CHAPTER 2  
REVIEW OF LITERATURE

2.1 FIBRIN FORMATION

Coagulation or clotting is the process by which blood changes from a liquid to a gel. It potentially results in hemostasis, the cessation of blood loss from a damaged vessel, followed by repair. The mechanism of coagulation involves activation, adhesion, and aggregation of platelets along with deposition and maturation of fibrin. Disorders of coagulation are disease states which can result in bleeding (hemorrhage or bruising) or obstructive clotting (thrombosis) [24].

Hemostasis is the body's way of stopping injured blood vessels from bleeding. Hemostasis includes clotting of the blood. Too much clotting can block blood vessels that are not bleeding. Consequently, the body has control mechanisms to limit clotting and dissolve clots that are no longer needed. An abnormality in any part of this system that controls bleeding can lead to excessive bleeding or excessive clotting, both of which can be dangerous [24]. When clotting is poor, even a slight injury to a blood vessel may lead to severe blood loss. When clotting is excessive, small blood vessels in critical places can become clogged with clots. Clogged vessels in the brain can cause strokes, and clogged vessels leading to the heart can cause heart attacks. Pieces of clots from veins in the legs, pelvis, or abdomen can travel through the bloodstream to the lungs and block major arteries there (pulmonary embolism) [24].

Hemostasis involves three major processes: Narrowing (constriction) of blood vessels; Activity of cell-like blood particles that help in blood clotting.
(platelets); Activity of proteins found in blood that work with platelets to help the blood clot (clotting factors) [3].

An injured blood vessel constricts so that blood flows out more slowly and clotting can start. At the same time, the accumulating pool of blood outside the blood vessel (a hematoma) presses against the vessel, helping prevent further bleeding. As soon as a blood vessel wall is damaged, a series of reactions activates platelets so that they stick to the injured area. As platelets accumulate at the site, they form a mesh that plugs the injury. The platelets change shape from round to spiny, and they release proteins and other substances that entrap more platelets and clotting proteins in the enlarging plug that becomes a blood clot [3].

Formation of a clot also involves activation of a sequence of blood clotting factors that generate thrombin. Thrombin converts fibrinogen, a blood clotting factor that is normally dissolved in blood, into long strands of fibrin that radiate from the clumped platelets and form a net that entraps more platelets and blood cells. The fibrin strands add bulk to the developing clot and help hold it in place to keep the vessel wall plugged. The reactions that result in the formation of a blood clot are balanced by other reactions that stop the clotting process and dissolve clots after the blood vessel has healed. Without this control system, minor blood vessel injuries could trigger widespread clotting throughout the body, which actually happens in some diseases [25].

The coagulation cascade of secondary hemostasis has two initial pathways which lead to fibrin formation. These are the contact activation pathway (also known as the intrinsic pathway), and the tissue factor pathway (also known as the extrinsic pathway) which both lead to the same fundamental reactions that produce fibrin. It was previously thought that the two pathways of coagulation cascade were of equal importance, but it is now known that the primary pathway for the initiation of blood coagulation is the tissue factor pathway. The pathways are a series of reactions, in which a zymogen (inactive enzyme precursor) of a serine protease and its glyco-protein co-factor are activated to become active components that then catalyze the next reaction in the cascade, ultimately resulting
in cross-linked fibrin. The coagulation factors are generally serine proteases (enzymes), which act by cleaving downstream proteins. There are some exceptions. For example, FVIII and FV are glycoproteins and Factor XIII is a transglutaminase. The coagulation factors circulate as inactive zymogens. The coagulation cascade is therefore classically divided into three pathways. The tissue factor and contact activation pathways both activate the "final common pathway" of factor X, thrombin and fibrin [25].

After Trauma or injury the body forms a protein called Fibrin, which is very essential to stop blood loss. There are more than twenty enzymes in the body that help in the clotting cascade of blood while, the only enzyme in the body that can break down the clot is the plasmin. Excess cross linked fibrin is also caused by the inflammatory conditions, which is triggered by Bacteria, viruses, fungi and toxins present in the blood. Since there is no danger of blood loss and trauma has not occurred, this cross-linked fibrin will circulate through the blood and will stick to the walls of blood vessels. This contributes to the formation of blood clots, slows blood flow and increases blood viscosity contributing to the elevation of blood pressure. The blockages in the heart is caused by the blood clots which partially cuts down the oxygen supply (ischemia) to the tissue and blocks the blood flow to the heart muscle tissue which results in angina and heart attacks, also death of heart muscle (necrosis) if prolonged. Senility/ Stroke are also resulted when the clots in the chambers of the heart mobilise to the brain, blocking blood and oxygen from reaching necessary areas [26].

2.2 FIBRINOLYSIS

High blood pressure is known to be one of the most important risk factors for cardiovascular disease (CVD) and it was shown that the reduction of highly or moderately elevated blood pressure levels resulted in decreased rates of stroke and myocardial infarction in 1993 and 2002 [27,28]. Furthermore, Iseki et al reported that hypertension is a significant predictor of stroke, acute myocardial infarction and end-stage renal disease [29]. Since the incidence of CVD in Asia is very low, traditional foods from Asia have been the subject of increased attention
recently [30,31]. For more than 1,000 years, people throughout Asia have consumed soybeans in a variety of traditional soy food products [32]. Hypertension has become a global public health challenge, affecting approximately 50 million individuals in the United States and one billion individuals worldwide [33,34]. The prevalence of hypertension has increased dramatically in developing countries in recent decades [35]. According to Choi et al [36], the prevalence of hypertension was 31.6% in the Korean population.

According to World Health Organization statistics, about 17.5 million people died from CVDs in 2005, which represented 30% of all global deaths. Almost 20 million people will die from CVDs mainly from heart disease and stroke, by 2015. One of the main causes of CVDs is the intravascular thrombosis due to fibrin aggregation in arteries. Plasminogen activators, such as tPA (tissue-type plasminogen activator), urokinase and streptokinase are the major thrombolytic agents. All these agents have undesirable side effects, and are also relatively expensive, despite their widespread use [37]. The world health organization (WHO) has reported that about 17 million people die of CVDs each year. [11], therefore, research is underway to develop cheaper and safer antithrombotic agents from various sources. During blood clot formation, fibrinogen is converted into fibrin via the proteolytic action of thrombin, this result in the formation of insoluble fibrin clots [38]. Over 80% of CVD deaths take place in disproportionally affected low & middle income countries & occur almost equally in men and women. The number of people, who die from CVDs, mainly from heart disease and stroke, will increase to reach 23.3 million by 2030. CVDs are projected to remain the single leading cause of death [39].

Although fibrin clot formation and fibrinolysis are maintained in balance by the biological system, thromboses such as myocardial infarction, occur when clots are not lysed as a result of a disorder in the balance. Blood clots are formed from fibrinogen by thrombin (EC 3.4.21.5) and are lysed by plasmin (EC 3.4.21.7), which is activated from plasminogen by tissue plasminogen activator (tPA). Intravenous administration of urokinase and streptokinase was widely used for thrombosis therapy but these enzymes have low specificity for fibrin. tPA was
developed for the treatment of thrombosis because of its efficiency and strong affinity for fibrin [40].

Accounts of cardiovascular diseases have become the leading cause of death in the Western world [41]. Many blood clot-dissolving agents, such as urokinase, streptokinase, and tissue plasminogen activator (t-PA), have been utilized in clinical treatments for cardiovascular diseases. Hemostasis is a complex process obtained through an optimal balance between bleeding and blood clot formation. The fibrin clots may not be lysed in an unbalanced state resulting in thrombosis. Thrombolytic agents from various sources have been extensively investigated. The major thrombolytic agents are classified into two types. One is plasminogen activators, such as urokinase, tPA (tissue type plasminogen activator), and streptokinase, which activate plasminogen to plasmin. The other type is plasmin-like proteins, such as fibrolase from snake venom and lumbrokinase [20] from earthworm, which can directly degrade the fibrin. However, these enzymes are often expensive, thermolabile and can produce undesirable side effects [42].

Streptokinase is currently used in clinical medicine as a therapeutic agent in the treatment of thromboembolic blockages, including coronary thrombosis [43,44]. Streptokinase is extracted from certain strains of beta hemolytic streptococcus, an extra cellular protein, is a non-protease plasminogen activator that activates plasminogen to plasmin - the enzyme that degrades fibrin cloth through its specific lysine binding site; it is used therefore as a drug in thrombolytic therapy [45]. Streptokinase (SK) a group of extracellular proteins produced by a variety of streptococci beta-hemolytic strains and is a plasminogen activator composed of 414 amino acids with a molecular mass of 47 kDa. Unlike urokinase or tissue-type plasminogen activator that performs direct proteolysis, SK forms a high affinity equimolar complex with a plasminogen [46].

Urokinase (UK) is a serine protease, which specifically cleaves the proenzyme/zymogen plasminogen to form the active enzyme plasmin. It specifically catalyzes the cleavage of the Arg-Val bond in plasminogen. The active plasmin is then able to break down the fibrin polymers of blood clots. Clinically,
UK is given to patients suffering from thrombolytic disorders. Among the plasminogen activators, UK provides a superior alternative for the simple reasons of its being more potent as compared to tissue-plasminogen activator and nonantigenic by virtue of its human origin unlike streptokinase. Based on these observations, UK is a strong plasminogen activator. Hence, UK, as one of the most potent plasminogen activators is attracting a great deal of attention [47]. Urokinase (UK) is given to patients suffering from thrombolytic disorders like deep vein thrombosis, thrombosis of the eye, pulmonary embolism, and myocardial infarction. This enzyme is a strong plasminogen activator which specifically cleaves the proenzyme/zymogen plasminogen to form the active enzyme plasmin [48].

In recent years, fibrinolytic enzymes from various sources, including microorganisms, worms, and animals, have been the subject of active researches because of their potential as novel agents in preventing or treating CVDs by dissolving fibrin blood clot [49,50]. Oral administration of the enzyme showed that it could enhance fibrinolytic activity in plasma and the production of tPA, and its fibrinolytic activity was retained in the blood for more than 3 h. These results suggest that nattokinase may be a potent natural agent for oral thrombolytic therapy. Subsequently, many fibrinolytic enzymes were identified in different traditional fermented foods, such as CK of Bacillus sp. strain CK 11-4 from Chungkook-Jang [51] and subtilisin DJ-4 of Bacillus sp. DJ-4 from Doenjiang [52] in Korea, a fibrinolytic enzyme of B. subtilis IMR-NK1 from Taiwanese soil [53], subtilisin DFE of B. amyloliquefaciens DC-4 from douchi in China [54] and a subtilisin-like fibrinolytic protease of B. subtilis TP-6 from Tempeh in Indonesia [55].

Thrombolytic enzymes are normally generated in the endothelial cells of the blood vessels, helps in breaking down blood clots. The production of Thrombolytic enzymes start declining as the body ages & makes body more prone to coagulation, leading to several conditions including cardiac or cerebral infarction. The poor production of thrombolytic enzymes can result in development of blood clots & the conditions caused by them anywhere in the body.
including arteries, veins, & lymphatic system as endothelial cells exist throughout the body [5].

It has recently been revealed that thrombotic clogging (blood clots) of the cerebral blood vessels may be a cause of dementia. It has been estimated that sixty percent of senile dementia patients in Japan is caused by thrombus. Thrombotic diseases typically include cerebral hemorrhage, cerebral infarction, cardiac infarction and angina pectoris, and also include diseases caused by blood vessels with lowered flexibility, including senile dementia and diabetes. If chronic diseases of the capillaries are also considered, then the number of thrombus related conditions might be much higher. Cardiac infarction patients may have an inherent imbalance. Their thrombolytic enzymes are weaker than their coagulant enzymes. Nattokinase holds great promise to support patients with such inherent weaknesses in a convenient and consistent manner, without side effects. [4,5,26].

The search for thrombolytic agents from other biological sources were necessary and important as the enzymes already available in the market had undesirable side effects in which patients suffered from gastrointestinal bleeding and allergic reactions. Recently, fibrinolytic enzymes have been discovered in fermented food products such as Japanese natto [37], Korean Chungkook-Jang soy sauce [56] and fermented shrimp paste [57].

2.3 FIBRINOLYTIC ENZYME (NATTOKINASE)

Dr H Sumi, a researcher of the Japan Ministry of Education and majoring in the physiological chemistry at the blood laboratory of the University of Chicago, had long researched thrombolytic enzymes. He was searching for a natural agent that could successfully dissolve thrombus associated with cardiac and cerebral infarction (blood clots associated with heart attacks and stroke). In 1980 while he was eating natto for lunch, he dropped a small portion into the artificial thrombus (fibrin) plate. The natto gradually dissolved the thrombus and had completely resolved it in 18 h. Dr. Sumi found that the sticky part of natto, commonly called "threads" exhibited a strong fibrinolytic (blood clot busting)
activity. He named the corresponding fibrinolytic enzyme, nattokinase. Dr. Sumi commented that nattokinase showed a potency matched by no other enzyme [5,26].

Dr. Sumi found that natto had the highest fibrinolytic (blood clot busting) activity among all 200 kinds of food he conducted the research from all over the world. There are many traditional foods for the prevention and treatment of thrombosis (e.g., azuki beans, Korean ginseng, and Japanese water dropwort) but most of these foods inhibit platelet aggregation similar to aspirin. Only nattokinase acts only on the fibrinolytic system to dissolve thrombi within the blood vessels [26]. In 1986, Dr. Sumi presented the results of his research in Japan for the first time at the Japan Agricultural Chemistry Society. Later, he wrote a similar article for the International Thrombolytic Association where the audience began to believe that the dietary intake of natto was the major contributor to the longevity of Japanese people. Nattokinase has been the subject of 17 studies, including two small human trials. Dr. Sumi and his colleagues induced blood clots in male dogs and orally administered either four capsules of Nattokinase (250 mg per capsule) or four placebo capsules to each dog. Angiograms revealed that the dogs who received nattokinase regained normal blood circulation (free of the clot) within five hours of treatment. Blood clots in the dogs who received only placebo showed no sign of dissolving in the 18 hours following treatment. [26,58].

Subtilisin nattokinase (NAT) (formerly designated Subtilisin BSP), produced by *Bacillus subtilis* natto, is a serine protease and is reported to have potent fibrinolytic activity [59]. Besides *in vitro* tests of fibrinolytic activity, many *in vivo* studies had been reported [15]. Fujita *et al* (1995) treated dogs with nattokinase by oral administration and the fibrinolytic activity in plasma increased and showed that subtilisin NAT could pass the rat intestinal tract and dissolve the chemically induced thrombosis [60]. The enzyme not only strongly hydrolyzes thrombi in vivo, but also converts plasminogen to plasmin [61]. Suzuki *et al* (2003) found that dietary supplementation of natto suppressed intimal thickening and modulated the lysis of mural thrombi after endothelial injury in rat femoral artery [62]. Sumi *et al* (1990) also reported a similar effect of dietary *Bacillus natto* productive protein on *in vivo* endogenous thrombolysis [15].
In 1995, researchers from Miyazaki Medical College and Kurashiki University of Science and Arts in Japan studied the effects of nattokinase on blood pressure in both animal and human subjects. In addition, the researchers confirmed the presence of inhibitors of angiotensin converting enzyme (ACE) within the test extract, which consisted of 80% ethanol extract of lyophilized viscous materials of natto. ACE causes blood vessels to narrow and blood pressure to rise by inhibiting ACE; nattokinase has a lowering effect on blood pressure. [5,26]. The same natto extract was then tested on human volunteers with high blood pressure. Blood pressure levels were measured after 30 grams of lyophilized extract (equivalent to 200 g of natto food) was administered orally for 4 consecutive days. In 4 out of 5 volunteers, the systolic blood pressure (SBP) decreased on average from 173.8 mmHg to 154.8 mmHg. Diastolic blood pressure (DBP) decreased on average from 101.0 mmHg to 91.2 mmHg. On average, this data represents a 10.9 percent drop in SBP and a 9.7 percent drop in DBP [4,5,26].

Fibrinolytic enzymes such as Nattokinase used as thrombolytic agent but too costly and also used through intravenous instillation, needs large scale production by some alternative methods and high purity [63]. Nattokinase supports healthy blood flow and circulation [64]. Nattokinase can hydrolyze fibrin in blood clots as fibrinogen acts as a very good substrate which hastens the production in the media [11]. Nattokinase may contribute to the regular healthy function of the heart and cardiovascular system by maintaining proper blood flow, thinning the blood and preventing blood clots [63]. Nattokinase produces a prolonged action in two ways by preventing the formation of thrombi & dissolving existing thrombus. Nattokinase orally administrated to twelve healthy adults indicated elevations of the breakdown products of the fibrin and the ability of the blood to breakdown fibrin called euglobulin fibrinolytic activity (EFA) suggest the ability of Nattokinase to accelerate fibrinolysis in the blood for a prolonged period of time. After the administration of Nattokinase FDP level in adults drastically increased in just 4 h indicating that fibrin within the blood vessels is gradually being dissolved with repeated intake of nattokinase.
By measuring EFA & FDP levels, the activity of nattokinase has been determined to last from 8 to 12 h. An additional parameter for confirming the action of Nattokinase following oral administration is a rise in blood levels of tissue plasminogen activator (TPA) antigen, which indicates a release of TPA from the endothelial cells and/or the liver and the endogenous production of plasmin (the body’s blood clotting buster) [4,5].

Nakamura et al [59] reported that the NK gene encodes a polypeptide with a 29 amino acids signal peptide, 77 amino acids propeptide, and whose mature peptide is 275 amino acids long. Sequence analysis showed that the nucleotide sequence of the NK gene is nearly identical to that of subtilisin E and subtilisin amylosacchariticus (only 13 and 27 nucleotide differences, out of the 1473 nucleotides sequenced, respectively). The primary structure of the NK mature peptide was 99.5% similar to that of mature subtilisin E and 99.3% similar to that of subtilisin amylosacchariticus. In spite of this near identity, only NK shows high substrate specificity for fibrin. The N-terminal 24 amino acid residues of subtilisin DFE were sequenced [54], and the sequence was almost identical (only one amino acid change) to that of subtilisin BPN, an extracellular serine protease from B. amyloliquefaciens.

Researchers from JCR Pharmaceuticals, Oklahoma State University and Miyazaki Medical College tested nattokinase on 12 healthy Japanese volunteers (6 men and 6 women, between the ages of 21 and 55). They gave the volunteers 200 g of natto (the food) before breakfast and then tracked fibrinolytic activity through a series of blood plasma tests. The tests indicated that the natto generated a heightened ability to dissolve blood clots. On average, the volunteers’ ELT (a measure of how long it takes to dissolve a blood clot) dropped by 48% within 2 h of treatment and volunteers retained an enhanced ability to dissolve blood clots for 2 to 8 h. As a control, researchers later fed the same amount of boiled soybeans to the same volunteers and tracked their fibrinolytic activity. The tests showed no significant change [4,26,58].
Fibrinolytic enzymes can be found in a variety of foods, such as Japanese Natto, Tofuyo, Korean soy sauce and edible honey mushroom. Fibrinolytic enzymes have been purified from these foods and their physiochemical properties have been characterized. A popular Asian seasoning, Fermented shrimp paste, was shown to have strong fibrinolytic activity. These novel fibrinolytic enzymes derived from traditional Asian foods are useful for thrombolytic therapy. Since large quantities of enzyme can be conveniently & efficiently be produced, they will efficiently provide an adjunct to the costly fibrinolytic enzymes that are currently used in managing heart disease. In addition, these enzymes have significant potential for food fortification and nutraceutical applications, such that their use could effectively prevent cardiovascular diseases [65].

Similar fibrinolytic enzyme producing bacteria have also been isolated from Japanese shiokara and Chinese douchi [43]. Nevertheless, it is still the most stable and economic way to obtain protein with fibrinolytic activity by B. subtilis natto. On the basis of its food origin, relatively strong fibrinolytic activity, stability in the gastrointestinal tract, and convenient oral administration, subtilisin NAT has advantages for commercially used medicine for preventative and prolonged effects [66]. Many fibrinolytic enzymes have been isolated from various foods such as Korean Chungkook-Jang [51], Chinese douchi [54], soybean grits [67] and Indonesian tempeh [68]. Endophytic bacteria such as Paenibacillus produce biotechnologically important enzymes. The genus Paenibacillus was created by Ash et al [69] to accommodate the “group 3” of the genus Bacillus. Fibrinolytic enzymes were identified and studied among many organisms including snakes, earthworms, and bacteria: Streptococcus pyogenes, Aeromonas hydrophila, Serratia E15, B. natto, Bacillus amyloliquefaciens, Actinomycetes and fungi: Fusarium oxysporum; Mucor sp, Armillaria mellea [70].

Isolation, production, purification, assay and characterization of fibrinolytic enzymes from bacterial sources are therefore, very effective and useful. In the future, the research will progress into the production of highly purified fibrinolytic enzymes from bacterial sources [53]. Other fibrinolytic enzymes from different traditional foods have since been discovered, such as katsuwokinase from
the traditional fermented Japanese food skipjack shiokara [71] and subtilisin CK and DJ-4 from the fermented food DoenJang [52]. The enzyme has a high substrate specificity for fibrin, can efficiently hydrolyze the thrombi in vitro and does not disrupt blood cells [72], suggesting that it could also be used as an oral thrombolytic agent.

Oral administration of the fibrinolytic enzyme Nattokinase (NK) [4], revealed to be the same as subtilisin NAT [59] and which is produced from Bacillus NAT in the traditional Japanese fermented food, Natto, has been reported to enhance fibrinolytic activity in plasma and the production of tPA [15]. A fibrinolytic enzyme produced from Bacillus subtilis has also been reported [73], but it did not show the same level of plasminogen activator activity as does NK.

The most popular soy foods in Western countries are tofu, soy milk (tonyu), soy burger, soy sauce, and miso. Natto, a traditional fermented vegetable cheese-like food, is another soy product. Natto extracts are known to include nattokinase (NK, formerly designated BSP, or subtilisin NAT, which is a serine proteinase from Bacillus subtilis natto), a potent fibrinolytic enzyme having an approximately 4-fold stronger activity than plasmin in clot lysis assays [61,74]. In addition, it has a direct effect on thrombus cleavage, the mechanism by which this enzyme potentiates fibrinolysis of its reactive site [75].

Mushroom extracts are widely used as nutritional supplements and medicines, with claimed human health benefits [76]. In the East Mediterranean countries, Ganoderma species are regarded as the herb of longevity. These fungi have been used in folk medicine for hundreds of years and stains are commercially cultivated for preparation of health tablets [77]. Medicinal benefits of Ganoderma spp have been reviewed by Jong and Birmongham [78]. Six novel triterpenoids, i.e. ganoderenic acid, furano ganodermic acid, ganoderic acid derivatives, were isolated from the fruit body of the fungus Ganoderma applanatum [79]. Choi and Sa had reported the presence of fibrinolytic enzyme from a Chinese isolate [80].

Bacillus species produce a variety of extracellular and intracellular proteases. An alkaline protease (subtilisin), a neutral metalloprotease, and an
esterase are secreted into media, whereas at least two intracellular serine proteases are produced within Bacillus spp. [81,82,83,84,85]. In particular, the production of subtilisin protease has been exploited commercially for use in laundry detergents and for other applications [86,87]. The usage of protease for thrombolytic therapy by oral administration has been assessed [18,19,88].

The catalytic centre of nattokinase, with 275 amino acids, contains three conserved residues, Asp-32, His-64, and Ser-221 [89], while the molecular mass and isoelectric point were 27.7 kDa and 8.7, respectively [61]. Its gene sequence is homologous to those of other members of subtilisin family (99.5% homology with subtilisin E, 86% with subtilisin BPN0 and 72% with subtilisin Carlsberg) [59]. Nattokinase has greater thrombolytic activity than plasmin [53,60,61], a natural thrombolytic protease in blood, and increases the production of plasmin from plasminogen due to its action on plasminogen activator.

Moreover, very few studies were reported on statistical optimization of fibrinolytic enzymes production in solid-state fermentation (SSF). Tao et al [90] optimized process parameters for the production of fibrinolytic enzymes by Fusarium oxysporum. Compared to submerged fermentation, SSF yields more enzyme and it could reduce the production cost of the enzyme. From an industrial point of view, around 30–40% of the production cost of enzymes is estimated to account the cost of the growth medium [91]. Therefore, optimization of the fermentation process parameters in SSF through a statistical approach is important for a significant improvement in yield as well as a decrease in the production cost of the enzyme. The selection of medium components is another critical factor for the production of fibrinolytic enzymes because each microbe requires unique nutrient components and environmental conditions for its growth and the production of fibrinolytic enzymes [67,92].

The traditional one-at-a-time optimization strategy is simple, but it fails to locate the region of optimal response because the comprehensive effect of factors is not taken into consideration for the production of fibrinolytic enzymes [93]. The statistical experimental design provides an universal language with which
experts from different areas such as academia, engineering, business, and industry can communicate for setting, performing, and analyzing experiments for research. Statistically designed experiments are more effective than other classical one-at-a-time optimization strategy because it can study many variables simultaneously with a low number of observations, saving time, and costs [94,95]. The statistical method such as factorial design, central composite design and response surface methodology (RSM) were frequently used to optimize the process parameters for the production of antimicrobial metabolites [96], bio-surfactants [97], and fibrinolytic enzymes [93, 98].

Solid-state fermentation (SSF) has emerged as a potential technology for the production of pharmaceutically significant products. Many agro-industrial residues were used for the production of these products using SSF. The agro-industrial residues such as pigeon pea, green gram husk, potato peel, and wheat bran were widely used for the production of proteases [99,100,101,102]. Utilization of these agro-industrial residues as substrates in SSF processes provides an alternate avenue and value addition. Product’s yield is mostly higher using SSF when compared with submerged fermentation. Selection of an appropriate substrate is another key aspect of SSF [102]. SSF was employed for the production of fibrinolytic enzymes with few solid substrates (rice chaff and Fusarium oxysporum [90] and Bacillus firmus NA-1 and soybean grits [67]. Response surface methodology (RSM) has been used widely for the production of various enzymes, including polygalacturonase [103], arginine deiminase [104], α-amylase [105], acid protease [106], and β-galactosidase [107]. However, there are few studies on the optimization of medium components for microbes to produce fibrinolytic enzymes via two-level full factorial design and RSM [108].

2.4 CLINICAL STUDIES

The relationship between drugs and the body's ability to control bleeding (hemostasis) is complicated. The body's ability to form blood clots is vital to hemostasis, but too much clotting increases the risk of a heart attack, stroke, or pulmonary embolism. Many drugs, either intentionally or unintentionally, affect the
body's ability to form blood clots. Some people are at high risk of forming blood clots and are intentionally given drugs to decrease the risk [3]. Drugs may be given that reduce the stickiness of platelets, so that they will not clump together to block a blood vessel. Aspirin, ticlopidine, clopidogrel, abciximab, and tirofiban are examples of drugs that interfere with the activity of platelets. Other people at risk of forming blood clots may be given an anticoagulant, a drug that inhibits the action of blood proteins called clotting factors. Although often called "blood thinners," anticoagulants do not really thin the blood. Commonly used anticoagulants are Warfarin-taken by mouth, and heparin- given by injection. People who take these drugs must be under close medical supervision [1].

Doctors monitor the effects of most of these drugs with blood tests that measure the time it takes for a clot to form, and they adjust the dose on the basis of test results. Doses that are too low may not prevent clots, while doses that are too high may cause severe bleeding. Another type of anticoagulant, low-molecular-weight heparin, does not require as much supervision. Lepirudin, bivalirudin, and argatroban are newer types of anticoagulants that directly act on thrombin, a potent protein that induces clotting. If a person already has a blood clot, a thrombolytic (fibrinolytic) drug can be given to help dissolve the clot. Thrombolytic drugs, which include streptokinase and tissue plasminogen activators, are sometimes used to treat heart attacks and strokes caused by blood clots. These drugs may save lives, but they can also put the person at risk of severe bleeding. Surprisingly, heparin, a drug given to reduce the risk of clot formation, sometimes has an unintended activating effect on platelets that increases the risk of clotting (heparin-induced thrombocytopenia). Estrogen, alone or in oral contraceptives, can have the unintended effect of causing excessive clot formation. Certain drugs used to treat cancer (chemotherapy drugs), such as asparaginase, can also increase the risk of clotting [109].

Some doctors in Japan started prescribing natto instead of warfarin on an experimental basis. Some patients with retinal-vein-blockage-disease, a disease causing blood clots to occur in retinal veins and haemorrhaging in the retina, were instructed to eat natto twice a week, and had very positive results [1]. Eating raw
natto gives the best protection since nattokinase enzyme is sensitive to heat and loses its effectiveness above 70 degrees C. Lecithin and linoleum acid are rich in soybeans contributes in purifying the blood. Soybeans' protein preserves the elasticity of blood vessels, and prevents coronary heart disease, brain apoplexy and high blood pressure as a result. It follows that these typical adult diseases may be prevented or improved, if natto made from soybeans is eaten as a staple food [110].

In 1989, Harvard University's research on 20,000 male American doctors concluded that one aspirin a day reduces heart failure due to blot clotting by 44%. However, recent research says that eating soybean products every day has the same effect. Aspirin tends to make blood easily soluble, and it is known to cause bleeding even from a healthy stomach. According to a Japanese joint research by the Ministry of Health and Gifu Medical University on 1,242 male and 3,596 female subjects from Takayama City's 31,000 residents, the more the subjects eat soybean products, the lower their cholesterol levels. A Japanese research reported that the level of K2 is low in the people with osteoporosis and high in the people without osteoporosis; although, the former and the latter have the same level of Vitamin K1 in their blood. It is statistically recognized that the regions with higher natto consumptions have lower rates of bone breakage. Japanese researchers credit natto for preventing osteoporosis [111].

According to Dr. Dean Ornish, Director of the Preventive Medicine Research Institute in Sausalito, California, the instances of prostate cancer in Japanese males is one quarter of their American counterparts [110]. However, for Japanese males who moved to America, the rate of prostate cancer abruptly rises. The reason for the low occurrence of cancer in Japanese males living in Japan is thought to be due to the high consumption of soybean products instead of animal products. This happens due to the anti-carcinogen effects of phytoestrogen and one type of flavanoid pigmentation ingredient called infrabin present in soybeans. Dr. Amy S. Lee of the University of Southern California's School of Medicine reported one type of isoflavone called genistein, present in soybeans in large quantity, slowed down the reproduction rate of cancer cells in mice [110]. Dr. Lee says that Asians consume 20 to 30 times as many soybeans as Americans, and it is thought that there is a reverse relationship between the instances of cancer of the breast, skin, large intestine, and prostate gland and the consumption of soybeans.
The Cancer Research Center in Hawaii reported in 1997 that genistein, daidzein and other types of isoflavones present in soybeans, were shown to effectively prevent uterine cancer. Other research has shown their effectiveness against kidney and breast cancer. In addition soybeans contain selenium that is an anti-cancer mineral. They also contain edible fibers that cleanse the intestines. Edible fibers are said to be effective in preventing large intestine cancer. There is also a report that not only soybeans, but also natto bacteria themselves have an anti-carcinogenic effect. According to the September 25, 1996 edition of the Daily Sports Newspaper in Japan, "A research conducted on mice by Professor Yukio Kameda of Kanazawa University, showed mice injected with natto bacteria either showed absolutely no growth of cancer cells, or slowed the growth rate to less than half the norm when implanted with carcinogenic protein [110]." No matter how you look at it, if you eat soybeans food products, especially natto, frequently, it is very effective in the prevention of various cancers.

Most of the bacteria beneficial to the intestines such as bifidus are killed in the stomach by the acid before they reach the intestines if taken orally. But natto bacteria are able to survive the journey and reproduce in the intestines where they aid digestion. A large amount of cellulose present in soybeans, which in combination with oligosaccharide the natto bacteria produce, help beneficial microbes such as bifidus to reproduce. The dietary fibers also help getting rid of waste materials and carcinogens. But modern Japanese eat an average dietary fiber of 17 g per day, falling short of the recommended amount of between 20 and 25 g. One hundred g of natto contains seven g of dietary fibers [110].

Natto is rich in Vitamin E and other forms of vitamins. Vitamin E is antioxidant and aids circulation of blood in the periphery vessels. Therefore, it prevents skin from damage and keeps it young. Other clinically thrombolytic agents, such as urokinase and streptokinase, are costly and unstable in the intestinal tract [64]. The use of oral administration of nattokinase in fibrinolytic therapy for thrombosis and prevention of atherosclerosis is therefore of interest. Accordingly, nattokinase is currently used as a nutrient supplement to improve the blood circulation in humans [26,63]. Although many bacteria, actinomycetes, algae, and fungi have been found to have fibrin-digestion abilities, the genus Bacillus, important GRAS strains from traditional fermented foods, could produce a high
yield of fibrinolytic enzymes for further commercial application [25]. These observations, together with the fact that it can be absorbed across the intestinal tract after oral administration [61,63] and subsequently induce fibrinolysis [63], make nattokinase to be a potential clot-dissolving agent for the treatment of cardiovascular disease. Because nattokinase suppresses the intimal thickening of arteries and leads to the lysis of mural thrombi observed after endothelial injury, the dietary supplementation with nattokinase-related foods has been considered to be safe and healthful for circulation system in human body [26].

2.5 MARKET STATUS

Urokinase, an enzyme extracted from urine, is being used as a drug to dissolve blood clots costs 20,000 yen (about USD 200) per dose, however it lasts only for about 30 min. In contrast to that, just 100 g of natto costs just near to USD 1.0, but gives similar effect. Moreover, once absorbed in our body, nattokinase continues to be effective for about 8 h possibly because it has fewer detrimental side effects than urokinase. The consumption of Nattokinase in Japan is shown below (Figure 2.1).

Figure 2.1 Consumption of Nattokinase
Approximately 234,000 tons per year of Soybean was produced in Japan. The quantity of Soybean used for the production of Nattokinase was 130,000 t. About 4.7 billion packages (50 g) of Nattokinase were manufactured in a year in Japan. The total household consumption of Nattokinase in 2006 was about 188.5 b Yen (USD 1,639 m) (Figure 2.2). The estimated total market size for Nattokinase was about Yen 193 b or USD 1,678 m [112].

![Figure 2.2 Market for Nattokinase in Japan](image)

The usage of soybean in Japan for the manufacture of nattokinase had slightly decreased and most of soybeans used for Nattokinase had imported from North America (Figure 2.3). The soybeans used for Nattokinase were of non-GMO premium varieties [112].

![Figure 2.3 Use of Soybean (tons) for Nattokinase Production](image)