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A new α-toxin monitoring method to optimize production efficiency in industrial cultures of *Clostridium novyi* B

Diana Pérez-Etcheverry*, Alberto Nieto-Cadenazzi and Iris Miraballes-Martínez
Laboratorio de Biotecnología, Polo Tecnológico-Facultad de Química, Universidad de la República, Uruguay

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This study presents fast and reliable immunoassays to provide appropriate toxin concentration data in 5 min to monitor industrial culture processes. A latex agglutination reagent for semi-quantification of *Clostridium novyi* B α-toxin was developed, characterized and validated in actual industrial conditions. This reagent represents a useful real-time assay, generating data during toxin preparation process and thus increasing efficiency of α-toxin industrial production from *C. novyi* B cultures.

Keywords: α-toxin, Clostridial toxins, *Clostridium novyi*, Latex agglutination tests

Introduction

Infection by clostridia is widespread among domestic animals[1]. Disease manifestations can range from mild food poisoning to very severe necrotic enteritis causing serious economic losses[2]. All diseases caused by clostridia can be prevented by vaccination[3-5]. *Clostridium novyi* type B based α-toxin (AT) has been described as a cause of gas gangrene. Once inactivated, it is very effective in formulation of vaccines[5]. Amino terminal region of *C. novyi* B AT [single peptide protein[6] (mol wt, 220-280 kDa)] shows glycosyl aminotransferase activity[7], while central hydrophobic region is responsible for translocation into the cell[8]. C-terminal region is responsible for initial interaction with target cell[8]. Its theoretical isoelectric point is 5.8-6.0[9]. Existing method[10] used to assay AT concentration in culture medium is through determination of lethal dose at 50% (LD_{50}). This method is labour-intensive, requires use of experimental animals, and data is available 72 h after sampling. Immunoassays (ELISA, latex agglutination, immunochromatography) have been described for toxin detection from *Clostridium* sp. to diagnose human infections[11-13] or monitor food contamination[14-17], besides toxin detection in culture media[18,19]. Difficulties in optimizing toxin production have already been analyzed[20]. For *C. novyi* B AT, an ELISA has been proposed by Pietrzykowski et al[19], but assay is labour-intensive and time-consuming.

This study presents development of a fast, reliable, and easy-to-use latex agglutination immunoassay to monitor real-time AT levels in industrial cultures of *C. novyi* B.

*Author for correspondence
E-mail: perezetcheverrydiana@gmail.com

Experimental Section

Purification of α-toxin (AT) from *C. novyi* B culture supernatant

AT was purified from culture supernatant samples (200 ml) by salting out[21]. Cells were separated from culture supernatant by centrifugation at 10,000 rpm for 20 min at 4°C (Beckman Avanti, USA). *C. novyi* B culture supernatant aliquots were adjusted to one of the 6 sets of pH/conductivity (mS/cm) values (7.0/20.2, 7.0/16.6, 7.5/20.2, 7.5/16.6, 8.0/20.2, and 8.0/16.6). These aliquots were used to analyze the best precipitation conditions to optimize yield and purity of toxin obtained after salting-out precipitation with ammonium sulphate (Sigma, USA). AT was precipitated from culture supernatant aliquots adjusted to those pH and conductivity conditions by adding ammonium sulphate up to final concentrations of 30%, 40%, 50%, and 60% of that of saturation. After centrifugation, precipitates were dissolved in phosphate buffered saline (PBS) and extensively dialyzed against PBS. Protein concentration of dialyzed samples was estimated using BCA Protein Assay Reagent (Pierce, Holland).

Purity of obtained AT samples was evaluated by SDS-PAGE using 8% polyacrylamide gel (PAG) under reducing conditions[22]. Briefly, purified AT (30 µg) was loaded into each gel lane, and electrophoresis was conducted for 1 h at 25 mA. Gels were then stained with Coomassie Blue R-250 (Sigma, USA) and washed. A digitalized gel image was obtained using an 800 dots per

inch resolution scanner (ColorPage-HR6, Genius, China), and image was analyzed using Phoretix 1D software (Total Lab Ltd.). Band putatively corresponding to AT was sliced from gel, and identity of AT was confirmed by mass spectrometry (MS) (MALDI-TOF/TOF 4800 Analyzer, Applied Biosystems, Framingham, USA). Conformational homogeneity was evaluated by light scattering, measuring turbidity in 300-340 nm range using a spectrophotometer (Ultrospec 3100 pro, Amersham, USA)[23,24]. A larger sample of purified AT was prepared using 2 l of *C. novyi* B culture supernatant.

Determination of LD_{50}

The 7 serial 10-fold dilutions of a sample were made, and 3 Balb/c mice were i.p. inoculated with 0.5 ml of each dilution. Results were observed within 3 days post-inoculation, and LD_{50} was calculated[10].

Preparation of a -Toxin-Specific Rabbit Polyclonal Antiserum

Purified AT was inactivated by mixing 1 µl of 37% formaldehyde with 0.3 mg AT diluted in 0.3 ml PBS buffer and stirring this mixture for 30 min at 37°C (Thermomixer, Eppendorf, Germany). Mixture was then extensively dialyzed against 10 mM Tris buffer, pH 7.5. Efficacy was evaluated by LD_{50}. A rabbit was immunized by administration of three 200-µg doses (first intra-dermal and other 2 intra-muscular) of inactivated AT in Freund's incomplete adjuvant on days 0, 20, and 50. After bleeding on day 60, controls of antiserum reactivity were performed by ELISA[25] and western blot[26]. Immunoglobulin fraction (Ig) was obtained from antiserum by salting-out using 37% ammonium sulphate saturation[21].

Western blot was performed as reported[26]. Purified AT was subjected to SDS-PAGE using an 8% PAG under reducing conditions, and transferred to a nitrocellulose membrane (pore size 0.45 µm; Amersham, USA). Blotted membrane strips were then incubated with 0.05% Tween 20 in PBS (PBS-T) containing 1% bovine serum albumin (BSA) (Sigma, USA) for 1 h at room temperature (RT). Strips were subsequently incubated with rabbit hyperimmune antiserum diluted 1/200 in PBS-T containing 0.1% BSA. After 2 h incubation at RT, membrane strips were washed 3 times with PBS-T. Strips were then incubated for 1 h at RT with alkaline phosphatase-conjugated anti-rabbit IgG (Sigma, USA), diluted in PBS-T containing 0.1% BSA. After incubation, strips were washed 3 times with PBS-T and treated with a substrate solution of 5-bromo-4-chloro-3-indolyl phosphate and Nitro Blue Tetrazolium (Sigma, USA)[21].

Preparation and onto Cards of Latex Agglutination Reagents

Latex particles AJ10, 0.1 µm, 10% solid content (Ikerlat, Spain) and K030, 0.3 µm, 10% solid content (Merck, France) were coated with AT-specific rabbit Ig. Coating trials were performed using 2 ratios of mg protein/mg latex for each type of latex assayed (0.050 and 0.075 mg Ig/mg AJ10 latex; 0.025 and 0.038 mg Ig/mg K030 latex). Coating was performed by incubating mixtures of latex particles with appropriate solutions of AT-specific Ig fraction in 0.1 M glycine and 150 mM NaCl, pH 8.2 over 2 h at 37°C to achieve mg protein/mg latex ratios. After addition of BSA (0.050 mg BSA/mg AJ10 latex and 0.025 mg BSA/mg K030 latex), mixtures were incubated overnight at 4°C and centrifuged at 20,000 rpm for 20 min at 4°C (Beckman, Germany). Once centrifuged, pellets were resuspended in 0.1 M glycine, 150 mM NaCl, pH 8.2 buffer to achieve a final particle concentration of 6 mg/ml. Particle size measurements were performed by using photocorrelation spectroscopy (PCS) using a Zetasizer Nano system (Malvern Instruments, UK). Samples were submitted to ultrasound pulses (cycles of 0.5 min, using an amplitude of 50%) (Hielscher UP 200S, Germany) to eliminate particle aggregates.

Agglutination tests onto cards were performed by stirring mixtures of 20 µl latex reagent and 20 µl sample dilutions in PBS, and agglutination was visually observed after 5 min stirring. Serial 2-fold dilutions of each sample were prepared in PBS. Samples tested were culture medium, culture supernatant with and without formaldehyde, and purified AT. Defined concentrations of purified AT diluted in PBS were assayed to estimate reagent sensitivity (detection limit). The detection limit of reagents was established as the lowest toxin concentration, at which agglutination was observed.

Stability of Latex Reagents

Latex reagent samples were stored for 1 or 12 months at 4°C, and for 1 week, 1 month, or 12 months at 37°C. After storage, detection limit, zeta potential, and particle size were determined.

Semiquantification of a -Toxin (AT) in Samples from Industrial Bioreactors

Agglutination of latex reagent prepared with a coating ratio of 0.0375 mg specific Ig/mg K030 latex was evaluated, using samples of culture supernatants obtained from an industrial bioreactor at different culture times to prepare titration curves of toxin release.

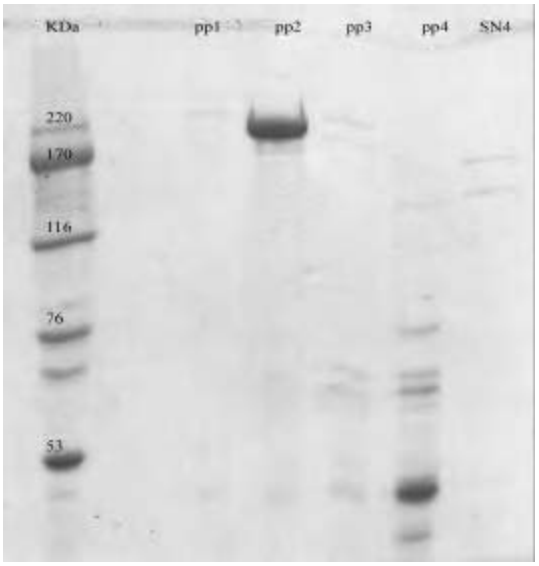

Fig. 1—SDS-PAGE patterns of precipitates obtained after precipitation of culture supernatants with different concentrations of ammonium sulphate (8% polyacrylamide under reducing conditions, Coomassie Blue stain) [(Salting out was performed using culture supernatant adjusted to pH 7.5 and 16.6 mS/cm ionic strength); Lanes: kDa, molecular weight marker; pp1, pp2, pp3, pp4, precipitates obtained at 30%, 40%, 50%, and 60% of ammonium sulphate saturation concentration, respectively; SN4, supernatant corresponding to a precipitation with 60% ammonium sulphate saturation]

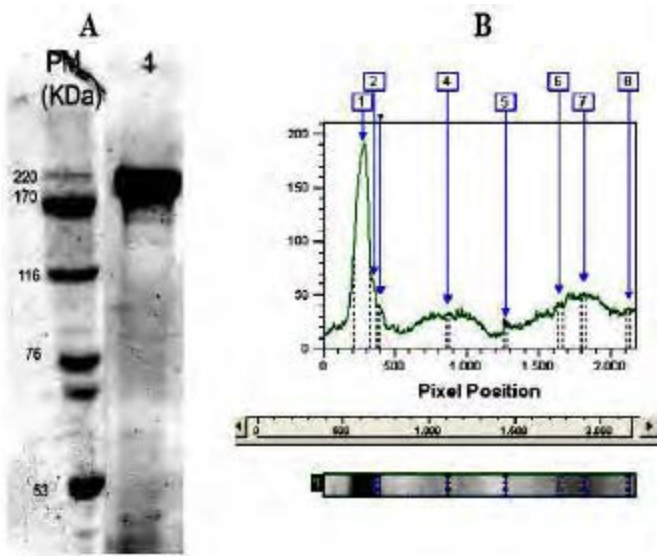

Fig. 2—Purity characterization by SDS-PAGE of α-toxin fraction (8% polyacrylamide under reducing conditions, Coomassie Blue stain) [A, SDS-PAGE patterns of molecular weight marker (kDa) and 30 µg α-toxin (lane 1); B, Densitometric analysis of lane 1 using Phoretix 1D software (TotalLab Ltd)]

Results and Discussion

Purification of α-Toxin (AT) from *Clostridium Novyi* B Culture Supernatant

In most cases, before chromatography, ultrafiltration or polyethylene glycol precipitation are needed[5,19,27]. Optimum precipitation conditions were identified by precipitation tests on aliquots of *C. novyi* B culture supernatants using different ammonium sulphate concentrations, after adjusting supernatants to different sets of pH and conductivity values. Efficiency of AT salting-out purification from *C. novyi* B culture supernatants is affected by protein concentration, ionic strength, and pH[21]. Maximum toxin purification yield was achieved when culture supernatant pH was 7.5 and conductivity to 16.6 mS/cm in the step of 30-40% ammonium sulphate saturation, which provided an AT-enriched fraction as precipitate (Fig. 1). Corresponding yield was 54 mg toxin from culture supernatant (2 l). This toxin preparation was subjected to SDS-PAGE, and 220-kDa band was excised from gel and analyzed by MS (MALDI-TOF/TOF). MS and MS/MS data allowed identification of protein band as corresponding to *C. novyi* B AT. Densitometry of stained gel estimated AT purity to be 70% (Fig. 2). Conformational homogeneity of

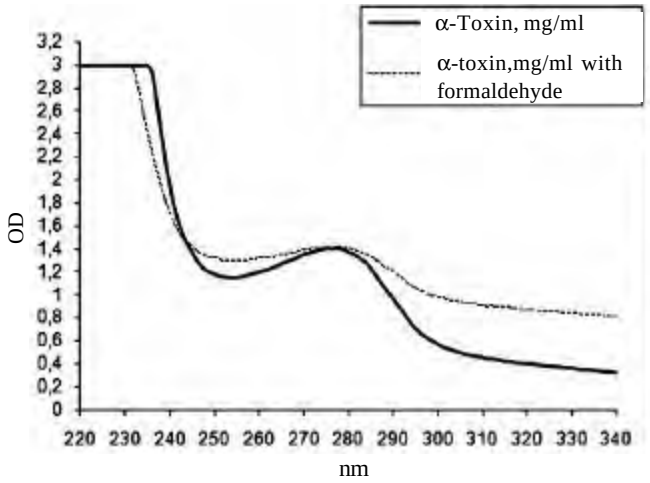

Fig. 3—UV Spectrum of α-toxin (Spectra were taken using 1 mg/ml purified α-toxin solution with and without formaldehyde inactivation treatment)

purified protein, estimated by turbidity associated with light scattering in 300-340 nm wavelength range[23], is not consistent with a 100% conformationally homogeneous protein (Fig. 3). This finding may indicate that some of the protein molecules are in a partially folded state or misfolded, exposing sites that lead to aggregation. Specific activity of purified AT evaluated by LD_{50}[10] was 1.76×10^4 LD_{50}/mg protein, while that of the culture supernatant used for its purification was 2.75×10^3 LD_{50}/mg protein.

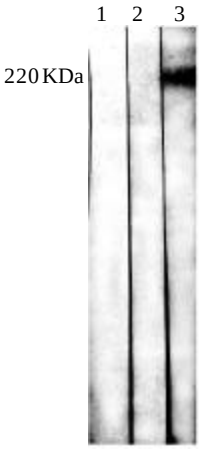

Fig. 4—Western blot analysis of rabbit antiserum reactivity [Western blot; lane 1: conjugate control; lane 2: pre-immune serum (dilution 1/100); lane 3: hyper-immune serum (dilution 1/200)]

With these data, an enrichment factor of 6.4 was estimated.

Preparation and Evaluation of Latex Agglutination Test and Its Components

Inactivation of toxin used as immunogen was confirmed by LD_{50} experiments. Presence of aggregates in inactivated AT was significantly higher than in purified active sample (Fig. 3). Specificity of rabbit anti-toxin-specific antiserum was assayed by western blot (Fig. 4). Despite SDS-PAGE results indicating that toxin preparation used as immunogen was 70% pure, western blot results demonstrated that majority of antibodies in rabbit antiserum recognized AT band, suggesting it was the major immunogenic component of toxin preparation. Particle size determinations of final reagents were assayed by PCS[28]. Light scattering-estimated polydispersity describes the width of particle size distribution and is derived from polydispersity index (PdI), a parameter calculated from PCS-derived intensity autocorrelation function. PdI (> 0.1) indicates that a sample is polydisperse, suggesting it contains large particles, aggregates, and/or dust. PdI (≤ 0.1) indicates that sample is monodisperse[29]. PCS data revealed that only reagents prepared with AJ10 latex particles coated with AT -specific rabbit Ig were monodisperse (PdI = 0.1), while those prepared with similarly coated K030 latex particles demonstrated PdI = 0.3. Ultrasound treatment was necessary to achieve monodispersity (Table 1).

Reagents were tested against serial dilutions of purified AT in PBS (from a 1 mg/ml PBS stock solution) using PBS as a control reference. Formaldehyde-inactivated toxin was also tested in serial PBS dilutions and demonstrated visible agglutination. Agglutination performance was not influenced by the use of culture medium instead of PBS. Reagent specificities were analyzed by testing their cross-reactivity with cultures of *C. sordellii, C. septicum, C. tetani, C. botulinum* type C and D, and *C. perfringens* C and D. No agglutination was observed in any of these cases (data not shown). A lower detection limit (4 μg/ml) was observed using the larger particle size latex reagent. Increase of mg protein/mg latex ratio did not decrease the lower detection limit of reagents (Table 2).

Stability of Latex Reagents

Aliquots of all reagents were stored for 12 months both at 4°C and at 37°C. All samples were easily resuspended and retained their white colour. No visible auto-agglutination was observed in any sample after stability tests. Zeta potential and particle size were determined using PCS after different storage times and temperatures. All reagents demonstrated a significant decrease in latex particle zeta potential after coating and throughout storage period, either at 37°C or at 4°C. Zeta potential decreased less in preparations stored at 4°C than those at 37°C (Table 3). Particle size distribution of different reagents indicated that increasing storage time led to PdI values greater than 0.1 in all cases (Table 1). In the case of reagents prepared with K030 latex particles, monodispersity was lost during coating. Particle size population corresponding to peak 1 diminished over time, while 1 or 2 larger particle size populations appeared in parallel to that process (Table 1). Monodispersity loss was greater for reagents stored at 37°C. If all particles in a colloidal system have a high zeta potential value, the system will be stable due to electrostatic forces. For stable systems, zeta potential values are generally higher than 30 mV (absolute value)[30,31]. Values of zeta potential (Table 3) and PdI (Table 1) for reagents are consistent evidence of unstability. However, detection limits of reagents generally remained unchanged after 1 y storage at 4°C. These results suggest that although zeta potential and particle size changed during storage, these changes were not sufficient to affect agglutination performance. Accelerated stability study demonstrated that 1 week of storage at 37°C is equivalent to 1 y at 4°C, and that a 50% decrease in detection limit is observed after 1 month of storage at 37ºC.

Table 1—Particle size determination (Malvern Instruments, UK) of AJ10-Ig anti- α -toxin latex reagents and K030-Ig anti- α -toxin latex reagents

Sample name	Pdl*	AJ10, %			Pdl	K030, %		
		Pk 1 Area Int	Pk 2 Area Int	Pk 3 Area Int		Pk 1 Area Int	Pk 2 Area Int	Pk 3 Area Int
Std	0.004	100	0	0	0	100	0	0
AJ10-Ig anti- α -toxin latex, 0.050 mg Ig/mg latex; and K030-Ig anti- α -toxin latex, 0.0375 mg Ig/mg latex								
Freshly prepared	0.071	100	0	0	0.361	90.7	9.3	0
Storage for 1 week at 37°C	0.468	93.2	6.8	0	0.416	74.1	25.9	0
Storage for 1 month at 37°C	0.493	68.7	31.3	0	0.708	68.8	23.9	7.3
Storage for 12 month at 37°C	0.745	49	46.5	4.5	0.866	64.9	26.5	6.9
Storage for 1 month at 4°C	0.33	100	0	0	0.579	87.6	12.4	0
Storage for 12 month at 4°C	0.394	86.5	6.9	6.6	0.356	87.6	7.2	5.1
A J10-Ig anti- a -toxin latex, 0.075 mg Ig/mg latex; and K030-Ig anti- a -toxin latex, 0.025 mg Ig/mg latex								
Freshly prepared	0.073	100	0	0	0.367	88.6	11.4	0
Storage for 1 week at 37°C	0.273	97	3	0	0.363	87.1	12.9	0
Storage for 1 month at 37°C	0.506	55.9	44.1	0	0.665	47.6	16.8	8.6
Storage for 12 month at 37°C	0.905	47.7	36.7	15.6	0.598	76.5	11.9	11.6
Storage for 1 month at 4°C	0.333	100	0	0	0.437	88	12	0
Storage for 12 month at 4°C	0.524	82.2	17.8	0	0.41	87	11.5	1.5

*PdI, Polydispersity index; PK1, 2 & 3–Peak means; All measurements at 25°C

Table 2—Detection limit determination of anti-α-toxin latex reagents K030-Ig and AJ10-Ig

Reagents	Detection limit, μg/ml					
	Freshly prepared	Storage for 1 month at 4°C	Storage for 12 months at 4°C	Storage for 1 week at 37°C	Storage for 1 month at 37°C	Storage for 12 months at 37°C
AJ10-Ig anti- α toxin latex						
0.0500 mg Ig/mg latex	8	8	8	8	16	64
0.0750 mg Ig/mg latex	8	8	8	8	16	64
K030-Ig anti- α toxin latex						
0.0250 mg Ig/mg latex	4	4	4	4	16	32
0.0375 mg Ig/mg latex	8	8	8	8	16	64

Semiquantification of a -**Toxin in Industrial Reactors**

Reagent prepared with K030 latex particles coated with 0.0375 mg AT-specific rabbit Ig/mg latex demonstrated the lowest detection limit and was therefore selected for trials in industrial bioreactor conditions. Samples of culture supernatants were taken from an industrial bioreactor at different times, and latex agglutination tests were performed to monitor toxin production process in culture. Toxin concentration *vs* time plot (Fig. 5) shows an example of the data obtained using this reagent to monitor a routine 24-h industrial culture of *C. novyi* B. In this particular culture, AT release was detected 14 h after inoculation, and its concentration increased for 6 h, when a plateau was reached and

Table 3—Zeta potential of AJ10-Ig anti- α -toxin latex reagents and K030-Ig anti- α -toxin latex reagents

Sample name	Zeta Potential, mV	
	AJ10	K030
Std	-50.08	-39.8
AJ10-Ig anti- α -toxin latex, 0.050 mg Ig/mg latex; and K030-Ig anti- α -toxin latex, 0.0375 mg Ig/mg latex		
Freshly prepared	-25.8	-26.1
Storage for 1 week at 37°C	-18	-11.7
Storage for 1 month at 37°C	-12.2	-10.5
Storage for 12 months at 37°C	-10.2	-10.1
Storage for 1 months at 4°C	-18.6	-21.7
Storage for 1 months at 4°C	-16.9	-13.1
AJ10-Ig anti- α -toxin latex, 0.075 mg Ig/mg latex; and K030-Ig anti- α -toxin latex, 0.025 mg Ig/mg latex		
Freshly prepared[b]	-26.8	-21.7
Storage for 1 week at 37°C	-22.4	-10.6
Storage for 1 month at 37°C	-16.08	-10.6
Storage for 12 months at 37°C	-10.4	-10
Storage for 1 months at 4°C	-22.9	-12.5
Storage for 12 months at 4°C	-20.3	-11.7

All measurements at 25°C

maintained for 7 h of additional culture. These results show usefulness of this reagent to monitor industrial process, since operator can make an informed decision to stop the process in just 5 min. Present method, as compared to existing *in vivo* method, does not use animals, thereby increase animal welfare and allow reduction of expenditures for controls in process. Additionally, it does not require sophisticated equipment or highly skilled personnel. Results are available in only 5 min, the titration curve of toxin release is provided in real time, and operator can make an informed decision to stop the process.

Conclusions

Latex reagent tested on cards against serial dilutions of purified toxin as well as of culture supernatants yielded good sensitivity and stability results. The use of larger particle size latex improved sensitivity. Increasing ratio of mg protein/mg latex particles did not necessarily lead to increased reagent sensitivity. Long-term stability of reagents was evaluated at 1 week, 1 month, and 12 months. Results demonstrated robustness of reagents over 1 y in the conditions of daily lab work when stored at 4°C. In accelerated stability study, a 50% increase of detection limit was observed after 1 month at 37°C.

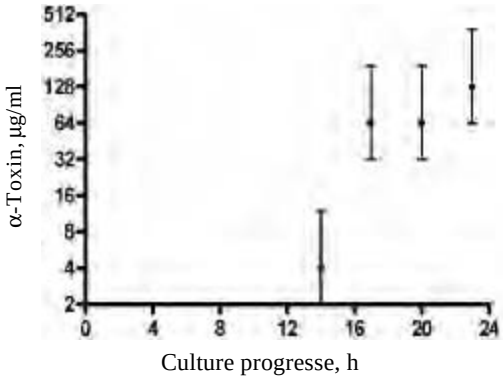

Fig. 5—Semi-quantification by latex agglutination of α-toxin content in samples extracted from the reactor as culture progressed (Toxin release was detected 14 h after inoculation and continued growing until 24 h, when culture was stopped for inactivation; Plotted data correspond to the means of triplicate tests with standard deviations)

Despite the data from PCS studies, which show colloidal unstability, reagent remains stable from the point of view of its detection limit over 1 y when stored at 4°C, indicating that changes in zeta potential and particle size during storage did not affect reagent agglutination performance. Latex agglutination reagent is a useful tool to monitor culture process and optimize AT yield. It performed successfully, showing no interference due to any component of culture medium as well as no cross-reactivity with any of the other *Clostridium* cultures tested. Present method has several advantages compared to existing *in vivo* method.

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