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FOREWORD

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Introduction

The report documents the research and development activities on BoNTC Hc, BoNTE Hc, and BoNTF Hc. BoNTC Hc research focused on characterizing the capture of the BoNTC Hc from *P. pastoris* lysate and the interference of DNA-type material on the binding of BoNTC Hc to an anion exchange column. BoNTF Hc process development was concerned with confirmation of a process developed by USARMIID transferred to Covance and tested at UN-L. UN-L found two steps needed further development, i.e. cell breakage conditions and one chromatographic condition. This process was scaled-up to the pilot scale and transferred back to USAMRIID. Fermentation research at the UN-L focused on developing standard fermentation protocols that can be easily transferred to Covance. The BoNTC₁ Hc *P. pastoris* fermentation process was successfully developed and optimized using a quantifiable Western blot procedure. Also, a fermentation process for BoNTE Hc express in *E. coli* was developed and conditions were determined for expression of soluble material. UN-L also provided fermentation support for BoNT D, B, and E Hc for both USAMRIID and UN-L purification activities.

The Quality Assurance/Quality Control group at UN-L established multiple pre-seeds for clones developed at USAMRIID and established batch records for the BoTNF Hc process.

UN-L will transfer BoNTC and BoNTE Hc to Covance over the next year. UN-L will also submit 4 to 6 papers for publication.

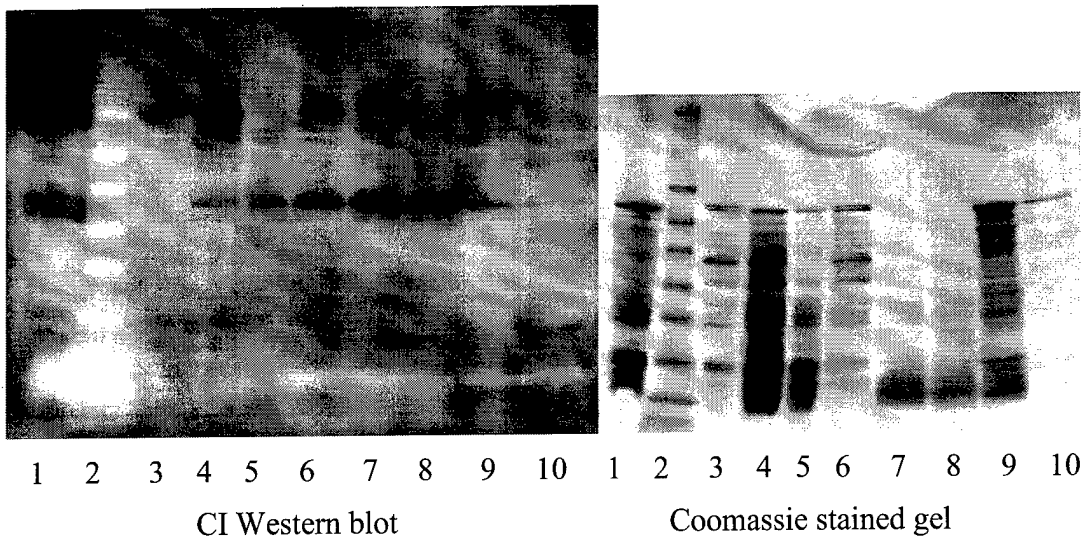
1. BoNT-C₁(Hc)

1.1. Early Process Development Activities on rBoNTC₁(Hc)

A method was received in September 1999 from Dr. Michael Byrne for recovery and purification of rBoNT-C₁(Hc) fragment from *Pichia pastoris* cells. This method was a three-step process, utilizing Poros HQ50 for the capture step, followed by Butyl Sepharose FF HIC, then Phenyl HIC (TosoHaas). The first test run was a scaled-up version of M. Byrne's method using Q Sepharose FF resin (30 mL column) instead of the Poros HQ. The method was tested with and without the use of DNase (Benzonase). Cells were from Lot ARC-FDL-004, strain A6. Cells were lysed in the same buffer used by MB, then the S/N split in half. One half was treated with DNase for ½ hour, then dialyzed against the dialysis buffer used by MB for 1.5 hours, and filtered before loading. The other half was diluted ½ with dialysis buffer, filtered, and loaded. The DNase treated sample filtered easier, but no difference was seen in the chromatographic profiles between the two runs (with and without DNase). Western blots did not turn out on this run, and no definitive information can be gained from looking at the gels (not shown).

Second, a small scale run was done using Streamline QXL resin in the packed-bed mode. Cells were from Lot ARC-FDL-004, strain A6. Lysis and running buffers were the same used by MB. No DNase was used, as it was noted that Benzonase requires Mg²⁺ for activity, and since the buffers had EDTA in them, it may not have been very active in previous runs. The western blots of fractions show a reactive 50 kDa band prominent in the 150 mM NaCl step elution, with some reactive 100 kDa in the FT and early elution. There was also both 50 kDa and 100 kDa reactive bands in the pellet. However, there

was no band (or very slight) seen on the Coomassie stain in the 50 kDa region in the 150 mM fractions (Figure 1).



Lanes: 1-washed pellet
2-MW Novex see blue
3-StrLn QXL FT 5
4-FT 7
5-FT 9
6-Peak 1 Fr 21 (150 mM Elute)
7-Peak 1 Fr 24 (150 mM Elute)
8-Peak 1 Fr 25 (150 mM Elute)
9-Peak 2 Fr 33 (400 mM Elute)
10-Peak 3 Fr 45 (1 M Elute)

Figure 1

(KP36-53W, KP36-53C)

The next run was the first to go beyond the capture step and include the two HIC steps. The capture resin was Q Sepharose FF (30 mL). Cells were from Lot ARC-FDL-004, strain A6. The 150 mM elution fraction was adjusted to 1M ammonium sulfate and loaded to a Butyl Sepharose 4FF HIC column (10 mL). The latest eluting peak (peak 4) from the Butyl column was readjusted with ammonium sulfate and loaded to a TosoHaas Phenyl column (10 mL). This peak was selected based on results from MB, and was not tested by western until after the Phenyl column was run. When the Phenyl fractions were tested, there was no signal by western in any fraction (not shown). It can be seen from the western of the Butyl fractions that the product is in the flow-through and peak 1 of the run (Figure 2).



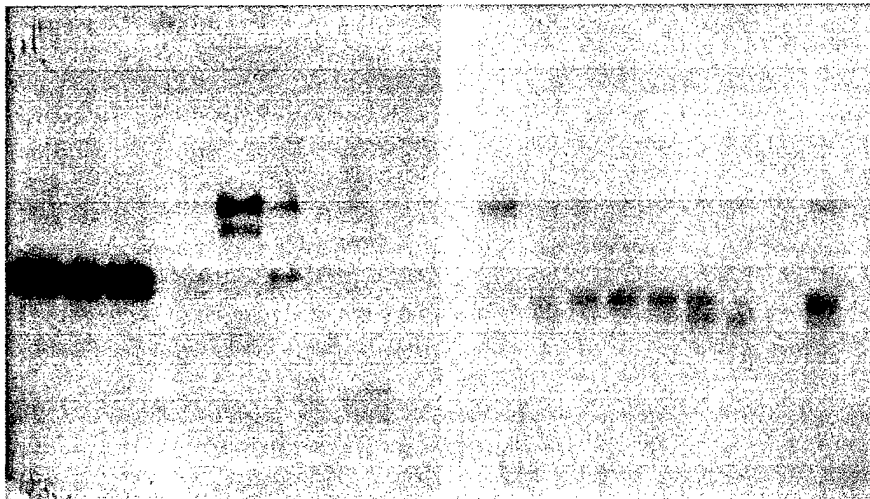
1 2 3 4 5 6 7 8 9 10
(KP36-56W)

Lanes: 1-butyl FT
2-Butyl Peak 1
3-Butyl Peak 2
4-Butyl Peak 3
5-Butyl Peak 4
6-Butyl Peak 5 (tail of 4)
7-MW see blue
8-Q load
9-Butyl load
10-Q product fraction

Figure 2

The Streamline QXL column was then run at the 100 mL expanded bed scale. Cells were from Lot ARC-FDL-004, strain A6. The MB lysis buffer was changed to 10 mM NaCl, and EDTA was omitted. The whole-cell lysate was treated with Benzonase for 40 min. prior to loading to the streamline column. The product was eluted with a step to 150 mM NaCl in MB equilibration buffer. A 50 kDa reactive band is detected strongly on the western blot, but no corresponding band (of similar intensity) is seen on the Coomassie stained blot. There is a 100 kDa reactive band detected in the FT and 1M fractions. There is also no definitive corresponding band on Coomassie to the 100 kDa reactive band (Figure 3). [KP36-69W, KP36-68W]

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20



Lanes: Figure 3
1 - 3: 150 mM elution peak
(3 fractions)
4 - MW
5 - 400 mM
6 - 1 M
7 - QXL load
9 - washed pellet
8, 10 are empty
11 - QXL load
12 - 18 FT samples taken
at increasing intervals during
sample loading
19 - washing after load
20 - MW

fractions also contained many contaminating bands. A thin band was seen on Coomassie in the 50 kDa region.

The Streamline QXL column (100 mL) was run again using lysate from Lot ARC-FDL-004, strain A6 cells. For this run, the streamline 150 mM fraction was loaded onto a 30 mL Butyl HIC column, then onto a 10 mL SP Sepharose to test a negative purification step. The 0.5M Butyl fraction was dialyzed to Tris buffer for SP. BoNT-C₁(Hc) (50 kDa reactive band) did flow through the SP column, but the banding pattern of the FT fraction did not look much different from the load material. It may have been better to use Tricine instead of Tris due to a possible interaction of Tris with the column that may have caused inhibition of binding. Further work with SP negative purification has not yet been pursued (Figure 4).

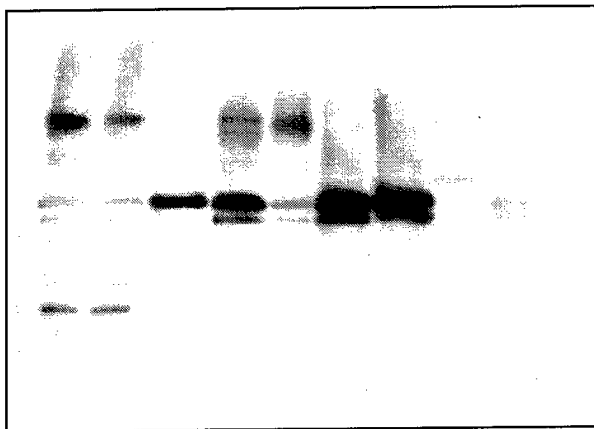
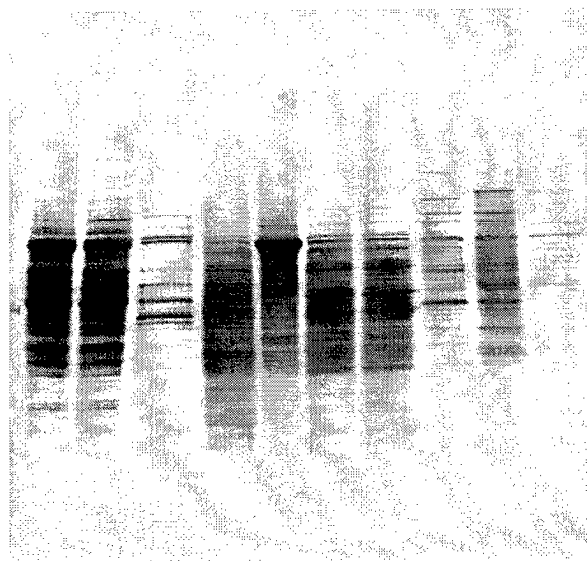


Figure 4

1 2 3 4 5 6 7 8 9 10 (JR95037W)



(JR95036WC)

- Lanes:
- 1 – QXL load
 - 2 – QXL FT
 - 3 – QXL wash
 - 4 – QXL product pool
 - 5 – 400 mM fraction
 - 6 – Dialyzed Butyl (SP load)
 - 7 – SP FT
 - 8 – SP Pk 1 front shoulder
 - 9 – SP Pk 1 apex
 - 10 – SP Pk 2

New fermentations were run using three different strains of BoNT-C₁(Hc) cells: A6, C5, and H2. A comparison between the A6 strain and the C5 strain was done. Cells from Lot Army-FDL-51, strain A6 were tested first. A Poros HQ column was used for capture, and Mike Byrne's original method was followed with the following exceptions: The Poros HQ50 column was 35 mL, 30g of cells were disrupted at a 20% cell/buffer ratio, no DNase was used, lysate S/N was dialyzed only 1 hour prior to loading, elution steps were 50, 150, 250, 400, and 1M NaCl. The 150 mM HQ fraction from was loaded to Butyl, but there was no reactivity on the western blot in any of the fractions (not shown). It started with this run that we began to see all of the western blot reactivity in the 100 kDa band and no reactivity at 50 kDa (Figure 5). This continued to be the case through all subsequent runs, and led to looking at the problems with the western blot.



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

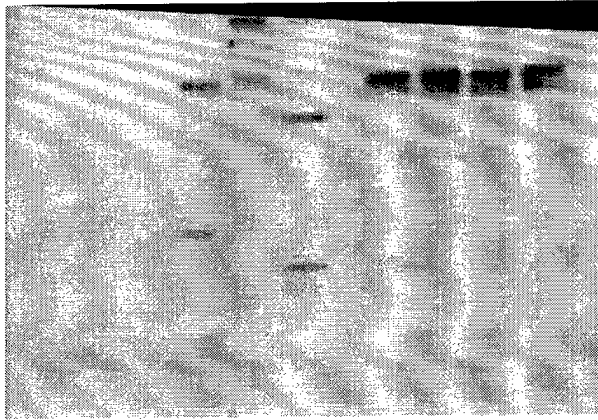
Lanes:

- 1 – 8: Poros HQ FT fractions taken across sample load
- 9 – HQ load
- 10 – MW
- 11 – Lysate
- 12 – HQ load
- 13 – MW
- 14 – HQ FT fr 20
- 15 – 50 mM elute
- 16 – 150 mM elute
- 17 – 250 mM elute
- 18 – 400 mM elute
- 19 – 1 M elute
- 20 – 150 mM product pool.

(KP36-88W, KP36-89W)

Figure 5

Cells from Lot Army-FDL-54, strain C5 were tested. The lysis and run conditions were the same as for the previous run with strain A6. The results are similar for this strain as for A6. There is no 50 kDa reactive band (Figure 6). [AY98-3-W1]



Lanes: Figure 6
1 – 50 mM HQ elute
2 – 150 mM elute
3 – 250 mM elute
4 – 400 mM elute
5 – 1 M elute
6 – MW
7 – HQ load
8 – 10 HQ FT fractions

(AY98-3-W1)

Figure 6

BoNT-C₁(Hc) cells of the H2 and A6 strains were processed under the same conditions as C5 for comparison. Results were inconclusive, and it was at this point that we started suspecting there was a problem with the Western blot procedure.

1.2. Optimization of BoNT-C₁ Western Blot Procedure

A series of experiments were conducted to determine four factors regarding the BoNT-C₁(Hc) western procedure. The goals were to: 1) determine an optimal level of primary and secondary antibodies (dilution factors) for good detection of the 50 kD BoNT-C₁(Hc) by chemiluminescence (CI) detection; 2) ascertain any non-specific reactivity to contaminating proteins; 3) compare the UNL CI method to Mike Byrne's colorimetric method for sensitivity; and 4) determine the source of a 100 kD reactive band detected on western blots that was not seen on Coomassie stains. It was also found after running all of these experiments that the lot of primary antibody received was bad, and was causing some of the non-specific interference problems. After receiving a new lot of antibody (the same lot used by Mike Byrne) many of these problems were eliminated. The dilution for the new lot of primary antibody was 1/2000, and incubation was overnight.

Methods: rBoNT-C₁(Hc), expressed in *Pichia pastoris*, was used as antigen. The primary antibody (1° Ab) was horse-anti-BoNT-C₁(Hc) (obtained from Intracel, Lot 4000-0001) reconstituted in 3 ml of 50% glycerol in PBS (with K), pH 7.2, and aliquots stored at -20°C (Lot 06035-PDL). The secondary antibody (2° Ab) was goat-anti-horse IgG (H+L) HRP conjugated (KPL). The following buffers and reagents were used in various tests:

PBS-T: Phosphate buffered saline + Tween-20 (40 mM NaPO₄ + 150 mM NaCl + 0.05% Tween 20, pH 7.2)

TBS: Tris buffered saline (10 mM Tris-HCl, 150 mM NaCl, pH 8.0)

GS-PBS-T: 5% normal goat serum (KPL) in PBS-T

NDM-PBS-T: 5% nonfat dry milk (BioRad) in PBS-T

NDM-TBS: 5% nonfat dry milk (BioRad) in TBS

ECL Plus: HRP substrate used for chemiluminescence detection (Pharmacia).

TMB: Single-component HRP substrate used for colorimetric detection (KPL).

BoNT-C₁(Hc) standard: 140 µg/ml purified BoNT-C₁(Hc) obtained from Michael Byrne.

GS115: *Pichia pastoris* host cell lysate supernatant, diluted to 1 mg/ml, boiled, and stored frozen at -20°C.

GS115+ BoNT-C₁(Hc): A mixture of 30 µl BoNT-C₁(Hc) standard, 22.5 µl GS115 lysate, 22.5 µl 4XR buffer.

4XR buffer: SDS-PAGE sample buffer, 4X with β-ME. (With the exception of GS115+ BoNT-C₁(Hc), samples were prepared for SDS-PAGE by mixing 3 parts sample with 1 part 4XR buffer). Samples were heated 10 minutes at 85 °C unless otherwise specified.

SDS-PAGE was done using 4-20% gradient Tris-glycine gels (Novex), 1.5 mm thick, 10 well, and Novex See Blue MW marker (250, 98, 64, 50, 36, 30, 16, 6, 4 kDa). The membrane was PVDF Immun-Blot (BioRad). Transfer was at 100V for 1h (BioRad Mini Transblot system).

The following different antibody dilutions were tested:

(A) 1° at 1/1,000 with 2° at 1/20,000, detection by CI

(B) 1° at 1/1,000 with 2° at 1/50,000, detection by CI

(C) 1° at 1/2,000 with 2° at 1/20,000, detection by CI

(D) 1° at 1/5,000 with 2° at 1/50,000 (dilutions previously used for CI detection), detection by CI

Primary and secondary antibodies were diluted in GS-PBS-T. Incubation times were 1 hour with each antibody.

To test for non-specific reactivity of 2° Ab to contaminating proteins, samples of GS115 only, GS115 spiked with BoNT-C₁(Hc) standard, and BoNT-C₁(Hc) standard alone were electrophoresed and transferred. Membranes were blocked with NDM-PBS-T, followed by incubation with 2° antibody for 1 hr. (no 1° Ab step). Detection was by CI.

Endogenous peroxidase activity was tested for by incubating a blot with ECL substrate after blocking, and visualizing by CI.

A comparison to Mike Byrne's method for sensitivity was done using GS115 only, GS115 spiked with BoNT-C₁(Hc) standard, and BoNT-C₁(Hc) standard alone. Membranes were blocked with NDM-PBS-T or NDM-TBS, followed by antibody incubation (overnight or 1 h). Detection was colorimetric with TMB.

It was found that with CI detection of BoNT-C₁(Hc), a 1/1,000 dilution of 1° Ab worked best, with a 1/20,000 or 1/50,000 dilution of 2° Ab, and a 1-2 minute exposure. 1/2,000 and 1/5,000 dilutions of 1° Ab were too dilute for good detection of the 50 kD BoNT-C₁(Hc) band by CI. There was non-specific binding of the secondary Ab to a 32 kD and a 15 kD band in *Pichia* lysate. This occurred in host cell lysate as well as on

host cell lysate spiked with BoNT-C₁(Hc) standard. There is also some other lower MW non-specific activity, though not as prominent as these two bands. The secondary Ab (alone) does not react (non-specifically) with any band in the 100 kD region. There was no signal detected on the blot incubated with substrate only.

With TMB detection 1/2000 dilution of the primary Ab is ok. An overnight incubation is required when using the TBS, pH 8.0 buffer system. If you use PBS-T, pH 7.2 the incubation time can be cut down to 1-2 hr on primary and secondary Ab. The TMB signal is greater when using the PBS-T system, however there is more non-specific binding (reactivity) at lower MW when using this system for testing lysate samples. A 1 hr incubation is not sufficient in the TBS system, thus pH appears to affect antibody affinity. The 100 kD band is reactive in the samples containing GS115.

Possible sources of the 100 kD reactive band include, a dimer of BoNT-C₁(Hc), an aggregate (binding) of BoNT-C₁ with another protein, the *Pichia* host cell lysate, a method dependent effect (gel, temperature, buffers, etc.), endogenous peroxidase activity from *Pichia*, or non-specific antibody binding. However, based on results from the experiments described above, the 100 kD band is not a BoNT-C₁(Hc) dimer, since it is detected in GS115 host cell lysate without BoNT-C₁(Hc) present, and it is not an aggregate, or binding of BoNT-C₁(Hc) to another protein. The 100 kD band is seen in the *Pichia* host cell lysate alone, and is detected using both Western methods (CI using PBS-T, and TMB detection systems using TBS or PBS-T), and is present when heating samples at different temperatures prior to gel loading. There is no endogenous peroxidase activity detected, as there is no reaction with substrate only in the absence of Ab. Though there is non-specific binding of 2° Ab to lower MW bands, non-specific reactivity in the 100 kDa region was not detected. The 100 kD band is only detected when both primary and secondary Ab are used thus it is specific for primary Ab.

Since the 100 kD shows up in samples of host cell GS115 lysate without BoNT-C₁(Hc), it is suspected that the prep used to raise the antibodies contained some of this contaminant. Note that this contaminant can not be seen with Coomassie, so a gel of the material used to raise the antibodies may not show a band in that area.

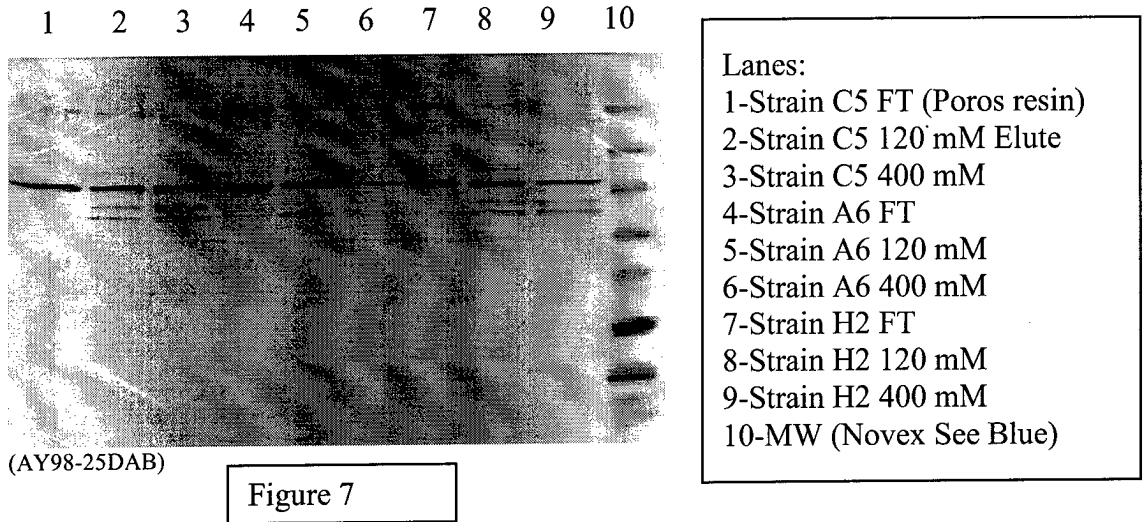
Subsequent to the above experiments, we received a new batch of primary antibody of the same lot that Mike Byrne was using. This new lot was not as reactive to the 100 kDa MW band, and was more reactive to the BoNT-C₁(Hc). The new lot of 1° Ab is used at a 1/2000 dilution. Additionally, a second vial of this same lot of 1° Ab was opened in July 2000 (stored lyophilized at 4°C) and was less reactive to BoNT-C₁(Hc), so dilution had to be reduced to 1/1000. Antibody (protein) concentration (by BioRad assay) of the newly reconstituted vial was only 0.5 mg/ml instead of the reported 1 mg/ml.

1.3. Recent Process Development Activities

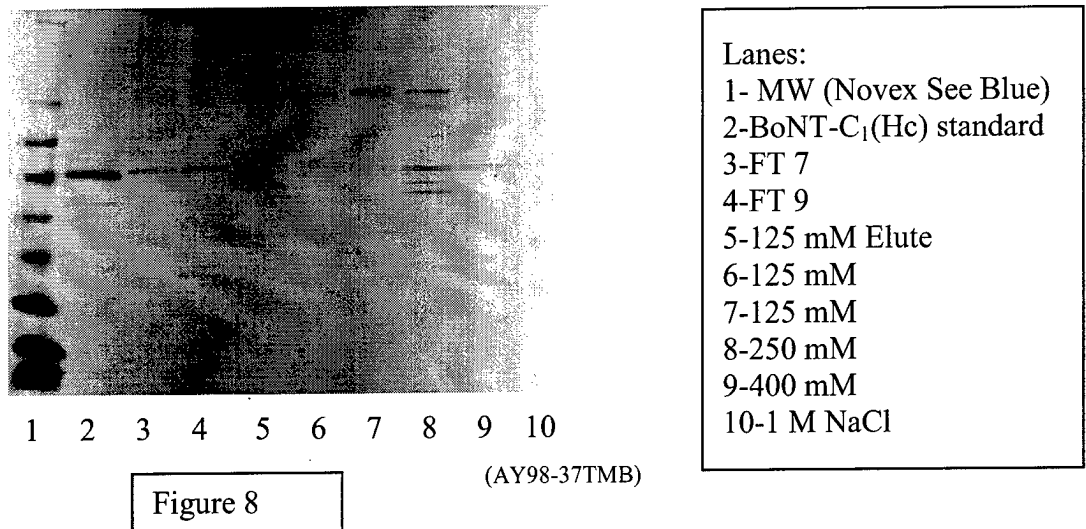
Strain and resin evaluation

In order to determine if there was a difference in protein expression between various clones, gravity flow columns were used to initially screen several resins using A6, H2, and C5 strains of *Pichia* expressing BoNT-C₁(Hc). Three resins were tested: Poros HQ, Q Sepharose FF, and Streamline QXL. Of these three resins, the Streamline QXL

required more NaCl to elute the product (Figure 7). Otherwise, there was not a significant difference seen between resins or the strains, and strain C5 was selected since that was the strain Mike Byrne had been working with at USAMRIID for previous development work.



We wanted to focus on Q Sepharose FF as a capture resin since it is relatively inexpensive and easily scalable. Strain C5 was used to generate lysates, and three buffers and pHs were tested: Tris pH 8.5, Mops, pH 7.5, and Tricine, pH 8.0 to determine maximum binding (capture). Using Tris buffer there was product in FT and 250 mM elution fraction. Using Tricine the results were not good, as there was a high degree of degradation seen in the fractions which reacted to western. The runs using Mops looked promising, with most of the product elution in the 250 mM fraction, though there was still a western signal detected in the flow through fractions (Figure 8). The Mops run was repeated using a 0-150 mM NaCl gradient instead of the 150 mM step, which resulted in all of the product in the 250 mM fraction (Figure 9).



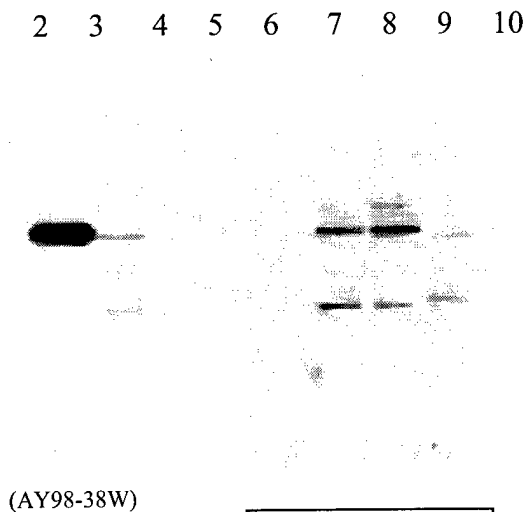


Figure 9

- Lanes:
- 1- MW (Novex See Blue)
 - 2-BoNT-C₁(Hc) standard
 - 3-FT Fr 5
 - 4-Gradient Fraction 22
 - 5-Gradient Fr 23
 - 6-Gradient Fr 25
 - 7-250 mM Step
 - 8-250 mM
 - 9-400 mM
 - 10-1 M NaCl

The Streamline QXL was also tested using Tricine buffer, but akin to the Q-Sepharose FF with Tricine buffer, there was not much signal in elution fractions compared to load. Apparently the Tricine buffer system is producing adverse results with this protein, but we are not sure why this would be.

The method that Mike Byrne sent using the Poros HQ and HIC resins with step elution was repeated and the fractions were tested using the new western blot (Figure 10). There was a significant amount of product in the FT fraction. Product was also detected in the 50, 130, and 250 mM fractions. Fractions were not very clean by SDS- PAGE (Figure 11).

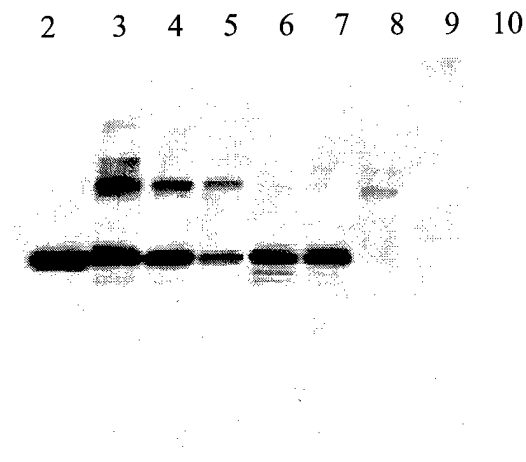
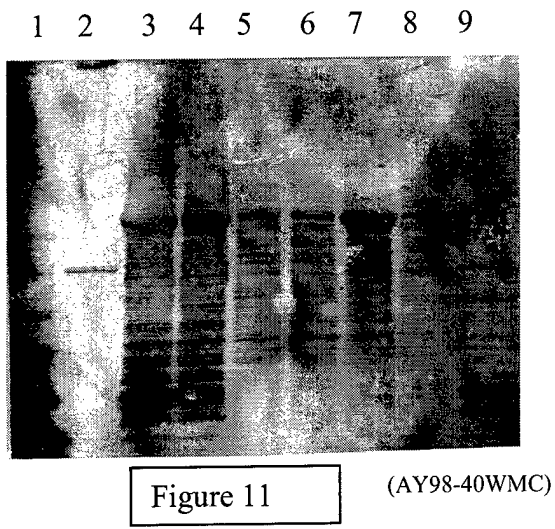


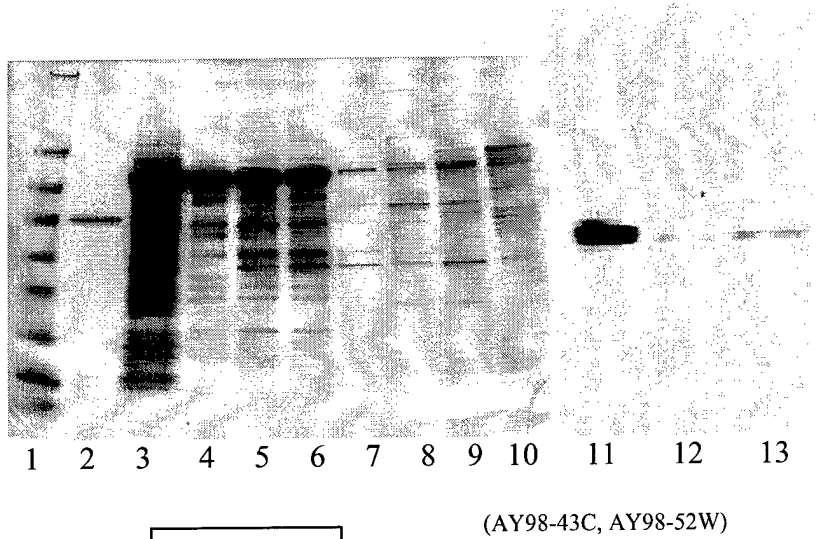
Figure 10

- Lanes:
- 1-MW (Novex See Blue)
 - 2- BoNT-C₁(Hc) standard
 - 3-Poros HQ Load
 - 4-HQ Flow Through
 - 5-50 mM NaCl Step
 - 6-130 mM
 - 7-250 mM
 - 8-400 mM
 - 9-1 M NaCl
 - 10-Empty



Lanes:
 1-MW (Novex See Blue)
 2- BoNT-C₁(Hc) standard
 3-Poros HQ Load
 4-HQ Flow Through
 5-50 mM NaCl Step
 6-130 mM
 7-250 mM
 8-400 mM
 9-1 M NaCl
 10-Empty

The 130 and 250 mM Q-fractions were pooled, adjusted to 1 M ammonium sulfate, and loaded to Poros Butyl HIC. The product was detected by western in the 0.2M ammonium sulfate step, but there was barely a signal seen on Coomassie gels, and the fraction still contains several contaminating bands of greater intensity (Figure 12) [AY98-43C]. The 0.2M Butyl fraction was then loaded to Phenyl HIC and product was present in the 0.2 and 0 M Phenyl fractions, however there was a product signal was barely detected by western. [AY98-52W].

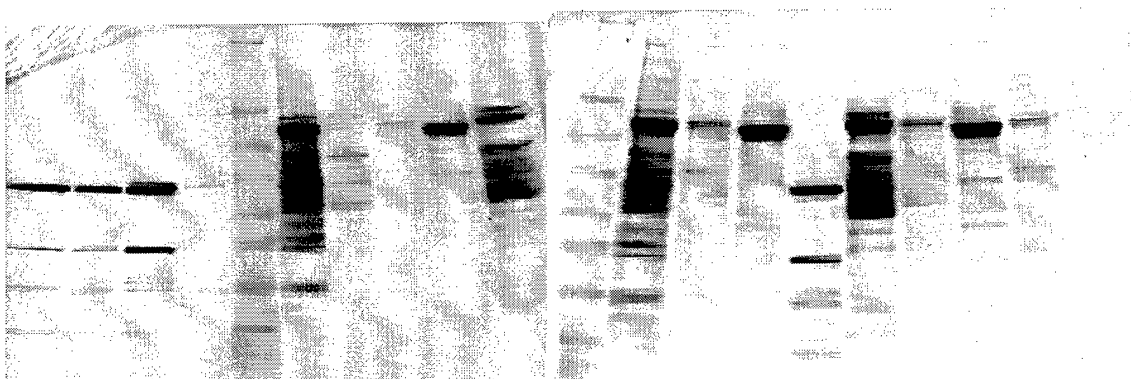


Lanes:
 1-MW (Novex See Blue)
 2-BoNT-C₁(Hc) standard
 3-Poros HQ Pool (Butyl Load)
 4-Butyl FT 7
 5-FT 12
 6-FT 16
 7-Butyl 0.8 M Elution
 8-0.4 M
 9-0.2 M
 10-0 M
 Western:
 11- BoNT-C₁(Hc) standard
 12-0.2M Phenyl
 13-0 M Phenyl

Based on the fact that we were unable to successfully reproduce the procedure that we had received from Mike Byrne, we decided to focus on the Q-Sepharose FF using MOPS buffer, since that had given us the most successful results to date. At column protein loads of 0.66, 1.3, and 2.6 mg/ml-resin, the product was consistently in the 250 mM

fraction (Figure 13, Coomassie), and none was detected in FT with these runs (Figure 14, Western). These runs were done using cell lot no. ARMY-FDL-54, WZ-75, C5 strain 10/28/99.

Lanes:	
1-0.66 mg/ml-r FT 6	11-MW (Novex See Blue)
2-0.66 mg/ml-r FT 8	12-Q Load
3-1.3 mg/ml-r FT6	13-1.3 mg/ml-r 250 mM Elute
4-1.3 mg/ml-r FT 12	14-1.3 mg/ml-r 400 mM Elute
5-MW (Novex See Blue)	15-2.6 mg/ml-r FT 7
6-Q Load	16-2.6 mg/ml-r 150 mM Elute
7-0.66 mg/ml-r 150 mM Elute	17-2.6 mg/ml-r 250 mM Elute
8-0.66 mg/ml-r 250 mM Elute	18-2.6 mg/ml-r 400 mM Elute
9-0.66 mg/ml-r 400 mM Elute	19-2.6 mg/ml-r 250 mM Elute
10-1.3 mg/ml-r 150 mM Elute	20-2.6 mg/ml-r 1 M Elute



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
(JL93-84C, JL93-85C)

Figure 13

(JL93-85W)

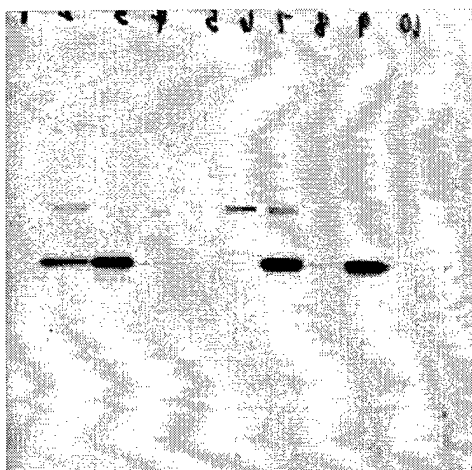


Figure 14

Western of lanes 11-20 (above)
 1-MW (Novex See Blue)
 2- Q Load
 3-1.3 mg/ml-r 250 mM Elute
 4-1.3 mg/ml-r 400 mM Elute
 5-2.6 mg/ml-r FT 7
 6-2.6 mg/ml-r 150 mM Elute
 7-2.6 mg/ml-r 250 mM Elute
 8-2.6 mg/ml-r 400 mM Elute
 9-2.6 mg/ml-r 250 mM Elute
 10-2.6 mg/ml-r 1 M Elute

We then switched to a fresher lot of cells, # ARC-FDL-014, WZ-93, strain C5, 3/2/00, which, based on western blot contained about 1% BoNT-C₁(Hc) in the soluble fraction of the lysate.

The Q-Sepharose was re-run using Mops and eluted with a 0-150mM gradient (to elute contaminants) previous to the 250 mM step (to elute product). There was a lot of product present in the flow-through fraction with a 7.7 mg/ml-resin load. When using a 150 mM step instead of 0-150 mM gradient, the product elutes both in the 150 and 250 mM fractions.

DNA removal

New information was obtained from Mike Byrne as to the importance of an overnight dialysis/precipitation step prior to Q chromatography to remove DNA from the lysate. We, therefore, started to look at a scalable process for DNA removal in order to stop competitive binding on Q resins. We looked at additives used to precipitate DNA and removal of DNA by exhaustive diafiltration.

We first tested polyethyleneimine (PEI) using w/cw addition, but it did not lower 260/280 ratio at all. Next a 0.25% w/v addition of PEI was tested, but it only lowered the ratio from 1.5 to 1.4. A 0.5% w/v PEI addition lowered 260/280 ratio to 1.1. With this sample, no western signal was detected in FT on Q load of 4.6 mg/ml-resin. However, product was detected in the 0-150 mM gradient elution instead of 250 mM fraction as before.

Other additives were also tested (protamine sulfate, protamine, MnCl₂, with and without NaCl) for DNA removal. The following treatments were tested:

- 1) PEI, 0.5% with and without 0.5M NaCl
- 2) PEI, 0.3% with 0.5M NaCl (NaCl supposed to help PEI work better)
- 3) MnCl₂, 0.5M with and without 0.2% PEI
- 4) protamine, 0.2%
- 5) protamine sulfate, 0.2%

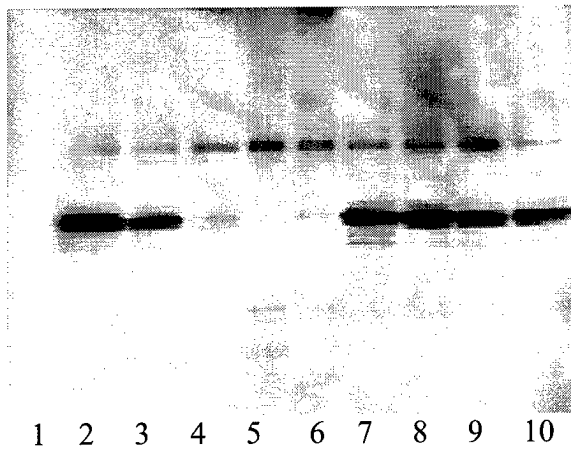
The control was no treatment (no additives). Treatments containing PEI (with exception of PEI + MnCl₂) resulted in the lowest 260/280 ratios (1.1-1.3) compared to 1.6 for the control. However, BoNT-C₁(Hc) coprecipitated with all treatments except the MnCl₂ preps. Treatments were all done at pH 7.5.

Next, lower pHs were tested (5.7 vs 6.5 using Mops/Mes or phosphate) with 0.2% PEI and at least 0.1M NaCl in all samples except controls.

Some problems occurred when adjusting the pH of some of the samples, such as extreme pH swings, which may have caused extraneous precipitate. Based on western blots, phosphate with pH 5.7 or 6.5 using 0.2% PEI + 0.5M NaCl was best as far as BoNT-C₁(Hc) recovery (Figure 15). Since S4 does not result in a BoNT-C₁(Hc) signal on western, it is possible that neither does S3, but since S3 was accidentally mixed with S2 it is not known if the signal is all from S2, or if some is contributed from S3. I suspect that, since the signal is weaker, that perhaps S2 is diluted by a sample with no signal (S3).

The following treatments were tested:

pH 5.7		pH 6.5	
Mops/Mes	PO ₄	Mops/Mes	PO ₄
Control 1	Control 3	Control 2	Control 4
0.2% PEI + 0.5M NaCl (S1)	0.2% PEI + 0.5M NaCl (S7)	0.2% PEI + 0.5M NaCl (S2)	0.2% PEI + 0.5M NaCl (S8)
0.2% PEI + 0.1M NaCl (S3)	0.2% PEI + 0.1M NaCl (S9)	0.2% PEI + 0.1M NaCl (S4)	0.2% PEI + 0.1M NaCl (S10)
0.2% PEI + 0.5M MnCl ₂ + 0.1M NaCl (S5)	0.2% PEI + 0.5M MnCl ₂ + 0.1M NaCl (S11)	0.2% PEI + 0.5M MnCl ₂ + 0.1M NaCl (S6)	0.2% PEI + 0.5M MnCl ₂ + 0.1M NaCl (S12)



Lanes:
 1-MW (Novex See Blue)
 2-S1
 3-S2+S3 (samples accidentally mixed)
 4-S4
 5-S5
 6-S6
 7-S7
 8-S8
 9-S9
 10-S10

Figure 15

(AY98-97W)

Lysate treated with 0.2% PEI + 0.5M NaCl pH 6 was run on a Q-Sepharose FF column with phosphate buffer to assess binding. Cells were lysed at pH 7.5 using phosphate buffer, then PEI was added and pH adjusted down to 6 with 1M phosphate). The preparation was split in half, and half was loaded to a SP-Sepharose column to remove residual PEI. The SP flow-through was dialyzed and loaded to Q-Sepharose FF. The other half was dialyzed and loaded to a Q-Sepharose column directly. The 260/280 ratio of these preps was only down to 1.3 after treatment. The BoNT-C₁(Hc) flowed through SP column and very little was lost to non-specific binding to the resin. Good binding to Q column was achieved in both cases (with or w/o SP column), but the product elutes from the Q column in both 150 mM gradient and 250 mM fractions. The Q product pool was then dialyzed to Tris buffer and loaded to a PI column. The column was eluted with pH gradient from pH 8 to pH 6. No peaks eluted during pH gradient, and BoNT-C₁(Hc)

product eluted in the salt wash at the end of the run. A further evaluation of the PI step has not yet been revisited.

A DEAE column was equilibrated with high salt (300 mM NaCl) to try to bind DNA while letting the BoNT-C₁(Hc) flow through. The 260/280 ratio of the DEAE load material was 1.5, and the flow-through was 1.46 (95% of load). This did not appear to be very successful, or possibly the column was severely overloaded with DNA, as the 1M elute did show a 260/280 ratio of 2. This process has also not been reevaluated.

Removal of DNA by dialysis or diafiltration

Mike Byrne had repeated success in removing DNA via room temperature, overnight dialysis through 10K MWCO slide-a-lyzers (formation of precipitate containing protein and DNA). We had been using 6-8K MWCO dialysis tubing instead and had not been seeing this precipitate, even at room temperature for ≥ 48 hours. Therefore, a comparison of dialysis of lysate using 6-8K MWCO tubing versus 10K slide-a-lyzer cassettes was done to see if the pore size really made that much of a difference. Treatments were with and w/o PEI, the sample used was the DEAE flow-through (containing BoNT-C₁(Hc)) obtained from the previous experiment. After dialysis, the 6-8K material had a 260/280=1.21, and 6-8K PEI treated was 260/280=0.93. The 10K treated material had a 260/280=0.84 and the 10K PEI treated was 260/280=0.87. Therefore, the 10K dialysis does appear to remove more DNA. This could be either a result of the different pore size, the different manufacturer of the membrane (though they were both regenerated cellulose), or the fact that the slide-a-lyzer design inherently allows for much greater surface contact between the membrane and the sample. In either case, this finding confirmed previous observations by Scott Johnson using the 10K slide-a-lyzers and 6-8K tubing.

Scott Johnson set up the OptiSep diafiltration system to test membranes having 20, 30 50K MWCO. All membranes were successful at removing DNA down to ratios ≤ 1.0 without loss of BoNT-C₁(Hc) in the permeate. Membranes having larger porosity would be preferred due to increased flux rates that can be achieved. The 30K membrane was selected for future runs due to the possibility of passing product through the 50K membrane under certain conditions (transmembrane pressure dependent).

Samples generated from Scott's diafiltration experiments were used to test a Mono Q column (the type Mike Byrne was using in his revised process). PEI treated lysate was used as a comparison to the diafiltered material. On the Mono Q column, the product clearly elutes in the 50 and 100 mM fractions (with some present in the flow-through, due to column overload). Sample was also loaded to a Q-Sepharose column, and no BoNT-C₁(Hc) was detected in the flow-through fraction. BoNT-C₁(Hc) elutes in a single step from the Q-Sepharose in the 50 mM fraction. As Mike Byrne suggested, it does appear that the dialysis step is critical to the process, and seems to work much more efficiently than when using PEI or other additives in a precipitation step.

1.4. Project plan for BoNT-C₁(Hc)

Note: Much of this work has been done, but the write up is not included in this final report. A report on this activity will be send under separate cover.

- I. Initial experiments testing the effect of pH on protein precipitation revealed that pH 4 (acetate) appears to reduce total protein concentration in the lysate by 10-fold with a 20% loss of BoNT-C₁(Hc). An 80kDa major contaminant is removed by ppt at pH 4.

Confirmation of this result by following experiments:

11. Break cells at pH 7.3-7.6 in 50 mM PO₄ buffer with no salt. Quickly adjust pH to 4.0 with 500 mM NaOAc. Stir at RT for 1 hour to ppt proteins. Centrifuge. Adjust pH back up to pH 7.3-7.6 quickly with 500 mM PO₄ or NaOH. Diafilter to lower conductivity and remove DNA. Load to Q column.
12. Break cells at pH 4.0 in NaOAc, run as above.
13. Break cells at pH 7.5 and 4.0 as above, but after ppt keep pH at 4.0, dilute and load to SP column

These experiments should answer the following:

- A. Is significant removal of contaminating proteins is worth the trade-off of 20% loss of BoNT-C₁(Hc)
- B. By breaking at pH 4 (not going through the pI in pH adjustment) may decrease or eliminate loss of BoNT-C₁(Hc) to ppt.
- C. Can run at pH 4 and capture with SP column, thus eliminating the DNA removal step.

- II. Initial experiments using 1M (NH₄)₂SO₄ to precipitate proteins in the cell lysate resulted in a 40% drop in total protein concentration without losing BoNT-C₁(Hc). At (NH₄)₂SO₄ concentrations greater than 1M, BoNT-C₁(Hc) losses were higher, and further total protein reduction was negligible. The ammonium sulfate-treated lysate was centrifuged and loaded to a butyl HIC column. No BoNT-C₁(Hc) was detected in the flow-through. Product eluted in a single peak at appx. 200 mM (NH₄)₂SO₄. This process looks promising as a way to easily remove a majority of proteins and DNA; and to capture the BoNT-C₁(Hc), prior to loading to a Q column for further purification.

- III. Based on data obtained by Mike Byrne, 5% cell/volume break ratio decreased loss of BoNT-C₁(Hc) to the cell debris pellet upon lysis. Breaking at higher ratio (e.g. 20%) caused greater loss of BoNT-C₁(Hc) to the pellet. To confirm this phenomenon, we will look at break ratios between 4 – 20% and quantitate BoNT-C₁(Hc) per unit cell mass obtained in the supernatant.

- IV. We will also be looking at other brands of Q resin, e.g. Tosohaas to obtain better binding affinity and/or selectivity of BoNT-C₁(Hc). To date, we have mainly been using Q-Sepharose-FF (Pharmacia). We will also determine if it is

necessary to break cells in the presence of NaCl. Methods have been used ranging from 0-100 mM NaCl.

2. BoNT-F(Hc)

2.1 Scale-up of the Fermentation and Purification of BoNT-F(Hc)

BoNT-F(Hc) was purified from soluble *Pichia* cell lysate using a modified method developed by Covance, Inc. employing ion-exchange chromatographic (IEC) and hydrophobic interaction chromatographic (HIC) methods. This process was performed at the pilot scale to for optimization of the scale-up. The total cellular protein recovered after homogenization was 72 mg per gram of cell paste. The modified process resulted in greater than 98% pure product consisting of two forms of BoNT-F(Hc) based on SDS-PAGE and yielded up to 205 mg/kg cells at the bench scale and 170 mg/kg cells at the pilot scale based on the BCA protein assay. N-terminal sequencing shows the two forms were recovered which are -11 (80%) and -14 (20%) from the full length form. The ratios of these two forms were consistently obtained from repeated bench scale and pilot scale runs.

A fermentation protocol utilizing glycerol batch, glycerol fed-batch and methanol fed-batch phases allowed high cell densities to be reached. The induction time was kept short to optimize BoNT-F(Hc) production while minimizing the proteolytic degradation. A purification method developed by Covance, Inc. was tested and some modifications were made, mainly in the harvesting step. Initial runs performed at the bench-scale resulted in much lower product yields than expected, which led us to optimize the cell breakage step. A total of three bench-scale runs were performed using cells generated from either a 60 L or 5 L fermentation. Two pilot-scale purification runs were performed using cells generated from the same 60 L fermentation to determine the ability of this method to be scaled-up. Unless otherwise noted, cells were processed immediately after harvesting.

Materials and Methods

5 Liter and 60 Liter Fermentation. A 1-L baffled shake flask containing 150 mL BMGY (buffered minimal glycerol complex medium, *Pichia* Expression Kit, Invitrogen Co., USA) was inoculated with 1 mL of frozen strain stock and incubated at 30 °C, 200 rpm until an OD₆₀₀ (optical density at 600 nm) between 4-8 was reached. The entire 150 mL was employed as an inoculum for a 5-L fermentor (Bioflo 3000, New Brunswick Scientific Co., USA) containing 3 L of BSM plus 0.435% v/v PTM1. BSM consists of (per L) 26.7 mL 85% H₃PO₄, 0.93 g CaSO₄, 18.2 g K₂SO₄, 14.9 g MgSO₄·7H₂O, 4.13 g KOH, and 40.0 g glycerol; and PTM1 consists of (per L) 6.0 g CuSO₄·5H₂O, 0.08 g NaI, 3.0 g MnSO₄·H₂O, 0.2 g Na₂MoO₄·2H₂O, 0.02 g H₃BO₃, 0.5 g CoCl₂, 20.0 g ZnCl₂, 65.0 g FeSO₄·7H₂O, 0.2 g biotin and 5.0 mL H₂SO₄). Prior to inoculation, the pH was adjusted to 5.0 with concentrated ammonium hydroxide. The fermentation was controlled at pH 5.0, temperature 30 °C and dissolved oxygen (D.O.) >20% of saturation. When the fermentation was grown to 30 - 50 g/L wet cell weight (WCW), the culture was transferred to a 80-L fermentor (MPP 80, New Brunswick Scientific Co.) containing 40 L of BSM plus 0.435% PTM1. When glycerol exhausted, which was indicated by a D.O.

spike, a glycerol feed was initiated and lasted for 4 hours. The feed rate has the following profile: 1st h, 20 g/h/L (g 50% w/v glycerol containing 1.2% v/v PTM1 per h per L initial medium); 2nd - 4th h, feed rate decreased linearly from 20 g/h/L to 0. At the end of 1st h, 1.5 g of methanol was added to the fermentor. At the end of 3rd h, a methanol feed was initiated and the feed rate was programmed to increase linearly using the following profile: 0 h, 4 g/h/L (g 100% methanol containing 1.2% v/v PTM1 per h per L initial medium); 2.4 h, 6 g/h/L; 3.8 h, 7 g/h/L; 8.5 h, 9 g/h/L. The methanol feed rate was adjusted using the dissolved oxygen spike method. Cell mass was harvested at 8.5 h. The total induction time on methanol was 10.5 h. The final cell density was about 170 g/L wet weight.

5 Liter Cell Harvest and Disruption. Cells harvested from a 5 L fermentation were spun at 8000 x g at 4 °C for 20 min using a Beckman J2-21 Centrifuge (Palo Alto, CA). Cell paste was then either frozen at -20 °C or processed immediately. Cell paste was resuspended in 25 mM sodium acetate + 5 mM EDTA, pH 5.0 to either 10 or 26% (w/v) solids. Cells were then homogenized using a Microfluidizer M-110EH (Microfluidics, Corp., Newton, CA) set at 21,000 psi. A total of 3 to 5 passes were performed to obtain at least 75% cell disruption. The homogenate was then brought to 0.25% Polyethyleneimine (PEI) using a 5% (w/v) stock solution at pH 7.0. This was allowed to mix for 30 min at 4 °C. The resulting mixture was processed by centrifugation at 10,000 x g for 20 min at 4 °C. The supernatant was then decanted, passed through a 0.2 micron CA filter and saved for further immediate processing.

Bench-scale Purification. All chromatographic separations were performed on a BioCad Workstation (PE Biosystems, Foster City, CA) at room temperature, with the load material on ice. Filtered supernatant was loaded onto a 100 mL SP Sepharose FF column (2.6 x 19 cm) (Amersham Pharmacia) equilibrated with 5 column volumes (CV) of 25 mM sodium acetate + 1 mM EDTA, pH 5.0, at a linear velocity of 250 cm/h. The columns were then washed with 5 CV equilibration buffer, followed by a wash step with 25 mM sodium phosphate + 1 mM EDTA, pH 6.8. The product was then eluted from the column using a 20 CV linear gradient from 0 to 1 M sodium chloride in 25 mM sodium phosphate + 1 mM EDTA, pH 6.8. The product begins eluting at about 20 mS conductivity.

The SP Sepharose product was then brought to 1 M ammonium sulfate using a 3.5 M ammonium sulfate stock. The resulting solution was filtered through a 0.2 micron filter. The filtered material was then loaded onto a 32 mL Butyl Sepharose 4 FF column (1.6 x 16 cm) (Amersham Pharmacia) equilibrated with 5 CV 1 M ammonium sulfate + 25 mM sodium phosphate + 1 mM EDTA, pH 6.8, at a linear velocity of 300 cm/h. The column was then washed with 5 CV of equilibration buffer. The product was then eluted using a 10 CV linear gradient from 1 to 0 M ammonium sulfate in sodium phosphate + 1 mM EDTA, pH 6.8. BoNT-F(Hc) elutes as the second major peak at 60 mS conductivity.

The Butyl Sepharose product was then dialyzed vs. 20 volumes of 25 mM sodium acetate + 1 mM EDTA, pH 5.0 using 10 kDa Slide-A-Lyzer cassettes (Pierce, Rockford, IL) at 4 °C. The dialyzed product was then loaded onto a 13 mL TosoPearl SP 650M column (1.0 x 17 cm) (TosoHaas, Montgomeryville, PA) equilibrated with 5 CV of 25 mM sodium acetate + 1 mM EDTA, pH 5.0, at 300 cm/h. After loading, the column was

washed with 5 CV of equilibration buffer. BoNT-F(Hc) was eluted from the column using a 20 CV linear gradient from equilibration buffer to 100 mM sodium phosphate + 1 mM EDTA, pH 7.0. The product elutes at the end of the gradient as the second major peak at about 13 mS conductivity.

60 L Cell Harvest and Disruption. Cells were harvested using a Westfalia CS-8 disk-stack separator (Oelde, Germany). Fermentation broth was diluted to 10% solids with distilled water. The resulting cell paste either frozen at $-20\text{ }^{\circ}\text{C}$ or processed immediately. The cell paste was brought to 21-26% (w/v) solids using 25 mM sodium acetate + 5 mM EDTA, pH 5.0. Cells were disrupted using an APV Gaulin 30-CD Homogenizer at 16000 (Everett, MA) psi at $10\text{ }^{\circ}\text{C}$. Cells were exposed to 3 to 5 passes through the homogenizer and cooled to $10\text{ }^{\circ}\text{C}$ after each pass using a water chiller. The resulting homogenate was brought to 0.25% PEI using a 5% (w/v) stock and allowed to mix for 30 min at $10\text{ }^{\circ}\text{C}$. The resulting mixture was then exposed to centrifugation using the Westfalia used above. The supernatant was collected for further immediate processing.

Pilot-scale Purification. All chromatographic separations were performed on a NC-SRT Pilot-scale Chromatography skid. For each chromatographic step, conditions were the same as the bench-scale work, ie. same linear velocity, equilibration, washing and elution methods. The SP Sepharose FF step was performed using a 4 L (20 x 13 cm) BPG 200/500 column (Amersham Pharmacia Biotech, Piscataway, NJ). The Butyl Sepharose 4 FF step was performed on a 1.3 L (10 x 16 cm) BPG 100/500 column (Amersham Pharmacia), while the final ToyoPearl SP 650M step was performed using a 526 mL (6.1 x 18 cm) Vantage 60A column (Millipore, Bedford, MA). The SP Seph product was brought to 1 M ammonium sulfate by addition of granular ammonium sulfate. The diafiltration step was performed using a 3 sq. ft. 10 kDa spiral wound membrane (Millipore). The material was diafiltered with 3 volumes of 25 mM sodium acetate + 1 mM EDTA, pH 5.0, at which time the retentate pH was 5.0 and the conductivity was 4.7 mS at $10\text{ }^{\circ}\text{C}$. The retentate was then concentrated about 2-fold and filtered through a 0.45 micron CA filter prior to loading onto the final column.

Protein Analysis. Total protein concentrations were determined using the BCA (Pierce) Standard Assay, using BSA to generate the standard curve. Purity was determined by SDS-PAGE using 4-20% polyacrylamide gels (Novex, San Diego, CA) stained with coomassie blue or silver stain. Western blot analysis was performed using polyclonal Protein G-Sepharose-purified horse anti-BoNT-F(Hc) antibody incubated at $1\text{ }\mu\text{g/mL}$ for 1 h. The secondary antibody was a horseradish peroxidase labeled affinity-purified goat anti-horse IgG (Kirkegaard & Perry Laboratories, Gainsburg, MD) incubated at $1\text{ }\mu\text{g/mL}$ for 1 h. SDS-PAGE-separated proteins were transferred to PVDF membranes (BioRad, Hercules, CA) at 100 V for 1 h, blocked with 5% non-fat dry milk for 1 h and washed with 0.05% Tween-20 in phosphate-buffered saline (PBS) prior to treatment with antibodies. Blots were then visualized by Chemiluminescence using the ELC plus Detection Kit (Amersham Pharmacia Biotech, Piscataway, NJ). N-terminal sequencing was performed by the University of Nebraska-Medical Center Protein Core Facility using automated Edman degradation performed on a Procise model 491-HT amino acid sequencer (PE Biosystems, Foster City, CA).

Results and Discussion

Bench-scale Fermentation and Purification. Fermentations performed at the 5 liter scale involved standard methanol feed rates, with up to a 10 h induction. This short induction time was performed to minimize the effect of proteolytic cleavage in the fermentation, yet still reach sufficient levels of expression. An initial run was performed following the process described by Covance Inc., which resulted in a significantly lower product yield than expected (54.35 mg BoNT-F(Hc) /kg cell) compared to published results. A review of the method suggested that breakage at a higher percent solids would result in a much higher amount of cell lysis. An experiment was performed to test the amount of total protein released by homogenization at different percent solids (see Figure 16). These results show a 6-fold increase in the amount of protein released by increasing the percent solids from 10 to 26%. We also found that for optimum release of protein during disruption, harvested cell paste should be processed immediately and not frozen. It is believed that the freezing of cells causes them to become rigid and more difficult to disrupt. Therefore, subsequent cell disruptions were performed at 26% solids and were conducted immediately after harvesting.

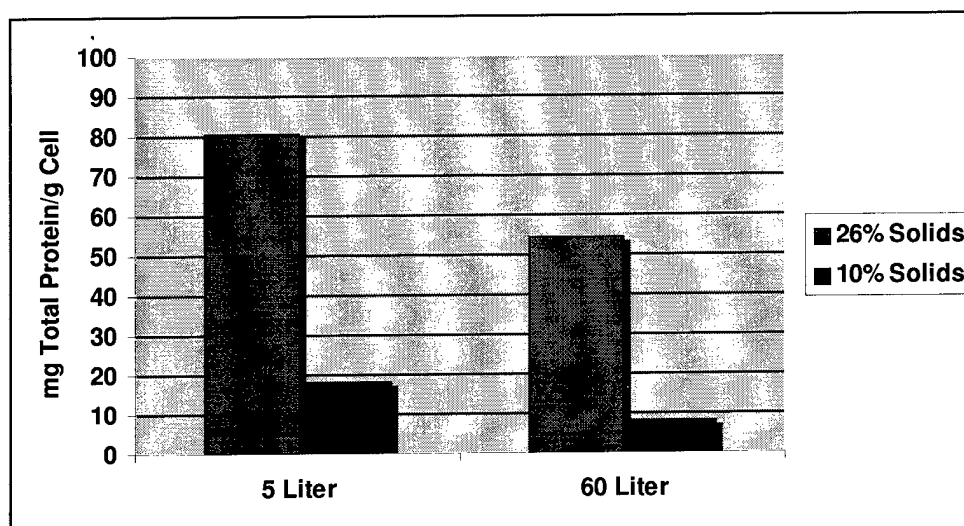


Figure 16. Effect of % Solids on Cell Breakage. Cells harvested from 5 L and 60 L fermentations were homogenized and exposed to centrifugation. Resulting supernatants were analyzed for total protein concentration using the Bradford protein assay (BioRad) using BSA as the standard.

A total of three purification processes were performed following the described method above, to determine reproducibility. Results of these purification runs were very similar in product yield, purity and elution profiles. The entire purification process, from harvesting to final sterile-filtering, was performed in 20 h. Purification runs resulted in final product yields, ranging from 155 mg to 205 mg BoNT-F(Hc) /kg cells (see Table 1). The maximum amount of total protein loaded onto the SP Sepharose FF column was 55.5 mg/mL resin. At this load less than 3% of the product was seen in the flow through. A maximum load of 4.8 mg/mL was used for the Butyl Sepharose 4 FF column and 2.7

mg/mL for the final ToyoPearl SP 650M column. No BoNT-F(Hc) was detected in flow through samples of the HIC or final SP columns.

Table 1. Bench Scale Purification of BoNT-F(Hc) ^a

Step	Vol (mL)	[Protein] _{Total} (mg/mL)	Total Protein (mg)	Purity ^b (%)	BoNT-F(Hc) ^c (mg)	Fold Purification
Lysate	820	13.26	10873	<0.5	54.4	1
PEI-treatment	740	7.65	5661	<1	45.3	1.6
SP Sepharose FF	270	0.64	174	25	43.4	50
Adjusted SP Seph FF	380	0.42	161	26	41.9	52
Butyl Sepharose 4 FF	87	N.D.	N.D.	87	N.D.	N.D.
Dialyzed Butyl Seph 4 FF	88	0.43	37.9	87	33.0	174
ToyoPearl SP 650M	65	0.36	23.3	98	22.9	196

^aPurification is from 150 g cells wet weight.

^bEstimated by visual inspection of SDS-PAGE.

^cDetermined by multiplication of total protein times purity.

N.D. Not determined

A typical purification resulted in at least 98% pure BoNT-F(Hc) which consisted of two forms of the product (see Figure 17). Analysis by N-terminal sequencing shows the prominent form (80% of the total) of the product to be missing the first 11 amino acids (a.a.) and a second form missing the first 14 a.a. of the amino terminus (see Table 2). This ratio of product forms was consistent for all purification processes performed at both the bench and pilot scales. SDS-PAGE analysis of in-process samples shows the two product forms to exist in the lysate, suggesting they may be the result of proteolytic degradation in the fermentor. A fermentation was performed with a shortened methanol induction time of 3 h, in an attempt to produce sufficient amounts of full-length BoNT-F(Hc). Immediate purification of this fermentation resulted in a 2-fold reduction of product yield and N-terminal sequence analysis shows two forms of the product, similar to previous purifications.

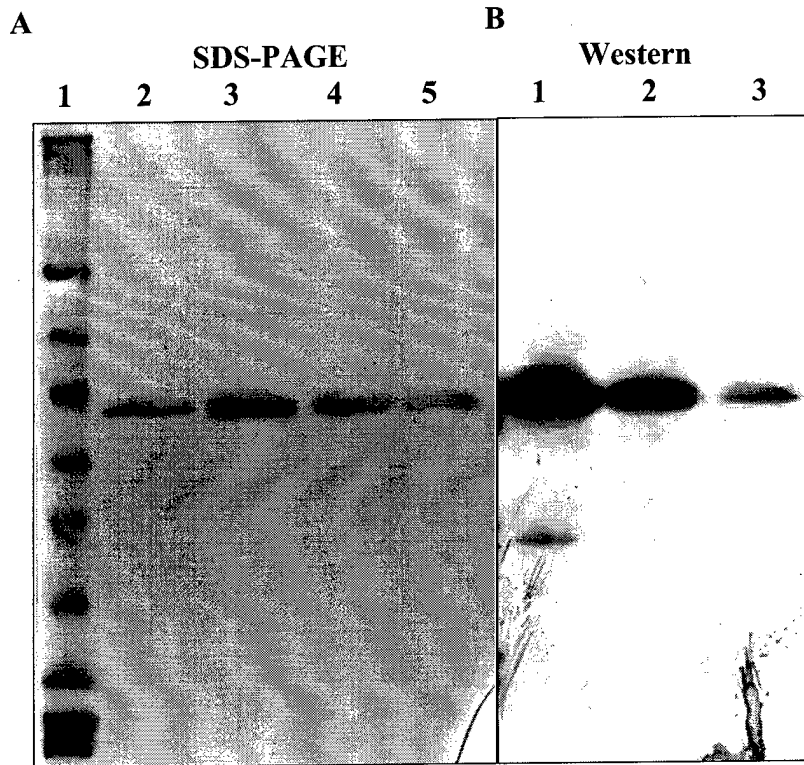


Figure 17. (A) Silver stained SDS-PAGE and (B) Chemiluminescent western blot of purified BoNT-F(Hc) from a typical bench-scale purification (PDL-004). (A) Lane 1 is MW standards, from top, 250 kDa, 98 kDa, 64 kDa, 50 kDa, 36 kDa, 30 kDa, 16 kDa, 6 kDa, 4 kDa, Lane 2 is μg BoNT-F(Hc) std (Covance, Inc), Lane 3 is 5 μg final product, Lane 4 is 2.5 μg final product, Lane 5 is 1 μg final product. (B) Lane 1 is 200 ng final product, Lane 2 is 50 ng final product, Lane 3 is 30 ng final product.

Table 2. N-terminal Sequence of Purified BoNT-F(Hc)

Sample	N-Terminal Sequence	
Intact BoNT-F(Hc)	MSYTN DKILI LYFNK LYKKI KDNSI	
Bench scale PDL-004	YFNK LYKKI K	80% (-11)
	K LYKKI XDNX	20% (-14)
PDL-005	YFNK LYKKI K	75% (-11)
	K LYKKI KDN	25% (-14)
PDL-006	YFNK LYKKI K	76% (-11)
	K LYKKI XDNX	24% (-14)
PDL-007	YFNK LYKKI K	80% (-11)
	K LYKKI KDN	20% (-14)
Pilot scale PPP-006	YFNK LYKKI K	89% (-11)
	K LYKKI KDN	11% (-14)
PPP-007	YFNK LYKKI K	77% (-11)
	K LYXXI XDNS	23% (-14)

An attempt to remove the minor high molecular weight contaminants present in the BoNT-F(Hc) final product was performed using the zwitterionic detergent, CHAPS as an additive in the initial column buffers. We reasoned that these contaminants may be involved in a protein-protein interaction with the product and therefore are not being separated during the multi-chromatographic process. We found that 0.25% CHAPS did not aid in the removal of these proteins (data not shown). Also unsuccessful was an attempt using a negative purification step with Q Sepharose FF following the capture step with CHAPS present in the buffers. BoNT-F(Hc) from this process did not show an increase in purity. These contaminants were removed however, by making a very detailed cut in collection of product eluting from the SP Sepharose FF column. Such detail in fraction collecting is difficult to perform at the pilot scale, and leads to lower product yields as the elution of these contaminants and BoNT-F(Hc) overlap.

Pilot Scale Fermentation and Purification. All work performed in the pilot plants employed use of batch records for process transfer to a GMP manufacturing plant. A total of two 60 liter fermentations and two purification runs were performed. Pilot scale fermentations resulted in final wet cell weights ranging from 21.2% to 30.8% with final volumes of 56 and 57 L.

A total of two purification runs were performed at the pilot scale. The initial run was performed immediately after harvesting of cells, and resulted in 179 mg BoNT-F(Hc) /kg cell (see Table 3). The product from the SP Sepharose FF capture column was collected in bulk with collection beginning as soon as the Abs₂₈₀ began to increase. Upon completion of this purification run, the final product was found to contain two minor higher molecular weight contaminants (data not shown). Further testing of the method showed these contaminants were the result of the type of cut made during product collection off the capture column. These contaminants co-elute significantly with BoNT-F(Hc) and proved difficult to remove during subsequent chromatography steps (see above). A second pilot run was performed using frozen cells from the same fermentation run and resulted in a highly pure product (see Figure 18), however, showed five-fold less BoNT-F(Hc) yield. To avoid collection of the higher molecular weight contaminants, product collection began only at the apex of the elution peak. This would account for approximately half of the product typically collected. The remaining product loss occurred during recovery of soluble material after treatment with PEI (data not shown). Without an HPLC or ELISA method for detection of BoNT-F(Hc) it is difficult to determine the actual product loss, however these estimates were generated based on total protein data.

Table 3. Pilot Scale Purification of BoNT-F(Hc) ^a

Step	Vol (L)	[Protein] _{Total} (mg/mL)	Total Protein (g)	Purity ^b (%)	BoNT-F(Hc) ^c (g)	Fold Purification
Lysate	23	13.72	316	<2	4.7	1
PEI-Treatment	90	1.56	140	<3	3.5	1.7
SP Sepharose FF	20	0.69	13.8	25	3.5	17
Adjusted SP Seph FF	29	0.24	7.0	33	2.3	22
Butyl Sepharose 4 FF ^d	5.6	0.41	2.3	80	1.9	56
Diafiltered Butyl Seph 4 FF	5	0.50	2.5	80	1.9	54
ToyoPearl 650M	4.1	0.26	1.1	98	1.0	61

^aPurification is from 5.95 kg cells wet weight.

^bEstimated by visual inspection of SDS-PAGE.

^cDetermined by multiplication of total protein times purity.

^dPrecipitation occurred during Amm. Sulfate addition.

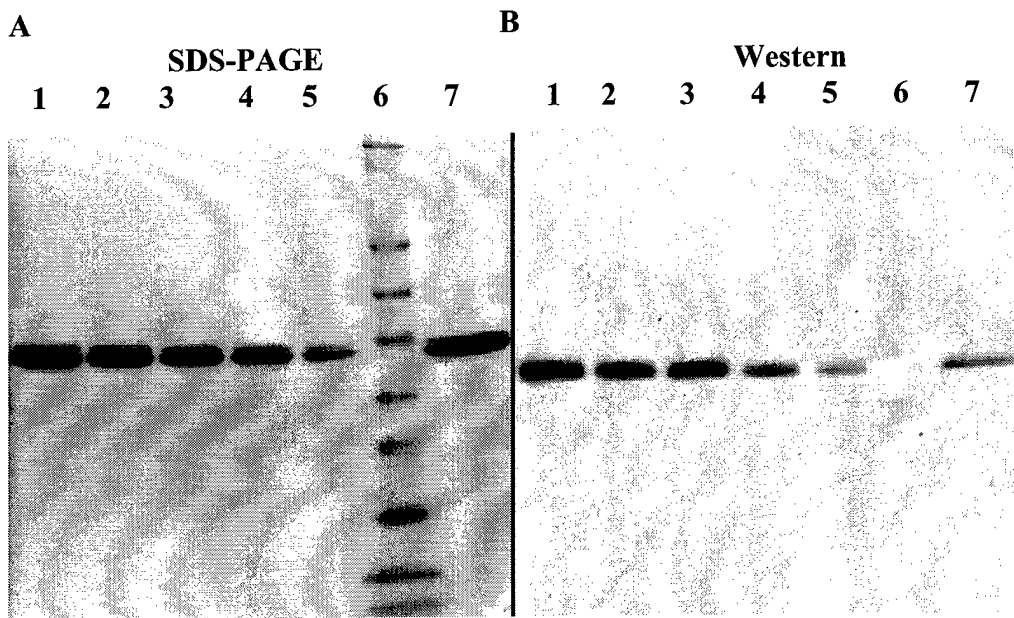


Figure 18. (A) Coomassie stained SDS-PAGE and (B) Chemiluminescent western blot of purified BoNT-F(Hc) from a pilot-scale purification (PPP-007). (A) Lane 1 is 5 µg final product, Lane 2 is 4 µg final product, Lane 3 is 3 µg final product, Lane 4 is 2 µg final product, Lane 5 is 1 µg final product, Lane 6 is MW standards, from top, 250 kDa, 98 kDa, 64 kDa, 50 kDa, 36 kDa, 30 kDa, 16 kDa, 6 kDa, 4 kDa, Lane 7 is 3µg BoNT-F(Hc) std (Covance, Inc.). (B) Lane 1 is 70 ng final product, Lane 2 is 50 ng final product, Lane 3 is 40 ng final product, Lane 4 is 20 ng final product, Lane 5 is 10 ng final product, Lane 6 is blank, Lane 7 is 15 ng BoNT-F(Hc) std.

The entire purification process from harvesting to final sterile-filtering took about 25 h. This is similar to the time required for small scale processing. Overall processing at the

pilot scale was smooth and resulted in a similar product yield per kg cell. The changes in processing relative to the bench scale were in the type of equipment used to perform certain tasks, i.e. laboratory centrifuge vs. disk-stacked separator or dialysis cassettes vs. diafiltration systems. In order to remove cell debris and flocculated nucleic acid from cellular homogenate, a disk-stacked separator was used rather than conventional laboratory centrifugation. Use of this equipment can result in a similar product yield obtained in the laboratory (see Tables 1 & 3). At the bench scale this step took 30 min, while at the pilot scale 1 h was required. Of the total protein released during homogenization of the initial pilot run, approximately 44% remained in the PEI-treated supernatant, however processing during a second run recovered only 9% of the total protein released.

To adjust the Butyl Sepharose 4 FF product to 25 mM sodium acetate + 1 mM EDTA, pH 4.5, a diafiltration system was used with a jacketed stainless-steel sample reservoir, chilled to 7 °C. This step resulted in an insignificant loss of product and took only 2 h to perform using a 10 kDa spiral-wound regenerated cellulose membrane. During adjustment of the SP Sepharose FF product to 1.0 M ammonium sulfate, some precipitation was seen initially, however if added slowly in increments over a 1 h period with continuous mixing, this was minimized. This phenomenon was more prominent at the pilot scale, possibly due to an increased sample temperature upon mixing. During adjustment at the pilot scale, the sample was approximately 17 °C, three times the temperature this step was performed at for the bench scale process, which may have caused the protein to be less stable.

Conclusion

The production and purification of recombinant BoNT-F(Hc) from *Pichia pastoris* using the process described above has been scaled-up and can result in a similar product yield and purity at both the bench and pilot scales. Fermentations achieved high cell densities, and reached optimum BoNT-F(Hc) production after 10 h induction. It was found that to achieve maximum BoNT-F(Hc) yield, purification should be performed immediately upon completion of the fermentation. The purification process involves three chromatographic steps, which is typical of methods used to purify non-secreted products from *Pichia*, and results in a consistent ratio of two nicked forms of pure BoNT-F(Hc) .

3. Supporting Activities from the Fermentation Development Laboratory (FDL)

Introduction:

The BPDF-FDL is responsible for supporting Army related *Pichia pastoris* fermentation research activities, providing cell mass to the downstream processing groups (both internal and external) and for the completion of the *E. coli* research activities. Section 3 of this report will summarize the *P. pastoris* support activities. The *E. coli* development work is summarized in the section 4.

3.1. Supporting Activities in *Pichia pastoris*.

The BPDF-FDL has completed forty (40) *P. pastoris* fermentations in support of fermentation development and purification activities. These runs are itemized in Table 4 and do not include fermentations completed by Dr. Zhang. The BoNT-A(Hc) strains FG-5/F3 and DI-17/B₁₂ fermentations were completed to provide material for downstream processing and were shipped to USAMRIID. Additional runs using the BoNT-A(Hc) strain were completed in order to develop and test a feed back program for controlling the methanol feed rate. The BoNT-C₁(Hc) fermentations were completed in order to provide material to the purification development laboratory (PDL). The BoNT-E(Hc) and BoNT-F(Hc) fermentations were completed to satisfy internal (research or development) and external (USAMRIID) requests.

Table 4. *Pichia pastoris* support activities

Strain	Fermentations Completed
BoNT-A(Hc)	8
BoNT-C ₁ (Hc)	15
BoNT-E(Hc)	6
BoNT-F(Hc)	11

3.2. BoNT-E(Hc) Expression in *E. coli*

It is considered desirable to develop fermentation methods for the expression of the BoNT-E(Hc) Hc protein in both *P. pastoris* and *E. coli*. This joint development pathway is necessitated by the low expression and recovery levels currently available for the toxin. Five strains of *E. coli* BL21(DE3) were delivered to the BPDF-FDL. These strains were identified as PET 24/LCA + BELT #11, PET 24/LCAL #14, PET 24/LCA + Xloc #1, PET24/LCAL #4, PET 24/LCA1 14/2. Each strain carries a different fragment of the BoNT-E(Hc) toxin. The initial goal of the project was to express protein without considering the formation of inclusion bodies or product yield. As the project progressed, it was determined that the protein must be expressed in a soluble form and that at least a preliminary attempt at optimizing expression and thus yield should be made. To this end, it was decided that a model for growth should be created which would allow predictive experiments to be conducted. As all fragments are contained within the BL21(DE3) strain, the model was to be constructed around the BoNT-E(Hc) Hc containing strain then modified as necessary for the remaining strains. Currently, the model has been defined for growth under both uninduced and induced conditions. The optimization of expression is still ongoing.

Preliminary Data

The fermentation development lab has worked to develop a fermentation method for the expression of the BoNT-E(Hc) Hc botulism toxin fragment in *E. coli*. Three main goals were defined for this project. The first was that no animal products, i.e., peptone, were to be used in the fermentation process. The second was that the protein should be expressed in soluble form. The third was that a minimal amount and preferably none of the selective agent, kanamycin, would be used. Preliminary data showed that a defined media would not support robust growth of this particular cell line. However, when supplemented with yeast extract and peptone, growth to high cell densities (O. D. > 100) was possible. When induced at 37 °C with IPTG, the heterologous gene product of interest was produced but was in the form of inclusion bodies. It was reported that, in shake flasks, induction at 18 - 20 °C resulted in the expression of soluble protein. With this information an experimental design was created that would test plasmid stability, isolate a combination of supplemental nutrients which supported growth, provide a model for growth and glucose consumption, and would yield a soluble protein.

Experimental Design

Four separate experimental series or "phases" were designed. The first phase was to test for the stability of the plasmid. It is known that plasmids containing selective markers such as kanamycin (Kan) exert an energy burden on the cell. If the burden is removed, i.e., growth in the absence of Kan, the cell will eject the plasmid. Cells that have lost the plasmid are at a growth advantage and will quickly overtake the culture, which will result in a loss of production. Although the use of a selective agent throughout the fermentation process is the simple solution, issues concerning proving removal of the selective agent, especially an antibiotic, are created. Thus, the first goal was to find the minimum concentration of Kan necessary to maintain plasmid stability.

The second phase of the research was to identify a nutritional supplement or supplements that would support the growth of the culture to high cell densities. Because of concerns with using animal derived products within the manufacturing of biologics, the elimination of such products is desirable. The alternatives to meat peptones are milk peptones and vegetable peptones. Although milk peptones are still animal derived, as long as they are certified to come from a country or area free from mad cow disease, their use is acceptable. To identify acceptable supplemental nutrients, an experiment was designed to first test the main effect of each supplement followed by the combined effect when used in conjunction with yeast extract. Tryptone with yeast extract was used as the baseline to compare growth on casamino acids and peptone-soytone. The responses were maximum specific growth rate (μ_m), maximum glucose consumption rate (v_m) and cell yield by O.D. per gram glucose ($Y_{od/x}$). Once this was completed the maximum cell density that the media can support could be determined which is necessary for targeting the induction cell density.

The third phase was to develop a growth model for the pre-induction and post-induction growth stages. These two stages have to be investigated independently as induction is known to greatly effect growth and since the temperature during induction is well below

the optimal growth temperature for *E. coli*, which is 37 °C. It is important to model growth before induction, as the pre-induction growth condition will effect expression of the target protein and is generally known to be best when growth is limited. Post-induction growth is characterized as being non-exponential which means using traditional Monod kinetics is not possible. Simple profiles that use linear or constant feed rate have been used as have more complicated profiles that rely on feed-back such as respiratory quotients. The problem with the first strategy is that feed rate is not linked to growth so knowing what feed rate to set is problematic. While the later strategy is preferable, the FDL does not posses the necessary equipment to implement a feed-back strategy. By linking feed rates to growth, just as in the pre-induction optimization, a more systematic approach to optimizing expression is possible. Therefore, the goal of the post-induction work would be to assume exponential growth to build a model then try to correct for error in glucose feed rates.

Once the above three phases of the project had been satisfied the fourth phase, expression optimization can begin. The optimal expression growth rate and duration, as dictated by the glucose feed strategy will first be determined followed by optimization of the cell density at which induction should start. The criteria to determine success or failure of any procedure will be the presence of soluble BoNT-E(Hc) Hc as determined by SDS-PAGE analysis. This work is currently ongoing.

Methods

Stability

The minimum required concentration of kanamycin was determined by varying the concentration of kanamycin in the fermentor from 0 ug/ml up to 50 ug/ml while holding the kanamycin concentration in the inoculum constant at 50 ug/ml. After completion of the fermentation run which included induction, samples were taken aseptically, diluted, then spread onto LB agar plates with and without 50 ug/ml kanamycin. After incubation colony counts were obtained from each plate. If the plates without kanamycin had a significantly higher number of colony forming units (CFUs) than the kan plates, the fermentation was determined to be "unstable".

Supplemental Nutrients

Using specific growth rate, glucose consumption rate and yield compared the effects of supplemental nutrients. Each of the following four nutrients were tested independently at their proposed supplemental levels; Yeast Extract, 5 g/L; Tryptone, 10 g/L; Peptone-Soytone, 10 g/L; Casamino acids, 10 g/L. The basal salts media consisted of (per liter) 2.8 g Na₂HPO₄, 2.0 g KH₂PO₄, 3.3 g (NH₄)₂SO₄ and 5.0 g NaCl and the supplemental nutrient(s) was added. After autoclaving 10 ml of 200 mM MgSO₄ – 7 H₂O, 10 ml of Trace Metals Salts and 20 ml of 82% wt/vol glucose was added per liter at then additional salts were added intermittently during the fermentation.

The value for u was determined graphically by plotting optical density (ABS_{600}) against elapsed fermentation time and selecting the region within the curve that most closely fit an exponential equation. By choosing the region of best fit, the lag phase and stationary phase effects did not influence the value for u_m . The value for v was determined by plotting glucose consumption against the changing cell density divided by specific growth rate $(X_t - X_0)/u$. The slope of the resulting line is the glucose consumption rate. The yield coefficient for glucose was calculated by dividing the cell yield (in O.D.) by the glucose consumed. Where appropriate, the stability of the expression cassette was determined by using the segregation test method as described above. The proposed fermentations for this section are shown in Table 5.

Table 5. Proposed fermentations for model.

Lot #	Run Conditions
ARE-FDL-034	15g/L Tryptone, 5 g/L YE, 10 ug/ml Kan
ARE-FDL-035	15g/L Tryptone, 5 g/L YE, 10 g/ml Kan
ARE-FDL-036	15g/L Tryptone 50 ug/ml kan
ARE-FDL-037	15g/L Tryptone 50 ug.ml kan
ARE-FDL-038	15g/L Soytone 10 ug.ml kan
ARE-FDL-039	15g/L Soytone 10 ug/ml kan
ARE-FDL-040	15g/L Tryptone 10 ug/ml kan
ARE-FDL-041	15g/L Tryptone 10 ug/ml kan
ARE-FDL-042	15g/L YE 10 ug/ml kan
ARE-FDL-043	15g/L YE 10 ug/ml kan
ARE-FDL-044	15g/L Tryptone, 5 g/L YE 10 ug/ml kan
ARE-FDL-048	15g/L Tryptone, 5 g/L YE 10 ug/ml kan
ARE-FDL-050	15g/L Casamino acids 10 ug/ml kan
ARE-FDL-051	15g/L Casamino acids 10 ug/ml kan
TBD	15 g Casa, 5 g YE 10 ug/ml kan
TBD	Max Cell density @ 50% u max
TBD	Max Cell density @ 25% u max
TBD	5 g/L YE

Growth Models

The coefficients for the pre and post-induction growth models were determined essentially as described above. To determine the coefficients for the post induction model, the cultures were grown to an O.D. of between 20 and 30. The temperature was then reduced to 20°C and the culture induced with IPTG. The optical density and glucose concentrations were followed until growth reached stationary phase, which typically occurred within one doubling of the cell density.

Analytical Methods.

To determine the presence of target protein, either SDS-PAGE or Western blotting was used depending to the necessary detection limit. To distinguish between soluble and insoluble protein, a combination of a freeze thaw cycle and the use of lysozyme in the presence of EDTA were used to lyse the cells. After lysis, viscosity was reduced by homogenization to shear the DNA. The resulting mixture was then centrifuged to separate soluble from insoluble fractions. To load a constant 10 μg /lane total protein, total protein was determined by using the Bio-Rad method. The gels were then electrophoresed and either transferred for probing or stained with Coomassie Brilliant Blue.

Results

Plasmid Stability

Two fundamental hypotheses were addressed when looking at plasmid stability. The first was what is the minimal concentration of Kan required to maintain selective pressure. The results of this study are shown in Table 6. From the data it is apparent that a minimum of 10 $\mu\text{g}/\text{ml}$ kan is required within the fermentors to assure retention of the plasmid. Once the minimum required concentration has been determined, there is no value in maintaining levels above the minimum. The second hypothesis was what is the effect of Kan on growth rate and cell yield. The answer to this question can be determined from the growth data for the 50 $\mu\text{g}/\text{ml}$ fermentations. No significant difference between growth rate and yield exists between the 50 $\mu\text{g}/\text{ml}$ fermentations and the 10 $\mu\text{g}/\text{ml}$ fermentations.

Table 6. Segregation frequency Study

Run Conditions	Log CFU LB + kan plates	Log CFU LB
0 $\mu\text{g}/\text{ml}$ kan	7.9	10.7
5 $\mu\text{g}/\text{ml}$ kan	8.6	10.34
10 $\mu\text{g}/\text{ml}$ kan	11.0	11.0
15 $\mu\text{g}/\text{ml}$ kan	10.0	11.0
50 $\mu\text{g}/\text{ml}$ kan	11.5	11.4

Supplemental nutrients

The results of preliminary work showed that tryptone - yeast extract gave similar growth results as animal peptone plus yeast extract. It was therefore decided to use tryptone-yeast extract as the baseline for comparisons between nutrients. For comparison the values for u_m , v_m and $Y_{od/g}$ for 3 runs was 0.70 +/- 0.07, 0.69 +/- 0.09 and 1.27 +/- 0.25 respectively.

The data for the complete study is shown in Table 7. Although the data for lot ARE-044 did not qualify as a statistical outlier, it did have a significant impact on the standard deviation for all categories and was thus eliminated. The supplemental nutrients tryptone and casamino acids yielded similar growth rates, glucose consumption rates and yield with soytone being significantly lower for growth rate. However, the final yield for soytone was actually higher than either tryptone alone or casamino acids alone. The fact that tryptone and casamino acids gave similar results is not unexpected as both are derived from casein. When the amino acid sources were combined with yeast extract, the reference data became statistically the same. Because the goal was to eliminate animal derived amino acid sources, it was decided to use soytone as the amino acid source for all subsequent fermentation development.

Table 7. Pre-Induction Growth Comparisons

Lot #	Nutrients	u	v	$Y_{x/g}$ (OD/g glc)
ARE-034	Tryp, YE	.73	.72	1.5
ARE-035	Tryp, YE	.62	.76	1.0
ARE-036	Tryptone *	1.041	.66	1.5
ARE-037	Tryptone*	1.053	.68	1.4
ARE-038	Soytone	.84	.51	1.7
ARE-039	Soytone	.81	.51	1.8
ARE-040	Tryptone	1.05	.59	1.7
ARE-041	Tryptone	.98	.56	1.7
ARE-042	YE 15 g/L	.88	.58	1.6
ARE-043	YE 15 g/L	.88	.50	1.8
ARE-044	Tryp, YE	1.02	.46	2.5
ARE-048	Tryp, YE	.74	.59	1.3
ARE-050	Casamino acids	.95	.54	1.6
ARE-051	Casamino acids	1.00	.62	1.5
ARE-056	YE 5 g/L	.72	.53	1.3
ARE-057	YE 5 g/L	.573	.54	1.1
ARE-059	Casa + YE	.93	.55	1.9
ARE-060	SoyT + YE	.89	.58	1.6
ARE-061	SoyT + YE	.91	.58	1.9

* These two run contained 50 ug/ml kanamycin as opposed to 10 ug/ml kanamycin in the rest of the runs.

Models

From the empirical data collected for growth on soytone and yeast extract, an estimation of the required glucose feed rate to support growth at a desired rate (u) was determined. For the purposes of this project, the maintenance coefficient was assumed to be negligible, which is false. In this case, the glucose feed rate (F) varies exponentially to maintain a constant u using the following equation:

$$F = v_{glc}(X_0 V_0)e^{ut} \quad \text{Eq. (1)}$$

where X_0 is the cell density and V_0 is the broth volume when initiating the feed profile ($t = 0$). Having determined the values for u_m and v_{glc} empirically under glucose excess conditions, v_{glc} is estimated for a desired u by the following equation:

$$v_{glc} = uv_{glc,m}/u_m \quad \text{Eq. (2)}$$

Eq. (2) is based on an assumption that $Y_{od/g} = u_m / v_{glc,m}$ and is independent of u and that the maintenance coefficient is negligible. As already mentioned, this assumption is false, but since the period under question is short and is used only to set-up the culture for induction, it was not felt that determining the maintenance coefficient was necessary. By substituting Eq. (2) into Eq.(1), F is estimated to deliver a constant desired u . Although the actual values of u and v_{glc} will differ from the estimated values, it is only important that they are reproducible. With an estimate of the parameters necessary to control and predict growth, a series of fermentations was completed to determine the maximum cell density that the media would support. Setting u to 40% of u_m a maximum cell concentration of between 112 and 124 ODU was obtainable.

The initial goal of the post-induction model work was to derive an equation that predicted glucose consumption rates and thus controllable growth rates under the target induction temperature of 18 – 20 °C. A predictive model was desirable, as the anticipated induction period was to be a minimum of 8 hours and up to 24 hours. Although several possible models had been previously described within the literature, they were typically designed to operate with automation beyond this facilities current capability, i.e., off gas analysis via mass spectrometry or automated glucose monitoring, temperatures closer to the optimal growth point of 37 °C, with much shorter induction lengths that would allow for manual adjustments or under significantly different fermentation conditions.

A process very similar to the method described for pre-induction optimization was followed. After dropping the temperature to 20 °C and inducing with 1.0 mM IPTG, the growth rate and glucose consumption rate was monitored at u_m . The resulting curves for lots ARE-064 and 065 are shown in Figures 19a and b. Although the growth curves were expected to be linear or quadratic, the fit for exponential was surprisingly close. The process was terminated once the cultures reached stationary phase, which occurred between 5 and 7 hours post-induction. For the two runs the value for u_m was .123 and the value for v_m was .087. With the parameters for controlling growth by limiting the

glucose feed rate now established, the process of optimizing induction conditions could begin.

Figure 19a. Post-Induction growth Curve

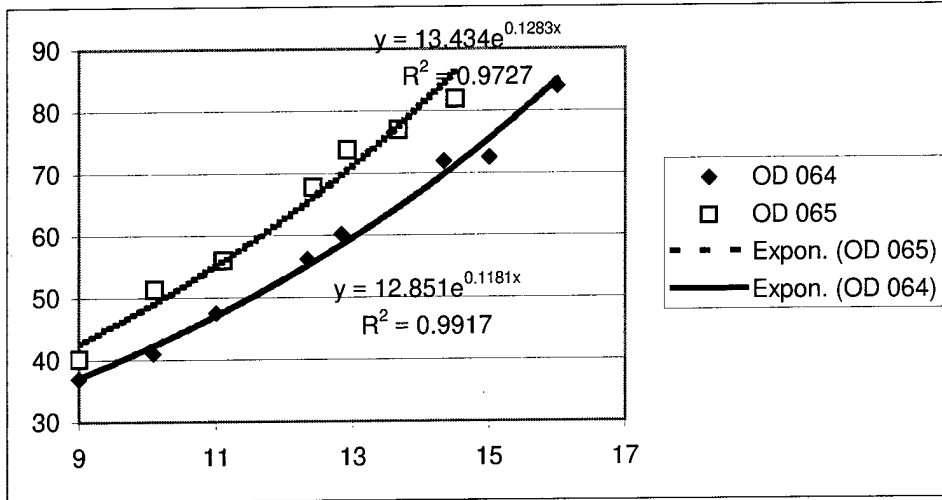
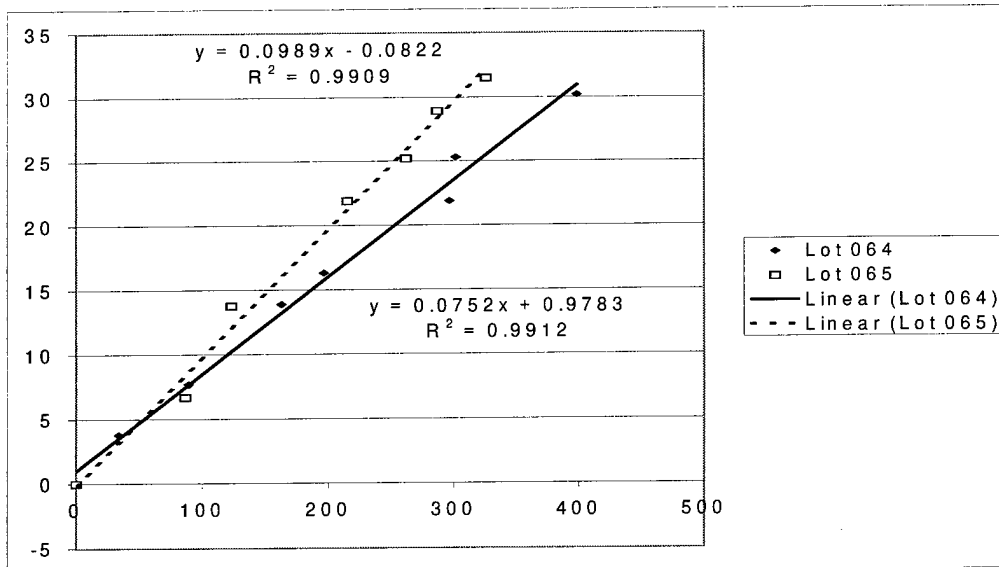


Figure 19b. Post-Induction glucose Consumption



Induction optimization (in progress)

With regards to growth rates, three primary factors need to be considered when optimizing induction conditions. The first is the O.D. at which to induce the culture. It is generally accepted that the culture should be induced while in exponential growth and when at least one additional doubling can occur. Considering a maximum obtainable culture density of 123 ODU, then the induction point should occur at or before 60 ODU. The actual induction point was set to 40 +/- 5 ODU for consistency. The second consideration is the rate at which the culture grows during the pre-induction phase. It is important that growth be limited as opposed to unlimited in order to prevent the formation of inhibitory compounds such as acetate. For this reason, pre-induction growth was set to 30% of u_m . With these two variables set, the growth rate during induction was or will be tested. The predicted growth rates were set to u_m , 50% of u_m and 25% of u_m . Additional feed profiles that ramped the glucose feed rate linearly or maintained a constant feed rate were or will be tested. Samples were taken as 1 ml aliquots during induction and stored frozen until prepared for analysis.

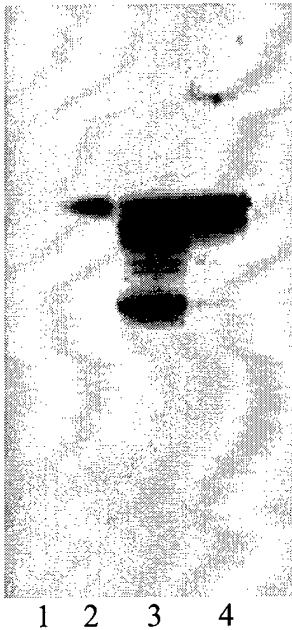
SDS-PAGE Analysis

Fermentation lots 68 and 70 were run on SDS-PAGE followed by staining with Coomassie Brilliant blue and probing with antibody for BoNT-E(Hc) Hc. The results of lot 070 are shown in Figure 20a and b lanes 3 and 4, and in Figure 21a and b lanes 2, 7, 9 and 10. It is apparent that expression is very low, as no BoNT-E(Hc) Hc band is visible by staining with Coomassie. However, when probed with antibody, expression is plainly visible in the soluble fraction. Although not confirmed, it is very likely that the separation procedure for soluble versus insoluble fractions works as very little background protein banding is present in the pellet fractions. Further work will need to be completed to determine if the BoNT-E(Hc) Hc fraction in the pellet is the result of inclusion body formation, trapped soluble protein or both. For lot 068, the results are shown in Figure 21a and b lanes 1, 3, 4 and 8 and the data confirms that protein is being expressed.



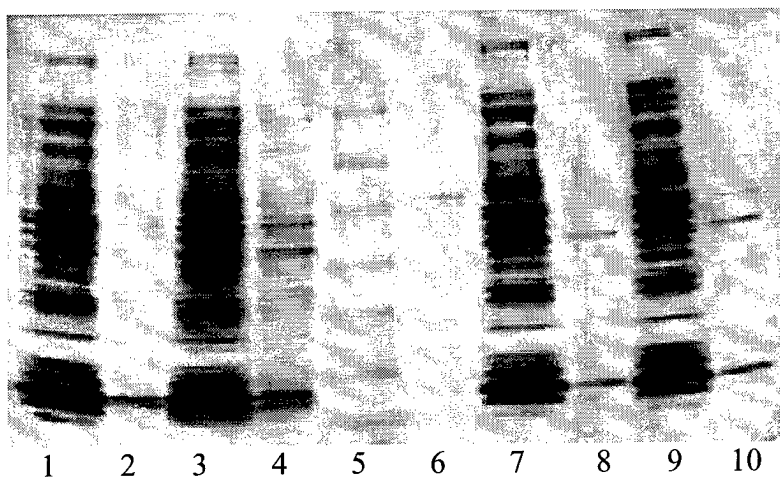
Lane 1: Molecular Weight Markers
 Lane 2: BoNT-E(Hc) Hc standard
 Lane 3: t = 8 supernatant
 Lane 4: t = 8 pellet

Figure 20a SDS-PAGE analysis of *E. coli* BoNT-E(Hc) Hc expression study lot 070 stained with Coomassie Brilliant Blue



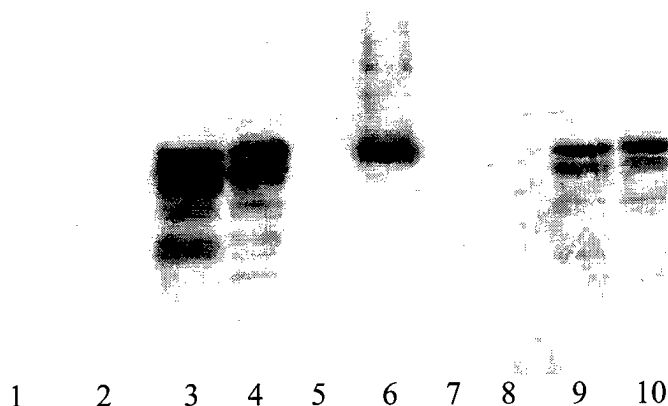
Lane 1: Molecular Weight Markers
 Lane 2: BoNT-E(Hc) Hc standard
 Lane 3: t = 8 supernatant
 Lane 4: t = 8 pellet

Figure 20b SDS-PAGE analysis of *E. coli* BoNT-E(Hc) hc expression study lot 070 probed with antibody (Western)



Lane 1: Lot 068 t = 0 supernatant
 Lane 2: Lot 070 t = 0 pellet
 Lane 3: Lot 068 t = 5 supernatant
 Lane 4: Lot 068 t = 5 pellet
 Lane 5: Molecular Weight markers
 Lane 6: BoNT-E(Hc) Hc standard
 Lane 7: Lot 070 t = 0 supernatant
 Lane 8: Lot 068 t = 0 pellet
 Lane 9: Lot 070 t = 2 supernatant
 Lane 10: Lot 070 t = 2 pellet

Figure 21a SDS-PAGE analysis of *E. coli* BoNT-E(Hc) Hc expression lots 068 and 070 coomassie brilliant blue.



Lane 1: Lot 068 t = 0 supernatant
 Lane 2: Lot 070 t = 0 pellet
 Lane 3: Lot 068 t = 5 supernatant
 Lane 4: Lot 068 t = 5 pellet
 Lane 5: Molecular Weight markers
 Lane 6: BoNT-E(Hc) Hc standard
 Lane 7: Lot 070 t = 0 supernatant
 Lane 8: Lot 068 t = 0 pellet
 Lane 9: Lot 070 t = 2 supernatant
 Lane 10: Lot 070 t = 2 pellet

Figure 21b SDS-PAGE analysis of *E. coli* BoNT-E(Hc) Hc expression lots 068 and 070 probed with antibody (Western).

Conclusions

The primary goal of this project was to express soluble BoNT-E(Hc) within *E. coli*. To accomplish this, a fermentation condition that maintained selective pressure on the plasmid with a minimum amount of antibiotic and did not use animal based products had to be established first. It was found that 10 $\mu\text{g/ml}$ kan was required to maintain the plasmid and that Peptone Soytone, a soy derived product, could replace animal or milk peptones. Once this had been completed, experiments were conducted to formulate a predictive model for growth on glucose for both pre and post induction conditions. Two predictive equations for feed rates were derived, one for pre-induction and one for post induction. For pre-induction the equation is:

$$F = u * .64(XV)e^{ut}$$

where F equals the feed rate, u equals the target growth rate, X equals cell density in O.D.U's, V equals fermentor volume and t equals time. For post induction the equation is:

$$F = u * .73(XV)e^{ut}$$

A preliminary induction run with an unlimited glucose feed rate resulted in the expression and accumulation of product but at very low amount. By continuing to test glucose feed rates and duration during the induction phase, better yields should be possible.

4. Summary Of 5-L Fermentation Research Activities by

Introduction

This report summarized our recent research achievements on 5-L fermentation of rBoNT(Hc) proteins. It contains three subsections: *Pichia* fermentation protocol, rBoNT-C(Hc) fermentation optimization, and rBoNT-B, -D, and -E fermentation results.

Note: Figure and Table numbering will start over again with this section.

4.1. *Pichia* Fermentation Protocol

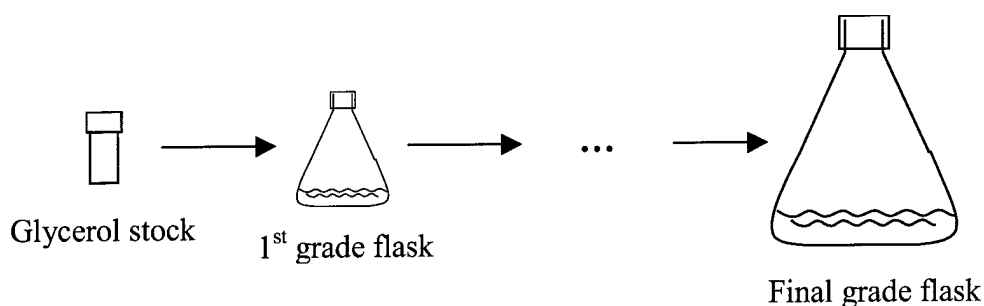
Introduction

This protocol describes the KEY controls and simulation of the Mut⁺ *Pichia* fermentation for the intracellular production of all types of BoNT(Hc) (botulinum neurotoxin heavy-chain fragment C). The protocol is based on the growth model and production optimization achieved from the studies on BoNT-A(Hc) fermentation (refer to the manuscript, Wenhui Zhang *et al.*, *Biotechnology and Bioengineering*, 2000, in press). All the operation procedures which are not detailed in this protocol can refer to the “*Pichia* Fermentation Process Guidelines” and “*Pichia* Expression Kit” of Invitrogen Co., fermentation SOPs of BPDF-UNL, “High Cell-Density Fermentation” in book “*Pichia* Protocols” (p107-120), and related manuals of applied equipment. This protocol proposed an "exponential schedule" control strategy for both glycerol and methanol fed-batch phases which is capable for optimizing the growth rate to maximize the production. It can be also used as a reference for studying the production of other intracellular or secreted recombinant proteins.

The degradation of rBoNT(Hc) proteins during fermentation, if any, needs to be studied furthermore. The P1 protocol proposed here provides a basic growth design to optimize the conditions, such as pH, temperature, addition of casamino acid, etc., to avoid the degradation.

Precultures

Precultures for bench-top fermentors (≤ 15 L working volume)

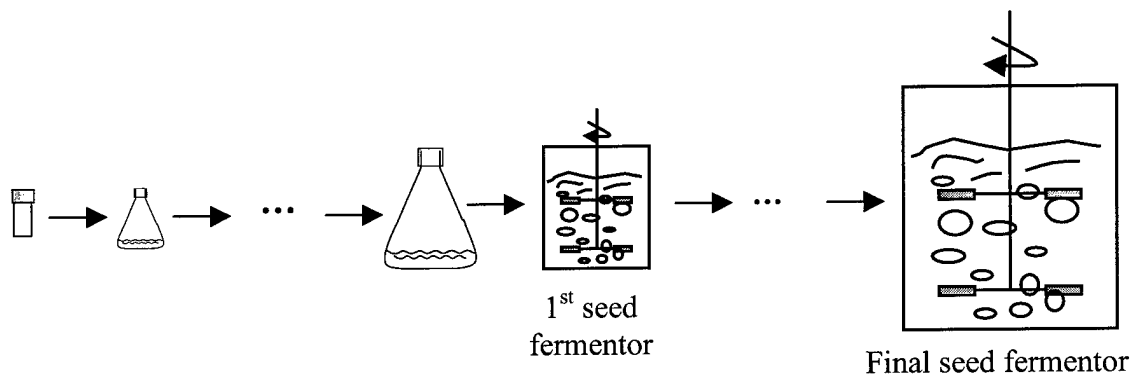


Preculture volume (V): V of the final grade flask is 5-10% of the initial fermentation volume. V increases 10-20 times when scaling up a grade of flask. V of the 1st grade flask is recommended not less than 20mL, so the number of grades can be decided when V of the final grade flask is known.

Medium: BMGY, refer to the "*Pichia* Expression Kit" of Invitrogen Co..

Growth: 30°C, 250-300rpm, grow to $OD_{600}=2-6$ before inoculate the next grade of flask.

Precultures for large-scale fermentors (≥ 15 L working volume)



Preparations of flask precultures: The same as that for bench-top fermentors, see 2.1. V of the final grade flask depends on V of the 1st seed fermentor.

Preparations of seed fermentors

Medium volume (V): The V of the final seed fermentor is 5-10% of the initial fermentation volume. V increases 10-20 times when scaling up a grade of seed fermentor. V of the 1st seed fermentor is recommended not less than 2 L, so the number of grades can be decided when V of the final seed fermentor is known.

Medium: BSM + PTM₁, the same as that for the batch phase of fermentation, see 4.1.

Growth conditions and control: The same as that for the batch phase of fermentation, see 3 and 4. Grow to WCW (centrifuge at 2000 × g for 10 min) = 30-50 g/L before inoculate the next grade of seed fermentor.

Control of Fermentation Parameters

pH: 5.0. Adjusted with 28% (undiluted) ammonium hydroxide.

Temperature: 30.0 °C.

Aeration: 1.0 vvm. The flow rate is calibrated with an air flow meter if necessary.

DO: 100% > DO > 20% of air saturation. Controlled by adjusting agitation, or by supplying pure oxygen when agitation is already at the maximum speed. The control is through the fermentor built-in microprocessor or fermentation software.

Agitation: Adjusted to maintain DO. Refer to DO control.

Pure oxygen: Supplied after agitation increases to the maximum, and adjusted to maintain DO. Refer to DO control.

Antifoam: The minimum needed to eliminate foam. Dissolved in methanol (50% v/v), and added manually with syringe + 0.45 µm mini-filter when needed. A common antifoam can be used, such as KFOTM 673 (food grade, KABO Chemicals, Inc., WY).

WCW: Wet cell weight indicating cell density, g/L. Obtained by centrifuging for 10 min at 2000 × g. 1 g WCW ≈ 0.28 g DCW (dry cell weight).

Glycerol Batch Phase

Medium: BSM + 4.35 mL PTM₁/L, refer to the "Pichia Fermentation Guidelines" of Invitrogen Co..

Preparation of BSM:

1 L BSM contains: 26.7 mL 85% H₃PO₄
0.93 g CaSO₄
18.2 g K₂SO₄
14.9 g MgSO₄·7H₂O
4.13 g KOH
40.0 g glycerol

Preparation of PTM₁:

1 L PTM₁ contains: 6.0 g CuSO₄·5H₂O
0.08 g NaI
3.0 g MnSO₄·H₂O
0.2 g Na₂MoO₄·2H₂O
0.02 g H₃BO₃
0.5 g CoCl₂
20.0 g ZnCl₂
65.0 g FeSO₄·7H₂O
0.2 g biotin
5.0 mL H₂SO₄

There may be a cloudy precipitate upon mixing of these ingredients. Make them dissolve as completely as possible. Store at room temperature. Filter-sterilize just before using.

BSM pH adjustment: After autoclaved and cooled down to 30°C, pH is adjusted to 5.0 with undiluted ammonium hydroxide. Recalibrate the pH probe by sampling and measuring the pH with an external pH meter. This is to confirm if the response of pH probe has any shift after autoclaving.

Addition of PTM₁: After pH is adjusted to 5.0, 4.35 mL filter-sterilized PTM₁ is added to per L BSM.

Initial volume (V₀): BSM volume before inoculation V₀ =

$$V_f - V_i - V_{gly} - V_{MeOH}$$

V_f: Fermentor working volume, ≈80% of the total volume.

V_i: Inoculum volume.

V_{gly}: Total fed volume of 50% (w/w) glycerol, see 5.2.3 and 6.3.

V_{MeOH}: Total fed volume of 100% methanol, see 7.3.3.2.

For P1 protocol: $V_{gly} + V_{MeOH} \approx [(20 + 30)/1129 + 60/788]V_0 = 0.12V_0$

For P2 protocol: $V_{gly} + V_{MeOH} \approx [(197 + 30)/1129 + 140/788]V_0 = 0.38V_0$

Note: 1129: density of 50% (w/w) glycerol, g/L

788: density of 100% methanol, g/L

So, For P1*: $V_0 = (V_f - V_i)/1.12$

For P2: $V_0 = (V_f - V_i)/1.38$

*P1 protocol is not an optimal protocol for maximum production, so V₀ is not necessary to be this value, but should be no more than this value.

The volume of fed NH₃H₂O, sampling and vaporization are considered to be negligible for V₀ calculation.

Inoculation: Aerate to make air saturated in the medium, and calibrate DO to 100% just before inoculation.

Growth

Growth kinetics on glycerol

$$\mu_{m, gly} = 0.177 \quad (\text{doubling time} = 3.9 \text{ h})$$

$$v_{m, gly} = 0.0688$$

$$Y_{x/g} = 2.57$$

μ_{gly} : specific growth rate on glycerol, h^{-1}

v_{gly} : specific glycerol consumption rate, g glycerol/g WCW/h

$Y_{x/g}$: cell yield to glycerol, g WCW/g glycerol

subscript m : maximum.

Note: These kinetic parameters are based on the recombinant *Pichia* strain expressing BoNT-A(Hc). They can be also used as a reference for other recombinant *Pichia* strains.

Predicted amount of final cell mass

Total cell mass: $Z = 40 \cdot 2.57 \cdot V_0 = 105V_0$ (g WCW)

Final cell density: $X = Z/(V_0 + V_i) = 105V_0/(V_0 + V_i)$ (g WCW/L)

Predicted time the batch phase ends (DO spikes)

Take a sample at t_i , measure the WCW as X_i , then the time the phase ends, t_e , can be predicted as:

$$t_e = t_i + \frac{1}{\mu} \ln \left(\frac{105V_0}{(V_0 + V_i)X_i} \right), \quad \mu = 0.177 \text{ h}^{-1}$$

t_i can be any time during the exponential growth. For example, if at $t_i = 10$ h, a sample is measured and the cell density $X_i = 10$ g WCW/L, assuming $V_i = 5\%$ of V_0 , then $t_e = 23$ h.

Glycerol Fed-Batch Phase

Glycerol feed: 50% w/w (=53% w/v) glycerol containing 12 mL PTM₁/L.

Feeding strategy

Feed rate

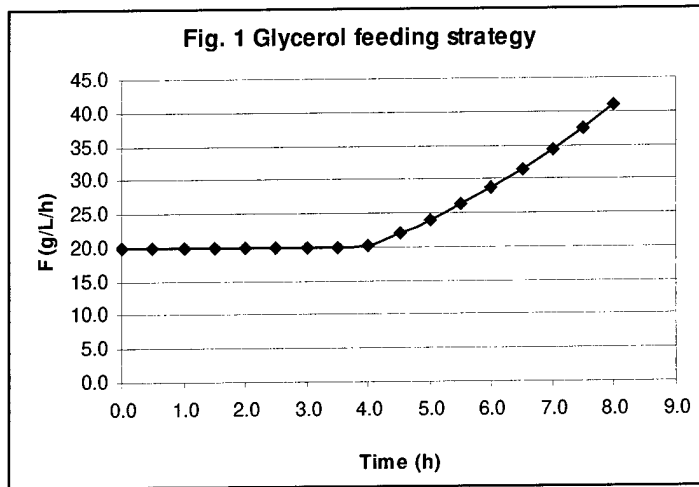
The glycerol feed rate, F_g , is expressed as gram of 50% glycerol per L of V_0 per h (g/L/h), and is set to:

$$t_g \leq 4 \text{ h: } F_g = 20 \text{ g/L/h,}$$

$$t_g > 4 \text{ h: } F_g = 14.1e^{0.177(tg-2)} \text{ g/L/h}$$

t_g : glycerol feeding time.

F_g can also be shown as in Fig 1:



Total amount of fed glycerol and predicted growth

The time course of total amount of fed 50% glycerol, ΣF_g , and predicted total cell mass, $Z (=XV)$, are calculated as:

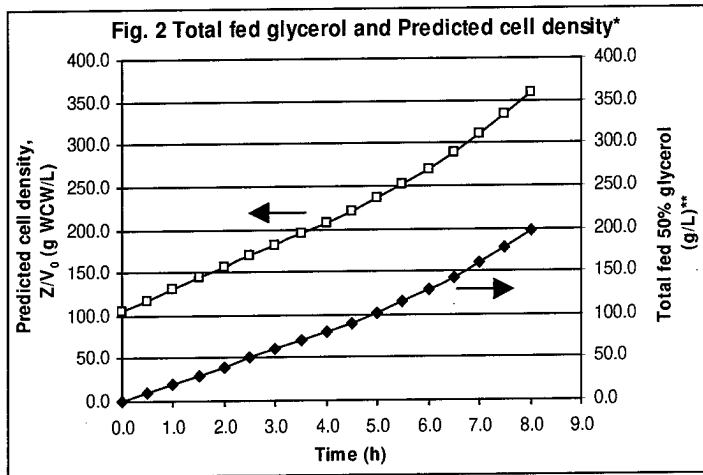
$$t_g \leq 4 \text{ h: } \Sigma F_g = 20 t_g V_0 \quad (\text{g 50\% glycerol})$$

$$Z = V_0 (105 + 25.7 t_g) \quad (\text{g WCW})$$

$$t_g > 4 \text{ h: } \Sigma F_g = 80 V_0 [e^{0.177(tg-2)} - 0.425] \quad (\text{g 50\% glycerol})$$

$$Z = V_0 \{105 + 102.8 [e^{0.177(tg-2)} - 0.425]\} \quad (\text{g WCW})$$

$\Sigma F_g/V_0$ and Z/V_0 can be also shown as in Fig. 2:



*The cell density is predicted based on V_0 and without considering any volume changes.

**g/L means total fed grams for per liter of V_0 , that is $\Sigma F_g/V_0$.

Length of the glycerol fed-batch phase

The length of the glycerol fed-batch phase, t_g , depends on the cell mass amount needed, and t_g is suggested not less than 1 h. Here gives two strategies, named P1 and P2 protocol, for the BoNT(Hc) production:

P1 protocol: $t_g = 1$ h

P2 protocol: $t_g = 8$ h

P1 does not give a maximum production for a batch of fermentation, but is a simple protocol to quickly obtain cell mass with high protein content. P1 is also the protocol used to build up a model for cell growth and protein production, as well as to optimize the fermentation conditions such as pH, temperature, addition of casamino acid, etc.

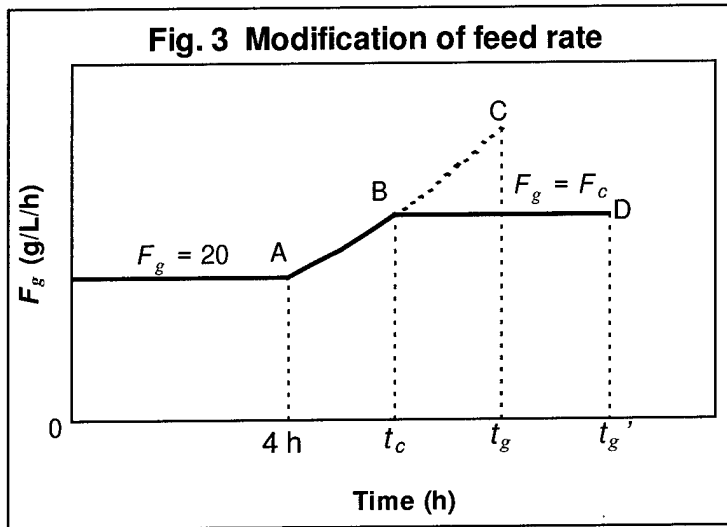
P2 is basically an optimal protocol to achieve a maximum production for a batch of fermentation in a shortest operation time.

Predicted growth for P1 and P2

	P1	P2
t_g (h)	1	8
Total fed 50% glycerol (g)	$20 V_0$	$197 V_0$
Final cell mass $Z (=XV)$ (g)	$\approx 130 V_0$	$\approx 360 V_0$

Modification of feed rate design

When feeding time $t_g > 4$ h, F_g increases exponentially (see 5.2.1). In some cases, fermentor operation conditions, such as oxygen supply and heat transfer abilities, will not support high growth rate in high cell density. In this case, F_g has to be limited to some level F_c after $t_g > t_c$, t_c is the time F_g reaches F_c exponentially, shown as in Fig. 3:

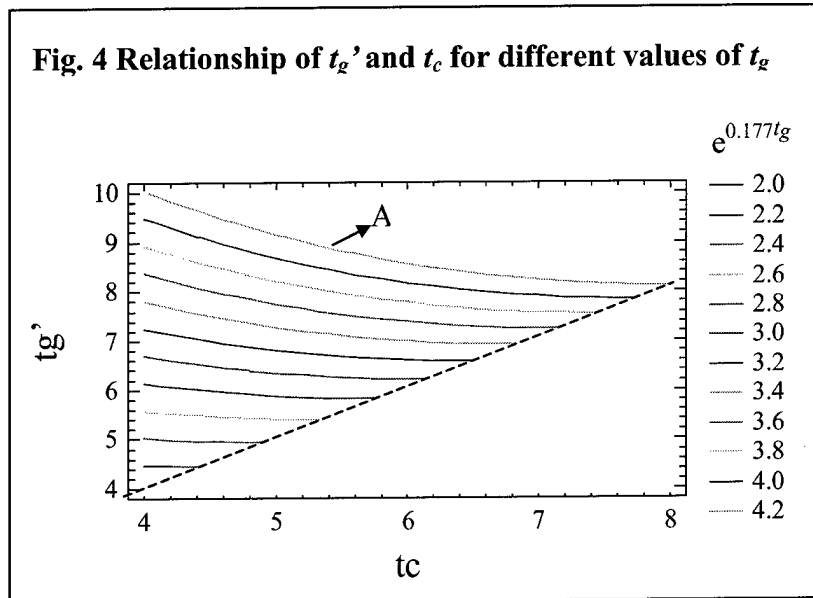


If t_g is the original designed length of the fed-batch phase, the total fed amount of glycerol during A-B-D should be the same as A-B-C, from this the following equations are derived:

$$e^{0.177t_c} \left[1 + 0.177(t_g' - t_c) \right] = e^{0.177t_g}$$

$$t_g' - t_c = 5.65 \left[e^{0.177(t_g - t_c)} - 1 \right]$$

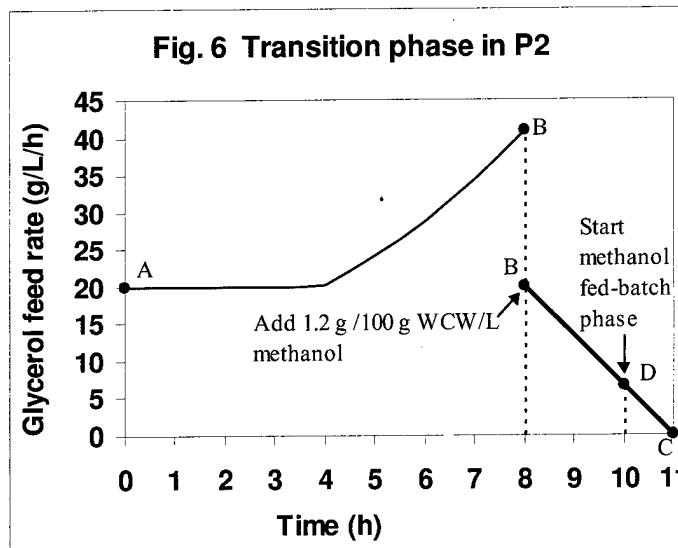
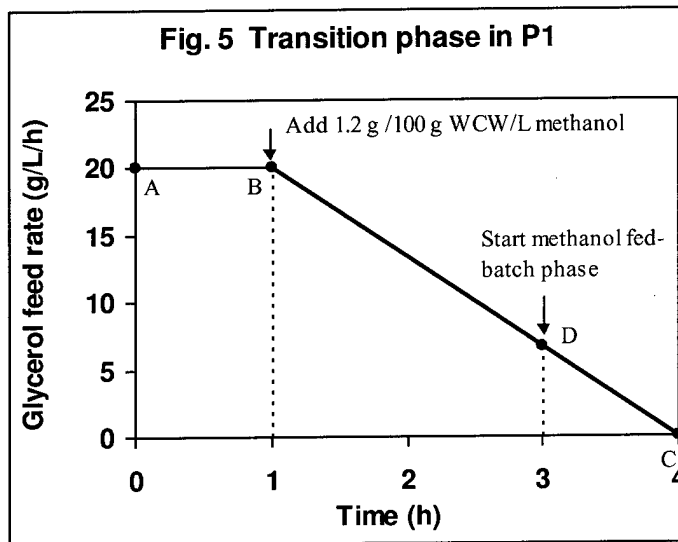
so t_g' can be calculated when t_g and t_c are known. For different values of t_g , the relationship between t_g' and t_c can be shown as in Fig. 4. Curve A is close to the case of P2 protocol in which $t_g = 8$ h, $e^{0.177t_g} = 4.12$.



Transition phase

Purpose: To shorten the time required for the cells to fully adapt to methanol.

Strategy: Following the glycerol fed-batch phase, the transition phase is initiated by the addition of 1.2 g /100 g WCW/L methanol which serves to initiate induction. The glycerol feed rate is simultaneously set to ramp down linearly from 20 g/L/h to 0 over a 3-hour period. Methanol fed-batch phase starts at the end of the 2nd hour after addition of the methanol. Fig. 5 and Fig. 6 show the transition phase for P1 and P2 protocols.



A-B: Glycerol fed-batch phase

B-C: Transition phase

B: Add 1.2g/100 g WCW/L methanol (≈ 1.5 g/L for P1, ≈ 4.5 g/L for P2)

D: Start methanol fed-batch phase (see 7)

Predicted growth in transition phase

In the transition phase, the glycerol feed rate, F_t , is expressed as the following equation:

$$F_t = V_0(20 - 6.67t_t) \text{ g/h,}$$

t_t : transition time, $t_t = 0$ at B point in Fig. 5 and 6.

The total fed 50% glycerol, ΣF_t , is expressed as:

$$\Sigma F_t = V_0(20 t_t - 3.33t_t^2) \text{ g}$$

The predicted cell mass, $Z=XV$, can be calculated as:

P1 protocol: $Z= V_0(130 + 25.7t_t - 4.28t_t^2)$

P2 protocol: $Z= V_0(360 + 25.7t_t - 4.28t_t^2)$

By the end of the transition phase, the total fed 50% glycerol and predicted cell mass are shown as in the following table:

	P1	P2
Total fed 50% glycerol (g)	$30 V_0$	$30 V_0$
Final cell mass (g)	$\approx 170 V_0$	$\approx 400 V_0$

Methanol Fed-Batch Phase

Methanol feed: 100% methanol containing 12 mL PTM1/L

Growth kinetics on methanol

$$v_{me} = 0.84\mu_{me} + 0.0071$$

$$\mu_{m,me} = 0.0709 \text{ (doubling time} = 9.8 \text{ h)}$$

$$v_{m,me} = 0.0682$$

$$Y_{x/me} = 1.19 - 1/(0.85 + 100\mu_{me})$$

μ_{me} : specific growth rate on methanol, h^{-1}

v_{me} : specific methanol consumption rate, g MeOH/g WCW/h

$Y_{x/me}$: cell yield to methanol, g WCW/g MeOH

subscript m : maximum.

Note: These kinetic parameters are based on the recombinant *Pichia* strain expressing BoNT-A(Hc). They can be also used as a reference for other recombinant *Pichia* strains.

Feeding strategy

Feed rate

The methanol feed rate, F_{MeOH} , is expressed as gram 100% methanol per h (g/h), and is set to:

$$F_{MeOH} = X_D V_D (0.84\mu_{set} + 0.0071)e^{\mu_{set}t}$$

X_D : cell density when initiating methanol feed at point D (see Fig. 5 and Fig 6, 6.2), g WCW/L

V_D : Medium volume at point D, L

μ_{set} : desired specific growth rate, h^{-1} ($\mu_{set} \leq \mu_m = 0.0709$)

t : methanol feeding time, h ($t = 0$ at point D)

For P1 protocol, $X_D V_D \approx 165V_0$, so

$$F_{MeOH} = 165V_0 (0.84\mu_{set} + 0.0071)e^{\mu_{set}t}$$

For P2 protocol, $X_D V_D \approx 395V_0$, so

$$F_{MeOH} = 395V_0 (0.84\mu_{set} + 0.0071)e^{\mu_{set}t}$$

For BoNT-A(Hc) production, the optimal $\mu_{set} = 0.0267 \text{ h}^{-1}$. For other BoNT(Hc) fragments, $\mu_{set} = 0.0267 \text{ h}^{-1}$ can be used as a reference before their optimal μ_{set} are achieved.

When $\mu_{set} = 0.0267 \text{ h}^{-1}$,

$$P1: F_{MeOH} = 4.87V_0 e^{0.0267t}$$

$$P2: F_{MeOH} = 11.66V_0 e^{0.0267t}$$

For other intracellular or secreted proteins, the optimal μ_{set} should be studied to maximize the production. If the optimal μ_{set} is not available yet, $\mu_{set} = 0.0267 \text{ h}^{-1}$ can be used as a reference. In 7.3.3, the predicted growth for $\mu_{set} = 0.04 \text{ h}^{-1}$ is also calculated as an example.

Length of methanol-fed batch phase

Length of the methanol fed-batch phase, t_{me} , should be optimized for different proteins. For BoNT-A(Hc) production, the optimal $t_{me} = 10 \text{ h}$. This can be also used as a reference for other BoNT(Hc) proteins.

Predict growth

Total fed methanol ΣF_{MeOH} :

$$\Sigma F_{MeOH} = X_D V_D (0.84\mu_{set} + 0.0071)(e^{\mu_{set}t} - 1) / \mu_{set}$$

$$\text{Predicted growth: } XV = X_D' V_D e^{\mu_{set}t}$$

$$\text{where } X_D' \approx 0.9X_D$$

Note: After methanol induction, it is observed that the cell density usually decreases to about 90% of that before induction under the same centrifuge conditions. This is possibly caused by the changing of the cell's shape, size and water content due to the induction.

Predict growth for P1 and P2

For BoNT(Hc) proteins, if $\mu_{set} = 0.0267 \text{ h}^{-1}$,

$$P1: \Sigma F_{MeOH} = 182.5V_0(e^{0.0267t} - 1)$$

$$XV=148.5V_0e^{0.0267t}$$

$$P2: \quad \Sigma F_{MeOH} = 436.8V_0(e^{0.0267t} - 1)$$

$$XV=355.5V_0e^{0.0267t}$$

For other proteins, if $\mu_{set} = 0.04 \text{ h}^{-1}$,

$$P1: \quad \Sigma F_{MeOH} = 167.9V_0(e^{0.04t} - 1)$$

$$XV=148.5V_0e^{0.04t}$$

$$P2: \quad \Sigma F_{MeOH} = 402.0V_0(e^{0.04t} - 1)$$

$$XV=355.5V_0e^{0.04t}$$

Predict final cell mass for P1 and P2

	$\mu_{set} = 0.0267 \text{ h}^{-1}$		$\mu_{set} = 0.04 \text{ h}^{-1}$	
	P1	P2	P1	P2
t_{me} (h)	10	10	10	10
Total fed methanol (g)	56 V_0	134 V_0	83 V_0	198 V_0
Final cell mass (g)	$\approx 194 V_0$	$\approx 464 V_0$	$\approx 222 V_0$	$\approx 530 V_0$

4.2. BoNT-C₁(Hc) Fermentation Optimization

A methanol with glycerol co-feeding strategy was applied to the BoNT-C₁(Hc) fermentation. Effects of methanol concentration (growth on excess methanol) or methanol feed rate (growth on limiting methanol), glycerol feed rate, T and pH on the expression were investigated for some level of optimization. BoNT-C₁(Hc) was quantitated from the band intensity of western blot (chemiluminescence detection) with software "UN-SCAN-IT Automated Digitizing System Version 5.1" (Silk Scientific Corporation, 1998). Details not described here refer to the SOPs of UNL-BPDF.

Methods

1. Strain: BoNT-C₁(Hc)/C5 (UNL-BPDF ID: PA01-4106)

2. Fermentation

(1) Batch phase

- Initial volume: 2 L
- Medium: BSM + 4.35 mL PTM1/L
- pH: 5.0 (Adjusted with concentrated ammonium)
- T: 30 °C
- DO: > 20 %
- Aeration: 1 vvm

(2) Glycerol fed-batch phase (1.5 h)

- Glycerol feed solution: 500 g/L + 12 mL PTM1/L
- Feed rate: 13.3 g/h/L or 12.1 mL/h/L (of initial volume)
- Feed length: 1.5 h
- pH, T, DO, and Aeration: the same as that in the batch phase

(3) Transition phase (2 h)

- Spiked MeOH: 1.54 g/L or 1.95 mL/L (of initial volume)
- Glycerol feed rate: $F_{\text{trg}} = 13.3(1-0.33t)$ g/h/L or $12.1(1-0.33t)$ mL/h/L (of initial volume)
- Feed length: $t = 2$ h
- pH, T, DO, and Aeration: the same as that in the batch phase

(4) Methanol fed-batch phase

- MeOH feed solution: 100% MeOH + 12 mL PTM1/L
- Feed rate or MeOH concentration: vary to study its effect on the expression
- pH: vary to study its effect on the expression
- T: vary to study its effect on the expression

- Glycerol feed rate: vary to study its effect on the expression
- Length: 40 - 45 h
- DO and Aeration: the same as that in the batch phase

3. Cell lysis

(1) Preparation of **precise 150 g/L** cell mass suspension for lysis

- ↳ Thaw the frozen cell mass in room temperature.
- ↳ Pipette 0.2 mL thawed cell paste of each sample into a pre-weighed (W_V) 2.5-mL vial. Make an extra vial (with any sample, marked EV) for determining the specific volume (mL/g) of cell paste later.
- ↳ Add 1.0 mL washing buffer (see compositions below) into each vial, mix well to wash cells.

Washing buffer: 31.5 mM sodium acetate
 18.5 mM acetic acid
 0.85 % NaCl
 pH 5.0

- ↳ Spin vials at $2000 \times g$, discard the supernatant and weigh vial + cell pellet (W_{VC}).
- ↳ Obtain pellet weight in each vial: $W_C = W_{VC} - W_V$. The weight of pellet in the vial EV (extra vial) is expressed as $W_{C, EV}$.
- ↳ Add 1 mL dH₂O into the vial EV, mix and suspend cells, take 1 mL of the suspension and get its weight expressed as W_{CW} .
- ↳ The pellet specific volume D , mL/g, can be calculated with the following equation where W unit is g:

$$D = \left(\frac{W_{C, EV} + 1}{W_{CW}} - 1 \right) / W_{C, EV}$$

- ↳ The pellet volume in each vial $V_C = W_C/D$
- ↳ The total volume of 150 g/L-suspension in each vial $V_T = W_C/0.15$ (mL)
- ↳ The volume of lysis buffer needed to add in each vial $V_B = V_T - V_C$
- ↳ Each sample has its V_B of lysis buffer added, then all samples' suspension prepared have exactly the same cell density of 150 g/L.

(2) Cell lysis with bead beater: the SOPs

4. Western blot

(1) Assay total protein in lysate solution: the SOPs.

(2) SDS-PAGE

- ↳ Calculate mean total protein of lysate, mTP (mg/mL), from all samples which are going to be loaded in the same gel.
- ↳ Dilute all lysate with the same dilution time mTP.
- ↳ Load 22.5 μ L of diluted lysate plus 7.5 μ L of loading buffer in each lane.
- ↳ For DAB detection method, the standard BoNT-C₁(Hc) loaded is 2.109 μ g; for chemiluminescence detection, 0.703 μ g.

(3) Western transfer and blot: the SOPs.

5. Quantification of BoNT-C₁(Hc) from Western results:

Sample bands in the film obtained with chemiluminescence detection are scanned to become a ".tif" file and digitized with software "UN-SCAN-IT Automated Digitizing System Version 5.1" (Silk Scientific Corporation, 1998). With the standard curve, samples' BoNT-C₁(Hc) are quantitated in mg/g WCW.

Results

1. Standard curve of BoNT-C₁(Hc) in Western blot

Five points with 703.13, 351.56, 175.78, 100.45 and 70.31 ng of BoNT-C₁(Hc) were loaded in duplication, two different developing time resulted in two sets of bands, shown in Fig. 1.

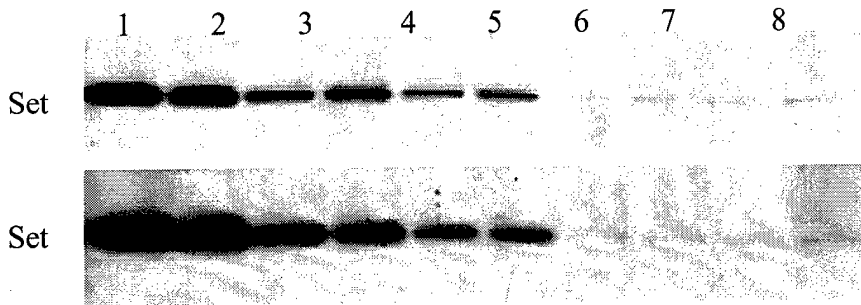


Fig. 1 Standard BoNT-C₁(Hc) Western blot with chemiluminescence detection. Set 2 had longer developing time than set 1. Loaded amount:

Lane	1	2	3	4	5	6	7	8	9	10
BoNT-C ₁ (Hc) (ng)	703.1	703.1	351.5	351.5	175.7	175.7	100.4	100.4	70.3	70.3
	3	3	6	6	8	8	5	5	1	1

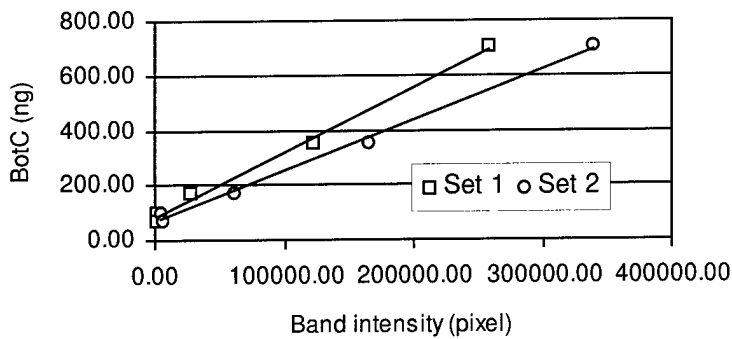


Fig. 2 BoNT-C₁(Hc) standard curve in western blot. Set 2 had longer developing time than set 1.

From Fig. 1, two standard curves, BoNT-C₁(Hc) (ng) vs. band intensity (pixel), were obtained, shown in Fig. 2, which gave the following linear equations:

$$\text{Set 1: BoNT-C}_1(\text{Hc}) = 0.002358\text{Int} + 86.289 \quad (1)$$

$$\text{Set 2: BoNT-C}_1(\text{Hc}) = 0.001842\text{Int} + 68.481 \quad (2)$$

Where BoNT-C₁(Hc): ng; Int: band intensity, pixel.

It was found that the slope ratio of these two equations ($0.002358/0.001842 = 1.28$) is approximately equal to the intercept ratio ($86.289/68.481 = 1.26$). Therefore, we may consider that the intensity is dependence of developing time, but all bands intensity in the same film will change in the same proportion with developing time. Based on equation (2), the standard curve can be expressed as the following equation:

$$\text{BoNT-C}_1(\text{Hc}) = k(0.001842\text{Int} + 68.481) \quad (3)$$

Where k is a factor decided by developing time. For set 1, $k = 1.27$. With a known standard point in a blot, the k can be calculated. In this research, a standard BoNT-C₁(Hc) of 703.13 ng was loaded in each gel. Assuming the intensity of this standard is Int_{std} after developing, k can be calculated by the following equation:

$$k = 703.13 / (0.001842 \text{Int}_{\text{std}} + 68.481) \quad (4)$$

With k known, the BoNT-C₁(Hc)/lane of samples in the same gel can be calculated with equation (3), and the BoNT-C₁(Hc) content in cells, α (mg/g WCW), is given by the following equation:

$$\alpha = D * \text{BoNT-C}_1(\text{Hc}) / 22.5 / 150 \quad (5)$$

D: Dilution time of cell lysate, which equals mTP (see SDS-PAGE in Methods 4 (2));

BoNT-C₁(Hc): ng per lane in gel;

22.5: Loaded volume of diluted lysate, 22.5 μL ;

150: Cell density of suspension for lysis, 150 g/L (see Methods 3 (1)).

2. Error of the quantification from Western blot

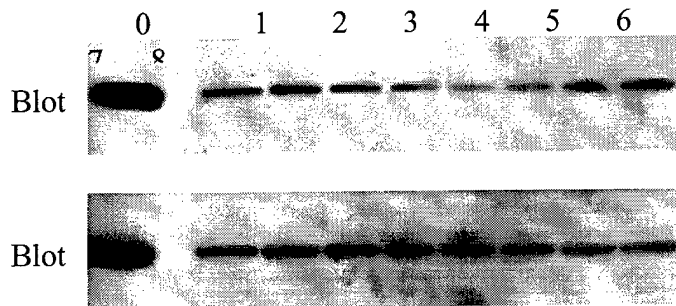


Fig. 3 Two blots with the same samples. The loaded amount in Blot 1 is half of that in Blot 2. Both had the same amount of standard BoNT-C₁(Hc) loaded in lane 0 (703.13 ng).

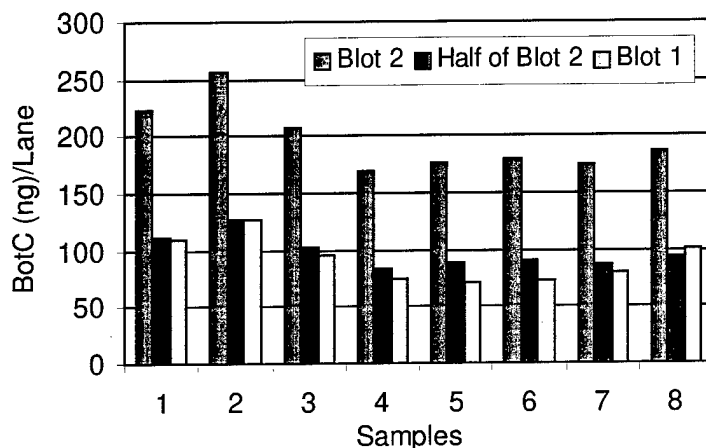


Fig. 4 BoNT-C₁(Hc) quantitated from Blot 1 and 2. The half value of Blot 2 was also plotted as a comparison to the value of Blot 1.

Two blots were loaded with the same eight samples, and the loaded amount in Blot 1 is half of that in Blot 2. Both had the same amount of standard BoNT-C₁(Hc) loaded in lane 0 (703.13 ng). Fig. 3 showed the bands obtained. With knowing the intensity of the standard (Int_{std}), k was calculated by equation (4), which is 0.695 and 1.33 for Blot 1 and 2, respectively. Then, BoNT-C₁(Hc) in the blots was quantitated by equation (3). The data obtained was showed in Fig. 4. The half amount of BoNT-C₁(Hc) in Blot 2, which is supposed to be theoretically the same as that in Blot 1, was calculated and also plotted in Fig. 4. The results showed that the value of Blot 1 is quite close to the half value of Blot 2. This illustrated that the standard curve and quantification method are acceptable

applicable. From the difference between the value of Blot 1 and the half value of Blot 2, an average error of quantification is estimated to be about 10% (the maximum error is about 20%).

3. Effect of pH on the α

Fermentations with pH 3.28, 5, 7.27 were run while methanol was controlled at 3.65 g/L, T 30 °C. The results in fig. 5 showed that pH 5.0 gave a highest α (1.62 mg/g).

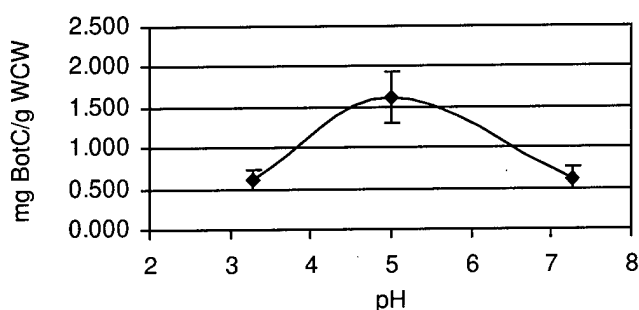


Fig. 5 Effect of pH on α (mg BoNT-C₁(Hc)/g WCW)

4. Effect of glycerol co-feeding on the α

Fermentation runs with and without glycerol co-feeding, shown in Table 1, were carried out. In Group 1 methanol was controlled to 20 g/L with methanol sensor (with PID feedback control). In Group 2, methanol was fed exponentially to achieve a limited growth rate $\mu_{\text{MeOH}} = 0.02 \text{ h}^{-1}$, in which the methanol feeding profile was decided with the growth model (See BoNT-A(Hc) protocol). The glycerol co-feeding profile in this research can be given by the following equation based on the growth kinetics on glycerol:

$$\text{For } \mu_{\text{gly}} = 0.03 \text{ h}^{-1}: F_{\text{gly}} = 3.5e^{0.03t} \quad (6)$$

$$\text{For } \mu_{\text{gly}} = 0.02 \text{ h}^{-1}: F_{\text{gly}} = 2.5e^{0.02t} \quad (7)$$

F_{gly} : 50% w/v glycerol/h/L initial volume

The result was shown in Fig. 6 which indicated that glycerol co-feeding could increase α when methanol was set to 20 g/L, while the increase was not obvious in the case of methanol-limited growth with $\mu_{\text{MeOH}} = 0.02 \text{ h}^{-1}$. This could be due to 20 g/L methanol resulting in a stronger induction to the AOX promoter and the co-feeding glycerol serving as a more efficient carbon and energy source.

Table 1 Fermentation runs with and without glycerol co-feeding, pH 5.0, T 30°C.

Group #	1		2	
Run #	1a	1b	2a	2b
Methanol	20 g/L	20 g/L	$\mu_{\text{MeOH}} = 0.02 \text{ h}^{-1}$	$\mu_{\text{MeOH}} = 0.02 \text{ h}^{-1}$
glycerol co-feeding	No	$\mu_{\text{gly}} = 0.03 \text{ h}^{-1}$	No	$\mu_{\text{gly}} = 0.02 \text{ h}^{-1}$

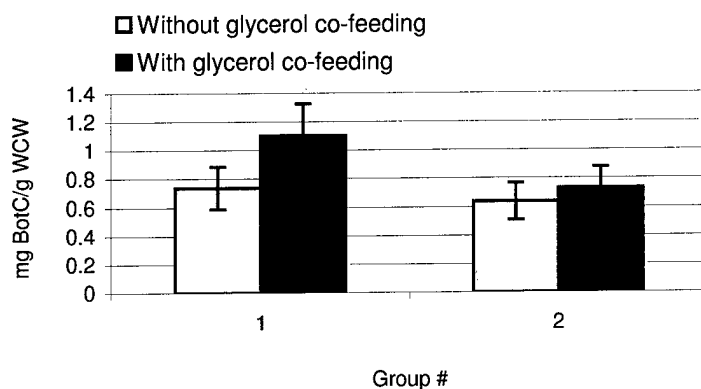


Fig. 6 Effect of glycerol co-feeding on the α

5. Effect of temperature on α

Fermentations at 26.5 °C, shown in Table 2, were run and compared with those at 30 °C. It was found, in Fig. 7, that 26.5 °C resulted in less α . But Fig. 8 showed that 26.5 °C gave more clear bands and less fragments. It needs to be further confirmed by N-terminal sequencing of the final purified product that if low temperature can result in a higher quality product.

Table 2 Fermentation runs at 26.5 °C and 30 °C, pH 5.0.

Group #	3		4	
Run #	3a	3b	4a	4b
Methanol	$\mu_{\text{MeOH}} = 0.02$ h^{-1}	$\mu_{\text{MeOH}} = 0.02$ h^{-1}	20 g/L	20 g/L
glycerol co-feeding	No	No	$\mu_{\text{gly}} = 0.03 \text{ h}^{-1}$	$\mu_{\text{gly}} = 0.03 \text{ h}^{-1}$
T	26.5 °C	30 °C	26.5 °C	30 °C

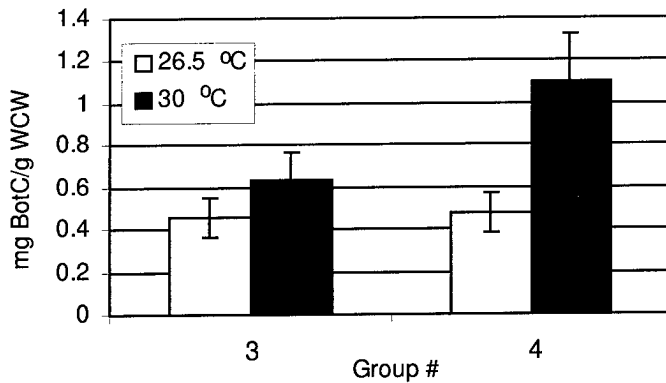


Fig. 7 Effect of temperature on α

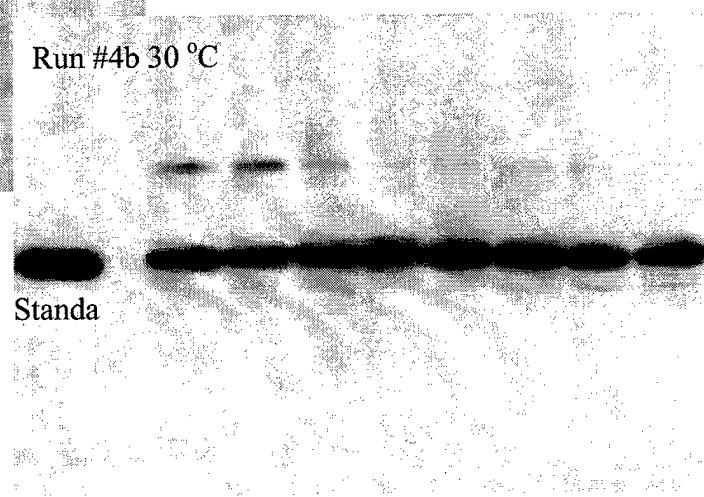
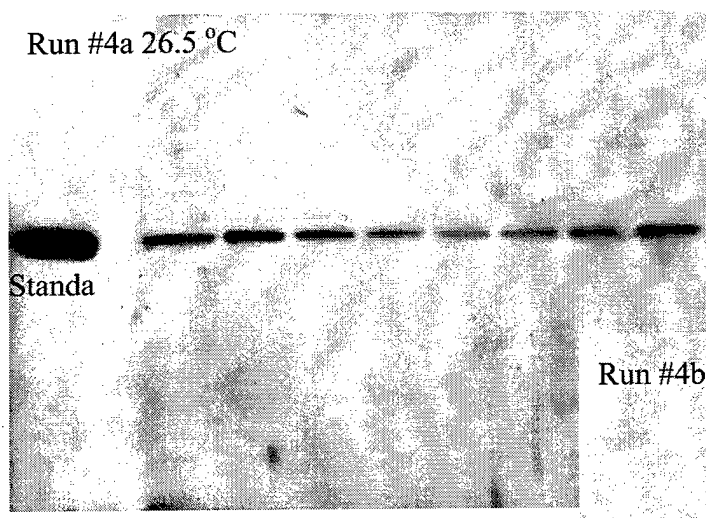
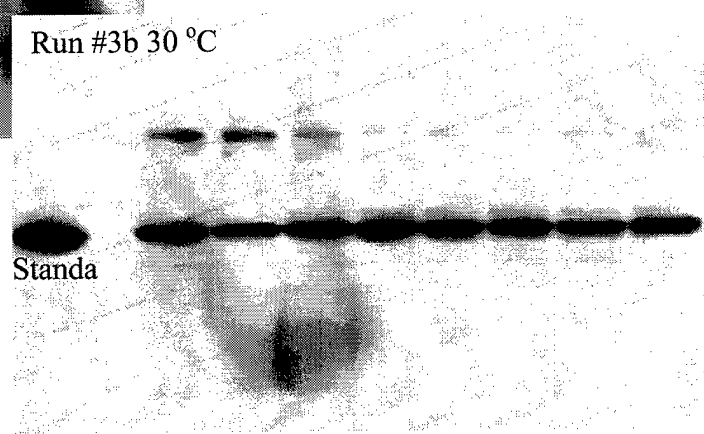
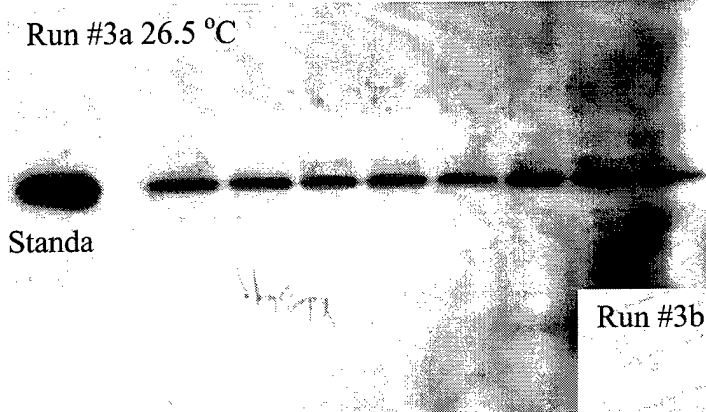


Fig. 8 Western blot comparison of fermentation at 26.5 °C to 30 °C

Conclusion

1. Highest α , 1.62 mg BoNT-C₁(Hc)/g WCW was obtained when MeOH = 3.65 g/L, pH 5.0, T 30 °C. This is close to the highest α of BoNT-A(Hc) (1.72 mg/g) achieved in the previous work which MeOH was at limited level, 0.338 g/L, resulting in $\mu = 0.0267 \text{ h}^{-1}$.
2. Glycerol co-feeding can enhance α at a high methanol concentration 20 g/L which resulted in an α of 1.11 mg BoNT-C₁(Hc)/g WCW. This was the first time to find that Pichia can also express well at high methanol concentration with co-feeding glycerol. Optimization of methanol level and glycerol co-feeding rate need to be further studied.
3. Lower temperature seems to be able to avoid the degradation efficiently, though the α achieved is lower than that at 30 °C. If this is confirmed true by the N-terminal sequencing, it will be a good strategy to run a fermentation at low temperature considering the quality of protein product.

4.3. BoNT-D(Hc), BoNT-B(Hc), and BoNT-E(Hc) Fermentations

4.3.1. BoNT-D(Hc) Fermentation (5/24/00 - 5/28/00)

Strains: BoNT-D(Hc) A-5 and BoNT-D(Hc) C-11

Lot#: wz95 (A-5) and wz96 (C-11)

Protocol applied: Basically based on BoNT-A(Hc) protocol

1. Shake-flask preculture:
 - Inoculum: 1 mL glycerol stock of A-5 (Lot# wz95) or C-11 (Lot# wz96)
BoNT-D(Hc) strain

- Medium: BMGY, refer to the "*Pichia Expression Kit*" of Invitrogen Co.
 - Medium volume: 200 mL in a 1000-mL baffle shake flask
 - T: 30°C
 - pH: 5.0
 - Shake rate: 250-300 rpm
 - Final OD₆₀₀ grown: 2-6
2. Fermentation conditions:
- T: 30°C
 - pH: 5.0, adjusted with 28% (undiluted) ammonium hydroxide
 - Aeration: 1.0 vvm
 - DO: 20-100%
 - Agitation: <1000 rpm, adjusted to maintain DO
 - Pure oxygen: Supplied and adjusted to maintain DO after agitation reaches the maximum
 - Antifoam: KFO 673 (KABO Chemicals Inc), 5% w/v in H₂O, the minimum needed to eliminate foam
3. Fermentation medium:
- Initial medium: BSM + 4.35 mL PTM1/L
 - BSM and PTM1: refer to the "*Pichia Fermentation Guidelines*" of Invitrogen Co.
 - Addition of PTM1: filter-sterilized and injected after the pH is adjusted to 5.0
 - Initial medium volume: 2 L
 - Glycerol feed solution: 50% w/v glycerol + 12 mL PTM1/L
 - Methanol feed solution: 100% methanol + 12 mL PTM1/L
4. Glycerol batch phase
- Growth rate: 0.177 h⁻¹ (maximum growth)
 - Length: 18 - 24 h, vary with the inoculum density and the initial medium volume
 - Cell density: ≈ 100 g WCW/L (centrifuging at 2000g, 10 min)
5. Glycerol fed-batch phase
- Feed rate F_G (g 50%-glycerol/h/L initial medium) and feed time T_G (h)

$$F_G = 13.3 \text{ g/h/L}$$

$$T_G = 4 \text{ h}$$

- Cell density at the end of the fed-batch phase: 178 g/L WCW

6. Transition phase

Following the glycerol fed-batch phase, a 2-hour transition phase is carried out to let cells adapt to methanol quickly and fully. The transition phase is initiated by adding a certain amount of methanol, simultaneously, the glycerol is fed with a feed rate decreasing linearly.

- Amount of added methanol at $t = 0$: 2 g/L
- Glycerol feed rate F_G (g 50%-glycerol/h/L initial medium):

$$F_G = 13.3(1-0.33t)$$

- Length: $t = 2 \text{ h}$

7. Methanol fed-batch phase

- Methanol concentration was controlled at 1 g/L (sensor read value) by MC-168 methanol controller during the whole production phase. The actual methanol concentration detected by GC was close to zero. Since methanol was fed by dropping to the broth, the methanol vapor from dropping caused the interference to the sensor response. This is why the actual methanol concentration was far from the sensor read value. This can be improved by inducing the methanol directly into the solution. The time course of actual methanol consumption is shown in Fig. 1.

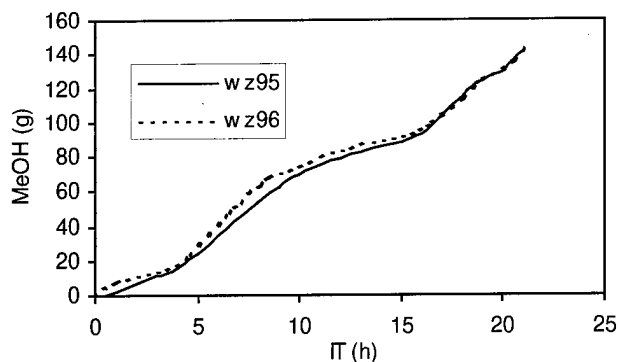


Fig. 1 w z95 and 96 consumed MeOH time course when controlled by MeOH sensor

- Induction length: 23 h

- Final cell density: wz95, 237 g/L; wz96, 222 g/L.
- Harvested cells were sent to USAMRIID and PDL-BPDF-UNL. Feedback of protein expression results is needed for further improvement of fermentation.

4.3.2. BoNT-B(Hc) Fermentation (6/6/00 - 6/9/00)

Strains: *P. pastoris* for BoNT-B(Hc) vaccine (BPR-182-00, Lot No. 0351, 02 Aug 96)

Lot#: wz97

Protocol applied: Basically based on BoNT-A(Hc) protocol and Karen Potter, *et al.*'s paper "Production and purification of the heavy-chain fragment C of botulinum neurotoxin, serotype B, expressed in the methylotrophic yeast *Pichia pastoris*. *Protein Expr. Purif.* 13: 357-65, 1998"

1. Shake-flask preculture: See BoNT-D(Hc).
2. Fermentation conditions: See BoNT-D(Hc).
3. Fermentation medium: See BoNT-D(Hc).
4. Glycerol batch phase: See BoNT-D(Hc).
5. Glycerol fed-batch phase
 - Feed rate F_G (g 50%-glycerol/h/L initial medium) = 15 mL/h/L
 - Feed time T_G (h) = 3 h
6. Transition phase: No
7. Methanol fed-batch phase
 - Temperature: Set to 25 °C.
 - Addition of casamino acids: 10g/L at the beginning of induction. 20% casamino acids solution was made and filter sterilized prior to the addition.
 - Methanol feeding profile: Feed rate = $(0.35t + 4)$ mL/h/L. Fig. 2 showed the time course of T, pH, fed amount of glycerol and methanol.

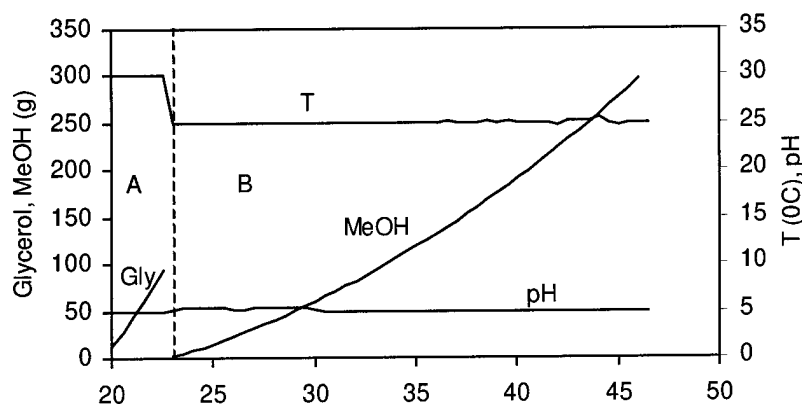


Fig. 2 T, pH, fed Glycerol and MeOH time course. A: glycerol fed-batch phase, B: methanol fed-batch phase.

- Induction length: 24 h
- Final cell density: 219 g/L.
- Harvested cells were sent to PDL-BPDF-UNL. Feedback of protein expression results is needed for further improvement of fermentation.

4.3.3. BoNT-E(Hc) Fermentation (6/13/00 - 6/16/00)

Strains: BoNT-E(Hc) C10

Lot#: wz98

Protocol applied: Basically based on BoNT-A(Hc) protocol

1. Shake-flask preculture: See BoNT-D(Hc).
2. Fermentation conditions: See BoNT-D(Hc).
3. Fermentation medium: See BoNT-D(Hc).
4. Glycerol batch phase: See BoNT-D(Hc).
5. Glycerol fed-batch phase
 - Feed rate F_G (g 50%-glycerol/h/L initial medium) = 13.3 g/h/L
 - Feed time T_G (h) = 1.5 h
6. Transition phase

Following the glycerol fed-batch phase, a 2-hour transition phase is carried out to let cells adapt to methanol quickly and fully. The transition phase is initiated by adding a certain amount of methanol, simultaneously, the glycerol is fed with a feed rate decreasing linearly.

- Amount of added methanol at $t = 0$: 2 g/L
- Glycerol feed rate F_G (g 50%-glycerol/h/L initial medium):
 $F_G = 13.3(1-0.33t)$

- Length: $t = 2$ h
7. Methanol fed-batch phase
- Temperature: Set to 25 °C.
 - Methanol concentration was controlled at 1.5 g/L (sensor read value) by MC-168 methanol controller during the whole production phase. The actual methanol concentration detected by GC was around 1.1 g/L. The methanol feed was induced directly into the broth through the harvest pipe, so the interference of methanol vapor from dropping was overcome. The working PID set values are: $P = 50$, $I = 5$, and $D = 100$. Fig. 3 show the time course of T, pH, fed glycerol and consumed methanol.

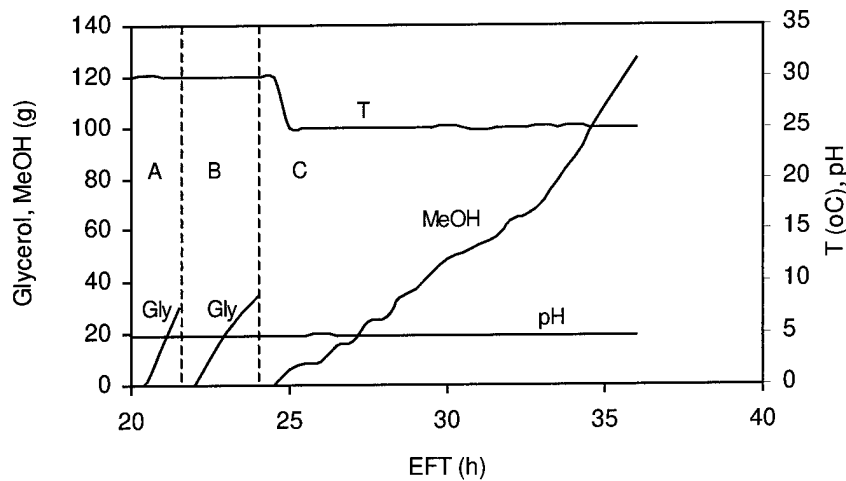


Fig. 3 T, pH, fed Glycerol and consumed MeOH time course. A: glycerol fed-batch phase, B: transition phase, C: methanol fed-batch phase.

- Induction length: 11.5 h
- Final cell density: 178.9 g/L.
- Harvested cells were sent to PDL-BPDF-UNL. Feedback of protein expression results is needed for further improvement of fermentation.

5. PRE-SEED CELL BANKS

The suitability of various expression systems for producing a given regulated product must be determined by respective development processes prior to the establishment of a Master Cell Bank (MCB) and Working Cell Bank (WCB). Nonetheless, quality attributes must also be designed into these types of banks even when a specific cell line has yet to be selected for long-term use. By initially preparing a bank of cultures that are derived under a limited degree of QC/QA control, the production and quality characteristics of several cell lines can be evaluated without producing the more rigorously tested, validated, and controlled MC/WC banks for every clone. Moreover, when a given cell line has finally been chosen for use in the industrial setting, such "pre-seed" banks ensure that the genealogy of a cell line can be tracked, as mandated by the FDA. These stock cultures also provide a foundation to further propagate the required MCB and WCB. In an effort to anticipate the future potential of each expression system, the BPDF has implemented a policy in which pre-seed stock banks are prepared for every cell line constructed by USAMRIID and subsequently transferred to our facility. Therefore, in this section of the report, a list of the "Army" pre-seed banks is provided as well as a description of the methods used to prepare, track, test, control, and maintain these banks.

As stated above, several pre-seed banks were established at the BPDF from expression systems received from USAMRIID. The following table cites these systems, the lot numbers assigned to the corresponding pre-seed banks, and the dates the banks were prepared.

When any of the above listed expression systems were received in-house, they were immediately routed into our "Culture Control and Traceability Program", which consists of documenting the date of the cultures' arrival, the number of vials received, any identifying information, the person(s) who handled/stored the culture(s), etc. The cultures were then stored

Expression System:	Pre-Seed Lot No.:	Date of Preparation:
Bot A (Hc) WRAIR	PA01-7876	7-12-99
Bot E (Hc) BG-2 Pet 24	PA01-9176	8-25-00
Bot A (Hc) D1-17/B12	PA01-14106	2-23-00
Bot A (Hc) FG-5/F3	PA01-25106	2-25-00
Bot F (Hc) D4	PA01 10660	3-29-00
Bot D (Hc) A5	PA01-1399	5-23-00
Bot D (Hc) C11	PA01-2899	5-28-00
Bot B WRAIR	PA01-2899	7-7-00
Bot E (Hc) C10	PA01-3399	7-8-00

in the vapor-phase of secured liquid nitrogen until the associated pre-seed banks were prepared. An inventory of the its contents and any activities associated with the dewar, i.e., monitoring the liquid nitrogen level, release/storage of a culture, etc., was recorded within a log book.

After the Certificate of Analysis accompanying an expression system was reviewed and approved, the process for actually deriving the pre-seed bank was initiated as follows. A loopful of cells obtained from the original stock culture was used to inoculate 10 mL of YPD, (yeast extract, peptone, dextrose). The inoculum was then placed within a shaker incubator set at a temperature of 30-32 °C and a rpm of 100-150 for 24 +/- 2 hours. Forty-five milliliters of fresh YPD media was inoculated with 5 mL of the cells grown in the first transfer stage. As determined by measuring the O.D.₆₀₀ of samples collected throughout this stage, the second transfer inoculum was allowed to grow to the late log phase by maintaining the temperature at 30-32 °C and the rpm at 100-150. The cells were subsequently harvested, spun down, and the resulting pellet was resuspended in fresh YPD media containing 15% glycerol.

From the bulk inoculum, twenty to thirty individual glycerol stock cultures were prepared by dispensing 1.0 mL aliquots into sterile 1.2-2.0 mL screw-topped vials. Each of these vials was gently agitated and the cells were allowed to equilibrate with the cryoperservative for at

least 15 min at room temperature. To prevent ice crystals from forming within the cells as a outcome of immediately cryostorage, the stock cultures were slowly frozen, by using a Nalgene Cryo 1 °C Freezing Containers and a -70 °C freezer for 4-5 hours. After the indicated time period had elapsed, the vials were finally stored in the vapor phase of liquid nitrogen.

Throughout the expansion process, routine quality-related procedures were conducted to ensure the control and integrity of the final stock cultures. Such measures included, but were not limited to:

- Evaluating the final inoculum for viability, cell morphology, colony morphology, non-host contaminants, and O.D,
- Assigning lot numbers to each pre-seed bank for traceability purposes,
- Propagating the culture in a controlled and monitored environment, with raw materials/media that are of high quality and traceable to Certificates of Analysis or other types of relevant documentation,
- Storing the pre-seed stocks and associated reserve cultures in a limited access locked dewar and in a -70 °C freezer, respectively,
- Documenting each step of this process, including the release of a stock to another department and tracking the culture thereafter.

6.0 DOCUMENTATION: Batch Records

Relevant documentation that describes the development and production of the BoNT-A(Hc) process was compiled at the BPDF. Such documents consisted of Master Batch Records, MBRs, Production Batch Records (PBRs), and related standard operating procedures (SOPs). As a result of the composition and use of these documents, several objectives were achieved, such as:

- The scale-up process(es) transferred from Covance for BoNT-F(Hc) is well-documented.
- cGMP and/or GLP documents are now available for transferring the various processes to another facility or for review by future and/or current regulatory auditing agents.

“Master Production Records” (MBRs) were the primary documents compiled for the pilot plant development of BoNT-F(Hc). In addition, batch records pertaining to Bot E derived from an E. coli-based system were also prepared and reviewed. However, these batch records were never used on the floor because the project proceeded in another direction.

Subsequent “Production Batch Records” (PBRs) for Bot F were then completed concurrently as qualified staff members performed a particular step in a process. During or after a given operation was conducted, the various processing steps and/or record-keeping items within the PBRs were reviewed for completeness and content by QA personnel and/or departmental supervisor(s). Finally, the QA Director reviewed the entire PBR and appropriate measures were then taken to approve the PBRs.

All original PBRs produced as an outcome of manufacturing, purifying, and/ or formulating/vialing BoNT-A(Hc) finished product are currently maintained within the QA unit at the BPDF. MPRs and/or PBRs are available from the following production/development runs of BoNT-F(Hc) pilot scale runs.

- Fermentation -- Lot #: AR-FPP-004.1, Lot # AR-FPP-004.2.
- Purification -- Lot #: AR-PPP-006, AR-PPP-007, AR-PPP-008.

7.0 Project Activities for Year 2000-2001

BoNTC₁ and E Hc processes will be transferred during this contract year.

7.1 Fermentation

During the next year the following fermentation research activities produce the following manuscripts.

- BotC paper:
Title "A Novel Strategy for Mut⁺ *Pichia pastoris* Fermentation Producing Heavy-Chain Fragment C of Botulinum Neurotoxin Serotype C: High Methanol Induction with Glycerol Co-Feeding". Will be submitted to "Biotechnology Letters" by the end of Oct 2000.

- Alpha-gal paper:
Title "Maximizing Production of Secreted Recombinant Proteins in *Pichia pastoris* Fed-Batch Fermentation". Will be submitted to "Biotechnology and Bioengineering" by the end of Nov 2000.

- BotF paper:
Title "Design of methanol feed controller and its application to *Pichia pastoris* fermentation producing heavy-chain fragment C of botulinum neurotoxin serotype F". Will be submitted to " Applied Microbiology and Biotechnology " by the end of Feb 2001.

- BotB paper:
Title "Induction optimization in *Pichia pastoris* fermentation producing heavy-chain fragment C of botulinum neurotoxin serotype B". Will be submitted to " Applied Microbiology and Biotechnology " by the end of May 2001.

7.2 Purification

During the next year the following purification manuscripts will be submitted on;

- BoNTF Hc paper by November 2000
- BoNTC₁ Hc paper by March 2001
- BoNTE Hc paper by July 2001