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Molecular identification of *Streptococcus equisimilis* SK-6 and evaluation of cultural parameters affecting streptokinase production

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ABSTRACT

Streptokinase is one of the best investigated fibrinolytic proteins of microbial origin and finds its application as a therapeutic. The enzyme is a single-chain polypeptide that exerts its fibrinolytic action indirectly by activating the circulatory plasminogen. The present study aims at the molecular identification of streptokinase producing SK-6 isolate by partial 16S rDNA sequencing and determination of various cultural parameters favouring the streptokinase production by the isolate under submerged fermentation. Effects of carbon and nitrogen supplements along with physical parameters such as initial pH of the medium and incubation temperature were considered. Streptokinase activity was determined by the casein digestion method, based on the amount of the liberated tyrosine from digested casein. The SK-6 isolate was found to be closely related to Streptococcus dysgalactiae subsp. equisimilis strain:A-yo with 99% sequence similarity. The 714 bp 16S rDNA nucleotide sequence was provided a GenBank accession number KF312378. Glucose (1% w/v) and tryptone (1.5% w/v) when used as supplements enhanced streptokinase production. Determination of the physical parameters revealed the optimum initial pH and incubation temperature for streptokinase production to be 7.0 and 37°C respectively. The current findings clearly denote that the cultural parameters have a profound effect on the production of streptokinase under submerged fermentation.

Key words: *Streptococcus equisimilis*, streptokinase, 16S rDNA sequencing, cultural parameters.

INTRODUCTION

A sudden rupture of a blood vessel may lead to profuse loss of blood. In order to counteract this condition and induce an effective vascular functioning, a blood clot or thrombus is developed in the circulatory system. However, in case of a faltered homeostasis, development of a thrombus can cause vascular blockage leading to serious consequences including death. Under normal body functioning, the development of a thrombus in circulation is usually suppressed [1, 2].

Thrombolytic therapy has reduced mortality and morbidity in patients with cardiac consequences like stroke, pulmonary embolism, deep vein thrombosis and acute myocardial infarction [3]. Thrombolytic agents have the unique ability to activate the components of the fibrinolytic system that converts plasminogen to a natural fibrinolytic agent called plasmin. The plasmin so produced lyses the clot by breaking down the fibrinogen and fibrin network in the blood clot [4].

Fibrinolytic agents are prevalent among many organisms including snakes, earthworms and bacteria [5]. Some commonly used fibrinolytic agents in thrombolytic therapy are streptokinase, urokinase and tissue type plasminogen activator. In comparison to streptokinase, tissue type plasminogen activator and urokinase are immunologically inert and possess significantly lower *in vivo* half-lives. Therefore, streptokinase is the drug of choice in thrombolytic treatment [6].

Streptokinase is an extra cellular protein, extracted from certain strains of β-haemolytic streptococci of human and animal origin [7, 8]. This plasminogen activator is composed of 414 amino acids with a molecular mass of 47 kDa [9]. It was the first thrombolytic agent for therapeutic use against acute myocardial infarction [10]. The exponential increase in the application of streptokinase in various fields in the last few decades demands extension in both qualitative improvement and quantitative enhancement [1].

The objectives of the present study include molecular identification of the potent streptokinase producing bacterial isolate and determination of its streptokinase production ability under the influence of various cultural parameters.

MATERIALS AND METHODS

Molecular identification of the selected isolate

The genomic DNA isolation, amplification by polymerase chain reaction (PCR) and partial 16S rDNA sequencing of the SK-6 isolate were performed at Chromous Biotech Pvt. Ltd., Bangalore, India.

Extraction of bacterial genomic DNA

24 h old culture of the selected bacterial isolate (grown on brain heart infusion agar) were inoculated into 20 ml of brain heart infusion broth and incubated at 37°C and 150 rpm for 24 h. Following incubation, bacterial biomass was harvested by centrifugation and used for genomic DNA isolation using Bacterial Genomic DNA isolation kit (Chromous Biotech Pvt. Ltd., Bangalore, India). 100 mg of bacterial culture was taken in a pre-cooled sterile mortar and ground to a fine paste with a sterile pestle and 750 µl of 1X suspension buffer. The slurry was taken in a 2 ml vial and 5 µl of RNase A solution was added to it and mixed well. The vial was given a heat treatment at 65°C for 10 min with intermittent mixing. To this 1 ml of lysis buffer was added, mixed well and incubated at 65°C for 15 min. The resultant suspension was centrifuged at 13,000 rpm for 1 min at room temperature. The clear supernatant was collected in a 2 ml vial and loaded on the spin column. Further, the DNA was extracted and purified according to the manufacturer's instructions. The eluted DNA was used for PCR amplification.

PCR amplification

DNA amplification by PCR was performed in a total volume of 100 µl. Each reaction mixture contained the following solutions: 1 µl template DNA, 400 ng forward 16S rDNA primer (5′-AGAGTTTGATCMTGGCTCAG-3′); 400 ng reverse 16S rDNA primer (5′-TACGGYTACCTTGTTACGACTT-3′); 4 µl of dNTPs (2.5 mM each); 10 µl of Taq DNA polymerase assay buffer and 1 µl Taq DNA polymerase (3 U/µl) (Chromous Biotech Pvt. Ltd., Bangalore, India) and water was added up to 100 µl. The ABI 2720 Thermal Cycler (Applied Biosystems, USA) was programmed as follows: 5 min initial denaturation at 94°C, followed by 35 cycles that consisted of denaturation for 30 sec at 94°C, annealing for 30 sec at 55°C and extension at 72°C for 1 min and a final extension of 5 min at 72°C. PCR products obtained were eluted from the gel using Gel Extraction Spin-50 kit (Chromous Biotech, Bangalore, India) according to the manufacturer's instructions. The PCR amplified product was detected by 1.2% agarose gel (with ethidium bromide) electrophoresis.

Partial 16S rDNA sequencing and analysis of sequenced data

Sequencing of the PCR amplified product was performed using Big Dye Terminator Version 3.1 cycles sequencing kit and ABI 3500 XL Genetic Analyzer (Applied Biosystems, USA). 10 µl of the sequencing reaction mixture contained 4 µl of Big Dye Terminator Ready Reaction Mix, 1 µl of rDNA amplification product (100 ng/µl), 2 µl primer (10 pmol/λ) and 3 µl Milli Q Water. The ABI 2720 Thermal Cycler (Applied Biosystems, USA) was programmed to perform initial denaturation at 96°C for 1 min, followed by 25 cycles that consisted of denaturation at 96°C for 10 sec, hybridization at 50°C for 5 sec and elongation at 60°C for 4 min. The resultant nucleotide sequence was analyzed using the software Seq Scape version 5.2. The bacterial species was identified by comparing the sequence with known 16S ribosomal sequences in the NCBI database using BLASTn [11]. The phylogenetic tree was constructed based on fast minimum evolution method [12] using BLAST pairwise alignment between the query and the database sequences. The nucleotide sequence was submitted to GenBank database (NCBI, USA) under an accession number.

Inoculum preparation
The SK-6 isolate was inoculated in 10 ml of mineral salt medium (MSM) (g/L: Glucose, 5.0; yeast extract, 5.0; KH_2PO_4, 2.5; $MgSO_47H_2O$, 0.4; $NaHCO_3$, 1.0; $CH_3COONa3H_2O$, 1.0; $FeSO_47H_2O$, 0.02; $MnCl_2.4H_2O$, 0.02; pH 7.4) and incubated at 37°C for 24 h. Following the development of turbidity, 1 ml of the culture was used as inoculum [13].

Effect of different carbon sources on streptokinase production
The SK-6 isolate was inoculated in the above mineral salt medium supplemented with different carbon sources such as glucose, fructose, maltose, sucrose, xylose and lactose (1% w/v). The concentrations of the best carbon source were also varied (0.1, 0.3, 0.5, 1.0, 3.0 and 5.0% w/v). All the flasks were incubated at 37°C for 24 h.

Effect of different nitrogen sources on streptokinase production
To determine the effect of various organic (peptone, tryptone, yeast extract, beef extract, and casein) and inorganic nitrogen supplements (ammonium chloride, ammonium sulphate, sodium nitrate, ammonium nitrate and sodium nitrite), the SK-6 isolate was inoculated in mineral salt medium. The concentrations of the best nitrogen source were also varied (0.1, 0.3, 0.5, 1.0, 1.5, 3.0 and 5.0% w/v). All the flasks were incubated at 37°C for 24 h.

Effect of initial pH of the media on streptokinase production
Following the optimization of the carbon and nitrogen sources, the effect of initial pH on streptokinase production by SK-6 was determined by adjusting the initial pH of the medium to 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0 and 9.5 using 1N HCL or NaOH. All the flasks were incubated at 37°C for 24 h.

Effect of incubation temperature on streptokinase production
To study the effect of incubation temperature on streptokinase production, the SK-6 isolate was inoculated in the modified mineral salt medium supplemented with the best carbon and nitrogen sources and incubated at 4, 10, 20, 25, 30, 37, 42 and 50°C for 24 h.

Streptokinase activity
Following incubation, the cultures were centrifuged at 10,000 *g* for 30 min. The cell free supernatant was filtered through 0.45 µm cellulose acetate filter and the filtrate was considered as crude enzyme [14].

Streptokinase activity was determined indirectly with casein digestion method, which is based on the determination of the liberated tyrosine from digested casein [15]. Reaction mixture containing 1.6 ml of 0.05 M phosphate buffer, 200 µl of the 1% (w/v) casein solution and 200 µl of the enzyme supernatant was incubated at 37°C for 15 min. The reaction was terminated by adding 5% trichloroacetic acid (10 ml). The precipitated casein was removed by filtration with Whatman No 41 filter paper (Whatman International Ltd., Maidstone, England). 5ml filtrate was removed and 10 ml of 0.5 N NaOH and 3 ml of diluted Folin-Ciocalteu phenol reagent were added. The absorbance at 650 nm was measured and converted to the amount of tyrosine equivalent.

One unit of caseinolytic activity (CU) was defined as the amount of enzyme releasing 1 µ mol of tyrosine equivalent/min. The soluble protein content of the enzyme sample was measured at 660 nm using a spectrophotometer (Chemiline CL 340, India). A standard curve was made using bovine serum albumin as a standard protein [16].

Statistical analysis
All the optimization studies were conducted in triplicate and the data were analyzed using single factor analysis of variance (ANOVA). All the data are graphically presented as the mean ± S.D. of triplicates (n=3). ANOVA was performed using Microsoft Excel 2007. *P* values < 0.05 were considered significant with a confidence limit of 95%.

RESULTS AND DISCUSSION

Microorganisms are capable of growing on a wide range of substrates and can produce a remarkable spectrum of products. For an industrial fermentation process, fermentation medium and fermentation process parameters play a critical role because they affect the formation, concentration and yield of a particular fermentation end product, thereby affecting the overall process economics. Thus it is important to consider the optimization of fermentation medium and process parameters in order to maximize the profits from fermentation process [17].

Most of the streptokinase is obtained from β-haemolytic streptococci of the Lancefield group A, C and G. Group A haemolytic streptococci are highly fastidious, commonly requiring complex media supplemented with various nutritional factors. The group C are preferred for producing streptokinase as they lack erythrogenic toxins [18].

Molecular identification of bacterial isolate SK-6

The molecular identification of the bacterial isolate SK-6 was performed by partial 16S rDNA sequencing. The apparent size of the PCR amplicon was ~ 1.5 kb, shown in **Fig. 1**.

Fig. 1: Agarose gel analysis of PCR amplification product using universal 16S rDNA primers. Lane M, 500 bp DNA molecular size marker; Lane 1, Bacterial isolate SK-6

16S rDNA sequencing is a powerful tool for rapid identification and phylogenetic analysis of bacterial species. The obtained 714 bp 16S rDNA nucleotide sequence was compared with available 16S ribosomal sequences in the NCBI database using BLASTn. The SK-6 isolate has been enrolled into a cluster containing *Streptococcus* sp. and was found to be closely related to *Streptococcus dysgalactiae* subsp. *equisimilis* strain:A-yo with 99% sequence similarity. Hence it was designated as *Streptococcus equisimilis* SK-6. The submitted nucleotide sequence, as depicted in **Fig. 2**, was provided a GenBank accession number KF312378. Based on 16S rDNA sequences, a fast minimum evolution tree revealed that the isolate SK-6 shares a same clade with *Streptococcus equisimilis* and occupies a distinct phylogenetic position within the representative members of the genus *Streptococcus*, as illustrated in **Fig. 3**.

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   1 aggtaaccct acctcatagc gggggataac tattggaaac gatagctaat accgcataaa
  61 agtgtttaac ccatgttaaa catttaaaag gtgcaattgc atcactaatg agatggacct
 121 gcgttgtatt agctagttgg tgaggtaacg gctcaccaag gcgacgatac atagccgacc
 181 tgagagggtg atcggccaca ctgggaactg agacacggcc cagactccta cgggaggcag
 241 cagtagggaa tcttcggcaa tggacggaag tctgaccgag caacgccgcg tgagtgaaga
 301 aggtttttcg gatcgtaaag ctctgttgtt agagaagaat gatggtggga gtggaaaatc
 361 caccatgtga cggtaactaa ccagaaaggg acggctaact acgttgccag cagccgcggt
 421 aatacgtagg tcccgagcgt tgtccggatt tattgggcgt aaagcgagcg caggcggttc
 481 tttaagtctg aagttaaagg caggtggctc aaccattgta cgctttggaa actggagaac
 541 ttgagtgcag aaggggagag tggaattcca tgtgtagcgg tgaaatgcgt agatatatgg
 601 agggaacacc ggtggcgaaa gcggctctct ggtctgtaac tgacgctgag gctcgaaagc
 661 gtggggagca aacaggatta gataccctgg tagtccacgc ccgtaaacga tgag
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Fig.2: The 714 bp 16S rDNA nucleotide sequence of bacterial isolate SK-6

Effect of different carbon sources on streptokinase production

It was reported that presence of sugars like lactose, xylose and sucrose usually prevented bacteria from producing fibrinolytic enzymes, whereas maltose, glucose and fructose, could decrease the catabolite repression and induce enzyme production [19].

The results indicated that among the different sugars supplemented in the liquid media, easily metabolizable sugar like glucose resulted in the highest yield of streptokinase (0.133 µ mol of tyrosine equivalent/min) followed by maltose and fructose **(Fig. 4)**. The probable reason for this result could be the rapid utilization of glucose. The cells can readily transport glucose from the medium inside the cells. Whereas for the other sugars, they have to be first broken down and then transported inside the cell.

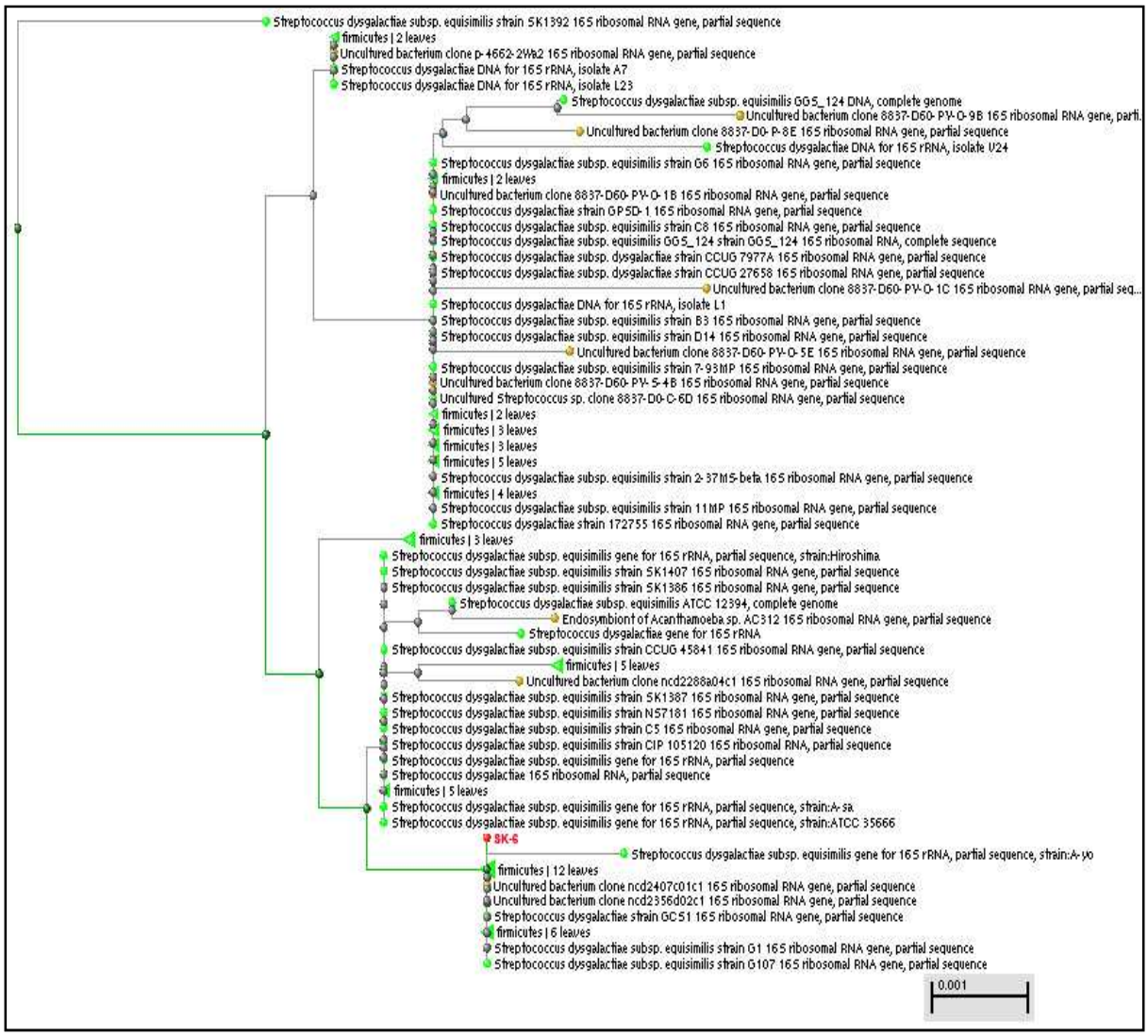

Fig.3: Phylogenetic tree showing the relationship between the bacterial isolate SK-6 (unknown) and related *Streptococcus* sp. from NCBI database based upon fast minimum evolution analysis of partial 16S rDNA sequences

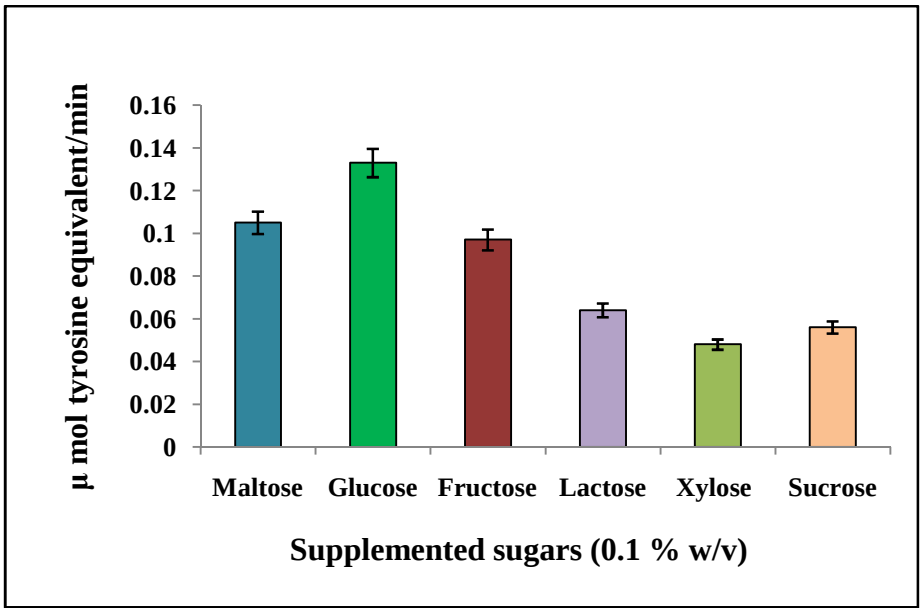

Fig.4: Effect of different sugar supplements on streptokinase production from *S. equisimilis* SK-6. Data represent mean ± S.D. (n=3); $P < 0.05$

In accordance to the findings of the present study, streptokinase production by both *S. pyogenes* and *S. equisimilis* was significantly affected by the carbon source incorporated in the medium. Both cultures of *Streptococcus* secreted the highest titre of streptokinase when grown on Strep-base medium supplemented with 0.5 % glucose. Mannitol or sorbitol did not support streptokinase production. Streptokinase production remained high even when a low concentration of glucose (0.25 %) was used. However, a higher concentration of glucose (1 %) did not help in improving the production level of the enzyme in both cultures [4]. Similarly, brain heart infusion broth medium with 1% glucose concentration gave the best results for streptokinase production from *S. dysgalactiae* subsp. *equisimilis* isolated from clinical cases suffering of tonsillitis or pharyngitis [20].

Earlier, glucose and casein hydrolysate medium supplemented with various salts was used for producing streptokinase from *S. equisimilis* [21]. Medium containing glucose, peptone, phosphate salts, biotin, riboflavin, tryptophan, glutamine and nucleic acids like thiamine, adenine and uracil was used to produce streptokinase from *S. equisimilis* H46A [22].

Effect of glucose concentration on streptokinase production

S. equisimilis SK-6 produced the highest titre of streptokinase (0.153 µ mol of tyrosine equivalent/min) in the presence of 1% (w/v) glucose in the medium. Both low and high concentration of the sugar reduced the streptokinase production **(Fig. 5)**. The reduction in the streptokinase production at higher glucose concentration may be attributed to the induction of Crabtree effect. Literatures suggest that high glucose causes Crabtree effect and leads to accumulation of acetate which is inhibitory to cell growth [23]. Simultaneously, the Crabtree effect results in wastage of glucose and generation of toxic by-products that often limit protein yields [24, 25].

Among the various expression systems used, the quantity of recombinant streptokinase produced in the medium containing 2.4% glucose was more than two-fold compared to the medium containing 0.2% glucose [26]. The production of streptokinase by *S. faecalis* was maximized when glucose was in excess and the other nutrients were present in limiting amounts [27]. However, low glucose concentration at inoculation allowed growth to become well established without excessive production of acid from sugar fermentation [22].

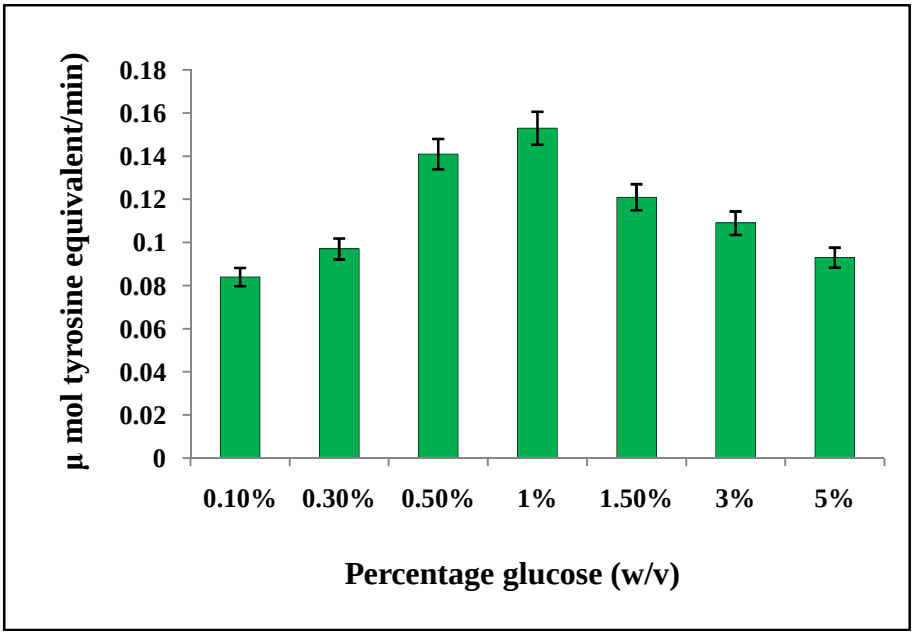

Fig.5: Effect of percentages of glucose on streptokinase production from *S. equisimilis* SK-6. Data represent mean ± S.D. (n=3); $P < 0.05$

Effect of different nitrogen sources on streptokinase production

It has been observed that the presence of nitrogen sources in the medium provides nutrients and precursors for synthesis of the building blocks of cells and helps to decrease the inhibitory effect of acetic acid [26].

Among the different nitrogen supplements, organic nitrogen sources proved to be superior in comparison to the inorganic sources with tryptone resulting in the highest streptokinase production (0.157 µ mol of tyrosine equivalent/min) followed by casein, yeast extract and peptone **(Fig. 6)**.

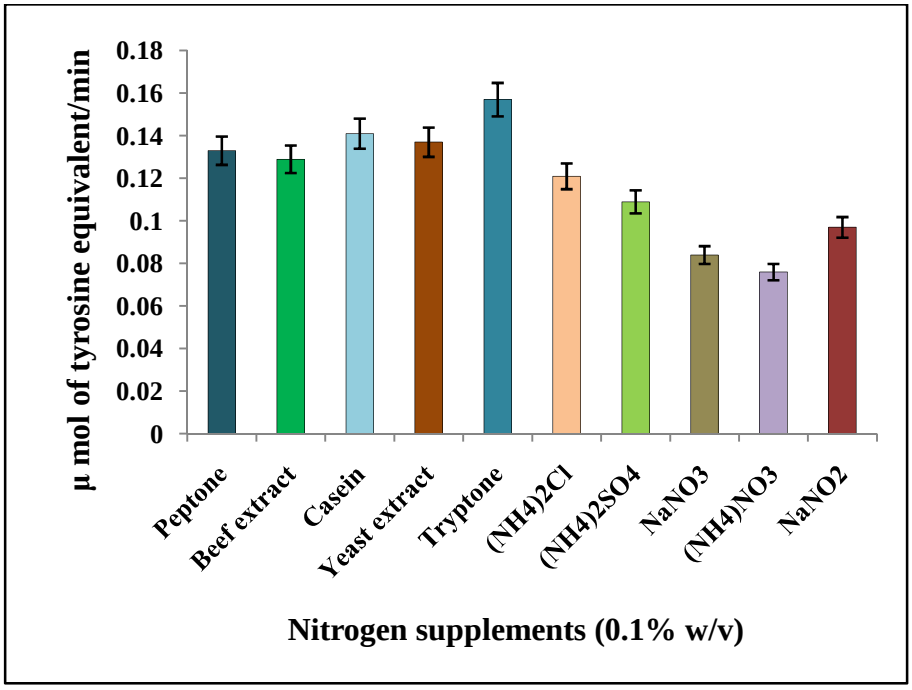

Fig.6: Effect of different nitrogen supplements on streptokinase production from *S. equisimilis* SK-6. Data represent mean ± S.D. (n=3); *P* < 0.05

Likewise, supplementation of production medium with yeast extract and tryptone and replacement of liquid ammonia with NaOH as pH control agent further enhanced streptokinase expression without affecting cell growth [28].

Effect of tryptone concentration on streptokinase production

S. equisimilis SK-6 produced the highest titre of streptokinase (0.153 µ mol of tyrosine equivalent/min) in the presence of 1.5% (w/v) tryptone in the medium. Both low and high concentration of tryptone reduced the streptokinase production **(Fig. 7)**.

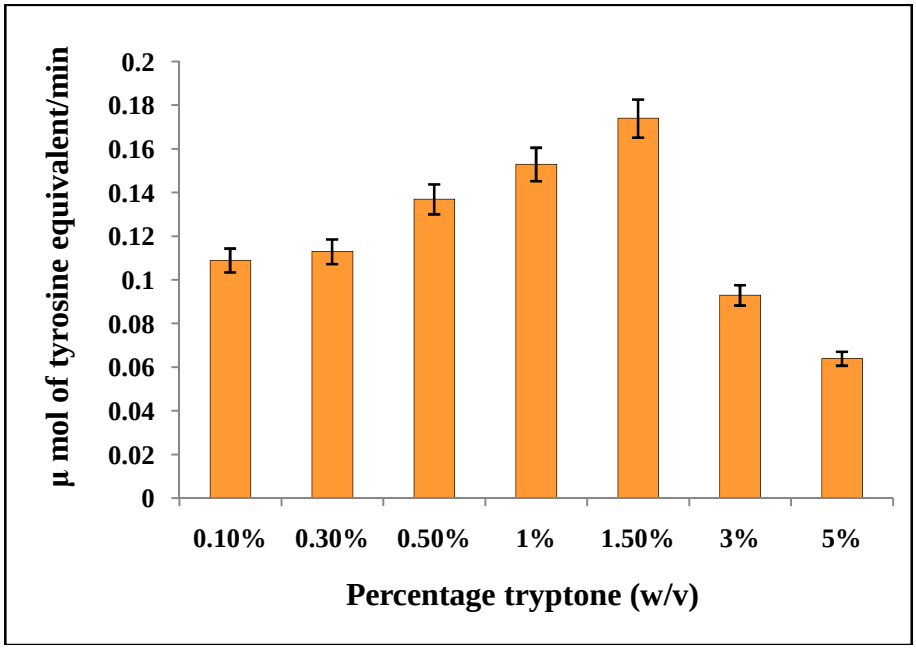

Fig.7: Effect of percentages of tryptone on streptokinase production from *S. equisimilis* SK-6. Data represent mean ± S.D. (n=3); *P* < 0.05

In order to enhance streptokinase yield, the concentration of organic nitrogen sources like tryptone in medium was increased [26]. In accordance to the present finding, while optimizing streptokinase production from two

streptococcal strains grown in a low cost growth medium, *S. equisimilis* gave the highest streptokinase production when grown on Strep-base medium supplemented with 1.5 % (w/v) tryptone [4].

Effect of initial pH of medium on streptokinase production

Among the physical parameters, pH of the growth medium plays an important role by inducing morphological changes in microbes and in the enzyme secretion. Microorganisms are sensitive to the concentration of hydrogen ions present in the medium. Thus pH change observed during the growth of microbes also affects product stability in fermentation medium [29]. Different organisms have different pH optima and decrease or increase in pH on either side of the optimum value results in poor microbial growth [30]. Studies on the growth of a β-hemolytic *Streptococcus* revealed that pH is a limiting factor [31].

The obtained results demonstrated that streptokinase production was maximum at pH 7.0 (0.206 µ mol of tyrosine equivalent/min). On the other hand, relatively low streptokinase production was recorded at extremes of pH **(Fig. 8)**. The comparison of the present study with other studies shows that the optimum pH for cell growth and streptokinase production is 7.0 [32]. A medium containing corn steep liquor, cerelose, KH_2PO_4 and $KHCO_3$ at pH 7.0 was used for producing streptokinase [33].

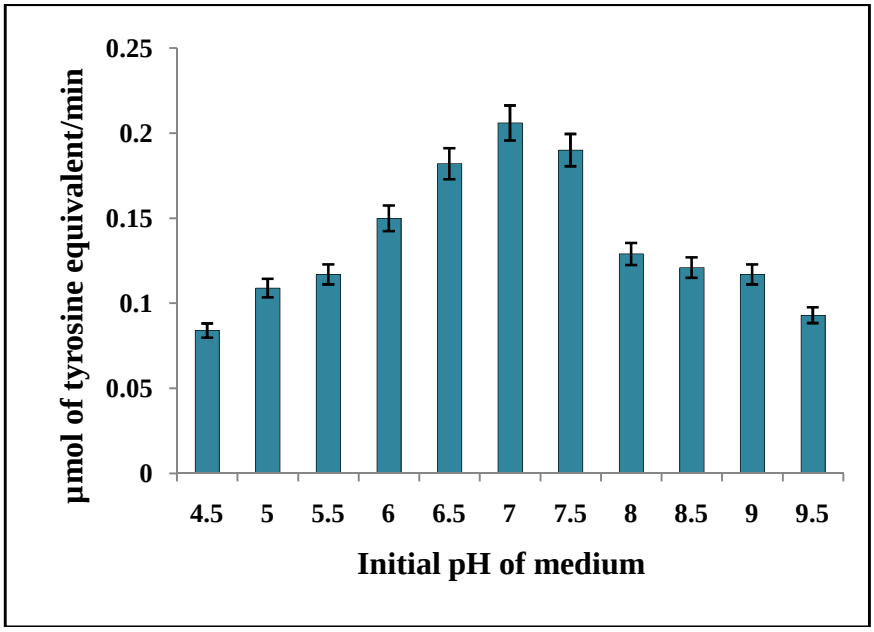

Fig.8: Effect of initial pH of the medium on streptokinase production from *S. equisimilis* SK-6.
Data represent mean ± S.D. (n=3); $P < 0.05$

Results from batch cultures indicated that strain *S. equisimilis* H46A produced relatively high yields of streptokinase when the pH in the culture was controlled within the range of 7.0 to 7.1. At pH levels lower than 6.5 or higher than 7.8, streptokinase production decreased to less than 25% of that obtained at neutral pH. By regulating the incubation period, glucose feeding and adjustment of the pH to neutrality, the rate of streptokinase increased up to three times [14].

Simultaneous adjustment of pH, inoculation rate and proper glucose feeding could produce relatively high yields of streptokinase. Because of high sensitivity of streptokinase to sudden pH and temperature changes, some part of this product is degraded at the same time [34].

Effect of incubation temperature on streptokinase production

Temperature influences cell growth, protein denaturation and enzyme inhibition, thereby playing a significant role in the development of the biological processes [35]. Influence of incubation temperature on streptokinase production by *S. equisimilis* SK-6 was studied, wherein 37°C supported the maximum streptokinase production (0.218 µmol of tyrosine equivalent/min). Streptokinase production was moderate over the mesophilic temperature range and drastically decreased at low and high temperatures **(Fig. 9)**.

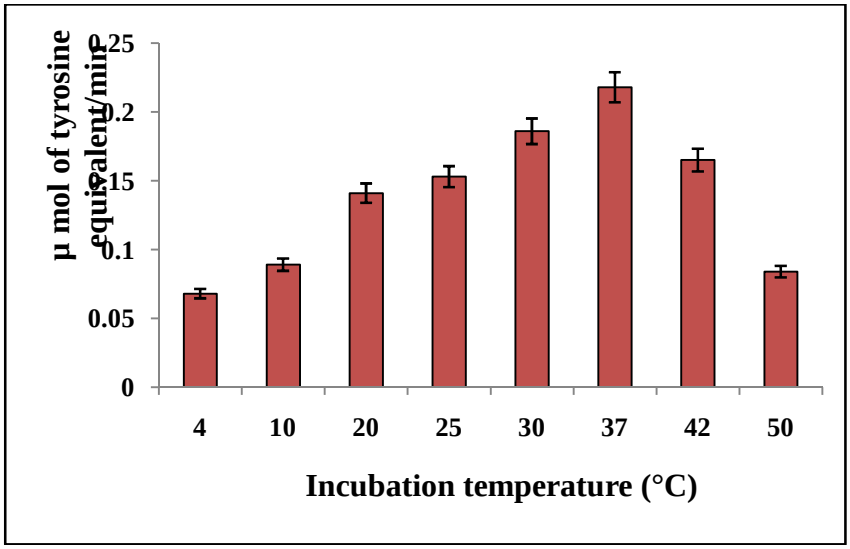

Fig.9: Effect of incubation temperature on streptokinase production from *S. equisimilis* SK-6. Data represent mean ± S.D. (n=3); $P < 0.05$

Copious quantities of streptokinase were produced using a mutant *Streptococcus.* The culture medium consisted of casein or serration peptidase hydrolysed casein, glutamine, cystine and yeast extract. The mutant was cultured at pH 6.8-7.2, 35-38°C, in broth aerated at 0.1-1.0 rpm. The titre of streptokinase exceeded 8,500 U/ml [36]. A previous study records the influence of temperature on the streptokinase production at two different pH levels. The dry weights seems to be slightly higher if the temperature is 1 or 2 degrees below 37°C, but the streptokinase production capacity was somewhat lower when calculated per mg dry weight of bacteria [37].

Influence of cultivation temperature on growth and streptokinase production of *S. equisimilis* H46A has been investigated over the temperature range of 28-43°C. The H46A strain was capable of growth over the entire temperature range, but 28°C was the optimal growth temperature [38].

CONCLUSION

In course of the study it was found that cultural parameters have a profound effect on the production of streptokinase under submerged fermentation. Glucose and tryptone when used as carbon and nitrogen sources can improve the streptokinase yield. Fermentation at neutral pH and 37°C facilitated the highest yield. The current findings clearly denote that *S. equisimilis* SK-6 has a remarkable potency for the production of streptokinase.

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