Synchronization of Zygote Production in *Saccharomyces cerevisiae*

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SUMMARY

A method of synchronous mass production of yeast zygotes is described. A yield of 30 to 40 % zygotes with the period of formation restricted to 1 h was achieved. This result was obtained by determining optimal parameters for different steps of sexual fusion of haploid *a* and α cells in yeast.

INTRODUCTION

The mechanism of sexual fusion in yeast is a very complicated process consisting of many steps. Yanagishima (1971) has shown that there are hormonal steps preparing the fusion of mating types *a* and α and formation of diploid zygotes. Recently Duntze, MacKay & Manney (1970) have demonstrated that the cells of α mating type excrete a diffusible peptide substance, α factor, which inhibits division in *a* cells. This factor specifically inhibits the initiation of DNA synthesis in *a* cells (Throm & Duntze, 1970; Bücking-Throm, Duntze, Hartwell & Manney, 1973). The α factor is produced constitutively by α cells and it may induce *a* cells to produce a diffusible factor which influences the α cells, preparing them for conjugation. To be competent for sexual fusion the cells of both mating types must be in an appropriate stage. They must not be budding, and DNA synthesis must be stopped so that the nuclei of both conjugating types are at the same stage (Hartwell, 1973).

A population of synchronously formed young zygotes is essential for any kind of future biochemical or genetical analysis of processes occurring in zygotes. The aim of the work described in the present paper was to determine the optimal physiological conditions for formation of yeast zygotes.

In 1962 Jacob described a method for synchronized formation of yeast zygotes. However, the synchronization was rather poor, as the yield of zygotes, although much higher than in Lindegren's classic method, was still low (only up to about 15 %) and the time from the appearance of the first zygotes to the maximum of their frequency was about three hours.

When studying the formation of diploid cells on selective media the results are much influenced by the presence of clumps formed as a result of sexual agglutination (Sakai & Yanagishima, 1971) and the yield obtained is overestimated owing to new zygote formation in these clumps.

The main advantage of Jacob's method was to enforce contact between competent haploid cells by centrifugation. This resulted in an almost hundredfold rise in the yield of zygotes. To raise the yield and to obtain a truly synchronous population of zygotes we have modified Jacob's basic procedure.

METHODS

Strains. The following strains were used: M/82-1 α leu₁ ade₁, M/82-2 a leu₁ thr₂₋₁; originating from cross 55R5/3c a ura₁ $\times$ S2615B α thr₂₋₁ tyr₄ leu₁ ade₁ ade₅. The strain 55R5 was obtained from Professor Slonimski (Gif-sur-Yvette) and strain S2615B from Professor Mortimer (Donner Laboratory, University of California, Berkeley).

Media. The basal medium YE 3 % for zygote formation was that used by Jacob (1962), and consisted of 3 % glucose and 1 % yeast extract. The lowering of glucose content or its removal lowered or prevented zygote formation. For yeast culture the YPG medium was used, consisting of 1 % yeast extract, 1 % Difco-Bacto-Peptone and 2 % glucose.

Estimation of zygote yield by selective plating. The number of zygotes was estimated by plating on minimal medium (Galzy & Slonimski, 1957) containing 2 % glucose and supplemented with leucine on which, in this cross, only diploids can grow. The total number of cells was estimated from simultaneous plating on YPG medium. The cells were sonicated before plating. The number of haploid a and α cells during conjugation was established by plating on minimal medium supplemented with leucine, leucine plus adenine, and leucine plus threonine and methionine, respectively.

Estimation of zygote yield by counting in a haemocytometer. The samples of cultures or of incubation mixtures were cooled in an ice bath, sonicated and immediately counted with a haemocytometer. Budded haploid cells were counted as two units, and single cells and budded or unbudded zygotes as one unit. At least 500 cells were scored for each determination.

Culture. Both haploid strains were cultivated in 350 ml Erlenmeyer flasks containing 100 ml of YPG medium for 18 h at 30 °C on an orbital shaker (120 rev./min). The concentration of cells in inocula was 3×10^5 for a strain and 2×10^6 for α strain.

Harvesting. The cells were collected at the beginning of stationary phase by centrifugation for 5 min at 3000 g (Sorvall SS-1) in 50 ml steel tubes, sterilized with 70 % ethanol.

RESULTS AND DISCUSSION

Sexual agglutination results in the formation of clumps which sediment, and also in a lower optical density of the suspension of conjugating haploid cells. The changes in optical density thus reflect the processes of sexual agglutination. Fig. 1 shows the dependence of changes in optical density of suspensions of a and α haploid cells on the pH of the incubation medium. The optimal pH for sexual agglutination of haploid cells was about 6. Beyond the pH range 5 to 7 the agglutination reaction dropped rapidly.

When the rate of zygote formation in samples of incubation medium was directly measured in the same conditions as for agglutination we obtained a very similar curve of dependence of zygote formation on the pH values of the incubation medium (Fig. 2) with the optimum at pH 6.

When the conjugating mixture was centrifuged and the conjugation took place in a pellet, the optimal pH for zygote formation was 4.5. The yield of zygotes after centrifugation was much higher, as one of the limiting factors in zygote formation in a suspension of haploid cells is the low chance of contact among the competent cells, and centrifugation increased contacts among cells. In such conditions the optimal pH was 4.5 which coincided with the optimal pH for the α factor action as determined by W. Duntze (personal communication).

The optimal density of haploid cells for zygote formation both for cells in suspension

Fig. 1. Effect of pH on cell agglutination in *Saccharomyces cerevisiae* after 2 h incubation of α and a strains. The suspensions of 10^8 cells/ml of a and α cells in YE 3 % were prepared and adjusted to pH 3.0 with acetic acid; to pH 4 to 4.5 with 0.1 M-acetate buffer; to pH 6.0 to 8.0 with phosphate buffer; to above pH 8.0 with 0.1 M-tris-HCl buffer. Strains were mixed 1:1 and incubated for 2 h at 30 °C in an orbital shaker. The details of culture, harvesting, incubation and conjugation conditions were as in Methods. After 2 h, 10 ml samples of conjugation mixture were poured into 10 ml graduated test-tubes and, after standing for 10 min, a 3 ml sample was taken from the top of the tube, diluted three times, and the absorbance was measured at 700 nm in 1 cm cuvettes with a Specol spectrophotometer. Treated in the same way, the initial suspension had an extinction of 1.14. At the end of the experiment pH values were checked, and differences were no more than 0.1 pH unit. The pH was measured with a Radiometer 22.

and after centrifugation was about 10^6 to 10^7 /ml of incubation medium. When the concentration rose above 10^8 /ml, the yield of zygotes was much lower.

Another important factor for effective zygote formation was an adequate relative proportion of opposite mating types in the mixture. We examined the influence of the concentration of opposite mating-type cells on the yield of zygotes, changing the concentration from 10^7 to 10^8 cells/ml and maintaining the concentration of the other mating type at 10^7 cells/ml. The resulting two curves (Fig. 3) show that when α cells were maintained at 10^7 cells/ml, and we raised the concentration of a cells from 10^7 to 10^8 cells/ml, the yield of zygotes rose up to a concentration of 5×10^7 cells/ml and then a plateau was reached. When the concentration of a cells was kept constant at 10^7 cells/ml and the concentration of α cells was raised from 10^7 to 10^8 cells/ml, the yield of zygotes with the stepwise increasing proportions of α cells decreased continuously. The excess of α cells inhibited in some way the formation of the zygotes.

As can be seen from Fig. 2, centrifugation of the conjugation mixture raised the yield of zygotes nearly a hundredfold. We have found that centrifugation was effective only when applied not later than 2 h after mixing a and α haploid cells. Contacts among cells may have

Fig. 2. Effect of pH on cell conjugation in *Saccharomyces cerevisiae*. ●—●, Conjugation without centrifugation; scale on the right. The culture and harvesting conditions and the medium were as for Fig. 1. The suspensions of 2×10^6 cells/ml of *a* and α strains were prepared in media with different pH values and mixed together in a 1:1 ratio. Ten ml portions of the mixture in 50 ml tubes were incubated on an orbital shaker at 30 °C for 2 h and without shaking at the same temperature for the next 2 h. After that time the samples were sonicated and plated on selective media to determine zygote formation frequency. ○----○, Conjugation with centrifugation: scale on the left. The preparation of cell suspensions and 2 h incubation were as for Fig. 1. Afterwards 1 ml of suspension was diluted ten times with the medium of the same pH value and centrifuged for 2 min at 3000 g in 10 ml tubes in a Sorvall SS1. After 30 min the tubes were gently shaken and then incubated at 30 °C without shaking. Four and a half hours after the initial mixing of both strains the samples were cooled on ice, sonicated and counted under a microscope.

been unnecessary for the early stage because of the ability of hormones to diffuse. Then, when cells were at a suitable stage for conjugation, effective contact between competent cells was necessary. Usually the first conjugation figures appeared 3 h after the mixing of both mating types and the maximum zygote formation was reached in the following 2 to 3 h.

The effect of pH during the first 2 h is shown in Fig. 4. There is a bimodal curve with two optima at pH 4.5 and 8.5 and a minimum at pH 6. In the conditions described in the legend to Fig. 4, the absolute number of cells after 5.5 h incubation was highest at pH 6.0 (1.6×10^7 cells/ml). At lower and higher pH values the number of cells decreased and at pH 8.5 it was nearly the same as at the beginning of incubation (1.2×10^7 cells/ml as compared with 10^7 cells/ml). At the same time the absolute number of zygotes was highest at pH 4.5 (3.4×10^6 zygotes/ml) and dropped at pH 6.0 (2×10^6 zygotes/ml) and in more alkaline conditions remained practically constant. Thus there is a real optimum for zygote formation at pH 4.5 and this bimodal curve may be the result of an interplay of many processes such as hormonal induction, conjugation, growth and divisions of haploid cells.

To obtain not only a high yield of zygotes but also a high degree of synchrony, it is necessary to restrict the period of their formation to a defined short period of time. At an early stage sexual agglutination occurs and clumps are formed. In clumps zygotes could

Fig. 3. Yield of zygotes after 4.5 h incubation of a mixture with different proportions of a and α strains of *Saccharomyces cerevisiae*. The culture and harvesting conditions were as for Fig. 1. Two series of mixtures of both strains in YE 3 % medium (Jacob, 1962) were prepared: $\bigcirc$ --- $\bigcirc$, with a constant level of α cells (10^7 cells/ml) and varying concentrations of a cells ranging from 10^7 to 10^8 cells/ml; $\bullet$ — $\bullet$, with a constant level of a -cells (10^7 cells/ml) and varying concentrations of α cells ranging from 10^7 to 10^8 cells/ml. The two mixtures were incubated for 2 h on an orbital shaker and without shaking at 30°C for a further 25 min. Afterwards the cells were cooled on ice, sonicated and plated on selective media to determine zygote formation frequency. Similar results were obtained with centrifugation.

Fig. 4. Effect of pH on preliminary steps of conjugation in *Saccharomyces cerevisiae*. The procedure was as for Fig. 1. After 2 h incubation in a given pH the cells were centrifuged and suspended again in the same volume of YE 3 % medium with pH 4.5. One ml of this suspension was diluted to 10 ml with fresh medium at pH 4.5 and centrifuged for 2 min at 3000 g (Sorval SS1) in 10 ml tubes. After 30 min the pellet was re-suspended and incubated at 30°C . Four and a half hours after the initial mixing of parental strains the cells were cooled on ice, sonicated and counted under a microscope.

Table 1. *Effect of sonication on zygote formation after various periods of incubation of a and α cells of Saccharomyces cerevisiae*

The culture and harvesting conditions were as in Methods; the conjugation medium was after Jacob (1962). A mixture of α cells (10⁷ cells/ml) and a cells (5 × 10⁷ cells/ml) was prepared; 100 ml were incubated for 2 h in 250 ml flasks at 30 °C on an orbital shaker and further incubated without shaking at 30 °C. Samples (10 ml) were taken each hour and centrifuged for 5 min at 3000 rev./min and suspended in 10 ml water. Samples (5 ml) were sonicated twice for 30 s (amplitude 2 μm from peak to peak with MSE 100 Watt Ultrasonic Disintegrator). The remaining 5 ml portions were not sonicated, and acted as controls. The samples were diluted and plated on selective media.

Time of incubation (h)	0	1	2	3	4	5	6
No. zygotes/ml							
Control	35	15	2 605	19 800	43 275	91 000	252 000
Sonication	1	3	14	3 415	34 500	74 000	166 500

Fig. 5. Formation of zygotes in the conjugation mixture. ○ --- ○, Prepared according to the procedure of Jacob (1962), cell concentration 10⁶/ml for each strain, scale on the right. ● — ●, Prepared according to the procedure of the authors, cell concentration 5 × 10⁶/ml, scale on the left. Estimation of the number of zygotes in both cases was by counting under the microscope in the haemocytometer.

arise immediately, but when after 2 h the suspension is sonicated, the clumps are disintegrated and thus formation of zygotes is prevented. The results of experiments on the influence of sonication on zygote formation are presented in Table 1. Sonication during the first 2 h almost completely prevented the formation of zygotes. The number of zygotes was tested by plating on selective media. When clumps were not disintegrated, then even after plating new zygotes were formed and new diploid colonies appeared on selective medium.

When the sonication was applied after 4 h incubation, the yield of zygotes was similar to that in unsonicated control samples. This indicates that sonication did not damage already-formed zygotes.

To obtain a high yield of synchronously formed zygotes the following procedure was finally applied. The haploid a and α cells were grown and harvested as indicated in Methods. Suspensions of 10^8 cells/ml in conjugation medium YE 3 % (yeast extract 1 %, glucose 3 %, at pH 4.5 adjusted with 1 M-acetic acid) were prepared. The suspensions of both mating-type cells in a 1:1 ratio were mixed and incubated for 1 h at 30 °C, in 50 ml steel Sorvall centrifuge tubes containing 35 ml of the mixture. Then the cells were centrifuged for 5 min at 3000 g (Sorvall SS1) and resuspended in 35 ml of YE 3 % at pH 8.5 (adjusted with 1 M- Na_2HPO_4). After 1 h incubation the cells were centrifuged in the same conditions, resuspended in 35 ml of fresh YE 3 % at pH 4.5 and sonicated twice for 30 s (amplitude 2 μm from peak to peak with an MSE 100 Watt Ultrasonic Disintegrator).

To 250 ml steel centrifuge bottles containing 180 ml of fresh YE medium, pH 4.5, 20 ml of sonicated suspension were added and immediately centrifuged on a MSE High Speed 18 with an angle rotor (6×250 ml) at room temperature for 2 min at 3000 rev./min. The bottles were then incubated at 30 °C for 30 min and then gently shaken to suspend the pellet. After another 30 min incubation they were again shaken to disintegrate the pellet and the suspension re-incubated without shaking.

With this procedure the first zygotes did not appear until 4.5 h incubation. During the next hour the number of zygotes rose abruptly, and then showed no further increase (Fig. 5). In Fig. 5 the kinetics of zygote formation by our procedure and that of Jacob (1962) are compared. We see not only that the yield of zygotes is much higher, but also that the synchronization of their formation is much improved, as practically all zygotes are formed during 1 h. After 5.5 h incubation the maximum percentage of zygotes in the whole yeast population is also reached. After that time the increase of diploid cell number is already the result of the separation of buds from the zygotes and their subsequent divisions.

Using this procedure we were able to increase the yield of zygotes to about 30 to 40 %. This percentage is calculated from the number of cells after 5.5 h conjugation during which some haploid cells have divided. About 60 to 70 % of the initial number of haploid cells are involved in conjugation. The high degree of synchrony results from preventing zygote formation during the first 2 h by creating conditions unfavourable for cell fusion, such as suboptimal pH, high cell concentration, and sonication. Then conditions are optimized for cell fusion and the cells are forced into contact. This yield could be raised further by disintegrating the pellet and afterwards centrifuging the cells several times, but then synchronization would be lost. It is quite possible that this yield of zygotes is nearly the highest possible by such a technique, since in a pellet of immobilized cells the spatial relations do not permit more effective pairing between competent cells. This assumption is supported by the fact that 95 % of α cells are competent. Using the conjugation technique described above we have found that only 5 % of α cells were still haploid when the a cells were more than four times as numerous in the initial mixture.

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