Protoplast Fusion of Koji-Mold *Aspergillus* spp. to Improve *Kecap* Production

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ABSTRACT

*Protoplast fusion of **Aspergillus oryzae** and **A. niger** for improving the enzyme productivity of desirable koji-molds for **kecap** (Indonesian soy sauce) was studied. Protoplasting of both mycellium aspergilli was obtained by treating them using Novozyme 234, at final concentration 5 mg/ml and the time of exposure for three and two hours. Regenerated protoplasts of **A. oryzae** on complete medium (RCMMPA) was 1.46-3.74 % and on minimal medium (RMMPA) was 0.93-4.93%, whereas **A. niger** was 0.44-7.64 % and 0.17-6.25 %, respectively. Protoplast fusion was conducted in 30 % PEG 6000 (Polyethylene Glycol) containing 0.01 M CaC12 and 0.05 % glycine, and at pH 7.5. For selecting the suspected fusant Dinikonasol 12.5%, at the final concentration 200 ppm and Cycloheximide at the final concentration 100 ppm was used as a marker. Results showed that hundred eighteen of suspected fusants were obtained and after screening found that seven as selected fusants. Based on the enzyme activities, finally, fusant number 77 was selected as a fusant with high proteolytic and glutaminase activities.*

Keywords: Protoplast fusion, Koji, *Kecap, Aspergillus.*

INTRODUCTION

The quality of *kecap* product are determined by several component such as soluble nitrogen and free amino acid from protein hydrolysis. Most of *kecap* factories used *Aspergillus oryzae* or *A. sojae* for fermenting soybean because their ability for hydrolyzing soy bean protein are higher than other aspergilli during kecap production (Rahayu 1991). In soy sauce fermentation, increasing the number of glutamic acid in the mash is an important phase for producing a delicious taste. The glutaminase from koji mold *A. oryzae* or *A. sojae* generally regarded as a key enzyme that controls the delicious taste of fermented foods such as soy sauce (Tomita *et al.*, 1988).

Generally, among the koji-molds, protease hyper-producer show low glutaminase activity, and on the contrary glutaminase hyper-producers show insufficient protease activity (Usijima and Nakadai, 1987). Further more, the research of the protoplast fusion between *A. oryzae* as a protease producer and *A. niger* as a glutaminase hyper-producer was done for obtaining a new strain with both enzyme activities in balanced during koji-mold fermentation process. This study deals with methods used for marker selection, optimization of protoplast formation, regeneration and protoplast fusion.

MATERIALS AND METHODS

Microorganisms and Chemicals

Aspergillus oryzae IFO 30113 is a protease hyper-producer, and *Aspergillus niger* IFO 6341 as a glutaminase hyper producer were obtained from The Institute of Fermentation Osaka, Japan. Stock cultures of these Aspergilli were maintained on Malt Extract Agar.
Novozyme 234 (Novo) was used as lytic enzyme. Dinikonasol 12.5% (Sumiate 12.5 WP), Mankoseb 80% (Dithane M-45), Metalaksi 35% (Ridomil 35 SD), Benomyl 50% (Benlate), Methyl thiofanat 70% (Topsin

M 70 WP), Tetrachloro isotaionitril (Klorotalonil 70%, Daconil 70 WD), and Cycloheximide were used as fungicide for markerring purpose.

Marker Selection

Fungicidal sensitivity test. Gradient plating technique was, as an initial step, applied to determine the Minimum Inhibitory Concentration (MIC) of fungi using PDA (Potato Dextrose Agar) as a growing medium (Bettina, 1983). Ten ml of PDA medium containing of each fungicide was poured and solidified in sloping position of the plate and then another ten ml PDA without fungicide was overlayered in the horizontal position. Spores suspension of each *Aspergilli* was inoculated and incubated in the plate for five days at 27^0 C. The growing colonies on the part of highest fungicidal concentration were picked up and transferred repeatedly into new gradient plates and fungicidal resistant strains were finally isolated from these plates as a markered aspergilli.

Minimal Inhibitory Concentration. The technique was determined by inoculating fungicidal resistant strains on PDA containing a different series dilution of fungicide, i.e. 0; 3.12; 6.25; 12.50; 25.00; 50.00; 100.00; 200.00; 400.00 ppm. Spore suspension of fungicidal resistant strains were inoculated on these PDA and incubated for 5 days at 27 C. The lowest fungicidal concentration which can inhibit the growth of spore was stated as minimal inhibitory concentration.

Cross resistance test. This step was done to determine crossing effect of a marker to the second strain and *vice versa.* The cross resistance was tested by inoculating the first fungicidal resistant strain on a series of PDA containing the second selected fungicide. The same method was also applied to the second fungicidal resistant strain inoculating it on the first selected fungicide.

Interference test. Interference test between two parental strains was done in order to know the relationship between these strains which can make new resistance and also to check whether the two selected fungicides cause the disappearance of the fungicidal effect toward parental strains. The medium PDA containing two selected fungicides was used for this study. Plates of this medium were divided into three sectors. The first sector was inoculated with *A. oryzae,* the second with *A. niger* and the third with *A. oryzae* and *A. niger.* The parameter of the result shows that there was no growth on the medium, it means that no interference between parental strains and between the two selected fungicides.

Protoplast Fusion

Protoplast fusion was done according to method of Matsushima and Baltz (1986). Protoplast were obtained from 17 hours cultured young mycelia of each strain. The preincubation medium (MPP) was used, containing (g/l) : 3 of $NaNO_3$, 0.52 KCl, 0.52 $MgSO_4$. $7H_2O$, 0.0001 $FeSO_4$. $7H_2O$, 0.0001 $ZnSO_4$. $7H_2O$, 20 glucose, 5 Yeast Extract and 2 casamino acid. The pH of medium was adjusted to 6.5 before autoclaving. Erlenmeyer Ca. 150 ml containing 30 ml of medium was inoculated with 1 ml of conidial suspension containing approximately 107 spores/ml. The young mycelia were harvested by centrifugation at 3000 rpm for 30 min, washed twice with buffer containing osmotic stabilizer (0.6 M NaCl in a 0.2 M phosphate buffer, pH 5.8) and finally resuspended in the same buffer containing an osmotic stabilizer. The suspension was mixed with Novozyme 234 solution with different concentration (2.5, 5.0 and 7.5 mg/ml), followed by incubation at 30 C with gentle shaking for 1-4 hr. The protoplasts were separated from the mycelial debris by centrifugation at 70 rpm for 10 min. and then washed twice with the osmotic stabilizer.

Protoplast fusion. Protoplasts of two parental strains were fused by mixing (5-10 x 10^6 spores / ml of each strain) and centrifuged at 700 rpm for 10 min. The pellet of the protoplast mixtures was resuspended in one ml of 30% (w/v) polyethylene glycol 6000 in 0.01 M $CaCl_2$ and 0.05 M glycine, pH 7.5. After incubation for 10-30 min at 30 C, the suspension was diluted with 6 ml of the minimal medium containing 3 g of $NaNO_3$, 0.5 g KCl, 0.5 g $MgSO_4$. 7 H_2O, 0.01 g $FeSo_4$. 7 H_2O, 1.0 g K_2PO_4 and 40 g glucose per liter water, followed by centrifugation at 700 rpm for 5 min. The protoplasts were washed twice with 8 ml osmotic stabilizer and then resuspended in 5 ml osmotic stabilizer.

Protoplast Regeneration. The pellet protoplast was resuspended in 0.6 M KCl and 0.2 M phosphate buffer. An aliquot of protoplasts suspension was poured on solid regeneration medium in a plate, and then 5 ml of soft regeneration medium (0.5 % agar, 45 C) was poured onto the plate and immediately mixed with the protoplast. The overlayered plates were incubated at 28 C for 2-3 days. The complete regeneration medium (RCMMPA) contained (g/l) : 6.0 NaNO3, 1.0 yeast extract, 1.0 peptone, 1.0 casamino acid, 10 ml vitamin solution, 0.52 $MgSO_4$. 7 H_2O, 0.52 KCl, 0.0001 $FeSO_4$. 7 H_2O, 0.0001 $ZnSO_4$. 7 H_2O, 15 agar and 0.6 M KCl as osmotic stabilizer. The pH of medium was adjusted at 6.5 before auto-

claving. The minimal regeneration medium (RMMPA) contained (g/l) : 6.0 $NaNO_3$ 0.52 KCl, 0.52 $MgSO_4$. 7 H_2O, 0.0001 $FeSO_4$. 7 H_2O, 0.0001 $ZnSO_4$ 7 H_2O,20 glucose and 0.6 M KCl for osmotic stabilizer.

RESULTS AND DISCUSSION

Selection of marker

Seven commercial fungicides were tested for marker, based on their activities toward *Aspergillus oryzae* and *A. niger* with gradient plating technique. They were dinikonasol 12.5% (Sumiate 12.5 WP), mankozeb 80% (Dithane M-45), metalaksil 35% (Ridomil 35 SD), benomyl 50% (Benlate), methyl thiofanat 70% (Topsin M 70 WP), tetrachloro isotaionitril (Klorotalonil 7%, Daconil 70 WD) and cycloheximide.

Table 1 showed that most of the fungicides have similarity in inhibitory activity patterns toward *A. oryzae* and *A. niger,* except dinikonasol 12.5% and cycloheximide. On the gradient plating technique containing 400 ppm of dinikonasol 12.5% the growth of *A. oryzae* was inhibited, whereas the growth of *A. niger* was not. On the contrary, in the gradient plate containing 200 ppm of cycloheximide the growth of *A. niger* was inhibited.

Table 1. The minimal inhibitory concentration of dinikonasol 12.5% to *A. oryzae* and cycloheximide to *A. niger.*

Fungicides and Molds	Concentration (ppm)								
	0	3.12	6.25	12.5	25	50	100	200	400
Dinikonasol 12.5% to *A. oryzae*	+	+	+	+	+	+	+	–	–
Cyclohexi-mide to *A. niger*	+	+	+	+	+	+	–	–	–

+ : Growth
– : No growth.

Minimal Inhibitory Concentration

Table 2 showed that minimal inhibitory concentration of dinikonasol 12.5% to *A. oryzae* was around 200 ppm and cycloheximide to *A. niger* was around 100 ppm. Therefore, the concentration of fungicides would be used as a marker for protoplast fusion purposes of those aspergilli.

Table 2. The Cross Resistance Test between Dinikonasol 12.5% and Cycloheximide to *A. oryzae* and *A. ninger.*

Strains of Fungi	Minimal Inhibitory Concentration (ppm)	
	Dinikonasol 12.5%	Cycloheximide
A. oryzae IFO 30113	200	600
A. niger IFO 6341	1000	1000

Cross resistance test

Results of the experiment were shown in Table 3. *A. oryzae* was resistant to cycloheximide concentration at 100 ppm, this concentration was also the minimal concentration which inhibits *A. niger.* On the other hand, *A. niger* was resistant to dinikonasol 12.5% concentration at 200 ppm, this concentration was also the minimal concentration which inhibits *A. oryzae.* The results indicated that the cross resistance between *A. oryzae* and *A. niger* toward both of the fungicides were determined.

Interference test

The results showed that there was no growth on the three sectors of plate test, it means that there was no indication of interaction between two parental strains occured. From the results of cross resistance test and the interference test could be withdrawn the conclusion that concentration 200 ppm of dinikonasol 12.5% and 100 ppm of cycloheximide could be applied as a marker in the protoplast fusion purpose of two parental strains of *A. oryzae* and *A. niger.*

Protoplast formation and regeneration

The protoplast formation of the parental strains were obtained using Novozyme 234 as a lytic enzyme and sufficient protoplast were released to give proper results in the following fusion steps, i.e. 3-10 x 10^5 protoplasts/ml for *A. oryzae* and 2-9 x 10^5 protoplasts / ml for *A. niger.* The time exposure and the concentration of Novozyme 234 were examined to determine their optimum.

Figure 1 showed that more concentration of enzyme used for protoplast formation of *A. oryzae* (Fig. 1 A) and *A. niger,* (Fig. 1B) more protoplasts would be released. However, based on the statistical analysis the results showed that the different concentration gave an insignificant effect. Therefore, it was concluded that the optimum concentration of Novozyme 234 for protoplast formation was 2.5 mg/ml.

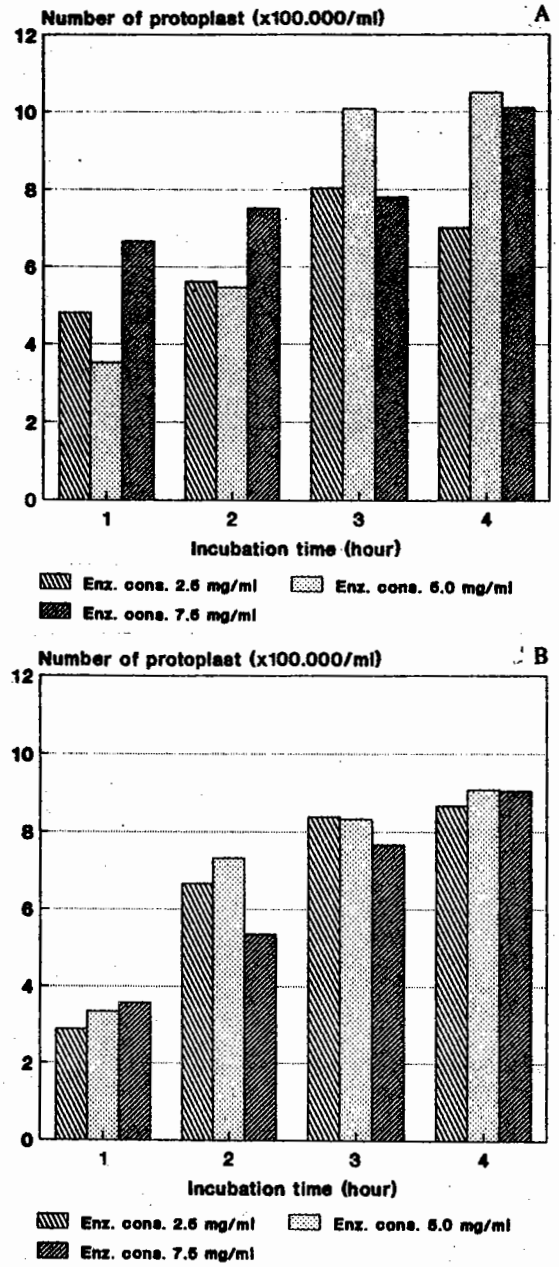

Figure 1. Effect of Novozym 234 concentration and incubation time toward protoplast isolation of *A. oryzae* (A) and *A. niger* (B).

The time exposure for each enzyme concentration showed a significant effect for protoplast formation. The longer time exposure, more protoplasts would be formed and the maximum time exposure of *A. oryzae* and *A. niger* was four hours. Since based on the statistical analysis, there was no significant differences on protoplast formation between three and four hours. Therefore, three hours of time exposure was done, for the next experiment.

The ratio of protoplast regeneration was calculated from the number of appeared colonies on the stabilizer containing medium and total protoplast counts of the original suspension. Protoplast formation affected the protoplast fusion because high number of protoplast would give high probability of fusion occur. Therefore, minimal (RMMPA) and complete regeneration media (RCMMPA) were used to know the effect of the enzyme concentration and time exposure which were then used at protoplast preparation towards protoplast regeneration.

Based on the statistical analysis, differences of time exposure for each enzyme concentration gave an insignificant effect for protoplast regeneration on the RCMMPA medium, but the differences of Novozyme 234 concentration used for protoplast preparation gave a significant effect. The result showed that Novozyme 234 concentration at 0.5 mg/ml gave maximum protoplast regeneration and the similarity pattern was obtained for the RMMPA medium (Fig. 2 and 3). Since enzyme concentration and time exposure affected the protoplast formation and regeneration, therefore, both factors should be determined to obtain the maximum regeneration of protoplast. From Figure 2, it was concluded that the optimal enzyme concentration and time exposure for protoplast formation of *A. oryzae* were 5 mg/ml and three hours, respectively.

Based on the statistical analysis, the differences of Novozyme 234 concentration and time exposure used for protoplast formation gave a significant effect for protoplast regeneration of *A. niger* on the RCMMPA medium. At the RMMPA medium, only the concentration of Novozyme gave a significant effect, whereas the time exposure gave no effect. From Figure 3, could be concluded that the optimal enzyme concentration and time exposure for protoplast formation of *A. niger* were 5 mg/ml and two hours, respectively.

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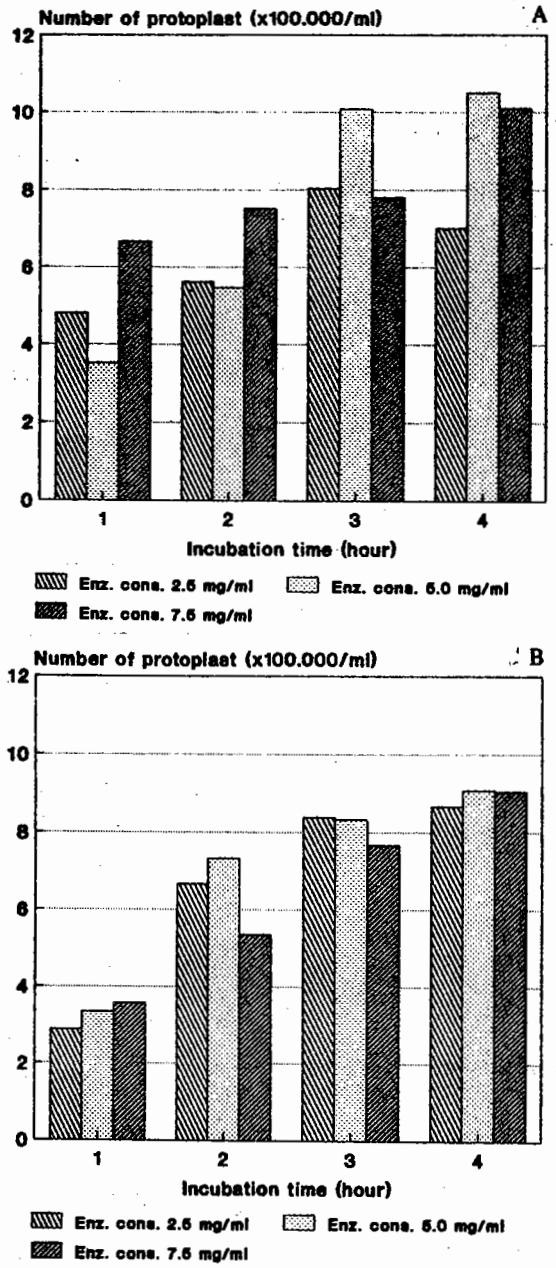

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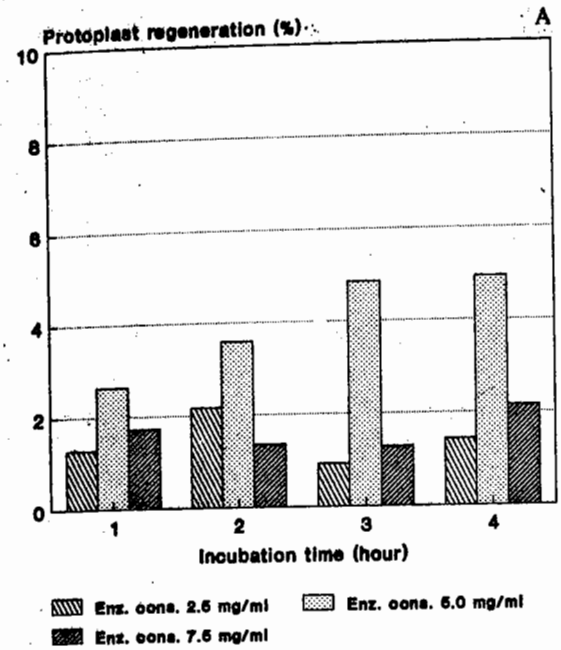


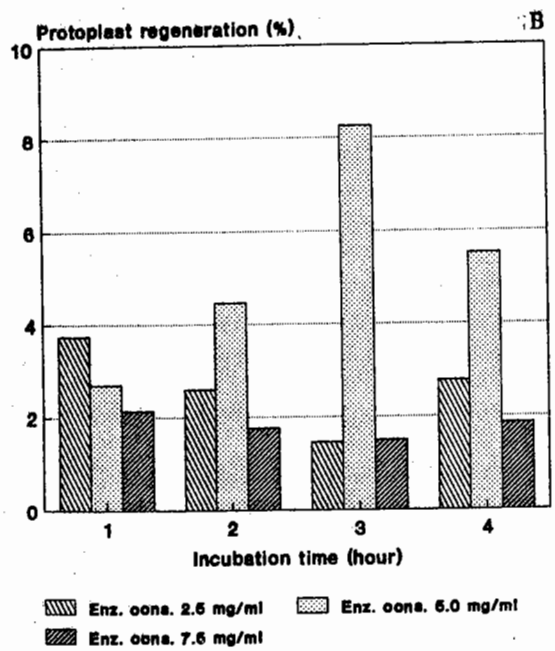
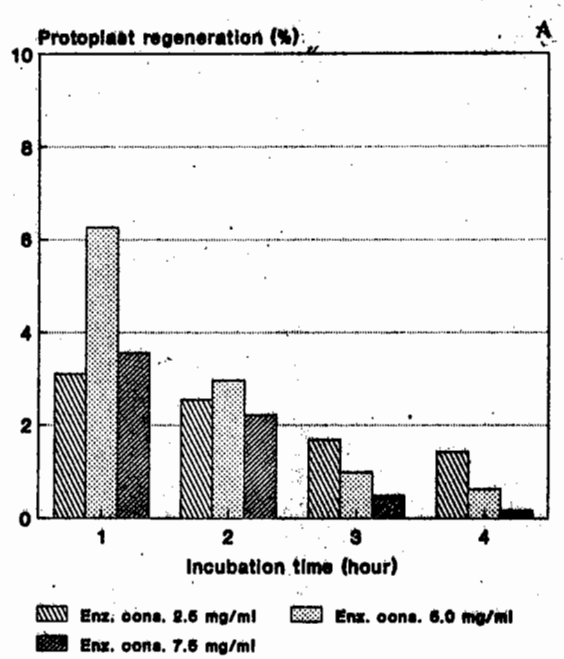


Figure 2. **Effect of Novozym 234 concentration and incubation time toward protoplast regeneration of *A. oryzae* on (A) minimal (RMMPA) and (B) complete (RCMMPA) media.**



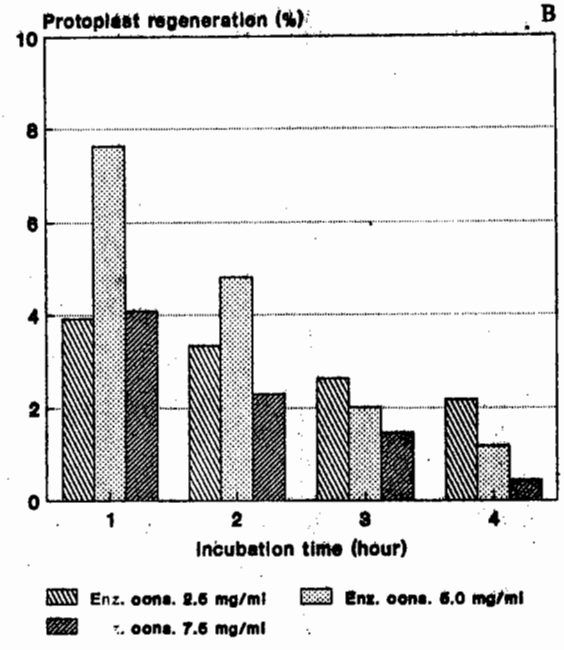


Figure 3. **Effect of Novozym 234 concentration and incubation time toward protoplast regeneration of *A. niger* on (A) minimal (RMMPA) and (B) complete (RCMMPA) media.**

Protoplast fusion

Results of several times fusion processes finally be found hundred eighteen suspected fusants, which could grow on both markers of dinikonasol 12.5% and cycloheximide. Screening was done based on the qualitative enzyme activities of proteolytic and glutaminase was found 7 suspected fusants and based on the quantitative analysis fusant number 77 had been selected as selected fusant with higher proteolytic and glutaminase activities than others (Figure 4).

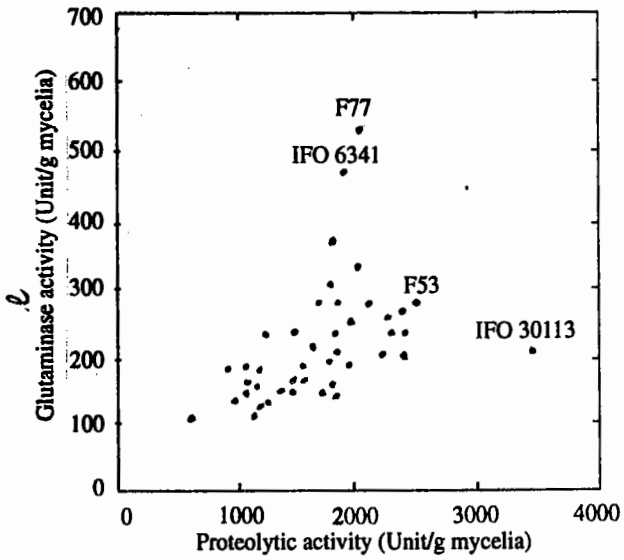


Figure 4. Proteoliytic activity and glutaminase activity of fusants.

CONCLUSION

Dinikonasol 12.5% at concentration of 200 ppm and cycloheximide at concentration of 100 ppm could be used as marker at protoplast fusion between *Aspergillus oryzae* and *A. niger*. Optimal concentration of Novozyme 234 and time exposure for protoplast preparation and regeneration of *A. oryzae* were 5.0 mg/ml and four hours, respectively, whereas for *A. niger* were 5.0 mg/ml and two hours, respectively. Selected fusant number 77 had a combination characters of parental strains of *A. oryzae* and *A. niger*, especially on proteolytic and glutaminase activities.

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