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ORIGINAL ARTICLE

Immobilization of *Clostridium perfringens* type D in calcium alginate beads: toxin production mimics free cell culture

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ABSTRACT

Background and Objectives: Cell-immobilization is used to maintain microbial culture to produce metabolites in repeated-batch or continuous fermentations, thereby reducing the time and resources spent on delivering mass production of microbe. The technique also enables shortening of the detoxification phase and the amount of formaldehyde required due to low incidence of viable bacteria in the extract.

Materials and Methods: A solution of sodium alginate containing *Clostridium perfringens* cells was dropped into stirring $CaCl_2$ solution via a sterile syringe needle. Optimizations resulted in reasonably uniform beads containing *C. perfringens.* Beads were externally stabilized by poly L-lysine, followed by immersion in a solution of Na-alginate to coat them with a new layer of alginate forming an alginate-PLL-alginate cortex.

Results: This study proved successful in immobilizing *C. perfringens* cells inside uniform alginate microspheres. Cell loading and cell propagation inside the beads were measured. The cell loaded beads were cultivable in liquid media producing 550 minimum lethal doses per milliliter (MLD/ml) in a 72 h.

Conclusion: The research paved the way for further investigations to optimize and establish an efficient bacterial encapsulation method. Thus, it seems possible to produce toxins from beads engulfing *C. perfringens* on larger scales via repeated-batch or continuous fermentation processes.

Keywords: *Clostridium perfringens*; Cell-immobilization techniques; Encapsulation; Calcium alginate beads; Toxin production

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INTRODUCTION

The anaerobic bacillus *Clostridium perfringens* (*C. perfringens* or CP) is responsible for pathologies such as hemorrhagic gastroenteritis, myonecrosis, enterotoxaemia, and diarrhea in a wide spectrum of domesticated species and imposes significant economic impact on animal agriculture (1, 2). Sudden changes in gut microbial flora may alter the physiological equilibrium of habitat in the intestinal environment and lead to *C. perfringens* excessive proliferation and occurrence of Clostridial disease (3). CP owes its virulence to the large arsenal of its toxins, including the α, β, ε, θ, κ, λ, ι, μ, and enterotoxin (4). Toxins α, β, ε, and ι are considered *C. perfringens*' main lethal toxins. Therefore, CP has been categorized into five toxinotypes, each associated with a particular disease due to production of different sets of toxins (5, 6). Type A, the predominant isotype, is the main cause of necrotic enteritis in poultry (7). On the other hand, types B, C, and D prevail in cases of livestock sudden death due to the fatal toxins β, ε. Toxinotypes B and C produce similar diseases characterized by sudden deaths accompanied by hemorrhagic or necrotic enterotoxemia in lamb and struck in adult sheep (8). Type D causes pulpy kidney disease and dysentery mainly in lambs due to overeating (9). Razi clostridial vaccine contains Types D (60%), C (15%), B (10%) and *C. septicum* (15%).

A mounting concern has been expressed over the antibiotics used in animal agriculture (10), therefore developing safe and practically sound preventive alternatives is an urgent need to control *C. perfringens* infections. Currently, an inactivated tetravalent enterotoxemia vaccine is available for the immunization of livestock by vaccine manufacturing branches of Razi Vaccine and Serum Research Institute, Iran. This product is obtained through a protracted period of longer than six weeks, involving inoculum preparation, incubation, detoxification, cold incubation, compounding and packaging steps. Consequently, a new course of toxoid vaccine preparation would involve all these steps once again. Such a procedure has disadvantages like being lengthened and costly, yielding a bulk of moderate quality but voluminous product.

Recently, cell immobilization techniques such as entrapment in polymers have been extensively used in many applications ranging from wastewater treatment (11, 12) to the production of valuable metabolites such as alcohol (13, 14), vitamins (15), antibiotics (16), and amino acids (17). The method of cell encapsulation in a matrix can provide an array of beneficial features (18), the most considerable being that metabolite production and purification processes would become simpler. Therefore, it offers an effective way to reduce the cost of complex and expensive product recovery steps (19). Additionally, the immobilized cells can be maintained easily for longer periods and employed repeatedly for production of the desired metabolite (20). This would lower the time and effort spent on preparative steps and of course, the production costs.

Although a variety of synthetic polymers or natural polysaccharides like carrageenan, agar, polyacrylamide and gelatin exist for the encapsulation purposes (21, 22), alginic acid is widely used for confining cells. This polysaccharide copolymer, also termed as algin, is extensively found in the cell walls of brown algae. The sodium salt of algin forms a viscous gum upon mixture with water, and if this is mixed with solutions containing divalent ions like Ca^{++}, the monovalent ion is replaced by the bivalent calcium ion to form insoluble calcium alginate. This phenomenon can readily be exploited to manufacture hydrogel spheres entrapping a desired object such as a compound or a bacterial cell (23). Gelling properties of Ca-alginate, however, rely strongly on the concentration of alginate and calcium ions. Ultimate physical characteristics of the microgels formed are determined by how the two solutions are mixed and agitated (24).

Bead stabilizing agents like chitosan and poly-L-lysine (PLL) are exploited to coat hydrogel beads, preventing re-dissolution of calcium alginate via replacement of monovalent ions like Na^{+} from culture media (25, 26). Thus, the main objective of this study was to develop an immobilization technique based on bacterial cell encapsulation in calcium alginate to pave for a continuous or repetitive toxin-producing culture.

MATERIALS AND METHODS

Reagents. Sodium alginate, calcium chloride, sodium citrate, Poly-l-Lysine, soy peptone and chitosan were obtained from Sigma Co. at purities above 99%. Bovine liver was collected from a domestic slaughterhouse. CP was provided by Razi Vaccine and Serum

Research Institute, Karaj, Iran (Collection number: CN 409).

Bead preparation. First, cell-free alginate beads were produced under different operational conditions to achieve the most suitable concentrations for stable microspheres. Briefly, aqueous solutions of sodium alginate at concentrations of 1, 2, and 3% w/v, were stirred for 1 h at room temperature to uniformity and autoclaved. Each solution was extruded dropwise from 2 cm height at approximately 10 mL/min speed via a 10 mL plastic syringe equipped with a 23G stainless steel needle into a stirring $CaCl_2$ solution of equal concentration. The capsular objects formed around each droplet were kept constantly agitated at 150 rpm using a magnetic stirrer for 30 min. The beads were then paper-filtered (Whatman® Grade 41) and washed 5 times with 100 ml of deionized distilled water (27).

Bead stabilization. For stabilization, calcium alginate spheres were transferred to 0.1% w/v chitosan or 0.05% w/v PLL solutions in separate sub-batches and stirred for 20 min. Finally, the beads were stirred in a 0.1% w/v sodium alginate solution for 10 min to provide alginate-PLL-alginate beads. The microspheres were washed 5 times with 100 ml of deionized distilled water (28).

Bead properties. To choose for best preparation condition, the beads formed under the above conditions were investigated visually for physical properties like size, shape, resilience, and uniformity.

Preparation of cell-loaded beads. A 24-h culture of type D of *C. perfringens* (CN 409) in nutrient broth was sub-cultured serially to contain 6×10^7 cells/ml and used to inoculate beads. Sodium alginate solutions (50 ml) charged with the clostridial inoculum (5 ml) was added dropwise to stirring $CaCl_2$ solutions of mutual concentrations. Beads underwent similar conditions applied to cell-free beads. Likewise, the two batches of beads obtained hereby were surface-treated by stirring 20 min in 0.1% w/v chitosan or 0.05% w/v PLL solutions.

Measurement of microbial loading. Ten cell-loaded beads were disrupted in a 5% sodium citrate solution (5 ml). Cell counting was performed using a Neubauer chamber and the total number of cells/µl of the sample was calculated, from which the number of cells per bead was deduced.

Culture media. BB medium contained soy peptone (25 gr), disodium hydrogen phosphate (8 gr), sodium chloride (3 gr), yeast extract powder (5 gr), cysteine hydrochloride (175 mg), and distilled water up to 1000 mL (pH 7.6). Liver extract broth medium (local routine) contained nutrient broth powder (8 g), minced beef liver (70 mL), and distilled water up to 1 L (pH 7.6).

Stability of beads in culture media. A number of 150 cell-free and cell-loaded beads with and without prior stabilization were inoculated into each of the culture media (400 ml) and deaerated for 4 min using an Anoxomat® under CO_2 (10%), H_2 (10%) and N_2 (80%). The culture was then incubated anaerobically at 37°C for 72 h. Rupture of the inoculated beads was visually investigated over time. Mineral content of media was changed in order to observe its effect on bead stability.

Bacterial proliferation in beads. After 24 h of cultivation of the beads in the liver extract medium (nutrient broth powder (8 g), minced beef liver (70 ml), and distilled water up to 1 L (pH 7.6), the cellular content of beads was measured in the same way described above to work out the number of cells proliferated inside the beads over 24 h.

Measuring minimum lethal dose (MLD). For this purpose, 25 ml of the liver extract medium inoculated with free Clostridial cells and cell-loaded beads were harvested after 24 h, centrifuged for 15 min at 2100 g. The supernatant was activated with trypsin and diluted with sterile PBS to obtain the dilutions of 1/10, 1/100, 1/1000, 1/2000, and 1/4000 required for MLD evaluation (29). Diluted solutions (500 µl) were intravenously injected into the tail vein of two mice (18-20 gr) and observed for 72 h.

RESULTS

Physical properties of the beads. When sodium alginate solution was dropped into the $CaCl_2$ solution, immediately spherical particles were formed and eventually hardened upon continued stirring. Final particles were white resilient spheres of approximate-

ly 3-4 mm in diameter (Fig. 1). No significant differences in these features were observed for the cell-free and cell-loaded beads.

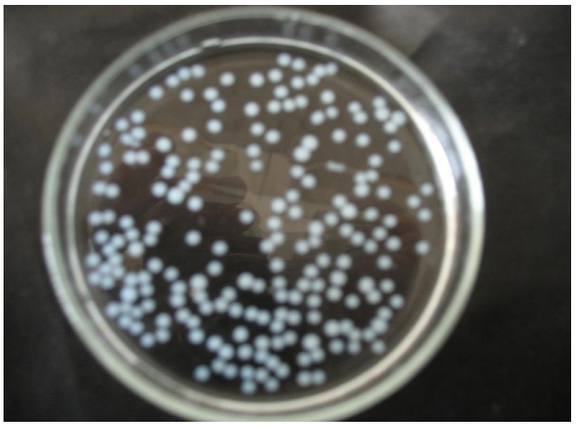

Fig. 1. Ca-Alginate beads formed with 2% w/v Na-alginate and 2% w/v $CaCl_2$ stirred at 150 rpm for 30 min.

Loading and proliferation of *C. perfringens* inside beads. Before and 24 h after cultivation of loaded bead, the clostridial cells were measured as 15×10^3 and 5×10^4 cells/bead, respectively.

Bead stability. Beads submitted to BB were disrupted very fast (Table 1 and Fig. 2). Omission of NaCl from the medium formula did not improve bead stability but its replacement with $CaCl_2$ delayed bead lysis, however cracked beads were still observable. Stabilization of the beads with PLL improved the stability of the beads to some extent better than chitosan. The rate of beads rupture in BB culture was not significantly different for free and CP-loaded beads. Data of these variations are not shown on the grounds of pointlessness.

On the other hand, PLL-coated hydrogel beads incubated in the liver extract medium were stable for 72 h without observable rupture and considerable bacterial

Table 1. Stability of beads (total No: 150) in BB medium at 37°C

Incubation time	No of Beads	
(min)	Intact	Lysed
0	150	0
60	95	55
120	50	45
135	25	25
150	0	25

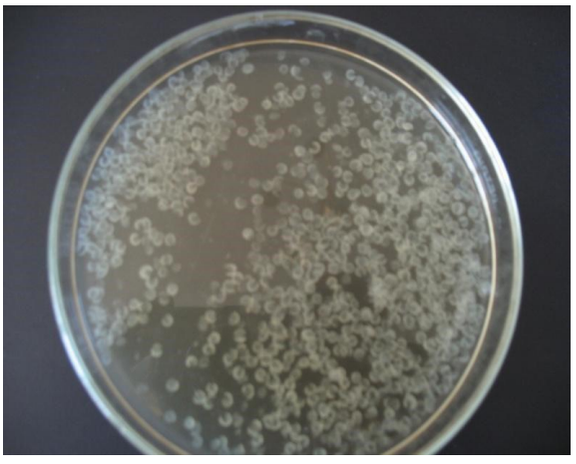

Fig. 2. Beads disrupted in BB medium at 37°C after 150 min.

leakage. The physical strength of beads was found to follow the order of PLL-coated > Chitosan-coated > non-coated, not differing for CP-loaded and CP-free beads.

Values of MLD experiments for both cultures inoculated with free CP cells and CP-containing beads were 550 MLD/ml (Table 2).

DISCUSSION

We hereby report the preparation and biological activities of *C. perfringens* entrapped in calcium alginate hydrogel beads as a preliminary means of biomass immobilization for continuous toxoid production. Among the four types of *C. perfringens* that are used for the production of the toxoid vaccine, type D has the highest proportion in the final vaccine formula. Therefore, this study was performed with *C. perfringens* type D. Since there are significant similarities between different types of CP (24), it is expected that the results of the present study can be generalized to other types with minor modifications.

Of all the different biopolymers, alginate is the most commonly used due to its ability to form hydrogel under mild conditions, non-toxicity and stability during the process (30). Solutions of Na-alginate and $CaCl_2$ at 2% w/v equal concentrations resulted in uniform and resilient spherical microparticles. The entrapment of cells in this matrix was simply achieved by dripping cell-alginate mixtures into a vessel containing $CaCl_2$ solution with a simple set of equipment available to almost any laboratory with basic facilities. The tech-

Table 2. MLD results from cultures of free CP cells and CP-containing beads in the liver extract medium after 24 h (dead/No of animals).

Groups	Dilutions/Culture type	1	1×10^{-1}	1×10^{-2}	1×10^{-3}	5×10^{-4}	2.5×10^{-4}
1	Free cells	2/2	2/2	2/2	0/2	0/2	0/2
2	Entrapped cells	2/2	2/2	2/2	0/2	0/2	0/2

nique of cell entrapment is mainly that described by Hannoun with some modification (31). The process resulted in the engulfment of approximately 15000 cells of CP per bead. However, the use of devices such as static pressure pumps and multi-needle nozzles could provide more speed and convenience (32). Nonetheless, the beads are prone to become gradually brittle with the passage of cultivation time highly depending on the medium components. Supporting the beads through surface treatment with materials such as poly-amino acids like poly-l-lysine (PLL) (33, 34) and poly-L-ornithine (PLO) (35) or natural polymers like chitosan (36-38) enhanced the beads stability. The simple procedure and mild conditions of coating microcapsules with poly-l-lysine make it the most commonly used material for this purpose in recent decades (39). In the present study, stability of beads treated with poly-L-lysine was relatively high in comparison with beads treated with chitosan. PLL is positively charged which might be able to pair with the negative charges of the alginate layer and contribute to the stability of the beads. Besides, the alginate beads stability is found to be varied in different culture media. While hydrogel microparticles exposed to the BB medium started to break after 1 h (more than 30%) and ended with complete rupture in 2.5 h, the beads showed to be stable for 72 h in the liver extract medium and no clostridial cell was detected in the medium. Proliferation of clostridial cells within the beads and excretion of toxin from the entrapped cells into the culture medium was investigated by measuring cell count and MLD, respectively. Propagation of cells to more than triple over 24 h was evident. In addition, toxin content of the liquid culture of entrapped cells was found to mimic that of free cell culture, where it was the 1×10^{-2} MLD/ml for both systems. To this end, we have not found a literature report on immobilization of *C. perfringens.* A few reports elaborate on immobilization of CP proteins like neuraminidase (40), and phospholipase C (41). The current project seems to promise that the entrapment of clostridial cells does not negatively affect toxin production whilst it enables prolonged active cultures. Nonetheless, differences in the effects of PLL and chitosan on toxin production remains to be addressed in future trials.

CONCLUSION

Overall, the results of this study demonstrated the practicality and feasibility of the applied *C. perfringens* immobilization strategy with efficient toxin production in setting up a semi-continuous system for the preparation of clostridial vaccines. Literature is rich in similar trials of entrapment experiments on other microorganisms for various purposes such as preservation of biomass for repeated or continuous processes, facilitation of product recovery and protection of compounds in degrading environments (42). The technique also devoid excessive use of formaldehyde for detoxification science no live cells are carried over throughout purification processes.

Obviously, the present study preliminarily paved the way for further investigations to optimize culture characteristics for an improved continuous clostridial vaccine production and to lessen current drawbacks further, provided it is funded and supported properly.

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