Biotechnology and Bioprocess Engineering 21: 537-543 (2016)
DOI 10.1007/s12257-016-0360-9

RESEARCH PAPER

Optimization of Diphtheria Toxin Production by *Corynebacterium diphtheriae* Using a Casein-based Medium in a Fermenter

Yong-Ju Chung, Jin-A Lee, Mi-Young Jung, Sang-Mi Lee, Tae-Yeon Kim, Yong-Kyung Choe, and Ik-Hwan Kim

Received: 31 May 2016 / Revised: 26 June 2016 / Accepted: 28 June 2016

Abstract The Td-based combined vaccine contains only small amounts of the diphtheria toxoid antigen. However, a high level of purity is necessary for this antigen. The diphtheria toxin is produced by growing *Corynebacterium diphtheriae* in a semisynthetic, casein-based medium in a fermenter. In order to obtain a highly pure diphtheria toxoid, the optimal conditions to express the toxin at 300 Lf/mL in a fermenter culture were determined. When *C. diphtheriae* was cultivated in a fermenter and a high concentration of toxin was obtained, specific patterns for the pH and dissolved oxygen levels identified. Overall, the fermenter cultivation process was divided into four stages according to variations in the pH. A specific range of K_La in the fermenter (0.0092 ~ 0.0093/sec) was required to produce high level expression of diphtheria toxin. The amount of toxin expression varied significantly according to culture conditions. Agitation and aeration in the fermenter affected toxin expression, even when the optimal K_La value for toxin production was maintained. A previous study has reported that the amounts of agitation and aeration are important factors when cultivating fungus in the fermenter to produce chitinolytic enzyme. A mass production of diphtheria toxoid with a purity level greater than 2,500 Lf/mgPN was obtained through purification and detoxification from this optimized toxin production.

Yong-Ju Chung, Jin-A Lee, Mi-Young Jung, Sang-Mi Lee, Tae-Yeon Kim
Boryung Biopharma Co., Ltd., Seoul, Korea

Yong-Kyung Choe
Korea Research Institute of Bioscience and Biotechnology, Daejeon, Korea

Yong-Ju Chung, Ik-Hwan Kim*
Department of Biotechnology, College of Life Sciences and Biotechnology, Korea University, Seoul, Korea
Tel: +82-2-3290-3447; Fax: +82-2-927-9028
E-mail: ihkkim@korea.ac.kr

Keywords: casein hydrolysate, diphtheria toxin, fermenter, semisynthetic media, Td vaccine

1. Introduction

Diphtheria is a localized disease of the respiratory tract and skin, caused by *Corynebacterium diphtheriae*, an aerobic Gram-positive bacterium. To prevent the disease, diphtheria vaccines have been used for decades, either alone or in combination with tetanus and pertussis vaccines (*e.g.,* the DTP, DTaP, and Tdap vaccines). Diphtheria toxoid application has expanded to include the use of diphtheria antigen in the Td vaccine for adults and as a carrier protein for polysaccharide conjugate vaccines [1]. Although the use of monovalent and combined diphtheria vaccines has significantly reduced the occurrence of diphtheria, the disease has reemerged in post-Soviet Union countries, causing more than 4,000 deaths [2].

C. diphtheriae Park-Williams No. 8 or its mutant strain has been used to obtain the diphtheria toxin for vaccination. The toxin is detoxified into a toxoid by chemical modification [3]. Surface static culture, a simple method developed by Mueller and Miller in 1941, can produce approximately 100 Lf/mL of toxin. The method involves placing a liquid synthetic medium containing acid hydrolysate casein in a Roux bottle and growing the bacterial cells on the liquid surface [4]. However, this method is labor intensive and requires a large production space, making it inappropriate for producing a large amount of highly pure toxin. One method to resolve these issues was a shaking culture technique using a medium that included enzyme hydrolyzed casein. Another technique enabled the production of 200 Lf/mL of toxin within 48 h by supplying air to the surface of an agitation tank, using a medium of beef digested with papain.

High level expression of toxin was achieved by culturing the bacteria in a medium containing inorganic phosphate, which is absent in casein, and introducing calcium chloride to induce calcium-phosphate precipitates in the medium [5].

Purifying the diphtheria toxin prior to detoxification yields toxoids with a higher purity level than that obtained by purification after detoxification. This also reduces vaccine side effects from medium-derived materials [6]. To ensure safety of the diphtheria vaccine for adults, the toxoids used as antigens must be of higher purity than those used for children. Diphtheria toxoid of high purity can safely be given to adults for basic immunization [7]. To produce highly pure diphtheria toxoids, the toxin expression level must be increased to improve the purification yield.

In this study, the fermenter culture conditions were optimized using casein-derived N-Z-Case for the diphtheria toxin production [8]. The toxin was produced at a high concentration when agitation and aeration were optimized while employing a specific $K_L a$ value for toxin expression in the fermenter. Toxin production was restricted when these conditions were not met. The optimal conditions for a four-stage process to produce a highly expressed diphtheria toxin in a fermenter are proposed.

2. Materials and Methods

2.1. Bacterial strain

The *C. diphtheriae* Park-Williams No. 8 strain was used for this study. The strain was maintained as a freeze-dried culture. A seed lot was prepared for use in the production of diphtheria toxin and kept frozen (-80°C). The master seed lot was produced by performing five rounds of subculturing from freeze-dried *C. diphtheriae*. The master seed lot underwent four subsequent rounds of subculturing to produce the working seed lot.

2.2. Preparation of N-Z-Case plus solution

A total of 500 g of N-Z-Case Plus powder (Kerry Bio-Science/Sheffield Pharma Ingr., Beloit, WI, USA) was dissolved in 4.2 L of distilled water. Subsequently, 19.5 g of anhydrous Na_2HPO_4 and 6.5 g of anhydrous KH_2PO_4 were added, and the pH was adjusted to 9.3 with 10 M NaOH. The mixture was heated to 79°C, and 20.46 g of anhydrous $CaCl_2$ was added. The mixture was cooled and stored in a 4°C refrigerator overnight. The final volume was adjusted to 4.2 L with distilled water, centrifuged, and the supernatant was stored in a 4°C refrigerator.

2.3. Preparation of modified Mueller's growth factor solution

Mueller replaced the original components of *C. diphtheriae* medium containing liver or meat extract with pimelic acid, nicotinic acid, and β-alanine [9]. The Mueller's modified growth factor solution contained the following: 1.15 g/L β-alanine, 1.15 g/L nicotinic acid, 0.075 g/L pimelic acid, 0.5 g/L $CuSO_4 \cdot 5H_2O$, 0.4 g/L $ZnSO_4 \cdot 7H_2O$, 0.15 g/L $MnCl_2 \cdot 4H_2O$, and 225 g/L $MgSO_4 \cdot 7H_2O$.

2.4. Media for cultivation

Wadsworth medium was prepared as previously described [10]. The semisynthetic broth for growing *C. diphtheriae* contained the following: 333 mL/L N-Z-Case Plus solution, 50 mL/L maltose (50% w/v), 5.66 mL/L sodium lactate (60% w/v), 12 mL/L Mueller's growth factor solution, 2 mL/L L-cystine (20% w/v), 1 mL/L $FeSO_4 \cdot 7H_2O$ (0.1% w/v), 2.88 mL/L of a mixture containing K_2HPO_4 (25.5% w/v) and KH_2PO_4 (8.5% w/v), and 6.83 mL/L $CaCl_2 \cdot 2H_2O$ (40% w/v). The N-Z-Case Plus solution, sodium lactate, Mueller's growth factor solution, L-cystine, $FeSO_4 \cdot 7H_2O$, phosphate mixture, and $CaCl_2 \cdot 2H_2O$ were sterilized by filtration and maltose was added after sterilization at 121°C for 15 min.

2.5. Seed culture preparation

C. diphtheriae was inoculated into Wadsworth media, cultured for 48 ~ 72 h at 35°C, and transferred to 500 mL Erlenmeyer flasks containing 200 mL Wadsworth (or semisynthetic) broth, as described above. The cultures were grown for 20 ~ 24 h at 35°C and with agitation at 200 rpm. The dry cell weight was measured as described in a previous study [11] and bacterial purity was confirmed by Gram staining. A 200 mL inoculum was used to start toxin production in the fermenter.

2.6. Production of toxin and toxoid

Laboratory-scale 10 L stainless steel vessels with a working volume of 7 L were used. These vessels are the typical stirred tank-type vessels with three six-blade turbine impellers attached to the top, middle, and end of the agitation rotor shaft. The vessels are 40 cm in height and 25 cm in diameter, and the impeller diameter is 8 cm. Sterile air was passed through a pressure regulator, flow meter, and removable filter. Cultivation was carried out for 48 ~ 72 h at 35°C using a recirculating water system equipped with a pump and an immersion heater. The pH, dissolved oxygen, and temperature were measured through a controller system. The pH of the media for toxin production was adjusted to 7.2 with 2 M HCl, which was not controlled during cultivation. Samples were taken to determine the dry cell weight of bacteria (bacterial density) and concentration of diphtheria toxin in the culture medium using a flocculation test. The agitation and aeration rates in the fermenter were 400 ~ 600 rpm and 0.7 ~ 1.75 L/min,

respectively. An antifoaming agent was added to the fermenter for foam control. Diphtheria toxoid was purified by subjecting the diphtheria toxin in the fermentation medium to ammonium sulfate fractionation and column chromatography, followed by detoxification with formaldehyde [12,13].

2.7. Maltose measurement

Maltose level in the fermentation medium was determined by either a colorimetric or fluorometric method (Maltose and Glucose Assay Kit; BioVision, Inc., Milpitas, CA, USA). The average maltose consumption rate (Qs,avg) was calculated by dividing the amount of maltose consumed during *C. diphtheriae* culture in the fermenter by the total culture duration time.

2.8. Measurement of limes flocculation (Lf)

The Lf value of the toxin solution was measured according to the Ramon method. In this method, 1 Lf unit is defined as the amount of toxin reacting with 1 unit of the antitoxin [14].

2.9. Nitrogen determination

Protein nitrogen was determined using the Kjeldahl method [15]. The toxin (or toxoid) purity was determined as the ratio of the Lf value to the protein nitrogen value, and expressed as Lf/mgPN (milligram protein nitrogen).

2.10. K_La measurement

The volumetric oxygen transfer coefficient, K_La (/sec), was measured using the static gassing-out method [16]. The pH of the culture was adjusted to 7.2 before the measurement, and the medium temperature was maintained at 35°C. The K_La was measured using 7 L of diphtheria toxin production medium inside the fermenter, under varying levels of agitation and aeration.

3. Results and Discussion

3.1. Conditions for the optimal diphtheria toxin expression

Conditions for the fermenter culture, using casein hydrolysate and calcium phosphate precipitation medium, for an optimal production of diphtheria toxin were determined. To examine the effects of dissolved oxygen in the culture medium on diphtheria toxin expression, the aeration and agitation rates were varied in the fermenter. When the rate of airflow sparging into the fermenter was 0.7 L/min and the agitation rate was varied from 400 to 500 and 600 rpm, the maximum expression of diphtheria toxin was 300 Lf/mL, which was obtained at 600 rpm (Fig. 1). As maltose level decreased, the pH decreased and the amount of diphtheria toxin began to increase. During diphtheria toxin production in a fermenter, rapid increases in pH and dissolved oxygen level during the final stage were signals to end the cultivation. This process was shown to result in an effective production of diphtheria toxin.

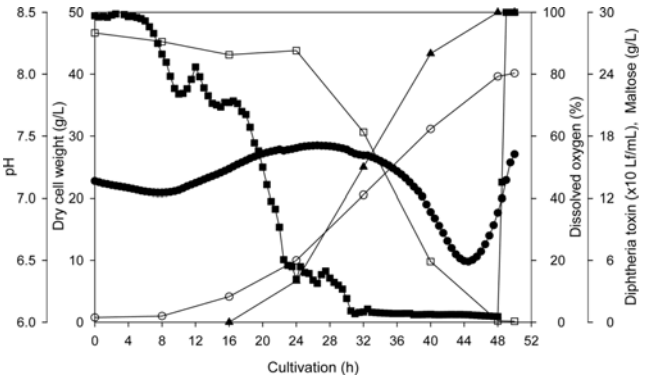

Fig. 1. Profiles of cell growth (○), toxin production (▲), pH (●), dissolved oxygen (■), and maltose concentration (□) during the fermenter culture of *C. diphtheriae*. The aeration and agitation rates in the fermenter were 0.7 L/min and 600 rpm, respectively. The K_La value of the culture medium in the fermenter was 0.0092/sec.

3.2. Variations of the fermentation parameters

The optimal aeration and agitation rates for diphtheria toxin expression are dependent on the specific conditions of the fermenter. With an aeration rate of 0.7 L/min and agitation rate of 600 rpm, the medium's K_La was 0.0092/sec. With aeration and agitation rates of 1.75 L/min and 500 rpm, respectively, a K_La of 0.0093/sec was observed. However, despite the near identical K_La values, a significant difference in diphtheria toxin expression was observed: 300 Lf/mL (Fig. 1) and 120 Lf/mL (Fig. 2), for the two conditions respectively. Even in fermenters with an identical K_La value, opitimal culture conditions by varying the combinations

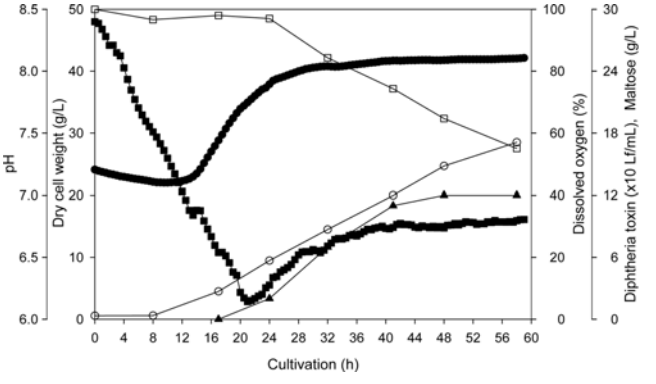

Fig. 2. Profiles of cell growth (○), toxin production (▲), pH (●), dissolved oxygen (■), and maltose concentration (□) during the fermenter culture of *C. diphtheriae*. The aeration and agitation rates in the fermenter were 1.75 L/min and 500 rpm, respectively. The K_La value of the culture medium in the fermenter was 0.0093/sec.

Table 1. Diphtheria toxin production *vs*. K_La, *Q*s,avg, *Y*p/s, and *Q*p according to the levels of aeration and agitation during diphtheria toxin production in the fermenter

Trial	Agitation (rpm)	Aeration (L/min)	K_La (/sec)	Toxin (Lf/mL) Average*	SD**	*Q*s,avg (g/L/h)	*Y*p/s (Lf/mL/g)	*Q*p (Lf/mL/h)
T1	400	0.7	0.0045	30	10	0.24	1.31	0.31
T2	500	0.7	0.0069	60	8.66	0.36	2.34	0.83
T3	600	0.7	0.0092	300	17.32	0.56	10.75	6
T4	400	1.75	0.0072	50	13.23	0.3	1.87	0.56
T5	500	1.75	0.0093	120	18.03	0.25	8.22	2.07
T6	600	1.75	0.0127	30	5	0.13	5.42	0.73

The volumetric oxygen transfer coefficient (K_La), diphtheria toxin at the end of fermenter cultivation (Lf/mL), average maltose consumption rate (*Q*s,avg), yield of diphtheria toxin on maltose consumed (*Y*p/s), and diphtheria toxin productivity (*Q*p) were measured. The fermenter's aeration rates were 0.7 and 1.75 L/min, and the agitation rates were 400, 500 , and 600 rpm.
*Average value of three independent trials.
**Standard deviation.

of parameters such as an aeration and agitation for the growth of *C. diphtheriae* and toxin expression exist.

Although maltose was completely converted into glucose and used as a carbon source when the K_La was less than optimal for diphtheria toxin expression, the level of dissolved oxygen in the medium was maintained at 0%. Under these conditions, bacterial growth was restricted owing to insufficient amount of dissolved oxygen. On the other hand, when the K_La exceeded the optimal level, bacterial growth was inhibited during cultivation owing to excessive dissolved oxygen amount in the medium, which resulted in increased pH and reduced maltose degradation, ultimately reducing toxin expression. Under fermenter culture conditions of 400 ~ 600 rpm and 0.7 L/min for the agitation and aeration rates, respectively, the *Q*s,avg decreased along with K_La. However, at 400 ~ 600 rpm and 1.75 L/min culture conditions, the *Q*s,avg decreased as K_La increased. When a highly pure diphtheria toxin yield of 300 Lf/mL or higher was obtained, the *Q*s,avg was greater than 0.5 g/L/h. The yield of diphtheria toxin on maltose consumed (*Y*p/s) is defined as the fraction of maltose converted to toxin (Lf/mL/g). The mass productivity (*Q*p) is defined as the amount of toxin produced in a unit of time (Lf/mL/h). Maximum *Q*s,avg, *Y*p/s, and *Q*p values were reached at the T3 fermenter cultivation condition, at which diphtheria toxin was produced at 300 Lf/mL (Table 1).

3.3. Shift in toxin expression conditions

Under 1.75 L/min aeration rate and 500 rev/min agitation rate, the pH steadily increased, reducing the expression of diphtheria toxin (Fig. 2). When the conditions were modified to 0.7 L/min and 600 rpm, as the pH in the fermenter reached 8, there was a shift to the typical toxin production conditions, where the pH level decreased and then increased (Fig. 3). This observation can be explained as follows: by maintaining the K_La in the medium and modifying the physical culture conditions, there was a change in pH profile. These conditions enabled *C. diphtheriae* to express a large amount of toxin. Under the cultivation conditions shown in Fig. 2, the toxin was expressed at approximately 120 Lf/mL. A switch to the cultivation conditions shown in Fig. 3 after 24 h resulted in increased toxin production to 240 Lf/mL, which was accompanied by increased dry cell weight. Immediate shift in culture conditions led to decreases in the pH level and maltose degradation. Under these conditions, the amount of diphtheria toxin exceeded those obtained under conditions without the shift. The pH profile at the specific stage displaying a K_La value that enabled high diphtheria toxin expression was a key factor that determined the increase or decrease in toxin expression.

The toxoids used as antigens in the Td vaccine must be of a high purity. To produce highly pure diphtheria toxoids, the yield of diphtheria toxin production must be increased. In this study, the fermenter culture conditions to produce

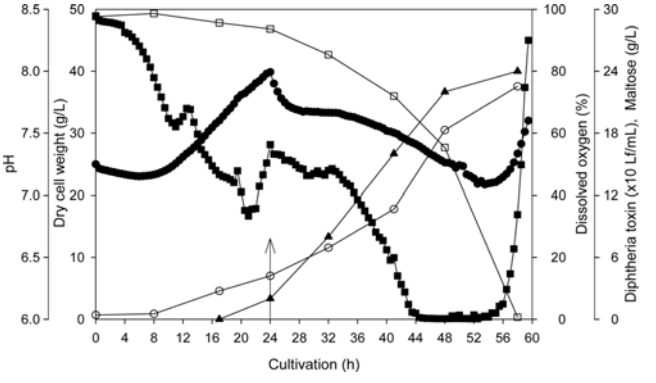

Fig. 3. Profiles of cell growth (○), toxin production (▲), pH (●), dissolved oxygen (■), and maltose concentration (□) during the fermenter culture of *C. diphtheriae*. The arrow indicates the change in the fermentation condition. At 24 h, the culture conditions in the fermenter shifted from 1.75 L/min and 500 rpm to 0.7 L/min and 600 rpm. The K_La value of the culture medium in the fermenter was 0.0093 and 0.0092/sec after the shift in cultivation conditions.

diphtheria toxin were optimized. The toxin was produced in a large quantity when agitation and aeration were optimized. The optimal conditions for a four-stage process to produce a highly expressed diphtheria toxin in a fermenter were described.

The casein hydrolysate concentration in the diphtheria toxin expression medium was 40 g/L. Other parameters, such as calcium - phosphate concentration and ratio, Fe^{2+}, maltose, lactate, and L-cystine in the mix, were described based on flask experiments [5]. Diphtheria toxin release typically begins during the exponential growth phase and continues for variable periods after exponential growth has ceased [8,17]. The amount of toxin produced varied significantly according to the physical cultivation conditions in the fermenter. A previous study has reported based on the results of RSM (Response Surface Methodology) analysis that the amounts of agitation and aeration are important factors when cultivating fungus in the fermenter to produce chitinolytic enzyme [18]. Although the amount of toxin production typically increases along with the growth of *C. diphtheriae* in the fermenter, the relationship is not always proportional. The cell amount was expressed as the dry cell weight because diphtheria toxin is secreted from inside of the bacteria into the medium, and produced proportionally to the cell growth period. Toxin production becomes stationary after exponential growth has ceased [11,17]. The maximum diphtheria toxin expression is affected more by the fermenter culture conditions than by the concentrations of the casein-based semisynthetic medium components [19]. Using the same medium, toxin expression can differ substantially depending on the fermenter culture conditions. In this study, a specific culture profile that resulted in the much higher level toxin production than reported was identified. As noted in a previous study [19,20], pH monitoring during culture was crucial in our study, too.

The fermenter cultivation process to produce diphtheria toxin was divided into four stage profiles according to cultivation time and pH variation. In Fig. 1, stages 1 through 4 indicate up to 10, 28, 44, and 50 h of cultivation, respectively. In Fig. 2, stages 1 and 2 indicate up to 12 and 28 h of cultivation, respectively. Stages 3 and 4 refer to those after 28 h of cultivation. In stage 1, cellular growth is slow, the pH in the medium gradually decreases, and the dissolved oxygen level remains saturated. In stage 2, the level of dissolved oxygen still exceeds the level required by the cells while pH of the medium increases. When the dissolved oxygen is exhausted, the pH reaches a plateau, signaling the start of exponential cellular growth and toxin expression. In stage 3, the dissolved oxygen level in the medium remains low, while maltose is rapidly converted into glucose, which is actively utilized by *C. diphtheriae* as a carbon source. Because organic acid is produced during the glucose metabolism, the pH falls drastically. This is the most important phase of the diphtheria toxin expression, as the cellular growth and toxin production peak in stage 3. In stage 4, cell activity decreases, which drastically reduces the oxygen requirement and thus rapidly increases the level of dissolved oxygen in the medium. The maltose concentration in the medium falls to 0 g/L while organic acid is no longer produced by *C. diphtheriae*, resulting in increased pH, and the dissolved oxygen level increases rapidly to 100%.

In order for diphtheria toxin to be expressed, the pH level must be lowered and dissolved oxygen level must be reduced and maintained at a low level in the transition phase between stage 2 and stage 3. When bacterial metabolism lowered the pH in the medium as the *C. diphtheriae* culture progressed from stage 2 to stage 3, maltose was steadily degraded (Fig. 1). However, the metabolism increased the pH level, and maltose degradation was reduced (Fig. 2). Maltose is broken down into glucose by maltase [21].

When diphtheria toxin production reached its peak under the cultivation conditions shown in Fig. 1, Qs,avg, Yp/sec, and Qp reached their maximum values. However, under the conditions shown in Fig. 2, toxin production was low with an identical K_La. Under the fermenter conditions that produced 30 Lf/mL toxin, Qs,avg, Yp/s, and Qp were at their minimum values. Conversely, Qs,avg, Yp/s, and Qp were high under the conditions where diphtheria toxin expression was maximized in the fermenter (Table 1). In addition, maltose was actively hydrolyzed into glucose, and the dry cell weight of *C. diphtheriae* was high. When diphtheria toxin expression reached its peak in the fermenter during stage 3 cultivation, the pH decreased, dissolved oxygen was maintained at a low level, the rate of maltose hydrolysis increased, and cell growth increased (Fig. 1). At the end of cultivation, the dry cell weight and diphtheria toxin production were 40 g/L and 300 Lf/mL, respectively in Fig. 1. In Fig. 2, the values were 30 g/L and 120 Lf/mL, respectively. After modifying the cultivation conditions to those shown in Fig. 3, dry cell weight increased to approximately 40 g/L, and toxin production reached 240 Lf/mL, which was higher than that observed in Fig. 2. Although diphtheria toxin production typically increases along with cell growth, the amount of toxin produced is not always proportional to the dry cell weight [11,19]. A high toxin expression occurred in the fermenter, the site of the first step in toxoid antigen production, only at a specific culture profile in the four stages. Thus, components of this profile can be used as effective parameters to monitor the production of diphtheria toxin antigen.

In a previous study, when a diphtheria toxin concentration of 150 ~ 200 Lf/mL was reached, a purity of 2,000 ~ 2,200 Lf/mgPN was obtained for the purified toxoid [8]. In

Table 2. Purity of diphtheria toxin and toxoid according to the purification process

Purification step	Purity (Lf/mgPN)		
	Batch 1	Batch 2	Batch 3
Culture toxin	140	202	155
Ultrafiltration	1,087	1,688	1,157
Ammonium sulfate fractionation	2,049	2,583	2,016
Chromatography	2,956	2,688	3,489
Toxoid	2,717	2,930	3,823

our study, diphtheria toxin was produced at a concentration of 250 Lf/mL or higher, from which a highly pure antigen with a purity of 2,500 Lf/mgPN or higher was obtained (Table 2). Although Tchorbanov, *et al*. used ultrafiltration to purify the toxoid [8], chromatography was used in our study. Increasing the concentration of diphtheria toxin produced and utilizing chromatography for the subsequent purification improved the toxoid purity. Compared to the DTP (or DTaP) vaccine, the Td vaccine requires a diphtheria toxoid antigen of a higher purity but in a smaller quantity. The reduced antigen content is designed to avoid the increasing reactogenicity historically seen with the fourth and fifth doses of infant vaccine [22]. Therefore, producing a highly expressed and pure diphtheria toxin could result in a highly pure antigen. By utilizing the described methods and column, up to 500,000 human doses of diphtheria toxoid could be obtained in a single operation of 10 L fermenter cultivation of *C. diphtheriae*. Diphtheria toxoid antigen was formulated into Td vaccine in a clinical study [23].

4. Conclusion

Diphtheria toxoid is an antigen used in the Td vaccine. A high concentration of diphtheria toxin was produced by culturing *C. diphtheriae* in a fermenter, which was then subjected to purification and detoxification. A specific cultivation profile that resulted in the production of highly expressed diphtheria toxin in the fermenter was identified. 300 Lf/mL of the diphtheria toxin was produced in a fermenter when a specific pH profile was used, and the production process was divided into four stages. A previous study reported that monitoring the pH level is very useful for producing toxin. The specific range of K_La values to produce the maximum diphtheria toxin amount was 0.0092 ~ 0.0093/sec in this fermentation system. When the K_La value was outside of this range, the amount of toxin produced in the fermenter varied significantly depending on the specific physical cultivation conditions, even at a constant K_La value. The diphtheria toxoid antigen used in the Td vaccine must be highly pure. Diphtheria toxoid antigen with purity greater than 2,500 Lf/mgPN was formulated into Td antigens and used in a clinical study of Td vaccine in Korea.

Acknowledgement

This work was partially supported by College of Life Sciences and Biotechnology, Korea University (K1608631).

References

1. Pina, L. M., E. Bassily, A. Machmer, V. Hou, and A. Reinhardt (2012) Safety and immunogenicity of a quadrivalent meningococcal polysaccharide diphtheria toxoid conjugate vaccine in infants and toddlers: Three multicenter phase III studies. *Pediatr. Infect. Dis. J*. 31: 1173-1183.
2. Zakikhany, K. and A. Efstratiou (2012) Diphtheria in Europe: current problems and new challenges. *Fut. Microbiol*. 7: 595-607.
3. Tchorbanov, A. I., J. D. Dimitrov, and T. L. Vassilev (2004) Molecular composition of diphtheria toxoid produced using semi-synthetic and meat extract-based broths. *World J. Microbiol. Biotechnol*. 20: 211-217.
4. Mueller, J. H. and P. A. Miller (1941) Production of diphtheric toxin of high potency (100 Lf) on a reproducible medium. *J. Immunol*. 40: 21-32.
5. Stainer, D. W., J. M. Corkill, and M. J. Scholte (1968) Preparation and properties of diphtheria toxoids in submerged culture. 3. Development of a new semisynthetic medium. *Can. J. Micobiol*. 14: 1155-1160.
6. Relyveld, E. H., B. Bizzini, and R. K. Gupta (1998) Rational approaches to reduce adverse reactions in man to vaccines containing tetanus and diphtheria toxoids. *Vaccine* 16: 1016-1023.
7. Bjorkholm, B., M. Granstrom, M. Wahl, C. E. Hedstrom, and L. Hagberg (1989) Increased dosage of diphtheria toxoid for basic immunization of adults. *Eur. J. Clin. Microbiol. Infect. Dis*. 8: 701-705.
8. Tchorbanov, A. I., J. D. Dimitrov, and T. L. Vassilev (2004) Optimization of casein-based semisynthetic medium for growing of toxigenic Corynebacterium diphtheriae in a fermenter. *Can. J. Microbiol*. 50: 821-826.
9. Dalby, A. and E. Holdsworth (1956) Growth factors for Corynebacterium diphtheriae strain Dundee. *J. Gen. Microbiol.* 15: 335-344.
10. Wadsworth, A. B. and M. W. Wheeler (1934) The attenuation and toxin production of the diphtheria bacillus. *J. Infect. Dis*. 55: 123-137.
11. Edwards, D. C. (1960) The growth and toxin production of Corynebacterium diphtheriae in submerged culture. *J. Gen. Microbiol*. 22: 698-704.
12. Cukor, G., J. D. Readio, and R. J. Kuchler (1974) Affinity chromatography purification of diphtheria toxin. *Biotechnol. Bioeng.* 16: 925-931.
13. Moyner, K. and G. Christiansen (1984) Comparison of gel filtration and ammonium sulphate precipitation in the purification of diphtheria toxin and toxoid. *Acta. Pathol. Microbiol. Immunol. Scand. C*. 92: 17-23.
14. Lyng, J. (1990) Quantitative estimation of diphtheria and tetanus

toxoids. 4. Toxoids as international reference materials definding Lf-units for diphtheria and tetanus toxoids. *Biologicals* 18: 11-17.
15. Hiller, A., J. Plazin, and D. D. Van Slyke (1948) A study of conditions for Kjeldahl determination of nitrogen in proteins; description of methods with mercury as catalyst, and titrimetric and gasometric measurements of the ammonia formed. *J. Biol. Chem*. 176: 1401-1420.
16. Cho, M. H. and S. S. Wang (1988) Enhancement of oxygen transfer in hybridoma cell culture by using a perfluorocarbon as an oxygen carrier. *Biotechnol. Lett*. 10: 855-860.
17. Righelato, R. C. and P. A. van Hemert (1969) Growth and toxin synthesis in batch and chemostat cultures of Corynebacterium diphtheriae. *J. Gen. Microbiol*. 58: 403-410.
18. Fenice, M., P. Barghini, L. Selbmann, and F. Federici (2012) Combined effects of agitation and aeration on the chitinolytic enzymes production by the Antarctic fungus Lecanicillium muscarium CCFEE 5003. *Microb. Cell Fact*. 11: 12.
19. Sundaran, B., Y. U. Rao, and R. Boopathy (2001) Process optimization for enhanced production of diphtheria toxin by submerged cultivation. *J. Biosci. Bioeng*. 91: 123-128.
20. Nikolajewski, H. E., S. Rustenbach, S. Mebel, and L. Meineke (1982) Production of Corynebacterium diphtheriae toxin in a bubble column fermentor. *J. Biol. Stand*. 10: 109-114.
21. Sugg, J. Y., W. L. Fleming, and J. M. Neill (1927) Studies on bacterial enzymes : VI. The maltase of the diphtheria bacillus. *J. Exp. Med*. 46: 909-916.
22. McCormack, P. L. (2012) Reduced-antigen, combined diphtheria, tetanus and acellular pertussis vaccine, adsorbed (Boostrix®): A review of its properties and use as a single-dose booster immunization. *Drugs* 72: 1765-1791.
23. Hong, T., Y. J. Chung, T. Y. Kim, I. H. Kim, Y. K. Choe, J. Lee, S. Jeon, S. Han, and D. S. Yim (2015) Safety and immunogenicity of a single intramuscular dose of a tetanus-diphtheria toxoid (Td) vaccine (BR-TD-1001) in healthy Korean adult subjects. *Hum. Vaccin. Immunother*. 11: 2440-2445.