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Optimization and comparative analysis of meat infusion in muller medium for the production of effective tetanus toxin

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Optimizations of fermentation medium for *Clostridium tetani* (MTCC 449) in the effective production of tetanus toxin were studied. Modified Muller Media with different concentration of Meat infusion has been used for the production of tetanus toxin . In this present work, the comparative study was carried out to determine the efficacy of tetanus toxin with the commercially prepared meat infusion broth compared and the meat infusion powder supplemented in Muller medium. The observed results showed the higher titer value of toxin in Muller media supplemented with meat infusion with higher concentration. The OD of the cell growth and pH changes due to buffering capacity were also studied. The better growth of the *C.tetani* was observed in the MM medium which contain higher rate of meat infusion broth. The level of the tetanus toxin was measured by Lf test. Minimal lethal dose was performed to check the presence of tetanus toxin.

Key words : Tetanus toxin, *Clostridium tetani*, Fermentation medium

INTRODUCTION

Tetanus is a life-threatening disease caused by infection with *Clostridium tetani* (Wassilak *et al.*,1994). *C. tetani* is a gram positive, spore forming, motile, anaerobic bacillus. The most common source of the environmental exposure to *C.tetani* are bacilli and spores in the soil, where the organism is widely but variably distributed. Typically measuring 0.3 to 0.5 micro meter in width and 2 to 2.5 micro meter in length, the vegetative form often develops long filament like cells in culture. Toxin has been traditionally prepared by growth of *C.tetani* in media containing animal and dairy products as a source of proteins, peptides and amino acids need for good growth. The growth medium traditionally used to produce tetanus toxin is MM (Mueller and Miller,1954),which contain glucose, beef heart infusion, a pancreatic digest of glucose, some amino acids and vitamins, uracil and inorganic salts. Mueller and Miller (1955) isolated three types of acid- labile components from casein digests by resin treatment, all of which were necessary for good toxin production, they were not chemically identified, however, meat infusion broths are good broths but the amount of peptone in them makes them expensive compared with digest broths.

The type of meat used is an important factor in determining the quality of the broth. It should be fresh, not frozen. Commercially available meat is used for the preparation of meat infusion broth. It makes costly and time consuming process. When the meat infusion broth is used in the media, the toxin production will vary depending upon the meat quality and health of the animal. So in order to maintain the consistency, use readymade meat infusion powder in the medium. The meat infusion powder contain 12% total nitrogen content. It reduce the time, cost and it make standard quality of meat protein. Since an infusion of beef heart is an essential component of the culture medium, the possibility exists that variations in different batches of this material may account for differences in toxin produced. Earlier work on the growth requirements of *C. tetani* (Feeney *et al.*, 1943) have shown the need for a fairly complex assortment of vitamins and extractives. The toxin is one of the most potent known poisons on a weight basis. As little as 1 nano gram / kg may kill a mouse; and 0.3 nano gram/kg will kill a guinea pig. The estimated minimum human lethal dose is less than 2.5 nano gram.

C. tetani produces two exotoxins, tetanolysin and tetanospasmin. Tetanus vaccines are based on tetanus toxoid. Conventional production includes growth of toxigenic strains of *C.tetani* in a liquid medium that favors toxin production, toxin harvest by filtration, detoxification by formaldehyde and several steps of purification and sterilization. The efficacy of the toxin has been analyzed by the antitoxin and the MLD test.

MATERIALS AND METHODS

Seed strain:

The strain of *Clostridium tetani* (MTTC 449) was obtained from Microbial Type Culture collection (MTTC)

center, Chandigarh. The strains were maintained in lyophilized state which ensures a long working life with the ability to yield potent vaccine. The seed copy was stored at 4^0C.

Seed revival and sub culture:

The seed was revived in Alternate Thioglycollate medium at 35^0C for 48 hrs. Further sub culture was prepared in Heart Infusion Glucose broth. The tubes with Heart infusion Glucose broth were kept in boiling water for 10 mins, rapidly cooled to 35^0C and taken for inoculation. The 24 hour culture was taken for inoculating into production medium.

Preparation of meat infusion broth:

The required amount of cleaned beef meat was taken and transferred into pots. The distilled water was added and kept at 4^0C for over night. The contents was transferred from the pots to the digestion kettle on the next day. Then added the distilled water, Increased the temperature gradually till the materials started boiling. Boiled for 20 mins. The material was filtered through the chain cloth. Finally the pH was adjusted to 7.4 to 7.6 using 40% sodium hydroxide solution and sterilized at 115 to 117 ^{0}C for 20 min. then stored at 4^0C.

Production of toxin:

MM medium contain meat infusion broth:

One litter Muller's medium was prepared in a two litre beaker which contain different concentration (40ml,50ml and 60ml) of commercially prepared meat infusion broth. The initial pH was adjusted to 7.4. The medium was sterilized at 120^0C for 20 mins. The sterilized medium was rapidly cooled to 35^0C by kepping the beaker in running cold water and taken for inoculation. The 1% of 24hr culture in HIGB was inoculated into the production medium. The beakers were kept at 35^0C keepng for 7 days. The OD was taken at 670nm and the pH variation was observed for all the 7 days.

MM medium contain meat infusion powder:

The HIMEDIA meat infusion powder RM192 was used for the meat infusion in the Muller medium. One litre Muller's medium was prepared in a two litre beaker which contain different concentration (40g,50g and 60g) of meat infusion powder. The initial pH was adjusted to 7.4. The medium was sterilized at 120^0C for 20 mins. The sterilized medium was rapidly cooled to 35^0C by keeping the beaker in running cold water and taken for inoculation. The 1% of 24hr culture in HIGB was inoculated into the production medium. The beakers were kept at 35^0C for 7 days. The OD was taken at 670nm and the pH variation was observed for all the 7 days.

Purity test:

Two tubes of Nutrient agar slants were inoculated with 2 to 3 drops of *Clostridium tetani*(MTTC 449) culture and incubated at 35^0C. Everyday the slants were observed.

Harvesting of toxin and precipitation of proteins:

At the seventh day the culture was harvested and centrifuged at 2500 rpm for 10 min. The supernatant contain the tetanus toxin. The 4.5ml of toxin was added with 0.5ml of 50%TCA and kept it in hot water bath for 5 mins.

Estimation of limes flocculation/ml(Lf/ml):

The toxin was distributed into 5 or 6 flocculation tubes in 1ml amount. Graded doses of standard antitoxin (100Lf/ml) were added. The contents were mixed properly by inverting the flocculation tubes 3 times and kept in a water bath maintained at 50^0C. The tubes which contain the optimum concentration of toxin and the antitoxin flocculates first and corresponding unit age of the antitoxin was taken as flocculation unit (Lf value) of the toxin. The time taken for flocculation was noted as Kf . This served as an useful indicator for testing quality of the toxin. If the Kf is short (below 10 mins) the quality of the toxin is good and *vice versa*.

Test for minimum lethal dose (MLD):

The quality of toxin was checked by minimum lethal dose (MLD).One ml of the culture supernatant was added to tube number 1 containing 9ml peptone water and mixed well. One ml from tube number 1 was transferred to tube number 2 containing 9ml peptone water and mixed well. One ml from the previous tube was transferred to the next tube until it reached tube number 6. All tubes contain 9 ml peptone water. From the tube number six, 5ml of mixture was transferred to the tube number 7 containing 5ml peptone water. One ml of each dilution from tube number 4 to tube number 7 was inoculated subcutaneously under the loose skin of the hind leg into two mice. The dilutions of the supernatant in the tubes were 10^{-4},10^{-5},10^{-6} and 2x 10^{-6}. Two lacca mice of 17-20gm each in weight were inoculated subcutaneously with 0.1ml of sample from tube number 5 to 9. The animals were observed for 4 days.

RESULTS AND DISCUSSION

Muller medium was prepared with different concentration

of meat infusion to determine their need for growth of *Clostridium tetani* (MTCC 449). The Table 1 shows the growth rate of *C. tetani* in MM medium at different concentrations of meat infusion broth. Table 2 shows the buffering capacity of Muller medium which contains different concentration of the meat infusion broth. The initial pH was adjusted to 7.4.The pH value was decreased upto 5th day in all concentration of meat infusion broth present in the Muller medium. The better growth of the *C.tetani* was observed in the MM medium which contained higher rate of meat infusion broth. On 6th and 7th day it showed the increased pH value and it described the lysis stage of *C.tetani* in the medium. The Table 3 and Table 4 showed the growth curve and buffering capacity of *C.tetani* in Muller medium which contained different concentrations of the meat infusion powder. The higher concentration of the meat infusion powder in the MM medium showed the higher growth rate of *C.tetani*. On 5th day of fermentation which reached the maximum growth of *C.tetani*. The lysis stage of the *C.tetani* was obtained on 5th and 6th day of fermentation. No growth was observed in the purity test. The protein was precipitated by the tetara chloride acetic acid.

Table 1 : Growth of *Clostridium tetani (MTCC 449)* at various concentration of meat infusion broth in MM medium

Days	40 (ml)	50 (ml)	60 (ml)
0th	0.81	0.81	0.81
1st	1.13	1.15	1.12
2nd	1.19	1.26	1.28
3rd	1.27	1.38	1.32
4th	1.36	1.55	1.36
5th	1.27	1.31	1.22
6th	1.21	1.18	1.13
7th	1.08	1.05	1.03

Table 2 : Buffering capacity of *Clostridium tetani (MTCC 449)* in MM medium at various concentration of meat infusion broth

Days	40 (ml)	50 (ml)	60 (ml)
0th	7.40	7.40	7.40
1st	7.24	7.21	7.20
2nd	7.19	7.14	7.10
3rd	6.93	6.88	6.82
4th	6.83	6.71	6.68
5th	6.23	6.18	6.13
6th	7.29	7.36	7.42
7th	7.38	7.46	7.47

Table 3 : Growth of *Clostridium tetani* at various concentration of meat infusion broth in MM medium

Days	40 (g)	50 (g)	60 (g)
0th	0.81	0.81	0.81
1st	1.13	1.16	1.18
2nd	1.19	1.24	1.28
3rd	1.27	1.36	1.39
4th	1.36	1.48	1.42
5th	1.32	1.31	1.36
6th	1.28	1.26	1.29
7th	1.23	1.21	1.19

Table 4 : Buffering capacity of *Clostridium tetani (MTCC 449)* in MM medium at various concentration of meat infusion broth

Days	40 (g)	50 (g)	60 (g)
0th	7.40	7.40	7.40
1st	7.24	7.21	7.19
2nd	7.18	7.12	7.09
3rd	6.98	6.96	6.95
4th	6.96	6.89	6.85
5th	7.09	6.96	6.65
6th	7.12	7.21	7.29
7th	7.20	7.32	7.38

Table 5 and Table 6 show the Lf value of tetanus toxin. The level of the tetanus toxin was measured by adding a standard antitoxin, and measuring the elapsed time before flocculation. The higher concentration of the meat infusion broth and powder in the MM medium showed the higher titter value of the toxin. The antitoxin react with the toxin prepared by the MM medium which contain different concentration of meat infusion broth was formed flocculation faster than the toxin prepared by the MM medium which contain different concentration of meat infusion powder. The MLD test was carried out tetanus toxins prepared by MM medium. The toxin prepared from the MM medium contain meat infusion broth showed paralysis within 16hrs in all the concentrations. The limp and tail paralysis were observed.

Table 5: Formation of flocculation with the tetanus toxin prepared from MM medium with the different concentration of meat infusion broth

Meat infusion Broth (ml)	Lf value	Kf in minutes
40	40/45	8/9
50	45/50	7/9
60	50/55	8/9

Table 6 : Formation of flocculation with the tetanus toxin prepared from MM medium with the different concentration of meat infusion broth

Meat infusion Broth (ml)	Lf value	Kf in minutes
40	35/40	9/11
50	40/45	9/12
60	40/45	8/9

But the tetanus toxin prepared from MM medium contain meat infusion powder, only showed the paralysis within 16hrs in the higher concentration of meat infusion powder. Finally, death occurred which indicated the presence of toxin. It is conformed that the stain was able to produce the tetanus toxin. The results showed that the meat infusion broth present in the Muller medium had the higher titer value of toxin than the meat infusion powder.

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REFERENCES

Feeney, R. E., Mueller, J. H., and Miller,P. A. (1943). Growth requirements of *Clostridium tetani*. III. A "synthetic" medium.*J. Bacteriol*, **46** : 563-571

Muller, J. Howard and Miller, Pauline A. (1954). Variable factors influnceing in the production of Tetanus Toxin. *J. Bacteriol.*, **67**(3) : 271–277.

Muller, J. Howard and Miller, Pauline, A. (1955). Separation from tryptic digests of casein of some acid-labile components essential in Tetanus Toxin formation. *J. Bacteriol.*, **69**(6) : 634–642.

Wassilak, S.G. F., Orenstein, W. A. and Sutter, R. W. (1994). Tetanus toxoid. In: Vaccines, S. A. Plotkin and E. A. Mortimer, eds., Philadelphia: Saunders, pp. 57-90,