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OPTIMIZATION OF A SIMPLE METHOD FOR THE HIGH YIELD PRODUCTION OF RECOMBINANT BONT/B LIGHT CHAIN PROTEIN IN *E. coli* HETEROLOCUS HOST SYSTEM

SWATI JAIN, PONMARIAPPAN S*., RICHA SIJORIA, GOEL A.K., OM KUMAR AND LOKENDRA SINGH

Biotechnology Division, Defence Research and Development Establishment,Jhansi Road, Gwalior- 474 002, MP, India.
*Corresponding Author: Email- ponmariappans@yahoo.com

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Abstract- Botulism is a neuroparalytic disease caused by *Clostridium botulinum*, which produces seven (A-G) different neurotoxins (BoNTs) and the most poisonous substances known to humans. To develop a low cost detection as well as therapeutics, in the preset study synthetic gene of BoNT serotype B light chain (BoNT/B LC) was constructed, Cloned in pQE30UA and pET28a+ vectors and was expressed in *E. coli* heterolocus hosts. To further increase the yield of the rBoNT/B LC different substrates were selected based on the nutritional composition of different media and the recombinant protein expression was evaluated in three different host system *E. coli* M15, SG13009 and BL21DE3. The rBoNT/B LC was purified by NiNTA affinity chromatography and confirmed by western blot using monoclonal antibody for BoNT/B. The results revealed that, the maximum recombinant protein yield was observed in YM9 glucose media (6.4 mg/gm cell pellet) followed by SB (5.69mg), TB (5.5mg), YM9 glycerol (5.2mg), LB1x (4.9mg) and SOC (3.6mg) in M15 host system. Whereas in *E. coli* (SG13009) host system the maximum recombinant protein yield was noticed in SB (4.47mg/gm cell pellet) followed by YM9 glucose (3.3mg), TB (3.03mg), SOC (2.61mg), YM9 glycerol (2.5mg) and LB1x (1.91mg). In BL21DE3 host system maximum recombinant protein yield was observed in Super broth 5.3mg/gm cell pellet followed by LB1x 4.6 mg/gm cell pellet, SOB 4.1 mg/gm cell pellet, SOC 3.7 mg/gm cell pellet, YM9 glucose 3.1 mg/gm cell pellet and YM9 glycerol 2.4 mg/gm cell pellet. Overall M15 host system better yielded followed by BL21DE3 and SG13009, and the yield varies from media to media. The results clearly indicate that using a simple optimization process increased ----fold yield of rBoNT/B protein. This study will be highly useful for cost effective mass production of these agents which will further helpful for the development of detection system and therapeutics for botulism.
Key Words- Botulinum toxins, BoNTs, Recombinant protein, Media.

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Introduction

Botulinum neurotoxin (BoNTs) is produced by *Clostridium botulinum* which induces a potentially fatal paralytic condition known as botulism (31). BoNT has been considered the most poisonous substance known to human (6). Based on their antigenic specificity, BoNTs are distinguished into seven serotypes (A-G) with BoNT A, B, E and F accounting for main causes of human disease (32). Selective and irreversible inhibition of acetylcholine release at neuromuscular junction is the characteristic feature of botulism (21). BoNTs are released as a 150 kDa single polypeptide which is cleaved endogenously or exogenously resulting in a 100 kDa heavy chain (HC) and 50 kDa light chain (LC) linked through a disulphide bond (31). The C terminal half of HC binds to the nerve membrane and the N terminal half facilitates the transport of light chain across the membrane of endosome to cytoplasm. The LC possesses the zinc endopeptidase activity which cleaves the SNARE (soluble N-ethylmaleimide factor attachment protein receptor) proteins involved in docking and fusion of acetylcholine containing vesicles to the plasma membrane (19, 22). Inactivation of any SNARE protein results in inhibition of acetylcholine secretion at the neurotransmitter junctions resulting in flaccid muscle paralysis (19, 27). BoNT A, C, E cleaves SNAP-25 (2, 3, 35). BoNT C also cleaves syntaxin (4, 5) and BoNT B, D, G and F are specific for VAMP/synaptobrevin (23, 24, 26). Botulinum toxin is the first biological toxin to become licensed for treatment of human diseases. Since its introduction into clinical use in the 1980's BTX-A has been successfully used to treat various dystonia, muscle spasms and spasticity, axillary hyperhidrosis and achalasia (8,

12, 29, 13, 28). More recently the U.S. FDA approved a BTX-B complex preparation (Myobloc TM, Elan South San Francisco, CA, USA) for clinical use in cervical dystonia patients (36). After its molecular structure was determined an understanding of mechanism and factors affecting it are still in process to allow its use as miracle drug.

Due to its extreme toxicity BoNTs have been considered as potential biological weapons for over 60 years. As well as in food industry, in particular refrigerated processed food with extended durability may represent a severe food borne poisoning hazard due to heat treatment at a lower temperature and the anaerobic atmosphere provided by the packaging (20). So it is imperative that therapeutic approaches and vaccines to be developed to counteract the threat posed by these agents and any engineered versions of these agents that might be developed in the future.

Commercialization of all these products needs large amount of botulinum toxin. *C. botulinum* culture growth requires utmost biosafety precautions and obligate anaerobic conditions thus several protocols are developed to obtain recombinant protein in *E .coli* as the heterologous host by cloning various gene fragment of *C. botulinum*. *E. coli* is considered for the rapid growth and faster multiplication and also the process is amenable to inexpensive scale up.

In the present study synthetic BoNT/B LC gene fragment was cloned in pQE30 UA and pET vectors and transformed in to *E. coli* expression host strains M15, SG13009 and BL21DE3. To study the effect of different substrates on the recombinant protein production ten different media were chosen for the expression of rBoNT/B LC gene, and find out which media yield highest recombinant protein so it can be used for mass production to develop therapeutics and diagnostics agents. Better protein expression translates to lower production costs for an industry making a therapeutic protein or diagnostic kit.

Materials and Methods

Qiagen Miniprep kit, Gel extraction kit, Ni-NTA spin column kit, Ni-NTA resin, anti His antibody, pQE30 UA expression vector, *E. coli* expression host M15 and SG13009 were purchased from Qiagen GmbH, Hilden, Germany. pET28a+ expression vector from Novagen and *E. coli* BL21DE3 cells from Stratagene. Prestained marker, Taq polymerase, dNTPs, and PCR buffer were purchased from MB1 Fermentas, Ontario, Canada. Kanamycin, ampicillin, IPTG, aluminium hydroxide gel adjuvant, Deoxy ribonuclease I, Protease inhibitor cocktail, BCA protein assay kit and BSA were purchased from Sigma, U.S.A. Nitrocellulose membrane (0.45 µM) was purchased from Millipore, Bangalore, India. Luria bertani broth, Super broth, terrific broth, M9 minimal was purchased from Difco laboratories, UK. Trptone, peptone and yeast extract were from Himedia laboratories, Mumbai, India. Monoclonal antibody for BoNT/B was purchased from Biodesign internationals, USA.

Cloning of synthetic BoNT/B LC

The synthetic gene coding the catalytic domain (amino acids 1-450) of the botulinum neurotoxin serotype B (BoNT/B LC) was constructed using PCR primer overlapping method as described in our previous paper (11). The PCR product was cloned in pQE30 UA vector using QIA express UA cloning kit according to the manufacturer's protocol. The ligated products were transformed into chemically competent *E. coli* M15 and SG13009 cells by heat shock method. The transformants were selected on Luria bertani (LB) agar plates supplemented with kanamycin (30µg/ml) and ampicillin (100µg/ml). Similarly the PCR product was cloned in pET28a+ vector using Novagen cloning kit according to the manufacturer's protocol. The ligated products were transformed into chemically competent *E. coli* BL21DE3 cells by the heat shock method. The transformants were selected on Luria bertani (LB) agar plates supplemented with kanamycin (30 µg/ml). Plasmids were extracted from the all clones using QIA miniprep kit according to the manufacture's protocol. The plasmids were then screened for confirmation of inserts using BoNT/B specific primers mentioned above and in frame were also checked by using combination of BoNT/B and vector specific primers. The synthetic gene sequence was verified and confirmed by double pass sequencing.

Media optimization

The positive transformants of SG13009 and M15 hosts were inoculated into 30 ml Luria Bertani (LB) containing 30µg/ml kanamycin and 100µg/ml ampicillin and BL21DE3 transformants were inoculated in LB media containing 30µg/ml kanamycin and grown overnight at 37ºC with 200 rpm shaking. These overnight grown cultures were further inoculated to the final concentration of 2% in 100ml of Super Broth (SB), Terrific Broth (TB), LB1x, LB5x, LB5x Glucose, LB5x glycerol, YM9 Glucose, YM9 glycerol, SOB and SOC (containing respective antibiotics). The media compositions of ten different media are described in Table 1. The cultures were grown at 37ºC at 200 rpm. Growth was monitored by absorbance measurements of optical density at 600nm (OD_{600}) at 2nd, 3rd and 4th h with respect to blank of respective media without bacteria (innoculum).

Table 1. Chemical Composition of ten different media used in the study

S.No	Media	Composition (gm/l)
1	LB	Tryptone, 10.0 g; Yeast extract, 5.0 g; NaCl, 5.0 g
2	LB5x	Tryptone, 50.0 g; Yeast extract, 25.0 g; NaCl, 25.0 g
3	LB5x glucose	Tryptone, 50.0 g; Yeast extract, 25.0 g; NaCl, 25.0 g
4	LB 5x glycerol	Tryptone, 50.0 g; Yeast extract, 25.0 g; NaCl, 25.0 g
5	SB	Soy Hydrolysate, 12.0 g; Yeast Extract, 24.0 g; Di-potassium phosphate, 11.4 g; Monopotassium phosphate, 1.7 g; Glycerol, 5 ml
6	TB	Pancreatic digest of casein, 12.0 g; Yeast Extract, 24.0 g; Di-potassium phosphate, 9.4 g; Monopotassium phosphate, 2.2 g; Glycerol, 4 ml
7	YM9 Glucose	M9, 11.3g; Yeast extract, 10g; Glucose, 4g
8	YM9 Glycerol	M9, 11.3g; Yeast extract, 10g; Glycerol, 4ml
9	SOC	Tryptone, 20.0 g; Yeast extract, 5.0 g; NaCl, 10 ml (1M); Kcl, 2.5 ml (1M); $Mgso_4$, 10 ml (2M); Glucose, 10 ml (2M)
10	SOB	Tryptone, 20.0 g; Yeast extract, 5.0 g; Kcl, 2.5 ml (1M); $Mgcl_2$, 5 ml (1M)

Expression of recombinant BoNT/B LC in selected media

Among the ten different media, six media showed better growth in which the cells reached near to log phase of their growth within four hour of inoculation which is optimum for induction of recombinant protein production. These six media were selected for protein expression studies. The positive transformants of SG13009, M15 and BL21DE3 hosts were inoculated into 30 ml of six different media having higher absorbance values i.e. Super Broth (SB),

Terrific Broth (TB), LB1x, YM9 Glucose, SOB and SOC containing respective antibiotics and were grown overnight at 37ºC with shaking at 200 rpm. The overnight grown cultures were further inoculated to the final concentration of 2% in 100ml of respective media (containing antibiotics). The cultures were grown at 37ºC at 200 rpm. Growth was monitored by absorbance measurements at 600nm. Approximately at OD_{600}~ 0.8 cultures were induced with 0.5 mM isopropylthiogalactoside (IPTG) and further the growth is resumed for 4 h under the same conditions. Prior to IPTG induction, 2-ml aliquots were taken out aseptically and used as uninduced control. The cells were harvested by centrifugation at 8000 x g for 10min at 4ºC. After 4h of induction, 2ml samples were drawn and cells were harvested by centrifugation at 8000 x g for 10min at 4ºC. All these samples were analyzed by 12% SDS-PAGE along with molecular weight marker (Fermentas SM0671).

Purification of His_6 tagged recombinant BoNT/B LC

The recombinant protein was purified under denatured conditions using affinity chromatography. Induced culture pellet (2 ml) at 4th h of induction of each selected media from both the hosts were resuspended separately in solubilizing/ equilibration buffer (8M urea, 0.1M NaH_2PO_4 and 0.01M Tris-Cl, pH 8.0) and the cell suspension was sonicated at 9.9 pulses on/off for 30 sec and centrifuged at 12000 x g for 20 min at 4ºC. The supernatant contains the solubilized inclusion bodies. Purification was carried out by Ni-NTA spin column as per manufacture's protocol with wash buffer (8M urea, 0.1M NaH_2PO_4 and 0.01M Tris-Cl, pH 6.3) and elution buffer (8M urea, 0.1M NaH_2PO_4 and 0.01M Tris-Cl pH, 4.5).

Estimation of recombinant BoNT/B LC yield

The protein concentration was determined with reference to standard BSA using the BCA protein assay kit in triplicates. Recombinant BoNT/B LC protein yield for each selected media was compared in both the host systems.

Characterization of rBoNT/B LC : Western Blot Analysis and ELISA

The purified recombinant BoNT/B LC was separated in SDS PAGE and then transferred on to a nitrocellulose membrane and blocked with 3% BSA overnight at 4ºC. After decanting, the blocking buffer, one strip of the membrane was incubated in monoclonal BoNT/B antibody (1:1000) in blocking buffer with gentle shaking at RT for 1 h to confirm the peptide encoded by the synthetic gene of the 450 amino acids of rBoNT/B. Another strip of the membrane was incubated with an 'anti His' monoclonal antibody (1:2000) to confirm the presence of the His tag at the N terminal and C terminal of rBoNT/B LC provided by vector pET28a+. After incubation these strips were washed three times with PBST (PBS containing 0.5% Tween20) of 5 min each. These strips were again incubated in rabbit anti-mouse IgG horse radish peroxidase (HRP) conjugate (1:2000) as a secondary antibody in a blocking buffer with gentle shaking at RT for 1 h. Colorimetric detection was carried out by using 3,3'- diaminobenzidine in PBS containing 8.8 mM H_2O_2 as a substrate.

Functionality of the recombinant BoNT/B LC protein was tested by an indirect enzyme linked immunoassay (ELISA) using protein specific antibodies raised against rBoNT/B LC as described earlier (11). For indirect ELISA, the purified recombinant BoNT/B LC was diluted to 5 µg/ml in a carbonate buffer (0.05 M, pH 9.6) and used to coat the wells of polystyrene plates (100 µl/well; Nunc-Immuno plate with Maxisorp surface). The plates were incubated overnight at 4ºC and the next morning the plates were washed three times with PBST. The remaining sites of absorption were blocked by the addition of 200 µl/well 3% BSA (made in PBS) for 2 h at 37ºC. The plates were washed thrice with PBST (PBS/0.05% Tween-20). Two fold serial dilution of rabbit serum containing rBoNT/B LC antibodies was prepared (1:1000 to 1:4,096,000) and examined in triplicate wells (100 µl/well) of the blocked antigen coated plates and incubated for 1 h at 37ºC. Pre-immunized serum of rabbit was used as the negative control. The plates were then washed five times with PBST and further incubated at 37ºC for 1 h with polyclonal Goat Anti-rabbit HRP (1:2000). The plate was washed five times with PBST and developed with 100 µl ortho-phenylenediamine (0.4 mg/ml) in a freshly prepared citrate phosphate buffer (0.1 M, pH 5.0) and H_2O_2 (0.4 µl/ml). The reaction was terminated by the addition of 50 µl of 2.5 N H_2SO_4/well. Absorbance was read at 492 nm with a microtiter plate reader. The cut-off value for assay was calculated as the mean specific optical density, plus three times the standard deviation (SD), for pre-immune serum assayed at a dilution of 1:1000. The titer of immune serum was calculated as the reciprocal of the highest serum dilution yielding a specific optical density above the cut-off value.

Results

Construction of synthetic BoNT/BLC gene fragment and its expression

Primers were designed to construct the catalytic domain of botulinum neurotoxin gene type B (BoNT/B LC) and synthesized commercially. Using these primers the synthetic gene of 1420bp was constructed using polymerase chain reaction (PCR). From this the catalytic domain of 1350bp was PCR amplified. The constructed synthetic gene fragment was sequenced and confirmed. The synthetic gene sequence was submitted in the Genbank Accession number HQ116625. After confirmation the synthetic construct was purified using agarose gel electrophoresis and the purified PCR products were cloned in pQE30 UA vector. The prelinearized pQE30 UA expression vector provide U overhang on each 3'end which allows the direct insertion of PCR products amplified by Taq DNA polymerase which add 3' A overhang to PCR products. The ligated product was transformed in *E. coli* M15 and *E. coli* SG13009. pQE30 UA vector provides ampicillin resistance and both hosts harbor pREP4 plasmid which provides kanamycin resistance. Positive transformants were selected on ampicillin and kanamycin supplemented plates. Transformants with inframe insert were screened by PCR and selected for protein expression studies. Similarly the PCR product was cloned in to pET28a+ vector. The ligated product was transformed in *E. coli* BL21DE3 transformants the presence of insert was checked by release of insert from the recombinant plasmid upon restriction digestion with NdeI and XhoI in *E. coli* BL21DE3. Both these vectors provides a 6X his tag at the N / C terminus which aids in purification of expressed recombinant protein by using metal affinity chromatography.

Expression of His_6 tagged recombinant BoNT/B LC in selected media

Growth was monitored by the absorbance measurement at 600nm at every one hour and showed different growth pattern in different media. In the case of *E. coli* M15 system the maximum OD was observed in YM9 glucose (3.44) at 4th hour of post inoculation followed by SB (3.3), TB (3.07), YM9 glycerol (2.56), SOC (1.89), LB1x (1.67), LB glucose (1.46), LB glycerol (1.07) LB5x (0.95) and SOB (0.70) [Figure1]. Whereas in *E. coli* (SG13009) transformant the maximum OD was noticed in YM9 glucose (1.73) followed by SB (1.58), SOC (1.39), TB (0.95), YM9 glycerol (0.42), LB1x (0.35), SOB (0.33) LB glucose (0.32), LB5x (0.26) and LB glycerol (0.25) [Figure2]. After 4 hrs of postinduction in *E. coli* BL21DE3 transformant the maximum O.D was noticed in noticed in SOC (1.28) followed by LB (1x) (1.13), SB (1.08), YM9 glycerol (1.08), YM9 glucose (0.91), SOB (0.83), LB glucose (0.72), TB (0.62), LB glycerol (0.61) and LB (5x) (0.39) The growth pattern of E. *coli* Bl21DE3 transformant in ten different media are shown in Fig 3. All the three host systems media with higher OD i.e. better biomass were selected for protein expression. Six media SB, TB YM9 glucose, SOC, YM9 glycerol and LB1x supports better growth than other media. Although O.D rises in *E. coli* (SG13009) transformant was much lower in comparison to *E. coli* (M15) transformant indicating host physiology play a vital role on growth. Amount of protein production was checked by inducing protein expression with 0.5mM IPTG when cells were in their log phase in respective media which supported better growth. After 4hr of induction, cells were harvested and the wet and dry weight was calculated for the respective media of both M15, SG 13009 and BL21DE3 host cells and the results are summarized in Table 2a - 2c. In the case of M15 host system the highest total protein yield was observed in YM9 Glucose 66.88 mg/gm of cell pellet followed by super broth 51.68 mg, Terrific Broth 50.19 mg, YM9 Glycerol 50.18 mg, SOC 49.91mg and LB(1X) 44.54mg/gm of cell pellet. Where as in SG13009 host system the highest total protein yield was observed in super broth 42.04 mg/gm of cell pellet followed by YM9 Glucose 44.54mg, SOC 40.79 mg, Terrific Broth 40.14mg, YM9 Glycerol 39.48mg and LB(1X) 37.50mg/gm of cell pellet. In BL21DE3 host system the highest total protein yield was observed in SOC 41.27 mg/gm of cell pellet followed by LB (1x) 40.79 mg, Super broth 40.22 mg, YM9 Glycerol 39.84 mg, YM9 Glucose 37.76 mg and SOB 35.5 mg/gm of cell pellet.

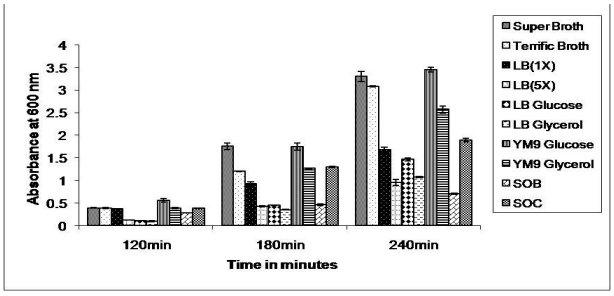

Fig. 1-Growth of *E. coli* M15 BoNT/B transformant at different time interval in various media.

Graph represents the variation in growth pattern in different media. Mean optical density of E. coli M15 transformant in ten different media at 120, 180 and 240 minutes after inoculation with respect to their respective media without inoculum were compared. The error bars indicates the standard deviation of triplicate values. Where not visible, the error bars are within the symbol.

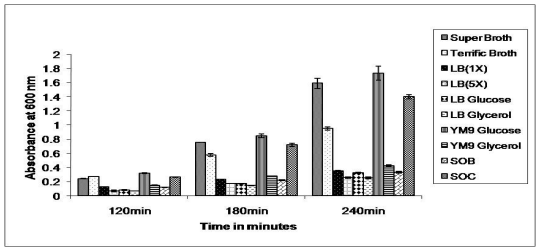

Fig. 2- Growth of *E. coli* SG13009 transformant at different time interval in various media.

Graph represents the variation in growth pattern in different media. Mean optical density of E. coli SG13009 transformant in ten different media at 120, 180 and 240 minutes after inoculation with respect to their respective media without inoculum were compared. The error bars indicates the standard deviation of triplicate values. Where not visible, the error bars are within the symbol.

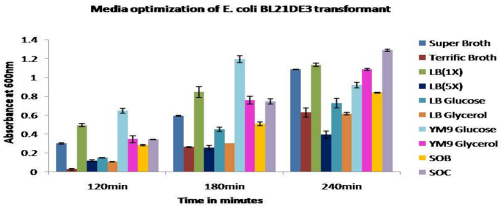

Fig. 3- Growth pattern of *E. coli* BL21DE3 transformant at different time interval using ten different media.

The error bars indicates the standard deviation of triplicate values. The error bars not visible are within the symbol.

Table 2a- Growth, biomass and the yield of recombinant protein in different media using E. coli M15 host systemPurification and yield of recombinant BoNT/B LC in different host syste

S. No	Media	Absorbance at 600nm	Wet weight (gm)	Dry weight (gm)	Total protein Yield (mg/gm cell pellet)	Purified recombinant protein yield (mg/gm cell pellet)
1	Super Broth	3.3	0.0624	0.00744	51.68	5.69
2	Terrific Broth	3.078333	0.0513	0.00722	50.19	5.5
3	LB(1X)	1.673334	0.0339	0.00424	44.54	4.9
4	YM9 Glucose	3.449667	0.0648	0.0077	66.88	6.4
5	YM9 Glycerol	2.568333	0.0510	0.00708	50.18	5.2
6	SOC	1.893333	0.0361	0.00472	49.91	3.6

Table 2b- Growth, biomass and the yield of recombinant protein in different media using E. coli SG 13009 host system

S. No	Media	Absorbance at 600nm	Wet weight (gm)	Dry weight (gm)	Total protein Yield (mg/gm cell pellet)	Purified recombinant protein yield (mg/gm cell pellet)
1	Super Broth	1.588	0.0312	0.00492	42.045	4.47
2	Terrific Broth	0.948333	0.027	0.00429	40.14	3.03
3	LB(1X)	0.350667	0.0199	0.00177	37.506	1.91
4	YM9 Glucose	1.729	0.0392	0.00594	44.54	3.3
5	YM9 Glycerol	0.423	0.0255	0.00186	39.48	2.5
6	SOC	1.893333	0.0295	0.00456	40.79	2.61

Table 2c- Growth, biomass and the yield of recombinant protein in different media using E. coli BL21DE3 host system

S. No	Media	Absorbance at 600nm	Wet weight (gm)	Dry weight (gm)	Total protein Yield (mg/gm cell pellet)	Purified recombinant protein yield (mg/gm cell pellet)
1	Super Broth	1.084	0.0289	0.00445	40.22	5.3
2	LB (1x)	1.131667	0.0312	0.00517	40.79	4.6
3	YM9 Glucose	0.918833	0.0198	0.00398	37.76	3.1
4	YM9 Glycerol	1.083667	0.0267	0.00439	39.84	2.4
5	SOB	0.839	0.0175	0.00345	35.51	4.1
6	SOC	1.287	0.0332	0.00543	41.27	3.7

pQE30UA vector provides his tag on the N terminal of the recombinant protein and it was used to purify the protein by Ni-NTA affinity chromatography. Purified protein was checked by SDS PAGE along with molecular marker. The recombinant protein purified in different media gave positive result when reconfirmed by western blot using BoNT/B specific monoclonal antibody. Figure 4a and 4b represents the SDS-PAGE of purified recombinant protein in selected media of *E. coli* M15 transformant and *E. coli* SG transformant respectively.

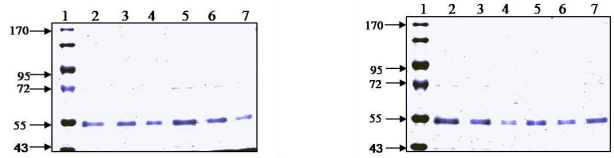

Fig. 4- SDS –PAGE profile of Purified recombinant BoNT/B LC protein in *E. coli* M15 and *E. coli* SG13009 transformant.

In Fig 3a and 3b the Lane 1 depicts protein molecular mass marker, Lane 2, 3, 4, 5, 6, 7 depicts the Coomassie-stained purified recombinant BoNT/B LC protein in SB, TB, LB, YM9 glucose, YM9 glycerol, and SOC media in *E. coli* M15 transformant and *E. coli* SG13009 transformant respectively. The molecular masses of the protein standards are indicated on the left in kilodaltons.

Figure 5a and 5b represents the western blot with BoNT/B specific antibody in selected media of *E. coli* M15 transformant and *E. coli* SG transformant respectively.

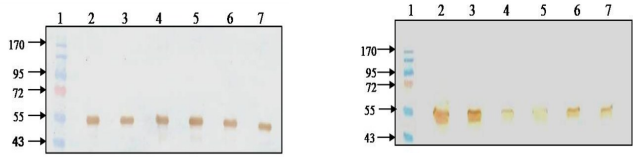

Fig. 5- Western blot of recombinant BoNT/B LC protein in *E. coli* M15 and *E. coli* SG13009 transformant with BoNT/B specific monoclonal antibody.

In Fig 4a and 4b the Lane 1 depicts protein molecular mass marker, Lane 2, 3, 4, 5, 6, 7 depicts the band of purified recombinant BoNT/B LC protein in SB, YM9 glucose, LB, YM9 glycerol, TB, and SOC media in *E. coli* M15 transformant and *E. coli* SG13009 transformant respectively . The molecular masses of the protein standards are indicated on the left in kilodaltons.

In the case of BL21DE3 system the SDS page and western blot are shown in Fig 6a & 6b. The BCA estimation showed the marked difference between the levels of recombinant BoNT/B LC protein expression in different media. *E. coli* M15 host system maximum recombinant protein yield was observed in YM9 glucose media (6.4 mg/gm cell pellet) followed by SB (5.69mg/gm cell pellet), TB (5.5mg/gm cell pellet), YM9 glycerol (5.2mg/gm cell pellet), LB1x (4.9mg/gm cell pellet) and SOC (3.6mg/gm cell pellet). Whereas in *E. coli* (SG13009) host system maximum recombinant protein yield was noticed in SB (4.47mg/gm cell pellet) followed by YM9 glucose (3.3mg/gm cell pellet), TB (3.03), SOC (2.61mg/gm cell pellet), YM9 glycerol (2.5mg/gm cell pellet) and LB1x (1.91mg/gm cell pellet). In BL21DE3 host system maximum recombinant protein yield was observed in Super broth 5.3mg/gm cell pellet followed by LB1x 4.6 mg/gm cell pellet, SOB 4.1 mg/gm cell pellet, SOC 3.7 mg/gm cell pellet, YM9 glucose 3.1 mg/gm cell pellet and YM9 glycerol 2.4 mg/gm cell pellet.

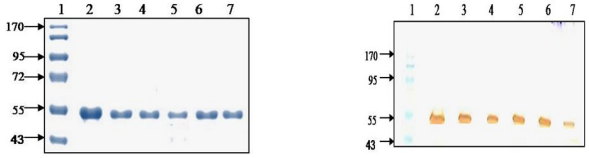

Fig. 6- Purified rBoNT/B LC proteins in six different media in *E. coli* BL21DE3. (a) SDS –PAGE profile (b) Western blot.

Lane 1:MWM, Lane 2, 3, 4, 5, 6, 7: depicts the purified recombinant BoNT/B LC protein in SB, TB, LB, YM9 glucose, YM9 glycerol, and SOC media in *E. coli* BL21DE3 transformant.

The purified recombinant BoNT/B LC protein yield among six different media in *E. coli* M15, SG13009 and BL21DE3 transformant are shown in Fig 7-9respectively. The reactivity of the purified rBoNT/B LC protein was characterized by western blot analysis using protein specific antibody raised in rabbits as described before (10). The antibody specific to rBoNT/B LC bound with the rBoNT/B LC protein in the blot and gave a single band at 55 kDa whereas the negative antibody did not react (result not shown). These results demonstrate that the protein expressed by batch fermentation was reactive to anti- rBoNT/B LC antibody.

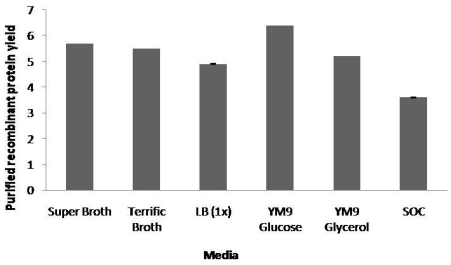

Fig. 7- Purified recombinant protein yield in *E. coli* M15 transformant in selected media.

Six media were selected for recombinant BoNT/B LC protein expression in *E. coli* M15 . Graph represents the purified recombinant protein yield in the selected media. The error bars indicates the standard deviation of triplicate values. Where not visible, the error bars are within the symbol.

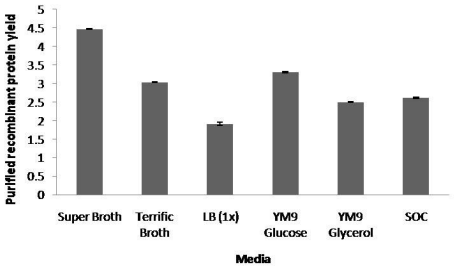

Fig. 8- Purified recombinant protein yield in *E. coli* SG13009 transformant in selected media.

Six media were selected for recombinant BoNT/B LC protein expression in *E. coli* SG13009. Graph represents the purified recombinant protein yield in the selected media. The error bars indicates the standard deviation of triplicate values. Where not visible, the error bars are within the symbol.

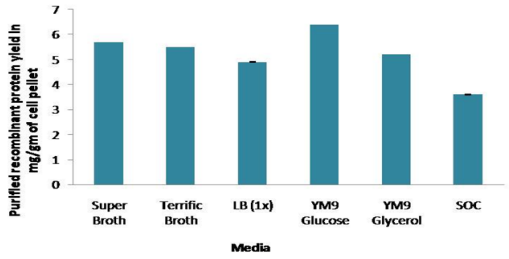

Fig. 9- Purified recombinant BoNT/B LC protein yield among six different media in *E. coli* BL21DE3 transformant.
The error bars indicates the standard deviation of triplicate values and the error bars not visible are within the symbol.

ELISA

Reactivity of this protein with specific antibody was also tested by indirect ELISA for evaluation of its diagnostic potential. For it, the antibodies against rBoNT/B LC protein were produced in rabbit as explained earlier(10). The rabbit was immunized with rBoNT/B LC protein. After 28th day of immunization blood was collected and the sera was separated and used for evaluating the immune response through indirect ELISA, taking preimmune sera as a negative control. The IgG antibody titer in mice was 512,000 with alum as adjuvant via intramuscular route and the results are depicted in Fig. 10 clearly demonstrate the reactivity of this protein. These results suggest that the rBoNT/B LC protein could indeed be used to detect the presence of Botulinum toxin in foods and hence, the optimization of high yield production of rBoNT/B LC may prove to be very beneficial. Being one of the major causes of food borne infections, detection of botulinum toxin in the sample is necessary. As BoNT/B is an attractive candidate for its application as a diagnostic reagent, its large scale production and purification is the need of time. Optimization of the cultivation medium and batch fermentation'strategy, as described in this report, should make this application more feasible. Also, we had shown previously that the antibodies produced against rBoNT/B LC are able to neutralize botulinum toxicity resulted by *C.botulinum* type in PC12 cells. Thus, optimization of rBoNT/B LC may also prove to be important and promising candidate for the bulk production.

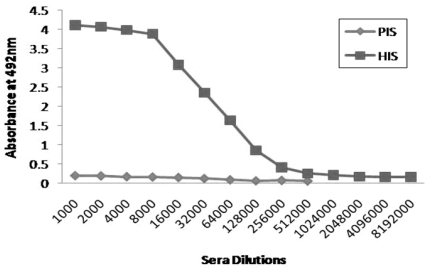

Fig. 10- Evaluation of immune response against BoNT/B LC protein in rabbit by indirect ELISA.
The graph represents the end point titer value of antibody. Where not visible, the error bars not visible are within the symbol.

Discussion

Botulinum toxins (BoNTs) with a lethal dose of 1 ng/kg pose a biological hazard to humans and a serious potential bio-weapon threat (1). The botulinum neurotoxins can be aerosolized and used directly as biological weapon. Concern about the potential biological warfare use of toxin has strengthened the need for rapid diagnosis and novel vaccination strategies. Also the list of conditions treated by BoNT has expanded to include both the dystonic and nondystonic involuntary movements, including excessive muscle contractions accompanying stroke, demyelinating disease, tremor, and certain cosmetic conditions (33). Clostridial proteins are the powerful tools for studying the mechanisms of synaptic vesicle exocytosis (4, 25). All these studies require ample amount of botulinum toxin. However one of the challenges in working with potently toxic proteins is to prepare variants that harness the biological function of the toxin whilst reducing the toxic effect to a minimum. To successfully achieve the solution coding sequence of variant are expressed in a nontoxic expressing host (9, 18).

E. coli is generally safe organism and has proved to be an economically viable means for producing protein products. Nevertheless, not all the proteins are accumulated to maximal levels in *E. coli* and production typically requires optimization. Hence the present study was designed for the optimization of the effect of different nutrient substrates and their recombinant protein yield. Many attempts to express fragments of clostridial neurotoxins in *E. coli* have failed because of unusually high AT content of Clostridial DNA. Leif Isaksson from Stockholm University explained the importance of codon optimisation when expressing heterologous proteins in *E.coli*, which can generate up to a 10-fold improvement in yield (30). De novo synthesis of optimized sequences for codon usage in *E. coli* has been already demonstrated for Clostridial species (17). Besides this to increase the translation efficiency of recombinant protein some rare tRNAs have been amplified in *E. coli*(38). These approaches are though laborious and expensive. With many attractive features of the *E. coli* gene expression system (34). The existing bioprocess for recombinant protein production is dominated by the use of this workhouse. Due to multiplicity of physiological impact arising from the high level gene expression and high cell density cultivation, the two production goals can hardly be achieved simultaneously. In this study a simpler approach which will significantly improve production of Clostridial neurotoxin serotype B light chain in *E. coli* has been tried. In BL21 DE3 system the highest recombinant protein yield was observed Super broth 5.3mg/gm cell pellet followed by LB1x 4.6 mg/gm cell pellet, SOB 4.1 mg/gm cell pellet, SOC 3.7 mg/gm cell pellet, YM9 glucose 3.1 mg/gm cell pellet and YM9 glycerol 2.4 mg/gm cell pellet. The present study results corroborated with the observation of Jan-Wilem de Gier, he did the genomic comparison of two workhorse strains of *E. coli*, K- 12 and BL21, revealed that they share 3978 genes in common. Combined transcriptomic and proteomic studies revealed that expression of 228 genes responded to the stress imposed by recombinant protein production. Increased recombinant protein expression in *E. coli* is known to induce the general stress response. Expression of membrane proteins is often an additional challenge due to their toxicity to the cell. Jan-Wilem de Gier from Centre for Biomembrane Research, Sweden reported the development of a BL21(DE3) host suitable for membrane protein expression that emerged from understanding of how membrane protein toxicity is overcome in Walker strains (C41 and C43) (30).

Though the expression of BoNT/B gene fragment have been reported (7, 39). But there have been no previous reports available

for optimization of culture media which facilitate expression of recombinant BoNT/B gene fragment for industrial applications. The present study results revealed that a significant increase in the yield of recombinant protein than previously reported results. Development of appropriate culture media is always favourable for industry as it is cost effective.

Transformants of two different expression host system of *E. coli* SG13009 and M15 were cultured in ten different media to find their optimal growth kinetics. In media which supports the good biomass were selected and further used for the expression and purification of recombinant BoNT/B LC. Few comparative studies had been carried out for the effect of nutrients and host cells for the recombinant protein production on *E. coli* BL21, BLR (DE3) and K12. These stains were optimized for pET series of vectors but for pQE UA vectors the recommended hosts are *E. coli* M15 and SG13009. So far there is no similar kind of studies were reported in *E. coli* M15 and SG13009. In *E. coli* M15 host system YM9 glucose, SB and TB had almost same O.D. after 240 min of inoculation but the total protein and recombinant protein yield was vary from media to media. But in the case of *E. coli* SG13009 host system there was a slight difference in pattern of growth and recombinant protein production as O.D was maximum in YM9 glucose but recombinant protein yield was maximum in SB. Surprisingly LB media which is generally used for expression of recombinant protein in *E. coli* and yielded a lower cell density and recombinant protein. The poor performance of LB compared to complex media may be due to lower amounts of readily available carbon and nitrogen (37, 16). In our study super broth, terrific broth and YM9 glucose supports good biomass. The high cell densities attained with complex medium are due to the medium being rich in tryptone, yeast extract, and phosphate salts. Yeast extract is a known source of trace components and can relieve cellular stress responses such as the production of proteases during synthesis of recombinant protein (15). Similarly, a high concentration of phosphate is known to be important for attaining high cell densities of *E. coli*, as phosphate can easily become a limiting nutrient when provided in low doses (14). Also phosphate salts provide a buffering capacity against pH fluctuations that could adversely affect normal metabolic activity.

Industrial scale protein production requires high cell density cultivation but the development of process intensification on a laboratory scale often provides unrealistic models of industrial scale production. Peter Neubauer from University of Oulu, Finland described a scaled-down fed-batch technology, EnBase, that can be realised in a variety of formats (microplates, shake flasks) and can be a useful tool for high-throughput screening. In this strategy, the nutrient availability and hence the growth rate are controlled by slow release of glucose from starch via a unique regulating gel, by action of glucoamylase. In *E. coli* and B. *Subtilis* expression systems, the EnBase technology produced up to 50 times more bacteria and up to 10 times more recombinant protein and more soluble protein than conventional microplate or shake flask cultures. Silvana Becerra from University of Valparaiso, Chile added to this by manipulating the environmental culture conditions (medium composition and cultivation temperature) during the proliferative stage of CHO cells cultivation, the yields of recombinant human tissue-type plasminogen activator in the second, productive, phase can be significantly increased. The proposed method of biphasic perfusion culture uses a slowly metabolised substrate, galactose, instead of glucose and decreased growth temperatures (30).

The growth, total biomass and the recombinant protein yield of both the host system M15 and SG 13009 suggested that the M15 host system is better suited for the expression of BoNT/B LC. In super broth medium the growth of M15 host cells are 2.2 fold higher than SG13009, the increased OD leads to 1.2 fold increase in the total protein which yield 1.3 fold increases in the recombinant protein when compared with SG13009. Similar trends were observed in all other media. It clearly indicated that host physiology also play a crucial role in the expression of recombinant protein yield. Jeff Cole from University of Birmingham, UK has stated that slow rates of recombinant protein accumulation combined with lower growth rate result in much better yields of soluble and total protein as well as cell biomass in *E. coli* BL21 (DE3) batch cultivations (30). But in the case of *E. coli* M15 and SG13009 host system we observed the reverse trend to BL21 (DE3).

The two high level expression systems selected *E. coli* M15 and SG13009 are promising for use in scale-up production. Both strains permits high-level expression and are easily handled. M15 and SG13009; both harbor the pREP4 plasmid thus expression is regulated tightly. The host systems varied in results in terms of media supporting better yield. Certain strains tolerate some protein better thus the yield of recombinant BoNT/B LC in *E. coli* M15 was 6.4mg/gm of cell pellet in YM9 glucose which was approximately 1mg/ml higher in comparison to *E. coli* SG13009 host system 4.47mg/gm of cell pellet in SB.

Developments of vaccines, therapeutic and diagnostic kits are the key features to fight against biothreat agents. These strategies are based on the production of neutralizing antibodies to passively inactivate the toxins in-vivo, or on the understanding of the mechanism by which these toxins enter cells, structural information on the toxin components and on the mechanisms of action of toxin enzymes. Availability of recombinant BoNT/B LC protein in large amount will facilitates studies, also the pure and concentrated immunogens may increase immunogenicity and protection efficacy. These data are of great interest as wider opportunities are available for Clostridial and other recombinant genes as simple experiment of media optimization will facilitate in their industrial scale up.

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