Classic techniques for improvement of industrial yeast strains: Part I – Construction of ethanol-resistant and osmophilic industrial strains of *Saccharomyces cerevisiae* by electrofusion

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Summary

The effective production of biomass or ethanol in industrial media of high osmolality requires new yeast strains. The present work focused on the development of such strains.

Genetic engineering methods using cytoplasmatically-marked yeast (electrofusion of protoplasts of heterothallic haploids; electrotransformation of killer dsRNA or VLPs into haploids; generation of *rho*$^-$) were used. The characteristics of the hybrids were evaluated by conventional analytical and instrumental methods, followed by statistical interpretation.

After screening for a minimum 10% increase in industrially-relevant parameters, 3 osmophilic hybrids of baker's yeast, as well as 8 improved strains of distillery yeasts were selected. The baker's yeasts showed optimum growth in a relatively concentrated molasses wort (1:5 ratio of molasses to final volume). The alcohol-resistant yeasts (including killer) produced up to 14.5% (w/w) ethanol in a medium containing 34% dissolved solids (a mixed mash of sucrose and potato). The characteristics of the alcohol-resistant and osmophilic yeasts were stable over several years of their industrial applications.

The results show that electrical techniques (fusion to obtain hybrids, with interpretation by computer-aided image analysis, and transformation to give marked strains) can be used effectively enough for the construction of some industrially-productive yeasts.

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1. Introduction

The use of improved strains of yeast in the baking and distillery industries offers increased productivity, as well as a reduction in waste products. Some or all of the following characteristics are required: osmophilicity, ethanol-resistance, possession of substrate-matched metabolic pathways. The latter require the synthesis of certain enzymes that are not typical of *Saccharomyces cerevisiae*, such as those for amylolysis or xylose fermentation. The ability to utilise all of the feedstock is very important for waste reduction (1-3).

The production of applicable strains with stable, desirable traits requires the use of genetic modification methods. In this case, it is still very difficult to use recombinant DNA techniques because the required characteristics are controlled by several unidentified genes. The best and fast approach seems to be somatic hybridisation using the fusion of protoplasts, for which polyethylene glycol (PEG) has usually been used as fusogenic agent (1,4-7).

Hybridisation between conventional and non-conventional yeasts for industrial purposes involves combination of the desirable/applicable traits of the parental strains, resulting in hybrids with outstanding metabolic abilities. In the evaluation of hybrids prior to the industrial application, the nature of their anabolic and catabolic process, as well as the stability of the specific traits in subsequent generations are usually assessed (1,2,4-7). However, only a small percentage of the genome of non-*Saccharomyces* strains could so far be incorporated into *S. cerevisiae* (8,9).

Therefore, it was decided to carry out hybridisation by means of protoplast electrofusion (10,11), because this technique is known to give a high frequency of recombinants (12,13). Even though the fusion of membranes between closely compatible types of cells is a well-known natural phenomenon, e.g. in conjugation, electrofusion may also be used on cells that are unable to undergo spontaneous fusion, such as yeasts of the same mating type (13,14). The method has already been used to construct new strains with desirable biological and technological properties, such as alcohol-tolerant yeasts (1,2,6,7). It has the further experimental advantage of allowing convenient electronic control and optical monitoring of the fusion process.

The goal of this study was to obtain osmophilic strains for the production of baker's yeast, and for the distillery industry. The former should give a high yield in relatively concentrated molasses wort (about 30% of the sugars may be raffinose, which is incompletely fermented by *Saccharomyces cerevisiae*) (4,5). The latter should be tolerant of high levels of ethanol, give rapid production of alcohol, and have the capacity to efficiently transform the sugar (and/or starch, if amylolytic enzymes are

present) into ethanol (1,6-8). Such strains would give a final product that would significantly reduce the costs of production and the problems associated with waste water (e.g. by reduction of the concentration of reducing sugars).

A further step would be to transfer killer dsRNA (as Virus Like Particles, VLPs) into the products of electrofusion (or haploids derived from tetrad analysis). This could be used to mark commercial strains and also to prevent contamination by other undesirable microorganisms in fermentation and baking. A similar idea (but using cytoduction) has been implemented by Chang et al. (5) and by Ouchi et al. (7) for Sake yeasts and for baker's yeasts. It is interesting that the quality of the Sake was unaffected by the killer trait. It is also relevant to mention that there is no evidence that yeast „killer toxin" is harmful to human beings, or indeed to any higher animals. Once again, electrical methods are used as the tool to accomplish classical hybridisation and transformation.

2. Materials and Methods

2.1. Selection of strains

Several strains of *Saccharomyces* spp. (see Tab. 1) were chosen as starting material because of certain morphological, physiological and biochemical characters appropriate for industrial applications (15). These strains were collected and stored by the Institute of Biotechnology of the Agricultural and Food Industry in Warsaw (Poland) over many years in collaboration with the Polish industry („Polmos").

Table 1

Parent yeast strains used for construction of industrial hybrids

Symbol of Strain	Ploidy	Derivation of strain	Genotype				Phenotype Sensitivity to antibiotics		
			Mating Type	rho	K	R	Erm	Cap	Acd
1	2	3	4				5		
G2-1a	heterothallic haploid	tetrad analysis of industrial polyploid G2	α	+	–	–	s	s	s
G3-1b	heterothallic haploid	tetrad analysis of industrial polyploid G3-1	α	+	–	–	s	s	s
HB3/3B$_1$	homothallic haploid	tetrad analysis of industrial polyploid HB3/3	α/a	+	–	–	r	r	s
HB3/3G$_1$	heterothallic haploid	tetrad analysis of industrial polyploid HB3/3	a	+	–	–	r	r	s

1	2	3	4				5		
$B3/5A_1$	heterothallic haploid	tetrad analysis of industrial diploid B3/5	a	+	–	–	s	s	s
$IB3/5A_1$	heterothallic haploid	electro-fusion of dsRNA into B3/A1 haploid	a	+	–	–	s	s	s
$B3/5B_2$	heterothallic haploid	tetrad analysis of industrial diploid B3/5	a	+	–	–	s	r	s
$AFrA_3$	heterothallic haploid	tetrad analysis of industrial triploid AFr	a	+	–	–	s	r	s
$AFrB_1$	heterothallic haploid	tetrad analysis of industrial triploid AFr	α	+	–	–	s	r	s
D_2	tetraploid	industrial strain	–	+	–	–	r	s	s
D_2-1	heterothallic haploid	tetrad analysis of industrial strain	a	+	–	–	r	s	s
AH 22	heterothallic haploid	hinnen A., his-leu	a	+	–	–	n.d.	n.d.	n.d.
$AS-4/H_2$	heterothallic haploid	tetrad analysis of hybrid AS-4	a	–	–	–	n.d.	s	n.d.
Sd21/45/3	heterothallic haploid	CCY – Tchecho-Slovak Collection	a	+	–	–	s	r	r
Sd21/45/6	heterothallic haploid	CCY – Tchecho-Slovak Collection	α	+	–	–	s	r	r
$AS-4/H_2$-1	heterothallic haploid	tetrad analysis of hybrid AS-4	α	–	–	–	n.d.	n.d.	n.d.

Key: Erm – antibiotic erythromycin, Cap – antibiotic chloramphenicol, Acd – actidion (cycloheximid), r – resistance to antibiotic, s – sensitivity to antibiotic, K – killer toxin producer, R – resistant (+) or sensitive (-) to killer toxin, n.d. – not determined.

The strains were selected from polyploid yeast clones according to the criteria of osmophilicity (see ref. 15,16) and of resistance to elevated (more than 10% w/w) ethanol concentrations. As discussed in ref. 16, these osmophilic strains were found to contain high levels of trehalose. The parental strains were all *S. cerevisiae* or *S. diastaticus*.

2.2. Media

2.2.1. Small-scale

Medium YPG contained: 2% bacto-peptone, 1% yeast extract, 2% glucose (and, if necessary – 2% agar); brought to pH 5.0 with HCl.

The regeneration medium used for protoplasts after electrofusion contained YNB (yeast nitrogen base, Difco), enriched by ATP, ergosterol and Tween 80. It has been described in detail elsewhere (17), as has the medium for evaluation of

osmophilic yeasts (16). Essentially, the latter contained molasses wort (1:3 ratio of concentrate to final volume) or YPS (1% yeast extract, 2% peptone) with 20% sucrose.

The respiratory-deficient mutants were detected by their inability to grow in RHO medium, which contained 1% yeast extract, 2% bacto-peptone, 2% glycerol and 2% agar by weight (this medium contains no fermentable carbon source). Alternatively, a medium which contained YNB, 3% glycerol (by weight) and 0.1% glucose was used.

For examination of the amylolytic ability of hybrids (*S. cerevisiae* x *S. diastaticus*) a medium containing a 0.67% solution of YNB, including 2% of a 1M potassium-phosphate buffer pH 6.0, 0.08% D-glucose, 0.32% soluble starch, 1.5% agar was used. After autoclaving, a solution of iodine (0.035% I_2) in 0.125% KI was added at 1/500 dilution. Hybrids which secreted amylolytic enzymes (mostly glucoamylase) showed a clear bright zone around growing clones.

Electrofusion Medium A contained: 1.2 M sorbitol, 0.1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1 mg/ml albumin, and 5 mM histidine.

Electrofusion Medium B contained: 1.2 M sorbitol, 0.1 mM Ca-acetate, 0.5 mM Mg-acetate, and 4.1 mM K-acetate (13).

2.2.2. Laboratory simulation of industrial conditions

Molasses-potato mash (dry mass up to 30%) and potato-sucrose mash (up to 34% dry mass) were used. Preparation of these media was described before (24). These media proved suitable for later scale-up in industrial applications using fermenters of 5000 l (Bydgoszcz, Poland) and 10 000 l (Sieradz, Poland) capacity.

2.3. Sporulation analysis

Tetrad analyses were carried out as described elsewhere (15), using a procedure based on the method of Mortimer & Hawthorne (18). This consisted of dissection of the ascospores from sporulated polyploid strains in order to obtain single-spore-colonies (haploid strains).

2.4. Mutation

Respiratory-deficiency mutants (rho-) were obtained by use of ethidium bromide at 100 μg/ml (19). This non-specific mutagen of transcription gave respiratory-deficient strains with non-specific deletions in the mitochondrial DNA.

2.5. DNA staining

Staining of mitochondrial and nuclear DNA *in vivo* in fixed cells used a DNA-specific fluorescent dye, DAPI (2,6-diamidino-phenylindole, Sigma D 9542). This procedure was described in part II and III of this paper.

2.6. Isolation of double-stranded RNA

The dsRNA (L_{1A}- and M_1-dsRNA) genetic information material for K_1 toxin was isolated from the killer strain of *S. cerevisiae* T 158C (***his***$^-$) according to the procedure of Fried & Fink (20), with the modifications (17).

Virus-like particles (VLPs, i.e. dsRNA with protein coat) were obtained from this strain by the method of Oliver et al. (21).

2.7. Formation of yeast protoplasts

Yeast cells were harvested from the early stationary phase by centrifugation (3000 rpm/10 min, 11 cm radius). Protoplasts were prepared using standard protocols with enzymes: helicase (*Helix pomatia*, snail digestive juice, Koch-Light Labs. Ltd., Colnbrook, Bucks, England) (15,22) or with Zymolyase-100T (Seikagaku Kogyo Co. Ltd., Japan) (13,17).

2.8. Electrofusion

This method of somatic hybridisation was based on the parameters described previously (Tab. 2) (13,15).

Table 2

Electrical conditions for electro-fusion

Electro-fusion Apparatus	Constructed at the Institute of Biotechnology, Poland	"Biojet" electrofusion system, Biomed GmbH Germany
Electrode Geometry	Parallel* d = 200 μm	Helical d = 200 μm
1	2	3
Alternating field frequency (MHz)	1.3	2.00
Alternating field (kV/cm)	2.66	0.75
Pulse duration (μsec)	80	10
Field pulse (kV/cm)	5.3	10.0

1	2	3
Number of pulses and intervals (sec)	2-3 0.5-3	2 0.5-2
Duration of pre-pulse alignment (min)	<3	<1
Duration of post-pulse alignment (min)	<5	1
Type of electro-fusion Medium (see methods) with conductivity (μS/cm)	A 600	B 200-300
Temperature (°C)	20	5
Cell density (per ml)	10^4	10^5
Volume of fusion chamber (μl)	50	250
Yield, hybrids per protoplast	10^{-4}	10^{-3}

* Ag on Ni layer (depth 50 μm) with a central slot, d = 150 μm.

2.8.1. Somatic hybridizer built in-house

During the initial electrofusion work the following equipment built at the Institute of Biotechnology of the Agricultural and Food Industry in Warsaw, Poland, was used:

– an electrofusion chamber, consisting of an Ag-coated Ni layer prepared by powder metallurgy on a glass microscope slide; in the layer, a slot of width 150 μm and depth 50 μm had then been milled away,

– a generator of high-frequency alternating current RC, type PO-25A (for dielectrophoresis),

– a generator of square pulses of direct current (built in-house),

– an electronic oscilloscope (type KR-7010) for measurement of the high-frequency and pulse voltages,

– a frequency counter (modified type KZ 2026 A-2).

The fusion sequence (pearl-chain formation followed by membrane fusion) was monitored through a microscope Ergaval (Zeiss, Jena), connected to a semi-professional video monitoring and recording system (videocamera F-10/configuration KT-100 and VHS videorecorder, both Panasonic; video monitor, Sony). The above system could be interfaced to an image analyser, consisting of:

– a CCD videocamera MTV 1801 CB, resolution 604x588, and

– an IBM PC/AT computer with a frame-grabber card of frame size 512x512 pixels (VFG-512-8-BC, Visionetics), and a library of application programs (written in Turbopascal 4.0).

2.8.2. Somatic hybridizer

Part of this work was carried out at the University of Würzburg, Germany (Department of Biotechnology). In this case a commercial system for electrofusion con-

sisting of helical chambers (3,23) and fusion power supply type „Biojet CF" (Biomed GmbH, 97531 Theres, Germany), was used. This apparatus integrates all the functions necessary for electrofusion (10). During the preliminary checks on the suitability of the protoplasts for fusion, a chamber consisting of two Pt-wires mounted in parallel at a distance d = 200 μm on a glass microscope slide was used.

2.8.3. Electrical conditions of electrofusion

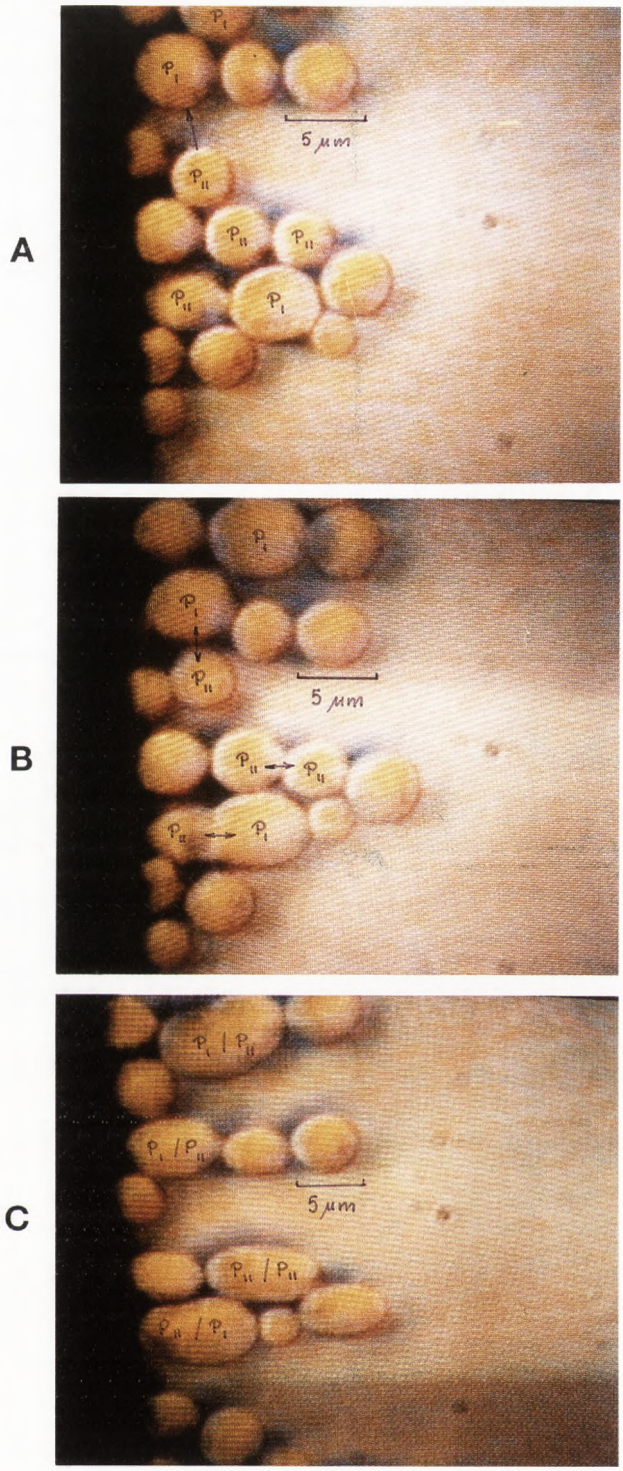
Table 2 details the parameters which were used in the two electrofusion systems. A suspension of protoplasts in 1.2 M sorbitol was placed between the electrodes which were supplied with a high-frequency voltage (30 V p-p at 1-2 MHz).

The alternating field is required to evoke dielectrophoresis (Fig. 1A): this causes movement of the cells towards each other (usually preceded by their movement towards the electrodes, if these are other than flat and parallel) (10-12,24-26). This step is necessary to give close apposition of the membranes of the two cells.

The movement occurs because each protoplast behaves as a field-induced dipole: this is due to the fact that charge displacement in its interior (which is highly-conductive (27)) occurs much more readily than in the medium, at frequencies of several MHz. Charge movement and polarisation in the medium are both much lower due to poor conductivity (600 μS/cm or below) and a relative permittivity rather below that of water (the value for a 1.2 M sorbitol solution at 23°C is 76 (24)). More highly-polarized objects are attracted towards regions of a higher field: such regions include the electrode-facing regions close to other polarisable cells (25,26), and especially electrodes of small radius of curvature. Especially in the case of sharp-edged or of wire electrodes, chains of protoplasts tend to form lines along the field, which emanate perpendicularly from the electrodes (Fig. 1A). It is also sometimes observed that objects of very different polarisability may form chains perpendicular to the field lines (28).

During dielectrophoresis, the force between the dipoles induced in adjacent cells is often sufficient to give a visible flattening of the two contacting membrane surfaces. Application of a short-duration high-intensity pulse now causes a limited breakdown of the membranes within the contact zone (the pulse should not be too long, or else cell lysis may occur). After the pulsing, the fusion aggregates are usu-

Fig. 1. Various stages in the electro-fusion of protoplasts of *Saccharomyces diastaticus* and *Saccharomyces cerevisiae*. The bar represents 5 μm: A – Formation of "pearl chains" of yeast protoplasts by dielectrophoresis; the black line to one side is one of the electrodes; the vast majority of protoplasts aligned in chains parallel to the field lines: these usually become attached to an electrode. B – After pulsing, four pairs of protoplasts (P_1 and P_{11}) have fused together. C – Alternating field still applied, just before its removal.


A
B
C
5 µm
5 µm
5 µm

ally held together by further application of the alignment field. In the case of very dense aggregations of cells the field distribution is so complex that pores may form anywhere on the protoplasts. This explains the fact that, under these conditions, fusion also occurs between adjacent chains (see Fig. 2).

2.9. Electrotransformation technique

Electrotransfection with killer yeast dsRNA was carried out as described in part II of this paper.

2.10. Killer activity assay

After electrofusion or after electrotransformation of killer activity, assessment of the transfected hybrids was carried out as described by Salek at al. (17), using the super-sensitive strain *S. cerevisiae* S · 6/1.

2.11. Mating type assay

Ascospore-haploid cell mating was carried out by *halo* assay with synthetic α-factor peptide (TRP-HIS-TRP-LEU-GLN-LEU-LYS-PRO-GLY-GLN-PRO-MET-TYR, MW 1684, acetate salt, α_1-mating factor of *S. cerevisiae*, Sigma T 6901).

A suspension of each strain to be characterised was overlaid on a small YPG-agar (pH 4.5) plate. A well of 8 mm diameter was formed in each plate, and filled with a solution (2.5 or 5 μM in YNB) of α-factor (stock solution: 50 mM in methanol). The plates were incubated at 30°C for 24 hours and then inspected. A clear zone (*halo*), indicated the temporary arrest of cell growth typical of an a-mating strain. If the strain was α, it grew without interruption.

2.12. Assessment of growth rate and of fermentative ability

Technical characteristics of yeast strains were assayed according to the methods described elsewhere (15,16).

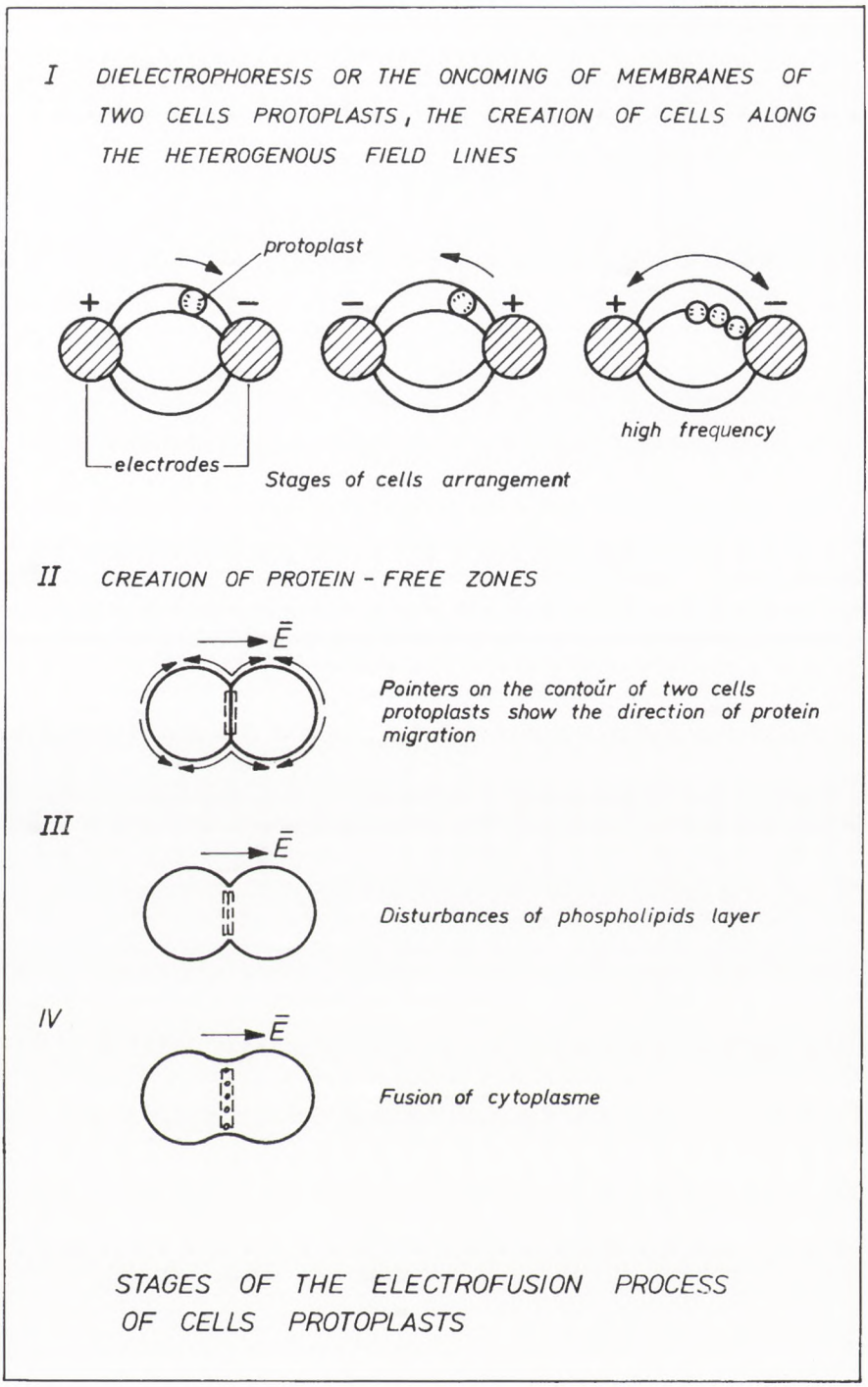


Fig. 2. Four stages of the electro-fusion of yeast protoplasts – scheme.

3. Results and discussion

3.1. Sporulation

Genetic analysis of the strains used here presented difficulties. Such problems are often encountered while working with industrial strains of *Saccharomyces* sp. (9). Sporulation of the industrial strains was poor. The ascospores that were obtained from dissection of "tetrads" (which did not always contain four spores, Fig. 3), germinated with difficulty and afforded slow-growing, small monospore colonies. The major part of the selected monospore cultures were homothallic, aneuploidal haploids. These data suggest that although the industrial parent-strains were usually polyploid (also indicated by their large size), a degree of aneuploidy was also present.

Screening of about 30 industrial strains, as well as genetic studies based on sporulation analysis of tetrads, afforded a group of heterothallic euploidal haploids. Results of genotype/ phenotype determinations on these strains are given in Table 3.

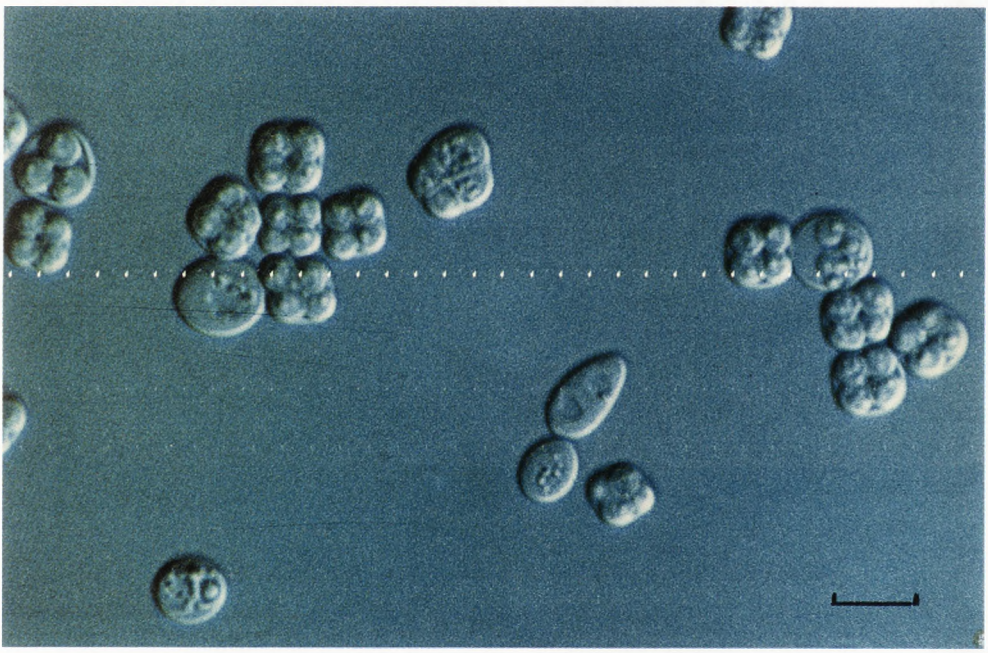


Fig. 3. Spores (mostly tetrads) formed from industrial strains of yeast *Saccharomyces cerevisiae* D_2 (as an established strain).

Table 3

Properties of the products of electro-fusion with killer strains and of the products of electro-transformation with M_1-dsRNA and L_{1A}-dsRNA

Symbol of Strain	Ploidy	Derivation of strain	Genotype				Phenotype		
			Mating type	Rho	K	R	Erm	Cap	Acd
IB3/5A$_1$ (K)	heterothallic haploid	electro-transformation of dsRNA into B3/5A$_1$	a	+	+	+	s	s	s
AS-3	diploid	electro-fusion of G3-1b x AFrA$_3$ (killer)	α/a	+	+	–	r	r	s
AS-4	diploid	electro-fusion of G$_2$-2a x D$_2$-1 (killer)	α/a	+	+	–	r	s	s
SL	tetraploid	electro-fusion of B3/5B2 x HB3/3B1 (killer)	α/aa	+	–	–	r	s	s
G$_1$	diploid	electro-fusion of AS-4/H$_2$ x AH 22	a/a	+	–	–	s	r	s
AS-4/H$_2$-1	heterothallic haploid	electro-transformation of dsRNA into AS-4/H$_2$-1	α	–	++	+	n.d.	n.d.	s
AS-4/H$_2$ (K)	heterothallic haploid	electro-transformation of dsRNA into AS-4/H$_2$-1	a	–	+	+	n.d.	n.d.	s
Sd21/45/6 (K)	heterothallic haploid	electro-transformation of dsRNA into Sd21/45/6	α	+	+	+	s	r	s
AMYL-1	polyploid	electro-fusion of Sd21/45/6(K) x B3/5A$_1$	α/a	+	+	+	s	r	s
AMYL-2	polyploid	electro-fusion of Sd21/45/6(K) x AS-4/H2	α/a	+	+	+	s	r	s
AMYL-3	polyploid	electro-fusion of Sd21/45/3 x AH 22	α/a	+	–	–	s	r	r

3.2. Attempts at marking the strains

The identification or introduction of markers in newly-obtained strains of technological value is a special problem. Markers are required to identify strains with suitable characteristics for particular problems in industrial populations, and also to detect inappropriate recombinants after protoplast fusion. In this study, the use of classical marking methods, e.g. the induction of auxotrophy, often led to a lowering of both the growth rate and cell viability (data not shown). These classical marking techniques were therefore not recommended. Determination of the inherent traits, mainly by assay for resistance and inhibition, were the preferred method of identification.

Spontaneous mutations in mitochondrial DNA which gave obvious phenotypic effects were found to be useful in this work. Among several possibilities for the parental strains, mutants with ant^R (antibiotic-resistant) point mutations were se-

lected (Tab. 1). The frequency of spontaneous, mitochondrial ant^R mutations was of the order of 10^{-6}-10^{-7}. They were sufficiently infrequent to be good markers. UV-irradiation of heterothallic haploids induced mutants with resistance to threshold doses of erythromycin (0.6%), chloramphenicol (0.5%) and cycloheximide (0.01%) at about 1-5% frequency. However, as many of these mutants were now also auxotrophic and not ***rho***$^-$, other methods for the introduction of mitochondrial deficiencies were preferred.

Ethidium bromide has been reported to give ***rho***$^-$ clones (products of somatic hybridisation) in good yield (see part II of this paper), which was confirmed here. It is also interesting that ***rho***$^-$ cells harvested from the exponential phase showed much greater rates of trehalose accumulation than similarly-grown *rho*$^+$ cells (data not shown). This suggested that respiratory-deficient mutants (***rho***$^-$, which could be identified by their lack of fluorescence from mitochondrial DNA after staining with DAPI (see part II of this paper), may be more resistant to high osmotic pressures (including alcohol stress, see ref. 29) than respiratory-competent (*rho*$^+$) strains. Previously published studies by Mansure et al. (30) also indicated a significant role of trehalose (although this was not the only factor) in promoting ethanol tolerance. In particular, the presence of trehalose on both sides of the cell membrane strongly inhibited ethanol-induced leakage (30).

Another marking technique involved the determination of resistance or sensitivity to the killer factor (a specific toxic protein, derived from super-killer strains T 158C (*his4C-864*, *rho*$^+$, MATα, *ski5*; authors: Vodkin, Fink, Katterman; (15)) of *S. cerevisiae*. Killer activity can be induced in non-killer strains by transformation with dsRNA-plasmids derived from VLPs of super-killer strains, as demonstrated by Bortol et al. (1) and Ouchi et al. (7). In contrast to the cytoduction used by those groups, in this study electrotransformation was used to introduce this "marker" (for frequency of transformation see part II of this paper). In some cases, advantage could be taken of the finding that the transformation efficiency with killer dsRNA is increased by the use of ***rho***$^-$ strains (19). Cells with mitochondrial defects cannot produce RNase (digested e.g. M_1- and L_{1A}-dsRNA) or DNase.

3.3. Conditions for electrofusion of protoplasts

The fusability of membranes is thought to depend mainly on their lipid bilayer structure, subject to the influence of trans-membrane proteins and of linkages to the cytoskeleton. It was therefore of some interest to compare the electrofusion of alcohol-tolerant strains of *Saccharomyces* sp. with that of normal strains, because alcohol-tolerance is known to be associated with changes in membrane-lipid composition. The phospholipids of ethanol-resistant yeasts have longer fatty-acyl residues (e.g. $C_{18:0}$) than sensitive strains and they also show a larger proportion of unsaturated fatty-acyl residues (i.e. $C_{18:1}$). In addition, the resistant strains tend to have

sterols with unsaturated alkyl chain (e.g. ergosterol) in place of saturated fatty-acyl residues (31-36). For review, see ref. 37. Posssibly, a higher degree of lipid unsaturation is able to offset the decrease in trans-membrane transport rates and in other manifestations of membrane fluidity, that are thought to be caused by ethanol (35).

As pointed out in the "Introduction", protoplast electrofusion permits step--by-step monitoring of the process (Fig. 1). After dielectrophoretic collection of protoplasts into chains (Fig. 1A), application of field pulse(s) led to fusion, usually of 2 but sometimes of 3 or more protoplasts (Fig. 1B). The first stage (not shown) was the formation of cytoplasmic bridges, usually within 100 ms after the pulses (see Fig. 2).

Finally, the hybrid assumed a spherical shape. At least in protoplasts of higher plants there is evidence that metabolic energy, which can be assessed as ATP, is required for this process (38). The rounding-up after electrofusion might last from a few to several dozens of minutes, depending on the size, nature and number of the protoplasts which had yielded the fusion product (Fig. 1C).

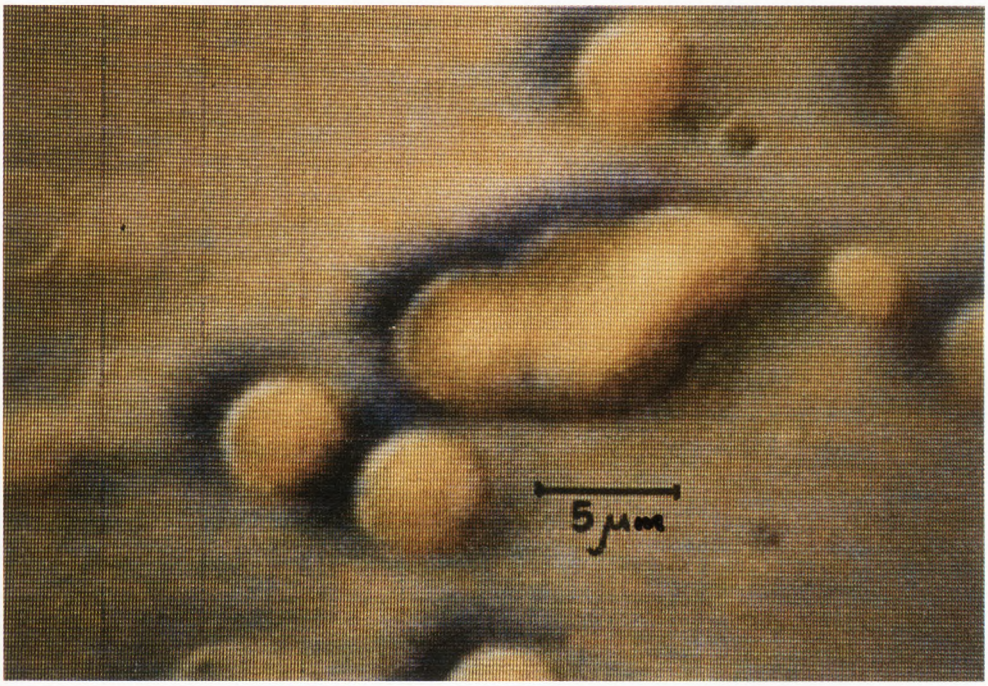


Fig. 4. Fusion products incorporating several protoplasts, produced by the electro-fusion of *Saccharomyces cerevisiae* x *Saccharomyces diastaticus* at relatively high protoplast density. The bar represents 5 µm.

Electron-microscopy of the products demonstrated that mixing of the cytoplasms (plasmogamy) of the cells to give cybrids was the usual result in the case of cells with the same mating type. In a few cases, fusion of the nuclei, i.e. karyogamy, could also be demonstrated (visualised by DAPI-staining of nuclei, data not shown).

Finally, the products were allowed to regenerate their cell walls (15,17). At higher-density suspensions of protoplasts in the electrofusion chamber, products formed from 4,5,6 or more protoplasts were common. At still higher densities, many contacting chains of protoplasts were formed (Fig. 4), which led to giant cells by the so-called three-dimensional fusion. Such cells may possibly be of technical or scientific interest.

3.4. Video-microscopy of the fusion process

Video-microscopy of electrofusion, especially when combined with computerised image-analysis (Fig. 5), also gave much information about the degree of protoplastisation and the efficiency of fusion (Fig. 6). The images were digitized and stored in the system memory, on a magnetic disc, or on a video tape for subsequent analysis. In this procedure, contrast-enhancement of the contour of the cell and protoplast revealed the sites of fusion as well as karyogamy when this occurred later. The method permitted rapid evaluation of the frequency of electrofusion.

3.5. Technological results

Polyploidisation of the parental haploids by protoplast electro-fusion afforded, depending on the parental strain (Tab. 1), the hybrids and products of transformation listed in Table 3. These new strains can be classified as:

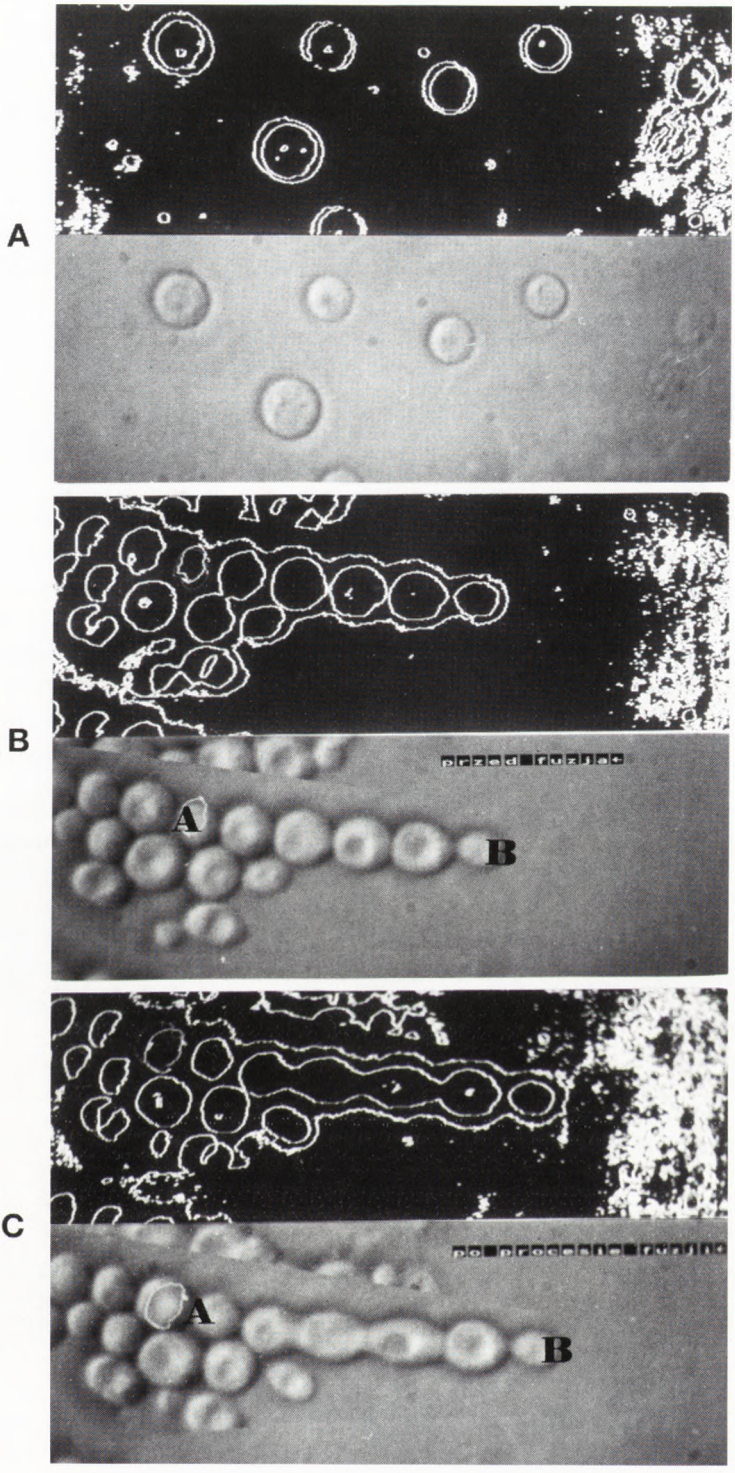
– ethanol-resistant. osmophilic. distillery yeasts, which were compared against their osmophilic-selected parental strains in two sorts of concentrated media (15);

– osmophilic baker's yeasts (15);

– interspecies hybrids (*S. cerevisiae* with *S. diastaticus*) containing the DEX gene complex which codes the amylolytic enzymes (Fig. 7).

Fig. 5. Photomicrographs/image analysis: stages of electro-fusion of yeast protoplasts of *Saccharomyces cerevisiae* x *Saccharomyces diaststicus:* A – protoplasts without alternating field; B – dielectrophoresis of protoplasts; C – electro-fusion of protoplasts. The pictures at the top show intensity contours derived from the photomicrograph. It can be seen that especially the inner (higher intensity) contour changes to include the newly-formed fusion product between point A and B (Fig. 5C), whereas other contours remain esentially unchanged.

→


A
B
A
B
C
A
B

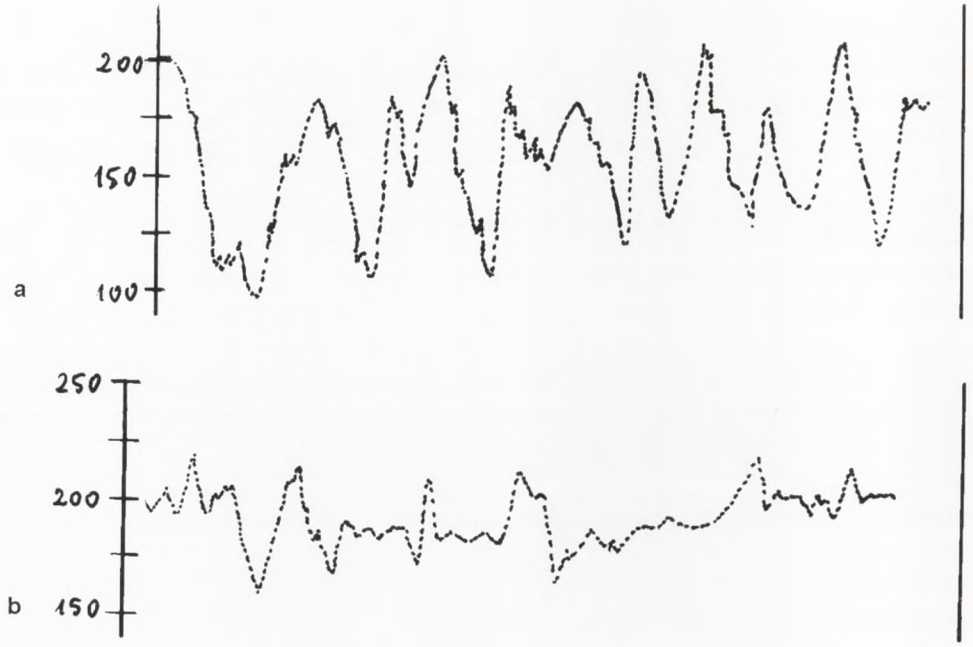


Fig. 6. Image analysis of the electro-fusion frequency. The pictures of intensities along the axial section a-b before electrofusion (a) and after electrofusion (b). They also show significant changes, but this sort of analysis is more difficult to interpret, especially when the chain is not strictly linear (as here).

Fig. 7. Colonies formed by some products of the electro-fusion of *Saccharomyces cerevisiae* with *Saccharomyces diastaticus* on starch-agar (free of mono- or disaccharides). The amylolytic nature of the hybrids is shown by the transparent (starch-free) zone.

The details of the strains in these three classes were:

1. In Table 4, the last 4 strains are products of electric-field manipulation. It can be seen that all 4 gave high yields (>10%) of ethanol (even after only 48 h fermentation). The diploids (AS-3 and AS-4) also gave very good rates of alcohol production (productivities > 2.2 ml A_{100}/l · h), whereas the two haploids of a killer character derived from diploid AS-4 showed lower productivity. However, in 72 h fermentations, one of them (AS-4/H_2 killer) showed extremely low residual sugars, and also the highest yield in this raffinose-containing medium. In the potato-sucrose mash fermentation (Tab. 5), the diploid hybrids AS-3 and AS-4 both gave consistently better values than the industry-standard osmophilic yeast D_2 with regard to all parameters of interest. Industrial-scale evaluation of these hybrids confirmed the laboratory results. In three distillery-yeast hybrids (AS-4 and related), which were utilised over a period of 8 years, the productivity exceeded that of the previously-used industrial strain (from 7% up to 14.5% w/w ethanol in mash medium contained 34% dissolved solids). The degree of conversion was the same, i.e. 56%.

Table 4

Biotechnological parameters of fermentations using the molasses-potato mash (up to 30% dry mass)

Strains	Ethanol (% w/w)		Reducing Sugars (%)		CO_2 production (% of theoretical)		Productivity (ml A_{100}/l h)	Specific fermentation rate (ml A_{100}/kg·glu·h)		Yield of ethanol (% of theoretical)
Duration:	48 h	72 h	48 h	72 h	48 h	72 h	72 h	72 h	48 h	72 h
G2-1a	9.80	10.30	3.3	3.0	97.3	99.8	2.2	9.5	42.1	43.3
G3-1b	9.50	9.30	3.4	3.1	93.7	98.7	2.1	9.2	40.8	42.6
HB3/3B_1	12.39	12.48	1.5	1.4	97.2	99.2	2.2	8.7	55.4	55.8
HB3/3G_1	11.42	11.95	1.8	1.7	93.7	98.0	2.0	8.1	51.0	53.4
AFrA_3	10.74	10.94	2.4	2.3	93.9	97.8	1.8	7.5	48.0	48.9
IB3/5A_1	11.42	11.93	1.9	1.8	94.5	98.0	2.0	8.2	51.0	53.3
B3/5B_2	11.04	12.12	1.9	1.8	90.7	97.3	2.0	7.5	49.3	54.2
B3/5A_1	12.45	12.57	1.4	1.3	94.1	97.9	2.2	8.7	55.6	56.2
D_2	11.10	12.40	2.6	2.2	89.5	95.5	2.4	10.4	48.2	54.1
D_2-1	11.00	12.39	2.6	2.2	89.2	95.3	2.4	10.3	48.1	54.0
AS-3	11.10	12.60	2.6	1.8	87.4	94.8	2.5	10.5	48.3	55.2
AS-4	10.80	12.70	2.6	1.8	88.2	94.7	2.5	10.5	46.9	55.2
AS-4/H_2 (K)	11.36	12.64	1.8	1.2	88.7	97.7	2.1	8.3	50.8	56.5
AS-4/H_2-1 (K)	10.23	10.71	2.3	2.2	92.8	97.6	1.8	7.3	45.7	47.9

ml A_{100}/l·h : ml absolute ethanol per liter and hour; ml A_{100}/kg·glu·h : ml absolute ethanol per kg glucose and hour.

Table 5

Parameters during fermentation of the potato-sucrose mash (34% dry mass)

Duration	Parameter	Strains		
		AS-3	AS-4	D_2 – control
24 h	CO_2 (g)	24.0	24.7	22.9
48 h	CO_2 (g)	36.7	38.0	35.9
72 h	CO_2 (g)	39.5	40.3	38.5
	ethanol [% w/w]	14.3	14.3	13.7
	reducing sugars [%]	0.7	0.6	0.8
	yield [%]	62.5	63.0	61.8

2. In the case of baker's yeast, Table 6 shows how adaptation of strains by repeated passage through hyperosmotic media resulted in up to 50% increases in biomass under industrial conditions (using a high-osmotic-pressure molasses broth). This was the case not only for standard strains such as B3/5, but also for haploids and for diploids formed sexually (16) or by electrofusion. With the exception of strain $AFrB_1$ (which had a very good yield as haploid), the yield of biomass increased with the degree of ploidy.

Table 6

Increases in biomass yield resulting from adaptation to higher osmotic pressure

Strains	Dilution of molasses in broth	During the process of adaptation: biomass after successive screenings [g of D_{100}/l]				
		I passage	II passage	III passage	IV-XI	XIII
B3/5 – control isolated from industrial diploid	1 : 5	7.6	8.7	9.9	–	9.9
	1 : 15	5.3	5.7	5.9	–	6.2
$AFrB_1$ heterothallic haploid	1 : 5	12.4	12.7	13.9	–	13.8
	1 : 15	4.9	5.5	6.0	–	9.6
F – diploid after sexual hybridization [OB-10 x OH]	1 : 5	10.9	11.8	13.4	–	14.0
	1 : 15	4.9	6.2	6.6	–	6.7
X – diploid after sexual hybridization [OH (a) x OH (α)]	1 : 5	11.2	11.4	12.2	–	14.6
	1 : 15	4.4	5.1	5.1	–	5.5
SL – tetraploid after electrofusion [B3/5B2 x HB3/3B1]	1 : 5	10.5	11.0	14.5	–	15.2
	1 : 15	8.2	8.6	8.6	–	8.9
a_1 – diploid after electrofusion [$B3/A_1$ x $IIB3/A_1$]	1 : 5	13.8	n.d.	n.d.	–	n.d.
b_1 – diploid after electrofusion [$B3/A_1$ x $HB3/B_1$]	1 : 5	14.1	n.d.	n.d.	–	n.d.
G_1 – diploid after electrofusion [AH22 x $AS\text{-}4/H_2$]	1 : 5	15.8	n.d.	n.d.	–	n.d.

3. Fig. 7 shows clones of amylolytic hybrids growing on starch agar (without mono- or di-saccharides). The products showed biomass production typical of hybrids *S. cerevisiae* x *S. diastaticus*. As with the hybrids obtained by Bortol and co-workers (1) by transformation of *S. diastaticus* parents, they contained the killer marker (see Tab. 3). The formation of hybrids (in this case AMYL-1 and AMYL-2) was encouraged by the use of fusion partners of opposite mating type (a x α). Three distinct clones could be isolated, one of which (AMYL-1) found industrial application.

These results, as well as the studies of Aarnio & Suihko (4), indicate that electrofusion and electrotransfection are rapid and effective methods to obtain new desirable strains.

Marking cells by electrotransformation with killer dsRNA had the additional advantage of preventing infection by exogenous yeasts during this breeding programme and in the subsequent development of killer hybrids, also for industrial application.

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