MICROBIOLOGY APPLICATIONS IN FOOD BIOTECHNOLOGY

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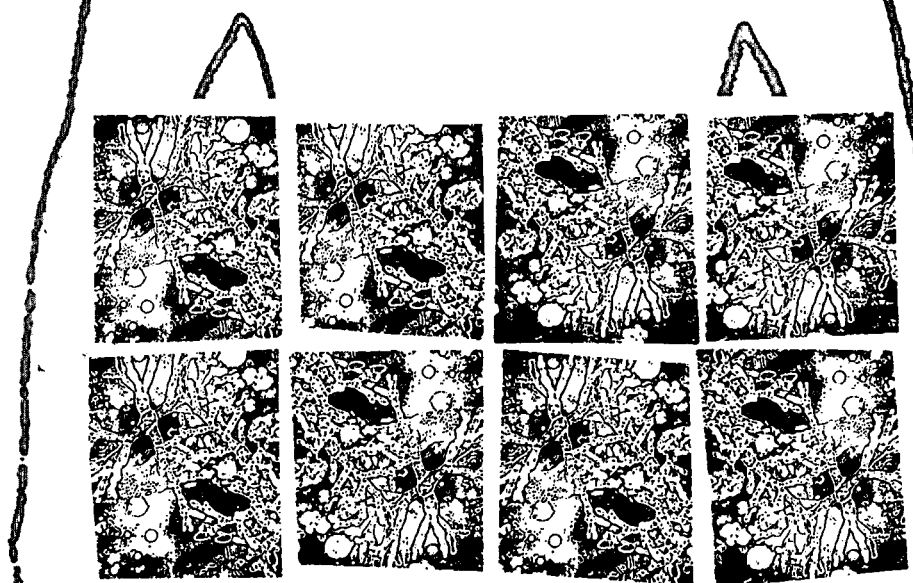

ELSEVIER APPLIED SCIENCE

CONTENTS

MITOTIC SEGREGATION IN INTERGENERIC HYBRIDS OF YEAST TO GIVE NOVEL GENETIC SEGREGANTS

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ABSTRACT

Two strains of yeast, Yarrowia lipolytica and Saccharomycopsis fibuligera, have a filamentous growth form in addition to budding cells. Y.lipolytica produces lipases and is used in the production of citric acid while S.fibuligera produces amylases and is used in the production of rice wine.

In the present report, we made a study of the following:

(i) karyotypes of the two yeast strains to obtain a better understanding of their genetic relatedness,

(ii) genetic crosses between the two strains to produce intergeneric hybrids, and

(iii) pattern of genetic segregation of the intergeneric hybrids via the mitotic process.

The results of our studies showed that the two yeast strains were genetically related and that putative

intergeneric hybrids were obtained by a genetic crossing of the strains. The hybrids were relatively stable in mitosis as compared to their parent strains. However, in prolonged vegetative propagation, the hybrids gave rise to genetic segregants, most of which were of one of the two parent phenotypes. A number of the segregants had phenotypes which combined those of the parental types. Of considerable significance was that yet a few others were novel as they exhibited phenotypes not hitherto seen for both parent strains.

INTRODUCTION

Yeast are well known to us as organisms used in the production of bread, beer, wine, yeast extract and alcohol. They are also used in the making of single cell protein and citric acid. A few yeasts are also known to be the causal organisms for human diseases. There are many different species of yeasts. Yeasts are eukaryotes. Yeasts reproduce by budding. Some species have a mycelial growth form in addition to the budding cells. In this report we made a study of two mycelial yeasts, Yarrrowia lipolytica and Saccharomycopsis fibuligera. Y.lipolytica produces lipases are useful in the hydrolysis of vegetable oils, including palm oil. Malaysia produces in excess of 4.5 million tonnes of palm oil a year. S.fibuligera produces amylases which hydrolyse starch. This makes it useful in the rice wine making industry. The genetic system for Y.lipolytica is well established and genetic cloning procedures for it is known [1]. Genetic cloning of genes for amylase of S.fibuligera is known from recent studies [2]. Previous studies of intergeneric hybrids in yeast have been reported [3]. We are interested in obtaining a better understanding of the genetic relatedness of the two yeasts [4].

Initially, we made a comparative study of the karyotypes of the two yeasts to establish their chromosome types. We then produced putative intergeneric hybrids of the two strains as they showed a good degree of similarity. Finally, we made a study of mitotic segregation of the intergeneric hybrids.

MATERIALS AND METHODS

Strains

The following strains of yeast were used for the OFAGE runs : Saccharomycopsis fibuligera strain 8014 (wild type) and Yarrowia lipolytica strain D29 (wild type). For subsequent intergeneric experiments, the parent yeast strains used were Saccharomycopsis fibuligera strain 193 met, and Yarrowia lipolytica strain 1529 A his_1.

Preparation of gel inserts containing yeast DNA for the OFAGE

To achieve this purpose, we used the method designed by Peter De Jonge et al. [5]. Other studies on the separation of chromosomal DNA by using OFAGE have also been reported [6-10].

We first obtained 10^{10} late-log-phase cells by growing a preculture for 24h at 30°C and then growing 0.5ml of this preculture in 40ml YEPD. A suitable volume containing the required number of cells was then spun down and the resulting cell pellet washed with 0.05M EDTA, pH 7.5 at room temperature. The mixture was then spun down again and the process repeated (resuspending and centrifuging) with CPE buffer and CPES buffer (CPE with sorbitol and dithiothreitol). The resulting cells were then acclimatized at 30°C for 5 min. To produce spheroplasts, 20mg of Novozym 234 was first dissolved in 5ml of 1% low gelling agarose mixed in CPE buffer. This was then mixed with the cell suspension and the mixture pipetted into a matrix cooled beforehand and allowed to gel at 0°C. The agarose blocks were then removed from the matrix and kept at 30°C for 1h in 20ml CPE buffer. 0.025g of proteinase K was added to 20ml NDS and the resulting mixture was then added to the agarose blocks. After 16h at 50°C without shaking, the blocks now contained sufficient yeast DNA for use in the OFAGE run. They may be stored for 3 weeks at 4°C in fresh NDS.

Crossing experiments

In our experiments, we obtained intergeneric hybrids of S.fibuligera and Y.lipolytica by crossing them naturally in mating media (YMC). The principle of this crossing method involves mixing compatible mating types of yeast with complementary nutritional requirements and plating this mixture onto minimal media (YNBG) in order to select hybrid prototrophs from parent auxotrophs.

Protoplast fusion

Strains, B leu_2 ade_1 xpr_2 and 193 met, were grown for 24h in 40ml YE media at 30°C with shaking. 10ml portions of these cultures were added to 90ml portions of YE media and grown to log phase. The cultures were harvested and washed in sterile distilled water twice. Cells were resuspended in 10ml PTP-sorbitol buffer which contained dithiothreitol. Cells of B leu_2 ade_1 xpr_2 were so treated for 5 min and of 193 met for 15 min. The tubes were kept in ice and then spun down and washed in MAP-sorbitol containing 70mg novozym and incubated at 30°C for 30 min. The protoplasts were harvested and washed in MAP-sorbitol containing 5% Ficoll. These were centrifuged and the protoplasts were resuspended in an osmotic stabilising buffer. Protoplasts from the two strains were mixed in equal volumes. These were centrifuged and the pellet was resuspended in PEG at 25% in OSB. This was incubated at 30°C for 30 min. Finally an equal volume of OSB was added to the mixture and the content was spun down. The pellet was washed once in OSB and resuspended in 2ml of OSB. Aliquots from this and diluted portions were plated onto regeneration media and incubated for 5-7d at 30°C.

Plating experiments

Spread plating was carried out as follows : 0.1ml of cell suspension containing about 1000 cells per ml was transferred onto the surface of respective media plates by a micropipette and spread evenly using a sterile glass spreader. The cells were then allowed to grow at 30°C for 3 days.

For auxanography test, petri-dishes of media containing YNBG supplemented with methionine (met) and histidine (his) was prepared. Before the agar solidified, a suspension containing the particular cells to be analysed was poured onto each plate, and the contents allowed to be homogenized with the media. After the agar solidified, the 4 supplements (hydrolyzed casein, nucleic acids, vitamins and an amino acid pool containing proline, arginine, glutamine and glutamic acid) were added on separate parts of the media plate using a micropipette. The cells were then allowed to grow for 3 days at 30°C.

Materials used for the preparation of DNA gel inserts for OFAGE runs

YEPD (yeast extract peptone dextrose) contained yeast extract 10g, trypticase peptone 20g, glucose 20g, distilled water 1000ml. EDTA buffer contained 0.05M EDTA solution 18.6g, Tris HCl 1.576g, dithiothreitol 1.542g, pH 7.5. Citric acid buffer contained citric acid 5.04g, sodium hydrogen phosphate 10.21g, distilled water 600ml, pH 6.0. CPE buffer consisted of citric acid buffer 1000ml, sorbitol 217.44g, dithiothreitol 0.771g. EDTA, disodium dihydrate, 0.05M, pH7.5 was EDTA 18.61g, distilled water 1000ml. Low melting agarose was low-melting point agarose (1%) 0.1g, CPE buffer 10ml. NDS solution contained EDTA 372.2g, Tris HCl 2.422g, pH 7.5. Spheroplasting solution consisted of novozym 20 mg, low-melting point agarose (1%) 5ml. Electrophoresis (running) buffer (TBE) was Tris base 32.7g, boric acid 16.69g, EDTA 2.79g, distilled water 1000ml, pH 8.2.

Media

Preparation for the various media used in the crossing experiments and the study of the pattern of genetic segregation of the intergeneric hybrids were as follows:

Mating media (YMC) was malt 3g, peptone 5g, yeast extract 3g, sodium citrate 0.5g, agar 25g, distilled water 1000ml. Simmons citrate agar was Simmons citrate agar 2.43g, distilled water 1000ml. Complete media (yeast extract agar, YEA) consisted of yeast extract 3g, glucose 18g, agar 9g, distilled water 600ml. Yeast peptone starch (YPSS) contained yeast extract 2.4g, potassium hydrogen phosphate 0.6g, magnesium sulphate 0.3g, agar 12g, soluble starch 9g, distilled water 600ml. Minimal media (yeast nitrogen base glucose, YNBG) contained yeast nitrogen base 2g, ammonium sulphate 1.5g, agar 1.5g, glucose 6g, distilled water 600ml.

Stock solutions of amino acid supplements to minimal media were prepared as follows : methionine - 48mg per 20ml of distilled water and histidine - 48mg per 20ml of distilled water. 5ml aliquots of stock amino acid supplements were added per 600ml of YNBG whenever necessary.

For auxanography test, the following were supplemented to YNBG which contained methionine and histidine in 10ul portions: vitamin complex (biotin,

calcium pantothenate, folic acid, niacin, p-aminobenzoic acid, pyridoxine hydrochloride); yeast nucleic acid (2.5ml per 1000ml of distilled water); hydrolysed casein (1.5g of casamino acids per 1000ml of distilled water); arginine, proline, glutamine, glutamic acid common pathway pool (48mg per 20ml of distilled water).

RESULTS

Results of OFAGE runs

The yeast strain Saccharomyces cerevisiae was used as a DNA size standard. It has small DNA molecules (less than 750kb). From the figure, it was observed that the chromosomal patterns of Y.lipolytica and S.fibuligera were similar. Certain bands of the 2 yeast strains were also located on similar positions, indicating similar chromosome sizes. Furthermore, both yeasts have approximately the same number of chromosome mobility groups, with 6 for S.fibuligera and 5 for Y.lipolytica.

Results of intergeneric cross between S.fibigerus and Y.lipolytica and subsequent study of the intergeneric hybrids

Four intergeneric hybrid strains, 7i, 9i, 14i and 15i, were obtained as described in the crossing methods. They were then inoculated onto centres of petri-dishes containing complete media containing starch (YPSS) along with the controls, S.fibuligera strain 193, met, and Y.lipolytica strain 1529, A his_1. The plates were incubated at 30°C for 2 weeks.

Samples of cells were taken from the centre of each hybrid culture after 7 days of growth and spread plated onto complete media (YEA) and minimal media (YNBG) plates. These were then incubated for 3 days at 30°C. The colonies that grew from the single cells or fragments of mycelia in these plates were classified according to their morphology as follows (Table 1):

On growing for 2 weeks at 30°C on YPSS, sectors were observed for the intergeneric hybrids 7i, 9i, 14i and 15i.

Table 1a

Plating of cells on YEA :

Hybrid strains	Number of colonies with phenotype of : *Y. lipolytica*	*S. fibuligera*	Total number of colonies
7i	525	107	632
9i	531	494	1025
14i	384	36	420
15i	9	531	540

Table 1b

Plating of cells on YNBG :

Hybrid strains	Number of colonies with phenotype of : *Y. lipolytica*	*S. fibuligera*	Total Number of colonies
7i	401	96	497
9i	357	514	871
14i	292	45	337
15i	2	538	540

The sectors were analysed by inoculating them onto media plates containing YPSS, citrate, YNBG, YNBG + met, YNBG + his, YNBG + met + his. The results obtained are given in Table 2.

TABLE 2

Spontaneous segregants from the hybrid; 193 met and A his_1 :

Hybrid strains	Number of Sectors met^-	his^-	$met^-his^-x^-$	met^+his^+	total
15i	232	0	8	0	240
9i	233	0	4	1	238
7i	86	0	2	1	89
14i	208	1	5	0	214

Hybrid strains	Number of Sectors met^-	his^-	met^-glu^-	$met^-his^-glu^-$	met^+his^+	total
15i	232	0	7	1	0	240
9i	233	0	3	1	1	238
7i	86	0	2	0	1	89
14i	208	1	3	2	0	214

In case of 7i, 9i, 14i and 15i, the 19 sectors which were not able to grow in media supplemented with methionine and histidine were subjected to further classification.

Auxanography test was then carried out for each of the 19 segregants. All 19 segregants gave growth on YNBG + his + met segregants. All 19 segregants gave growth on YNBG + his + met plate supplemented with hydrolysed casein. Supplementation with vitamin solution and nucleic acid solution did not give growth for any of the 19 segregants.

A classification of these segregants on YNBG media containing supplements of different combinations of the following chemicals : methionine, histidine and glutamic acid gave results as presented in the lower part of the Table.

In order to ascertain the genetic nature of the original S.fibuligera strain and S.fibuligera 193 met, these strains were inoculated onto YPSS plates and incubated for two weeks at 30°C. Cells of the Yarrowia lipolytica strain A his_1 were also inoculated onto YPSS plates and incubated at 30°C for two weeks. The results of this experiment are shown in Table 3.

TABLE 3

Spontaneous segregation from the original strain 8014 (S. fibuligera) :

Number of Sectors		Total
met^-	met^-glu^-	
250	0	250

Y. lipolytica 1529 A his_1 gave no spontaneous sectors.

Spontaneous segregants from the strain; 193 met

(a) Number of Sectors

met^-	his^-	met^-glu^-	$met^-his^-glu^-$	met^+his^+	total
233	0	7	0	0	240

(b) Number of Sectors

met^-	$met^-ade^-leu^-$	met^-ade^-	met^-leu^-	ade^-leu^-	ade^-	leu^-	met^-glu^-	total
112	0	0	0	0	0	0	3	115

S.fibuligera strain 193 met was obtained from the original strain 8014 by UV mutagenesis. A very low frequency of occurrence of mutants, mostly morphological, were obtained subsequent to UV mutagenesis of cells of the strain 8014. A plausible explanation of the present results is that the strain 193 is diploid and had a lesion in a gene for an enzyme in the glutamate biosynthetic pathway.

Protoplast fusants were obtained between the strain 193 met and 1529 A his_1 and between 193 met and B leu_2 ade_1 xpr_2. These hybrid strains were subjected to vegetative growth and spontaneous segregation on YPSS media. The results for the characteristics of the sectors of hybrid 193 met and 1529 A his_1 were similar to those obtained from the same hybrid from genetic crossing. The characteristics of the spontaneous segregants for the other hybrid are given in Table 4.

TABLE 4

Spontaneous segregants from the hybrid; 193 met and B leu_2 ade_1 xpr_2 :

Hybrid strains	YNBG	met^-	others
10		72	3
4		76	4
6	1	170	1
16	1	74	0

Fourteen spontaneous segregants from the hybrid 193 met and 1529 A his_1 were classified for their ability to utilise citrate, tributyrin and acetate as the carbohydrate source for growth. the results are given in Table 5.