



## *Pichia pastoris* as a Eukaryotic Protein Isotope-Labeling System

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The development of new systems for recombinant expression of isotopically labeled proteins is of significant interest to NMR spectroscopists. While many prokaryotic proteins can readily be over-expressed in *E. coli* and yield functional samples, over-expression of eukaryotic, and, more specifically, medically important mammalian proteins, often requires a different, non-prokaryotic, host.<sup>1-3</sup> Yeast is an alternative choice of eukaryotic expression system that has attracted considerable attention in recent years. Isotopic labeling of proteins in yeast for solution NMR studies began about 15 years ago.<sup>4</sup> Yeast is currently a second most popular expression system after *E. coli*, and seems to offer the best of the two worlds. These systems have the distinct advantages of low labeling costs, high expression yields, ease of genetic manipulation, ability to grow on deuterated media, and speeds of expression close to those in *E. coli*. At the same time, yeast expression allows native folding and an array of post-translational modifications, such as proteolytic truncation, formation of disulfide bonds, glycosylation, phosphorylation, and acylation, typical for eukaryotic cell cultures. Finally, the ability for secreted expression avoids problems with toxicity of the expressed proteins.<sup>1</sup>

Among several yeast species used for isotope labeling of proteins, the methylotrophic yeast *Pichia pastoris* accounts for the lion's share of produced structures (about 40 unique proteins).<sup>5</sup> Expression in *Pichia* usually results in higher expression yields (especially in fermenters) and more native patterns of glycosylation than in other yeast species.<sup>6</sup> Thus, *Pichia pastoris* is the yeast expression system of choice for today's NMR studies of eukaryotic proteins. Another popular choice, *Saccharomyces cerevisiae*, comes a distant second. So far, it has yielded nine unique protein structures, with no structures published in the last eight years.<sup>5</sup> Yet another yeast species, *Kluyveromyces lactis*, was recently evaluated for labeled protein expression with promising results.<sup>1</sup>

The uniform <sup>13</sup>C/<sup>15</sup>N isotopic labeling in *Pichia pastoris* is very straightforward and follows established protocols, both for shaker incubators and fermenters.<sup>6-8</sup> A large variety of strains and vectors designed for both secreted and non-secreted expression is available from Invitrogen.<sup>9</sup> Among the highlights are the

availability of protease-deficient strains, a number of vectors designed for efficient protein secretion (or membrane-targeting), protein tagging, and selection for multi-copy integration transformants. The protocols for the removal of covalently and non-covalently bound sugars are available as well.<sup>10</sup> *Pichia pastoris* grows well on minimal media, where <sup>15</sup>N can be supplied in the form of ammonium salts, and two different <sup>13</sup>C sources are employed in the pre-induction and the post-induction phase. As protein expression is induced by methanol and is conducted under the strong alcohol oxidase promoter, <sup>13</sup>C-labeled methanol is the main carbon source in the protein expression phase. Prior to the induction, <sup>13</sup>C-labeled glycerol or glucose should be employed.<sup>6</sup> Economical protocols to minimize the use of those labeled precursors have been developed.<sup>8</sup> Yields of hundreds of milligrams of soluble secreted proteins can be achieved in fermentors at extremely high cell densities. Even flask cultures can produce tens of milligrams of doubly isotopically labeled proteins per liter of culture.<sup>7</sup> Important for NMR applications, a number of deuteration protocols is available, both for efficient backbone deuteration using protonated carbon sources in D<sub>2</sub>O, as well as for full deuteration.<sup>11,12</sup> Finally, procedures for isotopic labeling of selected amino acid types (Cys, Leu, Lys, and Met) have been published, and *Pichia* strains for labeling aromatic sidechains have been developed.<sup>13,14</sup>

A number of interesting structures of proteins expressed in *Pichia pastoris* have been solved by solution NMR spectroscopy in the last 15 years, including the Man-6-P receptor diester recognition domain, cofactor-active fragment of thrombomodulin, collagen-binding domain of discoidin domain receptor 2, and many others.<sup>5</sup> One can expect that in the near future yeast expression systems will emerge as a very valuable tool in the NMR studies of eukaryotic membrane proteins, which are extremely hard to express functionally in bacterial systems and usually too expensive to produce in cell cultures. Multiple successful trials of functional expression of G-protein coupled receptors (GPCRs) and other membrane proteins in *Pichia pastoris* on a milligram scale<sup>15-17</sup> give us optimism on the future of yeast both for solution and solid-state NMR spectroscopy.

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