APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1984, p. 265–269
0099-2240/84/080265-05$02.00/0

Vol. 48, No. 2

# Fermentation of Cellodextrins by Different Yeast Strains

PIERRE GONDÉ, BRUNO BLONDIN, MARC LECLERC, ROBERT RATOMAHENINA, ALAIN ARNAUD, AND PIERRE GALZY*

*Chaire de Génétique et Microbiologie ENSA-INRA, 34060 Montpellier Cedex, France*

Received 8 November 1983/Accepted 15 May 1984

**The fermentation of cellodextrins by eight yeast species capable of fermenting cellobiose was monitored. Only two of these species, *Torulopsis molischiana* and *T. wickerhamii*, were able to ferment β-glucosides with a degree of polymerization between one and six. These two species showed exocellular β-glucosidase activity. Four other species were able to ferment cellotriose, and the last two species only fermented cellobiose. These latter six species produced a β-glucosidase capable of attacking cellodextrins, but this enzyme was endocellular.**

In a previous work (1, 5), we selected eight yeast species capable of fermenting cellobiose. Since then, another species, *Torulopsis molischiana* (Lodder), was selected. In the present work, we attempted to test the fermentation by these strains of soluble cellodextrins with a degree of polymerization (DP) between three and six. A similar study has already been performed by Freer and Detroy (3) with *Candida lusitaniae* and *T. wickerhamii*. *T. wickerhamii* was able to ferment soluble cellodextrins, whereas *C. lusitaniae* could only ferment glucose and cellobiose. These authors proposed the hypothesis that the excretion of β-glucosidase into the fermentation medium was responsible for the special aptitude of *T. wickerhamii*.

## MATERIALS AND METHODS

**Cellodextrin preparation.** We prepared the cellodextrins by the modified methods of Miller et al. (8), Huebner et al. (7), and Freer and Detroy (3). Cellulose (50 g; Sigmacell) was suspended in ice-cold fuming HCl (500 ml). This suspension was warmed up to 25°C and kept at this temperature with agitation so that hydrolysis would occur. After 2 to 2.5 h, HCl was partially removed under vacuum for 30 min while agitation was maintained. The soluble and insoluble cellodextrins were precipitated by the addition of 8 volumes of ice-cold ethanol (95% [vol/vol]). After being cooled for 2 h at −15°C, the precipitate was collected by centrifugation at 14,000 × *g* and washed three times with ice-cold ethanol. This precipitate was then suspended in 800 ml of deionized water, previously warmed to 50°C, and centrifuged. Insoluble cellodextrins were precipitated out, and soluble cellodextrins were kept in an ice-cold beaker. Once the solution was cold, it was adjusted to pH 4 to 5 by the slow addition of Dowex 2X-8-200 (Sigma Chemical Co.) ion-exchange resin that had been converted to the hydroxide form. The addition of the resin had to be performed slowly and under ice-cold conditions to avoid the formation of rearrangement products by localized heating effects. These by-products are toxic to yeast cells.

The cellodextrin preparation was concentrated with a rotary evaporator at 60°C. The evaporation also eliminated residual ethanol. The preparation was then filtered through a cellulose-acetate membrane (0.45-μm pore size; Millipore Corp.). The final preparation contained cellobiose, through cellohexaose, but very little glucose. It was possible to assay these β-glucosides (DP, two to six) by high-pressure liquid chromatography. Celloheptaose is soluble but was not detected by high-pressure liquid chromatography; it was probably present in our preparations. The total cellodextrin content was determined by weighing the dry matter obtained.

**Organisms.** The yeasts used are shown in Table 1.

**Culture conditions.** The basal culture medium used was G medium (4), which contained the following: distilled water (cellodextrin suspension), 1,600 ml; minerals, 200 ml; oligoelements (boric acid, copper sulfate, potassium iodide, manganese sulfate, sodium molybdate, zinc sulfate), 2 ml; vitamins, 10 ml; and ferric chloride, 2 ml. We also added yeast extract (Difco Laboratories) at a final concentration of 0.3% and ergosterol (final concentration, 0.05 g/liter) dissolved in Tween 80 (final concentration, 8.5 g/liter). The final cellodextrin concentration was 22.5 g/liter. The other fermentation conditions were identical to those previously described (1, 5). The fermentation mixtures were inoculated with a 24-h culture grown on yeast extract-cellobiose (0.5%). Enzymatic studies were performed on 24-h cultures grown on the same medium.

**Enzymatic studies.** Acellular preparations were made and enzyme assays were performed by techniques previously described (2). β-glucosidase activity was assayed with 4-nitrophenyl-β-D-glucopyranoside as the substrate. Activity was determined by adding 0.1 ml of enzyme solution to 4.9 ml of citrate-phosphate buffer (0.1 M; pH 5.0) containing 4-nitrophenyl-β-D-glucopyranoside (final concentration, $0.5 \times 10^{-2}$ M). The reaction mixture was incubated at 30°C. Aliquots (0.5 ml) were taken at regular intervals and added to 1.0 ml of carbonate buffer (0.2 M; pH 10.2). Liberated *p*-nitrophenol in this mixture was assayed by spectrophotometry at 400 nm. The molar extinction coefficient used was $\varepsilon = 18.300\ M^{-1}\ cm^{-1}$. One β-glucosidase activity unit was defined as the quantity of enzyme required for the hydrolysis of one micromole of substrate (4-nitrophenyl-β-D-glucopyranoside) per minute under the experimental conditions described above. Hydrolysis of cellodextrins was carried out by mixing the cellodextrin solution with an enzyme solution in citrate-phosphate buffer (0.1 M; pH 5.0). Samples were taken every 24 h and assayed by high-pressure liquid chromatography.

**Analytical methods.** Cell growth was monitored by direct count with a Thoma counting chamber. Ethanol was assayed by gas-solid chromatography with a Girdel 300 chromatograph, with propanol as the internal standard. Sugars were assayed by refractometric high pressure liquid chromatography with a Waters apparatus (carbohydrate column).

* Corresponding author.

TABLE 1. Yeasts used

| Yeast | Source | Strain |
|---|---|---|
| *Brettanomyces anomalus* | M. T. J. Custers | CBS 77 |
| *Brettanomyces clausenii* | M. T. J. Custers | CBS 76 |
| *Brettanomyces custersii* | G. Florenzano | CBS 5512 |
| *Brettanomyces intermedius* | J. P. Van der Walt and A. E. Van Kerken | CBS 73 |
| *Candida obtusa* | N. Van Udden and L. Do Carmo-Sousa | CBS 1944 |
| *Dekkera intermedia* | J. P. Van der Walt | CBS 2796 |
| *Torulopsis molischiana* | J. Lodder | CBS 136 |
| *Torulopsis wickerhamii* | A. Capriotti | CBS 2928 |

## RESULTS

**Fermentation of cellodextrins.** The fermentative capacities of the yeast are shown in Fig. 1–4. Three different types of behavior of these yeasts in the presence of cellodextrins under anaerobic conditions were observed.

**(i) Yeasts fermenting only glucose and cellobiose (Fig. 1).** Both *Candida obtusa* and *Brettanomyces anomalus* produced 0.32 g of ethanol per liter by 16 h. Only glucose and cellobiose were consumed, even after 7 days. The production of alcohol corresponded to known yields from these yeasts (5) for the fermentation of glucose and cellobiose.

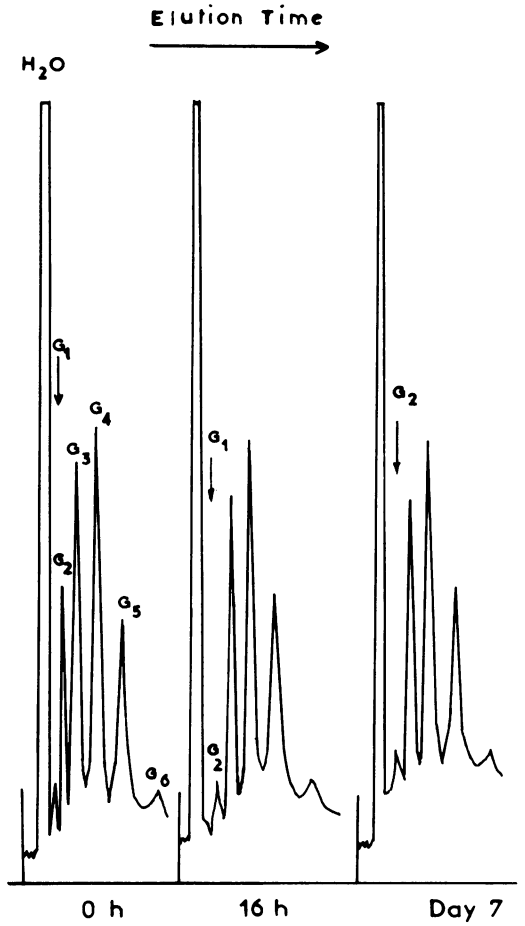


FIG. 1. Residual sugars present during the fermentation of cellodextrins (22.5 g/liter) by *B. anomalus* and *C. obtusa*.

**(ii) Yeasts fermenting glucose, cellobiose, and cellotriose (Fig. 2).** *Brettanomyces clausenii*, *Brettanomyces custersii*, *Brettanomyces intermedius*, and *Dekkera intermedia* produced, respectively, 0.88, 0.48, 0.72, and 0.56 g of ethanol per liter by 50 h. Again, other sugars were not utilized, even after 7 days.

**(iii) Yeasts fermenting soluble cellodextrins (Fig. 3–5).** *T. molischiana* and *T. wickerhamii* fermented sugars with a DP of one to six. The attack on cellodextrins by *T. molischiana* was rapid. After 19 h, the quantity of sugars with a DP of three to six had decreased, whereas the concentration of cellobiose had increased slightly. Glucose had disappeared completely. By 44 h, practically only cellobiose had remained. By 52 h, all sugars had disappeared. This species produced 8.8 g of ethanol per liter from 22.5 g of cellodextrins initially present per liter. The fermentation of cellodextrins by *T. molischiana* was faster and produced a better yield than that by *T. wickerhamii*. The latter produced only 6.4 g of ethanol per liter from 22.5 g of cellodextrins per liter after 65 h of fermentation. The attack also seemed different, as by 19 h, the concentrations of both cellobiose and cellotriose had increased. When both yeasts were incubated on glucose (200 g/liter), the ethanol yields were 95% for *T. molischiana* and 80% for *T. wickerhamii*.

**Localization of the β-glucosidase.** The results of β-glucosidase assays are summarized in Table 2. Again, we found different results for different yeasts. The two *Torulopsis* spp., which fermented cellodextrins, showed exocellular β-glucosidase activity, whereas the other yeasts only showed intracellular β-glucosidase activity.

**Attack on cellodextrins by intracellular enzymes.** We mixed cellodextrin solutions with cellular enzyme preparations from the six yeasts which did not ferment cellodextrins. The qualitative disappearance of sugars with a DP above two was monitored. The cellodextrins thus treated were attacked by the endocellular β-glucosidases of these six yeasts.

## DISCUSSION

The results presented above show that the limiting factor for the fermentation of cellodextrins by these yeasts was the contact between sugars and β-glucosidase. The yeast β-glucosidases studied here were able to hydrolyze soluble cellodextrins. Two species were able to excrete their β-glucosidases into the medium and, thus, could ferment soluble cellodextrins. The other strains behaved differently. Some strains (*B. clausenii*, *B. custersii*, *B. intermedius*, and *D. intermedia*) only allowed the penetration of cellobiose and cellotriose. It is noteworthy that these yeasts all belong to the physiologically related *Dekkera-Brettanomyces* group. This property is not, however, a taxonomic feature, as *B. anomalus*, like *C. obtusa*, allowed the penetration only of cellobiose and fermented only this sugar.

The total hydrolysis of cellulose can be achieved either by chemical or enzymatic means. Chemical hydrolysis is at present the most commonly used and fastest method. This technique, however, requires high pressure and temperature conditions and also produces by-products that are toxic to microorganisms. The fermentation of cellodextrins by yeasts increases the possibilities of use of cellulose residues, as this technique is a much milder method for the hydrolysis of cellulose. However, the results reported here only represent the beginning of a project for genetically improving strains and of a more complete enzymatic study, especially of the repression of β-glucosidases by glucose, which was observed during the fermentation of the glucose-cellobiose mixture (5).

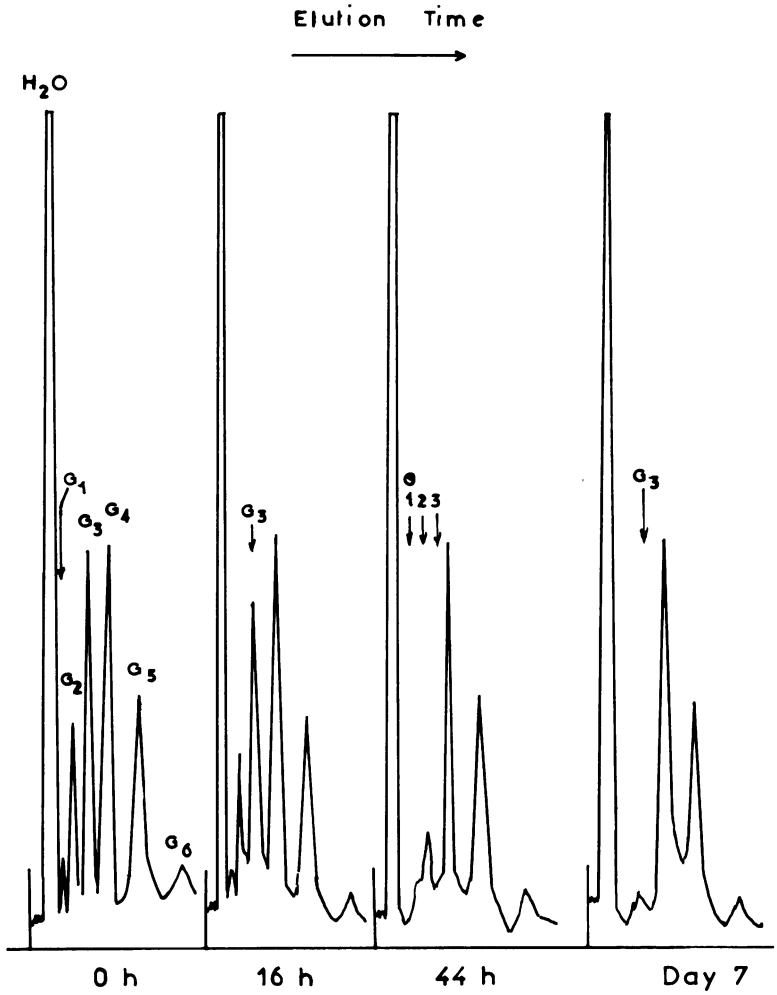


FIG. 2. Residual sugars present during the fermentation of cellodextrins (22.5 g/liter) by *B. clausenii*, *B. custersii*, *B. intermedius*, and *D. intermedia*.

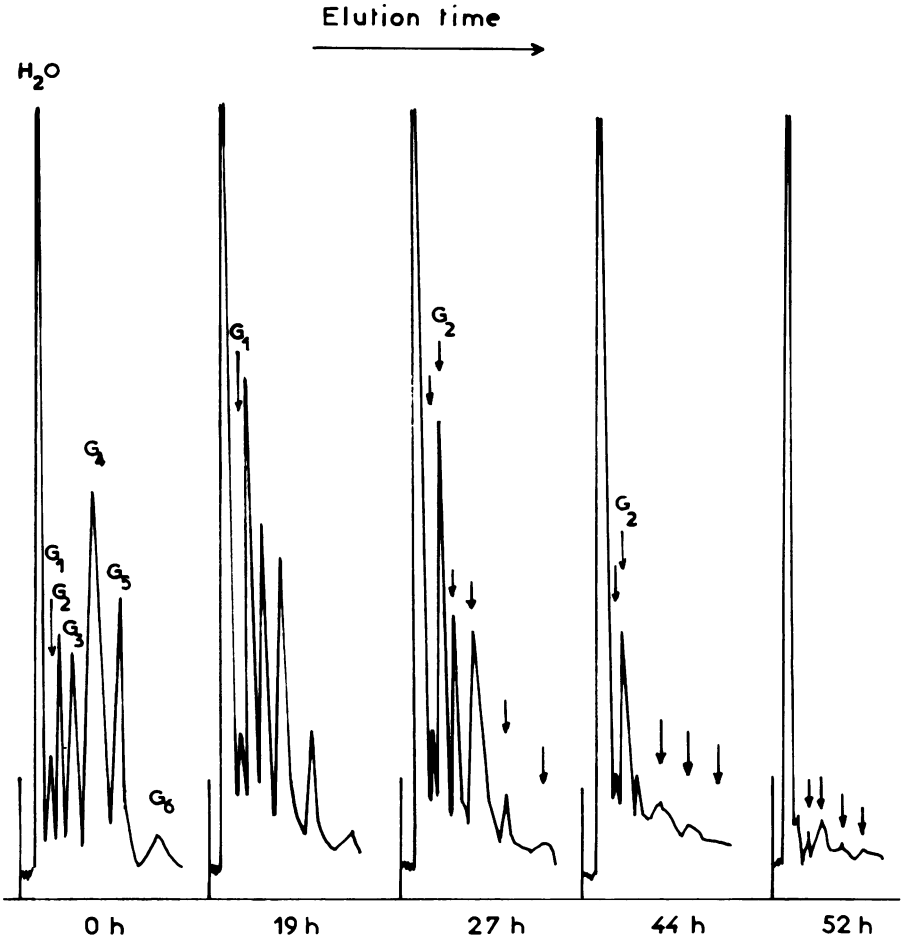


FIG. 3. Residual sugars present during the fermentation of cellodextrins (22.5 g/liter) by *T. molischiana*.

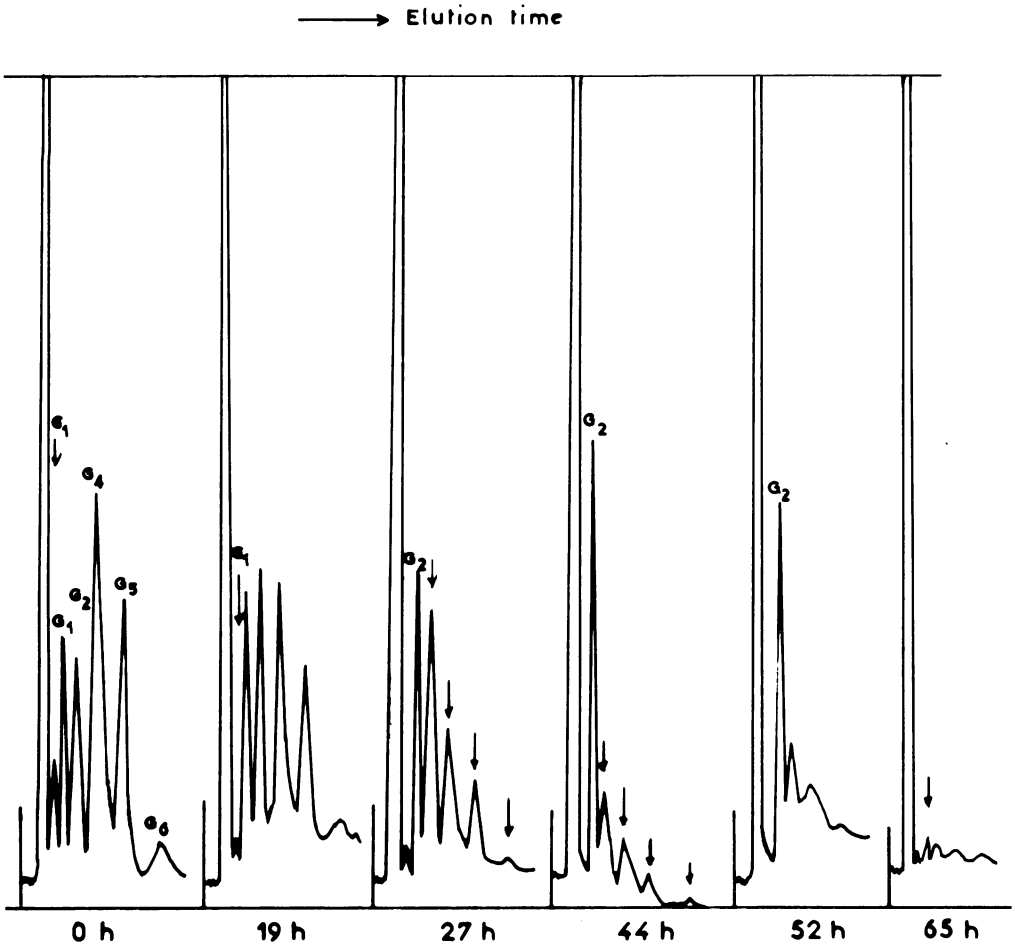


FIG. 4. Residual sugars present during the fermentation of cellodextrins (22.5 g/liter) by *T. wickerhamii*.

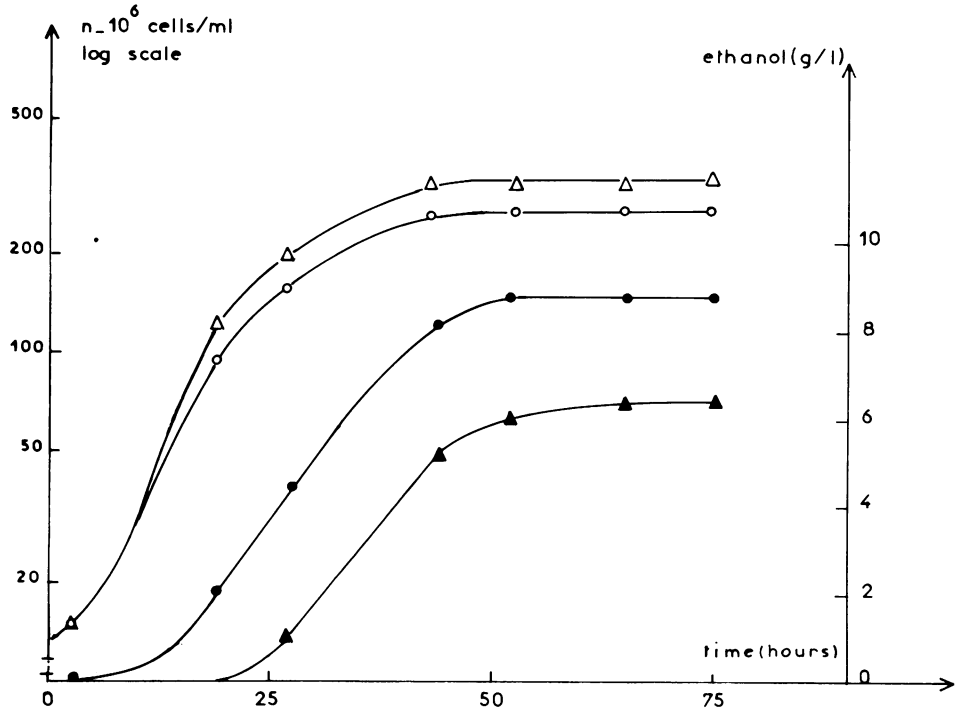


FIG. 5. Growth of and alcohol production by *T. molischiana* and *T. wickerhamii* over time. Symbols: ○, growth of *T. molischiana*; △, growth of *T. wickerhamii*; ●, ethanol production by *T. molischiana*; ▲, ethanol production by *T. wickerhamii*.

TABLE 2. Localization of β-glucosidase in the eight yeasts studied

| Yeast | β-Glucosidase activity[a] | |
|---|---|---|
| | Exocellular | Intracellular |
| *Brettanomyces anomalus* | − | + |
| *Candida obtusa* | − | + |
| *Brettanomyces clausenii* | − | + |
| *Brettanomyces custersii* | − | + |
| *Brettanomyces intermedius* | − | + |
| *Dekkera intermedia* | − | + |
| *Torulopsis molischiana* | + | + |
| *Torulopsis wickerhamii* | + | + |

[a] +, β-Glucosidase activity was found in the supernatant after centrifugation or in acellular preparations. −, No β-glucosidase activity was found.

## ACKNOWLEDGMENTS

We are grateful to P. Chemardin for technical assistance.

This work was supported by grants from Agence Française pour la Maitrise de l'Energie, INRA, and Ministère de l'Industrie et de la Recherche.

## LITERATURE CITED

1. **Blondin, B., R. Ratomahenina, A. Arnaud, and P. Galzy.** 1982. A study of cellobiose fermentation by a *Dekkera* strain. Biotechnol. Bioeng. **24**:2031–2037.
2. **Blondin, B., R. Ratomahenina, A. Arnaud, and P. Galzy.** 1983. Purification and properties of the β-glucosidase of a yeast capable of fermenting cellobiose to ethanol: *Dekkera intermedia* Van der Walt. Eur. J. Appl. Microbiol. Biotechnol. **17**:1–6.
3. **Freer, S. N., and R. W. Detroy.** 1982. Direct fermentation of cellodextrins to ethanol by *Candida wickerhamii* and *C. lusitaniae*. Biotechnol. Lett. **4**:453–458.
4. **Galzy, P., and P. P. Slonimski.** 1957. Variations physiologiques de la levure au cours de la croissance sur l'acide lactique comme seule source de carbone. C.R. Acad. Sci. **245**:2423–2426.
5. **Gondé, P., B. Blondin, R. Ratomahenina, A. Arnaud, and P. Galzy.** 1982. Selection of yeast strains for cellobiose alcoholic fermentation. J. Ferment. Technol. **60**:579–584.
6. **Hsu, T. A., G. S. Gong, and G. T. Tsao.** 1980. Kinetic study of cellodextrin hydrolysis by exocellulase from *Trichoderma reesei*. Biotechnol. Bioeng. **22**:2305–2320.
7. **Huebner, A., M. R. Ladisch, and G. T. Tsao.** 1978. Preparation of cellodextrins: an engineering approach. Biotechnol. Bioeng. **20**:1669–1677.
8. **Miller, G. L., J. Dean, and R. Blum.** 1960. A study of methods for preparing oligosaccharides from cellulose. Arch. Biochem. Biophys. **91**:21–26.