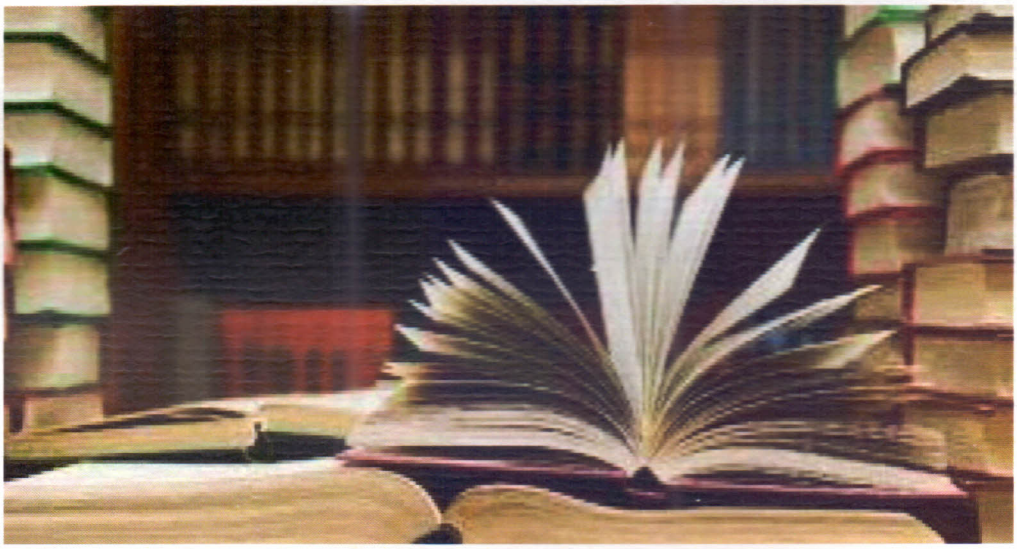
Chapter 2

Review of literature



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2.1. Historical

Earlier in 1880, Pasteur successfully cultured some small spherical shaped organisms from the pus of suppuration in broth. When he inoculated the culture into rabbits and formation of an abscess was observed. He also observed micrococci in isolated samples from the abscess. Similar results was found in the study of Ogston (1881) who investigated around eighty-two abscesses of different kind and demonstrated the presence of cocci in each. He showed that the yellow pus with the colour of the growth of *Staphylococci* indicates a dangerous form of infection.

The name of *Staphylococcus* (refers to a bunch of grapes) was introduced by Ogston (1883) for a group micrococci responsible for inflammation and suppuration in animal. He carried out the study further and became the first to distinguished two different kinds of pyogenic cocci: one arranged in groups was called "*Staphylococcus*" and another group appeared in chains was called "Billroth's *Streptococcus*" (Friedrich *et al.*, 2006). More details about the genus *Staphylococcus* was already revealed from the work of Rosenbach (1884) who divided the genus into the two species *Staphylococcus aureus* and *S. albus* (Zopf, 1885).

The genus *Staphylococcus* that showed Gram-positive and catalase-positive reaction was later putted in the family of *Micrococcaceae* (Crosslwy, 2009). Catalase enzyme is produced by Staphylococci that able to break down hydrogen peroxide H_2O_2 into water and oxygen, which can differentiate from the other group of gram positive cocci for example Streptococcus which cannot secrete catalase enzyme. Catalase is an important virulence factor as H_2O_2 is microbicidal and its degradation limits the ability of neutrophil to kill (Reuse and Bereswill, 2009).

Staphylococcus epidermis, Staphylococcus saprophyticus and Staphylococcus aureus are three major strains of staphylococcal human pathogens. A study conducted by Evans *et al.* (1955) proposed the separation of *staphylococci* from other micrococci on the basis of the relation towards oxygen. The strain found to be facultative anaerobic were kept in the genus *Staphylococcus*. Later a clear difference between

Ri Bhoi-793101

staphylococci and micrococci was made by Silvestri and Hill in 1965 on the basis of their DNA base composition.

Table I. Historic description of *Staphylococcus* genus reflecting the early detection of coagulase positive strains.

Staphylococcal Species	*Site of infection*	*Author of description and year*
S. aureus	Skin, hair, noses throats of people and animal	Rosenbach, 1884
S. intermedius	Normal skin and mucosal flora in a variety of animals, including dogs, cats, pigeons, minks, horses, foxes, raccoons, goats, and gray squirrels	Hájek, 1976
***S. schleiferi* subspecies coagulans**	Dogs and Human	Igimi *et al.*, 1990
S. delphini	Dolphins, Seals, Horses	Varaldo *et al.*, 1988
S. hyicus	Pigs and cattle. Not always coagulase positive	Sompolinsky, 1953; Devriese *et al.*, 1978
S. lutrae	Otters	Foster *et al.*, 1997
***S. aureus* subspecies anaerobius.**	Sheep	De La Fuente *et al.*, 1985

2.2. Characteristics of *Staphylococcal* strain

2.2.1. Virulence Factors:

The term "Virulence Factors "refers to a family of bacterial enzymes that can be affected the physical properties of tissue matrices and intercellular spaces and promoting the spread of the pathogen.

Blair in 1962 divided the virulence factors into two main categories one those enable the invasion of staphylococci and causes infection in the host e.g. leukocidin, hyaluronidase, coagulase and another which affect or damage the tissues of the host e.g. hemolysins or toxins. Among them coagulase and alpha hemolysin are the two factors that have received most attention probably due to the common occurrence of these substances associated with strains of known pathogenicity.

According to Blair coagulase is of primary importance to the *Staphylococci* only during the time in which the organisms are trying to establish themselves in the tissues, once they become established themselves in the early stages of infection after that the role of coagulase becomes less important. Earlier Menken and Walston (1935) suggested that lesion caused by *Staphylococci* was the result of the exotoxins produced during the metabolic activities of the organisms.

The virulence of the staphylococcal strain depends upon several physiological factors. The production of many diffusible products by *Staphylococci* has been implicated in varying degrees in the pathogenicity of Staphylococcal infections.

2.2.1. I. Hemolysins:

Several bacterial strains, including staphylococci produce Hemolysins, may be a type of channel-forming proteins or phospholipases or lecithinases that destroy red blood cells and other cells by lysis. There is no definite statement can be made regarding the relationship between alpha hemolysin produce by the *Staphylococci* and to pathogenesis. However, from the frequent production of the alpha hemolysin by these strains obtained from the site of lesions, one may draw the conclusion that it may play some role in the pathogenicity.

The existence of a close correlation between the production of alpha hemolysin and the coagulase production was established by several researchers. Different studies conducted by different investigators have confirmed this fact (Chapman *et al.*, 1934; Bryce and Roundtree, 1936; Cowan, 1938). Cowan *et al.* (1939) found that alpha lysin is produced by all coagulase positive strains and similar findings were also reported by Christie *et al.* (1946), Schwabacher *et al.* (1945). According to them

during their work about 91 percent of coagulase-positive strains studied produced alpha hemolysin. Marks in 1952 tested one hundred strains of *Staphylococcus aureus* and found that all strains produced alpha hemolysin and drew the conclusion that rather than the coagulase test, the production of alpha hemolysin was a more accurate and convenient criterion of the pathogenicity of staphylococci. Contrary to the finding, two different studies conducted by two different researchers (Elek and Levy, 1954; Lack and Wailling, 1954) concluded that alpha hemolysin detection was not reliable as coagulase testing as a measure of the pathogenicity of staphylococci. In fact Lack and Wailling showed in their survey that among those 435 strains examined by them only eighty-two percent has the ability of producing alpha hemolysin.

In 1935 Glenny and Stevens demonstrated a second type of serologically different haemolysis called beta hemolysin. The delta hemolysin was reported by Williams and Harper in 1947 while investigating the correlation between tube hemolysin tests and appearances on blood agar. The existence of two alpha hemolysins was claimed by Morgan and Graydon in 1936 they termed as alpha1 and alpha2. Smith *et al.* (1938) reported another type of hemolysin which they named as gamma hemolysin. In 1950 Elek and Levy reported that the gamma hemolysin is identical to the delta hemolysin. Therefore, it is a very difficult task to analyze the many claims because of the application of several methods and techniques have been employed in the demonstration of these various hemolysins.

2.2.1. II. Staphylococcal Hyaluronidase:

Hyaluronidase is the original virulence factor of the staphylococcal strain. The interstitial cement ("ground substance") of the connective tissue is affected by this enzyme by depolymerising hyaluronic acid. It has been studied extensively by Schwabacher *et al.* in 1945 and found that about 93. 6 per cent of the coagulase - positive strains produced hyaluronidase,and the absence of hyaluronidase in the coagulase negative strain was also noted. Under the conditions of in vitro culture, the early appearance of hyaluronidase and increased in amount for the first six days of incubation was reported by Davison *et al.* (1949).

It is a very difficult task to point out the role of hyaluronidase in the virulenceof *Staphylococci*. Though some investigators claim a relationship between hyaluronidase and virulence, while others claims that there is not enough evidence of it to draw any conclusion.

2.2.1. III. Leucocidin:

Leucocidin a metabolic product of *Staphylococci* that has been implicated in the virulence of staphylococcus strain. Earlier in 1894 Van de Velde noticed that the pleural exudate produced by the injection of virulent staphylococci showed a destructive effect on leucocytes of rabbit. Later in 1900 Neisser and Wechsberg demonstrated a strong toxin that able to kill polynlorphonuclear leucocytes within two minutes of contact and suggested that hemolysin and leucocidin were two different substances. These observations were made based on the effect of leucocidin upon rabbit white cells.

In 1932 Panton and Valentine used known leucocytes and found that a factor in staphylococcal toxin is capable of inhibiting their phagocytic ability. Later this type of leucocidin became known as the "Panton-Valentine leucocidin" to distinguish it from the one described by Neisser and Wechsberg in their report. Leucolysin is produced by a large number of coagulase positive *Staphylococcus* shares many properties in common with the delta lysine.

2.2.1. IV. Capsules:

Bacterial capsule is a Gelatinous layer made of polysaccharide, covering the bacteria. Capsules are a well-organized layer, firmly attached to the outer surface of the bacterium and not easily washed off. It can be the reason of numerous diseases in animal including human. Several investigations proved the fact that pathogenic bacteria would not able to cause disease without a capsule.

Earlier investigations have indicated that some species of the *Staphylococci* especially *Staphylococcus aureus* normally does not possess a capsule, but the work of Lyons (1937), claimed to have a capsule in cultures at the early stages. Though several

researchers failed to demonstrate the presence of capsules in various non-mucoid strains of *staphylococci* (Spink, 1939; Kleineberger-Nobel, 1948), but the work of Price and Kneeland (1954) obtained a capsulated *Staphylococcus aureus*. Now a day capsular material is used as an effective vaccine against some organisms.

2.2.2. Staphylococcal Coagulase:

2.2.2. I. Overview:

Coagulase or staphylococcal coagulase is mainly formed by some Staphylococcal strain. It is a cell-associated and diffusible enzyme that can be able to convert fibrinogen to fibrin that lead to clotting blood. Coagulase activity is often seen in the pathogenic strain which has led to the speculation as to its role as a determinant of virulence but despite rather widespread acceptance of coagulase as an index of pathogenicity, the mode of action of this protein and its significance in vivo has still remained hypothetical. The ability of clot plasma by certain Bacterial sp. was first reported by Loeb (1903), Much (1908), Gratia (1920) and Gross (1931). They have reported that clot formation of human plasma by a specific substance secreted by some *Staphylococcus* species then it was termed as Staphylocoagulase. Much emphasized the correlation between the clotting ability and pathogenicity of *Staphylococci* (Barber, 1958).The correlation has studied by Chapman *et al.* (1934) and Cruickshank (1937). Two very important findings in subsequent years were able to throw some light in the mode of action of coagulase. The discovery of Smith and Hale (1944) showed that coagulase performed the clotting of fibrinogen and convert fibrinogen to fibrin with the help of a third substance present in the plasma and tissues of several animals, and termed as a coagulase activator (Smith and Hale, 1944), coagulase reacting factor (Tager, 1948) or coagulase globulin (Miale, 1949). Secondly, the finding that may contrary to the earlier observations made by (Gross, 1931; Walston, 1935; Smith and Hale, 1944), that antibodies against coagulase can be produced (Tager and Hales, 1948; Rammelkamp *et al.*, 1950).

The existence of two different kinds of coagulase was reported by Duthie (1954). One is soluble coagulase and the other a bound coagulase the first kind of

coagulase is liberated into the medium and responsible for clotting of fibrinogen in plasma and the second is a bound coagulase which is responsible for clumping of the organisms by plasma or fibrinogen alone. This led Kapral and Li (1960) to determine the fact that either kind of coagulase is itself an essential virulence factor.

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Coagulase is tightly bound to the surface of the *Staphylococcus sp.* and can coat its surface with fibrin upon contact with blood. Coagulase is a traditional marker for identifying *S. aureus* in the clinical microbiology laboratory; however, there was no enough evidence that it is a virulence factor, although it is reasonable to speculate that

Ri Bhoi-793101

the bacteria could protect themselves from phagocytic and immune defences by causing localized clotting.

However, the widespread acceptance of coagulase as an index of pathogenicity, the mode of action and significance of this substance in vivo remain hypothetical for many years. Many theories have been postulated as to its mode of action. Later from the studies of the other researchers revealed the close correlation between the ability of the *Staphylococcal* strain to clot plasma and their disease producing capacity, and corresponding absence of the clotting property in non-pathogenic Strains by the same species, have led to the assumption that the coagulase enzyme can plays an important role in the pathogenesis of many disease caused by the strain. Then the discovery of "coagulase-reacting factor," which was not found in the insusceptible animal's blood supported the assumption with a solid ground (Blair, 1962).

In 1975, Hemker *et al.* reported that coagulase of certain *Staphylococcal* strain, induces the activity of thrombin in prothrombin by virtue of the changes brings in the prothrombin molecule in process of the formation of a stoichiometric complex between staphylocoagulase and prothrombin. The interaction between the molecules was an interesting example because in that process proteolysis or cleavage of a peptide bond was not demonstrated (Hemkar *et al.*, 1975). Later Hendrix (1983) demonstrated that activation of prothrombin by coagulase led to the formation of a stable protein-protein complex having thrombin-like activity. α-chymotryptic cleavage of staphylocoagulase has been found to produce remains that retain the ability to bind to prothrombin (Cheng *et al.*, 2010). Staphylocoagulase has also been reported to activate prothrombin by a unique mechanism known as 'molecular sexuality' (McDevitt *et al.*, 1992). The phenomenon of coagulase production has been reported to be so impulsive that it can be produced even by injured staphylococci i.e. even by those treated with heat shock at 54°C for 15min. They also recommended that growth of coagulase is independent of the molar strength of the buffer in which they were treated, but depends on the glucose, amino acid and phosphate for recovery of coagulase production (Kawabata *et al.*, 1986).

The production of staphylocoagulase has been also studied with continuous cultures of various *S. aureus* strains in a simple salt medium supplemented with mannitol, casein hydrolysate and three vitamins. An environment of low oxygen availability and magnesium-limitation were required for optimal steady-state staphylocoagulase production. It was also demonstrated that the precise rate of staphylocoagulase production was dependent on its growth rate (Bode and Huber, 1976). It has been suggested previously that the clumping of *Staphylococci* in plasma is caused by a protein molecule situated on the staphylococcal cell surface and that this molecule binds specifically and nonenzymatically to fibrinogen. This molecule, the so-called clumping factor, is supposed to be distinct from coagulase and, since it acts only on fibrinogen, also distinct from fibronectin-binding protein and protein (Friedrich *et al.*, 2003).

In a world of infections, wounds and diseases where blood clotting is a necessity, certain bacteria have been gifted with the power to naturally produce proteins that can clot blood and which can be utilized for our medical benefits. Bleeding at the surgical site is very disturbing both for the patient and the surgeon. Moreover certain wounds causing haemorrhage need immediate clotting agents. Staphylocoagulase can be described as a hidden tool which is still not easily accessible after so many years of research. However if it comes to play its role in human health care many health risks due to hemorrhage would be reduced. During the two and a half decades, many significant observations have been reported; but surely it would be unfair definitely to say that the concluding chapter on staphylocoagulase has been written. Staphylocoagulase does not provide clotting only in one way. Activated factor Xa is the position at which intrinsic and extrinsic coagulation cascades congregate. In staphylocoagulase clotting the protein acts at the prothrombin site producing a staphylothrombin. Thus the staphylocoagulase clotting reactions fall under both extrinsic and intrinsic pathways of normal coagulation (Mohamed *et al.*, 2012; Panizzi *et al.*, 2006; Shopsin *et al.*, 2000) . Ultimately the role of staphylothrombin is to convert fribrinogen to fibrin and to activate factor XIII to XIIIa. Factor XIIIa (also termed transglutaminase) cross-links fibrin polymers solidifying the clot. However,

coagulase has been found to have a limitation that it cannot activate all the factors responsible for coagulation. Therefore coagulase might require additional factors for quick clotting activity. A prior deep research is required for the same.

It was also reported that the protein binds to prothrombin with a 1:1 stoichiometry to form staphylothrombin, and the complex induces plasma coagulation by converting fibrinogen into fibrin. This would activate prothrombin bypassing the usual proteolytic cleavage. The mechanism markedly varies from the other clotting mechanism like activation of prothrombin by either blood-clotting factor Xa or snake venom procoagulant (Morita, 1976). Thus the interaction between staphylocoagulase and prothrombin represents an example to study the nonproteolytic activation of a zymogen (Kawabata, 1986).

The purification of staphylocoagulase found to be very difficult mainly because of problems arises with the stability of these activities. From different study conducted earlier showed that there are different molecular forms of the clotting activity of the *Staphylococcal* strain which differ in shape and size. Establishment of the multiple molecular nature of coagulase may helps to demonstrate the reasons, why the reported physical characterization of this protein has showed such diverse molecular weights. The characterization techniques that were used during the isolation of coagulase may consider as one important factor for the variation (Michael, 1981).

Using various bioinformatic tools it was found that coagulase protein in *Staphylococcus aureus* is containing around 690 units of amino acids having one possible transmembrane domain (Said, 2010). There was more than 75% similarity of amino acid sequence of coagulase in Staphylococcus aureus and other sequence that obtained in National Center for Biotechnology Information (NCBI). The similarities and difference between coagulase and the other six sequence of protein would able to through some light and important information about the function as well as the behaviour of coagulase protein. In a study it has been found that coagulase protein gene contains between 1800bp to 2100bp approximately (Kelmani Chandrakanth *et al.*, 2008).

Table II. Properties of Staphylocoagulase

Molecular Weight	56800-71000 Dalton
Iso - electric Point (pI)	5.4-6.4
Amino acids	670
Stability	below 4° C (Unstable at room temperature)

2.2.2. II. Production of Coagulase:

The simplest chemically defined medium used for the production of staphylocoagulase contained a wide range of amino acids and enrichment medium (Lominski *et al.,* 1950). In that particular medium a small amount of coagulase was produced and its maximum concentration was reached after incubation of 3 to 5 days. After the growth of staphylococcal strain in the digest broth or in infusion broth, the presence of coagulase after 24 hours incubation can be demonstrated easily. Several authors may have different opinion as to the length of time of incubation required for maximal production of coagulase, but, it is much more difficult to a draw comparison because of the use of different strains of organisms in different media and methods for detection of staphylocoagulase. *Staphylococcus aureus* was then taken by many authors as a standard to detect coagulase positive activity. It must be kept in mind that colonies of the same strains and even individual may vary in their ability to produce effective coagulase. The production of coagulase can be stimulated by adding egg yolk or serum into the media (Davies, 1951). It is possible that the presence of protein may protect against coagulas e destroying factor (Lominski *et al.,* 1953). Later in 1954 Duthie reported that the presence of serum actually enhanced coagulase production and the important factor was found in the albumin fraction.

The suitable conditions which favour the production of coagulase are not necessarily suitable for other products of *Staphylococci*. It is an established fact that incubation in an atmosphere of carbon dioxide is inhibitory to coagulase production, but favours the production of alpha hemolysin (Di-Rocco and Fulton, 1939).

The amount of coagulase produced by different staphylococcal strains in vitro shows no correlation with the ability of the strain to producing an infection in man.

The majority of the staphylococcal strains obtained from lesions show low activity (Tager and Hales, 1947).

2.2.2. III. Action of Coagulase on Plasma of Various Animal Species:

The basic requirement in all coagulase tests is suitable plasma. Since the action of coagulase is upon plasma that is why the plasma from many animal species have been studied by many researchers. The sources of plasma may be from a human or from another animal. Human have been susceptible to the action of coagulase (Walston, 1935; Pijoan, 1935). The plasmas of horse (Richou 1949), pig (Pijoan, 1935), dog (Walston, 1935) and goose (Loeb, 1903) can also clot.

2.2.2. IV. Techniques for Determination of Coagulase:

The main interest of coagulase to the bacteriologist is that, it forms the basis of a simple laboratory test for the recognition of the Staphylococcal strain, which involves the use of animal plasma, a variable material.

There are three methods described by researcher through which the production of coagulase can be easily detected.

The first method can be carried out by mixing a culture of *Staphylococci* with animal plasma and the visual observation of the clot.

The second method depends on the cultivation of the staphylococcal strain on nutrient agar medium containing plasma. Deposition of insoluble fibrin resulting from the action of coagulase appears as a zone of turbidity around the staphylococcal colony.

The observation of Much (1908) is the base of third method. Much observed that the addition of a suspension of staphylococci to plasma results in an immediate clumping of the organisms. This test can be easily carried out on a clean glass slide.

Routine coagulation assay is usually carried out by adding a certain volume of plasma to an equal staphylococcal suspension into a test tube and incubating the mixture at 37° C. The presence of coagulase will indicate through formation of a clot.

2.2.2. V. Chemical Nature of Coagulase:

Many researchers have found that coagulase activity is always associated with protein. Some investigators had made the Statements that coagulase are dialysable (Walston, 1935), while some others have different opinions (Tager, 1948). Though ultracentrifugation studies do not suggest that coagulase has an enormous particle size but gradocol filtration suggests an enormous particle size. Tager (1948) stated the existence of at least two components of different mobility. Some investigators (Fisher, 1936) suggested the alcohol precipitation technique whereas Walston (1935) suggested the saturation with ammonium sulphate to precipitate impurities from crude preparations. This material has a nitrogen content of 14 to 16 per cent, the amount generally found in proteins. Convincing evidence of the protein nature of coagulase is furnished by its behaviour towards proteolytic enzymes. Purified stephylocoagulase can inactivate by crystalline trypsin and chymotrypsin as well as trypsin and pepsin is also able to do so.

2.2.2. VI. Filterability of Coagulase:

Gonzenbach and Uemura (1916) noted that coagulase free from the living cell was more resistant to heat than the organisms. a cell free crude coagulase preparation was obtained by centrifuging the culture and sterilizing the supernatant by heating.but during the experiment the loss in activity of coagulase was occurring. According to Gratia (1920) that was due to removal of the bacterial cells

Earlier investigators while testing the filterability of coagulase found conflicting results. Some reported removed all activity (Walston, 1935) by Seitz filtration while others did not find the same results (Gross, 1928; Fisher, 1936). In 1944 Smith and Hale reported the advantages of gradocol membranes that coagulase can easily pass through those with an average pore diameter of 0.5 u. Mohamed *et al.* (2012) reported that the coagulase proteins can be extracted by the removal of cytosolic proteins under high salting in conditions and simultaneous excessive salts can be removed by salting out along with a chromatographic gel filtration technique using Sephadex .

However, the pH of the culture can affect the filterability of coagulase, since Seitz pads can be used to filtrate coagulase when it was produced in digest medium and the pH is adjusted to 6.7 or less (Lominski and Milne, 1947).

2.2.2. VII. Action of Physical Agents:

According to Carl Ross Anselmo (1964) purified coagulase can be stored for several months without losing its activity if kept in a desiccators at 5° C. Coagulase can be maintained at 15°C to 20°C for months but at higher temperatures it is rapidly deteriorating. They are most stable at a pH of 4.5 to 7 and in alkaline conditions it deteriorates markedly. Though the relative resistance of coagulase to heat was reported by several workers, it is generally agreed that crude coagulase is able to withstand 60° C for thirty minutes (Walston, 1935; Smith and Hale, 1944). Smith and Hale (1944) reported the degradation of coagulase within ten minutes at 120°C, while others reported the presence of the activity after autoclaving at 120°C for twenty minutes (Walker *et al.*, 1948).

2.2.2. VIII. Action of Chemical Agents:

Earlier the action of several substances on coagulase has been investigated by many researchers. The broth cultures of staphylococci in the presence of 0.15 percent phenol, 0.1 per cent permanganate can be stored without losing the activities of coagulase, but while using 0.3 percent formaldehyde at 37° C can completely inactivate coagulase within 48 hours (Gengou, 1933).

Oxidizing agents were found inhibitory (Farkas, 1947) but it was found that Coagulase reacts variably with several reducing agents. While thioglycollate exhibits no action, but propylene glycol and sodium azide are found to be inhibitory (Walker *et al.*, 1948).