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Effects of pH and Temperature on Recombinant Manganese Peroxidase Production and Stability

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Abstract The enzyme manganese peroxidase (MnP) is produced by numerous white-rot fungi to overcome biomass recalcitrance caused by lignin. MnP acts directly on lignin and increases access of the woody structure to synergistic wood-degrading enzymes such as cellulases and xylanases. Recombinant MnP (rMnP) can be produced in the yeast *Pichia pastoris* αMnP1-1 in fed-batch fermentations. The effects of pH and temperature on recombinant manganese peroxidase (rMnP) production by *P. pastoris* αMnP1-1 were investigated in shake flask and fed-batch fermentations. The optimum pH and temperature for a standardized fed-batch fermentation process for rMnP production in *P. pastoris* αMnP1-1 were determined to be pH 6 and 30 °C, respectively. *P. pastoris* αMnP1-1 constitutively expresses the manganese peroxidase (*mnp1*) complementary DNA from *Phanerochaete chrysosporium*, and the rMnP has similar kinetic characteristics and pH activity and stability ranges as the wild-type MnP (wtMnP). Cultivation of *P. chrysosporium* mycelia in stationary flasks for production of heme peroxidases is commonly conducted at low pH (pH 4.2). However, shake flask and fed-batch fermentation experiments with *P. pastoris* αMnP1-1 demonstrated that rMnP production is highest at pH 6, with rMnP concentrations in the medium declining rapidly at pH less than 5.5, although cell growth rates were similar from pH 4–7. Investigations of the cause of low rMnP production at low pH were consistent with the hypothesis that intracellular proteases are released from dead and lysed yeast cells during the fermentation that are active against rMnP at pH less than 5.5.

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Introduction

Microorganisms have evolved a variety of enzymes for degrading the different components of lignocellulosic material, including cellulose (cellulases), hemicellulose (xylanases), and lignin (heme peroxidases), and effectively recycle plant biomass in the environment to CO_2 and H_2O. Manganese peroxidase (MnP) is an extracellular heme peroxidase that catalyzes the H_2O_2-dependent oxidation of Mn(II) to Mn(III). Mn(III) chelates organic acids to create the diffusible oxidants that attack phenolic lignin structures [1]. Both wild-type MnP (wtMnP) from a white-rot fungus [2] and recombinant MnP (rMnP) from the yeast *Pichia pastoris* [3] have been shown to be effective for removing lignin from cellulose fibers in pulp bleaching experiments [4]. However, potential applications of rMnP in pulp and paper manufacture and biofuels production are limited by enzyme production costs.

Production of rMnP from *P. pastoris* for pulp bleaching experiments was previously conducted at pH 6 [4]. A medium pH of 4.5 resulted in very low rMnP concentrations, although the wtMnP is commonly produced in *P. chrysosporium* cultures at this pH. This observation was also surprising in that Gu et al. [3] found that rMnP purified from *P. pastoris* cultures shares similar characteristics with the wtMnP and is active at pH 4.5 and relatively stable from pH 3.0 to 6.0. Further experiments on the effect of pH on rMnP were thereby conducted in this study to determine why rMnP is not observed in low pH yeast cultures, although white-rot fungi typically produce wtMnP at low pH, and the purified *P. pastoris* rMnP appears to be as stable as the wtMnP at low pH.

The effects of pH, temperature, and buffer type on the stability of purified wtMnP from white-rot fungi have been previously investigated. Sutherland and Aust [5] found that wtMnP from the white-rot fungus *P. chrysosporium* was most stable at pH 5.5 and temperatures at or below 37 °C. They also found that the presence of Ca^{2+} is essential to maintain MnP activity. However, Mielgo et al. [6, 7] found that *P. chrysosporium* MnP stability and activity was optimal at pH 4.5 and 30 °C. Recently, the optimal pH for wtMnP from the white rot fungus *Irpex lacteus* was found to be from 5.5–6.5. [8, 9]. Banci et al. [10] observed that rMnP produced by *Escherichia coli* lost its activity immediately after treatment with buffers of pH<3.0 or pH>8.0.

P. pastoris fermentations are often conducted at pH 6, but *P. pastoris* also grows well at a pH range from 2.8 to 6.5 [11]. The optimal pH for expression of active recombinant proteins in *P. pastoris* fermentations has been found to vary widely, with even small changes having dramatic effects on expression. *P. pastoris* can also be grown over a broad temperature range, with 30 °C most commonly employed for fermentations. However, the production levels of some recombinant proteins at this temperature are unsatisfactory or even unobservable. Because of the instability of some recombinant proteins or susceptibility to proteolytic activity, decreasing the fermentation temperature below 30 °C often increases the yield of active antibodies and other proteins. For example, Jahic et al. [12] reported a doubling in the expression of a fusion protein when the temperature was lowered from 30 to 20 °C, although the observed cell growth rates were similar at both temperatures. The authors speculated that this was caused by a substantial reduction in serine protease activity at 20 °C.

Cassland and Jönsson [13] suggested that the higher recombinant protein yield often observed at lower temperatures is caused by a decrease in protein misfolding and aggregation, as well as a reduction in proteolytic degradation. The existence of proteases

has been widely observed during the expression of recombinant proteins in high-cell density *P. pastoris* fermentations [11, 12, 14–19], and addition of protease inhibitors has been shown to be effective in reducing proteolytic activity against certain recombinant proteins [11, 12, 17, 19]. The fermentation conditions reported for the optimized expression of various proteins in *P. pastoris* are summarized in Table 1 [20–32]. It can be concluded that the optimum pH and temperature for the production of recombinant proteins by *P. pastoris* strongly depends on the particular protein.

The current research describes the effect of pH and temperature on *P. pastoris* growth and rMnP production in both shake flask and bioreactor cultivation. Results of these experiments suggest that intracellular proteases are released from dead and lysed yeast cells during the fermentation that are active against rMnP at pH less than 5.5.

Materials and Methods

Analytical Methods

Cell Density by Spectrophotometry Yeast cell density was estimated by optical density at 600 nm (OD_{600}). Liquid broth samples were diluted in a normal saline solution (0.9% NaCl) or deionized water to achieve an optical density less than 0.4 in a final volume of 1 ml. The diluted cell solution was transferred to disposable semimicro cuvettes, and the absorbance at 600 nm was measured with a Spectronic Genesys 20 spectrophotometer (Thermo Electron, Waltham, MA). Normal saline solution or diluted heme solution were

Table 1 Optimal pH and temperature conditions reported for the expression of recombinant proteins by *Pichia pastoris*.

pH	Temperature (°C)	Protein expressed	Putative effect	Reference
3.0	25	A33 single-chain Fv antibody fragment	Protease inhibition	[20]
3.0	30	Mouse and rat collagen gelatins	Protease inhibition	[21]
4.0	20	Cellulose-binding *Cissus antartica* lipase B	Increased expression	[12]
4.5	30	Coffee bean α-galactosidase	Increased expression	[22]
5.0	–	120 kDa HIV-1 envelope protein	Protease inhibition	[23]
6.0	16	Bovine β-1,4-galactosyltransferase I	Increased expression	[24]
6.0	23	Herring anti-freeze protein (hAFP)	Increased expression	[25]
6.0	25	Galactose oxidase	Stabilized structure	[26]
6.0	28	Human cystatin C protein	Increased expression	[27]
6.0	30	*Trametes versicolor* laccase	Protease inhibition	[16]
–	19	*Trametes versicolor* laccase	Increased expression	[13]
6.0	–	Human serum albumin	Increased expression	[11]
6.0	–	Mouse epidermal growth factor (mEGF)	Increased expression	[14]
7.0	28	Hookworm anticoagulant peptide	Protease inhibition	[28]
7.5 – 8.0	15	Single-chain Fv antibody fragment	Increased expression	[17]
7.7 – 8.3	–	Prourokinase–annexin V chimeric protein	Protease inhibition	[29]
8.0	28	A/Victoria/3/75 (H_3N_2) hemagglutinin(1)	Increased expression	[30]
8.5	20	*Trametes versicolor* laccase(1)	Protease inhibition	[31]
10.0	20	Human μ-opioid receptor fusion protein(1)	Increased expression	[32]

Medium pH is outside of normal *P. pastoris* growth range (pH 2.8–6.5).

used to zero the spectrophotometer. A standard curve was prepared to correlate dry weight (g/l) to optical density. The resulting correlation was dry weight (g/l)=0.3×optical density.

MnP Activity Assay MnP activity was measured by monitoring the oxidation of 2,6-dimethoxyphenol (2,6-DMP) at 469 nm [33]. The reaction mixtures contained 0.4 mM $MnSO_4$, 50 mM sodium malonate (pH 4.5), 0.1 mM 2,6-DMP, and MnP enzyme. The extinction coefficient, ε_{469}, for the orange-brown dimeric product 2,2′,6,6′-tetramethoxydibenzo-1,1′-diquinone is 49,600 $m^{-1}\cdot cm^{-1}$ at 469 nm. MnP activity is expressed as the micromoles of product formed per minute (units)/liter (U/l). The culture samples (1 ml) were centrifuged at 10,000×*g* for 5 min to pellet yeast cells and heme particles, and the clear culture supernatant (10–100 μl) was added to the reaction mixture. Deionized water was added to a final volume of 1 ml. Addition of H_2O_2 to a concentration of 0.1 mM was used to initiate reactions at room temperature, and the absorbance at 469 nm was measured after 1 min.

Strains

P. pastoris SMD1168H (*his*$^+$ *pep4*$^-$) is a protease-deficient strain developed by Invitrogen, Carlsbad, CA. *P. pastoris* SMD1168H αMnP1-1 (*P. pastoris* αMnP1-1) was constructed by electroporating the plasmid pαAMNP [3] into *P. pastoris* SMD1168H. This plasmid contains *mnp1* complementary DNA (cDNA; *P. chrysosporium*) downstream of a constitutive glyceraldehyde-3-phosphate dehydrogenase promoter and a *Saccharomyces cerevisiae* á-factor secretion signal sequence. The plasmid integrates into the host chromosome and contains the *Sh ble* gene that confers resistance to the broad spectrum antibiotic Zeocin. The transformed *P. pastoris* αMnP1-1 was routinely cultured on YPD medium (1% yeast extract, 2% peptone, and 2% dextrose) in agar plates and shake flasks at 30 °C for inoculation of experimental cultures.

Shake Flask Cultivation

Shake flask experiments were performed in buffered glycerol-complex medium (BMGY), modified by substituting glucose for glycerol. The modified BMGY medium consisted of the following (weight percent): yeast extract (1%), peptone (2%), potassium phosphate buffer at pH 6.0 (200 mM) or sodium malonate buffer at pH 4.5 (200 mM), yeast nitrogen base (with ammonium sulfate, without amino acids) (1.34%), biotin (4×10^{-5}%), and glucose (2%).

Exogenous hemin (minimum 80%, Sigma-Aldrich, St Louis, MO) was added as dry powder to the modified BMGY medium after autoclaving. Zeocin (100 mg/l) was also added to inhibit bacterial contamination. Shake flask experiments were conducted with 20 ml medium in 125 ml baffled flasks on an Innoun 4230 refrigerated rotary shaker incubator (250 rpm; New Brunswick Scientific Co., Inc., Edison, NJ) at 25 or 30 °C. For pH experiments, 1 M sodium malonate buffer was used in pH 4.0, 4.5, and 5.0 media; 1 M potassium phosphate buffer was used for pH 5.5, 6.0, 6.5, and 7.0 media. HCl or NaOH (10 N) were added to adjust medium pH.

The *P. pastoris* αMnP1-1 inoculum was prepared with 100 ml YPD medium in a 500 ml shake flask at 250 rpm and 30 °C. After 24 h, the inoculum was separated through centrifugation and washed with sterile normal saline solution (0.9% NaCl). The washed inoculum was then added to each flask to achieve an initial cell density of approximately 0.15 g/l dry cell weight.

Bioreactor Fed-Batch Fermentation

Fed-batch fermentations with *P. pastoris* αMnP1-1 were performed using 3.0-l BioFlo 100 fermentors (New Brunswick Scientific Co. Inc.). The suspended cell, fed-batch cultivation was divided into two stages: batch and fed-batch cultivation with glucose. *P. pastoris* αMnP1-1 inoculum cultures (100 ml) were grown in YPD medium and used to inoculate 1 l of medium in the bioreactors.

Batch fermentations were conducted in basal salts medium (BSM) containing 4% glucose and were prepared as follows [31]:

Basal Salt Medium (BSM) For 11 final volume, the following ingredients were dissolved completely in distilled–deioinized water: phosphoric acid (85% H_3PO_4), 26.7 ml; calcium sulfate ($CaSO_4 \cdot 2H_2O$), 0.93 g; potassium sulfate (K_2SO_4), 18.2 g; magnesium sulfate ($MgSO_4 \cdot 7H_2O$), 14.9 g; potassium hydroxide (KOH), 4.13 g; glucose, 40.0 g. The medium was autoclaved, and 4 ml of PMT1 trace salt solution were aseptically added per liter of BSM.

PMT1 trace salts For 11 final volume, the following ingredients were dissolved completely in distilled–deionized water: cupric sulfate ($CuSO_4 \cdot 5H_2O$), 6.0 g; sodium iodide (NaI), 0.08 g; manganese sulfate·H_2O ($MnSO_4 \cdot H_2O$), 3.0 g; sodium molybdate ($Na_2MoO_4 \cdot H_2O$), 0.2 g; boric acid (H_3BO_4), 0.02 g; cobalt chloride ($CoCl_2 \cdot 6H_2O$), 0.5 g; zinc chloride ($ZnCl_2$), 20.0 g; ferrous sulfate ($FeSO_4 \cdot 7\ H_2O$), 65.0 g; biotin, 0.2 g; sulfuric acid (H_2SO_4), 5.0 ml. The solution was filter sterilized and stored at room temperature.

Fermentation conditions were set at 25 or 30 °C, with agitation (800 rpm) and 1.51 of air per liter of culture volume/minute. The pH was maintained at 6.0 by the addition of 30% ammonium hydroxide. For pH experiments, 30% phosphoric acid was added slowly for one-time pH adjustments from 6.0 to 4.5 either at the beginning or during the course of the fermentation as indicated. The level of dissolved oxygen was monitored using an Ingold galvanic electrode. One milliliter of Antifoam 204 (Sigma-Aldrich) was added before inoculation. When the carbon source was depleted as indicated by a sharp rise in oxygen concentration, the fed-batch cultivation was initiated. During glucose fed-batch, 50% glucose solution mixed with 12 ml of PMT1 per liter was continuously added at a rate of 12 ml/h to keep the dissolved oxygen in the medium broth above 30% saturation. Heme (0.1 g/l) was added as dried powder at the beginning of the fed-batch phase.

Enzyme Purification and Stability Experiments

Recombinant manganese peroxidase (rMnP) was concentrated from *P. pastoris* αMnP1-1 cultures (pH 6.0) by high-speed (10,000×*g*) centrifugation to remove the yeast cells and heme particulates, acetone precipitation (equal volumes of acetone and culture supernatant), dialysis (0.1 M pH 6.0 KH_2PO_4/K_2HPO_4 buffer) to concentrate the rMnP and remove salts, and freeze-drying. The final rMnP activity was concentrated from approximately 2,000 U/l in the fermentation broth to 30,000 U/l in the final concentrated solution [4]. For enzyme stability experiments in defined buffers, fermentation broth (with yeast cells) or supernatant fractions, the initial rMnP activity was adjusted at the start of the experiment through the addition of concentrated rMnP to about 1,500 U/l. Enzyme stability

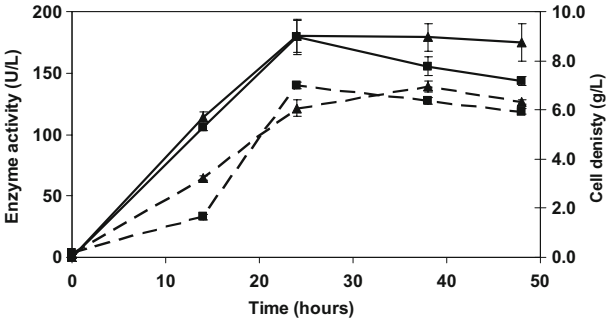

Fig. 1 Effects of temperature on cell growth and rMnP activity (U/l) in *P. pastoris* αMnP1-1 shake flask cultivation (pH 6). *Solid line* Enzyme activity (U/l), *dashed line* cell density (g/l). Symbols are as follows: *triangle* 25 °C, *square* 30 °C

experiments were conducted with 20 ml of solution in 125 ml baffled flasks on a rotary shaker incubator (250 rpm; New Brunswick Scientific Co., Inc.) at 30 °C.

Four different protease inhibitors [10 μM pepstatin, 20 μM E-64, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM ethylenediaminetetraacetic acid (EDTA)] were examined for their effects on rMnP stability. These inhibitors were dissolved in either distilled water or ethanol and then added into 20 ml of pH 4.5 culture supernatant either separately or in combination. The 125 ml baffled flasks were incubated on a rotary shaker as described above. The rMnP activities were measured at appropriate time intervals.

Results and Discussion

Production of MnP at Different Temperatures

Lowering the temperature of *P. pastoris* αMnP1-1 shake flask or bioreactor fermentations from 30 to 25 °C did not result in appreciable changes in cell growth rate or production of recombinant enzyme as indicated by cell density and rMnP activity measurements (Figs. 1 and 2). A higher cell density was achieved at 25 °C (52.6 g/l) than at 30 °C (42.1 g/l) in the fed-batch fermentations, but this did not result in a corresponding increase in rMnP activity (Fig. 2).

This is in contrast to many reports in which expression of active recombinant protein by *P. pastoris* was increased by up to 30 times by lowering the temperature from 30 to 15–25 °C

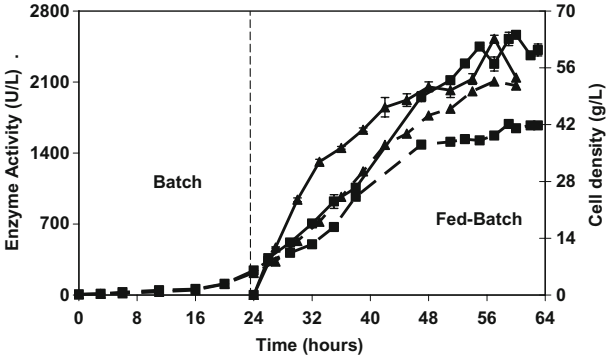

Fig. 2 Effects of temperature on cell growth and rMnP activity (U/l) in *P. pastoris* αMnP1-1 bioreactor fed-batch fermentations (pH 6). *Solid line* Enzyme activity (U/l), *dashed line* cell density (g/l). Symbols are as follows: *triangle* 25 °C, *square* 30 °C

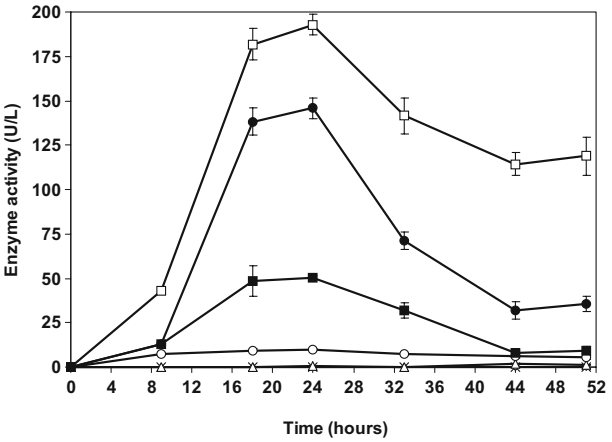

Fig. 3 Effects of pH on rMnP activity (U/l) in shake flask cultivation of *P. pastoris* αMnP1-1 (30 °C). Symbols are as follows: *cross* pH 4.0, *empty diamond* pH 4.5, *empty triangles* pH 5.0, *empty circles* pH 5.5, *empty squares* pH 6.0, *filled circles* pH 6.5, *filled squares* pH 7.0

[12, 13, 17, 20, 24, 25, 31]. The effect of lowering the temperature is typically attributed to decreased proteolysis and unfolding of the protein. The lack of a clear increase in the rMnP yield by *P. pastoris* αMnP1-1 upon lowering the fermentation temperature suggests that, at pH 6, spontaneous denaturation and protease activity do not limit the final active rMnP enzyme yield.

Production of MnP at Different pH

Varying the pH of shake flask cultures from pH 4.5 to 7 had little effect on *P. pastoris* cell growth. At pH 4, the cell density decreased to approximately half that of cultures at higher pH (data not presented). Little to no rMnP activity was observed during 48 h of cultivation at pH 4.0, 4.5, 5.0, or 5.5 (Fig. 3). After 24 h, the cultures at pH 6.0, 6.5, and 7.0 produced the highest enzyme activities (193, 147, and 51 U/l, respectively).

In fed-batch fermentations, growth rates at pH 4.5 and 6.0 (Fig. 4) were also similar, with short lag periods after large pH adjustments from pH 6 to 4.5 (Figs. 5 and 6). Growth of *P. pastoris* αMnP1-1 throughout this pH range is consistent with previously reported results [11, 17, 28].

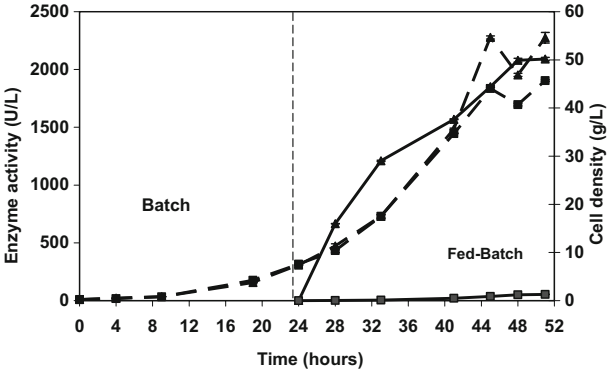

Fig. 4 Comparison of pH 4.5 and 6 *P. pastoris* αMnP1-1 fed-batch fermentations (30 °C). *Solid line* rMnP activity (U/l), *dashed line* cell density (dry weight, g/l). Symbols are as follows: *triangles* pH 6.0 during the whole cultivation, *squares* pH 4.5 during the whole cultivation

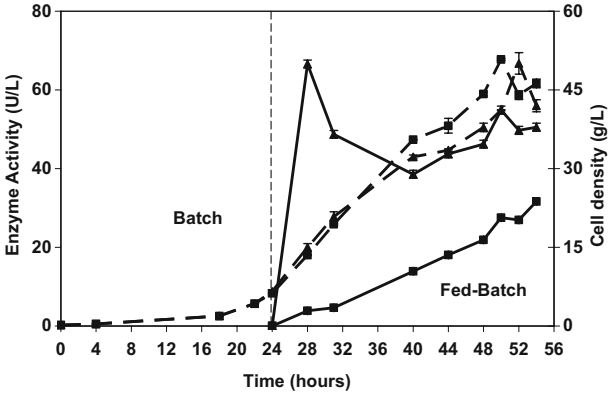

Fig. 5 Effects of reducing the pH from 6 to 4.5 at the beginning of the fed-batch phase. *Solid line* rMnP activity (U/l), *dashed line* cell density (dry weight g/l). Symbols are as follows: *triangle* pH 6.0 for batch/pH 4.5 for fed batch, *square* pH 4.5 during the whole cultivation

In bioreactors maintained at pH 4.5 throughout the batch and fed-batch phases, the rMnP enzyme activity was very low (54 U/l) compared with activities greater than 2,000 U/l in pH 6.0 fermentations at the end of the fed-batch phase (Fig. 4). Decreasing the culture pH from 6.0 to 4.5 at the start of the fed-batch phase also resulted in little rMnP accumulation in the medium (Fig. 5). Decreasing the culture pH from 6.0 to 4.5 after 20 h in the fed-batch phase resulted in a rapid loss of more than 1,000 U/l rMnP activity from the rMnP produced at pH 6 (Fig. 6). The failure of *P. pastoris* αMnP1-1 to produce appreciable amounts of rMnP at low pH and the decline in rMnP activity upon lowering the cultivation pH are somewhat unexpected. *P. pastoris* grows equally well at pH 6 and 4.5, and wtMnP and rMnP are both stable and active at pH 4.5 [3]. Furthermore, the wtMnP host (*P. chrysosporium*) is known to produce active wtMnP at pH 4.5 [34]. Because of the rapid inactivation of rMnP in cultures at pH 4.5, a pH of 6.0 was chosen for the routine cultivation of *P. pastoris* αMnP1-1, and further experiments were undertaken to investigate the cause of low rMnP production at low pH.

rMnP Stability

Recombinant MnP produced by *P. pastoris* αMnP1-1 in pH 6 fed-batch fermentations was not stable in pure pH 6.0 buffers (Fig. 7). In 0.01 M potassium phosphate buffer, rMnP activity decreased by 25% in 4 h, and a somewhat higher activity loss rate was observed in

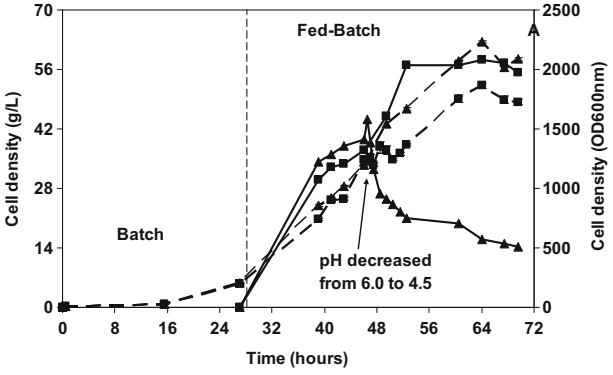

Fig. 6 Effects of reducing the pH from 6 to 4.5 during the middle of the fed-batch phase. *Solid line* rMnP activity (U/l), *dashed line* cell density (dry weight g/l). Symbols are as follows: *triangle* transition from pH 6.0 to 4.5 during the middle of fed-batch, *square* pH 6.0 during the whole cultivation

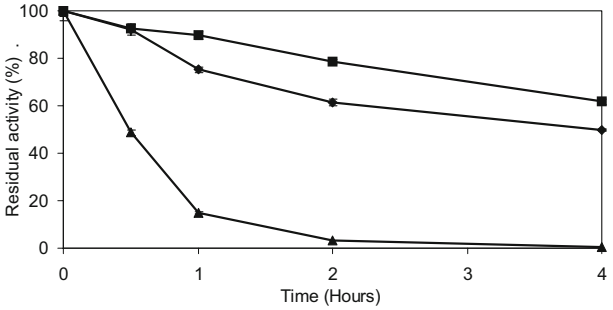

Fig. 7 Stability of rMnP in pH 6.0 buffers (30 °C). Symbols are as follows: *diamonds* 0.1 M phosphate buffer, *squares* 0.01 M phosphate buffer, *triangles* 0.01 M sodium citrate buffer

0.1 M potassium phosphate buffer. A much more rapid decrease in rMnP activity was observed in 0.01 M sodium citrate buffer (also pH 6); the enzyme half-life in this buffer was less than 30 min (Fig. 7).

These experimental data are well-described by first-order kinetics, with best-fit half-lives of 6.7, 3.4, and 0.4 h for 0.01 M phosphate, 0.1 M phosphate, and 0.01 M sodium citrate buffers, respectively. These purified buffers may lack essential co-factors that could stabilize the rMnP, such as calcium, manganese, and iron. As Ca^{2+} ion is essential for MnP activity, chelation of calcium ions by citrate (and to a lesser extent, phosphate) is probably the mechanism for the observed first-order kinetics of inactivation in these buffers. These attempts to determine the effect of pH on rMnP activity in defined buffers were ultimately deemed unsuccessful, as the rates of inactivation in potassium phosphate (0.1 and 0.01 M) and sodium citrate (0.01 M) buffers were observed to be considerably higher than in the bioreactor culture supernatant.

Recombinant MnP concentrated from pH 6 *P. pastoris* αMnP1-1 fed-batch fermentations was also added to pH 4.5 fed-batch fermentations and pH 4.5 and 6 cultures and culture supernatants. In culture supernatants obtained from pH 6.0 bioreactor fermentations, rMnP was stable for up to 6 h before a measurable loss of activity occurred, and only 25% of the initial activity was lost after 24 h incubation (Fig. 8). In supernatant from pH 4.5 fermentations, 25% of rMnP activity was lost during the first 6 h, and another 25% decrease occurred in the following 18 h. However, a much higher rate of loss of rMnP activity was observed in whole pH 4.5 culture, from which the yeast cells were not removed via centrifugation, than in either of the cell-free supernatants (Fig. 8). The rate of loss of rMnP activity in pH 4.5 bioreactor cultures and culture supernatant was higher in samples obtained after 72 h of fermentation than in samples taken after only 48 h. As before, the

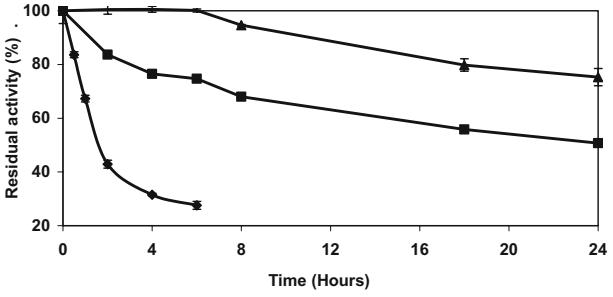

Fig. 8 rMnP stability in cultures and culture supernatants from pH 4.5 and 6.0 *P. pastoris* αMnP1-1 fed-batch fermentations (30 °C). Symbols are as follows: *triangles* pH 6.0 culture supernatant, *square* pH 4.5 culture supernatant, *diamond* pH 4.5 culture

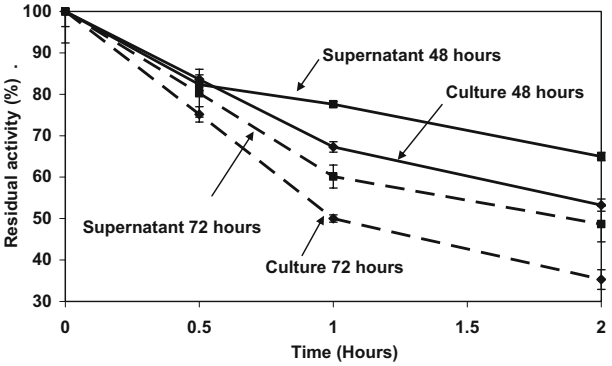

Fig. 9 rMnP stability in cultures and culture supernatants taken 48 and 72 h after the start of *P. pastoris* αMnP1-1 pH 4.5 fed-batch fermentations (30 °C). *Solid line* Samples at 48 h, *dashed line* samples at 72 h. Symbols are as follows: *diamonds* culture, *squares* culture supernatant

presence of yeast cells substantially increased the rate of loss of rMnP activity (Fig. 9). Also, the activity of concentrated rMnP added directly into the medium of an ongoing pH 4.5 bioreactor fermentation 20 h after the start of the fed-batch phase declined rapidly, with 50% of the activity lost within the first 2 h (Fig. 10).

Whereas it is known that the negatively charged yeast cell surface strongly adsorbs mono- and divalent ions, such as Ca^{2+} [35, 36], the cells in the whole culture were grown in calcium-rich media, and Ca^{2+} binding sites would likely be saturated. Thus, simple biosorption of calcium does not explain the very fast inactivation of rMnP in whole culture. A complex or cooperative mechanism is suggested by the failure of first-order kinetics to describe the inactivation of rMnP in any of these media (c.f. Fig. 7).

Proteases

The results of the rMnP stability experiments are consistent with the hypothesis that intracellular proteases released from dead cells during the fermentation are active against rMnP at pH 4.5. The rapid onset of rMnP inactivation during fermentation following a pH adjustment from 6.0 to 4.5 suggests that these proteases are produced at both pH 6 and 4.5, but are only active against rMnP at the lower pH. Lowering the temperature of the stability test in pH 4.5 culture supernatant from 30 to 20 °C slowed the rate of rMnP activity loss (Fig. 11), consistent with reduced activity of proteases against rMnP at lower temperatures. However, the lower temperatures would likely also favor the active enzyme conformation, so the observed rMnP inactivation cannot necessarily be attributed to the action of proteases.

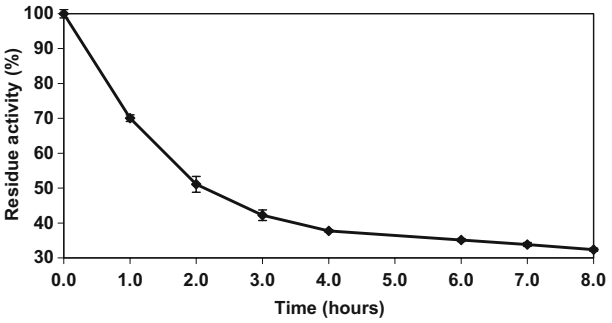

Fig. 10 Stability of rMnP added to pH 4.5 *P. pastoris* αMnP1-1 fed-batch fermentations (30 °C) 20 h after the start of the fed-batch phase

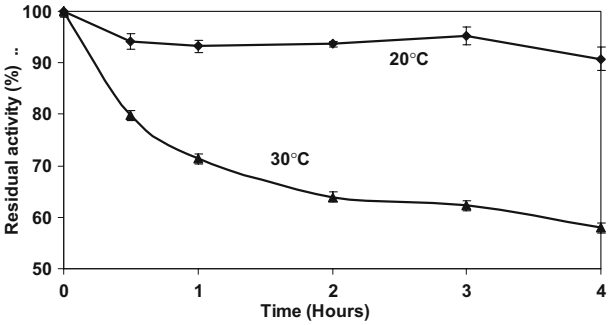

Fig. 11 rMnP stability in 20 and 30°C culture supernatant from *P. pastoris* αMnP1-1 pH 4.5 fed-batch fermentations (30 °C). Symbols are as follows: *diamond* 20 °C, *triangles* 30 °C

A comparison of the rate of inactivation of rMnP in whole culture and culture supernatants from *P. pastoris* αMnP1-1 fed-batch fermentations at pH 4.5 (Fig. 12) indicates that the rate of loss of activity in the presence of cells is approximately 100 U l^{-1} h^{-1} higher than in the culture supernatant. In both cases, the rate of rMnP degradation is initially high, presumably because of the action of proteases, and then decreases from approximately 650 to150 U l^{-1} h^{-1} during the first hour. The initial rapid inactivation is followed by a slower inactivation of rMnP over the next few hours, similar to the degradation rate observed in pH 6 culture supernatants (Fig. 8). This slower rate of loss of rMnP activity may be caused by denaturation of the enzyme.

Further attempts to obtain direct evidence for the action of intracellular acidic proteases against rMnP using protease inhibitors and defined buffers were inconclusive. Addition of the protease inhibitors pepstatin (10 μM), E-64 (20 μM), and PMSF (1 mM) to pH 4.5 culture supernatant did not decrease the rate of rMnP inactivation, suggesting that aspartic, cysteine, and serine proteases, respectively, in the culture supernatant were not active against rMnP. The presence of EDTA (1 mM) was found to inhibit rMnP activity, probably by chelation of essential Ca^{2+} ions or heme Fe^{3+}, and thus, could not be used to detect the activity of metalloproteases (data not shown). Analysis for peptide fragments by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was also inconclusive because of the highly variable molecular weight of the intact hyperglycosylated protein. Further progress in elucidating the role of proteases at low pH will probably require the preparation of substantially pure rMnP, as well as a suitable defined buffer or medium in which to conduct stability studies.

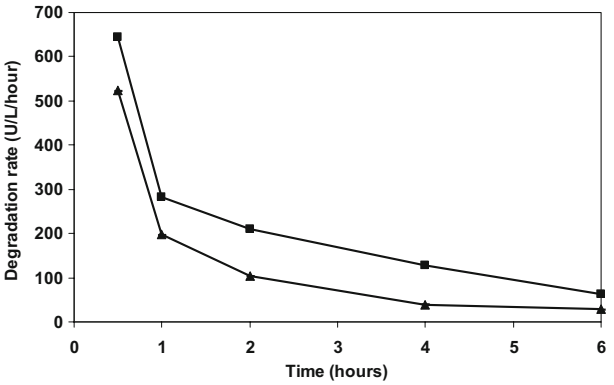

Fig. 12 Rate of degradation of rMnP added to cultures and culture supernatants from pH 4.5 *P. pastoris* αMnP1-1 fed-batch fermentations (30 °C). Symbols are as follows: *squares* pH 4.5 culture, *triangles* pH 4.5 supernatant

It does not appear that the production of active proteases limits the rMnP enzyme titer during pH 6 fermentations, but this possibility was not directly addressed and cannot be entirely eliminated. The production of rMnP in *P. pastoris* αMnP1-1 at pH 6 may be sufficiently practical for most industrial applications, such as pulp bleaching [2, 4]. Although rMnP is not produced effectively by *P. pastoris* αMnP1-1 at pH 4.5, the concentrated preparations of rMnP expressed at pH 6 exhibit activity at pH 4.5 similar to the wtMnP. Presumably these preparations could be used, perhaps with further purification to remove residual proteases, for low pH applications.

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