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Process optimization for an industrial-scale production of Diphtheria toxin by *Corynebacterium diphtheriae* PW8

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ABSTRACT

In this study, several parameters affecting the toxin production of *Corynebacterium diphtheriae* Parke Williams 8 (PW8) were investigated in detail. The comparison studies of amino acid profile in NZ Amine A-based medium (NZ medium) and beef digest-based medium (BD medium) suggested that an insufficient supply of amino acids was not responsible for low toxin yield observed in NZ medium. Supplementation of additional amino acids and growth promoting nutrient (in a form of yeast extract) into NZ medium enhanced only cell growth but not toxin production. Thus, BD medium was selected as the most suitable base medium for toxin production as it gave a significantly higher limit of flocculation (93 ± 0 Lf/ml) than NZ medium (46 ± 0 Lf/ml). Interestingly, a supplementation of 0.2% YE into BD medium resulted in a significant increase in growth as well as toxin production (235 ± 5 Lf/ml). In conclusion, consistently high toxin titer (174–239 Lf/ml) could be obtained from BD medium at a 5 L-scale production as long as 1) the protein content of BD medium was at least 24 g/L, 2) the iron content was below 0.15 ppm and 3) 0.2% YE was supplemented into the medium.

1. Introduction

While diphtheria is considered as a rare disease in many developed countries, its case fatality rates worldwide remain comparably high (>10%) [1]. Epidemics in different parts of the world were suspected as a consequence of waning immunity in adults, movements of large population groups, and an irregular supply of vaccines. Vaccination with the diphtheria toxoid (DTd) prepared from detoxified Diphtheria toxin (DTx) is therefore an indispensable strategy for a control of the disease. Such control requires not only a mass immunization of susceptible population, but also a repeated immunization of adults as a global vaccine policy for supporting immunization. For instance, the ministry of public health of Thailand recently launched a vaccination campaign against Diphtheria and Tetanus. Under this program, each healthy adult between 20 and 50 years of age has been encouraged to receive a booster injection of Diphtheria and Tetanus toxoid (dT) [2,3]. Efficient manufacturing of DTx for local usage is, therefore, essential for such program.

At present, nearly all manufacturers of DTx employ a variant of *Corynebacterium diphtheriae* Park-Williams 8 (PW8) [4] with a process based on a cultivation in either Pope-Lingood medium containing beef-derived proteins, or semi-synthetic medium such as casein hydrolysate [5]. This is largely because low growth and toxin yield are frequently reported for synthetic medium [6]. Previously, many attempts have been made to enhance the toxin production via medium optimization. For instance, while maltose was reported as the best carbon source for toxin production [7], four amino acids, namely cysteine (Cys), histidine (His), aspartate (Asp), and methionine (Met), were reported as critical for growth and toxin secretion of *C. diphtheriae* [8]. Moreover, the concentration of iron and calcium–phosphate complex in NZ medium was optimized in order to maintain a gradual supply of iron in the culture media, thus enhancing both growth and toxin production [6,9].

Up until now, BD medium based on Pope-Lingood medium [10] was still reported as the most suitable medium as it gave

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consistently high toxin titer [7]. There are, however, some advantages of replacing beef-derived protein with casein hydrolysate, for example, the consistent quality between batches and a less complicated purification process, due to the fact that most amino acids in casein hydrolysate are in a form of free amino acid which will not interfere with the purification of DTx [11]. Unlike the semi-synthetic medium, the protein residues from beef in BD medium might affect the purification step [5]. Besides, the appearance of bovine spongiform encephalopathy (BSE) or 'mad cow disease' epidemic has increased the profile of transmissible spongiform encephalopathies (TSEs) as a risk to human health and has already affected public health policy worldwide. As a result, WHO developed some guidelines on TSE in relation to biological and pharmaceutical products to minimize the risks [12,13]. The guidelines included a risk assessment based on a geographical category of BSE risks proposed by the World Organization for Animal Health (OIE). Therefore, if a vaccine manufacturer inevitably uses the animal-origin materials, such as beef muscle in the medium formula, these factors need to be seriously taken into account.

In this research, we focused on the amino acid compositions of the medium prepared from the beef digest (BD medium) and a casein hydrolysate NZ Amine A (NZ medium). The two types of media were compared, and nutrients supplementation was attempted in order to increase the toxin titer. Several factors affecting growth of *Corynebacterium diphtheria* and its toxin production were also investigated and optimized in order to obtain a consistently high toxin titer. Finally, the formula that gave the best toxin production was evaluated at a 5 L-scale production prior to a further scaling-up to a 50 L- and 500 L-scale bioreactor, respectively.

2. Methods

One month before the start of this research, all staffs and researchers received a booster injection of dT. All experiments were performed under a biosafety level-2 (BSL-2) facility provided by GPO.

2.1. Bacterial strain and growth conditions

C. diphtheriae PW8 was used in this study. The freeze-dried culture was inoculated onto Loeffler agar plate and the working seed lot was prepared according to WHO's guideline [14]. The inoculum was then prepared by subculturing from Loeffler slant into 200 ml of either NZ Amine A-based medium (NZ medium) or Beef digest medium (BD medium) in a 1 L-flask. The culture was incubated on an orbital shaker at 35 °C, 160 rpm for 24 h and then used as an inoculum for both flask-scale and bioreactor cultivation.

2.2. Media preparation

NZ medium was prepared according to Stainer et al. (1968) [6] except that the initial pH was adjusted to 7.2 instead. BD medium [10], containing beef meat 155 g (digested using L-cysteine 0.25 g and papain 1.5 g in 1 L distilled water and pH 7.2 adjusted with 40% (w/v) NaOH at 55 °C for 4 h, followed by an acid hydrolysis with conc. HCl 90 ml); yeast extract (YE) 0.15 g; sodium lactate 60% (w/v) 1.5 ml; $MgSO_4.7H_2O$ 0.7 g; maltose 18.7 g; nicotinic acid 2.1 mg; β-alanine 2.1 mg; pimelic acid 0.14 mg; $MnCl_2.4H_2O$ 0.28 mg; $ZnSO_4.7H_2O$ 0.74 mg; $CuSO_4.5H_2O$ 0.93 mg; distilled water 1 L; pH = 7.8, was prepared according to WHO's recommendation [14]. The papain beef digest was filtered by an ultrafiltration (MWCO 10 kDa) before use. Initial protein content in BD medium was controlled by adjusting the ratio of permeate and retentate during the ultrafiltration with further dilution using distilled water to obtain a desired value. All the chemicals used were purchased from Merck (Kenilworth, NJ, USA) or Sigma (St. Louis, MO, USA). Beef muscle used in this research was purchased from a certified slaughter house (KU beef, Thailand) with the following specifications. Beef muscle must be lean and comes from part farther away from brain and spinal cord (e.g. hinds). Meat traceability system must be operated by the vendor (e.g. bar codes or tags). The animal slaughtered in the slaughter house must be subject to ante-mortem and post-mortem examination. A TSE/BSE free declaration certificate must also be provided by the vendor [12,13]. That included other in-house specifications such as, color, odor, pH, bioburden limits, and etc. that must be drawn up as a standard operating procedure (SOP) for controlling the quality of beef meat in vaccine production. All medium was sterilized by autoclaving at 110 °C for 15 min. When specified, additional yeast extract (Difco™, BD Biosciences, Sparks, MD, USA) was supplemented into the medium (at 0.2–1.25%) prior to sterilization.

2.3. Flask-scale cultivation

Effects of medium type (1x NZ medium, 1.5x NZ medium or BD medium), aeration (round-bottom or baffled flask), sterilization (heat treatment or filtration), initial protein content (at 12, 24 or 36 g/L) as well as YE supplementation (at 0.2, 0.3, 0.75 or 1.25%) were evaluated in a flask-scale. Briefly, 200 ml of culture (with an initial OD650nm of 0.25–0.3) was cultivated in a 1L-flask (standard round-bottom flask or baffled flask) at 35 °C, 160 rpm for 48 h. Samples (10 ml) were taken every 12 h and analyzed for growth (as OD650nm). Supernatant was collected after a centrifugation at 11,337 × *g* for 10 min and then analyzed for pH and toxin titer by Ramon flocculation according to WHO's guideline [14] (Lf/ml; Lf or Limes flocculation is defined as an amount of toxin which, when mixed with one International Unit of antitoxin, gives a Ramon flocculation in the shortest time or Kf). The Diphtheria antitoxin used in this research was purchased from VINS Bioproducts Ltd (Telangana, India).

2.4. Bioreactor-scale cultivation

In this study, a 7.5 L-bioreactor (Labfors 5, Infors HT, Switzerland) was used for a toxin production at a working volume of 5 L. During cultivation, pH and temperature were controlled at 7.6 (using 50% glucose solution) and 35 °C, respectively. Air was supplied at a rate of 0.5–1 vvm while stirrer speed was controlled between 300 and 600 rpm using a cascade control. Percent dissolved oxygen (% DO) was monitored using a DO probe (Hamilton, Reno, NV, USA) calibrated according to the manufacturer's recommendation. Maximum oxygen transfer rate (OTR) obtained was approximately 2.5 mg/L min. Silicone antifoaming agent (5% solution of Silicolapse®) was used throughout the fermentation period to control foaming.

2.5. Analysis

Protein content was calculated from total nitrogen content determined by the method of Kjeldahl [15]. Iron content was quantified using Iron Test Kit (Aquaquant®, Merck, Germany) according to the manufacturer's recommendation. SDS-PAGE was carried out with a 4–12% Bis-Tris Gel (NuPAGE Novex, Thermo Scientific, IL, USA) at a constant current (15 mA/gel). PageRuler™ Prestained Protein Ladder (Thermo Scientific, IL, USA) was used as a molecular mass marker. After electrophoresis, the gel was stained with Coomassie brilliant blue R-250 [16] and de-stained by soaking in the methanol:acetic acid solution. HPLC analysis with precolumn phenylisothiocyanate derivatization [17] was used for