Optimal Conditions for the Enrichment and Isolation of Methanol-assimilating Yeasts

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INTRODUCTION

The existence of yeasts capable of utilizing methanol as the only source of carbon and energy for growth has been discovered comparatively recently by studying new isolates and screening culture collections (Ogata, Nishikawa & Ohsugi, 1969; Asthana, Humphrey & Moritz, 1971; Sahm & Wagner, 1972; Oki, Kouno, Kitai & Ozaki, 1972; Hazeu, de Bruyn & Bos, 1972; Levine & Cooney, 1973). The yeasts belonged to the genera *Kloeckera*, *Torulopsis*, *Candida*, *Pichia* and *Hansenula*.

We have isolated 36 different strains of *Candida boidinii* and *Pichia pinus* from more than 600 enrichment cultures prepared during a survey of conditions leading to the enrichment of methanol-assimilating yeasts.

METHODS

Media. These contained (per litre deionized water): $(\text{NH}_4)_2\text{SO}_4$, 0.5 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g; KH_2PO_4 , 3.0 g, and 0.2 ml of a trace element solution (Vishniac & Santer, 1957). The preferred enrichment medium contained in addition (per litre deionized water): vitamin mixture (Ogata, Nishikawa, Ohsugi & Tochikura, 1970), 10 ml; sodium penicillin G (Mycopharm, Delft, The Netherlands), 10^6 units; D-cycloserine (Sigma), 600 mg; and methanol, 5 ml. The pH was adjusted to 4.5 with NaOH. The medium was used without sterilization. The utilization of methanol by the isolated yeast strains was studied in the above solution at pH 5.5 in the absence of the antibiotics. In that case the vitamin mixture and methanol were filter-sterilized and added to the mineral medium which had been sterilized at 15 lb/in² for 15 min and cooled.

Organisms. The following yeasts were obtained from the Centraal Bureau voor Schimmelfcultures, Yeast Division, Delft, The Netherlands: *Hansenula capsulata* CBS1993, *H. non-fermentans* CBS5764, *H. polymorpha* CBS4732, *H. wickerhamii* CBS4307, *H. henricii* CBS5765, *H. glycozyma* CBS5766, *H. minuta* CBS1708, *Pichia pastoris* CBS704, *P. pinus* CBS5098, *P. trhalophila* CBS5361, *Candida boidinii* CBS2429.

These organisms were maintained on 1% Difco Malt agar and subcultured twice a year. Slopes were incubated overnight at 28 °C (except for *H. polymorpha* which was incubated at 37 °C) and then stored at 4 °C.

Enrichment and isolation. Conical flasks (100 ml) containing 30 ml of enrichment medium were inoculated with approximately 1 g of samples taken from soil, rotten wood, leaves, flowers and fruit. The flasks were put on a shaker (Model G25, New Brunswick Scientific Co.) at 28 or 37 °C. They were examined microscopically at 2-day intervals for 3 weeks. In positive enrichments growth of yeasts generally started after 5 to 8 days and after 10 days the cultures were transferred to another flask containing the enrichment medium. Growth

Table 1. *Optimal conditions for the isolation of methanol-assimilating yeasts*

Factors influencing enrichment	Optimum conditions
pH of the medium	4.5 (optimum pH for growth of yeasts on methanol is 5.0 to 6.0)
Antibiotics	Required in the physiological range of pH values for growth
Vitamin mixture	Necessary since most methanol-assimilating yeasts require one or more vitamins*
Methanol concentration	0.1 to 0.5 % (v/v). At methanol concentrations above 0.5 % inhibition of growth occurred in some species
Nature of the inoculum	Soil samples rich in organic matter were good sources

* Biotin and thiamin are especially important.

in the second flask was much faster, and 3 days after the transfer samples of the culture were plated on a modified enrichment medium without antibiotics, at pH 5.5, containing 20 g Difco Bacto-agar/l. Pure cultures of the yeasts were obtained by means of conventional techniques.

Criteria for methanol utilization. A 100 ml conical flask, containing 30 ml of the mineral salts medium + vitamins and supplemented with 0.2 % (w/v) glycerol, was inoculated from a 48 h old slope and incubated on a shaker at 28 °C (37 °C for *H. polymorpha*). When growth was apparent, 0.2 ml of the culture was transferred to another flask containing 30 ml of the modified enrichment medium at pH 5.5, containing 0.2 % (w/v) glycerol and 0.5 % (v/v) methanol. After 48 h of incubation, 0.2 ml of the culture was transferred to a similar solution containing only methanol as the carbon source. When after five subcultures in the latter medium growth was visible within 72 h, the yeast under study was considered to assimilate methanol.

Identification of the isolated yeasts. This was done by standard methods (Lodder, 1970).

RESULTS AND DISCUSSION

Preliminary work showed that at pH 4.5 penicillin G and D-cycloserine virtually prevented bacterial growth in the enrichment cultures. Yeasts were obtained from 15 to 25 % of these.

Of 36 strains eventually isolated in pure culture, 26 were identified as *P. pinus*, and 10 as *C. boidinii*. Only *C. boidinii* was isolated when $(\text{NH}_4)_2\text{SO}_4$ was replaced by 0.065 % (w/v) KNO_3 as the nitrogen source. *Pichia pinus* is unable to use nitrate as a nitrogen source for growth (Lodder, 1970). The enrichments were particularly successful when soil, rich in organic matter, from a tropical greenhouse was used as the inoculum. It is notable that when the incubation temperature during the enrichments was 37 °C no yeasts were isolated. A thermo-tolerant *Hansenula* strain has been isolated, however, by Levine & Cooney (1973) and *H. polymorpha* CBS4732 utilized methanol at 37 °C in our experiments. The optimal conditions for the isolation of methanol-assimilating yeasts are summarized in Table 1.

A number of yeast species taxonomically related to our isolates were obtained from the Delft collection. From the genera *Pichia* and *Hansenula* all the available type species were tested, whereas from the genus *Candida* only those species resembling *C. boidinii* were considered. By using the criteria outlined above the following species were found to grow with methanol as the sole source of carbon and energy: *H. capsulata*, *H. nonfermentans*, *H. polymorpha*, *H. wickerhamii*, *H. henricii*, *P. pastoris*, *P. pinus*, *P. trehalophila* and *C. boidinii*. In contrast to the results reported by Hazeu *et al.* (1972), *H. glycozyma* and *H. minuta*

failed to utilize methanol. This discrepancy may be due to the use of different criteria for methanol utilization.

All methanol-assimilating yeasts tested so far are unable to grow in batch or continuous culture with the following one-carbon compounds: methane, methylamine, formaldehyde and formate. The results obtained so far indicate that the utilization of organic C₁ compounds is not widespread among yeasts and is less common than among procaryotic organisms (Quayle, 1972).

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