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# Therapeutic and Anti-Thrombotic Properties of Some Naturally Fermented Soybean Foods of the Eastern Himalayas

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**Abstract:** Naturally fermented soybean foods have anti-thrombotic properties due to the presence of microbial fibrinolytic enzymes. However, fibrinolytic enzyme-producing bacteria from naturally fermented soybean foods of the Eastern Himalayas viz. *kinema*, *grep-chhurpi*, *peha*, *peron namsing*, *peruñyaan* and *bemerthu* have not been screened yet. Therefore, this study aims to screen the fibrinolytic enzyme-producing bacteria from these naturally fermented soybean foods and also to assess their therapeutic properties such as antioxidant, anti-inflammatory, flavones, isoflavones, anti-obesity and anti-diabetic properties of samples. A total of 877 bacteria were isolated, out of which *Bacillus subtilis* (ten strains), *B. velezensis* (three strains), *B. inaquosorum* (two strains) and *B. halotolerans* (one strain) showed fibrinolytic enzyme activity ranging from 775.70 U/mL to 1230.61 U/mL, with a blood clot-degrading property of more than 50%. A remarkable DPPH radical scavenging property (>50%), flavone (278.98 ± 7.06 µg QE/g) and isoflavone (394.13 ± 11.68 µg GEN/g) contents were observed. The Himalayan fermented soybean foods have shown anti-thrombotic, antioxidant, anti-inflammatory, anti-diabetic and anti-obesity properties indicating their therapeutic values.

**Keywords:** anti-thrombotic; fibrinolytic; *Bacillus*; naturally fermented soybeans; *kinema*



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## 1. Introduction

Naturally fermented soybean foods portray the distinct gastronomy of many ethnic Southeast Asians and the Himalayan people [1,2], which are flavoursome, heathy, nutritious and therapeutic in nature [3–5]. Consumption of naturally fermented, sticky soybean foods is restricted only to the eastern Himalayan regions of east Nepal, Northeast India and south Bhutan [6]. *Kinema* is the most popular fermented soybean food in the Eastern Himalayas, followed by other region-specific fermented soybean foods such as *hawaijar* of Manipur, *bekang* of Mizoram, *axone/aakhonii* of Nagaland, *tungrymbai* of Meghalaya, *grep-chhurpi*, *peha*, *peron aming* and *peruñyaan* of Arunachal Pradesh, *bemerthu* and *bekanthu* of Assam and *bezeithu* of Tripura [6–8]. Sticky, umami-flavoured [9] naturally fermented soybean foods are also the bio-reservoirs of microbial communities, mostly species of *Bacillus* [5,6,10,11], which have several biological functionalities, such as antioxidant, anti-inflammatory, anti-diabetic, anti-obesogenic activities, etc. [12]. Species of *Bacillus* play a crucial role in secreting various enzymes, especially fibrinolytic enzymes (serine protease) preventing fibrin clot formation and effectively attenuating the effects of blood pressure and cardiac disorders [13–15].

Thrombosis is caused due to aggregation of fibrin in blood vessels leading to myocardial infarction and other cardiovascular diseases (CVDs) [16] and as per the World Health Organisation (WHO), CVDs are the leading cause of death with an estimated 17.9 million lives lost each year (https://www.who.int/health-topics/cardiovascular-diseases accessed on 20 December 2022). Various commercial fibrinolytic enzymes are available for treatment of CVDs but their limitations, based on excessive cost and complications, lead to alternative and safer sources of fibrinolytic enzyme [17]. Fermented soybean foods containing microbial fibrinolytic enzymes have gained attention as one of the alternative treatments of CVDs with no after-effects, to eliminate the thrombus by degrading the fibrin

clot [18–20]. Microbial origin-fibrinolytic enzymes are more convenient and eco-friendlier, with high efficacy rates [21], among which nattokinase (NK), the first microbial fibrinolytic enzyme produced by *Bacillus subtilis* in *natto*, a fermented soybean food of Japan [22], is a potent blood-clot dissolving protein used for the treatment of CVDs [23]. Apart from *B. subtilis natto*, many microbial fibrinolytic enzyme-producing bacteria were isolated from some naturally fermented soybean foods such as *B. subtilis* DC27, *B. amyloliquefaciens* DC-4 and *B. mojavensis* LY-06 from *douche* of China [13,24,25]; *B. amyloliquefaciens, B. subtilis* and *B. licheniformis* from *cheonggukjang* of Korea [26,27]. Furthermore, *B. subtilis, B. amyloliquefaciens,* and *Vagococcus carniphilus*, isolated from some naturally fermented soybeans of North-east India such as *tungrymbai*; *hawaijar* and *bekang* were also reported to show fibrinolytic activities [28]. Fibrinolytic enzyme-producing bacteria from other Himalayan naturally fermented soybean foods such as *kinema*, *grep-chhurpi, peha, peron namsing, peruñyaan* and *bemerthu* have not been screened yet. Therefore, this study aims to screen the fibrinolytic enzyme-producing bacteria from some naturally fermented soybean foods of the Eastern Himalayas (Figure 1a–f) viz. *kinema* (India, eastern Nepal and Bhutan), *grep-chhurpi, peha, peron namsing, peruñyaan* (Arunachal Pradesh, India) and *bemerthu* (Assam, India). Samples were also assessed for their therapeutic properties such as antioxidant, anti-inflammatory, flavones and isoflavones, anti-obesity and anti-diabetic.

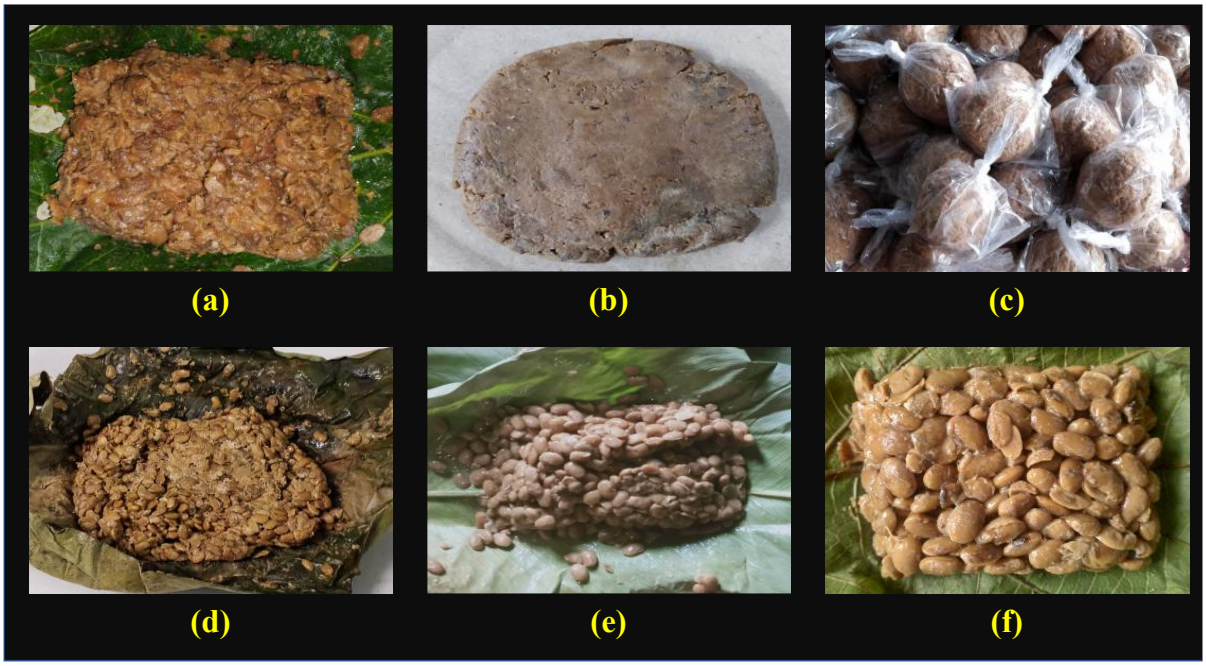


**Figure 1.** Naturally fermented soybean foods of the Eastern Himalayas (**a**) *kinema*, (**b**) *grepchhurpi*, (**c**) *peha*, (**d**) *peron naming*, (**e**) *perunyaan* and (**f**) *bemerthu*.

## 2. Materials and Methods

*Sample Collection*

A total of sixty-two dried samples of naturally fermented soybean foods were collected from different local markets viz. *kinema* (India), *kinema* (Nepal), *kinema* (Bhutan), *grep-chhurpi* (India), *peha* (India), *peron namsing* (India), *peruñyaan* (India) and *bemerthu* (India) (Table 1). Samples were packed in a pre-sterilised polybag, sealed, levelled, transported to the Microbiology Laboratory, Department of Microbiology, Sikkim University, Tadong, Gangtok (27.3106° N, 88.5976° E) and stored at 40 °C. The pH of the samples were determined by homogenizing 1 g of sample in 10 mL of sterile deionised water and the readings were taken using a digital pH-meter (Orion 910003, Thermo Fisher Scientific, Waltham, MA, USA).

**Table 1.** Location of sample collection, geographical coordinates and pH of *kinema*, *grep-chhurpi*, *peha*, *peron namsing* and *peruñyaan*.

| Product | Locations | Geographical Coordinates | pH |
|---|---|---|---|
| *Kinema* (India) | Gangtok, Sikkim, India | 27.3314° N, 88.6138° E | 7.19 ± 0.21 (6.92–7.46) |
| *Kinema* (Nepal) | Dharan, Eastern Nepal | 26.8065° N, 87.2846° E | 7.14 ± 0.14 (6.98–7.25), |
| *Kinema* (Bhutan) | Samtse, South Bhutan | 26.9131° N, 89.0836° E | 6.60 ± 0.48 (6.02–7.06) |
| *Grep-churpii* | Tawang, Arunachal Pradesh, India | 27.5861° N, 91.8594° E | 7.32 ± 0.10 (6.94–7.71) |
| *Peha* | Itanagar, Arunachal Pradesh, India | 27.1719° N, 93.7029° E | 7.31 ± 0.01 (5.46–8.43) |
| *Peron namsing* | Pasighat, Arunachal Pradesh, India | 28.0632° N, 95.3239° E | 8.24 ± 0.01 (7.72–8.53) |
| *Peruñyaan* | Ziro valley, Arunachal Pradesh, India | 27.6169° N, 93.8392° E | 7.55 ± 0.41 (7.15–8.15) |
| *Bemerthu* | North Bagetar, Dimahasao district, Assam, India | 24.98487° N 92.83307° E | 7.82 ± 0.04 (7.02–8.20) |

## 3. Therapeutic Properties

### *3.1. DPPH Radical Scavenging Assay*

Antioxidant properties of samples were evaluated using DPPH free radical scavenging activity following the method described by El Euch et al. [29]. Briefly, DPPH solution (0.2 mM) was prepared using 2,2-diphenyl-1-picrylhydrazyl (HiMedia, Mumbai, India) dissolved in ice-cold methanol. Then, 100 µL of each sample was mixed with 900 µL DPPH solution and incubated for 25 min in the dark. After incubation, the absorbance was measured at 524 nm using Eppendorf BioSpectrometer (Hamburg, Germany). The DPPH radical scavenging activity was calculated using the following equation:

$$\text{DPPH scavenging activity (\%)} = (A_{blank} - A_{sample})/A_{blank} \times 100$$

where, $A_{blank}$ is the absorbance of the control negative reaction without extract. $A_{sample}$ is the absorbance of the test sample.

### *3.2. Anti-Inflammatory Activity*

Assessment of anti-inflammatory activity of samples was determined by spectrophotometry measurement using linoleic acid (HiMedia, Mumbai, India) as the substrate treated with 5-LOX (Sigma Aldrich, St. Louis, MO, USA) [29]. Briefly, 20 µL of samples were mixed with sodium phosphate buffer (pH 7.4), containing 20 µL of 5-LOX and 60 µL of linoleic acid. In the blank, substrate was omitted, but instead buffer solution was added. Nordihydroguaiaretic acid (NDGA) (Sigma Aldrich, St. Louis, MO, USA) was used as the positive control. The mixture was incubated for 10 min at 25 °C, and after incubation, the result was observed at 234 nm (absorption change with the conversion of linoleic acid to 13-hydroperoxyoctadeca-9,11-dienoate) using Eppendorf BioSpectrometer (Hamburg, Germany).

### *3.3. Quantification of Total Flavone/Isoflavone Content*

Estimation of total flavonoid contents in samples was carried out following the method described by Bekir et al. [30]. Homogenised cell free supernatant of sample (100 µL) was

mixed with an equal volume of 2% aluminium trichloride (HiMedia, Mumbai, India) solution in methanol and incubated at room temperature for 15 min. After incubation, the absorption was measured at 415 nm using an Eppendorf BioSpectrometer (Hamburg, Germany). The total flavonoid contents in samples were expressed in micrograms of quercetin (HiMedia, Mumbai, India) equivalents per gram of dry weight (µg QE/g dw). Similarly, isoflavonoid contents of samples were also quantified and the amounts were expressed in micrograms of genistein (HiMedia, Mumbai, India) equivalents per gram of dry weight (µg Gen/g dw) by observing at 415 nm using an Eppendorf BioSpectrometer (Hamburg, Germany).

### *3.4. Anti-Obesity (Pancreatic Lipase Inhibition)*

The anti-obesity property of samples was assessed following the method described by Jaradat et al. [31]. A fresh solution of pancreatic lipase (Sigma Aldrich, St. Louis, MO, USA) enzyme (1 mg/mL) was prepared before use, followed by preparation of a stock solution of p-nitrophenyl butyrate (PNPB) (Sigma Aldrich, St. Louis, MO, USA) dissolved in acetonitrile (20.9 mg of PNPB in 2 mL acetonitrile (Merck, Darmstadt, Germany). Then, 0.2 mL of each sample (homogenate) or orlistat (positive control) was mixed with 0.1 mL of pancreatic lipase enzyme (1 mg/mL). The resulting mixtures were then added with Tris HCl solution (pH 7.4) to make up the volume to 1 mL and incubated for 15 min at 25 °C. After incubation, 0.1 mL of PNPB solution was added and again incubated at 37 °C for 30 min. The activity was determined using an Eppendorf BioSpectrometer (Hamburg, Germany) at 405 nm based on the hydrolysis of p-nitrophenyl butyrate to p-nitrophenol. The results were expressed in inhibition percentage (IP) using the following equation:

$$\text{IP } (\%) = (A_c - A_t)/A_t \times 100$$

where, $A_c$ is the absorbance of the positive control, $A_t$ is the absorbance of test samples.

### *3.5. Anti-Diabetic*

The inhibitory effect of samples on α-amylase (Sigma Aldrich, MO, USA) activity was determined following the method previously described by Erukainure et al. [32]. A total of 100 µL of α-amylase solution was mixed with 100 µL of sample homogenate or acarbose (positive control) or water (control) and incubated for 20 min at 37 °C. After incubation, 100 µL of 1% starch solution was then added to the mixture followed by addition of 400 µL 3,5-dinitrosalicylic acid (DNSA) (HiMedia, Mumbai, India) and boiled for 10 min. Addition of DNSA indicates the hydrolysis of starch using α-amylase which reduces 3,5-dinitrosalicylic acid (yellow) to 3-amino-5-nitrosalicylic acid (reddish-brown). After boiling, the reaction mixture was cooled, and the absorbance measured at 540 nm using an Eppendorf BioSpectrometer (Hamburg, Germany). The inhibition percentage (IP) was calculated as follows:

$$\text{IP } (\%) = (A_c - A_t)/A_t \times 100$$

where, $A_c$ is the absorbance of positive control, $A_t$ is the absorbance of test samples.

## 4. Bacterial Isolation

A total of 10g of the sample was mixed with 90 mL of sterile physiological saline (0.9% NaCl) and was homogenised using a stomacher blender (400 Circulator, Seward, UK). The homogenised samples were serially diluted, enumerated on nutrient agar plates (HiMedia, Mumbai, India) and incubated at 37 °C for 24 h. The pure culture of the isolates were obtained by streaking on a fresh nutrient agar plate, and phenotypically characterised based on the colony morphology, cell morphology and endospore staining [33].

**5. Screening of Proteolytic Isolates**

The bacterial strains were further subjected to primary screening (proteolytic activity) using skim milk agar plates (2% of skim milk powder (HiMedia, Mumbai, India) and 1.5% bacteriological agar (HiMedia, Mumbai, India)), incubated at 37 °C for 24 h [34].

**6. Qualitative Screening of Fibrinolytic Enzyme using Fibrin Plate Assay**

A loopful of fresh protease producing strains, grown in a sterile broth, was incubated at 37 °C for 24–48 h under shaking conditions (120 rpm) and was centrifuged at 6000× *g* for 30 min at 4 °C. The cell free supernatant (CFS) was collected and used as fibrinolytic enzyme for subsequent analysis. After collecting the CFS, a fibrin plate assay was used to determine the fibrinolytic enzyme activity following the method described by Hu et al. [13]. Briefly, a sterile 10 mL sodium phosphate buffer (pH 7.4) was mixed with fibrinogen (0.15%) (Sigma Aldrich, St. Louis, MO, USA). A sterile agarose gel (1.5%) was mixed with 1 mL thrombin (Sigma Aldrich, St. Louis, MO, USA). The two solutions were immediately mixed and poured into a sterile plate, followed by treatment at 37 °C for 30 min to allow the formation of fibrin clots, and heated at 60 °C to 80 °C for 30 min to inactivate the plasminogen. Additionally, the wells were punched and supplemented with 100 µL of CFS as a source of fibrinolytic enzyme and incubated at 37 °C for 24 h, where a clear zone was observed around the well indicating positive activity.

**7. Quantitative Screening of Fibrinolytic Enzyme Activity**

Bacterial cultures were centrifuged to obtain the crude extract that was considered as crude enzyme (fibrinolytic enzyme) [35]. In a cleaned sterile vial, 2.5 mL of 1% fibrin solution in Tris HCl (pH 7.8) was mixed with 0.5 mL of crude enzyme solution and incubated for 30 min at 37 °C. After incubation, the reaction was stopped by adding 110 mM trichloroacetic acid (HiMedia, Mumbai, India) and enzyme solution (220 mM sodium acetate and 0.33 M of acetic acid) followed by incubation at 37 °C for 30 min, and centrifugation at 10,000 rpm for 10 min. The supernatant obtained by centrifugation after the treatment was measured at 660 nm using Eppendorf BioSpectrometer (Hamburg, Germany). A single unit of protease activity (U) was defined as the amount of the enzyme that releases one µmol of tyrosine (HiMedia, Mumbai, India) equivalent per ml of enzyme per minute.

**8. In-Vitro Determination of Fibrinolytic Activity Using Whole Blood Clot**

Thrombolytic activity of the enzyme was performed using the whole blood clots following the method described by Lucy et al. [34]. Blood clots were cut into small pieces of about 1 $mm^3$, washed three times with 50 mM PBS (pH 7.4), and incubated at 37 °C for 5 min. One mL of the crude enzyme extract was added to each well with blood clot. One millilitre of streptokinase (2.5 mg/mL) was used as the positive control, and nutrient broth (HiMedia, Mumbai, India) used as negative control. After incubation, the dissolved clots were analysed qualitatively (by visual inspection) and photography. The remaining blood clots were dried at 80 °C for 20 min and weighed to obtain the final weights, and the percentage of blood clot degradation was calculated based on dry weight of the clot using the following formula:

$$\text{Blood clot degradation (\%)} = (\text{initial weight of blood clot} - \text{final weight of blood clot})/\text{initial weight of blood clot} \times 100\%$$

**9. Determination of Molecular Weight by SDS-PAGE**

The cell free supernatant was separated by acetone precipitation, followed by centrifugation at 6000× *g* for 15 min to obtain the proteins. The protein was dissolved in 20 mM Tris HCl buffer (pH 8.8) (HiMedia, Mumbai, India) and the molecular weight determined using SDS-PAGE as described by Syahbanu et al. [36] with minor modifications. Briefly, 12% separating and 5% stacking gel was prepared for SDS-PAGE. The cell free supernatant

was diluted in sample buffer 4 × (containing 60 mM Tris-HCl (pH 6.8), 25% glycerol (HiMedia, Mumbai, India), 2% sodium dodecyl sulphate (HiMedia, Mumbai, India), and 0.1% bromophenol blue (BPB) (HiMedia, Mumbai, India)), and the gel was run at 100 V, 100 mA for 1 h or up to the point where BPB reached the bottom. After electrophoresis, the protein bands were stained with Coomassie Blue (HiMedia, Mumbai, India) for 20 min, followed by sequential soaking in a de-staining solution (30% methanol in water with 10% acetic acid) to attain decoloration. The molecular mass of the protein was determined using standard pre-stained protein marker (HiMedia, Mumbai, India).

## 10. Genotypic Identification of Bacterial Isolates

### *10.1. DNA Extraction*

Genomic DNA of a pure bacterial culture was extracted following the method described by Shangpliang and Tamang [37]. A fresh bacterial culture inoculated in nutrient broth (2 mL) was transferred to a 2 mL micro-centrifuge tube and centrifuged at 8000× *g* for 5 min. The cell pellet was obtained after discarding the supernatant, and then washed two times with sterile 0.5 M sodium chloride (NaCl) (HiMedia, Mumbai, India), followed by immediate washing with sterile deionised water. The 1× TE buffer (pH 8) was used as the solution for pellet resuspension. Then, 10 µL of lysozyme (20 mg/mL) was added to the cell suspension and incubated for 30 min at 37 °C for enzyme activation, followed by heating at 98 °C for 15 min. The cell suspension was centrifuged at 10,000× *g* at 4 °C for 10 min to obtain the supernatant and transferred to a sterile micro-centrifuge tube. The DNA was quantified using an Eppendorf BioSpectrometer (Hamburg, Germany) with purity absorbance ($A_{260}/A_{280}$) of 1.8–2.2 and subjected to PCR amplification.

### *10.2. PCR Amplification*

The PCR amplification reaction was carried out in a 50 µL reaction volume using GoTaq® Master Mix (M7122, Promega, Wisconsin, WI, USA) containing Taq DNA polymerase, the required dNTPs (dATPs, dTTPs, dGTPs, dCTPs), $MgCl_2$ and the reaction buffers. A total of two sets of primers were used in the reaction, primers 27F 5′-AGAGTTTGATCATGGCTCAG-3′ and 1492R 5′-GTTACCTTGTTACGACTT-3′ [38]. The PCR amplification was performed in a Veriti™ Thermal cycler (4375305, Applied Biosystems, Thermo Fisher Scientific, Carlsbad, CA, USA) under the following conditions: initial denaturation at 94 °C for 5 min, followed by denaturation at 94 °C for 1 min (30 cycles), annealing process at 55 °C for 1 min, elongation process at 72 °C for 1.5 min, and lastly, final elongation process at 72 °C for 10 min, followed by a stop process at 4 °C.

### *10.3. Purification of PCR Amplicons*

The purification of PCR amplicons was performed following the method described by Shangpliang and Tamang [37] using PEG-NaCl (polyethylene glycol-sodium chloride) 20% (*w*/*v*) PEG, 2.5 M NaCl. Briefly, The PCR amplicons was mixed with 0.6 volumes of PEG-NaCl and incubated for 30 min at 37 °C. After incubation, the mixture was centrifuged at 10,000× *g* for 30 min at 4 °C to collect the pellet and the supernatant discarded. The pellet was washed two times using 70% freshly prepared ethanol and allowed to air dry overnight. A total of 30 µL of nuclease free water was added to resuspend the purified DNA pellet and the quality checked by agarose gel electrophoresis (1.2%) and visualisation using a Gel Doc™ EZ Imager (BioRad, Hercules, CA, USA).

### *10.4. 16S rRNA Gene Sequencing*

The purified PCR amplicons were processed for sequencing chemistry using the primer pairs 27F 5′-AGAGTTTGATCATGGCTCAG-3′; 1492R 5′-GTTACCTTGTTACGACTT-3′ [38], and two separate sequencing PCR reactions were carried out for each primer. A final volume of 50 µL reaction volume was prepared, containing 0.2 µM primer, 0.2 mM dNTPs (dATPs, dTTPs, dGTPs, dCTPs), 2.0 mM $MgCl_2$, 0.5 mg/mL and 0.04 U/µL Taq DNA polymerase. Further, the PCR reaction conditions for sequencing were performed starting

with an initial denaturation at 95 °C for 10 min, followed by a denaturation process at 95 °C for 1 min (35 cycles), annealing process at 40 °C for 2 min, elongation at 72 °C for 1 min and a final elongation at 72 °C for 10 min. Finally, the library prepared DNA was submitted for sequencing using an automated DNA analyser (ABI 3730XL Capillary Sequencers, Applied Biosystems, Foster City, CA, USA).

### 11. Bioinformatics Analysis

The quality of the raw sequence was evaluated using Sequence Scanner v2.0, Applied Biosystems (https://www.thermofisher.com/in/en/home/life-science/sequencing/sanger-sequencing/sanger-dna-sequencing/sanger-sequencing-data-analysis.html accessed on 20 December 2022.) as described by Shangpliang and Tamang [37]. The good quality sequences with a trace score >Q20 and with a length of more than 600 bp were assembled using ChromasPro v1.34 (http://technelysium.com.au/wp/chromas/ accessed on 20 December 2022), and the application of a programme called Mallard to remove the artifact sequences (chimera) [39]. The similarity identification of test sequences was acquired by aligning with BLAST (basic local alignment search tool) [40]. The relationships of the identified species were carried out by aligning the sequences using clustalW [41]. The phylogenetic relationships of the identified species were constructed using the neighbour-joining method [42] based on the Kimura 2-parameter model by Molecular Evolutionary Genetics Analysis 11 (MEGA 11.0.13) [43].

### 12. Statistical Analysis

All experiments were performed in triplicate sets with mean ± SD values. Data were analysed using PAST v4 (Paleontological Statistics Software Package for Education and Data Analysis) software version 3.26 [44].

### 13. Results

A total of 62 samples of naturally fermented soybean foods (6 samples of *kinema* (India), 6 samples of *kinema* (Nepal), 6 samples of *kinema* (Bhutan), 4 samples of *grep-chhurpi*, 10 samples of *peha*, 14 samples of *peron namsing*, 6 samples of *peruñyaan* and 10 samples of *bemerthu*) were collected from different regions of the Eastern Himalayas. The pH of *kinema* of India was 7.19 ± 0.21, *kinema* of Nepal 7.14 ± 0.14, *kinema* of Bhutan 6.60 ± 0.48, *grep-chhurpi* 7.32 ± 0.10, *peha* 7.31 ± 0.01, *peron namsing* 8.24 ± 0.01, *peruñyaan* 7.55 ± 0.41 and *bemerthu* 7.82 ± 0.04, respectively (Table 1).

#### *13.1. Therapeutic Properties*

Samples were assessed for therapeutic properties. The antioxidant property was higher in *kinema* of Bhutan (82.93 ± 7.32%) and *kinema* of Nepal (80.65 ± 8.20%), respectively, whereas *bemerthu* (28.09 ± 0.08%) had the least scavenging percentage (Table 1). The 5-LOX inhibition (anti-inflammatory) in *bemerthu* exhibited more inhibition with a concentration of 200.77 ± 0.15 µg NDGA/g followed by *peha* with 193.46 ± 18.96 µg NDGA/g, whereas *kinema* of Nepal (84.77 ± 0.72 µg NDGA/g) had the lowest concentration (Table 1). Furthermore, *peha* appeared to have higher flavone and isoflavone content with a concentration of 278.98 ± 7.06 µg QE/g and 394.13 ± 11.68 µg GEN/g, respectively (Table 1). The anti-obesity property was higher in *kinema* of India (52.40 ± 5.87%) and *kinema* of Nepal (51.37 ± 2.77%), respectively (Table 2). Additionally, the reducing sugars resulting from starch hydrolysis (anti-diabetic) was evaluated, and it was found that all the samples exhibited more than 40% inhibition with a maximum of 60% inhibition observed in *grep-chhurpi* (69.22 ± 4.76%), *kinema* of India (62.41 ± 1.51%), and *peruñyaan* (60.25 ± 7.09%), respectively (Table 2).

**Table 2.** Some therapeutic properties of naturally fermented soybean foods of the Eastern Himalayas.

| Samples | DPPH Radical Scavenging (%) | Anti-Inflammatory (µg NDGA/g) | Immunomodulatory Effects | | Anti-Obesity (IP %) | Anti-Diabetic (IP %) |
|---|---|---|---|---|---|---|
| | | | Flavones (µg QE/g) | Isoflavones (µg Gen/g) | | |
| *Kinema* (India) | 74.12 ± 10.20 | 137.42 ± 1.78 | 253.40 ± 100.16 | 358.91 ± 26.62 | 52.40 ± 5.87 | 62.41 ± 1.51 |
| *Kinema* (Nepal) | 80.65 ± 8.20 | 84.77 ± 0.72 | 113.22 ± 11.02 | 250.94 ± 24.37 | 51.37 ± 2.77 | 55.09 ± 1.05 |
| *Kinema* (Bhutan) | 82.93 ± 7.32 | 98.36 ± 5.41 | 204.65 ± 62.89 | 294.62 ± 18.82 | 42.26 ± 14.90 | 42.54 ± 3.21 |
| *Grep-chhurpi* | 64.23 ± 8.54 | 96.38 ± 0.19 | 194.90 ± 4.33 | 319.64 ± 24.72 | 36.39 ± 2.60 | 69.22 ± 4.76 |
| *Peha* | 55.28 ± 6.51 | 193.46 ± 18.96 | 278.98 ± 7.06 | 394.13 ± 11.68 | 39.74 ± 3.50 | 60.35 ± 1.04 |
| *Peron namsing* | 67.54 ± 9.95 | 108.02 ± 0.23 | 226.84 ± 59.73 | 301.80 ± 8.42 | 43.24 ± 10.98 | 56.56 ± 4.13 |
| *Peruñyaan* | 73.60 ± 6.36 | 157.85 ± 20.88 | 216.95 ± 122.20 | 370.73 ± 14.94 | 24.84 ± 2.88 | 60.25 ± 7.09 |

Experimental data sets; values were represented by mean ± standard deviation (SD). DPPH = 2,2 diphenylpicryl-hydrazyl; NDGA = Nordihydroguaiaretic Acid; QE = Quercetin; GEN = Genistein; IP = Inhibition Percentage.

### *13.2. Screening and Identification of Fibrinolytic Enzyme Producing Strains*

A total of 877 bacterial isolates (219 isolates from *kinema*, 53 from *grep-chhurpi*, 123 from *peha*, 161 from *peron namsing*, 75 from *peruñyaan* and 246 from *bemerthu*) were arbitrarily isolated from 62 samples of naturally fermented soybean foods of the Eastern Himalayas and were screened for proteolytic activity on skim milk agar. The isolates exhibiting a clear zone of >20 mm in diameter were selected and further subjected to the fibrin plate assay containing a combination of fibrinogen and thrombin for fibrin clot formation. Out of 58 proteolytic isolates, 16 isolates were able to digest the fibrin clot and formed a clear zone around the well on the fibrin plate. The fibrin clot degrading isolates were phenotypically and morphologically characterised, and tentatively identified as Gram-positive, rod-shaped, spore forming bacteria. Further identification of bacterial isolates was performed by the 16S rRNA gene sequencing analysis, which confirmed the identity of bacterial isolates mapped against the NCBI database with percentage of similarity (Table 3).

**Table 3.** Identifications of fibrinolytic-producing bacteria from naturally fermented soybean, of the Eastern Himalayas.

| Products | Identity with Sample Code | Type Species (% Similarity) | GenBank Accession Number |
|---|---|---|---|
| *Kinema* | *Bacillus subtilis* Ki01 | *Bacillus subtilis* NCIB 3610 (99.78) | OP776906 |
| | *Bacillus subtilis* Ki52 | *Bacillus subtilis* NCIB 3610 (99.79) | OP776907 |
| *Kinema* | *Bacillus subtilis* Kn16 | *Bacillus subtilis* NCIB 3610 (99.86) | OP776908 |
| *Kinema* | *Bacillus subtilis* Kb37 | *Bacillus subtilis* NCIB 3610 (99.71) | OP776909 |
| *Grep chhurpi* | *Bacillus subtilis* Gc06 | *Bacillus subtilis* NCIB 3610 (99.93) | OP776910 |
| *Peha* | *Bacillus subtilis* Ph07 | *Bacillus subtilis* NCIB 3610 (99.93) | OP776911 |
| *Peron namsing* | *Bacillus subtilis* Pn30 | *Bacillus subtilis* NCIB 3610 (99.58) | OP776912 |
| | *Bacillus subtilis* Pn88 | *Bacillus subtilis* NCIB 3610 (99.30) | OP776913 |
| *Peruñyaan* | *Bacillus subtilis* Py01 | *Bacillus subtilis* NCIB 3610 (99.93) | OP776914 |
| *Bemerthu* | *Bacillus velezensis* F6 | *Bacillus velezensis* CR-502 (99.59) | OP603227 |
| | *Bacillus halotolerans* F10 | *Bacillus halotolerans* ATCC 25096 (99.13) | OP603169 |
| | *Bacillus velezensis* F13 | *Bacillus velezensis* CR-502 (99.77) | OP603042 |
| | *Bacillus subtilis* F16 | *Bacillus subtilis* NCIB 3610 (99.12) | OP603123 |
| | *Bacillus velezensis* F50 | *Bacillus velezensis* CR-502 (99.65) | OP603145 |
| | *Bacillus inaquosorum* F90 | *Bacillus inaquosorum* KCTC 13429 (98.92) | OP602362 |
| | *Bacillus inaquosorum* F139 | *Bacillus inaquosorum* KCTC 13429 (97.02) | OP602954 |

*Bacillus* species with fibrinolytic activity were identified as *B. subtilis, B. velezensis, B. halotolerans* and *B. inaquosorum* (Figure 2a). *B. subtilis* was shared in all samples. Whereas *B. velezensis, B. halotolerans* and *B. inaquosorum* were identified only in *bemerthu* (Figure 2b).

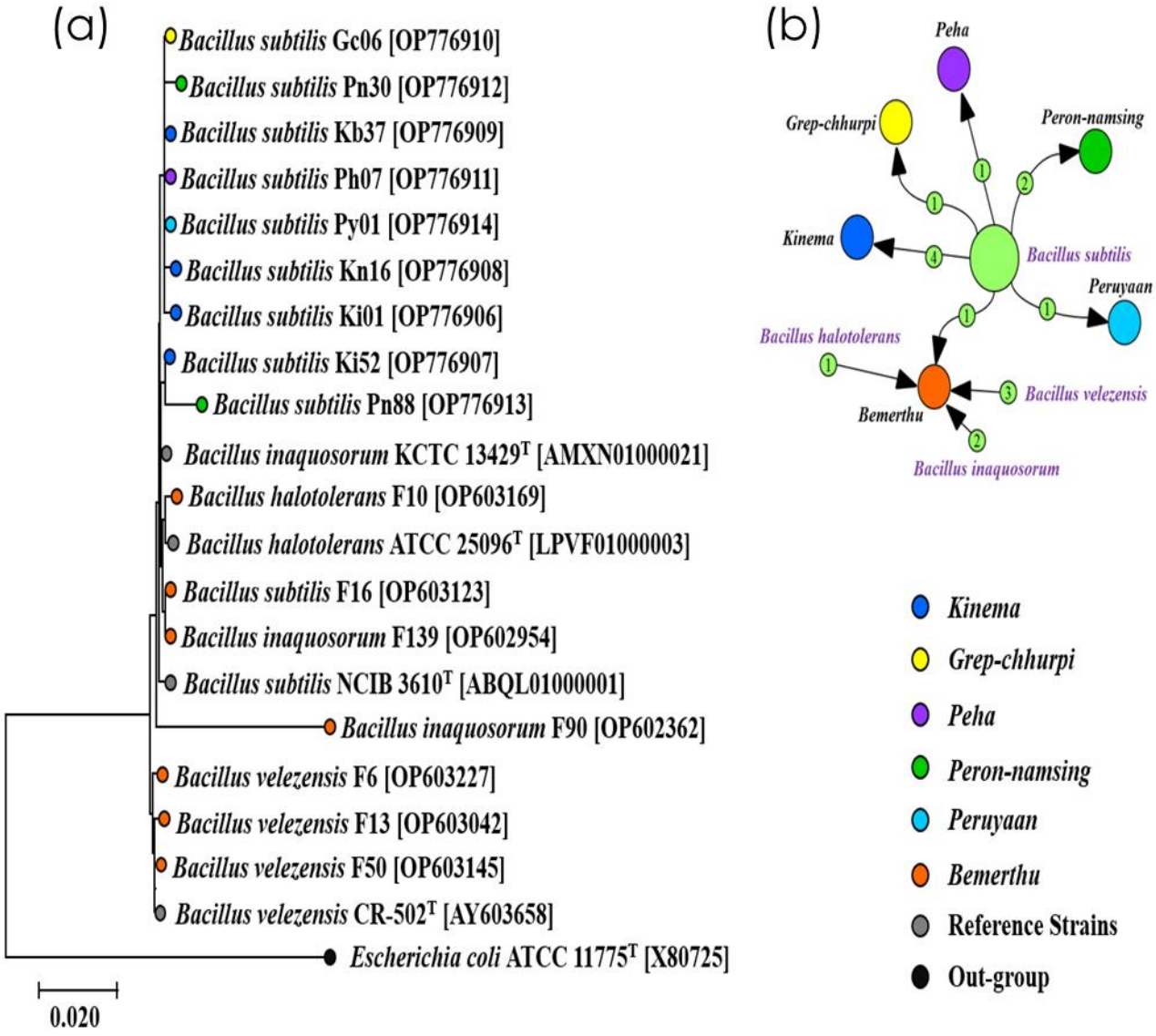


**Figure 2.** (**a**) Neighbour-joining phylogenetic analysis using Molecular Evolutionary Genetics Analysis 11 (MEGA 11.0.13) based on 16S rRNA gene sequences of bacterial strains isolated from naturally fermented soybean foods. The percentage of replicate trees in which the associated bacterial taxa clustered together was 1000 replicates in the bootstrap test with the evolutionary distances computed using the Kimura 2-parameter method. *Bacillus subtilis* NCIB 3610$^T$ [ABQL01000001], *Bacillus velezensis* CR-502$^T$ [AY603658], *Bacillus inaquosorum* KCTC 13429$^T$ [AMXN01000021] and *Bacillus halotolerans* ATCC 25096$^T$ [LPVF01000003] were used as the reference type strain acquired from NCBI database. *Escherichia coli* ATCC 11775$^T$ X80725 was used as the outgroup; (**b**) The shared and unique distribution of *Bacillus* species with fibrinolytic properties identified from naturally fermented soybean foods of the Eastern Himalayas, represented by a graphical constructed network.

The enzyme activity of fibrinolytic-producing strains was more than 700 U/mL (Table 4). *B. subtilis* Ki52, *B. subtilis* Kn16, (isolated from *kinema*) and *B. velezensis* F6 (isolated from *bemerthu*) showed the higher enzyme activity of 1230.61 U/mL, 1222.64 U/mL, and 1209.27 U/mL, respectively (Table 4). Furthermore, *Bacillus* species hydrolysed the whole blood clot with more than 40% using the crude enzymes in a bar graph (Figure 3a) and vi-

sual observation (Figure 3b). The activity of crude enzyme against the whole blood clot was significantly less compared to positive control (streptokinase) ($p < 0.05$). However, when compared among different species of *Bacillus*, the whole blood clot degrading percentage was observed to be higher in *B. subtilis* Ki52 (50.47%) and *B. subtilis* Kn16 (51.37%), isolated from *kinema*, and *B. velezensis* F6 (51.39%) isolated from *bemerthu*. The molecular weight of crude fibrinolytic enzyme ranged from 20 kDa to 75 kDa based on the size marker of a pre-stained protein marker (Table 4)

**Table 4.** In vitro fibrinolytic properties of *Bacillus* strains isolated from the Himalayan fermented soybean foods.

| Product | Bacterial Species | Proteolytic Activity | Fibrin Plate Assay | Enzyme Activity (U/mL) | Blood clot Degradation (%) ($A_0$–$A_1/A_0$) | Molecular Weight by SDS-PAGE (kDa) |
|---|---|---|---|---|---|---|
| *Kinema* | *Bacillus subtilis* Ki01 | +++ | ++ | 1033.92 | 46.70 | ≈27 |
| | *Bacillus subtilis* Ki52 | +++ | ++ | 1230.61 | 50.47 | ≈27 |
| | *Bacillus subtilis* Kn16 | +++ | ++ | 1222.64 | 51.37 | ≈25 |
| | *Bacillus subtilis* Kb37 | +++ | ++ | 1103.68 | 49.05 | ≈63 |
| *Grep-chhurpi* | *Bacillus subtilis* Gc06 | +++ | ++ | 1024.07 | 48.24 | ≈20 |
| *Peha* | *Bacillus subtilis* Ph07 | +++ | ++ | 1061.12 | 49.11 | ≈25 |
| *Peron namsing* | *Bacillus subtilis* Pn30 | +++ | ++ | 1032.16 | 48.99 | ≈25 |
| | *Bacillus subtilis* Pn88 | +++ | ++ | 1045.44 | 46.97 | ≈25 |
| *Peruñyaan* | *Bacillus subtilis* Py01 | +++ | ++ | 1057.76 | 44.65 | ≈27 |
| *Bemerthu* | *Bacillus velezensis* F6 | +++ | ++ | 1209.27 | 51.39 | ≈25 |
| | *Bacillus halotolerans* F10 | +++ | ++ | 934.82 | 46.67 | ≈25 |
| | *Bacillus velezensis* F13 | +++ | ++ | 984.68 | 47.37 | ≈63 |
| | *Bacillus subtilis* F16 | +++ | ++ | 1025.75 | 48.84 | ≈27 |
| | *Bacillus velezensis* F50 | +++ | ++ | 772.75 | 40.48 | ≈25 |
| | *Bacillus inaquosorum* F90 | +++ | ++ | 755.70 | 47.06 | ≈63 |
| | *Bacillus inaquosorum* F139 | +++ | ++ | 1086.98 | 49.75 | ≈20 |

+++ (zone of inhibition > 20 mm); ++ (zone of inhibition > 10 mm).

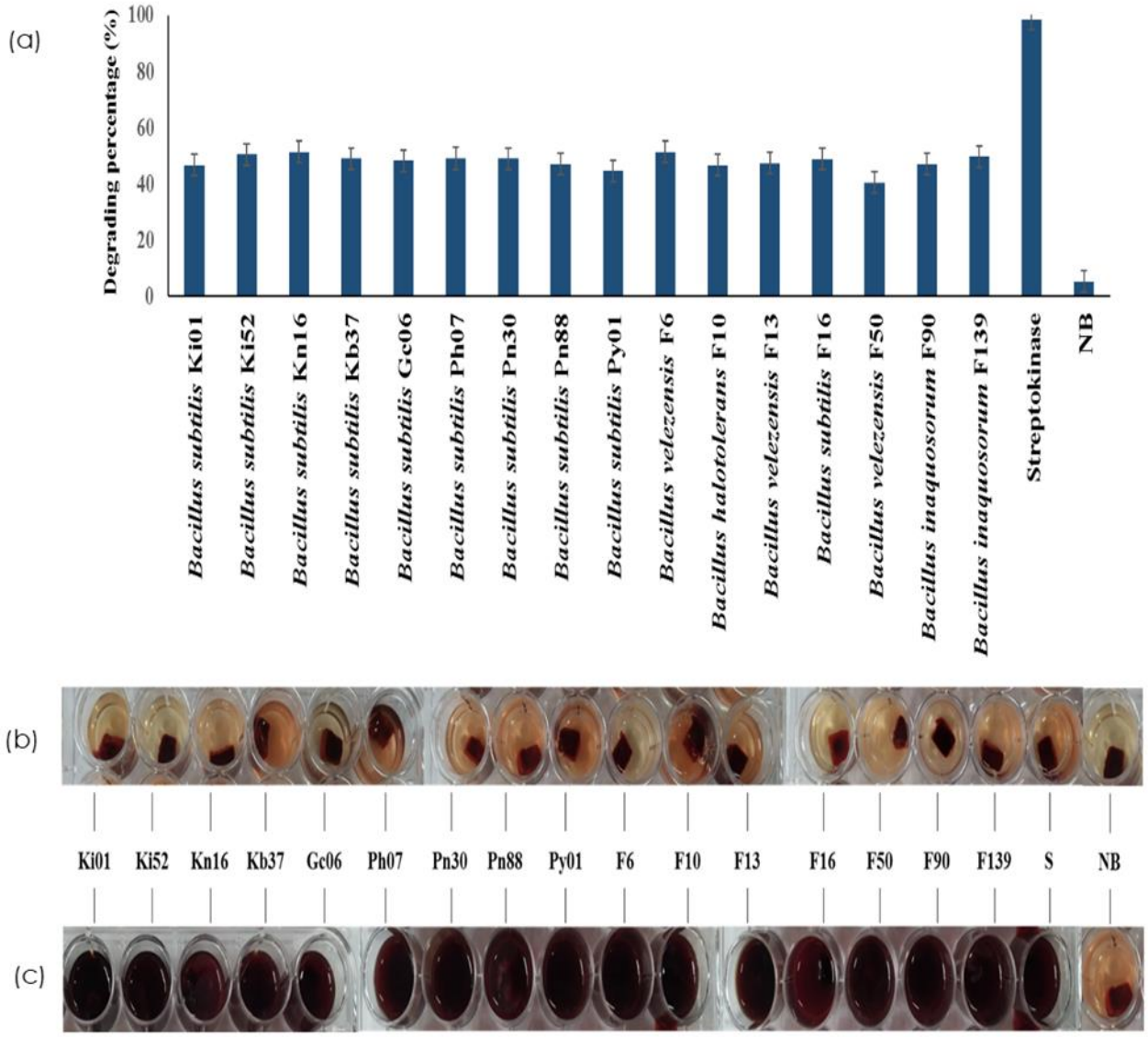


**Figure 3.** (**a**) Crude extracts of *Bacillus* spp. (as the source of fibrinolytic enzymes) exert properties that degrade the whole blood clot; Streptokinase (S) was used as positive control and nutrient broth (NB) as negative control (**b**) Visual observation of the results on whole blood clots treated with crude extracts of *Bacillus* spp. at 0 h incubation; (**c**) after 18–24 h incubation.

## 14. Discussion

The Himalayan people have been consuming the age-old traditional home-made fermented foods as delicacies, flavoursome and 'foods as medicines' with unclaimed therapeutic uses for many centuries. Sticky and flavoured fermented soybean foods are one of the popular inexpensive and high plant-protein dietary items among the ethnic communities of the Eastern Himalayas. In the current study, naturally fermented soybean foods of the Himalayan region revealed several properties such as antioxidant, anti-inflammatory, anti-obesity, and anti-diabetic effects with production of flavone and isoflavone. Among fermented soybean foods, *kinema* of Bhutan was observed to be highest in antioxidant activity, which indicates that the reduction in alkalinity could be the key factor in increasing the antioxidant activity [45]. Flavone and isoflavone contents were also found in adequate amounts, which may help in increasing antioxidant activity [46] and prevention of oxidative stress that causes human chronic diseases [47]. Fermented soybean foods are the sources of bio-peptides with several health benefits, and the availability of amino acids and significant levels of antioxidant activity could also contribute to the anti-inflammatory

effects [48,49]. A dominance of *Bacillus subtilis* in Himalayan fermented soybean foods has been reported [50], which may prevent obesity by improving transport processes and angiogenesis [51]. Interestingly, anti-diabetic properties have also been observed in some Himalayan fermented soybean foods, and probably delay the progression of type-2 diabetes [52]. A higher abundance of bioactive compounds such as isoflavones and peptides could reduce the impairments associated with diabetes [53]. *B. subtilis* in fermented soybean foods have been proven to have hypoglycaemic effect [53].

In the present study, *Bacillus subtilis*, *B. velezensis*, *B. halotolerans* and *B. inaquosorum* were identified as fibrinolytic enzyme-producing bacteria in samples of kinema, grep-chhurpi, peha, peron namsing, peruñyaan and bemerthu. *B. subtilis* was reported from all samples and exhibited anti-thrombotic activity, which was also reported in other fermented soybean foods [13,28]. Similarly, *B. inaquorum* (previously known as *Bacillus subtilis* subsp. inaquorum) and *B. halotolerans* [54], and *B. velezensis* [55] were also reported to exhibit a fibrinolytic property on fibrin clots. The fibrin degrading properties of *Bacillus* spp. in Himalayan fermented soybean foods were observed, indicating the production of fibrinolytic enzymes [15,34]. All isolates were able to hydrolyse insoluble fibrin, however, B. subtilis Ki52, *B. subtilis* Kn16 and *B. velezensis* F6 released the highest enzyme activities and whole blood clot degradation of more than >50% [34,56]. *Bacillus* spp. are known for their highly proteolytic properties [57] which maintain the alkalinity of the product [12] as well as enhance the presence of free amino acids and antioxidant properties [58,59].

## 15. Conclusions

Himalayan fermented soybean foods have shown antioxidant, anti-inflammatory, anti-diabetic and anti-obesity properties indicating their therapeutic values and health benefits. Himalayan fermented soybean food-origin *Bacillus subtilis*, *B. velezensis*, *B. halotolerans* and *B. inaquosorum* were identified as fibrinolytic enzyme-producing bacteria, imparting anti-thrombotic properties in their products. Further studies on optimization, purification and characterization of fibrinolytic enzymes from food-origin *Bacillus* spp. may be developed to use as a perceptible agent for oral fibrinolytic therapy as well as a functional food supplement.

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