STUDY OF INVITRO CLOT LYSIS POTENTIAL AND OPTIMIZATION OF STAPHYLOKINASE PRODUCTION

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Abstract: In recent years the rates of myocardial infarction have increased tremendously, which have increased the demand of producing an effective thrombolytic agents. The recent studies on Staphyokinase prove it to be a promising thrombolytic agent. The present work focuses on finding an effective Staphylokinase producing isolates by invitro clot lysis study. Out 28 isolates obtained, 10 isolates showed the highest invitro clot lysis activity. The optimization of Staphylokinase production was done in Satoh's medium and the result showed that the maximum production was obtained after 48 hours at room temperature and pH 5 for most of the isolates. Most of the isolates showed maximum Staphylokinase production with glucose and beef extract carbon and nitrogen source. Hence an effective Staphyokinase producing isolated can be studied in future for obtaining maximum yield.

Key Words: Thromobolytic, Staphylokinase, Invitro, Satoh's Medium.

I. INTRODUCTION

Fibrinolytic enzymes convert plasminogen to plasmin and lyse clots by breaking down the fibrin contained in the clot. Novel fibrinolytic enzymes derived from microbial source are useful for thrombolytic therapy. Fibrinolytic enzymes have been isolated from different sources and have been proved as effective thrombolytic agents. Fibrinolytic enzymes have been reported from various bacterial species such as *Bacillus, Staphylococcus, Aspergillus, Penicillium,* Mushrooms etc. There are different fibrinolytic enzymes available commercially such as Staphylokinase, Streptokinase, Nattokinase, t-PA, Thrombokinase, Urokinase.

Staphylokinase is an ideal fibrin specific plasminogen activator, which converts plasminogen to plasmin which in turn attacks on the fibrin clots. Staphylokinase is 136 aminoacid extracellular proteins produced during the late exponential phase by lysogenic strain of *Staphylococcus aureus* (Jaism, H. et al., 2015 a). SAK is one of the bacterial proteins having good clot specificity but it poses great risk in protein production as it is produced by pathogenic *S. aureus* (Pulicherla, K. *et al*., 2011). Staphylokinase when produced from non-pathogenic samples make production process safe; reduce the chances of cross contamination, and cost of downstream processing (Jin, T. *et al.*, 2004). Staphylokinase is a fibrin specific activator. It dissolves plasminogen to inactive proenzyme plasmin, thus acting as clot busters. It is relatively inexpensive due its easy production and presence in environmental samples, and has an effective role as an anti-clotting functioning (Lack, C. 1987; Gerheim, E. 1948 and Rajagopalan, S. *et al.,* 1985).

II. Materials and Methods

Production of Staphylokinase

The production medium was prepared according to Srinivasan, M. *et al*., (2013). The isolates were grown in Satoh's medium and incubated for 24 hours at 37°C. After incubation the medium was centrifuged at 7000 r.p.m for 20 minutes at 4°C and the supernatant was used for enzyme assay. Invitro clot lysis was carried out to determine enzyme efficiency to lyse the blood clot. All the assays were done in triplicates.

Invitro Clot Lysis Asssay

Invitro clot lysis assay was carried out as reported by Bhardwaj, S. *et al*., (2013). Sterile eppendroff tubes were labelled and their weights were determined (W_1). 1ml of blood was collected from a healthy volunteer and transferred to the tube. The tube was then incubated at 37°C for 45 minutes. After clot formation, the serum was completely removed, without disturbing the clot. The weights of tube with clots were determined (W_2). 1 ml of enzyme was added in the tube and then tubes were incubated at 37°C for 90 minutes and observed for clot lysis. In one tube 1ml sterile broth was added, which act as control. Following incubation, the lysed blood was removed, if any from the tube and the tube was weighed again (W_3) to observe the difference in clot weight. Percentage of clot lysis was calculated by using the following equation:

$$\% \text{ clot lysis} = 100 - \{[(W_3 - W_1)/ (W_2 - W_1)] \times 100\}$$

Optimization of Staphylokinase Production

Different parameter like Incubation time, Temperature, pH, Carbon source and Nitrogen sources were studied to observe the effect on Staphylokinase production using Satoh's medium. The parameters were optimized by varying one factor, while keeping the other parameters constant. The entire optimization procedure was carried out in triplicates and the result was recorded. After incubation, the medium was centrifuged at 7000 r.p.m for 20 minutes at 4°C. The supernatant obtained was proceeded for invitro clot lysis assay.

Effect of Incubation Time

To study the effect of incubation time on Staphylokinase production Satoh's medium was inoculated with 10 different isolates which were selected on the basis of the result of mannitol fermentation, haemolysis and giving highest clot lysis. The flasks were then incubated for 4 days and invitro clot lysis activity was studied after 24, 48, 72 and 96 hours of incubation.

Effect of Temperature

To study the effect of temperature on Staphylokinase production, Satoh's medium was inoculated with 10 different isolates and incubated at previously optimized time at 8°C, Room temperature, and 37°C.

Effect of pH

Satoh's medium with different pH (5, 7 and 9) was inoculated with 10 different isolates and incubated at previously optimized time and temperature.

Effect of Carbon Sources

Satoh's medium with above optimized parameters containing different carbon sources (Glucose, Lactose and Sucrose) were inoculated with 10 different isolates and incubated at previously optimized time and temperature.

Effect of Nitrogen Sources

Satoh's medium with optimized pH containing different Nitrogen sources (Beef Extract, Casein and Tryptone) were inoculated with 10 different isolates and incubated at previously optimized time and temperature.

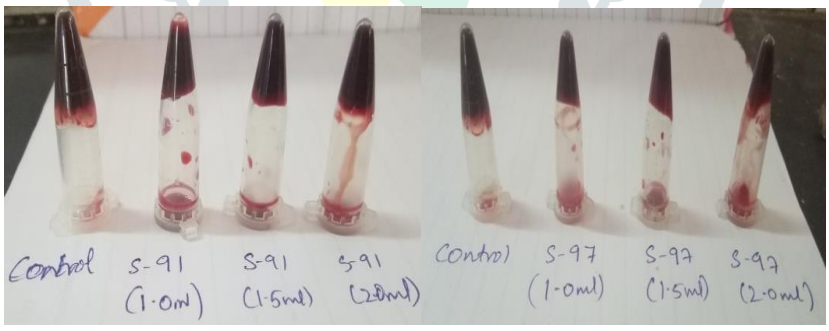
Statistical Analysis

The results obtained for the optimization of Staphylokinase production was statistically analysed using standard deviation in Microsoft excel.

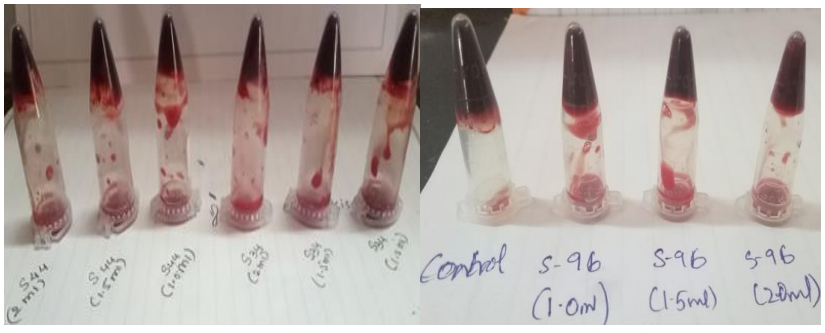
III. Results and Discussion

Table 1: Results of In-Vitro Clot Lysis for the Selected Isolates

Isolate/Aliquot of Supernatant	Clot Lysis (in %)		
	1 ml	1.5 ml	2.0 ml
S-34	39.6	39.3	38.5
S-42	34.9	34.9	34.6
S-44	18.0	17.2	17.2
S-56	12.6	12.0	12.0
S-90	21.9	21.8	21.8
S-91	25.2	23.2	23.3
S-94	48.3	43.9	46.7
S-95	18.8	17.5	15.2
S-96	29.8	29.6	29.3
S-97	22.7	22.7	22.2



(a) **(b)**



(c) **(d)**

Fig. 1 Invitro Clot Lysis Given by Few Selected Isolates (a) S-91, (b) S-97, (c) S-44 & S-34, (d) S-96

Invitro clot lysis studies showed that S-94 (48.3%) and S-34 (39.6%) showed maximum clot lysis with 1ml of aliquot of enzyme supernatant as compared to other isolates. Increase in aliquot from 1ml to 2ml did not show any significant change in clot lysis.

Optimization of Staphylokinase Production

The optimization of Staphylokinase was done using Satoh's medium prepared according to Srinivasan, M. *et al*., (2013).

Effect of Incubation Time

Figure 2 reveals the effect of incubation time on clot lysis activity of selected isolates. Incubation time help in proper interaction of organism with substrate and effective utilization of substrate. This in turn increases the growth of organisms. The studies showed that the optimum enzymes production was obtained after 48 hours for 8 isolates; S-34, 42, 56, 90, 91, 94, 95, 96 whereas 2 isolates S- 44 and S-97 gave optimum activity at 24 hours. For all isolates clot lysis activity was found to decrease at 72 hours and completely ceased after 96 hours. Similar results was obtained by Krishnaveni, K. *et al*., (2012), which showed that the maximum enzyme production in *Bacillus subtilis* was seen after 48 hours.

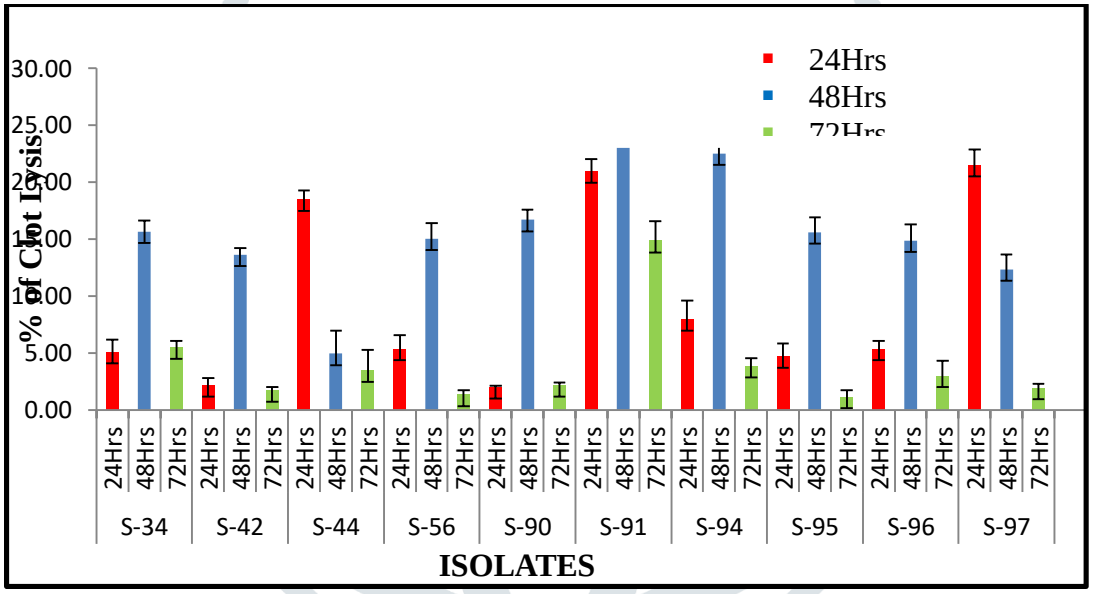


Fig. 2 Effect of Incubation Time on Clot Lysis Activity of Selected Isolates

Effect of Temperatures

Figure 3 reveals the effect of temperature on clot lysis activity on selected isolates. Temperature plays an important role in effective production of enzyme and also alters the growth of micro-organisms. The study showed that the optimum temperature for enzymes production for S-34, 44, 56, 90, 94, 95 and 96 was room temperature (29±1 °C), whereas for S-42, 91 and 97 was 37°C. As the human body temperature is 37°C, it is advantageous to have isolates with good clot lysis activity at this temperature. No growth and thereby nil enzyme activity was found at 8°C. The result obtained was compared with the work carried out by Jasim, H. *et al*., (2015). It was reported that the optimum temperature for Staphylokinase activity in genetically engineered *E. coli* JM 109 (DE3) transformant was 37°C, at this temperature the zone of hydrolysis on plasma agar plate was 30mm, while the enzyme activity was decreased at 30, 32, 34, 36, 39, and 40°C due to effect on tertiary structure and denaturation of the enzyme or decrease of activation energy for transforming the substrate to product. Similarly, Krishnaveni, K. *et al*., (2012) reported that the highest protease activity in *Bacillus subtilis* was found at 45°C.

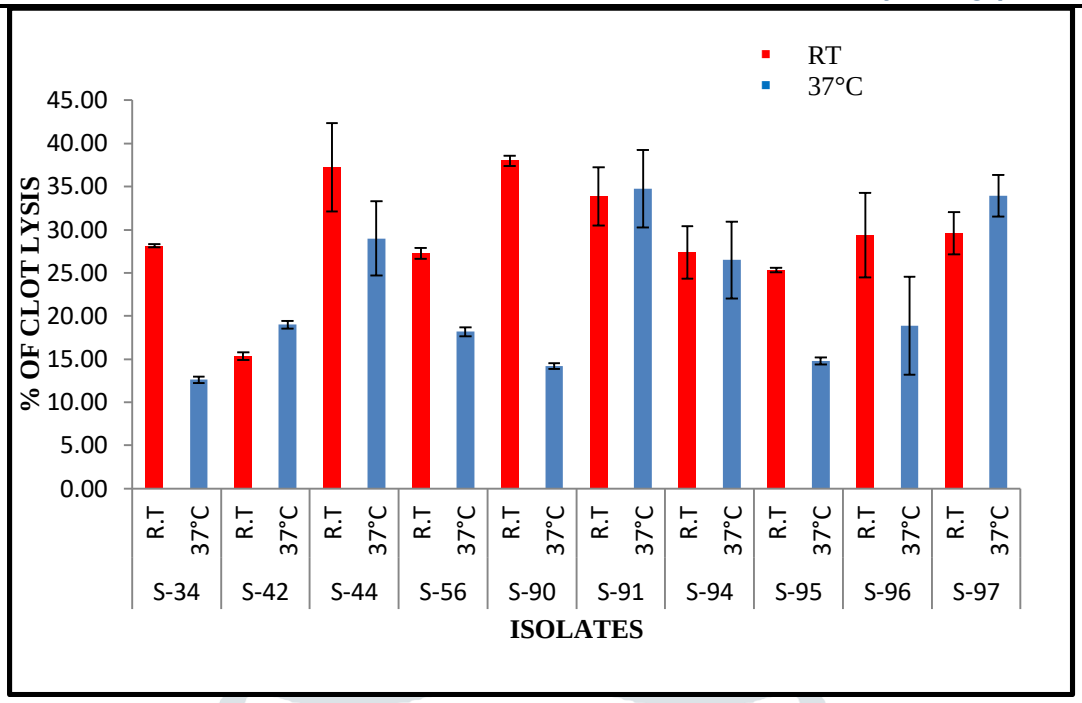


Fig. 3 Effect of Temperatures on Clot Lysis Activity of Selected Isolates

Effect of pH

Figure 4 reveals the effect of pH on enzyme activity selected isolates. pH plays an important role in maintaining enzyme activity and its production. The studies showed that the optimum pH for enzymes production for S-34, 56, 90, 95 is pH 5 and that of S-42, 44, 91, 94, 96 and 97 is pH 7. In this study no isolate gave optimum clot lysis at pH 9. The result obtained correlated with the work carried out by Das, G. *et al*., (2010), which indicated that the optimum pH required for production of protease from *Bacillus subtilis* was 8. Krishnaveni, K. *et al*., (2012) reported that the effective enzyme production at pH 9.0, which indicated that it, is an alkaline protease.

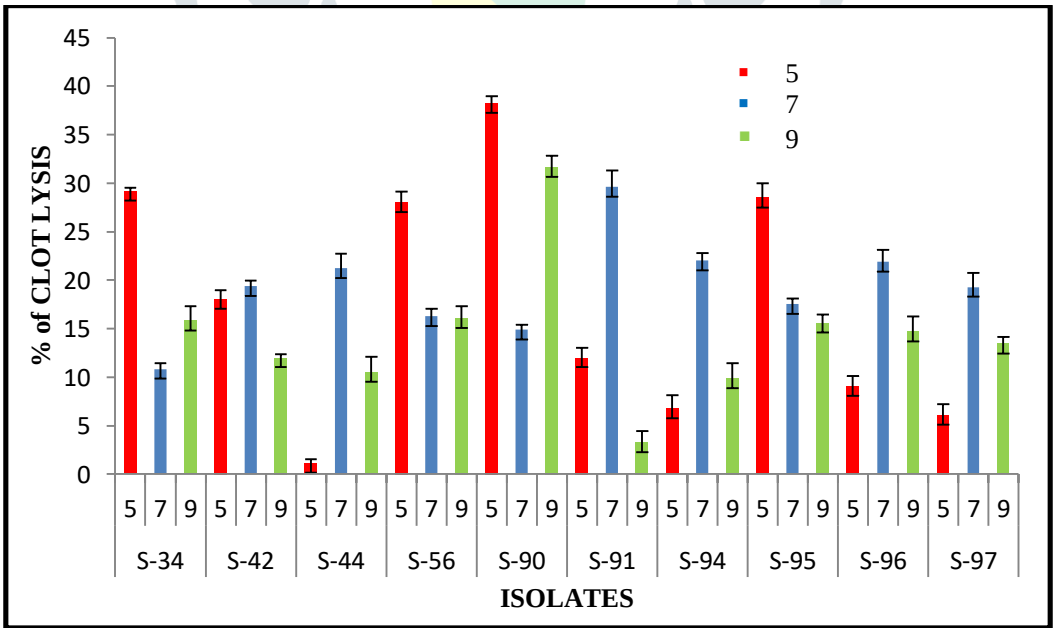


Fig. 4 Effect of pH on Clot Lysis Activity of Selected Isolates

Effect of Carbon Sources

Figure 5 reveals the effect of carbon sources on enzyme production of selected isolates. Carbon is important for organisms for the synthesis of biomolecules like carbohydrate, lipids, nucleic etc. and selection of carbon source help in increased production of enzyme. The studies showed that the optimum enzymes

production was obtained in Glucose as carbon source for S-34, 42, 44, 56, 90, 91, 95, 96, 97 and that of S-94 was obtained in Lactose as carbon source. Essam, F. *et al*., (2012) investigated the effect of 6 carbon sources: maltose, mannitol, fructose, glucose, sucrose and lactose for the growth of bacteria. Glucose was used as a reference. The results showed that fibrinolytic production was maximum when mannitol was used as a carbon source. Glucose, maltose and sucrose had positive effect, while sucrose and lactose showed negative effect. Similarly, Krishnaveni, K. *et al*., (2012) analysed the use of starch, mannose, fructose, glucose, sucrose and lactose as a carbon source for effective protease production in *Bacillus subtilis.* The result obtained revealed that glucose served as a better carbon source.

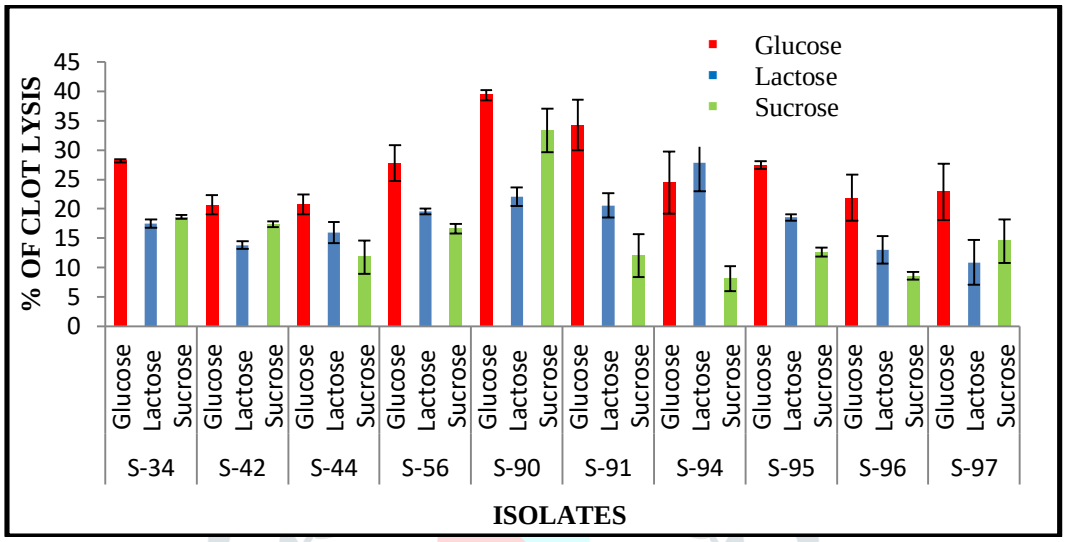


Fig. 5 Effect of Carbon Sources on Clot Lysis Activity of Selected Isolates

Effect of Nitrogen Sources

Figure 6 reveals the effect of nitrogen sources on enzyme production of selected isolates. Nitrogen sources play a vital role in production of amino-acids, which act as building blocks of proteins and nucleic acids. The study showed that the optimum enzymes production was obtained in Beef Extract as nitrogen source for S-34, 42, 44, 56, 90, 91, 94, 95, 97, whereas of S-96 was obtained in tryptone as nitrogen source. Work carried out by Essam, F. *et al*., (2012) showed that out of six nitrogen sources (casein, NH_4Cl, NH_3PO_4, KNO_3, peptone and soy peptone) used for fibrinolytic enzyme production in *Bacillus licheniformis*, soy peptone was found to be a promising nitrogen source. Similarly, Krishnaveni, K. *et al*., (2012) reported that Beef extract was found to be a better nitrogen source as compared to peptone, casein, yeast extract, ammonium chloride, ammonium carbonate, sodium nitrate and urea.

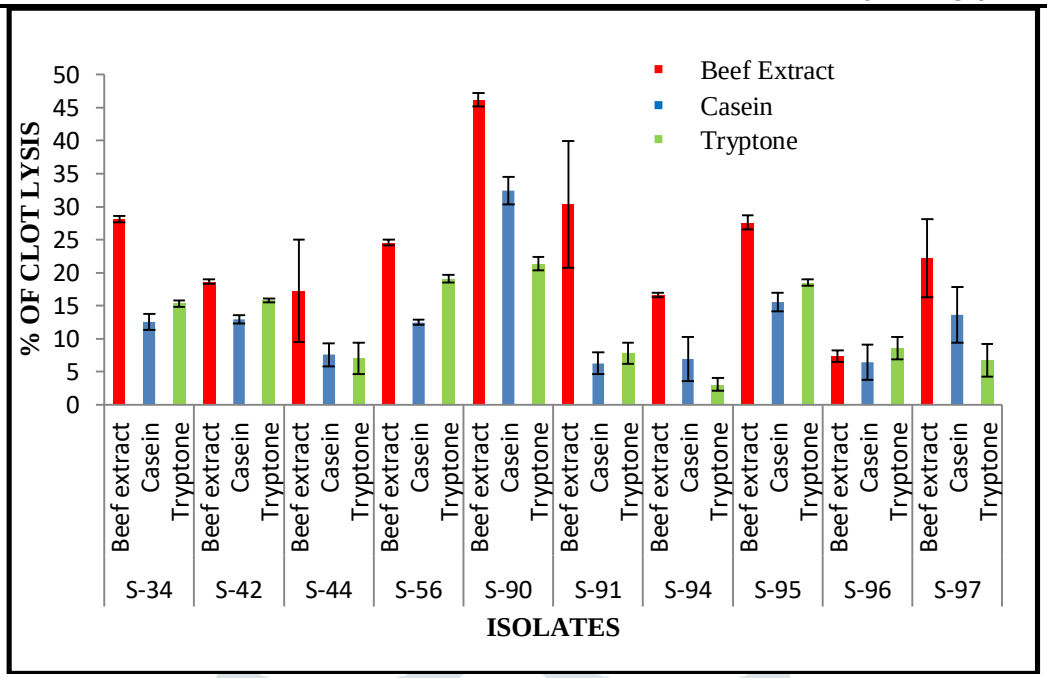


Fig. 6 Effect of Nitrogen Sources on Clot Lysis Activity of Selected Isolates

Medium optimization is an integral part of biopharmaceutical process development (http://www.sheffieldbioscience.com/media-optimization/). Medium optimization is a process where components of medium or different conditions either varied in concentration or changed to get better growth of the organisms for high productivity.

Based on the above result of optimization of production medium, the selected isolates were grown in Satoh's medium composed of best carbon and nitrogen source with optimum pH and incubated at temperature Fig 7 reveals that the clot lysis activity of enzyme S-34, S-42, S-56, S-90, S-91, S-94, S-95, S-96 and S-97 was found to increase when grown in optimized condition as compared to the result of invitro clot lysis before optimization except for isolate S-44. Hence the result obtained shows that by optimizing the parameters of Satoh's media, yield of Staphylokinase can be increased, which can ultimately result in more productivity of the enzyme.

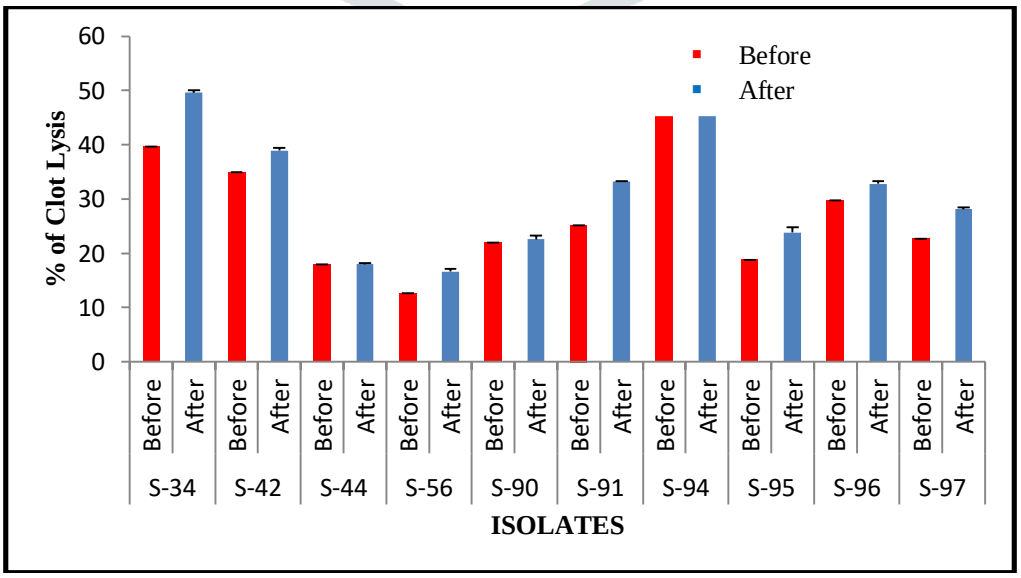


Fig. 7 Clot Lysis Activity Before and After Optimization of Satoh's Medium

Conclusion

The present study shows that Staphylokinase can act as an effective thrmobolytic agent with minimal risk factors and increased rate of production. Out of 28 isolates obtained, 10 isoaltes were found to have an ecellent clot lysis activity as comapared to others. Invitro clot lysis studies showed that S-94 (48.3%) and S-34 (39.6%) showed maximum clot lysis with 1ml of aliquot of enzyme supernatant. The optimum enzymes production was obtained after 48 hours for 8 isolates; S-34, 42, 56, 90, 91, 94, 95, 96 whereas 2 isolates S-44 and S-97 gave optimum activity at 24 hours. For all isolates clot lysis activity was found to decrease at 72 hours and completely ceased after 96 hours. The optimum temperature and pH for enzymes production for S-34, 44, 56, 90, 94, 95 and 96 was room temperature (29±1°C), whereas for S-42, 91 and 97 was 37°C. As the human body temperature was 37°C, it is advantageous to have isolates with good clot lysis activity at this temperature. No growth and thereby nil enzyme activity was found at 8°C and S-34, 56, 90, 95 was pH 5 and that of S-42, 44, 91, 94, 96 and 97 was pH 7. In this study no isolate gave optimum clot lysis at pH 9 respectively. The optimum enzymes production was obtained in Glucose as carbon source for S-34, 42, 44, 56, 90, 91, 95, 96, 97 and that of S-94 was obtained in Lactose as carbon source and Beef Extract as nitrogen source for S-34, 42, 44, 56, 90, 91, 94, 95, 97, whereas of S-96 was obtained in tryptone as nitrogen source respectively. After optimization of Satoh's medium; the clot lysis activity of enzyme was found to increase after optimization except for isolate S-40.

Hence it is proved from the research that Staphylokinase can be an efficient therapeutic agent for myocardial infarction and decrease the rate of heart stoke's. In future this enzyme can be produced at low cost with maximum clot lysis efficiency.

IV. References

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