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Liquid nitrogen storage of yeast cultures. II. Stability of characteristics of stored strains

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Nineteen strains of yeasts possessing different characteristics were stored in liquid nitrogen and after 5 years phenotypic characters were evaluated and compared with equivalent strains preserved under paraffin oil. All qualitative characters tested remained stable, and quantitative characters varied only within the range of natural variability.

INTRODUCTION

Five years ago we started to store yeast cultures under liquid nitrogen and reported the survival of selected sets of strains after 75 days of preservation (Hubálek and Kocková-Kratochvílová, 1978). The yeasts were suspended in a medium "M2" with calf serum and cryoprotective agent, dimethylsulfoxide (DMSO), using plastic ampoules submerged quickly and immediately into liquid nitrogen. Following storage, ampoules were quickly transferred into water held at 37 °C. All strains survived cryogenic conditions very well; in some cultures over 95% of cells survived. After 5 years of storage, parallel ampoules were opened and the survival of yeast strains was tested (Hubálek and Kocková-Kratochvílová, 1982). Here we report about the stability of different characteristics of the stored yeasts.

MATERIALS AND METHODS

Strains

The strains of yeasts were selected with the aim of including the largest number

Table 1. Some quantitative differences between strains stored under oil (A) and strains stored for 5 years in liquid nitrogen (B)

Strains	Radial growth rate ($\mu m \cdot h^{-1}$)		Sporulation (% of sporulation structures)		Growth in 10% glucose and 5% NaCl (growth in 2% glucose = 100%)		Growth in 60% sucrose (growth in 2% sucrose = 100%)		Growth in vitamine-free medium (growth in complete medium = 100%)	
	A	B	A	B	A	B	A	B	A	B
CCY 29-3-32	81	81	0	0	83.3	83.3	83.3	83.3	44.4	38.9
CCY 29-26-4	74	64	0	0	77.8	77.8	52.8	52.8	16.8	16.8
CCY 29-38-18	46	46	0	0	61.0	61.0	33.3	33.3	44.4	44.4
CCY 17-3-1	62	65	0	0	38.9	38.9	55.6	55.6	77.8	77.8
CCY 41-15-2	57	60	83.5	60.0	94.4	94.4	83.3	83.3	100	100
CCY 18-1-1	24	24	0	0	22.4	22.4	27.8	27.8	5.6	5.6
CCY 24-1-1	116	125	50.0	58.0	14.0	14.0	77.8	72.4	28.4	22.4
CCY 38-1-1	47	44	2.0	2.0	94.4	94.4	88.9	88.9	100	100
CCY 33-1-1	83	83	5.0	6.5	25.0	25.0	19.6	19.6	5.6	5.6
CCY 64-1-1	39	36	0	0	61.2	61.2	38.9	38.9	11.2	11.2
CCY 36-2-1	51	44	26.2	31.5	14.0	14.0	2.8	2.8	16.8	16.8
CCY 62-2-3	46	39	0	0	66.8	66.8	50.0	50.0	72.4	72.4
CCY 48-87	35	32	2.3	1.6	77.8	77.8	83.3	83.3	16.8	16.8
CCY 21-4-13	42	39	9.5	19.9	77.8	88.9	100	100	19.6	19.6
CCY 21-21-16	32	30	19.0	19.0	83.3	83.3	100	100	16.8	16.8
CCY 21-21-24	30	35	18.5	19.5	83.3	83.3	100	100	22.4	22.4
CCY 44-1-3	28	32	0.5	0.8	88.9	88.9	100	100	16.8	16.8
CCY 44-1-9	48	48	0	0	61.2	88.9	33.3	100	11.6	5.6
CCY 26-26-5	44	49	0	0	72.4	72.4	83.3	83.3	16.8	16.8

of features that could have been changed during storage. The selected strains possessed the following special characters:

1. *Candida albicans* CCY 29-3-32, a pathogenic yeast, producing pseudomycelium and chlamydospores, fermenting maltose.
2. *Candida lipolytica*[1] CCY 29-26-4, a type culture, utilizing *n*-alkanes; forming pseudomycelium and true mycelium.
3. *Candida utilis* CCY 29-38-18, a fodder yeast, utilizing nitrate; a non-chain former.
4. *Cryptococcus laurentii* var. *laurentii* CCY 17-3-1, producing heteropolysaccharide capsules with large amounts of slime.
5. *Debaryomyces formicarius*[1] CCY 41-15-2 (Golubev and Bab'eva, 1972), an abundantly sporulating strain.
6. *Dioszegia hungarica*[1] CCY 18-1-1 (Zsolt, 1957, 1961, 1972), producing carotenoids (Kocková-Kratochvílová and Bystrický, 1974); some growth factors are needed.
7. *Eremothecium ashbyi* CCY 24-1-1, forming true mycelium, many-spored asci and crescentiform spores, but no budding cells; production of riboflavine.
8. *Hansenula anomala* CCY 38-1-1, a pellicle-forming strain.
9. *Leucosporidium scottii* CCY 64-1-1 (haploid MATa), producing slime.
10. *Lipomyces starkeyi* CCY 33-1-1 (Q_9), producing slime abundantly; low survival after lyophilization.
11. *Nadsonia fulvescens* CCY 36-2-1, a sporulating culture; heterogamic conjugation.
12. *Rhodosporidium toruloides* CCY 62-2-3 (MATa, Q_9), producing carotenoids (Kocková-Kratochvílová and Bystrický, 1974).
13. *Saccharomyces carlsbergensis*[1] CCY 48-87, a production strain used in European breweries, fermenting the whole molecule of raffinose; triploid.
14. *Saccharomyces cerevisiae* CCY 21-4-13, the type species of the genus *Saccharomyces*, fermenting maltose and sucrose.
15. and 16. *Saccharomyces oviformis*[1] CCY 21-21-16 and 21-21-24, two different strains, deeply fermenting sugars. These strains were selected according to a taxometric study (Kocková-Kratochvílová, 1969).
17. *Schizosaccharomyces pombe* CCY 44-1-3 (Q_{10}), a fission yeast; self-sporulating culture.
18. *Schizosaccharomyces pombe* CCY 44-1-9 (mutant), a fission yeast, requiring growth factors.
19. *Torulopsis schatavii* CCY 26-26-5 (Kocková-Kratochvílová and Ondrušová, 1971), a type culture.

[1] The names of some yeast species used in this paper are not in agreement with those recommended by Lodder (1970) and Barnett and Pankhurst (1974). To avoid confusion the authors used the same names as in previous papers (Hubálek and Kocková-Kratochvílová, 1978, 1982).

Table 2. Relative growth in high concentrations of fructose (as % of growth in 2% fructose medium, measured photometrically at 560 nm)

Strains	g fructose added to 100 ml water													
	upon storage under paraffin oil							upon 5-year storage in liquid nitrogen						
	50	60	75	95	100	105	110	50	60	75	95	100	105	110
CCY 29-3-32	61.4	57.1	35.1	35.7	18.6	14.3	12.8	62.2	60.8	35.1	16.2	9.5	8.1	8.1
CCY 29-26-4	45.3	17.4	9.3	0	0	0	0	24.4	11.1	5.6	0	0	0	0
CCY 29-38-18	28.1	18.7	0	0	0	0	0	21.2	15.2	0	0	0	0	0
CCY 17-3-1	51.0	38.8	18.4	2.0	0	0	0	50.0	50.0	40.0	12.0	6.0	0	0
CCY 41-15-2	78.2	76.3	70.9	45.4	23.6	9.1	3.6	65.2	60.8	57.9	39.1	20.3	8.7	2.9
CYY 18-1-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CCY 24-1-1	5.4	0	0	0	0	0	0	5.1	0	0	0	0	0	0
CCY 38-1-1	79.5	67.9	55.7	33.3	31.6	20.2	20.2	75.6	67.1	56.1	34.1	32.9	24.4	24.4
CCY 33-1-1	50.0	0	0	0	0	0	0	60.0	50.0	0	0	0	0	0
CCY 64-1-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CCY 36-2-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CCY 62-2-3	24.4	24.4	20.0	15.6	15.6	0	0	20.8	20.8	20.8	14.6	14.6	0	0
CCY 48-87	64.7	38.2	20.6	0	0	0	0	65.6	37.5	21.8	0	0	0	0
CCY 21-4-13	76.5	70.6	58.8	38.2	35.3	26.5	26.5	72.2	63.9	58.3	36.1	27.8	27.8	27.8
CCY 21-21-16	72.9	67.6	64.9	24.3	8.1	0	0	83.8	75.7	67.6	27.0	8.1	0	0
CCY 21-21-24	69.2	58.9	56.4	35.9	23.1	23.1	17.9	56.8	56.5	43.2	25.0	20.5	18.2	11.4
CCY 44-1-3	94.8	89.7	74.3	46.1	46.1	38.5	38.5	95.0	87.5	72.5	47.5	42.5	37.5	37.5
CCY 44-1-9	60.0	60.0	54.0	36.0	34.0	24.0	0	94.7	89.4	68.4	42.1	39.5	34.2	34.2
CCY 26-26-5	45.3	17.4	9.3	0	0	0	0	24.4	11.1	5.6	0	0	0	0

Methods

The following characters were examined: morphological pattern of giant colonies; growth at various temperatures (5, 28, 37 and 42 °C); activity of sporulation (estimated on Fowell agar by counting the number of sporulation structures, viz. spores, asci, zygotes within a set of 500 cells); growth on vitamin-free medium; fermentation of saccharides (D-glucose, D-galactose, sucrose, lactose, maltose and raffinose); radial growth rate, assimilation of saccharides (L-arabinose, D-xylose, D-glucose, D-galactose, L-sorbose, sucrose, lactose, maltose, cellobiose, trehalose, melezitose, raffinose), assimilation of polyols (erythritol, D-arabitol, D-ribitol, D-glucitol, D-mannitol, D-galactitol, inositol); assimilation of polysaccharides (inulin, soluble starch); assimilation of potassium nitrate, L-lysine, DL-tryptophan; tolerance to high concentrations of sugars (D-glucose, D-fructose, sucrose); sensitivity to cycloheximide (actidione).

The methods have been previously described (Kocková-Kratochvílová, 1977; Kocková-Kratochvílová et al., 1978).

Results

All strains tested survived the 5 years of preservation in liquid nitrogen, but the viability of particular strains varied between 5 and 97% (average 62%) of that prior to freezing and storage in liquid nitrogen (Hubálek and Kocková-Kratochvílová, 1982).

Properties of strains tested in this study were compared with those of the same strains stored under paraffin oil. In a previous paper (Kocková-Kratochvílová et al., 1951*a*, *b*) we showed that storage under paraffin oil is one of the best methods used for maintaining the stability of properties of yeast strains.

Yeast populations which survived the preservation might be best evaluated using giant colonies (Kocková-Kratochvílová, 1973). Giant colonies differ from each other in size, appearance, formation of sectors, slime and colour production.

Table 1 shows the differences in radial growth rate of colonies after 100 h of incubation. Pigments (carotenoids, riboflavine) and slime formation remained stable.

A very important character is the degree of sporulation, which in both types of preservation is generally similar, although some strains (CCY 41-15-2 and CCY 48-87) showed a decrease (Table 1). The morphology of spores and their number per ascus are unchanged. There are no differences between strains stored under oil and strains stored in liquid nitrogen in sensitivity to low (5 °C) or high (42 °C) temperatures.

The fermentation of the six saccharides tested is similar for both groups of strains. The assimilation tests showed small differences, which can be accounted for by natural variability.

Table 3. Changes in sensitivity to cycloheximide (in mm of inhibition zone)

Strains	Upon storage under paraffin oil/ μg cycloheximide per disc				Upon 5-year storage in liquid nitrogen/ μg cycloheximide per disc			
	50	10	1	0.1	50	10	1	0.1
CYY 29-3-32	0[1]	0	0	0	12	0	0	0
CCY 29-26-4	0	0	0	0	0	0	0	0
CYY 29-38-18	24	19	10	0	36	24	12	0
CCY 17-3-1	18	11	0	0	19	13	0	0
CCY 41-15-2	0	0	0	0	0	0	0	0
CCY 18-1-1	12	0	0	0	12	0	0	0
CCY 24-1-1	0	0	0	0	0	0	0	0
CCY 38-1-1	30	22	14	0	35	31	20	14
CCY 33-1-1	0	0	0	0	0	0	0	0
CCY 64-1-1	19	16	8	0	22	20	0	0
CCY 36-2-1	56	48	24	0	59	52	8	0
CCY 62-2-3	0	0	0	0	7	0	0	0
CCY 48-87	12	30	21	10	48	44	30	13
CCY 21-4-13	62	46	42	22	33	30	24	11
CCY 21-21-16	38	33	25	14	36	30	23	12
CCY 21-21-24	46	35	22	11	46	38	23	7
CCY 44-1-3	N[1]	N	0	0	12	9	0	0
CCY 44-1-9	N	N	0	0	11	8	0	0
CCY 26-26-5	0	0	0	0	14	12	8	0

[1] 0, no inhibition; N, no growth.

As shown in Table 1 the vitamin requirements remained unchanged.

Injury, due to freezing and thawing, to membrane systems and its effects on active water content in the cells may be reflected in changes of osmolarity and in tolerance to higher concentration of saccharides. In order to detect differences of this kind three different tests were performed by growing the strains in (1) 10% glucose with 5% NaCl, (2) 60% sucrose and (3) different concentrations of fructose. In the first and second test (Table 1) only strain CCY 44-1-9 stored in liquid nitrogen showed an increased tolerance to higher concentration of sugars. The third test (Table 2) showed that there was a quantitative increase in tolerance to high concentrations of fructose in three strains (CCY 17-3-1, CCY 33-1-1 and CCY 44-1-9).

Some strains (CCY 38-1-1, CCY 62-2-3, CCY 26-26-5) showed increased sensitivity to cycloheximide upon 5-year storage in liquid nitrogen (Table 3).

Discussion

The success of cryopreservation depends on many factors including the techniques used in freezing and thawing. The extent of injury depends on lipid com-

position and degree of fatty acid saturation of membranes. Therefore, the preservation medium with the suitable cryoprotective agents added plays a very important role. The selection of cryoprotectants and their optimal concentration was investigated and the results have been published in a previous paper (Hubálek and Kocková-Kratochvílová, 1978).

Injury to membrane structures surrounding yeast cells could have far-reaching consequences such as changes in transport through cell membranes, membrane organization and structure, production and activity of enzymes located in the cell wall or in the periplasmic space.

It is necessary to draw attention to the low survival of the *Lipomyces* strain. We have experienced difficulties in the preservation of *Lipomyces* strains by lyophilization. Therefore, the survival of this strain after 5 years in liquid nitrogen, though low, is rather successful.

It can be concluded from this preliminary investigation that the storage of yeasts in liquid nitrogen by methods as used in our studies may well prove to be convenient for strains preserved in culture collections. Experiments will continue to extend the study to other genera, species and strains.

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