Journal of Microbiology and Biotechnology
Vol. 7. No. 2, 101 - 106, 1997

Strain Improvement for Enhanced Production of Streptokinase and Streptodornase in *Streptococcus* sp.

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Strain improvement for the enhanced production of streptokinase and streptodornase in *Streptococcus* sp. ATCC 12449 was performed. Strain UB111, a hyperproductive mutant which was isolated by use of nitrosoguanidine and selection of colonies with large clear zones on DNase test agar plates supplemented with 1% glucose and 0.5% ammonium chloride, produced about 3 fold more streptokinase and streptodornase than the wild type when tested in shake flask fermentations. The enhanced production of both streptokinase and streptodornase was achieved by cultivating the mutant in a pH-controlled fermentor containing fermentation medium enriched with yeast extract (2.1%). Under these conditions, the mutant produced 7300 units/ml of streptokinase and 800 units/ml of streptodornase.

Streptokinase is an extracellular protein exported by many strains of hemolytic streptococci to the growth medium and it is also a single-chain protein of 415 amino acids with a molecular weight of 47408, which is free of cysteine, phosphorus, carbohydrates and lipids (1, 2, 5, 8). Due to its capability of converting the enzymatically inert plasma plasminogen to the active plasmin (1, 4), streptokinase has been widely used as a therapeutic thrombolytic agent in some arterial diseases or for myocardial infarction (3, 6). Recently, the development of anisoylated plasminogen streptokinase activator complex (APSAC) further elevated the utility of streptokinase because of the superority of APSAC in terms of its clinical efficacy and tolerance to urokinase or tissue plasminogen activator (3).

Streptodornase is also an extracellular DNase which is produced with streptokinase by certain strains of hemolytic streptococcus group C (8, 10, 11). In combination with streptokinase, streptodornase has been therapeutically used as an antiinflammatory agent as well as an agent to break down blood clots and fibrinous exudates in closed spaces such as the joints or pleural cavity (10, 11).

In spite of the potential industrial utility of streptokinase and streptodornase, research related to their production has not been extensive except a few studies regarding their production conditions or strain development using recombinant DNA technology. Therefore, the purpose of the present research is to isolate hyperproductive mutant strains for the commercial production of both streptokinase and streptodornase.

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Key words: *Streptococcus*, streptokinase, streptodornase

MATERIALS AND METHODS

Bacterial Strain and Chemicals

Streptococcus sp. ATCC 12449 (Lancefields group C) used throughout this study was obtained from the American Type Culture Collection. All chemicals used were reagent grade. BHI (brain heart infusion) and DNase test agar were obtained from Difco (Detroit, MI, U.S.A.). Plasminogen, thrombin, fibrinogen, calf thymus DNA and streptokinase were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Media and Cultivation Conditions

The organism was routinely grown at 37°C in 500 ml-Erlenmeyer flasks containing 50 ml of BHI liquid medium that were shaken at 200 rpm. The cultures were mixed with 30% glycerol at a ratio of 1 : 1, frozen and stored in a deep freezer. For long term preservation, the cultures were lyophilized and stored in a refrigerator. For isolation of pure cultures, the organism was streaked onto plates of DNase test agar medium with methyl green (0.07 g/l) and incubated at 37°C. In order to measure the streptokinase and streptodornase productivity by shake flask culture, the seed cultures grown on BHI liquid medium

were transferred to a fermentation medium consisting of glucose 50 g/l, yeast extract 12.5 g/l, casein 1.5 g/l, KH_2PO_4 8.2 g/l, K_2HPO_4 2 g/l, $MgSO_4$ 1 g/l, glutamine 100 mg/l and NaOH 2.4 g/l. The inoculum size was 5% and the cultures were incubated for 16 h. Fermentation time course studies were conducted in a 5 liter-jar fermentor (Korea Fermentor Co., Incheon, Korea) that contained 2.5 liters of the fermentation medium. The fermentors were mixed at 150 rpm, not aerated, and controlled at pH 6.0 with 10% NaOH solution. In the experiments to identify growth stimulators, adenine, guanine, uracil and cytosine were dissolved in 1% KOH solution, and added to the fermentation medium to make 50 mg/l each. Vitamin mixtures consisting of biotin, riboflavin, nicotinic acd, pantothenic acid, pyridoxin and ascorbic acid were separately filter-sterilized and added to the fermentation medium so that the concentration of each vitamin was 100 μg/l.

Mutagenesis and Isolation of Mutants

The cultures grown on BHI medium until the mid logarithmic growth phase were centrifugated, suspended in fresh BHI medium, and then treated with N-methyl-N'-nitro-nitrosoguanidine (200 mg-NTG/l) at 37°C for 1 h. NTG treatment resulted in cell viability loss of greater than 99.9%. The treated cells were washed three times with saline solution, suspended in BHI medium, and incubated at 37°C for 2 h. The cells were appropriately diluted with saline solution, plated onto DNase test agar medium with methyl green (0.07 g/l), and incubated for 1 day. The colonies with large clear zones were selected and tested for their streptokinase and streptodornase productivity by shake flask culture.

Quantification of Growth and Fermentation Substrates

For the determination of culture turbidities, culture broths were appropriately diluted with distilled water and the optical densities were measured at 660 nm using a Gilford spectrophotometer. Glucose was enzymatically determined using Sigma diagnostic glucose reagents (Sigma, St. Louis, MO, U.S.A.).

Assays of Streptokinase and Streptodornase

Cell-free culture supernatants were prepared by centrifugation of culture broths at 10,000×g for 10 min. Streptokinase activity was assayed using a slight modification of the clot lysis method described originally by Christensen (2). Reaction mixtures that consisted of 0.1 ml of streptokinase solution which was serially diluted with 1/15 M phosphate buffer (pH 7.4) containing 3% bovine serum albumin, 0.4 ml of fibrinogen solution, 0.5 ml of plasminogen solution, and 0.1 ml of thrombin solution were placed in a water bath at 37°C. The lysis times which were taken as the time between formation and complete disappearance of the fibrin clot were measured and plotted against the reciprocal of the dilution on double logarithmic paper. By comparison with the standard curve prepared using a standard streptokinase solution purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), the streptokinase activities were determined. The fibrinogen solution was prepared by dissolving 90 mg of fibrinogen in 36 ml of borate buffer (pH 7.5) consisting of boric acid 11.1 g/l, sodium borate 7.0 g/l and sodium chloride 9.0 g/l. The plasminogen solution was prepared by dissolving 170 mg of plasminogen in 140 ml of the same borate buffer as above, and the thrombin solution was prepared by dissolving 42 mg of thrombin in 30 ml of 50 mM sodium acetate buffer (pH 5.6).

Streptodornase activity was assayed according to the procedures described by Taketo A. *et al.* (9). The reaction mixtures consisted of 0.15 ml of streptodornase solution that was appropriately diluted with 1/15 M phosphate buffer (pH 7.4) with 3% bovine serum albumin, and 1 ml of calf thymus DNA solution (0.5 g/l) in 0.16 mM glycin-KOH buffer (pH 9.0) with MgSO4 (1.6 μM) and $CaCl_2$ (1.6 μM). After incubation at 37°C for 10 min, the reaction was stopped by the addition of 0.1 ml of ice cold 5 N perchloric acid, kept in an ice bath for 30 min, and centrifuged at 10,000×g for 5 min. The optical density of the supernatant was measured at 260 nm. Standard curves were prepared using varidase tabulets (Yu Han Pharmaceutical Co., Korea) as a standard streptodornase.

RESULTS AND DISCUSSION

Isolation of Hyperproductive Mutants

In order to establish a screening method for the isolation of hyperproductive mutant strains, the effect of carbon sources and nitrogen sources on the formation of clear zones on DNase test agar plates in *Streptococcus* sp. wild type were studied. In *Streptococcus* sp., clear zones are formed around colonies grown on DNase test agar medium with methyl green only when it produces extracellular streptodornase. Clear zones, therefore, will not be formed under culture conditions where the enzyme is not produced. It was assumed that when mutagenized cells were plated and incubated under conditions in which the wild type did not form clear zones, the colonies with large clear zones would be hyperproductive mutants. As shown in Table 1, large clear zones were formed when none or ammonium chloride was added to the medium. However, the size of clear zones decreased when glucose was added to the medium and clear zones did not appear when both glucose and ammonium chloride were added. Based on these fundamental results, the colonies with large clear zones when mutagenized cells were plated on DNase test agar medium with glucose and ammonium chloride and incubated were isolated and tested for their productivity

Table 1. Effects of C-sources and N-sources on the formation of clear zones when *Streptococcus* sp. wild type was grown on DNase test agar medium.

Addition	Size of clear zones
None	Large
Glucose (1%)	Small
NH_4Cl (0.4 %)	Large
Glucose (1%)+NH_4Cl (0.5%)	Not detectable

Table 2. Comparison of streptokinase and streptodornase activities in *Streptococcus* sp. wild type and mutant strains.[a]

Strains	Growth (OD_{660})	Final pH	Streptokinase (units/ml)	Streptodornase (units/ml)
Wild type	2.00	4.4	320	85
U16-16	1.80	5.0	820	235
UB 111	1.65	4.4	820	270

[a]Cells were cultivated in an Erlenmeyer flask containing 50 ml of medium at 37°C for 16 h with shaking.

of streptodornase and streptokinase by shake flask culture.

The streptodornase and streptokinase activities of wild type and mutant strains grown in an Erlenmeyer flask containing 50 ml of fermentation medium are compared in Table 2. The mutants were confirmed to be stable by testing for both streptodornase and streptokinase productivities after at least 10 culture transfers. The mutant UB111 produced not only about 3-fold more streptodornase but also about 3-fold more streptokinase on fermentation medium than the wild type although it was isolated based on the size of clear zone indicating the streptodornase activity. The other mutant U16-16 also produced much more streptodornase and streptokinase than the wild type but it showed lower activity of streptodornase and almost the same level of streptokinase activity compared to the mutant UB111. The mutants were similar to the wild type in microscopic morphology but their colony sizes on DNase test agar medium were smaller than those of the wild type.

Comparison of Fermentation Time Courses in Wild Type and Hyperproductive Mutants in a Jar Fermentor

In order to evaluate the potential of mutant strains for industrial production of streptodornase and streptokinase, the fermentation time courses of both the wild type and mutants were studied in a jar fermentor because in a shake flask culture without pH control the early cessation of growth might occur due to the accumulation of proton or organic acids. Fig. 1A and 1B compare the fermentation time course of *Streptococcus* sp. wild type and mutant, respectively, when grown on fermentation medium under the conditions of agitation at 150 rpm and pH control. In the wild type strain, growth rate and glucose consumption rate were very high but the production rates of streptodornase and streptokinase were very low. On the other hand, in mutant strain UB111, growth rate and final cell concentration decreased as compared to that of the wild type while the activities of both streptodornase and streptokinase significantly increased. The mutant produced three fold more streptokinase and two fold more streptodornase. Also, the data suggests that an increase of growth rate or final cell concentration of the mutant by optimization of fermentation conditions will result in further enhancement of streptodornase and streptokinase production.

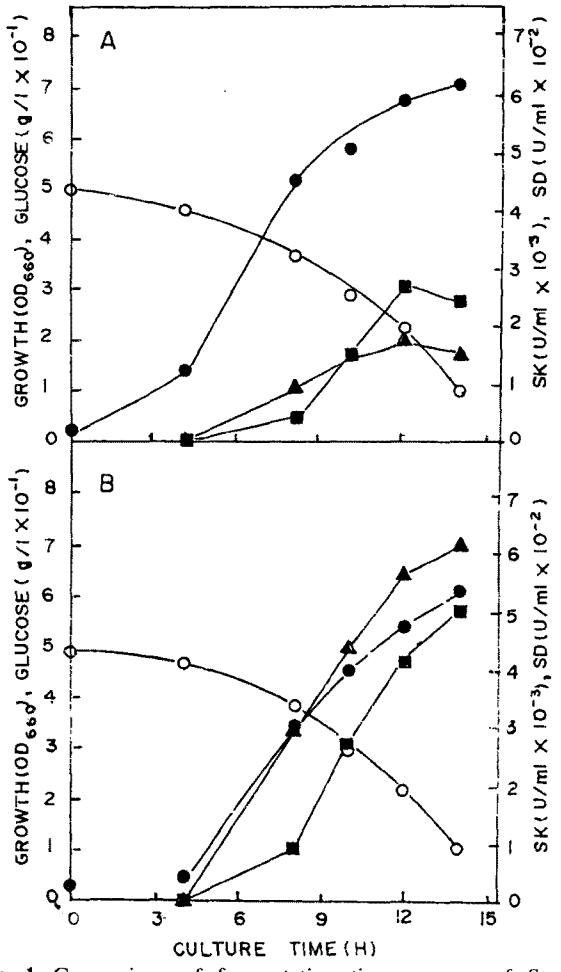

Fig. 1. Comparison of fermentation time courses of *Streptococcus* sp. wild type (A) and mutant UB111 (B).
Experiments were conducted in a fermentor containing 2.5 liter of fermentation medium, which was controlled at pH 6.0 with NaOH solution (10%), agitated at 150 rpm, and not aerated. ●, growth (OD_{660}); ○, glucose concentration; ▲, streptokinase; ■, streptodornase.

Optimization of Medium Composition for Growth Enhancement of the Hyperproductive Mutant

In order to investigate whether the mutant strains re-

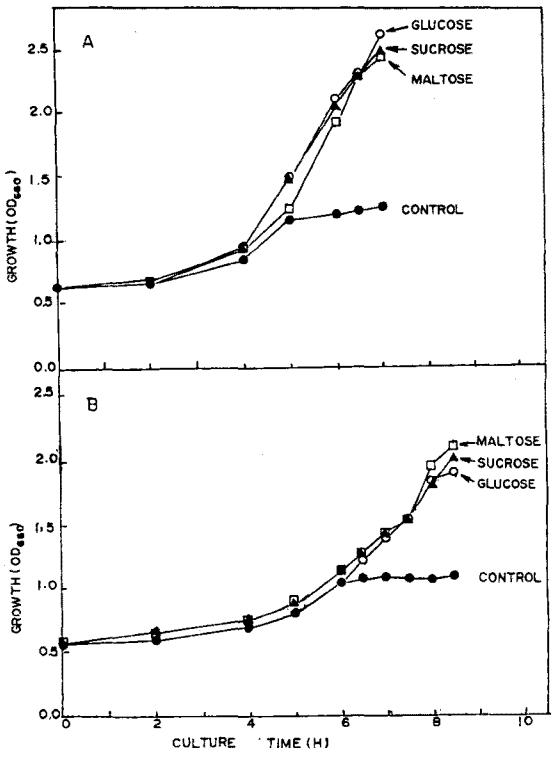

Fig. 2. Effects of carbon sources on the growth of *Streptococcus* sp. wild type (A) and mutant UB111 (B).
Cells were grown in an Erlenmeyer flask containing 50 ml of fermentation medium with the C-source (1.5%) as indicated.

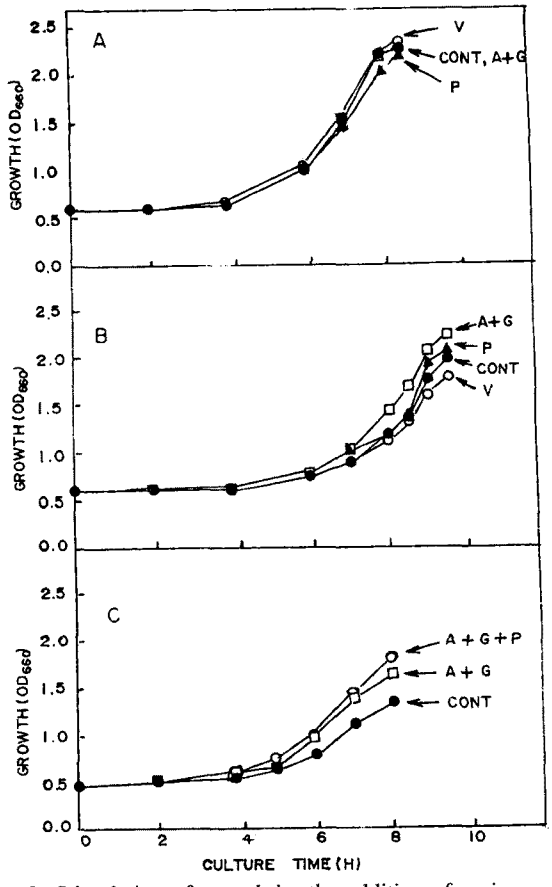

Fig. 3. Stimulation of growth by the addition of various compounds in *Streptococcus* sp. wild type (A) and mutant UB111 (B, C).
Cells were grown in an Erlenmeyer flask containing 50 ml of fermentation medium supplemented with those as indicated. CONT, no addition; V, vitamine mixture; A+G, adenine and guanine; P, peptone.

quire growth factors or stimulators for their enhanced growth, the effect of various medium components on the growth of mutants was examined. The addition of carbon sources such as glucose, sucrose and maltose did not affect the growth of both wild type and mutant strains (Fig. 2). The addition of yeast extract, beef extract or tuna extract, however, stimulated the growth of mutant UB111 whereas it did not stimulate the growth of the wild type (data not shown). We, therefore, tested which of amino acid mixture, vitamin mixture, and purine and pyrimidine base mixture stimulated growth of the mutant. As shown in Fig. 3A, the wild type strain grew at the same rate regardless of the addition of those components. In the mutant, however, the highest growth rate was observed when adenine and guanine were added to the fermentation medium (Fig. 3B). The addition of peptone stimulated the growth of the mutant but to a lesser extent as compared to the addition of adenine and guanine, whereas the addition of the vitamin mixture did not stimulate growth. The addition of cytosine or uracil did not affect the mutant's growth (data not shown). The additive effect of peptone and purine base mixture on the growth of the mutant was also investigated (Fig. 3C). The data indicates further stimulation of growth by the combined addition of peptone and purine bases, suggesting that the mutant requires at least more than three growth stimulators to obtain the highest growth rate.

Enhanced Production of Streptokinase and Streptodornase by the Hyperproductive Mutant

Experiments were performed to assess the potential of hyperproductive mutant UB111 for streptokinase and streptodornase production process application. Fig. 4 shows the fermentation time course of mutant UB111 when grown in a jar fermentor containing 2.5 liters of fermentation medium supplemented with adenine and guanine, which was controlled at pH 7.0 and agitated at 150 rpm without aeration. The data illustrates that supplementation of adenine and guanine resulted in an increase of growth rate but no change in final cell concentration when compared to the experimental results (Fig. 1B) achieved without supplementation. This result implies that growth factors other than adenine and

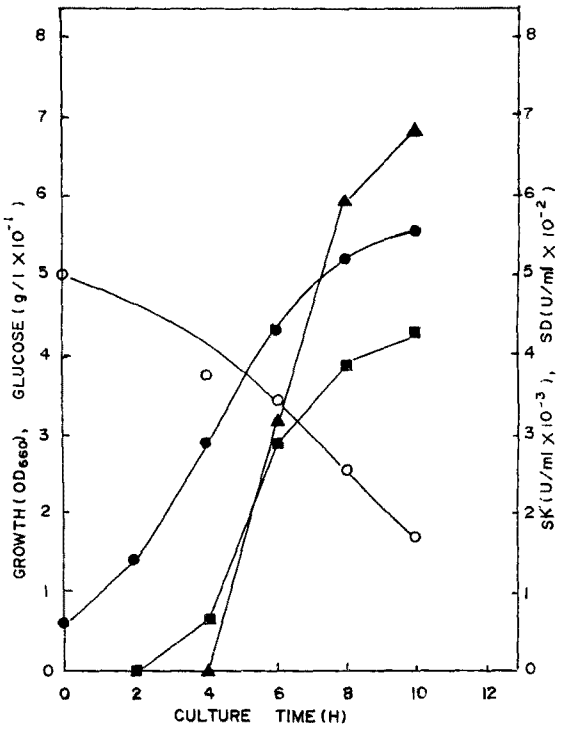

Fig. 4. Fermentation time course of *Streptococcus* sp. mutant UB111 when grown on fermentation medium supplemented with adenine and guanine.

Experiments were conducted in a fermentor containing 2.5 liter of fermentation medium with adenine (50 mg/l) and guanine (50 mg/l), which was controlled at pH 6.0 with NaOH solution (10%), agitated at 150 rpm, and not aerated. ●, growth (OD_{660}); ○, glucose concentration; ▲, streptokinase; ■, streptodornase.

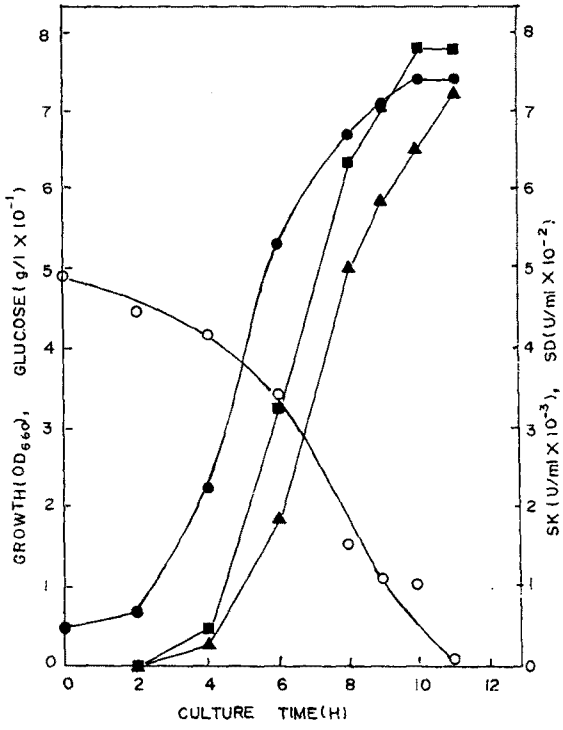

Fig. 5. Fermentation time course of *Streptococcus* sp. mutant UB111 when medium was enriched to contain 2.1% of yeast extract.

Experimental procedures were same as in Fig. 4. ●, growth (OD_{660}); ○, glucose concentration; ▲, streptokinase; ■, streptodornase.

guanine would be depleted during the fermentation period. It is also notable that streptodornase production decreased while streptokinase production was slightly increased when the medium was supplemented with adenine and guanine. The decrease of streptodornase production might be the result of catabolite repression by adenine or guanine. Therefore, in order to replace adenine and guanine and supply other growth stimulators, fermentation medium was enriched with yeast extract. The optimum concentration of yeast extract to maximize the production of streptokinase and streptodornase was determined through our experiments using jar fermentors. As shown in Fig. 5, the mutant displayed the highest production of streptodornase (800 units/ml) and streptokinase (7300 units/ml) when grown on fermentation medium containing 2.1% of yeast extract. The enhanced production of streptokinase and streptodornase is considered to be the result of growth promotion and derepression by the replacement of adenine and guanine with yeast extract. From these results, it is concluded that the mutant UB111 has great potential for the industrial production of streptokinase and streptodornase.

Acknowledgement

This study was supported by a research grant from the Bioproducts Research Center of Yonsei University (Project No. 95-K3-03-07-01-04-3)

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(Received Ocober 4, 1996)