Appl. Biochem. Biotechnol.

0273-2289/91/27(1): 65–74/$2.00

Isolation of Acetic Acid-Tolerant Baker's Yeast Variants in a Turbidostat

T. H. AARNIO, M.-L. SUIHKO,* AND V. S. KAUPPINEN
VTT, Biotechnical Laboratory, SF-02150 Espoo, Finland

Received January 19, 1990; Accepted February 28, 1990

ABSTRACT

A commercial baker's yeast was subjected to selection in a continuous turbidostat cultivation with increasing concentration of acetic acid. The final acetic acid content in fresh medium was 0.6% or 0.8% v/v. Two of seven selected variants were stable over 15 sequential shake flask cultivations without selection pressure. After laboratory scale production of baker's yeast, one of the variants also showed increased acetic acid tolerance in sour dough. The overall raising power (mL CO_2/h) in sour dough was improved 36%.

Index Entries: Turbidostat; baker's yeast; acetic acid tolerance; *Saccharomyces cerevisiae*.

INTRODUCTION

Systems of continuous cultivation, i.e. chemostats and turbidostats, have been used to study effects of different growth parameters, for selective enrichment of microorganisms with specific characteristics and for the isolation of spontaneously occurring genetic mutants (*1–4*). Chemostats are based on the limitation of some growth factor, whereas the operating principle of a turbidostat is maintenance of a constant vol of culture at a constant microbial population density (*5*). These methods have been used successfully for the isolation of yeast variants with altered ethanol tolerance (*6,7*), acid tolerance (*8*), xylose fermention (*9*) and for studying variants of temperature transitions (*10*).

*Author to whom all correspondence and reprint requests should be addressed.

The mechanism of acetic acid inhibition of ethanol fermentation by yeast has proved to be rather complex (*11–13*). Inhibition is initiated by rapid penetration of acetic acid into the yeast cells, where the dissociated acetate anion acts as an inhibitor by decreasing the activity of glucanolytic enzymes (*14*).

A baker's yeast with improved acetic acid tolerance would improve the economy of sour bread production. Furthermore, the yeastlike off-flavor, on account of the large amounts of commercial baker's yeast nowadays needed, would be avoided. The use of large amounts of yeast is currently necessary as a result of the poor acetic acid tolerance of commercial baker's yeast strains of *Saccharomyces cerevisiae* (*15,16*). Recombinant DNA methods cannot be used for improvement of this property, because acetic acid tolerance is controlled by several unidentified genes.

The aim of this study was to improve the acetic acid tolerance of an industrial baker's yeast *Saccharomyces cerevisiae* by selection in a turbidostat.

MATERIALS AND METHODS

Microorganisms and Media

The commercial baker's yeast strain *Saccharomyces cerevisiae* VTT-B-77044 (hereafter B44) and the sour dough yeast *Candida holmii* VTT-C-81116 (hereafter C116) were from the VTT Collection of Industrial Microorganisms.

YPD medium (1% w/v Difco yeast extract, 2% w/v Difco peptone, 2% w/v glucose) was used for shake flask and turbidostat cultivations. The final turbidostat cultures were plated on YPD agar plates containing 0% (YPDA), 4% (YPDA4), or 5% w/v (YPDA5) sodium acetate (with corresponding to 0%, 2.9%, or 3.6% w/v acetate, respectively). These two sodium acetate concentrations were chosen because the absolute acetic acid tolerance of baker's yeast lies between these values. Sodium acetate was added instead of acetic acid, that prevented agar solidification.

Turbidostat Cultivation

The inoculum was prepared by pipeting 100 μL of preinoculum (cultivated in 25 mL of Difco YM broth, 25°C, 3 d, 175 rpm) to 100 mL of YPD medium and incubating overnight at 30°C and 250 rpm. 60 mL of this culture was used to inoculate the turbidostat cultivations.

Experiments were carried out in a glass cultivation vessel (Kluyver-flask), constructed from a 1000 mL Erlenmeyer flask (Fig. 1). The working volume was 660 mL. The vessel was placed into a water bath at 30°C and supplied with a gas mixture (5% v/v oxygen in nitrogen) or air at a mean flow rate of 400 mL/min. Gas bubbles were used for both aeration and stirring of the culture. The pH and the growth of the culture were monitored continuously by a pH electrode with a continuous flow chamber (Ingold, Switzerland) and a flow-through cell in the Ratio/XR turbidimeter

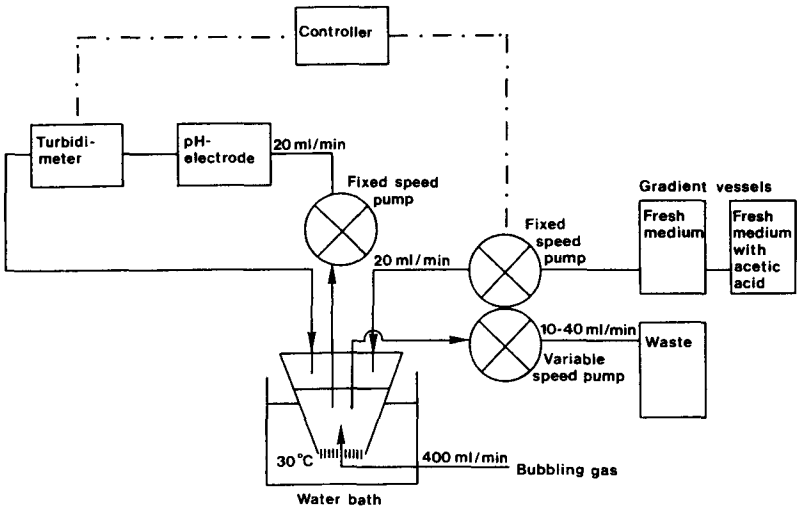

Fig. 1. Schematic illustration of the turbidostat cultivation system. Process line (——) and instrument line (—·—).

(Hach Co, USA), respectively. When the turbidity of the culture exceeded the set-point, the controller (West 2073; Gulton Industries Inc, USA) switched on a medium feed pump (Watson-Marlow 302SM/R; Smith and Nephew Pharmaceuticals Ltd, UK). Fresh medium was added at a mean flow rate of 20 mL/min until the turbidity of the culture decreased to the set-point 500 NTU (Nephelometric Turbidity Unit), corresponding to the middle stage of exponential growth.

The acetic acid concentration of the fresh medium was increased linearly by a gradient from 0% v/v to a final concentration of 0.6% or 0.8% v/v acetic acid, corresponding to pH values of 4.2 and 4.0, respectively. These pH values were chosen, because the pH of sour dough seed is normally 3.5–3.9 and the pH of sour dough lies between 4.2 and 4.5. The initial volume of fresh medium was 10 L. Half of it contained no acetic acid, whereas the other half contained the final acetic acid concentration (Fig. 1). No diffusion between these two gradient vessels was observed. The volume of the culture was maintained at a constant level by a pump-driven overflow. Samples were removed aseptically through a sampling port at the top of the vessel. The final cultures were plated on YPDA, YPDA4, and YPDA5 plates. Colonies growing on YPDA4 and YPDA5 plates were transferred to sugar wort slants containing 4% or 5% w/v sodium acetate as appropriate.

Acetic Acid Tolerance Tests

The isolated variants were tested in shake flask cultivations (50 mL of YPD medium, 30°C, 250 rpm, 12 h) at different acetic acid concentrations. Growth was defined as maximal specific growth rates, $\mu = (1/dt)$ (ln X_2/X_1), where t = time, h; X = cell dry wt, g/L.

Stability Tests

The yeast variants growing on sugar wort agar with 5% w/v sodium acetate were transferred to 100 mL of YPD medium with and without 0.6% v/v acetic acid and cultivated for 48 h. One mL of the culture was transferred to 100 mL of corresponding fresh medium and cultivated for 24 h. This cultivation cycle was repeated 15 times. The acetic acid tolerance of growing yeast cells was monitored by plating them on YPDA, YPDA4, and YPDA5 after the 1st, 6th, 10th, and 15th cultivation cycles. Plates were incubated for 5–7 d.

Baker's Yeast Production in Laboratory Scale

Yeast production was carried out by simulating the different production stages in the industrial production of baker's yeast. The method developed and used at VTT has been described in detail earlier (*15*). In this work, however, the process was shortened and instead of 3 only 1 aerated stage was used (*17*). This aerobic "commercial" stage was carreid out in an Escher-Wyss fermenter J100 equipped with an effective inferator aerator. In batch-fed cultivation, beet molasses was used as substrate and the final volme was 11–12 L. Growth was monitored by determining the dry weight of the culture. The yeast yield (kg compressed yeast, d.w. 27%) was calculated on the basis of the sugar (kg) used. The relative yields were calculated as percentages of the yield of the original baker's yeast strain.

Dough Raising Power Tests

Dough raising properties of the yeasts produced were determined using an SJA-fermentograph (*18*). In sour doughs, the added ingredients were 1.5 mL of 88% v/v lactic acid (BDH, Chemicals Ltd, UK) and 0.5 mL of glacial acetic acid (Merck, FRG). The relative raising powers were calculated as percentages of the raising power of the original baker's yeast strain. The acetic acid tolerance, however, was calculated as the raising power in sour dough expressed as a percentage of that in normal dough by the same yeast.

Determination of Ethanol and Acetic Acid

Ethanol was determined using an ethyl alcohol kit based on alcohol dehydrogenase (Boehringer Mannheim, FRG). Acetic acid was measured using an acetic acid kit based on malate dehydrogenase/citrate synthase/acetyl-CoA-synthetase (Boehringer Mannheim, FRG).

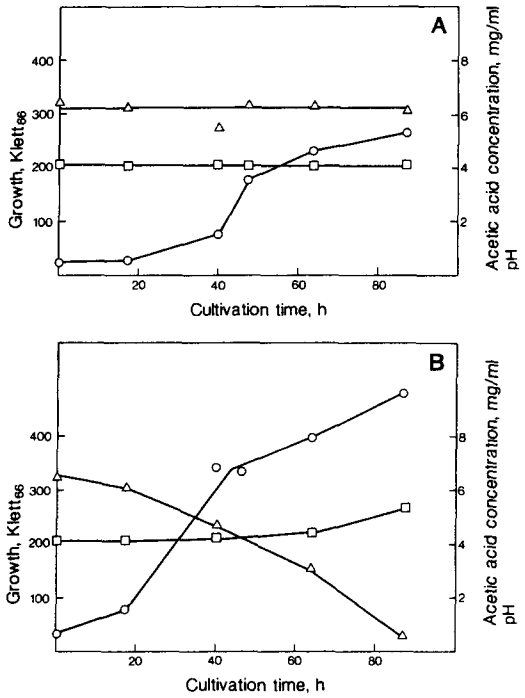

Fig. 2. Effect of 0.6% v/v acetic acid on the cultivation of baker's yeast (A) and sour dough yeast (B) in shake flasks. Acetic acid (△), growth (○), and pH (□).

RESULTS

Utilization of Acetic Acid

The effect of acetic acid in the cultivation medium was studied by monitoring pH, turbidity (growth) and acetic acid content of the broth during shake flask cultivations of the baker's yeast and sour dough yeast (100 mL of YPD medium with 0.6% v/v acetic acid) (Fig. 2). Baker's yeast was not able to utilize the added acetic acid, the pH remained constant and growth was considerably inhibited (Fig. 2a). By contrast the sour dough yeast immediately began to consume the added acetic acid and growth was initiated normally (Fig. 2b). The pH of the broth increased slightly during the cultivation.

Turbidostat Cultivation

A total of seven turbidostat cultivations were carried out, five with 0.6% (I, II, IV, V, and VI) and two with 0.8% v/v acetic acid (III and VII).

Table 1
Maximal Specific Growth Rate
of the Original Yeast Strain (B44), Sour Dough Yeast (C116)
and Isolated Variants (I-VI) at Different Acetic Acid Concentrations

Yeast	Acetic acid concentration, % v/v				
	0	0.4	0.5	0.6	0.8
B44	0.42	0	0	0	0
C116	0.41	0.35	0.26	0.18	nd
I	0.43	0.25	0.22	0.17	nd
II	0.38	0.31	0.25	0	nd
III	0.37	0.29	0.25	0	0
IV	0.41	0.27	0.18	0.17	0.12
V	0.41	0.23	0.21	0.16	0
VI	0.43	0.28	0.25	0.23	0

nd = not determined.
0 = no growth during the first 8 h.

The initial pH was 5.8. Except for cultivation III, growth was intensive in the beginning of cultivation. During the first 18 h, the pH decreased to 4.2, the average acetic acid concentration was 3.3 mg/mL, and on average 5 L of medium was used. During the subsequent 25-49 h, the remainder of the medium (about 5 L) was used, but the pH decreased only by 0.1–0.2 units and the acetic acid concentration increased on average by 1.5 mg/mL. Ethanol concentration remained constant at about 5.2 mg/mL throughout the cultivation.

Acetic Acid Tolerance of Variants

Without acetic acid the isolated variants (I-VI) and the original baker's yeast grew equally well (Table 1). Furthermore, the acetic acid tolerance of the variants was equal with that of the sour dough yeast C116. Variant VII did not grow during the first 8 h, but grew at all concentrations after 24 h (not shown in the Table 1). All the isolated variants were able to grow at 0.5% v/v acetic acid, suggesting that penetration of acetic acid through the cell membrane or acetate metabolism in cell had been altered. Four variants (I, VI, V, and VI) tolerated 0.6% v/v and only one (IV) 0.8% v/v acetic acid.

Stability of the Variants

The original yeast strain, B44, grew only without acetic acid (Table 2). The variants did not lose their acetic acid tolerance during the recycling without acetic acid. Except for the variant III, growth on the agar plates

Table 2
Growth of the Original Baker's Yeast Strain (B44) and Isolated Variants (I–VII) Plated on Agar Media with 4% w/v (YPDA4) or 5% w/v (YPDA5) Sodium Acetate After 15 Sequential Shake Flask Cultivations without and with 0.6% v/v Acetic Acid

Yeast	Shake flask		Percentage* colony count on	
	Acetic acid % v/v	Final $Klett_{66}$	YPDA4	YPDA5
B44	0	415	0	0
	0.6	31	0	0
I	0	405	100	100
	0.6	14	0	0
II	0	410	100	100
	0.6	216	100	100
III	0	400	36	19
	0.6	230	79	79
IV	0	405	100	86
	0.6	212	77	100
V	0	405	100	92
	0.6	195	72	3
VI	0	400	100	69
	0.6	200	92	36
VII	0	385	100	98
	0.6	208	86	80

*Calculated as a percentage of the colony count on control plates (YPDA).

containing 4% v/v acetate was as good as that on the control plates without acetate. All the variants except for I were able to grow in the presence of 0.6% v/v acetic acid. The variants III, V, VI, and VII, however, gradually lost their viability and acetic acid tolerance. The two most stable variants II and IV were tested further in a laboratory scale baker's yeast production process.

Yeast Yield and Dough Raising Powers

Variant II reverted to normal baker's yeast, whereas variant IV showed increased acetic acid tolerance from 28 to 51% (Fig. 3). The yeast yield of variant IV, however, was decreased by 15% and the raising power in normal dough by 25%. It appears that the production process would require adjustment when variants of this type are used. The overall rising power (mL CO_2/h) in sour dough was improved by as much as 36% compared with that of original baker's yeast. Thus, 36% less yeast would raise the dough within the same standard time used in bakeries. The use of acetic acid selection pressure (initial concentration 0.6% v/v; final 0.02% v/v) during the "commercial" cultivation stage did not further improve these

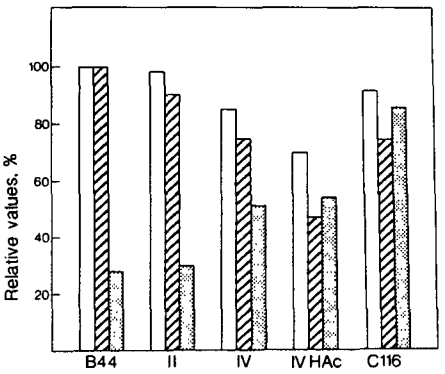

Fig. 3. Yeast yields and dough raising powers of baker's yeast (B44), turbidostat variants (II and IV) and sour dough yeast (C116) produced in a laboratory scale process. Yeast yield (□), raising power in normal dough (▨), and acetic acid tolerance (▩).

results (Fig. 3, IVHAc). In fact, the yeast yield and raising power were decreased further, whereas the acetic acid tolerance was not improved. For comparison the corresponding values of the sour dough yeast C116 are also presented. Because the process was optimized for baker's yeast, the yield of sour dough was slighly lower than that of the baker's yeast (Fig. 3). In these experiments, in which no glucose or sucrose was added to the doughs, the raising power in normal dough was significantly lower than that of the baker's yeast. This may be due to the inability of sour dough yeast to utilize maltose, the main sugar in flour. However, the raising power in sour dough was at least as good as that in normal dough, or even slightly stimulated.

DISCUSSION

Our results indicate that sour dough yeast is able to metabolize acetic acid efficiently, whereas baker's yeast cannot. However, during turbidostat selection the acetic acid metabolism of the original baker's yeast was altered. The measured acetic acid concentrations at the end of the turbidostat cultivations were lower than the calculated values (no evaporation was detected). Furthermore, the pH of the broth remained almost constant throughout the cultivation even though fresh medium with a higher acetic acid concentration was added intermittently. In this work, relatively short cultivation times (on average 3 d) were used and the changes in the acetic acid metabolism may therefore have been caused by temporary adaptation rather than permanent genetic mutation. Because one of seven cultivations gave the desired variant, the method can be considered efficient. Longer selection time in the turbidostat, e.g., one month, and the use of a mutagenic agent, e.g, NTG, in the cultivation medium would probably increase the mutation frequency and the stability of isolated variants or mutants.

In comparison with electrofusion (*19*), turbidostat cultivation, which is an old technique, is an effective, simple, and applicable method for the selection of acetic acid-tolerant yeast variants. Once constructed it is easy to handle and to modify. The probability of obtaining a permanently stable baker's yeast strain is much higher in turbidostat selection than in intergeneric protoplast fusion.

ACKNOWLEDGMENTS

This research was financed jointly by Oy Lahden Polttimo Ab and the Technical Research Centre of Finland (VTT). Anja Pallas and Taina Karhunen are warmly thanked for skillful technical assistance.

REFERENCES

1. Munson, R. J. (1970), in *Methods in Microbiology*, Vol. 2., Norris, J. R., and Ribbons, D. W., eds., Academic Press, London and New York, pp. 349–376.
2. Watson, T. G. (1972), *J. Appl. Chem. Biotechnol.* **22,** 229–243.
3. Harder, W., Kuenen, J. G., and Matin, A. (1977), *J. Appl. Bacteriol.* **43,** 1–24.
4. Dykhuizen, D. E. and Hartl, D. L. (1983), *Microbiol. Rev.* **47,** 150–168.
5. Bryson, V. (1952), *Science* **116,** 48–51.
6. Brown, S. W. and Oliver, S. G. (1982), *Eur. J. Appl. Microbiol. Biotechnol.* **16,** 119–122.
7. Korhola, M. (1983), in *Gene Expression in Yeast*, Korhola, M., and Väisänen, E., eds., Foundation for Biotechnical and Industrial Fermentation Research, Helsinki, pp. 231–242.
8. Andreeva, E. A., Blinova, O. P., and Rabotnova, I. L. (1985), *Microbiology* **54,** 1011–1014 (Engl. Translation of Mikrobiologiya).
9. Bolen, P. L. and Slininger, P. J. (1984), in *Developments in Industrial Microbiology*, vol. 25, Nash III, C. H., and Underkofler, L. A., eds., Arlington, VA, pp. 449–457.
10. Kalyuzhin, V. A. (1987), *Mikrobiologiya* **56,** 78–83 (Engl. Transl.).
11. Maesen, TH. J. M. and Lako, E. (1952), *Acta Biochim. Biophys.* **9,** 106, 107.
12. Samson, F. E., Katz, A. M., and Harris, D. L. (1955), *Arch. Biochem. Biophys.* **54,** 406–423.
13. Fencl, Z. (1960), in *Membrane Transport and Metabolism*, Kleinzeller, A., and Kotyk, A., eds., Czechoslovak Academy of Sciences, Prague, pp. 296–304.
14. Suomalainen, H. and Oura, E. (1955), *Exp. Cell Res.* **9,** 355–359.
15. Suihko, M.-L. and Mäkinen, V. (1984), *Food Microbiol.* **1,** 105–110.
16. Pons, M.-N., Rajab, A., and Engasser, J.-M. (1986), *Appl. Microbiol. Biotechnol.* **24,** 193–198.
17. Aarnio, T. H. (1989), Lic. Tech. Thesis, Helsinki University of Technology, Espoo, Finland.
18. Suihko, M.-L. and Mäkinen, V. (1981)), *Eur. J. Appl. Microbiol. Biotechnol.* **13,** 113–116.
19. Aarnio, T. H. and Suihko, M.-L. (1990), *Appl. Biochem. Biotechnol.*, submitted.