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Bioscience Research

Print ISSN: 1811-9506 Online ISSN: 2218-3973

Journal by Innovative Scientific Information & Services Network

RESEARCH ARTICLE BIOSCIENCE RESEARCH, 2018 15(1): 498-504. **OPEN ACCESS**

Factors affecting capability of toxin production of lyophilized *Clostridium tetani* (Harvard Strain)

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In order to preserve the characters of the highly toxigenic *C.tetani* (Harvard strain) as long as possible, a combined approach of pre-lyophilization treatment of microorganisms and subsequent storage was developed in order to improve cell survival and toxin production capability. Different stabilizers have been tried in combination with different storage temperatures. The study revealed that -20 °C is the best temperature for storing *C.tetani* (Harvard strain) and that stabilizer III (modified Pivnick) is the best stabilizer for lyophilization of *C.tetani* (Harvard strain) as it preserve the strain toxigenic capability till the 19th month post lyophilization with minimum reduction in toxin titer.

Keywords: ***C.tetani*** **- Harvard strain – Lyophilization – Stabilizer.**

INTRODUCTION

Tetanus is a life threatening disease, and is one of the most dramatic and globally prevalent diseases of human and animals caused by infection with *Clostridium tetani* (*C. tetani*) which is a Gram positive obligatory anaerobic spore forming bacillus showing terminal spores giving it the characteristic drum stick appearance. The organism is widely distributed in soil and large intestine of animals particularly equines. Spores of *C. tetani* are present in the large intestine of equine, cattle , dogs , cats , rats , chicken and nearly 10% of human (Bhatia et al. , 2002).

Tetanus toxin has been produced for many years by cultivation of C. *tetani* on complex medium consisting of enzymatic digests of beef meat and liver. Highly toxigenic C. *tetani* strain has been selected and special digests have been developed (NZ case, tryptose T or Ts) for large scale production of the toxin in high yields (Relyveld, 1996).

Tetanus toxin has been traditionally prepared by growth of *C. tetani* in Mueller and Miller medium (Mueller and Miller, 1954) or Latham medium (Latham et al., 1962).

Tetanus toxin is produced and inactivated, usually with formalin, to produce the toxoid. Immunization is completely effective and thus tetanus is rare in developed countries where vaccination rates can be as low as 20% (WHO, 2003).

Preservation of microorganisms is based on suspending the microorganisms in an anabiosis state, where metabolism activity is much lower. In this state, the bacteria can be stored away for longer time without the need of food. Drying works on the concept of lowering moisture content of bioproducts, usually to approximately 5–8%, where biodegradation that might be caused by activity of microorganisms, enzymes, or non-enzymic chemical reactions is inhibited. Moreover, putting microbiological cultures in dry state enables easier transportation, storage, reduced odor issue and even quality control of certain microbial cultures. (Tan et al., 2017)

Media that contain cryoprotectants such as carbohydrates and proteinaceous compounds are necessary to prevent death of bacterial cells during freezing. Sugars penetrate into the cells

and create a high osmotic pressure which impedes formation of ice crystals and cell destruction during the freezing process (Chervyakova et al., 2014).

The survival of six strains of bifido bacteria was analyzed using different concentrations of cryoprotectants in the process of their lyophilization. Sucrose and glucose at concentrations from 2 to 10% were tested as cryoprotectants. Glucose or sucrose at a concentration of 5% and above was the most effective cryoprotectants. (Kharchenko et al., 2017)

The present study is an attempt to find out a suitable cryoprotectant for the efficient lyophilization of the highly toxigenic C.tetani Harvard strain and determining the best temperature for its preservation after lyophilization.

MATERIALS AND METHODS

Strain:

Clostridium tetani,

Toxigenic Harvard strain was obtained as a lyophilized ampoule from Bacterial Sera and Antigens Research Department, Veterinary Serum and Vaccine Research Institute, Abbassia, Cairo, Egypt.

2- Media used:

i-Glucose beef heart infusion broth. **(Emara,1992)**
ii-Thioglycolate medium U.S.P.**(Oxoid)**
iii- Sabouraud dextrose agar.
iv- Nutrient agar 1.5% agar **(Oxoid).**
V-Mueller and Miller media. **(Abdel aziz, 2005).**

Table 1: Stabilizers used for lyophilization of bacteria:

Stabilizer	I	II	III	IV
Components				
1- Enzymatic digest of casein	2.5%	2 %	4 %	-
2- Sucrose	5 %	4 %	8 %	-
3-Sodium glutamate	1 %	1 %	2 %	-
				-
4- Gelatin	-	0.2%	0.4%	
5- Skimmed milk	-	-	-	10%
Sterilization	By filtration through Seitz filter			

Preparation of seed culture for lyophilization

Preparation of seed culture for lyophilization on thioglycolate broth

The content of a freeze dried ampoule of *Clostridium tetani* strain was reconstituted in thioglycolate broth (Oxoid) and incubated at 36˚C for 24-48 hrs. Purity check is done using nutrient agar and Sabouraud dextrose agar slopes. After proved pure, sub cultured once into four tubes then each tube was inoculated into 500 ml medium in one liter flask and incubated anerobically at 36˚C for 24-48 hrs. Culture was harvested from each flask by centrifugation for 30 min at 7000 rpm. The pellet of each flask was re suspended by stabilizer I, II, III or IV. Then this mixture was dispensed 3 ml into glass vials by dispenser for lyophilization.

Preparation of seed culture for lyophilization on glucose heart infusion broth

The content of a freeze dried ampoule of *Clostridium tetani* strain was reconstituted in glucose heart infusion broth and incubated anaerobically in anaerobic gas pack at 36˚C for 24-48 hrs. Then sub cultured once into four tubes then each tube was inoculated into 500 ml medium in one liter flask and incubated anaerobically at 36˚C for 24-48 hrs. Then treated as above.

Lyophilization of cultures:

Vials containing different mixture of cultures were admitted to Department of Biological lyophilization at Veterinary Serum and Vaccine Research Institute, Abassia, Cairo for lyophilization.

Evaluation of Lyophilized cultures

Capability of lyophilized cultures to produce tetanus toxin (According to Abdelaziz, 2005 and Maria et al. 1997):

Lyophilized vial was resuspended in a tube of the thioglycolate broth or glucose heart infusion broth culture and incubated at 35˚C for 24-48 hours. Then sub cultured again in the same media. Two ml were taken from either media to inoculate two tubes of Mueller and Miller media and incubated for 8 days at 36˚C. Incubation was stopped by exposing the cultures for two days at 4˚C to promote bacterial lysis. Then, centrifuged and checked for toxin production by flocculation test, Lf (Limits of flocculation) values were obtained.

Determination of Lf (Limits of flocculation) value of toxin: (According to WHO, 1997 and Demain *et al.,* 2005)

One milliliter aliquots of the supernatant fluid were added to tubes containing 0.1–0.8 ml of standard tetanus antiserum and the tubes were carefully shaken to mix their contents. The tubes were placed in a water bath at 45◦C-50◦C and the time

recorded. Tubes were checked frequently and the time at which flocculation began was recorded. The concentration of toxin in the tube in which flocculation was first initiated was recorded.

RESULTS

It appears that vials cultured pre and post lyophilization on Glucose heart infusion broth gave higher Lf values(50, 60, 70 and 40 Lf according to stabilizer used) than the one cultured on Thioglycolate broth (40,50, 60 and 30 Lf according to stabilizer used).

It is clear from table(2) that the culture preserved with stabilizer III kept its toxigenic power for longer periods (9 months) followed by stabilizer II(7 months),then stabilizer I (5 months) and lastly stabilizer IV(3 months).

Table (2): Results of limits of flocculation test (Lf/ml) for lyophilized strains with different stabilizers stored at 4 ˚c.

Culture Media	Glucose heart infusion broth				Thioglycolate broth			
	Stabilizer used for lyophilization							
Months after lyophilization	I	II	III	IV	I	II	III	IV
0	50	60	70	40	40	50	60	30
1	50	60	70	40	40	50	60	30
3	50	60	70	40	40	50	60	30
5	50	60	70	30	40	50	60	20
7	40	60	70	30	30	50	60	20
9	40	50	70	30	30	40	60	20
11	30	50	60	20	20	40	50	15
13	30	50	60	20	20	30	50	15
15	20	40	60	20	20	30	50	15
17	20	40	50	15	20	30	40	10
19	15	30	50	15	15	20	40	10
21	15	30	40	15	15	20	30	10
23	10	20	40	10	10	15	30	10

Table (3): Results of limits of flocculation test (Lf/ml) for lyophilized strains with different stabilizers stored at 0 ˚c

Culture Media	Glucose heart infusion broth				Thioglycolate broth			
	Stabilizer used for lyophilization							
Months after lyophilization	I	II	III	IV	I	II	III	IV
0	50	60	70	40	40	50	60	30
1	50	60	70	40	40	50	60	30
3	50	60	70	40	40	50	60	30
5	50	60	70	40	40	50	60	30
7	50	60	70	30	40	50	60	20
9	40	60	70	30	30	50	60	20
11	40	50	70	30	30	40	60	20
13	40	50	70	30	30	40	50	20
15	30	50	60	20	20	40	50	15
17	30	40	60	20	20	30	50	15
19	30	40	60	20	20	30	40	15
21	20	30	50	15	15	20	40	15
23	20	30	50	15	15	20	30	10

The table (3) showed that the culture lyophilized with stabilizer III preserved its toxigenic capacity for longer periods (13 months) followed by stabilizer II(9 months),then stabilizer I (7 months) and lastly stabilizer IV(5 months).

Table (4): Results of limits of flocculation test (Lf/ml) for lyophilized strains with different stabilizers stored at -20 ˚c.

Culture Media	Glucose heart infusion broth				Thioglycolate broth			
	Stabilizer used for lyophilization							
Months after lyophilization	I	II	III	IV	I	II	III	IV
0	50	60	70	40	40	50	60	30
1	50	60	70	40	40	50	60	30
3	50	60	70	40	40	50	60	30
5	50	60	70	40	40	50	60	30
7	50	60	70	40	40	50	60	30
9	50	60	70	30	40	50	60	20
11	40	60	70	30	30	50	60	20
13	40	60	70	30	30	40	60	20
15	40	60	70	20	30	40	60	15
17	40	50	70	20	30	40	60	15
19	30	50	70	20	20	40	50	15
21	30	50	60	20	20	40	50	15
23	30	40	60	15	20	30	50	10

The table demonstrated that the culture lyophilized with stabilizer III preserved its toxigenic capacity for 19 month producing tetanus toxin with high Lf titer (70Lf) followed by stabilizer II (15 month), then stabilizer I (9 month) and lastly stabilizer IV (7 month).

Table (5): Results of limits of flocculation test (Lf/ml) for lyophilized strains with different stabilizers stored at -84 ˚c

Culture Media	Glucose heart infusion broth				Thioglycolate broth			
	Stabilizer used for lyophilization							
Months after lyophilization	I	II	III	IV	I	II	III	IV
0	50	60	70	40	40	50	60	30
1	50	60	70	40	40	50	60	30
3	50	60	70	40	40	50	60	30
5	50	60	70	30	40	50	60	20
7	40	60	70	30	30	50	60	20
9	40	50	70	30	30	40	60	20
11	30	50	60	20	20	40	50	15
13	30	50	60	20	20	30	50	15
15	20	40	60	20	20	30	50	15
17	20	40	50	15	20	30	40	10
19	15	30	50	15	15	20	40	10
21	15	30	40	15	15	20	30	10
23	10	20	40	10	10	15	30	10

The data from the table (5) showed that the culture lyophilized with stabilizer III began losing some of its toxigenicity by the (11th month) followed by stabilizer II (9th month),then stabilizer I (7th month) and lastly stabilizer IV(5th month).

DISCUSSION

Although preventive medicine has progressed in recent decades, tetanus infection remains a life-threatening condition and is still an important health issue worldwide. Tetanus is caused by *C. tetani* which is an anaerobic, motile, gram positive rod found worldwide in the soil, as well as in animal and, occasionally, human feces. Although tetanus is ubiquitous, infections in certain developing regions of the world are associated with high mortality and morbidity largely because of a lack of rigorous immunization programs and available treatment options. Consequently,

tetanus has become one of the target diseases of the World Health Organization (WHO) Expanded Program on Immunization (Hatamabadi et al., 2011).

Control of tetanus is achieved by active immunization of animals by tetanus toxoid prepared from highly toxigenic strain of *C. tetani* (Harvard strain) which produces tetanospasmin toxin. So, it is important to have a potent toxin with high Lf values in order to produce tetanus toxoid and also to prepare hyper immune serum from horse

The characters of the highly toxigenic *C.tetani* (Harvard strain) needed to be preserved as long as possible. Lyophilization is often chosen as preservation method of microorganisms because of its multiple advantages; however, cells are damaged during the process resulting in viability and metabolic activity loss. Optimization of the survival rate during drying and subsequent storage is of outmost importance from technological and economic standpoint. (Carvalho et al., 2003)

Poor recovery of anaerobes from lyophiles is a common problem. Some anaerobes are very sensitive to the lyophilization process. In addition, the process for recovery of the lyophilized anaerobes needs to be taken into account, since those conditions can affect the viability of the restored microbe. They include the suspending diluent, exposure to oxygen during work-up, and other growth conditions **(**Pivnick et al., 1964). A combined approach to pre-lyophilization treatment of microorganisms and subsequent storage was developed in order to improve cell survival**.** Different stabilizers have been tried in combination with different storage temperatures. As shown in table (2) the tetanus toxin produced from strain cultured on glucose heart infusion broth before and after lyophilization showed higher titer (50 Lf,60 Lf,70 Lf,40 Lf) than the strain cultured on thioglycolate broth (40 Lf, 50 Lf, 60 Lf, 30 Lf). Influences of the composition of growth medium were reported. Beker and Rapoport found that yeast grown on a rich molasses medium survived drying better (90% survival) than yeast grown on synthetic medium (20-40% survival). (As cited by Lievense and Riet, 1994). Wright and Klaenhammer reported that inclusion of calcium in the growth medium favored the survival of *L. bulgaricus* after freeze-drying (19% vs. 0.4% without $Ca2^{+}$), probably by interaction of the Ca^{2+} ions with the bacterial membrane. (As cited by Lievense and Riet, 1994)

Also, Carvalho et al. (2003) investigated the effects of three different growth media (MRS, M17 and Lee's) on survival during freeze-drying and subsequent storage of six strains of *Enterococcus faecalis* and two strains of *E. durans*. The survival of the dried Enterococcus spp. tested during storage was shown to be strain-specific and dependent on the growth medium.

Tables (2, 3, 4 and 5) also show that the strain stored at -20ºc kept its toxigenic capacity for longer period than the other vials kept at higher temperatures followed by zero ºc ,4ºc ,-84ºc respectively. It was surprising to notice that the strain loss its toxigenic capacity rapidly in -84ºc.

The temperature change rate, controls transport of water around cell membranes and indirectly likelihood of intracellular freezing. If the cooling is too fast, the membranes cannot carry water out of the cell and freezing inside. Each cell has an optimum cooling rate, so cryoprotector is needed to reduce freezing damage (Criste et al. 2014), it appears from the tables (2, 3, 4 and 5) that stabilizer III gave the best result as it preserve the strain till the 19th month post lyophilization with minimum reduction in toxin titer (70Lf) followed by stabilizer II (60Lf) that start to decline at the 15th month post lyophilization.

Pivnick et al., 1964, mixed culture filtrate of *C. perfringens* type C with 2% casitone, 1% sodium glutamate, 4% sucrose, and 0.2% gelatin for lyophilization. This formula was tried for preservation of *C. tetani* (stabilizer II) and gave satisfactory result but the concentration of lyoprotectants needed to be changed. The concentration of sucrose was doubled to 8% as Kumar et. al (2013) stated that sucrose is inert and in 5-10% concentration is used as a lyoprotectant and provides good viability and stabilize liposome, protein, and virus formulations, especially when compared to skimmed milk**.**

Gelatin usually is used as a cryoprotectant in a concentration of 0.5% (Kaushik and Roos, 2007). Pivnick and his colleagues used a low concentration that needed to be adjusted.

Also sodium glutamate is increased to 2% in agreement with Lagoda and Bannikova (1975) who suggested that 10% saccharose, 5%gelatine and 2% sodium glutamate can be used as cryoprotectants for lactic acid bacteria.

CONCLUSION

It is concluded that preserving C. tetani by lyophilization using stabilizer III (modified Pivnick) retain its toxigenic power than other stabilizers for longer periods. Also, the optimum temperature for storing lyophilized *C. tetani* (Harvard strain) is at -

20 ^{o} c.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

ACKNOWLEGEMENT

The authors are very much thankful to Veterinary Serum and Vaccine Research Institute for providing all necessary facilities for conducting this research work.

AUTHOR CONTRIBUTIONS

MIE and WRA designed the experiment and reviewed the manuscript. SSA performed the experiments and wrote the manuscript. All authors read and approved the final version.

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