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Production of alkaline protease by a genetically engineered *Aspergillus oryzae* U1521

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The production of alkaline protease of *Aspergillus oryzae* U1521 was examined in liquid culture. In a culture of defatted soybean only, it gave satisfactory enzyme yields at 584,000 U/g defatted soybean. When various carbohydrates were supplemented, enzyme production was significantly increased. An increase in production by lactose was the most marked. Enrichment with casitone or casein increased productivity, but not cornsteep solid. Media formulation (g/L) of defatted soybean 10, lactose 5, casitone 1, and KH_2PO_4 5 enhanced alkaline protease production by *A. oryzae* U1521 to a maximum of 1,410,000 U/g defatted soybean. Scaling-up experiments indicated the flask-scale results could be reproduced at 40 g of substrate in 5-L fermenter. The enzyme activity was maximum between pH 8–9 and at a temperature of 45°C.

Key Words——alkaline protease; *Aspergillus oryzae*

Aspergillus proteases have been used in many fields, especially in food processing (Gerhartz, 1990). In Thailand, a yellow-green *Aspergillus* var. *columnaris* was first isolated from the soy sauce industry (Bhumiratana et al., 1980; Impoolsup et al., 1981). *Aspergillus oryzae* U212 obtained by two-step UV irradiation of *Aspergillus* var. *columnaris* produced twice as much protease as the wild-type did (Kalayanamitr et al., 1987). The major extracellular proteolytic activities of *A. oryzae* U212 appeared to be due to alkaline protease. Then the level of alkaline protease in *A. oryzae* U212 was improved further by cotransformation of the alkaline protease gene (Cheevadhanarak et al., 1991b). *A. oryzae* U1521, containing multiple copies of alkaline protease gene secreted five times more alkaline protease production than its parental strain, *A. oryzae* U212, did in solid state fermentation (Cheevadhanarak et al., 1991a).

As alkaline protease accounts for at least 25% of the total enzyme sales (Raq et al., 1998), *A. oryzae* U1521 has the potential to be an excellent candidate for large-scale production of this enzyme. In submerged culture, filamentous fungi generally produce smaller quantities of secretory enzymes than they do in solid-state culture. For the industrial production of enzymes, it is important to use an overproducing strain and to improve the cultural conditions to obtain better production of enzymes in liquid culture. This study explores the potential of using *A. oryzae* U1521, an overproducing strain, with an available low-cost complex medium, defatted soybean, for alkaline protease production.

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Materials and Methods

Organism and fermentation conditions. *A. oryzae* U1521 was obtained from the Molecular Biology and Gene Technology Laboratory, King Mongkut's University of Technology Thonburi, Bangkok, Thailand. The culture was maintained on malt extract agar (MEA) and contained (g/L) glucose 20, malt extract 20, peptone 1, and agar 20 at 4°C. Fungal spores were obtained from 5–7 day old culture grown on MEA at 30°C. The spores were collected in 0.01% (w/v) Tween-80 solution.

The basal defatted soybean (BDS) medium contained (g/L) defatted soybean 10 and KH_2PO_4 5, initial pH 5.0, unless otherwise stated. The fungus was grown aerobically in a 250 ml flask containing medium. The suspension of 1×10^8 spores were inoculated into the 50 ml medium, then incubated on a rotary shaker at 30°C, 250 rev/min. The mycelia were separated from the fermentation broth by centrifugation at 5,000 rev/min, and the clear supernatant was used for analysis. The amount of enzyme was expressed as unit per milliliter of the supernatant.

In the 5 L fermenter (LH fermenter, UK), the 8.0×10^9 spores suspension was inoculated into a 4 L medium, and the fermentation was carried out at 30°C with an aeration rate of 0.5 vvm and an agitation speed of 5,000 rev/min. Dissolved oxygen, pH, and temperature were monitored with appropriate on-line probes.

Analytical procedures. Alkaline protease activity was assayed by the method of Horikoshi (1971), with slight modification. The reaction condition was changed to pH 9.0 at 45°C. The 0.5 ml enzyme solution was added to the reaction mixture (2.5 ml) containing 0.6% casein in 50 mM boric acid-NaOH buffer (pH 9.0). After incubation at 45°C for 10 min, the reaction was halted by the addition of 2.5 ml of TCA mixture (0.11 M trichloroacetic acid, 0.22 M sodium acetate, and 0.33 M acetic acid). The reaction mixture was kept at 45°C for 10 min, then centrifuged. The absorbance of the supernatant was measured at 275 nm. One unit of the alkaline protease activity was defined as the amount of the enzyme that produces TCA mixture-soluble materials equivalent to 1 μg of tyrosine from casein per minute at pH 9.0 and 45°C, according to the above assay condition.

The biomass content was determined by measuring the dry weight of cells from a known amount of sample. In the culture of defatted soybean, however, the biomass content could not be determined because of the interference of defatted soybean solid.

The reducing sugar was measured by using the dinitrosalicylic acid method (Chaplin, 1986) with slight modification. The 0.5 ml of enzyme solution was added to 0.5 ml of DNS solution (0.04 M of 3,5-dinitrosalicylic acid, 1.0 M potassium sodium tartrate, and 0.4 M NaOH) and boiled for 10 min. The reaction was stopped by cooling it immediately. The absorbance was measured after adding 5 ml water at 570 nm. Glucose was used as a standard at a concentration ranging from 0.05 to 0.4 g/L.

The composition of defatted soybean was determined by using Jadah's method for protein analysis (Bassett et al., 1978) and Anthrone's method (Chaplin, 1986) for carbohydrate determined as glucose.

pH and temperature optima. The optimum pH and the optimum temperature of the enzyme solution were examined by using the similar assay condition for alkaline protease activity. The optimum pH was determined by measuring the activity in (50 mM) citrate buffer, KH_2PH_4-NaOH buffer, and boric acid-NaOH buffer at pH ranging 4–6, 7–8, and 9–10, respectively. The optimum temperature was determined at various temperatures (35–60°C).

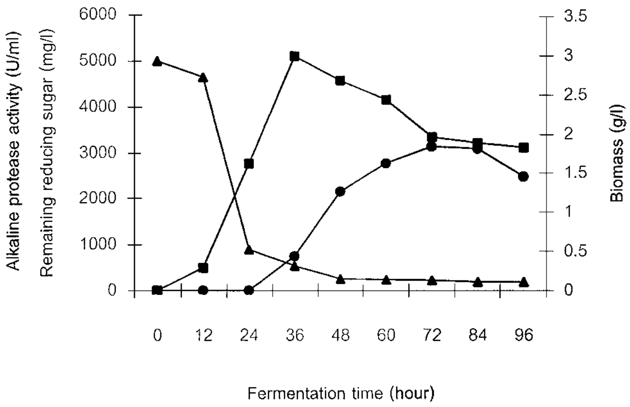

Fig. 1. Fermentation profile on alkaline protease production by *A. oryzae* U1521 in the culture of (g/L) glucose 5, yeast extract 5, KH_2PO_4 10, $MgSO_4\cdot7H_2O$ 0.1, and $ZnCl_2$ (trace).
(■) biomass, (●) alkaline protease activity, and (▲) remaining reducing sugar.

Results and Discussion

Time course analysis of alkaline protease production by A. oryzae U1521

The fermentation profile of *A. oryzae* U1521 in the medium consisting of (g/L) glucose 5, yeast extract 5, KH_2PO_4 10, $MgSO_4\cdot7H_2O$ 0.1, and $ZnCl_2$ (trace) is shown in Fig. 1. Enzyme production lagged during the first 24 h of fermentation. The investigation of alkaline protease gene expression using Northern blot analysis found no gene expression at 24 h of fermentation (data not shown). As soon as glucose concentration in the medium dropped to a low level, alkaline protease production increased sharply. The alkaline protease profile reached the maximum activity of 3,150 U/ml or 1,600 U/g dry cell at 72 h and leveled off thereafter. A similar alkaline protease profile was observed in the culture of BDS medium, as shown in Fig. 2. Unfortunately, cell growth could not be determined because of the interference of defatted soybean solid.

Defatted soybean as substrate for alkaline protease production

Our determination on the composition of defatted soybean indicated 45% protein and 23% carbohydrate determined as glucose. Therefore defatted soybean can be used as both carbon and nitrogen for cell growth. The production of alkaline protease in BDS medium was promising (Fig. 2). The production of proteases appeared to be subjected to control easily ei-

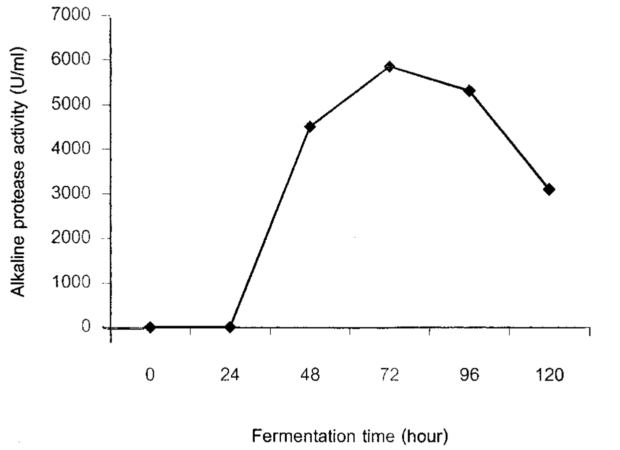

Fig. 2. Alkaline protease profile by *A. oryzae* U1521 in the culture of (g/L) defatted soybean 10 and KH_2PO_4 5.

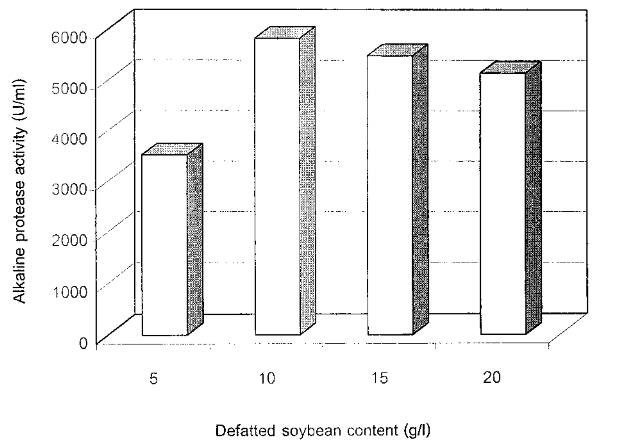

Fig. 3. Effect of defatted soybean content on alkaline protease production by *A. oryzae* U1521 in the culture of (g/L) defatted soybean, ranging from 5 to 20 and KH_2PO_4 5.

ther by metabolites repression/derepression or proteinaceous induction, or by the combination of both in several fungi (Cohen, 1973; Cohen et al., 1975; Farley and Ikasari, 1992; Lasure, 1980; North, 1982). Thus it is likely that the high production of alkaline protease in BDS medium involved the slow hydrolyzation and/or the proteinaceous component(s) of defatted soybean. *A. oryzae* U1521 secreted approximately 5,840 U/ml defatted soybean at 72 h of fermentation. This is much higher than the protease yields at the same unit definition produced by *A. oryzae* L-83 (10,320 U/g soybean meal) in submerged fermentation (Ueno et al., 1987). Therefore, the production of 584,000 U/g defatted soybean by *A. oryzae* U1521 assumes an industrial and economic importance.

Figure 3 shows that alkaline protease production increased with increasing defatted soybean content up to 10 g/L. This might be attributable to the increase in

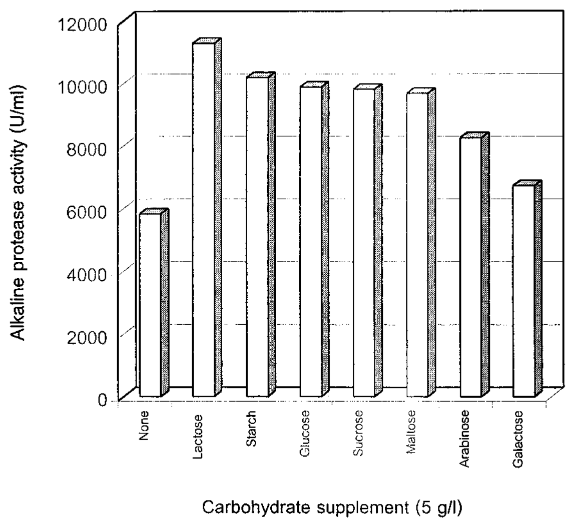

Fig. 4. Effect of carbohydrates (5 g/L) supplementing the medium contained (g/L) defatted soybean 10 and KH_2PO_4 5, on alkaline protease production by *A. oryzae* U1521.

growth yield at higher defatted soybean contents and to a further increase in total enzyme production. Candrall and Edward (1987) reported that in *C. albicans*, maximum specific activity was achieved with 0.2% glucose, but the total amount of protease increased as the glucose concentration and cell density increased, suggesting mild carbon catabolite repression of protease production. This was probably the explanation, since in defatted soybean composed of 23% carbohydrate determined as glucose, the BDS medium with 10 g/L defatted soybean assumed to contain about 2.3 g/L glucose. A content of defatted soybean higher than 10 g/L, however, prevented the increase in alkaline protease production by *A. oryzae* U1521. It was suggested that probably the alkaline protease production by *A. oryzae* in BDS medium was not dependent on the availability of defatted soybean content, but on the limitation of hydrolytic activity to break down the macromolecules of defatted soybean content in this range.

Enhancement of alkaline protease production by the addition of carbohydrates

To overcome the limitation of increasing alkaline protease production at high concentrations of defatted soybean, the BDS medium was supplemented with carbohydrates (5 g/L) (Fig. 4). An increase in alkaline protease production was observed when either glucose, starch, sucrose, maltose, or arabinose was added to the basal medium with different degrees of stimulation. Enhanced protease production in carbohydrate-enriched wheat bran in fungal solid state fermentation has also been documented by many researchers (Malathi and Chakraborty, 1991; Padmanabham et al., 1993; Ramana Murthy et al., 1993).

The addition of lactose was the most effective for the stimulation of alkaline protease production by *A. oryzae* U1521, followed by starch and glucose. The total alkaline protease activity was increased about twofold when lactose was added. Because the addition of glucose or galactose alone did not enhance the activity the same as lactose did, the induction should therefore be a direct effect from the intact lactose instead of from glucose or galactose. Ueno et al. (1987) reported the relation between enzyme induction and conidiation of the fungus by lactose and polysaccharides composed of galactosyl linkage.

Enhancement of alkaline protease production by the addition of casitone, casein, or cornsteep solid

From these experiments, the incorporation of lactose (5 g/L) into the BDS medium gave the highest protease production to 11,300 U/ml. Varying the concentration of added casitone, ranging from 1 to 5 g/L in the medium consisting of (g/L) defatted soybean 10, lactose 5, and KH_2PO_4 5, stimulated the alkaline protease production by 25% when casitone was added at 1 g/L and at only 7% when it was increased to 2.5 g/L (Fig. 5). It was suggested that amino acids and/or peptides in casitone may be served as specific inducers for increasing the total alkaline protease production. A similar result was found when casein was added. In contrast to casitone and casein suppliers, the addition of cornsteep solid failed to increase the alkaline protease production, even at low concentration. This could support the role of casitone and casein as a source of peptides and amino acids on the increase of alkaline protease by *A. oryzae* U1521. However, a trend on the inhibitory effect of casitone and casein in *A. oryzae* U1521 was noted on alkaline protease production at an increasing concentration. This might be due to nitrogen metabolite repression, reported as a regulatory on the synthesis of protease in many yeasts (Ogrydziak, 1993).

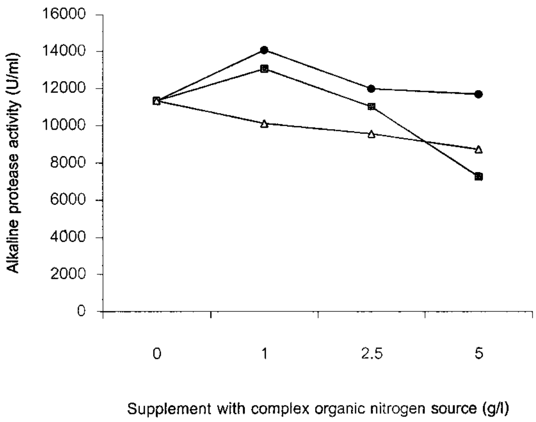

Fig. 5. Effect of casitone (●), casein (■), and cornsteep solid (▲) supplementing the medium contained (g/L) defatted soybean 10, lactose 5, and KH_2PO_4 5 at a concentration ranging from 1 to 5 g/L, on alkaline protease production by *A. oryzae* U1521.

Enzyme characteristic

Protease produced by *A. oryzae* U1521 inhibited by PMSF was indicated to be a serine protease. Figure 6a shows that the enzyme is effective in the alkaline pH range with an optimum pH of 8.0–9.0. The reduction in enzyme activity was 19 and 36% at pH 7.0 and 10.0, respectively. The enzyme exhibited an optimum temperature of 45°C (Fig. 6b). The activity was reduced by 15 and 66% at 50°C and 55°C, respectively.

In conclusion, the optimization of alkaline protease production by *A. oryzae* U1521 was successful in the medium consisting (g/L) of defatted soybean 10, lactose 5, casitone 1, and KH_2PO_4 5. Alkaline protease production was enhanced up to 14,100 U/ml or 1,410,000 U/g of defatted soybean. Scaling-up experiments in a 5 L fermenter in which the medium con-

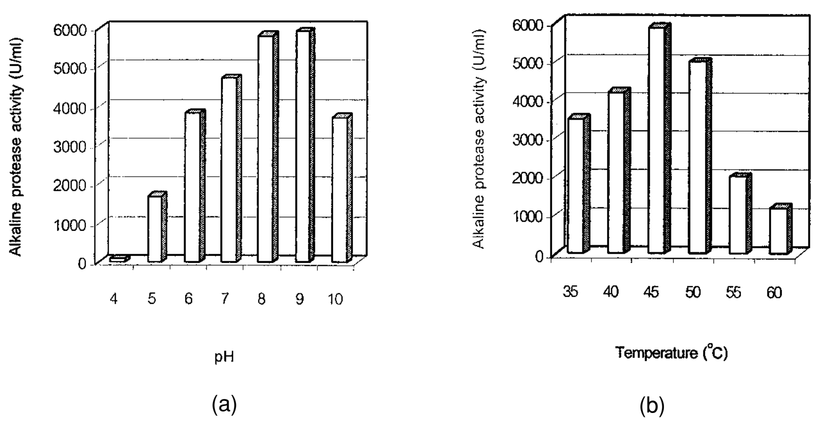

Fig. 6. (a) pH and (b) temperature profile of protease production by *A. oryzae* U1521.

sisted of up to 40 g of defatted soybean was reproducible. The enzyme exhibited maximum activity at pH 8–9, temperature optimum at 45°C, and is similar to those of commercially available fungal proteases employed in animal feed and food processing. Studies are in progress in bioreactors under controlled conditions employing various fermentation strategies to further improve the enzyme yields.

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