THE DESIGN OF A CULTURE MEDIUM WHICH WILL FACILITATE THE STUDY OF DIMORPHISM IN *CANDIDA ALBICANS*

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Introduction

Candida albicans is of growing importance in the medical field. Candida spp. cause superficial infections of the skin, scalp, nails, and reproductive system. However, Candida spp. may also cause systemic infections, especially when an underlying disease or condition has weakened the body's defenses. While both the yeast (blastospores) and hyphal forms are pathogenic, there is some evidence which suggests that the hyphal form is more virulent. What triggers this switch in the body is as yet unknown.

For these reasons, Candida spp. have been studied extensively to determine what causes the shift in cell type. Many factors that are believed to have an affect on the dimorphism of Candida spp. have been tested. For example, it has been found that a 12°C temperature increase during experiments with Candida maximizes the induction of the shift (yeast → hyphae) (Lee, et al., 1975; Barlow et al., 1974; Mitchell and Soll, 1979; Mardon et al., 1969). In these experiments, yeast phase growth was attained at 25°C, and hyphal phase growth at 37°C.

In addition to temperature, another factor that apparently affects morphogenesis of Candida spp. is the pH of the culture medium. Many researchers have found that a pH change can

facilitate dimorphic change in Candida spp. For example, a slightly alkaline pH (7.0-7.9) has been found to favor the hyphal form of Candida (Barlow, Aldersley, and Chattaway, 1974; Mitchell and Soll, 1979; Evans et al.,1974; Evans et al., 1975), while a more acidic pH (4.5-6.0) favors the yeast phase (Elin and Wolff, 1973; Lee et al., 1975). Often, a pH change and a temperature change were used in concert in order to maximize morphogenesis (Lee et al., 1975). Others (Auger and Joly, 1977) used a constant temperature and varied the pH.

In analyzing the research literature pertaining to temperature and pH-induced shifts, we began to question the use of such factors to facilitate the study of dimorphism in Candida spp. We felt that a 12°C increase in temperature and/or a 3-4 unit increase in pH will have a very general effect on the cell's physiology, not merely morphology, and this complicates (instead of facilitates) the study of dimorphism. For these reasons we designed experiments, employing constant temperature and pH, which would allow us to examine other variables (such as addition of albumin or amino acids) that might be used to elicit a change in cell type.

Materials and Methods

Organism

Candida albicans strain #304 was obtained from the UCLA Medical School, Los Angeles, California. The stock cultures were maintained on Sabouraud Dextrose agar slants at 5°C.

Medium

Semi-Defined medium (Peters and Sypherd, 1978) consisting of 0.13% aspartate, 0.09% alanine, 0.17% glutamate, 0.05% yeast nitrogen base, and 2.0% dextrose dissolved in distilled water was used as the base medium in all experiments. A pH of 4.5 was used for all experiments except as noted. The pH was adjusted with H_2SO_4 or NaOH prior to autoclaving.

Culture Conditions

Starter cultures were grown in 125-ml Erlenmeyer flasks containing 25 ml of sterile Semi-Defined medium, pH 4.5. These cultures were incubated at 37°C in a rotary water bath for 12 to 24 hours, depending on experimental design. The starter culture was checked after this growth period to insure 100% yeast phase growth (blastospores). To standardize the inoculum, a haemocytometer was used to count cells, and the desired number of cells (1×10^6 cell/ml or 2×10^6 cells/ml, depending on the experiment) were collected from the starter culture with an automatic pipette. These cells were then filtered through a membrane filter (to remove collected starter culture medium) and the filter transferred to a 125-ml Erlenmeyer flask containing 25 ml of fresh Semi-Defined medium. These test cultures were incubated in a rotary water bath

at 37°C for up to three hours.

Quantification of Germ Tube Formation

Small samples of cells were collected with sterile pasteur pipettes every thirty minutes after the inoculation of the test culture. Cells were placed on a haemocytometer and examined microscopically to quantitate germ tube formation.

Positive germ tube formation was indicated by the presence of germ tubes at least three yeast cell diameters in length. Percent germ tube formation was determined by the ratio of germ tube formers to the total number of cells present.

Results

The Effect of Serum Albumin on Growth of Germ Tubes at Constant Temperature and pH

Experiments were performed using serum albumin to stimulate germ tube formation while keeping temperature (37°C) and pH (4.5) constant. The serum albumin concentration was 400 µg/ml. The inoculum concentration was 1 x 10^6 to 2 x 10^6 cells/ml and the age of the starter culture varied from 12 to 24 hr.

Figure 1 shows that the presence of serum albumin had very little effect on the percentage of blastospores switching to hyphae. In fact, a high percentage of germ tube formation was seen in the non-albumin control cultures. In three of the four test cultures, inoculation of cells into fresh test medium was enough to cause germ tube formation. In the 12-hr test culture, no shift was seen in either the experimental or control flask.

In light of these results, it was theorized that the age of the starter culture might have an effect on the dimorphism of Candida albicans. Experiments were designed to test this hypothesis.

Age of the Starter Culture vs. Percent Germ Tube Formation

To test the idea that the age of the starter culture might affect dimorphism in Candida albicans, starter cultures were grown in Semi-Defined medium, pH 4.5 (in a 37°C water bath) for 12 hr, 24 hr, and 48 hr. 1 x 10^6 cells/ml were collected from each and transferred to three flasks of fresh Semi-Defined medium, pH 4.5. These test cultures were then allowed to grow at

37°C in a rotary water bath for two hours.

Figure 2 shows that the 24-hr and 48-hr cultures were stimulated to germ tube growth by mere transferral to fresh medium; the 12-hr culture, on the other hand, again showed no germ tube formation after two hours.

In view of these results, the possibility of the presence of a "germ tube inhibitor" in the young culture (12 hr) was noted. Experiments were designed to test this hypothesis.

The Possibility of an Inhibitor of Germ Tube Formation in Young Starter Cultures

After observing that young (12 hr) starter cultures exhibited no germ tube formation upon inoculation into fresh media, an experiment was designed to test for the possible presence of a "germ tube inhibitor" released into the starter culture medium during the growth of the yeast culture.

Two starter cultures, one 24-hr and one 12-hr, were grown in 25 ml of Semi-Defined medium, pH 4.5, at 37°C. The 12-hr medium was then filter-sterilized and poured back into a sterile Erlenmeyer flask; this was done to filter out cells and debris in order to test the medium for the presence of a germ tube inhibitor. 1×10^6 cells/ml were collected from the 24-hr culture and filtered; these cells were then added to the filter-sterilized 12-hr medium. The age of 24 hr was selected because in previous experiments, 24-hr cells were shown to undergo switching by merely transferring them to fresh medium.

As a control, 1×10^6 cells/ml from the 24-hr culture were

also added to 25 ml of fresh Semi-Defined medium, pH 4,5. The cultures were incubated in a rotary bath at 37°C for 2 1/2 hours.

Figure 3 illustrates that there is indication of a possible germ tube inhibitor in the 12-hr medium. Even though 24-hr yeast cells had been shown to switch upon inoculation into fresh medium in previous experiments, 17% less switching occurs when they are transferred into filter-sterilized 12-hr medium than when they are transferred to fresh medium. However, pseudohyphae (an intermediate form) were formed instead of germ tubes in this experiment. This supports the hypothesis that a germ tube inhibitor may exist in young cultures.

The Effect of pH on Germ Tube Formation Under Constant Temperature

While conducting these experiments, it became evident that researchers had reported conflicting results concerning the pH at which Candida spp. should be grown in order to obtain a high degree of germ tube formation. Some optimum pH values proposed are: pH 7.7 (Odds and Evans, 1975); pH 7.4-7.5 (Evans and Odds, 1974); pH 7.4 (Barlow and Aldersley, 1974); pH 6.8 (Lee, Buckley, and Campbell, 1975); pH 6.5 (Mitchell and Soll, 1979); pH 6.0 (Elin and Wolff, 1973), and pH 4.5 (Peters and Sypherd, 1978). Obviously, pH 4.5 to pH 7.7 is a very wide range for an optimum pH value. For this reason, experiments were designed to examine the pH value at which Candida albicans yields maximum germ tube formation at 37°C.

A starter culture in Semi-Defined medium, pH 4.5, was incubated at 37°C for 12 hours. Five experimental flasks were then

prepared, each having 25 ml of fresh Semi-Defined medium at different pH values ranging from pH 4.5 to pH 6.5. 1 x 10^6 cells/ml from the starter culture were transferred into each experimental flask, and the flasks were incubated at 37° C for 3 1/2 hours.

After the incubation period, the cultures were examined and it was determined that all failed to form true germ tubes. However, after two hours the cells in all flasks of pH 5.0 to pH 6.5 exhibited abnormal growth in that blastospore buds appeared elongated and unusually shaped (pseudohyphae-like). The flask with pH 4.5 medium contained cells in which no switching was seen.

The Effect of Amino Acids on Germ Tube Formation of Candida albicans During Growth at Constant Temperature and pH

Chattaway et al., (1976) and Lee et al., (1975) as well as others, have tried to develop a growth medium consisting of amino acids, glucose, and inorganic phosphate that will yield a high percentage of germ tube formation in Candida spp. However, these experiments also included a temperature shift from 25° C to 37° C upon incubation of the experimental culture. Under these conditions, 90% germ tube formation was achieved by Chattaway et al. (1976).

Since the purpose of this experiment was to develop a growth medium that would support shifting at a constant temperature, an experiment was designed to observe the effects of amino acids on a shift while maintaining a constant temperature (37° C) and pH (4.5).

Stock solutions of five amino acids (aspartate, threonine,

proline, lysine, and histidine) were prepared at a concentration of 20µg/ml. These five amino acids were chosen based on Chattaway's work (1976).

A starter culture, pH 4.5, was incubated at 37°C for 12 hours. 1 x 10^6 cells/ml were then transferred to each of two flasks; one containing Semi-Defined medium (pH 4.5) plus the five amino acids, and the other containing only Semi-Defined medium (pH 4.5). These flasks were then incubated at 37°C for 2 1/2 hours.

Figure 4 shows that the results were inconclusive. Instead of observing a normal yeast → hyphae change, the yeast switched to pseudohyphae (an intermediate form). However, the flasks containing the amino acid mixture showed 35% more yeast cells switching to pseudohyphae that the control flask. This suggests that the amino acids had some effect on dimorphism.

Discussion

As Figure 1 shows, the effect of serum albumin on dimorphism of Candida albicans is negligible. A high percentage of switching was obtained in both (+) albumin flasks and (-) albumin flasks. However, no switching occurred in the 12-hr flasks, leading us to hypothesize that the age of the starter culture might have an effect on dimorphism. To test this idea, experiments were performed using 12-hr, 24-hr, and 48-hr cultures, and percent germ tube formation was observed. Figure 2 shows that 24-hr cells show 100% more germ tube formation after 2 hours than 48-hr cells. Again, no shifting was seen in the 12-hr cultures, which led us to postulate that there might be a germ tube inhibitor being released into the culture medium of young (12 hr) cultures. Experiments were designed to test this theory, using 24-hr cells (which had shown a high percentage of switching upon inoculation into fresh medium) inoculated into filter-sterilized 12-hr medium (which would inhibit germ tube formation of the 24-hr cells, if the germ tube inhibitor existed). Figure 3 shows that even though no true germ tubes were seen in the test or control flasks, the control flask showed 17% more pseudohyphae (an intermediate form) than the test flask. These experiments show some promise, yet further work is needed in order to examine this phenomenon.

Experiments studying the effect of pH on germ tube formation were based on work done by other researchers who reported conflicting pH values for obtaining maximum germ tube formation. We decided to test the 12-hr culture in media having different pH

values (pH 4.5 to 6.5) since it had previously shown no shifting when inoculated into fresh medium. No true germ tube formation was observed in any of the flasks after 2 1/2 hours of incubation. However, flasks with media of pH 5.0, 5.5, 6.0, and 6.5 showed cells with pseudohyphae-like growth on the blastospores after 2 hours. These results are in agreement with the current theory that acidic pH favors the yeast phase, since the more alkaline pH values caused a pseudohyphae-like growth of yeast cells.

In view of all the results, i.e., that pH 4.5 favors the yeast phase and that a 12-hr culture will not switch upon mere transferral into fresh medium, we decided to test the amino acid combination reported by Chattaway (1976) to see if it would stimulate germ tube formation in a 12-hr culture at pH 4.5 and constant temperature (37°C). Figure 4 shows that, although no true germ tube formation was achieved, the percentage of pseudohyphae formation in the (+) amino acids flask was 35% higher than the control flask containing no amino acids. This suggests that amino acids may have some effect on the dimorphism of Candida albicans, and further studies need to be conducted in this area.

Overall, the experiments on the effects of different substances on the germ tube formation of Candida albicans, using constant pH and temperature, need to be extended. It may be possible to achieve true germ tube formation by raising the pH slightly (to 4.6-4.8) and using a starter culture older than 12 hr but younger than 24 hr, in order to stimulate the formation of true hyphae. Under these conditions, Chattaway's amino acid

mixture (1976) might stimulate a high percentage of true germ tube formation.

Also, the amino acid combination used by Chattaway (1976) needs to be studied more and possible modified in order to yield maximum germ tube formation under constant temperature and pH.

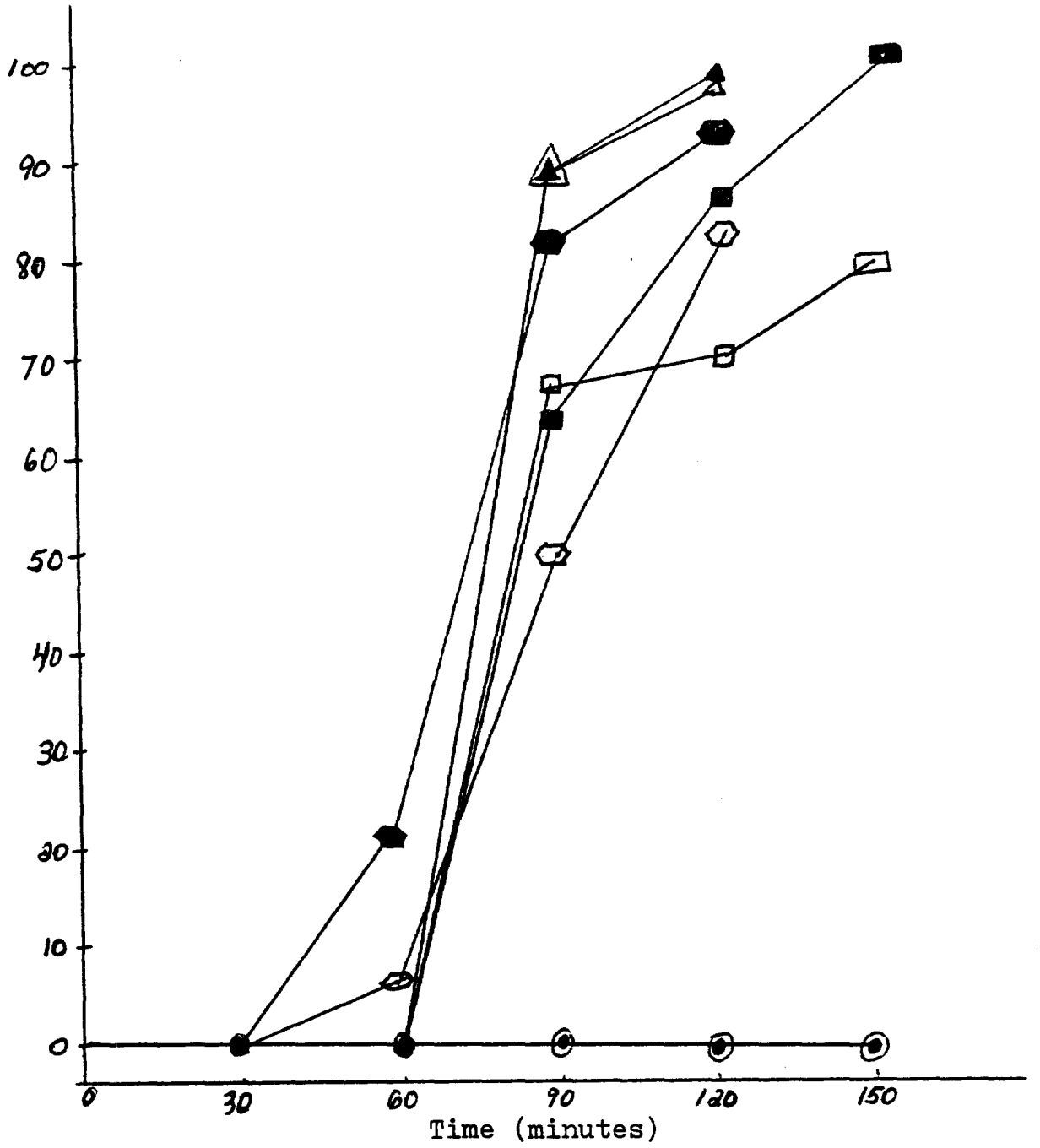

Figure 1. The percent germ tube formation by cells as a function of time. Cells from starter cultures of ages 12 hr (●), 18 hr (▲), and 24 hr (■ and ⬣) were used. In the 24-hr culture, 1 x 10^6 cells/ml (■) and 2 x 10^6 cells/ml (⬣) were tested. Solid figures denote (+) albumin; clear figures denote (-) albumin. Serum albumin (400 µg/ml) was used to stimulate germ tube formation.

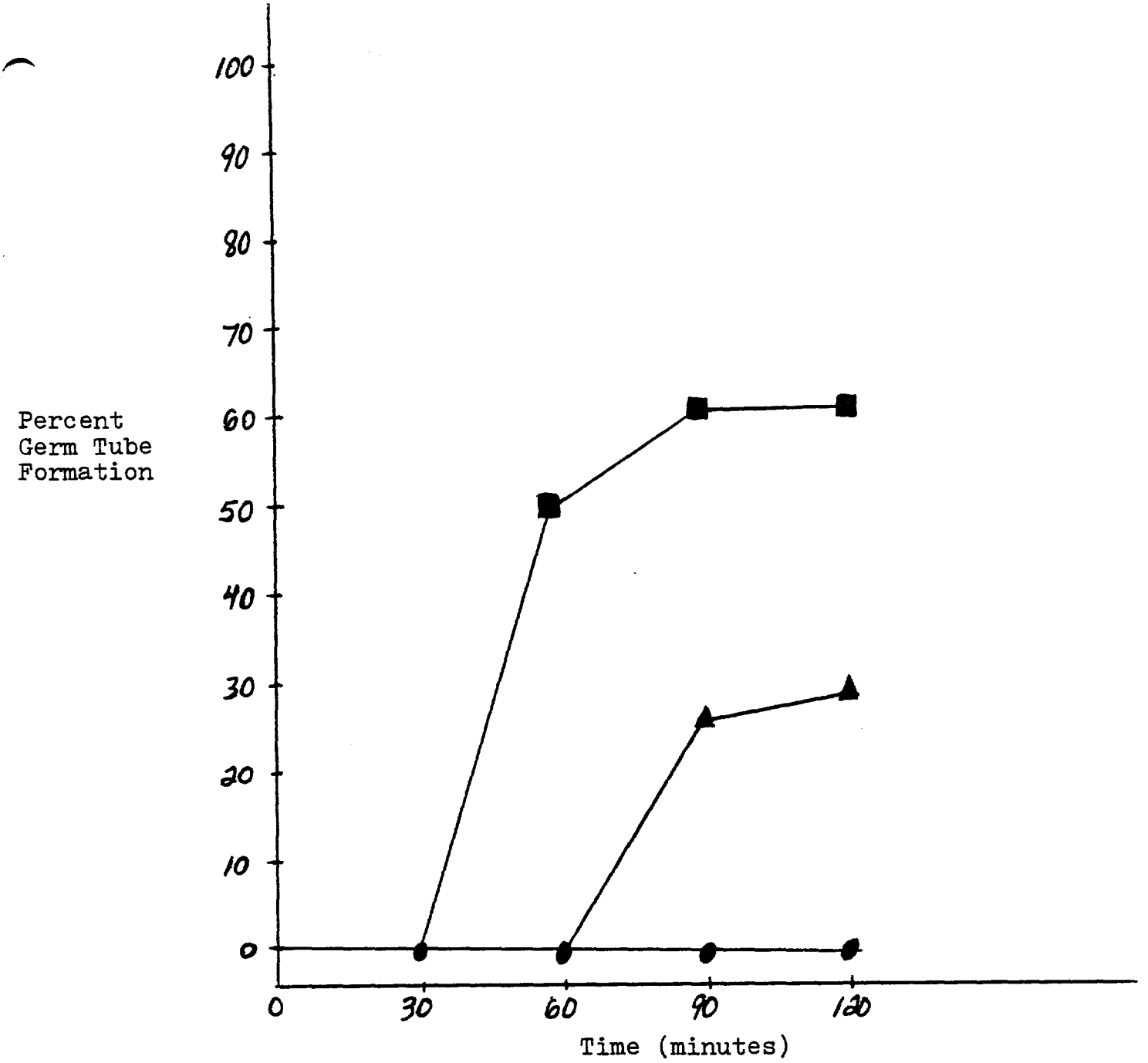

Figure 2. The percent germ tube formation by Candida albicans as a function of time. Starter cultures were grown for 12 hr (●), 24 hr (■), and 48 hr (▲), and then transferred to fresh growth medium; temperature was kept constant (37°C). The 12-hr culture showed no germ tube formation after two hours.

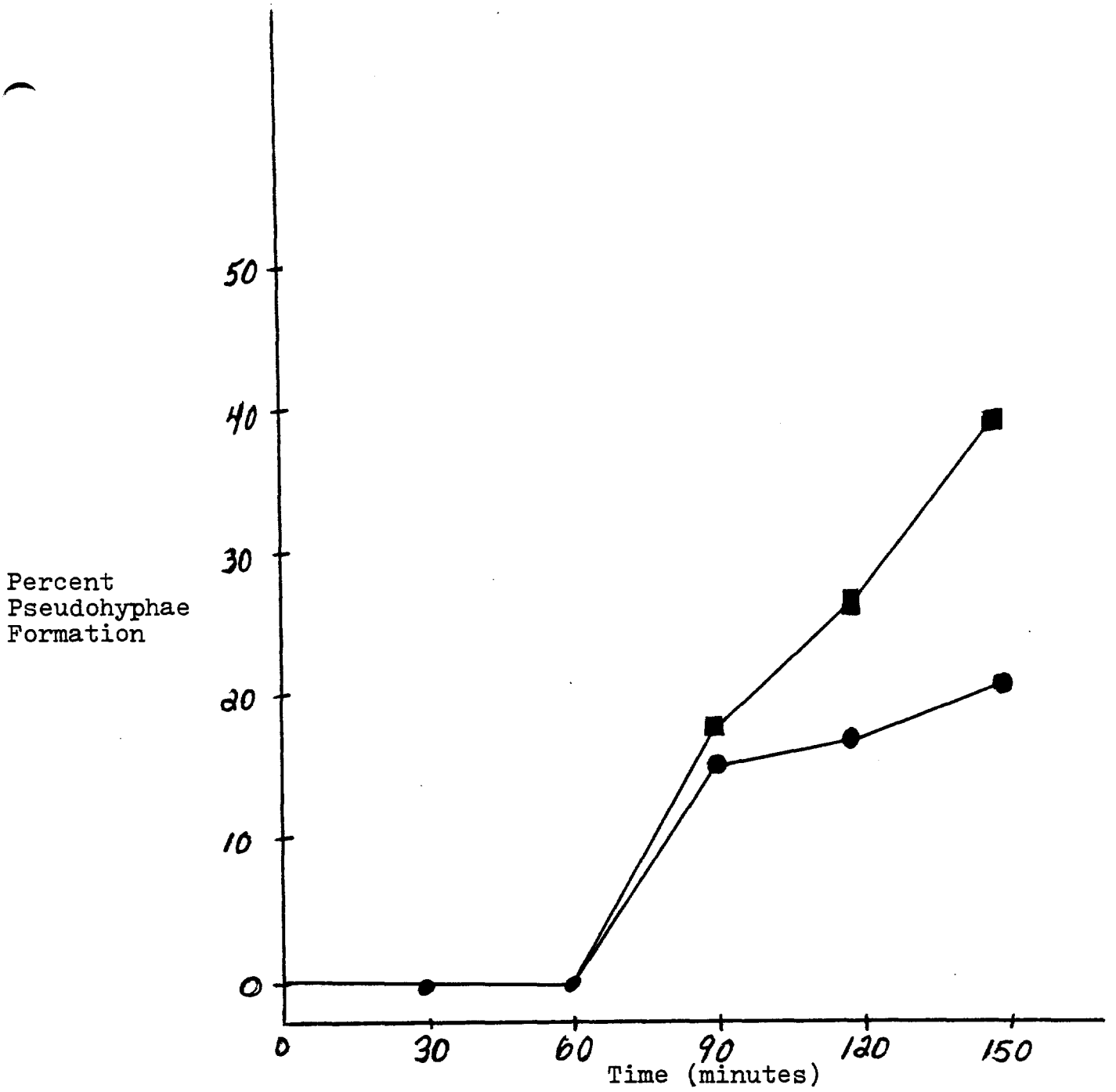

Figure 3. The percent pseudohyphae formation as a function of time. 1 x 10^6 cells/ml from a starter culture of 24-hr were inoculated into filter-sterilized 12-hr medium (●) and fresh medium (■). The filter-sterilized 12-hr medium shows evidence of containing a germ tube inhibitor released into it by 12-hr cells.

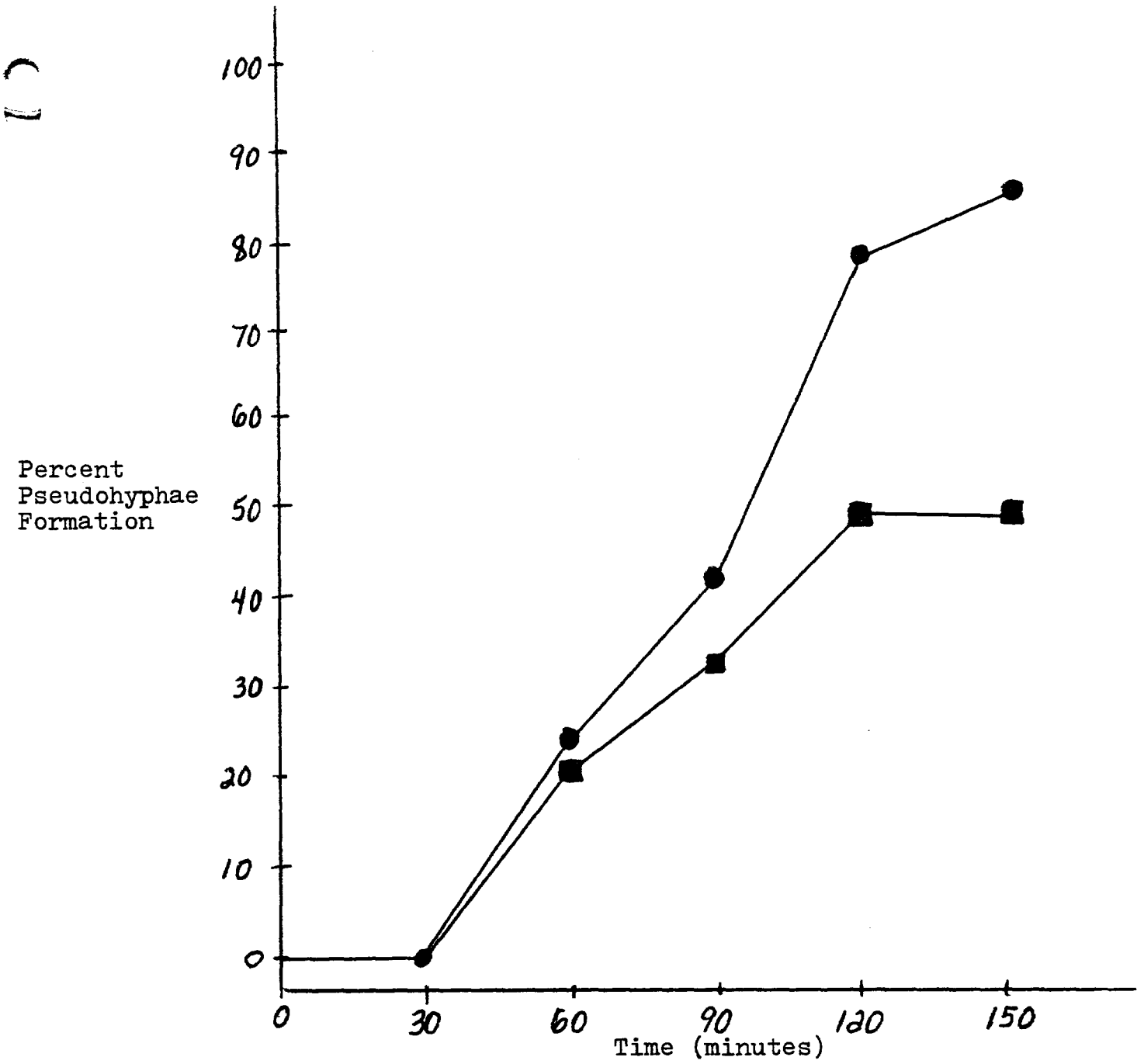

Fig. 4. The percent germ tube formation as a function of time. 1 x 10^6cells/ml were transferred from a starter culture (containing no amino acids) to experimental flasks containing medium plus amino acids (●) and medium minus amino acids (■). No true germ tubes appeared, only pseudohyphae.

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