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MONITORING STREPTOKINASE AND SOME FACTORS THAT AFFECT ITS PRODUCTION DURING GROWTH CURVE OF TWO STREPTOCOCCAL STRAINS

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ABSTRACT

Streptokinase enzyme is one of the most important drugs used in the treatment of strokes and myocardial infarction. Enzyme production was monitored during the growth curve of both S. pyogenes and S. equisimilis grown on different media. Adjustment of the medium pH for cultures of S. pyogenes and S. equisimilis every 12 hours on incubation - when both microbes were grown on Strep-base medium - significantly increased the enzyme production level. Streptokinase production was almost doubled in the pH-adjusted cultures after three days of incubation.Glucose at 0.5 g% was found to be the best source of carbon that supports the streptokinase production by both S. pyogenes and S. equisimilis. On the other hand, both mannitol and sorbitol were found to be improper carbon sources for the enzyme production by both microbes.

Tryptone, yeast extract, and casein could be used as the primary source of organic nitrogen to produce streptokinase from S. pyogenes when the microbe is allowed to grow on Strep-base medium. The higher level of the enzyme production by S. equisimilis was obtained when 1.5 g% Tryptone was used as the organic nitrogen source.Streptokinase produced by S. pyogenes and S. equisimilis was assayed upon growing both bacterial strains on different media by the common casein digestion method and the more sensitive Chromozym substrate digestion method. Moreover, the enzyme was monitored electrochemically during microbial growth of both microbes using the protamine-sensitive electrode to compare between different methods of detection. Results obtained from electrochemical method were so close to that obtained from the

spectrophotometric method when both S. pyogenes and S. equisimilis were tested for their streptokinase activity during bacterial growth curve. These results offer an alternative and reliable method for streptokinase detection during microbial growth. Moreover, electrochemical method provide a faster and less expensive technique for streptokinase determination especially when there is a need to detect the enzyme in turbid media like bacterial cultures.

KEYWORDS: STREPTOKINASE, COLORIMERTIC ASSAY, ELECTROCHEMICAL ASSAY

INTRODUCTION

Disorders of the circulatory system such as stroke and myocardial infarction are very widely distributed diseases that affect the human health especially in the last few decades. 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 treatment was limited with no reduction in mortality rates 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 and Marder, 1991). Plasminogen activators are fibrinolytic agents that work by converting plasminogen to the natural fibrinolytic agent plasmin, 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 stochiometric 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 specially 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 □-hemolytic streptococci. It is a single-chain polypeptide with a molecular weight of 47 kDa and is made up of 414 amino acid residues (Malke and Ferretti, 1984). The enzyme does not contain conjugated carbohydrates and lipids (Banerjee *et al.*, 2004). Streptokinase has a multiple domains structure with α-, β- and γ-domains. The three domains have different associated functional properties (Welfle *et al.*, 1992). Gamma domain is essential for plasminogen activation (Wu *et al.*, 2001), and β 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 residue 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 dissolving drugs are obtained from β-hemolytic streptococci especially those strains which are isolated from human origin as they lack erythrogenic toxins (Hagenson *et al.*, 1989; Wong *et al.*, 1994).

Several attempts were made to increase streptokinase production by growing β-hemolytic streptococci on different media supplemented with various nutritional factors for growth (Christensen, 1945; Holmstrom, 1968; Hyun *et al.*, 1997), but commonly, the enzyme is produced from β-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.

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 caseinonlysis 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.

MATERIALS AND METHODS

Bacterial growth and streptokinase production

The activity of extracellular streptokinase was monitored throughout the growth of the bacterial strains *S. pyogenes* ATCC number 21060 and *S. equisimilis* ATCC number 35666. Cells were activated by growing them overnight on Strep-base medium containing 10 g tryptone, 5 g yeast extract, 2 g K_2HPO_4, 5 g NaCl, and 5 g glucose per liter. The pH was adjusted to 7.4 by the addition of diluted NaOH. Cells were allowed to grow at 37°C with shaking at 100 rpm using a shaker incubator. One ml of the previously grown cultures was used to inoculate 100 ml of the different media used for enzymatic production. Cells were allowed to grow at 37°C with shaking at 100 rpm. 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* upon growing 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 curve

Both *S. pyogenes* and *S. equisimilis* were activated over night 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 K2HPO4, 5 g NaCl, and appropriate weight of sugar under investigation per liter. The pH was adjusted to 7.4 by the addition of diluted 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_2HPO_4, 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 diluted 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 µl of a preincubated (5 min) sample of centrifuged bacterial culture solution (40 µl), 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 a 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 (ΔEMF) 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 µl of the propitiate buffer and 5 ml of protein reagent.

RESULTS AND DISCUSSION

Bacterial growth and effect of different media on streptokinase production

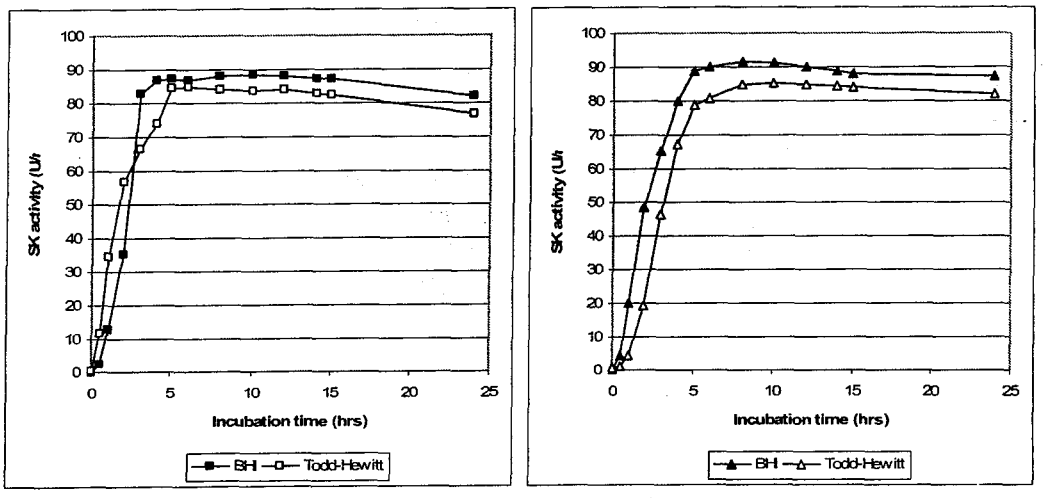
S. pyogenes and *S. equisimilis* are streptokinase hyperproducing Streptococcal strains (Steiner and Malke, 2002; Banerjee *et al.*, 2004). The two strains show a Beta hemolysis which is caused by one or more streptolysins (Wolef and Liljemark, 1978). To monitor the enzyme production during the bacterial 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 researches for the cultivation of *Streptococcus* as well as the production of streptokinase. Streptokinase reached 88.5 units per ml when *S. pyogenes* was grown on BHI medium. 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 (40.8 units per ml) when *S. equisimilis* was grown on Strep-base medium (Table 2), but the production level reached 83 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). Enzyme production patterns when both *S. pyogenes* and *S. equisimilis* are grown on previous media are shown in Figure 2.

Table (1): Streptokinase production during growth curve of *S. pyogenes* and *S. equisimilis* upon growing both microbes on BHI and Todd-Hewitt media

Incubation Time (hrs.)	Streptokinase production (U/mg protein) upon growing *S. pyogenes* on		Streptokinase production (U/mg protein) upon growing *S. equisimilis* on	
	BHI	Todd-Hewitt	BHI	Todd-Hewitt
0	0.86	1.16	0.27	3.74
0.5	2.92	14.82	7.85	2.63
1	13.01	23.36	21.04	7.46
2	21.94	25.17	31.73	15.88
3	27.48	27.87	33.56	25.36
4	27.04	28.74	35.57	32.25
5	24.6	27.29	32.04	30.63
6	23.41	24.85	32.75	28.59
8	24.59	22.53	29.37	26.06
10	24.45	22.51	25.89	25.29
12	24.41	23.15	27.12	23.38
14	25.31	23.94	23.96	22.2
15	22.06	22.19	21.93	20.49
24	20.32	18.12	22.04	20.17

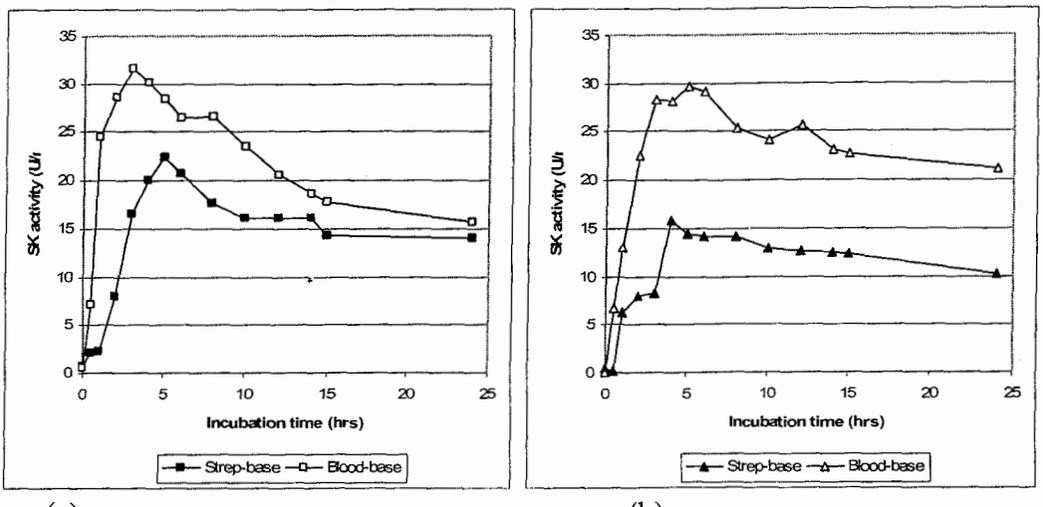


(a) (b)

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

Table (2): Streptokinase production during growth curve of *S. pyogenes* and *S. equisimilis* upon growing both microbes on Strep-base and blood base media

Incubation Time (hrs.)	Streptokinase production (U/mg protein) upon growing *S. pyogenes* on		Streptokinase production (U/mg protein) upon growing *S. equisimilis* on	
	Strep-base	Blood base	Strep-base	Blood base
0	0.64	0.54	0.46	0.02
0.5	2.08	7.17	0.1	6.73
1	2.23	24.52	6.35	13.04
2	8.04	28.50	8.03	22.55
3	16.58	31.70	8.32	28.22
4	20.07	30.15	15.78	28.10
5	22.42	28.48	14.44	29.67
6	20.66	26.45	14.11	29.05
8	17.61	26.63	14.08	25.31
10	16.08	23.49	12.94	24.15
12	16.06	20.58	12.55	25.68
14	16.11	18.56	12.41	23.11
15	14.27	17.77	12.36	22.74
24	14.04	15.73	10.27	21.13



(a) (b)

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

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 curve 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; 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 of growth both of *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 *pyogenes* and *S. equisimilis* are allowed to grow in a continuous fermentation system.

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

Incubation Time (hrs)	*S. pyogenes* SK activity (U/ml)		*S. equisimilis* SK activity (U/ml)	
	without pH adjustment	with pH adjustment	without pH adjustment	with pH adjustment
24	52.25	50.37	42.15	41.8
48	35.13	46.30	28.9	36.58
72	20.80	39.40	17.69	33.1

*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

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 primary 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.

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

Carbon source	*S. pyogenes* SK activity (U/ml)			*S. equisimilis* SK activity (U/ml)		
	0.25g %	0.5g %	1g %	0.25g %	0.5g %	1g %
Sucrose	7.4	7.1	4.7	7.7	5.3	2.8
Glucose	52.2	51.7	47.3	38.1	42.4	33.6
Mannitol	0.0	0.2	0.0	0.0	0.0	0.0
Sorbitol	0.1	0.1	0.0	0.0	0.0	0.0
Lactose	17.7	15.4	10.1	24.1	22.4	16.4

*Cultures were grown at 37°C using the indicated media composition (as mentioned in Materials and Methods).
*Enzyme activity represents (U/ml) measured in samples taking after the end of the exponential phase.

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 as described in Materials and Methods. 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 (5): Effect of organic nitrogen source on streptokinase production by *S. pyogenes* and *S. equisimilis.*

Organic nitrogen source	*S. pyogenes* SK activity (U/ml)			*S. equisimilis* SK activity (U/ml)		
	0.5g %	1g %	1.5 g %	0.5g %	1g %	1.5g %
Tryptone	36.2	39.2	52.1	29.2	32.5	41.6
Yeast Extract	25.3	32.4	51.1	11.4	18.7	32.4
Peptone	16.5	22.7	26.9	9.3	13.2	17.4
Casein	35.6	37.4	55.6	28.3	36.4	39.2
Beef Extract	22.3	31.8	43.6	15.6	26.8	28.2

*Cultures were grown at 37°C using the indicated media composition (as mentioned in Materials and Methods).

*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 curve 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.

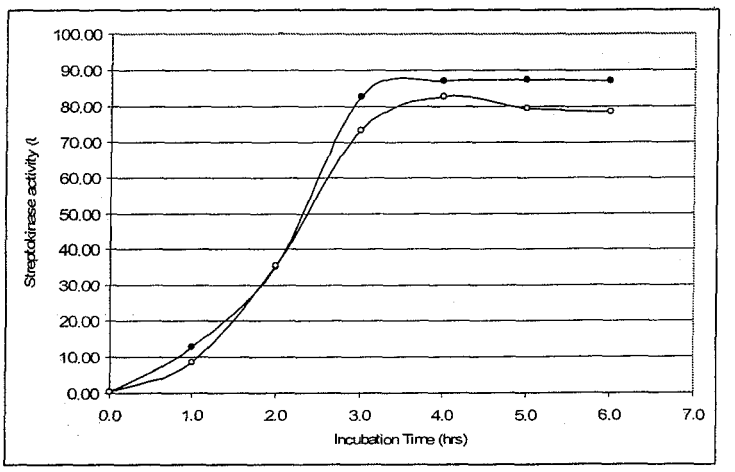


Figure (3). Streptokinase production when *S. pyogenes* was grown on BHI medium. Enzyme was assayed with Chromozym substrate method (•), and casein digestion method (○), respectively.

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 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 librated plasmin. Figure (4) shows the average potentiometric responses of tubular dinonylnaphthalene sulfonate (DNNS)-based protamine-sensitive membrane electrodes toward 25

μg/ml protamine and the effect of adding increased concentrations of standard streptokinase. Enzyme samples were first incubated with plasminogen for 5 minutes.

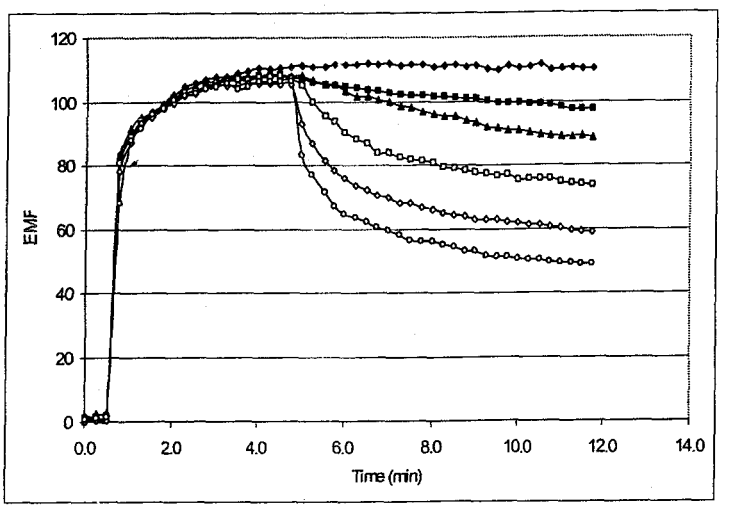


Figure (4): Potentiometric response of (DNNS)-based protamine-sensitive membrane electrodes towards 25 μg/ml protamine and subsequent addition of 100 μl of standard streptokinase. Enzyme aliquots: (♦) 0; (■) 10; (▲) 30; (□) 100; (◊) 300; and (○) 500 U/ml were preincubated with plasminogen for 5 minutes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

Monitoring bacterial streptokinase production with 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 curve 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 curve. These results offer an alternative and reliable method for streptokinase detection during microbial growth.

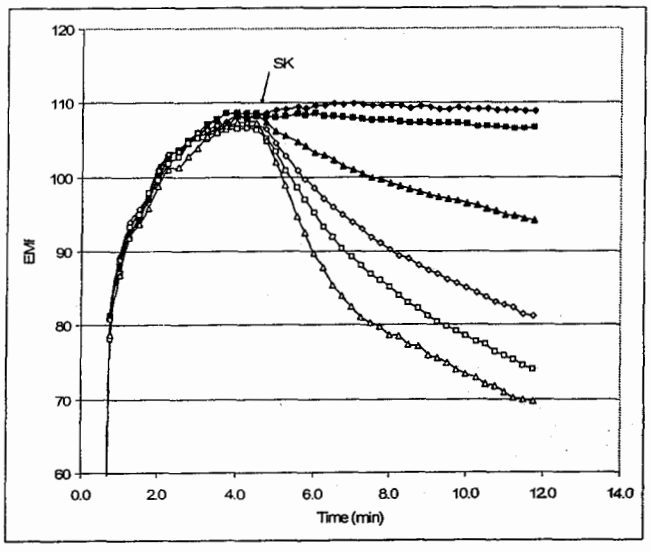


Figure (5). Potentiometric response of (DNNS)-based protamine-sensitive membrane electrodes towards 25 μg/ml protamine and subsequent addition of 100 μl of cell-free bacterial culture after growing *S. pyogenes* on BHI medium at 37°C for : (♦) 0; (■) 1; (▲) 2; (◊) 3; (□) 4; and (Δ) 6 hours. Samples were preincubated with plasminogen for 5 minutes at room temperature.

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

Incubation time (hrs)	*S. pyogenes* Streptokinase activity (U/ml)		*S. equisimilis* Streptokinase activity (U/ml)	
	Protamine-sensitive electrode method	Chromozym substrate method	Protamine-sensitive electrode method	Chromozym substrate method
0	0	0	0	0
1	5.13	4.17	3.73	4.07
1.5	ND	ND	10.31	9.74
2	18.29	16.91	20.03	19.11
2.5	40.33	43.08	38.83	38.2
3	54.79	52.98	51.14	50.22
3.5	73.03	70.15	69.41	71.63
4	81.03	83.28	83.08	84.87

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.

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رصد إنزيم الإستربتوكينيز و دراسة بعض العوامل التي تؤثر على إنتاجه أثناء النمو الميكروبي لسلالاتين من الإستربتوكوكاس

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يعتبر إنزيم الإستربتوكينيز من أهم الأدوية المستخدمة في علاج الأزمات و الذبحات الصدرية. و قد تم رصد إنتاج هذا الإنزيم أثناء نمو الميكروبات *S. pyogenes* و *S. equisimilis* على بيئات معملية مختلفة. و قد وجد أن ضبط درجة الحمضية و القاعدية ل *S. pyogenes* و *S. equisimilis* المنماه على بيئة *Strep-base* كل ١٢ ساعة من التحضين يؤثر بدرجة ملحوظة على زيادة إنتاج الإنزيم في الوسط. فقد وجد أن إنتاج الإنزيم قد تضاعف بعد ثلاث أيام من التحضين في البيئة المضبوط درجة الحمضية و القاعدية لها. وقد وجد أن إستخدام الجلوكوز بتركيز ٠,٥ جرام % في البيئة يعتبر أفضل مصدر كربوني لإنتاج الإنزيم من السلالات المستخدمة. و على النقيض, لم يكن المانتول و لا السوربيتول مصادر كربونية مناسبة لإنتاج الإنزيم. و قد وجد أن التربتون و مستخلص الخميرة و الكازيين يمكن إستخدامها كمصادر أولية للنيتروجين العضوي عند إنتاج إنزيم الإستربتوكينيز من *S. pyogenes* المنماه على بيئة ال *Strep-base*. و قد وصل الإنتاج الإنزيمي لأعلى معدل عند إستخدام التربتون بتركيز ١,٥ جرام% كمصدر النيتروجين العضوي. و قد تم رصد الإنزيم المنتج بواسطة *S. pyogenes* و *S. equisimilis* أثناء النمو البكتيري على بيئات معملية مختلفة بواسطة طريقة تحليل الكازيين الشهيرة بالإضافة إلى طريقة تحليل ال *Chromozym* الأكثر حساسية. بالإضافة إلى ذلك تم رصد الإنزيم أثناء النمو الميكروبي بالطريقة الكهروكيمائية بإستخدام المجس الحساس للبروتامين و ذلك للمقارنة بين الطرق المختلفة للرصد الإنزيمي. و قد كانت النتائج المتحصل عليها من الطريقة الكهروكيمائية شديدة الحساسية كالنتائج المتحصل عليها من طرق التحليل الطيفي و ذلك عند رصد الإستربتوكينيز المنتج بواسطة كلا من *S. pyogenes* و *S. equisimilis* أثناء منحنى النمو البكتيري. و بذلك تتيح النتائج المتحصل عليها طريقة بديلة يمكن الإعتماد عليها لرصد الإنزيم أثناء منحنى النمو البكتيري. بالإضافة إلى ذلك, فإن الطريقة الكهروكيمائية توفر تقنية أسرع و أقل تكلفة لرصد إنزيم الإستربتوكينيز خاصة عند وجود الحاجة لمعرفة كمية الإنزيم المنتج في وسط عكر كوسط البيئات البكتيرية.