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Enzyme production and growth of *Aspergillus oryzae* S. on soybean koji fermentation

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Abstract

Soybean koji is an important ingredient for traditional fermented food in South-East Asia and East Asia. It provides large amount of enzyme from koji mold, *Aspergillus oryzae* S., to digest nutrients in substrates. This study was aimed at production of certain enzymes in soybean koji and potential to be applied for accelerating fish sauce fermentation. Koji containing 60% soybean was used as substrate to investigate the enzyme production by *A. oryzae* S. The growth of this mold was enumerated by scanning electron microscope. During koji fermentation, pH increase of soybean koji was caused by enzymes production. The highest protease and amylase activities were 84.38 and 200 unit/g of dry weight, respectively. Moreover, growing of enzyme activities on soybean koji correlated with the growth of this mold. Electron micrograph showed that spores of *A. oryzae* S. were formed after 48 h of cultivation period. Additionally, the highest enzymes activities were also shown in this stage.

Keywords: *Aspergillus oryzae* S., soybean koji, protease, amylase

1. Introduction

Koji is cooked wheat and/or soybean that has been inoculated with a fermentation culture or koji mold. The first step in making fermented foods, such as soy sauce, miso, mirin, sake, and other fermented foods is

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creating the koji. During the koji making, the koji mold (*Aspergillus oryzae* S.) produces a variety of amylases and proteases to break down carbohydrates and proteins in wheat and soybean [1, 2, 3].

A. oryzae S. is a filamentous fungus, which has an ability to secrete large amounts of hydrolytic enzymes. It is widely used in the manufacture of traditional fermented soy sauce in Asia. The extracellular proteins in soybean koji inoculated with *A. oryzae* S. contain different protein profiles including neutral and alkaline protease, amylase, glutaminase, and metallopeptidase [4]. Moreover, *A. oryzae* S. is genomically well characterized and considered to be a safe organism for producing of food enzymes because it lacks expressed sequence tags for the genes responsible for aflatoxin production [5].

In traditional fermentation, solid-state fermentation (SSF) is suitable for fungi growth because of its low moisture content and permitting of the penetration of fungi mycelium through the solid substrates. Fungal mycelium can penetrate into the solid substrate as 4 layers of mycelium penetration. The first layer is areal hyphae, followed by aerobic wet hyphae and anaerobic wet hyphae, the last layer is penetrative hyphae [6]. Low humidity in a solid-state fermentation makes microorganisms more capable of producing certain enzymes and metabolites which usually will not be produced in a submerge fermentation [6, 7].

As same as traditional soy sauce production, protease and amylase are also used to hydrolyze substrate in fish sauce fermentation to create amino nitrogen and other nutrients. However, this process takes up to one to two years for fermentation. Accordingly, there are many acceleration techniques, such as acid or alkaline hydrolysis, enzyme degradation, and bacterial inoculation [8, 9]. The acceleration of fish sauce fermentation will be advantageous if the fermentation period can be reduced. In this study, we investigated the production of certain enzymes in koji containing mixture of soybean and wheat bran which have been used in soy sauce production and the potential to be applied for accelerating fish sauce production. Moreover, the growth of *A. oryzae* S. during koji fermentation was also investigated.

2. Materials and methods

2.1. Koji preparation

Sixty percent of soybean was soaked in water for 6-8 h then autoclaved at 121°C for 40 min. Forty percent of wheat bran was roasted to dark brown and broken. The 32% of total wheat bran was ground to powder and mixed with culture before spreading to raw material to ensure mixing through of raw material. Spores of *A. oryzae* S. NPUST-FS-206-A1 (0.1%) were inoculated into raw material and incubated at 30°C for 72h.

2.2. Enzyme extraction

The fermented matter was mixed with 0.1 M phosphate buffer (pH 6.9) (1:2 w/v) using a shaking incubator (150 rpm, 30°C, 30 min). The supernatant was collected as crude enzyme extract by centrifugation at 10,000 xg, 4°C for 15 min.

2.3. Analytical methods

The moisture content of a fermented sample was determined by infrared moisture determination balance (FD-720, Kett Electric Laboratory, Tokyo, Japan). The fermented matter was mixed with deionized water (1:2 w/v) by blender and the pH value was measured by pH meter (DKK-TOA, HM-25G, Japan).

Casein solution (0.65%) in 0.05 M phosphate buffer (pH 6.9) and 0.05 M carbonate buffer (pH 10) were used as a substrate to analyze neutral and alkaline protease activity, respectively. One mL of crude enzyme extract and 5 mL of casein were mixed and incubated at 30°C for 10 min. The reaction was arrested by adding

5 mL of trichloroacetic acid (TCA) and incubated for 30 min. After centrifugation at 10,000 xg for 10 min at 4°C, the supernatant was collected. The supernatant (2 mL) was reacted with 5 mL of 500 mM Na_2CO_3 and 1 mL of five-fold diluted Folin-Ciocalteau reagent for 10 min. Absorbance was read against a blank at 660 nm. One unit of enzyme activity was defined as the amount of enzyme that liberated 1μg of tyrosine per minute under assay conditions [10].

Amylase activity was measured with 1.0% soluble starch in 0.1 M sodium acetate buffer (pH 5.0) as a substrate. One mL of crude enzyme extract and 1 mL of soluble starch were mixed and incubated at 30°C for 10 min. After incubation, one mL of 3, 5-dinitrosalicylic acid was added, reacted in a boiling water bath for 15 min, cooled to room temperature and added with 9 mL of deionized water. The amylase activity was analyzed spectrophotometrically at 540 nm. One unit of enzyme activity was defined as the amount of enzyme that released 1 mM of reducing sugar as maltose per minute under the assay condition [11]. The enzyme activity was reported per gram of dry koji used in the initial extraction. All the samples were analyzed in triplicate.

2.4. Scanning electron microscope

Samples were fixed by 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.0), dried by a critical point dryer (HCP-2, Hitachi, Japan) and mounted on stub holders. The samples were then coated with gold in a coater (E-1010, Hitachi, Japan) and investigated on a scanning electron microscope (Model S-3000N, Hitachi, Japan).

3. Results and discussions

3.1. Physical properties of koji

As shown in Fig. 1a, the initial pH of koji was 6.32, then the pH decreased to 6.12 after 24 h of fermentation. However, it increased to 6.97 at the end of fermentation period. Similar results have also been reported for *A. oryzae* in soybean koji by Liang et al. [4]. The increasing of pH of fermented matter was due to the microbial metabolic activities especially various extracellular proteins production [4].

Moisture in substrates brings a suitable water activity and swells substrates for mold growth [12]. The results showed that the initial moisture content of koji was 40% then increased to 45% at 12 h cultivation. However, it decreased to 22% after 72 h of fermentation (Fig. 1a). The high moisture content at the beginning of fermentation period resulted in decreasing the porosity of substrates and reduction of heat transfer. Consequently, low enzymes activities at the beginning of cultivation period (Fig. 1b) might be caused by the increasing of temperature (data not shown) [13].

3.2. Enzyme production

The changes of neutral protease, alkaline protease and amylase production during soybean koji fermentation were investigated (Fig. 1b). Neutral and alkaline protease activities of koji increased rapidly after 24 h of fermentation. At 48 h of fermentation, soybean koji showed the highest neutral protease activity at 84.38 unit/g of dry weight. However, alkaline protease activity was slightly increased and the activity was lower than neutral protease. The highest alkaline protease activity was shown at the end of fermentation period at 41.35 unit/g of dry weight. Amylase activity showed the highest activity (200 unit/g of dry weight) at 72 h of fermentation. These results were similar to the previous study that extracellular proteins from *A.*

oryzae on soybean koji were deduced by the intensity of time dependence during 48 h by digesting nutrients from substrates [4].

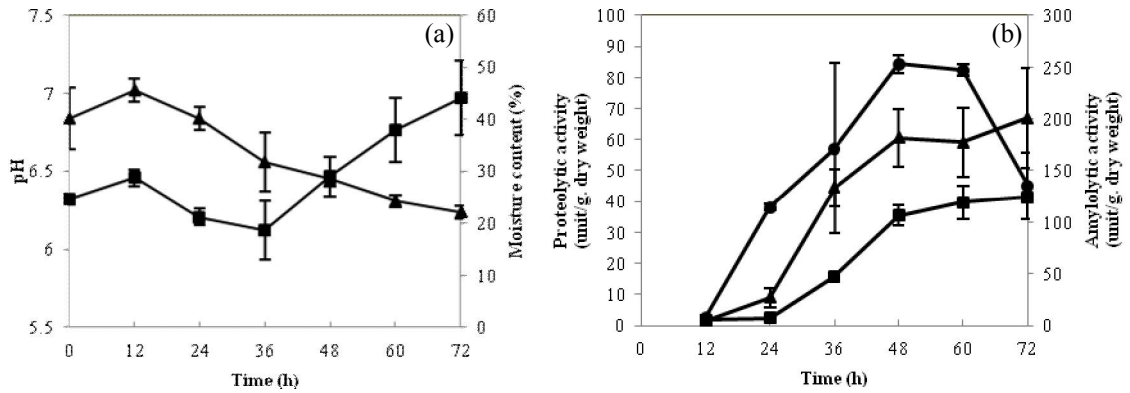

Fig. 1. Changes of pH (■) and moisture content (▲) (a); neutral protease (●), alkaline protease (■), and amylase (▲) production (b) during koji fermentation.

3.3. Growth of A. oryzae S. *during koji fermentation*

The growth of *A. oryzae* S. during koji fermentation was observed by a scanning electron microscope. As shown in Fig. 2, *A. oryzae* S. grew continuously during cultivation period. The growth of mold also showed the contrasting relation with the moisture content (Fig. 1a). The decrease of moisture content in koji is due to the utilization of water in substrate by mold to generate mycelia [12].

Furthermore, the increasing of enzyme activity was also related to the growth of *A. oryzae* S. (Fig. 1b). Sanchez and Pilosof (2000) have indicated that development of conidia and protease production of *A. niger* are correlated during the fermentation period [14]**.** Additionally, the results showed that neither high content of enzyme activity nor spore forming of mold was observed at the beginning of cultivation (24 h). However, we found the formation of spores and the highest enzyme activities at 48 h of cultivation. The reason was reported that the asexual cycle or spore forming is related to the secondary metabolite production, such as enzyme and organic acid [3].

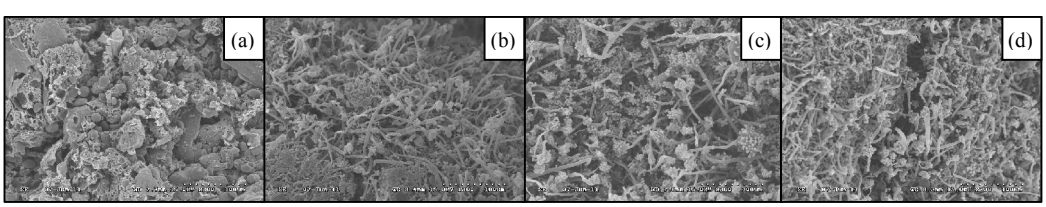

Fig. 2. Scanning electron micrographs of mycelium propagation on soybean koji fermentation. at 0 h (a), 24 h (b), 48 h (c) and 72 h (d).

4. Conclusion

The results indicated that production of enzymes from *A. oryzae* S. was related to the physical properties of soybean koji during fermentation. Moreover, the mycelium growth of fungi was also related to the increasing of enzyme production. Particularly, both the spore forming stage of *A. oryzae* S. and the highest enzyme production were at 48 h of fermentation period. It can summarize that fermented soybean koji at 48 h might be used as an activated enzyme source to accelerate fish sauce fermentation.

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