Ex. 1032 Guarna 1997

Volume 56, Number 3, November 5, 1997

Biotechnology and Bioengineering



ISSN 0006-3592

WILEY
Publishers Since 1807

Biotechnology and Bioengineering

Editor
Douglas S. Clark
Department of Chemical Engineering, University of California
201 Gilman Hall, Berkeley, CA 94720-1462

Associate Editors

James D. Bryers
Montana State University
Bozeman, Montana

Pauline Doran
University of New South Wales
Sydney, Australia

Jonathan S. Dordick
University of Iowa
Iowa City, Iowa

George Georgiou
University of Texas
Austin, Texas

Jeffery A. Hubbell
ETH—Zürich and
University of Zürich
Zürich, Switzerland

Maria-Regina Kula
Heinrich-Heine Universität
Düsseldorf
in der KFA Jülich, Germany

Lee R. Lynd
Dartmouth College
Hanover, New Hampshire

Eleftherios T. Papoutsakis
Northwestern University
Evanston, Illinois

John Villadsen
Technical University of Denmark
Lyngby, Denmark

Editorial Board

James E. Bailey
ETH–Zürich
Zürich, Switzerland

Harvey W. Blanch
University of California
Berkeley, California

Isao Karube
University of Tokyo
Tokyo, Japan

Malcolm D. Lilly
University College
London, United Kingdom

Bernhard Ø. Palsson
University of California at
San Diego
La Jolla, California

Michael L. Shuler
Cornell University
Ithaca, New York

Daniel I.C. Wang
Massachusetts Institute of
Technology
Cambridge, Massachusetts

Founding Editor: Elmer L. Gaden, Jr., *University of Virginia, Charlottesville, Virginia*

Biotechnology and Bioengineering (ISSN: 0006-3592) is published 24 times a year, semimonthly, by John Wiley & Sons, Inc., 605 Third Avenue, New York, NY 10158.

Copyright © 1997 John Wiley & Sons, Inc. All rights reserved. No part of this publication may be reproduced in any form or by any means, except as permitted under section 107 or 108 of the 1976 United States Copyright Act, without written permission of the publisher, authorization through the Copyright Clearance Center, 222 Rosewood Drive, Danvers, MA 01923, 508-750-8400, fax (508) 750-4470. Periodicals postage paid at New York, NY, and at additional mailing offices.

The code and copyright notice appearing at the bottom of the first page of an item in the journal indicates the copyright holder's consent that copies may be made for personal or internal use, or for the personal or internal use of specific clients, on the condition that the copier pay for copying beyond that permitted by Sections 107 and 108 of the United States Copyright Law. The per copy fee for each item is to be paid through the Copyright Clearance Center. This consent does not extend to other kinds of copying, such as copying for general distribution, for advertising or promotional purposes, for creating new collective works, or for resale. Such permission requests and other permission inquiries should be addressed to the Permissions Dept.

Subscription Price (Volumes 53–56, 1997): $2204.00 in US, $2,444.00 in Canada and Mexico, $2,600.00 outside North America. All subscriptions outside US will be sent by air. Personal rate: $445.00 in US, and $589.00 outside North America. Subscriptions at the personal rate are available to individuals. Payment must be made in US dollars drawn on a US bank. Claims for undelivered copies will be accepted only after the following issue has been received. Please enclose a copy of the mailing label. Missing copies will be supplied when losses have been sustained in transit and where reserve stock permits. Please allow four weeks for processing a change of address. For subscription inquiries, please call (212) 850-6645, E-mail: SUBINFO@wiley.com

Postmaster: Send address changes to Biotechnology and Bioengineering. Susan Swanson, Director, Subscription Fulfillment and Distribution, John Wiley & Sons, Inc., 605 Third Avenue, New York, NY 10158.

Advertising Sales: Inquiries concerning advertising should be forwarded to Susan Levey, Advertising Sales, John Wiley & Sons, Inc., 605 Third Avenue, New York, NY 10158; (212) 850-8832. Advertising Sales, European Contact: Michael Levermore, Advertising Manager, John Wiley & Sons, Ltd., Baffins Lane, Chichester, Sussex PO19 1UD, England.

Reprints: Reprint sales and inquiries should be directed to the Customer Service Department, John Wiley & Sons, Inc., 605 Third Avenue, New York, NY 10158. Tel: (212) 850-8776.

Manuscripts should be submitted to the B&B Editor, Professor Douglas S. Clark, Dept. of Chemical Engineering, University of California, 201 Gilman Hall, Berkeley, CA 94720-1462.

All Other Correspondence: Address to Biotechnology and Bioengineering, Publisher, Interscience Division, Professional, Reference, and Trade Group, John Wiley & Sons, Inc., 605 Third Ave., New York, NY 10158.

Information for Contributors appears in the first and last issue of each volume. The contents of this journal are indexed or abstracted in Biological Abstracts, BIOSIS, Chemical Abstracts, Chemical Titles, Current Contents/Life Sciences, Current Contents/Agriculture, Biology, and Environmental Science, Engineering Index, Excerpta Medica, Research Alert (ISI), Science Citation Index (ISI), and SCISEARCH Database (ISI).

Editorial Production, John Wiley
Diane Gern

♾ **This paper meets the requirement of ANSI/NISO Z39.48-1992 (Permanence of Paper)**

Case No.: IPR2023-00321
U.S. Patent No. 10,689,656

Biotechnology and Bioengineering

Volume 56, Number 3, November 5, 1997

Contents

239 Expression of Green Fluorescent Protein in Insect Larvae and Its Application for Heterologous Protein Production
H.J. Cha, M.-Q. Pham, G. Rao, and W.E. Bentley

248 Predicting the Partition Coefficients of a Recombinant Cutinase in Polyethylene Glycol/Phosphate Aqueous Two-Phase Systems
M.J. Sebastião, P. Martel, A. Baptista, S.B. Petersen, J.M.S. Cabral, and M.R. Aires-Barros

258 Biological Breakdown of RDX in Slurry Reactors Proceeds with Multiple Kinetically Distinguishable Paths
D.M. Young, C.L. Kitts, P.J. Unkefer, and K.L. Ogden

268 Hydrogen Transfer Between Methanogens and Fermentative Heterotrophs in Hyperthermophilic Cocultures
V. Muralidharan, K.D. Rinker, I.S. Hirsh, E.J. Bouwer, and R.M. Kelly

279 On-Line Monitoring and Control of Methanol Concentration in Shake-Flask Cultures of *Pichia pastoris*
M.M. Guarna, G.J. Lesnicki, B.M. Tam, J. Robinson, C.Z. Radziminski, D. Hasenwinkle, A. Boraston, E. Jervis, R.T.A. MacGillivray, R.F.B. Turner, and D.G. Kilburn

287 Morphometric Evaluation of the Specific Growth Rate of *Aspergillus niger* Grown in Agar Plates at High Glucose Levels
C.P. Larralde-Corona, F. López-Isunza, and G. Viniegra-González

295 Performance of Three Phase Fluidized Bed Reactor for Quinoline Degradation on Various Supports at Steady State and Dynamic Conditions
C. Buchtmann, U. Kies, W.-D. Deckwer, and V. Hecht

304 Crossflow Microfiltration of Recombinant *Escherichia coli* Lysates after High Pressure Homogenization
S.M. Bailey and M.M. Meagher

311 Polymer-Induced Phase Separation in Aqueous Micellar Solutions of Octyl-β-D-Thioglucoside for Extraction of Membrane Proteins
H. Tani, T. Saitoh, T. Kamidate, T. Kamataki, and H. Watanabe

319 Prediction of Average Biofilm Density and Performance of a Spherical Bioparticle under Substrate Inhibition
A. Tanyolaç and H. Beyenal

330 Shift from Growth to Nutrient-Limited Maintenance Kinetics during Biofilter Acclimation
R.S. Cherry and D.N. Thompson

(continued on the next page)

LINDA HALL LIBRARY OCT 7 1997

Motif Exhibit 1059, 363 of 653

COMMUNICATIONS TO THE EDITOR

340 Thermodynamic Analysis of Solvent Effect on Substrate Specificity of Lyophilized Enzymes Suspended in Organic Media
C.R. Wescott and A.M. Klibanov

345 Boron Based Separations for In Situ Recovery of L-Erythrulose from Transketolase-Catalyzed Condensation
R.P. Chauhan, L.W. Powell, and J.M. Woodley

352 Forthcoming Articles

Cover: Baculovirus-infected insect larvae. Courtesy of the Center for Agricultural Biotechnology, University of Maryland. Photo by William E. Bentley, University of Maryland.

On-Line Monitoring and Control of Methanol Concentration in Shake-Flask Cultures of *Pichia pastoris*

M. M. Guarna,[1] G. J. Lesnicki,[1] B. M. Tam,[2] J. Robinson,[1,3] C. Z. Radziminski,[1,3] D. Hasenwinkle,[1] A. Boraston,[1,3] E. Jervis,[1,4] R. T. A. MacGillivray,[2] R. F. B. Turner,[1,5] D. G. Kilburn[1,3]

[1]*The Biotechnology Laboratory, The University of British Columbia, Vancouver, BC V6T 1Z3, Canada; telephone: 604-822-4182; fax: 604-822-2114; e-mail: Kilburn@unixg.ubc.ca*
[2]*Department of Biochemistry, The University of British Columbia, Vancouver, BC V6T 1Z3, Canada*
[3]*Department of Microbiology and Immunology, The University of British Columbia, Vancouver, BC V6T 1Z3, Canada*
[4]*Department of Chemical Engineering, The University of British Columbia, Vancouver, BC V6T 1Z3, Canada*
[5]*Department of Electrical Engineering, The University of British Columbia, Vancouver, BC V6T 1Z3, Canada*

Received 6 September 1996; accepted 13 March 1997

Abstract: The methylotrophic yeast *Pichia pastoris* can be used to express recombinant genes at high levels under the control of the methanol-inducible alcohol oxidase 1 (AOX1) promoter. Accurate regulation of the methanol concentration in *P. pastoris* cultures is necessary to maintain induction, while preventing accumulation of methanol to cytotoxic levels. We developed an inexpensive methanol sensor that uses a gas-permeable silicone rubber tube immersed in the culture medium and an organic solvent vapor detector. The sensor was used to monitor methanol concentration continuously throughout a fed-batch shake-flask culture of a *P. pastoris* clone producing the N-lobe of human transferrin. The sensor calibration was stable for the duration of the culture and the output signal accurately reflected the methanol concentration determined off-line by HPLC. A closed-loop control system utilizing this sensor was developed and used to maintain a 0.3% (v/v) methanol concentration in the culture. Use of this system resulted in a fivefold increase in volumetric protein productivity over levels obtained using the conventional fed-batch protocol. © 1997 John Wiley & Sons, Inc. *Biotechnol Bioeng* **56**: 279–286, 1997.

Keywords: methanol sensor; methanol monitoring and control; methylotrophic yeast fermentation; *Pichia pastoris;* transferrin; shake-flask cultures

INTRODUCTION

Pichia pastoris has proven to be an effective host for the production of recombinant proteins (reviewed by: Buckholz and Gleeson, 1991; Cregg et al., 1993; Faber et al., 1995; Romanos, 1995; Romanos et al., 1992). This methylotrophic yeast combines several advantages of both prokaryotic and animal cell expression systems. It is readily amenable to genetic manipulation, can be easily grown to high cell densities using minimal media, and is able to introduce some eukaryotic posttranslational modifications. *P. pastoris* is also capable of expressing heterologous genes to high levels under the control of the strong and tightly regulated alcohol oxidase 1 (AOX1) promoter (Tschopp et al., 1987). Methanol serves as the inducer and as the carbon source during the production phase of *P. pastoris* cultures. A challenge associated with methanol feeding is that the methanol concentration must be maintained within a relatively narrow range. We have observed that recombinant protein production decreases when the methanol concentration exceeds ca. 1.0% (v/v); this concentration is only ca. 0.5% (v/v) higher than that suggested in the *Pichia* Expression Kit (Invitrogen, San Diego, CA) to initiate induction.

While it is possible to monitor methanol concentration using HPLC or GC, these methods are expensive and not easily implemented on-line. Alternatively, methanol concentration can be estimated indirectly by interrupting the feed and measuring the time required for methanol to be exhausted, as indicated by a sharp increase in dissolved oxygen (i.e., ''DO spike''). This approach, however, repeatedly exposes the cells to potentially noninducing levels of methanol and it is restricted to clones having a wild-type phenotype for methanol utilization (Mut^+). Clones with a disrupted AOX1 gene, exhibiting a slow methanol utilization phenotype (Mut^S), consume methanol at a rate that is too slow for this technique to be practical. Furthermore, in shake-flask cultures, DO monitoring is difficult to implement using currently available probes.

The protocol for shake-flask cultures provided with the *Pichia* Expression Kit recommends 0.5% (v/v) methanol to

Correspondence to: D. G. Kilburn
Contract grant sponsor: The National Institutes of Health
Contract grant number: RO1 DK 21739

© 1997 John Wiley & Sons, Inc.
CCC 0006-3592/97/030279-08
This material may be protected by Copyright law (Title 17 U.S. Code)

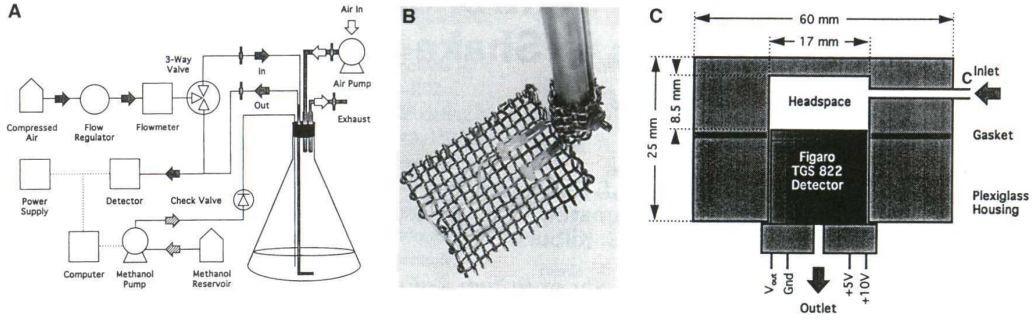


Figure 1. Methanol monitoring and control system for shake-flask cultivations of *Pichia pastoris.* (A) Schematic diagram of instrumentation and fluid/sample handling system components. (B) Photograph of silicone rubber tubing sensor probe showing stainless steel support fixture for shake-flask applications. (C) Drawing of in-house fabricated flow cell and detector housing.

start induction and the additions of methanol to 0.5% (v/v) every 24 h. However, the methanol consumption rate is strongly dependent on culture variables such as cell density, and hence the actual methanol concentration in a shake-flask culture after methanol adaptation is normally not known with any precision. A simple, inexpensive, and reliable on-line system for monitoring and controlling methanol concentration would be advantageous for optimizing and maintaining induction conditions in *P. pastoris* cultures both in shake-flask cultures and large-scale fermentations.

We designed and characterized a practical closed-loop methanol control system based on a commercially available organic solvent vapor detector, an in situ silicone rubber tubing probe, and a computer-actuated methanol feeding system. In this report we describe the components and operation of this control system and present data demonstrating its utility and performance in cell-free aqueous methanol solutions and in fed-batch shake-flask cultures of *P. pastoris* expressing recombinant human transferrin N-lobe (hTF/2N). Recombinant protein levels observed in the methanol-regulated cultures are compared to non-methanol-regulated cultures as a first step toward assessing the advantages of on-line control of methanol concentration in cell culture environments.

MATERIALS AND METHODS

Methanol Monitoring and Control System

The methanol sensor and methanol feeding system with associated instrumentation developed for use in this work are shown schematically in Figure 1A. The sensor itself comprises two components: a probe with a segment of silicone rubber tubing that remains immersed in the culture medium and facilitates conversion of the solution-phase methanol concentration to a partial pressure of methanol vapor in a carrier stream of air perfusing the lumen of the tubing (Fig. 1B); and a solid-state methanol vapor detector connected in-line with the outlet of the methanol:air flow stream (Fig. 1C). The voltage output signal from the sensor is digitized and fed to a personal computer where it is compared to a manually entered set-point in order to generate an actuating signal to drive the methanol feed pump. The headspace of the shake flask is continuously flushed with sterile filtered air to maintain a constant gas-phase oxygen concentration.

The silicone rubber membrane probe was designed based on principles described earlier in work involving measurements of pCO_2 (Kilburn, 1967), pO_2 (Phillips and Johnson, 1961), and ethanol (Yamada et al., 1987; Yamane et al., 1981) using silicone rubber and polytetrafluoroethylene tubing probes. Physically, the methanol probe consists of a 20-cm length of silicone rubber tubing (Sani-Tech, Lafayette, NJ) with a lumen diameter of 0.31 mm and a wall thickness of 0.15 mm supported by a stainless steel wire mesh, as shown in the photograph of Figure 1B. The probe remains immersed in the liquid phase while the lumen of the probe is continuously perfused with filtered air. The flow rate is regulated by a needle valve and monitored using an Omega model FMA 1804 mass flow meter (Omega Engineering, Stamford, CT). Methanol dissolved in the culture medium diffuses across the gas-permeable silicone rubber "membrane" of the probe. Vapor-phase methanol in the lumen of the probe tubing, at a partial pressure determined by the concentration in the bulk liquid phase, is carried by the flowing stream of air to the downstream methanol detector element. The methanol–air sample mixture passes through a 0.2-μm pore size hydrophobic filter (Gelman Sciences, Ann Arbor, MI) inserted upstream of the detector to remove aerosol moisture from the flow stream, which can lead to instability in the detector signal. The carrier stream can be switched to bypass the methanol probe using a manually actuated three-way valve to provide a methanol-free sample to the detector at any time to check the baseline. For all media tested, the detector baseline voltage measured in methanol-free medium was within ±1% of the value measured in the bypass air stream.

The methanol vapor detector utilizes a Figaro model TGS 822 SnO_2 organic vapor sensor (Figaro Engineering, Wilmette, IL), which is sensitive to a broad range of alcohols and organic solvent vapors. The operating characteristics and instrumentation required for the detector element are

described in the manufacturer's products catalog (Figaro Engineering, Inc., 1990). The detector is mounted in a Plexiglas housing as shown in Figure 1C. The methanol–air sample mixture flows through an inlet port (1.7-mm diameter) on the side wall of the housing to an interior "headspace" (approximate volume 1.9 cm^3) where it is exposed to the detector; the sample exhaust is vented through a port in the base of the detector element. The power supply for the detector, detector heater, and the conductance measuring circuit were designed and fabricated according to the manufacturer's specifications and application notes.

The sensor (probe plus detector) was calibrated on-line by adding prescribed aliquots of 100% methanol to a known volume of distilled water or culture medium in an agitated thermostated shake flask. Calibration was verified by withdrawing samples and determining the methanol concentration off-line by HPLC using a Shimadzu model SCL 6B auto-injector and LC 6A pump (Shimadzu Inc., Kyoto, Japan) with an HPX-87H column (BioRad, Hercules, CA).

In fed-batch operation, the methanol feed stock (100% CH_3OH) was delivered using a Gilson Minipuls 3 peristaltic pump (Gilson, Villiers-Le-Bel, France) with an in-line check valve inserted between the pump and shake flask. Because the methanol consumption rate is slow compared to the slowest practical feeding rate, a simple on/off pulsed-feed control algorithm was used. When the methanol concentration, as determined by the sensor output signal, fell below the set-point value, the controller actuated the feed pump for a prescribed (user-defined) time interval to deliver a precise dose of methanol. The controller then entered a wait state for 5–7 min (a time slightly longer than the characteristic response time of the probe) before reevaluating the concentration. These feed-pulse + delay cycles were repeated until the methanol concentration exceeded the set-point value.

Pichia pastoris Cultures

To evaluate the methanol control system in *P. pastoris* cultures, two parallel 2-L baffled shake flasks, each containing 500 mL of BMGY medium (buffered minimal glycerol complex medium, *Pichia* Expression Kit) and 0.1 mL of antifoam 289 (Sigma Chemical Co., St. Louis, MO), were inoculated. The inoculum consisted of 20 mL of an overnight culture (6.2 optical density units at 600 nm, OD_{600}) of a Mut^+ *P. pastoris* GS115 clone producing hTF/2N (Mason et al., 1996). The cultures were grown in BMGY for 24 h to a cell density of 28 OD_{600}. The cells were then separated by centrifugation and resuspended in 500 mL of basal salts medium (26.7 mL L^{-1} 85% o-phosphoric acid, 0.82 g L^{-1} calcium sulfate anhydrous, 18.2 g L^{-1} potassium sulfate, 14.9 g L^{-1} magnesium sulfate-$7H_2O$, and 4.13 g L^{-1} potassium hydroxide), containing 1 mL of PTM4 trace salts with biotin (2 g L^{-1} cupric sulfate-$5H_2O$, 0.08 g L^{-1} sodium iodide, 3.0 g L^{-1} manganese sulfate-H_2O, 0.2 g L^{-1} sodium molybdate-$2H_2O$, 0.02 g L^{-1} boric acid, 0.5 g L^{-1} cobalt chloride, 7.0 g L^{-1} zinc chloride, 22.0 g L^{-1} ferrous sulfate-$7H_2O$, 1 mL L^{-1} sulfuric acid, and 0.2 g L^{-1} D-biotin).

Two different strategies were employed for feeding methanol to the cultures. For one culture, the standard (Invitrogen) protocol was followed: 2.5 mL of pure methanol was added daily. In the parallel culture, methanol was maintained at 0.3% (v/v) using the closed-loop control system based on the feedback signal provided by the methanol sensor. Culture samples were taken periodically to determine the OD_{600}. Additional samples were collected and filtered through a 0.2-μm pore size filter; the filtrate was used for off-line analysis of methanol and hTF/2N concentrations. Culture supernatants were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and protein bands were visualized with Coomassie brilliant blue stain and quantified by scanning densitometry using a Fast-Scan Computing Densitometer (Molecular Dynamics).

Chemicals and Reagents

All chemicals used in the characterization of the sensor and in the preparation of culture media were obtained from Fisher Scientific (Nepean, ON), BDH Chemicals (Toronto, ON), Anachem Science (Montréal, PQ), and Sigma Chemical Co. and were used as received. All solutions were prepared using distilled water.

RESULTS AND DISCUSSION

Methanol Probe Characterization

Sensitivity to Methanol

A typical strip-chart record of the time-dependent response to methanol additions and the resulting methanol calibration curve are shown in Figure 2A and 2B, respectively. The baseline sensor voltage (i.e., the reading in a methanol blank solution) under the conditions used throughout this work was 1.39 ± 0.01 V; the baseline voltage periodically measured using the bypass stream of air remained within this range during all experiments. The sensor response time (to reach 90% of steady state) at 30°C in methanol–water solutions was ca. 4 min, using a carrier air-stream flow rate of 5 mL min^{-1} with probe dimensions as indicated in the Materials and Methods section. In terms of the calibration characteristic and response time, the behavior of the probe was virtually the same whether measuring in methanol–water solutions or culture media, with or without cells. In the present configuration, the methanol concentration detection limit (defined as the concentration corresponding to a signal 3 standard deviations of the noise above the mean baseline response) at 30°C was approximately 1.2×10^{-4}% (v/v).

The nonlinearity evident in the sensor response function results from a combination of two effects: one is an artifact of the electronic measuring circuit that employs a simple voltage divider configuration to measure conductance

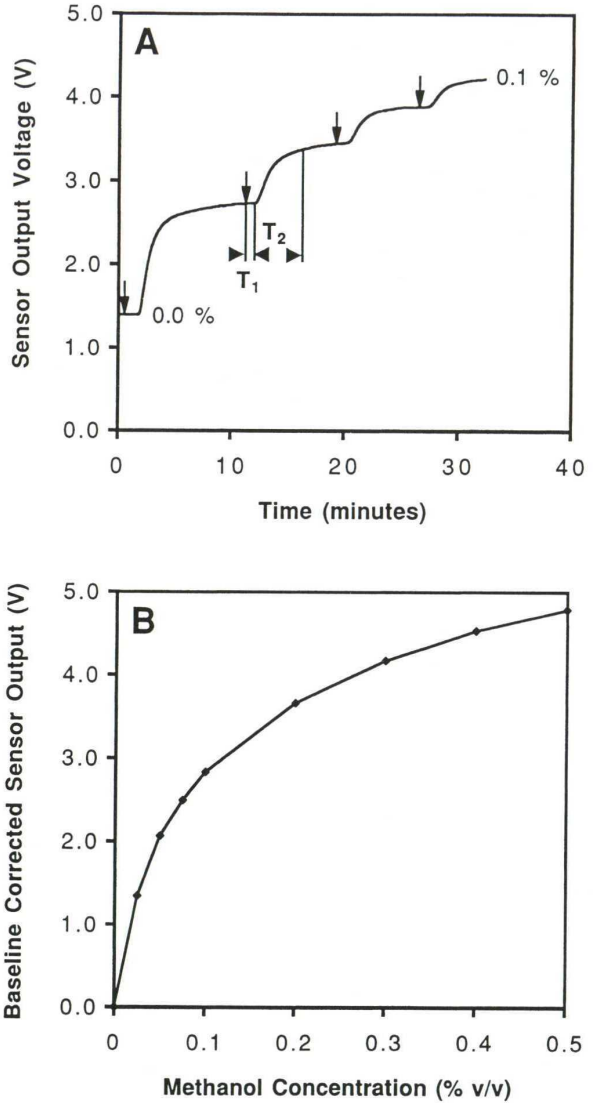


Figure 2. Methanol sensor response characteristics measured at 30°C with a carrier air flow rate of 5 mL min^{-1}. (A) Strip-chart record showing characteristic response time to 2.5 mL methanol injections (arrows); component T1 (0.71 ± 0.19 min) is the time for the sensor to initiate a response and component T2 (3.15 ± 0.63 min) is the additional time required to reach 90% of the steady-state signal. (B) Sensor response versus methanol concentration in methanol–water solution.

changes in the detector element, yielding a hyperbolic relationship between output voltage and conductance. The other is itself a composite effect that results from the fundamental physical chemistry and physical electronic principles of semiconducting metal-oxide gas sensors. These principles have been described in detail elsewhere (e.g., Heiland, 1982; Jones et al., 1985; Morrison, 1982; Watson, 1984); however, a summary of some of the salient points may be helpful here. Briefly, the conductivity of sintered metal oxides is mediated primarily by electrons populating surface states that arise due to a deficit of lattice oxygen at the grain boundaries and lie energetically near the edge of the conduction band. The conductivity is modulated by any process that alters the availability of these surface states. In the case of the methanol probe described here, chemisorption of oxygen in the absence of methanol depletes the conduction electrons available at the surface and lowers the macroscopic conductivity. In the presence of methanol, which coadsorbs to the surface, the semiconductor catalyzes the reduction of surface-bound oxygen, forming the more weakly interacting formaldehyde that desorbs and frees surface conduction band electrons that, in turn, increases the observed conductivity. All of the processes involved are nonlinear over an appreciable range of methanol concentrations and hence so is the resulting response function, even though consideration of mass transfer limited by the diffusion of methanol across the silicone rubber membrane would lead to the expectation of a linear response.

Sensitivity to Other Organic Vapors

The detector element employed in this work is not intrinsically selective for methanol. Some extrinsic selectivity can be engineered in this family of sensors by the incorporation of appropriate dopant species in the sintered metal oxide pellet and by the choice of surface temperature (Gentry and Jones, 1986; Yamazoe et al., 1983). The particular model of detector element used here is specified for applications involving detection of a range of alcohols, as well as toluene, xylene, benzene, acetone, carbon monoxide, and several small hydrocarbons (Figaro Engineering, 1990). In the applications considered here under aerobic conditions, none of these potential interfering species exist in the culture medium in significant concentrations. However, if mix feeding of glycerol and methanol is used or if the culture becomes oxygen limited, low levels of ethanol can be produced by *P. pastoris.* Although the sensor is slightly more sensitive to ethanol than to methanol, the maximum ethanol concentration reported in mix-fed *P. pastoris* cultures was 0.02% (v/v) and only 0.003% in methanol-fed cultures (Brierley et al., 1990). Ethanol interference is thus of no practical concern when methanol is controlled at 0.3% (v/v), a level that seems adequate to maintain induction (see below).

Probe Dimensions and Air Flow Rate

The main physical parameters of the probe that dictate both the kinetics and the sensitivity of the response are the silicone rubber tubing length, tubing diameter and wall thickness, and the flow rate of the carrier stream of air. In the present work, the tubing diameter and wall thickness as specified in the Materials and Methods section were chosen to yield reasonable durability with a minimal compromise of sensitivity and response time. The tubing length and air flow rate were then optimized based on the performance needed for the intended application. The dependence of tubing length and air flow on the sensor response is shown in Figure 3A and 3B, respectively. Longer tubing lengths

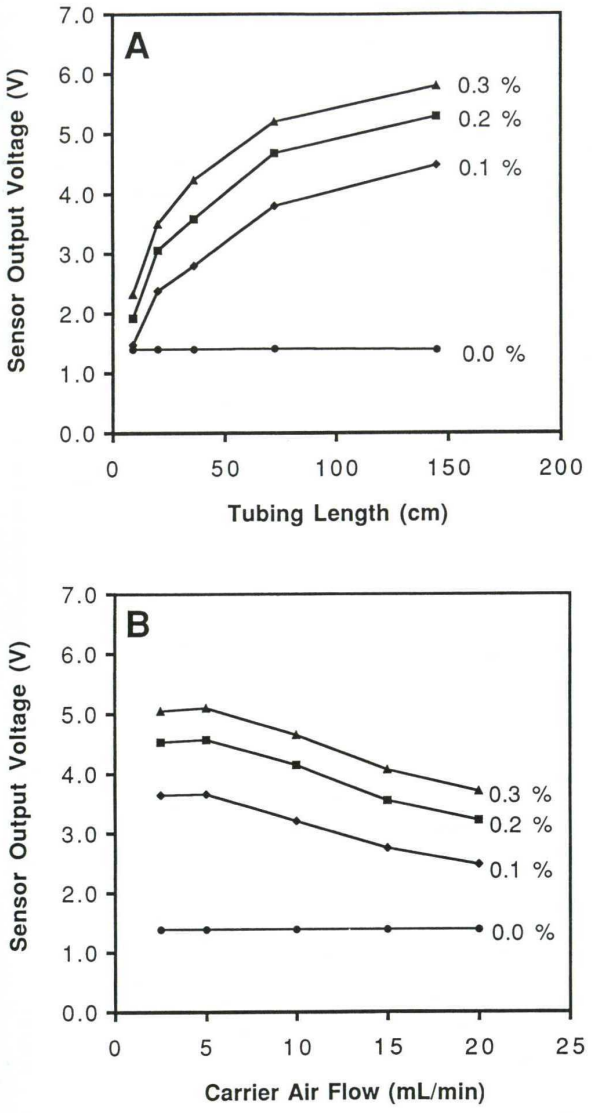


Figure 3. Tubing length and carrier air flow-rate dependence determined at 30°C. (a) Sensor response versus silicone rubber sensor tubing length for a methanol blank solution and three nonzero methanol concentrations. (b) Sensor response versus carrier air flow rate for the same methanol concentrations as in (a).

clearly yield higher sensitivity and larger output signals, albeit with rapidly diminishing returns beyond ca. 50 cm; however, the response time beyond 40–50 cm increases (data not shown) to a point where the system becomes impractical for use in *P. pastoris* fermentations. A length of 20 cm was chosen as the optimal compromise between sensitivity and response time. Response time is also (inversely) dependent on the carrier air flow rate. However, as shown in Figure 3B, the sensitivity drops as a function of air flow above 5 mL min^{-1}; hence, a rate of 5 mL min^{-1} was again chosen as the optimal compromise between sensitivity and response time.

Temperature and pH Dependence

The temperature and pH dependence of the sensor, measured in a methanol–water solution, is shown in Figure 4A and 4B, respectively. Essentially all of the physical and chemical processes involved in the operation of the sensor are inherently temperature dependent. However, the detector element is remotely located and thereby thermally isolated from the culture environment; the detector also incorporates an integrated heating coil that maintains the surface temperature at ca. 400°C. No significant cooling of the detector is effected by the methanol–air gas flow through the

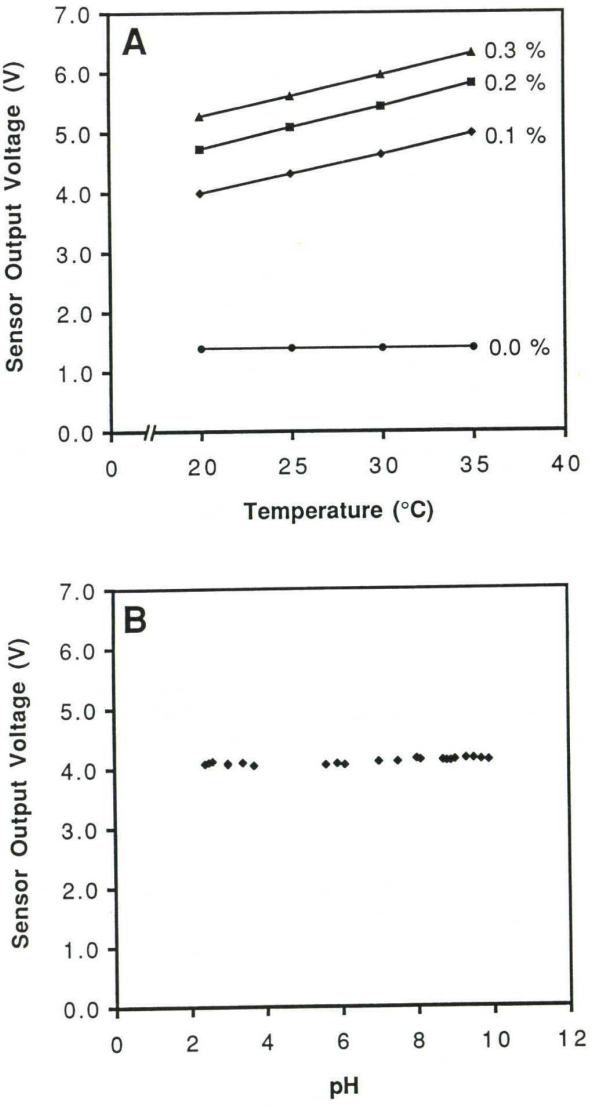


Figure 4. Effect of variations in temperature and pH determined at 30°C using a carrier air flow rate of 5 mL min^{-1} and a tubing length of 20 cm. (a) Sensor response versus temperature of the methanol–water analyte matrix. (b) Sensor response versus pH of the methanol–water analyte matrix with a methanol concentration of 0.1%.

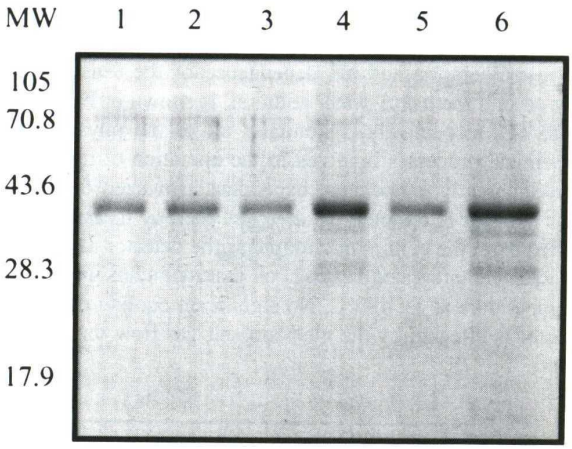


Figure 5. SDS-PAGE analysis of hTF/2N produced by *P. pastoris*. Culture medium after (lanes 1, 2) 10 h, (lanes 3, 4) 33 h, and (lanes 5, 6) 56 h of production was sampled from (lanes 1, 3, 5) the flask receiving daily additions of methanol and (lanes 2, 4, 6) the flask with constant (ca. 0.3%) methanol concentration.

housing as evidenced by the flat response as a function of flow rate for a methanol blank solution shown in Figure 3B. Furthermore, no temperature dependence is observed in the absence of methanol. The observed temperature dependence of the sensor is therefore dominated by effects relating to the mass transfer of methanol across the silicone rubber membrane of the probe. With regard to pH dependence, it was important to establish that the sensor response is independent of fluctuations in culture pH, as demonstrated by the data in Figure 4B, because pH is normally not controlled in shake-flask cultures.

Increased Protein Productivity in *P. pastoris* Cultures

The results obtained using the two different methanol feeding protocols in parallel shake-flask cultures are shown in Figures 5, 6, and 7. SDS-PAGE showed that recombinant hTF/2N comprises the majority of the total protein in the medium under both conditions (Fig. 5). In the production phase of one 500-mL culture, the standard Invitrogen protocol was followed with daily additions of 2.5 mL of methanol. After each dose, methanol was rapidly consumed such that its concentration was lower than 0.3% (v/v) for more than half of the production phase and the methanol was consistently exhausted by the culture before the next scheduled feeding time was reached. In response to the first methanol dose, recombinant protein concentration in the culture medium increased to 32 mg L^{-1}. However, as the run progressed, hTF/2N not only ceased to accumulate, but its concentration actually declined, presumably due to protein degradation in the medium. After the third dose of methanol, however, recombinant protein accumulation resumed but only to 82% of the hTF/2N concentration obtained after the first methanol dose. In the parallel culture, the methanol control system was used to maintain a methanol concentration of 0.3% (v/v) in the production phase. In contrast to the uncontrolled culture, recombinant protein accumulated continuously for 2 days, reaching a maximum concentration of 120 mg L^{-1}.

Under the experimental conditions used, the standard protocol of daily methanol additions was insufficient to maintain induction. The concentration of hTF/2N in the medium reached a maximum 10 h after the first methanol addition. Maintaining the culture for longer times with daily additions did not result in any further increase in volumetric protein yield. In contrast, control of methanol concentration

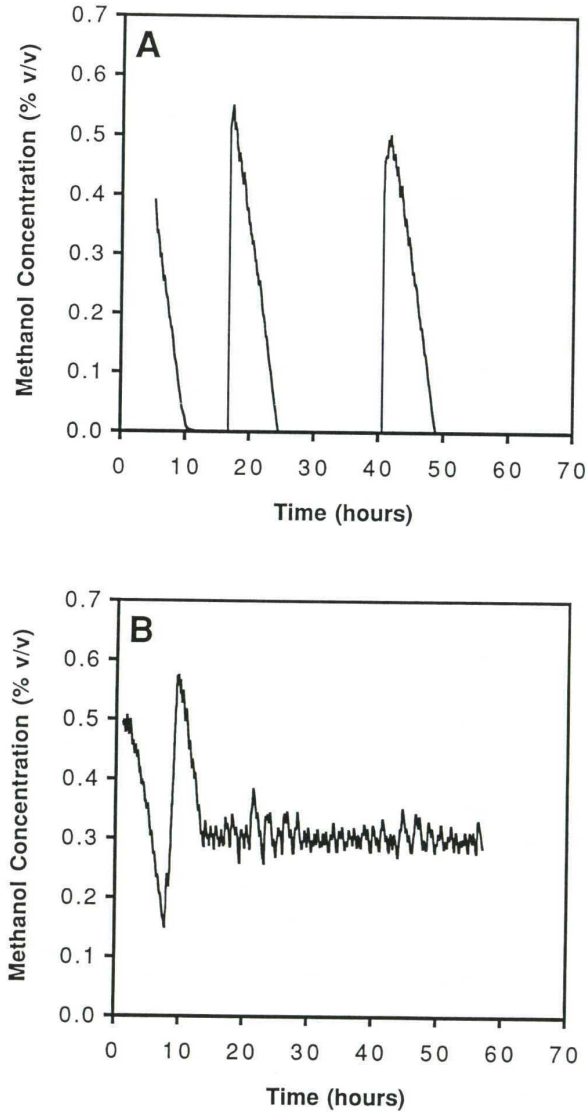


Figure 6. Methanol concentration versus time in two parallel *P. pastoris* shake-flask cultures. (a) the 500-mL culture was fed daily with 2.5 mL methanol. (b) The closed-loop control system was set to maintain the methanol concentration at 0.3% (v/v), as determined by in situ calibration of the sensor.

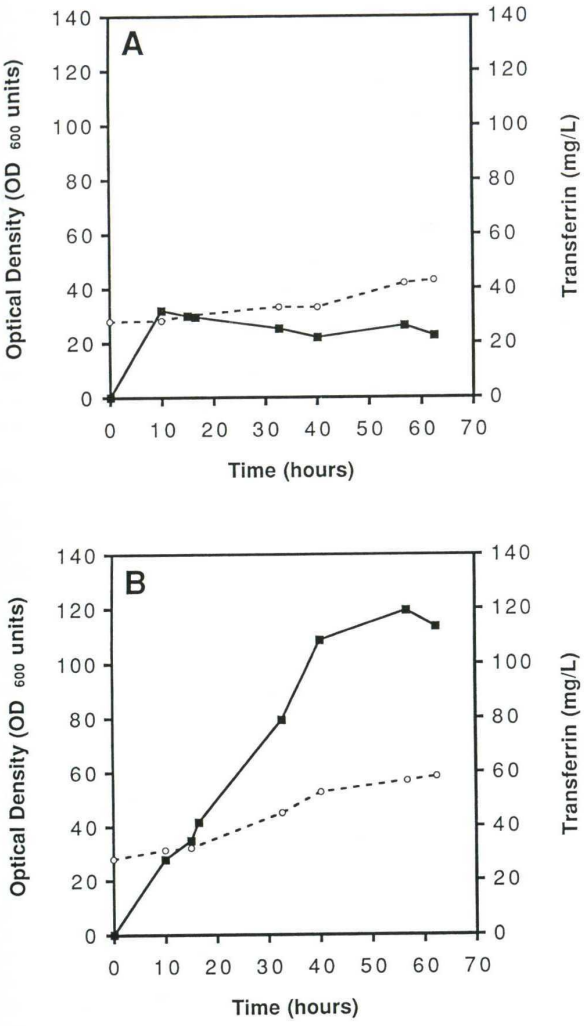


Figure 7. Effect of on-line control on protein productivity. (○--○) Cell density and (■—■) recombinant protein production were analyzed in (a) a culture receiving daily doses of methanol and (b) in a controlled culture in which the methanol concentration was maintained at 0.3% (v/v).

at 0.3% (v/v) resulted in continuous culture growth and hTF/2N production, reaching levels fivefold higher than in the flask that received daily methanol feedings.

CONCLUSIONS

A practical and effective system for on-line monitoring and control of methanol concentrations in *P. pastoris* shake-flask cultures has been presented. The sensor element, based on a commercially available organic vapor detector and a custom designed gas-permeable methanol probe, has been optimized with respect to performance-determining physical parameters (e.g., carrier stream flow rate, tubing length) and characterized as a function of the key fermentation environmental variables (e.g., methanol concentration, temperature, pH) over their typical respective ranges. The probe is autoclavable, can be calibrated in situ, and is stable for several days under routine operating conditions for *P. pastoris* fermentations.

Shake-flask cultures in which the sensor was used to control the methanol concentration exhibited higher recombinant protein productivity than simultaneously run cultures carried out using the conventional protocol. These experiments also provided the first quantitative data demonstrating the drastic variability in the methanol concentration profile that can result from the conventional protocol. The higher productivity observed in the controlled cultures was attributed to elimination of this variability.

On-line monitoring of substrate concentration is seldom, if ever, used in simple shake-flask cultures and we know of no similar example of controlled fed-batch operation in this type of system. These features have previously been limited to much more expensive and complex bioreactor systems not routinely available to most investigators. The ability to monitor and control methanol concentration in shake-flask cultures will expedite the early selection and characterization of recombinant clones of methanol utilizing organisms (e.g., *P. pastoris, Hansenula polymorpha*).

Although beyond the scope of this work, the methanol sensor described here provides a valuable tool for further optimization of *P. pastoris* expression systems, particularly with respect to target methanol concentrations during the induction phase of the culture. This sensor system can easily be adapted for use in applications involving larger scale cultures in stirred-tank bioreactors of methylotrophic organisms or for monitoring production in biomass to ethanol processes. Continuing work in our laboratories includes application of this system to Mut^+ and Mut^S mixed-feed fermentations of *P. pastoris* for enhanced productivity of recombinant proteins.

The authors wish to thank Mr. Randy Deane for assisting with the fabrication of electronic components used in this work. Financial support was provided by the Natural Sciences and Engineering Research Council of Canada, The Medical Research Council of Canada, and Ciba–Geigy Corporation.

References

Brierley, R. A., Bussineau, C., Kosson, R., Melton, A., Siegel, R. S. 1990. Fermentation development of recombinant *Pichia pastoris* expressing the heterologous gene: Bovine lysozyme. Ann. NY Acad. Sci. **589**: 350–362.

Buckholz, R. G., Gleeson, M. A. G. 1991. Yeast systems for the commercial production of heterologous proteins. Bio/Technology **9**: 1067–1072.

Cregg, J. M., Vadvick, T. S., Raschke, W. C. 1993. Recent advances in the expression of foreign genes in *Pichia pastoris.* Bio/Technology **11**: 905–910.

Faber, K. N., Harder, W., Ab, G., Veenhuis, M. 1995. Review: Methylotrophic yeasts as factories for the production of foreign proteins. Yeast **11**: 1331–1344.

Figaro Engineering Inc. 1990. Figaro gas sensors. Figaro Products Catalog pp. 2–12.

Gentry, S. J., Jones, T. A. 1986. The role of catalysis in solid-state gas sensors. Sensors Actuators **10**: 141–163.

Heiland, G. 1982. Homogeneous semiconducting gas sensors. Sensors Actuators **2**: 343–361.

Jones, T. A., Firth, J. G., Mann, B. 1985. The effect of oxygen on the electrical conductivity of some metal oxides in inert and reducing atmospheres at high temperature. Sensors Actuators **8**: 281–306.

Kilburn, D. G. 1967. Some factors affecting growth of mammalian cells in suspension cultures. Ph.D. thesis. Department of Chemical Engineering, University College, London, Chap. 2.

Mason, A. B., Woodworth, R. C., Oliver, R. W. A., Green, B. N., Lin, L.-N., Brandts, J. F., Tam, B. M., Maxwell, A., MacGillivray, R. T. A. 1996. Production and isolation of the recombinant N-lobe of human serum transferrin from the methylotrophic yeast *Pichia pastoris.* Protein Expr. Purif., to appear.

Morrison, S. R. 1982. Semiconductor gas sensors. Sensors Actuators **2**: 329–341.

Phillips, D. H., Johnson, M. J. 1961. Measurement of dissolved oxygen in fermentations. J. Biochem. Microb. Technol. Eng. **111**: 261–275.

Romanos, M. A. 1995. Advances in the use of *Pichia pastoris* for high-level gene expression. Curr. Opin. Biotechnol. **6**: 527–533.

Romanos, M. A., Scorer, C. A., Clare, J. J. 1992. Foreign gene expression in yeast: A review. Yeast **8**: 423–488.

Tschopp, J. F., Brust, P. F., Cregg, J. M., Stillman, C. A., Gingeras, T. R. 1987. Expression of the *lacZ* gene from two methanol regulated promoters in *Pichia pastoris.* Nucl. Acids Res. **15**: 3859–3876.

Watson, J. 1984. The tin oxide gas sensor and its applications. Sensors Actuators **5**: 29–42.

Yamada, M., Mizuno, M., Tsukamoto, Y., Yamada, K. 1987. Method for a measurement of alcohol concentration in acetic acid fermenting broth. U.S. Pat. 4,656,140.

Yamane, T., Matsuda, M., Sada, E. 1981. Application of porous Teflon tubing method to automatic fed-batch culture of microorganisms. II. Automatic constant-value control of fed substrate (ethanol) concentration in semibatch culture of yeast. Biotechnol. Bioeng. **13**: 2509–2524.

Yamazoe, N., Kurokawa, Y., Seiyama, T. 1983. Effects of additives on semiconductor gas sensors. Sensors Actuators **4**: 283–289.

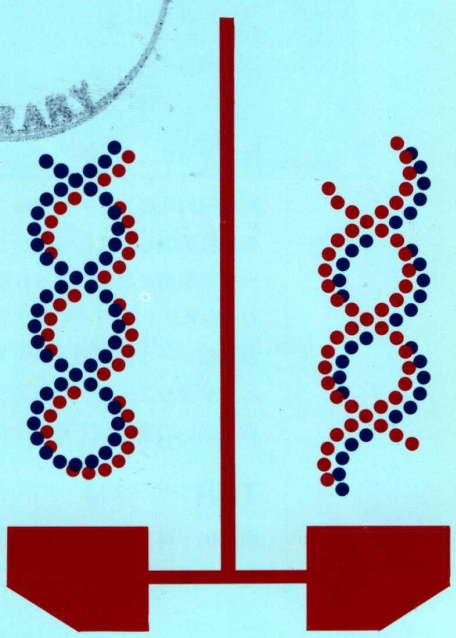
Ex. 1033 Katakura 1998

ISSN 0922-338X
CODEN: JFBIEX
平成10年1月16日学術刊行物認可

Vol.86 No.5 1998

Journal of Fermentation and Bioengineering

DEC 30 1998
LIBRARY



FOUNDED 1923

Published monthly by the Society for Fermentation and Bioengineering
Distributed outside Japan by Elsevier Science

This Journal is supported by a scientific grant-in-aid for publishing of research papers from the Ministry of Education, Science, Sports and Culture of Japan.
Note to contributors: Instructions to authors are published in detail in the first issue of each volume. A copy of these instructions will be sent upon request to the Business Office of the Society.

For subscribers from abroad:

"The subscription price for 1998, Volumes 85–86 (12 issues) is Dfl. 1713.00 (US$ 984.00). Prices include postage and handling. The Dutch Guilder (Dfl.) price quoted applies worldwide, except in the Americas (North, Central and South America). The US Dollar price quoted applies in the Americas only. Non VAT registered customers in the European Community should add the appropriate VAT rate applicable in their country to the price. Subscriptions are accepted on a pre-paid basis only unless different terms have previously been agreed upon.
Subscription orders can only be entered by calendar year (Jan.-Dec.) and should be sent to:

Elsevier Science B.V.
P.O. Box 2227, 1000 CE Amsterdam,
The Netherlands
Telephone (+31–20) 4853642, Telefax (+31–20) 4853598.

In the USA and Canada contact:

Elsevier Science Inc.
Journal Information Center,
655 Avenue of the Americas,
New York, NY 10010, USA
Telephone: (212) 633–3750, Telefax: (212) 633–3990, Telex: 420–643 AEP UI.

"Journals are sent by Surface Mail to all countries except the following where Air delivery via SAL mail is ensured at no extra cost to the subscriber: Argentina, Australia/New Zealand, Brazil, Hong Kong, India, Israel, Japan, Malaysia, Mexico, Pakistan, P.R. China, Singapore, S. Africa, S. Korea, Taiwan, Thailand, USA and Canada. Claims for missing issues must be made within six months of our publication (mailing) date, otherwise such claims cannot be honoured free of charge.



Distributed outside Japan
by Elsevier Science
Amsterdam, The Netherlands

The Journal of Fermentation and Bioengineering is abstracted/cited in:
Current Contents,
Elsevier BIOBASE/Current Awareness in Biological Sciences,
ISI Biotechnology Citation Index,
EMBASE/Excerpta Medica,
Chemical Abstracts,
BIOSIS.

Dues for individual active members of the Society are ¥8,000 per year for residents of Japan and ¥10,000 per year for active members from abroad. In order to be active members of the Society, send the application form in the last page in each issue for admission to the Society for Fermentation and Bioengineering, Japan directly. Active member can receive each issue of this journal and Seibutsu-kogaku Kaishi in calender year (Jan.–Dec.) delivered by surface mail.

Advertisements: Application for advertising space is accepted. Please send your inquiry to MYU RESEARCH, 2–32–5 Sendagi, Bunkyo-ku, Tokyo 113, Japan. Copy is required five weeks before publication date. Rates are available on request.

JOURNAL OF FERMENTATION AND BIOENGINEERING

Vol. 86, No. 5, 1998

REGULAR PAPERS

***SSU1-R*, a Sulfite Resistance Gene of Wine Yeast, is an Allele of *SSU1* with a Different Upstream Sequence.** Nami Goto-Yamamoto, Kazuyoshi Kitano, Kunio Shiki, Yuichi Yoshida, Takashi Suzuki, Tomoko Iwata, Yoshiharu Yamane, and Shodo Hara 427–433

PCR Cloning and Expression of the F/10 Family Xylanase Gene from *Streptomyces olivaceoviridis* E-86. Atsushi Kuno, Daisuke Shimizu, Satoshi Kaneko, Yoshinori Koyama, Shigeki Yoshida, Hideyuki Kobayashi, Kiyoshi Hayashi, Kazunari Taira, and Isao Kusakabe 434–439

Construction of Chimeric Proteins between Protein G and Fluorescence-Enhanced Green Fluorescent Protein, and Their Application to Immunoassays. Ryoichi Arai, Hiroshi Ueda, and Teruyuki Nagamune 440–445

Characterization of the Biotin Synthase Reaction from *Bacillus sphaericus* Using the Photoreduced Deazaflavin System. Takashi Ohshiro, Takaaki Kishimoto, Miwako Arase, and Yoshikazu Izumi 446–450

Ethanol-Induced Water Stress and Fungal Growth. John E. Hallsworth, Yoshiyuki Nomura, and Masayoshi Iwahara 451–456

Trehalose Metabolism and Leavening Ability of Bakers' Yeast Grown in the Presence of Sodium Chloride. Nazneen Bagum, Kumio Yokoigawa, Yuka Isobe, and Hiroyasu Kawai 457–460

β-Mannanase and Xylanase of *Bacillus subtilis* 5H Active for Bleaching of Crude Pulp. Chartchai Khanongnuch, Kouhei Asada, Hideo Tsuruga, Toshihiko Ooi, Shinichi Kinoshita, and Saisamorn Lumyong 461–466

Interaction between Homoacetogens and Methanogens in Lake Sediments. Jiunn-Jyi Lay, Yu-You Li, and Tatsuya Noike 467–471

Determination of the Content of Water and Rice Bran in Solid Media Used for Mushroom Cultivation Using Near-Infrared Spectroscopy. Takuo Yano, Ken-ichiro Suehara, and Yasuhisa Nakano 472–476

Renewal Rate of Semicontinuous Cultures of the Microalga *Porphyridium cruentum* Modifies Phycoerythrin, Exopolysaccharide and Fatty Acid Productivity. Jaime Fábregas, Digna García, Ever Morales, Adolfo Domínguez, and Ana Otero 477–481

Effect of Methanol Concentration on the Production of Human β_2-Glycoprotein *I* Domain *V* by a Recombinant *Pichia pastoris*: A Simple System for the Control of Methanol Concentration Using a Semiconductor Gas Sensor. Yoshio Katakura, Wenhui Zhang, Guoqiang Zhuang, Takeshi Omasa, Michimasa Kishimoto, Yuji Goto, and Ken-ichi Suga 482–487

Continued on following page

Continued from preceding page

Effect of Product Removal by a Pervaporation on Ethanol Fermentation. Kaseno, Ichiro Miyazawa, and Takao Kokugan 488–493

NOTES

Cloning and Nucleotide Sequence of the Nitric Oxide Reductase Locus in *Paracoccus denitrificans* IFO 12442. Kiyohito Murai, Katsuhide Miyake, Jun Andoh, and Shinji Iijima 494–499

Display of Heterologous Gene Products on the *Escherichia coli* Cell Surface as Fusion Proteins with Flagellin. Satoshi Ezaki, Manae Tsukio, Masahiro Takagi, and Tadayuki Imanaka 500–503

Lipid Composition of a Newly Isolated Polyunsaturated Fatty Acid-Producing Fungus, *Achlya* sp. ma-2801. Tsunehiro Aki, Yukimasa Matsumoto, Tsutomu Morinaga, Seiji Kawamoto, Seiko Shigeta, Kazuhisa Ono, and Osamu Suzuki 504–507

Lipase-Catalyzed Kinetic Resolution of 4-Hydroxy-5-Dodecynonitrile and Its Application to Facile Synthesis of a Cupreous Chafer Beetle Sex Pheromone. Ei-ichiro Fukusaki, Shiro Satoda, Shuji Senda, and Tetsuo Omata 508–509

Improved Adsorption of *Aspergillus niger* 589 Spores on High-Density Polyethylene for Progesterone Biotransformation. Aparna G. Kulkarni, Smita S. Lele, and Pushpa R. Kulkarni 510–512

A New Method for the Production of L-Lyxose from Ribitol Using Microbial and Enzymatic Reactions. Shakhawat Hossain Bhuiyan, Zakaria Ahmed, Masaru Utamura, and Ken Izumori 513–516

Immobilization of *Rhodobacter capsulatus* on Cellulose Beads and Water Treatment Using a Photobioreactor. Shigeki Sawayama, K. Krishna Rao, and David O. Hall 517–520

Hydrogen Peroxide as an Antibacterial Factor in Zinc Oxide Powder Slurry. Jun Sawai, Shinobu Shoji, Hideo Igarashi, Atsushi Hashimoto, Takao Kokugan, Masaru Shimizu, and Hiromitsu Kojima 521–522

TECHNICAL NOTE

Non-Radiochemical 3-Hydroxy-3-Methylglutaryl-Coenzyme A Synthase Assay by Reversed-Phase HPLC without Using Ion-Pair Reagent. Akira Tachibana, Yoshihisa Yano, Shuzo Otani, and Makoto Taniguchi 523–526

Case No.: IPR2023-00321
U.S. Patent No. 10,689,656

This material may be protected by Copyright law (Title 17 U.S. Code)

JOURNAL OF FERMENTATION AND BIOENGINEERING
Vol. 86, No. 5, 482–487. 1998

Effect of Methanol Concentration on the Production of Human β_2-Glycoprotein *I* Domain *V* by a Recombinant *Pichia pastoris*: A Simple System for the Control of Methanol Concentration Using a Semiconductor Gas Sensor

YOSHIO KATAKURA,[1]* WENHUI ZHANG,[1]§ GUOQIANG ZHUANG,[1] TAKESHI OMASA,[1] MICHIMASA KISHIMOTO,[1] YUJI GOTO,[2] AND KEN-ICHI SUGA[1]

Department of Biotechnology, Graduate School of Engineering,[1] Institute for Protein Research,[2] Osaka University, Suita, Osaka 565–0871, Japan

Received 15 May 1998/Accepted 17 August 1998

The methylotrophic yeast *Pichia pastoris* is one of the best hosts for the production of foreign proteins because of the presence of the strong *AOX*1 promoter induced by methanol. Methanol feeding during the production phase of the foreign proteins is important because methanol not only induces protein production but also provides energy source for the host cells. Excess methanol inhibits the growth of host cells, while an insufficient amount of energy source and/or methanol starvation lead to poor growth and production. We constructed a simple methanol control system consisting of a semiconductor gas sensor and a relay. Using this system, we studied the effect of methanol concentration on the production of a model foreign protein, human β_2-glycoprotein *I* domain *V*. The methanol concentrations were kept constant at 1.5, 10, 17, or 31 g·l^{-1} (±5%) during the production phase. Although the specific rates of growth and methanol consumption decreased with increase in the methanol concentration, the specific production rates increased, indicating that the energy for the production competed with that for cell growth. Accordingly, we provided glycerol as an extra energy source during the production phase, with the result that the specific production rate increased two times. Our simple and inexpensive system will help bioengineering studies on the production of recombinant proteins in *P. pastoris*, the growth and production of objective proteins in which are dependent on the methanol concentration.

[**Key words:** β_2-glycoprotein *I*, *Pichia pastoris*, methanol control system, semiconductor sensor]

The methylotrophic yeast *Pichia pastoris* is one of the best hosts for the production of foreign proteins, especially of eukaryotic secretory proteins which require post-translational modifications. Many eukaryotic proteins have been produced using the *Pichia* expression system (1–4) because this system is more efficient, simpler and less expensive than other eukaryotic expression systems. This expression system is useful not only for the production of industrial proteins but also for biochemical researchers who are interested in tertiary structure analysis of proteins by X-ray crystallography or nuclear magnetic resonance (4). This yeast has two alcohol oxidase genes, *AOX*1 and *AOX*2, which are induced strongly and weakly, respectively, by methanol (5, 6). The gene of the foreign protein is inserted between the *AOX*1 promoter and the *AOX*1 terminator of shuttle vectors between *P. pastoris* and *Escherichia coli*. When the vector is integrated into the chromosome of *P. pastoris*, the *AOX*1 genotype of the yeast remains, which grows fast on methanol (integration-type). When the *AOX*1 structural gene of the yeast genome is replaced by that of the foreign protein, the resultant yeast genotype is *aox*1, which grows slowly on methanol (replacement-type), because alcohol oxidase, a key enzyme for methanol utilization, is expressed only from *AOX*2. The transformed yeast is usually cultivated on glycerol, a non-inducible and non-repressive substrate, in the growth phase, and thereafter, protein production is induced by methanol feeding. In both types of transformants, the methanol concentration in the medium affects not only the level of production of foreign proteins but also the growth of the host cell. However, no biochemical engineering studies have so far been conducted on the production of foreign proteins in the presence of constant methanol concentrations. Although some methanol feeding programs during the production phase were applied to the production of bovine lysozyme and human epidemal growth factor (7; Brerley, R. A. *et al.*, US patent 5,324,639, 1994; Siegel, R. S. *et al.*, International patent application, WO 90/10697, 1990; Buckholz, R. G. *et al.*, International patent application, WO 90/03431, 1989), it is difficult to keep the methanol concentration constant because the methanol consumption rate increases with cell growth. These programs are probably not suitable for being employed for the production of other proteins because the specific methanol consumption rate depends on the species of foreign proteins and the copy number of the gene.

It has been reported that a semiconductor sensor, which has been used as a gas-leakage sensor, can be used for monitoring the ethanol and methanol concentrations in microbial culture (8, 9), including recombinant *P. pastoris* culture (10). The sensor consists of a semiconductor and a heater unit which heats up the semiconductor. Since the heat increases the mobility of free electrons in the semiconductor, the density of free electrons at the surface of the semiconductor increases. When an oxygen molecule touches the free electrons, it detaches

* Corresponding author.
§ Present address: Department of Food Science and Technology, University of Nebraska-Lincoln, Lincoln, NE 68583–0919, USA.

Motif Exhibit 1059, 378 of 653

Case No.: IPR2023-00321
U.S. Patent No. 10,689,656

itself into atoms and traps the free electrons. As a result, since free electrons are the carriers of electric current, the resistance of the sensor increases. When flammable gas molecules are introduced into the sensor, the atomic oxygen reacts easily with them and releases the free electrons, the density of free electrons in the semiconductor then increases and the resistance of the sensor decreases according to the concentration of the flammable gas molecules (11, 12).

By combining this sensor with a simple circuit, a relay and a pump, we constructed a feedback system for the control of methanol concentration. Recently Guarna *et al.* used this sensor to keep the methanol concentration constant in a flask-fed batch culture of recombinant *P. pastoris* (10). However, they emphasized the expedition of early selection and characterization of recombinant clones by their shake-flask system and did not discuss kinetic parameters such as specific rates. The purposes of this study are (i) to establish a simple methanol control system which is easy to use, and (ii) to investigate the effects of methanol concentration on the level of production of a foreign protein in a genetically engineered *P. pastoris* culture using the system for the control of methanol concentration.

MATERIALS AND METHODS

Strains and plasmid Human β_2-glycoprotein *I* (β_2GP*I*) domain *V* was used as the representative foreign protein. β_2GP*I* is a glycosylated plasma protein and its fifth domain (β_2GP*IdV*) was reported to interact with negatively charged phospholipids *in vivo* (13). β_2GP*IdV* is a basic protein (isoelectric point 10) which consists of 86 amino acid residues with three disulfide bonds (molecular weight, 9.7 kDa). The plasmid pNPD5 (13), containing the β_2GP*IdV* gene inserted in the vector pPIC9 (Invitrogen, San Diego, USA), was digested by *Sal*I and introduced into *P. pastoris* GS115 (*his*4, *AOX*1, *AOX*2) by electroporation. Most of the transformants grew fast on the minimum agar plate containing methanol as the sole carbon source (MM plate), because the *Sal*I digestion resulted in integration-type transformants (*HIS*4, *AOX*1, *AOX*2). The β_2GP*IdV* expression level in the transformants on the MM plate was assayed by colony Western blotting using the anti-human β_2GP*I* antibody from rabbit and alkaline phosphatase conjugated anti-rabbit immunoglobulins antibody from goat (Biosource, Camarillo, USA) as the primary and secondary antibodies, respectively. Ten clones which showed strong signals were selected and the production of β_2GP*IdV* in a test tube culture was assayed by sodium dodecyl sulfate polyacrylamide gel electrophoresis according to the manufacturer's instructions (Invitrogen). A clone, designated SP216, which gave the highest level of β_2GP*IdV* among the transformants was finally selected. SP216 had at least 3 copies of the β_2GP*IdV* gene as judged from the results of Southern hybridization using the β_2GP*IdV* gene as a probe (data not shown).

Analytical methods Dry cell weight was calculated based on the optical density at 660 nm and the β_2GP*IdV* expression level was measured by cation-exchange chromatography. The culture supernatant was diluted three times with 20 mM acetic acid. The diluted sample was filtered (0.22 μm filter) and loaded onto the SP-5PW column (4.6 mm × 75 mm, Tosoh Co., Tokyo) equilibrated with 20 mM acetic acid at a flow rate 1.0 ml·min^{-1} at room temperature. β_2GP*IdV* was eluted with a linear gradient, from 20 mM acetic acid to 50 mM sodium acetate containing 0.5 M sodium chloride (pH 5.5), and the concentration was calculated from the extinction coefficient of 10,185 at 276 nm (13). Glycerol concentrations were measured by F-kit glycerol (Boehringer Mannheim, Tokyo). Methanol concentrations in the culture broth were measured off-line by gas chromatography (G-3000, Hitachi Co., Tokyo) on the TC-1 column (GL Sciences Co., Tokyo) at a column temperature of 60°C, using 0.5 g·l^{-1} *n*-propanol as the internal standard.

Methanol control system The semiconductor sensor model TGS822 was purchased from Figaro Engineering Inc. (Minoh, Osaka). As shown in Fig. 1, the heater of the sensor was connected to a 5 V stabilized power supply (the heater consumed 660 mW). The sensor, with a series resistance R_1 and variable resistance R_2, was connected to a 10 V stabilized power supply. The sensor was connected in parallel to a conventional high-impedance recorder and to the detect relay model CR1 (Tsuruga Co. Ltd., Tokyo). Since the resistance of the sensor responds to the concentration of flammable gases and varies below about 10 kΩ, the output voltage range can be adjusted from 0 to 5 V by tuning R_2. The methanol feeding pump is switched on or off by the detect relay corresponding to a desired voltage, which can be changed by rotating its dial. Methanol feeding was monitored by a conventional weight sensor. All of the exhaust gas from the jar fermentor was introduced into the sensor box. The sensor box and gas line from the fermentor were heated to 50–60°C by a ribbon heater to avoid the condensation of dew which delays the response and may damage the sensor. Methanol was introduced into the culture broth to avoid its direct evaporation to the gas phase. An automatic antifoam system was used to avoid overflow of the culture which could damage the sensor.

Preparation of inoculum The preculture medium was composed of 10 g yeast extract (Difco, Detroit, USA), 20 g tryptone (Difco), 100 mM potassium phosphate, 6.7 g yeast nitrogen base (without amino acids, Difco), 0.4 mg biotin and 18 g glycerol in 1 *l* of deionized water. The yeast grown in 50 ml of preculture medium was inoculated into a 5-*l* jar fermentor (MDL-

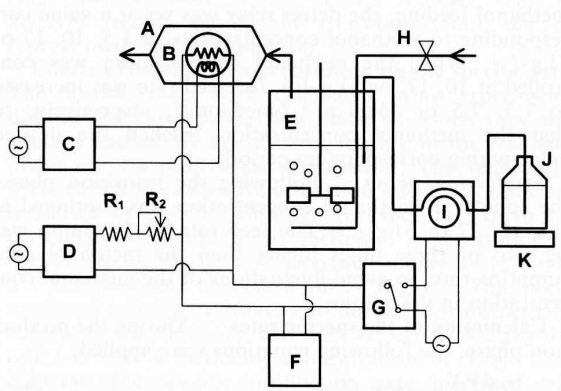


FIG. 1. Outline of the system for the control of methanol concentration. A, Sensor box; B, semiconductor sensor; C and D, stabilized direct current power supply (5 and 10 volts, respectively); E, jar fermentor; F, recorder; G, detect relay; H, flow control valve; I, peristaltic pump; J, methanol reservoir; K, digital weight sensor; R_1, 10 kΩ resistance; R_2, 10 kΩ variable resistance.

Motif Exhibit 1059, 379 of 653

Case No.: IPR2023-00321
U.S. Patent No. 10,689,656

500S; Marubishi Bioeng. Co. Ltd., Tokyo) containing 2 *l* of the preculture medium and was cultivated to OD_{600}= 50 (11.7 g dry-cell·l^{-1}) at 30°C, air flow rate of 3 *l*·min^{-1}, dissolved oxygen (DO)>20% and pH 6.0 (with 28% ammonium hydroxide). The cells were centrifuged at 2000×*g* for 5 min and suspended in the fresh preculture medium containing 150 g·l^{-1} glycerol at a cell density of about OD_{600}=200. The suspension was dispensed in sterilized tubes of 15 ml volume and stored at −80°C as an inoculum stock.

Culture conditions

Growth phase Two liters of the Basal Salts Medium (BSM; containing 26.7 ml H_3PO_4 (85%), 0.93 g $CaSO_4$, 18.2 g K_2SO_4, 14.9 g $MgSO_4 \cdot 7H_2O$, 4.13 g KOH and 40.0 g glycerol in 1 *l* of deionized water) was sterilized in the 5-*l* jar fermentor and its pH was adjusted to 5.0 by 28% ammonium hydroxide. After addition of 8.7 ml of the trace salts solution, PTM_1 (containing 6.0 g $CuSO_4 \cdot 5H_2O$, 0.08 g NaI, 3.0 g $MnSO_4 \cdot H_2O$, 0.2 g $Na_2MoO_4 \cdot 2H_2O$, 0.02 g H_3BO_3, 0.5 g $CoCl_2$, 20.0 g $ZnCl_2$, 65.0 g, $FeSO_4 \cdot 7H_2O$, 0.2 g biotin and 5.0 ml H_2SO_4 in 1 *l* of deionized water), the cultivation was started with addition of the inoculum stock described above. The temperature, pH, air flow rate and DO concentration were 30°C, 5.0 (with 28% ammonium hydroxide), 3 *l*·min^{-1}, >20%, respectively.

Transition phase After 15–20 min of glycerol exhaustion (identified by a rapid increase in DO), a glycerol solution (500 g·l^{-1}, containing 12 ml·l^{-1} PTM_1) was fed at 20 ml·h^{-1}·*l*-medium^{-1} for two hours. Glycerol feeding was continued for an additional 3 h at the flow rate 10, 7.5 and 5 ml·h^{-1}·*l*-medium^{-1} in the first, second and third hour, respectively. Glycerol starvation is believed to allow the cells to consume metabolites which promote the toxicity of methanol, and glycerol feeding helps the cell to adapt to grow and adapt to the production on methanol. When the glycerol feed rate was reduced from 20 to 10 ml·h^{-1}·*l*-medium^{-1}, feeding of methanol containing 12 ml·l^{-1} PTM_1 was started, using a conventional peristaltic pump and the detect relay was set at a value corresponding to a methanol concentration 1.5 g·l^{-1}. To avoid a rapid increase of methanol concentration which could damage the cells, the feed rate was set at 3.5 ml·h^{-1}·*l*-medium^{-1}. After 2 h of methanol feeding, the detect relay was set at a value corresponding to methanol concentrations of 1.5, 10, 17 or 31 g·l^{-1}. When the methanol concentration was controlled at 10, 17, or 31 g·l^{-1}, the feed rate was increased to 7.5, 7.5 or 15 ml·h^{-1}·*l*-medium^{-1}, respectively, so that the methanol concentration reached the desired value within corresponding periods.

Production phase Following the transition phase, the control of methanol concentration was continued at 1.5, 10, 17 or 31 g·l^{-1}. The feed rate of the pump was set two or three times higher than the methanol consumption rate, to avoid fluctuations of the methanol concentration in the culture.

Calculation of the specific rates During the production phase, the following equations were applied.

$$\frac{dVX}{dt} = \mu VX \quad (1)$$

$$\frac{dV_sP}{dt} = \rho VX \quad (2)$$

$$\frac{dV_sS}{dt} = FS_f + \nu VX = 0 \quad (3)$$

Since the cell concentrations in the production phase were more than 50 g dry-cell·l^{-1}, the total cell volume was not negligible compared with the culture volume. Therefore, the amounts of the product were calculated using the volume of the culture supernatant.

$$V_s = V - aVX \quad (4)$$

where V and V_s are culture volume and the volume of the culture supernatant, and a is the cell volume per dry-cell. We provided a=0.003 *l*·g dry-cell^{-1} in this study. Since equations 1–3 can be rewritten as follows under constant specific rates:

$$\ln(VX) = \mu t + \ln(V_0X_0) \quad (5)$$

$$V_sP = \rho \int_{t_0}^{t} VX\,dt + V_{s0}P_0 \quad (6)$$

$$\int_{t_0}^{t} FS_f\,dt = -\nu \int_{t_0}^{t} VX\,dt \quad (7)$$

the specific rates μ, ρ and ν were calculated from t vs $\ln(VX)$, $\int_{t_0}^{t} VX\,dt$ vs V_sP, and $\int_{t_0}^{t} VX\,dt$ vs $\int_{t_0}^{t} FS_f\,dt$ plot, respectively (14), where $\int_{t_0}^{t} FS_f\,dt$ and $\int_{t_0}^{t} VX\,dt$ were obtained from the weight change of the methanol reservoir and the area of t vs VX plot, respectively.

RESULTS AND DISCUSSION

Response of the sensor The sensor was calibrated using various dilutions of methanol in distilled water under the same conditions as for the yeast culture (at 30.0±0.1°C, air flow rate at 3 *l*·min^{-1} using the 5-*l* jar fermentor). A good linearity between the output voltage of the sensor and methanol concentration was observed (Fig. 2, circles). It was reported that flammable gases as well as ethanol in the exhaust gas affected the response of the sensor in the case of baker's yeast production (8). In our experiments, however, the methanol concentrations in the culture, as determined by gas chromatography, agreed well with those determined by the sensor (triangles in Fig. 2). Accordingly, we concluded that the effect of other flammable gases on the on-line monitoring of methanol concentration, if any, was negligible in our experiments. It was reported that oxygen concentration in the exhaust gas also affects the

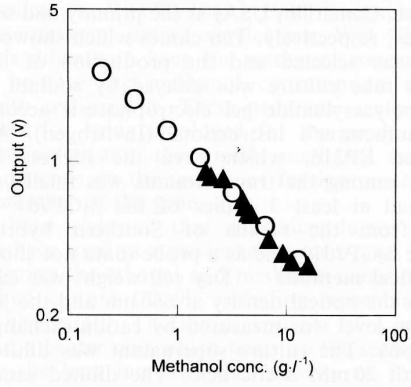


FIG. 2. Relationship between the output voltage of the sensor and methanol concentration. Symbols: circles, in distilled water; triangles, in the culture.

Motif Exhibit 1059, 380 of 653

Case No.: IPR2023-00321
U.S. Patent No. 10,689,656

response of the sensor (9). When the oxygen concentration in the exhaust gas decreases, the resistance of the sensor decreases because the density of free electrons increase. In our case, even at the highest cell concentration (80 g·l^{-1}), the relationship between methanol concentration, as determined by gas chromatography, and the output voltage of the sensor agreed well with that determined from the calibration curve shown in Fig. 2 (see also Fig. 3). This result indicates that the effect of decrease in the oxygen concentration in the exhaust gas due to respiration of the host cell, on the sensor response was negligible for our purposes. The temperature drift of the culture significantly influenced the sensor response, because the drift directly affects the methanol equilibrium between liquid and gas phase. We controlled the culture temperature at 30.0±0.1°C to minimize the effect of the drift. The agitation rate, which was increased during the fermentation from 300 rpm to 1000 rpm to keep the DO from exceeding 20%, did not affect the on-line monitoring of methanol concentration. Since the sensor resistance decreased with increasing air flow, the air flow rate was fixed at 3 l·min^{-1}; all of the exhaust gas was introduced into the sensor box in our study to avoid the effect of air flow rate on the sensor response.

Yamane *et al.* (15) and Guarna *et al.* (10) controlled the ethanol and methanol concentrations using a Teflon and a silicon tubing system, respectively, in which the ethanol and methanol permeating across the tubes are introduced into the detector by a carrier gas. Although this system can prevent the effect of foaming in aerobic processes and be used for anaerobic processes, parameters such as tube length and flow rate of the carrier gas must be optimized to obtain a suitable response and sensitivity of the sensor, even when a tube with suitable properties is selected. We introduced the exhaust gas from the fermentor directly into the sensor to simplify the system. In conclusion, when the temperature of the fermentor is kept at 30.0±0.1°C, the exhaust line is heated up by the ribbon heater, and the air flow rate is constant, the methanol concentration in the culture could be maintained within ±5% of the set point even when the agitation rate was increased with cell growth.

Time course of the fermentation The time-courses of fermentation in which methanol concentrations were kept at 10 and 31 g·l^{-1} are shown in Fig. 3. The growth of the cells in the production phase at a methanol concentration of 31 g·l^{-1} was slower than that at 10 g·l^{-1}. After 60 h, the specific production rate decreased. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the culture supernatants showed that the decrease was due to proteolytic degradation of the protein (data not shown). Although it might be possible to avoid degradation by the addition of casamino acid to the medium (16) or by changing the pH of the culture (Brerley, R. A. *et al.*, US patent 5,324,639, 1994; Siegel, R. S. *et al.*, International patent application W/O90/10697), we evaluated the effects of methanol concentration on the production of β_2GP/dV by the specific rates before the proteolysis.

Trends of the specific rates The specific rates of growth, methanol consumption and production of β_2 GP/dV were calculated from the experimental results under the methanol concentrations of 1.5, 10, 17, and 31 g·l^{-1} based on equations (5–7). The tendency for transition of the specific rates was almost similar at all methanol concentrations. The specific growth rates during the transition phase were higher than those during the production phase because not only methanol but also glycerol were fed as a carbon source (Fig. 3). The specific production rates during the transition phase were also higher than those during the early production phase (Fig. 4A, squares). The decrease in specific production rates in the late production phase was due to proteolytic degradation as described above. The specific methanol consumption rates during the transition phase were slightly higher than those during the production phase (Fig. 4B, squares). These results indicate that the glycerol feeding during the transition phase did not repress methanol consumption.

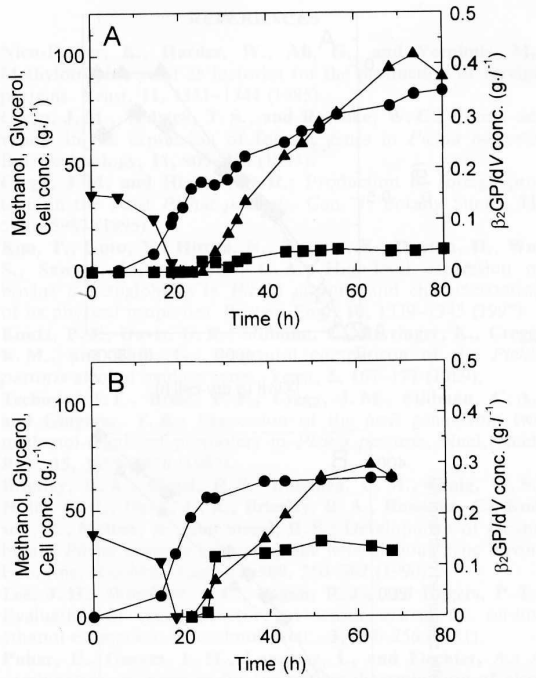


FIG. 3. Time-course of the culture. Methanol concentration in the culture during the production phase was controlled at 10 (A) and 31 g·l^{-1} (B), respectively. Symbols: ●, cell; ▲, β_2GP/dV; ■, methanol; ▼, glycerol.

Effects of methanol concentration on the specific rates The specific rates were calculated using experimental data in the early production phase in which they were affected neither by the glycerol feeding in the transition phase nor by proteolytic degradation. The relationship between the methanol concentration and the specific rate is shown in Fig. 5. Although the specific growth rate decreased gradually as the methanol concentration increased up to 17 g·l^{-1}, it decreased markedly to 0.0009 h^{-1} at a methanol concentration of 31 g·l^{-1}. The specific methanol consumption rate also decreased with methanol concentration, however, the rate was still 0.042 g·g dry-$cell^{-1}$·h^{-1} at a methanol concentration of 31 g·l^{-1}. In contrast, the specific production rate increased with the methanol concentration. At high methanol concentrations, cell growth was markedly inhibited and the consumption of methanol was inhibited slightly, but β_2GP/dV production increased. The remarkable inhibition of cell growth at a methanol concentration of 31 g·l^{-1} appeared to be mainly due to the toxic effects of

Motif Exhibit 1059, 381 of 653

Case No.: IPR2023-00321
U.S. Patent No. 10,689,656

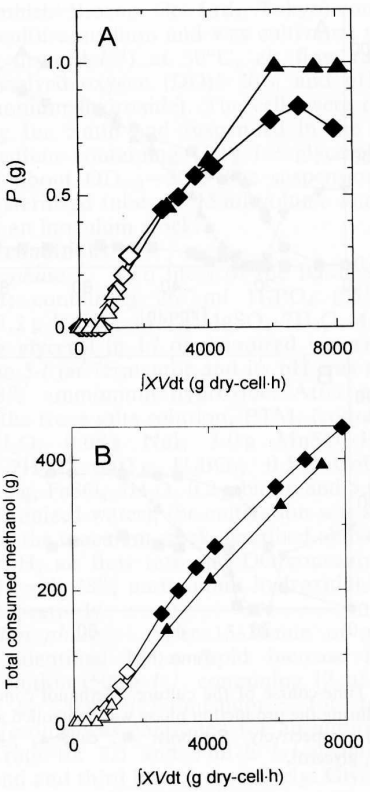


FIG. 4. Calculation of the specific rates. A, The specific production rate; B, the specific methanol consumption rate. Symbols: squares, methanol concentration controlled at 10 g·l^{-1}; triangles, methanol concentration controlled at 5.5 g·l^{-1} and glycerol feeding at the rate 5.0 ml·l-medium^{-1}·h^{-1} during the production phase. Filled symbols indicate the production phase.

methanol on DNA replication and membrane synthesis, because metabolism and protein biosynthesis were not significantly inhibited. The following three interpretations are proposed to explain the increase in β_2GP*IdV* production in the presence of the high methanol concentrations.

The first is an increase of β_2GP*IdV* gene transcription, the second is an increase of β_2GP*IdV* permeability across the cell membrane, and the third is an increase in energy supply for β_2GP*IdV* production. The transcription of β_2GP*IdV* and alcohol oxidase, a key enzyme for methanol utilization, are both under the control of the *AOX*1 promoter. It was reported that the specific alcohol oxidase activity decreased at methanol concentrations of greater than 20 g·l^{-1} (17), and the specific methanol consumption rate decreased at such high methanol concentrations (Fig. 5). Accordingly, the increase in β_2GP*IdV* production could not be due to increase in the level of transcription. Although it is known that solvents including methanol permeabilize cell membranes, if permeabilization of the membrane promotes the secretion of β_2GP*IdV* of a large molecular size, low-molecular-size metabolites should leak also from the cell and such leakage could seriously damage cell activities. Therefore, β_2GP*IdV* production is increased neither due to an increase in the level of transcription nor by the permeabilization. In contrast, the third interpretation is reasonable. The energy for cell growth and β_2GP*IdV* production is supplied by methanol oxidation, and the growth and production compete with each other for the energy in the production phase. When the cell growth is inhibited at high methanol concentrations, the energy for cell growth could be diverted for β_2GP*IdV* production, resulting in an increase in the productivity. This interpretation is supported by the observation that the specific production rates in the transition phase (clear squares in Fig. 4A) were higher than those in the production phase (filled squares in Fig. 4A). Since glycerol was fed in addition to methanol during the transition phase, the energy supply for β_2GP*IdV* production increased. Methanol is not only an inducer of but also an energy source for the production of foreign proteins. However, these results indicate that the energy from the added methanol was insufficient for the production.

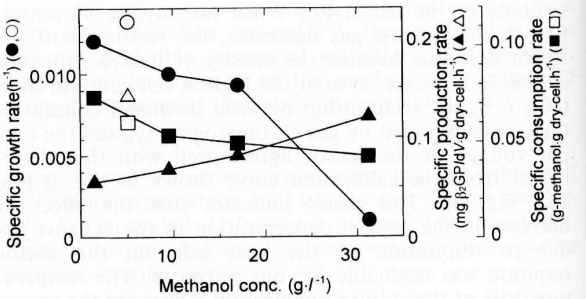


FIG. 5. Effects of methanol concentration on the specific rates. Symbols: circles, the specific growth rates; squares, the specific methanol consumption rates; triangles, the specific production rates. Clear symbols indicate the case under which the glycerol solution was fed at 5.0 ml·h^{-1}·l-medium^{-1} during the production phase.

Mixed feeding in the production phase The simplest method to increase the energy supply to recombinant cells is to increase the concentration of the carbon sources in the culture broth. However, if the methanol concentration is increased, cell growth would be inhibited in the case of recombinant *P. pastoris*. Although glucose was reported to repress the *AOX*1 promoter (2), glycerol does not repress its expression as stated above. Therefore, the glycerol solution was fed at 5.0 ml·l-medium^{-1}·h^{-1} throughout the production phase during which the methanol concentration was controlled at 5.5 g·l^{-1}. The specific rates during the early production phase were almost constant (clear symbols in Fig. 5) although the glycerol feed rate was constant. The specific methanol consumption rate was almost the same as that in the absence of glycerol feeding (see also Fig. 4B). The specific growth rate at a methanol concentration of 5.5 g·l^{-1} as estimated from the data in the absence of glycerol feeding (filled circles in Fig. 5) was 0.011 h^{-1}. The specific growth rate in the presence of glycerol feeding (clear circles in Fig. 5) was about 20% higher than that in the absence of glycerol feeding. The specific production rate of 0.14 mg·g dry-cell^{-1}·h^{-1} (clear triangle in Fig. 5) was 2.3 times higher than that in the absence of glycerol feeding (filled triangles in Fig. 5). In the absence of glycerol feeding, the specific production rates in the early production phase were lower than those in the transition phase (squares in Fig. 4A). In contrast, in the presence of glycerol (triangles in Fig. 4B),

Motif Exhibit 1059, 382 of 653

Case No.: IPR2023-00321
U.S. Patent No. 10,689,656

the specific production rate in the early production phase was almost same as that in the transition phase. These results demonstrated that the glycerol feeding increased the energy supply and increased the productivity of foreign proteins in *Pichia* fermentation.

Conclusions The glycerol feed rate and the methanol concentration should be optimized individually for the production of each foreign protein, because the optimum conditions should depend on the species of the protein, the copy number of the gene, and how the gene(s) is introduced into the chromosome of the host cell. For example, when a gene of interest is introduced by replacement or integration, the recombinants have the *aox1* or *AOX*1 genotype, that is, low or high alcohol oxidase activity, respectively. Therefore, the glycerol feed rate for replacement-type recombinants would be higher than that for integration-type recombinants, because methanol is oxidized by only the weak *AOX*2 product. Furthermore, it is experimentally known that the tolerance of integration-type recombinants to methanol is usually higher than that of the replacement-type, indicating that the tolerance varies with the specific activity of alcohol oxidase.

In conclusion, (i) a simple, practical and inexpensive methanol control system was constructed, (ii) the effect of methanol concentration on the specific growth, methanol consumption and production rate in recombinant *P. pastoris* culture was first studied and (iii) it was demonstrated that the specific production rate of foreign proteins could be increased by glycerol feeding during the production phase.

NOMENCLATURE

a : cell volume per dry-cell, $l \cdot$g dry-cell^{-1}
F : methanol feed-rate, g$\cdot$h^{-1}
P : β_2GP*Id*V concentration in the culture, g$\cdot l^{-1}$
S : methanol concentration in the culture, g$\cdot l^{-1}$
S_f : methanol concentration of the feed, g$\cdot l^{-1}$
V : volume of the culture, l
V_s : volume of the culture supernatant, l
X : dry cell concentration, g$\cdot l^{-1}$
μ : specific growth rate, h^{-1}
ν : specific methanol consumption rate, g methanol$\cdot$g dry-cell$^{-1}\cdot$h^{-1}
ρ : specific β_2GP*Id*V production rate, g β_2GP*Id*$V\cdot$g dry-cell$^{-1}\cdot$h^{-1}
Subscripts indicate the time, h

ACKNOWLEDGMENTS

We thank Dr. Russ Brierley, Cephalon Inc., for kind guidance on glycerol feeding in the transition phase, Mr. Hiroki Ema for technical assistance, Dr. Yoshihisa Hagiwara for providing purified β_2GP*Id*V and Dr. Mikio Nakajima for helpful suggestions.

REFERENCES

1. **Nico-Farber, K., Harder, W., Ab, G., and Veenhuis, M.:** Methylotrophic yeast as factories for the production of foreign proteins. Yeast, **11,** 1331–1344 (1995).
2. **Cregg, J. M., Vedvick, T. S., and Raschke, W. C.:** Recent advances in the expression of foreign genes in *Pichia pastoris*. Bio/Technology, **11,** 905–910 (1993).
3. **Cregg, J. M. and Higgins, D. R.:** Production of foreign proteins in the yeast *Pichia pastoris*. Can. J. Botany Supp., **73,** 5981–5987 (1995).
4. **Kim, T., Goto, Y., Hirota, N., Kuwata, K., Denton, H., Wu, S., Sawyer, L., and Batt, C. A.:** High-level expression of bovine β-lactoglobulin in *Pichia pastoris* and characterization of its physical properties. Protein Eng., **10,** 1339–1345 (1997).
5. **Koutz, P. J., Davis, G. R., Stillman, C., Barringer, K., Cregg, K. M., and Thill, G.:** Structural comparison of the *Pichia pastoris* alcohol oxidase genes. Yeast, **5,** 167–177 (1989).
6. **Tschopp, J. F., Brust, P. F., Cregg, J. M., Stillman, C. A., and Gingeras, T. R.:** Expression of the *lacZ* gene from two methanol-regulated promoters in *Pichia pastoris*. Nucl. Acids Res., **15,** 3859–3876 (1987).
7. **Brierley, R. A., Siegel, R. S., Bussinea, C. M., Craig, W. S., Holtz, G. C., Davis, G. R., Brierley, R. A., Bussinea, C., Kosson, R., Melton, A., and Siegel, R. S.:** Development of recombinant *Pichia pastoris* expressing the heterologous gene bovine lysozyme. Biochem. Eng. VI, **589,** 350–362 (1990).
8. **Lee, J. H., Woodard, J. C., Pagan, R. J., and Rogers, P. L.:** Evaluation of semiconductor gas sensor system for on-line ethanol estimation. Biotechnol. Lett., **3,** 251–256 (1981).
9. **Puhar, E., Guerra, L. H., Lorencez, I., and Fiechter, A.:** A combination of methods for the on-line determination of alcohols in microbial cultures. Eur. J. Appl. Microbiol. Biotechnol., **9,** 227–233 (1980).
10. **Guarna, M. M., Lesnicki, G. J., Tam, B. M., Robinson, J., Radzimiski, C. Z., Hasenwinkle, D., Boraston, A., Jervis, E., MacGllivaray, R. T. A., Turner, R. F. B., and Kilburn, D. G.:** On-line monitoring and control of methanol concentration in shake-flask cultures of *Pichia pastoris*. Biotechnol. Bioeng., **56,** 279–286 (1997).
11. **Morrison, D. H.:** Semiconductor gas sensors. Sensors Actuators, **2,** 329–341 (1982).
12. **Heiland, G.:** Homogeneous semiconducting gas sensors. Sensors Actuators, **2,** 343–361 (1982).
13. **Hagiwara, Y., Enjoji, K., Omasa, T., Katakura, Y., Suga, K., Igarashi, T., and Goto, Y.:** Structure and function of the recombinant fifth domain of human β_2-glycoprotein I: Effects of specific cleavage between Lys77 and Thr78. J. Biochem., **121,** 128–137 (1996).
14. **Omasa, T., Higashiyama, K., Shioya, S., and Suga, K:** Effect of lactate concentration on hybridoma culture in lactate-controlled fed-batch operation. Biotechnol. Bioeng., **39,** 556–564 (1992).
15. **Yamane, T., Matsuda, M., and Sada, E.:** Application of porous Teflon tubing method to automatic fed-batch culture of microorganisms. II. Automatic constant-value control of fed substrate (ethanol) concentration in semibatch culture of yeast. Biotechnol. Bioeng., **13,** 2509–2524 (1981).
16. **Clare, J. J., Rayment, F. B., Ballantine, S. P., Sreekrishna, K., and Romanos, M. A.:** High-level expression of tetanus toxin fragment C in *Pichia pastoris* containing multiple tandem integrations of the gene. Bio/Technology, **9,** 455–460 (1991).
17. **Duff, S. J. B. and Murry, W. D.:** Production and application of methylotrophic yeast *Pichia pastoris*. Biochem. Bioeng., **31,** 44–49 (1988).

Motif Exhibit 1059, 383 of 653

Ex. 1034 Hong 2002

Volume 79, Number 4, August 20, 2002
Articles published online in Wiley InterScience 12 June 2002–18 June 2002

Biotechnology and Bioengineering



articles in this journal are available online ahead of the print is
Wiley InterScience
EarlyView®
www.interscience.wiley.co

Biotechnology and Bioengineering

Editor
Douglas S. Clark
Department of Chemical Engineering, University of California
Berkeley, California

Associate Editors

James D. Bryers
University of Connecticut
Farmington, Connecticut

Pauline Doran
University of New South Wales
Sydney, Australia

Jonathan S. Dordick
Rensselaer Polytechnic Institute
Troy, New York

Charles A. Haynes
University of British Columbia
Vancouver, BC, Canada

Jeffrey A. Hubbell
ETH—Zürich and
University of Zürich
Zürich, Switzerland

Jay D. Keasling
University of California
Berkeley, California

Lee R. Lynd
Dartmouth College
Hanover, New Hampshire

Tadashi Matsunaga
Tokyo University of Agriculture and Technology
Tokyo, Japan

Jens Nielsen
Technical University of Denmark
Lyngby, Denmark

Eleftherios T. Papoutsakis
Northwestern University
Evanston, Illinois

Editorial Board

Harvey W. Blanch
University of California
Berkeley, California

Ho Nam Chang
Korea Advanced Institute of Science & Technology
Taejon, Korea

George Georgiou
University of Texas
Austin, Texas

Isao Karube
University of Tokyo
Tokyo, Japan

Alexander M. Klibanov
Massachusetts Institute of Technology
Cambridge, Massachusetts

Maria-Regina Kula
Heinrich-Heine Universität Düsseldorf
in der KFA Jülich, Germany

Douglas A. Lauffenburger
Massachusetts Institute of Technology
Cambridge, Massachusetts

Bernhard Ø. Palsson
University of California at San Diego, La Jolla, California

Michael L. Shuler
Cornell University
Ithaca, New York

John Villadsen
Technical University of Denmark
Lyngby, Denmark

Daniel I.C. Wang
Massachusetts Institute of Technology
Cambridge, Massachusetts

Founding Editor: Elmer L. Gaden, Jr., *University of Virginia, Charlottesville, Virginia*

Biotechnology and Bioengineering (Print ISSN: 0006-3592; Online ISSN: 1097-0290 at Wiley InterScience, www.interscience.wiley.com) Volumes 77–80 are published 28 times a year, semi-monthly, except tri-monthly in March, June, September, and December, by Wiley Subscription Services, Inc., a Wiley Company, 605 Third Avenue, New York, NY 10158.

Copyright © 2002 Wiley Periodicals, Inc., a Wiley Company. All rights reserved. No part of this publication may be reproduced in any form or by any means, except as permitted under section 107 or 108 of the 1976 United States Copyright Act, without written permission of the publisher, authorization through the Copyright Clearance Center, 222 Rosewood Drive, Danvers, MA 01923, 978-750-8400, fax 978-750-4470. Periodicals postage paid at New York, NY, and at additional mailing offices.

The copyright notice appearing at the bottom of the first page of an article in the journal indicates the copyright holder's consent that copies may be made for personal or internal use, or for the personal or internal use of specific clients, on the condition that the copier pay for copying beyond that permitted by Sections 107 and 108 of the United States Copyright Law. The per copy fee for each item is to be paid through the Copyright Clearance Center. This consent does not extend to other kinds of copying, such as copying for general distribution, for advertising or promotional purposes, for creating new collective works, or for resale. Such permission requests and other permission inquiries should be addressed to the Permissions Department, c/o John Wiley & Sons, Inc., 605 Third Avenue, New York, NY 10158-0012; (212) 850-6011, fax (212) 850-6008, e-mail: permreq@wiley.com.

Subscription Price (Volumes 77–80, 2002): Print only: $4125.00 in US, $4405.00 in Canada and Mexico, $4643.00 outside North America. Electronic only: $4125.00 worldwide. A combination price of $4322.00 in U.S., $4612.00 in Canada and Mexico, $4850.00 outside North America, includes the subscription in both electronic and print formats. Personal rate: $495.00 in North America, and $663.00 rest of world. Subscriptions at the personal rate are available to individuals. All subscriptions containing a print element, shipped outside U.S., will be sent by air. Payment must be made in U.S. dollars drawn on U.S. bank. Claims for undelivered copies will be accepted only after the following issue has been delivered. Please enclose a copy of the mailing label. Missing copies will be supplied when losses have been sustained in transit and where reserve stock permits. Please allow four weeks for processing a change of address. For subscription inquiries, please call (212) 850-6645; E-mail: SUBINFO@wiley.com.

Postmaster: Send address changes to Biotechnology and Bioengineering. Caroline Rothaug, Director, Subscription Fulfillment and Distribution, c/o John Wiley & Sons, Inc., 605 Third Avenue, New York, NY 10158.

Advertising Sales: Inquiries concerning advertising should be forwarded to Advertising Sales, c/o John Wiley & Sons, Inc., 605 Third Avenue, New York, NY 10158; (212) 850-8832. Advertising Sales, European Contacts: Jackie Sibley, c/o John Wiley & Sons, Ltd., Baffins Lane, Chichester, West Sussex PO19 1UD, England. Tel: 44 1243 770 351; Fax: 44 1243 770 432; e-mail: adsales@wiley.co.uk.

Reprints: Reprint sales and inquiries should be directed to the Customer Service Department, c/o John Wiley & Sons, Inc., 605 Third Avenue, New York, NY 10158. Tel: (212) 850-8789.

Manuscripts should be submitted to Biotechnology and Bioengineering, Editorial Receiving Office, c/o John Wiley & Sons, Inc., 605 Third Avenue, New York, NY 10158-0012. Phone: (212) 850-6297; Fax: (212) 850-8881; E-mail: biotechbioeng@wiley.com

All Other Correspondence: Address to Biotechnology and Bioengineering, Publisher, c/o John Wiley & Sons, Inc., 605 Third Ave., New York, NY 10158.

The contents of this journal are indexed or abstracted in Analytical Abstracts, BIOSIS Previews, Biological Abstracts, Biotechnology Citation Index®, Chemical Abstracts, Current Contents®/Agriculture, Biology, and Environmental Sciences, Current Contents®/Life Sciences, International Abstracts in Operations Research (IAOR), IAOR-Online, ISI Alerting Services (includes Research Alert®), Reference Update®, Science Citation Index®, and SciSearch® (also known as Science Citation Index-Expanded).

Editorial Production, John Wiley
Merilee Croft Olson

This paper meets the requirement of ANSI/NISO Z39.48-1992 (Permanence of Paper)

Biotechnology and Bioengineering

Volume 79, Number 4, August 20, 2002

Contents

367 Protein–Protein Interactions in Concentrated Electrolyte Solutions. *Hofmeister-Series Effects*
R.A. Curtis, J. Ulrich, A. Montaser, J.M. Prausnitz, and H.W. Blanch
Published online 12 June 2002

381 Ultra Scaledown to Predict Filtering Centrifugation of Secreted Antibody Fragments From Fungal Broth
N. Boulding, S.S.S. Yim, E. Keshavarz-Moore, P.A. Shamlou, and M. Berry
Published online 12 June 2002

389 Effect of Complexing Agents on Reduction of Cr(VI) by *Desulfovibrio vulgaris* ATCC 29579
A.N. Mabbett, J.R. Lloyd, and L.E. Macaskie
Published online 12 June 2002

398 The Response of GS-NS0 Myeloma Cells to Single and Multiple pH Perturbations
J.J. Osman, J. Birch, and J. Varley
Published online 12 June 2002

408 The Effect of Ajmalicine Spiking and Resin Addition Timing on the Production of Indole Alkaloids from *Catharanthus roseus* Cell Cultures
C.W.T. Lee-Parsons and M.L. Shuler
Published online 18 June 2002

416 A Two-Phase Model for Water and Heat Transfer Within an Intermittently-Mixed Solid-State Fermentation Bioreactor with Forced Aeration
O.F. von Meien and D.A. Mitchell
Published online 13 June 2002

429 Differential Rates of Gene Expression Monitored by Green Fluorescent Protein
C. Lu, C.R. Albano, W.E. Bentley, and G. Rao
Published online 13 June 2002

438 Fermentation Strategies for Improved Heterologous Expression of Laccase in *Pichia pastoris*
F. Hong, N.Q. Meinander, and L.J. Jönsson
Published online 13 June 2002

(continued on the next page)

This Journal is Online
WILEY InterScience®
www.interscience.wiley.com

Volume 79, Number 4 was mailed the week of July 22, 2002.

(continued from the previous page)

450 Protein-Mediated Isolation of Plasmid DNA by a Zinc Finger-Glutathione S-Transferase Affinity Linker
J. Woodgate, D. Palfrey, D.A. Nagel, A.V. Hine, and N.K.H. Slater
Published online 13 June 2002

457 Anchorage of Cyclodextrin Glucanotransferase on the Outer Membrane of *Escherichia coli*
H.-M. Wan, B.-Y. Chang, and S.-C. Lin
Published online 13 June 2002

465 Polymer Surfaces Derivatized with Poly(Vinyl-*N*-Hexylpyridinium) Kill Airborne and Waterborne Bacteria
J.C. Tiller, S.B. Lee, K. Lewis, and A.M. Klibanov
Published online 13 June 2002

472 Selective Recovery of Lactate Dehydrogenase Using Affinity Foam
S. Fernandes, R. Hatti-Kaul, and B. Mattiasson
Published online 13 June 2002

481 Forthcoming Articles

Case No.: IPR2023-00321
U.S. Patent No. 10,689,656

Fermentation Strategies for Improved Heterologous Expression of Laccase in *Pichia pastoris*

Feng Hong,[1,2] Nina Q. Meinander,[3] Leif J. Jönsson[2*]

[1]*Department of Applied Microbiology, Centre for Chemistry and Chemical Engineering, Lund Institute of Technology/Lund University, P.O. Box 124, SE-22100 Lund, Sweden*
[2]*Division for Chemistry, Karlstad University, SE-651 88 Karlstad, Sweden; telephone: +46 54 7001801; fax: +46 54 7001457; e-mail: Leif.Jonsson@kau.se*
[3]*Molecular Sciences, AstraZeneca R&D Lund, SE-22187 Lund, Sweden*

Received 30 October 2001; accepted 7 February 2002

Published online 13 June 2002 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/bit.10297

Abstract: Improved expression of recombinant laccase by *Pichia pastoris* carrying the *lcc1* cDNA isolated from *Trametes versicolor* was achieved by optimization of the cultivation conditions in a fermentor equipped with a methanol sensor system. The results indicated that the activity obtained in fermentor cultivations was at least 7 times higher than in shake-flask cultures. Three different strategies for fermentor cultivations were compared: A (30°C, 1.0% methanol), B (20°C, 1.0% methanol), and C (20°C, 0.5% methanol). The laccase activity, particularly the specific activity, could be improved by decreasing the cultivation temperature. The mechanisms behind the temperature effect on the laccase activity may be ascribed to poor stability, release of more proteases from dead cells, and folding problems at higher temperature. The results showed that the methanol concentration had a marked effect on the production of active heterologous laccase. A fivefold higher volumetric laccase activity was obtained when the methanol concentration was kept at 0.5% instead of 1.0%. The detrimental effect of methanol on the production of recombinant laccase may be attributed to lower laccase stability, a higher proteolytic activity, and folding problems due to higher growth rate at 1.0% methanol. © 2002 Wiley Periodicals, Inc. *Biotechnol Bioeng* **79**: 438–449, 2002.
Keywords: laccase; heterologous expression; *Pichia pastoris*; temperature effect; methanol concentration

INTRODUCTION

Laccases are blue copper-containing phenol oxidases (EC 1.10.3.2) that are widely distributed in plants and certain fungi (Reinhammar, 1997). Laccases have been ascribed diverse biological functions in different organisms. They catalyze the oxidation of a variety of aromatic compounds, in particular phenols, with the concomitant reduction of molecular oxygen to water. White-rot fungi, such as *Trametes* (*Coriolus*, *Polyporus*) *versicolor*, are generally good producers of laccases. *T. versicolor* laccase is a secreted glycosylated enzyme with two disulphide bridges and four copper ions, one of which gives the protein its characteristic greenish-blue color (Reinhammar, 1984; Jönsson et al., 1995).

Recently, there has been great interest in laccase in relation to both the structure of the active site and the catalytic mode of action (Ducros et al., 1998) as well as the possibilities of using the enzyme for various applications. Potential applications include (i) delignification and biobleaching of pulp (Bourbonnais et al., 1997; Smith et al., 1997), (ii) detoxification of lignocellulose hydrolysates for ethanol production by yeast (Jönsson et al., 1998; Larsson et al., 1999), (iii) treatment of wastewater from industrial plants (Bergbauer et al., 1991), (iv) enzymatic removal of phenolic compounds in beverages (Cantarelli et al., 1989; Servili et al., 2000), (v) enzymatic modification of fibers and dye-bleaching in the textile and dye industries (Abadulla et al., 2000), (vi) construction of biosensors (Ghindilis, 2000), and (vii) manufacture of new compounded materials by using laccase and lignin wastes (Huttermann et al., 2001). All these applications require large quantities of enzyme, which makes the expression of laccase in heterologous systems an important issue.

The methylotrophic yeast *Pichia pastoris* can be used to express recombinant proteins under the control of the strong, tightly regulated, and methanol-induced alcohol oxidase promoter *AOX1* (Cereghino and Cregg, 2000). *P. pastoris* has the potential for high expression levels (Buckholz and Gleeson, 1991; Romanos et al., 1992; Cregg et al., 1993), efficient secretion of extracellular protein, post-translational modifications such as glycosylation (Tschopp et al., 1987) and growth to high cell densities on defined minimal medium (Brierley et al.,

*Correspondence to: L. J. Jönsson
Contract grant sponsor: Carl Trygger's Foundation

© 2002 Wiley Periodicals, Inc.

This material may be protected by Copyright law (Title 17 U.S. Code)

1990). A possible advantage with *P. pastoris* compared to many filamentous fungi is that it does not produce cellulolytic enzymes and laccase produced in this host could, therefore, potentially be applied directly in the pulp and paper industry without any purification. In addition, molecular genetic methods for *P. pastoris* are rapid and well developed, and the organism is easy to cultivate in large scale.

Previously, the *lcc1* cDNA, isolated from the white-rot fungus *T. versicolor*, was expressed in *P. pastoris* and was found to provide active secreted laccase (Jönsson et al., 1997). However, the expression levels obtained in shake-flask cultures were too low to be convenient for biochemical characterization of the recombinant enzyme. The objective of this work was, therefore, to optimize the expression of recombinant laccase by *P. pastoris* by using controlled conditions in fermentor cultivations and to find new strategies to improve the laccase activity.

Regulation and induction of heterologous gene expression by methanol has been shown to be simple, easy to scale-up, and cost-effective for industrial fermentations (Cregg et al., 1993). However, high concentrations of methanol are toxic to *P. pastoris* because of the accumulation of formaldehyde and hydrogen peroxide inside the cells (Couderc and Baratti, 1980; Murray et al., 1989). Therefore, accurate regulation of the methanol concentration in *P. pastoris* cultures is necessary not only to maintain the induction of genes under the control of the *AOX1* promoter but also to prevent accumulation of methanol to levels that are toxic to the cells. To obtain high cell densities while keeping a low level of methanol, a fed-batch strategy is commonly used. In the past, several complex feeding schemes were devised by using the dissolved oxygen (DO) spike method (Stratton et al., 1998). However, the DO spike protocol has several drawbacks. For example, it is complicated to implement and may repeatedly expose the cells to non-inducing levels of methanol because it is designed to maintain the residual methanol concentration close to zero, which is not necessarily optimal for maximum protein production (Zhang et al., 2000). Moreover, Mut^S clones, which have a disrupted *AOX1* gene and exhibit a slow methanol utilization rate, consume methanol too slowly for the "oxygen spike" technique to be practical (Guarna et al., 1997). Similarly, this technique cannot be used when mixed feed of glycerol and methanol is used to increase protein productivity during fermentation. Alternatively, it is of course possible to monitor the methanol concentration using HPLC or GC, but these methods are expensive and not easily implemented on-line. Therefore, in this study, a methanol sensor system, consisting of a probe with a gas-permeable silicone rubber tubing connected to an air carrier line, was used to conveniently monitor and control the methanol concentration on-line (Guarna et al., 1997; Wagner et al., 1997).

Cultivation of *P. pastoris* in fermentors is generally advisable because several fermentation parameters, such as pH and dissolved oxygen, can be adjusted and controlled to achieve higher levels of the desired protein (Cregg et al., 1993). In this work, the effects of temperature and methanol concentration on the expression of laccase by a Mut^+ transformant of *P. pastoris* were investigated. Possible mechanisms behind the findings are discussed.

MATERIALS AND METHODS

Chemicals

All chemicals were of spectral or analytical grade. Unless otherwise stated, all chemicals used in the preparation of culture media were obtained from Merck Eurolab AB (Stockholm, Sweden). Distilled/deionized water (Millipore, Bedford, MA) was used in all the experiments.

Organism

The *Pichia pastoris* strain SMD 1168 (*his4*, *pep4*) was transformed with the *lcc1* laccase gene (Jönsson et al., 1997) under control of the *AOX1* promoter by using the vector pHIL-D2, which contains both the promoter and transcription terminator of the *AOX1* gene. A Mut^+ phenotype transformant was maintained on YPD agar plates [Yeast Extract Peptone Dextrose medium: 10 g/L yeast extract, 20 g/L peptone (Oxoid Ltd. Hampshire, UK), 20 g/L dextrose (BDH, Poole, UK), and 20 g/L agar].

Culture Media

(1) BMGY/BMMY/BMM medium (buffered complex glycerol medium, buffered complex methanol medium, and buffered minimal methanol medium): BMGY contained 10 g/L yeast extract, 20 g/L peptone, 100 m*M* potassium phosphate buffer (pH 6.0), 13.4 g/L yeast nitrogen base with ammonium sulfate without amino acids (Difco, Detroit, MI), 400 μg/L biotin (Sigma-Aldrich, Steinheim, Germany), and 10 mL/L glycerol. For BMMY, the glycerol was substituted by 5 mL/L methanol. For BMM, yeast extract and peptone were omitted.

(2) PTM_1 trace salts contained 6.0 g/L cupric sulfate · $5H_2O$, 0.08 g/L sodium iodide, 3.0 g/L manganese sulfate · H_2O, 0.2 g/L sodium molybdate · $2H_2O$, 0.02 g/L boric acid, 0.5 g/L cobalt chloride, 20.0 g/L zinc chloride, 65.0 g/L ferrous sulfate · $7H_2O$, 0.2 g/L biotin, and 5.0 mL/L sulfuric acid.

(3) Fermentation basal salt medium contained 26.7 mL/L phosphoric acid (85%, Sigma, St. Louis, MO), 1.0 g/L calcium chloride · 2 H_2O, 18.2 g/L potassium sulfate, 14.9 g/L magnesium sulfate · $7H_2O$, 4.13 g/L potassium hydroxide, 40 g/L glycerol, 4.4 mL/L PTM_1

trace metal salts, and 0.5 mL/L antifoam emulsion (Dow Corning Antifoam RD Emulsion, Dow Corning, Midland, MI).

(4) The methanol feed solution consisted of filter-sterilized methanol (100%, BDH) supplemented with 12 mL/L PTM_1 trace salts. A concentrated aqueous solution of ammonium hydroxide (28–30%, Sigma) was used to adjust the pH to 5.0 and to provide the yeast with a nitrogen source.

Preparation of Inocula

Cells were transferred from YPD agar plates into 250-mL baffled Erlenmeyer flasks containing 50 mL of BMGY medium and 0.1 m*M* $CuSO_4$, and grown at 30°C in a rotary water bath-shaking incubator (Infors AG, Bottmingen, Switzerland) at 250 rpm. The cells were harvested in log-phase growth and were used as inoculum for the shake-flask and fermentor cultivations.

Shake-Flask Cultivations

Shake-flask cultivations were performed in 1000-mL baffled flasks containing 100 mL BMM or BMMY medium supplemented with 0.1 m*M* $CuSO_4$ at 20 or 30°C in the rotary shaker (250 rpm). The inoculum was harvested aseptically by centrifugation at 3000 g for 5 min under sterile conditions at room temperature. The pellet was then resuspended in BMM or BMMY medium to an OD_{600} of 1.0, as measured with a U-1100 spectrophotometer (Hitachi Ltd., Tokyo, Japan). An OD_{600} of 1.0 corresponds to a concentration of approximately 0.2 g (dry weight) per liter. Filter-sterilized 100% methanol was added to a final concentration of 0.5–2.0% daily to maintain induction. Samples were taken daily for spectrophotometric determination of cell growth and for assay of laccase activity.

Fermentor Cultivations

To scale up the expression of laccase in *P. pastoris*, a BioFlo III bench-top fermentor (New Brunswick Scientific Co. Inc., Edison, NJ) with a 2.5-L working volume water-jacketed glass vessel was used for batch and fed-batch fermentations. The fermentor was equipped with an Ingold pH electrode, an Ingold dissolved oxygen electrode (Mettler Toledo GmbH, Urdorf, Switzerland), and a methanol sensor system (Raven Biotech. Co., Vancouver, Canada) (Guarna et al., 1997) with a peristaltic pump (Model VS2-10R, Alitea AB, Stockholm, Sweden).

The fermentation of *P. pastoris* included two phases: first a growth phase on glycerol followed by an induction phase on methanol. All fermentations began with a batch growth phase in 1.0 L of basal salt medium containing 40 g/L glycerol as carbon source at 30°C to obtain high cell density. Before inoculation, the pH was adjusted to 5.0 with concentrated ammonium hydroxide, and 4.35 mL PTM_1 trace salts were supplemented aseptically. An inoculum (as described above) of 50 mL was added to the fermentor so that a final optical density of approximately 1.0 at 600 nm was obtained. The oxygen was supplied by using a constant flow of air (0.5 L/min) that was controlled by mass flow meters (type E6222A-2RH; Bronkhorst High-Tech, Ruurlo, The Netherlands). The agitation was set to be controlled automatically by the microprocessor of the fermentor in a range from a minimum of 500 rpm to a maximum of 1000 rpm to keep the dissolved oxygen (DO) level well over 20% of the saturation level. In addition to air, pure oxygen was supplied during the late period of batch cultivation on glycerol so that the DO was kept in the range of 20–30% of saturation. The pH of the medium was automatically maintained at 5.0 with ammonium hydroxide. The composition of the exhaust gas was continuously monitored by using a carbon dioxide and oxygen analysis system based on acoustic detection (model 1308; Brüel & Kjaer, Copenhagen, Denmark).

After depletion of the glycerol, methanol containing 12 mL of PTM_1 trace salts per liter was fed to start the methanol fed-batch phase (the induction phase or production phase when the *AOX1* promoter is used). The methanol was the sole carbon source. Pure oxygen was supplemented to keep the DO level above 20% during the whole induction phase on methanol. The methanol concentration was monitored and regulated on-line by the methanol sensor system. Samples were withdrawn every 12 h for optical density measurement, gravimetric analysis of biomass, laccase activity assay, and determination of total soluble protein and gel electrophoresis analysis. Antifoam was injected manually as required throughout the fermentation. Three fermentation strategies (as described below) were investigated.

Fermentation Strategies

Three different fermentation strategies, denoted A, B, and C, were used. In strategy A, the temperature was maintained at 30°C throughout the whole cultivation. Methanol was fed stepwise from 0.1 to 0.5% (v/v) at the beginning of the induction phase for half a day to adapt the culture to growth on methanol. Then, the methanol concentration was increased to 1.0% and kept at this level. In strategy B, the methanol feeding was the same as in strategy A, but the temperature was decreased from 30 to 20°C in the beginning of the induction phase on methanol and maintained at 20°C throughout the induction phase. In strategy C, the temperature was adjusted as described in strategy B. Methanol was added to the fermentor to reach a final concentration of 0.5% immediately after the glycerol was consumed and maintained at 0.5% during the entire induction phase.

Analyses of Fermentation Results

Cell Mass and Growth Rate

The optical density (OD) of the samples was measured against distilled water at 600 nm with a spectrophotometer (U-1100; Hitachi Ltd., Tokyo, Japan) after appropriate dilution ensuring a value within the linear range (0.1–1.0). The biomass was determined gravimetrically (as dry weight) for each time point. To determine the cell dry weight (CDW), 2.5 mL of the cell suspension was filtered through predried and preweighed Supor-200 membrane filters with a pore size of 0.45 μm (Gelman Laboratory, Pall Co., MI). The filtered pellets were then washed with 7.5 mL double-deionized water (Millipore) and dried in a microwave oven (Whirlpool, Benton Harbor, MI) at a power setting of 350 W for 15 min. The maximum growth rate (μ_{max}) was determined from the slope of the logarithmic dry weight (ln [CDW]) obtained in the log-phase versus time.

Determination of Protein Concentration

The concentration of protein in cell-free samples was determined by using the Coomassie Protein Assay Reagent (Pierce, Rockford, IL) following the manufacturer's instructions. Bovine serum albumin was used as the standard.

Effect of Temperature on the Stability of the Laccase

To determine the stability of laccase, both native laccase prepared and purified from *T. versicolor* (Fåhraeus and Reinhammar, 1967) and recombinant laccase in the crude culture broth of *P. pastoris* (strategy C) were assessed. The samples were first diluted with distilled water to an activity of around 9 U/mL. One-milliliter aliquots were then incubated in 1.5-mL tubes in water baths (Infors AG, Bottmingen, Switzerland) with 250 rpm shaking for 24 h at temperatures ranging from 10 to 30°C. After incubation, the laccase activity was measured spectrophotometrically at 414 nm.

Effect of Methanol Concentration on the Stability of the Laccase

The effect of the methanol concentration on the stability of recombinant laccase was assessed by incubation of 10 U of laccase from the supernatant of the fermentation (strategy C) mixed with deionized water and methanol. The native laccase from *T. versicolor* was used as control. Additional methanol was added to 0.5%, 1.0%, and 2.0% in a final volume of 1.0 mL in 1.5-mL tubes. The laccase samples were incubated at room temperature (≈23°C) for 24 h. The enzyme activity was measured spectrophotometrically at 414 nm as described below. The measurements were performed twice.

Laccase Activity Assay

Cell-free samples were obtained from the shake-flask cultivations and fermentations by centrifugation. Because BMMY contains interfering compounds, the supernatants from BMMY medium (for shake flask cultivations) were dialyzed before laccase activity assays. For dialysis of BMMY culture supernatants, Spectra/Pro membrane tubing (Spectrum Laboratories, Rancho Dominguez, CA) with a molecular weight cutoff of 12,000–14,000 was used. The samples were dialyzed against deionized water at 8°C overnight.

The detection of laccase activity was performed with a spectrophotometer (U-2000, Hitachi, Tokyo, Japan) using a reaction medium containing 0.4 m*M* ABTS (2, 2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid)), 50 m*M* acetate buffer (pH 5.2), supernatant from the fermentations after appropriate dilution in a total volume of 1.0 mL. The change in absorbance at 414 nm was recorded with the spectrophotometer for 5 min at 25°C. For calculating the activity, an extinction coefficient (ε) of $3.6 \times 10^4\ M^{-1} \cdot cm^{-1}$ was used (Childs and Bardsley, 1975). Laccase activity was expressed in units per milliliter of sample. One unit was defined as the amount of enzyme, which forms 1 micromole ABTS radical cation per minute under the conditions of the assay. The specific activity was expressed as the laccase activity in the supernatant as a function of the total amount of protein (in units per milligram of protein).

Protease Activity Assay

Proteolytic activity in the *P. pastoris* cultures was assayed by using the QuantiCleave protease assay kit (Pierce). This assay is based on the use of succinylated casein in conjunction with trinitrobenzenesulfonic acid (TNBSA) (Hatakeyama et al., 1992; Bubnis and Ofner, 1992). Proteases will cleave peptide bonds in the succinylated casein and expose primary amines (predominantly α-amines). Thereafter, TNBSA will react with the primary amines to generate an orange-yellow color. The color change was quantitated at 450 nm by using a spectrophotometer (U-2000, Hitachi). The assay was performed at 37°C for 20 min at pH 5.0 (acetate buffer, 50 m*M*) or pH 8.5 (borate buffer, 50 m*M*).

SDS-PAGE Analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) was performed by using 10% (w/v) polyacrylamide gels under reducing and denaturing conditions with a Mini-PROTEAN II system (Bio-Rad, Hercules, CA). Fifteen-microliter supernatant was loaded on the gel together with 5 μL of sample buffer. The protein in the gel was stained with Coomassie Brilliant Blue R-250. A silver stain kit (Bio-Rad) was used to examine the time course of protein secretion. The molecular weight of the enzyme was estimated

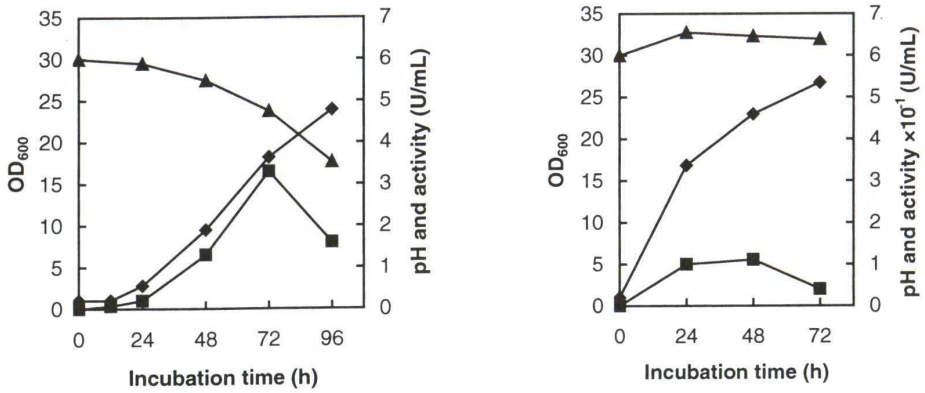


Figure 1. Time course of shake-flask cultivations in BMM (left) and BMMY (right) medium at 30°C. Methanol was added daily to a final concentration of 0.5%. The pH (▲), the OD_{600} (◆), and the laccase activity (■) are shown.

by using the low molecular weight marker (Pharmacia Biotech, Uppsala, Sweden) containing: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa).

Native-PAGE Analysis

Native-PAGE analysis was performed by using a 4–15% gradient gel (Bio-Rad) under nondenaturing conditions (i.e., SDS and 2-mercaptoethanol were omitted in the sample buffer and the samples were not boiled). The culture supernatant was concentrated 10–12 times by using Centricon centrifugal filter devices with YM-10 membranes (Millipore). Two native PAGE gels containing the same samples were stained with Coomassie Brilliant Blue R-250 and ABTS, respectively. The ABTS staining was performed by incubating the gel in a solution containing 0.4 m*M* ABTS and 50 m*M* acetate buffer, pH 5, on a shaker for 15–30 min at room temperature. During incubation, the pH of the ABTS staining solution was checked with pH paper and adjusted to 5 with acetic acid to avoid alkaline pH.

RESULTS

Heterologous Laccase Expression in Shake-Flask Cultivations

Heterologous laccase expression was initially assessed in shake-flask cultivations with BMM and BMMY medium at 30°C (Fig. 1). The pH of the culture broth declined gradually. With BMM medium, the highest laccase activity was 3.3 U/mL, which was observed after 3 days of induction; after that it declined to 1.6 U/mL (Fig. 1). With BMMY medium, the highest activity observed was only 0.11 U/mL (Fig. 1). The BMMY medium contained compounds interfering with the ABTS laccase activity assay. Therefore, the supernatants from cultivations with BMMY medium were dialyzed before the assay was performed (as described in Materials and Methods).

Effect of Methanol on Laccase Expression in Shake-Flask Cultivations

Variation of methanol concentration between 0.5 and 2.0% caused much less difference in cell growth (μ_{max} was approximately 0.044–0.047 h^{-1}) than in laccase activity (2.0–11.5 U/mL) (Fig. 2). The methanol accumulated gradually after 1 day when the concentration was 1.0% or higher (data not shown). However, this did not affect the growth to any greater extent (Fig. 2). At the end of the cultivations, the cell density achieved by daily methanol addition to 1.0% exceeded that obtained by daily methanol addition to 0.5%, which means that growth was not inhibited by the 1.0% methanol protocol. The laccase activity increased with lower level of methanol (Fig. 2). The highest activity, 11.5 U/mL, was obtained at 20°C by using 0.5% methanol additions and was around fourfold higher than at 30°C (Fig. 1).

Stability of Recombinant Laccase

Effect of Temperature

The stability of recombinant laccase decreased with increasing temperature (Fig. 3). If the residual activity at 10°C was set to 100%, 88% and 42% of the activity remained after incubation for 24 h at 20°C and 30°C, respectively. The stability of native laccase showed a similar correlation to temperature (data not shown).

Effect of Methanol Concentration

The recombinant laccase activity decreased with increasing methanol concentration (Fig. 4). Compared to the sample with 0.5% methanol, 90% and 80% of the activity remained after 24 h at room temperature in the samples treated with 1.0% and 2.0% methanol, respec-

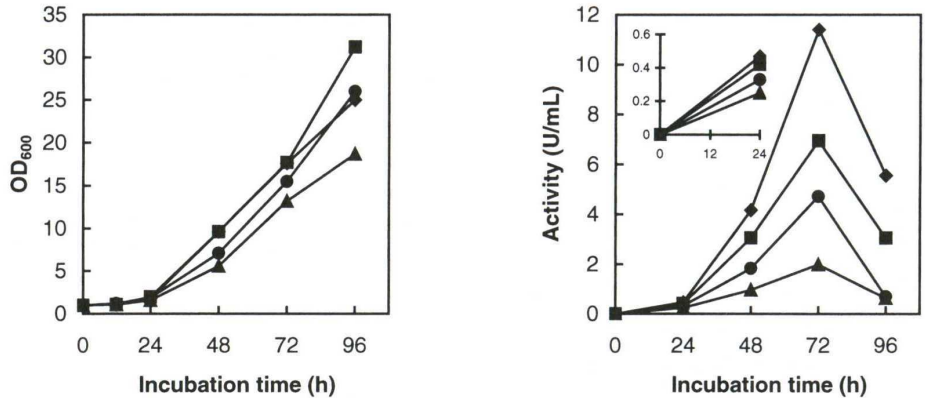


Figure 2. Effect of methanol concentration on laccase expression in shake-flask cultivations with BMM medium at 20°C. Methanol was added daily to a concentration of 0.5% (◆), 1.0% (■), 1.5% (●) and 2.0% (▲). The OD_{600} (left) and the laccase activity (right) are shown. The laccase activity during the first 24 h is indicated in the inset (right).

tively. In contrast, the activity of the native laccase was not negatively affected by methanol.

Scale-Up of Recombinant Laccase Production in Fermentors

Strategy A

The end of the glycerol batch phase was indicated by a spike in the DO caused by the exhaustion of glycerol and by a decrease in CO_2 in the off-gas to a level close to that in the beginning of the fermentation. Within 24–26 h after inoculation, the cells had consumed all the glycerol and had reached 20–22 g/L cell dry weight, corresponding to an OD value at 600 nm of around 100–110. After the growth phase on glycerol finished, the induction phase was initiated by feeding methanol with three different fermentation strategies.

In strategy A, the fermentation was performed at 30°C with 1.0% (v/v) methanol. Methanol was the sole carbon source and was automatically kept at 1.0% by the methanol sensor system. After 4 days, the fermentation yielded 120 g of dry biomass per liter (Fig. 5A). The consumption of methanol was fast owing to the Mut^+ phenotype of the strain. The maximum specific growth rate (μ_{max}) calculated for logarithmic growth was 0.035 h^{-1}.

The highest laccase activity, 24 U/mL, was reached after 3.5 days at a cell dry weight of 100 g/L. This activity was about 7 times higher than in shake-flask cultures with BMM medium at 30°C (3.3 U/mL, Fig. 1). The total soluble extracellular protein concentration at the time of the highest volumetric activity was 550 mg/L, giving a specific laccase activity of only 43 U/mg protein. However, the highest specific activity obtained at an earlier time-point was 150 U/mg protein (Fig. 5A). Both laccase activity and total amount of extracellular

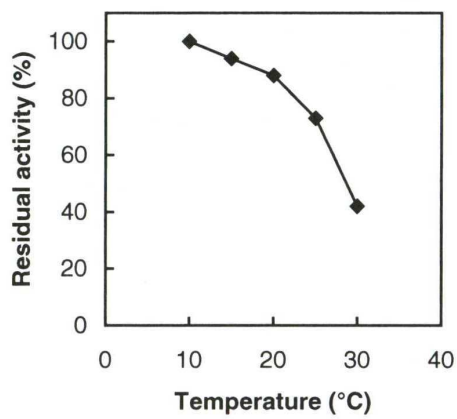


Figure 3. Stability of crude recombinant laccase at different temperatures. Laccase was incubated for 24 h in the culture medium after centrifugation. The residual activity of the 10°C sample was set to 100%.

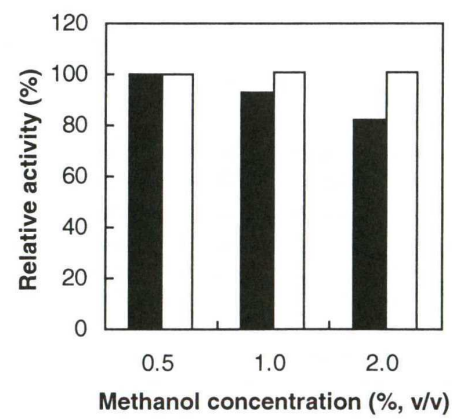


Figure 4. Laccase activity after incubation in different concentrations of methanol. The residual activity of the 0.5% sample was set to 100%. Recombinant laccase (■) and native laccase (□) are shown.

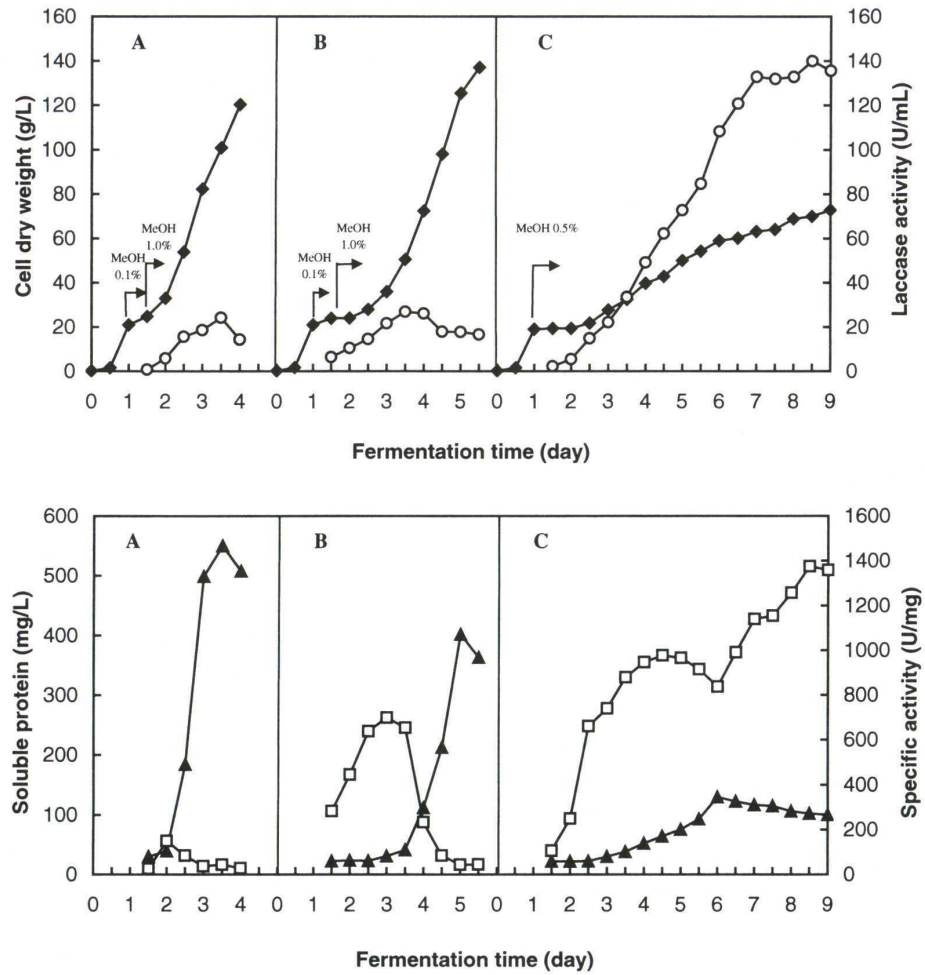


Figure 5. Fermentation of laccase-expressing *P. pastoris* using three different fermentation strategies A (30°C, 1%), B (20°C, 1%), and C (20°C, 0.5%). The cell dry weight (◆), the laccase activity (○), the total soluble protein (▲), and the specific activity (□) are indicated.

protein decreased significantly at the end of the fed-batch fermentation. The activity decreased even though the biomass increased further from 100 to 120 g/L.

Strategy B

In strategy B, 20°C and 1.0% methanol was used. The yeast still grew fast (Fig. 5B). However, compared to strategy A, the growth lag in the induction phase was 1 day longer when the temperature was decreased from 30 to 20°C. The growth rate after the lag phase reached a μ_{max} of 0.026 h^{-1}, which was slightly lower than in strategy A. The final biomass concentration was also beyond 100 g/L and the highest activity obtained (27 U/mL) was slightly higher than in strategy A (24 U/mL). The total soluble extracellular protein reached was 400 mg/L, giving a specific laccase activity of 44 U/mg protein at that time point. However, the highest specific activity obtained was 700 U/mg protein (Fig. 5B). The activity and amount of total protein decreased at the end of the fed-batch fermentation, as also observed for strategy A.

Although the difference in volumetric laccase activity between strategy A and B was slight, the level of total soluble protein at 20°C was lower than at 30°C. This contributed to the specific activity obtained at 20°C (700 U/mg protein), which was fivefold higher than at 30°C (150 U/mg protein). Therefore, it can be concluded that cultivation at 30°C was more beneficial for the growth of the yeast cells and the production of extracellular protein than cultivation at 20°C, but not for obtaining high laccase activity. In contrast, the laccase activity was improved by decreased cultivation temperature, particularly the specific activity.

Strategy C

In strategy C, 0.5% methanol was investigated at 20°C (Fig. 5C). The results showed that 0.5% methanol was

much better for producing active laccase than 1.0%. More than fivefold higher volumetric laccase activity (140 U/mL) was reached at 0.5% methanol compared to 1.0% (27 U/mL). The μ_{max} was only 0.012 h^{-1}, the lowest among the three fermentation strategies. The final dry cell weight reached only around 70 g/L. The total soluble protein concentration reached only 130 mg/L, and the highest specific laccase activity was 1380 U/mg protein. Furthermore, the results indicated that the total extracellular protein decreased slightly during the late period of the fermentation, as in the other fermentation strategies. The volumetric activity was at least 12-fold higher than in shake-flask cultures with BMM medium containing 0.5% methanol at 20°C (11.5 U/mL, Fig. 2).

Analyses of Culture Supernatants

SDS-PAGE Analysis

To determine the molecular weight of the recombinant laccase expressed by *P. pastoris*, SDS-PAGE was performed (Fig. 6). The M_r of the recombinant laccase (~67 kDa) was slightly higher than that of the native *T. versicolor* laccase, which is 64 kDa (Fåhraeus and Reinhammar, 1967). Figure 6 also indicates that a few other proteins were present in the *P. pastoris* cultures. For instance, bands around 30 and 43 kDa were found on the gel. Some protein bands, such as the ones around 43 kDa, were found also in the culture of the host strain SMD 1168 without any laccase gene inserted (data not shown).

To explain why the specific activity declined in the later phase of the fermentations, SDS-PAGE analysis of samples taken after different time points was performed. A more sensitive staining method, silver staining, was used. The amount of protein (0.5 μg) loaded on the gel was the same for all samples. The results indicate the appearance of many other proteins than laccase during the progress of the fermentations (not shown). The protein bands between 30 and 43 kDa were stronger at 30 than at 20°C, indicating that more protein was released into the culture medium at higher temperature. This result confirms the observation regarding the differences in total protein and specific activity between strategy A and B (Fig. 5A and B).

Zymogram Analysis

Zymogram analysis indicated that the molecular weight of the recombinant laccase was higher than that of native laccase (Fig. 7). Other protein bands were not associated with any activity. By using this novel zymogram staining technique, it was possible to identify and distinguish laccase from other proteins.

Proteolytic Activity Assay

Proteolytic activity was detected in the culture supernatants of all fermentations (Table I). The proteolytic activity decreased in the order strategy A, B, and C. The assay is generally performed at alkaline pH (8.5), but pH 5.0 was also tested because it was the pH of the fermentations. The proteolytic activity was higher at pH 5.0 than at pH 8.5, which indicates that most of the proteases, which were released by *P. pastoris* under the prevalent conditions, had acidic optimum.

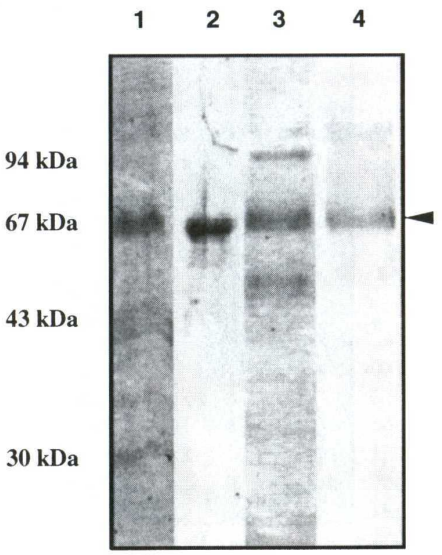


Figure 6. Proteins secreted by laccase-expressing *P. pastoris* analysed with SDS-PAGE and stained with Coomassie Brilliant Blue R-250. Culture supernatants (15 μL) from strategy A after 3.5 days (protein concentration: 550 mg/L) (lane 1), from strategy B after 5.0 days (400 mg/L) (lane 3), and from strategy C after 6.0 days (130 mg/L) (lane 4) were loaded on the gel. Purified native laccase from *T. versicolor* was used for comparison (lane 2). The arrow indicates the recombinant laccase. The positions of the markers are shown to the left.

DISCUSSION

Effects of pH

The decrease in laccase activity during the late period in shake-flask cultivation might be due to the low pH (Fig. 1). Previous results have indicated that low pH affects both recombinant (Jönsson et al., 1997) and native (Larsson et al., 2001) *T. versicolor* laccase

Table I. Proteolytic activity (arbitrary units) in the culture supernatants.

	Fermentation strategy		
Assay pH	A (30°C, 1%, 4.0 days)	B (20°C, 1%, 5.5 days)	C (20°C, 0.5%, 9.0 days)
pH 8.5	0.056	0.032	n.d.[a]
pH 5.0	0.30	0.27	0.21

[a]n.d.: not detectable.

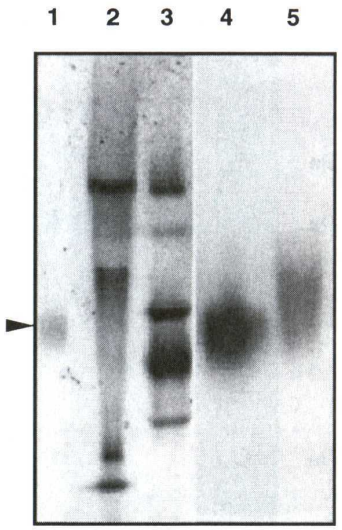


Figure 7. Native PAGE and zymogram analysis of laccase. Bands visualized by Coomassie Brilliant Blue R-250 staining (lanes 1, 2, and 3) and ABTS staining of active protein (lanes 4 and 5). Concentrated culture supernatants (15 μL) from strategy C after 8.5 days (activity was about 1400 U/mL) (lanes 2 and 5) were loaded on the gel. Purified native laccase from *T. versicolor* was used for comparison [lanes 1 (1.6 μg) and 4 (0.016 μg)]. Lane 3 contains marker proteins (30, 43, 67, and 94 kDa). The arrow indicates the native laccase.

negatively. Expression of other laccase isoforms in *P. pastoris*, for instance, laccase *LCCI* from *T. versicolor* (Gelo-pujic et al., 1999) and *lac1* from *Pycnoporus cinnabarinus* (Otterbein et al., 2000) has also been negatively affected by low pH.

Effects of Cultivation Temperature in the Induction Phase on Recombinant Laccase Expression

The temperature is an important parameter for optimization of laccase expression in yeast systems (Cassland and Jönsson, 1999). The activity of heterologous laccase expressed by *S. cerevisiae* cultivated in shake-flasks was found to be improved by lowering the temperature to approximately 19°C. The results also indicated that the laccase activity in *P. pastoris* shake-flask cultures was much higher at 16–19°C than at 28°C (Cassland and Jönsson, 1999), close to what is commonly used for heterologous expression in *P. pastoris*. The present study confirmed that cultivation at low temperature in shake flasks improved laccase activity (3.3 U/mL at 30°C, Fig. 1 and 11.5 U/mL at 20°C with 0.5% methanol additions, Fig. 2), and, in addition, showed that this was also the case under controlled conditions in a fermentor (Fig. 5).

P. pastoris is a widely used system for heterologous protein expression, but few results regarding production improvement by temperature optimization have been documented so far. Inan et al. (1999) showed that the temperature did not have any significant effect on the production of recombinant hookworm anticoagulant peptide (rAcAP-5) by *P. pastoris*. Li et al. (2001) showed that lower temperature increased the yield of recombinant herring antifreeze protein dramatically, which was attributed to improved protein folding as well as increased cell viability. Our results revealed that the cultivation temperature in the induction phase particularly influenced the specific laccase activity (Fig. 5). Together with the other observations, our results indicate that the effect of temperature on expression of heterologous proteins in the *P. pastoris* system is specific and due to the character of the expressed protein.

There may be several mechanisms behind the effect of fermentation temperature on laccase activity. First, poor thermostability of laccase may have led to lower activity in the 30°C cultures (Fig. 3). A second possible explanation is the action of proteases. Proteases can either be secreted or be released from dead cells. Both the Coomassie-stained and the silver-stained gels showed more low molecular weight protein bands in the culture medium at 30°C (strategy A) than at 20°C (strategy B). These low molecular weight protein bands could possibly be degradation products of proteins of higher molecular weight because protein bands were visible around 94 and 50 kDa at 20°C but not at 30°C (Fig. 6). Moreover, the protease assay confirmed that the proteolytic activity was higher in the samples from the fermentation performed at 30°C than at 20°C (Table I). Because the results presented in Table I were obtained by using the same incubation temperature (37°C) for all samples, the actual difference in proteolytic activity between the 20 and 30°C fermentation should be even greater because of a higher reaction rate at 30°C. Therefore, cultivation at 20°C decreases the degradation of secreted laccase compared to cultivation at 30°C. A third possibility is improper folding of the recombinant laccase at higher temperature. Cultivating the cells at lower temperature would slow the rate of protein synthesis as well as cell growth (μ_{max} was 0.026 and 0.035 h^{-1} at 20 and 30°C, respectively) and allow more time for the peptide chains to fold properly if folding is rate-limiting. Brierley et al. (1990) found that low growth rates could be desirable for production of certain recombinant proteins by Mut^S strains of *P. pastoris*. The fact that lower cultivation temperature may improve the production of heterologous protein in *E. coli* (Schein and Noteborn, 1988) has also been ascribed to aggregation problems at higher temperatures. In production of recombinant proteins using *E. coli*, lowering the cultivation temperature is routinely used for increasing the amount of properly folded protein.

Susceptibility to Acidic Proteases

One possible explanation for the decrease in laccase activity and total protein concentration in the later part

Case No.: IPR2023-00321
U.S. Patent No. 10,689,656

of the fermentations is susceptibility to acidic proteases. The pH was kept at 5.0 in the fermentor cultivations, and in the protease assay higher proteolytic activity was found at pH 5.0 than at pH 8.5 (Table I). A previous investigation indicated that the proteolytic activity played an important role for laccase expression by *P. pastoris* (Jönsson et al., 1997), because the protease deficient SMD 1168 strain performed better than GS115. The strain SMD 1168 is deficient in protease A and, therefore, also deficient in carboxypeptidase Y and protease B1, which are activated by protease A.

Although pH 5.0 is preferable for heterologous expressions of many proteins (Sreekrishna and Kropp, 1996; Stratton et al., 1998), the yield of recombinant protein has in some cases been improved by increasing the pH. For instance, production of human serum albumin (HSA) was improved more than threefold by using pH 5.85 instead of the generally used pH of 5.0 (Sreekrishna et al., 1990). Because pH 5.0 might reduce the level of active recombinant laccase due to susceptibility to acidic proteases as well as lower the enzyme stability (laccase was recently shown to be most stable at pH 6.0 and 7.0, Larsson et al., 2001), the optimal pH for the production of recombinant laccase needs to be investigated in more detail in the future.

Influence of Methanol Concentration on Recombinant Laccase Expression

The influence of two different methanol concentrations, 1.0% (v/v) and 0.5% (v/v) on recombinant laccase expression, was investigated at 20°C by comparing the feeding strategies B and C. A direct positive correlation between the amount of methanol consumed and the quantity of protein produced has been observed (Murray et al., 1989). In the present study, a positive correlation between total soluble protein and methanol consumption was evident. However, the activity of recombinant laccase did not correlate with the methanol concentration (Fig. 5B and C). The analyses showed that the concentration of native extracellular proteins increased with higher methanol concentration (Fig. 6).

The use of 1.0% methanol to induce the expression of foreign genes with *P. pastoris* has been recommended (Murray et al., 1989; Jiménez et al., 1997). Cell proliferation was not inhibited until the methanol concentration reached >3% (Murray et al., 1989). Our study also indicated that growth was not impaired with 1.0% methanol compared to 0.5%, and the cells grew twice as fast with 1.0% (μ_{max} 0.026 h^{-1}) as with 0.5% methanol (μ_{max} 0.012 h^{-1}) at 20°C (Fig. 5B and C). Similar observations were made for the shake-flask cultivations (Fig. 2).

Although 1.0% methanol was beneficial for the growth of the cell, the laccase activity was negatively affected. The lower laccase activity obtained may be partially due to lower enzyme stability in presence of increasing methanol concentration (Fig. 4). In addition, induction of the *AOX1* promoter with a lower methanol concentration would slow the rate of protein synthesis as well as the growth rate and allow more time for the peptide chains to fold properly. This effect may be similar to the temperature effect. Previously, Sreekrishna and Kropp (1996) concluded that longer fermentation times might be necessary to allow for accumulation of high levels of a slowly secreted protein in the broth. Furthermore, large amounts of methanol and a rapid metabolic rate when *P. pastoris* grows on 1.0% methanol may result in higher levels of formaldehyde and hydrogen peroxide, which could lead to the death of cells. This phenomenon occurs with another methylotrophic yeast, *Hansenula polymorpha* (Veenhuis et al., 1983). Thus, lower viability of cells in 1.0% methanol could lead to higher proteolytic activity (Table I) and release of more native proteins (Fig. 6). The possible effect of toxic products from methanol should also be kept in mind with regard to the effect of methanol on laccase stability in *P. pastoris* culture supernatant (Fig. 4), in particular because laccase was not negatively affected by up to 2% methanol in an aqueous solution without culture supernatant.

Gel Electrophoresis Analysis

The higher molecular weight of recombinant laccase compared with the native enzyme is likely due to glycosylation (Figs. 6 and 7). Compared to *S. cerevisiae*, *P. pastoris* is considered to be less likely to hyperglycosylate heterologous protein (Romanos et al., 1992). Previous studies showed that laccase expressed in *S. cerevisiae* was heterogeneous, and its M_r was much higher than that of the native enzyme (Cassland and Jönsson, 1999). Both *S. cerevisiae* and *P. pastoris* display N-linked glycosylation of the high-mannose type. Nevertheless, the length of the oligosaccharide chains added post-translationally to proteins in *P. pastoris* (average 8–14 mannose residues per side chain) is much shorter than in *S. cerevisiae* (50–150 mannose residues) (Tschopp et al., 1987; Grinna and Tschopp, 1989).

The other proteins appearing at 30°C might have been released from dead cells because of faster metabolic rate and/or comprise native secreted proteins from the host strain. Cell viability could be higher at lower temperature than 30°C (Li et al., 2001). Some additional protein bands were found around 30 and 43 kDa. Zymogram analysis with specific activity staining confirmed that the M_r of recombinant laccase was higher than the native, although some heterogeneity in electrophoretic mobility was apparent.

CONCLUSIONS

Laccase is a biotechnologically important enzyme, and it is of great interest to find simple and efficient systems for

Motif Exhibit 1059, 398 of 653

heterologous expression. We have explored the use of advanced fermentation control and different fermentation strategies to optimize the expression of laccase in the methylotrophic yeast *P. pastoris*. This work is the first in which recombinant laccase expression by *P. pastoris* is investigated in detail under controlled conditions in fermentor, especially concerning the effect of the cultivation temperature and methanol concentration. Production of laccase in *P. pastoris* was found to be affected by several factors, including the cultivation temperature, the pH, the proteolytic activity, and the methanol concentration. It was possible to greatly enhance both the volumetric and specific laccase activity in *P. pastoris* cultures using carefully controlled conditions and a strategy based on low-temperature and low-methanol concentration.

References

Abadulla E, Tzanov T, Costa S, Robra KH, Cavaco-Paulo A, Gübitz GM. 2000. Decolorization and detoxification of textile dyes with a laccase from *Trametes hirsuta*. Appl Environ Microbiol 66:3357–3362.

Bergbauer M, Eggert C, Kraepelin G. 1991. Degradation of chlorinated lignin compounds in a bleach plant effluent by the white-rot fungus *Trametes versicolor*. Appl Microbiol Biotechnol 35:105–109.

Bourbonnais R, Paice MG, Freiermuth B, Bodie E, Borneman S. 1997. Reactivities of various mediators and laccases with kraft pulp and lignin model compounds. Appl Environ Microbiol 63:4627–4632.

Brierley RA, Bussineau C, Kosson R, Melton A, Siegel RS. 1990. Fermentation development of recombinant *Pichia pastoris* expressing the heterologous gene: bovine lysozyme. Biochemical Engineering VI, Ann NY Acad Sci 589:350–362.

Bubnis WA, Ofner CM III. 1992. The determination of epsilon-amino groups in soluble and poorly soluble proteinaceous materials by a spectrophotometric method using trinitrobenzenesulfonic acid. Anal Biochem 207:129–133.

Buckholz RG, Gleeson MAG. 1991. Yeast systems for the commercial production of heterologous proteins. Biotechnology 9:1067–1072.

Cantarelli C, Brenna O, Giovanelli G, Rossi M. 1989. Beverage stabilization through enzymatic removal of phenolics. Food Biotechnol 3:203–214.

Cassland P, Jönsson LJ. 1999. Characterization of a gene encoding *Trametes versicolor* laccase A and improved heterologous expression in *Saccharomyces cerevisiae* by decreased cultivation temperature. Appl Microbiol Biotechnol 52:393–400.

Cereghino JL, Cregg JM. 2000. Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. FEMS Microbiol Rev 24:45–66.

Childs RE, Bardsley WG. 1975. The steady-state kinetics of peroxidase with 2, 2′-azino-di-(3-ethyl-benzthiazoline-6-sulfonic acid) as chromogen. Biochem J 145:93–103.

Couderc R, Baratti J. 1980. Oxidation of methanol by the yeast, *Pichia pastoris*. Purification and properties of the alcohol oxidase. Agric Biol Chem 44:2279–2289.

Cregg JM, Vedvick TS, Raschke WC. 1993. Recent advances in the expression of foreign genes in *Pichia pastoris*. Biotechnology 11:905–910.

Ducros V, Brzozowski AM, Wilson KS, Brown SH, Ostergaard P, Schneider P, Yaver DS, Pedersen AH, Davies GJ. 1998. Crystal structure of the type-2 Cu depleted laccase from *Coprinus cinereus* at 2.2 A resolution. Nat Struct Biol 5:310–316.

Fåhraeus G, Reinhammar B. 1967. Large scale production and purification of laccase from cultures of the fungus *Polyporus versicolor* and some properties of laccase A. Acta Chem Scand 21:2367–2378.

Gelo-pujic M, Kim HH, Butlin NG, Palmore GTR. 1999. Electrochemical studies of a truncated laccase produced in *Pichia pastoris*. Appl Environ Microbiol 65:5515–5521.

Ghindilis A. 2000. Direct electron transfer catalysed by enzymes: application for biosensor development. Biochem Soc Trans 28:84–89.

Grinna LS, Tschopp JF. 1989. Size distribution and general structural features of N-linked oligosaccharides from the methylotrophic yeast, *Pichia pastoris*. Yeast 5:107–115.

Guarna MM, Lesnicki GJ, Tam BM, Robinson J, Radziminski CZ, Hasenwinkle D, Boraston A, Jervis E, MacGillivray RTA, Turner RFB, Kilburn DG. 1997. On-line monitoring and control of methanol concentration in shake-flask cultures of *Pichia pastoris*. Biotechnol Bioeng 56:279–286.

Hatakeyama T, Kohzaki H, Yamasaki N. 1992. A microassay for proteases using succinylcasein as a substrate. Anal Biochem 204:181–184.

Huttermann A, Mai C, Kharazipour A. 2001. Modification of lignin for the production of new compounded materials. Appl Microbiol Biotechnol 55:387–394.

Inan M, Chiruvolu V, Eskridge KM, Vlasuk GP, Dickerson K, Brown S, Meagher MM. 1999. Optimization of temperature-glycerol-pH conditions for a fed-batch fermentation process for recombinant hookworm (*Ancylostoma caninum*) anticoagulant peptide (AcAP-5) production by *Pichia pastoris*. Enzyme Microb Technol 24:438–445.

Jiménez ER, Sánchez K, Roca H, Delgado JM. 1997. Different methanol feeding strategies to recombinant *Pichia pastoris* cultures producing high level of dextranase. Biotech Techniques 11:461–466.

Jönsson L, Sjöström K, Häggström I, Nyman PO. 1995. Characterization of a laccase gene from the white-rot fungus *Trametes versicolor* and structural features of basidiomycete laccases. Biochim Biophys Acta 1251:210–215.

Jönsson LJ, Saloheimo M, Penttilä M. 1997. Laccase from the white-rot fungus *Trametes versicolor*: cDNA cloning of *lcc1* and expression in *Pichia pastoris*. Curr Genet 32:425–430.

Jönsson LJ, Palmqvist E, Nilvebrant NO, Hahn-Hägerdal B. 1998. Detoxification of wood hydrolysates with laccase and peroxidase from the white-rot fungus *Trametes versicolor*. Appl Microbiol Biotechnol 49:691–697.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

Larsson S, Reimann A, Nilvebrant NO, Jönsson LJ. 1999. Comparison of different methods for the detoxification of lignocellulose hydrolysates of spruce. Appl Biochem Biotechnol 77–79:91–103.

Larsson S, Cassland P, Jönsson LJ. 2001. Development of a *Saccharomyces cerevisiae* strain with enhanced resistance to phenolic fermentation inhibitors in lignocellulose hydrolysates by heterologous expression of laccase. Appl Environ Microbiol 67:1163–1170.

Li Z, Xiong F, Lin Q, d'Anjou M, Daugulis AJ, Yang DSC, Hew CL. 2001. Low-temperature increases the yield of biologically active herring antifreeze protein in *Pichia pastoris*. Protein Express Purif 21:438–445.

Murray WD, Duff SJB, Lanthier PH. 1989. Induction and stability of alcohol oxidase in the methylotrophic yeast *Pichia pastoris*. Appl Microbiol Biotechnol 32:95–100.

Otterbein L, Record E, Longhi S, Asther M, Moukha S. 2000. Molecular cloning of the cDNA encoding laccase from *Pycnoporus cinnabarinus* I-937 and expression in *Pichia pastoris*. Eur J Biochem 267:1619–1625.

Reinhammar B. 1984. Laccase. In: Lontie R, editor. Copper proteins and copper enzymes, Vol. 3. Boca Raton: CRC Press. p 1–35.

Reinhammar B. 1997. Kinetic studies on *Polyporus* and tree laccases. In: Messerschmidt A, editor. Multi-copper oxidases. Singapore: World Scientific. p 167–200.

Romanos MA, Scorer CA, Clare JJ. 1992. Foreign gene expression in yeast: a review. Yeast 8:423–488.

Schein CH, Noteborn MHM. 1988. Formation of soluble recombinant proteins in *Escherichia coli* is favored by lower growth temperature. Biotechnology 6:291–294.

Servili M, De Stefano G, Piacquadio P, Sciancalepore V. 2000. A novel method for removing phenols from grape must. Am J Enol Viticulture 51:357–361.

Smith M, Thurston CF, Wood DA. 1997. Fungal laccases: role in delignification and possible industrial applications. In: Messerschmidt A, editor. Multi-copper oxidases. Singapore: World Scientific. p 201–224.

Sreekrishna K, Barr KA, Hoard SA, Prevatt WD, Torregrosa RE, Levingston RE, Cruze JA, Wegner GH. 1990. Expression of human serum albumin in *Pichia pastoris*. 15th Int Congr on Yeast Genetics and Molecular Biology, Hague, The Netherlands, Topic No. 09–37B.

Sreekrishna K, Kropp KE. 1996. *Pichia pastoris*. In: Wolf K, editor. Noncon Yeasts Biotechnol. Berlin, Germany: Springer. p 203–253.

Stratton J, Chiruvolu V, Meagher M. 1998. High cell-density fermentation. In: Higgins DR, Cregg JM, editors. Methods in molecular biology, Vol. 103: *Pichia* protocols. Totowa, NJ: Humana Press Inc. p 107–120.

Tschopp JF, Sverlow G, Kosson R, Craig W, Grinna L. 1987. High level secretion of glycosylated Invertase in the methylotrophic yeast *Pichia pastoris*. Biotechnology 5:1305–1308.

Veenhuis M, Dijken JP van, Harder W. 1983. The significance of peroxisomes in the metabolism of one-carbon compounds in yeasts. Adv Microb Physiol 24:1–82.

Wagner LW, Matheson NH, Heisey RF, Schneider K. 1997. Use of a silicone tubing sensor to control methanol concentration during fed batch fermentation of *Pichia pastoris*. Biotechnol Techniques 11:791–795.

Zhang W, Bevins MA, Plantz BA, Smith LA, Meagher MM. 2000. Modeling *Pichia pastoris* growth on methanol and optimizing the production of a recombinant protein, the heavy-chain fragment C of botulinum neurotoxin, serotype A. Biotechnol Bioeng 70:1–8.