Lab Manual on Non-conventional Yeasts

Genetics, Biochemistry, Molecular Biology and Biotechnology

K. Wolf, K. Breuning, G. Barth (eds.)

Title of experiment:

Use of a differential culture medium for the enumeration of *Zygosaccharomyces bailii*, *Saccharomyces cerevisiae* and *Pichia membranifaciens* in wine

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Aim of the experiment:

In this experiment a differential culture medium is used for the rapid and efficient detection and enumeration of *Z. bailii* in the presence of other yeast species encountered in wines such as *S.cerevisiae* and *P. membranifaciens*.

Materials

Strains

Zygosaccharomyces bailii	IGC 4806
Pichia membranifaciens	IGC 2486
Sacharomyces cerevisiae	IGC 4072

The strains can be obtained from the

Portuguese Yeast Culture Collection (PYCC)

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Apparatus

Membrane filtration unit with sterile funnels (for example MICROFIL filtration system from Millipore)

Sterile membrane filters (47 mm diameter, 0.45µm porosity)

General equipment of a microbiology laboratory (balance, autoclave, pH meter, incubator, magnetic stirring plate)

Media

(1) YPD Medium

Peptone	1% (w/v)
Yeast Extract	0.5% (w/v)
Glucose	2% (w/v)
Agar	2% (w/v)

(2) Differential culture medium according to the composition described in Table 1:

Table 1: Culture medium composition for the detection of *Z. bailii*

Compound			Concentration (%)	
Base Medium	Ammonium sulphate	$(NH_4)_2SO_4$	0.5	(w/v)
	Potassium dihydrogenophosphate	KH_2PO_4	0.5	(w/v)
	Magnesium sulphate hetpahydrate	$MgSO_4 \cdot 7H_2O$	0.05	(w/v)
	Calcium chloride dihydrate	$CaCl_2 \cdot 2\ H_2O$	0.013	(w/v)
	Bromocresol green	$C_{21}H_{14}Br_4O_5S$	0.005	(w/v)
	Agar	-	2.0	(w/v)
Glucose	-	$C_6H_{12}O_6$	0.1	(w/v)
Formic acid	-	CH_2O_2	0.2	(v/v)
Oligoelements Solution A	(Composition according to Table 2)	-	0.05	(v/v)
Oligoelements Solution B	(Composition according to Table 2)	-	0.05	(v/v)
Vitamin Solution	(Composition according to Table 2)	-	0.05	(v/v)

Table 2: **Oligoelements and vitamin solutions composition**

Compound			Concentration (%)	
Oligoelements Solution A	Boric acid	H_3BO_3	1.0	(w/v)
	Potassium Iodide	KI	0.2	(w/v)
	Sodium molibdate dihydrate	$Na_2MoO_4 \cdot 2H_2O$	0.4	(w/v)
Oligoelements Solution B	Copper sulphate pentahydrate	$CuSO_4 \cdot 5H_2O$	0.08	(w/v)
	Iron chloride hexahydrate	$FeCl_3 \cdot 6\ H_2O$	0.4	(w/v)
	Manganese sulphate tetrahydrate	$MnSO_4 \cdot 4H_2O$	0.8	(w/v)
	Zinc sulphate heptahydrate	$ZnSO_4 \cdot 7H_2O$	0.8	(w/v)
	Hydrochloric acid	$HCl\ 10^{-3}$ N	0.8	(v/v)
Vitamin Solution	Biotin	$C_{10}H_{16}N_2O_3S$	0.001	(w/v)
	Calcium panthotenate	$C_9H_{16}NO_5 \cdot 1/2\ Ca$	0.08	(w/v)
	Mioinositol	$C_6H_{12}O_6$	4.0	(w/v)
	Niacin	$C_6H_5NO_2$	0.16	(w/v)
	Pyridoxine hydrochloride	$C_8H_{11}NO_3 \cdot HCl$	0.16	(w/v)
	Thiamin hydrochloride	$C_{12}H_{17}ClN_4OS \cdot HCl$	0.16	(w/v)

The base medium compounds are dissolved in 4/5 of the estimated deionized water volume, and the pH value is adjusted to 4.5 using HCl 1M. Autoclave at 121°C for 20 minutes.

The other medium compounds (glucose, formic acid, oligoelements solution A, oligoelements solution B, and vitamin solution) are dissolved in the remaining water volume so that the final concentration of these compounds equals the values mentioned in Table 1. The pH must be adjusted to 4.5 with HCl 1M. The sterilization is accomplished by filtration. This solution and the base medium are mixed at 50±5°C and dispensed into Petri dishes (ca. 5 cm diameter).

Note: The filter-sterilized oligoelements solutions A and B, as well as the vitamin solution can be stored at 4°C up to 1 year. They should be discarded when they become cloudy.

Reagents

Glycerol, 30% (v/v).

This solution is distributed in 1 ml volumes in cryopreservation vials. Autoclave at 121°C for 20 minutes.

Introduction

Spoilage due to the proliferation of yeasts in an ongoing concern in the food and beverage industries. Therefore, the use of differential media for rapid detection and enumeration of preservative resistant yeasts is of most importance in the quality control. This is particularly relevant for the case of *Zygosaccharomyces bailii,* which is responsible for considerable economic losses (Thomas and Davenport 1985, Loureiro and Querol, 1999;).

The low permeability of *Z. bailii* to weak acid preservatives at low pH values and its ability to metabolize acid compounds, even in the presence of glucose, are some of the physiological traits associated to its high tolerance to acidic environments (Fernandes *et al*., 1997; Sousa *et al*., 1996; Sousa *et al*., 1998). Infections with pellicle-forming yeasts, such as *Pichia* spp., may irrevocably damage wine by production of high concentrations of volatile esters, especially ethyl acetate.

The classical yeast identification methods are based in a series of vegetative and sexual reproduction characteristics, and comprise a large range of physiological and biochemical tests. In the present experiment, a medium containing a mixture of glucose and formic acid as sole carbon and energy sources, with the incorporation of bromocresol green as acid-base indicator will be used for the enumeration of *Z. bailii*, *P. membranifaciens* and *S. cerevisiae*. The methodology and the culture medium described in this experiment is a fast and economic alternative to the classical procedures (Schuller *et al*., 2000).

Procedure

Note

It is convenient to store the 3 reference yeast strains in glycerol solution (30%, v/v) at a temperature of – 60°C or less.

Day 1 (To be done by the instructor)

Revive the 3 yeast strains by transferring with a sterile toothpick a portion of the frozen sample onto a YPD plate. Incubate for about 2 days at 26 – 30°C.

Day 3

From each of the 3 strains grown on YPD plates, prepare a dense cell suspension (A_{640} ~ 0,7) in a tube containing sterile water. From this initial suspension prepare a 10^{-1} and 10^{-2} dilution for *S. cerevisiae* IGC 4072 and *P. membranifaciens* IGC 2487, and a 10^{-1} to 10^{-4} dilution for *Z. bailii* IGC 4806, using tubes containing 9 ml of deionized water. Transfer 0.1 ml from different dilutions to various 100 ml aliquots of wine or water, according to table 3. The inoculation of water should be performed as a control, as *P. membranifaciens* and *S. cerevisiea* may grow poorly on the differential medium after being exposed to the high ethanol concentrations that are found in wines. Agitate vigorously to homogenize the inoculated wine. (To be done by the instructor).

Table 3: **Preparation of inoculated wine and water aliquots**

Group Nº	Aliquot Nº (100 ml wine)	Aliquot Nº (100 ml water)	Inoculation with			Filtration volume (ml)
			Species	Dilution	Volume (ml)	
1	1	7	*S. cerevisiae*	10^{-2}	0.1	5 10 50
2	2	8	*P. membranifaciens*	10^{-2}	0.1	5 10 50
3	3	9	*Z. bailii*	10^{-4}	0.1	5 10 50
4	4	10	*S. cerevisiae* *Z. bailii*	10^{-2} 10^{-3}	0.1 0.1	1 5 10
5	5	11	*P. membranifaciens* *Z. bailii*	10^{-2} 10^{-3}	0.1 0.1	1 5 10
6	6	12	*S. cerevisiae* *P. membranifaciens* *Z. bailii*	10^{-2} 10^{-2} 10^{-3}	0.1 0.1 0.1	1 5 10

Perform membrane filtration of the inoculated wine(s) through membrane filters (0.45-µm pore size, Millipore) with the aid of partial vacuum. Consider hereby the indicated volumes in table 3. In order to obtain a uniform distribution of cells on the surface of the membrane, add 50 ml of sterile, deionized water to the wine sample in the funnel before filtration. Place the filters on the surface of the differential culture medium. Incubate the Petri dishes at 30°C.

Additionally, samples of spoiled wines that were obtained from local wineries can also be examined without prior inoculation.

Note: The recuperation of the 3 yeast species can depend on the characteristics of the wine that was inoculated. It might therefore be necessary to use higher or lower filtration volumes than those indicated above. A previous test may be helpful.

Day 5, 6, 7, 8 and 9

Examine the plates for the development of colonies with the characteristic colonies illustrated in Figure 1. As the medium is selective for *Z. bailii*, growth of *S. cerevisiae* and *P. membranifaciens* will appear only after a longer incubation period (4-5 days). Calculate the number of colony forming units (cfu) per ml of wine for each yeast species, and conclude about the microbiological stability of the wine samples that were examined.

References

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Figure legends

Figure 1

Morphology of the colonies of *Zygosaccharomyces bailii* in pure or mixed culture with other wine contaminating yeasts on membrane filters placed on the surface of the differential medium after incubation for 96 h at 30°C.

A *Zygosaccharomyces bailii* IGC 4806 (*ZB*) – blue colonies (here: grey);

B *Saccharomyces cerevisiae* IGC 4072 (*SC*) – white or light-green colonies (the acid-base indicator, bromocresol green, might be incorporated into some cells (here: white to light grey);

C *Pichia membranifaciens* IGC 2487 (*PM*) – dark green colonies (here: grey);

D *Z. bailii* IGC 4806 (*ZB*) + *S. cerevisiae* IGC 4072 (*SC*) - several colonies can be light blue, (here: light grey) resulting possibly from *S. cerevisiae* cells which were able to incorporate the acid-base indicator (of blue color due to the alkalization performed by *Z. bailii*);

E *Z. bailii* IGC 4806 (*ZB*) + *P. membranifaciens* IGC 2487 (*PM*) – dark colonies of *P. membranifaciens,* formed in the presence of *Z. bailii* can be distinguished clearly from each other by size and intensity of the blue (here: grey) color.