Journal of Industrial Microbiology, 10 (1992) 117–122

Published by Elsevier

SIM 00423

Improvement of the cell volume of *Candida blankii* through protoplast fusion

Mariekie Gericke and Willem Heber Van Zyl

Division of Food Science and Technology, CSIR, Pretoria, South Africa

(Received 11 October 1991; revision received 17 March 1992; accepted 23 March 1992)

Key words: Single-cell protein; Protoplast fusion; *Candida blankii*; Cell volume

SUMMARY

The yeast *Candida blankii* ESP-94, capable of utilizing xylose as substrate, was isolated for the production of single-cell protein (SCP) on bagasse hydrolysates. However, the small cell volume of strain ESP-94 would complicate harvesting of the cells during a continuous fermentation process. Auxotrophic mutants of strain ESP-94 were generated and intraspecific protoplast fusion experiments performed in an attempt to increase the cell volume of strain ESP-94. The fusion products were characterised with respect to cell volume, DNA content and genetic stability. Six genetically stable fusants with bigger cell volumes and higher DNA contents were obtained. One such fusant, fusant F17, had a cell volume 3-times that of strain ESP-94, while exhibiting similar growth rates to strain ESP-94 on D-xylose as carbon source.

INTRODUCTION

Protein sources, such as fish meal and soyabean meal, have traditionally been used to supplement animal feeds, particularly poultry feeds. Limited supplies of these feed proteins prompted a search for alternative protein sources. The production of yeast single-cell protein (SCP) on sugar-cane bagasse hydrolysate, a biowaste substrate, presented an attractive alternative source of protein. The fermentable sugars in bagasse hydrolysate contain about 70% D-xylose and 5% L-arabinose [26]. Yeast strains were isolated from soil and insect frass and screened for protein production on minimal medium with D-xylose as sole carbon source. One of the 267 yeasts isolated, *Candida blankii* ESP-94, exhibited protein yields 15% superior to that of fodder yeast (*Candida utilis* ATCC 9950) on D-xylose [26]. Strain ESP-94 also had the physiological advantage of being able to utilize L-arabinose.

Unfortunately, strain ESP-94 had a small cell volume, which is a drawback when considering this yeast for commercial SCP-production. The small cell volume would complicate harvesting of the yeast during the industrial production of SCP. It is known that an increase in nuclear ploidy is accompanied by an increase in the cell size [16]. For example, Sasaki and Oshima [19] took advantage of the yeast cell size-ploidy relationship and constructed hybrids with higher ploidy and larger cell volumes from *Torulaspora delbrueckii* by means of protoplast fusion.

It was decided to investigate the possibility of increasing the cell size of strain ESP-94 through intraspecific protoplast fusion. The determination of the ploidy of strain ESP-94, the isolation of auxotrophic mutants and the protoplast fusion experiments performed with some of the mutants are presented in this paper. The protoplast fusants generated are compared with the wild-type strain ESP-94 with regard to cell volume, DNA content and genetic stability.

Correspondence to: M. Gericke, Division of Food Science and Technology, CSIR, P.O. Box 395, Pretoria 0001, South Africa.

MATERIALS AND METHODS

Strains

Candida blankii ESP-94 (isolated by J.P. van der Walt, CSIR, Pretoria) was used as parental strain for fusion experiments. *Saccharomyces cerevisiae* strains S150-2B, 294 and 8Dip (this laboratory) were used as reference strains in the determination of cell ploidy and DNA content.

Media

Media were prepared according to Sherman et al. [20]. Growth media used in this study were YP medium supplemented with 2% (w/v) glucose (YPD) or 2% (w/v) D-xylose (YPX). Minimal media (Difco yeast nitrogen base

without amino acids) were prepared with 2% (w/v) glucose (SD) or 2% (w/v) D-xylose (SX) as carbon sources. SD-minimal medium with the addition of 1 M sorbitol and 3% (w/v) agar was used as regeneration medium (MMR).

Ploidy determination

The ploidy of stationary phase cells of strain ESP-94 and fusant F17 was determined according to the X-ray inactivation method described by Beam et al. [1]. Both a haploid and a diploid strain of *Saccharomyces cerevisiae* (strains S150-2B and 8Dip, respectively) were included as reference strains.

Mutagenesis

Cells of strain ESP-94 were mutagenised with 3% (v/v) ethyl methylsulphonate (EMS) as described by Fink [5]. The mutagenised cells were allowed to develop into colonies on complete YPD medium. The colonies were replica plated to SD-minimal plates to identify auxotrophic mutants unable to grow. Auxotrophic mutants were characterized for their nucleic acid base and amino acid requirements by plating them on SD-minimal plates, each supplemented with one of the 20 amino acids, as well as adenine and uracil, respectively. Amino acid concentrations were prepared as described by Sherman et al. [20]. The genetic stability of the auxotrophs was tested by growing them through approximately 20 generations in non-selective complete medium (YPD), before plating on SD-minimal medium to assess the spontaneous reversion frequencies.

Protoplast formation

Yeast cultures were grown in shake flasks (150 rpm) for 9 to 10 h at 37 °C in SD-minimal medium, supplemented with 0.05% (w/v) DL-homocysteine, 0.04% (w/v) methionine, 0.3% (w/v) L-cysteine, and the necessary amino acids. 2-D-deoxyglucose was added to the cultures to a concentration of 0.065% (w/v) and the cultures were grown for a further 1.5 h [4].

The cells were harvested, washed with sterile distilled water and suspended in PTM-solution (1 M sorbitol, 0.1 M sodium citrate, 0.06 M EDTA and 0.8% (v/v) β-mercaptoethanol) at a cell density of 5×10^8 cells/ml. After pre-incubation for 15 min at 37 °C, the cells were harvested and resuspended at the same cell density in 5 mg/ml Novozym (Sigma) and 1 M sorbitol with gentle shaking. Protoplast formation was followed microscopically and upon completion the protoplasts were centrifuged for 10 min at $1000 \times g$ and washed with 1 M sorbitol.

The efficiency of protoplasting was measured by comparing the number of protoplasts suspended in 1 M sorbitol with the number of osmotically resistant cells. The ability of protoplasts to regenerate their cell walls was evaluated by comparing the haemocytometer cell count of the protoplasts with the viable count of the protoplasts suspended in 1 M sorbitol and plated on regeneration medium (MMR), supplemented with amino acids.

Protoplast fusion

Equal numbers of protoplasts of each strain (approx. 10^6 cells as determined by haemocytometer counts) were mixed, centrifuged and resuspended in a solution of 2 ml 30% polyethylene glycol (PEG) MW 3350 (w/v) in 10 mM $CaCl_2$. The protoplast mixtures were incubated for 30 min at 37 °C to allow protoplast fusion to occur. The protoplasts were harvested for 10 min at $1000 \times g$ and resuspended in 1 M sorbitol. The suspensions were mixed with MMR-medium at 50 °C and poured as thin layers onto the surfaces of MMR-plates. The plates were incubated for 4–7 days to allow protoplast fusants to regenerate.

Cell volume

Cells were stained with 0.5% (w/v) methyl violet and the cell dimensions determined with a Quantimet 520 Image Analyzer (Cambridge Instruments, Inc.). The cell volumes were calculated using the formula for an ellipsoid ($V = 4/3\pi a^2 b$), where a and b are half the lengths of the short and long axis, respectively. Buds were not included in the calculations.

DNA content

The cellular DNA of stationary phase cells (about 5×10^9 cells per sample) was extracted with perchloric acid and the DNA content determined in duplicate according to the diphenylamine method described by Stewart [24]. Herring sperm DNA (Boehringer Mannheim) was used as DNA standard and *S. cerevisiae* 294, a haploid strain, as reference strain.

Genetic stability

Yeast cultures were grown through 80 generations at 37 °C and 150 rpm in shake flasks containing YPX medium. The cultures were diluted, plated on YPD-plates and replica-plated to SD-minimal plates. Prototrophic growth on SD-plates was interpreted as an indication of genetic stability.

RESULTS AND DISCUSSION

The isolation of protoplast fusants generated by rare fusion events necessitate an effective selection scheme. Auxotrophic mutants of strain ESP-94 were isolated to allow counterselection of prototrophic fusants in protoplast fusions experiments.

Ploidy

Prior to the isolation of auxotrophic mutants, the ploidy of strain ESP-94 was determined by X-ray inactivation. When plotting the logarithm of the yeast surviving fraction against the X-ray radiation dosage, strain ESP-94 and the haploid *S. cerevisiae* strain S150 gave linear curves (Fig. 1A) characteristic for haploid strains [14]. The curve for the diploid *S. cerevisiae* strain 8Dip showed a shoulder at low X-ray dosages (higher X-ray inactivation resistance), which is characteristic for a diploid strain. It was concluded that strain ESP-94 is a haploid strain.

Mutagenesis

The haploid nature of strain ESP-94 simplified the isolation of auxotrophic mutants. Cells of strain ESP-94 were exposed to mild EMS treatment with an effective mutation frequency of about 1×10^{-4}. The mutation frequency was calculated as the fraction of mutagenised colonies that turned red on complete YPD medium due to mutations at a single *ADE* locus, resulting in the accumulation of red pigment [3,18]. A total of 1.3×10^4 mutagenised colonies were screened. Sixty-one mutants had spontaneous reversion frequencies of less than 10^{-5} and were retained as stable auxotrophs. Seven different auxotrophic phenotypes (Ade^-, Arg^-, Cys^-, His^-, Leu^-, Lys^-, and Met^-) were identified. An Ade^- and two Arg^- auxotrophs were used in protoplast fusion experiments.

Protoplast formation and fusion

The fusion experiments were carried out with protoplasts of Ade26, Arg1 and Arg2 auxotrophic strains. Great difficulty was experienced in removing the cell walls of ESP-94 auxotrophs. The cells were subjected to a variety of lysing enzymes including Glusulase (*Helix pomatia*), Lyticase (*Arthrobacter luteus*), Novozym (*Trichoderma harzianum*) and lytic enzymes from *Rhizoctonia solani* and *Oerskovia xanthineolytica*. However, no more than 30% of the cells could be converted to protoplasts.

The growth conditions and media for culturing strain ESP-94 prior to protoplasting were modified, based on the idea that the inclusion of sulphur-containing amino acids in the medium and the addition of 2-D-deoxyglucose would lead to inhibition of cell wall synthesis [4]. Short exposure of growing organisms to 2-D-deoxyglucose has no effect on respiration [7] but inhibits cell wall synthesis [21]. Subsequently, β-mercapthoethanol was added to reduce the disulphide bonds in the cell wall proteins, as this could lead to a severe weakening of cell wall conformation and rendered cells significantly more vulnerable to lytic enzymes [4]. By employing this method, the rate of protoplast formation increased and protoplasting rates of 80 to 88% were achieved (Table 1) after treatment with Novozym for approx. 1.25 h. The regeneration ability of the protoplasts was low and only about 16 to 17.5% of the protoplasts could regenerate their cell walls (Table 1). The low regeneration rate is not unusual and equally low rates were also reported by Stahl [23], Sarachek et al. [18], and Gupthar and Garnett [9]. The fusion frequency of 8.8×10^{-4} to 5.9×10^{-4} (Table 2) is within the range reported for other intraspecific fusions [8,19,22]. Reversion of the auxotrophic mutants to the prototrophic stage was less than 10^{-7} (Table 2). The prototrophic colonies that regenerated were thus not merely the result of back reversions, but true fusion products.

A total of 182 protoplast fusants were isolated. Cultures of the fusants were grown through approximately 20 generations in SX-minimal medium to allow their cell sizes to stabilize before the cell volumes were determined. Twenty-seven fusants with bigger cell volumes than strain ESP-94 were identified and retained for further characterization.

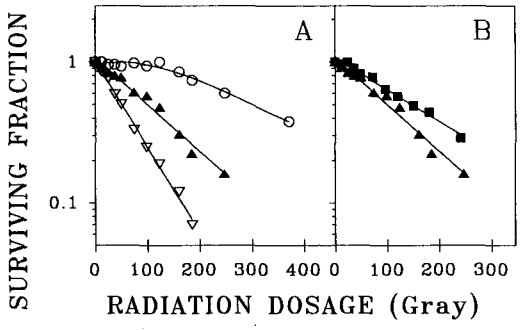

Fig. 1. X-ray survival curves of (A) strain ESP-94 and a haploid and diploid *S. cerevisiae* strain, and (B) strain ESP-94 and fusant F17. Stationary phase cells of *S. cerevisiae* strains S150-2B (▽), 8Dip (○), ESP-94 (▲), and fusant F17 (■) were irradiated for 11 different time intervals at a fixed radiation dosage. Surviving cells were determined and the surviving fraction for each strain plotted against time.

TABLE 1

Efficiency of protoplast formation and cell wall regeneration of three auxotrophic strains of strain ESP-94

Strain	Efficiency of protoplast formation (%)	Cell wall regeneration ability (%)
Ade 26	88	16.7
Arg 1	85	17.5
Arg 2	80	16.4

TABLE 2

Frequency of reversion to prototrophy by strain ESP-94 auxotrophic mutants and PEG-induced prototrophic colony formation between these mutants

Strain	Frequency of reversion to prototrophy	Fusion fusion frequency
Ade 26	$<10^{-7}$	–
Arg 1	$<10^{-7}$	–
Arg 2	$<10^{-7}$	–
Arg 1 × Ade 26	–	8.8×10^{-4}
Arg 2 × Ade 26	–	5.9×10^{-4}

Characterization of fusants

To determine the true hybrid nature of the fusants, they were grown through 80 generations in non-selective complete medium (YPX) and characterised with respect to cell volume, DNA content and genetic stability. The results obtained for the six best fusants are compiled in Table 3. The cell volumes of the six fusants (fusants F17, F20, F23, F42, F53 and F86) varied between 2–3-times that of strain ESP-94. The fusants had DNA content values between 1–2-times that of strain ESP-94 (Table 3). In an attempt to verify the ploidy of one of the fusants, the X-ray inactivation curve for fusant F17 was determined (Fig. 1B). Fusant F17, like the haploid ESP-94, was sensitive to low X-ray dosages, suggesting that at least part of the genome of fusant F17 is haploid [14]. This correlates with the DNA content of fusant F17 that was about 1.5-times that of ESP-94 (Table 3). In *S. cerevisiae*, the cell volume increases proportionally to the ploidy level [17]. However, the fusants obtained in this study did not follow this pattern. Although the DNA content of the fusants, like their cell volumes, exceeded that of strain ESP-94, no clear correlation could be found between the cell volume and ploidy of the different fusants.

The lower than diploid DNA content in the fusants can be attributed to aneuploidy. Support for this assumption is found in results recorded for hybrids of *Candida tropicalis* [6], *Kluyveromyces lactis* [15], *Schwanniomyces alluvius* [27] and *Pichia guilliermondii* [10,11]. In all these cases the lower than expected DNA content was ascribed to the loss of chromosomes after fusion. Hybrids are in a heterokaryotic state directly after fusion. Stable hybrids can only be formed if the cytoplasmic fusion is followed by karyogamy. During these events, cells can either stabilize, undergo spontaneous haploidization (re-establish auxotrophs) or partial haploidization to various states of aneuploidy [18]. The fusants were vigorously grown through 80 generations under non-selective conditions before the DNA contents were determined, leaving ample opportunity for stabilization and formation of aneuploids.

Higher SCP yields are obtained by continuous fermentation processes than are possible in batch cultures [13]. When considering SCP production by continuous fermentation, the genetic stability of the organism is crucial. The

TABLE 3

A comparison between the cell volume, DNA content and genetic stability of the parental strain, ESP-94, and the six best fusants after growth through 80 generations in YPX-medium

Strain	Cell volume (μm^3)	DNA content (fg/cell)	Stability (%)
ESP-94	16.02 ± 6.62	25.98	–
F17	47.99 ± 15.99	41.02	99.7
F20	45.47 ± 10.18	38.29	99.9
F23	45.48 ± 14.55	33.98	> 99.9
F42	32.90 ± 8.66	32.29	> 99.9
F53	40.98 ± 12.61	34.83	> 99.9
F86	38.79 ± 12.41	41.02	99.9

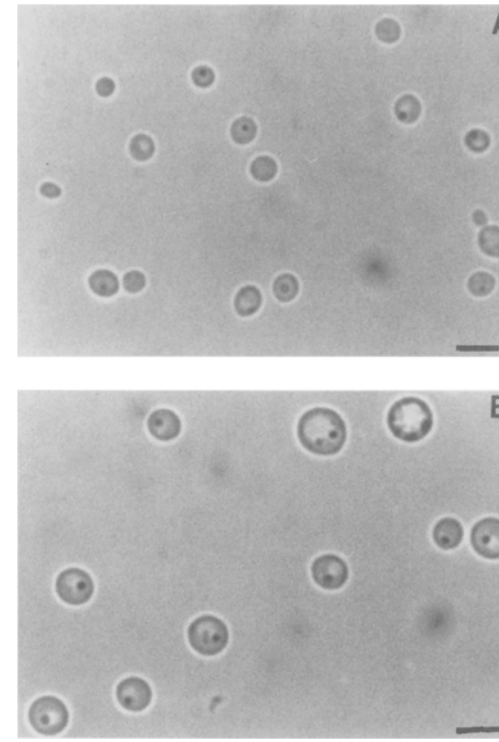

Fig. 2. Photographs showing the difference in cell size between (A) the wild-type strain ESP-94 and (B) fusant F17 after growth through 80 generations in non-selective complete YPX medium. The magnification scales are indicated on the photographs by black bars representing 5.0 μm.

genetic stability of the fusants was determined as the percentage of stable prototrophic fusants retained after growth through 80 generations in complete YPX medium. In most cases more than 99% of the fusants remained prototrophic after 80 generations. A possible explanation for the high stability is that unstable fusants were shed during vigorous growth and disappeared from the population. Only the fast-growing, more stable progeny was retained. Several workers reported that stabilization occurred over a period of time, during which the fusion products give rise to fewer auxotrophs [6,27].

Fusant F17 was identified as the most promising fusion product. It was confirmed by standard criteria for yeast identification [25] that fusant F17 is a *Candida blankii* strain. Fusant F17 has a cell volume three times that of the parental strain, when grown in YPX (Fig. 2). The large cell volume, but low DNA content and X-ray sensitivity of fusant F17 suggests that only a subset of its chromosomes/genes (including those determining cell volume) was retained at multiple copy with the remainder of the genome being reduced to the haploid state. Nevertheless, fusant F17 and the wild-type strain ESP-94 still showed the same growth rates (about 0.5 h^{-1}) in shake flasks on D-xylose as carbon source. Fusant F17 is currently evaluated for genetic stability, cell volume and protein content under continuous fermentation conditions.

The work done on *C. blankii* ESP-94 showed that intraspecific protoplast fusions can yield very stable fusants, whereas intergeneric fusions often give unstable fusants [2,27]. Intraspecific protoplast fusion could, therefore, be a very useful technique for producing stable industrial yeasts with improved industrial-related properties.

REFERENCES

1 Beam, C.A., R.K. Mortimer, R.G. Wolfe and C.A. Tobias. 1954. The relation of radioresistance to budding in *Saccharomyces cerevisiae*. Arch. Biochem. Biophys. 49: 110–122.

2 Chang, M.C., H.K. Jung, T. Suzuki, M. Takagi and K. Yano. 1984. Ploidy in the asporogenous yeast *Candida maltosa*, isolation of its auxotrophic mutants and their cell fusion. J. Gen. Appl. Microbiol. 30: 489–497.

3 Corner, B.E. and R.T.M. Poulter. 1989. Interspecific complementation analysis by protoplast fusion of *Candida tropicalis* and *Candida albicans* adenine auxotrophs. J. Bacteriol. 171: 3586–3589.

4 Evans, C.T. and D. Conrad. 1987. An improved method for protoplast formation and its application in the fusion of *Rhodotorula rubra* with *Saccharomyces cerevisiae*. Arch. Microbiol. 148: 77–82.

5 Fink, G.R. 1970. The biochemical genetics of yeast. Methods Enzymol. 17 (A): 59–78.

6 Fournier, P., A. Provost, C. Bourguignon and H. Heslot. 1977. Recombination after protoplast fusion in the yeast *Candida tropicalis*. Arch. Microbiol. 115: 143–149.

7 Foury, F. and A. Goffeau. 1973. Combination of 2-deoxyglucose and snail-gut enzyme treatments for preparing spheroplasts of *Schizosaccharomyces pombe*. J. Gen. Microbiol. 75: 227–229.

8 Fujii, T., H. Yamamoto, E. Takenaka, K. Fujinami, A. Ando and M. Yabuki. 1988. Intraspecific hybridization of a methanol-utilizing yeast, *Candida* sp. N-16, through protoplast fusion. Agric. Biol. Chem. 52: 1661–1667.

9 Gupthar, A.S. and H.M. Garnett. 1987. Hybridization of *Pichia stipitis* with its presumptive imperfect partner *Candida shehatae*. Curr. Genet. 12: 199–204.

10 Klinner, U. and F. Bottcher. 1984. Hybridization of yeasts by protoplast fusion: Ploidy level of hybrids resulting from fusions in haploid strains of *Pichia guilliermondii*. Z. Allg. Mikrobiol. 24: 533–537.

11 Klinner, U. and F. Bottcher. 1985. Hybridization of yeasts by protoplast fusion: Attempts to polyploidize *Pichia guilliermondii*. J. Basic Microbiol. 25: 233–241.

12 Lawford, G.R., A. Kligerman, T. Williams and H.G. Lawford. 1979. Production of high-quality edible protein from *Candida* Yeast grown in continuous culture. Biotechnol. Bioeng. 21: 1163–1174.

13 Litchfield, J.H. 1977. Comparative technical and economic aspects of single-cell protein. Adv. Appl. Microbiol. 22: 267–305.

14 Lucke, W.H. and A. Sarachek. 1953. X-Ray inactivation of polyploid *Saccharomyces*. Nature 171: 1014–1015.

15 Morgan, A.J., A. Brunner and P.A. Whittaker. 1980. Protoplast fusion in a *petite*-negative yeast, *Kluyveromyces lactis*. Curr. Genet. 2: 87–93.

16 Murray, L.E., L.M. Veinot-Drebot, P.J. Hanic-Joyce, R.A. Singer and G.C. Johnston. 1987. Effect of ploidy on the critical size for cell proliferation of the yeast *Saccharomyces cerevisiae*. Curr. Genet. 11: 591–594.

17 Pina, A., I.L. Calderon and T. Benitez. 1986. Intergeneric hybrids of *Saccharomyces cerevisiae* and *Zygosaccharomyces fermentati* obtained by protoplast fusion. Appl. Environ. Microbiol. 51: 995–1003.

18 Sarachek, A., D.D. Rhoads and R.H. Schwarzhoff. 1981. Hybridization of *Candida albicans* through fusion of protoplasts. Arch. Microbiol. 129: 1–8.

19 Sasaki, T. and Y. Ohshima. 1987. Induction and characterization of artificial diploids from the haploid yeast *Torulaspora delbrueckii*. Appl. Environ. Microbiol. 53: 1504–1511.

20 Sherman, F., G.R. Fink and J.B. Hicks. 1986. Laboratory course manual for methods in yeast genetics, Cold Spring Harbor Laboratory, New York.

21 Sipiczki, M. and L. Ferenczy. 1977. Protoplast fusion of *Schizosaccharomyces pombe* auxotrophic mutants of identical mating-type. Mol. Gen. Genet. 151: 77–81.

22 Sipiczki, M., W.-D. Heyer and J. Kohli. 1985. Preparation and regeneration of protoplast and spheroplasts for fusion and transformation of *Schizosaccharomyces pombe*. Curr. Microbiol. 12: 169–174.

23 Stahl, U. 1978. Zygote formation and recombination between like mating types in the yeast *Saccharomycopsis lipolytica* by protoplast fusion. Mol. Gen. Genet. 160: 111–113.

24 Stewart, P.R. 1975. Analytical methods for yeasts. In: Meth-

ods in Cell Biology, Vol. XII (Prescott, D.M., ea), pp. 111–147, Academic Press, New York.

25 Van der Walt, J.P. and D. Yarrow. 1984. Method for isolation, classification and identification of yeasts. In: The Yeasts, a Taxonomic Study (Kreger-van Rij, N.J.W., ed), Elsevier Science Publishers, Amsterdam.

26 Watson, T.G. 1989. Single-cell protein production. CSIR Division of Food Science and Technology Progress Report, C-Voed 231, November 1989.

27 Wilson, J.J., G.G. Khachatourians and W.M. Ingledew. 1982. Protoplast fusion in the yeast, *Schwanniomyces alluvius*. Mol. Gen. Genet. 186: 95–100.