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ORIGINAL ARTICLE

Standardization of process for increased production of pure and potent tetanus toxin

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ABSTRACT

Objectives: The aim of the study was to increase the yield of tetanus toxin in short time fermentor cultivation and also to produce pure and potent tetanus toxin replacing initial nitrogen source (N.Z Case) with papain digest broth in the modified Mueller Miller medium (MMMM).

Methods: A fermentor, using a vibromixer and optimum supply of sterile air to the headspace of the fermentor to flush out the accumulated gases was used. The MMMM containing initial N.Z Case was replaced with papain digest broth was used successfully.

Results: It was found that under optimal conditions of temperature, vibromixing, surface aeration, and an alkaline pH favored toxin release. Furthermore, to enhance the production volume, fermentor culture is more suitable. The tetanus toxin was produced with good Limes flocculation (Lf) titre and high antigenic purity. A significant increase in the tetanus toxin yield in short time cultivation (about 5 to 6 days against 8 days) was noticed even with MMMM containing papain digest broth instead of N.Z.Case.

Conclusions: For large-scale production of purified and potent (antigenic purity) tetanus toxin, the use of fermentor technology can be utilized under optimal conditions. The production medium using indigenously available ingredients containing high level of aminonitrogen as in the case of PDM can be substituted in place of N.Z Case, which is being imported and expensive, in addition to lot-lot variation. *J Microbiol Infect Dis 2013; 3(3): 133-140*

Key words: *Clostridium tetani*, modified mueller miller medium, papain digest, limes flocculation

Saf ve potent tetanoz toksin eldesi için standardizasyon işlemi

ÖZET

Amaç: Bu çalışmanın amacı nitrojen kaynağı papain digest broth içeren N. Z Case yerine modifiye Mueller Miller medium (MMMM) kullanarak tetanoz toksin üretiminin artırılabileceğini göstermektir.

Yöntemler: *Clostridium tetani*'nin sabit pot kültürü metodu bir vibromikser ve fermentör boşluğuna steril hava sağlayan düzenek kullanılarak fermentörde kültüre edilmesi esasına dayanan daldırma kültürü metodu ile değiştirildi.

Bulgular: Isı, vibromiksing, yüzey havalandırması ve alkalin pH'nın optimmal şartlarının sağlanması ile toksin salınımı sağlandı. Ek olarak üreme hacminin arttırılmasında fermentör kültürü daha uygun idi. Üretilen tetanoz toksininin Limes flokülasyon (Lf) titresi iyi ve yüksek antjienik saflığa sahip idi. Tetanoz toksin üretiminde N. Z Case yerine modifiye Mueller Miller medium (MMMM) kullanarak kısa süreli kültivasyonla (8 güne karşılık 5-6 gün) belirgin artış sağlandı.

Sonuç: Büyük miktarlarda saf ve potent (saf antijenik) tetano toksin üretimi için optimal şartlarda fermentor teknolojisi kullanılabilir. Saf ve potent tetanoz toksini eldesi için N.Z Case yerine papain dijest broth besiyerinin kullanılabileceğini düşünmekteyiz.

Anahtar kelimeler: *Clostridium tetani*, modifiye mueller miller besiyeri, papain dijest, limes flokülasyon

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INTRODUCTION

Tetanus is a frequently fatal infectious disease caused by highly toxigenic strains of *Clostridium tetani* bacillus. The disease remains as an important public health problem in many parts of the world, particularly in the poorest districts of tropical developing countries, where tetanus morbidity and mortality are dominated by maternal and neonatal tetanus. Tetanus is readily preventable through immunization and tetanus toxoid-containing vaccines, which are included in childhood immunization programs all over the world. In the international market, tetanus toxoid is available as single antigen vaccine (TT), combined with diphtheria toxoid (DT) or low-dose diphtheria toxoid (dT) and in combination with diphtheria and pertussis vaccines (DTwP, DTaP, dTaP or dTap). Tetanus toxoid is adsorbed onto aluminium salts (aluminium hydroxide or aluminium phosphate) to increase its immunogenicity. Tetanus toxoid is stable and can withstand exposure to room temperature for months and to 37°C for few weeks without any significant loss of potency.[1] In most studies, the efficacy of tetanus toxoid vaccine, ranged from 70% to 100%.[2] Hence, immunization with tetanus toxoid is one of the most effective prophylactic procedures to control tetanus and NT in particular in less developed and developed countries.

The classical variant of the strain, *C. tetani* (Harvard strain), and an anaerobic organism is being employed to produce tetanus exotoxin, which is inactivated by formaldehyde to tetanus toxoid yield. The most suitable medium is modified Mueller Miller medium (MMMM), which consistently gives a very good titer.[3] In this study papain digested medium (PDM), normally used for the production of diphtheria toxin has been successfully substituted in place of N.Z. Case to produce tetanus toxin economically under optimal conditions using a fermentor.

The usual way to grow *C. tetani* in artificial medium is by stationary pot culture method. Variation in toxin yield (Lf mL-1) and purity are invariably noticed in this system besides intensive labor and occupation of more space in the incubator room maintained at 35°C. For mass scale immunization program the requirement of final vaccine is very high and hence the stationary pot culture method cannot be economically accomplished. The problems encountered in the stationary pot culture method can be solved by submerging cultivation in fermentor. Processes such as sterile aeration and use of vibromixer were standardized to get high rate of bacterial mass growth and antigenic purity in terms of Lf per mg of nitrogen protein. In the present study, both MMMM and PDM were used in the fermentor of 500L capacity (Bioengineering) to scale up the production of tetanus toxin with high purity and potency.

METHODS

1. Modified Mueller Miller medium

For the preparation of 525 L of modified Mueller Miller medium (4), about 308.25L of distilled water was taken in a tank and the following salts and solutions were dissolved and mixed in the order given below:

No.	Substances	Vol
1.	Glucose	5775 g
2.	Sodium chloride	1312.5 g
3.	Di-sodium hydrogen orthophosphate	525 g
4.	Potassium di-hydrogen orthophosphate	78.75 g
5.	Magnesium sulphate in 25 *L* of distilled water	78.75 g
6.	L-cystine (10 g %)	1312.5 mL
7.	Uracil (25 mg %)	5250 mL
8.	Calcium d- pentothenate (100 mg %)	525 mL
9.	Thiamine (25 mg %)	525 mL
10	Pyridoxine (25 mg %)	525 mL
11.	Riboflavin (25 mg %)	525 mL
12.	Biotin (1.25 mg %)	105 mL
13.	Meat infusion (50%)	26.250 L
14.	N.Z. Case (10% preparations in boiling water and filtered through chain Cloth after the treatment with 200 g of activated charcoal).	131.25 L

The N.Z. Case (Tryptic digest of casein) was supplied by M/s Avonchem and M/s Avondale, England. The pH of medium was adjusted to 7.4 to 7.6 with 40% sodium hydroxide solution. About 400 L of prepared medium was transferred to the fermentor and sterilized at 115°C for 20 min. About 15 L of prepared medium was transferred to each stainless steel pot for steam under pressure sterilization at 115°C for 20 min.

2. Papain digest beef muscles medium

About 25 kg of lean beef was minced and transferred to the digestion kettle, containing 165 L of distilled water. The mixture´s temperature was raised to 50°C. The pH was adjusted to 7.2 and 100 mL of papain solution (20 % in distilled water) was added. The addition of 100 mL of papain solution for seven more times at an interval of 30 min was done after ascertaining the pH of 7.2. The digestion was completed within 3.5 h with adequate stirring during digestion. Then the pH was adjusted to 5.2 with hydrochloric acid and the broth was boiled vigorously for 10 min. The pH was further brought down to 4.5 with glacial acetic acid and filtered hot through chain Cloth. Samples were collected for amino ni-

trogen estimation by the Sorensen titration method.[4] This medium was used in place of N.Z. Case (tryptic digest of casein) and mixed with other ingredients as in the case of MMMM.

3. Preparation of seed inoculum

The seed lot system was used in the preparation of master seed copies of *C. tetani*. The working seed copies were also prepared in a lyophilized form and stored at 8°C. The seed copy was revived in alternate thioglycollate medium, incubated at 35°C for 48 h. Further sub-culturing was done in heart infusion glucose broth, duly incubated at 35°C for 24 h. An inoculum of 1% was used in stationary pot culture method as well as in fermentor culture method.

4. Stationary pot culture method

Presterilized stainless steel pots having smoothed inner surface and corners with a total capacity of 20 L were used as production vessels and about 15 L of production media was taken in each pot. These pots were covered with a layer of non-absorbent cotton wool of about one inch thick, held between two layers of muslin cloth and tied with thread at the pots´neck to create anaerobic condition. A small glass tube of about 10 cm long with 15 mm diameter, open at both ends was passed through the center of the covering pad and held in position by rubber washers which fitted snugly to the tube. The open end of the glass tube above the pad was plugged with cotton wool.

5. Fermentor (Bioengineering) culture method

The fermentor vessel of 500 L capacity (M/s Bioengineering ltd) was used. The top plate harbors a vibromixer. The vibromixer is an electromagnetic stirrer, which generates a vertical vibration. The vibration corresponds to the frequency of the AC-current (50 or 60 Hz). The stirrer moves up and down at a rate of 50 times (50 Hz) or 60 times (60 Hz) per second. The vibrostirrer consists of a shaft with a centrically mounted stirrer disc. The stirrer disc is perforated with round conical form holes. The stirring and pumping effect depends on which way the narrow end of the cone points. The stirring and pumping effect is upwards in case of cones pointing upwards and vice versa in case of cones pointing downwards (Fig. 1). In case of cone pointing upward the stirring and pumping effect is upwards. The operative range is blending, emulsifying and homogenization of liquids. In case of the cone pointing downward, the stirring and pumping effect is downwards. The operative range is dissolving solid matter in a liquid and keeping solid matter in suspension.

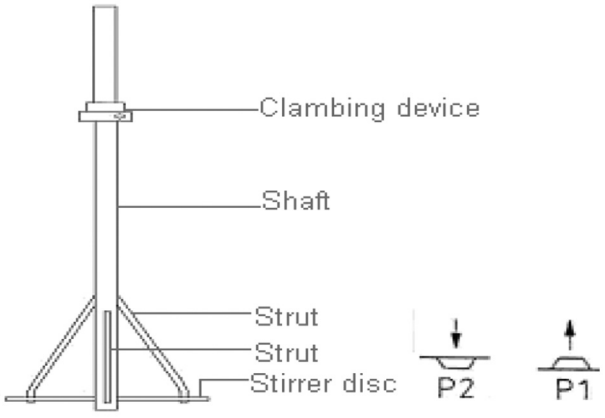

Figure 1. Schematic diagram of vibromixer with P1 and P2 stirrer discs.

6. Optimization of culture parameters

For stationary pot culture, stainless steel pots containing 15 L production medium (MMMM and PDM) inoculated with the seed were incubated in a hot room maintained at 35°C for seven days. During the cultivation the gases evolved were apparently let out using exhaust fan provided in the culture hot room. Since *C. tetani* is an anaerobic organism, there was no supply of filtered sterile air and no buffers addition into the pot culture during the cultivation period. After ascertaining the sterility, tetanus toxin from the pot cultures was harvested on the 8th day after seed inoculation. For fermentor culture method, both the production media were used. The quantity of medium to be sterilized in the fermentor is about 400 L. During sterilization the vibromixer was adjusted to 190V and the medium was sterilized at 115°C for 20 min. The production media (MMMM and PDM) in the fermentor and in the stainless steel pots were immediately cooled down soon after sterilization. The seed inoculum in heart infusion glucose broth (24 h) was used at the rate of 1% for both the culture conditions. The agitations using vibromixer and the rate of supply of sterilized, filtered air for the fermentor culture system during submerged cultivation of C .tetani were standardized. The temperature was maintained at 35°C and the pH was constantly recorded. The vibromixer was adjusted so that the rate of vibration was very minimal to nil on the day of toxin harvest from fermentor culture. Sampling was done on the 7th and 5th day in the case of stationary pot culture system and fermentor culture system, respectively. After ascertaining the Lf/mL of the toxin samples, the fermentor culture was harvested by sterile filtration at the end of the 5th day or beginning of 6th day and the stationary pot culture was harvested on the 8th day of culture.

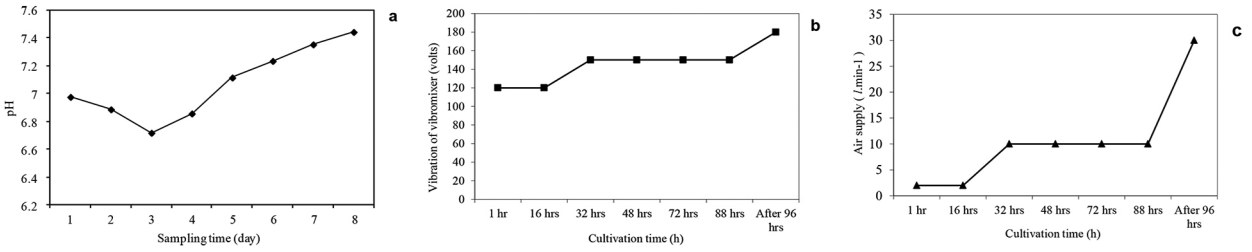

Figure 2. (a) pH of the fermentor culture on different days. (b) Status of vibromixer on different days of fermentor culture. (c) Sterile air supply at flow meter reading on different days of fermentor culture.

7. Production media analysis

The amino nitrogen content for MMMM and PDM was determined by the Sorensen titration method. Since the content of iron is not that critical for the production of tetanus toxin, the iron content of both the production media was not controlled, though both the production media contained iron to the extend of 10-20 ug/100 mL of medium.

8. Culture analysis

Samples from the stationary pot cultures and fermentor cultures were drawn at different time intervals to determine the optical density of the bacterial mass using Beckman spectrophotometer. The other parameters analyzed were pH and toxin flocculation unit (Fig. 2a, b, and c).

9. Toxin analysis

The total protein nitrogen content of the toxin is determined by the method described by Kjeldahl.[6] The ratio of toxin concentration (Lf/mL) to protein nitrogen concentration (mg PN/mL) denoted the antigenic purity of toxin and was expressed as Lf per mg of protein nitrogen. The pH of the medium prior to inoculation and harvesting was also recorded.

DISCUSSION

The amino nitrogen content of papain-digest of beef was on an average of 65mg/100 mL and that of N.Z Case was about 210 mg 100 mL-1. The MMMM containing N.Z Case yielded on an average 66 Lf mL-1 and 44 Lf mL-1in fermentor culture and stationary pot culture, respectively. The production medium containing papain digest of beef yielded on an average 64 Lf mL-1 and 44 Lf mL-1 in fermentor culture and stationary pot culture, respectively. This clearly showed that the indigenously prepared production medium containing papain digest of beef was found to be equally good for the production of tetanus toxin, in spite of the fact that the amino nitrogen content is far low. It was observed from our experience that the optimal level of amino nitrogen is essentially required to get a good Lf titer. However, high level of amino nitrogen content has no impact on the production of the toxin. Our earlier studies have indicated that the quality of papain is directly attributed to the level of amino nitrogen in the digested beef muscles and variation from batch to batch. The papain digest of beef muscles was prepared according to the procedures laid down by WHO and as suggested by Stainer6 and ensured that the PDM did not contain any extraneous substances of bovine origin, responsible for allergic reaction. The papain digest of beef with the addition of hydrochloric acid to bring down the pH to 5.1 for boiling resulted in the degradation of peptides and devoid of bovine protein.

The use of PDM in place of MMMM was mooted in order to cut down the cost of production. For the preparation of MMMM, pancreatic digest of casein is required, which is expensive and is being imported to India in the trade name of N. Z. Case. The preparation cost of one liter of PDM is about 0.30 US$ whereas the preparation cost of one litre of 10% N. Z Case solution is about 6 US$. For the preparation of final production medium, the quantity of papain digest broth to be used is about one and a half times more than the quantity of 10% N. Z Case solution. Since this PDM is already in use for the production of diphtheria toxin the same was tested for its suitability in producing tetanus toxin and used accordingly.

C. tetani can easily be cultivated in enrichment culture medium, especially when these are supplemented with reducing substances at a neutral or slightly alkaline pH. Growth is usually accompanied by the active formation of gas and is associated with a characteristic fetid odor, reminiscent of that of volatile fatty acids.[7] *C. tetani* is not proteolytic but gelatin is slowly liquified. The glucidolytic activity is strongly dependent on the strain. The breakdown of glucides is accompanied by escaping gases, without any concomitant acidification of the medium. Glu-

cose and maltose are actively fermented, whereas sucrose and galactose are only weakly fermented. During the production of tetanus toxin, there is production of CO_2, H_2, NH_3, H_2S, indole, acetic acid, butyric acid, ethanol, and butanol. The accumulation of these gases, hamper the further growth of the organisms. It is important that gasses should be allowed to escape freely from the culture, and for this reason, Mueller and Miller used wide-mouthed vessels (cylinders holding 6L of medium). Any technical process incorporated in the fermentation vessel to flush out these gases is useful in getting high titer of tetanus toxin. In this study, the supply of sterile air was continuously made to this fermentor to flush out the gases that come up to the headspace of the fermentor while the culture being continuously agitated by vibromixer. Thomson[8] grew 70 liter cultures in 80 liter tanks with continuous stirring. The speed of the vibromixer, in this study was adjusted according to the level of gases production during log phase culture (Table1).

The main source of carbon in the production medium was dextrose anhydrous purified. The growth pattern, change in the pH and toxin yield were investigated. The change in the pH was due to the production of organic acid from readily available dextrose in the broth. The maintenance of neutral pH through out the culture period indicated normal microbial growth and toxin production. The vibromixer helped in the lysis of the bacterial mass and in the escape of gasses from the culture to the headspace in the fermentor to be flushed out during aeration.[5]

Table 1. Optimal level of vibromixer and aeration used in fermentor culture

Hours	Agitation (in volts)	Air supply(in / min^{-1})
During sterilization	190	0
Up to 16 hrs	120	2.0
After 16 hrs	150	10
After 96 hrs	180	30

The growth of *C. tetani* and toxin production was compared between stationary pot culture and fermentor culture using MMMM and PDM. The growth and toxin production were significantly higher in the fermentor culture than in the stationary pot culture. In stationary pot culture and fermentor culture there was no significant difference in the OD value of the culture growth in different days. However, toxin yield was on the higher side of the fermentor culture (70 Lf mL-1 for MMMM and 60 Lf mL-1 for PDM). In stationary pot culture the Lf mL-1value was 40 for both MMMM and for PDM, even though the OD value for the bacterial growth was found to be equivalent to that of in fermentor culture. It was ascertained that in addition to bacterial mass, for the production and release of toxin, factors such as agitation by vibromixer, optimal level of air supply, favorable pH and conductive temperature had a definite impact on the lysis of the bacilli and release of toxin to the surrounding medium. The cultivation time was considerably less (5 to 6 d) than cultivation time for stationary pot culture (8 d) (Table 2 and 3).

Table 2. Growth rate, pH and toxin yield in stationary pot culture

Medium	Parameters	Sampling time (d)							
		1	2	3	4	5	6	7	8
MMMM	pH	6.97	6.88	6.71	6.85	7.11	7.23	7.35	7.44
	Growth (OD)	0.91	1.53	1.55	1.55	1.58	1.44	1.41	1.41
	Lf titre	NT	NT	NT	NT	30	30	40	40
	Flocculation time (kf) in min	ND	ND	ND	ND	10	9	9	9
PDM	pH	6.95	6.90	6.81	6.87	6.95	7.20	7.26	7.27
	Growth (OD)	0.87	1.23	1.46	1.51	1.55	1.45	1.43	1.43
	Lf titre	NT	NT	NT	NT	30	40	40	40
	Flocculation time (kf) in min	ND	ND	ND	ND	10	9	9	9

NT, No toxin; ND, Not detected

Table 3. Growth rate, pH and toxin yield in fermentor culture

Medium	Parameters	Sampling time (d)							
		1	2	3	4	5	6	7	8
MMMM	pH	7.14	6.88	6.75	6.83	7.31	7.42	7.45	7.55
	Growth (OD)	1.62	2.23	2.25	2.26	2.29	2.14	2.05	2.00
	Lf titre	NT	NT	NT	NT	60	70	70	70
	Flocculation time (kf) in min	ND	ND	ND	ND	7	6	6	6
PDM	pH	7.21	6.90	6.81	6.66	7.01	7.21	7.30	7.31
	Growth (OD)	1.51	1.78	2.10	2.11	2.20	2.15	2.02	1.90
	Lf titre	NT	NT	NT	NT	50	60	60	60
	Flocculation time (kf) in min	ND	ND	ND	ND	8	7	7	7

NT, No toxin; ND, Not detected

Table 4. Toxin yield and antigenic purity obtained in stationary pot and fermentor cultures in different production media

Culture method	Medium	Trial	Lf/ml	PN2 mg/ml	Antigenic Purity (Lf/mg PN)
Stationary Pot Culture	MMMM	1	40	0.066	606
		2	50	0.071	704
		3	40	0.059	678
		4	50	0.073	685
		5	40	0.056	714
Stationary Pot Culture	PDM	1	40	0.067	597
		2	40	0.063	635
		3	40	0.059	678
		4	50	0.070	714
		5	50	0.069	725
Fermentor Culture	MMMM	1	60	0.063	952
		2	70	0.071	986
		3	70	0.075	933
		4	70	0.069	1014
		5	60	0.070	857
Fermentor Culture	PDM	1	70	0.073	959
		2	60	0.066	909
		3	60	0.063	952
		4	70	0.073	959
		5	60	0.064	938

The antigenic purity of the toxin produced in fermentor culture and stationary pot culture methods using MMMM and PDM for five independent trials was analyzed (Table 4).

The antigenic purity average (Lf mg-1 PN) in stationary pot culture was 677.2 and 669.8 for MMMM and PDM, respectively, whereas in case of fermentor culture the antigenic purity average (Lf mg-1 PN) was 948.4 for MMMM and 943 for PDM. The antigenic purity of toxoid directly determines the quality of the product to be immunogenic. The WHO requirement of antigenic purity for the concentrated and purified tetanus toxoid should be more than 1000 Lf mg-1 of protein nitrogen.[9] The toxins thus prepared in fermentor culture after concentration and purification is bound to have a very high antigenic purity, as they are already purer in crude status. This is of primary importance as for as the post vaccinal reactions are concerned. From these observations it was ascertained that purified

and high quality tetanus toxins could be prepared using fermentor culture system having provision for vibromix and sterile air supply. Even though the OD of tetanus bacilli growth in stationary pot culture method was almost equal to that of fermentor culture, yet the toxin yield was very low in stationary pot culture compared to fermentor culture. The production and release of exotoxin after lysis of the tetanus bacilli were undoubtedly influenced by agitation and aeration.

In this study the PDM was successfully used as a substitute for MMMM with less cost of production. It was observed that some of the lots of N.Z. Case yielded good titer of toxin and some lots were poor in the toxin production. N.Z. Case lots procured from two different manufacturers were used in different batches of toxin production. During our earlier study (unpublished data), it was observed that simple chemical analysis of various lots of N.Z. Case did not explain why some lots should be good and others poor. Individual lots showed differences in iron and phosphate levels but there was no significant overall pattern related to these two substances (Table 5). N.Z. Case is prepared by digestion of casein with a mixture of enzymes. Any differences in enzyme composition (particularly relative to levels of proteinases and peptidases) could result in alteration in the nitrogen distribution between long chain peptides and free amino acids. Analysis of the ratio of total nitrogen to amino nitrogen is indicative of the average number of amino acid residues per peptide molecule and consequently of the degree of hydrolysis. Latham[10] pointed out that the effect of heat input to the medium during autoclaving was an important physicochemical factor in the toxigenecity of tetanus culture. This factor was also ascertained from the fact that there was no proper growth in the production medium when the medium was sterilized by sterile filtration using Seitz filter in our earlier studies.

The influence of medium composition on the growth and metabolism of bacteria has been well known. It is imperative to know not only the growth of the bacteria but also the production of specific metabolites. Mueller was using a mixture of two complex materials, beef heart infusion and N.Z Case. Latham's work resulted in the removal of one of these (beef heart infusion), only to emphasize how toxin yield seemed to depend on lot-to-lot reproducibility of N.Z Case.[10] The amino nitrogen content of N.Z Case did not truly indicate the quality as in the case of poor lots of N.Z Case, which did not yield good Lf titer. The lot-to-lot variability of N.Z Case was noticed as described in Table 5. Since the N.Z Case is being imported the variability during enzyme digestion could not be ascertained.

Table 5. Chemical analysis of good and poor lots of N.Z. Case and toxin yield in pot culture

Lot No. N.Z. Case	% Free Phosphate	Free Iron (ppm)	Alpha amino Nitrogen mg/ml	Lf/ml
Good Lots				
AC-1	0.31	16	2.33	40
AC-2	0.34	20	2.10	40
AC-3	0.36	23	2.10	40
AC-4	0.40	24	2.41	50
AC-5	0.32	21	2.32	50
Poor Lots				
AD-1	0.31	16	2.22	30
AD-2	0.34	20	2.31	30
AD-3	0.36	23	2.33	20
AD-4	0.40	24	2.10	20
AD-5	0.32	21	2.10	30

Optimal production of tetanus toxin depended on various factors such as the fermentor design, proper blending, emulsification and liquid homogenation, dissociation of solid matter in a liquid, the status of solid matter in suspension using vibromix, sterile aeration to flush out the accumulated gasses and the quality of raw ingredients in the medium. In this study besides the complexity of raw materials, a direct link between the processes such as aeration and agitation using vibromix and the release of tetanus exotoxin into the surrounding medium was demonstrated and confirmed. The submerged fermentor culture was found to be more suitable than stationary pot culture for obtaining toxin of high Lf titer and antigenic purity. Toxins obtained from fermentor culture had very high minimum lethal dose (MLD) and after detoxification with formalin treatment and purification by ultra filtration and fractional precipitation with ammonium sulphate yielded toxoid of high purity and potency, meeting the requirements of Indian Pharmacopoeia (unpublished data).

In conclusion for large-scale production of purified and potent (antigenic purity) tetanus toxin, the use of fermentor technology as described above can be utilized with minimal cost. It is also observed that any production medium using indigenously available ingredients containing optimum level of aminonitrogen as in the case of PDM can be substituted in place of N.Z Case, which is being imported and expensive, in addition to lot-lot variation.

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