Research Article

Trace Elements Optimization for Production of Fibrinolytic Protein from Wild and Mutant *Streptococcal* Strains

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Abstract | Streptokinase is a fibrinolytic enzyme produced from various bacterial species especially beta hemolytic *Streptococci*. Among these beta hemolytic strains the *Streptococcus equisimilis* is being used for industrial streptokinase production. It has 414 amino acids globular chain with 47000 D molecular weight. The fibrinolytic drug is comparatively cheap as compared to all other fibrinolytic drugs. It is the drug of choice in all low income nations and developing countries. The mortality rate with cardiovascular diseases is 85% in less developed countries and 75% prevalence is in women. The wild and mutant strains of *Streptococcus mutans* were used for this trace element optimization study. There are some salts like KH_2PO_4, K_2HPO_4, $NaHCO_3$, $CaCO_3$, $CH_3COONa.3H_2O$ and $FeSO_4.7H_2O$ have been optimized for the production of SK. The K_2HPO_4 has shown the highest enzymatic activity with UV-Et mutant 680.0 UmL^{-1} and with $CaCO_3$ UV-Et mutant has been found to give the relatively lowest enzyme activity 652.01UmL^{-1}. While UV-mutant has shown maximum enzyme activity 275 UmL^{-1} with the addition of K_2HPO_4 and this mutant shown minimum 57 UmL^{-1} with the addition of $FeSO_4.7H_2O$. he parental strain expressed maximum enzyme activity with 108 UmL^{-1} $FeSO_4$ minimum enzymatic activity 5 UmL^{-1} with the addition of $CH_3COONa.3H_2O$.

Received | October 14, 2022; **Accepted** | November 22, 2022; **Published** | December 26, 2022
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Citation | Akbar, G., Zia, M.A., Jamil, A., and Joyia, F.A., 2022. Trace elements optimization for production of fibrinolytic protein from wild and mutant *Streptococcal* strains. *Journal of Innovative Sciences*, 8(2): 301-310.
DOI | https://dx.doi.org/10.17582/journal.jis/2022/8.2.301.310
Keywords | Fibrinolytic, Streptokinase, Trace elements, Optimization, Enzyme production

1. Introduction

It was investigated that SK produced by various *Streptococcal* strains activates the plasminogen by different ways because of its different structural domains make up (α, β and γ) having various functional activity. It was also suggested that SK protein is made up of two structural domains analyzed by calorimetric scanning analysis. One domain has NH2-terminal including a minimum plasminogen affinity and a protein chain of 60-414 amino acids is responsible for the synthesis of plasminogen complex. The other is CO2-terminal domain which plays important role in recognition and activation of substrate: Plasminogen complex (Rafipour *et al.*, 2020) and specifically the section of Asp 41 to His 48 interlink to plasminogen (Kim *et al.*, 2000). For the activation of plasminogen the important region is the γ-domain coiled region

of the SK with the proper conformational structure where SK links with plg by lysine residue (Wu *et al.*, 2001; Sharma *et al.*, 2020).

The SK directly has no proteolytic effect however, its combination to form complex with plasminogen with 1:1 ratio breaks down the Arg 561-Val 562 of circulating plasminogen to transform them into plasmin and this activated plasmin degrades the fibrin (Figure 1) (de Souza *et al.*, 2013). As it was already described that SK is a non-specific in its action however, its not only activate plasminogen but also have ability to induce hyper-plasminemia, which is condition of exhaustion of circulating fibrinogen and coagulation factors V and VIII (Figure 2) with the relative enhancement of degradation products of plasminogen (Lin *et al.*, 2020). SK dose of 1500 000 IU despite the systemic lytic condition have an equal chance of hemorrhagic complications like other fibrinolytic drugs which exhibit higher degree interaction for fibrin (Aslanabadi *et al.*, 2018). The removal of SK from the circulatory system is biphasic. First is very fast phase in which SK inactivation occurs by particular antibodies that is about of four minutes. In 2nd phase when once SK-Plg complex is synthesized then after half hour of its average life SK is degraded and removed from the blood circulation (Erdoğan *et al.*, 2006).

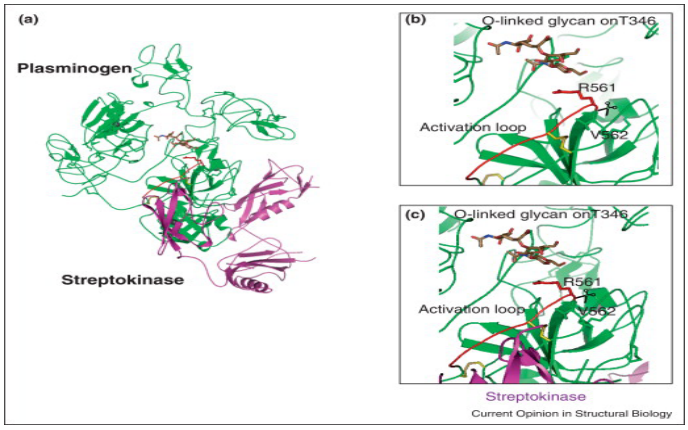

Figure 1: Proteolytic activation by breaking R561-V562 bond (a) Green color full-length plasminogen (plg) and SK superposition where SK covered the plasminogen catalytic domain. (b) Clear picture of plg activation domain of O-linked glycan on T346. (c) streptokinase-plasminogen binding view without steric hindrance and in yellow colored sticks disulphide bonds are expressed (Law *et al.*, 2012).

The two basic problems of SK therapy are bleeding, which depends on dose and administration duration and immunogenicity of SK, due to its bacterial source, which may cause allergic reactions. On the basis of carbohydrate composition of bacterial antigens found on their cell wall lance grouping have been arranged which includes coagulase-negative and catalase negative bacteria. This system made by Rebecca Lancefield was established for different species of *Streptococcaeae* in which *Streptococcus* and *Lactococcus* genera included. However, now it is exceeded due to identification of much number of *Streptococci*. Kozińska and Sitkiewicz (2020) used the both classical and molecular analysis techniques for the detection and characterization of group A *S. pyogenes* bacterial strain. The most of group A, C and G species are beta hemolytic which cause complete hemolysis of red blood cells.

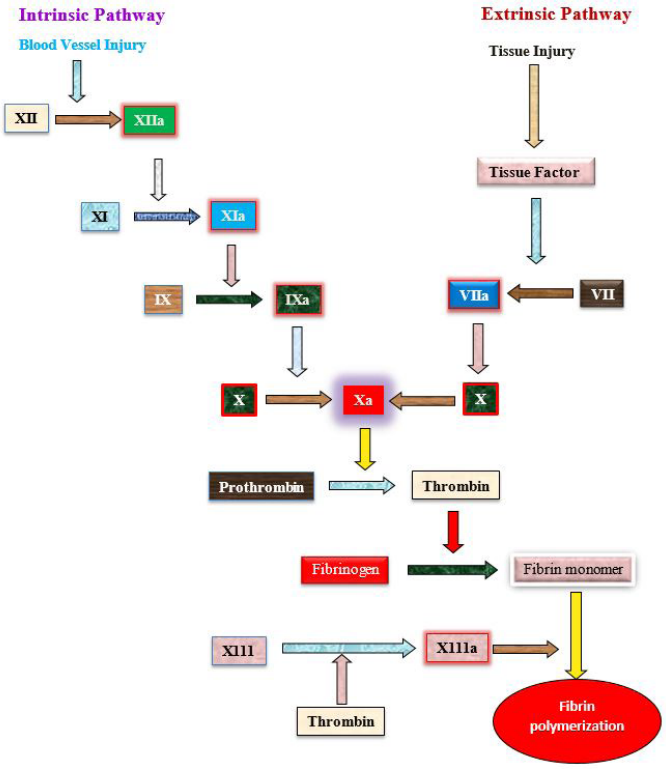

Figure 2: Extrinsic and intrinsic pathway involving in blood clotting (Schmaier *et al.*, 2011).

Thrombolytic enzyme activity depends upon the capacity of enzyme to convert plasminogen into plasmin and this capability of plasmin hydrolysis for specific duration is directly linked to the concentration of streptokinase. There are many substrates that are cleaved by the plasmin have a composition of fibrin clot, casein and esters that are good for finding the enzymatic activity (Arshad *et al.*, 2018). SK is the major fibrinolytic drug which is commonly used for myocardial infarction treatment in several countries

and about 0.5 patients have been successfully cured with SK treatment per year (Couto *et al.*, 2004). The mortality and the morbidity rates occurred from cardiovascular diseases are very high throughout the World and especially in underdeveloped and undeveloped countries are more than 80%. However, acute myocardial infarction (AMI), ischemic heart disease, arrhythmias and stroke are primary reasons of mortality (Go *et al.*, 2013). The blockage of arteries and veins may cause the indication of blood clots with serious conditions which can leads to death. In some clinical studies streptokinase and tPA were compared but there was no clear preference was observed by any one of them. SK is more efficient and cost-effective as compared to the tissue plasminogen activators (tPA). The use of streptokinase directly into the coronary artery was uncommon until 1979. However, direct administration of streptokinase into coronary vessels causes serious harmful side effects such as brain hemorrhage and digestive tract bleeding. These side effects generally appear in the older heart patients treated with high doses of streptokinase (Aslanabadi *et al.*, 2018). There is a balance between blood clot formation and prevention and this process which equilibrates both these conditions is called hemostasis. Outside the body blood forms blood clot while inside the body fibrinolytic agents are present which prevent blood clot formation. Imbalance in hemostasis causes either bleeding or thrombus formation. There are many fibrin degradation enzymes like urokinase, streptokinase (SK), plasminogen activators (tPA) are the main medicines used for the treatment of cardiovascular diseases (Nedaeinia *et al.*, 2019). Tissue plasminogen activators are expensive but more specific for plasminogen which made it popular in advanced countries like British, Germany, America and Japan. While streptokinase is nonspecific and less costly due to its microbial origin so it's widely used in undeveloped and under developed countries such as continent African and Asian continents. The most of the people in these countries are poor and can't afford expensive medicine. So patients of heart diseases in these low income areas preferred the use of streptokinase. This is the main reason of saving lives of million to trillions of cardiovascular disorders patients (Yan *et al.*, 2020).

In the blood circulatory system, the formation of a thick and sticky clump which blocks the flow of blood through vessels this process is known as thrombosis. After injury of a blood vessel, platelets, fibrin and red blood cells play a key role in the formation of a blood clot to stop bleeding. A piece of the blood clot broken from thrombus start to travel in the blood vessels is named as embolus (Furie and Furie, 2008). Blood clot formation may occur in the veins or in arteries. Insoluble Fibrin is formed from soluble fibrinogen by the action of thrombin protein. This fibrinogen is polypeptide made up of two subunits which are connected by disulphide linkages consisting of molecular weight, 340 kDa. Each subunit contains three large peptide chains (Aα, Bβ, and γ) (Vasilyeva *et al.*, 2020).

The fibrin protein in blood matrix clots the blood in blood vessels is known as thrombosis. Thrombolytic medications such as streptokinase, urokinase and plasminogen activator (tPA) act with plasminogen to form plasmin which acts on this clot and breaks down it (Table 1). This procedure is called thrombolysis. In mammalian cells, the protein responsible for thrombolysis is plasmin, which is a serine protease analog to trypsin (Emberson *et al.*, 2014).

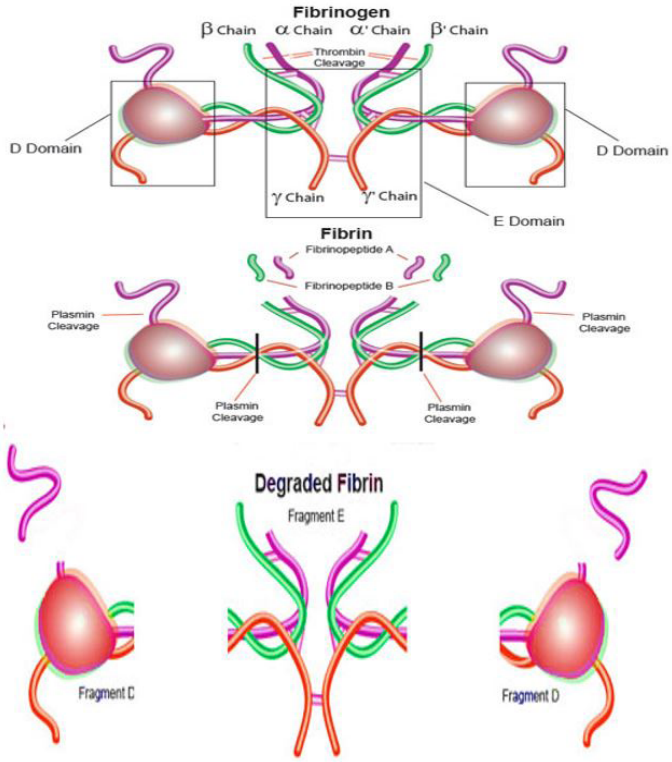

Figure 3: Fibrinogen transformation to fibrin by thrombin and fibrin cleavage by plasmin (https://www.sigmaaldrich.com/life-science/metabolomics/enzyme-explorer/analytical-enzymes/fibrinogen-and-fibrin.html**).**

Plasmin is functional form modified from inactive zymogen (plasminogen) and this transformation of

Table 1: Properties of thrombolytic enzymes.

Enzyme	Mol. mass (kDa)	Substrate specificity	Source of origin	Half-life (Min)	Cure for diseases	References
Streptoki-nase	47	Plasmin	*Streptococcus* species	20	Myocardial infarction, pulmonary embolism,	Tillet *et al.*, 1933
Nattokinase	27.7	Plasmin	Bacillus subtilis	240	Remedy for heart diseases	Fujita *et al.*, 1993
Alteplase	59	Plasmin	Human melanoma cell line	5	Ischemic stroke, pulmonary emboli. myocardial infarction	McCartney *et al.*, 2019
Urokinase	31	Plasmin	Human urine	15	Pulmonary embolism, myocardial infarction	Degryse, 2011
Reteplase	39	Plasmin	*Escherichia coli*	15	Myocardial infarction, ischemic stroke.	Noble *et al.*, 1996
Anistreplase	131	Plasmin	*Streptococcus* + plasma product	70	Myocardial infarction and pulmonary embolism	Hannaford *et al.*, 1995
Tenecteplase	70	Plasmin	Mammalian cell line	15	Myocardial infarction, pulmonary emboli.	Melandri *et al.*, 2009
Lanoteplase	39	Plasmin	Recombinant	30	Investigated for use/treatment in myocardial infarction.	Hazzard *et al.*, 1999
Amediplase	346 Da	Plasmin	Animal source	16	Myocardial infarction, thrombosis.	Guimarães *et al.*, 2001
Saruplase	46	Plasmin	Kidney and liver	9	Arterial thrombosis	Grignani *et al.*, 1994
Subtilisin DJ-4	29	Plasmin	*B. licheniformis*	30	Myocardial, cardiovascular infarction	Kim and Choi 2000
Subtilisin QK-2	28	Plasmin	*Bacillus subtilis*	30	Prevent and control thrombosis diseases.	Ko *et al.*, 2004
Subtilisin DFE	28	Plasmin	*Bacillus amyloliquefacien*	10.5	Deep venous thrombus and Athero-sclerosis	Peng *et al.*, 2004

plasminogen into plasmin the Arg 561-Val 562 bond is the site of proteolytic cut by several plasminogen activators and this activated plasmin degrades fibrin molecules as shown in Figure 3. The most of the group C beta hemolytic *Streptococcal* species produce streptokinase protein which is not found in human body only appears after medical treatment. There is no direct effect of fibrinolytic agents such as, tPA, uPA and SK, however their clinical effect is performed by the transformation of the plasminogen into plasmin (Law *et al.*, 2012).

2. Materials and Methods

The wild *Streptococcus mutans* was subjected to physical mutation by UV-radiations and Ethidium bromide with chemical random mutations. The optimization trace elements in the nutrient agar medium of all these strains were performed and obtained optimum levels of these elements on wild and mutants' bacterial strains.

2.1 Trace element K_2HPO_4

The different concentrations of K_2HPO_4 (Spectrum Chem. Mfg. Corp.) (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0%) were added in the fermentation medium containing 250mL Erlenmeyer flasks. These flasks were incubated for 24h and after that centrifugation were performed. The supernant and pellet were subjected for enzyme activity (Arshad *et al.*, 2018).

2.2 Trace element $CH_3COONa.\ 3H_2O$

The various concentrations of hydrated sodium acetate ($CH_3COONa.\ 3H_2O$) (Elsen Golden Laboratories, assay 99%) (0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, 1.0%) were added in the fermentation medium (nutrient agar medium) containing Erlenmeyer flasks. These flasks were incubated for 24h and then centrifugation was done. The supernant and pellet were subjected for enzyme activity.

2.3 Trace element KH_2PO_4

The various quantities of KH_2PO_4 (Elsen Golden Laboratories, assay 99%) (0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, 1.0 %) were added in the fermentation medium (nutrient agar medium) containing 250mL Erlenmeyer flasks. These flasks were incubated for 24h and then centrifugation was

done. The supernant and pellet were subjected for enzyme activity.

2.4 Trace element $NaHCO_3$

The NaHCO3 (Pure Chems™ M.W: 84) (0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, 1.0 %) were added in the fermentation medium (nutrient agar) containing 250mL Erlenmeyer flasks. These flasks were incubated for 24h and then centrifugation was done. The supernant and pellet were subjected for enzyme activity.

2.5 Trace element $FeSO_4.7H_2O$

The $FeSO_4.7H_2O$ (Sigma Aldrich ACS reagent, ≥99.0%) (0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, 1.0 %) were added in the fermentation medium containing 250mL Erlenmeyer flasks. These flasks were incubated for 24h and then centrifugation was done. The supernant and pellet were subjected for enzyme activity.

2.6 Trace element $CaCO_3$

The $CaCO_3$ (Minday Materials, assay 99%) (0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, 1.0 %) were added in the fermentation medium (nutrient agar) containing 250mL Erlenmeyer flasks. These flasks were incubated for 24h and then centrifugation was done. The supernant and pellet were subjected for enzyme activity.

2.7 Statistical analysis

The data was statistically observed in one way ANOVA by using Graph Pad InStat software.

3. Results and Discussion

The bacterial strain *Streptococcus mutans* was collected from EBL stock University of Agriculture Faisalabad. This strain was re-cultured on nutrient agar media again and again to get a healthy and pure strain culture. The optimization of trace elements was performed after the mutagenesis of wild strain.

3.1 Optimization of trace elements in the fermentation medium

3.1.1 Optimization of K_2HPO_4

In a fermentation medium with the addition of dipotassium hydrogen phosphate (K_2HPO_4) it was shown that the amount of enzymes produced was observed in an elevated level. In this research work various concentrations of K_2HPO_4 from 0.1 to 1.0% have been added in the fermentation media of parental and mutant *Streptococcus mutans* shown in Figure 4 and Table 2. The largest amount of SK was found from UV-EtBr-mutant with enzyme activity 675.01 UmL^{-1} by adding 0.3 % K_2HPO_4.

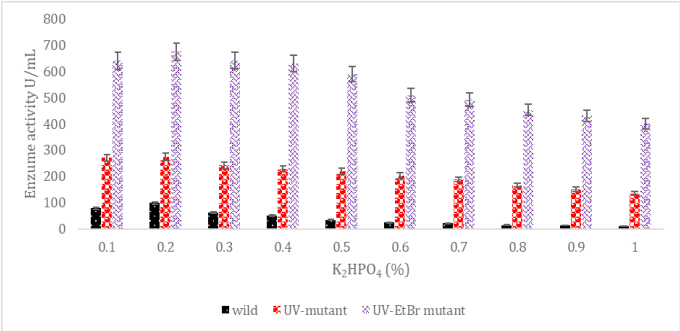

Figure 4: SK production from wild and mutated *Streptococcus mutans* strains from various K2HPO4 amounts.

Table 2: ANOVA for influence of K2HPO4 on SK yield from wild and mutagenic *Streptococcus mutans*.

S.O.V	DF	Sum of square	Mean square	F-value	p-value
Source	2	1162355	581178	181.98	0.0000**
Levels	1	69685	69685	21.82	0.0000**
Source×Level 2	22574	11287	3.53	0.0336*	
Erro	84	268270	3194		
Total	89				

*, Significant (P<0.05); **, Highly significant (P<0.01).

3.1.2 Optimization of $CH_3COONa.3H_2O$

The $CH_3COONa.3H_2O$ had a significant effect in the production of SK in optimized fermentation media. The amount of $CH_3COONa.3H_2O$ from 0.01 to 0.1 was applied to optimize its concentration. The optimum production of SK 676.0 U/ml was observed by using 0.01% of $CH_3COONa.3H_2O$ in the UV-EtBr *Streptococcus mutans* fermentation medium (Figure 5 and Table 3).

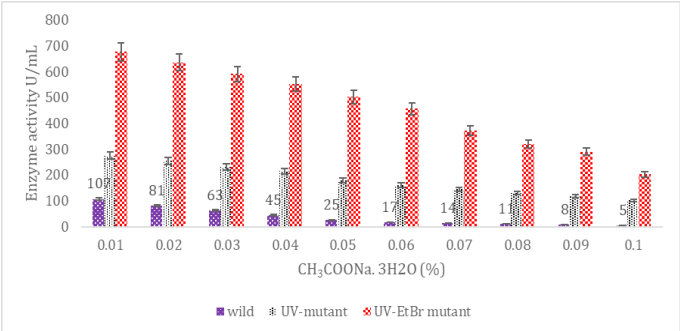

Figure 5: SK yield from wild and mutagenic *Streptococcus mutans* on diverse CH3COONa.3H2O amount.

Table 3: ANOVA for influence of $CH_3COONa.3H_2O$ on SK yield from wild and mutagenic *Streptococcus mutans*.

S.O.V	DF	Sum of square	Mean square	F value	p-value
Source	2	568320	284160	42.09	0.0000**
Level	1	148759	148759	22.03	0.0000**
Source ×level	2	93513	46756	6.93	0.0016**
Error	84	567098	6751		
Total	89				

*, Significant (P<0.05); **, Highly significant (P<0.01).

3.1.3 Optimization of KH_2PO_4

Throughout the continual improvement of enzymes from bacterial strains in the fermentation process, salt concentrations even play a crucial role. In various trials, a spectrum of KH_2PO_4 concentrations was introduced around 0.01 to 0.1 in the fermentation medium of *S. mutans* (Figure 6 and Table 4). The 0.06% quantity of such a salt as given for the maximum SK enzyme output with an activity of 680.0 UmL^{-1}.

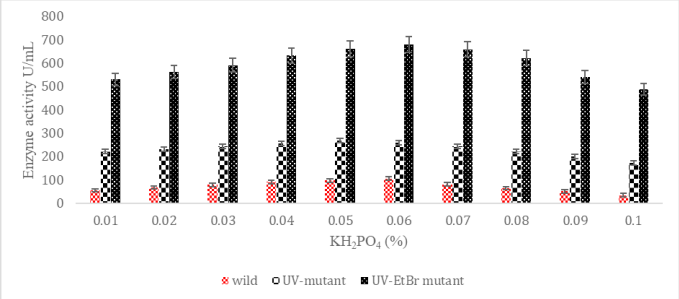

Figure 6: SK yield from wild and mutagenic *Streptococcus mutans* on diverse KH2PO4 amount.

Table 4: SK yield from wild and mutagenic *Streptococcus mutans* on diverse KH2PO4 amount.

S.O.V	DF	Sum of square	Mean square	F value	p-value
Source	2	1301099	650549	592.23	0.0000**
Level	1	25056	25056	22.81	0.0000**
Source× level	2	17811	8906	8.11	0.0006**
Error	84	92271	1098		
Total	89				

*, Significant (P<0.05); **, Highly significant (P<0.01).

3.1.4 Optimization of $NaHCO_3$

The Influence of $NaHCO_3$ was detected for the utmost production of SK from wild and mutant bacteria in the fermented medium. The ten various concentrations were applied from 0.01 to 0.1 percent (Figure 7 and Table 5). An amount of 0.04% $NaHCO_3$ in the fermentation medium showed the extreme yield of SK with activity of 675.0 UmL^{-1}.

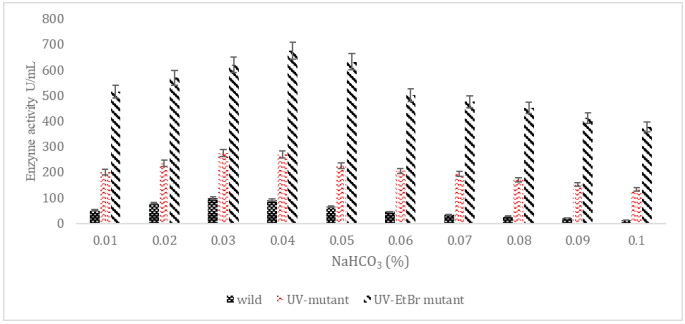

Figure 7: SK yield from wild and mutagenic *Streptococcus mutans* on diverse NaHCO3 amount.

Table 5: ANOVA for influence of $NaHCO_3$ on SK yield from wild and mutagenic *Streptococcus mutans*.

S.O.V	DF	Sum of square	Mean square	F-value	p-value
Source	2	1006447	503224	166.89	0.0000**
Level	1	73845	73845	24.49	0.0000**
Source× level	2	19929	9965	3.30	0.0415*
Error	84	253279	3015		
Total	89				

*, Significant (P<0.05); **, Highly significant (P<0.01).

3.1.5 Optimization of $FeSO_4.7H_2O$

The effect of $FeSO_4.7H_2O$ on enhanced SK output in the fermentation process was also identified. In the fermented medium, the different concentrations of this salt were analyzed from 0.01 to 0.1 percent shown in Figure 8 and Table 6. A concentration of 0.01% added in the fermentation medium expressed the highest quantity of enzyme with activity of 663.02UmL^{-1}.

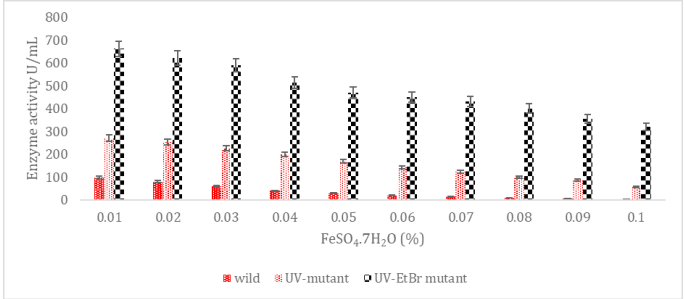

Figure 8: SK yield from wild and mutagenic *Streptococcus mutans* on diverse $FeSO_4.7H_2O$ amount.

3.1.6 Optimization of $CaCO_3$

The impact of calcium carbonate in the fermented product on SK yield through bacterial strains has been reported. The calcium carbonate concentrations range has been examined between 0.01 and 0.1% and the

highest production with SK activity of 652.01UmL^{-1} was found with a level of 0.02% $CaCO_3$ (Figure 9 and Table 7).

Table 6: ANOVA for influence of $FeSO_4.7H_2O$ on SK yield from wild and mutagenic *Streptococcus mutans*.

S.O.V	DF	Sum of square	Mean square	F-value	p value
Source	2	891739	445869	97.54	0.0000**
Level	1	96694	96694	21.15	0.0000**
Source× level	2	28724	14362	3.14	0.0483*
Error	84	383988	4571		
Total	89				

*, Significant (P<0.05); **, Highly significant (P<0.01).

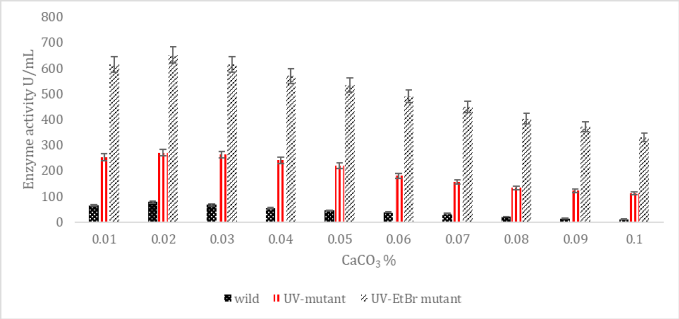

Figure 9: SK yield from wild and mutagenic *Streptococcus mutans* on diverse $CaCO_3$ amount.

Table 7: ANOVA for influence of $CaCO_3$ on SK yield from wild and mutagenic *Streptococcus mutans*.

S.O.V	DF	Sum of square	Mean square	F value	p-value
Source	2	835333	417667	150.35	0.0000**
Level	1	75864	75864	27.31	0.0000**
Source× level	2	23453	11727	4.22	0.0179*
Error	84	233349	2778		
Total	89				

*, Significant (P<0.05); **, Highly significant (P<0.01).

El-Mongy and Taha (2012) and Faran *et al.* (2015) who found maximum SK production (331.71UmL^{-1}) and (467. 73 UmL^{-1}) from mutated *Streptococcus equisimilis* by adding 0.25% and 0.05% K_2HPO_4 in the fermentation medium.

Faran *et al.* (2015) obtained maximum SK production (365.33U/mL) by using 0.15% $CH_3COONa.3H_2O$ in the fermented medium of mutated *S. equisimilis* and Madhuri *et al.* (2011) obtained highest amount of SK production by using 0.1% $CH_3COONa.3H_2O$ in the medium. Abdelghani *et al.* (2005) and Faran *et al.* (2015) are significantly associated who observed optimum SK output at 0.25% and 0.15% KH_2PO_4 concentrations and analyzed the decrease in SK output by raising the concentrations.

Patel *et al.* (2011), who got the highest output of SK from bacterial strain by adding 0.2% $NaHCO_3$ in the fermentation medium, Madhuri *et al.* (2011) who observed maximum production streptokinase by using 0.15% $NaHCO_3$ in the media and also correlates with the findings of Faran *et al.* (2015) who obtained maximum SK quantity with activity (457.01UmL^{-1} by using 0.15% $NaHCO_3$ in the optimized fermentation medium. Yazdani and Mukherjee (2002) who achieved optimum SK output in the fermentation process by adding 0.06% $FeSO_4.7H_2O$ concentration and also related to the results of Madhuri *et al.* (2011) and Faran *et al.* (2015) that accomplished optimum SK activity upon the addition of 0.04% $FeSO_4.7H_2O$ in the fermentation media.

Baewald *et al.* (1975) and Faran *et al.* (2015) that found better SK yield from the fermented medium by the addition of 0.004% $CaCO_3$ and also narrowly interlinked with the findings of Elmongy and Taha (2012) who attained maximum SK yield upon addition of 0.005% of $CaCO_3$ in the fermentation medium.

Conclusions and Recommendations

The addition of trace elements like KH_2PO_4, K_2HPO_4, $NaHCO_3$, $CaCO_3$, $CH_3COONa.3H_2O$ and $FeSO_4.7H_2O$ in the fermentation medium expressed the highly significant results. Among all these trace elements, K_2HPO_4 has shown the highest enzymatic activity with UV-Et mutant 680.0 UmL^{-1} and with $CaCO_3$ UV-Et mutant has been found to give the relatively lowest enzyme activity 652.01UmL^{-1}. While UV-mutant has shown maximum enzyme activity 275 UmL^{-1} with the addition of K_2HPO_4 and this mutant shown minimum 57 UmL^{-1} with the addition of $FeSO_4.7H_2O$. The parental strain expressed maximum enzyme activity with 108UmL^{-1} $FeSO_4$ minimum enzymatic activity 5UmL^{-1} with the addition of $CH_3COONa.3H_2O$. However, the trace elements play key role for the production of specific metabolites from microbial source.

Acknowledgement

I am thankful to Enzyme Biotechnology Laboratory for the facilitation of this research study.

Novelty Statement

As for as the novelty is concerned, first time I have developed this strain from locally isolated strain. In enzyme biotechnology use of novel strain for enzyme production and enhancement in enzyme activity is its novelty as well. Hence, developing a novel strain and achieving higher levels of enzyme production than the previous ones is the novelty of my work.

Author's Contribution

Ghulam Akbar performed all the research work, Muhammad Anjum Zia Supervised research work, Amer Jamil provided research plan and technical assistance, Faiz Ahmad Joyia helped research work to perform some part in his lab and also provided guide lines in paper writing and reviewed this article before submission.

Conflict of interest

The authors have declared no conflict of interest.

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