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Recombinant Human Interleukin-3 Production in *Streptomyces lividans* Fermentation

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Streptomyces lividans NCIMB 11416/IL3 p002 secreting recombinant human interleukin-3 was used as the host organism for the study of improving target protein production in a filamentous host. At constant nominal glucose concentration in the medium, low C:N ratios seemed to promote tppA action causing a dramatic increase in compromised rHuIL-3 concentration. The cultures were grown in shake flasks, airlift and stirred bioreactor Fermentation at high agitation rates (200 rpm) gave rise to more dispersed filamentous growth and higher occurrence of cell damage, contaminating the culture broth with cell lysate. In air-sparged systems, productivity reached a plateau at an aeration rate of 1.5 vvm. Volumetric productivity of rHuIL-3 could be increased via the introduction of the temperature downshift and oxygen-enriched cultures, each producing a maximal rHuIL-3 titre of about 250 mg/L. Fluorescence microscopy of pellets treated with live/dead staining confirmed complete cell viability throughout the production phase of the fermentation.

Introduction

Human proteins are increasingly used today in pharmaceutical applications rather than extracts from animals or human plasma due to the lower immune response and reduced risk of transmission of diseases. *Escherichia coli* remains the microbial system of choice presently for heterologous protein production. One of the important reasons for its success is the number of higher-level expression of heterologous protein products, typically representing 10-30% of the total cellular protein [3]. Its lack of an efficient secretion mechanism, however, leads to products trapped in inclusion bodies inside the cell.

Established protocols for intracellular inclusion body isolation are frequently complex, multistage, require polypeptide denaturation and refolding. Recently, much interest has been generated in *Streptomyces* mainly due to its ability to liberate vast amounts of enzyme and proteinaceous enzyme inhibitors, having molecular weights in the range of about 4000 to 100,000. This interest is supported by the recent development of the transformation systems, plasmids and the accumulation of knowledge about gene regulation in *Streptomyces*.

The technology for cultivating *Streptomyces* and downstream processing is supported by more than 50

years of experience in antibiotic and native enzyme production. Secreting bacteria such as *Streptomyces* spp. may be used as hosts for expression of products in which glycosylation is not crucial [1]. *S. lividans* 66 (strain 1326), with its reduced restriction-modification barrier and low level of endogenous peptidases, is particularly suitable amongst known *Streptomyces* species for the expression of recombinant proteins [2].

The literature is replete with reports of heterologous protein yield enhancement achieved by genetic manipulations of the host. In contrast, very little attention has been given to the role of bioprocess and reactor engineering in improving the heterologous protein production. Thus, in this research project, the efficacy of bioprocessing strategies in enhancing the yield of heterologous protein in a filamentous prokaryotic host was assessed. This includes addressing the protease problem, which may be less compared to fungal fermentations, but nonetheless has significant room for improvements.

This study attempts to investigate the effects of production physiological factors such as medium formulations and culture conditions on protease repression or inhibition and authentic heterologous protein yield in *S. lividans* including.

Materials and Methods

The recombinant *S. lividans* 66 strain was kindly provided by Cangene Corporation, Canada. The plasmid carries a thostrepton-resistance marker. The rhIL-3 expression system and the casein peptone hydrolysate glucose salts medium formulation are proprietary.

The inoculum was prepared in 250 ml Erlenmeyer flask containing thiostrepton dissolved in 40 ml seed medium. An aliquot, 2 ml was transferred to a 250 ml Erlenmeyer flask containing 40 ml production medium. Initially, all the flasks were incubated at 32℃ on rotary shakers set at 20 rpm. The temperature was reduced to 19°C for half of the flasks at 18 h post-inoculation. The experiment was performed in duplicate.

Samples, 10 ml, were centrifuged for 5 min at 5000 g. The biomass pellets were washed twice and then dried in an oven at 110°C to constant weight. The culture supernatant was analysed for glucose using a commercial kit (Sigma).

Supernatant samples were subjected to SDS-PAGA analysis using 15% (w/v) polyacryl amide gel. After running for 70 min at 120 V, the gel was stained with Coomassie Blue. The amount of rhIL-3 was estimated by comparing the intensity of its band to a gel with known concentrations of rhIL-3 concentrations.

Azocaseinase activity was used to measure proteolytic activity in the culture medium, and was quantified using the method described by Sarath *et al.* [4]. One activity unit is defined as the amount of enzyme required to produce an absorbance change of enzyme required to produce an absorbance change of 0.001 in a 1 cm cuvette under the conditions of the assay.

Results and Discussion

The concentration of authentic rhIL-3 is dependent on the type of carbon source available in the culture

medium. The growth on fructose resulted in higher overall growth rates at the expense of elevated protease. Figure 1 illustrates the profiles of the two rhIL-3 forms in cultures with glucose, fructose as the main carbon source.

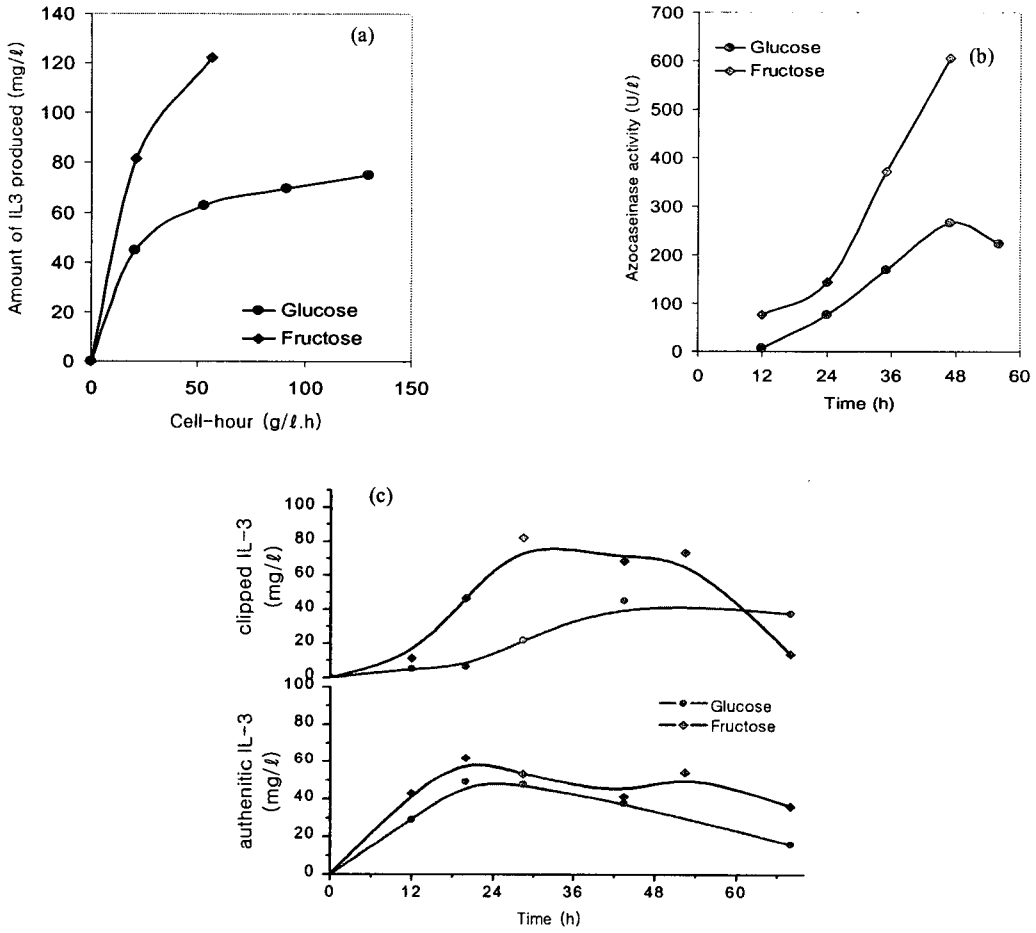

Fig. 1. Effect of carbon source on (a) rhIL-3 productivity (b) azocaseinase activity (c) authentic profiles of rhIL-3.

The concentration profiles of authentic rhIL-3 were relatively similar in all cases. However, the concentration of clipped rhIL-3 in the fermentation with fructose was almost three-fold at 20 h compared to that with sucrose. This implies the presence of elevated levels of tppA when *Streptomyces lividans* was cultivated on fructose. The concentration of clipped rhIL-3 continued to increase reaching about 80 mg/ ℓ at 28 h before declining. In contrast, the concentration of the clipped form of rhIL-3 in the cultures grown on sucrose and glucose continued to increase towards the end of the experiment. This observation agrees with the previous results shown Fig 1c, where the increasing levels of azocaseinase activity, especially in the case with fructose, caused the disappearance of both clipped and complete forms of rhIL-3 in the culture broth as the fermentation progressed. It was concluded that growth on fructose did not offer any advantage in terms of achieving higher titre of authentic rhIL-3 since clipped rhIL-3 contributed a major portion of the increase in total rhIL-3 titre.

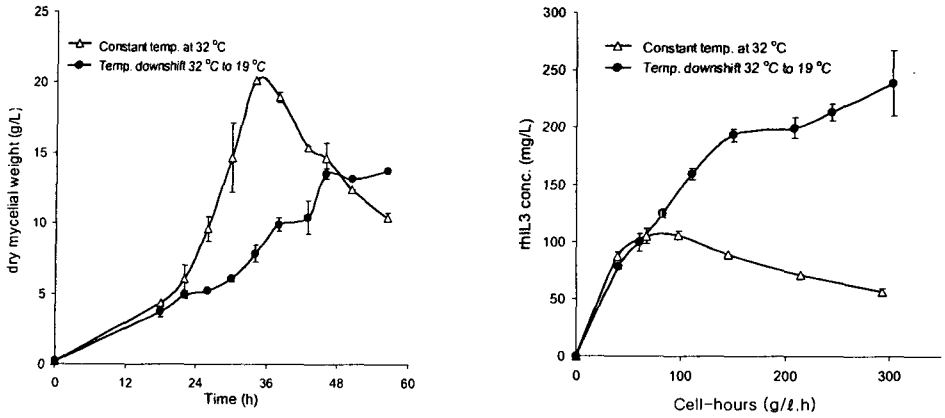

Fig. 2. Effect of temperature downshift on (a) dry cell weight and (b) rhIL-3 productivity.

Figure 2a illustrates the slower growth of *Streptomyces lividans* in shake flasks after lowering the temperature from 32°C continued to grow, reaching a maximum at 35 h, after which biomass started to decline. In contrast, biomass at 19°C continued to increase, albeit at a slower rate, until the experiment was terminated. In another experiment, *Strepmyces lividans* was cultivated in conventional shake flask at a temperature of 32°C. After 18 h of fermentation, the temperature was decreased to 19°C. Figure 2a illustrates the slower growth rate of *Streptomyces lividans* in shake flasks after lowering the temperature from 32°C to 19°C at 18 h. The culture grown at 32°C continued to grow, reaching a maximum at 35 h, after which biomass started to decline. In contrast, biomass at 19°C continued to increase, albeit at a slower rate, until the experiment was terminated.

The effect of growth temperature on rhIL-3 levels in the supernatant is evident in Fig. 2b. The level of rhIL-3 began to decrease from the maximum value of about 120 mg/L as the culture grown at 32°C entered late log phase and drew closer to stationary phase. In contrast, following temperature downshift the rhIL-3 titre continued to increase until the experiment was terminated at 46 h, finally reaching about 250 mg/ℓ. the maximum specific concentration of rhIL-3 was determined to be 4 mg/g and 17 mg/g for cultures grown at 32°C and downshifted to 19°C, respectively.

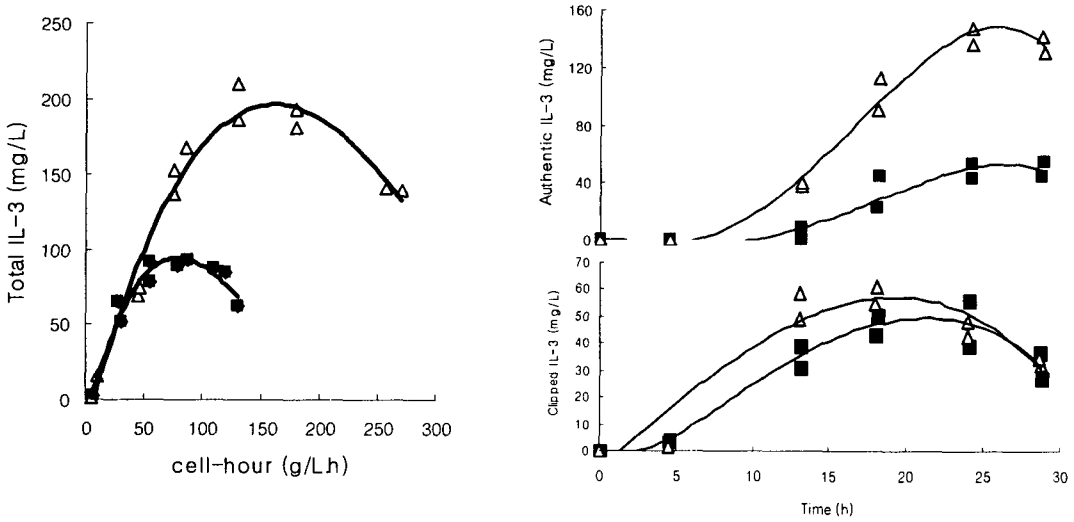

Fig. 3. Effect of oxygen (a) total IL-3 productivity (b) rhIL-3 authenticity. ■, air; △, oxygen.

The findings indicate that although oxygen-enriched fermentation can increase the overall product titre, the instantaneous specific productivity remains unchanged. The volumetric rhIL-3 concentration increased by 2-fold when supplied with oxygen, rather obtained in less than 24 h. The results showed that the production process of rhIL-3 is tightly coupled with growth and is highly dependent on oxygen level (Fig. 3a). They also show the undesirable corresponding increase in general proteolytic activity with increasing biomass. However, the expression of general protease typically lags behind rhIL-3 expression. In practice, the cultures would be terminated well before approaching the stationary phase to avoid condition that may potentially lead to secondary metabolite production and sporulation.

The higher biomass also meant increasing production of rhIL-3 and intrinsically higher tppA activity. Presumably, the elevated concentration of tppA in higher density cultures soon took its toll on the authenticity of rhIL-3, reducing the concentration of authentic rhIL-3. As the results, 70% of the maximal 150 mg/L rhIL-3 produced were clipped in the bubble column culture aerated at 2.0 vvm (Fig. 3b). It seemed that the optical balance between increasing total rhIL-3 with relatively lower tppA was, in this case, at an aeration rate of 1.5 vvm. This compromise resulted in a moderately high total rhIL-3 titre at 118 mg/L, but maintained a ratio lower than 50% of clipped rhIL-3 throughout the fermentation.

Conclusion

Recombinant human interleukin-3 has been successfully secreted in 100-200 mg/ℓ levels in *Streptomyces lividans* fermentations. The course of *Streptomyces lividans* fermentation shows a wide variation under different growth conditions. The results clearly demonstrate the need for the establishment of strict culture conditions to ensure reproducibility of subsequent production schemes for heterologous proteins. Although the secretion levels are still very low, the possibility of enhancing rHuIL-3 secretion shown in this work offers significant bioprocessing advantages.

References

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