

【DESCRIPTION】

【Invention Title】

MEDIUM COMPOSITION FOR PREPARING BOTULINUM TOXIN

【Technical Field】

[1] The present invention relates to a medium composition for production of botulinum toxin, and more specifically, to a medium composition for culturing *Clostridium* sp. strains capable of producing botulinum toxin. The medium composition of the present invention is characterized by containing plant peptones, including wheat peptone and peptone from pea, D-(+)-glucose, and yeast extract.

[2]

【Background Art】

[3] A variety of *Clostridium* sp. strains that secrete neurotoxins have been discovered since the 1890s, and characterization of toxins secreted from these strains has been conducted for the past 70 years (Schant, E. J. *et al.*, *Microbiol. Rev.*, 56:80, 1992).

[4] Neurotoxins derived from the *Clostridium* sp. strains, that is, botulinum toxins, are classified into seven types (types A to G) depending on their serological properties. Each of the toxins has a toxin protein having a size of about 150 kDa and naturally contains a complex of several non-toxic proteins. A medium complex (300 kDa) is composed of a toxin

protein and a non-toxic non-hemagglutinin protein, and a large complex (450 kDa) and a large-large complex (900 kDa) are composed of the medium complex bound to hemagglutinin (Sugiyama, H., *Microbiol. Rev.*, 44:419, 1980). Such non-toxic hemagglutinin proteins are known to function to protect the toxin from low pH and various proteases in the intestines.

[5] The toxin is synthesized as a single polypeptide having a molecular weight of about 150 kDa in cells, and then is cleaved, at a position 1/3 from the N-terminal end, by the action of intracellular protease or treatment with an artificial enzyme such as trypsin into two units: a light chain (L; molecular weight: 50 kDa) and a heavy chain (H; molecular weight: 100 kDa). The cleaved toxin has greatly increased toxicity compared to the single polypeptide. The two units are linked to each other by a disulfide bond and have different functions. The heavy chain binds to a receptor of a target cell (Park. M. K. *et al.*, *FEMS Microbiol. Lett.*, 72:243, 1990) and functions to interact with a biomembrane at low pH (pH 4) to form a channel (Mantecuccio, C. *et al.*, *TIBS.*, 18:324, 1993), and the light chain has pharmacological activity, and interferes with the secretion of a neurotransmitter when cells have been permeabilized using a detergent or the light chain has been introduced into cells by, for example, electroporation (Poulain, B. *et al.*, *Proc. Natl. Acad. Sci. USA.*, 85:4090, 1988).

[6] The toxin inhibits the exocytosis of acetylcholine at the cholinergic presynapse of a neuromuscular junction, causing asthenia. It has been believed that treatment with a very small amount of the toxin exhibits toxicity, suggesting that the toxin has any enzymatic activity (Simpson, L. L. et al., *Ann. Rev. Pharmacol. Toxicol.*, 26:427, 1986).

[7] According to a recent report, the toxin has metallopeptidase activity, and its substrate is composed of synaptobrevin, syntaxin, a synaptosomal associated protein of 25 kDa (SNAP25) and the like, which are the unit proteins of an exocytosis machinery complex. Each type of toxin uses one of the above-described three proteins as its substrate, and it is known that type B, D, F and G toxins cleave synaptobrevin at a specific site, type A and E toxins cleave SNAP25 at a specific site, and type C toxin cleaves syntaxin at a specific site (Binz, T. et al., *J. Biol. Chem.*, 265:9153, 1994).

[8] Particularly, type A botulinum toxin is known to be soluble in a dilute aqueous solution at a pH of 4.0 to 6.8. It is known that a stable non-toxic protein is separated from neurotoxin at a pH of about 7 or higher, and as a result, the toxicity is gradually lost. Particularly, it is known that the toxicity decreases as pH and temperature increase.

[9] Botulinum toxin is fatal to humans even in small amounts and is easy to produce in large amounts. Thus, it

constitutes four major bio-terror weapons together with *Bacillus anthracis*, *Yersinia pestis*, and smallpox virus. However, it has been found that, when type A botulinum toxin is injected at a dose that does not systematically affect the human body, it can paralyze local muscle around the injection site. Based on this characteristic, type A botulinum toxin can be used in a wide range of applications, including wrinkle removing agents, agents for treating spastic hemiplegia and cerebral palsy, etc. Thus, the demand for type A botulinum toxin has increased, and studies on methods of producing botulinum toxin so as to satisfy the demand have been actively conducted.

[10] A current typical commercial product is BOTOX® (a purified neurotoxin complex of type A botulinum toxin) that is commercially available from Allergan, Inc., USA. A 100-unit vial of BOTOX® is composed of about 5 ng of a purified type A botulinum toxin complex, 0.5 mg of human serum albumin, and 0.9 mg of sodium chloride, is provided in a vacuum-dried form, and is reconstituted using sterile saline without a preservative (injection of 0.9% sodium chloride). Other commercial products include Dysport® (a *Clostridium botulinum* type A toxin-hemagglutinin complex which has lactose and human serum albumin in a pharmaceutical composition containing botulinum toxin and is reconstituted using 0.9% sodium chloride before use) that is commercially

available from Ipsen Ltd., UK, MyoBloc® (an injectable solution with a pH of about 5.6 comprising botulinum type B toxin, human serum albumin, sodium succinate, and sodium chloride) that is commercially available from Solstice Neurosciences, Inc.

[11] A medium for culturing a *Clostridium botulinum* strain, which is generally used in a method for production of botulinum toxin as disclosed in Korean Patent No. 10-1339349, contains animal-derived components. Thus, if an animal-derived abnormal prion known as an agent that causes transmissible spongiform encephalopathy is contained in the animal components due to contamination, it poses problems in a process for producing botulinum toxin.

[12] Transmissible spongiform encephalopathy (TSE) is a type of a neurodegenerative disorder causing serious degeneration of neurons, and examples thereof includes bovine spongiform encephalopathy (BSE), Scrapie, Creutzfeldt-Jakob disease (CJD), Gerstmann-Straussler-Scheinker syndrome, Kuru, transmissible mink encephalopathy, chronic wasting disease, feline spongiform encephalopathy, etc., which affect humans and animals. It was reported that BSE crosses the species barrier and affects even humans.

[13] The agent that causes transmissible spongiform encephalopathy is characterized in that it is non-immunogenic and has a long incubation period. From post-mortem analysis

of the brains of cattle infected with bovine spongiform encephalopathy, it can be seen that a unique pattern of spongiform vacuoles was formed in the brain due to the destruction of neurons and the deposition of abnormal protein fibers.

[14] The pathogen believed to be the cause of transmissible spongiform encephalopathy is an infectious protein called an abnormal prion. Unlike regular viruses that require nucleic acids, the abnormal prion is an infectious particle composed only of protein and containing no nucleic acid. Regarding TSE, it is known that, when an abnormal prion (PrPsc) that is an infectious particle binds to a normal prion (PrPc), it is converted to a pathogenic prion which then accumulates in the brain (Prusiner SB, *Alzheimer Dis Assoc Disord.*, 3:52-78, 1989).

[15] Creutzfeldt-Jakob disease is a rare neurodegenerative disorder of human transmissible spongiform encephalopathy (TSE) where the transmissible agent is apparently an abnormal isoform of a prion protein. An individual with Creutzfeldt-Jakob disease can deteriorate from apparent perfect health to akinetic mutism within six months. Thus, when a pharmaceutical composition containing a biologic, such as botulinum toxin, obtained using animal-derived products, there is a risk of developing a prion-mediated disease such as Creutzfeldt-Jakob disease. Thus, if

a pharmaceutical composition is prepared using stock produced using animal-derived components, there is a risk that the patient will receive various pathogens or infectious agents.

[16] Accordingly, it has been reported that adding a casein hydrolysate (e.g., TSE-certificated casein hydrolysate) to an animal protein-free (APF) medium to prevent TSE infection improves the growth rate of strains and the production of botulinum toxin (Korean Patent No. 17-1729251, etc.). However, since the APF medium to which the casein hydrolysate has been added still contains casein, which is an animal-derived component, research is needed to achieve high growth rates and high toxin production of *Clostridium botulinum* strains in APF medium that does not contain animal-derived components.

[17] Accordingly, the present inventors have made extensive efforts to develop a medium that contains plant peptones causing no risk of TSE infection when culturing *Clostridium botulinum* strains and does not contain animal-derived components in order to prevent the risk of prion-mediated disease as described above, and as a result, have found that the medium can improve the growth rate of *Clostridium botulinum* strains and the toxin production of the strains compared to conventional media, thereby completing the present invention.

[18]

【Disclosure】

【Technical Problem】

[19] An object of the present invention is to provide a medium composition for culturing *Clostridium botulinum* strains that does not contain animal-derived components.

[20] Another object of the present invention is to provide a method of improving the production of botulinum toxin by culturing *Clostridium botulinum* in the above-described medium composition.

[21]

【Technical Solution】

[22] To achieve the above objects, the present invention provides a medium composition for culturing *Clostridium botulinum* strains, which contains plant peptones, including wheat peptone and peptone from pea, D-(+)-glucose, and yeast extract, and does not contain animal-derived components.

[23] The present invention also provides a method for producing botulinum toxin, comprising steps of: (a) producing botulinum toxin by culturing *Clostridium botulinum* using the medium composition; and (b) recovering the produced botulinum toxin.

[24]

【Advantageous effects】

[25] When the medium according to the present invention, which contains plant peptones, glucose, and yeast extract

and does not contain animal-derived components, is used to culture *Clostridium botulinum* strains, the medium may increase the growth rate of the strains compared to conventional media, and increase the production of botulinum toxin by culturing the strain in a safe manner because it does not contain animal-derived components.

[26]

【Description of Drawings】

[27] FIG. 1 shows a JMP custom design to determine the appropriate proportion of plant peptones using three plant peptones (peptone from pea, wheat peptone, and peptone from potatoes No. 2) as factors and an OD_{540nm} value of 3 or higher as a response.

[28] FIG. 2 shows the results of design evaluation by power analysis of the custom design and the fraction of design space plot.

[29] FIG. 3 shows media containing APF medium components.

[30] FIG. 4a is a graph showing the results of measuring the growth rate of a *Clostridium botulinum* strain over time in APF media containing dextrin as a carbon source, and FIG. 4b is a graph showing the results of measuring the growth rate of a *Clostridium botulinum* strain over time in APF media containing glucose as a carbon source.

[31] FIG. 5 is a graph showing the results of measuring the growth rate of a *Clostridium botulinum* strain over time in 16 different types of APF media.

[32] FIG. 6 shows distribution analysis of D-optimal design experiments, and it can be seen that the experimental results are generally evenly distributed.

[33] FIG. 7 shows scatterplot matrix analysis of D-optimal design results, and it can be seen that wheat peptone and peptone from pea have a weak positive correlation with OD_{540nm} and toxin concentration.

[34] FIG. 8 shows the results of outlier analysis, and it can be seen that there are no outliers in the experimental results.

[35] FIG. 9 shows the process for establishing a model of OD_{540nm}, and it can be seen that the VIF value does not exceed 10 and is at level 1, suggesting that there is no problem with the model, and that all studentized residuals results are included in the 95% confidence interval.

[36] FIG. 10 shows the prediction formula for OD_{540nm}.

[37] FIG. 11 shows the interaction profiles of OD_{540nm}.

[38] FIG. 12 shows the process for establishing a model of OD_{540nm}, and it can be seen that the VIF value does not exceed 10 and is at level 1, suggesting that there is no problem with the model, and that all studentized residuals results are included in the 95% confidence interval.

[39] FIG. 13 shows the prediction formula for toxin concentration.

[40] FIG. 14 shows interaction profiles.

[41] FIG. 15 shows the process of finding medium conditions for OD_{540nm} and toxin concentration using a Prediction Profiler based on the prediction formula for each response value.

[42] FIGS. 16a and 16b show the distribution of Y in Spec (FIG. 16a) and edge of failure analysis (FIG. 16b) after performing 5,000 simulations as a function of the random variation in the factors and model noise using the simulator function.

[43] FIG. 17 shows the analysis of pea capability with the range corresponding to 3σ set as NORs and the range corresponding to 4.5σ set as PARs.

[44] FIG. 18 shows the analysis of wheat capability with the range corresponding to 3σ set as NORs and the range corresponding to 4.5σ set as PARs.

[45] FIG. 19 shows the contour profiler results of the APF medium, and it can be seen that, when NORs (3σ , red line) and PARs (4.5σ , blue line) were plotted on a contour plot, they did not exceed the white range, which is the range satisfying the model.

[46] FIG. 20 shows the growth curve of a botulinum strain in each APF medium.

[47] FIG. 21 shows the level of toxin expression for each APF medium.

[48]

【Best Mode】

[49] Although an animal protein-free (APF) medium composition exhibited an improved growth rate of *Clostridium botulinum* strains compared to conventional media, there is a need to add medium components that further improve the growth rate of the strains without the risk of infection such as TSE infection. Recently, a casein hydrolysate (e.g., TSE-certificated casein hydrolysate) that has not been reported to cause TSE infection has been added to APF medium to grow strains and produce toxins.

[50] However, casein hydrolysate is a component extracted from milk, and casein accounts for about 80% of the total protein contained in milk. Although APF medium containing casein hydrolysate does not contain animal-derived components such as fetal bovine serum (FBS), casein hydrolysate itself is an animal-derived component, and thus there is a need for a highly safe medium for culturing *Clostridium botulinum* strains, which contains plant peptones without containing animal-derived components such as casein hydrolysate.

[51] Accordingly, in one aspect, the present invention relates to a medium composition for culturing *Clostridium*

botulinum strains, which contains plant peptones, including wheat peptone and peptone from pea, D-(+)-glucose, and yeast extract, and does not contain animal-derived components. Here, "plant peptone" means peptone extracted from wheat or pea, and preferably may be commercially available Millipore™ 96174, or Millipore™ 93492-500G-F, without being limited thereto.

[52] In the present invention, the term "conventional medium" refers to a medium containing casein hydrolysate, yeast extract, and thioglycolate medium, which are animal-derived components. Additionally, the term "animal protein-free (APF) medium" refers to a medium not containing animal-derived proteins.

[53] In one example of the present invention, APF media were prepared containing various plant-based medium ingredients, including peptone from pea, wheat peptone, peptone from potatoes No. 2, corn steep solid, gluten from wheat, potato peptone E210, banana powder, tomato juice, and malt extract, without animal-derived components, under transmissible spongiform encephalopathy (TSE)-free conditions, and the growth rates and toxin expression levels of the strain in the media were compared. As a result, it could be confirmed that the plant-derived medium components for efficiently culturing the *Clostridium botulinum* strain included peptone from pea, wheat peptone, and peptone from potatoes No. 2, and in this case, it was confirmed that the

growth and toxin expression of the strain were excellent. Finally, as shown in Tables 4 and 5 below and FIG. 4, it was determined that the plant peptones contained in the final selected medium composition for culturing the *Clostridium botulinum* strain were peptone from pea, wheat peptone, and potato peptone.

[54] In another example of the present invention, APF media were prepared containing peptone from pea, wheat peptone, and potato peptone at various compositional ratios without animal-derived components under transmissible spongiform encephalopathy (TSE)-free conditions, and the growth rates and toxin expression levels of the strain in the media were compared. As a result, it could be confirmed that the medium composition for efficiently culturing the *Clostridium botulinum* strain contained at least one plant peptone selected from the group consisting of wheat peptone and peptone from pea, in addition to a carbon source (e.g., glucose) and a nitrogen source (e.g., yeast extract), and in this case, the growth and toxin expression of the strain were excellent. Finally, as shown in Tables 8 to 12 below and FIGS. 5 to 19, the contents of plant peptones in the final selected medium composition for culturing the *Clostridium botulinum* strain were 18 g/L of peptone from pea and 7 g/L of wheat peptone.

[55] In another example of the present invention, an experiment was conducted to compare the growth pattern and toxin concentration of the *Clostridium botulinum* strain between when grown in an APF medium containing the final selected plant peptone of the present invention and when grown in a medium (Comparative Example 7) described in Korean Patent Application Publication 10-2022-0140696, which was published prior to the present invention. As a result, as shown in Tables 13 to 15 below and FIGS. 20 and 21, in the case of the medium of the present invention, the OD_{540nm} value was 7.1021 after 28 hours of culture of the *Clostridium botulinum* strain, and then the OD value gradually decreased, the OD_{540nm} value was 5.5636 after 48 hours, and the toxin concentration in the supernatant of *Clostridium botulinum* toxin was 588.8 µg (10 mL of culture medium) after 48 hours of culture. Meanwhile, as shown in Tables 13 to 15 below and FIGS. 20 and 21, in the case of the medium of Comparative Example 7, the OD_{540nm} value was 6.7739 after 28 hours of culture of the *Clostridium botulinum* strain, and the toxin concentration in the supernatant of *Clostridium botulinum* toxin was 263.7 µg (10 mL of culture medium) after 48 hours of culture, suggesting the toxin expression level decreased by 55.2% compared to that in the case of the medium of the present invention.

[56] In another embodiment of the present invention, an experiment was conducted to compare the growth pattern and toxin concentration of the *Clostridium botulinum* strain between the medium of the present invention and media (Comparative Examples 1 to 3) further containing L-cysteine hydrochloride monohydrate and/or medical antifoam C emulsion (Dow Corning®), which are/is the compositional component(s) of the medium described in Korean Patent Application Publication No. 10-2022-0140696, in addition to the medium of the present invention. As a result, as shown in Tables 13 to 15 below and FIGS. 20 and 21, it can be confirmed that, in the case of Comparative Examples 1 to 3, the OD_{540nm} value was lower than that in the medium of the present invention, and the toxin expression level after 48 hours of culture decreased by 14.9 to 76.6% compared to that in the medium of the present invention.

[57] In another example of the present invention, an experiment was conducted to compare the growth pattern and toxin concentration of the *Clostridium botulinum* strain between the medium of the present invention and media (Comparative Examples 4 to 6) containing soy peptone in place of peptone from pea and/or wheat peptone in the medium of the present invention and further containing L-cysteine hydrochloride monohydrate and medical antifoam C emulsion (Dow Corning®). As a result, as shown in Tables 13 to 15

below and FIGS. 20 and 21, it can be confirmed that, in the case of Comparative Examples 4 to 6, the OD_{540nm} value was significantly lower than that in the medium of the present invention, and the toxin expression level after 48 hours of culture decreased by 87.0 to 94.9% compared to that in the medium of the present invention.

[58] In the present invention, the medium composition for culturing *Clostridium botulinum* strains may contain at least one plant-derived component selected from the group consisting of peptone from pea, wheat peptone, peptone from potatoes No. 2, corn steep solid, gluten from wheat, potato peptone E210, banana powder, tomato juice, and malt extract, preferably may contain peptone from pea, wheat peptone, and peptone from potatoes No. 2, and more preferably may contain peptone from pea and wheat peptone, without being limited thereto.

[59] As used herein, the term "plant peptone" or "plant hydrolysate" refer to the digestion products of proteins obtained from plants. For example, wheat peptone (wheat hydrolysate) refers to the product obtained by digesting the total protein obtained from wheat. Also, "casein hydrolysate" refers to the digestion product of casein protein.

[60] The digestion of the plant protein or casein protein is preferably carried out by partial digestion. The digestion of the protein is preferably carried out by acid treatment,

base treatment, enzyme treatment, high-pressure treatment, heat treatment, or physical treatment, and more preferably, the plant peptone or casein hydrolysate may be enzyme-treated. The physical treatment is, for example, grinding.

[61] The plant peptone or casein hydrolysate in the present invention is a partial degradation product of protein, and is in the form of a mixture containing not only amino acids as single molecules but also peptides composed of several to tens of amino acids and whole protein molecules.

[62] In the present invention, the content of the plant peptones may be 0.1 to 10 w/v% (1 to 100 g/L), preferably 0.2 to 5 w/v% (2 to 50 g/L), more preferably 0.5 to 2 w/v% (5 to 20 g/L).

[63] In the present invention, the content of the wheat peptone may be 0.1 to 4.0 w/v% (1 to 40 g/L), preferably 0.45 to 0.95 w/v% (4.5 to 9.5 g/L), preferably 0.48 to 0.92 w/v% (4.8 to 9.2 g/L), preferably 0.55 to 0.85 w/v% (5.5 to 8.5 g/L), more preferably 0.7 w/v% (7 g/L).

[64] In the present invention, the content of the pea peptone may be 0.1 to 3.5 w/v% (1 to 35 g/L), preferably 1.55 to 2.05 w/v% (15.5 to 20.5 g/L), preferably 1.58 to 2.03 w/v% (15.8 to 20.3 g/L), preferably 1.65 to 1.95 w/v% (16.5 to 19.5 g/L), more preferably 1.8 w/v% (18 g/L).

[65] In the present invention, the medium composition for culturing *Clostridium botulinum* strains may not contain L-

cysteine hydrochloride monohydrate and/or an antifoaming agent.

[66] As used herein, the term "antifoaming agent" means a substance that completely or partially prevents the formation of foam and is used interchangeably with "foam-preventing agent". The antifoaming agent may include a medical antifoaming agent (Dow Corning®), without being limited thereto.

[67] In the present invention, the medium composition for culturing *Clostridium botulinum* strains may contain a carbon source and a nitrogen source.

[68] In the present invention, the carbon source may be a monosaccharide (e.g., glucose, fructose, etc.), a disaccharide (e.g., maltose, sucrose, etc.), an oligosaccharide, a polysaccharide (e.g., dextrin, cyclodextrin, starch, etc.), or a sugar alcohol (e.g., xylitol, sorbitol, erythritol, etc.), preferably D-(+)-glucose, without being limited thereto.

[69] In the present invention, the content of the carbon source may be 0.1 to 1.5 w/v% (1 to 15 g/L), preferably 0.25 to 1.5 w/v% (2.5 to 15 g/L), preferably 1.0 w/v% (10 g/L), without being limited thereto.

[70] As used herein, the term "nitrogen source" means an organic or inorganic substance containing nitrogen atoms essential for the synthesis of proteins, purines, pyrimidines,

and polysaccharide chitin in the growth of strains. The nitrogen source is preferably yeast extract, without being limited thereto.

[71] In the present invention, the nitrogen source may be yeast extract, polypeptone, tryptone, malt extract, corn steep liquor, or soytone, preferably yeast extract, without being limited thereto.

[72] In the present invention, the content of the nitrogen source may be 0.5 to 2.0 w/v% (5 to 20 g/L), preferably 0.5 to 1.0 w/v% (5 to 10 g/L), preferably 1.0 w/v% (10 g/L), without being limited thereto.

[73] In another aspect, the present invention relates to a method for producing botulinum toxin, comprising steps of: (a) producing botulinum toxin by culturing *Clostridium botulinum* using the medium composition; and (b) recovering the produced botulinum toxin.

[74] In the present invention, the culturing may be performed under anaerobic conditions, the culturing may be performed under temperature conditions of 33°C to 37°C, the culturing may be performed for 20 to 48 hours, and the toxin may be selected from the group consisting of botulinum toxins A, B, C, D, E, F and G.

[75]

[76] Hereinafter, the present invention will be described in more detail through examples. These examples are intended

only to illustrate the present invention, and it will be obvious to those skilled in the art that the scope of the present invention is not to be construed as being limited by these examples.

[77]

[78] **Example 1: Selection of plant peptones**

[79] **1-1: Composition of CYG medium used for culture (control)**

[80] CYG medium (casein, yeast extract and glucose) for culturing *Clostridium botulinum* was prepared by dissolving 2% casein hydrolysate [20 g/L], 1% yeast extract [10 g/L], and 1% glucose [10 g/L] in 90 mL of distilled water, adjusting the final volume to 100 mL, and dispensing 10-mL aliquots of the prepared medium into 15-mL culture tubes. Thereafter, the medium was autoclaved at 121°C for 30 minutes and then kept under anaerobic conditions in an anaerobic incubator.

[81] **1-2: Compositions of APF media used for culture**

[82] As shown in Tables 1 and 2 below, 9 types of 2% plant peptone candidates (Sigma™ C8160-500G, Millipore™ 96174, Millipore™ 93492-500G-F, Sigma-Aldrich™ G5004-500G, Millipore™ 38143-500G, Organotechnie™ 19425, Sigma-Aldrich™ B4032-500G, Sigma-Aldrich™ 17218-500G, and Merck™ 1.05391.0500) [10 g/L] were each added to 1% yeast extract [10 g/L] and 1% dextrin or glucose [10 g/L], and then dissolved in 90 mL of water for injection. Then, the final

volume was adjusted to 100 mL, and the pH of each medium was adjusted to 7.3. Then, 10-mL aliquots of the prepared media were dispensed into 15-mL culture tubes, respectively. Thereafter, the media were autoclaved at 121°C for 30 minutes and kept under anaerobic conditions in an anaerobic incubator.

[83] [Table 1]

Category	Medium (g/L)		1	2	3	4	5	6	7	8	9
Carbon source	Dextrin		10	10	10	10	10	10	10	10	10
	Glucose										
Plant-derived components	Corn steep	Corn steep solid	10								
	Pea	Peptone from pea		10							
	Wheat gluten	Wheat peptone			10						
		Gluten from wheat				10					
	Potato	Peotone from potatoes No. 2					10				
		Potato peptone E210						10			

	Banana	Banana powder							10		
	Tomato	Tomato juice								10	
	Malt	Malt extract									10
Nitrogen source	Yeast extract		10	10	10	10	10	10	10	10	10

[84] [Table 2]

Category	Medium (g/L)		10	11	12	13	14	15	16	17	18
Carbon source	Dextrin		10	10	10	10	10	10	10	10	10
	Glucose										
Plant-derived components	Corn steep solid	Corn steep solid	10								
	Pea	Peptone from pea		10							
	Wheat gluten	Wheat peptone			10						
		Gluten from wheat				10					
	Potato	Peotone from					10				

		potatoes No. 2									
		Potato peptone E210						10			
	Banana	Banana powder							10		
	Tomato	Tomato juice								10	
	Malt	Malt extract									10
Nitrogen source	Yeast extract		10	10	10	10	10	10	10	10	10

[85] 1-3: Culture of *Clostridium botulinum* strain

[86] Culture tubes containing medium compositions 1 to 18 shown in Tables 1 and 2 above were inoculated with 20 μ L of *Clostridium botulinum* (Korea Center for Disease Control and Prevention, control No. 4-029-CBB-MF-2020001), and samples were taken while the strain was stationary-cultured under anaerobic conditions at 35°C $\pm$ 1°C for 20, 24, 28, and 48 hours. Thereafter, the growth rate of the strain was determined by measuring the OD_{540nm} value of the culture sampled at each time using a UV spectrophotometer, and the culture medium was used as a blank. Based on the measured OD_{540nm} value, plant peptone candidates for the medium were selected by comparison with the control.

[87] 1-4: Strain growth rate

[88] Among the plant peptone candidates, corn extract solid, banana powder, and gluten from wheat were excluded from the plant peptone candidates because they formed precipitates after sterilization of the media, making it difficult to perform the filtration process and accurately measure the OD_{540nm} value. The medium compositions containing the plant peptone candidates are shown in Table 3 below.

[89] [Table 3]

Medium (g/L)		1	2	3	4	5	6	7	8	9	10	11	12
Dextrin		10	10	10	10	10	10						
Glucose								10	10	10	10	10	10
Pea	Peptone from pea	10						10					
Wheat gluten	Wheat peptone		10						10				
Potato	Peptone from potatoes No. 2			10						10			
	Potato peptone E210				10						10		
Tomato	Tomato juice					10						10	

Malt	Malt extract						10						10
Yeast extract		10	10	10	10	10	10	10	10	10	10	10	10

[90] The OD_{540nm} values of cultures sampled at each time point for medium compositions 1 to 12 shown in Table 3 above and two control media (CYG media) were measured (Table 4), and the graphs of the OD_{540nm} values of the carbon source (dextrin or glucose) of each medium over time were compared (FIG. 4).

[91] [Table 4]

Medium	OD _{540nm} value over time			
	20hr	24hr	28hr	48hr
1	1.8730	2.3500	3.2304	3.2722
2	1.8599	2.1685	2.5121	4.0037
3	1.4141	1.5862	2.0204	2.4978
4	1.4811	2.0280	2.3678	2.6301
5	1.0544	1.1912	1.3145	0.8669
6	1.1858	1.6250	1.8331	1.4966
7	2.2260	3.0544	3.7671	4.0443
8	2.3389	3.2501	3.6515	2.6177
9	1.5869	1.9006	1.9675	2.0508
10	1.2805	1.2364	1.1067	1.8789
11	0.9147	1.0896	1.1602	1.3564
12	1.0836	1.4444	1.6244	0.5030
Control 1	2.0969	2.8093	3.0028	2.3717
Control 2	1.1039	1.6976	2.0191	2.2898

[92] As a result, as shown in Table 4 above and FIG. 4, it was confirmed that the strain showed high growth rates

commonly in media 1, 2, 3, 7, 8 and 9 containing components derived from pea, wheat, and potato.

[93] In addition, it was confirmed that, even when the same plant peptones derived from pea, wheat, and potato were used, the strain showed higher growth rates in media 7, 8 and 9 containing glucose as a carbon source than in media 1, 2 and 3 containing dextrin. In particular, it was confirmed that, in the case of wheat-derived components, the strain tended to grow even after 48 hours after inoculation in medium 2 containing dextrin (FIG. 4). This growth trend may unnecessarily delay the production time of the stock solution in the future, suggesting that using glucose as a carbon source for the medium is suitable.

[94] 1-5: Botulinum toxin concentration

[95] The amounts of toxin protein in media 1, 2, 3, 7, 8, and 9, which showed high growth rates of the strain in Examples 1-4, were measured. Since previous studies recover toxins during the death phase, toxin concentrations were measured by the ELISA method for the quantitative analysis of botulinum toxin type A culture medium at 28 and 48 hours, which are the latter parts of the culture, and the results are shown in Table 5 below (ICH guideline, Q2(R1)).

[96] Antitoxin was diluted and dispensed into each well of a 96-well plate, which was then coated for 16 hours or more. The contents of the plate were removed, each well was

blocked with blocking solution, and then the plate was washed three times with wash buffer. The standard and the culture were diluted and dispensed into each well in triplicate, and the plate was incubated and then washed three times with wash buffer. Primary antibody dilution solution was dispensed into each well, and the plate was incubated and then washed three times with wash buffer. Substrate solution was dispensed into each well and incubated, and then stop solution was dispensed into each well to stop the reaction. Absorbance was measured at wavelengths of 450 to 540 nm.

[97] [Table 5]

Medium	Plant-/animal-derived component	Carbon source	Toxin concentration (ng/mL)	
			28 hr	48 hr
Control (CYG)	Casein	Glucose	17826.0	20007.7
1	Pea	Dextrin	11716.8	6676.7
2	Wheat		4294.4	5703.5
3	Potato		14732.2	9945.9
7	Pea	Glucose	20775.2	11772.9
8	Wheat		5135.8	17415.1
9	Potato		14440.7	16602.9

[98] As a result, as shown in Table 5 above, the toxin expression levels were highest in the order of pea, potato, and wheat. In addition, it was confirmed that, when the same plant peptones were used, the toxin expression levels in the

media containing glucose as the carbon source were higher than those in the medium containing dextrin, and that using glucose as the carbon source for the medium for producing toxin from the *Clostridium botulinum* strain was more suitable.

[99] Based on the experimental results of Example 1, three plant peptones were selected as final plant peptone candidates: peptone from pea, wheat peptone, and potato peptone (peptone from potatoes No. 2).

[100]

[101] Example 2: Selection of proportion of plant peptones

[102] 2-1: Method for selection of proportion of plant peptones

[103] In order to select the proportion of the three final plant peptone candidates finally selected in Examples 1-4 and 1-5, a JMP custom design was used to set the OD_{540nm} value as a response to 3 or higher and the proportion of the three plant peptones as factors to 2% or less. In addition, in the same manner as in Examples 1-1 and 1-2, the proportion of each of the carbon source and the yeast extract was fixed at 1% and the experiment was conducted in duplicate per medium condition (FIG. 1; Peptone 1: peptone from pea; Peptone 2: wheat peptone; Peptone 3: potato peptone).

[104] For design evaluation, power analysis was performed and the fraction of design space plot was examined (FIG. 2;

Peptone 1: peptone from pea; Peptone 2: wheat peptone; Peptone 3: potato peptone).

[105] The experimental design for OD_{540nm} values according to the proportion of plant peptones obtained through custom design is shown in Table 6 below, and the toxin concentration after the completion of culture under each condition was measured and added to the analysis items (Peptone 1: peptone from pea; Peptone 2: wheat peptone; Peptone 3: potato peptone).

[106] [Table 6]

	Peptone 1	Peptone 2	Peptone 3	OD540nm
1	10	20	0	
2	10	0	10	
3	20	10	0	
4	10	0	0	
5	10	10	20	
6	0	10	0	
7	0	0	0	
8	20	20	0	
9	0	20	20	
10	0	20	10	
11	20	20	20	
12	20	0	0	
13	20	0	20	
14	0	20	0	
15	20	10	10	
16	0	0	20	

[107] 2-2: Compositions of APF media used for culture

[108] As shown in Table 7 below, to 1% glucose [10 g/L] as a carbon source and 1% yeast extract [10 g/L], 2% three plant peptones [10 g/L] were added. Each of the mixtures was dissolved in 90 mL of water for injection, and the final volume was adjusted to 100 mL. 10-mL aliquots of the prepared

media were then dispensed into 15-mL culture tubes, respectively. Thereafter, the media were autoclaved at 121°C for 30 minutes and then kept under anaerobic conditions in an anaerobic incubator.

[109] [Table 7]

	Medium composition (g/L)															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Carbon source	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
Yeast extract	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
Peptone from pea	10	10	20	10	10	0	0	20	0	0	20	20	20	0	20	0
Wheat peptone	20	0	10	0	10	10	0	20	20	20	20	0	0	20	10	0
Potato peptone	0	10	0	0	20	0	0	0	20	10	20	0	20	0	10	20

[110] 2-3: Culture of *Clostridium botulinum* strain

[111] Culture tubes containing medium compositions 1 to 16 shown in Table 7 above were inoculated with 20 µL of *Clostridium botulinum* (Korea Center for Disease Control and Prevention, control No. 4-029-CBB-MF-2020001), and samples were taken while the strain was stationary-cultured under anaerobic conditions at 35°C±1°C for 20, 24, 28, and 48 hours.

Thereafter, the growth rate of the strain was determined by measuring the OD_{540nm} value of the culture sampled at each time using a UV spectrophotometer, and the culture medium was used as a blank. Based on the measured OD_{540nm} value, plant peptone candidates for the medium were selected by comparison with the control. Based on the measured OD_{540nm} value, the proportion of addition of plant peptones for the medium was selected.

[112] 2-4: Strain growth rate

[113] The OD_{540nm} values of the cultures sampled at each time point for medium compositions 1 to 16 shown in Table 7 above were measured (Table 8), and the graphs of the OD_{540nm} values over time for each medium were compared (FIG. 5).

[114] [Table 8]

Medium	OD _{540nm} value over time			
	20hr	24hr	28hr	48hr
1	4.2407	6.0792	6.6703	7.6256
2	2.9021	3.1707	3.7849	4.5212
3	4.3292	5.5489	6.7432	5.1707
4	2.3007	2.6335	3.6120	3.9954
5	3.3414	4.4627	4.8113	4.5013
6	2.2590	2.8932	3.7034	3.1049
7	0.2857	0.1835	1.1849	0.6199
8	4.2728	6.7621	8.1035	4.7047
9	4.2576	4.2045	5.4406	5.8829
10	3.3709	3.7021	5.1411	4.3266
11	5.1641	5.6140	6.6381	2.2498
12	3.7988	4.7715	5.9342	4.2629
13	5.3545	5.1057	6.5547	2.0944
14	2.8511	3.3602	3.7440	4.6341
15	4.8876	3.4032	5.8843	5.2539
16	2.2215	2.8578	2.7791	3.8910

[115] 2-5: Analysis of results of Design of Experiment (DoE)

[116] For medium compositions 1 to 16 shown in Table 7 above, the OD_{540nm} value at 24 hours, which is the primary seed culture time in previous studies on *Clostridium botulinum*, was used for analysis, and since the toxin is recovered during the death phase, the amount of toxin protein, the value measured by ELISA at 48 hours were used for the amount of toxin protein (Table 9). Based on these values, the results were statistically analyzed using JMP.

[117] [Table 9]

	Pea	Wheat	Potato	OD at 24hr	Toxin at 48hr
1	10	20	0	6.0792	42041.2
2	10	0	10	3.1707	56296.2
3	20	10	0	5.54885	64887.3
4	10	0	0	2.6335	44518.3
5	10	10	20	4.46265	44377.2
6	0	10	0	2.89315	35791.2
7	0	0	0	0.18351	16783
8	20	20	0	6.7621	56713.4
9	0	20	20	4.2045	19848.6
10	0	20	10	3.7021	34152.4
11	20	20	20	5.61395	34592.6
12	20	0	0	4.7715	50099.6
13	20	0	20	5.10565	49023.4
14	0	20	0	3.36015	20371.4
15	20	10	10	3.4032	35153.9
16	0	0	20	2.8578	25383.1

[118] Through distribution analysis of the results for each condition as shown in Table 9 above, it was confirmed that the experimental design was D-optimal and that the experimental results were generally evenly distributed (FIG. 6).

[119] According to scatterplot matrix analysis, all correlation values were 0.75 or lower, indicating a weak correlation. The R values between the X variables (pea, wheat, and potato) were close to 0, indicating a very weak correlation, so there was no interaction. It was confirmed that there was a weak positive correlation between the Y variables (OD_{540nm}, and toxin concentration), between pea and the Y variable, and between wheat and the OD_{540nm} value (FIG.

7). Outlier analysis confirmed that there were no outliers in the experimental results (FIG. 8).

[120] 2-6: Analysis of OD_{540nm} value depending on type of plant peptone

[121] A fit model was established based on the results of analysis of OD_{540nm} value depending on the type of plant peptone. The model was established by the effect leverage method using least squares, and the X variables were analyzed using the response surface method. To select the optimal model, the R-squared value and RMSE value were considered together, and after checking effect summary and the p-value of effect test, terms that were not significant were deleted.

[122] As a result of checking the VIF value of the optimal established model, it was confirmed that the value did not exceed 10 and was at level 1, suggesting that there was no problem with the model. In residual analysis, it was confirmed that points were evenly distributed up and down and all studentized residuals results were included in the 95% confidence interval (FIG. 9).

[123] As a result of identifying the factors that significantly affect the model through the effect test results, it was found that pea and wheat had an effect on the model (Table 10). A prediction formula for OD_{540nm} was obtained using this model (FIG. 10). As a result of examining the main effects and interaction profiles, it was confirmed

that there were interactions between pea and potato, and between wheat and potato, but there were no interactions between the other factors (FIG. 11).

[124] [Table 10]

Effect Tests					
Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Pea	1	1	19.476398	42.6410	0.0006*
Wheat	1	1	12.520622	27.4122	0.0019*
Potato	1	1	0.147585	0.3231	0.5904
Pea*Pea	1	1	0.695719	1.5232	0.2633
Pea*Wheat	1	1	0.789833	1.7292	0.2365
Wheat*Wheat	1	1	0.170263	0.3728	0.5639
Pea*Potato	1	1	2.003438	4.3863	0.0811
Wheat*Potato	1	1	2.272906	4.9762	0.0672
Potato*Potato	1	1	2.029966	4.4443	0.0796

[125] 2-7: Analysis of toxin expression level depending on type of plant peptone

[126] A fit model was established based on the results of ELISA. The model was established by the effect leverage method using least squares, and the X variables were analyzed using the response surface method. To select the optimal model, the R-squared value and RMSE value were considered together, and after checking effect summary and the p-value of effect test, terms that were not significant were deleted.

[127] As a result of checking the VIF value of the optimal established model, it was confirmed that the value did not exceed 10 and was at level 1, suggesting that there was no problem with the model. In residual analysis, it was confirmed that points were evenly distributed up and down

and all studentized residuals results were included in the 95% confidence interval (FIG. 12).

[128] As a result of identifying the factors that significantly affect the model through the effect test results, it was found that pea had an effect on the model (Table 11). A prediction formula for OD_{540nm} was obtained using this model (FIG. 13). As a result of examining the main effects and interaction profiles, it was confirmed that there were interactions between pea and potato, and between wheat and potato, but there were no interactions between the other factors (FIG. 14).

[129] [Table 11]

Effect Tests					
Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Pea	1	1	1432636114	15.5150	0.0043*
Wheat	1	1	16083929.8	0.1742	0.6874
Potato	1	1	107423198	1.1634	0.3122
Pea*Pea	1	1	239492040	2.5936	0.1460
Wheat*Wheat	1	1	43348778.2	0.4695	0.5126
Pea*Potato	1	1	151433498	1.6400	0.2362
Wheat*Potato	1	1	94093681.4	1.0190	0.3423

[130] 2-8: Robust optimization

[131] Based on the prediction formula established for each response value, robust optimization conditions were identified using a profiler to determine the medium conditions that can obtain the appropriate OD_{540nm} and the maximum toxin concentration. In the actual culture process, for the OD_{540nm} value, a cell state is selected that can

continue to grow after passage while maintaining the exponential phase. Therefore, as the minimum cell concentration for passage in the exponential phase was at an OD_{540nm} value of 3 to 7, the appropriate conditions were explored by setting the OD_{540nm} value to a match target of 5 based on the established model formula. For the toxin concentration, the ELISA value was set to maximum value to find the composition that produces the highest concentration of toxin. As a result of performing an analysis to maximize desirability for the two response values, the use of only pea and wheat was determined, and potato was excluded from subsequent analyses (FIG. 15).

[132] After performing 5,000 simulations as a function of the random variation in the factors and model noise using the simulator function (FIG. 16a), the simulation results were presented through edge of failure analysis. It was confirmed that all response values fell within the spec limit except for one case out of 5,000 (FIG. 16b).

[133] To obtain results under appropriate conditions, the acceptable ranges for each component were determined. The range corresponding to 3σ was set as NORs (a range representing 99.7% of the actual result distribution), and the range corresponding to 4.5σ was set as PARs (FIGS. 17 and 18). Based on this, the determined acceptable ranges for each component are shown in Table 12 below.

[134] [Table 12]

Component (g/L)	Set point	NORs (3σ)	PARs (4.5σ)
Peptone from pea	18 g/L	16.5 to 19.5	15.8 to 20.3
Wheat peptone	7 g/L	5.5 to 8.5	4.8 to 9.2

[135] For the convenience of the operator upon application to the actual process, the set point was 18 g/L for peptone from pea and 7 g/L for wheat peptone. As a result of plotting NORs (3σ , red line) and PARs (4.5σ , blue line) on a contour plot to find the range satisfying the model, it was confirmed that NORs and PARs did not exceed the white range satisfying the process conditions, suggesting that the NORs and PARs set by this test and statistical analysis were suitable (FIG. 19).

[136]

[137] **Example 3: Comparative measurement of absorbance and toxin expression level for APF media**

[138] **3-1: Compositions of media used for culture**

[139] To APF medium for culturing *Clostridium botulinum*, peptone from pea and wheat peptone were added in amounts selected in Examples 2-8, and D-(+)-glucose as a carbon source and yeast extract as a nitrogen source were added in amounts identical to those in Examples 1-2.

[140] Meanwhile, to determine the effect of L-cysteine hydrochloride monohydrate or antifoaming agent emulsion on the APF medium, 0.2 g/L L-cysteine hydrochloride monohydrate

and/or 0.24 g/L medical antifoam C emulsion (Dow Corning®) was added to the APF medium. In addition, to determine the effect of soy peptone on the APF medium, 18 g/L soy peptone was added instead of the pea peptone and/or wheat peptone of the APF medium, and additionally, 0.2 g/L L-cysteine hydrochloride monohydrate and 0.24 g/L medical antifoam C emulsion (Dow Corning®) were added.

[141] 10-mL aliquots of the media were dispensed into 15-mL culture tubes, respectively. Thereafter, the media were autoclaved at 121°C for 30 minutes and then kept under anaerobic conditions in an anaerobic incubator. The APF media for culturing *Clostridium botulinum* and the specific compositions of the media are shown in Table 13 below.

[142] [Table 13]

Component (g/L)	APF medium	Comp. Example 1	Comp. Example 2	Comp. Example 3	Comp. Example 4	Comp. Example 5	Comp. Example 6	Comp. Example 7
Peptone from pea	10	10	10	10	-	-	-	-
Wheat peptone	7	7	7	7	-	7	7	20
Yeast extract	10	10	10	10	10	10	10	20
D-(+)- glucose	10	10	10	10	10	10	10	10

Soy peptone	-	-	-	-	10	10	10	
L-cysteine HCl	-	0.2	-	0.2	-	-	0.2	0.2
Antifoam C emulsion	-	-	0.24	0.24	-	-	0.24	0.24

[143] 3-2: Culture of *Clostridium botulinum* strain

[144] Culture tubes containing the media shown in Table 13 were inoculated with 20 μ L of *Clostridium botulinum* (Korea Center for Disease Control and Prevention, control No. 4-029-CBB-MF-2020001), and samples were taken while the strain was stationary-cultured under anaerobic conditions at 35°C $\pm$ 1°C for 20, 24, 28, and 48 hours. Thereafter, the growth rate of the strain was determined by measuring the OD_{540nm} value of the culture sampled at each time using a UV spectrophotometer, and the culture medium was used as a blank.

[145] 3-3: Strain growth rate and toxin expression level

[146] The OD_{540nm} value of the cultures sampled at each time for the media shown in Table 13 above and the toxin expression levels after 48 hours of culture were measured (Tables 14 and 15, and FIGS. 20 and 21).

[147] [Table 14]

	APF medium	Comp. Example 1	Comp. Example 2	Comp. Example 3	Comp. Example 4	Comp. Example 5	Comp. Example 6	Comp. Example 7
		1	2	3	4	5	6	7

OD at 20 hr	4.0474	3.7144	1.7721	3.9557	2.2205	2.8330	1.7535	3.7871
OD at 24 hr	5.9563	5.3636	2.0319	5.6034	2.9668	3.4903	2.7539	5.4549
OD at 28 hr	7.1021	6.2576	1.7567	7.1667	3.5252	4.2801	3.2109	6.7739
OD at 48 hr	4.5636	5.3044	1.5032	4.5062	4.4182	5.3889	3.7297	7.8900
Toxin (μg) at 48 hr	588.8	452.1	137.9	501.1	75.2	76.4	30.1	263.7
Toxin expression (%) compared to control	-	-23.2	-76.6	-14.3	-87.2	-87.0	-94.9	-55.2

[148] [Table 15]

		Measured value (ng/mL)	Average value	Culture (mL)	Toxin amount (μg)
APF medium	Tube 1	46894.1	58880.0	10	588.8
	Tube 2	70867.4			
Comparative Example 1	Tube 1	51505.6	45208.9	10	452.1
	Tube 2	38912.1			
Comparative Example 2	Tube 1	13052.2	13972.6	10	137.9
	Tube 2	14533.0			

Comparative	Tube 1	57599.9	50110.8	10	501.1
Example 3	Tube 2	42621.6			
Comparative	Tube 1	6196.3	7522.7	10	75.2
Example 4	Tube 2	8849.1			
Comparative	Tube 1	7051.5	7644.9	10	76.4
Example 5	Tube 2	8238.4			
Comparative	Tube 1	2696.2	3012.2	10	30.1
Example 6	Tube 2	3328.3			
Comparative	Tube 1	29852.4	26366.1	10	263.7
Example 7					

[149] As a result, as shown in Table 14 above and FIGS. 20 and 21, it was confirmed that, when *Clostridium botulinum* was cultured in the APF medium of the present invention, the growth of APF was best at 20 to 28 hours, and the level of toxin expression was highest at 48 hours after the growth of the strain.

[150] Meanwhile, it was confirmed that, in the cases of Comparative Example 1, in which only L-cysteine HCl monohydrate was added to the APF medium of the present invention, Comparative Example 2, in which only medical antifoam C emulsion was added to the APF medium of the present invention, and Comparative Example 3, in which L-cysteine HCl monohydrate and medical antifoam C emulsion were added to the APF medium of the present invention, strain growth was lower than that in the APF medium of the present invention,

and the levels of toxin expression decreased by 23.2%, 76.6%, and 14.9%, respectively. Accordingly, it can be confirmed that, even if L-cysteine HCl monohydrate and/or medical antifoam C emulsion are added to the APF medium composition of the present invention, strain growth and toxin expression in the APF medium composition of the present invention are excellent.

[151] Meanwhile, it was confirmed that, in the cases of Comparative Example 4, in which soy peptone in place of peptone from pea and wheat peptone was added to the APF medium of the present invention, Comparative Example 5, in which soy peptone in place of peptone from pea was added to the APF medium of the present invention, Comparative Example 6, in which soy peptone in place of peptone from pea was added to the APF medium of the present invention and additionally L-cysteine HCl monohydrate and medical antifoam C emulsion were added, and Comparative Example 7, which has the same composition as that in Korean Patent Application Publication No. 10-2022-0140696, strain growth was lower than that in the APF medium of the present invention, and the levels of toxin expression decreased by 87.2%, 87.0%, 94.9%, and 55.2%, respectively. Accordingly, considering that the time required to reach the death phase where the toxin can be recovered is expected to be 72 hours or more, it can be

confirmed that strain growth and toxin expression in the APF medium composition of the present invention are excellent.

【CLAIMS】

【Claim 1】 A medium composition for culturing *Clostridium botulinum* strains, comprising: plant peptones, including wheat peptone and peptone from pea; D-(+)-glucose; and yeast extract.

【Claim 2】 The medium composition according to claim 1, not comprising an animal-derived component.

【Claim 3】 The medium composition according to claim 2, wherein the animal-derived component is casein hydrolysate.

【Claim 4】 The medium composition according to claim 1, wherein a content of the wheat peptone is 0.1 to 4.0 w/v%.

【Claim 5】 The medium composition according to claim 1, wherein a content of the peptone from pea is 0.1 to 3.5 w/v%.

【Claim 6】 The medium composition according to claim 1, wherein contents of the D-(+)-glucose and the yeast extract are each 0.005 to 2 w/v%.

【Claim 7】

A method for producing botulinum toxin, comprising steps of:

(a) producing botulinum toxin by culturing *Clostridium botulinum* using the medium composition according to any one of claims 1 to 6; and

(b) recovering the produced botulinum toxin.

【Claim 8】 The method according to claim 7, wherein the culturing is performed under anaerobic conditions.

【Claim 9】 The method according to claim 7, wherein the culturing is performed under temperature conditions of 33°C to 37°C.

【Claim 10】 The method according to claim 7, wherein the culturing is performed for 20 to 48 hours.

【Claim 11】 The method according to claim 7, wherein the toxin is selected from the group consisting of botulinum toxins A, B, C, D, E, F and G.