Mathematical Modeling the Dynamics of Recombinant Endonuclease Production in Microsystems

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ABSTRACT

A mathematical model for the growth of genetically modified strain of *Pichia pastoris* and the expression of recombinant human DNase I, which is a clinically useful enzyme for cystic fibrosis, was analyzed to investigate the production of it in micron-size biotechnological devices. DNase I catalyses the degradation of DNA, and hence an extracellular production of it from *Pichia pastoris* was designed. First, human DNase I gene was inserted into pPIK9K and expressed in *P.pastoris*. The activity in culture supernatant reached 2.06 mU/μL at 120 h. Second, kinetics for the growth and the product formation was modeled. Calculation, based on the mathematical model, resulted in the maximum productivity of 0.901 mU/μL· h for steady-state plug flow micro-bioreactors. The space-time at which the maximum productivity occurred was 37 h. Experiments for the separation of recombinant human DNase I in culture supernatant on a micro-device were also performed. The partition coefficient of the run with 90 μg/μL polyethylene glycol and 80 μg/μL dextran was 0.32.

Keywords: DNase I, *Pichia pastoris*, recombinant DNA, growth kinetics, product formation kinetics, mathematical model, micron-size biotechnological process, partition coefficient, micro-bioreactor, cystic fibrosis.

1 INTRODUCTION

Mathematical modeling the dynamics of the production of a clinically useful protein on micron-size biotechnological devices requires the simultaneous consideration of the inherent kinetics along with the mass transfer effects created by the configuration of biocatalyst and the fluid-surface interactions in micro flow systems. Genetically engineered microorganisms in free or immobilized form would play a vital role in such micro scale productions. To construct a mathematical model, a set of equations are needed. The element of this set includes the equations of fluid dynamics, continuity, the mass transport equations, the energy balance equations [1], the equations of slip phenomena, wetting, adsorption and electrokinetics [2], and the rate equations of microbial cell growth, substrate consumption [3-7], DNA replication, transcription, translation [8,9] and post-translational processes[10]. The principal modeling equations for simulating the process dynamics for the production of clinically useful protein are the rate equations for microbial cell growth and the desired protein formation.

The construction of DNA molecule for the micro scale production of recombinant human DNase I and the formulation of a mathematical model describing the kinetics of DNase I production are the subjects of this study. DNase I is an endonuclease that cuts within a polynucleotide chain to yield 5'-phosphorylated di- and oligo- nucleotides. The fact that no recombinant plasmid coding for the DNase I gene is exempt from the deleterious effect of its products presents serious problems to the development of recombinant DNase I production systems. To cope with this problem, study was designed to achieve an extracellular production of human DNase I gene product from methylotrophic yeast *Pichia pastoris*. This enzyme holds promise of an agent for the lung disease caused by cystic fibrosis that is a genetic disease causing the body to generate an abnormally thick, sticky mucus. Patients with this disorder are approximately 20-30 in Japan and 30,000 in the United States.

2 EXPERIMENTAL

The methylotrophic yeast used for heterologous expression was *Pichia pastoris* GS115 (ATCC20864). *P.pastoris* was grown in buffered methanol-complex medium (BMMY; 10g/L yeast extract, 20g/L Bacto Peptone, 13.4g/L Difco's Yeast Nitrogen Base without amino acid, 100mmol/L potassium phosphate at pH6.0, 40 μg/L biotin, 0.5% methanol added daily) or in buffered glycerol-complex medium (BMGY; 10g/L yeast extract, 20g/L Bacto Peptone, 13.4g/L Yeast Nitrogen Base without amino acid, 100mmol/L potassium phosphate at pH6.0, 40 μg/L biotin, 10 g/L glycerol).

The *Escherichia coli* strain XL-1 blue was used in DNA manipulations for PCR.

The plasmid pHumanDNase1 cording for the cDNA of human DNase I gene was kindly supplied by Dr.Toshihiro Yasuda, Medical School, Gunma University. The *P.pastoris* vector pPIC9K(Invirogen) was used for expression.

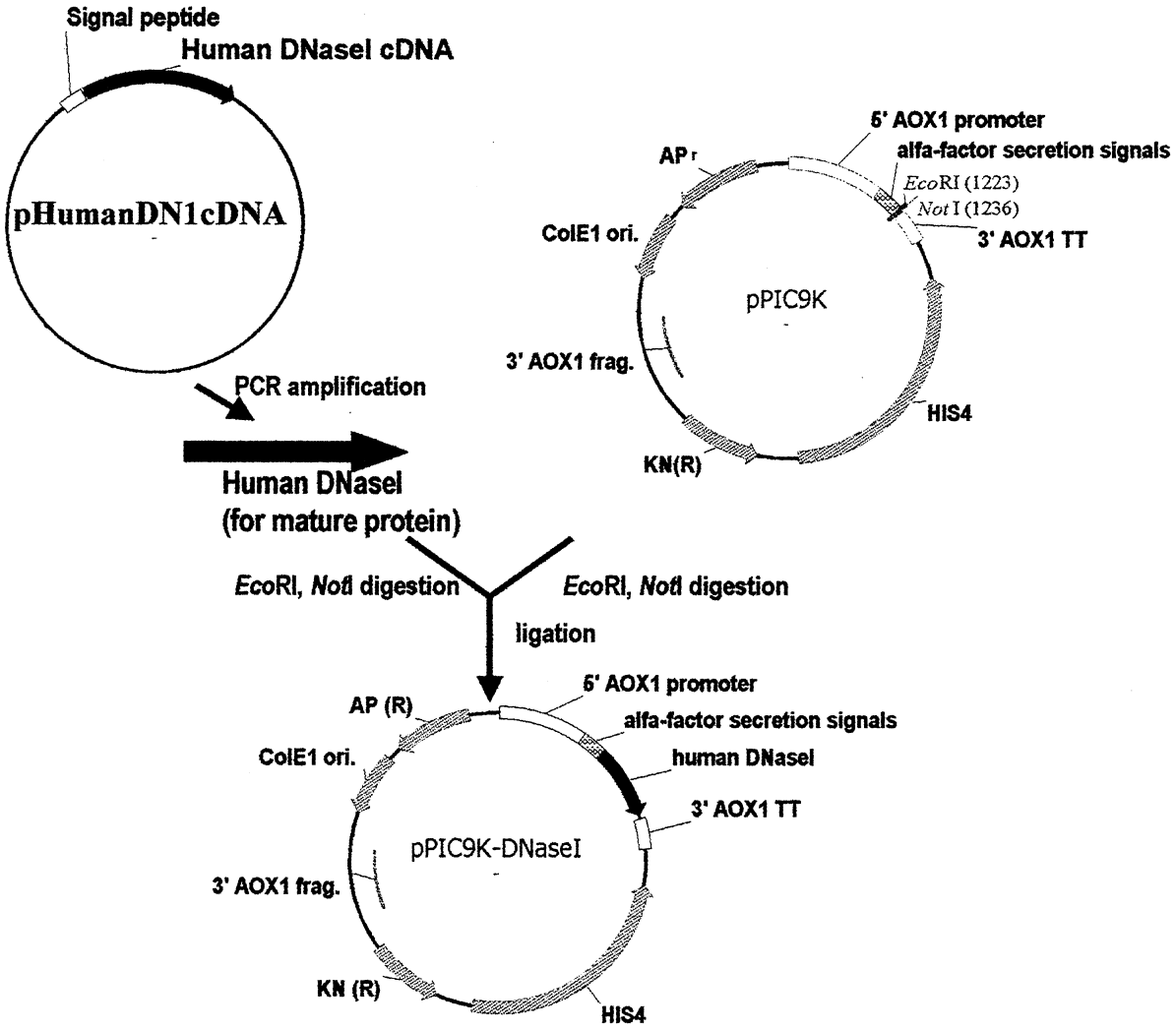

Figure 1. Construction of the human DNase I bearing plasmid pPIC9K-DNase I.

For expression, *P.pastroris* was incubated at 303K in a 500 mL Erlenmeyer flask containing 40 mL BMMY solution. Flasks were shaken at 250 rpm on a rotary-reciprocating shaker. The assay of human DNase I activity was performed according to single radial enzyme-diffusion (SRED) method [11]. For the assay, agar plate (2 mm thick) containing DNA, ethidium bromide and reaction buffer for DNase I at pH6.5 was prepared. Sample wells were punched in each agar plate using 1 mm diameter cork borer. Culture aliquots were collected daily and cells were removed by centrifugation. Human DNase I activity in the culture supernatant was determined by loading 1 μL of it into the well of the agar plate. The plate was incubated for 2h at 310K and photographed using UV field illumination for 1/3 second. The total diameter of clearing around each well was measured and units of human recombinant DNase I were read off from a calibration plot, constructed using a set of data from the test with bovine DNase I of which activity was known

3 RESULTS AND DISCUSSION

Signal sequence was removed from human DNase I genes (849 bp). PCR technique was used for amplification. In developing human DNase I excretion systems, we have constructed the plasmid pPIC9K-DNase I (Fig.1) by inserting a 783 bp *EcoRI/NotI* fragment carrying the human DNase I gene without signal sequence into the *EcoRI-NotI* sites of the expression vector pPIC9K. The plasmid pPIC9K-DNase I was integrated at the AOX1 region in the chromosome of *P.pastroris* GS115.

Figure 2 compares the size of total diameter of clearing around each well, by SRED method, that was due to

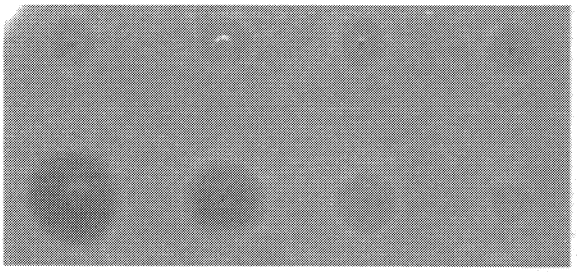

Figure 2. Comparison of human DNase I activities in the supernatant of culture of *P.pastroris* GS115/pPIC9K-DNase I with those of bovine DNase I.

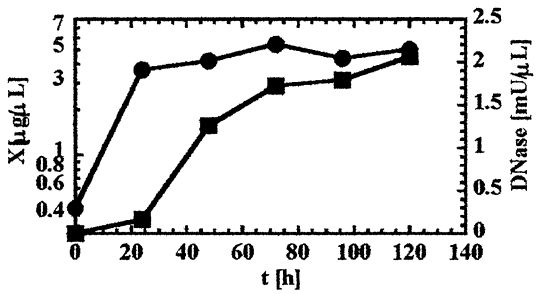

Figure 3. Time courses of the concentration of *P.pastoris* dry cell mass (●) and the activity of human DNase I (■).

recombinant human DNase I with that due to bovine DNase I. Four wells shown at the top of the photograph contained the recombinant human DNase I activity in the supernatant of culture of *P.pastroris* GS115/pPIC9K-DNase I, and those shown at the bottom contained bovine DNase I. The activities of bovine DNase I used were, from left to right, 2600, 260, 26 and 2.6 nU/μL. The extracellular production of recombinant human DNase I was quite evidently recognizable in this photograph.

Shown in Fig. 3 is the batch culture time courses of the concentration of *P.pastoris* biomass measured by optical density at 660 nm and the activity of the recombinant human DNase I activity in the supernatant of culture of *P.pastroris* GS115/pPIC9K-DNase I. The activity of the secreted endonuclease was low during the logarithmic growth, while it increased significantly during the late logarithmic growth. The activity of DNase I reached 2.06 mU/μL at 120 h.

The kinetic model for cell growth and DNase I formation based on this experimental observation is

$$dX/dt = \mu\phi X \quad (1),$$

$$\phi = 1 \quad (2)$$

for logarithmic growth phase,

$$\phi = a_1 - a_2 X/X_f \quad (3),$$

for late logarithmic growth phase,

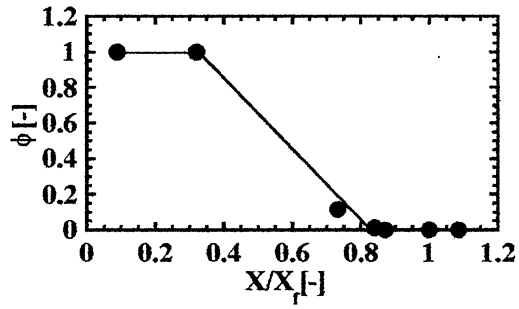

Figure 4. Growth kinetics of *P.pastoris* in terms of the relative cell mass concentration.

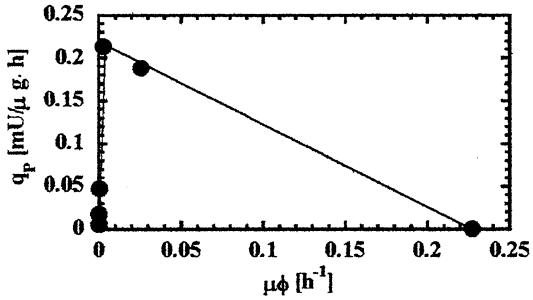

Figure 5. Product formation kinetics of DNase I in terms of the growth activity of *P.pastoris*.

$$dc_P/dt = q_P X \quad (4),$$

and

$$q_P = b_1 + b_2\mu\phi \quad (5)$$

where X is the concentration of *P.pastoris* bimass, X_f the concentration of *P.pastoris* bimass at the end of cell growth, c_P the activity of DNase I in a unit volume of culture, μ the specific growth rate at the logarithmic growth phase, ϕ the coefficient defined by Eq.(1), q_P the specific expression rate, in terms of the change of activity in a unit volume of culture, of DNase I, and a_1, a_2, b_1 and b_2, model parameters. The specific growth rate in the logarithmic growth phase was 0.227 $h^{-1}$and the attainable level of cell mass concentration was 5.02 μg/μL. Experimental results confirming the profiles predicted by Eqs.(2), (3) and (5) are shown in Figs.4 and 5. Calculations by the least squares method on Fig.4 resulted in the following parameters; a_1=0.162 and a_2=1.97. Those on Fig.5 resulted in the parameters; b_1=0.0154 mU/μg·h, b_2=67.6 mU/μg for $\mu\phi$ being not more than 0.0259 h^{-1} and b_1=0.215 mU/μg·h, b_2=-0.946 mU/μg for $\mu\phi$ between 0.0259h^{-1} and 0.227 h^{-1}.

A micro scale production of recombinant human DNase I on a steady-state plug flow micron-size device for bioreaction was modeled mathematically. Simulation result suggests that the space time at which the maximum

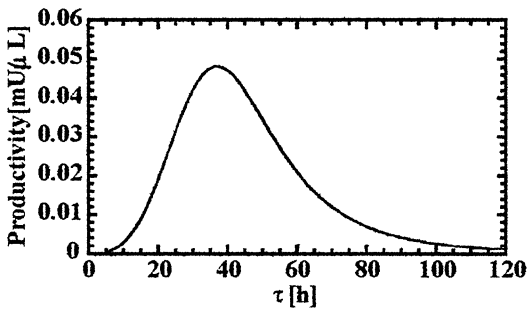

Figure 6. Simulation of productivity of recombinant human DNase. τ: space time.

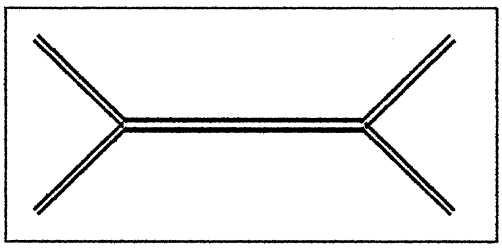

Figure 7. Micro-size device for the separation of recombinant human Dnase.

productivity of the gene product occurs is 37h (Fig.6). The maximum productivity was 0.901 mU/μL.

Micro scale unit to separate recombinant human DNase I from cells was also designed using liquid-liquid extraction The device was fabricated on acrylic plastic using a desktop engraver (model EGX-300, Roland) (Fig.7). The typical microchannel was 300 μm wide and 100 μm deep. Along the micro-channel of this device aqueous polyethylene glycol solution containing DNase I was designed to contact with aqueous dextran solution.

Effect of the concentrations of polyethylene glycol and dextran upon partition coefficient was studied using BSA as a model protein. Concentration of BSA was set at 0.1 μg/μL. Concentration of polyethylene glycol or dextran ranged from 80 μg/μL to 110 μg/μL. The highest partition coefficient was 0.32. It was resulted in the run with 90 μg/μL polyethylene glycol and 80 μg/μL dextran. When 0.1 μg/μL Na_2SO_4 was added, partition coefficient increased to 0.34.

4 CONCLUSIONS

In this paper, we described the molecular construction of the *P.pastoris* vector for the expression of human DNase I and the formulation of the mathematical model on the product formation kinetics of recombinat human DNase I. The proposed mathematical model can be used to estimate the growth and the product formation in micron-size biotechnological process. The model can be used to find the optimum size and flow rate of the micro-bioreactors. The partition coefficient for the separation of protein found in this study is also useful for the design of micro scale separators.

ACKNOWLEDGMENTS

The authors thank Mr. K. Kuwabara for his excellent technical assistance. We also thank Dr.T.Yasuda for supplying a plasmid. This work was supported by grant on High Efficiency Micro Chemical Process Technology Project from The Research Association of Micro Chemical Process Technology, New Energy and Industrial Technology Development Organization, Japan.

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