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Alkaline Protease of a Genetically-Engineered *Aspergillus oryzae* for the Use as a Silver Recovery Agent from Used X-Ray Film

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Abstract *Aspergillus oryzae* U1521, which was a genetically engineered strain, produced 1,000,600 U per g · glucose of extracellular alkaline protease within 72 h in a submerged fermentation. However, the alkaline protease was not detected during the first 24 h. Northern blot analysis indicated that the enzyme synthesis was repressed at the transcriptional level during the lag period. Both catabolite repression and pH of the growth medium significantly affected the enzyme production. Use of this enzyme as a silver recovery agent from used X-ray film was confirmed by experiments in the shake-flask scale.

Key words: *Aspergillus oryzae*, alkaline protease, silver recovery, X-ray film

Mineral natural resources are rapidly decreasing throughout the world, therefore, the valuable minerals have been reused for the purpose of slowing down the mineral exhaustion. Used X-ray films contain a large amount of fine silver particles in its gelatin layers, however, they are usually discarded after being stored for a certain period. Presently, the most common method for silver recovery of the used X-ray film is a combustion method in which used film is incinerated in a furnace and the silver is recovered from the ashes. However, it is expensive to recover silver by this method, because maintaining the furnace and treating the effluent soot and smoke is tremendously costly. Furthermore, silver in base film made of polyester cannot be recovered by this method. Therefore, an inexpensive and effluent enzymatic method that does not pollute the enviroment has been widely suggested to be a sound alternative to the chemical process. Fujiwara and Yamamoto [7] suggested that alkaline protease from alkalophilic *Bacillus* sp. is capable of decomposing the gelatin layers of the used X-ray films, and this enzyme reaction can be used for recovering the silver.

Aspergillus oryzae U1521 used in this study is known as a genetically engineered strain [2]. This fungus secreted a high level of alkaline protease in both the solid state [3] and in submerged fermentation due to its high stability of additional copies of the inserted alkaline protease gene. The enzyme has its optimum activity at pH 9 and 45°C.

The main purpose of this research was to use alkaline protease of *A. oryzae* U1521 as an enzyme to hydrolyze the gelatin layer of the used X-ray films and to recover the silver particles without further purification. We explored the a potential use of alkaline protease overproduced in the strain, *A. oryzae* U1521, as a silver recovery agent for the used X-ray film commercially, depending on the experimental outcome. Studies of effects of glucose and pH of the growth medium are were included.

MATERIALS AND METHODS

Organism and Fermentation Conditions

A. oryzae U1521 was obtained from the Molecular Biology and Gene Technology Laboratory, King Mongkuts University of Technology Thonburi, Bangkok, Thailand. The culture was maintained on a malt extract agar (MEA) at 4°C. Fungal spores were obtained from a 5 day old-culture grown on MEA at 30°C. The spores were collected in 0.01% (w/v) Tween-80 solution.

The basal medium contained (g/l) glucose, 5; yeast extract, 10; KH_2PO_4, 10; $MgSO_4 \cdot 7H_2O$, 0.1; and $ZnCl_2$ (trace). The fungus was grown in a 250-ml flask containing 50 ml of medium. The suspension of 1×10^8 spores were inoculated and then incubated on a rotary shaker at 30°C, 250 rpm. The mycelia were separated from the fermentation broth by

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centrifugation at 5,000 rpm and the clear supernatant was used for analysis. The amount of enzyme was expressed as a unit per ml of the supernatant.

Optimization Studies

Growth period ranging from 12 to 96 h, effect of glucose (1 to 10 g/l) concentrations, and effect of pH of the medium (pHs 5-8) were evaluated in relation to enzyme yields. Appropriate buffers of citric acid-sodium citrate (pHs 5 and 6), KH_2PO_4-NaOH (pH 7), and Tris-HCl (pH 8) at 50 mM were used for controlling pH throughout the shake flask fermentation. These experiments were carried out in triplicate, and the results were taken as in the averages of three independent experiments.

Analytical Procedures

Alkaline protease activity was assayed by the method of Horikoshi [8], with a slight modification of optimum temperature and pH. One unit of the protease activity was defined as the amount of the enzyme that produced TCA mixture-soluble materials equivalent to 1 µg per min of tyrosine from casein at pH 9 at 45°C.

The biomass content was determined by measuring the dry weight of cells from a known amount of the sample.

The reducing sugar was measured by using a dinitrosalicylic acid (DNS) method. One-half ml of enzyme solution was added to 0.5 ml of DNS solution (0.04 M 3,5-dinitrosalicylic acid, 1.0 M potassium sodium tartate, and 0.4 M NaOH) and boiled for 10 min. The reaction was stopped by immediately cooling the reaction mixture, and absorbance was measured at 570 nm after adding 5 ml of water. Glucose was used as a standard at concentration ranging from 0.05 to 0.4 g/l.

Molecular Biological Studies

The mycelia in the basal medium was harvested by filtration, and RNA was extracted from the sample. Alkaline protease gene expression was investigated by Northern blot hybridization by probing with a specific alkaline protease gene, which was obtained from the 700-bp *SalI*/*EcoRI* fragment from pAP71 [2]. Most techniques used have been described by Maniatis *et al.* [13].

Enzymatic Hydrolysis Trials

The raw film used was RX-type X-ray film (Fuji Photo Film Co., Ltd., Tokyo). The films consisted of a 175-µm thick polyester base with 4.4 µm emulsion layers on both sides in which particles of silver halide with an average diameter of 1.2 µm were suspended before processing. On each of the emulsion layers, there was a protective overcoat with a thickness of about 1.2 µm [9]. The emulsion layers and the overcoats were made of gelatin, and their thickness increased by about three times when soaked in water compared with the dry state. From these raw films, the following two types of films were prepared: (i) film which was developed without being exposed to light and thus did not show any indications that any silver particle (unexposed film) was used in the controlled experiments, and (ii) film which was developed after being exposed evenly to light and thus contained fine silver particles in the emulsion layers (exposed film).

In order to use the enzyme as a silver recovery agent of X-ray film, the maximally expressed alkaline protease at the 72-h sample of the growth period of *A. oryzae* U1521 was filtered, and the clear supernatant was then used in the experiments without further purification. The 125-ml flask, with a sheet of X-ray film (a certain weight from 0.05 to 0.25 g/sheet) was first filled with boric acid-NaOH buffer of pH 9.0 and, then shaken for about 10 min at a speed of 250 rpm and 45°C. Then, the enzyme was added to the solution to start the hydrolysis reaction for 10 min. The total 10-ml solution consisted of 50 mM boric acid-NaOH buffer of pH 9.0 contained the enzyme at a concentration of about 1,000 U/ml. The content of silver in the solution was determined by using a Shimadzu AA-6501 atomic absorption spectrophotometer.

Acid Digestion Procedure

A sheet of X-ray film with a certain weight was filled with 10 ml containing 4:1 HNO_3:H_2SO_4 and heated at 80-100°C for 20 min under a fume hood. Then, the solution was filtered and adjusted to 50 ml in a volumetric flask. The content of the silver in the solution was determined by using a Shimadzu AA-6501 atomic absorption spectrophotometer.

RESULTS AND DISCUSSION

Growth and Alkaline Protease Production

The fermentation profile of *A. oryzae* U1521 in the basal medium is shown in Fig. 1. The lag period of enzyme

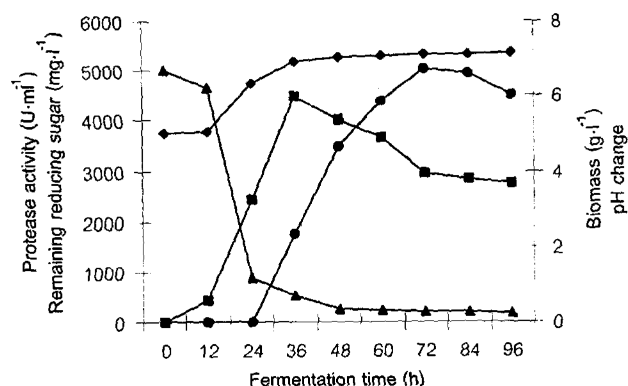

Fig. 1. Fermentation profile on alkaline protease production by *A. oryzae* U1521.
(■) biomass; (●) alkaline protease activity; (▲) remaining reducing sugar; (◆) pH change.

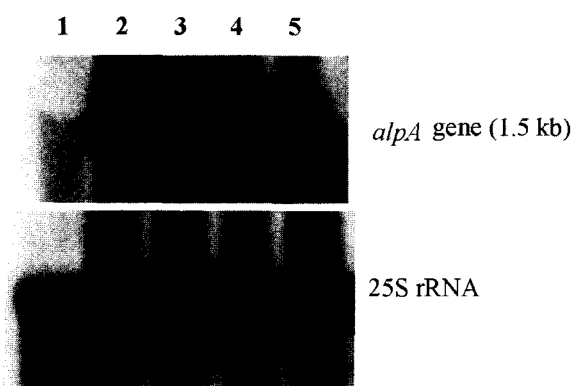

Fig. 2. Northern blot analysis of *A. oryzae* U1521 during batch fermentation.

Each lane contained 10 μg total RNA from mycelium grown in the basal medium. Alkaline protease-specific probe, a 0.7-kb *SalI*-*Eco*RI fragment from pAP 71, and 25S rRNA probe from *Mucor rouxii* ATCC 24905 were used. Lanes 1, 2, 3, 4, and 5 were from mycelium collected at 24, 36, 48, 60, and 84-h old samples, respectively.

production during the first 24 h of fermentation was apparent. As soon as glucose concentration in the medium dropped to a low level and the pH of the medium was changed to about 7, alkaline protease production increased sharply until 72 h, with the maximum activity of 5,030 U/ml (1,000,600 U/g glucose). Then, the alkaline protease activity declined. The decrease of the enzyme might be due to deactivation or to self-digestion [4]. The biomass reduction was detected after 36 h and microscopic examination revealed lysis of some mycelia, as reported in *Streptomyces exfoliatus* [12].

The *alpA* Gene Expression during the Fermentation

Northern blot analysis of alkaline protease gene expression detected no trace of gene expression at 24 h of fermentation (Fig. 2). This suggests that the synthesis of alkaline protease by *A. oryzae* U1521 was repressed at the transcriptional level during the lag period of the protease production. Therefore, the rapid increase in protease activity which appeared after 24 h resulted from a rapid *de novo* protein synthesis.

Effect of pH of the Medium

One of the reasons for the lag period of protease synthesis in *A. oryzae* U1521 might be that the production required an appropriate pH value which did not exist until about 24 h [11]. In order to study the effect of pH on the protease production, *A. oryzae* U1521 cultures were grown in the basal media at a range of pH values between 5-8. Figure 3 shows that the higher the pH of the growth medium, the lower the biomass yield. The specific protease activity reached its maximum level at pH 7.0. Northern blot analysis (Fig. 4) shows that the amount of mRNA was higher at pH 7.0 than at pH 5.0. It can be concluded that

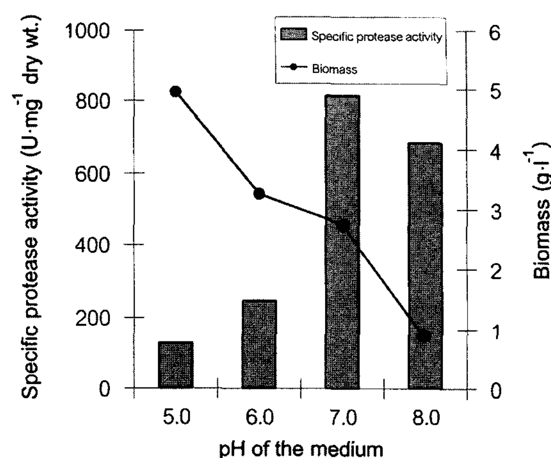

Fig. 3. Effect of pH of the medium on *A. oryzae* U1521 alkaline protease production and biomass.

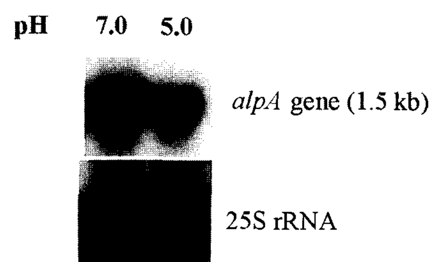

Fig. 4. Northern blot analysis of RNA extracted from *A. oryzae* U1521 batch culture grown in the basal medium at different controlled pH values.

Each lane contained 10 μg total RNA from the 72-h-sample controlled at pHs 7.0 and 5.0, respectively. Alkaline protease-specific probe, a 0.7-kb *SalI*-*Eco*RI fragment from *pAP* 71, and 25S rRNA probe from *Mucor rouxii* ATCC 24905 were used.

the pH of the culture regulated gene expression of alkaline protease.

Effect of Glucose

Catabolite repression effect might also be an another explanation for the lag period, since it was reported to be a common regulation of protease synthesis in many microorganisms [1, 5-6]. To investigate this effect on protease production, *A. oryzae* U1521 was cultivated in the media containing glucose at different concentrations of 1 to 10 g/l. The specific activity increased to a maximum at 5 g/l glucose (Fig. 5). The low specific activity at higher glucose concentration (>5 g/l) indicated the catabolite repression effect.

The Recovery of Silver from an X-Ray Film by Using an Acid Digestion Method

Results of a chemical procedure for recovering silver from an X-ray film was carried out by acid digestion of silver particles covered with gelatin layer in an exposed film.

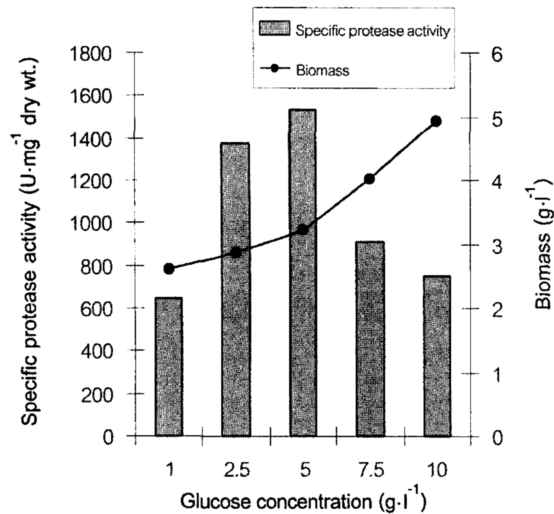

Fig. 5. Effect of glucose concentration on *A. oryzae* U1521 alkaline protease production and biomass.

The results strongly indicated the amount of silver being present at 2.00±0.05% by weight.

Enzymatic Hydrolysis of Gelatin and Release of Silver Particles

Figure 6 shows the enzyme capability to hydrolyze gelatin and the resulting release of silver particles. The efficiency of the enzyme to release silver particles in the exposed film was at its optimum at 0.20 g of X-ray film. The silver content released was 1.88±0.04% by weight. This means that alkaline protease of *A. oryzae* U1521 could almost completely hydrolyze the gelatin layer at a maximum of 0.20 g of X-ray film per 10,000 U of the enzyme solution.

In conclusion, both pH of the medium and catabolite repression significantly affected the alkaline protease production by *A. oryzae* U1521. The results also suggested that the enzyme synthesis occurred under conditions of carbon/energy starvation [10]. Furthermore, the enzymatic hydrolysis trials for evaluating the use of an alkaline protease of *A. oryzae* U1521 as a silver recovery agent from used X-ray film indicates that this enzymatic hydrolysis is an effective alternative procedure. However, further studies are required before undertaking an industrial exploitation process.

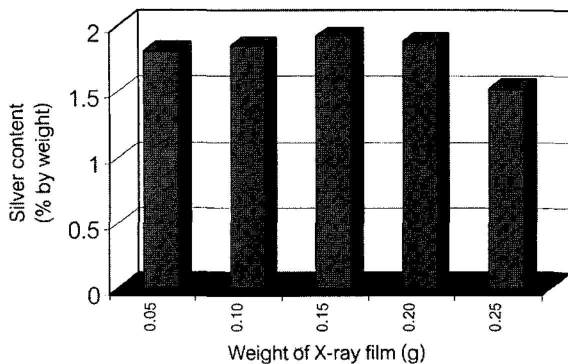

Fig. 6. The efficiency of releasing silver particles in the exposed film by using alkaline protease of *A. oryzae* U1521.

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