# 2

# Isotopic Labeling of Recombinant Proteins From the Methylotrophic Yeast *Pichia pastoris*

**Andrew R. Pickford and Joanne M. O'Leary**

## Summary

The methylotrophic yeast *Pichia pastoris* is now an established expression system for the production of recombinant protein for nuclear magnetic resonance (NMR) studies. It is capable of expressing high levels of heterologous proteins and possesses the ability to perform many of the posttranslational modifications of higher eukaryotes. Here, we describe efficient methods for the production of uniformly $^{13}C$,$^{15}N$-labeled proteins in shake flasks and of uniformly $^{13}C$,$^{15}N$-labeled and $^{2}H$,$^{13}C$,$^{15}N$-labeled proteins in fermenters. We also provide details of two chromatographic procedures, cation exchange and concanavalin A lectin affinity, that have proved useful in purifying *P. pastoris*-expressed proteins for NMR studies.

**Key Words:** *Pichia pastoris*; isotopic labeling; fermentation; nuclear magnetic resonance; recombinant protein.

## 1. Introduction

In recent years, the methylotrophic yeast *Pichia pastoris* has succeeded the baker's yeast *Saccharomyces cerevisiae* as the most-favored lower eukaryotic host for the recombinant expression of heterologous proteins ***(1)***. Like *S. cerevisiae*, *P. pastoris* can easily be manipulated at the molecular genetic level ***(2)*** and is capable of many higher eukaryotic protein modifications, such as glycosylation, disulfide-bond formation, and proteolytic processing ***(1)***. However, *P. pastoris* has numerous significant advantages over *S. cerevisiae*. First, *P. pastoris* prefers a respiratory rather than a fermentative mode of growth and, therefore, does not accumulate the toxic levels of ethanol and acetic acid that restrain cultures of *S. cerevisiae* ***(3)***. Second, unlike *S. cerevisiae*, which tends to hyperglycosylate *N*-linked sugars, *P. pastoris* glycosylation is more similar to that of higher eukaryotes with 8–14 mannose residues per chain ***(4)***. Finally,

From: *Methods in Molecular Biology, vol. 278: Protein NMR Techniques*
Edited by: A. K. Downing © Humana Press Inc., Totowa, NJ

in *P. pastoris*, the unusually efficient and tightly regulated promoter from the alcohol oxidase 1 gene (*AOX1*) can be used to drive high-level transcription of the foreign gene, resulting in protein-expression levels many fold higher than those achievable in *S. cerevisiae* ***(1)***.

The early reports of the extraordinary protein-expression levels achievable with *P. pastoris* ***(5)***, together with the simplicity of the cell culture media, which easily allows for isotopic labeling, soon made the system highly attractive to the nuclear magnetic resonance (NMR) community ***(6)***. Its popularity grew rapidly to the point where it is now the second most-used expression host for NMR studies behind *Escherichia coli*.

This chapter describes methods for the production of uniformly $^{13}C$,$^{15}N$- and $^{2}H$,$^{13}C$,$^{15}N$-labeled proteins for NMR studies using shake-flask and fermenter cultures of *P. pastoris*. Particular attention is paid to the differences in methodology from non-NMR applications that are imposed by the limited availability of isotopically labeled carbon and nitrogen sources and by the specific requirements of deuteration. It is beyond the scope of this chapter to discuss features of the *P. pastoris* expression system in detail. Comprehensive guides and detailed methodologies for the construction and analysis of recombinant strains of *P. pastoris* can be found elsewhere ***(7–10)***. However, given the number of strains and vectors available (and their differing compatibility with the isotopic labeling procedures described here), we begin this chapter with a survey of the *P. pastoris* expression system options.

### 1.1. *The* P. pastoris *Expression System*

A wide variety of expression vectors are available from Invitrogen (Carlsbad, CA) offering varying promoters, secretion signal sequences, affinity tags, and antibiotic/antifungal resistance. However, not all of these are compatible with the methods described here for efficient production of isotopically labeled proteins. We do not recommend using the vectors pPIC9, pHIL-D2, or pHIL-S1 supplied in the original *Pichia* expression kit, as these do not allow for the selection of multicopy transformants that is necessary for the highest levels of recombinant expression ***(8)***. Instead, we recommend using either pPIC9K, pPICZα, or pPIC6α, each of which contains a dose-dependent antibiotic resistance gene allowing for multicopy transformant selection through resistance to the antifungals G418 (for pPIC9K), Zeocin® (for pPICZα), or blasticidin (for pPIC6α). Each of these vectors contains the *AOX1* promoter for high-level transcription of the foreign gene and incorporates the α-factor pre-pro signal sequence from *S. cerevisiae* for secretion into the culture medium. In addition, pPICZα and pPIC6α contain polyhistidine tags C-terminal to their multiple cloning sites for convenient purification using metal chelating resins.

A wide variety of *P. pastoris* host strains are available, not all of which are compatible with all vectors. For pPIC9K constructs, we recommend using GS115 (*his4*) or the protease-deficient SMD1168 (*his4 pep4*) as the host strain. Completed pPIC9K constructs should *not* be linearized with the restriction enzyme *Bgl*II prior to transformation, as this may result in replacement of the endogenous *AOX1* gene ***(7)***. The resulting methanol utilization slow phenotype (*mut*$^S$) transformants are incompatible with the methods described here. Instead, the restriction enzymes *Sac*I or *Sal*I should be used for linearization, each of which will produce the required wildtype methanol utilization (*mut*$^+$) phenotype ***(7)***. For pPICZα and pPIC6α constructs, the wildtype X-33 or the protease-deficient SMD1168H (*pep4*) should be used as the host strain. With these vectors and host strains, all transformants will have the required *mut*$^+$ phenotype regardless of the choice of restriction enzyme for linearization.

### *1.2. Shake-Flask Cultures of* **P. pastoris**

The most convenient method of producing recombinant protein from *P. pastoris* transformants is in shake-flask culture because no specialized equipment is required. This chapter describes a method for $^{13}$C,$^{15}$N-labeling of protein from *mut*$^+$ transformants, which uses $^{13}$C-glucose and $^{13}$C-methanol as carbon sources and $^{15}$N-ammonium sulfate as the nitrogen source.

### *1.3. Fermentation of* **P. pastoris**

Although recombinant expression of heterologous proteins from *P. pastoris* can be done in shake-flask culture, the expression levels achievable in fermenters are typically much higher ***(3)***. For most applications, a three-phase fermentation scheme is employed ***(11,12)***. In the first phase, the engineered strain is batch-cultured (in a simple, defined medium) with the repressing carbon source, glycerol, to accumulate biomass. The second phase is a fed-batch transition stage in which the glycerol is fed to the culture at a growth-limiting rate to further increase the biomass. Finally, in the induction phase, methanol is added to the culture at a slow rate to induce expression of the recombinant protein. The methanol feed rate is then adjusted upward periodically until the desired growth rate and expression level are reached. Throughout the fermentation, ammonium hydroxide is used as a nitrogen source and pH control solution.

The methods for producing $^{13}$C,$^{15}$N- and $^{2}$H,$^{13}$C,$^{15}$N-labeled protein by fermentation described in this chapter differ from the previously mentioned scheme in three important ways. First, because there is little to be gained in accumulating isotopically labeled biomass, the batch phase of the fermentation is kept short (typically 24 h), and there is no fed-batch phase. Thus, in this application, the role of the fermenter is not to produce ultra-high cell densities but simply to allow the pH, aeration, and methanol feeding rate of the culture to be tightly controlled and so optimize the protein-expression level. Second, because of the greater expense of $^{13}$C-glycerol compared to $^{13}$C-glucose, $^{13}$C-glucose is used as the repressing carbon source during the batch phase. Finally, given the impracticality and expense of using $^{15}$N-ammonium hydroxide in cell culture, $^{15}$N-ammonium sulfate is used as the nitrogen source with potassium hydroxide as the pH control solution.

High-density fermentation of *P. pastoris* does have one significant disadvantage—not only does the concentration of recombinant protein increase with cell density, so does that of the debris from cell lysis, in particular vacuolar proteases ***(1)***. Three strategies have proved effective in minimizing the proteolytic instability of foreign proteins secreted into the *P. pastoris* culture medium. One is the addition of amino acid-rich supplements, such as peptone or Casamino acids, to the culture medium. These supplements appear to reduce product degradation by acting as excess substrates for one or more problem proteases ***(13)***. However, this strategy is inappropriate for isotopic labeling as the additives themselves would need to be labeled. A second is changing the culture medium pH. *P. pastoris* is capable of growing across a relatively broad pH range from 3.0 to 7.0, which allows considerable flexibility in adjusting the pH to one that is not optimal for a problem protease. A third is the use of a protease-deficient *P. pastoris* host strain, such as SMD1168 (*his4 PEP4*) or SMD1163 (*his4 PEP4 PRB1*) ***(14)***. The *PEP4* gene encodes proteinase A, a vacuolar aspartyl protease required for the activation of other vacuolar proteases, such as carboxypeptidase Y and proteinase B. Proteinase B, prior to processing and activation by proteinase A, has about half the activity of the processed enzyme. The *PRB1* gene codes for proteinase B. Therefore, *PEP4* mutants display a substantial decrease or elimination in proteinase A and carboxypeptidase Y activities and a partial reduction in proteinase B activity. The *PEP4 PRB1* double mutant shows a substantial reduction or elimination in all three of these protease activities.

### 1.4. Purification of Recombinant Protein

Because *P. pastoris* secretes only low levels of endogenous proteins and because its culture medium contains no added proteins, a secreted heterologous protein makes up the vast majority of the total protein in the medium. Thus, in separating the foreign protein from the bulk of cellular proteins, secretion serves as a major first step in purification. Subsequent purification steps must be tailored to the characteristics of the foreign protein. However, here we have included two purification methods that we have found to be particularly well suited to recombinant proteins expressed from *P. pastoris*, cation exchange and concanavalin A (Con A) affinity chromatography.

### *1.5. Disclaimer*

The shake-flask and fermentation methods described in this chapter are adapted from those published previously ***(6,10,12,15)***. They have been developed to make sufficient quantities of recombinant proteins for NMR structural and functional studies, using cost-effective amounts of labeled substrates. However, they remain "first-shot" approaches, which should be tried with unlabeled reagents to assess the performance of a given clone; the scale of the culture and/or the feed times can be adjusted to compensate for clones that perform poorly. As with all other protein-expression systems, the results are protein specific. Fermentation of *P. pastoris* is not difficult, but we strongly advise anyone attempting it to consult a fermentation scientist for general information about safe handling and disposal; also, there are variations in fermentation systems that may necessitate small adaptations to our protocols.

## 2. Materials

All unlabeled reagents were standard laboratory reagent grade; all solutions were prepared using Milli-Q water (Millipore, Billerica, MA). $D_2O$ (99.9 At %), $^{15}$N-ammonium sulfate (>98 At %), U-[$^{13}$C]-glucose (>99 At %), $^{13}$C-methanol (>99 At %) and $^{2}$H,$^{13}$C-methanol (>99 At %) were obtained from CK Gas Products (Finchampstead, UK).

### *2.1. Seed Culture for Uniform $^{13}$C,$^{15}$N-Labeling in Shake Flasks or a Fermenter*

1. Potassium phosphate solution, pH 6.0 (per liter): Dissolve 23 g potassium dihydrogen phosphate and 118 g dipotassium phosphate in water. If necessary, adjust the pH to 6.0 ± 0.1 with potassium hydroxide (corrosive) or phosphoric acid (85% wt; corrosive) (*see* **Note 1**). Autoclave and store at room temperature for up to 1 yr.
2. Yeast nitrogen base (YNB) solution (per liter): Dissolve 34 g YNB without amino acids or ammonium sulfate (Difco, UK) in water. Filter-sterilize and store at 4ºC for up to 1 yr.
3. Biotin solution (per 100 mL): Dissolve 20 mg D-biotin in 10 m*M* sodium hydroxide. Filter-sterilize and store at 4ºC for up to 3 mo.
4. $^{13}$C-glucose solution (per 25 mL): Dissolve 1.25 g U-[$^{13}$C]-D-glucose in water. Filter-sterilize and store at 4ºC for up to 1 yr (*see* **Notes 2** and **3**).
5. $^{15}$N-ammonium sulfate solution (per 50 mL): Dissolve 5 g $^{15}$N-ammonium sulfate in water. Filter-sterilize and store at 4ºC for up to 1 yr (*see* **Notes 2** and **3**).
6. $^{13}$C,$^{15}$N-labeled buffered minimal glucose ($^{13}$C,$^{15}$N-BMD), pH 6.0 (per 50 mL): Autoclave 30 mL of water and allow to cool to room temperature. Aseptically, add 5 mL of potassium phosphate, pH 6.0, solution, 5 mL of YNB solution, 0.1 mL of

biotin solution, 5 mL of $^{13}C$-glucose solution, and 5 mL of $^{15}N$-ammonium sulfate solution. Store at 4ºC for up to 1 mo.

## 2.2. Uniform $^{13}C,^{15}N$-Labeling in Shake Flasks

1. $^{13}C,^{15}N$-BMD, pH 6.0 (per 200 mL): Scale-up from the 50-mL recipe in **Subheading 2.1.**, **step 6.**
2. $^{13}C$-methanol feed solution (per 100 mL): Add 10 mL $^{13}C$-methanol (toxic) to 90 mL water. Filter-sterilize and store at 4ºC for up to 1 mo (*see* **Notes 2** and **3**).
3. $^{13}C,^{15}N$-labeled buffered minimal methanol ($^{13}C,^{15}N$-BMM), pH 6.0 (per liter): Autoclave 600 mL of water and allow to cool to room temperature. Aseptically, add 100 mL of potassium phosphate, pH 6.0, solution, 100 mL of YNB solution, 2 mL of biotin solution, 100 mL of $^{13}C$-methanol solution, and 100 mL of $^{15}N$-ammonium sulfate solution. Store at 4ºC for up to 1 mo.

## 2.3. Fermenter Vessel Preparation for Uniform $^{13}C,^{15}N$-Labeling

A 1-L glass fermentation vessel was obtained from Electrolab (Tewkesbury, UK) with fittings for variable-speed impeller, temperature control via "cold finger" and heater mat, adjustable sterile air supply introduced through a sparger, and an air outlet fitted with a condenser. The vessel head plate has ports for temperature, pH, dissolved oxygen ($dO_2$), and foam probes, for acid-, base-, antifoam-, and nutrient-feed lines, and for inoculation and sampling. The fermenter was operated using a controller (from Electrolab) with feedback regulation of temperature, pH (via a base pump), aeration (via the impeller), and foaming (via an antifoam pump). A manually controlled, variable-speed, nutrient-feed pump (from Electrolab) was used to deliver methanol.

1. Basal salts medium (per 400 mL): Dissolve 0.47 g calcium sulfate, 7.5 g magnesium sulfate heptahydrate, and 13.4 mL orthophosphoric acid (85% wt; corrosive) in water. Adjust the pH to 3.0 with solid potassium hydroxide (corrosive) (*see* **Note 4**). Sterilize *in situ* by autoclaving (*see* **Subheading 3.3.**, **step 3**).
2. *Pichia* trace metals ($PTM_1$) (per 100 mL): Dissolve 0.6 g copper (II) sulfate, 8 mg sodium iodide, 0.3 g manganese sulfate monohydrate, 20 mg sodium molybdate dehydrate, 2 mg boric acid, 50 mg cobalt chloride, 2 g zinc chloride, 6.5 g iron (II) sulfate, 0.5 mL sulfuric acid (98%; corrosive) in water. Store at room temperature for up to 1 yr. Filter-sterilize before use.

## 2.4. Uniform $^{13}C,^{15}N$-Labeling in a Fermenter

1. $^{13}C$-methanol feed solution (per 100 mL): Prepare as in **Subheading 2.2.**, **step 2**.
2. Antifoam solution: Autoclave polypropylene glycol 1025, and store at room temperature indefinitely (*see* **Note 5**).

3. Base control solution: Prepare and autoclave 2 *M* potassium hydroxide (corrosive), and store at room temperature indefinitely (*see* **Note 6**).

## *2.5. Seed Culture for Uniform $^2$H,$^{13}$C,$^{15}$N-Labeling in a Fermenter*

1. Deuterated potassium phosphate solution, pH 6.0 (per 10 mL): Prepare potassium phosphate solution (*see* **Subheading 2.1.**, **step 1**). Lyophilize 10 mL of the solution, and resuspend in 10 mL $D_2O$. Filter-sterilize and store at room temperature for up to 1 yr (*see* **Note 2**).
2. Deuterated YNB solution (per 10 mL): Prepare YNB solution (*see* **Subheading 2.1.**, **step 2**). Lyophilize 10 mL of the solution, and resuspend in 10 mL $D_2O$. Filter-sterilize and store at room temperature for up to 1 yr (*see* **Note 2**).
3. Deuterated biotin solution (per 10 mL): Prepare biotin solution (*see* **Subheading 2.1.**, **step 3**). Lyophilize 10 mL of the solution, and resuspend in 10 mL $D_2O$. Filter-sterilize and store at room temperature for up to 1 yr (*see* **Note 2**).
4. Deuterated $^{13}$C-glucose solution (per 30 mL): Dissolve 1.5 g U-[$^{13}$C]-glucose in 30 mL $D_2O$. Lyophilize the solution, and resuspend in 30 mL $D_2O$. Filter-sterilize and store at 4ºC for up to 1 yr (*see* **Notes 2** and **3**).
5. Deuterated $^{15}$N-ammonium sulfate solution (per 55 mL): Dissolve 5.5 g $^{15}$N-ammonium sulfate in 55 mL $D_2O$. Lyophilize the solution, and resuspend in 55 mL $D_2O$. Filter-sterilize and store at 4ºC for up to 1 yr (*see* **Notes 2** and **3**).
6. 70%-deuterated $^{13}$C,$^{15}$N-BMD (70%-$^2$H,$^{13}$C,$^{15}$N-BMD), pH 6.0 (per 50 mL): Filter-sterilize 30 mL $D_2O$. Aseptically, add 5 mL of deuterated potassium phosphate, pH 6.0, solution, 5 mL of deuterated YNB solution, 0.1 mL of deuterated biotin solution, 5 mL of deuterated $^{13}$C-glucose solution, and 5 mL of deuterated $^{15}$N-ammonium sulfate solution. Store at 4ºC for up to 1 mo.
7. 100%-deuterated $^{13}$C, $^{15}$N-BMD (100%-$^2$H,$^{13}$C,$^{15}$N-BMD), pH 6.0 (per 50 mL): Filter-sterilize 30 mL $D_2O$. Aseptically, add 5 mL of deuterated potassium phosphate, pH 6.0, solution, 5 mL of deuterated YNB solution, 0.1 mL of deuterated biotin solution, 5 mL of deuterated $^{13}$C-glucose solution, and 5 mL of deuterated $^{15}$N-ammonium sulfate solution. Store at 4ºC for up to 1 mo.

## *2.6. Fermenter Vessel Preparation for Uniform $^2$H,$^{13}$C,$^{15}$N-Labeling*

1. Deuterated BSM (per 390 mL): Dissolve 27.2 g potassium dihydrogen phosphate and 12.4 g magnesium sulfate heptahydrate in 100 mL $D_2O$. Lyophilize the solution, and resuspend in 390 mL $D_2O$ (*see* **Note 4**). Filter-sterilize and store at room temperature for up to 1 yr (*see* **Note 2**).
2. Deuterated calcium chloride solution (per 10 mL): Dissolve 1.47 g calcium chloride dihydrate in 10 mL $D_2O$. Lyophilize the solution and resuspend in 10 mL $D_2O$. Filter-sterilize and store at room temperature for up to 1 yr (*see* **Note 2**).
3. Deuterated $PTM_1$ (per 10 mL): Prepare $PTM_1$ solution (*see* **Subheading 2.3.**, **step 2**). Lyophilize 10 mL of the solution, and resuspend in 10 mL $D_2O$. Filter-sterilize and store at room temperature for up to 1 yr (*see* **Note 2**).

### 2.7. Uniform $^{2}H,^{13}C,^{15}N$-Labeling in a Fermenter

1. Deuterated $^{2}H,^{13}C$-methanol feed solution (per 100 mL): Add 10 mL $^{2}H,^{13}C$-methanol (toxic) to 90 mL $D_2O$. Filter-sterilize and store at 4°C for up to 1 mo (*see* **Notes 2** and **3**).
2. Antifoam solution: Prepare as in **Subheading 2.4.**, **step 2**.
3. Deuterated base control solution (100 mL): Dissolve 21.2 g tri-potassium phosphate (corrosive) in 100 mL $D_2O$. Filter-sterilize and store at room temperature for up to 1 yr (*see* **Notes 2** and **6**).

### 2.8. Protein Purification by Cation-Exchange Chromatography

SP-Sepharose Fast Flow was supplied by Amersham Biosciences (Little Chalfont, UK).

1. Cation-exchange equilibration buffer (per liter): Prepare 20 m*M* sodium citrate, 0.02% (w/v) sodium azide (highly toxic), pH 3.0. Store at room temperature for up to 1 yr.
2. Cation-exchange elution buffer (per liter): Prepare 20 m*M* sodium citrate, 1 *M* sodium chloride, 0.02% (w/v) sodium azide (highly toxic), pH 3.0. Store at room temperature for up to 1 yr.

### 2.9. Glycoprotein Purification by Lectin Affinity Chromatography

Con A Sepharose 4B was supplied by Amersham Biosciences (Little Chalfont, UK).

1. Con A regeneration buffer: Prepare 20 m*M* Tris, 0.02% (w/v) sodium azide (highly toxic), pH 8.5. Store at room temperature for up to 1 yr.
2. Con A equilibration buffer: Prepare 20 m*M* Tris, 0.5 *M* sodium chloride, 1 m*M* manganese (II) chloride, 1 m*M* calcium chloride, 0.02% (w/v) sodium azide (highly toxic), pH 7.4. Store at room temperature for up to 1 yr.
3. Con A elution buffer: Prepare 0.5 *M* methyl-α-D-glucopyranoside, 20 m*M* Tris, 0.5 *M* sodium chloride, 0.02% sodium azide (w/v) (highly toxic), pH 7.4. Store at room temperature for up to 1 yr.

## 3. Methods

### 3.1. Seed Culture for Uniform $^{13}C,^{15}N$-Labeling in Shake Flasks or a Fermenter

1. Thaw an aliquot of a *P. pastoris* strain that has been stored as a 20% (v/v) glycerol stock at –80°C. Pellet the cells by centrifugation at 3000*g* for 5 min at room temperature. Resuspend the cell pellet in 1 mL of $^{13}C,^{15}N$-BMD.
2. Inoculate a sterile 250-mL baffled flask containing 49 mL of $^{13}C,^{15}N$-BMD with the cell suspension and grow, shaking at 30°C for 18–24 h, until the optical density $(OD)_{600}$ >2 (*see* **Note 7**). This will constitute the seed culture for a 1-L shake-flask growth (*see* **Subheading 3.2.**, **step 1**) or 0.5-L fermentation (*see* **Subheading 3.4.**, **step 2** and **Note 8**).

### *3.2. Uniform $^{13}C,^{15}N$-Labeling in Shake Flasks*

1. Inoculate a sterile 2-L baffled flask containing 200 mL of $^{13}C,^{15}N$-BMD with the 50 mL seed culture (*see* **Subheading 3.1.**, **step 2**). Incubate with shaking at 30ºC for 48–60 h, until the $OD_{600}$ = 10–20 (*see* **Note 7**).
2. Pellet the cells in sterile containers at 3000*g* for 5 min at room temperature. Discard the supernatant.
3. Gently resuspend the cells in 1 L of $^{13}C,^{15}N$-BMM. Equally divide the cell suspension into four 2-L baffled flasks (*see* **Note 9**). Incubate with shaking at 30ºC, until the optimal induction time is reached (*see* **Note 10**). Take samples regularly (every 8–12 h) for analysis of cell density ($OD_{600}$) and of expression level by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (*see* **Note 11**).
4. Every 24 h during the induction phase, feed the cultures by adding 12.5 mL $^{13}C$-methanol feed solution.
5. At the end of the induction phase, pellet the cells by centrifugation at 10,000*g* for 10 min at 4ºC (*see* **Note 10**). Remove the yellow-green supernatant, measure its volume, and store it at 4ºC (*see* **Note 12**). Resuspend the cell pellet in a volume of 100 m*M* HCl equivalent to the original supernatant volume. Pellet the cells by centrifugation, as above; combine this supernatant with the original supernatant, and clarify this solution by passing through a 0.2 µ*M* filter (*see* **Note 13**). Start purification immediately to minimize proteolytic degradation (*see* **Subheading 3.8.**, and *see* **Note 14**).

### *3.3. Fermenter Vessel Preparation for Uniform $^{13}C,^{15}N$-Labeling*

1. Calibrate the fermenter pH probe with pH 4.0 and pH 7.00 buffers, rinse it with deionized $H_2O$, and introduce it into one of the vessel's ports (*see* **Note 15**).
2. Test the fermenter $dO_2$ probe with zeroing gel (available from Mettler-Toledo, Leicester, UK), rinse it with deionized $H_2O$, and introduce it into one of the vessel's ports (*see* **Note 16**). If available, connect a polarization module to the electrode (*see* **Note 17**).
3. Mask the external ends of all ports and lines of the fermenter vessel with aluminum foil and autoclave tape, clamp any lines that will be in contact with the media (e.g., the air inlet and sampling line), and sterilize the vessel containing 400 mL of BSM. Also sterilize three empty aspirators for base control, antifoam, and methanol feed (*see* **Note 5**).
4. Assemble the vessel on the fermentation system, insert the temperature probe, and connect the facilities for temperature control (e.g., heating mat and cold finger, or vessel jacket with circulating water bath). Allow the media to equilibrate to 30ºC.
5. Connect the impeller and the $dO_2$ probe to the controller. If a polarization module has not been used, allow 6 h for probe polarization before inoculating the vessel with the starter culture (*see* **Note 17**). The $dO_2$ level can now be controlled by feedback regulation of the impeller speed (*see* **Note 18**).
6. Aseptically, fill the base control aspirator with base control solution, and connect it to the vessel via the base control pump. Connect the pH probe to the controller.

The system can now regulate the culture pH (*see* **Note 6**). Allow the media to equilibrate to the desired pH for fermentation (*see* **Notes 1**, **4,** and **19**).

7. Aseptically, fill the antifoam aspirator with antifoam solution, and connect it to the vessel via the antifoam pump. Connect the foam probe to the controller to allow the system to manage excessive frothing (*see* **Note 5**).
8. Set the impeller speed to 250 rpm to aid temperature equilibration. Aseptically, introduce 20 mL $^{13}$C-glucose solution, 45 mL $^{15}$N-ammonium sulfate solution, 2.5 mL biotin solution, and 2.5 mL PTM$_1$ via the inoculation port (*see* **Note 19**).
9. Connect the sterile air-line, open the air outlet, and increase the impeller speed and air-flow rates to the maximum value to be used during the fermentation, typically 1000 rpm and 5 vol of air per volume of culture per min (vvm). After 10 min, calibrate the dO$_2$ probe at 100% saturation. Set the air-flow rate to 2 vvm.

### 3.4. Uniform $^{13}$C,$^{15}$N-Labeling in a Fermenter

1. Activate the aeration feedback control with a target dO$_2$ of 20%, and maximum and minimum impeller speeds of 1000 rpm and 200 rpm, respectively (*see* **Note 18**). The saturated media should cause the impeller speed to drop to 200 rpm.
2. Inoculate with the 50-mL seed culture (*see* **Subheading 3.1.**, **step 2**), and start the controller to monitor dO$_2$, impeller speed, temperature, and pH. Take a 10-mL sample via the sample port for assay and determination of wet cell weight and/or OD$_{600}$ (*see* **Note 11**).
3. Aseptically, fill the methanol feed aspirator with the $^{13}$C-methanol feed solution, and connect the aspirator to the vessel via the nutrient-feed pump.
4. When the dO$_2$ "spikes" (typically 18–24 h after inoculation), take a 10-mL sample for analysis, and add 50 mL $^{13}$C-methanol feed solution (toxic) via the inoculation port (*see* **Note 20**). Start the methanol feed at 2.5 mL/h, increasing to 5 mL/h after 6 h. Take samples regularly (every 8–12 h) for analysis of cell density (OD$_{600}$) and of expression level by SDS-PAGE (*see* **Note 11**). Continue the fermentation until it has consumed at least 250 mL methanol feed solution (*see* **Note 3**).
5. To prevent methanol accumulating in the vessel, but at the same time maintaining optimal cell growth, the feed rate of methanol can be increased with time, but it must remain limiting. Under conditions of limiting carbon source, the respiration rate rises and falls as methanol is constantly depleted, then repleni- shed, in the vessel; this is signaled by multiple dO$_2$ "spikes" (*see* **Note 21**).
6. Harvest the expressed protein by centrifugation as described in **Subheading 3.2.**, **step 5**, and start purification immediately to minimize proteolytic degradation (*see* **Subheading 3.8.** and **Note 14**).

### 3.5. Seed Culture for Uniform $^{2}$H,$^{13}$C,$^{15}$N-Labeling in a Fermenter

To produce significant quantities of $^{2}$H,$^{13}$C,$^{15}$N-labeled protein, *P. pastoris* cells must be "conditioned" for growth in a deuterated environment. This is achieved by subculturing the cells into first 70-, then 100%-deuterated media.

1. Thaw an aliquot of a *P. pastoris* strain that has been stored as a 20% (v/v) glycerol stock at −80°C. Pellet the cells by centrifugation at 3000*g* for 5 min at room temperature. Resuspend the cell pellet in 1 mL of 70%-$^{2}H,^{13}C,^{15}N$-BMD.
2. Inoculate a sterile 250-mL baffled flask containing 49 mL of 70%-$^{2}H,^{13}C,^{15}N$-BMD with the cell suspension and grow, shaking at 30°C for 36–48 h, until the $OD_{600}$ >2 (*see* **Note 7**). Pellet a 10-mL aliquot of the cells by centrifugation at 3000*g* for 5 min at room temperature. Resuspend the cell pellet in 1 mL of 100%-$^{2}H,^{13}C,^{15}N$-BMD.
3. Inoculate a sterile 250-mL baffled flask containing 49 mL of 100%-$^{2}H,^{13}C,^{15}N$-BMD with the cell suspension and grow, shaking at 30°C for 36–48 h, until the $OD_{600}$ >2 (*see* **Note 7**). This will constitute the seed culture for a 0.5-L fermentation (*see* **Subheading 3.7.**, **step 2** and **Note 8**).

### *3.6. Fermenter Vessel Preparation for Uniform $^{2}H,^{13}C,^{15}N$-Labeling*

To achieve the highest levels of deuteration, every care should be taken not to introduce water into the fermenter vessel.

1. Calibrate the fermenter pH probe with pH 4.0 and pH 7.00 buffers, and immerse its electrode in a measuring cylinder of deionized $H_2O$ in preparation for autoclaving (*see* **Note 15**).
2. Test the fermenter $dO_2$ probe with zeroing gel, rinse it with deionized $H_2O$, and immerse its electrode in a measuring cylinder of deionized $H_2O$ in preparation for autoclaving (*see* **Note 16**). If available, connect a polarization module to the electrode (*see* **Note 17**).
3. Mask the external ends of all ports and lines of the fermenter vessel with aluminum foil and autoclave tape, and clamp any lines that will be in contact with the media (e.g., the air inlet and sampling line). Cover the ports to be used for the pH and $dO_2$ probes with gauze. Sterilize the vessel, the pH and $dO_2$ probes, and three empty aspirators for deuterated base control, antifoam, and deuterated methanol feed.
4. Dry the sterile fermenter vessel in an oven at approx 80°C for 24–48 h to remove any traces of water. Once cooled, aseptically fill the dry vessel with 390 mL deuterated BSM and 10 mL of deuterated calcium chloride solution.
5. Aseptically, air-dry the sterile pH and $dO_2$ probes for 10 min, rinse their electrodes with filter-sterilized $D_2O$ to remove any traces of water, and introduce them into the gauze-covered ports on the vessel.
6. Assemble the vessel on the fermentation system, and connect the facilities for temperature, $dO_2$, pH, and foam control as described in **Subheading 3.3.**, **steps 4–7**.
7. Set the impeller speed to 250 rpm to aid temperature equilibration. Aseptically, introduce 20 mL deuterated $^{13}C$-glucose solution, 45 mL deuterated $^{15}N$-ammonium sulfate solution, 2.5 mL deuterated biotin solution, and 2.5 mL deuterated $PTM_1$ via the inoculation port (*see* **Note 19**).
8. Calibrate the $dO_2$ probe as described in **Subheading 3.3.**, **step 9**.

### 3.7. Uniform $^2H,^{13}C,^{15}N$-Labeling in a Fermenter

1. Activate the aeration feedback control as described in **Subheading 3.4.**, **step 1**.
2. Inoculate with the 50-mL seed culture (*see* **Subheading 3.5.**, **step 3**) and start the controller to monitor $dO_2$, impeller speed, temperature, and pH. Take a 10-mL sample via the sample port for assay and determination of wet cell weight and/or $OD_{600}$ (*see* **Note 11**).
3. Aseptically, fill the methanol feed aspirator with the $^2H,^{13}C$-methanol feed solution and connect the aspirator to the vessel via the nutrient-feed pump.
4. When the $dO_2$ "spikes" (typically 36–48 h after inoculation), take a 10-mL sample for analysis, and add 50 mL deuterated $^2H,^{13}C$-methanol feed solution (toxic) via the inoculation port (*see* **Note 20**). Start the methanol feed at 1.25 mL/h, increasing to 2.5 mL/h after 6 h. Take samples regularly (every 16–24 h) for analysis of cell density ($OD_{600}$) and of expression level by SDS-PAGE (*see* **Note 11**). Continue the fermentation until it has consumed at least 250 mL methanol feed solution (*see* **Note 3**).
5. *See* **Subheading 3.4.**, **step 5**.
6. Harvest the expressed protein by centrifugation as described in **Subheading 3.2.**, **step 5**, and start purification immediately to minimize proteolytic degradation (*see* **Subheading 3.8.** and **Note 14**).

### 3.8. Protein Purification by Cation-Exchange Chromatography

The following protocol is a convenient first-step procedure that serves not only to partially purify the recombinant protein but also to concentrate the sample volume for subsequent steps. However, if the heterologous protein is acid labile, insoluble at low pH, or is highly acidic (has a low isoelectric point) an alternative purification method should be used.

1. Dilute the crude supernatant twofold with Milli-Q water. Adjust the pH to 3.0 with 2 *M* hydrochloric acid (corrosive) or 2 *M* sodium hydroxide (corrosive).
2. Equilibrate the cation-exchange column of SP-Sepharose FF with 5 column volumes of cation-exchange equilibration buffer.
3. Apply the diluted protein sample onto the column at 10–20 mL/min. Collect the yellow-green nonbinding material that contains alcohol oxidase (AOX) and other acidic proteins (*see* **Note 12**).
4. Wash the column with 2 column volumes of cation-exchange equilibration buffer.
5. Elute the bound proteins by applying a gradient of 0–100% cation-exchange elution buffer and collecting fractions equivalent to 0.1 column volumes.
6. Analyze the fractions and the nonbinding material by SDS-PAGE, and pool those containing the recombinant protein.

### 3.9. Glycoprotein Purification by Lectin Affinity Chromatography

Separation of glycosylated from nonglycosylated proteins secreted from *P. pastoris* can be achieved using affinity chromatography with immobilized jack bean Con A lectin. Con A has specificity for branched mannoses and carbohydrates with terminal mannose or glucose, and therefore, it binds to both N- and O-linked oligosaccharides produced by *P. pastoris.*

1. Regenerate a column of Con A-Sepharose (to remove any bound glycoproteins or sugar analogs) by washing with 10 column volumes of Con A regeneration buffer, followed by 10 column volumes of Con A equilibration buffer.
2. Add $CaCl_2$, $MgCl_2$, and $MnCl_2$ to the protein sample, each to a final concentration of 1 m*M*. Adjust the sample pH to 7.4 with solid Tris base (*see* **Note 22**).
3. Apply the protein sample to the column at 0.1–0.5 mL/min, and wash the column with 5 column volumes of Con A equilibration buffer. Collect the nonbinding fraction that contains the nonglycosylated proteins.
4. To elute the glycoproteins and free oligosaccharides, wash the column with 5 column volumes of Con A elution buffer. Analyze the eluted glycoproteins and the nonbinding aglycosyl proteins by SDS-PAGE.

### 3.10. Enzymatic Deglycosylation of Glycoproteins

The large hydrodynamic radius of *N*-linked, high-mannose sugars on recombinant proteins greatly increases their rotational correlation time ($\tau_c$) leading to extensive line-broadening in their NMR spectra. In the majority of cases, the sugar moiety is not essential for structural integrity or functional activity of the protein and can be trimmed using the enzyme endoglycosidase (Endo) $H_f$ (*see* **Note 23**). Endo $H_f$ cleaves the chitobiose core of high-mannose and hybrid type *N*-linked oligosaccharides to leave a single *N*-linked *N*-acetylglucosamine (GlcNAc). Although enzymatic removal of *O*-linked oligosaccharides is possible and has been described in detail elsewhere ***(16)***, in our experience it is not economically viable to do so on the quantity of protein required for an NMR sample.

1. Adjust the protein sample to pH 5.5 with 1 *M* sodium hydroxide (corrosive).
2. Add 1 kU of Endo $H_f$ (supplied by New England Biolabs) per milliliter of glycoprotein solution. Incubate at 37°C for 1–3 h.
3. Analyze the glycosylated and aglycosyl protein by SDS-PAGE. *N*-linked glycosylation results in an increase in apparent molecular weight of approx 7 kDa per high-mannose chain, and a significant smearing of the protein band.
4. The removed oligosaccharides may be separated from the deglycosylated protein by Con A affinity chromatography (*see* **Subheading 3.9.**).

## 4. Notes

1. Selection of the growth and induction pH of *P. pastoris* can help to overcome proteolysis and insolubility of the recombinant product. The optimum value should be determined in small-scale cultures prior to isotopic labeling. *P. pastoris* is capable of growing across a relatively broad pH range from 3.0 to 7.0, which allows considerable leeway in adjusting the pH to one that is not optimal for a problem protease.

2. Solutions of ammonium sulfate and D-glucose can be sterilized by autoclaving. Filter-sterilization is used instead as a precaution against accidental loss of the isotopically labeled compounds. $D_2O$ solutions should never be sterilized by autoclaving.
3. The amounts of $^{15}N$-,$^{13}C$-, and $^{2}H$,$^{13}C$-labeled isotopes given here have resulted in good yields in our experience, but given their high cost, it is highly recommended that a "dry run" be performed using unlabeled ammonium sulfate, D-glucose, and methanol to establish the minimum quantities needed for the required expression level.
4. Final adjustment of the fermentation media pH is performed after autoclaving as inorganic salts may precipitate during the sterilization process if the BSM pH is >3.5. Brady et al. ***(17)*** have solved this problem by reducing the concentration of all salts in the BSM to one-quarter of those given here without any adverse effect on cell growth rate, biomass yield, or the level of their recombinant protein. The reduced ionic strength may be beneficial when using ion-exchange chromatography as a first step in protein purification.
5. Different molecular-weight preparations of polypropylene glycol are available; we routinely use 1025 obtained from Merck (Poole, UK). The disadvantage of polypropylene glycol as an antifoam reagent is that its presence, even at low concentrations, can significantly reduce flow rates through certain ultrafiltration membranes. The addition of antifoam is usually necessary, but when growing *P. pastoris* at relatively low cell densities only small quantities are required. If the methanol feed is continued for longer periods, more antifoam solution may be needed. If the culture foams excessively, it is a sign of carbon source limitation, low pH, or ill health of the culture. If automated antifoam addition is unavailable, add the antifoam manually, but use the minimum amount to control foaming.
6. In general, no acid is required for *Pichia* fermentation because a healthy culture always acidifies the media. If the pH of the culture is increasing, it is a sign of carbon source depletion or ill health of the culture.
7. An $OD_{600} = 1$ is equivalent to $5 \times 10^7$ *P. pastoris* cells.
8. The volume of seed culture can be scaled according to the volume of the fermentation. In our experience, good results are obtained from using a seed culture that is 10% of the fermentation volume.
9. Aeration, particularly during the induction phase, is the most important parameter for efficient expression in shake flasks. Baffled flasks should always be used, and the volume of the culture should not exceed 30% of the flask volume.
10. Typical induction times range from 36 to 72 h, but will depend on the desired expression level, the stability of the expressed product, and the amount of $^{13}C$- or $^{2}H$,$^{13}C$-methanol feed solution available. Optimal induction times should be determined from SDS-PAGE analysis of culture samples taken during the extended induction phase of a "dry run" using unlabeled D-glucose and methanol.
11. Regular sampling is important to monitor the growth of the fermentation, allows assay for any recombinant product, and permits microscopic inspection of the culture for contaminating organisms. Any precipitated inorganic salts in the sample

can interfere with these tests but can be dissolved by the addition of an equal volume of 100 m*M* HCl without leading to cell lysis. If the recombinant product is acid labile, then a sample of supernatant should be removed for assay before addition of the HCl.

12. The yellow-green color of the culture supernatant is the result of AOX that, although not actively secreted, accumulates in the media because of leakage from the cells. The AOX can be conveniently purified away from the recombinant material by cation-exchange chromatography (*see* **Subheading 3.8.**).
13. The purpose of washing the cells is threefold: (a) to increase the yield of recombinant protein; (b) to dilute the supernatant and, therefore, to reduce its ionic strength prior to ion-exchange chromatography; and (c) to reduce the pH to approx 3.0 for cation exchange. If the recombinant protein is acid sensitive, then the cells should be washed with sterile $H_2O$ instead of 100 m*M* HCl.
14. To minimize proteolysis in the crude supernatant, add phenylmethylsulfonyl fluoride (PMSF) (toxic) and ethylenediaminetetraacetic acid to a concentration of 1 m*M*. The PMSF should be refreshed every few hours.
15. The performance of the gel-filled autoclavable pH probes will deteriorate with repeated sterilization but can be prolonged by periodic cleaning as recommended by the manufacturer. Probes should be stored in 3 *M* KCl.
16. We have only used polarographic oxygen probes; information on the use of galvanic oxygen probes should be obtained from the manufacturer. Polarographic oxygen probes require routine maintenance to prolong their working life, and these details should also be obtained from the manufacturer.
17. It takes approx 6 h to completely polarize a $dO_2$ electrode. The polarization module applies the same voltage to the $dO_2$ electrode as the controller. Autoclavable modules offer the speed advantage of performing electrode polarization during sterilization.
18. The fermenter impeller speed can be controlled manually, but this is not recommended given the extended duration of *P. pastoris* fermentations. During the induction phase, the $dO_2$ level must remain above 15% at all times to prevent the buildup of methanol in the vessel, but it must also stay below 50% to avoid methionine oxidation of the recombinant protein.
19. As the pH rises above 3.5, and particularly on addition of $PTM_1$ salts, some inorganic salts will precipitate. This does not affect cell growth.
20. When a culture is growing normally and consumes the entire carbon source, respiration will slow dramatically, causing the $dO_2$ level to rise sharply or “spike.” Addition of more carbon source should lead to an almost instantaneous rise in respiration signaled by a concomitant fall in $dO_2$. Many fermenter controllers (including ours) have automated feedback control, which adjusts the impeller speed to maintain the $dO_2$ at a preset level. This is very convenient when the system is left unattended for long periods of time but can mask $dO_2$ fluctuations. In this case, test for carbon limitation by fixing the impeller speed at the current variable level and watch for $dO_2$ “spikes.”
21. The $dO_2$ “spike” method of ensuring growth-limiting methanol concentration is labor intensive and repeatedly exposes the cells to noninducing levels of methanol.

This can be avoided through the use of a methanol probe that allows continuous measurement and control of the methanol concentration in the media.

22. Con A is a metalloprotein that contains four metal binding sites. To maintain the binding characteristics of Con A, both $Mn^{2+}$ and $Ca^{2+}$ must be present in the binding buffer and protein sample.
23. Endo $H_f$ is a protein fusion of Endo H (cloned from *Streptomyces plicatus*) and maltose binding protein. It has identical activity to Endo H but is less expensive. PNGase F may be used as an alternative to Endo $H_f$. This enzyme cleaves between the innermost GlcNAc and asparagine residues of high mannose, hybrid, and complex oligosaccharides from N-linked glycoproteins. However, the activity of this enzyme is more susceptible to steric hindrance of the scissile bond.

## Acknowledgments

The authors thank the Wellcome Trust for financial assistance and Prof. Iain D. Campbell and Dr. A. K. Downing for their support and direction.

## References

1. Higgins, D. R. and Cregg, J. M. (1998) Introduction to *Pichia pastoris.* In *Methods in Molecular Biology*, vol. 103: *Pichia Protocols* (Higgins, D. R. and Cregg, J. M., eds.), Humana, Totowa, NJ, pp. 1–15.
2. Cregg, J. M., Shen, S., Johnson, M., and Waterham, H. R. (1998) Classical genetic manipulation. In *Methods in Molecular Biology,* vol. 103: *Pichia Protocols* (Higgins, D. R. and Cregg, J. M., eds.), Humana, Totowa, NJ, pp. 17–26.
3. Lin Cereghino, G. P., Lin Cereghino, J., Ilgen, C., and Cregg, J. M. (2002) Production of recombinant proteins in fermenter cultures of the yeast *Pichia pastoris*. *Curr. Opin. Biotechnol.* **13,** 1–4.
4. Grinna, L. S. and Tschopp, J. F. (1989) Size distribution and general structural features of N-linked oligosaccharides from the methylotrophic yeast, *Pichia pastoris*. *Yeast* **5,** 107–115.
5. Cregg, J. M., Vedvick, T. S., and Raschke, W. C. (1993) Recent advances in the expression of foreign genes in *Pichia pastoris*. *Biotechnology* **11,** 905–910.
6. Laroche, Y., Storme, V., De Meutter, J., Messens, J., and Lauwereys, M. (1994) High level secretion and very efficient isotopic labelling of tick anticoagulant peptide (TAP) expressed in the methylotrophic yeast *Pichia pastoris*. *Biotechnology* **12,** 1119–1124.
7. Cregg, J. M., Barringer, K. J., Hessler, A. Y., and Madden, K. R. (1985) *Pichia pastoris* as a host system for transformations. *Mol. Cell. Biol.* **5,** 3376–3385.
8. Scorer, C.A., Clare, J. J., McCombie, W. R., Romanos, M. A., and Sreekrishna, K. (1994) Rapid selection using G418 of high-copy number transformants of *Pichia pastoris* for high-level foreign gene expression. *Biotechnology* **12,** 181–184.
9. Romanos, M. A. (1995) Advances in the use of *Pichia pastoris* for high-level gene expression. *Curr. Opin. Biotechnol.* **6,** 527–533.

10. Invitrogen Corporation. *Pichia Expression System: Manual of Methods for Expression of Recombinant Proteins in Pichia pastoris*, Version G. Available at http://www.invitrogen.com/content/sfs/manuals/pich_man.pdf. Accessed 03/01/04.
11. Stratton, J., Chriuvolu, V., and Meagher, M. (1998) High cell-density fermentation. In *Methods in Molecular Biology,* vol. 103: *Pichia Protocols* (Higgins, D. R. and Cregg, J. M., eds.), Humana, Totowa, NJ, pp. 107–120.
12. Invitrogen Corporation. *Pichia Fermentation Process Guidelines.* Available at http://www.invitrogen.com/content/sfs/manuals/pichiaferm_prot.pdf. Accessed 03/01/04.
13. Clare, J. J., Romanos, M. A., Rayment, F. B., Rowedder, J. E., Smith, M. A., Payne, M. M., et al. (1991) Production of mouse epidermal growth factor in yeast: high-level secretion using *Pichia pastoris* strains containing multiple gene copies. *Gene* **105,** 205–212.
14. Gleeson, M. A. G., White, C. E., Meininger, D. P., and Komives, E. A. (1998) Generation of protease-deficient strains and their use in heterologous protein expression. In *Methods in Molecular Biology,* vol. 103: *Pichia Protocols* (Higgins, D. R. and Cregg, J. M., eds.), Humana, Totowa, NJ, pp. 81–94.
15. Bright, J. R., Pickford, A. R., Potts, J. R., and Campbell, I. D. (1999) Preparation of isotopically labelled recombinant fragments of fibronectin for functional and structural study by heteronuclear nuclear magnetic resonance spectroscopy. In *Methods in Molecular Biology,* vol. 139: *Extracellular Matrix Protocols* (Streuli, C. and Grant, M., eds.), Humana, Totowa, NJ, pp. 59–69.
16. Cremata, J. A., Montesino, R., Quintero, O., and Garcia, R. (1998) Glycosylation profiling of heterologous proteins. In *Methods in Molecular Biology,* vol. 103: *Pichia Protocols* (Higgins, D. R. and Cregg, J. M., eds.), Humana, Totowa, NJ, pp. 95–105.
17. Brady, C. P., Shimp, R. L., Miles, A. P., Whitmore, M., and Stowers, A. W. (2001) High-level production and purification of $\text{P30P2MSP1}_{19}$, an important vaccine antigen for malaria, expressed in the methylotrophic yeast *Pichia pastoris*. *Protein Expr. Purif.* **23,** 468–475.

http://www.springer.com/978-1-58829-246-9

Protein NMR Techniques
Downing, A.K. (Ed.)
2004, XIII, 487 p., Hardcover
ISBN: 978-1-58829-246-9
A product of Humana Press