Simple Method for Inducing Ascospore Formation in Yeasts

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A new method for inducing ascospore formation in yeasts is described and compared with conventional methods for its performance. The method has the advantage of simplicity, reproducibility, and saving of time.

More than 15 different methods for inducing ascosporulation in yeasts have been recommended following the first report by DeSeynes in 1868 describing the formation of ascospores by yeasts on the surface of water (2). These methods are based either on starvation of cells or their cultivation on restricted amounts of directly assimilable carbohydrates; certain methods are specific for particular species or genera. It is usually necessary to apply almost all the methods to ascertain the sporulating ability of an unknown yeast culture. In this note, a simple and effective method for inducing ascospore formation in yeasts is reported.

Complete growth of a 24-hr yeast culture from a yeast extract-malt extract (YM) agar slant (3) was transferred to a 250-ml Erlenmeyer flask containing 30 to 40 ml of mineral salt medium (6) to which 1 ml of separately sterilized gas oil (a petroleum fraction supplied by Assam Oil Company, Digboi, Assam, India) was added. The gas oil has the following characteristics: bp, between 250 and 350 C; sp gr, 0.8658; carbon in paraffins, naphthenes, and aromatics in weight percentage of total carbon, 55, 24, and 21, respectively; n-alkane content, 25 to 30%. The flask was incubated on a gyrotary shaker at optimum growth temperature of the yeast, and examination for ascospore formation was made at 15, 24, 48 and 72 hr of incubation. To detect spore formation, a smear was prepared and air dried. The thin film or droplets of gas oil were removed from the air-dried smear by pressing the smear gently with blotting paper. The smear was stained for spores by using Bartholomew and Mitter's cold method (5). Every observation on spore staining by Bartholomew and Mitter's cold method was confirmed by staining spores by Dorner's method (5). Other approved spore-staining methods, i.e., Snyder's modification of Dorner's method (5), Conklin's modification of Wirtz method (5), and Schaeffer-Fulton's method (1), were used with Endomycopsis lipolytica Y-3. By using all these staining methods, it was observed that the growth of the cells in liquid medium containing gas oil did not change the staining properties of spores or vegetative cells.

The use of gas oil induced ascospore formation both in hydrocarbon- as well as nonhydrocarbon-utilizing yeasts. Positive results were obtained even in stock cultures maintained for 2 to 3 years on YM slants. Ascospores were observed in 17 hydrocarbon-utilizing strains belonging to nine species of three genera. These include Endomycopsis javanensis, E. lipolytica, Endomycopsis sp., E. fibuliger, Saccharomyces fructuum, S. chevalieri, S. waram, S. marxianus, and Schwanniomyces occidentalis. Ascospore formation was also detected in 11 strains of nonhydrocarbon-utilizing yeasts belonging to five species of four genera, including Pichia polymorpha, P. farinosa, E. capsularis, Saccharomyces cerevisiae, and Debaryomyces hansenii. Ascospores were observed at 15 hr of incubation in most of the strains and at 24 hr of incubation in all the strains (Fig. 1).

To test the comparative performance of the proposed method and conventional methods (4) for induction of ascospores, strains of hydrocarbon-utilizing and nonhydrocarbon-utilizing yeasts were grown on gas oil and on Gorodkowa, carrot, and potato slants and examined. The results are depicted in Table 1.

Spores were found in none of these strains when they were grown on YM slant and were stained for spores at 18, 24, and 48 hr of incubation at 30 C. It is evident that the proposed method is more efficient as compared to con-
FIG. 1. Induction of ascospore formation in the presence of gas oil. Saccharomyces chevalieri W-9, 0 hr (A), 24 hr (B). Phase contrast, x1,250. Endomycopsis lipolytica Y-15, 0 hr (C), 24 hr (D). Stained with Bertholomew and Mitter’s cold method. x1,250. Spores are marked with arrows.

TABLE 1. Comparative efficiency of the proposed method with conventional methods for induction of ascospores in yeasts

| Yeast                        | Incubation for 15 hr* | Incubation for 40 hr |
|------------------------------|-----------------------|----------------------|
|                              | Gorod-kowa agar slant | Carrot slant | Potato slant | Gas oil | Gorod-kowa agar slant | Carrot slant | Potato slant | Gas oil |
| Gas oil utilizier            |                       |              |             |         |                      |              |             |         |
| Schwanniomyces occidentalis NCYC 133 | -                      | -            |             | ++      | -                      | -            | -            | ++      |
| Endomycopsis lipolytica Y-15 | +                      | +            | ++          | +       | +                      | +            | ++          | ++      |
| E. javanensis Y-20           | -                      | +            | ++          | +       | +                      | -            | +            | ++      |
| Saccharomyces fructuum Y-21  | -                      | ++           | ++          | -       | ++                     | -            | ++          | ++      |
| S. chevalieri W-9            | -                      | ++           | ++          | +       | +                      | +            | ++          | ++      |
| Non-gas oil utilizier        |                       |              |             |         |                      |              |             |         |
| Saccharomyces cerevisiae Y-40 | +                      | +            | ++          | +       | +                      | +            | ++          | ++      |
| Pichia farinosa Y-41         | +                      | +            | ++          | +       | +                      | +            | ++          | ++      |

* Symbols: -, no spores detected; +, few spores; ++, many spores.

Conventional methods. Wickerham, Kurtzman, and Herman (7) also reported that, up to 72 hr of incubation on YM agar, their strain of *E. lipolytica* was not able to produce ascospores though spores were observed after 96 to 120 hr of incubation. As the 24-hr-old growth from YM slant was used as inoculum in the present method, it was very unlikely that spores would be present in the inoculum.

It is well known that yeasts when grown on hydrocarbons develop numerous lipid vacuoles which may be confused with spores in unstained cells. In the present study, the positive results for ascospore formation were recorded only when the results were positive with both of the spore-staining methods used. Since lipid vacuoles and artifacts may give false results for spore formation by taking spore stain, an aspo-
rogenous hydrocarbon-utilizing yeast *Candida tropicalis* NCIM 3122, which produces numerous lipid vacuoles when grown on hydrocarbons, was stained with both spore-staining methods after cultivation for 15, 24, and 30 hr. The lipid vacuoles were not stained with either method.

Of the hydrocarbon-utilizing yeast strains reported in this communication, only two strains, Y-14 and Y-15, showed ascospore formation in association with mycelium formation on conventional ascospore-inducing solid medium (4). These strains also gave a similar type of ascospores in association with mycelium formation when grown on gas oil-containing liquid medium. Other yeast strains did not become hyphal in hydrocarbon medium.

Limitation of assimilable carbon substrate may not be the reason for the induction of spore formation in all these cases because a number of strains studied grow profusely on gas oil. It is possible that gas oil contains a spore-inducing factor. However, by using *n*-alkanes of 95 to 99% purity as carbon source, induction of ascospore formation was observed in these yeasts. The *n*-alkanes studies included *n*-decane, *n*-tetradecane, *n*-pentadecane, *n*-octadecane, and *n*-eicosane (supplied by BDH Chemicals Ltd., Poole, England). Induction of ascospores was also observed when the yeasts were cultured on other petroleum fractions such as high-speed diesel oil and white kerosene (both supplied by Assam Oil Company, Digboi, Assam, India). The possible spore-inducing factor or factors present in hydrocarbons are under investigation.

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