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Alcoholic Fermentation by 'Non-fermentative' Yeasts*

JOHANNES P. VAN DIJKEN, EDUARD VAN DEN BOSCH, JOHN J. HERMANS, LENNART RODRIGUES DE MIRANDA† AND W. ALEXANDER SCHEFFERS†

Department of Microbiology and Enzymology, Delft University of Technology, Julianalaan 67, NL-2628 BC Delft, The Netherlands
**Centraalbureau voor Schimmelcultures, Yeast Division, Delft, The Netherlands*

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All type strains of 'non-fermentative' yeasts, available in the culture collection of the Centraalbureau voor Schimmelcultures, were reinvestigated for their capacity to ferment glucose in the classical Durham tube test. Although visible gas production was absent, nearly all strains produced significant amounts of ethanol under the test conditions. Under conditions of oxygen-limited growth, even strong alcoholic fermentation may occur in a number of yeasts hitherto considered as non-fermentative. Thus, shake-flask cultures of *Hansenula nonfermentans* and *Candida silvae* fermented more than half of the available sugar to ethanol. It is concluded that the taxonomic test for fermentation capacity, which relies on detection of gas formation in Durham tubes, is not reliable for a physiological classification of yeasts as fermentative and non-fermentative species.

KEY WORDS — Yeasts; fermentation; ethanol; Durham tube test.

INTRODUCTION

Approximately one-third of the 439 yeast species described by Barnett *et al.* (1979) are listed as non-fermentative. So far, little is known about the enzymological background of the apparent inability of this group of yeasts to convert sugars to ethanol. During a comparative study on the enzymology of facultatively fermentative and non-fermentative yeasts we noticed that a variety of yeasts, described as non-fermentative, possessed pyruvate decarboxylase (EC 4.1.1.1), the key enzyme of alcoholic fermentation. This prompted us to reinvestigate the fermentative capacities of this group of yeasts. The results described below reveal that the capacity for alcoholic fermentation may occur in many yeast species hitherto considered as non-fermentative.

MATERIALS AND METHODS

Organisms

Type strains of 148 'non-fermentative' yeast species (negative in the fermentation of D-glucose, according to Barnett *et al.* (1979)), were examined. Besides, type strains of the following taxa were investigated: *Bullera aurantiaca, B. globospora, B. grandispora, Candida hordei, Dipodascus ovetensis, Holtermannia corniformis, Rhodosporidium diobovatum, Rhodosp. sphaerocarpum, Rhodosp. toruloides, Rhodotorula glutinis* var. *dairenensis, Rh. minuta* var. *texensis, Rh. sinensis,* and *Sirobasidium magnum.* For comparative purposes *Candida utilis* CBS 621 and *Saccharomyces cerevisiae* CBS 8066 were also studied.

Standard fermentation test

Fermentation tests were performed by the established technique (Van der Walt, 1970). Cell material from 24-h-old slant cultures was used to inoculate Durham tubes containing 6 ml medium. The medium consisted of 1 per cent (w/v) yeast extract with 2 per cent (w/v) glucose. Cultures in 1 per cent yeast extract served as a blank. In addition, fermentation tests were performed under 'anaerobic' conditions in culture tubes, containing 6 ml 1 per cent yeast extract with or without 2 per cent glucose. After inoculation, the liquid was covered with a 2 cm layer of plain agar, on top of which a 2 cm layer of molten paraffin was deposited. After static incubation at 25°C for 10 days all tubes were analysed for gas production and ethanol content.

*Dedicated to Dr Nelly J. W. Kreger-van Rij.
†Author to whom correspondence should be addressed.

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Table 1. Ethanol production by 'non-fermentative' yeasts in the standard fermentation test.

Ethanol concentration after 10 days	No. of species	Examples
<0·1 g/l	25	*Hansenula nonfermentans* CBS 5764
0·1–1·0 g/l	130	*Cryptococcus laurentii* CBS 139
1·0–5·0 g/l	6	*Candida silvae* CBS 5498

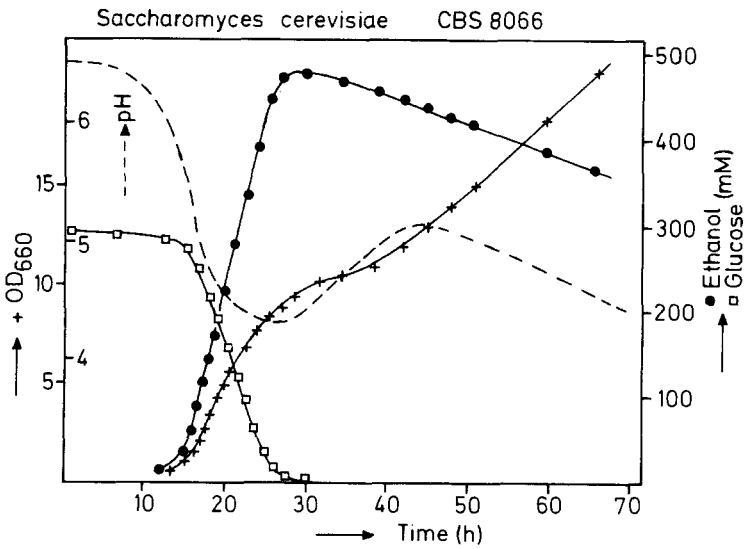

Figure 1. Growth, glucose consumption, ethanol production and pH in shake-flask culture of *Saccharomyces cerevisiae* CBS 8066.

Shake-flask cultures

For shake-flask cultures organisms were pre-grown in 100 ml shake-flasks, containing 25 ml medium consisting of 1 per cent (w/v) yeast extract with 2 per cent (w/v) glucose and incubated on a rotatory shaker at 150 rpm and 30°C for 24 h. These cultures were used to inoculate 500 ml Erlenmeyer flasks, containing 250 ml medium consisting of 1 per cent (w/v) yeast extract with 5 per cent (w/v) glucose; cultures were incubated on a rotatory shaker at 150 rpm and 30°C.

Analytical procedures

Growth was followed by measuring the optical density at 660 nm in a colorimeter (Vitatron, Dieren, The Netherlands). Glucose was assayed by the GOD-Perid method (Boehringer). Ethanol was determined either by gas chromatography as described by Toivola *et al.* (1984), or colorimetrically according to Verduyn *et al.* (1984).

Chemicals

Yeast extract was from Difco. Ingredients for the colorimetric determination of ethanol were obtained from Boehringer, except the alcohol oxidase, which was prepared according to Verduyn *et al.* (1984).

RESULTS

Apart from the 148 species listed as non-fermentative by Barnett *et al.* (1979), another 13

species described from 1979 onwards were tested for their fermentative capacity. All type strains except three (*Candida amylolenta, Candida austromarina* and *Trichosporon terrestre*) failed to develop visible gas within the incubation period. At the end of the test period, however, most yeasts had produced significant amounts of ethanol (Table 1) in open or in sealed tubes, and generally in both. The rate of ethanol production was rather low as compared to that of typical fermentative yeasts. Whereas yeasts considered as non-fermentative produced at most 5 g/l ethanol after a 10-day incubation, *Saccharomyces cerevisiae* and *Candida utilis* produced 5–10 g/l within 3 days under identical conditions.

Further studies with two selected species demonstrated that alcoholic fermentation by so-called non-fermentative yeasts can be considerable. In

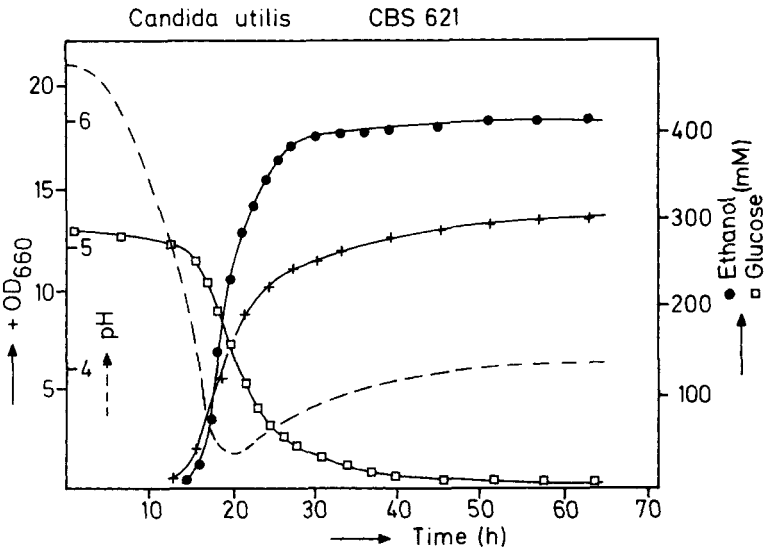

Figure 2. Growth, glucose consumption, ethanol production and pH in shake-flask culture of *Candida utilis* CBS 621.

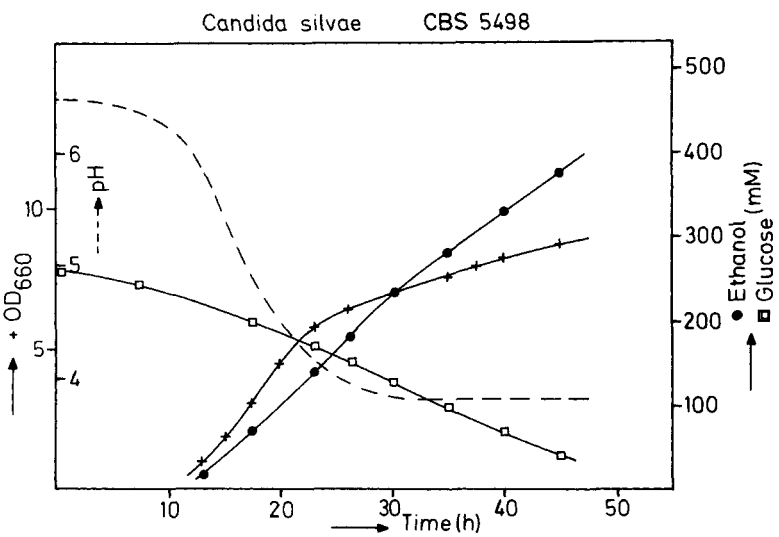

Figure 3. Growth, glucose consumption, ethanol production and pH in shake-flask culture of *Candida silvae* CBS 5498.

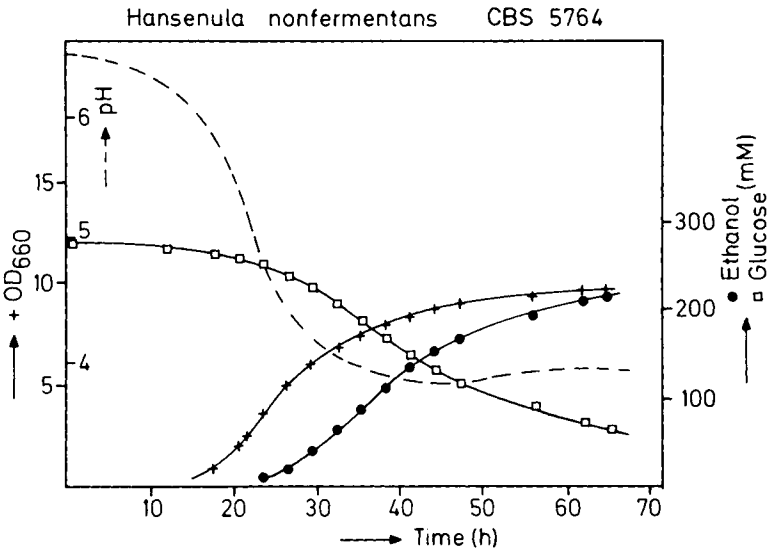

Figure 4. Growth, glucose consumption, ethanol production and pH in shake-flask culture of *Hansenula nonfermentans* CBS 5764.

Table 2. Ethanol production in shake-flask cultures of various yeasts growing on 1% (w/v) yeast extract with 5% (w/v) glucose.

Organism	Maximum rate of ethanol production (mmol/l. h)	Ethanol yield*
Saccharomyces cerevisiae CBS 8066	42	83
Candida utilis CBS 621	55	70
Candida silvae CBS 5498	12	95
Hansenula nonfermentans CBS 5764	9	53

*Amount of glucose fermented to ethanol/amount of glucose consumed × 100%.

shake-flask cultures, the 'non-fermentative' yeasts *Hansenula nonfermentans* and *Candida silvae* exhibited a pattern of growth and ethanol production similar to that of the typical facultatively fermentative yeasts *S. cerevisiae* and *C. utilis* (Figures 1–4). Both *H. nonfermentans* and *C. silvae* fermented more than half of the sugar to ethanol, albeit at a lower rate than either of the other yeasts (Table 2).

DISCUSSION

With the fermentation test commonly used for the identification of yeasts we found that most strains of 'non-fermentative' species produced significant amounts of ethanol (Table 1), despite absence of visible gas production. With respect to this apparent contradiction it should be kept in mind that 'in Durham and Einhorn tubes, which are examples of open vessels, a weak or slow fermentation may be unnoticed if the carbon dioxide diffuses out as quickly as it is formed' (Kreger-van Rij, 1962). This, of course, is particularly true for yeasts which tend to grow at the surface of static cultures, a phenomenon observed for a great number of strains in our study. Therefore, absence of gas formation is not a reliable criterion for the absence of fermentation capacity. Indeed, it has been noted before that in closed systems, such as in Warburg vessels, a slow fermentation may be observed in certain

yeasts that give negative results in Einhorn and Durham tube tests (Diddens and Lodder, 1942).

At present, there is no simple alternative for Durham tubes as a means of detecting gas formation. We noticed that even in static tube cultures, sealed with agar and paraffin, ethanol was produced by large numbers of 'non-fermentative' yeasts, although gas formation was not detectable. Apparently, the rate of ethanol and CO_2 production under these conditions is lower than the rate of CO_2 diffusion through the agar and solid paraffin. It is concluded, therefore, that the colorimetric spot test for ethanol, as developed by Verduyn *et al.* (1984), is to be preferred as a simple method for the detection of a low rate of alcoholic fermentation.

Although the absence of gas formation in the Durham tube test is of taxonomic importance, a negative outcome of this test has, as pointed out above, no decisive physiological significance. Moreover, it should be realized that the amount of ethanol produced in this test is of restricted significance only. This is evident from our results with *H. nonfermentans*. This yeast produced negligible amounts of ethanol in the standard fermentation test (Table 1) but exhibited appreciable alcoholic fermentation in shake-flask cultures (Figure 4). Further studies are required to determine whether, and to what extent, other typical oxidative yeasts are capable of alcoholic fermentation under appropriate conditions.

Our results further confirm that the occurrence and intensity of alcoholic fermentation strongly depend on cultivation conditions. It is well known that *S. cerevisiae* exhibits a Crabtree effect: performance of alcoholic fermentation under strictly aerobic conditions in the presence of excess glucose (Fiechter *et al.*, 1983). This phenomenon is known to be absent in *C. utilis*. It must be concluded, therefore, that the almost identical behaviour of *S. cerevisiae* and *C. utilis* in shake-flask cultures (Figures 1 and 2) is due to oxygen limitation. Limited oxygen supply is known to enhance alcoholic fermentation in *C. utilis* (Moss *et al.*,1969; van Dijken and Scheffers, 1986). The same apparently holds for *H. nonfermentans* and *C. silvae*, as in shake-flask cultures ethanol production was considerable, whereas in well-aerated fermenter cultures alcoholic fermentation was absent (J. P. van Dijken *et al.*, unpublished results). Therefore, with respect to the regulation of alcoholic fermentation, *H. nonfermentans* and *C. silvae*, like *C. utilis*, fall into the class of Crabtree-negative, facultatively fermentative yeasts.

The present studies were restricted to tests with glucose as the fermentable substrate for 'non-fermentative' yeasts. According to the Kluyver rule, a yeast that does not ferment glucose does not ferment any sugar (Kluyver, 1914). From the data presented above it will be clear that a more detailed evaluation of the fermentative capacities of these 'non-fermentative' yeasts is required, not only with respect to glucose fermentation and its regulation, but also with respect to fermentation of other sugars.

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