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ORIGINAL PAPER

Jason Pritchett · Susan A. Baldwin

The effect of nitrogen source on yield and glycosylation of a human cystatin C mutant expressed in *Pichia pastoris*

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Abstract Human cystatin C is a cysteine proteinase inhibitor with potential applications as an anti-viral agent, cancer tumor growth inhibitor, and in prevention of proteolysis during food processing. A glycosylated cystatin C mutant with increased temperature stability was developed for the latter application [Nakamura et al. (1998) FEBS Lett 427:252–254]. A recombinant variant of cystatin C [Nakamura et al. (2000) International patent no. PLTCA99/00717] with two potential sites for N-linked glycosylation was expressed in *Pichia pastoris* Mut^{s} . Little of the cystatin C produced was in the glycosylated form under fermentation conditions of pH 6, temperature 28°C, methanol only feed, and ammonium hydroxide as a nitrogen source. Thus, the effect of addition of complex nitrogen sources, peptone and amino acid supplements, on the yield and glycosylation of this mutant cystatin C were investigated. A full factorial design experiment using 2-l fermenters was performed with three factors: ammonium hydroxide, peptone, and an amino acid mix, at two levels, absent or present. Peptone addition was found to have a positive, and the most significant, effect on cell specific cystatin C yield. A maximum mutant cystatin C yield of 0.82 μmol (g-dry cell weight)$^{-1}$ min^{-1} was obtained when all three nitrogen sources were used together. However, under these conditions only 16% of protein was in the glycosylated form since ammonia was found to have a significant negative effect on glycosylation extent. The maximum extent of glycosylation was 30% when peptone and amino acid mix were the only nitrogen sources added.

J. Pritchett · S. A. Baldwin (✉)
Chemical and Biological Engineering,
University of British Columbia, 2357 Main Mall,
Vancouver, BC, V6T 1Z4, Canada
E-mail: sbaldwin@interchange.ubc.ca
Tel.: +1-604-8221973
Fax: +1-604-8225407

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Introduction

Yeasts are cost-effective, robust host organisms for producing high titers of recombinant proteins [10]. Yeasts can carry out post-translational modifications such as N- and O-linked glycosylation. Most studied yeasts undergo glycan trimming resulting in a core structure of $Man_8GlcNAc_2$, which undergoes only mannose addition of the α-1,2-linked, α-1,3-linked, or α-1,6-linked branches [6]. *Saccharomyces cerevisiae* is known to hyperglycosylate by adding glycan structures with a very high number of mannose residues, often greater than 50 residues. Hyperglycosylation has been associated with antigenicity and rapid clearance of glycosylated proteins from the blood by the liver, and is a problem for therapeutic protein production in *S. cerevisiae*. On the other hand, *Pichia pastoris*, in most cases, does not hyperglycosylate, and does not produce glycans with α-1,3-linkages [22]. *P. pastoris* also produces glycosylated chain lengths similar to mammalian host systems, which makes this yeast a potential organism for screening new recombinant glycoprotein candidates. Recently, the glycosylation pathway in *P. pastoris* has been re-engineered to enable the yeast to produce a complex human N-glycan, $GlcNAc_2Man_3GlcNAc_2$, in vivo. This is a significant step towards using *P. pastoris* in the actual manufacture of therapeutic human glycoproteins [16]. Another advantage of *P. pastoris* is that it can be grown at high cell productivities. Extensive work was done in the 1980s to optimize fermentation conditions for growth of *P. pastoris* on methanol when it was being considered as a single cell protein source [30].

Glycosylation of proteins affects their stability and function. It is beneficial in some cases, for example, when it improves the stability of recombinant proteins secreted into the fermentation medium [26, 28]. Cystatin

C is a cysteine proteinase inhibitor that serves many functions in the human body [1]. Some potential therapeutic applications of cystatin C include defense against bacteria and viruses [4], and inhibition of cancer metastasis [9]. Another potential use of cystatin C is to prevent proteolysis during food processing. For this application, it was shown that glycosylated mutant forms of cystatin C improved its stability and activity [23]. The literature, with respect to the effect of process conditions on glycosylation of recombinant proteins expressed by *P. pastoris*, is limited. Work has been conducted on glycosylation of recombinant proteins expressed by mammalian cells, since mammalian cells are often used for producing human therapeutics requiring glycosylation. Process factors that have been found to influence glycosylation in mammalian expression systems include ammonia concentration, pH, temperature, carbon source, and culture time. Extracellular ammonia in the concentration range 2–50 mM has been shown to decrease the amount of glycosylation (percent site occupancy or macro-heterogeneity) and degree of sialylation (structure of the glycan groups or microheterogeneity) [15, 31]. For mammalian cells, it has been widely hypothesized that this is due to local intracellular pH alterations as a result of importing ammonia into the cell. This shift in pH can alter the enzymatic activity of transferases involved in oligosaccharide and protein processing. For example, β-1,4 galactosyltransferase was identified as having optimum activity at pH 6.5, whereas an extracellular ammonia concentration of 10–15 mM can increase the trans-Golgi pH to 7–7.2 [14]. The pH of mammalian cultures has been shown to have a large effect on glycosylation; however, only in the presence of ammonia. Thus, it has been postulated that pH indirectly affects glycosylation via ammonia transport into the cell [5]. Studies have shown that decreasing mammalian culture temperature results in increased site occupancy [11]. It appears as if this is due to decreased growth rate and the fraction of cells in the G(0)/G(1) phase of the cell cycle. Glucose starvation of mammalian cultures has also been shown to effect glycosylation, resulting in shorter chain oligosaccharides [27]. However, the effects of ammonia, pH, temperature and carbon source starvation on glycosylation of proteins expressed in *P. pastoris* has not been extensively explored.

In this work, *P. pastoris* was used to express a variant of human cystatin C, invented by Nakamura et al. [24], with two potential glycosylation sites. The main goal of our work was to investigate process conditions that affect glycosylation of proteins in *P. pastoris* and this mutant cystatin C was used a model protein for this purpose. Under standard fermentation conditions of 1.49 g l^{-1} h^{-1} methanol, pH = 6 and T = 28°C, very little protein was expressed in either of the glycosylated forms (single or double). A preliminary set of shake flask experiments performed in our laboratory indicated that nitrogen source affected the percentage of cystatin C expressed in the glycosylated form more significantly than pH and temperature (data not shown). Thus, we investigated further the effect of three different nitrogen sources, ammonium hydroxide, peptone and amino acid supplements, on glycosylation of cystatin C in *P. pastoris* Mut^s .

Materials and methods

Cell strain

P. pastoris strain X-33 (Mut+, His+; obtained from Invitrogen, Carlsbad, Calif.) was transformed by electroporation with the pPICZα vector containing the gene for the cystatin C variant. The cystatin C gene had been mutated to introduce a consensus sequence for N-linked glycosylation (N-X-S/T) at two sites on the gene [24]. Transformed cells were selected by plating on zeocin (Invitrogen)-containing agar. Transformants were shown to be Mut^s His^-.

Culture media

All chemicals were from Sigma-Aldrich (St. Louis, Mo.) unless otherwise stated. For culture plates and inocula, yeast peptone dextrose (YPD) with or without zeocin was used with the following composition (amounts given in brackets are in g l^{-1} unless otherwise stated): yeast extract (10), Bacto-peptone (20), dextrose (20), agar (20) and zeocin (0.1–0.5). The Bacto-peptone was obtained from Difco (BD Diagnostic Systems, Sparks, Md.). Fermentation was carried out with basal salts medium (BSM; Invitrogen): H_3PO_4 (85%) (26.7 ml l^{-1}), $CaSO_4 \cdot H_2O$ (0.93), K_2SO_4 (18.2), $MgSO_4 \cdot H_2O$ (14.9), KOH (4.13), and glycerol (40). Histidine (1) and PTM1 (4.35 ml l^{-1}) trace salts (Invitrogen): $CuSO_4 \cdot 5H_20$ (6), NaI (0.08), $MnSO_4 \cdot H_2O$ (0.2), H_3BO_3 (0.02), $CoCl_2$ (0.5), $ZnCl_2$ (20), $FeSO_4 \cdot 7H_2O$ (65), biotin (0.2), and H_2SO_4 (5 ml l^{-1}) were added after sterilization. NH_4OH, Bacto-peptone and an amino acid mix were added, in differing amounts, as nitrogen source. The amino acid mixture was based on a recipe for *S. cerevisiae* [2]: adenine sulfate (0.66), L-arginine (0.32), L-aspartic acid (1.59), L-glutamic acid (1.59), L-histidine (0.32), L-leucine (0.95), L-lysine (0.48), L-methionine (0.32), L-phenylalanine (0.80), L-serine (5.96), L-threonine (3.18), L-tryptophan (064), L-tyrosine (0.48), L-valine (2.39), and uracil (0.32).

Fermentation conditions

Fermentation was performed using two 2-l working volume glass fermenters with temperature, pH and dissolved oxygen (DO) control (a bioreactor system from LH, formerly of San Francisco, Calif., and a Biobundle, from Applikon, Schiedam, The Netherlands). Three factors were varied: NH_4OH (0–8 g l^{-1}),

peptone (0–20 g l^{-1}) and amino acid mixture (0–20 g(total) l^{-1}). All nitrogen sources produced the same level of nitrogen (3.2 g l^{-1}). The inoculum was prepared by selecting one colony from the YPD plate. The colony was grown in 50 ml YPD medium at 28°C in a shaking incubator (Innova 4000, New Brunswick Scientific, Edison, N.J.) at 300 rpm for 20 h. This inoculum was added to 1-l BSM, including 0.1 g l^{-1} antifoam 289 (Sigma-Aldrich). The pH was controlled at pH 5 by NaOH addition. The temperature was maintained at 28°C and impeller speed at 1,000 rpm. Medical grade oxygen was fed to the bioreactor at 0.3 l min^{-1} to maintain the DO concentration above 30% saturation. The pH was measured with a Mettler Toledo (Columbus, Ohio) pH electrode or an Applikon AppliSens gel filled pH sensor. The oxygen concentration was measured with a Mettler Toledo Ingold electrode or an Applikon AppliSens DO electrode. The bioreactor was operated in batch mode for 18–20 h until glycerol depletion was identified by an abrupt increase in DO reading. The glycerol fed-batch phase was 4 h with a glycerol feed of 9.5 g l^{-1} h^{-1} and PTM1 trace salts fed to the reactor at a rate of 15 ml l^{-1} h^{-1} . The fed-batch phase was stopped after the cell density reached 200 g wet cell weight (WCW) l^{-1} . Prior to induction and methanol feeding, the pH was adjusted to 6 with NaOH. During induction, a methanol feed of 1.49 g l^{-1} h^{-1} 100% methanol with 12 ml l^{-1} PTM1 trace salts was maintained for up to 96 h. In previous experiments where methanol was monitored with a Methanol Sensor Model 2.1 probe (Raven Biotech, Vancouver, BC, Canada), this methanol feed rate was shown to maintain a constant methanol concentration of 0.2% (v/v) in the culture broth.

Cell density measurement

For dry cell weight (DCW), samples were dried in an oven at 100°C for 24 h before weighing on a Mettler Toledo AB104-S balance. WCW was determined by centrifuging 10 ml samples at 1,160 *g* (5810R centrifuge; Eppendorf, Hamburg, Germany) for 3 min and the supernatant aspirated using a pipette. A calibration curve was used to convert WCW to DCW ($r^2 = 0.9945$).

Cystatin C assay

Supernatant samples from the fermenter experiments were stored at −20°C until analysis. The storage period was shown to have no significant effect on the cystatin C assay (data not shown). Active cystatin C in the broth was measured in duplicate using a papain inhibition assay [3] with *N*-α-benzoyl-DL-arginine *p*-nitroanaline hydrochloride (BAPNA) as the substrate. Nitroanaline liberated by the residual papain activity was measured by spectrophotometry at 410 nm (Pharmacia Biotech Ultrospec 1000; Pfizer, New York, N.Y.). Sample blanks were prepared by heating at 100°C for 10 min to denature the cystatin C. A sample volume of 0.1 ml containing cystatin C was added to the assay buffer and 5.6×10^{-5} g papain (MW = 21,000 Da). Cystatin C concentration (μM) was calculated using the following formula, assuming that 1 mol cystatin C inhibits 1 mol papain:

$$\left(\frac{OD_{450}control - OD_{450}sample}{OD_{450}control}\right)\left(\frac{5.6\times10^{-5}\,g\,papain}{21,000\,g/mol}\right)\left(\frac{10^{6}\,\mu mol}{mol}\right)\left(\frac{1}{10^{-4}\,l}\right)$$

SDS gel electrophoresis

Cystatin C in the culture supernatant was concentrated by adding trichloroacetic acid (TCA) to a concentration of 20% (v/v). The samples were incubated for 10 min at 4°C, then centrifuged at 20,800 *g* for 5 min and the pellet washed twice with ice-cold acetone. To prepare the pellet for electrophoresis, 20–40 μl sample buffer (20 mM Tris-HCl (pH 6.8), 20 g l^{-1} SDS, 20 g l^{-1} β-mercaptoethanol, and 400 g l^{-1} glycerol) were mixed followed by incubation for 3 min at 100°C. A standard low molecular weight marker (product 161-0304; Bio-Rad, Hercules, Calif.) was prepared using the same heating procedure. The lanes were loaded with 20 μl sample and the system was electrophoresed at 50 mA using a Hoeffer SE 250 Mighty Small II apparatus from Amersham Biosciences (Piscataway, N.J.). Running buffer contained (in g l^{-1}): Tris-HCl (6), glycine (28), and SDS (1). After running the gel, it was stained overnight (Coomassie blue dye (0.25 g l^{-1}), methanol (50% v/v) and acetic acid (10% v/v)) and then destained (methanol (20% v/v) and acetic acid (10% v/v). Scion imaging software (Scion, Frederick, Md.) was used to quantify the intensity of the gel bands.

Statistical analysis

Statistical analysis was performed using the R 1.9.0 statistical analysis software package on a Macintosh Powerbook G3 500 MHz.

Results and discussion

In the preliminary shake flask experiments, temperature and pH were examined over the ranges of 21–35°C and 5.2–6.8, respectively. The maximum cystatin C yield was obtained at 28°C and pH 6 (data not shown). Thus, these conditions were selected for fermentation experiments. Cell densities reached approximately 200 g-WCW l^{-1} (equivalent to 49 g-DCW l^{-1}) following the glycerol-fed batch phase, and remained relatively constant throughout the induction period, as seen in Fig. 1.

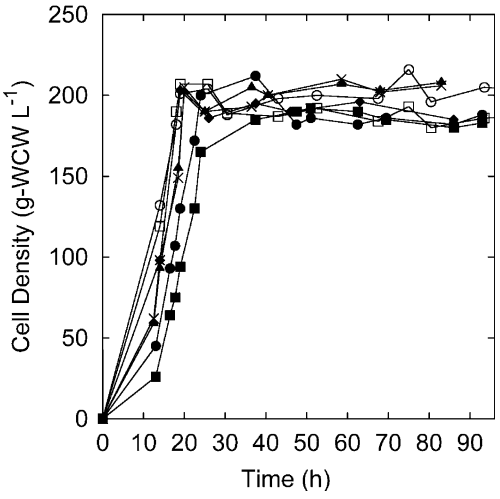

Fig. 1 *Pichia pastoris* growth curves in fermentation runs with different nitrogen sources: *open circles* NH_4OH; *crosses* amino acid mix; *filled squares* peptone; *open squares* NH_4OH and amino acid mix; *filled circles* NH_4OH and peptone; *filled triangles* amino acid mix; *filled diamonds* peptone, NH_4OH, peptone and amino acid mix

During the induction phase, in each fermentation, the average cell density varied only ± 9 g-WCW l^{-1}. Cystatin C yields (μmol g-DCW^{-1}) increased with time and then, after ~50 h, leveled off or decreased as seen in Fig. 2. Cystatin C productivities (in nmol g-DCW^{-1} h^{-1}) measured after 48 h are presented in Table 1. An ANOVA comparing cystatin C productivities for fermentations with and without peptone determined that peptone addition had a significant effect ($P = 0.0000374$). Similar analyses determined that the presence or absence of ammonium hydroxide or amino acid mix did not have significant effects on cystatin C productivity. The highest cystatin C productivities were obtained for fermentations with peptone, even in the presence of other nitrogen sources. When ammonium hydroxide was the only nitrogen source added, the maximum cystatin C yield obtained was 0.30 μ mol g-DCW^{-1} after 96 h, whereas when ammonia was replaced with peptone, the cystatin C yield increased more than 2-fold to 0.72 μmol g-DCW^{-1} . Slightly higher cystatin C yields were obtained when both ammonia and peptone were used as nitrogen sources. The amino acid mixture was used as a defined nitrogen source replacement for peptone. However, cystatin C yields were very low when amino acids were used as the only source of nitrogen.

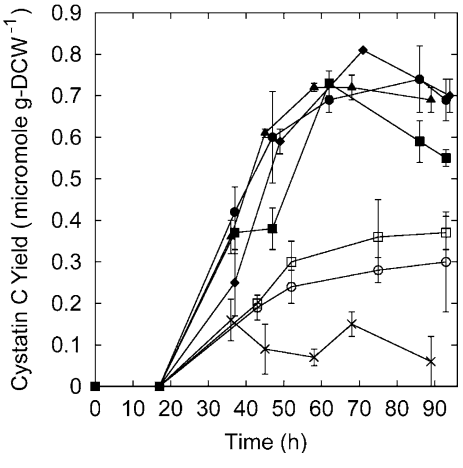

Fig. 2 Production of mutant cystatin C (μmol g-DCW^{-1}) in fermentation runs with different nitrogen sources: *open circles* NH_4OH; *crosses* amino acid mix; *filled squares* peptone; *open squares* NH_4OH and amino acid mix; *filled circles* NH_4OH and peptone; *filled triangles* amino acid mix and peptone; *filled diamonds* NH_4OH, peptone and amino acid mix. *Error bars* were obtained from duplicate cystatin C assays

Table 1 Cell specific total cystatin C productivities and percentages of un-glycosylated (*UG*), single (*SG*) and double glycosylated (*DG*) cystatin C after 48 h. *AA* Amino acid mix

Nitrogen source	Productivity (nmol g-DCW^{-1} h^{-1})	Percentage glycosylation		
		UG	SG	DG
NH_4	4.7	94	6.0	0.0
Peptone + AA	13.5	70	19.5	10.5
Peptone	11.8	74	16.1	9.9
NH_4 + AA	5.7	90	5.0	5.0
NH_4 + peptone	12.8	88	6.5	5.5
Peptone + AA + NH_4	12.1	84	8.3	7.7
AA	4.4	77	11.5	11.5

The positive effect of peptone addition on cell-specific cystatin C yield is similar to trends observed for mammalian cell cultures. For example, addition of protease or meat peptone to defined serum-free media increased both hybridoma cell concentration and monoclonal antibody production in batch, continuous and perfusion cultures [18]. Vegetable-derived protein hydrolysates have also improved therapeutic protein production in mammalian cell cultures [17]. These peptones were added to eliminate animal or human products in the medium. Peptone, in addition to being a nutritional supplement, has the potential to decrease proteolytic degradation of the product, which may lead to higher protein titers. Although peptone, used in this study, contains free amino acids, it is the complex oligopeptide composition of peptone that is thought to be responsible for the increased cystatin C cell-specific yield. Short peptides, rather than free amino acids, have been shown to increase protein yield in mammalian cell cultures. Work by Franek and Katinger [12] has revealed that short peptides are bioactive and those containing a basic amino acid, lysine or histidine, suppressed cell growth and enhanced monoclonal antibody yield. For example, addition of the tri-peptide Gly–Lys–Gly increased monoclonal antibody concentration from 31 mg l^{-1} for the control to 49 mg l^{-1} [13].

The relative amounts of un-glycosylated and glycosylated cystatin C were determined from SDS-PAGE gels of concentrated broth samples taken at 48 h. Three bands on the gel at approximately 15, 19, and 27 kDa for un-glycosylated, single and double glycosylated cystatin C, respectively, had been previously confirmed

as cystatin C by western blot analysis using rabbit antiserum (Dako, Carpinteria, Calif.; M. Ogawa—personal communication). All three bands for un-glycosylated, single, and double glycosylated cystatin C can be seen for most fermentations in Fig. 3, although the bands vary in intensity. The relative amounts of un-glycosylated, single and double glycosylated cystatin C as calculated from the intensity peak areas of the scanned images are presented in Table 1. Statistical *t*-tests were performed comparing the total glycosylation [single (SG) + double (DG) glycosylation in Table 1] achieved in fermentations with NH_4 to those where NH_4 was excluded. A similar analysis was performed for peptone and the amino acid mix. The presence of ammonia was found to have a significant negative effect ($P = 0.00357$) on the extent of glycosylation. Significantly better glycosylation results were obtained for fermentations that did not contain ammonium hydroxide but contained either peptone or amino acid mix or both. The presence of peptone or amino acid mix did not have a significant effect on the extent of total glycosylation. The best result was 30% total glycosylation obtained in the fermentation with both peptone and amino acid mix but no ammonium hydroxide.

The negative effect of ammonium hydroxide on the extent of glycosylation seen in our work is compatible with the trends observed for mammalian expression systems, as discussed in the Introduction. Thus, it is beneficial to use an alternative nitrogen source, like peptone, to increase glycosylation of cystatin C in *P. pastoris*. Although amino acid mix addition increased the extent of total glycosylation (26%) over that observed in the ammonium hydroxide fermentation (6%), it had a negative effect on cystatin C productivity and therefore cannot be recommended. However, more work needs to be done to optimize the amino acid mix composition for *P. pastoris*.

There are not many studies in the literature that report the effect of growth medium composition on glycosylation of proteins expressed in *P. pastoris*. In one such study [8], buffered minimal glycerol and methanol media maintained the culture pH at 6, under which condition more beta-casein was expressed in the glycosylated form than in non-buffered medium. The same study showed that alteration of non-buffered medium with 2% peptone resulted in slightly increased amounts of glycosylated protein. However, in this experiment, the pH of the non-buffered medium varied from 5.5 to 3 over the course of the fermentation. In another study, substitution of minimal medium with a molasses-based medium did not result in any changes in composition of glycan groups on proteins expressed in *P. pastoris* [22]. However, this paper does not mention the effect of the medium change on extent of glycosylation.

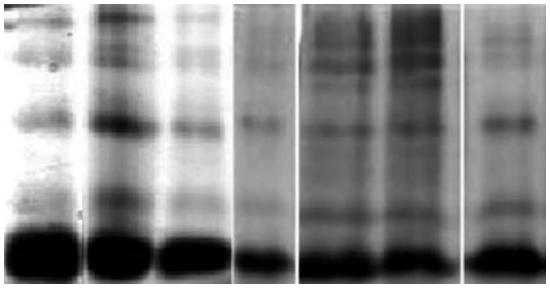

Fig. 3 SDS-PAGE gels for the concentrated protein products in broth samples taken at 48 h from fermentation runs with different nitrogen sources: *N* ammonium hydroxide, *P* peptone, *A* amino acid mix. *UG*, *SG* and *DG* indicate un-glycosylated, single and double glycosylated mutant cystatin C, respectively

Glycosylation levels, where reported, for some *P. pastoris*-expressed proteins vary from 10 to 35% depending on the protein [22]. The protein structure and location of the consensus sequence sites, Asn–X–Ser/Thr, have some effect on the extent of glycosylation [25]. Since *P. pastoris* expresses high levels of recombinant protein through the *AOXI* promoter, the post-translational apparatus in the cell may be unable to handle large amounts of protein, resulting in low glycosylation levels. Only 10% of the protein Bm86 (five potential N-linked glycosylation sites, MW = 72 kDa), which has many disulfide bridges and a particulated morphology, was expressed in the glycosylated form [21], whereas higher levels (30–35%) of α-amylase (six potential N-linked glycosylation sites, MW = 45 kDa), were glycosylated when expressed in *P. pastoris* [25]. The mutant cystatin C expressed by us is a low molecular weight protein (MW = 13 kDa) with one alpha helix and six beta sheets; however, in minimal medium very little of the glycosylated form was expressed. Thus, it is difficult to predict the extent of glycosylation based purely on protein structure.

Glycosylation involves addition of sugar groups with the lipid dolichol acting as the key carrier, thus supplemental sugars [29] and lipid precursors [7, 20] have been found to increase glycosylation in mammalian systems. We were unable to find literature reports quantifying the effects of defined amino acid mixtures or complex protein hydrolysates on glycosylation levels of recombinant proteins expressed in either mammalian or yeast cells. However, the preliminary work here suggests that substituting peptone instead of an ammonium salt as the nitrogen source increases glycosylation extent in a *P. pastoris*-expressed protein. Like carbon source supplements, the effect of peptone may be to increase cell metabolism and the induction of certain glycosyltransferases [19].

In conclusion, peptone supplementation of minimal medium was found to increase *P. pastoris*-expressed mutant cystatin C cell-specific yield. Addition of a defined amino acid mixture did not have a similar effect to that of peptone supplementation, indicating that the complex nature of peptides in peptone may be responsible for enhancing cell specific yield of this protein. The presence of ammonium hydroxide in the medium had a significant negative effect on glycosylation extent, as expected from experience reported in the literature for

mammalian cell cultures. The greatest extent of glycosylation for mutant cystatin C, obtained in the fermentation supplemented with both peptone and amino acid mix, but without ammonium hydroxide, was 30%.

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