

*Full Length Research Paper*

# **In vitro detection and optimization of streptokinase production by two streptococcal strains in a relatively low cost growth medium**

**Mahmoud Abd El-Mongy<sup>1</sup> and Taher M. Taha<sup>2</sup>**

<sup>1</sup>Microbial Biotechnology Department, Genetic Engineering and Biotechnology Research Institute, Minufiya University, Sadat City, Egypt

<sup>2</sup>Botany and Microbiology Department, Faculty of Science, AL-Azhar University, Assiut, Egypt

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**This study sought to demonstrate the optimization of Streptokinase Production. Enzyme production was monitored during the growth of both *Streptococcus pyogenes* and *Streptococcus equisimilis* in different media. Adjustment of the pH for culture media of *S. pyogenes* and *S. equisimilis*, every 12 hours during incubation, significantly increased the enzyme production levels, when both microbes were grown on Strep-base medium. The best carbon source for streptokinase production was glucose of both *S. pyogenes* and *S. equisimilis*, while mannitol and sorbitol were found to be improper carbon sources. Yeast extract, and casein could be used as the primary source of organic nitrogen for streptokinase production, when the microbes were allowed to grow on Strep-base medium. The highest levels of the enzyme production were obtained with 1.5 % (w/v) tryptone and 1.5 % (w/v) casein for *S. equisimilis* and *S. pyogenes*, respectively. Detection of streptokinase produced was by the common casein digestion method and by the more sensitive chromozym substrate digestion method. Moreover, the enzyme was assayed electrochemically using the protamine-sensitive electrode to compare different methods of detection. Results obtained from electrochemical method were very close to that obtained with other methods. These results offer alternative and reliable method for streptokinase detection during microbial growth. It provides a faster and less expensive technique for streptokinase determination especially when there is a need to detect the enzyme in turbid media.**

**Keywords:** Streptococcus, streptokinase, optimization.

## **INTRODUCTION**

Streptokinase is an enzyme produced by streptococcus bacteria which is used in hospitals to dissolve the fibrin of blood clots, especially those in the arteries of the heart and lungs. It is also used on the clots formed in shunts during kidney dialysis. A fast-acting drug, streptokinase is most effective in dissolving newly-formed clots and is often released at the site of the clot via a catheter inserted into an artery. Administered in the early stages of a heart attack to dissolve a clot in the coronary arteries (thrombosis) it can reduce the amount of damage to heart

muscle. Because excessive bleeding is a common side effect treatment is closely supervised. The drug is also used to treat wounds and ulcers in combination with another enzyme, streptodornase, for which use it is applied locally the SK-plasminogen complex is an efficient proteolytic activator of plasminogen (Malke *et al.*, 1984).

The blood clot or thrombus, which is the cause of strokes, consists of blood cells trapped in a matrix of the protein fibrin. Finding the cure for strokes and myocardial infarction has been taken into consideration since early decades of the twentieth century (Baruah *et al.*, 2006). Anticoagulants have been used for treatment of myocardial infarction in the early 1940s, but this treatment was limited with no reduction in mortality rates

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\*Corresponding Author E-mail: [m\\_abdelmongy@yahoo.com](mailto:m_abdelmongy@yahoo.com)

in patients treated with oral administration of the drug (Wasserman *et al.*, 1966). On the other hand, plasminogen activators were found to be the most effective drugs used in treatment of strokes and myocardial infarction (Collen *et al.*, 1988; Francis; Marder, 1991; and Zivin 2009). Plasminogen activators are fibrinolytic agents that work by converting plasminogen to the natural fibrinolytic agent, consequently the produced plasmin lyses clot by breaking down the fibrinogen and fibrin contained in a clot (Feied and Handler, 2004). Plasminogen activators can be classified into direct and indirect (Iqbal *et al.*, 2002). Direct activators (e.g. tissue plasminogen activator) are highly specific limited serine proteases that directly cleave a single Arg 561-val 562 bond in the plasminogen molecule to yield plasmin (Baruah *et al.*, 2006). Indirect plasminogen activators (e.g. streptokinase) are kinase enzymes that are mainly produced by bacteria but do not have proteolytic action. They forms a 1:1 stoichiometric complex with plasminogen that can convert additional plasminogen to plasmin (Iqbal *et al.*, 2002; Feied and Handler, 2004; Banerjee *et al.*, 2004).

Streptokinase is the most widely distributed plasminogen activator especially in the world's poorer health care system because of its low relative cost (Banerjee *et al.*, 2004; Feied and Handler, 2004). The enzyme has a relatively long half-life period comparing to other plasminogen activators and it has a high affinity for circulatory plasminogen (Banerjee *et al.*, 2004). The enzyme was first isolated in 1933 and entered clinical use in mid 1940s (Feied and Handler, 2004).

It is an extracellular enzyme that is produced by various strains of  $\beta$ -hemolytic streptococci. It is a single-chain polypeptide with a molecular weight of 47 kDa (Malke and Ferretti, 1984 and Dubey *et al.*, 2011) is made up of 414 amino acid residues (Malke and Ferretti, 1984). The enzyme does not contain conjugated carbohydrates or lipids (Banerjee *et al.*, 2004).

Streptokinase has a multiple domains structure with,  $\alpha$ -,  $\beta$ - and  $\gamma$ -domains. The three domains have different associated functional properties (Welfle *et al.*, 1992). Gamma domain is essential for plasminogen activation (Wu *et al.*, 2001), while  $\beta$ -domain is responsible for the formation of streptokinase-plasminogen complex which is in turn responsible for activating the plasminogen (Banerjee *et al.*, 2004). It has been found that the N-terminal domain (residues 1–59) is responsible for the high plasminogen activation ability rather than the 60–414 amino acid residues domain of the protein (Nihalani *et al.*, 1998). Moreover, streptokinase has an unstable secondary structure as well as a greatly reduced activity of the remaining enzyme fragment without these N-terminal 1-59 amino acids residues (Young *et al.*, 1995). Most of the streptokinases that are used as clot dis-

solving drugs are obtained from  $\beta$ -hemolytic streptococci especially those strains which are isolated from human origin as they lack erythrogenic toxins (Hagenson *et al.*, 1989; Wong *et al.*, 1994).

Streptokinase determination depends on its ability to activate plasminogen to plasmin which in turn, hydrolyzes an indicator substrate and the extent of hydrolysis over a given period of time is related back to the concentration of streptokinase. Substrates for plasmin may include the fibrin clot, casein, other proteins, and various synthetic esters (e.g., lysine methyl ester, lysine ethyl ester, L-arginine methyl ester), which have been used successfully for the sensitive detection of the enzyme activity (Pratap and Dikshit, 1998; Pratap *et al.*, 2000; Mundada *et al.*, 2003). Radial caseinolysis method of agarose gel containing both casein and plasminogen is commonly used for simple detection of the enzyme (Saksela, 1981). The method is simple but it lacks accuracy and it is time consuming. Chromogenic detection for plasmin using the plasmin specific tripeptide H-D-valyl-leucyl-lysine-p-nitroaniline ((Kulisek *et al.*, 1989; Wohl *et al.*, 1980; Leigh *et al.*, 1998; Ringdahl *et al.*, 1998; Wang, S.G., *et al.*, 1999) or, tosyl-glycyl-prolyl-lysine-4-nitroanilide (Wong *et al.*, 1994; Yazdani and Mukherjee, 1998, 2002) has been reported as a sensitive method for enzyme assay. Plasmin activity could be determined electrochemically by detecting its proteolytic activity on the arginine-rich peptide protamine (Abd-Rabboh *et al.*, 2003). The assay was based on the use of a macromolecule polycation/polyanion substrate; a complex of protamine and pentosan polysulfate (PPS), a highly sulfated polysaccharide. As plasmin cleaves the protamine within the complex, free PPS is generated and potentiometrically detected via a polyanion sensitive membrane electrode. The method could be used to detect streptokinase indirectly. The most desirable advantages of detecting the enzyme using biosensors are their accuracy, speed, and easy automation (Wang, 1999). These analytical devices give great promises in clinical and industrial applications.

Several attempts were made to increase streptokinase production by growing  $\beta$ -hemolytic streptococci on different media supplemented with various nutritional factors for growth (Christensen, 1945; Holmstrom, 1968; Hyun *et al.*, 1997; and Patel *et al.*, 2011), but commonly, the enzyme is produced from  $\beta$ -hemolytic streptococcal strains by growing cells on the rich complex Brain Heart Infusion (BHI) (Malke and Ferretti, 1984), or Todd Hewitt (Vieira *et al.*, 1998) media. In this work, we monitored the streptokinase production by two streptococcal strains in four different growth media and optimized some conditions for effective enzyme production, by the studied streptococcal strains, in relatively cheap medium. Comparison of different determination methods of streptokinase is also done.

## MATERIALS AND METHODS

### Bacterial growth and streptokinase production

Two bacterial strains, *S. pyogenes* ATCC 21548 and *S. equisimilis* ATCC 12388, were used in this work as the source of extracellular streptokinase. Cells were activated by growing them overnight on Strep-base medium containing 10 g tryptone, 5 g yeast extract, 2 g K<sub>2</sub>HPO<sub>4</sub>, 5 g NaCl, and 5 g glucose per liter. The pH was adjusted to 7.4 by the addition of 1N NaOH. Cultures were grown under 100 rpm shaking conditions at 37°C. One ml of the previously grown cultures was used to inoculate 100 ml of the different media used for enzymatic production. Cultures were grown under 100 rpm shaking conditions at 37°C. Growth was monitored by measuring the absorbance at 600 nm (Ko *et al.*, 1995), which was very much correlated with the number of viable cells count. At the indicated time 1.5 ml of each of the growing cultures was taken and centrifuged in a microcentrifuge at 7,000 rpm for 2 minutes. The supernatants were used as a crude sample to measure the activity of streptokinase enzyme.

To monitor streptokinase production by both *S. pyogenes* and *S. equisimilis* grown on different microbiological media, the same previous procedure was carried out to inoculate Brain Heart Infusion medium (BHI), Todd-Hewitt medium, Blood Base medium, and Strep-base medium.

### Effect of pH on streptokinase production during growth

Both *S. pyogenes* and *S. equisimilis* were activated overnight by growing them on Strep-base medium at 37°C with shaking at 100 rpm. For each bacterium, a hundred ml BHI medium was inoculated with one ml of the previously activated culture. Growth curve and streptokinase production were monitored by taking samples at the desired time intervals. At the indicated time, a sample was taken for pH measurement. The pH of the growing culture was adjusted by the addition of 5N NaOH.

### Effect of different carbon sources on streptokinase production

Strep-base medium was used to study the effect of different carbon sources on streptokinase production by both *S. pyogenes* and *S. equisimilis*. Sucrose, glucose, mannitol, sorbitol, and lactose were used as pure soluble sugars for supplement of primary carbon source in the medium. Sugars were supplemented at concentrations of 2.5 g, 5 g, and 10 g per liter. Modified Strep-base contained 10 g tryptone, 5 g yeast extract, 2 g K<sub>2</sub>HPO<sub>4</sub>, 5 g NaCl, and appropriate weight of sugar under investigation per liter. The pH was adjusted to 7.4 by the addition of 1N NaOH.

### Effect of different organic nitrogen sources on streptokinase production

Strep-base medium was used to study the effect of different organic nitrogen sources on SK production by both *S. pyogenes* and *S. equisimilis*. Tryptone, yeast extract, peptone, casein, and beef extract were used as the primary source of organic nitrogen in the medium at concentrations of 5 g and 10 g per liter. Modified Strep-base contained 2 g K<sub>2</sub>HPO<sub>4</sub>, 5 g NaCl, and 5 g glucose, and appropriate weight of the organic nitrogen source under investigation per liter. The pH was adjusted to 7.4 by the addition of 1N NaOH.

### Enzyme activity

#### Casein digestion method

Streptokinase activity was determined indirectly with casein digestion method, which is based on the determination of the liberated tyrosine from digested casein after plasminogen activation (Mounter and Shipley, 1957). Activity was determined according to a modified method of Sutar *et al.* (1986). Reaction mixture (2 ml) containing 10 mg casein, 50 mM Tris-HCl, pH 8.0, containing 0.1 ml (or an appropriate dilution) of supernatants. The reaction was carried out at 37°C for 20 minutes then it was terminated by the addition of 2.6 ml 5% w/v trichloroacetic acid (TCA) and 0.4 ml 3.3 M HCl. Reactions were then kept on ice for 30 minutes after which they were filtered using Whatman paper #1. The absorbance of the TCA soluble fractions were measured at 280 nm. Units of enzyme activity were calculated with a standard SK curve.

#### Chromozym activity test

Streptokinase activity was determined by the colorimetric method with N-p-tosyl-glycyl-prolyl-lysine-p-nitroanilide acetate (Chromozym PL; Boehringer) as the substrate for Plasmin enzyme (Wong *et al.*, 1994; Yazdani and Mukherjee, 2002). Samples were mixed with Plasminogen and the mixture was incubated at 37°C for about 5 minutes. The substrates mixture containing the chromozym dissolved in 50 mM Tris-HCl buffer pH 8.0 was then added to the enzyme-substrate mixture. The reaction was incubated for 20 minutes at 37°C and the change in absorbance at 405 nm was monitored at 37°C by using a spectrophotometer. Units of enzyme activity were calculated with a standard streptokinase curve.

#### Electrochemical assay of streptokinase

Three separate DNNS-based protamine-sensitive membrane electrodes were used simultaneously to monitor the initial decrease in protamine levels (Chang *et al.*, 1999). Experiments were performed by adding 5 µl of

a 5 mg/ml protamine (Sigma, St. Louis, MO) solution to 1 ml of Tris working buffer (50 mM Tris and 120 mM NaCl, pH 7.4). After reaching a steady-state/ non-equilibrium response (3 min), 100  $\mu$ l of a preincubated (5 min) sample of centrifuged bacterial culture solution (40  $\mu$ l), human plasminogen (0.45 U, Sigma), all in Tris working buffer. The decrease in the EMF response toward protamine was monitored over a 5-min period by each of the sensors. A calibration plot for streptokinase was constructed by graphing the initial rate of the potential decrease, in mV/min against streptokinase activity, in IU/ml sample.

### Preparation of polycation-sensitive electrode

Protamine-sensitive membrane electrodes were prepared according to the method described by (Ramamurthy *et al.*, 1998). Electrochemical EMF measurements were made vs an Ag/AgCl reference electrode using a VF-4 (World Precision Instruments, Sarasota, FL). Sensors were calibrated for protamine by adding different concentrations of 1 mg/ml protamine solution, to 4.9 ml of Tris-HCl buffer with continuous stirring of the test solution at a constant rate. The EMF changes of the membrane electrodes were measured 2 min following each sequential addition of protamine solution, at which time the non-equilibrium steady-state potential had been reached. Calibration curves were plotted using the net potential change from the baseline value ( $\Delta$ EMF) versus the concentration of the polycation.

### Protein determination

Protein concentrations in different fractions were determined as described by (Bradford, 1976). Protein content was measured at 595 nm using a spectrophotometer. A standard curve was made using Bovine serum albumin as a standard protein. Blank reagent was 100  $\mu$ l of the propitiate buffer and 5 ml of protein reagent.

## RESULTS

Streptokinase production in different growth media by two streptococcal strains *S. pyogenes* and *S. equisimilis* are streptokinase hyperproducing streptococcal strains (Steiner and Malke, 2002; Banerjee *et al.*, 2004). These two strains show a Beta hemolysis which is caused by one or more streptolysins (Wolef and Liljemark, 1978). To monitor the enzyme production during the bacterial growth curve of both strains, they were grown on different media. Brain Heart Infusion (BHI) (Malke and Ferretti, 1984; Gase *et al.*, 1995) and Todd-Hewitt (Vieira *et al.*, 1998; Caballero *et al.*, 1999) are both specific media that

have been used in several reports for cultivation of streptococci and streptokinase production. Streptokinase reached 88.5 units per ml when *S. pyogenes* was grown on BHI medium (streptokinase unit is the amount of streptokinase that catalyzes the production of 1  $\mu$ mol of plasmin from plasminogen in one minute at standard conditions). Specific activity reached 27.48 units per mg protein and remained over 20 units per mg protein for 24 hours of cultivation. A similar result was observed when *S. pyogenes* was grown on Todd-Hewitt medium; the production level was 85.12 units after 7 hours of cultivation. Specific activity also reached a maximum level of 28.74 units per mg protein with a steady state pattern over 15 hours of cultivation (Table 1). Streptokinase production level was 91.6 units per ml and 85.2 units per ml (Table 1) when *S. equisimilis* was grown on BHI and Todd-Hewitt media, respectively. The high level of production remained over 80 units per ml along the incubation period for both media. The specific activity of the produced enzyme reached over 30 units per mg protein for both cultures. Production patterns of streptokinase when both *S. pyogenes* and *S. equisimilis* are grown on previous media are shown in Figure 1.

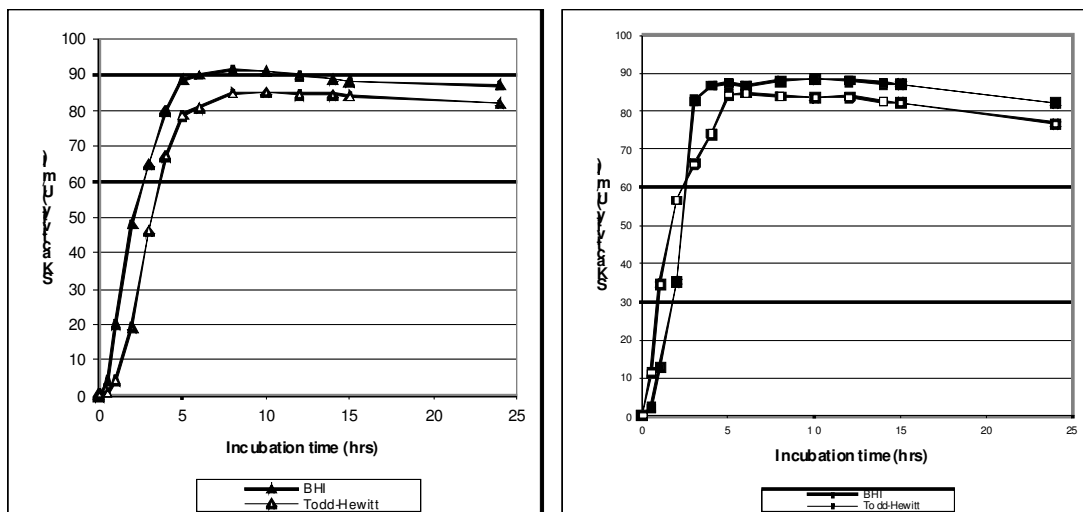
In an attempt to produce streptokinase from *S. pyogenes* and *S. equisimilis* using relatively inexpensive media, Strep-base and blood base media were used for the microbial growth. When *S. pyogenes* was grown on Strep-base medium, its enzymatic production did not reach its maximum activity at the end of the exponential phase like it did upon growing the microbe on BHI medium. The maximum enzyme production was relatively low, 54.96 units per ml (Table 2). On the other hand, streptokinase production reached 74.3 units per ml (Table 2) when *S. pyogenes* was grown on Blood base media. Specific activity reached 31.70 units per mg protein in Blood base medium (i.e., the specific activity values are too close to that obtained upon growing the bacterium on BHI and Todd Hewitt media). The enzyme production was relatively low (40.82 units per ml) when *S. equisimilis* was grown on Strep-base medium (Table 2), but the production level reached 83.07 units per ml when the microbe was grown on Blood-base medium. The specific activity for streptokinase in blood base medium exceeds 29 units per mg protein (Table 2). Streptokinase production patterns when both *S. pyogenes* and *S. equisimilis* are grown on previous media are shown in Figure 2.

### Effect of pH on streptokinase production during bacterial growth

Streptokinase production by both *S. pyogenes* and *S. equisimilis* was greatly affected by the change of the medium pH during the growth of the microbes. Samples were taken from all cultures after three days of incubation, the measured pH in all samples ranged from 5.6 to 6.1;

**Table 1.** Streptokinase production during growth of *S. pyogenes* and *S. equisimilis* grown on BHI and Todd-Hewitt media

Incubation Time (hrs.)	Streptokinase production (U/mg protein), <i>S. pyogenes</i> grown on		Streptokinase production (U/mg protein), <i>S. equisimilis</i> grown on	
	BHI	Todd-Hewitt	BHI	Todd-Hewitt
0	0.87	1.15	0.28	3.75
0.5	2.92	13.82	7.87	2.64
1	12.01	22.36	21.04	7.46
2	20.94	25.17	31.73	15.88
3	26.48	26.87	32.55	24.33
4	26.04	27.74	34.57	31.25
5	24.6	26.29	31.04	29.63
6	22.41	23.85	31.75	27.55
8	23.56	21.54	28.34	25.05
10	23.44	21.52	24.87	24.28
12	23.42	22.18	26.15	22.35
14	24.31	22.84	22.91	21.3
15	21.06	21.19	20.93	19.45
24	19.35	17.14	21.08	19.19

**Figure 1.** Streptokinase production during growth of (a) *S. pyogenes* and (b) *S. equisimilis*

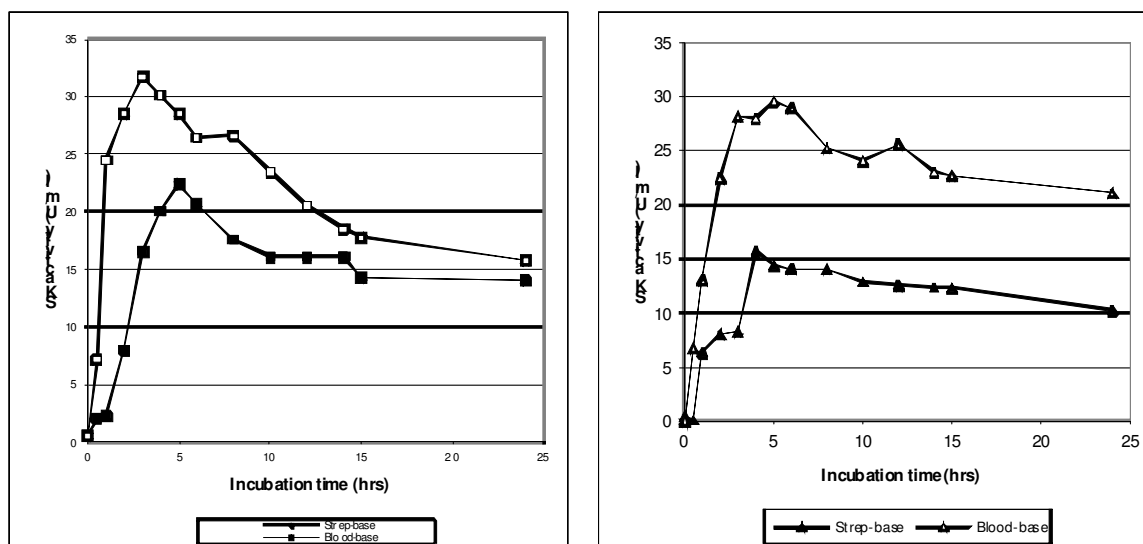
that drop of pH may affect the bacterial growth as well as the SK production. Acidification of the medium occurs as a result of bacterial growth and metabolic consumption of sugars found in the medium (Mickelson, 1964). When the pH was adjusted every 12 hours for both *S. pyogenes* and *S. equisimilis* grown on Strep-base medium, streptokinase production was almost doubled at the end of the 72 hours of incubation (Table 3). These results suggest a longer and more controllable production of the

enzyme if both *S. pyogenes* and *S. equisimilis* are allowed to grow in a continuous fermentation system.

Effect of different carbon sources on streptokinase production during bacterial growth. Streptokinase production by both *S. pyogenes* and *S. equisimilis* was significantly affected by the carbon source found in the medium. Maximum production of the enzyme was obtained when both microbes were grown on Strep-base medium in which 0.5 g% glucose was used as the sole

**Table 2.** Streptokinase production during growth of *S. pyogenes* and *S. equisimilis* on Strep-base and blood base media

Incubation Time (hrs.)	Streptokinase production (U/mg protein), <i>S. pyogenes</i> grown on		Streptokinase production (U/mg protein), <i>S. equisimilis</i> grown on	
	Strep-base	Blood base	Strep-base	Blood base
0	0.54	0.44	0.36	0.01
0.5	1.98	6.16	0.1	5.63
1	2.41	23.42	5.55	12.06
2	7.78	27.40	7.05	21.85
3	15.78	30.80	7.35	27.29
4	19.09	29.18	14.98	27.20
5	21.72	27.58	13.55	28.87
6	19.77	25.65	13.21	28.09
8	16.82	25.64	13.07	24.35
10	15.06	22.56	11.88	23.17
12	15.09	19.77	11.66	24.55
14	15.22	17.44	11.50	22.33
15	13.66	16.87	11.67	21.82
24	13.07	14.85	9.87	20.18

**Figure 2.** Streptokinase production during growth of (a) *S. pyogenes* and (b) *S. equisimilis*

source of carbon. The enzyme production was not supported by using 0.5 g% mannitol or sorbitol as the primary source of carbon (Table 4). Streptokinase production remained high even when a low concentration of glucose (0.25 g %) was used. However, a higher concentration of glucose (1 g %) did not help in improving the production level of the enzyme in both cultures.

Effect of different organic nitrogen sources on streptokinase production during bacterial growth both *S. pyogenes* and *S. equisimilis* were grown on Strep-base medium which contained different sources of organic

nitrogen. Generally, 1.5g % of organic nitrogen gave the highest SK production in both cultures (Table 5). Both casein and yeast extract supported a high level of enzyme production when *S. pyogenes* was grown on the modified Strep-base medium suggesting that these two sources of organic nitrogen could be used as replacement for tryptone in the original Strep-base medium.

*S. equisimilis* gave the highest SK production upon growing on Strep-base medium supplemented with 1.5g % tryptone (Table 5).

**Table 3.** Effect of pH on streptokinase production by *S. pyogenes* and *S. equisimilis*

Incubation Time (hrs)	<i>S. pyogenes</i> SK activity (U/ml)		<i>S. equisimilis</i> SK activity (U/ml)	
	without pH adjustment	with pH adjustment	without pH adjustment	with pH adjustment
24	55.33	52.48	44.32	42.7
48	37.15	47.43	29.78	37.56
72	21.44	40.43	18.75	34.22

Both microbes were grown on Strep-base medium to the indicated time  
pH was adjusted every 12 hours of incubation by 5N NaOH  
The final pH value in the adjusted flasks was 7.4

**Table 4.** Effect of carbon source on streptokinase production by *S. pyogenes* and *S. equisimilis*

Carbon source	<i>S. pyogenes</i> SK activity (U/ml)			<i>S. equisimilis</i> SK activity (U/ml)		
	0.25g %	0.5g %	1g %	0.25g %	0.5g %	1g %
Sucrose	8.5	8.3	5.6	8.6	6.4	3.7
Glucose	53.4	52.8	48.6	39.2	43.5	34.7
Mannitol	0.0	0.2	0.0	0.0	0.0	0.0
Sorbitol	0.2	0.2	0.0	0.0	0.0	0.0
Lactose	18.6	16.7	11.3	25.2	23.6	17.6

\*Cultures were grown at 37°C using the indicated media composition.

\*Enzyme activity represents (U/ml) measured in samples taking after the end of the exponential phase.

**Table 5.** Effect of organic nitrogen source on streptokinase production by *S. pyogenes* and *S. equisimilis*

Organic nitrogen source	<i>S. pyogenes</i> SK activity (U/ml)			<i>S. equisimilis</i> SK activity (U/ml)		
	0.5g %	1g %	1.5 g %	0.5g %	1g %	1.5g %
Tryptone	37.4	40.3	53.4	30.12	33.6	42.16
Yeast Extract	26.23	33.24	52.21	12.14	17.17	33.24
Peptone	17.25	23.17	27.29	10.4	14.3	18.5
Casein	36.7	38.6	56.16	29.13	37.24	40.12
Beef Extract	23.23	32.18	44.7	16.8	27.9	29.3

\*Cultures were grown at 37°C using the indicated media composition.

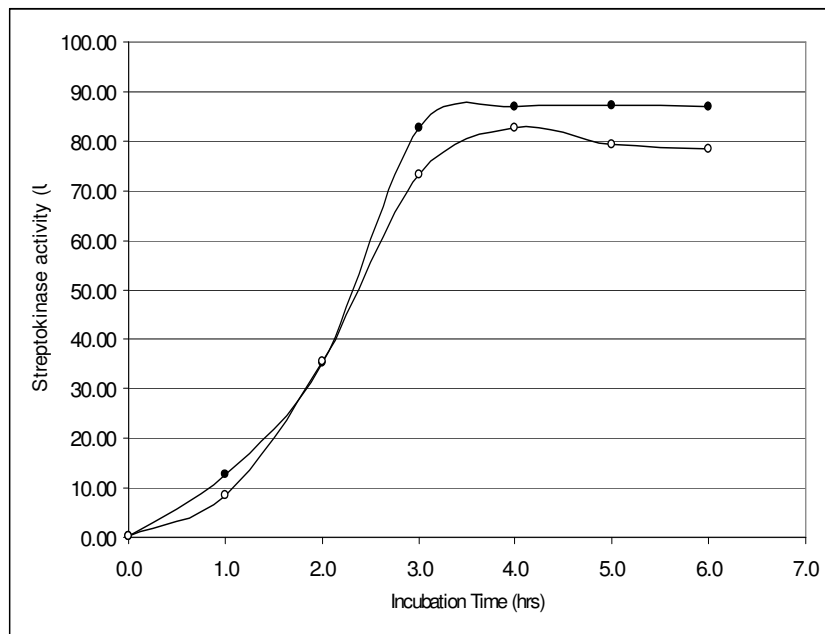
\*Enzyme activity represents (U/ml) measured in samples taking after the end of the exponential phase.

### Detection of streptokinase

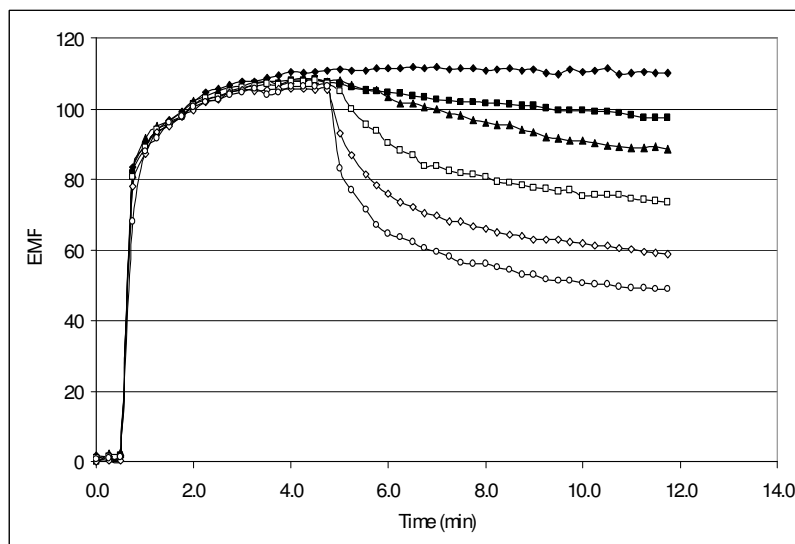
To monitor enzymatic activity during growth of the microbe, detection method should be fast and accurate. Streptokinase assay with radial caseinolytic activity is one of the oldest methods used for this purpose (Saksela, 1981). The method depends on direct measurement of the area of the transparent lysis zone in an agarose-casein plate that contains plasminogen. Although it is relatively inexpensive method, it is slow and can not be used to monitor the enzyme activity in short time intervals. It was used mainly for the qualitative determination of the enzyme, but for an accurate and quantitative method for streptokinase assay, other methods were used. The enzyme was assayed upon growing bacterial strains on different media by the common casein digestion method (Muller *et al.*, 1989) and the more sensitive Chromozym

substrate digestion method (Wong *et al.*, 1994) (Figure 3). The two methods are based on detecting the amidolytic activity of plasmin – librated after plasminogen activation by streptokinase – upon synthetic chromogenic substrates (Chang *et al.*, 1999). Although these methods are accurate and reliable, they are expensive and their total reaction time is relatively high.

Assaying streptokinase with electrochemical detectors, such as polymer membrane-based ion-selective electrodes offers a number of advantages over spectrophotometric methods especially in cases where samples are highly colored or turbid like bacterial cultures. Protamine-sensitive membrane electrodes have been used to measure trypsin activity (Yun *et al.*, 1995). Protamine is an arginine-rich protein which is known to be an excellent substrate to proteases (Ong and Johnson, 1976). Polyion-sensitive electrodes have a high response



**Figure 3.** Streptokinase production when *S. pyogenes* was grown on BHI medium. Enzyme was assayed with Chromozym substrate method (●), and casein digestion method (○), respectively



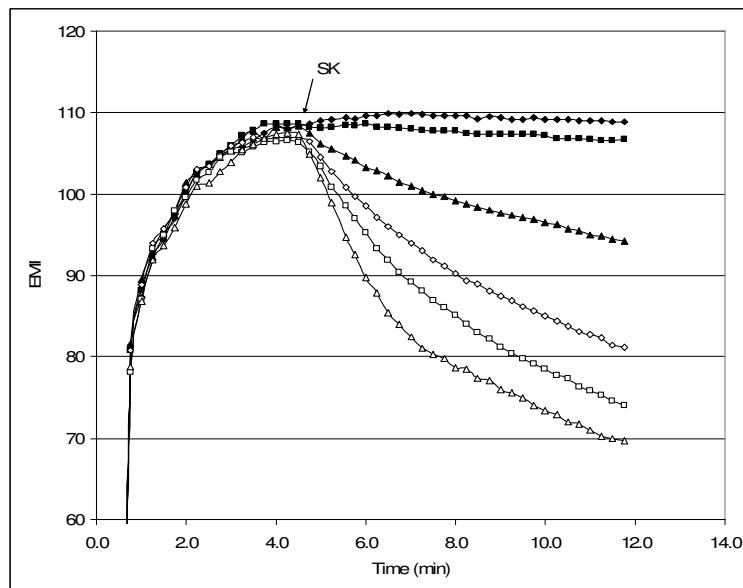
**Figure 4.** Potentiometric response of (DNNS)-based protamine-sensitive membrane electrodes towards 25 µg/ml protamine and subsequent addition of 100 µl of standard streptokinase. Enzyme aliquots: (◆) 0; (■) 10; (▲) 30; (◻) 100; (◊) 300; and (○) 500 U/ml were preincubated with plasminogen for 5 minutes at room temperature.

towards protamine, but smaller polycationic fragments formed after the action of plasmin on protamine can not be detected by these electrodes (Chang *et al.*, 1999).

Streptokinase activity can be detected by measuring the initial rate of decrease in the potentiometric response

of the polycation-sensitive membrane electrode towards protamine degradation by the action of liberated plasmin. Figure 4 shows the average potentiometric responses of tubular dinonylnaphthalene sulfonate (DNNS)-based protamine-sensitive membrane electrodes toward 25





**Figure 5.** Potentiometric response of (DNNS)-based protamine-sensitive membrane electrodes towards 25  $\mu\text{g/ml}$  protamine and subsequent addition of 100  $\mu\text{l}$  of cell-free bacterial culture after growing *S. pyogenes* on BHI medium at 37°C for : (◆) 0; (■) 1; (▲) 2; (◇) 3; (□) 4; and (△) 6 hours. Samples were preincubated with plasminogen for 5 minutes at room temperature.

$\mu\text{g/ml}$  protamine and the effect of adding increased concentrations of standard streptokinase. Enzyme samples were first incubated with plasminogen for 5 minutes.

Monitoring bacterial streptokinase production polycation-sensitive electrode. To assay for streptokinase during microbial growth using the protamine-sensitive electrode, *S. pyogenes* and *S. equisimilis* were grown on BHI medium at 37°C and at different time intervals, samples were taken to be tested for streptokinase activity. To compare between different methods of detection, enzyme assay was carried out with both electrochemical and spectrophotometric methods. Figure 5 shows response of the protamine-sensitive electrode to streptokinase samples – taken at different time intervals – during growth of *S. pyogenes*.

Results obtained from electrochemical method were so close to that obtained from the traditional spectrophotometric method when both *S. pyogenes* and *S. equisimilis* (Table 6) were tested for their streptokinase activity during bacterial growth. These results offer an alternative and reliable method for streptokinase detection during microbial growth.

## DISCUSSION

Streptokinase is one of the most important enzymes produced by certain streptococci strains. This enzyme is

used as a treatment for stroke and myocardial infarction. Decreasing the production costs of this enzyme is a strong demand for many users all over the world as it will make this effective treatment for such dangerous diseases in the reach of poor patients. Optimization the production process is the first step toward decreasing the production costs. In this work we used two strains, *S. pyogenes* ATCC 21548 and *S. equisimilis* ATCC 12388, for enzyme production. These strains were grown on four different media Brain Heart Infusion medium (BHI), Todd-Hewitt medium, Blood Base medium, and Strep-base medium. When streptococci strains were cultured in the first two media, they produced much enzyme, however these media are expensive. In Stripe-base which is relatively cheap medium, enzyme production was weak. In this work we tried to improve the enzyme productivity in the relatively cheap Stripe-base medium by some trials such as adjusting pH and selecting the optimum concentrations of carbon and nitrogen sources.

The results showed that, enzyme production in the adjusted cultures did not dramatically decreased as that without pH adjustment and after three days the enzyme activities of the adjusted cultures were as much as duple of the non adjusted cultures. These results match the results obtained by Babashamsi (Babashamsi *et al.*, 2009) and also suggested the efficiency of continues fermentation method, whereby the pH value will be maintained optimum along the production process, for enzyme production. Fortunately, low concentration

**Table 6.** Streptokinase detection during *S. pyogenes* and *S. equisimilis* growth with both Protamine-sensitive electrode and spectrophotometric methods

Incubation time (hrs)	<i>S. pyogenes</i> Streptokinase activity (U/ml)		<i>S. equisimilis</i> Streptokinase activity (U/ml)	
	Protamine-sensitive electrode method	Chromozym substrate method	Protamine-sensitive electrode method	Chromozym substrate method
0	0	0	0	0
1	6.15	5.27	4.53	3.09
1.5	ND	ND	10.31	9.74
2	19.19	17.88	21.05	20.22
2.5	41.34	44.09	39.93	39.3
3	55.68	53.87	52.33	51.24
3.5	734.06	71.23	70.54	72.56
4	82.05	84.29	84.09	85.92

Microbes were grown on BHI at 37°C and at time intervals, samples were centrifuged at 8000 g for 2 minutes then, cell-free supernatants were tested for the streptokinase activity.

(0.25%) glucose was superior over other carbon sources for enzyme production. These results suggest that, the fed-batch fermentation method will be more suitable for supplying the carbon source for efficient enzyme production. Concerning the organic nitrogen sources, results showed that high concentration 1.5% of both casein and yeast extract as well as tryptone were suitable organic nitrogen sources for enzyme production by the two studied streptococcal strains. These results suggested that the set-batch fermentation process is more suitable for supplying the organic nitrogen source for the enzyme production.

Monitoring the enzyme activity during the enzyme production process plays a very important role for efficient enzyme production. Finding reliable, easy, fast, and cheap method for streptokinase detection in bacterial culturing broth will facilitate monitoring and consequently improve production process. There are many methods for detection of streptokinase, such as casein digestion method (Muller *et al.*, 1989) and the more sensitive Chromozym substrate digestion method (Wong *et al.*, 1994). Although these methods are accurate and reliable, their total reaction time is relatively long and cannot be used to monitor the enzyme activity in short time intervals. The electrochemical detectors, such as polymer membrane-based ion-selective electrodes are easy, fast, and relatively cheap methods for streptokinase assay. In this study the ion-selective electrode assaying method compared to the more sensitive Chromozym method, the results showed complete accordance between the two methods. These results confirmed the reliability of the ion-selective electrode assaying method.

## CONCLUSION

In conclusion, *S. pyogenes* ATCC 21548 and *S.*

*equisimilis* ATCC 12388 can be used for streptokinase production in the relatively cheap Stripe-base medium. Results recommended continues fermentation process at which glucose as sole carbon source, is supplied by fed-batch technique, while organic nitrogen source and other nutrients can be supplied by the set-batch. The fast, easy, and cheap ion selective electrode is a reliable assaying method for detecting the enzyme production activity along the fermentation process. Using such conditions will lead to much enzyme production with lower costs.

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