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(71) Applicant (for all designated States except US): **INDUSTRY-ACADEMIC COOPERATION FOUNDATION, KEIMYUNG UNIVERSITY** [KR/KR]; #1000 Sindang-dong, Dalseo-gu, Daegu 704-701 (KR).

(71) Applicant and

(72) Inventor (for all designated States except US): **LEE, Sam Pin** [KR/KR]; 102-306 Mujigae Apt., Igok-dong, Dalseo-gu, Daegu 704-787 (KR).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **SEO, Ji-Hyun** [KR/KR]; #789-12 Daemyeong 1-dong, Nam-gu, Daegu 705-031 (KR). **LEE, In Seon** [KR/KR]; 1416-1803, Lotte hwaseong Castle Gold Park, 368, Hwanggeum-dong, Suseong-gu, Daegu 706-850 (KR).

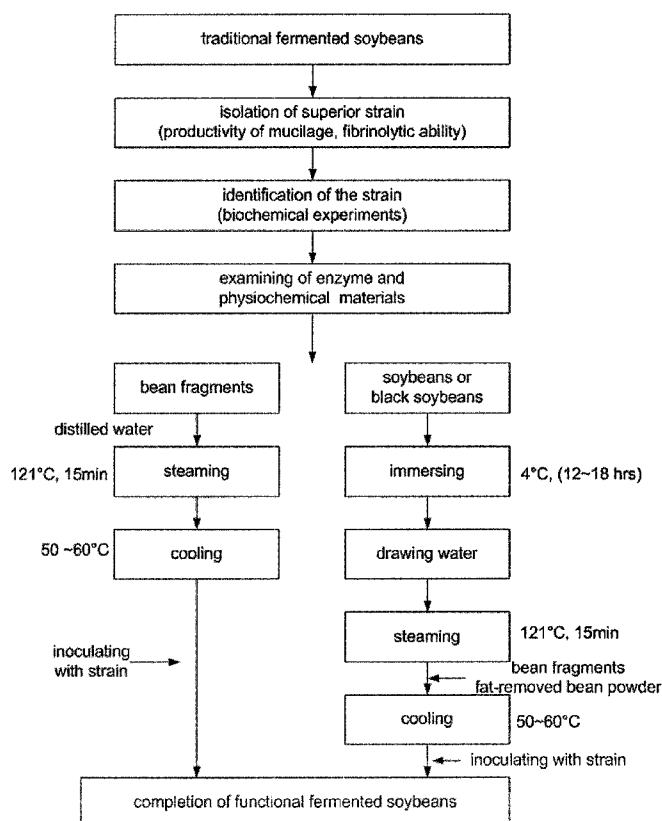
(74) Agent: **KIM, Il-Hwan**; 5th Fl. Oksan Bldg., 1627-10, Bongcheon-7-dong, Gwanak-gu, Seoul 151-818 (KR).

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(54) Title: **BACILLUS SUBTILIS HA PRODUCING FIBRINOLYTIC ENZYME AND MUCILAGE HIGHLY, METHOD OF PREPARING FERMENTED SOYBEANS USING THE SAME STRAIN, AND SOYBEANS PREPARED BY THE METHOD**



(57) Abstract: The present invention provides *Bacillus subtilis* HA KCCM- 10775P having a high productivity of protease, fibrinolytic enzyme and mucilage, a method preparing fermented soybeans using the same strain, in which bean fragments, soybeans and black soybeans are fermented, and fermented soybeans produced according to the said method. The *Bacillus subtilis* HA KCCM- 10775P isolated from traditional fermented soybeans and identified is the superior strain having a high productivity of protease, fibrinolytic enzyme and mucilage. When preparing fermented soybeans using the strain of the present invention, it has good sensory properties by removing a peculiar smell thereof, as well as a high productivity of fibrinolytic enzyme and mucilage.



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***Bacillus subtilis* HA producing fibrinolytic enzyme and mucilage highly,
method of preparing fermented soybeans using the same strain, and
soybeans prepared by the method**

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TECHNICAL FIELD

The present invention relates to a microorganism having a high productivity of protease, fibrinolytic enzyme and mucilage, and a method for preparing fermented
10 soybeans using the said microorganism, and the fermented soybeans prepared by the said method,

BACKGROUND ART

Soybeans have been used in various traditional foods such like soybean paste
15 including soy sauce. Bean curd which is the primary processed food of soybeans is concentrated materials of protein and has been used as a source of protein in oriental people.

According to a traditional method, soybeans have been used to prepare fermented
20 soybeans, and various foods such like fresh fermented soybeans, powder and pill have been produced and sold since an excellent effect of the fermented soybeans was known to public. Especially, the superior fermented soybeans having a high productivity of mucilage can be prepared by using black soybeans or mixing with subsidiary materials.

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It has been estimated that fermented soybeans is good fermented food for health, which is caused by *Bacillus subtilis*, the leading role in fermentation. Besides lactic acid, *Bacillus subtilis* as probiotics plays several roles to maintain good health of intestine and liver.

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Bacillus subtilis decreases a production of carcinogen and absorbs these harmful materials to excrete together with excrement. Furthermore, it has an anti-germ property and produces organic acids to stimulate an intestine for digestion.

- 5 Additionally, vitamin B2 contained richly in fermented soybeans helps to detoxify smoothly. It has been reported that various enzymes are produced in the process of the fermentation of beans by *Bacillus subtilis*, and among them, especially, the fibrinolytic enzyme lyse fibrins, thus it is useful for preventing myocardial infarction and cerebral thrombosis etc.

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Until now, the study about fermentation by *Bacillus subtilis* relates to the preparation of fermented soybeans using beans. In fermentation according to traditional method, there are difficulties of contamination by sundry germ and non-uniformity in fermented foods. Especially, fermented soybeans using bean
15 fragments and fat-removed bean powder have been hardly reported.

Since it has been reported that γ -PGA, main mucilage produced from fermented soybeans has various physical properties and physiologic activities and the mucilage production in the Natto (Japanese fermented soybeans) is emphasized, it
20 becomes more important to secure superior strains and use industrially.

These days, it has been reported that in order to optimize the production of mucilage, the strain producing γ -PGA was liquid-cultured using monosodium glutamate (MSG) to obtain 50g/L of γ -PGA. In case of solid-state fermentation, the
25 productivity of mucilage depends on the composition of culture media. Especially, when adding with MSG, the strain intakes fermented material as it is, resulting in not converting into γ -PGA and finally remaining MSG.

Accordingly, the present inventors have isolated *Bacillus subtilis* HA KCCM-
30 10775P having a high productivity of protease, fibrinolytic enzyme and mucilage

from fermented soybeans. Moreover, to enhance a productivity of mucilage, the present inventors added bean fragments and fat-removed bean powder to soybeans or black soybeans and then added *Bacillus subtilis* HA strain thereto and followed by performing a fermentation to obtain fermented beans (fresh fermented soybeans)
5 which have an increased mucilage-productivity and functionality and comprise various physiochemical materials, thereby completing the present invention

SUMMARY OF THE INVENTION

10 A main object of the present invention is to provide *Bacillus subtilis* HA KCCM-10775P having a high productivity of protease, fibrinolytic enzyme and mucilage.

Another object of the present invention is to provide a method preparing fermented soybeans using the same strain, which have good sensory properties by removing a
15 peculiar smell thereof, as well as a high productivity of fibrinolytic enzyme and mucilage.

Another object of the present invention is to provide fermented soybeans produced according to the above method, which have good sensory properties by removing a
20 peculiar smell thereof, as well as a high productivity of fibrinolytic enzyme and mucilage

In order to achieve the above objects, in one aspect, the present invention provides a *Bacillus subtilis* HA KCCM-10775P having a high productivity of protease,
25 fibrinolytic enzyme and mucilage.

The *Bacillus subtilis* HA KCCM-10775P can be used in fermenting soybeans.

In one aspect, the present invention provides fermented soybeans produced by
30 fermenting soybeans or black soybeans using the *Bacillus subtilis* HA KCCM-

10775P

In another aspect, the present invention provides a method for preparing fermented soybeans, the said method comprises the steps of steaming bean fragments;
5 inoculating 100 weight parts of the steamed bean fragments with 0.2~5 weight parts of *Bacillus subtilis* HA KCCM-10775P; and fermenting the beans inoculated with *Bacillus subtilis* HA KCCM-10775P.

In still another aspect, the present invention provides a method for preparing
10 fermented soybeans, the said method comprises the steps of steaming soybeans or black soybeans; inoculating 100 weight parts of the steamed soybeans or black soybeans with 0.2~5 weight parts of *Bacillus subtilis* HA KCCM-10775P; and fermenting the soybeans or black soybeans inoculated with *Bacillus subtilis* HA KCCM-10775P.

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In still another aspect, the method for preparing fermented soybeans further comprises a step of mixing 5~20 weight parts of bean fragments or fat-removed bean powder with 100 weight parts of soybeans or black soybeans after steaming the soybeans or black soybeans, and then inoculating 100 weight parts of the
20 mixture with 0.2~5 weight parts of *Bacillus subtilis* HA KCCM-10775P.

The step of fermentation may be performed at 30~35 °C for 10~48 hrs.

In still another aspect, the present invention provides fermented soybeans produced
25 by the said method for preparing fermented soybeans.

BRIEF DESCRIPTION OF DRAWINGS

30 FIG. 1 is a flowchart showing isolation and identification process of the present

strain and a method for preparing fermented soybeans using the strain.

FIG. 2 is a photograph of scanning electron microscope for *Bacillus subtilis* HA according to the present invention.

FIG. 3 is 16S rDNA base sequences (SEQ ID NO.:1) of *Bacillus subtilis* HA according to the present invention.

FIGs. 4 is a phylogenetic analysis based on 16S rDNA base sequences of *Bacillus subtilis* HA according to the present invention.

FIG. 5 shows comparison results of fibrinolytic ability between *Bacillus subtilis* HA according to the present invention and type strain (A: no heat-treated HA strain of the present invention, B: 50°C heat-treated HA strain of the present invention, C: 60°C heat-treated HA strain of the present invention, D: 70°C heat-treated HA strain of the present invention; E: no heat-treated type strain, F: 50°C heat-treated type strain, G: 60°C heat-treated type strain, H: 70°C heat-treated type strain).

FIG. 6 is a graph showing a resistance to NaCl in liquid culture technique of *Bacillus subtilis* HA according to the present invention.

FIG. 7 shows a consistency and fibrinolytic ability of fermented soybeans using the present strain and type strain respectively.

FIG. 8 shows a consistency and mucilage content of fermented soybeans produced by adding fat-removed bean powder and bean fragments to soybeans or black soybeans.

DETAILED DESCRIPTION OF THE INVENTION, AND PPRFERRED EMBODIMENTS

Another features and embodiments of the present invention will be more clarified from the following “detailed description” and the appended “claims” referring to the Drawings.

5

FIG. 1 is a flowchart showing isolation and identification process of the present strain and a method for preparing fermented soybeans using the strain

Referring to the FIG. 1, the present inventors isolated and identified a new strain
10 having a high productivity of fibrinolytic enzyme and mucilage from the traditional fermented soybeans on the market.

More specifically, Astrup and Mullertz methods as kinds of fibrin plate method were performed to isolate a new strain having high fibrinolytic activity and high
15 mucilage productivity from the traditional fermented soybeans on the market.

Then, the isolated strain was identified and characterized. Cultural characteristics, physiological characteristics, biochemical characteristics (refer to Table 1), an availability of saccharide (refer to Table 2) and morphological characteristics were
20 studied, as a result, it is confirmed that the isolated strain belongs to *Bacillus* genus. The Gene Manipulation Techniques are performed to clarify a correlation of the various *Bacillus* genuses.

FIG. 3 is 16S rDNA base sequences (SEQ ID NO.:1) of *Bacillus subtilis* HA
25 according to the present invention. FIGs. 4 are a phylogenetic analysis based on 16S rDNA base sequences of *Bacillus subtilis* HA according to the present invention. The base sequences of the isolated strain have 98.3% homology to *Bacillus subtilis* Z99104.

30 On the basis of the results, the isolated strain was named as *Bacillus subtilis* HA.

Accordingly, the HA strains was identified as *Bacillus subtilis* HA and deposited to Depositary Authority as a new strain [Korean Culture Center of Microorganisms, (KCCM); deposition day: August 30, 2006; KCCM 10775P].

- 5 The *Bacillus subtilis* HA of the present invention has a very high fibrinolytic activity. When *Bacillus subtilis* HA is treated with heat for 10 min at 50~70°C, especially 60°C, the fibrinolytic activity thereof is decreased by about 25%. Furthermore, the type strain of *Bacillus subtilis* Z99104 has a low fibrinolytic activity compared to the present strain (refer to FIG. 5). It is confirmed that the
- 10 *Bacillus subtilis* HA of the present invention can grow at the condition of 7% NaCl (refer to FIG. 6).

In the method for preparing fermented soybeans of the present invention, the *Bacillus subtilis* HA is used to ferment bean fragments, soybeans or black soybeans.

15

According to one aspect of the present invention, bean fragments are steamed after adding with the same weight of distilled water. The condition of the said steaming is not limited to 121 °C for 15 min in autoclave.

- 20 Then, the steamed bean fragments may be cooled to, for example, 50 ~ 60°C at room temperature.

- Subsequently, the 100 weight parts of the cooled bean fragments are inoculated with 0.2~5 weight parts of *Bacillus subtilis* HA KCCM-10775P. When inoculated with
- 25 less than 0.2 weight parts thereof, it could be not enough to ferment. When inoculated with more than 5 weight parts thereof, an efficiency of fermentation can not be increased.

Following inoculating, the inoculated bean fragments are fermented. When the

fermentation is performed at 30~35 °C for 10 ~ 48 hrs, the efficiency of fermentation is the highest.

According to still another aspect of the method for preparing fermented soybeans of the present invention, at first, soybeans or black soybeans are immersed in water. The immersion may be performed for 12~18 hrs at 4 °C.

After taking water out, the resultants are steamed. The condition of the said steaming is not limited to 121 °C for 15 min in autoclave.

10

Then, the 100 weight parts of soybeans or black soybeans are mixed with 5~20 weight parts of bean fragments or fat-removed bean powder. As described above in detail, an addition of the 5~20 weight parts of bean fragments or fat-removed bean powder to soybeans or black soybeans can lead to produce fermented stuffs having improved mucilage.

Subsequently, the 100 weight parts of soybeans or black soybeans, or the 100 weight parts of the mixture of 'the soybeans or black soybeans' and 'the bean fragments or fat-removed bean powder' are inoculated with 0.2~5 weight parts of *Bacillus subtilis* HA KCCM-10775P. When inoculated with less than 0.2 weight parts thereof, it could be not enough to ferment. When inoculated with more than 5 weight parts thereof, an efficiency of fermentation can not be increased.

Next, the inoculated bean fragments are fermented. When the fermentation is performed at 30~35 °C for 10 ~ 48 hrs, the efficiency of fermentation is the highest.

The fermented soybeans produced according to the present invention using *Bacillus subtilis* HA KCCM-10775P have good sensory properties by removing a peculiar smell thereof, as well as have a high productivity of fibrinolytic enzyme and mucilage.

Examples

Hereinafter, the present invention will be described in more detail by examples.

- 5 However, it is obvious to a person skilled in the art that these examples are for illustrative purpose only and are not construed to limit the scope of the present invention.

Example 1: Isolation and identification of the strain of the present invention

10

1-1 Isolation of the strain

- A sample 1g from the traditional fermented soybeans on the market was added to 9ml of sterilized distilled water to suspend and located for 5 min. The upper layer was extracted and diluted to be $10^4 \sim 10^7$ (CFU/mL), followed by smearing on MRS
15 agar plate and culturing for 24hrs at 37°C to isolate the strain which producing a lots of mucilage individually.

- The isolated strain was purified by smearing on MRS agar plate respectively, and then activated for 24hrs in 42°C thermostat. Among the occurred colonies, one
20 colony was taken out to inoculate into sterilized NB medium (0.3% of beef extract, 0.5% of peptone) and shaking-cultured for 48hrs at 180rpm in 42°C thermostat, and then centrifuged for 15 min at 150,000rpm to abstain a supernatant as a crude enzyme.

- 25 A fibrinolytic activity was measured by Astrup and Mullertz methods which is one kinds of fibrin plate method. Fibrin plate was prepared by dissolving 0.5% of fibrinogen in 0.067M of sodium phosphate buffer (pH 7.4) and adding 10ml thereof to Petri dish with diameter of 9cm. 0.1 ml of thrombin (100NIH/ml) dissolved in 0.067M of sodium phosphate buffer was added thereto and mixed rapidly to prepare
30 a uniform plate and followed by locating for 30 min at room temperature.

After pointing a drip location of sample to the fibrin plate, the supernatant (crude enzyme) was dripped by $20\mu\ell$ at the location pointed above to measure an activity of enzyme by an area of lysis.

5

To gain a Standard Curves of fibrinolytic enzyme, the standard plasmin solution was prepared to be 0.6, 1.6, 2.6 and 5 unit/mL with Tris-lysine buffer (pH 9.0) and added to the fibrin plate by $20\mu\ell$ to form an area of lysis and a standard curves of the activity of plasmin enzyme therefrom.

10

For the standard curves, the fibrinolytic enzyme in broth was changed into plasmin unit and calculated the activity of the enzyme using the below formula to select a strain having a high fibrinolytic ability.

15 [Formula 1]

$$\text{fibrinolytic activity(\%)} = \text{lysis area of sample} / \text{lysis area of plasmin} \times 100$$

1-2 Identification and characterization of the isolated strain

20

FIG.1 show the successive processes including isolating microorganism from sample (traditional fermented soybeans), identifying and characterizing probiotic features thereof. The cultural characteristics, physiological characteristics and biochemical characteristics of selected HA strain according to the present invention were shown in Table 1 as follows, and the availability of saccharides thereof were shown in Table 2.

25

FIG. 2 is a photograph of scanning electron microscope for *Bacillus subtilis* HA according to the present invention. When examining a morphological characteristics of the isolated strain referring to FIG. 2, it could be confirmed that the cell is a kinds

30

of bacillus having the size of 0.7~0.8 μ m (thickness) and 1.38~1.42 μ m(length) and the shape of short bar.

As cultural characteristics, the isolated strain is aerobic and grows well in the condition of 30~55 $^{\circ}$ C temperature. In MRS agar medium, the milk-white colonies were formed, which are mucoid and have externals with wrinkle, sawlike edge. Furthermore, the results of gram staining and catalase test were positive, which means *Bacillus* genus.

10 **Table 1** The cultural and physiological characteristics of HA strain according to the present invention

characteristics	HA strain of the present invention
gram staining	Positive
Temperature for growth	30~55 $^{\circ}$ C
Growth at pH 5.7	+
Growth at 3,5,7% of NaCl	+
Growth at aerobic condition	+
Growth at anaerobic condition	-
Motility	-
Voges-proskauer test	+
O/F	+
nitrate reduction	+
Indole formation	-
oxidase production	-
catalase production	+
Urease production	-
β -Galactosidase production	+
Gelatinase production	+
tryptophan amide enzyme production	-
starch degrading capability	+
Casein degrading capability	+
citrate availability	-

Table 2 Availability of carbon source according to HA strain of the present invention

carbon source	availability	carbon source	availability
L-arabinose	+	Adonitol	-
D-cellobiose	+	D-fructose	+
D-galactose	-	D-glucose	+
D-mannitol	+	Raffinose	-
L-rhamnose	-	Salicin	+
D-sucrose	+	D-xylose	-

- 5 As shown in Table 2, with respect to the availability of saccharides according to HA strain of the present invention, D-galactose, L-rhamnose, Adonitol, Raffinose and D-xylose do not have the availability of saccharides, compared to L-arabinose, D-cellobiose, D-mannitol, D-sucrose, D-fructose, D-glucose and Salicin having the availability of saccharides.

10

- GC-content of the isolated strain was analyzed with HPLC, then it was confirmed that *Bacillus subtilis* Z99104 which is a type strain (46.18 mol%) is similar to the HA strain of the present invention (46.16 mol%). Furthermore, in order to identify the strain more exactly, fatty acids of cell wall were analyzed. The results
- 15 comparing *Bacillus subtilis* Z99104 and HA strain of the present invention were shown in Table 3. Consequently, the fatty acids of cell wall according to the present HA strain were similar to those of type strain, and C15:0 ISO and C15:0 ANTEISO were confirmed as main fatty acids.

- 20 **Table 3** Analysis of cellular Fatty Acids according to HA strain of the present invention (unit:%)

cellular Fatty Acids	HA strain of the present invention	type strain <i>Bacillus subtilis</i> Z99104
C15:0 ISO	24.69	34.55

C15:0 ANTEISO	35.54	36.03
C16:0	6.62	4.11
C17:0 ISO	12.7	4.11
C17:0 ANTEISO	8.87	6.42

As described above, the chemotaxonomical characteristics of the present HA strain of *Bacillus* genus isolated from fermented soybeans are similar to those of the type strain of *Bacillus subtilis*. To clarify the correlation of the species, Gene Manipulation Techniques was performed.

The present HA strain was identified by a molecular classical taxonomy analysis based on with the determination of the base sequences of 16S ribosome DNA, the specific region of DNA.

10

FIG. 3 is 16S rDNA base sequences (SEQ ID NO.:1) of *Bacillus subtilis* HA according to the present invention. FIGs. 4 is a phylogenetic analysis based on 16S rDNA base sequences of *Bacillus subtilis* HA strain according to the present invention. The comparison of homogeneity between 16S rDNA base sequences of *Bacillus subtilis* HA strain and those of other strains belonging to *Bacillus* genus registered in data bases (DDBJ, EMBL, GenBank) is shown in Table 4.

15

Table 4 The comparison of homogeneity between HA strain and other strain.

strain	(%)														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
<i>Bacillus subtilis</i> HA															
<i>Bacillus subtilis</i> Z99104	98.3														
<i>Bacillus</i>	88.9	89.2													

<i>benzoevorans</i> D78311														
<i>Bacillus circulans</i> AY043084	91.7	93.1	92.2											
<i>Bacillus cohnii</i> X76437	92.0	93.4	91.1	95.0										
<i>Bacillus cereus</i> AE017013	92.2	93.6	89.2	93.5	94.7									
<i>Bacillus acidogenensis</i> AF547209	94.0	95.4	91.0	94.4	94.8	95.1								
<i>Bacillus</i> <i>amyloliquefaciens</i> AF045057	93.8	94.3	91.7	88.2	88.5	88.6	90.1							
<i>Bacillus vallismortis</i> AB021198	97.9	99.6	89.0	92.9	93.5	93.6	95.1	94.4						
<i>Bacillus mojavenensis</i> AB021191	98.2	99.6	89.1	93.1	93.3	93.6	95.3	94.2	99.4					
<i>Bacillus atrophaeus</i> AB021181	97.7	99.2	88.9	92.8	93.7	93.9	95.3	94.2	99.4	99.3				
<i>Bacillus licheniformis</i> AE017333	96.7	98.2	89.3	93.2	93.4	93.5	95.7	93.0	98.0	98.0	98.0			
<i>Bacillus sonorensis</i> AF302118	93.5	94.0	92.9	89.5	89.8	89.6	91.7	96.6	93.7	93.9	94.0	95.6		
<i>Bacillus alcalophilus</i> X76436	90.9	92.4	88.0	91.7	92.5	91.9	93.3	87.7	92.5	92.2	92.4	92.4	88.4	
<i>Bacillus acidovorans</i> X77789	86.4	87.2	86.0	87.4	87.6	86.7	87.6	84.8	87.4	87.3	87.4	86.9	85.1	85.5

Among them, based on the base sequences of the present *HA* strain have 98.3% homology to *Bacillus subtilis* Z99104 of type strain, the *HA* strain isolated from fermented soybeans was named as *Bacillus subtilis HA* by molecular classical taxonomy analysis (morphological, cultural, physiological, chemical and Molecular
5 Ecological analysis).

As described in above, the present *HA* strain was identified as *Bacillus subtilis* and deposited as a new strain [Korean Culture Center of Microorganisms, (KCCM); deposition day: August 30, 2006; KCCM 10775P].

10

The *Bacillus subtilis HA* according to the present invention has a high fibrinolytic ability.

To evaluate a fibrinolytic enzyme activity and thermal stability, the *Bacillus subtilis*
15 *HA* and *Bacillus subtilis* Z99104 were inoculated into NB medium (0.3% of beef extract, 0.5% of peptone) by one colony and cultured for 48hrs to obtain a supernatant for measuring a fibrinolytic ability.

FIG. 5 shows comparison photographs of fibrinolytic ability between *Bacillus*
20 *subtilis HA* according to the present invention and type strain.

In the fibrin plate of FIG. 5, A~D are *Bacillus subtilis HA* according to the present invention and E~F are *Bacillus subtilis* Z99104 of type strain. After treating with heat for 10 min at 50~70°C, the fibrinolytic ability was measured.(A: no heat-
25 treated *HA* strain, B: 50°C heat-treated *HA* strain, C: 60°C heat-treated *HA* strain. D: 70°C heat-treated *HA* strain; E: no heat-treated type strain, F: 50°C heat-treated type strain, G: 60°C heat-treated type strain, H: 70°C heat-treated type strain).

Referring to FIG. 5, the *Bacillus subtilis HA* strain according to the present
30 invention has a high fibrinolytic ability. When the *Bacillus subtilis HA* strain treated

with heat for 10 min at 50~70 °C was observed, the *Bacillus subtilis* HA strain treated with heat at about 60 °C decreased their fibrinolytic ability by 25%, but the *Bacillus subtilis* Z99104 got to lose the fibrinolytic ability over 60 °C. In conclusion, it was confirmed that the fibrinolytic enzyme of *Bacillus subtilis* HA strain is stable up to 5 60 °C and unstable over 70 °C .

The the *Bacillus subtilis* HA strain was cultured for 24hrs in NB medium added with NaCl of various concentration, and then measured an absorbance at 660nm for observing a growth of the strain.

10

FIG. 6 is a graph showing a resistance to NaCl in liquid culture of *Bacillus subtilis* HA according to the present invention.

Referring to FIG. 6, the *Bacillus subtilis* HA strain according to the present 15 invention can grow at 7% NaCl.

Example 2: Preparation of fermented beans using bean fragments

Fermented soybeans producing fibrinolytic enzyme and mucilage highly were 20 prepared from bean fragments using *Bacillus subtilis* HA isolated and identified in Example 1.

2-1 Treatment of raw materials

Bean fragments were added with the same amount of distilled water and then 25 steamed for 15min at 121 °C in autoclave. The steamed bean fragments were cooled to 50~60 °C at room temperature to use the following steps.

2-2 Preparation of *Bacillus subtilis* starter

30 To prepare *Bacillus subtilis* starter, the superior strain of *Bacillus subtilis* HA

isolated from fermented soybeans was activated in MRS agar medium for 24hrs using 42°C thermostat, and one colony was taken out to inoculate into 5% of sterilized solution of bean powder and then shaking-cultured for 24hrs at 180rpm in 42°C thermostat to use as a starter spawn.

5

2-3 Preparation of fresh fermented soybeans using *Bacillus subtilis*

100 weight parts of the material composition prepared in the step of 2-1 were inoculated by 1 weight part of the starter of *Bacillus subtilis* HA prepared in the step of 2-2 to ferment for 20 hrs at 42°C to obtain fresh fermented soybeans.

For comparison, other fermented soybeans were prepared by inoculating with the type strain of *Bacillus subtilis* Z99104.

15 FIG. 7 shows a consistency and fibrinolytic ability of fermented soybeans according to the present strain and the type strain.

Referring to FIG. 7, the fermented soybeans by *Bacillus subtilis* HA strain according to the present invention have a high consistency, compared to the fermented soybeans by the type strain of *Bacillus subtilis* Z99104. Furthermore, the fermented soybeans by *Bacillus subtilis* HA strain have more than twice fibrinolytic ability compared to the fermented soybeans by *Bacillus subtilis* Z99104.

Example 3: Preparation of fermented beans by adding subsidiary materials to soybeans and black soybeans

25

3-1 Treatment of raw materials

Soybeans and black soybeans were selected, washed, immersed in water, and then drawn with water. The resultants were steamed for 15min at 121°C in autoclave and cooled. Then the 100 weight parts thereof were mixed with 10 weight parts of

bean fragments and 2 weight parts of fat-removed bean powder, respectively.

3-2 Preparation of *Bacillus subtilis* starter

- 5 To prepare *Bacillus subtilis* starter, the superior strain of *Bacillus subtilis* HA isolated from fermented soybeans was activated for 24hrs in MRS agar medium using 42°C thermostat, and one colony was taken out to inoculate into 5% of sterilized solution of bean powder and then shaking-cultured for 24hrs at 180rpm in 42°C thermostat to use as a starter spawn.

10

3-3 Preparation of fresh fermented soybeans using *Bacillus subtilis*

- 100 weight parts of the material composition prepared in the step of 3-1 were inoculated by 1 weights part of the starter of *Bacillus subtilis* HA prepared in the
15 step of 3-2 to ferment for 20 hrs at 42°C to obtain fresh fermented soybeans. As a control, black soybeans were steamed and inoculated with the starter to ferment.

Experiment 1: Physicochemical characteristics of fresh fermented soybeans

- 20 A consistency of the fermented soybeans prepared in Examples 2 and 3 was measured by a measuring cup DG43 using Rheometer System (HAAKE RheoStress 1, Germany) equipped with a spindle (Rotor DG43 DIN 53544 Titan). The value of consistency could be expressed as shear rate (1/s) and shear stress (Pa). As a result of measuring the consistency, it was confirmed that the higher the value of
25 consistency was, the higher the shear stress was. At 20°C, fluid characteristics were examined by the shear rate of the range of 1~100 s⁻¹. Moreover, flow behavior index and consistency index were measured by Power law model ($\tau = arb$).

- The contents of mucilage in the fermented soybeans were measured as follows. The
30 fermented soybeans were added with 10 times volume of distilled water to obtain

extracts and centrifuged to take a supernatant. The supernatant was added with double volume of alcohol to aggregate polysaccharides and centrifuge them. The solid contents of mucilage were crystallized by air-oven method at 105 °C.

- 5 FIG. 8 shows consistency and mucilage content of fermented soybeans produced by adding fat-removed bean powder and bean fragments to the soybeans or black soybeans.

Referring to FIG. 8, the fermented soybeans produced by adding with fat-removed
10 bean powder and bean fragments respectively showed a high productivity of mucilage from *Bacillus subtilis* HA strain compared to the control. Additionally, the case of adding with fat-removed bean powder showed better effects than the case of adding with bean fragments. The case of adding with fat-removed bean powder produced more than twice mucilage compared to the control.

15

To prepare a sample for measuring a fibrinolytic ability, 1g of fermented soybeans were mixed with 19 mL of 0.1M phosphate buffer (pH 7.5) to filter and then centrifuged to obtain supernatant as a crude enzyme.

- 20 As a result of measuring a fibrinolytic ability, the activity of fibrinolytic enzyme in case of fresh fermented soybeans added with bean fragments or fat-removed bean powder showed a similar tendency to the control (just fermenting the soybeans or black soybeans, not adding with bean fragments or fat-removed bean powder) as shown in Table 5.

25

30

Table 4 The comparison of fibrinolytic ability in cases of adding with bean fragments and fat-removed bean powder (unit: %)

	Soybeans	Black soybeans
Control	70	68
bean fragments	66	68
fat-removed bean powder	69	65

INDUSTRIAL APPLICABILITY

The *Bacillus subtilis* HA, KCCM-10775P according to the present invention is new superior strain having a high productivity of protease, fibrinolytic enzyme and
5 mucilage. The new strain according to the present invention is effective to produce various food including fermented soybeans and medicinal stuff for treating diseases.

Although the present invention has been described in detail with reference to the specific features, it will be apparent to those skilled in the art that this description is
10 only for a preferred embodiment and does not limit the scope of the present invention. Thus, the substantial scope of the present invention will be defined by the appended claims and equivalents thereof.

THE CLAIMS

What is Claimed is:

- 5 1. *Bacillus subtilis* HA KCCM-10775P having a high productivity of protease, fibrinolytic enzyme and mucilage.
2. The *Bacillus subtilis* HA KCCM-10775P according to claim 1, wherein the *Bacillus subtilis* HA KCCM-10775P is used in fermentation of fermented soybeans.
- 10 3. A method for preparing fermented soybeans, the method is characterized in that bean fragments, soybeans or black soybeans is fermented by *Bacillus subtilis* HA KCCM-10775P
- 15 4. The method for preparing fermented soybeans according to claim 3, the method comprises the following steps of:
 - steaming bean fragments;
 - inoculating the 100 weight parts of the steamed bean fragments with 0.2~5 weights part of *Bacillus subtilis* HA KCCM-10775P; and
 - 20 fermenting the inoculated bean fragments.
5. The method for preparing fermented soybeans according to claim 3, the method comprises the following steps of:
 - steaming soybeans or black soybeans;
 - 25 inoculating the 100 weight parts of the steamed soybeans or black soybeans with 0.2~5 weights part of *Bacillus subtilis* HA KCCM-10775P; and
 - fermenting the inoculated soybeans or black soybeans.
6. The method for preparing fermented soybeans according to claim 5, wherein the
30 method further comprises a step of mixing the 5~20 weight parts of bean fragments

or fat-removed bean powder with the soybeans or black after the step of steaming soybeans or black soybeans, and inoculating the 100 weight parts of the mixture with 0.2~5 weights part of *Bacillus subtilis* HA KCCM-10775P.

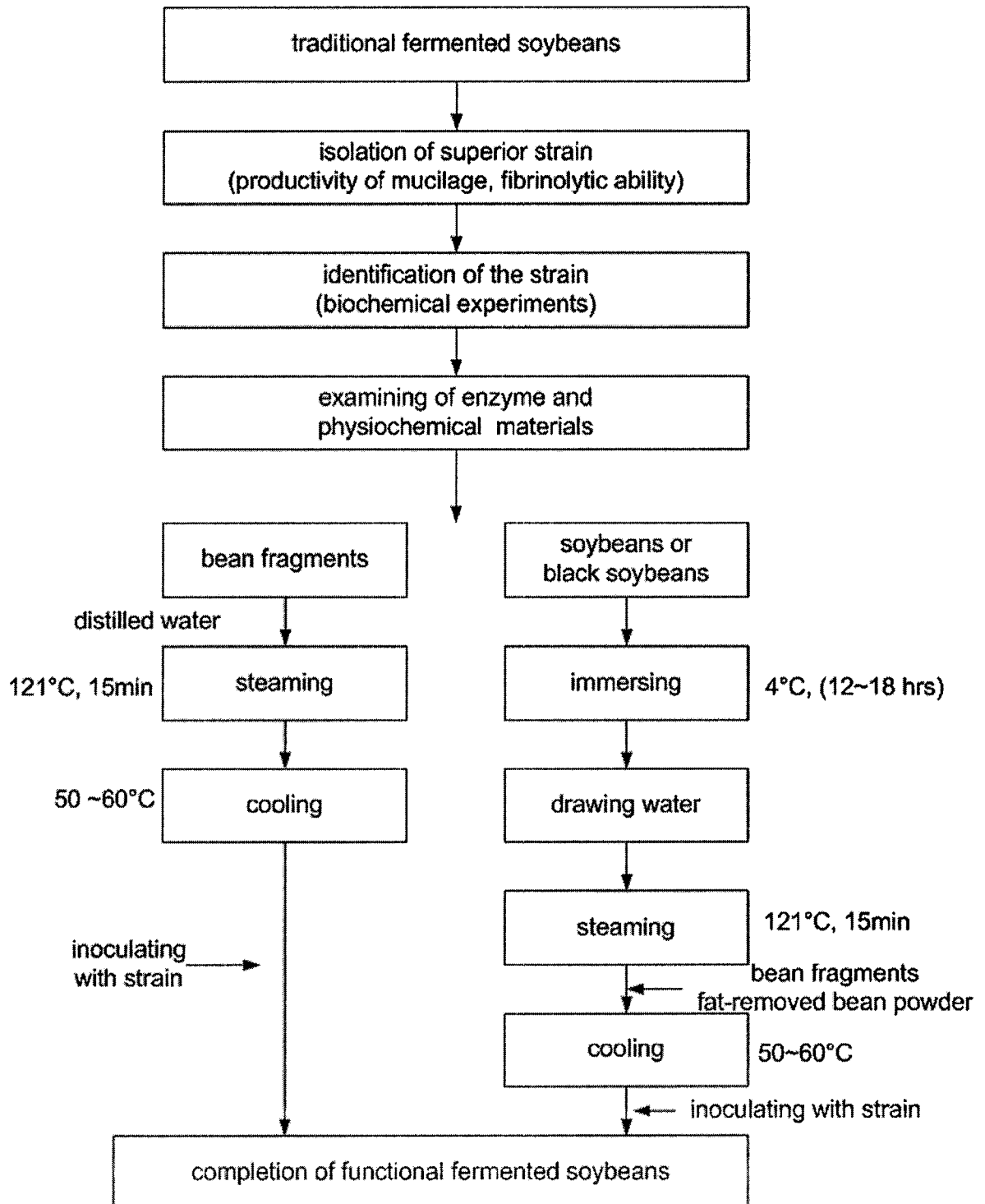
- 5 7. The method for preparing fermented soybeans according to claim 5, the step of fermenting the inoculated soybeans or black soybeans is performed for 10~48 hrs at 30~55 °C .

8. Fermented soybeans prepared by the method according to any one claims 3 to 7.

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FIG. 1



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FIG. 2

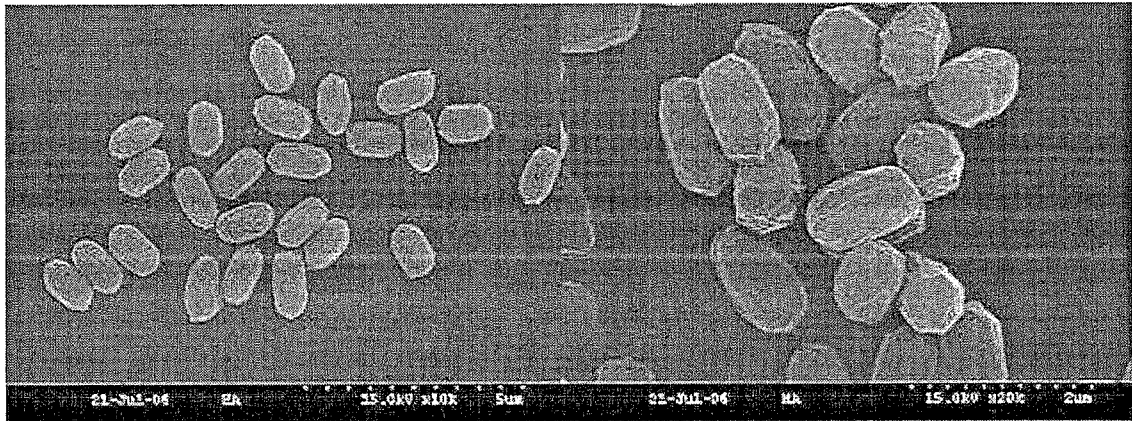


FIG. 3

GCGCTATCTGCAGTCGAGCGGACGATGGGAGCTTGCTCCCTGATGTTAGC
 GGCGGACGGGTGAGTAACACGTGGGTAACTGCCCTGTAAGACTGGGATAA
 CTCGGGAAACCGGGGCTAATACCGGATGGTTGTTTGAACCGCATGGTTC
 AAACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCGCGGCGCA
 TTAGCTAGTTGGTGAGGTAACGGCTCAACCAAGGCAACGATGGGTAGCCGA
 CCTGAGAGGGGTGATCGGCCACACTGGGACTGAGACACGGCCACAGACTCCT
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 GCAACGCCCGCGTGAGTGATGAAGGTTTTTCGGATCGTAAAGCTCTGTTGTT
 AGGGAAGAACAAGTACCGTTTCGAATACGGCGGTACATTGACTCCACCTAA
 CCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGT
 GGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTC
 TTAAGTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCAATTEGAAA
 CTGGGGAACTTGAGTGCAAGAGAGGAGAGTGGAATTCCACGTGTAGCGGT
 GAAATGCCGTAGAGATGTGGAGGAACAACAGTGGCGAAGGCGACTCTCTGG
 TCTGTAACTGACCGCTGAGGAGCGAAAGCOTGGGGAGCGAACAGGATTAGA
 TACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTT
 TCCGCCCCCTTAGTGCTGCAGCTAACGCCATTAAGCACTCCGCCCTGGGGAGT
 ACGGTCCGAAGACTGAAACTCAAAGGAATTGACGGGGGGCCCCGACAAGCG
 GTGGAGCATGTGGTTTAATTTCGAAGCAACGCGAAGAACCTTACCAGGTCT
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 TGACAGGTGGTGATGGTTGTGCTCAGCTCGTGTCGTGAGATGTTGGGTT
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 GGCACCTCTAAGGTGACTGCCGCTGACAAACCGGAGGAAGGTGGGGATGAC
 GTCAAATCATCATGCCCTTATGACCTGGGCTACACACGTGCTACAATGG
 ACAGAACAAAGGGCAGCGAAACCGCGAGGTTAAGCCAATCCCAACAATCT
 GTTCTCAGTTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAAGCTGGAATC
 GCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCCTTG
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FIG. 4

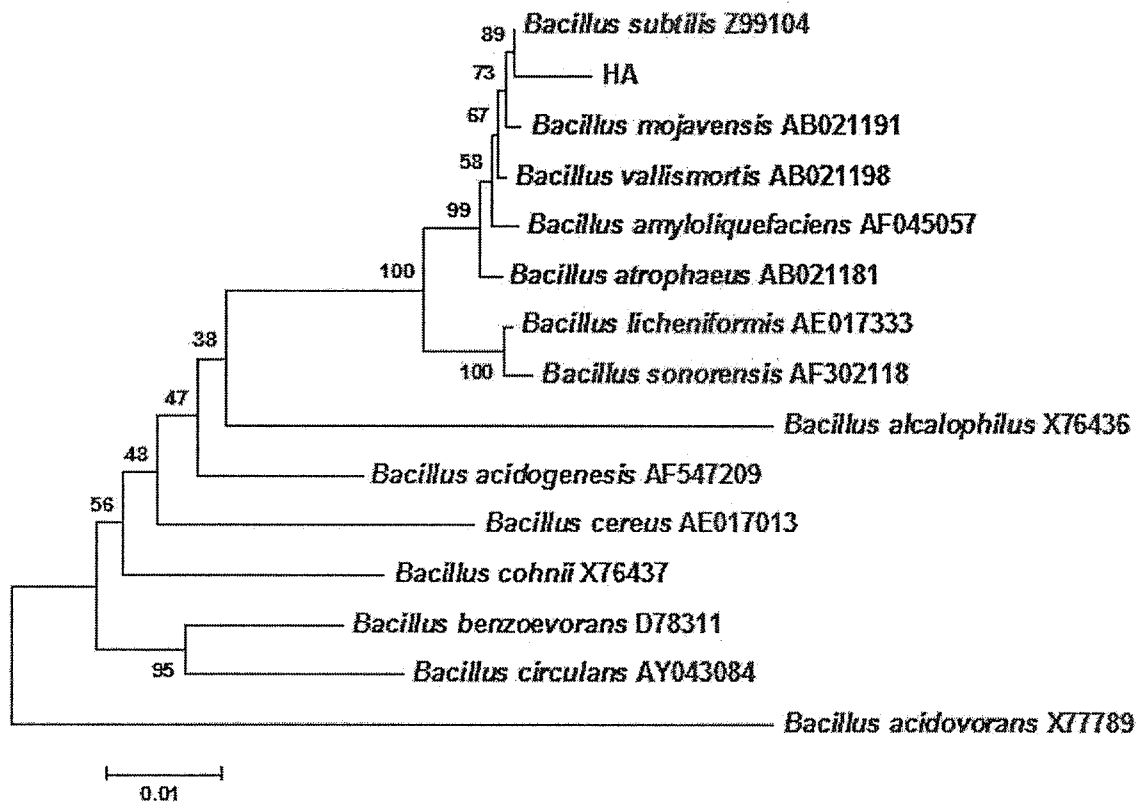
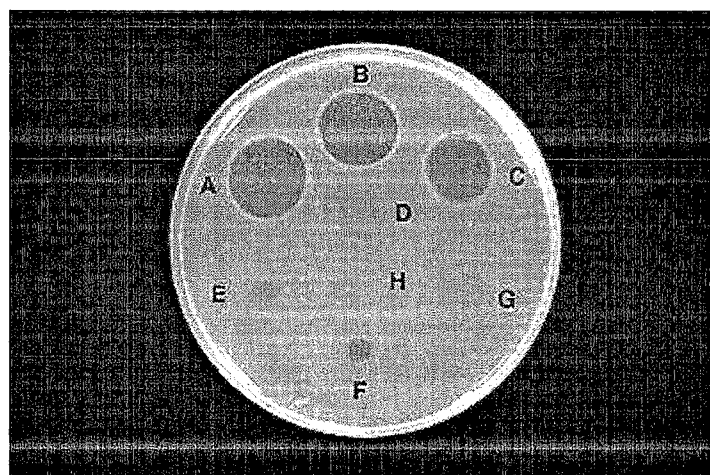
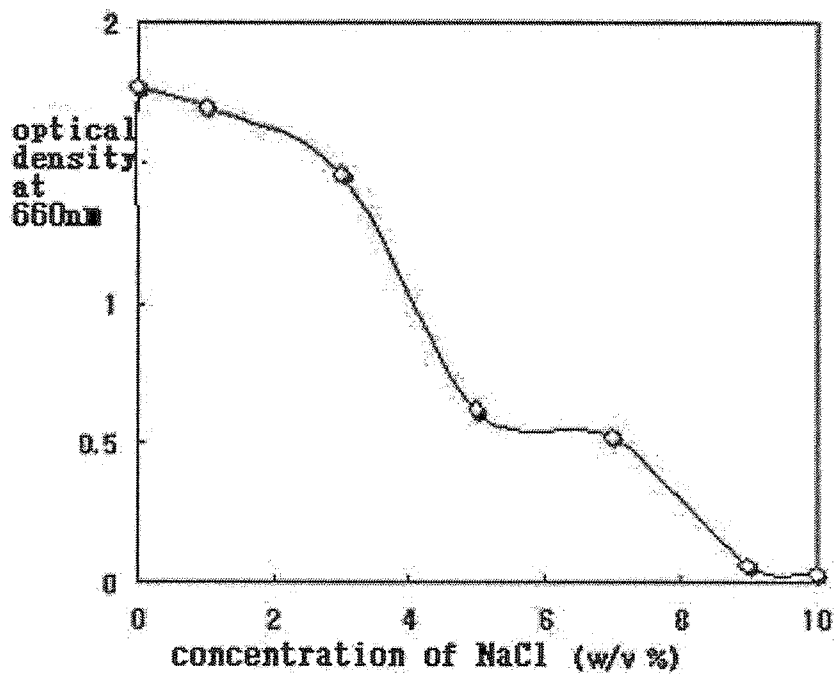


FIG. 5



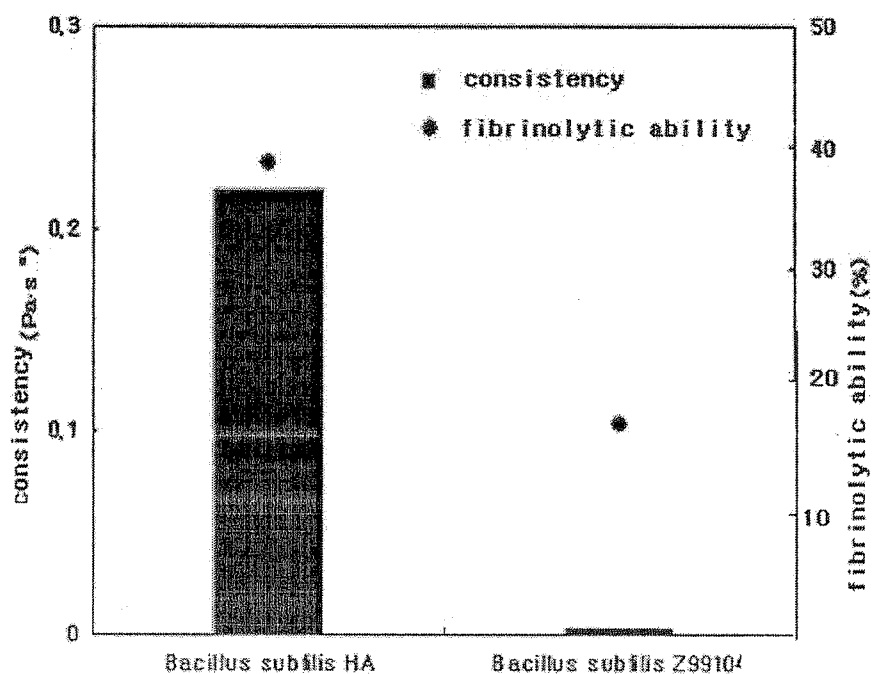
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FIG. 6



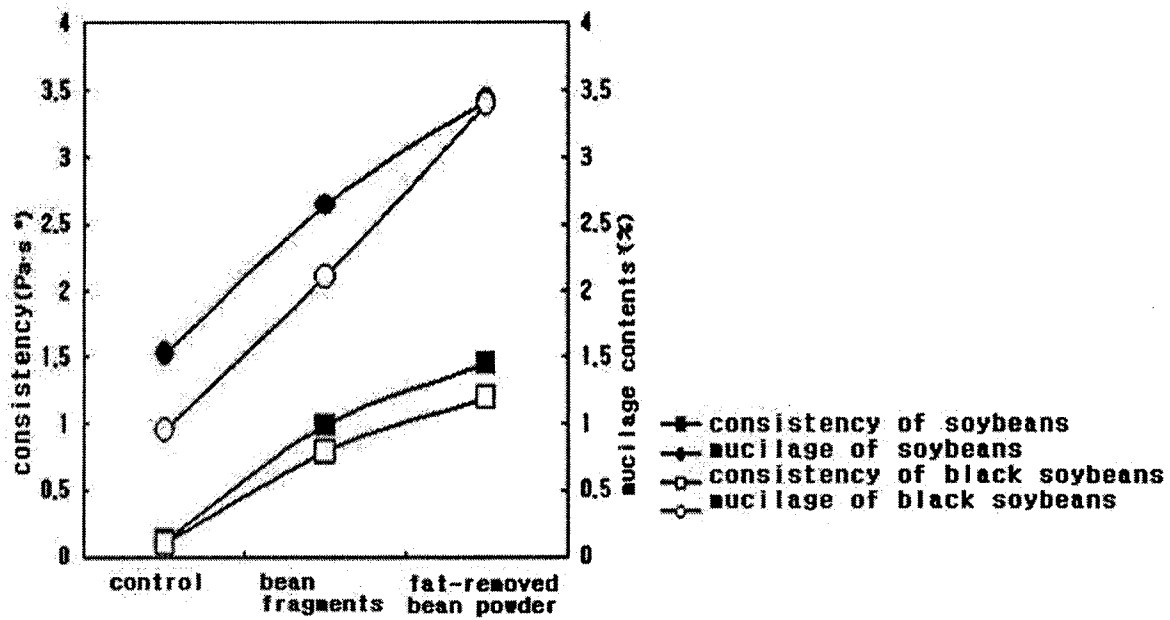
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FIG. 7



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FIG. 8



A. CLASSIFICATION OF SUBJECT MATTER*C12N 1/20(2006.01)i, A23L 1/20(2006.01)i*

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC8 C12N 1/20, A23L 1/20, A23L 1/202

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

NCBI PubMed, Esp@cenet, CA, e-KIPASS(KIPO internal) "Bacillus subtilis, fermented soybean, chungkukjang, protease, fibrinolysis, viscous extract"

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KIM I.J. et al., 'Study of functional chungkukjang containing fibrinolytic enzyme', In: Korean J. Life Science, 2002, Vol. 12(3), pp. 357-362 See the abstract.	1-8
A	OMURA K. et al., 'A newly derived protein from Bacillus subtilis natto with both antithrombotic and fibrinolytic effects', In: J. Pharmacol. Sci., 2005, Vol. 99, pp. 247-251 See the abstract.	1-8
A	KR 2005/094081 A (Park et al.) 27 SEPTEMBER 2005 (27.09.2005) See the whole document.	1-8
A	KR 2005/106303 A (Cha et al.) 9 NOVEMBER 2005 (09.11.2005) See the whole document.	1-8
A	KR 2005/052418 A (Gal et al.) 2 JUNE 2005 (02.06.2005) See the whole document.	1-8
A	JP 2002/065294 A (Hiroyuki) 5 MARCH 2002 (05.03.2002) See the abstract.	1-8

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search

21 AUGUST 2007 (21.08.2007)

Date of mailing of the international search report

21 AUGUST 2007 (21.08.2007)

Name and mailing address of the ISA/KR

Korean Intellectual Property Office
920 Dunsan-dong, Seo-gu, Daejeon 302-701,
Republic of Korea

Facsimile No. 82-42-472-7140

Authorized officer

CHO, YOUNG GYUN

Telephone No. 82-42-481-8132



INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR2007/000991

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KR 2005/042277 A (Cho et al.) 6 MAY 2005 (06.05.2005) See the whole document.	1-8
A	CN 1647689 A (Zhang) 3 AUGUST 2005 (03.08.2005) See the abstract.	1-8

INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR2007/000991

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of :

a. type of material



a sequence listing



table(s) related to the sequence listing

b. format of material



on paper



in electronic form

c. time of filing/furnishing



contained in the international application as filed



filed together with the international application in electronic form



furnished subsequently to this Authority for the purposes of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/KR2007/000991

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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KR 2005/052418 A	02.06.2005	None	
JP 2002/065294 A	05.03.2002	None	
KR 2005/042277 A	06.05.2005	None	
CN 1647689 A	03.08.2005	None	