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Optimization of Staphylokinase Production in *Bacillus subtilis* Using Inducible and Constitutive Promoters

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Abstract Staphylokinase (SAK) was produced in *B. subtilis* using two different promoter systems, *i.e.* the P43 and *sacB* promoters. To maximize SAK expression in *B. subtilis*, fermentation control strategies for each promoter were examined. SAK, under P43, a vegetative promoter transcribed mainly by σ^B containing RNA polymerase, was overexpressed at low dissolved oxygen (D.O.) levels, suggesting that the *sigB* operon is somewhat affected by the energy charge of the cells. The expression of SAK at the 10% D.O. level was three times higher than that at the 50% D.O. level. In the case of *sacB*, a sucrose-inducible promoter, sucrose feeding was used to control the induction period and induction strength. Since sucrose is hydrolyzed by two sucrose hydrolyzing enzymes in the cell and culture broth, the control strategy was based on replenishing the loss of sucrose in the culture. With continuous feeding of sucrose, WB700 (pSAKBQ), which contains the SAK gene under *sacB* promoter, yielded *ca.* 35% more SAK than the batch culture. These results present efficient promoter-dependent control strategies in *B. subtilis* host system for foreign protein expression.

Keywords: staphylokinase, *B. subtilis*, P43 promoter, *sacB* promoter

INTRODUCTION

B. subtilis is one of the most important host systems for the production of industrial enzymes and therapeutic foreign proteins due to its ability to express and secrete proteins. To develop *B. subtilis* as a host for the production of recombinant foreign protein, various approaches have been adopted, such as the development of strong promoters [1], the enhancement of plasmid stability [2], the improvement of protein secretion using a fusion signal sequence [3], the harnessing of secretory machinery to the host cell [4], the deletion of various host proteases [5] and the prolongation of production time using spore negative mutants [6].

SAK is a promising, attractive thrombolytic agent [7], which activates human plasminogen into active plasmin with which fibrin clots can be lysed. In several clinical trials, SAK was shown to be safe and potent [8]. It can lyse clots effectively in a fibrin-specific manner without causing a significant depletion of plasma proteins and has the ability to lyse even platelet-rich clots. Given these properties, large quantities of SAK are being needed for research and clinical trials. There are several reports about SAK expression in two microbial hosts. Using *E. coli*, expression of 50 mg/L of secreted SAK has been reported [9]. However, retardation of cell growth, the accumulation of the SAK precursor in the membrane fraction, and the microheterogeneity of N-terminal sequence [9,10] are identified problems. Recently, intracellular production of SAK in *E. coli* was reported with a yield of 175 mg/L [11]. In contrast, using *B. subtilis* (GB500 and DB104), a 25-50 mg/L yield of SAK was obtained, and problems of microheterogeneity were experienced [10]. However, our results showed the yield of 337 mg/L of authentic SAK using WB700, and provided a detailed biological characterization of the protein [12]. In the same paper, we systemically compared four different promoters (*i.e.*, *amyE*, P43, *sacB* and the original SAK promoter of *S. aureus*) for the production of intact SAK and investigated the protocols of fed-batch fermentation using two most promising promoters (*i.e.*, P43 and *sacB*). We also attempted to maximize SAK production using the P43 promoter (*i.e.*, 225 mg/L) and the *sacB* promoter (*i.e.*, 337 mg/L) using 2X modified super rich (MSR) media. The two promoters have distinctive characteristics in terms of their transcriptional regulation. P43 promoter is regulated by the intracellular [ATP]/[ADP] ratio, which depends upon growth conditions and, the *sacB* promoter is inducible with sucrose. Therefore, here, we report upon the development of precise control strategies, which can be applied to each promoter for the production of any recombinant proteins including SAK.

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MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Bacillus subtilis strain WB700 (trpC2 nprE aprE epr bpf Δmpr::ble ΔnprB::bsr Δvpr::ery), which contains pSAKP or pSAKBQ, was used as the expression host for the secretory production of SAK [12]. While plasmid vector pSAKP has a P43 promoter for the expression of SAK, pSAKBQ has a *sacB* promoter for the expression of SAK and an additional P43-*sacQ* cassette, which is a positive transcription regulator of the *sacB* promoter [12]. Both plasmids have a *B. subtilis sacB* signal sequence for the extracellular secretion of expressed SAK.

Only 1X modified super rich (MSR) media was used for all types of cultivation [12]. MSR media contains 2.5% yeast extract, 1.5% Bacto tryptone, 0.3% K_2HPO_4 and 1.0% glucose. 10 μg/mL of kanamycin was used as a selection marker for all types of culture except the main 2 L fermentation run. For batch flask culture, 20 mL of MSR medium was used in 250-mL Bellco flasks, at a shaker speed and a culture temperature of 250 rpm and 37°C, respectively. Cell O.D. was determined at 600 nm.

Quantitative Analysis of SAK

To determine the amount of SAK secreted in the media, samples were taken at the appropriate times, and frozen directly at -70°C. Total amounts of secreted proteins in the supernatant were measured using a Bio-Rad Bradford assay kit with bovine serum albumin as a reference. Ten μL of the supernatant of each sample was analyzed by a SDS-PAGE and the resulting acrylamide gel was analyzed using an UltraScan XL densitometer (Pharmacia LKB, Uppsala, Sweden). Purified SAK was also used to accurately measure the amount of SAK expressed, when necessary [12].

Fed-Batch Fermentation

For fermentation study, 2 mL of MSR broth from overnight culture was inoculated into 50 mL of the MSR media in a 250-mL shake flask. When the cell density of the flask had reached at 6.0–9.0 about four hours after inoculation, the culture was transferred to a BIOFLO IIC fermenter (New Brunswick Scientific, NJ, USA) with a working volume of 2 L. Glucose level in the fermenter was monitored every 30 minutes with a glucose analyzer (Johnson & Johnson, IL, USA) using disposable tips. In the case of WB700 (pSAKP), fermentation was started with 10 g/L of glucose concentration, and 20% (w/w) glucose stock solution was used for on-off feeding. In the case of WB700 (pSAKBQ), fermentation was started with 1 g/L of glucose and the same 20% (w/w) glucose stock solution was fed into the fermenter to meet the needs of the cell growth. Glucose was maintained below 2 g/L before the sucrose was fed. Culture was induced with a 50% (w/w) sucrose solution, which was fed using a Masterflex peristaltic pump (Model 7523-10, Cole-Palmer Inc., Barrington, VT, USA) according to different induction schemes. After the sucrose induction, the exogenous glucose feed was discontinued, because the glucose liberated from the added sucrose then acted as the carbon source.

By-Product Analysis

Fermentation by-products were analyzed by gas chromatography (GC-14B, SHIMADZU, Kyoto, Japan) with flame ionization detector (FID). The GC column was packed with PoraPak (Waters, 34 Maple Street, Milford, MA, USA). N_2 was used as the carrier gas with flow rate of 80 mL/min. The injector temperature was maintained at 220°C, the column at 200°C and the detector at 250°C. Analytical grade acetic acid, acetoin and 2,3-butanediol were used as standards for peak identification and area calculations.

RESULTS AND DISCUSSION

Batch Culture of WB700 (pSAKP) at Different Agitation Rates

The objective of this study was to develop precise control strategies for these two strong and distinctive promoters, in terms of their ability to express and secrete the therapeutic protein, SAK. P43 promoter was originally found using a promoter probing vector in *B. subtilis* [13]. With the completion of the *B. subtilis* genome sequencing project, it was recognized as the cytidine/deoxycytidine deaminase promoter. P43 promoter is transcribed by σ^A and σ^B containing RNA polymerases, but mainly by σ^B-RNA polymerase holoenzyme [14]. The main RNA polymerase for P43 promoter, E-σ^B, is known to transcribe a subset of genes activated as part of general stress response [15]. The key factors in *sigB* regulation are RsbV, RsbW and SigB. σ^B-dependent transcription occurs when σ^B is released from RsbW-mediated inhibition. This mode of regulation, which is negatively regulated by anti-σ factor proteins, is commonly found in stress-stimulated operons such as σ^F and σ^G in *B. subtilis* and σ^F in *Salmonella typhimurium* [16-19]. Many factors activate *sigB* operon, such as high salt concentration, EtOH addition, heat shock, stationary phase and glucose and phosphate limitation, via [ATP]/[ADP] change. A low [ATP]/[ADP] ratio is known to be the intracellular signal for the release of SigB (σ^B) from RsbW, and it also increases the concentration of σ^B for the transcription of many E-σ^B transcribed genes. Among these various external stresses, D.O. levels in the fermenter was chosen because the internal [ATP]/[ADP] can be strictly modulated by D.O. level in the fermenter. [21,22]. The D.O. level variation is not as harmful as other forms of stress, and moreover, D.O. levels can be simply controlled by nutrient feeding or more directly by air flow rate and agitation speed.

At first, we tried to modulate the D.O. level by direct rotor rpm changes. Three fermentations were carried

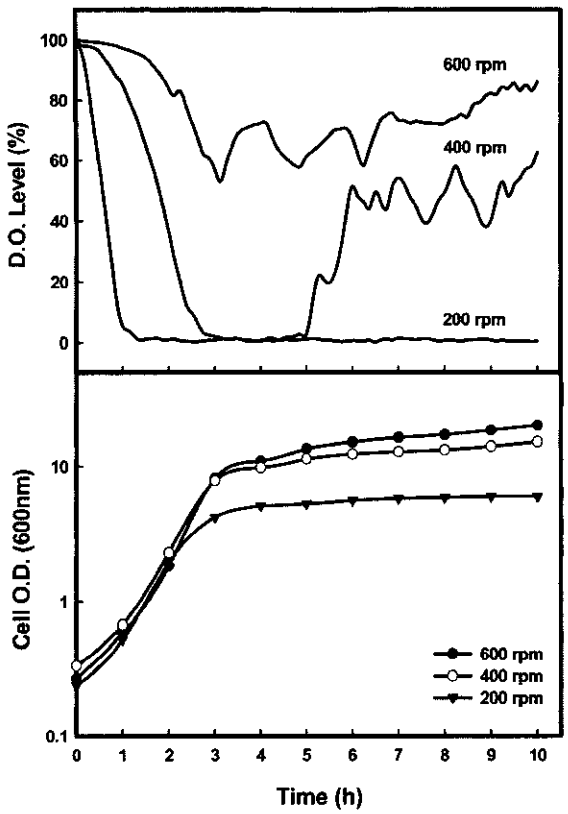

Fig. 1. Time course of D.O. profiles and cell growth of the runs at different rpms.

out with variation only in agitation speeds, namely 200, 400 and 600 rpm. In the case of 200 and 400 rpm fermentation runs, the D.O. level in the fermenter decreased to its lowest level (*ca.* 0%). This value (0%) was maintained to the end of the fermentation (200 rpm) or increased to 60% (400 rpm) (Fig. 1). But, more rapid decreases in D.O. level and lower final cell density (about 1/3 that of the other runs, Table 1) were obtained at 200 rpm, due to probably the lower oxygen transfer rate, suggesting that the oxygen transfer rate at 200 rpm is insufficient for the cell growth. In the case of 600 rpm, the lowest D.O. level ever reached was 58% and this level increased to 86% at the end of the run. The quantity of SAK expressed and the final cell densities are shown in Table 1. The highest level of specific expression of SAK per cell O.D. was obtained at 200 rpm.

Fed-batch Culture of WB700 (pSAKP)

Having completed the previous section, we carried out fed-batch fermentation to determine the effect of the D.O. level on the expression of the P43 promoter. Two experiments at D.O. level of 10% and 50% were compared. The lower D.O. level (10%) was used to maintain a low [ATP]/[ADP], and was chosen because reportedly cell growth is not affected above 5% D.O.

Table 1. Comparison of SAK yields obtained using WB700 (pSAKP) at various agitation speeds at 8 h after inoculation

RPM	200	400	600
Cell O.D.	6.0	15.2	20.2
SAK yield (mg/L)	57	113	13.5
Specific SAK yield (mg/L/O.D.)	9.5	7.4	0.7

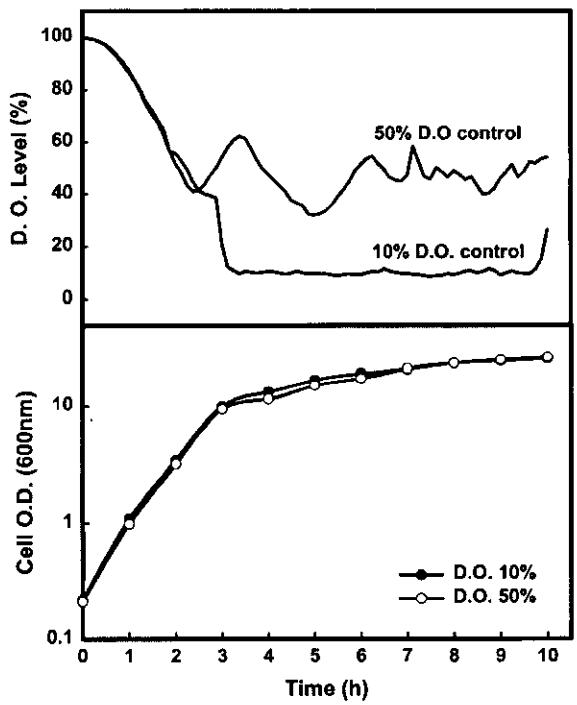

Fig. 2. Time course of D.O. profiles and cell growth of the two runs controlled at 10% and 50%.

level [20] and our results on cell growth also demonstrates that this is the case (Fig. 2). The upper limit of D.O. level, 50%, was determined somewhat arbitrarily. However, in many cases of *B. subtilis* fermentation, a 50% D.O. level in the fermenter is believed to support the full aerobic growth of *B. subtilis*, thereby maintaining high a [ATP]/[ADP] ratio.

The D.O. profiles and cell growth observed are shown in Fig. 2. The final cell O.D.s were 25.4 (10% D.O.) and 26.1 (50% D.O.), which suggest that the cell growths were almost the same in both runs. Advanced Fermentation Software (AFS, New Brunswick Scientific, NJ, USA) was used to maintain the D.O. level by on-off glucose feeding and by agitation speed. In the case of the 10% D.O. control, the D.O. level in the fermenter decreased gradually. And from 3 h after the inoculation, the agitation speed was adjusted to control the D.O. level in the fermenter. Glucose on-off feeding was also started at this time. In the case of the 50% D.O. control, the glucose on-off control was started 2 h after inoculation. SDS-PAGE analysis of the expression levels of SAK during the runs is shown in Fig. 3. The amounts of the

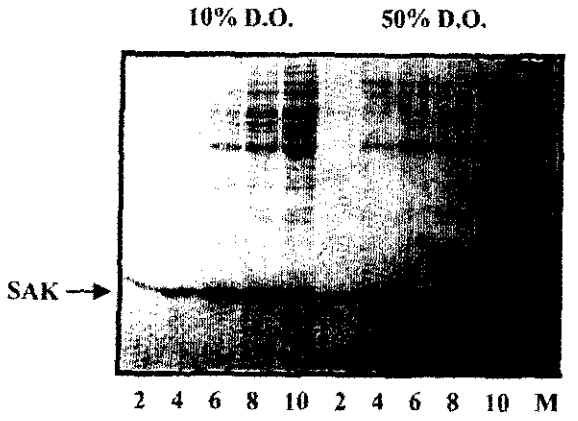

Fig. 3. SDS-PAGE analysis of the secreted SAK levels from WB700 (pSAKP) with two different D.O. levels. The numbers in the lower panel denote the sampling times (h) after induction; five different time points are compared. M denotes protein size marker with M.W. of 97.4, 66, 45, 31, 21.5 and 14.5 kDa from top to bottom.

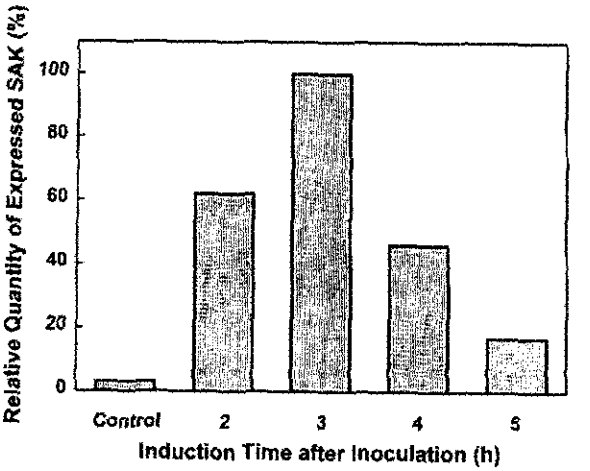

Fig. 4. Comparison of the relative SAK expression levels at different sucrose induction initiation times. 5% final concentration of sucrose was added in total to induce WB700 (pSAKBQ). Lane 1 denotes the control culture, with no added sucrose. Lanes 2, 3, 4 and 5 are cultures induced at 2 h, 3 h, 4 h and 5 h after inoculation, respectively. Samples were collected 10 h after inoculation, regardless of induction time.

total secreted protein differed by about 15% (*i.e.* 457 mg/L for 10% and 397 mg/L for 50%). However, the Bradford assay of total secreted protein and densitometric measurement of SAK in SDS-PAGE indicated that the SAK expression level at 10% D.O. was 184 mg/L and that at 50% was 59 mg/L 10 h after the inoculation.

Though the relation between D.O. level and [ATP]/[ADP] was not shown directly in this experiment, the relation has been studied by many researchers. In the fermentation of *Pseudomonas fluorescens* for extracellular enzyme production, intracellular ATP level changed rapidly upon varying the D.O. level. The intracellular ATP level decreased to either 20% or 0% of its maximum value with different culture type, as the D.O. level was reduced [21]. Shifting *E. coli* from aerobic to anaerobic condition by purging with an anaerobic gas mixture (85% nitrogen, 10% hydrogen and 5% carbon dioxide) resulted in a 67% decrease in [ATP]/[ADP] within 5 minutes [22]. Another example of this relationship is that cellular ATP contents in cultured hepatocytes in hypoxia gradually declined, corresponding to the decrease in oxygen tension, and the cellular ATP level at the 5% oxygen level was approximately 20% of that at an oxygen level of 20% [23].

The direct D.O. stat strategies, controlling D.O. level in the fermenter 10% and 50%, which was proved to be effective for the P43 promoter system in this experiment, could be a general fermentation strategy for σ^B, σ^F and σ^G transcribed genes in *B. subtilis*, and other genes in other microorganism under the control of a σ^B-like regulation.

In our previous paper [12], SAK expression of 225 mg/L (2X MSR medium) and 180 mg/L (1X MSR medium) using P43 promoter was reported. In this paper, though only similar quantity of SAK (186 mg/L with 1X MSR medium) was obtained, the mechanism related to the P43 promoter expression system was proved on a fermentation scale and higher value of D.O. level was proved to be detrimental for the P43 promoter-driven expression of SAK. Considering our previous result [12] and result obtained in this experiment, the value of about 180 mg/L is postulated to be the maximized expression level with 1X MSR media using the P43 promoter.

Optimization of *sacB* Promoter Induction in Flask Culture

SacB promoter, originating from the *B. subtilis* levansucrase gene, has been fully characterized [24]. It needs a transcription activator, SacQ, for strong expression, and is transcribed by σ^A RNA polymerase holoenzyme and induced by sucrose. Two factors, sucrose concentration and induction time were optimized for WB700 (pSAKBQ) in flask cultures. In our experiments, the higher the concentration of sucrose was the better for the induction of *sacB* promoter up to 5% (at induction time) of sucrose concentration. But above 7% of sucrose concentration at induction time (*i.e.* 7% or 10% sucrose), final cell O.D. started to decrease and the expression level of SAK gradually decreased. Therefore, 5% of sucrose was chosen as the optimal concentration for the flask culture of WB700 (pSAKBQ).

Induction time is also important for the proper induction of the *sacB* promoter. For the optimization of *sacB* promoter in *B. subtilis* in flask culture, four times were chosen. The cell growth was not affected by varying the induction time (data not shown). Fig. 4 shows that the culture induced at 3 h showed the highest yield of SAK expression, and samples collected at another

Table 2. Comparison of various runs using WB700 (pSAKBQ) for the production of SAK

No.	O.D.	SAK (mg/L)	Sucrose induction method
1	22.4	184	100 g sucrose addition at O.D. 4
2	21.9	158	20 g sucrose addition at O.D. 4, then 4 h induction with 20 g/h
3	27.4	242	100 g sucrose addition at O.D. 4, then 4 h induction with 25 g/h
4	28.4	255	100 g sucrose addition at O.D. 4, then 4 h induction with 100 g/h.

times (9 h and 11 h) gave similar results (data not shown). At 3 h, culture is in the state of the exponential growth. Thus 5% sucrose induction at the exponential growth phase appears to be the optimum condition for *sacB* promoter induction in flask culture.

Fed-bath Culture of WB700 (pSAKBQ)

One of the interesting aspects of *sacB* promoter is its induction mechanism. Sucrose induces not only cloned-SAK expression in WB700 (pSAKBQ), but also the expression of sucrase (SacA) and levansucrase (SacB) in the host cell. While the internally expressed sucrase hydrolyses sucrose transported into the host cell, externally secreted levansucrase hydrolyses sucrose in the media. Because of the interplay of between these two enzymes, the sucrose concentration cannot be maintained at the initial level of the induction in the fermenter, but the sucrose concentration gradually decreases in the fermentation. Therefore, continuous external feeding of sucrose to maintain the induction level of sucrose until the end of fermentation is important in fed-batch culture. Table 2 summarizes the fermentation runs with different induction schemes. Referring to Table 2, run No.1 is a control experiment; as in the flask culture, a single addition of sucrose resulting in a concentration of 5% (w/v) (100 g of sucrose/2 L working volume) was done when cell O.D. reached *ca.* 4.0. Run No. 2 represents continuous induction with same amount of sucrose as run No. 1 (100 g). Run Nos. 3 and 4 involved continuous inductions with increased amount of sucrose, achieved by using a higher feed rate. By increasing the amount of sucrose (100 g to 500 g of sucrose), the amount of SAK expressed increased from 184 mg/L (run No. 1) to 255 mg/L (run No. 4). We did not examine higher amount sucrose for the induction, but we believe that further optimization would be worthwhile.

Our result clearly demonstrates the importance of continuous induction during fed-batch fermentation to maintain the induction level. A suggested development of the *sacB* promoter induction system might be the development of *sacA* (sucrase) and *sacB* (levansucrase) deleted strains. In this case, the added sucrose is not metabolized and its concentration in the fermenter and in *B. subtilis* will be constant throughout fermentation. Though higher expression level might not be expected, the induction strategy would be simplified.

Though the amount of levan polymer was not measured directly, its existence is suspected by the increased glucose concentration (70 g /L) at the end of fermentation in the case of run No. 4. Sucrose is hydrolyzed to glucose and fructose, and then the fructose is polymerized to levan by the same enzyme. The effect of the presence of levan polymer on cell growth and target protein expression remains to be seen.

In the light of the results previously published [12], *sacB* was assumed to be a stronger promoter than P43, but it must be emphasized that a simple comparison is of limited value, because of large differences between the regulation mechanisms of these promoters. *SacB* promoter would stand lower without *sacQ* cassette. In this experiment with WB700 (pSAKQB), and the appropriate fermentation strategies, about 255 mg/L of secreted SAK with the 1X MSR medium was obtained.

By-product Formation

Acetic acid, acetoin and 2,3-butanediol were found to be major by-products. In terms of the inhibitory effects of these by-products as shown by other workers [25, 26] it is apparent that the levels of by-product encountered during our experiments are likely to inhibit cell growth, but the reason for this remains uncertain. This problem will be solved by more accurate glucose control.

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