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Optimization Studies for Enhanced Production of Streptokinase by *Streptococcus equisimilis* UVM6

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The main focus of this current research is to enhance the efficiency of *Streptococcus equisimilis* (MTCC3522) using random mutagenesis for enhanced streptokinase production. The influence of environmental parameters for the growth and streptokinase production of the UV mutated strain UVM6 was determined by optimizing various conditions. Five different parameters were assigned for optimization studies carbon source, salt concentration, pH, temperature and growth medium. The maximum streptokinase activity of 0.3U/ml was obtained in the culture conditions, glucose (1%) as carbon source, 2.5g/l, NaCl concentration, at 37ºC for 24 h with an initial pH 7.5 at 120 rpm. The blood clot lysis by mutated strain exhibited maximum enzyme activity. The maximum production of streptokinase activity was shown by mutated strain *Streptococcus equisimilis* UVM6. The results showed that UV irradiation was one of the effective mutagenic agents for strain improvement studies of *Streptococcus equisimilis* for streptokinase production.

Key words: Streptokinase, optimization, thrombolytic drugs, UV mutation.

The blood clot (Thrombus) developed in the circulatory system may leads to vascular blockage which may result to death. The blood clots occur in coronary, cerebral or pulmonary vessels which lead to myocardial infarctions, strokes, respiratory and cardiac failure therefore it is important to diagnose and treat blood clots. Finding the cure for strokes and myocardial infarction has been taken into consideration since early decades of the twentieth century[1]. Thrombolytic drugs are used to dissolve (lyse) blood clots (thrombi). It is included in the World Health Organization (WHO) Model List of Essential Medicines[2]. Streptokinase (SK) is a group of extracellular proteins produced by a variety of beta-hemolytic *Streptococcus* sp. The enzyme was first isolated in 1933 and entered clinical use in mid 1940s[3]. The plasminogen activator composed of 414 amino acids with a molecular mass of 47 kDa[4] which has a multiple domains structure with, a-, b- and g-domains with different associated functional properties[5]. Gamma domain is essential for plasminogen activation[6], Most group A, C, and G *Streptococci* isolated from human hosts secrete a plasminogen activator known as streptokinase which catalyzes the conversion of the plasma zymogen, plasminogen, to the serine protease plasmin. Human plasminogen and streptokinase form a 1:1 stoichiometric complex that hydrolyzes other plasminogen molecules to generate plasmin, which subsequently can degrade fibrin, the primary protein component of blood clots. Streptokinase commercially marketed in different brand names globally. As per market research 2011 report - Asia, Europe and North America are the leading countries worldwide in manufacturing and supplying

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streptokinase. Unlike urokinase or tissue-type plasminogen activator that performs direct proteolysis, SK forms a high affinity equimolar[7]. Over the years, thrombolytic therapies via injecting or orally administrating thrombolytic agents to lyse thrombi in blood vessels have been extensively investigated[8, 9]. Most of the research has focused on the streptokinase secreted by a human isolate of the group C *Streptococcus*[10]. In this study we have monitored the influence of cultural parameters for the growth and streptokinase production of UV mutated *S. equisimilis* UVM6.

MATERIALS AND METHODS

Streptococcus equisimilis (MTCC 3522) was obtained from the Culture Collection Center (Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology (IMTECH, India) and was maintained in Sheep Blood Agar Medium at 37°C.

Mutagenesis by UV treatment

The ultra violet mutagenesis was carried out for the wild type Streptococcus *equisimilis* (MTCC3522). The UV treated mutants after appropriate dilutions were spread onto specialized media and exposed to UV light (254 nm) for variable time intervals ranging from 5 to 120 min in UV chamber keeping the distance of UV source fixed at 11.5 cm. The growth colonies were selected randomly and tested for their enzyme productivity. The exposure time and the distance between the Petri dish and UV sources were determined experimentally.

Casein-plasminogen overlay for detection of Streptokinase (SK) activity

The UV mutated strain was grown overnight in 3 different medium - Brain Heart Infusion medium (BHI), Todd- Hewitt medium[11], Baewald production medium [12]. Preliminary screening for bacterial colonies producing streptokinase was detected by overlay on casein and human plasminogen in soft agar [13]. 10 ml soft agarose mixture consisting of 0.8% agarose, 10% skimmed milk, 200 µl of human plasma, 150 mM NaCl, and 50 mM Tris- HCl (pH 8.0) was poured on top of the plates and streaked, incubated at 37° C for 2-6 h. Positive streptokinase activity was indicated by the appearance of zones of clearance (halo formation) around the colonies[14].

Streptokinase activity - Modified Holmstrom method

Streptokinase activity was determined indirectly with Modified Holmstrom method[15]. 5ml of fibrinated human blood was taken in 10 tubes and the filtrate was added in various concentrations and incubated at 37°C for 18 h. One unit is defined as the quantity at which the enzyme will liquefy the clot completely. The strain with maximum activity was selected for growth curve analysis.

Growth pattern of *Streptococcus equisimilis* UVM6

The UV mutated strain was scrapped with 5 ml of sterile distilled water from blood agar slants, and transferred into 250 ml Erlenmeyer flask containing 45 ml of Baewald production medium. The flasks were incubated at 37°C for 24 h in a shaker incubator (120 rpm). The growth was observed at regular intervals of time varied as 0, 30, 60, 90, 120, 150, 180, 210,240,270min. The generation time of the isolate was determined. Growth was monitored by measuring the absorbance at 600 nm[16].

Effect of supplementary carbon sources and salt concentration (NaCl)

Carbon sources such as 1% of glucose, fructose, maltose and sucrose, were supplemented separately into the fermentation medium. Similarly, sodium chloride (NaCl) was added to the fermentation medium at varying concentrations at 1.5%, 2.5%, 3.5% and 5%. The enhancement of streptokinase production was investigated by casein enzyme digestion method.

Effect of initial pH and incubation temperature

In order to study the impact of pH the initial pH levels of the growth media were adjusted from 5.5 to 8.5, As well the optimum temperature for the production medium at temperatures ranging 32°C, 37°C, 40°C and 45°C. The maximum streptokinase production was characterized by casein enzyme digestion method.

RESULTS

In the present study an attempt was made to improve the yield of streptokinase production from *Streptococcus equisimilis* (MTCC3522) by UV mutation, the screening of *Streptococcus* by Casein-plasminogen overlay method was reliable

and quick method for the detection of streptokinase by zone of clearance in order as well to monitor enzymatic activity during the growth of microbe (Fig.1). When compared to wild parental strain, enhanced streptokinase production was observed in mutated strain. The important inducers of the strain improvement were found to be UV rays and the strain improvement studies showed increased SK production and showed higher stability of the organism. The maximum enzyme activity of the mutated strain was found to produce threefold increase in streptokinase enzyme 0.3 IU and while the parental was found to be 1.8IU. (Fig. 2) these results suggest that UV mutation is a reliable method for streptokinase production. The betterment of growth was observed by subjecting to various media. In comparison with Todd Hewitt broth and Brain heart infusion broth, streptokinase production was high in the Baewald production medium. The growth of the mutant was monitored over a period of 12h. Cell density was measured at regular intervals of 1h in early phases of growth. The mid log phase (A600=0.4), (Late-log=A600=0.5), early stationary (A600=0.5). The stationary phase of *S.equisimilis* UVM6 was extended from 120 min to 210 min of incubation. The mean generation time (g) = k^{-1} = 3.78 hours. The growth phase study was ideal for the detection of streptokinase in various stages of growth phase (Fig.3). In order to design the effective medium,

Fig. 1. Casein-plasminogen overlay for detection of Streptokinase activity by *S.equisimilis* UVM6

Fig. 2. Blood clot lysis – 0.3ml of crude extract showed complete dissolution of blood clot by *S.equisimilis* UVM6

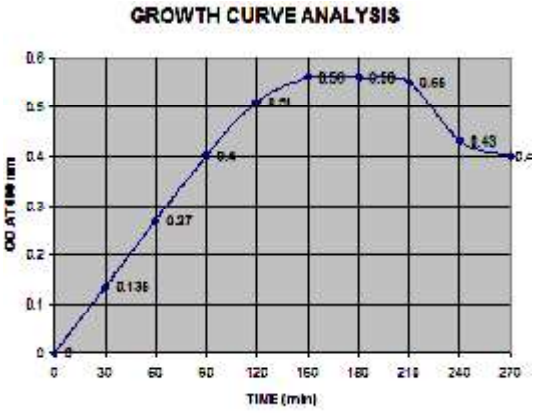

Fig. 3. Growth pattern of *S.equisimilis* UVM6

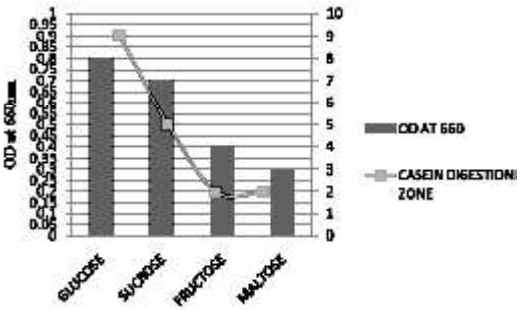

Fig. 4. Effect of different carbon sources

the roles of different carbon and salt concentrations were evaluated for their impact on growth and SK production. Among the various carbon sources tested, glucose was the best carbon source for both biomass and Streptokinase production. 2% glucose supplemented in the medium promoted the highest biomass production, whereas 1% glucose was found to be the best for production of SK (Fig. 4). The impact of sodium chloride concentration on SK production was investigated (Fig. 5). The production of SK was very low with 1.5g/l of salt concentration in the medium. Maximum SK activity was obtained in culture filtrates supplemented with 2.5g/l of sodium chloride in the medium. The environmental requirements and cultural conditions for growth and SK production have been studied. Maximum growth, as well as SK activity were obtained at pH 7 (Fig. 6), suggesting the neutrophilic characteristics of the strain. Similar results have been reported for several *Streptococcus* spp. The

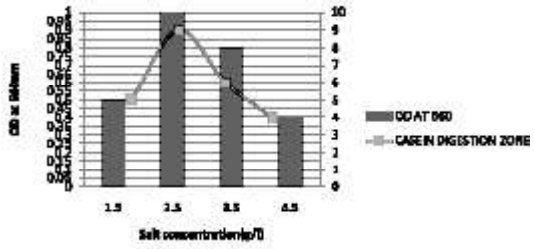

Fig. 5. Effect of salt concentration

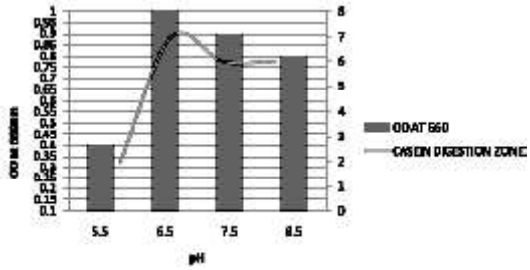

Fig. 6. Effect of pH

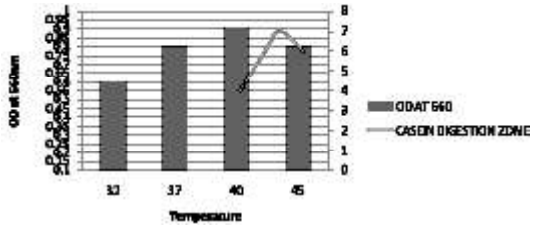

Fig. 7. Effect of incubation temperature

maximum streptokinase production of the mutated strain was obtained at 37°C (Fig.7). In terms of its optimum temperature for growth, the organism appeared to be mesophilic. Hence the study had proven and demonstrated the improvement of streptokinase production ability by UV mutagenesis.

DISCUSSION

Rapid vascular reperfusion plasminogen activators are currently applied as thrombolytic therapy[24]. Although commercially valuable sources of streptokinase are available, there is an urge and increasing potential of SK application for screening and strain improvement studies for the betterment of streptokinase production. Also the application of SK reached exponential increase in various fields. There is a demand in extension of both quantitative enhancement and qualitative improvement. Optimization of the production process is the first step towards decreasing the production costs. As the production rate of wild strains are usually too low, strain improvement and medium optimization for the overproduction of the enzyme depends on the quantitative enhancement. Hence the success of strain improvement in industry is mostly dependent on selection and mutational studies. According to the literature studies the wild strain of *S. equisimilis* T3 subjected to strain improvement studies resulted in higher streptokinase production with greater stability of about 821 U/ml which is 120% higher yield than the wild type. In our study the rate of production supports previous reports and observation with highest of about 0.3U/ml by S. *equisimilis* UV10, which is a significant increase of yield. Hence the present study demonstrated the improvement of strain and its ability to produce enzymes and the study was successful in detecting productive levels of streptokinase at various stages. It's anticipated that this research may aid in gaining a deeper insight into other functional properties of the mutant.

CONCLUSION

The results had proven that UV was an effective mutagenic agent for strain improvement of *S.equisimilis* (MTCC3522) for enhanced SK

productivity. Hence the mutants strain *S. equisimilis* UVM6 can be exploited commercially for large scale industrial production of streptokinase production. The hyper-productive strain by mutagenic treatments can reduce the cost of the enzyme in a bioprocess manufacturing. Also future observations are yet focused on *in vivo* studies of streptokinase, cloning, sequencing, and expression.

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