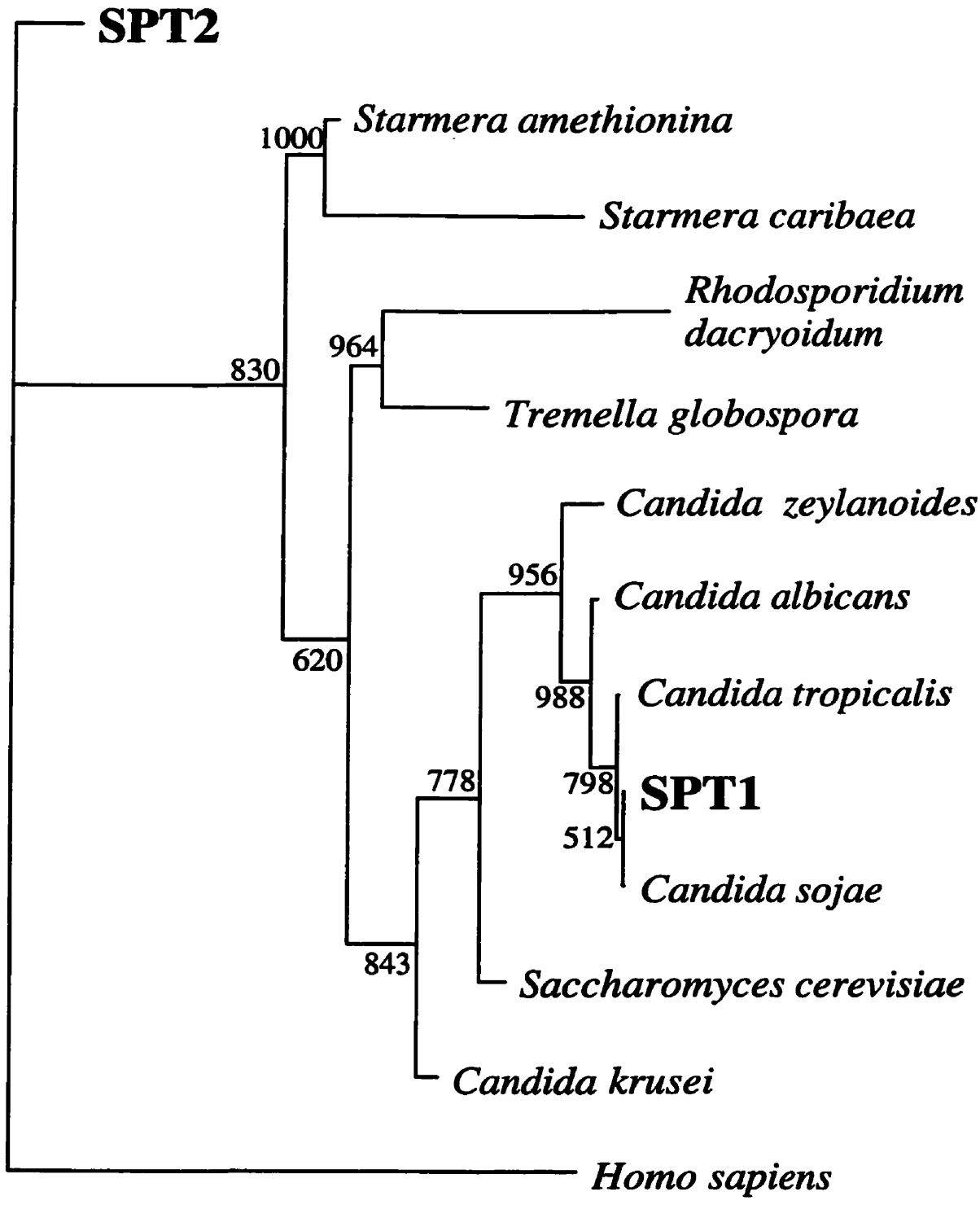

Figure 3.4 Dendrogram showing the phylogenetic relationships of isolates SPT1 and SPT2 to their closest matching strains (derived from physiological and biochemical testing, fatty acid analysis, and BLAST searches) based on 18S rRNA gene sequences. The unrooted tree was constructed by using the neighbour-joining method (Saitou & Nei 1987). Bootstrap values, based on 1000 replications, are given at branch points.

metabolic capabilities occur between the two strains. When compared to the description of *C. sojae* given by Nakase et al. (1994), SPT1 differs by its inability to assimilate four compounds (cadaverine, ethylamine, glycerol, and soluble starch), and its ability to ferment maltose and grow at 42 °C (both of which *C.sojae* can not do). It should be noted that SPT1 was isolated from the primary clarifier effluent of a pulp mill in Québec, while *C. sojae* was isolated from the extraction water of defatted soybean flakes in Japan. It is not improbable that such vast differences in habitat and geographic location have lead to strain variation. Despite this possibility, it must be noted that Nakase et al. (1994) base their distinction of *C. sojae* from *C. tropicalis* and *C. albicans* on its inability to ferment maltose and grow at 40 °C. Based on these criteria, SPT1 cannot be defined as *C. sojae.* While the intricacies of these metabolic differences are important, a definitive relationship of SPT1 to the other members of cluster III may be made only once the entire length of the 18S rRNA is sequenced and compared. Stackebrandt and Rainey (1995) do not recommend making inferences on evolutionary relationships from phylogenetic trees based on partial 18S rRNA sequences since it only allows for intergenic delineation. Furthermore, the creation of dendrograms using different regions of rRNA can result in different tree topologies (James et al., 1998).

Analysis of sequence distance matrix (not shown) revealed that isolate SPT2 is not closely related to any previously characterized species. Phylogenetic comparison (Figure 3.3) to *S. amethionina*, *C. zeylanoides*, and *C. krusei* (the nearest matches obtained from the biochemical tests, fatty acid analysis and BLAST searching, respectively) did not result in the grouping of SPT2 with any of these organisms. James et al. (1998) have stated that an 18S rRNA sequence divergence of as little as ~2 % can be enough to suggest separate genera. This indicates that the genus of isolate SPT2 is not represented in Figure 3.3. However, conclusions regarding genera classification are questionable if only one representative of the lineage is known (van de Peer and Wachter 1995). For example, the branching of SPT2 may be an artifact caused by a lack of 18S rRNA sequence information in the various databases for the species to which it is most closely related. Despite this, the preliminary indications are that SPT2 is a representative of an

Table 3.4 Summary of the results obtained from the various yeast characterization schemes.

Characterization technique	SPT1	SPT2
Physiological/ biochemical	*Candida tropicalis*	*Starmera amethionina*
Fatty acid analysis*	*Candida albicans*(0.688) *Candida famata* (0.419) *Candida guiliermondii* (0.372)	*Candida zeylanoides* (0.617) *Candida famata* (0.487) *Candida krusei* (0.400)
18S rRNA BLASTn searches†	*Candida sojae* (99.8 %) *Candida viswanathii* (99.8 %) *Candida tropicalis* (99.6%)	*Candida krusei* (76.0 %) *Pichia membranifaciens*(74.1%) *Candida mesenterica* (75.6 %)

* numbers in parentheses indicate the similarity indices
† numbers in parentheses indicate the percent homology

uncharacterized genus. A summary of the various characterization schemes attempted is presented in Table 3.4.

3.2 Mediator Screening

Cyclic voltammetry was employed to screen for potential mediators and the selection of mediators was based primarily on previous published results (Table 3.5). Ten mediators exhibiting a wide range of redox potentials were screened (eight water-soluble and two water-insoluble mediators in a hp-β-CD solution). However, only potassium ferricyanide and HMF^+ exhibited significant mediating capabilities. The responses of the microbial sensors in the absence of mediator were negligible, indicating that the mediators were responsible for electron shuttling from the yeasts to the electrode.

Figure 3.4 depicts typical cyclic voltammograms of isolates SPT1 and SPT2 in the absence and presence of 500 ppm (BOD) GGA using 5 mM potassium ferricyanide as mediator. Within the range of potential scanned (-0.2 to +1 V), potassium ferricyanide exhibited voltammograms that corresponded well with a one electron redox compound (Hill and Sanghera, 1990). In the absence of GGA, the yeast did not contribute a response, and only the electrochemical behaviour of potassium ferricyanide was noted. Upon addition of 500 ppm (BOD) GGA a large increase in the oxidation current was observed. This is indicative of catalytic regeneration of the mediator by the reduced cellular enzymes of the yeasts. The increase in the oxidation peak is a consequence of the oxidation of ferrocyanide ($Fe(CN)_6^{4-}$) from ferricyanide ($Fe(CN)_6^{3-}$) by the yeast cellular enzymes in a reduced form and subsequent reoxidation of the reduced mediator at the surface of the electrode. An excess of GGA was used to ensure that the yeast cellular enzymes were fully reduced. The regeneration of $Fe(CN)_6^{4-}$ to $Fe(CN)_6^{3-}$ by the electrode was rapid since the use of the mediator at 5 mM should not lead to significant diffusional limitation. No change in either the cathodic or anodic current was observed at the glassy carbon electrode with the addition of GGA in the absence of yeast (data not shown). The latter observation strengthens the argument that yeast enzymes are

Table 3.5 Summary of results obtained from the various mediators screened for electron mediation via cyclic voltammetry.

Water-Soluble Mediators	Mediation	Reference
2,6-dichlorophenol-indophenol	insignificant	Kaláb and Skládal (1994)
1,1'-dimethylferricinium	insignificant	Luong (personal communication)
Fe^{III} EDTA	insignificant	Kaláb and Skládal (1994)
ferricinium hexafluorophosphate	none, cytotoxic	Luong (personal communication)
ferricinium tetrafluoroborate	none, cytotoxic	Luong (personal communication)
hydroxymethylferricinium	significant reversible	Luong (personal communication)
phenazine ethosulphate	none	Roller et al. (1984)
potassium ferricyanide	significant quasi-reversible	Rawson et al. (1989) Bennetto et al. (1983)
Water-Insoluble Mediators		
1,1'-dimethylferricinium	insignificant	Luong et al., (1993)
3,3',5,5'-tetramethylbenzidine	insignificant cytotoxic	Cattaneo & Luong (1994)

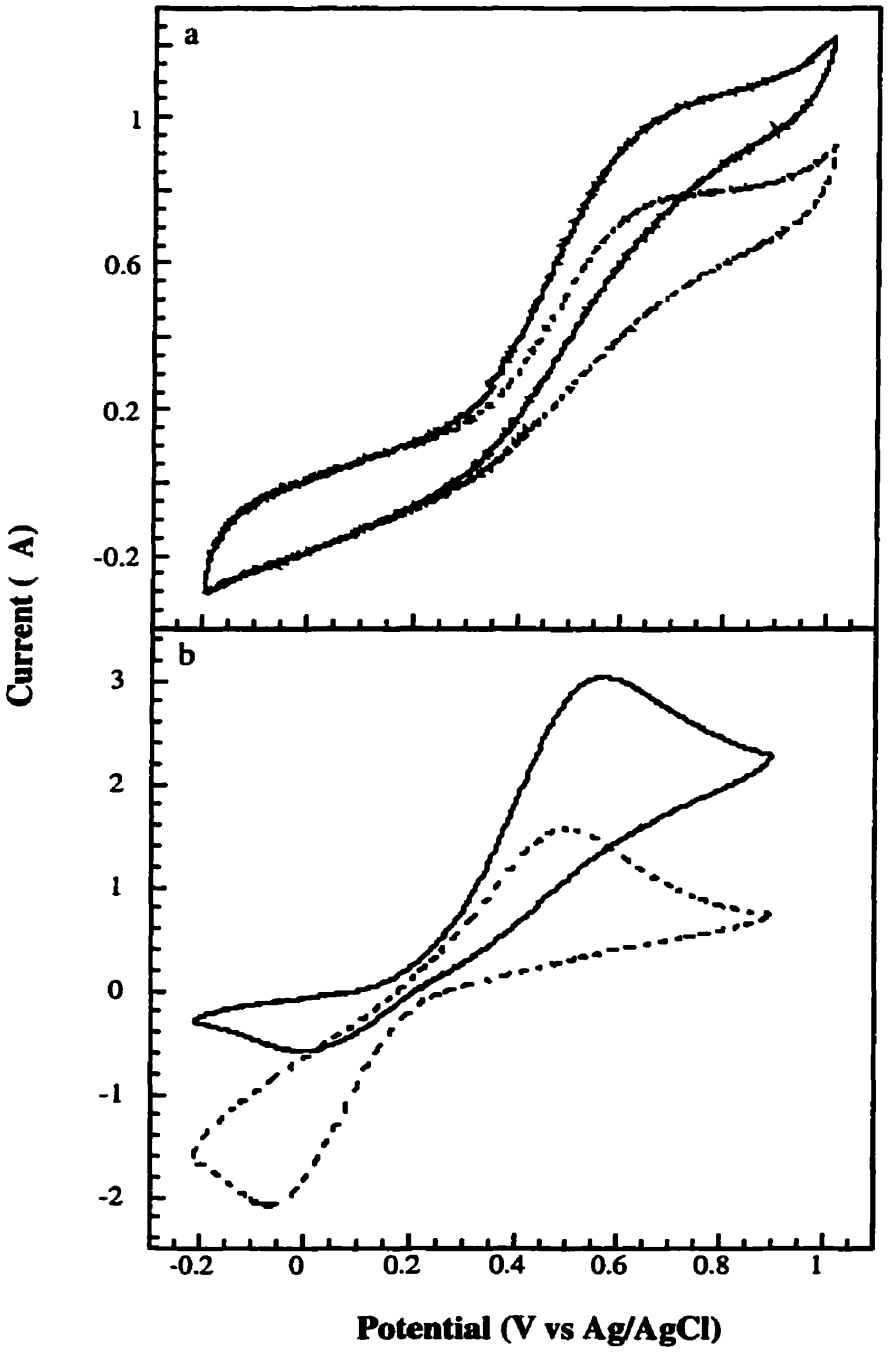

Figure 3.4 Cyclic voltammogram with isolates (a) SPT1 and (b) SPT2 using potassium ferricyanide in the presence (———) and absence (---------) of 500 ppm BOD GGA(2 mV/s, 5 mM $K_3Fe(CN)_6$, 100 mM PBS, pH 7.2, 22 °C).

mainly responsible for mediator reduction.

The increase in oxidation current for the SPT1 sensor was not accompanied by an increase in the reductive current, thus the reaction is only quasi-reversible (Figure 3.4a). According to Cass et al., (1984) the lack of an increase in the reduction current is a consequence of an enzyme-dependent catalytic reduction of $Fe(CN)_6^{3-}$ at the oxidizing potential.

The potassium ferricyanide voltammogram for SPT2 was slightly different from that of SPT1 in that the reaction was more reversible (Figure 3.4b). After substrate addition, there was a noticeable decrease in the reduction current as well as an increase in the oxidation current. The height of the anodic peak current (i_{pa}) was much greater than the cathodic peak current (i_{pc}) indicating that the reaction was not reversible ($i_{pa}/i_{pc} = 9.7$). This seems to indicate that the reduced enzymes of SPT1 were able to interact with $Fe(CN)_6^{3-}$ in a more efficient manner than the reduced enzymes of SPT2. Cass et al. (1984) noted that the rate limiting step in mediated electron shuttling is the interaction of reduced enzymes and the oxidized mediator. Unlike SPT2, the reason behind why SPT1 did not exhibit quasi-reversible redox reactions with ferricyanide is not clear. However, it may have been related to different outer membrane compositions (as indicated by the fatty acid analysis). A less permeable cell membrane may create a electron recycling bottleneck by increasing the time it takes for $Fe(CN)_6^{3-}$ to come into contact with reduced enzymes. Kaláb and Skládal (1994) indicated that mediators such as ferrocene exhibit increased diffusion times through bacterial cell walls. With regards to diffusion rates, both the composition of the cell wall as well as the mediator type are important factors governing the reversibility of reactions.

With HMF^+ mediation, the cyclic voltammograms of SPT1 exhibited a more reversible reaction ($i_{pa}/i_{pc} = 0.91$) with well pronounced oxidation and reduction peaks (Figure 3.5a). With the SPT1 sensor the addition of 500 ppm (BOD) GGA resulted in a distinct shift in the electrochemical profile of HMF^+. The increase in oxidation current was nearly matched by the decrease in the reduction current resulting in a very similar profile located 0.25 μA higher. Under the same conditions, the SPT2 sensor produced a higher oxidation current peak (1.4 μA) than SPT1

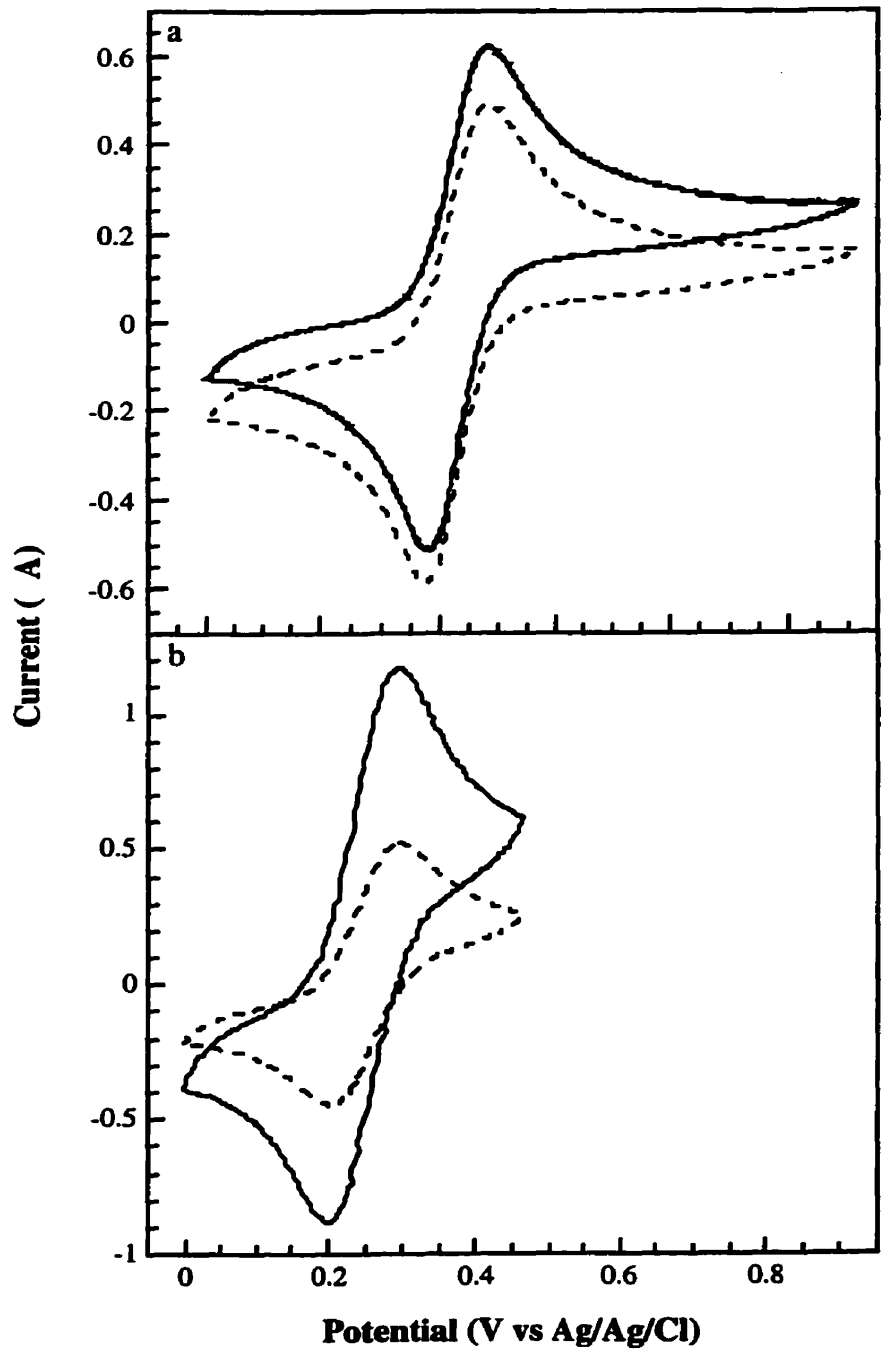

Figure 3.5 Cyclic voltammogram of isolates (a) SPT1 and (b) SPT2 using hydroxymethylferricinium the presence (———) and absence (---------) of 500 ppm BOD GGA (2 mV/s, 5 mM $K_3Fe(CN)_6$,100 mM PBS, pH 7.2, 22 ℃).

(Figure 3.5b). The reaction was almost completely reversible (i_{pa}/i_{pc} = 1.0). In the absence of GGA, only the typical oxidation and reduction peaks for HMF^+ were observed. No oxidation current increase was noted with GGA addition when yeast cells were not retained on the electrode. Consequently, the rise in oxidation current upon addition of substrate was interpreted as the reduction of HMF^+ by reduced yeast enzymes to hydroxymethylferrocene and the subsequent reoxidation of hydroxymethylferrocene to HMF^+ at the surface of the electrode.

Despite the promising results obtained for the remaining mediators in Table 3.5 by other researchers, none of these exhibited any clear increases in oxidation peaks. Luong et al. (1995) suggested that water insoluble mediators (i.e. DMF^+, TMB) possess excellent mediator qualities for various oxidoreductases when solubilized in CD. Mediators solubilized by CDs have been demonstrated to effectively mediate electrons between enzymes (i.e. glucose oxidase) and electrodes (Cattaneo and Luong, 1994; Luong et al., 1993; Zhao and Luong, 1993). Effective reversible mediation at low potentials, eliminating many electrochemical interferents, could potentially be achieved using water soluble CD-mediator complexes and whole cells. Unfortunately, no mediation was exhibited by any of the water-insoluble mediators. Takayama et al. (1996) remarked that two important determining factors controlling the effectiveness of a compound as an electron acceptor are solubility and permeability of the cellular membrane. It is believed mediators solubilized with CDs do not necessarily have any more access to the catalytic site within cells. One reason may be related to strength and type of CD-mediator complex, this possibility is subject to further investigation.

3.2.1 Effect of cyclodextrin on GGA response

The inhibitory effect of CD, if any, on the yeast response to GGA was investigated using a microbial oxygen electrode in the presence and absence of hp-β-CD. An oxygen electrode was used in place of the mediated electrode in order to eliminate any possibility of water soluble mediator entrapment by cyclodextrin effecting responses. No significant difference was found between

electrode responses in the absence of cyclodextrin and in the presence of 10 mM hp-β-CD (data not shown). In the presence of hp-β-CD, SPT1 exhibited only a 12.2% decrease in mean response to 5 ppm (BOD) GGA (as compared to responses in the absence of CD). Similarly, mean responses of SPT2 to 5 ppm (BOD) GGA additions were 9.6% lower in the presence of 10 mM hp-β-CD. The reasons for the lower response heights for both strains in the presence of cyclodextrin is not clear. Cyclodextrin is considered to be relatively nontoxic (Szejtli, 1982), therefore hp-β-CD should not exhibit any direct negative effects on the yeasts. Furthermore, cyclodextrins generally cannot be assimilated by yeasts (Szejtli, 1982). The approximately ten percent decrease in signal is considered to be insignificant and within the allowable error for this type of measurement. Consequently, it is concluded that cyclodextrin does not inhibit the response of the isolates to the GGA additions.

3.3 Biosensor Optimization

3.3.1 Mediator concentration optimization

The responses to 20 ppm (BOD) injections of GGA increased linearly with increasing concentrations of mediator until approximately 10 mM $K_3Fe(CN)_6$ (Figure 3.6). The K_s (as determined from a Hanes plot - not shown) of potassium ferricyanide was fairly high (10.5 mM), and the V_{max} was 2.5 μA. Partially for reasons of toxicity, 20 mM $K_3Fe(CN)_6$ was chosen as the optimal concentration to use for measurements at a set potential. Increasing $K_3Fe(CN)_6$ to 40 mM would only effect an ~20 % increase in the signal response. Diffusional constraints causing electron shuttle lags due to limiting $K_3Fe(CN)_6$ were not expected to occur at this concentration. As a result, 20 mM $K_3Fe(CN)_6$ concentration was considered sufficient to account for electron shuttling and any increase in mediator concentration was avoided owing to possible negative effects on the yeast cells. Atkinson and Haggett (1993) suggested that prolonged exposure to potassium ferricyanide can induce a toxic response in cells. This is likely a consequence of excessive diversion of the electron transport chain via mediator reduction (Ramsay and Turner, 1988). An investigation into the toxic effects of potassium ferricyanide on the isolates (via standard plate counting) revealed that

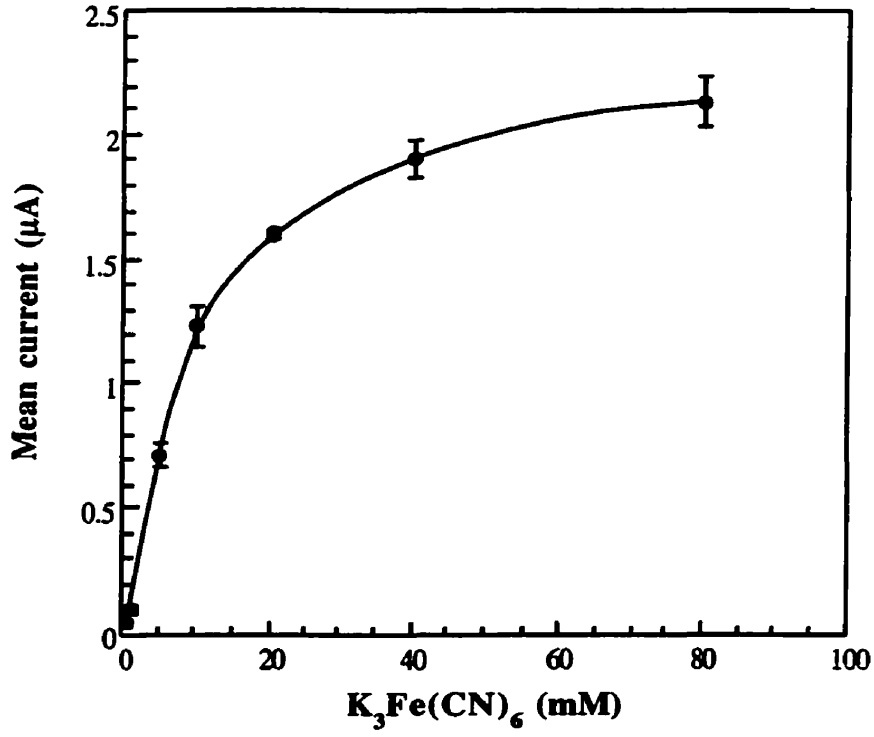

Figure 3.6 Optimization curve indicating standard deviations for potassium ferricyanide (+400 mV, 100 mM PBS buffer pH 7.2, 22°C)

growth was not observed for SPT1 in concentrations above 15 mM. Mediator concentrations ranging from 1 to 5 mM were sufficient to inhibit growth for SPT2.

Owing to solubility, the preparation of HMF^+ does not allow for concentrations exceeding 8 mM. The solution prepared in our laboratory according to a patented procedure (U.S. patent no. 5, 432, 274) had a concentration of 6.33 mM, and thus limited the range over which the HMF^+ optimization could be measured. The response heights to 20 ppm (BOD) additions of GGA were measured from 0.1 mM to 4 mM HMF^+ (Figure 3.7). Fortunately, the K_s, of HMF^+ was 7-fold lower (1.5 mM) than for $K_3Fe(CN)_6$, indicating better mediator efficiency. However, the V_{max} using HMF^+ as mediator was ~25-fold lower (0.1 μA) than that of $K_3Fe(CN)_6$. A concentration of 3 mM HMF^+ was determined to yield the maximal response height. Increasing HMF^+ concentrations beyond this resulted in a decreased mean signal. HMF^+ concentrations were held at 3 mM for all future experiments.

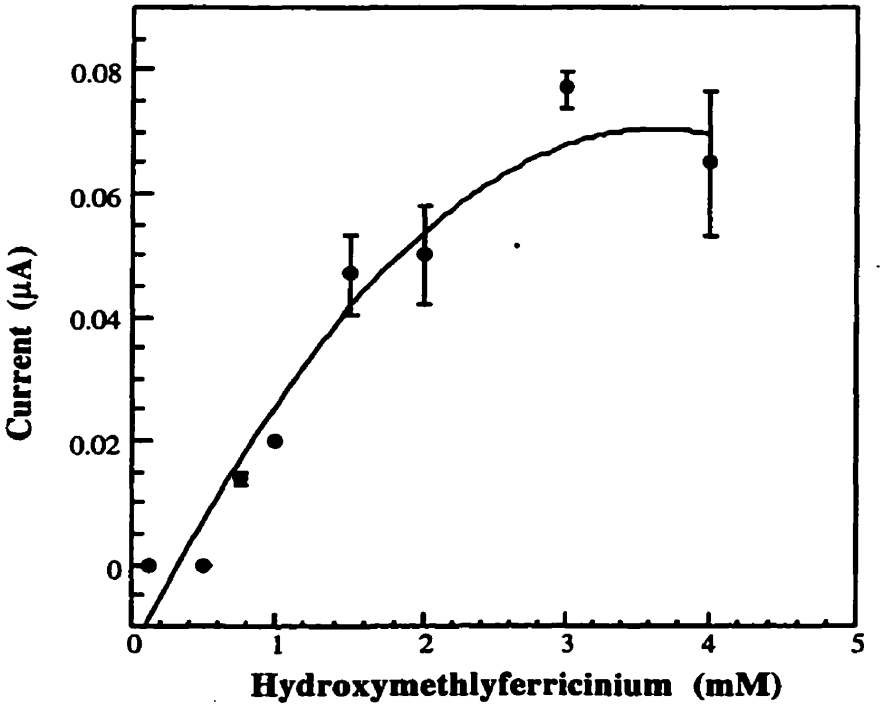

Figure 3.7 Optimization curve showing standard deviations for hydroxymethylferricinium (+285 mV, 100 mM PBS buffer pH 7.2, 22 °C)

3.3.2 Optimal yeast loading

Increasing the number of cells at the surface of an electrode often results in increased signals as a consequence of a greater number of redox reactions occurring (Chan et al., 1999; Li et al., 1994; Tanaka et al., 1994). However, such behaviour was not observed with the two isolates in this study (Figure 3.8). Response heights increased with increasing yeast slurry volume up to 15 μL, for both SPT1 and SPT2. This quantity represented a mean (n=10) wet weight of 15.0 mg for both strains. Increasing the slurry volume beyond this point resulted in decreasing signals, likely due to mass transfer diffusion. Since only those yeasts in intimate contact with the electrode were presumed to contribute to the signal response, this may have been a consequence of mediator and substrate diffusional constraints. Similarly, Richardson et al. (1991) observed that increasing the microbial load past an optimal density (i.e. 2.25×10^8) created diffusional barriers that limited the access of substrate to the cells and mediator to the cells and the electrode. Large quantities of yeasts retained behind a dialysis membrane may have directly interfered with the signal response by creating a diffusional barrier. This was evidenced by the increased response times observed for the electrodes with higher volumes of retained yeast. Substrate utilization by the outermost yeasts (not in intimate contact with the surface of the electrode) may have indirectly contributed to decreased signals. The latter hypothesis may account for the decreased signal observed for higher concentrations of yeast. To be effective the substrate must first be in close proximity to the surface of the electrode and the biocatalyst. Therefore, diffusional barriers or premature consumption by the outermost yeast not in intimate contact with the surface of the electrode will naturally cause delayed and decreased height responses.

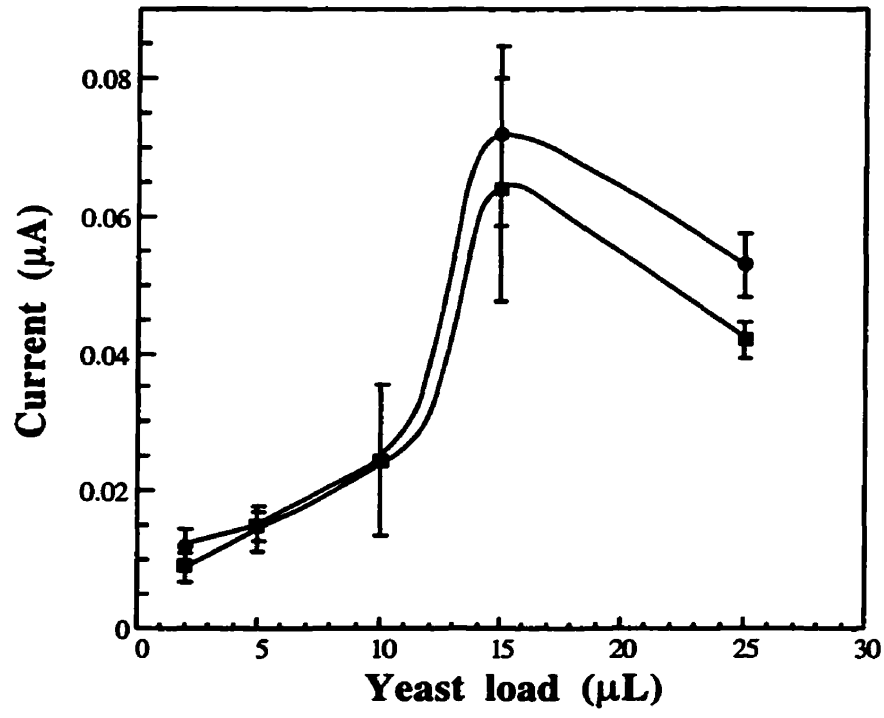

Figure 3.8 Determination of optimal loading for isolates (—●—) SPT1 and (—■—) SPT2 indicating mean values (n=3) on glassy carbon electrode (+450 mV, 20 mM $K_3Fe(CN)_6$, 100 mM PBS pH7.2, 22 °C, error bars = standard deviation).

3.3.3 Preconditioning of Electrode

Preconditioning of freshly prepared electrodes was necessary because no mediation was observed upon addition of pulp mill effluent. Newly prepared sensors did not exhibit sensitive responses to pulp mill samples and only minimal responses to GGA additions. Current increases were observed only after the injection of 1000 ppm GGA. This indicated that mediation was indeed occurring, yet at an extremely minimal level. Repeated use of the electrode over a number of days resulted in increasing response heights. Investigation of the literature revealed that this was not an unusual phenomenon. Li et al. (1994) observed that preconditioning of their BOD sensor was necessary to obtain optimal responses. They concluded from their study that the time required for preconditioning was determined by the quantity of microorganisms on the electrode (i.e. a minimum of 20 hours for 2.2 X 10^8 cells) and the concentration of GGA exposed to the microorganisms (70 ppm was optimal). Yang et al. (1996) also concluded that their BOD sensor required 24 hours of preconditioning (i.e. five minute substrate measurements every thirty minutes). Praet et al. (1995) noted that regeneration of their electrode, after storage, took 2 to 4 days. In this study, both the preconditioning time and procedure needed to be experimentally established for each microorganism.

Preconditioning via starvation

Initial investigations to determine the length of preconditioning time involved starving the cells in a stirred solution of 100 mM PBS buffer pH 7.2 while taking daily measurements in triplicate. Figure 3.9 shows the time needed to obtain the optimal response height via starvation for the sensor using strain SPT1. Up to 10 days of starvation in 100 mM PBS buffer pH 7.2 were needed for SPT1 to attain the maximum response height via this method. For the first five days of effluent measurement responses only increased from 0.03 μA to 0.065 μA. After this point the responses increased linearly to 0.17 μA for a 20 ppm (BOD) of Thurso primary settling basin effluent. Preconditioning beyond this point resulted in a slight increase in the mean

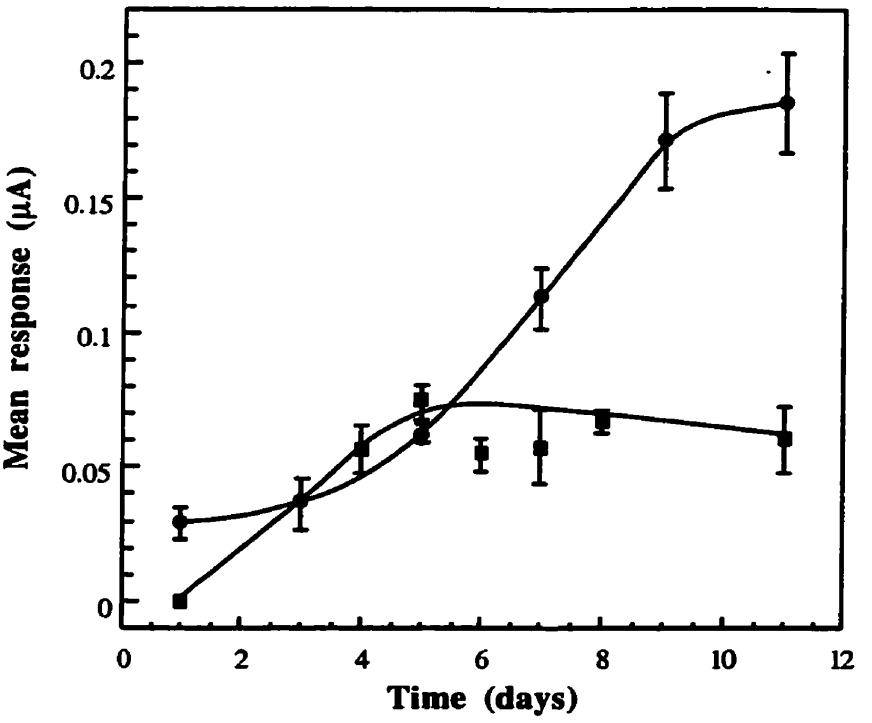

Figure 3.9 Response of isolates (—●—) SPT1 and (—■—) SPT2 to additions of 20 ppm BOD effluent after several days of preconditioning via starvation in 100 mM PBS buffer, pH 7.2 (+450 mV, 20 mM $K_3Fe(CN)_6$, 22 °C, error bars = standard deviation)

response (to 0.185 μA) that was within the standard deviation of the result obtained after 10 days.

The time needed for the SPT2 sensor to attain a maximal response to 20 ppm (BOD) effluent from the Thurso primary clarifier was 60 percent less then the time needed by the SPT1 sensor (Figure 3.9). After four days of starvation in 100 mM PBS buffer pH 7.2, responses from the SPT2 sensor increased from no response after one day of preconditioning, to the maximal value of approximately 0.06 μA. This result is similar to that obtained by Tan et al. (1993) who observed that three to four days of preconditioning was needed by their microbial (*Bacillus* spp.) BOD sensor to obtain optimal responses. Chan et al. (1999) had similarly found that a period of five days was needed to obtain a maximal signal from their yeast based BOD sensor. They claimed that a gradual increase in the sensor signal for the first five days was a consequence of small but significant growth of the yeast (*Arxula adeninivorans*) and that a gradually decreasing background current "proves" this assumption. This is likely not the case with two yeast isolates used in this study since preconditioning via starvation did not provide any opportunity for yeast growth. In this case, increased signals were more likely related to metabolic changes within the yeasts. Heim et al. (1999) reported that preconditioning of their *Rhodococcus erythropolis* and *Issatchenkia orientalis* sensor via overnight starvation effected higher signals than starvation from 0 - 6 hours. They attributed this to the requirement of cells to exhaust internally stored carbon sources prior to the acceptance of external supplies. A similar phenomenon was believed to have occurred here.

Preconditioning via incubation with 50 % effluent

Riedel et al. (1990) determined that preincubation of an oxygen-based BOD biosensor (using the yeast *Trichosporon cutaneum*) allows for better correlations to actual BOD_5 values as a result of increased sensor responses. Preincubation of 12 hours in various wastewaters was sufficient to increase responses (i.e. ~200 % and ~300 % for food factory and chemical industry wastes, respectively) such that they correlated well with BOD_5 values (i.e. R^2 =0.933). A similar result was obtained by Tan et al. (1993) who deduced that supplementing the culture growth

medium with 10 % of the desired organic solute resulted in significantly improved signals of their oxygen-based BOD sensor. However, this modification of the growth medium did not alter the period of preconditioning. The objective of this investigation into preconditioning was to alter the sensor activity by increasing sensor sensitivity and selectivity for the substrate. In a process called direct induction, preincubation of the sensor in the desired substrate is expected to induce uptake systems and metabolic degradation pathways of the microorganisms for that substrate (Riedel 1991). On the basis of such results, preincubation with effluent from the primary settling basin of the Thurso mill was investigated.

The SPT1 sensor attained a maximal response to 20 ppm (BOD) injections of effluent by the fourth day (Figure 3.10). This represents a decrease in preconditioning time of six days as compared to the starvation technique. This agreed well with the findings of Chan et al. (1999) and Tan et al. (1993), however, the lower mean responses upon reaching the plateau stage contradicted the aforementioned results obtained by Riedel et al. (1990). One possible explanation for this stems from effluent toxicity. The presence of heavy metal ions in an effluent sample can have deleterious effects on the biocatalyst if exposed to them for extended periods of time (Kim and Kwon; 1999, Li and Tan, 1994b; Riedel 1998b). In particular, Ag^{+}, Cd^{2+}, Cu^{2+}, Hg^{2+}, and Mn^{2+} exhibited various levels of inhibition for different microorganisms. When Riedel et al. (1990) conducted their preconditioning experiment, the microorganisms were exposed to the effluent for a period of only 12 h. Extended exposure to effluent (such as in this study), while still inducing metabolic systems, may also have negative impacts manifested by decreased overall signals - especially if the sole biocatalyst is susceptible to effluent toxicity. The heavy metal concentrations in the effluent from the Thurso primary clarifier were not measured in this study.

The results obtained from the preconditioning test involving isolate SPT2 are also displayed in Figure 3.10. Interpretation of the result was difficult since the preconditioning time had increased by one day. Preconditioning of the SPT2 sensor in the presence of a 50 % effluent

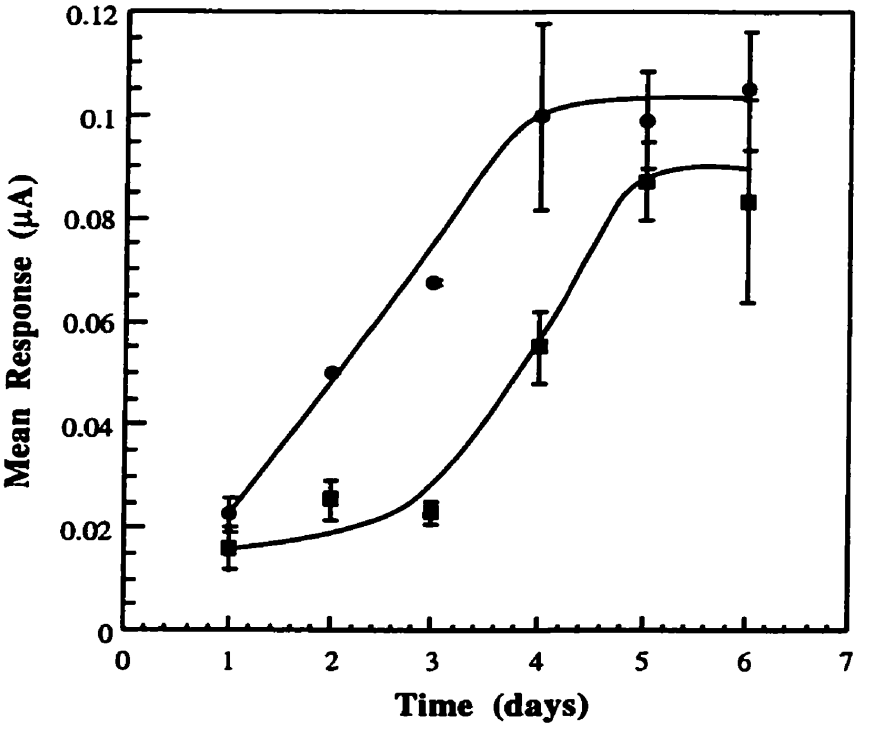

Figure 3.10 Reponse of isolates (—●—) SPT1 and (—■—) SPT2 to additions of 20 ppm BOD Thurso effluent after several days of preconditioning via 50 % effluent and 50 % 100 mM PBS buffer, pH 7.2 (+450 mV, 20 mM $K_3Fe(CN)_6$, 22 °C, error bars = standard deviation)

solution required five days, as opposed to the four days needed under starvation conditions. After a period of four days of incubation in 50 % effluent the mean response height was 0.056 μA. This corresponded very well with the result obtained for SPT2 after four days of preconditioning via starvation (i.e. 0.057 μA). The mean response obtained for day five increased to 0.088 μA, slightly higher than the response obtained after five days of starvation preconditioning (0.074 μA). A decrease in the mean signal was observed after the fifth day. These results indicated that the SPT2 isolate did not respond significantly to metabolic response induction via incubation with the target analyte.

The reasons behind why isolate SPT2 did not respond to direct induction were not clear. The difference could be related to the use of a mediated sensor as opposed to the oxygen-based electrodes used in previous investigations. For example, mediated sensors which use ferrocenes have been shown to require a preconditioning period that other mediated sensors do not (Cass et al., 1984; Gunasingham and Tan, 1990). However, considering isolate SPT1 did respond well to direct induction, the reasons behind the lack of response by SPT2 are probably related to it simply being another strain with other metabolic requirements.

3.3.4 pH Response Range

The optimal pH range for sensor operation was determined by measuring the responses of both isolates to 20 ppm (BOD) GGA under varying pH conditions (Figure 3.11). Measurements were taken over a pH range of 5 to 8 using 100 mM buffer concentrations. Considering these strains were isolated via acidic media (pH 3.5) both strains were expected to prefer pH conditions below the 7.2 used in previous measurements. Isolate SPT1 displayed a clear maximum current response at pH 6.0, and decreasing the pH from 6.0 to 5.0 resulted in decreased signals (i.e. 0.165 vs 0.044). The SPT2 sensor exhibited increased responses with decreasing pHs, suggesting that SPT2 was an acidophile. The maximum response for isolate SPT2 was obtained in PBS buffer at pH 5.0. Attempts to take measurements at lower pHs with buffers such as phthalate, acetate, and

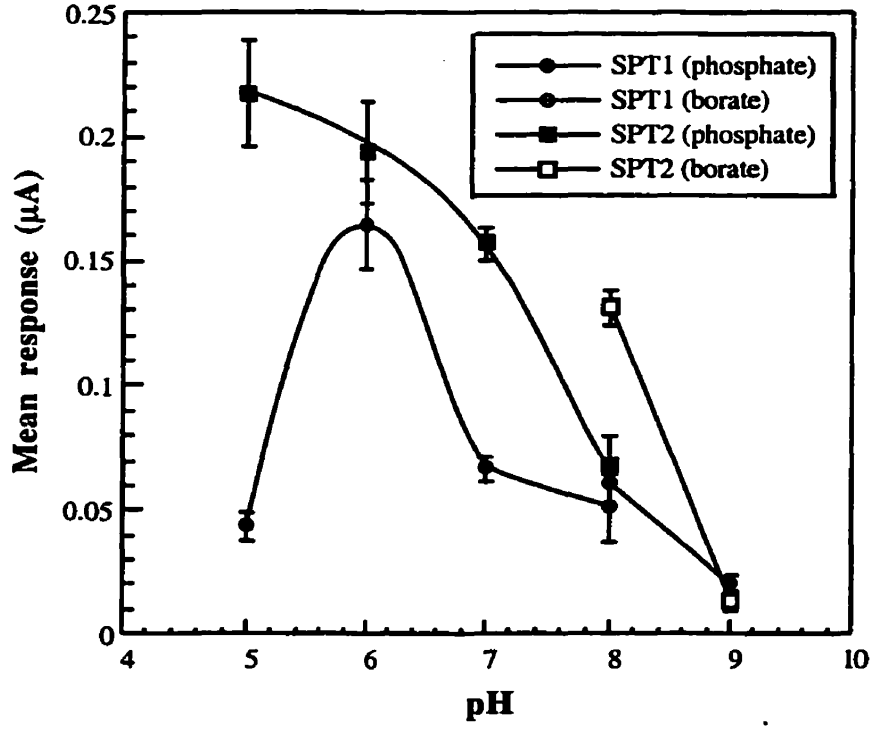

Figure 3.11 Reponse of isolates SPT1 and SPT2 to additions of 20 ppm GGA under varying pH conditions in 100 mM buffers (+450 mV, 20 mM $K_3Fe(CN)_6$, 30 °C, error bars = standard deviation).

citrate yielded no responses. For indeterminate reasons, SPT2 did not respond to substrate additions when buffers other than PBS were used in acidic conditions with potassium ferricyanide. Since the standard deviation at pH 5.0 overlapped the mean response at pH 6.0 (for the SPT2 sensor), the latter pH was chosen as the optimal pH for all measurements. This permitted us to maintain an equilibrium between the two sensors.

3.3.5 Temperature Response Range

The optimal operating temperature for both isolates was determined via responses to 20 ppm (BOD) GGA under varying temperature conditions. The temperature range used to monitor the responses for both strains ranged from 5 °C to 50 °C. A comparison of the temperature response ranges of the two isolates is presented in Figure 3.12. The maximum response height for isolate SPT2 was several times higher than that of SPT1 (about 7-fold higher). Since all conditions were identical for both strains, the higher response heights for SPT2 were presumed to be related to metabolic processes. Both isolates exhibited a maximum mean response at 40 °C. In the case of SPT1 there was a 6 % increase in mean response from 30 °C to 40 °C. Isolate SPT2 displayed a response increase of approximately 17 % from 30 °C to 40 °C. In both cases, there was an increase in the standard deviation in the responses with increasing temperature. Owing to increased standard deviations and the possibility that extended measurement periods at higher temperatures could lead to a shortened service life of the sensor, 30 °C was chosen as the optimal operating temperature.

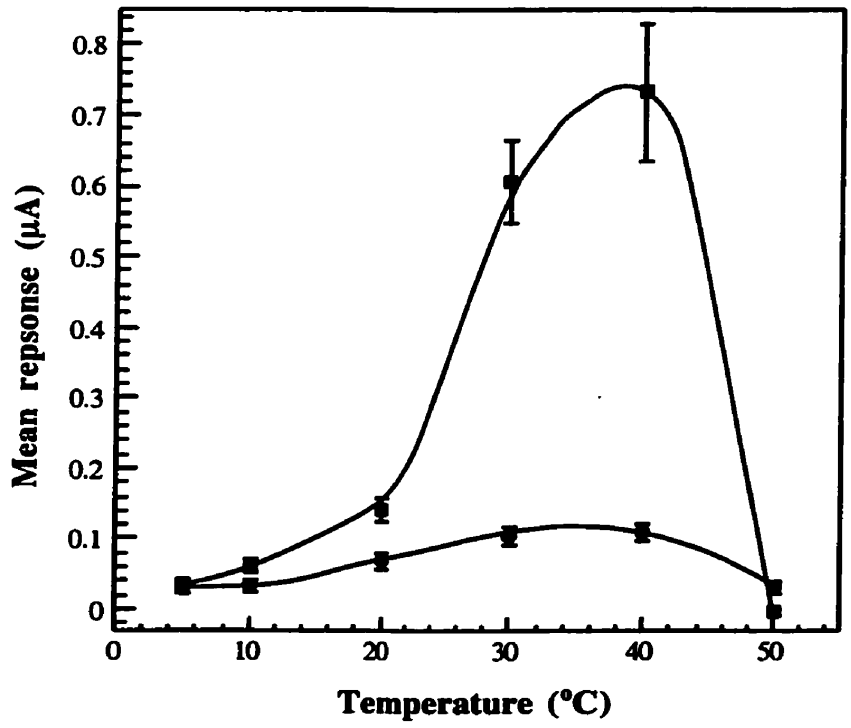

Figure 3.12 Reponse of isolates (—●—) SPT1 and (—■—) SPT2 to additions of 20 ppm GGA under varying temperature conditions (+450 mV, 20 mM $K_3Fe(CN)_6$, 100 mM PBS pH 7.2, error bars = standard deviation).

3.4 Standard Solution Calibration Curve

The responses to various concentrations of standard solution at a set potential was measured for both isolates using the only two mediators (potassium ferricyanide and HMF^+) which exhibited significant increases in oxidation currents during CV scanning.

3.4.1 GGA calibration using potassium ferricyanide

The mean response heights of the SPT1 and SPT2 sensors to various concentrations of GGA under optimal conditions (450 mV, 30 °C, pH 6.0, 20 mM $K_3Fe(CN)_6$, and 15 µL yeast load) are presented in Figure 3.13. Measurements were taken in sets of three using one electrode. The SPT1 sensor had a detection limit of 2 ppm (BOD), while the SPT2 sensor was able to respond to GGA concentrations as low as 0.5 ppm. The standard error of the mean at 10 ppm BOD was 10.1 % and 3.9 % for the SPT1 and SPT2 sensors, respectively. A clear distinction could be found in the mean responses to various concentrations of GGA between isolates SPT1 and SPT2 in that the responses of SPT2 were more robust. Not only is isolate SPT2 more sensitive to low concentrations of GGA, it also displayed a greater maximum response to GGA than SPT1 (i.e. ~600 vs ~200 ppm BOD, respectively). The mean response height of the SPT1 sensor to 600 ppm (BOD) GGA was 1.8 µA while the SPT2 sensor gave an 8.0 µA response to the same quantity of GGA. The reasons for this difference in response values has not been investigated but is likely related to differences in substrate assimilation capabilities.

Another important difference between the two isolates lies in their respective capacities to linearly assimilate GGA (Figure 3.14). The SPT2 sensor exhibited linearity to higher concentrations vs the SPT1 sensor (i.e. 200 vs 100 ppm BOD, respectively). The linear response range of the SPT2 sensor extended from 0.5 ppm to 200 ppm, while that of the SPT1 sensor extended from 2 to 100 ppm. This represents an 8-fold broader range for the SPT2 sensor. Linear regression analysis indicated that the response ranges of both sensors were highly significant. The coefficient of determination for the SPT1 based sensor was 0.998, while the SPT2

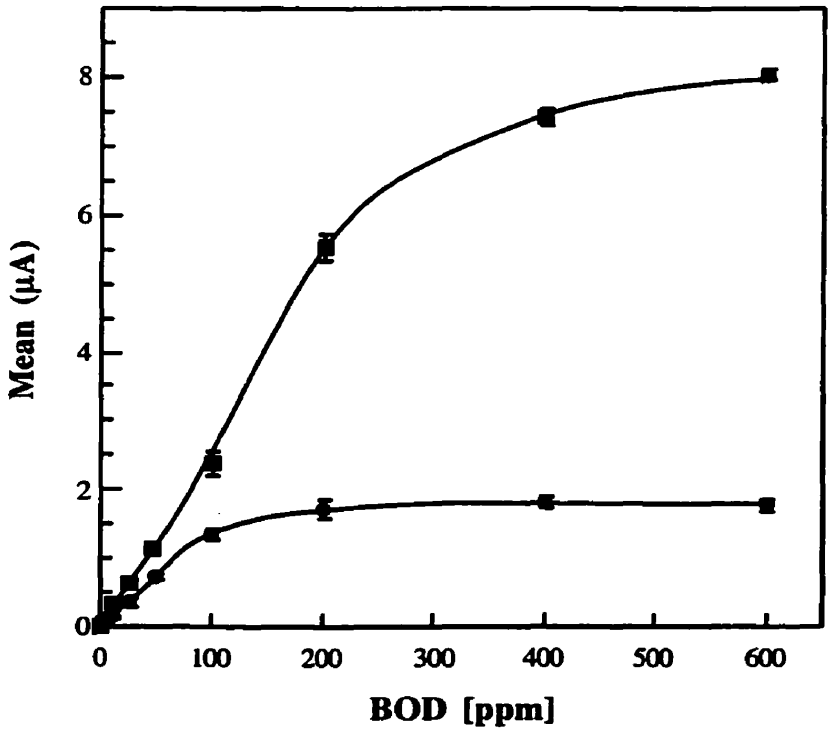

Figure 3.13 GGA calibration curve for isolates (—●—) SPT1 and (—■—) SPT2 using potassium ferricyanide as a mediator (+450 mV, 20 mM $K_3Fe(CN)_6$, 100 mM PBS pH 6.0, 30 °C, error bars = standard deviation).

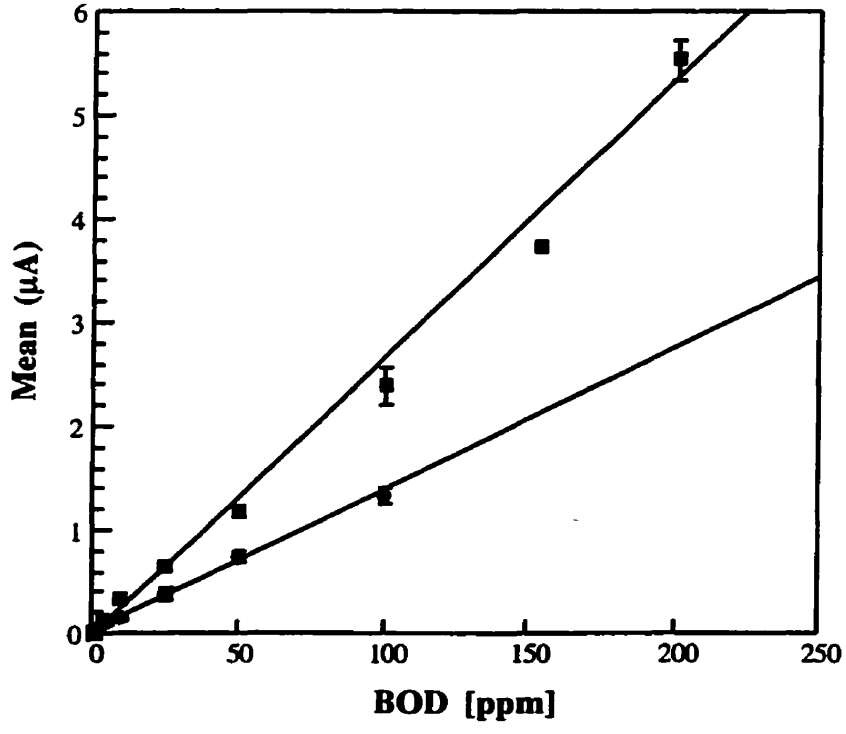

Figure 3.14 Linear response range to GGA for isolates (—●—) SPT1 and (—■—) SPT2 using potassium ferricyanide mediation (+450 mV, 20 mM $K_3Fe(CN)_6$, 100 mM PBS pH 6.0, 30 °C, error bars = standard deviation).

sensor had an R^2 value of 0.995 over the linear response range. The mean standard deviations throughout the linear range were fairly low for both SPT1 and SPT2 (0.038 and 0.055, respectively). These results compared very well with the findings of other authors (see Riedel, 1998). As previously mentioned, the average linear range for a standard solution was only 80 ppm (BOD) (Yang et al., 1996). Furthermore, using the mediated sensor system has enabled these new isolates to detect very low concentrations of GGA. The sensor using isolate SPT2 is able to detect GGA concentrations as low as those obtained by Yang et al. (1996), and was as sensitive as the BOD sensor developed by Chee et al. (1999).

3.4.2 GGA calibration using hydroxymethylferricinium

The potential of using HMF^+ as an electron mediator was demonstrated in the cyclic voltammetric investigations. Owing to a substantially lower anodic peak, measurements involving HMF^+ were taken at + 285 mV as opposed to + 450 mV for potassium ferricyanide. The lower operating potential of HMF^+ offered the possibility of decreased interference from electrochemically active compounds in effluent samples.

The calibration curves of both strains using HMF^+ are shown in Figure 3.15. Both sensors exhibited substantially decreased signals as compared to responses using potassium ferricyanide mediation. This was likely a consequence of the ~25-fold lower V_{max} exhibited with HMF^+ mediation. As with the measurement using potassium ferricyanide, the SPT2 sensor exhibited a much more robust response to the various GGA concentrations then the SPT1 sensor. The response plateau of SPT1 was reached at a substrate concentration of 300 ppm (BOD). At this concentration the mean response of SPT1 was only 0.26 μA. The SPT2 sensor attained the point of substrate saturation at a concentration of 250 ppm (BOD) of GGA (note that a 300 ppm measurement was not taken). At this concentration, the mean response height of the SPT2 sensor was 1.85 μA. This represents a seven-fold higher current then SPT1, suggesting that SPT2 is capable of more sensitive substrate utilization. Considering these trends were persistent for both of

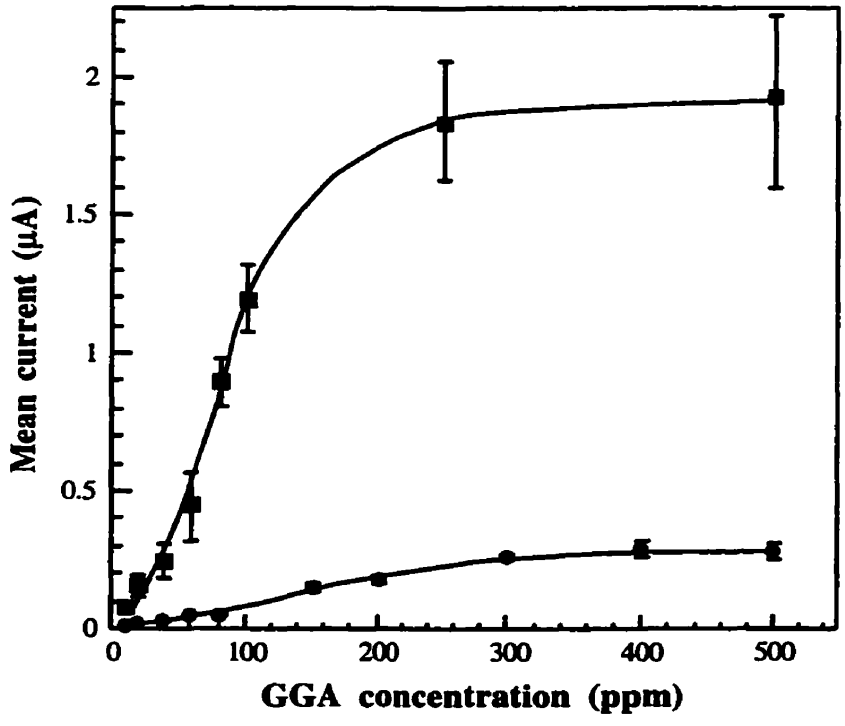

Figure 3.15 GGA calibration curve for isolates (—●—) SPT1 and (—■—) SPT2 using hydroxymethylferricinium as a mediator (+285 mV, 3 m HMF^+, 100 mM phthalate pH 6.0, 30 °C, error bars = standard deviation).

the mediators used in this study, the higher level of responsiveness exhibited by isolate SPT2 could not be an artifact of mediator specificity but a consequence of metabolic processes.

Both isolates exhibited a higher detection limit of 10 ppm with HMF^+ mediation. This represented a 5 to 20-fold decrease in sensitivity as compared to potassium ferricyanide (section 3.4.1). Although 3 mM HMF^+ was optimal, a possible cause for decreased signal responses was related to the heterogenous electron transfer reaction between hydroxymethylferrocene and hydroxymethylferricinium at the surface of the electrode. Such a problem could not be alleviated owing to the mediator solubility problems alluded to earlier. The standard error of the mean was 9.7 % and 21.0 % for the SPT1 and SPT2 sensors, respectively.

The linear response ranges of the sensor with HMF^+ mediation is given for both strains in Figure 3.16. The SPT1 sensor exhibited a higher upper range of linearity with HMF^+ (i.e. 200 vs 100 ppm BOD). However, the relative mean response was 20-fold lower (i.e. 0.146 μA vs 0.007 μA at 10 ppm BOD) and total linearity was decreased 2.5-fold from potassium ferricyanide mediation. The coefficient of determination was slightly lower (R^2 =0.973) but was still considered significant. For the SPT2 sensor, the upper range of linearity was shortened from 200 ppm (BOD) to 100 ppm (BOD) with HMF^+ mediation. Furthermore, the total linear response range was decreased 40-fold. The coefficient of determination was considered insignificant (R^2 =0.906).

Clearly, in comparison to strain SPT1, isolate SPT2 was not as well suited to HMF^+. A similar phenomenon was observed by Kaláb and Skládal (1994) who reported that certain mediators function better with some microorganisms than with others. The reasons for this were not discussed, however, variable responses to mediators by different microorganisms could be attributed to the different modes and sites of mediator interactions. While potassium ferricyanide reduction via interaction with NADPH dehydrogenase (at the electron transport chain) has been shown to occur, the mode of HMF^+ reduction is not known (Atkinson and Haggett 1993; Rawson et al., 1989). Decreased signals and poorer linearity for the SPT2 sensor may have been a consequence of morphological differences in areas such as the plasma membrane.

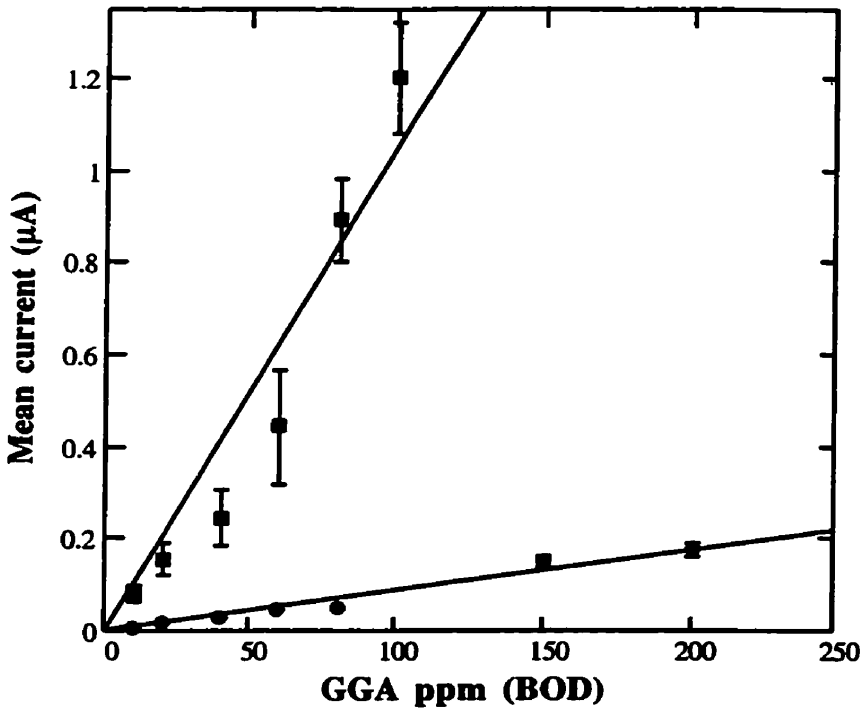

Figure 3.16 Linear response range of isolates (—●—) SPT1 and (—■—) SPT2 to GGA using hydroxymethylferricinium as a mediator (+285 mV, 3 mM HMF^+, 100 mM phthalate pH 6.0, 30 °C, error bars = standard deviation).

3.5 Measurement of Pulp Mill Effluent

3.5.1 Linear response range to Thurso effluent

The focus of this study was to design a mediated sensor applicable for measuring the multiple changes in BOD concentration in pulp mill effluent over time. Initial cooperation with a pulp mill in Thurso, Québec, led to the availability of abundant supplies of effluent from that mill. Consequently, effluents from the Thurso site were used for the determination of the linear response range using real samples. Since the two strains were isolated from Thurso effluent, they were considered to be best adapted to the carbon sources available in effluent from Thurso.

Wastewater emanating from the secondary treatment facility was initially used for sensor BOD measurement. This measurement was desirable since it was effluent in this form that was released into the environment, potentially resulting in elevated BOD levels in the Ottawa river. Unfortunately, the BOD of effluents emanating from the secondary treatment facility was too low to allow for measurement via the current system design. The effluent from the primary clarifier was subsequently used instead as it was possible to dilute these samples for measurement. A BOD_5 measurement determined that the sample used in this study had a BOD_5 value of 229 ppm. All measurements were taken under optimized conditions with both potassium ferricyanide and HMF^+.

Linear response range using potassium ferricyanide mediation

The linear response range for sensor BOD measurements of the Thurso primary clarifier effluent using potassium ferricyanide are presented in Figure 3.17. The sensor containing strain SPT1 was able to measure BOD concentrations down to 2 ppm, while the SPT2 sensor could measure the effluent at a BOD as low as 1 ppm. The standard error of the mean was 3.6 % and 14.3 % for the SPT1 and SPT2 sensors, respectively. There existed a tendency for increasing standard deviations with increasing BOD concentrations. At 2 ppm (BOD) the standard

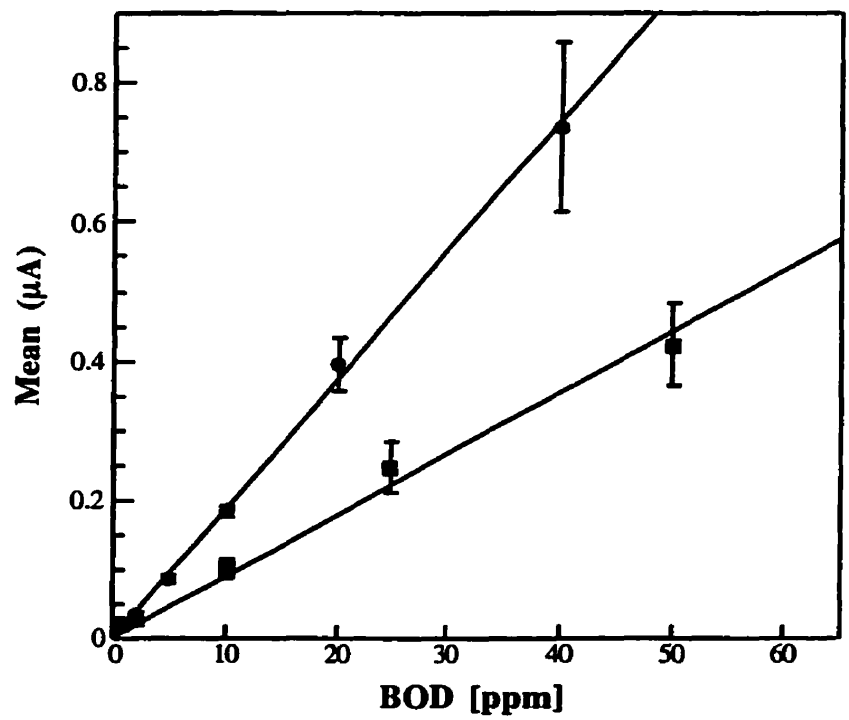

Figure 3.17 Linear response ranges of (—●—) SPT1 and (—■—) SPT2 sensors to pulp mill effluent from Thurso using potassium ferricyanide mediation (+450, 20 mM $K_3Fe(CN)$, 100 mM PBS pH 6.0, 30 °C, error bars = standard deviation).

deviations were 0.007 and 0.004 for the SPT1 and SPT2 sensors, respectively. These values increased to 0.122 for SPT1 and 0.061 for SPT2 at the upper limits of the respective measurements. Both sensors exhibited excellent coefficients of determination of 0.998 and 0.988 for SPT1 and SPT2, respectively. Despite the more robust nature of isolate SPT2, it was not a surprise to find a better correlation coefficient and higher mean responses from the SPT1 sensor. As determined by the carbon assimilation tests (Tables 3 and 4), strain SPT1 has a much broader substrate assimilation range than SPT2. Several authors (Racek, 1995; Riedel et al., 1998; Sangeetha et al., 1995) have commented that a good BOD sensor should incorporate a microorganism strain possessing a broad substrate spectrum as this allows for the increased probability that the microorganism will be able to assimilation the numerous compounds present in wastewaters. A similar observation was obtained here.

Linear response range using hydroxymethylferricinium mediation

The linear response ranges to Thurso primary clarifier effluent for sensors using HMF^+ mediation are presented in Figure 3.18. The results from these experiments were similar in nature to those obtained with sensors using potassium ferricyanide in that sensors incorporating isolate SPT1 had higher overall mean responses and a longer response range then when SPT2 was used as the biocatalyst. This finding was once again in agreement with the observations of other researchers regarding biocatalyst substrate spectra (Racek, 1995; Riedel et al., 1998; Sangeetha et al., 1995). The SPT1 sensor had a linear range extending from 10 - 60 ppm (BOD). When SPT2 was used as the biocatalyst, the linear response range for the effluent extended from 5 ppm (BOD) to only 20 ppm (BOD). The standard error of the mean was 13.3 % and 22.2 % for the SPT1 and SPT2 sensors, respectively. The standard deviations values for both sensor measurements was low, however, this was a consequence of very low overall responses likely due to weak mediation. At 10 ppm, SPT1 and SPT2 sensors using HMF^+ mediation had responses approximately 10 and

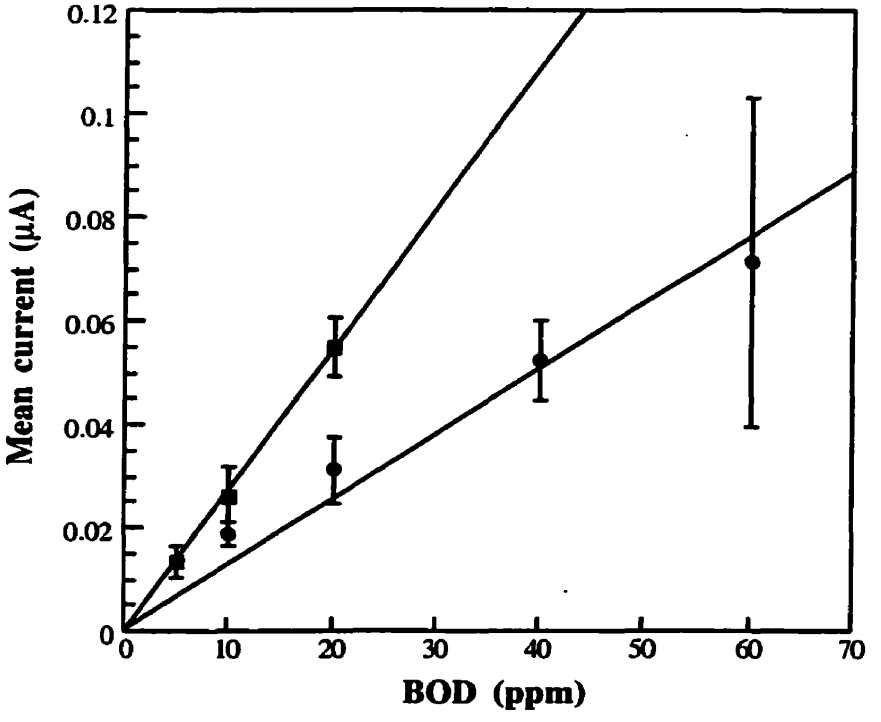

Figure 3.18 Linear response range of isolates (—●—) SPT1 and (—■—) SPT2 to primary effluent from Thurso using hydroxymethylferricinium as a mediator (+ 285 mV, 3 mM HMF^+, 100 mM phthalate pH 6.0, 30 °C, error bars = standard deviation).

4-fold lower than measurements taken with potassium ferricyanide mediation (for the SPT1 and SPT2 sensors, respectively). This could again be a consequence of slow mediator cycling owing to diffusional problems caused by low mediator concentrations, as was perhaps the case with GGA measurement (section 4.2). The coefficients of determination with HMF^+ also were not as good as with potassium ferricyanide mediated sensors. The SPT1 sensor had an R^2 value of only 0.939 while sensors using SPT2 had a better R^2 value of 0.996 (although this could be an artifact caused by a low sample number).

3.5.2 Measurement of various effluents

Due to the complexity and the ever changing nature of pulp mill effluents (Dence and Reeve, 1996; Lloyd et al., 1997; Suntio et al., 1988), a BOD sensor must have a wide substrate spectrum. Several authors have shown such sampling flexibility with their BOD sensors. The BOD concentration of various municipal wastewaters (Marty et al., 1997; Princz and Olah, 1990; Tan et al., 1992) along with factory effluents (Li and Chu, 1991; Princz and Olah, 1990; Riedel et al., 1990; Sangeetha et al., 1995) were successfully monitored with oxygen based biosensors. A practical BOD sensor for pulp mill effluents should exhibit good reproducibility with changing effluent composition. Consequently, the universality of the mediated BOD sensor was examined.

Effluent samples from a total of eight different mills were analyzed. In all measurements, potassium ferricyanide was used since it displayed better properties than HMF^+. All parameters were optimized. Preconditioning of all sensors was done via the starvation technique to avoid any possibility of reduced signals due to effluent toxicity, and to prevent skewed results due to habituation to any one specific substrate.

Reproducibility within effluent samples

The sensor reproducibility results were based on a minimum of four 200 µL measurements of each effluent sample. The mean response heights for each effluent were correlated to a sensor

BOD value by means of the slope formulae obtained from the GGA calibration plots for each respective sensor. Response times for these measurements ranged from approximately 5 to 10 min for both sensor types. Bar graphs of the response reproducibility for the various effluents are presented in Figures 3.19 and 3.20 for the SPT1 and SPT2 sensors, respectively. The mean sensor BOD value for each effluent is given along with the upper and lower confidence intervals. Both the SPT1 and the SPT2 sensors had excellent response reproducibility for each effluent sample. The measurements within effluent samples for both sensors never deviated more than 1 ppm (BOD) except for the SPT1 sensor measurement of the Beaupré sample (1.2 ppm). The standard deviation in response signals from the sensor incorporating isolate SPT1 had a maximum standard deviation of 0.071 for the effluent sample from Thurso and a minimum standard deviation of 0.040 for the effluent originating from Madawaska. Response reproducibility between three electrodes was also determined for the SPT1 sensor only. The sensor responses had a standard error of the mean of 10.6 % when 20 ppm BOD Thurso effluent were measured.

The standard deviations in the responses were higher for the sensor using SPT2 as the biocatalyst. The maximum standard deviation for the SPT2 sensor was observed for the Beaupré sample (0.265), while the least amount of standard deviation came from the Madawaska sample (0.022). Overall, the SPT1 sensor exhibited higher responses then the SPT2 sensor to the various effluents. The SPT1 sensor responses correlated to roughly 5 ppm (BOD) (except for the Beaupré sample), while the SPT2 sensor gave responses ranging from under 1 ppm to nearly 6 ppm (BOD). Once again, the differences in response heights could be related to the metabolic capacities of each isolate. These differences were examined in the correlation of sensor BOD to the BOD_5 value.

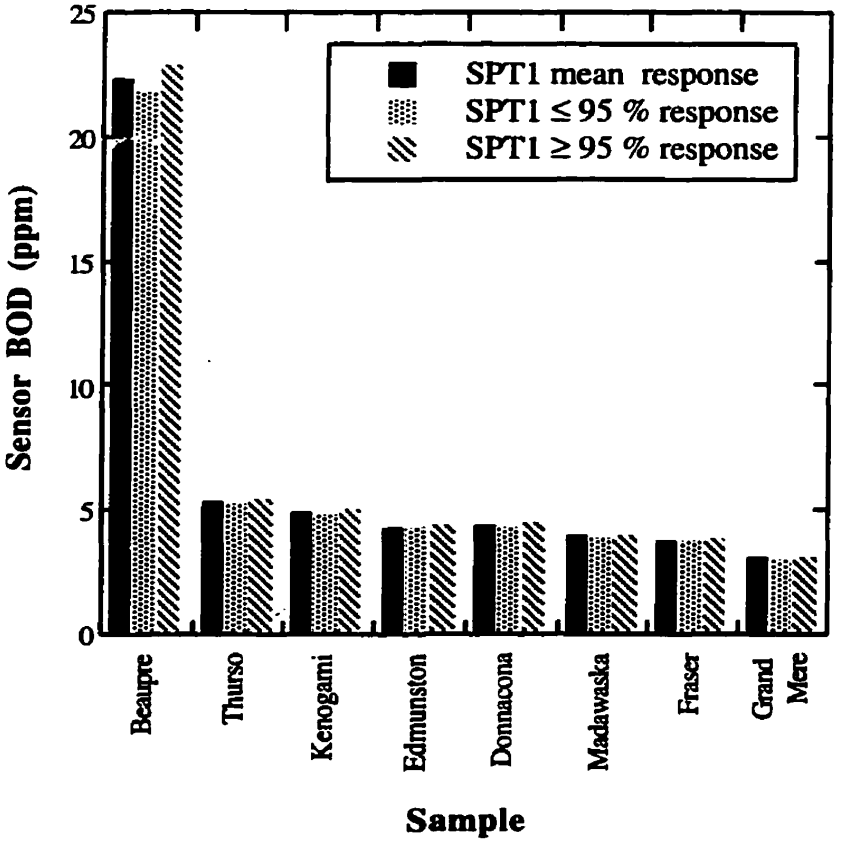

Figure 3.19 Response reproducibility of the SPT1 sensor to 200 µL additions of eight different effluents showing the upper and lower 95% confidence interval limits (+450 mV, 20 mM $K_3Fe(CN)_6$, 100 mM PBS pH 6.0, 30 °C).

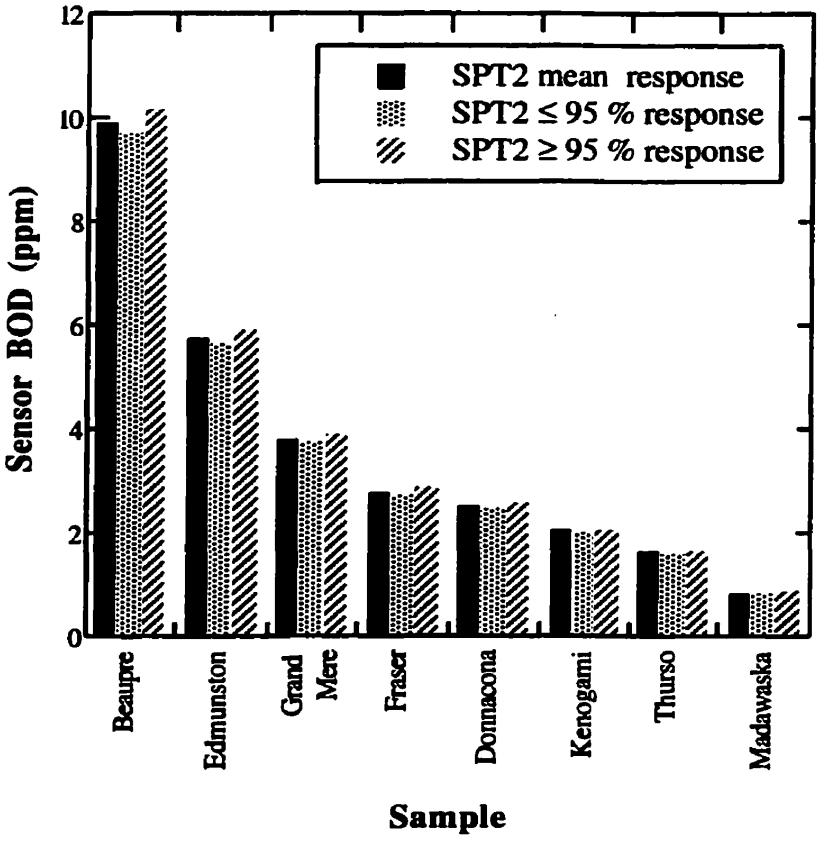

Figure 3.20 Response reproducibility of the SPT2 sensor to 200 µL additions of eight different effluents showing the upper and lower 95 % confidence interval limits (+450 mV, 20 mM $K_3Fe(CN)_6$, 100 mM PBS pH 6.0, 30 °C).

Correlation of sensor BOD value with BOD_5 value

For the validation of the results obtained in Figures 3.19 and 3.20 with respect to true BOD values, as measured by the standard BOD_5 test, a percent correlation was calculated (Table 3.6). Between the two sensors, only the SPT1 sensor was able to yield a measurement with less than 10 % error. The best correlation (103 %) was found with the SPT1 measurement of the sample from the Fraser mill. The only other effluent measurement which resulted in sensor BOD value correlations close to the standard BOD_5 values came from the Thurso effluent which had a 116 % correlation. The correlations for all other effluents were either too high (Beaupré, Kenogami, Madawaska) or too low (Donnacona, Edmunston, Grand Mère) to be significant.

Possible reasons for why the SPT1 sensor yielded results up to almost 200 % (Beaupré) those of the BOD_5 values were not clear. The responses were initially attributed to electrochemical interferences, however, examination of the data (not shown) obtained from the negative controls refuted this notion. When these effluents were measured using a bare electrode (i.e. no cells only a dialysis membrane) the responses to 200 µL effluent injections were negligible in comparison to those obtained in the presence of viable cells. Given that the microorganisms on an electrode have only minutes to assimilate a complex combination of substrates present in the effluent, while the standard BOD_5 test involved many microbial species over a period of five days, the reasons behind why some sensor responses were higher than the BOD_5 responses has not been determined. A similar phenomenon was encountered by Lehmann et al. (1999) with their *A. adeninivorans* -based biosensor. They suggested that the exceptionally large substrate range of *A. adeninivorans* caused it to be hypersensitive to substrates (as compared to the mixed populations used in the BOD_5) resulting in higher sensor values. This hypothesis was consistent with the findings in this study, where the SPT1 (wit h its greater substrate spectrum) sensor exhibited higher sensor - BOD_5 ratios than the SPT2 sensor. Another important factor demonstrated by Liu et al. (2000) was that sensor - BOD_5 ratios can be dependant upon the composition of the standard solution used for calibration. They argued that when GGA is used as the standard solution over-estimations of BOD values will

Table 3.6 Correlation of sensor BOD and BOD_5 values for various effluents

SPT1 sensor (95% confidence interval, slope* = 0.0137)

Sample	Sensor BOD	BOD_5 (ppm)	Ratio (%)
Beaupre	22.3	11.3	198
Donnacona	4.38	7.68	57.0
Edmunston	4.33	7.00	61.8
Fraser	3.78	3.68	103
Grand Mere	3.06	13.3	23.0
Kenogami	4.88	3.50	140
Madawaska	3.93	3.00	131
Thurso	5.32	4.58	116

SPT2 sensor (95% confidence interval, slope* = 0.0267)

Sample	Sensor BOD	BOD_5 (ppm)	Ratio (%)
Beaupre	9.91	11.3	87.8
Donnacona	2.52	7.68	32.9
Edmunston	5.76	7.00	82.3
Fraser	2.79	3.68	75.7
Grand Mere	3.81	13.3	28.7
Kenogami	2.03	3.50	58.0
Madawaska	0.83	3.00	27.7
Thurso	1.62	4.58	35.3

* The slopes were obtained from the linear response ranges to standard solution (Fig. 3.14) and used to calculate the sensor BOD values (n=5).

occur. This is due to the sudden stimulation of several distinct metabolic pathways required for the assimilation of the complex mixture of substrates in wastewaters. It was suggested that using synthetic wastewater was more appropriate for sensor calibration as it contained numerous compounds and did not lead to sensor BOD over-estimations.

Readings obtained with the SPT2 sensor were consistently lower then the values yielded by the conventional BOD_5 method as also seen with the sensor developed by Tan et al. (1992). This was not unexpected given the results obtained for the linear response range of the Thurso effluent. If the criterion given by Riedel et al. (1998) is applied here (in which a 30 % deviation is permitted), then three mill samples (Beaupré, Edmunston, and Fraser) were in good agreement with their BOD_5 values. The SPT2 sensor also demonstrated low correlations with the Donnacona and Grand Mère samples. This was probably related to the lack of easily assimilable compounds in these samples. The relatively poor correlation between the SPT2 sensor and BOD_5 value for the Thurso effluent was a surprise considering strain SPT2 was isolated from that effluent.

As Princz and Olah (1990) concluded, BOD values obtained by these two different methods could not be compared unless the sensor can be calibrated in a standard solution identical in nature to the given sample. Considering the complex and variable nature of pulp mill effluent composition, this is not normally possible. As stated earlier, the inherent differences between sensor and BOD_5 measurement dictates that results from either method were not truly comparable (Marty et al., 1997; Praet et al., 1995; Riedel, 1998; Tanaka et al., 1994). In view of this, one must question the importance of good sensor to BOD_5 correlations. What is likely to be far more important is the consistency of the correlation ratios. It is of interest to investigate the response of the sensor described here to different samples from one specific mill.

3.6 Determination of Biosensor Operating Life

Tanaka et al. (1994) pointed out that it is important that biocatalysts selected for sensor BOD measurements exhibit high stability. Variations in microbial responses to measurement stimuli

can cause misleading measurements of BOD values. For this reason, it is essential that sensor response stability is measured over time in order to determine its reproducibility. The stability of the mediated microbial sensors and the reproducibility of the results were examined over the lifetime of the sensors (Figure 3.21). Sensors were stored in 100 mM PBS buffer, pH 6.0 and kept at ambient temperatures (22 °C). Storage conditions also included 20 ppm BOD of GGA between measurements to reduce the possibility of cell death due to extended periods of starvation. A minimum of three measurements were taken per day. The low readings obtained for both sensors for the first few days can be attributed to the required preconditioning period. Maximum responses were obtained by day 7 and 6 for the SPT1 and SPT2 sensors, respectively. Both sensors failed to establish extended periods of stability. The SPT1 sensor displayed a steadily increasing mean response until day 12, whereupon it began to gradually decrease until day 22. The fluctuation in response over this 10 day period was greater than 25 %. The SPT2 sensor failed to produce a clearly definable response plateau throughout the 14 day measurement period. It is believed that the poor reproducibility exhibited by the two sensors is a reflection of cell loss rather than a decreased metabolism or cell death. A gradual reduction in the thickness of the yeast paste on the electrodes was observed over time. This cell leakage was attributed to micro tears in the dialysis membrane retaining the yeasts. The lack of data points after day 22 and 14 for the SPT1 and SPT2 sensors, respectively, was due to complete cell loss due to large tears in the dialysis membrane. The tears were attributed to deterioration over time. A similar problem was noted by Liu et al. (2000) when using the same dialysis membrane. It was observed that sensor stability decreased over time due to erosion of the cellulose dialysis membrane. This was attributed to enzymes secreted by the biocatalyst, and microorganisms contaminating the surface of the dialysis membrane.

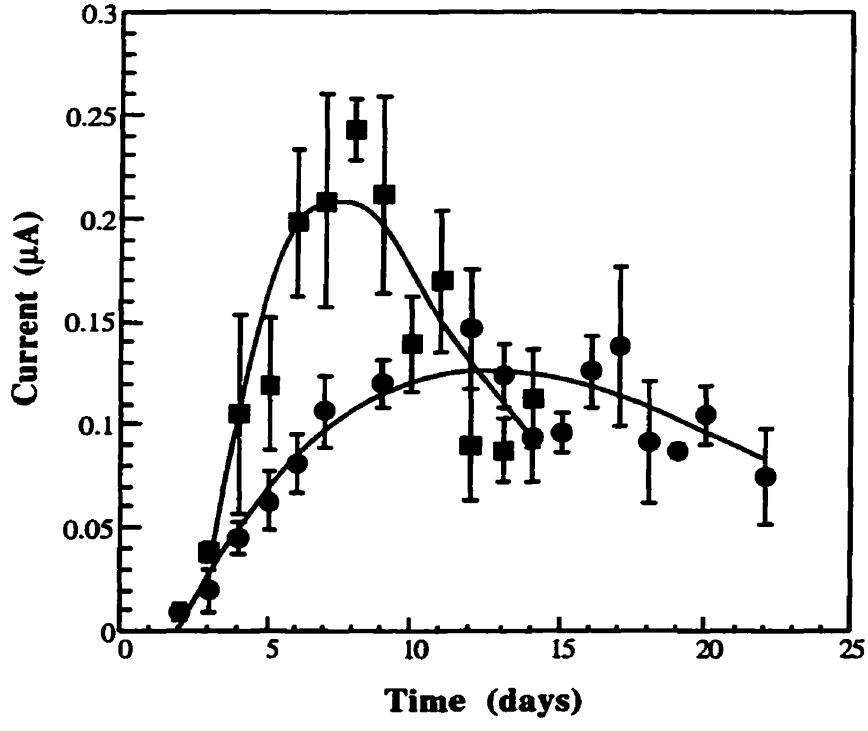

Figure 3.21 Response stability of (—●—) SPT1 and (—■—) SPT2 sensor over several days in optimal conditions (+450 mV, 20 mM $K_3Fe(CN)_6$, 100 mM PBS pH 6.0, 30 °C). Error bars represent standard deviations.

CONCLUSION

The results gathered in this study to determine the feasibility of using a mediator based biosensor to effectively measure the BOD concentration of wastewater showed that it is possible to obtain both sensitive measurements and practical linear response ranges using a mediated biosensor rather than an oxygen based biosensor. BOD measurements of real samples could be determined in 5 to 10 min, compared with the conventional 5 days required for the standard BOD test. Using potassium ferricyanide as a mediator, two yeast strains (SPT1 and SPT2, respectively) isolated from pulp mill effluent and retained on a glassy carbon electrode, exhibited low detection limits of 2 ppm to 0.5 ppm BOD for the GGA standard solution, and 2 to 1 ppm for a pulp mill effluent originating from Thurso, Québec. The SPT1- and SPT2-based sensors also exhibited linear response ranges of up to 200 ppm BOD for glucose-glutamic acid (GGA) using either hydroxymethylferricinium or potassium ferricyanide as mediators. Highly reproducible (<10 % error) measurements of pulp mill and paper mill samples were obtained, however, the sensor BOD value and the BOD_5 value generally resulted in poor correlations, indicating that the two measuring systems are not entirely comparable. Since the biosensors developed here measured substrate metabolism rather than the change in oxygen concentration, they are not true BOD sensors - which may have contributed to the poor comparison. Presently, stability over time continues to be the main problem of these mediated BOD sensors. Based on the results obtained in this study, it is concluded that SPT2 is the most suitable biocatalyst for pulp mill wastewater analysis. This conclusion is based on the broader linear response range and lower detection limit exhibited by the SPT2 sensor, along with the uncertainty attached to the excessively high sensor BOD / BOD_5 correlation values for the SPT1 sensor.

Characterization via traditional (physiological and biochemical) and fatty acid methods, as well as 18S rDNA sequence analysis, narrowed the identity of SPT1 to a member of the *Candida* genus. Sequence comparison, via BLAST searches, to the newly classified yeast *C. sojae*

indicated that SPT1 was most closely related to it (99.8 %). This observation was supported, via bootstrap (1000 x) analysis, by the formation of a highly significant (95.6 %) subcluster comprised of SPT1 and *C. sojae*. However, a single basepair (bp) mismatch along with important biochemical differences (the ability of SPT1 to ferment maltose and grow at 40 °C) suggested that SPT1 is a previously uncharacterized species of the *Candida* genus. Sequencing of the internal transcribed spacer region would better delineate SPT1 at the species level.

The three characterization techniques did not yield comparable results for SPT2. BLAST searching revealed that SPT2 was most closely matched to *Candida krusei*, but with only 76 % sequence homology. Furthermore, when the sequences of the closest matches from these characterization schemes were used to create a dendrogram, SPT2 did not cluster with any of them. These observations led to the conclusion that SPT2 is a previously uncharacterized species, perhaps belonging to a novel genus. To confirm that SPT2 is a novel species, and to create a more accurate phylogenetic tree, the complete 18S rDNA gene must be sequenced. Furthermore, to achieve accurate taxonomic conclusions based on tree topologies, a reasonable number of differentially related species must be compared.

Further investigations into the applicability of mediated BOD sensors in place of oxygen based sensors should first concentrate on improving the biocatalyst retention mechanism. For example, a sturdier dialysis membrane should be employed as this may allow for improved sensor stability. The reproducibility of sensor response to effluent samples, from the same mill, with varying BOD concentrations should be determined to establish the overall reproducibility of the sensor to one effluent type. The universality of the sensor can then be determined by measuring response reproducibility to different mill effluents.

Another obstacle that needs to be studied is the extended preconditioning time required for mediated BOD sensors. It is possible that a different mediator-microorganism combination, or preconditioning technique, could decrease preconditioning times. Improved sensitivity may be achieved via better mediator-biocatalyst combinations. It must be stressed that finding a suitable

combination is quite difficult given the large number of potential mediators an the even bigger pool of microorganisms. An improved understanding of the cellular enzymes responsible for mediator reduction may facilitate mediator-microorganism selection. This would likely entail detailed knowledge of the outer membrane structures of potential biocatalysts.

For circumstances that call for low limits of detection, a platinum electrode may be substituted for the glassy carbon electrode used here. The relatively high concentrations of potassium ferricyanide that were required suggest that increased sensitivity could be attained if a more efficient mediator was used. Comparisons should be made of the mediated biosensors developed in this study with those containing proven biocatalysts (i.e. *T. cutaneum, A. adeninivorans*) to obtain more detailed information on their relative performances. In all cases, the toxicity of the mediator to the biocatalyst should be monitored. Future sensor comparisons should involve wastewaters similar to those tested in other studies (i.e. from the food industry). There is also a need to devise a mediated BOD sensing method that would allow for direct analysis of wastewater emanating from treatment facilities. The low detection limit of the biosensors developed here should in theory allow for the monitoring of the usually low BOD loads found in secondary treatment effluents. These future studies should not ignore the potential benefits of using cyclodextrin in combination with whole cells. A more detailed analysis to better understand its feasibility is required.

Wastewater monitoring should ideally be done continuously to control accidents causing high BOD wastewater discharges. Due to potential mediator and effluent toxicity, a flow injection system, with measurements at regular intervals, would best suit the biosensors developed here. Important adaptations for flow injection analysis would include the yeast retention mechanism as well as the mediator delivery mechanism.

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