

## RECURRENT MUTATION-SELECTION TO IMPROVE RENNET PRODUCTION IN *Candida tsukubaensis*

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### ABSTRACT

The yeast *Candida tsukubaensis* is of industrial importance for the production of microbial milk-clotting enzyme. Milk-clotting enzyme is an enzymatic complex capable of coagulating milk for cheese manufacturing. High clotting activity (CA) and low proteolytic activity (PA) are desirable qualities. To study the genetic nature of the CA and PA traits, we analyzed 179 colonies obtained after mutagenic treatment. Analysis of the data obtained for this populations showed that CA and PA are traits controlled by polygenes and that they are correlated ( $r = 0.3$ ). The existence of a positive correlation indicates that selection for one trait without considering the other may alter one of the traits in an undesirable direction, since the objective of selection would be an increase in clotting activity and a decrease in proteolytic activity. Three cycles of recurrent mutation-selection were carried out to obtain improved strains. The ultraviolet light dose permitting a 5% rate of cell survival was sufficient to generate genetic variability in the three selection cycles. At the end of the third cycle there was an increase of about 98% in clotting activity and a decrease of about 20% in proteolytic activity. Analysis of variance of the selective cycles showed that the linear effects were significant ( $P < 0.01$ ) for both traits. Estimates of genetic variances and heritabilities of the three selection cycles are presented.

### INTRODUCTION

Milk-clotting enzyme, also called rennet, is an enzyme mixture extracted from the fourth stomach of cattle. It is used by the dairy industry to coagulate milk for cheese manufacturing. For many years, this enzyme mixture was the only clotting agent employed in cheese manufacturing. However, with the increasing demand for meat and cheese consumption, it became necessary to look for alternative sources of clotting enzymes. Several substitutes of animal rennet have been found and today some milk-clotting enzymes of microbial origin are being used commercially (Arima *et al.*, 1967; Sardinias, 1976; Prins and Nielsen, 1970).

One of the requirements for a rennet substitute is related to the organoleptic quality of cheese. Marked proteolysis is an undesirable factor because it may result in the formation of hydrophobic peptides normally

containing high proline levels that impart a bitter taste to the product. Thus, a substitute of animal rennet should have high clotting and low proteolytic activity. The establishment of a relationship between clotting activity (CA) and proteolytic activity (PA) has been used by several researchers as a parameter for the selection of more promising microbial strains for milk-clotting enzyme production (Shrinivasan *et al.*, 1964; Arima *et al.*, 1967; Menezes and Nagato, 1974; D'Souza and Pereira, 1982). The higher the CA/PA ratio, the more promising will be the strain producing milk-clotting enzymes.

Menezes and Nagato (1974) isolated from soil the yeast *Candida tsukubaensis*, which is capable of producing a milk-clotting enzyme with a higher CA/PA ratio than that found for other microbial clotting enzymes commercially available abroad. This clotting enzyme was denoted Renital and is currently being studied in order to improve the technology of its production.

One of the strategies for obtaining a clotting enzyme of better quality and of reduced cost would be the development of programs for the genetic improvement of the yeast *C. tsukubaensis*. Two major methods can be used for the genetic improvement of microorganisms, i.e.,

mutation-selection and recombination followed by selection. The option for one of the two methods will depend on certain biological characteristics of the microorganism involved, as well as on the genetic nature of the trait to be improved.

For traits of a polygenic nature, the recurrent mutation-selection method has been successfully employed in various species of microorganisms. In *Aspergillus nidulans*, an increase of about 300% in the production of penicillin was obtained after six cycles of mutation-selection (Simpson and Caten, 1979). Three mutation-selection cycles were sufficient to generate an *A. nidulans* population 76% superior compared to the original one for the production of citric acid (Silva and Azevedo, 1978).

The objective of the present study was to carry out three mutation-selection cycles in order to increase the clotting activity and to decrease the proteolytic activity of the clotting enzyme produced by *C. tsukubaensis*.

## MATERIAL AND METHODS

### Strain

The *C. tsukubaensis* strain was isolated from soil by the Industrial Fermentation Section of the Food Technology Institute (ITAL), Campinas, SP, Brazil, and identified by the Micological Institute of Universidade Federal de Pernambuco, Recife, Brazil.

### Variability induction

Genetic variability was achieved by irradiation of a cell suspension with UV light (Mineralight 254 nm, 1 A, 69 W, 115 V, distance 15 cm) for 3 min 30 sec, giving a 5% survival rate.

### Clotting activity evaluation (CA)

Clotting activity was evaluated in assay tubes containing 10 ml of maize flour medium (Menezes and Menezes, 1977) inoculated with a  $10^5$  cell suspension and incubated at 30 C on a rotary shaker (0.38 g) for 68 hours. Clotting activity was measured according to Sternberg (1971) in the supernatant fraction resulting from centrifugation of the culture broth at 1500 g.

### Evaluation of proteolytic activity (PA)

Protease production was assayed by blastospore inoculation in Petri dishes containing 16 ml of minimal medium (Reaume and Tatum, 1949) plus 4 ml of a 1% solution of non-hydrolyzed casein. After 96 hours, 5 ml of a saturated ammonium sulfate solution was added to each

plate in order to improve the visualization of the casein degradation circles. The colony diameters and their respective degradation circles were measured and PA was estimated as follows:  $PA = (\text{colony diameter} + \text{circle}) / \text{colony diameter}$ .

### Genetic-statistical methodology

The genetic variability of the original strain was evaluated in a population of 30 colonies. CA was evaluated using random blocks and PA was evaluated in a fully randomized experiment, both with three replications.

A population of 179 colonies originating from the parental strain treated with UV was evaluated for CA and PA in randomized blocks with three replications. For the PA trait, all colonies were evaluated in a single experiment; for the CA trait, the colonies were evaluated in seven experiments because of the technical difficulties related to the simultaneous evaluation of various colonies. For the CA trait, the analyzes of variance of the individual experiments were later grouped into a single analysis.

Using the data for cycle I, estimates of genetic variance ( $\sigma_g^2$ ) and phenotypic variance ( $\sigma_p^2$ ) for colony means were obtained from least squares of the analyses of variance as follows:  $\sigma_g^2 = (Q_1 - Q_2)/r$  and  $\sigma_p^2 = Q_1/r$ , where  $Q_1$ ,  $Q_2$  and  $r$  are colony mean square, error and number of replications, respectively. We then estimated heritability:  $h^2 = (\sigma_g^2/\sigma_p^2) \times 100$ ; expected gain by selection:  $G_s = d_s \cdot h^2$ , where  $d_s$  is the selection differential; genetic correlation between PA and CA:  $r_g = \text{cov}_g(\text{PA}, \text{CA}) / (\sigma_g(\text{PA}) \cdot \sigma_g(\text{CA}))$ , where genetic covariance between PA and CA ( $\text{cov}_g(\text{PA}, \text{CA})$ ) was estimated from the means of the two traits; the correlated responses in traits PA and CA with selection in traits CA and PA, respectively:  $R_{\text{PA}/\text{CA}} = d_{\text{SCA}} \cdot \text{cov}_g(\text{PA}, \text{CA}) / \sigma_p(\text{CA})^2$ , and  $R_{\text{CA}/\text{PA}} = d_{\text{SPA}} \cdot \text{cov}_g(\text{PA}, \text{CA}) / \sigma_p(\text{PA})^2$ .

The data for cycle I were used to determine the number of colonies to be evaluated in the subsequent cycles. We used Table A.2 of Steel and Torrie (1960) which contains the values for parameter  $A/\sigma_p$  for each sample size, where  $A$  and  $\sigma_p$  refer to the phenotypic amplitude and standard deviation, respectively. The  $\sigma_p$  values for each trait were used to estimate the  $A/2$  values which, when summed and subtracted from the overall mean, permitted us to obtain the maximum and minimum phenotypic values, respectively, for each sample size. By simulating various sample sizes, we determined the number of colonies to be used in the subsequent selection cycles.

### Selection cycles

Three mutation cycles followed by selection were carried out. A sample from the colonies submitted to

mutagenic treatment was evaluated in each cycle. Colonies with the highest CA values were selected and, among them, the colony with PA equal to or lower than the population mean was selected. The colony selected in each cycle was used as the parental colony for the subsequent cycle. The population sample of cycle I was 179 colonies, whereas this number was reduced to 70 in the subsequent cycles. CA and PA were evaluated in randomized blocks with three replications in the three selection cycles.

After the selection cycles were completed, the parental and selected strains were evaluated simultaneously using a fully randomized experimental design with seven replications. Estimates of genetic variance, environmental variance and heritability were calculated for each selection cycle.

### RESULTS AND DISCUSSION

The mean squares for the analyses of variance of clotting activity and proteolytic activity data for the population not treated with the mutagen, indicated that the original *C. tsukubaensis* strain presents no significant genetic variability. Thus, in order to investigate the genetic nature of these traits, variability was induced by submitting the original strain to UV light irradiation.

The population obtained after mutagenic treatment showed significant differences ( $P < 0.01$ ) among colonies for both traits (Table I), indicating that the mutagenic treatment utilized was efficient. Continuous variation spectra were detected in the frequency distributions of CA and PA (Figure 1), indicating that these traits are under the control of polygenes. The amplitude of variation of the means for this population was 58.06 to 193.37, with a population mean of 103.31%, for CA, and 2.10 to 4.25, with an overall mean of 2.67, for PA.

Irradiation was effective in generating sufficient genetic variances ( $\sigma_g^2 CA = 233.94$  and  $\sigma_g^2 PA = 3.23$ ) to permit selection for the two traits. This was demonstrated by the high heritabilities ( $h^2_{CA} = 79.54\%$  and  $h^2_{PA} = 86.24\%$ ) and expected gains by selection of the superior colony for each trait:  $GS_{(CA)} = 68.56\%$  and  $GS_{(PA)} = -18.59\%$ . The

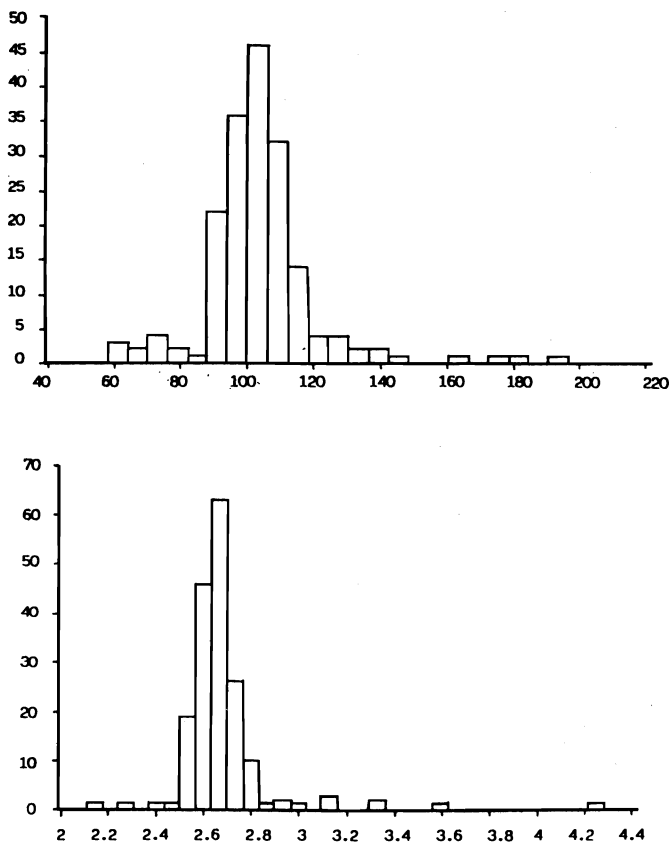


Figure 1 - Distribution of the average clotting activity (above) and proteolytic activity (below).

Table I - Values and significances of the mean squares of variance analysis for cycles I, II and III for clotting and proteolytic activities and general average and experimental coefficient of variation (CV%).

SV	Clotting activity			Proteolytic activity		
	MS			MS+		
	Cycle I	Cycle II	Cycle III	Cycle I	Cycle II	Cycle III
Blocks	884.63	11712.08	15242.46	472.29	3552.28	734.04
Colonies	882.37**	2633.58**	2662.51**	112.23**	162.34**	85.04*
Error	180.54	284.94	871.41	15.45	70.48	59.00
Average	103.31	215.61	198.86	2.67	1.96	2.63
CV (%)	13.00	7.82	14.84	4.64	13.56	9.14

\*Values times  $10^3$ ; \* $P < 0.05$ ; \*\* $P < 0.01$ .

Table II - Estimates of genetic variance ( $\sigma_g^2$ ), error ( $\sigma_e^2$ ), and heritability ( $h^2$ ) for clotting activity and proteolytic activity for the three mutation-selection cycles.

Selection cycles	Clotting activity			Proteolytic activity		
	$\sigma_g^2$	$\sigma_e^2$	$h^2$	$\sigma_{g+}^2$	$\sigma_{e+}^2$	$h^2$
I	233.94	60.18	79.54	32.26	5.15	86.24
II	782.88	89.18	89.18	30.62	23.49	56.58
III	597.04	67.27	67.27	9.01	19.33	31.79

<sup>†</sup>Values times  $10^3$ .

mean for the strain selected for CA would be 174.14 and the mean of the strain selected for PA would be 2.18.

Genetic correlation between the two traits was  $r_g = 0.30$ , indicating the existence of pleiotropy in the control of these traits. The estimate of the correlated response in PA when selection is applied to CA was  $RC_{PA/CA} = 0.25$  or 9.22% and  $RC_{CA/PA} = -12.54$  or -12.14%. This means that selection for CA involve a 9.22% increase in PA, and selection for PA will involve a 12.14% decrease in CA. Thus, selection performed without considering the two traits at the same time may alter one of them in an undesirable direction.

A mutation-selection program should consider the size of the population evaluated ( $N_1$ ), the number of colonies selected ( $N_2$ ), and selection intensity ( $N_2/N_1$ ). When determining sample size ( $N_1$ ), one should keep in mind that the frequency of desirable mutants tends to be reduced, though there are technical difficulties involved in evaluating large numbers of colonies (Simpson and Caten, 1979).

In the present study, we investigated the possibility of reducing the number of colonies to be evaluated in each selection cycle. For a sample size ( $N_1$ ) of 200 colonies, the maximum and minimum values were 150.47 and 56.19 for CA and 3.20 and 2.19 for PA. For  $N_1 = 70$ , the maximum and minimum values were 144.47 and 62.15 for CA and 3.13 and 2.21 for PA. Thus, it can be seen that reducing sample size from 200 to 70 colonies did not result in a significant decrease in the genetic variability obtained by inducing mutation under the conditions used here. Therefore, a sample size of 70 colonies was used in the two subsequent cycles of mutation-selection.

One of the colonies from the first mutagenic treatment was selected and utilized as the parental line for the second selection cycle. The mean squares of the analyses of variance for clotting activity and proteolytic activity of this new population were significant, indicating genetic variability for both traits. A third population, also showing genetic variability for both traits, was obtained

after mutagenic treatment of the colony selected in the previous cycle (Table I).

Genetic variance estimates for PA decreased significantly from the first to the third selection cycle and the same occurred with heritability (Table II). An increase in genetic variance occurred in CA, and inconsistent changes in heritability were observed.

Simultaneous evaluation of parental and selected lines showed that CA increased by 98.29% and PA decreased by 20% after three selection cycles.

The mean squares for the selection cycles were highly significant (Table III). The sums of squares for cycles in the analysis of variance were partitioned to determine whether the responses to the selection cycles were linear. For CA, the linear effect was significant ( $P < 0.01$ ), the deviations were not significant, and the determination coefficient was high. For PA, the linear effect was significant ( $P < 0.01$ ) but the deviations also were significant ( $P < 0.05$ ) despite the relatively high value of the determination coefficient.

Table III - Values and significances of the mean squares of variance analysis of the selective cycles for clotting activity and proteolytic activity and the respective experimental coefficient of variation (CV%) and determination coefficient ( $R^2\%$ ).

SV	DF	MS	
		Clotting activity	Proteolytic activity <sup>†</sup>
Cycles	3	38,631.32**	397.84**
linear	1	111,689.21**	1,027.03**
deviations	2	2,702.38 <sup>ns</sup>	83.14*
Error	24	676.73	21.74
CV (%)		9.36	6.31
$R^2$ (%)		96.41	86.35

<sup>†</sup>Values times  $10^3$ ; \* $P < 0.05$ ; \*\* $P < 0.01$ .

Figure 2 graphically shows the response of CA and PA to the three selection cycles, as well as the equations that express the linear selection response. The progressive improvement detected over the selection cycles indicates that there was an accumulation of small favorable mutations during the mutation-selection cycles. The gain obtained for PA was much lower than that obtained for CA. This difference was expected since the selection criterion was based on preference for colonies with higher CA values but with PA values equal to or lower than the population mean.

The high gains obtained by selection for CA were due to the high heritability values obtained in the three selection cycles. Gains were not so high for PA despite

relatively high heritabilities, since PA was selected as a secondary trait.

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## RESUMO

A levedura *Candida tsukubaensis* apresenta importância industrial para a produção de coalho microbiano. O coalho é um complexo enzimático capaz de coagular o leite destinado à fabricação de queijos. A alta atividade coagulante (AC) e baixa atividade proteolítica (AP) são qualidades desejáveis. Visando estudar a natureza genética dos caracteres AC e AP, foram analisadas 179 colônias obtidas após tratamento mutagênico. A análise dos dados desta população mostrou que os caracteres AC e AP são controlados por poligenes e apresentam-se correlacionados ( $r_g=0,3$ ). A existência de correlação positiva indica que a seleção sem levar em consideração os dois caracteres conjuntamente poderá alterar no sentido indesejável um dos caracteres, uma vez que o objetivo da seleção seria o aumento da atividade coagulante e a diminuição da atividade proteolítica. Visando a obtenção de linhagens melhoradas, três ciclos de mutação-seleção recorrente foram conduzidos. A dose de ultravioleta, que permitiu 5% de células sobreviventes, foi suficiente para gerar variabilidade genética nos três ciclos de seleção. Ao final do terceiro ciclo houve um acréscimo de cerca de 98% na atividade coagulante, e um decréscimo de cerca de 20% na atividade proteolítica. A análise dos ciclos de seleção mostrou que os efeitos lineares foram não significativos ( $P < 0,01$ ) para ambos os caracteres. Estimativas de variâncias genéticas e coeficientes de herdabilidade dos três ciclos de seleção são apresentadas.

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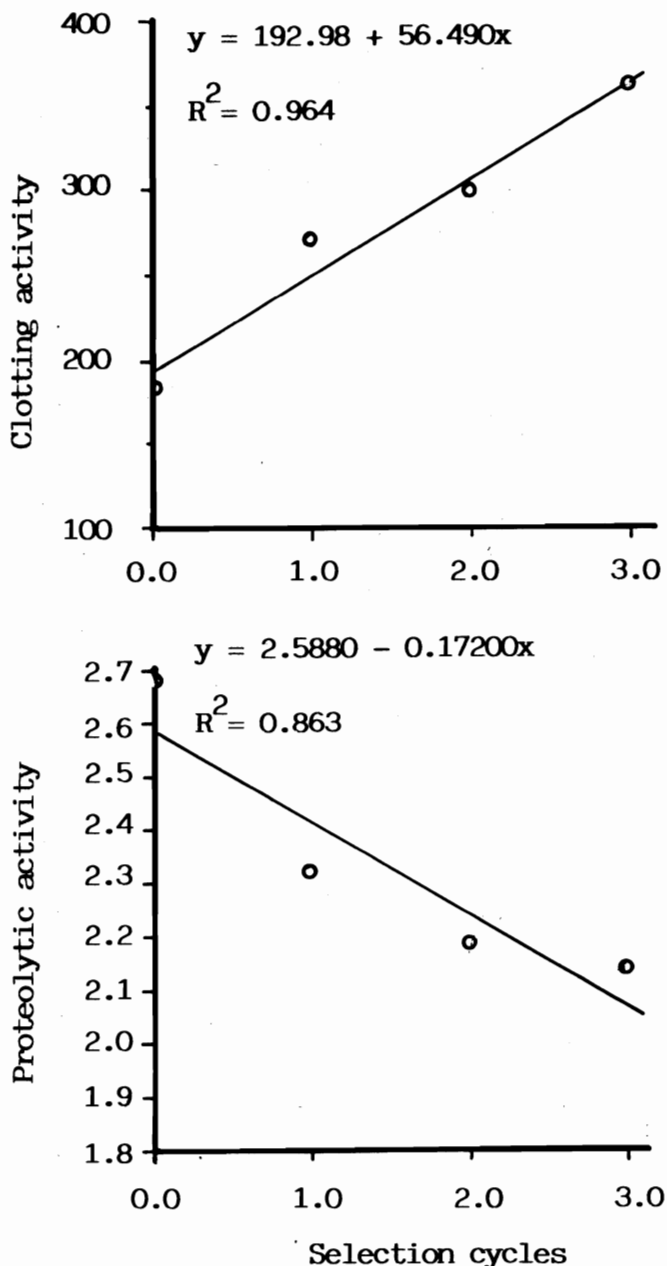


Figure 2 - Responses of clotting activity (upper figure) and proteolytic activity (lower figure) to three selection cycles.

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