Original Research Article Microbiology

International Journal of Pharma and Bio Sciences

ISSN
0975-6299

DIPHTHERIA TOXIN PRODUCTION IN PILOT SCALE USING CASEIN BASED MEDIA

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ABSTRACT

In diphtheria vaccine production, a high level purity of the antigen is imperative. *Corynebacterium diphtheriae* is an exotoxin producing infectious bacteria. It is cultivated in a media which supports its growth. For decades the media used was meat based in nature. Presently due to WHO CDS guidelines, and also due to presence of undesirable protein presence, the use of meat based media is at disputation leading to a few countries opting to avoid meat. Thus a need for an alternative media has arisen. In the present study a complete meat free, a milk protein-casein based media was used. The adaptability of the bacteria in the media and its growth and toxin release was studied using cultural and toxin analysis parameters such as Optical density, amino nitrogen estimation and Limes of flocculation (Lf titre), Protein estimation respectively. In this study it was observed that the media supports the growth with a steady increase in the optical density denoting growth and Lf of 30-50 units with a short flocculation time denoted as Kf in a shaking culture method. The produced toxin has a significant antigenic purity.

KEYWORDS: ***Corynebacterium diphtheriae, Casein, NZ Amine AS, Diphtheria toxin, Toxoid, Lf***

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Received on: 29-09-2018
Revised and Accepted on: 25-10-2018
DOI: http://dx.doi.org/10.22376/ijpbs.2018.9.4.b228-237

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INTRODUCTION

Diphtheria is one among the deadly infectious diseases globally, to have caused disastrous epidemics, mainly affecting children.[1,2] Diphtheria is caused by *Corynebacterium diphtheriae*, a Gram positive bacilli. The most important virulence factor is exotoxin, affecting the respiratory tract and occasionally the skin (Cutaneous diphtheria). Absorption of diphtheria toxin into the bloodstream results in toxic damage to organs such as the heart, kidneys and peripheral nerves[3]. After the inclusion of diphtheria vaccine in the Expanded Programme of Immunization (EPI) in 1974, the incidence of Diphtheria decreased dramatically worldwide[4-6]. However, Diphtheria remains a significant health problem in countries with poor routine vaccination coverage[7]. The largest reported cases during the period 2011-2015 was from India with a 5 year total of 18350 cases, followed by Indonesia with 3203 and Madagascar 1633 cases. The South-East Asia Region was the source of 55-99% of all the reported cases each year during this period[4]. The control of diphtheria is based on primary prevention of disease by ensuring high herd immunity through vaccination[3]. In the 1940s diphtheria toxoid, tetanus toxoid and pertussis antigens were combined to form diphtheria-tetanus-pertussis vaccine (DTP) and used widely throughout the world[2]. Currently, for pediatric use, diphtheria toxoid is almost exclusively available in combination with tetanus toxoid (T) as DT, or with tetanus and pertussis antigens (DTP). Though Toxoid containing vaccines are mostly in use even now, formation of protein polymer due to the linking of the toxin molecules with the animal proteins present in the medium leads to nontoxoid protein, which reduces the purity[8-12]. Thus careful selection of the source (ruminant) starting materials to be used in the production vaccines, pharmaceutical products is an important criterion in the TSE (Transmissible Spongiform Encephalopathy) risk assessment[13-14]. At this time, the only known food animal species with BSE is cattle, and hence the principal target for BSE (Bovine Spongiform Encephalopathy) eradication is cattle[13]. In the recent times a few countries have initiated the banning of meat, thus the use of meat as a culture medium is being regulated. Replacing beef-derived protein with casein hydrolysate is advantageous with consistent quality between batches and free form of amino acids leads to less complex purification process [14-15]. Using different media components, the growth and toxin production of *Corynebacterium diphtheriae* have been studied. The use of beef muscle yielded toxin titer of 200 flocculation units [Lf] or more in 48 hours especially in the fermentor culture method[16]. When Media containing only three amino acids, were used under proper cultivation conditions, a toxin of high potency was obtained, but they were too expensive for routine use[17]. Thus a need for an ideal media preparation has arisen which yields good toxin release without meat ingredients. A tryptic - digest of casein branded as N.Z. Amine type AS, with addition of inorganic phosphate solution, calcium chloride and ferrous sulphate under controlled levels was shown to have yielded high levels of toxin[16]. This study is an attempt to produce diphtheria toxin using a Casein based-NZ Amine AS medium in comparison with the globally used Papain Digest Medium (PDM).

MATERIALS AND METHODS

In this study, Parke Williams 8 (PW8) strain of *Corynebacterium diphtheriae* was used to produce the exotoxin. The globally used Papain Digest Medium (PDM) was used as standard and compared with the Casein based-NZ Amine AS medium. pH adjustment was not done with PDM, whereas, NZ Amine AS batch I had pH adjustment only at 48 hours of cultivation and batch II at intervals of 6 hours. The main goal of this study was to observe the support of casein hydrolysate NZ Amine AS medium in the growth of *Corynebacterium diphtheriae* and its toxin release in a 3L scale.

Media Preparation

Preparation of Papain Digest Medium[18-20]

The required quantity of minced beef muscle was suspended in sterile water (1: 7 w/v) and digestion process was carried out by 7 additions of the powdered Papain dissolved in distilled water at 30 min intervals under controlled conditions of temperature (50˚C ± 1˚C) and pH (7.1 ± 0.1). The duration of the digestion was 3.5 hours with adequate stirring throughout. Sodium hydroxide solution (12.5 N) was used for pH maintenance at 7.0-7.2. When digestion was complete, concentrated hydrochloric acid was added to reduce the pH to 5.1 and the mixture was boiled for 10min. Glacial acetic acid was added to reduce the pH to 4.1 and the mixture was filtered using a cloth filter. This broth can be stored at 2-8°C for about 4 weeks without significant loss in quality. The medium pH was raised to 8.0 with 12.5 N sodium hydroxide solution and baker's yeast was added (4g/l). The temperature was maintained at 30-35˚C for 60 min while stirring. After 60min the temperature was raised to 80°C and maintained for 10min to kill the yeast and the broth was clarified .To the clarified media was added yeast extract, 60% sodium lactate solution, Mueller's growth factor solution, magnesium sulphate and Maltose as the energy source. The pH of the medium was adjusted to 8.0 and the medium sterilized by filtration.

Preparation of Loeffler's Serum Medium using PDM[18-20]

One part of sterile Papain Digest Medium (PDM) was mixed with three parts of sterile calf serum. A sterility test was done on nutrient agar slope. 7 ml was distributed in sterile Mac-Cartney bottles incubated at 85˚C for one hour in a slanting position. This was left overnight in the incubator, removed and incubated at 35˚C ± 0.5˚C for 48 hours to check the sterility and then stored at 2 to 8˚C. The seed strain-starting material was revived in the Loefflers slope which was prepared with one part of Papain Digest Medium (PDM) and three parts of New Born Calf Serum.

Preparation of NZ Amine AS Medium

N.Z Amine type AS powder (Sheffield Chemical Corporation, Norwich, New York.) 233.1grams was dissolved in 15.75 litres of distilled water at 60ºC. To this were added 27.3 grams of anhydrous Na_2HP0_4 and 9.1 grams of anhydrous KH_2P0_4 and the pH adjusted to 9.3 with 50% NaOH. The mixture was heated to 80ºC, and 28.42 grams of anhydrous calcium chloride dissolved in 400 ml distilled water, was added to the media with vigorous stirring. The temperature was raised to 85ºC and the mixture was allowed to cool to ambient temperature and stored at 4˚C overnight. The precipitate

was removed through centrifugation. The supernatant pH was adjusted to 8.8 with glacial acetic acid, followed by the addition of 175 grams of maltose (50%), 11.9 ml of 60% sodium lactate and 56 ml of the modified Muller's Growth Factors solution. The volume was adjusted to 6.3 liters with distilled water, 14 ml of the iron-free 10% Cystine hydrochloride solution was added, the pH adjusted to 7.5, and the volume was finally brought to 7 liters. The medium was sterilized by filtration followed by the addition of Phosphate solution, 20% Calcium chloride solution and 0.1% Ferrous sulphate solution and steamed at 95˚C to <100˚C for 10 min.

Preparation of Loeffler's Serum Medium using NZ Amine AS Medium

One part of sterile NZ Amine AS medium was mixed with three parts of sterile calf serum. A sterility test was done on nutrient agar slope. 7 ml was distributed in sterile Mac-Cartney bottles incubated at 85˚C for one hour in a slanting position. This was left overnight in the incubator, removed and incubated at 35˚C ± 0.5˚C for 48 hours to check the sterility and then stored at 2 to 8˚C. The seed strain-starting material was revived in the Loefflers slope which was prepared with one part of media-NZ Amine AS in the place of Papain Digest Medium (PDM) and three parts of New Born Calf Serum.

Medium Analysis

Estimation of Amino Nitrogen

The α-amino nitrogen content was determined by the Sorensen titration method[21].

Spectrophotometer Estimation of Amino Acid in Media

A solution of ninhydrin was prepared by dissolving 2 grams in 25 ml of acetone. To this solution was added 25ml of 0.2M acetate buffer, pH 5.5. A standard solution of one amino acid (l-lysine) was prepared by dissolving 5 milligrams of amino acid in 10 ml of 0.1N HCl. Aliquots of the amino acid was pipetted out into a series of test tubes, water was added to each tube to make the total volume to 4.0ml. The ninhydrin reagent (1.0ml) was added to each tube, mixed well, and the tubes were kept in boiling water bath for 15 minutes, and then cooled and 1 ml of 50% ethanol added to each tube. The tubes were then cooled and 1 ml of 50% ethanol was added to each tube. The pink color developed was measured in a spectrophotometer at 550 nm[22].

Bacterial strain and growth conditions

Corynebacterium diphtheriae Parke William (PW 8) strain was used throughout the study. The lyophilized working seed was emulsified with a few drops of condensate from Loefflers slope and inoculated into 2 Loefflers slopes. The slopes were incubated at 35˚C for 48 hours. Then it was subcultured into seed flasks containing 100 ml of the medium for enabling the seed to adapt to the medium. The flasks were kept on rotary shaker at a speed of 160±10 rpm for 24 hours at 35˚C.The bacterial purity was confirmed by Gram staining and then used as an inoculum for flask scale cultivation.

Flask-scale cultivation

The Toxin production was assessed in the flask scale in which 3L production medium was taken in a 10L capacity round bottom flask. The flasks were shaken at 140 rpm under incubation temperature of 35^0C.The pH of the medium for toxin production was adjusted between 7.0 to 7.5 with 50% NaOH, at different time intervals during cultivation. Samples were taken periodically and analyzed for bacterial purity, pH, growth (OD at 600nm), optical density assessed based on visual comparison of opacity unit between appropriate diluted culture samples using McFarland standards. On centrifugation, the supernatant was collected and toxin titre analyzed (according to WHO guidelines) by Ramon flocculation method.

Toxin Analysis

Protein Nitrogen Estimation

Protein nitrogen was determined using the Kjeldahl method. The antigenic purity of the toxin was determined as the ratio of the Lf[23] value to the protein nitrogen value (expressed as Lf/PN).

Total Protein Estimation by Bicinchoninic acid test

The BCA working reagent was prepared by mixing copper sulphate with Bicinchoninic acid respectively, in the ratio 1:20. The standard dilution and the toxin samples were mixed with BCA working reagent in the wells of microtitre plate. The mixture was incubated at 37˚C for 30 to 60 minutes. Further the mixtures were subjected to spectrophotometer analysis at 520nm, and the transmittance for each mixture was determined.

Electrophoresis

Sodium dodecyl sulfate (SDS)-polyacrylamide gel (12%) electrophoresis (PAGE) was carried out at a constant current (80Volts/gel). Protein Marker (Puregene-Genetix Protein Marker ranging 10kDa to 315 kDa) was used as a molecular marker. After electrophoresis, the gel was stained with Coomassie brilliant blue R-250 and de-stained by soaking in the methanol:acetic acid solution[24].

Experimental animal

The albino Guinea pig in the weight range of 250-350 grams bred in Pasteur Institute of India, Coonoor breeding facility which is registered under CPCSEA (Committee for the purpose of control and supervision of experiments on animals). The animals were maintained according to the guidelines of CPCSEA and use of these laboratory animals has the approval of Institutional Animal Ethics Committee (IAEC) of Pasteur Institute of India, Coonoor.

Detoxification of Toxin and test for detoxification

Formalin (formaldehyde) at a final volume concentration of 0.6% was added aseptically to filtered toxin for six days and shaken well and stored in room temperature and shifted to 35˚C±0.5˚C and incubated for 6 weeks. The sample collected after 6 weeks incubation was divided into two portions (1 ml each) and one portion was heated to 80˚C and maintained for 30 minutes. About 0.2ml from each portion was injected into the depilated skin of white guinea pig intradermally and observed at 24 and 48 hours.

Test for free formaldehyde

Standard formaldehyde solution is diluted with distilled water to obtain the following dilutions

a. 0.1ml of CHHO (formaldehyde) +9.9ml Distilled water (3600µg/ml)
b. 0.5ml of (a) +8.5ml Distilled water (200 µg /ml)
c. 1.0ml of (b) +9.0ml Distilled water (20 µg /ml)
d. 4.0ml of (c) +1.0ml Distilled water (16 µg /ml)
e. 2.0ml of (d) +2.0ml Distilled water (8 µg /ml)
f. 2.0ml of (e) +2.0ml Distilled water (4 µg /ml)
g. 2.0ml of (f) +2.0ml Distilled water (2 µg /ml)

The dilutions (b) to (g) of the standard formaldehyde was taken for the test.

The toxoid samples were centrifuged and the supernatant solution was used for the test. 1.0ml of aliquots was used for the test. 1.0ml aliquots of test sample and 1.0ml of aliquots of standard formaldehyde dilutions (b) to (g) were transferred to separate test tubes. To each of these tubes, 4.0ml of Distilled water and 5.0ml of acetylacetone reagent were added and incubated in a water bath at 40°C for 40 minutes. The greenish yellow color that developed in the standard formaldehyde solutions were then visually compared with the colour that appeared in the test sample solutions.

RESULT

Media Analysis

The amino nitrogen content of the meat medium was found to be above 0.5mg per ml (Table 1), effective for the cultivation of *Corynebacterium diphtheriae.* In the case of NZ Amine AS medium the amino nitrogen content was fairly high (Table 1). A spectrophotometer estimation of amino acid present in the medium was performed using amino acid (L-Lysine) as a standard (Table 2). The amino acid content of NZ Amine AS medium was double the content of meat medium (Table 3) (Graph 1). The Total Protein content of medium was estimated by Bicinchoninic acid test using BSA as standard (Table 8) and it was observed that the protein was found more in meat based medium (Table 9) (Graph 2).

Table 1
Amino Nitrogen analysis of PDM and NZ Amine AS

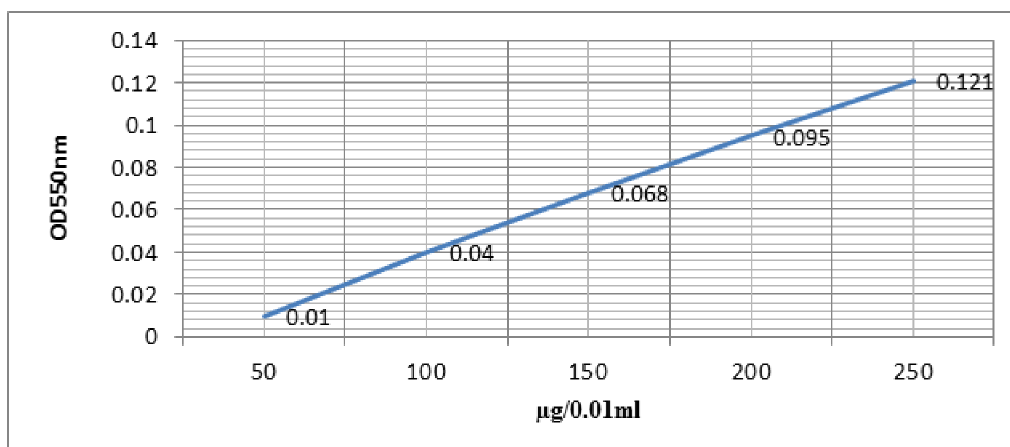
Media	Amino Nitrogen (mg/ml)
PDM	0.8
NZ Amine AS Batch 1	3.08
NZ Amine AS Batch 2	3.64

Table 2
Spectrophotometer Estimation of Amino Acid in PDM and NZ Amine AS

S.No.	Lysine Concentration (µg)	OD 550nm
1.	50	0.010
2.	100	0.040
3.	150	0.068
4.	200	0.095
5.	250	0.121

Standard: L- Lysine

Graph 1
Standard Graph of L-Lysine



(X axis-Concentration of L-lysine; Y axis- OD value of different concentrations of L-lysine)

Table 3
Spectrophotometer Estimation of Amino Acid in PDM and NZ Amine AS

Media	Sample Volume (ml)	OD 550nm	Amino Content µg/0.01ml
PDM	0.01	0.035	75
NZ Amine AS Batch 1	0.01	0.120	250
NZ Amine AS Batch 2	0.01	0.119	240

Culture Analysis

The starter culture used for inoculation of the medium were checked for its purity by Grams staining. A pH range of 7.0 to 7.5 supported the toxin production and the acidic pH of the medium inhibited the toxin production even though the growth was satisfactory. The initial pH of the medium before inoculation was kept in the range of 7.2 and 7.5 and decrease in pH was found during the growth phase. pH adjustment to optimal pH 7.5 to study the Lf with increase in time yielded a better Lf titre (Table 4). Presence of toxin was noted around 12 hours and increase in concentration observed thereafter. The Lf per ml of meat based medium was 70 and that of the NZ Amine AS medium batches was found to be 30 and 50.The NZ Amine AS culture with pH adjustment showed toxin yield close to that of PDM (Table 4,5,6).

Table 4
The growth and production of diphtheria toxin in Papain digest medium with no pH adjustment

TIME (Hr)	Bacterial Purity	pH initial	Opacity	OD (600nm)	Lf (unit)	Kf (minutes)
24	+	6.8	6	1.824	20	11
48	+	6.0	9	2.211	30	8
72	+	6.5	>9	2.602	80	2

After sterile filtration of the toxin the Lf unit was: 70 unit; and Kf: 3min
(+: good; OD-Optical Density; Lf-Lime of flocculation; Kf: Time of flocculation; Opacity- McFarland standards tube.)

Table 5
The growth and production of diphtheria toxin in NZ AMINE AS medium (Batch 1) with adjustment of pH only once.

TIME (Hr)	Bacterial Purity	pH initial	pH final	Opacity	OD (600nm)	Lf (unit)	Kf (minutes)
24	+	6.5	NA	7	1.714	18	15
48	+	6.5	7.5	9	2.1463	35	60
72	+	7.0	NA	>9	2.599	35	6

Note: pH adjusted with 50%NaOH, pH Adjustment to optimal 7.5 pH to study the increase in Lf with time, After sterile filtration of the toxin the Lf unit was: 30 unit; and Kf: 7min, (NA-no addition; +: good; OD-Optical Density; Lf-Lime of flocculation; Kf: Time of flocculation; Opacity- McFarland standards tube)

Table 6
The growth and production of diphtheria toxin in NZ AMINE AS medium (Batch 2) with adjustment of pH at intervals of 6 hours.

TIME (Hr)	Bacterial Purity	pH initial	pH final	Opacity	OD (600nm)	Lf (unit)	Kf (minutes)
18	+	6.3	7.2-7.5	4	0.679	5	Overnight
	+	6.3	7.2-7.5	3	0.614	5	Overnight
24	+	6.5	7.2-7.5	4	0.734	20	Overnight
	+	6.6	7.2-7.5	4	0.778	20	Overnight
42	+	6.3	7.2-7.5	5	1.043	40	9
	+	6.3	7.2-7.5	5	1.041	40	9
48	+	6.8	7.2-7.5	6	1.150	50	5
	+	6.8	7.2-7.5	6	1.176	50	5

Note: pH adjusted with 50%NaOH, pH Adjustment to optimal 7.5 pH to study the increase in Lf with time, Adjustment of pH at intervals of 6 hours showed an increase in Lf, After sterile filtration of the toxin the Lf unit was: 50 unit; and Kf: 5min, (NA-no addition; + : good; OD-Optical Density; Lf-Lime of flocculation; Kf: Time of flocculation; Opacity- McFarland standards tube.)

Toxin Analysis

The antigenic purity of each batch of toxin depends entirely on the Lf and the protein nitrogen content of the toxin. The antigenic purity of the toxin was estimated and found to be above 1000 which is of a suitable range (Table 7). The Total Protein content of toxin produced from meat and NZ AMINE AS media was estimated by Bicinchoninic acid test using BSA as standard (Table 8) and it was observed that the toxin produced from meat based media contain double the protein level of the toxin produced from NZ AMINE AS media (Table 9)(Graph 2).

Table 7
Analysis of Protein Nitrogen, Lf and Antigenic Purity of the toxin prepared in different media

Media	TIME (hr)	Protein Nitrogen (mg/ml)	Lf (unit)	Antigenic Purity
PDM	72	0.0434	70	1612
NZ AMINE-AS-1	72	0.0196	30	1530
NZ AMINE-AS-2	48	0.028	50	1785

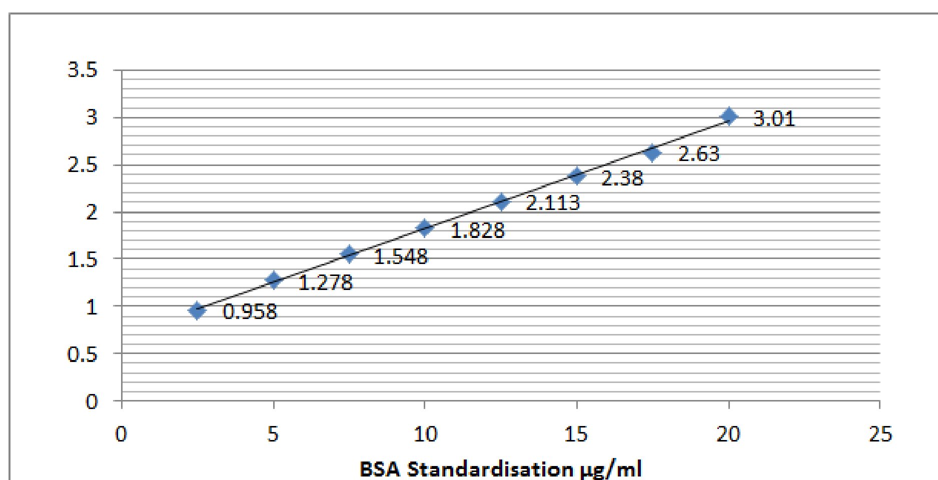
(PDM- Papain Digest Medium, Lf-Lime of flocculation)

Table 8
Estimation of Total protein of PDM, NZ Amine AS media and Toxin

S.No.	BSA Concentration (µg)	OD 550nm
1.	2.5	0.958
2.	5.0	1.278
3.	7.5	1.548
4.	10	1.828
5.	12.5	2.113
6.	15	2.38
7.	17.5	2.63
8.	20	3.01

Standard: Bovine Serum Albumin (BSA)

Graph 2
Standard Graph of BSA

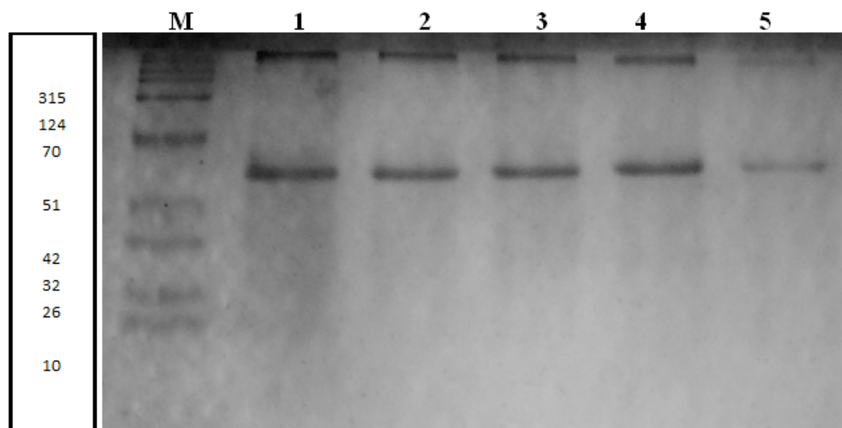


(X axis-Concentration of BSA; Y axis- OD(520nm) value of different concentrations of BSA) (BSA-Bovine Serum Albumin)

Table 9
Estimation of Total protein of PDM, NZ Amine AS media and Toxin

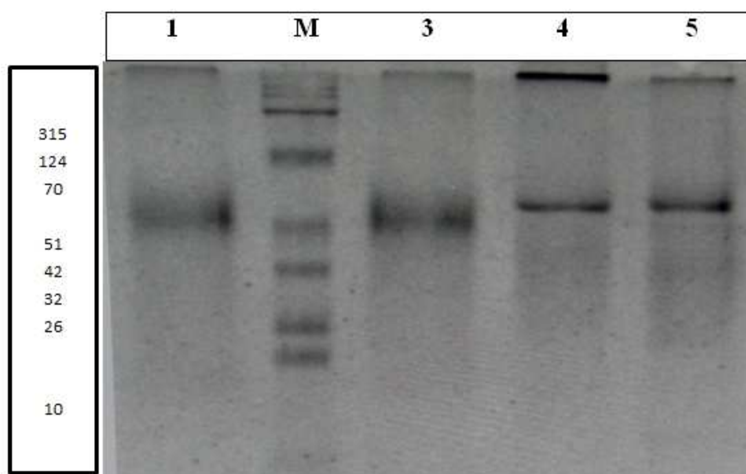
Sample	OD 562nm	Protein Content µg/ml
PDM MEDIA	0.962	2.5
NZ Amine	0.456	<2.5
PDM TOXIN	2.743	18.5
NZ AMINE AS TOXIN 30 LF	1.524	7.0
NZ AMINE AS TOXIN 50 LF	1.507	6.7

The intensity of DTx protein band (58 kDa) observed on SDSPAGE is in correlation with the titer in terms of Lf/ml (15);(ie) an increase in toxin Lf, showed an increase in intensity of the band (Figure 1). On addition of formaldehyde-the detoxifying agent, the individual protein molecules lost their identity and form large molecular complexes within broad molecular mass limits, in agreement to this the Toxoid showed diffused band (Figure 2).



Lane1: protein ladder 10-315 kDa, Lane2: 84hours, Lane3: 48hours, Lane4: 42hours, Lane5: 24hours, Lane6: 18hours. Band observed at 58kDa. The intensity of the band is in correlation with the Lf titre ie. with increase in toxin Lf, intensity of the band increases.

Figure 1
SDS-PAGE of diphtheria toxin samples



Lane1: 30Lf Toxoid, Lane2: protein ladder 10-315 kDa, Lane3: 50Lf Toxoid, Lane 4: 30Lf Toxin, Lane 5: 50Lf Toxin. Toxoid shows diffused band.

Figure 2
SDS-PAGE of diphtheria Toxin and Toxoid

Detoxification Test
In detoxification test the toxoid sample were injected by intradermal route in the shaved region of the guinea pig (Figure 3). Observation at 24 to 48 hours there was no redness or necrosis which showed the absence of toxicity in the sample (Figure 4).

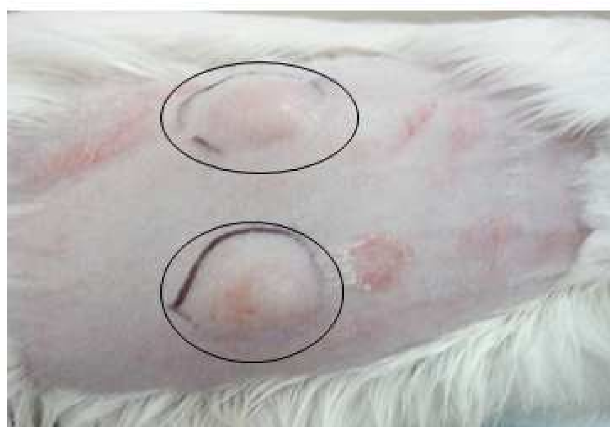
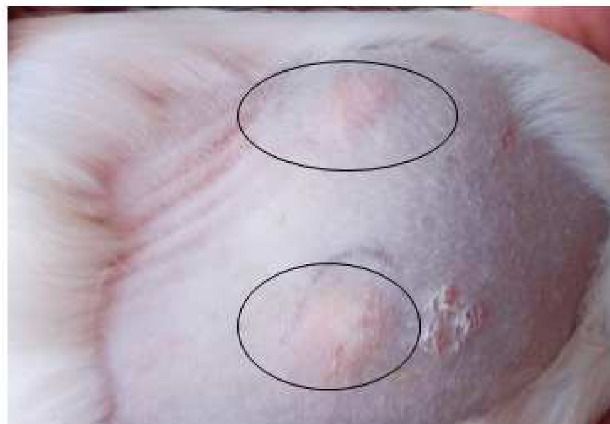


Figure 3
Detoxification Test- intradermal route of injection



no redness or necrosis

Figure 4
Detoxification Test - Observation after 24 to 48hours

Free formalin test
A free formalin test was performed with the Toxoid produced from meat based media and NZ AMINE AS media and a minimal amount of ~8µg/ml (Table 10) which is nonreactive in animals and human (Table 11).

Table 10
Free formalin test-Standard-formaldehyde

S.no.	Formaldehyde	Free formalin Content µg/ml
1	1	3600
2	2	200
3	3	20
4	4	16
5	5	8
6	6	4
7	7	2

Table 11
Free formalin content in PDM & NZ AMINE AS

S.No.	Sample	Test Performed	Result
1	PDM	Free formalin content	~ 8µg/ml
2	NZ AMINE-AS-1	Free formalin content	~ 8µg/ml
3	NZ AMINE-AS-2	Free formalin content	~ 8µg/ml

DISCUSSION

Diphtheria vaccine production follows seed lot system. *Corynebacterium diphtheriae,* Parke William No.8 strain was used for the study. A meat free casein based medium-NZ Amine AS was used as growth medium. For the preparation of Loefflers medium-the revival medium, NZ Amine AS media was used in the place of Papain Digest Medium (PDM) with calf serum in the ratio 1:3, in which cream colored colonies depicting *Corynebacterium diphtheriae* was observed. Thus the presence of undesirable proteins and hazard of variant Creutzfeldt-Jakob Disease (vCJD) as a result of BSE could be avoided[13]. For culturing in NZ Amine AS medium, the shake culture method was followed with agitation rate at 140 rpm with no aeration. Two pH conditions were maintained – A one time pH adjustment to 7.5 with NaOH yielded fairly good titre of diphtheria toxin of Lf unit 30 and a controlled pH range of 7.2 to 7.5 via the addition of NaOH at 6 hours interval, yielded Lf unit 50 which is close to the PDM based toxin Lf unit 70. Though diphtheria toxin production increases with cell growth, the amount of toxin produced is not equivalent to the cell weight[25-26]. Previous studies have reported that the diphtheria toxin concentration of 150~200Lf/ml was obtained with the iron concentration in the range from 0.05 to 0.41 µg/ml and at a pH range of 7.2 [14]. A toxin concentration of 300Lf/ml was obtained in fermentor with 0.7L/min air flow sparging, and agitation rate from 500 and 600 rpm[27]. With reference to previous studies, monitoring of pH is critical in the regulation of toxin production[14,25]. Casein, a phosphoprotein, having the phosphate may be in a bound form, which does not precipitate with calcium chloride. On addition of inorganic phosphate before the calcium chloride forms a calcium phosphate precipitate. Addition of calcium chloride solution of 20% along with neat Phosphate solutions at a Ca/P ratio 2.23 and a 0.1% ferrous sulphate solution (within a range of 0.05-0.3µg/ml) were found to produce good and consistent growth and toxin production. There is no requirement of pH adjustment when aeration is given, provided the other conditions are maintained, as pH adjustment with acid/alkali has an impact on the quality of the toxin.

This article can be downloaded from www.ijpbs.net

(data unpublished). In casein based media the iron is held in loose adsorption complex by the calcium phosphate and released during the growth period. It dissolves leaving no bulky precipitate after 24 hours and releases the adsorbed iron during the period of growth. On the removal of the phosphate precipitate, poor toxin production is observed. Thus the precipitate must be left behind in the final medium[18]. In this study an approach was made to use casein based medium to observe the growth and toxin production of *Corynebacterium diphtheriae*. Apart from the use of NZ Amine AS in culturing and toxin production, the media preparation process is less labor intensive in comparison with the preparation of Papain digest medium (PDM).

CONCLUSION

Based on the results, it was observed that N-Z AMINE AS media with growth factors supported the growth of *Corynebacterium diphtheriae* and the addition of calcium and phosphate of specific volume was essential for the release of toxin. In the present study casein based media was used throughout from seed revival to culturing, without the measurement of maltose utilization and the iron content during the culturing. With the manual adjustment of pH there was more toxin yield compared with non adjusted pH culture. Further standardization of the study can be done using a small scale Fermentor for production of diphtheria toxin. To observe the pH maintenance by montoring of pH with aeration-dissolved oxygen, agitation, or need for addition of acid/alkali to obtain maximum toxin yield as these are critical for an enhanced toxin yield and for large scale production.

ACKNOWLEDGEMENT

Authors are thankful to the Officer and Staffs of the Diphtheria Laboratory of Pasteur Institute of India, Coonoor for their technical discussion and constant assistance.

AUTHORS CONTRIBUTION STATEMENT

Study conception and design:Balaraman Sekar, Bheeman Sundaran, Preethi S. Acquisition of data: Bheeman Sundaran, Preethi S. Analysis and interpretation of data and Drafting of manuscript: Preethi S., Supervision in development of work, data interpretation and manuscript evaluation: Bheeman Sundaran, Manuscript evaluation and Critical revision: Balaraman Sekar.

CONFLICT OF INTEREST

Conflict of interest declared none.

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