Application of fermentor technology in production of diphtheria toxin

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Abstract
The present study shows the production of diphtheria toxoid in fermentor as compared to old static method of production. With using 45l glass fermentor which has the provision for growth of the organisms under controlled conditions with regard to temperature, pH, aeration and addition of maltose which can be automatically monitored and maintain microorganism growth toxin production and recording all of the conditions during growth. We succeeded to produce diphtheria toxin with 115 Lf (Rammon flucolation titer) within 40h time period in a minimum possible space, then the toxin was detoxified with formaldehyde, concentrated and purified and finally quality controlled and compared with toxin produced with static method. Fermentor production showed a remarkable increase in volume, titer and immunogenecity in a shorter period of time, lower contamination and minimum labor work and requirements. Therefore this technique can be industrially used for production of diphtheria Toxin (DT) and Diphtheria Pertussis Toxin (DPT) vaccines.

Keywords: Fermentor, Diphtheria, Toxin, Toxoid, DT vaccine, DPT vaccine

Introduction

Diphtheria is a toxigenic infection that is caused by *Corynebacterium diphtheria*. Klebs *et al.* demonstrated *C. diphtheria* as etiologic agent of diphtheria. One year later, Loeffler found that organism could only be cultured from nasopharyngeal cavity, and postulated that damage to internal organs resulting from a soluble toxin [1]. By 1888 Roux and Yersin [2] showed that animals injected with sterile filtrate of *C. diphtheria* developed organ pathology indistinguishable from that of human diphtheria. This was demonstrated that a potent exotoxin is the major virulent factor which lead to the development of both an effective antitoxin based therapy for acute infection and a highly successful toxoid vaccine [3]. Since then the conventional technology for vaccine production in static culture was started and was undergoing rapid changes and recently being replaced by fermentor technology.

Fermentor production offers considerable economics in medium, time and space. A 350 liter fermentor can yield 300 titers of toxin [3-6] at 250-300 lf/ml (toxin titer), after 48h of incubation involving only a few aseptic manipulations. This remarkable increase in production rate can supply the country's need for DT and DPT vaccines [6, 7]. The present study shows the initial trial for the production of diphtherin toxin in fermentor in comparison to old static method of production. The production in fermentor showed increased volume, toxin titer and toxicity. However,

the amount of production in each run showed decrease in production time, contamination, space and personals required. Because certain nutrients or their metabolite may inhibitory effect on toxin production in static culture but in fermentor monitoring of pH of fermentation culture and the levels of maltose are balanced automatically which support bacterial growth, toxin production and reduces this inhibitory effects [8].

Material and Method

Preparation of seed

The lyophilized strain of *C. diphtheria* (CN-2000) was obtained from CRI India. The content of the freeze dried strain was emulsified in a tube with few drops of nutrient broth and incubated on two large slopes of Loeffler Serum (LS) and were incubated for 72h at 35°C. Cultures were checked by gram staining smear [9]. Seed cultures were prepared in one liter cornical flask containing 200ml of the linggood medium (Sigma). Each flask was inoculated with one slope of LS after 72h incubation. The cotton plugged flask were placed on a shaker and incubated for 24h at 35°C [2, 7, 10]. Gram stained smear was made for each flask, lf/ml, pH and opacity of seed culture were also checked and it was used for the inoculation of production medium [4, 5].

Culture media for the production of toxin

Diphtheria toxin can be obtained in consistently good yield based on papain digest of Meat (all the chemicals used were Merk or Sigma products). Linggood medium should be free from ingredients that are known to cause toxic or allergic reactions in man. Neither mammalian products nor human blood group substances should be present in the final vaccine [3, 7]. Linggood medium contained: Beef meat (fat free), 15g; Yeast extract, 0.15g; Sodium lactate 60% w/v, 1.5ml; Maltose 50% (solution), 50ml; $MgSO_4$ $7H_2O$, 0.62g; β–Alanine, 1.75g; Nicotinic acid, 1.75; Pimelic acid, 0.11g; Cu So_4 $5H_2O$, 0.75g; Zn SO_4 $7H_2O$, 0.60g; Mn Cl_2 $4H_2O$, 0.22g; distilled water 1L; pH=8. Digestion of meat involves the approximate amounts of the following. Papain, 1.2-1.5g; NaOH 40% w/v, 8ml; HCl (conc), 2.5ml; Glacial acetic acid, 6ml; L-Cysteine HCl, 0.25g. Medium is sterilized at 100°C for 30 minutes in fermentor [3, 9-11].

Inoculation of the batch

About 200ml seed prepared in production medium was added to 40L medium in 45L fermentor (Beethoven, Netherlands) as inoculums seed after making proper connection with aseptic precautions [12].

Toxin Production

In this experimental production, toxin was produced in 45L glass fermentor. This fermentor has the provision for growth of the organisms under controlled conditions with regard to temperature, pH, aeration which can be automatically monitored and record during growth. Airflow was maintained at 20-22l/h throughout the production time and temperature was maintained at 35ºC. The stirrer speed for first 24h was kept at 700rpm and thereafter increased to 900rpm. After 40-44h samples were taken from the fermentor and the following tests were carried out; Lf/ml from the supernatant for determination of titer and Gram stained smear from sediment for visual checking. We were succeeded to obtain 115 lf/ml in the initial trials. Gram smear did not show any contaminations, pH was about 7.8 and opacity test showed satisfactory growth (Fig. 1).

Harvestings, detoxification and purification

After ascertaining the purity of the culture and quality of the toxin the bacterial was filtered using diatomaceous earth to separate the bacterial mass [7].

Detoxification of toxin was carried out by the addition of 0.6% formalin (40%). This quantity was added in two parts of 0.3% each at 24h interval. The formalized toxin was kept at room temperature for seven days and pH raised to 7.5 at the end of seven days it was thereafter stored at 35ºc

for six more weeks [1]. After six weeks samples were taken for detoxification test and no reaction was seen in the formalized toxin was marked labeled as toxoid and stored at cold and dark place pending purification [13].

Purification was carried out using Zinc chloride precipitation and ammonium sulfate fractionation.

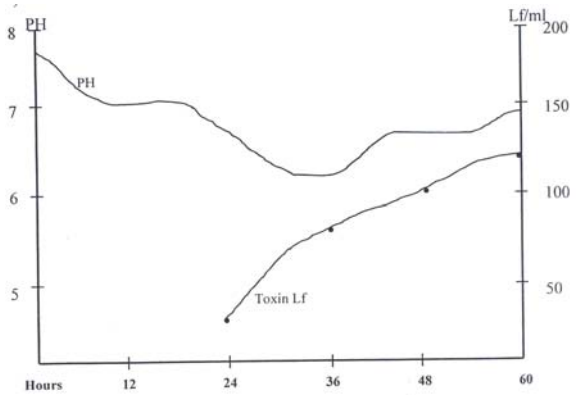

Fig. 1: Relation of pH and titer toxin Lf with time of incubation

Table 1: Comparison of diphtheria toxids produced by fermentor and old static method

Culture method	Culture		Toxin titer lf/ml	Toxicity of product MLD times	Contamination	Amount of product in each run times	Required space (m^2)
	Volume (l)	Time (h)					
Fermentor method	350	40	100-250	4	0-5%	6	Max 2×2
Old static method	100	168	50-100	1	25%	1	Min 6×4

Result and Discussion

Diphtheria toxin produced in fermentor, for the first trial as shown in table 1 compared with diphtheria toxin produced by old static method. This showed higher production volume, toxin titer, toxicity (mouse weight gain test) and amount of product in each run and lower contamination, culture time with minimum space and workers (Table 1). Therefore this method can be replaced by old static method of diphtheria toxin production due to the following advantages: very large scale of production; economical cost of production; production under controlled commercial conditions; homogenous high titer production supplies the country needs for DT and DPT vaccines; few aseptic manipulation; the use of one type of standard equipment for making different type of vaccines, decreased labor work; reduced space required. This technique of production is suggested for commercial production of diphtheria toxin, DT and DPT vaccines.

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