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Isolation, production, and application of fibrinolytic enzyme from fermented food sources: a review

Un análisis de aislamiento, producción y aplicación de la enzima fibrinolítica a partir de alimentos fermentados

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ABSTRACT

The accumulation of the fibrin in bacterial fibrinolytic enzymes finds applications to treat and prevent cardiovascular diseases which fail in hemostasis that leads to the formation of undesirable blood clots in the blood vessels leading to condition called thrombosis. The fibrinolytic enzymes from food grade organisms are useful for thrombolytic therapy. Conventional thrombolytic agents such as streptokinase, nattokinase etc. Nattokinase is one such fibrinolytic enzyme with a wide range of applications in Pharmaceutical industry, health care and medicine etc. Hence, potent blood-clot dissolving protein used for the treatment of cardiovascular diseases is produced by the bacterium Bacillus subtilis during the fermentation of soybeans to produce Natto. The health benefits of some fermented foods are synthesis of nutrients, prevention of cancer, diabetes due to presence of functional microorganisms, which possess probiotics properties, antimicrobial, antioxidant, etc. The first report of fibrinolytic enzyme production of cow dung used as a cheap substrate from Bacillus species in SSF has been given earlier. This review describes different isolation methods which enable the screening and selection of promising organisms for industrial production. The purification and properties of these fibrinolytic proteases is discussed, and the use of fibrinolytic enzyme. In order to obtain Bacillus species producing fibrinolytic enzymes, the fermented food sample such as sprouted grain and processed grain etc were used. The heat tolerant isolates initially were selected for catalase test. Fibrinolytic activity of the selected isolates was determined by using Fibrin plate assay. From the above work, it can be concluded that the fibrinolytic enzyme produced by Bacillus from fermented food samples had the ability to degrade the fibrin and hence can be used for functional food formulation.
Keywords: Fibrin, myocardial infarction, thrombolytic agent, Bacillus, Fibrinolytic activity.

RESUMEN

La acumulación de fibrina en enzimas fibrinolíticas bacterianas encuentra aplicaciones para tratar y prevenir enfermedades cardiovasculares que fallan en la hemostasia que conducen a la formación de coágulos sanguíneos indeseables en los vasos sanguíneos que conducen a una condición llamada trombosis. Las enzimas fibrinolíticas de organismos de calidad alimentaria son útiles para la terapia trombolítica. Agentes trombolíticos convencionales como estreptoquinasa, nattoquinasa, etc. La nattoquinasa es una enzima fibrinolítica con una amplia gama de aplicaciones en la industria farmacéutica, la atención médica y la medicina, etc. la bacteria Bacillus subtilis durante la fermentación de la soja para producir Natto. Los beneficios para la salud de algunos alimentos fermentados son síntesis de nutrientes, prevención del cáncer, diabetes por presencia de microorganismos funcionales, que poseen propiedades probióticas, antimicrobianas, antioxidantes, etc. El primer reporte de producción de enzimas fibrinolíticas de estiércol de vaca utilizado como sustrato barato de especies de Bacillus en SSF. Esta revisión describe diferentes métodos de aislamiento que permiten el cribado y la selección de organismos prometedores para la producción industrial. Se discute la purificación y las propiedades de estas proteasas fibrinolíticas, y el uso de la enzima fibrinolítica. Para obtener especies de Bacillus que produzcan enzimas fibrinolíticas, se utilizó la muestra de alimento fermentado, como el grano germinado y el grano procesado, etc. Los aislados tolerantes al calor se seleccionaron inicialmente para la prueba de catalasa. La actividad fibrinolítica de los aislados seleccionados se determinó usando un ensayo de placa de fibrina. A partir del trabajo anterior, se puede concluir que la enzima fibrinolítica producida por Bacillus a partir de muestras de alimentos fermentados tenía la capacidad de degradar la fibrina y, por lo tanto, puede usarse para la formulación funcional de alimentos.

Palabras clave: fibrina, infarto de miocardio, agente trombolítico, bacilo, actividad fibrinolítica.

INTRODUCTION

Fibrinolytic enzyme is considered as the potent thrombolytic agent to treat and prevent cardiovascular diseases (CVDs) (Mine *et al*., 2005). The commercially available thrombolytic agents such as urokinase plasminogen activator and tissue plasminogen activator are generally safe but are very expensive and based on working mechanism, thrombolytic agents are of two types, one is plasminogen activator which activates plasminogen into active plasmin to degrade fibrin and the other is plasmin like proteins which directly degrade fibrin. Moreover, on the basis of catalytic mechanism, microbial fibrinolytic enzymes are classified into three types, serine protease (eg. Nattokinase), metallo protease (eg. Armillaria mellea metallo protease), mixture of both serine and metallo protease (eg.Protease from Streptomyces). Proteases, also known as peptidyl-peptide hydrolases are industrially useful enzymes which catalyze the hydrolysis of a peptide bond in a protein molecule. However, the bacterial streptokinase is a cheap thrombolytic agent but causes undesirable side effects such as bleeding complications (Banerjee *et al*., 2004).

Hence, searching of a potent thrombolytic agent to prevent and treat CVDs continues, fibrinolytic proteases continue to be attractive as their potent activity on blood clot (He *et al.* 2007). Cardio vascular such as high blood pressure, acute myocardial infarction, vascular heart disease, peripheral vascular disease, arrhythmias, stroke etc. are the primary causes of death. Among these CVDs, thrombosis is one of the most important diseases (Wang *et al.* 2006).

However, these fibrinolytic agents cause allergic reactions, bleeding complications and short half-lives (Blann *et al*., 2002; Bode *et al*., 1996; Turpie *et al*., 2002). Several studies have shown that B. subtilis produces a variety of fibrinolytic enzyme. However, the high price of soy peptone and yeast extract currently used for fibrinolytic enzyme production has restricted its industrial application as a feedstock. Thus, in the study, the use of low-cost and easy-to-obtain nitrogen sources were investigated to reduce the cost of medium. Inexpensive inorganic nitrogen sources and agricultural byproducts have previously been used to replace soy peptone.

In one of the further study, single factor experiments combined with an L9 orthogonal design were performed to improve fibrinolytic enzyme production by B. subtilis WR350, a mutant derived from B. subtilis HQS-34. First, culture medium containing inexpensive CSP was optimized in 250-mL shake flasks. Second, various OS conditions were evaluated for enhancing the production of fibrinolytic enzyme in a 100-L fermenter with the optimized medium. Finally, using the optimized and initial conditions, techno-economic assessments of the processes for fibrinolytic enzyme production were performed. The low-cost medium and suitable OS developed for fibrinolytic enzyme production by aerobic fermentation of B. subtilis WR350 may facilitate process optimization for the economical production of microbial fibrinolytic enzymes at an industrial scale.

Solid-state fermentation (SSF) is defined as the growth of microorganisms on solid materials for the production of biomolecules in the absence or near absence of free water (Pandey *et al.*, 2000). SSF is a useful technique for utilization of low-cost agro residues in large volumes in biosynthesis of enzymes and metabolites. The agro industrial wastes such as pigeon pea (Johnvesly *et al*., 2002), green gram husk (Prakasham *et al*., 2006), potato peel (Mukherjee *et al*., 2008), sesame oil cake (Rajendran and Thangavelu, 2013), and ground nut husk (Salihu *et al*., 2014) were recently used as the substrate for the production of hydrolytic enzymes.

The ideal agro-wastes for enzyme production in SSF process mainly depend on the cost and the availability of the substrate material (Pandey *et al*., 2000).

Cow dung is one of such nutritive-rich feed stocks that was unexploited for the production of fibrinolytic enzymes by Bacillus sp. Cow dung contains ash, nitrogen, carbon, cellulose, hemicelluloses, magnesium, manganese, calcium, zinc, and trace elements (Fulhage *et al*., 2000). Cow dung manure is rich in carbon and nitrogen, which indicated that it could be a promising feedstock for the growth of microbes (Adegunloye *et al.*, 2007).

In recent years, cow dung was used as the substrate for the production of proteolytic enzymes from Halomonas sp. PV1 and Bacillus sp. and fibrinolytic enzymes from Shewanella sp. IND20 (Vijayaraghavan and Vincent *et al.*, 2015). Unlike other solid substrates, cow dung has high moisture-holding capacity, which was preferred by the bacterial species for their growth and production of bimolecular (Vijayaraghavan and Vincent *et al.*, 2015). Although cow dung was utilized for the production of proteolytic enzymes from Bacillus sp., the production of fibrinolytic enzyme from the genus Bacillus using cow dung substrate has not yet been reported. Considering the nutrient compositions, availability, and cheap cost, cow dung was used as the substrate for the production of fibrinolytic enzyme from the newly isolated Bacillus sp. IND7, and the process parameters were optimized. In addition, the in vitro clot lytic activity of this enzyme was evaluated.

Existing scientific data show many fermented foods have both nutritive and non-nutritive components in foods, which have the potential to modulate specific target functions in the body relevant to well-being and health of the consumers. However, 90% of naturally fermented foods and alcoholic beverages in different countries and regions of the world are still at home production under traditional conditions. Naturally fermented foods and beverages contain both functional and non-functional microorganisms (Tamang *et al*., 2016).

Functional microorganisms transform the chemical constituents of raw materials of plant/animal sources during food fermentation thereby enhancing the bio-availability of nutrients, enriching sensory quality of the food, imparting bio-preservative effects and improvement of food safety, degrading toxic components and anti-nutritive factors, producing antioxidant and antimicrobial compounds, stimulating the probiotic functions, and fortifying with some health-promoting bioactive compounds (Tamang *et al.*, 2009, 2016; Farhad *et al.*, 2010; Bourdichon *et al*., 2012; Thapa and Tamang *et al.*, 2015).

Functional properties of microorganisms in fermented foods include probiotics properties (Hill *et al.*, 2014), antimicrobial properties (Meira *et al*., 2012), antioxidant (Perna *et al*., 2013), peptide production (De Mejia and Dia *et al*., 2010), fibrinolytic activity (Kotb *et al*., 2012), poly-glutamic acid (Chettri and Tamang *et al*., 2014), degradation of antinutritive compounds (Babalola *et al*., 2014), etc. which may be important criteria for selection of starter culture(s) to be used in the manufacture of functional foods (Badis *et al*., 2004). The present paper is aimed to review the information on some functional properties of the microorganisms associated with fermented foods and beverages, and their health-promoting benefits to consumers.

TABLE-1: Microorganisms used as commercial starter in fermentation

Group	Genera/species	Product/application(s)
Bacteria	Acetobacter aceti subsp. Aceti	Vinegar
	A. pasteurianus subsp. Pasteurianus	Vinegar, cocoa
	Bacilllus acidopullulyticus	Pullulanases (food additive)
	B. licheniformis	Protease (food additive)
	B. subtilis	Fermented soybeans, protease, glycolipids, riboflavin-B_2 (food additive)
	Bifidobacterium animalis subsp. lactis, B. breve	Fermented milks with probiotic properties; common in European fermented milks
	Corynebacterium ammoniagenes	Cheese ripening
	Enterobacter aerogenes	Bread fermentation
	Enterococcus durans	Cheese and sourdough fermentation
	E. faecium	Soybean, dairy, meat, vegetables
	L. acidophilus	Fermented milks, probiotics, vegetables
	L. brevis	Bread fermentation; wine; dairy
	L. buchneri	Malolactic fermentation in wine; sourdough
	L. fermentum	Fermented milks, sourdough, urease (food additive)
	L. ghanensis	Cocoa
	L. helveticus	Starter for cheese; cheese ripening, vegetables
	L. hilgardii	Malolactic fermentation of wine
	L. kimchii	Kimchi
	L. oeni	Wine
	L. pentosus	Meat fermentation and biopreservation of meat; green table olives; dairy, fruits, wine
	L. salivarious subsp. Salivarius	Cheese fermentation
	Lactococcus lactis subsp. Lactis	Dairy starter, Nisin (protective culture)
	Oenococcus oeni	Malolactic fermentation of wine
	Propionibacterium acidipropionici	Meat fermentation and biopreservation of meat
	P. arabinosum	Cheese fermentation; probiotics
	P. freudenreichii subsp. Freudenreichii	Cheese fermentation (Emmental cheese starter)
	Streptococcus natalensis	Natamycin (food additive)

Probiotic Microorganisms

Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (Hill *et al*., 2014). Probiotic organisms used in foods must have the ability to resist gastric juices, exposure to bile, and be able to proliferate and colonize the digestive tract (Saad *et al*., 2013). The beneficial effects of probiotic foods on human health and nutrition are constantly increasing (de LeBlanc *et al.*, 2007; Monteagudo-Mera *et al*., 2012), and probiotics are popularly using bio-ingredients in many functional fermented foods (Chávarri *et al*., 2010). The most commonly used probiotic bacteria belong to the heterogeneous

group of LAB (Lactobacillus, Enterococcus) and to the genus Bifidobacterium, however, yeasts and other microbes have also been developed as potential probiotics during recent years (Ouwehand *et al*., 2002). Products containing probiotic bacteria generally include foods and supplements (Varankovich *et al*., 2015). Fermented milk products are the most traditional source of probiotic strains of lactobacilli (Bernardeau *et al*., 2006; Shah, 2015); however, commercial probiotic lactobacilli have also been added to meat products, snacks, fruit juice, etc. (Ranadheera *et al*., 2010). Probiotic properties of Lactobacillus plantarum isolated from kimchi, Korean fermented vegetable product, has been reported (Ji *et al*., 2013), and is also found to prevent the growth of Helicobacter pylori (Lim and Im, 2009). Probiotic strain L. acidophilus La-5 produces conjugated linoleic acid (CLA), an anti-carcinogenic agent (Macouzet *et al*., 2009).

Antimicrobial Properties

Many species of LAB isolated from fermented vegetable and milk products have antimicrobial activities due to production of antimicrobial compounds such as bacteriocin and nisin (Tamang *et al*., 2009; Khan *et al*., 2010; Gaggia *et al*., 2011; Jiang *et al*., 2012; Grosu-Tudor and Zamfir, 2013). Many strains of LAB isolated from kimchi produce antimicrobial compounds such as bacteriocin by L. lactis BH5 (Hur ***et al***., 2000) and L. citreum GJ7 (Chang *et al*., 2008), and pediocin by P. pentosaceus (Shin *et al*., 2008). Species of LAB isolated from kimchi show strong antimicrobial activity against Listeria monocytogenes, Staphylococcus aureus, E. coli, and Salmonella typhimurium (Lee *et al*., 2009). Weissella cibaria isolated from fermented cabbage product shows antimicrobial activity against Gram-positive and Gram-negative pathogens (Patel *et al*., 2014). Lactococcus lactis isolated from dahi, Indian curd, produces nisin Z that inhibits L. monocytogenes and S. aureus (Mitra *et al*., 2010). Several LAB species isolated from Romanian traditional fermented fruits and vegetables have antimicrobial activity against L. monocytogenes, E. coli, Salmonella, and Bacillus (Grosu-Tudor and Zamfir, 2013). Microorganisms as protective cultures, e.g., bacteriocin producers, may have several advantages, as they can contribute to the flavor, texture and nutritional value of the product besides the production of bacteriocin (Gaggia *et al*., 2011).

Antioxidant Activity

Antioxidant activities in fermented foods include 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging activity, 2,2'-azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid; ABTS) radical scavenging activity, total phenol content (TPC) estimation, and reducing power assay (Liu and Pan, 2010; Abubakr *et al*., 2012). Many Asian fermented soybean foods have antioxidant properties, e.g., natto, Bacillus-fermented soybean food of Japan (Ping *et al*., 2012), chungkokjang and jang, fermented soybean foods of Korea (Shon *et al*., 2007; Shin and Jeong, 2015), douchi, a fermented soybean food of China (Wang *et al*., 2007a), kinema, Bacillus-fermented soybean food of India and Nepal (Moktan *et al*., 2008; Tamang, 2015), bekang and tungrymbai, Bacillus-fermented soybean foods of India (Chettri and Tamang, 2014), thua nao, Bacillus-fermented soybean food of Thailand (Dajanta *et al*., 2013), and tempe mold-fermented soybean food of Indonesia (Nurrahman *et al*., 2013). Antioxidant activities have also been observed in kimchi (Park *et al*., 2011) and yogurt (Sabeena *et al*., 2010).

Production of Enzymes by Microorganisms

Another important reason to ferment foods is to coax microorganisms into producing enzymes that also provide very useful services. During food fermentation microorganisms produce enzymes to break down complex compounds to simple bio-molecules for several biological activities such as proteinase, amylase, mannase, cellulase, and catalase in many Asian fermented soybean foods by Bacillus spp. (Tamang and Nikkuni, 1996; Chettri and Tamang, 2014). Common genera of mycelial fungi in fermented foods and beverages such as Actinomucor, Amylomyces, Aspergillus, Monascus, Mucor, Neurospora, and Rhizopus produce various carbohydrases such as α-amylase, amyloglucosidase, maltase, invertase, pectinase, ß-galactosidase, cellulase, hemi-cellulase; acid and

alkaline proteases; and lipases (Nout and Aidoo, 2002). Taka-amylase A (TAA), an enzyme produced by Aspergillus oryzae in koji has many uses in industry (Suganuma *et al*., 2007). Dry, solid, cake-like mixed amylolytic starters used for alcohol production in the Himalayas have yeasts Saccharomycopsis fibuligera, S. capsularis and Pichia burtonii with high amylase activities (Tsuyoshi *et al*., 2005; Tamang *et al*., 2007).Bacillus subtilis subsp. natto in natto produces nattokinase showing fibrinolytic activity (Mine *et al*., 2005; Kotb, 2012). Among bacteria isolated from fermented foods, B. subtilis and B. amyloliquefaciens (Chang *et al*., 2012; Zeng *et al*., 2013; Singh *et al*., 2014), Vagococcus carniphilus, V. lutrae, Enterococcus faecalis, E. faecium, E. gallinarum, and P. acidilactici (Singh *et al*., 2014), and Virgibacillus halodenitrificans SK1-3-7 isolated from fish sauce fermentation (Montriwong *et al*., 2012) produce fibrinolytic enzymes.

Prevention of Hypertension and Heart Disease

Antihypertensive properties of many fermented milk products have been validated using animal models and clinical trials (Seppo *et al*., 2002; Sipola *et al*., 2002). Consumption of some fermented foods reduces the cholesterol level in temp (Hermosilla *et al*., 1993), fermented soybean foods (Lee, 2004), and kefir (Otes and Cagindi, 2003). Monascus purpureus in fermented red-rice of China locally called angkak, prohibits creation of cholesterol by blocking a key enzyme, HMG-CoA reductase due to presence of mevinolin citrinin (Pattanagul *et al*., 2008). Drinking of fermented tea of China prevents heart disease (Mo *et al*., 2008). Some Asian fermented soybean foods have antihypertensive properties as observed in natto (Nagai, 2015) and tempe (Astuti, 2015). Isoflavone in doenjang, mold-fermented soybean food of Korea, plays an important role in preventing cardiovascular diseases (Kwak *et al*., 2012; Shin *et al*., 2015). Fermented whole-grain intake appears to protect from development of heart disease and diabetes (Anderson, 2003). Moderate consumption of wine is healthier (Walker, 2014). Polyphenols in red wine probably are synergists of the tocopherol (Vitamin E) and ascorbic acid (Vitamin C), thus they inhibit lipid peroxidation (Feher *et al*., 2007).Fermented foods, which are rich in fibrinolytic enzymes, are useful for thrombolytic therapy to prevent rapidly emerging heart diseases (Mine *et al*., 2005; Singh *et al*., 2014).

Prevention from Cancer

Some LAB-fermented foods have antimutagenic and anticarcinogeinc activities (Lee *et al*., 2004). Kefir is used for the treatment of cancer (Otes and Cagindi, 2003; Yanping *et al*., 2009). Sauerkraut, fermented vegetable of Germany, contains s-methylmethionine, which reduces tumourigenesis risk in the stomach (Kris-Etherton *et al*., 2002). Similarly, Indian dahi has anti-carcinogenic property (Mohania *et al*., 2013). Cancer preventive potential of W. cibaria, and L. plantarum has been reported in kimchi (Kwak *et al*., 2014).

Protection from Diabetes and Osteoporosis-

Intake of high fiber foods may decrease the insulin requirements in diabetic persons (Meyer *et al*., 2000), and may increase the sensitivity to insulin for non-diabetic persons (Fukagawa *et al*., 1990; Anderson, 2003). Probiotic dahi-supplemented diet significantly delays the glucose intolerance, hyperglycemia, hyperinsulinemia, oxidative stress and dyslipidemia indicating a lower risk of diabetes (Yadav *et al*., 2007). Daily consumption of chungkokjang may increase the insulin resistivity thus controls diabetics (Shin *et al*., 2011; Tolhurst *et al*., 2012).Vitamin K2 present in natto stimulates the formation of bone, which may help to prevent osteoporosis in older women in Japan (Yanagisawa and Sumi *et al*., 2005).

Health Benefits of Fermented Foods

Ethnic foods have in-built systems both as foods and medicine to meet up hungry and also curative (Shin and Jeong, 2015; Thapa and Tamang, 2015). The highest longevity observed among the people of Okinawa

prefecture in Japan is mostly due to their traditional and cultural foods such as natto, miso, tofu, shoyu, fermented vegetables, cholesterol-free, low-fat, and high bioactive-compounded foods in addition to active physical activity, sound environment, happiness and other several factors (Willcox *et al*., 2004). Korean kimchi has been claimed to possess health-promoting benefits (Cheigh, 1999; Lee *et al*., 2011; Park *et al*., 2014; Han *et al*., 2015). Kimchi has also anti-aging effect (Kim *et al*., 2002). Natto has several health benefits such as high contents of nattokinase, isoflavones, saponins, vitamin K, unsaturated fatty acids, probiotics and immunomodulating activities mostly produced by B. subtilis (natto; Tsubura *et al*., 2012; Nagai *et al*., 2015). Kinema has also some health promoting benefits (Omizu *et al*., 2011; Tamang, 2015). Indian popular fermented milk dahi has anti-carcinogenic property (Arvind *et al*., 2010). Lactic acid produced in kimchi may prevent fat accumulation and to improve obesity-induced heart diseases (Park *et al*., 2008). Anti-obesity effects have been reported in kimchi (Kim *et al*., 2011; Park *et al*., 2012) and in doenjang (Kwak *et al*., 2012) based on clinical trials (Cha *et al*., 2012; Jung *et al*., 2014).

TABLE-2: Bioactive compounds in fermented foods and their health benefits.

Bioactive compounds	Synthesized in fermented foods	Health benefits	Reference
Genistein	Doenjang	Facilitates the β-oxidation of fatty acid, reducing body weight	Kwak *et al*., 2012
Lipoteichoic acid from L. rhamnosus GG	Fermented milk	Oral photoprotective agent against UV-induced carcinogenesis	Weill *et al*., 2013
Isocyanate and sulphide indole-3-carbinol	Kimchi	Prevention of cancer, detoxification of heavy metals in liver, kidney, and small intestine	Kwak *et al*., 2014
Ornithine	-	Anti-obesity efficacy	Park *et al*., 2012
Vitamin A, Vitamin C, fibers	-	Suppression of cancer cells	Han *et al*., 2015
Capsaicin, Allicin	-	Prevention of cancer, suppression of Helicobacter pylori	Lim and Im, 2009
Chlorophyll	-	Helps in prevention of absorbing carcinogen	Ferruzzi and Blakeslee, 2007
S-adenosyl-L-methionine (SAM)	-	Treatment of depression	Lee and Lee, 2009
HDMPPA (an antioxidant)	-	Therapeutic application in human atherosclerosis	Kim *et al*., 2007
Nattokinase, antibiotics, Vitamin K	Natto	Antitumor, immunomodulating	Nagai, 2015
Vitamin C	Sauerkraut	Scurvy	Peñas *et al*., 2013
Glucosinolates	-	Activation of natural antioxidant enzymes	Martinez-Villaluenga *et al*., 2012
Antioxidant genestein, daidzein, tocopherol, superoxide dismutase	Tempe	Prevents oxidative stress causing non-communicable disease such as hyperlipidemia, diabetes, cancer (breast and colon), prevents the damage of pancreatic beta cell	Astuti, 2015

Phenolics- resveratrol	Wine (red)	Anti inflammatory	Jeong *et al*., 2010
Phenolics, succinic acid	-	Digestive aid	Jackson, 2008
Phenolics, resveratrol, flavonoids – quercitin, Vitamins C and E, mineral selenium	-	Prevent cardiovascular diseases, reduce incidence of heart attacks and mortality rate	Walker, 2014
Melatonin, resveratrol	-	Antioxidant and anti-aging property	Fernández-Mar *et al*., 2012
Resveratol	-	Anti-diabetic	Ramadori *et al*., 2009

Health Risk of Fermented Foods

One of the important health risks in fermented foods is presence of biogenic amines. Biogenic amines are low molecular weight organic compounds by microbial decarboxylation of their precursor amino acids or by transamination of aldehydes and ketones by amino acid transaminases (Zhai *et al*., 2012), which are present in some fermented foods such as sauerkraut, fish products, cheese, wine, beer, dry sausages, etc. (Halász *et al*., 1994; Suzzi and Gardini, 2003; Spano *et al*., 2010; Visciano *et al*., 2014). Enterobacteriaceae and enterococci are major biogenic amine producers in foods (Nout, 1994). Foods with high levels of biogenic amines could be considered as unhealthy (Latorre-Moratalla *et al*., 2010). Fermentation of cabbage with certain lactic starters such as L. casei subsp. casei, L. plantarum and L. curvatus could reduce the biogenic amine content of sauerkraut (Rabie *et al*., 2011).

Biotechnological applications of fibrinolytic enzymes

The Bacillus species have a huge potential to secrete variety of proteases and most of these have the ability to degrade fibrin. Potent fibrinolytic enzyme have been expressed in other Bacillus spp. (Xiao *et al*., 2004) and E. coli (Kho *et al*., 2005) for ease of purification, better yield, and in lactic acid bacterial system (Liang *et al*., 2007) for possible starter cultures. Further, these fibrinolytic enzymes can be encapsulated in nano-capsules for higher stability and easy oral applications. (Law and Zhang *et al*., 2006) have encapsulated NKCP in Shellac and about 60% retention in the enzyme activity after encapsulation. Shellac particles showed low permeability to acid. (Ko *et al*., 2008) prepared alginate microparticles to assess the effect on fibrinolytic enzymes of Korean fermented soybean paste. (Wei *et al*. 2012) have produced nattokinase from B. subtilis LSSE- 22 on chickpeas as substrate and employed ethanol for extraction and precipitation of Nattokinase. Methacrylic acid –thylacrylate copolymer was used to encapsulate the nattokinase and to increase the stability at acidic pH. A variety of fibrinolytic enzymes have been reported from genus Bacillus that differ in their properties like molecular weight and substrate specificity and have the potential to become cost effective and orally administrable, direct acting thrombolytic agents. Experimental results on the effect of orally administered NK on canine (Sumi *et al*., 1990) and rat models (Suzuki *et al*., 2003) as well as human trails (Sumi *et al*., 1989; Omura *et al*., 2004) indicated their efficacy and safety and hence, NK has been commercially produced. These includes NSK-SD™, Cardiokinase, Natto-K, Nattokinase NSK-SD, Orokinase, Nattozyme, Best – Nattokinase, Nattokinase- plus, Serracor-NK and Nattobiotic. Genome sequence of Bacillus subtilis168 indicates the presence of genes which codes for several peptidases and proteases (Kunst *et al*., 1997). Thus, genomic comparison of related bacterial may help in analysing and exploration of new fibrinolytic enzymes. The N-terminal sequence of many fibrinolytic enzymes has showed many conserved amino acid sequences. However, significant changes are observed in their properties.

Kimchi

The unique geographical location of Korea and the isolation from neighboring countries imposed by rugged mountains from the north and rocky ocean from the east, south, and west, largely contributed to the development of a distinct ethnic group with unique culture. This simplicity is also reflected in the food habits. A fundamental aspect of this culture has been the preservation of fish, meat, pulses, and vegetables from times of abundance to times of scarcity through lactic acid fermentation; a process applied for more than 1500 years (Han *et al*., 1998; Surh *et al*., 2008; Oh *et al*., 2014). Intake of a specific dose of fruits and vegetables in the daily food to prevent different types of chronic pathologies and diseases such as coronary heart problems, hypertension, and risk of strokes has been recommended by the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) (Swain *et al*., 2014). The process of fermentation scientific technology has been developed in order to preserve different types of fruits and vegetables by organic acid and alcohols during their harvesting season and use them at the time of scarcity. Fermented foods not only provide important sources of nutrients but have also great potential in maintaining health and preventing diseases along with the addition of desirable flavor, texture, reduction of toxicity, and decrease in cooking time (Rolle and Satin, 2002; Kabak and Dobson, 2011). Generally, the fermentation process is the slow breakdown of organic substances that is prompted by a group of microorganisms or enzymes and results in the alteration of carbohydrates to organic acids or alcohols (FAO, 1998). A number of lactic acid bacteria (LAB) including Lactobacillus brevis, Lb. fermentum, Lb. plantarum, Leuconostoc mesenteroides, Weissella confusa and Pediococcus pentosaceus are regularly retrieved and have been widely used in the fermentation process (Jung *et al*., 2014). A number of fermented food products including cereal-based fermented food and non-alcoholic beverages, fermented milks, fermented fruits and vegetables and fermented meat products etc. have been consumed in most parts of the world (Kabak and Dobson *et al*., 2011). In the present review, all available literature on the afore mentioned Korean fermented foods with particular focus on scientific research regarding preparation, processing, structure of the microecosystem and health benefits of kimchi are integrated and critically reviewed.

Fermented Food of Korea

There are numerous fermented foods and beverages, which are the essential element of the Korean cuisine and are consumed by the Koreans as well as many people around the world throughout the year. The major fermented food items, except the alcoholic beverages that are consumed nowadays in Korea, are basically divided into three broad categories (Surh *et al*., 2008).

1. The soy-based products, that includes chongkukjang (quick fermented soybean paste), doenjang (soybean paste), ganjang (soy sauce), and gochujang (hot pepper-soybean paste) (Surh *et al*., 2008). Traditionally, these types of fermented products are prepared once in a year, stored in large clay pots and consumed throughout the year.

- Chongkukjang is a fermented product manufactured by short term fermentation of boiled soybean seeds using Bacillus subtilis in rice straw. It is one of the favorite traditional foods in Korea (Su *et al*., 2007; Kwon *et al*., 2011; Shin and Jeong, 2015). It contains a number of useful microorganisms and bioactive compounds that are absent from unfermented soybean products. Chongkukjang has the shortest fermentation period of 2–4 days and is fermented at a high (40–43°C). The soybean proteins are degraded during fermentation process by the protein degrading enzymes of B. subtilis, and flavonoid glycosides are converted into aglycones by hydrolysis during fermentation, resulting in production of free amino acids along with related peptides (Nakajima *et al*., 2005; Kim N. Y. *et al*., Wei *et al*., 2015). The Koreans have been consuming Chongkukjang for hundreds of years. Significant amount of data suggests that Chongkukjang contains a number of proteins and minerals that can stimulate the generation and growth of human cells and strengthen the immune system (Choi *et al*., 2014; Shin and Jeong, 2015). Moreover, there are several reports on the bioactive potential of Chongkukjang such as antidiabetic, antiinflammatory, antimicrobial, antioxidant, blood pressure lowering activities, and neuroprotective

effects (Kang *et al*., 1998; Cho *et al*., 2000; Yang *et al*., 2003; Kim *et al*., 2004; Kim N. Y. *et al*., 2008; Wei *et al*., 2015).

- Doenjang is traditionally used in Korea as a basic seasoning (Kim *et al*., 2009; Nam *et al*., 2015). It is produced by the fermentation of cooked and crushed soybean seeds or blocks, Meju, by naturally occurring bacteria and fungi with brine in a container such as a porcelain pot and has been consumed for centuries as a source of protein and flavoring ingredient in Korea (Kim *et al*., 2009; Kwon *et al*., 2010). During recent times, the doenjang has been prepared commercially by various local firms around the Korean peninsula using a slightly modified procedure that varies in the quality as affected by the fermentation process, microbiota involved, and by the basic ingredients used, such as soybeans or a combination of soybeans and grains (Yoo *et al*., 2000; Park *et al*., 2002). Doenjang has attracted much attention due to its health-related beneficial properties such as anticancer, anti-mutagenicity, antioxidative, and fibrinolytic activity (Lim *et al*., 1999; Ra *et al*., 2004).A number of reports have stated that B. subtilis and B. licheniformis are the dominant microorganisms in doenjang along with Aspergillus, Mucor, and Rhizopus species (Kang *et al*., 2000; Nam *et al*., 2015). Daily intake of doenjang has been reported to suppress the body weight gain, cytokine levels, and serum oxidative stress high-fat-fed mice (Nam *et al*., 2015). Similarly, the anti-inflammatory and anti-oxidative stress effects of doenjang have also been reported in the adipose tissue (Nam *et al*., 2015).
- Ganjang is a kind of Korean soybean sauce made from fermented soybeans (Hong-beum, 2004). It contains approximately 15–20% salt, 50–70% water, free sugars, isoflavones, peptides, and organic acids that are produced from the soybeans during the fermentation process (Jeon *et al*., 2002; Shim *et al*., 2008). The sauce has a characteristic black color due to the presence of melanoidins, which are formed when carbonyl compounds and amino compounds combine together (Kim *et al*., 2008).The melanoidins present in soybean sauce are responsible for its antioxidant potential (Choi *et al*., 1990). Ganjang is prepared from the soybeans blocks, meju, which is dried for about 1 week and then tied with straw and dried for another 40 days. After the meju have dried, they are then fermented in a specially made clay pot mixed with salt and water. When the fermentation is complete, dark liquid separates, which is called ganjang (soy sauce or soya sauce) (Hong-beum *et al*., 2004).
- Gochujang is a fermented paste made of red chili powder, glutinous rice powder, pureed soybeens and salt, seasonings like garlic and onion, sweetened with a little sugar syrup and fermented for long period in specially designed earthen vessels (Choi *et al*., 2012). It is an essential part of the Korean cuisine and is used in almost all the Korean foods like bibimbap, noodles etc. It is a basic ingredient for other sauses and pastes, it is mixed with the doenjang to make samjang, it is used to prepare the chogochujang, salad dressing etc. (Choi *et al*., 2012).

2. The popularly consumption that is prepared from fish and shellfish. These products are consumed as such or are combined with kimchi (Surh *et al*., 2008). Fish, shellfish and their products provide a healthy source of essential vitamins, high quality proteins, minerals, and polyunsaturated fatty acids (Prester *et al*., 2011).

3. The kimchi, which is most widely and popularly consumed not only in Korean peninsula but around the world. It is a major Korean traditional fermented food. Kimchi is prepared from the Chinese cabbage (Brassica rapa L. spp. pekinensis Han) and/or radish as its main ingredient, along with different kind of vegetables (Surh *et al*., 2008). The fermentation process is completed within short period of time.

Sources of fibrin (ogen)olytic enzyme(s)- Fibrinolytic enzymes are widely found in nature. Among the variety of fibrinolytic enzyme sources, microorganism and snake venom are considered as natural sources of fibrinolytic enzymes. Besides, a variety of resources such as earthworm, algae, and insects have been screen for fibrino(geno)lytic enzymes. In recent years, many fibrinolytic enzyme producing microbes have been extracted from both food and non-food sources.

Fibrinolytic enzymes producing food borne microbes- The genus Bacillus isolated from traditional fermented foods is an important group of microorganisms that has been found as a prominent candidate for fibrinolytic protease producer. For example, nattokinase (NK) produced by Bacillus natto was the first screened fibrinolytic enzyme from a traditional Japanese soybean-fermented food named natto. Subsequently, other species of Bacilli found in different fermented foods have been characterized and identified as fibrinolytic enzyme producers. Moreover, traditional fermented foods consumed all over the world have been considered as excellent source of fibrinolytic enzyme producing microbes, which are generally regarded as safe (GRAS) category. Different species of fibrinolytic enzyme, producing microbes isolated from traditional foods are summarized in Table-1.

TABLE-3: Fibrinolytic enzyme producing microbes isolated from traditional foods Microorganism

Microorganism	Fibrinolytic enzyme	Food	Reference
Bacillus natto	Nattokinase	Natto (fermented soybean), Japan	Sumi *et al*.
Bacillus sp.	SMCE	Tofuyo (coagulating fermented soy juice), Japan	Fujita *et al*.
Bacillus sp.	Bacillus protease	Kimchi (fermented vegetables with seasonings),	Noh *et al*.
Bacillus subtilis IMR-NK1	Fibrinolytic enzyme	Natto	Chang *et al*.
Bacillus sp. DJ-4	Subtilisin DJ-4	Doen-jang (fermented soybean), Korea	Kim and Choi
Bacillus amyloliquefaciens DC-4	Subtilisin DFE	Douchi (fermented soybean), China	Peng *et al*.
Bacillus subtilis QK02	QK-1 and QK-2	Fermented soybean	Ko *et al*.
Bacillus firmus NA-1	N.A.	Natto	Seo and Lee
Bacillus sp. DJ-2	Subtilisin DJ-2	Doen-jang	Choi *et al*.
Bacillus subtilis TP-6	TPase	Tempeh (fermented soybean), Indonesia	Kim *et al*.
Bacillus subtilis DC33	Subtilisin FS33	Ba-bao Douchi	Wang *et al*.
Bacillus vallismortis Ace02	Ace-02	Chungkook-jang	Kim *et al*.
Flammulina velutipes	FVP-1b	Local culture collection	Park *et al*.
Bacillus subtilis LD-8547	LD-8547	Douchi	Wang *et al*.
Schizophyllum commune	Cultured mycelia of mushroom	Bioresource Collection and Research Center (Taiwan)	Lu *et al*.

MATERIAL AND METHODS

Screening of Fibrinolytic Enzyme- Producing Bacterial Strains from Fermented Rice

Screening of organism producing fibrinolytic enzyme

About 1 g fermented rice was mixed with 100 ml distilled water. Sample was taken from it and screened for organisms showing proteolytic activity using skimmed milk agar plates. Ten bacterial cultures, which showed clear zone in the casein agar plates, were further screened for fibrinolytic enzyme producing ability. The protease positive isolates were cultured in a medium containing peptone (3.0%), glucose (1.0%), $CaCl_2$ (0.50%) and $MgSO_4$ (0.20%). The pH of culture medium was brought to 7.0, and incubated at 37 °C for 72 h at 150 rpm (Gad *et al*. 2014). After 72 h of incubation, the bacterial biomass was separated by centrifugation (8000 rpm, 10 min, and 4

°C) and the extract devoid of cells was used to screen fibrinolytic enzyme activity. Fibrin plate assay was used to assess the fibrinolytic activity of the extracts. The fibrinolytic protease activity appeared as a halo zone around the fibrin clot after incubation at 37 °C for a period of 5 h.

Primary Screening

Cooked rice was used as the sample source to screen protease enzyme-producing bacterial strains. Rice was boiled in drinking water for 1 h approximately. After that, the boiled rice was allowed under aerobic fermentation at room temperature (30 ± 2°C) for 48–72 h. sampling was made twice in each experimental trial (after 48–72 h fermentation). This procedure was repeated five times. About 1.0 g of cooked rice was suspended in 99 ml of double-distilled water and serially diluted (10-1–10-7) using sterile double-distilled water and plated on skimmed milk agar plates (g/l; agar 15, skim milk 10, peptone 5, yeast extract 5, and NaCl 1.5 (g/l). These plates were incubated at 37°C for 24–72 h. The protease-producing bacterial isolate shows a clear zone on skimmed milk plates. In each trial, one organism was selected and further screened (secondary screening) for fibrinolytic enzyme production.

Secondary Screening

The selected five protease-producing bacterial isolates were cultured in the liquid medium composed of casein 10, yeast extract 5, peptone 5, and NaCl 1.5 (g/l). The culture medium was sterilized at 121°C for 20 min. Then, a loopful culture of the selected isolates was individually inoculated. Fermentation was carried out in 250-ml Erlenmeyer flasks, and these were kept on an orbital shaker (175 rpm) for 48 h at 37°C. After 48 h, all cultures were centrifuged at 10,000 rpm for 10 min, and the clear supernatant was used as the crude enzyme for determination of fibrinolytic activity on fibrin–agarose plates. The fibrin–agarose plate was made with 1% (w/v) agarose, 50 µl thrombin (100 NIH U/ml), and 0.5% (w/v) fibrinogen. These were mixed immediately and allowed to stand for 1 h at room temperature (30°C ± 2°C) to form a fibrin clot. Then 15 µl of enzyme from the bacterial isolates was dropped individually into the wells and incubated for 5 h at room temperature (30°C ± 2°C), and the lytic halo zone was measured (Astrup and Müllertz *et al*., 1952). The strain that showed the largest halo zone on the fibrin plate was selected for further studies. This selected strain was subjected to morphological and biochemical characteristics and 16S rDNA sequencing.

Bacterial strain

The B. subtilis strain WR350 used in this study was previously generated in our laboratory via UV mutagenesis of B. subtilis HQS-324, which produced the same fibrinolytic enzyme as strain HQS-324, and resulted in a significant improvement in fibrinolytic enzyme production. The previously described procedures for obtaining B. subtilis WR350 were as follows. A culture of B. subtilis HQS-3 was spread onto a preliminary screening agar plate containing 15 g/L casein and other essential nutrients. The plate was subsequently exposed to a germicidal lamp for 0–3 min, and the resulting mutant colonies were further screened on a Luria Bertani (LB) agar plate supplemented with 0.5 g/L fibrinogen and 0.5 U/mL thrombin. The stable mutant B. subtilis strain WR350 with the highest observed fibrinolytic activity was obtained and stored in equal volumes of LB broth and 50% (v/v) glycerol at −80 °C.

Substrate

The agro-wastes such as banana peel, cow dung, wheat straw, wheat bran, rice bran, rice straw, and green gram husk were collected locally and dried under sunlight for 7 days. These dried agro-wastes were powdered using a mixer grinder and used as the substrate.

Fibrinolytic enzyme assay

The enzyme (0.1 ml) was mixed with 2.5 ml of tris–HCl buffer (pH 7.8) containing calcium chloride (0.01 M). Fibrinolytic activity was assayed on fibrin substrate. The absorbance was read at 275 nm (Anson 1938; Chang *et al*. 2000). Enzyme activity was calculated based on the calibration curve drawn for standard solution of L-Tyrosine. One unit of fibrinolytic enzyme activity (U) was defined as the amount of enzyme which liberates 1 µg of tyrosine per min under the standard assay condition. The results of the determination of fibrinolytic activity were described in units of activity/gram of substrate (U/g).

Fibrinolytic activity assay

Fibrinolytic enzyme activity was determined with the method described by Astrup and Mullertz25 using plasminogen-rich fibrin plates. Urokinase, which catalyzed the generation of plasmin from plasminogen, was used as a control. The dimension of the clear zone on each fibrin plate was measured, and fibrinolytic activity was calculated as per the standard curve generated using the control, where urokinase concentration (20–120 U/mL) was plotted against the transparent zone area.

In Vitro Blood Clot Lytic Activity of Fibrinolytic Enzyme

About 0.5-ml aliquots of goat blood were collected from the slaughter house at Nagercoil, India and allowed to form a clot. The clot was washed thrice with phosphate-buffered saline and incubated with crude fibrinolytic enzyme (120 µg). Streptokinase was used as a positive control, and phosphate-buffered saline was used as the negative control. All tubes were incubated at room temperature (30°C ± 2°C) for 6 h, and the results were observed for every 30 min.

DISCUSSION

In the study, a fibrinolytic enzyme-producing bacterium was screened and isolated from cooked rice sample. Fibrinolytic enzyme screening from various sources, such as Japanese shiokara (Sumi *et al*., 1995), Indonesian tempeh (Kim *et al*., 2006), and fermented red bean (Chang *et al*., 2012), has been carried out. The bacterial fibrinolytic enzymes are considered as the safe thrombolytic agent, and the administration of these fibrinolytic agents upon oral administration could increase fibrinolytic activity in human plasma (Sumi *et al*., 1990). Hence, the studies on bacterial fibrinolytic enzyme, especially from the genus Bacillus, could be useful to develop potent thrombolytic agents. Enzymes can be produced by submerged fermentation (SmF) and SSF. SSF has many advantages over SmF (Pandey *et al*., 2000). Hence, in the study, SSF was performed the production of fibrinolytic enzyme. In SSF, many substrates have used for the production of fibrinolytic enzyme. Comparing the reported agro residues, cow dung substrate is cheap. Cow dung medium may be considered as a promise and in expensive substrate for fibrinolytic enzyme production. Cow dung contains high amounts of nutrients (Misra *et al*., 2003). It is rich in total organic carbon (42.5%), nitrogen (0.65%), phosphorus (0.5%), potassium (0.125%), sodium (0.138%), calcium, iron, copper, zinc and cadmium (Yadav *et al*., 2013). So, the bacteria can be grown on this medium for the production of fibrinolytic enzymes. Banana peel is rich of organic matter (91.5 ± 0.05%), carbohydrate (59 ± 1.36%), crude fiber (31.7 ± 0.25%), crude lipid (1.7 ± 0.1) and low amount of crude protein (0.9 ± 0.25%) (Anhwange *et al*., 2009). Rice straw contains less amount of protein (0.469%), fiber (3.289%), cellulose (4.374%), hemicelluloses (1.368%) and traces of sodium, potassium, calcium, phosphorus, and magnesium (Akinfemi and Ogunwole, 2012). Wheat straw is rich in cellulose (33.7%), hemicellulose (21%), lignin (11%), fiber (54%), protein (3.6%), calcium (0.18%) and phosphorus (0.05%) (Yasin *et al*., 2010). Rice bran contains more quantity of protein (16.16%), total lipids (17.87%), carbohydrates (33.24%) and traces of calcium, iron, sodium, zinc, and potassium (Faria *et al*., 2012). Considering the cheap cost and its availability, cow dung could spearhead enzyme bioprocess in an industrial scale. Reports on fibrinolytic enzyme production using cow dung substrate in SSF from Bacillus sp. are

limited or perhaps not available. The first report on the production of fibrinolytic enzyme from Bacillus sp. IND7 using cow dung substrate in SSF. Hence, the present study is useful to utilize the cow dung substrate for the production of fibrinolytic enzyme by Bacillus sp. The agroresidues such as rice bran and wheat bran are considered as waste, but these substrates are useful to make feed for aquatic organisms and poultry (Vijayaraghavan and Vincent *et al*., 2012). In SSF, moisture content is one of the critical factors where cow dung substrate has high moisture-holding capacity, which facilitates the production of fibrinolytic enzyme from bacterial species.

Optimization of pH and nutrient content in any bioprocess is primarily important to develop efficient process. Initially, the pH of the medium and the nutrient components were optimized by one-variable-at-a-time approach. Fibrinolytic enzyme production was maximum after 72 h of incubation, at pH 9.0, and with 90% moisture level. The fermentation period requirement of the strain IND7 for the maximum fibrinolytic activity is comparable with that of different fibrinolytic enzyme-producing bacteria. In the present study, optimum pH for enhanced production of fibrinolytic enzyme was found to be 9.0, and fibrinolytic activity was 2717.2 ± 79.2 U/g.

Hence, the pH of culture medium was maintained as 8.0 for further studies. In SSF, moisture is one of the critical factors that strongly influence the enzyme yield. The optimum moisture content for enzyme production could vary depends on the organism and substrate used in SSF process (Prakasham *et al*., 2006). In the study, various carbon and nitrogen sources were used for enhanced production of fibrinolytic enzyme. Results revealed that starch favors maximum fibrinolytic enzyme production, followed by sucrose. However, very little studies were reported to use RSM to optimize the fibrinolytic enzyme production in SSF. To the best of our knowledge, this is the first study that deals the optimization of medium components for the production of fibrinolytic enzyme from Bacillus sp. in SSF by statistical method.

CONCLUSION

In the present study, the enhanced production of fibrinolytic enzyme from Bacillus sp. IND7 was achieved using cow dung under SSF. The interactions of pH, starch, and beef extract were investigated by RSM. This clearly implies that enhanced production of fibrinolytic enzyme can be achieved by simply adjusting the pH of solid medium of the cow dung substrate. These kinds of studies help to utilize cow dung substrate in enzyme bioprocess. In vitro clot lysis revealed its activity on blood clot of thrombolytic diseases is today a major cause of morbidity and mortality worldwide. Microbial fibrinolytic enzymes have apparent significance in thrombosis therapy. Therefore great attention has been directed towards a search for microbial thrombolytic agents of various origins with particular reference to agents with more specificity and less toxicity. This review information helps in isolation of promising microbial fibrinolytic enzymes producers for industrial production, optimization, purification, and characterization.With the available information on fibrinolytic enzymes and as a future prospective, research need to be focussed on exploring novel fibrinolytic enzymes. A huge diversity is seen in species of Bacillus with their fibrinolytic enzymes. The detailed understanding of their sequences and properties may contribute to the development of novel affordable and safer thrombolytic agents to address the limitations of conventional thrombolytic such as cardiovascular diseases affects not only individual health but also the quality of life due to their high cost of treatment. The food-grade Bacillus spp. with the ability to produce fibrinolytic enzymes can be used for the formulation of functional foods and their enzyme preparation may become an alternative for conventional fibrinolytic molecules

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