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# Production of recombinant botulism antigens: A review of expression systems

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ABSTRACT

Botulism is a paralytic disease caused by intoxication with neurotoxins produced by *Clostridium botulinum*. Despite their similar mechanism of action, the botulinum neurotoxins (BoNT) are classified in eight serotypes (A to H). As to veterinary medicine, the impact of this disease is essentially economic, since different species of production animals can be affected, especially by BoNT/C and D. In human health, botulism is feared in a possible biological warfare, what would involve mainly the BoNT/A, B, E and F. In both cases, the most effective way to deal with botulism is through prevention, which involves vaccination. However, the current vaccines against this disease have several drawbacks on their process of production and, besides this, can be dangerous to producers since it requires certain level of biosafety. This way, recombinant vaccines have been shown to be a great alternative for the development of vaccines against both animal and human botulism. All BoNTs have a 50-kDa light chain (LC) and a 100-kDa heavy chain (HC). The latter one presents two domains of 50 kDa, called the N-terminal ($H_N$) and C-terminal ($H_C$) halves. Among these regions, the $H_C$ alone seem to confer the proper immune response against intoxication. Since innumerous studies describe the expression of these distinct regions using different systems, strategies, and protocols, it is difficult to define the best option for a viable vaccine production. Thereby, the present review describes the problematic of botulism and discusses the main advances for the viable production of vaccines for both human and veterinary medicine using recombinant antigens.



## 1. Introduction

Botulism is an intoxication caused by potent botulinum neurotoxins (BoNTs) that are secreted during the multiplication and sporulation of *Clostridium botulinum*, a mobile, anaerobic Gram-positive bacilli [1]. *C. botulinum* spores are ubiquitous in the environment, surviving for extended periods under adverse environmental conditions. In appropriate anaerobic conditions, germinating spores produce, grow and secrete BoNTs [2]. Based on the antigenic properties of BoNTs, *C. botulinum* strains are grouped into one of eight serotypes: A, B, C, D, E, F, G and H. The recently isolated serotype H was isolated from a patient with infant botulism, and it secretes a proteolytic toxin that is not neutralized by any of the seven monovalent antitoxins (Anti-A to Anti-G), hence the name of H toxin [3].

*C. botulinum* strains compose four genotypically and phenotypically distinct groups of organisms, designated I to IV [4]. Groups I and II cause botulism in humans, while group III is involved with animal botulism; however, exceptions occur. Group IV, formed by *Clostridium argentinense* [5], is not commonly associated with the occurrence of disease [6]. Group I strains, which are pathogenic for humans, produce the proteolytic BoNT/A, B and F, while Group II strains produce non-proteolytic BoNTs B, E and F. Strains from Group III, which are pathogenic for animals, produce BoNTs C and D. Finally, Group IV strains secrete serotype G neurotoxins, and some Group IV strains were reported to produce two types of toxin. However, strains that are PCR-reactive for both type A and B toxins may generally be considered to be type A toxin-producing strains [7].

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The BoNTs, which are the most poisonous biological substance known, have $LD_{50}$ values in mice ranging from 0.5 to 5 ng/kg, depending on the serotype [8]. The BoNTs are released as progenitor toxin complexes (PTCs), which have distinct molecular compositions. The PTCs are multiprotein complexes composed of a BoNT and several non-toxic neurotoxin-associated proteins (NAPs) that are also called non-toxic-associated proteins [9]. These PTCs cause food poisoning, and BoNT serotype A PTC has $LD_{50}$ values in humans of approximately 0.09–0.15 mg by intravenous administration, 0.7–0.9 mg by inhalation and 70 mg by oral administration, indicating the miniscule amounts of toxin necessary to cause disease in humans [10]. Associated non-toxic proteins (ANTPs) include hemagglutinin (HA) and non-toxic non-hemagglutinin (NTNH), as well as proteins with unknown function, called OrfX.

The genes encoding the BoNTs and ANTPs are clustered in a DNA segment, called the botulinum locus, which is located chromosomally. The gene encoding the NTNH component immediately precedes it. Both genes form an operon located in the 3' region of the botulinum locus that is highly conserved in different species of *C. botulinum*. The BoNTs genes are present in various genetic elements, including phages, plasmids or chromosomes in the different groups of *C. botulinum* and other *Clostridium* species. Plasmids of various sizes and bacteriophages have been found in *C. botulinum* A, B, E, and F. However, these plasmids have not been associated with the toxicity of the strains, but instead with the expression of chromosomal genes [11]. However, in *C. botulinum* C and D, it is clear that BoNT is encoded by bacteriophages [12].

BoNTs are synthesized as inactive 150-kDa single-chain proteins that are activated by proteolytic cleavage to form a disulfide-linked dimer consisting of a 100-kDa heavy chain (HC) and a 50-kDa light chain (LC) [13]. The heavy chain is divided into an amino- ($H_N$) and a carboxy-terminal domain ($H_C$). While the $H_N$ portion is highly homologous among various clostridium neurotoxins, the $H_C$ domain presents more variability [2]. The mechanism of BoNT action can be divided into four stages: binding, internalization, membrane translocation, and proteolysis of specific SNARE proteins. In the first step, the $H_C$ binds on the presynaptic terminal of the neuronic membrane to specific receptors comprising gangliosides (GD1a, GD1b, GT1b, GQ1a, GM1a) and specific proteins including synaptotagmins (Syt-I, Syt-II) and synaptic vesicle associate proteins (SV2A, SV2B, SV2C) [14,15]. After binding, the lateral presynaptic membrane movements trap the neurotoxin inside the array of protein receptors, increasing its interaction with additional binding molecules and making the BoNT activity irreversible. The role of $H_C$ explains the higher rate of protective epitopes in this region when compared with any other part of the molecule. Therefore this domain can induce strong humoral response and consequent protection against the native toxin [16].

The next step, toxin internalization, involves trafficking via vesicles, which is an energy-dependent and temperature-sensitive mechanism that is favored by the high rates of vesicle recycling from the hyperactive nerve terminal. The influx of calcium triggers protein-mediated events that cause synaptic vesicles docked at the presynaptic membrane near the calcium channels to fuse with the membrane and release acetylcholine. The resultant vesicle recycling induces BoNT internalization, which starts a new process of BoNT exocytosis and endocytosis. Once BoNTs are in the lumen of the endosomes, it acidifies and the HC undergoes a conformational change that results in the formation of a HC protein pore in the endosomal membrane. This pore enables the translocation of the LC to the cytosol. Then, it cleaves its cognate SNARE in different places, resulting in the inhibition of acetylcholine release [2]. The lack of this neurotransmitter in the synaptic cleft governs the flaccid paralysis of skeletal muscles. Each serotype cleaves specific peptide bonds in one or more of the SNARE proteins [17].

Different animal species have different susceptibilities to BoNTs and botulism. Equines seem to be sensible to all serotypes [18]. Bovines are susceptible to serotypes C and D with acute and subacute cases, and some description of types A and B intoxication [19]. On the other hand, the ovine disease is commonly chronic [20,21]. Canines are less sensitive and disease occurrence is associated with types A, B and C, but does not occur frequently [22]. In addition, birds are affected by type C and, more rarely, by types A and E, but they can eliminate all serotypes in their excreta [23–25].

Cattle with high nutritional requirements, such as pregnant or lactating females, in inadequate pastures without proper mineral supplementation, especially phosphorus, can develop the habit of eating corpses and its bones [26,27]. Simultaneously, they intake preformed BoNTs on the decomposing carcasses, which lead to large outbreaks resulting in the death of thousands of animals [28–30]. The incubation time and severity of botulism is dependent on the amount toxin ingested and the susceptibility of the animal species involved. In cattle, the disease course can range from hours to a few days, with a mortality rate near 100%. The first clinical signs are difficulty in walking and incoordination of the hind limbs with cranial progression to flaccid paralysis. The animal enters preagonal state, and death, preceded by coma, occurs due to cardiac arrest. Throughout the course of the disease, the physical attributes of the animal remain unchanged. Gross lesions are rare and limited to petechiae in the myocardium as a result of the respiratory distress that precedes death [30–32].

Vaccination with C and D toxoids is still the primary way to control botulism in cattle. In Brazil only, over 150 million vaccine doses with these toxoids are produced annually. Although these immunogens may be effective in preventing disease [1], their quality varies greatly between countries and manufacturers, which consequently results in a large range of immune responses in vaccinated animals [33,34]. BoNT production is one of the most important factors to be considered in the industrial production of botulinum toxoids, because it requires the use of toxigenic strain as well as specific culture media, pH, time and atmosphere suitable for cultivation and incubation [35]. Furthermore, the risk of toxoid handling, the high manufacturing cost and the chemical inactivation of the botulinum toxoid all stimulated research on alternative approaches. To this end, recombinant vaccines have garnered the attention of researchers as an alternative to those made by native toxoids [36]. Preliminary studies with recombinant protein antigens representing one or more of the three domains of the BoNTs were evaluated as potential vaccine candidates. The recombinant protein antigens can be produced in large quantities using expression systems, such as yeast or *Escherichia coli*. These antigens may be purified using conventional chromatography methods or they may not even require this step [37,38]. Recombinant formulations are stable, safe and well tolerated in animal models, as well as target animal species [39–41]. Generally, they induce protective immunity after two to three vaccinations, even when the challenges are generated using large amounts of active neurotoxin. However, these factors were not tested in humans yet [36], being limited to preliminary results in monkeys [42]. The aim of this review is to discuss systems, strategies and protocols to produce recombinant vaccines for controlling botulism.

## 2. Overview of recombinant botulism antigens expressed in *E. coli*

The *E. coli* expression system is a robust way to express heterologous proteins, because its genetic manipulation is simple and well described. Innumerable active proteins have been obtained by this system, which have been useful for many medical applications [43]. The field of immunology has also benefited from the

production of antigenic proteins, which are tested as vaccines [44,45]. Because of the vast options for protein engineering and expression, there are no fixed rules to produce a protein. The BoNTs are no exception, because multiple protocols are described for each type, which does not provide a clear conclusion regarding the best industrial production option for either human or animal applications. Herein, we review the recent advances for recombinant vaccines production in *E. coli* and identify options for possible large-scale production of the different antigens employed against botulinum toxins using this expression system.

Essentially, protein expression using *E. coli* can result in two protein states: (1) soluble, where the protein is maintained in the native conformation almost exclusively; and (2) insoluble, where the protein forms aggregates called inclusion bodies, which require denaturing agents for solubilization and additional refolding steps. In vaccine development, both states have advantages and disadvantages. Soluble proteins can be more immunogenic and thus serve as a more efficient antigen, but because most *E. coli* proteins will be part of the cell lysate, they entail a more intensive purification procedure. Insoluble proteins may not have the same immunogenicity as soluble ones; therefore, protein refolding should be performed to avoid this shortcoming. However, the subsequent purification step is much less laborious, because few *E. coli* proteins will be present after using denaturing agents on inclusion bodies [46]. Furthermore, inclusion bodies may have adjuvant activity, attenuating the conformational antigen deficiency. Although insoluble proteins are commonly obtained, the soluble ones are preferred for vaccine development. Therefore, many of the strategies used in recombinant protein expression for vaccines using the *E. coli* system aim to obtain soluble antigens.

Most of the recombinant antigens against botulism expressed in *E. coli* involve the use of pET plasmids. These commercial vectors possess the T7 promoter and the pBR322 origin of replication. Therefore, a promoter with high translation efficiency does not injure the solubility of the proteins obtained and a low-copy origin of replication fits this type of expression. However, in some cases, other plasmid strategies may be useful. For example, the use of the T5 promoter decreases expression levels and thus increases the probability that the expressed protein will be soluble, but this expression is not entirely effective [47–49]. Another alternative is the use of soluble tags [50], such as glutathione S-transferase (GST) [39,51], thioredoxin (TRX) [52–54] and maltose binding protein (MBP) [55], which help protein expression into the soluble fraction and have been successful in the expression of soluble botulism antigens.

In large-scale production, it is recommended that all components be tested to obtain their optimal concentration. This way, wasted materials are avoided and the production can be optimized to result in a higher yield. The first component to be optimized is the culture medium. In this context, the use of the traditional Luria–Bertani (LB) broth or other rich broths, such as 2 YT, phytone yeast extract or Super Broth (SB) and Terrific Broth (TB), are not viable for industrial production, because the components are complex and therefore augment production expenses. Furthermore, these broths are comprised of peptone of animal origin, which improves the risk of prion contamination. Alternatively, M9 medium can be successfully applied for recombinant botulism antigens production [56,57]. This medium is composed of a combination of salts, which provide the essential growth nutrients, such as phosphates, ammonia and metal ions. In addition, M9 medium contains glucose as the carbon source, but the used concentration of this carbohydrate does not interfere with protein expression using the widely utilized *lac* operator. Another component is the inducer, which controls the promoter and then initiates protein expression. The most common inducer, isopropyl β-D-1-thiogalactopyranoside (IPTG), is used in varying concentrations (0.05–1 mM) and the optimal concentration was only determined in a few cases. Together with the optimization of the inducer, the culture density must be investigated. Most published protocols follow the standard for *E. coli* expression, which involves the initiation of induction when the culture reaches an $OD_{600}$ between 0.5 and 0.8. Few sources use a higher absorbance [57,58]. However, when it comes to high cell density cultures (HCDCs), the $OD_{600}$ is much higher, reaching 10.0–20.0 in batch fermentations and 90.0 in fed-batch ones [56]. Moreover, selective agents, such as ampicillin and kanamycin, also deserve attention regarding large-scale optimization. The removal of antibiotics from the cultures is being studied for botulism antigens by determining plasmid stability, which consists on the period a plasmid is kept inside the *E. coli* cells without the use of antibiotics.

Another important point for industrial applications is the culture temperature, which can control the metabolism of the organism and thus provide the perfect conditions to express soluble proteins. Two temperatures are important in recombinant vaccine production against botulism: (1) the pre-induction temperature, which is when the culture is acquiring biomass (reaching the correct $OD_{600}$) to further expression; and (2) the post-induction temperature, which is when the culture does not need more biomass and the focus is predominantly on protein expression. During the pre-incubation period, the culture temperature is consistent with the purpose of this period; therefore, the temperature used is based on the optimal growth temperature for *E. coli*, which is 37 °C with few works using 30 °C [59,60]. While the pre-induction temperature is well established, the post-induction temperature is variable for botulism antigens. This disparity arises from the intrinsic properties of the recombinant proteins being expressed, which make the exploration of the optimal expression temperature more complicated. The most commonly used post-induction temperatures are low (16–18 °C), medium (25–30 °C) or normal (37 °C) (Table 1). The latter is the one preferred by HCDC, because it preserves the perfect metabolic conditions of the microorganism. However, in some cases, this temperature can impair the expression of soluble proteins [61]. The post-induction time is also important in the production process as it can be optimized to obtain greater amounts of protein in less time, which results in a high production yield. The post-induction time for

**Table 1**
Summary of the protocols used for recombinant antigens against BoNTs expressed in *E. coli*.

| Targeted serotype | Production strategy | Post-induction temperature (°C) | Expression time (h) | Yield (mg/L of culture) | References |
|---|---|---|---|---|---|
| BoNT/A-F | Shake flask | Low (16–18) | 15–22 | 12–50 | [59,60,62] |
| | | Medium (25–30) | 12–30 | 10–125 | [37,39,51,52,57,58,63–65] |
| | | Normal (37) | 3–6 | 1–220 | [38,47–49,53–55,66–68] |
| | | | 18 | 12 | [69] |
| BoNT/A and B | Batch | Low (16) | 22 | 96 | [70] |
| | | Normal (37) | 4 | 62 | [56] |
| BoNT/A | Fed-batch | Normal (37) | 3 | 468 | [56] |

botulism antigens expression exhibits two options: (1) large periods (12–30 h) for low or medium post-induction temperatures and (2) short periods (3–6 h) for normal post-induction temperatures (Table 1). The use of a short expression period results in an easier process and fast production, because fewer hours are necessary to obtain protein.

After expression, the cells must be processed to obtain the purified recombinant protein. Currently, recombinant botulism vaccines are often engineered with a 6xHis-tag, which allows purification by $Ni^{+2}$ or $Co^{+2}$ affinity columns. Other procedures, such as ion exchange, gel filtration, hydrophobic interaction and microcentrifugation, might be used together with affinity chromatography or individually for further purification [53,54,59,70,71]. Taking this into consideration, the protein yield after purification ranges from 1 to 220 mg per liter of culture in shaking flasks (Table 1). Nonetheless, when it comes to large-scale production, batch and fed-batch cultures should be considered, because the botulism antigens yield is often higher, reaching 62–96 and 468 mg per liter of culture, respectively, in optimized conditions.

Despite the variation in the protocols for botulism antigens expression in *E. coli*, all methods showed that vaccines containing the $rH_C$ were able to elicit effective protection in mice. However, limited sources describe vaccine testing in target species. For the subtypes that typically affect humans, A, B, E, and F, none were tested in clinical studies, although protection in model animals has been thoroughly reported. In contrast, vaccines against the subtypes that generally affect production animals, C and D, have been tested in some of the target species. A recombinant vaccine consisting of the GST tag fused with the entire heavy chain of BoNT/C was tested subcutaneously in ducks in a two-dose regimen (50 μg/dose) using $Al(OH)_3$ as adjuvant, generating protection against 10 MLD [39]. Another study involved the $rH_C$ in horses using different vaccine formulations. The best one, with GPA (GERBU Pharma Adjuvant®, Gerbu Biotechnik GmbH, Gaiberg, Germany) as adjuvant and a three-dose regimen (100 μg/dose), generated reliable protection against BoNT/D only [40]. In cattle, a recent work described a chimeric antigen composed of the adjuvant LTB and both $rH_CC$ and $rH_CD$. This antigen was injected subcutaneously in a two-dose regimen (100 μg/dose) inducing effective protection against both toxins as determined by a serum neutralization assay in mice [41]. The summarized information of these three vaccine tests is shown in Table 2. Until now, no work has described the expression of the serotype G and the recently reported serotype H in *E. coli*.

Of the studies reported in Table 2, the one involving ducks obtained 10 MLD protection only against BoNT/C, which is below the expected when compared to the experiment in mice (at least $10^3$ MLD protection). Similarly, the formulations tested in horses showed reliable results only against BoNT/D. On the other hand, the vaccine tested in cattle generated protection values that matches the requirements for vaccines against both BoNT/C and D. Since none dose-dependent studies were conducted in these works, it is possible to improve the achieved results by using a higher amount of antigen per dose. In contrast, the route of immunization should not make difference a in the results, since subcutaneous route is the standard for botulism vaccines. Furthermore, the use of combined adjuvants, like LTB and $Al(OH)_3$ in the cattle experiment, can also improve the immune response of vaccines in the target species. Testing other adjuvants to enhance the protection is still a field to be explored on vaccines against botulism. Nonetheless, oil adjuvants, such as mineral oil, are not attractive for these vaccines once they can cause adverse effects due to exacerbated inflammatory responses. This way, the preferred adjuvant still is $Al(OH)_3$.

## 3. Overview of BoNT expression in *P. pastoris*

Yeasts are a widely-used expression system for recombinant proteins. Among them, the methylotrophic yeast *P. pastoris* has been used to express $rH_C$ domains from BoNT serotypes A to F. The same way as for *E. coli*, there are no reports of the cloning and expression of serotype G and the recently described serotype H [3] using this expression system. Many interesting characteristics, such as recombinant protein secretion, post-translational modifications and soluble intracellular heterologous protein expression, are available using expression in yeast. This section discusses the many aspects of heterologous gene expression in *P. pastoris* and underscores its use for the production of vaccines against botulism.

The clostridial genome is rich in AT. Therefore, the coding sequences for all serotypes of $H_C$ domains contain codons rarely used by *P. pastoris*, which may result in undesirable low expression levels or truncated proteins [72]. Consequently, synthetic coding sequences with the codon usage optimized for *P. pastoris* [73–77] or, more specifically, with the *aox1* codon bias [78,79] should be used to achieve higher levels of recombinant protein expression. Indeed, no works were found that used PCR to clone the $H_C$ domain of the BoNT coding sequences in the *P. pastoris* expression system, which indicates that optimized codons are a unanimous choice among all authors. Furthermore, the Kozak sequence plays a key role in initiating translation in eukaryotic organisms [80], which is an important aspect to consider when designing synthetic genes to express recombinant proteins in *P. pastoris* or any other eukaryotic system [76,78].

The transformation process in *P. pastoris* results in the integration of exogenous DNA into the host genome. As an alternative to the use of antibiotics, auxotrophic vectors may be used to select recombinant clones. Indeed, certain authors used the pHIL-D4 expression vector [42,73,75,81,82], which contains a coding sequence for histidinol dehydrogenase 4 (HIS4) to complement the defective gene in *P. pastoris* strain GS115. Other authors [74,76–79] used plasmids from the pPICZ family (Invitrogen), which select the transformants by resistance to the antibiotic Zeocin.

Alcohol oxidase (AOX) is an enzyme produced when methanol is the only source of carbon, representing up to 30% of protein production [83]. Vectors used in the works discussed here utilized the AOX promoter, which regulates the expression of recombinant protein in *P. pastoris*. Constitutive promoters were not found in any of the investigated literature. Inducible promoters provide a clear advantage over constitutive ones, because the former allow the culture to reach high cell density before commencing recombinant

**Table 2**
Summary of the studies involving vaccine tests against botulism in target species.

| Targeted species | Targeted serotype | Vaccination strategy (no. of doses *X* amount of antigen) | Route | Adjuvant | Level of protection | References |
|---|---|---|---|---|---|---|
| Duck | BoNT/C | 2 × 50 μg | s.c. | $Al(OH)_3$ | 10 MLD | [39] |
| Horse | BoNT/D | 3 × 100 μg | s.c. | GPA | 1000–2000 UI/mL | [40] |
| Cattle | BoNT/C and D | 2 × 200 μg[a] | s.c. | $Al(OH)_3$ | 5 ± 0 IU/mL (C) and 3.85 ± 1.35 IU/mL (D) | [41] |

s.c., subcutaneous.
[a] Approximately 100 μg of each serotype antigen were used.

protein expression, which optimizes the process. In contrast with IPTG induction in the *E. coli* expression system, which is expensive, methanol induction in yeast is cheaper. However, it is important to note that *E. coli* expression system can use heat, lactose or salt, which are even cheaper, to induce protein expression. Additionally, long periods of induction are needed when using *P. pastoris*, varying from 9 h to more than 74 h [74,81], which is a drawback when compared to the *E. coli* expression system [84]. Although most works do not specify, the methanol concentration varies from 0.2% [74] to 1.1% by the end of the fermentation process [42]. The only works describing variation in methanol feeding within the fermentation are Boles et al. (2006) [42] and Byrne et al. (1998) [81], which began with the addition of methanol at a rate of 4 mL per culture liter per hour (mL/L/h) and ended at 11 mL/L/h. Johnson et al. (2003) [75] and Byrne et al. (2000) [73] also described the methanol feeding rate, beginning at 4 g/h/L and finishing at 9 g/h/L 8.5 h after the feed started. The methanol feeding rate in these cases was adjusted according to the dissolved oxygen spike.

*P. pastoris* allows the secretion of the recombinant proteins. The secretion process forces the heterologous protein to go through the Golgi apparatus and the rough endoplasmic reticulum, resulting in protein glycosylation. If $rH_C$ secretion is desired, the glycosylation sites must be removed by direct mutagenesis; however, this strategy was not tested for the expression of botulism antigens. Because native bacterial proteins are not glycosylated, it is desirable that the recombinant protein is as similar to the native one as possible to induce an appropriate immunological response. Thus, the glycosylated $rH_C$/B failed to induce protective immunity in mice [44]. However, this same author was able to induce protective immunity in mice with the glycosylated $rH_C$/A. Glycosylation in *P. pastoris* can be avoided using vectors without signal peptides and it is advisable to do so. This modification results in intracellular protein expression, typically in a soluble form with no refolding required, which eliminates the need of denaturing agents in the purification process and favors a better immunological response. Indeed, several works corroborate this, reporting the expression of soluble intracellular $rH_C$ from serotypes A to F [42,73–75,77,78,85]. However, it is more difficult to lyse yeast cells compared to *E. coli* cells. Alternatively, it is possible to enzymatically remove the sugars from the recombinant proteins [86], but it is an expensive process. Furthermore, choosing the correct vaccination strategy to help better mimic intoxication or infection and the consequent antigen presentation to the immune system allied with properly folded protein provides a stronger immunological response, especially if conformational epitopes are involved. Additionally, chromatography steps during purification are required in both *E. coli* and *P. pastoris*, even though the supernatant of cell lysate and unpurified antigens have been used to successfully immunize animals (data not published). Although such strategy has only been tested with *E. coli* (data not published), it may work with *P. pastoris* as well.

Taking advantage of the aforementioned strategies, it is possible to achieve expression levels as high as 2 g/kg of wet cell mass [74] and 3 g/kg [85] of heterologous protein using 5 L fermenters. Johnson et al. (2003) [75] were able to attain 179 mg of recombinant $H_C$ serotype F per kilogram of wet cell mass in a 60 L fermentation process, highlighting the feasibility of scaling-up the production process in *P. pastoris*.

Notably, no works using strains with the $Mut^S$ phenotype were found. The $Mut^+$ phenotype presents a high growth rate when methanol is the sole source of carbon, while $Mut^S$ has a slow growth rate. However, Krainer et al. (2012) [87] showed that the $Mut^S$ phenotype, while it grows slower than $Mut^+$, might achieve a better production rate of recombinant proteins. Choosing the correct phenotype to express recombinant proteins is still a very controversial matter, highlighting the need to test both phenotypes of each protein before choosing the one that has a better expression pattern.

Among the authors mentioned in this section, Sinha et al. (2007) [85] performed the only fermentation assays to enhance the bioprocess. All $rH_C$ produced in *P. pastoris* were able to induce protective immunity in mice [74,78,81,82]. Furthermore, rhesus monkeys were vaccinated with $rH_C$ produced in *P. pastoris* and challenged via aerosol with BoNT/B. The vaccinated animals survived the challenge, while the control groups died. Lastly, it was still possible to detect significant levels of neutralizing antibodies on the blood of a third group of monkeys up to 24 months post vaccination [42].

A recombinant holotoxin with three point mutations to ablate toxicity was also expressed in *P. pastoris* and used to immunize mice, achieving protective immunity against three subtypes of BoNT/A [79]. Another strategy employed to express recombinant vaccines against botulism was to fuse $H_C$ to Adenovirus fiber protein 2 (Ad2F) to mediate antigen uptake and increase mucosal and humoral immune response. Mice vaccinated with this fusion protein exhibited a faster onset of sIgA and plasma IgG supported by a $T_H1/T_H2$ mixed response when compared to the response of mice vaccinated with $H_C$ alone [76]. Similarly, rabbits were nasally immunized with the aforementioned fusion protein, with $H_C$ alone or with CT or C40/80 as adjuvants [77]. In accordance with Maddalone et al. (2006) [76], the rabbits vaccinated with the fusion protein elicited a higher titer of antibodies as determined by ELISA when compared to the animals vaccinated solely with the $H_C$.

It is particularly difficult to compare literature regarding the potency test for recombinant vaccines against botulism, because many different methodologies are employed, including ELISA [77], challenge in mice [60] and mice neutralization bioassays [41,84].

## 4. Other expression systems

Another strategy, explored by few authors, is the use of recombinant virus and cell-free expression systems [53,88–90]. Although efficient in protecting animals against challenge, such expression strategies are expensive. Culturing cells and/or buying cell-free expression kits makes these strategies way too expensive to be employed on an industrial scale of antigen production.

## 5. Conclusions

*E. coli* and *P. pastoris* are the most commonly used expression systems to produce recombinant vaccines due to the low costs and biosafety. However, *E. coli* present several drawbacks, such as pyrogens on the cell wall and inclusion body formation. Nonetheless, the use of $rH_C$ as antigen has shown to induce protective response no matter the state of the protein. Proteins expressed in *P. pastoris* may be hyperglycosylated when directed to secretion, which might affect the immunogenicity of these proteins if the native structures do not contain sugars. Nonetheless, this drawback can be bypassed by using vectors that lack the signal peptide for secretion. Additionally, the lysis of yeast cells is a notably toilsome process that may be disadvantageous on an industrial scale. Because of the high AT content in the clostridial DNA, it is imperative that synthetic genes with optimal codon usage for the applied expression system are used to improve expression levels.

Even though all vaccine prototypes described in this review presented successful results with laboratory animals, regardless of expression system, only few of them was carried out on the target species. Thereby, we conclude that all expression systems can express recombinant toxins capable of eliciting a protective immune response against botulism. When considering production costs, *E. coli* is the best system. Alternatively, *P. pastoris* has an easier

scale-up process, although a bacterial system can also be scaled-up to an industrial scale. Cell-free and viral systems are not indicated for large-scale production due to high costs and low recombinant protein yield, despite being academically interesting.

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