HYDROLYSIS OF SOYBEAN PROTEIN BY *ASPERGILLUS SOJAE, A ORYZAE* AND *RHIZOPUS OLIGOSPORUS*

By

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Abstract

Three species of molds, i.e. *Aspergillus sojae, A. oryzae* and *Rhizopus oligosporus* were used to hydrolyze soybean proteins. Whole soybeans were soaked overnight and cooked in boiling water for an hour, drained, sterilized at 121°C for 15 minutes, then cooled and inoculated with *A. oryzae, A. sojae,* and *R. oligosporus*. As a control treatment another batch of soybeans was prepared for spontaneous fermentation. Fermentation lasted for five days.

Based on the colony forming units (CFU), *A. sojae, A. oryzae, R. oligosporus* and the control reached the stationary phase on the third day of fermentation. The CFU of *A. sojae* and *A. oryzae* were higher than that of the others, with 10^7 — 10^8 CFU/g dry weight for *A. sojae* and *A. oryzae* and 10^6 — 10^7 CFU/g dry weight for the others.

The proteolytic activity of *A. sojae, A. oryzae, R. oligosporus* and the control was maximum on the third day. The proteolytic activity of *A. sojae* and *A. oryzae* using soybean as substrate was higher (180 — 200 units tyrosine/g dry weight) than the other two groups (100 — 120 UT/g dry weight).

Based on the efficiency of hydrolysis, *A. sojae* and *A. oryzae* had a higher hydrolysis percentage, giving 32 — 33% and total soluble nitrogen of 2.1 — 2.2 percent/g dry weight. The hydrolysis efficiency of *R. oligosporus* and the control was 21 — 22%, with the total soluble nitrogen 1.4 — 1.5 percent/g dry weight. The difference in initial pH i.e. pH 5.0; pH 6.0 and pH 6.7 did not affect the soluble nitrogen production significantly.

Introduction

Molds have been used for fermented food production since they are capable of hydrolysing the complex-component of raw materials to the lower molecular weight and extractable substances which in turn contribute the specific flavor of fermented food. Hydrolysis of soybean protein by mold enzymes is an important part in some fermented food production, such as 'kecap' (Indonesian soy sauce), 'tempe' and 'shoyu'.

"Kecap' is a popular condiment in the Indonesian diet. It is produced by a rather primitive method involving two stages of fermentation, mold fermentation and brine fermentation. For the first stage black soybeans are soaked in water for 12 hours (over night), boiled for 3 — 4 hours until soft, drained and spread on bamboo trays, then covered with rice straw or gunny sack. Since the trays and gunny sack are used repeatedly, they are permeated with mold spores important for natural inoculation. This spontaneous mold fermentation is allowed for 3 — 12 days at 20 — 30°C (room temperature). The next stage is fermentation in brine; fermented soybeans are sundried then submerged in container with 20 — 30 percent salt solution for 14 — 120 days. The product is then boiled and filtered and after addition of spices, 'kecap' is finally produced.

According to Sri Hartadi *et al* (1978), fermentation of 'kecap' is due to various molds. From 32 samples collected in Jawa, 325 strains of molds were isolated. Among these, 210 were identified as *Aspergillus* spp., 27 isolates as *Rhizopus* spp. and 88 isolates identified as other species. Based on the proteolytic and amylolytic activity, only 89 of the 210 *Aspergillus* spp. isolates have a high level of activity.

Producing 'kecap' by spontaneous mold fermentation has many disadvantages

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such as; contamination by undesirable microorganisms, long fermentation time and poor quality based on nitrogen content. *The extent of protein hydrolysis is generally* acknowledged to be the most important factor governing the quality of 'kecap', since flavor and nutritional value all depend on the extent to which the soybean protein are brought into the stable solution. Hydrolysis of protein is affected by many factors such as; molds and their enzyme productivity, moisture, temperature and pH of the raw material. The proteolytic enzymes of mold have been classified on the basis of their pH optima. *They are acid, neutral and alkaline proteases.* According to Yokotsuka (1981) acid and neutral proteases with the pH optima 4 — 5 and 6 — 7 are important for "shoyu" production.

This study is aimed at minimizing the disadvantages by using three selected molds with high proteolytic activity; *Aspergillus oryzae, A. sojae* and *Rhizopus oligosporus* as inoculum. Three different initial pH of soybeans were used for medium. The proteolytic activity and soluble nitrogen content were monitored during fermentation. Chemical hydrolysis of soybeans using HCl was also done in this study to compare with enzymatic hydrolisis.

Materials and Methods

Raw material and microorganisms

Black soybean of unknown variety, bought from a local market in Yogyakarta, Indonesia was used as raw material. The microorganisms used were *Aspergillus oryzae, A. sojae* and *Rhizopus oligosporus* obtained from the collection of the National Biology Research Center Bogor. Inoculum was prepared by growing the three molds on potato dextrose agar (PDA) slant at room temperature for 4 days.

Fermentation

Black soybeans were soaked for about 12 hours, in water without/with addition H_2SO_4 to adjust the pH 5.0 & 6.0, then cooked for one hour, drained and put into erlenmeyer flask and sterilized for 121°C, 1.5 atm, for 15 minutes. After sterilization, each flask was inoculated separately with spore of *A. oryzae* and *A. sojae* and *R. oligosporus* from PDA slants after flooding the surface with sterile water. Fermentation was allowed for 5 days.

Chemical hydrolysis

Black soybeans were put into erlenmeyer flask, added 6N HCl in 1 : 3 proportion, then allowed to be hydrolyzed for 6 hours at 100°C. After filtration and neutralization until pH 6,5 using $NaHCO_3$, the soluble nitrogen in filtrate was determined.

Analytical Procedure

Determination of water content and pH

Water content of samples (raw and fermented soybeans) were determined by measuring the weight loss of samples dried at 105°C for 24 hours. The hydrogen ion concentration of samples were determined using "Beckman digital" pH meter by mixing mashed samples with distilled water in a 1 : 3 proportion.

Nitrogen content analysis

Nitrogen content (total and soluble) of samples were determined by using micro. Kjeldahl method (Villegas and Mertz, 1981). For soluble nitrogen analysis, samples of about 5 — 10 g were mashed then transfered into a flask and extracted using 100 ml of distilled water for 45 minutes with shaking at room temperature, then centrifuged at 10.000 g for 30 minutes (modified from

Shieh *et al*, 1982). The supernatant was filtered through Whatman paper no. 42 and the nitrogen in known volumes of filtrate was determined using micro-Kjeldahl method.

Proteolytic activity

Proteolytic activity was determined by spectrophotometric method (FAO, 1982, Se and Ueda, 1979 and Bhumiratana, 1980). 10 — 20 grams of samples were crushed and extracted as above using 50 ml of phosphate buffer (pH 6,5) by shaking for 15 minutes at 10°C. After filtration using Whatman paper no. 42, the filtrate was used as enzyme solution. 0.2% Casein in phosphate buffer (pH 6,5) was used as substrate for the analysis of enzyme activity. Casein solution (10 ml) added with 2 ml enzyme solution, was incubated in a shaking water bath, at 40°C, for 3 minutes. Proteolysis was stopped by adding 10 ml trichloracetic acid (14%), and the precipitate was removed by filtration. The optical density of filtrate was measured using the "double beam spectrophotometer UV-210" at 275 nm. One unit of proteolytic activity was defined as the amount of enzyme that liberates 1 μ g tyrosine equivalent per minute under the analysis condition.

Fractionation of soluble nitrogen component

Gel filtration using Sephadex-150 was used for fractionation of soluble nitrogen component in samples, based on their molecular weight. Samples were prepared as in soluble nitrogen analysis, using phosphate buffer pH 6,5 instead of distilled water. 4 ml soluble nitrogen samples were applied to the top of column of Sephadex G-150 (2.0 × 75 cm) which was equilibrated with the same buffer. The effluent fractions (5 ml each) were collected at 10 ml per hour. The absorbancy of effluent solutions were analyzed using "double beam spectrophotometer UV-210" at 280 nm (Obara and Kimura, 1967, with sligth modification).

Enumeration of molds

Samples (1 g) after crushing and diluting at 10^2 — 10^8 fold was used for determination. Potato dextrose agar (PDA, pH 5,6) suplemented with 100 ppm of chloramphenicol was used as medium. Plates after inoculated with diluted samples and mixed with medium were incubated at 30°C for 1 — 2 days. Enumeration of colonies was made before plates become overgrown with mold mycelia.

Discussion

Proteolytic activity, soluble nitrogen and colony forming unit

The proteolytic activities of *A. sojae, A. oryzae* and *R. oligosporus* and the control on various initial pH of medium as shown in fig. 1, 2, 3, and 4 increased rapidly within 1 — 3 days. Maximum activity was obtained within 3 — 4 days, followed by steady decline in activity for the rest of the fermentation. *Rhizopus oligosporus* and the control produced lower activity than the other two molds.

Yong and Wood (1977) reported, that using 3 strains of *Aspergillus* for soybean fermentation mixed with wheat in a 1 : 1 proportion reached the maximum proteolytic activity around 40 — 50 hours. Bhumiratana (1980), on the other hand, used soybean and 3 strains of *Aspergillus*, reported that maximum proteolytic activity was attained at 3 — 4 days. *This result is the same as in this present study.*

Moistening of soybean during soaking and cooking brings substrate to swell, and resulting in easier utilization of subtrate by mold because of easy penetration of mycelia into the substrate. Narahara *et al* (1981)

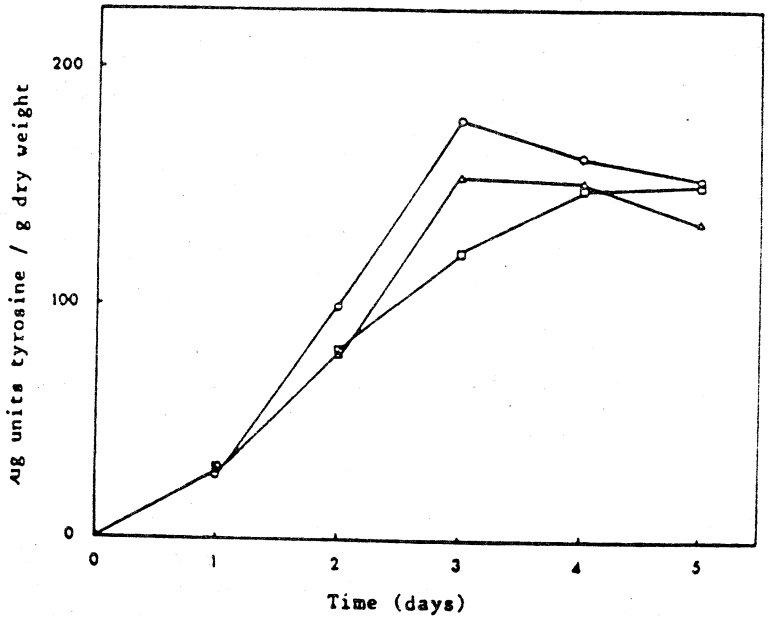

Figure 1. **Proteolytic activity in fermented soybean by *A. sojae*. Initial pH 5.0 o—o; pH 6.0 Δ—Δ and pH 6.7 □—□ (unadjusted)**

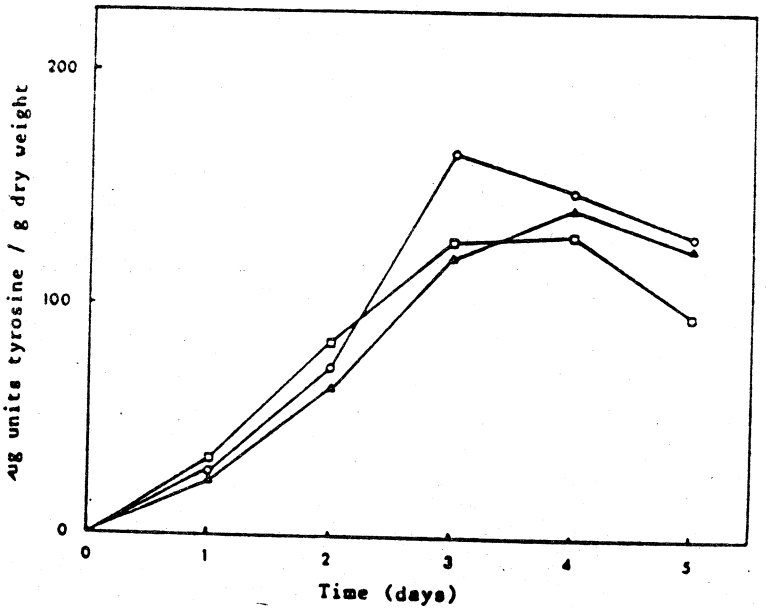

Figure 2. **Proteolytic activity in fermented soybean by *A. oryzae*. Initial pH 5.0 o—o; pH 6.0 Δ—Δ and pH 6.8 o-o (unadjusted)**

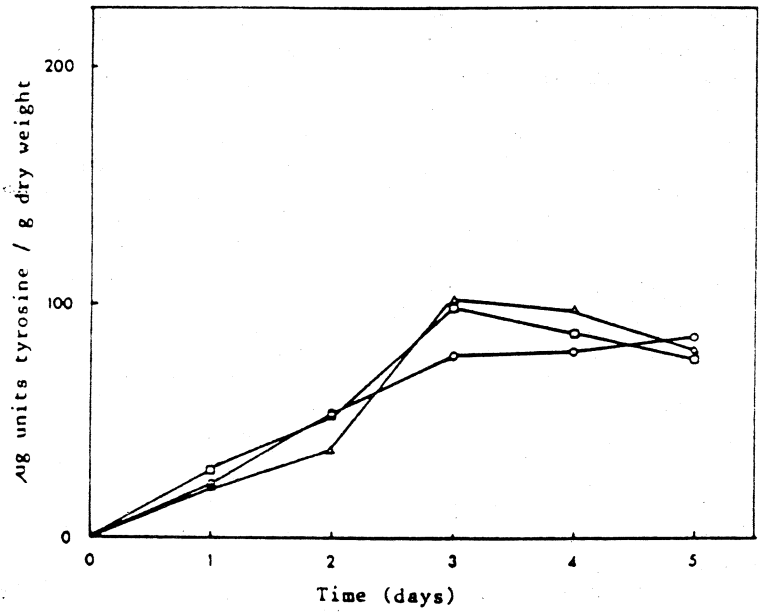

Figure 3. **Proteolytic activity in fermented soybean by *R. oligosporus*. Initial pH 5.0 o—o; pH 6.0 Δ—Δ and pH 6.7 □—□ (unadjusted)**

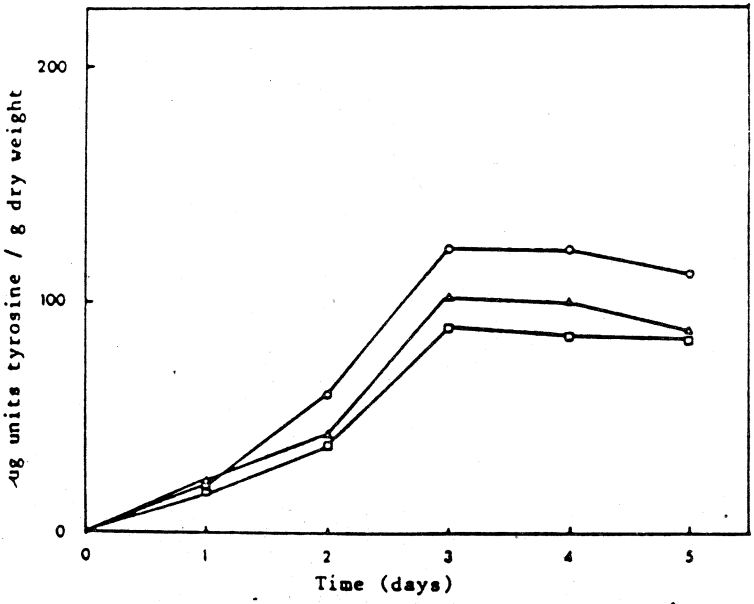

Figure 4. **Proteolytic activity in fermented soybean in control. Initial pH 5.0 o—o—o; pH 6.0 Δ—Δ and pH 6.7 □—□ (unadjusted)**

reported that the moisture level in the soybean affected the enzyme activity to a certain extent, as it was observed that high water content suppressed proteolysis. It was also reported that the highest proteolytic activity level of *Aspergillus oryzae* inoculated on steamed rice was obtained at the initial water content of 35%.

In this study, the initial water content was about 57 — 58%. This high water content may be one reason for the delayed maximum proteolytic activity. Yokotsuka (1977) have shown that by adding wheat to the mixture, the water content is decreased from 60% to 40%, thus, shortening the time to reach maximum proteolytic activity.

This result showed that the high proteolytic activity was obtained by *A. oryzae* and *A. sojae* with the level 180 — 200 unit tyrosine/g dry weight. The proteolytic activity of the two other fermentation was lower (100 — 120 UT/g dry weight).

As shown in the results (figure 5, 6, 7 and 8), there is a significant change in soluble nitrogen level as fermentation progressed. All sets of fermentation gave rapid increase during 1 — 3 days, and then followed by a steady level until the end of fermentation. This result is similar with the pattern for proteolytic activity, that is, soluble nitrogen content is maximum at maximum proteolytic activity.

Visual observations showed that during fermentation, only a few mycelia of *Rhizopus oligosporus* grew and penetrated into the beans. *R. oligosporus* known as "tempe" mold generally grow rapidly on the dehulled soybeans during "tempe" production, eventually covering the beans with its mycelia and resulting in a compact cake. In this study, however mycelia of this mold only grew on the surface of the undehulled beans and the penetration into the beans was not as well as in the dehulled beans. At the end of fermen-

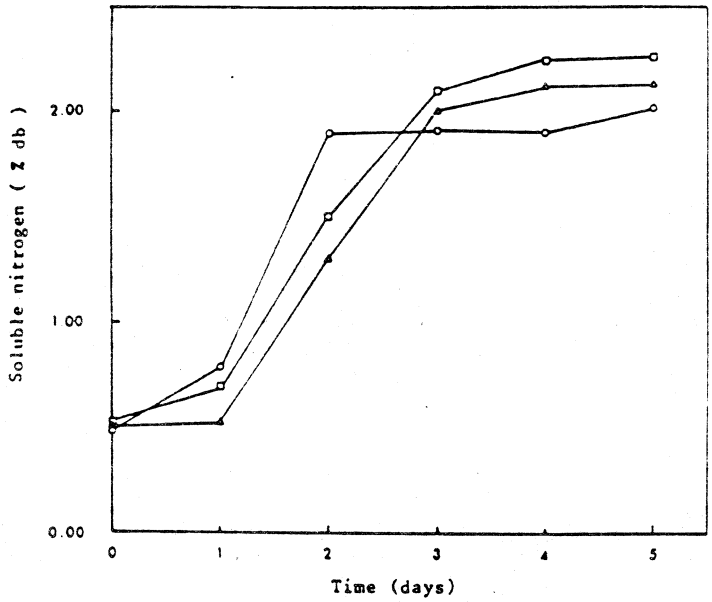

Figure 5. **Soluble nitrogen in fermented soybean by *A. sojae*. Initial pH 5.0 o—o; pH 6.0 Δ—Δ and pH 6.7 □—□ (unadjusted)**

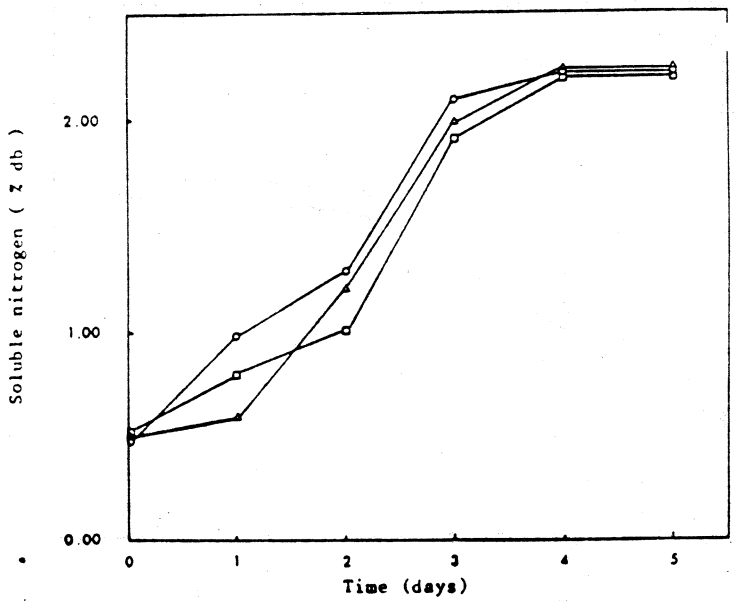

Figure 6. **Soluble nitrogen in fermented soybean by *A. oryzae*. Initial ph 5.0 o—o; pH 6.0 Δ—Δ and pH 6.7 □—□ (unadjusted)**

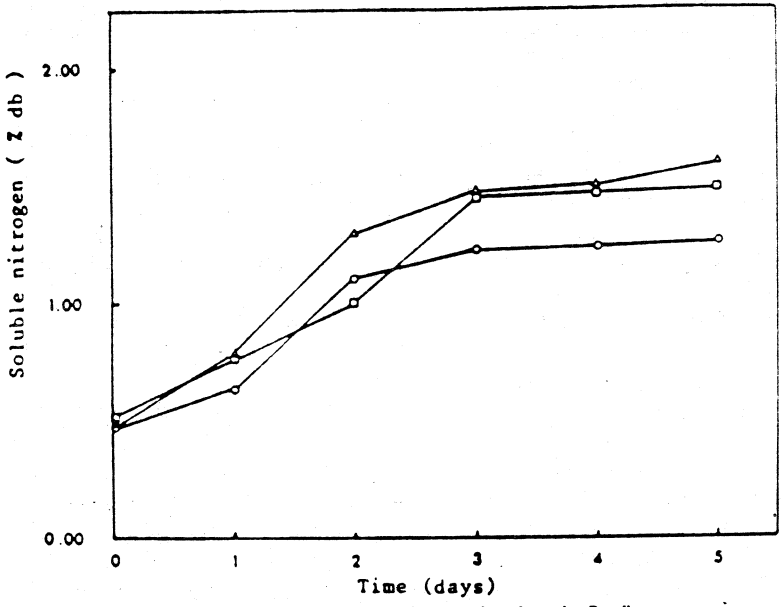

Figure 7. **Soluble nitrogen in fermented soybean by *R. oligosporus*. Initial pH 5.0 o—o; pH 6.0 Δ—Δ and pH 6.7 □—□ (unadjusted)**

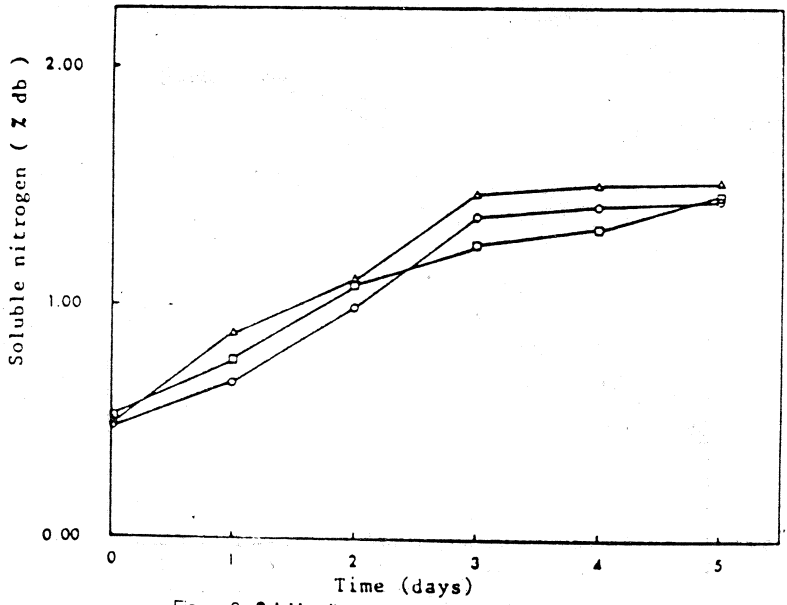

Figure 8. **Soluble nitrogen in fermented soybean in control. Initial pH 5.0 o—o; pH 6.0 Δ—Δ and pH 6.7 □—□ (unadjusted)**

tation a compact cake covered with mycelia was not obtained. The unsuccessfull mycelia penetration is thought to be due to the lack of cellulase by this mold responsible for breaking-down the cellulose component of the bean wall. This condition lowered the transfer of proteolytic enzyme into the beans, thus causing a low soluble nitrogen level. On the other hand *A. sojae* and *A. oryzae* grew very well and their mycelia penetrated the undehulled beans because these molds produce enzyme cellulase as reported by Wood and Yong (1975).

Colony forming units were enumerated to present the molds population, and is shown on figure 10. The initial population of *A. oryzae* was 3.2×10^3 CFU/g dry weight and *A. sojae* was 5.1×10^3 CFU/g dry weight. The population increased rapidly during 1 — 2 days and reached the stationary phase during 2 — 3 days, with the maximum population of 8.2×10^7 CFU/g dry weight for *A. oryzae* and 1.1×10^8 CFU/g dry weight for *A. sojae.*

Initial population of *R. oligosporus* was 8.2×10^2 CFU/g dry weight and reached maximum level at 5 days fermentation with 8.2×10^6 CFU/g dry weight. This mold did not grow as well as *A. sojae* and *A. oryzae*, as shown by a low number of CFU. This slow growth may be due to the presence of hull in the bean that inhibits the penetration of mycelia. The population of the control was detectable after one day with the initial population 1.1×10^2 CFU/g dry weight and increased up to 2.7×10^7 CFU/g dry weight after 5 days fermentation. Both *Rhizopus oligosporus* and molds grown on the control reached the stationary phase during 2 — 3 days fermentation.

From the result was shown that the maximum proteolytic activity was attained during the stationary phase. A similar observation was also reported by Volesky and Luong (1985), where they obtained a maximum proteolytic activity during the stationary phase. On the fourth day of fermentation, the molds began to sporulate and the

proteolytic activity decreased, Chey (1978) also concluded that during sporulation the enzyme productivity of mold become very low.

Soluble nitrogen before and after hydrolysis

Determination of hydrolysis efficiency is based on the soluble nitrogen, thus further study on the soluble nitrogen before and after hydrolysis was done (table 1). In this study, the fermentation was followed only for 3 days, because based on the earlier result, after 3 days the soluble nitrogen has reached steady stage, which means that the proteolytic activity is no longer effective even in longer fermentation periods.

After 3 days fermentation with *A. sojae* and *A. oryzae*, soluble nitrogen increased from 0.48 — 0,53% to 1.91 — 2.26% or about 32 — 33% of protein complex in soybean hydrolyzed to soluble form. Yong (1971) in Wood and Yong (1975) reported that during fermentation using mixed soybeans and wheat flour, the soluble nitrogen increase only from 0,57 to 1,58%. In this present study, a higher yield of soluble nitrogen was obtained using soybean without any additional substrate. Yokotsuka (1977) also concluded that using wheat as additional substrate decreased the soluble nitrogen at the end of fermentation. Thus despite the benefits of adding wheat to increase mold growth and enzyme production by lowering the moisture content on substrate, it present another problem, that is lowering of soluble nitrogen.

Soluble nitrogen in soybeans fermented by *R. oligosporus* was increase from 0.48 — 0,53% to 1.22 — 1.46% or around 18 — 21% of protein complex in raw soybean was hydrolyzed to soluble form. Shurtleff and Aoyagi (1979) reported that *R. oligosporus* fermentation on dehulled soybean during "tempe" production, increase the nitrogen soluble from 0.5% to 2%. In this study, however, this mold did not grow well on the undehulled beans and thus soluble nitrogen was very low.

The increase of soluble nitrogen in the control is also very low, from 0.48 — 0,53% to 1.26 — 1.47% or only 19 — 21% soybean protein was hydrolyzed to soluble form.

Table 1, also shows that the pH was increase during fermentation in a various value. Using statistical analysis, the difference in initial pH (5.0; 6.0 and 6.7 or unadjusted) of soybean before fermentation did not give a significant result on the soluble production for each mold used.

Chemical hydrolysis of soybean using 6N HCl as shown in figure 1 gave a high efficiency around 60%, with the increase of soluble nitrogen from 1.07% to 4.19%. However this hydrolysis is not advisable to produce "kecap", since the product of hydrolysis has a strong and undesirable odor (Yokotsuka, 1977).

Fractionation Soluble Nitrogen Component

The extractable proteins component of soybean before hydrolysis was fractionated by Sephadex G-150 into four peaks (A, B, C and D), as shown in figure 10. Obara and Kimura (1967) reported that they also found 4 peaks of proteins during fractionating water-extractable soybean protein.

During hydrolysis, proteolytic enzymes split peptida-peptida bonds producing polypeptide and amino acids of lower molecular weight. It was shown at fractionation of the soluble nitrogen after hydrolysis which gave a different pattern, where peak B and C were not detected, however several smaller molecular weight substances fractionated at peak D. It was therefore assumed that most of the soluble nitrogen were obtained from peak B and C, were then eluted slower and fractionated at peak D area.

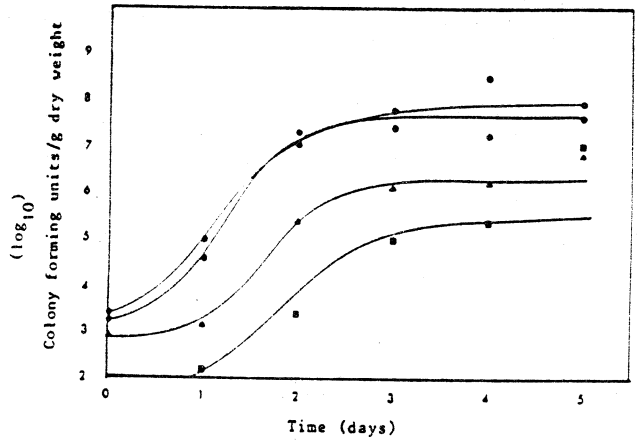

Figure 9. **Total mold population in fermented soybean by *A. sojae* •—•; *A. oryzae* •—• *R. oligosporus* ▲—▲: control ■—■ (pH unadjusted)**

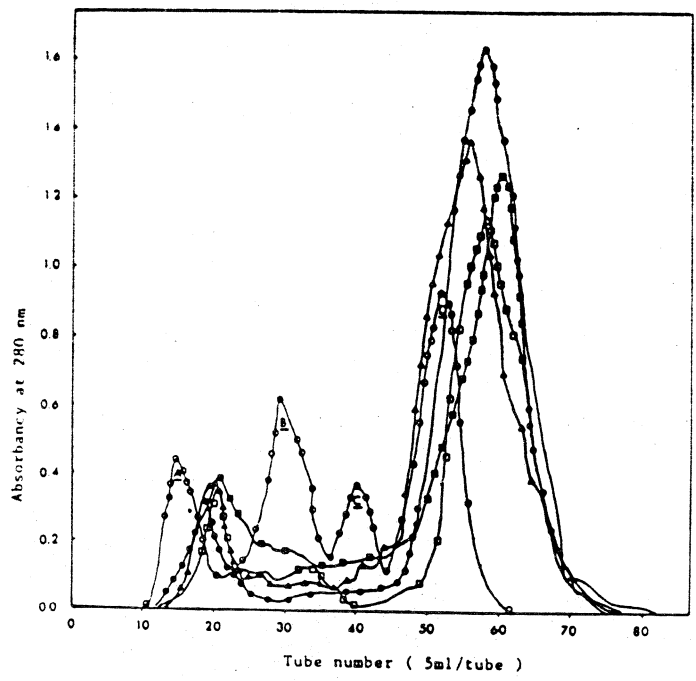

Figure 10. **Fractionation soluble nitrogen in soybean using Sephadex G-150 before hydrolysis o—o; after hydrolysis using *A. sojae* •—•; *A. oryzae* Δ—Δ *R. oligosporus* □—□ and Control ■—■.**

Table 1. **Total and soluble nitrogen content and pH in soybean before and after hydrolysis**

Treatment	No.	Before Hydrolysis		After Hydrolysis		% Hydrolyzed N-component	Before After Hydrolysis pH	
		Total-N	Sol-N	Total-N	Sol-N			
A. sojae	1	6.48	0.48	6.39	1.92	29.63	5.08	6.22
	2	6.64	0.52	5.89	2.26	32.02	6.03	6.91
	3	6.59	0.53	6.40	2.10	31.87	6.67	7.13
A. oryzae	1	6.48	0.48	6.35	2.12	32.72	5.08	6.25
	2	6.64	0.52	5.91	1.91	28.76	6.03	6.87
	3	6.59	0.53	6.45	2.03	30.80	6.67	7.05
R. oligosporus	1	6.48	0.48	6.42	1.22	18.83	5.08	5.84
	2	6.64	0.52	6.27	1.46	21.99	6.03	6.85
	3	6.59	0.53	5.95	1.38	20.94	6.67	6.79
Control	1	6.48	0.48	6.32	1.35	20.83	5.08	6.12
	2	6.64	0.52	6.36	1.47	22.14	6.03	6.91
	3	6.59	0.53	6.37	1.26	19.14	6.67	6.92
Chemical		6.91	1.07	—	4.19	60.49	—	—

Result

Based on the colony forming units (CFU), *Aspergillus sojae, A. oryzae, Rhizopus oligosporus* and molds on the control reached the stationary phase on the third day of fermentation on soybeans. *Aspergillus sojae* and *A. oryzae* have a higher number of colony (10^7 — 10^8 CFU/g dry weight) than the two others (10^6 — 10^7 CFU/g dry weight).

The proteolytic activity of *A. sojae, A. oryzae, R. oligosporus* and the control was maximum on the third day, with the activity of *A. sojae* and *A. oryzae* (180 — 200 units tyrosine/g dry weight) was higher than the two others fermentation (100 — 120 UT/g dry weight).

Based on the efficiency of hydrolysis, *A. sojae* and *A. oryzae* gave a better result than two others fermentation. The hydrolysis efficiency of *A. sojae* and *A. oryzae* was 32 — 33%, with the total soluble nitrogen of 2.1 — 2.2 percent/g dry weight and the hydrolysis efficiency of *R. oligosporus* and the control was 21 — 22%, with the total soluble nitrogen of 1.4 — 1.5 percent/g dry weight. The difference in initial pH i.e. pH 5.0; pH 6.0 and pH 6.7 did not affect the soluble nitrogen production.

Chemical hydrolysis using HCl was higher than enzymatically, with the efficiency of hydrolysis 60% and the increase of soluble nitrogen from 1.07 to 4.19%.

The extractable proteins component of soybean before hydrolysis was fractionated into four fractions by Sephadex G-150. After hydrolysis using mold enzymes, two fractions of a lower molecular weight.were obtained..

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